Short Note

Phenolic composition of extra virgin olive oil samples from Istria (Croatia)

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Croatia is a Mediterranean country with a widespread production of olive oil in three different regions: Istria, Kvarner and Dalmatia. Each region has specific olive cultivars and different traditions in olive oil production. The objective of this study was to characterise Istrian olive oils obtained from local cultivars based on their bioactive components. Therefore, for the first time, we determined specific phenolic compounds in Istrian olive oils by using liquid chromatography coupled to triple the quadrupole mass spectrometer (LC-QQQ) and the antioxidant activity by DPPH assay.
Results revealed that secoiridoid derivatives are the most represented phenolic compounds in Istrian olive oils. Among them, 3,4-DHPEA-EA was presented at the highest concentrations, ranging from 13.15±1.30 to 132.42±1.49 mg/kg. In all samples, a high content was also found for p-HPEA-EA and 3,4-DHPEA-EDA with concentrations ranging from 4.82±0.33 to 68.69±15.89 mg/kg and from 2.93±0.11 to 28.11±1.41 mg/kg, respectively. Additionally, the antioxidant capacity was reported in the range from 0.66±0.05 to 4.12±0.06 mmol TE/kg. Principal component analysis (PCA) on obtained results indicated that oleuropein aglycon derivatives are the most important phenols for the antioxidant activity of Istrian olive oils.

Keywords: Extra virgin olive oil, LC-QQQ, Antioxidant capacity, Secoiridoid derivatives.

1. INTRODUCTION

Extra virgin olive oil (EVOO) is the most commonly used type of oil in Mediterranean countries notably responsible for well-known the health benefits of the Mediterranean diet. This finding is attributed to high diversity of bioactive constituents i.e. phenols that have proven wide spectrum of positive effects on human health [1]. Additionally, it was shown that these constituents can impact the oxidative state of oils and influence their organoleptic properties [2]. However, it is important to mention that the polyphenol composition in plants is usually highly affected by climate changes and by the geographical region [3], suggesting that same plants from different regions can have different biological and pharmacological effects. Additionally, when talking about olive oils specifically, production process, as well as the time of harvesting and olive cultivar, also have a strong influence on olive oil quality and affect the phenolic composition [4-7].
Croatia is one of the Mediterranean countries with a long-established olive oil production. There are three main geographical regions that are putting their EVOOs on the markets: Istria, Kvarner and Dalmatia. In our previous work [8], we showed that there are differences between EVOOs in these regions regarding triacylglycerol and the fatty acid profile. Also, we showed that this region specificity can be easily and quickly determined by using the Near-infrared spectroscopy (NIRS). However, here we wanted to analyse minor constituents

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as well, i.e. phenolic compounds to add additional value to Croatian EVOOs. For this task, we selected Istrian EVOO samples made from autochthonous or the most represented olive cultivars.
Furthermore, since the phenolic profile of EVOOs from Istria was not studied extensively, but only selected phenols with commercially available standards [9], we wanted to determine all secoiridoid derivatives for which it was previously shown that they have high shares in olive oils: monoaldehydic form of oleuropein-aglycone (3,4-DHPEA-EA), dialdehydic form of decarboxymethyl oleuropein aglycone, also known as oleacein (3,4-DHPEA-EDA), dialdehydic form of decarboxymethyl ligstroside aglycone or oleocanthal (p-HPEA-EDA), monoaldehydic form of ligstroside aglycone (p-HPEA-EA) and hydroxytyrosol acetate (3,4-DHPEA-AC) [10,11]. The only paper where all secoiridoid derivatives were analysed in Croatian EVOOs was from Šarolić et. al. (2015), where authors analysed Croatian monovarietal EVOOs from Oblica, Lastovka and Levantinka [12]. However, these olive cultivars are specific for another Croatian geographical region – Dalmatia, and the results obtained on these samples can't be transferred to Istrian olive oils, considering all previously mentioned facts on the influence of different factors on EVOOs phenolic composition.
In this paper, quality parameters were first determined for all olive oil samples. Then, liquid chromatography (LC) coupled to triple-quadrupole mass spectrometry (QQQ) was used for the identification and quantitative analysis of phenols with commercially available standards. For the analysis of secoiridoid derivatives, the LC-QQQ method was developed using Precursor and Product ion scan modes due to the absence of commercial standards. Lastly, the phenolic profile was completed with antioxidant activity determined with DPPH assay.

