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Metody speciační analýzy sloučenin arsenu
Methods for elemental speciation of arsenic compounds

Zkrácená verze PhD Thesis

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CONTENT

1 INTRODUCTION

Humans are unavoidably exposed to arsenic through drinking water or the diet. Although the World Health Organization has recommended a Maximum Permissible Concentration for arsenic in drinking water of 10 µg/l, millions of people in the world are exposed to significant quantities of inorganic arsenic through drinking water for example in West Bengal (India). This exposure contributes to cancers of skin, bladder and lung in addition to having noncancer health effects include nausea, vomiting, diarrhoea, blindness and skin pigmentation. The toxicity of arsenic is highly dependent on its chemical form, and most research has been focused on inorganic arsenic as it was believed to be more toxic than organic arsenic species. The major arsenic species in drinking water are arsenate As^{V} and arsenite As^{III}, both of which are highly toxic (WHO 2003, Yoshida 2004).

Further humans are exposed to arsenic through seafood; such as fish, algae and molluscs. In organisms and hence in foods arsenic occurs as water soluble and lipid soluble arsenic species (arsenolipids). Marine sources of food are known to contain relatively high levels of arsenic compared to other foods. The presence of arsenic in marine samples was first reported over 100 years ago. Marine organisms and algae, because of biotransformation and accumulation, contain high contents of arsenic, typically in the range 1-100 mg/kg (Francesconi, 1997). Most arsenic found in seafood is associated with relatively nontoxic organoarsenic species such as arsenobetaine or arsenosugars. In fish and molluscs is arsenic presented mainly as arsenobetaine and in algae as arsenosugars.

Rice has attracted a lot of attention because it is staple food in many countries, the major arsenic species in rice can be inorganic arsenic or dimethylarsinate (Stone, 2008).

It has been considered that determination of total arsenic concentration provide not enough information. Therefore, for evaluation of the toxic potential to humans and the environment, it is necessary to investigate not only the total arsenic concentrations but also to differentiate each arsenic species. Arsenic speciation is necessary to evaluate the risk assesment of food products and analytical methods are needed to distinguish between toxic and non-toxic arsenic species in food (EFSA, 2005).

2 LITERATURE REVIEW

2.1 ARSENIC CHEMISTRY

Arsenic is a metalloid with metallic and non-metallic properties. In biological samples arsenic occurs mainly in oxidation states +3 and +5. The only, natural occurring arsenic isotope is ^{75}As. Arsenic belongs to the group 15 of the periodic table and lies directly below P on the periodic table and so the two elements share many chemical properties. Due to these similarities, arsenic can often substitute for phosphorous in biological systems (Greenwood, 1997)

Arsenic can enter into the environment via anthropogenic processes including mining, smelting, combustion, the production and the use of pesticides, herbicides, insecticides and natural processes as weathering, volcanism, and dissolution of soils and sediments rich in arsenic. Arsenic is used mainly in agriculture (pesticides, wood preservation agents) and as the feed additive (Roxarsone) to improve growth of poultry although arsenic use in these applications have been reduced in recent years because of health concerns. Further, arsenic trioxide is used in medicine for treatment of certain type of leukemia (Cullen, 1989).

2.2 TOXICITY OF ARSENIC COMPOUNDS

The toxicity of arsenic is dependent on its chemical species. Furthermore the toxicity of arsenic species depends also on how they are metabolized in the body (Hansen, 2003). Therefore the toxicity and metabolic pathway of arsenic species need to be considered.

Inorganic arsenic compounds are generally more toxic than the organic arsenic compounds. Trivalent arsenic is considered more toxic than pentavalent arsenic. (Mandal, 2002). The best known victim of arsenic poisoning is French emperor Napoleon Bonaparte.

Two types of arsenic toxicity have been described:

Acute: Caused by ingestion of high amounts of inorganic forms of arsenic. A common parameter to evaluate acute toxicity is the LD_{50} value. The LD_{50} values of several arsenic species are displayed in Table 1.

Chronic: Humans are chronically exposed to inorganic arsenic mainly through their drinking water. Chronic exposure to inorganic arsenic may lead to skin lesions, Black foot disease, diabetes mellitus and various forms of cancers (Chaterjee, 1995).

Table 1. Acute toxicity of some arsenic compounds in laboratory animals

Arsenic species	Animal	Route	LD_{50} (mgAs/kg)	Reference
As^{III}	mouse	oral	26	Kaise, 1989
As^{V}	mouse	im*	22	Bencko, 1978
MA^{III}	hamster	ip**	2	Petrick, 2001
MA^{V}	mouse	oral	916	Kaise, 1989
DMA^{V}	mouse	oral	648	Kaise, 1989
Arsenobetaine	mouse	oral	>10 000	Kaise, 1985

* intramuscular
** intraperitoneal injection

2.3 ARSENIC SPECIATION

The IUPAC has defined elemental speciation in chemistry as follow:

Chemical species: specific form of an element defined as to isotopic composition, electronic or oxidation state, and/or complex or molecular structure

Speciation analysis: analytical activities of identifying and/or measuring the quantities of one or more individual chemical species in a sample

Speciation of an element (speciation): distribution of an element amongst defined chemical species in a system (Templeton, 2000).

For toxicological studies total arsenic concentrations provide not enough information because the acute toxicity depends on arsenic species. Arsenic provides a very good example of the importance of speciation in relation to toxicity. Nowadays many techniques and their combination are used for arsenic speciation analysis.

A fundamental tool for speciation analyses has been the combination of chromatographic separation techniques with element specific detector (ICPMS). Further hydride generation coupled with atomic absorbtion, atomic emission, atomic fluorescence is common method (Gong, 2002).

In 1986 Thompson and Houk were the first who combined HPLC with ICPMS. Up to this time arsenic speciation analyses were limited to certain types of samples or to samples with very high arsenic concentrations. The combination became the most powerful and commonly used method for arsenic speciation analysis.

2.3.1 Separation techniques

Hydride generation method

Hydride generation remains one of the most frequently used methods for arsenic determination. This instrumentation is a very efficient way for measuring arsenic species which can form hydrides (As^{III}, As^{V}, MA, DMA, TMAO) but it is not suitable for arsenic determination especially when most of arsenic is present as arsenobetaine or other non-hydride forming arsenic species. For this reason the HG technique is used mainly for samples that contain iAs, MA, DMA (drinking water, natural waters and human urine).

It is a derivatization process based on the production of volatile arsines formed from reaction with Zn/HCl or more commonly with sodium borohydride ($NaBH_4$) in acidic medium. As^{III} and As^{V} give AsH_3, MMA gives CH_3AsH_2, and DMA gives $(CH_3)_2AsH$. The volatile arsines are transported by an inert gas to the detection system. Only gaseous hydrides are introduced to the detector, and the matrix effect is minimized. The reaction involves the reduction of As^{V} to As^{III} and formation of arsine (AsH_3). The hydride generation technique for the determination of arsenic was first applied by Holak, who combined HG with AAS (Holak, 1969). In 1973 Braman and Foreback used a combination of selective reduction and cold trapping to individually analyse arsenic species. The boiling points of the arsines are different AsH_3, −55°C; CH_3AsH_2, 2°C; and $(CH_3)_2AsH$, 35.6°C; therefore the arsenic species can be differentiated by using HG with cryogenic trapping (Braman, 1973)

Arsenobetaine is a non-hydride forming As species, but it is possible to be detected, by introducing on-line UV photo-oxidation with persulfate $K_2S_2O_8$ prior to HG. The UV photo oxidation step is important for compounds that can not produce hydrides under reducing conditions (Rubio, 1993).

