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Factors Limiting the Synthesis of Virgin Olive Oil Volatile Esters

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Supporting Information

ABSTRACT: The aim of the present work was to establish the limiting factors affecting the biosynthesis of volatile esters present in virgin olive oil (VOO). Oil volatile fractions of the main Spanish olive cultivars, Arbequina and Picual, were analyzed. It was observed that acetate esters were the most abundant class of volatile esters in the oils, in concordance with the high content of acetyl-CoA found in olive fruit, and that the content of C6 alcohols is limited for the synthesis of volatile esters during the production of VOO. Thus, the increase of C6 alcohol availability during VOO production produced a significant increase of the corresponding ester in the oils in both cultivars at two different maturity stages. However, the increase of acetyl-CoA availability had no effect on the VOO volatile fraction. The low synthesis of these C6 alcohols seems not to be due to a shortage of precursors or cofactors for alcohol dehydrogenase (ADH) activity because their increase during VOO production had no effect on the C6 alcohol levels. The experimental findings are compatible with a deactivation of ADH activity during olive oil production in the cultivars under study. In this sense, a strong inhibition of olive ADH activity by compounds present in the different tissues of olive fruit has been observed.

KEYWORDS: *Virgin olive oil, olive fruit, aroma, volatile, ester*

INTRODUCTION

Olive oil, one of the main components of the Mediterranean diet, is related to protection against cardiovascular diseases and cancer because of its fatty acid profile and the presence of minor amounts of phenolic constituents.[1,2] A large increase in the demand for high-quality virgin olive oil during recent years can be attributed to not only its potential health benefits but also its excellent organoleptic properties. The aim of increasing the quality standards for virgin olive oil (VOO) is continuously stimulating the study of biochemical pathways related to organoleptic properties and the development of technological procedures to improve those organoleptic properties. In this context, our group established the participation of the lipoxygenase (LOX) pathway in the biosynthesis of compounds of six straight-chain carbons (C6 compounds) in olive oil aroma.[3] 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.[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 (ADHs) to form C6 alcohols[3,8] and can finally be esterified with carboylic acids in the form of acyl-CoA derivatives, a reaction catalyzed by alcohol acyltransferase (AAT).[3,9]

Volatile esters, which are important constituents of the aroma of many fruits,[10] are minor components of the aroma of oils from most olive cultivars. The VOO ester fraction is formed mainly by acetate esters with alcohol moieties synthesized through the LOX pathway, such as hexan-1-ol, (*E*)-hex-2-enol, and (*Z*)-hex-3-enol. These alcohols, especially the unsaturated alcohols, and their corresponding esters are grouped among those VOO aroma compounds with green-fruity perceptions and in the boundaries of the sweet perception according to canonical correlations reported by Aparicio et al.[11] Some of them are among the most potent odorants in VOO, characterized by having high odor activity values.[12]

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.[13] This radical would undergo subsequent non-enzymatic β-scission in a homolytic way to form a 1,3-pentene allylic radical that could be chemically dimerized to form pentene dimers or react with a hydroxyl radical to form C5 alcohols. The latter would be the origin of C5 carbonyl compounds present in the aroma of olive oil through an enzymatic oxidation by ADH, as suggested to occur in soybean leaves.[14]

There are many different factors, either biochemical or technological, that can modify the activity level of enzymes of the LOX pathway during the industrial process to obtain the

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oil. In this context, there are quite a number of studies describing the way technological procedures affect the VOO aroma compound profile or the biosynthetic pathway determining this profile.[15−20] However, studies devoted toward identifying limiting factors for the biosynthesis of VOO aroma compounds are scarce. Considering the low level of esters present in the VOO volatile fraction of the most important Spanish olive cultivars (Arbequina and Picual) and their high sensorial significance, the aim of the present work was to establish the limiting factors affecting the biosynthesis of volatile esters present in VOO.

MATERIALS AND METHODS

Chemicals and Reagents. Compounds used for volatile identification and substrate and cofactor addition experiments as well as yeast ADH were supplied by Sigma-Aldrich (St. Louis, MO), except for (*Z*)-hex-3-enyl acetate purchased from Givaudan Co. (Clifton, NJ) and (*Z*)-hex-3-enal generously supplied by S.A. Perlarom (Louvaine-La-Neuve, Belgium).

Plant Material. Olive fruits (*Olea europaea* L.), cultivars Picual and Arbequina, were harvested at the 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). Upon each sampling day, around 6 kg of olive fruits at the desired maturity stage was sampled from 12 trees per cultivar and oil was extracted using an Abencor analyzer in 1 kg batches. Malaxation was carried out for 30 min with the Abencor thermobeater operating at 30 °C. 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 and stored at −18 °C until analysis.

When indicated, a fruit homogenate approach was followed. 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 (24 000 rpm) for 1 min. After an equilibrium period of 5 min at 25 °C, 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 −18 °C until analysis.

Modification of Metabolic Factor Availability during Oil Processing. To increase the availability of enzymatic activity or substrates for ADH and AAT activities during processing, different amounts of yeast ADH or C6 or C5 compounds were added to the olive fruit during the milling step in the process of VOO extraction or to fruit mesocarp homogenization in the case of the pyridine nucleotides and acetyl-CoA. Duplicate experiments were carried out for each cultivar and maturity index.

ADH Extraction and Activity Measurement. Acetone powders were prepared from mesocarp and seed tissues of fresh harvested olive fruits. Typically, 10 g of tissue was ground in 150 mL of cold acetone (−20 °C) using a Waring blender. The residue obtained, after filtration, was re-extracted twice with 20 mL of cold acetone (−20 °C). The whitish powder obtained was finally rinsed with diethyl ether, dried, and stored at −20 °C. ADH enzyme extracts were prepared according to Salas and Sanchez[8] from 0.1 g of acetone powder in 3 mL of a buffer consisting of 0.5 M sodium phosphate buffer at pH 7.2, 7 mM mercaptoethanol, 0.5 M dithiothreitol (DTT), and 10% glycerol using an Ultraturrax homogenizer. The resulting homogenate was centrifuged at 27000*g* for 20 min at 4 °C, and the clear supernatant was used as a crude extract. ADH activity was measured by monitorization of reduced nicotinamide adenine dinucleotide (NADH) oxidation at 338 nm using acetaldehyde as the substrate. A total of 1 unit of ADH activity is defined as the amount of enzyme oxidizing 1 μmol of NADH/min at 25 °C, taking into account a molar extinction coefficient of 6160 M^{-1} cm^{-1} for this compound.

