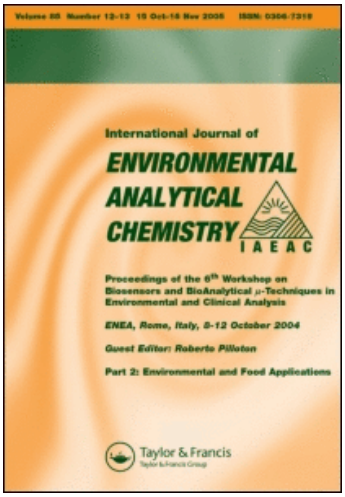

International Journal of Environmental Analytical Chemistry
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713640455

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To cite this Article Mattusch, Jürgen , Cimpean, Maria and Wennrich, Rainer(2006) 'Enzyme-assisted extraction of arsenic species from plant material', International Journal of Environmental Analytical Chemistry, 86: 9, 629 — 640
To link to this Article: DOI: 10.1080/03067310600557489
URL: http://dx.doi.org/10.1080/03067310600557489

Intern. J. Environ. Anal. Chem.
Vol. 86, No. 9, 10 August 2006, 629–640

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Enzyme-assisted extraction of arsenic species from plant material

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(*Received 7 June 2005; in final form 4 October 2005*)

Investigations regarding the transfer and metabolism of arsenic species in plants require mild extraction conditions to conserve the original composition of arsenic species. Beside the use of water or water/methanol for extraction of arsenic species from plant samples, enzymes can assist this procedure by digestion of cellulose and other constituents of cell walls, resulting in a faster, more efficient extraction technique which preserves the arsenic species. The investigations presented here were focused on the stability of certain arsenic species in enzymatic solutions, optimal conditions for their chromatographic separation and detection namely by means of ion chromatography–inductively coupled plasma mass spectrometry and improvements with respect to extraction efficiency. With commercially available enzymes and enzyme mixtures, the digestion rate of soluble starch as model cellulose was determined using high-performance anion exchange chromatography–pulsed amperometric detection analysis of glucose as the major digestion product. The most effective digestion rate (80% within 4 h) was obtained with Viscozyme®. This enzyme mixture was applied to extracted arsenic species from algae and terrestrial plant materials. Qualitative and quantitative differences in the results between enzyme-assisted and water extractions were obtained and discussed. The results show that the application of enzymes in mild extraction protocols should be evaluated as an additional step for the identification of As-metabolics in organisms. Careful selection of suitable enzyme mixtures can overcome the disadvantage that extraction efficiency is very organism-specific.

Keywords: Arsenic species; Enzyme; Extraction

1. Introduction

For the accurate determination of inorganic and organic arsenic species in plant samples, 'mild' and efficient extraction techniques [1] must be used for sample preparation prior to ion chromatography coupled with inductively coupled plasma mass spectrometry (IC-ICP-MS) analysis.

Water and/or water/methanol as elution agents [2–6] are usually combined with supporting techniques such as microwave [7, 8], ultrasound [9, 10] or pressurized solvent

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International Journal of Environmental and Analytical Chemistry
ISSN 0306-7319 print/ISSN 1029-0397 online © 2006 Taylor & Francis
DOI: 10.1080/03067310600557489

extraction [4, 11]. The extraction yields vary strongly depending on the plant structure, the degree to which they are cut up, and the extraction method used. Therefore, the results are often not reproducible and/or comparable [6, 12–15]. This can be remedied by freezing the sample under liquid N_2 with immediate homogenization, which results in both an increase in extraction efficiency and an improvement in reproducibility [12]. The disadvantages of all the well-known extraction methods are the high labour intensity and the fact that the procedure cannot easily be automated.

Enzyme-assisted extractions like the application of trypsine for As-species extraction from fish samples [16] (extraction efficiencies of 80–130%), extraction from baby food [17] with trypsine and pancreatine with efficiencies of about 100%, and the use of α-amylase for extraction from frozen apples [18] (80–104%) are described. Short extraction times for arsenic species in rice were achieved through enzymatic digestion combined with ultrasonic pre-treatment [19].

The advantages of the use of enzymes as mediator in the extraction of arsenic species could be (1) the careful and/or selective hydrolysis of cellulose, the major component of the cell walls, and the enzymatic digestion of cell components, (2) the possibility of decreasing significantly the mass of the plant material, (3) a small dilution of the sample, and (4) the opportunity for analysis of arsenic species which are not accessible with conventional techniques (water or water/methanol).