Hydride generation method can be easily connected to various detection systems such as AAS, AES, ICPMS. Moreover HG can be incorporated between HPLC and AAS or ICPMS and improves the detection limits.

Capilary Electrophoresis

CE has been applied for arsenic speciation studies. It offers high separation efficiency, small sample consumption and rapid analysis. The electric field is applied along the column, typically between 20-30 kV, and the analytes are moving with different velocity in the electric field. Buffer composition, concentration and pH affect the separation of arsenic species.

Early report used UV detection and silica capillary column with an acidic phosphate buffer to separate As^{III}, As^{V}, DMA, MMA (Morin, 1992). CE was also coupled to ICPMS and As^{III}, As^{V}, DMA, MMA, AsB, AsC were separated and LODs were in µg/L range (Olesik, 1995). The crucial point in coupling CE with ICPMS is the interface. Generally the flow rate of CE does not match with the flow rate for an efficient nebulisation. Therefore low flow microconcentric nebulizers have to be used. CE is able to separate cationic, anionic and neutral species but is

mostly limited to pure standard solutions or simple matrix. The application of CE to real sample has been reported only in few papers (Michalke, 2005).

Chromatographic separation of arsenic species

Liquid chromatography (LC) is the most popular technique for arsenic speciation. In LC a mobile phase is used to transport the sample into the column where individual species are selectively retained in the stationary phase and thus separated. Most of the speciation studies in biological samples are performed by High Performance Liquid Chromatography (HPLC) because it can determine both organic and inorganic arsenic species. HPLC can be easily connected with many other detection techniques such as ICPMS, HGAFS, MS which makes the system most useful for As speciation. After HPLC separation HG can be used to increase the sensitivity.

The chromatographic separation of arsenic species depends on pH. Arsenic species can be positively or negatively charged depending on the pH of solution, and consequently, they can be separate on cation- and anion-exchange columns. At neutral pH, As(V), (pKa1 = 2.3), MMA (pKa = 3.6) and DMA (pKa = 6.2) are present as anions; AC, (TMAO, pKa = 3.6) and TETRA as cations; AB (pKa = 2.18) as zwitterion; As(III), (pKa1 = 9.3) as an uncharged species (Raber, 2000; Goessler, 2002).

Ion exchange chromatographic separation (IC) is frequently used in arsenic speciation analysis. IC is based on the competition of the analyte ions and ions of the mobile phase for the oppositely charged sites (e.g. HSO_3^-, NH_3^+) bound to the stationary phase. The retention of the analyte is determined by several factors like, degree of ionisation, ionic strength, pH of mobile phase, temperature and flow rate.

Several types of chromatography can be used. With anion exchange chromatography is possible to separate As^V, MA, DMA and four common arsenosugars. A polymer-based column PRP-X 100 is used for the separation with 20 mM ammonium dihydrogen phosphate at pH 5.6. In this method arsenosugar glycerol and As^{III} are coeluting (Raber, 2000).

A method for the separation of cationic species, TMAO, DMAE, AB, AC and TETRA is performed on cation-exchange column. For the separation, a silica based Ionosphere 5C column is used and 10 mM pyridine at pH 2.6 as mobile phase (Hansen, 1992).

One of the most important parameter what is neccesary to calculate in HPLC analysis is the column recovery. Column recovery is the quantity of arsenic injected onto the HPLC column is accounted for by the sum of arsenic species eluting from the column. If 50 % or more of the arsenic is lost on the HPLC column, the arsenic speciation picture provided by HPLC is incomplete (Francesconi, 2004).

Gas chromatography was also applied for the measurement of volatile species. Since many compounds are not volatile or can not be transformed to volatile derivates without loss of information LC is without doubt the most commonly applied separation technique in arsenic specition analysis.

2.3.2 Detection techniques

Atomic Absorbtion Spectrometry

In atomic absorption spectrometry (AAS), the element analyte is thermally decomposed to atoms which absorb light at a particular wavelength characteristic of the element. The decomposition is usually performed by a flame or in an electrically heated graphite tube.

Until the 1980s, flame atomic absorption spectrometry (FAAS) was used as an HPLC detector for arsenic speciation but FAAS suffers from low sensitivity (LOD for As: 1 mg/L) and high background noise from the flame, GF-AAS was introduced. An improvement by a factor of 10–100 in analytical sensitivity was obtained by using a small heated graphite tube. However, a direct coupling of HPLC to GFAAS is difficult because it is necessary to use a long analytical cycle, including drying and ashing the sample prior to furnace atomization. The problem of losing arsenic during the ashing stage (arsenic sublimes at 613°C) was overcome by adding the matrix modifers such as paladium nitrate to sample solution (Shan, 1984).

In 1969 Holak discribed HGAAS in which AsH_3 was generated and collected in a cold trap and passed into the burner of an AA spectrometer. Atomic absorption spectrometry, in combination with a hydride generation step, was the most common method for the determination of total arsenic content during the 1970s and 1980s, and is still widely used. With hydride generation AAS, arsenic content can be determined bellow 0.02 mgAs/kg (Holak, 1969).

Atomic Fluorescence Spectrometry

In atomic fluorescence spectrometry (AFS), atoms are excited to higher energy state by absorption of electromagnetic radiation, and the fluorescnece emission from these excited atoms is measured at a specific wavelength. By measuring the intensity of the emitted light it is possible to determine the concentration. In combination with hydride generation, AFS provides excellent sensitivity for arsenic with reported quantitative measurements bellow to 0.01 mg arsenic/kg. Early work by Woller employed HPLC coupled to AFS detector for the determination of As^{III}, As^{V}, MMA, DMA (Woller, 1995). The coupling of HPLC with HGAFS was applied for arsenic speciation analysis in wide range of biological and environmental samples (Mester, 1996; Vilano, 2000).

Atomic Emission Spectrometry

Generally in atomic emission spectrometry, element is thermally excited to a high energy state, and as it returns to lower energy states it emits light at a wavelength characteristic of the element. For arsenic the major emission lines are 193.7, 197.2 and 228.0 nm. There are several types excitation including microwave-induced plasma, inductively coupled plasma, dirrect current plasma etc. The favored

excitation source in AES is ICP. Inductively coupled plasma atomic emission spectrometry (ICP AES) has been coupled to HPLC for use in arsenic speciation but for samples containing lower levels of arsenic, HPLC–ICPAES does not provide sufficient sensitivity for arsenic speciation. The technique is applicable to arsenic rich samples such as marine samples (Twyman, 2005). In several studies was HG incorporated between HPLC and ICPAES to enhance the sensitivity (Rubio, 1992).

ICPAES is a robust technique but compared to ICPMS, its application is prefered for analysis of major elements. For ultra trace level analysis ICPMS is without doubt the technique of choise.

