

Synthesis of Volatile Compounds of Virgin Olive Oil Is Limited by the Lipoxygenase Activity Load during the Oil Extraction Process

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S Supporting Information

ABSTRACT: The aim of this work was to determine whether the lipoxygenase (LOX) activity is a limiting factor for the biosynthesis of virgin olive oil (VOO) volatile compounds during the oil extraction process. For this purpose, LOX activity load was modified during this process using exogenous LOX activity and specific LOX inhibitors on olive cultivars producing oils with different volatile profiles (Arbequina and Picual). Experimental data suggest that LOX activity is a limiting factor for the synthesis of the oil volatile fraction, this limitation being significantly higher in Picual cultivar than in Arbequina, in line with the lowest content of volatile compounds in the oils obtained from the former. Moreover, there is evidence that this limitation of LOX activity takes place mostly during the milling step in the process of olive oil extraction.

KEYWORDS: *Olea europaea*, lipoxygenase, olive oil, volatiles

■ INTRODUCTION

Olive oil is one of the oldest known plant oils, and it is unique among them because it can be consumed as a fruit juice called virgin olive oil (VOO). This oil, 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.^{1,2} 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 biochemical pathways related to organoleptic properties and the development of technological procedures to improve them. In this sense, our group established the participation of the lipoxygenase (LOX) pathway in the biosynthesis of compounds of six straight-chain carbons (C6 compounds) in the olive oil volatile fraction.³ C6 aldehydes and alcohols and the corresponding esters are the most important compounds in the VOO aroma, from either a quantitative or qualitative point of view.^{4,5} These compounds are synthesized from 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 produces the corresponding 13-hydroperoxide derivatives that are subsequently cleaved heterolytically by hydroperoxide lyase (HPL) to C6 aldehydes.^{3,6,7} C6 aldehydes can then undergo reduction by alcohol dehydrogenases (ADH) to form C6 alcohols^{3,8} and can finally be transformed into the corresponding esters by means of an alcohol acyltransferase.^{3,9} Moreover, Angerosa et al.⁵ also demonstrated the relevance of compounds of five straight-chain 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-alkoxyl radical by LOX as demonstrated in soybean seeds.¹⁰ This radical would undergo subsequent nonenzymatic β -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 a 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.¹¹ 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.¹²

There are quite a number of studies describing the way technological procedures affect VOO volatile compound profile or the biosynthetic pathway determining this profile.^{13–18} However, as far as we know, the studies devoted to identifying limiting factors for the biosynthesis of VOO aroma are scarce. LOX activity is the first enzyme acting in the homonymous pathway producing VOO volatile compounds. Taking into account differences in volatile contents in Arbequina and Picual oils that are not satisfactorily explained by the availability of nonesterified polyunsaturated fatty acids during VOO extraction process,¹⁹ the aim of the present work was to study whether the LOX activity load is a limiting factor for the biosynthesis of VOO volatile compounds during the process to obtain this oil.

■ MATERIALS AND METHODS

Chemicals and Reagents. Soybean LOX-1 and LOX inhibitors octyl gallate, phenidone, and phenylbutazone were purchased from Sigma-Aldrich (St. Louis, MO). Reference compounds used for volatile identification were also supplied by Sigma-Aldrich 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 PD were tentatively identified on

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Table 1. Volatile Contents^a (Nanograms per Gram Olive Oil) of the Oils Obtained by the Addition of Different Amounts of Lipoxygenase Activity during the Oil Extraction Process of Arbequina Fruits at Different Maturity Stages (MI 1 and 5)

