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Determination of Esters of Fatty Acids with Low Molecular Weight Alcohols in Olive Oils

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A simple and precise analytical method was developed for the simultaneous determination of squalene and methyl, ethyl, propyl, and butyl esters of fatty acids present in olive and olive pomace oils. A fraction containing squalene and fatty acid alkyl esters was isolated from the oil by solid phase extraction on silica gel cartridges and quantitatively analyzed by gas chromatography. A modification of the procedure allowed the isolation of squalene and esters separately. Repeatability and recovery of the method were good. The method was applied to extra and lampant virgin olive oil categories and also to oils obtained from olive pomace by second centrifugation and solvent extraction. Extra virgin olive oils contained low amounts of fatty acid methyl and ethyl esters, while oils obtained from altered olive or olive pomace showed high concentrations of fatty acid alkyl esters, mainly ethyl esters. Correlation between oil acidity and ethyl esters concentration was poor.

KEYWORDS: Solid phase extraction−GC analysis; olive oil; olive pomace oil; fatty acid low molecular weight alkyl esters; squalene

INTRODUCTION

Minor amounts of fatty acid ethyl esters (FAEEs) were found in the unsaponifiable matter of rapeseed and sunflower oils, but they were considered to be byproducts of the transesterification that took place during the saponification of triacylglycerols (TAGs) (*1*). Mellidis and Papageorgiou (*2*) studied the lipids from the roots of a shrub, which grows in the North of Greek, and assumed that those ethyl esters are of particular importance since they are connected with the biosynthesis of fatty substances in plants. Although these authors assured that these compounds are present in rare cases in the lipids of higher plants, Mariani et al. found methyl and ethyl esters in olive oils (*3*−*5*). Recently, we have detected significant amounts of ethyl esters of palmitic, oleic, and linoleic acids by gas chromatographic (GC) analysis of the fatty acid methyl esters (FAMEs) fraction obtained from some olive oils by transesterification with cold methanolic solution of KOH (*6*). However, the composition of fatty acid alkyl esters (FAAEs) and their significance for the olive oil quality have not been yet studied.

The FAEEs show a polarity similar to squalene, and both compounds have been isolated together from the oil by thin-layer chromatography (TLC) on silica gel plates (*1*). The squalene is a minor constituent of vegetable oils that is rather abundant (800−12 000 mg/kg) in pumpkin and olive oils (*1*, *7*). The official method of analysis of the Association of Official Analytical Chemists (AOAC) for determining this polyunsaturated hydrocarbon in the oil is time-consuming since it requires saponification of the oil, fractioning of the unsaponifiable matter on an alumina column, and analysis by iodometric titration (*8*). The fractioning of the unsaponifiable on a silica gel column and analysis by GC have also been reported (*7*).

In this paper, an analytical procedure for the quantitative determination of FAAEs together with squalene in vegetable oils is developed. The fraction containing these compounds was isolated from the oil by solid phase extraction (SPE) and then directly analyzed by GC. A modification of the SPE procedure allowed the isolation of FAAEs fraction free of squalene. The precision of the analytical method is determined.

The origin of FAAEs in virgin olive oils of different quality is investigated. Finally, the determination of FAAEs by methylation of the oil with cold methanolic potash is also discussed.

MATERIALS AND METHODS

Samples. Virgin olive oils of different origins, varieties, and qualities were obtained from oil mills. "Fusty" olive oils were obtained from olive fruits (500 kg) piled under atmospheric conditions for 14, 28, 42, and 50 days, using the Abencor system (Comercial Abengoa, S. A., Sevilla, Spain) (*9*).

Second centrifugation olive oils were obtained from the olive pomace produced in an industrial oil mill operating in two phase centrifugation mode. The olive pomace was again mixed for 30 min at 40 °C in an Abencor mixer and later centrifuged. Aliquots of the olive pomace were processed after they were obtained and after storage at room temperature for 1 and 2 months. Samples of olive pomace oils obtained by solvent extraction and second centrifugation oils were supplied by a local refining industry.

Materials and Reagents. All reagents were of analytical reagent grade unless otherwise stated. Standards of heptadecanoic acid ethyl

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ester (C17:0 EE), octadecenoic acid ethyl ester (C18:1 EE), squalene, and squalane were purchased from Sigma (St. Louis, MO). The SPE cartridge (6 mL), packed with silica gel phase (1000 mg), was from Varian (EA Middelburg, The Netherlands). Dye Sudan I was supplied by Aldrich (Steinheim, Germany).

