

Determination of Arsenic Species in *P. calomelanos* and *N. biserrata*

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O sítio industrial abandonado na Baía de Sepetiba (SE-Brasil) está entre os maiores passivos ambientais contaminados com Zn no mundo e o As também se encontra em concentrações alarmantes. A vegetação no local é escassa e das espécies que sobrevivem no local *Pityrogramma calomelanos* e *Nephrolepis biserrata* são as mais abundantes. Concentrações de As de até 2271 mg kg⁻¹ foram detectadas na espécie *Pityrogramma calomelanos*, sendo que essa espécie foi mais eficiente que *N. biserrata* no acúmulo desse elemento. Utilizou-se cromatografia de exclusão por tamanho (SEC) com detecção UV-Vis e espectrometria de massas com plasma indutivamente acoplado (ICP-MS) em sistema “on line” para monitorar a distribuição de As na fração orgânica. As maiores concentrações de As foram determinadas na fração extraída com TRIS-HCl, pH 7,4 (entre 60-100% da concentração total de As) mostrando uma evidente associação desse elemento com bioligantes, entre eles, fitoquelatinas e glutatona.

The abandoned industrial site at the Sepetiba Bay (SE-Brazil) is one of the most heavily contaminated sites on the earth's surface concerned to Zn, but also presents As in alarming concentrations. Vegetation on this site is scarce and under the surviving species *Pityrogramma calomelanos* and *Nephrolepis biserrata* are the most abundant ones. Total As concentrations up to 2271 mg kg⁻¹ in *P. calomelanos* were determined, being this specie more efficient in arsenic accumulation than *N. biserrata*. The size exclusion chromatography (SEC) with UV-Vis detector coupled to inductively coupled plasma mass spectrometry (ICP-MS) was used to screen the distribution of As between different molar mass fractions. Highest concentration of As was found after extraction with TRIS-HCl, pH 7.4 (about 60-100% of total As) characterizing an association of this element with bioligands including phytochelatin and glutathione.

Keywords: terrestrial plants, phytochelatin, arsenic, speciation, SEC-UV-Vis-ICP-MS

Introduction

Sepetiba Bay is located 60 km from Rio de Janeiro city. Large population inflows and the construction of a harbour in the early 70's made the area further attractive for industrial development. In the past, the lack of environmental protection laws, society pressure and industrial social responsibilities had brought as a consequence a series of spots containing open-air waste piles resulting from the industrial activities. One such spot is located in an abandoned Zn-producing in the Engenho Inlet inside Sepetiba Bay. In this area, Zn was extracted from calamine or willemite and arsenic trioxide

(As₂O₃) was used in a step of the purification process. An estimated amount of about 3 million tons of toxic waste has been accumulated in Sepetiba Bay since 1969, and concentrations of Zn, Cd, Pb and As in soil reach up 15%, 0.1%, 0.5% and 250 mg kg⁻¹, respectively.^{1,2}

Vegetation in this site is scarce and under the surviving species *Pityrogramma calomelanos* and *Nephrolepis biserrata* are the most abundant. Studies regarding to the presence of arsenic species in plants are especially important considering their appearance in the food chain. The high level of arsenic in the studied area is a unique opportunity to investigate arsenic uptake by terrestrial plants naturally growing on a highly contaminated site.

It is well known from the literature that in mammals and marine organisms inorganic arsenic suffers

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biotransformation by two main processes: biomethylation or arsenic binding in organic molecules such as arsenobetaine and arsenosugars, which are considered non-toxic.³⁻⁵ However in plants arsenic detoxification seems to be related to phytochelatin that are peptides biosynthesised from glutathione GSH (γ -Glu-Cys)_n-Gly with n varying from 2 to 11, which are enzymatically synthesized by PC-sintetase.⁶⁻¹¹

The most common techniques for separation and detection of biomolecules are a combination of separation techniques (such as liquid chromatography (LC), gas chromatography (GC), electrophoresis) with ICP-MS or graphite furnace atomic absorption spectrometry (GF AAS) for arsenic detection and UV-Vis for molecule screening.¹²

The aim of this work was to characterise arsenic species in *P. calomelanos* and *N. biserrata* growing in a highly contaminated area in order to provide some insight into the mechanisms involved in arsenic tolerances by plants species.

