



METHOD FOR THE DETERMINATION OF THE PERCENTAGE OF 2-GLYCERYL MONOPALMITATE

1. PURPOSE AND FIELD OF APPLICATION

This method describes the analytical procedure for the determination of the percentage of palmitic acid at the 2-position of the triacylglycerols by means of 2-glyceryl monopalmitate evaluation.

WARNING: This method may require the use of dangerous apparatus and chemicals as well as the performance of dangerous operations. It does not specify all the safety issues connected with its use. Users are therefore responsible for taking all appropriate safety measures beforehand and for observing any legal requirements.

Note: This method is applicable to vegetable oils that are liquid at ambient temperature (20 °C)

2. PRINCIPLE

After suitable preparation the oil sample 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 silanisation, by capillary gas chromatography.

3. LABORATORY APPARATUS AND WARE

- 3.1. Erlenmeyer flask, 25 mL.
- 3.2. Beakers, 100, 250 and 300 mL.
- 3.3. Glass chromatographic column, 21–23 mm internal diameter, 400 mm in length, with septum and stopcock.

- 3.4. Graduated cylinders, 10, 50, 100 and 200 mL.
- 3.5. Round-bottomed flasks, 100 and 250 mL.
- 3.6. Rotary evaporator.
- 3.7. Centrifuge tubes, conical bottom, 10 mL, with ground-glass stopper.
- 3.8. Centrifuge suitable for 10 and 100 mL tubes.
- 3.9. Thermostat bath for maintaining the temperature at $40 \pm 0.5^\circ\text{C}$.
- 3.10. Graduated pipettes, 1 and 2 mL.
- 3.11. Hypodermic syringe, 1 mL.
- 3.12. Micro-syringe, 100 μL .
- 3.13. Separating funnel, 1000 mL.
- 3.14. Gas chromatograph, suitable for capillary columns, with cold on-column injection system and oven capable of maintaining the set temperature within $\pm 1^\circ\text{C}$.
 - 3.14.1. Cold on-column injector.
 - 3.14.2. Flame ionisation detector equipped with converter–amplifier.
 - 3.14.3. Recorder–integrator for operation with converter–amplifier, with a response time not exceeding 1 sec and variable chart speed.
- 3.15. Glass or fused silica capillary column, 8–12 m in length, 0.25–0.32 mm internal diameter, coated with methylpolysiloxane or 5% phenyl methylpolysiloxane, with a film thickness of 0.10–0.30 μm , suitable for use at 370°C .
- 3.16. Micro-syringe, 10 μL , with hardened needle, 7.5 cm in length at least, suitable for on-column injection.

4. REAGENTS

- 4.1. Silica gel with a particle size of 0.063–0.200 mm (70/280 mesh), prepared as follows: put the silica gel into a porcelain cup, dry in an oven at 160°C for 4 hours, then cool at ambient temperature in a desiccator. Add a volume of water equivalent to 5% of the weight of the silica gel as follows: weigh 152 g of silica gel into a 500 mL Erlenmeyer flask and add 8 g of distilled water, stopper and homogenise carefully. Leave to settle for 12 hours at least before using.

- 4.2. n-hexane (chromatography grade) (see B.2). (Hexane may be replaced by isooctane (2,2,4- trimethylpentane in chromatography grade, see B.3), provided that comparable precision values are achieved (see Precision values of the method with the used of isooctane in page 17).
- 4.2.1 Isopropanol.
- 4.2.2. Isopropanol/water mixture 1:1 (v/v).
- 4.3. Pancreatic lipase¹, activity between 2.0 and 10 lipase units per mg.
- 4.4. Buffer solution of tris(hydroxymethyl)aminomethane: 1 M aqueous solution with pH adjusted at 8 (potentiometric check) with concentrated HCl (1:1 v/v).
- 4.5. Sodium cholate, special enzyme grade: 0.1% aqueous solution (this solution must be used within 15 days of preparation).
- 4.6. Calcium chloride, 22% aqueous solution.
- 4.7. Diethyl ether, chromatography grade (see B.1).
- 4.8. Elution solvent: n-hexane/diethyl ether mixture 87:13 v/v.
- 4.9. Sodium hydroxide, 12% solution by weight..
- 4.10. Phenolphthalein, 1% ethanolic solution.
- 4.11. Carrier gas: hydrogen or helium, gas chromatography grade.
- 4.12. Auxiliary gases (see B.7): - hydrogen, 99% minimum purity, free from moisture and organic substances – and air, gas chromatography grade, of the same purity.
- 4.13. Silanisation reagent: mixture pyridine/hexamethyldisilazane, trimethylchlorosilane 9/3/1 (v/v/v)² (see B.4, B.5, B.6).
- 4.14. Reference samples: pure monoacylglycerols and mixtures of monoacylglycerols with a known percentile composition similar to that of the sample.

