Arch Biol Sci. 2019;71(1):145-157 https://doi.org/10.2298/ABS181004056B

Element accumulation capacity of *Vaccinium myrtillus* from Montenegro: Comparison of element contents in water and ethanol extracts of bilberry plant parts

Snežana Brašanac Vukanović[1], Vanja M. Tadić[2], Nada Blagojević[1], Vesna Vukašinović Pešić[1], Slađana Đurđić[3], Milica Stanković[4], and Jelena Mutić[3,5,*]

[1] *Faculty of Metallurgy and Technology, University of Montenegro*, Džordža Vašingtona bb, 20000, Podgorica, Montenegro
[2] *Institute for Medicinal Plant Research "Dr Josif Pančić"*, Tadeuša Košćuškog 1, 11000, Belgrade, Serbia
[3] *Faculty of Chemistry, University of Belgrade*, Studentski trg 12-16, 11001, Belgrade, Serbia
[4] *University of Niš, Faculty of Medicine*, Dr Zorana Đinđića 81, 18000 Niš, Serbia
[5] *Ghent University Global Campus*, Incheon, South Korea

***Corresponding author**: jmutic@chem.bg.ac.rs

Received: October 4, 2018; **Revised**: November 28, 2018; **Accepted**: November 30, 2018; **Published online**: December 14, 2018

Abstract: Bilberry (*Vaccinium myrtillus* L., Ericaceae) is a rich source of different mineral elements that are important as essential trace elements, or that can be potentially toxic, depending on their concentration. The contents of sixteen elements: the major (Al, Ca, Fe, K and Mg) and trace elements (As, Ba, Cd, Co, Cu, Cr, Mn, Ni, Pb, Sr and Zn) in roots, stems, leaves and fruits of bilberry and the corresponding soil substrate were determined in order to investigate element mobility and availability in soil. Soil was subjected to sequential extraction procedure as proposed by the Community Bureau of Reference in order to fractionate acid-soluble/exchangeable, reducible, oxidizable and residual fractions. Analysis of water and ethanol extracts of bilberry aerial parts revealed a significant transfer of elements during the extraction procedure, with corresponding extraction coefficient values of up to 95.12%. Results showed that bilberry fruits could serve as a good dietary source of essential elements for humans, especially Mn and Cu.

Keywords: *Vaccinium myrtillus*; wild bilberry; extraction coefficients; ethanol extracts; daily metal intake

INTRODUCTION

Medicinal plants and their extracts deserve special attention due to their benefits for human health. Bilberry (*Vaccinium myrtillus* L., Ericaceae) is classified as a Class 1 herb by the American Herbal Products Association, meaning that it can be safely consumed when used appropriately [1]. Its fruits and leaves are rich in the phenolic compounds, including flavonols, tannins, ellagitannins, phenolic acids and anthocyanins [2]. Although most attention has focused on flavonoids in relation to the health benefits, the investigation of mineral content in different plant parts and in corresponding extracts could be of great importance as an addition to previous studies [3]. The content of heavy metals is one of the criteria for the use of plant materials in the production of traditional medicines and herbal infusions [4]. Bilberries are a rich source of several metal elements that are important in human health as essential trace elements, or they can be potentially toxic, depending on their concentrations.

Data regarding the metal content of bilberries have been published in many papers [5-11]. Generally, the main source of metals in plants is their growth media (e.g. soil, nutrient solution) from which the metals are taken up by the roots. Many single or sequential extraction procedures have been applied to soil to fractionate metals using different extractants or reagents [12-15]. One of the most widely applied extraction procedures is the original three-step [16] or modified four-step Community Bureau of Reference (BCR) extraction procedure [17,18]. This allows metals to be divided into acid-soluble/exchangeable, reducible and oxidiz-

How to cite this article: Brašanac-Vukanović S, Tadić VM, Blagojević N, Vukašinović-Pešić V, Đurđić S, Stanković M, Mutić J. Element accumulation capacity of *Vaccinium myrtillus* from Montenegro: Comparison of element contents in water and ethanol extracts of bilberry plant parts. Arch Biol Sci. 2019;71(1)145-57.

able fractions. Soluble metals in water solution and exchangeable fractions are considered to be bioavailable, while those in reducible and oxidizable fractions may only be potentially bioavailable. Generally though, metal mobility in the residual fraction is strongly dependent on the environmental conditions, and the metals here are unavailable to both plants and microorganisms.

Determination of metals in plants is important from two aspects: (i) as an estimate of their nutritional value, and (ii) to guard against any possible ill-effects due to metal intake. The content of metals is one of the criteria used to determine the acceptability of a plant for the production of herbal beverages or other traditional medicaments. Bilberry has been used in traditional medicine in the treatment of many diseases; bilberry leaves are used in antiinflammatory mixtures, especially for the urinary tract, and in mixtures to decrease glucose concentration [19]. Bilberry fruit has protective properties towards blood vessels, and antiedema and immunostimulatory properties. However, to the best of our knowledge, there is no available peer-reviewed literature providing information on which part of the bilberry (roots, stems, leaves or fruits) accumulates which elements, and especially on how the elements taken up from soil might be translocated from the roots to the above-ground parts of the plant. This is of paramount importance, since wild bilberry is collected seasonally for use in traditional medicines, but generally without regard for whether the area is polluted or not. Since both water and ethanol extracts of bilberry leaves and fruits have been used in traditional medicine, our work gives insight into the content of elements, not only in water extracts discussed in the literature to date regarding the levels of metals in bilberry plants, but in ethanol extracts as well. This is of great importance due to the frequent use of ethanol as a solvent in herbal products.

Therefore, the aims of this investigation were to (i) determine the concentrations of sixteen elements including major (Al, Ca, Fe, K and Mg) and trace (As, Ba, Cd, Co, Cu, Cr, Mn, Ni, Pb, Sr and Zn) elements in the roots, stems, leaves and fruits of wild bilberry and in corresponding soil from the growth site, in order to estimate relationships between these elements in bilberry and the soil substrate; (ii) to calculate translocation factors (*TF*) and bioconcentration factors (*BCF*) for each element in order to understand the accumulation patterns and potential of bilberry plants to uptake elements from the soil substrate; (iii) to compare the content of elements in leaves and fruits of wild and commercial bilberry plants; (iv) to determine the content of elements in both extracts (water and ethanol) of leaves and fruits of wild and commercially-grown bilberry plants; (v) to determine the extraction coefficients of elements in the two solvents, and (vi) to estimate the nutritional value of the bilberry fruit for dietary intake.

MATERIALS AND METHODS

Reagents and chemicals

All chemicals were of analytical grade and were supplied by Merck (Darmstadt, Germany). All glassware was soaked in 10% HNO_3 for a minimum 12 h and rinsed well with distilled water. Ultra-pure water was prepared by passing double deionized water from a Milli-Q system (Millipore Simplicity 185 System incorporating dual UV filters (185 and 254 nm) to remove carbon contamination). Multi-element Stock Solution (Alfa Aeser, Thermo Fisher, UK) containing 1000 g/L of major and trace elements and Semiquantitative Standard Solution (Alfa Aeser, Thermo Fisher, UK) containing 10 μg/mL were used to prepare intermediate multi-element standard solutions for ICP-OES measurements.

