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Comparison of mild extraction procedures for determination of arsenic compounds in different parts of pepper plants (*Capsicum annum*, L.)

Jiřina Száková[1]*, Pavel Tlustoš[1], Walter Goessler[2], Daniela Pavlíková[1] and Jiří Balík[1]

[1]Department of Agrochemistry and Plant Nutrition, Czech University of Agriculture, 165 21 Prague 6, Suchdol, Czech Republic
[2]Institute of Chemistry, Analytical Chemistry, Karl-Franzens-University Graz, Universitätsplatz 1, A-8010 Graz, Austria

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Eight extraction agents (water, methanol–water mixtures in various ratios, methanol, a 20 mmol l^{-1} ammonium phosphate buffer, and a methanol–phosphate buffer) were tested for the extraction of arsenic compounds from fruits, stems + leaves, and roots of pepper plants grown on soil containing 17.2 mg kg^{-1} of total arsenic. The arsenic compounds in the extracts were determined using high-performance liquid chromatography–hydride generation inductively coupled plasma mass spectrometry. Whereas pure water was the most effective extraction agent for fruits (87 ± 3.3% extraction yield) and roots (96 ± 0.6% extraction yield), the 20 mM ammonium phosphate buffer at pH 6 extracted about 50% of the arsenic from stems + leaves. Decreasing extractability of the arsenic compounds was observed with increasing methanol concentrations for all parts of the pepper plant. In pepper fruits, arsenic(III), arsenic(V), and dimethylarsinic acid (DMA) were present (25%, 37%, and 39% respectively of the extractable arsenic). Arsenic(V) was the major compound in stems + leaves and roots (63% and 53% respectively), followed by arsenic(III) representing 33% and 42% respectively, and small amounts (not exceeding 5%) of DMA and methylarsonic acid were also detected. Hence, for a quantitative extraction of arsenic compounds from different plant tissues the extractant has to be optimized individually. Copyright © 2005 John Wiley & Sons, Ltd.

KEYWORDS: arsenic compounds; arsenite; arsenate; dimethylarsinic acid; methylarsonic acid; HPLC–ICPMS; pepper; extraction procedures

INTRODUCTION

The occurrence of different arsenic compounds in soil can significantly affect the plant availability of arsenic, as well as the toxicity of arsenic for plants.[1] Although inorganic arsenic compounds predominate in the soil, both inorganic and organic were detected in different parts of higher plants, usually the simple methylated arsenicals, i.e. methylarsonic acid (MA) and dimethylarsinic acid (DMA). Transformation and translocation of arsenic compounds among plant tissues is strongly dependent on plant species and/or soil characteristics.[2] Arsenic(III), arsenic(V), DMA, MA, trimethylarsine oxide, the tetramethylarsonium ion, and one arsenoribose were identified in 12 green plant species grown at an arsenic-contaminated site.[3] Geiszinger *et al*.[4] determined mainly organic arsenicals, with dominating MA in the extracts of *Trifolium pratense* grown on the top of an ore vein, whereas inorganic arsenic compounds were detected mostly in extracts of above-ground biomass of *Dactylis glomerata* and *Plantago lanceolata* grown on the same site. These results demonstrate that arsenic compounds and their concentrations differ with plant species.

The differences in distribution of arsenic compounds within the radish plant were investigated by Tlustoš *et al*.[5] Arsenic(III) was the dominant compound (63%) with the presence of arsenic(V) and DMA (20% and 17% respectively) in radish roots planted at the untreated soil in which arsenate

*Correspondence to: Jiřina Száková, Department of Agrochemistry and Plant Nutrition, Czech University of Agriculture, 165 21 Prague 6, Suchdol, Czech Republic.
E-mail: szakova@af.czu.cz
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was confirmed as the dominant arsenic compound (91%). In leaves, however, most of the arsenic present was arsenate (42%) and arsenite (40%), and DMA was also detected (18%), indicating the role of biomethylation processes within the plant. Transformation of mainly inorganic arsenic compounds in different plant tissues, and the various distributions of these compounds, has been described for different terrestrial plant species.[6–8] Investigation of arsenic methylation activities in *Agrostis tenuis* suggested that arsenate in the plant growth medium was taken up by the roots and converted to arsenite before methylation in the leaves. These observations were supported by increased activity of arsenic methyltransferase in plants.[8] Differences in the speciation of arsenic within one plant species were influenced by the total arsenic concentration in the plant and/or the nutrient status. In particular, the concentration of phosphorus in soil influenced the uptake and transformation of individual arsenic species in plant tissues.[9,10]

