

Article

The Use of Olive Leaves in Buža Olive Cultivar Oil Production: Exploring the Impact on Oil Yield and Chemical Composition

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Abstract: The effect of the addition of different amounts of olive leaf (1, 2.5, and 5%, m/m) during Buža olive cv. oil production on the quantitative production parameters, composition, and sensory characteristics of the obtained oils were investigated in this study. The addition of leaf during oil extraction increased oil yield and extractability index by 97% compared to the control oil. The addition of leaf during extraction increased the concentration of pigments in oils, and the oil positive sensory attributes intensities, such as fruitiness and green grass/leaf notes. The influence on oil phenolic composition was dependent on the amount of leaf added. When 1% leaf was added, most of the phenolic compounds were preserved, while the addition of leaf at 5% decreased the concentration of the majority of phenols, especially secoiridoids by 45% compared to the control oil. The addition of leaf slightly increased the concentration of fatty acid ethyl esters and waxes in the oils. The obtained results indicate that particular importance should be given to the amount of olive leaf present in olive paste during oil extraction, since it apparently can increase the extractability of oil, but can also have negative effects on phenolic composition when added in excess.

Keywords: *Olea europaea* L.; olive leaf; oil yield; oil composition; phenolic compounds; sensory characteristics



Citation: Novoselić, A.; Klisović, D.; Lukić, I.; Lukić, M.; Brkić Bubola, K. The Use of Olive Leaves in Buža Olive Cultivar Oil Production: Exploring the Impact on Oil Yield and Chemical Composition. *Agriculture* **2021**, *11*, 917. <https://doi.org/10.3390/agriculture11100917>

Academic Editors: Franc Štampar and Robert Veberič

Received: 26 August 2021

Accepted: 23 September 2021

Published: 24 September 2021

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1. Introduction

Virgin olive oil (VOO) is produced solely from olive fruits (*Olea europaea* L.) by mechanical or physical processes that include fruit crushing, olive paste malaxation, and oil extraction [1]. VOO is an important source of fat in the Mediterranean diet and is a valued product with rich nutritional properties and unique aroma and taste [2]. It is considered a functional food due to its high content of monounsaturated fatty acids and bioactive compounds, such as phenolic compounds [3]. Phenols, in addition to contributing to the taste of VOO, are responsible for its oxidative stability as well as shelf life of the product [4].

Olive leaves can be found in fruits after harvesting in high amounts (10% of the total olive weight) and could be partially or totally removed before crushing of olives. Olive leaves also accumulate in the total biomass as a result of annual pruning of the olive trees [5]. Olive leaves consist of water, carbohydrates, proteins, minerals, and lipids [6]. The lipid fraction is mainly consisting of omega-3 fatty acids, especially α -linolenic fatty acids (34–41% of total fatty acids) [7]. It also includes significant amounts of biologically active ingredients, such as phenolic compounds, chlorophylls, tocopherols, β -carotene, squalene, triterpenes, and sterols [8]. Therefore, olive leaves can be considered as a rich natural source of antioxidants, due to their high content of phenolic compounds especially oleuropein and its derivative hydroxytyrosol [6].

Leaf removal is a common practice in the VOO production process. However, a small portion of the leaves remains present during the oil processing. Some authors have found that the presence of olive leaves in the olive oil production process could influence mechanical problems related to the flow of olive paste through the pump [9]. On the contrary, Di Giovacchino et al. [10] reported that mixing leaves and olives did not cause

such problems when fixed hammer metal crusher was used, which reduced leaves to small pieces that became better merged with the olive paste. The influence of the presence of olive leaves during oil extraction on the oil composition and quality has been poorly investigated. Previous studies focused on the effect of the addition of leaf on basic quality parameters (free fatty acids, peroxide value, K232 and K270 UV extinction coefficients), total phenols, pigments, and antioxidant activity of oils thus produced and have found only a slight decrease in quality but an increase in pigments content, total phenols, and antioxidant activity [9,11–13]. However, the effect of the addition of leaf on particular phenols in olive oil has not been thoroughly investigated up to date. A few previous studies that considered phenolic composition found that the influence of the addition of leaf depended on olive variety and the proportion of leaves added [13,14]. Moreover, olive leaves, besides naturally occurring compounds, could also contain pesticide residues. Therefore, investigation on potential positive or negative health effects are needed to evaluate the safety of addition of leaf during oil production from olive fruits.

Since olive leaves contain a certain number of enzymes [15], it can be assumed that their addition during oil extraction could accelerate hydrolytic deterioration of oil [9]. To the best of our knowledge, there is a lack of studies related to the effect of the olive leaf addition on the content of fatty acid ethyl esters (FAEE), a very important parameter for VOO quality recently introduced in the IOC and EU regulations [16,17].

Further, olive leaf also contains waxes that cover its outer surface protecting it from wetting and loss of moisture [18]. Since waxes are very important parameters for authenticity control of VOO [19] and were also found to positively correlate with quality [20], it is important to determine the effect of the addition of leaf during olive processing on their content in the obtained oils, which was not investigated so far.

The aim of this study was to investigate the effect of the addition of different amounts of olive leaf (0%, 1%, 2.5%, and 5%, m/m) during olive processing on chemical composition (FAEE, waxes, phenolic compounds, and pigments), as well as on sensory characteristics of the obtained oils. For this purpose, Croatian autochthonous cultivar (cv.) Buža was chosen in an attempt to improve its relatively low phenolic content as well as low extractability and industrial oil yield caused by an unfavorable ratio between water and oil content in the fruits [21,22]. Due to high economic importance of Buža olive cv. for the oil production in the Istria region of Croatia, an additional objective was to investigate the influence of leaf addition, in the context as a drainage material, on the quantitative parameters of the obtained oils, such as yield and extractability index (EI). However, it is important to emphasize that intentional addition of leaves during oil extraction could be a way to produce potentially enriched new oil product, which cannot be considered as VOO according to EU regulations [16], since it is not produced solely from olive fruits.

