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The effect of soil properties on cadmium bonds to organic substances of spinach biomass†

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The main objective of this study was focused on sequential fractionation of spinach biomass, partial characterization of cadmium-binding compounds and determination of the cadmium concentration in these fractions. Spinach was cultivated after application of sewage sludge on two soils of different properties and total cadmium content in a model pot experiment. Two non-polar solvents (light petroleum and ethyl acetate), a polar solvent (methanol) and two solvent mixtures (methanol + H_2O & H_2O and methanol + H_2O + HCl) were used in the extraction series. Isolated fractions were characterized by IR spectroscopy. The lower weight of light petroleum (aliphatic hydrocarbons), ethyl acetate (fatty acids), and methanol (carboxylic acids, mainly acids of citric cycle, pyrrole substances) fractions in sludge treatments compared with the controls showed that the metabolism of plant natural products was inhibited by toxins from sludge. The weight of methanol fraction was dominant for all treatments. The cadmium determined in these fractions was low. The majority of cadmium was found in the methanol + H_2O & H_2O (oligopeptides, mainly phytochelatins, extractable polypeptides, proteins) and methanol + H_2O + HCl (compounds isolated from cell walls and cytoskeleton after hydrolysis) fractions. The effect of toxin stress only appears to be on the quantity of substances, as there is no qualitative change observed in the isolated fractions (measured values of IR spectra bands did not differ for treatments tested). The application of sequential analysis for spinach biomass extraction makes it possible to investigate the complex effects of cadmium and other toxins on plant metabolism.

KEYWORDS: cadmium; cadmium-binding compounds; sequential analysis; spinach biomass

Cadmium is a widespread heavy metal released into the environment by thermal power and heating plants, metals industries, urban traffic, sewage sludge and phosphate fertilizers. High concentrations of cadmium in soil solution can be toxic for plants. Cadmium can alter the synthesis of ribonucleic acid (RNA), inhibit ribonuclease activity of plant,[1] and also reduce the absorption of nitrate and its transport from roots to shoots by inhibiting the nitrate reductase activity in the shoots.[2] The inhibition of root iron(III) reductase induced by cadmium led to iron(II) deficiency, and this affects photosynthesis.[3] Cadmium interacts with the water balance[4] and damages the photosynthetic apparatus (photosystems II and I).[5] Cadmium inhibits oxidative mitochondrial phosphorylation,[6] reduces activity of plasma membrane ATPase[7] and strongly affects the activity of several enzymes, such as glucose-6-phosphate dehydrogenase, malic enzyme, isocitrate dehydrogenase,[8] Rubisco and carbonic anhydrase.[9] Cadmium ions can inhibit the activity of several antioxidative enzymes.[10]

In response to cadmium stress the plant can (as a defence against cadmium stress) develop the biosynthesis of ligands such as phenolic compounds, organic acids or oligo- or poly-

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Table 1. Soil type, cadmium content, amount of organic matter and sorption capacity of soils

Treatment	Soil type	pH_{KCl}	Cd_T (mg kg^{-1})	Available Cd (% of Cd_T)	C_{ox} (%)	CEC (mval kg^{-1})
1	Chernozems	7.2 ± 0.2	0.321 ± 0.065	0.3	1.83 ± 0.41	258 ± 4
2	Fluvisols	4.8 ± 0.4	0.147 ± 0.033	11.4	0.82 ± 0.15	77 ± 13

peptides. Phytochelatins, a class of oligopeptides, were found to play a crucial role in detoxification within plants. Phytochelatins form various complexes with cadmium, due to presence of the thiol groups of cysteine, which chelate cadmium and, as a result, prevent it from circulationg as free Cd^{2+} inside the cytosol.[11] The complexation of metals occurs through the cysteine sulfur atom, leading to a number of relatively poorly characterized metal complexes.[12]

