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Second edition
2022-08

Olive oils and olive-pomace oils — Determination of the 2-glyceryl monopalmitate content

Huiles d'olive et huiles de grignons d'olive — Détermination de la teneur en 2-glycéryl monopalmitate

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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,* in collaboration with the European Committee for Standardization (CEN) Technical Committee CEN/TC 307, *Oilseeds, vegetable and animal fats and oils and their by-products — Methods of sampling and analysis,* in accordance with the Agreement on technical cooperation between ISO and CEN (Vienna Agreement).

This second edition cancels and replaces the first edition (ISO 12872:2010), which has been technically revised.

The main changes are as follows:

— the use of iso-octane as an alternative to hexane has been added;

— precision data of the method using iso-octane compared with hexane have been added.

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.

Introduction

As part of the *Trade standard applying to olive oils and olive-pomace oils*, the International Olive Council (IOC) published COI/T.20/Doc. No 23:2006[6].

COI/T.20/Doc. No 23 was applicable to olive and olive-pomace oils and was used to distinguish between lampante virgin olive oils and crude olive-pomace oils. Olive pomace is the residual paste which still contains a variable amount of water and oil after pressing or centrifuging.

In 2008, the IOC submitted the document to ISO/TC 34/SC 11 for adoption as an International Standard.

In 2017, the IOC published a revision of COI/T.20/Doc. No 23/Rev.1[7], and this revised document is an adoption of the IOC revised method.

Olive oils and olive-pomace oils — Determination of the 2-glyceryl monopalmitate content

1 Scope

This document specifies a procedure for the determination of the content, as a percentage mass fraction, of 2-glyceryl monopalmitate in olive oils and olive-pomace oils that are liquid at ambient temperature (20 °C).

NOTE This document is based on COI/T.20/Doc. No 23/Rev.1:2017[Z].

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 terminology databases for use in standardization at the following addresses:

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

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

3.1
2-glyceryl monopalmitate content
mass fraction of 2-glyceryl monopalmitate in the monoacylglycerol fraction

Note 1 to entry: This is determined according to the method specified in this document.

Note 2 to entry: The 2-glyceryl monopalmitate content is expressed as a percentage.

4 Principle

The oil, after suitable preparation, is subjected to the action of pancreatic lipase. A partial hydrolysis takes place that is specific for positions 1 and 3 of the triacylglycerol molecule so that 2-monoacylglycerols are obtained as reaction products. The percentage of 2-glyceryl monopalmitate in the monoacylglycerol fraction is determined, after silylation, by capillary gas chromatography.

5 Reagents

WARNING — 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 Silica gel, with a particle size of 0,063 mm to 0,200 mm (70/280 mesh), prepared as follows:

— put the silica gel into a porcelain cup, dry in an oven at 160 °C for 4 h, then cool at ambient temperature in a desiccator;

— add a volume of water equivalent to 5 % of the mass of the silica gel as follows:

  — weigh 152 g of silica gel into a 500 ml Erlenmeyer flask, add 8 g of water, stopper and homogenize carefully;

  — leave to settle for at least 12 h before using.

5.2 *n*-Hexane, chromatography grade.

Hexane may be replaced by iso-octane (2,2,4-trimethylpentane of chromatography grade), provided that comparable precision values are achieved (see the precision values of the method with the use of iso-octane in Annex B).

5.3 Isopropanol.

5.4 Isopropanol-water mixture, volume fractions 50 ml/100 ml.

5.5 Pancreatic lipase, activity between 2,0 and 10 lipase units per milligram (see Annex C).

NOTE Pancreatic lipase with an activity between 2 and 10 units per mg of enzyme is available commercially.

5.6 Buffer solution of tris(hydroxymethyl)aminomethane: prepare an aqueous solution (1 mol/l) with pH 8 and mix with concentrated HCl, volume fractions 50 ml/100 ml.

5.7 Sodium cholate, special enzyme grade, aqueous solution, mass fraction 0,1 g/100 g.

Use this solution within 15 days of preparation.

