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# Phenolic Potential of Olive Leaves from Different Istrian Cultivars in Croatia

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**Abstract:** For the first time the effects of different sampling periods and their interaction with five major autochthonous Croatian Istrian olive cultivars and the Italian cultivar 'Leccino' on the quantity and composition of olive leaf phenolic compounds and mineral nutrients were investigated. For that purpose, olive leaves were sampled in two collecting periods, in October and March, coinciding with the harvesting and pruning periods, respectively. All selected cultivars had a higher oleuropein leaf content in the pruning collecting period, with the highest levels noted for the 'Leccino' and 'Buža' cultivars. Cultivar significantly affected almost all the investigated phenols, with higher concentrations of these valuable compounds in the pruning than in the harvesting period. Differences observed in leaf mineral composition were closely related to the differences in phenolic profiles and were significantly affected by genotype. Some of the studied mineral nutrients, such as P, Cu and B, were found to be significantly correlated with the most abundant olive leaf phenolic compounds, oleuropein and verbascoside.

**Keywords:** *Olea europaea* L.; phenols; minerals; 'Leccino'; 'Buža'; oleuropein



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## 1. Introduction

The olive tree (*Olea europaea* L.) is traditionally grown on the Croatian coast, where it plays an important social, economic and environmental role. Generally, olive oil production generates a large amount of waste. Olive leaves generated mostly during pruning and harvesting make up the largest part of this representing 10% of the weight of olives collected for oil extraction, while olive pruning annually produces 25 kg/tree of by-products (twigs and leaves) [1]. It is well known that olive leaves contain a high number of phenolic compounds, such as secoiridoids (represented by oleuropein), simple phenolic alcohols such as tyrosol and hydroxytyrosol, flavonoids (represented by luteolin-7-*O*-glucoside) and hydroxycinnamic acid derivatives such as verbascoside [2–4]. Phenolic compounds are known to exhibit antimicrobial [5], antioxidant [6], antihypertensive [7], anti-proliferative, apoptotic [8] and many other properties potentially beneficial to human health. Because

of this, a part of olive leaves is used in the cosmetic and pharmaceutical industries and as a supplement in the food industry [9]. Nevertheless, only a small fraction is actually exploited, and the huge amount of this residual material is usually burnt or otherwise discarded. Among several reasons for that is the unavailability of precise data regarding the distribution of phenols and their possible recovery from highly heterogeneous leaf material (cultivar, region, collection time, etc.), which disables their proper valorization. Thus, there is an urgent need to continue the research in this field and contribute to the knowledge that would allow better exploitation of these valuable compounds from easily available starting materials, such as olive leaves.

It is known that the composition of leaves varies significantly during the agronomic cycle and strongly depends on several factors, such as climate conditions and collecting period, with genotype perhaps the most important factor [10,11]. Cultivar origin usually determines the capability of a plant to face environmental changes and to synthesize protective metabolites that regulate their future growth and productivity. Even under suitable and similar growing conditions, the synthesis of major olive phenols seems to be regulated differently between different olive genotypes. For example, Palmeri et al. [12] reported that the Sicilian cultivars 'Biancolilla' and 'Nocellara' contained twice the number of total phenols as the 'Coratina' and 'San Benedettese' cultivars, while Talhaoui et al. [13] found a similar trend of shifts in phenol concentrations during ripening for six Spanish cultivars. In our previous paper we reported that, among six studied cultivars, the domestic Croatian cultivar 'Istarska bjelica' had the highest oleuropein potential [14]. In addition to the aforementioned factors, optimal nutritional status is crucial for correct plant development and strongly affects phenol concentrations in leaves, as shown earlier.

The olive growing area in Croatia consists of six regions: Istria, Kvarner, Northern Dalmatia, Central Dalmatia, South Dalmatia and the Dalmatian Hinterland. Our previous papers have reported olive leaf cultivar phenol variations in the most common cultivars in Kvarner, North Dalmatia and South Dalmatia regions [14–16]. However, to date there is a serious lack of sufficient data on the olive leaf phenolic profiles of autochthonous olive cultivars from the Croatian Istria peninsula, one of the best-known olive regions worldwide, with a long history of olive cultivation and prime quality olive oils [13,14,17]. Specifically, the Istria region has a large number of autochthonous cultivars, the most common ones including 'Puntoža' (Syn. 'Buža puntoža', 'Puntuža'), 'Buža' (Syn. 'Buga', 'Burgaca', 'Morgaca', 'Domaća', 'Gura', 'Feminuškula', 'Buža ženska') and 'Istarska bjelica' (Syn. 'Istarska Belica', 'Bianchera'), as well as 'Karbonaca' (Syn. 'Karbonasa', 'Karbonera') and 'Rošinjola' ('Rosulja', 'Rosinjola', 'Rovinješka', 'Rušinjola') [18,19]. In addition, the most widespread and economically important allochthonous cultivar in Istria is the Italian 'Leccino'.

Based on everything stated above, it is clear that a more in-depth investigation of the distribution of olive leaf phenols over the Istrian olive gene pool would be of great importance. The aim of this work was to compare the content and composition of phenols and mineral macro- and micronutrients in the leaves of olive cultivars commonly grown in the Istria region of Croatia collected during the harvesting and pruning periods. Besides contributing to the knowledge in the field by clarifying the extents of the interactive effects of olive cultivar and collecting period, the results are also expected to have significant practical importance, since they would allow for the identification of the most promising cultivar × collection period combinations for more efficient olive leaf exploitation.

## 2. Materials and Methods

### *2.1. Olive Leaf Sampling*

The sampling of olive leaves was conducted in an olive grove located in Poreč (Istria, Croatia) included in the experimental collection of the Institute of Agriculture and Tourism, located 0.5 km from the coast (45°22′ N; 13°60′ E) and 35 m above sea level. The climate area is classified as Cfa according to Köppen [20]. The grove is planted on the southern slope in Rhodic cambisol soil (Table S1). Soil properties were determined as described

previously [14]. Rhodic cambisols, well known as Terra rossa, are heavy, clay-rich soils, strongly red, developed on limestone or dolomite and typically found in Istria.

All sampled trees, which were up to 10 years old, had a similar tree density, and olives were grown without irrigation. Integrated pest management as well as standard fertilization practices were applied each year [14]. Data about average daily temperatures and rainfall (Figure S1) were provided by the Croatian Meteorological and Hydrological Service. Only well developed and similarly conditioned trees of six cultivars ('Puntoža', 'Buža', 'Istarska bjelica', 'Karbonaca', 'Leccino' and 'Rošinjola') were selected for sampling. Set as a completely randomized design (Photo S1), with each of the six cultivars represented by three trees, the total number of trees in the experiment was 18. Leaves from the central part of olive shoots were collected evenly around the tree in two sampling periods, during the olive harvest in October 2017 (CP1) and during pruning in March 2018 (CP2). All the samples were carefully washed sequentially with tap water, 1% acetic acid solution with deionized water, and deionized water. After air drying the olive leaves in clean and open paper bags, at 35 °C (Fan 80%) in a dryer (Memmert GmbH + Co.KG, Büchenbach, Germany) up to constant mass, the samples were milled to a fine powder using a Retsch ZM 200 mill (Retsch GmbH, Haan, Germany) before analysis [4].

### *2.2. Chemicals*

Methanol and acetonitrile (HPLC grade) were purchased from Merck (Darmstadt, Germany), phosphoric acid (HPLC grade) from Sigma–Aldrich (St. Louis, MO, USA) and standards of all described phenolic compounds (HPLC grade) from Extrasynthese (Genay, France), while hydrochloric acid (Suprapure) was procured from Merck (Darmstadt, Germany). Deionized water was obtained by the Siemens UltraClear apparatus (Siemens AG, München, Germany). Multi-element standard solution was purchased from Perkin Elmer (NexION Setup Solution, Waltham, MA, USA). Argon used to form plasma for the inductively coupled plasma mass spectrometric analysis (ICP-MS) was of purity 6.0 and, together with acetylene, was supplied by Messer (Messer Croatia Plin d.o.o., Zaprešić, Croatia).

### *2.3. High-Performance Liquid Chromatography (HPLC)*

Olive leaf phenols were extracted and then analyzed by high-performance liquid chromatography (HPLC) with simultaneous UV/Vis detection at four different wavelengths on the Thermo Ultimate 3000 HPLC System (ThermoFischer Scientific, Waltman, MA, USA) as described in our previous work [15]. Briefly, after drying and grounding, 500 mg of leaves were suspended in a mixture of methanol and water (8:2) and then ultrasonicated for 20 min. The obtained mixture was centrifuged and then filtered using a cellulose acetate syringe filter with 0.45 µm pores. A detailed description of HPLC conditions was given in our previous work [15]. The identification of phenolic compounds was based on the comparison of their retention times with those of pure standards, while they were quantified by the external standard method using the corresponding calibration curves.