Standard Solutions. *Standard Solution A.* A solution of 0.5 mg/mL of C17:0 EE in hexane was used as internal standard for determining esters (for extra virgin olive oils a solution of 0.05 mg/mL was used).

Standard solution B. A solution of 0.5 mg/mL of squalane, in hexane, was used as internal standard for determining squalene. A solution of dye Sudan I (1 mg/mL) in hexane/ethyl ether 99:1 was prepared.

SPE. Olive oil (0.1 ± 0.001 g) was weighed into a 3 mL vial, and 500 μL of both standard solutions A and B and two drops of Sudan I solution were added. The vial was screwed up, and the mixture was homogenized. A silica SPE cartridge (1000 mg) was placed in a vacuum elution apparatus and conditioned by the passing of 6 mL of hexane. The oil solution in hexane containing the standards was applied to the column, and the solvent was pulled through, leaving the sample, the standards, and the stain on the top of the column. The sample container was washed with two 0.5 mL portions of hexane that were added and run out the cartridge. The column was eluted with approximately 10 mL of the solvent mixture hexane/ethyl ether 99:1 until the dye reached the lower part of the cartridge. The eluate was evaporated in a rotary evaporator at room temperature under vacuum until dryness. The residue was redissolved with 200 μL of hexane, and a volume (1 μL) of the solution was injected into the GC.

For the analysis of a fraction containing only the esters, the standard B was not added. The oil sample was applied to the SPE cartridge as indicated in the above paragraph, the column was eluted first with 12 mL of hexane, and the fraction was rejected. Next, the column was eluted with approximately 10 mL of the solvent mixture hexane/ethyl ether 99:1 until the dye reached the lower part of the cartridge. The procedure follows as previously described.

GC Analysis. Chromatographic analysis of the esters and squalene was performed using a Chrompack (Middelburg, Netherlands) CP9000 GC fitted with a flame ionization detector and a split injection system (split ratio 1:30). Separations were carried out on an Rtx-65TG capillary column (30 m × 0.25 mm id) coated with 35% dimethyl−65% diphenyl polysiloxane (Restek Corporation, Bellefonte, PA). The operating conditions were the following: oven temperature, 160 °C for 10 min and then increased at 10 °C/min up to 325 °C and maintained for 10 min; injector and detector temperatures, 320 °C. Hydrogen was used as the carrier gas at a column head pressure of 110 psi.

For identification purposes, the GC analysis of FAAEs was carried out according to the method described for the determination of FAMEs in olive oils (*10*). A fused silica capillary column (SP-2380, 60 m × 0.25 mm id, Supelco, Bellefonte, PA) coated with cyanopropylsilicone (0.20 μm film thickness) and hydrogen as a gas carrier was used. Operating conditions were as follows: initial oven temperature, 160 °C for 13 min and then increased at 1.5 °C/min until 190 °C and maintained for 5 min; temperatures of injector and detector temperature, 225 and 250 °C, respectively. Data acquisition and processing were carried out using a Chrom-Card Data System (Fisons, Altrincham, U.K.).

Repeatability and Recovery. For the determination of recovery, stock solutions of C18:1 EE and squalene were prepared in hexane. Increasing quantities of both stock solutions were added to crude sunflower oil. For each concentration, six replicates were prepared. The oil samples were processed according to the analytical procedure, and the results were compared with those obtained by direct injection of the stock solutions.

GC−MS Identification. A mass spectrometer MAT 95-S (Finnigan, Manchester, U.K.) was coupled directly to a GC HP-5890 (Hewlett-Packard, Wilmington, DE) fitted with an Rtx-65TG fused silica capillary column. Electron impact ionization at 70 eV and resolution 2500 were used. The GC operating conditions were as indicated in the analytical procedure.

Methylation of Total Oil. Into 5 mL screw top test tubes, aliquots of an oil (0.10 g) were weighted and dissolved in 2 mL of heptane. Volumes (0.20 mL) of 2 N methanolic KOH solution were added, and the tubes were tightened with a screw cap provided with a PTEF joint.

