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Olive oils and olive-pomace oils — Determination of aliphatic and triterpenic alcohols content by capillary gas chromatography

Huiles d'olive et huiles de grignons d'olive — Détermination de la teneur en alcools aliphatiques et triterpéniques par chromatographie en phase gazeuse sur colonne capillaire

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Foreword

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The procedures used to develop this document and those intended for its further maintenance are described in the ISO/IEC Directives, Part 1. In particular, the different approval criteria needed for the different types of ISO documents should be noted. This document was drafted in accordance with the editorial rules of the ISO/IEC Directives, Part 2 (see www.iso.org/directives).

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For an explanation of the voluntary nature of standards, the meaning of ISO specific terms and expressions related to conformity assessment, as well as information about ISO's adherence to the World Trade Organization (WTO) principles in the Technical Barriers to Trade (TBT) see www.iso.org/iso/foreword.html.

This document was prepared by Technical Committee ISO/TC 34, *Food products*, Subcommittee SC 11, *Animal and vegetable fats and oils*.

This second edition cancels and replaces the first edition (ISO 12871:2010), which has been technically revised. The following change has been made:

— the determination of triterpenic alcohols has been introduced.

Any feedback or questions on this document should be directed to the user's national standards body. A complete listing of these bodies can be found at www.iso.org/members.html.

Olive oils and olive-pomace oils — Determination of aliphatic and triterpenic alcohols content by capillary gas chromatography

1 Scope

This document specifies a procedure for the determination of the content, as a mass fraction expressed as milligrams per kilogram, of aliphatic and triterpenic alcohols in olive oils and olive-pomace oils.

NOTE This document is based on COI/T.20/Doc. 26 Rev.2:2017[4].

2 Normative references

The following documents are referred to in the text in such a way that some or all of their content constitutes requirements of this document. For dated references, only the edition cited applies. For undated references, the latest edition of the referenced document (including any amendments) applies.

ISO 661, *Animal and vegetable fats and oils — Preparation of test sample*

3 Terms and definitions

For the purposes of this document, the following terms and definitions apply.

ISO and IEC maintain terminological databases for use in standardization at the following addresses:

— ISO Online browsing platform: available at https://www.iso.org/obp

— IEC Electropedia: available at http://www.electropedia.org/

3.1
aliphatic alcohols content
sum of the aliphatic alcohols with carbon number C22, C24, C26, and C28, as a mass fraction, determined according to the method specified in this document

4 Principle

The oil, to which 1-eicosanol has been added as an internal standard, is saponified with ethanolic potassium hydroxide and the unsaponifiable matter extracted with diethyl 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 (TMSE) and analysed by capillary gas chromatography.

5 Reagents

Technical, organizational and personal safety measures shall be followed.

During the analysis, unless otherwise stated, use only reagents of recognized analytical grade, and distilled or demineralized water or water of equivalent purity.

5.1 Potassium hydroxide, ethanolic solution, *c*(KOH) approximately 2 mol/l.

Dissolve, while cooling, 130 g potassium hydroxide [*w*(KOH) = 85 % mass fraction minimum] in 200 ml water and make up to 1 l with ethanol. Store the solution in a well-stoppered opaque glass bottle.

5.2 Potassium hydroxide, ethanolic solution, *c* (KOH) approximately 0,2 mol/l.

Dissolve 13 g potassium hydroxide in 20 ml water and make up to 1 l with ethanol.

5.3 Diethyl ether.

5.4 Anhydrous sodium sulfate.

5.5 Glass plates, coated with silica gel, without fluorescence indicator, 0,25 mm thick.

Suitable ready-for-use products are available commercially.

5.6 Acetone, chromatography grade.

5.7 Hexane, chromatography grade.

5.8 Diethyl ether, chromatography grade.

5.9 Chloroform, chromatography grade.

5.10 Reference solution for thin-layer chromatography: C20 to C28 aliphatic alcohols 0,5 g/100 ml solution in chloroform or a fraction of alcohols obtained as indicated in 9.2 from the unsaponifiable matter of an olive-pomace oil.

5.11 2′,7′-Dichlorofluorescein in ethanol, 0,2 g/100 ml solution. Make slightly basic by adding a few drops of alcoholic potassium hydroxide solution (5.1).