Experimental

Equipments

For speciation studies a chromatographic system composed by an isocratic bomb LC-200 (Perkin Elmer, CT, USA), a SEC column Superdex peptide 10/300 GL (Tricorn), an UV-Vis detector (Perkin Elmer) and an ICP-MS ELAN 5000A (Perkin-Elmer-Sciex) was used. Arsenic determination in extracts was performed by HG-ICP-MS using flow injection system (FIAS 200, Perkin Elmer) coupled to ICP-MS. Additionally, an ICP-MS model ELAN 6000 (Perkin Elmer-Sciex) was also used for total arsenic determination in plant samples.

Reagents and standards

Suprapur acids from Merck (Darmstadt, Germany) and water doubly distilled in quartz were used for the preparation of samples and standard solutions. All other reagents were of analytical reagent grade. A stock standard solution of As (1000 mg kg⁻¹ in 5% v/v HNO₃) used for calibration in ICP-MS determinations was obtained from Merck. Fresh working standard solutions were prepared daily by dilution of stock solutions with water. The buffer solution used for extraction consisted of 10 mmol L⁻¹ TRIS-HCl buffer (Sigma-Aldrich, Steinheim, Germany). The buffer pH was adjusted to 7.4 with an appropriate amount of 1 mol L⁻¹ hydrochloric acid. Cystein (104 Da), metallothionein (7 kDa), insulin (6 kDa) and Dextran blue (20 kDa) were used for column calibration.

Sampling and sample preparation

Plants were collected in a highly contaminated site located in Sepetiba Bay, SE-Brazil. This area has been widely studied mainly in relation to toxic elements contamination, identifying arsenic,¹³ chromium, cadmium and zinc as the main pollutants.¹⁴ Two plant species (*Pityrogramma calomelanos* and *Nephrolepis biserrata*), showing similar sizes, were collected in two sampling sites: along drainage channel (DC) and in a waste pile (WP). Then, they were packed into paper bags and transported to the laboratory. Immediately, samples were washed several times with deionised water (Milli-Q from Milipore, specific resistivity of 18 M Ω cm) and air-dried. Microscopic inspection of plant did not show any visible deposits of solid matter on the surface of them. For each group of 4 plants a composite sample was prepared. Each composite sample was mechanically ground using a stainless steel grinder, and then by careful homogenisation of the powder. These composite samples were stored in clean polyethylene tubes and kept frozen (-4 °C) until analysis.

Total arsenic determination

Four replicates of 500 mg of each composite plant samples were digested with 6 mL of 2-fold sub-boiled (Berghof, model Distill acid BSB-939-IR, Eningen, Germany) HNO₃ (Merck, 65% m/m) and 3 mL of H₂O₂ (Merck Suprapur[®], 30% v/v). For digestion, samples were placed in 50 mL graduated closed polypropylene tubes, which were placed into a heating block and kept at 90 °C for 12 h. After cooling, 3 mL of H₂O₂ (Merck Suprapur[®], 30% v/v) were added and digestion continued during 3 h. Sample extracts were then completed to 10 mL and centrifuged to separate small insoluble portion. This insoluble fraction was discharged and not considered for the results. Digests were diluted 10 times before the determination. The total arsenic determination was performed by ICP-MS, using ¹⁰³Rh as internal standard. A certified reference material (CRM) of tomato leaves (NIST, SRM 1573a, Gaithersburg, MD, USA) was used to check the accuracy of the analytical technique.

