VYSOKÉ UČENÍ TECHNICKÉ V BRNĚ

BRNO UNIVERSITY OF TECHNOLOGY

FAKULTA CHEMICKÁ
ÚSTAV CHEMIE POTRAVIN A BIOTECHNOLOGIÍ

FACULTY OF CHEMISTRY
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METHODS FOR ELEMENTAL SPECIATION OF ARSENIC COMPOUNDS.

DIZERTAČNÍ PRÁCE
DOCTORAL THESIS

AUTOR PRÁCE Ing. JANA NAVRÁTILOVÁ
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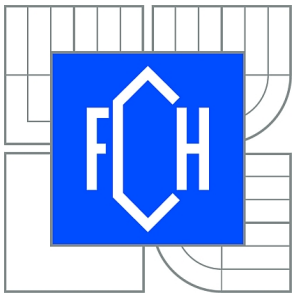

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METODY SPECIAČNÍ ANALÝZY SLOUČENIN ARSENU.

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DOCTORAL THESIS

AUTOR PRÁCE — Ing. JANA NAVRÁTILOVÁ
AUTHOR

VEDOUCÍ PRÁCE — prof. Ing. PETER ŠIMKO, DrSc.
SUPERVISOR

BRNO 2011

Vysoké učení technické v Brně
Fakulta chemická
Purkyňova 464/118, 61200 Brno 12

Zadání dizertační práce

Číslo dizertační práce:	**FCH-DIZ0045/2010**	Akademický rok:	**2010/2011**
Ústav:	Ústav chemie potravin a biotechnologií		
Student(ka):	**Ing. Jana Navrátilová**		
Studijní program:	Chemie a technologie potravin (P2901)		
Studijní obor:	Potravinářská chemie (2901V021)		
Vedoucí práce	**prof. Ing. Peter Šimko, DrSc.**		
Konzultanti:			

Název dizertační práce:

Metody speciační analýzy sloučenin arsenu.

Zadání dizertační práce:

Stanovení jednotlivých spécií sloučenin arsenu ve vybraných biologických materiálech a potravinách

Termín odevzdání dizertační práce: 15.8.2011

Dizertační práce se odevzdává ve třech exemplářích na sekretariát ústavu a v elektronické formě vedoucímu dizertační práce. Toto zadání je přílohou dizertační práce.

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Ing. Jana Navrátilová	prof. Ing. Peter Šimko, DrSc.	doc. Ing. Jiřina Omelková, CSc.
Student(ka)	Vedoucí práce	Ředitel ústavu

V Brně, dne 1.2.2011

- -
prof. Ing. Jaromír Havlica, DrSc.
Děkan fakulty

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%.

Klíčová slova: arsen, speciace, HPLC-ICPMS, dimethylarseničná kyselina

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%).

Key words: arsenic, speciation, HPLC- ICPMS, dimethyl arsenic acid.

Navrátilová, J. *Metody speciační analýzy sloučenin arsenu.* Brno: Vysoké učení technické v Brně, Fakulta chemická, 2011. 81 s. Vedoucí dizertační práce prof. Ing. Peter Šimko, DrSc.

PROHLÁŠENÍ

Prohlašuji, že jsem dizertační práci vypracovala samostatně a že všechny použité literární zdroje jsem správně a úplně citovala. Dizertační práce je z hlediska obsahu majetkem Fakulty chemické VUT v Brně a může být využita ke komerčním účelům jen se souhlasem vedoucího práce a děkana FCH VUT.

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podpis studenta

DECLARATION

I declare that this doctoral thesis has been worked out by myself and all the quotations from the literary sources are accurate and complete. The content of this doctoral thesis is the property of the Faculty of Chemistry of Brno University of Technology and all commercial uses are allowed on the base of approval by both the supervisor and the dean of the Faculty of Chemistry, BUT.

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student signature

Acknowledgements

I am thankful to my supervisor Prof. Ing. Peter Šimko, DrSc. for his wise advise and encouragement.

I am deeply grateful to Prof. Kevin A. Francesconi for the chance to work with him and to be a part of his research group.

I would like to give my special regards to Dr. Georg Raber for his help and constructive critique.

I would like to thank also to all colleagues from University of Graz.

Financial support from the Austrian Exchange Servise and from Ministry of Education Youth and Sport of Czech Republic is gratefully acknowledged.

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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

The literature review is divided in two parts, the first part is dealing with general information about arsenic chemistry including arsenic toxicity and its metabolism in human. The description of chosen arsenic compounds relevant to PhD thesis is also given. The second part is focused on speciation analysis and its importance to evaluate the toxicity of arsenic, commonly used technique such coupling HPLC-ICPMS used for arsenic speciation study is described in details. Briefly are mentioned the pitfalls of quality assurance with respect to arsenic speciation analysis and current legislative situation.

2.1. General information

2.1.1. Arsenic chemistry

Arsenic is a metalloid with metallic and non-metallic properties. Arsenic can exist in several forms - the molecular non-metallic yellow form, however, black and gray is the most common one. Arsenic is commonly found in sulfide-rich mineral the most abundant is arsenopyrite (FeAsS). Elemental arsenic has the atomic number of 33 and molecular weight of 74.92 g/mol. Arsenic is widely distributed in the earth's crust and can exist in four oxidizing states; –3, 0, +3, +5 and in a variety of inorganic and organic forms (Merian, 2004).

In biological systems, arsenic occurs mainly in oxidation states +3 and +5. The only, natural occurring arsenic isotope is ^{75}As. (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).

Arsenic belongs to the group 15 of the periodic table and lies directly below P on the periodic table and so these two elements have similar chemical properties. Due to these similarities, arsenic can often substitute for phosphorous in biological systems (Greenwood, 1997).

2.1.2. 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 drinking water. Chronic exposure to inorganic arsenic may lead to skin lesions, Black foot disease, diabetes mellitus and various forms of cancer (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

2.1.3. Mechanisms of toxicity of inorganic arsenic species

Trivalent arsenic compounds such as arsenite (As^{III}) have high affinity for thiol groups and can interact with active centre of number of enzymes. Lewisit is an example of toxic trivalent compound which was used in second word war as the chemical weapon.

The most studied arsenic-inhibited enzyme is the pyruvate dehydrogenase (PDH) complex. PDH is a multi subunit complex consisting of E_1, E_2, E_3 that requires for enzymatic activity the cofactor lipoic acid, a dithiol (Hu, 1998). Arsenical inhibition of PDH is caused by arsenic binding to these dithiols. Inhibition of PDH leads to decreased production of ATP (Adamson, 1984).

PDH is a complex of three enzymes activities. E_1 is a pyruvate decarboxylase that removes the carboxyl group from the pyruvate substrate. E_2 is a dihydrolipoyl transacetylase that transfers an acetyl moiety to CoA. E_3 is a dihydrolipoyl dehydrogenase.

PDH catalyzes the oxidative decarboxylation of pyruvate after glycolysis to form acetyl-CoA, which enters the citric acid cycle. PDH catalyzes the reaction: Pyruvate + CoA-SH + $NAD^+ \rightarrow$ Acetyl-Co-A + NADH + CO_2.

Pentavalent arsenic compounds such as arsenate (As^V) can be reduced in the body to the trivalent form that has toxic effects on the body. Another possibility is the substitution of phosphate (P^V) by arsenate (As^V) in ADP or glucose-6-phosphate. Arsenate competes with phosphate for the enzyme which than disrupts ATP production and ultimately uncouples oxidative phosphorylation. ATP is generated during glycolysis in the presence of phosphate, but not in the presence of arsenate (Aposhian, 1989).

The presence of arsenic also increases hydrogen peroxide production, which can lead to the formation of reactive oxygen species (ROS) (Eblin, 2006).

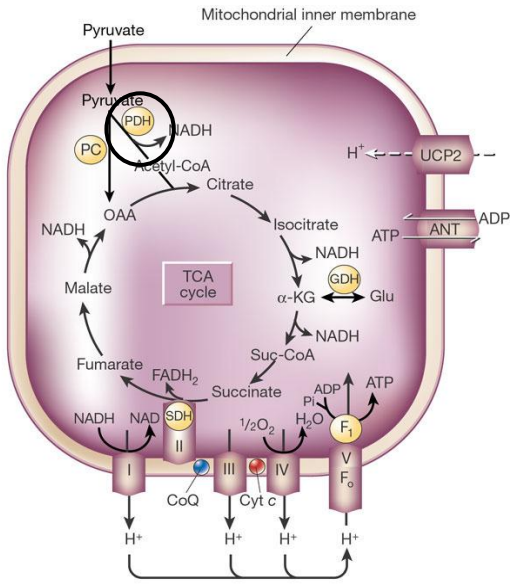


Figure 1. Mitochondrial structure

The Krebs cycle and the electron transport chain in the mitochondrion. PDH is located in the matrix of mitochondria and provides an important link between glycolysis and the Krebs cycle (Maechler, 2001).

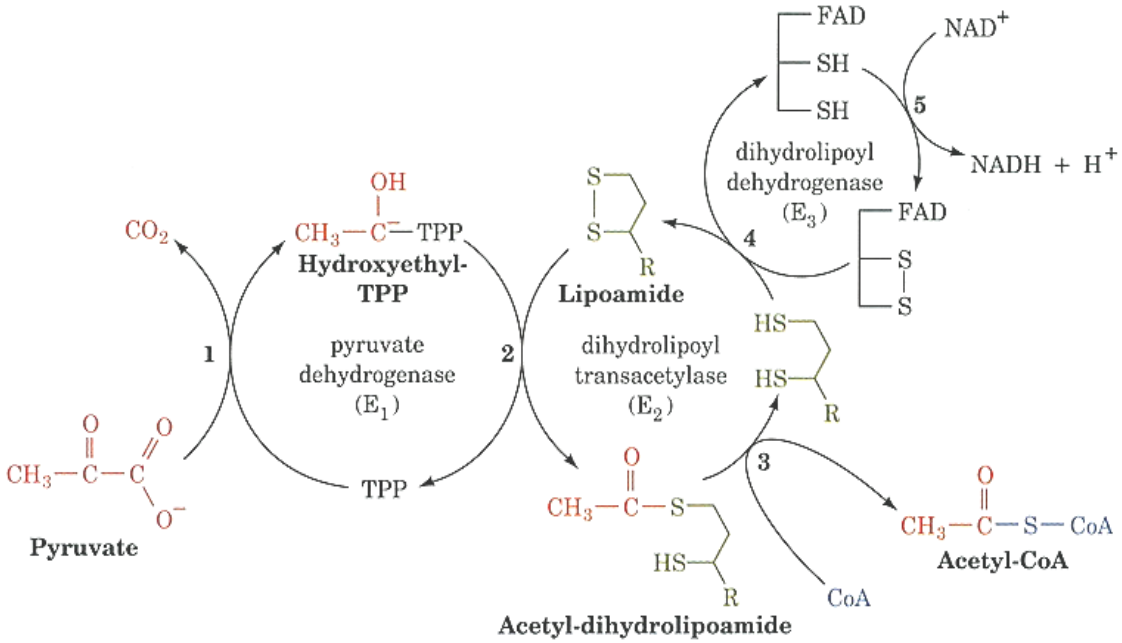


Figure 2. Structure of pyruvate dehydrogenase complex
(available online <http://chemistry.uah.edu/faculty/ciszak/ research_e1.htm> accessed 05 Jul 2011)

2.1.4. Metabolism of arsenic in human

Generally the metabolism of inorganic arsenic in humans involves two types of reactions, the reduction of pentavalent arsenicals to trivalent and the oxidative methylation of trivalent arsenicals to yield methylated pentavalent metabolites. This pathway was first described by Challenger to explain microbial methylation of arsenic by the bread mould *Scopulariopsis brevicaulis* as shown in Figure 2, (Challenger, 1945). In humans this process stops with the formation of DMA^{V} and probably DMA^{III}. Trimethylarsine oxide TMAO is not usually detected in human urine, however, it was found in the urine of humans after ingestion of high dose of dimethylarsinate (Marafante, 1987). In microorganisms, the final product is mainly trimethylarsine oxide TMAO with some trimethylarsine TMA^{III}.

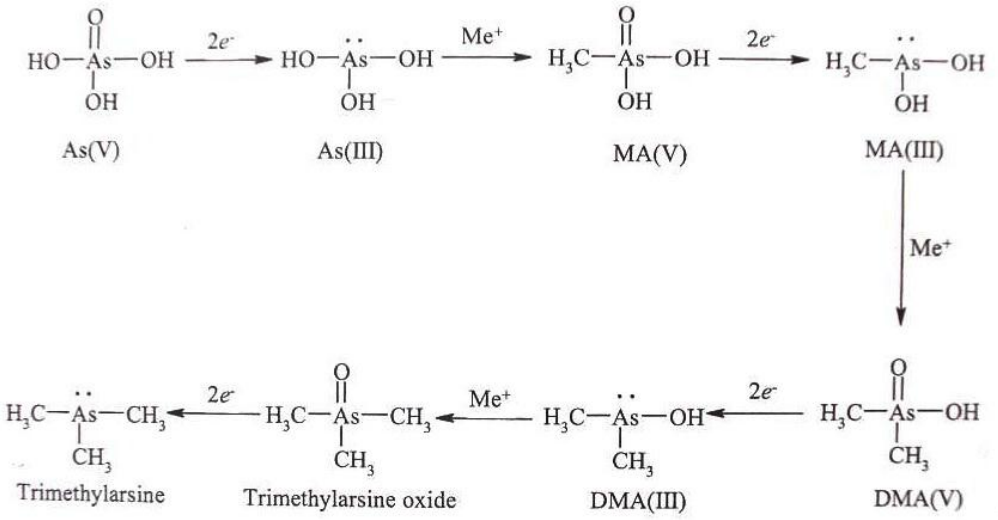


Figure 3. Challenger´s pathway (Challenger, 1945)

The substrate for methylation is As^{III}, therefore As^{V} is firstly reduced to As^{III} via glutathione-S-transferase-ω (Zakharian, 2001). As^{III} is then methylated to MA^{V} by arsenic methyltransferase (Cyt 19). This requires the methyl donor S-adenosylmethionine (SAM) (Lin, 2002).

The liver is the primary site of arsenic metabolism in mammals. Minor routes of arsenic elimination from the body are through the faeces, sweat, and incorporation into hair and nails. Arsenic speciation in urine has been considered a biomarker of exposure. The major metabolites found in human urine are the methylated pentavalent forms DMA and MA and inorganic arsenic. The typical profile of urinary arsenic metabolites consist of 10-30 % inorganic arsenic, 10-20 % methylarsonate and 60-70 % dimethylarsinate. The rate of excretion in urine varies is depending on the chemical form of arsenic and species exposed (Vahter, 1999).

The trivalent metabolites monomethylarsonous acid (MMA^{III}) and dimethylarsinous acid (DMA^{III}) have been detected for the first time in the urine of people exposed to arsenic concentrations in drinking water after administration of a chelating agent, sodium 2,3-dimercapto-1-propanesulfonate (DMPS) (Aposhian, 2000). Later, these compounds were identified also in the urine of exposed populations without administration of DMPS (Mandal, 2001) and of promyelocytic leukaemia patients undergoing arsenic trioxide treatment (Wang,

2004). Some studies have shown that these metabolites are more toxic and more potent enzyme inhibitors and cytotoxins than inorganic arsenic (Styblo, 2000).

Presence of the trivalent methylated arsenic species in human urine raised several questions. The biomethylation of arsenic in humans had previously been considered to be detoxification pathway because the MA^V and DMA^V are both less toxic than inorganic arsenic. Now it is believed that the methylation of inorganic arsenic may not be a detoxification mechanism and could be an activation process (Kitchin, 2001; Hughes, 2002).

Another pathway was proposed by Hayakawa et al., suggesting that trivalent methylated arsenic species may be formed before the respective end products of pentavalent species (Hayakawa, 2005). In this pathway, As^{III} reacts with glutathione, becoming arsenic triglutathione which has been identified in the bile of rats treated either with arsenate or arsenite. Arsenic triglutathione is then methylated by arsenic-methyltransferase by transfer of the methyl group from S-adenosylmethionine, resulting in monomethylarsenic diglutathione, which is further methylated by arsenic-methyltransferase to dimethylarsenic glutathione or it becomes methylarsonite after reacting with glutathione. Monomethylarsenic diglutathione and dimethylarsenic glutathione are biliary metabolites of arsenic detected in rats (Cui, 2008).