Sample collection

Samples of soil and wild bilberry (*Vaccinium myrtillus* L., Ericaceae) were collected on Bjelasica Mountain (Montenegro) at an altitude of 1200 m in a geographical location without direct exposure to any source of pollution (Supplementary Fig. S1.). The location is a wilderness area populated by spruce forest. Soil samples were collected at a depth of 0-20 cm. After sampling, soil and plant materials were stored in polyethylene bags until transfer to the laboratory. Samples of commercially grown bilberry plants, *Vaccinii myrtlii folium* and *fructus*, were obtained from the "Josif Pančić" Institute in Belgrade, Serbia. The voucher specimens (No VMF_121215, and VML_111215) were deposited at the Faculty of Pharmacy, University of Belgrade, where identification was performed.

Soil preparation for determination of element content

Soil samples were air-dried at room temperature for three weeks, mechanically ground and sieved to <2.5-mm mesh diameter size. One g of each soil sample were analyzed by the BCR sequential extraction procedure. The following solutions for extraction were used: phase 1 (F1) 0.11 M acetic acid (HOAc extractable fraction; acid soluble fraction); phase 2 (F2) 0.5 M hydroxylamine hydrochloride adjusted to pH 1.5 (reducible fraction); phase 3 (F3) 8.8 M hydrogen peroxide stabilized at pH 2 and 1 M ammonium acetate adjusted to pH 2 (oxidizable fraction); phase 4 (R) aqua regia 15 mL 37% HCl and 5 mL 65% HNO_3, at 80°C during 5 h (residual fraction). For pseudo-total element content determination, 0.5 g of soil were digested in the same way as the residual fraction using the BCR sequential extraction procedure. After digestion, samples were filtered through Whatman no. 42 paper and diluted to 100 mL in a volumetric flask. The element recovery during the sequential extraction procedure was determined by comparison of the sum of each element from all four fractions with the pseudo-total element concentrations, and was in the range of 85-111%.

Preparation of wild and commercial bilberry for determination of the element content

Wild bilberry samples were divided into roots (WBR), stem (WBS), leaves (WBL) and fruits (WBF). After drying, samples were ground in an electric mill. Each of the parts (0.5 g amounts) was transferred into a separate PTFE cuvette and 7 mL of 65% HNO_3 and 1 mL 30% H_2O_2 were added. Microwave digestion was performed under the following program: warm up for 10 min to 180°C and maintain for 15 min at the same temperature. After a cooling period, samples were quantitatively transferred into volumetric flasks (50 mL) and diluted with distilled water. Commercial bilberry samples, leaves (CBL) and fruits (CBF), were prepared in the same way.

Determination of bioconcentration and translocation factors

Bioconcentration factors (*BCF*) were calculated for each element by summing the element concentrations from the first three extraction phases, assuming that these fractions were bioavailable for the plant. BCFs were calculated as follows:

$$BCF = \frac{C_{part\ of\ plant}}{C_{soil}}$$

where $C_{part\ of\ plant}$ is the total concentration of the target element in the plant part and C_{soil} is the concentration of the same element in the first three extraction phases of soil. BCF_r, BCF_s, BCF_l and BCF_f were calculated for roots, stems, leaves and fruits, respectively. Bioconcentration factors were calculated for roots, stems, leaves and fruits (BCF_r, BCF_s, BCF_l and BCF_f respectively). The ability of plants to transport elements from the roots to stems, leaves or fruits was estimated using the translocation factor (*TF*). The *TF* was calculated as the ratio of the concentration of target element in stems (TF_s), leaves (TF_l), fruits (TF_f) and roots (TF_r) as follows:

$$TF = \frac{C_{above\ ground\ part\ of\ plant}}{C_{root}}$$

Preparation of water and ethanol bilberry extracts for element determination

To produce water extracts, 0.5 g of wild or commercial bilberry (leaves or fruits) was added to 50 mL of deionized water and heated at 50°C for 1 h. This water extraction process produced WBLW (wild bilberry leaf extract), WBFW (wild bilberry fruit extract), CBLW (commercial bilberry leaf extract) and CBFW (commercial bilberry fruit extract). To produce ethanol extracts, 10 g of wild or commercial bilberry (leaves or fruits) was added to 50 mL of 70% ethanol and heated at 40°C for 1 h. This ethanol extraction process produced WBLE (wild bilberry leaf extract), WBFE (wild bilberry fruit extract), CBLE (commercial bilberry leaf extract) and CBFE (commercial bilberry fruit extract). After cooling to room temperature for 3 h, extracts were filtered through Whatman no. 42 paper and diluted to 50 mL in volumetric flasks. Before determining the element content in the extracts, water extracts were diluted 1:10 and ethanol extracts were diluted 1:50.

Arch Biol Sci. 2019;71(1):145-157

Determination of extraction coefficient

Extraction coefficient (*EC*) was calculated using:

$$EC = \frac{c_{\text{extracts of plants}}}{c_{\text{plants}}}$$

With respect to the extraction efficiencies, elements can be classified into three groups [20]: highly extractable (>55%), moderately extractable (20-55%) and poorly extractable (<20%).

Determination of dietary daily mineral intake and daily intake

A 100-g fresh-weight portion of bilberry fruits per meal was used to estimate element intakes per day. This typical portion contained about 16 g of dry matter. Daily mineral intake (*DMI*, %) and recommended daily allowance (*RDA*) values were calculated according to the European Economic Community Directive [21]. The *DMI* was calculated using:

$$DMI = \frac{C \times 100}{RDA}$$

where *C* is the element content (mg) in 100 g of fruits. According to EEC regulation 90/496, the contribution is important if 100 g contains at least 15% of the *RDA*.

Daily Intake (*DI*, %) for As, Cd and Pb was calculated for 100 g of bilberry fruits using:

$$DI = \frac{C \times 100}{MDI}$$

where *MDI* corresponds to the maximum tolerable daily intake established by EFSA.

Instrumentation

An inductively coupled plasma-optical emission spectrometer (ICP-OES) (Thermo Scientific, United Kingdom), model 6500 Duo, equipped with a CID86 chip detector, was used for determination of elements. This instrument operates sequentially with both radial and axial torch view. The entire system was controlled with iTEVA software. Instrument conditions are given in Supplementary Table S1. Microwave digestion was used for destruction digestion of bilberry plant parts. Digestion was performed in a microwave oven equipped with a rotor holding 10 PTFE cuvettes (Ethos 1, Advanced Microwave Digestion System, Milestone, Italy).

Statistical analysis

Parameters of descriptive statistics (mean value and standard deviation) were obtained using a demo version of the NCSS statistical software (www.ncss.com).

