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## Labeled extra virgin olive oil as food supplement; phenolic compounds in oils from some autochthonous Croatian olives

C. Jakobušić Brala<sup>a,✉</sup>, Đ. Benčić<sup>b,✉</sup>, Z. Šindrak<sup>b</sup>, M. Barbarić<sup>a</sup> and S. Uršić<sup>a</sup><sup>a</sup>Faculty of Pharmacy and Biochemistry, University of Zagreb, A. Kovačića 1, 10 000 Zagreb, Croatia<sup>b</sup>Faculty of Agriculture, University of Zagreb, Svetošimunska 25, 10 000 Zagreb, Croatia✉Corresponding author: [cjakobus@pharma.hr](mailto:cjakobus@pharma.hr), [bencic@agr.hr](mailto:bencic@agr.hr)

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**SUMMARY:** Within the framework of an incentive to provide labeled extra virgin olive oils as a food supplement in pharmacies, the phenolic profile analysis of extra virgin olive oils obtained from Croatian olive cultivars has been reported. With the aim of increasing the consumption of EVOO-s in northern Croatia, the varieties Bjelica, Buža and Italian Leccino have been studied involving two different agroclimatic locations, over two harvest years differing significantly in the amount of rainfall. The Croatian cultivars Plominka, Žižolera, Oblica and Lastovka, were also examined. Correlation tests and the insight from PCA reveal that the cultivars are highly individualized in character with regard to relationships among phenolic compounds. Some elements of an innovative labeling aimed to better present the authenticity, quality, excellence and uniqueness of the EVOO-s were suggested.

**KEYWORDS:** *Autochthonous Croatian olive varieties; Extra virgin olive oil; Labeling; Phenolic compounds*

**RESUMEN:** *Aceites de oliva virgen extra etiquetados como suplemento alimenticio; compuestos fenólicos en aceites de aceitunas autóctonas croatas.* En el marco de los incentivos que se han considerado para proporcionar el etiquetado de aceites de oliva virgen extra como suplemento alimenticio en farmacias, se reporta el análisis del perfil fenólico de aceites de oliva vírgenes extra obtenidos a partir de variedades croatas. Para ampliar el consumo de AOVE-s en el norte de Croacia, se han estudiado las variedades Bjelica, Buža y Leccino italiana procedentes de dos lugares agroclimáticos diferentes que difieren significativamente en la cantidad de lluvia y obtenidos en dos cosechas. También fueron examinados los cultivares croatas Plominka, Žižolera, Oblica y Lastovka. Los test de correlación y los resultados de PCA revelan que las variedades están altamente individualizadas en su carácter en lo que respecta a las relaciones entre los compuestos fenólicos. Se sugirieron algunos elementos innovadores para un etiquetado dirigido a presentar mejor la autenticidad, la calidad, la excelencia y la singularidad de los AOVE-s.

**PALABRAS CLAVE:** *Aceite de oliva virgen extra; Compuestos fenólicos; Etiquetado; Variedades de aceitunas autóctonas croatas*

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## 1. INTRODUCTION

The evidence suggesting many beneficial health effects associated with the dietary consumption of extra virgin olive oils (EVOO-s) has steadily accumulated for decades. More recent studies and reviews, for example, by Oliveras-Lopez *et al.* (2014), Garcia-Martinez *et al.* (2014a, 2014b), Estruch *et al.* (2013), Sofi *et al.* (2010) and Fung *et al.* (2009) fit the same general picture presented by Keys (1970) and Martinez-Gonzales *et al.* (2002) and López-Miranda *et al.* (2010) or Lipworth *et al.* (1997). The phenolic compounds, naturally present in EVOO-s are deemed to be of central importance for these beneficial cardiovascular, according to Estruch *et al.* (2013), Fung *et al.* (2009), Valls *et al.* (2015), anti-atherogenic/antiinflammatory, Scoditti *et al.* (2014), Lu *et al.* (2013), Cabrerizo *et al.* (2013), anticancer, Hashim *et al.* (2008), Lamy *et al.* (2014), Hashim *et al.* (2014), antioxidant and antimicrobial effects Cicerale *et al.* (2012), Karaosmanoglu *et al.* (2010), Paiva-Martins *et al.* (2013), Giordano *et al.* (2014).

Olive cultivation and olive oil consumption are traditional in southern Croatia. In contrast to the millenary tradition on the Croatian coast and islands of the Adriatic, olive oil consumption is substantially lower in the northern continental part of Croatia. Although people in northern Croatia traditionally use sunflower oil more frequently than olive oil, a lack of enforced standards for EVOO in the consumer market also may play a role. As Clodoveo *et al.* (2014) noted, consumers place a high value on the accurate and defensible labeling of EVOO-s, also involving voluntary information such as the olive variety used. In order to extend the consumption of EVOO-s in northern Croatia, an incentive has been considered to provide labeled extra virgin olive oils as a food supplement in pharmacies, where a number of food supplements associated with some health effect expectations, such as blackberry wine, for example, had already been well marketed. This should involve an innovative labeling that includes, among others, the contents of essential phenolics such as hydroxytyrosol, tyrosol and oleuropein as well as total phenolics and possibly some important minor phenolics. In addition, according to prevailing practices and popular traditions in the country, extra virgin olive oils from autochthonous olive cultivars are preferred. Here, the results of the analysis of the phenolic profile of the extra virgin olive oils obtained from the three autochthonous Croatian olive cultivars, *Buža*, *Plominka* and *Žižolera*, and the domesticated cultivar *Bjelica*, being a part of the above mentioned incentive, are reported for the first time. Furthermore, the determination also involves two more autochthonous cultivars i.e. *Oblica* and *Lastovka* and the Italian cultivar *Leccino*. The investigation includes the biomedicinally most significant simple phenolic compounds, hydroxytyrosol and tyrosol, along with nine other simple phenolics

and the complex phenolic compound oleuropein in the oils from two successive crop years and different agroclimatic areas.

