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Article

Bioactive Compounds in Olive Oils of Autochthonous Slovenian Olive Varieties 'Buga', 'Črnica' and 'Drobnica'

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Abstract: The fatty acid composition, biophenol, tocopherol, sterol and triterpenic dialcohol content and composition of the autochthonous Slovenian olive varieties 'Buga', 'Črnica' and 'Drobnica' were studied during a three-year period with the aim to valorise the characteristics of the three olive varieties. Standardized and accredited analytical methods were applied. The results of the investigation showed that the highest average amount of oleic acid (75.75%) was determined in oils of the variety 'Črnica', followed by 'Drobnica' (72.06%) and 'Buga' (68.73%). All the three varieties are a good source of total biophenols ('Buga' 616 mg/kg, 'Drobnica' 569 mg/kg and 'Črnica' 427 mg/kg) and α -tocopherol ('Buga' 378 mg/kg, 'Drobnica' 279 mg/kg and 'Črnica' 243 mg/kg). 'Buga' and 'Drobnica' are characterised by high amounts of total sterols, 2468 mg/kg and 2391 mg/kg, respectively, 'Črnica' oils, in comparison, showed a lower average value of total sterols (1351 mg/kg). The acquired data of the autochthonous Slovenian olive varieties 'Buga', 'Črnica' and 'Drobnica' are very important to valorise the characteristics of the produced oils. The studied varieties showed a great potential for further cultivation and valorisation of the traditional olive oil production of the region and are important for preserving the biodiversity and tradition of the territory.

Keywords: 'Buga'; 'Črnica'; 'Drobnica'; chemistry; characterisation

1. Introduction

The olive (*Olea europaea* L.) is one of the most important crops in Slovenia and is cultivated in the Primorska region, which is characterised by a Mediterranean climate. The last edition of the Fruit Selection for Slovenia [1] reported that based on the number of cultivated hectares, the olive tree is the second most common culture among fruit species in Slovenia, despite the limited possibilities of spreading. Official data for the year 2023 [2] shows that there are 2571 ha of olive orchards in Slovenia. Most of them are located in the region of Slovenian Istria (96%), while the remainder (4%) are in the regions of Goriška Brda, the Vipava Valley and the Karst. Slovenia is one of the northernmost regions of the Mediterranean where olives are still cultivated. Due to climate change and new climatic conditions, the northern Mediterranean countries could now become important oil producers. Tanasajevic et al. [3], based on regional climate models driven by ECHAM5 for the A1B scenario of the Special Report on Emission Scenarios (SRES) and the Agro Eco-logical Zoning method, have shown that the potentially cultivable areas for olive cultivation are expected to expand northwards and to higher altitudes, increasing by 25% in 50 years. In Slovenia the average yield of olives is 2.5 t/ha, the average olive oil production is 400 kg/ha, with a total average annual production of 700 t of extra virgin olive oil. Despite the relatively low production levels, the geographical area of Slovenian Istria enables the production of high-quality extra virgin olive oil.

In the northern part of the Adriatic region, the most widespread olive variety is 'Istrska Belica', which is cultivated intensively in the regions of Slovenian Istria and Friuli Venezia Giulia (Italy). According to oral tradition, the 'Istrska Belica' variety began to spread in Slovenian Istria after the severe frost of 1929, as it is resistant to low temperatures, produces good and regular yields and has a high oil content [4]. Before 'Istrska Belica', the varieties 'Buga' ('Burla'), 'Drobnica' ('Komuna'),

'Črnica' ('Carbon'), 'Štorta', 'Mata', 'Žižula' and 'Zmartel' were the most spread olive varieties in the area, which were replaced by new, higher-yielding varieties due to their low and alternative yields [5]. This led to accelerated genetic erosion, the abandonment of traditional olive groves and centuries-old olive trees, the overgrowth of the cultivated landscape and the establishment of intensive, economically profitable plantations. Nowadays, however, the production of monovarietal oils obtained from a specific local autochthonous variety with specific characteristics that emphasise its identity and uniqueness is being promoted [6]. This leads to the preservation of autochthonous and traditional varieties of agricultural plants and protects the genetic diversity that enables better adaptation to new and rapidly changing environmental conditions, which is a prerequisite for the survival of a species in a given climatic environment.

The aim of this study was to systematically analyse the main bioactive compounds of 'Buga', 'Črnica' and 'Drobnica' olive oil, during a three-year period from 2018 to 2020. The acquired data are very important to valorise the characteristics of the autochthonous varieties that represent a typicality of the territory and can certify the protected designation of origin and to evaluate the compliance of the produced oils with international standards such as the Trade standard applying to olive oils and olive pomace oils, released by the International Olive Council [7], and the EU legislation on olive oil, Commission Delegated Regulation (EU) 2022/2104 [8].