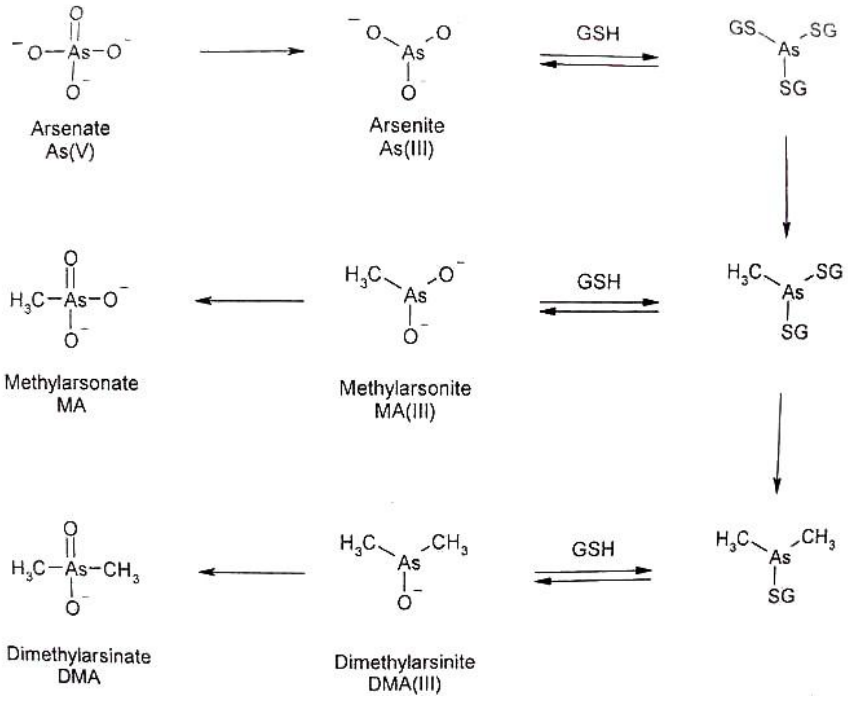


Figure 4. Pathway proposed by Hayakawa (Hayakawa, 2005)

2.1.5. Arsenic species relevant to the PhD thesis and their chemical structure

The pattern of arsenic species differs in marine organisms. For example, arsenobetaine in fish is dominant specie usually presenting to more than 80% of total arsenic. In marine algae, arsenosugars are the major species (Francesconi, 2002).

Arsenic species differ significantly in their toxicity; therefore there is an importance to know the species distribution and their addition to the total arsenic concentration in a biological system to assess correctly an measure of toxicological risks.

Inorganic arsenic species: Arsenite (As^{III}), Arsenate (As^{V})

Arsenate can exist in several pH-dependent forms from the fully protonated arsenic acid, H_3AsO_4 to the fully deprotonated AsO_4^{-3} though in most environmental systems, while the charged $H_2AsO_4^-$ and $HAsO_4^{-2}$ species dominate.

Seawater contains inorganic arsenic largely as arsenate and arsenite. Arsenate is mainly present as $HAsO_4^{-2}$ but traces of $H_2AsO_4^-$ are present as well. With the uptake of arsenate and arsenite from marine organisms, organoarsenic species are formed (Francesconi, 1993).

Usually inorganic arsenic content in seafood is low, less than 5% of the total arsenic, although there are some clear exceptions. Arsenate is present at high concentrations (60 μg/g) in the brown alga *Hizikia Fusiformis* (commercially available as "hijiki"), which is commonly eaten in Japan. Therefore, in 2004, the Food Standard Agency (FSA) of the United Kingdom advised consumers to avoid eating hijiki (Edmonds 1987; FSA, 2004; Shimoda, 2010). In 2008 Sloth et al. reported unusual high level of inorganic arsenic (constituted up to 42%) in samples of blue mussels *Mytilus edulis L*. (Sloth, 2008).

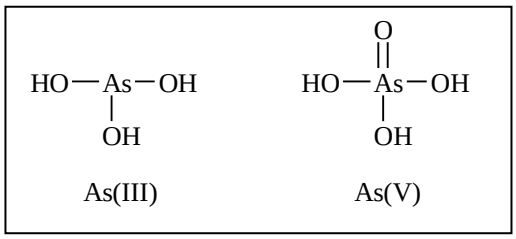


Figure 5. Inorganic arsenic species

Methylated arsenic species: Methylarsonate (MA^{V}), Dimethylarsinate (DMA^{V})

Methylarsonate and dimethylarsinate are common arsenic metabolites found in marine samples as the products of biotransformations (Francesconi, 1997).

Arsenocholine (AC)

Arsenocholine is a common arsenic species in marine organisms presented usually as a minore constituent. It is a probable metabolic precursor of arsenobetaine in marine organisms. This presumption has been based on laboratory experiments, where fish was fed by arsenocholine and it was readily biotransformed to arsenobetaine (Francesconi, 1989). In terrestrial organisms can be sometimes found as a major water soluble arsenic species, for

example in the fungus *Sparassi crispa* (Slejkovec, 1977). Arsenocholine is considered to be a non-toxic compound (Francesconi, 1994).

Oxo-dimethylarsenoethanol (oxo-DMAE)

Dimethylarsenoethanol was first identified in marine samples in 2005 (Sloth, 2005).

Tetramethylarsonium ion (TETRA)

Tetramethylarsonium ion is generally considered as a minor arsenic species in marine organisms, but may be a major species in some molluscs (Cullen, 1989).

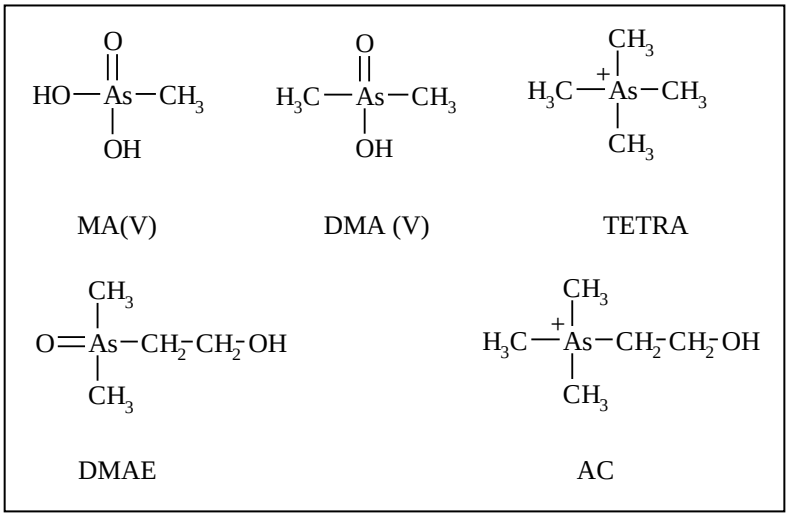


Figure 6. Structures of arsenic species

Arsenobetaine (AB)

In marine organisms, the predominant form of arsenic is arsenobetaine, which usually represents more than 80 % of water extractable arsenic compounds. Edmonds and co-workers in 1977 identified arsenobetaine in the tail muscle of the rock lobster *Panulirus Cygnus* what was the first organoarsenic compound identified in seafood. Although arsenobetaine is present in almost all marine organisms, it has not been detected in seawater (Edmonds, 1977).

In 2005, arsenobetaine was found in marine algae, *Laminaria digitata* and *Fucus vesiculosus* for the first time (Nischwitz, 2005).

Arsenobetaine is not metabolized in the mammalian body and is excreted unchanged in urine within a few hours after intake (Cannon, 1981). The fact that arsenobetaine is rapidly excreted in the urine without metabolic change and therefore does not present any risk for the human health is really notable. Arsenobetaine is considered to be a nontoxic with regatd to the data LD_{50} in mice of >10 000 mg/kg (Kaise, 1985).

It has also been proposed that some marine organisms transform oxo-arsenosugars into arsenobetaine. The biotransformation starts with oxo-arsenosugars which degradate to give oxo-DMAE, followed by an oxidation and subsequent methylation via the proposed intermediate oxo-DMAA to the end product arsenobetaine. In another pathway the key

intermediate arsenocholine is oxidize to arsenobetaine (Fig.7a). The formation via the oxo-arsenosugars was supported by a study showing that oxo DMAE was formed after anaerobic decomposition of a brown alga, *Ecklonia radiata* (Edmonds, 1982).

Another proposed pathway (Fig.7b) is based on the amino acid synthesis and involves an "arsenylation" between DMA(III), which can be formed from As(V), and glyoxylic acid to oxo-DMAA (Edmonds, 2000).

Arsenobetaine is structurally similar to the osmolyte glycinebetaine, which suggests that arsenobetaine may be associated with osmoregulation but clear evidence has not been observed yet (Clowes, 2004).

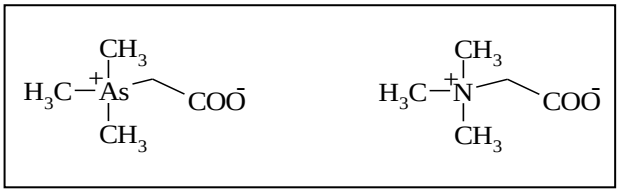


Figure 7. Structures of arsenobetaine and glycinebetaine

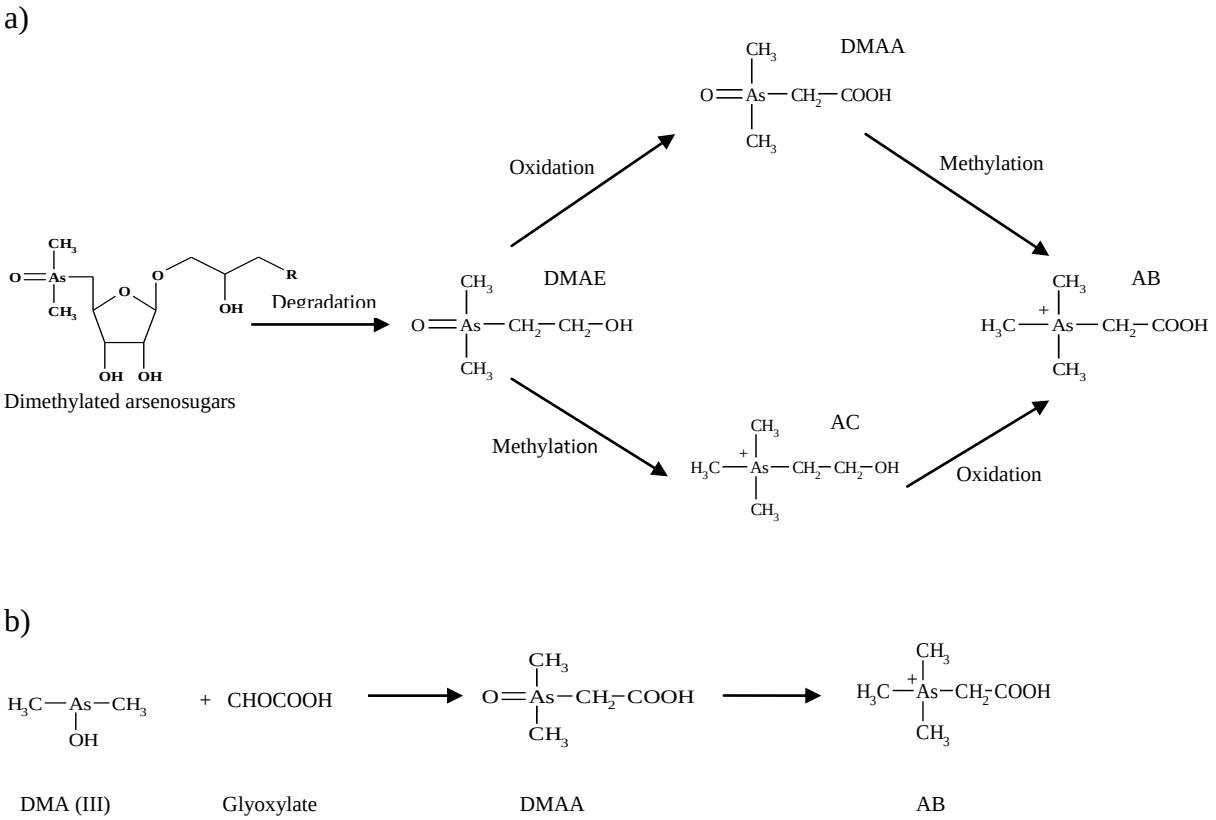


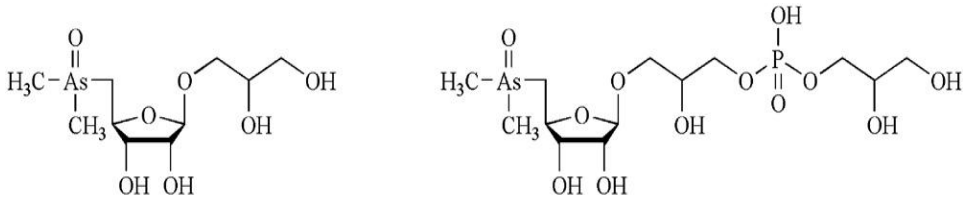
Figure 8. Possible pathways for the biogenesis of AB

a) Biotransformation of arsenobetaine from arsenosugars (Edmonds, 1982)
b) Biotransformation of arsenobetaine by arsenylation of 2-oxo acid (Edmonds, 2000)

Arsenosugars

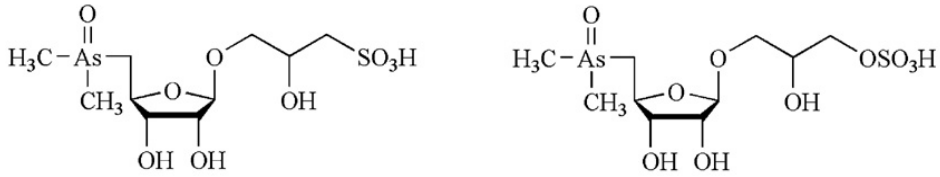
Oxo -arsenosugars

The oxo-arsenosugars are compounds containing a 5-deoxypentose moiety and an arsinoyl group, attached to the C5 atom. These compounds are substituted by a variety of side chains.



Oxo-arsenosugar glycerol
oxo-OH

Oxo-arsenosugar phosphate
oxo-PO_4



Oxo-arsenosugar sulfonate
oxo-SO_3

Oxo-arsenosugar sulfate
oxo-SO_4

Figure 9. Molecular structures of the four most common arsenosugars found in algae

Arsenosugars are the major organoarsenic species present in marine algae where they can occur at concentration exceeding 50 mg/kg dry mass. Commonly occuring are dimethylarsenosugars, but trimethylarsoniosugars are known, as well. The first arsenosugars were isolated from brown algae *Ecklonia radiata* in 1981. These compounds were oxo-arsenosugar-sulfonate and oxo-arsenosugar-glycerol (Edmonds, 1981). Oxo-arsenosugar-sulfate was isolated from the kidney of the giant clam, *Tridacna maxima* in 1982 (Edmonds, 1982). The last common arsenosugar, oxo-arsenosugar-phosphate was identified in 1983 and was also isolated from *Ecklonia radiata* (Edmonds, 1983). In marine animals arsenosugars are also present, but their origin is most probably the algae on which the food chain is based (Francesconi, 1997). Trimethylarsonoribosides are usually minor constituents (Francesconi, 1998).

Instrumental methods used for the analysis of arsenosugars are based on high-performance liquid chromatography (HPLC), especially ion-exchange chromatography, hyphenated with an arsenic specific detector (Raber, 2000).

The exposure of humans to arsenosugars is relatively high in the Asian world because of the frequent consumption of seaweed. Arsenosugars are largely metabolized in mammals, and the metabolites are excreted relatively slowly. Individual variation in urinary arsenic excretion after intake of inorganic arsenic by humans is known, and 46-66% of a dose has been reported to be excreted within a few days. The corresponding value for a chemically synthesized

arsenosugar is 80% during 4 days. The major metabolites excreted in both cases are DMA^V and several thio-arsenicals (Raml, 2009).

