DEVELOPEMENT AND APPLICATION OF ANALYTICAL METHOD FOR ARSENIC SPECIATION OF ENVIRONMENTAL SAMPLES

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CSILLA SÖRÖS

Corvinus University of Budapest

Department of Applied Chemistry

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PhD School/Program

Name: **PhD School of Food Science**

Field: **Food Science**

Head: **Prof. András Fekete university professor, DSc**
Corvinus University of Budapest, Faculty of Food Science
Department of Physics and Automation

Supervisor: **Dr. Péter Fodor university professor, DSc**
Corvinus University of Budapest, Faculty of Food Science
Department of Applied Chemistry

The applicant met the requirement of the PhD regulations of the Corvinus University of Budapest and the thesis is accepted for the defence process.

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Head of PhD School **Supervisor**

1. INTRODUCTION

1.1 Speciation analyses of arsenic

Speciation analysis is a quite new branch of the analytical chemistry originating from the middle of the 20th century. The approach of the field is based on the fact that the bio-geochemical mobility, essentiality and toxicity of an element is primary depends on their chemical environment. Hence, the biological effect of an element can only be evaluated by the determination of the molecular forms rather than the total concentration of the element. The history of speciation analysis originates from the determination of Hg-, Sn-, Se- and the As-species.

Arsenic has been thought to be a toxic element and even now the total arsenic concentration in the food materials are well controlled. However, it was cleared in 1977, that the typically high-arsenic-content foods of marine origins (fish and mussels) contain arsenic in a non-toxic molecular form, such as arsenobetain (AB). Since then numerous arsenic molecules were proven to be present in our environment. On the other hand, arsenic is not such a "safe element" in all cases. It was also demonstrated by toxicological investigations that the inorganic arsenic forms (arsenite and arsenate) are bioactive species, and have strong carcinogen, mutagen and teratogen effect for the human cells.

Based on the above-mentioned experience, the speciation researchers focus on the development of analytical methods suitable for selective determination of the arsenic species. There are two main purposes of such an analytical works.

Important research area is the toxicological investigations, by which the concentration of the toxic inorganic arsenic compounds are determined in frequently consumed foodstuffs. Drinking water is the most important matrix in that question, because it can be polluted with high amount of arsenic (mostly in the form of inorganic arsenic) from geochemical origin. The topic is highlighted in Hungary, because the southeast part of the country is well known about the arsenic-polluted drinking water supply. Therefore arsenic-removal is a big challenge for the present/future engineers, and these techniques require the

knowledge of the oxidation states of arsenic present in the water. The question can be answered by the modern arsenic speciation methods. In the case of the marine animals, the speciation literature claims the opposite: the non-toxic AB constitutes the most part of the cumulated arsenic. The Hungarian legislation has also been convinced by the arsenic speciation results: since 2003 the speciation approach appears in the Hungarian food-regulation. While earlier there was a strict threshold limit for the total arsenic concentration of the marine fish and mussels, it was deleted in 2003. In the case of other foodstuffs, such as mushrooms and rice, the speciation data are not so uniforms, hence the threshold limits for total arsenic content are still valid.

Studying the biochemical process of arsenic is the other important research field of the topic. The aim of the investigations is to understand the arsenic cycle of the environment, focusing especially on the human metabolism of arsenic. These types of works require very sensitive and selective analytical techniques capable of determining trace amount of molecules proving the presence of certain biochemical process. Studies emphasize newly identified arsenic species, such as thio-arsenic compounds. It can be also seen, that the research line is orienting into the direction of bigger-molecules preserving the original chemical character of arsenic - it often means for instance the identification of arsenic containing proteins (>500 kDa size). In this process the determination of thio-arsenic species can be an intermediate step, which indicates the presence of arsenic-cysteine connection in the sample matrix.

Very fast developing was experienced in the arsenic speciation analytical methods during the past five years. While the HPLC is the most frequently used separation technique, the conventional columns have been changed by the micro/nano-filled columns having favorable analytical parameters, less sample- and solution demand. The ion-chromatographic techniques are the most popular methods for the analysis of small arsenic molecules. The separation of arsenic-containing peptides and proteins can be solved based on their size (size-exclusion-chromatography), iso-electric point (isoelectric focusing) or using both techniques (multi-dimensional separation).

