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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

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[^{F1}ANNEX XIX U.K.]

Textual Amendments

- F1** Substituted by [Commission Implementing Regulation \(EU\) 2019/1604 of 27 September 2019 amending Regulation \(EEC\) No 2568/91 on the characteristics of olive oil and olive-residue oil and on the relevant methods of analysis.](#)

DETERMINATION OF THE STEROL COMPOSITION AND CONTENT AND ALCOHOLIC COMPOUNDS BY CAPILLARY GAS CHROMATOGRAPHY

1. SCOPE U.K.

The method describes a procedure for determining the individual and total alcoholic compound content of olive oils and olive pomace oils as well as of blends of these two oils.

The alcoholic compounds in olive and olive pomace oils comprise aliphatic alcohols, sterols and triterpenic dialcohols.

2. PRINCIPLE U.K.

The oils, with added α -cholestanol and 1-eicosanol as internal standards, are saponified with potassium hydroxide in ethanolic solution and the unsaponifiable matter is then extracted with ethyl ether.

The different alcoholic compounds fractions are separated from the unsaponifiable matter either by thin-layer chromatography on a basic silica gel plate (reference method) or by HPLC with a silica gel column. The fraction recovered from the silica gel separation is transformed into trimethylsilyl ethers and then analysed by capillary column gas chromatography.

PART 1 U.K.

PREPARATION OF THE UNSAPONIFIABLE MATTER

1. SCOPE U.K.

This Part describes the preparation and extraction of the unsaponifiable matter. It includes the preparation and extraction of the unsaponifiable matter from olive and olive-pomace oils.

2. PRINCIPLE U.K.

A test portion is saponified by boiling under reflux with an ethanolic potassium hydroxide solution. The unsaponifiable matter is extracted with diethyl ether.

3. APPARATUS U.K.

The usual laboratory equipment and in particular the following:

- 3.1. Round bottomed flask fitted with a reflux condenser with ground-glass joints, 250 mL.
- 3.2. Separating funnel, 500 mL.
- 3.3. Flasks, 250 mL.
- 3.4. Microsyringes, 100 μ L and 500 μ L.

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- 3.5. Cylindrical filter funnel with a G3 porous septum (porosity 15-40 μm) of diameter approximately 2 cm and a depth of 5 cm, suitable for filtration under vacuum with male ground-glass joint.
- 3.6. Conical flask with ground-glass female joint, 50 mL, which can be fitted to the filter funnel (3.5).
- 3.7. Test tube with a tapering bottom and a sealing glass stopper, 10 mL.
- 3.8. Calcium dichloride desiccator.
4. REAGENTS **U.K.**
- 4.1. Potassium hydroxide minimum titre 85 %.
- 4.2. Potassium hydroxide ethanolic solution, approximately 2 M.
Dissolve 130 g of potassium hydroxide (4.1) with cooling in 200 ml of distilled water and then make up to one litre with ethanol (4.7). Keep the solution in well-stoppered dark glass bottles and stored maximum 2 days.
- 4.3. Ethyl ether, for analysis quality.
- 4.4. Anhydrous sodium sulphate, for analysis quality.
- 4.5. Acetone, for chromatography quality.
- 4.6. Ethyl ether, for chromatography quality.
- 4.7. Ethanol of analytical quality.
- 4.8. Ethyl acetate of analytical quality.
- 4.9. Internal standard, α -cholestanol, purity more than 99 % (purity must be checked by GC analysis).
- 4.10. Internal standard solution of α -cholestanol, 0,2 solution (m/V) in ethyl acetate (4.8).
- 4.11. Phenolphthalein solution, 10 g/L in ethanol (4.7).
- 4.12. A 0,1 % (m/v) solution of 1-eicosanol in ethyl acetate (internal standard).

5. PROCEDURE **U.K.**

Using a 500 μL micro-syringe (3.4) introduce into the 250 mL flask (3.1) a volume of the α -cholestanol internal standard solution (4.10) and a volume of 1-eicosanol (4.12) containing an amount of cholestanol and eicosanol corresponding to approximately 10 % of the sterol and alcohol content of the sample. For example, for 5 g of olive oil sample add 500 μL of the α -cholestanol solution (4.10) and 250 μL of 1-eicosanol solution (4.12). For pomace olive oils add 1500 μL of both α -cholestanol solution (4.10) and 1-eicosanol (4.12). Evaporate until dryness with a gentle current of nitrogen in a warm water bath. After cooling the flask, weigh $5,00 \pm 0,01$ g of the dry filtered sample into the same flask.

Note 1: Animal or vegetable oils and fats containing appreciable quantities of cholesterol may show a peak having a retention time identical to cholestanol. If this case occurs, the sterol fraction will have to be analysed in duplicate with and without internal standard.

Add 50 mL of 2M ethanolic potassium hydroxide solution (4.2) and some pumice, fit the reflux condenser and heat to gentle boiling until saponification takes place (the solution becomes

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clear). Continue heating for a further 20 minutes, then add 50 mL of distilled water from the top of the condenser, detach the condenser and cool the flask to approximately 30 °C.

Transfer the contents of the flask quantitatively into a 500 mL separating funnel (3.2) using several portions of distilled water (50 mL). Add approximately 80 ml of ethyl ether (4.6), shake vigorously for approximately 60 seconds, periodically releasing the pressure by inverting the separating funnel and opening the stopcock. Allow standing until there is complete separation of the two phases (Note 2). Then draw off the soap solution as completely as possible into a second separating funnel. Perform two further extractions on the water-alcohol phase in the same way using 60 to 70 mL of ethyl ether (4.6).

Note 2: Any emulsion can be destroyed by adding small quantities of ethanol (4.7).

Combine the three ether extracts in one separating funnel containing 50 mL of water. Continue to wash with water (50 mL) until the wash water no longer gives a pink colour on the addition of a drop of phenolphthalein solution (4.11). When the wash water has been removed, filter on anhydrous sodium sulphate (4.4) into a previously weighed 250 mL flask, washing the funnel and filter with small quantities of ethyl ether (4.6).