### *2.4. Inductively Coupled Plasma Mass Spectrometry (ICP-MS)*

Dried and finely grounded leaves (500 mg) were dry ashed at 550 °C for 8 h, then suspended in hydrochloric acid, filtered and quantitatively diluted with deionized water. Mineral nutrients were then analyzed by ICP-MS as described in our previous study [14,15]. In particular, for boron (B), copper (Cu), manganese (Mn) and zinc (Zn) analysis a NexION 300× system (PerkinElmer Instruments, Waltham, MA, USA) was used, while calcium (Ca), magnesium (Mg), potassium (K) and iron (Fe) were analyzed by flame atomic absorption spectrometry (FAAS) using a PerkinElmer AAS800 system (PerkinElmer Instruments, Waltham, MA, USA), using acetylene-air as an oxidant, and phosphorus (P) was analyzed by UV/Vis spectrophotometry (Carry UV/Vis 50 spectrophotometer, Varian Inc., Palo Alto, CA, USA).

### *2.5. Statistical Analysis*

One-way analysis of variance (ANOVA), with cultivar as the predictor, was conducted for soil analysis. Two-way ANOVA, with cultivar and sampling time as the main factors, was performed followed by Tukey's post hoc test. For significant interactions simple effects were computed at each level of the other factor as noted in our previous work [21]. Furthermore, a Pearson's correlations matrix was made for the most abundant phenolic compounds (verbascoside, oleuropein, luteolin-7-*O*-glucoside) and total phenols versus olive leaf mineral elements. All previously listed statistical analyses were performed using the Statistica 13.2 software (StatSoft®, Palo Alto, CA, USA). The data were further processed by multivariate statistical analysis using hierarchical cluster analysis (HCA) and partial least squares–discriminant analysis (PLS–DA). HCA is an unsupervised multivariate statistical method in which distances between samples (cases) are calculated and samples are grouped into categories based on similar characteristics defined by the variables (e.g., phenols or elements). PLS–DA is a supervised multivariate statistical method that minimizes the variance within and maximizes the variance between different categories (e.g., varieties or collecting periods) and gives information about the most useful variables (e.g., phenols or elements) in the form of variable importance in projection (VIP) scores. HCA and PLS-DA were performed by MetaboAnalyst v. 5.0 [22].

## 3. Results

The results for chemical soil properties at the beginning of the experiment, for each of the selected cultivars, are shown in Table S1. The results of one-way ANOVA had confirmed that there were no significant differences in the determined soil parameters between different cultivars at the beginning of the experiment.

The results of two-way ANOVA for phenols in olive leaves collected from the six studied cultivars in two collecting periods are presented in Tables 1–3.

A significant level of interaction between cultivar and collecting period was noted for hydroxytyrosol and tyrosol concentration (Table 1). The concentration of hydroxytyrosol in both collecting periods showed a similar trend, being the highest in 'Leccino' leaves while having significantly higher values in the pruning collecting period CP2 for all the cultivars except 'Puntoža'. In the first collecting period, during the harvest (CP1), the concentration of tyrosol was higher in leaves of 'Puntoža', 'Istarska bjelica' and Leccino' than in leaves of the 'Karbonaca' and 'Rošinjola' cultivars. The difference in tyrosol concentration in the CP2 leaves was expressed by higher levels in 'Leccino' compared to those in 'Karbonaca', 'Buža' and 'Puntoža'.

The concentrations of vanillin, 4-hydroxybenzoic acid and all the detected flavonoids were strictly cultivar- and/or collecting period-dependent, without significant interactions. The concentration of vanillin was higher in 'Leccino' leaves than in 'Istarska bjelica' and 'Karbonaca' leaves (Table 1), while between two collection periods the highest concentration was determined in CP2. 'Karbonaca' yielded higher 4-hydroxybenzoic acid levels compared to all the other cultivars, while the CP1 leaves contained a higher concentration than the CP2 leaves (Table 2).

Significant interactions were determined for the majority of major phenols. The concentration of oleuropein was the highest in CP1 'Leccino', but not statistically different from that found in 'Puntoža' leaves, while the lowest value was determined in 'Rošinjola', although in some cases without statistical significance. In the CP2 leaves, 'Buža' and 'Leccino' had the highest and 'Karbonaca' the lowest oleuropein concentration. For all the investigated cultivars, the concentration of oleuropein was higher in the pruning collecting period, CP2 (Table 1).

**Table 1.** Concentrations of simple phenols, oleuropein and total phenols (mg/100 g DW) in leaves of six olive cultivars collected at different collecting periods.

| *Source of Variability* | *Simple phenols* | | | **Secoiridoids** | |
|---|---|---|---|---|---|
| | ***Hydroxytyrosol*** | ***Tyrosol*** | ***Vanillin*** | ***Oleuropein*** | ***Total Phenols*** |
| ***Cultivar (cv.)*** | | | | | |
| *Puntoža* | 28.78 ± 1.58 cd | 7.91 ± 0.8 abc | 1.56 ± 0.29 ab | 4919.26 ± 722.13 a | 3747.69 ± 590.23 bcd |
| *Buža* | 32.89 ± 4.36 bc | 6.59 ± 0.89 bc | 1.34 ± 0.46 ab | 5258.78 ± 1332.27 a | 4373.40 ± 548.45 ab |
| *I. bjelica* | 22.51 ± 3.23 d | 9.24 ± 0.30 ab | 0.63 ± 0.10 b | 2733.04 ± 653.41 b | 3045.22 ± 364.60 cd |
| *Karbonaca* | 29.61 ± 6.20 bcd | 4.76 ± 1.50 c | 0.56 ± 0.08 b | 1149.39 ± 249.34 c | 3178.26 ± 273.08 cd |
| *Leccino* | 68.90 ± 10.26 a | 10.36 ± 1.20 a | 1.87 ± 0.48 a | 6194.95 ± 627.58 a | 4687.47 ± 461.50 a |
| *Rošinjola* | 39.37 ± 9.19 b | 5.73 ± 1.54 c | 0.75 ± 0.13 ab | 2782.62 ± 1028.16 b | 3956.97 ± 524.53 abc |
| ***Collecting period (CP)*** | | | | | |
| *Harvest (CP1)* | 24.91 ± 2.88 b | 6.00 ± 0.78 b | 0.78 ± 0.12 b | 2228.51 ± 416.30 b | 2883.34 ± 158.04 b |
| *Pruning (CP2)* | 49.11 ± 5.18 a | 8.86 ± 0.58 a | 1.45 ± 0.24 a | 5450.84 ± 543.68 a | 4779.67 ± 205.33 a |
| ***Cv. × CP*** | | | | | |
| *Puntoža × Harvest* | 25.93 ± 2.09 $^{b}$ | 8.09 ± 1.40 $^{a}$ | 1.18 ± 0.22 | 3389.23 ± 452.35 $^{ab}$ | 2453.63 ± 140.18 $^{ab}$ |
| *Puntoža × Pruning* | 31.62 ± 0.19 $^{CD}$ | 7.73 ± 1.08 $^{B}$ | 1.93 ± 0.49 | **6449.28 ± 248.56 $^{AB}$** | **5041.76 ± 218.15 $^{A}$** |
| *Buža × Harvest* | 24.95 ± 4.04 $^{b}$ | 6.69 ± 1.88 $^{ab}$ | 0.77 ± 0.31 | 2453.51 ± 964.06 $^{bc}$ | 3192.12 ± 259.01 $^{ab}$ |
| *Buža × Pruning* | **40.84 ± 3.94 $^{CD}$** | 6.49 ± 0.65 $^{B}$ | 1.90 ± 0.79 | **8064.04 ± 275.26 $^{A}$** | **5554.67 ± 203.69 $^{A}$** |
| *I. bjelica × Harvest* | 15.99 ± 1.48 $^{b}$ | 8.98 ± 0.17 $^{a}$ | 0.58 ± 0.10 | 1394.17 ± 219.83 $^{bc}$ | 2270.87 ± 206.15 $^{b}$ |
| *I. bjelica × Pruning* | **29.03 ± 2.73 $^{D}$** | 9.50 ± 0.60 $^{AB}$ | 0.69 ± 0.19 | **4071.90 ± 542.06 $^{C}$** | **3819.57 ± 150.21 $^{B}$** |
| *Karbonaca × Harvest* | 16.13 ± 2.38 $^{b}$ | 1.85 ± 0.74 $^{b}$ | 0.45 ± 0.07 | 671.04 ± 139.62 $^{c}$ | 2790.83 ± 351.68 $^{ab}$ |
| *Karbonaca × Pruning* | **43.09 ± 2.25 $^{C}$** | **7.68 ± 1.47 $^{B}$** | 0.66 ± 0.11 | **1627.73 ± 250.10 $^{D}$** | 3565.69 ± 314.75 $^{B}$ |
| *Leccino × Harvest* | 47.07 ± 6.35 $^{a}$ | 8.03 ± 0.75 $^{a}$ | 1.21 ± 0.54 | 4962.29 ± 346.07 $^{a}$ | 3765.28 ± 456.85 $^{a}$ |
| *Leccino × Pruning* | **90.73 ± 3.10 $^{A}$** | **12.69 ± 1.11 $^{A}$** | 2.53 ± 0.67 | **7427.62 ± 574.51 $^{A}$** | **5609.66 ± 76.06 $^{A}$** |
| *Rošinjola × Harvest* | 19.38 ± 4.00 $^{b}$ | 2.36 ± 0.51 $^{b}$ | 0.51 ± 0.09 | 500.80 ± 167.15 $^{c}$ | 2827.28 ± 299.65 $^{ab}$ |
| *Rošinjola × Pruning* | **59.37 ± 2.58 $^{B}$** | **9.09 ± 0.49 $^{AB}$** | 0.99 ± 0.12 | **5064.45 ± 225.64 $^{BC}$** | **5086.67 ± 98.31 $^{A}$** |
| ***(Cv.) p-value*** | <0.001 | <0.001 | 0.010 | <0.001 | <0.001 |
| ***(CP) p-value*** | <0.001 | <0.001 | 0.007 | <0.001 | <0.001 |
| ***(Cv. × CP) p-value*** | <0.001 | 0.003 | 0.567 | <0.001 | 0.018 |

