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

THE COMMISSION OF THE EUROPEAN COMMUNITIES,

Having regard to the Treaty establishing the European Community,

Having regard to Council Regulation (EC) No 865/2004 of 29 April 2004 on the common organisation of the market in olive oil and table olives and amending Regulation (EEC) No 827/68 [1], and in particular Article 5(3) thereof,

Whereas:

(1) Commission Regulation (EEC) No 2568/91 [2] defines the physical and chemical characteristics of olive oils and olive-residue oils and the methods of analysis of these characteristics. These methods, and the limit values for the characteristics of oils, must be updated on the basis of the opinion of chemical experts and in line with the work carried out within the International Olive Oil Council.

(2) In particular, the chemical experts have concluded that the quantification of the percentage of 2-glyceryl monopalmitate is more precise for the detection of esterified oils. Decreasing the limit value for stigmastadiene in virgin olive oils also makes it possible to achieve better separation of virgin olive oils and refined olive oils.

(3) In order to allow a period of adjustment to the new standards, to give time for introducing the means of applying them and to avoid disturbance to commercial transactions, the amendments to this Regulation should not apply until 1 January 2008. For the same reasons, provision should be made for olive oil and olive-residue oils that are legally manufactured and labelled in the Community or legally imported into the Community and released for free circulation before that date to be marketed until all stocks are used up.

(4) The measures provided for in this Regulation are in accordance with the opinion of the Management Committee for Olive Oil and Table Olives,

HAS ADOPTED THIS REGULATION:

Article 1

Regulation (EEC) No 2568/91 is amended as follows:

1. The sixth indent of Article 2(1) is replaced by the following:

   '— for the determination of the percentage of 2-glyceryl monopalmitate, the method set out in Annex VII,'.

2. The Annexes are amended in accordance with the Annex to this Regulation.

Article 2

This Regulation shall enter into force on the third day following its publication in the *Official Journal of the European Union*.

This Regulation shall apply from 1 January 2008.

However, products which have been legally manufactured and labelled in the Community or legally imported into the Community and released for free circulation before 1 January 2008 may be marketed until all stocks are used up.

This Regulation shall be binding in its entirety and directly applicable in all Member States.

Done at Brussels, 21 June 2007.

For the Commission
Mariann FISCHER BOEL
Member of the Commission

[1] OJ L 161, 30.4.2004, p. 97, corrected by OJ L 206, 9.6.2004, p. 37.
[2] OJ L 248, 5.9.1991, p. 1. Regulation as last amended by Regulation (EC) No 1989/2003 (OJ L 295, 13.11.2003, p. 57).

ANNEX

The Annexes to Regulation (EEC) No 2568/91 are amended as follows:

1. the summary is amended as follows:

   (a) the title of Annex II is replaced by the following:

   'Determination of free fatty acids, cold method'

   (b) the title of Annex VII is replaced by the following:

   'Determination of the percentage of 2-glyceryl monopalmitate';

2. Annex I is replaced by the following:

'ANNEX I

OLIVE OIL CHARACTERISTICS

Category	Acidity (%) (*)	Peroxide index mEq O_2/kg (*)	Waxes mg/kg (**)	2-glyceril monopalmitate (%)	Stigmastadiene mg/kg (1)	Difference: ECN42 (HPLC) and ECN42 (theoretical calculation)	K_{232} (*)	K_{270} (*)	Delta-K (*)	Organoleptic evaluation median defect (Md) (*)	Organoleptic evaluation fruity median (Mf) (*)
1. Extra virgin olive oil	≤ 0,8	≤ 20	≤ 250	≤ 0,9 if total palmitic acid % ≤ 14 ≤ 1,0 if total palmitic acid % > 14	≤ 0,10	≤ 0,2	≤ 2,50	≤ 0,22	≤ 0,01	Md = 0	Mf > 0
2. Virgin olive oil	≤ 2,0	≤ 20	≤ 250	≤ 0,9 if total palmitic acid % ≤ 14 ≤ 1,0 if total palmitic acid % > 14	≤ 0,10	≤ 0,2	≤ 2,60	≤ 0,25	≤ 0,01	Md ≤ 2,5	Mf > 0
3. Lampante olive oil	> 2,0	—	≤ 300 (3)	≤ 0,9 if total palmitic acid % ≤ 14 ≤ 1,1 if total palmitic acid % > 14	≤ 0,50	≤ 0,3	—	—	—	Md > 2,5 (2)	—
4. Refined olive oil	≤ 0,3	≤ 5	≤ 350	≤ 0,9 if total palmitic acid % ≤ 14 ≤ 1,1 if total palmitic acid % > 14	—	≤ 0,3	—	≤ 1,10	≤ 0,16	—	—
5. Olive oil composed of refined and virgin olive oils	≤ 1,0	≤ 15	≤ 350	≤ 0,9 if total palmitic acid % ≤ 14 ≤ 1,0 if total palmitic acid % > 14	—	≤ 0,3	—	≤ 0,90	≤ 0,15	—	—
6. Crude olive-residue oil	—	—	> 350 (4)	≤ 1,4	—	≤ 0,6	—	—	—	—	—
7. Refined olive-residue oil	≤ 0,3	≤ 5	> 350	≤ 1,4	—	≤ 0,5	—	≤ 2,00	≤ 0,20	—	—
8. Olive-residue oil	≤ 1,0	≤ 15	> 350	≤ 1,2	—	≤ 0,5	—	≤ 1,70	≤ 0,18	—	—