2. Results

2.1. Fatty Acids Composition

The fatty acid composition was determined in olive oils produced from the varieties 'Buga', 'Črnica' and 'Drobnica'. The results of the means and the standard deviation are presented in Table 1. Statistically significant differences (ANOVA, $p < 0.05$) between the three olive varieties were found for most of the fatty acids, whereas there were no significant differences between 'Buga', 'Črnica' and 'Drobnica' for myristic (C 14:0), heptadecenoic (C 17:1) and lignoceric (C 24:0) acids.

The LSD multiple comparisons test showed statistically significant differences ($p < 0.05$) in the average content of palmitic (C 16:0), stearic (C 18:0), oleic (C 18:1) and behenic (C 22:0) acids between the cultivars 'Buga' and 'Črnica', 'Buga' and 'Drobnica', and 'Črnica' and 'Drobnica'. The highest average content of palmitic acid was determined in samples of the variety 'Buga' (16.45%), followed by the varieties 'Drobnica' (14.52%) and 'Črnica' (14.08%). The variety 'Črnica' showed the highest average amount of oleic (75.75%) and stearic (2.12%) acids, followed by 'Drobnica' (72.06% oleic acid and 1.97% stearic acid) and 'Buga' (68.73% oleic acid and 1.60% stearic acid). The differences in behenic acid content were less pronounced between the three varieties: 0.12% in 'Drobnica', 0.11% in 'Črnica' and 0.10% in 'Buga' samples.

Statistically significant differences (LSD, $p < 0.05$) between 'Buga' and 'Črnica', and 'Buga' and 'Drobnica' were determined in the case of palmitoleic (C 16:1), heptadecanoic (C 17:0), linolenic (C 18:3) and arachidic (C 20:0) acids, whereas there were no significant differences for the studied compounds between 'Črnica' and 'Drobnica'. The olive oil samples of 'Buga', compared to 'Črnica' and 'Drobnica', showed the highest amount of palmitoleic (2.84%) and linolenic (0.92%) acids, and the lowest amount of heptadecanoic (0.03%) and arachidic (0.32%) acids, although the differences between the three varieties for heptadecanoic and arachidic acids are minimal.

Statistically significant differences (LSD, $p < 0.05$) between 'Buga' and 'Črnica', and 'Črnica' and 'Drobnica' were determined for linoleic acid (C 18:2), whereas there were no significant differences between 'Buga' and 'Drobnica'. The lowest average amount of linoleic acid (4.54%) was determined in 'Črnica' samples, while 'Drobnica' and 'Buga' samples showed higher content of linoleic acid, 7.87% and 8.58%, respectively.

Statistically significant differences (LSD, $p < 0.05$) between 'Buga' and 'Drobnica', and 'Črnica' and 'Drobnica' were determined for eicosenoic acid (C 20:1), whereas there were no significant differences between 'Buga' and 'Črnica'. The highest amount of eicosenoic acid (0.33%) was determined in 'Drobnica' samples, followed by 'Buga' (0.28%) and 'Črnica' (0.27%), as it is shown in Table 1.

Total saturated fatty acids (SFA), total monounsaturated fatty acids (MUFA), total polyunsaturated fatty acids (PUFA), PUFA/SFA ratio and oleic acid/linoleic acid ratio were calculated. Statistically significant differences (ANOVA and LSD, $p < 0.05$) between the studied olive varieties were determined for all the mentioned parameters. 'Črnica' showed the highest value of MUFA, the lowest values of SFA and PUFA, the highest ratio between PUFA and SFA and between oleic and linoleic acids.

Table 1. Fatty acid composition of 'Buga', 'Črnica' and 'Drobnica' olive oils.