2. Materials and Methods

2.1. Samples Preparation

Olive fruits and olive leaves of Buža cv. non-irrigated trees were harvested at the beginning of November 2018 from the experimental olive grove of the Institute of Agriculture and Tourism in Poreč, Croatia. Fruits were collected in an earlier ripening stage (ripening index 1.72 as determined by the method of Beltrán et al. [23]), which is common ripening stage for harvesting of Buža cv. fruits in Croatia. Oil samples were extracted from olive fruits without (B-0) and with the addition of different amounts of leaves: 1% (B-1), 2.5% (B-2.5) and 5% (B-5) using an Abencor laboratory oil mill (MC2 Ingeniería y Sistemas, Sevilla, Spain). Olive fruits were divided into twelve batches containing 3 kg of olives per batch. Three batches of fruits were processed into oil without the leaf addition and three batches per treatment were processed with the addition of different amounts of leaves. For all the samples, fruits with or without leaves were crushed using a hammer mill. Olive paste was malaxed 45 min in the thermoheater at 25 ± 1 °C and centrifuged during 1 min at 3500 rpm. The extracted oil was separated after natural sedimentation and stored in dark glass bottles at 4 °C until analysis.

2.2. Oil Yield and Extractability Index

Oil yield (%) was determined multiplying by 100 the mass ratio of obtained oil (g) and processed olive paste (g) from three separated processing repetitions [22]. EI was determined using the formula [24]:

$$EI = (V \times d) / (W \times F) \times 100$$

where V (mL) represents the extracted oil volume, d (0.915 g/mL) the mean density of olive oil, W (g) the mass of olive paste, and F (%) the oil content on fresh mass. Theoretical oil content in fresh fruit was determined by Soxtec Avanti 2055 apparatus (Foss Tecator, Sweden) using the method presented in Brkić et al. [21].

2.3. Analysis of Waxes and Fatty Acid Ethyl Esters

Analysis of waxes and FAEE (mg/kg of oil) in oil samples were performed according to the IOC method [25] using a gas chromatograph Varian 3350 GC (Varian Inc., Harbour City, CA, USA) with a flame-ionization detector, and equipped with a 15 m long metal column coated in Crossbond 5% diphenyl/95% dimethyl polysiloxane, 0.25 mm ID, 0.25 µm df (MXT-5) from Restek (Bellefonte, PA, USA). As internal standards methyl heptadecanoate and lauryl arachidate of purity ≥ 99% (Sigma, Saint Louis, MO, USA) were used.

2.4. Analysis of VOOs Pigments

Chlorophylls and carotenoids in oil samples were determined according to Mínguez-Mosquera et al. [26] using a UV/Vis spectrophotometer (Varian Cary 50, Varian, Harbour City, CA, USA) and expressed as pheophytin a and lutein content (mg/kg of oil), respectively.

2.5. Analysis of Phenolic Compounds

Extraction and analysis of phenolic compounds in oil samples was performed according to the method proposed by Jerman Klen et al. [27] and slightly modified by Lukić et al. [28] using an HPLC-DAD Agilent Infinity 1260 System (Agilent Technologies, Santa Clara, CA, USA). HPLC operating conditions were exactly the same as proposed by Jerman Klen et al. [27]. A Kinetex PFP column (2.6 µm, 100 mm × 4.6 mm) with a PFP guard column (Phenomenex, Sydney, Australia) was used at 27 °C. A glacial acetic acid–water (50 mL/L) (A) and methanol (B) solvents were used, with a flow rate of 1 mL/min. Ten µL of the oil sample extract were injected. Identification of phenols were carried out by comparison with retention times and UV/Vis spectra with those of pure standards, and with UV/Vis spectra from the literature, as well as full names and isomer numbers as designated of by Jerman Klen et al. [27]. Detection wavelengths were 280 nm (for simple phenols, lignans, secoiridoids, and vanillic acid) 320 nm (vanillin and *p*-coumaric acid), and 365 nm (flavonoids). The standard calibration curves were made for tyrosol, hydroxytyrosol, vanillic acid, vanillin, *p*-coumaric acid, luteolin, apigenin, pinoreosin, and oleuropein quantification and the concentrations were expressed as mg/kg oil. The amounts of hydroxytyrosol acetate, acetoxypinoreosin, and secoiridoids were obtained by semi-quantitative analysis, where their concentrations were expressed as equivalent to those of hydroxytyrosol, pinoreosin, and oleuropein, respectively, assuming a response factor equal to one. For example, the concentration of hydroxytyrosol acetate was calculated according to the following equation: ((hydroxytyrosol peak area)/(tyrosol peak area)) × (tyrosol concentration calculated using a corresponding tyrosol calibration curve). Total phenolic content was presented as the sum of all identified phenolic compounds.

2.6. Sensory Analysis

Sensory analysis of oils was performed according to the IOC method presented in the European Commission Regulation [16] by a panel composed of eight trained and experienced assessors in VOO sensory analysis. In order to better explain sensorial profile of oil, the panel used a profile sheet expanded with particular positive aroma and taste

attributes (green grass/leaves, green apple, almond, aromatic herbs, chicory/rocket, sweet and astringent), which differs from the standard one.

2.7. Statistical Analysis

The results were reported as the means of three technical repetitions $\pm$ standard deviations. To study the influence of the leaves addition on the oil parameters, chemical and sensory analysis data were subjected to one-way analysis of variance (ANOVA) at 5% significance level. The homogeneity of variance was tested by the Levene test. Data were additionally processed by principal component analysis (PCA). Variables used for PCA were chemical and sensory analysis data. The mean values were compared using the Tukey's honest significance difference test at the level of $p \leq 0.05$. Statistical data elaboration was performed by software package Statistica v. 13.2 (Stat-Soft. Inc., Tulsa, OK, USA).

3. Results and Discussion

3.1. Influence of the Addition of Olive Leaf on Oil Yield and Extractability Index

Oil yield and EI are one of the main parameters for the determination of the economic efficiency and overall oil extraction performance [24,29]. EI is influenced by cultivar and ripening degree of olive fruits but also depends on the extraction process variables [24]. Most of the Spanish olive cultivars can achieve an extractability index above 0.40 (41.7% of the investigated cultivars) or even above 0.60 (45.8% of the investigated cultivars) [24]. In our study the extractability of oil from Buža cv. paste without the addition of olive leaf was 0.20 (Figure 1). This result confirmed the relatively low industrial oil yield of Buža cv. due to unfavorable ratio water-oil content in its fruit harvested in earlier ripening degrees, found in previous studies [21,30]. For this reason, the improvement of Buža cv. oil yield and extractability using processing aids (talc, sodium chloride) has been an aim for scientists and experts familiar with this problem for quite some time [22].