Table 1. Concentration (mg/kg) of trace and major elements in soil from Bjelasica Mountain (Montenegro)

Element	Phase 1	Phase 2	Phase 3	Phase 4	Pseudo-total	Recovery (%)
As	0.061±0.006	0.053±0.002	0.82±0.10	0.9±0.2	1.6±0.2	107.56
Ba	14.30±0.08	30.51±0.10	9.54±0.03	11.11±0.04	73.5±0.4	89.11
Cd	0.048±0.009	0.065±0.009	0.08±0.01	0.25±0.02	0.48±0.04	92.16
Co	0.78±0.10	4.92±0.05	2.18±0.03	5.73±0.03	14.56±0.08	93.41
Cr	6.0±0.4	2.18±0.04	6.96±0.09	212.6±2.3	265.8±5.6	85.65
Cu	1.37±0.05	0.46±0.02	7.44±0.07	12.41±0.04	19.66±0.09	110.31
Mn	135.9±1.2	236.0±2.4	97.7±0.5	96.5±2.1	657.8±7.5	86.06
Ni	0.71±0.02	5.50±0.10	4.81±0.04	13.74±0.05	23.0±0.2	107.76
Pb	0.20±0.04	25.65±0.08	4.45±0.07	3.27±0.06	34.9±0.8	96.13
Sr	5.65±0.10	3.24±0.04	2.46±0.03	13.8±0.2	22.7±0.5	110.58
Zn	1.17±0.02	7.72±0.03	9.04±0.04	24.65±0.02	44.8±0.4	95.09
Al	469±9	2750±20	2435±14	19155±200	23313±183	106.42
Ca	13774±214	883±16	841±5	8537±177	22433±80	107.14
Fe	262±9	2238±18	2394±18	12289±240	17345±210	99.06
K	1478±27	1172±14	1165±19	5662±126	10490±143	90.36
Mg	1816±19	1039±9	714±6	2323±14	5940±28	99.23

RESULTS

Profile of elements in soil

The results obtained for each element after soil was subjected to the BCR sequential extraction procedure, pseudo-total element content obtained from aqua regia digestion, and recovery values, are given in Table 1. The distribution of the elements from the mountain soil among fractions: (phase 1) exchangeable and associated with carbonates, (phase 2) associated with easily and moderately reducible iron and manganese oxyhydroxides, (phase 3) associated with organic matter and sulfides, (and phase 4) aqua regia extractable residual fraction, are presented in Fig. 1A and B. Contents of trace and major elements (% of total content of each element) are given in Supplementary Figs. S2 and S3, respectively.

Bioconcentration and translocation factors in bilberry plants and tissues

For better understanding of the relationship between available concentrations of elements in soil and element content in plant tissue, the *BCF* was calculated. Higher *BCF* values imply a greater phytoaccumulation ability of the plant. The ability of plants to transport elements from the roots to stems, leaves or fruits was estimated using the *TF*. A higher *TF* value corresponds to higher translocation ability. The *BCF* and *TF* values obtained for bilberry are presented in Table 2.

The element content of wild and commercial bilberry

The element contents in the different plant parts of wild and commercial bilberries are presented in Table 3.

The element content of water and ethanol bilberry extracts

People mostly utilize bilberry leaves or fruits, and so accordingly, in this work we determined the content of major and trace elements and the coefficients of water and ethanol extraction from these plant parts (Tables 4 and 5). Extraction coefficients depend on the extraction medium and on the plant species extracted [31]. Additionally, the duration and temperature of the process of extraction could highly influence the *EC* value.

Table 2. Calculated bioconcentration (*BCF*) and translocation (*TF*) factors in bilberry plant parts.

Element	BCFr	BCFs	BCFl	BCFf	TFs	TFl	TFf
As	* n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
Ba	1.08	2.76	2.34	0.18	2.56	2.17	0.17
Cd	0.71	0.57	0.41	0.21	0.81	0.58	0.30
Co	0.01	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
Cr	0.23	0.10	0.13	0.03	0.46	0.57	0.15
Cu	0.62	0.70	0.61	0.55	1.13	0.99	0.89
Mn	3.62	6.45	12.54	0.03	1.78	3.46	0.01
Ni	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
Pb	0.17	0.13	0.14	0.01	0.77	0.81	0.04
Sr	1.60	4.80	5.31	0.12	3.01	3.32	0.07
Zn	0.83	2.80	0.93	0.40	3.39	1.13	0.49
Al	0.07	0.06	0.12	0.02	0.86	1.81	0.33
Ca	0.20	0.85	1.51	0.04	4.33	7.70	0.21
Fe	0.07	0.04	0.13	0.01	0.57	1.77	0.09
K	1.39	3.35	5.27	1.39	2.42	3.81	1.00
Mg	0.14	0.40	0.92	0.12	2.83	6.53	0.83

* n.a. – not applicable

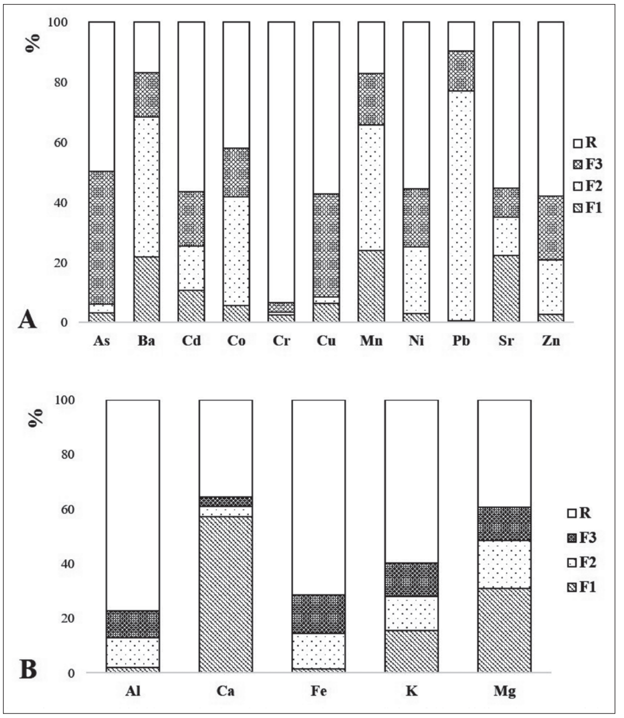

Fig. 1. Profile of elements in soil. Fractions (% of total content of each element) of (**A**) trace and (**B**) major elements in soil from Mount Bjelasica, Montenegro, determined using the BCR sequential extraction procedure.

Table 3. Element content (mg/kg) in the roots, stems, leaves and fruits of wild bilberry (WBR, WBS, WBL, WBF, respectively) and in commercial bilberry leaves (CBL) and commercial bilberry fruits (CBF).