2. MATERIALS AND METHODS

2.1. SAMPLES

23 Istrian olive oil samples from the harvest year 2015 were collected directly from producers. To best represent tradition of the cultivation area, olive oils obtained from single olive cultivars or a mixture of cultivars were selected. Quality parameters were determined for all samples.

2.2. CHEMICALS AND MATERIALS

Hydroxytyrosol, vanillic acid, pinoresinol, luteolin, oleuropein, 3,4-dihydroxybenzoic acid (3,4-DHBA) and tyrosol were purchased from Sigma Aldrich (St. Louis, MO, USA). Gallic acid was obtained from Alfa Aesar (Thermo Fischer Scientific, Massachusetts, SAD).
Chemical reagents, methanol (HPLC grade) and acetonitrile (LC-MS grade) were supplied by Honeywell. Research chemicals, formic acid (LC-MS grade), Trolox and 2,2-diphenyl-1-picrylhydrazyl (DPPH) were supplied by Sigma Aldrich. Ultra-pure water was obtained using Mili-Q water purification system (<0.058 μS/cm, Mili-Q model Pacific TII 12, Thermo Scientific, Massachusetts, SAD).
All sample extracts were filtrated before analysis with Chromafil cellulose acetate microfilters (0.45 μm, 25 mm) (Macherey-Nagel, Germany).

2.3. QUALITY PARAMETERS

Determined quality parameters were percentage of free fatty acid (expressed as a percentage of oleic acid), peroxide value (expressed as milliequivalents of active oxygen per kilogram of analysed oil – meq O2/kg) and specific extinction coefficients calculated from the absorption at 232 and 270 nm (K_{232} and K_{270}). All analyses were performed according to standard methods described in the European Union Commission Regulation EEC 2568/91 and its later amendments.

2.4. ANTIOXIDANT ACTIVITY ASSAY

50 mg of olive oil samples were extracted with 1 ml of methanol, mixed for one minute and sonicated for 15 min at constant temperature of 25°C. Afterwards, samples were filtrated and prepared for the analysis.
The antioxidant activity was tested using a stable radical 2,2-diphenyl-1-picrylhydrazyl (DPPH•). Initially, 800μL of a DPPH• methanol solution (4.0×10^{-6} g/mL) was mixed with 200 μL of a diluted extract. Afterwards, 200 μL of each prepared mixture was transferred into a 96-well microplate. The absorbance was measured at 515 nm after 60 min incubation in the dark. A calibration curve was prepared with Trolox (0-0.21 mM; R^2=0.9971) and the scavenging activity was expressed as Trolox equivalents (TE). All measurements were done in triplicate.

2.5. LC-QQQ

3 g of olive oil samples were extracted with 2 ml of methanol, mixed for two minutes (IKA vortex 3, Sigma Aldrich), sonicated (Sonorex digitec, Bandelin, Germany) for 15 min at a constant temperature of 25°C, centrifuged (Centrifuge 5427R, Eppendorf, Germany) for 10 min at 4000 rpm and frozen for 20 min at -80°C. Methanol layer was decanted and stored. The procedure was repeated on the olive oil residue and both methanol layers were mixed together and filtrated.
An Agilent 1260 series HPLC chromatograph equipped with a degasser, binary pump, auto-sampler and column oven coupled to an Agilent 6460 triple quadrupole mass spectrometer equipped with jet stream electrospray (AJS ESI) source (Agilent Technologies, Palo Alto, CA, USA) was used for

Table I - LC-QQQ method parameters of phenolic acids and flavonoids analysis of extra virgin olive oil extracts

Phenolic compound	RT/min	Polarity	Fragmentor/V	*m/z* Precursor ion	*m/z* Product ion	Collision energy/V	Y=ax+b		Linearity range µg/ml	R^2	LOD mg/kg	LOQ mg/kg
							Slope (a)	Intercept (b)				
Method A												
Hydroxytyrosol	1.7	-	100	152.9	123.0 95.0*	18 20	756.94	18.41	0.1-2.5	0.994	0.0803	0.2432
Oleuropein	8.1	-	170	538.9	377.3 307.3 275.1	12 18 18	15681.77	-10.76	0.001-2.5	0.991	0.0023	0.0069
Vanillic acid	5.2	-	90	166.9	151.8 122.9 107.9	10 6 14	260.86	19.43	0.5-5.0	0.991	0.2458	0.7448
Luteolin	9.1	-	180	284.9	150.9 133.0	24 36	26778.66	63.89	0.01-5.0	0.994	0.0079	0.0239
3.4-DHBA	1.8	-	80	152.9	108.0 81.0 53.0	20 16 18	3761.51	15.01	0.01-7.5	0.998	0.0132	0.0399
Method B												
Tyrosol	1.7	-	100	136.8	119.0 107.0 105.9 93.0	10 10 12 40	61.54	-19.73	1.0-5.0	0.993	1.058	3.206
Pinoresinol	15.3	-	170	357.0	175.0 151.0 136.0 123.0	40 40 36 40	982.04	-470.63	2.5-5.0	0.983	1.581	4.792

*) Quantifier ions are underlined

quantitative analysis. All data processing was obtained using the Mass Hunter workstation software (version B.07.00).