Crude aqueous extracts from fresh fruit mesocarp and seed tissues were prepared by homogenization with distilled water (2 mL/g of fresh tissue) and centrifugation at 27000*g* for 20 min. Supernatants were heated to 100 °C for 1 h and, after centrifugation, constituted the thermally inactivated crude extracts.

Analysis of Acyl-CoAs. Mesocarp and seed tissues from olive fruit and pastes generated in the process to obtain VOO were frozen in liquid nitrogen and lyophilized. Acyl-CoAs were extracted as described by Larson and Graham[21] from 10 mg of lyophilized material. Acyl-CoA extracts were reconstituted in 40 mL of chloroacetaldehyde-derivitizing reagent and analyzed by reversed-phase high-performance liquid chromatography (HPLC) using a quaternary gradient system and a fluorescence detector described by Larson et al.[22] Quantification was carried out by reference to response factors calculated from separately injected standard mixtures and known concentrations of internal standards (heptanoyl and heptadecanoyl-CoA; Sigma) included in the sample extracts. Quintuplicate experiments were carried out for each cultivar and maturity index.

Analysis of Volatile Compounds. Olive oil and homogenate 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 solid-phase microextraction (SPME) fiber DVB/Carboxen/PDMS 50/30 μm (Supelco Co., Bellefonte, PA) according to Luaces et al.[19] The sampling time was 50 min at 40 °C, and it was carried out in triplicate. Desorption of volatile compounds trapped in the SPME fiber was performed directly into the gas chromatograph (GC) injector. Volatiles were analyzed using a HP-6890 GC equipped with a DB-Wax capillary column (60 m × 0.25 mm inner diameter; film thickness, 0.25 μm; J&W Scientific, Folsom, CA). Operating conditions were as follows: N_2 as the carrier gas; injector and detector at 250 °C; and column held for 6 min at 40 °C and then programmed at 2 °C min^{-1} to 128 °C (see Figure A in the Supporting Information). Quantification was performed using individual calibration curves for each identified compound in each matrix (olive oil and olive mesocarp homogenate). In some cases, 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 is the sum of the content of the following compounds:

C6/LnA aldehydes: (*E*)-hex-2-enal, (*Z*)-hex-3-enal, (*Z*)-hex-2-enal, and (*E*)-hex-3-enal.
C6/LnA alcohols: (*E*)-hex-2-enol, (*Z*)-hex-3-enol, and (*E*)-hex-3-enol.
C6/LA aldehyde: hexanal.
C6/LA alcohol: hexan-1-ol.
C5/LnA carbonyls: pent-1-en-3-one, (*E*)-pent-2-enal, and (*Z*)-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: pentene dimers.
C5/LA carbonyls: pentan-3-one, pentan-2-one, and pentanal.
C5/LA alcohol: pentanol.
LOX esters: hexyl acetate and (*E*)-hex-2-en-1-yl acetate.
Non-LOX esters: methyl acetate, ethyl acetate, methyl hexanoate, and ethyl hexanoate.

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

RESULTS AND DISCUSSION

Analysis of the composition of the VOO volatile fraction obtained from the main Spanish olive cultivars Arbequina and Picual revealed low levels of C6 alcohols compared to their metabolic precursors, the C6 aldehydes (Table 1). This observation highlights the possibility of a bottleneck in the LOX pathway for synthesis of esters of sensorial significance (LOX esters in Table 1).

Table 1. Average Contents of Volatile Compounds[a] (ng/g of Oil) in Olive Oils from Arbequina and Picual Fruits in Four Seasons (2006−2009)

volatile class	Arbequina	Picual
C6/LnA aldehydes	18824	7954
C6/LnA alcohols	421	402
∑C6/LnA	19245	8358
C6/LA aldehyde	713	463
C6/LA alcohol	132	73
∑C6/LA	843	538
C5/LnA carbonyls	403	593
C5/LnA alcohols	573	723
PD	5351	4656
∑C5/LnA	6327	5972
C5/LA carbonyls	35	48
C5/LA alcohol	14	12
∑C5/LA	49	59
LOX esters	110	128
non-LOX esters	143	75
∑esters	253	204
total volatiles	26716	15128

[a]Data are the mean value from three determinations. The average coefficient of variance was 18 and 34% for Arbequina and Picual oils, respectively. Compounds are clustered into different volatile classes according to the polyunsaturated fatty acid and the LOX pathway branch origin.

AAT is the LOX pathway enzyme responsible for synthesis of esters by condensing an acyl-CoA and an alcohol. A study of the possible limitations concerning substrates for AAT activity in olive fruit has been carried out by increasing the availability of C6 alcohols and acyl-CoAs during the synthesis of the oil volatile fraction in Arbequina and Picual fruits at two maturity stages (MI 1 and MI 5). To this end, first, the composition and content of acyl-CoAs in the main fruit tissues, mesocarp and seed, and in olive fruit pastes arising during VOO production were assessed. Tables 2−4 display the acyl-CoA contents in olive fruit mesocarp and seed and in olive pastes arising during the oil extraction process. As shown, among the long-chain acyl-CoAs, the major proportions were those of palmitoyl-CoA (16:0-CoA) and oleyl-CoA (18:1-CoA), in concordance with the highest proportion of their corresponding fatty acids in olive fruit and VOO triacylglycerides. Acetyl-CoA (2:0-CoA) is the main short-chain acyl-CoA present in fruit mesocarp and paste, resulting from the milling of the fruit. This would justify that VOO volatile ester fraction has, in general, a higher content of acetate esters derived from acetyl-CoA (Tables 5−7). On the other hand, the hexanoates derived from hexanoyl-CoA (6:0-CoA) form the second volatile ester group from a quantitative point of view. Although hexanoyl-CoA is present in fruit mesocarp in a low proportion, this compound was not detected in olive pastes. These results suggest their complete catabolism during the fruit milling and paste kneading steps in the process of VOO production, suggesting a greater specificity of olive AAT for hexanoyl-CoA than for acetyl-CoA, in agreement with what was found for AAT enzyme activities in other fruit, such as strawberry.[23]

