INTERNATIONAL

OLIVE OIL

COUNCIL

COI/T.20/Doc. nº. 24
2001

ENGLISH
Original: ENGLISH

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METHOD OF ANALYSIS

PREPARATION OF THE FATTY ACID METHYL ESTERS FROM OLIVE OIL AND OLIVE-POMACE OIL

The following two methods are recommended for preparing the fatty acid methyl esters from olive oils and olive-pomace oils:

METHOD A. Trans-esterification with cold methanolic solution of potassium hydroxide

METHOD B. Methylation by heating with sodium methylate in methanol followed by esterification in acid medium

Each method will be applied according to the analytical parameter to be determined and the oil category as indicated below:

a) Determination of ΔECN42 (difference between actual and theoretical content of triglycerides with ECN42)

- Method A will be applied to samples of all the oil categories after purification of the oil by passing it through a silica gel column.

b) Determination of the fatty acid composition

- Method A will be applied directly to samples of the following oil categories:

  - Virgin olive oil with an acidity of not more than 3.3%
  - Refined olive oil
  - Olive oil (blend of virgin and refined olive oil)
  - Refined olive-pomace oil
  - Olive-pomace oil (blend of virgin olive oil and refined olive-pomace oil)

- Method B will be applied directly to samples of the following oil categories:

  - Virgin olive oil with an acidity of more than 3.3%
  - Crude olive-pomace oil

c) Determination of *trans*-isomers of fatty acids

- Method A will be applied directly to samples of the following oil categories:

  - Virgin olive oil with an acidity of not more than 3.3%
  - Refined olive oil
  - Olive oil (blend of virgin and refined olive oil)
  - Refined olive-pomace oil
  - Olive-pomace oil (blend of virgin olive oil and refined olive-pomace oil)

- Method A will be applied to the following categories of oils after purification of the oil by passing it through a silica gel column:

  - Virgin olive oil with an acidity of more than 3.3%
  - Crude olive-pomace oil

PURIFICATION OF OIL SAMPLES

When necessary, the samples will be purified by passing the oil through a silica gel column, eluting with hexane/diethyl ether (87:13, v/v) as described in IUPAC method 2.507.

Alternatively, solid-phase extraction on silica gel phase cartridges can be used. A silica gel cartridge (1 g, 6 mL) is placed in a vacuum elution apparatus and washed with 6 mL of hexane. The vacuum is released to prevent the column from becoming dry and then a solution of the oil (0.12 g approx.) in 0.5 mL of hexane is loaded into the column and vacuum is applied. The solution is pulled down and then eluted with 10 mL of hexane/diethyl ether (87:13 v/v) under vacuum. The combined eluates are homogenised and divided in two similar volumes. An aliquot is evaporated to dryness in a rotary evaporator under reduced pressure at room temperature. The residue is dissolved in 1 mL of heptane and the solution is ready for fatty acid analysis by GC. The second aliquot is evaporated and the residue is dissolved in 1 mL of acetone for triglyceride analysis by HPLC, if necessary.

METHODS FOR PREPARING THE FATTY ACID METHYL ESTERS

1. METHOD A. Trans-esterification with cold methanolic solution of potassium hydroxide

1.1. Purpose

This rapid method is applicable to olive oils and olive-pomace oils with a free fatty acid content of not more than 3.3%. Free fatty acids are not esterified by potassium hydroxide. Fatty acid ethyl esters are trans-esterified at a lower rate than glyceridic esters and may be only partially methylated.

1.2. Principle

Methyl esters are formed by trans-esterification with methanolic potassium hydroxide as an intermediate stage before saponification takes place (title 5 in ISO-5509:2000, title 5 in IUPAC method 2.301).

1.3. Reagents

Methanol containing not more than 0.5% (m/m) water.
Heptane, chromatographic quality.
Potassium hydroxide, approximately 2 N methanolic solution: dissolve 11.2 g of potassium hydroxide in 100 mL of methanol.

1.4. Apparatus

Screw-top test tubes (5-mL volume) with cap fitted with a PTFE-joint.
Graduated or automatic pipettes, 2 mL and 0.2 mL

1.5. Procedure

In a 5-mL screw-top test tube weigh approximately 0.1 g of the oil sample. Add 2 mL of heptane, and shake. Add 0.2 mL of 2 N methanolic potassium hydroxide solution, put on the cap fitted with a PTFE-joint, tighten the cap, and shake vigorously for 30 seconds. Leave to stratify until the upper solution becomes clear. Decant the upper layer containing the methyl esters. The heptane solution is suitable for injection into the gas chromatograph. It is advisable to keep the solution in the refrigerator until gas chromatographic analysis. Storage of the solution for more than 12 hours is not recommended.

2. METHOD B. Methylation by heating with sodium methylate in methanol followed by esterification in acid medium

2.1. Purpose

This method is applicable to olive oils and olive-pomace oils with a free fatty acid content of more than 3.3%.

2.2. Principle

Neutralisation of the free fatty acids and alkaline methanolysis of the glycerides, followed by esterification of the fatty acids in acid medium (title 4.2. in IUPAC method 2.301).

2.3. Reagents

Heptane, chromatographic quality.
Methanol containing not more than 0.05% (m/m) water.
Sodium methylate, 0.2 N methanolic solution: dissolve 5 g of sodium in 1,000 mL of methanol (this may be prepared from commercial solutions).
Phenolphthalein, 0.2% methanolic solution.
Sulphuric acid, 1 N in methanolic solution: add 3 mL of 96% sulphuric acid to 100 mL of methanol.
Saturated solution of sodium chloride in water.

