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Synthesis of aroma compounds of virgin olive oil: significance of the cleavage of polyunsaturated fatty acids hydroperoxides during the oil extraction process

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ABSTRACT

Synthesis of the aroma compounds of virgin olive oil (VOO) occurs through the lipoxygenase (LOX) pathway comprising mainly the actuation of LOX and hydroperoxide lyase (HPL) enzymes. The aim of this work was to determine whether the cleavage of polyunsaturated fatty acid hydroperoxides catalyzed by HPL is a limiting factor for the biosynthesis of VOO volatile compounds during the oil extraction process. For this purpose, HPL activity and the availability of substrates for this activity were modified during the oil extraction process from olive fruits of cultivars Arbequina and Picual, which give rise to oils with quantitatively different volatile profiles. Experimental data suggest that the HPL enzyme activity is just slightly limited during the oil extraction process in both cultivars, being this limitation apparently more significant during processing of Arbequina fruits than of Picual fruits. However, this difference in HPL limitation seems to be more related to the differences in the amount of hydroperoxides produced in each cultivar than to the level of HPL activity during the olive processing.

Keywords: hydroperoxide lyase, olive oil, extraction, aroma, volatiles

1. Introduction

Virgin olive oil (VOO), one of the main components of the Mediterranean diet, is related to protection against cardiovascular diseases and cancer, due to its fatty acid profile and the presence of minor amounts of phenolic constituents (Ruíz-Gutiérrez, Muriana, & Villar, 1998; Visioli & Galli, 1998). However, the increase in the demand for high-quality VOO can be attributed not only to its potential health benefits but also to its excellent organoleptic properties. The aim of increasing the quality standards for VOO is continuously stimulating the study of the biochemical pathways related to the organoleptic properties and the search for olive cultivars with an improved quality. Thus, the participation of the lipoxygenase (LOX) pathway in the biosynthesis of straight-chain six-carbons (C6) compounds was established (Olías, Pérez, Rios, & Sanz, 1993). C6 aldehydes and alcohols and the corresponding esters are the most important compounds in the VOO aroma, from either a quantitative or a qualitative point of view (Morales, Aparicio, & Ríos, 1994; Angerosa, Mostallino, Basti, & Vito, 2000). These compounds are synthesized from polyunsaturated fatty acids containing a (*Z*,*Z*)-1,4-pentadiene structure such as linoleic (LA) and linolenic (LnA) acids (Fig. 1). In a first step of this pathway, LOX produces the corresponding 13-hydroperoxide derivatives that are subsequently cleaved heterolytically by hydroperoxide lyase (HPL) to C6 aldehydes (Olías et al., 1993; Salas, Williams, Harwood, & Sánchez, 1999; Salas & Sánchez, 1999). C6 aldehydes can then undergo reduction by alcohol dehydrogenases (ADH) to form C6 alcohols (Olías et al., 1993; Salas & Sánchez, 1998) and can finally be transformed into the corresponding esters by means of an alcohol acyltransferase activity (Olías et al., 1993; Salas, 2004). Moreover, Angerosa et al. (2000) also demonstrated the relevance of

straight-chain five-carbons (C5) compounds in the aroma of olive oil. C5 compounds would be generated through an additional branch of the LOX pathway that would involve the production of a 13-alcoxyl radical by LOX as demonstrated in soybean seeds (Gardner, Grove, & Salch, 1996). This radical would undergo subsequent nonenzymatic ß-scission in a homolytic way to form a 1,3-pentene allylic radical that could be chemically dimerized to form pentene dimers (PD) or react with an hydroxyl radical to form C5 alcohols. The latter would be the origin of C5 carbonyl compounds present in the volatile fraction of olive oil through an enzymatic oxidation by ADH as suggested to occur in soybean leaves (Fisher, Grimes, & Fall, 2003). The lack of HPL activity gives rise to an accumulation of hydroperoxides and a subsequent increase of the homolytic LOX branch activity, producing higher contents of C5 compounds as demonstrated in antisense-mediated HPL-depleted tomato plants (Vancanneyt, Sanz, Farmaki, Paneque, Ortego, Castañera, & Sánchez-Serrano, 2001).

Synthesis of volatile compounds responsible for VOO aroma occurs when enzymes and substrates meet as olive fruit tissues are disrupted during olive oil processing. The experimental data suggest that this synthesis takes place mainly during the milling step in the process of olive oil extraction (Sánchez-Ortiz, Romero, Pérez, & Sanz, 2008). Moreover, there is evidence that the LOX activity level in the fruit is an important limiting factor for the synthesis of the oil volatile fraction and that it is cultivar dependent (Sánchez-Ortiz, Romero-Segura, Sanz, & Pérez, 2012). The aim of the present work was to study the significance of the enzymatic step catalyzed by HPL activity, within the LOX pathway, for the biosynthesis of VOO aroma compounds during olive fruit processing. Thus, HPL activity may be consider as a trait for the selection of interesting genotypes with improved aroma quality in olive breeding programs.

2. Materials and methods

2.1. Chemicals and reagents

LA, LnA, soybean LOX, and reference compounds used for volatile identification were supplied by Sigma-Aldrich (St. Louis, MO) except for (*Z*)-hex-3-enal, which was generously supplied by S. A. Perlarom (Louvaine-La-Neuve, Belgium). Compounds such as (*E*)-hex-3-enal, (*Z*)-hex-2-enal, (*Z*)-pent-2-enal, and pentene dimers were tentatively identified on the basis of mass spectra and their concentrations approximately quantified according to their available isomers. The 13-hydroperoxide derivatives from LA (13-HPOD) and from LnA (13-HPOT) were prepared using soybean LOX according to the method of Hamberg & Samuelsson (1967).

2.2. Plant material

Olive fruits (*Olea europaea* L.) cultivar Picual and Arbequina, were harvested at matured-green stage, maturity index 1 (MI 1), and ripe stage, maturity index 5 (MI 5), in the experimental fields of the Instituto de la Grasa (Seville, Spain).

Olive oil extraction was performed using an Abencor analyzer (Comercial Abengoa, S.A., Seville, Spain) that simulates at laboratory scale the industrial process of VOO production. Milling of olive fruits (1 kg) was performed using a stainless steel hammer mill operating at 3000 rpm provided with a 5 mm sieve. The resulting olive pastes were immediately kneaded in a mixer at 50 rpm for 30 min at 30 ºC. Centrifugation of the kneaded olive pastes was performed in a basket centrifuge at 3500 rpm for 1 min. After

centrifugation, oils were decanted and paper-filtered. Samples for volatile compound analyses (0.5 g each) were stored under nitrogen atmosphere at -20 ºC until analysis.