An element or molecule specific detector is coupled to the separation unit. The difference between such detectors appears in the ion-source: the former is mostly flame or inductively-coupled-plasma (ICP), in which the arsenic molecules are transformed to arsenic atoms/ions. In the latter case the ion-source is much more "soft", which let the species getting through the detector in their original molecular forms. Electro-spray is the most popular ion-source in the molecule-selective arsenic speciation.

The ions generated in the ion-source are passing into the detector. Nowadays, the elemental specific optical detectors (such as AAS – atomic absorption spectroscopy, AFS – atomic fluorescence spectroscopy, AES-atomic emission spectroscopy) has been changed by the modern mass-spectrometric detectors, by which much better detection limit can be achieved. In that case mostly quadrupole analysators are used. Besides the quadrupoles, TOF analysators are often installed into the molecular-specific mass spectrometers.

Derivatisation steps, such as hydride generation, can complete the above-mentioned coupled techniques. The gas-phase sample-introduction is more effective than the solid-phase methods. However, due to the small number of volatile arsenic species, this technique is kept back.

Arsenic speciation is a very intensively improving research area, which offers a huge number of unanswered questions to the researchers in the near future.

1.2 Objectives

The objectives of the study can be defined as the follows:

- Developing arsenic speciation chromatographic methods, which is capable of the selective determination of the most frequently investigated arsenic species.
- Application of the developed methods for the investigation of the following samples:
  - Hungarian drinking water samples,
  - marine mussels,
  - mushrooms,
  - Hungarian farmed fish,
  - fresh-water mussels.

- Determination of total arsenic as well as arsenite/arsenate ratio in arsenic polluted drinking water samples collected in the southeast part of Hungary.
- Answering the question: „Is there any proportion between the total arsenic content and the AB concentration of the marine mussels?".
- Based on the high AB concentration found in the marine mussels, investigation of the species-pattern in freshwater mussels was also the purpose of the work. Emphasis was laid on the presence of thio-arsenic molecules in the mussels.
- Extraction techniques applied for the arsenic speciation of mushrooms to be compared.
- Investigation of arsenic accumulation of *Agaricus bisporus.*
- Arsenic speciation in farmed fish collected from the southeast part of Hungary, including two main groups of fish: african catfish raised in thermal water and carps grown in surface water of the region. Comparing the results with the marine fish.
- Performing the quality assurance of the applied methods.

2. MATERIALS AND METHODS

2.1 Reagents, standards and reference materials

The following reagents and standards were used during the work: deionized water, K_2HPO_4, Na_2HPO_4, $(NH_4)_2HPO_4$, KH_2PO_4, NaH_2PO_4, $NH_4H_2PO_4$, NaOH, formic acid, hydrochloric acid, pyridine, NH_4OH, DDAB ion-pairing reagent, methanol, $NaBH_4$, $K_2S_2O_8$, HNO_3, H_2O_2, arsenate stock solution, As_2O_3, CH_3AsO_3Na x 6 H_2O, $(CH_3)_2AsO_2Na$ x 3 H_2O, AC, AB, TMAP, TMAO, TETRA standards, DOLT-2, DORM-2, NIST 2711 CRMs, and BCR-710 reference material.

2.2 Instrumentation

The following instruments were used during the work: Christ Alpha 1-4 freeze-drying system, laboratory grinder, house-made teflon bombs, Mutiwave 3000 microwave digestion system, HPA-S digestion system, Milestone ultra CLAVE II digestion system, ICP-AES, ICP-TOF-MS and ICP-Q-MS instruments, Maxi Dry Lyo vacuum evaporator, HPLC-HG-AFS, HPLC-PO-HG-AFS, HPLC-ICP-Q-MS, HPLC-HG-ICP-Q-MS coupled systems, LiChrospher 100 RP-18 reverse phase HPLC column, Zorbax 300-SCX and SupelCosil LC-SCX cation exchange columns, Hamilton PRP-X100 anion-exchange column.

2.3 Samples

The developed analytical methods were applied for arsenic speciation of Hungarian drinking water samples, marine mussels (*Mytilus edulis, Mytilus galloprovincialis*), mushrooms (*Amanita muscaria,*

Agaricus bisporus), freshwater fish (*Clarias gariepinus, Cyprinus carpio*), and freshwater mussels (*Unio sp., Anadonta sp.*).

3. RESULTS

3.1 Development of arsenic speciation chromatographic methods

For the selective determination of different arsenic species HPLC methods were developed, which were further improved parallel with the new instruments available to us. The work was based on the literature data applying ion-exchange as well as ion-pairing methods for the speciation of arsenic.