Element	Wild bilberry				Commercial bilberry	
	roots	stems	leaves	fruits	leaves	fruits
As	* n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Ba	58.5±0.5	150.0±0.7	127.2±0.5	9.70±0.04	119.5±0.4	14.05±0.05
Cd	0.136±0.007	0.111±0.004	0.079±0.005	0.041±0.004	0.073±0.005	0.021±0.005
Co	0.087±0.002	n.d.	n.d.	n.d.	0.600±0.001	0.534±0.002
Cr	3.47±0.06	1.58±0.08	1.98±0.04	0.52±0.02	1.99±0.06	0.833±0.005
Cu	5.73±0.04	6.50±0.04	5.68±0.04	5.11±0.07	6.78±0.05	4.378±0.004
Mn	1701±25	3031±11	5887±30	13.61±0.02	5955±23	14.07±0.05
Ni	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Pb	5.09±0.05	3.90±0.04	4.11±0.06	0.20±0.02	4.29±0.08	0.237±0.006
Sr	18.12±0.08	54.5±0.4	60.2±0.2	1.32±0.02	52.3±0.5	2.057±0.009
Zn	14.84±0.08	50.3±0.2	16.76±0.07	7.24±0.04	29.19±0.07	13.83±0.06
Al	380±2	328±2	689±2	126.8±0.6	594.0±0.8	130.6±1.0
Ca	3028±9	13116±105	23330±210	651±8	17264±180	1419±15
Fe	349±2	200.0±0.5	620±2	32.5±0.5	580±4	45.2±0.2
K	5285±10	12796±57	20113±210	5290±50	16894±80	5474±42
Mg	504±3	1424±12	3286±14	419±7	2324±20	653±7

* n.d. – not detected

Table 4. Element content (mg/kg) in water and ethanol bilberry extracts.

	Water extract of wild plant		Water extract of commercial plant		Ethanol extract of wild plant		Ethanol extract of commercial plant	
	Leaves (WBLW)	Fruits (WBFW)	Leaves (CBLW)	Fruits (CBFW)	Leaves (WBLE)	Fruits (WBFE)	Leaves (CBLE)	Fruits (CBFE)
As	* n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Ba	10.5±0.2	2.36±0.02	13.60±0.07	4.3±0.4	0.066±0.008	n.d.	n.d.	n.d.
Cd	0.074±0.002	0.039±0.002	0.061±0.006	0.020±0.001	0.065±0.004	0.026±0.002	0.05±0.03	0.013±0.001
Co	n.d.	n.d.	0.22±0.02	0.20±0.02	n.d.	n.d.	0.192±0.007	0.062±0.004
Cr	0.525±0.008	0.193±0.004	0.557±0.003	0.298±0.001	n.d.	n.d.	0.109±0.001	n.d.
Cu	1.62±0.02	1.568±0.002	2.03±0.03	1.5±0.2	0.60±0.03	0.17±0.05	1.25±0.05	0.40±0.05
Mn	683±6	2.73±0.03	697±3	2.76±0.05	88.2±0.2	2.573±0.010	45.9±0.3	1.49±0.03
Ni	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Pb	1.179±0.001	0.083±0.004	1.51±0.07	0.115±0.008	0.069±0.008	0.03±0.03	0.26±0.05	0.044±0.010
Sr	3.93±0.03	0.770±0.005	8.68±0.02	1.15±0.04	0.092±0.001	0.080±0.001	1.19±0.08	0.38±0.03
Zn	11.11±0.02	5.00±0.05	17.17±0.03	10.94±0.02	0.044±0.003	0.042±0.004	n.d.	n.d.
Al	61.3±0.6	39.8±0.3	70±1	48±1	57±2	37.8±1.0	40.1±0.2	40±2
Ca	1039±12	184±2	1075±28	391±11	21.4±0.2	4.3±0.2	24±1	17±2
Fe	3.68±0.06	19.5±0.2	2.5±0.2	26.0±0.3	n.d.	n.d.	n.d.	n.d.
K	1459±10	2743±15	1603±2	2901±9	763±6	1111±25	239±8	1101±9
Mg	673±3	237.1±0.5	598±4	400±2	321±5	21.5±0.6	154±2	18.1±0.7

* n.d. – not detected

The most abundant major element in all investigated bilberry extracts (WBLW, WBLE, WBFW, WBFE, CBLW, CBLE, CBFW and CBFE) was K (Table 4), although we found a low extraction coefficient for K in the water and ethanol extracts of leaves (up to only 9.49%, Table 5). In extracts WBLW and CBLW, Ca levels were higher than those of Mg, even though the extraction coefficients for Ca were low (up to 6.23%), while for Mg, they were medium (up to 25.73%). In WBFW and CBFW, Ca was the third most abundant

Table 5. Extraction coefficients (%) of elements in extracts (water and ethanol) of bilberry plant parts.

	Water extract of wild plant		Water extract of commercial plant		Ethanol extract of wild plant		Ethanol extract of commercial plant	
	Leaves (WBLW)	Fruits (WBFW)	Leaves (CBLW)	Fruits (CBFW)	Leaves (WBLE)	Fruits (WBFE)	Leaves (CBLE)	Fruits (CBFE)
As	n.a.*	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
Ba	8.26	24.33	11.38	30.37	0.05	n.a.	n.a.	n.a.
Cd	93.67	95.12	83.56	95.24	81.91	62.94	74.44	60.05
Co	n.a.	n.a.	36.50	36.89	n.a.	n.a.	32.00	11.70
Cr	26.50	37.48	27.95	35.77	n.a.	n.a.	5.46	n.a.
Cu	28.54	30.71	29.99	34.99	10.52	3.38	18.35	9.18
Mn	11.61	19.85	11.70	19.62	1.50	18.91	0.77	10.6
Ni	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
Pb	28.69	41.59	35.25	48.67	1.68	15.00	5.96	19.00
Sr	6.53	58.38	16.60	55.81	0.15	6.08	2.28	18.37
Zn	66.30	69.00	58.83	79.10	0.26	0.59	n.a.	n.a.
Al	8.91	31.39	11.83	37.03	8.34	29.80	6.76	30.73
Ca	4.45	28.23	6.23	27.54	0.09	0.67	0.14	1.20
Fe	0.59	59.99	0.43	57.60	n.a.	n.a.	n.a.	n.a.
K	7.25	51.86	9.49	53.00	3.79	21.00	1.41	20.11
Mg	20.47	56.55	25.73	61.33	9.77	5.12	6.61	2.77

* n.a. – not applicable

Table 6. Calculated element concentrations in bilberries, recommended daily allowance and daily mineral intake of a) essential and b) toxic elements for bilberry fruits.

a)

Element	Average concentration (mg/g)	Average concentration in 100 g of fresh bilberry fruits	RDA (mg per day)	DMI (%)
K	5.290	84.640	2000	4.23
Mg	0.419	6.704	375	1.79
Ca	0.651	10.416	800	1.30
Fe	0.0325	0.520	14	3.71
Zn	0.0072	0.115	10	1.15
Cu	0.0051	0.081	1	8.17
Mn	0.1361	0.217	2	10.88

b)

Element	Average concentration (mg/kg)	Average concentration in 100 g of fresh bilberry fruits	MDI (µg per day)	DI (%)
As	0.000	0.000	150	* n.a.
Cd	0.041	0.656	25	2.62
Pb	0.200	3.200	250	1.28

* n.a. – not applicable

major element (Table 4). In these cases, Ca showed medium (up to 28.23%) while Mg showed high extraction coefficients (up to 61.33%, Table 5). Ca and Mg were poorly extractable from bilberry plant parts with ethanol (contents were all <20%). The content of Al varied from 37.8 mg/kg in WBFE to 70.0 mg/kg in CBLW. High Fe contents were found in the water fruit extracts (19.5 mg/kg in WBFW and 26.0 mg/kg in CBFW), and we also calculated a high extraction coefficient (up to 59.99%). Significantly lower Fe content was detected in water leaf extracts (3.68 mg/kg in WBLW and 2.5 mg/kg in CBLW). The content of Fe in all ethanol extracts was below the limit of detection (<0.06 mg/kg).

