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Influence of Phenols Mass Fraction in Olive (*Olea europaea* L.) Paste on Volatile Compounds in Buža Cultivar Virgin Olive Oil

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ABSTRACT: The influence of the phenolic content in olive paste of cv. Buža increased by the addition of an aqueous solution of phenolic extract of freeze-dried olive pulp (cv. Istarska bjelica) on the final products of the lipoxygenase pathway in oil was studied. Increases by 12, 38, and 56% for ripe fruits (maturity index = 4.0) and by 38% for unripe fruits (maturity index = 1.2) were examined. Phenols in the olive paste were determined according to the HPLC method, whereas volatiles in oil were determined according to SPME-GC-MS. A significant negative effect on *Z*-3-hexenal and *E*-2-hexen-1-ol (Tukey's test, $p < 0.05$) was found for ripe fruits (average decreases of 55 and 60%, respectively), but not for the unripe sample. Positive effects in both ripening levels were found for *Z*-3-hexenyl acetate (average increase of 68% for ripe and a double increase for unripe fruits) and total C5 compounds (average increase of 32% for ripe and an increase of 30% for unripe fruits).

KEYWORDS: *Olea europaea L., Buža cultivar, phenols in olive paste, virgin olive oil, volatile compounds*

INTRODUCTION

Volatile and phenolic compounds are the main contributors to the complex and desirable flavor of virgin olive oils (VOOs). The synthesis and biotransformation of these compounds are directly related to the activity of several endogenous enzymes released during fruit tissue disruption in the milling step of olive fruits.[1] The most important components of VOO aroma are low molecular weight aldehydes, alcohols, esters, and ketones, giving the pleasant green and fruity odor notes to high-quality VOOs. These compounds are synthesized through the lipoxygenase pathway from nonesterified polyunsaturated fatty acids such as linoleic and linolenic acid from glycerolipids and phospholipids in cell membranes.[2,3] Linoleic and linolenic fatty acid, detached by acyl hydrolase, are oxidized by lipoxygenase (LOX) and cleaved by hydroperoxide lyase (HPL) into C6 aldehydes, which can be later reduced to C6 alcohols by alcohol dehydrogenase (ADH) and transformed to C6 esters by alcohol acyl transferase (AAT).[4] Besides hydroperoxides formation, LOX can cleave them, producing alkoxy radicals, which lead to C5 volatile compounds formation that are contributors to green, sweet, and pungent sensory perceptions.[5,6]

A few studies deal with factors that could be limiting for VOO volatile compound biosynthesis.[7−10] Sanchez-Ortiz et al. have reported that the availability of nonesterified fatty acids and the enzymatic load of LOX and HPL are the main limiting factors in the biosynthesis of VOO aroma compounds.[7−9] The same authors have found that the synthesis of volatile esters is mainly influenced by low C6 alcohol levels and proposed the deactivation of ADH during VOO production as a possible cause of low precursor levels in the synthesis of esters.[10] Difficulties that phenolic compounds can cause during the enzyme extraction from plant tissues arise from irreversible interactions of oxidized phenols with proteins.[11] Therefore, some authors have hypothesized that one of the inhibitory factors of the enzymatic activity during VOO processing could be the phenols and their oxidized products present in olive paste.[12,13] Numerous papers describe the inhibition of various enzymes by different phenolic compounds, attributed to the redox properties and the ability of phenols to directly bind to target proteins.[14] Recently, Sanchez-Ortiz et al. have investigated the effect of a relatively high dose (32 g/kg in olive paste) of synthetic antioxidants with phenolic structure, known as LOX inhibitors, on the biosynthesis of Picual and Arbequina VOO volatile compounds. A notable reduction of volatile compound content in such obtained oils was observed. Authors have concluded that a generally low rate of volatile compound synthesis during malaxation is most probably associated with the deactivation of the LOX activity by phenols in the olive paste.[9]

To our knowledge, there are no data concerning the effect of phenolic compounds naturally present in olive drupes and paste on the restriction of VOO volatile synthesis during malaxation. Therefore, a dried methanolic extract of Istarska bjelica fruits, as a rich source of phenolic compounds, has been prepared and used as an aqueous solution to increase the total phenols content of olive paste. The impact of different olive paste phenols levels on the mass fraction of VOO volatile compounds has been explored on an autochthonous Croatian cultivar Buža, known for its relatively high content of volatile compounds and low

