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DEVELOPEMENT AND APPLICATION OF COUPLED METHODS FOR ARSENIC SPECIATION STUDY

Theses of the doctoral (Ph.D.) dissertation of

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1 INTRODUCTION

The number of arsenic speciation studies has been quite numerous during the last decades, since the toxicity of arsenic highly depends on the chemical form in which it is present. Studies in laboratory animals have demonstrated that the toxicity of arsenic is depended on its form and oxidation states.

Humans are exposed to many different forms of inorganic and organic arsenic species in food, water and other environmental media. Each of the forms has different physicochemical properties and bioavailability and therefore the study of the kinetics and the metabolism of arsenicals in animals and humans is a complex matter.

Arsenic speciation studies in environmental and biological samples are of expanding interest in the last decades parallel with the recognition of the dissimilar toxicity and biochemical behavior of the different arsenic molecules. Investigations of the chemical constituents of aquatic organisms can provide useful information about the environment as well as toxicologically relevant data about the composition of biological species consumed by humans. It is well known that different arsenic compounds can be accumulated by aquatic biota, especially by marine algae, fish and bivalves, and at the endpoint of the food chain contaminants can reach terrestrial animals and humans. It is also well established that the toxicity of arsenic highly depends on the chemical form in which it is present. For instance, marine fish contain mostly arsenobetaine (AB, Fig. 1), which is non-toxic, and hence arsenic accumulated in this form represents no health hazard. On the other hand, some organisms contain considerable amounts of inorganic arsenic (known to be toxic) or arsenosugars (the toxicity of which is still unknown): The use of these organisms as human food may have toxicological consequences.

During the last decades most of the arsenic speciation studies in aquatic systems have focussed on marine ecosystems, while reports about freshwater ecosystems are still scarce. One reason for this is that the arsenic concentrations in marine organisms are usually much higher then in freshwater organisms, which facilitates arsenic analysis in the marine ecosystem. Although "seafood" products are primarily of marine origin, there are numerous countries, like Hungary, where fish of freshwater origin form a substantial part of the local diet. The possible human health consequences of arsenic in food have resulted in several countries imposing a maximum permissible concentration for this element. Usually this concentration is based on total arsenic, although it is well recognized that the various arsenic species found in food items vary greatly in their toxicity, from the non-toxic AB to the toxic inorganic arsenic species arsenite (As^{III}) and arsenate (As^{V}). Future discussions in the European Union on trace elements and food

safety are likely to include consideration of the elemental species present, and legislation will be framed accordingly.

For determination of arsenic species in biological samples, high-performance liquid chromatography (HPLC) by reversed-phase and ion exchange is generally used followed by an element specific detection by inductively coupled plasma mass spectrometer (ICPMS) on occasion equipped with hydride generation unit. This approach, however, has some key drawbacks manifested mainly in the identification of the analytes, which is based on matching chromatographic retention times with those of standard arsenic compounds. For some samples, separation of all arsenic compounds present is not possible, even more, many samples contain arsenic compounds that do not match any of the available standards. For this reason, molecular mass spectrometry is an attractive alternative to HPLC-ICPMS for arsenic speciation analysis. HPLC-electrospray-tandem-mass-spectrometry (HPLC-ESMS/MS) has been enjoying growing popularity in the application of speciation of organoarsenic species in the last years. Because of its soft ionization, the species can be transformed to their protonated molecular form providing molecular-mass information of a certain molecule. By the use of different fragmentation techniques additional structural information can also be achieved of a molecule.

Irrespective of the applied method quality control is necessary to prove the accuracy of our speciation analysis. Beside calculating the mass-balance that can provide us useful information about the extraction efficiency and column recovery of the arsenic compounds we can make certain of the suitability of the applied method with certified reference materials (CRMs). Although there is very important to choose the right matrix contained the required analytes CRMs are available limited and reference value are mostly given only for total element content and one arsenic species. Because of the growing interest of arsenic speciation analysis much research continues on improving our understanding of the health effects and environmental chemistry of arsenic species.

