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DETERMINATION OF COMPOSITION OF TRIACYLGLYCEROLS AND COMPOSITION AND CONTENT OF DI-ACYLGLYCEROLS BY CAPILLARY GAS CHROMATOGRAPHY, IN VEGETABLE OILS

1 SCOPE

This method describes determination of composition and content of the composition of triacylglycerol and of the composition and content of di-acylglycerols (DAG) by capillary gas chromatography in vegetable oils with a lauric acid content below 1 %.

2 PRINCIPLE OF THE METHOD

The fatty substance, after adding the internal standard and silylating reagent, is solved in a suitable reagent and directly injected in the gas chromatographic apparatus. Triacylglycerols are resolved on the basis of their carbon atom number, while di-acylglycerols are resolved in function of their Carbon atom number and structure. 1,2 structures show a lower retention time than 1,3 ones.
Unsaturated di-acylglycerol structures don't affect retention time therefore saturated and unsaturated diglycerides are eluted together. 1,2 and 1,3 di-acylglycerol structures are identified through retention time and the percentage content of 1,2 structure is determined through the ratio of 1,2 diglyceride peaks on the sum of all di-acylgycerol peaks; total diglycerides are quantified through the ration between the sum of all di-acylglycerol peaks areas and the internal standard.

3 MEANING OF DETERMINATION

The profile of triglycerides is characteristic for each kind of oil, as it depends on the fatty acid composition, as well as on the biosynthesis rules, so that it can be used to check for the presence of extraneous oils.

Triglyceride biosynthesis pathway develops through 1,2 di-acylglycerols. However this reaction isn't 100 % completed therefore little 1,2 diglyceride amounts remain. During storage or the technological processes, partial hydrolysis can occur with also 1,3 diglyceride origin; it is also possible to get this result through the transposition from 1,2 to 1,3 di-acylglycerol. The speed of this transposition depends on oil acidity. During technological treatments a transposition from 1,2 to 1,3 usually occurs up to reach the equilibrium (≈ 33% of 1,2-DAG - 66% of 1,3-DAG). Therefore by assessment of 1,2 percentage content it's related the olive oil freshness or eventual carried out treatments.

4 APPARATUS

4.1 **Analytical balance** suitable to perform weighing to an accuracy of within +/- 0,1 mg.

4.2 **Gas chromatograph** for use with a capillary column, equipped with a system for direct on-column for cold injection

4.2.1 **Thermostat-controlled oven** with temperature programming.

4.2.2 **Cold injector** for on-column injection or PTV injector with on-column insert" (liner guiding the syringe needle into the column inlet).

4.2.3 **Flame-ionisation detector** and converter-amplifier.

4.2.4 **Recorder-integrator** for use with the converter-amplifier (4.2.3), rate of response below 1 second, with variable paper speed.

4.2.5 **Capillary column, fused silica,** 3 – 7 m length, 0,25 – 0,32 mm internal diameter, internally coated with SE 52, SE 54 liquid phase (5% diphenyl dimethyl polysiloxane) to a uniform thickness of 0,10 – 0,15 µm.

(note: thermal degradation may affect analysis by diminishing recovery on C58 an C60, if this happens a shorter column should be used) – Normally a 5 m column leads to acceptable results.

4.3 **Microsyringe**, 10 µl, suitable for on-column injection.

4.4 **Microsyringe,** 100 µl

4.5 Usual laboratory glassware.

5 REAGENTS

5.1 **Carrier gas** : hydrogen or helium, pure, for gas chromatography.

5.2 **Auxiliary gases** :

- Hydrogen, pure, for gas chromatography;
- Air, pure, for gas chromatography;
- Nitrogen, pure for gas chromatography.

5.3 **Silylating reagent :** mix equal volumes of:

1) pyridine
2) bistrimethylsilylfluoroacetamide/trimethylchlorosilane (BSTFA/TMCS 99:1, *v/v*).

Note: ready to use solution are available; the silylating solution should be freshly made.

5.4 **n-Heptane** for analysis

5.5 **Reference samples :** pure diglycerides and triglycerides and their mixtures, with known composition.

5.6 **Methyl-terbutyl-ether**

5.7 **Dinonadecanoine sample solution** (internal standard), 0,1 % w/v in methyl-terbutyl-ether.