Various extraction procedures have been described for determination of arsenic compounds in plant material, ranging from water extraction at ambient temperature to pressurized liquid extraction or microwave-assisted extraction at elevated temperatures using different extractants. In the case of the marine alga *Hijiki fusiforme*, the extractability of arsenic compounds increased from 33% with a methanol/water (9/1) mixture to a 74% extraction yield with 1.5 mol l^{-1} orthophosphoric acid.[11] For the freshwater alga *Chlorella vulgaris*, the recoveries of total arsenic ranged from 74% for a water extraction to 100% with methanol–water mixtures in which the methanol concentration was above 40% (v/v). A 0.03 mol l^{-1} phosphate buffer extracted 84% of total arsenic.[12] The extractability of arsenic from green parts of submerged water plants with methanol/water (9/1) was found not to exceed 16.1% of the total arsenic content.[13]

To improve the extraction efficiency, microwave-heated extraction using various extraction solutions (e.g. water, modified protein extraction procedure, and 10% (v/v) tetramethylammonium hydroxide;[14] 0.3 mol l^{-1} orthophosphoric acid;[15] or 2 mol l^{-1} trifluoroacetic acid[16]) was tested, with almost 100% removal of arsenic from different parts of higher plants. However, the application of trifluoroacetic acid for extraction of rice grain samples led to partial reduction of arsenate to arsenite during the extraction process.[16] Sonication[17] and a pressurized solvent-extraction procedure[9,18] were recommended as suitable methods for extraction of arsenic compounds from higher plants.

The influence of small changes in the composition of the extractants for releasing the arsenic compounds from individual parts of the pepper plant (*Capsicum annum*, L.) was investigated in our study to evaluate the effect of the extraction procedure on the interpretation of analytical data obtained by high-performance liquid chromatography (HPLC)–hydride generation (HG) inductively coupled plasma mass spectrometry.

MATERIAL AND METHODS

Pot experiments

Pepper var. California Wonder was cultivated in pot experiments (pot volume 12 l, 7 kg of substrate per pot) in a greenhouse under controlled conditions in a substrate prepared by mixing of peat, soil, and sand in the ratio 12 : 8 : 3. Substrate was treated by N, P, K fertilizer in the amount representing 3.0 g nitrogen, 1.45 g phosphorus and 2.9 g potassium per pot. The main characteristics of the substrate were as follows: pH 7.3, available phosphorus 205 mg kg^{-1}, available potassium 193 mg kg^{-1}, available magnesium 344 mg kg^{-1}, available calcium 5381 mg kg^{-1}, total arsenic 17.2 mg kg^{-1}. Available contents of nutrient elements (calcium, magnesium, potassium, phosphorus) were determined by the Mehlich II soil extraction procedure.[19] The experiment was divided into six replications. The plants were watered by deionized water, and soil humidity was kept at 60% of its maximum water holding capacity. Fruits were continually harvested during the vegetation period, and roots, stems and leaves of plants were sampled twice during vegetation. The pepper roots were freed from adhering soil by washing with deionized water. The stems were separated from the roots with a stainless steel knife. Fruits, stems, leaves and roots were dried at 60 °C to constant mass and then separately ground to a fine powder in a mixer. The samples were mixed together according to individual plant tissues (fruits, stems, leaves and roots) to obtain representative amounts of samples for analysis. After determination of the total arsenic concentration in individual samples, stems and leaves were mixed together to obtain a sufficient amount of sample for testing with the set of extraction agents.

