



METHOD OF ANALYSIS

DETERMINATION OF ALIPHATIC AND TRITERPENIC ALCOHOLS CONTENT BY CAPILLARY GAS CHROMATOGRAPHY

1. PURPOSE

The procedure describes a method for the determination of aliphatic and triterpenic alcohols content in oils and fats.

2. PRINCIPLE OF THE METHOD

The fatty substance, with 1-eicosanol added as internal standard, is saponified with ethanolic potassium hydroxide and then the unsaponifiable matter extracted with ethyl ether. The alcoholic fraction is separated from the unsaponifiable matter by chromatography on a basic silica gel plate; the alcohols recovered from the silica gel are transformed into trimethylsilyl ethers and analysed by capillary gas chromatography.

3. EQUIPMENT

- 3.1. 250 ml round-bottomed flask fitted with a reflux condenser having ground-glass joints.
- 3.2. 50.0 ml separating funnel.
- 3.3. 250 ml round-bottomed flasks.
- 3.4. Chromatographic tank for thin-layer chromatographic analysis, for glass plates of dimensions 20 x 20 cm.
- 3.5. Ultraviolet lamp having a wavelength of 366 or 254 nm.
- 3.6. 100 µl and 500 µl microsyringes.
- 3.7. A cylindrical filter funnel with a G3 porous septum (porosity 15 to 40 µm) of diameter approximately 2 cm and a depth of some 5 cm, with an attachment suitable for filtration under vacuum and a 12/21 male ground glass joint.

- 3.8. 50 ml vacuum conical flask with a 12/21 ground-glass female joint which can be fitted to the filter funnel (3.7).
- 3.9. A 10 ml test tube with a tapering bottom and a sealing stopper.
- 3.10. Gas chromatograph for use with capillary columns, provided with:
 - 3.10.1. Thermostatic chamber for columns (column oven) to hold the temperature desired with a precision of $\pm 1^{\circ}\text{C}$.
 - 3.10.2. A temperature-adjustable split injection unit with a persilanised glass vaporising element, or an on-column unit.
 - 3.10.3. A flame ionisation detector and converter-amplifier.
 - 3.10.4. Recorder-integrator for operation with the converter-amplifier (3.10.3), with response time not exceeding one second and with variable paper-speed.
- 3.11. Glass or fused silica capillary column, of length 20 to 30 m, internal diameter 0.25 to 0.32 mm, with SE-52 or SE-54 liquid phase or equivalent, with a film thickness between 0.10 and 0.30 μm .
- 3.12. Microsyringe for gas chromatography, of 10 μl capacity with hardened needle.
- 3.13. Analytical balance sensitive to 1 mg (with 0.1 mg display).

4. REAGENTS

- 4.1. Potassium hydroxide, approximately 2 N ethanolic solution: 130 g potassium hydroxide (minimum concentration 85%) is dissolved, with cooling, in 200 ml distilled water and then made up to one litre with ethanol. The solution should be stored in a well-stoppered opaque glass bottle.
- 4.2. Ethyl ether, pure for analysis.
- 4.3. Anhydrous sodium sulphate, analytical purity.
- 4.4. Glass plates coated with silica gel, without fluorescence indicator, thickness 0.25 mm (commercially available ready for use).
- 4.5. Potassium hydroxide, approximately 0.2 N ethanolic solution; 13 g of potassium hydroxide are dissolved in 20 ml of distilled water and made up to one litre with ethanol.
- 4.6. Benzene, for chromatography. (See 5.2.2)
- 4.7. Acetone, for chromatography. (See 5.2.2)

- 4.8. Hexane, for chromatography. (See 5.2.2)
- 4.9. Ethyl ether, for chromatography. (See 5.2.2)
- 4.10. Chloroform, for chromatography.
- 4.11. Reference solution for thin-layer chromatography: C₂₀-C₂₈ alcohols 0.5% in chloroform, or a fraction of alcohols obtained as indicated in 5.2. from the unsaponifiable matter of an olive-pomace oil.
- 4.12. 0.2% solution of 2', 7'-dichlorofluorescein in ethanol. Make slightly basic by adding a few drops of 2 N alcoholic potassium hydroxide solution.
- 4.13. Anhydrous pyridine, for chromatography.
- 4.14. Hexamethyl disilazane.
- 4.15. Trimethylchlorosilane.
- 4.16. 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.
- 4.17. A 0.1% (m/v) solution of 1-eicosanol in chloroform (internal standard).
- 4.18. Carrier gas: hydrogen or helium, gas-chromatographic purity.
- 4.19. Auxiliary gas: nitrogen, gas-chromatographic purity.