The most abundant trace element in water leaf extracts was Mn followed by Zn, while in water fruit extracts, Zn levels were higher than Mn levels. The Mn content in WBLW and CBLW was 683 and 697 mg/kg, respectively, while in fruit, it was much lower (2.73 and 2.76 mg/kg, respectively). However, we found low extraction coefficients for Mn in all our extracts (up to only 19.85% was extracted). The content of Zn in water extracts of leaves and fruits was from 5.00 mg/kg in WBFW to 17.17 mg/kg in CBLW. In addition, a high extraction coefficient was calculated for Zn in WBFW and CBFW (79.10%). The contents of Zn in WBLE and WBFE were very low (0.044 and 0.042 mg/kg, respectively), while the concentration of this element in CBLE and CMFE was undetectable (<0.020 mg/kg). In the ethanol extracts, the most abundant trace element was also Mn (from 1.49 mg/kg in CBFE to 88.2 mg/kg in WBLE), but the extraction coefficient of Mn in ethanol was low (up to 18.91%). The second most abundant trace element in ethanol fruit and leaf extracts was Cu (from 0.17 in WBFE to 1.25 mg/kg in CBLE), with the extraction coefficients being poor, only up to 18.35%. In water extracts of leaves and fruits, the Cr content ranged from 0.193 mg/kg in WBFW to 0.557 mg/kg in CBLW, with moderate extraction coefficients (up to 37.48%). In ethanol, Cr was poorly extractable (5.46%) or even unextractable and so remained undetected (<0.026 mg/kg).

Toxic metals were also present in the bilberry extracts. The Pb content in the water extracts of leaves and fruits ranged from 0.083 mg/kg in WBFW to 1.51 mg/kg in CBLW, with coefficient extraction being up to 48.67%. In ethanol, the Pb content was from 0.03 mg/kg in WBFE to 0.26 mg/kg in CBLE and the extraction coefficients were low (up to 19%). On the other hand, we measured high percentages of Cd from both bilberry leaves and fruit transferred into ethanol and water (extraction coefficients from 60.05% in CBFE to 95.24% in CBFW). The determined high extraction coefficients for Cd could, however, be considered insignificant, since our research also revealed that this metal accumulated neither in the leaves nor in the fruit of the bilberry (*BCF*s were up to 0.71, Table 2). In addition, the *TF* determined for Cd (0.81) pointed to this metal translocating more to plant stems than to fruit or leaves. In the investigated plant parts, the Cd content measured was up to ten times less than the World Health Organization (WHO) recommended upper limit for Cd in medicinal plants, meaning that in terms of Cd content, the bilberry examined would be considered safe for medicinal use.

The water extracts had higher extraction coefficients and higher concentrations of all studied elements in comparison to ethanol extracts obtained from the same parts of bilberry (Tables 4 and 5). The water and ethanol extracts of leaves contained higher amounts of almost all studied elements in comparison to the extracts of fruit. K and Fe, being exceptions, were higher in water extracts of fruit rather than in leaf extracts. Additionally, the content of K was higher in the ethanol fruit extracts than in leaf extracts. On the other hand, the water extracts of fruit showed higher extraction coefficients for all investigated elements than did the leaf extracts (Table 5).

Dietary elemental intake

Calculated *RDA* and *DMI* values of essential trace elements and *DI* and *MDI* values of toxic metals for bilberry fruits are given in Table 6a and b. We calculated a good *DMI* for Mn (10.88% of *RDA*). The calculated *DMI* for Cu was 8.17%, whereas for Zn, it was 1.15%. These findings reveal the positive significance of bilberry consumption as part of a normal daily diet. In the case of major elements, the calculated *DMI* was highest for K (4.23% of *RDA*), while for Fe it was 3.71% of *RDA*. The calculated *DMI* for Mg was 1.78% of *RDA*, whereas the calculated *DMI* for Ca was 1.30%. Provisional tolerable weekly intake (*PTWI*) for inorganic As is 15 µg per kg body weight (b.w.), which corresponds to an intake of 2.1 µg per kg b.w per day [34]. This daily intake is equivalent to an intake of 150 µg per day for the average consumer (70 kg b.w.). For Cd, EFSA's [35] Panel on Contaminants in the Food Chain (CONTAM Panel) set the tolerable weekly intake (*TWI*) of 2.5 µg per kg b.w. in order to ensure a high level of protection for all consumers, which corresponds 25 µg per day for an adult person weighing 70 kg. The provisional *TWI* for Pb is 25 µg per kg b.w. [36]. We found that consumption of 100 g of bilberries would result in Cd intake of just 2.62% and Pb intake of just 1.28% of their respective *TDIs*. We did not detect As in the bilberries examined. Based on our findings, therefore, the bilberry fruits possessed no toxicological risk with respect to the examined metals.

DISCUSSION

Large percentages of Ca and Mg in the mountain soil were associated with the acid soluble fraction (57.31 and 30.84%, respectively). Elements extracted in this fraction would include weakly taken-up element species, particularly those caught on the soil surface by relatively weak electrostatic interactions and those that can be released by ion-exchange processes. The predominant chemical form of Pb was associated with the reducible fraction (76.41%) followed by the oxidizable (13.27%), and, lastly, the residual (9.73%) fraction. Taking into account that the fractionation profile of Pb in the first three fractions comprised more than 90% of the total content of this metal, it could be considered as the most easily mobilized, i.e. the most bioavailable metal. Although the pseudo-content of Pb in the investigated soil was in the range defined as safe by Serbian national regulations [24], this does not mean the same safety criteria could be applied to plants, taking into account the mobility we observed. In fact, this highly mobile Pb could be a problem if taken up by bilberry. This level of metal mobilization indicates that Pb could have been remobilized, becoming more easily bioavailable following a slight lowering of pH [15, 25]. Furthermore, our results showed that Ba and Mn had similar behavior, meaning that the highest percentages of these metals were found in the reducible fraction (46.61 and 41.69%, respectively). Metals in this fraction, in reducible conditions, can bind to hydrous oxides of Mn and Fe. As, Pb, Ba and Mn can be considered easily mobilized elements, based on the fact that more than 80% of the total content of these elements in soil was distributed in the first three fractions. The soil Cr concentration was higher than the maximum recommended value [24] for this metal (100 mg/kg). The average content of this metal in soils worldwide was established to be 60 mg/kg, although in soils derived from basic rocks and serpentines, it can be even higher [22]. However, we found that more than 90% of the Cr in the Montenegrin soil was bound to the residual fraction, which indicates that this metal, being strongly bound to crystalline structures of minerals, would be the least available to bilberry. Our results confirmed that the highest percentages of Al and Fe were present in the residual fraction (77.21 and 71.52%, respectively), which showed that these metals were also immobile in soil. The contents of Cd, As, Ni, Cu, Zn and Co in the soil were below the maximum allowed limits, according to national regulations [24]. The greatest percentages of As, Cd, Cu and Zn were extracted in the residual fraction (46.91, 56.49, 57.23, 57.89%, respectively) followed by the oxidizable fraction (46.60, 18.08, 34.32, 21.23%, respectively). In the case of Co and Ni, both metals were extracted in the residual fraction (42.09; 55.48%, respectively), as well as being associated with the reducible fraction (36.17; 22.22%, respectively), while K and Sr were extracted in the residual fraction (59.74 and 54.83%, respectively), followed by the acid soluble fraction (15.60 and 22.50%, respectively). The results obtained indicated that there were no anthropogenic inputs of these elements in the soil from the investigated site. Our pseudo-total contents were in accordance with data published by Kabata-Pendias [22], who stated that the metal content might vary within different ranges in various soil groups.