Arsenosugars appear to be the key intermediates in the biochemical cycling of arsenic. They may serve as precursors to arsenobetaine, the major form of arsenic in marine animals. It is assumed that algae absorb As^V from seawater and accumulate it as arsenosugars. In seawater arsenic exists primaly as arsenate and a major seawater nutrient is phosphate. Algae have a membrane transport system to take up the essential phosphate from seawater, but this can not distuinquish. To eliminate the toxicity of arsenic algae have developed a process of converting it to arsenosugars. A biosynthetic pathway was proposed, which is based on the methylation pathway of arsenic by microorganisms (Challanger, 1945). In this pathaway the third methylation step is replaced by an adenosylation step followed by glycosidation (Edmonds, 1987). This scheme was supported by the identification of the key intermediate oxo-aresenosugar-nucleoside in the kidney of the giant clam *Tridacna maxima* (Francesconi, 1992)

The example of the detoxification process was reported for the brown alga *Fucus serratus* by Geiszinger et al. The study shows that at low (20 µgAs/l) arsenate concentration the alga takes up arsenate readily and converts it efficiently to arsenosugars while at the high exposure (100 µgAs/l) the detoxification process was overloaded, the toxic arsenic species (presented mainly as arsenite and methylarsonate) accumulated to levels fatal to the alga and arsenosugars were not significantly produced (Geiszinger, 2001).

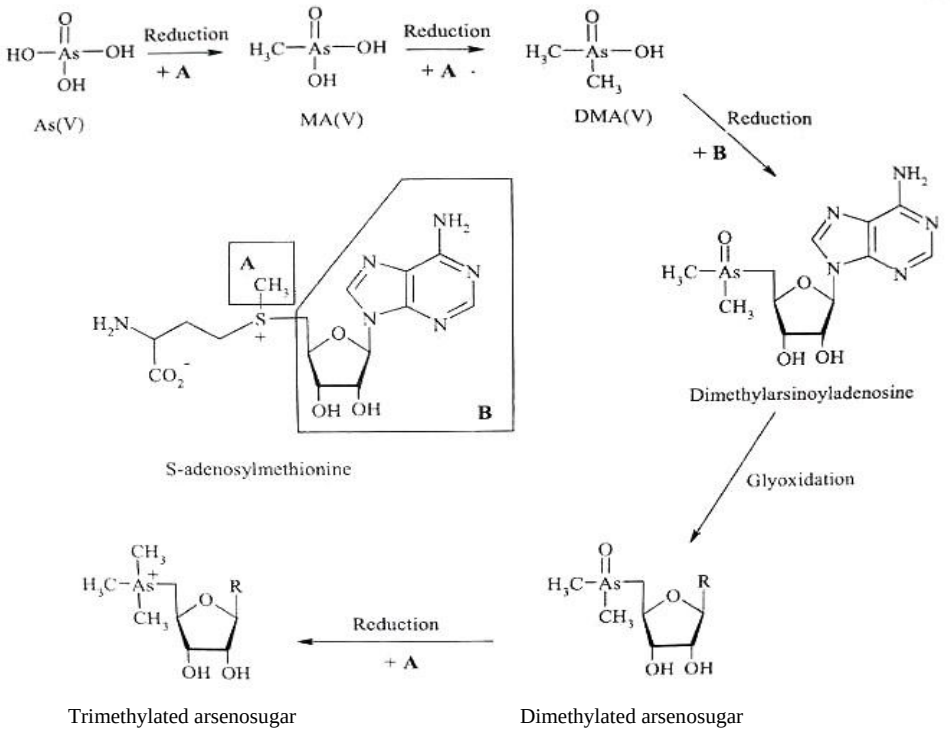


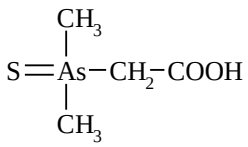
Figure 10. Proposed biosynthetic pathway for arsenosugars from arsenate in algae (Edmonds, 2003)

Thio-arsenosugars

Some arsenosugars can exist in two different forms, oxo- and thio- arsenosugars. These compounds are the sulfur analogues of oxo-arsenicals, where the arsinoyl (As=O) group is substituted by an arsinothioyl group (As=S)

The first identified thio- arsenical was (thio-dimethylarsinoyl) acetate DMAAS in 2004 by Hansen in the urine of sheep feed on algae (Hansen, 2004). After that report several other thio-arsenic species were identified in molluscs, algae, and human urine (Raml, 2005).

Reversed-phase conditions proved to be suitable for the analysis of thio-arsenosugars. Most of them have long retention times and resulting broad peaks when determined under the routine anion exchange conditions established for oxo-arsenosugars analysis. Raml *et al.* developed a chromatographic method uses a Water Atlantis TM dC18 column. This column is a difunctionally bonded silica column to provide increased retention of polar compounds (Raml, 2006).



Thio – DMAA

Figure 11. Chemical structure of (thio-dimethylarsinoyl) acetate

Arsenolipids

Arsenolipid is a synonym for lipid-soluble (organic solvent soluble) arsenic. The occurrence of lipid soluble arsenic was first reported by Lunde. In 1968, Lunde did preliminary investigations on arsenic compounds in oils from cod liver and herring and also investigated the lipid soluble arsenicals in algae (Lunde, 1968; Lunde, 1973).

However, the data on lipid-soluble arsenic compounds were very limited. The major reason was that the analytical techniques developed for measuring polar arsenicals were not suitable for arsenolipids. The necessity to employ organic solvents as mobile phases for HPLC separation of non polar compounds creates considerable problems in terms of stability the plasma.

In 2005 the method for the direct measurement of lipid soluble arsenic species by HPLC ICPMS was reported. The problems connected with plasma stability were overcome by using low flow rate in the HPLC, smaller torch diameter, cooled spray chamber, and the addition of oxygen directly to plasma. (Schmeisser, 2005)

In 2008 six arsenolipids were isolated from cod liver oil namely arsenic containing fatty acids with following molecular masses 334, 362, 390, 418, 388, 436; 20% of the total arsenolipids content of cod liver oil. In this work cod-liver was partitioned between hexane and aqueous methanol. The polar phase was subjected to preparative chromatography with size-exclusion and anion exchange media to yield a fraction enriched with the polar arsenolipids. The HPLC/ICPMS analysis showed presence of at least 15 arsenolipids. The unidentified arsenolipids were less polar arsenolipids (Rumpler, 2008).

Another group of three arsenolipids arsenic containing hydrocarbons was isolated from the fish capelin (arsenolipid hydrocarbons and their molecular mass 332, 360, 404). They constituted about 70% of the total arsenic content. One of the identified hydrocarbons was the dimethylarsinoyl alkene, an arsenic containing analog of docosahexaenoic acid (DHA) a common omega-3 fatty acid (Taleshi, 2008). Arsenic containing hydrocarbons were also identified in sashimi tuna muscle tissue (Taleshi, 2010).

Arsenolipids are of human health interest because they are present in fish oils supplements recommended by nutritionists due to their high content of omega-3 fatty acids. The investigation of human metabolism of arsenolipids showed that the arsenolipids are bioavailable and are quickly converted to DMA by humans (Schmeisser, 2006).

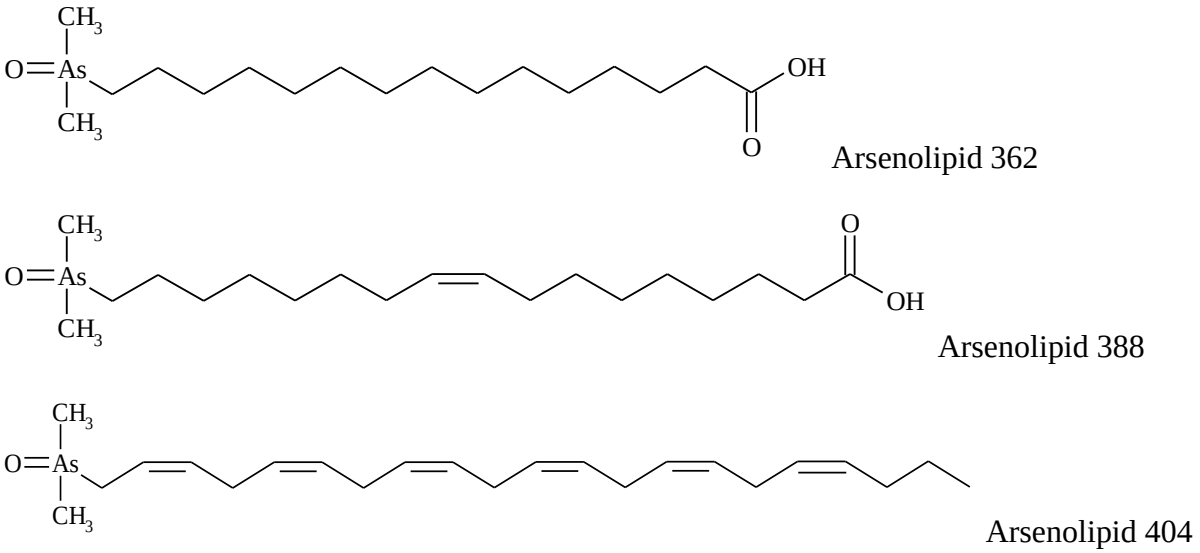


Figure 12. Structures of some arsenolipids identified in fish oils and their molecular masses. Arsenolipid 404 is dimethylarsinoyl alkene (1-dimethylarsinoyl all-cis-4,7,10,13,16,19-docosahexane) analog to all-cis-4,7,10,13,16,19-docosahexaenoic acid [DHA, 22:6 (n = 3)], a common fatty acid in fish.

2.2. 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 determination provides 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 combined HPLC with ICPMS. Up to this time, arsenic speciation analyses were limited to certain types of samples or to samples with high arsenic concentrations. In general, this combination has become the most powerful and commonly used method for arsenic speciation analysis.

2.2.1. Separation techniques

2.2.1.1. 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.

2.2.1.2. 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).

2.2.1.3. 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 OH 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.

2.2.2. Detection techniques

2.2.2.1. 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).

2.2.2.2. 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).

2.2.2.3. 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 emit 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.

2.2.2.4. 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.

Quadrupole based ICPMS often suffers from spectral and non-spectral interferences. Nonspectral interferences are connected with matrix-induced signal suppression and spectral

with overlap of the signals ions showing a difference with mass. In most cases nonspectral interferences could be easily solved by sample dilution or by internal standard addition.

The spectral interferences are divided into three categories: isobaric, polyatomic, and doubly charged species. The isobaric interferences apper when two isotopes have the same mass (^{116}Sn and ^{116}Cd). These interferences can not be resolved with a quadrupole mass spectrometer and high resolution mass spectrometers must be used or if possible other isotope which is not influenced by an isobaric overlap must be chosen. Arsenic is monoisotopic (^{75}As), which leads to an excellent ICPMS signal intensity. With respect to arsenic during analysis polyatomic ions ($^{40}Ar^{35}Cl$) can be formed with the same mass as the analyte (^{75}As) that can not be distinguished by the quadrupole mass filter. Polyatomic interferences occur when two or more atoms are measured at the same *m/z* as the analyte. Chloride rich samples can disturb the analysis of arsenic due to $^{40}Ar^{35}Cl$ formation, which can interfere with the detection of ^{75}As. One possibility to overcome these interferences is an octopole reaction cell (ORC), which consists of an octopole placed between the ion optics and the mass filter. The ORS uses reaction (H_2) or collision gas (He) therefore can work in collision or reaction mode (Niu, 1996). Polyatomic ions (e.g. $^{40}Ar^{35}Cl$) have a larger cross section than single ions of the same nominal mass. The polyatomic species will collide with the collision gas and loose their energy. Hellium used as collision gas can remove essentially all polyatomic interferences either by one of two proceses: collision induced dissociation or energy discrimination. Collision induced dissociation is basically limited to polyatomic species such as ArO^+ with bond energies lower than impact energy between the helium and the polyatomic species. Energy discrimination is possible because polyatomic ions being larger than the analyte atoms collide with the cell gas more frequently and consequently loose more kinetic energy compared to the smaller monoatomic analytes. A positive field at the cell exit transmits only those ions which have enough energy- the lower energy polyatomic interfering species cannot enter the quadrupole mass analyser and as result they are not detected. Elements with their first ionization energy below the first ionization potential of argon (15.76 eV) will be singly charge positive ions. For a number of elements (e.g. Ba and Ce) the second ionisation potential is also lower than the first ionisation potential of argon. Therefore the doubly charged ions are formed instead of singly charged. Mass analyzer separates atoms based on their mass to charge ratio e.g. $^{90}Zr^{++}$ interfers with ^{45}Sc because their mass charge ratio is identical. The formation of doubly charged species can be generally minimised by optimising instrument conditions (Agilent Primer, 2005; Linge, 2009).

ICPMS is a powerful and versatile technique for ultratrace analysis characterized by extremely low limits of detection, a wide linear dynamic range and multi element capabilities.

The coupling of ICPMS and HPLC has been successful for the speciation of many elements. The major advantage of using ICPMS as a detector is that it can selectively detect element of interest in a complex matrix and that the elemental response is usually independent of species, so it is often possible to quantify species even when their structures are unknown. One limitation of HPLC–ICPMS is the fact that no structural (molecular) information is obtainable because of the harsh ionization process. Electrospray ionozation mass spectrometry (ES–MS) is a soft ionization technique what has become the most common method to obtain structural information in order to identify unknown peaks. The use of variable fragmentor voltage gives simultaneous elemental and molecular information. First who applied ES-MS for arsenic speciation studies were Corr and Larsen in 1996 (Corr, 1996).

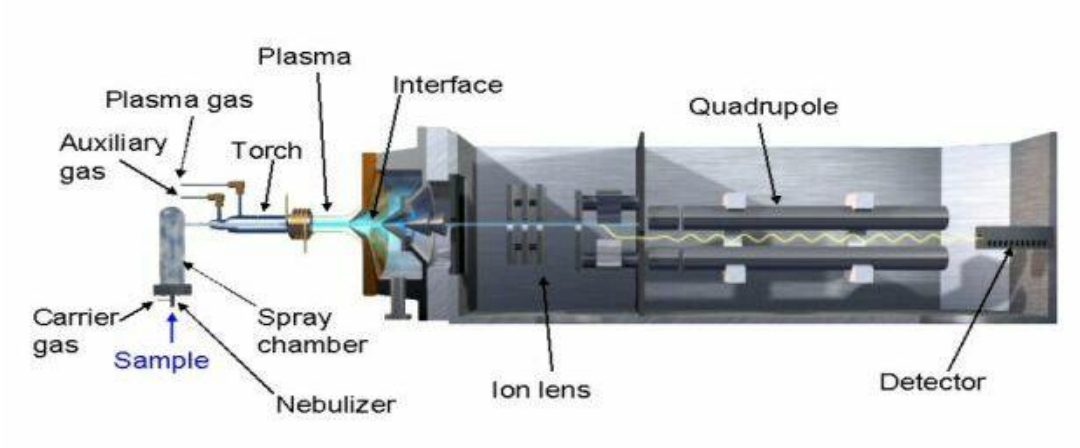


Figure 13. Schematic picture of an ICPMS system (Agilent Primer, 2005)

Signal enhancement by introduction of carbon to plasma

The degree of ionisation of an element in ICPMS is partly depended on plasma temperature and partly on an elements first ionisation potential which is specific for each element. Elements with ionisation potential higher than 9 eV are hard to ionise, and less than 50% of the element is ionised under standard ICPMS conditions (Jarvis, 1992). It has been proved that for elements with an ionisation potential between 9 eV and 11 eV, addition of carbon (CH_3OH) to samples for ICPMS analyses enhances the sensitivity. Variation of the carbon content in the mobile phase leads to changes in the ICPMS response. It is proposed that an increased population of carbon ions (ionisation potential 11.26 eV) in the plasma, improved the ionization of elements lower in ionisation energy than that of carbon. This effect is generally attributed to charge transfer reactions in the plasma between positively charged carbon ions and the analyte atoms, which are then ionized to higher extents. For elements with an ionisation potential lower than 9 eV, the enhancement effect was not observed, or was not significant becasuse they are already almost completely ionised. Furthermore, sensitivity enhancement for elements with an ionisation potential higher than that of carbon was not observed (Larsen, 1994). Arsenic with its ionisation potential of 9.81 eV, lies in the range where carbon has an enhancing effect.