The aim of this study was to describe the additional activity of 'ready-to-use enzyme preparations' as the extraction agent on the efficiency of extraction, without species interconversion. Special emphasis was given to the stability of the encompassed analytes, arsenious acid (As(III)), dimethylarsinic acid (DMA), arsenate (As(V)), arsenobetaine (AsB), and trimethylarsine oxide (TMAO), in the enzymatic solution, as well as the effects of the enzymatic solutions on the chromatographic separation efficiency. The kinetics and efficiency of the enzymatic hydrolysis were estimated by measuring the glucose, the major product of cellulase activity. The application of the enzyme-assisted extraction was demonstrated on algae and the cultivated, arsenic-exposed terrestrial plant, nasturtium, *Tropaeolum majus*.

2. Experimental

2.1 *Chemicals and enzymes*

2.1.1 Chemicals. For microwave digestion, 65% Suprapur nitric acid and 30% Suprapur hydrogen peroxide (both from Merck) were used. DMA(V) [$(CH_3)_2As(O)ONa \cdot 3H_2O$] (Sigma), AsB [$(CH_3)_3AsCH_2COOH$] (Fluka), As(III) [$As_2O_3$] (Fluka), and As(V) (arsenic acid in water) (Titrisol, Merck) were used to prepare the standard solutions. Concentrations of arsenic species are referred to as elemental arsenic. Solutions of Roxarson (Sigma) were used to irrigate the model plant, nasturtium, *Tropaeolum majus*.

2.1.2 Enzymes. The investigations were accomplished with commercial, 'ready-to-use' enzyme solutions provided by the National Centre for Biotechnology, NCBE (University of Reading, UK) [20] in different dilutions with bidistilled water or acetate buffer. *Viscozyme L*, the most effective multi-enzyme mixture, consisted of a wide range

of carbohydrases, including arabanase, cellulase, β-glucanase, hemicellulase, and xylanase, and had a declared activity of 100 FBG g^{-1}.

3. Methods

3.1 *Extraction procedures*

3.1.1 Water extraction. For water extraction 0.2 g of the freeze-dried algae samples or 2 g of fresh plant material roughly hackled was suspended in 10 mL of bidistilled water and horizontally shaken for 24 h. The supernatant were centrifuged for 5 min with a revolution speed of 5000 rpm.

3.1.2 Enzymatic-assisted extraction. For enzymatic-assisted extraction, the algae and fresh plant samples were treated with 10 mL of acetate buffer at a pH of 5.4 and with 10 µL of the enzyme mixture. All the following procedures are similar to that for water extraction.

3.2 *Digestion procedure*

The microwave digestion was performed with a Multiwave system (Perkin Elmer) using a mixture of nitric acid and hydrogen peroxide as a reagent. Algae and fresh plant material (0.5 and 3 g, respectively) were suspended in the digestion reagent and stored overnight. Then, the samples were digested at 500 W for 10 min followed by 1000 W for 30 min.

3.3 *Arsenic species analysis*

Ion chromatography coupled with inductively coupled plasma mass spectrometry (ICP-MS) via a micro-concentric nebulizer was used to determine the arsenic species. The ion chromatograph consisted of a binary HPLC pump 126 (Gold, Beckman, Fullerton, CA) and an autosampler 507 using an injection volume of 50 µL. For fast and effective separation of the arsenic species, arsenite, dimethylarsinic acid, arsenate arsenobetaine, and trimethylarsine oxide, a gradient elution using 0.4 mM HNO_3 (eluent A) and 50 mM HNO_3 (eluent B) combined with a high-capacity anion-exchange guard and analytical column IonPac AG7/AS7 (Dionex, Sunnyvale, CA) was used [21]. Chromatograms were integrated using Microcal^{TM} Origin Peak Fitting Module software. The detailed instrumentation parameters for the ICP-MS detection and ion chromatography are summarized in table 1.

3.4 *Glucose analysis*

High-performance anion exchange chromatography–pulsed amperometric detection (HPAEC-PAD) was performed with a DX 500 consisting of an autosampler AS3500, an isocratic pump IP20, an eluent generator EG40, and an electrochemical detector ED40 equipped with a gold electrode (Dionex). Sugars such as fucose, glucose, and mannose were separated using a CarboPac PA1 (250×4 mm) analytical column

Table 1. Instrumentation parameters.

