Chemical Structure of Long-Chain Esters from "Sansa" Olive Oil

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The major objective of this study was to determine the chemical structure of long-chain esters present in lower-grade olive oil. The classes of esters composing the hexane–diethyl ether (99:1) extract of the wax fraction from a pomace olive oil were: (i) esters of oleic acid with C_1–C_6 alcohols, (ii) esters of oleic acid with long-chain aliphatic alcohols in the range C_{22}–C_{28} and (iii) benzyl alcohol esters of the very long-chain saturated fatty acids C_{26} and C_{28}. The analysis and the structure assignments were carried out by gas chromatography coupled with mass spectrometry and by comparison with synthetic authentic model compounds. This work provided precise data on the chemical nature of the wax esters present in olive oil and should represent a means to detect adulteration of higher-grade olive oil with less expensive pomace olive oil and seed oils.

KEY WORDS: Benzyl alcohol esters, GC–MS, long-chain aliphatic esters, olive oil, olive oil wax esters.

The International Olive Oil Council defines olive oil categories as follows: "Virgin olive oil is the oil obtained from the fruit of the olive tree solely by mechanical or other physical means under conditions that do not lead to alteration of the oil. The oil has not undergone any treatment other than washing, decantation, centrifugation and filtration. Olive oil or pure olive oil (or 100% pure olive oil) is the oil consisting of a blend of virgin olive oil, fit for consumption as it is, and refined olive oil."

Two oils are commercially important: (i) pomace or "sansa" olive oil, obtained from olive residue by extraction with solvent, followed by evaporation of the solvent, dilute alkali washing, bleaching and deodoration and (ii) "Lampante" olive oil, whose lower grade is caused by the state of the olives to be extracted, harvested when almost decayed and having gone bad. According to many reports in the literature, a reliable means to detect "sansa" oil and to distinguish it from olive oil of a different grade is the presence of wax esters (1,2). In a more general sense, the presence of long-chain esters in a vegetable oil is safe evidence of the presence of solvent-extracted material. These findings are now being examined by the European Economic Community Olive Committee, which is considering including the presence and the content limits of wax esters among the chemical parameters on which to base the purity and classification of the various grades of olive oils. Accordingly, higher-grade virgin olive oil should not contain wax ester, while lower-grade olive oils that contain wax esters in varying proportions should be considered mixtures of olive oil with solvent-extracted oil, such as "sansa" and seed oils. In this respect, a recent study in our laboratory on the cuticular substances of both green and black "Coratina" cv. olives has revealed the presence of wax esters, composed of fatty acids bound to fatty alcohols. During that study a series of benzyl alcohol esters also accompanied the wax esters (3). In the benzyl alcohol esters, the alcohol moiety consisted exclusively of the benzyl alcohol, which was esterified with the normal very long-chain acids in the range C_{26}–C_{32}. Regarding the long-chain aliphatic esters, only a preliminary investigation of their composition was carried out in that study. The isolation of the wax esters and benzyl alcohol esters from a "sansa" olive oil, the separation and identification of positional isomers by capillary gas chromatography–mass spectrometry (GC–MS) and the synthesis of standard compounds are the subject of this report.

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EXPERIMENTAL PROCEDURES

Extraction of wax ester mixtures. Purchased in a local Italian supermarket, 521.0 mg of "sansa" olive oil, to which 0.5 mg of arachidyl laurate was added as an internal standard, was dissolved in hexane and subjected to silica gel column chromatography (15 g of silica gel; Merck, Darmstadt, Germany), according to the published procedure (4). The ester fractions were eluted with 150 mL of a mixture of hexane/diethyl ether, 99:1. The solvent was evaporated under reduced pressure in a rotary evaporator to give a crude residue (3.5 mg, 0.67% yield, w/w). The ester mixture was used as such for the GC and GC–MS analyses.