Because acetyl-CoA is the most abundant short-chain acyl-CoA in olive fruit and the acetates are the most important volatile esters in VOO from a quantitative point of view, this compound was selected to study the limitation of acyl-CoA for the synthesis of volatile esters present in VOO. Thus, the availability of acetyl-CoA for ester synthesis was increased using the homogenization approach of fruit mesocarp from Arbequina and Picual cultivars. As shown in Table 5, the increase of the acetyl-CoA content did not produce any significant change in the content of the main esters found in olive mesocarp homogenates, either with or without a LOX

Table 2. Composition (%) and Total Content (fmol/mg of Dry Weight)[a] of Acyl-CoA in Olive Fruit Mesocarp of Cultivars Picual and Arbequina at Different Maturity Stages (MI 1 and MI 5)

	Arbequina		Picual	
Acyl-CoA	MI 1	MI 5	MI 1	MI 5
2:0	31.1 ± 2.7	27.2 ± 1.9	26.3 ± 6.0	28.3 ± 4.4
4:0	0.4 ± 0.1	1.1 ± 0.1	nd[b]	0.9 ± 0.5
5:0	0.3 ± 0.1	0.7 ± 0.2	nd	0.6 ± 0.4
6:0	0.0 ± 0.0	0.1 ± 0.1	0.4 ± 0.1	0.1 ± 0.1
8:0	0.4 ± 0.2	0.6 ± 0.3	0.7 ± 0.3	0.3 ± 0.2
10:0	nd	nd	nd	nd
12:0	nd	nd	nd	nd
14:0	1.6 ± 0.1	1.1 ± 0.1	1.6 ± 0.1	1.1 ± 0.1
16:0	27.8 ± 0.9	18.6 ± 0.8	17.3 ± 1.7	18.3 ± 1.0
16:1	4.8 ± 0.4	5.2 ± 0.2	2.5 ± 0.2	5.1 ± 0.5
18:0	1.4 ± 0.1	5.1 ± 0.2	1.2 ± 0.1	4.9 ± 0.2
18:1	22.4 ± 2.4	33.3 ± 1.2	42.2 ± 3.2	33.4 ± 2.5
18:2	5.2 ± 0.2	4.8 ± 0.2	3.1 ± 0.2	4.7 ± 0.2
18:3	0.2 ± 0.0	0.6 ± 0.1	0.5 ± 0.4	0.6 ± 0.2
20:0	0.7 ± 0.1	0.9 ± 0.0	0.5 ± 0.1	0.9 ± 0.1
20:1	nd	nd	nd	nd
20:2	nd	nd	nd	nd
20:3	nd	nd	nd	nd
22:0	1.7 ± 0.1	0.4 ± 0.0	2.4 ± 0.3	0.4 ± 0.2
22:1	1.9 ± 0.2	0.3 ± 0.3	1.9 ± 0.3	0.4 ± 0.4
total content (fmol/mg of dry weight)	2787.4 ± 54.3	4444.8 ± 111.3	2270.1 ± 54.3	4252.1 ± 240.8

[a]Data represent the mean value ± standard deviation from five determinations. [b]nd = not detected.

Table 3. Composition (%) and Total Content (fmol/mg of Dry Weight)[a] of Acyl-CoA in Olive Fruit Seeds of Cultivars Picual and Arbequina at Different Maturity Stages (MI 1 and MI 5)

	Arbequina		Picual	
Acyl-CoA	MI 1	MI 5	MI 1	MI 5
2:0	nd[b]	nd	nd	nd
4:0	nd	nd	nd	nd
5:0	nd	nd	nd	nd
6:0	nd	nd	nd	nd
8:0	nd	nd	nd	nd
10:0	nd	nd	nd	nd
12:0	nd	nd	nd	nd
14:0	8.4 ± 0.7	2.9 ± 0.7	6.4 ± 1.0	4.1 ± 0.6
16:0	15.2 ± 1.1	19.3 ± 1.8	17.7 ± 1.6	21.8 ± 0.4
16:1	nd	1.3 ± 0.2	0.8 ± 1.2	2.4 ± 1.0
18:0	5.3 ± 0.7	5.5 ± 0.7	6.2 ± 0.5	6.0 ± 0.6
18:1	35.2 ± 5.6	46.7 ± 1.5	28.1 ± 5.1	38.1 ± 2.0
18:2	11.7 ± 2.8	12.8 ± 0.9	9.7 ± 2.2	12.6 ± 3.2
18:3	nd	nd	nd	nd
20:0	7.9 ± 1.1	4.5 ± 0.5	6.4 ± 1.1	4.8 ± 0.8
20:1	nd	1.3 ± 0.2	2.2 ± 0.7	1.2 ± 0.3
20:2	nd	nd	nd	nd
20:3	nd	nd	nd	nd
22:0	16.2 ± 8.2	5.6 ± 3.1	22.4 ± 2.5	8.8 ± 1.1
22:1	nd	nd	nd	nd
total content (fmol/mg of dry weight)	149.8 ± 32.2	491.7 ± 36.7	262.6 ± 41.8	407.6 ± 41.3

[a]Data represent the mean value ± standard deviation from five determinations. [b]nd = not detected.

