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POLYPHENOLS AND ANTHOCYANIN PROFILE OF BILBERRIES (*VACCINIUM MYRTILLUS* L.) FROM NORTH MACEDONIA

SUMMARY

In this study, physico-chemical properties, phenolic compounds, and antioxidant capacity of bilberries (*Vaccinium* spp.) fruits grown in three main localities in Sharr Mountain were investigated. The bilberries localities are: B1 (Kobilica, 1936m altitude), B2 (Kodra e Diellit, 1698 m), B3 (Tetovo, 467m). B3 locality is characterized by the significant ($P<0.05$) higher dry matter values (18 g/100g) as well as higher total acidity (1.32%) than the other localities. Based on the environmental conditions that prevailed during the vegetation, the B1 bilberries location recorded significantly higher values ($P<0.05$) of polyphenol content 651.36±0.50mg GAE/100g. Also, bilberries B1 and B2 localities showed higher amounts of antioxidant values (717.72±17.17 and 634.99±15.61 mg Trolox equivalents (TE)/100 g) compared to B3 locality (447.68±18.73 mg TE/100 g). These findings indicate that growing altitude is a major environmental factor determining the concentration of phenolic compounds and antioxidant activity in wild *Vaccinium myrtillus* L. populations in North Macedonia.

Keywords: natural; HPLC; DPPH; tannins; phenolic acids; flavonoids.

INTRODUCTION

The bilberry fruit belongs to the botanical family of Ericaceae that grows in the form of bushes in temperate climates on soils with pH 3.5-4.0 (Michalska and Łysiak 2015; Krzewinska, 2004). Bilberry (*Vaccinium myrtillus* L.), also known as European blueberry, is a low-growing deciduous shrub of the Ericaceae family native to northern Europe and widespread in mountainous regions of the western Balkans (Nestby *et al*. 2011; Zhang *et al*. 2016; Zorenc *et al*. 2016; Skupień, 2006). Numerous studies have compared the phenolic content and antioxidant capacity of wild bilberries (*V. myrtillus*) with those of cultivated highbush blueberries (*V. corymbosum*) (Taruscio *et al.,* 2004). Studies have shown that bilberry fruits contain numerous components that benefit human health especially vitamin C, carotenoids and polyphenols showing strong antioxidant capacity (Shivembe and Ojinnaka, 2017).

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Bilberries are available on the market in the form of berries and are consumed as such (fresh, frozen, or dried), and bilberries are also available in the form of semi-finished products (juices, concentrates, canned). Phenolic compounds in bilberries vary greatly, depending on the cultivar, season, and growing location. It is reported that the plant's growth location and interaction with the ecosystem influence the phytochemical compounds in blueberry fruits (Zoratti *et al*., 2015; Correia *et al.,* 2016). Bilberries are recognised for their exceptionally high anthocyanin and phenolic content, which confer strong antioxidant, anti-inflammatory, and cardioprotective properties (Zhang *et al.* 2016, Može *et al*. 2011, Lätti *et al*. 2009; Brambilla *et al.* 2008; Cho *et al*. 2004). Information regarding the high antioxidant efficiency of wild bilberries has aroused considerable popular interest in these fruits from consumers who want to know how bilberries can contribute to the nutritional quality of their diet. Antioxidants are necessary to prevent and protect the body against the action of variable free radicals, which cause damage to various biomolecules in the body and lead to many health problems (Bayazid *et al.* 2021). Natural antioxidants are preferred by consumers because of the quality and safety compared to unnatural colorants, antioxidants and nutraceuticals (Wang *et al.* 2017).

