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Characterization of the Lipoxygenases in Some Olive Cultivars and Determination of Their Role in Volatile Compounds Formation

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Enzymatic extracts from olive pulp (*Olea europea* L.) were used to characterize lipoxygenase (LOX) activity in order to determine its role in the biogenesis of the volatile compounds that influence the aroma of extra virgin olive oil. The LOX activity was tested spectrophotometrically at an optimal pH of 6.0 in three olive cultivars, Ascolana Tenera, Kalamata, and FS17. The trend of the LOX activity was determined as a function of pH and temperature; the kinetic constants of the enzyme were also determined. The highest LOX activity was observed in the FS17 fruit, which had the highest concentrations of C_5 and C_6 compounds (aldehydes, alcohols, and ketones), followed by Kalamata and Ascolana T., respectively. Given the direct relationship between enzymatic activity and the quantity of aromas measured in the fruit, it is hypothesized that olive LOX is involved in the formation of C_5 and C_6 volatile compounds. To study the mechanism of the movement of the aromas from the fruit to the oil, which was obtained by simple mechanical extraction, the headspace of the oil for each cultivar was analyzed as well as the aromatic composition in order to compare it with the aromas of the fruit.

KEYWORDS: ***Olea europaea*** **L.; olive; lipoxygenase; kinetics, volatile biosynthesis; volatile compounds; virgin olive oil**

INTRODUCTION

A complex mixture of volatile compounds, including aldehydes, ketones, alcohols, and esters, gives rise to the aroma of extra virgin olive oil. These compounds can be identified and quantified by gas chromatographic analysis of the static or dynamic headspace (*1*−*4*). The olfactory and organoleptic characteristics of the oils differ greatly according to the origin of the cultivar and, in some cases, meet the requirements needed to confer typicality to the oil. This has compelled studies on the mechanisms of the synthesis of the volatile compounds to determine if, besides being quality indicators, they can be used as markers of the place of origin of the oils.

Lipoxygenases (LOX; EC 1.13.11.12) are a group of enzymatic proteins that can oxidize free polyunsaturated fatty acids, characterized by an (*E*,*E*)-1,4-pentadienic system (linoleic, linolenic, and arachidonic acid), to obtain the corresponding conjugated hydroperoxides (6- and 10-hydroperoxide) in which the double bonds are in a *cis*−*trans* configuration (*5*). These enzymes catalyze the first reaction of the complex metabolic pathway commonly known as the "lipoxygenase pathway" that leads to the formation of C_5 and C_6 volatile compounds (*6*−*8*). In some plant species (*9*−*12*) these compounds originate from free polyunsaturated fatty acids via a chain reaction that requires the sequential action of LOX and the hydroperoxide-lyase enzyme that involves cleavage of the hydroperoxides, giving rise to C_6 aldehydes. The enzymatic reduction of the aldehydes, catalyzed by alcohol dehydrogenase, leads to the formation of the corresponding alcohols.

The aromatic compounds present in the volatile fractions of fruits and vegetables (*13*) give them their characteristic odors. The aim of this work was to determine the quantity of C_5 and C_6 volatile compounds in the fruit of the new olive cultivar FS17 (patent IRO-CNR 1165/nv) and of the cultivars Ascolana Tenera and Kalamata and relate them to the LOX activity in order to determine the role of this enzyme in the biogenesis of these compounds. The LOX activity was partially characterized; the kinetic constants of the enzyme, substrate specificity, pH, and optimal catalysis temperature were also determined. To study the mechanism of the movement of the aromas from the fruit to the oil, obtained by simple mechanical extraction, the headspace of the oils of the respective cultivars was analyzed and the aromatic composition was determined for comparison with the fruit.

