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METHOD OF ANALYSIS

DETERMINATION OF THE DIFFERENCE BETWEEN ACTUAL AND THEORETICAL CONTENT OF TRIACYGLYCEROLS WITH ECN 42

1. Scope

Determination of the absolute difference between the experimental values of triacylglycerols (TAGs) with equivalent carbon number 42 ($ECN42_{HPLC}$) obtained by determination in the oil by high performance liquid chromatography and the theoretical value of TAGs with an equivalent carbon number of 42 ($ECN\ 42_{theoretical}$) calculated from the fatty acid composition.

2. Field of application

The standard is applicable to olive oils. The method is applicable to the detection of the presence of small amounts of seed oils (rich in linoleic acid) in every class of olive oils.

3. Principle

The content of triacylglycerols with ECN 42 determined by HPLC analysis and the theoretical content of triacylglycerols with ECN 42 (calculated on the basis of GLC determination of fatty acid composition) correspond within a certain limit for genuine olive oils. A difference larger than the values adopted for each type of oil points out that the oil contains seed oils.

4. Method

The method for the calculation of the theoretical content of triacylglycerols with ECN 42 and of the difference with respect to the HPLC data is essentially by the co-ordination of analytical data obtained by means of other methods. It is possible to distinguish three phases: determination of fatty acid composition by capillary gas chromatography, calculation of theoretical composition of triacylglycerols with ECN 42, HPLC determination of ECN 42 triacylglycerols.

4.1. Apparatus

4.1.1 Round-bottomed flasks, 250 and 500 mL.
4.1.2 Beakers 100 mL.
4.1.3 Glass chromatographic column, 21 mm internal diameter, 450 mm length, with cock and normalised cone (female) at the top.
4.1.4 Separating funnels, 250 mL, with normalised cone (male) at the bottom, suitable for connection to the top of the column.
4.1.5 Glass rod, 600 mm length.
4.1.6 Glass funnel, 80 mm diameter.
4.1.7 Volumetric flasks, 50 mL.
4.1.8 Volumetric flasks, 20 mL.
4.1.9 Rotary evaporator.
4.1.10 High performance liquid chromatograph, allowing thermostatic control of column temperature.
4.1.11 Injection units for 10 µl delivery.
4.1.12 Detector: differential refractometer. The full scale sensitivity should be at least 10^{-4} units of refractive index.
4.1.13 Column: stainless steel tube 250 mm length x 4.5 mm internal diameter packed with 5 µm diameter particles of silica with 22 to 23% carbon in the form of octadecylsilane*.
4.1.14 Data processing software.
4.1.15 Vials, of about 2 mL volumes, with Teflon-layered septa and screw caps.

*Examples: Lichrosorb (Merck) RP 18 Art 50333
Lichrosphere (Merck) 100 CH18 Art 50377 or equivalent

4.2. Reagents

The reagents should be of analytical purity. Elution solvents should be de-gassed, and may be recycled several times without effect on the separations.

4.2.1 Petroleum ether 40-60°C chromatographic grade or hexane. Hexane may be replaced by iso-octane (2,2,4-trimethyl pentane in chromatography grade), provided that comparable precision values are achieved (see Precision values of the method with the used of isooctane in page 23), Solvents with higher boiling point than n-hexane take longer to evaporate. However, they are preferred due to the toxicity of hexane.

4.2.2 Ethyl ether, peroxide-free, freshly distilled.

4.2.3 Elution solvent for purifying the oil by column chromatography mixture petroleum ether/ethyl ether 87/13 (v/v).

4.2.4 Silica gel, 70-230 mesh, type Merck 7734, with water content standardised at 5% (w/w/).

4.2.5 Glass wool.

4.2.6 Acetone for HPLC.

4.2.7 Acetonitrile or propionitrile for HPCL.

4.2.8 HPLC elution solvent: acetonitrile + acetone (proportions to be adjusted to obtain the desired separation; begin with 50:50 mixture) or propionitrile.

4.2.9 Solubilisation solvent: acetone.

4.2.10 Reference triglycerides: commercial triglycerides (tripalmitin, triolein, etc.) may be used and the retention times then plotted in accordance with the equivalent carbon number, or alternatively reference chromatograms obtained from soya oil, mixture 30:70 soya oil - olive oil and pure olive oil (see notes 1 and 2 and figures 1, 2, 3, 4).

4.2.11 Solid phase extraction column with silica phase 1 g, 6 mL.

4.2.12 Heptane, chromatographic quality. Heptane may be replaced by iso-octane (2,2,4-trimethyl pentane in chromatography grade.

4.3. Sample preparation

As a number of interfering substances can give rise to false positive results, the sample must always be purified according to either procedure 4.3.1 & 4.3.2 (purification on silica gel column) or procedure 4.3.3 (purification silica gel SPE).

4.3.1 Purification on silica gel column - Chromatographic column preparation

Fill the column (4.1.3.) with about 30 mL of elution solvent (4.2.3.), then introduce inside the column some glass wool (4.2.5.) pushing it to the bottom of the column by means of the glass rod (4.1.5.)

In a 100 mL beaker, suspend 25 g of silica gel (4.2.4.) in 80 mL of elution mixture (4.2.3.), then transfer it to the column by means of a glass funnel (4.1.6.).

To ensure the complete transfer of the silica gel to the column, wash the beaker with the elution mixture and transfer the washing portions to the column too.

Open the cock and let the solvent elute from the column until its level is about 1 cm over the silica gel.

4.3.2 Purification on silica gel column- Column chromatography

Weigh with the accuracy of 0.001 g, 2.5 ± 0.1 g of oil, previously filtered, homogenised and anhydrified, if necessary, in a 50 mL volumetric flask (4.1.7.). Dissolve it in about 20 mL of elution solvent (4.2.3.). If necessary, slightly heat it to make the dissolution easily. Cool at room temperature and adjust the volume with elution solvent.