Analyses of samples. An HPGC Carlo Erba series 4160 gas–liquid chromatograph (Milano, Italy), fitted with a flame-ionization detector (FID) and equipped with an OV-1 fused-silica glass capillary column of 10 m length, 0.32 mm i.d. and 0.25 μm film thickness, was used for GC analyses. The samples were introduced by means of an "on column" injector with a starting temperature of 60°C for 1 min, programmed to 150°C at 25°C min^{-1}, and then programmed to 340°C at 15°C min^{-1} for 20 min. The detector temperature was 350°C.

GC–MS analyses were carried out on a capillary GC instrument, equipped with a mass-selective detector (HP 5970B CGC–MS apparatus; Hewlett-Packard, Palo Alto, CA). The capillary column used (DB-5, 30 m, 0.32 mm i.d. and 0.25 μm film thickness) was directly introduced into the ion source, which was operated in the electron impact mode. The chromatographic conditions were: interface 300°C and temperature programs as previously reported. The mass detector was optimized under Auto-Tune (Hewlett Packard, Palo Alto, CA) conditions with perfluorotributylamine calibration. Mass spectra were acquired over a 40–800 amu range at 1 scan s^{-1} with an ionizing electron energy of 70 eV, electron current of 0.3 mA, and ion source at 200°C and 15^{-5} Torr.

The ^1H and ^{13}C spectra were obtained with a Bruker AC 300 MHz apparatus. The samples were dissolved in $CDCl_3$. Tetramethylsilane was used as internal standard.

Synthesis of aliphatic esters. A number of esters were synthesized according to the following method. Approximately 5.0 millimole of alcohol and oleyl or palmitoyl chlorides were dissolved in 2 mL CCl_4 in the presence of a few drops of pyridine. The solution was left at room temperature overnight. The reaction mixture was transferred to a 50-mL separatory funnel and diluted with cold water. The organic layer was separated, dried over Na_2SO_4 and evaporated to dryness in a rotary evaporator. The residue (70–90% yields) was purifed by silica-gel column chromatography preparated in hexane/diethyl

ether (99:1). Each synthesized ester was eluted from the column with hexane/diethyl ether 99:1 to give a pure sample of the desired compound. Their physical purity was determined by gas-liquid chromatography. GC-MS analysis was carried out to obtain data useful for comparison purposes.