¹ Pancreatic lipase with an activity between 2 and 10 units per mg of enzyme is available commercially.

² Ready-to-use solutions are available commercially. Other silanisation reagents can be used, e.g. bis trimethylsilyl trifluoroacetamide + 1% trimethylchlorosilane, diluted with an identical volume of anhydrous pyridine.

5. PROCEDURE

5.1. Preparation of the sample

5.1.1. Oils with a free acidity below 3% do not require neutralisation prior to silica gel column chromatography. Oils with a free acidity above 3% do require neutralisation as described in the procedure (5.1.1.1.).

5.1.1.1 Into a 1000 mL separating funnel (3.13) introduce 50 g of the oil and dissolve in 200 mL of n-hexane. Add 100 mL of isopropanol and an amount of 12% sodium hydroxide solution (4.9) corresponding to the free acidity of the oil plus 5% excess. Shake vigorously for 1 minute, add 100 mL of distilled water, shake again and leave to settle.

After separation, remove the bottom soap layer. An intermediate layer of mucilage and insoluble matter often forms and must also be removed. Wash the hexane solution of the oil with successive 50–60 mL portions of the isopropanol/water mixture 1:1 (v/v) (4.2.2) until the wash phase is neutral to phenolphthalein.

Remove most of the hexane by distillation under vacuum (e.g. use a rotary evaporator) and transfer the oil to a 100 mL flask (3.5); dry it under vacuum to complete solvent removal.

By the end of this procedure the acidity of the oil must be below 0.5 %.

5.1.2. Introduce 1.0 g of the oil prepared as described into a 25 mL Erlenmeyer flask (3.1) and dissolve in 10 mL of elution mixture (4.8). Leave the solution to settle for 15 minutes at least before silica gel column chromatography. If the solution is cloudy, centrifuge it to ensure optimal conditions for chromatography (*note 1*).

Note 1: Ready-to-use 500 mg silica gel SPE cartridges may be used.

5.1.3. Preparation of the chromatography column

Fill the column (3.3) with about 30 mL of elution solvent (4.8), insert a wad of cotton at the bottom of the column with the aid of a glass rod; press to remove the air.

In a beaker prepare a suspension of 25 g of silica gel (4.1.) in about 80 mL of elution solvent and transfer to the column by means of a funnel.

Make sure that all the silica gel has been transferred to the column; wash with elution solvent (4.8), open the stopcock and allow the solvent to flow until the solvent level is about 2 mm above the silica gel.

5.1.4. Column chromatography

In a 25 mL Erlenmeyer flask (5.1.2.), weigh exactly 1.0 g of sample, prepared as described in 5.1.

Dissolve the sample in 10 mL of elution solvent (4.8). Transfer the solution to the chromatographic column, prepared as described (5.1.3.). Avoid moving the surface of the column.

Open the stopcock and let the sample solution flow until it reaches the level of the silica gel. Elute with 150 mL of elution solvent. Regulate the flow to a rate of 2 mL/min (in this way, 150 mL will pass through the column in about 60–70 minutes).

Recover the eluate in a 250 mL flask, previously weighed. Evaporate the solvent under vacuum and remove the last traces of solvent under a stream of nitrogen.

Weigh the flask and calculate the recovery (*note 2*).

Note 2: If ready-to-use silica SPE cartridges are used (note 1), proceed as follows:

Introduce 1 mL of solution (5.1.2.) into the cartridges, previously conditioned with 3 mL of n-hexane.

After percolation of the solution, elute with 4 mL of n-hexane/diethyl ether 9/1 (v/v).

