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Separation of organic compounds binding trace elements in seeds of *Leuzea carthamoides* (Willd.) DC†

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The distribution of trace elements into important groups of compounds in seeds was investigated using a seven-step sequential extraction of seed biomass (solvents used: petroleum ether, ethyl acetate, butanol, methanol, methanol + H_2O (1 + 1; v/v), H_2O, methanol + H_2O + HCl (49.3 + 49.3 + 1.4; v/v/v)). Isolated fractions were partially characterized using IR spectroscopy. Results of sequential analysis showed different portions of the elements investigated in individual fractions. The dominant portions of cadmium (60.6% of total content), lead (41%), zinc (77.8%) and copper (33.9%) were found in the methanol + H_2O + HCl fractions (compounds isolated from cell walls and cytoskeleton after hydrolysis—phytic acid and its salts, proteins). The second most significant fractions for cadmium, zinc and lead were in the water fractions (pectin, phytin) and for copper in the methanol fraction (acids of citric cycle). The ethyl acetate fraction, mainly containing lignans and phospholipids, had the highest portion of arsenic (34.2%). Lignans are common compounds for seeds of *Leuzea carthamoides*. Therapeutic compounds of *L. carthamoides* (20-hydroxyecdysone, *N*-feruloylserotonin isomers) were confirmed in the first four fractions by thin-layer chromatography. Copyright © 2004 John Wiley & Sons, Ltd.

KEYWORDS: arsenic; cadmium; copper; lead; zinc; organic compounds; binding; trace elements; sequential analysis; *Leuzea carthamoides* (Willd.) DC

INTRODUCTION

The majority of articles describing elements bound in plants are focused on amidic compounds binding trace elements, mainly metallothioneins and phytochelatins.[1–4] Cations of trace elements are bound into carboxylic groups of acids, e.g. citric acid, malic acid,[5,6] amino acids, phenylpropane acids, and fatty acids forming adequate salts. Salts of phytic acid represent a specific group.[7,8] Many analytical laboratories have recently been involved in the determination of individual compounds or binding types of elements in biological material. However, most papers are focused on the separation of one group of macromolecular compounds, mainly proteins or polypeptides (phytochelatins, metallothioneins).[9–12] The extraction solvents frequently used for isolation of these compounds are H_2O or buffer solutions (e.g. 10 mM Tris–HCl). The difficult-to-extract constituents, e.g. polypeptides or proteins, can be extracted after hydrolysis using diluted HCl. The determination of individual organometallic compounds is frequently discussed because of their different plant toxicities (e.g. between inorganic arsenic compounds and organoarsenicals).[13–16] However, the determination of one group of compounds from such a wide spectrum of compounds, or the changes in these compounds in plants under stress conditions, is difficult.

The distribution of trace elements into significant groups of compounds in plants can be investigated using sequential extraction of plant biomass followed by determination of compounds incorporating the elements in plants and

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concentrations of elements in these compounds.[17,18] This method leads to detailed explanations of the behavior of element compounds into individual parts of plant cells as well as the behavior of important substances in plant stress metabolism dependent on stress level.

The aim of our work was to investigate the spectrum of organic compounds binding trace elements in seeds of *Leuzea carthamoides* (Willd.) DC, the Siberian medicinal plant.[19,20] Various important therapeutic compounds (phytoecdysteroids, flavonoids, stilbene, sesquiterpene lactones (guaianolides), polyacetylenes and *N*-feruloylserotonin isomers) are contained in different parts of this plant. Their role in plant stress metabolism is not fully explained.

EXPERIMENTAL

Seeds of *L. carthamoides* were harvested from perennial (3 years) plants. After harvesting, the seeds were ground up.