Evaporate the solvent by distillation in a rotary evaporator at 30 °C under vacuum. Add 5 mL of acetone (4.5) and remove the volatile solvent completely in a gentle current of nitrogen. Dry the residue in the oven at 103 ± 2 °C for 15 min. Cool in desiccators and weigh to the nearest 0,1 mg.

PART 2 U.K.

SEPARATION OF THE ALCOHOLIC COMPOUNDS FRACTIONS

1. SCOPE U.K.

The unsaponifiable matter prepared in Part 1 is fractionated in the different alcoholic compounds, aliphatic alcohols, sterols and triterpenic dialcohols (erythrodiol and uvaol).

2. PRINCIPLE U.K.

The unsaponifiable matter can be fractionated using basic thin layer chromatography (reference method), revealed and the corresponding bands scratched and extracted. As an alternative method of separation, HPLC using a silica gel column and UV detector and the different fractions collected. The aliphatic and triterpenic alcohols as well as the sterol and triterpenic dialcohols are isolated together.

3. APPARATUS U.K.

The usual laboratory equipment and in particular the following:

- 3.1. Complete apparatus for analysis by thin-layer chromatography using 20×20 cm glass plates.
- 3.2. Ultraviolet lamp with a wavelength of 366 or 254 nm.
- 3.3. Microsyringes, 100 μ L and 500 μ L.
- 3.4. Cylindrical filter funnel with a G3 porous septum (porosity 15-40 μ m) of diameter approximately 2 cm and a depth of 5 cm, suitable for filtration under vacuum with male ground-glass joint.
- 3.5. Conical flask with ground-glass female joint, 50 mL which can be fitted to the filter funnel (3.4).

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- 3.6. Test tube with a tapering bottom and a sealing glass stopper, 10 mL.
- 3.7. Calcium dichloride desiccator.
- 3.8. HPLC system, consisting of:
 - 3.8.1. Binary pump.
 - 3.8.2. Manual or automatic injector equipped with 200 µL injection loop.
 - 3.8.3. In-line degasser.
 - 3.8.4. UV-VIS or IR detector.
- 3.9. HPLC column (25 cm × 4 mm i.d.) with silica gel 60 (5 µm particle size).
- 3.10. Syringe filter, 0,45 µm.
- 3.11. Conical flask 25 mL.
4. REAGENTS **U.K.**
 - 4.1. Potassium hydroxide minimum titre 85 %.
 - 4.2. Potassium hydroxide ethanolic solution, approximately 2 M.
Dissolve 130 g of potassium hydroxide (4.1) with cooling in 200 ml of distilled water and then make up to one litre with ethanol (4.9). Keep the solution in well-stoppered dark glass bottles and stored maximum 2 days.
 - 4.3. Ethyl ether, for analysis quality.
 - 4.4. Potassium hydroxide ethanolic solution, approximately 0,2 M.
Dissolve 13 g of potassium hydroxide (4.1) in 20 ml of distilled water and make up to one litre with ethanol (4.9).
 - 4.5. Glass 20x20 plates coated with silica gel, without fluorescence indicator, thickness 0,25 mm (commercially available ready for use).
 - 4.6. Acetone, for chromatography quality.
 - 4.7. n-Hexane, for chromatography quality.
 - 4.8. Ethyl ether, for chromatography quality.
 - 4.9. Ethanol of analytical quality.
 - 4.10. Ethyl acetate of analytical quality.
 - 4.11. Reference solution for thin-layer chromatography: cholesterol, phytosterols, alcohols and Erythrodiol 5 % solution in Ethyl acetate (4.10).
 - 4.12. Solution of 2,7-dichlorofluorescein, 0,2 % in ethanolic solution. Make slightly basic by adding a few drops of 2 M alcoholic potassium hydroxide solution (4.2).
 - 4.13. n-Hexane (4.7)/ethyl ether (4.8) mixture 65:35 (V/V).
 - 4.14. HPLC mobile phase n-hexane (4.7)/ethyl ether (4.8) (1:1) (V/V).
5. REFERENCE METHOD: SEPARATION OF THE ALCOHOLIC COMPOUNDS BY BASIC THIN-LAYER CHROMATOGRAPHY (TLC) PLATE **U.K.**

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Preparation of the basic thin layer chromatography plates. Immerse or dip the silica gel plates (4.5) about 4 cm in the 0,2 M ethanolic potassium hydroxide solution (4.4) for 10 seconds, then allow to dry in a fume cupboard for two hours and finally place in an oven at 100 °C for one hour.

Remove from the oven and keep in a calcium chloride desiccator (3.7) until required for use (plates treated in this way must be used within 15 days).

Place hexane/ethyl ether mixture (4.13) (Note 3) into the development chamber, to a depth of approximately 1 cm. Close the chamber with the appropriate cover and leave thus for at least half an hour, in a cool place, so that liquid-vapour equilibrium is established. Strips of filter paper dipping into the eluent may be placed on the internal surfaces of the chamber. This reduces developing time by approximately one-third and brings about more uniform and regular elution of the components.

Note 3: The developing mixture should be replaced for every test, in order to achieve perfectly reproducible elution conditions. Alternative solvent 50:50 (V/V) n-hexane/ethyl ether may be used.

Prepare an approximately 5 % solution of the unsaponifiable prepared in Part 1 in ethyl acetate (4.10) and, using the 100 µL microsyringe (3.3), depose 0,3 ml of the solution on a narrow and uniform streak on the lower end (2 cm) of the chromatographic plate (4.5). In line with the streak, place 2 to 3 µL of the material reference solution (4.11), so that the sterol, triterpene dialcohols and alcohols bands can be identified after developing.

Place the plate in the developing chamber (3.1). The ambient temperature should be maintained between 15 and 20 °C (Note 4). Immediately close the chamber with the cover and allow eluting until the solvent front reaches approximately 1 cm from the upper edge of the plate. Remove the plate from the developing chamber and evaporate the solvent in a flow of hot air or by leaving the plate for a short while, under a hood.

Note 4: Higher temperature could worsen the separation.

