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## **GLOBAL METHOD FOR THE DETECTION OF EXTRANEOUS OILS IN OLIVE OILS**

### **1. SCOPE**

The method is used to detect the presence of extraneous vegetable oils in olive oils. High linoleic vegetable oils (soybean, rapeseed, sunflower, etc), and some high oleic vegetable oils - such as hazelnut, high oleic sunflower and olive-pomace oils - are detected. The detection level depends on the type of extraneous oil and the variety of olive. For hazelnut oil, a detection level between 5 and 15% is usually achieved. The method is unable to identify the type of extraneous oil, and only indicates if the olive oil is genuine or non-genuine.

### **2. PRINCIPLE**

The oil is purified by solid phase extraction (SPE) on silica gel cartridges. The triacylglycerol (TAG) composition is determined by reverse phase high resolution liquid chromatography using a refractive index detector and propionitrile as the mobile phase. Fatty acid methyl esters (FAMES) are prepared from purified oil by methylation with a cold solution of KOH in methanol (Method A in COI/T.20/Doc. no. 24) and then the esters are analysed by capillary gas chromatography using high polar columns (COI/T.20/Doc.no 17). The theoretical triacylglycerol composition is calculated from the fatty acid composition by a computer program assuming a 1,3-random, 2-random distribution of fatty acids in the triacylglycerol, with restrictions for saturated fatty acids in the 2-position. The calculation method is a modification of the procedure described in COI/T.20/Doc. no. 20. Several mathematical algorithms are calculated from theoretical and experimental (HPLC) triacylglycerol compositions, and the resulting values are compared with those contained in a data base built from genuine olive oils.

### 3. MATERIAL AND REAGENTS

#### 3.1. Oil purification

- 3.1.1. 25-ml conical flasks.
- 3.1.2. 5-ml screw top glass tubes and caps fitted with PTFE joint.
- 3.1.3. Silica gel cartridges, 1 g (6 ml), for solid phase extraction (for example, Waters, Massachusetts, USA).
- 3.1.4. *n*-hexane, analytical grade.
- 3.1.5. Solvent mixture of hexane/diethyl ether (87:13, v/v).
- 3.1.6. *n*-heptane, analytical grade.
- 3.1.7. Acetone, analytical grade.

#### 3.2. HPLC analysis of triacylglycerols

- 3.2.1. Micro syringes (50  $\mu$ L) and needles for HPLC injection
- 3.2.2. Propionitrile, super purity or HPLC grade (for example, ROMIL, Cambridge, United Kingdom), used as mobile phase.
- 3.2.3. HPLC column (25 cm x 4 mm i.d.), packed with RP-18 phase (4 $\mu$ m particle size).

#### 3.3. Preparation of fatty acid methyl esters

(See Method A in COI/T.20/Doc. no. 24)

- 3.3.1. Methanol containing not more than 0.5% water.
- 3.3.2. Heptane, analytical grade.
- 3.3.3. A 2N solution of potassium hydroxide in methanol. Dissolve 1.1 g of potassium hydroxide in 10 ml of methanol.
- 3.3.4. 5-ml screw top glass tubes and caps provided with PTFE joint.

#### 3.4. GC analysis of FAMES

(See method for the determination of *trans*-unsaturated fatty acids by capillary column gas chromatography; COI/T.20/Doc. no. 17).

- 3.4.1. Micro syringes (5  $\mu$ L) and needles for GC injection.
- 3.4.2. Hydrogen or helium as carrier gas.
- 3.4.3. Hydrogen and oxygen for FID detector.
- 3.4.4. Nitrogen or helium as auxiliary carrier gas.
- 3.4.5. Fused silica capillary column (50-60 m x 0.25 – 0.30 mm i.d.) coated with cyanopropylpolysiloxane or cyanopropylphenylsiloxane phases (SP-2380 or similar) with 0.20-0.25  $\mu$ m of film thickness.

### 4. APPARATUS

- 4.1. Vacuum apparatus for solid phase extraction.
- 4.2. Rotary evaporator.

- 4.3. HPLC equipment composed of:
  - 4.3.1. Degasser for the mobile phase.
  - 4.3.2. Rheodyne injector valve with a 10  $\mu$ L loop.
  - 4.3.3. High pressure pump unit.
  - 4.3.4. Thermostatic oven for the HPLC column capable of maintaining sub-ambient temperatures (15-20  $^{\circ}$ C), (for example, Peltier type).
  - 4.3.5. Refractive index detector.
  - 4.3.6. Computerised data acquisition system provided with an integration program.
  
- 4.4. Capillary gas chromatography equipment described in COI/T.20/Doc. no. 17, provided with
  - 4.4.1. Split injector.
  - 4.4.2. Flame ionisation detector (FID).
  - 4.4.3. Oven with programmable temperature.
  - 4.4.4. Computerised data acquisition system provided with an integration program.
  