5. PROCEDURE

5.1. Preparation of the unsaponifiables.

- 5.1.1. Using a 500 µl microsyringe place, into a 250 ml round-bottom flask, a volume of 0.1% 1-eicosanol solution in chloroform (4.17) containing a quantity of 1-eicosanol approximately equal to 10% of the aliphatic alcohols content in that portion of sample to be taken for analysis. For example, to 5 g of sample add 250 µl of the 0.1% 1-eicosanol solution if olive oil and 1 500 µl if olive pomace oil.

Evaporate to dryness in current of nitrogen and then weigh accurately 5 g of the dry filtered sample into the same flask.

- 5.1.2. Add 50 ml of 2 N potassium hydroxide ethanolic solution, fit the reflux condenser and heat the apparatus to slight boiling on a steam bath, stirring continuously throughout the heating process until saponification has taken place (the solution becomes clear). Continue heating for a further 20 minutes and then add 50 ml of distilled water through the condenser. The condenser is then disconnected and the flask cooled to approximately 30°C.

- 5.1.3. The contents of the flask are quantitatively transferred to a separating funnel of 500 ml capacity by adding distilled water several times, using a total of around 50 ml distilled water. Add approximately 80 ml of ethyl ether, shake vigorously for approximately 30 seconds and allow to settle (Note 1).

Separate off the lower aqueous phase collecting it in a second separating funnel. Two further extractions are effected on the aqueous phase, in the same manner, using each time 60 to 70 ml ethyl ether.

Note 1: Emulsions may be eliminated by adding, using as a spray, small quantities of ethyl alcohol or methyl alcohol.

- 5.1.4. The ethyl ether extracts are combined in a separating funnel and washed with distilled water (50 ml at a time) until the washing water gives a neutral reaction.

Discard the washing water, dry with anhydrous sodium sulphate and filter, into a flask of 250 ml capacity which has been weighed beforehand, the funnel and filter being washed with small quantities of ethyl ether which are added to the total.

- 5.1.5. Distil the ether down to a few ml, then bring to dryness under a slight vacuum or in a current of nitrogen, completing drying in an oven at 100°C for approximately a quarter of an hour, and then weigh after cooling in a desiccator.

5.2. Separation of alcoholic fractions.

- 5.2.1. Preparation of basic TLC plates: the silica gel plates (4.4) are immersed completely, in 0.2 N potassium hydroxide solution (4.5) for 10 seconds, and then left to dry for two hours under an extractor hood and finally placed in an oven at 100°C for one hour (Note 2).

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

Note 2: When basic silica gel plates are used to separate the alcoholic fraction there is no need to treat the unsaponifiables with alumina. It follows that all acid compounds (fatty acids and others) are retained at the origin thereby obtaining both aliphatic alcohol and triterpenic alcohol bands which are both separated distinctly from the sterol band.

- 5.2.2. Place a 65/35 by volume hexane/ethyl ether mixture in the plate-developing chamber to a depth of approximately 1 cm(*).

(*) In these cases in particular, a 95/5 by volume benzene/acetone eluent mixture must be used to obtain distinct band separation.

Close the chamber using an appropriate cover and leave for half an hour to allow equilibration between vapour and liquid. Strips of filter paper dipping into the eluent may be affixed to the inside surfaces of the tank to reduce the development time by approximately one-third and obtain more uniform, regular elution of the components (Note 3).

Note 3: The developing solution must be replaced for each analysis in order to obtain reproducible developing conditions.

5.2.3. An approximately 5% solution of unsaponifiable matter (5.1.5) in chloroform is prepared and 0.3 ml of the solution is streaked as a uniform strip of minimum thickness, using the 100 µl microsyringe, on a TLC plate at approximately 2 cm from the bottom of the TLC plate. Aligned with the origin, 2 to 3 µl of the aliphatic alcohols reference solution (4.11) are spotted for the identification of the aliphatic alcohols band after development has been completed.

5.2.4. Place the plate inside the development tank as stated in 5.2.2. The ambient temperature should be maintained between 15 and 20°C. Immediately close the chamber with the cover and allow to elute until the solvent front reaches approximately 1 cm from the upper edge of the plate.