Results are expressed as means ± standard errors (n = 3). For main factors (*Cv., CP*) different lowercase letters in a column represent statistically significant differences (stat. sign. diff.) between mean values for each main effect at $p < 0.05$ obtained by a two-way ANOVA and Tukey's test. Only for significant interactions (*Cv. × CP*, $p < 0.05$) simple main effects were computed at each level of the other factor and bold results in a column designate stat. sign. diff. (n = 3, Tukey's test, $p < 0.05$) between specific cultivar separately at each CP, while different superscript lower- and upper-case letters designate stat. sign. diff. (n = 3, Tukey's test, $p < 0.05$) between cultivars at *CP1* and *CP2* respectively.

**Table 2.** Concentrations of phenolic acids (mg/100 g DW) in leaves of six olive cultivars collected at different collecting periods.

| *Source of Variability* | **Phenolic Acids** | | | | |
|---|---|---|---|---|---|
| | ***4-Hydroxybenzoic Acid*** | ***Caffeic Acid*** | ***Ferulic Acid*** | ***Vanillic Acid*** | ***Verbascoside*** |
| ***Cultivar (cv.)*** | | | | | |
| *Puntoža* | 0.74 ± 0.17 b | 1.94 ± 0.39 | 0.97 ± 0.35 ab | 3.10 ± 1.20 a | 572.09 ± 170.49 a |
| *Buža* | 1.15 ± 0.25 b | 1.04 ± 0.21 | 0.64 ± 0.11 b | 0.80 ± 0.00 c | 470.53 ± 170.77 ab |
| *I. bjelica* | 0.84 ± 0.28 b | 1.05 ± 0.24 | 1.49 ± 0.47 ab | 2.4 ± 0.53 ab | 136.59 ± 34.06 c |
| *Karbonaca* | 2.66 ± 0.40 a | 1.14 ± 0.20 | 1.32 ± 0.49 ab | 2.43 ± 0.39 ab | 121.98 ± 49.95 c |
| *Leccino* | 1.61 ± 0.60 b | 1.61 ± 0.12 | 1.85 ± 0.11 a | 1.44 ± 0.30 bc | 316.77 ± 115.50 b |
| *Rošinjola* | 1.12 ± 0.42 b | 1.11 ± 0.39 | 1.62 ± 0.17 ab | 3.84 ± 0.29 a | 326.57 ± 144.41 b |
| ***Collecting period (CP)*** | | | | | |
| *Harvest (CP1)* | 2.00 ± 0.26 a | 1.33 ± 0.14 | 1.55 ± 0.18 a | 3.21 ± 0.42 a | 81.59 ± 18.34 b |
| *Pruning (CP2)* | 0.71 ± 0.13 b | 1.30 ± 0.19 | 1.08 ± 0.21 b | 1.46 ± 0.24 b | 556.58 ± 72.69 a |
| ***Cv. × CP*** | | | | | |
| *Puntoža × Harvest* | 0.97 ± 0.25 | 1.89 ± 0.42 | 1.48 ± 0.59 $^{a}$ | **5.40 ± 1.40 $^{a}$** | 205.47 ± 28.48 $^{a}$ |
| *Puntoža × Pruning* | 0.51 ± 0.14 | 1.99 ± 0.77 | 0.46 ± 0.14 | 0.80 ± 0.00 $^{B}$ | **938.70 ± 100.61 $^{A}$** |
| *Buža × Harvest* | 1.49 ± 0.44 | **1.44 ± 0.19** | 0.80 ± 0.00 $^{ab}$ | 0.80 ± 0.00 $^{c}$ | 94.47 ± 34.65 $^{ab}$ |
| *Buža × Pruning* | 0.80 ± 0.00 | 0.64 ± 0.16 | 0.48 ± 0.18 | 0.80 ± 0.00 $^{B}$ | **846.59 ± 56.48 $^{AB}$** |
| *I. bjelica × Harvest* | 1.30 ± 0.43 | **1.53 ± 0.24** | **2.44 ± 0.38 $^{ab}$** | **3.43 ± 0.20 $^{a–c}$** | 66.69 ± 12.85 $^{ab}$ |
| *I. bjelica × Pruning* | 0.39 ± 0.08 | 0.58 ± 0.05 | 0.55 ± 0.25 | 1.36 ± 0.54 $^{B}$ | **206.49 ± 27.39 $^{C}$** |
| *Karbonaca × Harvest* | 3.51 ± 0.30 | 0.99 ± 0.08 | 0.80 ± 0.00 $^{ab}$ | 3.10 ± 0.25 $^{a–c}$ | 22.30 ± 14.83 $^{b}$ |
| *Karbonaca × Pruning* | 1.81 ± 0.06 | 1.30 ± 0.40 | 1.84 ± 0.96 | 1.75 ± 0.48 $^{B}$ | **221.66± 48.14 $^{C}$** |
| *Leccino × Harvest* | 2.67 ± 0.80 | 1.72 ± 0.20 | **2.09 ± 0.01 $^{a}$** | **2.07 ± 0.19 $^{bc}$** | 80.21 ± 56.92 $^{ab}$ |
| *Leccino × Pruning* | 0.55 ± 0.25 | 1.49 ± 0.12 | 1.62 ± 0.10 | 0.80 ± 0.00 $^{B}$ | **553.32 ± 86.63 $^{BC}$** |
| *Rošinjola × Harvest* | 2.03 ± 0.25 | 0.44 ± 0.09 | 1.72 ± 0.09 $^{b}$ | **4.45 ± 0.05 $^{ab}$** | 20.43 ± 3.47 $^{b}$ |
| *Rošinjola × Pruning* | 0.21 ± 0.04 | 1.79 ± 0.53 | 1.52 ± 0.35 | 3.24 ± 0.23 $^{A}$ | **632.72 ± 102.67 $^{AB}$** |
| ***(Cv.) p-value*** | <0.001 | 0.072 | 0.036 | <0.001 | <0.001 |
| ***(CP) p-value*** | <0.001 | 0.853 | 0.037 | <0.001 | <0.001 |
| ***(Cv. × CP) p-value*** | 0.101 | 0.031 | 0.019 | 0.002 | <0.001 |

Results are expressed as means ± standard errors (n = 3). For main factors (*Cv.*, *CP*) different lowercase letters in a column represent statistically significant differences (stat. sign. diff.) between mean values for each main effect at $p < 0.05$ obtained by a two-way ANOVA and Tukey's test. Only for significant interactions (*Cv.* × *CP*, $p < 0.05$) simple main effects were computed at each level of the other factor and bold results in a column designate stat. sign. diff. (n = 3, Tukey's test, $p < 0.05$) between specific cultivar separately at each *CP*, while different superscript lower- and upper-case letters designate stat. sign. diff. (n = 3, Tukey's test, $p < 0.05$) between cultivars at *CP1* and *CP2* respectively.