2.2.3. Quality assurance in speciation analysis

The reliability of speciation data depends on the accuracy of the speciation procedure. The need for quality control in speciation analysis is given by errors which can occur during sampling, sample preparation, separation and detection. A common way to verify analytical procedure is to check it with certified reference materials (CRMs). CRMs are materials as similar as possible to real sample analysed by several laboratories using a variety of analytical techniques. They offer the best way in which to ensure that the employed method provides acceptable results.

To test the quality the CRM samples should be treated in the same way as the other samples (Larsen, 1998; Szpunar, 2003). CRMs are not available for all analytes and matrices, especially for trace metals speciation. Only a few species certified reference materials are commercially available mostly with certified value for arsenobetaine. In table 2 are displayed

chosen certified materials DORM-2 (Corr, 1997), BCR-627 (Lagarde, 1999), No.18 (Yoshinaga, 2000) with their certified values.

Table 2. Reference materials certified for arsenic species

Origin	Code	Supplier	Certified arsenic species	Certified value
Dogfish muscle	DORM-2	NRCC*	Arsenobetaine	16.4 ± 1.1[a)]
			Tetramethylarsonium ion	0.248 ± 0.054[a)]
Tuna fis	BCR-627	IRMM**	Arsenobetaine	3.9 ± 0.2[a)]
			Dimethylarsinate	0.15 ± 0.01[a)]
Human urine	No.18	NIES***	Arsenobetaine	0.069 ± 0.012[b)]
			Dimethylarsinate	0.036 ± 0.009[b)]

* NRCC: National Research Council of Canada (Canada)
**IRMM: Institute for Reference Materials and Measurements (Belgium)
*** NIES: National Institute for Environmental Studies (Japan)
a) mg As/kg dry mass
b) mg As/ l

2.2.4. 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 rice was analyzed to get fundamental data for evaluation of risk assessment coming from 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 it was necessary to validate analytical methods by using certified reference materials. 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 experiments were designed to follow the transformation of arsenic compounds in algae, and proved 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.

4. EXPERIMENTAL

4.1. Chemicals

- Arsenic standard solution (for total measurement), 1000 ± 3 μg/ml in 2% HNO_3 CPI International Amsterdam Netherlands
- Arsenite prepared from $NaAsO_2$ Merck (Darmstadt, Germany)
- Arsenate prepared from $Na_2HAsO_4.7H_2O$ Merck (Darmstadt, Germany)
- Standard arsenic compounds (for speciation analysis) were synthesised at the Institute of Chemistry –Analytical Chemistry, Karl- Franzens University Graz by organic research group
- Acetic acid, CH_3COOH, p.a., 96%, Merck (Darmstadt, Germany)
- Acetone, $CH_3CO\ CH_3$, Roth, (Graz, Austria)
- Ammonium acetate,CH_3COONH_4, puriss p.a., ≥ 98%, Fluka (Buchs, Switzerland)
- Ammonium dihydrogen phosphate, $NH_4H_2PO_4$, puriss p.a., ≥ 99%, Fluka (Buchs, Switzerland)
- Aqueous ammonia solution, NH_3, p.a. min. 25% NH_3, Merck (Darmstadt, Germany)
- Chloroform, CH_3Cl, Roth, (Graz, Austria)
- Ethanol, CH_3CH_2OH, Merck (Darmstadt, Germany)
- Formic acid, HCOOH, puriss p.a., ~ 98%, Fluka (Buchs, Switzerland)
- Germanium standard solution (for total measurement), 1000 ± 3 ug/mL in 2% HNO_3 CPI International Amsterdam Netherlands
- Hexane, C_6H_{14}, Roth, (Graz, Austria)
- Hydrogen sulphide, H_2S Messer Griesheim, (Krefeld,Germany)
- Hydrogen peroxide, H_2O_2 30% Merck (Darmstadt, Germany)
- Indium standard solution (for total measurement), 1000 ± 3 ug/mL in 2% HNO_3 CPI International, Amsterdam, Netherlands
- Malonic acid $CH_2(COOH)_2$, puriss p.a., >98%, Fluka (Buchs, Switzerland)
- Methanol, CH_3OH, p.a. ≥ 99,9%, ROTIPURAN®, Carl Roth (Karlsruhe, Germany)
- MilliQ water, deionized water (18MΩ*cm), prepared with a Milipore System (Milipore, Bedford, MA,USA)

- Nitric acid, HNO_3, p.a., 65%, Merck (Darmstadt, Germany), further purified with a sub-boiling distillation unit
- Pyridine, C_5H_5N, Merck, (Darmstadt, Germany)
- Trifluoric acetic acid, CF_3COOH, Karls Roth (Karlsruhe, Germany)

4.2. Materials

- HPLC polypropylene vials and polytetrafluorethylene caps (Agilent, Waldbronn, Germany)
- Polystyrene vial, 12ml, Brand (Wertheim, Germany)
- Quartz tubes with Teflon caps, MLS- Mikrowellenlaborsysteme GmbH (Leutrich, Germany)
- Syringe, 2ml, Terumo Europe (Leuven, Belgium)
- Syringe filter, polyamide, pore size 0.22 μm, Markus Bruckner Analysentechnik (Linz, Austria)
- Volumetric polypropylene tubes, 15ml, 50ml, Greiner Bio-One GmbH (Frickenhausen, Germany)

4.3. Instruments

- Autoclave- ultraCLAVE III, microwave heated, MLS- Mikrowellenlaborsysteme GmbH (Leutrich, Germany)
- ICPMS- Agilent 7500ce Inductively Coupled Plasma Mass Spectrometer System (Agilent Technologies, Waldbronn, Germany)
- Autosampler- ASX-500, CETAC Technologies (Ohama, Nebraska, USA)
- Centrifuge- JOUAN C 422, JOUAN (Saint Mazaire, France)
- Freeze dryer- Christ (Osterode am Harz, Germany)
- HPLC system- Agilent 1100 system (Agilent Technologies, Waldbronn, Germany)
- Micro-HPLC system - Agilent 1100 system (Agilent Technologies, Waldbronn, Germany)
- Retch mill (0.25 mm steel mesh, Retch ZM 200, Haan, Germany)

4.4. Methods

4.4.1. Microwave acid digestion

Digestion was performed with an Ultraclave III system at a loaded pressure of 4 000 kPa (40 bars), and operated under the following temperature program:

(i) room temperature to 75°C in 5 min

(ii) 75 – 150°C in 20 min

(iii) 150 – 250°C in 20 min

(iv) 250°C for 30 min

Every digestion run included 3 digestion blanks. Up to 40 samples could be mineralized within one run. After mineralization, the samples were diluted with water to 10 mL or 50 mL in polypropylene tubes (Greiner, Bio-one, Frickenhausen, Germany). The method was used to obtain quantitative data on solid samples and extracts.

4.4.2. Determination of total arsenic content

The arsenic standards for external calibration were prepared from 1 000 mg As/l arsenic standard solution in the same acid concentration as the samples. Before the measurement the ICPMS was tuned with 10 µg/l solution of ^{7}Li, ^{89}Y, ^{205}Tl for maximum sensitivity, specifically ^{89}Y which has the closest mass to arsenic. The most important values monitored were CeO/Ce ratio (156/140) and for the doubly charged species (70/140). Generally the doubly charged ions increase when the plasma temperature decreases. The CeO/Ce ratio is a criterium for plasma robustness which indicates the efficiency with which the plasma can decompose the strong Ce-O bond. If the plasma is unable to decompose CeO efficiently the residual energy would not be high enough for an efficient ionisation of elements with high first ionization potentials such as arsenic (9.81 eV). The CeO/Ce ratio is typically between 0.3 - 3%.

For internal standardisation, a solution containing germanium and indium, each at 500 µg/L, measured at *m/z* 74 (^{74}Ge) and *m/z* 115 (^{115}In), was constantly introduced to the ICPMS. Relatively high concentrations of the internal standards were used to ensure the stability of the signals. The method of standard addition was used as a confirmation of concentrations as it is more effective way to quantify when there are variations in the matrix composition. Generally the requires for internal standards are: the internal standard should not be present in the sample to avoid errors in the normalization, should have similar chemical behaviour as the analyte during analysis (e.g. ionization potential) and the mass-to-charge ratio should be near the mass-to-charge ratio of the element of interest.

The ICPMS measurements were performed in collision cell mode with helium at 4 ml/ min. Arsenic was monitored at m/z 75 and additionally m/z 77 was monitored to detect possible $^{40}Ar^{35}Cl^{+}$ interferences. Typical instrumental settings are given in Table 3.

The Certified Reference Material, *Trace Elements in Water 1643e* (National Institute of Standars and Technology, Gaithersburg, Maryland, USA) was measured to check the accuracy of the calibration curve. Precision was evaluated by monitoring drift to gauge within

a day's variability. Tipacally the standard with the middle concentration (usually 10 µgAs/l) was measured every tenth sample as a drift control.

Table 3. ICPMS parameters for total arsenic measurement

Parameter	Setting
Nebulizer type	Burgener Ari Mist HP Nebulizer
Spray chamber type	Peltier cooled, Scott type, double pass
Spray chamber temperature	2°C
Torch	2.5 mm ID
RF power	1550 W
Plasma gas flow	15 l/min
Carrier gas flow	0.9 l/min
Auxiliary gas flow	0.23 l/min
Sample cone	Nickel
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
Reaction cell	He (4 ml/min)

4.4.3. Speciation analysis

Speciation analysis was done using an Agilent 1100 Series HPLC system which consisted of a vacuum degasser, a binary pump, a thermostated autosampler and a thermostated column.

Anion-exchange HPLC separations were performed on a PRP-X100 column (4.1 mm x 150 mm, 10 µm particles; Hamilton, Reno USA) at 40°C with a mobile phase of 20 mM aqueous ammonium dihydrogen phosphate at pH 6 (adjusted with aqueous ammonia) and a flow rate of 1.5 ml/min. In later work the method was slightly modified and 10 mM malonic pH 5.6 was used as mobile phase and flow rate was decreased 1.2 ml/min.

Cation-exchange HPLC separations were performed on a Chrompack Ionospher 5C column (3 x 100 mm; Varian, Middelburg, Netherlands) at 40 °C with a mobile phase of 10 mM aqueous pyridine at pH 3 (adjusted with formic acid) and a flow rate of 1.5 ml/min. In early work cation-exchange column Zorbax 300 SCX (4.6 x 250 mm, 10 µm particle size; Hewlett-Packard, Waldbronn, Germany) maintained at a temperature 30°C, mobile phase 10mM pyridine, pH 2.3, flow rate 1.5 ml/min, was used however under these conditions DMAE and TMAO were coeluting. Better separation was achieved with Ionospher 5C column.

Reversed phase HPLC was used for determination of thio-arsenicals and separations were perfomed at 30°C with an Atlantis®dC18 column (4.6 x 150mm; Waters, Massachusetss,

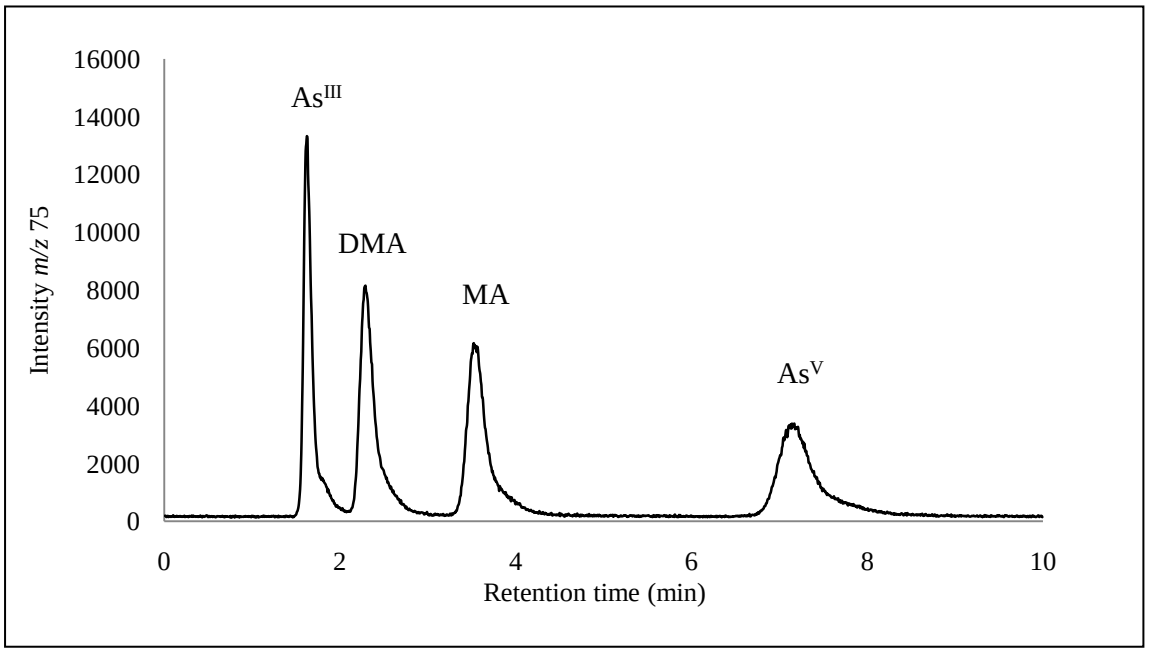
USA); the flow rate was 1 ml/min, mobile phase 20 mM $NH_4H_2PO_4$, pH 3. The calibration curve was established using solution of DMA (1-100 µg As/ DMA standards).

Before the measurement the columns were equilibrated with the mobile phase at the flow rate used for analysis for at least 30 min.

The outlet of the HPLC column was directly connected by PEEK (polyether ether ketone) tubing (0.125 mm i.d.) into the Burgener Ari Mist HP nebulizer (Burgener Research Inc, Mississauga, Canada) of an ICPMS. HPLC and ICPMS systems were connected through a remote cable that allowed the simultaneous start of the chromatographic run. After the separation of the arsenic compounds an Agilent ICPMS 7500ce system was used as an element-selective detector in the time resolved analysis mode. Arsenic was monitored at m/z 75 with an integration time of 0.3 sec. Aditionally m/z 77 was monitored to detect possible ArCl interference. The data evaluation was carried out with chromatographic software G1824C VersionC.01.00 (Agilent, Waldbronn, Germany). The quantification was based on peak areas. Arsenic species in extracts were identified by retention time matching against standard arsenic compounds, and by spiking experiments. The species were quantified by external calibration against the standard arsenic species prepared in the extractant solution.

Under the anion-exchange conditions used in this work, arsenite elutes near the void volume and hence cannot be reliably measured; its presence or absence in a sample was determined by treating a portion of the extract with H_2O_2, whereby it is oxidised to arsenate. The level of arsenite was assessed indirectly by comparing the quantities of arsenate before and after oxidation. To effect this oxidation, 10 µl of H_2O_2 (30%) was added to 90 µl of extract, and the mixture was briefly warmed (*ca* 40º C). The same conditions were applied to a solution of the arsenic standards at concentrations of 100 µg As/l.