2 OBJECTIVES

The last three decades of arsenic speciation studies were characterized by continuous improvement and this tendency will be increase in the future. With the increase of sensitivity and selectivity several new arsenic species, not found in the environment before can bee detected. For the selective determination of these new arsenic species new separation and detection techniques are needed. The objectives of my study can be conceived as follows:

- Development of chromatographic separation methods for the most frequent arsenic species focusing on the oxo- and thio-arsenosugar analogous.
- Beside element specific detection a need arises for a technique, which is able to generate a signal, that would be specific not to an element, but to a species. The aim was to develop an HPLC-ESMS/MS coupling based speciation method. The method allows us providing not only quantitative but also structural information from the target compounds.
- The application of the developed methods for environmental samples:
    - Marine mussels and fish
    - Range of freshwater samples (sediment, water, algae, plants, sponge, mussels, frog and fish species)
- One of the main objectives of this work was to analyze seafood from Greece that may pose a risk to consumer health by their high arsenic content and investigate arsenic speciation in these samples.
- Determine the arsenic accumulation ability of freshwater organism and establish the arsenic species distribution of the investigated samples.
- Characterize a certified reference material (BCR-710, oyster tissue) from the point of view of arsenic species distribution in extract. The characterization can be helpful for identification of arsenic compounds found in biological samples by retention time when standard solutions are not available.
- Performing the quality assurance of the applied methods.

3 MATERIALS AND METHODS

All solutions were prepared with Milli-Q water (18.2 MΩ·cm). For preparing the mobile phases ammonium dihydrogen phosphate (p.a.), ammonium hydrogen carbonate (p.a.), methanol (puriss p.a) formic acid (puriss p.a.) aqueous ammonia solution (25%, suprapur) and pyridine (p.a.) were used. The wet digestion of the samples was carried out with HNO_3 and H_2O_2. In the case of hydride generation technique NaOH, $NaBH_4$, HCl and $K_2S_2O_8$ were used. For the identification and quantification of arsenic species in the samples, 9 standard solutions (1000 mg L^{-1}As) were used. Oxo-arsenosugar standard solutions were kindly donated by Kevin A. Francesconi (Austria). Thio-arsenosugars were obtained by addition of a saturated aqueous solution of H_2S to a standard solution containing 100 µg As/L of the respective oxo-analogue. Quality control was carried out using DOLT-2, DORM-2 and BCR-710 certified reference materials.

Samples were freeze-dried in a Christ Alpha 1-4 freeze-drying system. The dry samples were pulverized in a laboratory grinder. Samples were digested in house-made teflon bombs and in a Milestone ultraCLAVE II microwave digestion system. Arsenic speciation analyses were performed with the following coupled techniques: HPLC-(UV)-HG-AFS, HPLC-ICPMS and HPLC-ESMS/MS. The separation of the arsenic species was achieved on strong anion- and cation-exchange column.

4 RESULTS

4.1 Development of separation and detection techniques

In the frame of my Ph.D. study different type of coupled methods were developed for the determination of inorganic and organic arsenic compounds occurring in the aquatic environment. The separation of the arsenic species was performed by high performance liquid chromatography (HPLC) while the detection was carried out using atomic fluorescence spectrometer (AFS), inductively coupled plasma mass spectrometer (ICPMS) and electrospray tandem mass spectrometer (ESMS/MS).

In the first part of my work an HPLC-(UV)-HG-AFS coupled method was developed for separation and detection of twelve arsenic compounds including the oxo-arsenosugar compounds. The twelve arsenicals were separated and determined on the basis of their difference

in two properties: (i) the pKa values and (ii) hydride generation capacity. The separation was carried out both with an anion- and a cation-exchange column, with and without photo-oxidation.

Then, the directly hydride-generating species (As^{III}, As^{V}, DMA^{V}, MA^{V} and TMAO) may be determined by the HPLC-HG-AFS system. However, this system is not suitable for the determination of the arsenosugars, AB, TMAO, AC and TETRA, because these organoarsenic compounds contain either three or four methyl-groups and therefore are unable to form volatile hydrides directly. Thus, a photo-oxidation treatment should be used to break them down into smaller molecules and thus make them suitable to form volatile hydrides. Therefore they were determined with the HPLC-(UV)-HG-AFS instrument combination. The efficiency of the hydride generation was investigated and it was found that the best yield could be achived using 6 m/v% $K_2S_2O_8$ solution as an oxidant.