## 2. MATERIALS AND METHODS

### 2.1. Preparation of EVOO samples

Fruits from the autochthonous Croatian olive cultivar *Buža*, the domesticated (since 1929) cultivar *Bjelica* and the Italian cultivar *Leccino* (cultivated on the Istra peninsula) were collected during two successive olive-oil years, from two significantly different agroclimatic locations of the Istra peninsula, denoted as agroclimatic location “north”, N (Sovinjak, the northeastern Istra peninsula, about 300 m above the sea-level and 25 km from the sea coast) and agroclimatic location “south”, S (Pula/Barbariga and Vodnjan, the southern Istra peninsula, about 10 m above sea-level, close to the sea coast). Samples from the autochthonous olive cultivars *Plominka* from the island of Cres, *Žižolera* from the Istra peninsula (agroclimatic location “south”), *Oblica* and *Lastovka* from the island of Hvar, obtained in the second harvest year, were also examined. The two harvest years differ significantly regarding rainfall: the first year was extremely rainy and the second one suffered extreme drought. The fruits were hand-picked. Ordinarily, about 8–14 kg were taken from one olive tree. Olive trees were chosen randomly, though of similar age and with other apparent characteristics of olive trees. The samples from the same trees were taken in the successive olive-oil years for the sake of comparison. Only healthy fruits were processed within 24 hours after harvesting. The oil was obtained using a small extracting system (Olio mio-baby, Enologia Mori, Tuscany, Italy). Crushing, malaxation, mixing (30 min.) and the centrifuge separation was performed at a temperature no higher than 27–30 °C, using very small quantities of cold water or no water in the processing. The olive oil samples were stored in the dark in glass bottles.

### 2.2. Extraction of phenolic compounds

Phenolic compounds were extracted from the oil samples by methanol, according to the procedure of Owen *et al.* (2000). 20 g of EVOO were mixed with 4 mL of methanol for 30 min using a magnetic stirrer. The methanolic layer was separated by centrifugation, 30 min at 5000 rot·min<sup>-1</sup>. This procedure was repeated twice. The extracts were evaporated under reduced pressure and in a nitrogen atmosphere at 32 °C. The residues were suspended in acetonitrile (5 mL), and lipid substances were removed by shaking the suspension with hexane (10 mL). Acetonitrile was evaporated using a stream of nitrogen. The residues for HPLC analysis were stored at –20 °C.

### 2.3. HPLC analysis of phenolic compounds

The standard compounds gallic acid, tyrosol, 3,4-dihydroxybenzoic acid, *p*-hydroxybenzoic acid, homovanillyl alcohol, vanillic acid, caffeic acid, syringic acid, *p*-coumaric acid, ferulic acid and *o*-coumaric acid were obtained from Fluka, and oleuropein was from Extrasynthese (Z.I. Lion Nord, Genay). Hydroxytyrosol was prepared by the enzymatic hydroxylation of tyrosol using the mushroom tyrosinase (Espin *et al.*, 2001).

An HPLC analysis was performed in essence by applying the procedure of Owen *et al.* (2000). The HPLC system, consisting of a Spectra Physics chromatograph equipped with a 250×4.0 mm (5 μm) C<sub>18</sub> Restek column was used at room temperature. Dry extracts of the samples were dissolved in 1.0 mL methanol and 20 μL of the solution were injected into the system. The flow rate was 1 mL·min<sup>-1</sup>. The mobile phase used was 2% acetic acid in water (A) vs. methanol (B) for a total running time of 45 min. The gradient changes were: 95% A-5% B for 2 min, 75% A-25% B in 8 min, 60% A-40% B in 10 min, 50% A-50% B in 10 min, and 0% A-100% B until the end of the run. The UV absorption of eluates at 278 nm was detected. The retention times of the standards in this system were (min): Gallic acid, 9.69; hydroxytyrosol, 12.71; 3,4-dihydroxybenzoic acid, 14.15; tyrosol, 16.82; *p*-hydroxybenzoic acid, 18.85; homovanillyl alcohol, 19.09; vanillic acid, 21.13; caffeic acid, 21.57; syringic acid, 22.55; *p*-coumaric acid, 26.73; ferulic acid, 28.20; *o*-coumaric acid, 30.66 and oleuropein, 31.25. The phenolic compounds were identified by comparing their retention times with those of the standards. The corresponding concentrations of the compounds were determined from the integrated peak areas using the appropriate calibration curves and gallic acid as the internal standard. The recovery study was also carried out according to the method of Owen *et al.* (2000). The extraction method used gave recoveries in the range of 75–105% of the starting values. All EVOO samples were measured at least twice to ensure reproducibility.