**Table 3.** Concentrations of flavonoids (mg/100 g DW) in leaves of six olive cultivars collected at different collecting periods.

| *Source of Variability* | **Flavonoids** | | | | | |
|---|---|---|---|---|---|---|
| | ***Apigenin*** | ***Apigenin-7-O-Glucoside*** | ***Luteolin*** | ***Luteolin-7-O-Glucoside*** | ***Rutin*** | ***Catechin*** |
| ***Cultivar (cv.)*** | | | | | | |
| *Puntoža* | 2.00 ± 0.56 | 53.42 ± 3.43 b | 19.19 ± 3.13 c | 537.28 ± 24.17 a | 31.36 ± 3.55 cd | 30.03 ± 4.34 a |
| *Buža* | 6.00 ± 2.02 | 40.60 ± 5.03 b | 25.80 ± 5.73 bc | 538.28 ± 69.17 a | 58.78 ± 5.17 bc | 23.99 ± 4.64 ab |
| *I. bjelica* | 5.46 ± 1.61 | 41.14 ± 2.99 b | 21.71 ± 3.94 c | 299.82 ± 22.15 b | 114.83 ± 5.03 a | 17.24 ± 3.51 b |
| *Karbonaca* | 4.32 ± 0.82 | 14.26 ± 2.89 b | 46.00 ± 4.50 a | 231.22 ± 37.75 b | 27.93 ± 4.59 d | 13.99 ± 3.89 b |
| *Leccino* | 6.49 ± 3.67 | 105.16 ± 20.17 a | 36.48 ± 7.57 ab | 516.62 ± 39.54 a | 77.15 ± 12.86 b | 28.70 ± 5.67 a |
| *Rošinjola* | 6.86 ± 2.25 | 30.78 ± 6.03 b | 41.85 ± 6.40 a | 313.83 ± 62.60 b | 23.48 ± 5.34 d | 24.62 ± 7.52 ab |
| ***Collecting period (CP)*** | | | | | | |
| *Harvest (CP1)* | 8.58 ± 1.22 a | 42.68 ± 7.78 | 42.14 ± 3.43 a | 341.16 ± 41.20 b | 55.03 ± 9.55 | 13.44 ± 2.10 b |
| *Pruning (CP2)* | 1.80 ± 0.20 b | 52.44 ± 8.96 | 21.53 ± 2.29 b | 471.19 ± 31.06 a | 56.15 ± 7.80 | 32.75 ± 1.97 a |
| ***Cv. × CP*** | | | | | | |
| *Puntoža × Harvest* | 3.06 ± 0.52 | 57.34 ± 5.78 | 24.35 ± 4.50 | 550.16 ± 39.08 | 33.51 ± 4.02 | 23.30 ± 6.20 |
| *Puntoža × Pruning* | 0.94 ± 0.40 | 49.50 ± 3.17 | 14.03 ± 1.49 | 524.40 ± 35.05 | 29.21 ± 6.51 | 36.77 ± 3.19 |
| *Buža × Harvest* | 10.51 ± 0.17 | 40.87 ± 10.48 | 37.01 ± 5.94 | 456.18 ± 113.78 | 63.96 ± 5.82 | 15.20 ± 5.09 |
| *Buža × Pruning* | 1.48 ± 0.11 | 40.33 ± 4.09 | 14.59 ± 1.73 | 620.39 ± 65.05 | 53.60 ± 8.53 | 32.78 ± 2.15 |
| *I. bjelica × Harvest* | 9.03 ± 0.44 | 39.41 ± 4.13 | 30.15 ± 1.45 | 265.90 ± 35.65 | 120.89 ± 8.15 | 9.81 ± 1.65 |
| *I. bjelica × Pruning* | 1.88 ± 0.25 | 42.86 ± 4.90 | 13.26 ± 2.08 | 333.74 ± 5.70 | 108.77 ± 4.84 | 24.66 ± 1.98 |
| *Karbonaca × Harvest* | 5.58 ± 1.34 | 9.37 ± 3.98 | 54.00 ± 5.57 | 155.91 ± 36.25 | 20.72 ± 4.10 | 5.81 ± 0.98 |
| *Karbonaca × Pruning* | 3.06 ± 0.07 | 19.14 ± 1.47 | 37.99 ± 2.46 | 306.53 ± 11.85 | 35.15 ± 6.03 | 22.18 ± 2.79 |
| *Leccino × Harvest* | 11.87 ± 6.19 | 90.46 ± 28.12 | 51.36 ± 7.62 | 440.29 ± 43.97 | 94.54 ± 29.56 | 18.44 ± 6.17 |
| *Leccino × Pruning* | 1.12 ± 0.45 | 119.86 ± 32.07 | 21.61 ± 2.70 | 592.96 ± 7.53 | 93.48 ± 33.02 | 38.96 ± 4.20 |
| *Rošinjola × Harvest* | 11.40 ± 2.16 | 18.62 ± 4.25 | 55.99 ± 0.86 | 178.54 ± 31.20 | 12.95 ± 2.17 | 8.09 ± 2.51 |
| *Rošinjola × Pruning* | 2.32 ± 0.15 | 42.94 ± 4.02 | 27.71 ± 1.99 | 449.13 ± 17.67 | 34.01 ± 5.18 | 41.14 ± 1.81 |
| ***(Cv.) p-value*** | 0.173 | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 |
| ***(CP) p-value*** | <0.001 | 0.212 | <0.001 | <0.001 | 0.847 | <0.001 |
| ***(Cv. × CP) p-value*** | 0.164 | 0.695 | 0.119 | 0.076 | 0.468 | 0.128 |

Results are expressed as means ± standard errors (n = 3). For main factors (*Cv., CP*) different lowercase letters in a column represent statistically significant differences (stat. sign. diff.) between mean values for each main effect at $p < 0.05$ obtained by a two-way ANOVA and Tukey's test.

In the CP1 leaves, 'Leccino' had more total phenols than 'Istarska bjelica', while in CP2 'Istarska bjelica' and 'Karbonaca' were characterized by the lowest total phenol concentration. Similar to the case of oleuropein, all the cultivars except 'Karbonaca' accumulated more total phenols in the pruning (CP2) than in the harvesting (CP1) collecting period (Table 1).

Leaves of 'Leccino' and 'Puntoža' contained more ferulic acid compared to 'Rošinjola' in CP1, while 'Buža' and 'Istarska bjelica' were richer in this phenol in CP1 than in CP2 (Table 2). 'Puntoža' leaves accumulated more vanillic acid than those of 'Buža' and 'Leccino' cultivars in CP1, while in CP2 'Rošinjola' leaves contained the highest vanillic acid concentration. The majority of cultivars exhibited a higher concentration of vanillic acid in CP1 compared to CP2. 'Puntoža' reached higher verbascoside levels in CP1 compared to 'Karbonaca' and 'Rošinjola', as well as higher levels in CP2 compared to 'Leccino', 'Karbonaca' and 'Istarska bjelica'. Again, all the studied cultivars accumulated more verbascoside in CP2 than in CP1 (Table 2).

The content of luteolin was higher in 'Karbonaca', 'Rošinjola' and 'Leccino' than in leaves of other cultivars. 'Leccino' had the highest concentration of apigenin-7-O-glucoside. The concentration of luteoline-7-*O*-glucoside was higher in 'Leccino', 'Buža' and 'Puntoža' leaves than in the other cultivars; 'Istarska bjelica' was the most and 'Karbonaca' and 'Rošinjola' the least abundant in rutin, while 'Puntoža' and 'Leccino' were characterized by a higher concentration of catechin compared to 'Istarska bjelica' and 'Karbonaca' leaves (Table 3).

The concentration of macro- and micronutrients found in leaves of the studied cultivars in the two collecting periods are reported in Table 4. No interactions between the two studied factors were observed for phosphorus (P), magnesium (Mg), manganese (Mn) or boron (B). The concentration of P was higher in 'Karbonaca' then in 'Buža' leaves. The highest Mg concentration was found in 'Rošinjola'. 'Rošinjola' leaves contained the highest and those of 'Istarska bjelica' the lowest level of Mn, although with some exceptions regarding statistical significance, while the latter cultivar had the highest and 'Karbonaca' and 'Rošinjola' the lowest B level. The levels of P and B were higher in the harvest (CP1) than in the pruning (CP2) leaves, while the opposite was determined for Mn.

'Leccino' had a higher concentration of potassium (K) when compared to 'Puntoža', 'Istarska bjelica', 'Karbonaca' and 'Rošinjola' in CP1. The concentration of K was higher in CP1 than in CP2 for all the cultivars. A higher concentration of calcium (Ca) was found in 'Karbonaca' and 'Rošinjola' than in 'Buža' and 'Istarska bjelica' in the CP1 leaves, while Buža had the lowest Ca concentration in CP2. More sodium (Na) was found in leaves of 'Leccino' than in those of the 'Buža', 'Istarska bjelica', 'Karbonaca' and 'Rošinjola' cultivars in the harvest period, CP1. All the cultivars except 'Istarska bjelica' and 'Karbonaca' exhibited higher Na levels in CP1 compared to CP2.