volatile compound class ^b	Arbequina MI 1 LOX activity added				Arbequina MI 5 LOX activity added			
	0 U/g fruit	5 U/g fruit	10 U/g fruit	20 U/g fruit	0 U/g fruit	5 U/g fruit	10 U/g fruit	20 U/g fruit
C6/LnA aldehydes	15114 ± 673 a	16001 ± 362 b	16783 ± 114 bc	16916 ± 307 c	23103 ± 56 a	24205 ± 607 b	23872 ± 242 ab	24043 ± 345 b
C6/LnA alcohols	146 ± 15 a	148 ± 14 a	143 ± 8 a	141 ± 6 a	183 ± 2 a	200 ± 5 ab	190 ± 13 b	172 ± 5 b
∑ C6/LnA	15260 ± 687 a	16150 ± 357 ab	16926 ± 107 b	17057 ± 309 b	23285 ± 58 a	24405 ± 612 b	24062 ± 235 ab	24215 ± 350 b
C6/LA aldehyde	462 ± 16 a	441 ± 2 a	438 ± 33 a	369 ± 9 b	500 ± 42 a	407 ± 22 b	430 ± 50 ab	425 ± 42 ab
C6/LA alcohol	7 ± 1 a	11 ± 1 b	9 ± 0 ab	8 ± 2 a	49 ± 2 a	64 ± 2 b	50 ± 1 ab	49 ± 2 ab
∑ C6/LA	469 ± 16 a	452 ± 1 a	447 ± 32 a	377 ± 8 b	550 ± 41 a	471 ± 22 a	480 ± 50 ab	474 ± 41 ab
C5/LnA carbonyls	306 ± 5 a	420 ± 10 b	430 ± 14 b	489 ± 18 c	292 ± 13 a	360 ± 10 b	421 ± 6 c	457 ± 8 d
C5/LnA alcohols	647 ± 4 a	587 ± 21 b	548 ± 9 c	481 ± 25 d	453 ± 7 a	332 ± 1 b	313 ± 5 c	304 ± 3 d
PD	7848 ± 273 a	7202 ± 247 b	6675 ± 299 b	5825 ± 362 c	6108 ± 182 a	4697 ± 90 b	4088 ± 107 c	3832 ± 57 d
∑ C5/LnA	8801 ± 277 a	8209 ± 252 b	7653 ± 287 b	6795 ± 390 c	6852 ± 203 a	5389 ± 81 b	4823 ± 102 c	4593 ± 51 d
C5/LA carbonyls	15 ± 2 a	15 ± 1 a	14 ± 1 a	15 ± 1 a	16 ± 1 a	20 ± 1 b	21 ± 1 b	20 ± 1 b
C5/LA alcohol	4 ± 2 ab	5 ± 1 ab	6 ± 1 b	3 ± 1 a	14 ± 1 a	12 ± 1 a	12 ± 1 a	11 ± 3 a
∑ C5/LA	20 ± 4 a	20 ± 1 a	19 ± 1 a	18 ± 1 a	29 ± 1 a	32 ± 2 a	33 ± 1 a	31 ± 3 a
LOX esters	20 ± 2 a	29 ± 2 b	18 ± 3 a	11 ± 3 c	59 ± 1 a	58 ± 2 ab	50 ± 3 a	46 ± 3 b
non-LOX esters	99 ± 7 a	106 ± 5 a	104 ± 17 a	95 ± 2 a	135 ± 12 a	146 ± 4 ab	147 ± 4 a	178 ± 21 b
∑ esters	119 ± 5 ab	135 ± 6 c	122 ± 14 bc	106 ± 2 a	194 ± 13 a	204 ± 4 ab	197 ± 6 a	224 ± 22 b
total volatiles	24668 ± 989 a	24966 ± 618 a	25167 ± 442 a	24354 ± 710 a	30910 ± 314 a	30501 ± 721 a	29595 ± 393 b	29537 ± 467 b

^aMean 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 ($P \leq 0.05$). ^bVolatile compounds were identified using reference compounds except as indicated and clustered into different classes as follows: C6/LnA aldehydes, (E)-hex-3-enal (tentatively identified), (Z)-hex-3-enal, (Z)-hex-2-enal (tentatively identified), and (E)-hex-2-enal; C6/LnA alcohols, (E)-hex-3-enol, (Z)-hex-3-enol, (Z)-hex-2-enol, and (E)-hex-2-enol; C6/LA aldehyde, hexanal; C6/LA alcohol, hexan-1-ol; C5/LnA carbonyls, pent-1-en-3-one, (Z)-pent-2-enal (tentatively identified), and (E)-pent-2-enal; C5/LnA alcohols, pent-1-en-3-ol, (E)-pent-2-en-1-ol, and (Z)-pent-2-en-1-ol; PD, seven pentene dimers (tentatively identified); C5/LA carbonyls, pentan-3-one + pentan-2-one and pentanal; C5/LA alcohol, pentan-1-ol; LOX esters, hexyl acetate and (E)-hex-2-en-1-yl acetate; non-LOX esters, methyl acetate, ethyl acetate, methyl hexanoate, and ethyl hexanoate.

Table 2. Volatile Contents^a (Nanograms per Gram Olive Oil) of the Oils Obtained by the Addition of Different Amounts of Lipoxygenase Activity during the Oil Extraction Process of Picual Fruits at Different Maturity Stages (MI 1 and 5)

volatile compound class ^b	Picual MI 1 LOX activity added			Picual MI 5 LOX activity added		
	0 U/g fruit	5 U/g fruit	10 U/g fruit	0 U/g fruit	5 U/g fruit	10 U/g fruit
C6/LnA aldehydes	8292 ± 1116 a	10017 ± 947 b	10487 ± 382 b	11595 ± 970 b	7883 ± 304 a	10495 ± 151 b
C6/LnA alcohols	183 ± 7 ab	197 ± 5 b	192 ± 9 ab	180 ± 12 b	531 ± 27 b	584 ± 10 bc
∑ C6/LnA	8475 ± 1113 a	10215 ± 943 b	10679 ± 374 b	11775 ± 961 b	8413 ± 330 a	11080 ± 160 b
C6/LA aldehyde	607 ± 10 a	565 ± 27 b	444 ± 15 c	433 ± 9 c	201 ± 22 b	241 ± 7 c
C6/LA alcohol	15 ± 2 a	17 ± 1 a	17 ± 1 a	18 ± 2 a	95 ± 3 b	116 ± 4 c
∑ C6/LA	622 ± 12 a	582 ± 27 b	461 ± 15 c	450 ± 8 c	295 ± 19 b	357 ± 11 a
C5/LnA carbonyls	654 ± 15 a	681 ± 29 a	726 ± 18 b	763 ± 9 c	633 ± 13 b	681 ± 11 a
C5/LnA alcohols	932 ± 51 a	685 ± 5 b	598 ± 14 c	527 ± 23 d	655 ± 13 b	592 ± 8 c
PD	4975 ± 178 a	4650 ± 96 a	4165 ± 121 b	3767 ± 272 c	4574 ± 122 b	4330 ± 112 b
∑ C5/LnA	6561 ± 242 a	6017 ± 63 b	5489 ± 135 c	5057 ± 285 d	5862 ± 145 b	5602 ± 125 b
C5/LA carbonyls	21 ± 1 a	22 ± 3 a	21 ± 1 a	16 ± 1 b	70 ± 4 a	61 ± 3 b
C5/LA alcohol	13 ± 1 a	11 ± 1 a	11 ± 2 a	12 ± 1 a	20 ± 4 b	21 ± 2 b
∑ C5/LA	33 ± 1 a	33 ± 3 a	32 ± 1 a	27 ± 2 b	89 ± 2 a	81 ± 5 b
LOX esters	33 ± 1 ab	36 ± 9 b	25 ± 1 a	26 ± 1 a	146 ± 4 a	157 ± 9 ab
non-LOX esters	80 ± 4 ab	76 ± 12 a	75 ± 12 a	72 ± 4 a	52 ± 3 a	57 ± 3 ab
∑ esters	112 ± 3 ab	113 ± 3 a	99 ± 12 b	98 ± 4 b	198 ± 2 a	215 ± 9 ab
total volatiles	15803 ± 1371 a	16959 ± 1039 ab	16760 ± 538 ab	17408 ± 1260 b	14859 ± 498 b	17335 ± 309 c