### 2.4. Statistical analysis

Different statistical techniques such as analysis of variance (ANOVA) using Duncan's multiple range test ( $p < 0.05$ ), Pearson's correlation test ( $p < 0.01$ ;  $p < 0.05$ ) and multivariate analysis based on principal component analysis (PCA) were carried out to evaluate cultivar, crop year and location dependent differences regarding the phenolic compound composition and interrelations among them. Results are shown as the mean values and standard deviations of *n* EVOO samples (*n* was at least 3, commonly 5). The statistical analysis was performed using the XLSTAT 2011 software, version 13.2.05 (Addinsoft).

## 3. RESULTS AND DISCUSSION

The selected data obtained during the investigation of the phenolic fraction of the EVOO-s from the autochthonous Croatian olive cultivars *Buža*, *Plominka*, *Žižolera*, *Oblica* and *Lastovka*, the domesticated (since 1929.) cultivar *Bjelica* and the Italian cultivar *Leccino* (cultivated at the Istra peninsula) are given in Tables 1 and 2. The phenolic profiles of the EVOO-s from the autochthonous cultivars *Buža*, *Plominka*, *Žižolera* and the domesticated cultivar *Bjelica* were examined for the first time. All the EVOO-s examined were obtained from conventional cultivation. In Table 1 the data obtained for the EVOO-s of *Bjelica*, *Buža* and *Leccino* cultivars for the two olive-oil years are presented. The data enable a comparison of the phenolic contents of the EVOO-s of *Bjelica*, *Buža* and *Leccino* cultivars with regard to one rainy and a successive drought olive-oil year, also involving two significantly different agroclimatic locations. The locations (Istra peninsula) differ in their height above sea-level and their distance from the sea *i.e.* the southern location (Pula/Barbariga and Vodnjan), is about 10 m above sea-level and close to the sea coast and the northern location (Sovinjak), is 300 m above sea-level and 25 km from the sea coast. In Table 2, the results from one crop year (corresponding to the second year denoted in Table 1) for the autochthonous cultivars *Plominka*, *Žižolera*, *Oblica* and *Lastovka* are presented.

The data presented in the Tables reveal the commonly observed difference in the contents of hydroxytyrosol and tyrosol, and other simple phenolics; whereas concentrations of hydroxytyrosol are in the range 1–12 mg·kg<sup>-1</sup> and tyrosol between 3 and 140 mg·kg<sup>-1</sup> (the latter value in the case of two samples), “minor” phenolics appeared in most cases in concentrations less than 1 mg·kg<sup>-1</sup> of the oil. The concentrations of hydroxytyrosol in the EVOO-s of *Bjelica* and *Buža* are in a commonly observed range, found for instance by Tura *et al.* (2007), Šarolić *et al.* (2014), Ramos-Escudero *et al.* (2015), Bajoub *et al.* (2015), and Ballus *et al.* (2015). Hydroxytyrosol and tyrosol in the EVOO of *Leccino* are present in concentrations similar to that obtained by Šarolić *et al.* (2014), for the same cultivar from the city of Zadar (about 200 km to the south-east of the Istra peninsula). The total phenolic content is effectively the same in that case (*Leccino*, Zadar 246 mg·kg<sup>-1</sup>) as for *Leccino* in the rainy crop year in this study (254 mg·kg<sup>-1</sup>). The concentrations of the complex phenolic compound oleuropein are in the range of 1.5 and 4 mg·kg<sup>-1</sup> and could be similar, for example, to those reported by O. Garcia-Martinez *et al.* (2014a), for the Sicilian varieties Biancolilla, Passulunara, Tonda iblea etc., or Ilyasoglu *et al.* (2010), for the Turkish variety *Ayvalik*.

It could be seen that phenolic profile is characteristic of a particular olive cultivar. This can also

TABLE 1. Phenolic compounds (mg·kg<sup>-1</sup>) in virgin olive oils from three cultivars from Croatia: Bjelica, Buža, Leccino, from two agroclimatic locations in two harvest years