- 4.5. PC computer with Microsoft EXCEL program, version 2000 or XP.

## **5. ANALYTICAL PROCEDURE**

### **5.1. Oil purification**

An SPE silica gel cartridge is placed in a vacuum elution apparatus and washed under vacuum with 6 ml of hexane. The vacuum is released to prevent the column from drying and a conical flask is placed under the cartridge. A solution of the oil (0.12 g, approximately) in 0.5 ml of hexane is loaded into the column and the solution is pulled through and then eluted with 10 ml of the solvent mixture (3.1.5) of hexane-diethyl ether (87:13 v/v) under vacuum. The eluted solvent is homogenised and approximately half of the volume is poured into another conical flask. Both solutions are separately evaporated to dryness in a rotary evaporator under reduced pressure at room temperature. For triacylglycerol analysis, one of the residues is dissolved in 1 ml of acetone (See first paragraph of 5.2) and poured into a 5-ml screw top glass tube. The other residue is dissolved in 1 ml of *n*-heptane and poured into a second 5-ml screw top glass tube for preparing the fatty acid methyl esters.

Note: Oil purification may be done using a silica gel column, as described in IUPAC method 2.507.

## 5.2. HPLC analysis of triacylglycerols

Set up the HPLC system, maintaining the column temperature at 20°C and using propionitrile as the mobile phase at a flow rate of 0.6 ml/min. When the baseline is stable run a solvent injection; if the base line appears disturbed in the region from 12 to 25 min, use another type of acetone or a mixture of propionitrile/acetone (25:75) to resolve the sample.

Note: Some types of acetone produce disturbances of the baseline in the above-mentioned region.

Inject a 10 µl aliquot of the solution of purified oil in acetone (5%). The run takes approximately 60 min. Oven temperature and/or flow rate must be adjusted to achieve a chromatogram similar to that depicted in Figure 1 where trilinolein (peak 1) elutes at 15.5 min and the resolutions between the pairs LLL/OLLn (peaks 1 and 2) and OLL/OOLn (peaks 4 and 5) are good.

The height of peak 2 (OLLn+PoLL) must reach at least 3% of the full scale.

## 5.3. Preparation of fatty acid methyl esters.

Add 0.1 mL of a 2N solution of potassium hydroxide in methanol to the solution of purified oil in 1 mL of *n*-heptane. Cap the tube and screw tight. Shake the tube vigorously for 15 seconds and leave to stratify until the upper layer becomes clear (5 minutes). The *n*-heptane solution is ready to be injected into the gas chromatograph. The solution may be left at room temperature for a maximum of 12 hours.

## 5.4. GC analysis of fatty acid methyl esters

The procedure described in the method for the determination of *trans*-unsaturated fatty acids must be used (COI/T.20/Doc. no. 17).

The GC system is set up at an oven temperature of 165 °C . The recommended oven temperature is isothermal at 165 °C for 10 min, then raising it to 200 °C at 1.5 °C/min. An injector temperature between 220 °C and 250 °C is recommended to minimise the formation of *trans*-fatty acids (See IOOC method). Detector temperature 250 °C. Hydrogen or helium must be used as the carrier gas at a column head pressure of 130 kPa, approximately. Injection volume 1µL in split injection mode.

A GC profile similar to that shown in Figure 2 must be obtained. Special attention must be paid to the resolution between C18:3 and C20:1 (the C18:3 peak must appear before the C20:1). To achieve these conditions, the initial temperature and/or the column head pressure must be optimised. Adjust the injector conditions (temperature, split ratio and volume injection) to minimise the discrimination of palmitic and palmitoleic acid.

The height of the C20:0 peak must be about 20% of full scale to quantify the *trans* isomers. If the C18:0 peak appears distorted, reduce the sample amount.

## 5. INTEGRATION OF CHROMATOGRAPHIC PEAKS

### 5.1. HPLC chromatogram

Figure 1 shows a typical HPLC chromatogram of the triacylglycerols of a purified olive oil. For peak integration, three baselines must be traced: the first between the start of peak 1 and the end of peak 3; the second between the start of peak 4 and the valley before peak 8; the third between the valley preceding peak 8 and the end of peak 18.

The total area is the sum of the areas of all the peaks (identified and not identified) from peak 1 to peak 18. The percentage of each peak is given by

$$\text{TAG}_x (\%) = 100 (A_x + A_T)$$

The percentages have to be given to two decimal figures.