For sequential analysis, 50 g of seeds was weighed into a column with a fritted disc. Extraction solvent was then added and stirred with the sample. Samples were extracted in sequence from nonpolar to polar solutions. Sequential analysis of seeds was conducted according to an extraction scheme that allowed us to determine toxic elements in seven fractions (Fig. 1). The extraction time of each of first three fractions was 24 h and the time for following four extractions was 48 h. Extraction was performed at laboratory temperature (22–24 °C). Water temperature for extraction was 55–60 °C. Fractions of each solvent were collected and evaporated to dryness (40 °C). Extraction by each solvent was complete at a constant weight of each individual fraction. Evaporated isolated fractions (A–F) were dissolved in a mixture of 1 ml concentrated HNO_3 + 1 ml H_2O using an ultrasonic bath. Fraction (G), methanol + H_2O + HCl, was decomposed in a mixture of concentrated HF + concentrated HNO_3 (1 : 2) at a temperature of 150 °C. The mixture was evaporated to dryness and the residue was dissolved in 1 ml of 1.5% HNO_3 using an ultrasonic bath. Non-extractable residues (fraction H) were decomposed by a dry ashing procedure in a mixture of oxidizing gases ($O_2 + O_3 + NO_x$) using an Apion Dry Mode Mineralizer (Tessek, CZ) and the ash was dissolved in 1 ml of 1.5% HNO_3.

Plant material was decomposed by a modified dry ashing procedure in a mixture of oxidizing gases ($O_2 + O_3 + NO_x$) using an Apion Dry Mode Mineralizer (Tessek, CZ). Ash was dissolved in 1.5% HNO_3.

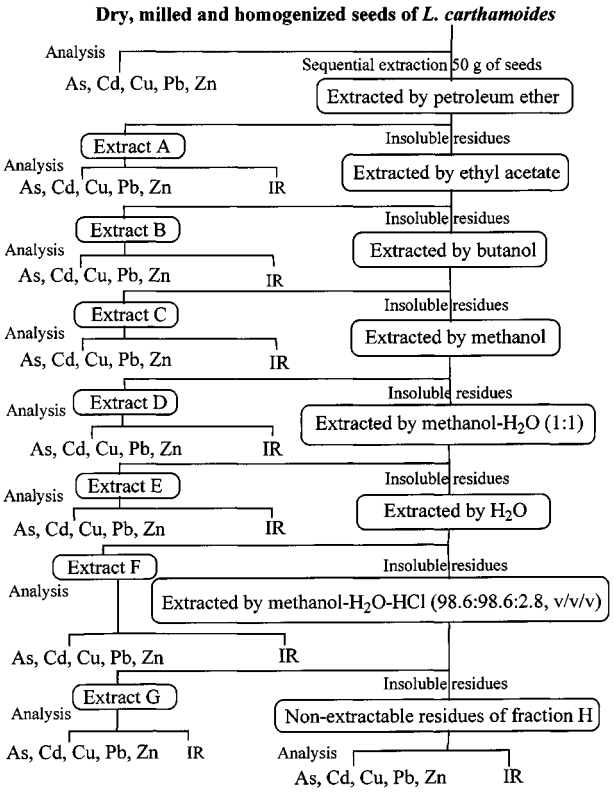

Figure 1. Sequential extraction scheme of *L. carthamoides* seeds.

Arsenic was determined in the digests of individual fractions by a continual hydride generation technique using a Varian SpectrAA-300 (Australia) atomic absorption spectrometer equipped with a VGA-76 hydride generator. A mixture of potassium iodide and ascorbic acid was used for pre-reduction of the sample and the extract was acidified with HCl before measurement.

A Varian SpectrAA-400 (Australia) atomic absorption spectrometer equipped with a GTA-96 graphite tube atomizer was used for cadmium, lead, and copper determination. A pyrolytically coated tube with L'vov platform was used for all measurements.

For the determination of zinc, flame atomization (air–acetylene flame) was applied (Varian SpectrAA-300 atomic absorption spectrometer).

The quality of plant analyses was verified by use of reference material RM 12-02-03 Lucerne (Table 1).