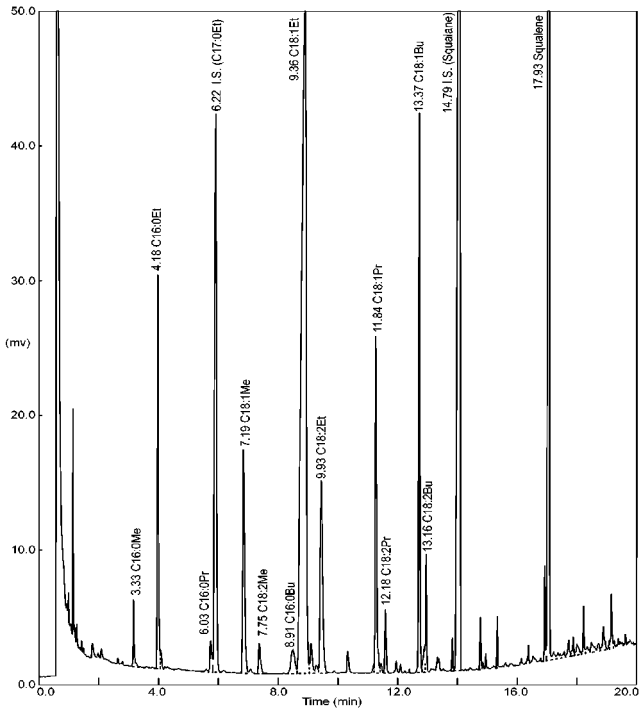

Figure 1. GC chromatogram of FAAEs and squalene from crude olive pomace oil using a RTx-65TG fused silica capillary column (30 m × 0.25 mm id) coated with 35% dimethyl–65% diphenyl polysiloxane.

Each tube was vigorously shaken during different times (from 5 to 60 s), and then, 0.5 mL of standard solution (1.5 mg/mL) of C17:0 EE was added. The solvent mixtures were left to stratify until the upper solutions became clear. The upper layers were separated and immediately analyzed by GC.

Acidity Determination. Acidity was determined by titration following European Commission Regulation (EEC) 2568/91 (*11*). Results were expressed as a percentage of oleic acid.

RESULTS AND DISCUSSION

Determination of FAAEs and Squalene. In olive oils, the nonpolar minor compounds include hydrocarbons, FAAEs, and waxes. The TLC analysis on silica gel plates of a mixture of these compounds in oils run with a solvent mixture of hexane/diethyl ether (99:1) showed that they could be isolated from the oil. Therefore, the use of SPE silica cartridges using the same solvent mixture allowed the isolation of the hydrocarbons, FAEEs, and waxes from the oil. The elution volume was rather variable depending on the cartridge batches, temperature, and elution flow rate. To prevent incomplete elution of esters or partial elution of TAGs, the dye Sudan I was added to visualize the elution of the compounds. This dye has a similar retention time to TAGs (*12*); consequently, the elution was stopped when the colored band reached the bottom of the cartridge. When the silica cartridge was eluted with 10 mL of hexane prior to the elution with hexane/diethyl ether 99:1, a first fraction containing only hydrocarbons was obtained. A second fraction containing FAAEs and waxes was isolated by subsequent elution with hexane/diethyl ether 99:1. The composition of both fractions was checked by TLC and GC.

For the GC analysis of the fractions containing the FAAEs, a capillary column coated with a polar phase (35% dimethyl−65% diphenyl polysiloxane) working at high temperatures (160−325 °C) was chosen, because it is possible to elute the waxes by increasing the oven temperature (up to 325 °C). The

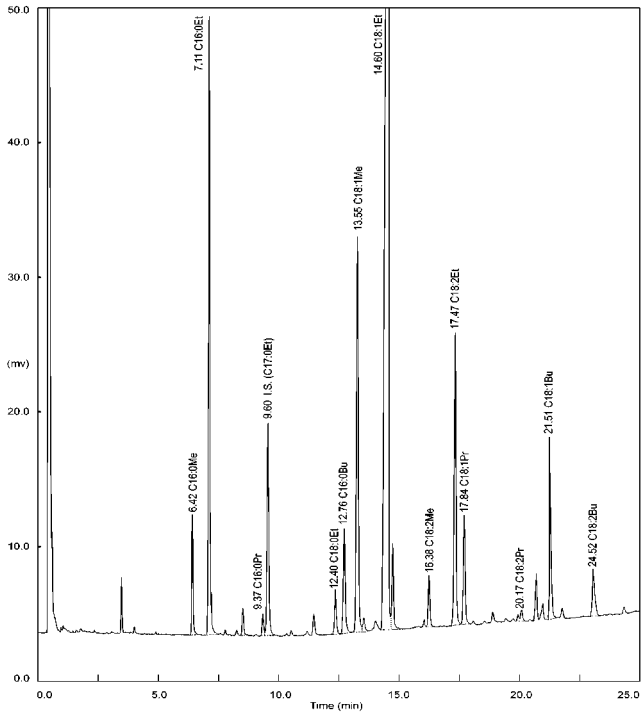

Figure 2. GC chromatogram of FAAEs from crude olive pomace oil using a SP-2380 fused silica capillary column (60 m × 0.25 mm id) coated with cyanopropylsilicone.