By means of a volumetric pipette, introduce 20 mL of solution inside the column prepared according to 4.3.1., open the cock and let the solvent elute to the silica gel layer level.

Then elute with 150 mL of elution solvent (4.2.3.), adjusting the solvent rate at about 2 mL/min (150 mL will take about 60-70 minutes to pass through the column).

The eluate is recovered in a 250 mL round-bottomed flask (4.1.1.) previously tared in an oven and exactly weighed. Eliminate the solvent at reduced pressure in a rotary evaporator (4.1.9) and weigh the residue that will be used to prepare the solution for HPLC analysis and for methyl ester preparation.

The sample recovery from the column must be 90% at least for the extra virgin, virgin, ordinary, refined and olive oil categories, and a minimum of 80% for lampante and olive-pomace oils.

4.3.3 SPE purification

Silica SPE column (4.2.11) is activated by passing 6 mL of hexane (4.2.1) under vacuum, avoiding dryness.

Weigh to an accuracy of 0.001 g, 0.12 g in a 2 mL vial (4.1.15) and dissolve with 0.5 mL of hexane (4.2.1).

Load the SPE column with the solution and elute with 10 mL of hexane-diethyl ether (87:13 v/v) (4.2.3) under vacuum. The combined eluates are homogenised and divided in two similar volumes. The collected fractions are evaporated to dryness in a rotary evaporator (4.1.9) under reduced pressure at room temperature. The first aliquot is dissolved in 1 mL of heptane and the solution is ready for fatty acid methyl ester preparation and analysis by GC.

The second aliquot is evaporated and the residue is dissolved in 1 mL of acetone (4.2.6) for triglyceride analysis by HPLC (4.4).

4.4. HPLC analysis

4.4.1 Preparation of the samples for chromatographic analysis

A 5% solution of the sample to be analysed is prepared by weighing 0.5 ± 0.001 g of the sample into a 10 ml graduated flask and making up to 10 ml with the solubilisation solvent (4.2.9.).

4.4.2 Procedure

Set up the chromatographic system. Pump elution solvent (4.2.8) at a rate of 1.5 ml/min to purge the entire system. Wait until a stable base line is obtained. Inject 10 µl of the sample prepared as in 4.3.

4.4.3 Calculation and expression of results

Use the area normalisation method, i.e. assume that the sum of the areas of the peaks corresponding to TAGs from ECN 42 up to ECN 52 is equal to 100%. Calculate the relative percentage of each triglyceride using the formula:
% triglyceride = area of peak x 100/ sum of peak areas.
The results should be given to at least two decimal places.
See notes 1, 2, 3 and 4.

4.5. Calculation of triacylglycerols composition (moles %) from fatty acid composition data (area %)

4.5.1 Determination of fatty acid composition

Fatty acid composition is determined following “Determination of Fatty Acid Methyl Esters by Gas Chromatography” COI/T.20/Doc.No33/Rev.1 2017.

4.5.2 Fatty acids for calculation

Glycerides are grouped by their Equivalent Carbon Number (ECN), taking into account the following equivalencies between ECN and fatty acids. Only fatty acids with 16 and 18 carbon atoms were taken into consideration, because only these are important for olive oil. The fatty acids should be normalised to 100%.

Fatty acid (FA)	Abbreviation	Molecular weight (MW)	ECN
Palmitic acid	P	256.4	16
Palmitoleic acid	Po	254.4	14
Stearic acid	S	284.5	18
Oleic acid	O	282.5	16
Linoleic acid	L	280.4	14
Linolenic acid	Ln	278.4	12

4.5.3 Conversion of area % into moles for all fatty acids

$$\left.\begin{array}{lll} \text{moles P} = \dfrac{\text{area \% P}}{\text{MW P}} & \text{moles S} = \dfrac{\text{area \% S}}{\text{MW S}} & \text{moles Po} = \dfrac{\text{area \% Po}}{\text{MW Po}} \\ \text{moles O} = \dfrac{\text{area \% O}}{\text{MW O}} & \text{moles L} = \dfrac{\text{area \% L}}{\text{MW L}} & \text{moles Ln} = \dfrac{\text{area \% Ln}}{\text{MW Ln}} \end{array}\right\} \quad (1)$$

4.5.4 Normalisation of fatty acid moles to 100%

$$\left.\begin{array}{ll} \text{moles \% P (1,2,3)} = & \dfrac{\text{moles P} * 100}{\text{moles (P + S + Po + O + L + Ln)}} \\ \text{moles \% S (1,2,3)} = & \dfrac{\text{moles S} * 100}{\text{moles (P + S + Po + O + L + Ln)}} \\ \text{moles \% Po (1,2,3)} = & \dfrac{\text{moles Po} * 100}{\text{moles (P + S + Po + O + L + Ln)}} \\ \text{moles \% O (1,2,3)} = & \dfrac{\text{moles O} * 100}{\text{moles (P + S + Po + O + L + Ln)}} \\ \text{moles \% L (1,2,3)} = & \dfrac{\text{moles L} * 100}{\text{moles (P + S + Po + O + L + Ln)}} \end{array}\right\} \quad (2)$$

$$\text{moles \% Ln (1,2,3)} = \frac{\text{moles Ln} * 100}{\text{moles (P + S + Po + O + L + Ln)}}$$

The result gives the percentage of each fatty acid in moles % in the overall (1, 2, 3–) position of the TAGs.

Then the sum of the saturated fatty acids P and S (SFA) and the unsaturated fatty acids Po, O, L and Ln (UFA) are calculated:

$$\left.\begin{array}{l}\text{moles \% SFA} = \text{moles \% P} + \text{moles \% S} \\ \text{moles \% UFA} = 100 - \text{moles \% SFA}\end{array}\right\} \quad (3)$$

4.5.5 Calculation of the fatty acid composition in 2- and 1,3- positions of TAGs

The fatty acids are distributed to three pools as follows: two identical for 1- and 3-positions and one for 2-position, with different coefficients for the saturated (P and S) and unsaturated acids (Po, O, L and Ln).