2.4. Apparatus

50-mL flat-bottomed volumetric flask with long, narrow, ground neck
Reflux condenser: air condenser (1 m long) with ground joint appropriate to the neck of the flask.
Boiling chips.
Glass funnel.

2.5. Procedure

Transfer about 0.25 g of the oil sample into a 50-mL ground-necked volumetric flask. With the aid of a funnel, add 10 mL of 0.2 N sodium methylate in methanol and the boiling chips. Fit a reflux condenser, shake, and bring to the boil. The solution should become clear, which usually occurs in about 10 minutes. The reaction is complete after 15 minutes. Remove the flask from the source of heat, wait until the reflux stops, remove the condenser, and add two drops of phenolphthalein solution. Add a few mL of 1 N sulphuric acid in methanol solution until the solution becomes colourless and then add 1 mL in excess. Fit the condenser and boil again for 20 minutes. Withdraw from the source of heat and cool the flask under running water. Remove the condenser, add 20 mL of saturated sodium chloride solution, and shake. Add 5 mL of heptane, plug the flask, and shake vigorously for 15 seconds. Leave to settle until the two phases have separated. Add saturated sodium chloride solution again until the aqueous layer reaches the lower end of the flask neck. The upper layer containing the methyl esters fills the flask neck. This solution is ready to be injected in the GC.

Caution: Methylation by method B must be done under a hood.

2.6 ALTERNATIVES TO METHYLATION METHOD B

2.6.1 METHOD C.

2.6.1.1. Principle

The fatty matter undergoing analysis is treated with methanol-hydrochloric acid, in a sealed vial, at 100º C.

2.6.1.2. Apparatus

Strong glass vial of a capacity of about 5 mL (height 40 to 45 mm, diameter 14 to 16 mm).
1 and 2 mL graduated pipettes.

2.6.1.3. Reagents

Solution of hydrochloric acid in 2% methanol. This is prepared from gaseous hydrochloric acid and anhydrous methanol (Note 1).
Hexane, chromatographic quality.

2.6.1.4. Procedure

Place in the glass vial 0.2 g of the fatty matter, which has previously been dried out on sodium sulphate and filtered, and 2 mL of hydrochloric acid-methanol solution. Heat seal the vial.
Immerse the vial at 100ºC for 40 minutes.
Cool the vial under running water, open, add 2 mL of distilled water and 1 mL of hexane. Centrifuge and remove the hexane phase, which is ready for use.

Note 1: *Commercial solutions of hydrogen chloride in methanol can be used. Small amounts of gaseous hydrochloric acid can easily be prepared in the laboratory by simple displacement from the commercial solution (p = 1.18) by dripping concentrated sulphuric acid (p =1.84). The liberated gas is easily dried by bubbling through conc. sulphuric acid. Since hydrochloric acid is very rapidly absorbed by methanol, it is advisable to take the usual precautions when dissolving it, e.g. introduce the gas through a small inverted funnel with the rim just touching the surface of the liquid. Large quantities of methanolic hydrochloric acid solution can be prepared in advance, as it keeps perfectly in glass-stoppered bottles stored in the dark. Alternatively, this reagent can be prepared by dissolution of acetyl chloride in anhydrous methanol.*

2.6.2. METHOD D.

2.6.2.1. Principle

The fatty matter undergoing analysis is heated under reflux with methanol-hexane-sulphuric acid. The methyl esters obtained are extracted with petroleum ether.

2.6.2.2. Apparatus

Test tube of a capacity of about 20 mL, fitted with an air reflux condenser approximately 1 m in length, with ground glass joints.
5 mL graduated pipette.
50 mL separating funnel.
10 mL and 25 mL measuring beakers.
15 mL test tube with conical base.

2.6.2.3. Reagents

Methylation reagent: anhydrous methanol-hexane-concentrated sulphuric acid (p = 1.84) in the ratio 75:25:1 (V/V/V).
40 to 60º C petroleum ether.
Anhydrous sodium sulphate.

2.6.2.4. Procedure

Place 0.1 g of oil in the 20 mL test tube and add 5 mL of methylation reagent.
Fit the reflux condenser and heat for 30 minutes in a boiling water bath (Note 2).
Transfer quantitatively the mixture into a 50 mL separating funnel, with the aid of 10 mL distilled water and 10 mL petroleum ether. Shake vigorously, and allow the phases to separate, remove the aqueous phase and wash the ether layer twice with 20 mL distilled water. Add to the separating funnel a small quantity of anhydrous sodium sulphate, shake, allow to settle for a few minutes and filter, collecting the filtrate in a 15 mL test tube with a conical base.

Evaporate the solvent over a water bath in a current of nitrogen.

Note 2: *To control the boiling point insert a glass rod into the test tube and limit the temperature of the water bath to 90º C.*

3. Precision parameters

The statistical evaluation of the precision of methods A and B was published in Grasas y Aceites, Vol. 51, Fasc. 6 (2000), 447-456, by A. Cert, W. Moreda and M.C. Pérez-Camino.

The collaborative test was carried out by 16 laboratories from seven countries on five samples:

1. Extra virgin olive oil (free acidity 0.18%)
2. Virgin olive oil (free acidity 2.0%)
3. Virgin olive oil (free acidity 3.3%)
4. Olive oil (free acidity 0.88%)
5. Crude olive-pomace oil (free acidity 15.8%)

The collaborative trial was set up following the directions given by W. Horwitz (Horwitz 1988). The statistical analysis of the repeatability and reproducibility was performed according to ISO 5725 (ISO 1986) and AOAC Regulation (AOAC 1995) where the procedures for the identification of outliers and the mathematical procedures are described, using a computer program developed by the authors. The Cochran and Grubbs tests were applied to identify outliers and determined respectively the laboratories that gave very different results between replicates and those with exceedingly high and low values.

