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STUDY ON THE ELEMENTAL COMPOSITION OF *CLINOPODIUM VULGARE* L. – MEDICINAL PLANT, COLLECTED FROM DIFFERENT REGIONS OF BULGARIA

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Abstract

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The elemental composition of the medicinal plant *Clinopodium vulgare* L., collected from 15 regions of Bulgaria, was studied by ICP-MS and FAAS techniques and data for the concentrations of 19 elements in leaves, blossoms and stems of the plant are presented. Percentage of water extractable part of elements from their total content in plant organs are calculated as well, permitting assessment of the intakes for elements, when the plant is used as tea. Highest concentrations for most of the elements were measured in leaves, where the order of concentrations is Ca > K > Mg > Al > Fe > Na > Zn > Mn > B > Sr > Cu > Cr > Ni > Pb > Ce > La > Cd. Cadmium as a toxic ingredient has highest (up to 95% from leaves) extractability in water infusions which implies the necessity to control its concentration in the herb. The investigated plants were collected from industry free regions and the data can be used as mean background values for this plant in Bulgaria.

Key words: Clinopodium vulgare L., elemental composition, ICP-MS analysis, medicinal plant, water infusions

Introduction

Plants are natural resources of nutritional and biologically active ingredients, synthesized during their growth and accumulated from the environment. Despite of the use of many plants as everyday food, some of them, known as "medicinal plants" (MP), have been historically used in traditional medicine (TM) as therapeutic resources as well – either as home-made teas and remedies, as crude extracts or in varial pharmaceutical preparations (De Pasquale, 1984). Across the world, TM is either the mainstay of health care delivery or serves as a complement to it (WHO. Traditional Medicine strategy, 2014-2023). The long history of MP use results in abundant empirical information about their therapeutic effects and applications, and directs attention of scientists to investigate prospective representatives aiming controlled and more effective practical use.

After a period of neglecting the exploitation of medical plants in favor of synthetic drugs, nowadays attention to MP use reinforce due to their mild features, low side effects (Rates, 2001) and "environment-friendly" production. Based on the scientific arguments, supplementation of diet with various plants is recommended among individual consumers both for their healing properties and nutritive value (Ernst, 1998).

The interest in chemical composition of medicinal plants and their products is growing because of ongoing development in nutrition and in biochemical surveying and mineral prospecting (DeMan, 1999; Karak et al., 2010), concerning organic ingredients and elements composition.

Essential elements are necessary for growth, normal physiological functioning and maintaining of plants and human life, and their content in the plants and plants' products is important for composition of balanced diet. Some elements are co-factors of enzymes and, by activating them, influence in essential fashion the biochemical processes in living cells. Whereas some are macronutrients such as Ca, K, Mg and Na, others occur in trace or ultra trace quantities. Cu, Fe, Ni, Zn, and Mn are at the top end of this trace scale and play an important role in biological systems (WHO, 1996). Also, Cr, Co and Se are essential for normal development and function of human cells, but only in defined quantitative limits (Kosalec,

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2009; Basgel, 2006; WHO, 2007). Content of toxic elements like Cd, Pb, As, Hg etc, because of possible intake by food and/or plants' preparations, should be regulated (DeMan, 1999; Basgel et al., 2006; Fraga, 2005). Elemental content in plants is affected by many factors of the environment but also by the ability of concrete plant species to selectively accumulate and manage certain elements.

Chemical content of many plants and their preparations remain unidentified and unstudied. Such is the herb *Clinopodium vulgare* L., family *Labiatae,* which is the object of this study. It is widespread in different regions of the world and in Bulgaria as well (Kofidis et al., 2007). It has been used in TM, and is known with antibacterial (Opalchenova et al., 1999), antiinflamatory (Burk et al., 2009) and antitumor (Dzhambazov et al., 2002) activities of its preparations, which make it a prospective medicinal plant. It is known that major part of essential elements pass into water infusions but also Cd represents health risk for consumers being extractable up to 90% from leaves. *Clinopodium vulgare* total elements content is still not investigated in wider regional scale (Bardarov et al., 2014).