Determination of total arsenic by HG atomic absorption spectrometry

The plant samples were decomposed by a dry ashing procedure as follows: an aliquot (~1 g) of the dried and powdered fruits, leaves or roots was weighed to 1 mg and placed in a borosilicate glass test-tube and decomposed in a mixture of oxidizing gases ($O_2 + O_3 + NO_x$) at 400 °C for 10 h in a Dry Mode Mineralizer Apion[20] (Tessek, Czech Republic). The ash was dissolved in 20 ml of 1.5% HNO_3 (electronic grade purity, Analytika Ltd, Czech Republic) and kept in glass tubes until measurement. Aliquots of the certified reference material RM 12-02-03 Lucerne (pb-anal, Slovakia) were mineralized under the same conditions for quality assurance of the total arsenic contents in experimental plants. The total arsenic concentrations in the fruits, stems, roots and leaves of pepper decomposed by the dry ashing procedure were determined by HG atomic absorption spectrometry (Varian SpectrAA-300, Australia), equipped with a VGA-76 continuous hydride generator.[21]

Determination of arsenic compounds by HPLC–HG-ICPMS

Extraction procedures

Aliquots (~500 mg) of the dried and powdered fruits, above-ground green biomass (stems were mixed together with leaves to obtain sufficient sample mass for all extraction procedures) or roots weighed to 0.1 mg were placed into 10 mL screw-capped polyethylene tubes and 10 ml of one of the extraction agents was added. The extractants were as follows: (1) water, 10 ml; (2) methanol/water, 2/8 ml; (3) methanol/water, 4/6 ml; (4) methanol/water, 6/4 ml; (5) methanol/water, 8/2 ml; (6) methanol; (7) methanol/20 mmol l^{-1} ammonium phosphate buffer, 5 + 5 ml; (8) 20 mmol l^{-1} ammonium phosphate buffer, 10 ml. The closed tubes were fastened to the arms of a cross-shaped rotor and turned top over bottom at 45 rpm for 14 h. Methanol was evaporated from the solutions and the samples were resuspended in 10 ml of water. The mixtures were then centrifuged for 10 min at 3000 rpm and filtered through a 0.22 μm cellulose-nitrate ester filter (Millex-GS, Milipore, Bedford, MA, USA). Aliquots of this solution (20 μl) were chromatographed.

Chromatographic system

A Hewlett Packard 1100 solvent delivery unit (Germany) together with a Hamilton PRP-X100 (USA) anion-exchange column (250 mm × 4.1 mm i.d., spherical 10 μm particles of a styrene–divinylbenzene copolymer with trimethylammonium exchange sites) was used for the separation of arsenic(III), DMA, MA, and arsenic(V). An aqueous 0.020 mol l^{-1} $NH_4H_2PO_4$ solution, pH 6.0, at a flow rate of 1.5 ml min^{-1} served as the mobile phase. The column effluent was reduced by 0.7% $NaBH_4$ solution in 3 mol l^{-1} HCl and the volatile hydrides produced were introduced into the plasma of the ICPMS instrument (Hewlett Packard 4500) for arsenic-selective detection.[22] Calibration was in the range of 1–100 μg l^{-1} of each compound and detection limits were 1.2 μg ml^{-1} 3.3 μg ml^{-1} 1.2 μg ml^{-1} and 1.4 μg kg^{-1} for arsenic(III), DMA, MA and arsenic(V) respectively.

Statistics

The concentrations of total arsenic and arsenic compounds in solutions obtained by individual extraction procedures were evaluated by analysis of variance (Statgraphics 5.0 plus) at an $\alpha = 0.05$ significance level.

RESULTS AND DISCUSSION

The total arsenic concentrations in the different parts of the pepper plant were 0.056 ± 0.012 mg kg^{-1} (fruits), 0.26 ± 0.02 mg kg^{-1} (leaves), 0.74 ± 0.16 mg kg^{-1} (stems) and 10.0 ± 1.3 mg kg^{-1} (roots). Owing to the fact that there was not sufficient material available, the leaf samples and the stem samples were combined and a weighed mean of 0.46 mg kg^{-1} was calculated and the different extractions were carried out with these samples. The low arsenic concentrations in the above-ground parts of the pepper plant and the high concentration in the roots show that the pepper belongs to the plants avoiding the transport of this element to other parts of the plant. A similar behavior was reported for tomatoes, whereas bush beans and radish have been shown to transport arsenic amended to soil or soilless cultures efficiently.[23,24]