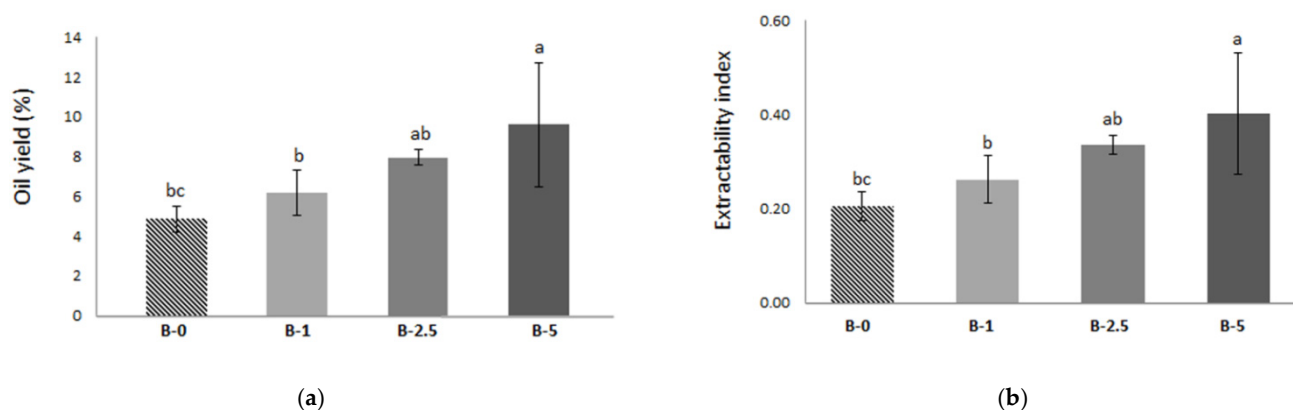


Figure 1. (a) Oil yield (%) and (b) extractability index determined in the production of Buža olive cultivar oil obtained without (B-0) and with the addition of different amounts of olive leaf: 1% (B-1), 2.5% (B-2.5) and 5% (B-5). Results are presented as mean values of three technical repetitions $\pm$ standard deviations. Different letters above bars represent significant differences among the treatments (Tukey's test, $p < 0.05$) for each parameter separately.

In this study, the addition of 1%, 2.5% and 5% of olive leaf during oil extraction increased the EI and consequently oil yield by 28%, 64% and 97%, respectively, compared to the control B-0 oil. However, only when 5% leaf was added the increase was significant (Figure 1). On the contrary, Sari & Ekinci [31] have found that a treatment with 2% and 5% leaves had no influence on the oil yield of Ayvalık cultivar, even though in the case of the addition of leaves at 5% a slight decrease in oil extractability was determined. Our results pointed out the possibility that olive leaf addition may improve oil yield and EI in the case of Buža cultivar.

3.2. Influence of the Addition of Olive Leaf on Fatty Acid Ethyl Esters

High FAEE concentration in olive oil can be connected with the low quality fruits that previously undergone hydrolytic and fermentative processes in which additional amounts of free fatty acids (FFA) and alcohols were produced [17]. Our results have shown that the addition of leaf in all the investigated amounts increased the concentration of FAEE in the obtained oils due to an increase in ethyl palmitate (EEC16), although in the case of 2.5% leaf addition the increase was not significant (Figure 2). Despite all, the determined values did not exceed the limit proposed for the EVOO category [16] in any of the investigated samples regardless of the amount of leaf added. Ammar et al. [9] have found that the olive leaves addition increases the amount of lipolytic enzymes which leads to the increase in FFA. Since FAEE are a result of esterification of FFA with a low molecular weight alcohol ethanol [32], it is reasonable to assume that an increased amount of FFA could lead to an increased value of FAEE.

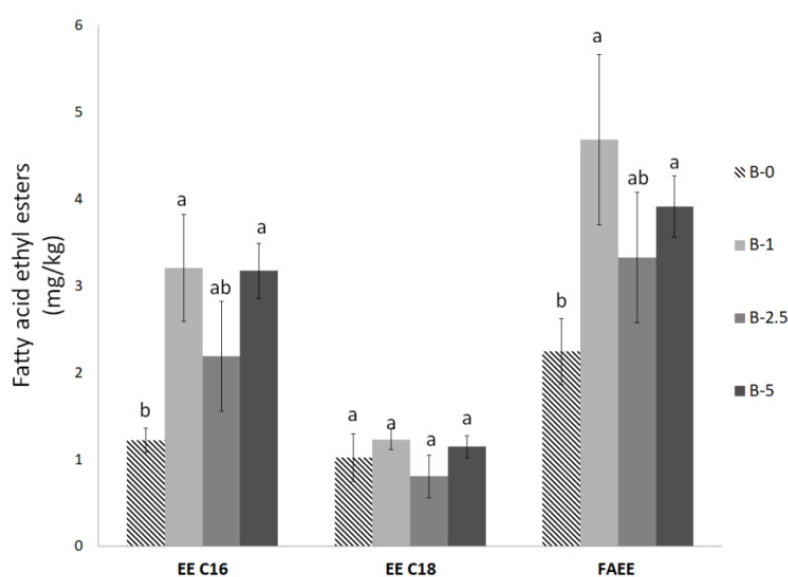


Figure 2. Concentration (mg/kg) of fatty acid ethyl esters (FAEE) in Buža olive cultivar oil obtained without (B-0) and with the addition of different amounts of olive leaf: 1% (B-1), 2.5% (B-2.5) and 5% (B-5). Results represent mean values of three technical repetitions $\pm$ standard deviations. Different letters above bars represent significant differences among the treatments (Tukey's test, $p < 0.05$) for each parameter separately. FAEE represents the sum of ethyl palmitate (EE C16) and ethyl stearate (EE C18).

