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Oxygen Concentration Affects Volatile Compound Biosynthesis during Virgin Olive Oil Production

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The effect of O_2 concentration on oil volatile compounds synthesized during the process to obtain virgin olive oil (VOO) was established. The study was carried out either on the whole process or within the main steps (milling and malaxation) of this process with two olive cultivars, Picual and Arbequina, at two ripening stages. Data show that O_2 control during milling has a negative impact on VOO volatile synthesis. This effect seems to depend on cultivar and on the ripening stage in cultivar Picual. Because most VOO volatiles are synthesized during olive fruit crushing at the milling step, O_2 control during malaxation seems to affect just slightly the volatile synthesis. The highest effect was observed when control of O_2 concentration was performed over the whole process. In this case, the content of volatile compounds of oils obtained from both cultivars and ripening stages showed quite similar trends.

KEYWORDS: Lipoxygenase pathway; oxygen concentration; olive oil; volatiles

INTRODUCTION

A large increase in the demand for high-quality virgin olive oil (VOO) during the past few years can be attributed not only to its potential health benefits (*1*) but also to its particular organoleptic properties. Benefits of VOO consumption are related to protection against cancer and cardiovascular diseases mainly due to the presence of phenolic compounds (*2, 3*). The synthesis of these phenolic compounds is directly related to the activation of endogenous enzymes during the mechanical extraction process (*4–6*). However, different oxidoreductases are also activated during olive fruit crushing and oxidize phenolic compounds, reducing their concentration in the pastes and oils (*4, 7–9*). During recent years several works were performed selectively to control endogenous oxidoreductases in the pastes during the technological process. In this sense, the use of inert gas to remove O_2 in the headspace of the mixer has been studied (*8, 10–12*). This new processing approach for improving nutritional quality may have, however, a deleterious effect on the organoleptic quality, mainly on oil volatile compound biosynthesis, that should be carefully considered.

Aldehydes and alcohols of six straight-chain carbons (C6) and the corresponding esters are the most important volatile compounds in VOO, from both qualitative and quantitative points of view (*13, 14*). The participation of the lipoxygenase (LOX) pathway in the biosynthesis of C6 volatile compounds of olive oil was established in the early 1990s (*15*). These compounds are synthesized from nonesterified polyunsaturated fatty acids containing a (*Z*,*Z*)-1,4-pentadiene structure such as linoleic (LA) and linolenic (LnA) acids. In a first step of this pathway LOX, using molecular O_2 as cosubstrate, produces 13-hydroperoxide derivatives that are subsequently cleaved heterolytically by hydroperoxide lyase (HPL) to C6 compounds (*15–17*). More recently, Angerosa et al. (*18*) demonstrated the relevance of C5 compounds in the volatile fraction of olive oil. C5 compounds would be generated through an additional branch of the LOX pathway that would involve the production of a 13-alkoxyl radical by LOX in a homolytic way as demonstrated in soybean preparations (*19*).

The first step in the process for olive oil extraction is the milling of olive fruits to produce olive pastes by means of different types of crushers. Olive pastes are then kneaded during the malaxation step to favor the separation of the oily and aqueous phases. This last step in the process to obtain olive oil has been thoroughly studied in order to understand the way VOO acquires its organoleptic and nutritional properties (*11, 12, 14, 20–23*). However, considerably less attention has been given to the milling step, especially in terms of the organoleptic properties of the resulting oils. In this sense, Angerosa and Di Giacinto (*24*) found that the crushing system influenced the oil quality, and we have recently found that the temperature of olive fruit at milling is the main factor responsible for the content of VOO pigments and volatile compounds and that it is closely related to the activity load of the LOX/HPL enzyme system (*25, 26*).