The various extractants resulted in different extraction yields, as illustrated in Fig. 1. Generally, a similar trend can be observed for fruits, stems + leaves, and for the roots. Increasing methanol concentrations in the extractants result in a decreasing extractability of the arsenic compounds. The decrease is more pronounced when the methanol concentration exceeds 50%. With pure methanol, only ~35% of the total arsenic concentration is extractable in fruits, ~8% in (stems + leaves), and ~5% in roots, whereas with water ~87% of total arsenic content in fruits, ~45% in stems + leaves, and ~96% in roots is extracted. The methanol–20 mmol l^{-1} ammonium phosphate buffer mixture was very efficient in removing the arsenic compounds from the fruits (93%), whereas only 34% was removed from the stems + leaves sample and 70% from the roots. The phosphate buffer was the most efficient extractant for the extraction of the arsenic from the stems + leaves sample (~50%), and reasonable extraction yields were obtained for the fruits (~74%) and for the roots (~81%). For all three plant tissues, water was very effective in removing the arsenic. For roots, water was the best extractant. Relatively poor extractability of arsenic by methanol–water mixtures from above-ground biomass and roots of higher plants compared with plants growing in a water environment has been observed in many previous experiments.[2,4,25,26] The influences of plant species, the individual plant tissues, and the arsenic compounds present are evident in this context.

In order to investigate these influences, we systematically studied the arsenic compounds extractable with the various extraction agents from the different plant tissues. The results are summarized in Tables 1–3. In all three plant tissues, arsenic(III), DMA, and arsenic(V) were extractable at various ratios. MA was only detectable in stems + leaves and root extracts. In stems + leaves and roots, arsenate and arsenite were the dominant arsenic compounds, and low portions of MA and DMA were determined (Fig. 2). Whereas the DMA concentration in stems + leaves slightly exceeded that of MA, the opposite behavior was observed in roots. The dominant abundance of arsenite and arsenate in above-ground biomass and roots of higher plants with the presence of small amounts of organic arsenic compounds has already been described,[1,2,4,13,26] with exceptions such as *T. pratense*[3] dominating in MA content in above-ground biomass. Because plant-available arsenic in soil is mostly present in the inorganic state (arsenate) in oxidizing soil conditions,[3,4,27,28] the distribution of arsenic compounds in the individual parts of pepper plants supports the ability of the plant roots to reduce arsenate from soil solution