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Table 1. Mass Fraction[a] (mg/kg) and Contribution of Single Phenolic Compounds to Total Phenols (%) in Ripe Buža Olive Paste Samples (Maturity Index = 4.0) Enriched with Phenol Extract (Malaxation Time = 0 min)

	level of olive paste enrichment (w/w)							
	0%		12%		38%		56%	
phenolic compound	mg/kg	%	mg/kg	%	mg/kg	%	mg/kg	%
Tyr-OH*[b]	839 ± 54 ab	8.7	749 ± 58 b	7.0	816 ± 46 ab	6.2	845 ± 16 a	5.6
Tyr**	214 ± 11 a	2.2	209 ± 12 a	1.9	217 ± 26 a	1.6	212 ± 9 a	1.4
DMO-Agl-dA*	1920 ± 107 a	20.0	2320 ± 255 b	21.6	2850 ± 131 c	21.6	3160 ± 239 c	21.1
iso-oleuropein*	204 ± 12 a	2.1	171 ± 10 b	1.6	203 ± 10 a	1.5	252 ± 11 c	1.7
O-Agl-dA*	78 ± 11 a	0.8	241 ± 22 b	2.2	449 ± 30 c	3.4	720 ± 84 d	4.8
ligstroside*	211 ± 14 a	2.2	270 ± 24 b	2.5	341 ± 18 c	2.6	409 ± 32 d	2.7
verbascoside**	709 ± 61 a	7.4	814 ± 111 ab	7.6	946 ± 69 bc	7.2	1050 ± 55 c	7.0
luteolin-7-O-glu**	243 ± 6 a	2.5	265 ± 19 a	2.5	329 ± 20 b	2.5	356 ± 22 b	2.4
luteolin**	168 ± 7 a	1.8	190 ± 15 b	1.8	219 ± 9 c	1.7	238 ± 10 d	1.6
apigenin**	4 ± 0 a	0.0	6 ± 1 b	0.1	8 ± 0 c	0.1	9 ± 1 d	0.1
total unidentified[c]	5010 ± 150 a	52.2	5540 ± 318 b	51.4	6820 ± 250 c	51.7	7700 ± 430 d	51.5
total phenols	9590 ± 339 a		10800 ± 707 b		13200 ± 458 c		15000 ± 866 d	

[a]Results are means of eight values (two independent repetitions of oil preparation × quadruplicate analyses) ± SD and are presented with up to three significant figures; means within each row marked with different letters are significantly different (Tukey's test, $p < 0.05$). [b]Identification of compounds marked with ** is based on reference compound; compounds marked with * are tentatively identified on the basis of their respective relative retention times, UV maxima, and UV spectra. [c]Sum of the mass fraction of all peaks of unidentified phenolic compounds on chromatogram.

phenol content.[15,16] Differences in the responses of ripe and unripe fruits have also been checked.

MATERIALS AND METHODS

Chemicals. Sodium carbonate p.a., sodium hydroxide p.a., caffeic acid (purity = 99%), and methanol p.a. used for the extraction of phenols from olive fruits were purchased from Panreac (Barcelona, Spain). Folin−Ciocalteu reagent was supplied from Merck KGaA (Darmstadt, Germany) and formic acid from Riedel de Haën (Seelze, Germany). Acetonitrile and methanol for HPLC analysis, as well as 4-methyl-2-pentanol (purity > 98%), were obtained from Sigma-Aldrich (Steinheim, Germany). Tyrosol (purity > 99.5%), syringic acid (purity ≥ 95%), and squalene (purity > 97%) were procured by Fluka (Buchs, Switzerland), luteolin (purity > 99%) and apigenin (purity > 98%) from Sigma (St. Louis, MO, USA), and luteolin-7-*O*-glucoside (purity ≥ 98%) and verbascoside (purity ≥ 99%) from Extrasynthese (Genay, France).

Olive Fruits. Olive (*Olea europaea* L.) fruits were handpicked in the western part of the Istria region (Croatia) during October and November 2010. The maturity index (MI) of the fruits was determined by applying the method described by Gutierrez et al., based on the evaluation of the olive skin and pulp color.[17] Fruits of Buža cultivar were sorted by hand into two groups of maturity index (4.0 and 1.2) and then divided into a total of 12 batches of 1 kg each and used for the preparation of olive paste matrices. Fruits of Istarska bjelica cultivar (MI = 0.7), as a rich source of phenolic compounds, were used to obtain an aqueous solution intended to increase the phenols content in Buža olive paste matrices.

Preparation of Aqueous Solution of Phenolic Compounds. The aqueous solution of phenolic compounds was prepared from previously freeze-dried olive fruits of cultivar Istarska bjelica. Destoned and milled dry pulp was shaken with two portions of methanol (mass/volume ratio = 1:5) in a glass cylinder for 5 min. The suspension was transferred to test tubes and centrifuged at 1450 rpm for 3 min. Supernatant was filtered through filter paper, and methanol was eliminated under reduced pressure at 40 °C until the residue reached a viscous consistency. The residue was immediately dissolved in distilled water (mass ratio = 1:2), and the mass fraction of total phenols in the obtained aqueous solution was determined colorimetrically as described below.