Fatty acid	Unit	'Buga'	'Črnica'	'Drobnica'
Myristic acid (C 14:0)	%	0.01 ± 0.001 ^a	0.01 ± 0.001 ^a	0.01 ± 0.001 ^a
Palmitic acid (C 16:0)	%	16.45 ± 0.62 ^a	14.08 ± 0.75 ^b	14.52 ± 0.75 ^c
Palmitoleic acid (C 16:1)	%	2.84 ± 0.44 ^a	1.77 ± 0.36 ^b	1.77 ± 0.27 ^b
Heptadecanoic acid (C 17:0)	%	0.03 ± 0.01 ^a	0.04 ± 0.01 ^b	0.04 ± 0.01 ^b
Heptadecenoic acid (C 17:1)	%	0.08 ± 0.01 ^a	0.08 ± 0.01 ^a	0.08 ± 0.01 ^a
Stearic acid (C 18:0)	%	1.60 ± 0.11 ^a	2.12 ± 0.14 ^b	1.97 ± 0.13 ^c
Oleic acid (C 18:1)	%	68.73 ± 2.34 ^a	75.75 ± 1.04 ^b	72.06 ± 2.48 ^c
Linoleic acid (C 18:2)	%	8.58 ± 1.58 ^a	4.54 ± 0.64 ^b	7.87 ± 1.87 ^a
Linolenic acid (C 18:3)	%	0.92 ± 0.11 ^a	0.79 ± 0.14 ^b	0.78 ± 0.10 ^b
Arachidic acid (C 20:0)	%	0.32 ± 0.01 ^a	0.38 ± 0.04 ^b	0.39 ± 0.03 ^b
Eicosenoic acid (C 20:1)	%	0.28 ± 0.02 ^a	0.27 ± 0.03 ^a	0.33 ± 0.02 ^b
Behenic acid (C 22:0)	%	0.10 ± 0.01 ^a	0.11 ± 0.02 ^b	0.12 ± 0.01 ^c
Lignoceric acid (C 24:0)	%	0.06 ± 0.01 ^a	0.06 ± 0.01 ^a	0.07 ± 0.01 ^a
SFA	%	18.57 ± 0.64 ^a	16.80 ± 0.84 ^b	17.11 ± 0.76 ^c
MUFA	%	71.92 ± 2.02 ^a	77.87 ± 0.89 ^b	74.25 ± 2.33 ^c
PUFA	%	9.50 ± 1.58 ^a	5.33 ± 0.55 ^b	8.65 ± 1.83 ^c
PUFA/SFA ratio	-	4.39 ± 0.18 ^a	4.97 ± 0.30 ^b	4.86 ± 0.26 ^c
Oleic acid/Linoleic acid ratio	-	8.32 ± 1.75 ^a	16.99 ± 2.36 ^b	9.67 ± 2.34 ^c

Abbreviations: SFA: saturated fatty acids; MUFA: monounsaturated fatty acids, PUFA: polyunsaturated fatty acids. Data are expressed as means ± standard deviation, $n = 70$, values in the same row with different superscript letters are statistically significantly different (ANOVA and LSD, $p < 0.05$).

2.2. Biophenols and Tocopherols Content and Composition

The results of the determination of biophenols and tocopherols in the samples of the varieties 'Buga', 'Črnica' and 'Drobnica' are shown in Table 2. Statistically significant differences (ANOVA and LSD, $p < 0.05$) between the three olive varieties were determined for total ligstroside biophenols and lignans. The olive variety 'Buga' is characterised by the highest average amount of total ligstroside biophenols (188 mg/kg), followed by 'Črnica' (156 mg/kg) and 'Drobnica' (125 mg/kg). The 'Črnica' variety shows the highest content of lignans (83 mg/kg), compared to 'Buga' (26 mg/kg) and 'Drobnica' (19 mg/kg).

Statistically significant differences (ANOVA and LSD, $p < 0.05$) between 'Buga' and 'Črnica', and 'Črnica' and 'Drobnica' were determined in the case of total oleuropein biophenols, total biophenols and the following oleuropein derivatives: the dialdehydic form of decarboxymethyl oleuropein aglycone (DMO-Agl-dA), the dialdehydic form of oleuropein aglycone (O-Agl-dA) and the aldehydic

form of oleuropein aglycone (O-Agl-A), whereas there were no significant differences between 'Buga' and 'Drobnica' in the biophenol compounds. The results presented in Table 2 shows that 'Buga' and 'Drobnica' have the highest average amounts of total oleuropein biophenols ('Buga' 373 mg/kg, 'Drobnica' 403 mg/kg) and total biophenols ('Buga' 616 mg/kg, 'Drobnica' 569 mg/kg), compared to 'Črnica' (170 mg/kg of total oleuropein biophenols and 427 mg/kg of total biophenols). 'Buga' and 'Drobnica' are also characterised by higher amounts of DMO-Agl-dA, O-Agl-dA and O-Agl-A compared to 'Črnica', as it is shown in Table 2.

Table 2. Biophenol and tocopherol content and the composition of 'Buga', 'Črnica' and 'Drobnica' olive oils.

Biophenols and tocopherols	Unit	'Buga'	'Črnica'	'Drobnica'
Total biophenols	mg/kg	616 ± 152 ^a	427 ± 113 ^b	569 ± 193 ^a
Total oleuropein biophenols	mg/kg	373 ± 103 ^a	170 ± 64 ^b	403 ± 167 ^a
Total ligstroside biophenols	mg/kg	188 ± 40 ^a	156 ± 54 ^b	125 ± 31 ^c
Lignans	mg/kg	26 ± 12 ^a	83 ± 14 ^b	19 ± 9 ^c
DMO-Agl-dA	mg/kg	190 ± 52 ^a	110 ± 38 ^b	180 ± 86 ^a
DML-Agl-dA	mg/kg	47 ± 19 ^a	55 ± 27 ^a	23 ± 12 ^b
O-Agl-dA	mg/kg	53 ± 27 ^a	14 ± 15 ^b	75 ± 59 ^a
L-Agl-dA	mg/kg	31 ± 17 ^a	15 ± 14 ^b	21 ± 16 ^b
O-Agl-A	mg/kg	36 ± 13 ^a	10 ± 8 ^b	46 ± 34 ^a
L-Agl-A	mg/kg	11 ± 5 ^a	7 ± 5 ^b	10 ± 5 ^{ab}
α-tocopherol	mg/kg	378 ± 67 ^a	243 ± 37 ^b	279 ± 55 ^c
γ-tocopherol	mg/kg	14 ± 8 ^a	4 ± 2 ^b	8 ± 2 ^c