4.5.5.1 Saturated fatty acids in 2-position [P(2) and S(2)]

$$\left.\begin{array}{l}\text{moles \% P(2)} = \text{moles \% P(1,2,3)} * 0.06 \\ \text{moles \% S(2)} = \text{moles \% S (1,2,3)} * 0.06\end{array}\right\} \quad (4)$$

4.5.5.2 Unsaturated fatty acids in 2-position [Po(2), O(2), L(2) and Ln(2)]:

$$\left.\begin{array}{l}\text{moles \% Po(2)} = \dfrac{\text{moles \% Po(1,2,3)}}{\text{moles \% UFA}} * (100 - \text{moles \% P(2)} - \text{moles \% S(2)} \\[2ex] \text{moles \% O(2)} = \dfrac{\text{moles \% O(1,2,3)}}{\text{moles \% UFA}} * (100 - \text{moles \% P(2)} - \text{moles \% S(2)} \\[2ex] \text{moles \% L(2)} = \dfrac{\text{moles \% L(1,2,3)}}{\text{moles \% UFA}} * (100 - \text{moles \% P(2)} - \text{moles \% S(2)}\end{array}\right\} \quad (5)$$

$$\text{moles \% Ln(2)} = \frac{\text{moles \% Ln(1,2,3)}}{\text{moles \% UFA}} * (\text{100-moles \% P(2)-moles \% S(2)}$$

4.5.5.3 Fatty acids in 1,3-positions [P(1,3), S(1,3), Po(1,3), O(1,3), L(1,3) and Ln(1,3)]:

$$\left.\begin{aligned}
\text{moles \% P(1,3)} &= \frac{\text{moles \% P(1,2,3)-moles \% P(2)}}{2} + \text{moles \% P(1,2,3)} \\
\text{moles \% S(1,3)} &= \frac{\text{moles \% S(1,2,3)-moles \% S(2)}}{2} + \text{moles \% S(1,2,3)} \\
\text{moles \% Po(1,3)} &= \frac{\text{moles \% Po(1,2,3)-moles \% Po(2)}}{2} + \text{moles \% Po(1,2,3)}
\end{aligned}\right\} \quad (6)$$

$$\left.\begin{aligned}
\text{moles \% O(1,3)} &= \frac{\text{moles \% O(1,2,3)-moles \% O(2)}}{2} + \text{moles \% O(1,2,3)} \\
\text{moles \% L(1,3)} &= \frac{\text{moles \% L(1,2,3)-moles \% L(2)}}{2} + \text{moles \% L(1,2,3)} \\
\text{moles \% Ln(1,3)} &= \frac{\text{moles \% Ln(1,2,3)-moles \% Ln(2)}}{2} + \text{moles \% Ln(1,2,3)}
\end{aligned}\right\} \quad (6)$$

4.5.6 Calculation of triacylglycerols

4.5.6.1 TAGs with one fatty acid (AAA, here LLL, PoPoPo)

$$\text{moles \% AAA} = \frac{\text{moles \% A(1,3)} * \text{moles \% A(2)} * \text{moles \% A(1,3)}}{10{,}000} \quad (7)$$

4.5.6.2 TAGs with two fatty acids (AAB, here PoPoL, PoLL)

$$\text{moles \% AAB} = \frac{\text{moles \% A(1,3)} * \text{moles \% A(2)} * \text{moles \% B(1,3)} * 2}{10{,}000} \quad (8)$$

$$\text{moles \% ABA} = \frac{\text{moles \% A(1,3)} * \text{moles \% B(2)} * \text{moles \% A(1,3)}}{10{,}000}$$

4.5.6.3 TAGs with three different fatty acids (ABC, here OLLn, PLLn, PoOLn, PPoLn)

$$\left.\begin{aligned}
\text{moles \% ABC} &= \frac{\text{moles \% A(1,3)} * \text{moles \% B(2)} * \text{moles \% C(1,3)} * 2}{10{,}000} \\
\text{moles \% BCA} &= \frac{\text{moles \% B(1,3)} * \text{moles \% C(2)} * \text{moles \% A(1,3)} * 2}{10{,}000} \\
\text{moles \% CAB} &= \frac{\text{moles \% C(1,3)} * \text{moles \% A(2)} * \text{moles \% B(1,3)} * 2}{10{,}000}
\end{aligned}\right\} \quad (9)$$

4.5.6.4 Triacylglycerols with ECN42

The triacylglycerols with ECN42 are calculated according to equations 7, 8 and 9 and are then given in order of expected elution in HPLC (normally only three peaks).

LLL
PoLL and the positional isomer LPoL

OLLn and the positional isomers OLnL and LnOL
PoPoL and the positional isomer PoLPo
PoOLn and the positional isomers OPoLn and OLnPo

PLLn and the positional isomers LLnP and LnPL
PoPoPo
SLnLn and the positional isomer LnSLn
PPoLn and the positional isomers PLnPo and PoPLn

The triacylglycerols with ECN42 are given by the sum of the nine triacylglycerols including their positional isomers. The results should be given to at least two decimal places.

5. Evaluation of the results

The calculated theoretical content and the content determined by the HPLC analysis are compared. If the difference in the absolute value of the HPLC data minus the theoretical data is greater than the values stated for the appropriate oil category in the standard, the sample contains seed oil.
Results are given to two decimal figures.