Virgin Olive Oil Sample Preparation. Oil samples from Buža fruits were extracted using the laboratory olive mill (Abencor, MC2 Ingeneria y Sistemas, Seville, Spain), which consisted of a hammer crusher, thermostated vertical olive paste mixers, and centrifuge. Immediately after fruit milling, an appropriate volume of the aqueous solution of phenolic compounds was added into the olive paste to increase the phenolic content of the olive pastes up to 45% (w/w) according to colorimetric determination of the total phenol mass fraction. The same volume of distilled water was added to the control sample. Olive pastes were malaxed for 45 min at 25 ± 0.5 °C. After centrifugation at 3600 rpm for 70 s, extracted oil samples were stored in fully filled dark bottles at room temperature until analysis. Two independent oil extractions were performed for each fruit sample.

Colorimetric Determination of Total Phenol Mass Fraction in Olive Paste and Aqueous Solution. To determine the required amounts of aqueous solution to be added to the olive paste, the total phenol mass fractions in both matrices were determined by the rapid colorimetric method. Olive paste was homogenized with methanol (mass/volume ratio = 1:10) using an Ultraturax homogenizer (11000 rpm for 3 min). After 30 min of solids deposition in darkness, the upper methanol layer was taken into a colorimetric reaction. An aliquot of the obtained methanolic extract from olive paste, or aqueous solution obtained from freeze-dried olive pulp, was used for the colorimetric reaction, according to the procedure of Koprivnjak et al.[18] The aliquot was diluted with 30 mL of distilled water, and then Folin−Ciocalteu reagent (2.5 mL) and 7.5 mL of sodium carbonate solution (25% w/v) were added. The reaction mixture was filled with deionized water to 50 mL and left in the dark for 2 h. The absorbance at 765 nm (HACH spectrophotometer DR/400) was measured against the blank solution. Total phenol mass fraction was calculated from a caffeic acid calibration curve and expressed in milligrams per kilogram as caffeic acid equivalents. Three independent determinations for olive paste and aqueous solution were performed, respectively.

HPLC Analysis of Phenols in Olive Pastes. Phenols were extracted from freeze-dried olive paste samples according to the procedure described by Valenčič et al.[19] Prior to extraction, a solution of syringic acid (0.15 mg/mL) in a mixture of methanol/water (80:20, v/v) was added to freeze-dried olive paste (4 mL/g) as the internal standard. Dried samples were repeatedly mixed with methanol (mass/volume ratio = 1:20), each time for 2 min, until complete extraction of phenols (verified by negative reaction to sodium hydroxide solution, 20% w/v) was reached. Obtained phenolic extracts were filtered and dried using a rotary evaporator at 40 °C, reconstructed with 1 mL of methanol, and filtered through 0.45 μm syringe filters. The reconstructed phenolic fraction was analyzed by reverse

phase high-performance liquid chromatography (RP HPLC) using an Agilent Technologies (Waldbronn, Germany) 1100 series HPLC system chromatograph equipped with a thermostated autosampler, a binary pump (BinPump G1312A), a thermostated column compartment (COLCOM G1316A), and a diode array detector (G1315B). A Phenomenex Synergi 4 μm Hydro-RP 80 Å (250 × 4.6 mm i.d.) column (Torrance, CA, USA) was used. The determinations were performed according to a modified method published by the International Olive Council (IOC).[20] A binary elution gradient of 0.1% formic acid in water with acetonitrile and methanol was used. The column compartment temperature was kept at 15 °C. Detection was performed at 280 nm, with the exception of the flavonoids luteolin and apigenin, which were recorded at 340 nm. Calibration curves for tyrosol (mass fraction range from 30 to 800 mg/kg; $y = 0.0811a$), luteolin (mass fraction range from 30 to 250 mg/kg; $y = 0.0134a$), and apigenin (mass fraction range from 2 to 30 mg/kg; $y = 0.0052a$) were constructed using standard compounds. All other phenol compounds were assigned according to the IOC publication on the basis of their respective relative retention times, UV maxima, and UV spectra, or on the basis of their standards (luteolin-7-*O*-glucoside and verbascoside), and quantitated using the response factor for tyrosol.[20] Two independent extraction procedures for each olive paste sample and four HPLC determinations for each extract of olive paste were performed.