Spray the plate lightly and uniformly with the 2,7-dichlorofluorescein solution (4.12) and then leave to dry. When the plate is observed under ultraviolet lamp (3.2), the sterols, triterpene dialcohols and alcohols bands can be identified through being aligned with the spots obtained from the reference solution (4.11). Mark the limits of the bands along the edges of the fluorescence with a black pencil (see TLC plate in Figure 1).

By using a metal spatula, scrape off the silica gel of the marked area. Place the finely comminuted material removed into the filter funnel (3.4). Add 10 mL of hot ethyl acetate (4.10), mix carefully with the metal spatula and filter (under vacuum if necessary), collecting the filtrate in the conical flask (3.5.) attached to the filter funnel.

Wash the residue in the flask three times with ethyl ether (4.3) (approximately 10 mL each time), collecting the filtrate in the same flask attached to the funnel, evaporate the filtrate to a volume of 4 to 5 mL, transfer the residual solution to the previously weighed 10 mL test tube (3.6), evaporate to dryness by mild heating, in a gentle flow of nitrogen, make up again using a few drops of acetone (4.6), evaporate again to dryness. The residue contained in the test tube consists of the sterol and triterpene dialcohols or the alcohols and triterpenic alcohols fractions.

6. SEPARATION OF THE ALCOHOLIC FRACTION BY HPLC U.K.

The unsaponifiable obtained from Part 1 is dissolved in 3 mL of the mobile phase (4.14), filter the solution with a syringe filter (3.10) and reserve.

Inject 200 µL of the filtered unsaponifiable solution in the HPLC (3.8).

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Run the HPLC separation at 0,8 mL/min, discard the first 5 min. and collect in 25 mL conical flasks (3.11) between the 5 and 10 min. for aliphatic and triterpenic alcohols and between 11 and 25 min for sterols and erythrodiol and uvaol (Note 5).

The separation can be monitored with an UV detector at 210 nm wavelengths or a refractive index detector (see Figure 6).

The fractions are evaporated until dryness and prepared for chromatographic analysis.

Note 5: Carefully control the pressure of the HPLC pump, the ethyl ether can increase the pressure, adjust the flow to keep the pressure under control.

PART 3 U.K.

GAS CHROMATOGRAPHIC ANALYSIS OF THE ALCOHOLIC COMPOUNDS FRACTIONS

1. SCOPE U.K.

This part gives general guidance for the application of capillary column gas chromatography to determine the qualitative and quantitative composition of the alcoholic compounds isolated in accordance with the method specified in Part 2 of this method.

2. PRINCIPLE U.K.

The fractions collected from the unsaponifiable matter using TLC or HPLC are derivatized into trimethylsilyl ethers and analysed by capillary column gas chromatography with split injection and flame ionization detector.

3. APPARATUS U.K.

The usual laboratory equipment and in particular the following:

- 3.1. Test tube with a tapering bottom and a sealing glass stopper, 10 mL.
- 3.2. Gas chromatograph suitable for use with a capillary column with split injection system, consisting of:
 - 3.2.1. A thermostatic chamber for columns capable of maintaining the desired temperature with an accuracy of ± 1 °C;
 - 3.2.2. A temperature-adjustable injection unit with a persilanised glass vaporising element and split system;
 - 3.2.3. A flame ionisation detector (FID);
 - 3.2.4. Data acquisition system suitable for use with the FID detector (3.10.3.), capable of manual integration.
- 3.3. Fused-silica capillary column of length 20 to 30 m, internal diameter 0,25 to 0,32 mm, coated with 5 % Diphenyl - 95 % Dimethylpolysiloxane (SE-52 or SE-54 stationary phase or equivalent), to a uniform thickness between 0,10 and 0,30 μm .
- 3.4. Microsyringe, of 10 μL capacity, for gas chromatography, with cemented needle suitable for split injection.

4. REAGENTS U.K.

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- 4.1. Anhydrous pyridine, for chromatography quality.
- 4.2. Hexamethyl disilazane of analytical quality.
- 4.3. Trimethylchlorosilane of analytical quality.
- 4.4. Sample solutions of sterol trimethylsilyl ethers. To be prepared at the time of use from sterols and erythrodiol obtained from oils containing them.
- 4.5. Standard solutions of trimethylsilyl ethers of aliphatic alcohols from C20 to C28. They may be prepared from mixtures of pure alcohols at the time they are required for use.
- 4.6. Carrier gas: hydrogen or helium, gas-chromatographic purity.
- 4.7. Auxiliary gases: hydrogen, helium, nitrogen and air, of gas-chromatographic purity.
- 4.8. Silylation reagent, consisting of a 9:3:1 (V/V/V) mixture of pyridine/hexamethyl disilazane/trimethylchlorosilane.
- 4.9. n-Hexane, for chromatography quality.
5. PREPARATION OF THE TRIMETHYLSILYL ETHERS **U.K.**

Add the silylation reagent (4.8) (Note 6), in the ratio of 50 µl for every milligram of alcoholic compound, in the test tube (3.1) containing the alcoholic compound fraction, avoiding any uptake of moisture (Note 7).

Note 6: Ready for use solutions are available commercially. Other silylation reagents, such as, for example, bistrimethylsilyl trifluor acetamide + 1 % trimethylchlorosilane, which has to be diluted with an equal volume of anhydrous pyridine, are also available. Pyridine can be replaced by the same amount of acetonitrile.

Note 7: The slight opalescence, which may form, is normal and does not cause any anomaly. The formation of a white flock or the appearance of a pink colour is indicative of the presence of moisture or deterioration of the reagent. If these occur the test must be repeated (only if hexamethyldisilazane/trimethylchlorosilane is used).

Stopper the test tube (3.1), shake carefully (without overturning) until the compounds are completely dissolved. Leave to stand for at least 15 minutes at ambient temperature and then centrifuge for a few minutes. The clear solution is ready for gas chromatographic analysis.

6. GAS CHROMATOGRAPHIC ANALYSIS **U.K.**

6.1. Preliminary operations, capillary column conditioning **U.K.**

Fit the column (3.3) in the gas chromatograph, by attaching the inlet end to the split injector and the outlet end to the detector.