The plate is then removed from the development chamber and the solvent evaporated under a hot air current or the plate is left for a while under the extractor hood.

5.2.5. The plate is sprayed lightly and evenly with the solution of 2', 7'-dichlorofluorescein when the plate is observed under ultra violet light. The aliphatic alcohols band can be identified through being aligned with the stain obtained from the reference solution: mark the limits of the band with a black pencil; outlining the band of aliphatic alcohols and the band immediately above that, which is the triterpenic alcohols band, together (Note 4).

Note 4: The aliphatic alcohols band and the triterpenic alcohols band are to be grouped together in view of the possible migration of some aliphatic alcohols into the triterpenic alcohols band. An example of the TLC separation is given in Annex 1

5.2.6. Using a metal spatula scrape off the silica gel in the marked area. Place the finely comminuted material removed into the filter funnel (3.7). Add 10 ml of hot chloroform, mix carefully with the metal spatula and filter under vacuum, collecting the filtrate in the conical flask (3.8) attached to the filter funnel.

Wash the silica gel in the flask three times with ethyl ether (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 (3.9), evaporate to dryness by mild heating in a gentle flow of nitrogen, make up again using a few drops of acetone, evaporate again to dryness, place in an oven at 105°C for approximately 10 minutes and then allow to cool in a desiccator and weigh.

The residue inside the test tube is composed of the alcoholic fraction.

5.3. Preparation of the trimethylsilyl ethers.

5.3.1. The reagent for silylation, consisting of a mixture of 9:3:1 by volume (Note 5) of pyridine-hexamethyldisilazane-trimethylchlorosilane in the proportion of 50 µl for each milligram of aliphatic alcohols, is added to the test tube containing the alcoholic fraction, avoiding all absorption of moisture (Note 6).

Note 5: Solutions which are ready for use are available commercially. Other silanising reagents such as, for example, bis-trimethylsilyl, trifluor acetamide + 1% trimethyl chlorosilane, which has to be diluted with an equal volume of anhydrous pyridine, are also available.

Note 6: The slight opalescence which may form is normal and does not cause any interference. The formation of a white floc or the appearance of a pink colour are indicative of the presence of moisture or deterioration of the reagent. If these occur the test must be repeated.

5.3.2. Stopper the test tube, shake carefully (without overturning) until the aliphatic alcohols are completely dissolved. 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.

5.4. Gas chromatography analysis.

5.4.1. Preliminary operations, column packing.

5.4.1.1. Fit the column in the gas chromatograph, attaching the inlet end to the injector connected to the splitting system and the outlet end to the detector. Carry out a general check of the gas chromatography assembly (tightness of gas fittings, efficiency of the detector, efficiency of the splitting system and of the recording system, etc.).

5.4.1.2. If the column is being used for the first time it is recommended that it should be subjected to conditioning. A little carrier gas is passed through the capillary column and then the gas chromatography assembly is switched on and gradually heated until a temperature not less than 20°C above the operating temperature (see Note 7) is attained. That temperature is held for not less than two hours and then the assembly is brought to the operating conditions (regulation of gas flow, split flame ignition, connection to the electronic recorder, adjustment of the temperature of the capillary column oven, the detector and the injector, etc.) and the signal is adjusted to a sensitivity not less than twice the highest level contemplated for the execution of the analysis. The course of the base line must be linear, without peaks of any kind, and must not drift. A negative straight-line drift indicates leakage from the column connections; a positive drift indicates inadequate conditioning of the column.

Note 7: The conditioning temperature shall be at least 20°C less than the maximum temperature contemplated for the liquid phase employed.

5.4.2. Choice of operating conditions.

5.4.2.1. The guideline operating conditions for a chromatographic system with a split injection unit are as follows:

- column temperature: the initial isotherm is set at 180°C for eight minutes and then programmed at 5°C/minute to 260°C and a further 15 minutes at 260°C,
- temperature of injector: 280°C,
- temperature of detector: 290°C,
- linear velocity of carrier gas: helium 20 to 35 cm/s, hydrogen 30 to 50 cm/s,
- splitting ratio: 1:50 to 1:100,

- sensitivity of instrument: 4 to 16 times the minimum attenuation,
- sensitivity of recording: 1 to 2 mV fs,
- paper speed: 30 to 60 cm/h,
- quantity of substance injected: 0.5 to 1 µl of TMSE solution.