ICPMS - Indouctively Coupled Plasma Mass Spectrometry

The ICPMS is the detection method of choise for interfacing with HPLC. The ICP-MS instrument uses Inductively Coupled Plasma ICP as the ionization source and a mass spectrometer MS to analyze the ions produced. It can perform qualitative, semi-quantitative, and quantitative analysis, and since it employs a mass analyzer, it can also measure isotopic ratios. The plasma is generated inside a quartz torch, surrounded by a copper coil and radio-frequency power is applied to the load coil, which creates a magnetic field. Liquid samples are introduced by a peristaltic pump, to the nebulizer where the sample aerosol is formed, passed through a cooled spray chamber, where the small droplets of aerosol are separated from larger droplets and is introduced via the inner tube of the torch. The sample introduction takes place at atmospheric pressure which is an advantage of the technique because the coupling with HPLC is easily done. The elemental response is essentially independent of species, so it is usually possible to quantify a species without having that particular species as standard. The sample aerosol is decomposed in the plasma (plasma temperature lies between 6000 – 10 000 K) to form atoms which are simultaneously ionized. The ions are extracted from the plasma into the mass spectrometer region which is held at high vacuum (typically 10^{-4} Pa) provided by turbo pumps (Jarvis, 1992). The ions pass through the sampling and skimmer cones then are focused by a series of ion lenses into a mass analyzer, which separates the ions based on their mass/charge ratio. The quadrupole mass filter has been the most widely used mass analyzer for ICPMS. The ions are counted using an electron multiplier. When the ion beam strikes the surface of the first dynode, secondary electrons are released. The electrons are accelerated to the next dynode and release secondary electrons again. 12 or 24 dynodes are typical for a discrete-dynode electron multiplier (Agilent, 2005).

2.3.3 Legislation

Arsenic presents an example of the importance of speciation analysis in relation to legislation concerning food safety. The chemical forms of arsenic in most seafoods, arsenobetaine and arsenosugars, are generally considered to be not toxic,

whereas inorganic arsenic is highly toxic and should be monitored and controlled in relevant foods and in drinking water.

The EU has set a maximum limit of 10 µgAs/l in drinking water based on the recommendation of WHO. There are no EU-wide regulations for arsenic content in food. The average adult intake from food has been estimated to be 30-120 µg/day. The intake may be higher in countries with large consumption of seafood (Berg, 1999). For example in England arsenic levels are regulated by the Arsenic in Food Regulations 1959, the limit for total arsenic in food was set as 1 mg As/kg.

The toxicity of arsenic is expressed by the FAO/WHO Joint Expert Committee on Food Additives and Contaminants (JECFA) as a Provisional Tolerable Weekly Intake (PTWI) at 15 µg/kg body mass for inorganic arsenic. The WHO set a Provisional Maximum Tolerable Daily Intake (PMTDI) at 2 g/kg to cover risks from water and food however no specific guidelines are given.

European Commission has requested the European Food Safety Authority (EFSA) to issue a scientific opinion on human health related to the presence of arsenic in foodstuffs and provide a risk assessment (EFSA, 2009).

Future legislative limits for arsenic must be based on those arsenic species which are of toxicological concern, such as inorganic arsenic and not only on the total arsenic content.

3 GOAL OF THE THESIS

The main goal of the thesis is arsenic speciation analysis especially in marine organisms such algae, fish and mussels. Moreover were analyzed rice samples with relatively low arsenic content in order to evaluate risk assessment of rice available in Czech market.

Beside calculating the mass balance that also provide useful information about the extraction efficiency and column recovery of the arsenic compounds was necessary to validate analytical method by using certified reference material. Very important was to choose the right matrix that contained the required analyte. Suitable CRMs are limited and certified values are mostly given only for total arsenic content and one arsenic species.

The laboratory experiment was designed to follow the transformation of arsenic compounds in decomposing algae, and was showed that the arsenosugars originally present in algae were converted primarily to arsenate and DMA during 25 days. The results showed significant amount of insoluble or recalcitrant arsenic therefore the subsequent laboratory work was focused on different extraction procedures as microwave assisted extraction to extract recalcitrant arsenic and identification of unknown arsenic compound.

The outcome of the work supposes to help with further understanding of arsenic metabolisms in marine organisms.

4 OVERVIEW OF RESULTS AND DISCUSSION

This chapter presents an overview of the most important findings of the PhD thesis.

4.1 STUDY ON ALGAL DECOMPOSITION

The results from this section were published in following publication: *Navratilova, J., Raber, G., Fisher, S., Francesconi, K.A. Arsenic cycling in marine systems: Degradation of arsenosugars in decomposing algae to arsenate and evidence for recalcitrant arsenic. Environ Chem 8(1) 44-51, 1448-2517, doi: 10.1071/EN10107* therefore only additional results were selected.

The aim of the study was to follow the degradation of arsenosugars present in marine algae under simulated natural condition. The chosen alga was brown alga *Ecklonia radiata* which contains a mixture of three major arsenosugars: arsenosuger glycerol, arsenosugar PO4 and arsenosugar SO3. The laboratory experiments were designed to follow the transformation of arsenic compounds in decomposing algae showed that the arsenosugars originally present were converted primarily to arsenate and DMA. The study demonstrates a conversion of organic arsenicals back to inorganic arsenic.

Total arsenic concentrations were determined in freeze-dried alga powders and reference materials, and in their aqueous-methanol extracts by ICPMS after microwave-assisted acid digestion (procedure describes above). Arsenic species were extracted from the dry alga samples as follows: about 100 mg of powdered material was weighed (to a precision of 0.2 mg) directly into a 15 ml polypropylene tube and 2.0 ml of a water/methanol solution (9+1, v/v) was added. The contents were shaken top-over-bottom overnight, then centrifuged (3000 g x 15 min). The supernatant was filtered (0.22 µm nylon) and aliquots (2 x 100 µl) were removed for the duplicate determination of total arsenic to calculate extraction efficiency.

Arsenic species were determined on aqueous-methanol extracts of the samples using anion- and cation-exchange HPLC/ICPMS

Additionally the total arsenic concentration in the extracts of part D was measured by flow injection method. The determination of column recovery usually requires digestion of an aliquot of the injected sample solution and subsequent total arsenic analysis. The advantage of the flow injection method for detremination of total arsenic content is that there is no need for further sample pre-treatment, like for classical digestion analysis. It is supposed that the response of the ICPMS is not significantly influenced by the type of arsenic species (Beauchemin, 1989). 20 mM ammonium dihydrogen phosphate pH 5.6 10% methanol was used as eluent. Standard solutions of arsenic (1–500 mg/ml) were used for external calibration. All samples were injected three times. The outlet of the injector port was directly

connected to the nebulizer of the ICPMS by a polyetheretherketone capillary tubing and quantification was done using peak area.

Quality control for total arsenic measurements and arsenic speciation analyses

The reference materials used were DORM-2, a dogfish Certified Reference Material (CRM) available from the National Research Council of Canada, Ottawa, Canada; and CRM IAEA-140/TM, a "Seaweed", *Fucus sp*., from the International Atomic Energy Agency, Vienna, Austria. In addition, an in-house reference alga extract was used, which has well-documented arsenosugar content, as a check sample to validate the analysis procedure (Madsen, 2000).

Total arsenic

The accuracy of the method for determining total arsenic content was tested by the analysis of an alga reference material CRM IAEA-140/TM, which has a certified arsenic value of 44.3 ± 2.1 mg As/kg dry mass (95% confidence interval); the obtained value was 45.8 ± 1.1 mg As/kg dry mass (mean ± SD, n=9).