Where indicated, a fruit homogenate approach was used that mimics at a smaller scale the biosynthesis of volatile compounds during oil extraction process. For this purpose, 4 g of olive fruit mesocarp was homogenized with 8 mL of distilled water by means of an Ultraturrax at the highest speed (24000 rpm) for 1 min. After an equilibrium period of 5 min at 25 ºC, homogenate aliquots of 1.5 mL were taken into 10-mL vials containing 1.5 mL of a saturated $CaCl_2$ solution, which were sealed and stored at –20 °C until analysis.

2.3. Preparation of fatty acid hydroperoxides and measurement of hydroperoxide lyase activity

For 13-HPOD and 13-HPOT synthesis, 10 mg of LA or LnA, respectively, and 4 mg of soybean LOX were added to 100 mL of oxygenated 0.2 M borate buffer, pH 9. The reaction was carried out at 2 °C for 15 min under a constant flow of oxygen and then stopped by adjusting the pH to 3 with 12 N HCl. Reaction products were extracted from the incubation mixture on reverse-phase C18 Sep-Pak cartridges (Waters, Milford, MA) and eluted with methanol. Hydroperoxides were further purified using activated TLC plates (Silica gel 60 F_{254}, Merck, Darmstadt, Germany) developed with hexane:diethyl ether:acetic acid (50:50:1). The bands at Rf=0.32 were scraped off, the hydroperoxides extracted with diethyl ether, and their purity assessed by HPLC according to the method of Sanz, Pérez, Ríos, & Olías (1993). Hydroperoxides were stored in methanol at -20 ºC until use as HPL substrate or to increase their availability during the oil extraction process.

115 HPL activity was tested by monitoring the hydroperoxide decomposition at 234 nm
116 according to Olías, Ríos, Valle, Zamora, Sanz, & Axelrod (1990). The reaction mixture
117 contained 1.5 mL of 100 mM sodium phosphate buffer pH 8, 8 µL of 10 mM 13-HPOT,
118 and the appropriate amount of enzyme extract (10-20 µL). Changes in absorbance at 234
119 nm were recorded for 60 s, five times each extract, and one unit of HPL activity was
120 expressed as the amount of enzyme consuming 1 µmol of 13-HPOT in 1 min. An
121 extinction coefficient of 25000 M^{-1} cm^{-1} was used for 13-HPOT.
122
123 *2.4. Extraction of HPL from olive fruit mesocarp, paste, and olive leaf*
124
125 To assess the level of HPL activity of olive fruit mesocarp and paste, obtained during
126 the oil extraction process, 4 g were homogenized at 4 °C with an Ultra-Turrax T-25
127 homogenizer with 20 mL of HEPES-NaOH buffer (50 mM, pH 7.5) containing 20 mM
128 KCl, 2 mM $MgCl_2$, 2 mM Na_2-EDTA, 7 mM dithiothreitol, 2 mM $Na_2S_2O_5$, 0.1% Na-
129 ascorbate, 0.5% (v/v) Triton X-100 and 12.5% (w/v) PVPP. The extract was vacuum-
130 filtered through two layers of Miracloth (Calbiochem, La Jolla, CA) and centrifuged (20
131 min, 27000*g*). The supernatant was filtered through four layers of gauze and centrifuged
132 again (20 min, 27000*g*). The supernatant constituted the crude extract.
133 To obtain an enriched extract of HPL activity for increasing this enzymatic activity
134 during the synthesis of VOO volatile compounds, olive leaves (20 g) were extracted as
135 described above with 200 mL of HEPES-NaOH buffer but containing 1% (v/v) Triton X-
136 100 and 50% (w/v) PVPP. PEG 8000 (30%, w/v) was added slowly to the resulting crude
137 extract. After 1 h of slow agitation, the extract was centrifuged (20 min, 27000*g*). The
138 residue was solubilized in 25 ml of Tris-HCl buffer (20 mM, pH 9) containing 0.5% (w/v)

139 Triton X-100 and centrifuged again (20 min, 27000*g*). The supernatant was the semi-
140 purified extract rich in HPL activity and deprived of LOX activity.
141
142 *2.5. Modification of metabolic factors*
143
144 To increase the proportion of polyunsaturated fatty acid hydroperoxides availability
145 during the process to obtain the oil, different amounts of either 13-HPOD or 13-HPOT in
146 the range 0-22.6 mg/kg fruit were added as sodium salts to the olive fruits during the
147 milling step following the procedure used by Sánchez-Ortiz, Pérez, & Sanz (2007). To
148 increase the HPL enzymatic activity load during processing, enriched olive leaf HPL
149 extracts were added during fruit homogenization approach in the range 0-6.5 U/g fruit.
150 Controls were performed with thermally deactivated (1h, 100 ºC) HPL extracts. Duplicate
151 experiments were carried out for each cultivar and maturity stage.
152
153 *2.6. Analysis of volatile compounds*
154
155 Olive oil and homogenates samples were conditioned to room temperature and then
156 placed in a vial heater at 40 °C. After 10 min of equilibrium time, volatile compounds
157 from headspace were adsorbed on a SPME fiber DVB/Carboxen/PDMS 50/30 µm
158 (Supelco Co., Bellefonte, PA). Sampling time was 50 min at 40 °C. Desorption of volatile
159 compounds trapped in the SPME fiber was done directly into the GC injector. Volatiles
160 were analyzed three times in duplicate experiments using a HP-6890 gas chromatograph
161 equipped with a DB-Wax capillary column (60 m × 0.25 mm i.d., film thickness=0.25
162 µm; J&W Scientific, Folsom, CA). Operating conditions were as follows: N_2 as carrier

gas; injector and detector at 250 °C; column held for 6 min at 40 °C and then programmed at 2 °C min^{-1} to 128 °C. Quantification was performed using individual calibration curves for each identified compound in each matrix (olive oil and olive mesocarp homogenate). Compound identification was carried out on a HRGC-MS Fisons series 8000 equipped with a similar stationary phase column and two different lengths, 30 and 60 m, matching against the Wiley/NBS Library, and by GC retention time against standards.