Recover the eluate in a 10 mL tube and evaporate to dryness in a stream of nitrogen..

Subject the dry residue to the action of pancreatic lipase (5.2.). It is essential to check the fatty acid composition before and after passage through the SPE.

5.2. Hydrolysis with pancreatic lipase

5.2.1. Into the centrifuge tube weigh 0.1 g of the oil, prepared as described in 5.1. Add 2 mL of buffer solution (4.4.), 0.5 mL of sodium cholate solution (4.5.) and 0.2 mL of calcium chloride solution, shaking well after each addition.

Cover the tube with the glass stopper and place it in the thermostat bath at 40 ± 0.5 °C.

5.2.2. Add 20 mg of lipase, mix carefully (avoid wetting the stopper) and place in the thermostat bath for exactly 2 minutes, then remove, shake vigorously for exactly 1 minute and cool.

5.2.3. Add 1 ml of diethyl ether, stopper and shake vigorously, then centrifuge and transfer the ether solution to another clean, dry tube by means of a micro-syringe.

5.3. Preparation of the silanised derivatives and gas chromatography

5.3.1. Take 100 µL of solution (5.2.3) with the aid of a micro-syringe and transfer to a 10 mL tube with a conical bottom.

Eliminate the solvent under a gentle stream of nitrogen, add 200 µL of silanisation reagent (4.13), stopper the tube and leave to settle for 20 minutes.

After 20 minutes, add 5 mL of n-hexane: the resultant solution is ready for gas chromatography.

5.4. Gas chromatography

The guideline operating conditions are as follows:

Injector temperature: below solvent boiling point (68 °C)

Detector temperature 350 °C

Oven temperature programming: 60 °C for 1 minute, increased to 180 °C at a rate of 15 °C/min, then increased to 340 °C at a rate of 5 °C/min, 13 minutes at 340 °C.

Carrier gas: hydrogen or helium, regulated to the suitable linear speed in order to obtain the resolution shown in Figure 1. The retention time of triacylglycerol C54 must be within 40±5 minutes (see Figure 2) (*note 3*).

Injection volume: 0.5–1 µl of the (5.3.1) solution.

Note 3: The above operating conditions are for guidance. Each operator has to optimise these conditions to achieve the desired resolution. The height of the peak for 2-glyceryl monopalmitate must be at least 10% of the full-scale value.

5.4.1. 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.

5.4.2. Quantitative evaluation

The area of each peak is calculated by electronic integration.

6. EXPRESSION OF RESULTS

The percentage of glyceryl monopalmitate is calculated from the ratio between the area of the corresponding peak and the sum of the peak areas of all the monoacylglycerols (see Figure 2), given by the formula:

$$\text{Glyceryl monopalmitate (\%)} : \frac{A_x}{\sum A} \times 100$$

where: A_x = area of the glyceryl monopalmitate peak
 ΣA = sum of the peak areas of all the monoacylglycerols