### 5.2. GC chromatogram

Figure 2 shows a GC chromatogram of fatty acid alkyl esters obtained from a purified olive oil. Percentages of the following fatty acids must be calculated:

Palmitic;	P (C16:0) = methyl ester + ethyl ester
Stearic;	S (C18:0) = methyl ester
Palmitoleic;	Po (C16:1) = sum of methyl esters of the two <i>cis</i> -isomers
Oleic;	O (C18:1) = sum of methyl esters of the two <i>cis</i> -isomers + ethyl ester + <i>trans</i> -isomers
Linoleic;	L (C18:2) = methyl ester+ ethyl ester + <i>trans</i> -isomers
Linolenic;	Ln (C18:3) = methyl ester + <i>trans</i> -isomers
Arachidic;	A (C20:0) = methyl ester
Eicosenoic (gondoic);	G (C20:1) = methyl ester

Ethyl and *trans*-isomers esters may be absent in the GC chromatogram.

Total area (AT) is the sum of all the peaks appearing in the chromatogram from C14:0 to C24:0, except that corresponding to squalene. The percentage of each peak is calculated as follows:

$$\text{FA}_x (\%) = 100 (A_x + A_T)$$

The results have to be expressed to two decimal places.

For the calculations of the computer programs, it is not necessary to normalise to 100 because this is done automatically.

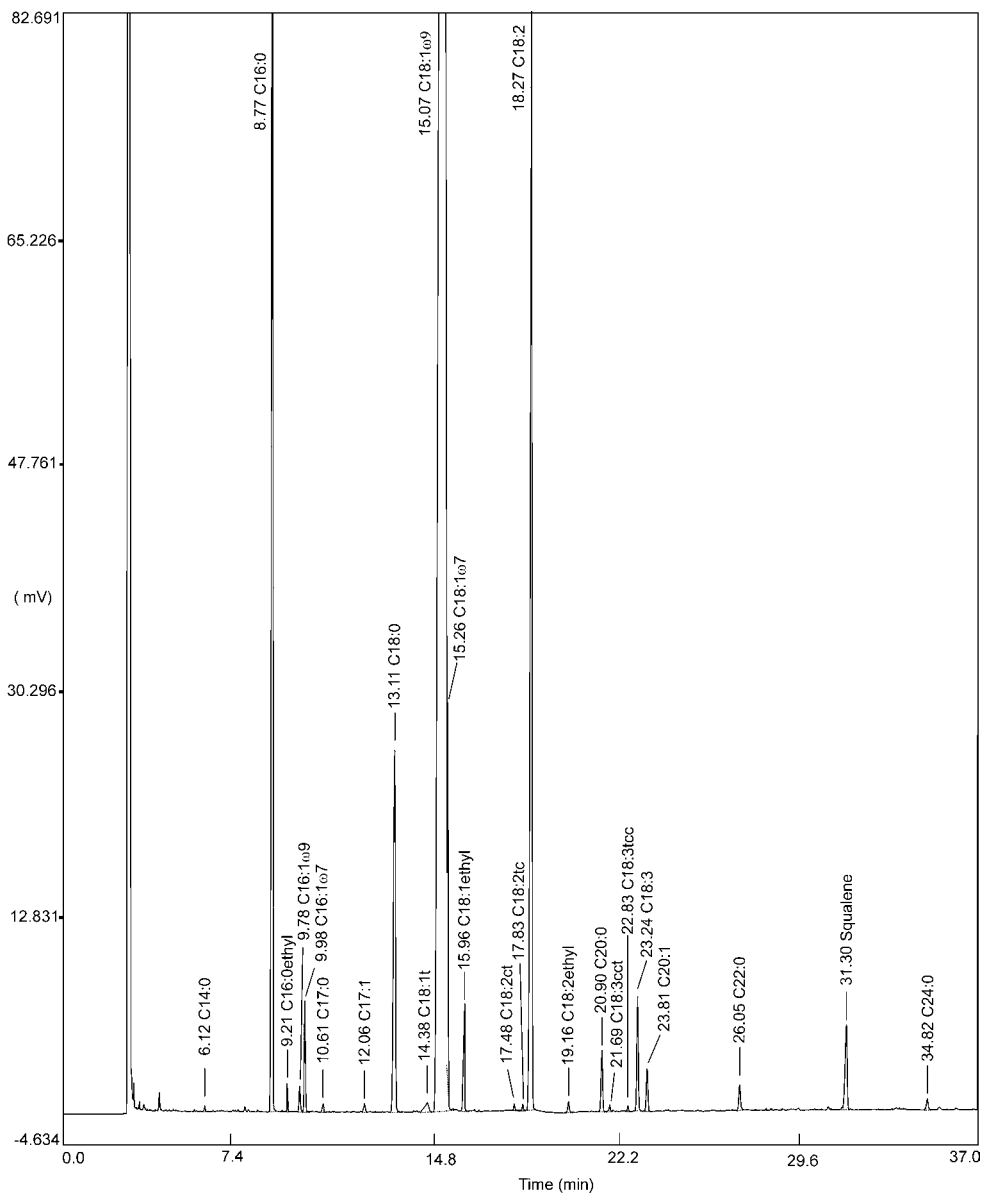


**Table 1:** Repeatability data of the determination of virgin olive oil TAGs by HPLC at a column temperature of 20° C and using propionitrile as mobile phase.