A)



B)

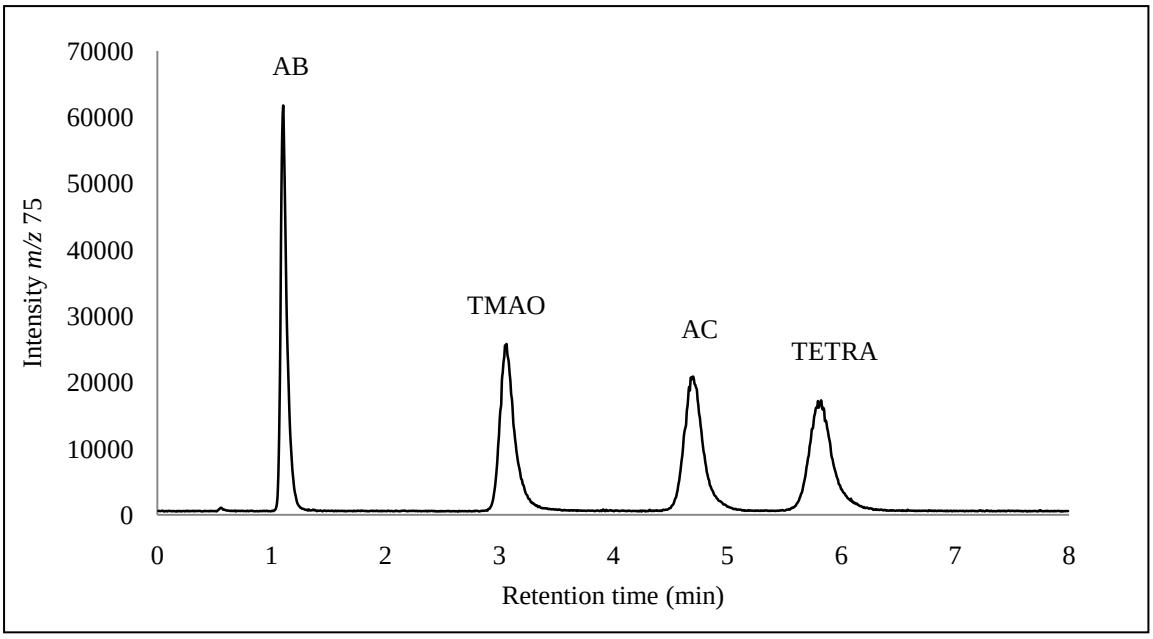


Figure 14 HPLC ICPMS chromatograms of standard solutions of arsenic compounds 100 µg/L of each compound

A) Separation of As^{III}, DMA, MA, As^{V} on a PRP-X100 column (4.1 mm x 150 mm, 10 µm particles; Hamilton, Reno USA) at 40°C with a mobile phase of 20 mM aqueous ammonium dihydrogen phosphate at pH 6 (adjusted with aqueous ammonia) and a flow rate of 1.5 ml/min

B) Separation of AB, TMAO, AC, TETRA on a Chrompack Ionospher 5C column (3 x 100 mm; Varian, Middelburg, Netherlands) at 40 °C with a mobile phase of 10 mM aqueous pyridine at pH 3 (adjusted with formic acid) and a flow rate of 1.5 ml/min.

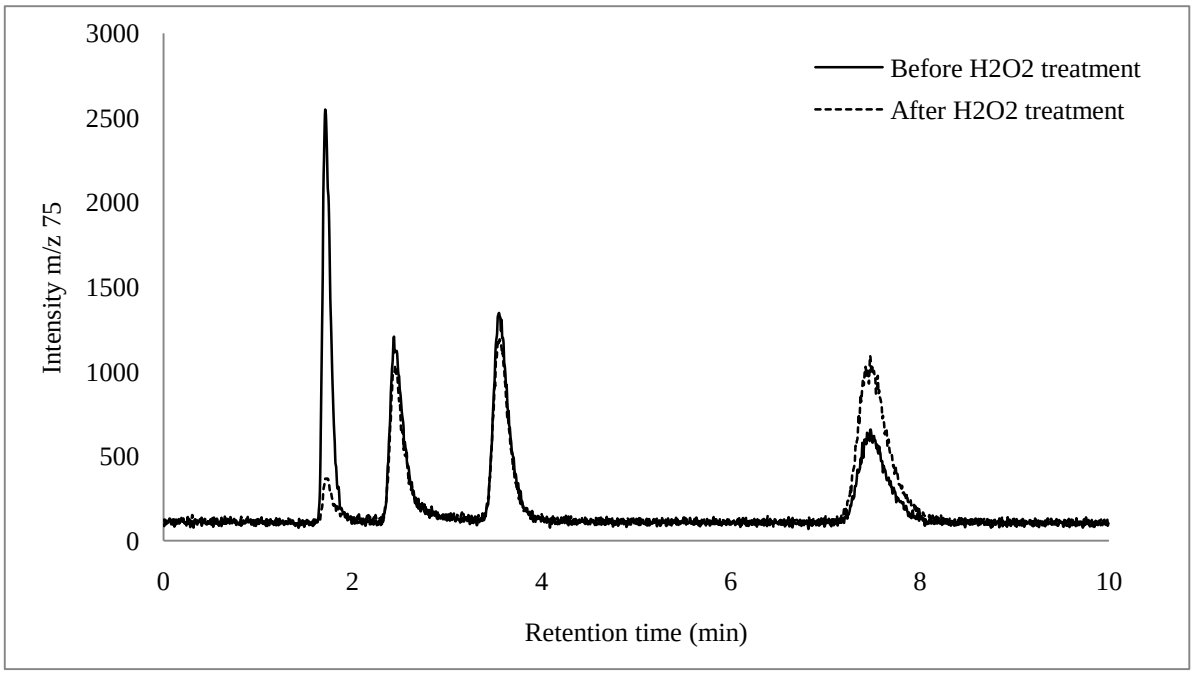


Figure 15. HPLC/ICPMS chromatograms of standard solutions of arsenic compounds (100 µg/l of each compound) before and after H_2O_2 treatment.

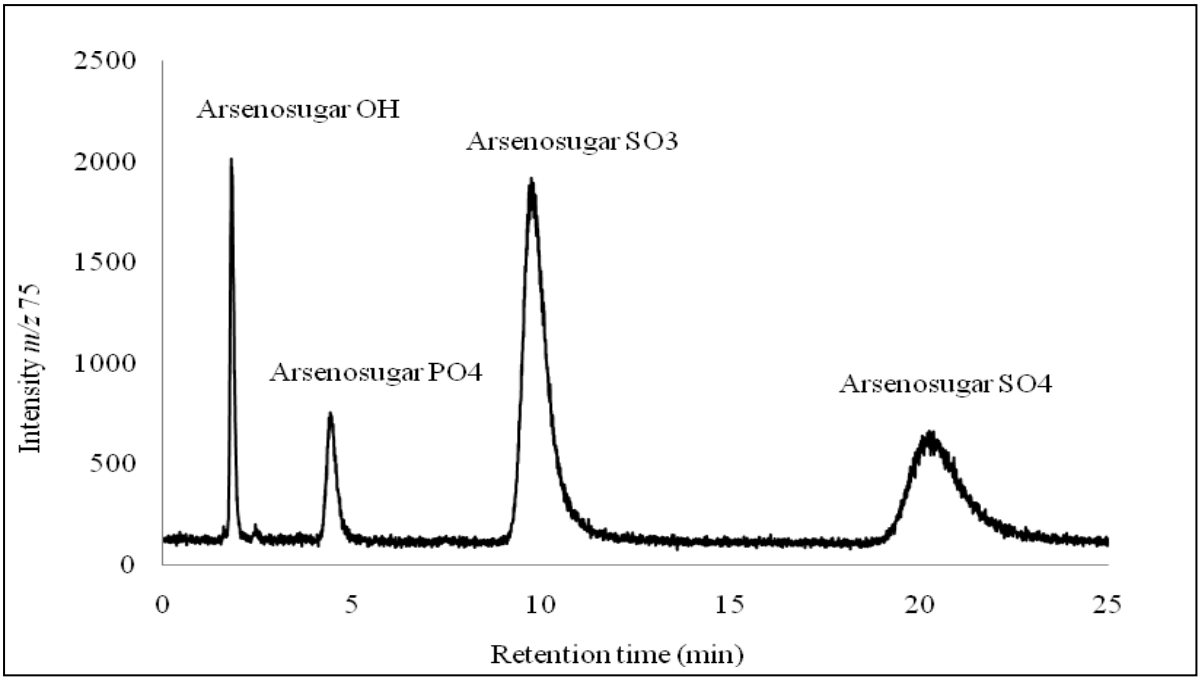


Figure 16. HPLC ICPMS chromatogram of water/methanol extract from brown algae Fucus seratus
PRP-X100 column (4.1 mm x 150 mm, 10 µm particles; Hamilton, Reno USA) at 40°C with a mobile phase of 20 mM aqueous ammonium dihydrogen phosphate at pH 6 (adjusted with aqueous ammonia) and a flow rate of 1.5 ml/min.

5. RESULTS AND DISCUSSION

5.1. STUDY ON ALGAE DECOMPOSITION

This chapter is based on paper:

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

Aim of the study

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.

Collection and preparation of samples

The samples of *Ecklonia radiata* were collected in the Marmion lagoon area north of Perth, Western Australia and immediately transported to the aquarium facilities at the nearby Fisheries and Marine Research Laboratories at Hillarys marina where the alga was packed into 12 plastic tubes, which were then closed at one end by placing them vertically in a plastic tank containing a 5 cm layer of beach sand to a depth of *ca* 3 cm. The top of the PVC tubes were left open. In this way, experimental alga samples were maintained under the aquarium room conditions of fluorescent lighting with a 12 h (light/dark) daily cycle, and a controlled temperature of 22 ± 1ºC. Single tubes were removed at intervals of 1 to 4 days over the following 25 days. The contents (cores) were extruded and cut into four equal sections (*ca* 8-10 cm lengths), which were labelled A (bottom, reduced air contact), B, C, and D (top, direct contact with air); the sections were then separately freeze-dried to constant mass. The dry samples were ground and homogenised in a Retch mill (0.25 mm steel mesh) and stored in polypropylene tubes until analysis. In total 48 alga samples were analysed for total arsenic content and arsenic species.

Total arsenic content

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). A portion (*ca* 100 mg) of the dry sample was weighed (to a precision of 0.2 mg) directly into a quartz digestion tube, and nitric acid (1 ml) and water (4 ml) were added. The digests were then cooled, quantitatively transferred with water to polypropylene tubes (15 ml capacity) to a final volume of 10 ml, and their arsenic content measured by ICPMS.

The suppression of the arsenic signal by the matrix was investigated by the analysis of CRM IAEA-140/TM (Figure 18).

Digestion versus flow injection analysis performed on extracts of part D

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 PEEK (polyetheretherketone) capillary tubing (0.125 cm i.d.100 cm) and quantification was done using peak area.

The obtained concentrations with different sample preparation procedure are showed in Table 5.

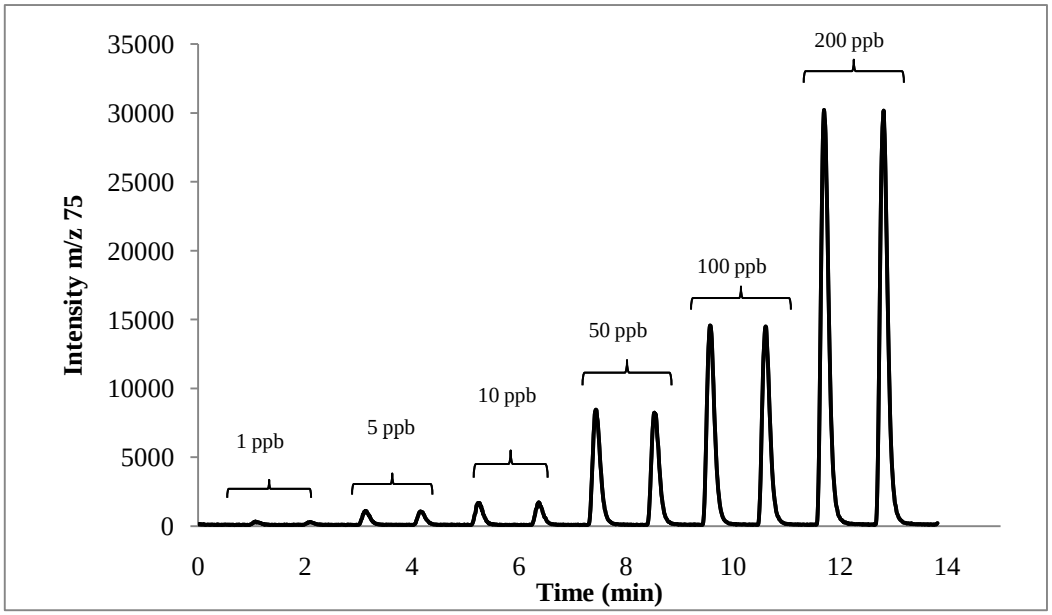


Figure 17. Typical flow injection signals for DMA standard

Extraction procedure

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.

Determination of arsenic species

Arsenic species were determined on aqueous-methanol extracts of the samples using anion- and cation-exchange HPLC/ICPMS under the conditions described above (4.4.3). The presence of thio arsenosugars was checked by reverse phase HPLC. All extractions were performed in duplicate. Extracts from the early samples (Day 0) were diluted 10-fold before HPLC; later sample extracts were injected undiluted.

Fractionation of arsenic

A sequential extraction (fractionation) procedure was employed for four samples, A0 (section A, day 0), A21, D0 and D21 (triplicates of each), in the following way. About 100 mg of the dry powder was weighed (to a precision of 0.2 mg) into a polypropylene tube and treated sequentially with 2 x aqueous methanol (9+1 v/v, 2 ml), acetone (2 ml), and hexane (2 ml). After each of the four extractions, the mixtures were centrifuged, the supernatant removed, and the pellet dried and weighed. The pellet obtained after the hexane extraction was then extracted under more forcing conditions with 0.1 M trifluoroacetic acid (2 x 2 ml) and microwave heating (95ºC, 30 min), and the mixture centrifuged. From all supernatants, one half of the sample was removed for microwave-assisted acid mineralisation and total arsenic analysis as described above; the pellet remaining after trifluoroacetic acid treatment was dried, weighed and then mineralised prior to total arsenic measurement. This arsenic was called as recalcitrant arsenic.

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.

Summary

The alga, packed in 12 tubes, was left to decompose for periods ranging from 1 to 25 days. Each tube, representing a different decomposition time, was further divided into four sections (A-D, from the closed bottom of the tube to the open top), giving a total of 48 samples for analysis.

The complete data set (total arsenic, extraction efficiencies, arsenic species and column recoveries) for the four sections is shown in Table 4. The data for the samples could be viewed in two parts: sections A-C were similar to each other whereas section D showed clear differences from the other three. Thus, the following discussion will focus on sections A and D.

Total arsenic and arsenic species in alga samples: changes with time and core depth

The temporal sampling showed quite variable arsenic content, which was a reflection of the natural variation between *Ecklonia* fronds and the fact that the samples were independent from each other and hence would have experienced non-uniform microbial conditions. There were clear temporal patterns in arsenic content of the decomposing algae. The day 0 sample of *Ecklonia* had a total arsenic content of about 45 μgAs/g, of which about 80% was extracted into aqueous-methanol. For section A samples, the arsenic content of the alga decreased with the time of sampling; for example, arsenic concentrations at days 0, 6 and 21 were 48.8 μg/g, 30.6 μg/g, and 23.9 μg/g, respectively. For section D samples, however, there was only a slight trend to lower arsenic content with time. The decreasing arsenic contents may have represented a partial "washing-out" of the arsenic, or perhaps production of some volatile products. The proportion of non-extractable arsenic also tended to increase with time for section A, and, again, this trend also was less apparent for section D samples (Figure 20). Although this effect was partly a consequence of the total arsenic level decreasing with time in the section A samples, there also appeared to be an absolute increase in the non-extractable arsenic which suggested the formation of recalcitrant arsenic. In the initial (Day 0) alga samples, the major extractable arsenic species were arsenosugar SO3 (*ca* 65% of the sum of species measured by HPLC/ICPMS), arsenosugar PO4 (25%), and arsenosugar OH (*ca* 12%). There was considerable variability in arsenic species between individual samples, but the trend with time was nevertheless very clear. To simplify the data presentation, results for arsenic species and non-extractable arsenic have been averaged over the periods 1-5 days, 6-11 days, 13-18 days, and 21-25, and are presented for sections A and D (Figure 21).

Section A: The arsenosugars originally present begin to degrade after several days to dimethylarsinoylethanol (DMAE), a result reported in the first experiments on arsenosugar degradation. With increasing time, however, DMAE is further degraded to DMA, which in turn is demethylated to arsenate. Interestingly, arsenosugar OH increased during the early stages before decreasing. Presumably, increased levels of arsenosugar OH resulted from degradation of arsenosugar PO4. It is interesting that both the P and the S containing arsenosugars were quickly degraded; this might reflect their preferred utilisation (because of their P/S content) by microbes involved in the decomposition processes.