MATERIALS AND METHODS

Sampling of Fruits and Oil Extraction. Fruits from the cultivars FS17, Kalamata, and Ascolana Tenera were obtained from the experimental field in Bettona (PG), set up with drip irrigation according

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	ALDEHYDES			ALCOHOLS			Mean peak area aldehyde + alcohol	St. Dev.
mg/kg	trans-2-Hexenal Area (counts)	trans-2-Heptenal Area (counts)	(E,E)-2,4-Hexadienal Area (counts)	3-Methyl-1-butanol Area (counts)	trans-2-Penten-1-ol Area (counts)	cis-3-Hexen 1-ol Area (counts)	(counts)	(%)
10	104188	119946	104576	100979	102132	120403	108704	8.26
5	49398	62868	46666	48570	47541	58155	52200	12.78
1	10453	14403	8701	9626	9971	11633	10798	18.65
0.5	4600	8814	4493	4986	5681	6425	5833	27.95

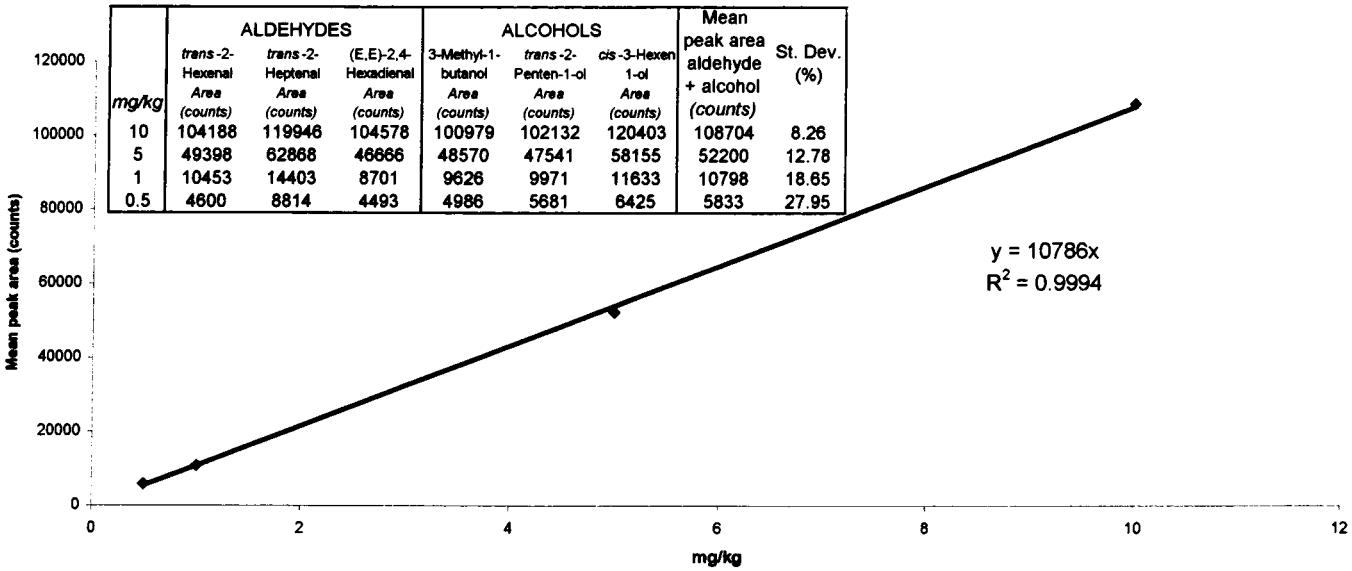

Figure 1. Mean coefficient of response (counts/mg·kg^{-1}) of C_5 and C_6 aldehydes and alcohols determined with an FID.

to the intensive olive culture model, IRO-CNR (*14*). The olives were harvested in October at the commercially ripe stage. A part of the sample was frozen in liquid nitrogen and stored at −80 °C until the time of biochemical analysis. The remaining olives (2 kg) were immediately milled in a Pieralisi micropress (malaxation time, 30 min; temperature, 25 °C; separation by centrifugation).