ECN	HPLC peaks	TAGs	Sample 1		Sample 2		Sample 3		Sample 4		Sample 5	
			Mean (%)	RSD <sub>r</sub> (%)	Mean (%)	RSD <sub>r</sub> (%)	Mean (%)	RSD <sub>r</sub> (%)	Mean (%)	RSD <sub>r</sub> (%)	Mean (%)	RSD <sub>r</sub> (%)
42	1	LLL	0.020	7.23	0.066	5.18	0.095	4.10	0.113	0.95	0.34	1.05
	2	OLLn+ PoLL	0.085	7.44	0.24	1.78	0.26	2.25	0.35	2.02	0.50	2.83
	3	PLLn	0.023	15.74	0.039	5.51	0.057	5.62	0.082	4.35	0.12	6.15
44	4	OLL	0.47	1.52	1.53	0.42	2.62	0.98	3.35	1.05	4.37	1.13
	5	OOLn+ PoOL	1.07	2.01	1.54	0.46	1.61	0.71	1.72	1.07	1.77	2.40
	6	PLLn+ PoPoO	0.11	12.86	0.24	4.37	0.65	1.32	1.35	0.73	2.28	1.24
	7	POLn+ PpoPo+ PpoL	0.42	5.11	0.49	2.89	0.55	2.01	0.85	1.83	1.09	1.96
46	8	OOL+ LnPP	6.72	0.63	8.79	0.31	11.21	0.42	13.25	0.33	15.24	0.23
	9	PoOO	1.24	2.86	1.49	0.95	1.63	0.85	2.12	0.45	2.52	0.56
	10	SLL+ PLO	2.70	0.65	4.05	0.70	6.02	0.65	9.86	0.53	11.53	0.31
	11	PoOP+ SpoL+ SOLn+ SpoPo	0.64	4.42	0.69	3.02	0.79	1.23	1.53	0.89	1.70	1.66
48	12+13	OOO+ PLP+ PoPP	49.60	0.07	48.15	0.06	42.93	0.06	33.25	0.10	24.16	0.06
	14	SOL	0.82	1.72	0.92	1.56	1.05	1.32	1.25	1.05	1.60	1.77
	15	POO	22.75	0.25	21.80	0.20	21.05	0.30	20.36	0.35	20.17	0.14
50	16	POP	3.05	0.46	4.56	0.42	4.98	0.52	5.26	0.41	5.57	0.38
	17	SOO	6.87	0.21	5.56	0.33	4.86	0.43	4.12	0.72	3.09	0.69
	18	POS+ SLS	1.73	1.23	1.65	1.10	1.54	0.99	1.49	1.10	1.41	1.00

n = 3 replicates

RSD<sub>r</sub> = Relative Standard Deviation of the repeatability



**Figure 2.** GC chromatogram of fatty acid alkyl esters obtained from an olive-pomace oil by transesterification with a cold solution of KOH in methanol.



## **ANNEX 1**

### **DETECTION OF EXTRANEIOUS OILS IN OLIVE OILS BY MEANS OF R42/R44 RATIO**

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#### **1. CALCULATION AND EXPRESSION OF RESULTS**

##### **1.1 Triacylglycerol composition**

Use the area normalisation method, i.e. assume that the sum of the areas of the peaks corresponding to TAGs from ECN42 up to ECN52 is equal to 100%. Calculate the relative percentage of each triacylglycerol using the formula:

$$\% \text{ triacylglycerol} = \text{area of peak} \times 100 / \text{sum of peak areas.}$$

The results are to be given to at least two decimal places.

##### **1.1.1. Triacylglycerols with ECN42**

The triacylglycerols with ECN42 are calculated according to equations COI T.20/Doc. 20 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 are to be given to at least two decimal places.

### 1.1.2. Triacylglycerols with ECN44

The triacylglycerols with ECN44 are calculated according to the same mathematical equations and are then given in order of expected elution in HPLC.