The determination of the total concentration of heavy metals in plants is a routine method to monitor the exposure of a plant to environmental metal pollution. To increase our knowledge of the ecotoxicity of heavy metals, their pathways in the ecosystem and their metabolism in plants requires the identification, characterization and quantification of the particular metal species concerned. Such analyses are mostly focused on isolation of one group of compounds, mainly proteins, oligo- or poly-peptides. Extraction solvents frequently used for isolation of these compounds are H_2O or a buffer solution (e.g. 10 mM Tris–HCl).[12] Difficult constituents, e.g. polypeptides or proteins, can be extracted after hydrolysis (hot HCl).[13] Application of isolation techniques included extraction of matrices such as tea leaves,[14] fruits and vegetables.[15,16]

The main objective of our study was focused on sequential fractionation of spinach biomass, partial characterization of cadmium-binding compounds and determination of the cadmium concentration in these fractions. Separation analyses have mostly been focused on the isolation of one group of compounds and relatively few studies have been concerned with the sequential fractionation of plant biomass.

EXPERIMENTAL

The cadmium accumulation in spinach biomass was investigated on Fluvisol and Chernozem soils (5 kg) treated by sewage sludge (32 g dry weight) in a model pot experiment (Table 1). Fresh homogeneous sewage sludge with 26–28% of dry matter and total cadmium content of 3.68 ± 1.03 mg kg^{-1} was used in this experiment. Spinach (*Spinacia oleracea* L.) var. Monores was grown to full leaf development stage. The pot experiment was described by Tlustoš *et al.*[17] After harvest the above-ground biomass was washed gently with deionized water, dried, ground and analysed for total cadmium content by a dry ashing procedure.[18] For sequential analysis, 0.5 g of ground spinach dry matter was weighed onto a column with a fritted disc. Extraction solvent was added into it and stirred with the sample. The time of each of the first three extractions was 24 h and the time of the following extractions was 48 h. Extraction was performed at laboratory temperature (22–24 °C). Fractions of each solvent were collected and evaporated to dryness (40 °C). Extraction by solvent was finished at constant weight of individual fraction. The determination of cadmium concentration was performed by atomic absorption spectrometry (VARIAN SpectrAA-300) with flameless atomization. The accuracy of plant analyses was verified by reference material RM 12-02-03 Lucerne with certified contents of 0.136 ± 0.003 mg kg^{-1} for which we obtained a cadmium content of 0.143 ± 0.021 mg kg^{-1}.

Sequential analysis of spinach dry matter to determine cadmium was conducted according to the extraction scheme outlined in Table 2. Typically non-polar, highly lipophilic solvents immiscible with water are used at the beginning of this series (fractions A, and B); polar hydrophilic solvents completely miscible with water are found in fraction C. At the end of the series are the solvent mixtures of methanol + H_2O & H_2O (fraction D) and methanol + H_2O + HCl (fraction E). This extraction series was used to characterize natural plant substances in each of the isolated fractions. IR spectra of fractions were determined (Brucker IFS 88) by evaporation and incorporation of the residue KBr into discs.

Fractions A, B, C, and D were dissolved in a mixture of 1 ml HNO_3(conc.) + 1 ml H_2O using an ultrasonic bath. Fraction E (methanol + H_2O + HCl) was decomposed in a mixture of HF(conc.) + HNO_3(conc.) (1:2) at 150 °C. The mixture was evaporated to dryness and the residue dissolved in 1 ml 1.5% HNO_3 by sonication. Non-extractable residues (fraction F) were decomposed by dry ashing, the ash being dissolved in 1 ml of 1.5% HNO_3. The cadmium concentration was determined by atomic absorption spectrometry.