6. **Example** (The numbers refer to the sections in the text of the method)

6.1. Calculation of moles % fatty acids from GLC data (normalised area %)

The following data are obtained for the fatty acid composition by GLC:

FA MW	**P** 256.4	**S** 284.5	**Po** 254.4	**O** 282.5	**L** 280.4	**Ln** 278.4
Area %	10.00	3.00	1.00	75.00	10.00	1.00

6.2 Conversion of area % into moles for all fatty acids

$$\text{moles P} = \frac{10}{256.4} = 0.03900 \text{ moles P}$$

$$\text{moles S} = \frac{3}{284.5} = 0.01054 \text{ moles S}$$

$$\text{moles Po} = \frac{1}{254.4} = 0.00393 \text{ moles Po}$$

$$\text{moles O} = \frac{75}{282.5} = 0.26549 \text{ moles O}$$

$$\text{moles L} = \frac{10}{280.4} = 0.03566 \text{ moles L}$$

$$\text{moles Ln} = \frac{1}{278.4} = 0.00359 \text{ moles Ln}$$

Total = 0.35821 moles TAGs

See formula (1)

6.3 Normalisation of fatty acid moles to 100%

$$\text{moles \% P(1,2,3)} = \frac{0.03900 \text{ moles P*100}}{0.35821 \text{ moles}} = 10.887\%$$

$$\text{moles \% S(1,2,3)} = \frac{0.01054 \text{ moles S*100}}{0.35821 \text{ moles}} = 2.942\%$$

$$\text{moles \% Po(1,2,3)} = \frac{0.00393 \text{ moles Po*100}}{0.35821 \text{ moles}} = 1.097\%$$

See formula (2)

$$\text{moles \% O(1,2,3)} = \frac{0.26549 \text{ moles O*100}}{0.35821 \text{ moles}} = 74.116\%$$

$$\text{moles \% L(1,2,3)} = \frac{0.03566 \text{ moles L*100}}{0.35821 \text{ moles}} = 9.955\%$$

$$\text{moles \% Ln(1,2,3)} = \frac{0.00359 \text{ moles Ln*100}}{0.35821 \text{ moles}} = 1.002\%$$

Total moles % = 100.0%

Sum of the saturated and unsaturated fatty acids in the 1,2,3-position of TAGs:

moles % SFA = 10.887% + 2.942% = **13,829%**
moles % UFA = 100.000% - 13.829% = **86,171%**

See formula (3)

6.4 Calculation of the fatty acid composition in 2- and 1,3-positions of the TAGs

6.4.1 Saturated fatty acids in 2-position [P(2) and S(2)]

moles % P(2) = 10.887% * 0.06 = 0.653 moles %
moles % S(2) = 2.942% * 0.06 = 0.177 moles %

See formula (4)

6.4.2 Unsaturated fatty acids in 2-position [Po(1,3), O(1,3), L(1,3) and Ln(1,3)]

$$\text{moles \% Po(2)} = \frac{1.097\%}{86.171\%} * (100-0.653-0.177) = 1.262 \text{ moles \%}$$

$$\text{moles \% O(2)} = \frac{74.116\%}{86.171\%} * (100-0.653-0.177) = 85.296 \text{ moles \%}$$

See formula (5)

$$\text{moles \% L(2)} = \frac{9.955\%}{86.171\%} * (100-0.653-0.177) = 11.457 \text{ moles \%}$$

$$\text{moles \% Ln(2)} = \frac{1.002\%}{86.171\%} * (100-0.653-0.177) = 1.153 \text{ moles \%}$$

6.4.3 Fatty acids in 1,3-positions [P(1,3), S(1,3), Po(1,3), O(1,3), L(1,3) and Ln(1,3)]

$$\text{moles \% P(1,3)} = \frac{10.887 - 0.653}{2} + 10.887 = 16.004 \text{ moles \%}$$

$$\text{moles \% S(1,3)} = \frac{2.942 - 0.177}{2} + 2.942 = 4.325 \text{ moles \%}$$

$$\text{moles \% Po(1,3)} = \frac{1.097 - 1.262}{2} + 1.097 = 1.015 \text{ moles \%}$$

See formula (6)

$$\text{moles \% O(1,3)} = \frac{74.116 - 85.296}{2} + 74.116 = 68.526 \text{ moles \%}$$

$$\text{moles \% L(1,3)} = \frac{9.955 - 11.457}{2} + 9.955 = 9.204 \text{ moles \%}$$

$$\text{moles \% Ln(1,3)} = \frac{1.002 - 1.153}{2} + 1.002 = 0.927 \text{ moles \%}$$

6.5 Calculation of triacylglycerols

From the calculated fatty acid composition in sn-2- and sn-1,3-positions:

FA in	1,3-pos	2-pos
P	16.004%	0.653%
S	4.325%	0.177%
Po	1.015%	1.262%
O	68.526%	85.296%
L	9.204%	11.457%
Ln	0.927%	1.153%
Sum	100.0%	100.0%

the following triacylglycerols are calculated:

LLL
PoPoPo

PoLL with 1 positional isomer
SLnLn with 1 positional isomer
PoPoL with 1 positional isomer

PPoLn with 2 positional isomers
OLLn with 2 positional isomers

PLLn with 2 positional isomers
PoOLn with 2 positional isomers

6.5.1 TAGs with one fatty acid (LLL, PoPoPo) See formula (7)

$$mol\%LLL = \frac{9.204\% * 11.457\% * 9.204\%}{10,000} = \mathbf{0.09706\ \ mol\ LLL}$$

$$mol\%PoPoPo = \frac{1.015\% * 1.262\% * 1.015\%}{10,000} = \mathbf{0.00013\ \ mol\ PoPoPo}$$