Carry out general checks on the gas chromatograph unit (leaks from the gas circuits, detector efficiency, efficiency of the splitting system and recording system, etc.).

If the column is being used for the first time, it is recommended that it should be subjected to conditioning: passing a gentle flow of gas through the column itself, then switch on the gas chromatography unit and begin a gradual heating, up to a temperature of at least 20 °C above the operating temperature (Note 8). Hold this temperature for at least two hours, then place the entire unit in operating mode (adjustment of gas flows and splitting, ignition of the flame, connection with the computing system, adjustment of the column, detector and injector temperature, etc.) and then record the signal with a sensitivity at least two times greater than that one intended for the analysis. The course of the base line must be linear, without peaks of any kind, and must

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not show drift. A negative straight-line drift indicates leakage from the column connections; a positive drift indicates inadequate conditioning of the column.

Note 8: The conditioning temperature must always be at least 20 °C less than the maximum temperature specified for the stationary phase used.

6.2. Operating conditions U.K.

Optimize the temperature programme and the carrier gas flow so that chromatograms similar to Figures 3 to 6 are obtained.

The following parameters were tested and found useful:

6.2.1. Aliphatic alcohols U.K.

Oven Program	180 °C (8 min.) → 260 °C (at 5 °C/min) → 260 °C (15 min)
Injector Temperature	280 °C
Detector Temperature	290 °C
Linear Velocity of Carrier gas	Helium (20 to 30 cm/s); Hydrogen (30 to 50 cm/s)
Split Ratio	1:50 to 1:100
Volume Injected	0,5 to 1 µL of TMSE solution

6.2.2. Sterol and triterpenic dialcohols U.K.

Oven Program	260 ± 5 °C Isothermal
Injector Temperature	280 – 300 °C
Detector Temperature	280 – 300 °C
Linear Velocity of Carrier gas	Helium (20 to 30 cm/s); Hydrogen (30 to 50 cm/s)
Split Ratio	1:50 to 1:100
Volume Injected	0,5 to 1 µL of TMSE solution

These conditions may be changed according to the characteristics of the column and gas chromatograph, so as to obtain chromatograms, which meet the following requirements:

- Alcohol C26 retention time shall be 18 ± 5 minutes.
- Alcohol C22 peak shall be 80 ± 20 % of the full-scale value for olive oil and 40 ± 20 % of the full-scale value for olive-pomace oil.
- The retention time for the β-sitosterol peak should be at 20 ± 5 min.
- The campesterol peak should be: for olive oil (mean content 3 %) 20 ± 5 % of full scale.
- All the present sterols must be separated. In addition to being separated, the peaks must also be completely resolved, i.e. the peak trace should return to the base line before leaving for the next peak. Incomplete resolution is, however, tolerated, provided that the peak at RRT 1,02 (Sitostanol) can be quantified using the perpendicular.

6.3. Analytical procedure U.K.

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By using the 10 µl microsyringe (3.4), take 1 µl of hexane, draw in 0,5 µl of air and then 0,5 to 1 µl of the sample solution. Raise the plunger of the syringe further, so the needle is emptied. Push the needle through the membrane of the injector and after one to two seconds, inject rapidly, and then slowly remove the needle after around five seconds. An automatic injector can be used as well.

Carry out the recording until the TMSE of the corresponding alcoholic compounds present are completely eluted. The base line must continue to meet the requirements of the corresponding operating conditions (6.2.1 or 6.2.2).

6.4. Peak identification **U.K.**

Identify individual peaks on the basis of retention times and by comparison with the mixture of the aliphatic and triterpenic alcohols or the sterol and triterpene dialcohols TMSE, analysed under the same conditions. A chromatogram of the aliphatic and triterpenic alcohols fraction is showed in Figure 3 and the corresponding chromatograms for sterols and triterpenic dialcohols are showed in Figure 2.

The aliphatic alcohols are eluted in the following order: C20-ol (I.S.), C22-ol, C23-ol, C24-ol, C25-ol, C26-ol, C27-ol and C28-ol.

The sterols and triterpene dialcohols are eluted in the following order: cholesterol, brassicasterol, ergosterol, 24-methylen-cholesterol, campesterol, campestanol, stigmasterol, Δ7-campesterol, Δ5,23-stigmastadienol, clerosterol, β-sistosterol, sitostanol, Δ5-avenasterol, Δ5,24-stigmastadienol, Δ7-stigmastenol, Δ7-avenasterol, erythrodiol and uvaol.

6.5. Quantitative evaluation **U.K.**

The peak areas of 1-eicosanol and of the aliphatic alcohols C22, C24, C26, C28 are calculated by a data acquisition system. The response factor for 1-eicosanol should be considered equal to 1.

Calculate the areas of the α-cholestanol and the sterol and triterpene dialcohols peaks by using the computing system. Ignore peaks for any compound, which are not included (ergosterol must not be calculated) among those listed in Table 1. The response factor for α-cholestanol should be considered equal to 1.

Calculate the concentration of each individual alcoholic compound, in mg/kg of fatty material, as follows:

$$\text{Alcoholic compound } x = \frac{A_x \times m_s}{A_s \times m} \times 1000$$

where:

- A_x = Peak area for alcoholic compound x, in computing system counts.
- A_s = Area of the 1-eicosanol/α-cholestanol peak, in computing system counts.
- m_s = Mass of added 1-eicosanol/α-cholestanol, in milligrams.
- m = Mass of the sample used for determination, in grams.

7. EXPRESSION OF THE RESULTS **U.K.**

Report individual aliphatic and triterpenic alcohols concentrations as mg/kg of fatty material and their sum as 'total aliphatic alcohol content'. The total content is the sum of C22, C24, C26 and C28.

The composition of each of the individual alcoholic compounds should be expressed to one decimal point.

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Total sterol concentration should be expressed without any decimal point.