5.12 Anhydrous pyridine, chromatography grade.

5.13 Hexamethyldisilazane (HMDS).

5.14 Trimethylchlorosilane (TMCS).

5.15 Standard solutions of trimethylsilyl ethers (TMSE), of aliphatic alcohols from C20 to C28. Prepare from mixtures of pure alcohols immediately prior to use.

5.16 Internal standard solution: solution of 1-eicosanol in chloroform, mass concentration 0,1 g/100 ml.

5.17 Carrier gas: hydrogen or helium, gas chromatography grade.

5.18 Auxiliary gas: nitrogen, gas chromatography grade.

6 Apparatus

Usual laboratory equipment and, in particular, the following.

6.1 Round-bottomed flask, of capacity 250 ml, fitted with a reflux condenser having ground-glass joints.

6.2 Separating funnel, of capacity 500 ml.

6.3 Round-bottomed flasks, of capacity 250 ml.

6.4 Chromatographic chamber for thin-layer chromatography, suitable for glass plates of dimensions 20 cm × 20 cm.

6.5 Ultraviolet lamp, of wavelength 366 nm or 254 nm.

6.6 Microsyringes, of capacities 100 µl and 500 µl.

6.7 Cylindrical filter funnel with a G3 porous septum (porosity 15 µm to 40 µm), of approximate dimensions: diameter 2 cm and depth 5 cm, with an attachment suitable for filtration under vacuum and a 12/21 male ground-glass joint.

6.8 Vacuum conical flask, of capacity 50 ml, with a 12/21 female ground-glass joint which can be fitted to the filter funnel (6.7).

6.9 Test-tube, of capacity 10 ml, with a tapering bottom and a sealing stopper.

6.10 Gas chromatograph, suitable for use with capillary columns, equipped with the components specified in 6.11 to 6.14.

6.11 Column oven, capable of maintaining a temperature to within ±1 °C.

6.12 Split injection unit, temperature-adjustable, with a persilylated glass vaporizing element, or an on-column unit.

6.13 Flame ionization detector.

6.14 Integration system.

6.15 Fused silica capillary column, of length 20 m to 30 m, internal diameter 0,25 mm to 0,32 mm, with SE-52 or SE-54[1)] liquid phase or equivalent, with a film thickness between 0,10 µm and 0,30 µm.

6.16 Microsyringe for gas chromatography, of capacity 10 µl, with hardened needle.

6.17 Analytical balance, sensitive to 1 mg (with 0,1 mg display).

6.18 Desiccator, with calcium chloride as desiccant.

6.19 Drying oven.

7 Sampling

Sampling is not part of the method specified in this document. A recommended sampling method is given in ISO 5555.

It is important that the laboratory receive a truly representative sample that has not been damaged or changed during transport or storage.

1) SE-52 and SE-54 are examples of suitable products available commercially. This information is given for the convenience of users of this document and does not constitute an endorsement by ISO of these products.

8 Preparation of the test sample

Prepare the test sample in accordance with ISO 661.

9 Procedure

9.1 Preparation of the unsaponifiable matter

9.1.1 Using a 500 µl microsyringe (6.6), transfer to a 250 ml round-bottomed flask (6.1) a volume of internal standard solution (5.16) containing a quantity of 1-eicosanol approximately equal to 10 % of the content of aliphatic alcohols in the test portion. For example, to 5 g of sample, add 250 µl of the internal standard solution for olive oil and 1 500 µl for olive-pomace oil.

Evaporate to dryness under a stream of nitrogen, then weigh (6.17) accurately 5,000 g of the dry filtered sample into the same flask.

9.1.2 Add 50 ml of 2 mol/l ethanolic potassium hydroxide solution (5.1), fit the reflux condenser and boil gently on a steam bath, stirring continuously throughout the heating process until saponification has taken place, i.e. until the solution becomes clear. Continue heating for a further 20 min and then add 50 ml of water through the condenser. The condenser is then disconnected and the flask cooled to approximately 30 °C.

9.1.3 Transfer the contents of the flask quantitatively to a 500 ml separating funnel (6.2), progressively adding portions of about 50 ml water. Add approximately 80 ml of diethyl ether (5.8), shake vigorously for approximately 30 s, and allow to settle.

NOTE Emulsions are broken by spraying small quantities of diethyl ether or methanol into the funnel.