3.3. Influence of the Addition of Olive Leaf on Waxes

Olive fruit skin is made up of a layer of epidermal cells above which is a cuticle covered with a fat-wax coating containing about 10% of waxes. A fraction of these compounds is usually extracted into olive oil during processing where fruit coating comes in contact with the oil. In the olive oil, three classes of waxes can be found according to chain length: (i) lower than 40 (C36 and C38), (ii) C40 and C42 waxes, and (iii) with 44 or more carbon atoms (C44 and C46 [33]. Among these, to test the quality and authenticity of olive oil, the C40, C42, C44 and C46 waxes are assessed [25]. However, when testing the compliance with the requirements of the EVOO and VOO categories, only the sum of C42, C44 and C46 waxes (C246) is considered, with the established maximum limit of 150 mg/kg [16,25].

Only few studies characterized the epicuticular waxes that cover olive leaves [34,35]. Bianchi et al. [36], found differences between Coratina cv. olive leaf and fruit wax content. In the olive fruit more C40 (10–24% of the total waxes) and C42 (14% of the total waxes) waxes have been found than in the leaf of the same cultivar (C40 with 4% and C42 with 8% of total content). On the other hand, 14% of C44 and 19% of C46 waxes were found in the leaf, which was significantly higher than the percentage found in the Coratina cv. fruits,

where it was only 3% for both C44 and C46. Generally, olive leaf addition slightly increased the wax content in the investigated oils, except in the case of C44 wax (Figure 3). Waxes found in the waxy surface layer of olive leaves, together with those from olive fruits, were probably extracted more efficiently due to better drainage and increased leaf and fruit cells breakage during joint milling, therefore releasing more waxes into oil. The addition of 1% of olive leaf increased the content of C40, C42 and C46 waxes, and consequently the sum of waxes C246 and C0246 in a greater proportion than in the other two treatments, probably due to more favorable solvent (oil)/matrix (waxes) ratio during oil extraction in the B-1 treatment. Considering the difference between treatments, the greatest amount of C246 was found in B-1 treatment, but it was an increase from 4 mg/kg (B-0) to 6 mg/kg (B-1), which even if it was statistically significant, was negligible respect to EVOO limits. Although the concentrations of particular waxes slightly increased, in all the investigated samples the sum of waxes C246 was much below the limits proposed for EVOO and VOO categories, while the sum of waxes C0246 was much below the limit for lampante oil category [25].

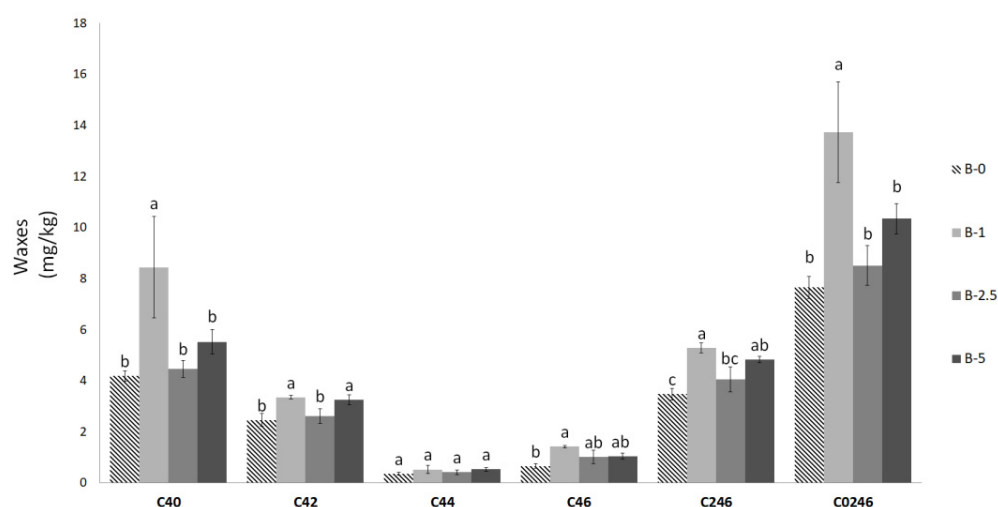


Figure 3. Concentrations (mg/kg) of waxes (C40, C42, C44, and C46) as well as C246 (sum of C42, C44 and C46 waxes) and C0246 (sum of C40, C42, C44, and C46) in Buža olive cultivar oil obtained without (B-0) and with the addition of different amounts of olive leaf: 1% (B-1), 2.5% (B-2.5) and 5% (B-5). Results represent mean values of three technical repetitions $\pm$ standard deviations. Different letters above bars represent significant differences among the treatments (Tukey's test, $p < 0.05$) for each parameter separately.

3.4. Influence of the Addition of Olive Leaf on Chlorophylls and Carotenoids

By increasing the amount of olive leaf added during Buža cv. oil extraction the chlorophylls and carotenoids concentration in the obtained oils increased (Figure 4). The addition of olive leaf at 1%, 2.5% and 5% increased the chlorophyll content by 38%, 375% and 1183% regarding to B-0 oil, respectively. Such findings are in agreement with the results of other authors who also established an increase in chlorophyll [11] and total pigment content [12,13] in oils obtained with leaves added during production. This could be due to the increased degree of breakage of leaf cells during simultaneous milling of leaves and olive fruits, which facilitated the release of chlorophyll and pheophytin into oil [10]. Such an increase in chlorophyll content could improve the antioxidant properties of oil, when stored in dark, and consequently prolong its shelf-life [11]. Since chlorophylls act as prooxidants under light [37], special attention should be paid during storage of oils enriched with olive leaf chlorophylls. Hence, it is highly advisable to preserve such products in the dark and in non-transparent bottles. When added at 1%, 2.5% and 5%, olive leaf increased the content of carotenoids by 23%, 168% and 485% regarding to B-0 oil, respectively (Figure 4). In addition to their known property of increasing oil stability [38],

increased carotenoids concentration in oils is appealing from a nutritional perspective because of their pro-vitamin A activity [12].