5.8 Calcium chloride, aqueous solution, mass fraction 22 g/100 g.

5.9 Diethyl ether, chromatography grade.

5.10 Elution solvent 1: mixture of *n*-hexane-diethyl ether, volume fraction of *n*-hexane 87 ml/100 ml and of diethyl ether 13 ml/100 ml.

5.11 Sodium hydroxide, aqueous solution, mass fraction 12 g/100 g.

5.12 Phenolphthalein, ethanolic solution, mass concentration 1 g/100 ml.

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

5.14 Auxiliary gases: hydrogen, free from moisture and organic substances, and synthetic air, gas chromatography grade.

5.15 Silylation reagent: mixture of pyridine, hexamethyldisilazane (HMDS) and trimethylchlorosilane (TMCS); volume fractions: 9 ml/13 ml, 3 ml/13 ml, and 1 ml/13 ml, respectively.

NOTE Ready-to-use solutions are available commercially. Other silylation reagents can be used, e.g. bis trimethylsilyl trifluoracetamide + 1 % trimethylchlorosilane, diluted with an identical volume of anhydrous pyridine.

5.16 Reference samples: pure monoacylglycerols and mixtures of monoacylglycerols with a known composition similar to that of the sample.

5.17 Elution solvent 2: mixture of *n*-hexane-diethyl ether, volume fraction of *n*-hexane 90 ml/100 ml and of diethyl ether 10 ml/100 ml.

6 Apparatus

The usual laboratory equipment and, in particular, the following shall be used.

6.1 Erlenmeyer flasks, of capacity 25 ml.

6.2 Beakers, of capacities 100 ml, 250 ml and 300 ml.

6.3 Glass chromatography column, 21 mm to 23 mm internal diameter, 400 mm in length, with septum and stopcock.

6.4 Measuring cylinders, of capacities 10 ml, 50 ml, 100 ml and 200 ml, ISO 4788[2] class A.

6.5 Round-bottomed flasks, of capacities 100 ml and 250 ml.

6.6 Rotary evaporator.

6.7 Centrifuge tubes, conical bottom, of capacity 10 ml, with ground-glass stopper.

6.8 Centrifuge, suitable for 10 ml and 100 ml tubes.

6.9 Water bath, capable of maintaining a temperature of (40 ± 0,5) °C.

6.10 Graduated pipettes, of capacities 1 ml and 2 ml, ISO 835[1] class A.

6.11 Hypodermic syringe, 1 ml.

6.12 Microsyringe, 100 µl.

6.13 Separating funnel, 1 000 ml.

6.14 Gas chromatograph, suitable for use with capillary columns, equipped with the components specified in 6.14.1 to 6.14.5.

6.14.1 Cold on-column injector.

6.14.2 Flame ionization detector.

6.14.3 Column oven, capable of maintaining the temperature to within ± 1 °C.

6.14.4 Computer-based integration system.

6.14.5 Fused silica capillary column, of length 8 m to 12 m and internal diameter 0,25 mm to 0,32 mm, coated with methylpolysiloxane or 5 % phenyl methylpolysiloxane, with a film thickness of 0,10 µm to 0,30 µm, suitable for use at 370 °C.

6.15 Microsyringe, 10 µl, with hardened needle, of minimum length 7,5 cm in length, suitable for on-column injection.

7 Sampling

Sampling is not part of the method specified in this document. A recommended sampling method is given in ISO 5555[3].

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

8 Preparation of the test sample

Prepare the test sample in accordance with ISO 661. Oils with a free acidity greater than 3 % require a neutralization step as specified here.

Introduce 50 g of the oil into a 1 000 ml separating funnel (6.13) and dissolve it in 200 ml of *n*-hexane (5.2). Add 100 ml of isopropanol (5.3) and a volume of sodium hydroxide solution (5.11) corresponding to the free acidity of the oil plus an excess of 5 %. Shake vigorously for 1 min, add 100 ml of water, shake again and leave to settle.