^aMean 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 ($P \leq 0.05$). ^bEach class of volatile compound comprises compounds listed in Table 1 and under Materials and Methods.

the basis of mass spectra and their concentrations approximately quantified according to their available isomers.

Plant Material. Olive fruits (*Olea europaea* L.) of cultivars Picual and Arbequina were harvested in CIFA Cabra-Priego orchards (Cabra, Cordoba, Spain) during the 2007–2009 olive fruit seasons (October–December) at maturity index (MI) 1 (fruits with green yellowish skin) and at MI 5 (fruits with black skin and <50% purple flesh), according to the method of Garcia and Yousfi.²⁰

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 resulting olive pastes were immediately kneaded in a mixer at 50 rpm for 30 min at 30 °C. 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 at –18 °C until analysis.

To increase or decrease the LOX activity load during the process to obtain the oil, different amounts of soybean LOX-1 or LOX inhibitors such as octyl gallate, phenidone, and phenylbutazone were added to the olive fruits during the milling step or at the beginning of the kneading step. Duplicate experiments were carried out for each cultivar and maturity index.

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: N₂ 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 reodorized 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 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, and 1083].

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

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

LOX esters (esters having alcoholic moieties synthesized through the LOX pathway): hexyl acetate [1293] and (*E*)-hex-2-en-1-yl acetate [1337].

Non-LOX esters (esters having alcoholic moieties that were not synthesized through the LOX pathway): methyl acetate [716], ethyl acetate [846], methyl hexanoate [1185], and ethyl hexanoate [1249].

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.

RESULTS AND DISCUSSION

Previous findings suggest that the biosynthesis of VOO volatile compounds from cultivars Arbequina and Picual depends on the availability of substrates to be catabolized through the LOX pathway during the process to obtain this oil.¹⁹ This availability seems to be cultivar-dependent, and it is comparatively lower in Arbequina than in Picual fruits. Thus, the lower contents of volatile compounds found in Picual oils might be the result of a lower LOX activity load during the process to obtain the oil, despite the apparently higher availability of substrates as compared to Arbequina fruits. To explore this possibility, LOX activity in the different olive tissues and in olive pastes during the VOO extraction process was determined. However, measurements of this enzyme activity proved not to be useful to assess the effective LOX activity load during the oil extraction process because the enzymatic extracts displayed quite similar levels of LOX activity when obtained from fresh tissues (1.1–1.5 U/g FW in Picual fruits; 1.2–1.8 U/g FW in Arbequina fruits) and no activity when obtained from olive pastes. In this sense, there are several references in the literature on the interference of many of the components of the fruits, mainly phenolic compounds, on LOX activity,^{21–23} and it is well-known that phenolics interact with proteins through different mechanisms.^{24,25} To overcome these circumstances, we used an indirect procedure in which was studied the effect of modifying the LOX activity load during the oil extraction process on the synthesis of volatile compounds. First, the effect of increasing the level of LOX activity on the synthesis of VOO volatile compounds was assessed by the addition of exogenous LOX during fruit milling, the first step in the olive oil extraction process. Soybean LOX-1 was selected because it is commercially available and it is a type 13-LOX producing 13-hydroperoxides from polyunsaturated fatty acids. This catalytic characteristic is essential for the synthesis of C6 and C5 compounds present in the VOO volatile fraction.³ As shown in Tables 1 and 2, the effect of increasing LOX activity during fruit milling promoted a low modification of the total content of volatile compounds of the oils, irrespective of the ripening stage of the olive fruit, but a significant change in the volatile compound profile that may have an important impact on the oil from a sensory point of view. Thus, an increase in the content of the main volatile compounds determining VOO aroma, the C6 compounds derived from LnA (C6/LnA), was observed when the level of exogenous LOX was increased during the oil extraction process. For the highest dose (20 U/g fruit), significant increases ($P \leq 0.05$) for this group of compounds were found, 12 and 4% for Arbequina fruits at MI 1 and 5, respectively. However, the same dose of exogenous LOX during the oil extraction of Picual fruits reached increases of around 39 and 54% for fruits at MI 1 and 5, respectively. The content of C6 compounds derived from LA (C6/LA) did not show this trend, remaining unaltered or displaying even a slight decrease. These results imply that olive LOX activity seems to be limiting the synthesis of volatile compounds during the oil extraction process as previously suggested and that this limitation is significantly higher in Picual fruits than in Arbequina fruits, regardless their ripening stage. By contrast, the content of C5 compounds derived from LnA (C5/LnA)