Table 1. Repeatability Standard Deviation and Recovery Data of Fatty Acids Alkyl Esters and Squalene Determination by SPE-GC

	$C_{18:1}$ ethyl ester			squalene		
sample	mean (mg/kg)	recovery (%)	RSD_r[b] (%) ($n = 6$)[a]	mean (mg/kg)	recovery (%)	RSD_r[b] (%) ($n = 6$)[a]
1	1135	99.9	5.5	9350	99.5	3.5
2	727	99.6	6.5	8059	98.6	3.7
3	345	98.7	5.6	5798	99.3	4.5
4	129	96.9	5.9	3859	97.9	4.3
5	59	95.5	7.3	1990	98.9	6.0
6	27	90.5	8.4	440	99.7	5.5

[a] n, number of replicates. [b] RSD_r, relative standard deviation of the repeatability.

GC capillary column coated with a more polar phase (cyanopropylsilicone) widely used for FAMEs analysis was only used for identification purposes since the column temperature limit (≈225 °C) does not allow the elution of waxes.

In the GC chromatogram of the fraction isolated by SPE from crude olive pomace oil (**Figure 1**), the main peaks were the ethyl esters of palmitic, oleic, and linoleic acids and the methyl, propyl, and butyl esters of oleic acid. The GC peaks corresponding to the methyl and ethyl esters were identified by comparison with standards, while those assigned to propyl and butyl esters were identified by GC−MS analysis. To confirm the peak identification, the FAAEs fraction free of squalene was analyzed by GC on a more polar phase (**Figure 2**).

For quantitative determination of esters, C17:0 EE was used as internal standard, since it did not overlap with other peaks. For all of the esters, the same response factor was adopted according to Ulberth et al. (*13*). The determination of squalene using C17:0 EE as internal standard resulted in a rather low precision. The use of squalane as internal standard resulted in better precision since both hydrocarbons have similar volatility.

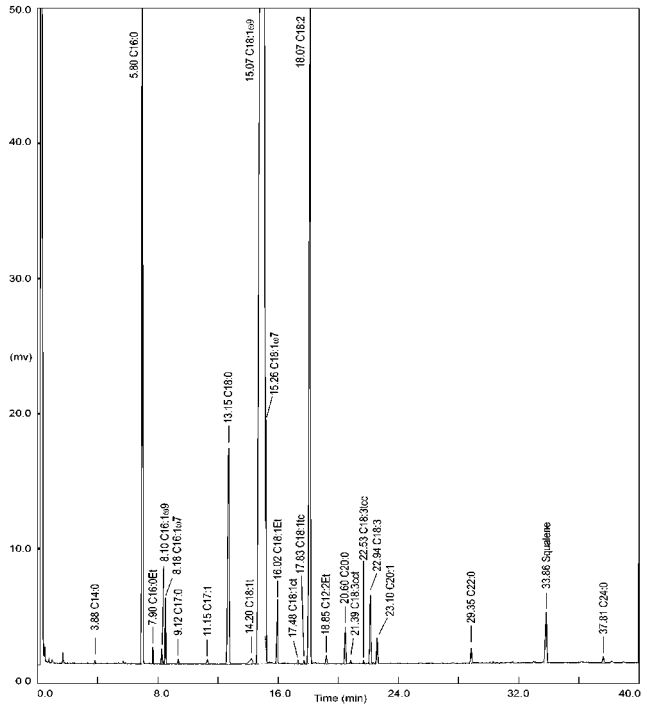

Figure 3. GC chromatogram of FAAEs obtained by cold methylation with methanolic potash from crude olive pomace oil using a SP-2380 fused silica capillary column (60 m × 0.25 mm id) coated with cyanopropylsilicone.