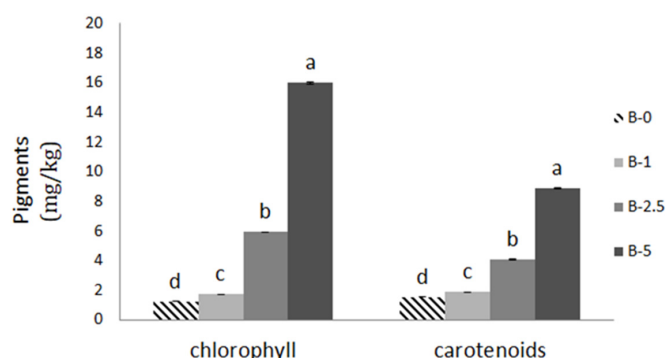


Figure 4. Concentration (mg/kg) of pigments (chlorophylls and carotenoids) in Buža olive cultivar oil obtained without (B-0) and with the addition of different amounts of olive leaf: 1% (B-1), 2.5% (B-2.5) and 5% (B-5). Results represent mean values of three technical repetitions $\pm$ standard deviations. Different letters above bars represent significant differences among the treatments (Tukey's test, $p < 0.05$) for each parameter separately.

3.5. Influence of the Addition of Olive Leaf on Phenolic Compounds

Influence of the addition of olive leaf during extraction of oil from Buža cv. fruits on the oil phenolic composition was dependent on the amount of leaf added (Table 1).

Table 1. Concentrations of phenolic compounds in Buža olive cultivar oil obtained without (B-0) and with the addition of different amounts of olive leaf: 1% (B-1), 2.5% (B-2.5) and 5% (B-5).

Phenolic Compounds (mg/kg)	B-0	B-1	B-2.5	B-5
Simple phenols				
Tyrosol	5.8 $\pm$ 1.2	6.0 $\pm$ 0.1	5.9 $\pm$ 0.1	5.3 $\pm$ 0.5
Hydroxytyrosol	4.6 $\pm$ 0.6 a	4.9 $\pm$ 1.2 a	3.4 $\pm$ 0.6 ab	1.8 $\pm$ 0.5 b
Hydroxytyrosol acetate	2.0 $\pm$ 0.8	4.2 $\pm$ 1.6	2.1 $\pm$ 1.2	1.5 $\pm$ 0.5
Vanillin	0.4 $\pm$ 0.0 a	0.4 $\pm$ 0.1 a	0.2 $\pm$ 0.0 b	0.3 $\pm$ 0.0 a
Total simple phenols	12.7 $\pm$ 0.6 ab	15.5 $\pm$ 2.7 a	11.6 $\pm$ 1.8 ab	8.9 $\pm$ 1.2 b
Secoiridoids				
Elenolic acid glucoside (isomer)	1.4 $\pm$ 0.1	1.4 $\pm$ 0.0	1.5 $\pm$ 0.1	1.5 $\pm$ 0.2
3,4-DHPEA-EDA *	238.5 $\pm$ 17.9 a	230.0 $\pm$ 22.7 a	174.9 $\pm$ 24.8 b	124.0 $\pm$ 6.9 c
Oleuropein aglycone (isomer I)	63.5 $\pm$ 4.2 a	64.9 $\pm$ 3.7 a	44.2 $\pm$ 3.4 b	28.8 $\pm$ 2.3 c
p-HPEA-EDA *	90.3 $\pm$ 6.0 a	84.2 $\pm$ 3.1 a	61.9 $\pm$ 7.5 b	51.0 $\pm$ 1.2 b
Oleuropein + ligstroside aglycones I & II	26.9 $\pm$ 2.8 a	24.2 $\pm$ 1.3 a	20.1 $\pm$ 3.4 ab	16.2 $\pm$ 3.9 b
Oleuropein aglycone (isomer II)	25.1 $\pm$ 3.4 a	25.5 $\pm$ 0.6 a	23.7 $\pm$ 1.9 a	17.2 $\pm$ 0.8 b
Ligstroside aglycone (isomer II)	17.7 $\pm$ 1.5	17.4 $\pm$ 1.9	22.1 $\pm$ 13.7	15.3 $\pm$ 1.1
Oleuropein aglycone (isomer III)	4.0 $\pm$ 0.8 a	4.1 $\pm$ 0.3 a	3.0 $\pm$ 0.3 ab	2.4 $\pm$ 0.5 b
Total secoiridoids	467.3 $\pm$ 31.9 a	451.6 $\pm$ 25.6 a	351.4 $\pm$ 46.6 b	256.3 $\pm$ 12.0 c
Flavonoids				
Luteolin	4.4 $\pm$ 1.3	4.4 $\pm$ 0.4	4.5 $\pm$ 0.6	5.1 $\pm$ 1.5
Apigenin	0.7 $\pm$ 0.2	0.8 $\pm$ 0.1	1.6 $\pm$ 0.8	0.8 $\pm$ 0.2
Total flavonoids	5.1 $\pm$ 1.5	5.2 $\pm$ 0.4	6.1 $\pm$ 1.3	6.0 $\pm$ 1.7
Lignans				
Pinoresinol	21.8 $\pm$ 0.8 a	19.0 $\pm$ 0.6 a	18.5 $\pm$ 1.4 ab	14.9 $\pm$ 2.4 b
Acetoxypinoresinol	1.6 $\pm$ 0.5	2.0 $\pm$ 0.4	1.9 $\pm$ 0.2	2.4 $\pm$ 0.4
Total lignans	23.4 $\pm$ 1.2 a	21.0 $\pm$ 0.6 ab	20.4 $\pm$ 1.6 ab	17.3 $\pm$ 2.8 b
Phenolic acids				
Vanillic acid	1.1 $\pm$ 0.1 ab	1.1 $\pm$ 0.0 a	0.9 $\pm$ 0.1 ab	0.9 $\pm$ 0.1 b
p-coumaric acid	1.9 $\pm$ 0.3	1.8 $\pm$ 0.1	1.7 $\pm$ 0.1	1.5 $\pm$ 0.2
Total phenolic acids	3.0 $\pm$ 0.3 a	3.0 $\pm$ 0.1 ab	2.6 $\pm$ 0.2 ab	2.4 $\pm$ 0.3 b
TOTAL PHENOLIC CONTENT	511.5 $\pm$ 31.1 a	496.2 $\pm$ 28.7 a	392.7 $\pm$ 48.6 b	290.9 $\pm$ 16.6 c

Results represent mean values of three technical repetitions $\pm$ standard deviations. Different letters in a row represent significant differences among the treatments (Tukey's test, $p < 0.05$). * Abbreviations: Decarboxymethyl elenolic acid linked to hydroxytyrosol—3,4-DHPEA-EDA corresponding to isomer II according to the work of Jerman Klen et al. [27]; Decarboxymethyl elenolic acid linked to tyrosol—p-HPEA-EDA.