(1) Total isomers which could (or could not) be separated by capillary column.
(2) Or where the median defect is less than or equal to 2,5 and the fruity median is equal to 0.
(3) Oils with a wax content of between 300 mg/kg and 350 mg/kg are considered to be lampante olive oil if the total aliphatic alcohol content is less than or equal to 350 mg/kg or if the erythrodiol and uvaol content is less than or equal to 3,5 %.
(4) Oils with a wax content of between 300 mg/kg and 350 mg/kg are considered to be crude olive-residue oil if the total aliphatic alcohol content is above 350 mg/kg and if the erythrodiol and uvaol content is greater than 3,5 %.

Category	Acid content (1)						Total trans-oleic isomers (%)	Total trans-linoleic + trans-linolenic isomers (%)	Sterols composition						Total sterols (mg/kg)	Erythrodiol and uvaol (%) (**)
	Myristic (%)	Linolenic (%)	Arachidic (%)	Eicosenoic (%)	Behenic (%)	Lignoceric (%)			Cholesterol (%)	Brassicasterol (%)	Campesterol (%)	Stigmasterol (%)	Betasitosterol (%) (2)	Delta-7-stigmastenol (%)		
1. Extra virgin olive oil	≤ 0,05	≤ 1,0	≤ 0,6	≤ 0,4	≤ 0,2	≤ 0,2	≤ 0,05	≤ 0,05	≤ 0,5	≤ 0,1	≤ 4,0	< Camp.	≥ 93,0	≤ 0,5	≥ 1 000	≤ 4,5
2. Virgin olive oil	≤ 0,05	≤ 1,0	≤ 0,6	≤ 0,4	≤ 0,2	≤ 0,2	≤ 0,05	≤ 0,05	≤ 0,5	≤ 0,1	≤ 4,0	< Camp.	≥ 93,0	≤ 0,5	≥ 1 000	≤ 4,5
3. Lampante olive oil	≤ 0,05	≤ 1,0	≤ 0,6	≤ 0,4	≤ 0,2	≤ 0,2	≤ 0,10	≤ 0,10	≤ 0,5	≤ 0,1	≤ 4,0	—	≥ 93,0	≤ 0,5	≥ 1 000	≤ 4,5 (3)
4. Refined olive oil	≤ 0,05	≤ 1,0	≤ 0,6	≤ 0,4	≤ 0,2	≤ 0,2	≤ 0,20	≤ 0,30	≤ 0,5	≤ 0,1	≤ 4,0	< Camp.	≥ 93,0	≤ 0,5	≥ 1 000	≤ 4,5
5. Olive oil composed of refined and virgin olive oils	≤ 0,05	≤ 1,0	≤ 0,6	≤ 0,4	≤ 0,2	≤ 0,2	≤ 0,20	≤ 0,30	≤ 0,5	≤ 0,1	≤ 4,0	< Camp.	≥ 93,0	≤ 0,5	≥ 1 000	≤ 4,5
6. Crude olive-residue oil	≤ 0,05	≤ 1,0	≤ 0,6	≤ 0,4	≤ 0,3	≤ 0,2	≤ 0,20	≤ 0,10	≤ 0,5	≤ 0,2	≤ 4,0	—	≥ 93,0	≤ 0,5	≥ 2 500	> 4,5 (4)
7. Refined olive-residue oil	≤ 0,05	≤ 1,0	≤ 0,6	≤ 0,4	≤ 0,3	≤ 0,2	≤ 0,40	≤ 0,35	≤ 0,5	≤ 0,2	≤ 4,0	< Camp.	≥ 93,0	≤ 0,5	≥ 1 800	> 4,5
8. Olive-residue oil	≤ 0,05	≤ 1,0	≤ 0,6	≤ 0,4	≤ 0,3	≤ 0,2	≤ 0,40	≤ 0,35	≤ 0,5	≤ 0,2	≤ 4,0	< Camp.	≥ 93,0	≤ 0,5	≥ 1 600	> 4,5