Table 3. Volatile Contents^a (Nanograms per Gram Olive Oil) of the Oils Obtained by the Addition of Lipoxygenase Activity (20 U/g Paste) during the Kneading of Pastes from Arbequina and Picual Fruits at Different Maturity Stages (MI 1 and 5)

volatile compound class ^b	Arbequina				Picual			
	MI 1		MI 5		MI 1		MI 5	
	control	+ LOX	control	+ LOX	control	+ LOX	control	+ LOX
C6/LnA aldehydes	16189 ± 320 a	17123 ± 395 b	18698 ± 607 a	19529 ± 640 a	12579 ± 54 a	17490 ± 313 b	7984 ± 190 a	10184 ± 190 b
C6/LnA alcohols	89 ± 12 a	124 ± 11 b	99 ± 8 a	90 ± 2 a	312 ± 2 a	195 ± 7 b	241 ± 19 a	1891 ± 19 b
∑ C6/LnA	16278 ± 325 a	17247 ± 406 b	18798 ± 615 a	19619 ± 638 a	12891 ± 52 a	17685 ± 320 b	8226 ± 209 a	12076 ± 209 b
C6/LA aldehyde	821 ± 27 a	828 ± 17 a	891 ± 83 a	959 ± 22 a	759 ± 13 a	480 ± 14 b	347 ± 11 a	390 ± 31 a
C6/LA alcohol	10 ± 0 a	13 ± 1 b	36 ± 1 a	38 ± 7 a	22 ± 1 a	8 ± 1 b	66 ± 4 a	11 ± 0 b
∑ C6/LA	831 ± 27 a	841 ± 18 a	927 ± 84 a	997 ± 29 a	781 ± 12 a	489 ± 13 b	413 ± 15 a	401 ± 31 a
C5/LnA carbonyls	225 ± 3 a	308 ± 10 b	509 ± 43 a	617 ± 21 b	329 ± 3 a	182 ± 0 b	780 ± 29 a	1009 ± 21 b
C5/LnA alcohols	484 ± 17 a	352 ± 10 b	594 ± 22 a	588 ± 14 a	440 ± 20 a	268 ± 1 b	1075 ± 414 a	1278 ± 70 a
PD	6074 ± 168 a	5762 ± 138 a	9998 ± 538 a	10588 ± 479 a	4841 ± 23 a	4324 ± 27 b	6952 ± 374 a	3901 ± 105 b
∑ C5/LnA	6783 ± 181 a	6422 ± 157 a	11100 ± 604 a	11794 ± 515 a	5610 ± 46 a	4774 ± 29 b	8807 ± 817 a	6188 ± 196 b
C5/LA carbonyls	8 ± 0 a	8 ± 0 a	15 ± 1 a	16 ± 1 a	10 ± 1 a	7 ± 0 b	40 ± 2 a	64 ± 12 b
C5/LA alcohol	9 ± 2 a	10 ± 2 a	8 ± 5 a	11 ± 2 a	3 ± 1 a	4 ± 2 a	4 ± 1 a	14 ± 1 b
∑ C5/LA	17 ± 2 a	18 ± 1 a	23 ± 6 a	26 ± 1 a	13 ± 0 a	11 ± 2 a	44 ± 1 a	78 ± 13 b
LOX esters	14 ± 2 a	15 ± 2 a	24 ± 4 a	25 ± 2 a	10 ± 0 a	14 ± 0 b	25 ± 1 a	34 ± 2 b
non-LOX esters	88 ± 4 a	105 ± 9 b	87 ± 10 a	86 ± 9 a	100 ± 11 a	117 ± 14 a	61 ± 3 a	92 ± 20 a
∑ esters	102 ± 3 a	120 ± 10 b	111 ± 7 a	111 ± 7 a	111 ± 10 a	131 ± 14 a	86 ± 2 a	126 ± 23 a
total volatiles	24010 ± 537 a	24648 ± 592 a	30960 ± 1315 a	32547 ± 1189 a	19405 ± 121 a	23090 ± 401 b	17576 ± 1043 a	18868 ± 472 a

^aMean value from three determinations in two different experiments. Values for the main volatile classes with different letters in the same row within each cultivar and ripening index are significantly different ($P \leq 0.05$). ^bEach class of volatile compound comprises compounds listed in Table 1 and under Materials and Methods.