Abbreviations: Lignans: sum of pinoresinol and 1-acetoxy-pinoresinol; DMO-Agl-dA: Decarboxymethyl oleuropein aglycone, dialdehyde form; DML-Agl-dA: Decarboxymethyl ligstroside aglycone, dialdehyde form; O-Agl-dA: Oleuropein aglycone, dialdehyde form; L-Agl-dA: Ligstroside aglycone, dialdehyde form; O-Agl-A: Oleuropein aglycone, aldehyde form; L-Agl-A: Ligstroside aglycone, aldehyde form; β- and δ-tocopherol were not detected in any samples. Data are expressed as means ± standard deviation, n = 70, values in the same row with different superscript letters are statistically significantly different (ANOVA and LSD, p < 0.05).

The olive variety 'Drobnica' has the lowest average content (23 mg/kg) of the dialdehydic form of decarboxymethyl ligstroside aglycone (DML-Agl-dA), and it is statistically different (LSD, p < 0.05) from 'Buga' (47 mg/kg) and 'Črnica' (55 mg/kg). The highest amount of the dialdehydic form of ligstroside aglycone (L-Agl-dA) was determined for the variety 'Buga' (31 mg/kg), followed by 'Drobnica' (21 mg/kg) and 'Črnica' (15 mg/kg), whereas the aldehydic form of ligstroside aglycone (L-Agl-A) varied from 7 mg/kg in 'Črnica' to 10 mg/kg in 'Drobnica' and 11 mg/kg in 'Buga' olive oil samples (LSD, p < 0.05).

Statistically significant differences (ANOVA, p < 0.05) between the three studied olive varieties were determined in the content of α- and γ-tocopherol. The LSD multiple comparisons test showed statistically significant differences (p < 0.05) between 'Buga' and 'Črnica', 'Buga' and 'Drobnica', and 'Črnica' and 'Drobnica' in the content of both tocopherol isomers. The highest average content of α-tocopherol was determined in samples of the variety 'Buga' (378 mg/kg), followed by 'Drobnica' (279 mg/kg) and 'Črnica' (243 mg/kg). All three varieties showed a lower content of γ-tocopherol compared to α-tocopherol. Results showed 14 mg/kg of γ-tocopherol in 'Buga', 8 mg/kg in 'Drobnica' and 4 mg/kg in 'Črnica' samples. β- and δ-tocopherols were not detected in any samples of the three varieties (below the limit of detection 3 mg/kg).

2.3. Sterols and Triterpenic Dialcohols

The results of the determination of sterols and triterpenic dialcohols erythrodiol and uvaol are presented in Table 3. Statistically significant differences (ANOVA and LSD, $p < 0.05$) between 'Buga', 'Črnica' and 'Drobnica' were determined in the content of sitostanol, Δ -5-avenasterol, Δ -5,24-stigmastadienol and Δ -7-avenasterol. The olive variety 'Drobnica' is characterised by the highest content of Δ -5-avenasterol (11.72%), Δ -5,24-stigmastadienol (1.26%) and Δ -7-avenasterol (0.68%), compared to the other two varieties, while a higher value of sitostanol (3.51%) was present in 'Črnica' samples. The variety 'Drobnica' is statistically different from 'Buga' and 'Črnica' also in the content of clerosterol and β -sitosterol. In the case of clerosterol there were minor differences (from 0.99% to 1.06%) between the olive varieties, whereas for β -sitosterol, the most abundant sterol, higher values were determined in 'Buga' (84.67%) and 'Črnica' (85.23%) than in 'Drobnica' (79.45%) samples.

Table 3. Sterol content and composition, and erythrodiol and uvaol content of 'Buga', 'Črnica' and 'Drobnica' olive oils.