After separation, remove the lower soapy layer. Also remove any intermediate layer of mucilage and insoluble matter which often forms. Wash the *n*-hexane solution of the oil with successive 50 ml to 60 ml portions of the isopropanol-water mixture (5.4) until the washed phase is neutral to phenolphthalein (5.12).

Remove most of the hexane by distillation under vacuum, e.g. by using a rotary evaporator (6.6), and transfer the oil to a 100 ml round-bottomed flask (6.5). Dry it under vacuum to complete solvent removal.

By the end of this procedure, ensure that the acidity of the oil is below 0,5 %.

9 Procedure

9.1 Preparatory steps

9.1.1 Introduce 1,0 g of the oil, pretreated (see Clause 8) where necessary, into a 25 ml Erlenmeyer flask (6.1) and dissolve it in 10 ml of elution solvent 1 (5.10). Leave the solution to settle for at least 15 min before starting the silica gel column chromatography procedure.

If the solution is cloudy, centrifuge it to ensure optimal conditions for chromatography.

Ready-to-use 500 mg silica gel solid phase extraction (SPE) cartridges may be used. If SPE cartridges are used, go directly to 9.2.2.

9.1.2 Fill the chromatography column (6.3) with about 30 ml of elution solvent 1 (5.10). Insert a wad of cotton wool at the bottom of the column using a glass rod. Press to remove the air.

In a beaker, prepare a suspension of 25 g of silica gel (5.1) in about 80 ml of elution solvent 1 and transfer it to the column by means of a funnel.

Make sure that all the silica gel is transferred to the column. Wash with elution solvent 1, open the stopcock and allow the solvent to drain until the solvent level is about 2 mm above the silica gel.

9.2 Column chromatography

9.2.1 Conventional procedure

Transfer the solution prepared in 9.1.1 on to the chromatography column (see 9.1.2). Avoid moving the surface of the column.

Open the stopcock and let the sample solution drain until it reaches the level of the silica gel. Elute with 150 ml of elution solvent 1 (5.10). Regulate the flow to a rate of 2 ml/min (150 ml shall pass through the column in about 60 min to 70 min).

Collect the eluate in a previously weighed 250 ml round-bottomed flask (6.5). Evaporate the solvent under vacuum and remove the last traces of solvent under a stream of nitrogen.

Weigh the flask and calculate the mass recovered by difference.

9.2.2 Procedure when ready-to-use silica SPE cartridges are employed

Introduce 1 ml of the solution (see 9.1.1) into the cartridges, previously conditioned with 3 ml of *n*-hexane (5.2). After percolation of the solution, elute with 4 ml of elution solvent 2 (5.17). Recover the eluate in a 10 ml tube and evaporate to dryness under a stream of nitrogen. Subject the dry residue to the action of pancreatic lipase (5.5). Check the fatty acid composition before and after passage through the SPE cartridge.

9.3 Hydrolysis with pancreatic lipase

9.3.1 Weigh 0,1 g of the oil prepared as described in 9.2 into a centrifuge tube (6.7). Add 2 ml of buffer solution (5.6), 0,5 ml of sodium cholate solution (5.7) and 0,2 ml of calcium chloride solution (5.8), shaking well after each addition. Cover the tube with the glass stopper and place it in the water bath (6.9) at (40 ± 0,5) °C.

9.3.2 Add 20 mg of lipase (5.5), mix carefully (to avoid wetting the stopper) and place in the water bath for exactly 2 min, then remove, shake vigorously for exactly 1 min and cool.

9.3.3 Add 1 ml of diethyl ether (5.9), stopper and shake vigorously, then centrifuge and transfer the ether solution to another clean, dry tube by means of a microsyringe.

9.4 Preparation of the silylated derivatives and gas chromatography

Take 100 µl of solution (see 9.3.3) using a microsyringe and transfer to a 10 ml tube with a conical bottom (6.7). Eliminate the solvent under a gentle stream of nitrogen, add 200 µl of silylation reagent (5.15), stopper the tube and leave to settle for 20 min.