(1) Other fatty acids content (%): palmitic: 7,5-20,0; palmitoleic: 0,3-3,5; heptadecanoic: ≤ 0,3; heptadecenoic: ≤ 0,3; stearic: 0,5-5,0; oleic: 55,0-83,0; linoleic: 3,5-21,0.

(2) Total: Delta-5,23-stigmastadienol+chlerosterol+beta-sitosterol+sitostanol+delta-5-avenasterol+delta-5,24-stigmastadienol.

(3) Oils with a wax content of between 300 mg/kg and 350 mg/kg are considered to be lampante olive oil if the total aliphatic alcohol content is less than or equal to 350 mg/kg or if the erythrodiol and uvaol content is less than or equal to 3,5 %.

(4) Oils with a wax content of between 300 mg/kg and 350 mg/kg are considered to be crude olive-residue oil if the total aliphatic alcohol content is above 350 mg/kg and if the erythrodiol and uvaol content is greater than 3,5 %.

Notes:

(a) The results of the analyses must be expressed to the same number of decimal places as used for each characteristic.
The last digit must be increased by one unit if the following digit is greater than 4.

(b) If just a single characteristic does not match the values stated, the category of an oil can be changed or the oil declared impure for the purposes of this Regulation.

(c) If a characteristic is marked with an asterisk (*), referring to the quality of the oil, this means the following:
— for lampante olive oil, it is possible for both the relevant limits to be different from the stated values at the same time;
— for virgin olive oils, if at least one of these limits is different from the stated values, the category of the oil will be changed, although they will still be classified in one of the categories of virgin olive oil.

(d) If a characteristic is marked with two asterisks (**), referring to the quality of the oil, this means that for all types of olive-residue oil, it is possible for both the relevant limits to be different from the stated values at the same time.'

3. Appendix 1 is amended as follows:

(a) the first indent is replaced by the following:

'— Acidity	Annex II	Determination of free fatty acids, cold method'

(b) the thirteenth indent is replaced by the following:

'— Saturated fatty acids in position 2	Annex VII	Determination of the percentage of 2-glyceryl mono-palmitate';

4. the title of Annex II is replaced by the following:

'DETERMINATION OF FREE FATTY ACIDS, COLD METHOD';

5. Annex IV is replaced by the following:

'ANNEX IV

DETERMINATION OF WAX CONTENT BY CAPILLARY COLUMN GAS CHROMATOGRAPHY

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

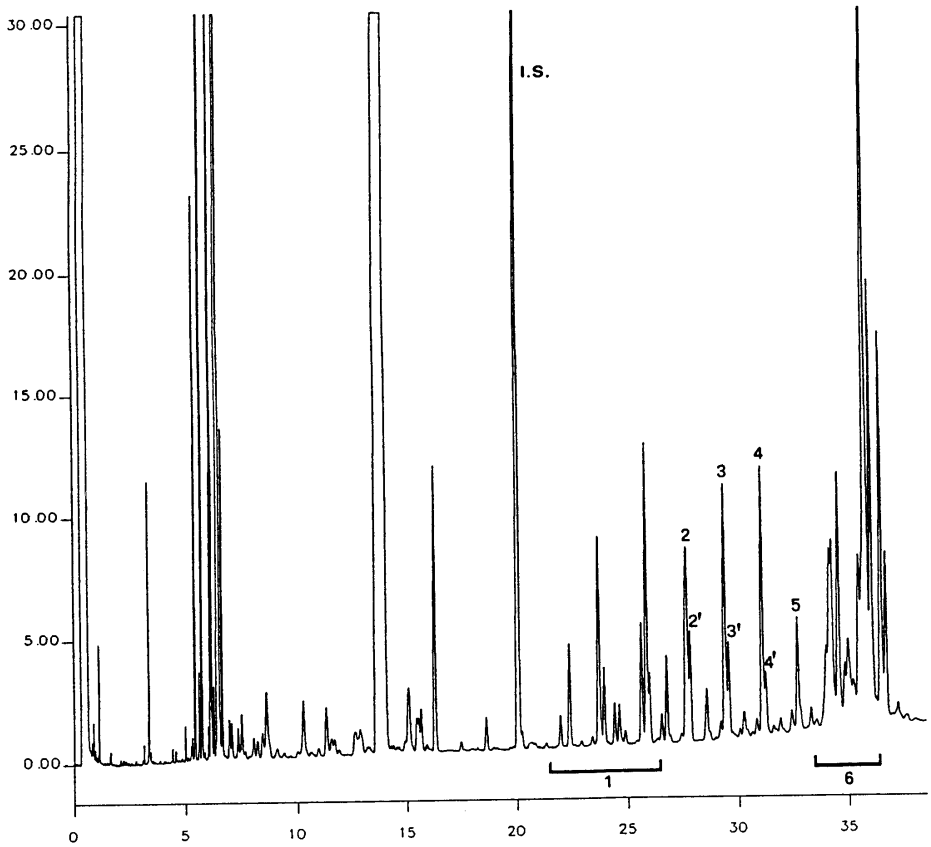
Indicate the total of the contents of the various C_{40} to C_{46} waxes in mg/kg fat (ppm).