Table 1. Quality control of plant analyses

Reference material RM 12-02-03 Lucerne	As_T	Cd_T	Cu_T	Pb_T	Zn_T
Certified content (mg kg^{-1})	0.263 ± 0.007	0.136 ± 0.003	11.6 ± 0.4	1.84 ± 0.08	33.2 ± 0.5
Content obtained (mg kg^{-1})	0.270 ± 0.001	0.147 ± 0.007	11.4 ± 0.8	1.99 ± 0.03	33.2 ± 1.4

The IR spectrum of isolated fractions was measured using a Brucker IFS 88 spectrometer. Evaporated isolated fractions were analysed in micro-tablets amended by KBr.

All isolated fractions were solubilized in methanol and analysed by thin-layer chromatography (TLC) analyses. The plates were developed once by the eluent. Eluted substances were detected under UV light, sprayed with sulfuric acid and heated by open flame. 20-Hydroxyecdysone and *N*-feruloylserotonin were detected in the analysed fraction using an internal standard.[21,22]

RESULTS AND DISCUSSION

The distribution of trace elements into important groups of compounds in plant seeds was investigated using sequential extraction of seed biomass. The results of sequential analysis showed different portions of the elements investigated and substances contained in individual fractions (Tables 2 and 3).

Typical bands of the functional groups of organic compounds in the IR spectrum and knowledge of the nonspecific and specific (chemotaxonomic characteristics) occurrence of compounds in plant species and in different plant parts were used to investigate the organic compounds binding trace elements. Use is also made of the published physical and chemical characteristics of individual compounds arising from their isolation and identification.

Esters of fatty acids (mainly glycerides) were determined in petroleum-ether fraction (A) by sequential extraction of *L. carthamoides* seeds. Substances of this fraction are an important source for plant growth. Fatty acids were originated from cleaved lipids by lipase. The first product of this reaction, acetyl-CoA, enters into the citric cycle and the second product, i.e. glycerol, is used for saccharide metabolism. Fraction A contained low of cadmium, copper and zinc contents (Table 2). Seeds of *L. carthamoides* have a higher content of lipids. The nonpolar lipid portion content is reported as about 20.4% of dry seed matter and about 3.4% of dry root matter.[23] The nonpolar lipid portion was extracted mainly in the petroleum ether fraction (A: 18.8% of dry seed matter) and also in the ethyl acetate fraction (B: 2.9% of dry seed matter) in this procedure. A portion of slightly polar lipids is also contained in fraction B. Some important therapeutic compounds are detected in fraction B. According to the IR spectrum there are ecdysteroids present: the band at 1653 cm^{-1} is typical of a ketone on the sixth carbon with a conjugated C═C bond of 7-cholesten. The band at 1763 cm^{-1} belongs to a γ-lactone corresponding to lignans,[33] and bands at 1516 and 1512 cm^{-1} correspond to aromatic substances similar to four *N*-feruloylserotonin isomers[21] (Table 3). Oil seeds contain fatty acids and lipids, mainly phospholipids (e.g. lecithin). Lipid compounds, mainly phospholipids, are important substances in this fraction. In this context, the high arsenic content in this fraction (34.2% of total content) is of interest. From Table 4 we can see bands from three arsenic chemical compounds (Fluka Chemie GmbH). These bands can be compared with bands 805 and 762 cm^{-1} in fraction B. There is a competitive interaction between arsenic and phosphate for the same uptake system in plants. Arsenate behaves as a phosphate analogue and is taken up by the phosphate transport system.[34] According to the IR spectrum (Tables 3 and 4), we can observe arsenic analogues of phospholipids (mainly arsenic analogue of lethicin). Arsenolipids (including arsenolecithin) are identified mainly after hydrolysis.[25] The results in Tables 2 and 3 show that cadmium, copper, lead and zinc can form salts of fatty acids in this fraction. *L. carthamoides* seeds contain mainly free fatty acids or fatty acids and glycerol with ester binding. Cadmium, copper, lead and zinc form salts of linoleic, oleic, palmitic, linolenic and stearic acids. We propose that salts of these acids are contained in the ethyl acetate fraction (B) and also in the butanol fraction (C).[24]