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

9.1.4 The diethyl ether extracts are combined in a separating funnel and washed with water (50 ml at a time) until the washing water gives a neutral reaction against phenolphthalein.

Discard the washing water, dry with anhydrous sodium sulfate (5.4) and filter into a 250 ml round-bottomed flask (6.3) that has previously been weighed, and wash the funnel and filter with small quantities of diethyl ether which are added to the total.

9.1.5 Distil the ether down to a few millilitres, then bring to dryness under a slight vacuum or under a stream of nitrogen, completing the drying process in an oven (6.19) maintained at 103 °C for approximately 15 min. Weigh (6.17) after cooling in a desiccator (6.18).

9.2 Separation of alcoholic fractions

9.2.1 Prepare basic TLC plates by immersing the silica gel plates (5.5) completely in an 0,2 mol/l potassium hydroxide solution (5.2) for 10 s, leaving them to dry for 2 h under an extractor hood and finally placing them in an oven (6.19) at 100 °C for 1 h.

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

Remove the plate from the oven and keep in a desiccator (6.18) until required for use (plates treated in this way shall be used within 15 days).

9.2.2 Place a hexane-diethyl ether mixture (volume fraction of hexane 65 ml/100 ml and of diethyl ether 35 ml/100 ml) in the plate development chamber to a depth of approximately 1 cm.

Close the chamber using an appropriate cover and leave for half an hour to allow equilibration between vapour and liquid. Strips of filter paper partially immersed in the eluent may be affixed to the inside surfaces of the chamber to reduce the development time by approximately one-third and obtain a more uniform and regular elution of the components.

Use fresh developing solution for each analysis in order to obtain reproducible developing conditions.

9.2.3 Prepare a solution of approximately 50 mg/ml unsaponifiable matter (see 9.1.5) in chloroform and streak 0,3 ml of the solution as a uniform strip of minimum thickness, using a 100 µl microsyringe (6.6), at approximately 2 cm from the bottom of a TLC plate. Aligned with the origin, 2 µl to 3 µl of the aliphatic alcohols reference solution (5.10) are spotted for the identification of the aliphatic alcohols band after development has been completed.

9.2.4 Place the plate inside the development chamber (see 9.2.2). The ambient temperature shall be maintained between 15 °C 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.

Remove the plate from the development chamber and either evaporate the solvent under a hot air stream or leave the plate for a while under the extractor hood.

9.2.5 Spray the plate lightly and evenly with the solution of 2′,7′-dichlorofluorescein (5.11) when the plate is placed under ultraviolet light (6.5) for observation. Identify the aliphatic alcohols band by aligning against the stain obtained from the reference solution: using a black pencil, outline both the band of aliphatic alcohols and the band immediately above that, which is the triterpenic alcohols band.

NOTE The aliphatic alcohols band and the triterpenic alcohols band are grouped together because of the possible migration of some aliphatic alcohols into the triterpenic alcohols band. An example of the TLC separation is given in Figure A.1.

9.2.6 Using a metal spatula, scrape off the silica gel in the marked area. Place the finely comminuted material into the filter funnel (6.7). Add 10 ml of boiling chloroform, mix carefully with the metal spatula and filter under vacuum, collecting the filtrate in a conical flask (6.8) attached to the filter funnel.

Wash the silica gel in the flask three times with diethyl ether (5.3), using approximately 10 ml each time, collecting the filtrate in the flask attached to the funnel. Evaporate the filtrate to a volume of 4 ml to 5 ml, transfer the residual solution to a previously weighed 10 ml test-tube (6.9), evaporate to dryness by heating under a gentle stream of nitrogen, make up again using a few drops of acetone, evaporate again to dryness, place in an oven (6.19) at 103 °C for approximately 10 min, then allow to cool in a desiccator (6.18) and weigh (6.17).

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

9.3 Preparation of the trimethylsilyl ethers

9.3.1 The silylation reagent (13 ml in total) is made up by mixing pyridine (5.12), hexamethyldisilazane (HMDS) (5.13) and trimethylchlorosilane (TMCS) (5.14) in volume fractions of 9 ml/13 ml; 3 ml/13 ml and 1 ml/13 ml, respectively. The silylation reagent is added to the alcoholic fraction in the test tube (see 9.2.6) in the proportion of 50 µl silylation reagent for each milligram of aliphatic alcohols, avoiding all absorption of moisture.