Preparation of Enzymatic Extracts. Ten grams of olive pulp was homogenized at 4 °C in an Ultra Turrax with 40 mL of 50 mM sodium phosphate buffer, pH 6.8, containing 0.3 mM dithiothreitol (DTT), 0.2 mM ethylenediaminetetraacetic acid (EDTA), 10 mM sodium metabisulfite, 0.1% Triton X-100, and 5 g of insoluble polyvinylpolypyrrolidone (PVPP) (*15*). The homogenate was shaken for 30 min at 4 °C to facilitate enzyme extraction. After the mixture had been filtered through cheesecloth under vacuum, the sample was centrifuged at 27000*g* for 30 min and filtered through paper. After the pellet was removed, the supernatant was precipitated with 75% saturated ammonium sulfate, shaken at 4 °C for 30 min, and set aside for 1 h to facilitate protein precipitation. The sample was then centrifuged for 90 min at 20000*g*. The pellet was resuspended in a small volume of 50 mM sodium phosphate, pH 6.8, and dialyzed overnight. The dialysate (AS) was used for the enzymatic trials.

Substrate Preparation. For the linoleic and arachidonic substrates, a mother solution of 10 mM and Tween 20 (0.1% v/v) in sodium tetraborate buffer, pH 9, was prepared. The preparation was carried out under a continuous nitrogen flow to maintain anoxic conditions. The substrate was then stored in the dark at −20 °C.

Enzymatic Assay. The LOX activity was determined spectrophotometrically (Perkin-Elmer Lambda 10) at 30 °C by measuring for 2 min the increase in absorbance at a wavelength of 234 nm for the oxidation of linoleic acid and at 238 nm for arachidonic acid (*12*). The reaction mixture (3 mL) contained sodium phosphate buffer (0.2 M, pH 6.0) with Tween 20 (0.1% v/v) and a quantity of enzymatic extract equal to 1.5 μg/mL of sample mixture. The final concentration of linoleic and arachidonic acids was 0.8 mM. Tween 20 was added to the sample mixture to prevent the development of turbidity that normally occurs when the substrates are added. Specific activity is expressed as units of enzyme per milligram of total protein, where a unit of enzyme is defined as the amount of enzyme capable of causing an absorption increment of 0.001/min at 30 °C.

The protein concentration was determined according to the method of Bradford (*16*), using bovine serum albumin as standard, to calculate specific enzyme activity.

Effect of pH and Temperature. The optimum pH for lipoxygenase activity, using linoleic acid as substrate, was determined in a pH range from 4 to 9 at 30 °C using the following buffers: sodium phosphate, sodium borate, and glycine−HCl, all at a final concentration of 0.2 M. Because enzymatic activity is temperature-dependent, the protein extracts were treated at different temperatures (10−80 °C) for 10 min. After cooling in ice, the LOX activity was determined under standard assay conditions.

Determination of Kinetic Parameters. The kinetic constants of LOX were measured under standard assay conditions using linoleic and arachidonic acid concentrations ranging from 25 to 800 μM; the enzyme concentration was always equal to 1.5 μg/mL of reaction mixture. All of the determinations were repeated two times, and the respective kinetic parameters were evaluated by plotting the data on a Lineweaver−Burk double-reciprocal graph (*17*).

Analysis of Volatile Compounds. The volatile compounds were determined on samples of olive pulp and olive oil. The pulp was pulverized with liquid nitrogen in a mortar and heated to 100 °C for 1 min to denature the proteins and deactivate the enzymes. Static headspace gas chromatographic (SHS-GC) analysis was performed with a Perkin-Elmer Auto System XL gas chromatograph equipped with a flame ionization detector (FID) and an HS 40 XL headspace sampler (Perkin-Elmer).

The vials, containing 10 mL of oil or 1.5 g of olive pulp, were equilibrated for 45 min at 80 °C and pressurized with carrier gas for 30 s. The pressurized headspace was transferred onto a transfer capillary (150 °C) before injection into a Stabilwax capillary column (60 m, 0.25 mm i.d., 0.25 μm film thickness). The injector was set at 180 °C. The oven temperature was programmed from 40 to 70 °C at 2 °C/min and subsequently heated to 160 °C at 4 °C/min; the final temperature was held for 15 min.

The C_5 and C_6 volatile compounds were tentatively identified by determining the retention times of the standard compounds (Sigma Aldrich) added to the rectified oils.