6 PROCEDURE

6.1 Check the gas chromatographic apparatus and capillary column condition

Fit the column to the gas chromatograph (4.2), connecting the inlet port to the on-column system and the outlet port to the detector. Check the gas chromatography apparatus (operation of gas loops, detector and recorder efficiency, etc.).

Run a light flow of gas through the column, then switch on the gas chromatography apparatus. Gradually heat until a temperature of 350 °C is reached after approximately 4 hours.

Maintain this temperature for at least 2 hours, then regulate the apparatus to the operating conditions (regulate gas flow, light flame, connect to electronic recorder, regulate oven temperature for column, regulate detector, etc.). Record the signal at a sensitivity at least twice as high as that required for the analysis. The base line should be linear, with no peaks of any kind, and must not have any drift.

Negative straight-line drift indicates that the column connections are not correct ; positive drift indicates that the column has not been properly conditioned.

6.2 Choice of operating conditions

The operating conditions are generally as follows, *but temperature program can be modified according to column and instrument characteristics* (note 1) :

- The injector temperature has to be at least 10 °C below the vaporization temperature (99 °C) of the employed solvent (n-Heptane);
- Detector temperature: 350 °C;
- Column temperature: 80 °C at first (1') ----------> 220 °C (20 °C/min) ----------> 340 °C (10') (5 °C/min);
- Carrier gas : hydrogen or helium at the optimal linear speed for the gas chosen:
- Amount of injected substance : 0,5 – 1 µl of solution prepared as in 6.3.

Note 1: in order to correctly calculate 1,2 / total DAG ratio resolution should be able at least to separate each couple of isomers down to a 10% valley.

6.3 Procedure of analysis

Weigh (4.1) with precision about 100 mg of oil in a glass bottom conical tube and add 1 ml of internal standard solution (5.7). Shake the sample up to a complete solution, take up with a microsyringe (4.4) 20 – 30 µl of solution, put it inside a new glass tube (with a stopper) and dry by a gentle nitrogen stream.
Add 200 µl of silylation reagent and allow the mixture to stand until silylation is complete (note 2). Dry by a soft nitrogen flow (note 1), add 2 ml of n-Heptane and shake. Inject (4.3) from 0,5 to 1 µl of solution, at the conditions reported in 6.2 point.

Note 2 : To determine when derivatization is complete, analyze aliquots of the sample at selected time intervals until no further increase in product peaks is observed. Normally at room temperature complete silylation is achieved in 20 minutes. Heating the sample at 60 °C for 20-30 minutes should be evaluated when derivatization is not complete.

6.4 Peak identification

The triglycerides peaks identification is carried out from the retention times by comparing them with mixtures of known composition; they are eluted in the following order: C50, C52, C54, C56, C58, C60, C62.

The diglycerides peak identification is carried out from the retention times by comparing them with mixtures of known di-acylglycerols. Diglycerides are eluted according to the following order :
1,2 C32; 1,3 C32; 1,2 C34; 1,3 C34; 1,2 C36 1,3 C36 (see figures 1,2,3 and 4).

6.5 Determination of percentage content of each triacylglycerol

Calculate the areas of each peak through an electronic integrator.
Results are expressed as percentage of C50, C52, C54, C56, C58, C60, C62
Other peaks showed by gas chromatographic run haven't to be considered.

All triacylglyceroles, including those lying in between the major peaks, must be integrated.
Each odd triglycerides (see figure 5) is combined to the one that precedes in order to have the sum of all triglycerides normalized to 100%.

6.6. Determination of percentage content of each 1,2 di-acylglycerol

Calculate the areas of each peak through electronic integrator.
Results are expressed as percentage of 1,2 C32, 1,3 C32, 1,2 C34, 1,3 C34, 1,2 C36, 1,3 C36 on the sum of DAG areas. Other eventual peaks showed by gas chromatographic run haven't to be considered :

$$\% \text{ of single diglyceride} = \frac{A_x}{\sum A_{DAG}}$$

Where :
A_x : area corresponding to the peak of diglyceride x,
$\sum A_{DAG}$: sum of areas of all diglyceride peaks.

Express these results to one decimal place.