As mentioned before O_2 is a cosubstrate of the LOX pathway. Reduction of this gas concentration during the industrial process to obtain VOO could produce, besides its beneficial effect on the nutritional quality, through a decrease of phenolic oxidation, an alteration of the organoleptic properties of the oil. In the

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context of our current studies to assist in the design of new oil extraction equipment and procedures for improving VOO quality, the aim of the present work was to study the effect of O_2 concentration during the industrial process on the content of VOO volatile compounds and, specifically, in each of the main steps in this process, milling of olive fruit and malaxation of the resulting pastes.

MATERIALS AND METHODS

Plant Material. Olive fruits (*Olea europaea* L.) cultivars Picual and Arbequina were harvested in CIFA Cabra-Priego orchards (Cabra, Cordoba, Spain) during the 2006 and 2007 olive fruit seasons (October−December) at ripening index (RI) 1 (fruits with green-yellowish skin) and at RI 5 (fruits with black skin and <50% purple flesh), according to the method of Garcia and Yousfi (*27*).

Olive Oil Extraction. 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 mill was modified to allow the milling process under reduced O_2 concentrations (0.1−21%) by means of a polyethylene flexible container (120 L) sealed around the hammer head. The container was equipped with an inlet and an outlet to allow a continuous gas flow (8 L/min) of the desired O_2 concentration by mixing air and N_2 in the right proportion by means of pressure regulators. O_2 concentration was monitored using a portable electrochemical O_2 analyzer (Abiss, Viry Chantillon, France) with a 0.1% resolution.

The resulting olive pastes were immediately submitted to malaxation by kneading in a mixer at 50 rpm for 30 min at 30 °C in 1.2 L bowls. These bowls were also adapted to carry out malaxation under reduced O_2 concentrations (0.1−21%). For this purpose bowls were covered with a polyethylene lid allowing a continuous flow (1 L/min) of the desired O_2 concentration by mixing air and N_2 in the right proportion and monitoring O_2 concentration with the O_2 analyzer described above.

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 analyses (0.5 g) were taken in 10 mL vials, which were sealed under N_2, stored at −18 °C, and analyzed within 2 months.

Analysis of Volatile Compounds. Olive oil samples were conditioned to room temperature and then placed in a vial heater at 40 °C. After 10 min of equilibrium time, volatile compounds from headspace were adsorbed on a SPME fiber DVB/Carboxen/PDMS 50/30 μm (Supelco Co., Bellefonte, PA). Sampling time was 50 min at 40 °C. Desorption of volatile compounds trapped in the SPME fiber was done directly into the GC injector. Volatiles were analyzed three times in duplicate experiments using a HP-6890 gas chromatograph equipped with a DB-Wax capillary column (60 m × 0.25 mm i.d., film thickness = 0.25 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 by adding known amounts of different compounds to redeodorized high-oleic sunflower oil. 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 compounds: (*E*)-hex-3-enal [1137], (*Z*)-hex-3-enal [1156], (*Z*)-hex-2-enal [1218], (*E*)-hex-2-enal [1233], (*E*)-hex-3-enol [1364], (*Z*)-hex-3-enol [1383], and (*E*)-hex-2-enol [1399].

C6/LA compounds: hexanal [1074] and hexan-1-ol [1355].

C5/LNA compounds: pent-1-en-3-one [1018], (*Z*)-pent-2-enal [1100], (*E*)-pent-2-enal [1127], pent-1-en-3-ol [1168], (*E*)-pent-2-en-1-ol [1322], (*Z*)-pent-2-en-1-ol [1327], and seven pentene dimers [965, 970, 1009, 1023, 1077, 1081, 1083].

C5/LA compounds: pentan-3-one + pentan-2-one [978], pentanal [980], and pentan-1-ol [1261].

Esters: methyl acetate [716], ethyl acetate [846], methyl hexanoate [1185], ethyl hexanoate [1249], hexyl acetate [1293], and (*E*)-hex-2-en-1-yl acetate [1337].