ICP-MS	
Instrument	PQ ExCell (Thermo)
Rf-power	1400 W
Ar flow rate	Coolant: 14 L min^{-1}
	Nebulizer: 0.9 L min^{-1}
	Auxiliary: 1.2 L min^{-1}
Measurement mode	Peak area of ^{75}As
Detection mode	Transient
Integration time	1 s
Ion chromatography	
Instrument	HPLC Gold (Beckman)
Anionic column	IonPac AS7 (250 × 4 mm)
Guard column	IonPac AG7 (50 × 4 mm)
Mobile phase	A: 0.4 mM HNO_3
	B: 50 mM HNO_3

(Dionex) with a potassium hydroxide gradient elution [22, 23]. To determine the enzymatic digestion efficiency, 1 g of soluble starch (Merck) was diluted in 100 mL of 0.1 M acetate buffer, pH 5.4, and 0.1 mL of the enzyme solution was added to start the reaction. The solution was thermostated at 37°C during horizontal shaking. The digestion efficiency η was calculated by equation (1)

$$\eta\ (\%) = \left(C_{\text{Glucose}} \times \frac{18.02}{m} \right) \times 100, \tag{1}$$

where C_{Glucose} is the glucose concentration (M) measured and m the weight (g) of soluble starch.

3.5 *Microscopy*

For the microscopic analysis of the enzymatic digestion process, an Olympus stereomicroscope equipped with a digital camera was used.

4. Results and discussion

4.1 *Selection of enzymes*

For the enzymatic digestion of plant materials, three types of 'ready-to-use' enzyme solutions were selected as potential candidates for the hydrolysis of the cellulose membranes of the plant cells:

- Celluclast®, a preparation containing cellulase used for breaking down cellulose into glucose, cellobiose, and longer glucose polymers (pH 4.5–6, 50–60°C);
- Thermamyl®, a preparation containing α-amylase used for liquefaction of starch (pH 7, up to 110°C);
- Viscozyme®, a carbohydrase mix prepared for the extraction of useful products from plant materials (pH 3.3–5.5, 25–50°C).

The optimal pH and temperature range is enzyme-specific and was adjusted according to the values recommended by the producer.

4.2 *Stability of arsenic species in the presence of enzymes*

First, it was necessary to clarify whether the enzymes themselves or the matrix in the enzymatic solutions have any influence on the ion-chromatographic separation of the arsenic species as well as their own stability. Therefore, an appropriate As-species-standard solution containing As(III), As(V), DMA, AsB, and TMAO was mixed with an enzyme solution at a ratio of 1 : 1 and chromatographed using ion chromatography—ICP-MS. It was found that the enzyme solutions added to the arsenic standard solution had a dramatic influence on the chromatograms, as shown in figure 1, for a solution containing Celluclast. The reason for the disturbance of the chromatographic separation can be attributed to the matrix of the enzymatic solutions that contain very high concentrations of anions and cations (table 2) like chloride, sulphate, sodium, and potassium, which are necessary for the long-term stabilization of the enzymes. An additional influence of the proteins on the separation cannot be excluded. This fact could be a disadvantage for the application of enzymes for arsenic-species extraction. However, the disturbance action of the enzyme matrix on the chromatographic separation decreased with increasing dilution factor.