Analysis of Volatile Compounds. The solid-phase microextraction (SPME) sampling procedure described by Vichi et al. was used.[21] VOO samples were weighed in a 10 mL vial and sealed with a PTFE/silicone septum. After 30 min of sample conditioning at 40 °C in a vial heater, volatile compounds from olive oil headspace were adsorbed on a SPME fiber DVB/Carboxen/PDMS 50/30 μm, 2 cm length (Supelco, Bellefonte, PA, USA), during 30 min at 40 °C. Before each sampling, the fiber was reconditioned for 10 min at 260 °C in an injecting port of the gas chromatograph. A Shimadzu 2010 gas chromatograph (Tokyo, Japan) equipped with a Supelco equity 5 capillary column 60 m × 0.25 mm i.d., 1 μm film thickness (Supelco), and a quadrupole mass detector Shimadzu QP2010 (Tokyo, Japan) were used. The column temperature was held at 40 °C for 10 min, increased to 220 °C at 5 °C/min and to 260 °C at 15 °C/min, and held for 10 min. The injector temperature was 260 °C, and the time of desorption of the fiber was fixed at 1 min with the splitless injection mode. The carrier gas was helium at a linear velocity of 30 cm/s. The temperatures of ion source and transfer line were 200 and 260 °C, respectively. Volatile compounds were tentatively identified by comparison of their mass spectra with those contained in the mass spectra libraries Wiley 6 and NIST 21 and by comparison of their Kovats retention indices (KI) with those reported in the literature.[21−23] KI values were calculated for each volatile compound using a mixture of homologous *n*-alkanes (C5−C15). Volatile compounds were semiquantitated using the relative response factor of internal standard 4-methyl-2-pentanol. Forty microliters of 4-methyl-2-pentanol solution in squalene (5.2 μg/mL) was added to 1.5 g of oil sample. Each sample was analyzed in two parallel repetitions, and results were expressed in milligrams per kilogram of oil. Odor activity values were calculated on the basis of threshold values in oil reported in the literature.[25−27]

Statistical Analysis. Differences among samples at four different levels of olive paste enrichment, as well as between samples obtained from ripe and unripe fruits, were tested by a one-way analysis of variance at the 5% significance level. The homogeneity of variance was tested by the Brown−Forsythe test. The mean values were compared by Tukey's honest significant difference test ($p \leq 0.05$). Statistical analyses were performed using the software package Statistica 10.

RESULTS AND DISCUSSION

Olive fruits of Buža, an autochthonous Croatian cultivar, were used to study the effect of phenolic compounds naturally present in olive drupes on the VOO volatile compounds created through the LOX pathway. Buža cultivar was selected on the basis of its relatively high content of volatile compounds and low phenols content.[15,16] For the purpose of olive paste enrichment, phenolic compounds were obtained from the pulp of freeze-dried unripe fruits (MI = 0.7) of cultivar Istarska bjelica, known to have a high phenols content.[19] The mass fraction of total phenolic compounds in the original ripe Buža olive paste sample was 9.6 g/kg (Table 1) and was enriched at three levels by adding 1.2, 3.6, and 5.4 g/kg of natural olive phenolic compounds, respectively.

