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Accumulation of microelements Cd, Cu, Fe, Mn, Pb, Zn in walnuts (*Juglans regia* L.) depending on the cultivar and the harvesting year

Sv. Momchilova[1,*], S. Arpadjan[2], E. Blagoeva[3]

[1]*Department of Lipid Chemistry, Institute of Organic Chemistry with Centre of Phytochemistry, Bulgarian Academy of Sciences, Acad. G. Bonchev Str., bl. 9, 1113 Sofia, Bulgaria*
[2]*Department of Analytical Chemistry, Faculty of Chemistry and Pharmacy, University of Sofia, J. Bourchier Blvd. 1, 1164 Sofia, Bulgaria*
[3]*Agricultural Experiment Station, 1 Minjorska Str., 6600 Kardzhali, Bulgaria*

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Microelements Cd, Cu, Fe, Mn, Pb and Zn were determined by flame and electrothermal AAS in six walnut cultivars during four consecutive years. Analysis of variance showed that accumulation of Cu, Fe, Mn and Zn depended on the cultivar whereas the harvesting year of samples influenced significantly none of the investigated six microelements. The contents of Cd, Cu, Fe, Mn and Zn were two (Cd) to six (Zn) fold higher in kernels than in shells while Pb content was higher in shells than in kernels. In addition, a correlation study was performed on Cd and Pb contents in kernels and in the corresponding shells, involucres, leaves and soil extracts. Strong positive correlation was observed between i) Cd content in kernels and in soil fractions; ii) Pb content in kernels and in corresponding shells, involucres and leaves, indicating the soil as the main source for Cd accumulation and the air pollution – for Pb.

Keywords: bioaccumulation, contamination sources, cultivar, harvesting year, microelements, walnuts.

INTRODUCTION

Walnuts (*Juglans regia* L.) are recognized as a healthy food [1] and are consumed as pure nuts or as ingredients in various food products [2]. Regular consumption of their kernels improves nutrient intake in humans. Whereas most nuts contain mainly monounsaturated fatty acids, walnuts are rich in essential polyunsaturated fatty acids as linoleic (18:2, omega-6) and linolenic (18:3, omega-3) acids at levels of above 500 g/kg and 130 g/kg, respectively [3]. Also, walnuts contain many other bioactive compounds as proteins, minerals, fibers, tocopherols, phytochemicals [4–8]. The unique chemical composition, proved health benefits and suitable climatic conditions in Bulgaria leads to increased interest in harvesting new walnut trees with the help of EU subsidy projects on the agriculture area. Until now, systematic investigations on the content of essential and toxic elements in Bulgarian walnuts are missing. Although many walnut trees in the country grow on both sides of roads or in industrially contaminated areas, the influence of traffic or industrial pollution on the accumulation of microelements in walnuts has not been studied yet. In order to estimate the effects of environmental pollution, as well as to make the right choice in organizing new walnut orchards, it is important to investigate whether the walnut cultivar has an influence on the elements accumulation extent. In the present paper we studied the accumulation of essential (Cu, Fe, Mn, Zn) and toxic (Cd, Pb) elements in six walnut cultivars grown in antropogenically polluted area in Bulgaria during four consecutive harvests (2008–2011). The toxic elements Cd and Pb were also measured in the walnut shells, involucres, leaves and soils in order to elucidate the sources of contamination.

EXPERIMENTAL

Samples and reagents

Six walnut (*Juglans regia* L.) cultivars were studied, namely Adams, Hartley, Izvor 10, Pedro, Sheynovo and Tehama. They were grown in the orchard of the Agricultural Experiment Station – Kardzhali, South-Eastern Bulgaria, near a lead/zinc smelter. The walnut trees were cultivated without any irrigation and fertilization. From 2008 to 2011, samples were taken from the same cultivar trees. The total number of nut samples for analysis was 24 (6 cultivars × 4 years). The nuts with the corresponding involucres and leaves were picked up randomly from under the trees during the first half of October. The samples were air dried and the

* To whom all correspondence should be sent:
E-mail: svetlana@orgchm.bas.bg

nuts were unshelled before analysis. Shells, involucres and leaves were analysed without preliminary washing. Soil samples (0–30 cm) were taken under the studied trees according to ISO 10381-2002. All reagents used were of analytical-reagent grade (p.a. Merck, Darmstadt, Germany). Milli-Q water (Millipore, Bedford, MA, USA) was used throughout.