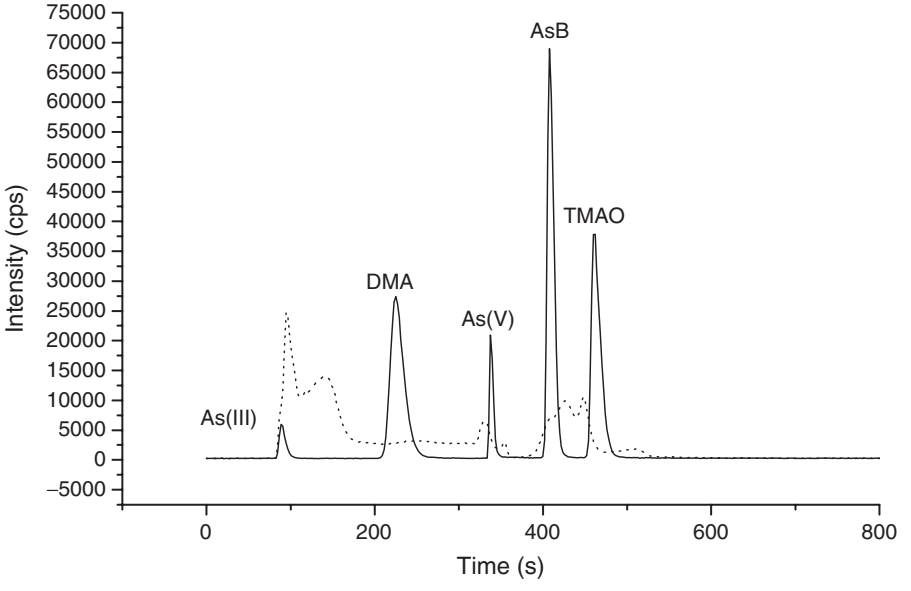

Figure 1. IC-ICPMS of an arsenic standard solution (solid line) and an arsenic-standard/enzyme (Celluclast) mixture (1 : 1) (dotted line). Conditions: 20 $\mu g\,L^{-1}$ As (arsenite, arsenate), 200 $\mu g\,L^{-1}$ As (DMA, AsB, TMAO).

Table 2. Concentration of selected anions and cations in different enzyme solutions.

Enzyme	Cl^- ($g\,L^{-1}$)	Na ($g\,L^{-1}$)	K ($g\,L^{-1}$)	Ca ($g\,L^{-1}$)	Mg ($mg\,L^{-1}$)	Fe ($mg\,L^{-1}$)	P ($mg\,L^{-1}$)	S ($mg\,L^{-1}$)
Celluclast	107	20.9	0.6	0.5	40	1	272	9.5
Viscozyme	75	53.0	2.1	nd	96	nd	950	810
Termamyl	333	69.4	2.1	2.2	77	1.8	375	13

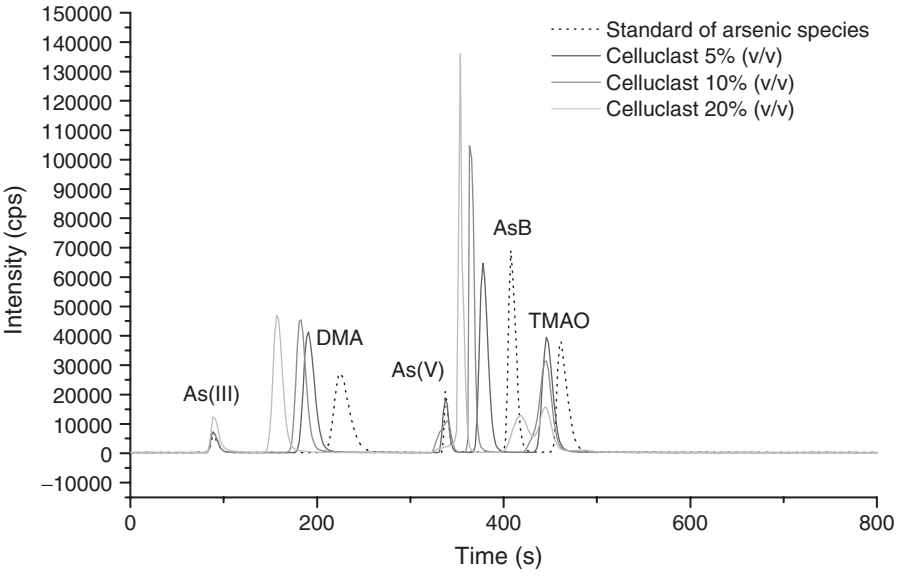

Figure 2. IC-ICPMS of arsenic standard solution without and with different amounts of enzyme Celluclast. Conditions: 20 μg L^{-1} As (arsenite, arsenate), 200 μg L^{-1} As (DMA, AsB, TMAO).

Figure 2 illustrates the shift of the peaks of the arsenic species depending on the applied enzyme concentration (Celluclast) immediately after sample preparation. At a high dilution (1 : 20) of the enzyme solution, a shift of the peak position to shorter retention times of DMA, AsB, and TMAO could be observed. The retardation of As(III) (near the void volume) and As(V) was not influenced by a dilution of 1 : 20 of the enzyme solution. At a lower dilution factor (1 : 5), As(V) seems to be reduced to As(III) to some extent, as can be seen in the increase in the arsenite signal. Two possible explanations can be given for this phenomenon: the enzyme mixture contains enzymes (impurities) that are able to reduce arsenate (reductases), or the enzymes are composed of sugars with reducing properties.

With dilutions, of more than 1 : 100 (enzyme : sample), no influence on either the chromatographic separation or the stability within 24 h (not shown) was found. Therefore, the question arose as to whether the highly diluted enzymes are able to hydrolyse the membrane cellulose fast and with a high level of efficiency.

4.3 *Kinetics and efficiency of cellulose digestion*

In a second trial, the kinetics and the efficiency of the enzymatic cellulose hydrolysis were examined using model substances (e.g., cellulose, starch, soluble starch).