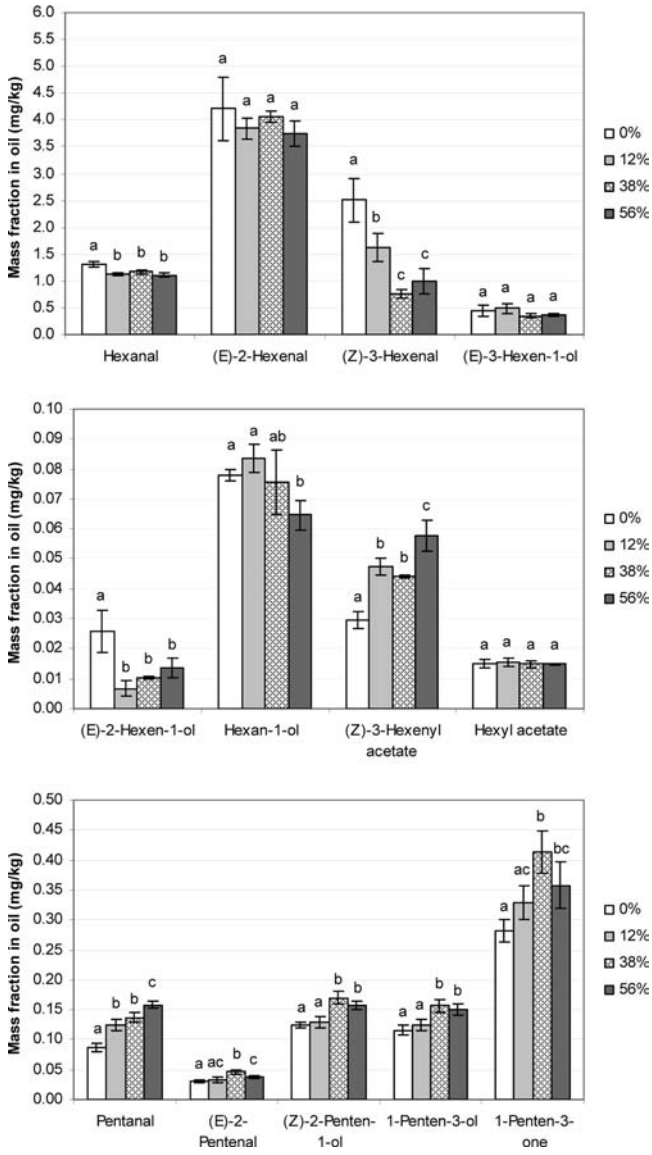

Figure 1. Mass fraction (mg/kg) of volatile compounds in Buža VOO samples obtained from olive pastes enriched with phenol extract (levels of olive paste enrichment: 0, 12, 38, and 56%; malaxation time = 45 min). Compounds are tentatively identified by comparing their mass spectra with mass spectra libraries Wiley 6 and NIST 21 and Kovats' retention indices in the literature.[21−23] Results are means of four values (two independent repetitions of oil preparation × duplicate analyses) ± SD. Means within each volatile compound, marked with different letters, are significantly different (Tukey's test, $p < 0.05$).

The composition of the phenolic fraction in olive paste samples enriched with phenol extract before malaxation is shown in Table 1. Among identified or tentatively identified compounds, the most abundant (20% of total phenols mass fraction) was demethyl oleuropein derivative (DMO-Agl-dA), probably a product of β-glucosidase and methylesterase activity on oleuropein at the moment of fruit crushing.[28,29] Gomez-Rico et al. have reported for cultivar Cornicabra that a few

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minutes after crushing, about 95% of oleuropein in olive fruits was transformed in derivatives, among which DMO-Agl-dA was the major compound.[30] In the Buža olive paste sample, a group of unidentified phenolic compounds, which also included some oxidized forms, contributed to the greatest extent (52%) to the total phenols mass fraction. Rovellini and Cortesi have identified oxidized forms of oleuropein and ligstroside derivatives in VOO by mass spectrum analysis, stating that the oxidation refers to the acidic structure but not the aromatic alcoholic moiety.[31] The composition of phenolic fraction in olive paste samples enriched with phenol extract was similar to that of the original sample. The main changes along with the increase of phenol enrichment level were a moderate decrease of contribution of hydroxytyrosol (from 8.7 to 5.6%) and an increase of contribution of O-Agl-dA (from 0.8 to 4.8%).

Considering volatile compounds (Figure 1), the most abundant volatiles in the original oil sample were C6 aldehydes and C6 alcohols (>90% of total volatile mass fraction), the same as in other high-quality virgin olive oils.[24] Among C6 aldehydes, *Z*-3-hexenal showed a statistically significant decrease (on average, reduction of 55%) by increasing the level of phenols enrichment up to 56%. This compound, with the highest odor activity value (OAV) among all volatile compounds in analyzed VOOs (Table 2), underwent up to a 3-fold mass fraction reduction. A statistically significant, although less extensive, decrease (on average, a mass fraction reduction of 15% at the three enrichment levels) was observed for hexanal, the second-placed among volatile compounds according to OAV. Reduction of some of C6 aldehydes might be the result of reduced activity of LOX and HPL by phenolic compounds added to olive paste. According to Sanchez-Ortiz et al., the enzymatic activity load of these two enzymes seems to have the main influence on the biosynthesis of VOO volatile compounds.[8,9] Besides, phenolic compounds with potent free radical scavenging ability are capable of breaking the propagation chain of lipoperoxidation induced by LOX.[32]

With regard to volatile C6 alcohols, which are the products of ADH activity, two components showed statistically significant decreases. A mass fraction reduction of *E*-2-hexen-1-ol was on average 60% at the three enrichment levels with regard to the original sample. A mass fraction of hexan-1-ol was significantly, although just slightly, reduced (by 17%) only at the highest enrichment level of olive paste. This decrease could be the consequence of either LOX or ADH activity inhibition. Sanchez-Ortiz et al. have reported a significant decrease of C6

Table 2. Odor Activity Values[a] (OAV) of Volatile Compounds in Ripe Buža VOO Samples (Maturity Index = 4.0) Obtained from Olive Pastes Enriched with Phenol Extract (Malaxation Time = 45 min)