RESULTS AND DISCUSSION

The total cadmium content in plant biomass was affected by the availability of cadmium in the soils (Table 3). The readily available cadmium in Fluvisols led to a significant increase in accumulation of cadmium in spinach compared with Chernozems. Cadmium accumulation in spinach increased after application of sludge to both the soils. This increase in cadmium accumulation in plant biomass after application of sewage sludge to the soil was also confirmed by Balik and coworkers.[17,19]

Sequential analysis of spinach biomass showed varying

Table 2. Sequential extraction scheme of spinach biomass

Fraction	Extraction solvent	Fractionation process
A	Light petroleum (free water)	5 × 20 ml
	↓	
B	Ethyl acetate (free water)	5 × 20 ml
	↓	
C	Methanol (free water)	7 × 20 ml
	↓	
D	Methanol + H_2O(1 + 1; v/v)	3 × 20 ml
	H_2O	1 × 30 ml
	↓	
E	Methanol + H_2O + HCl (36%) (49.3 + 49.3 + 1.4; v/v/v)	1 × 15 ml
	↓	1 × 5 ml
F	Non-extractable residues	Dry ashing

yields of isolated fractions (Table 4) and different accumulations of cadmium in these fractions according to treatment (Table 3). Light petroleum and ethyl acetate fractions taken together formed the lowest percentage isolated fractions—1.9–2.9% without sludge; 1.0–1.5% with sludge treatment—and the lowest cadmium amount—0.6–1.4% without sludge; 0.1–0.5% with sludge treatment—(Table 3). These fractions consisted of the least polar substances (Table 5). Cadmium ions in the light petroleum fraction were probably located either in intracellular spaces or were bound to long-chain hydrocarbons in membranes by chelation. Cadmium in fraction B (ethyl acetate) was bound to saturated and unsaturated fatty acids. These organic compounds in both fractions can be significant for membrane transport.

The methanol fraction (C) mainly contained amino[20] and carboxylic acids, aromatic and pyrrole substances (Table 5). This fraction was dominant for all treatments. The highest proportion was obtained in spinach cultivated on soils without sewage sludge (33.7–36.2%). This fraction was decreased by 8–16% on treatment with sewage sludge. Cadmium associated with fraction C represented only 1.4–3.9% of the total cadmium content. The cadmium amounts in fraction C were not significantly different in sludge treated and untreated plants.

The lower amounts of fractions A, B, and C after sludge treatment may mean that metabolism of plant natural products (primary and secondary metabolites) is inhibited by toxins in the sludge. A strong influence of toxins on the metabolism of different substances (cytochrome P450, glutamate kinase—biosynthesis of free proline) observed in fractions after sludge treatment.[21]

IR spectroscopy of the isolated fractions revealed some characteristic absorbance bands. Although there are quantitative differences between the respective fractions from treated and untreated plants there were no qualitative differences in the IR spectra. Toxins can influence the biosynthesis of stress metabolites, e.g. phytoalexins,[22] but in this case no changes in functional groups that would indicate the presence of new metabolites were seen in the IR spectra. The results suggest that biosynthesis of all components of spinach fractions A (aliphatic hydrocarbons), B (fatty acids), and C (carboxylic acids, mainly acids of citric cycle,[8] pyrrole substances) were suppressed by sludge

Table 3. Total cadmium content and portion of cadmium in fractions isolated from spinach biomass

Extraction solvent	Treatment[a]			
	1	1 + ss	2	2 + ss
Light petroleum (%)	0.07	0.08	0.11	0.03
Ethyl acetate (%)	1.36	0.40	0.46	0.11
Methanol (%)	3.80	3.85	1.86	1.37
Methanol + H_2O & H_2O (%)	65.00	51.49	38.78	45.83
Methanol + H_2O + HCl (%)	17.51	36.60	54.86	49.88
Non-extractable residues (%)	12.21	7.63	3.92	2.78
Total cadmium content (mg kg^{-1})	2.11	2.77	3.12	4.24

[a] SS: Sewage Sludge.