Phenolic compound (mg·kg <sup>-1</sup> )	A.L.	1 <sup>st</sup> year			2 <sup>nd</sup> year		
		Bjelica	Buža	Leccino	Bjelica	Buža	Leccino
Hydroxytyrosol	S	2.15 <sup>c,d</sup> (1.64)	3.36 <sup>c</sup> (2.49)	1.27 <sup>d,e</sup> (1.67)	12.10 <sup>a</sup> (2.26)	2.48 <sup>c,d</sup> (1.08)	8.67 <sup>b</sup> (4.71)
	N	0.08 <sup>e</sup> (0.03)	5.00 <sup>c,d,e</sup> (6.09)	0.25 <sup>e</sup> (0.30)	8.67 <sup>b</sup> (3.88)	3.58 <sup>e</sup> (2.02)	2.06 <sup>c,d</sup> (1.43)
Tyrosol	S	59.49 <sup>a</sup> (18.37)	6.28 <sup>f</sup> (1.51)	13.73 <sup>e</sup> (5.94)	27.83 <sup>b</sup> (8.67)	4.70 <sup>f</sup> (0.75)	17.68 <sup>d</sup> (6.63)
	N	21.77 <sup>c</sup> (12.23)	11.97 <sup>e</sup> (7.55)	10.19 <sup>f</sup> (10.08)	11.62 <sup>e</sup> (5.64)	2.66 <sup>f</sup> (0.59)	2.85 <sup>f</sup> (0.92)
3,4-dihydroxy benzoic acid	S	0.21 <sup>b</sup> (0.11)	0.16 <sup>b,c</sup> (0.01)	2.17 <sup>b,c</sup> (3.12)	0.03 <sup>d,e</sup> (0.00)	0.03 <sup>e</sup> (0.01)	0.04 <sup>c,d,e</sup> (0.02)
	N	0.13 <sup>c,d,e</sup> (0.01)	0.27 <sup>a</sup> (0.18)	0.15 <sup>b,c,d</sup> (0.05)	0.04 <sup>c,d,e</sup> (0.03)	0.07 <sup>c,d,e</sup> (0.04)	0.06 <sup>c,d,e</sup> (0.05)
<i>p</i> -hydroxy benzoic acid	S	0.43 <sup>a</sup> (0.14)	0.32 <sup>b</sup> (0.04)	0.16 <sup>d</sup> (0.09)	0.10 <sup>e</sup> (0.07)	0.25 <sup>e</sup> (0.20)	0.03 <sup>f</sup> (0.02)
	N	0.21 <sup>b,c,d</sup> (0.13)	0.22 <sup>e</sup> (0.03)	0.23 <sup>e</sup> (0.14)	0.02 <sup>f</sup> (0.01)	0.04 <sup>f</sup> (0.02)	0.01 <sup>f</sup> (0.01)
Homovanillyl alcohol	S	1.17 <sup>a</sup> (0.49)	0.32 <sup>d,e,f</sup> (0.26)	0.70 <sup>a,b</sup> (0.37)	0.41 <sup>c,d</sup> (0.14)	0.47 <sup>c,d</sup> (0.10)	0.44 <sup>c,d</sup> (0.11)
	N	0.39 <sup>c,d,e</sup> (0.01)	0.64 <sup>b</sup> (0.12)	0.80 <sup>a</sup> (0.35)	0.25 <sup>d,e,f</sup> (0.03)	0.59 <sup>b,c</sup> (0.09)	0.16 <sup>f</sup> (0.05)
Vanillic acid	S	1.02 <sup>c,d</sup> (0.43)	2.20 <sup>a</sup> (0.58)	1.29 <sup>b</sup> (0.41)	0.60 <sup>e</sup> (0.13)	1.20 <sup>b,c,d</sup> (0.26)	2.04 <sup>a</sup> (0.51)
	N	0.48 <sup>e</sup> (0.05)	1.26 <sup>b,c</sup> (0.41)	1.45 <sup>b</sup> (0.67)	0.48 <sup>e</sup> (0.16)	0.84 <sup>d,e</sup> (0.10)	0.93 <sup>d</sup> (0.71)
Caffeic acid	S	0.24 <sup>b</sup> (0.21)	0.33 <sup>b</sup> (0.16)	0.10 <sup>d,e</sup> (0.08)	0.15 <sup>c,d</sup> (0.04)	n.a.	0.16 <sup>c,d</sup> (0.19)
	N	0.09 <sup>d,e</sup> (0.09)	0.23 <sup>b,c</sup> (0.04)	0.10 <sup>d,e</sup> (0.04)	0.08 <sup>d,e</sup> (0.05)	0.03 <sup>e</sup> (0.03)	0.03 <sup>e</sup> (0.01)
Syringic acid	S	0.36 <sup>e</sup> (0.22)	0.79 <sup>a</sup> (0.29)	0.58 <sup>e</sup> (0.18)	0.01 <sup>g</sup> (0.01)	0.40 <sup>d</sup> (0.04)	0.08 <sup>g</sup> (0.09)
	N	0.21 <sup>e,f</sup> (0.01)	0.63 <sup>b,c</sup> (0.08)	0.67 <sup>b</sup> (0.12)	0.09 <sup>f,g</sup> (0.15)	n.a.	0.04 <sup>g</sup> (0.04)
<i>p</i> -coumaric acid	S	0.40 <sup>c</sup> (0.18)	0.30 <sup>d,e</sup> (0.16)	0.09 <sup>h</sup> (0.04)	0.53 <sup>b</sup> (0.08)	0.22 <sup>f</sup> (0.04)	0.11 <sup>g</sup> (0.05)
	N	0.23 <sup>e,f</sup> (0.09)	0.23 <sup>e,f</sup> (0.07)	0.08 <sup>h</sup> (0.04)	0.36 <sup>c,d</sup> (0.20)	n.a.	0.12 <sup>g</sup> (0.04)
Ferulic acid	S	0.59 <sup>a</sup> (0.28)	0.47 <sup>a</sup> (0.30)	0.31 <sup>b</sup> (0.20)	0.42 <sup>a,b</sup> (0.15)	0.26 <sup>b</sup> (0.10)	0.25 <sup>b</sup> (0.16)
	N	0.50 <sup>a</sup> (0.09)	0.51 <sup>a</sup> (0.04)	0.33 <sup>b</sup> (0.08)	0.57 <sup>a</sup> (0.01)	n.a.	0.14 <sup>b</sup> (0.06)
<i>o</i> -coumaric acid	S	0.27 <sup>a</sup> (0.23)	n.a.	0.27 <sup>a</sup> (0.23)	n.a.	n.a.	n.a.
	N	0.07 <sup>a,b</sup> (0.02)	0.28 <sup>a</sup> (0.08)	0.05 <sup>c</sup> (0.04)	n.a.	n.a.	0.34 <sup>a</sup> (0.18)
Oleuropein	S	2.37 <sup>a,b</sup> (1.84)	n.a.	3.49 <sup>a</sup> (3.23)	2.89 <sup>a,b</sup> (0.66)	1.07 <sup>b</sup> (0.44)	3.35 <sup>a</sup> (2.31)
	N	1.53 <sup>b</sup> (0.35)	n.a.	3.02 <sup>a,b</sup> (0.86)	4.18 <sup>a</sup> (3.15)	3.18 <sup>a,b</sup> (0.26)	3.10 <sup>a,b</sup> (2.06)
Total phenols***	S	516	301	254	1589	1246	1230
	N	419	365	254	1481	1071	1339
Ripening index	S	1.33	3.73	3.53	1.2	4.56	4.87
	N	1.27	4.57	4.13	1.2	3.97	4.36