'Leccino' was characterized by a higher concentration of iron (Fe) compared to 'Istarska bjelica' and 'Karbonaca' in CP1, while 'Karbonaca' contained the lowest iron concentration among the cultivars in CP2. The concentration of zinc (Zn) was the highest in 'Rošinjola' but not significantly different from that found in 'Puntoža' leaves. 'Puntoža', 'Buža' and 'Rošinjola' had more Zn in CP1 than in CP2. While no differences between cultivars were found in CP1, 'Buža' and 'Istarska bjelica' leaves contained more copper (Cu) than leaves of the 'Leccino' and 'Karbonaca' cultivars in CP2. The leaves of all the studied cultivars contained more Cu in CP2 than in CP1.

**Table 4.** Concentrations of macro- and micronutrients (mg/100 g DW) in leaves of six olive cultivars collected at different collecting periods.

| *Source of Variability* | **Macronutrients (g/kg DW)** | | | | | **Micronutrients (mg/kg DW)** | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | ***Phosphorus*** | ***Pottasium*** | ***Calcium*** | ***Magnesium*** | ***Sodium*** | ***Iron*** | ***Zinc*** | ***Manganese*** | ***Copper*** | ***Boron*** |
| ***Cultivar (cv.)*** | | | | | | | | | | |
| *Puntoža* | 1.53 ± 0.08 ab | 4.99 ± 0.42 b | 19.36 ± 0.45 ab | 0.93 ± 0.03 b | 0.5 ± 0.1 ab | 76.16 ± 5.11 a | 24.96 ± 0.77 ab | 38.78 ± 1.4 cd | 56.27 ± 13.39 ab | 19.66 ± 1.3 ab |
| *Buža* | 1.47 ± 0.04 b | 5.49 ± 0.42 ab | 14.81 ± 0.96 c | 0.88 ± 0.02 b | 0.44 ± 0.08 bc | 72.20 ± 4.44 ab | 20.55 ± 0.56 c | 38.83 ± 0.44 cd | 61.40 ± 18.29 a | 21.22 ± 2.08 a |
| *I. bjelica* | 1.57 ± 0.04 ab | 4.86 ± 0.31 b | 17.20 ± 1.20 bc | 1.07 ± 0.05 b | 0.37 ± 0.06 bc | 64.72 ± 4.33 bc | 21.86 ± 1.16 bc | 32.08 ± 1.87 d | 62.01 ± 17.79 a | 16.85 ± 1.27 c |
| *Karbonaca* | 1.72 ± 0.06 a | 4.87 ± 0.33 b | 20.81 ± 0.31 a | 0.89 ± 0.02 b | 0.31 ± 0.05 c | 60.32 ± 5.22 c | 24.24 ± 1.14 abc | 46.73 ± 1.88 ab | 43.82 ± 10.32 bc | 18.9 ± 1.74 b |
| *Leccino* | 1.6 ± 0.05 ab | 5.88 ± 0.68 a | 18.47 ± 0.97 ab | 0.86 ± 0.03 b | 0.60 ± 0.13 a | 79.41 ± 8.23 a | 22.96 ± 1.21 bc | 44.58 ± 2.36 bc | 36.37 ± 7.39 c | 18.12 ± 1.44 bc |
| *Rošinjola* | 1.55 ± 0.09 ab | 4.94 ± 0.52 b | 20.77 ± 0.15 a | 1.48 ± 0.14 a | 0.46 ± 0.06 abc | 68.09 ± 4.20 abc | 27.95 ± 2.52 a | 53.27 ± 0.61 a | 51.42 ± 13.07 ab | 16.48 ± 1.35 c |
| ***Collecting period (CP)*** | | | | | | | | | | |
| *Harvest (CP1)* | 1.66 ± 0.04 a | 6.12 ± 0.17 a | 17.6 ± 0.78 b | 1.02 ± 0.07 | 0.61 ± 0.04 a | 80.30 ± 2.86 a | 25.44 ± 1.07 a | 41.50 ± 1.96 b | 22.87 ± 0.78 b | 21.84 ± 0.55 a |
| *Pruning (CP2)* | 1.49 ± 0.03 b | 4.23 ± 0.09 b | 19.54 ± 0.40 a | 1.02 ± 0.05 | 0.29 ± 0.01 b | 60.00 ± 1.46 b | 22.07 ± 0.55 b | 43.26 ± 1.72 a | 80.90 ± 4.94 a | 15.24 ± 0.39 b |
| ***Cv. × CP*** | | | | | | | | | | |
| *Puntoža × Harvest* | 1.63 ± 0.14 | **5.83 ± 0.37** $^{b}$ | 18.75 ± 0.71 $^{ab}$ | 0.91 ± 0.04 | **0.72 ± 0.03** $^{ab}$ | **86.75 ± 3.56** $^{ab}$ | **26.13 ± 0.72** $^{ab}$ | 38.16 ± 1.63 | 27.31 ± 2.13 | 22.38 ± 0.71 |
| *Puntoža × Pruning* | 1.44 ± 0.03 | 4.15 ± 0.18 | 19.97 ± 0.39 $^{A}$ | 0.95 ± 0.05 | 0.28 ± 0.01 | 65.57 ± 2.36 $^{A}$ | 23.80 ± 1.06 | 39.41 ± 2.59 | **85.23 ± 7.28** $^{AB}$ | 16.95 ± 0.78 |
| *Buža × Harvest* | 1.52 ± 0.07 | **6.39 ± 0.20** $^{ab}$ | 13.11 ± 0.44 $^{c}$ | 0.85 ± 0.02 | **0.60 ± 0.08** $^{bc}$ | **80.97 ± 4.35** $^{ab}$ | **21.59 ± 0.45** $^{b}$ | 38.50 ± 0.75 | 20.85 ± 2.34 | 25.71 ± 0.78 |
| *Buža × Pruning* | 1.42 ± 0.05 | 4.58 ± 0.17 | 16.52 ± 1.24 $^{B}$ | 0.91 ± 0.01 | 0.29 ± 0.05 | 63.43 ± 1.62 $^{A}$ | 19.50 ± 0.50 | 39.17 ± 0.53 | **101.96 ± 4.79** $^{A}$ | 16.73 ± 0.87 |
| *I. bjelica × Harvest* | 1.59 ± 0.04 | **5.44 ± 0.08** $^{b}$ | 14.76 ± 1.10 $^{bc}$ | 1.04 ± 0.08 | 0.47 ± 0.10 $^{bc}$ | 68.88 ± 8.72 $^{b}$ | 22.86 ± 2.37 $^{b}$ | 29.21 ± 2.13 | 22.84 ± 0.83 | 19.61 ± 0.28 |
| *I. bjelica × Pruning* | 1.55 ± 0.07 | 4.28 ± 0.37 | **19.64 ± 0.29** $^{A}$ | 1.11 ± 0.07 | 0.27 ± 0.03 | 60.57 ± 0.55 $^{A}$ | 20.87 ± 0.29 | 34.94 ± 2.19 | **101.18 ± 6.91** $^{A}$ | 14.08 ± 0.56 |
| *Karbonaca × Harvest* | 1.83 ± 0.06 | **5.60 ± 0.08** $^{b}$ | 21.02 ± 0.54 $^{a}$ | 0.88 ± 0.03 | 0.39 ± 0.07 $^{c}$ | **71.37 ± 3.56** $^{b}$ | 25.27 ± 1.53 $^{b}$ | 45.59 ± 2.85 | 23.27 ± 1.70 | 22.76 ± 0.41 |
| *Karbonaca × Pruning* | 1.62 ± 0.06 | 4.14 ± 0.14 | 20.60 ± 0.37 $^{A}$ | 0.89 ± 0.05 | 0.24 ± 0.01 | 49.27 ± 1.08 $^{B}$ | 23.20 ± 1.76 | 47.87 ± 2.87 | **64.38 ± 10.33** $^{BC}$ | 15.05 ± 0.30 |
| *Leccino × Harvest* | 1.66 ± 0.09 | **7.39 ± 0.11** $^{a}$ | 17.16 ± 1.67 $^{a\text{-}c}$ | 0.84 ± 0.05 | **0.89 ± 0.02** $^{a}$ | **97.45 ± 0.43** $^{a}$ | 23.52 ± 2.28 $^{b}$ | 44.47 ± 3.62 | 20.35 ± 1.31 | 21.13 ± 0.35 |
| *Leccino × Pruning* | 1.54 ± 0.04 | 4.36 ± 0.11 | 19.78 ± 0.42 $^{A}$ | 0.89 ± 0.05 | 0.31 ± 0.05 | 61.37 ± 3.64 $^{A}$ | 22.40 ± 1.35 | 44.69 ± 3.85 | **52.40 ± 3.83** $^{C}$ | 15.10 ± 1.08 |
| *Rošinjola × Harvest* | 1.74 ± 0.03 | **6.04 ± 0.28** $^{b}$ | 20.81 ± 0.24 $^{a}$ | 1.58 ± 0.20 | **0.59 ± 0.02** $^{bc}$ | **76.37 ± 3.78** $^{ab}$ | **33.27 ± 0.86** $^{a}$ | 53.08 ± 0.72 | 22.58 ± 0.84 | 19.44 ± 0.33 |
| *Rošinjola × Pruning* | 1.36 ± 0.05 | 3.84 ± 0.21 | 20.73 ± 0.22 $^{A}$ | 1.38 ± 0.20 | 0.33 ± 0.00 | 59.80 ± 2.29 $^{A}$ | 22.63 ± 1.63 | 53.46 ± 1.15 | **80.27 ± 4.56** $^{A\text{-}C}$ | 13.51 ± 0.42 |
| ***(Cv.) p-value*** | 0.025 | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 |
| ***(CP) p-value*** | <0.001 | <0.001 | <0.001 | 0.925 | <0.001 | <0.001 | <0.001 | 0.205 | <0.001 | <0.001 |
| ***(Cv. × CP) p-value*** | 0.208 | 0.004 | 0.015 | 0.713 | 0.002 | 0.027 | 0.022 | 0.844 | <0.001 | 0.051 |