Our findings revealed that among the major elements, Ca and Mg mostly accumulated in bilberry leaves (*BCF* values were 1.51 and 0.92, respectively), while K was present in all parts of bilberry (including fruits), with the highest amount in the leaves (*BCF* between 1.39 and 5.27). It is well known that Mg is a constituent of chlorophyll and essential for photosynthesis, while Ca accumulates in the stems and leaves. Lack of Ca might cause poor development of roots and drying of leaves. K, being located in all parts of the plant, is especially important because of its capacity to enable better nitrogen use, which affects the synthesis of protein, enables the synthesis of carbohydrate in leaves, facilitates the transport of the resulting compounds to other parts of the plant, and increases the resistance of plants to cold [26].

In the case of trace elements, we determined that Mn and Zn accumulated in roots, stems and leaves of bilberry, with *BCF*s from 3.62 to 12.54 for Mn, and from 0.83 to 2.80 for Zn. This level of Mn accumulation might be of great importance, taking into account its role as an essential nutrient for plants. Mn is a functional component for the assimilation of nitrate, and it is an important component of many enzyme systems in plants. Zn is an important and useful element for plants, mainly as part of the active site of various metalloenzymes; it participates in the process of photosynthesis and the biosynthesis of cytochrome superoxide dismutase and catalysis, responsible for the protection of plants from stress [26]. Interestingly, our results differed in comparison to the published data, where lower accumulation of Zn was found [10]. In contrast to Mn and Zn, Cu was distributed approximately equally through all parts of the bilberry, including fruits (*BCF* was from 0.55 to 0.70). A similar *BCF* value for Cu in bilberry from northern Europe was observed previously [10].

Cu participates in physiological processes in plants, including photosynthesis, respiration, and the distribution of carbohydrate and nitrogen; it is present in the metabolic pathways of plants as an enzyme activator and regulator and contributes to plant resistance to disease [26]. Ba and Sr were highly concentrated in the roots, stems and leaves of our bilberry plants (*BCF* was from 1.08 to 5.31). Therefore, Sr was easily absorbed by bilberry. Although it is known that Sr is not essential for the growth and multiplication of most plants, it is necessary for the metabolic demands of Ca in plants [27].

In the case of toxic elements, As was not detected in any part of the bilberry plants examined, while *BCF* values for Pb were very low (*BCF* from 0.01 to 0.17). On the other hand, a slightly higher *BCF* value for Cd occurred in the roots of plants, although it remained below one (up to 0.71).

Our results showed that bilberry did not accumulate As or Ni. The absorption of Ni by the plant and its transfer from root to shoot might be inhibited by the presence of Cu, Zn, Fe, and Co [28]. Competition kinetic studies showed Cu and Zn were capable of competitively preventing Ni absorption, suggesting that Ni, Cu, and Zn were absorbed using the same carrier site. We found that Al, Co, Cr, Fe and Pb were present only in relatively low amounts in all parts of the plant (*BCF* was from 0.01 to 0.23), which was in agreement with previously published results [10].

Based on the translocation factors obtained, it was assumed that the K, Mg, and Cu (*TF* values were from 0.83 to 1.00) were transported from the roots to fruits in the highest amounts. The most significant finding was that there was no translocation of toxic trace elements to the bilberry fruits. They translocated to the stems or leaves, although *TF* values were less than 1. In the case of essential metals, all the major elements, as well as Mn and Zn, were transported from bilberry roots to leaves, with *TF* values from 1.13 to 7.70. Taking into account that in traditional medicine only the leaves and fruits are historically used for treatment of health conditions, our findings provide insight into the safety of application and use of these plant parts.

The presence of major and trace metals (i.e. elements) in bilberries has been reported in a number of previous studies [5-11]. Differences in the metal content of plant tissues in different countries might be dependent on metal availability in soil as well as on plant species, growth stage and morphological features [9]. In addition, the accumulation might depend on seasonal variation [7].

In the current study, WBS, WBL and WBF contained Co in quantities below the limit of detection (<0.011 mg/kg), while WBR (0.087 mg/kg) and the commercial bilberry parts contained a significantly higher amount of this metal (0.600 mg/kg and 0.534 mg/kg in CBL and CBF, respectively). Li et al. [29] indicated that plants can accumulate small amounts of Co, and that its absorption and distribution depend on the species and are controlled by different mechanisms. Co distribution could involve organic complexes, although the low mobility of Co in the plants restricts its transportation from the roots to the shoot, as seen in the current study in the case of wild bilberry. However, the concentrations of other investigated elements in both our wild and commercial bilberries were similar and followed the same trend, being lower in fruits than in leaves. In all studied parts of wild and commercial bilberry, the contents of As and Ni were below the limits of detection (0.047 mg/kg and 0.012 mg/kg, respectively).

The most abundant major element in WBR and WBF was K (5285 and 5290 mg/kg, respectively), followed by Ca (3028 and 651 mg/kg, respectively), while in the WBS and WBL, Ca was the most abundant (13116 and 23330 mg/kg, respectively), along with K (12796 and 20113 mg/kg, respectively). Mg was the third most abundant major element in all investigated bilberry plant parts (from 419 in WBF to 3286 mg/kg in WBL), followed by Al (from 126.8 in WBF to 689 mg/kg in WBL) and then Fe (from 32.5 in WBF to 620 mg/kg in WBL).

Distribution of trace elements (adopting the order Mn>Zn>Cu>Cr) and toxic metals (Cd>Pb) in all investigated parts of wild bilberry followed the same trends. WBF had the lowest contents of Mn, Zn, Cu and Cr (13.61, 7.24, 5.11 and 0.52 mg/kg, respectively) compared with the other plant parts. WBL had the highest content of Mn (5887 mg/kg), Zn and Cu were highest in WBS (50.3 and 6.50 mg/kg, respectively), while the lowest content of Cr was measured in WBR (3.47 mg/kg), compared with the other plant parts.

