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Methods of preparation of fatty acid methyl esters (FAME). Statistical assessment of the precision characteristics from a collaborative trial

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RESUMEN

Métodos de preparación de ésteres metílicos de ácidos grasos (FAME). Evaluación estadística de la precisión del método mediante un estudio colaborativo.

Los métodos oficiales para el control del aceite de oliva y de orujo de oliva de la Unión Europea (UE) y del Comité Oleícola Internacional (COI) incluyen la determinación de ácidos grasos en la aplicación de varios criterios de pureza.

La determinación de ácidos grasos requiere la preparación de los ésteres metílicos de los ácidos grasos (FAME) y su posterior análisis mediante cromatografía de gases con una buena repetibilidad y reproducibilidad.

Entre los muchos métodos usados por los laboratorios de la industria y de los organismos oficiales de control, se seleccionaron los siguientes: 1) metilación en frío con potasa metanólica y 2) metilación en caliente con metilato sódico seguido de acidificación con ácido sulfúrico en metanol y calentamiento.

Se realizó una evaluación estadística de la precisión de la composición de ácidos grasos obtenidos usando ambos métodos de metilación, mediante un estudio colaborativo siguiendo las indicaciones recogidas por la AOAC (AOAC 1995).

En aceites con baja acidez, los resultados obtenidos usando ambos métodos de metilación fueron equivalentes. Sin embargo, en la muestra de aceite de orujo crudo de oliva (acidez 15.5%) se apreciaron diferencias significativas en la composición de ácidos grasos obtenidos usando ambos métodos.

Finalmente, el uso del método de metilación en caliente no dio lugar a un aumento de la concentración de isómeros *trans*.

PALABRAS-CLAVE: Aceite de oliva - Análisis estadistico - Esteres Metílicos Acidos Grasos - Estudio colaborativo - Métodos de preparación.

SUMMARY

Methods of preparation of fatty acid methyl esters (FAME). Statistical assessment of the precision characteristics from a collaborative trial.

The official regulations for the control of the olive and olive pomace oils of the European Union (EU) and International Olive Oil Council (IOOC) include the determination of fatty acids in order to be applied to several purity criteria.

The determination of fatty acids require the preparation of the fatty acid methyl esters (FAME) for the subsequent analysis by gas chromatography with good precision and reproducibility.

Among the methods used in the laboratories of both the industries and the official institutions looking after the olive oil control, the ones selected were: 1) cold methylation with methanolic potash and 2) hot methylation with sodium methylate followed by acidification with sulphuric acid in methanol and heating.

A statistical assessment of the precision characteristics were performed on the determination of fatty acids using both methods by a collaborative trial following the directions included in the AOAC regulation (AOAC 1995).

In oils with low acidities, the results obtained for both methylation methods were equivalent. However, the olive-pomace oil sample (acidity 15.5%) showed significative differences between the fatty acid compositions obtained using both methylation methods.

Finally, the methylation with the acidic+basic method did not yield an increase of the *trans*-isomers of the fatty acids.

KEY-WORDS: Collaborative analysis - Fatty Acid Methyl Esters (FAME) - Methods of preparation - Olive oil - Statistical analysis.

1. INTRODUCTION

The official regulations for the control of the olive and olive pomace oils of the European Union (EU) (EEC 1991) and International Olive Oil Council (IOOC) (IOOC 1998) include the fatty acid composition from C14:0 to C24:0 and the *trans*-isomers of the fatty acid (*t*-C18:1 and *t*-C18:2+*t*-C18:3) as criteria for the oil genuineness. Furthermore, the fatty acid composition, in particular the C16:0, C16:1, C18:0, C18:1, C18:2 and C18:3 are used in the determination of a purity criterion, that is the difference between the experimental value of the triacylglycerols of equivalent carbon number 42 (ECN42) determined experimentally by HPLC and the theoretical one obtained from the fatty acid composition.

The determination of fatty acid requires the preparation of the fatty acid methyl esters (FAME), in order to improve volatility and to reduce peak tailing, and subsequent analysis by gas chromatography with good precision and reproducibility.