LLO and the positional isomer LOL  
PoLO and the positional isomers OPoL and LOPo  
PoPoO and the positional isomer PoOPo  
LnOO and the positional isomer OLnO  
LnOP and the positional isomer OPLn and PLnO  
PLnP and the positional isomer LnPP  
LLP and the positional isomer LPL  
PoLP and the positional isomers LPoP and LPPo  
PoPoP and the positional isomers PoPPo  
LLnS and the positional isomers LSLn and LnLS  
PoLnS and the positional isomers LnPoS and LnSPo

The triacylglycerols with ECN44 are given by the sum of the eleven triacylglycerols including their positional isomers. The results are to be given to at least two decimal places.

### 1.2 Fatty acid composition

The theoretical composition of the triacylglycerols is calculated from the composition of the C16 and C18 fatty acids – palmitic acid, stearic acid, oleic acid, linoleic acid and linolenic acid – according to method COI/T.20/Doc. no. 20.

## 2. EVALUATION OF THE RESULTS

The calculated theoretical content and the content determined by the HPLC analysis for ECN42 and ECN44 are compared. The following ratios must then be calculated:

$$r_{\text{ecn42}} = \frac{\text{ecn42}_{\text{hplc}}}{\text{ecn42}_{\text{theor}}}$$

$$r_{\text{ecn44}} = \frac{\text{ecn44}_{\text{hplc}}}{\text{ecn44}_{\text{theor}}}$$

The genuineness of all olive oils, virgin and refined (except for olive-pomace oils), is defined by the ratio R:

$$R = r_{\text{ecn42}}/r_{\text{ecn44}}$$

**The oil is genuine when:**

<b>For oils with an oleic acid /linoleic acid ratio <math>\leq 5</math></b>	<b><math>R \leq 0.95</math></b>
<b>For oils with an oleic acid /linoleic acid ratio <math>&gt; 5 &lt; 15</math></b>	<b><math>R \leq 1.05</math></b>
<b>For oils with an oleic acid /linoleic acid ratio <math>\geq 15</math></b>	<b><math>R \leq 1.10</math></b>

### **3. APPLICATION OF THE COMPUTER PROGRAM**

3.1 Load EXCEL on the computer.

3.2 Open the file EXEMPLA.xls, which contains three examples of calculations.

Data for the calculations can **only** be entered in the blue boxes. **Do not type in any part of the program** other than in the areas indicated.

3.3 Data entry in the program begins at sample # where the sample code is typed on the yellow band.

3.4 Next, in the row corresponding to **FA found** enter the data for the fatty acids.

The fatty acids are arranged in the following sequence:

P = 16:0 = palmitic; M = 16:1 = palmitoleic; S = 18:0 = stearic;  
O = 18:1 = oleic; L = 18:2 = linoleic; T = 18:3 = linolenic.

Other fatty acids are NOT necessary. Only enter those specified, which are automatically normalised and come up in the **FA corr** row.

3.5 Enter the data for the HPLC ECN42, called **T42**, in the appropriate box, under **HPLC42**.

3.6 Enter the data for the HPLC ECN44, called **T44**, in the appropriate box under **HPLC44**.

3.7 The program concludes whether or not the olive oil contains extraneous oils: genuine – adulterated.

sample#	2144						
<b>INTRODUCE FATTY ACID ANALYSIS(FOUND)</b>							
	P	M	S	O	L	T	Tot
FA found	8,96	0,63	3,05	25,00	3,50	0,54	41,7
FA corr	23,13	1,64	7,10	58,59	8,26	1,28	100,0

<b>INTRODUCE TGC ANALYSIS VALUES</b>			
GLI	HPLC	GLI	HPLC
T42	0,18	T44	2,21

<b>RESULTS</b>	
r42	0,26
r44	0,43
r42/r44	0,60
O/L	7,09

<b>CONSIDERATIONS</b>		<b>LIMITS</b>	
IF	O/L > 15	r42/r44	< = 1,10
IF	O/L > 5 < 15	r42/r44	< = 1,05
IF	O/L < = 5	r42/r44	< = 0,95

<b>CONCLUSIONS</b>	
The sample is:	GENUINE

**Legend**  
P=16:0  
M=16:1  
S=18:0  
O=18:1  
L=18:2  
T=18:3

## ANNEX 2

### DETECTION OF EXTRANEOUS OILS IN OLIVE OILS BY MEANS OF A COMPARISON OF MATHEMATICAL ALGORITHMS WITH A DATA BASE BUILT FROM GENUINE OLIVE OILS

#### 1. CALCULATION

##### 1.1. Triacylglycerol composition

###### **Peaks of ECN42 TAGs**

LLL = peak 1

OLLn = peak 2+shoulder 2a must be summed (*Note 1*)

PLLn = peak 3

*Note 1. In some oils, and in very good chromatographic conditions, the shoulder may be resolved as a separate peak. In this case, the area must be summed to that of peak 2.*

###### **Peaks of ECN44 TAGs**

OLL = peak 4

OOLn = peak 5 + posterior shoulder, if it is present (In Figure 1, this shoulder is not present).