Nuclear magnetic resonance (NMR) data of the synthesized esters. Tetracosyl palmitate ($C_{40}H_{80}O_2$; m.p. 68-69°C; ^{1}H: 0.89δ, 6H, *t*, J = 7.0Hz; 1.25δ, 66H, *s*; 1.61δ, 4H, *m*; 2.29δ, 2H, *t*, J = 7.0Hz; 4.03δ, 2H, *t*, J = 7.0Hz; ^{13}C: C=O, 173.94δ; CH_2-O, 64.29δ; CH_2C=O, 34.31δ; CH_3, 14.02δ). Hexacosyl palmitate ($C_{42}H_{84}O_2$; m.p. 69-71°C; ^{1}H: 0.88δ, 6H, *t*, J = 7.0Hz; 1.25δ, 70H, *s*; 1.60δ, 4H, *m*; 2.28δ, 2H, *t*, J = 7.0Hz; 4.04δ, 2H, *t*, J = 7.0Hz; ^{13}C: C=O, 174.04δ; CH_2-O, 64.40δ; CH_2C=O, 34.42δ; CH_3, 14.12δ). Octacosyl palmitate ($C_{44}H_{88}O_2$; m.p. 73-74°C; ^{1}H: 0.90δ, 6H, *t*, J = 7.0Hz; 1.25δ, 74H, *s*; 1.62δ, 4H, *m*; 2.30δ, 2H, *t*, J = 7.0Hz; 4.03δ, 2H, *t*, J = 7.0Hz; ^{13}C: C=O, 173.94δ; CH_2-O, 64.30δ; CH_2C=O, 34.31δ; CH_3, 14.03δ). Triacontanyl palmitate ($C_{46}H_{92}O_2$; m.p. 76-77°C; ^{1}H: 0.89δ, 6H, *t*, J = 7.0Hz; 1.25δ, 78H, *s*; 1.62δ, 4H, *m*; 2.29δ, 2H, *t*, J = 7.0Hz; 4.04δ, 2H, *t*, J = 7.0Hz; ^{13}C: C=O, 174.02δ; CH_2-O, 64.39δ; CH_2C=O, 34.41δ; CH_3, 14.10δ). Dotriacontanyl palmitate ($C_{48}H_{96}O_2$; m.p. 79-80°C; ^{1}H: 0.90δ, 6H, *t*, J = 7.0Hz; 1.25δ, 82H, *s*; 1.61δ, 4H, *m*; 2.29δ, 2H, *t*, J = 7.0Hz; 4.03δ, 2H, *t*, J = 7.0Hz; ^{13}C: C=O, 173.90δ; CH_2-O, 64.30δ; CH_2C=O, 34.32δ; CH_3, 14.03δ). Tetracosyl oleate ($C_{42}H_{82}O_2$; m.p. 49-51°C; ^{1}H: 0.88δ, 6H, *t*, J = 7.0Hz; 1.25δ, 62H, *s*; 1.60δ, 4H, *m*; 2.02δ, 4H, *m*; 2.28δ, 2H, *t*, J = 7.0Hz; 4.05δ, 2H, *t*, J = 7.0Hz; 5.35δ, 2H, *m*; ^{13}C: C=O, 173.93δ; C=C, 129.92δ, 129.73δ; CH_2-O, 64.35δ; CH_2C=O, 34.37δ; CH_3, 14.09δ). Hexacosyl oleate ($C_{44}H_{86}O_2$; m.p. 52-54°C; ^{1}H: 0.89δ, 6H, *t*, J = 7.0Hz; 1.25δ, 66H, *s*; 1.62δ, 4H, *m*; 2.02δ, 4H, *m*; 2.29δ, 2H, *t*, J = 7.0Hz; 4.05δ, 2H, *t*, J = 7.0Hz; 5.33δ, 2H, *m*; ^{13}C: C=O, 173.90δ; C=C, 129.66δ, 129.63δ; CH_2-O, 64.30δ; CH_2C=O, 34.28δ; CH_3, 14.02δ). Octacosyl oleate ($C_{46}H_{90}O_2$; m.p. 56-57°C; ^{1}H: 0.90δ, 6H, *t*, J = 7.0Hz; 1.25δ, 70H, *s*; 1.60δ, 4H, *m*; 2.01δ, 4H, *m*; 2.29δ, 2H, *t*, J = 7.0Hz; 4.05δ, 2H, *t*, J = 7.0Hz; 5.32δ, 2H, *m*; ^{13}C: C=O, 173.90δ; C=C, 129.88δ; 129.64δ; CH_2-O, 64.30δ; CH_2C=O, 34.30δ; CH_3, 14.01δ). Triacontanyl oleate ($C_{48}H_{94}O_2$; m.p. 58-60°C; ^{1}H: 0.90δ, 6H, *t*, J = 7.0Hz; 1.26δ, 74H, *s*; 1.61δ, 4H, *m*; 2.01δ, 4H, *m*; 2.30δ, 2H, *t*, J = 7.0Hz; 4.03δ, 2H, *t*, J = 7.0Hz; 5.34δ, 2H, *m*; ^{13}C:C=O, 173.91δ; C=C, 129.67δ, 129.63δ; CH_2-O, 64.30δ; CH_2C=O, 34.28δ; CH_3, 14.02δ). Dotriacontanyl oleate ($C_{50}H_{98}O_2$; m.p. 60-62°C; ^{1}H: 0.90δ, 6H, *t*, J = 7.0Hz; 1.25δ; 78H, *s*; 1.63δ, 4H, *m*; 2.01δ, 4H, *m*; 2.30δ, 2H, *t*, J = 7.0Hz; 4.05δ, 2H, *t*, J = 7.0Hz; 5.34δ, 2H, *m*; ^{13}C: C=O, 174.00δ; C=C, 129.92δ, 129.74δ; CH_2-O, 64.38δ; CH_2C=O, 34.37δ; CH_3, 14.08δ).