To quantify the enzymatic digestion, glucose was selected as an indicator substance for two reasons: first, glucose is, as mentioned above, a major digestion product in the hydrolysis of cellulose; second, glucose can be determined sensitively and selectively beside other saccharides by means of HPAEC-PAD. The calculation of reaction efficiencies was based on the determination of glucose with respect to the initial concentration of soluble starch, which contains only glucose units. In a first experiment, the production of glucose by enzymes that contain only one enzyme

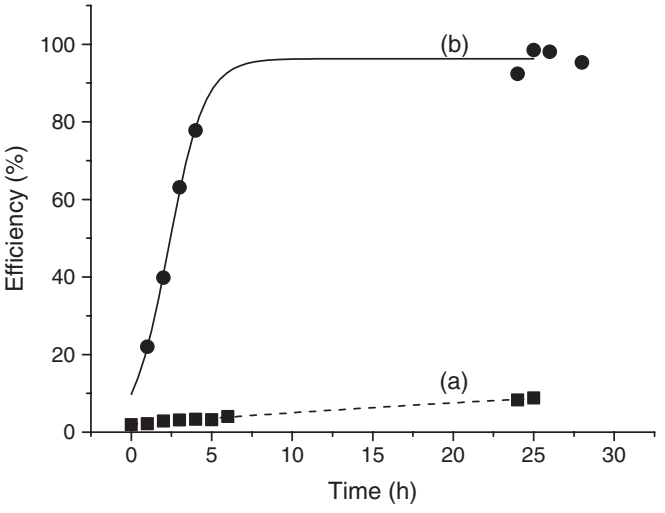

Figure 3. Dependence of the degradation of soluble starch (digestion efficiency) on reaction time. Conditions: (a) Enzyme: 0.1% (v/v) Termamyl, substrate: soluble starch, pH = 5.4, $T = 37^{\circ}C$; (b) Enzyme: 0.1% (v/v) Viscozyme, substrate: soluble starch, pH 5.4, $T = 37^{\circ}C$.

(Celluclast—(cellulase) and Termamyl—(α-amylase)) was investigated. With a 0.1% percentage solution of cellulase (Celluclast), only 2% of the assigned quantity of soluble starch was digested to glucose within a time interval of 15 h.

Somewhat higher efficiencies (approx. 9%) were obtained with α-amylase (Termamyl) as the hydrolysis enzyme and soluble starch as the model cellulose (figure 3a).

Unfortunately, these results are unsatisfactory and indicate that the enzymatic process appears time-consuming combined with a low digestion efficiency.

During a further experiment, a ready-to-use mixture of different enzymes (Viscozyme®) was used to digest soluble starch. The mix of enzymes in Viscozyme combines the properties of carbohydrases like cellulase, hemicellulase, arabase, arabinase, xylanase, and β-glucanase [20]. The enzyme mix enhances the availability of starch by degrading the non-starch polysaccharides and reduces the viscosity of the extracted material. The last parameter is important for efficient filtration, centrifugation, and subsequently injection of the plant extracts into the liquid chromatographic system.

Using this preparation as a 0.1% solution, about 70% of the initial glucose units in the form of starch could be digested within 4 h, and an efficiency of 98% was reached after 24 h as shown in figure 3(b).

With the same preparation and the same concentration, the cell-wall dismantling process was visible with microscopy, as shown with an onion membrane treated with Viscozyme (figure 4). The digestion of the cell membranes already began after 4 h, and the membranes were nearly completely destroyed after 24 h. The results show that such a rapid and efficient hydrolysis of cellulose in plant materials can be realized. To test the stability of the arsenic species and the performance of chromatographic separation, a 0.1% (v/v) Viscozyme solution containing different arsenic species was prepared. In comparison with the pure arsenic standard solution, the Viscozyme solution showed