Table 4. Portion of fractions isolated from spinach biomass (%)

Extraction solvent	Treatment[a]			
	1	1 + ss	2	2 + ss
Light petroleum	1.2	0.4	1.9	0.8
Ethyl acetate	0.7	0.6	1.0	0.7
Methanol	33.7	25.8	36.2	20.3
Methanol + H_2O & H_2O	13.7	14.2	12.1	19.4
Methanol + H_2O + HCl	9.8	11.4	10.0	12.7
Non-extractable residues	38.2	44.8	37.0	46.8

[a] SS: sewage sludge.

Table 5. IR bands of substances in isolated fractions

Fraction	Characterization of IR bands	Measured values of bands $\nu(cm^{-1})$	Substances in isolated fractions
A	νCH	2955, 2920, 2849 vs	Saturated and unsaturated long-chain hydrocarbons with or without keto group
	δCH	1473, 1463, 1378	
	δCH_2 beside νC=O	1412	
	$\delta(CH_2)_n$ $n \geq 4$	719, 730,	
	νC=O ketones	1712, 1737	
B	νOH	3350	Saturated and unsaturated fatty acids
	νC−O	1050	
	δCH	1453, 1379	
	νC−O, ?−OAc	1246	
	νC=O	1724	
	νC=O, ? amides	1656, 1545	
C	νCOO^-	1621–1630, 1398–1408	Different carboxylic acids, probably not fatty acids, but acids for example from citric cycle or free amino acids, aromatic substances and pyrrole substances
	νOH	3360 or 3392–3393 or 3415	
	νC−O	1052–1056	
	ν? aromatic, ? pyrroles	1514–1516	
	νC=O ? ester	1718 or sh 1720 or 1720 w	
	νC=O	1666–sh 1670	
D	νOH, NH	3563, 3400–3415, sh 3240	Substances with amide bonds, for example oligopeptides or extractable polypeptides and proteins, extractable soluble oligosaccharides
	νCH	2924–2934, 2953, 2854–2857	
	νC=O, ? ester, COOH	sh 1720–sh 1725	
	amides	1638–1646, sh 1630–1632, 1311–1318 or 1326–1335, 617–618	
	νC−O	1070–1075, 1102–1115	
	νarom.	780 or 820	
E	νOH, NH	3399–3414, 3239–sh 3240–3244	Substances with amide bonds, extractable hydrolysate substances from cell walls or cytoskeleton (oligosaccharides, polysaccharides, proteins, glycoproteins)
	νCH	sh 2950 vw–2950 vw–2960 vw, 2924 vw–2927, 2834 vw	
	νC=O, ? ester, COOH	sh 1715–1725	
	amides	1630–1635, 1326, 610–614	
	νC−O	1074–1077	
	ν?arom.	789	

treatment. We cannot eliminate a possible change in the amount of isolated free amino acids.

The majority of cadmium was found in fraction D (methanol + H_2O & H_2O) after treatments 1 (65%) and 1 + ss (60%) and in fraction E (methanol + H_2O + HCl) after treatments 2 (55%) and 2 + ss (50%). Both these fractions contained the bulk of the total cadmium amount (82.5–95.7%). The cadmium content in spinach was significantly higher after sludge treatment compared with control plants, and this was reflected in the greater dry weights of these fractions. The major compounds detected in IR spectra of fraction D were compounds with amide bonds — oligopeptides, extractable polypeptides, proteins, phytochelatins and metallothioneins.[23–25] Phytochelatins, cadmium-binding compounds, in this fraction play a crucial role in detoxification within plants, but they are active only in the presence of the free metal ion. Fraction E (methanol + H_2O + HCl) contained compounds isolated from cell walls and the cytoskeleton after hydrolysis. Cadmium ions seem to be mostly bound by pectic sites (cell wall) and histidyl groups (cytoskeleton), leading to immobilization of these ions in roots and leaves.[11]

In fraction F the non-extractable residues were determined as a percentage of the fractions as a whole. These amounted to 37.0–38.2% for untreated spinach and 44.8–46.8% after sludge treatment (Table 4). The amount of cadmium in this fraction was highest (12.2%) after exposure to the lowest total cadmium content (treatment 1) and was lowest (2.8%) following treatment with the highest total cadmium content (Table 3). Toxins from sludge are probably elicitors of

biosynthesis of compounds that form cytoskeleton but, on the other hand, these toxins may inhibit the cadmium-binding compounds.