Table 4. Composition (%) and Total Content (fmol/mg of Dry Weight)[a] of Acyl-CoA in Olive Fruit Pastes of Cultivars Picual and Arbequina at Different Maturity Stages (MI 1 and MI 5)

	Arbequina		Picual	
Acyl-CoA	MI 1	MI 5	MI 1	MI 5
2:0	24.8 ± 3.0	18.2 ± 2.0	26.8 ± 2.5	17.2 ± 2.7
4:0	nd[b]	0.5 ± 0.1	nd	0.4 ± 0.1
5:0	nd	nd	nd	0.4 ± 0.1
6:0	nd	nd	nd	nd
8:0	nd	nd	nd	0.4 ± 0.1
10:0	nd	nd	nd	nd
12:0	nd	nd	nd	nd
14:0	1.0 ± 0.3	0.6 ± 0.2	1.1 ± 0.0	1.1 ± 0.0
16:0	21.9 ± 1.0	14.0 ± 2.2	26.6 ± 4.0	22.3 ± 1.0
16:1	1.7 ± 0.1	2.0 ± 0.2	3.2 ± 0.2	4.4 ± 0.2
18:0	1.6 ± 0.1	3.7 ± 0.1	1.7 ± 0.2	4.1 ± 0.3
18:1	43.7 ± 2.2	57.2 ± 1.4	32.7 ± 2.4	45.0 ± 1.7
18:2	1.6 ± 0.1	1.6 ± 0.1	4.0 ± 0.1	2.3 ± 0.2
18:3	nd	nd	nd	nd
20:0	0.9 ± 0.1	1.0 ± 0.1	1.0 ± 0.1	0.9 ± 0.0
20:1	nd	nd	nd	nd
20:2	nd	nd	nd	nd
20:3	nd	nd	nd	nd
22:0	1.9 ± 0.3	1.0 ± 0.0	1.4 ± 0.3	1.0 ± 0.1
22:1	0.8 ± 0.0	0.4 ± 0.1	1.5 ± 0.2	0.5 ± 0.1
total content (fmol/mg of dry weight)	3843.7 ± 411.0	4345.1 ± 222.1	4077.9 ± 125.4	5391.8 ± 249.8

[a]Data represent the mean value ± standard deviation from five determinations. [b]nd = not detected.

pathway origin. Therefore, the acyl-CoA content does not seem to be a limiting factor for ester synthesis during VOO production.

The availability of C6 alcohols, either saturated or unsaturated, was also modified during VOO production to assess whether the content of C6 alcohols is a limiting factor for the synthesis of the most relevant esters in VOO from a sensorial point of view. As shown in Tables 6 and 7, the increase of (*E*)-hex-2-en-1-ol availability during the milling step produced an increase in the synthesis of the corresponding ester, (*E*)-hex-2-en-1-yl acetate. These results suggest that unsaturated C6 alcohols are limited during VOO production. In contrast, a significant reduction of the total content of esters not derived from the LOX pathway is observed, possibly

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Table 5. Content of Esters[a] (ng/g of Oil) Obtained after the Addition of Different Amounts of Acetyl-CoA during Homogenization of Olive Fruit Mesocarp from Cultivars Arbequina and Picual at Different Maturity Stages (MI 1 and MI 5)

	Arbequina MI 1 Acetyl-CoA added (μg/kg of fresh weight)				Arbequina MI 5 Acetyl-CoA added (μg/kg of fresh weight)			
	0	0.16	0.33	0.66	0	0.16	0.33	0.66
hex-1-yl acetate	8.7 ± 1.1 a	7.7 ± 1.6 a	7.2 ± 0.8 a	7.7 ± 0.5 a	17.8 ± 0.8 a	20.7 ± 0.7 a	22.6 ± 1.4 a	20.5 ± 2.7 a
(*E*)-hex-2-en-1-yl acetate	37.4 ± 7.3 a	35.0 ± 3.3 a	32.6 ± 0.9 a	33.6 ± 2.6 a	145.3 ± 26.6 a	102.1 ± 2.3 b	130.9 ± 5.4 ab	122.5 ± 17.9 ab
methyl acetate	6.4 ± 0.3 a	5.2 ± 2.6 a	7.7 ± 0.3 a	7.5 ± 0.9 a	5.3 ± 0.4 a	4.8 ± 0.7 a	5.1 ± 0.9 a	5.0 ± 1.5 a
ethyl acetate	149.2 ± 12.3 a	118.9 ± 23.4 a	137.4 ± 25.3 a	126.7 ± 18.5 a	6.1 ± 3.1 a	6.6 ± 1.5 a	7.5 ± 0.3 a	7.5 ± 0.3 a
methyl hexanoate	52.8 ± 9.2 a	50.2 ± 6.0 a	61.8 ± 2.2 a	65.0 ± 7.2 a	39.1 ± 5.0 a	45.6 ± 2.9 a	42.6 ± 0.4 a	40.5 ± 2.7 a
ethyl hexanoate	11.9 ± 1.6 a	10.2 ± 0.1 a	9.5 ± 0.1 a	11.6 ± 2.7 a	23.1 ± 5.8 a	18.8 ± 2.7 a	23.7 ± 1.5 a	20.8 ± 1.6 a
	Picual MI 1 Acetyl-CoA added (μg/kg of fresh weight)				Picual MI 5 Acetyl-CoA added (μg/kg of fresh weight)			
	0	0.16	0.33	0.66	0	0.16	0.33	0.66
hex-1-yl acetate	46.4 ± 5.1 a	53.4 ± 3.7 a	53.4 ± 7.3 a	48.3 ± 3.8 a	10.7 ± 0.6 a	13.5 ± 2.7 a	10.9 ± 0.7 a	10.5 ± 1.3 a
(*E*)-hex-2-en-1-yl acetate	35.9 ± 2.1 a	31.8 ± 1.6 a	38.4 ± 6.2 a	37.2 ± 2.4 a	117.6 ± 15.8 a	135.2 ± 5.1 a	131.6 ± 2.1 a	130.7 ± 0.2 a
methyl acetate	2.6 ± 0.9 a	3.4 ± 0.3 a	3.3 ± 0.1 a	3.4 ± 0.0 a	2.4 ± 0.3 a	3.5 ± 0.2 a	3.0 ± 1.0 a	5.7 ± 0.5 a
ethyl acetate	349.2 ± 65.9 a	246.4 ± 65.7 a	342.9 ± 32.1 a	366.7 ± 62.2 a	279.3 ± 14.5 a	308.2 ± 9.9 a	287.5 ± 7.1 a	289.7 ± 2.3 a
methyl hexanoate	15.0 ± 2.7 a	19.2 ± 2.9 a	16.5 ± 1.4 a	18.0 ± 0.5 a	38.3 ± 12.1 a	47.8 ± 0.9 a	48.0 ± 0.2 a	43.3 ± 5.1 a
ethyl hexanoate	3.9 ± 0.5 a	2.6 ± 1.3 a	3.2 ± 0.8 a	3.4 ± 0.2 a	26.9 ± 3.0 a	30.7 ± 9.4 a	26.0 ± 8.1 a	20.0 ± 4.9 a

[a]Data represent the mean value ± standard deviation from three determinations in two different experiments. Values with different letters in the same row within each cultivar and ripening index are significantly different ($p \leq 0.05$).