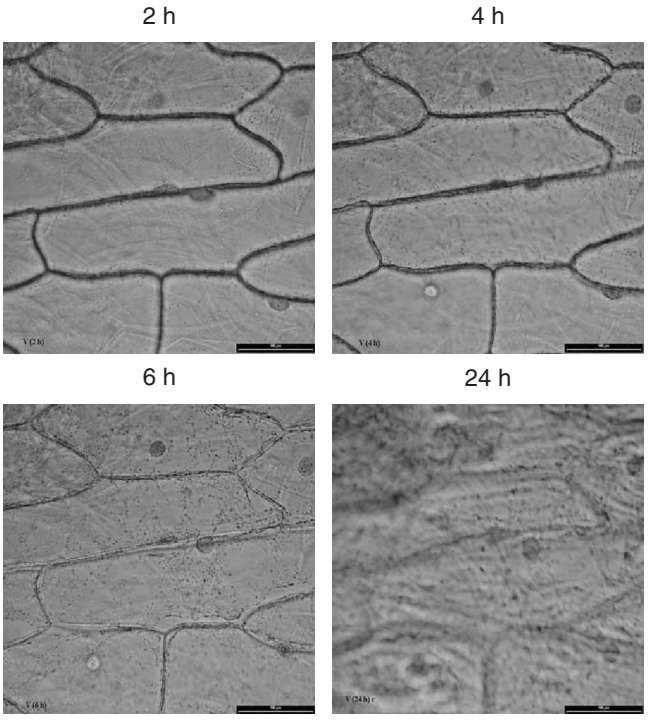

Figure 4. Time-dependent dismantling of cell walls of an onion membrane. Conditions: 0.1% (v/v) Viscozyme solution, pH 5.4.

a slight shift in the peaks to shorter retention times but a sufficient reproducibility of the peak areas and retention times for repetitive measurements to be undertaken.

4.4 *Application*

Based on the previous findings, 0.1% (v/v) Viscozyme solution with an optimal and stable pH value of 5.4 (acetate buffer) was used as a solvent to extract arsenic species from freeze-dried algae powder (figure 5) and a terrestrial plant, nasturtium, *Tropaeolum majus* (figure 6), irrigated with an aqueous solution containing roxarsone during growth under controlled conditions in a phytochamber within 6 weeks [12]. The extraction was carried out at room temperature (25°C). As can be seen in both figures and the amplifications therein, the enzyme-assisted extraction of arsenic species compared with the aqueous extraction method resulted in:

(1) differences in concentrations of arsenic species indicated at different peak heights; and
(2) slight distinctions in the arsenic species composition of the extracts, as can be seen in the additional peaks or absence of peaks, respectively.

The major components containing arsenic are present as unbound species. Beyond these compounds, arsenic species are also bound to high molecular structures that are only extractable after exoneration from the matrix. A broad spectrum of high-molecular weight organic compounds forming cell walls and membranes of plastides

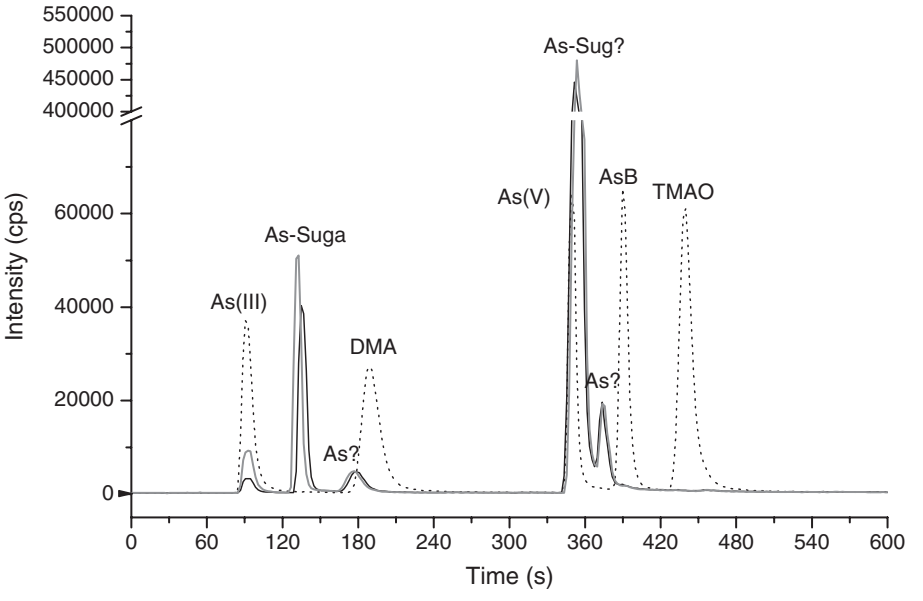

Figure 5. Comparison of water- and enzyme-assisted extraction of brown algae. Conditions: extraction time 24 h, enzyme concentration 0.1% (v/v) Viscozyme, $T = 25°C$; dotted line: arsenic standard solution: 200 $\mu g\,L^{-1}$ As of each compound As(III), DMA, As(V), AsB, TMAO; grey line: with enzyme; black line: without enzyme.