Section D: Samples showed clear differences from the A samples - primarily, the arsenosugars degraded more slowly, and they remained significant species even after 25 days. Furthermore, DMAE, although present, was not a significant intermediate for the D section samples. Rather, DMA was the major intermediate, and its further degradation to arsenate was less marked than that recorded for section A samples. These results entirely support the earlier observations by Pengprecha who investigated arsenosugar breakdown in sediments (Pengprecha, 2005). In that study, the arsenosugars were degraded quickly in deeper anaerobic sediments, with DMAE being the major metabolite constituting 60-80% of the total arsenic.

Results from this study also suggested that the formation of DMAE is favoured by conditions where oxygen concentration is relatively low - peak DMAE percentages for the four sections were A (35%), B (30%), C (13%), and D (4%).

The oxidation procedure was performed in order to check the presence of arsenite. This procedure was also used to check for any thio-arsenicals, but they were not present in significant amounts.

Are arsenosugars in decomposing algae partly converted to recalcitrant arsenic?

Although HPLC/ICPMS is a valuable method for speciation analysis, it can only provide information on the extractable species, and then, usually only on water-soluble species. The advantages of using a multi-method approach, and including a solid-phase method such as X-ray absorption spectroscopy, have been clearly presented in a recent review (Feldmann, 2009). In this study was not performed speciation analysis on solid-phase samples, however it was necessary to obtain some information on the non-extractable arsenic from fractionation experiments. Sequential extraction with polar (water/methanol) and non-polar (acetone and then hexane) solvents, and a mild trifluoroacetic acid extraction was performed on four of the samples: section A days 0 and 21; and section D days 0 and 21 (Table 6). A most striking difference between the days 0 and days 21 data was the quantity of arsenic remaining in the residue (pellet) after the fractionation procedure - the final residues for the day 21 samples contained about 4-5-fold greater (absolute) amounts of arsenic compared with the day 0 samples.

These remarkable results showed that the amount of insoluble or recalcitrant arsenic dramatically increased in the decaying algae. The apparent formation of significant amounts non-extractable or recalcitrant arsenic was an unexpected result that demands further investigation. The result suggests that about 10% of the original arsenic in the alga was converted to recalcitrant arsenic after 21 days. Nonextractable arsenic cannot be ignored if the complete pattern of arsenic metabolism in marine organisms desires to be clearly understood.

The observed fractionation data showed that the recalcitrant arsenic is largely solubilised by treating the pellet with hot aqueous TFA.

Table 4. Total arsenic content and arsenic species in extracts of *Ecklonia radiata*

	µg As/ g	Extraction efficiency	As species µg As/ g in algae powder						Column recovery %	
Sample	**dry mass**	**%**	**OH**	**PO4**	**SO3**	**DMAE**	**DMA**	**AsV**	**Anion exch.**	**Cation exch.**
A0	48,8	86	2,1	7,3	23,1	<0,1	<0,1	<0,1	85	75
B0	52,1	76	1,2	6,7	17,4	<0,1	0,6	<0,1	73	43
C0	43,4	85	<0,1	7,7	19,0	<0,1	0,4	<0,1	84	-
D0	44,5	79	1,4	6,0	22,0	<0,1	<0,1	<0,1	89	89
A1	38,8	81	2,5	4,5	18,5	<0,1	<0,1	<0,1	84	76
B1	46,3	73	2,5	4,5	18,3	<0,1	0,6	<0,1	78	97
C1	46,3	84	2,9	4,4	22,2	<0,1	0,9	<0,1	86	79
D1	42,9	72	4,1	3,1	15,4	<0,1	0,7	<0,1	85	87
A4	38,8	69	3,4	0,1	7,2	5,4	1,6	0,6	88	69
B4	32,9	56	1,0	<0,1	4,2	<0,1	1,2	<0,1	73	53
C4	32,5	66	2,7	1,1	7,1	2,7	2,3	<0,1	82	78
D4	45,3	86	0,3	1,4	17,4	1,4	3,5	0,5	86	87
A5	37,3	81	3,5	0,2	18,2	0,4	1,6	<0,1	92	77
B5	41,8	63	4,9	0,5	14,3	<0,1	<0,1	<0,1	78	104
C5	46,0	71	4,6	0,9	15,6	0,4	4,5	<0,1	87	73
D5	48,0	85	4,0	1,3	15,2	1,0	7,5	<0,1	88	68
A6	30,6	73	1,5	0,4	3,1	3,2	0,9	<0,1	80	36
B6	34,7	61	4,2	<0,1	10,2	0,7	1,3	<0,1	81	101
C6	45,6	69	5,1	0,3	15,4	<0,1	2,4	<0,1	75	73
D6	45,8	71	2,7	0,9	5,1	<0,1	16,2	<0,1	91	79
A8	33,5	64	2,9	0,4	2,3	7,0	1,3	<0,1	77	76
B8	35,0	61	2,4	1,1	4,0	3,7	2,9	<0,1	77	72
C8	31,1	64	<0,1	0,7	<0,1	0,8	7,7	0,1	77	77
D8	42,8	72	2,3	1,1	5,9	<0,1	13,3	<0,1	90	71

A11	21,3	54	<0,1	0,5	<0,1	3,2	2,7	0,5	77	81
B11	22,9	51	<0,1	1,0	<0,1	4,0	3,5	0,4	81	110
C11	20,0	63	<0,1	0,5	<0,1	0,5	3,7	1,1	78	78
D11	25,8	56	1,0	0,7	0,9	<0,1	6,4	1,1	89	66
A13	35,9	69	1,2	0,9	<0,1	8,1	7,6	<0,1	81	79
B13	35,5	55	1,6	0,9	<0,1	0,7	5,9	<0,1	75	91
C13	22,3	65	<0,1	0,1	<0,1	0,5	9,2	0,4	79	82
D13	54,4	74	3,2	<0,1	7,8	<0,1	23,6	0,5	94	78
A15	20,6	47	<0,1	0,9	<0,1	1,8	2,1	1,5	82	65
B15	18,4	45	0,8	0,7	<0,1	1,8	2,2	1,3	92	102
C15	13,5	60	<0,1	0,1	<0,1	0,5	1,9	3,6	90	81
D15	34,5	77	2,0	<0,1	6,7	0,4	7,9	2,6	84	74
A18	23,2	56	<0,1	0,4	<0,1	0,8	7,8	0,2	79	77
B18	33,2	60	0,7	1,1	<0,1	<0,1	12,2	0,5	77	95
C18	18,3	58	<0,1	0,1	<0,1	0,2	5,6	2,5	81	76
D18	39,1	62	1,8	<0,1	3,8	<0,1	13,0	2,3	90	68
A21	23,9	37	<0,1	0,5	<0,1	1,7	1,1	2,5	79	78
B21	20,8	35	<0,1	0,4	<0,1	1,5	0,9	3,0	84	92
C21	21,2	53	<0,1	0,3	<0,1	<0,1	1,0	6,8	83	78
D21	36,2	64	2,7	<0,1	3,5	<0,1	4,0	7,6	89	83
A25	17,6	42	<0,1	0,3	<0,1	0,6	1,3	2,2	72	76
B25	21,1	40	<0,1	0,1	<0,1	<0,1	0,8	4,9	85	104
C25	21,6	60	<0,1	0,1	<0,1	<0,1	1,9	8,1	83	80
D25	53,6	71	5,4	<0,1	12,6	<0,1	10,0	4,4	91	85

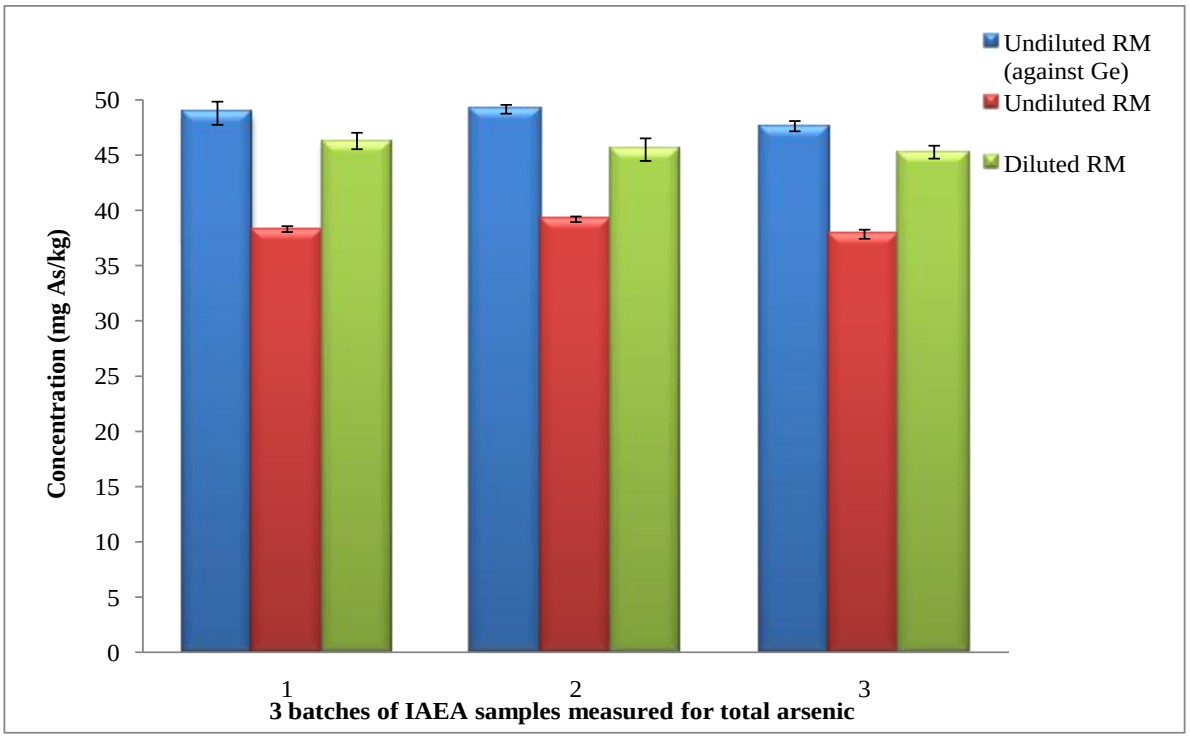


Figure 18. Determined arsenic concentration in CRM IAEA-140/TM (Fucus sp., Sea Plant Homogenate) showing non-normalized and normalized data against ^{74} Ge

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.

Table 5. 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.

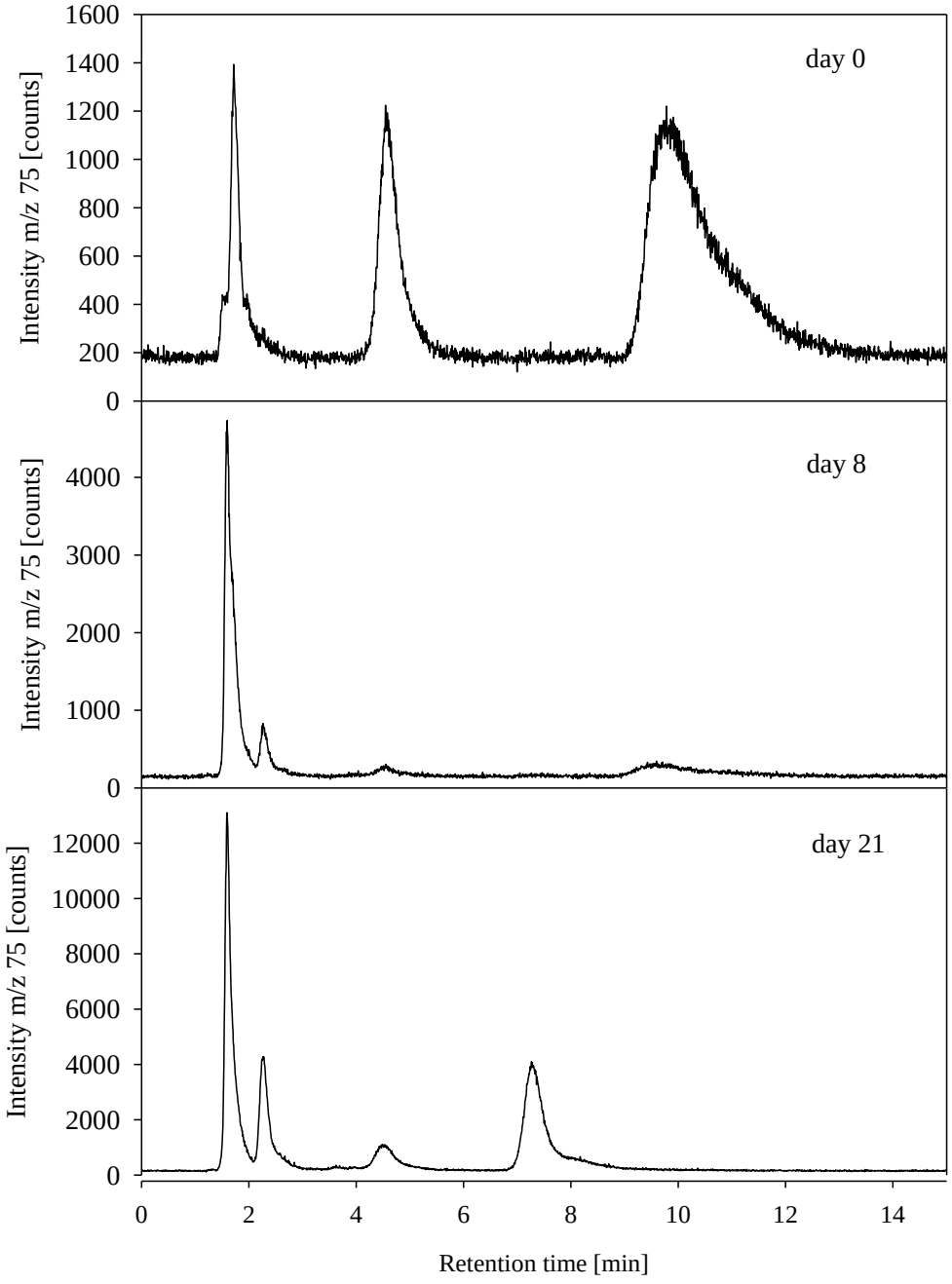


Figure 19. HPLC/ICPMS chromatograms for extracts of alga from Section A, days 0, 8, 21.

Anion-exchange showing the rapid decrease in arsenosugar SO3 (RT 9.8 min) with concomitant increase in cationic metabolites (void volume, RT 1.6 min), and then the increase in arsenate (RT 7.3 min) at Day 21.
Conditions: PRP-X100 column (4.1 mm x 150 mm) at 40°C with a mobile phase of 20 mM aqueous ammonium dihydrogen phosphate at pH 6; flow rate 1.5 ml/min; 10 µl injected. Day 0 sample was diluted 10-fold before injection.