NOTE Ready-for-use solutions are available commercially. Other silylating reagents such as bis-trimethylsilyl trifluoroacetamide +1 % trimethyl chlorosilane, which has to be diluted with an equal volume of anhydrous pyridine, are also available.

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 shall be repeated.

9.3.2 Stopper the test-tube, shake carefully (without overturning) until the aliphatic alcohols are completely dissolved. Stand for at least 15 min at ambient temperature and then centrifuge for a few minutes. The clear TMSE solution is ready for gas chromatographic analysis.

9.4 Gas chromatographic analysis

9.4.1 Preliminary operations and column packing

9.4.1.1 Fit the column (6.15) in the gas chromatograph (6.10), attaching the inlet end to the injector connected to the splitting system (6.12) and the outlet end to the detector (6.13). 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.).

9.4.1.2 Condition the column on the first time of use. Circulate a little carrier gas through the capillary column, then switch on the gas chromatography assembly and heat gradually until a temperature of not less than 20 °C above the operating temperature is reached. Maintain that temperature for not less than 2 h. Then bring the assembly to operating conditions [by regulation of gas flow, split flame ignition, connection to the electronic recorder, adjustment of the temperature of the capillary column oven (6.11), the detector and the injector, etc.] and adjust the signal to a sensitivity that is at least twice as high as that planned for the analysis.

The base line shall be linear, with no peaks of any kind, and shall not drift. A negative straight-line drift indicates leakage from the column connections. A positive drift indicates inadequate conditioning of the column.

The conditioning temperature shall be at least 20 °C less than the maximum temperature planned for the liquid phase employed.

9.4.2 Operating conditions

9.4.2.1 The following operating conditions are recommended for a chromatographic system with a split injection unit:

a) column temperature: the initial temperature is set to 180 °C for 8 min, then programmed to increase at a rate of 5 °C/min to 260 °C, and the final temperature is maintained for a further 15 min at 260 °C;

b) temperature of injector: 280 °C;

c) temperature of detector: 290 °C;

d) linear velocity of carrier gas: helium 20 cm/s to 35 cm/s, hydrogen 30 cm/s to 50 cm/s;

e) splitting ratio: 1:50 to 1:100;

f) injection quantity: 0,5 µl to 1 µl of TMSE solution (see 9.3.2).

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:

— the retention time for C26 alcohol shall be (18 ± 5) min;

— the peak of the C22 alcohol shall be (80 ± 20) % of the full-scale value for olive oil and (40 ± 20) % of the full-scale value for olive-pomace oil.

9.4.2.2 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.

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

9.4.3 Analytical procedure

9.4.3.1 Using the 10 µl microsyringe (6.16), draw in 1 µl of hexane followed by 0,5 µl of air and subsequently 0,5 µl 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 1 s to 2 s, inject rapidly. Slowly remove the needle after approximately 5 s.

9.4.3.2 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 9.4.1.2.

9.4.4 Peak identification

The individual peaks are identified according to their retention times and by comparison with the standard TMSE mixture analysed under the same conditions.

Examples of chromatogram of the alcoholic fraction of a refined olive oil is shown in Figures A.2 and A.3.

9.4.5 Quantitative evaluation

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

9.4.5.2 The content of each aliphatic alcohol i, w_i, as a mass fraction expressed in milligrams per kilogram of oil, is calculated as shown by Formula (1):

$$w_i = \frac{A_i \cdot m_s \cdot 1\,000}{A_s \cdot m} \tag{1}$$

where

A_i is the peak area due to alcohol i;

A_s is the peak area of 1-eicosanol;

m_s is the mass, in milligrams, of 1-eicosanol;

m is the mass, in grams, of the test portion.

The contents of the individual aliphatic alcohols in milligrams per kilogram of oil and the sum of the "total aliphatic alcohols" are reported.

10 Precision

10.1 Interlaboratory test

Details of an interlaboratory test on the precision of the method are summarized in Annex B. The values derived from this interlaboratory test may not be applicable to concentration ranges and matrices other than those given.

10.2 Repeatability

The absolute difference between two independent single test results, obtained with the same method on identical test material in the same laboratory by the same operator using the same equipment within a short interval of time, should, in not more than 5 % of cases, exceed the values of the repeatability limit, *r*, given in Table B.2.