Sterols	Unit	'Buga'	'Črnica'	'Drobnica'
Cholesterol	%	0.09 \pm 0.04 ^a	0.13 \pm 0.04 ^b	0.09 \pm 0.02 ^a
24-methylene-cholesterol	%	0.19 \pm 0.10 ^a	0.08 \pm 0.04 ^b	0.18 \pm 0.10 ^a
Campesterol	%	2.55 \pm 0.18 ^a	3.70 \pm 0.29 ^b	2.60 \pm 0.16 ^a
Campestanol	%	0.08 \pm 0.03 ^a	0.34 \pm 0.08 ^b	0.11 \pm 0.05 ^a
Stigmasterol	%	0.78 \pm 0.25 ^a	1.00 \pm 0.60 ^a	0.75 \pm 0.36 ^a
Clerosterol	%	1.06 \pm 0.05 ^a	1.05 \pm 0.07 ^a	0.99 \pm 0.06 ^b
β -sitosterol	%	84.67 \pm 1.99 ^a	85.23 \pm 1.90 ^a	79.45 \pm 4.03 ^b
Sitostanol	%	1.04 \pm 0.30 ^a	3.51 \pm 0.84 ^b	1.94 \pm 0.56 ^c
Δ -5-avenasterol	%	7.94 \pm 1.84 ^a	3.55 \pm 1.74 ^b	11.72 \pm 4.15 ^c
Δ -5,24-stigmastadienol	%	0.85 \pm 0.12 ^a	0.63 \pm 0.16 ^b	1.26 \pm 0.23 ^c
Δ -7-stigmastenol	%	0.20 \pm 0.05 ^a	0.29 \pm 0.06 ^b	0.24 \pm 0.08 ^a
Δ -7-avenasterol	%	0.57 \pm 0.05 ^a	0.49 \pm 0.09 ^b	0.68 \pm 0.09 ^c
Apparent β -sitosterol	%	95.56 \pm 0.45 ^a	93.98 \pm 0.56 ^a	95.35 \pm 0.36 ^a
Total sterols	mg/kg	2468 \pm 251 ^a	1351 \pm 270 ^b	2391 \pm 314 ^a
Erythrodiol + uvaol	%	0.79 \pm 0.33 ^{ab}	0.66 \pm 0.25 ^a	1.06 \pm 0.75 ^b

Brassicasterol, Δ -7-campesterol and Δ -5,23-stigmastadienol were not detected in any samples. Erythrodiol and uvaol are expressed as a percentage of the total sterols. Data are expressed as means \pm standard deviation, $n = 46$, values in the same row with different superscript letters are statistically significantly different (ANOVA and LSD, $p < 0.05$).

The olive variety 'Črnica' is statistically different (ANOVA and LSD, $p < 0.05$) from 'Buga' and 'Drobnica' regarding the content of cholesterol, 24-methylene-cholesterol, campesterol, campestanol, Δ -7-stigmastenol and total sterols. The variety 'Črnica' is characterised by higher values of cholesterol (0.13%), campesterol (3.70%), campestanol (0.34%), Δ -7-stigmastenol (0.29%) and lower values of 24-methylene-cholesterol (0.08%) and total sterols (1351 mg/kg), compared to 'Buga' and 'Drobnica'.

There were no statistically significant differences between the three studied varieties in the content of stigmasterol and apparent β -sitosterol, whereas 'Črnica' showed a higher content of stigmasterol (1.00%) and a lower content of apparent β -sitosterol (93.98%) compared to 'Buga' (stigmasterol 0.78%, apparent β -sitosterol 95.56%) and 'Drobnica' (stigmasterol 0.75%, apparent β -sitosterol 95.35%).

The varieties 'Črnica' and 'Drobnica' showed statistically significant differences (ANOVA and LSD, $p < 0.05$) in the content of the triterpenic dialcohols erythrodiol and uvaol, which varied from 0.66% in 'Črnica' to 1.06% in 'Drobnica' samples.

3. Discussion

3.1. Fatty Acids Composition

According to the methodology for secondary characterisation by the International Olive Council [9] and considering the variation of the data, the olive varieties 'Črnica' and 'Drobnica' are characterised by a high content of oleic acid (C 18:1), 75.75% and 72.06%, respectively, whereas the 'Buga' variety has a medium content (68.73%) of oleic acid. The opposite situation was observed in the case of linoleic acid (C 18:2): 'Črnica' has a very low content (4.54%) of linoleic acid, and 'Drobnica' (7.87%) and 'Buga' (8.58%) have a low content of linoleic acid which, consequently, affects the ratio between oleic and linoleic acids. 'Črnica' showed the highest oleic/linoleic ratio (16.99), followed by 'Drobnica' (9.67) and then 'Buga' (8.32), which indicates that the oleic/linoleic acid ratio is influenced by olive varieties. Our results are in accordance with Hernández et al. [10], who stated that oleic and linoleic acid contents displayed the highest degree of variability of the different fatty acids present in olive oils. Also, Brkić-Bubola et al. [11] reported a high oleic/linoleic ratio in the Croatian 'Rosinjola' and 'Istarska bjelica' oils, which indicated their higher oxidative stability when compared to other investigated oils. It was found that the content of oleic acid of the variety 'Buga' is in the same range of the Croatian varieties 'Buža' (67.73%), 'Buža puntoža' (67.81%) and 'Bova' (64.51%), as reported by Brkić-Bubola et al. (2018), whereas the content of oleic acid of 'Črnica' and 'Drobnica' is in the same range of Slovenian 'Istrska belica' (75.35%) and 'Leccino' (72.74%) as reported by Bešter et al. [12], and Croatian 'Istarska bjelica' (73.93%) and 'Rosinjola' (74.82%).