Results are expressed as means ± standard errors (n = 3). For main factors (*Cv.*, *CP*) different lowercase letters in a column represent statistically significant differences (stat. sign. diff.) between mean values for each main effect at $p < 0.05$ obtained by a two-way ANOVA and Tukey's test. Only for significant interactions (*Cv.* × *CP*, $p < 0.05$) simple main effects were computed at each level of the other factor and bold results in a column designate stat. sign. diff. (n = 3, Tukey's test, $p < 0.05$) between specific cultivar separately at each *CP*, while different superscript lower- and upper-case letters designate stat. sign. diff. (n = 3, Tukey's test, $p < 0.05$) between cultivars at *CP1* and *CP2* respectively.

Significant correlations of the concentrations of the most abundant phenolic compounds and total phenol concentration with those of macronutrients and micronutrients in olive leaves were calculated and are reported in Table 5. In both collecting periods, a positive, moderate to strong correlation between K, Fe, Zn, Cu, B, Na and total phenol concentration was observed. Furthermore, a positive moderate to strong correlation of Zn, Cu and B with oleuropein, Zn with luteolin-7-O-glucoside and Cu with verbascoside concentration was noted. On the other hand, the P content exhibited a strong negative correlation with verbascoside, oleuropein and total phenol concentration, while P, K, Na, Zn and B were negatively proportional to verbascoside content.

**Table 5.** Correlation matrix between minerals and the most abundant phenolic compounds or total phenols in olive leaves.

| | Verbascoside | Luteolin-7-*O*-Glucoside | Oleuropein | Total Phenols |
|---|---|---|---|---|
| **Phosphorus** | **r = −0.654** * $p < 0.001$ | **r = −0.636** $p < 0.001$ | **r = −0.621** $p < 0.001$ | **r = −0.617** $p < 0.001$ |
| **Potassium** | **r = −0.617** $p < 0.001$ | r = 0.202 $p = 0.236$ | r = 0.352 $p = 0.035$ | **r = 0.533** $p < 0.001$ |
| **Calcium** | r = 0.110 $p = 0.524$ | r = 0.233 $p = 0.171$ | r = 0.034 $p = 0.842$ | r = 0.125 $p = 0.467$ |
| **Magnesium** | r = −0.098 $p = 0.568$ | r = 0.371 $p = 0.026$ | r = 0.250 $p = 0.142$ | r = 0.113 $p = 0.510$ |
| **Sodium** | **r = −0.492** $p = 0.002$ | r = 0.016 $p = 0.927$ | r = 0.201 $p = 0.239$ | **r = 0.462** $p = 0.005$ |
| **Iron** | r = −0.392 $p = 0.018$ | r = 0.044 $p = 0.797$ | r = 0.121 $p = 0.482$ | r = 0.413 $p = 0.012$ |
| **Zinc** | **r = −0.402** $p = 0.015$ | **r = 0.470** $p = 0.004$ | **r = 0.476** $p = 0.003$ | **r = 0.474** $p = 0.004$ |
| **Manganese** | r = 0.009 $p = 0.960$ | r = 0.121 $p = 0.483$ | r = 0.055 $p = 0.751$ | r = 0.208 $p = 0.223$ |
| **Copper** | **r = 0.710** $p < 0.001$ | r = 0.342 $p = 0.041$ | **r = 0.592** $p = <0.001$ | **r = 0.657** $p = <0.001$ |
| **Boron** | **r = −0.536** $p < 0.001$ | r = 0.194 $p = 0.257$ | **r = 0.456** $p = 0.005$ | **r = 0.614** $p = <0.001$ |

* Pearson correlation coefficients (r) are bolded only for moderate (absolute r = 0.40–0.50) to strong (absolute r > 0.50) significant correlations.

Hierarchical clustering analysis (HCA) was conducted and included all the leaf samples grouped into two groups based on the collecting period, with phenols and elements as variables. A clear clustering of the groups was obtained, as shown on the heatmap diagram in Figure 1. The two investigated harvest periods were related mostly to the characteristic phenols/elements previously determined by ANOVA (Tables 1–4). In general, the samples collected during pruning (CP2) were characterized by Cu and the majority of phenols, including the major ones, while the harvest leaves (CP1) were clustered together mostly based on the highest amounts of all the other elements and particular phenols, such as vanillic acid, apigenin, 4-hydroxybenzoic acid and luteolin. As for the inter-cultivar differences, among the pruning samples (CP2), 'Leccino' and 'Karbonaca' formed a subcluster separated from all the other cultivars, while similar was noted for 'Rošinjola' and 'Karbonaca' leaves among the samples collected in the harvest (CP1).