The quantitative analysis of the volatile compounds and the determination of the response factor were determined using the substances reported in **Figure 1**. The mean response factor of the aldehydes was 10889 counts/mg·kg^{-1} (count is measurement of the chromatographic peak's area associated with concentration of volatile compound), whereas for the alcohols it was 10683 counts/mg·kg^{-1}. Considering that the difference between the two values was 1.89%, which is less than the experimental error, an average response coefficient was calculated for both classes of compounds (10786 counts/mg·kg^{-1}).

RESULTS AND DISCUSSION

Characterization of LOX Activity. It is known that it is difficult to isolate the enzymes from olive plant material due to the cross-reactions between the proteins and the phenolic compounds that are present in high concentrations in olive fruit (*18*). Therefore, insoluble PVPP was added to complex, at least

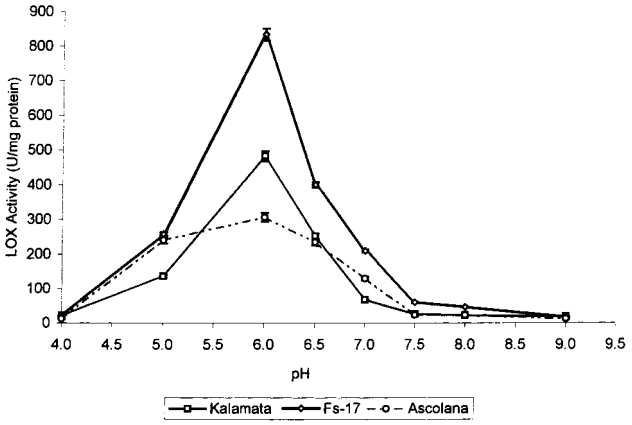

Figure 2. Effect of pH on olive LOX activity (cvs. FS17, Kalamata, and Ascolana Tenera) using linoleic acid as the substrate.

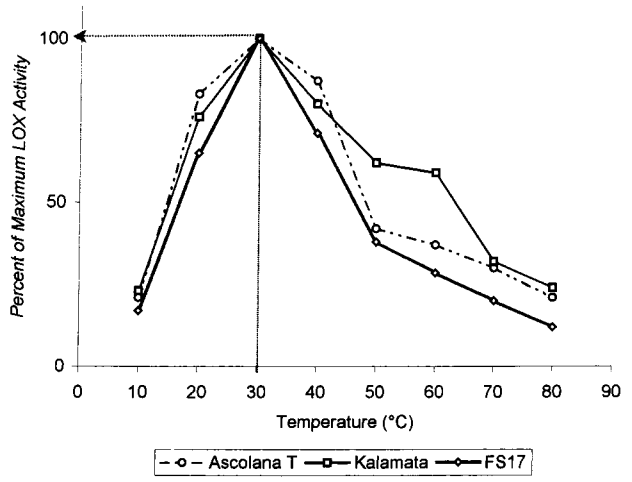

Figure 3. Effect of temperature on LOX activity.

in part, these substances in the extract, thereby reducing the inhibitory action on LOX activity (*19*). To isolate the protein fraction of the membrane, Triton X-100 was used; it is a nonionic detergent with lipohilic chains able to bond to the hydrophobic portions of the protein and break the protein−membrane interactions. To maintain the functionality of the macromolecule, it was preferable to use a buffer containing DTT and EDTA, a chelater that complexes Ca^{2+} and Mg^{2+} ions, thereby inhibiting any possible enzyme contaminants. The addition of sodium metabisulfite caused the extract to turn dark rapidly due to the chemical and enzymatic oxidation of the polyphenols.

LOX pH Profile. LOX was assayed using linoleic acid as substrate in a pH range from 4.0 to 9.0. LOX activity showed its maximum at pH 6.0 in 0.2 M sodium phosphate buffer, but it was also appreciable in the pH range from 5.0 to 7.0 (**Figure 2**). This is comparable to what has been reported in the literature for the LOX of peach (*20*) and bean with an optimum at pH 6.0 (*21*), for peppers at pH 6.5 (*22*), and for the LOX of olive (*19, 23*). Salas et al. (*24*) reported slightly different results with olive fruit that showed an optimum pH between 5.0 and 5.5. This difference could be due to different methods used to extract the enzymes as well as the cultivars tested (cv. Picual), which presumably has an aromatic−organoleptic profile that is different from that of the cultivars tested in this study.