After 20 min, add 5 ml of *n*-hexane (5.2). The solution is then ready for gas chromatography.

9.5 Gas chromatography

9.5.1 Operating conditions

The following operating conditions are recommended:

a) injector temperature: below solvent boiling point (68 °C);

b) detector temperature: 350 °C;

c) oven temperature programming: isothermal at 60 °C for 1 min; then increase at a rate of 15 °C/min to 180 °C, then at a rate of 5 °C/min to 340 °C; finally maintain at 340 °C for 13 min;

d) carrier gas: hydrogen or helium, regulated to the suitable linear speed in order to obtain the resolution shown in Annex A (see Figures A.1 to A.3);

e) retention time of triacylglycerol C_{54}: (40 ± 5) min (see Figure A.2);

f) injection volume: 0,5 µl to 1 µl of the solution obtained in 9.4.

Optimize the separation conditions to get the required resolution. Ensure that the height of the peak for 2-glyceryl monopalmitate is at least 10 % of the full-scale value.

9.5.2 Identification of peaks

The individual monoacylglycerols are identified by comparing the retention times obtained with those obtained for standard monoacylglycerol mixtures analysed under the same test conditions (see Figures A.1 to A.3).

9.5.3 Quantitative evaluation

The area of each peak is calculated by electronic integration.

10 Expression of results

The content of glyceryl monopalmitate, w_{MP}, expressed as a percentage mass fraction, is calculated from the ratio between the area of the relevant peak and the sum of the peak areas of all the monoacylglycerols (see Figure A.2) as shown by Formula (1):

$$w_{MP} = \frac{A_{MP}}{\sum A} \times 100 \quad (1)$$

where

A_{MP} is the peak area of the glyceryl monopalmitate peak;

ΣA is the sum of the peak areas of all monoacylglycerols.

Report the result to one decimal place.

11 Precision

11.1 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 are not necessarily applicable to concentration ranges and matrices other than those given.

11.2 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 *r* given in Table B.1.

11.3 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 *R* given in Table B.1.

12 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 International Standard used, with reference to this document, i.e. ISO 12872, including its year of publication as well as the method used (if the standard includes several);

d) the result(s) obtained, including a reference to the clause which explains how the results were calculated;