The statistical parameters used were the following:

- S_r: Standard deviation of the repeatability
- r: Repeatability ($2.8 \sqrt{S_r^2}$)
- RSD_r: Relative standard deviation of the repeatability
- S_R: Standard deviation of the reproducibility
- R: Reproducibility ($2.8 \sqrt{S_R^2}$)
- RSD_R: Relative standard deviation of the reproducibility
- Ho_R: Horwitz ratio $\left[\frac{RSD_R}{RSD_{R\,th}} \right]$ where, $RSD_{R\,th} = 2^{(1\ -\ 0.5\ \log\ C)}$, C being the concentration of the analytes expressed to the power 10.

Table 1. Statistical parameters from C14:0 acid determination in olive and olive –pomace oils										
Sample	**Extra virgin**		**Virgin**		**Lampante**		**Olive oil**		**Crude olive-pomace oil**	
Method	**KOH in methanol**	**Basic+acidic methylation**	**KOH in methanol**	**Basic+acidic methylation**	**KOH in methanol**	**Basic+acidic methylation**	**KOH in methanol**	**Basic+acidic methylation**	**KOH in methanol**	**Basic+acidic methylation**
Participants	15	16	15	16	15	16	15	16	15	16
Outliers	0	1	0	0	1	3	1	0	3	3
Mean (%)	0.0090	0.0087	0.0127	0.0138	0.0118	0.0098	0.0100	0.0100	0.0181	0.0296
Repeatability										
S_r	0.0018	0.0026	0.0026	0.0043	0.0042	0.0034	0.0038	0.0043	0.0020	0.0059
r	0.0051	0.0072	0.0072	0.0121	0.0118	0.0092	0.0106	0.0121	0.0055	0.0165
***RSDr*(%)**	20	30	20	31	36	37	38	43	11	20
Reproducibility										
S_R	0.0041	0.0044	0.0059	0.0071	0.0062	0.0060	0.0047	0.0057	0.0058	0.0060
R	0.0114	0.0123	0.0166	0.0200	0.0173	0.0161	0.0133	0.0160	0.0162	0.0168
***RSD_R* (%)**	45	51	47	52	52	64	42	57	32	20
Ho_R	0.5	0.6	0.5	0.6	0.6	0.7	0.5	0.6	0.4	0.3

S_r: Standard deviation of the repeatability; r: Repeatability ; RSD_r: Relative standard deviation of the repeatability; S_R: Standard deviation of the reproducibility; R: Reproducibility; RSD_R: Relative standard deviation of the reproducibility; Ho_R: Horwitz ratio.

Table 2. Statistical parameters from C16:0 acid determination in olive and olive –pomace oils										
Sample	**Extra virgin**		**Virgin**		**Lampante**		**Olive oil**		**Crude olive-pomace oil**	
Method	**KOH in methanol**	**Basic+acidic methylation**	**KOH in methanol**	**Basic+acidic methylation**	**KOH in methanol**	**Basic+acidic methylation**	**KOH in methanol**	**Basic+acidic methylation**	**KOH in methanol**	**Basic+acidic methylation**
Participants	15	16	15	16	15	16	15	15	15	16
Outliers	2	3	3	4	1	3	0	2	0	4
Mean (%)	7.96	8.06	10.32	10.51	10.35	10.62	10.51	10.41	9.67	10.28
Repeatability										
S_r	0.04	0.09	0.06	0.13	0.15	0.13	0.10	0.08	0.14	0.09
r	0.12	0.26	0.18	0.35	0.42	0.35	0.29	0.22	0.38	0.25
***RSDr*(%)**	0.53	1.1	0.62	1.2	1.5	1.2	0.98	0.75	1.4	0.87
Reproducibility										
S_R	0.24	0.14	0.16	0.18	0.33	0.17	0.46	0.42	0.45	0.15
R	0.68	0.40	0.44	0.49	0.93	0.48	1.3	1.2	1.3	0.41
***RSD_R*(%)**	3.0	1.8	1.5	1.7	3.2	1.6	4.4	4.0	4.7	1.4
Ho_R	0.09	0.05	0.05	0.05	0.10	0.05	0.14	0.13	0.15	0.05

S_r: Standard deviation of the repeatability; r: Repeatability; RSD_r: Relative standard deviation of the repeatability; S_R: Standard deviation of the reproducibility; R: Reproducibility; RSD_R: Relative standard deviation of the reproducibility; Ho_R: Horwitz ratio.

Table 3. Statistical parameters from C16:1 acid determination in olive and olive –pomace oils										
Sample	**Extra virgin**		**Virgin**		**Lampante**		**Olive oil**		**Crude olive-pomace oil**	
Method	**KOH in methanol**	**Basic+acidic methylation**	**KOH in methanol**	**Basic+acidic methylation**	**KOH in methanol**	**Basic+acidic methylation**	**KOH in methanol**	**Basic+acidic methylation**	**KOH in methanol**	**Basic+acidic methylation**
Participants	15	16	15	16	15	16	15	16	15	16
Outliers	0	1	2	2	0	1	1	1	1	2
Mean (%)	0.504	0.501	0.675	0.662	0.735	0.718	0.906	0.870	0.636	0.674
Repeatability										
S_r	0.014	0.017	0.010	0.027	0.026	0.020	0.012	0.012	0.014	0.018
r	0.041	0.049	0.027	0.077	0.074	0.057	0.034	0.034	0.040	0.050
***RSDr*(%)**	2.9	3.5	1.4	4.1	3.6	2.8	1.3	1.4	2.3	2.7
Reproducibility										
S_R	0.034	0.034	0.027	0.047	0.047	0.044	0.44	0.057	0.046	0.049
R	0.966	0.095	0.077	0.131	0.132	0.122	0.123	0.159	0.128	0.138
***RSD_R*(%)**	6.8	6.8	4.1	7.0	6.4	6.1	4.9	6.5	7.2	7.3
Ho_R	0.14	0.14	0.08	0.15	0.13	0.13	0.11	0.14	0.15	0.15