Data were statistically evaluated using Statgraphics Plus 5.1 (Manugistic Inc., Rockville, MD). Analysis of variance (ANOVA) was applied, and comparison of means was done by the Student−Newman−Keuls/Duncan test at a significance level of 0.05.

Chemicals and Reagents. 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.

Table 1. Contents of the Different Volatile Classes (Nanograms per Gram of Oil)[a] of Olive Oils from Arbequina and Picual Fruits at Two Ripening Stages (RI 1 and 5) Obtained after Malaxation at 30 °C for 30 min or without Malaxation (0 min)

	Arbequina				Picual			
	RI 1		RI 5		RI 1		RI 5	
volatile[b]	0 min	30 min	0 min	30 min	0 min	30 min	0 min	30 min
Σ C6/LnA	20043	20876	18134	19057	2012	2241	4435	4223
Σ C6/LA	502	708	1399	1312	250	160	470	413
Σ C5/LnA	5763	6046	8971	9723	4212	4710	4087	4454
Σ C5/LA	42	38	64	64	119	132	124	121
Σ esters	176	184	1057	1031	107	110	435	402
total	26526	27852	29625	31187	6699	7352	9550	9613

[a] Mean value from three determinations in two different experiments. Mean values were not significantly different at $P \leq 0.05$. [b] Each class of volatile compound comprises compounds listed under Materials and Methods.

RESULTS AND DISCUSSION

Picual and Arbequina olive cultivars, the oils of which are quite different in terms of volatile compound profile (*28*), were selected to study the effect of O_2 concentration on the biosynthesis of VOO volatile compounds through the LOX pathway during the industrial process to obtain the oil. For this purpose, the contribution of each of the main steps of this process, milling of olive fruits and malaxation of resulting pastes, to the biosynthesis of VOO volatile compounds was first studied. As shown in **Table 1**, most of the volatile compounds found in the oils from Arbequina and Picual fruits were synthesized during the milling step (0 min malaxation), whereas only slight nonsignificant modifications of the volatile content took place during malaxation (30 min). These data are in good agreement with those found in the literature in oils and pastes, although in some cases a dependency on olive cultivar was found (*11, 20, 21, 23, 29*). Angerosa et al. (*20*) pointed out that after the very fast biosynthesis of volatiles 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 malaxation step. The reasons for this apparent low rate of volatile compound synthesis during the malaxation step remain unclear but might be associated with a shortage of substrates, nonesterified polyunsaturated fatty acids, for the LOX pathway, occurring as a consequence of the depletion of these substrates during the previous milling step. One more reason might be a deactivation of the LOX/HPL enzymatic system by oxidized

Table 2. Volatile Content (Nanograms per Gram of Olive Oil)[a] of Oils from Arbequina Fruits at Two Ripening Stages Obtained at Different Oxygen Concentrations during Milling and Using Standard Malaxation Conditions (30 °C, 30 min, 21% O_2)

	Arbequina, RI 1						Arbequina, RI 5					
volatile[b]	0.1%	2.5%	5%	10%	15%	21%	0.1%	2.5%	5%	10%	15%	21%
Σ C6/LnA	6881 a	9047 b	12067 c	13003 c	12369 c	12321 c	12471 a	13267 ab	14407 bc	14697 bc	14238 c	15056 c
Σ C6/LA	445 a	553 ab	640 b	625 b	621 b	648 b	833 a	1040 ab	1333 c	1223 bc	1209 bc	1015 ab
Σ C5/LnA	2014 a	2983 b	4354 c	4441 cd	4501 cd	4759 d	3984 a	4720 a	6255 b	6268 b	6406 b	6933 b
Σ C5/LA	23 a	23 a	30 b	36 c	33 bc	31 bc	58 a	55 a	68 ab	60 ab	70 ab	78 b
Σ esters	78 a	106 a	199 b	186 b	184 b	190 b	232 a	259 ab	298 c	284 bc	281 bc	286 bc
total	9441 a	12713 b	17291 c	18291 c	17707 c	17949 c	17579 a	19342 a	22361 b	22533 b	22204 b	23369 b

[a] Mean value from three determinations in two different experiments. Values for the main volatile classes with different letters in the same row within each ripening index are significantly different ($P \leq 0.05$). [b] Each class of volatile compound comprises compounds listed under Materials and Methods.