The above conditions may be modified according to the characteristics of the column and of the gas chromatograph to obtain chromatograms satisfying the following conditions:

- alcohol C₂₆ retention time shall be 18 ± 5 minutes,
- the alcohol C₂₂ peak shall be $80 \pm 20\%$ of the full scale value for olive oil and $40 \pm 20\%$ of the full scale value for olive-pomace oil.

5.4.2.2. The above requirements are checked by repeated injection of the standard TMSE mixture of alcohols and the operating conditions are adjusted to yield the best possible results.

5.4.2.3. The parameters for the integration of peaks shall be set so that a correct appraisal of the areas of the peaks considered is obtained.

5.4.3. Analytical procedure.

5.4.3.1. Using the microsyringe of 10 µl capacity draw in 1 µl of hexane followed by 0.5 µl of air and subsequently 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 injection unit and after one to two seconds inject rapidly, then slowly remove the needle after some five seconds.

5.4.3.2. Recording is effected until the TMSE of the aliphatic alcohols present have been eluted completely. The base line shall always correspond to the requirements of 5.4.1.2.

5.4.4. Peak identification.

The identification of individual peaks is effected according to the retention times and by comparison with the standard TMSE mixture, analysed under the same conditions.

Examples of chromatograms of the alcoholic fraction of a refined olive oil are shown in Annex 1.

5.4.5. Quantitative evaluation.

5.4.5.1. The peak areas of 1-eicosanol and of the aliphatic alcohols C₂₂, C₂₄, C₂₆ and C₂₈ are calculated by electronic integration.

5.4.5.2. The contents of each aliphatic alcohol, expressed in mg/1000 g fatty substance, are calculated as follows:

$$\text{alcohol x} = \frac{A_x \cdot m_s \cdot 1000}{A_s \cdot m}$$

where:

A_x = area of the alcohol peak x;

A_s = area of 1-eicosanol;

m_s = mass of 1-eicosanol in milligrams;

m = mass of sample drawn for determination, in grams.

6. EXPRESSION OF THE RESULTS

The contents of the individual aliphatic alcohols in mg/1000 g of fatty substance and the sum of the 'total aliphatic alcohols' are reported.

ANNEX 1

TLC SEPARATION EXAMPLE AND CHROMATOGRAM EXAMPLES

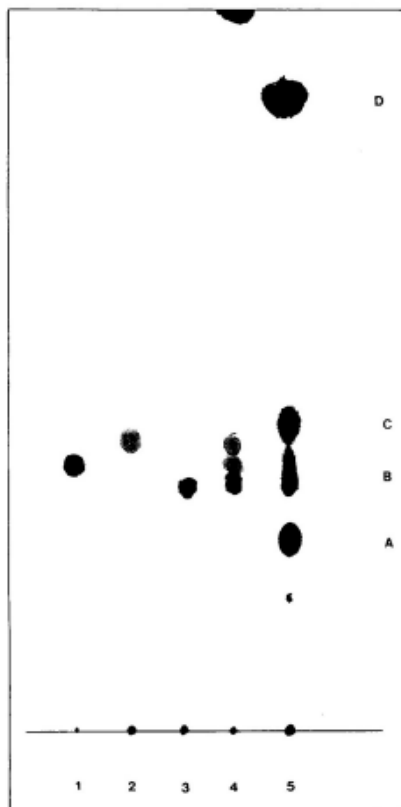


Figure 1- *Thin-layer chromatography plate of the unsaponifiable fraction from olive oil eluted with hexane/ethyl ether (65/35)*

1	Alcohol C26	A	Sterols
2	Alcohol C30	B	Aliphatic alcohols
3	Alcohol C20	C	Triterpenic alcohols
4	Mix Alcohols C20-22-26-30	D	Squalene
5	Extra virgin unsaponifiable		