A.L., agroclimatic location; S, south (about 10 m above sea level); N, north (about 300 m above sea level); n.a., not accessed, \*from Šindrak *et al.* (2007), \*\*mg caffeic acid equivalent·kg<sup>-1</sup> olive oil. Results are means of at least three VOO values (standard deviation), the means within each row (including both S,N producing area), labeled by different letters, are significantly different (Duncan's test,  $p \leq 0.05$ ). Some data are missing, related to poorly defined peaks in chromatograms, due to the presence of other complex phenolic compounds.

be seen from the correlation test and PC analysis (see below). Thus, although the ripening index was significantly lower in the case of the cultivar *Bjelica* than that for *Buža* and *Leccino*, both hydroxytyrosol and tyrosol and total phenolic content of *Bjelica* are greater than those for *Buža* and *Leccino* and this holds for the rainy and drought crop year as well. Furthermore, while the observed content of hydroxytyrosol and total phenolics for cultivars *Bjelica* and *Leccino* were much greater in the drought than in the rainy year, the opposite could be seen for tyrosol and there is a difference in the content of hydroxytyrosol with regard to the *Buža* cultivar. Following an

investigation comprising four successive crop seasons, Romero *et al.* (2003) concluded that phenolic profiles were mainly influenced by cumulative rainfall. Similarly, Ilyasoglu *et al.* (2010) found that the total phenolic contents of some Aegean VOO-s are greater in the drought than in the rainy crop year, but their observed range of variation is much smaller than that observed in this study. It seems that the total phenolic content could depend less on the plantation area but is much more characteristic of the cultivar and strongly depends on the climate characteristics of the year (see also the above mentioned case of *Leccino*). In this context some findings of



TABLE 2. Phenolic compounds (mg·kg<sup>-1</sup>) in virgin olive oils from the Croatian cultivars Plominka, Žižolera, Oblica and Lastovka

Phenolic compound <sup>a</sup> (mg·kg <sup>-1</sup> )	Plominka <sup>b</sup>	Žižolera <sup>c</sup>	Oblica <sup>d</sup>	Lastovka <sup>d</sup>
Hydroxytyrosol	7.22	5.07	6.24	16.29
Tyrosol	5.26	4.35	32.61	12.46
3,4dihydroxybenzoic acid	0.02	0.16	0.37	n.a.
<i>p</i> -hydroxybenzoic acid	0.05	0.10	n.a.	n.a.
Homovanillyl alcohol	0.25	0.35	0.72	0.30
Vanillic acid	1.90	0.56	0.18	0.30
Caffeic acid	0.17	0.09	n.a.	n.a.
Syringic acid	0.02	0.05	n.a.	n.a.
<i>p</i> -coumaric acid	0.32	0.43	0.81	0.80
Ferulic acid	n.a.	n.a.	0.52	0.24
Oleuropein	7.61	3.97	2.37	n.a.

<sup>a</sup>2<sup>nd</sup> year; <sup>b</sup>island Cres; <sup>c</sup>Pula (agroclimatic location south), about 300 year old trees; <sup>d</sup>island Hvar

Tura *et al.* (2009) may be of interest; they suggested that the cultivation sites did not affect oil composition and profile of EVOO of the same cultivar in the same crop year. However, Table 1 shows the concentrations of a majority of individual phenols in the EVOO-s to be greater in oils from the southern location. This seems not to be unexpected since environments where olive trees grow may likely influence plant physiology as well as the olive ripening process and subsequently the phenolic profiles. Here, it might be noted that, for example, the finding of a total phenolic content of 1589 mg·kg<sup>-1</sup> of EVOO in the *Bjelica* cultivar from Pula (Barbariga) is consistent with the historically well known great appreciation that the olive oil from the Istra peninsula received from the ages of the Roman Empire. To emphasize this issue, as well as, for example, the unusually high concentration of tyrosol in the EVOO (see below) could be additional line within the intended framework for a more innovative labeling of EVOO aimed at being presented as a food supplement.

The examined EVOO-s from the cultivar *Bjelica* have very high concentrations of tyrosol in some cases. While the observed concentrations range from 3 mg·kg<sup>-1</sup> to 140 mg·kg<sup>-1</sup> of tyrosol, the reported average value of the tyrosol content in VOO-s from the 23 samples from Spain, Italy, Greece and Tunisia (Hrncirik *et al.*, 2004) is tenfold or even lower than our upper value for this phenol. This upper value presents one of the highest observed contents of tyrosol in an EVOO and values well below 13 mg·kg<sup>-1</sup> of tyrosol were often reported (Ramos-Escudero *et al.*, 2015, Bajoub *et al.*, 2015, Ballus *et al.*, 2015). Given the great biomedical potential of tyrosol suggested which, among others involves antioxidants, Cicerale *et al.* (2012), neuroprotective, Vauzour *et al.* (2010), cardiovascular Nakbi *et al.* (2011), antimicrobial, Karaosmanoglu *et al.* (2010), anti-inflammatory, Lu *et al.* (2013), Cárdeno *et al.* (2014), antigenotoxic,

Anter *et al.* (2014), to mention only a few, future selection of the olives based on the presented results could be of use to obtain oils which are more abundant in particular simple phenol compounds.