Table 6. Content of Esters[a] (ng/g of Oil) in Oils Obtained after the Addition of Different Amounts of (*E*)-hex-2-en-1-ol during Processing of Olive Fruits from Cultivars Arbequina and Picual at Different Maturity Stages (MI 1 and MI 5)

	Arbequina MI 1 (*E*)-hex-2-en-1-ol added (mg/kg of fruit)				Arbequina MI 5 (*E*)-hex-2-en-1-ol added (mg/kg of fruit)			
	0	4	8	16	0	4	8	16
hex-1-yl acetate	11.1 ± 0.9 a	13.6 ± 2.0 b	13.9 ± 1.0 b	15.3 ± 0.8 b	143.4 ± 7.3 a	143.1 ± 7.9 a	131.5 ± 2.0 a	138.6 ± 9.8 a
(*E*)-hex-2-en-1-yl acetate	15.0 ± 1.0 a	53.9 ± 2.8 b	78.7 ± 0.1 c	127.8 ± 17.0 d	36.5 ± 4.8 a	64.4 ± 3.3 a	137.6 ± 11.5 b	183.6 ± 30.9 b
methyl acetate	12.6 ± 0.1 a	8.5 ± 0.7 b	12.3 ± 0.2 a	12.9 ± 1.9 a	27.6 ± 1.9 ab	36.5 ± 9.0 a	28.5 ± 0.0 a	15.1 ± 0.6 b
ethyl acetate	0.5 ± 0.1 ab	0.7 ± 0.1 a	0.4 ± 0.1 b	0.6 ± 0.1 ab	127.0 ± 7.2 a	67.0 ± 6.3 b	15.4 ± 1.3 c	10.2 ± 0.8 c
methyl hexanoate	7.7 ± 0.7 a	7.5 ± 1.6 a	6.7 ± 0.3 ab	5.2 ± 0.5 b	11.7 ± 1.0 a	15.2 ± 9.2 a	16.0 ± 4.3 a	15.9 ± 0.5 a
ethyl hexanoate	17.0 ± 3.9 ab	18.7 ± 1.4 ab	15.7 ± 2.3 a	22.0 ± 2.9 b	20.4 ± 2.5 a	20.6 ± 2.6 a	23.0 ± 1.8 a	23.0 ± 5.5 a
	Picual MI 1 (*E*)-hex-2-en-1-ol added (mg/kg of fruit)				Picual MI 5 (*E*)-hex-2-en-1-ol added (mg/kg of fruit)			
	0	4	8	16	0	4	8	16
hex-1-yl acetate	15.6 ± 2.4 a	15.4 ± 1.2 a	14.3 ± 0.1 a	16.4 ± 0.5 a	9.5 ± 2.1 a	7.1 ± 1.3 ab	7.2 ± 0.4 b	6.8 ± 1.1 b
(*E*)-hex-2-en-1-yl acetate	7.2 ± 0.3 a	15.9 ± 0.7 b	17.0 ± 0.7 bc	22.6 ± 4.4 c	12.1 ± 4.7 a	17.1 ± 0.1 ab	23.6 ± 5.8 ab	28.3 ± 8.0 b
methyl acetate	1.1 ± 0.1 a	1.8 ± 0.1 a	1.8 ± 0.6 a	1.5 ± 0.1 a	16.9 ± 0.2 a	13.7 ± 0.1 b	11.6 ± 0.4 c	6.1 ± 0.5 d
ethyl acetate	4.9 ± 1.0 a	1.8 ± 0.3 b	1.9 ± 0.1 b	1.4 ± 0.1 b	3.9 ± 0.1 a	3.5 ± 0.3 ab	2.9 ± 0.3 bc	2.3 ± 0.2 c
methyl hexanoate	5.5 ± 0.4 a	5.3 ± 0.5 a	4.8 ± 0.3 a	5.0 ± 0.3 a	9.9 ± 1.7 a	8.8 ± 0.2 ab	9.1 ± 0.7 ab	7.2 ± 0.1 b
ethyl hexanoate	5.5 ± 1.0 a	5.4 ± 0.5 a	5.4 ± 0.1 a	4.4 ± 0.1 a	124.5 ± 10.6 a	78.8 ± 3.7 b	93.1 ± 6.1 bc	99.8 ± 3.0 c

[a]Data represent the mean value ± standard deviation from three determinations in two different experiments. Values with different letters in the same row within each cultivar and ripening index are significantly different ($p \leq 0.05$).

because of an effect of competition for substrate. Similarly, the increase of hexan-1-ol availability during the process of VOO production gave rise to an increase in the content of the corresponding ester, hex-1-yl acetate. This increase depended upon the cultivar, being greater in Arbequina oils than in Picual oils, which suggests that the level of AAT activity is more limited in Picual fruits than in Arbequina fruits. Moreover, the trend in ester synthesis by increasing the availability of saturated and unsaturated C6 alcohols during VOO production seems to be quite similar. Salas[9] found that AAT activity from olive mesocarp was more effective at esterifying hexan-1-ol than (*E*)-hex-2-en-1-ol. However, he found a similar substrate specificity for hexa-1-ol and (*Z*)-hex-3-en-1-ol. Our observations indicate that most of the AAT activity in olive fruit seems to reside in the seed rather than the mesocarp. This is in good agreement with the important role of this fruit tissue in ester formation during VOO production, which has been demonstrated to be quite substrate-unspecific.[19] Our data suggest that the origin of the low-volatile ester content arising from the LOX pathway in oils of cultivars Arbequina and Picual is largely

Table 7. Content of Esters[a] (ng/g of Oil) in Oils Obtained after the Addition of Different Amounts of Hexan-1-ol during Processing of Olive Fruits from Cultivars Arbequina and Picual at Different Maturity Stages (MI 1 and MI 5)