Table 4. Volatile Contents^a (Nanograms per Gram Olive Oil) of the Oils Obtained by the Addition of Octyl Gallate (32 mg/g Fruit) during the Oil Extraction Process of Arbequina and Picual Fruits at Different Maturity Stages (MI 1 and 5)

volatile compound class ^b	Arbequina				Picual			
	MI 1		MI 5		MI 1		MI 5	
	control	+ octyl gallate	control	+ octyl gallate	control	+ octyl gallate	control	+ octyl gallate
C6/LnA aldehydes	14283 ± 539 a	2909 ± 500 b	22723 ± 394 a	15590 ± 160 b	6923 ± 735 a	1667 ± 331 b	8441 ± 44 a	3976 ± 43 b
C6/LnA alcohols	306 ± 41 a	95 ± 15 b	264 ± 12 a	191 ± 3 b	157 ± 2 a	93 ± 10 b	465 ± 25 a	311 ± 6 b
∑ C6/LnA	14588 ± 579 a	3004 ± 485 b	22987 ± 381 a	15781 ± 158 b	7080 ± 735 a	1760 ± 326 b	8906 ± 28 a	4287 ± 49 b
C6/LA aldehyde	513 ± 448 a	97 ± 9 b	455 ± 18 a	103 ± 2 b	941 ± 112 a	31 ± 21 b	347 ± 18 a	76 ± 2 b
C6/LA alcohol	8 ± 0 a	7 ± 1 b	39 ± 1 a	39 ± 1 a	25 ± 1 a	13 ± 3 b	85 ± 3 a	85 ± 2 a
∑ C6/LA	779 ± 81 a	104 ± 9 b	494 ± 19 a	142 ± 2 b	967 ± 112 a	44 ± 24 b	432 ± 18 a	161 ± 3 b
C5/LnA carbonyls	694 ± 105 ^{a,b}	587 ± 58 a	254 ± 1 a	243 ± 3 a	1178 ± 95 a	581 ± 35 b	661 ± 3 a	390 ± 6 b
C5/LnA alcohols	1108 ± 64 a	325 ± 30 b	424 ± 4 a	289 ± 4 b	1467 ± 100 a	283 ± 20 b	840 ± 14 a	508 ± 7 b
PD	8517 ± 170 a	7860 ± 61 b	5621 ± 70 a	6248 ± 66 b	5482 ± 96 a	2985 ± 111 b	5459 ± 192 a	4466 ± 111 b
∑ C5/LnA	10319 ± 296 a	8772 ± 66 b	6298 ± 75 a	6780 ± 67 b	8127 ± 278 a	3849 ± 165 b	6960 ± 205 a	5364 ± 124 b
C5/LA carbonyls	36 ± 4 a	109 ± 11 b	14 ± 1 a	34 ± 1 b	40 ± 4 a	78 ± 20 b	58 ± 4 a	66 ± 3 b
C5/LA alcohol	6 ± 1 a	10 ± 1 b	18 ± 1 a	7 ± 1 b	22 ± 4 a	14 ± 4 b	23 ± 2 a	11 ± 1 b
∑ C5/LA	42 ± 5 a	120 ± 9 b	32 ± 1 a	40 ± 2 b	62 ± 4 a	93 ± 24 b	81 ± 2 a	78 ± 4 a
LOX esters	24 ± 4 a	24 ± 5 a	30 ± 1 a	38 ± 1 b	24 ± 2 a	29 ± 5 a	125 ± 4 a	172 ± 5 b
non-LOX esters	137 ± 20 a	67 ± 6 b	119 ± 1 a	104 ± 6 b	116 ± 57 a	8 ± 3 b	51 ± 5 a	29 ± 2 b
∑ esters	161 ± 16 a	96 ± 10 b	149 ± 1 a	142 ± 6 a	172 ± 13 a	37 ± 6 b	176 ± 8 a	201 ± 7 b
total volatiles	25888 ± 978 a	12095 ± 579 b	29961 ± 478 a	22886 ± 235 b	16407 ± 1142 a	5783 ± 544 b	16555 ± 261 a	10091 ± 188 b

^aMean 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 ($P \leq 0.05$). ^bEach class of volatile compound comprises compounds listed in Table 1 and under Materials and Methods.

Table 5. Volatile Contents^a (Nanograms per Gram Olive Oil) of the Oils Obtained by the Addition of Phenidone (32 mg/g Fruit) during the Oil Extraction Process of Arbequina and Picual Fruits at Different Maturity Stages (MI 1 and 5)