Chemical analysis

The microelements Cu, Fe, Mn and Zn were determined by flame atomic absorption spectrometry (flame AAS) on a Perkin Elmer AAnalyst 400 equipment (Perkin Elmer, Norwalk, Connecticut, USA) using appropriate aqueous standard solutions for external calibration. Electrothermal AAS (Zeeman Perkin Elmer 3030/HGA-600) was applied for determination of cadmium and lead using standard addition calibration mode. Kernels, shells, involucres and leaves were digested with 65% HNO_3 and 30% H_2O_2 (modified USGS Method B-9001-95) [9]. With each run of these samples two certified reference materials CRM NCS ZC73011 soy bean and INCT-MPH-2 Mixed Polish Herbs (LGC Standards, Łomianki, Poland) were analyzed in parallel in order to check the accuracy of the entire analytical procedure. The obtained mean values for the analytes were not significantly different (p>0.05) from their certified values. Accuracy of the data for element content in nut kernels was also checked by analysis of spiked walnut (cv. Adams, Pedro) samples. The difference between the obtained and spiked values was not significant (p>0.05). The soil samples were digested with *aqua regia* and HF (ISO 13656) for determination of the total content [10]. The accuracy of determination of total Cd and Pb in soils was checked measuring Soil CRM NCS DC 73386 and the differences between the experimental and certified values were not significant (p>0.05). The bias calculated from the means of certified and measured values was 0.8% for Cd and -0.3% for Pb. EDTA and acetic acid soil extracts were prepared according to a harmonized BCR protocol [11]. The quality control of the procedure for evaluation of soil EDTA and acetic acid extractable Cd and Pb was performed using certified reference material BCR–700. Recoveries from the analysis of BCR–700 were in the range of 95–101% for Cd and in the range of 93–104% for Pb, which was considered as satisfactory [12].

Statistical analysis

For each sample, three independent analytical portions were weighed and analyzed together with corresponding procedural blanks. The statistical data calculations were performed using the Microsoft Excel (Microsoft, Redmond, Washington, USA) and STATISTICA 7.0 (StatSoft, Tulsa, Oklahoma, USA) software packages.

RESULTS AND DISCUSSION

The results for microelements content, corresponding to 24 walnut samples, are summarized in Table 1 (different cultivars) and Table 2 (different harvesting years). The statistical analysis of data was performed by analysis of variance as the cultivar and the harvesting year of samples were the variables. Table 1 shows the mean content of analytes for four harvesting years (2008–2011) depending on the walnut cultivar, the standard deviation between varieties (SDv), the standard deviation (SD) of triplicate analysis of all 24 samples (N=72) and the calculated Fisher's F-test ($F_{calc}=SDv^2/SD^2$) values. It can be seen that in relation to the cultivar, significant differences ($F_{calc}>F$) were registered for Cu, Fe, Mn and Zn whereas cadmium and lead had F_{calc} (1.93 and 1.96, respectively) lower than F (2.40) at p=0.05 significance level. Influence of variety on the microelements content was reported also for walnuts from Turkey [13,14] and Romania [15,16].

Table 2 shows the mean values for the six walnut cultivars obtained during four consecutive years. In relation to the harvesting year, no significant differences were found for the contents of Cd, Cu, Fe, Mn, Pb and Zn, i.e. the accumulation of these elements had not been affected by climatic conditions as rainfall amounts, wind direction, etc. It could be assumed that the uptake of these six elements is only from the soil and proceeds through mechanisms that are independent of the soil moisture content. To the best of our knowledge no data have been published yet concerning the influence of harvesting year on the accumulation of microelements in walnuts.

Furthermore, the correlation between the contents of cadmium and lead in kernels, shells, involucres, leaves, and soils as total (S-total), EDTA (S-EDTA) and acetic acid (S-acetic) extractable forms was studied.

Table 1. Content of microelements in walnuts in dependence on the cultivar (mean values for four harvesting years); F (P=0.95; F_1=5; F_2=48) = 2.40.