Volatile compounds were clustered into different classes according to the polyunsaturated fatty acid and the LOX pathway branch origin. Quantitative data for every volatile class are the sum of the content of the following compounds; Kovats indices are given in brackets:

C6/LnA aldehydes: (*E*)-hex-3-enal [1137], (*Z*)-hex-3-enal [1156], (*Z*)-hex-2-enal [1218], and (*E*)-hex-2-enal [1233].

C6/LnA alcohols: (*E*)-hex-3-enol [1364], (*Z*)-hex-3-enol [1383], and (*E*)-hex-2-enol [1399].

C6/LA aldehyde: hexanal [1074].

C6/LA alcohol: hexan-1-ol [1355].

C5/LnA carbonyls: pent-1-en-3-one [1018], (*Z*)-pent-2-enal [1100], and (*E*)-pent-2-enal [1127].

C5/LnA alcohols: pent-1-en-3-ol [1168], (*E*)-pent-2-en-1-ol [1322], and (*Z*)-pent-2-en-1-ol [1327].

PD: seven pentene dimers [965, 970, 1009, 1023, 1077, 1081, 1083].

C5/LA carbonyls: pentan-3-one + pentan-2-one [978] and pentanal [980].

C5/LA alcohol: pentan-1-ol [1261].

187 LOX esters (esters whose alcoholic moieties were synthesized through the LOX
188 pathway): hexyl acetate [1293] and (*E*)-hex-2-en-1-yl acetate [1337].
189 Non-LOX esters (esters whose alcoholic moieties were not synthesized through the
190 LOX pathway): methyl acetate [716], ethyl acetate [846], methyl hexanoate [1185], and
191 ethyl hexanoate [1249].
192
193 *2.7. Statistical analysis*
194
195 Data were statistically evaluated using Statgraphics Plus 5.1 (Manugistic Inc.,
196 Rockville, MD). Analysis of variance (ANOVA) was applied, and comparison of means
197 was done by the Student-Newman-Keuls/Duncan test at a significance level of 0.05.
198

3. Results and discussion

As mentioned in the Introduction, there are experimental evidences which point out that the LOX activity level in the olive fruit is an important limiting factor for the synthesis of the VOO volatile fraction. This limitation is significantly higher during processing of Picual fruits than of Arbequina fruits, in good agreement with the lower contents of volatile compounds in the oils obtained from Picual fruits than from Arbequina fruits (Sánchez-Ortiz et al., 2008). However, the synthesis of VOO volatile compounds may also depends on the level of the HPL activity cleaving the polyunsaturated fatty acid hydroperoxides produced during the oil extraction process.

To determine the biochemical constraints associated with this HPL activity, the level of this enzyme was measured in olive fruits and the olive pastes produced during the milling step in the oil extraction process. On average, HPL activity was slightly higher in Arbequina fruits than in Picual fruits, in the range of 6-7 U/g fruit and 4-6 U/g fruit, respectively. This HPL activity is rapidly inactivated during the milling step. No HPL activity could be detected in the olive fruit pastes immediately obtained after milling of olive fruits. Thus, these results are compatible with the assumption that most of the VOO volatile compounds produced through the LOX pathway are synthesized in the milling step of the oil extraction process (Sánchez-Ortiz et al., 2008). In this sense, Angerosa, d'Alessandro, Basti, & Vito (1998) pointed out that after the very fast synthesis of volatile compounds occurring during cell disruption at milling, the partition phenomena between the oily and aqueous phases would be the main factor responsible for the variations of the volatile content in the oils during the olive paste malaxation. The reasons for this apparent lack of volatile compound synthesis during malaxation remain unclear

223 but it might be associated to a deactivation of the enzymes of the LOX pathway by
224 components in the olive paste, probably oxidized phenolics arisen during the milling step.
225 This inactivating role of oxidized phenolics on enzymatic activity is well-established
226 (Loomis & Battaille, 1966; Loomis, 1969; Chedea, Braicu, & Socaciu, 2012) and can
227 contribute to reduce the effective enzyme activity load during the oil extraction process.
228 In this regard, the role of olive polyphenol oxidase and peroxidase has been reported to
229 act as major factors oxidizing phenolics during VOO production (García-Rodríguez,
230 Romero-Segura, Sánchez-Ortiz, Sanz, & Pérez, 2011).

231 In order to find out whether the HPL activity is a limiting factor for the synthesis of
232 VOO aroma compounds, the effect of modifying the level of HPL activity on the
233 synthesis of volatile compounds was studied. For this purpose, different amounts of
234 enriched HPL extracts from olive leaf were added to homogenates of olive fruit mesocarp
235 taking into account the levels of HPL activity measured previously in the olive fruit in
236 both cultivars. Increasing the HPL activity causes an increased synthesis of volatile
237 compounds in both cultivars and ripening stages. As displayed in Table 1, the increase is
238 produced mainly in the content of C6 compounds, being slightly higher in Arbequina
239 fruits than in Picual fruits. Among the major C6 compounds, an average 84% increase in
240 the contents of C6/LnA compounds was observed for cultivar Arbequina at both ripening
241 stages and at the highest amount of HPL activity added. Similarly, an average increase of
242 51% was observed for Picual cultivar. On the other hand, the increase of the HPL activity
243 led to an average 15% decrease in the synthesis of C5/LnA compounds in fruit mesocarp
244 homogenates of both cultivars and ripening stages. This decrease of the synthesis of
245 C5/LnA compounds could be attributed to a lower availability of substrates for the LOX
246 homolytic activity synthesizing C5 compounds as a consequence of a decrease of the

hydroperoxide contents due to the exogenous HPL activity. The overall data suggest that the HPL enzyme activity might be slightly limited during the oil extraction process in both cultivars, working as a constitutive enzyme activity in the olive fruit tissues, although for other reasons more limited during processing of Arbequina fruits than of Picual fruits. In this sense, we have found earlier that there is just one gene coding for HPL enzyme in the olive fruit which displays a low and constitutive expression pattern during development and ripening of both Arbequina and Picual fruits (Padilla, Hernández, Pérez, Sanz, & Martínez-Rivas, 2010). This constitutive role for HPL enzyme has been proposed in the plant tissues to cope with a hypothetical rise of deleterious fatty acid hydroperoxides as consequence of biotic or abiotic stresses (Vancanneyt et al., 2001).