Table 2. FAAEs and Squalene in Extra Virgin Olive Oils Obtained from Different Olive Varieties

olive variety	acidity[a] (%)	FAMEs[a] (mg/kg)	FAEEs[a] (mg/kg)	squalene[a] (mg/kg)
Arbequina 1	0.16	nd[b]	nd	2374
Arbequina 2	0.18	19	14	1968
Picual	0.20	42	31	5445
Manzanilla	0.25	nd	nd	6511
Frantoio	0.25	nd	nd	3695
Carolea	0.31	13	6	5730
Hojiblanca	0.34	15	9	4627
Blanqueta	0.40	29	25	4320
Chamlali	0.42	16	17	1828
Koroneiki	0.49	11	8	4361
Picholine Marrocaine	0.93	40	32	539
Cornicabra	0.96	42	21	4219

[a] Each result is the average from two replicates. [b] nd, not detected.

The response factor was 0.9876. Recovery and precision data obtained from analysis of six replicates of sunflower oil spiked with several amounts of C18:1 EE and squalene are shown in **Table 1**. Repeatability and recovery of both esters and squalene were excellent.

Methylation of Total Oil. In the GC chromatogram obtained from crude olive pomace oils by methylation with cold solution of KOH in methanol (**Figure 3**), peaks of ethyl esters were observed (*6*). Nevertheless, the amount of oleic acid ethyl ester obtained by analysis of the same sample carried out by several laboratories was very variable. Methylations of an oil containing 21.7 mg/kg of ethyl oleate during variable times (5−60 s) yielded ethyl oleate concentrations from 21.9 to 1.6 mg/kg. These results indicated that a partial methylation of ethyl esters occurred depending on the methylation conditions. In addition, the FAMEs present in the oil could not be determined. Therefore, this methylation method is not suitable for determining FAAEs in vegetable oils.

Table 3. FAAEs in Lampant Virgin Olive Oils

sample	acidity[a] (%)	methyl esters (%)			ethyl esters (%)			FAMEs[a] (mg/kg)	FAEEs[a] (mg/kg)	squalene[a] (mg/kg)
		P	O	L	P	O	L			
L1	1.0	25.1	74.9	nd[b]	10.8	83.2	6.0	33	171	3239
L2	2.2	18.8	73.1	8.1	21.6	70.2	8.2	456	642	512
L3	3.8	15.3	73.8	10.9	17.5	72.6	9.9	218	870	3046
L4	5.1	17.5	67.4	15.1	16.0	72.3	11.7	121	231	6549
L5	6.6	11.8	69.2	19.0	14.7	74.8	10.5	349	834	6079
L6	7.3	12.3	77.8	9.9	14.7	74.2	11.1	240	1121	5596

[a] Each result is the average from two replicates. [b] nd, not detected.

Table 4. FAAEs in Fusty Olive Oils Obtained from Olives Piled under Atmospheric Conditions

sample	piling time (days)	acidity (%)	FAMEs (%)[e]			FAEEs (%)[f]			FAMEs[a] (mg/kg)	FAEEs[a] (mg/kg)	FAPEs[a,b] (mg/kg)	FABEs[a,c] (mg/kg)	squalene[a] (mg/kg)
			P	O	L	P	O	L					
initial	0	0.1	30.6	69.4	nd[d]	34.1	65.9	n.d.	24	18	nd	nd	4022
1	14	8.9	13.2	79.8	7.0	13.1	81.1	5.8	24 323	177 703	434	905	4043
2	28	8.8	11.4	82.8	5.8	11.9	83.7	4.5	24 963	88 925	380	693	4163
3	42	10.8	11.1	84.8	4.1	11.3	83.9	4.8	30 014	167 783	735	1052	4169
4	50	12.0	10.7	85.2	4.1	11.2	83.9	4.9	38 923	278 260	1027	1543	4004

[a] Each result is the average from two replicates. [b] FAPEs, fatty acid propyl esters. [c] FABEs, fatty acid butyl esters. [d] nd, not detected. [e] Percentages on total FAMEs. [f] Percentages on total FAEEs.

Table 5. FAAEs in Olive Oils Obtained by Second Centrifugation from Olive Pomace

sample	FAMEs (%)			FAEEs (%)			FAMEs[a] (mg/kg)	FAEEs[a] (mg/kg)	squalene[a] (mg/kg)
	P	O	L	P	O	L			
first centrifugation	20.2	67.5	12.3	22.5	77.5	nd[b]	167	34	3074
second centrifugation storage time (months)									
0	18.1	69.4	12.5	21.2	78.8	nd	178	41	2989
1	17.3	70.4	12.3	15.5	71.7	12.8	229	1424	2861
2	13.5	76.4	10.1	13.9	74.4	11.7	318	2514	2510

[a] Each result is the average from two replicates. [b] nd, not detected.