RESULTS

Chemical composition of the "sansa" olive oil wax ester extract. The chromatogram of the capillary GC separation of the components of the hexane/diethyl ether (99:1)-soluble classes of compounds from a commercial sample of "sansa" olive oil is shown in Figure 1. The capillary GC-MS total ion-current chromatogram of the same fraction is given in Figure 2. As will be discussed later, the gas chromatogram shows the presence of four classes of

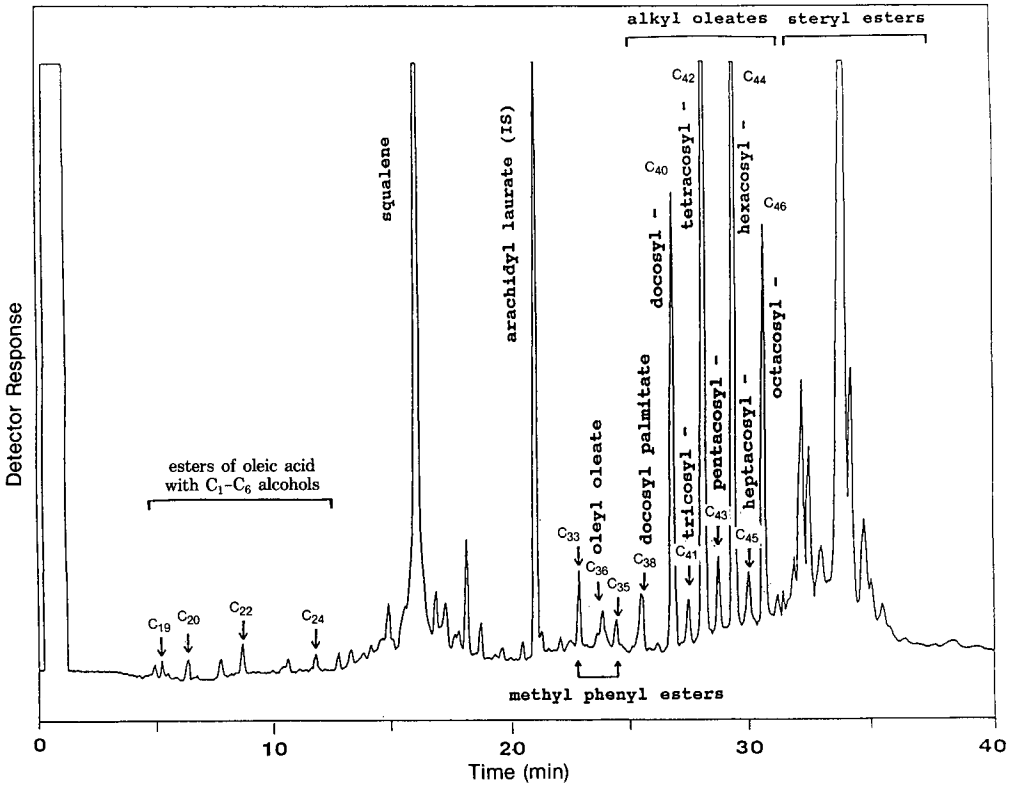

FIG. 1. Gas chromatogram of the hexane/diethyl ether (99:1)-soluble fraction from a commercial "sansa" oil. The brackets group together the predominant wax classes.