The apparent contradiction between the different degree of HPL limitation and the level of HPL activity in each cultivar mentioned above might be explained if considering the amount of hydroperoxides to be metabolized during the oil extraction process. The amount of hydroperoxides produced during processing would be higher in Arbequina fruits than in Picual fruits according to the slightly higher LOX activity level (1.2-1.8 U/g Arbequina fruit; 1.1-1.5 U/g Picual fruits) (Sánchez-Ortiz et al., 2012). Consequently, a higher HPL activity would be needed (higher limitation) for metabolizing the polyunsaturated fatty acid hydroperoxides produced during processing of Arbequina fruits. Similarly, the lower LOX activity during the oil extraction process of Picual fruits compared to that of Arbequina fruits would result in a more reduced production of the hydroperoxides which explains the apparent lower HPL activity need (lower limitation) during the oil extraction process of Picual fruits compared to that of Arbequina fruits. To verify this point, the substrate availability for HPL activity (13-HPOD and 13-HPOT) was modified during the oil extraction process of these two cultivars. Table 2 shows the result

271 of increasing the availability of 13-HPOD during the olive oil extraction process in the oil
272 volatile fraction. The contents of C6/LA compounds in the oils increased when increasing
273 the availability of 13-HPOD during processing of both cultivars and maturity stages under
274 study. This increase was mainly due to a rise of the contents of the C6/LA aldehyde
275 (hexanal), the main product of HPL enzyme acting on 13-HPOD. Moreover, this increase
276 was higher in Picual oils than in Arbequina oils regardless of the maturity index. Thus,
277 around 500% and 150% increases were observed in oils from Picual and Arbequina fruits,
278 respectively, at MI 5 and the highest dose of 13-HPOD added. Despite the changes
279 observed in the contents of C6/LA compounds, the total volatile compounds content was
280 affected just slightly due to the low relevance of these compounds with respect to the total
281 volatile compounds of VOO from a quantitative standpoint. On the other hand, increasing
282 the availability of 13-HPOT during the oil extraction process resulted in a higher increase
283 of the contents of C6/LnA compounds in the oils obtained from Picual fruits (Table 3).
284 This increase was mainly due to a rise in the content of C6/LnA aldehydes and it was
285 higher in the oils obtained from ripe fruits (MI 5), 230%, than in oils from matured-green
286 fruits (MI 1), 30%, for the highest doses of 13-HPOT. However, just a 10% increase was
287 measured in the oils extracted from Arbequina fruits at MI 5, whereas no effect was
288 observed in the oils obtained from Arbequina fruits at MI 1. The overall experimental
289 data reveal a major limitation of substrates for HPL activity during the oil extraction
290 process of Picual fruits than of Arbequina fruits. As mentioned above, these differences
291 are consequence of the higher limitation of LOX activity (lower LOX activity) in Picual
292 fruits compared to Arbequina fruits, producing a lower amount of polyunsaturated fatty
293 acid hydroperoxides during the oil extraction process. Moreover, the increases of the
294 contents of C6 compounds observed in the different experiments showed a very good

linearity, suggesting that no inhibition of the LOX/HPL enzymatic system was occurring in good agreement with the kinetic parameters found for the recombinant HPL enzyme (Padilla et al., 2010).

The contents of C5/LnA compounds in the oils increased significantly when increasing the availability of 13-HPOT during the oil extraction process (Table 3). This increase was primarily due to an increased synthesis of pentene dimers that reached, for the highest doses of 13-HPOT, around 600% in the oils obtained from Picual fruits and 150% in the oils extracted from Arbequina fruits, both at MI 5. The oils from both cultivars at MI 1 displayed lower increases of these compounds but, again, higher in the oils from Picual fruits than from Arbequina fruits (173% and 95%, respectively). Taking into account that the C5 compounds are synthesized through a homolytic activity of LOX, these findings are compatible with a proportionally higher LOX homolytic activity in Picual fruits than in Arbequina fruits. Besides, these findings also suggest that the pentene dimers have a different metabolic origin than the other C5/LnA compounds. Pentene dimers might be synthesized directly from 13-HPOT since the other C5/LnA compounds did not show the same trend when increasing the availability of 13-HPOT during the oil extraction process. On the other hand, Table 2 shows that the contents of C5/LnA compounds decreased when increasing the availability of 13-HPOD during the oil extraction process. This decrease of C5/LnA was mainly due to a reduction of the content of the pentene dimers fraction, around 20% for the highest doses in both cultivars and maturity stages, which suggest that whereas 13-HPOT is the substrate of LOX homolytic activity synthesizing C5 compounds, 13-HPOD might be a competitive inhibitor of this LOX homolytic activity.

4. Conclusions

There are experimental evidences supporting that the differences in LOX activity limitation during the oil extraction process explains the differences of aroma compounds contents in Picual and Arbequina oils. The HPL activity level, that metabolizes the hydroperoxides formed by LOX activity, displays no major differences between Arbequina and Picual olive fruits. The apparent differences found in the limitation of HPL activity during processing of Arbequina and Picual fruits could be ascribed to the different amounts of polyunsaturated fatty acid hydroperoxides to be metabolized by this enzyme in both cultivars as a consequence of the differences in the limitation of LOX activity. Thus, HPL limitation seems to be higher in cultivar Arbequina than in cultivar Picual, in line with the higher LOX activity (lower LOX limitation) and the highest content of volatile compounds in the oils obtained during processing of Arbequina fruits.

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409 **Figure legend**

410

411 **Fig. 1**. Lipoxygenase pathway on linolenic acid (LnA) for the synthesis of main volatile
412 compounds in virgin olive oil. (**a**), 13-hydroperoxide-LnA (13-HPOT); (**b**), (Z)-hex-3-
413 enal; (**c**), (E)-hex-2-enal; (**d**), (Z)-hex-3-enol; (**e**), (E)-hex-2-enol; (**f**), (Z)-hex-3-en-1-yl
414 acetate; (**g**), (E)-hex-2-en-1-yl acetate; (**h**), pent-1-en-3-ol; (**i**), (Z)-pent-2-en-1-ol; (**j**),
415 pent-1-en-3-one; (**k**), (Z)-pent-2-enal.