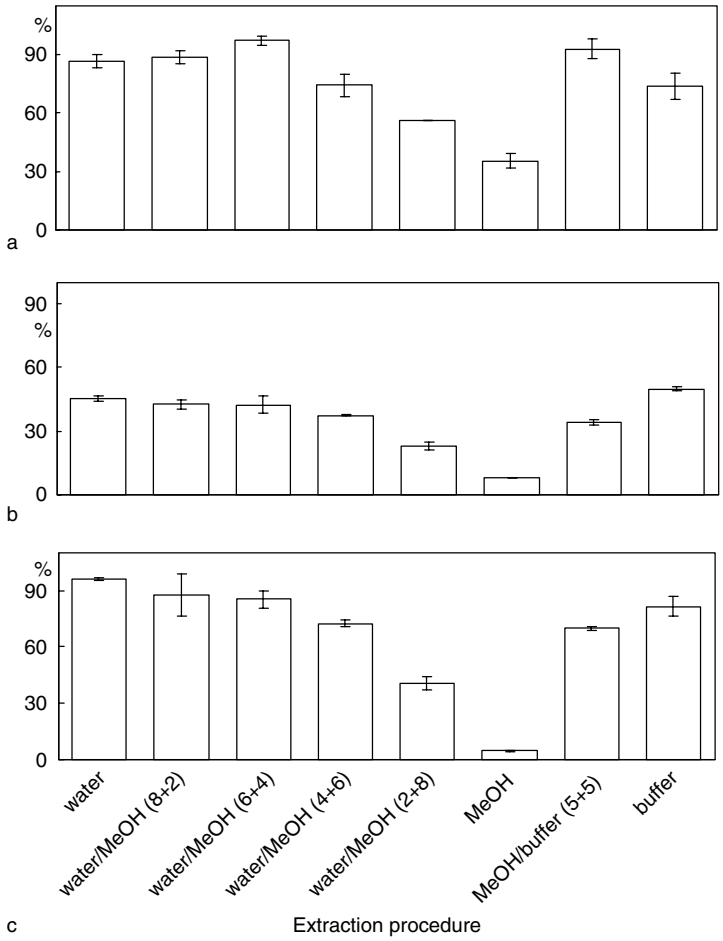

Figure 1. Average recoveries of arsenic from (a) pepper fruits, (b) stems + leaves and (c) roots by individual extraction agents (100% represents total arsenic content determined in individual plant parts).

Table 1. The extractable arsenic content and content of individual arsenic species in the extracts of pepper fruits according to individual extraction procedures; $n = 3$; data marked by the same superscript letter do not differ significantly at $\alpha = 0.05$ within individual columns of data

	Arsenic species content (μg kg^{-1})									
	Sum		As(III)		As(V)		MA		DMA	
Extraction agent	AVG	SD	AVG	SD	AVG	SD	AVG	SD	AVG	SD
Water	48.5^{d}	1.9	11.9^{e}	2.01	17.9^{c,d}	4.6	<1.2	—	18.8^{b}	0.8
Water/MeOH (8/2)	49.6^{d,e}	1.9	11.5^{e}	0.09	17.2^{c,d}	2.2	<1.2	—	20.9^{b}	0.4
Water/MeOH (6/4)	54.5^{e}	1.3	10.6^{d,e}	0.53	23.1^{d,e}	1.8	<1.2	—	20.8^{b}	0.1
Water/MeOH (4/6)	41.5^{c}	3.1	8.19^{c}	0.73	11.8^{b,c}	0.6	<1.2	—	21.5^{b}	1.8
Water/MeOH (2/8)	31.4^{b}	0.1	3.60^{b}	0.75	6.66^{a,b}	0.9	<1.2	—	21.1^{b}	1.7
MeOH	19.8^{a}	2.2	0.423^{a}	0.05	1.16^{a}	0.7	<1.2	—	18.2^{a,b}	2.9
MeOH/buffer (5/5)	52.1^{d,e}	2.8	8.62^{c,d}	0.89	26.4^{e}	7.5	<1.2	—	17.0^{a,b}	3.8
Buffer	41.4^{c}	3.8	11.7^{e}	0.09	15.7^{c,d}	2.2	<1.2	—	14.0^{a}	1.7

AVG: average; SD: standard deviation.

to arsenite followed by methylation in above-ground plant biomass.[5,7,9]

Similar trends for the extractability of the arsenicals could be observed for the different plant tissues. Increasing concentrations of methanol result in a decreasing extractability of the four arsenic compounds. This effect is more pronounced for the inorganic arsenic compounds, i.e. arsenic(III) and arsenic(V). As documented in Table 3, in

Table 2. The extractable arsenic content and content of individual arsenic species in the extracts of pepper stems + leaves according to individual extraction procedures; $n = 3$; data marked by the same letter do not differ significantly at $\alpha = 0.05$ within individual columns of data

	Arsenic species content (μg kg^{-1})									
	Sum		As(III)		As(V)		MA		DMA	
Extraction agent	AVG	SD	AVG	SD	AVG	SD	AVG	SD	AVG	SD
Water	208^{d}	6	66.9^{d}	2.6	131^{e,f}	3.6	4.53^{a}	0.49	6.01^{b}	0.26
Water/MeOH (8/2)	196^{d}	9	62.0^{d}	2.1	124^{d,e}	7.8	4.08^{a}	0.11	5.84^{b}	0.50
Water/MeOH (6/4)	195^{d}	19	62.7^{d}	4.1	123^{d,e}	15.0	3.59^{a}	0.11	5.47^{a,b}	0.17
Water/MeOH (4/6)	172^{c}	2	54.1^{c}	1.3	108^{d}	0.8	3.66^{a}	0.56	5.73^{a,b}	0.06
Water/MeOH (2/8)	106^{b}	9	34.6^{b}	0.9	63.5^{b}	9.4	2.94^{a}	0.21	5.04^{a}	0.68
MeOH	36.9^{a}	1	10.4^{a}	0.3	18.0^{a}	0.4	2.41^{a}	1.17	6.02^{b}	0.01
MeOH/buffer (5/5)	158^{c}	6	67.1^{d}	1.9	80.5^{c}	0.5	5.06^{a}	3.19	5.77^{a,b}	0.28
Buffer	208^{d}	4	66.9^{d}	2.7	131^{e,f}	0.2	4.53^{a}	1.01	6.01^{b}	0.05