The addition of 1% olive leaf had no influence on the phenolic composition nor on the total phenolic content, while the additions of 2.5% and 5% leaf caused a decrease in both particular and total phenolic content, respectively.

Regarding simple phenols, the addition of leaf caused a mild decrease in the concentration of vanillin (B-2.5) and hydroxytyrosol (B-5). Consequently, a slight decrease in total simple phenols content was observed (B-5) (Table 1). Tyrosol and hydroxytyrosol acetate remained preserved in oils after all the treatments. Differently, Ammar et al. [14] reported an increase in the concentration of hydroxytyrosol, tyrosol, and hydroxytyrosol acetate after the addition of leaf during olive processing for three of four investigated cultivars. The exception was Zalmati cv., where the concentration of the mentioned compounds decreased together with that of vanillin [14]. Such results, along those from Tarchoune et al. [13], indicate that the influence of the addition of olive leaf on the concentration of simple phenols is cultivar depended. The loss of particular simple phenols could be explained by their hydrophilic character responsible for good solubilization in water phase during oil processing [13,39].

The main phenolic compounds with antioxidant properties responsible for the VOO shelf life are secoiridoids, in the major part aglycones and other derivatives of oleuropein and ligstroside [40,41]. The addition of leaf generally caused a decrease in the total secoiridoids concentration (Table 1). The addition of 2.5% leaf caused a decrease in total secoiridoid concentration of about 25%, while the addition of 5% leaf decreased it for about 45% compared to B-0. Regarding particular secoiridoids, most of the oleuropein derivatives were reduced after the addition of 5% leaf, while adding 2.5% caused only the concentration of oleuropein aglycone isomer I to decrease (Table 1). On the contrary, Tarchoune et al. [13] found that the addition of 3% olive leaf increased the concentration of oleuropein derivatives in Ouselati and Neb Jmel cv. oils. A decrease in concentration was also observed for p-HPEA-EDA and 3,4-DHPEA-EDA after the addition of 2.5% and 5% leaf. Particular secoiridoids, such as 3,4-DHPEA-EDA, one of the components in olive leaves [42], are better soluble in water than in oil and probably ended up in a greater proportion in the vegetable water [10] due to the addition of leaf. During the malaxation process in the Abencor laboratory system open containers were used, in which the presence of olive leaf particles in olive paste increased the surface in contact with air compared to the more homogenous surface of control paste. Therefore, the observed decrease in secoiridoids could have also been caused by enzymatic oxidation during the malaxation process due to the activation of polyphenol oxidase and peroxidase, [43]. Also, several authors have found that the addition of olive leaf led to an increase in peroxide value in obtained oils because such a drainage favors the exchange of gases and availability of oxygen that accelerates the peroxidation process [9,12].

Although flavonoids luteolin and apigenin were previously found to be abundant in Buža cv. leaf extract [44], an increase in their concentration after the addition of Buža cv. leaves was not detected in the oils obtained in this study (Table 1). Also, there is a possibility that the concentration of several flavonoids previously found to be characteristic for Buža cv. leaf, such as quercetin and diosmetin [45], increased in the oils obtained with the addition of leaf. However, the two compounds were not detected due to limitations of the method used. Several authors reported different trends for flavonoids concentration in oils obtained with 3% leaf addition. Tarchoune et al. [13] observed an increase in total flavonoids in Neb Jmel and Oueslati oils, while Ammar et al. [14] reported an increase in apigenin and a decrease in luteolin in oils extracted with the addition of leaf.

The concentrations of pinoresinol and total lignans decreased only in the oils with 5% leaf addition (around 26% compared to B-0). Lignans are more stable in oils when compared to other phenolic compounds probably due to their lipophilic character causing them less susceptible to partitioning into the water, and also due to their low antioxidant activity causes them less susceptible to oxidation [46].

The total phenolic acids decreased only in B-5 sample (around 22% compared to B-0), mostly due to a decrease in vanillic acid (Table 1). Ammar et al. [14] reported a decrease

in vanillic acid in the case of Zalmati cv. oil obtained with the addition of 3% leaf, while Tarchoune et al. [13] did not observe a significant effect of the addition of 3% leaf during olive processing on phenolic acids in oils from Tunisian Ouselati and Neb Jmel varieties.

A decrease in several phenolic compounds concentration previously mentioned resulted in a decrease of total phenolic compounds in B-2.5 (about 23% compared to B-0) and in B-5 (about 43% compared to B-0) oil samples (Table 1). Besides the already mentioned reasons, the loss of phenolic compounds in oils obtained with the addition of leaf could be also tentatively explained as a result of enzymatic reactions accelerated by different compounds from leaf, such as enzymes, metals, etc. The presence of oxidative enzymes in olive leaf chloroplasts, such as polyphenol oxidase (PPO) and peroxidases (POD) [47,48] catalyzes the oxidation of phenolic compounds. In addition, Yuan et al. [49] reported that content of phenolic compounds in olive leaf decreased after enzymatic hydrolysis biocatalyzed by hemicellulose, present in chloroplasts. Therefore, there is a possibility that the addition of leaf during oil extraction could increase the concentration of hemicellulose and consequently reduced the concentration of phenols in oils. Olive leaves contain significant amounts of minerals: potassium, manganese, magnesium, copper, and iron being the most common ones [50,51]. Several metals, as iron and copper, could have an important role in degradation of phenolic compounds [52]. Therefore, olive leaf metals transferred into olive paste during simultaneous malaxation could contribute to the phenols oxidation in oils.