The aim of the present work is to give mean values for elemental composition of the plant collected from 15 regions in Bulgaria, their variation in plant organs - blossoms, stems and leaves and variability between these regions, as well as migration of elements to water infusion. Inductively coupled plasma mass spectrometry (ICP-MS) is selected as a tool for this study, which is known as an ultimately sensitive, fast multi-element analytical technique (Basgel et al., 2006; Lozak et al., 2002; Arceusz et al., 2010) giving up a large-scale picture of elements in the analysed object.

Materials and Methods

Reagents and chemicals

- Nitric acid, hydrogen peroxide and hydrofluoric acid with ICP-MS compatible purity (Fisher Scientific, Trace Metal Grade) were used as solvents and for mineralization;
- Milli-Q water (Milipore, Bedford, MA, USA) was used as solvent;
- A multielement standard stock solution containing 29 elements in 3%HNO_3, concentration of 10 mg/l (Ultra Scientific), was used for preparation of calibration solutions by appropriate dilution for water infusions analyses.
- One-point calibration using certified reference material (CRM) were performed for total element determination after plant samples microwave digestion procedure with the CRM passed trough the whole sample preparation in total plant elemental determinations.
- CRM "bush branches and leaves" certified reference material DC73349 (China National Analysis Center) was used for calibration;
- INCT-MPH-2 mixed polish herbs reference material (Institute of nuclear Chemistry and Technology, Warsaw-Poland) was used for accuracy check.

Instrumentation

A Perkin Elmer SCIEX Elan DRC-e quadrupole ICP-MS system with cross-flow nebulizer and autosampler was exploited for all measurements. The working parameters are presented in Table 1. The spectrometer is optimized to provide minimal values of the ratio CeO^+/Ce^+ and Ba^{2+}/Ba^+ and optimal intensities of the analyte ions. Measurements were conducted in two optimized modes – under "hot plasma" (higher energy of the RF generator) and "cold plasma" (lower plasma energy) with different nebulizer gas flow rates. Among the available isotopes of those elements that exist with more than one natural isotope, the most interference-free isotopes were used for analysis: ^{27}Al, ^{112}Cd, ^{59}Co, ^{53}Cr, ^{63}Cu , ^{208}Pb, ^{86}Sr, ^{67}Zn, ^{140}Ce, ^{139}La, ^{96}Mo, ^{62}Ni, ^{11}B, ^{26}Mg, ^{23}Na, ^{43}Ca, ^{40}K, ^{57}Fe, ^{55}Mn.

The elements Na, K and Mn were analysed by flame atomic absorption spectrometry (FAAS) using Perkin Elmer AAnalyst 400 equipment (Perkin Elmer, Norwalk, Connecticut, USA) and appropriate aqueous standard solutions for external calibration. Microwave digestion was carried-out using an Anton Paar, Perkin Elmer, Multiwave 3000 system equipped with 8 Teflon® digestion vessels. Working conditions are presented in Table 2.

Table 1
ICP-MS working paramanders

Paramander	Value
Argon plasma gas flow	15 l/min
Auxiliary gas flow	1.2 l/min
Nebulizer gas flow	0.9 l/min *(1.3 l/min)
Lens voltage	6.00 V
ICP RF power	1100 W *(600 W)
Pulse stage voltage	950 V
Integration time	2000 ms
Dwell time	50 ms
Acquisition mode	Peak hopping
Peak pattern	One point per mass at max. peak
Sample uptake rate	2 ml/min
Number of runs	6
Rinse time	180 s
Rinse solution	3% HNO_3

*Values used for "cold plasma" conditions

Plant samples

Aerial parts of batches of the plant *Clinopodium vulgare* L. samples were collected from 15 regions in Bulgaria (Figure 1 and Table 3) in July-August 2013, in the period of flowering. Plants were air-dried in clean, dark and airy room and stored in ventilated paper boxes before analysis. Plant organs (leaves, blossoms and stems) were gently separated and treated separately. Representative parts were randomly taken from these collections, each from more than 0.4 kg dried total mass. The representative samples were milled/homogenized in a grinder-mill before analysis. 0.5 g from each sample type were taken for microwave digestion and 1,5 g for water infusions preparation.