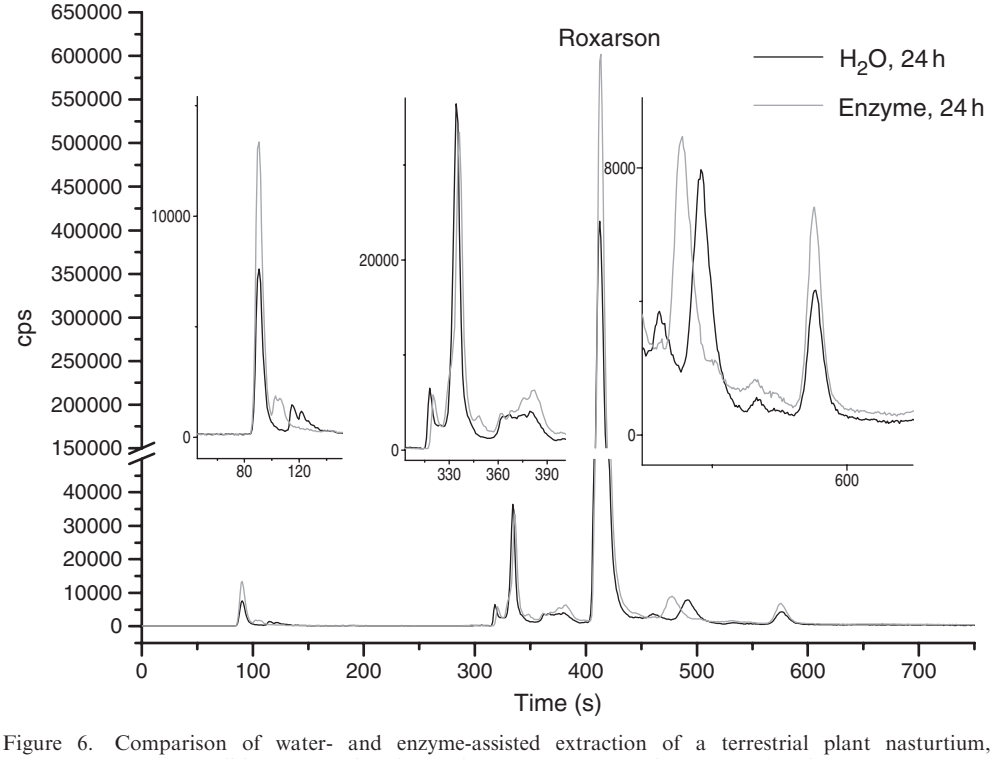

Figure 6. Comparison of water- and enzyme-assisted extraction of a terrestrial plant nasturtium, *Tropaeolum majus*. Conditions: extraction time 24 h, enzyme concentration 0.1% (v/v) Viscozyme, $T = 25°C$.

like phospholipids and cellulose derivatives can act simultaneously as potential candidates for arsenate incorporation in their structure. The occurrence of arsenolipids [24], arsenosugars [25] arsenic phytochelatine complexes [26] and a number of other compounds containing arsenic are the evidence that this transfer takes place, and therefore novel compounds containing arsenic should be detected after enzymatic treatment of the plants.

The identification of these novel enzyme-extracted arsenic species is of interest for two reasons: (1) Are the arsenic compounds rudiments of higher molecular mass units and where they are bound? (2) Are the detected compounds containing arsenic naturally occurring, and from which plastids do they originate?

To answer these questions, on the one hand more selective enzymes must be included in the investigations, and on the other hand methods for separations of different cellular constituents are needed to locate their binding.

With current technology, it is very difficult to identify the unknown arsenic species because of both the lack of reference substances and their occurrence at very low concentrations insufficient for their identification by means of ESI-MS.

Semi-quantitative results for the extracted arsenic species obtained after an extraction time of 24 h using water and enzyme solution, respectively, are given in figure 7. Here, the number of species occurring in different enzymatic algal extracts is indicated