AVG: average; SD: standard deviation.

Table 3. The extractable arsenic content and content of individual arsenic species in the extracts of pepper roots according to individual extraction procedures; $n = 3$; data marked by the same letter do not differ significantly at $\alpha = 0.05$ within individual columns of data

	Arsenic species content (μg kg^{-1})									
	Sum		As(III)		As(V)		MA		DMA	
Extraction agent	AVG	SD	AVG	SD	AVG	SD	AVG	SD	AVG	SD
Water	9591^{f}	64	4034^{f}	110	5092^{f}	174	445^{d}	1	19.3^{b,c}	0.9
Water/MeOH (8/2)	8751^{e,f}	1127	3672^{e,f}	537	4674^{e,f}	487	378^{b,c,d}	92	26.6^{c}	10.9
Water/MeOH (6/4)	8499^{e,f}	458	3626^{e,f}	219	4442^{d,e}	215	407^{c,d}	22	23.9^{c}	0.8
Water/MeOH (4/6)	7220^{c,d}	177	2754^{c,d}	62	4015^{c,d}	115	423^{c,d}	6	28.0^{c}	5.5
Water/MeOH (2/8)	4063^{b}	323	1569^{b}	160	2133^{b}	129	351^{b,c}	31	11.1^{a,b}	2.4
MeOH	472^{a}	32	78.5^{a}	11	302^{a}	10	86.2^{a}	11.4	5.18^{a}	0.4
MeOH/buffer (5/5)	6964^{c}	83	2722^{c}	121	3837^{c}	61	392^{b,c,d}	23	12.9^{a,b}	0.1
Buffer	8143^{d,e}	513	3304^{d,e}	262	4509^{d,e}	246	323^{b,d}	4	6.39^{a}	0.7

AVG: average; SD: standard deviation.

root samples, where the water extractable content of arsenite was 4.03 mg kg^{-1}, the methanol-extractable content was only 0.079 mg kg^{-1} (~2% of water extractable). In the case of MA, the methanol-extractable content dropped only from 0.45 mg kg^{-1} (water extractable) to 0.086 mg kg^{-1} (~19% of water extractable). The solubility of the arsenic compounds cannot be the reason for this, because MA (and its salts) are completely soluble in water and also highly soluble in methanol. Arsenous acid also readily dissolves in water (12 g l^{-1}). The arsenic concentrations determined are at least three orders of magnitude lower than the theoretical solubilities.

The different arsenic compounds can be present in the plant cell as free molecules, adsorbed to various cell compartments (such as the cell wall, mitochondria, and vacuoles), or chemically bound as a part of arsenic–phytochelatin complexes.[29] In the first case, the extraction yield is only dependent on the penetration of the extractant through the cell wall as long as the theoretical solubility is not reached. Assuming that all the examined extractants can penetrate the cell wall in the same manner, differences in the extraction yield should not be observable. Our results show that the different plant compartments are penetrated differently. For roots and fruits we observed similar, for some extractants even quantitative, extraction yields, whereas for stems + leaves the extractability never exceeded 50%. As the grinding of the samples was the same for all plant tissues, the differences in the cell structure must be the reason for these observations.