Calculate the percentage of each individual sterol from the ratio of the relevant peak area to the total peak area for sterols:

$$\text{Sterol } z = \frac{A_z}{\Sigma A} \times 100$$

where:

A_x = Peak area for sterol x.
 ΣA = Total peak area for sterols.

Apparent β -sitosterol: $\Delta 5,23$ -stigmastadienol + clerosterol + β -sitosterol + sitostanol + $\Delta 5$ -avenasterol + $\Delta 5,24$ -stigmastadienol.

Calculate the percentage of erythrodiol and uvaol:

$$\text{Erythrodiol} + \text{Uvaol} = \frac{A_{Er} + A_{Uv}}{\Sigma A_T} \times 100$$

where:

A_{Er} = Area of Erythrodiol in computing system counts.
 A_{Uv} = Area of Uvaol in computing system counts.
 ΣA_T = Sum area for sterol + erythrodiol + uvaol in computing system counts.

Besides the calculation of relative percentage of single sterols and triterpenic dialcohols and the total concentration of sterols, the concentration of erythrodiol and of uvaol and their sum, in mg/kg of fatty material must be calculated, according the following expressions:

$$\text{Erythrodiol} = \frac{A_{Er} \times m_s}{A_s \times m} \times 1000$$

$$\text{Uvaol} = \frac{A_{Uv} \times m_s}{A_s \times m} \times 1000$$

where:

A_{Er} = Peak area of Erythrodiol, in computing system counts.
 A_{Uv} = Area of Uvaol in computing system counts.
 A_s = Area of the α -cholestanol peak, in computing system counts.
 m_s = Mass of added α -cholestanol, in milligrams.
 m = Mass of the sample used for determination, in grams.

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Appendix U.K.

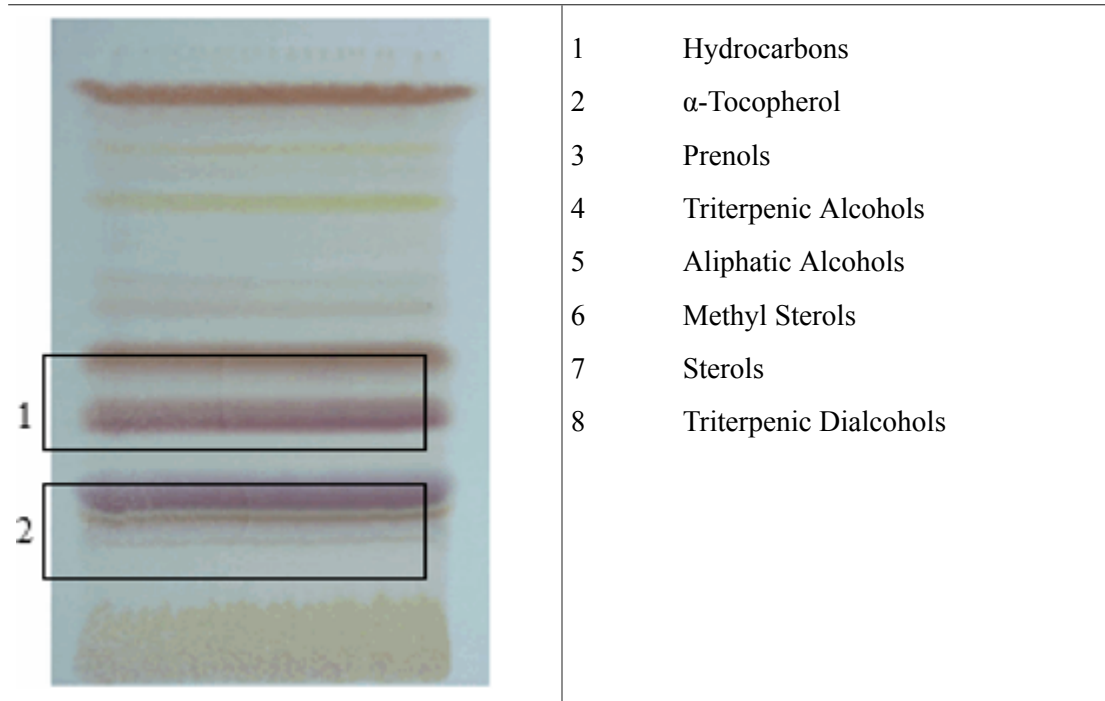


Figure 1 — TLC of the unsaponifiable fraction from olive pomace oil eluted twice with hexane:diethyl ether (65:35), developed with SO₄H₂ (50 %) and heated. The bands that should be scrapped are the ones contained within the rectangle, 1 are the bands for aliphatic alcohols and 2 for the sterols and triterpenic dialcohols.

TABLE I — RELATIVE RETENTION TIMES FOR STEROLS

Peak	Identification		Relative retention time	
			SE 54 column	SE 52 column
1	Cholesterol	Δ -5-cholesten-3 β -ol	0,67	0,63
2	Cholestanol	5 α -cholestan-3 β -ol	0,68	0,64
3	Brassicasterol	[24S]-24-methyl- Δ -5,22-cholestadien-3 β -ol	0,73	0,71
*	Ergosterol	[24S]-24-methyl- Δ -5,7,22-cholestatrien-3 β -ol	0,78	0,76
4	24-methylene-cholesterol	24-methylene- Δ -5,24-cholestadien-3 β -ol	0,82	0,80