Usually, FAME can be conveniently prepared by reaction of lipids with a large excess of methanol with either acid- or base-catalytic reagents. There are several described methods to prepare FAME (EEC 1991), IUPAC 1987), (ISO 1998) from fats and oils. These methods are based in different chemical principles and therefore, the results are not completely equivalent. We can divide the methods in two main groups: a) methods causing only transesterification of glyceridic compounds and waxes and which do not usually form FAME from free fatty acids (Glass 1971) (basic methylation, $(CH_3)_2SO_4$ and cold methanolic potash) and b)

methods that methylate glyceridic compounds and free fatty acids (Christie 1992) (F_3B, acidic methylation, trimethylsulfonium hydroxide, basic + acidic methylation and 1,1,3,3-tetramethylguanidine (Schuchardt *et al.* 1988)). However, in virgin olive oils and specifically in crude pomace oils, there are fatty acid ethyl esters which behaviour in the different methylation methods are not completely studied. With the aim of standardizing the FAME preparation method for olive and olive pomace oils, to obtain repeatable and reproducible measures, the Expert Chemist Group of the International Olive Oil Council chose one method of each group bearing in mind at it easiness and low toxicity of the reactants and solvents used. The methods selected for being used in the laboratories of both the industries and the official institutions looking after the olive oil control were: 1) cold methylation with methanolic potash and 2) hot methylation with sodium methylate followed by acidification with sulphuric acid in methanol and heating.

To standardize these methods the IOOC organized a collaborative trial with the participation of laboratories of different countries. The results obtained are exposed.

2. EXPERIMENTAL

2.1. Sample descriptions

The oil samples are as follows:

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

2.2. Instructions

Samples were sent, together with the following instructions. The methyl esters solution should be analysed as soon as possible. If it is necessary, the heptane solution may be stored under an inert gas in a refrigerator to protect the methyl esters from autoxidation.

The gas chromatographic analysis of the heptane solutions must be done according to the method for the determination of fatty acid trans-isomers (EEC 1992).

Each sample must be analysed using both A and B methylation methods. Each method must be done by duplicate. In total, four analyses by each oil sample.

The *cis*-isomers of the FAME from C14:0 up to C24:0 and the *trans*-isomers of the C18:1, C18:2 and C18:3 must be considered. Likewise, the ethyl esters of C16:0, C18:1 and C18:2 must be considered (Figure 1). The results must be expressed as the percentages on total area of peaks, excluding the area of squalene, with two decimal figures.

2.3. Methods description

A) Methylation with cold methanolic solution of potassium hydroxide (IUPAC 1987)

In a 5 -ml screw top test tube, weigh 0.10 g of the oil sample. Add 2 ml of heptane and stir. Add 0.20 ml of 2N methanolic potassium hydroxide solution, put on the cap provided with a PTFE (polytetrafluoroethylene)-joint, tighten the cap, and shake vigorously for 15 seconds. Leave to stratify until the upper solution becomes clear. Decant the upper layer containing the methyl esters.

B) Methylation by heating with sodium methylate in methanol followed by heating in acidic medium (IUPAC 1987)

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 0.2N sodium methylate in metanol and boiling chips. Fit a reflux condenser, stir, and bring to the boil. The solution should become clear, which usually occurs in about 10 minutes. The reaction is complete after 15 minutes. Take away the flask from heating, wait until the reflux stops, remove the condenser, and add two drops of 1% of phenolftalein solution in methanol. Add a solution of 1N sulphuric acid in methanol until the solution becomes colourless and, then, add 1 ml in excess. Fit the condenser and boil again for 20 minutes. Withdraw the source of heat and cool the flask under running water. Remove the condenser, add 20 ml of

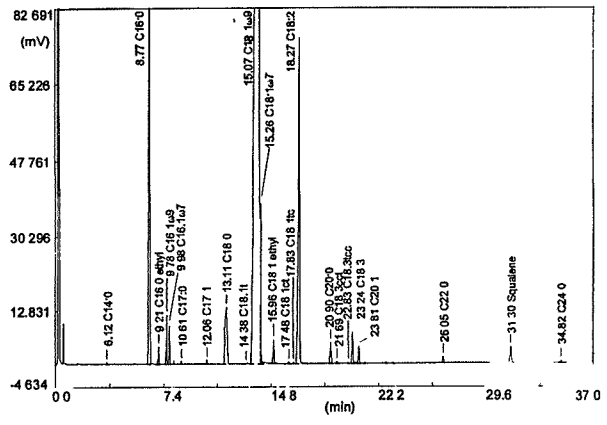

Figure 1
Gas chromatographic profile of fatty acid esters obtained by the method A from a mixture of crude olive-pomace oil and refined olive oil. The peaks correspond to the methyl esters otherwise indicated.