Arsenic species

There is currently no reference material certified for arsenosugars. The reliability of the arsenic speciation procedure was assessed in two ways. First, by the analysis of the CRM DORM-2 certified for arsenobetaine (16.4 ± 1.1 mg As/kg) and tetramethylarsonium ion (0.248 ± 0.054 mg As/kg); the following results for (mean ± SD, n=3), arsenobetaine 15.6 ± 0.6 mg As/kg, and tetramethylarsonium ion 0.24 ± 0.02 mg As/kg were obtained. Second, (n=4) an in-house algal reference extract (Fucus serratus) was analysed, for which published values are available for the four arsenosugars commonly found in algae (Madsen, 2000); the results agreed to within 5% of the published data for each of the four arsenosugars.

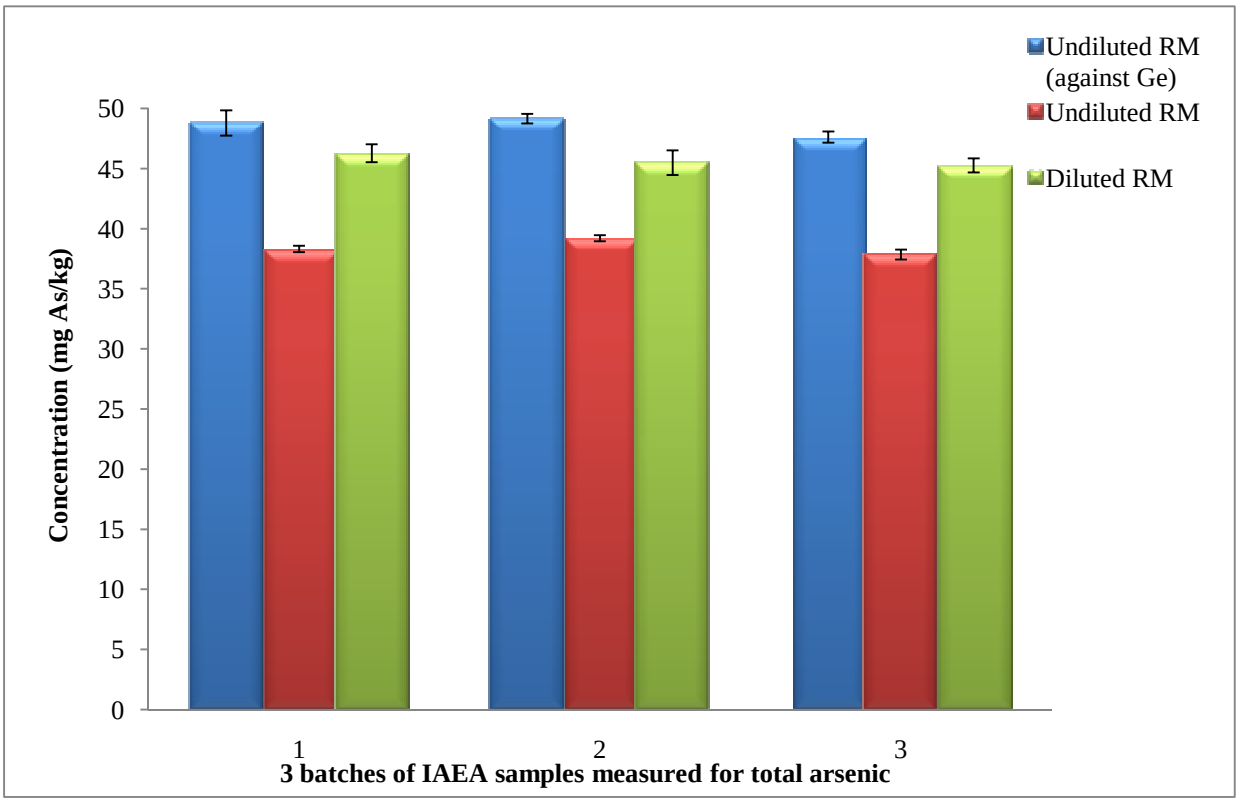


Figure 1. Determined arsenic concentration in CRM IAEA-140/TM (Fucus sp., Sea Plant Homogenate) showing non-normalized and normalized data against ^{74} Ge

The suppression of the arsenic signal by the matrix was investigated by the analysis of CRM IAEA-140/TM (Figure 1).

The results showed that in case of measurement undiluted IAEA samples (DF 100) was observed significant matrix effect and the arsenic signal was suppressed. Therefore it was necessary to normalised data against internal standard ^{74}Ge or to further dilute the samples (final DF 1000). The diluted sample (DF 1000) solutions showed clearly differences compared to the concentration of arsenic in the undiluted samples solutions. The signal was influenced by the sample matrix and matrix effect was observed. It is obvious that the signal suppression can be easily eliminated by the standard adition method.

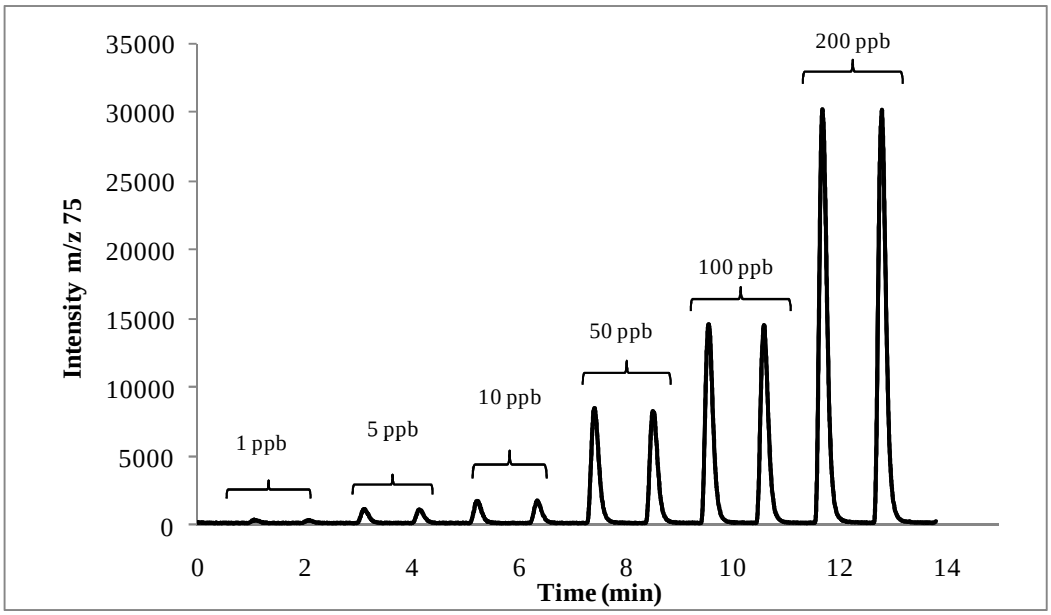


Figure 2. Typical flow injection signals for DMA standard

Table 2. Comparison of digestion method and flow injection analysis performed on the extracts of section D

Sample D	Digestion µg As g ± SD	FIA µg As g ± SD	%
D0	1.94 ± 0.05	1.81 ± 0.01	93
D1	1.67 ± 0.02	1.63 ± 0.02	98
D2	2.17 ± 0.11	2.02 ± 0.02	93
D3	2.21 ± 0.11	2.03 ± 0.01	92
D4	1.74 ± 0.04	1.64 ± 0.01	94
D5	1.65 ± 0.06	1.42 ± 0.01	86
D6	0.77 ± 0.03	0.69 ± 0.01	90
D7	2.09 ± 0.01	2.08 ± 0.01	98
D8	1.70 ± 0.04	1.58 ± 0.01	93
D9	1.34 ± 0.01	1.32 ± 0.01	99
D10	1.25 ± 0.05	1.12 ± 0.01	90
D11	2.04 ± 0.01	1.92 ± 0.01	94

Results from both digestion method and flow injection analysis were in good agreement with each other. FIA is an appropriate procedure for the total arsenic determination.