The presence of biomedically important “minor” phenolic compounds such as *p*-coumaric, caffeic acid, homovanillyl alcohol, vanilic acid and others in the examined EVOO-s (see Table 1 and 2) should be of particular interest. Because of, for instance, beneficial anticancer effects, Nichenametla *et al.* (2006), anti-inflammatory potential, Pragasam *et al.* (2013) potential beneficial effects in treating metabolic disorders, Yoon *et al.* (2013), antibacterial effects, Lou *et al.* (2012) and a possible preventive effect on myocardial infarct size, Roy *et al.* (2013) of *p*-coumaric acid, or the beneficial effects of caffeic acid, El Seedi *et al.* (2012), Vauzour *et al.* (2010), da Cunha *et al.* (2004), these and other “minor” phenolics should be an inevitable part in the new labeling framework.

Here, a feasibility of using a back-label on EVOO bottles to additionally quote scientific references (perhaps including citation of short statements from such references) is under consideration. Namely, in the case of olive oil, European Regulation 432/2012 states that the health effect can be claimed only if the oil contains more than 5mg of hydroxytyrosol and its derivatives (e.g., oleuropein complex and tyrosol) in 20 g of oil, but the quotation of scientific references is not explicitly forbidden.

The phenolic profiles of EVOO-s of the autochthonous cultivars *Plominka*, *Žižolera*, *Oblica* and *Lastovka* (Table 2) are not very different from those of *Buža* and *Bjelica*. It could be noted that *Oblica* is the most represented olive variety in Croatia. Table 2 shows fairly high concentrations of tyrosol, hydroxytyrosol and *p*-coumaric acid both in EVOO-s of *Oblica* and *Lastovka*. The case of *Žižolera* could be of some interest because oil from three hundred year olives was examined. Namely, there are at the

island of Pag (Lun, about 50 km to the south-east of the Istra peninsula) about 1000 very old olive trees (mainly of the autochthonous Croatian varieties *Mastrinka* and *Oblica*); the majority of these olives comprise trees older than 700 years, and there is also a sizable group of trees more than 1000 years old. Their location is of special interest within the framework of the incentive mentioned at the beginning. It should be noted that the European Community (EC) Council of Regulation established compulsory standards for olive oil production with regard to labeling (European Commission Regulation 182/2009, *cf.* in references); the regulation has compelling virgin and extra-virgin olive oil producers to indicate the geographical location of

olive harvesting and oil production on the label, but the age of the olive trees is not involved. There are thousands of very old autochthonous olive orchards across the Croatian Adriatic coast and islands.

A correlation test involving data obtained from the analysis of the phenolic profile of EVOO-s from the cultivars *Bjelica*, *Buža* and *Leccino* (Tables 1, 3, 4, 5) shows significant characteristic correlation profiles pertaining to a particular cultivar. It could be speculated that the relationships among phenolic compounds reflect more complex relationships between genetic and biosynthetic patterns, thus describing the “identity” of a particular olive variety.

Recently, Tura *et al.* (2007) and El Riachy *et al.* (2012a) reported the correlation between tyrosol

TABLE 3. Pearson correlation among individual phenols, data obtained for the cultivar *Bjelica*

	OHTyr	tyr	3,4OHb	pOHb	hva	va	ca	sa	pcoa	fa	ole
<b>OHTyr</b>	1										
<b>tyr</b>	n.s.	1									
<b>3,4OHb</b>	n.s.	n.s.	1								
<b>pOHb</b>	n.s.	n.s.	0.827**	1							
<b>hva</b>	n.s.	0.827**	0.822**	0.916**	1						
<b>va</b>	n.s.	n.s.	n.s.	0.698**	n.s.	1					
<b>ca</b>	n.s.	n.s.	n.s.	0.800**	n.s.	0.795**	1				
<b>sa</b>	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	1			
<b>pcoa</b>	n.s.	n.s.	n.s.	n.s.	n.s.	0.761**	n.s.	n.s.	1		
<b>fa</b>	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	0.695**	1	
<b>ole</b>	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	1

n.s., not significant; \*\*, significant at  $p \leq 0.01$ .

OHTyr, hydroxytyrosol; tyr, tyrosol; 3,4OHb, 3,4dihydroxybenzoic acid; pOHb, *p*-hydroxybenzoic acid; hva, homovanillyl alcohol; va, vanillic acid; ca, caffeic acid; sa, syringic acid; pcoa, *p*-coumaric acid; fa, ferulic acid; ole, oleuropein.