S_r: Standard deviation of the repeatability; r: Repeatability; RSD_r: Relative standard deviation of the repeatability; S_R: Standard deviation of the reproducibility; R: Reproducibility; RSD_R: Relative standard deviation of the reproducibility; Ho_R: Horwitz ratio.

Table 4. Statistical parameters from C18:0 acid determination in olive and olive –pomace oils										
Sample	**Extra virgin**		**Virgin**		**Lampante**		**Olive oil**		**Crude olive-pomace oil**	
Method	**KOH in methanol**	**Basic+acidic methylation**	**KOH in methanol**	**Basic+acidic methylation**	**KOH in methanol**	**Basic+acidic methylation**	**KOH in methanol**	**Basic+acidic methylation**	**KOH in methanol**	**Basic+acidic methylation**
Participants	15	16	15	16	15	16	15	16	15	16
Outliers	2	1	0	1	0	1	0	0	1	0
Mean (%)	2.883	2.871	2.490	2.508	2.618	2.676	3.492	3.495	3.118	3.256
Repeatability										
S_r	0.032	0.018	0.012	0.017	0.030	0.027	0.034	0.035	0.038	0.047
r	0.089	0.049	0.034	0.047	0.084	0.075	0.094	0.972	0.107	0.133
***RSDr*(%)**	1.1	0.61	0.49	0.66	1.1	1.0	0.96	0.99	1.2	1.5
Reproducibility										
S_R	0.061	0.110	0.092	0.109	0.088	0.106	0.131	0.147	0.117	0.128
R	0.171	0.308	0.259	0.306	0.246	0.297	0.367	0.411	0.328	0.358
***RSD_R*(%)**	2.1	3.8	3.7	4.4	3.4	4.0	3.8	4.2	3.8	3.9
Ho_R	0.05	0.10	0.09	0.11	0.09	0.10	0.10	0.11	0.10	0.10

S_r: Standard deviation of the repeatability; r: Repeatability; RSD_r: Relative standard deviation of the repeatability; S_R: Standard deviation of the reproducibility; R: Reproducibility; RSD_R: Relative standard deviation of the reproducibility; Ho_R: Horwitz ratio.

Table 5. Statistical parameters from C18:1 acid determination in olive and olive –pomace oils										
Sample	**Extra virgin**		**Virgin**		**Lampante**		**Olive oil**		**Crude olive-pomace oil**	
Method	**KOH in methanol**	**Basic+acidic methylation**	**KOH in methanol**	**Basic+acidic methylation**	**KOH in methanol**	**Basic+acidic methylation**	**KOH in methanol**	**Basic+acidic methylation**	**KOH in methanol**	**Basic+acidic methylation**
Participants	15	16	15	16	15	16	15	16	15	16
Outliers	0	1	0	1	1	1	1	1	0	1
Mean (%)	79.42	79.39	74.55	74.56	75.55	75.41	76.14	76.22	75.80	75.02
Repeatability										
S_r	0.15	0.10	0.11	0.16	0.14	0.14	0.08	0.14	0.16	0.11
r	0.42	0.29	0.30	0.45	0.39	0.40	0.23	0.40	0.46	0.32
***RSDr*(%)**	0.19	0.13	0.15	0.21	0.19	0.19	0.11	0.19	0.21	0.15
Reproducibility										
S_R	0.49	0.42	0.45	0.49	0.45	0.48	0.47	0.48	0.64	0.57
R	1.37	1.18	1.26	1.37	1.26	1.34	1.33	1.33	1.80	1.58
***RSD_R*(%)**	0.61	0.53	0.61	0.66	0.60	0.63	0.62	0.63	0.85	0.75
Ho_R	0.03	0.02	0.03	0.03	0.03	0.03	0.03	0.03	0.04	0.03

S_r: Standard deviation of the repeatability; r: Repeatability; RSD_r: Relative standard deviation of the repeatability; S_R: Standard deviation of the reproducibility; R: Reproducibility; RSD_R: Relative standard deviation of the reproducibility; Ho_R: Horwitz ratio.

Table 6. Statistical parameters from C18:2 acid determination in olive and olive –pomace oils										
Sample	**Extra virgin**		**Virgin**		**Lampante**		**Olive oil**		**Crude olive-pomace oil**	
Method	**KOH in methanol**	**Basic+acidic methylation**	**KOH in methanol**	**Basic+acidic methylation**	**KOH in methanol**	**Basic+acidic methylation**	**KOH in methanol**	**Basic+acidic methylation**	**KOH in methanol**	**Basic+acidic methylation**
Participants	15	16	15	16	15	16	15	16	15	16
Outliers	2	2	1	2	0	1	1	1	0	1
Mean (%)	7.33	7.25	9.66	9.60	8.52	8.44	7.18	7.12	8.75	8.85
Repeatability										
S_r	0.02	0.04	0.03	0.06	0.06	0.08	0.04	0.04	0.05	0.04
r	0.07	0.12	0.08	0.16	0.17	0.21	0.12	0.11	0.13	0.10
***RSDr*(%)**	0.33	0.58	0.28	0.59	0.70	0.90	0.62	0.56	0.55	0.41
Reproducibility										
S_R	0.12	0.17	0.19	0.20	0.18	0.19	0.16	0.12	0.21	0.17
R	0.34	0.47	0.52	0.55	0.50	0.52	0.45	0.33	0.59	0.48
***RSD_R*(%)**	1.7	2.3	1.9	2.0	2.1	2.2	2.2	1.6	2.4	1.9
Ho_R	0.05	0.07	0.06	0.06	0.06	0.07	0.07	0.05	0.07	0.06