416

417

418 **Figure 1**

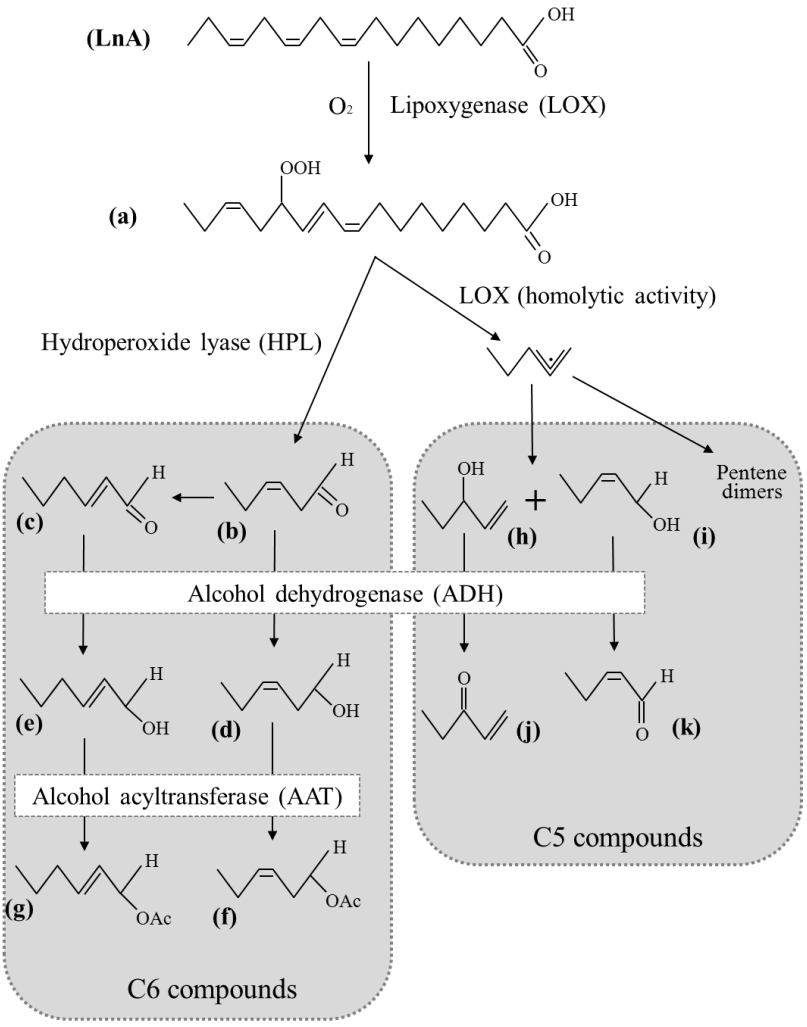
(LnA)
OH
O
O2
Lipoxygenase (LOX)
OOH
(a)
OH
O
LOX (homolytic activity)
Hydroperoxide lyase (HPL)
H
O
(c)
(b)
OH
+
H
OH
(h)
(i)
Pentene dimers
Alcohol dehydrogenase (ADH)
H
OH
(e)
(d)
O
(j)
H
O
(k)
Alcohol acyltransferase (AAT)
H
OAc
(g)
(f)
C6 compounds
C5 compounds

Table 1 Volatile contents* (ng/g olive oil) obtained after addition of different amounts of hydroperoxide lyase activity (U/g fruit) during homogenization of olive fruit mesocarp from cultivars Arbequina and Picual at different maturity stages (MI 1 and 5)

Volatile class**	Arbequina MI 1 HPL activity added (U/g fruit)				Arbequina MI 5 HPL activity added (U/g fruit)			
	0	3.25	4.37	6.50	0	3.25	4.37	6.50
C6/LnA aldehydes	20255 ± 2388 a	26994 ± 1111 b	33860 ± 1023 c	40727 ± 935 d	30457 ± 5739 a	50462 ± 5654 b	52761 ± 8500 b	55060 ± 11346 b
C6/LnA alcohols	1724 ± 130 a	1253 ± 65 b	1242 ± 44 b	1232 ± 22 b	1121 ± 144 a	1753 ± 393 b	1844 ± 217 c	934 ± 40 a
∑ C6/LnA	21979 ± 2288 a	28247 ± 1177 b	35103 ± 1067 c	41959 ± 957 d	31578 ± 5595 a	52215 ± 6047 b	54605 ± 8676 b	55994 ± 11305 b
C6/LA aldehyde	2648 ± 408 a	3526 ± 356 ab	4287 ± 331 b	4047 ± 305 b	3250 ± 193 a	4850 ± 709 b	5605 ± 1088 b	4360 ± 467 b
C6/LA alcohol	434 ± 50 a	232 ± 6 b	214 ± 11 b	195 ± 15 b	275 ± 32 a	212 ± 7 b	198 ± 8 ab	184 ± 9 b
∑ C6/LA	3082 ± 447 a	3758 ± 350 ab	4500 ± 335 b	4242 ± 320 b	3525 ± 168 a	5062 ± 1716 b	5803 ± 1087 bc	4543 ± 458 b
C5/LnA carbonyls	9194 ± 1170 a	8360 ± 902 a	8114 ± 913 a	7869 ± 923 a	19761 ± 1627 a	17070 ± 225 a	17058 ± 634 a	17046 ± 1043 a
C5/LnA alcohols	2891 ± 78 a	2722 ± 255 a	2885 ± 267 a	3048 ± 279 a	4887 ± 244 a	4082 ± 91 a	3877 ± 175 b	3672 ± 260 b
PD	4621 ± 395 a	3191 ± 586 b	3058 ± 457 b	2926 ± 328 b	6094 ± 497 a	5354 ± 134 b	5176 ± 95 b	4997 ± 57 c
∑ C5/LnA	16706 ± 1592 a	14272 ± 571 ab	14057 ± 1051 b	13842 ± 1530 b	30741 ± 2147 a	26506 ± 450 ab	26111 ± 848 a	25715 ± 1246 a
C5/LA carbonyls	453 ± 14 a	421 ± 18 a	425 ± 27 a	430 ± 35 a	869 ± 55 a	759 ± 84 a	753 ± 62 a	747 ± 40 a
C5/LA alcohol	20 ± 1 a	15 ± 3 a	25 ± 3 a	36 ± 2 a	176 ± 19 a	195 ± 35 a	240 ± 48 ab	284 ± 61 b
∑ C5/LA	473 ± 12 a	436 ± 15 a	451 ± 26 a	465 ± 37 a	1046 ± 70 a	954 ± 119 a	993 ± 70 a	1031 ± 20 a
LOX esters	52 ± 4 a	94 ± 1 c	76 ± 4 bc	58 ± 6 ab	45 ± 8 a	28 ± 0 b	56 ± 3 a	83 ± 6 c
Non-LOX esters	248 ± 17 a	272 ± 4 a	231 ± 20 a	390 ± 35 b	277 ± 6 a	429 ± 91 a	664 ± 103 b	900 ± 114 c
∑ Esters	299 ± 20 a	367 ± 5 a	307 ± 23 a	448 ± 41 a	322 ± 8 a	457 ± 91 a	720 ± 99 b	983 ± 108 c
Total volatiles	42540 ± 4360 a	47080 ± 2118 ab	54419 ± 2502 bc	60957 ± 2886 c	67213 ± 7987 a	85194 ± 7424 ab	88230 ± 10781 b	88267 ± 13138 b