PLL = peak 6

POLn = peak 7 + posterior shoulder if it is present (In Figure 1, this shoulder is not present)

###### **Peaks of ECN46 TAGs**

OOL = peak 8

##### 1.2. Fatty acid composition

A GC chromatogram of fatty acid alkyl esters obtained by cold transmethylation with a methanolic solution of KOH from a purified olive oil is shown in Figure 2. Percentages of P, Po, S, O, L, Ln, A, and G acids must be calculated.

## 2. MATHEMATICAL ALGORITHMS TO DISCRIMINATE THE OILS.

### 2.1. Parameters to classify the oils in groups

$$LLL_{Theor.}$$

$$\Delta OOL = OOL_{Theor.} - OOL_{HPLC}$$

$$\Delta LLL = LLL_{HPLC} - LLL_{Theor}$$

$$Ln = \% \text{ linolenic acid}$$

$$\Delta ECN44_p = (PLL + POLn)_{HPLC} - (PLL + POLn)_{Theor}$$

The oil sample is classified in one of the eight groups according to its  $LLL_{Theor}$  value:

- a)  $\leq 0.018 \%$
- b)  $> 0.018 - \leq 0.040 \%$
- c)  $> 0.040 - \leq 0.090 \%$
- d)  $> 0.090 - \leq 0.15 \%$
- e)  $> 0.15 - \leq 0.25 \%$
- f)  $> 0.25 - \leq 0.35 \%$
- g)  $> 0.35 - \leq 0.55 \%$
- h)  $> 0.55 \%$

In each group the oils are classified in subgroups according to the values of  $\Delta OOL$  or  $\Delta LLL$ .

### 2.2. Parameters for comparison with the data base

#### 2.2.1. Legal limit: $\Delta ECN42 = ECN42_{HPLC} - ECN42_{Theor.}$

For information purposes only because this limit is not used to verify the genuineness of the oil.

#### 2.2.2. Previous criterion for groups a, b and h. $R1_{exp} = LLL_{HPLC}/OLLn_{HPLC}$

If  $R1_{exp} \leq \text{limit}$ , the oil is genuine.

#### 2.2.3. 1<sup>st</sup> criterion:

$$K1 = (LLL_{HPLC} + OLLn_{HPLC}) * (OLL_{Theor} + OOLn_{Theor}) / (LLL_{Theor} + OLLn_{Theor}) * (OLL_{HPLC} + OOLn_{HPLC})$$

If  $K1 \leq \text{limit}_{tw}$ , the oil is genuine

If  $K1 > \text{limit}_{up}$ , the oil is non-genuine

For groups b, c, and d, there is an intermediate limit. If  $K1$  lies between the intermediate and upper limits, and  $L3$  (see further on) is higher than 0.50 the oil is not genuine.

- 2.2.4. 2<sup>nd</sup> criterion:  $\Delta R1 = [LLL / OLLn]_{HPLC} - [LLL / OLLn]_{Theor}$ .  
If  $\Delta R1 \leq \text{limit}_{lw}$ , the oil is genuine  
If  $\Delta R1 > \text{limit}_{up}$ , the oil is non-genuine
- 2.2.5. 3<sup>rd</sup> criterion:  $\Delta R3 = [OLL / OOLn]_{HPLC} - [OLL / OOLn]_{Theor}$ .  
If  $\Delta R3 \leq \text{limit}_{lw}$ , the oil is genuine  
If  $\Delta R3 > \text{limit}_{up}$ , the oil is non-genuine
- 2.2.6. 4<sup>th</sup> criterion:  $L4 = [\Delta LLL - \Delta OLLn] / LLL_{Theor}$ .  
If  $L2 \leq \text{limit}_{lw}$ , the oil is genuine  
If  $L2 > \text{limit}_{up}$ , the oil is non-genuine
- 2.2.7. 5<sup>th</sup> criterion:  $L3 = [\Delta LLL - \Delta OLLn] / OLLn_{Theor}$   
If  $R2 \geq \text{limit}$ , the oil is genuine  
If  $R2 < \text{limit}$ , the oil is non-genuine
- 2.2.8. 6<sup>th</sup> criterion:  $R2 = [\Delta OLL * LLL_{Theor}] / [\Delta LLL * OLL_{Theor}]$

Where

$$\begin{aligned}\Delta LLL &= LLL_{exp} - LLL_{theor} \\ \Delta OLLn &= OLLn_{exp} - OLLn_{theor} \\ \Delta OLL &= OLL_{exp} - OLL_{theor}\end{aligned}$$

### 2.3. Determination of limits

For each subgroup, the limits for some parameters are given by the equation:

limit versus  $\Delta ECN44p$  value

The 1<sup>st</sup>, 2<sup>nd</sup>, 3<sup>rd</sup>, 4<sup>th</sup>, and 5<sup>th</sup> criteria have lower and upper limits ( $Lwlimit$  and  $Uplimit$ ), whereas the 6<sup>th</sup> criterion has only one limit.