The content of palmitic acid (C 16:0) is also interesting, as extra virgin olive oils from 'Buga' have a very high content (16.45%) of palmitic acid, whereas 'Drobnica' (14.52%) and 'Črnica' (14.08%) oils have a high content of C 16:0, which is consequently related to the synthesis and content of oleic acid, because palmitate is the precursor of oleic acid and longer-chain fatty acids that are formed through the elongation and desaturation biosynthesis of fatty acids [13,14]. The fatty acid composition of the three olive varieties is in accordance with the limit values for extra virgin olive oils set in Commission Delegated Regulation (EU) 2022/2104 [8]. The variety 'Buga' shows a high average value (0.92%) of linolenic acid (C 18:3), which in some cases, usually at the end of September (data not shown), can be at the upper border of the limit value set in the legislation, i.e. $\leq 1.0\%$ (Commission Delegated Regulation (EU) 2022/2104) [8]. Nevertheless, it is still in accordance with the latest revision of the International Olive Council's Trade standard applying to olive oils and olive pomace oils [7], which prescribes that a virgin olive oil with a content of linolenic acid that lies between 1.00% and 1.40% is considered authentic if the ratio between apparent β -sitosterol and campesterol is greater than or equal to 24 and all other purity criteria lie within the official limits. The ratio between apparent β -sitosterol and campesterol can be calculated from the data of the research, and in the case of 'Buga' is 37.5, 'Črnica' 25.4, and 'Drobnica' 36.7. The other purity parameters were not determined, because it was not the aim of this work, and in this case the traceability from the olive fruits to the produced oils is guaranteed, because the olive samples were manually picked by the laboratory staff and all the production process was under control in the laboratory olive mill.

'Črnica' extra virgin olive oils are very interesting due to their potential to be included in the certification of the protected designation of origin (PDO) 'Ekstra deviško oljčno olje Slovenske Istre', as they are characterised by a high level of oleic acid (75.75%) and low level of linoleic acid (4.54%), and they also fall within the limits set in the 'Specification of PDO Ekstra deviško oljčno olje Slovenske Istre' [15], which prescribes a minimum of 72% oleic acid and a maximum of 8% linoleic acid. The use of 'Drobnica' oils for the PDO is limited and mainly depends on the other varieties present in the mixture for the PDO that can affect the fatty acid composition of the prepared oil and consequently the content of oleic and linoleic acids. Due to the low content of oleic acid and high content of linoleic acid, the use of 'Buga' oils is not recommended for the PDO, although we must consider that this variety has high content of total biophenols (616 mg/kg) and produces good quality olive oils that can be successfully present on the market.

3.2. Biophenols and Tocopherols Content and Composition

The olive varieties 'Buga' and 'Drobnica' have a high content of total biophenols, 616 mg/kg and 569 mg/kg, respectively, while the variety 'Črnica' is among the oils with a medium content (427 mg/kg) of total biophenols according to the methodology for secondary characterisation by the International Olive Council [9]. In Slovenia, the 'Istrska belica' variety with 598 mg/kg of total biophenols and 'Leccino' variety with 399 mg/kg of total biophenols are also present [12]. A high

content of total biophenols was also determined in the Croatian varieties 'Buža' (511.5 mg/kg), as reported by Novoselic et al. [16], and 'Oblica' (from 537 mg/kg to 788 mg/kg, depending on the degree of ripening) as reported by Lukić et al. [17].

In the 'Buga' and 'Drobnica' oils, the total oleuropein biophenol levels are predominant (373 mg/kg and 403 mg/kg, respectively), followed by total ligstroside biophenols (188 mg/kg and 125 mg/kg, respectively). The difference between the total oleuropein and total ligstroside compounds are less expressed in the 'Črnica' variety (170 mg/kg of total oleuropein biophenols and 156 mg/kg of total ligstroside biophenols). In general, all three studied varieties are characterised by the predominance of oleuropein derivatives in comparison to ligstroside derivatives, and between them, the most present is the dialdehydic form of decarboxymethyl aglycons derivatives, followed by the dialdehydic and aldehydic forms of oleuropein and ligstroside aglycons.

In relation to the 'Specification for the Ekstra deviško oljčno olje Slovenske Istre' with PDO, the Slovenian varieties 'Buga', 'Črnica' and 'Drobnica' show appropriate biophenol content, as the minimum prescribed amount of total biophenols is 150 mg/kg; however, it is also necessary to take into account the restrictions regarding the fatty acid composition, as already stated in the previous chapter.