volatile compound class ^b	Arbequina				Picual			
	MI 1		MI 5		MI 1		MI 5	
	control	+ phenidone	control	+ phenidone	control	+ phenidone	control	+ phenidone
C6/LnA aldehydes	14283 ± 539 a	3647 ± 460 b	22737 ± 1112 a	11730 ± 93 b	7614 ± 250 a	2256 ± 60 b	8441 ± 44 a	2561 ± 75 b
C6/LnA alcohols	306 ± 41 a	45 ± 3 b	253 ± 20 a	138 ± 2 b	157 ± 2 a	169 ± 10 b	466 ± 26 a	405 ± 23 b
∑ C6/LnA	14588 ± 579 a	3692 ± 463 b	22990 ± 1097 a	11868 ± 91 b	7771 ± 251 a	2425 ± 59 b	8907 ± 30 a	3278 ± 65 b
C6/LA aldehyde	704 ± 7 a	346 ± 7 b	462 ± 18 a	139 ± 13 b	941 ± 112 a	72 ± 41 b	347 ± 18 a	110 ± 8 b
C6/LA alcohol	8 ± 0 a	31 ± 1 b	39 ± 1 a	63 ± 1 b	25 ± 1 a	26 ± 1 a	85 ± 3 a	110 ± 4 b
∑ C6/LA	712 ± 8 a	377 ± 8 b	501 ± 18 a	202 ± 13 b	967 ± 112 a	98 ± 42 b	432 ± 18 a	219 ± 8 b
C5/LnA carbonyls	694 ± 105 a	211 ± 4 b	255 ± 2 a	66 ± 3 b	1178 ± 95 a	172 ± 31 b	661 ± 3 a	124 ± 6 b
C5/LnA alcohols	1108 ± 64 a	138 ± 13 b	435 ± 19 a	103 ± 3 b	1467 ± 100 a	62 ± 12 b	840 ± 14 a	349 ± 7 b
PD	8517 ± 170 a	247 ± 15 b	4652 ± 73 a	90 ± 4 b	5482 ± 96 a	79 ± 38 b	5459 ± 192 a	344 ± 22 b
∑ C5/LnA	10319 ± 296 a	596 ± 3 b	5342 ± 92 a	259 ± 8 b	8127 ± 278 a	313 ± 28 b	6960 ± 205 a	816 ± 26 b
C5/LA carbonyls	36 ± 4 a	1643 ± 45 b	14 ± 1 a	86 ± 12 b	40 ± 4 a	1941 ± 345 b	58 ± 4 a	366 ± 11 b
C5/LA alcohol	6 ± 1 a	304 ± 5 b	18 ± 1 a	18 ± 2 a	22 ± 4 a	407 ± 33 b	23 ± 2 a	57 ± 5 b
∑ C5/LA	42 ± 5 a	1947 ± 46 b	32 ± 1 a	103 ± 13 b	62 ± 4 a	2349 ± 374 b	81 ± 2 a	425 ± 14 b
LOX esters	26 ± 2 a	47 ± 2 b	30 ± 1 a	58 ± 3 b	24 ± 2 a	40 ± 4 b	129 ± 8 a	147 ± 5 b
non-LOX esters	211 ± 10 a	296 ± 179 a	119 ± 1 a	106 ± 6 a	133 ± 23 a	181 ± 25 b	51 ± 5 a	40 ± 3 b
∑ esters	237 ± 8 a	343 ± 181 a	148 ± 1 a	163 ± 4 b	157 ± 22 a	221 ± 27 b	180 ± 6 a	187 ± 3 a
total volatiles	25897 ± 896 a	6955 ± 306 b	29014 ± 1209 a	12595 ± 129 b	17083 ± 667 a	5406 ± 530 b	16560 ± 261 a	4925 ± 116 b

^aMean 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 ($P \leq 0.05$). ^bEach class of volatile compound comprises compounds listed in Table 1 and under Materials and Methods.

Table 6. Volatile Contents^a (Nanograms per Gram Olive Oil) of the Oils Obtained by the Addition of Phenylbutazone (32 mg/g Fruit) during the Oil Extraction Process of Arbequina and Picual Fruits at Different Maturity Stages (MI 1 and 5)

volatile compound class ^b	Arbequina				Picual			
	MI 1		MI 5		MI 1		MI 5	
	control	+ phenylbutazone	control	+ phenylbutazone	control	+ phenylbutazone	control	+ phenylbutazone
C6/LnA aldehydes	13896 ± 654 a	9154 ± 246 b	23205 ± 1075 a	19561 ± 606 b	5338 ± 290 a	2290 ± 148 b	954 ± 116 a	313 ± 51 b
C6/LnA alcohols	199 ± 9 a	127 ± 2 b	252 ± 29 a	189 ± 13 a	84 ± 2 a	71 ± 1 b	50 ± 5 a	50 ± 4 a
∑ C6/LnA	14096 ± 645 a	9281 ± 249 b	23458 ± 1047 a	19749 ± 619 b	5423 ± 291 a	2361 ± 148 b	1005 ± 121 a	363 ± 55 b
C6/LA aldehyde	660 ± 29 a	266 ± 23 b	458 ± 24 a	361 ± 24 b	786 ± 53 a	247 ± 6 b	275 ± 6 a	79 ± 7 b
C6/LA alcohol	16 ± 0 a	9 ± 1 b	39 ± 0 a	32 ± 3 a	11 ± 1 a	9 ± 1 b	27 ± 2 a	29 ± 1 a
∑ C6/LA	676 ± 29 a	275 ± 23 b	497 ± 24 a	392 ± 24 b	797 ± 53 a	257 ± 5 b	303 ± 8 a	108 ± 8 b
C5/LnA carbonyls	364 ± 10 a	332 ± 1 b	255 ± 3 a	215 ± 5 b	728 ± 18 a	432 ± 10 b	124 ± 2 a	71 ± 2 b
C5/LnA alcohols	830 ± 36 a	789 ± 6 a	439 ± 25 a	474 ± 23 a	632 ± 18 a	564 ± 16 b	500 ± 12 a	504 ± 10 a
PD	7838 ± 6 a	7546 ± 177 a	4643 ± 101 a	5099 ± 338 a	3187 ± 103 a	2555 ± 6 b	1152 ± 46 a	734 ± 15 b
∑ C5/LnA	9032 ± 52 a	8667 ± 184 a	5338 ± 130 a	5788 ± 363 a	4547 ± 95 a	3551 ± 18 b	1777 ± 60 a	1311 ± 27 b
C5/LA carbonyls	22 ± 3 a	25 ± 1 a	15 ± 0 a	59 ± 4 b	23 ± 1 a	42 ± 2 b	29 ± 1 a	34 ± 1 b
C5/LA alcohol	1 ± 0 a	3 ± 0 b	18 ± 0 a	58 ± 13 b	36 ± 3 ab	29 ± 2 b	11 ± 1 a	13 ± 1 a
∑ C5/LA	23 ± 3 a	29 ± 1 a	33 ± 1 a	117 ± 13 b	60 ± 4 a	72 ± 2 b	41 ± 1 a	48 ± 3 b
LOX esters	40 ± 16 a	30 ± 1 a	30 ± 1 a	36 ± 2 a	41 ± 7 a	42 ± 1 a	382 ± 11 a	401 ± 17 a
non-LOX esters	59 ± 0 a	42 ± 2 b	118 ± 1 a	112 ± 6 a	160 ± 10 a	41 ± 3 b	67 ± 1 a	98 ± 3 b
∑ esters	99 ± 16 a	72 ± 3 b	148 ± 2 a	148 ± 5 a	201 ± 12 a	84 ± 2 b	450 ± 11 a	500 ± 21 b
total volatiles	23926 ± 745 a	18323 ± 460 b	29474 ± 1203 a	26194 ± 1024 b	11029 ± 455 a	6326 ± 177 b	3577 ± 203 a	2332 ± 116 b