4.2 ARSENIC SPECIATION IN RICE

The purpose of the study was to evaluate arsenic species distribution in rice available in Czech market in order to improve understanding of the health risk posed by arsenic in rice available in Czech Republic. Terrestial foods have low levels of arsenic. There are some exceptions for example rice can typically contain about 0.1 - 0.4 µgAs/g (Williams, 2005). Rice is an important route of human exposure to arsenic and dietary studies in Europe and the US showed that rice is the primary source of arsenic in nonseafood diet (Schoof, 1999; Tao, 1999). The health risk of arsenic in rice is based on its inorganic content because these species are generally considered to be more toxic than methylated species. Ten commercially available rice samples from various locations worldwide were chosen and analysed for arsenic total content and inorganic arsenic. The key step in arsenic speciation analysis is the extraction procedure. In term of rice speciation mostly trifluoroacetic acid is used however As^{V} in the samples could be reduced to As^{III} during the extraction procedure. In this work microwave assisted extraction with 20 mM TFA. TFA hydrolyzes starch in the grain during extraction, which can result in a more successful recovery than a methanol/water extraction, usually used for arsenic extraction.

Samples were homogenized to fine powder. For digestion 250 mg of sample was weighted and 2 ml HNO_3 and 2 ml water were added. After digestion the digests were transfered to polypropylene tubes (15 ml) and fill up with milliQ water to 9 mL. To all samples and also to calibration standards 1 ml of 100 µg/l of ^{74}Ge, ^{115}In in 50% CH_3OH was added. The final volume was 10 ml. Each sample was digested and analysed in triplicates.

A portion (*ca* 250 mg) of rice powder was weighted for extraction and 5 ml of 20 mM TFA. The tubes were shortly vortexed and placed in an ultrasonic bath at 40°C for 15 min before extraction. Extraction was performed with an Ultraclave III system at a loaded pressure of 40 bars, at 95°C for 1 h. After extraction, samples were centrifuged for 15 min at 3000 g and the supernatant was injected directly onto HPLC column. Each sample was extracted and analysed in triplicates.

Arsenic speciation analysis was performed on the extracts by HPLC/ICPMS under anion-exchange conditions. In this study was necessary to use CO_2 as an optional gas for arsenic signal enhancement considering the low arsenic concentration. The calibration curve was estabilished from 0.2-10 µg As/l of As^{III}, DMA, MA, As^{V}.

Quality control

Total arsenic content was determined in SRM 0.29 ± 0.04 µg As/g (mean ± SD, n=3) and agreed well with the certified value 0.29 ± 0.03 µg As/g.

The accuracy of the arsenic speciation procedure was determined by the analysis of the certified reference material NIST SRM 1568a Rice Flour (National Institute of Standards and Technology, NIST, USA) even though not certified for arsenic

species, the sum of the species recovered from HPLC separation was compared with the total arsenic concentration. Resulted sum of species 0.27 ± 0.02 µg As/g (mean ± SD, n=3) was in good agreetment with certified value 0.29 ± 0.03 µg As/g.

The limit of detection was determined by the 3σ method at the concentration of 0.2 µg As/l. The HPLC/ICPMS analysis detection limit for speciation ranged between 0.04 and 0.05 µgAs /l, based on 20 µl injection volume, determined as three times the standard deviation of the measured 0.2 µg As/l standard solution.

Table 3. Concentrations of total arsenic and arsenic species in Czech market rice

Country of origin	Total arsenic (µg As/kg ± SD)	Arsenic Species (µgAs/kg ± SD)		
		DMA	MA	AsV
Thailand	126.50 ± 1.87	49.70 ± 0.09	1.87 ± 0.30	68.40 ± 1.33
Italy	211.29 ± 2.23	37.49 ± 0.12	2.28 ± 0.25	141.38 ± 0.99
Pakistan	49.31 ± 0.63	17.28 ± 0.15	1.68 ± 0.25	30.16 ± 0.83
India	93.14 ± 1.30	13.09 ± 0.97	>LOD	72.33 ± 0.39
EU	36.06 ± 2.32	10.67 ± 0.14	>LOD	25.36 ± 1.00
Italy	87.08 ± 2.79	21.91 ± 0.45	>LOD	58.54 ± 0.39
Poland	156.45 ± 1.88	35.80 ± 0.51	1.73 ± 0.15	114.04 ± 1.31
EU	128.60 ± 2.95	41.20 ± 1.28	3.58 ± 0.87	77.29 ± 0.40
Italy	107.88 ± 0.41	21.61 ± 0.21	>LOD	76.75 ± 0.82
Italy	218.11 ± 4.67	29.56 ± 0.97	>LOD	170.77 ± 2.33

Total arsenic concentrations found in the samples were between 36.06 µg As/kg for Basmati Uncle Bans rice and 218.11 µg As/kg for sample Bio long grain natural Harmony. The content of inorganic arsenic varied from 54% (Thai yasmine rice) to 78% (Bio long grain natural Harmony). The main species detected were DMA and inorganic arsenic, quantifiable amounts of MA were observed in five samples (Table 3). Thailand is the largest exporter of rice in the world, only one sample of Thai rice was analysed, which had a total arsenic concentration of 126.50 µg As/kg and 54% of iAs.

The conclusion of the rice survey is that there is a clear variation in As species distribution and total As concentration in rice grown in different countries.