Considering the average amount of tocopherols and the variation of the data, the variety 'Buga' is characterised by a high amount of α -tocopherol and total tocopherols (sum of α - and γ -tocopherols), whereas 'Črnica' and 'Drobnica' have a medium content of tocopherols. Tocopherol content can be influenced by the olive variety and crop year, as is shown in literature data [18] of the neighbour Croatian varieties 'Buža' (228–260 mg/kg), 'Leccino' (241–389 mg/kg) and 'Rosulja' (211–351 mg/kg), that showed a low to middle content of tocopherols for the years 2010 and 2011. The content of tocopherols can be affected by several factors; for example, Borges et al. [19], who studied the 'Arbequina' variety in Spain and Brazil, reported that climatic and geographic factors of the production zones seem to greatly affect the content of tocopherols and biophenols. The varieties 'Buga', 'Črnica' and 'Drobnica' are a good source of tocopherols, which also contribute to the oxidative stability of olive oils. This finding is supported by Franco et al. [20], who reported a high positive correlation with oxidative stability for seven Spanish olive varieties.

3.3. Sterols and Triterpenic Dialcohols

The sterol content and composition of the three olive varieties is in accordance with the limit values for extra virgin olive oils as set in Commission Delegated Regulation (EU) 2022/2104 [8] and IOC Trade standard applying to olive oils and olive pomace oils [7]. 'Buga' and 'Drobnica' are characterised by high amounts of total sterols, 2468 mg/kg and 2391 mg/kg, respectively, while 'Črnica' oils, in comparison, showed a lower average value of total sterols (1351 mg/kg), and are more similar to 'Istrska belica', the most widespread variety in Slovenia, which has 1265 mg/kg of total sterols [21]. Compared to 'Buga' and 'Drobnica', the 'Črnica' variety has also higher average values of campesterol (3.70%) and Δ -7-stigmastanol (0.29%). Regarding sterol composition, β -sitosterol, Δ -5-avenasterol and campesterol were the predominant sterols for all the three studied varieties, which is in accordance with Yorulmaz et al. [22]. The β -sitosterol content of 'Buga' and 'Drobnica' varieties is comparable to the Croatian varieties 'Bova' (87.89%), 'Buža' (87.51%), 'Buža puntoža' (84.08%) and Rosinjola (82.05%), as reported by Brkić-Bubola et al. [11], and exert antioxidant effects on human tissues [23]. The variety 'Buga' shows 7.94% Δ -5-avenasterol and is comparable, considering the variation of the data, to the Croatian 'Buža' (6.18%) and 'Buža puntoža' (9.97%), while 'Drobnica' (11.72%) is comparable to 'Rosinjola' (10.61%). The campesterol content of 'Buga' (2.55%) and 'Drobnica' (2.60%) is in the same range of the Croatian 'Buža' (2.87%).

Brassicasterol, Δ -7-campesterol and Δ -5,23-stigmastadienol were not detected in any samples, while triterpenic dialcohols erythrodiol and uvaol were slightly higher in the 'Drobnica' variety (1.06%) compared to 'Buga' (0.79%) and 'Črnica' (0.66%), but far from the limit value of 4.5% as set in Commission Delegated Regulation (EU) 2022/2104 [8].

4. Materials and Methods

4.1. Material

Olive fruits of the varieties 'Buga', 'Črnica' and 'Drobnica' were collected at 3 locations: Purissima (collection plantation established between 2004 and 2006 in Slovenian Istra), Sečovlje (traditional productive plantation established before 1929 in Slovenian Istra) and Šempeter (collection plantation established between 2007 and 2014 in Vipava Valley), in 2018, 2019 and 2020 in three periods from 20 September to 5 November (the 38th, the 41st and the 44th week in each year). For each sample, approximately 1 kg of olive fruits were manually collected. The olive oils were produced in an Abencor system MC2 laboratory olive mill (MC2 Ingenieria y Sistemas, Sevilla, Spain).

4.2. Methods

All the methods used for the determination of fatty acids, biophenols, tocopherols, sterols and triterpenic dialcohols were accredited in accordance with SIST EN ISO/IEC 17025:2017 [24]. All the chemicals reported in the following subsections met the requirements of the official methods and were purchased from Sigma-Aldrich Chemie GmbH (Munich, Germany).

4.2.1. Determination of fatty acids

Fatty acids were determined in accordance with Commission Regulation (EEC) No 2568/91, Annex X [25]. Fatty acid methyl esters were prepared in heptane with 2-M methanolic potassium hydroxide solution and determined by gas chromatography. An Agilent HP 6890 Series (Agilent Technologies, Santa Clara, CA, USA), equipped with Supelco 2560 Capillary GC Column (100 m × 0.25 mm ID, df 0.20 µm; Supelco Inc., Bellefonte, PA, USA) and FID detector was used. Fatty acids were assigned by comparing the retention times with those of the reference standard Supelco 37 Component FAME Mix.