3.6. Influence of the Addition of Olive Leaf on Sensory Profile

The addition of olive leaf increased the intensity of particular positive sensory characteristics of olive oil aroma, such as fruitiness and green grass/leaves (Figure 5). Such findings are in agreement with the results of other authors who have found improvements of oil sensory aroma characteristics after the addition of leaves during processing, mainly green notes and fruitiness [9,10,53]. During lipoxygenase (LOX) pathway enzymatic conversion of 13-L-hydroperoxides from linoleic and linolenic acids into Z-3-hexenal, E-2-hexenal, hexanal, and corresponding alcohols occurs in the lamellae of chloroplasts [54]. Since more chlorophylls were extracted into oil obtained with the addition of leaf (Figure 4), a greater enzymatic load from chloroplasts was possibly involved in the LOX pathway, thus generating higher concentrations of the mentioned odoriferous C6 and C5 volatiles, which are mainly responsible for green and fruity attributes of olive oil aroma. In addition, certain other positive sensory attributes of oil increased by the addition of leaf, such as almond in B-1 oil. Furthermore, phenols may act as inhibitors of LOX enzymatic activity [55], so the decreased phenol concentrations determined in the oils obtained with the addition of olive leaf possibly allowed higher LOX activity and generation of higher amounts of C6 and C5 volatiles compared to the control B-0 oil, which resulted in increased intensities of particular positive sensory attributes.

Regarding the oil taste attributes, leaf addition influenced a slight increase in sweetness (Figure 5) probably due to a slight, although not significant, decrease in bitterness. Ammar et al. [9] reported a slight increase of the bitter and pungent taste in four Tunisian cultivar oils obtained by addition of 3% leaf. Bitter and pungent attributes are connected to the phenolic compounds extracted, specifically secoiridoids [33,56]. According to authors Mateos et al. [57], 3,4-DHPEA-EDA and oleuropein aglycon are the principal compounds responsible for bitter taste, while other authors stated that 3,4-DHPEA-EDA and p-HPEA-EDA are mostly responsible for pungency [58,59]. In our study, the concentration of 3,4-DHPEA-EDA and p-HPEA-EDA decreased in the oils obtained after the addition of 2.5% and 5% leaf (Table 1), probably affecting the detected changes in the taste of the obtained oils.

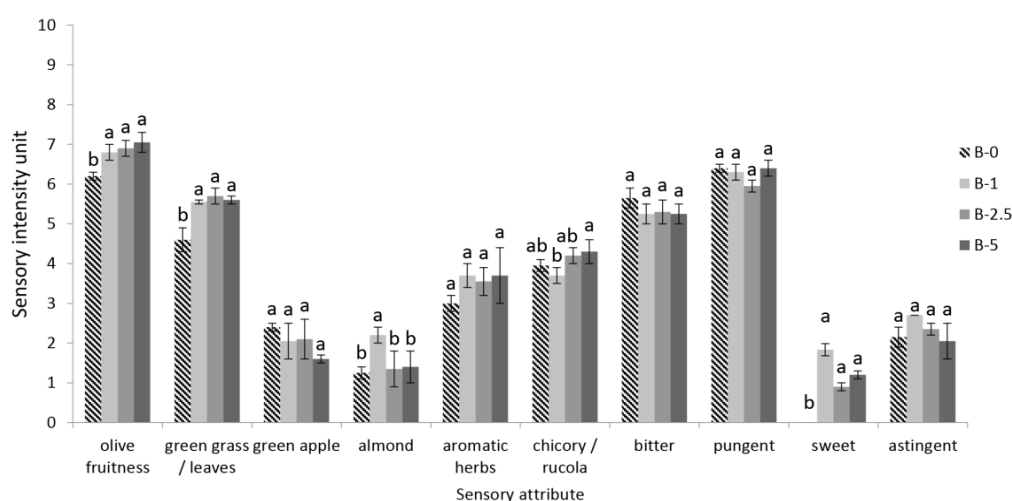


Figure 5. Results of descriptive sensory analysis of Buža olive cultivar oils obtained without (B-0) and with the addition of olive leaf in different amounts: 1% (B-1), 2.5% (B-2.5) and 5% (B-5). Results represent the mean values of medians of three technical repetitions $\pm$ standard deviations. Different letters above bars represent significant differences among the treatments (Tukey's test, $p < 0.05$) for each sensory attribute separately.

Although mild changes in sensory characteristics of oils obtained with the addition of leaf were determined, it is important to emphasize that panelists did not recognize any defects in the investigated oils.

3.7. Multivariate Statistical Analysis

Principal component analysis (PCA) was applied as a multivariate method and allowed a good differentiation of oil samples produced without (B-0) or with leaf addition (B-1, B-2.5 and B-5) (Figure 6a,b).