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 (*)



Key:

I.S. = Lauryl arachidate

1. = Diterpenic esters

2 + 2′ = C_{40} esters

3 + 3′ = C_{42} esters

4 + 4′ = Esters C_{44}

5. = C_{46} esters

6. = Sterol esters and triterpenic alcohol

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

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.';

6. Annex VII is replaced by the following:

'ANNEX VII

DETERMINATION OF THE PERCENTAGE OF 2-GLYCERYL MONOPALMITATE

1. PURPOSE AND SCOPE

This method describes the analysis procedure for determining the percentage of palmitic acid in position 2 of the triglycerides by evaluating 2-glyceryl monopalmitate.

This method can be applied to liquid vegetable oils at ambient temperature (20 °C).

2. PRINCIPLE

After preparation the oil sample is subjected to the action of pancreatic lipase: partial and specific hydrolysis in positions 1 and 3 of the triglyceride molecule causes monoglycerides to appear in position 2. The percentage of 2-glyceryl monopalmitate in the monoglyceride fraction is determined after silylation by capillary-column gas chromatography.

3. APPARATUS AND MATERIALS

3.1. 25 ml Erlenmeyer flask

3.2. 100, 250 and 300 ml beakers

3.3. Glass chromatograph column, internal diameter 21-23 mm, length 400 mm, fitted with a sintered glass disc and a stopcock

3.4. 10, 50, 100 and 200 ml measuring cylinders

3.5. 100 and 250 ml flasks

3.6. Rotary evaporator

3.7. 10 ml conical-bottomed centrifuge tubes with groundglass stopper

3.8. Centrifuge for 10 and 100 ml tubes

3.9. Thermostat permitting a stable temperature of 40 ± 0,5 °C

3.10. 1 and 2 ml graduated pipettes

3.11. 1 ml hypodermic syringe

3.12. 100 µl microsyringe

3.13. 1 000 ml funnel

3.14. Capillary gas chromatograph with an on-column cold injector for direct injection of the sample into the column and a furnace able to maintain the selected temperature to approximately 1 °C

3.15. On-column cold injector for direct injection of the sample into the column

3.16. Flame ionisation detector and electrometer

3.17. Recorder-integrator adapted to the electrometer with a response rate no greater than 1 sec and a variable paper roll rate

3.18. Capillary column made of glass or fused silica 8-12 metres long, 0,25-0,32 mm internal diameter, covered with methylpolysiloxane or phenyl methylpolysiloxane 5 %, 0,10-0,30 µm thick, useable at 370 °C

3.19. 10 µl microsyringe fitted with a hardened needle, at least 7,5 cm long for direct on-column injection.

4. REAGENTS

4.1. Silica gel with a grain size of between 0,063 and 0,200 mm (70/280 mesh) prepared as follows: Place the silica gel in a porcelain capsule, dry in an incubator at 160 °C for four hours, then leave to cool at room temperature in a desiccator. Add water equivalent to 5 % of the mass of the silica gel as follows: Weigh 152 g silica gel into an Erlenmeyer flask then add 8 g of distilled water, stopper and shake gently to distribute the water evenly. Leave to stand for at least 12 hours before use.