Table 3. Volatile Content (Nanograms per Gram of Olive Oil)[a] of Oils from Picual Fruits at Two Ripening Stages Obtained at Different Oxygen Concentrations during Milling and Using Standard Malaxation Conditions (30 °C, 30 min, 21% O_2)

	Picual, RI 1						Picual, RI 5					
volatile[b]	0.1%	2.5%	5%	10%	15%	21%	0.1%	2.5%	5%	10%	15%	21%
Σ C6/LnA	3390 a	4387 b	4765 b	5268 bc	6090 c	7236 d	2498 a	2463 a	2861 a	3994 b	3962 b	4076 b
Σ C6/LA	476 a	495 a	535 ab	541 ab	546 ab	595 b	338 a	405 ab	439 bc	520 c	454 bc	441 bc
Σ C5/LnA	5552 a	6680 b	6883 b	7634 c	8223 d	8299 d	3118 a	3245 a	4286 bc	4089 b	4707 c	4581 c
Σ C5/LA	23 a	23 a	29 bc	25 ab	30 c	28 bc	73 a	111 a	102 a	109 a	97 a	77 a
Σ esters	70 a	94 a	71 a	72 a	100 a	165 b	180 a	206 ab	192 a	259 bc	306 c	284 bc
total	9510 a	11679 b	12284 b	13540 c	14989 d	16323 e	6206 a	6430 a	7880 b	8971 bc	9527 c	9459 c

[a] Mean value from three determinations in two different experiments. Values for the main volatile classes with different letters in the same row within each ripening index are significantly different ($P \leq 0.05$). [b] Each class of volatile compound comprises compounds listed under Materials and Methods.

phenolics arisen during the milling step. This inactivating role of oxidized phenolics on enzymatic activity is well-established (*30*) and can contribute to reduce the effective enzyme activity load during the process.

During the past decade several works were performed selectively to control endogenous oxidoreductases in the pastes to increase phenolic concentrations in the oils. In this sense, there are quite a number of studies describing the way malaxation conditions (temperature, time, and O_2 concentration) affect both VOO phenolic and volatile compound profiles or the biosynthetic pathways determining these profiles (*10–12, 14, 20–23*). According to the low rate of volatile compound synthesis produced during the malaxation step, we have previously observed that modification of the O_2 concentration during malaxation had almost no effect on the volatile compound profiles of oils from different olive cultivars (unpublished results). This is also in good agreement with results reported by Servili et al. (*10*) for cultivar Frantoio and the quantitative data published by Migliorini et al. (*22*) for the main volatile compounds in oils from mixed Frantoio and Moraiolo fruits. However, the former authors found a significant decrease of the content of VOO volatile compounds when flushing fruit pastes from cultivar Moraiolo with N_2 during malaxation (*11*).

Fixing the malaxation conditions in terms of time (30 min), temperature (30 °C), and O_2 content (atmospheric 21%), the effect of O_2 concentration on volatile compound synthesis during the milling step was studied. As displayed in **Tables 2** and **3**, O_2 concentration during milling affects the content of all classes of volatile compounds. Cultivar Arbequina is characterized by an enhancement of volatile synthesis as O_2 concentration increased during the milling step up to approximately 5% O_2 (**Table 2**). Higher O_2 concentrations during milling did not give rise to a higher level of volatile synthesis in the oils both from ripe (RI 5) and unripe (RI 1) fruits, especially for the main classes of volatile compounds, C6 and C5 from LnA. Cultivar Picual displayed quite similar behavior (**Table 3**), although with slight differences with respect to Arbequina fruits. Thus, processing of unripe Picual fruits at increasing O_2 concentrations during milling gave rise to a linear augment of total volatile synthesis, mainly due to an increase of the content of C6 and C5 compounds from LnA. However, oils obtained from ripe fruits showed an increment of total volatile content as O_2 concentration increases during milling up to 10−15% O_2, whereas no further changes were observed in the biosynthesis of volatile compounds at higher O_2 concentrations.