Virgin Olive Oils. Extra virgin olive oils showed only low concentrations of FAEEs (≤32 mg/kg) and FAMEs (≤42 mg/kg) (**Table 2**). In each sample, the formers present lower or similar concentrations than the latter. At very low concentrations, only esters of oleic acid were found, while at higher concentrations, minor amounts of palmitic and linoleic esters were also detected.

In lampant virgin olive oils (**Table 3**), the FAEEs and FAMEs were much more abundant than in extra virgin olive oils, and esters of oleic, palmitic, and linoleic acids were always detected. Concentrations of propyl and butyl esters were negligible. Opposite to extra virgin olive oils, the amounts of ethyl esters found in lampant oils were greater than those of methyl esters. In each group of esters, oleic alkyl esters were the most abundant and few differences in the fatty acid compositions could be observed.

As might have been expected, high values of acidity did not imply high quantities of FAAEs, as can be seen in **Tables 2** and **3**; therefore, no relationship between FAAEs and acidity could be established. The squalene concentrations were very variable in agreement with data earlier reported (*7*).

Fusty Olive Oils. The oils obtained from olive fruits piled before the oil extraction (Fusty olive oils) showed very high concentrations of ethyl and methyl esters; the ethyl esters were much more abundant than the methyl esters (**Table 4**). Besides, significant amounts of propyl and butyl esters were also detected. The methyl esters increased with the piling time, but the amounts of ethyl, propyl, and butyl esters were variable. In each sample, the fatty acid compositions of methyl and ethyl esters were similar (**Table 4**). These results indicated that the increase of FAAEs is related to alterations occurring in the olive fruits before the milling, and they explain the higher content of FAEEs than FAMEs in poor quality olive oils (lampant oils). The squalene remained practically constant along the pile time, indicating that this compound was not affected drastically by the alteration of olive fruits.

Second Centrifugation and Olive Pomace Oils. Oils produced by a second centrifugation of just obtained olive pomace showed similar content of FAMEs, FAEEs, and squalene like oils obtained in the first centrifugation (**Table 5**). Nevertheless, oils obtained after storage of the olive pomace showed an increase of FAAEs, the concentrations of ethyl esters being greater than those of methyl esters. In the oil obtained from olive pomace stored for 2 months, small amounts of propyl and butyl oleate were also detected. The same results were observed in the set of second centrifugation oils supplied by the industry, which suggested that olive pomace was not processed immediately (**Table 6**).

In crude olive pomace oils obtained from the olive pomace by extraction with hexane, high concentrations of FAAEs were found, including significant concentrations of propyl and butyl esters (**Table 7**). In all of the samples analyzed, the FAEEs were the most abundant. These results are in agreement with the fact that olive pomace is stored for a long time before the oil extraction. The variability of the FAAEs content is attributed to the different alteration of the raw material before the oil

Table 6. FAAEs in Olive Oils Industrially Obtained by Second Centrifugation of Olive Pomace

second centrifugation olive oils	FAMEs[a] (mg/kg)	FAEEs[a] (mg/kg)	squalene[a] (mg/kg)
C1	184	494	4960
C2	245	895	4829
C3	793	5807	1530
C4	386	2456	3132
C5	597	3935	2979
C6	169	959	3080
C7	149	832	3000
C8	147	846	2857

[a] Each result is the average from two replicates.

Table 7. FAAEs and Squalene in Crude Olive Pomace Oils

sample	FAMEs[a] (mg/kg)	FAEEs[a] (mg/kg)	FAPEs[a] (mg/kg)	FABEs[a] (mg/kg)	squalene[a] (mg/kg)
OP-1	8036	40 175	4763	1090	972
OP-2	1667	5 573	153	51	417
OP-3	2628	13 917	654	313	305
OP-4	3586	12 656	784	655	61
OP-5	2753	5 511	175	253	616
OP-6	884	2 100	32	67	51

[a] Each result is the average from two replicates.

extraction. In olive pomace oils, squalene concentrations were lower than those found in olive oils.

All above results indicate that extra virgin olive oil contains low concentrations of fatty acid methyl and ethyl esters, the former being usually more abundant than the latter. The presence of high concentrations of FAAEs, particularly ethyl esters, is due to alterations occurring in either olive fruit or olive pomace before the oil extraction. If the low concentration of methyl and ethyl esters is confirmed in extra virgin olive oils obtained from other olive varieties growing in different countries, these parameters might be additionally used to assess the quality of extra virgin olive oil.

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