Sample preparation and analysis

500 mg aliquots of herbal material were accurately weighed into a Teflon digestion vessel. 8 ml concentrated HNO_3, 4ml H_2O_2 and 1ml HF were added. Digestion of samples was carried out at the conditions described in Table 2 with maximum power of 800 W. After cooling the samples were quantitatively transferred into clean Teflon vessels and the acids were evaporated on a sand bath allowing the volume to reduce to around 0.5 ml removing the HF. The samples were quantitatively transferred again in polypropylene tubes, diluted to 14 ml, capped and homogenized. With each set of digested samples, a blank sample and a CRM sample were passed through the entire procedure.

Plants' infusions were prepared by adding 150 ml hot (95^{o}C) Milli-Q water to 1.5 g plant material (air dried leaves, stems and blossoms). The suspensions ware stirred with plastic stirrer for 10 min and then filtered through blue ribbon filter paper. The infusions obtained were left to cool at room temperature and analyzed immediately.

Table 2
Microwave digestion program_

Step	Time, min	Microwave power, W
1	5	450
2	5	800
3	5	450
4	5	300
5	20	Vent and cooling

Fig. 1. Map of Bulgaria with sampling sites

Table 3
Description of collection sites

Sample Number	Region of collection	Sample Number	Region of collection
1	German village, Region in Sofia field, in the foot of Lozen mointain collected 2013	8	Laki town, Region of Rodopi mountain
2	German village, Region in Sofia field, in the foot of Lozen mointain, collected 2012	9	Drianovo village, Region of Rodopi mountain
3	Mirkovo village, Region in the foot of Central-East Balkan mountain	10	Balgarski izvor village 15m aside from road with intensive trafic
4	Near Ognianovo lake, flat country region in Sofia Field	11	Makocevo village Sredna gora mountain, clean region
5	Dragalevci, Region in Vitosha mountain, north side, near Sofia, clean region	12	Kupen village Central Balkan mountain, clean environment
6	Near Goliama Rakovica village, flat country region in Sofia Field	13	Balgarski izvor village At the road banket of road with intensive traffic
7	Lukovo village, Region of Balkan mountain, near Sofia	14	On the road from Laki to Drianovo, Region of Rodopi mountain, clean region
		15	Belica village Rodopi mountain, clean region

Table 4
Accuracy and precision of the mandhod

Element anlaysed	Accuracy, %	Precision RSD, %
Al	20	10
Cd	1.5	4,2
Co	1.5	3.9
Cr	1.9	5.2
Cu	6.3	5.0
Pb	1	5.5
Sr	0.6	1.3
Zn	2.8	5.4
Ce	12	9.4
La	18	2.3
Mo	13	3.4
Ni	5	1.6
B	19	15
*Mg, %	2	0.9
*Na (FAAS)	2,5	5
*Ca, %	23	2.8
*K, % (FAAS)	2,8	6
*Fe	14	1.6
*Mn (FAAS)	1	4

*Elements analyzed under "cold plasma conditions" or AAS

Data quality

Acuracy and precision of the results was checked analyzing the certified reference material INCT-MPH-2 with matrix close to that of the samples. Accuracy and precision of overall analytical procedure are given in Table 4.

Total precision of the method expressed as relative standard deviations (RSD) for microwave digested solid plant samples was ≤ 15%, much less than elemental variations in different plant samples, and the acuracy checked with the SRM varied between 1 and 23% for the different analysed elements.

Results

Elemental concentrations in the separated Clinopodium vulgare L. organs

The elemental concentrations in the plant organs, presented by their maximal, minimal and mean values, are described in Table 5.

Figure 2 presents the variations in the elemental concentrations in the organs of the plants, collected from different regions.

Elemental composition of Clinopodium vulgare in dependence of its regional origin

The values for leaves elemental composition of the plants, collected from different regions, are presented separately in Table 6.