4.2. n-hexane (for chromatography)

4.3. Isopropanol

4.4. Isopropanol, 1/1 (v/v) aqueous solution

4.5. Pancreatic lipase. It must have an activity of between 2,0 and 10 lipase units per mg. (Pancreatic lipases with an activity of between 2 and 10 units per mg enzyme are commercially available.)

4.6. Buffer solution of trishydroxymethylaminomethane: 1 M aqueous solution adjusted to pH 8 (potentiometric control) by conc. HCl (1/1 v/v)

4.7. Enzyme-quality sodium cholate, 0,1 % aqueous solution (this solution must be used within two weeks of its preparation)

4.8. Calcium chloride, 22 % aqueous solution

4.9. Diethyl ether for chromatography

4.10. Developer solvent: mixture of n-hexane/diethyl ether (87:13 v:v)

4.11. Sodium hydroxide, 12 % by weight solution

4.12. Phenolphthalein, 1 % solution in ethanol

4.13. Carrier gas: hydrogen or helium, for gas chromatography

4.14. Auxiliary gases: hydrogen, 99 % minimum purity, free from moisture and organic substances, and air, for gas chromatography, of the same purity

4.15. Silanisation reagent: mixture of pyridine/hexamethyldisilazane, trimethylchlorosilane 9/3/1 (v/v/v). (Ready-to-use solutions are commercially available. Other silylation reagents may be used, particularly bis-trimethylsilyl trifluoracetamide + 1 % trimethylchlorosilane, diluted with an identical volume of anhydrous pyridine.)

4.16. Reference samples: pure monoglycerides or monoglyceride mixtures with a known percentage composition similar to that of the sample.

5. METHOD

5.1. **Sample preparation**

5.1.1. Oils with a free acidity of less than 3 % do not need to be neutralised before chromatography on a silica gel column. Oils with a free acidity of more than 3 % must be neutralised as per point 5.1.1.1.

5.1.1.1. Pour 50 g of oil and 200 ml n-hexane into the 1 000 ml funnel (3.13). Add 100 ml of isopropanol and a quantity of 12 % sodium hydroxide solution (4.11) equivalent to the free acidity of the oil plus 5 %. Shake vigorously for one minute. Add 100 ml of distilled water, shake again and leave to stand.

After decanting, remove the lower layer containing the soaps. Remove any intermediate layers (mucilage and insoluble substances). Wash the hexane solution of the neutralised oil with successive portions of 50-60 ml of the 1/1 (v/v) isopropanol/water solution (4.4) until the pink colouration of the phenolphthalein disappears.

Remove most of the hexane by vacuum distillation (use a rotary evaporator, for example) and transfer the oil into a 100 ml flask (3.5). Dry the oil in vacuum until the solvent is completely removed.

After that procedure is completed, the acidity of the oil should be less than 0,5 %.

5.1.2. Put 1,0 g of the oil prepared as above into a 25 ml Erlenmeyer flask (3.1) and dissolve in 10 ml of developer mixture (4.10). Leave the solution to stand for at least 15 minutes before silica gel column chromatography.

If the solution is cloudy centrifuge it to ensure optimum conditions for chromatography. (Ready-to-use 500 mg silica gel SPE cartridges can be used).

5.1.3. *Preparation of the chromatography column*

Pour about 30 ml of the developer solvent (4.10) into the column (3.3), insert a piece of cotton into the bottom part of the column using a glass rod; press to eliminate the air.

In a beaker prepare a suspension of 25 g of silica gel (4.1) in about 80 ml of developer solvent and pour it into the column using a funnel.

Check that all the silica gel is in the column; wash with developer solvent (4.10), open the stopcock and allow the liquid to reach a level about 2 mm above the level of the silica gel.

5.1.4. *Column chromatography*

Weigh accurately 1,0 g of sample prepared as in point 5.1 into a 25 ml Erlenmeyer flask (3.1).

Dissolve the sample in 10 ml of developer solvent (4.10). Pour the solution into the chromatography column prepared as in point 5.1.3. Avoid disturbing the surface of the column.

Open the stopcock and pour the sample solution until it reaches the level of the silica gel. Develop with 150 ml of the developer solvent. Adjust the flow rate to 2 ml/min (so that 150 ml enters the column in about 60-70 minutes).

Recover the eluate in a previously weighed 250 ml flask. Evaporate the solvent under vacuum and remove the final traces of the solvent under a nitrogen current.

Weigh the flask and calculate the recovered extract.