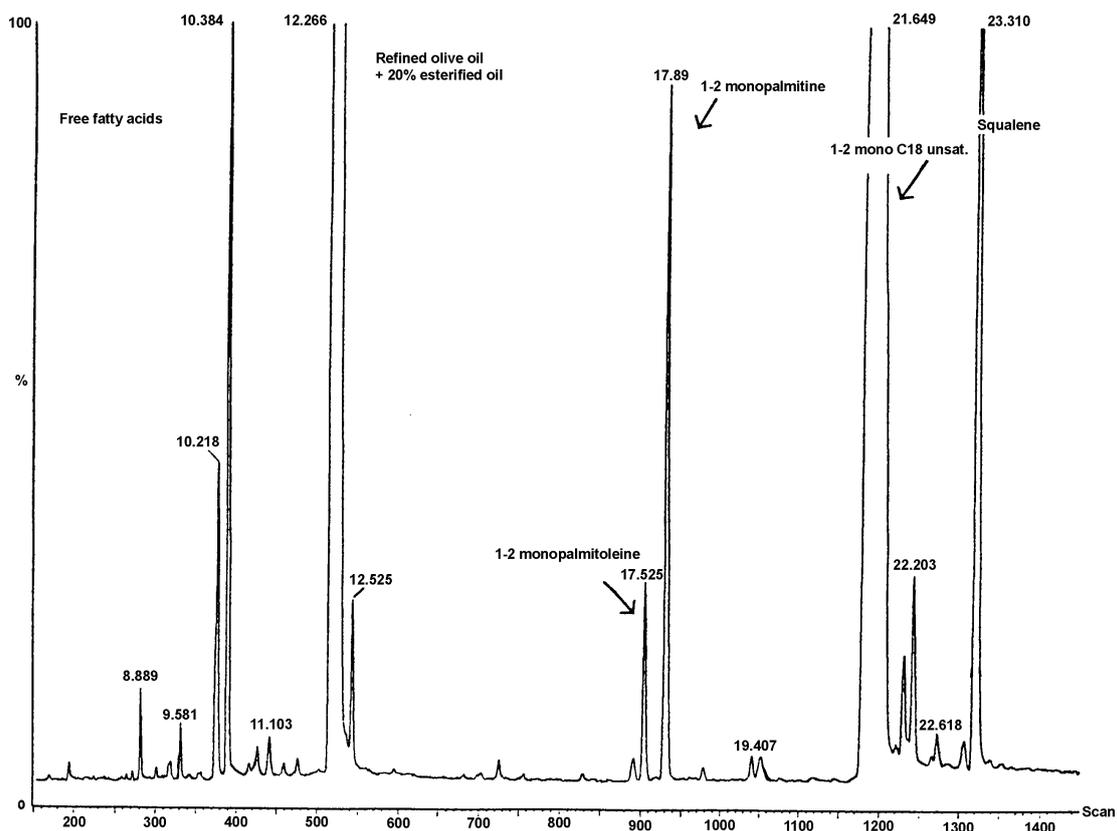
The result is to be given to one decimal place.

7. TEST REPORT

The test report shall specify:

- the reference of this method;
- all the information necessary for the complete identification of the sample;
- the test result;
- any departure from this method, made by agreement between the parties concerned or for any other reason;
- the identification details of the laboratory, the date on which the test was performed and the signature of the test supervisors.

Figure 1:



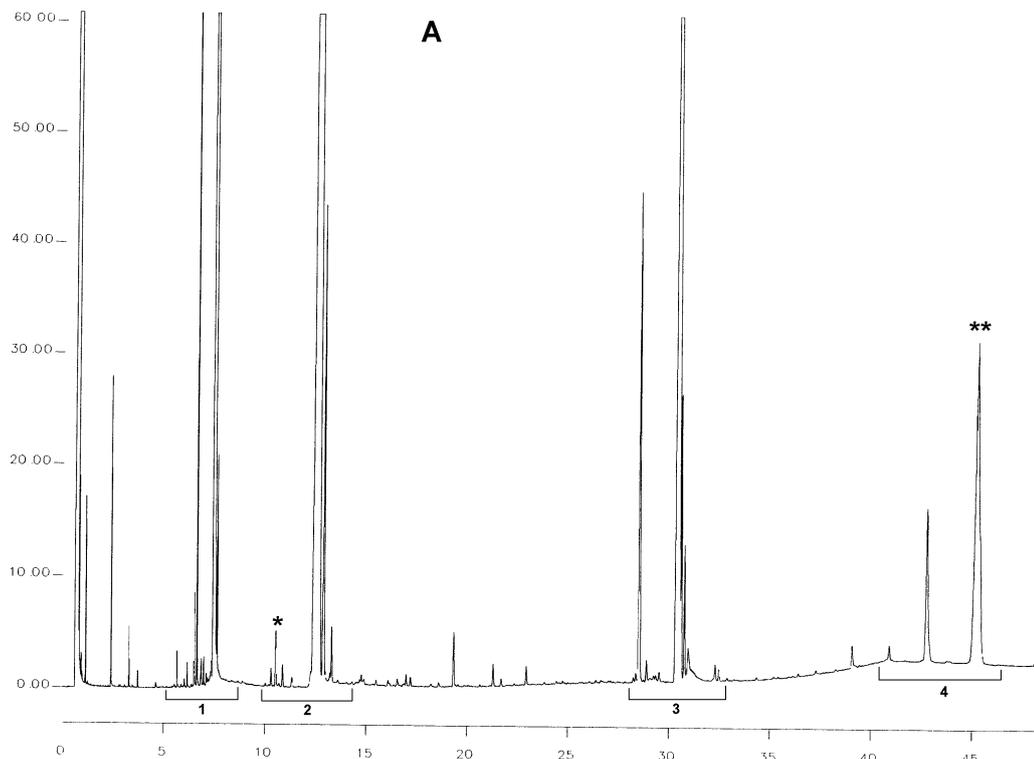
Chromatogram of the silanisation reaction products obtained by lipase action on a refined olive oil spiked with 20% esterified oil (100%).