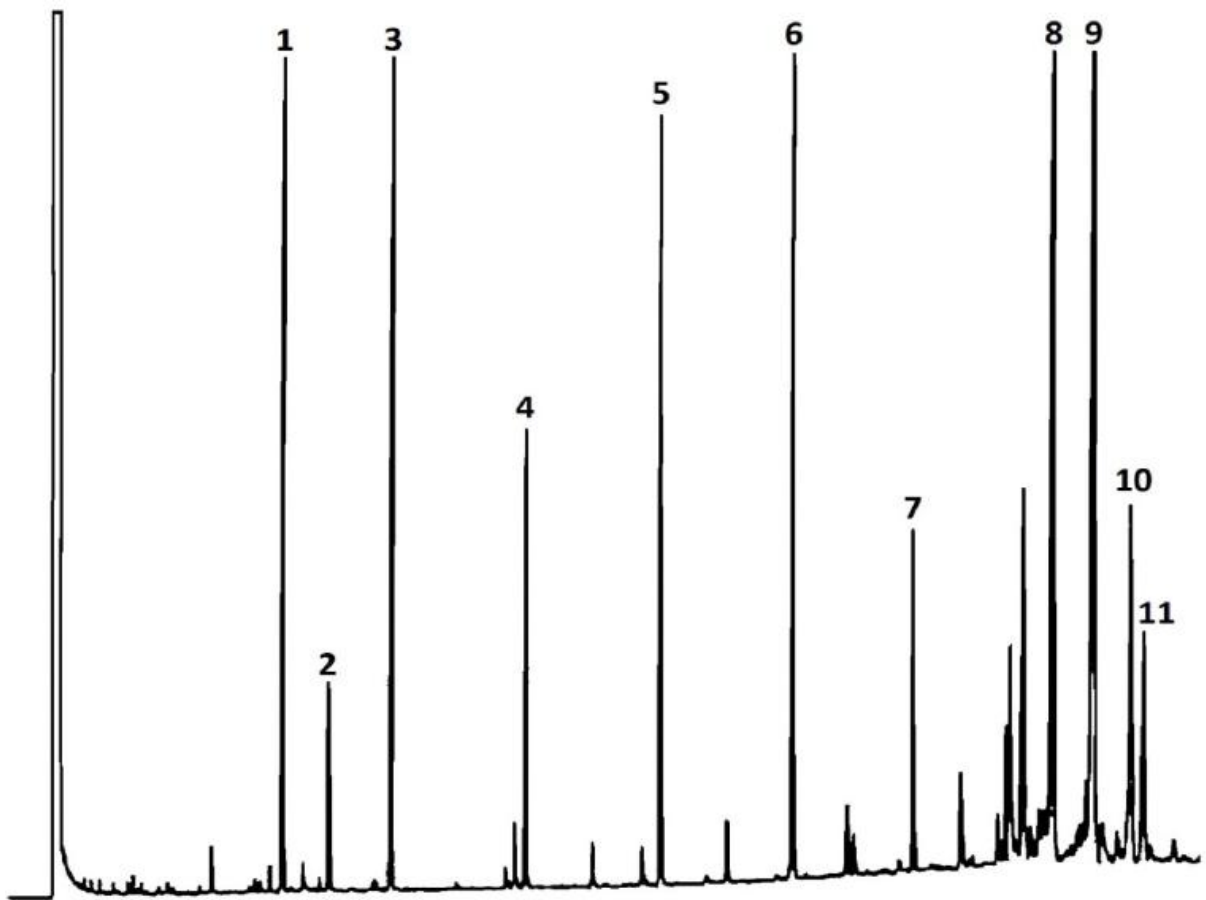


Figure 2- *Chromatogram of the alcoholic fraction of a refined olive oil*

1 = Phytol
2 = Geranyl geraniol
3 = Alcohol C20 (IS)
4 = Alcohol C22

5 = Alcohol C24
6 = Alcohol C26
7 = Alcohol C28
8 = Cycloartenol

9 = 24-Methylen-cycloartenol
10 = Citrostadienol
11 = Cyclobranol

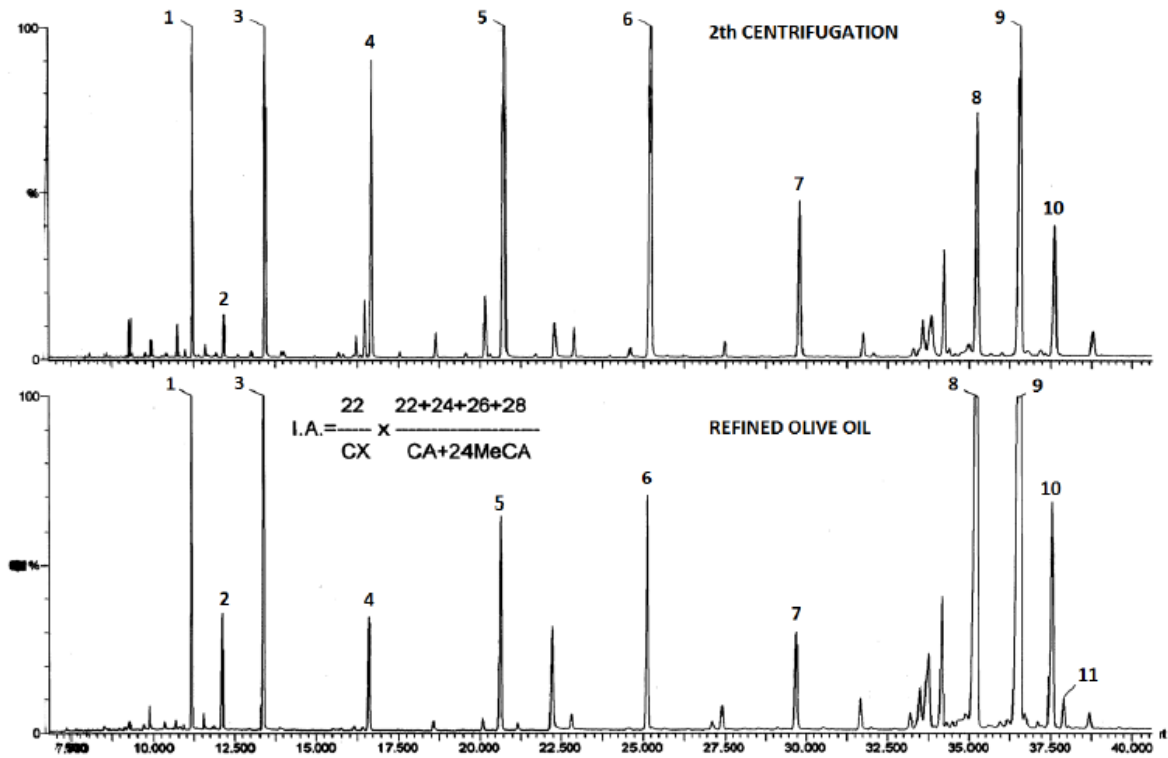


Figure 3- Aliphatic and triterpenic alcohols of a refined olive oil and a second centrifugation olive oil

- | | | |
|---------------------------|-----------------------|---------------------------------------|
| 1 = Phytol | 5 = Alcohol C24 | 9 = 24-Methylen-cycloartenol (24MeCA) |
| 2 = Geranyl geraniol (CX) | 6 = Alcohol C26 | 10 = Citrostadienol |
| 3 = Alcohol C20 | 7 = Alcohol C28 | 11 = Cyclobranol |
| 4 = Alcohol C22 | 8 = Cycloartenol (CA) | |

ANNEX 2
PRECISION VALUES OF THE METHOD

1. Analysis of the collaborative test results

The precision values of the method are given in the table on the next page.

Fifteen laboratories holding IOOC recognition at the time took part in the collaborative test organised in May–June 2003 and coordinated by Mr Arturo Cert, Fats & Oil Institute, Seville (Spain). The laboratories were from seven countries.