Effect of Temperature. The maximum LOX activity was recorded at 30 °C (**Figure 3**). Similar results were reported by Jadhar et al. (*25*) in tomato, where the LOX activity increased with increasing temperature up to 30 °C. The lipoxygenases of the olive cultivars examined were active between 20 and 40 °C, maintaining ∼80% of their maximum activity. An increase in temperature inhibited the production of hydroperoxides. At 50 °C there was a 60−70% inactivation of LOX in cvs. Ascolana Tenera and FS17, whereas the decrease was less marked in cv. Kalamata (∼40%). At 80 °C, 80−90% of the activity was inhibited. When the enzymatic extract was heated at 80 °C for 15 min, the LOX activity was completely eliminated.

Table 1. Substrate Specificity of the Olive Lipoxygenase in Three Different Cultivars: FS17, Ascolana Tenera, and Kalamata

substrate	cultivar	V_{max} (units^{-1}/mg of protein)	K_M (μM)	K_{cat} (s^{-1})	K_{cat}/K_M ($s^{-1}\,\mu M^{-1}$)
linoleic acid	FS17	77	84.12	48.26	0.573
	Ascolana	80	96.35	56.15	0.582
	Kalamata	130	20.08	61.32	3.053
arachidonic acid	FS17	4	118.00	2.59	0.022
	Ascolana	4	117.60	2.94	0.025
	Kalamata	8	62.21	3.11	0.050

Kinetic Analysis and Substrate Specificity. The kinetic constants, K_M and K_{cat}, are known for some plants; for example, for soy (*26*), $K_M = 11.25\ \mu M$ and $K_{cat} = 156.26\ s^{-1}$ for linoleic acid and $K_M = 16.27\ \mu M$ and $K_{cat} = 7.88\ s^{-1}$ for arachidonic acid. The values for these parameters are not well-known for olive, so the enzyme kinetics were determined.

The kinetic characterization of olive lipoxygenases was carried out with two different substrates, linoleic acid (C18:2, a compound found in olive oil in percentages between 4 and 15%) and arachidonic acid (C20:4, found in olive oil in percentages between 0.3 and 0.4%), using sodium−phosphate buffer (0.2 M, pH 6). The kinetic constants, K_M and K_{cat}, determined for the three cultivars, are reported in **Table 1**. The K_M values for linoleic acid were between 20.08 and 96.35 μM. These values indicate that there is a good affinity of the enzyme for the substrate. The lowest K_M value was recorded in the cv. Kalamata extracts ($K_M = 20.08\ \mu M$), which is about one-fifth of the value reported for the other cultivars; it had the highest V_{max} value (130 units^{-1}/mg of protein). This indicates that in this cultivar the LOX has the highest affinity for the substrate and is particularly active. This constant did not differ significantly in cvs. FS17 and Ascolana Tenera.

The K_M values were highest in all of the enzymatic extracts when arachidonic acid was used as substrate. Also in this case, the LOX isolated from cv. Kalamata fruit showed kinetic values that were very different from the other two cultivars ($K_M = 62.21\ \mu M$ and $V_{max} = 8$ units^{-1}/mg of protein). These data support the idea that there is a different isoenzymatic form in this cultivar with respect to the other two.

The K_{cat}/K_M ratio, which measures the catalytic efficiency and enzyme specificity, was also determined. The values reported in **Table 1** show that linoleic acid was the preferred substrate for the three cultivars; in fact, arachidonic acid was a poor substrate with the highest K_M value and the lowest K_{cat} value.