4.3 DETERMINATION OF WATER SOLUBLE AND LIPID SOLUBLE ARSENICALS IN ESCOLAR

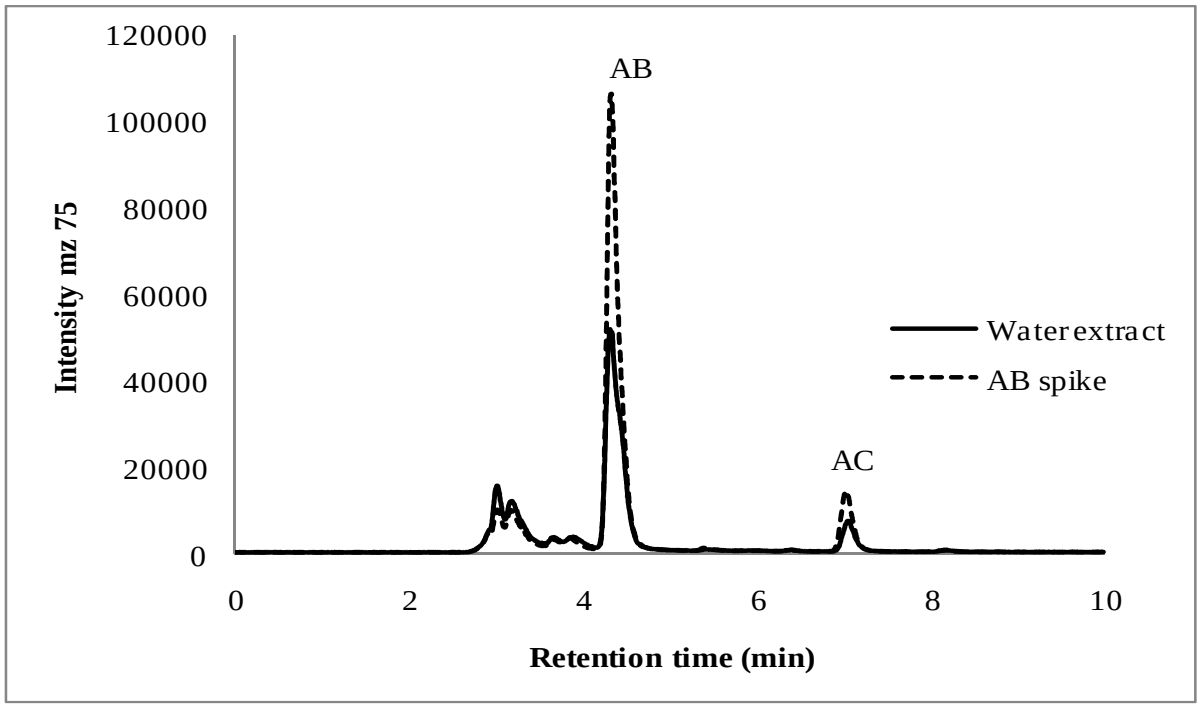
The aim of the study was the determination of water and lipid soluble arsenicals in fish tissue of escolar (*Lepidocybium flavobrunneum*) belonging to the family of Gempylidae. The fish is known for its high content of wax ester (>90%), which can cause diarrhoea and other acute gastro-intestinal symptoms. A fish steak (0.4 kg) was purchased from local market in Austria labelled as "Buttermakrele". Skin was excluded and only meat was used for further analysis. Fish tissue was washed with milliQ water and cut into small pieces with a scalpel and transferred to clean and dry vials. The fish tissue was freeze dried to constant mass in order to determine the moisture content (60%). The dried tissue was ground under liquid nitrogen with a mortar and pestle to fine powder. Portions of sample (0.2 g) were weighted for digestion and 1 ml of HNO_3 was added, in total 3 replicates were digested and final volume after digestion was 10 ml. Arsenic content was determined in freeze dried powders of fish tissue by ICPMS method. The determined concentration in fish tissue was 1.8 ± 0.1 mg As/kg **(**mean ± SD, n=3). Three different extraction procedures were performed on the fish tissue (hexane, water and mixture of $CHCl_3$/methanol; 2:1) and extraction efficiency was calculated for each fraction. About 4 g of sample was weighted for extraction and 50 ml of extractant solvent was added and the mixtures were shaken overnight at room temperature. An aliquat 500 µl of hexane fraction, 500 µl of $CHCl_3$/methanol fraction and 2 ml of water fraction was removed and digested with 1ml HNO_3. Total arsenic was determined in all extracts by ICPMS in order to calculate extraction effieciencies. The hexane and water fractions were further evaporated to dryness using rotar evaporater and the residues were dissolved in 1 ml of hexane and water, respectively.

Table 4. Percentage of extractable As

Extractant	Extractable As (%)
Hexane	22
$CHCl_3$/MeOH (2/1)	112
Water	17

Arsenic species were determined by anion- and cation-exchange HPLC/ICPMS in water extract. The water fraction was preconcentrated prior HPLC/ICPMS by rotar evaporater and the residue was redissolved in 1ml of water. Anion-exchange HPLC separations was performed on a PRP-X100 column (150×4.1 mm, 10µm particles; Hamilton, Reno, USA) at 40°C with a mobile phase of 20 mM aqueous ammonium dihydrogen phosphate at pH 5.6 (adjusted with aqueous ammonia) at a flow rate of 1.5ml/min. Cation-exchange HPLC separations were performed on a Zorbax 300-SCX column (150×4.6 mm, 5 µm particles; Agilent Technologies) at 40°C with 10mM aqueous pyridine at pH 2.6 (adjusted with formic acid) at a flow rate of 1.5 ml/min.

a)



b)

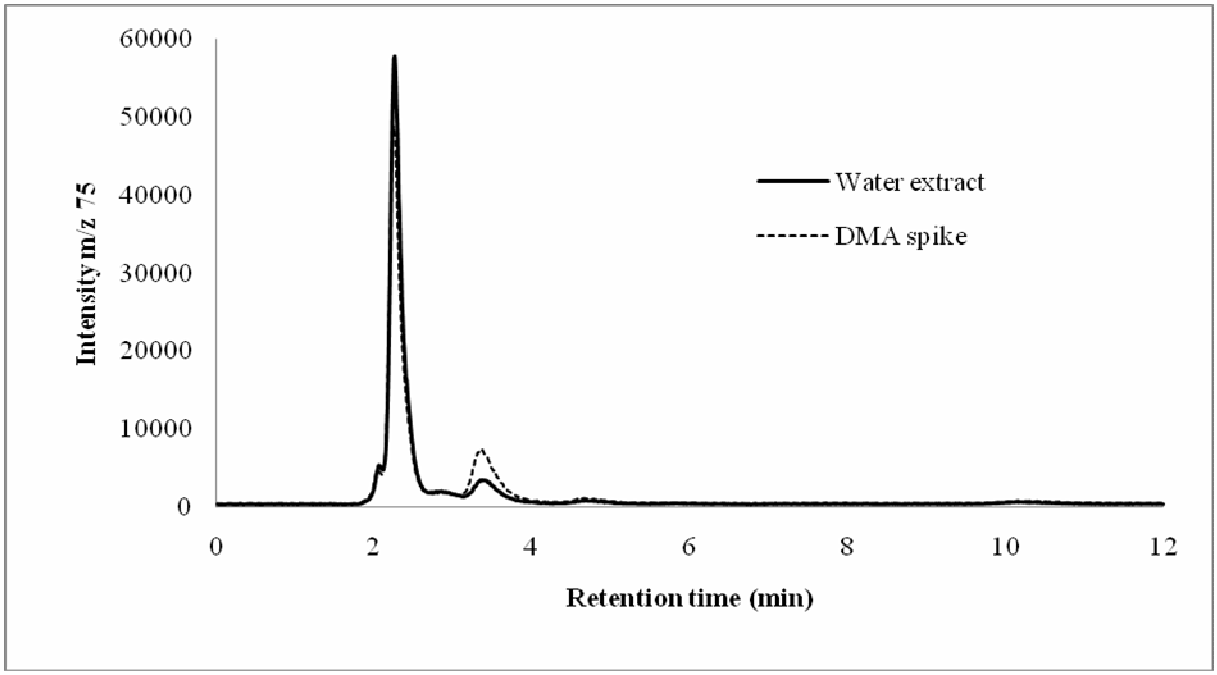


Figure 3. Overlayed HPLC/ICPMS chromatograms of spiked water extracts

a) anion exchange separation: PRP-X100 column (150×4.1 mm, 10µm particles; Hamilton, Reno, USA) at 40°C with a mobile phase of 20 mM aqueous ammonium dihydrogen phosphate at pH 5.6, flow rate of 1.5 ml/min, 20 µl injection volume,column recovery: 87%

b) cation-exchange separations: Zorbax 300-SCX column (150×4.6 mm, 5µm particles; Agilent Technologies) at 40°C with 10 mM aqueous pyridine at pH 2.6, flow rate of 1.5 ml/min, 20 µl injection volume, column recovery: 95%

In past was very difficult to analyze arsenolipids directly by HPLC-ICPMS, therefore the research focus was mainly on arsenic water soluble species. In 2005 was published method for direct measurement of arsenolipids (Schmeisser, 2005).