	Arbequina MI 1 hexan-1-ol added (mg/kg of fruit)				Arbequina MI 5 hexan-1-ol added (mg/kg of fruit)			
	0	4	8	16	0	4	8	16
hex-1-yl acetate	9.4 ± 0.7 a	75.3 ± 3.2 b	161.4 ± 0.5 c	254.2 ± 6.8 d	147.1 ± 2.6 a	364.6 ± 6.3 b	374.1 ± 12.4 b	435.7 ± 3.9 c
(*E*)-hex-2-en-1-yl acetate	15.1 ± 2.4 a	13.1 ± 2.4 a	13.7 ± 1.6 a	13.8 ± 0.7 a	18.1 ± 2.0 a	17.3 ± 1.5 a	19.4 ± 1.2 a	20.4 ± 1.2 a
methyl acetate	12.6 ± 0.1 a	9.3 ± 0.9 b	30.8 ± 6.1 c	28.7 ± 2.5 d	29.2 ± 0.9 a	21.3 ± 7.8 a	30.9 ± 9.0 a	21.8 ± 2.0 a
ethyl acetate	0.5 ± 0.1 a	0.5 ± 0.0 a	0.4 ± 0.0 b	0.4 ± 0.1 b	130.6 ± 2.5 a	7.7 ± 1.0 b	6.2 ± 0.4 b	5.7 ± 0.7 b
methyl hexanoate	7.7 ± 0.7 a	8.3 ± 0.4 a	6.6 ± 0.6 b	3.3 ± 0.4 c	12.3 ± 0.4 a	11.4 ± 2.0 a	9.3 ± 1.5 a	10.0 ± 1.4 a
ethyl hexanoate	13.1 ± 0.7 a	10.5 ± 1.2 b	8.2 ± 0.7 b	27.5 ± 1.9 c	163.9 ± 2.3 a	147.2 ± 29.7 a	132.2 ± 42.1 a	153.1 ± 8.3 a
	Picual MI 1 hexan-1-ol added (mg/kg of fruit)				Picual MI 5 hexan-1-ol added (mg/kg of fruit)			
	0	4	8	16	0	4	8	16
hex-1-yl acetate	8.3 ± 1.2 a	19.4 ± 1.4 b	21.5 ± 2.6 b	22.8 ± 1.6 b	7.9 ± 0.7 a	8.1 ± 2.0 a	12.1 ± 1.2 b	16.4 ± 1.1 c
(*E*)-hex-2-en-1-yl acetate	6.6 ± 0.5 ab	8.0 ± 0.3 a	7.7 ± 1.0 ab	6.1 ± 0.5 b	20.4 ± 3.4 a	20.7 ± 4.9 a	21.3 ± 1.5 a	22.1 ± 1.3 a
methyl acetate	0.9 ± 0.2 a	0.6 ± 0.1 a	0.8 ± 0.2 a	0.9 ± 0.0 a	16.7 ± 0.4 a	5.9 ± 0.5 b	9.1 ± 0.2 c	1.4 ± 0.1 d
ethyl acetate	5.3 ± 1.5 a	4.7 ± 0.9 a	5.9 ± 0.2 a	6.5 ± 1.5 a	4.7 ± 1.5 a	53.3 ± 0.6 b	34.8 ± 1.5 c	25.8 ± 1.5 d
methyl hexanoate	11.7 ± 1.8 a	6.2 ± 2.2 a	9.7 ± 0.6 a	10.9 ± 3.6 a	30.0 ± 3.6 a	40.7 ± 2.5 b	35.4 ± 3.5 ab	37.3 ± 4.9 ab
ethyl hexanoate	5.7 ± 0.7 a	5.1 ± 1.0 a	5.5 ± 0.6 a	5.1 ± 0.2 a	94.3 ± 28.7 a	103.2 ± 11.1 a	100.8 ± 16.4 a	78.7 ± 6.1 a

[a]Data represent the mean value ± standard deviation from three determinations in two different experiments. Values with different letters in the same row within each cultivar and ripening index are significantly different ($p \leq 0.05$).

due to a limitation of alcohol synthesis during VOO production.

As commented in the Introduction, ADH activity catalyzes the reversible reduction of C6 aldehydes to C6 alcohols using reduced pyridine nucleotides as cofactors. In addition, as described for soybean seed,[14] this activity could also be responsible for the synthesis of C5 carbonyl compounds present in the VOO volatile fraction through the oxidation of the C5 alcohols to the corresponding aldehydes and ketones using oxidized pyridine nucleotides as cofactors. To study the possible limitation concerning substrates for ADH, the availability of the main substrates of this enzymatic activity within the LOX pathway [(*Z*)-hex-3-enal, (*E*)-hexen-2-enal, and hexanal] was increased during VOO production. No significant modifications of the C6 alcohol content were observed in the oils extracted from either Arbequina or Picual fruits (see Figure B in the Supporting Information). The most remarkable effect of increasing the availability of these C6 aldehydes in the oil volatile profile was in relation to the expected increase in the C6 aldehyde content. Instead, a significant decrease of all classes of volatile compounds was observed, which might be ascribed to an inhibition of olive HPL activity by its main reaction product. A suicide-like mechanism for HPLs has been reported.[24] It has also been observed that (*Z*)-hex-3-enal is totally isomerized into (*E*)-hex-2-enal during this process, so that the volatile compound profiles of the oils were similar to those of the oils obtained after the addition of (*E*)-hex-2-enal. These results suggest that there is no limitation concerning the main substrates for olive ADH activity during the VOO extraction process.

As mentioned above, ADH activity could also be responsible for the synthesis of C5 carbonyl compounds present in the VOO volatile fraction, through the oxidation of the C5 alcohols. The availability of (*Z*)-pent-2-enol was increased during the process of VOO production, but the content of the corresponding C5 carbonyl compounds remained unalterable (see Figure C in the Supporting Information). Similar results were found when investigating the limitation of substrate for ADH in the opposite reaction by increasing the availability of (*E*)-pent-2-enal. Finally, the effect of the level of pyridine nucleotides during VOO extraction was also studied. As mentioned in the Introduction, three proteins with ADH activity in olive mesocarp have been characterized, one NADH- and two NADPH-dependent proteins.[8] Increasing amounts of reduced forms of NADPH and NADH were studied using the homogenization approach. Similarly, the effect of increasing the content of the NAD oxidized form was studied. Again, no significant modifications of the level of the corresponding theoretical products were observed in the oils (see Figure D in the Supporting Information), which suggests that there is no limitation concerning pyridine nucleotides for volatile compound synthesis during the process of VOO extraction.