The butanol fraction (C) contains similar structures of compounds as in fraction B. The cadmium (1%), copper (5.3%), lead (3.4%) and zinc (0.7%) contents in this fraction were low (Table 2). Arsenic content binding in the extracted compounds formed 10% of the total content. Important bands at 763 and 811 cm^{-1}, corresponding to arsenic species, were also detected in fraction C. Bands at about 931, 951 and 1000 cm^{-1} corresponded to organophosphate compounds[35] (Table 3). *p*-Coumaric acid, ferulic acid and their salts, originating from the phenylpropanoid pathway, were extracted in this fraction. Phenylpropanoid products originating from these acids (lignins, flavonoids, anthocyanins) were detected in *L. carthamoides*. Cadmium, copper, lead and zinc can form salts of *p*-coumaric and ferulic acids. Salicylic-acid-containing carboxylic groups can be extracted in fraction C. Cadmium, copper, lead and zinc are bound to the carboxylic group of the acid.

According to IR and TLC analysis (using the method of internal standards), the methanol fraction (D) contained 20-hydroxyecdysone, *N*-feruloylserotonin isomers and lignans (slight band at about 1764 cm^{-1}; Table 3). Fraction D had

Table 2. Total content of toxic elements and their amounts in fractions isolated from seeds of *L. carthamoides*

Fraction	Amount of toxic element in fraction (%)				
	As	Cd	Cu	Pb	Zn
A	6.8	0.5	2.1	9.4	0.5
B	34.2	0.1	0.4	4.8	0.3
C	10.1	1.0	5.3	3.4	0.7
D	6.3	7.7	31.1	12.2	3.2
E	10.0	4.7	10.9	3.8	1.1
F	13.3	24.9	13.0	19.1	14.3
G	11.5	60.6	33.9	41.0	77.8
H	7.9	0.5	3.4	6.3	2.1
Total content (mg kg^{-1})	0.144	0.522	11.34	1.147	32.57

Table 3. IR bands of substances in isolated seeds fractions and proposed assignments important compounds for binding of trace elements

Fraction	ν	(cm^{-1})	Substances in isolated fractions
A	=C–H	3010	
	C=O	1746	Ester, probably esters of fatty acids—glyceride
	C–O	1167	Ester, probably esters of fatty acids—glyceride
	$\delta_{(CH_2)_n}$	721	$n \geq 4$
Compounds binding trace elements			Salts of fatty acids[23]
B	OH	3428	Substances with hydroxy group
	C–O	1025, 1077	Substances with acyl in molecule
	Arom.	1516, 1592	Aromatic compounds
	C=O	1653, 1649	Substances with conjugated ketone, e.g. ecdysteroids
	C=O	1745	Probably ester
	C=O	1763	Probably γ-lactone
	AsO	811, 764	Detecting bands equal to P and As compounds or $\delta_{Car.H}$ (phospholipids, arsenolipids); it is not possible to eliminate aromatic substances
Compounds binding trace elements			Salts of fatty acids (linoleic, oleic, palmitic, linolenic, stearic acid),[24] arsenolipids (arsenolecithin)[25]
C	OH	3400, 3392	Substances with hydroxy group
	C–O	1026, 1073	Substances with acyl in molecule
	Arom.	1515	Aromatic compounds
	Arom.	1598	Aromatic compounds
	C=O	1647	Substances with conjugated ketone, e.g. ecdysteroids
	C=O	1740	Probably ester
	C=O	1764	Probably γ-lactone
	AsO	811, 763	Detecting bands equal to P and As compounds or $\delta_{Car.H}$ (phospholipids, arsenolipids); it is not possible to eliminate aromatic substances
Compounds binding trace elements			Salts of more polar fatty acids, salts of *p*-coumaric acid and ferulic acid, salts of salicylic acid, arsenolipids[25]
D	OH	3390	Substances with hydroxy group
	C–O	1028, 1071	
	Arom.	1515, 1603	Aromatic compounds
	C=O	1654	Conjugated ketone, e.g. ecdysteroids, lignans
	C=O	1727	Slight band
	C=O	1764	Probably γ-lactone
	AsO	804, 763	Detecting bands equal to P and As compounds or $\delta_{Car.H}$ (polar phospholipids, arsenolipids), it is not possible to eliminate aromatic substances
Compounds binding trace elements			Salts of acids of citric cycle,[5,6] avenic acid,[26] mugineic acid[27]
E	OH	3400	Substances with hydroxy group
	C–O	1046, 1070	
	C–O	1126	
	Amide	1654, 1603	Amide I, II
	Amide	1312	Amide III
	COO^-	1398, 1603	Band of carboxyl group ($-COO^-$)
	Arom.	1516	Aromatic compounds
	C=O	1654	Typical band for conjugated ketone, e.g. ecdysteroids, lignans
	C=O	SH 1720	+wide carboxyl group (–COOH band $\sim$ 3000 cm^{-1})
Compounds binding trace elements			Salts of avenic[26] and mugineic acids[27]
F	OH	3392	Substances with hydroxy group
	C–O	1000, 1130	