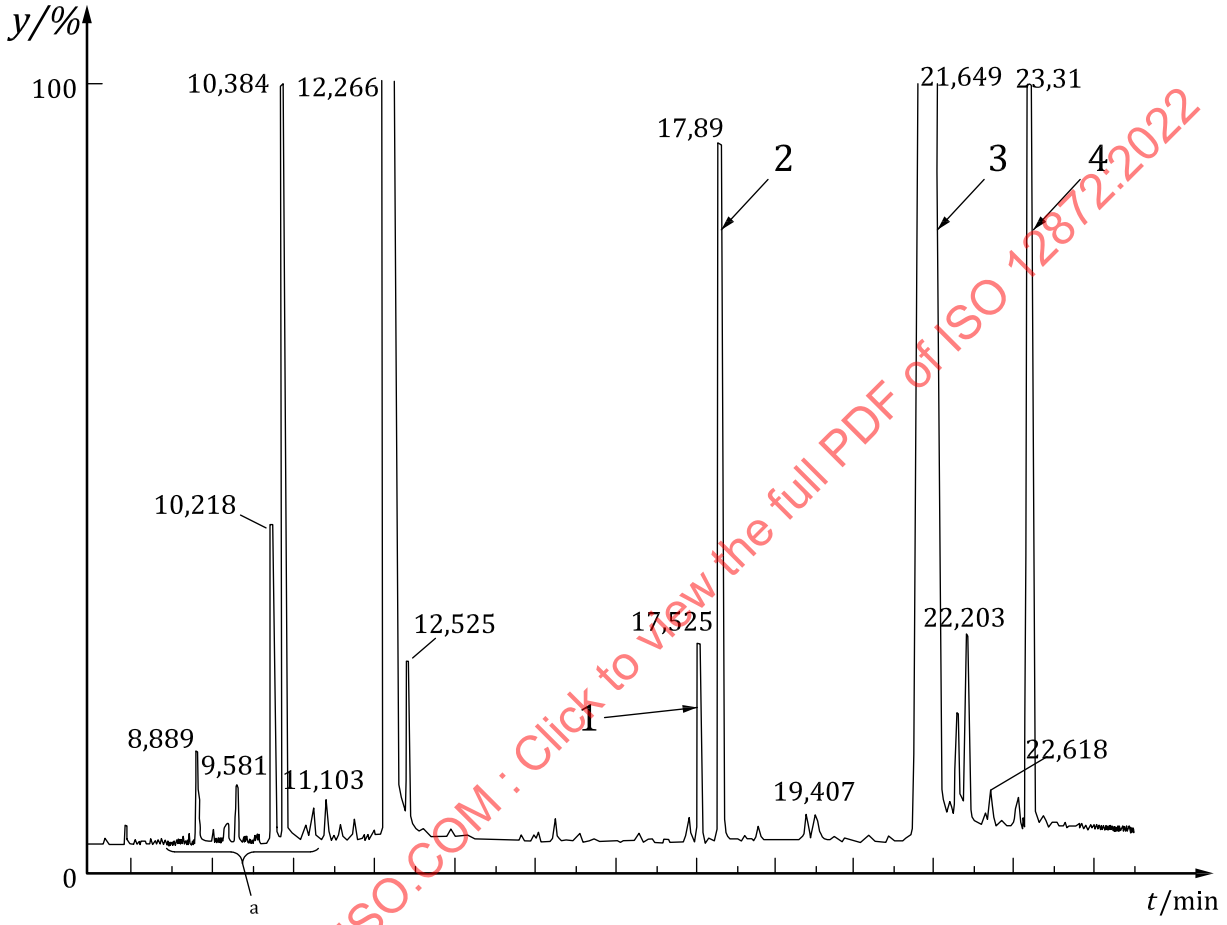
e) if the repeatability has been checked, the final quoted result obtained;

f) any deviations from the procedure detailed in this document, or operating details regarded as optional, together with details of any incidents or unusual features observed which can have influenced the test result(s);

g) the date of the test.

Annex A
(informative)