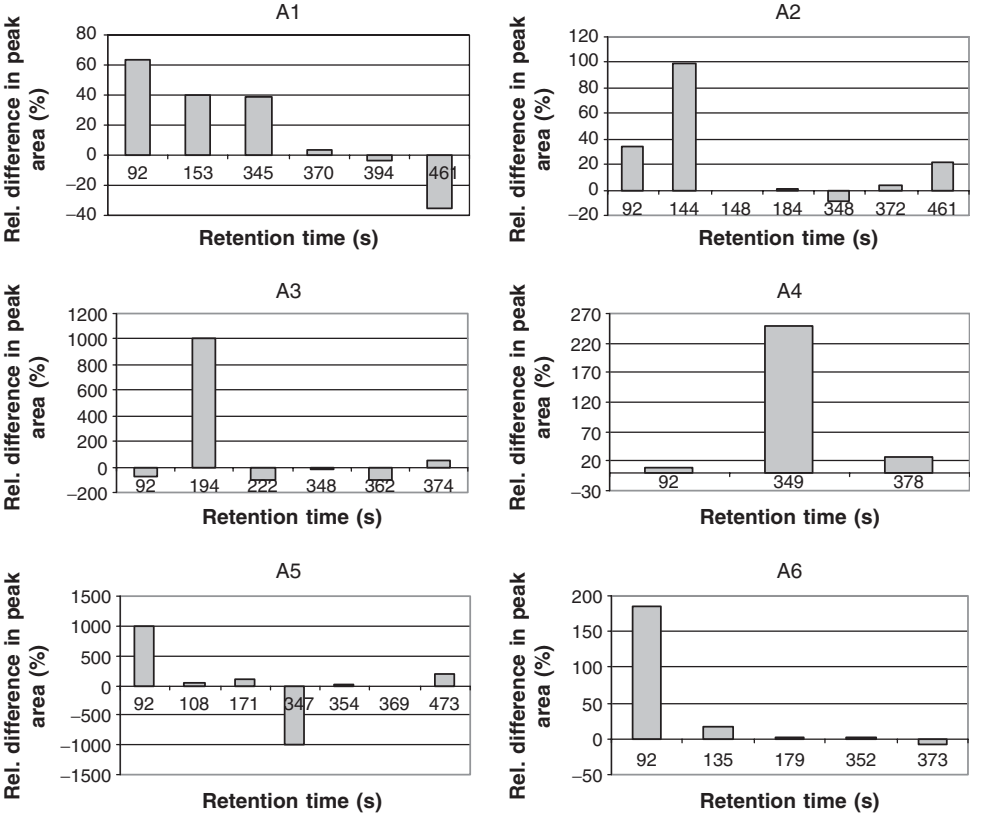

Figure 7. Comparison of water and enzymatic extraction of algae. For identification of A1–A6, see table 3.

Table 3. Comparison of the total arsenic concentration in the water extract ('Water'), enzyme-assisted extract ('Enzyme'), and after microwave digestion (MW).

Algae names	Sample number	Arsenic concentration ($mg\,kg^{-1}$)		
		Water	Enzyme	MW
BCR 279	A1	3.1	4.3	2.9
Hijiki A	A2	77.2	82.4	103
Sechee	A3	26.6	28.6	33.4
Black moss	A4	1.9	2.3	27.2
Hijiki B	A5	29.7	32.3	68.9
Hijiki C	A6	52.0	54.5	115

by the retention times and their relative concentrations related to the water extract, given as the difference between the peak areas obtained by enzyme extraction and water extraction. Negative columns suggest a lower extraction efficiency using enzymes and vice versa. The results seem to be confusing, because they cannot be summarized under one uniform scheme. Each sample has to be observed separately because the plants investigated are quite different in structure and consistency. Also, the metabolism of arsenate in the plants is itself versatile and depends on the growth conditions as well as plant functions. The extracts of six freeze-dried algae samples were analysed and the results compared with the total arsenic concentrations resulting from a microwave digestion (table 3). In general, the concentration of arsenic enzymatically extracted (and calculated from the sum of the As-species detected) is somewhat higher than with water extraction but is still much lower than total arsenic. Especially in the case of black moss (dulse), the enzymatic extraction efficiency was only 8%. This special behaviour is explained by the fact that the cell walls of this algae consist of lime [27] among other substances. Therefore, it can be assumed that arsenic is also bound as insoluble $Ca_3(AsO_4)_2$.

5. Conclusion

The results of this study show that low enzyme concentrations have no influence on the stability of different arsenic species and do not influence the chromatographic determination of arsenic species by means of HPLC(IC)-ICP-MS. It was also demonstrated that arsenic species contained in different plant materials were more effectively extracted by enzymes than by using only water as eluent. A higher efficiency than single enzymes seems to be achieved with mixtures of enzymes, as can be seen in the fast digestion of soluble starch using *Viscozyme L*. The enzyme-assisted extractions lead to supplementary results in comparison with the aqueous extraction method. Further investigations are necessary to optimize the enzyme combinations and the reaction conditions.

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