Method A: Chromatographic analysis for hydroxytyrosol, oleuropein, 3,4-DHBA, vanillic acid and luteolin was carried out on Zorbax SB-C18, Rapid resolution HT, 600 bar column (2.1 mm × 50 mm I.D, 1.8 µm, Agilent Technologies). The mobile phase was composed of (A) 0.1% formic acid in miliQ and (B) 0.1% formic acid in ACN. The gradient elution was modified as follows: 0-0.9 min linear gradient from 1% to 10%B, 0.9-3 min from 10% to 20%B, 3-4.5 min from 20% to 25%B, 4.5-6 min from 25% to 30%B, 6-7.5 30%B, 7.5-9 min from 30% to 90%B, 9-9.30 90%B, 9.30-9.60 from 90% to 10%B and 9.60-15 min from 10 to 1%B. Post time was set to 2 minutes. The flow rate was 0.33 ml/min. The column oven was maintained at 30°C. The sample injection volume was 2.5 µl. For AJS-ESI-QQQ, the parameters were set as follows: capillary voltage was 3.5 kV in both positive and negative mode, nozzle voltage 0.5 kV, ion source temperature was set to 300°C, gas flow was 5 l/min, nebuliser pressure was 45 psi, drying gas temperature was 250°C and sheath gas flow was 11 l/min. Nitrogen was used as collision gas and collision energies were from 0 V to 40 V.

Method B: For pinoresinol and tyrosol analysis, the chromatographic method was developed on the same column and mobile phases, but the gradient elution was modified as follows: 0-0.1 linear gradient from 1% to 10%B, 0.1 to 13.39 from 10% to 90%B, 12.39-14.59 min from 90% to 10%B, from 14.59-16 min from 10% to1%B and 16-20 min 1%B. Post time was set to 4 minutes. The flow rate was 0.1 ml/min. The column oven was maintained at 30°C. The sample injection volume was 10 µL. For AJS-ESI-QQQ, the parameters were set as follows: capillary voltage was 4 kV in both positive and negative mode, nozzle voltage 0.5 kV, ion source temperature was set to 300°C, gas flow was 10 l/min, nebulizer pressure was 35 psi, drying gas temperature was 250°C and sheath gas flow was 11 l/min. Nitrogen was used as collision gas and collision energies were from 0 V to 40 V.

In order to establish the calibration curves, reference phenolic compounds were dissolved in methanol and diluted in appropriate concentrations. For quantitative analysis, 15 different concentrations for each standard were made and linearity ranges of their calibration curves are shown in Table I. The concentrations of all the standards were injected in triplicates. Calibration curves were constructed using linear regression and were not forced to pass through zero. Also, a 1/x statistical weight was applied in order to obtain the most reliable calibration curves for all the phenolics. The calibration curves ranges were achieved based on the linearity of the responses for each individual metabolite (Tab. I). Linearity was determined using the coefficient of determination (R^2).

The limit of detection (LOD) and limit of quantification (LOQ) were determined using quantifier transition according to the International conference on Harmonisation (ICH) guidelines:

$$LOD = 3.3 \times \frac{b}{a}$$

$$LOQ = 10 \times \frac{b}{a}$$

Table II - List of analysed phenolic compounds without available standards and their MRM parameters for LC-QQQ analysis

Phenolic derivative	RT/min	Polarity	Fragmentor/V	*m/z* precursor ion	*m/z* product ion[a]	Collision energy/V	Calibration curve
3,4-DHPEA-EA derivative	8.0	-	104	409	274.8 138.9 95.0	10 20 20	oleuropein calibration curve
3,4-DHPEA-EA	10.9	-	104	377	307.0 275.1 139.0 95.1	12 0 10 12	
p-HPEA-EA	11.2	-	110	361	291.0 259.1 101.0	12 10 12	
3,4-DHPEA-EDA	8.1	-	110	319	183.1 139.0 94.8 69.0 59.0	10 12 10 32 12	
p-HPEA-EDA	9.3	-	128	303	285.0 137.0 68.9 59.0	0 12 20 8	
3,4-DHPEA-AC	6.7	-	128	196	59.0	8	