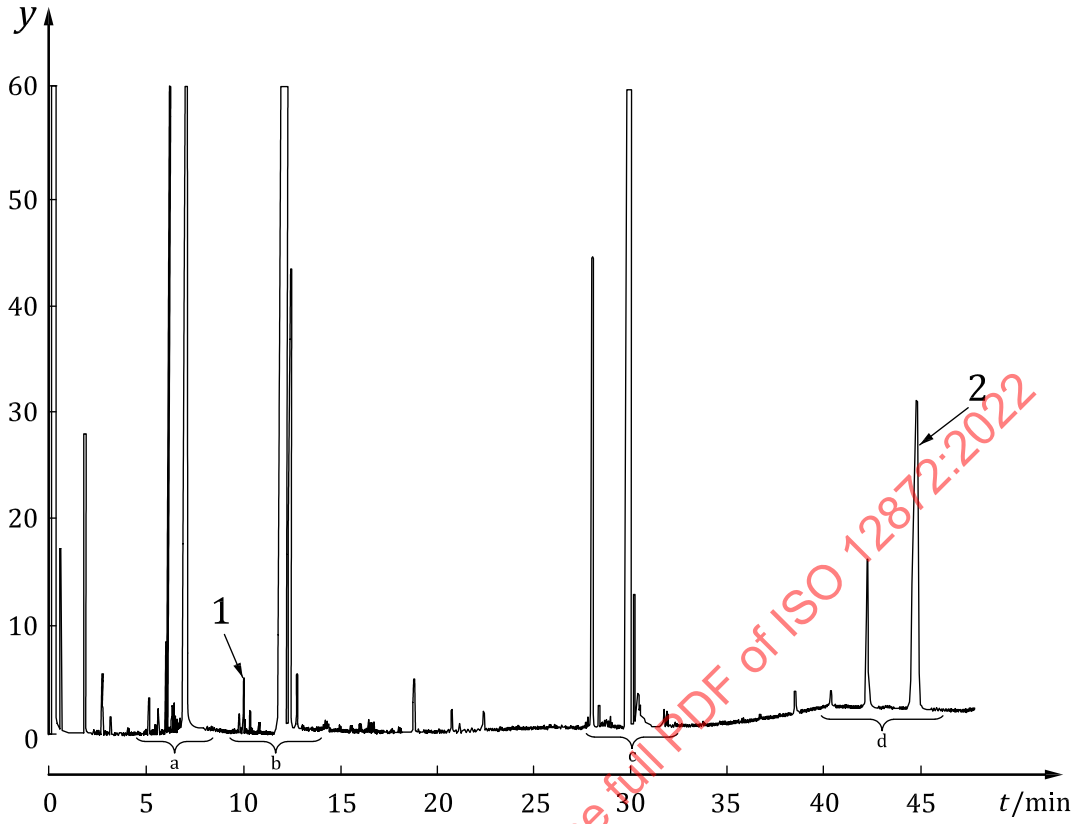
Chromatograms



Key

1	2-monopalmitoleine	*t*	time, in min
2	2-monopalmitine	*y*	measure of relative peak size, in %
3	2-mono C_{18} unsaturated	a	Free fatty acids.
4	squalene		

Figure A.1 — Chromatogram of the silylation reaction products obtained by lipase action on a refined olive oil spiked with 20 % volume fraction esterified oil