North Macedonia, spanning 2,571,000 ha, values its forests as a key part of its national heritage. Forests cover 835,056 ha and host about 16% of all Balkan endemic or subendemic species. Favorable natural conditions-its climate, terrain, geology, and hydrology-support rich biodiversity, including over 3,000 higher plant species, about 1,000 with medicinal uses, and roughly 500 fully medicinal plants; (https://mk.macedonism.org/Makedonska-Enciklopedija/shumarstvo/). The Sharr Mountain range in North Macedonia, with its broad altitudinal gradient and diverse microclimates, hosts extensive natural populations of *V. myrtillus* and offers an ideal setting to study environmental effects on fruit quality. (Abdi *et al.,* 2016). Limited published data are available on the phenolic composition and antioxidant properties of wild bilberry (*Vaccinium myrtillus* L.) fruits from the Balkan region. Existing studies have focused mainly on populations from Slovenia (Može *et al*., 2011; Mikulic-Petkovsek *et al*., 2012), Croatia (Jakobek *et al.,* 2007), Romania (Bunea *et al*., 2011), Serbia (Jovancevic *et al.,* 2011), Montenegro (Brašanac-Vukanović *et al.,* 2018), and Bosnia and Herzegovina (Akagić *et al.,* 2014). These wild types, distinguished by their higher acidity and unique flavor yet still underutilized compared to cultivated varieties, present significant scientific and economic interest for value-added products, especially amid growing regional and global demand for organic and functional foods (Veliu *et al.,* 2025).

No published data exist on the phenolic composition or antioxidant properties of bilberry fruits from North Macedonia. Therefore, the purpose of this research is to investigate differences and similarities among localities at various latitudes and elevations, with special emphasis on the antioxidant values of bilberries, including their anthocyanin and phenolic compound content.

MATERIAL AND METHODS

Material

Bilberry fruits from three different locations were collected: two natural populations growing at altitudes of 1829 m and 1936 m, and one population located at 467 m. The fruits were harvested during the first and second weeks of August 2022 (Figure 1). Following collection, five samples from each location were vacuum-sealed and refrigerated until analyses were conducted. The samples were evaluated for various parameters, including pH, dry matter, total acidity, color, total phenolic content, individual phenolic profile, and antioxidant capacity, using established analytical methods. Depending on the requirements of each method, the samples were either homogenized, diluted, or analyzed in their solid form.

Figure 1. Sampling localities in the Sharr Mountain, North Macedonia: B1 – Kobilica (1936 m), B2 – Kodra e Diellit (1829 m), B3 – Tetovo (467 m).

Methods

Chemical composition, Total phenolic and Antioxidant capacity assays

Dry matter content, pH, and total acidity were measured using standard AOAC procedures (AOAC, 1990). Samples (5 g) were extracted twice with 50 mL of 80 % aqueous methanol by homogenisation (Ultra-Turrax, 10 000 rpm, 2 min) followed by shaking (2 h, 25 °C). Extracts were combined, centrifuged (2800×g, 10 min), and filtered (0.45 µm) before analysis (Goztepe *et al.,* 2022). The Total Phenolic Content (TPC) of the bilberry samples was analyzed following a modified version of the method described by Singleton *et al*. (1999). In summary, 2.5 mL of Folin–Ciocalteu reagent (diluted 1:10 v/v) and 2 mL of Na_2CO_3 solution (7.5 g/100 g) were added to the methanol-diluted cranberry extracts. The mixture was placed in a test tube, sealed, and incubated in the dark for 30 minutes. After incubation, the absorbance was measured at 760 nm using a

UV–vis spectrophotometer (Shimadzu UV-1800, Kyoto, Japan). Total phenolic content was expressed as gallic acid equivalents (GAE) in mg per gram of dry matter (Figure 2).