For the quantification of 3,4-DHPEA-EA derivative, 3,4-DHPEA-EA, *p*-HPEA-EA, 3,4-DHPEA-EDA, *p*-HPEA-EDA and 3,4-DHPEA-AC, analysis was carried out over the precursor phenolic compound and was set with the same parameters as in method A. A semiquantitative procedure was carried out using the calibration curve of the phenolic compound with a similar chemical structure (oleuropein). This was done in order to convert all phenolic derivatives to a form which has a standard available, i.e. to estimate their equivalent values. Parameters, as well as phenolic compounds by which the calibration was done, are given in Table II.
Each EVOO extraction was repeated three times and samples were injected in duplicates. Results of the semiquantitative analysis in Tables were given as mean values with standard deviation (SD) and are expressed as oleuropein equivalents (mg/kg).

2.6. STATISTICAL ANALYSIS

Principal component analysis (PCA) was performed using the UNSCRAMBLER software version 10.4 from CAMO (Computer Aided Modelling, Trondheim, Norway).

3. RESULTS AND DISCUSSIONS

Table III shows the results of analysed quality parameters (free acidity given as % of oleic acid, peroxide value expressed in milliequivalents of active oxygen per kg of oil (meq O_2/kg), K_{270}, and K_{232} values) of Istrian olive oils collected for further analysis. All the obtained results were within limits for the extra virgin olive oil set by the Commission of the European Community (Commission Regulation (ECC) 2568/91 and its further modifications). Therefore, all the olive oils under study, considering only the analysis that were performed (organoleptic evaluation and analysis of fatty acid ethyl ester content were not performed), could be classified as EVOOs for qualitative aspects.
There were two developed LC-QQQ methods for analysis of the phenolic compound with commercially available standards in olive oil samples: Method A and Method B. Method B was established for the analysis of pinoresinol and tyrosol to enhance their low ionisation efficiency. The problem with the ionisation of these compounds was already confirmed by other researchers (Jerman Klen, Golc Wondra, Vrhovšek, & Mozetič Vodopivec, 2015; Obied, Bedgood, Prenzler, & Robards, 2007). An additional chromatographic method for the analysis of secoiridoid derivatives was the same as method A, but MS detection was developed using specific fragments from the literature with the modification of fragmentor values and collision energies [10, 16, 17]. That way we were able to quantitatively analyse 3,4-DHPEA-EA derivative, 3,4-DHPEA-EA, p-HPEA-EA, 3,4-DHPEA-EDA, p-HPEA-EDA, 3,4-DHPEA-AC in Istrian EVOOs.
LC-QQQ quantitative analysis of phenolic compounds

Table III - Results of quality parameters of extra virgin olive oils expressed as mean value ± standard deviation (SD)

Sample	Olive cultivar	Free fatty acid (%)	Peroxide value (meq O_2/kg)	K_{232}	K_{270}
1	Leccino	0.28±0.00	2.08±1.26	1.77±0.09	0.12±0.01
2	Leccino	0.32±0.02	2.92±0.42	1.79±0.20	0.13±0.01
3	Leccino	0.31±0.00	2.08±0.42	1.82±0.11	0.11±0.00
4	Leccino	0.30±0.02	4.58±2.08	1.92±0.18	0.12±0.00
5	Leccino	0.28±0.00	1.24±0.42	1.95±0.11	0.14±0.00
6	Leccino	0.27±0.02	2.50±0.00	1.74±0.01	0.12±0.01
7	Leccino	0.28±0.00	2.08±0.42	1.76±0.07	0.13±0.01
8	Mixture	0.28±0.00	2.08±0.42	1.79±0.07	0.12±0.00
9	Leccino	0.28±0.00	4.16±2.50	1.76±0.07	0.11±0.00
10	Istarska bjelica	0.32±0.02	1.46±1.04	1.89±0.29	0.16±0.03
11	Mixture	0.31±0.00	3.34±2.50	2.02±0.09	0.12±0.00
12	Buža	0.25±0.04	8.72±1.22	2.21±0.11	0.11±0.01
13	Žižolera	0.28±0.04	2.90±1.24	1.74±0.02	0.11±0.01
14	Istarska bjelica	0.25±0.04	4.58±0.44	1.71±0.06	0.13±0.00
15	Buža	0.23±0.00	4.16±0.84	1.89±0.00	0.13±0.00
16	Mixture	0.17±0.00	5.20±1.04	1.91±0.01	0.13±0.00
17	Mixture	0.21±0.02	5.40±0.42	2.27±0.24	0.13±0.01
18	Mixture	0.53±0.04	10.78±3.28	2.11±0.31	0.14±0.02
19	Istarska bjelica, Buža	0.25±0.04	1.46±0.62	1.70±0.04	0.12±0.01
20	Rosinjola	0.23±0.00	1.24±0.42	1.87±0.05	0.12±0.00
21	Mixture	0.27±0.02	12.84±1.24	1.90±0.12	0.13±0.00
22	Mixture	0.28±0.00	4.86±0.08	1.89±0.20	0.15±0.05
23	Mixture	0.21±0.24	1.46±0.62	1.57±0.38	0.09±0.03