4.2.2. Determination of Biophenols

Biophenols were determined in accordance with the method of the International Olive Council (IOC), COI/T.20/Doc. No 29/Rev. 1 [26]. The extraction was done from 2.0 g of oil with the addition of 1 mL of internal standard solution (syringic acid 0.15 mg/mL). The sample was shaken with the aid of a Vortex-genie 2 G-560 E agitator (Scientific Industries Inc., Bohemia, New York, USA) for 30 seconds, then 5 mL of methanol/water 80/20 (V/V) extraction solution was added and sacked for 1 minute. Biophenols were extracted using the ultrasonic bath ELMA D-78244 (ELMA, Singen, Germany) for 15 minutes at room temperature, followed by centrifugation with the aid of the Eppendorf centrifuge 5430R (Eppendorf SE, Hamburg, Germany) at 5000 rpm for 25 min. An aliquot of the supernatant phase was filtered through a 5 mL plastic syringe with a 0.45 µm PVDF filter in a 2 mL vial. Biophenols were determined by HPLC analysis as set out in the IOC method. An Agilent 1200 Series HPLC System (Agilent Technologies, Santa Clara, CA, USA) equipped with a binary pump and automatic liquid sampler, C18 reversed-phase column (Phenomenex synergi hydro, 250 × 4.6 mm, 4 µm; Phenomemex, Inc, Torrance, CA, USA), operating at 20 °C, with DAD detection at 280 nm was used. Spectral data for the peaks were recorded in the range of 200–600 nm. The mobile phase used was a gradient consisting of 0.2% aqueous H₃PO₄ (by volume) (A) and methanol/acetonitrile 1/1 (by volume) (B). The initial gradient composition was A at 96% and B at 4%. After forty minutes, the ratio of B increased to 50%, to 60% in the next five minutes, and to 100% in the last fifteen minutes. After 72 min, the concentration of B was put at an initial value of 4%. The column was then equilibrated for 10 min before the next injection. A volume of 10 µL of the methanolic extract was injected into the system; the flow rate was 1 mL/min. An external calibration solution of tyrosol (0.030 mg/mL) and syringic acid (0.015 mg/mL) was prepared. All biophenol compounds were quantified using the response factor for tyrosol and assigned by comparing their relative retention times to the retention time of the internal standard syringic acid.

4.2.3. Determination of Tocopherols

Tocopherols were determined in accordance with SIST EN ISO 9936:2016 [27]. 100 mg of oil was weighed into a 10 mL volumetric flask and dissolved with n-heptane. An Agilent 1100 Series HPLC

System (Agilent Technologies, Santa Clara, CA, USA) equipped with a binary pump and automatic liquid sampler, C18 column (Phenomenex Luna 5 μ Silica(2), 250 \times 4.6 mm, 5 μ ; Phenomenex, Inc, Torrance, CA, USA), operating at 25 °C, with fluorescence detector with excitation wavelength set at 290 nm and emission wavelength at 330 nm was used. The mobile phase used was n-heptane : tetrahydrofuran (96.2 : 3.8, by volume) at a flow-rate of 1.0 mL/min. α -, β -, γ - and δ -tocopherol were determined. The method is validated in the range from 3 to 2220 mg/kg.

4.2.4. Determination of Sterols and Triterpenic Dialcohols

Sterols and triterpenic dialcohols were determined in accordance with Commission Regulation (EEC) No 2568/91, Annex XIX [28], and were extracted from 5.0 g of oil in the presence of the internal standard α -cholestanol (0.2%, m/V). The sample preparation involved saponification with 2-M ethanolic potassium hydroxide solution, solvent extraction of unsaponifiable matter with diethyl ether, separation of sterol and triterpenic dialcohols from the unsaponifiable matter with thin-layer chromatography, derivatisation into trimethylsilyl ethers, and determination by gas chromatography. An Agilent HP 6890 Series (Agilent Technologies, Santa Clara, CA, USA), equipped with Supelco SPB-5 Capillary GC Column (60 m \times 0.53 mm ID, df 5.00 μ m; Supelco Inc, Bellefonte, PA, USA) and FID detector was used. The sterols and triterpenic dialcohols were assigned by comparing their relative retention times to the retention time of the internal standard α -cholestanol.

4.2.5. Statistical Analysis

All the data were statistically analysed and expressed as mean values \pm standard deviation. Significance of the difference was analysed by ANOVA for equality of means, and a post-hoc Fisher's least significant difference (LSD) test to find out the differences between each group were performed with SPSS Statistics v. 26 (SPSS, Chicago, IL, USA), with statistical significance set at $p < 0.05$.

5. Conclusions

The autochthonous varieties 'Buga', 'Črnica' and 'Drobnica' are characterised by a medium to high content of oleic acid, tocopherols and biophenols, and a low content of linoleic acid. The varieties are well adapted to climatic conditions of the local environment and are a good source of natural antioxidants (biophenols and tocopherols), monounsaturated fatty acids and sterols. 'Buga', 'Črnica' and 'Drobnica' show a great potential for further cultivation and valorisation of the traditional olive oil production of the region and are important for preserving the biodiversity and tradition of the territory. In particular, the 'Črnica' variety has adequate chemical characteristics to be included in the certification of the PDO 'Ekstra deviško oljčno olje Slovenske Istre' [15], the other two varieties could be included with limitations, which may potentially contribute to the greater visibility of the oils of the region.

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