(If ready-to-use silica gel SPE cartridges are used use the following method: Put 1 ml of solution (5.1.2) into the prepared cartridges with 3 ml of n-hexane.

After percolating the solution develop with 4 ml of n-hexane/diethyl ether 9/1 (v/v).

Recover the eluate in a 10 ml tube and evaporate to dry in a nitrogen current.

Expose the dry residue to pancreatic lipase (5.2). (It is essential to check the fatty acid composition before and after crossing the SPE cartridge.)

5.2. **Hydrolysis by pancreatic lipase**

5.2.1. Weigh into the centrifuge tube 0.1 g of the oil prepared as in point 5.1. Add 2 ml of buffer solution (4.6), 0,5 ml of the sodium cholate solution (4.7) and 0,2 ml of the calcium chloride solution, stirring well after each addition. Close the tube with the groundglass stopper and place in the thermostat at 40 + 0,5 °C.

5.2.2. Add 20 mg of lipase, shake carefully (avoid wetting the stopper) and place the tube in the thermostat for exactly two minutes. Then remove it, shake vigorously for exactly 1 minute and leave to cool.

5.2.3. Add 1 ml of diethyl ether, stopper and shake vigorously, then centrifuge and transfer the ether solution into a clean, dry tube using a microsyringe.

5.3. **Preparation of the silanised derivatives and gas chromatography**

5.3.1. With a microsyringe insert 100 µl of solution (5.2.3) into a 10 ml conical-bottomed tube.

5.3.2. Remove the solvent under a slight nitrogen current, add 200 µl of silanisation reagent (4.15), stopper the tube and leave to stand for 20 minutes.

5.3.3. After 20 minutes, add 1 to 5 ml of n-hexane (depending on the chromatography conditions): the resulting solution is ready for gas chromatography.

5.4. **Gas chromatography**

Operating conditions:

— Injector temperature (on-column injector) lower than solvent boiling point (68 °C);

— Detector temperature: 350 °C;

— Column temperature: programming of furnace temperature: 60 °C for 1 minute, increasing by 15 °C per minute up to 180 °C, then by 5 °C per minute up to 340 °C, then 340 °C for 13 minutes;

— Carrier gas: hydrogen or helium, set at a linear velocity sufficient to obtain the resolution reflected in Figure 1. The retention time of the C_{54} triglyceride must be 40 ± 5 minutes (see Figure 2). (The operating conditions indicated above are indicative. Operators will have to optimise them to obtain the desired resolution. The peak corresponding to 2-glyceryl monopalmitate must have a minimum height equal to 10 % of the recorder scale.)

— Quantity of substance injected: 0,5-1 µl of the n-hexane solution (5 ml) (5.3.3).

5.4.1. *Identification of the peaks*

The individual monoglycerides are identified from their retention times and by comparison with those obtained for standard monoglyceride mixtures under the same conditions.

5.4.2. *Quantitative evaluation*

The area of each peak is calculated using an electronic integrator.

6. EXPRESSION OF RESULTS

The percentage of glyceryl monopalmitate is calculated from the ratio between the area of the corresponding peak and the areas of the peaks of all the monoglycerides (see Figure 2) using the formula:

glyceryl monopalmitate (%): $\frac{A_x}{\Sigma A} \times 100$

where:

A_x = area of the peak corresponding to glyceryl monopalmitate

ΣA = sum of the areas of all the monoglyceride peaks

The result must be to one decimal place.

7. ANALYSIS REPORT

The analysis report must specify:

— reference to this method,

— all the information needed for a full identification of the sample,

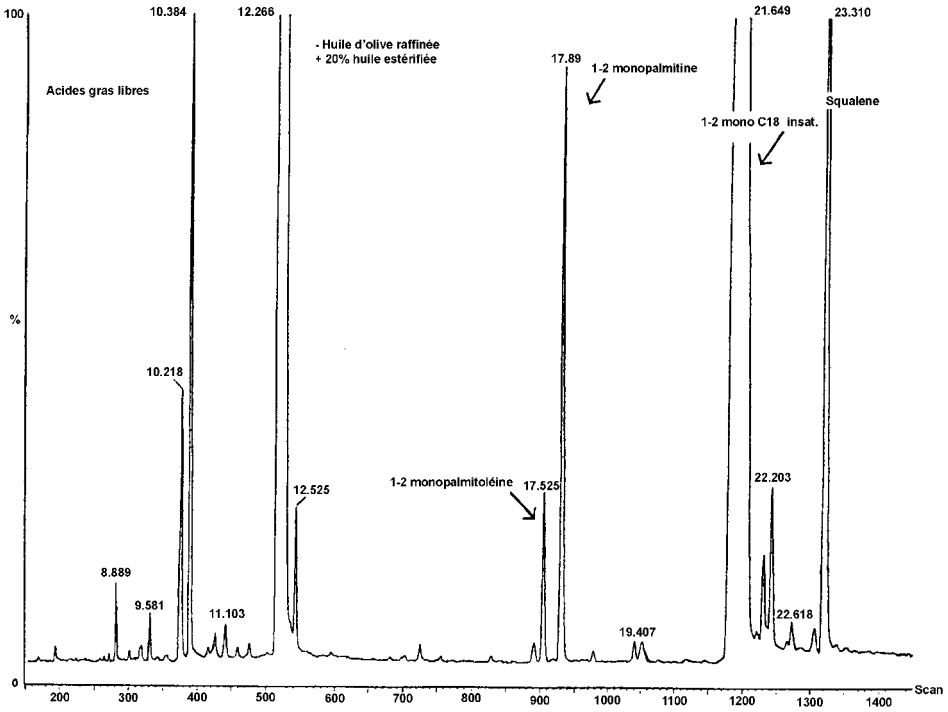
— the analysis result,

— any deviation from the method, whether as the result of a decision by the parties concerned or for another reason,

— details to identify the laboratory, the date of the analysis and the signatures of those responsible for the analysis.

Figure 1

Chromatogram of the products of the silanisation reaction obtained by the action of lipase on a refined olive oil with 20 % esterified oil added (100 %)



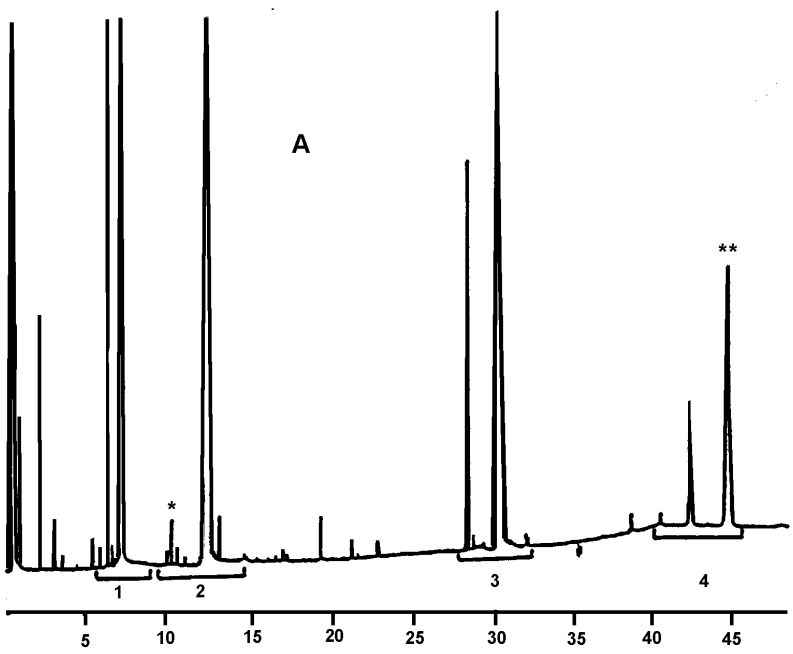
Key: "acides gras libres" = free fatty acids; "Huile d'olive raffinée + 20 % huile estérifiée" = refined olive oil + 20 % esterified oil; "1-2 monopalmitoléine" = 1-2 monopalmitolein; "1-2 mono C_{18} insat." = unsaturated 1-2 mono C_{18}

Figure 2

Chromatogram of:

(A) unesterified olive oil, after lipase; after silanisation; under these conditions (8-12 m capillary column) the wax fraction is eluted at the same time as the diglyceride fraction or slightly afterwards.