S_r: Standard deviation of the repeatability; r: Repeatability; RSD_r: Relative standard deviation of the repeatability; S_R: Standard deviation of the reproducibility; R: Reproducibility; RSD_R: Relative standard deviation of the reproducibility; Ho_R: Horwitz ratio.

Table 7. Statistical parameters from C18:3 acid determination in olive and olive –pomace oils										
Sample	**Extra virgin**		**Virgin**		**Lampante**		**Olive oil**		**Crude olive-pomace oil**	
Method	**KOH in methanol**	**Basic+acidic methylation**	**KOH in methanol**	**Basic+acidic methylation**	**KOH in methanol**	**Basic+acidic methylation**	**KOH in methanol**	**Basic+acidic methylation**	**KOH in methanol**	**Basic+acidic methylation**
Participants	15	16	15	16	15	16	15	16	15	16
Outliers	2	1	0	2	0	0	0	1	4	1
Mean (%)	0.730	0.719	0.896	0.876	0.860	0.834	0.744	0.720	0.753	0.852
Repeatability										
S_r	0.013	0.012	0.017	0.013	0.010	0.015	0.014	0.010	0.020	0.018
r	0.036	0.025	0.049	0.037	0.029	0.042	0.039	0.029	0.055	0.051
***RSDr*(%)**	1.8	1.7	1.9	1.5	1.2	1.8	1.9	1.4	2.6	2.2
Reproducibility										
S_R	0.029	0.032	0.041	0.039	0.036	0.043	0.028	0.030	0.041	0.035
R	0.080	0.089	0.116	0.110	0.101	0.120	0.079	0.083	0.115	0.097
***RSD_R*(%)**	3.9	4.4	4.6	4.5	4.2	5.2	3.8	4.1	5.4	4.1
Ho_R	0.08	0.09	0.10	0.10	0.09	0.11	0.08	0.09	0.12	0.09

S_r: Standard deviation of the repeatability; r: Repeatability; RSD_r: Relative standard deviation of the repeatability; S_R: Standard deviation of the reproducibility; R: Reproducibility; RSD_R: Relative standard deviation of the reproducibility; Ho_R: Horwitz ratio.

Table 8. Statistical parameters from C20:0 acid determination in olive and olive –pomace oils										
Sample	**Extra virgin**		**Virgin**		**Lampante**		**Olive oil**		**Crude olive-pomace oil**	
Method	**KOH in methanol**	**Basic+acidic methylation**	**KOH in methanol**	**Basic+acidic methylation**	**KOH in methanol**	**Basic+acidic methylation**	**KOH in methanol**	**Basic+acidic methylation**	**KOH in methanol**	**Basic+acidic methylation**
Participants	15	15	15	15	15	15	15	15	15	15
Outliers	1	1	0	1	0	1	1	1	0	4
Mean (%)	0.394	0.405	0.441	0.465	0.440	0.464	0.424	0.423	0.425	0.429
Repeatability										
S_r	0.015	0.016	0.018	0.015	0.013	0.017	0.013	0.013	0.019	0.013
r	0.041	0.044	0.050	0.042	0.037	0.047	0.037	0.037	0.053	0.038
***RSDr*(%)**	3.8	3.9	4.0	3.2	3.0	3.7	3.1	3.1	4.4	3.1
Reproducibility										
S_R	0.029	0.037	0.032	0.041	0.031	0.039	0.042	0.038	0.036	0.028
R	0.080	0.104	0.089	0.114	0.086	0.108	0.117	0.107	0.102	0.080
***RSD_R*(%)**	7.3	9.1	7.2	8.8	7.0	8.4	9.8	9.0	8.6	6.6
Ho_R	0.14	0.18	0.14	0.17	0.14	0.16	0.19	0.18	0.17	0.13

S_r: Standard deviation of the repeatability; r: Repeatability; RSD_r: Relative standard deviation of the repeatability; S_R: Standard deviation of the reproducibility; R: Reproducibility; RSD_R: Relative standard deviation of the reproducibility; Ho_R: Horwitz ratio.

Table 9. Statistical parameters from C20:1 acid determination in olive and olive –pomace oils										
Sample	**Extra virgin**		**Virgin**		**Lampante**		**Olive oil**		**Crude olive-pomace oil**	
Method	**KOH in methanol**	**Basic+acidic methylation**	**KOH in methanol**	**Basic+acidic methylation**	**KOH in methanol**	**Basic+acidic methylation**	**KOH in methanol**	**Basic+acidic methylation**	**KOH in methanol**	**Basic+acidic methylation**
Participants	15	16	15	16	15	16	15	16	15	16
Outliers	1	1	1	0	1	2	0	1	1	4
Mean (%)	0.372	0.375	0.388	0.400	0.370	0.379	0.280	0.284	0.296	0.298
Repeatability										
S_r	0.009	0.013	0.011	0.016	0.013	0.009	0.017	0.011	0.026	0.013
r	0.026	0.038	0.032	0.046	0.036	0.024	0.047	0.032	0.073	0.037
***RSDr*(%)**	7.8	3.6	3.0	4.1	3.5	2.3	6.0	4.0	8.9	4.4
Reproducibility										
S_R	0.029	0.032	0.034	0.032	0.023	0.027	0.028	0.024	0.027	0.016
R	0.082	0.091	0.095	0.091	0.064	0.077	0.079	0.069	0.077	0.045
***RSD_R*(%)**	7.9	8.7	8.7	8.1	6.2	7.2	10	8.6	9.3	5.4
Ho_R	0.15	0.17	0.17	0.16	0.12	0.14	0.18	0.16	0.17	0.10