Moreover, an upper limit for the synthesis of volatile compounds specific for each cultivar and ripening stage is inferred from the data. This limit might be a consequence of the activity load of the LOX/HPL system and the substrate availability for each cultivar/ripening stage. In this sense, we observed previously (*28*) that substrate availability was the main limiting factor for volatile synthesis during olive fruit processing from Arbequina fruits, whereas the enzymatic activity load seemed to be the main limiting factor in the case of Picual fruits. Thus, both substrates of the LOX/HPL system, nonesterified polyunsaturated fatty acids and molecular O_2, would be involved in the limitation of volatile synthesis during milling of Arbequina fruits at low O_2 concentrations (up to 5%). At higher O_2 concentrations (5−21%), volatile synthesis would be limited only by the availability of nonesterified polyunsaturated fatty acids. However, volatile synthesis during the processing of Picual fruits, characterized by a higher availability of nonesterified polyunsaturated fatty acids (*28*), would be almost limited only by the O_2 concentration during milling, at least for oils from unripe fruits, explaining the linear enhancement of this synthesis observed at increasing O_2 concentrations during this process step.

Once the specific effect of O_2 concentration on the volatile synthesis during milling and malaxation was established, the effect of O_2 concentration during the whole oil extraction process was studied. Thus, volatile compounds of the oils obtained from fruits of both cultivars and both ripening stages, maintaining identical O_2 concentrations during the milling and malaxation

Table 4. Volatile Content (Nanograms per Gram of Olive Oil)[a] of Oils from Arbequina Fruits at Two Ripening Stages Obtained at Different Oxygen Concentrations during Milling and Malaxation

	Arbequina, RI 1						Arbequina, RI 5					
volatile[b]	0.1%	2.5%	5%	10%	15%	21%	0.1%	2.5%	5%	10%	15%	21%
Σ C6/LnA	1592 a	6399 b	7170 c	8691 d	10213 e	11148 f	1968 a	4990 b	7993 c	8894 d	10483 e	14279 f
Σ C6/LA	149 a	266 ab	323 bc	396 cd	506 de	586 e	337 a	500 b	707 c	744 c	934 d	919 d
Σ C5/LnA	352 a	1977 b	2699 c	3037 d	3886 e	4498 f	727 a	2138 b	4138 c	4972 d	5257 d	6273 e
Σ C5/LA	23 ab	21 a	21 a	21 a	25 ab	28 b	55 a	52 a	75 a	78 a	71 a	71 a
Σ esters	101 a	136 ab	122 ab	113 a	147 ab	172 b	208 a	235 ab	280 c	255 bc	239 ab	259 bc
total	2216 a	8799 b	10335 c	12257 d	14777 e	16432 f	3296 a	7915 b	13193 c	14943 d	16984 e	21801 f

[a] Mean value from three determinations in two different experiments. Values for the main volatile classes with different letters in the same row within each ripening index are significantly different ($P \leq 0.05$). [b] Each class of volatile compound comprises compounds listed in Materials and Methods section.