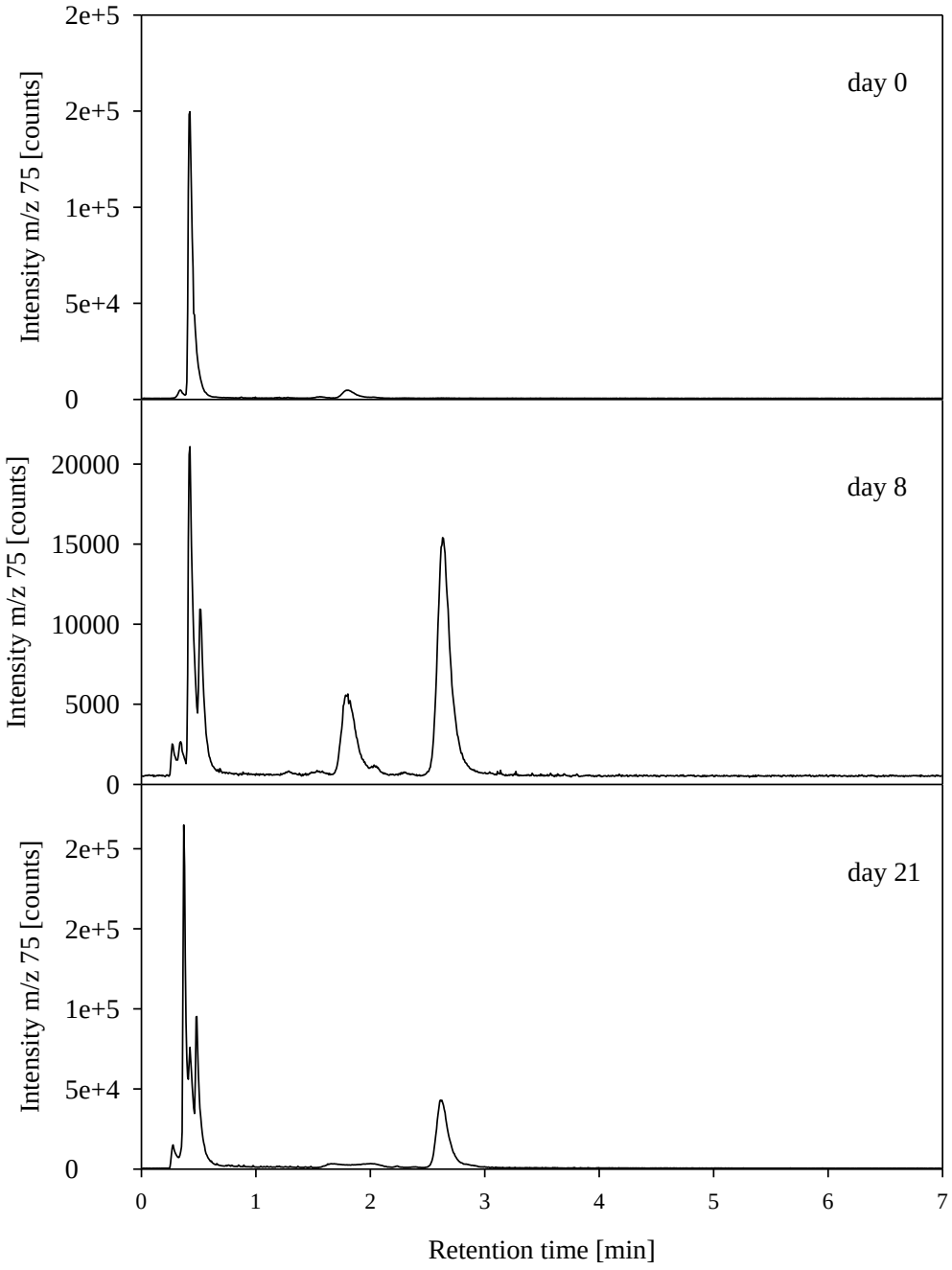


Figure 20. HPLC/ICPMS chromatograms for extracts of alga from Section A, days 0, 8, 21.

Cation-exchange showing the initial increase in arsenosugar OH (RT 1.8 min) and DMAE (RT 2.6 min), and then their decrease at Day 21.
Conditions: Chrompack Ionospher 5C column (3 x 100 mm) at 40 °C with a mobile phase of 10 mM aqueous pyridine at pH 3; flow rate 1.5 ml/min; 10 µl injected**.**

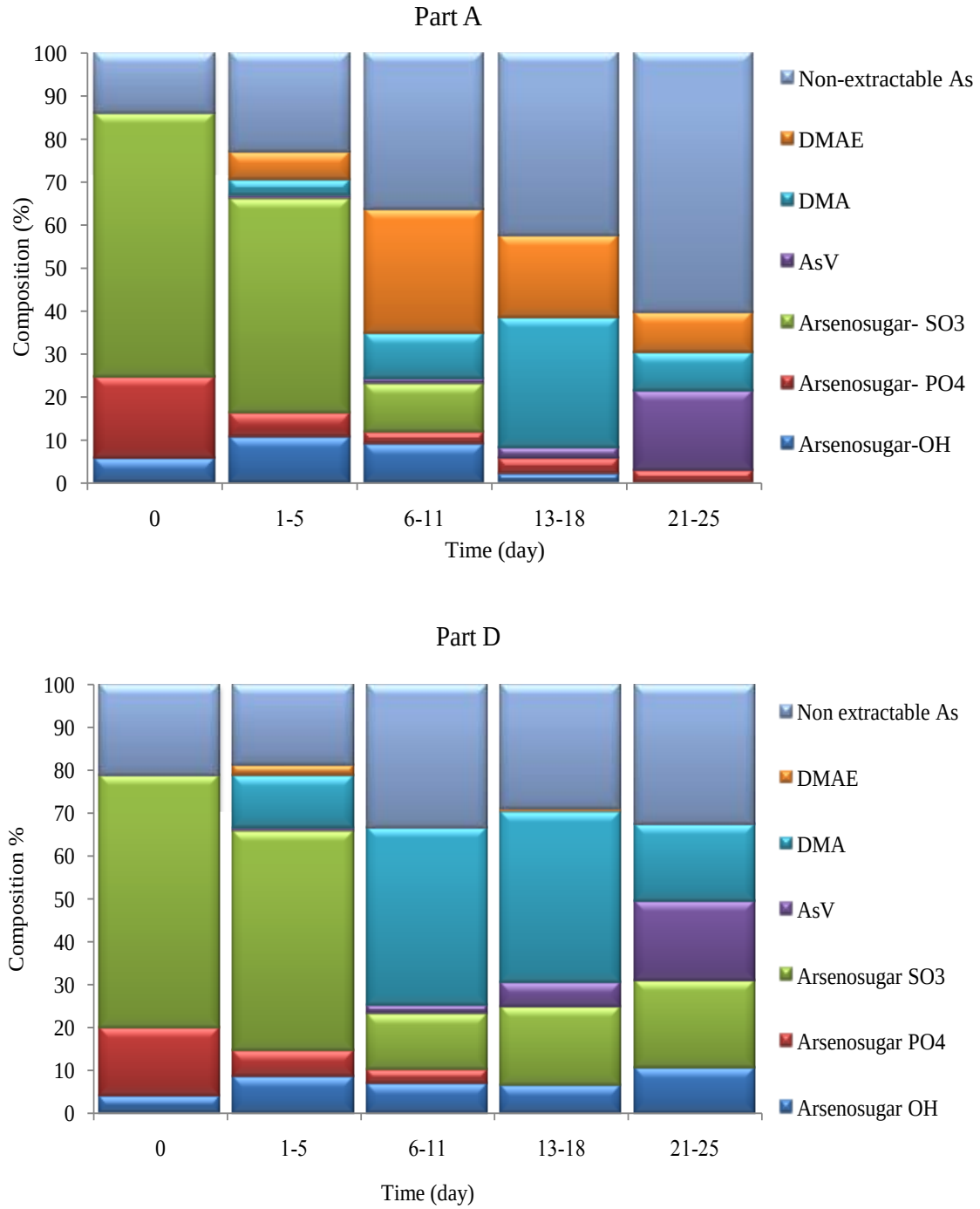


Figure 21. Variation with time in the proportion of arsenic species for section A and D in decomposing *Ecklonia radiata*.

Table 6. Quantity of arsenic
Quantity of arsenic at each stage of a sequential fractionation procedure performed on Day 0 and Day 21 samples for sections A and D.
Results are mean ± SD of three replicate extractions given in µg As based on an initial sample of 100 mg of algal powder.

Treatment	**Arsenic (µg)**			
	A0	**A21**	**D0**	**D21**
Water /methanol 9+1	5.26 ± 0.06	0.99 ± 0.02	4.27 ± 0.18	2.12 ± 0.02
Acetone	0.24 ± 0.01	0.17 ± 0.01	0.47 ± 0.03	0.05 ± 0.01
Hexane	< 0.01	< 0.01	< 0.01	< 0.01
0.1 M TFA	0.33 ± 0.01	0.45 ± 0.03	0.32 ± 0.02	0.46 ± 0.03
Residue	0.14 ± 0.01	0.60 ± 0.02	0.12 ± 0.01	0.61 ± 0.06

Investigation of TFA fraction

Sequential extraction revealed significant concentration of arsenic in TFA fraction The TFA fraction was examined by HPLC/ICPMS and surprisingly it was not inorganic arsenic but it was present as one major cationic species which did not match any of the arsenic species commonly found in marine samples.

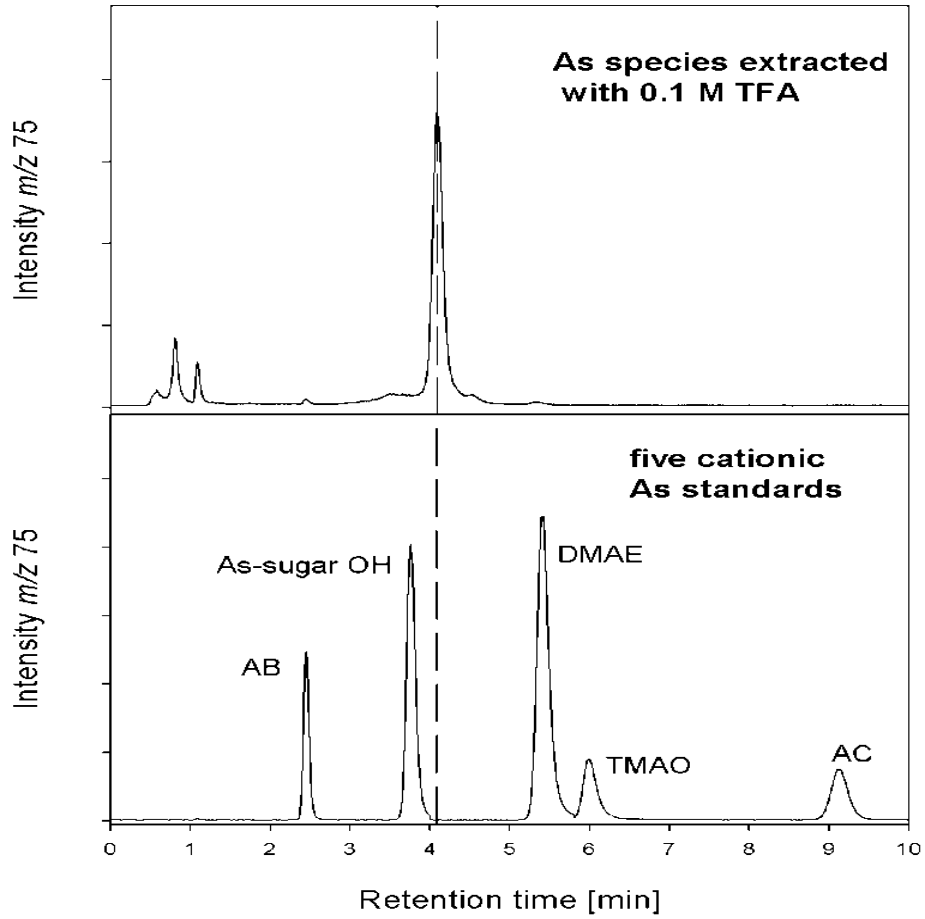


Figure 22. HPLC/ICPMS chromatograms of TFA fraction
Chrompack Ionospher 5C column (3 x 100 mm; Varian, Middelburg, Netherlands) at 40 °C with a mobile phase of 10 mM aqueous pyridine at pH 3 (adjusted with formic acid) and a flow rate of 1 ml/min

Thin layer chromatography laser ablation ICPMS (TLC-LA-ICPMS) of TFA fraction (performed by Mag. Christoph Kurta)

The aim of this experiment was to separate and purify the unknown compound with TLC and its quantitavive determination with LA-ICPMS. Briefly the laser ablation ICPMS is a technique used for the in situ analysis of trace elements on solid samples. Solid particles are physically ablated due to the interaction of a high power laser beam with the surface of the sample. The particles are carried in a stream of argon into plasma where they are ionised prior to measurement in a quadrupole mass spectrometer.

For TLC experiment was used as stationary phase: Cellulose (0.1 mm) Merck (Darmstadt, Germany) and mobile phase composition butanol/acetic acid/water; 60/15/25, respectively. LA-ICPMS analysis of the TLC plate suggested very strong interaction with the solid support, because the compound retained 1 cm above the start line.

HPLC/ESMS of TFA fraction

ICPMS is an element specific detector and it does not provide molecular information. The settings of the ESMS were chosen so that arsenic was detected with a high fragmentor voltage (400V) at m/z 91 $(AsO)^+$ in SIM mode and a mass range was scanned simultaneously with low fragmentor voltage (100V). The HPLC conditions were same as for the detection with ICPMS: Column Chrompack Ionospher 5C (3 x 100 mm; Varian, Middelburg, Netherlands) at 40 °C with a mobile phase of 10 mM aqueous pyridine at pH 3 (adjusted with formic acid) and a flow rate of 1 mL min^{-1}. The species could be not directly identified.

The isolation of the HPLC/ICPMS peak of interest was performed by collection the HPLC effluent. The fraction was evaporated to dryness and redissolved in 500 µl of water prior to ESI/MS analysis. The mass spectrum still contained several additional signals and a clear assignment of a molecular mass could not be made even preconcentration step was applied therefore would be necessary to apply suitable clean up procedure e.g. size exclusion chromatography. From the investigations of the chromatographic behaviour of the unknown cationic compound performed so far the following assumption could be made:
The unknown cationic coumpouds contains arsinoyl group ($Me_2As=O$). The suggestion was confirmed by treatment of TFA fraction with H_2S. The oxygen present in arsinoyl group was replaced by sulphur (Figure 23).

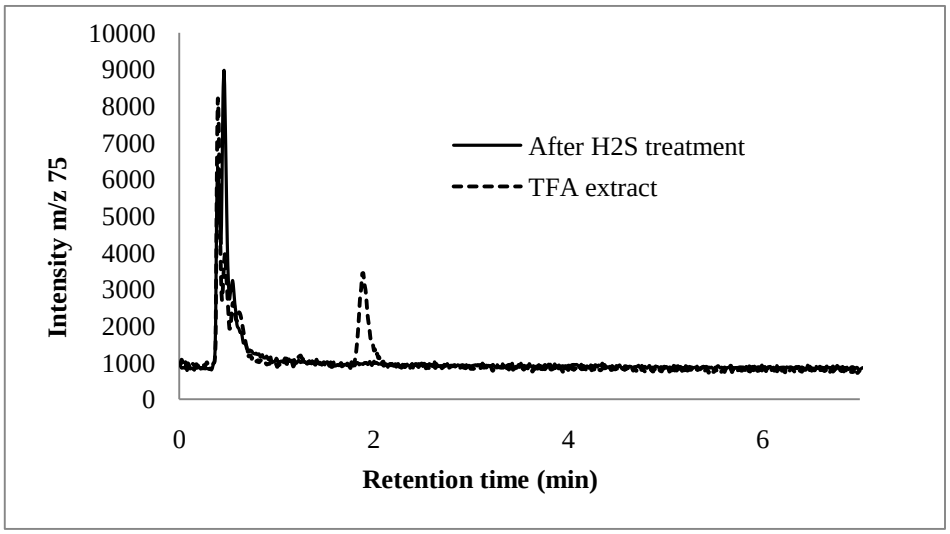


Figure 23. HPLC/ICPMS chromatograms of TFA fraction

Chrompack Ionospher 5C column (3 x 100 mm; Varian, Middelburg, Netherlands) at 40 °C with a mobile phase of 10 mM aqueous pyridine at pH 3 (adjusted with formic acid) and a flow rate of 1.5 ml/min

5.2. ARSENIC SPECIATION IN RICE

Aim of the study

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). 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. 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 1% H_2O_2 as extraction solvent was used. 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. H_2O_2 was used in order to oxidize As^{III} to As^{V} and determine inorganic arsenic in As^{V} form. The method is only suitable for determination the levels of total inorganic arsenic

Sample preparation and total arsenic determination

Samples were homogenized in Retch mill 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.

Microwave-assisted extraction for speciation

A portion (*ca* 250 mg) of rice powder was weighted for extraction and 5 mL of 20 mM TFA 1% H_2O_2 was added. 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.

Speciation analysis of arsenic

Arsenic speciation analysis was performed on the extracts by HPLC/ICPMS under anion-exchange conditions: PRP-X 100 anion exch. column, 10 mM malonic acid pH 5.6 as a mobile phase, flow rate 1.2 ml/l and 20 μl injection volume. The arsenite was oxidized during extraction procedure due to the H_2O_2 presence and inorganic arsenic was determined as As^{V}.