When the different arsenic compounds are not present as free molecules, but rather are adsorbed to cell compartments, additional energy in the form of conventional or microwave-assisted heating, sonication, and more intensive agitation should improve extraction yields. As we observed almost

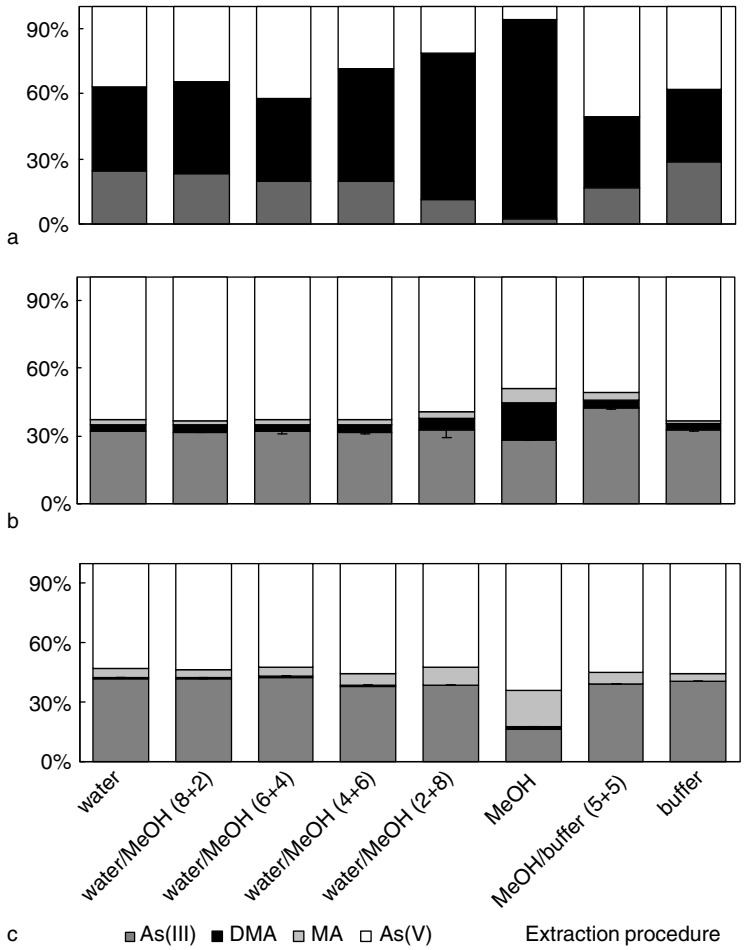

Figure 2. Distribution of arsenic species within individual extracts of (a) pepper fruits, (b) stems + leaves and (c) roots (100% represents sum of arsenic compounds determined in individual extracts).

quantitative extraction yields for fruits and roots of pepper, more-intensive extraction procedures seem to be unnecessary. In the case of stems + leaves, modification and improvement of the extraction procedure is needed for quantitative extraction of arsenic compounds. Within this experiment, the dominant role of the extraction mixture on the extraction yield, as well as on the distribution of arsenic compounds present in individual extracts, was documented. Even small changes in the experimental conditions can lead to large variations in the extractability and arsenic speciation, which makes it difficult to compare results between individual laboratories.

We cannot exclude the presence of arsenic–phytochelatin complexes, because the chromatographic conditions used throughout this work would disintegrate these complexes. Raab *et al*.[29] investigated intensively methods of extraction and separation of arsenic–phytochelatin complexes in *Holcus lanatus* and *Pteris cretica*. Only 13% of arsenic was present in phytochelatin complexes for *H. lanatus* and 1% for *P. cretica*, whereas arsenic was present dominantly in non-bound inorganic forms and DMA (*H. lanatus*). These results suggested that phytochelatins do not play a major role in arsenic storage in plant tissues.

Our results confirm the importance of sample preparation for arsenic speciation analysis in plants and, subsequently, on interpretation of analytical data. Analyzing a pooled sample (fruits and green biomass) could lead to erroneous interpretation of the data, because the different parts of a plant material can have completely different arsenic speciations and requirements for extraction. Practical assessment of the environmental impact of arsenic compounds in terrestrial plants, as affected by arsenic content in soil, can be partially limited by using different extraction procedures among individual experiments or experimental sites. Sample preparation for arsenic speciation analysis has to be optimized with respect to the part of the plant species, the parts analyzed and the arsenic compounds present, as emphasized by Francesconi.[30]

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