Figure 2:

Chromatogram of:

- (A) a genuine olive oil after the action of the lipase, following silanisation. In these conditions (capillary column 8–12 m), the wax fraction elutes with the diacylglycerol fraction or soon after it.

After lipase action, triacylglycerol content should not exceed 15%.



Legend:

1 = Free fatty acids

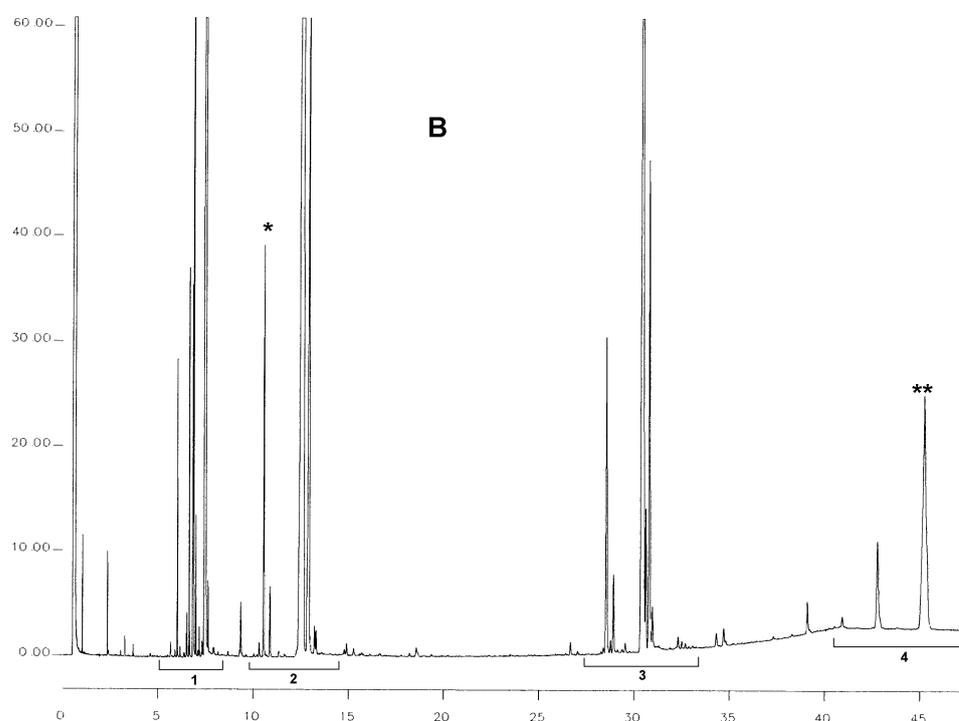
2 = Monoacylglycerols

3 = Diacylglycerols (*) = 2-monopalmitine

4 = Triacylglycerols (**) = Triacylglycerol C54

(B) an esterified oil after the action of the lipase, following silanisation. In these conditions (capillary column 8–12 m), the wax fraction elutes with the diacylglycerol fraction or soon after it.

After lipase action, triacylglycerol content should not exceed 15%.



Legend:

1 = Free fatty acids

2 = Monoacylglycerols

3 = Diacylglycerols (*) = 2-monopalmitine

4 = Triacylglycerols (**) = Triacylglycerol C54

ANNEX A

(informative)

A.1. PREPARATION OF THE LIPASE

Lipase is available commercially, but may also be prepared in the laboratory as follows:

Take 5 kg of fresh pig pancreas that has been chilled at 0°C. Remove the surrounding solid fat and connective tissue and triturate in a blender so as to obtain a fluid paste. Stir this paste for 4–6 hours with 2.5 L of anhydrous acetone and centrifuge. Extract the residue three times more with the same volume of anhydrous acetone, then twice with 1/1 (V/V) mixture of acetone and diethyl ether, and twice with diethyl ether.