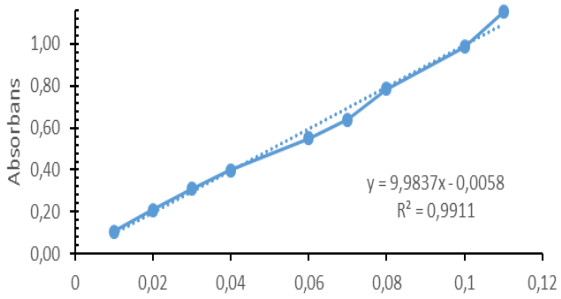

Figure 2. Gallic acid standard calibration curves

The antioxidant capacity of the extracts was evaluated using the 1,1-diphenyl-2-picrylhydrazyl (DPPH) assay. A 0.1 mL aliquot of blueberry extract was combined with 4.9 mL of DPPH• solution (0.1 mmol/L in methanol). After incubating the mixture in the dark for 30 minutes, the absorbance was recorded at 517 nm (Singh *et al*., 2002). Results were expressed as milligrams of Trolox equivalents per gram of dry matter (mg TE/g DM). For the ABTS assay, the procedure was based on Arnao *et al.* (2001) with minor modifications. A fresh $ABTS^+$ radical solution was prepared for each measurement. A 100 µL portion of blueberry extract was mixed with 2 mL of the $ABTS^+$ solution and left to react in the dark for 2 hours. Absorbance was then measured at 734 nm using a spectrophotometer, and antioxidant activity was expressed as milligrams of Trolox equivalents (TE) per gram of dry matter. The Trolox standard curve showed linearity within the ranges of 100–1998 µM for DPPH and 10–799 µM for ABTS (Figure 3a, 3b).

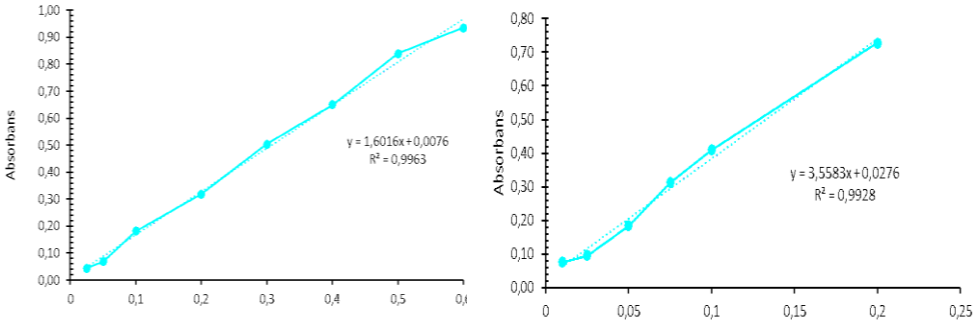

Figure 3. DPPH and ABTS standard troloxa calibration curves (a, b)

HPLC analysis of phenolics and anthocyanin profiles

Individual phenolic compounds were quantified by high-performance liquid chromatography with diode-array detection (HPLC-DAD) following the method of Özcan *et al*. (2023) with minor modifications, using a Shimadzu LC-20AD system (Shimadzu Corp., Kyoto, Japan). Extracts were prepared in methanol/water (50:50, v/v) at a liquid-to-solid ratio of 10:1 (v/w), centrifuged, and filtered through a 0.45 μm PTFE membrane prior to injection. Separation of phenolic acids was performed on a Waters Atlantis C18 column (250 mm×4.6 mm, 5 μm) using a linear gradient elution program with solvent A (formic acid/water, 1:99, v/v) and solvent B (formic acid/acetonitrile, 1:99, v/v) at a flow rate of 1 mL/min. The gradient conditions were as follows: 10–60% B from 0–15 min; 60% B (isocratic) from 15–20 min; 60–10% B from 20–25 min; and 10% B (isocratic) from 25–30 min. Chromatograms were monitored at 278, 320, and 360 nm, with spectral data collected from 190–400 nm. Phenolic acids were identified by comparing retention times and UV–Vis spectra of sample peaks with those of authentic standards. Quantification of phenolic compounds—including gallic acid, protocatechuic acid, catechin, 4-hydroxybenzoic acid, syringic acid, ellagic acid, m-coumaric acid, o-coumaric acid, chrysin, caffeic acid, p-coumaric acid, ferulic acid, myricetin, quercetin, kaempferol, rutin, sinapic acid, and chlorogenic acid—was performed using calibration curves generated for each standard. Results are expressed as mg per 100 g dry matter; compounds below the limit of quantification were reported as not detected (nd).