(Table 1 Continued)

	Picual MI 1 HPL activity added (U/g fruit)				Picual MI 5 HPL activity added (U/g fruit)			
	0	3.25	4.37	6.50	0	3.25	4.37	6.50
C6/LnA aldehydes	29493 ±3401 a	27333 ±2615 ab	34915 ±2321 b	42497 ±2027 c	24107± 2660 a	32708± 511 b	36414± 3203 b	40121± 5895 b
C6/LnA alcohols	1547 ±96 a	1326 ±73 ab	1189 ±67 bc	1052 ±61 c	1154± 385 a	1667± 119 a	1350± 172 a	1032± 226 a
∑ C6/LnA	31039 ±3328 a	28659 ±2542 ab	36104 ±2254 b	43549 ±1966 c	25261± 2500 a	34375± 630 b	37764± 3149 b	41153± 5669 b
C6/LA aldehyde	1753 ±330 a	3432 ±570 b	3752 ±544 b	3072 ±519 b	2306± 648 a	2204± 111 a	2944± 380 ab	3683± 650 b
C6/LA alcohol	225 ±35 a	171 ±5 a	160 ±5 a	149 ±4 a	256± 35 a	228± 9 ab	205± 7 ab	182± 6 b
∑ C6/LA	1978 ±336 a	3603 ±575 b	3912 ±545 b	3221 ±515 b	2562± 682 a	2433± 120 a	3149± 382 ab	3864± 644 b
C5/LnA carbonyls	8862 ±456 a	8723 ±1712 ab	7883 ±983 ab	7043 ±253 b	14083± 4110 a	11800± 1953 b	13100± 2039 b	12400± 2126 b
C5/LnA alcohols	3199 ±359 a	3202 ±327 a	2728 ±227 ab	2253 ±127 b	4954± 550 a	3824± 376 b	4295± 541 b	4767± 705 b
PD	3221 ±516 a	2944 ±712 a	2896 ±493 a	2849 ±273 a	4777± 839 a	3871± 11 b	3412± 296 b	3953± 581 b
∑ C5/LnA	15282 ±1176 a	14870 ±1327 a	13507 ±990 ab	12145 ±653 b	23814± 4049 a	18495± 2317 b	20807± 2284 b	21119± 2250 b
C5/LA carbonyls	400 ±57 a	399 ±3 a	380 ±6 a	361 ±9 a	828± 233 a	719± 138 a	630± 94 a	541± 51 a
C5/LA alcohol	131 ±128 a	139 ±8 a	113 ±15 a	87 ±23 a	159± 38 a	136± 17 a	141± 9 a	145± 2 a
∑ C5/LA	531 ±177 a	538 ±10 a	493 ±12 a	449 ±14 a	987± 268 a	855± 121 a	771± 87 a	686± 52 a
LOX esters	69 ±17 a	53 ±15 a	49 ±9 a	45 ±3 a	33± 6 a	83± 2 b	90± 3 bc	97± 4 c
Non-LOX esters	79 ±19 a	247 ±56 b	173 ±30 c	99 ±5 a	224± 29 a	686± 169 b	436± 201 ab	1187± 233 c
∑ Esters	148 ±18 a	300 ±71 b	222 ±39 ab	144 ±8 a	256± 32 a	769± 171 b	527± 204 ab	1284± 237 c
Total volatiles	48978 ±5034 a	47970 ±4525 a	54238 ±3840 ab	59508 ±3156 b	52880± 7533 a	56927± 3359 a	63017± 6106 a	68107± 8852 a

* Mean value from three determinations in two different experiments.
** Volatile compounds were clustered into different classes as described in Materials and Methods.
[a-d] Values for the main volatile classes with different letters in the same row within each ripening index are significantly different ($P \leq 0.05$).

Table 2 Volatile contents* (ng/g olive oil) of the oils obtained after addition of different amounts of 13-hydroperoxide-linoleic acid (13-HPOD) during processing of olive fruits from cultivars Arbequina and Picual at different maturity stages (MI 1 and 5)

Volatile class**	Arbequina MI 1 13-HPOD added (mg/kg fruit)				Arbequina MI 5 13-HPOD added (mg/kg fruit)			
	0	5.7	11.1	22.6	0	5.7	11.1	22.6
C6/LnA aldehydes	26682 ±952 a	25432 ±667 a	25303 ±385 a	25399 ±1142 a	24592± 667 a	24040± 158 a	24128± 52 a	25489± 531 b
C6/LnA alcohols	182 ±8 a	229 ±34 ab	237 ±12 b	199 ±2 ab	663± 10 a	575± 15 b	789± 13 c	661± 11 a
∑ C6/LnA	26864 ±944 a	25660 ±633 a	25541 ±372 a	25598 ±1140 a	25255± 677 a	24614± 161 a	24916± 60 a	26150± 537 b
C6/LA aldehyde	689 ±1 a	739 ±30 b	931 ±1 c	930 ±15 c	307± 16 a	409± 10 b	503± 15 c	749± 35 d
C6/LA alcohol	13 ±1 a	25 ±1 c	25 ±1 c	21 ±1 b	339± 1 a	295± 4 b	320± 3 c	304± 3 d
∑ C6/LA	701 ±1 a	764 ±30 b	956 ±2 c	951 ±15 c	646± 16 a	704± 6 b	823± 12 c	1053± 33 d
C5/LnA carbonyls	358 ±18 a	316 ±32 ab	295 ±10 b	278 ±21 b	413± 2 a	382± 2 b	348± 2 c	314± 1 d
C5/LnA alcohols	517 ±2 a	484 ±6 ab	511 ±12 a	462 ±20 b	429± 3 a	465± 3 b	466± 13 b	460± 5 b
PD	7127 ±164 a	5905 ±117 b	5878 ±7 b	5128 ±115 c	4690± 15 a	4315± 52 b	3943± 189 c	4076± 36 c
∑ C5/LnA	8002 ±180 a	6705 ±79 b	6685 ±29 b	5868 ±156 c	5532± 10 a	5162± 55 b	4757± 202 c	4850± 33 c
C5/LA carbonyls	12 ±1 a	15 ±1 a	15 ±1 a	15 ±2 a	27± 1 a	22± 1 b	31± 1 c	26± 1 a
C5/LA alcohol	12 ±2 a	35 ±2 ab	46 ±1 b	21 ±20 ab	11± 2 a	14± 1 a	13± 1 a	15± 1 a
∑ C5/LA	24 ±1 a	50 ±2 ab	61 ±1 b	35 ±22 ab	38± 1 a	35± 1 a	45± 1 b	41± 2 a
LOX esters	76 ±1 a	78 ±4 a	97 ±1 b	94 ±4 b	113± 2 a	139± 6 b	133± 6 b	135± 4 b
Non-LOX esters	74 ±3 a	126 ±8 b	123 ±5 b	111 ±24 b	124± 12 a	133± 8 a	136± 11 a	161± 13 b
∑ Esters	150 ±2 a	204 ±4 c	220 ±5 c	205 ±20 b	237± 14 a	272± 4 b	269± 6 b	296± 9 c
Total volatiles	35741 ±1129 a	33384 ±748 a	33462 ±409 a	32657 ±1353 a	31707± 717 a	30788± 227 a	30810± 281 a	32390± 214 a