S_r: Standard deviation of the repeatability; r: Repeatability; RSD_r: Relative standard deviation of the repeatability; S_R: Standard deviation of the reproducibility; R: Reproducibility; RSD_R: Relative standard deviation of the reproducibility; Ho_R: Horwitz ratio.

Table 10. Statistical parameters from C22:0 acid determination in olive and olive –pomace oils										
Sample	**Extra virgin**		**Virgin**		**Lampante**		**Olive oil**		**Crude olive-pomace oil**	
Method	**KOH in methanol**	**Basic+acidic methylation**	**KOH in methanol**	**Basic+acidic methylation**	**KOH in methanol**	**Basic+acidic methylation**	**KOH in methanol**	**Basic+acidic methylation**	**KOH in methanol**	**Basic+acidic methylation**
Participants	15	15	15	15	15	15	15	15	15	15
Outliers	0	1	1	0	1	1	1	1	3	2
Mean (%)	0.111	0.114	0.135	0.141	0.135	0.143	0.116	0.114	0.185	0.205
Repeatability										
S_r	0.008	0.011	0.013	0.008	0.014	0.008	0.016	0.010	0.013	0.015
r	0.022	0.032	0.036	0.022	0.039	0.021	0.045	0.029	0.036	0.041
***RSDr*(%)**	7.0	9.9	9.6	5.5	10	5.3	14	9.0	6.9	7.2
Reproducibility										
S_R	0.014	0.014	0.016	0.020	0.018	0.018	0.020	0.017	0.015	0.024
R	0.038	0.039	0.044	0.056	0.050	0.050	0.056	0.047	0.043	0.067
***RSD_R*(%)**	12	12	12	14	13	12	17	15	8.3	12
Ho_R	0.19	0.20	0.19	0.23	0.22	0.20	0.27	0.23	0.14	0.20

S_r: Standard deviation of the repeatability; r: Repeatability; RSD_r: Relative standard deviation of the repeatability; S_R: Standard deviation of the reproducibility; R: Reproducibility; RSD_R: Relative standard deviation of the reproducibility; Ho_R: Horwitz ratio.

Table 11. Statistical parameters from C24:0 acid determination in olive and olive –pomace oils										
Sample	**Extra virgin**		**Virgin**		**Lampante**		**Olive oil**		**Crude olive-pomace oil**	
Method	**KOH in methanol**	**Basic+acidic methylation**	**KOH in methanol**	**Basic+acidic methylation**	**KOH in methanol**	**Basic+acidic methylation**	**KOH in methanol**	**Basic+acidic methylation**	**KOH in methanol**	**Basic+acidic methylation**
Participants	15	16	15	16	15	16	15	16	15	16
Outliers	1	1	0	2	0	2	0	2	3	3
Mean (%)	0.040	0.047	0.062	0.076	0.058	0.075	0.049	0.056	0.075	0.125
Repeatability										
S_r	0.006	0.014	0.005	0.004	0.012	0.010	0.012	0.006	0.014	0.013
r	0.017	0.039	0.0153	0.012	0.033	0.029	0.033	0.017	0.040	0.036
***RSDr*(%)**	15	30	8.9	5.6	20	14	24	11	19	10
Reproducibility										
S_R	0.020	0.021	0.026	0.014	0.026	0.016	0.019	0.015	0.014	0.024
R	0.055	0.059	0.073	0.040	0.072	0.045	0.054	0.043	0.040	0.068
***RSD_R* (%)**	49	44	42	19	45	21	39	27	19	19
Ho_R	0.67	0.62	0.61	0.28	0.64	0.32	0.55	0.39	0.29	0.31

S_r: Standard deviation of the repeatability; r: Repeatability; RSD_r: Relative standard deviation of the repeatability; S_R: Standard deviation of the reproducibility; R: Reproducibility; RSD_R: Relative standard deviation of the reproducibility; Ho_R: Horwitz ratio.

Table 12. Statistical parameters from *trans*-C18:1 acids determination in olive and olive –pomace oils										
Sample	**Extra virgin**		**Virgin**		**Lampante**		**Olive oil**		**Crude olive-pomace oil**	
Method	**KOH in methanol**	**Basic+acidic methylation**	**KOH in methanol**	**Basic+acidic methylation**	**KOH in methanol**	**Basic+acidic methylation**	**KOH in methanol**	**Basic+acidic methylation**	**KOH in methanol**	**Basic+acidic methylation**
Participants	15	15	15	15	15	15	15	15	15	15
Outliers	1	2	1	1	1	1	1	3	2	1
Mean (%)	0.0100	0.0115	0.0114	0.0129	0.0107	0.0114	0.0127	0.0117	0.1173	0.0961
Repeatability										
S_r	0.0038	0.0078	0.0046	0.0027	0.0027	0.0046	0.0045	0.0050	0.0158	0.0109
r	0.0106	0.0220	0.0130	0.0075	0.0075	0.0130	0.0125	0.0140	0.0443	0.0304
***RSDr*(%)**	38	68	41	21	25	41	35	43	13	11
Reproducibility										
S_R	0.0096	0.0097	0.0098	0.0103	0.0107	0.0106	0.0113	0.0088	0.0559	0.0270
R	0.0268	0.0273	0.0276	0.0289	0.0300	0.0297	0.0316	0.0247	0.1566	0.0756
***RSD_R* (%)**	96	84	86	80	100	93	89	76	48	28
Ho_R	1.1	0.95	0.97	0.92	1.1	1.1	1.0	0.85	0.76	0.44