Table 3. *Continued*

Fraction	ν	(cm^{-1})	Substances in isolated fractions
	Amide	1662, 1548	Amide I, II
	Amide	1325	Amide III
	AsO	848, 828, 802, 785	Detecting bands equal to P and As compounds or $\delta_{Car.H}$; it is not possible to eliminate glucoronic acid
Compounds binding trace elements			Pectins[28,29] (arsenopectin), phytic acid[7,8,30] (arsenophytic acid), metallothioneins,[1–4] storage proteins, trace elements and nicotinamine complex[27,31]
G	OH	3392	Substances with hydroxy group
	C═O	1653	Amide I, probably significant content of proteins
	δ_{NH}	1542	Amide II, probably significant content of proteins
	C–O	1074, 1140	
	C–O	1242	
	C═O	SH 1730	Probably ester
	AsO	925, 892	Detecting bands equal to P and As compounds or $\delta_{Car.H}$
	AsO	835, 778	Detecting bands equal to P and As compounds or $\delta_{Car.H}$
	OH	3401	
	C–O	1045, 1065	
	NH	3153, 3051	
	C═O	1738	Ester
	C–O	1133	Ester
	C═O	1658	Amide I, probably significant content of proteins
	δ_{NH}	1520	Amide II, probably significant content of proteins
	COO^-	1404	Band of carboxyl group ($-COO^-$)
Compounds binding trace elements			Phytic acid[7,8,30] (arsenophytic acid), proteins,[32] lignans, polysaccharides
H	OH	3390	Polysaccharides
	C–O	1045	
	C═O	1735	Ester, no salt
	C–O	1167	Ester, no salt
	C═O	1653	Amide I, probably proteins
	δ_{NH}	1517	Amide II, probably proteins
	AsO	896	Detecting bands equal to P and As compounds or $\delta_{Car.H}$
Compounds binding trace elements			Polysaccharides, proteins[32]

Table 4. IR bands of arsenic compounds (Fluka Chemie GmbH)

Compound	ν	(cm^{-1})
Na_2HAsO_4	AsO	715, 847, 870
$NaAsO_2$	AsO	693, 830, 855
$(CH_3)_2As(O)OH$	AsO	753, 830, 867
		608, 652, 895, 919, 984

the lowest portion of arsenic (6.3%); the contents of the other elements measured ranged from 3.2% (zinc) to 12.2% (lead). According to the IR spectrum, the elements are bound to salts of organic acids (bands at 1727 and ~3000 cm^{-1} corresponding to carboxylic groups of organic acids). The copper portion represented 31.1% of the total content in this fraction. Copper bound to carboxylic groups of phenylpropanoid acids and/or acids of the citric cycle probably caused this increase. In fractions D and E we can extract acids that form specific chelating agents for iron(III) and copper(II), e.g. avenic acid and mugineic acid.[26,27]

Important therapeutic compounds of *L. carthamoides* were shown in the first four fractions (A–D) by TLC.[19,21] The results showed a high arsenic content (57.4% of total content) in these fractions. The fractions (A–D) were also important for copper (38.9%) and lead (29.8%). Cadmium and zinc contents were not significant (being 10.2% and 4.7% of total content respectively). The results of Pavlíková and co-workers,[17,18] describing the extraction of spinach biomass, also showed low contents of cadmium and zinc in these fractions.