(Table 2 Continued)

	Picual MI 1 13-HPOD added (mg/kg fruit)				Picual MI 5 13-HPOD added (mg/kg fruit)			
	0	5.7	11.1	22.6	0	5.7	11.1	22.6
C6/LnA aldehydes	7458 ± 267 a	6727 ± 156 a	7145 ± 87 a	7444 ± 627 a	1649 ± 88 a	1700 ± 61 a	1751 ± 35 a	1724 ± 36 a
C6/LnA alcohols	170 ± 1 a	170 ± 5 a	171 ± 1 a	197 ± 2 b	57 ± 2 a	62 ± 3 a	63 ± 3 a	62 ± 3 a
∑ C6/LnA	7628 ± 266 a	6897 ± 151 a	7316 ± 88 a	7641 ± 629 a	1706 ± 86 a	1762 ± 59 a	1815 ± 35 a	1786 ± 34 a
C6/LA aldehyde	394 ± 21 a	481 ± 1 b	624 ± 22 c	818 ± 14 d	79 ± 9 a	176 ± 12 b	324 ± 16 c	491 ± 1 d
C6/LA alcohol	7 ± 1 a	9 ± 1 ab	9 ± 1 ab	11 ± 1 b	38 ± 1 a	40 ± 1 ab	43 ± 4 b	43 ± 2 b
∑ C6/LA	401 ± 21 a	490 ± 0 b	633 ± 22 c	829 ± 13 d	118 ± 10 a	216 ± 12 b	366 ± 20 c	535 ± 1 d
C5/LnA carbonyls	646 ± 1 a	766 ± 15 b	589 ± 2 c	545 ± 16 d	92 ± 1 a	78 ± 1 b	80 ± 1 c	69 ± 1 d
C5/LnA alcohols	603 ± 7 ab	675 ± 15 c	635 ± 14 bc	581 ± 21 a	409 ± 7 a	398 ± 19 a	422 ± 32 a	402 ± 15 a
PD	5572 ± 62 a	4547 ± 190 bc	4814 ± 133 b	4363 ± 165 c	1140 ± 98 ab	1180 ± 54 a	1050 ± 21 b	884 ± 8 c
∑ C5/LnA	6821 ± 68 a	5988 ± 221 b	6039 ± 149 b	5489 ± 202 c	1641 ± 91 a	1657 ± 67 a	1552 ± 13 a	1356 ± 14 b
C5/LA carbonyls	21 ± 1 a	22 ± 2 a	20 ± 4 a	17 ± 1 a	31 ± 1 a	32 ± 4 a	29 ± 1 a	28 ± 3 a
C5/LA alcohol	17 ± 1 a	19 ± 3 a	21 ± 3 a	22 ± 1 a	1 ± 1 a	2 ± 1 a	2 ± 1 a	2 ± 1 a
∑ C5/LA	39 ± 1 a	41 ± 5 a	41 ± 1 a	39 ± 1 a	32 ± 1 a	34 ± 4 a	31 ± 1 a	30 ± 3 a
LOX esters	26 ± 1 a	24 ± 1 a	20 ± 1 a	26 ± 4 a	331 ± 3 a	315 ± 11 a	313 ± 19 a	324 ± 9 a
Non-LOX esters	160 ± 19 a	139 ± 20 a	138 ± 16 a	145 ± 43 a	75 ± 1 a	92 ± 3 c	78 ± 2 ab	82 ± 1 b
∑ Esters	186 ± 18 a	162 ± 20 a	159 ± 16 a	171 ± 39 b	407 ± 3 a	408 ± 9 a	391 ± 19 a	406 ± 10 a
Total volatiles	15075 ± 374 a	13578 ± 397 b	14188 ± 277 ab	14168 ± 883	3903 ± 192 a	4076 ± 151 ab	4155 ± 88 b	4113 ± 61 b

* Mean value from three determinations in two different experiments.

** Volatile compounds were clustered into different classes as described in Materials and Methods.

[a-d] Values for the main volatile classes with different letters in the same row within each ripening index are significantly different ($P \leq 0.05$).