saturated sodium chloride aqueous solution, and stir. Add 5 ml of heptane, plug the flask, and shake vigorously for 15 seconds. Leave to settle until the two phases have separated. Add again saturated sodium chloride solution until the aqueous layer reaches the lower end of the flask neck. The upper layer containing the methyl esters fills the flask neck.

2.4. Results organization

2.4.1. Fatty acid composition

For calculation of the fatty acid composition, the following peaks were considered: C14:0 methyl ester, C16:0 as sum of methyl and ethyl esters, C16:1 as sum of two *cis*-methyl esters (C16:1ω9 and C16:1ω7), C17:0 methyl ester, C17:1 methyl ester, C18:0 methyl ester, C18:1 as the sum of two *cis*-methyl esters (C18:1ω9 and C18:1ω7), ethyl ester and *trans*-methyl ester, C18:2 as sum of *cis*-methyl, ethyl esters and *trans*-methyl esters, C18:3 as sum of *cis*- and *trans*-methyl esters, C20:0 methyl ester, C20:1 methyl ester, C22:0 methyl ester and C24:0 methyl ester.

2.4.2. Fatty acid trans-isomers

The *trans*-isomers of the fatty acids C18:1, C18:2 (as sum of the *trans-cis*-isomer, *cis-trans*-isomer and *trans-trans*-isomer) and C18:3 (as sum *of trans-cis-cis*-isomer, *cis-trans-cis*-isomer, *cis-cis-trans*-isomer and *trans-cis-trans*-isomer) were determined following the method included in the Regulation EEC/1492/92 (EEC 1992).

2.5. Statistical interlaboratory study

The collaborative trial was set up following the directions gave by W. Horwitz (Horwitz 1988). The statistical analysis of repeatability and reproducibility was performed following the ISO5725 (ISO 1986) and AOAC Regulation (AOAC 1995) where the procedures of identification of outliers and mathematical procedures are described, using a computer program developed by the authors. The criterion used to identify outliers was the Cochran and Grubbs test, determining respectively the laboratories that gave results quite different among replicates and those with outstandingly high and low values.

The statistical parameters used were the followings:

- 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
- H_{OR}: Horwitz ratio $\left[\frac{RSD_R}{RSD_{R\,th}}\right]$ where,

  $RSD_{R\,th} = 2^{(1-0.5 \log C)}$, being C the concentration of the analytes expressed in 10 power.

Among these parameters is important to point up the statistical meaning of the repeatability, within-laboratory variance, which indicate that the values obtained in two successive determinations of the same sample, using the same analytical method, do not differ more than the value of r. Likely, the reproducibility, interlaboratory variance, means that the results obtained by two laboratories using the same sample and analytical method, do not differ in more of the R value. Besides, it must be highlighted the meaning of the Horwitz ratio (H_{OR}) that take in account the analytes concentration figures. The value is obtained by the ratio between the RSD_R experimental and theoretical in such way that a value equal or less than 1 means that the analytical method has a good reproducibility (Pocklington 1991).

The comparison between both methylation methods was done by an analysis of variance (ANOVA) with repeated measures, being significative differences higher than 95%. The analysis was performed using the statistical package STATISTICA (Statsoft Inc., USA).

2.6. Operating conditions

Data shown in Table 1 were reported by collaborators on the operating conditions for the GC analysis. The majority of laboratories used columns of 50 or 60 m length, 0.25 μm of internal diameter, coated with cyanopropylpolysiloxane or cyanopropylphenylsiloxane (0.20-0.25 μm of film thickness).