With the establishment that substrate limitation does not appear to be limiting C6 alcohol synthesis in olive fruit during the process to obtain VOO, ADH activity levels in olive tissues were investigated as an alternative explanation. It was not possible to detect ADH activity in crude extracts from fresh tissues. ADH activity has only been measured in crude extracts prepared from acetone powders of olive mesocarp and seed tissues of Arbequina and Picual cultivars. According to the results, fruits of cultivar Picual display ADH activity levels higher than those of cultivar Arbequina, in both mesocarp and seed tissues, with average values of 0.45 unit/g of mesocarp and 1 unit/g of seed in Arbequina and of 0.75 unit/g of mesocarp and 1.25 units/g of seed in Picual. When those values are taken into account, different amounts of yeast ADH have been used to increase the effective enzymatic load of ADH activity during the process to obtain VOO. It has been observed that the addition of up to 60 units of ADH/g of fruit during the milling step of fruits of Picual and Arbequina cultivars did not produce any significant difference in the content of C6 alcohols or esters in the oils (see Figure E in the Supporting Information). This amount of activity represents around 100 times the average ADH activity measured in olive fruit mesocarp. Then, the level

of exogenous ADH activity was increased up to an average of 400 times (240 units of ADH/g of fruit) using the mesocarp homogenization approach. Again, the increase of ADH activity did not produce any significant effect on volatile compound synthesis. These results suggested that either ADH activity is not a limiting factor for C6 alcohol synthesis or this enzymatic activity is strongly inactivated during the process of VOO production. The difficulties experienced in obtaining crude extracts from fresh olive tissues with detectable ADH activity suggest the presence in mesocarp and seed tissues of compounds that can inhibit endogenous ADH activity during the process of VOO production. In this context, we have previously observed that, while mesocarp tissue slices were able to reduce exogenously added C6 aldehydes, we failed to detect any ADH activity in crude extracts from mesocarp tissues.[3] It is well-known that phenolics interact with proteins reversibly by hydrogen bonding and irreversibly by oxidation to quinones followed by covalent condensation of the quinones with reactive groups of proteins or even by oxidation of essential groups of proteins.[25,26] Also, the role of olive polyphenol oxidase and peroxidase acting as major factors oxidizing phenolics during VOO production has been reported.[27]

It was observed that the addition of thermally inactivated crude extracts from fresh olive mesocarp and seed tissues to active ADH reaction media inhibits this activity (Figure 1). As

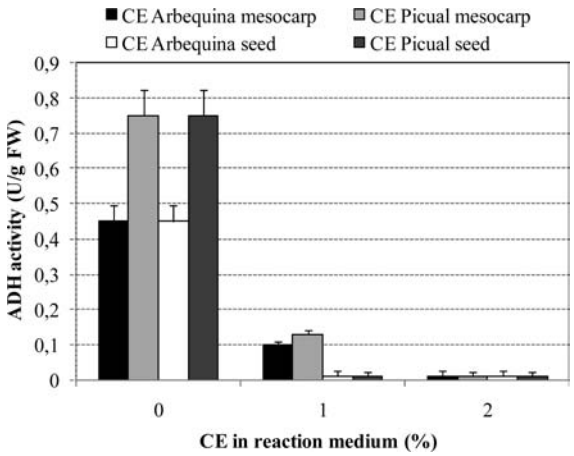

Figure 1. Effect of the addition of crude extracts (CE) from different olive fruit tissues and cultivars on reaction media of olive mesocarp ADH activity.

mentioned, active ADH extracts from olive fruit tissues were prepared from acetone powders that are deprived of lipids, pigments, and phenolics. The percentage of inhibition was very similar for extracts from both cultivars (around 80%) when 1% of deactivated crude extracts was added. The inhibition was complete when increasing the proportion of crude extracts in the reaction media to 2% (Figure 1). These results suggest that ADH activity is severely limited during the process of VOO production because of the presence of inhibitors, probably phenolics, in both fruit mesocarp and seed tissues. This limitation of ADH activity seems to be independent of the olive cultivar and fruit ripeness stage, and it might be the reason for the low C6 alcohol levels found in VOO from Arbequina and Picual olive cultivars and, consequently, the level of esters of sensorial significance in VOO. Moreover, inhibition of ADH activity during the production of VOO would also explain the lack of effect on the VOO volatile fraction when the availability of substrates and cofactors for ADH activity is increased.

The data presented suggest that synthesis of volatile esters during the production of VOO is limited by the levels of C6 alcohols. The low levels of C6 alcohols do not however appear to be determined by a shortage of precursors or cofactors. In fact, one such precursor [(*E*)-hex-2-enal] is the most important volatile compound in VOO from a quantitative point of view. Instead, we propose that deactivation of ADH activity during olive oil production in the cultivars under study is responsible for the low levels of C6 alcohols and important sensorial esters in VOO from Arbequina and Picual olive cultivars.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Typical chromatograms of volatile compounds identified in the headspace of virgin olive oil of Arbequina and Picual cultivars (Figure A), contents of C6/LnA and C6/LA alcohols obtained after the addition of different amounts of (*E*)-hex-2-enal and hexanal, respectively, during the oil extraction process (Figure B), contents of C5/LnA carbonyls and C5/LnA alcohols obtained after the addition of different amounts of (*Z*)-pent-2-en-1-ol and (*E*)-pent-2-enal, respectively, during the oil extraction process (Figure C), contents of total alcohols obtained after the addition of different amounts of NADPH and NADH during the oil extraction process (Figure D), and contents of total alcohols and LOX esters obtained after the addition of different amounts of yeast ADH during the oil extraction process (Figure E). This material is available free of charge via the Internet at http://pubs.acs.org.