S_r: Standard deviation of the repeatability; r: Repeatability; RSD_r: Relative standard deviation of the repeatability; S_R: Standard deviation of the reproducibility; R: Reproducibility; RSD_R: Relative standard deviation of the reproducibility; Ho_R: Horwitz ratio.

Table 13. Statistical parameters from *tras*-C18:2 acid determination in olive and olive –pomace oils										
Sample	**Extra virgin**		**Virgin**		**Lampante**		**Olive oil**		**Crude olive-pomace oil**	
Method	**KOH in methanol**	**Basic+acidic methylation**	**KOH in methanol**	**Basic+acidic methylation**	**KOH in methanol**	**Basic+acidic methylation**	**KOH in methanol**	**Basic+acidic methylation**	**KOH in methanol**	**Basic+acidic methylation**
Participants	15	15	15	15	15	15	15	15	15	15
Outliers	1	2	0	1	2	1	2	1	0	1
Mean (%)	0.0061	0.0058	0.0111	0.0096	0.0081	0.0089	0.0073	0.0086	0.0133	0.0129
Repeatability										
S_r	0.0042	0.0034	0.0050	0.0019	0.0020	0.0019	0.0020	0.0053	0.0045	0.0038
r	0.0118	0.0095	0.0140	0.0053	0.0055	0.0053	0.0055	0.0150	0.0125	0.0129
***RSDr*(%)**	70	59	45	20	24	21	27	62	34	29
Reproducibility										
S_R	0.0064	0.0051	0.0079	0.0076	0.0058	0.0085	0.0068	0.0086	0.0120	0.0099
R	0.0178	0.0143	0.0223	0.0212	0.0162	0.0237	0.0190	0.0240	0.0337	0.0278
***RSD_R*(%)**	105	88	72	79	71	95	93	100	90	77
Ho_R	1.1	0.90	0.81	0.86	0.76	1.0	0.98	1.1	1.0	0.89

S_r: Standard deviation of the repeatability; r: Repeatability; RSD_r: Relative standard deviation of the repeatability; S_R: Standard deviation of the reproducibility; R: Reproducibility; RSD_R: Relative standard deviation of the reproducibility; Ho_R: Horwitz ratio.

Table 14. Statistical parameters from *trans*-C18:2 + *trans*-C18:3 acid determination in olive and olive –pomace oils										
Sample	**Extra virgin**		**Virgin**		**Lampante**		**Olive oil**		**Crude olive-pomace oil**	
Method	**KOH in methanol**	**Basic+acidic methylation**	**KOH in methanol**	**Basic+acidic methylation**	**KOH in methanol**	**Basic+acidic methylation**	**KOH in methanol**	**Basic+acidic methylation**	**KOH in methanol**	**Basic+acidic methylation**
Participants	15	15	15	15	15	15	15	15	15	15
Outliers	3	3	3	3	4	2	2	2	3	1
Mean (%)	0.0054	0.0054	0.0100	0.0088	0.0077	0.0092	0.0088	0.0081	0.0254	0.0186
Repeatability										
S_r	0.0046	0.0035	0.0050	0.0046	0.0021	0.0028	0.0102	0.0044	0.0061	0.0038
r	0.0128	0.0099	0.0140	0.0128	0.0060	0.0078	0.0285	0.0123	0.0171	0.0106
***RSDr*(%)**	84	65	50	52	28	30	115	54	24	20
Reproducibility										
S_R	0.0067	0.0051	0.0079	0.0069	0.0063	0.0095	0.0115	0.0086	0.0211	0.0151
R	0.0186	0.0144	0.0221	0.0193	0.0175	0.0267	0.0321	0.0241	0.0590	0.0423
***RSD_R* (%)**	123	95	79	79	81	103	130	117	83	81
Ho_R	1.2	0.96	0.87	0.85	0.86	1.1	1.4	1.1	1.1	0.99

S_r: Standard deviation of the repeatability; r: Repeatability; RSD_r: Relative standard deviation of the repeatability; S_R: Standard deviation of the reproducibility; R: Reproducibility; RSD_R: Relative standard deviation of the reproducibility; Ho_R: Horwitz ratio.

RECOMMENDATIONS FOR GAS CHROMATOGRAPHIC ANALYSIS OF THE FATTY ACID ESTERS FROM OLIVE OIL AND OLIVE-POMACE OIL

1. Procedure

The gas chromatographic analysis of solutions of fatty esters in heptane will be done according to standard ISO-5508 using a capillary column (50 m length x 0.25 or 0.32 mm i.d.) impregnated with cyanopropylsilicone phase as indicated for the determination of fatty acid *trans*-isomers (COI/T.20/Doc. no. 17).

Figure 1 gives the typical gas chromatographic profile of an olive-pomace oil containing methyl and ethyl esters of fatty acids, and *trans*-isomers of methyl esters.