Sequential analysis of spinach biomass is an ideal means to investigate the complex effects of cadmium and other toxins on plant metabolism. This analysis provides the chance to isolate new metabolites formed in response to toxins. It should also be possible to investigate those biosynthetic processes that are decreased on exposure to toxins.

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REFERENCES

1. Shah K and Dubey RS. *Plant Physiol. Biochem.* 1995; **33**: 577.
2. Hernandez LE, Carpena-Ruitz R and Garate A. *J. Plant Nutr.* 1996; **19**: 1581.
3. Alcantra E, Romera FJ, Canete M and De La Guardia MD. *J. Exp. Bot.* 1994; **45**: 1893.
4. Costa G and Morel J-L. *Plant Physiol. Biochem.* 1994; **32**: 561.
5. Siedlecka A and Krupa Z. *Plant Physiol. Biochem.* 1996; **34**: 833.
6. Kessler A and Brand MD. *Plant Physiol. Biochem.* 1995; **33**: 519.
7. Obata H, Inoue N and Umebayashi M. *Soil Sci. Plant Nutr.* 1996; **42**: 361.
8. Van Assche F and Clijsters H. *Plant Cell Environ.* 1990; **13**: 195.
9. Siedlecka A, Krupa Z, Samuelsson G, Öquist G and Gardeström P. *Plant Physiol. Biochem.* 1997; **35**: 951.
10. Gallego SM, Benavides MP and Tomaro ML. *Plant Sci.* 1996; **121**: 151.
11. Sanita di Toppi L and Gabbrielli R. *Environ. Exp. Bot.* 1999; **41**: 105.
12. Vacchina V, Polec K and Szpunar J. *J. Anal. At. Spectrom.* 1999; **14**: 1557.
13. Pavlikova D, Pavlik M, Vasickova S, Szakova J, Tlustos P, Vokac K and Balik J. *Rostl. Výr.* 2001; **47**: 549.
14. Oedegard KE and Lund W. *J. Anal. At. Spectrom.* 1997; **12**: 403.
15. Szpunar J, Pellerin P, Makarov A, Doco T, Williams P and Lobinski R. *J. Anal. At. Spectrom.* 1999; **14**: 639.
16. Gunther K, Ji G and Kastenholz B. *Fresenius J. Anal. Chem.* 2000; **368**: 281.
17. Tlustoš P, Balík J, Dvořák P, Száková J and Pavlíková D. *Rostl. Výr.* 2001; **47**: 129.
18. Mader P, Kučera J, Cibulka J and Miholová D. *Chem. Listy* 1989; **83**: 765.
19. Balík J, Tlustoš P, Pavlíková D, Száková J, Kaewrahun S and Hanč A. *Rostl. Výr.* 2000; **46**: 273.
20. Oncel I, Keles Y and Ustun AS. *Environ. Pollut.* 2000; **107**: 315.
21. Talanova VV, Titov AF and Boeva NP. *Russ. J. Plant. Physiol.* 1999; **46**: 141.
22. Kim MS, Kim Ch, Jo DH and Ryu YW. *J. Microbiol. Biotechnol.* 1999; **9**: 661.
23. Leopold I, Günther D, Schmidt J and Neuman D. *Phytochemistry* 1999; **50**: 1323.
24. Kotrba P, Macek T and Ruml T. *Collect. Czech Chem. Commun.* 1999; **64**: 1057.
25. Rauser WE. *Cell Biochem. Biophys.* 1999; **31**: 19.