Table 5
Mean, minimal and maximal element concentrations in air dried *Clinopodium vulgare* organs

Element measured	Average concentration (minimal÷maximal value)		
	Stems, µg/g	Blossoms, µg/g	Leaves, µg/g
Cd	0.05 (0.008÷0.235)	0.03 (0.060÷0.153)	0.04 (0.010÷0.251)
Co	0.08 (0.04÷0.13)	0.13 (0.04÷0.20)	0.24 (0.10÷0.37)
Cr	4.87 (1.27÷7.39)	2.38 (0.58÷5.00)	7.10 (0.43÷16.13)
Cu	5.47 (4.31÷7.97)	9.59 (4.49÷12.63)	7.47 (4.50÷10.92)
Mn	20.3 (11.4÷40.0)	33.4 (18.9÷60.0)	60.2 (35.5÷100.0)
Pb	0.97 (0.15÷2.08)	1.31 (0.28÷4.72)	1.78 (0.39÷5.73)
Sr	24.6 (24.0÷25.0)	24.6 (23.7÷25.0)	24.4 (23.6÷24.9)
Zn	41.0 (29.7÷84.5)	39.4 (7.5÷50.8)	60.5 (37.6÷100.3)
Ce	0.35 (0.07÷0.70)	0.36 (0.07÷0.77)	0.85 (0.08÷1.70)
La	0.24 (0.06÷0.54)	0.22 (0.04÷0.40)	0.56 (0.06÷1.31)
Mo	0.43 (0. 07÷1.01)	0.75 (0.12÷0.12)	1.19 (0.25÷0.25)
Ni	1.85 (0.54÷3.71)	2.64 (1.09÷1.09)	2.43 (1.01÷3.49)
B	24.08 (19.7÷27.3)	30.7 (20.8÷38.4)	41.7 (29.1÷53.5)
Mg	3472 (2415÷5705)	6372 (4415÷7455)	7004 (4343÷11745)
Na	94 (71÷192)	101 (20÷176)	202 (139÷307)
Ca	10044 (6512÷15204)	12877 (6135÷19537)	23387 (19012÷35193)
K	14717 (13642÷15859)	11111 (7123÷14450)	13698 (12230÷17741)
Fe	549 (376÷890)	190 (81÷336)	413 (126÷604)
Al	333 (92÷1071)	257 (113÷598)	480 (152÷1042)

Table 6
Elemental composition of air dried leaves of *Clinopodium vulgare* L., collected from 15 regions of Bulgaria