The main benefit of the ICPMS detection based on its element-specific feature: the signal of a species measured is that of an elemental ion, by which it is rather robust toward co-eluting matrix compounds. As the signal intensity is proportional to the concentration of As-atom in the molecule, using isocratic elution profile there is no need standard of a certain species for quantification. Further advantage of the ICPMS detection is that the samples can be introduce without any prior derivatization steps. For separation of the cationic arsenic compound a newly developed cation-exchange column was used. In this case the separation was achieved within five minutes. Take advantage of the ICPMS detection the separation of the anionic arsenic compounds used for HPLC-HG-AFS coupled method was also improved: decreased retention times, sharper peaks and lower detection limits. Beside the separation of the four oxo-arsenosugar a second anion-exchange chromatographic method was developed for the determination of the four thio-arsenosugar derivatives.

In case of an HPLC - single-quadrupole-ESMS system, after the recognition of the molecular ion ($[M+H]^+$), fragmentation of the molecule using variable fragmentor voltage can be achieved in the ion-source. These two procedures are separated in time. However, application of tandem MS in MRM mode is more favorable, by which the selection of the molecular ion and the fragmentation process by collision induced dissociation (CID) are separated in space, therefore they can be achieved in one chromatographic run. This benefit of a tandem MS instrument is expressed in the "multiple reaction monitoring (MRM)" mode, which has been reported as a very sensitive on-line detection technique. My efforts initially concentrated on optimizing the enhanced product ion (EPI) mass spectra for the investigated organoarsenic molecules. On the mass spectra the most intensive and characteristic six-to-seven MS/MS transitions were chosen for further optimizations. Firstly, the source-dependent parameters were optimized. The component-dependent parameters including collision energy (Coll. E), cell

entrance potential (CEP), cell exit potential (CXP), de-clustering potential (DP) and entrance potential (EP) were also optimized for all of the chosen transitions of the each analytes. Based on these curves the two most intensive and most characteristic transitions were chosen, which finally resulted in the development of a very sensitive and specific selected reaction monitoring approach. As the performance of the ESMS technique is negatively affected by the salt content as well as the presence of ion-pairing reagents of the mobile phase, the conventional chromatographic separation methods used for arsenic speciation by ICP-MS had to be changed. Volatilized buffers, such as NH_4HCO_3 or $HCOONH_4$ were chosen adjusting the ionic-strength and the pH of the anion-/cation-exchange chromatographic separation of the species. Additionally, in the ES process, formation of the molecular ions is known to be enhanced when high concentration of organic solvents - such as methanol - is present during sample introduction. But, on the other hand, methanol can influence the apolar interaction of certain molecules to the stationary phase as well, by which the retention of these compounds can be altered. Because of this, the influence of methanol on the signal intensity as well as on the retention of the species was investigated. In the case of arsenosugars the chemical properties of the thio-arsenosugars allow determining them only in a separate chromatographic run by HPLC-ICPMS, which is time-consuming in the routine analysis. In my work a new HPLC method was developed that is ble to separate the anionic arsenic compounds together with the oxo- as well as the four thio-arsenosugars in one chromatographic run. Theoretically, the very selective ESMS/MS detection does not require complete HPLC separation of the investigated molecules. However, it should be noted, that certain groups of arsenicals - such as oxo- or thio-arsenosugars - have very similar CID behavior. Thus, in order to avoid any possibility for misidentification, chromatographic separation of these compounds is suggested prior to ESMS detection.

4.2 Determination of arsenic species in seafood samples from the Aegean Sea

In this study arsenic compounds were determined in mussels (*Mytulis galloprovincialis*), anchovies (*Engraulis encrasicholus*), sea-breams (*Sparus aurata*), sea bass (*Dicentrarchus labrax*) and sardines (*Sardina pilchardus*) collected from Aegean Sea. Since the final aim of this work was to investigate the content of total arsenic and arsenic speciation in seafood intended for consumption and to estimate the possible dietary exposure of Greek population to arsenic, the collection of animals was based on the available consumption data and their production. In all the samples arsenobetaine (AB) was detected as the major compound with trace amounts of arsenite, (As^{III}), dimethylarsinic acid (DMA^{V}) and arsenocholine (AC). Although arsenosugars are mainly present in marine algae, high concentration of arsenosugar compounds were found in