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5	Campesterol	(24R)-24-methyl- Δ -5-cholesten-3 β -ol	0,83	0,81
6	Campestanol	(24R)-24-methyl-cholestan-3 β -ol	0,85	0,82
7	Stigmasterol	(24S)-24-ethyl- Δ -5,22-cholestadien-3 β -ol	0,88	0,87
8	Δ -7-campesterol	(24R)-24-methyl- Δ -7-cholesten-3 β -ol	0,93	0,92
9	Δ -5,23-stigmastadienol	(24R,S)-24-ethyl- Δ -5,23-cholestadien-3 β -ol	0,95	0,95
10	Clerosterol	(24S)-24-ethyl- Δ -5,25-cholestadien-3 β -ol	0,96	0,96
11	β -sitosterol	(24R)-24-ethyl- Δ -5-cholesten-3 β -ol	1,00	1,00
12	Sitostanol	24-ethyl-cholestan-3 β -ol	1,02	1,02
13	Δ -5-avenasterol	(24Z)-24-ethylidene- Δ -cholesten-3 β -ol	1,03	1,03
14	Δ -5,24-stigmastadienol	(24R,S)-24-ethyl- Δ -5,24-cholestadien-3 β -ol	1,08	1,08
15	Δ -7-stigmastenol	(24R,S)-24-ethyl- Δ -7-cholesten-3 β -ol	1,12	1,12
16	Δ -7-avenasterol	(24Z)-24-ethylidene- Δ -7-cholesten-3 β -ol	1,16	1,16
17	Erythrodiol	5 α -olean-12-en-3 β ,28-diol	1,41	1,41
18	Uvaol	Δ 12-ursen-3 β ,28-diol	1,52	1,52

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. (See end of Document for details) View outstanding changes

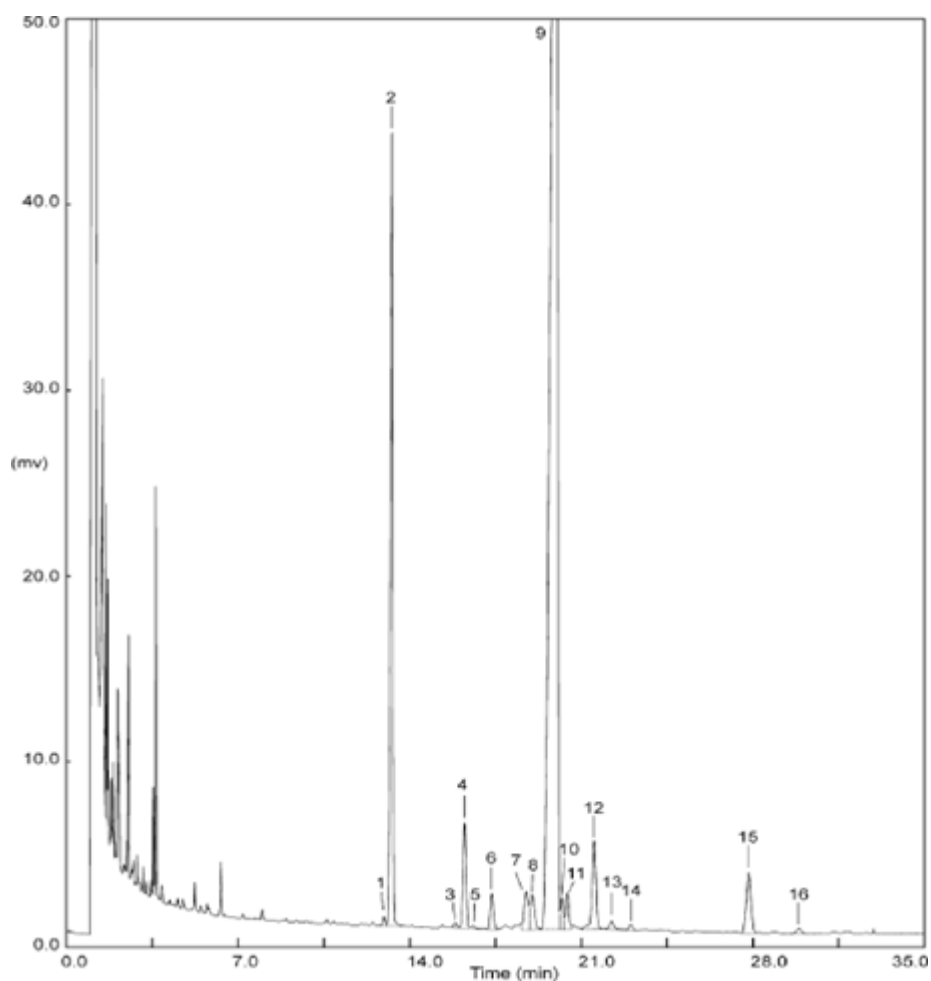


Figure 2 — GC-FID chromatographic profile of the sterol and triterpenic dialcohols from refined olive oil. (1) Cholesterol, (2) α -cholestanol (I.S.), (3) 24-methylencholesterol, (4) campesterol, (5) campestanol, (6) stigmasterol, (7) Δ 5,23-stigmastadienol, (8) clerosterol, (9) β -sitosterol, (10) sitostanol, (11) Δ 5-avenasterol, (12) Δ 5,24-stigmastadienol, (13) Δ 7-stigmastenol, (14) Δ 7-avenasterol, (15) erythrodiol, (16) uvaol.

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. (See end of Document for details) View outstanding changes