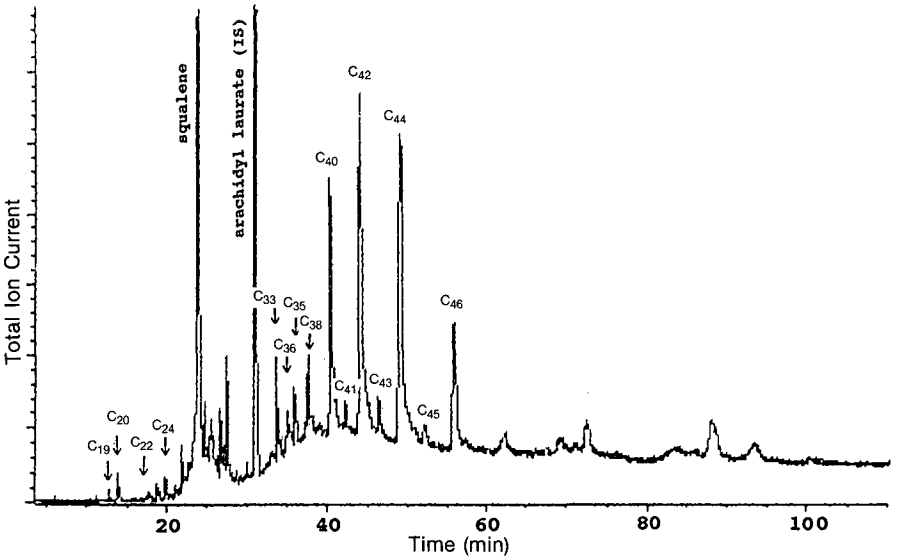

FIG. 2. Total ion-current chromatogram of the capillary gas chromatography/mass spectrometry analysis of the components of the hexane/diethyl ether (99:1)-soluble substances from "sansa" oil.

compounds with increasing retention times. Squalene is well evidenced by a sharp intense peak. The first group of compounds is represented by esters of oleic acid with short-chain alcohols (C_1–C_6), the second by benzyl alcohol esters of very long-chain fatty acids, the third by long-chain aliphatic esters and the last eluted group of substances is made up of steryl esters. The last class of compounds was not studied further. The composition and the homologue composition of the various classes of compounds are shown in Table 1.

Short-chain alcohol esters of oleic acid. A minor group of four medium-chain esters, C_{19}–C_{24}, was identified as being composed of oleic acid esterified with C_1, C_2, C_4 and C_6 alcohols.

TABLE 1

Composition and Relative Distribution of Identified Esters from "Sansa" Oil Extract

Carbon number	Acid-alcohol moiety	% in the extract[a]	mg/g of oil
19	$C_{18:1}$–C_1	0.7	0.010
20	$C_{18:1}$–C_2	1.0	0.015
22	$C_{18:1}$–C_4	1.5	0.019
24	$C_{18:1}$–C_6	0.7	0.010
36	$C_{18:1}$–$C_{18:1}$	3.8	0.053
38	C_{16}–C_{22}	4.5	0.062
40	$C_{18:1}$–C_{22}	40.8	0.564
41	$C_{18:1}$–C_{23}	4.0	0.055
42	$C_{18:1}$–C_{24}	97.5	1.351
43	$C_{18:1}$–C_{25}	5.5	0.077
44	$C_{18:1}$–C_{26}	100.0	1.386
45	$C_{18:1}$–C_{27}	3.8	0.053
46	$C_{18:1}$–C_{28}	41.5	0.575
33	C_{26}—benzyl alcohol	5.0	0.069
35	C_{28}—benzyl alcohol	2.2	0.029

[a]Relative to the major wax ester hexacosyl oleate.

Benzyl alcohol esters. The benzyl alcohol ester fraction represents a minor proportion of the substances extracted by hexane from the olive oil. GC–MS analysis permitted the detection and identification of the two benzyl alcohol esters of hexa- and octacosanoic acid.

Very long-chain aliphatic esters. The capillary gas chromatogram of the very long-chain aliphatic ester fraction of Figure 1 showed four major peaks, due to C_{40}, C_{42}, C_{44} and C_{46} chains, making up the largest proportion of the total esters in the extract (Table 1). The GC–MS data revealed that the fatty acid moiety of these aliphatic esters was constituted by oleic acid, with the sole exception of minor percentages of palmitic acid, esterified with 1-docosanol and homologues. The ^1H and ^{13}C data provided further evidence of the structural assignment (5). Definitive proof of the structure of these two classes of compounds was obtained by the synthesis of a number of models (see Experimental Procedures).