3. RESULTS AND DISCUSSION

The collaborative study was performed with the results of 17 laboratories of different countries for evaluating the reproducibility and repeatability of the FAME preparation methods.

The results sent by the laboratory 17 were discarded, because the application of response factor for each individual FAME. The determination performed by the laboratory 16 using the method A was also discarded, because the lack of identification of the ethyl esters, neither the FAME C22:0 obtained by the method B. From the results sent by the laboratory 4, the results corresponding to the *trans*-isomers obtained by the method B were also discarded, because the use of a gas

Table 1
GC Operating condition of the laboratories

Laboratory	Column	Lenght (m x mm)	Film Thickness (μm)	Carrier Gas	Initial		Final		Rate (°C/min)	Injector Temp. (°C)	Detector Temp. (°C)
					Temp. (°C)	Time (min)	Temp. (°C)	Time (min)			
1	SP-2380	60 x 0.25	0.20	He	165	35	210	10	5	230	250
2	BPX-70	60 x 0.30	0.25	He	185	—	—	—	—	220	260
3	SP-2380	60 x 0.25	0.20	H_2	160	13	190	4	1.5	220	250
4	CPSIL-88	50 x 0.25	0.20	H_2	160	—	180	—	—	220	250
4	CW-20	25 x 0.25	0.20	He	170	—	220	—	3	250	250
5	SP-2380	60 x 0.25	0.20	H_2	167	5	175	5	1	240	260
5	SP-2380	60 x 0.25	0.20	H_2	167	5	200	8	2	240	260
6	SP-2340	60 x 0.32	0.20	He	150	18	175	5	2	230	230
6	SP-2340	60 x 0.32	0.20	He	150	18	200	10	5	230	230
7	RT-2330	60 x 0.32	0.20	He	170	7	190	—	2	240	240
7	RT-2330	60 x 0.32	0.20	He	170	7	220	3	3	240	240
8	CPSIL-88	100 x 0.20	0.20	H_2	150	—	220	—	1.5	250	250
9	CPSIL-88	50 x 0.25	—	—	170	—	—	—	—	230	250
10	RT-2330	60 x 0.25	0.20	H_2	170	21	200	—	5	250	260
11	SP-2380	60 x 0.25	0.20	He	170	25	210	—	5	250	260
12	CPSIL-88	60 x 0.25	0.20	He	185	12	210	—	5	280	290
13	SP-2330	60 x 0.32	0.20	He	180	15	210	—	5	220	220
14	BPX-70	50 x 0.33	0.25	He	160	30	210	—	5	250	280
15	BPX-70	30 x 0.25	0.25	H_2	170	—	—	—	—	250	250
16	BPX-70	50 x 0.22	0.25	—	180	—	—	—	—	250	230
17	SP-2380	60 x 0.25	0.25	He	155	2	182	—	1	220	250
17	SP-2380	60 x 0.25	0.25	He	155	2	210	—	3	220	250

chromatographic stationary phase quite different (Carbowax 20M).

The determination of the ethyl esters of the fatty acids by the method A (Table 2) showed results with a great variability of values, in both within-laboratory and inter-laboratory determinations, indicating that transesterification of the ethyl esters of the fatty acids is a slow reaction and hence, the results depend on the operating mode. Therefore, to calculate the percentages of C16:0, C18:1 and C18:2 the sum of areas corresponding to the peaks of both the methyl and the ethyl esters and trans-isomers were taken in account.

Table 2
Results of ethyl esters (%) determination by cold methanolic KOH method (A) in crude olive-pomace oil sample (5).

Participant	C16:0 Ethyl		C18:1 Ethyl		C18:2 Ethyl	
1	0.03	0.03	0.25	0.26	0.03	0.03
2	0.62	0.65	3.49	3.81	0.60	0.65
3	0.19	0.45	0.98	2.24	0.14	0.35
4	0.26	0.27	1.43	1.45	0.22	0.23
5	0.50	0.48	2.55	2.47	0.40	0.38
6	0.12	0.13	0.76	0.80	0.08	0.09
7	0.10	0.11	0.65	0.66	0.09	0.09
8	0.47	0.39	2.46	2.11	0.56	0.46
9	0.73	0.77	3.43	3.72	0.57	0.57
10	0.43	0.60	2.46	3.22	0.20	0.23
11	0.42	0.42	2.08	2.08	0.36	0.36
12	0.78	1.27	3.31	6.38	0.63	1.04
13	0.67	0.45	0.14	0.10	0.59	0.44
14	0.15	0.12	0.94	0.84	0.14	0.12
15	0.00	0.00	0.85	0.78	0.15	0.12

On the other hand, methylation by the method B yielded negligible amounts of ethyl esters, indicating that transesterification was completed.