The test was performed on five samples of lampante virgin olive oil (LVOO), crude-olive pomace oil obtained on centrifuging the pomace from two-phase decanters (COPO) and blends thereof:

- A: 100 % LVOO
- B: 85% LVOO + 15% COPO
- C: 75% LVOO + 25% COPO
- D: 60% LVOO + 40% COPO
- E: 100% COPO.

The results of the collaborative test were statistically processed by the coordinator according to the recommendations of the Collaborative Study Guidelines in the *J. of AOAC International 78, 143A-160A, 1995*. Outliers were examined by applying Cochran's and Grubbs' test to the laboratory results for each determination (replicates a and b) and each sample.

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
- RDS_r (%)** Repeatability coefficient of variation ($S_r \times 100/\text{mean}$)

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

RDS_R (%) Reproducibility coefficient of variation ($S_R \times 100/\text{mean}$)

Total aliphatic alcohol content C22 + C24 + C26 + C28 (mg/kg)

	A	B	C	D	E
n	15	15	15	15	15
outliers	3	1	1	1	1
mean	245	363	440	541	997
r	16	28	29	36	54
S_r	6	10	10	13	19
RSD_r(%)	2,4	2,8	2,3	2,4	1,9
R	47	78	161	150	198
S_R	17	28	58	54	71
RSD_R(%)	6,9	7,6	13,1	9,9	7,1