different mussel species as well, mostly in the form of glycerol and phosphate sugars. Since the major As content in the examined mussel and fish samples is in non-toxic forms, it could be stated that consuming seafood originated from the Aegean Sea does not pose any health hazard to humans. The mean total As in analyzed seafood is 14.3 μg g^{-1} dw, which corresponds to a content of 4.3 μg g^{-1} wet mass (a moisture content of 70% is considered). If an average fish consumption of 18 g d^{-1} is considered for the general Greek population, then the dietary exposure of the population in total As could be estimated as 1.1 μg d^{-1} Kg^{-1} for an average body weight of 70 Kg. This value is lower than the proposed TDI (2 μg d^{-1} Kg^{-1} bw) for inorganic arsenic. Taking into consideration the speciation results, where it was proved that about 90% of the incorporated arsenic is arsenobetaine, which is nontoxic, it can be concluded safely that there is no risk posed by the consumption of seafood originating from the Aegean Sea.

4.3 Arsenic speciation of freshwater organism from the river Danube

The aim of this work was to determine the arsenic speciation in several diverse species of biota collected from the same freshwater ecosystem. Additionally, mass balance calculations were carried out at each stage of the analysis to investigate possible reasons for the low overall recovery of arsenic species previously reported for freshwater organisms. Water, sediment and biological samples were collected in June 2004 from the river Danube in Hungary at the City of Paks, which is one of the most important fishing-grounds along the Hungarian Danube. The organisms collected were: one species of green alga; five species of freshwater plants, seven fish species; one species of mussels; one sponge and one frog species. Water samples contained only 1.1 ± 0.2 μg As L^{-1}. This value is at the low end of the range of arsenic concentrations usually reported for river water and is well below the arsenic concentrations recently reported for surface-water in Hungary, especially those in the eastern part which typically show arsenic concentrations of 10-100 μg L^{-1}. Despite the low arsenic concentrations in the water samples, many of the freshwater organisms collected from the sampling site contained appreciable concentrations of arsenic.

The dominating arsenic species in the extracts of freshwater algae were arsenosugars, whereas arsenate was present only as a minor constituent. On the other hand, plant extracts contained only inorganic arsenic, except for two samples which contained trace amounts of dimethylarsinate and the tetramethylarsonium cation. The oxo-arsenosugar-phosphate (*ca* 35% of extractable arsenic) and the oxo-arsenosugar-glycerol (*ca* 20%) as well as their thio-analogues (1-10%) were found in the mussel extracts, while arsenobetaine was present as a minor species

only. In general, fish extracts contained only traces of arsenobetaine, and the oxo-arsenosugar-phosphate was the major arsenic compound. In addition, samples of white bream contained thio-arsenosugar-phosphate; this is the first report of a thio-arsenical in a fish sample. The frog presented an interesting arsenic speciation pattern because in addition to the major species, arsenite (30%) and the tetramethylarsonium cation (35%), all three intermediate methylation products, methylarsonate, dimethylarsinate, and trimethylarsine oxide, and arsenate were also present. Collectively, the data indicate that arsenobetaine, the major arsenical in marine animals, is virtually absent in the freshwater animals investigated, and this represents the major difference in arsenic speciation between the two groups of organisms.

The dominance and virtual absence of AB in marine and freshwater animals, respectively, may also explain the low recoveries observed for arsenic species in freshwater animals. AB is a polar molecule readily soluble in both methanol and water, the two most common solvents used for extraction in arsenic speciation analysis. Thus, when AB is the dominant arsenical in the sample (such as in most samples of marine animals) high extraction yields are obtained. In freshwater animals, however, AB may not be present at all, and the presence of other, non-extractable, arsenicals becomes significant. Future studies focusing on the currently non-extractable arsenic might be informative and perhaps reveal further similarities between arsenic species in marine and freshwater samples.