6.5.2 TAGs with two fatty acids (PoLL, SLnLn, PoPoL) See formula (8)

$$mol\%PoLL{+}LLPo = \frac{1.015\% * 11.457\% * 9.204\% * 2}{10,000} = 0.02141$$

$$mol\%LPoL = \frac{9.204\% * 1.262\% * 9.204\%}{10,000} = 0.01069$$

0.03210 mol PoLL

$$mol\%SLnLn{+}LnLnS = \frac{4.325\% * 1.153\% * 0.927\% * 2}{10,000} = 0.00092$$

$$mol\%LnSLn = \frac{0.927\% * 0.177\% * 0.927\%}{10,000} = 0.00002$$

0.00094 mol SLnLn

$$mol\%PoPoL{+}LPoPo = \frac{1.015\% * 1.262\% * 9.204\% * 2}{10,000} = 0.00236$$

$$mol\%PoLPo = \frac{1.015\% * 11.457\% * 1.015\%}{10,000} = 0.00118$$

0.00354 mol PoPoL

6.5.3 TAGs with three different fatty acids (PoPLn, OLLn, PLLn, PoOLn) See formula (9)

$$\text{mol\%PPoLn} = \frac{16.004\%*1.262\%*0.927\%*2}{10,000} = 0.00374$$

$$\text{mol\%LnPPo} = \frac{0.927\%*0.653\%*1.015\%*2}{10,000} = 0.00012$$

$$\text{mol\%PoLnP} = \frac{1.015\%*1.153\%*16.004\%*2}{10,000} = 0.00375$$

<u>0.00761 mol PPoLn</u>

$$\text{mol\%OLLn} = \frac{68.526\%*11.457\%*0.927\%*2}{10,000} = 0.14556$$

$$\text{mol\%LnOL} = \frac{0.927\%*85.296\%*9.204\%*2}{10,000} = 0.14555$$

$$\text{mol\%LLnO} = \frac{9.204\%*1.153\%*68.526\%*2}{10,000} = 0.14544$$

<u>0.43655 mol OLLn</u>

$$\text{mol\%PLLn} = \frac{16.004\%*11.457\%*0.927\%*2}{10,000} = 0.03399$$

$$\text{mol\%LnPL} = \frac{0.927\%*0.653\%*9.204\%*2}{10,000} = 0.00111$$

$$\text{mol\%LLnP} = \frac{9.204\%*1.153\%*16.004\%*2}{10,000} = 0.03397$$

<u>0.06907 mol PLLn</u>

$$mol\%PoOLn = \frac{1.015\% * 85.296\% * 0.927\% * 2}{10,000} = 0.01605$$

$$mol\%LnPoO = \frac{0.927\% * 1.262\% * 68.526\% * 2}{10,000} = 0.01603$$

$$mol\%OLnPo = \frac{68.526\% * 1.153\% * 1.015\% * 2}{10,000} = 0.01604$$

0.04812 mol PoOLn

ECN42 = 0.69512 mol TAGs

Note 1: The elution order can be determined by calculating the equivalent carbon numbers, often defined by the relation ECN = CN - 2n, where CN is the carbon number and n is the number of double bonds; it can be calculated more precisely by taking into account the origin of the double bond. If n_o, n_l and n_{ln} are the numbers of double bonds attributed to oleic, linoleic and linolenic acids respectively, the equivalent carbon number can be calculated by means of the relation of the formula:

$$EN = CN - d_o n_o - d_l n_l - d_{ln} n_{ln}$$

where the coefficient d_o, d_l and d_{ln} can be calculated by means of the reference triglycerides. Under the conditions specified in this method, the relation obtained will be close to:

$$ECN = CN - (2.60\ n_o) - (2.35\ n_l) - (2.17\ n_{ln})$$

Note 2: With several reference triglycerides, it is also possible to calculate the resolution with respect to triolein:

$$\alpha = RT^1 / RT\ triolein$$

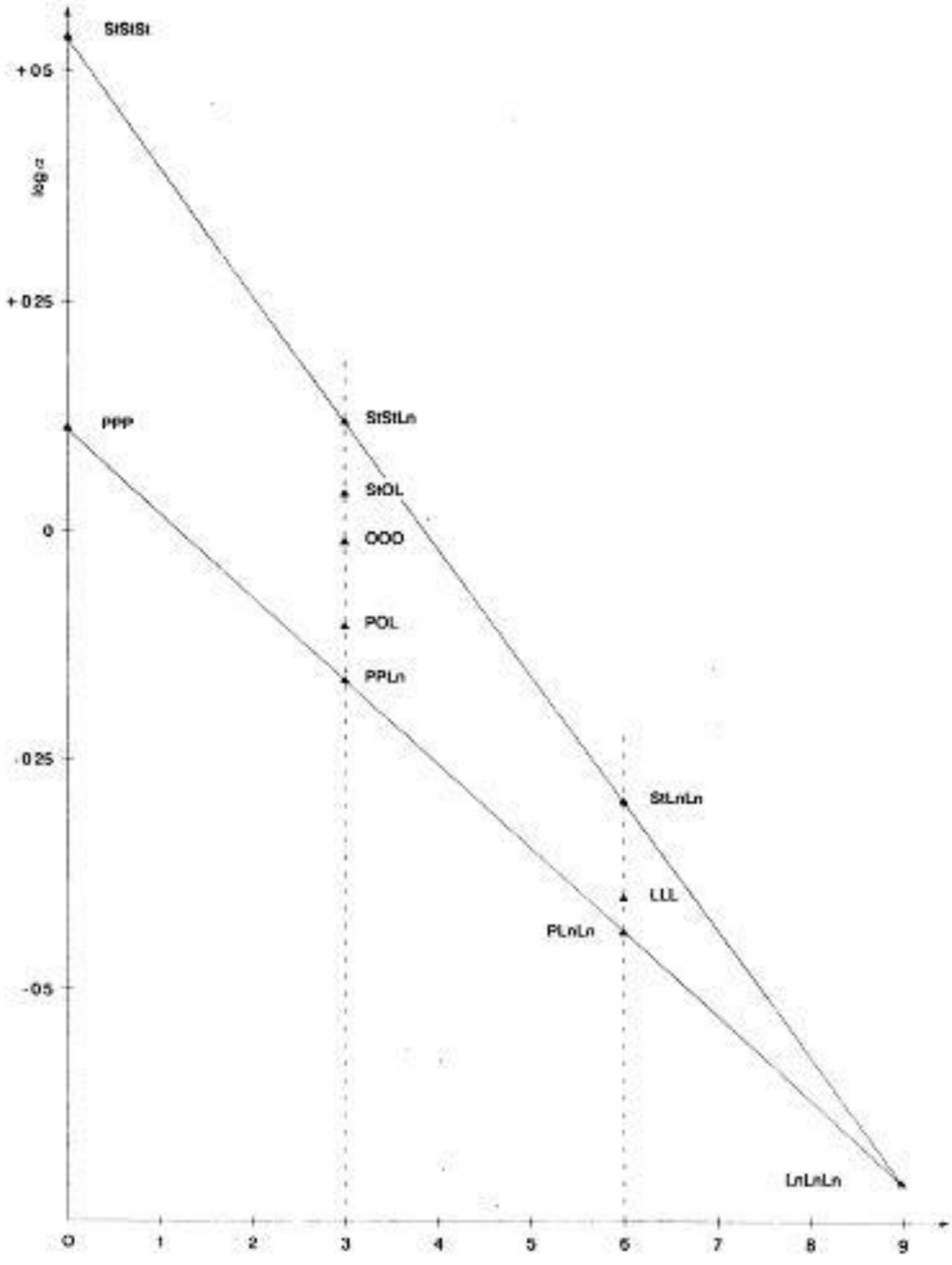
by use of the reduced retention time $RT^1 = RT - RT\ solvent$