Mass spectral analysis. Diagnostic ions for the structure assignment of saturated and unsaturated esters of long-chain alcohols and long-chain fatty acids generally originate from cleavage at the ester grouping. The predominant double hydrogen rearrangement peak in the mass spectrum of the saturated esters may be detected as a decomposition of the parent ion, following the rearrangement of two hydrogen atoms to the acid moiety and subsequent elimination of the alkyl portion of the alcohol moiety, to give rise to a protonated acid ionic species with a mass equal to the acid + 1. This accounts for the base peak of *m/z* 257 in the spectrum of both the synthetic and of the natural component of the oil sample, docosanyl palmitate, shown in Figure 3. The protonated acid is often the base peak (6–10). In the mass spectra of esters of oleic acid with long-chain alcohols, the characteristic fragmentation pattern is represented by ion *m/z* 264, due to the loss of the alcohol group, and the ion 222 (Fig. 4). The latter peak has been reported to stem from a

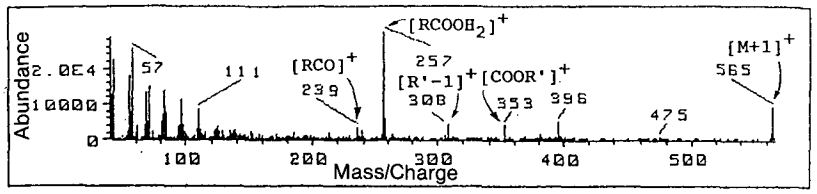

FIG. 3. Mass spectra of docosanyl palmitate (C_{16}–C_{22}).

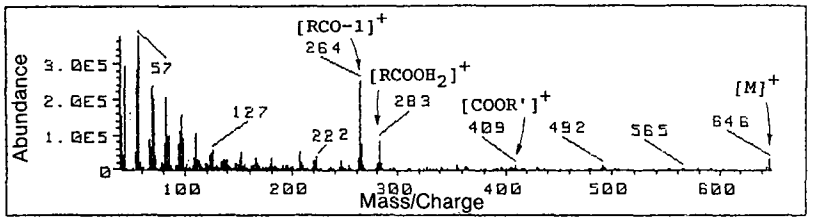

FIG. 4. Mass spectra of hexacosanyl oleate ($C_{18:1}$–C_{26}).

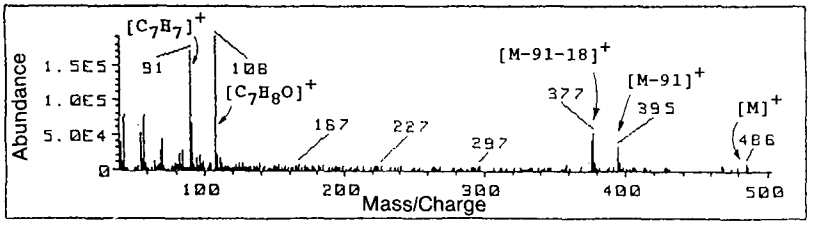

FIG. 5. Mass spectra of benzyl hexacosanoate (C_{26}–C_7).

McLafferty-type cleavage (7). Thus, in summary, the acid + 1 for saturated esters, the acid − 18 for unsaturated esters with the unsaturation on the acid moiety and the molecular ion are the most useful diagnostic peaks in the mass spectra of these classes of compounds because they permit also the easily deduction of the esterified alcohols (10). The latter, however, show up in the mass spectrum with ions arising from cleavage next to the carbonyl of the carboxylic group. Such a fragmentation leads to an ion composed of the alcohol moiety plus the carbonyl, and usually appears at low intensity. A further useful peak for the identification of the alcohol moiety is that arising from the fragmentation leading to $[R' - 1]^+$ ion (Figs. 3 and 4).

For the mass spectra of benzyl alcohol esters, the fragmentation pattern appeared characterized by ions arising from the benzyl alcohol moiety. Among the most abundant fragments observed were those corresponding to tropylium ion $[C_7H_7]^+$, a peak at *m/z* 108 formally corresponding to benzyl alcohol and the ions $[M - 91]^+$ and $[M - 91 - 18]^+$ (11,12) (Fig. 5). The characteristic mass spectral data are shown in Table 2.