The most abundant major element in all investigated bilberry extracts was K. However, the extraction coefficient of K from fruit in water and ethanol extracts (up to 53.00%) indicated only moderate extraction efficiency from this plant part. We calculated a high extraction coefficient (up to 59.99%) for Fe in the water fruit extracts. The extraction coefficients obtained for Fe in leaf water extracts were low (up to only 0.59%), which was in accordance with values found in the literature [6]. The second most abundant trace element in fruit and leaf ethanol extracts was Cu, with low extraction coefficients, only up to 18.35%. On the other hand, the extraction coefficients for Cu in fruit and leaf water extracts were similar to published values (29.99%) [6]. The obtained extraction coefficients for Mn in all our extracts were up to only 19.85%, while Gallaher et al. [6] found a higher extraction coefficient for Mn in leaf water extracts (39.30%). The extraction coefficient we measured for Zn in WBLW and CBLW was high (up to 66.30%), and was also higher than the values found in the literature [6]. Obtained Pb content in the water extracts of leaves and fruits with coefficient extraction being up to 48.67% is in agreement with the literature [20]. High extraction coefficients of Cd in water extracts (95.24% in CBFW) were also reported in the literature [20]. The water extracts of fruit showed higher extraction coefficients than did the leaf extracts, which could be explained by the fact that most elements in water extract of leaves could be complexed by flavonols, catechols, tannins and polyphenols found in the leaves [20]. Tannins, especially, have been known to strongly bind Al, Cr, Cu and Fe.

The highest concentrations of toxic metals (Cd and Pb) were found in WBR (0.136 and 5.09 mg/kg, respectively), while WBF did not contain these metals in significant quantities. For medicinal plants, the permissible limits for Cd and Pb set by the WHO are 0.3 and 10 mg/kg, respectively [30]. All the studied parts of wild and commercial bilberry accumulated these metals in quantities that were below these limits.

Overall, the results revealed that levels of toxic metals were below the upper permissible limit set by the WHO, while essential metals, i.e. the trace elements, were present in significant quantities important for exhibiting beneficial pharmacological effects.

Cr functions as a cofactor in insulin-regulating activities [32,33]. It might facilitate insulin binding and subsequent uptake of glucose into the cell, and therefore, cause decreases in fasting glucose levels, which might improve glucose tolerance, lower insulin levels and decrease total cholesterol in type II diabetic subjects.

It is known that Mn activates the antioxidant enzyme manganese superoxide dismutase, which protects cell membranes and tissues from disruption and degeneration. Mn helps the body to catabolize lipids, carbohydrates, and proteins and assists in energy production. It is also involved in the modulation of glucose transport across cell membranes.

The heart and blood vessels are dependent upon Cu, which is also used for the transportation of oxygen by Fe. Cu might act as an antioxidant, as support for giving the skeleton strength and elasticity and it is important for the immune system. It was earlier established that Zn enables the function of many enzymes. Amongst others, the transport of carbon dioxide from the tissues to the lungs and the production of protein are dependent on Zn. Zn also works together with the hormone insulin that regulates carbohydrate conversion in the body [32,33].

Ca and Mg are needed for building the skeleton, for nerve and muscle functions and in order for cells to function.

The health benefits derived from all the above mentioned elements are clear indications that deficiencies in these metals could be considered an additional risk factor in the development, progress and complications of human disease. Therefore, daily intake of essential elements provided by consuming bilberry fruit might have beneficial effects on impaired metabolic processes.

CONCLUSION

The results of the applied BCR sequential extraction procedure revealed that Pb was the most easily mobilized, bioavailable metal in the investigated soil. However, Pb had a low bioconcentration in all examined parts of bilberry (*BCF* values were only up to 0.17). In the case of Cd, the *BCF* for roots was 0.71 but this metal translocated more to the stems than to leaves or fruit. As from the investigated soil did not accumulate in bilberry at all, since levels in the plants were below the limit of detection. Therefore, although available to the plants from the soil, because of the low *BCF* value, As would not be considered as a risk to the health of human consumers of these bilberries or bilberry extracts. A high *BCF* value in bilberry fruit was only found for K (*BCF* of 1.38). Interestingly, the results obtained revealed that only this metal was translocated in a significant quantity to the fruit. On the other hand, Ca, Mg, K, Fe, Al, Sr, Ba, Mn, Cu and Zn were translocated mostly from roots to leaves. Ca, Mg, K, Sr, Ba, Mn and Zn also exhibited high capability of being accumulated in leaves.

We determined that the bilberry extract preparation method had an effect on the element content. Water and ethanol extracts of leaves or fruits were rich in all essential metals. However, the water extracts had higher metal concentrations than the ethanol extracts. In addition, the water and ethanol extracts of leaves contained higher amounts of almost all studied metals than the fruit extracts. Also, the water extracts of fruit showed higher extraction coefficients for all investigated metals than the leaf extracts. This was possibly due to metals binding to flavones, catechols, tannins and polyphenols, which are present in the leaves in higher quantities than in the fruit. The calculated dietary intakes showed that bilberries could serve as a good dietary source of essential elements, especially Mn (10.88% of *RDA)* and Cu (8.17% of *RDA*).

Acknowledgements: The Ministry of Education, Science and Technological Development (Serbia) and Ministry of Science (Montenegro) supported this study through the research project "Chemical, biochemical and microbiological characterization of *Vaccinium myrtillus* as potential source of valuable phytoconstituents in functional food and dermocosmoceutics *(Vaccinium myrtillus* L., Ericaceae – old remedy, new application)", (Project **No** 451-03-01414/2016-09/1) and Ministry of Education, Science and Technological Development, projects No III 45017 and OI 172030.

Author contributions: Snežana Brašanac Vukanović and Slađana Đurđić: soil and plant analysis, determination of elements using ICP OES; Nada Blagojević and Vesna Vukašinović Pešić: statistical analysis and manuscript preparation; Vanja Tadić: plant sampling and sample preparation for extraction; Milica Stanković: extract preparation. Jelena Mutić supervised the study, analyzed the data and prepared the manuscript.

Conflict of interest disclosure: The authors declare no conflicts of interest.