The graph of log α against f (number of double bonds) enables the retention values to be determined for all the triglycerides of fatty acids contained in the reference triglycerides - see Figure 1.

Note 3: The efficiency of the column should permit clear separation of the peak of trilinolein from the peaks of the triglycerides with an adjacent RT. The elution is carried out up to ECN 52 peak.

Note 4: A correct measure of the areas of all peaks of interest for the present determination is ensured if the second peak corresponding to ECN 50 is 50% of full scale of the recorder.

Figure 1: Graph of log α against f (number of double bonds)

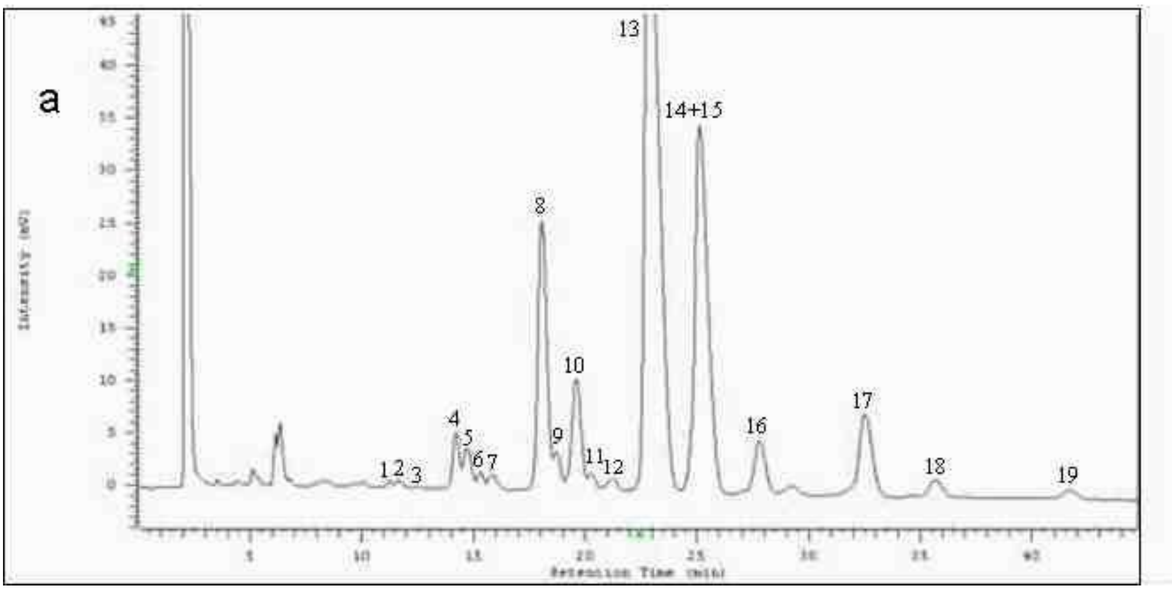


Number of double bonds

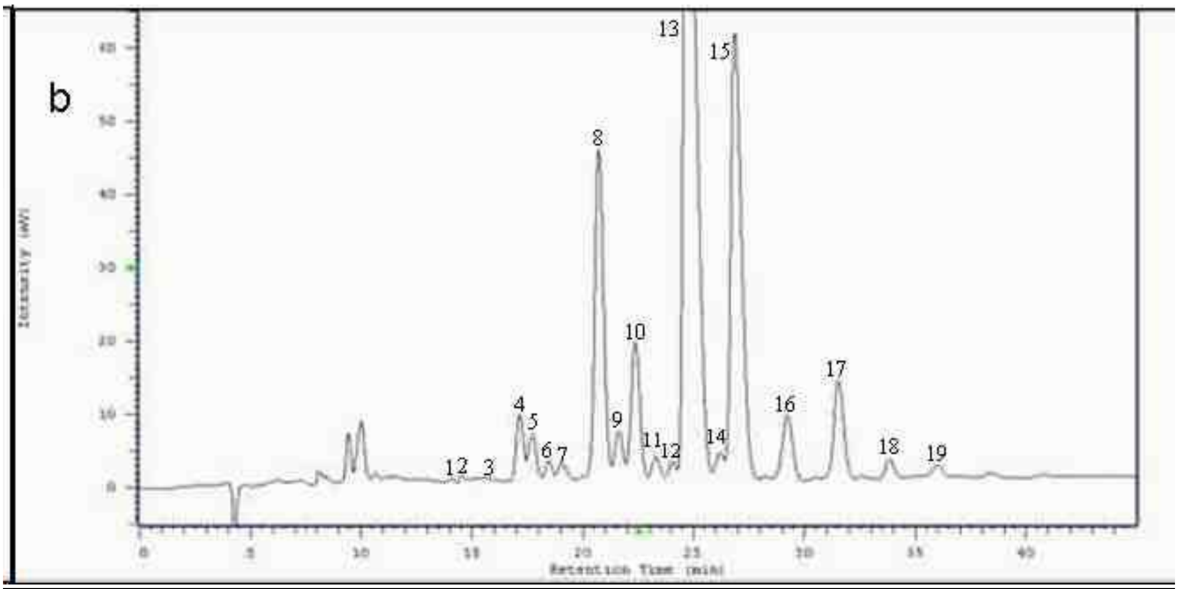
La: lauric acid; My: myristic acid; P: palmitic acid; St: stearic acid; O: oleic acid;