The results obtained were analysed statistically as stated in the experimental section and the results showed in Tables 3 to 16 for each fatty acid.

From the preliminary examination of the results, it can be pointed out that in general there are good acceptances of the results, except for the fatty acid C16:0 (Table 4) and for the *trans*-C18:2+*trans*-C18:3 (Table 16), in which there are more outliers than expected, probably due in the former to the mass discrimination produced during the GC split injection and in the latter to the different gas chromatographic operating modes result in erroneous assignments.

Regarding the repeatability, the results are acceptable, although the C14:0 (Table 3), C24:0 (Table 13), *trans*-C18:1 (Table 14), *trans*-C18:2 and *trans*-C18:2+*trans*-C18:3 (Tables 15 and 16) showed high *RSDr* values, due to that in olive oils these FAME gave very small peaks (less than 0.05%), close to the detection limit.

Similarly, the reproducibility is good for the major components since their H_{OR} are lower than 0.27, meaning that the variance is according to the concentration. For those components with values close to the detection limit: C14:0 (Table 3), C24:0 (Table 13), *trans*-C18:1 (Table 14), *trans*-C18:2 (Table 15) and *trans*-C18:2+*trans*-C18:3 (Table 16); the RSD_R

Table 3

Statistical parameters from C14:0 acid determination in olive and olive-pomace oils

Sample	Extra virgin		Virgin		Lampant		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										
Sr	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	23
H_{Or}	0.5	0.6	0.5	0.6	0.6	0.7	0.5	0.6	0.4	0.3

Table 4

Statistical parameters from C16:0 acid determination in olive and olive-pomace oils

Sample	Extra virgin		Virgin		Lampant		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										
Sr	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
H_{Or}	0.09	0.05	0.05	0.05	0.10	0.05	0.14	0.13	0.15	0.05

Table 5

Statistical parameters from C16:1 acid determination in olive and olive-pomace oils

Sample	Extra virgin		Virgin		Lampant		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										
Sr	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	0.044	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
H_{Or}	0.14	0.14	0.08	0.15	0.13	0.13	0.11	0.14	0.15	0.15

Table 6
Statistical parameters from C18:0 acid determination in olive and olive-pomace oils

Sample	Extra virgin		Virgin		Lampant		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										
Sr	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	0.097	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
H_{Or}	0.05	0.10	0.09	0.11	0.09	0.10	0.10	0.11	0.10	0.10

Table 7
Statistical parameters from C18:1 acid determination in olive and olive-pomace oils

Sample	Extra virgin		Virgin		Lampant		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										
Sr	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
H_{Or}	0.03	0.02	0.03	0.03	0.03	0.03	0.03	0.03	0.04	0.03

Table 8
Statistical parameters from C18:2 acid determination in olive and olive-pomace oils

Sample	Extra virgin		Virgin		Lampant		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										
Sr	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
H_{Or}	0.05	0.07	0.06	0.06	0.06	0.07	0.07	0.05	0.07	0.06

Table 9

Statistical parameters from C18:3 acid determination in olive and olive-pomace oils

Sample	Extra virgin		Virgin		Lampant		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										
Sr	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
H_{Or}	0.08	0.09	0.10	0.10	0.09	0.11	0.08	0.09	0.12	0.09

Table 10

Statistical parameters from C20:0 acid determination in olive and olive-pomace oils

Sample	Extra virgin		Virgin		Lampant		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										
Sr	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
H_{Or}	0.14	0.18	0.14	0.17	0.14	0.16	0.19	0.18	0.17	0.13

Table 11

Statistical parameters from C20:1 acid determination in olive and olive-pomace oils