Element	Adams	Hartley	Izvor 10	Pedro	Shey novo	Teha ma	Mean of the six cultivars	SDv^{*}	SD^{**}	F_{calc}^{***}
Cd (µg/kg)	29.8	29.7	23.5	28.5	25.5	26.5	27.3	2.5	1.8	1.93
Cu (mg/kg)	15.7	17.2	15.7	16.7	15.7	16.3	16.2	0.6	0.3	4.00
Fe (mg/kg)	24.8	28.2	24.2	29.2	31.8	32.8	28.5	3.5	2.0	3.06
Mn (mg/kg)	39.5	42.5	35.2	32.0	37.0	35.2	36.9	3.7	2.0	3.42
Pb (µg/kg)	95.0	120	115	102	116	95.0	107	11.2	8.0	1.96
Zn (mg/kg)	26.8	28.5	27.5	27.8	28.0	34.2	28.8	2.7	1.0	7.29

*SDv: standard deviation between the varieties

**SD: standard deviation of triplicate measurements of six samples during four consecutive harvests (N=72; n=24)

***$F_{calc} = SDv^2/SD^2$

Table 2. Content of microelements in walnuts in dependence on the harvesting year (mean values for six walnut cultivars); F (P=0.95; F_1=3; F_2=48) = 2.80.

Element	2008	2009	2010	2011	Mean of the four years	SD_Y^{*}	F_{calc}^{**}
Cd (µg/kg)	29.7	27.7	25.3	25.8	27.1	2.0	1.23
Cu (mg/kg)	16.3	16.1	16.3	16.2	16.2	0.1	0.11
Fe (mg/kg)	26.0	27.0	28.0	29.0	27.5	1.3	0.42
Mn (mg/kg)	34.0	37.0	40.0	36.5	36.9	2.5	1.56
Pb (µg/kg)	98.0	114.	99.0	110.	105.	8.0	1.00
Zn (mg/kg)	29.0	28.0	29.0	29.0	28.8	0.5	0.25

*SD_Y – standard deviation between harvest years

**$F_{calc} = SD_Y^2/SD^2$; SD – see Table 1

Table 3. Correlation between the content of Cd in walnut kernels, in corresponding shells, involucres, leaves, in soils as total (S-total), EDTA (S-EDTA) and acetic acid (S-acetic) extractable cadmium.

		Shells	Involucres	Leaves	S-total	S-EDTA	S-acetic
Kernels	*r*	0.24	0.15	0.21	**0.82**	**0.62**	**0.60**
	p	NS*	NS	NS	**0.0002**	**0.001**	**0.002**
Shells	*r*		0.04	0.76	0.34	0.19	-0.01
	p		NS	0.0001	NS	NS	NS
Involucres	*r*			0.45	0.13	0.21	0.14
	p			0.027	NS	NS	NS
Leaves	*r*				0.19	-0.04	0.25
	p				NS	NS	NS
S-total	*r*					0.80	0.58
	p					0.0001	0.003
S-EDTA	*r*						0.51
	p						0.011

*NS – not significant

Table 4. Correlation between the content of Pb in walnut kernels, in corresponding shells, involucres, leaves, in soil as total (S-total), EDTA (S-EDTA) and acetic acid (S-acetic) extractable lead.

		Shells	Involucres	Leaves	S-total	S-EDTA	S-acetic
Kernels	*r*	**0.97**	**0.68**	**0.52**	0.23	0.21	0.20
	p	**< 0.001**	**< 0.001**	**0.01**	NS*	NS	NS
Shells	*r*		0.71	0.53	0.22	0.21	0.20
	p		< 0.001	0.008	NS	NS	NS
Involucres	*r*			0.66	0.29	0.29	0.31
	p			< 0.001	NS	NS	NS
Leaves	*r*				-0.11	-0.11	-0.11
	p				NS	NS	NS
S-total	*r*					0.96	0.98
	p					< 0.001	< 0.001
S-EDTA	*r*						0.98
	p						<0.001

* NS – not significant

The results for the correlation coefficient (*r*) and significance level (*p*) are shown in Table 3 for cadmium and in Table 4 for lead. According to the results (Table 3) Cd in kernels correlated positively only with the Cd content in all soil fractions: total soil Cd (r=0.82; p=0.0002); EDTA extractable Cd (r=0.62; p=0.001) and acetic acid extractable Cd (r=0.60; p=0.002). This confirms the assumption made from the analysis of variance that walnuts accumulate mainly soil Cd.