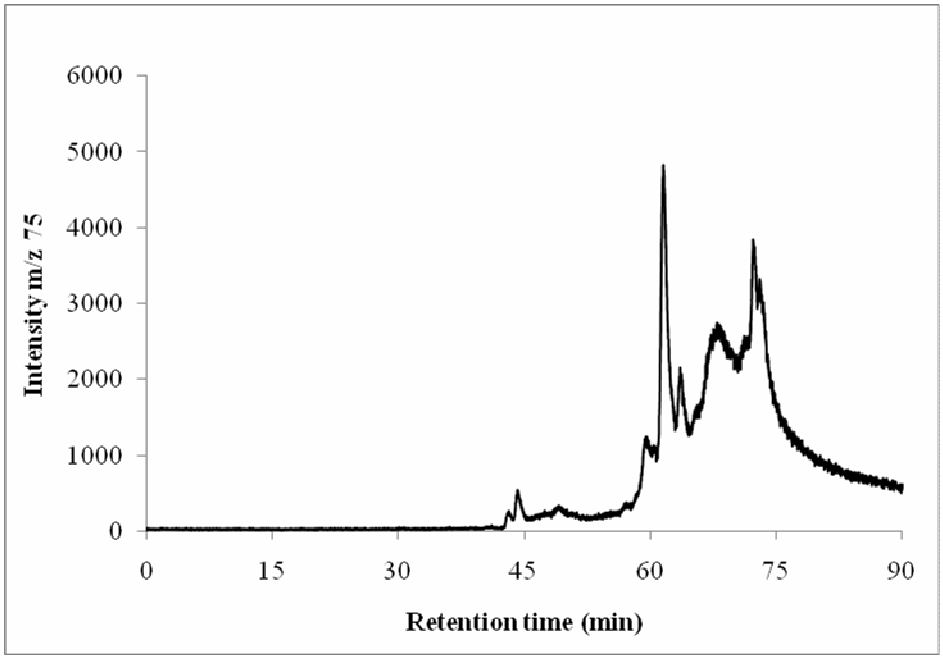
In this study the separation of arsenolipids by HPLC/ICPMS was performed with an Atlantis®dC18 column (4.6 x 150 mm; Waters, Massachusetss, USA) at 30°C with ethanol/20 mM ammonium acetate pH 6 as mobile phase and a flow rate of 100 µl. Chromatography was performed with gradient elution 0 - 60 min for 35% - 100% ethanol and then 30 min at 100% ethanol. The hexane fraction was preconcentrated prior HPLC/ICPMS by rotar evaporater and the residue was redissolved in 1ml of hexane. Injection volume was 5 µl.

Following ICPMS conditions were used: radio frequency (RF) power 1600 W, the spray chamber was set at -5°C to limit the amount of organic solvent organic solvent reaching the plasma, because it can cause the problems of plasma instability, torch with a narrow i.d. (1.5 mm). The ICPMS was tuned with a solution of 10 µgAs/L in the mobile phase to obtain maximum sensitivity. The signals at m/z 75 and m/z 77 were monitored using an integration time of 300 ms, and the carbon content in the plasma was monitored at m/z 53 ($^{40}Ar^{13}C$) using an integration time of 100 ms. To prevent deposition of carbon on interface cones an optional gas (20% oxygen in argon) was introduced through a T-piece connecting the spray chamber and the torch. Since added oxygen promotes corrosion of interface cones, a platinum sampler cone was used. The optimization of the appropriate level of oxygen was performed by seting up the default flow of optional gas (5% of carrier gas flow) and by increasing the flow rate of the HPLC until carbon began to appear on the sample cone. Then the oxygen flow rate was increased until the carbon deposits were decomposed and the green emission visible in the central channel of plasma was observed. This was repeated until the flow rate 100 µl was reached.

Table 5. ICPMS setting for organic measurement

Parameter	Setting
Nebulizer type	Burgener Ari Mist HP Nebulizer
Spray chamber type	Peltier cooled, Scott type, double pass
Spray chamber temperature	5°C
Torch	1.5 mm ID
RF power	1600 W
Plasma gas flow	15 l/min
Carrier gas flow	0.53 l/min
Auxiliary gas flow	0.25 l/min
Optional gas (20% O_2 in argon)	8%
Sample cone	Platinum
Skimmer cone	Nickel
Extract lens 1	1
Extract lens 2	-132
Omega Bias ce	-28
Omega lens ce	0
Cell Entrance	-34
QP focus	4
Cell exit	-36

a)



b)

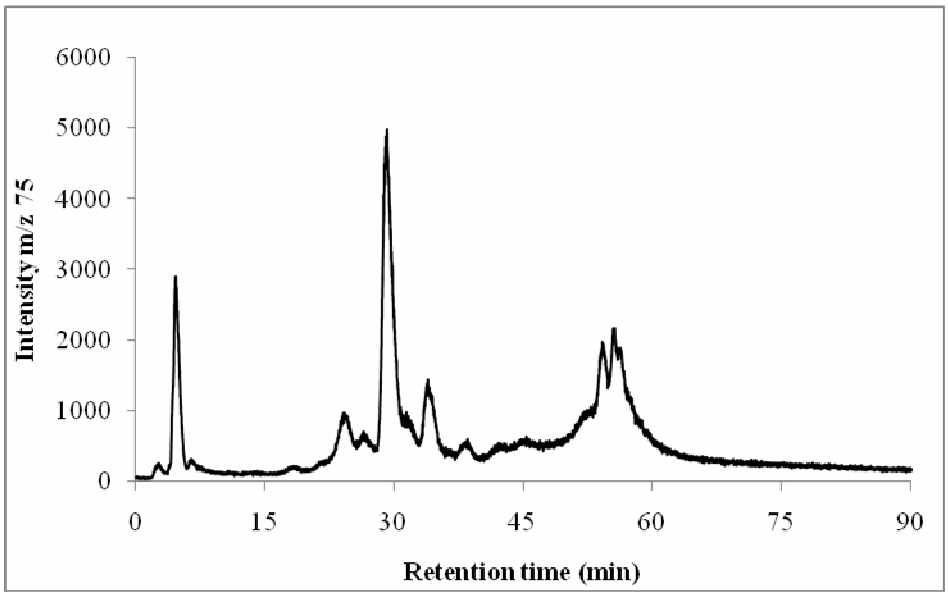


Figure 4. Reversed-phase HPLC/ICPMS chromatogram of the nonpolar arsenolipids (hexane extract) from escolar fish.

a) conditions: Atlantis dC18 (150 x 1.0mm, 5 um) at 30°C , mobile phase 20 mM NH_4OAc pH 6 and etanol at a flow rate of 100 µl/min. The chromatography was performed with linear gradient elution: 0-60 min for 35% - 100% ethanol and then 30 min at 100% ethanol, injection volume 5 µl.

b) conditions: Atlantis dC18 (150 x 1.0mm, 5 um) at 30°C , mobile phase 20 mM NH_4OAc pH 6 and etanol at a flow rate of 100 µl/min. The chromatography was performed with linear gradient elution: 0-60 min for 75% - 100% ethanol and then 30 min at 100% ethanol, injection volume 5 µl.