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■ REFERENCES

(1) Ruiz-Gutierrez, V.; Muriana, F. J. G.; Villar, J. Virgin olive oil and cardiovascular diseases. Plasma lipid profile and lipid composition of human erythrocyte membrane. *Grasas Aceites* **1998**, *49*, 9−29.

(2) Visioli, F.; Galli, C. Olive oil phenols and their potential effects on human health. *J. Agric. Food Chem.* **1998**, *46*, 4292−4296.

(3) Olias, J. M.; Perez, A. G.; Rios, J. J.; Sanz, C. Aroma of virgin olive oil: Biogenesis of the green odor notes. *J. Agric. Food Chem.* **1993**, *41*, 2368−2373.

(4) Morales, M. T.; Aparicio, R.; Rios, J. J. Dynamic headspace gas chromatographic method for determining volatiles in virgin olive oil. *J. Chromatogr., A* **1994**, *668*, 455−462.

(5) Angerosa, F.; Mostallino, R.; Basti, C.; Vito, R. Virgin olive oil odour notes: Their relationships with the volatile compound from the lipoxigenase pathway and secoiridoid compounds. *Food Chem.* **2000**, *68*, 283−287.

(6) Salas, J. J.; Williams, M.; Harwood, J. L.; Sánchez, J. Lipoxygenase activity in olive (*Olea europaea*) fruit. *J. Am. Oil Chem. Soc.* **1999**, *76*, 1163−1169.

(7) Salas, J. J.; Sánchez, J. Hydroperoxide lyase from olive (*Olea europaea*) fruits. *Plant Sci.* **1999**, *143*, 19−26.
(8) Salas, J. J.; Sánchez, J. Alcohol dehydrogenases from olive (*Olea europaea*) fruit. *Phytochemistry* **1998**, *48*, 35−40.
(9) Salas, J. J. Characterization of alcohol acyltransferase from olive fruit. *J. Agric. Food Chem.* **2004**, *52*, 3155−3158.
(10) Sanz, C.; Perez, A. G. Plant metabolic pathways and flavor biosynthesis. In *Handbook of Fruit and Vegetable Flavors*; Hui, Y. H., Ed.; John Wiley and Sons, Inc.: New York, 2010; pp 129−155.
(11) Aparicio, R.; Morales, M. T.; Alonso, M. V. Relationship between volatile compounds and sensory attributes of olive oils by the sensory wheel. *J. Am. Oil Chem. Soc.* **1996**, *73*, 1253−1264.
(12) Reiners, J.; Grosch, W. Odorants of virgin olive oils with different flavor profiles. *J. Agric. Food Chem.* **1998**, *46*, 2754−2763.
(13) Gardner, H. W.; Grove, M. J.; Salch, Y. P. Enzymic pathway to ethyl vinyl ketone and 2-pentenal in soybean preparations. *J. Agric. Food Chem.* **1996**, *44*, 882−886.
(14) Fisher, A. J.; Grimes, H. D.; Fall, R. The biochemical origin of pentenol emissions from wounded leaves. *Phytochemistry* **2003**, *62*, 159−163.
(15) Morales, M. T.; Angerosa, F.; Aparicio, R. Effect of the extraction conditions of virgin olive oil on the lipoxygenase cascade: Chemical and sensory implications. *Grasas Aceites* **1999**, *50*, 114−121.
(16) Angerosa, F.; d'Alessandro, N.; Basti, C.; Vito, R. Biogeneration of volatile compounds in virgin olive oil: Their evolution in relation to malaxation time. *J. Agric. Food Chem.* **1998**, *46*, 2940−2944.
(17) Angerosa, F.; Mostallino, R.; Basti, C.; Vito, R. Influence of malaxation temperature and time on the quality of virgin olive oils. *Food Chem.* **2001**, *72*, 19−28.
(18) Perez, A. G.; Luaces, P.; Rios, J. J.; Garcia, J. M.; Sanz, C. Modification of volatile compound profile of virgin olive oil due to hot-water treatment of olive fruit. *J. Agric. Food Chem.* **2003**, *51*, 6544−6549.
(19) Luaces, P.; Perez, A. G.; Sanz, C. Role of olive seed in the biogenesis of virgin olive oil aroma. *J. Agric. Food Chem.* **2003**, *51*, 4741−4745.
(20) Luaces, P.; Perez, A. G.; Sanz, C. Effect of the blanching process and olive fruit temperature at milling on the biosynthesis of olive oil aroma. *Eur. Food Res. Technol.* **2006**, *224*, 11−17.
(21) Larson, T. R.; Graham, I. A. A novel technique for the sensitive quantification of acyl CoA esters from plant tissues. *Plant J.* **2001**, *25*, 115−125.
(22) Larson, T. R.; Edgell, T.; Byrne, J.; Dehesh, K.; Graham, I. A. Acyl CoA profiles of transgenic plants that accumulate medium-chain fatty acids indicate inefficient storage lipid synthesis in developing oilseeds. *Plant J.* **2002**, *32*, 519−527.
(23) Olias, R.; Perez, A. G.; Sanz, C. Catalytic properties of alcohol acyltransferase in different strawberry species and cultivars. *J. Agric. Food Chem.* **2002**, *50*, 4031−4036.
(24) Santiago-Gómez, M. P.; Vergely, C.; Policar, C.; Nicaud, J. M.; Belin, J. M.; Rochette, L.; Husson, F. Characterization of purified green bell pepper hydroperoxide lyase expressed by *Yarrowia lipolytica*: Radicals detection during catalysis. *Enzyme Microb. Technol.* **2007**, *41*, 13−18.
(25) Loomis, W. D.; Battaille, J. Plant phenolic compounds and the isolation of plant enzymes. *Phytochemistry* **1966**, *5*, 423−438.
(26) Loomis, W. D. Removal of phenolic compounds during the isolation of plant enzymes. *Methods Enzymol.* **1969**, *13*, 555−563.
(27) Garcia-Rodriguez, R.; Romero-Segura, C.; Sánchez-Ortiz, A.; Sanz, C.; Perez, A. G. Role of polyphenol oxidase and peroxidase in shaping the phenolic profile of virgin olive oil. *Food Res. Int.* **2011**, *44*, 629−635.