Separation of cationic/protonated arsenic compounds was solved by two cation-exchange stationer phases. In the first case SuperCosil LC-SCX column was used, by which AB and AC could be separated during 25 min at pH = 2.0 by gradient elution (deionized water – 300 mM pyridine-formiate). As the number of the arsenic species is continuously expanding, a newly developed cation-exchange column (Zorbax 300 SCX) was also tried in order to separate the bigger group of the arsenic species (namely oxo-glicerol sugar, AB, TMAO, TMAP, AC and TETRA) The separation was carried out in 4 min at pH = 2.6 with isocratic elution (20 mM pyridine-formiate).

For the separation of anionic/deprotonated arsenic compounds ion-pairing as well as ion-exchange chromatographic methods were developed. In the case of ion-pairing LiChrospher 100 RP-18 LiChroCART silica-based reverse stationer phase was used. The mobile phase consisted of 10^{-5} M DDAB ion-pairing reagent, 0.5 v/v% MeOH, and 50 mM $H_2PO_4^-/HPO_4^{2-}$ buffer (pH = 6.0). By this method it was solved to separate As(III), DMA, MA and As(V) during 7 min.

In the second part of the work PRP-X100 organic polymer-based anion-exchange column was applied for the separation of As(III), DMA, MA, oxo-phosphate-arsenosugar, As(V), oxo-sulfonate and oxo-sulfate arsenosugars. The separation was solved in 15 min at pH = 5.6 with the use of 20 mM buffer.

3.2 Arsenic speciation in drinking water

The knowledge of arsenite/arsenate ratio of the water supply (i) let us estimate the subsurface geo-chemical processes as well as (ii) it is an important indicator for the arsenic-removal techniques. In the doctoral work the ratio of As(III)/As(V) was determined in different drinking water samples collected in the arsenic polluted region of Hungary. Prior to the sample collection, the optimal sample-handling/storage process was measured and defined, avoiding the change of the species oxidation states.

It was concluded that the total arsenic concentration in the case of all samples was significantly higher, than the valid threshold limit (10 ng As/ml). In order to preserve the original oxidation state of the species, the analyses should be adjusted in one day after the sampling and the samples should be stored at +4 ^{o}C until analysis.

3.3 Arsenic speciation in marine mussels

Marine mussels are known to be able to accumulate high amount of arsenic in the form of dominantly the non-toxic AB. In the doctoral study marine mussels were collected from the Venice gulf as well as around the Sardinia-island of Italy and were investigated for speciation analyses of arsenic. Relationship was determined between total arsenic and accumulated AB concentrations of the samples. Is the AB-dominance general or does the species pattern depend on the accumulated amount of arsenic? It was presumed that the bigger arsenic accumulation results more inorganic arsenicals in the mussel tissue as the „detoxification" process is overdosed.

Based on the results the following statements can be drawn.

(i) There is a direct proportional relationship between the accumulated AB and the total arsenic content of the mussel tissue. In the case of anionic compounds proportionality was not found.

(ii) In respects of biomonitoring, the pollution state (based on arsenic) of the environment can be well characterized by the total arsenic concentration in the samples; arsenic speciation is not necessary.

(iii) AB-dominance is a general statement in the case of marine mussels, and is proportional with the arsenic accumulation rate.

3.4 Arsenic speciation in mushrooms

Mushrooms are able to accumulate high amount of arsenic. It has been also pointed out, that the species-pattern in the mushrooms can be very different, but dominantly organic species has been detected in the wild-grown samples. Literature data provide us numerous alternatives for the extraction of arsenicals from the mushrooms. During the research work extraction methods were compared regarding the species extracted as well as the extraction yield. In the second part of the work, *Agaricus bisporus* was grown on artificial arsenic polluted media and the arsenic accumulation was investigated.

The results of this study revealed that there is no significant difference between the four most efficient extraction techniques, they are all suitable for arsenic speciation purposes. It was proven that *Agaricus bisprus* can accumulate arsenic from the arsenate-enriched medium, but there was no significant arsenic metabolism experienced.

3.5 Arsenic speciation in freshwater environment I. – freshwater fish

Unlike marine fish, the literature data about arsenic speciation in freshwater fish is scarce, and no uniform consequence can be outlined. However, due to the arsenic polluted Hungarian water supply (southeast part of the country) arsenic speciation of the farmed fish is especially important.