Dry the residue under vacuum for 48 hours to obtain a stable powder. When stored in the refrigerator and kept away from damp, it will keep for a long time.

A.2. CHECKING OF LIPASE ACTIVITY

Prepare an olive oil emulsion as follows:

Shake a mixture of 165 mL of gum arabic solution at 100 g/l, 15 g of crushed ice and 20 mL of neutralised olive oil in a suitable agitator for 10 minutes.

In a 50 mL beaker, place 10 mL of this emulsion, followed successively by 0.3 mL of sodium cholate solution at 0.2 g/mL and 20 mL of distilled water.

Put the beaker into a thermostat maintained at 37°C; insert the electrodes of a pH meter and a spiral stirrer.

By means of a burette, add a 0.1 N sodium hydroxide solution, drop by drop, until the pH reaches 8.3.

Add an aqueous suspension of the lipase being checked (0.1 g/mL of lipase). Check the pH. As soon as the pH reaches 8.3 start the stopwatch and drip in the sodium hydroxide solution at such a rate as to maintain the pH at 8.3. Note down the volume of solution consumed every minute.

Record the observations in graph form, plotting the time readings as abscissae and the mL of 0.1 N alkaline solution required to maintain the pH constant as ordinates. A linear graph should be obtained.

The activity of the lipase, measured in lipase units per mg, is given by the formula:

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

Where:

- A is the activity in lipase units/mg;
- V is the volume of the 0.1 N sodium hydroxide solution consumed per minute (calculated from the graph), in mL;
- N is the normality of the sodium hydroxide solution;
- m is the mass of the lipase used in the test, in mg.

The lipase unit is defined as the amount of enzyme that will liberate 10 μ -equivalents of acid per minute.

ANNEX B

OPERATING PRECAUTIONS

B.1. Diethyl ether

Highly inflammable. Moderately toxic and irritates the skin. Pernicious if inhaled. Can damage the eyes. Effects may be delayed. It can form explosive peroxides. The fumes may ignite. Keep away from sources of heat, sparks, or naked flames. Make sure bottles are properly closed. Ensure proper ventilation during usage. Avoid build-up of fumes and remove any possible fire risk, such as heaters or electric apparatus not manufactured from non-inflammable material. Do not evaporate to dryness or quasi-dryness. The addition of water or a reducing agent may reduce peroxide formation. Do not swallow. Avoid breathing in the fumes. Avoid prolonged or repeated contact with the skin.

B.2. n-hexane

Highly inflammable. Fumes may ignite. Keep away from sources of heat, sparks, or naked flames. Make sure bottles are properly closed. Ensure proper ventilation during usage. Avoid build-up of fumes and remove any possible fire risk, such as heaters or electric apparatus not manufactured from non-inflammable material. Pernicious if inhaled since it can damage the cells of the nervous system. Avoid breathing in the fumes, using a suitable respiratory apparatus if necessary. Avoid contact with the eyes and skin.

TRANSLATE INTO ENGLISH

B.3. Iso-octane (2,2,4-triméthylpentane)

L'isooctane est un liquide inflammable qui présente des risques d'incendie. Les limites d'explosivité dans l'air sont de 1,1 % à 6,0 % (fraction volumique). Il est toxique par ingestion et inhalation. Utiliser une hotte ventilée en bon état de marche pour travailler avec ce solvant.

B.4. Pyridine

Highly inflammable. Pernicious if inhaled, if it comes into contact with the skin or if swallowed. If the product comes into contact with the eyes, rinse with abundant water and

seek medical advice. If it comes into contact with the skin, wash straight away with abundant water.

B.5. Hexamethyldisilazane

Highly inflammable liquid. Keep in a well-sealed container away from damp. Keep away from flames or sparks. Do not smoke.

B.6. Trimethylchlorosilane

Readily inflammable liquid substance. Irritates eyes, respiratory tract and skin. Keep in a well-sealed container away from damp.