The determined total arsenic concentration in the fish muscle tissue with ICPMS was 1.8 ± 0.1 mg As/kg (mean ± SD, n=3). Solvent partitioning showed that 17% of arsenic was water exractable. The major water-soluble arsenic compound was identified as arsenobetaine (89%), which was confirmed by spiking experiment.

Lipid-soluble arsenicals were then extracted from portions of the fish tissue with hexane because it was expected their higher content in nonpolar fraction. About 22% from the total arsenic was extractable into hexane. Examination of the hexane fraction using HPLC-ICPMS with gradient elution revealed a number of unknown peaks. Due to their chromatographic behavior (nonpolarity) was assumed that these compounds are neither arsenic containing fatty acids nor arsenic containing hydrocarbons. The quantification of arsenolipids was impossible because of the lack of standard and because of gradient elution with different carbon composition. The identification of nonpolar arsenolipids by HPLC/electrospray MS was not informative because of large matrix interference and suppression of the molecular ion signals. To clarify the structures of lipid-soluble arsenicals would be necessary to design suitable clean up procedures to the point where electrospay MS data could be obtained. The stability of the compounds during the purification procedure must be taken in account.

5 CONCLUSION

The key task of this PhD thesis has been a development of speciation analysis of arsenic and the outcome of this thesis should highlight the necessity to distinguish between different chemical species of arsenic.

Arsenic speciation analysis represents a field that will without doubt continue to grow in importance in the future even though regulations still focus on total arsenic concentrations and not on arsenic species.

The overall objective of this thesis was to gain a better understanding of the fate of arsenic taken up by marine organisms and their ability to transform it. The laboratory experiments were designed to follow the transformation of arsenic compounds in decaying algae showed that the arsenosugars originally present were converted primarily to arsenate and DMA.

Rice samples from Czech market were analysed in term of the risk associated with low level arsenic exposure. The arsenic species identified in rice by HPLC/ICPMS after TFA-extraction method were iAs (54-78%) and DMA. Higher content of inorganic arsenic might have implication for public health.

The important findings from the investigation of arsenolipids present in fish escolar are the data observed from the extraction procedures with solvents of different polarities. The hexane fraction, represents 22%, was analysed by HPLC/ICPMS and the analysis revealed the number of unknown peaks however the instrument does not provide structural information. For structural elucidation will be necessary to apply appropriate clean up procedure which enable to obtain clear ESI/MS mass spectrum. The chemical synthesis of the coumpounds would be necessary as well. The water soluble fraction was analyzed by HPLC/ICPMS and

showed presence mainly of arsenobetaine with a traces of DMA. These results are consistent with several previous studies for water soluble arsenic compounds obtained for a number of marine animals. Arsenobetaine is excreted by humuns unchanged in the urine and is consider to be nontoxic. The human health risk associated with arsenic is usually disussed in terms of iAs however organic arsenic may represent potential risk and it should be noted that the metabolism of arsenolipids in human is little understood. The work so far indicate that a reasonable amount of arsenic in fish escolar is lipid bound.

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7 CURRICULUM VITAE

Education
2007-present: Doctoral study
Brno University of Technology, Faculty of Chemistry, Department of Food Science and Biotechnology
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Research visits
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Granted by specific research grant
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Project: Arsenosugar degradation
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2002: Awarded price of Jaroslav Heyrovsky for young student for 1st place in National Student Competition: "The detection of genetically modified soya by PCR"

Publication
Navratilova, J., Raber, G., Fisher, S., Francesconi, K.A. Arsenic cycling in marine systems: Degradation of arsenosugars in decomposing algae to arsenate and evidence for recalcitrant arsenic. *Environ Chem* 8(1) 44-51, 1448-2517, doi: 10.1071/EN10107.

Conference Presentation

Navratilova, J., Simko, P. Arsenic speciation in rice: Survey of the Czech market. Oral presentation. Chemistry and Life, 2011, Brno, Czech Republic

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8 ABSTRACT

Arsenic speciation analysis was carried out by high performance liquid chromatography coupled with inductively coupled plasma mass spectrometry (HPLC/ICPMS). The toxicity of arsenic considerably depends on its oxidation state and physico-chemical character of given compounds. The knowledge of speciation is critical to evaluate correctly the bioavailability and toxicity of arsenic as well. Generally, the inorganic species are more toxic than organic ones.

The degradation products of arsenosugars in marine algae were investigated under simulated natural conditions and the arsenosugars originally present were converted primarily to arsenate and dimethyl arsenic acid.

Because of possible arsenic adsorption by plants from soils and water, the arsenic speciation analysis was perfomed on rice available at Czech market in order to determine particulary inorganic arsenic. The total arsenic content ranged between 36.06 µg/kg - 218.11 µg/kg. The main species detected were dimethyl arsenic acid and inorganic arsenic (54-78%).

Marine fish and oils contains significant amount of lipid soluble arsenical compounds. The study was focused on the analysis of arsenic species bound to the lipids of the fish Escolar (*Lepidocybium flavobrunneum)*. As found, the total arsenic content was 1.8 mg/kg and contained 22% of arsenic extractable by hexane while the water soluble arsenic was predominantly arsenobetaine (89%).

9 ABSTRAKT

Speciační analýza arsenu v různých matricích s využitím HPLC/ICPMS byla předmětem této práce. Toxicita arsenu závisí na oxidačním stavu a formě, ve které je přítomen. Znalost zastoupení specií arsenu je nutná k hodnocení toxicity a biodostupnosti. Obecně, anorganické specie jsou více toxické než organické.

V práci byla studována degradace arsenocukrů v mořských řasách za simulovaných přirodních podmínek. Původní arsenocukry byly transformovány na arseničnan a kyselinu dimethylarseničnou.

Arsen vstupuje do rostlin z půdy a vody a následně může vstoupit do potravního řetězce. S ohledem na tuto skutečnost byla speciační analýza provedena u vybraných vzorcích rýže, zakoupených v české obchodní síti. Stanovený celkový obsah arsenu se pohyboval v rozmezí 36.06 µg/kg - 218.11 µg/kg a hlavními speciemi byla kyselina dimethylarseničná a anorganický arsen (54-78%).

Mořské ryby a tuky obsahují významnou část arsenu ve formě zvané arsenolipidy. Část práce byla zaměřena na analýzu arsenolipidů u máslové ryby (*Lepidocybium flavobrunneum*) s celkovým obsahem arsenu 1.8 mg/kg a 22% celkového arsenu bylo vyextrahováno pomocí hexanu, což potvrzuje lipofilni charakter těchto sloučenin. Hlavní specií stanovenou ve vodném extraktu byl arsenobetain, představující 89%.