Element measured	Region of collection (see Table 3 and Fig. 1)															
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	SD, %
Cd, mg/Kg	0.006	0.011	0.010	0.014	0.012	0.045	0.021	0.029	0.011	0.251	0.017	0.041	0.231	0.038	0.034	160
Co, mg/Kg	0.11	0.10	0.26	0.14	0.19	0.19	0.19	0.19	0.37	0.30	0.34	0.21	0.32	0.29	0.40	38
Cr, mg/Kg	0.43	1.10	6.19	3.22	4.34	7.19	7.82	5.46	12.09	16.13	5.56	5.50	16.53	7.98	14.73	67
Cu, mg/Kg	9.80	8.55	8.09	7.67	6.87	5.50	6.14	6.70	5.63	10.92	6.82	6.65	11.12	4.50	9.91	27
Mn, mg/Kg	62	40	18	62	42	109	106	100	31	35	79	29	45	47	57	51
Pb, mg/Kg	0.4	0.6	1.0	0.9	1.1	3.6	1.8	1.6	2.1	2.4	1.7	5.7	2.9	1.3	4.6	71
Sr, mg/Kg	24.1	24.7	24.6	24.6	23.6	24.7	24.4	24.7	24.6	24.9	23.1	24.5	24.9	24.8	24.7	2
Zn, mg/Kg	42.5	44.0	60.2	37.6	55.7	77.8	60.7	93.2	64.6	11.5	74.6	76.9	11.8	100.3	64.0	45
Ce, mg/Kg	0.08	0.33	0.87	0.32	0.65	0.69	0.69	1.06	1.70	1.24	0.67	0.66	1.14	1.37	1.40	52
La, mg/Kg	0.06	0.28	0.56	0.16	0.40	0.48	0.44	0.84	0.99	0.71	0.40	0.39	0.71	1.31	0.69	57
Mo, mg/Kg	0.6	0.2	0.5	2.2	0.7	1.6	1.5	0.3	1.2	1.5	3.9	2.9	1.5	0.3	0.8	79
Ni, mg/Kg	3.5	3.5	2.2	1.0	1.2	1.1	1.4	4.5	3.0	2.3	2.2	1.9	2.2	2.9	2.8	42
B, mg/Kg	33.2	30.0	44.9	46.5	29.1	41.3	46.1	48.0	53.5	38.9	34.1	52.5	36.9	45.0	51.4	19
Mg, g/Kg	6.44	4.34	5.49	7.04	8.12	5.43	7.75	6.05	4.52	7.63	8.08	7.85	7.63	8.43	11.74	26
Na, mg/Kg	201	185	139	205	196	189	198	268	270	301	275	283	307	300	185	23
Ca, g/Kg	19.0	19.7	23.2	19.2	27.1	21.1	19.0	26.3	26.2	35.2	21.4	20.3	17.4	22.0	24.7	20
K, g/Kg	12.2	11.0	12.8	13.5	17.7	15.5	10.0	12.2	13.0	12.0	14.8	15.0	11.8	13.0	20.0	19
Fe, g/Kg	0.44	0.20	0.32	0.38	0.58	0.30	0.90	0.34	0.60	0.13	0.42	0.50	0.51	0.50	0.27	44
Al, g/Kg	0.76	0.15	0.57	0.39	0.53	0.44	1.04	0.61	0.25	0.23	0.45	0.25	0.68	0.37	0.45	48