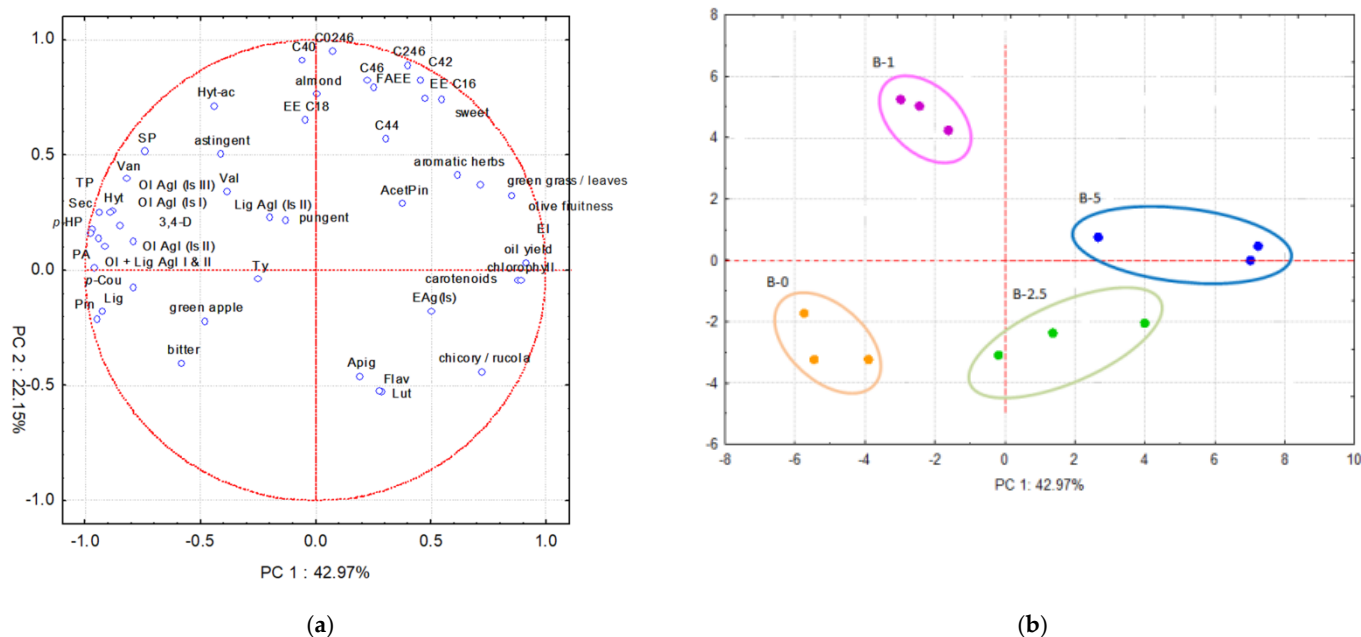


Figure 6. (a) Factor loadings of selected variables (quantity parameters, pigments, waxes and FAEE, the intensities of odor sensory attributes and concentrations of phenolic compounds) along the directions of principal components PC1 and PC2. (b) Separation of Buža olive cultivar oils according to the rate of leaf addition on PC1 and PC2 (full orange circles—oils produced without leaf addition (B-0), full violet circles—oils produced with 1% of leaf addition (B-1), full blue circles—oils produced with 2.5% of leaf addition (B-2.5), full green circles—oils produced with 5% of leaf addition (B-5). Abbreviations:

SP—total simple phenols; Hyt—hydroxytyrosol; Ty—tyrosol; Hyt-ac—hydroxytyrosol acetate; Val—vanilin; PA—total phenolic acids; Vac—vanilic acid; *p*-Cou—*p*-coumaric acid; Flav—total flavonoids; Lut—luteolin; Apig—apigenin; Lig—total lignans; Pin—pinosresinol; AcetPin—acetoxypinosresinol; Sec—total secoiridoids; EAg—elenolic acid glucoside; 3,4-D—3,4-DHPEA-EDA; Ol Agl (Is I)—oleuropein aglycone (isomer I); *p*-HP—*p*-HPEA-EDA; Ol + Lig Agl I & II—oleuropein + ligstroside aglycones I & II; Ol Agl (Is II)—oleuropein aglycone (isomer II); Lig Agl (Is II)—ligstroside aglycone (isomer II); TP—total phenolic content; FAEE—fatty acid ethyl esters; EE C16—ethyl palmitate; EE C18—ethyl stearate.

Dataset comprised 12 cases (oil samples) and 49 variables (quantity parameters, pigments, waxes and FAEE, the intensities of odor sensory attributes and concentrations of phenolic compounds). Oil samples without leaf addition (B-0) and with only 1% of leaf addition (B-1) were clearly differentiated from the others oil samples with leaf addition (B-2.5 and B-5) along the direction of PC1. B-0 oil samples were characterized mostly by green apple, bitter, and pinosresinol, as well as lignans, which were loaded high on the negative sides of both PC1 and PC2 in the third quadrant. Clearly, B-1 oil samples were separated from other oil samples by the concentration of most secoiridoids, which was probably related to the increased intensity of astringent. B-2.5 oil samples were associated most strongly with the compounds in the fourth quadrant characterized mainly by flavonoids and chicory/rucola. Green grass/leaves and olive fruitiness descriptors, oil pigments, as well as oil yield and EI were more characteristic for the B-5 oil samples.

4. Conclusions

The results achieved indicated that leaf addition could improve the extractability of Buža cultivar olive fruits and consequently increase the obtained oil yield. This very important production parameter for the oil industry was improved, most probably as a result of the drainage and percolation effect of olive leaves during malaxation of olive paste.

Oil samples obtained with leaf addition had more pronounced positive sensory attributes, such as fruitiness and green grass/leaf notes and higher concentration of chlorophylls and carotenoids. Influence of olive leaf addition on the oil phenolic composition was dependent on the amount added. While the addition of 1% leaf did not exhibit any influence, addition in the amount of 5% decreased the concentration of almost all the investigated phenolic compounds. The addition of leaves caused an increase in FAEE and waxes content in the obtained oils, but with values not exceeding maximum limits for EVOO and VOO categories. Since apparently leaf addition during extraction has a significant impact on quantity, composition and quality of oil, particular importance should be given to the amount of olive leaf present with olive fruits prior to extraction.

Since VOO is obtained exclusively from olive fruits, oil obtained with intentional addition of olive leaf during extraction could be considered a new product outside the current official VOO categorization. Moreover, extensive studies on potential positive or negative health effects are needed, as well as toxicity studies to evaluate the safety of addition of leaf during oil production from olive fruits with respect to naturally occurring compounds and pesticide residues.

Author Contributions: Conceptualization, K.B.B.; methodology, K.B.B., A.N. and I.L.; formal analysis, A.N., D.K., K.B.B. and M.L.; investigation, K.B.B. and A.N.; resources, K.B.B.; data curation, K.B.B., I.L. and A.N.; writing—original draft preparation, K.B.B. and A.N.; writing—review and editing, K.B.B., A.N., D.K., I.L. and M.L.; supervision, K.B.B.; project administration, K.B.B. All authors have read and agreed to the published version of the manuscript.

Funding: This research has been supported in part by the Croatian Science Foundation (DOK-2018-09-2293 and DOK-2018-01-4693) and co-financed by the European Union from the European Social Fund (DOK-2018-01-4693).

Conflicts of Interest: The authors declare no conflict of interest.

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