4.4 Application of the new HPLC-ESMS/MS method for determination of organoarsenic compounds in marine and freshwater samples

Cation- and anion-exchange HPLC online with ESMS/MS mode was used for the identification of arsenic species in freshwater and marine samples, including TORT-2 certified reference material, freshwater mussel, freshwater fish, and marine algae. The sample selection was based on two viewpoints. (i) Arsenic speciation of these samples were earlier carried out by HPLC-ICPMS systems and was published, by which the results of this study could be compared.

In my work additional three non-published arsenic species were identified in the certified sample, namely TMAO, oxo-glycerol- and oxo-sulfate-arsenosugars. The reason, that these compounds were not detected by HPLC-ICPMS may be the co-elution of these species at the applied chromatographic condition. In the case of freshwater mussels and fish the characteristic arsenosugar compounds, namely oxo-glycerol-, oxo-phosphate-, thio-glycerol- and thio-phosphate-arsenosugars were identified by the HPLC-ESMS/MS method well agreeing with the data used HPLC-ICPMS. On the other hand, in this work DMA, oxo-sulfate-arsenosugar and

TMAO were also detected in the sample. The reason that DMA could not been detected by HPLC-ICPMS is presumably the high amount of oxo-arsenosugars present in the sample overlapping the signal of the DMA on the anion-exchange column. Based on these results it can be concluded, that the developed HPLC-ESMS/MS method is suitable for the qualitative analysis of the organoarsenic species in crude extracts. The results furthermore show that the species-selective detection allows the identification of known arsenic species, which was "hidden" so far from the ICPMS detection.

It has been fully established that the ionization efficiency of ESMS is suppressed by co-existing matrix, which may prevents the successful application of the technique for the quantification of arsenic species in crude extracts. First of all I aimed to investigate weather the developed chromatographic method can sufficiently separate the matrix from the analytes. As the matrix effects on the ES signal can sophisticate the quantification by external calibration, method of standard addition calibration was also performed. As the standard addition calibration is a complicate and time-consuming method for quantification in the routine analysis, different sample-clean-up methods were also tried in order to reduce the matrix effect on ESMS. Based on the results, it is cleared that the signal suppression caused by matrix effect means a significant limitation in the quantification of arsenicals by ES-MRM detection. This drawback is manifested especially in the case of the slightly retained species, such as AB, DMA and oxo-phosphate arsenosugar. The three methods tried for separation of the signal-suppressive matrix from the analytes proved to be ineffective. Standard addition calibration seems to be the solution for such problems.

4.5 Minor arsenic species in a standard reference material BCR-710 Oyster tissue

The aim of this work was to caracterize a certified reference material BCR-710 Oyster tissue from the point of view of arsenic species distribution in extract. Anion- and cation-exchange column were used to determine arsenic compounds in the sample. Three extraction methods (extraction with water, methanol-water 1:1 and methanol) were applied for extraction of arsenic species. BCR-710 is certified for arsenobetaine (AB, 13.89 ± 1.79 mg As kg-1) and indicative values are given for total arsenic (25.7 ± 2.7 mg As kg-1) and for dimethylarsinic acid (DMA, 0.446 ± 0.098 mg As kg-1)). 14 arsenic compounds including oxo- and thio-arsenosugars (7.1-12.2 %) and two unknown species (1.0-4.3 %) were found in the extracts. Extraction yield was between 70-78 % and the highes efficiency was achieved with methanol-water extraction. It was established that the extraction efficiency of several arsenic compounds depends on the applied

extraction method. The characterisation can be helpful for identification of arsenic compounds found in biological samples by retention time when standard solutions are not available.

4.6 Quality assurance in speciation analysis

The quality and usefulness of the data from speciation analysis can often be compromised by low overall recovery of arsenic species. Arsenic speciation analysis of biological samples involves two main steps, namely extraction of species and their separation by HPLC, and it is important to know where arsenic losses are occurring. During my Ph.D. study, I investigated this by determining arsenic mass balances at each stage. In the case of HPLC-ESMS/MS beside the retention time two additional approaches are available. For the identification of the arsenic species at the low μg/L level the HPLC-ESMS/MS method was set up to monitor two transitions for each molecule. For quality control the intensity ratios expressed by peak area of the two transition signals were calculated in the case of standards as well as for the species found in the samples. The fact that the two identical transition rations of the extracted and standard species agree well, in combination with the matching chromatographic retention time, indicate the good quality analysis of the arsenic species. On the other hand analyzing the EPI spectra of the analytes and comparing it with the EPI spectra of a standard molecule help us to confirm the component identification. In my work an EPI spectra of each detected compounds were recorded showing good fingerprint matching with the corresponding standard species.