Extraction procedures and efficiency

Aliquots of 400 mg (n = 3) of each composite plant sample were used for organic complexes extraction in three sequential steps, according to Połéc-Pawlak *et al.*¹⁵ The extraction procedure consisted basically of three steps: (i) (E1) - extraction with 10 mmol L⁻¹ TRIS-HCl (pH 7.4) for aqueous complexes removal located at cytoplasm and vacuoles; (ii) (E2) - extraction with 1% (m/v) sodium dodecyl

sulfate (SDS) solution in 10 mmol L⁻¹ TRIS-HCl (pH 7.4) for hydrophobic proteins removal and (iii) (E3) - extraction with 10 mmol L⁻¹ ammonium acetate solution (pH 4.6) for removal of small organic acids with low molecular mass. The extraction procedures were performed with 10 mL of extracting solution under agitation during 1 h with shaker, followed by centrifugation during 40 min (12000 rpm) at 5 °C in the same vessel.

In order to investigate the extraction efficiency, an aliquot of the extract obtained was digested as previously described. The determination of total arsenic concentration in those extracts was performed by hydride generation, ICP-MS (HG-ICP-MS). The extracts were diluted 100 times before the determination.

Determination of inorganic arsenic species

In order to obtain complementary information about As chemical forms present in the plant extracts, arsenic concentration was determined by HG, using FIAS 200 system with NaBH₄ solution (0.4% m/v, Merck) coupled to ICP-MS. Hydride generation allows elimination of most part of the sample matrix and because of that, the possible spectral polyatomic interference caused by the presence of chloride in the samples (⁴⁰Ar³⁵Cl⁺ interferes on ⁷⁵As⁺) is also avoided.

The procedure used for arsenic determination in extracts was similar to described by Stroh *et al.*¹⁶ Firstly, the As(III) was determined by direct injection of the original extracts in the HG-ICP-MS system. A second aliquot of the original extract was used to determine total inorganic As concentration (III + V) by pre reduction of As(V) to As(III) with 1 mL of KI (5% m/v, Merck) solution plus ascorbic acid (5% m/v, Sigma-Aldrich) and 3 mL of concentrated HCl (Merck, 37% m/m), allowing this mixture to stand during 15 min before the HG-ICP-MS analysis. The concentration of As(V) was obtained by the difference between total inorganic arsenic and As(III) concentration. The original extract was used to determine total arsenic concentration (As(III) + As(V) + As organic), as follows: (i) arsenic oxidation in closed polypropylene tubes with concentrated sub-boiled HNO₃ placed in a digestion block at 80 °C overnight; evaporation of HNO₃ in excess; (ii) pre reduction of As(V) to As(III) and arsenic determination by HG-ICP-MS. Comparing the results obtained after complete oxidation of the extracts with HNO₃ show information about the arsenic fraction associated to organic compounds (proteins, peptides, etc.).

Organoarsenicals characterization

For organoarsenicals characterization, the extracts obtained were immediately submitted to chromatographic

analysis using SEC-UV-Vis-ICP-MS. For connection of UV-Vis detector output to ICP-MS a PEEK® tube was used. A Superdex peptide 10/300 GL column, UV-Vis detector for macromolecules monitoring and ICP-MS detector for ⁷⁵As⁺ monitoring were used. The elution times of cystein (104 Da), metallothionein (7 kDa), insulin (6 kDa) and Dextran blue (20 kDa) were used to estimate the molecular mass. The analytical curve was plotted by injection of solutions containing adequate arsenic concentrations, prepared in the same medium used to extract the analyte from sample. This medium was also used as blank solution to compensate possible non-spectral interferences caused by saline matrix. The accuracy and repeatability of the results obtained were evaluated by analysis of a certified reference material (CASS-4, seawater from National Research Council, Ottawa, ON, Canada) in each set of samples.

Results and Discussion

For evaluation of method accuracy, including samples digestion and arsenic determination by ICP-MS, certified reference material (SRM 1573a NIST) was analysed. Acceptable agreement was obtained (114%, n = 4) with similar repeatability to that recommended by NIST. Accuracy verification of HG-ICP-MS method was made by analysis of a seawater certified reference material (NRCC CASS-4) with good agreement (105%, n = 5) and RSD about 14% for 1 µg L⁻¹ solution. The limit of detection (LOD) was calculated by the ratio of three times the standard deviation of the blank (n = 10) and the slope of calibration curve. The limit of quantification (LOQ) was obtained by 3.3 times the LOD. The LOD and LOQ were 0.010 and 0.033 µg kg⁻¹, respectively.