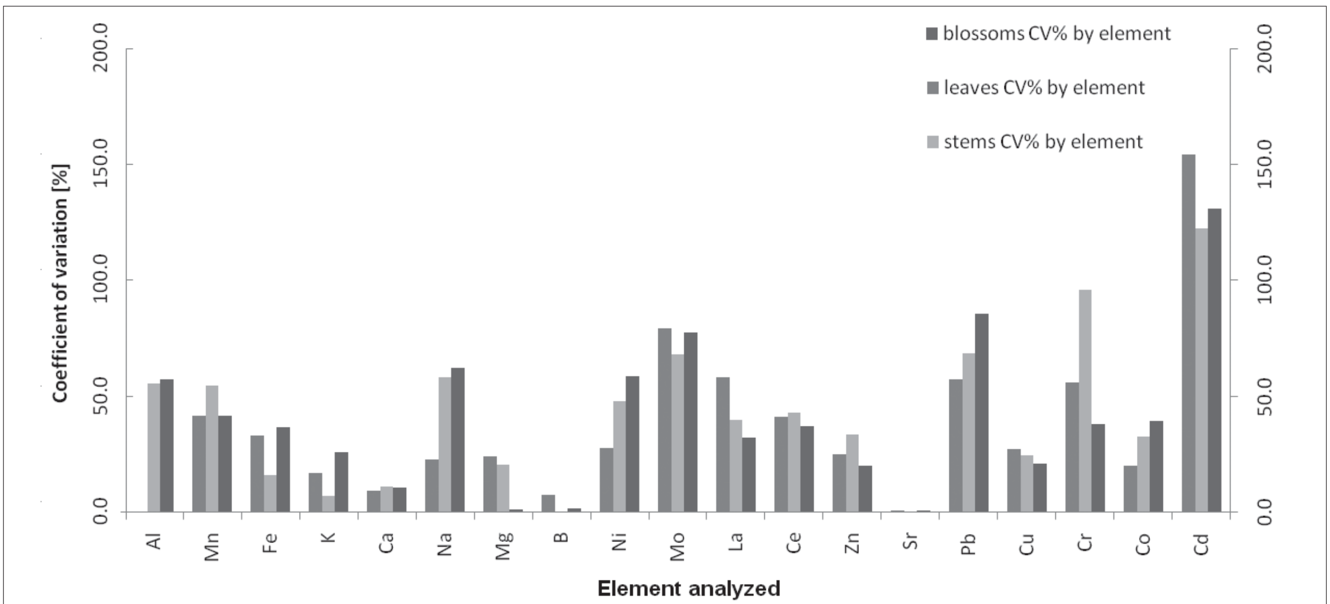

Fig. 2. Variation of elements concentrations in plant organs among regions

Extraction of the elements in herbal infusions and Intake of the elements

The percentage of elements from plant's organs migrating to water extract was calculated and the data are presented in Table 7.

Discussion

Elemental concentrations in the separated Clinopodium vulgare L. organs

Highest concentration of K, Ca and Mg, followed by Fe, Al, Na, Zn, Mn, Sr, B, Cu, Cr, Ni, Pb in descending order can be observed, and Mo, Ce, La, Co and Cd in traces.

While for almost all elements highest concentrations were observed in leaves, Cu and Ni show highest concentrations in blossoms and Cd has highest concentration in the stems.

The content of the elements in the total areal part of the plant can be calculated using the approximate weight proportion of stems, leaves and blossoms in the dried plant which we determined as 45:25:30.

Variation between regions is much higher than total bias and RSD of the method (Table 3). Most variable elements were Cd, varying within 130-150% between regions, followed by Cr and Pb (70-100%). These elements are usually connected with environment pollution and variation between regions is expected, even though in our case plants were collected from sites with no industrial activity. Mo, Mn, Na and Al are characterized with coefficients of variation above 50%. Highest variation was observed for Na and Al in stems and blossoms.

Table 7
Migration of elements from the plant organs into water infusions

Element	Element amount in the infusion, %		
	Stems	Blossoms	Leaves
Cd	36	36	95
Co	26	70	72
Cr	5	21	34
Cu	40	34	30
Mn	14	33	39
Pb	13	15	25
Sr	5	14	20
Zn	4	9	10
Ce	15	32	30
La	40	40	32
Mo	40	40	32
Ni	60	52	50
B	18	40	17
Mg	30	40	60
Na	40	90	13
Ca	50	25	42
K	40	77	95
Fe	10	50	60

No literature data were found for the elemental concentrations in *Clinopodium vulgare*. For this reason, comparison was done with similar herbs as those in *Lamiceae* family.

Cindric et al. (2013) do not find any Cd, Co and Cr in the aerial parts of similar plants (*Salvia officinalis* L. and *Teucrium montanum* L.). The present mean values for cadmium are 0.05, 0.03 and 0.04 µg/g in stems, blossoms and leaves respectively; and for Co - 0.08 µg/g in stems, 0.13 µg/g in blossoms and 0.24 µg/g in leaves. These concentrations are probably below LOD of ICP-OES technique that has been used by the authors. For chromium the concentrations determined are 4,9 µg/g (stems), 2.4 µg/g (blossoms) and 7.1 µg/g (leaves) in the different parts of the plant.

Similar concentrations to ours for the major elements are reported by Arceusz (Arceusz et al., 2010) when analysed plant samples belonging to *Lamiaceae* family. Nevertheless, concentrations of the elements in plants depend on environmental conditions, soil, genotype, stage of growth, geochemical and other factors (Aminzadeh et al., 2010), and most importantly - on the plant species - so by such comparison only similarity for plants belonging to one family could be expected.

Despite the lower concentrations for Pb and Cd in *Clinopodium vulgare* than the WHO tolerances - 0.3 mg/kg for Cd, 10 mg/kg for Pb (WHO, 1996), it should be stressed that concentration values for Cd (0.008-0.250 µg/g) and for Pb (0.15-5.7 µg/g) are measured for plants, collected from environmentally not poluted sites. That might be a concern, since in the course of preparation of any products from this herb, these two toxic elements could be engulfed concentratad. That is why the content of Cd and Pb in the herb, intended for consumption, should be controlled. Similar results and tendencies for *Lamiceae* family medicinal plant species were reported in Poland (Lozak et al., 2002), Egypt (Abou-Arab et al., 1999), Argentina (Gomez, 2006), United States (Khan et al., 2001) and Brasil (Caldas and Machado, 2004).