The values of each criterion are sequentially compared with the upper and lower limits. Values between them indicate that the next parameter has to be compared. Figure 3 shows the flow diagram of the sequential procedure.

The criteria are compared in the order indicated above, except for groups g and h where  $\Delta R3$  is compared after  $L3$ .

## 3. APPLICATION OF THE COMPUTER PROGRAM.

- 3.1. Load EXCEL on the computer.
- 3.2. Open the file GLOBAL.XLS contained in the diskette.
- 3.3. Activate "Macro" if it is necessary.
- 3.4. Click the "**Press to initiate calculation**" button.

- 3.5. Enter data in **yellow cells** only (Figure 4)
  - 3.5.1. In “**Sample code**”, type the description of the sample
  - 3.5.2. In “**Oil category**” type:
    - “**EV**” for edible virgin olive oils (extra, and virgin categories)
    - “**L**” for lampante olive oil
    - “**R**” for the refined olive and olive (blend of virgin and refined olive oils) categories.This code is necessary to calculate the official limit for  $\Delta\text{ECN}42$ .
  - 3.5.3. In “**Introd. FAMES-GC**”, type the fatty acid composition. The program automatically normalises the percentages to the sum of the eight fatty acids. If **Warning** comes up, please revise the fatty acid data.
  - 3.5.4. Type the triacylglycerol data in the corresponding cells. Please revise the data.
- 3.6. The program calculates the theoretical triacylglycerol composition from the normalised fatty acid composition.
- 3.7. Using the experimental and theoretical triacylglycerol percentages, the program calculates the values of the mathematical algorithms corresponding to the oil sample and the values of the limits.
- 3.8. The command “**Press control+letter**” appears in the red band, indicating the  $LLL_{\text{Theor}}$  range in which the oil is classified.
- 3.9. To see the results, press simultaneously “control” and the specified letter keys; the results sheet for the oil sample then comes up (Figure 5).
- 3.10. Read the result in the red bands.
  - “**The oil is correct**” indicates that the oil is a genuine olive oil or that the adulteration level is low.
  - “**The oil is not correct**” indicates that the oil is a non-genuine olive oil.For each criterion, the values of the algorithms are typed in bold figures. The higher and lower limits and the values of other parameters are also indicated.
- 3.11. Click the button “**Press to print the results**” to print the results. Two sheets will be printed: the experimental data and the values and limits for the sample.
- 3.12. Click the button “**Press for a new calculation**” to reinitialise the program. All data will be erased.