After lipase, the triglyceride content should not exceed 15 %



Key:

1 = Free fatty acids

2 = Monoglycerides

3 = Diglycerides

4 = Triglycerides

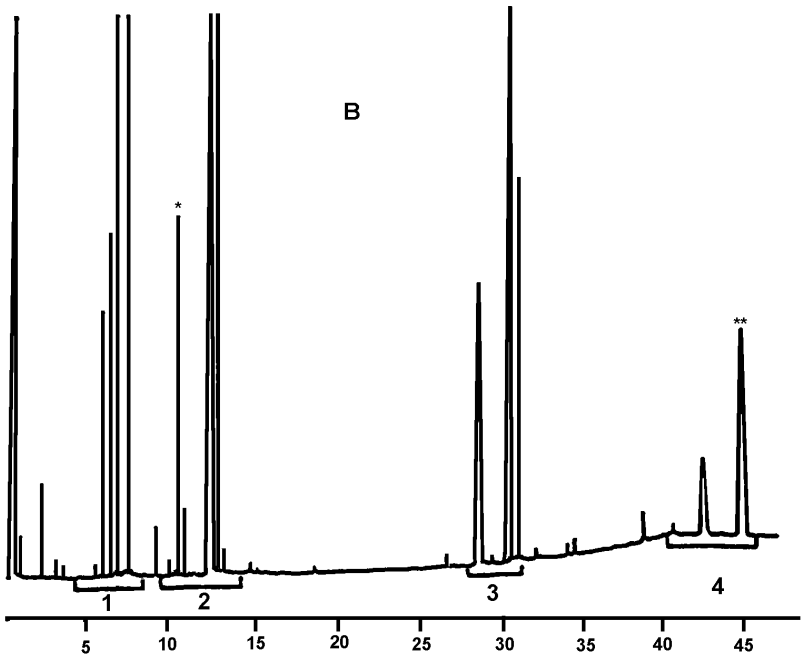
* = 2-monopalmitine

** = Triglyceride C_{54}

Chromatogram of:

(B) unesterified oil after lipase; after silanisation; under these conditions (8-12 m capillary column) the wax fraction is eluted at the same time as the diglyceride fraction or slightly afterwards.

After lipase, the triglyceride content should not exceed 15 %.



Key:

1 = Free fatty acids

2 = Monoglycerides

3 = Diglycerides

4 = Triglycerides

* = 2-monopalmitine

** = C_{54} triglyceride

8. NOTES

Note 1. PREPARATION OF THE LIPASE

Lipases with satisfactory activity are commercially available. They can also be prepared in the laboratory in the following manner:

Cool to 0 °C 5 kg of fresh pig's pancreas. Remove the surrounding solid fat and the connective tissue and grind to a liquid paste in a blender. Stir the paste with 2,5 litres of anhydrous acetone for 4-6 hours, then centrifuge. Extract the residue three more times with the same volume of anhydrous acetone, then twice with an acetone/diethyl ether mixture (1/1 v/v) and twice with diethyl ether.

Vacuum-dry the residue for 48 hours to obtain a stable powder which can be stored for a long time in a refrigerator away from moisture.

Note 2. MONITORING LIPASE ACTIVITY

Prepare an olive oil emulsion as follows:

In a mixer stir for 10 minutes a mixture of 165 ml of a 100 g/l gum arabic solution, 15 g of crushed ice and 20 ml of a previously neutralised olive oil.

Pour 10 ml of the emulsion into a 50 ml beaker, then 0,3 ml of a 0,2 g/ml sodium cholate solution and then 20 ml of distilled water.

Put the beaker in a thermostat set at 37 °C; introduce the electrodes of the pH meter and the screw agitator.

Using a burette, add a 0,1 N sodium hydroxide solution drop by drop until a pH of 8,3 is obtained.

Add an aliquot of the lipase powder suspension in water (0,1 g/ml of lipase). As soon as the pH meter reads 8,3, start the chronometer and add the sodium hydroxide solution drop by drop at a rate which maintains the pH at 8,3. Note every minute the volume of solution consumed.

Record the data on an x/*y* graph with the time on the x-axis and millilitres of 0,1 N alkaline solution consumed to keep a constant pH on the *y*-axis. A linear graph should be obtained.

Lipase activity, expressed in lipase units per mg, is given by the following formula:

$$A = \frac{V \times N \times 100}{m}$$

where:

A is activity in lipase units/mg

V is the number of millilitres of 0,1 N sodium hydroxide solution per minute (calculated on the basis of the graph)

N is the titre of the sodium hydroxide solution

m is the mass in mg of the test lipase.

A lipase unit is defined as the quantity of enzyme which releases 10 micro-equivalents of acid per minute.';

7. in Annex XA, point 6.2 is replaced by the following:

'6.2. The methyl esters are prepared using procedure B set out in Annex XB. Fatty substances having a free acidity over 3 % must first be neutralised in accordance with point 5.1.1 of Annex VII.'