		OAV at level of olive paste enrichment (w/w)			
volatile compound[b]	Kovats retention index	0%	12%	38%	56%
hexanal	800	17.6	15.0	15.6	14.8
E-2-hexenal	861	9.9	9.1	9.6	8.8
Z-3-hexenal	799	835.6	545.5	256.8	332.7
E-3-hexen-1-ol	864	<1.0	<1.0	<1.0	<1.0
E-2-hexen-1-ol	872	<1.0	<1.0	<1.0	<1.0
hexan-1-ol	874	<1.0	<1.0	<1.0	<1.0
Z-3-hexenyl acetate	1004	<1.0	<1.0	<1.0	<1.0
hexyl acetate	1009	<1.0	<1.0	<1.0	<1.0
pentanal	696	<1.0	<1.0	<1.0	<1.0
E-2-pentenal	758	<1.0	<1.0	<1.0	<1.0
Z-2-penten-1-ol	770	<1.0	<1.0	<1.0	<1.0
1-penten-3-ol	682	nc[c]	nc	nc	nc
1-penten-3-one	686	5.6	6.6	8.3	7.2

[a]Odor activity values were calculated on the basis of threshold values reported in the literature.[24−27] Volatile compounds with OAV > 1 have direct contribution to the olive oil aroma. [b]Compounds are tentatively identified by comparing their mass spectra with mass spectra libraries Wiley 6 and NIST 21 and Kovats retention indices in the literature.[21−23] [c]nc, not calculated because of lack of the threshold values reported in the literature.

Table 3. Mass Fraction[a] (mg/kg) and Contribution of Single Phenolic Compounds to Total Phenols (%) in Buža Olive Paste Samples (Malaxation Time = 0 min) Obtained from Unripe (Maturity Index = 1.2) and Ripe Fruits (Maturity Index = 4.0)

	level of olive paste enrichment (w/w)							
	0%				38%			
	unripe fruits (MI = 1.2)		ripe fruits (MI = 4.0)		unripe fruits (MI = 1.2)		ripe fruits (MI = 4.0)	
phenolic compound	mg/kg	%	mg/kg	%	mg/kg	%	mg/kg	%
Tyr-OH*[b]	1100 ± 113 a	11.1	839 ± 54 b	8.7	1140 ± 29 a	8.4	816 ± 46 b	6.2
Tyr**	263 ± 24 a	2.6	214 ± 11 b	2.2	287 ± 7 a	2.1	217 ± 26 b	1.6
DMO-Agl-dA*	1920 ± 72 a	19.4	1920 ± 107 a	20.0	2820 ± 72 b	20.7	2850 ± 131 b	21.6
iso-oleuropein*	225 ± 20 a	2.3	204 ± 12 a	2.1	265 ± 12 b	2.0	203 ± 10 a	1.5
O-Agl-dA*	106 ± 12 a	1.1	78 ± 11 a	0.8	472 ± 59 b	3.5	449 ± 30 b	3.4
ligstroside*	199 ± 12 a	2.0	211 ± 14 a	2.2	353 ± 17 b	2.6	341 ± 19 b	2.6
verbascoside**	511 ± 28 a	5.1	709 ± 61 b	7.4	682 ± 9 b	5.0	946 ± 69 c	7.2
luteolin-7-O-glu**	164 ± 6 a	1.7	243 ± 6 b	2.5	208 ± 7 c	1.5	329 ± 20 d	2.5
luteolin**	124 ± 13 a	1.2	168 ± 7 b	1.8	194 ± 13 c	1.4	219 ± 9 d	1.7
apigenin**	3 ± 0 a	0.0	4 ± 0 b	0.0	8 ± 1 c	0.1	8 ± 0 c	0.1
total unidentified[c]	5310 ± 192 a	53.5	5010 ± 150 a	52.2	7170 ± 659 b	52.7	6820 ± 250 b	51.7
total phenols	9930 ± 423 a		9600 ± 339 a		13600 ± 750 b		13200 ± 459 b	

[a]Results are means of eight values (two independent repetitions of oil preparation × quadruplicate analyses) ± SD and are presented with up to the three significant figures; means within each row marked with different letters are significantly different (Tukey's test, $p < 0.05$). [b]Identification of compounds marked with ** is based on reference compound; compounds marked with * are tentatively identified on the basis of their respective relative retention times, UV maxima, and UV spectra. [c]Sum of the mass fraction of all peaks of unidentified phenolic compounds on chromatogram.