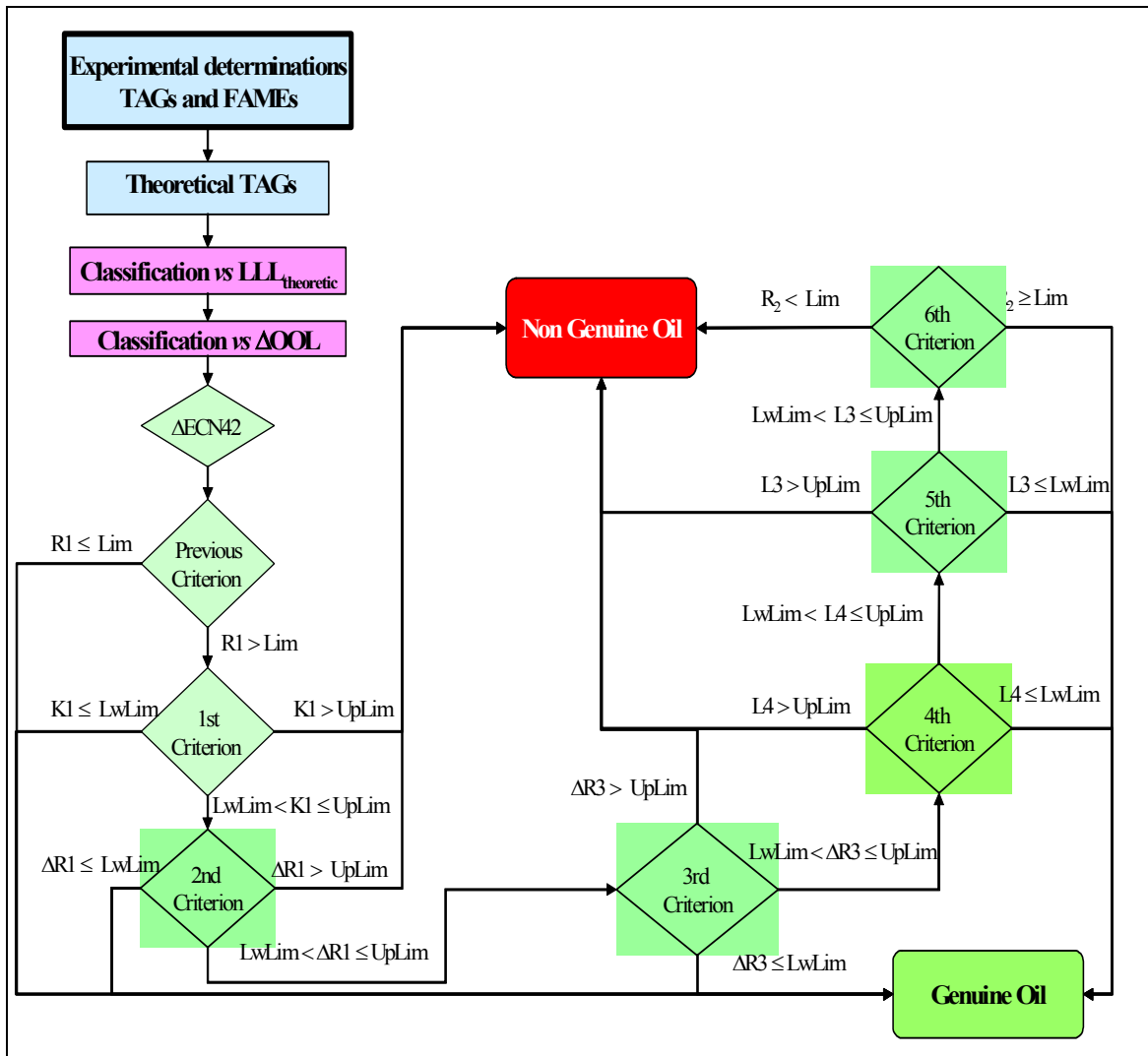


Figure 3. Flow diagram of the sequential procedure

EXPERIMENTAL DATA									
Fill only yellow cells									
Sample Code	VOO + VHO								
Oil Category	R	Refined Olive Oil							
Area % (GLC)	C16:0	C18:0	C16:1	C18:1	C18:2	C18:3	C20:0	C20:1	Sum
FAMES-Comp. Corr.	9,62	2,94	0,41	79,39	6,52	0,49	0,33	0,30	100,00
Introd. FAMES-GC	9,62	2,94	0,41	79,37	6,52	0,49	0,33	0,30	99,98
HPLC Experimental Data									
ECN42			ECN44				ECN46		
LLL	OLLn	PLnL	OLL	OOLn	PLL	POLn	OOL		
0,12	0,19	0,04	1,55	1,28	0,22	0,47	12,57		
Press CTRL+b to see criteria for LLL theor. 0,018 - 0,040									

Figure 4. Data sheet of the calculation program

Criteria for LLL theor. 0,018 - 0,040			
Calculation Parameters			
Sample Code	VOO + VHO		
Oil Category	R	Refined Olive Oil	
$\Delta ECN42 =$	0,14	0,35	Proceed to the Early Criterion
Early Criterion R1 exp.			
Parameter	Value	Limit	
R1 exp.	0,63	0,40	Proceed to the Criterion 1
Criterion 1 K1			
Parameter	Value	Higher Limit	Lower Limit
K1	1,15		
L3	0,41	1,35	0,95
Proceed to the Criterion 2			
Criterion 2 $\Delta ECN44p$ vs $\Delta R1$			
Parameter	Value	Higher Limit	Lower Limit
$\Delta R1 =$	0,46		
$\Delta OOL =$	-0,69		
$\Delta ECN44p$	0,26	0,51	0,37
Proceed to the Criterion 3			
Criterion 3 $\Delta ECN44p$ vs $\Delta R3$			
Parameter	Value	Higher Limit	Lower Limit
$\Delta R3 =$	0,26		
$\Delta OOL =$	-0,69		
		0,50	0,25
Proceed to the Criterion 4			
Criterion 4 L4			
Parameter	Value	Higher Limit	Lower Limit
L4 =	2,52		
$\Delta OOL =$	-0,69		
		6,00	4,00
The oil is correct			
Criterion 5 L3			
Parameter	Value	Higher Limit	Lower Limit
L3 =	0,41		
$\Delta OOL =$	-0,69		
		0,60	0,40
Criterion 6 R2			
Parameter	Value	Limit	

Press to Print Results

Press for a New Calculation

Figure 6. Results sheet