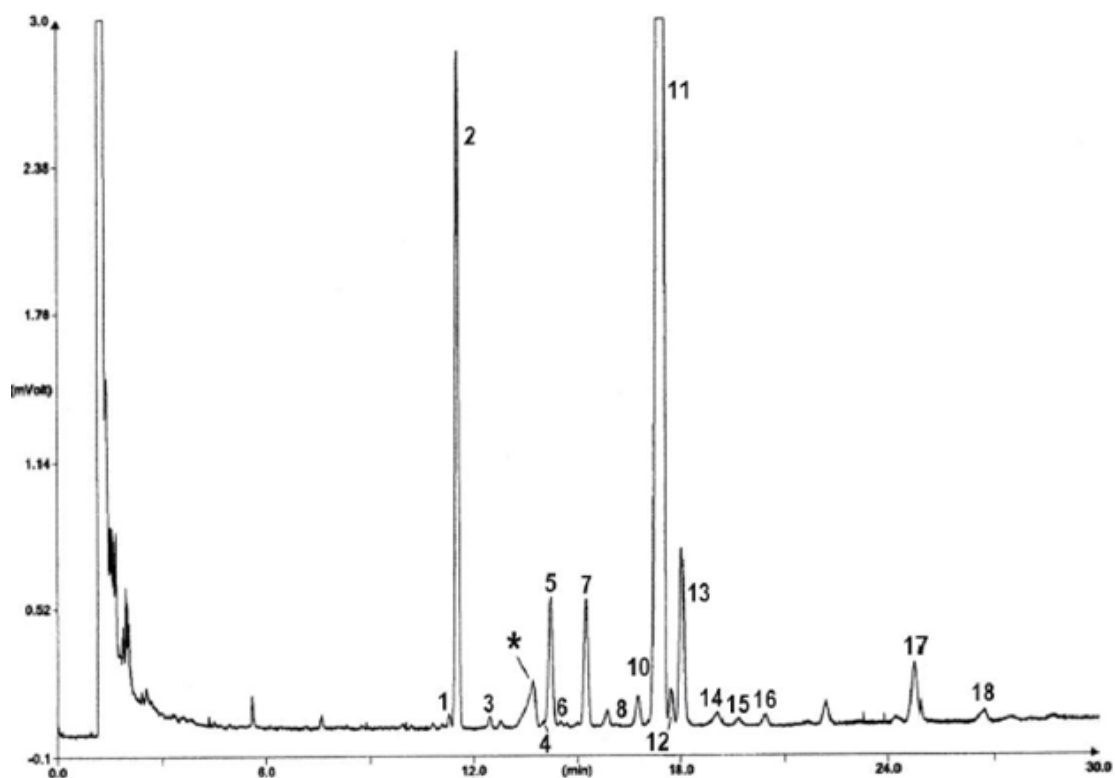
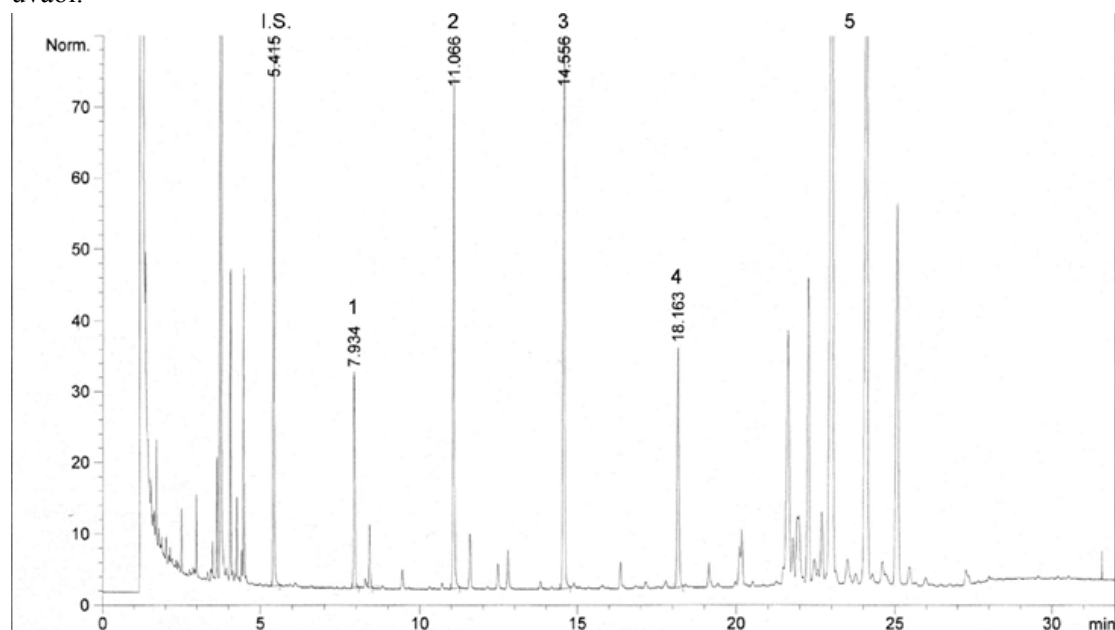


Figure 3 — GC-FID chromatographic profile of the sterol and triterpenic dialcohols from a lampante olive oil. (1) Cholesterol, (2) α -cholestanol, (3) brassicasterol, (4) 24-methylencholesterol, (5) campesterol, (6) campestanol, (7) stigmasterol, (8) Δ^7 -campesterol, (9) $\Delta^{5,23}$ -stigmasteradienol, (10) clerosterol, (11) β -sitosterol, (12) sitostanol, (13) Δ^5 -avenasterol, (14) $\Delta^{5,24}$ -stigmasteradienol, (15) Δ^7 -stigmasterol, (16) Δ^7 -avenasterol, (17) erythrodiol, (18) uvaol.



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Figure 4 — GC-FID chromatographic profile of aliphatic alcohols and triterpenic alcohols of olive oil. (I.S.) C20-ol, (1) C22-ol, (2) C24-ol, (3) C26-ol, (4) C28-ol, (5) triterpenic alcohols.