Table 3 Volatile contents* (ng/g olive oil) of the oils obtained after addition of different amounts of 13-hydroperoxide-linolenic acid (13-HPOT) during processing of olive fruits from cultivars Arbequina and Picual at different maturity stages (MI 1 and 5)

Volatile class**	Arbequina MI 1 13-HPOT added (mg/kg fruit)				Arbequina MI 5 13-HPOT added (mg/kg fruit)			
	0	5.7	11.1	22.6	0	5.7	11.1	22.6
C6/LnA aldehydes	27527 ±1400 a	26925 ±570 a	26923 ±1106 a	27205 ±1639 a	21437± 542 a	23605± 17 b	23802± 137 b	23705± 319 b
C6/LnA alcohols	182 ±6 ab	178 ±13 a	217 ±16 c	199 ±3 bc	652± 20 a	648± 29 a	705± 18 b	644± 11 a
∑ C6/LnA	27709 ±1399 a	27102 ±563 a	27140 ±1117 a	27405 ±1642 a	22089± 558 a	24252± 23 b	24506± 139 b	24349± 329 b
C6/LA aldehyde	675 ±24 a	684 ±19 a	686 ±58 a	732 ±27 a	379± 25 a	379± 15 a	396± 21 a	423± 49 a
C6/LA alcohol	13 ±1 a	13 ±1 a	15 ±1 a	16 ±3 a	337± 24 a	305± 10 b	313± 3 b	330± 11 a
∑ C6/LA	688 ±24 a	697 ±18 a	701 ±58 a	748 ±26 a	716± 41 a	685± 19 a	709± 18 a	753± 59 a
C5/LnA carbonyls	362 ±14 a	358 ±27 ab	332 ±12 ab	326 ±21 b	409± 7 a	409± 11 a	305± 11 b	299± 3 b
C5/LnA alcohols	530 ±23 a	491 ±9 b	516 ±24 ab	531 ±20 a	428± 30 a	478± 13 a	442± 4 a	468± 12 a
PD	7306 ±331 a	8705 ±567 b	10698 ±873 c	14269 ±629 d	3660± 52 a	5522± 151 b	6434± 36 c	8953± 485 d
∑ C5/LnA	8198 ±363 a	9553 ±550 b	11545 ±890 c	15126 ±669 d	4497± 87 a	6409± 175 b	7181± 33 c	9720± 495 d
C5/LA carbonyls	13 ±1 a	12 ±1 a	15 ±1 b	14 ±1 ab	26± 3 a	22± 1 a	22± 2 a	22± 1 a
C5/LA alcohol	12 ±2 a	16 ±2 a	8 ±1 a	14 ±8 a	11± 1 a	12± 2 a	20± 9 a	15± 4 a
∑ C5/LA	24 ±1 a	28 ±2 a	23 ±1 a	28 ±8 a	37± 2 ab	33± 3 a	41± 10 a	38± 4 a
LOX esters	78 ±4 a	93 ±9 a	78 ±2 a	80 ±9 a	117± 7 a	126± 9 ab	144± 15 b	190± 17 c
Non-LOX esters	76 ±4 a	117 ±20 b	134 ±13 b	143 ±20 b	120± 11 a	127± 14 ab	150± 16 b	146± 13 b
∑ Esters	154 ±8 a	210 ±14 b	212 ±14 b	224 ±12 b	236± 10 a	253± 22 a	294± 20 b	336± 24 c
Total volatiles	36773 ±1795 a	37591 ±1147 a	39621 ±2081 a	43530 ±2357 b	27576± 699 a	31632± 242 b	32732± 222 c	35195± 914 d

(Table 3 Continued)

	Picual MI 1 13-HPOT added (mg/kg fruit)				Picual MI 5 13-HPOT added (mg/kg fruit)			
	0	5.7	11.1	22.6	0	5.7	11.1	22.6
C6/LnA aldehydes	6458 ±377 a	7431 ±856 ab	8148 ±980 ab	8399 ±58 b	1056± 29 a	1999± 195 b	2293± 367 b	3481± 182 c
C6/LnA alcohols	130 ±1 a	159 ±5 b	184 ±9 c	183 ±6 c	57± 4 a	61± 1 a	60± 5 a	81± 9 b
∑ **C6/LnA**	6588 ±377 a	7590 ±854 ab	8332 ±971 b	8582 ±66 b	1113± 33 a	2060± 194 b	2353± 362 b	3562± 173 c
C6/LA aldehyde	394 ±29 a	435 ±45 a	454 ±27 a	443 ±34 a	88± 4 a	96± 9 a	79± 10 a	102± 8 a
C6/LA alcohol	7 ±3 a	8 ±1 a	9 ±1 a	11 ±2 a	38± 2 a	37± 1 a	33± 4 a	34± 1 a
∑ **C6/LA**	401 ±32 a	443 ±44 a	464 ±27 a	473 ±11 a	126± 6 a	133± 10 a	112± 12 a	136± 7 a
C5/LnA carbonyls	646 ±2 a	587 ±26 b	622 ±31 ab	417 ±18 c	92± 10 a	105± 11 a	117± 20 a	112± 3 a
C5/LnA alcohols	603 ±9 a	625 ±14 a	618 ±16 a	543 ±2 b	409± 64 a	458± 26 a	504± 44 a	470± 19 a
PD	5572 ±88 a	8958 ±252 b	11314 ±654 c	15187 ±446 d	844± 15 a	2509± 49 b	4175± 272 c	6162± 250 d
∑ **C5/LnA**	6821 ±96 a	10170 ±246 b	12554 ±646 c	16147 ±444 d	1345± 54 a	3073± 65 b	4797± 317 c	6744± 271 d
C5/LA carbonyls	21 ±1 a	18 ±2 a	19 ±1 a	17 ±3 a	41± 7 a	34± 4 a	33± 1 a	33± 1 a
C5/LA alcohol	17 ±1 a	17 ±3 a	15 ±1 a	15 ±2 a	2± 1 a	1± 0 a	1± 0 a	1± 0 a
∑ **C5/LA**	39 ±1 a	35 ±4 a	34 ±2 a	33 ±5 a	42± 7 a	35± 4 a	34± 1 a	34± 1 a
LOX esters	26 ±2 a	22 ±1 a	23 ±1 a	26 ±6 a	331± 5 a	351± 24 a	339± 36 a	323± 7 a
Non-LOX esters	160 ±27 a	182 ±6 ab	236 ±5 b	308 ±50 c	76± 3 a	69± 5 a	73± 1 a	70± 2 a
∑ **Esters**	186 ±25 a	204 ±6 ab	259 ±5 b	334 ±51 c	407± 8 a	420± 26 a	412± 37 a	393± 8 a
Total volatiles	14035 ±530 a	18442 ±1155 b	21643 ±1651 c	25570 ±577 d	3034± 108 a	5721± 300 b	7709± 729 c	10870± 460 d

* Mean value from three determinations in two different experiments.
** Volatile compounds were clustered into different classes as described in Materials and Methods.
[a-d] Values for the main volatile classes with different letters in the same row within each ripening index are significantly different ($P \leq 0.05$).