aldehydes and C6 alcohols, caused by the addition of specific synthetic lipoxygenase inhibitors with the phenolic structure (octyl gallate, phenidone, and phenylbutazone), during the malaxation of Arbequina and Picual olive pastes.[9] On the other hand, Sanchez-Ortiz et al. have observed a strong inactivation of ADH by the addition of crude extracts from olive mesocarp and olive seeds to ADH reaction media.[10]

Increased total phenols content in olive pastes caused a positive effect on the synthesis of volatile C6 ester *Z*-3-hexenyl acetate. Its mass fraction increased in enriched samples in the range from 49 to 95% (on average = 68%) with respect to the original sample. AAT, which is the enzyme responsible for the synthesis of volatile esters through the condensation of alcohols and acyl-CoA, seems to have maintained its activity despite increased levels of total phenols in olive pastes.[33] On the other hand, unaffected activity of AAT might contribute, at least partly, to the previously discussed decrease of *E*-2-hexen-1-ol in the oil samples obtained from phenols-enriched olive pastes.

A positive effect of increased phenols content in olive pastes was also evident in the mass fraction of all C5 compounds in oil samples. In most cases, a statistically significant increase of mass fraction with regard to the original sample appeared at enrichment levels of 38 and 56% and ranged from 27 to 81%. The average increase for total C5 compounds mass fraction was 32%. An increment of C5 volatiles content could be a consequence of increased homolytic activity of lipoxygenase. Vancanneyt et al. have reported a higher content of C5 compounds in the leaves of HPL-deficient transgenic line of potato plants and suggested lipoxygenase involvement in hydroperoxide cleavage.[34] Reduced HPL activity and accumulation of 13-hydroperoxides in enriched olive pastes could result in a higher mass fraction of C5 compounds in oils. Although C5 compounds are present in VOOs in a lower quantity than C6 aldehydes and alcohols, some of them could significantly contribute to VOO sensory attributes because of their relatively low odor threshold values.[3,6] For instance, the odor activity value of 1-pentene-3-one, which was positively related to bitterness and pungency, increased from 5.6 in the original sample to 8.3 in the sample enriched by 38% of phenols (Table 2). If a sum of all analyzed volatile compounds mass fraction is concerned, a reduction by 14, 20, or 22% with regard to the original sample is evidenced along with the increase of phenol enrichment level (data not shown). These results suggest that several enzymes in the LOX pathway might be partially deactivated by added phenolic compounds, particularly by oxidized phenols, as proposed by some authors.[11−13] These oxidation products could be formed during VOO production due to the activity of polyphenol oxidase and peroxidase.[35]

The content and composition of phenolic compounds in olive drupes, as well as of volatile compounds in olive oils, are influenced by the ripening level of olive fruits.[2,29] To check if there are differences in the responses of ripe and unripe fruits on an increased level of phenolic compounds, the experimental design was complemented by a trial with unripe Buža fruits (MI = 1.2), applying the phenols enrichment level of 38%. The total phenols content in the original olive paste samples obtained from ripe and unripe fruits was not significantly different (Table 3), although there were some differences in the composition. Green olive paste had significantly higher values of phenolic alcohols and lower values of verbascoside and flavonoids luteolin-7-*O*-glucoside, luteolin, and apigenin. Considering the contribution of a single compound to the total phenols