Determination of total arsenic concentration

Table 1 shows total arsenic concentration in plant species in both sampling points. It can be seen that arsenic concentrations are higher in *P. calomelanos* when compared to *N. biserrata*, regardless of the sample location.

P. calomelanos belongs to the same order (Pteridales) of *Pteris vitatta*, which is worldly recognised as specie that hipperaccumulates arsenic. The potential of this plant for phytoremediation of soil contaminated with arsenic has already been reported.¹⁷ The lower and similar arsenic concentrations in *N. Biserrata* observed in both sampling points (Table 1) suggests that some specie did not appear reflecting the arsenic contamination of those sites, as already observed.¹⁸

Although the number of samples was small, *P. calomelanos* showed large difference in arsenic

Table 1. Total As concentration (n = 4) in sample plants and in each extract (n = 3) and extraction efficiency

Sample	Total As / (mg kg ⁻¹)	E1 / (mg kg ⁻¹)	E2 / (mg kg ⁻¹)	E3 / (mg kg ⁻¹)	Extraction efficiency / (%)
<i>P. calomelanos</i> (DC)	2271.0 ± 47.0	854.0 ± 24.0	278.0 ± 12.0	266.0 ± 21.0	60
<i>P. calomelanos</i> (WP)	510.0 ± 32.0	202.0 ± 9.8	78.9 ± 0.8	35.8 ± 1.3	60
<i>N. biserrata</i> (DC)	26.4 ± 5.8	7.6 ± 0.3	1.95 ± 0.20	1.2 ± 0.2	40
<i>N. biserrata</i> (WP)	36.7 ± 2.7	23.7 ± 0.6	13.2 ± 0.6	5.4 ± 0.3	115

concentration between the drainage channel (DC) and the waste pile (WP) sampling points. Considering that the samples were collected at the same polluted sites, these variations may reflect differences in arsenic bioavailability between sampling location. According to Bech *et al.*,¹⁹ in soils with high total arsenic concentration, the available arsenic fraction seems to be strongly influenced by soil texture and pH. Moreover, differences in mineral content, especially Fe and Mn content in sampling locations may also interfere in arsenic bioavailability.¹⁷

Table 1 also shows mean (n = 3) of total arsenic concentration in each extract of the plants. Usually, extraction of arsenic species from plants is carried out with water/methanol mixtures¹⁶ but the extraction efficiency is low. The results obtained show that the main portion of total arsenic is extracted with 10 mmol L⁻¹ TRIS-HCl (pH 7.4, E1). When compared to the total arsenic concentration in the plant species it can be seen that approximately 60% of As in *P. calomelanos* is extractable, regardless arsenic concentration. These results are in agreement with data recently reported²⁰ in a study of arsenic speciation analysis using sequential extraction (water/SDS).

However, it is important to notice that for *N. biserrata* arsenic extraction efficiency is different (40% and 115% for DC and WP, respectively) depending on the sample location. Considering that this species shows lower arsenic tolerance compared to *P. calomelanos*, this is probably related to differences in arsenic species distribution in the environment. The higher anaerobic conditions in the drainage channel (DC) sampling point compared to waste pile (WP) one probably favours As(III) presence.²¹

Arsenic speciation

Based on the previous results we investigated which arsenic species were present in sample extracts. Table 2

shows the results obtained only from extract 1 (E1) due to its higher arsenic extraction efficiency.

The distribution of arsenic species shows that inorganic arsenic is present in all samples, with As(V) being the predominant species when compared to As(III). The only exception is for *N. Biserrata* in the drainage channel (DC) where As(III) concentration is approximately twice higher than As(V).