^aMean 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 ($P \leq 0.05$). ^bEach class of volatile compound comprises compounds listed in Table 1 and under Materials and Methods.

experienced a decline when exogenous LOX activity increased during the oil extraction process from fruits from the two cultivars and ripening stages under study, reaching around a 30% decrease for the highest LOX dose. This decrease is observed for all classes of compounds within the C5/LnA compounds except for the minor C5/LnA carbonyls, which, in general, increased as the LOX dose increase. There are no experimental clues to explain the decrease in the content of C5 compounds, but it might be related to differences between olive LOX and exogenous LOX in the C5 synthesizing homolytic activity, taking into account that both compete for the same substrates. The other minor groups of volatile compounds, C5/LA and esters, did not show precise trends when LOX doses were increased.

A similar effect was observed in general when LOX activity load was increased during the kneading step of the oil extraction process. Table 3 shows the contents of volatile compounds in the oils extracted after the addition of the highest dose of exogenous LOX activity used above (20 U LOX/g olive paste) during the olive paste kneading. Again, the effect of increasing the level of the LOX activity load gave rise to different responses depending on the olive cultivar. The increase in LOX activity at the beginning of the kneading step causes a significant increase (average 42%) in the content of C6/LnA compounds in the oils from Picual fruits, regardless their ripening stage. However, the addition of exogenous LOX at the beginning of the kneading step of Arbequina fruit pastes only promoted a slight but significant increase ($P \leq 0.05$) in the oils obtained from fruits at MI 1 (6%). Moreover, whereas the contents of C5/LnA compounds in the oils from Arbequina fruits, at either of the ripening stages under study, did not change significantly, a decrease was observed in the case of Picual oils, being higher when obtained from fruits at MI 5 (30%) than from fruits at MI 1 (15%). The other groups of volatile compounds displayed no major changes. These data suggest again a greater limitation of the LOX activity load over the whole oil extraction process from Picual fruits than from Arbequina fruits. Besides, the fact that increasing the LOX activity load during the kneading step of Picual fruits produced an increase in the volatile compound synthesis suggests that there is still substrate available for this synthesis. However, the almost complete lack of effect on the content of oil volatile compounds in Arbequina suggests a depletion of substrate at the beginning of the kneading step to be catabolized by the LOX activity. These results will support the above-mentioned previous findings¹⁹ suggesting that substrate availability for LOX activity is comparatively lower in Arbequina than in Picual fruits.

To further confirm these findings, the effect of reducing the LOX activity load during the VOO extraction process on the synthesis of oil volatile compounds was assessed. For this purpose, different specific LOX inhibitors (octyl gallate, phenidone, and phenylbutazone) were added during the oil extraction process. Octyl gallate is a potent competitive inhibitor of LOX activity,²⁶ and it is currently permitted for use as an antioxidant additive in food.²⁷ Ruddat et al.²⁸ stated that the inhibition caused by octyl gallate is unlikely due to its capability of scavenging polyunsaturated fatty acid derived free radicals because these free radical intermediates that are formed during the catalytic cycle of LOX remain tightly bound at the active site and, thus, are not accessible for free radical scavengers. As shown in Table 4, oils obtained from Picual fruits displayed, in general, a greater reduction of the content of

volatile compounds than those from Arbequina fruits as a consequence of the presence of the octyl gallate during the oil extraction process, the oils obtained from fruits at MI 1 being the most affected. Thus, theoretical half-maximal inhibitory concentrations (IC_{50}) calculated for total volatile synthesis were found to be 20 and 26 mg/g fruit at MI 1 for Picual and Arbequina, respectively, whereas IC_{50} values were 39 and 77 mg/g fruit at MI 5 for Picual and Arbequina, respectively. The contents of C6 aldehydes and C5 alcohols, direct products of the LOX/HPL enzymatic system, are the most affected as a result of the addition of octyl gallate during the milling of olive fruits. The contents of volatile compounds of the highest sensory importance in VOO, C6/LnA, were reduced up to an average 77% in both cultivars at MI 1 as a consequence of the presence of octyl gallate during the milling of the fruit. However, oils obtained from fruits at MI 5 displayed reductions of 52 and 31% of the contents of C6/LnA compounds in cultivars Picual and Arbequina, respectively. The other groups of volatile compounds, C5 compounds and esters, have no clear trend, although the greatest reductions in the synthesis of these classes of compounds were always found in cultivar Picual.