10.3 Reproducibility

The absolute difference between two single test results, obtained with the same method on identical test material in different laboratories by different operators using different equipment, should, in not more than 5 % of cases, exceed the values of the repeatability limit, *R*, given in Table B.2.

11 Test report

The test report shall include at least the following information:

a) all information necessary for the complete identification of the sample;

b) the sampling method used, if known;

c) the test method used, with reference to this document, i.e. ISO 12871:2019;

d) the result(s) obtained;

e) if the repeatability has been checked, the final quoted result obtained;

f) any operating details not specified in this document, or regarded as optional, together with details of any incidents which may have influenced the test result(s);

g) the date of the test.

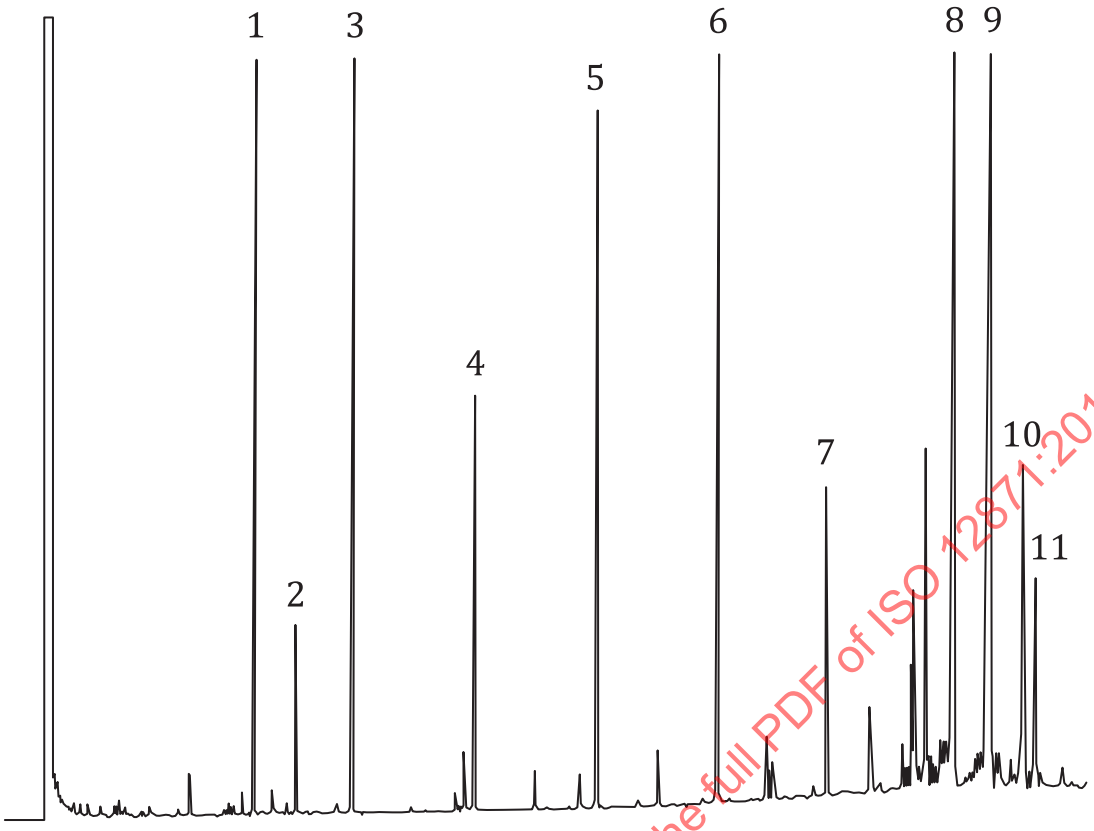
Annex A
(informative)

Thin-layer chromatography separation example and chromatogram examples

Key

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 matter		

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



Key

1	phytol	7	alcohol C28
2	geranyl geraniol	8	cycloartenol
3	alcohol C20 (IS)	9	24-Methylen-cycloartenol
4	alcohol C22	10	citrostadienol
5	alcohol C24	11	cyclobranol
6	alcohol C26		

Figure A.2 — Chromatogram of the alcoholic fraction of a refined olive oil