Data from literature shows that, due to its similarity to phosphate, arsenate can be taken up by plants through phosphate transporters on the plasma membrane of root epidermal cells.²²⁻²⁵ Once inside the plant As(V) is reduced to As(III) and subsequently reacts with SH-groups.²⁶ So, it seems that the different behaviour presented by *N. biserrata* in both sampling locations suggests that this species has more efficient control mechanisms of arsenite directly uptaken by the roots. The highest As(III) concentration in DC is probably associated to As(V) reduction inside the plant.

In general, partial mass balance indicates that As present in these plants (Table 2) is mainly associated to organic fraction, varying from 62 to 88% of total As mass present in extract 1 (E1).

In order to characterize whose organoarsenicals species were predominantly present, all plant extracts (E1, E2 and E3) were analysed by SEC-UV-Vis-ICP-MS. In general, the UV-Vis chromatograms for *P. calomelanos* and *N. biserrata* showed peaks with similar retention times regardless sample location.

The SEC-UV-Vis and the ICP-MS chromatograms (Figures 1 and 2) reveal that *Pityrogramma calomelanos* species presents four peptides peaks associated to As. The retention times obtained by these peaks were used to identify molecular mass range of those peptides and the results are shown in Table 3. Peak 1 presents an elution volume too close to the exclusion limit which makes it

Table 2. As speciation by HG-ICP-MS in the extract 1 (TRIS-HCl 10 mmol L⁻¹, pH 7.4) from all sample plants (mg kg⁻¹)

Extract 1 (E1)	As-total	As (III)	As(III) + As(V)	As(V)	As-organic
<i>P. calomelanos</i> (DC)	854.0	42.6	150.0	107.0	704.0
<i>P. calomelanos</i> (WP)	202.0	21.4	75.0	53.6	127.0
<i>N. biserrata</i> (DC)	7.6	0.8	0.9	0.5	6.7
<i>N. biserrata</i> (WP)	23.7	1.1	6.4	5.4	17.3

difficult to characterize. Peak 2 corresponds to metallic complexes with ligands of molecular mass ranging from 529 to 567 Da, characteristics of phytochelatins, probably PC_2 ($(\gamma\text{-Glu-Cys})_n\text{-Gly}$, $n = 2$, $MM = 539$ Da).

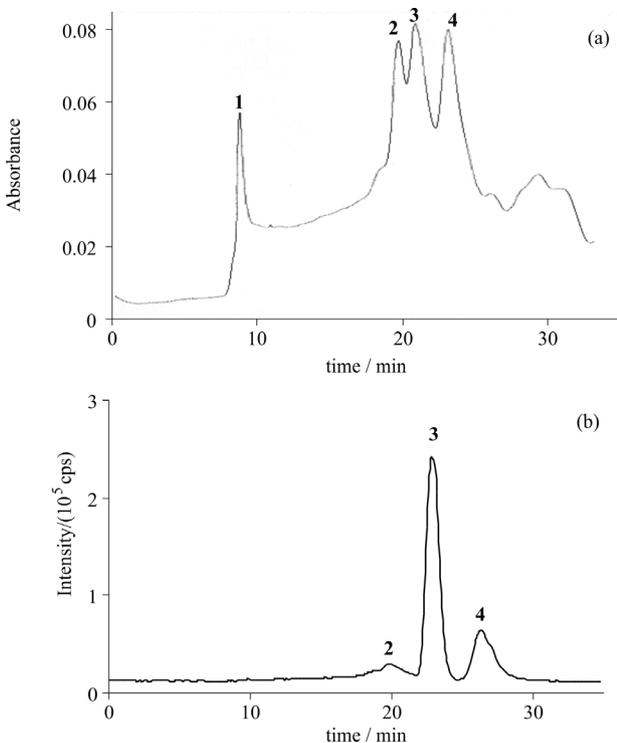


Figure 1. Chromatograms obtained by SEC-UV-Vis-ICP-MS (Superdex peptide column) for extract 1 (E1) of *Pityrogramma calomelanos* specie. (a) UV-Vis detector monitoring peptides; (b) ICP-MS detector monitoring arsenic.