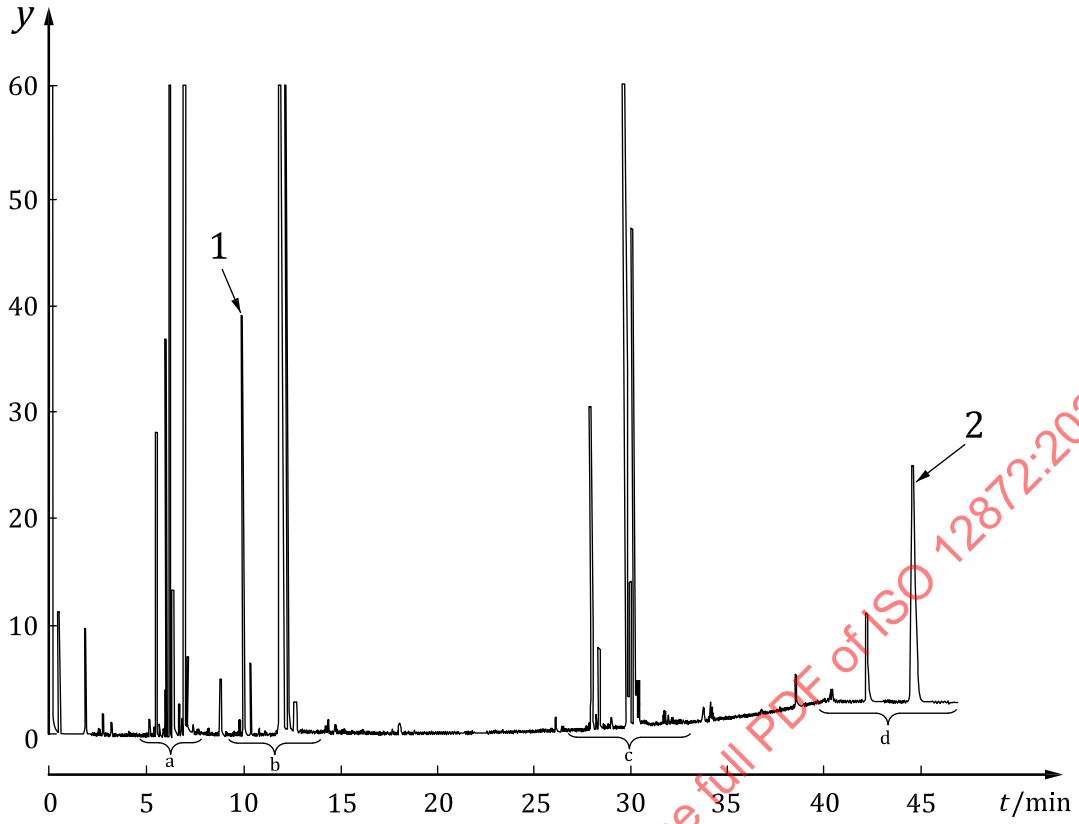


Key

1	2-monopalmitate	a	Free fatty acids.
2	triacylglycerol C_{54}	b	Monoacylglycerols.
t	time, in min	c	Diacylglycerols.
y	measure of relative peak size, arbitrary units	d	Triacylglycerols.

In these conditions (capillary column 8 m to 12 m), the wax fraction elutes with the diacylglycerol fraction or soon after it. After lipase action, triacylglycerol content should not exceed 15 % mass fraction.

Figure A.2 — Genuine olive oil after the action of the lipase and after silylation



Key

1	2-monopalmitate	a	Free fatty acids.
2	triacylglycerol C_{54}	b	Monoacylglycerols.
t	time, in min	c	Diacylglycerols.
y	measure of relative peak size, arbitrary units	d	Triacylglycerols.

In these conditions (capillary column 8 m to 12 m), the wax fraction elutes with the diacylglycerol fraction or soon after it. After lipase action, triacylglycerol content should not exceed 15 % mass fraction.

Figure A.3 — An esterified oil after the action of the lipase and silylation

Annex B
(informative)

Results of interlaboratory test and comparison study

The precision data in Table B.1 are derived from the results of an international collaborative trial.

The interlaboratory test was organized by the International Olive Council and was coordinated from the University of Genoa. Participants included twelve laboratories from five countries.

The test results were subjected to statistical analysis in accordance with ISO 5725-1[4] and ISO 5725-2[5] to give the precision data shown in Table B.1. Outliers were examined by applying the tests of Cochran and Grubbs to the laboratory results for each determination (replicates a and b) and each sample.

Table B.1 — Statistical results of the international collaborative trial

Parameter	Sample				
	A Extra virgin olive oil	B Lampante virgin olive oil	C Refined olive oil	D Refined olive oil and re-esterified oil (90+10) % volume fractions	E Refined olive oil and re-esterified oil (80+20) % volume fractions
No. of participating laboratories, n_P	12	12	12	12	12
No. of laboratories retained after eliminating outliers, n_p	12	12	12	12	12
No. of test results in all laboratories, n_t	24	24	24	24	24
Mean glyceryl monopalmitate content, $\bar{w}_{MP}$, % mass fraction	0,46	0,75	0,90	1,81	2,82
Repeatability standard deviation, s_r, %	0,04	0,04	0,06	0,04	0,09
Coefficient of variation of repeatability, $C_{V,r}$, %	8,91	5,44	6,77	1,95	3,32
Repeatability limit, r, %	0,11	0,11	0,17	0,10	0,26
Reproducibility standard deviation, s_R, %	0,05	0,10	0,09	0,20	0,31
Coefficient of variation of reproducibility, $C_{V,R}$, %	11,07	12,66	10,16	11,07	10,88
Reproducibility limit, R, %	0,14	0,27	0,26	0,56	0,86

The results obtained either with *n*-hexane or iso-octane were compared during a collaborative study organized by the International Olive Council in 2017 (see Table B.2). Only one sample of virgin olive oil, adulterated with 10 % refined olive oil and 2 % animal fat was tested.

The comparison of the results was focused on the comparative evaluation of the variances, both under conditions of reproducibility, as well as the existence of a significant bias or not among the assigned values after applying the method with *n*-hexane and after using the alternative solvent (iso-octane).

For this, the F Fisher of two variances obtained, in both conditions, as well as the Student t statistic of the two populations studied, which compares the two means obtained and their respective variances, under conditions of reproducibility, were calculated.