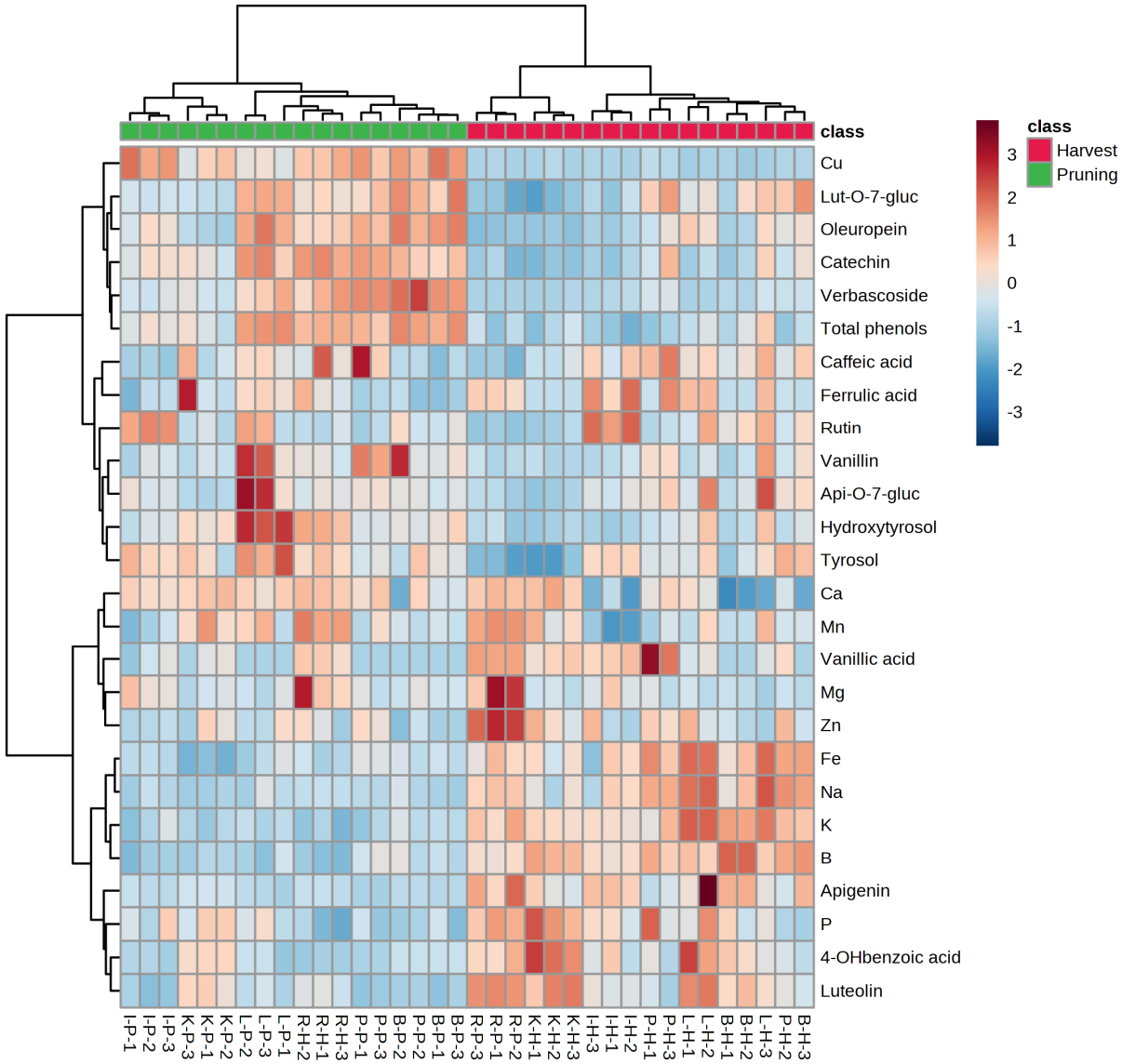


**Figure 1.** Clustering of olive leaf samples by hierarchical cluster analysis (HCA) based on the composition of phenols and elements (variables). The rows in the heatmap diagram represent phenols/elements, and the columns represent samples. Sample codes were formed on the following principle: 'Buža' (B) leaves collected during harvest (H) in the first replicate (1) were coded as B-H-1 and so on. Colors of the heatmap cells indicate low (dark blue), medium (white) and high (dark red) abundance of a particular phenol/element.

To better identify particular phenols and elements characteristic of the investigated cultivars and collecting periods, which are therefore most useful for their mutual separation, the data were subjected to partial least squares–discriminant analysis (PLS–DA). Separation according to cultivar was not very clear, probably partly because of the large number of cultivars (six). The variables with the highest variable importance in projection (VIP) scores, and therefore those most useful for separation according to cultivar, were elements such as Cu, Mn and Zn (VIP scores > 1.5), followed by rutin, vanillic acid and Mg (VIP scores > 2.0). PLS–DA differentiation according to collecting period was very successful, as can be seen in Figure 2. Again, elements such as Cu, B and K contained most information useful for the separation, followed by total phenols, Na, catechin, verbascoside and Fe, while other variables contributed less. The abundance of all the elements selected according to the VIP scores, except Cu, were more characteristic for the harvest period (CP1), while major

phenols, such as oleuropein and verbascoside, as well as total phenols, were more abundant in leaves collected during pruning (CP2) (Figure 2).

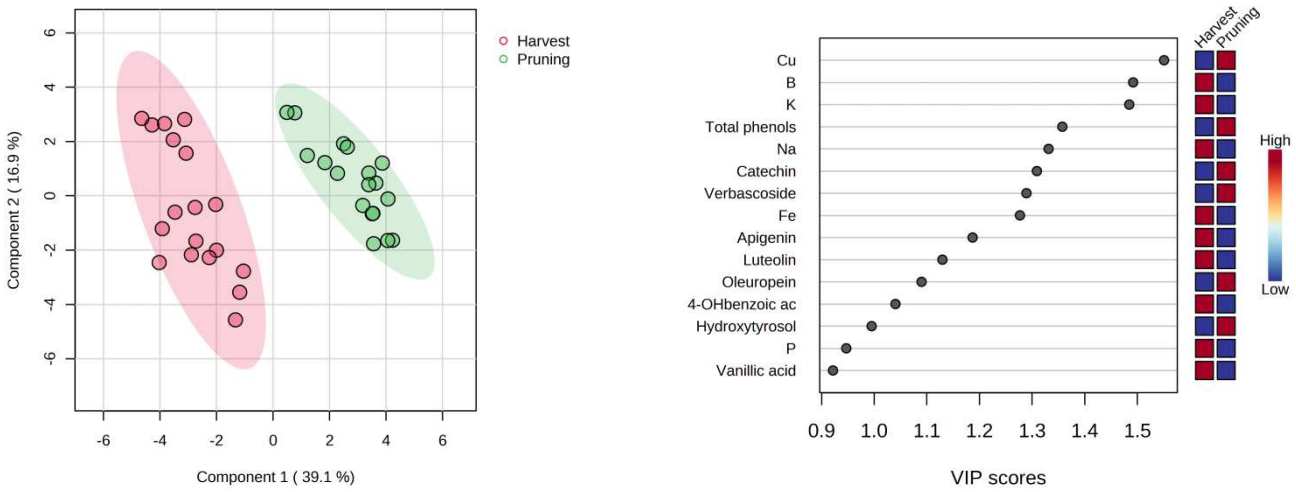


**Figure 2.** (**a**) Separation of olive leaves according to collecting period in two-dimensional space by partial least squares−discriminant analysis (PLS−DA); (**b**) variable importance in projection (VIP) scores of phenols and elements (variables) most useful for the separation.

## 4. Discussion

The results of this study showed that phenolic compounds found in the leaves of the different cultivars and in different phenological phases do not vary qualitatively but only quantitatively. The concentration of most of the studied phenols was cultivar-dependent, with an increase in the second collecting period CP2 (vegetative recovery phase) (Tables 1–3). Talhaoui et al. [13] reported that differences in phenolic content are often associated with an increase in the content and activity of polyphenol protein oxidase (PPO) in leaves, which is related to genotype and to external factors such as geographical location, stress and ultraviolet radiation. Thus, the fact that differences were observed was in line with the findings of many other authors [23,24]. The most abundant phenol in leaves of all the investigated cultivars in both collecting periods was oleuropein (Table 1), which was in line with different cultivars worldwide [3]. While some publications reported a significantly negative correlation between oleuropein and hydroxytyrosol and tyrosol [25], explaining this by the fact that hydroxytyrosol and tyrosol are products of oleuropein hydrolysis, in this study such a relation was not observed. However, according to our previous studies [14,15] and according to Blasi et al. [26], hydroxytyrosol and oleuropein content is highly dependent on the type of cultivar and harvesting period. Blasi et al. [26] reported that Leccino was among the cultivars with the highest concentration of hydroxytyrosol and oleuropein in leaves, especially in March [26]. Such an increase in oleuropein concentration in March was also found in other cultivars [27,28]. On the other hand, in the autumn and winter months a decrease in oleuropein content was observed for many cultivars, which could be associated with a decrease in the enzymatic activity of L-phenylalanine ammonia-lyase (PAL) causing the degradation of oleuropein [29]. Such results partially coincide with those obtained in this study, since concentrations of oleuropein and hydroxytyrosol were much higher in March (CP2) than in October (CP1), and, in the majority of interaction combinations, the 'Leccino' cultivar was shown to be more abundant in these compounds, although 'Buža' also showed high secoiridoid potential, but mostly in the pruning period, CP2 (Table 1, Figure 1). Knowing that oleuropein has previously been linked to olive cultivar resistance to freezing temperatures [30], one of the possible triggers for the higher oleuropein concentration in CP2 was a pre-sampling lower-temperature period (Figure S1). Surprisingly, for cultivars grown in Poreč the highest concentration of oleuropein

was not found in 'Istarska bjelica' leaves, although it was previously reported that this cultivar has exceptionally high oleuropein potential [14]. On the contrary, the oleuropein content of leaves of 'Istarska bjelica' was mostly lower than in leaves of the other studied cultivars, with the exception of 'Rošinjola'. Such a result could be explained by the possible impact of terroir and cultivar interaction on this and other leaf phenolics through cultivar adaptability to climate and soil parameters in the region in question, since 'Istarska bjelica' has been marked as one of the most known and represented autochthonous cultivars in Istria [31]. The 'Karbonaca' cultivar was once again confirmed as a cultivar with a low content of oleuropein and consequently a lower content of total phenols [3] since, as it could be expected, in this study the levels of these two compounds correlated strongly ($r$= 0.836, $p < 0.001$).

In our previous studies we reported significantly higher levels of tyrosol and vanillin in leaves of 'Buža' [3] as well as high levels of tyrosol in leaves of the 'Istarska bjelica' cultivar [14], but in this study no such relations were confirmed, with higher concentrations of these phenols mostly observed in leaves of the 'Leccino' cultivar (Table 1).