Sample	Extra virgin		Virgin		Lampant		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										
Sr	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	10.0	8.6	9.3	5.4
H_{Or}	0.15	0.17	0.17	0.16	0.12	0.14	0.18	0.16	0.17	0.10

Table 12
Statistical parameters from C22:0 acid determination in olive and olive-pomace oils

Sample	Extra virgin		Virgin		Lampant		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										
Sr	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	10.0	5.3	14.0	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 *(%)*	12.0	12.0	12.0	14.0	13.0	12.0	17.0	15.0	8.3	12.0
H_{Or}	0.19	0.20	0.19	0.23	0.22	0.20	0.27	0.23	0.14	0.20

Table 13

Statistical parameters from C24:0 acid determination in olive and olive-pomace oils

Sample	Extra virgin		Virgin		Lampant		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										
Sr	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	0.015	0.012	0.033	0.029	0.033	0.017	0.040	0.036
RSDr(%)	15.0	30.0	8.9	5.6	20.0	14.0	24.0	11.0	19.0	10.0
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 *(%)*	49.0	44.0	42.0	19.0	45.0	21.0	39.0	27.0	19.0	19.0
H_{Or}	0.67	0.62	0.61	0.28	0.64	0.32	0.55	0.39	0.29	0.31

Table 14
Statistical parameters from *trans*-C18:1 acid determination in olive and olive-pomace oils

Sample	Extra virgin		Virgin		Lampant		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										
Sr	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(%)	38.0	68.0	41.0	21.0	25.0	41.0	35.0	43.0	13.0	11.0
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 *(%)*	96.0	84.0	86.0	80.0	100.0	93.0	89.0	76.0	48.0	28.0
H_{Or}	1.10	0.95	0.97	0.92	1.10	1.10	1.00	0.85	0.76	0.44

Table 15
Statistical parameters from trans-C18:2 acid determination in olive and olive-pomace oils

Sample	Extra virgin		Virgin		Lampant		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										
Sr	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(%)	70.0	59.0	45.0	20.0	24.0	21.0	27.0	62.0	34.0	29.0
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 *(%)*	105.0	88.0	72.0	79.0	71.0	95.0	93.0	100.0	90.0	77.0
H_{Or}	1.10	0.90	0.81	0.86	0.76	1.0	0.98	1.10	1.0	0.89

Table 16
Statistical parameters from *trans*-C18:2 +*trans*-C18:3 acid determination in olive and olive-pomace oils

Sample	Extra virgin		Virgin		Lampant		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										
Sr	0.0046	0.0035	0.0050	0.0046	0.0021	0.0028	0.0102	0.0044	0.0061	0.038
r	0.0128	0.0099	0.0140	0.0128	0.0060	0.0078	0.0285	0.0123	0.0171	0.0106
RSDr(%)	84.0	65.0	50.0	52.0	28.0	30.0	115.0	54.0	24.0	20.0
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 *(%)*	123.0	95.0	79.0	79.0	81.0	103.0	130.0	117.0	83.0	81.0
H_{Or}	1.20	0.96	0.87	0.85	0.86	1.10	1.40	1.10	1.10	0.99

values range between 43 to 123%, although the H_{Or} was close to 1.

In respect to the comparison of the FAME preparation methods, the fatty acid compositions were similar in samples 1 to 4. However, in sample 5 (crude olive-pomace oil) the variance analysis showed that there are significative differences *(p-level < 0.005) between both methods in the* FAME percentages, probably due that by method A the abundant free fatty acids are not methylated.

Finally, in reference to the *trans*-isomers, there are not significative differences between both methods, indicating that the acidic methylation does not yield *trans*-isomers in this conditions.

4. CONCLUSIONS

The results obtained showed a good repeatability and reproducibility in both methods. Concluding that, in olive oils with low acidity (virgin, olive, refined olive oil, refined pomace oil and olive-pomace oil) is recommended to use the method A, bearing in mind that the ethyl esters have to be added to the corresponding fatty acid methyl esters. On the other hand, in olive oils with high acidity is recommended to use the method B, in order to assume the methylation of free fatty acids. In respect to the trans-isomers determination, the method A is the most appropriate method, although, the basic+acidic method can be also used.

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