5 NEW SCIENTIFIC ACHIEVEMENTS

1. Anion and cation-exchange high performance liquid chromatographic method was developed for selective determination of inorganic and organic arsenic compounds based on HG-AFS, ICPMS és ESMS/MS detection techniques.

2. In my PhD study, seafood samples collected from the Aegean Sea in Greece, an area characterized by high seafood consumption, were analyzed extensively for the first time.
   - The total arsenic concentrations found in the samples ranged between 5.3-34 mg/kg.
   - It vas concluded, that in the case of fish the more than 90% of the accumulated arsenic was present on the form of the non toxic AB. On the other hand high concentration of arsenosugar compounds were found in different mussel species, mostly in the form of glycerol and phosphate sugars.
   - Taking into consideration the speciation results, that about 90% of the incorporated arsenic is arsenobetaine, which is nontoxic, it can be concluded safely that there is no risk posed by the consumption of seafood originating from the Aegean Sea.

3. Total arsenic concentration and the species distribution was determined in samples from freshwater origin.
   - Despite the low arsenic concentrations in the water samples (1.1 ng/ml), many of the freshwater organisms collected from the sampling site contained appreciable concentrations of arsenic. There was, however, a considerable range of concentrations between the different types of organisms.
   - In accordance with data obtained from marine algae, arsenosugars were also detected in the fresh green *alga Cladophora* sp. from the Danube where oxo-arsenosugar-glycerol was the main arsenic compound in the extracts constituting 84% of the extractable arsenic.
   - Arsenic speciation studies in the terrestrial environment show clearly that the dominating water soluble arsenic compounds in plant species are inorganic As^{III} and As^{V}
   - The investigated frog sample showed an interesting species distribution pattern; arsenite (30%) and TETRA (35%) were major species and all three intermediate methylation products (MA^{V}, DMA^{V}, TMAO) and arsenate were also detected. Thus the frog contained all the arsenic species in the proposed arsenic biomethylation pathway that begins with arsenate and has TETRA as the ultimate end product

- Out of accordance with the marine mussels freshwater mussels investigated in this study contained oxo- and thio-arsenosugars as the major extractable arsenic species, and arsenobetaine was present only as a minor species (1-2%).
- All the fish samples contained oxo-arsenosugar-phosphate as the major arsenic compound while AB was present only in trace amounts. Of particular interest was the presence of the thio-arsenosugar-phosphate in both samples of bream. This is the first report of a thio arsenical in fish.

4. Cation- and anion-exchange high-performance-liquid-chromatography electrospray tandem mass spectrometric (HPLC-ESMS/MS) methods were developed for the determination of 15 organoarsenic compounds in marine and freshwater samples.
   - Based on enhanced product ion spectrum the source-dependent and component-dependent parameters were optimized for all of the chosen transitions.
   - In my PhD work I developed an HPLC method that was able to separate the anionic arsenic compounds together with the oxo- as well as the four thio-arsenosugars in one chromatographic run.
   - It can be concluded, that the detection limits for the most species are absolutely comparable or even better than those reported by elemental-specific detection methods.
   - It was demonstrated, that certain groups of arsenicals have very similar CID behavior. Thus, in order to avoid any possibility for misidentification, chromatographic separation of these compounds is suggested prior to ESMS/MS detection.
   - The results obtained in this study demonstrate that the developed HPLC-ESMS/MS method is a powerful approach for the identification of organoarsenic species in crude sample extracts.
   - The three methods tried for separation of the signal-suppressive matrix from the analytes proved to be ineffective. Standard addition calibration seems to be the solution for such problems.

5. A certified reference material (BCR-710) was characterized from the point of view of arsenic species distribution in extract.
   - Beside AB 13 minor arsenic compounds were quantified in BCR-710 oyster CRM.
   - Oxo arsenosugars are more water soluble, while in the case of thio arsenosugars higher extraction efficiency was achieved with methanol.

6 PUBLICATION RELATED TO THE DISSERTATION

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