Table 5. Volatile Content (Nanograms per Gram of Olive Oil)[a] of Oils from Picual Fruits at Two Ripening Stages Obtained at Different Oxygen Concentrations during Milling and Malaxation

	Picual, RI 1						Picual, RI 5					
volatile[b]	0.1%	2.5%	5%	10%	15%	21%	0.1%	2.5%	5%	10%	15%	21%
Σ C6/LnA	1522 a	2352 b	3730 c	3868 c	5571 d	6547 e	1416 a	1527 a	2039 b	2800 c	3089 d	3688 e
Σ C6/LA	172 a	273 a	395 b	413 b	547 c	626 c	229 a	253 a	313 b	353 bc	360 bc	371 c
Σ C5/LnA	1632 a	3363 b	5538 c	5997 c	7504 d	7255 d	1832 a	2025 a	2818 b	3339 c	4111 d	4274 d
Σ C5/LA	26 ab	29 a	24 ab	27 ab	26 ab	23 b	80 a	71 a	83 a	77 a	71 a	69 a
Σ esters	48 a	68 b	65 ab	69 b	107 c	149 d	186 a	203 ab	201 a	281 bc	353 c	304 c
total	3399 a	6084 b	9750 c	10374 c	13755 d	14600 e	3743 a	4079 a	5454 b	6851 c	7984 d	8707 e

[a] Mean value from three determinations in two different experiments. Values for the main volatile classes with different letters in the same row within each ripening index are significantly different ($P \leq 0.05$). [b] Each class of volatile compound comprises compounds listed under Materials and Methods.

steps were quantified. As displayed in **Tables 4** and **5**, the contents of all volatile classes increased with O_2 concentration for the two cultivars and ripening stages under study. Data suggest that this substrate (O_2) of the LOX/HPL system is the main limiting factor for the synthesis of volatile compounds when O_2 control was performed over the whole oil extraction process. That would also mean that the main substrate for volatile synthesis, nonesterified polyunsaturated fatty acids, is not completely depleted when VOO is obtained at O_2 concentrations lower than 21%. Moreover, data showed that the contents of volatiles were significantly lower at $P \leq 0.05$ (statistical significance not shown) when O_2 concentration below 21% was applied to the whole process (**Tables 4** and **5**) than when performed only during the milling step (**Tables 2** and **3**) for the same O_2 concentration. This observation seems to be general, as it was common for the two cultivars and ripening stages, and would demonstrate that volatile synthesis is still partially active during malaxation in atmospheric conditions (21% O_2) when O_2 concentration is controlled during milling. This fact would support the above-mentioned suggestion that the fatty acid substrates for volatile synthesis were still available to be metabolized through the LOX pathway during malaxation in these processing conditions and that the content of these substrates limits volatile synthesis in each cultivar and ripening stage in normal oil industrial extraction processes.

On the other hand, it seems that the content of O_2 dissolved in the fruit tissues is enough to trigger the LOX pathway, producing volatiles. As shown in **Tables 4** and **5**, oils obtained under the lowest O_2 concentration (0.1%) displayed an appreciable volatile synthesis rate. This synthesis seems to be cultivar dependent. Thus, oils obtained from Arbequina fruits in these low O_2 conditions showed an average 14% of volatile synthesis for both ripening stages compared to control oils obtained in atmospheric conditions (21% O_2), whereas an average 33% of volatile synthesis was found for Picual oils. These differences in the volatile synthesis rates could be related to the higher availability of nonesterified polyunsaturated fatty acid in Picual fruits compared to Arbequina fruits mentioned above (*28*).

In summary, reduction of oxidoreductase activities during olive fruit processing seems to be of great interest for the nutritional and organoleptic quality of VOO. This control has been carried out so far by reducing the O_2 level during the malaxation step of the process. A similar or even greater effect is presumed by controlling O_2 concentration during milling. In this sense, data arising from this work point out that O_2 control during malaxation seems to affect just slightly volatile synthesis, because most VOO volatiles are synthesized during olive fruit crushing at the milling step. However, O_2 control during milling seems to have a negative impact on VOO volatile synthesis, being higher when this control is carried out through the whole process (milling plus malaxation). Thus, work is under progress to find minimum levels of O_2 during VOO extraction process that, while affecting minimally the volatile synthesis, increase the level of phenolics in the oils in order to design olive oil extraction equipment allowing the production of flavorful oils with improved nutritional quality.

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