According to the IR spectra, higher concentrations of organic acids are found in the methanol + H_2O fraction (E). These acids were also extracted into fraction D. 20-Hydroxyecdysone, *N*-feruloylserotonin isomers and lignans

were not determined here by TLC analysis. Substances containing carboxylic groups (bands at 1398 and 1603 cm^{-1}) and substances with amide bonds (oligopeptides, extractable polypeptides) were isolated (bands at 1654 cm^{-1} (amide I), 1603 cm^{-1} (amide II), and 1312 cm^{-1} (amide III)); see Table 3. This fraction contained low amounts of cadmium, zinc and lead (1.1–4.7% of total content). These elements can be bound into amino acids indicated in this fraction (bands at 1398 and 1603 cm^{-1}). The elements can be also bounded into oligopeptides and form specific chelates (bands at 1654, 1604 and 1312 cm^{-1}). Cadmium, zinc and lead contents increased significantly in the following water fraction (F), from 14.3% to 24.9% of total content. Arsenic and copper contents were similar in both factions (10–13%; Table 2). The major compounds in both fractions according to the IR spectra were amidic bound compounds: oligopeptides, extractable polypeptides, proteins.[17,18] Finally, chelating agents[31] were present in this fraction. Nicotinamine has an optimal molecular structure for chelating iron ions[27] and also forms stable anionic complexes with several toxic elements. Pectins,[28] myo-inositol hexaphosphoric acid (phytic acid) and its salts are important substances isolated in fraction F. Pectin substances contain carboxylic groups. Cations of trace elements can be bound to carboxylic groups of D-galacturon acid, forming pectin substances. Phosphoric acid and the OH^- group of D-galacturon acid form ester bonds. The IR spectra (Table 3) and arsenic determination (Table 2) show that arsenic acid (v) can probably form the same ester bonds. According to Yuldasheva *et al.*,[29] pectins were isolated from roots by water after extraction of therapeutic compounds. Phytin and phytic acid are typical substances for seed, and they are also important substances for binding of trace elements. Bands from 1000 to 1100 cm^{-1} demonstrate the presence of organophosphate compounds and also compounds contained C–O groups. Bands at 785, 802, 828, 848 and 915 cm^{-1} confirm arsenic compounds in this fraction (Table 4).

The highest of cadmium (60.6%), copper (33.9%), lead (41%) and zinc (77.8%) contents were determined in the methanol + water + HCl fraction (G). According to the IR spectroscopic analysis, these elements can be bound to proteins and acids from myo-inosytol monophosphoric acid to myo-inositol hexaphosphoric acid.[30] We can also explain the occurrence of phytic acid and some its salts in this fraction by stronger bonds of some of these substances in seeds. Binding to carboxylic groups of phytic acid is typical for zinc (element with highest content, 77.8% of total content) and other cations in fraction G (band at 1404 cm^{-1}). The high arsenic content in this fraction can be explained by the formation of the arsenic analogue of phytic acid (arsenophytic acid). We cannot eliminate the occurrence of arsenosaccharides.[36] The occurrence of organophosphate compounds is confirmed by bands similar to bands in fraction F. Bands of amide I and II (Table 3) show the presence of proteins. Cations of trace elements can be bound to COOH and NH_2 groups of proteins.[32]

Non-extractable residues were determined in fraction H. Compounds contained in residues were difficult to extract. Low contents of all elements were determined (0.5–7.9% of total content) in fraction H. This H fraction contained polysaccharides and tight binding of proteins to the cell cytoskeleton detected by IR analysis.

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