Good correspondance with data in (Basgel et al., 2006; Cindric, 2013) was observed for minor components Al (around 250 µg/g) and Mn (20-60 µg/g). All other elements concentrations were higher, except that for Co and Cr, which are in lower concentrations in *Clinopodium vulgare* L. according our results.

Elemental composition of Clinopodium vulgare in dependence of its regional origin

Because leaves could be perceived as the most "environmentaly sensitive" organ of the usable plant part, the values for elements concentrations and their RSDs could represent regional-dependance for the plant samples. The higher measured values are highlighted.

No drastic differences can be observed among the values for different regions. The highest RSD show values for Cd and the highest values are measured in the plans from region 10 and 13; they are collected near to a main road with intensive traffic. The high variation of the concentrations of this toxic element confirms the necessity of its control.

The next element showing relatively high RSD is Mo. Its highest value is measured in plants from region 11 (Sredna gora mountain). The values for Pb show relatively high RSD, as well; its highest values are measured in the plants from regions 12 (Balkan mountain) and 15 (Rodopi mountain).

Among the sides of samples collection, there are no places, known as highly polluted. That fact permits to classify the results presented for elemental composition of *Clinopodium vulgare* as background values for the plants of Bulgarian origin.

Extraction of the elements in herbal infusions and Intake of the elements

Traditional use of *Clinopodium vulgare* is as tea. An intake of elements from the plant usually comes as extracted from the plant in the tea preparation procedure. The quantitative assessment of such intake of chemical elements is important to be done. Aiming this, water infusions were prepared as described in 2.4 and analyzed.

Different elements pass to the water extracts with extraction efficiencies from 5 to 95% of the total concentration in the air dried plant material, depending on the element and the plant organ extracted.

It is important to note the high extraction efficiency of Cd, especially from leaves (95%), most of which in low molecular cationic and bio accessible chemical form (Bardarov et al., 2014). The Pb extractability is significant as well (13 ÷ 25%), but most of it – bound to big molecules with no electric charge (Bardarov et al., 2014). This should be taken in account and the Cd and Pb content in the herb intended for human use should be controlled.

Relatively high extractabilities are observed for K (40-95%), Mg (30-60%), Ca (25-50%), Ni (50-60%), Mo (30-40%), La (30-40%) and Co (26-70%) as well. Mn (14-39%), Zn (4-10%) and Cu (30-40%) are represented with lower extraction efficiencies but still enough to ensure a balanced essential mineral uptake for the consumers of the herbal infusion.

Conclusion

The elemental composition of the plant *Clinopolium vulgare* has been studied by ICP/MS and AAS techniques. Such data are published for the first time for this plant, which seems to be a pharmaceutical plant candidate based on its bioactive effects. The elemental composition is determined in its total aerial part, and separately in its organs (stems, leaves and blossoms) as well. 19 elements are quantitatively measured in the plant organs and their water infusions. Mean values are given for elemental composition of the plant (plant organs) collected from non polluted environmental sites in Bulgaria, together with elemental variations, aiming to give background values for *Clinopolium vulgare* elemental composition. Data for the elemental compositions of the plant's leaves – as the most "environment-dependant" plant organ – are presented for the plants collections from 15 regions in Bulgaria.

The elements extracted in water infusions were measured and the extracteble part of the elements were presented. For most essential elements, the composition is well balanced in terms of a good mineral nutritive impact, when this plant is in consumption. Attention should be adverted however on the toxic elements Cd and Pb: their concentrations in the herbal extracts, collected from some of the regions, are considerable and claim to be bioaccessible, because significant portion of the total elemental content pass into water infusions. These facts show the necessity of control of these elements in the plant, and especially on the environmental and agricultural conditions when *Clinopodium vulgare* is cultivated.

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