6.7 Determination of weigh percentage total content of di-acylglycerols

By using the integrator, calculate the areas of internal standard and diglycerides compounds, by considering only the peaks that are reported in previous 6.5 point.
You compute each single diglyceride, in % weight (gr/100g) of fat matter, as follows :

$$\text{Single diglyceride, \% in weight (g./100g)} = \frac{A_x \times m_s \times 100}{A_s \times m}$$

where :
A_x : area corresponding to the peak of single diglyceride,
A_s : area corresponding to internal standard peak,
m_s : added amount (mg) of internal standard,
m : amount (mg) of sample, take up for the determination.

Express these results to one decimal place.

7 EXPRESSION OF RESULTS

Single triglyceride percentage. Compute the sum of percentages of triglycerides. Results have to be expressed to two decimal place.

1*,2 diglyceride percentage*. Compute the sum of percentages of 1,2 diglycerides. Results have to be expressed to one decimal place.

Weight percentage. Sum the amounts of all detected diglycerides. Results have to be expressed to one decimal place.

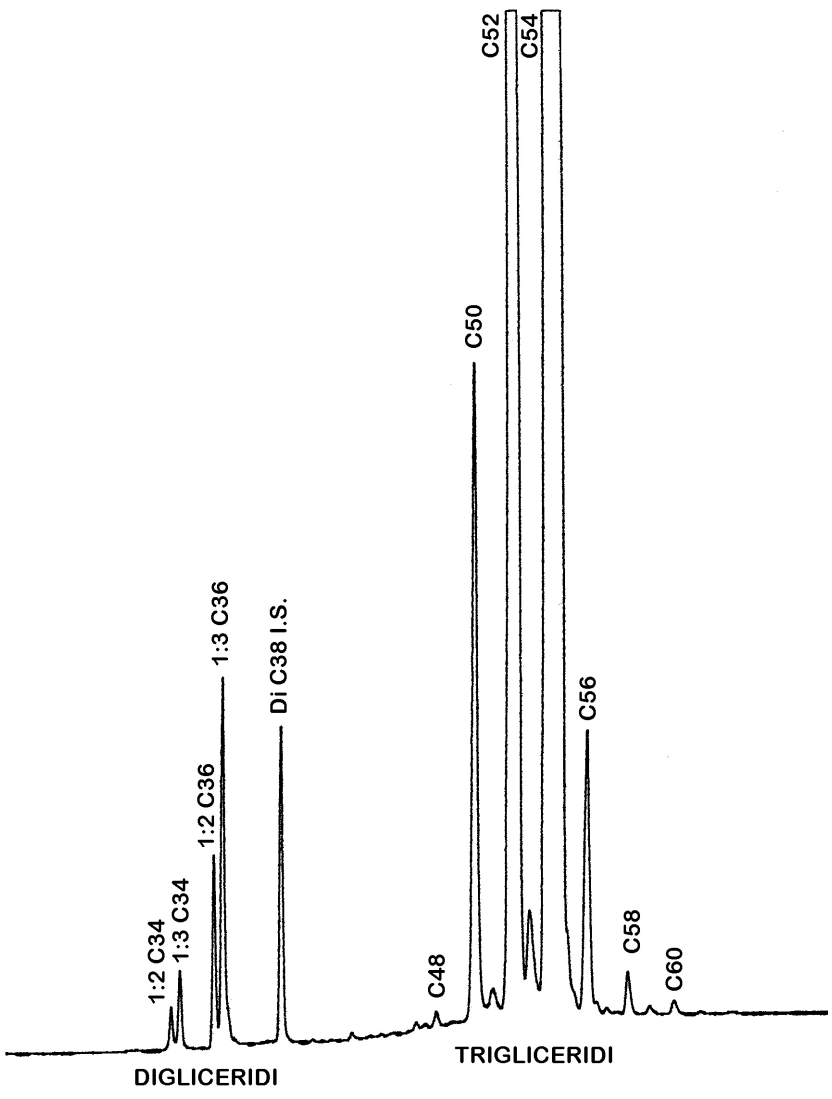

Figure 1: GLC trace of diacylglycerol an triacylglycerol of an olive oil sample

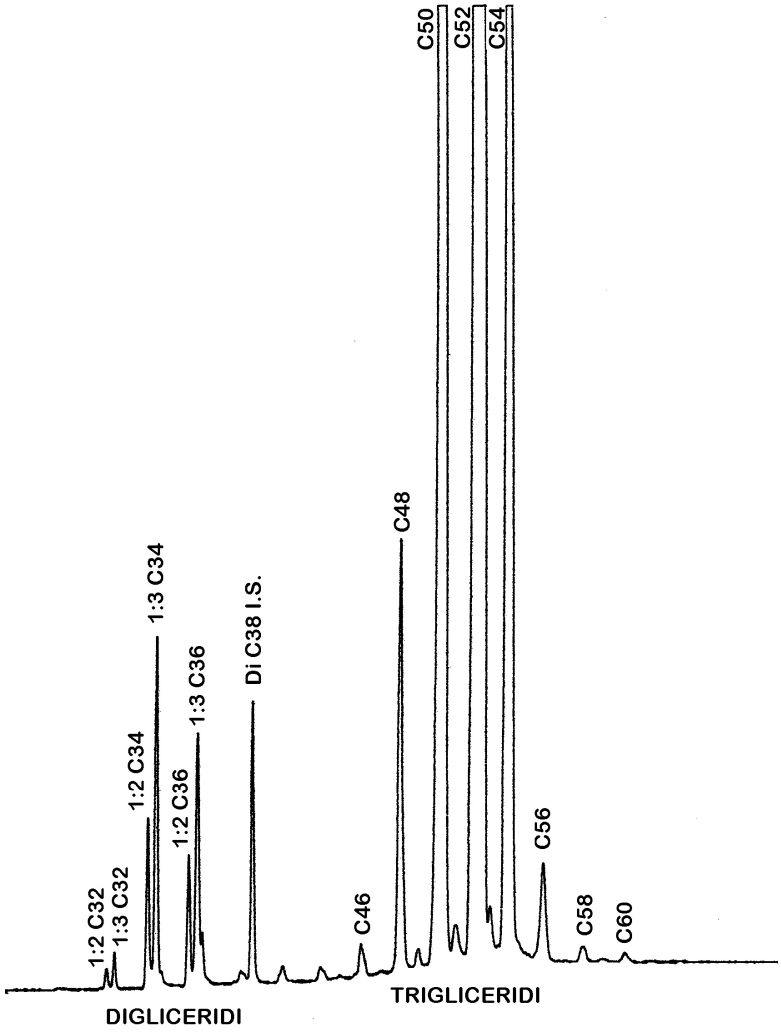

Figure 2: GLC trace of diacylglycerol an triacylglycerol of a palm olein sample

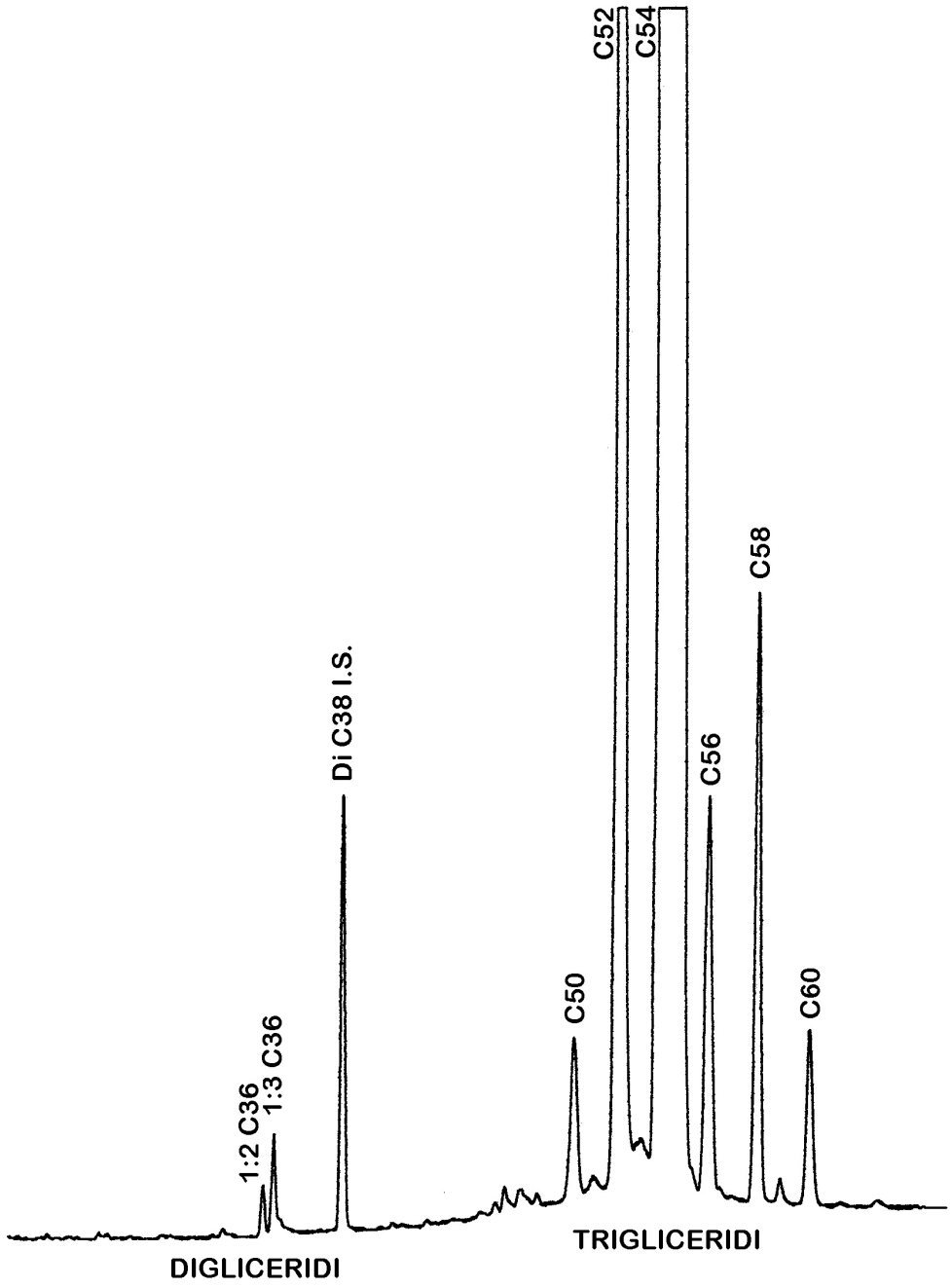

Figure 3: GLC trace of diacylglycerol an triacylglycerol of an high oleic sunflower oil sample