Table IV - Results of quantification of specific phenols in extra virgin olive oils expressed as mean value ± standard deviation (SD)

Sample	Hydroxytyrosol (mg/kg)	Tyrosol (mg/kg)	Oleuropein (mg/kg)	Pinoresinol (mg/kg)	Vanillic acid (mg/kg)	Luteolin (mg/kg)	3,4-DHBA (mg/kg)
1	3.89±0.07	1.46±0.13	0.01±0.00	0.66±0.00	0.27±0.09	3.21±0.00	0.21±0.00
2	11.42±0.13	1.91±0.13	0.01±0.00	0.65±0.00	0.33±0.06	2.75±0.03	0.61±0.03
3	6.20±0.44	1.46±0.21	0.01±0.00	0.66±0.01	0.36±0.09	3.47±0.06	0.34±0.01
4	6.47±0.04	1.37±0.12	0.01±0.00	0.65±0.00	0.33±0.10	4.28±0.01	0.35±0.01
5	8.02±0.36	1.62±0.02	0.01±0.00	0.65±0.00	0.24±0.01	3.41±0.04	0.46±0.00
6	4.11±0.08	1.31±0.19	0.01±0.00	0.66±0.00	0.31±0.09	3.12±0.03	0.25±0.01
7	12.42±0.35	1.92±0.15	0.01±0.00	0.66±0.01	0.60±0.03	3.71±0.00	0.72±0.02
8	6.35±0.01	0.89±0.00	0.01±0.00	0.28±0.02	0.21±0.07	3.94±0.06	0.35±0.01
9	19.25±0.24	1.53±0.07	0.01±0.00	0.67±0.00	0.25±0.01	4.76±0.02	1.08±0.02
10	14.21±0.07	1.61±0.30	0.01±0.00	0.65±0.00	0.39±0.07	4.12±0.06	0.78±0.02
11	19.47±0.35	1.77±0.01	0.01±0.00	0.68±0.01	0.18±0.00	2.98±0.04	1.15±0.02
12	0.86±0.05	1.37±0.08	0.01±0.00	0.69±0.03	0.12±0.01	2.89±0.12	0.74±0.03
13	2.68±0.12	0.99±0.03	0.01±0.00	0.68±0.01	0.19±0.00	4.89±0.06	0.78±0.05
14	3.51±0.11	1.13±0.10	0.01±0.00	0.65±0.01	0.15±0.01	3.54±0.05	1.10±0.01
15	12.58±0.18	1.58±0.12	0.01±0.00	0.69±0.01	0.70±0.22	4.28±0.15	0.05±0.01
16	4.31±0.22	1.46±0.06	0.01±0.00	0.65±0.00	0.51±0.17	3.66±0.12	0.14±0.03
17	3.86±0.09	1.44±0.11	0.01±0.00	0.66±0.02	0.41±0.14	3.38±0.01	0.21±0.04
18	3.09±0.04	1.53±0.12	0.01±0.00	0.72±0.00	0.20±0.07	1.40±0.01	0.12±0.01
19	2.38±0.08	1.62±0.05	0.01±0.00	0.66±0.00	0.44±0.07	3.76±0.03	0.72±0.03
20	4.19±0.08	1.51±0.26	0.01±0.00	0.72±0.03	0.30±0.07	1.94±0.07	0.27±0.00
21	5.47±0.07	1.19±0.00	0.01±0.00	0.65±0.01	0.29±0.08	4.32±0.05	0.23±0.01
22	4.88±0.03	1.38±0.08	0.01±0.00	0.66±0.01	0.44±0.04	5.59±0.04	0.29±0.03
23	1.80±0.02	1.61±0.09	0.01±0.00	0.65±0.00	0.28±0.07	5.05±0.11	0.12±0.00

revealed high differences in concentrations of specific phenols in Istrian EVOOs. This was expected as different olive cultivars (with different and unique phenolic profile) were considered for the analysis of phenolic compounds. However, some common characteristics for Istrian olive oils were found.