TABLE 4. Pearson correlation among individual phenols, data obtained for the cultivar *Buža*

	OHTyr	tyr	3,4OHb	pOHb	hva	va	ca	sa	pcoa	fa	ole
<b>OHTyr</b>	1										
<b>tyr</b>	n.s.	1									
<b>3,4OHb</b>	n.s.	0.963**	1								
<b>pOHb</b>	n.s.	n.s.	n.s.	1							
<b>hva</b>	n.s.	n.s.	n.s.	n.s.	1						
<b>va</b>	n.s.	n.s.	n.s.	n.s.	n.s.	1					
<b>ca</b>	n.s.	n.s.	n.s.	0.824**	n.s.	n.s.	1				
<b>sa</b>	n.s.	n.s.	n.s.	n.s.	n.s.	0.751**	n.s.	1			
<b>pcoa</b>	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	0.661*	1		
<b>fa</b>	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	0.727*	0.800**	n.s.	1	
<b>ole</b>	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	1

n.s., not significant; \*, significant at  $p \leq 0.05$ ; \*\*, significant at  $p \leq 0.01$ .

OHTyr, hydroxytyrosol; tyr, tyrosol; 3,4OHb, 3,4dihydroxybenzoic acid; pOHb, *p*-hydroxybenzoic acid; hva, homovanillyl alcohol; va, vanillic acid; ca, caffeic acid; sa, syringic acid; pcoa, *p*-coumaric acid; fa, ferulic acid; ole, oleuropein.

TABLE 5. Pearson correlation among individual phenols, data obtained for the cultivar Leccino

	OHTyr	tyr	3,4OHb	pOHb	hva	va	ca	sa	pcoa	fa	ole
OHTyr	1										
tyr	n.s.	1									
3,4OHb	n.s.	0.400*	1								
pOHb	n.s.	n.s.	n.s.	1							
hva	n.s.	n.s.	n.s.	0.702**	1						
va	n.s.	n.s.	n.s.	n.s.	0.653**	1					
ca	n.s.	n.s.	n.s.	n.s.	0.588**		1				
sa	n.s.	n.s.	n.s.	0.593**	0.666**	0.899**	n.s.	1			
pcoa	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	0.764**	1		
fa	n.s.	n.s.	n.s.	n.s.	n.s.	0.741**	n.s.	n.s.	n.s.	1	
ole	n.s.	n.s.	n.s.	n.s.	n.s.	0.798**	n.s.	0.875**	n.s.	0.946**	1

n.s., not significant; \*, significant at  $p \leq 0.05$ ; \*\*, significant at  $p \leq 0.01$ .

OHTyr, hydroxytyrosol; tyr, tyrosol; 3,4OHb, 3,4-dihydroxybenzoic acid; pOHb, *p*-hydroxybenzoic acid; hva, homovanillyl alcohol; va, vanillic acid; ca, caffeic acid; sa, syringic acid; pcoa, *p*-coumaric acid; fa, ferulic acid; ole, oleuropein.

and vanillic acid, and El Riachy *et al.* (2012b) the correlation between vanillic acid and *p*-coumaric acid, ascribing the findings to the same biosynthetic pathway of these compounds.

A principal component analysis (PCA) also was carried out in an attempt to explore some underlying pattern that may be taken as a source of variation in EVOO phenolic compound composition.

The data set for different cultivars, involving two different agroclimatic locations and obtained over two harvest years that differ significantly in the amount of rainfall, regarding the variables of phenolics was subjected to PCA to investigate possible grouping of samples on the basis of cultivar, harvest year and agroclimatic location.

PCA showed that 68% of the total variance was associated with the first three principal components. The first component accounted for 33.5% of the total variance, with a high positive correlation with 3,4-dihydroxybenzoic acid, *p*-hydroxybenzoic acid, homovanillyl alcohol and syringic acid; the second component accounted for 21.5% of the total variance and was positively correlated to tyrosol, hydroxytyrosol, *p*-coumaric and ferulic acid and the third component accounted for 13.4% of the total variance with a high positive correlation with vanillic and caffeic acid.

The biplot on PC1 and PC2 (Figure 1), explaining 55% of the total variance, allows for differentiation of the two groups according to harvest year mainly on the basis of the first principal component values. Almost all EVOO samples from the rainy harvest year have positive scores for the first component, while almost all drought year EVOO samples have negative scores. There is also obvious differentiation taking into regard cultivar, mainly on the basis of the second principal component values. All EVOO

samples corresponding to the *Bjelica* cultivar have positive scores for the second component, while *Buža* and *Leccino* samples have negative scores. Furthermore, there is differentiation between *Buža* and *Leccino* samples, with *Buža* samples found between *Bjelica* and *Leccino*. The biplot on PC1 and PC3 (Figure 2), explaining 47% of the total variance, also shows good separation of samples regarding harvest year, while in the biplot on PC2 and PC3 (Figure 3), explaining 35% of the total variance, the grouping is less obvious. Regarding cultivar, the biplot on PC2 and PC3 shows good grouping of the *Bjelica* cultivar and some overlap between *Leccino* and *Buža*; while in the

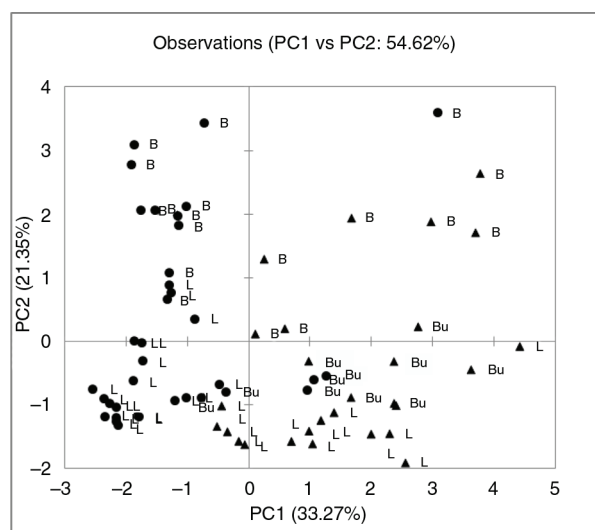


FIGURE 1. The score-plot of samples from principal component analysis (PC1 vs PC2); rainy year (▲), drought year (●); B, *Bjelica*; Bu, *Buža*; L, *Leccino*.