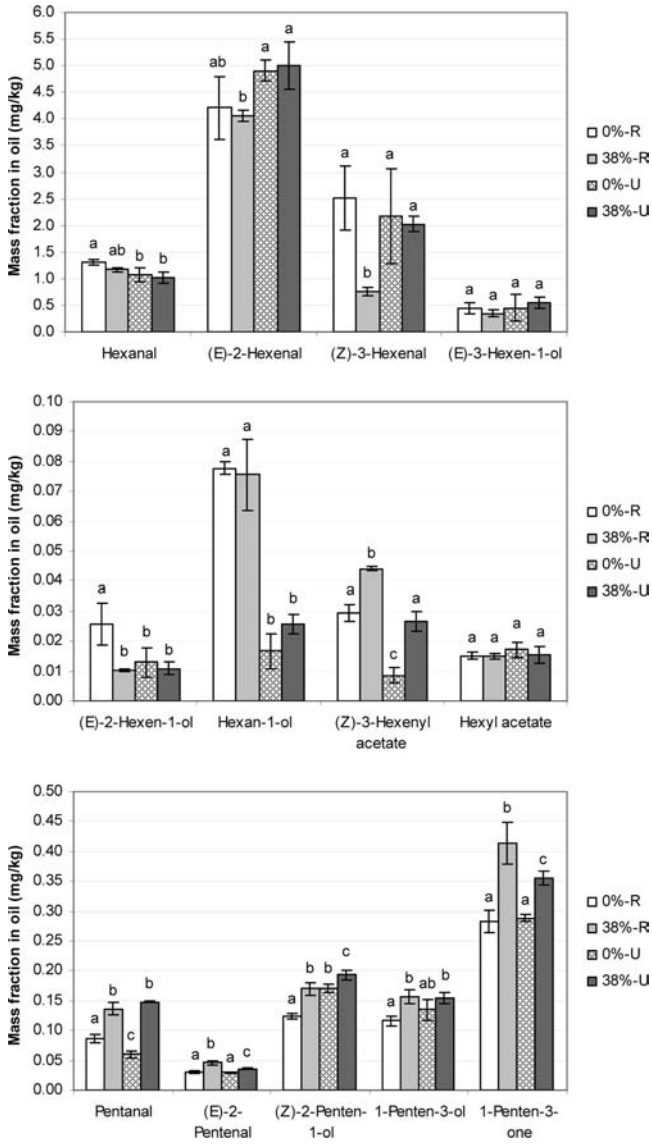

Figure 2. Mass fraction (mg/kg) of volatile compounds in Buža VOO samples obtained from ripe (R; maturity index = 4.0) and unripe (U; maturity index = 1.2) olive pastes enriched with phenol extract (level of olive paste enrichment = 0 and 38%; malaxation time = 45 min). Compounds are tentatively identified by comparing their mass spectra with mass spectra libraries Wiley 6 and NIST 21 and Kovats retention indices in the literature.[21−23] Results are means of four values (two independent repetitions of oil preparation × duplicate analyses) ± SD. Means within each volatile compound, marked with different letters, are significantly different (Tukey's test, $p < 0.05$).

mass fraction, the main differences between two ripening levels were observed for hydroxytyrosol (11.1% in unripe sample vs 8.7% in ripe sample) and verbascoside (5.1% in unripe sample vs 7.4% in ripe sample). The same as in the original samples, the total phenols content in enriched olive paste samples from ripe and unripe fruits was not significantly different. Also, a similar contribution of a single compound to the total phenols mass fraction can be observed. Thus, these olive pastes could be considered an appropriate medium for comparison of the influence of increased phenols content on volatile compounds in oils obtained from different fruit ripening degrees.

Results relating to volatile mass fraction are presented in Figure 2. Despite similar quantitative and qualitative phenolic

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profiles, the enrichment of the unripe olive paste sample by 38% of phenols content showed a weaker negative impact on analyzed volatile compounds in oil in comparison with the ripe sample. It can be seen that there is no statistically significant negative influence on *Z*-3-hexenal and *E*-2-hexen-1-ol mass fraction. The reasons for this different behavior remain unclear, but it should be stressed that similar results of C6 aldehydes and alcohols deriving from linolenic acid have been observed by Sánchez-Ortiz et al. in the case of synthetic phenol addition to ripe and unripe Arbequina and Picual olive pastes during malaxation.[9] On the other hand, similar to the oil sample from ripe fruits, a significant increase was found in the case of *Z*-3-hexenyl acetate (doubled value), as well as in the case of C5 compounds pentanal, *E*-2-pentenal, *Z*-2-penten-1-ol, and 1-penten-3-one. The total C5 compounds mass fraction increased by 30% compared to the original sample.

Results suggest that the phenols level in olive paste could modify the activity of single enzymes of the LOX pathway, although significant changes appear mainly at relatively high positive deviation (total phenols content higher for a third and more). Thus, an increased phenols mass fraction leads to higher levels of C6 esters and C5 compounds, although for most of them at concentrations in oil below their odor thresholds (Table 2). On the other hand, increased total phenols content in olive paste suppresses the synthesis of C6 aldehydes and alcohols in VOO obtained from ripe fruits. Therefore, during olive processing, attention should be given to circumstances that may lead to the increased level of phenols in olive paste, such as the reuse of vegetable water or the combination of cultivars with markedly different total phenols content.

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Notes

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ABBREVIATIONS USED

AAT, alcohol acyl transferase; ADH, alcohol dehydrogenase; DMO-Agl-dA, dialdehydic form of decarboxymethyl oleuropein aglycone; HPL, hydroperoxide lyase; KI, Kovats retention index; LOX, lipoxygenase; MI, maturity index; O-Agl-dA, dialdehydic form of oleuropein aglycone; OAV, odor activity value; RP HPLC, reverse phase high-performance liquid chromatography; SPME, solid-phase microextraction; Tyr, tyrosol; Tyr-OH, hydroxytyrosol; VOO, virgin olive oil

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