Arsenic complexes in *Pteris vittata* bound to PC_2 have already been identified.²⁷ Other authors studying cells suspensions of *R. serpentina* showed that in the presence of arsenite/arsenate predominates PC_2 , while PC_3 was predominated in presence of cadmium.⁶ Peak 3 corresponds to metallic complexes with ligands of molar mass between 342 and 363 Da, probably glutathione (GSH), a tri-peptide containing glutamic acid, cystein and glycine. On the other

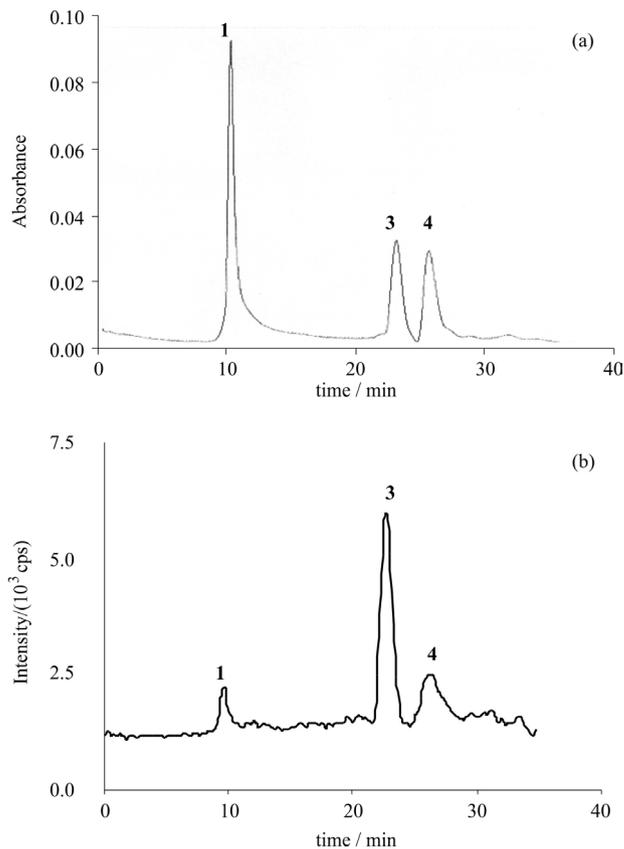


Figure 2. Chromatograms obtained by SEC-UV-Vis-ICP-MS (Superdex peptide column) for extract 3 (E3) of *Pityrogramma calomelanos* specie. (a) UV-Vis detector monitoring peptides; (b) ICP-MS detector monitoring arsenic.

hand, metallic complexes of molecular mass below 300 Da (peak 4) may be a result of proteins degradation. According to Poléc-Pawlak *et al.*,¹⁵ in a study of Cd speciation in *Arabidopsis thaliana*, this peak may correspond to metallic complexes with cysteine.

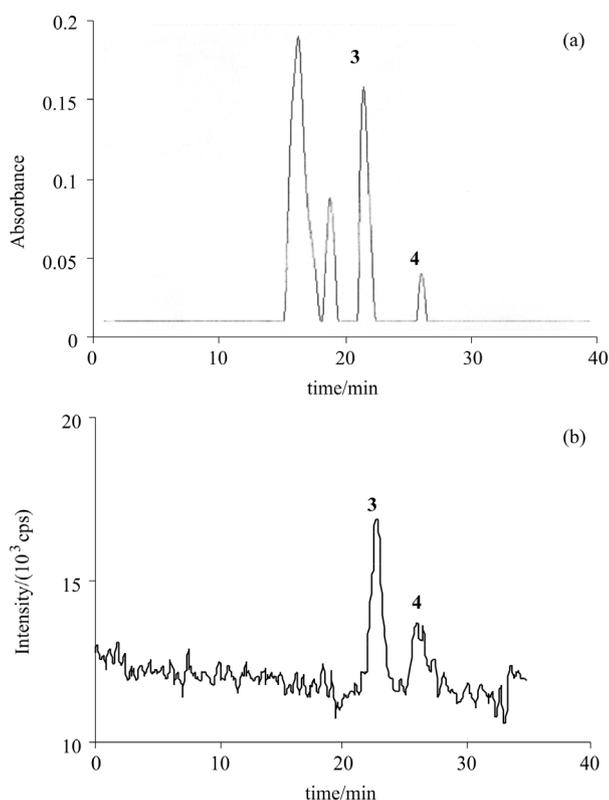
The SEC-UV-Vis and the ICP-MS chromatograms (Figure 3) reveal that *Nephrolepis biserrata* specie presents two proteins peaks associated to As. The retention times obtained by these peaks were used to identify those proteins (Table 3). Peak 3 corresponds to As associated to GSH and