Relationship between LOX Activity and the Quantity of C_5 and C_6 Volatile Compounds. The C_5 and C_6 compounds derived from the LOX-catalyzed oxidation of the free polyunsaturated fatty acids and from the successive enzymatic transformations can modify the primary formed volatile compounds. Consequently, a broad variety of volatile compounds can be created by the primary action of LOX (**Figure 4**). Linoleic and linolenic acid are oxidized by LOX to give the hydroperoxides

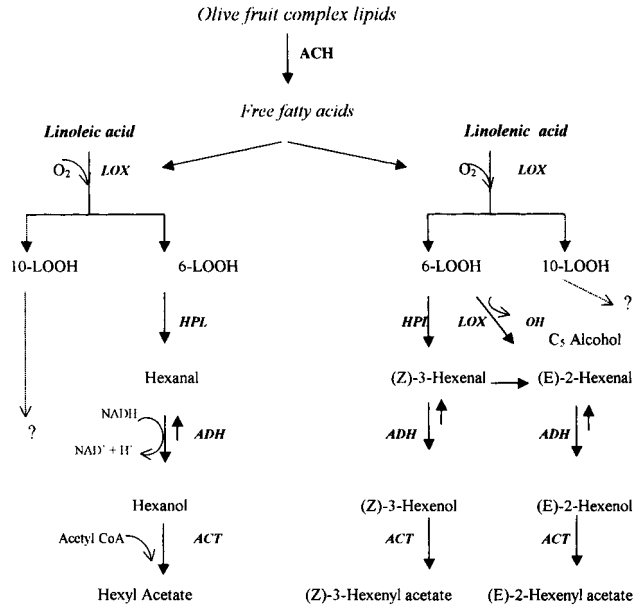

Figure 4. Proposed biosynthetic pathway for the formation of some volatile compounds (C_5 and C_6) starting from linoleic and linolenic acids. Abbreviations: LOOH, hydroperoxide; HPL, hydroperoxide lyase; ADH, alcohol dehydrogenase; ACT, acyl-transferase.

(6-LOOH and 10-LOOH). The 6-hydroperoxides are then split by hydroperoxide lyase (HPL) into aldehydes, which, once reduced to alcohols by alcohol dehydrogenase (ADH), undergo a series of reactions by alcohol acyl-transferase (ACT) that transforms them into esters. Moreover, the 6-hydroperoxides could be submitted to an enzyme (LOX)-mediated cleavage giving rise to C_5 alcohol and C_{13}-oxoacid. The amount of C_5 and C_6 volatile compounds was compared to the LOX activity assayed in the enzymatic extracts (AS) of the three olive cultivars under optimal conditions of pH, temperature, and substrate specificity, determined in the previous part of the enzyme characterization.

The data reported in **Figure 5** show that in the fruit of cv. FS17 there is a high LOX activity (833.4 units/mg of protein) that corresponds to a greater quantity of volatile C_5 and C_6 compounds (60.14 mg/kg), whereas in cv. Ascolana Tenera the low enzymatic activity corresponds to the lowest quantity of

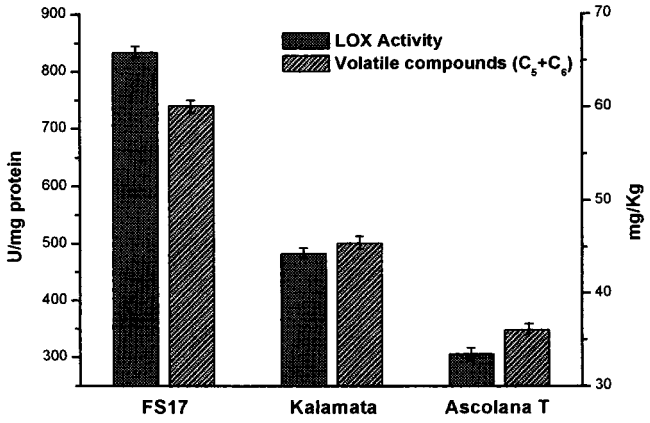

Figure 5. LOX activity in ammonium sulfate precipitated extracts (AS extract) relationship to the C_5 and C_6 volatile compounds in different olive fruits (FS17, Kalamata, and Ascolana Tenera). Activity was measured using linoleic acid as the substrate: results are means of three determinations ± SD (3%). The quantitative values of the volatile compounds are the means of five determinations ± SD (5%).

C_5 and C_6 aromas. Cv. Kalamata lies between these two with respect to enzymatic activity and the quantity of volatile compounds. There appears to be a relationship between the quantity of C_5 and C_6 compounds and LOX activity, which confirms the involvement of this enzyme in the biogenesis of C_5 and C_6 aldehydes and alcohols.