Regarding phenolic acids, which constitute an important fraction of the non-flavonoid phenolic fraction in leaves, the highest levels of 4-hydroxybenzoic acid among the investigated cultivars was found in 'Karbonaca', confirming our previous results [3]. Levels of caffeic acid, an extremely strong antioxidant, remained unchanged in leaves of olives grown in this trial (Table 2). Caffeic acid, together with its glycoside verbascoside, plays an important role in scavenging free radicals, and thus it is of crucial importance to determine the cultivars and collection periods with high amounts of these phenols. In our previously published study, verbascoside was found to be an important differentiator of leaf collection periods [14]. This was additionally confirmed in this research since among the listed phenolic compounds, along with catechine, verbascoside emerged in the same role (Figure 2b). Other authors reported a significantly higher concentration of verbascoside after olive exposure to cold stress [32], which could explain the results obtained in this study with higher verbascoside concentrations of all cultivars in March (CP2) than in October (CP1). Thus, from Figure 1 it can be seen that verbascoside as the main hydroxycinnamic derivative in olives was dominant in CP2 [33], while a higher concentration of 4-hydroxybenzoic acid as well as of vanillic acid, which are benzoic acid derivatives [34,35], were connected to CP1.

Flavonoids are considered an important fraction of olive leaf phenolics, with a great superoxide scavenging ability compared to non-flavonoids. Our data revealed that apigenin and its derivative luteolin [34] were primarily linked to the harvest period (CP1) (Figure 1). In general, the most prevalent leaf flavonoid identified was luteolin-7-*O*-glucoside (Table 3), as was previously reported for Croatian cultivars [15]. Its concentration was significantly cultivar-dependent and differentiated 'Buža', 'Puntoža', and 'Leccino', with higher values, from the 'Istarska bjelica', 'Karbonaca' and 'Rošinjola' cultivars (Tables 1 and 2). In addition, in the leaves of olives grown in this study concentrations of luteolin were higher in 'Karbonaca', while luteolin-7-*O*-glucoside dominated in 'Buža' (Table 3), showing a similar trend between these two cultivars as that observed in our previous study [3]. As determined in many of our previous studies [14–16], rutin concentration was confirmed to be highest in 'Istarska bjelica' leaves.

It is known that an increase in flavonoid content is regulated by the environment [36] and thus mainly linked to growing season period. In our previous research on the temporal dynamics of the accumulation of all the flavonoids studied in this study similar trends were reported [14], with higher values of lutolin-7-*O*-glucoside, as the most abundant flavonoid, in the pruning period.

The mineral status of plants is essential in predicting the nutritional requirements of olive trees, while also providing co-factors for many enzymes involved in the synthesis of phenolic compounds. Among macronutrients, the levels of P, Ca and Na in all the analyzed leaf samples were above the deficiency threshold, which was also observed for the concentrations of K in the first collecting period, CP1 (Table 4). However, in the second collecting period, CP2, the concentration of K decreased and, in some cases, dropped under

the deficiency limit of 4 g/kg DW (Table 4) [37]. It has been reported that low temperatures reduce the mobility and uptake of P and K [38]. This fact could explain the somewhat higher P content and much higher K content found in October (CP1) than in March (CP2), which was previously reported in other studies [32]. Significantly positive and negative correlations between P and particular phenolic compounds [15,39] as well as between K and oleuropein concentration [15] were observed in other studies. In this study, strong negative correlations between P and most plentiful phenolic compounds, as well as total phenolic content, was observed (Table 5). On the other hand, potassium in our research had shown a positive correlation with total phenolic content. The concentration of Mg was adequate, higher than 0.8 g/kg DW [37], in leaves of all the selected cultivars. Magnesium, together with Mn, is essential for correct PAL function and thus affects phenol synthesis [40]. In one of our previous studies we reported a negative correlation between Mg and flavonoids [15], which was partially confirmed by this study, since Mg was correlated negatively with luteolin-7-*O*-glucoside. Nevertheless, the concentration of Mg was found to be highly cultivar-dependent, with 'Rošinjola' the most abundant one (Table 4). The deficiency threshold of Ca is 10 g/kg, and in this study its concentration was in the optimal range [37]. A higher Ca content is typical for olives grown in the Mediterranean region [32].

As can be seen in Figure 1, the harvest period is characterized by almost all the determined microelements, with the exception of Cu. Therefore, the concentration of Fe was higher in CP1 than in CP2 for all the studied cultivars except 'Istarska bjelica', with a negative correlation between Fe and verbascoside observed in this and in a previous study [14]. Zinc concentrations were sufficient, more than 10 mg/kg DW [31,37], in leaves of all the studied cultivars, with moderate positive correlations with oleuropein, luteolin-7-*O*-glucoside and total phenol concentration. Zinc applied as a constituent of Brotomax foliar fertilizer increased the level of leaf polyphenols in citrus and olive in previous studies [41,42]

The role of Mn in the synthesis of phenols was highlighted previously, but no correlation was observed between this micronutrient and the most abundant phenols in this study. The concentration of Mn varied significantly among the cultivars and was time-dependent (Table 4). In all the cases its level was above the corresponding deficiency threshold of 20 mg/kg [37]. The concentration of Cu was cultivar-dependent only in March (Table 4), and it was far above its deficiency threshold of 4 mg/kg [37], probably because it was used extensively as a fungicide added in this olive grove. Furthermore, Cu was found to have a strong positive correlation with oleuropein and verbascoside, the most abundant phenolic components in CP2, as well as with total phenolic concentration (Table 5) (Figure 2). On the contrary, Cu and K showed a strong negative correlation ($r = -0.785$, $p < 0.001$), which put these two minerals among the most potent collecting period differentiators in this study (Figure 2b). Ferreira et al. [43] reported that different Cu based pesticide formulations lowered olive leaf total phenol concentration. However, increased Cu concentration upgraded total phenol content in tea leaves [44]. According to Beutel et al. [45], the deficiency limit for B is 14 mg/kg, while Fernández-Escobar et al. [46] proposed the limit of 33 mg/kg. In this study, the B content was lower than 14 mg/kg only in leaves of the 'Rošinjola' cultivar in CP2 (Table 4). Despite the fact that in all the other investigated samples the B content was lower than 33 mg/kg, no visible B deficiency symptoms were observed. According to Karioti et al. [47], a B nutrient deficiency in leaves increases the content of some secoiridoids, while Liakopoulos and Karabourniotis found a higher oleuropein concentration in B-sufficient leaves [48]. In our previous study, B-treated leaves were found to have a higher oleuropein concentration [4]. In this study, a medium-strength correlation of B with oleuropein content was noticed (Table 5). A higher concentration of Cu in the samples collected during pruning at CP2, and of B and K in the samples collected during harvesting at CP1, is clearly visible on Figures 1 and 2 as the most distinguishing variables between the two sampling times, which could be linked to the usual fertilization (K and B) and plant protection (Cu) practices in the selected olive orchard.

## 5. Conclusions

Olive leaves are a valuable source of phenols, whose quantity is highly dependent on the cultivar; however, the same cultivar may be richer or poorer in phenols depending on the sampling period. For this reason, it is quite complicated to classify beyond doubt a particular cultivar as one with a high or low phenolic content. In this study, however, the highest concentration of oleuropein was found during the pruning period in leaves of 'Buža' and 'Leccino' compared to all the other cultivars studied, with the exception of 'Puntoža'. For all the cultivars the concentration of this valuable secoiridoid was significantly higher in the spring pruning collecting period. In general, oleuropein and the other most abundant olive leaf phenolics, such as vebascoside and luteolin-7-*O*-glucoside, were represented by higher total values during pruning in March than after the harvest in October, which was possibly partly linked to the pre-sampling cold period or the common gradual degradation of oleuropein during the autumn harvest period. The obtained results provided valuable confirmation that the month of March, which coincides with pruning, could be a suitable period for the recovery of larger amounts of bioactive phenols, while differences among cultivars could be utilized to valorize the autochthonous olive tree byproducts rich in particular phenols useful for application in several fields. The effect of cultivar and collecting period on the mineral composition of olive leaves was also noticed, with particular mineral nutrients, such as P, K, Na, Zn, Cu and B, clearly linked to all or some of the most abundant phenolic compounds (oleuropein, verbascoside and luteolin-7-*O*-glucoside) either in a positive or negative correlation. This can lead to a new perspective which can define future hydroponically based trials in order to enhance biochemical farming plant nutrition practices.

**Supplementary Materials:** The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/horticulturae9050594/s1, Table S1. Chemical properties of the Rhodic cambisol used in the study, Figure S1. Average daily temperatures (°C) and rainfall (mm) measured in the sampling period from the beginning of October 2017 until the end of March 2018 in Poreč, Istria, Croatia., Photo S1. Experiment design scheme.

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