2. Calculations

2.1. For the calculation of the fatty acid composition and ΔECN42, all the following fatty acids will be taken into account:

Myristic (C14:0)
Palmitic (C16:0). Sum of the areas of the peaks corresponding to the methyl and ethyl esters.
Palmitoleic (C16:1). Sum of the areas of the peaks corresponding to the ω9 and ω7 isomers of the methyl ester.
Margaric (C17:0).
Margaroleic (C17:1).
Stearic (C18:0).
Oleic (C18:1). Sum of the areas of the peaks corresponding to the ω9 and ω7 isomers of the methyl ester, ethyl ester, and *trans*-isomers of the methyl ester.
Linoleic (C18:2). Sum of the areas of the peaks corresponding to the methyl and ethyl esters, and the *trans*-isomers of the methyl ester.
Arachidic (C20:0).
Linolenic (C18:3). Sum of the areas of the methyl ester and the *trans*-isomers of the methyl ester.
Eicosenoic (C20:1).
Behenic (C22:0).
Lignoceric (C24:0).

Squalene will not be taken into account for the calculation of the total area.

2.2. For the calculation of the percentage of *trans*-C18:1 the peak corresponding to the methyl esters of this fatty acid will be used. For the sum [*trans*-C18:2 + *trans*-C18:3], all the peaks corresponding to the *trans*-isomers of these two fatty acids will be summed. For the calculation of the total area, all the peaks mentioned in 2.1. will be taken into account (see COI/T.20/Doc. no. 17).

The calculation of the percentage of each fatty acid will be done according to the formula

$$\% X = (\text{Area } X \times 100) / (\text{total area})$$

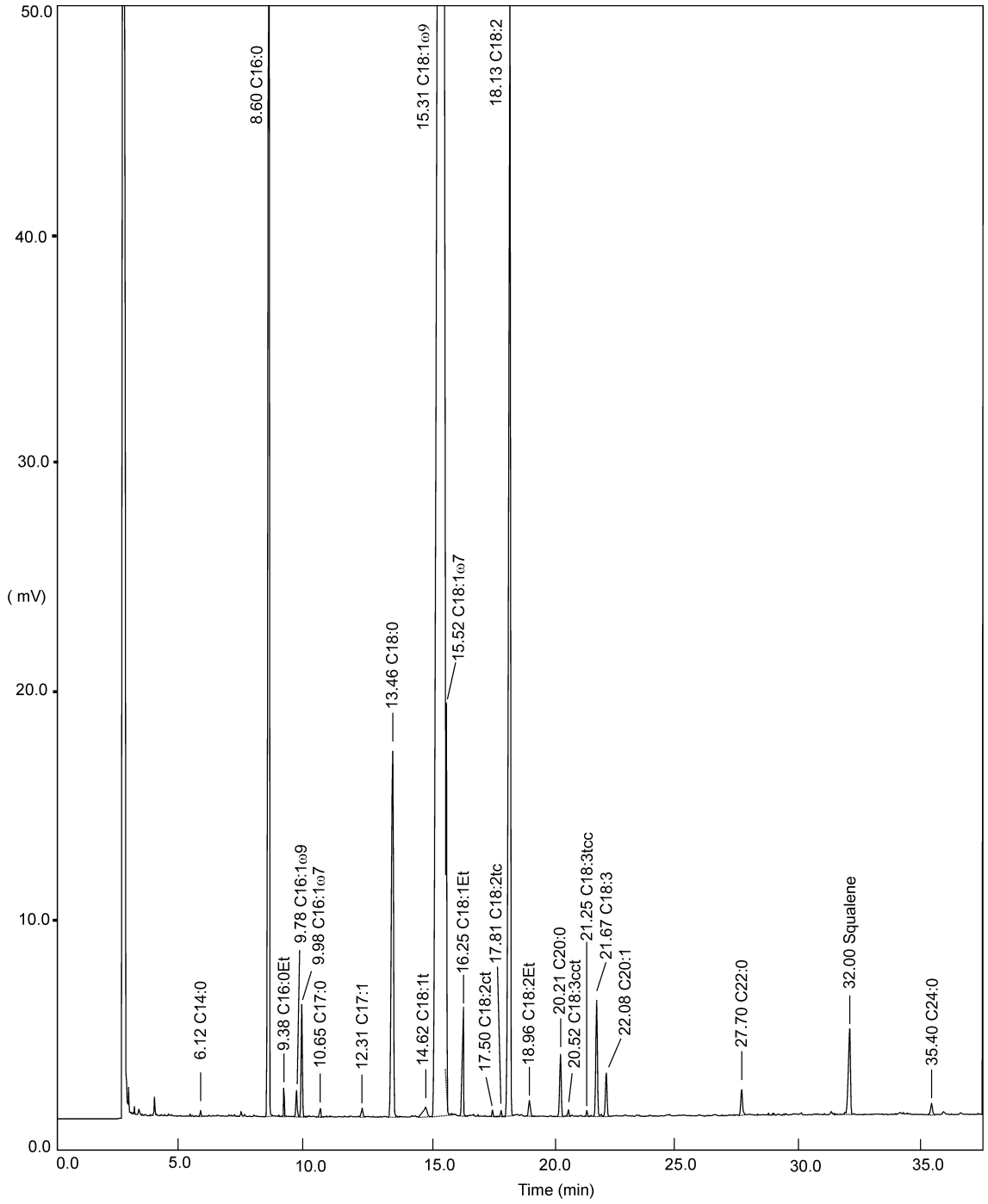

Figure 1 Gas chromatographic profile obtained by the cold methylation method from olive-pomace oil. The chromatographic peaks correspond to the methyl and ethyl esters except where otherwise indicated.