Cadmium in the leaves and involucres resulted from air pollution and had no influence on the element content in kernels. It could be concluded that cadmium content in walnuts mainly depends on the soil pollution. The measured contents of cadmium (mean for six walnut cultivars in four consecutive years) were 1.7, 0.7 and 0.03 mg/kg, respectively in the total soil, EDTA- and acetic acid-extractable soil fractions, which were below the maximal allowed content (2 mg/kg Cd in soil) according to the Bulgarian legislation [17]. The lead contents in the investigated soil fractions (97.6, 46.7 and 0.17 mg/kg, respectively in the total soil, EDTA- and acetic acid-extractable fractions) were also lower than the maximal allowed content (100 mg/kg Pb in soil [17]). However, unlike cadmium, lead showed opposite correlation behaviour (Table 4), as no significant correlation was registered between Pb content in kernels and in soil fractions. This is probably because walnut tree roots act as a barrier for translocation of soil lead to the kernels. The strong positive correlation between Pb in kernels and in corresponding shells (r=0.97; p<0.001), involucres (r=0.68; p<0.001) and leaves (r=0.52; p=0.01) shows that some lead accumulation could proceed through polluted environment during kernels ripening.

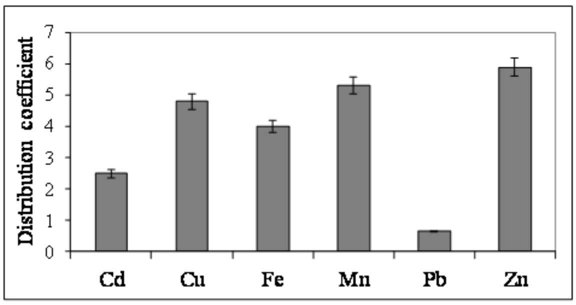

Fig. 1. Distribution of microelements between kernels and corresponding shells as mean values for six walnut cultivars and for four harvesting years.

Figure 1 presents the distribution coefficients of all six studied elements, calculated as a ratio between elements content in kernels and that in the corresponding shells. As can be seen, only lead has a distribution coefficient below 1, i.e. its content in shells is higher than in kernels. Thus, in order to prevent high levels of lead in walnut kernels, special care must be taken to avoid air pollution with lead containing aerosol particles.

CONCLUSIONS

The accumulation of Cu, Fe, Mn and Zn in walnuts is dependent on the cultivar. The harvesting year and the herewith connected particular climatic conditions do not significantly influence the microelements contents. Lead content is higher in walnut shells than in corresponding kernels. The contents of Cd, Cu, Fe, Mn and Zn are two (Cd) to six (Zn) fold higher in kernels in comparison to shells. Correlation analysis showed that the main source for Cd accumulation was soil cadmium, as long as for lead it could be also the polluted environment.

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НАТРУПВАНЕ НА МИКРОЕЛЕМЕНТИ (Cd, Cu, Fe, Mn, Pb, Zn) В ОРЕХИ (*Juglans regia* L.) В ЗАВИСИМОСТ ОТ СОРТА И ГОДИНАТА НА ОТГЛЕЖДАНЕ

Св. Момчилова[1,*], С. Арпаджян[2], Е. Благоева[3]

[1]*Институт по органична химия с Център по фитохимия, Българска Академия на Науките, ул. „Акад. Георги Бончев“, бл. 9, 1113 София, България*
[2]*Факултет по химия и фармация, СУ „Св. Кл. Охридски“, бул. „Джеймс Баучер“ 1, 1164 София, България*
[3]*Опитна Станция по Земеделие, ул. „Миньорска“ 1, 6600 Кърджали, България*

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(Резюме)

Микроелементите кадмий, мед, желязо, манган, олово и цинк бяха определени чрез пламъкова и електротермична атомно-абсорбционна спектрометрия в шест сорта орехи през четири последователни години. Анализът на вариациите показа, че натрупването на мед, желязо, манган и цинк зависи от сорта, докато годината на отглеждане на орехите не влияе значимо върху никой от изследваните шест микроелемента. Съдържанието на кадмий, мед, желязо, манган и цинк беше от два (при кадмия) до шест (при цинка) пъти по-високо в ядките, в сравнение с черупките, докато съдържанието на олово беше по-високо в черупките, отколкото в ядките. Също така беше проведено корелационно изследване на количествата кадмий и олово в ядките и в съответните им черупки, мезокарп, листа и почвени екстракти. Резултатите разкриха силна положителна корелация между: а) съдържанието на кадмий в ядките и в почвените фракции; б) съдържанието на олово в ядките и в съответните им черупки, мезокарп и листа, което показва, че основен източник за натрупване на токсични елементи в ядките е почвата – за кадмий и замърсяването във въздуха – за олово.