Following the analysis of the volatile compounds found in the olive pulp, a preliminary analysis was conducted on the concentration of aromas in the oil samples. The three cultivars showed a significant decrease in the total aromas (88.53% in cv. FS17, 90.11% in cv. Ascolana Tenera, and 91.14% in cv. Kalamata) as well as a partial qualitative change (**Table 2**). In olive pulp *trans*-2-hexenal, which gives the typical "green note" to extra virgin olive oil, accounted for ~50% of the total aromas present, whereas 1-pentanol, responsible for the pungent taste, was the second most important compound (10.71−13.03 mg/kg). In oil, *trans*-2-hexen-1-ol was the most represented compound (in cv. FS17 it accounted for 41.26% of the analyzed compounds); *trans*-2-hexenal was present in a much lower concentration than in the fruit (~98% less). Some substances were found in the oil that were not found in the fruit including

Table 2. Quantitative Determination of C_5 and C_6 Volatile Compounds Present in Static Headspace of Olive Fruits and Oils of Different Cultivars: FS17, Kalamata, and Ascolana Tenera

			FS17			Kalamata			Ascolana		
volatile compound[a]	sniffing[b]	retention time (min)	olive fruit (mg/kg)	oil (mg/kg)	variation (%)	olive fruit (mg/kg)	oil (mg/kg)	variation (%)	olive fruit (mg/kg)	oil (mg/kg)	variation (%)
3-pentanone	sweet	13.054	3.78	0	100	0.40	0	100	1.00	0	100
1-penten-3-one	sweet, strawberry	15.382	0	1.50		0	0.06		0	0.13	
hexanal	green, apple	18.932	8.20	0.11	98	1.97	0.18	89	2.49	0.05	97
1-penten-3-ol	wet earth	24.112	0	0		0	0.19		0	0.28	-
3-methyl-1-butanol (isoamyl alcohol)	unpleasant	26.795	1.05	0.11	83	0.95	0.06	94	1.77	0.14	90
trans-2-hexenal	green-fruity	27.514	31.13	0.78	96	23.44	0.24	98	17.69	0.14	99
1-pentanol	pungent	29.244	10.71	0.42	94	13.03	0.86	89	11.32	0.84	88
trans-2-penten-1-ol	green apple	32.283	0	0.23		0	0.18		0	0.20	
1-hexanol	fruity, aromatic, soft	34.132	0	0.61		0	0.33		0	0	
trans-3-hexen-1-ol	green	34.658	0.93	0.27	71	0.81	0.06	93	0	0.27	
cis-3-hexen-1-ol	grass, banana	35.598	0	0	-	1.99	0	100	0	0	
trans-2-hexen-1-ol	green, grassy	36.443	4.34	2.62	34	1.95	1.54	26	1.78	1.15	46
(*E*,*E*)-2,4-hexadienal	ripe fruit	36.658	0	0.24		0.89	0.33	63	0	0.37	
total			60.14	8.90	88	45.43	4.03	91	36.05	3.57	89

[a] Tentatively identified by GC retention time. [b] The sensory characterization of each volatile compound by sniffing (olfactometry) was described by Morales et al. (*1*).

1-penten-3-one, 1-penten-3-ol, *trans*-2-penten-1-ol, and (*E*,*E*)-2,4-hexadienal, which were probably formed during the malaxation of the paste (*27*).

The data obtained in this preliminary study show that in the olive cultivars examined, there is a significant quantitative and qualitative difference in the aromas in the fruit pulp compared to the oil. A similar phenomenon has been observed for the phenolic substances. It is known that during the process of oil extraction these substances, which are present in high concentrations in the fruit, undergo different repartitioning, in relationship to the degree of solubility, between the oil, pomace, and vegetation water, which notably reduces the quantity in the oil matrix.

An analogous mechanism could be proposed for the aromas. It is necessary, however, to determine which of the phases of the physical process leading to extraction of oil from the olive, from grinding to centrifugation, determine the content and quality of these substances. It should also be noted that there were differences within the varieties, with the prevalence of the aromas being in the oil of cv. FS17, following the trend already observed in the fruit.

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