Results on the quantification of phenols with available standards are shown in Table IV, where it can be seen that hydroxytyrosol and luteolin are phenolic compounds with the highest concentrations in tested EVOOs. Their share in Istrian EVOOs range from 1.80±0.02 to 19.47±0.35 mg/kg and 1.40±0.01 to 5.59±0.04 mg/kg, respectively. A large range of hydroxytyrosol concentration values in Istrian EVOOs was also reported in paper from Jakobušić Brala et al., (2015) [9]. There they reported that, even the same olive variety (Istarska bjelica), but from two different years, can have different hydroxytyrosol concentrations. As for other determined phenols, our results were lower than the results from Jakobušić Brala et al., (2015). We reported tyrosol concentrations from 0.99±0.03 to 1.77±0.01 mg/kg, while in their work the lowest tyrosol concentration was 4.70±0.75 mg/kg for Buža olive cultivar. Results of vanillic acid concentrations were just slightly lower with concentrations ranging from 0.12±0.01 to 0.70±0.22 mg/kg. Lastly, oleuropein concentrations are really low in analysed EVOO samples, which was expected since oleuropein degraded during the olive oil processing to secoiridoid aglycon derivatives [18, 19]. Results of antioxidant activity and the quantification of secoiridoid derivatives (expressed as oleuropein equivalents) are shown in Table V and their average ratios at Figure 1. These are the first results for these compounds in Istrian EVOO samples. Concentrations

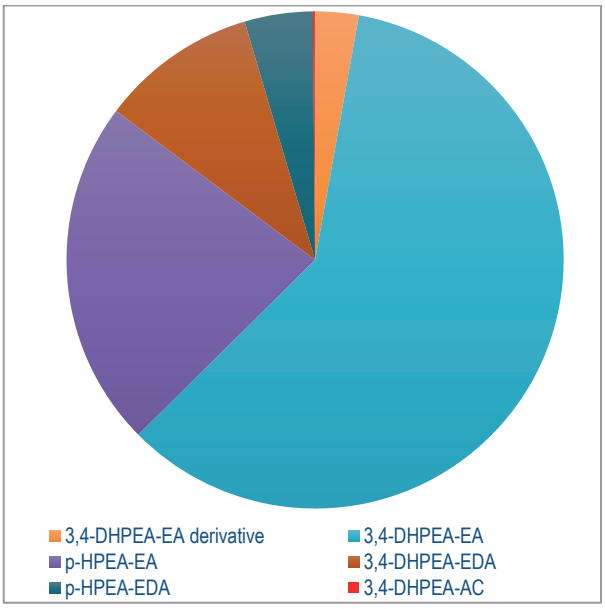

Figure 1 - Average ratio of secoiridoid derivatives in Istrian EVOOs

Table V - Result of antioxidant activity and analysis of secoiridoid derivatives quantified with calibration curve of oleuropein and expressed as a oleuropein equivalent mean value ± standard deviation (SD).