B.7. Bottled, compressed hydrogen

Highly inflammable, under pressure. Keep away from sources of heat, sparks, naked flames or electric apparatus not manufactured from non-inflammable material. Make sure the bottle valve is shut when not in use. Always use with a pressure reducer. Release the tension of the reducer spring before opening the bottle valve. Do not stand in front of the bottle outlet when opening the valve. Ensure proper ventilation during usage. Do not transfer hydrogen from one bottle to another.

Do not mix gas in the bottle. Make sure the bottles cannot be knocked over. Keep away from sunlight and sources of heat. Store in a corrosion-free environment. Do not use damaged or unlabelled bottles.

B.8. Bottled, compressed air

Compressed gas at high pressure. Use with caution in the presence of combustible substances since the self-ignition temperature of most of the organic compounds in the air is considerably lower under high pressure. Make sure the bottle valve is always closed when not in use. Always use a pressure reducer. Release the tension of the reducer spring before opening the bottle valve. Do not stand in front of the bottle outlet when opening the valve.

Do not switch the gas from one bottle to another. Do not mix gas in the bottle. Make sure the bottles cannot be knocked over. Keep away from sunlight or sources of heat. Store in a corrosion-free environment. Do not use damaged or unlabelled bottles. Air intended for technical purposes must not be used for inhaling or for respiratory apparatus.

PRECISION VALUES OF THE METHOD

1. Analysis of the collaborative test results

The precision values of the method are given in the table 1 below.

The interlaboratory test carried out to obtain these data was organised by the International Olive Council and coordinated by Enrico Tiscornia and Raffaella Boggia from the University of Genoa. Twelve laboratories took part from five countries.

The test was carried out on five different samples and their corresponding replicates (a and b). The identification of the oils was as follows:

- A Extra virgin olive oil
- B Lampante virgin olive oil
- C Refined olive oil
- D Refined olive oil + re-esterified oil (90:10)
- E Refined olive oil + re-esterified oil (80:20)

The test results underwent statistical analysis according to the rules laid down in the ISO 5725 standards **Accuracy (trueness and precision) of measurement methods and results**. Outliers were examined by applying the Cochran and Grubbs tests to the laboratory results for each determination (replicates a and b) and each sample.

The table contains the following terms, which mean:

n number of laboratories which participated in the test;

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%. (Repeatability.);
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%.(Reproducibility.);
S_R	Reproducibility standard deviation;
RDS_R (%)	Reproducibility coefficient of variation ($S_R \times 100/\text{mean}$).

Table 1 :

	A	B	C	D	E
n	12	12	12	12	12
outliers	0	0	0	0	0
mean	0,5	0,8	0,9	1,8	2,8
r	0,11	0,11	0,17	0,10	0,26
S_r	0,04	0,04	0,06	0,04	0,09
RSD_r(%)	8,9	5,4	6,8	2,0	3,3
R	0,14	0,27	0,26	0,56	0,86
S_R	0,05	0,10	0,09	0,20	0,31
RSD_R(%)	11,1	12,7	10,2	11,1	10,9

2. Analysis of the collaborative IOOC test results in 2017 for the aptitude test

Only a sample of Virgin olive oil with a quantifiable content of alkyl ester, adulterated with 10% refined olive oil and 2% animal fat has been tested.

Table 2 : 2-Glyceryl monopalmitate

2-Glyceryl monopalmitate (%)	n	Consensus mean	S _r	S _R
Regulatory method	23	1,8	0,03	0,08
Alternative solvent method	23	1,8	0,03	0,08
Evaluation	Calculated	Limit	Conclusion/Comments	
Difference (Regulatory method- Alternative solvent method)	0,0			
Test F repeatability	1,00	2,08	F cal < F limit	
Test F reproducibility	1,13	2,08	F cal < F limit	
Current reproducibility Regulatory	0,08	0,23	S _R obtained is smaller than current S _R	
T Student	1,29	2	t cal < t limit	

Where Regulatory Method is with the use of hexane as solvent;

Where Alternative solvent method is with the use of isooctane as solvent.

The comparison of the results has 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 regulated method and the obtained after using the alternative solvent.

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, was calculated.

The currently published precision value for the studied level has also been compared, and that obtained with the use of alternative solvents.