Table 3. Retention times (average and standard deviations, $n = 6$); elution volumes; constant distribution (K_{av}) and molar mass range of the proteins from *Pityrogramma calomelanos* and *Nephrolepis biserrata* obtained using SEC-UV-Vis (a Superdex peptide 10/300 GL column was used)

Peak	Retention time / min	Elution volume / mL	K_{av}	Molecular Mass range / Da
<i>Pityrogramma calomelanos</i>				
2	20.3 ± 0.1	16.2-16.4	0.525-0.535	529.0-567.0
3	21.6 ± 0.1	17.2-17.3	0.586-0.590	342.0-363.0
4	24.8 ± 0.6	19.3-20.3	0.716-0.778	89.0-140.0
<i>Nephrolepis biserrata</i>				
3	21.7 ± 0.1	17.2-17.4	0.590-0.601	327.0-354.0
4	25.9 ± 0.2	20.6-20.9	0.794-0.811	70.1-79.2

Table 4. Average As concentration (mg kg⁻¹, n = 3) associated with protein peaks from *Pityrogramma calomelanos* and *Nephrolepis biserrata* obtained using SEC-UV-Vis-ICP-MS (a Superdex peptide 10/300 GL column was used)

Sample	Peak 1	Peak 2	Peak 3	Peak 4	Total As	Column recovery / (%)
<i>P. calomelanos</i> (DC)	10.21	28.90	492.9	154.5	1398.0	49
<i>N. biserrata</i> (DC)	-	-	4.6	2.5	10.8	66
<i>P. calomelanos</i> (WP)	2.58	15.34	84.6	27.0	316.7	40
<i>N. biserrata</i> (WP)	-	-	16.0	10.1	42.3	64

**Figure 3.** Chromatograms obtained by SEC-UV-Vis-ICP-MS (Superdex peptide column) for extract 1 (E1) of *N. biserrata* specie. (a) UV-Vis detector monitoring peptides; (b) ICP-MS detector monitoring arsenic.

peak 4 corresponds to As complexes with bioligands of molecular mass below 300 Da, probably cystein.¹⁵

As distribution among plant species

Table 4 shows arsenic concentration in each fraction obtained after separation in Superdex peptide column compared to arsenic total concentration presented in the original extract. These results show that the sum of As mass in extract 1 (E1) from *P. calomelanos* in both sampling locations corresponds to approximately 45% of total As present in this extract. On the other hand, for *N. biserrata* this percentage was higher (about 65%). It is uncertain the reason for this incomplete chromatographic recovery. However we can speculate that the lower arsenic recoveries obtained for *P. calomelanos* specie are possibly

related to its higher As mass present and perhaps, a fraction of this element may be retained in the column. On the other hand, arsenic peaks quantification in the *N. biserrata* species may be a difficult task, considering that their intensity is close to the baseline and peaks 1 and 2 were not observed.

Conclusions

The behaviour of *N. Biserrata* suggests that this specie is physiologically tolerant to As due to its lower total As concentration compared to *P. calomelanos* and relatively higher content of As bound to organic fraction. This may suggest that this specie controls arsenic uptake. Moreover, once inside the plant inorganic arsenic is reduced by a detoxification mechanism that involves proteins and/or peptides. Whether or not phytochelatins play an important role in the arsenic detoxification mechanisms of plants is still a matter of debate, but the results obtained in this study confirm data reported in the literature which indicate that PCs are induced in the presence of arsenic.

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