Similar experiments to reduce LOX activity load during the oil extraction process were carried out using the specific LOX inhibitors phenidone and phenylbutazone. These potent LOX inhibitors are not used in the food industry, but they are currently employed in anti-inflammatory drug therapy due to their dual cyclooxygenase and LOX inhibitor characteristics.^{29,30} Tables 5 and 6 show the results of the addition of these inhibitors during the milling of fruits of the cultivars and ripening stages under study. In general, the modification of the contents of the oil volatile compounds showed a trend similar to those found when using octyl gallate. The degree of inhibition caused by phenidone (Table 5) on the synthesis of C6/LnA compounds was quite similar to that of octyl gallate, >70% in both cultivars at MI 1 and 63 and 48% in cultivars Picual and Arbequina at MI 5, respectively. On the other hand, oils obtained from Picual fruits displayed, in general, a greater reduction of the content of total volatile compounds than those from Arbequina fruits as a consequence of the presence of phenylbutazone during the oil extraction process, the oils obtained from fruits at MI 1 being again the most affected. As observed in Table 6, the degree of inhibition caused by phenylbutazone on the synthesis of volatile compounds was, in general, and particularly on C6/LnA compounds, significantly lower than that caused by octyl gallate or phenidone. Data obtained in experiments with inhibitors showed a higher modification of the synthesis of VOO volatile compounds in cultivar Picual than in Arbequina, so that they point again to an apparent greater limitation of LOX activity in fruits of cultivar Picual than in cultivar Arbequina.

The inhibition characteristics seem to be different depending on the inhibitor as shown when the effect on the synthesis of each class of volatile compounds is examined. For instance, phenidone reduces by >90% the synthesis of C5/LnA compounds, especially of the PD compounds, whereas octyl gallate and phenylbutazone showed inhibition percentages lower than those observed for the synthesis of C6/LnA compounds. Besides, phenidone strongly enhanced the synthesis of C5/LA compounds, especially in the milling of olive fruits at MI 1 from both cultivars, whereas the other two inhibitors showed different trends depending on the cultivar and ripening stage. The reason for these different inhibition characteristics might be related to the different capacities of

scavenging intermediate free radicals formed during the catalytic cycle of LOX.

The addition of LOX inhibitors during the kneading step in the oil extraction process also led to a reduction of the contents of oil volatile compounds (Tables A and B in the Supporting Information). The pattern of the synthesis inhibition for each group of volatile compounds showed a trend similar to that observed when the addition was carried out during the milling step, but the reduction of the contents of the oil volatile compounds was lower. Data showed that octyl gallate gives rise to an average 40% decrease in the synthesis of C6/LnA compounds in both cultivars, whereas the average decrease is higher in Picual than in Arbequina cultivar when using phenidone and, especially, phenylbutazone. Again, experimental data point to a higher limitation of LOX activity in cultivar Picual than in Arbequina. On the other hand, the fact that there is an inhibition of the synthesis of volatile compounds when the inhibitor is present during the kneading step indicates that there is some LOX activity in the recently obtained olive paste, although probably it is lost rapidly when advancing the paste kneading. Thus, volatile compound synthesis might be still feasible during this step in the oil extraction process. Thus, results are compatible with the assumption that most of the volatile compounds produced through the LOX pathway are synthesized in the milling step of the VOO extraction process, as suggested earlier.³¹ In this sense, Angerosa et al.¹⁴ 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 kneading step. The reasons for this apparent low rate of volatile compound synthesis during the kneading step remain unclear, but our experimental data suggest that it is not entirely associated with a shortage of substrates for the LOX pathway, occurring as a consequence of the depletion of these substrates during the previous milling step as previously suggested,³¹ but more likely with a deactivation of the LOX activity by components in the olive paste, probably oxidized phenolics arising during the milling step. The role of olive polyphenol oxidase and peroxidase acting as major factors oxidizing phenolics during VOO production has been reported.³² This inactivating role of oxidized phenolics on enzymatic activity is well-established^{24,25} and can contribute to reduce the effective enzyme activity load during the oil extraction process.

In summary, there is evidence that the LOX activity level in the fruit is a limiting factor for the synthesis of the oil volatile fraction, this limitation being significantly higher in olive cultivar Picual than in Arbequina, in line with the lowest content of volatile compounds in the oils being obtained from Picual fruits. Moreover, experimental data suggest that this limitation of LOX activity takes place mainly during the milling step in the process of olive oil extraction because most of the VOO volatile compounds seem to be synthesized during this step.

■ ASSOCIATED CONTENT

📄 Supporting Information

Volatile contents of the oils obtained by addition of different inhibitors (32 mg/g paste) during the kneading of Arbequina (Table A) and Picual (Table B) fruit pastes at different maturity stages (MI 1 and 5). This material is available free of charge via the Internet at <http://pubs.acs.org>.

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