REFERENCES

1. Chu W, Cheung SCM, Lau RAW, Benzie IFF. Bilberry (*Vaccinium myrtillus* L.). In: Benzie IFF, Wachtel-Galor S, editors. Herbal medicine biomolecular and clinical aspects. Boca Raton: CRC Press; 2011. p. 55-73.
2. Može S, Polak T, Gašperlin L, Koron D, Vanzo A, Poklar Ulrih N, Abram V. Phenolics in Slovenian bilberries (*Vaccinium myrtillus* L.) and blueberries (*Vaccinium corymbosum* L.). J Agric Food Chem. 2011;59(13):6998-7004.
3. Brasanac-Vukanovic S, Mutic J, Stankovic DM, Arsic I, Blagojevic N, Vukasinovic-Pesic V, Tadic VM. Wild Bilberry (*Vaccinium myrtillus* L., Ericaceae) from Montenegro as a Source of Antioxidants for Use in the Production of Nutraceuticals. Molecules. 2018;23(8):1864-83.
4. Kostić D, Mitić S, Zarubica A, Mitić M, Veličković J, Randjelović S. Content of trace metals in medicinal plants and their extracts. Hem Ind. 2011;65(2):165-70.
5. Demczuk M, Garbiec K. Heavy metals in edible fruits. A case study of bilberry *Vaccinium myrtillus* L. Environ Prot Nat Resour. 2009;40:307-12.
6. Gallaher RN, Gallaher K, Marshall AJ, Marshall AC. Mineral analysis of ten types of commercially available tea. J Food Compos Anal. 2006;19:53-7.
7. Kandziora-Ciupa M, Ciepał R, Nadgórska-Socha A, Barczyk G. A comparative study of heavy metal accumulation and antioxidant responses in *Vaccinium myrtillus* L. leaves in polluted and non-polluted areas. Environ Sci Pollut Res. 2013;20(7):4920-32.
8. Kozanecka T, Chojnicki J, Kwasowski W. Content of heavy metals in plant from pollution-free regions. Polish J Environ Stud. 2002;11(4):395-9.
9. Parzych A. The heavy metal content of soil and shoots of *Vaccinium myrtillus* L. in the Słowiński national park. For Res Pap. 2014;75(3):217-24.
10. Reimann C, Koller F, Frengstad B, Kashulina G, Niskavaara H, Englmaier P. Comparison of the element composition in several plant species and their substrate from a 1500000-km^2 area in Northern Europe. Sci Total Environ. 2001;278(1–3):87-112.
11. Skesters A, Kleiner D, Blázovics A, May Z, Kurucz D, Szentmihályi K. Mineral element content and antioxidant capacity of some Latvian berries. Eur Chem Bull. 2013;3(1):98-101.
12. Alvarez JM, Lopez-Valdivia LM, Novillo J, Obrador A, Rico MI. Comparison of EDTA and sequential extraction tests for phytoavailability prediction of manganese and zinc in agricultural alkaline soils. Geoderma. 2006;132(3-4):450-63.
13. Kubová J, Matúš P, Bujdoš M, Hagarová I, Medveď J. Utilization of optimized BCR three-step sequential and dilute HCl single extraction procedures for soil–plant metal transfer predictions in contaminated lands. Talanta. 2008;75(4):1110-22.
14. Zimmerman AJ, Weindorf DC. Heavy metal and trace metal analysis in soil by sequential extraction: a review of procedures. Int J Anal Chem. 2010;2010(3-4):387803.
15. Stefanović V, Trifković J, Mutić J, Tešić Ž. Metal accumulation capacity of parasol mushroom (*Macrolepiota procera*) from Rasina region (Serbia). Environ Sci Pollut Res. 2016;23(13):13178-90.

16. Davidson CM, Duncan AL, Littlejohn D, Ure AM, Garden LM. A critical evaluation of the three-stage BCR sequential extraction procedure to assess the potential mobility and toxicity of heavy metals in industrially-contaminated land. Anal Chim Acta. 1998;363(1):45-55.
17. Rauret G, López-Sánchez JF, Sahuquillo A, Barahona E, Lachica M, Ure AM, Davidson CM, Gomez A, Lück D, Bacon J, Yli-Halla M, Muntau H, Quevauviller P. Application of a modified BCR sequential extraction (three-step) procedure for the determination of extractable trace metal contents in a sewage sludge amended soil reference material (CRM 483), complemented by a three-year stability study of acetic acid and EDTA extractable metal content. J Environ Monit. 2000;2(3):228-33.
18. Mossop KF, Davidson CM. Comparison of original and modified BCR sequential extraction procedures for the fractionation of copper, iron, lead, manganese and zinc in soils and sediments. Anal Chim Acta. 2003;478(1):111-8.
19. Rosłon W, Osińska E, Ewelina Pióro-Jabrucka E, Grabowska A. Morphological and chemical variability of wild populations of bilberry (*Vaccinium myrtillus* L.). Polish J Environ Stud. 2011;20(1):237-43.
20. Szymczycha-Madeja A, Welna M, Pohl P. Elemental analysis of teas and their infusions by spectrometric methods. Trac-Trend Anal Chem. 2012;35:165-81.
21. European Economic Community (EEC). Commission Directive 2008/100/EC. Amending Council Directive 90/496/EEC on nutrition labelling for food stuffs as regards recommended daily allowances, energy conversion factors and definitions. Off J Eur Commun. 2008;L285:9.
22. Kabata-Pendias A. Trace Elements in soils and plants. 4th ed. Boca Raton: CRC Press; 2011. 520 p.
23. Sungur A, Soylak M, Ozcan H. Investigation of heavy metal mobility and availability by the BCR sequential extraction procedure: relationship between soil properties and heavy metals availability. Chem Speciat Bioavailab. 2014;26(4):219-30.
24. Rulebook on permissible quantities of dangerous and harmful substances in soil and water for irrigation and methods of their testing. Official Gazette of the Republic of Serbia. 1994;23.
25. Jain CK. Metal fractionation study on bed sediments of River Yamuna, India. Water Res. 2004;38(3):569-78.
26. Chizzola R. Metallic mineral elements and heavy metals in medicinal plants. Med Aromat Plant Biotechnol. 2012;6:39-53.
27. Ozgen S, Busse SJ, Palta PJ. Influence of root zone calcium on shoot tip necrosis and apical dominance of potato shoot: simulation of this disorder by ethylene glycol tetra acetic acid and prevention by strontium. Hortscience. 2011;46(10):1358-62.
28. Cataldo DA, Garland TR, Wildung RE. Nickel in plants: I. Uptake kinetics using intact soybean seedlings. Plant Physiol. 1978;62(4):563-5.
29. Li HF, Gray C, Mico C, Zhao FJ, McGrath SP. Phytotoxicity and bioavailability of cobalt to plants in a range of soils. Chemosphere. 2009;75(7):979-86.
30. Patel P, Patel NM, Patel PM. Who guidelines on quality control of herbal medicines. Int J Res Ayurveda Pharm. 2011;2(24):1148-54.
31. Donkora A, Kuranchiea C, Osei-Fosua P, Nyarkoa S, Doamekpora L. Assessment of essential minerals and toxic trace metals in popularly consumed tea products in Ghana, A Preliminary Study. Res J Chem Environ Sci. 2015;3(1):49-55.
32. Arika WM, Ogola PE, Nyamai DW, Mawia AM, Wambua FK, Kiboi NG, Wambani JR, Njagi SM, Rachuonyo HO, Emmah KO, Lagat RC, Muruthi CW, Abdirahman YA, Agyirifo DS, Ouko RO, Ngugi MP, Njagi ENM. Mineral elements content of selected Kenyan antidiabetic Medicinal Plants. Adv Tech Biol Med. 2015;4(1):1-5.
33. Khan AR, Awan FR. Metals in the pathogenesis of type 2 diabetes. J Diabetes Metab Disord. 2014;13(1):16.
34. European Food Safety Authority (EFSA). Dietary exposure to inorganic arsenic in the European population. EFSA J. 2014;12(3): 3597.
35. European Food Safety Authority (EFSA). Cadmium dietary exposure in the European population. EFSA J. 2012;10(1): 2551.
36. European Food Safety Authority (EFSA). Scientific opinion on lead in food. EFSA panel on contaminants in the food chain (CONTAM). EFSA J. 2010;8(4): 1570.

Supplementary Data

Supplementary data are available at: http://serbiosoc.org.rs/NewUploads/Uploads/Mutic%20et%20al_3485_Supplementary%20Data.pdf