Determination of color in bilberries

The color of the three bilberry types was measured using a chromameter (Konica Minolta CR-400, NJ, USA). Color parameters included L* (lightness), where positive values indicate greater lightness and negative values indicate darkness; a* (green–red axis), where positive values reflect stronger green tones and negative values stronger red tones; and b* (yellow–blue axis), where positive values denote more intense yellow hues and negative values more intense blue hues. Additional color characteristics, Hue (overall color tone) and Chroma (color intensity), were also calculated to quantify color properties.

The color shades (Chroma) were calculated according to the formula:

$$C^* = \sqrt{(a^*)^2 + (b^*)^2}$$

a* and b* are the components of chroma of the color in the CIE color space.
a* represents the green-red axis.
b* represents the blue-yellow axis.

Statistical analyses

Three localities (samples B1, B2, and B3) were included in a randomized design, with five samples per locality and two independent replicates (trials), to analyze response variables related to composition, color, and phenolic compounds. Analysis of variance (ANOVA) was performed using the general

linear model procedure (SAS, 1995), with locality treated as a fixed effect and replicates as a random effect, and significance ($P<0.05$) determined using Duncan's test. Correlation analyses were conducted using the Kruskal-Wallis test and Pearson correlation as a non-parametric approach. To investigate relationships among phenolics within localities, principal component analysis (PCA) and heat mapping were carried out using SPSS software (IBM Corporation, 21.0, Armonk, NY, USA).

RESULTS AND DISCUSSION

Physicochemical parameters, total antioxidant and phenolics

The physicochemical properties of bilberry samples from different locations are presented in Table 1. Dry matter content was 18.0±0.26% for the B1 location, 17.6±0.14% for B2, and 16.8±0.29% for B3. These values are higher than those reported by Yörük (2019), who found dry matter levels ranging from 11.70 to 13.11 g/100 g. The pH of the bilberries varied between 2.40 and 2.77 across locations, which is slightly lower than values reported by Türkben *et al*. (2008) (2.77–2.95) and Yörük (2019) (3.03–3.16). Titratable acidity ranged from 1.56 to 1.77 g/100 g, with B3 showing the highest acidity and B1 the lowest (0.76±0.04). Yıldız (2012) reported acidity values of 0.27–1.65 g tartaric acid/100 g in 13 bilberry varieties collected from various regions of Turkey. Similarly, Türkben *et al*. (2008) found total acidity levels of 0.90–1.23 g citric acid/100 g in *Vaccinium myrtillus* L. samples from different areas of Uludağ. Overall, these results support previous findings by Zeng *et al.* (2020) and Correia *et al.* (2016), confirming that growing location significantly influences dry matter content, pH, and total acidity.

Table 1. Physical-chemical parameters of bilberries

Paremeter	***Unit***	***B1***	***B2***	***B3***
Dry matter	g/100 g	18.0 ± 0.26^{c}	17.6 ± 0.14^{b}	16.8 ± 0.29^{a}
pH	-	2.77 ± 0.02^{b}	2.87 ± 0.02^{c}	2.49 ± 0.01^{a}
Total acidity	% citric acid	1.56 ± 0.021^{a}	1.59 ± 0.043^{b}	1.77 ± 0.021^{c}
L	-	32.47 ± 1.15^{c}	25.18 ± 2.94^{b}	22.33 ± 1.58^{a}
a	-	3.60 ± 0.39^{a}	6.74 ± 0.17^{c}	4.83 ± 1.47^{b}
b	-	-2.89 ± 0.92^{c}	-4.75 ± 0.17^{a}	-3.36 ± 0.80^{b}
C	-	4.61 ± 0.008^{a}	8.23 ± 0.43^{c}	5.88 ± 0.057^{b}

Means ± SD, n = 3. Values in the same row with different superscript letters differ significantly (Duncan test, P < 0.05).

The measured *L, a,* and *b* values of the bilberry fruits revealed notable differences in their color characteristics. The *L* values ranged from 22.33 to 32.47, while the *a* values varied between 3.60 and 6.74, and the b values ranged from –2.89 to –4.75 (Table 1). Among the samples, B1 exhibited the highest lightness (32.33±1.15), followed by B2 (25.18±2.94) and B3 (22.33±1.58). Sample B2 showed the greatest a value (6.74±0.17) and the highest chroma (C=8.23±0.43). Overall, these results demonstrate that location has a clear impact on the color attributes of the different bilberry samples.