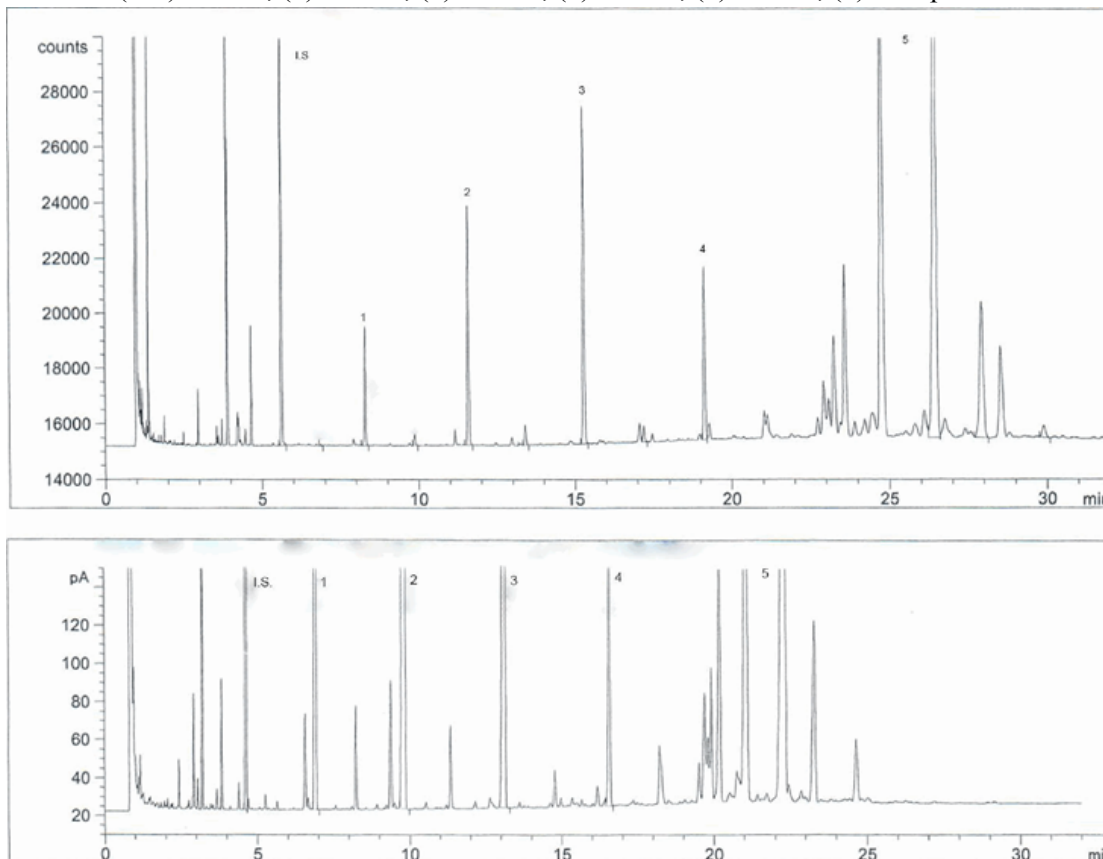


Figure 5 — GC-FID chromatographic profile of aliphatic alcohols and triterpenic alcohols of a refined olive oil and a second centrifugation olive oil. (I.S.) C20-ol, (1) C22-ol, (2) C24-ol, (3) C26-ol, (4) C28-ol, (5) triterpenic alcohols.

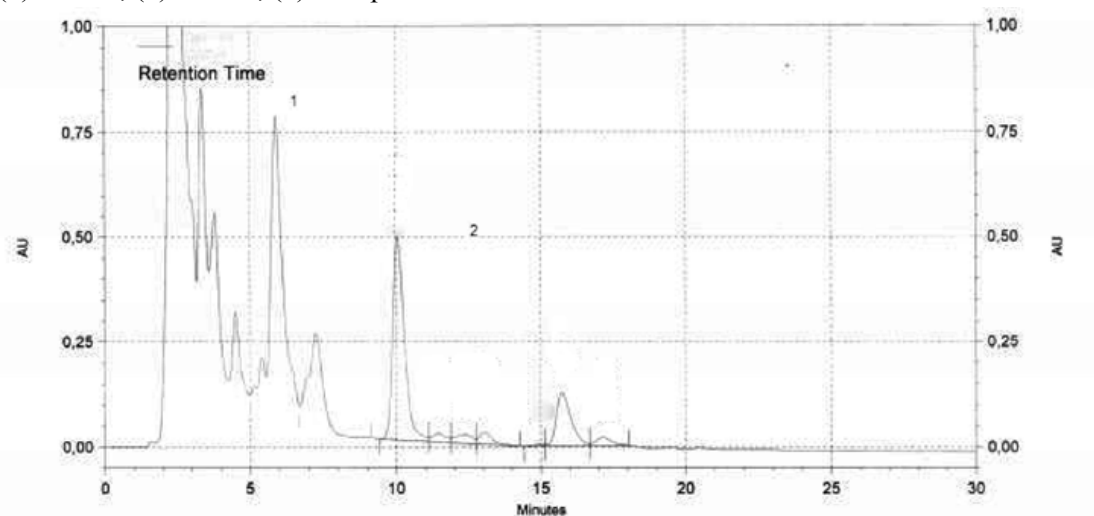


Figure 6 — HPLC Chromatogram of an olive oil unsaponifiable separated by HPLC using a UV detector. (1) Aliphatic and triterpenic alcohols; (2) Sterols and triterpenic dialcohols]

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.

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Changes and effects yet to be applied to the whole legislation item and associated provisions

- Signature words omitted by [S.I. 2019/1422 reg. 6\(10\)](#)
- Art. 1(8) inserted by [S.I. 2019/1422 reg. 6\(2\)\(b\)](#)
- Art. 1(8)(a)(ii)(bb) omitted in earlier amending provision S.I. 2019/1422, reg. 6(2)(b) by [S.I. 2020/1453 reg. 14\(16\)\(a\)\(i\)](#)
- Art. 1(8)(b) words substituted in earlier amending provision S.I. 2019/1422, reg. 6(2)(b) by [S.I. 2020/1453 reg. 14\(16\)\(a\)\(ii\)](#)
- Art. 1(8)(c)(ii) omitted in earlier amending provision S.I. 2019/1422, reg. 6(2)(b) by [S.I. 2020/1453 reg. 14\(16\)\(a\)\(iii\)](#)
- Annex 1a para. 1.1 words substituted by [S.I. 2019/1422 reg. 6\(11\)\(a\)](#)
- Annex 1a para. 1.2 words substituted by [S.I. 2019/1422 reg. 6\(11\)\(b\)](#)
- Art. 2a(3)(e) words substituted by [S.I. 2019/1422 reg. 6\(4\)\(c\)](#) (This amendment not applied to legislation.gov.uk. Reg. 6(4)(c) substituted immediately before IP completion day by S.I. 2020/1453, regs. 1(2)(b), 14(16)(c)(ii))
- Art. 2a(3)(e) words substituted by S.I. 2019/1422, reg. 6(4)(c) (as substituted) by [S.I. 2020/1453 reg. 14\(16\)\(c\)\(ii\)](#)
- Art. 3 words substituted by [S.I. 2019/1422 reg. 6\(5\)\(a\)](#)
- Art. 3 words substituted by [S.I. 2019/1422 reg. 6\(5\)\(b\)](#)