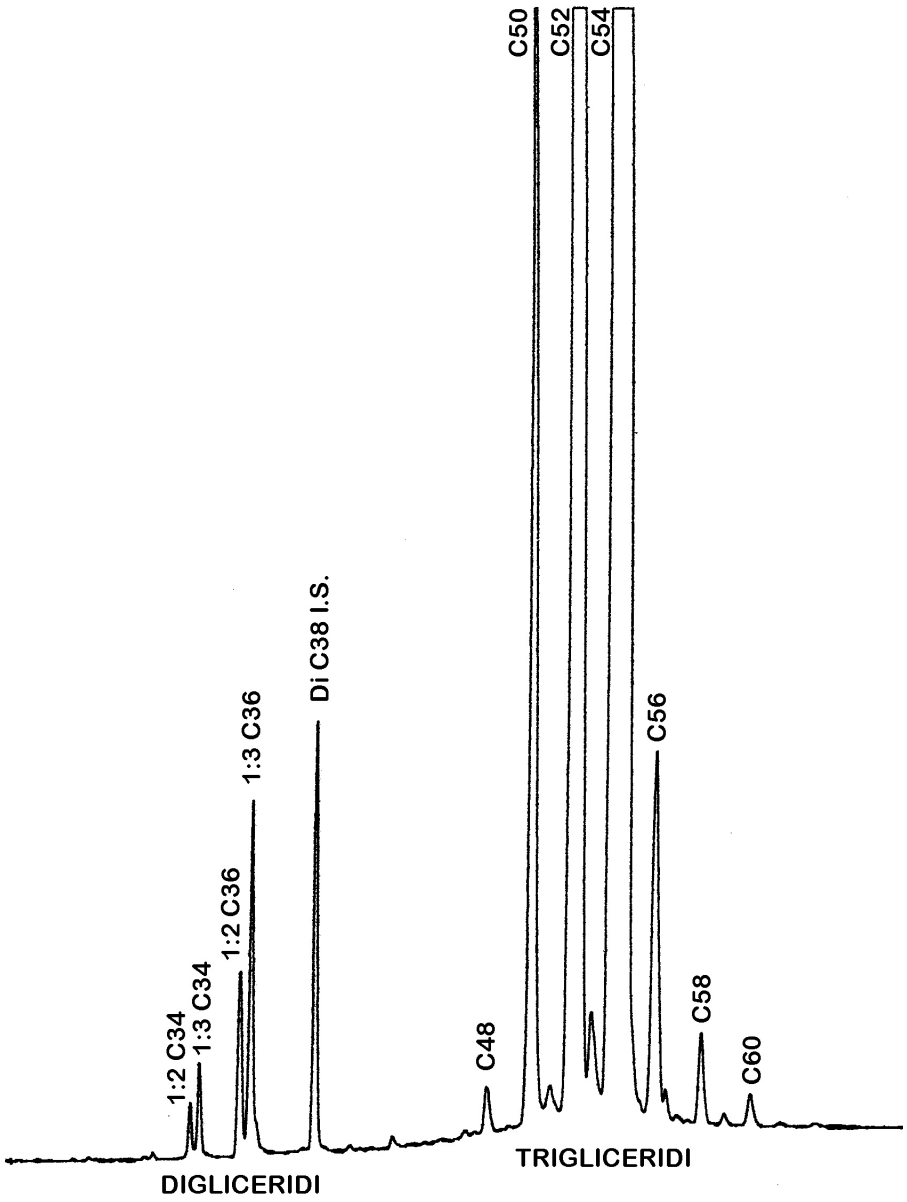

Figure 4: **GLC trace of diacylglycerol an triacylglycerol of an olive oil mixed with palm olein and high oleic sunflower oil**

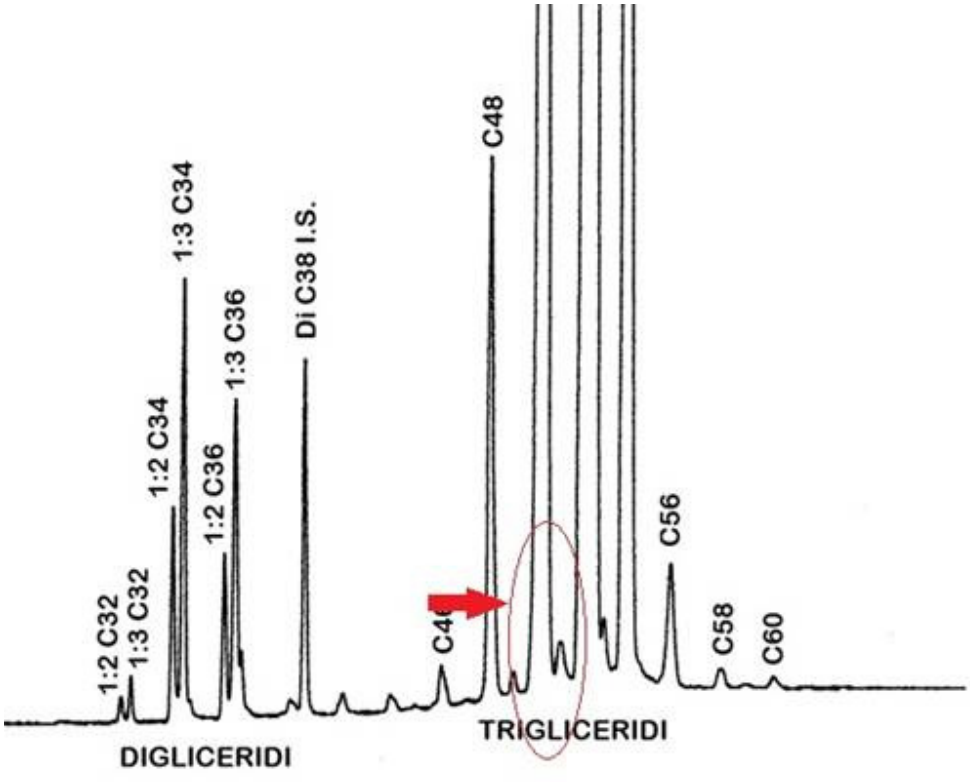

Figure 5: example of peaks integration

PRECISION VALUES OF THE METHOD FOR DETERMINATION OF COMPOSITION OF TRIACYLGLYCEROLS AND COMPOSITION AND CONTENT OF DI-ACYLGLYCEROLS

1. Analysis of the collaborative test results

The precision values of the method are given in the table overleaf.