Sample	Antioxidant activity (mmol TE/kg)	3,4-DHPEA-EA derivative (mg/kg)	3,4-DHPEA-EA (mg/kg)	*p*-HPEA-EA (mg/kg)	3,4-DHPEA-EDA (mg/kg)	*p*-HPEA-EDA (mg/kg)	3,4-DHPEA-AC (mg/kg)
1	2.96±0.73	1.65±0.40	32.13±0.56	5.56±2.22	21.88±1.34	7.25±0.12	0.43±0.03
2	2.79±0.05	3.18±0.51	82.02±4.52	17.12±4.48	18.91±1.19	6.30±0.36	0.38±0.04
3	2.49±0.01	4.30±0.27	94.63±3.37	22.80±3.05	24.24±0.82	8.99±0.41	0.49±0.01
4	2.81±0.04	2.84±0.25	62.69±3.60	13.17±0.67	20.99±1.27	7.65±0.46	0.41±0.05
5	3.59±0.03	5.18±0.19	119.50±3.79	26.74±2.76	20.65±0.11	8.04±0.20	0.43±0.01
6	2.27±0.10	4.97±0.29	89.19±5.65	19.50±0.61	28.11±1.41	12.85±0.57	0.52±0.02
7	2.84±0.73	2.17±0.23	46.07±1.95	9.59±0.97	17.44±0.91	6.01±0.21	0.33±0.02
8	2.45±0.18	1.71±0.00	55.67±0.94	15.42±0.11	18.72±0.12	7.71±0.16	0.35±0.02
9	1.78±0.06	1.90±0.04	50.86±0.54	22.51±4.34	11.08±0.23	8.26±0.25	0.22±0.02
10	2.55±0.05	4.11±0.36	93.96±4.32	34.27±4.84	17.24±0.95	8.26±0.50	0.37±0.05
11	1.46±0.16	1.65±0.20	39.42±4.07	21.86±4.46	5.72±0.44	7.02±0.53	0.12±0.02
12	0.66±0.05	0.69±0.01	17.21±0.54	24.34±2.51	2.93±0.11	5.22±0.10	0.06±0.00
13	1.61±0.17	2.69±0.27	42.36±2.66	28.01±1.28	5.45±0.38	2.56±0.16	0.14±0.03
14	1.86±0.01	3.22±1.13	95.56±7.92	68.69±15.89	6.82±0.51	4.80±0.40	0.25±0.06
15	2.61±0.54	4.96±0.16	102.99±17.32	48.85±0.01	12.03±0.02	5.59±0.03	0.33±0.02
16	2.73±0.02	4.67±0.00	96.63±23.48	26.28±1.67	12.48±0.29	4.70±0.16	0.33±0.02
17	2.13±0.07	3.95±0.54	94.48±19.14	53.99±3.59	7.55±0.02	4.73±0.13	0.26±0.08
18	0.95±0.11	0.32±0.01	13.15±1.30	4.82±0.33	8.41±0.81	3.86±0.36	0.21±0.03
19	1.81±0.65	2.13±0.40	65.90±1.02	53.09±0.03	5.00±0.03	3.26±0.03	0.13±0.01
20	1.95±0.09	2.99±0.22	51.32±1.55	20.73±1.39	10.51±0.34	4.04±0.08	0.22±0.02
21	2.39±0.15	5.18±0.24	132.42±1.49	40.10±2.09	20.27±0.79	9.63±0.24	0.54±0.02
22	4.12±0.06	4.59±0.07	104.52±0.85	27.43±3.81	24.24±0.26	8.85±0.18	0.59±0.00
23	1.41±0.05	2.70±0.05	62.45±18.07	46.47±0.04	10.59±0.33	6.57±0.17	0.28±0.01

of all analysed derivatives were in very high share, directing the importance of analysing these constituents in EVOO profiling studies. As shown in Table V, abundant secoiridoid derivatives in Istrian EVOOs were 3,4-DHPEA-EA and p-HPEA-EA, ranging from 13.15±1.30 to 132.42±1.49 mg/kg and 4.82±0.33 to 68.69±15.mg/kg, respectively. All Istrian EVOOs (except sample 12) had 3,4-DHPEA-EA in the largest concentrations, followed by p-HPEA-EA or 3,4-DHPEA-EDA. The average ratio of secoiridoid derivatives in samples under study is presented in Figure 1 pointing out the dominance of 3,4-DHPEA-EA. Our results are in line with previous researches on olive oil phenolic composition. Namely, de la Torre-Carbot et al. (2005) and Medina et. al. (2015) already confirmed oleuropein and ligstroside aglycon derivatives as the most abundant compounds in olive oils [17, 20].

Additionally, all samples were characterised according to their antioxidant activity. DPPH radical scavenging assay showed divers antioxidant activity from 0.66±0.05 to 4.12±0.06 mmol TE/kg. To better explore the connection between single phenolic compounds determined by LC-QQQ and measured antioxidant activity by DPPH radical scavenging assay in Istrian olive oil Principal component analysis (PCA) was performed. For the PCA with all tested olive oils as cases, concentrations of individual phenolic compounds and antioxidant activity were used as variables (Fig. 2). The first principal component explains 33% and the second principal component explains 20% of the total variance, together accounting for 53% of the total variance. In the space of the first two principal components, derivatives with 3,4-dihydroxy moiety linked to an aromatic ring, i.e. oleuropein aglycon derivatives and antioxidant activity (AA) are grouped together, showing highest positive loadings on the first principal component. The results of PCA suggest that oleuropein aglycon derivatives can be considered as positively correlated with AA variable. This finding highlights oleuropein aglycon derivatives as compounds that contribute mostly to the EVOO antioxidant activity. The only phenolic compound showing an opposite loading along the first principal component was pinoresinol from the lignin group.