Table 2. Total phenolic compounds, antioxidant capacity of bilberries

Localities	*Total polyphenol content (mgGAE/100g)*	*DPPH antioxidant capacity (mg TE/100 g)*	*ABTS antioxidant capacity (mg TE/100g)*
B1	2034.12±25.29[b]	717.72±17.17[b]	651,36±0.50[c]
B2	2008.82±22.48[b]	634.99±15.61[b]	409.97±2.50[a]
B3	1317.48±84.31[a]	447.68±18.73[a]	498.11±1.50[b]

aAbbreviation: DM, dry matter. Values are means ± SD; means within a column with different superscript letters differ significantly (P<0.05).

Samples from the highest altitude (B1, 1936 m) exhibited significantly higher total phenolic content than those from B2 and B3 (P<0.05). The antioxidant capacity of the bilberries from the three localities was calculated using the DPPH free radical and the ABTS free radical. The results showed that the B1 bilberries sample has higher antioxidant capacity compared to the B2 and B3 samples, whichh showed slightly lower antioxidant properties compared to the B1 sample. The marked increase in total phenolic content and antioxidant capacity with altitude agrees with previous reports and is attributed to greater UV-B exposure and cooler temperatures that enhance phenylpropanoid pathway activity (Zeng *et al*., 2020; Rossi *et al*., 2022).

Similarly, in samples harvested from localities exposed to the sun were detected higher amounts of total phenolics in comparison with those grown in shadow with total phenol from 3.92-5.24 mg GA/g dm (Jovancevic *et al.* 2011). Değirmencioğlu *et al.* (2017) evaluated the antioxidant capacity of bilberries (*Vaccinium myrtillus*) grown in Balıkesir using the DPPH and ABTS assays. They reported DPPH values ranging from 8.56 to 19.23 μmol TE/g and ABTS values between 4.26 and 9.56 μmol TE/g. In a similar study, Jakobek *et al*. (2007) measured the antioxidant capacity of Slovenian bilberries and found much higher values: 125.52 μmol TE/g using the DPPH method and 53.28 μmol TE/g using ABTS. Bunea *et al*. (2011) analyzed wild bilberries from Romania using the ABTS assay and reported antioxidant capacities ranging from 43.08 to 56.65 μmol TE/g. HPLC-DAD analysis revealed slight differences in the anthocyanin profiles of the first two locations (Figure 4; B1, B2). Peak identification was based on spectral characteristics and retention times. Although the qualitative anthocyanin composition was similar across sites, the peak areas varied. The B1 location exhibited 17 distinct anthocyanin peaks, while the third location (B3) showed fewer and weaker peaks, with a total of 14 identified anthocyanins.