DISCUSSION

A previous work has dealt with the study of the chemical composition of surface lipids of olive fruits. The major surface components from green and black "Coratina" cv. olive were: (i) aliphatic hydrocarbon, (ii) aliphatic long-chain esters, (iii) benzyl alcohol esters, (iv) aldehydes, (v) alcohols, (vi) acids, (vii) triacylglycerols and (viii) cyclic triterpene alcohols and acids. During that study, a preliminary GC–MS analysis of the aliphatic esters in the range C_{36}–C_{56} disclosed that the acid moiety consisted of either the saturated C_{16} palmitic acid or $C_{18:1}$ monounsatured oleic acid. The saturated–unsaturated series were found in the mean ratio 92.5:7.5%. The two fatty acids were esterified with saturated normal alcohols in the range C_{22}–C_{28}. Over 95% of the ester fraction was made up of even-carbon chains, the remaining being constituted by homologues that were neither further investigated nor cited (3).

The present work has shown that the aliphatic linear esters present in the "sansa" oil studied are almost entirely esters of oleic acid with short- and long-chain alkanols. The only odd-chain esters identified in the oil

TABLE 2

Diagnostic Mass-Spectral Ions[a]

Carbon	Oleic and palmitic acid esters						
	$[M]^+$	$[M+1]^+$	$[RCOOH_2]^+$	$[RCO]^+$	$[RCO-1]^+$	$[R'-1]^+$	$[COOR']^+$
19	296(5)[b]	—	—	265(16)	264(25)	—	—
20	310(9)	—	—	265(22)	264(28)	—	—
22	—	—	—	265(12)	264(13)	56(7)	—
24	—	—	283(7)	265(9)	264(18)	84(21)	—
36	532(1)	533(3)	283(7)	265(8)	264(18)	250(9)	295(3)
38	564(6)	565(32)	257(100)	239(8)	238(2)	308(13)	353(14)
40	590(9)	591(3)	283(22)	265(23)	264(78)	308(1)	353(2)
41	604(1)	605(3)	283(15)	265(18)	264(100)	322(−)	367(4)
42	618(9)	619(3)	283(19)	265(17)	264(63)	336(1)	381(3)
43	632(trace)	633(1)	283(15)	265(22)	264(100)	350(−)	395(2)
44	646(8)	647(3)	283(22)	265(19)	264(67)	364(1)	409(3)
45	660(−)	661(−)	283(10)	265(13)	264(70)	378(3)	423(−)
46	674(1)	675(4)	283(19)	265(26)	264(100)	392(2)	437(3)
	Benzyl esters						
	$[M]^+$	$[M+1]^+$	$[M-91]^+$	$[M-91-18]^+$	$[COOR']^+$	$[C_7H_7]^+$	$[C_7H_8O]^+$
33	486(3)	487(1)	395(17)	377(27)	135(2)	91(89)	108(100)
35	514(1)	515(−)	423(11)	405(25)	135(3)	91(97)	108(100)

[a]R and R′ are the alkyl moieties of acid and alcohol, respectively.
[b]Relative abundances.

studied were esters of oleic acid with C_{23}, C_{25} and C_{27} alcohols. That the three odd-carbon chain C_{41}, C_{43} and C_{45} had linear structure was proved by their mass spectral data, which were similar to the even homologues and in which no ions were observed that arose from *iso* or *anteiso*-branched isomers (8). The acid moiety was that of oleic acid esterified with linear odd-chain alcohols. The dominance of saturated esters in the surface lipids of the olive drupes may be reasonably explained as being due to their greater stability to oxidation compared to those of the unsatured series. The almost complete lack of the latter esters in the olive oil, instead, implies that (i) the synthesis of long-chain aliphatic esters is not restricted to the epicarp of the olives and (ii) the biosynthetic machinery responsible for the synthesis of long-chain esters operates on the two substrate acids, the saturated C_{16} and the monounsaturated oleic $C_{18:1}$, depending on the part of the fruit. A further point worth emphasizing is the finding that each carbon chain of the olive fruit esters is made up of a single isomer. This is quite unusual when comparing it with the composition of the numerous ester fractions found in nature from various sources. In fact, in the majority of the cases studied, each ester chain was comprised of several positional isomers (13).

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