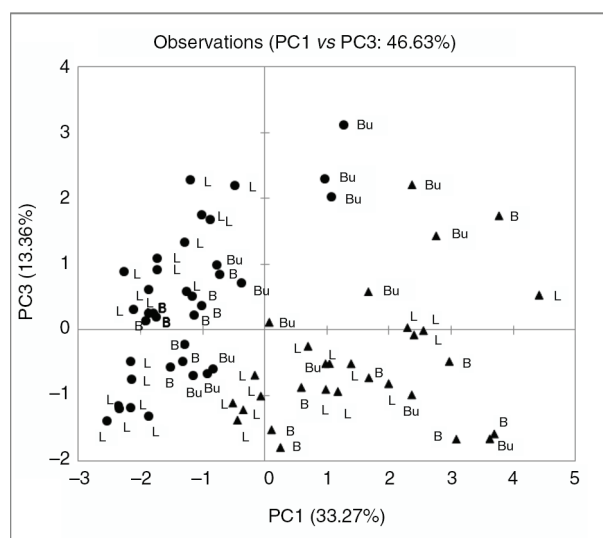


FIGURE 2. The score-plot of samples from principal component analysis (PC1 vs PC3); rainy year (▲), drought year (●); B, Bjelica; Bu, Buža; L, Leccino.

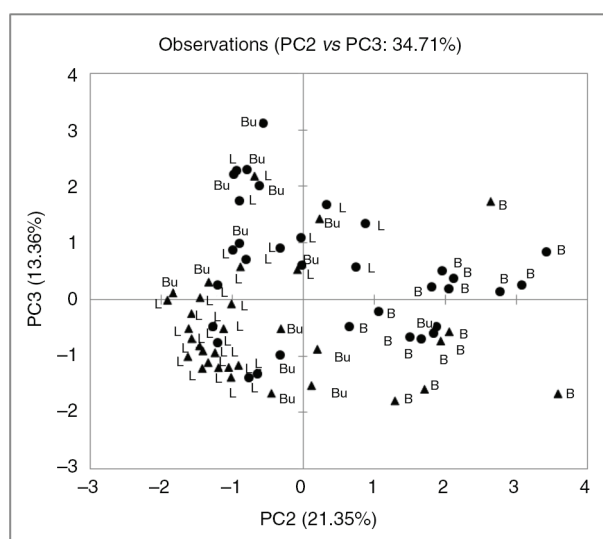


FIGURE 3. The score-plot of samples from principal component analysis (PC2 vs PC3); rainy year (▲), drought year (●); B, Bjelica; Bu, Buža; L, Leccino.

biplot on PC1 and PC3 there is no obvious grouping of different cultivars. Interestingly, and consistent with some of the mentioned considerations (see above) regarding agroclimatic location, there is no significant differentiation in the score plots.

#### 4. CONCLUSIONS

Phenolic profile analyses of the extra virgin olive oils obtained from the three autochthonous Croatian olive cultivars, *Buža*, *Plominka* and *Žižolera*, and the

domesticated cultivar *Bjelica*, are reported for the first time. The analyses have been performed within the framework of an incentive that has been considered to provide labeled extra virgin olive oils as a food supplement in pharmacies, in order to extend the consumption of EVOO-s in northern Croatia. The obtained data, also involving a correlation test and the insight from PCA reveal that the cultivars are highly individualized in character with regard to relationships among phenolic compounds. This finding as well as the observation of very significant differences in the content of phenolic compounds between the rainy and successive drought crop years will be of importance for targeted acquisition and perhaps the price of the oils. The observation of an extremely high concentration of tyrosol in some cases can be useful not only for future selection of the olives to obtain oils which are more abundant in a particular simple phenol compound. While the information on the most important phenolic compounds and the total phenolic content should be a part of the main label, on the back-label of EVOO bottles appropriate biomedicinally important scientific references (perhaps including citation of short statements from such references) with regard to selected and especially abundant phenolic compound(s) in the oil could be additionally quoted. The information on the variety used, the age of very old olive trees, the location of orchard and the harvest year should follow a tendency toward an innovative labeling aimed at better presenting the authenticity, quality, excellence and uniqueness of the EVOO-s. Finally, the issue reported also seems to be consistent with the The Framework Programme for Research and Innovation - 2014-2020 of the European Commission, promoted as a research program aimed at preserving the image of olive oil and to guarantee its quality and authenticity. A very important issue related to the uniqueness, authenticity and quality of EVOO-s is the question of the aromatic profile of oils (Reboredo-Rodríguez *et al.*, 2012, 2013a,b,c, 2014a,b); although not elaborated in this study, it should be an unavoidable part of subsequent future work in the matter.

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