4. CONCLUSIONS

In this study, phenolic compounds of Istrian EVOO were characterised by LC-QQQ and DPPH assay. In all analysed EVOOs, 13 phenolic compounds were identified and quantified. This is the first analysis of secoiridoid derivatives in Istrian EVOOs. Results revealed very low concentrations of oleuropein in

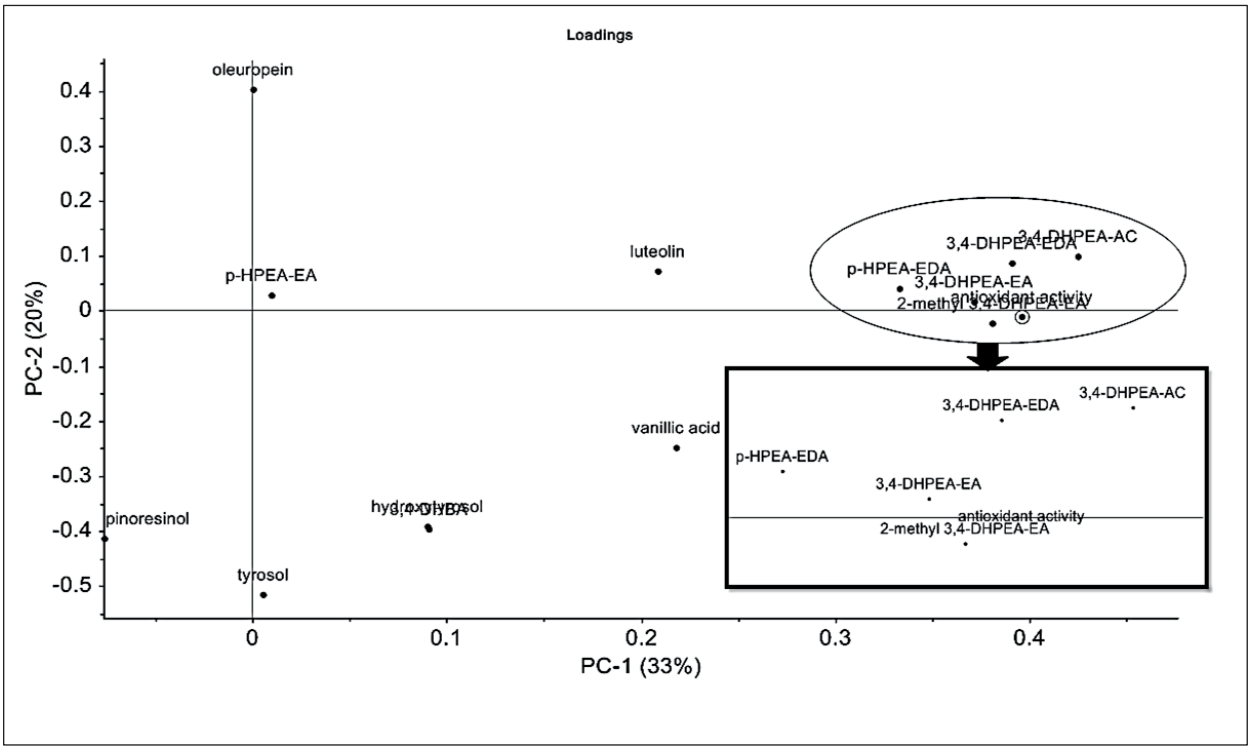

Figure 2 - Loading plot of the concentrations of the individual phenolic compounds determined by LC-QQQ and antioxidant activity determined by DPPH radical scavenging assay in the space of principal component 1 and principal component 2 (PC 1 to PC 2) with enlarged area around variable antioxidant activity.

samples, but higher concentrations of secoiridoid compounds and derivatives. Specifically, 3,4-DHPEA-EA was in the largest concentrations, followed by p-HPEA-EA and 3,4-DHPEA-EDA. Among the analysed phenols with commercially available standards, the most abundant phenolic alcohol was hydroxytyrosol and, from the flavonoid group, luteolin. Overall results revealed no qualitative differences in the phenolic composition, however quantitative differences were expressed in a wide number of phenolic compounds in olive oils from the same region.

Acknowledgement
We greatly appreciate the access to equipment in possession of the University of Rijeka within the project "Research Infrastructure for Campus-based Laboratories at University of Rijeka", financed by the European Regional Development Fund (ERDF).

Conflict of interest
The authors have no conflict of interest to declare.

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Received: December 11, 2018
Accepted: May 23, 2019