During the work arsenic speciation analysis was carried out in the farmed freshwater fish of the area. Based on the results it can be seen that African catfish can accumulate high amount of AB from food origin. On the other hand the accumulation capability of the carp is not significant (*ppb* range), and the arsenic speciation pattern of the fish muscle is not known as only the 2-29% of the accumulated arsenic was successfully identified.

3.6 Arsenic speciation in freshwater environment II. – freshwater mussels

Based on the results of freshwater fish samples, the aim was the investigation of freshwater mussels as well. It has been already known that the freshwater mussels are suitable for biomonitoring as they accumulate high amount of pollutants. Important to mention their role in the aquatic food web: the pollutants can migrate relatively fast to higher animals and at the endpoint to the humans. Compared to the freshwater fish, arsenic speciation in the mussels is an easier task, as they can accumulate arsenic in the *ppm* range. Hence, even besides the low extraction yields, it remains sufficient arsenic in the extract for quantification.

In this chapter of the PhD work arsenic speciation was carried out in freshwater mussels collected from the river Danube. The results show that the species pattern of the freshwater mussels are very different that those in the marine mussels: AB was present only trace amount, while the dominant arsenic forms were the arsenosugars (namely oxo-glycerol and oxo-phophate sugars), which constitute 30-30% of the identified compounds.

This is the first study identifying thio-arsenic compounds in freshwater samples. The reason is probably the strong interaction of these compounds to the chromatographic stationer phase and the fact that these compounds can be oxidized very fast to their oxo-analogues.

4. NEW SCIENTIFIC ACHIEVEMENTS (THESES)

1. Arsenic speciation chromatographic methods were developed for the determination of arsenic compounds including As(III), As(V), DMA, MA, oxo-glycerol-, oxo-phosphate-, oxo-sulfonate-, oxo-sulfate-, thio-glycerol-, thio-phosphate arsenosugars, TMAP, AB, TMAO, AC and TETRA. By the use of HPLC-HG-AFS system, application of NH_4 ion (as $NH_4H_2PO_4$) in the mobile phase is more favorable than Na or K ions (as NaH_2PO_4 and KH_2PO_4), assuring better signal-to-noise ratio than the later ones.
2. Arsenic speciation was carried out on 15 drinking water samples collected at the southeast part of Hungary. It was concluded that the total arsenic concentration in all of the samples was significantly higher, than the presently valid threshold limit (10 ng As/ml). In order to preserve the original oxidation state of the species, the analyses should be adjusted in one day after the sampling and the samples should be stored at +4 ^{o}C until analysis.
3. The marine mussel samples collected from the Venice gulf and around the Sardinia-island contain 7.5-30 mg/kg total arsenic, but more than 90% of the accumulated arsenic is present in form of the non-toxic AB. Higher accumulation rate of arsenic does not mean higher toxicity.
4. Ten widely used extraction methods were tried out for arsenic speciation in mushroom sample (*Amanita muscaria*). There was no significant difference between the four most effective methods, nevertheless water agitation is suggested most of all due to its relative simplicity. *Agaricus bisporus* grown on artificial arsenate-polluted media can accumulate high amount of arsenic in the form of mostly inorganic species.
5. While the Hungarian natural fish-lakes contain similar amount of total arsenic than the sea (1–2 ng/ml), the arsenic accumulation capability of the carps is two orders of magnitude less than the marine fish. In the carp samples <11 μg/kg arsenic was identified in the form of inorganic, mono- and dimethylated arsenic species. African catfish collected from the southeast region of the country were raised in thermal water polluted with high amount of arsenic. The catfish samples contained 2510–4720 μg/kg arsenic, but 96% of the accumulated semi-metal was detected in the form of AB. It was proven that the high amount AB originates from the fish-food.
6. The freshwater mussels collected from the Danube accumulate similar amount of arsenic than the marine mussels. On the other hand their species-pattern are very different from those: AB constitutes only the trace amount of the identified arsenicals, while the dominant arsenic forms were the arsenosugars. We identified firstly thio-arsenic compounds in the freshwater environment.

7. The importance of the quality control (internal standards, CRMs, internal-external calibrations, total material balance, drift monitoring) of speciation analysis was also proven. All of the quality control processes used in the work are suitable for assuring the quality of the speciation analysis.

5. PUBLICATION RELATED TO THE DISSERTATION

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