Ten laboratories took part in the collaborative test arranged by Executive Secretariat in 2013. The laboratories were from five countries.

The test was performed in duplicate on five samples:

Sample 1 – Extra virgin olive oil with 5% of extraneous oils (desterolate sunflower, avocado, peanut, rapeseed and hazelnut oils)
Sample 2 – Extra virgin olive oil with 10% of extraneous oils (desterolate sunflower, avocado, peanut, rapeseed and hazelnut oils)
Sample 3 – High quality extra virgin olive oil
Sample 4 – Extra virgin olive oil from retail
Sample 5 – Extra virgin olive oil with palm, desterolate sunflower, avocado and hazelnut oils

The results of the collaborative test organised by the IOC Executive Secretariat underwent statistical analysis according to ISO 5725 "Accuracy (trueness and precision) of measurement methods and results". Outliers were examined by applying Cochran's and Grubbs's tests to the laboratory results for each determination (replicates a and b) .

The tables 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

RSD_r (%) repeatability coefficient of variation (S_r x 100 / 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

RSD_r (%) reproducibility coefficient of variation (S_r x 100 / mean)

Single triglyceride percentage (%)

TAG 48

	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5
N	10	10	10	10	10
outliers	1	1	2	2	1
mean	0,088	0,115	0,044	0,055	0,224
r	0,023	0,021	0,020	0,020	0,044
S_r	0,0083	0,0074	0,0070	0,0070	0,0157
RSD_r(%)	9,4%	6,5%	16,0%	12,8%	7,0%
R	0,0545	0,0808	0,0683	0,0589	0,0858
S_R	0,0195	0,0289	0,0244	0,0210	0,0306
RSD_R(%)	22,0%	25,2%	55,9%	38,4%	13,7%

TAG 58

	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5
N	10	10	10	10	10
outliers	0	0	0	0	1
mean	0,305	0,405	0,255	0,221	0,369
r	0,034	0,062	0,077	0,029	0,040
S_r	0,012	0,022	0,028	0,010	0,014
RSD_r(%)	3,9%	5,5%	0,8%	4,7%	3,8%
R	0,15	0,19	0,15	0,14	0,19
S_R	0,052	0,068	0,054	0,050	0,068
RSD_R(%)	17,0%	16,8%	21,2%	22,6%	18,4%

1,2 diglyceride percentage (%)

1*,2 DAG*

	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5
N	10	10	10	9	8
outliers	1	1	1	1	0
mean	36,83	31,36	49,85	29,43	29,24
r	1,5	4,3	4,8	4,8	1,7
S_r	0,53	1,5	1,7	1,7	0,62
RSD_r(%)	1,4%	4,9%	3,5%	5,9%	2,1%
R	16	11	6,9	7,9	9,4
S_R	5,8	4,0	2,5	2,8	3,4
RSD_R(%)	15,7%	12,8%	5,0%	9,5%	11,6%

Diglycerides content (g/100g))

Total DAG

	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5
N	10	10	10	10	10
outliers	1	1	1	1	1
mean	1,48	1,62	1,23	1,47	2,33
r	0,16	0,21	0,19	0,15	0,21
S_r	0,058	0,074	0,067	0,053	0,076
RSD_r(%)	3,9%	4,6%	5,5%	3,6%	3,2%
R	0,45	0,59	0,48	0,50	0,67
S_R	0,16	0,21	0,17	0,18	0,24
RSD_R(%)	10,8%	13,0%	13,8%	12,2%	10,3%

3. References

ISO 5725 – Accuracy (trueness and precision) of measurement methods and results – Part 1: General principles and definitions.

ISO 5725 - Accuracy (trueness and precision) of measurement methods and results - Part 2: Basic method for the determination of repeatability and reproducibility of a standard measurement method.

ISO 5725 – Accuracy (trueness and precision) of measurement methods and results – Part 5: Alternative methods for the determination of the precision of a standard measurement method.

ISO 5725 – Accuracy (trueness and precision) of measurement methods and results – Part 6: Use in practice of accuracy values.

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