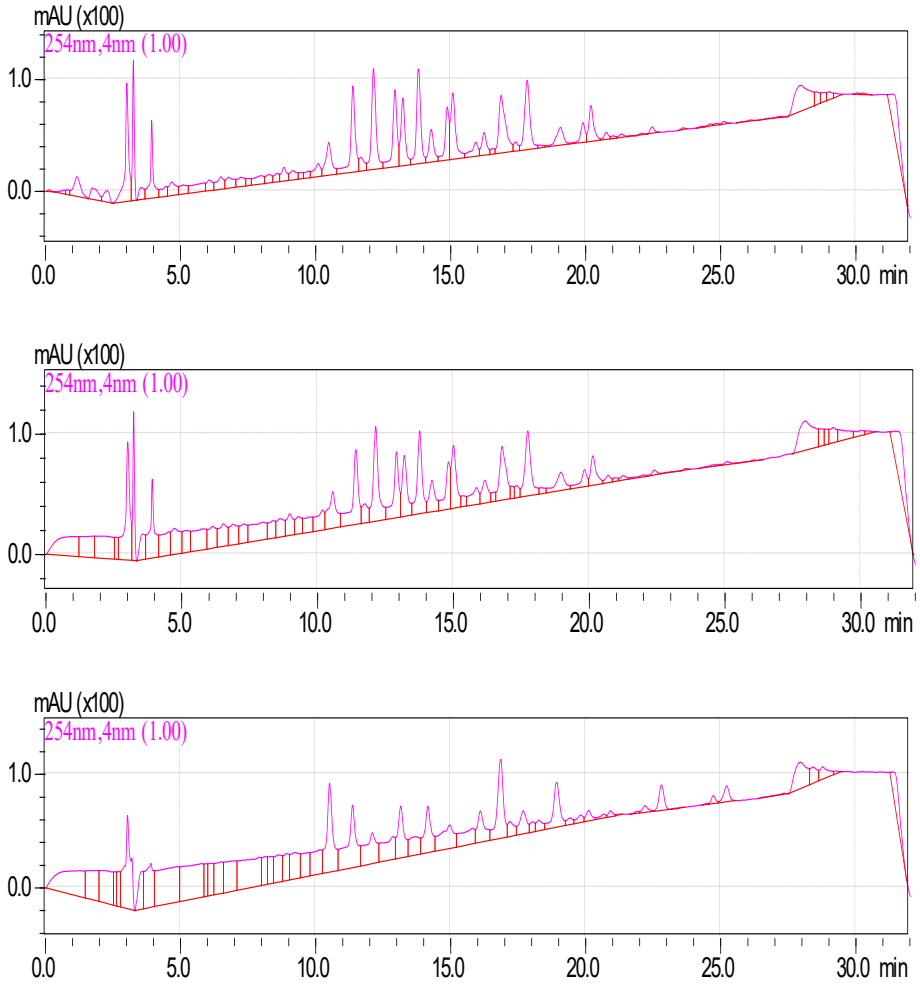

Figure 4. Representative HPLC-DAD anthocyanin chromatograms (520 nm) of bilberry extracts from localities B1, B2, and B3.

Phenolic compounds

A total of 17 phenolic compounds were identified in the bilberry fruits (Table 2). Flavonoids represented the largest group, with catechin, myricetin, and rutin occurring at notably higher concentrations in samples from the B3 location. Phenolic acids formed the second major group, with chlorogenic acid being most abundant in B3, while protocatechuic acid was present at higher levels in B1. The third group of polyphenols consisted of tannins, represented primarily by ellagic acid. Previous studies have consistently demonstrated that wild fruits are rich sources of health-promoting compounds such as gallic acid, chlorogenic acid, hydroxybenzoic acids, and rutin. Other important contributors to their nutritional value include quercetin-3-galactoside, kaempferol-3-O-glucoside, ellagic acid,

and various ellagic acid derivatives. These findings emphasize the nutritional benefits and dietary importance of wild fruits (Deng *et al*., 2014; Natić *et al*., 2019; Drkenda *et al.,* 2014). The phenolic compound profiles varied among the bilberry samples from different locations. Catechin levels were significantly higher ($P<0.01$) in the B3 sample, while protocatechuic acid and ellagic acid were most abundant in the B1 sample. Only small amounts of quercetin and caffeic acid were detected, which aligns with previous reports indicating that these compounds typically occur in minor quantities in bilberries (Okan *et al*., 2018; Zimmer *et al*., 2014). Jakobek *et al.* (2007) analyzed methanolic extracts of bilberries (*Vaccinium myrtillus*) from Slovenia using HPLC and reported concentrations of 54.90 mg/kg p-coumaric acid, 9.57 mg/kg ferulic acid, and 136.97 mg/kg quercetin. In another study, Değirmencioğlu *et al*. (2017) examined bilberries grown in Balıkesir and identified a wide range of phenolic compounds, including 9.25–532.97 mg/kg vanillic acid, 28.79–995.15 mg/kg syringic acid, 1.81–20.05 mg/kg p-coumaric acid, 4.13–24.87 mg/kg ferulic acid, 1.62–6.93 mg/kg quercetin, and 4.42–79.37 mg/kg naringin.