L: linoleic acid; Ln: linolenic acid

Figure 2: Low linoleic olive oil



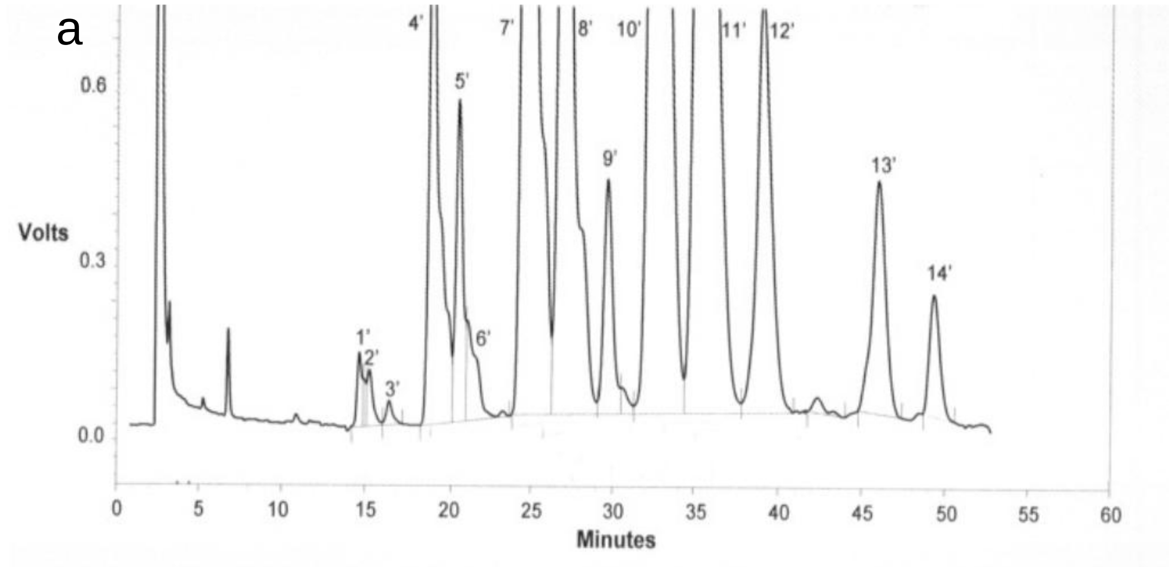
With solvent: Acetone/Acetonitrile.
PROFILE a: Main components of chromatographic peaks: **ECN42:** (1) LLL+PoLL; (2) OLLn+PoOLn; (3) PLLn; **ECN44:** (4) OLL+PoOL; (5) OOLn +PLL; (6) POLn+PPoPo; (7) OOL+PoOO; **ECN46:** (8) OOL+LnPP; (9) PoOO; (10) SLL+PLO; (11) PoOP+SPoL+SOLn+SPoPo; (12) PLP; **ECN48:** (13) OOO+PoPP; (14+15) SOL+POO; (16) POP; **ECN50:** (17) SOO; (18) POS+SLS.



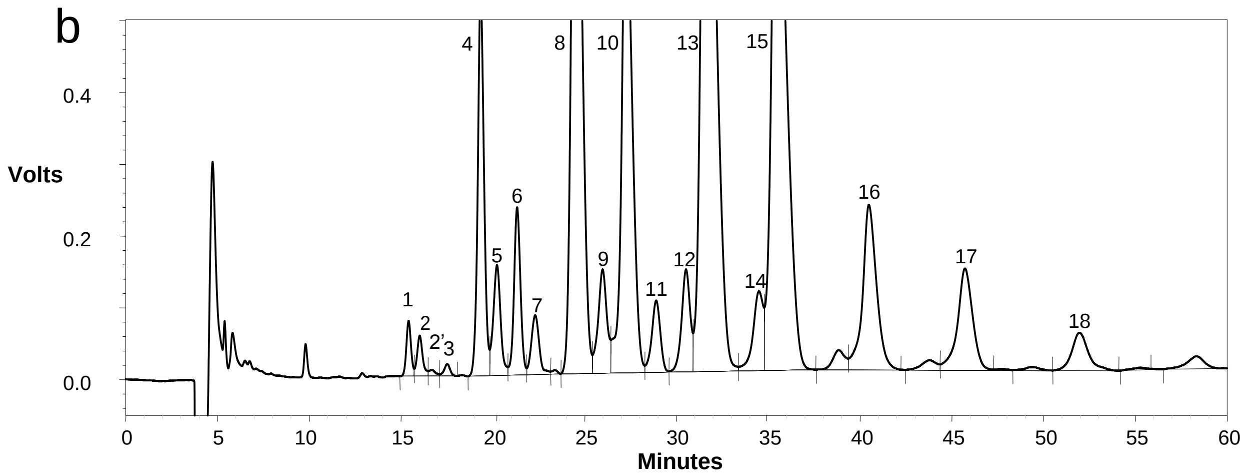
With solvent: Propionitrile
PROFILE b: Main components of chromatographic peaks: **ECN42:** (1) LLL; (2) OLLn+PoLL; (3) PLLn; **ECN44:** (4) OLL; (5) OOLn+PoOL; (6) PLL+PoPoO; (7) POLn+PPoPo+PPoL; **ECN46:** (8) OOL+LnPP; (9) PoOO; (10) SLL+PLO; (11)

PoOP+SPoL+SOLn+SPoPo; (12) PLP; **ECN48:** (13) OOO+PoPP; (14) SOL; (15) POO; (16) POP; **ECN50:** (17) SOO; (18) POS+SLS

Figure 3: High linoleic olive oil



With solvent: Acetone/Acetonitrile (50:50).
Profile a: Main components of chromatographic peaks: **ECN42:** (1') LLL+PoLL; (2') OLLn+PoOLn; (3') PLLn; **ECN44:** (4') OLL+PoOL; (5') OOLn +PLL; (6') POLn+PPoPo; **ECN46:** (7') OOL+PoOO; (8') PLO+SLL+ PoOP; (9') PLP+PoPP; **ECN48:** (10') OOO; (11') POO+SLL+PPoO; (12') POP+PLS; **ECN50:** (13') SOO; (14') POS+SLS



With solvent: Propionitrile.
Profile b: Main components of chromatographic peaks: **ECN42:** (1) LLL; (2+2') OLLn+PoLL; (3) PLLn; **ECN44:** (4) OLL; (5) OOLn+PoOL; (6) PLL+PoPoO; (7) POLn+PPoPo+PPoL; **ECN46:** (8) OOL+LnPP; (9) PoOO; (10) SLL+PLO; (11) PoOP+SPoL+SOLn+SPoPo; **ECN48:** (12) PLP; (13) OOO+PoPP; (14) SOL; (15) POO; (16) POP; **ECN50:** (17) SOO; (18) POS+SLS; **ECN52:** (19) AOO.

PRECISION VALUES OF THE METHOD

1. Analysis of the collaborative test results determined with acetone and acetonitrile

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

Nineteen laboratories holding IOOC recognition at the time took part in the collaborative test arranged by the Executive Secretariat in 1999. The laboratories were from eight countries.

The test was performed on five samples:

A: extra virgin olive oil

B: virgin olive oil + refined sunflower oil

C: virgin olive oil + refined olive-pomace oil

D: virgin olive oil + refined soybean oil + refined sunflower oil

E: refined olive oil + refined olive-pomace oil + refined soybean oil + lampante virgin olive oil

The results of the collaborative test organised by the IOOC Executive Secretariat have been statistically processed according to the rules laid down in the international standards ISO 5725 **Accuracy (trueness and precision) of measurement methods and results.** 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 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

RDS_R (%) Reproducibility coefficient of variation (S_R x 100/mean)

Table 1 : Difference between actual and theoretical content of triglycerides with ECN42 determined with acetone and acetonitrile

	A	B	C	D	E
n	19	19	19	19	19
outliers	1	0	0	0	3
mean	0,04	1,66	0,04	0,18	0,82
r	0,08	0,12	0,09	0,11	0,11
S_r	0,02	0,04	0,03	0,04	0,04
RSD_r(%)	82,2$_{(not\ sig.)}$	2,8	76,1$_{(not\ sig.)}$	22,5	5,1
R	0,12	0,25	0,16	0,22	0,24
S_R	0,05	0,09	0,05	0,08	0,08
RSD_R(%)	127,6$_{(not\ sig.)}$	5,4	132,2$_{(not\ sig.)}$	46,2	10,9

2. Analysis of the collaborative test results determined with propionitrile

The test was performed on four samples:

A: 70% virgin olive oil + 10% refined olive-pomace oil + 20% high oleic sunflower oil

B: 80% high campesterol virgin olive oil + 20% palm olein

C: 100% virgin olive oil

D: 70% virgin olive oil + 15% refined olive-pomace oil + 15% high oleic sunflower oil

Table 2 : Validation data of the ΔECN42 calculated from the experimental ECN42 determined with propionitrile.

	A	B	C	D
n	16	16	11	11
outliers	0	2	0	0
mean	1,07	0,10	0,06	0,84
r	0,05	0,02	0,06	0,06
S_r	0,02	0,01	0,02	0,02
RSD_r(%)	1,6	7,9	36,6	2,7
R	0,33	0,11	0,12	0,35
S_R	0,12	0,04	0,04	0,12
RSD_R(%)	11,2	36,8	78,6$_{(not\ sig.)}$	14,8

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

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

ΔECN42	n	Consensus mean	S_r	S_R
Regulatory method	21	0,03	0,004	0,013
Alternative solvent method	23	0,03	0,006	0,019
Evaluation	**Calculated**	**Limit**	**Conclusion/Comments**	
Difference (Regulatory method- Alternative solvent method)	0,00			
Test F repeatability	2,05	2,14	F cal < F limit	
Test F reproducibility	2,12	2,14	F cal < F limit	
Current reproducibility Regulatory	0,02	0,03	S_R obtained is smaller than current S_R	
T Student	0,44	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.

4. Normative references

ISO 5725-1: 1994 Accuracy (trueness and precision) of measurement methods and results – Part 1: General principles and definitions

ISO 5725-2: 1994 Accuracy (trueness and precision) of measurement methods and results – Part 2: Basic method for the determination of the repeatability and reproducibility of a standard measurement method

ISO 5725-5: 1994 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 5725-6: 1994 Accuracy (trueness and precision) of measurement methods and results – Part 6: Use in practice of accuracy values

COMPUTER PROGRAM