Table 3. Phenolic compounds (mg/100g) of bilberries

Poliphenolic compounds	***B1***	***B2***	***B3***
PHENOLIC ACIDS			
Protocatechuic acid	52.35	49.61	n.d
4-Hydroxybenzoic acid	4.83	1.88	n.d
Syringic acid	0.91	0.32	9.73
m-Coumaric acid	0.01	n.d	n.d
o-Coumaric acid	n.d	0.17	n.d
Caffeic acid	1.60	1.51	2.20
p-Coumaric acid	1.29	1.06	0.80
Ferulic acid	1.50	0.48	0.38
Chlorogenic Acid	38.10	7.58	111.00
Sinapic Acid	1.23	0.14	0.03
FLAVONOIDS			
Catechin	66.67	45.81	164.56*
Myricetin	8.79	8.76	8.71
Quercetin	4.31	0.00	0.00
Kaempferol	1.73	1.69	0.00
Rutin	7.01	5.71	4.15
TANINS			
Ellagic acid	29.54	21.18	7.04

**significance at p<0.01*

3.5. Principal component analysis (PCA) and heatmap

Principal component analysis showed that the first two components accounted for 99.3% of the total variance (PC1: 82.9%, PC2: 16.4%). PC1 was strongly and positively associated with protocatechuic acid, ellagic acid, total

phenolics, and antioxidant capacity, effectively separating the high-altitude samples (B1 and B2) from B3. PC2 was mainly influenced by catechin and chlorogenic acid, providing additional distinction of the B3 samples (Figure 5).

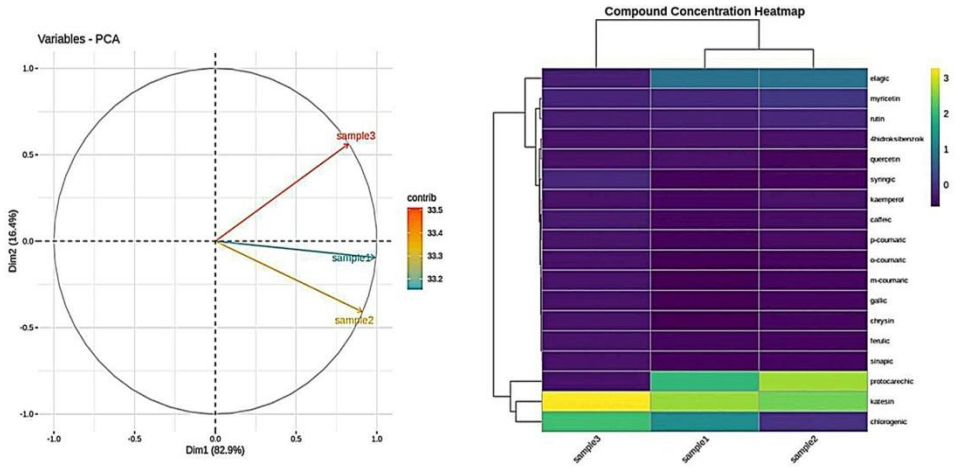

Figure 5. Principal component analysis (PCA) biplot (left) and heatmap (right) of phenolic compounds in bilberry fruits from the three localities

CONCLUSIONS

Altitude was the main factor influencing the phenolic content and antioxidant capacity of wild bilberry (*Vaccinium myrtillus* L.) fruits in the Sharr Mountain, North Macedonia. Fruits collected at 1829–1936 m exhibited significantly higher total phenolic content (589–651 mg GAE/100 g DM) and antioxidant capacity (612–718 mg TE/100 g DM by DPPH; 612–689 mg TE/100 g DM by ABTS) than fruits from 467 m. Individual phenolic compounds and anthocyanin profiles also varied with altitude, with higher concentrations of protocatechuic acid, ellagic acid, and total anthocyanins at the two higher sites. Protecting natural bilberry stands across the full altitudinal gradient is therefore relevant for preserving biodiversity and maintaining a source of high-quality raw material for the regional food and nutraceutical sectors. This study provides the first characterisation of phenolic and antioxidant properties of *V. myrtillus* fruits from North Macedonia.

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