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}.

Table 7. ICPMS setting for arsenic speciation analysis

Parameter	Setting
Nebulizer type	Burgener Ari Mist HP Nebulizer
Spray chamber type	Peltier cooled, Scott type, double pass
Spray chamber temperature	2°C
Torch	2.5 mm ID
RF power	1550 W
Plasma gas flow	15 l/min
Carrier gas flow	0.9 l/min
Auxiliary gas flow	0.12 l/min
Optional gas (1% CO_2 in argon)	11%
Sample cone	Nickel
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

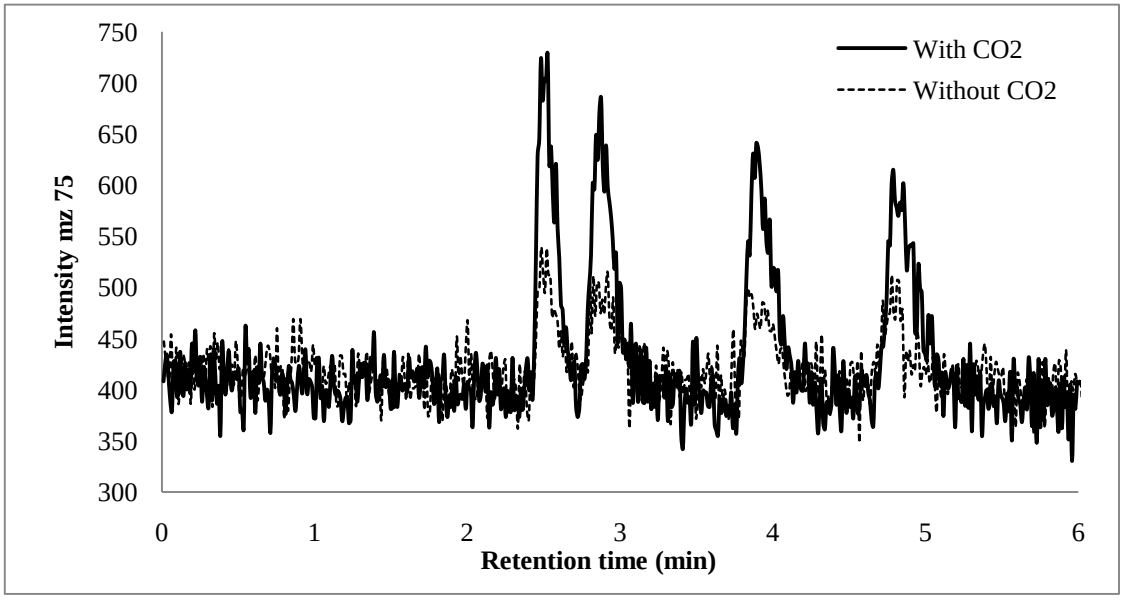


Figure 24. 0.2 µg As/l standard of As^{III}, DMA, MA, As^{V} with and without signal enhancement
Conditions: PRP-X 100 anion exch. column, 10 mM malonic acid pH 5.6 as a mobile phase, flow rate 1.2 ml/l and 20 µl injection volume

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.

Limit of detection

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 8. Concentrations of total arsenic and arsenic species in Czech market rice

Sample specification	Country of origin	Total arsenic (µg As/kg ± SD)	Arsenic Species (µgAs/kg ± SD)			Species sum (µgAs/kg)	Recovery (%)	Inorganic As (%)
			DMA	MA	As^V			
Thai yasmine rice	Thailand	126.50 ± 1.87	49.70 ± 0.09	1.87 ± 0.30	68.40 ± 1.33	119.97	94.83	54.06
Bio quality Thaibonnet natural	Italy	211.29 ± 2.23	37.49 ± 0.12	2.28 ± 0.25	141.38 ± 0.99	181.15	85.73	66.91
Basmati F.W. Tandoori	Pakistan	49.31 ± 0.63	17.28 ± 0.15	1.68 ± 0.25	30.16 ± 0.83	49.12	99.61	61.17
Tesco Basmati	India	93.14 ± 1.30	13.09 ± 0.97	>LOD	72.33 ± 0.39	85.42	91.71	77.65
Basmati Uncle Bens	EU	36.06 ± 2.32	10.67 ± 0.14	>LOD	25.36 ± 1.00	36.03	99.93	70.33
Long grain Menu	Italy	87.08 ± 2.79	21.91 ± 0.45	>LOD	58.54 ± 0.39	80.45	92.39	67.23
Long grain white rice Euro shopper	Poland	156.45 ± 1.88	35.80 ± 0.51	1.73 ± 0.15	114.04 ± 1.31	151.58	96.88	72.89
Long grain Uncle Bens	EU	128.60 ± 2.95	41.20 ± 1.28	3.58 ± 0.87	77.29 ± 0.40	122.07	94.93	60.10
Long grain natural Lagris	Italy	107.88 ± 0.41	21.61 ± 0.21	>LOD	76.75 ± 0.82	100.52	93.17	71.14
Bio long grain natural Harmony	Italy	218.11 ± 4.67	29.56 ± 0.97	>LOD	170.77 ± 2.33	200.33	91.85	78.30

Summary

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 $DMA^V$and iAs, quantifiable amounts of MA^V were observed in five samples (Table 8). 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.

5.3. DETERMINATION OF THIO - ARSENOSUGARS IN DEEP VENT MUSSEL

The aim of the study:

In deep water organisms are expected anaerobic conditionds under these conditionds anaerobic metabolism is favoured and oxygen is substitued by sulphur. The following study demonstrates this suggestion. The role of thio arsenicals in marine organisms is unknown.

For separation of thio arsenosugars the chromatographic method developed by Raml et al. was used (Raml, 2006).

Total arsenic analysis in mussel powder

Total asrenic concentrations in the sample were determined by ICPMS after mineralization of the samples with microwave assisted acid digestion (10 mg of sample was weighted for digestion + 1 ml HNO_3). The digested sample solutions were quantitatively transferred to 15 ml polypropylene tubes and diluted to 10 ml with Milli-Q water. Arsenic concentrations in the digest solutions were determined by ICPMS. The accuracy of the total measurement was tested by the analysis of the reference material DORM-2 (10-20 mg) which has a certified value of 18 ± 1.1 mg As/kg dry mass. The obtained value was 19.2 ± 0.6 mg As/kg dry mass (mean ± SD, n=3).

Extraction procedure

Portion of the mussel (40 mg) was transferred together with water (2 ml) to 15 ml polypropylene tubes and the mixture was shaken overnight at room temperature, the same procedure was aplied to the reference material. The water phase was filtered (0.22 μm) prior to total arsenic determination and arsenic speciation analysis.

For total arsenic determination aliquots (100 μl) of the extract were digested and analysed by ICPMS in order to calculate extraction efficiency.

Arsenic speciation analysis

Arsenic speciation analysis was performed on the extracts by HPLC/ICPMS under anion- and cation- exchange, and reversed-phase conditions.

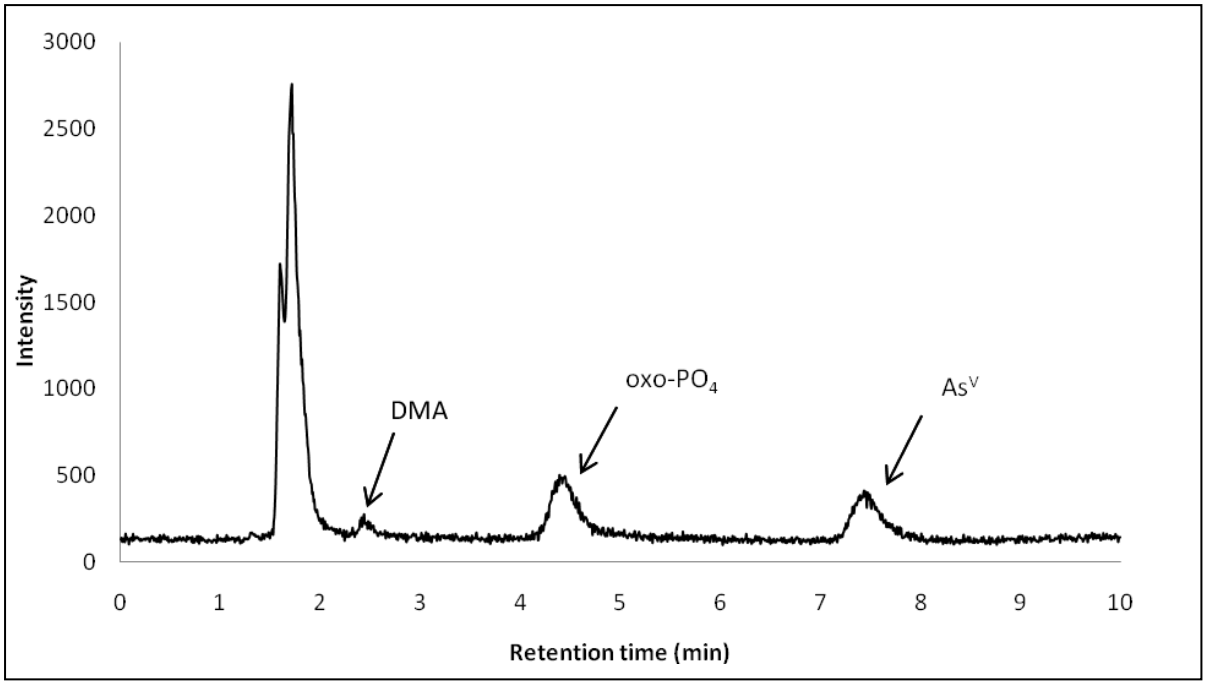
- *oxidation procedure*

Under anion-exchange conditions As^{III} elutes near the void volume therefore the extract was treated with H_2O_2 (30%) to oxidaze As^{III} to As^{V} and detremine As^{III} indirectly. This oxidation reaction showed the interconversion among thio-arsenosugars and oxo-arsenosugars.

Quality control

The method for arsenic speciation was validated by analysis of arsenic species in certified reference material DORM-2. The concentrations (mean ± SD, n=3) of AB (15.4± 0.58 mg/kg) and TETRA (0.235± 0.024 mg/kg) were in good agreement with certified values (AB, 16.4 ± 1.1 mg As/ kg; TETRA, 0.248 ± 0.054 mg/kg).

(a)



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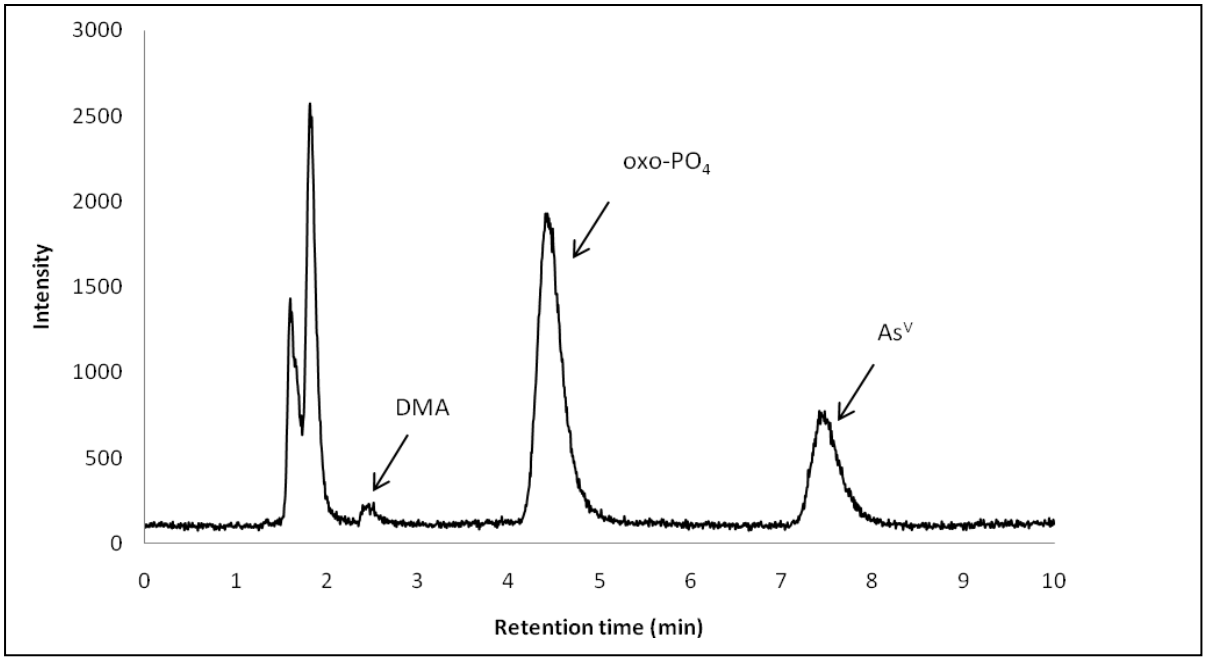


Figure 25. Anion-exchange HPLC/ICPMS chromatograms

(a) mussel extract (column recovery 40%);
(b) mussel extract oxidized with H_2O_2 (column recovery 68%).
Conditions: PRP-X100 column (250 mm x 4.1 mm id; Hamilton Company, Reno, Nevada, USA) mobile phase 20 mM $NH_4H_2PO_4$ pH 6; flow rate 1.5 ml/min, injection volume 20 µl, column temperature 40°C

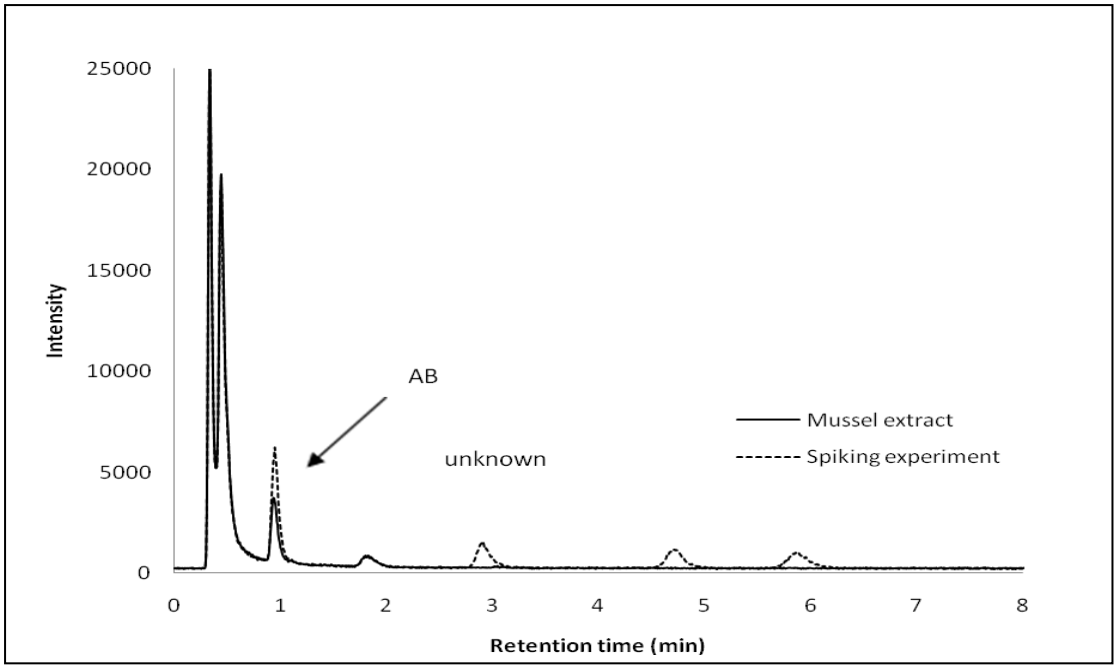


Figure 26. Cation-exchange HPLC/ICPMS chromatogram of mussel extract - column recovery 96%.
Conditions: Chrompack Ionospher 5C (3 × 100 mm; Varian, Middelburg, Netherlands) mobile phase 10 mM pyridine pH 3; flow rate 1.5 ml/min, injection volume 20 µl, column temperature 40°C

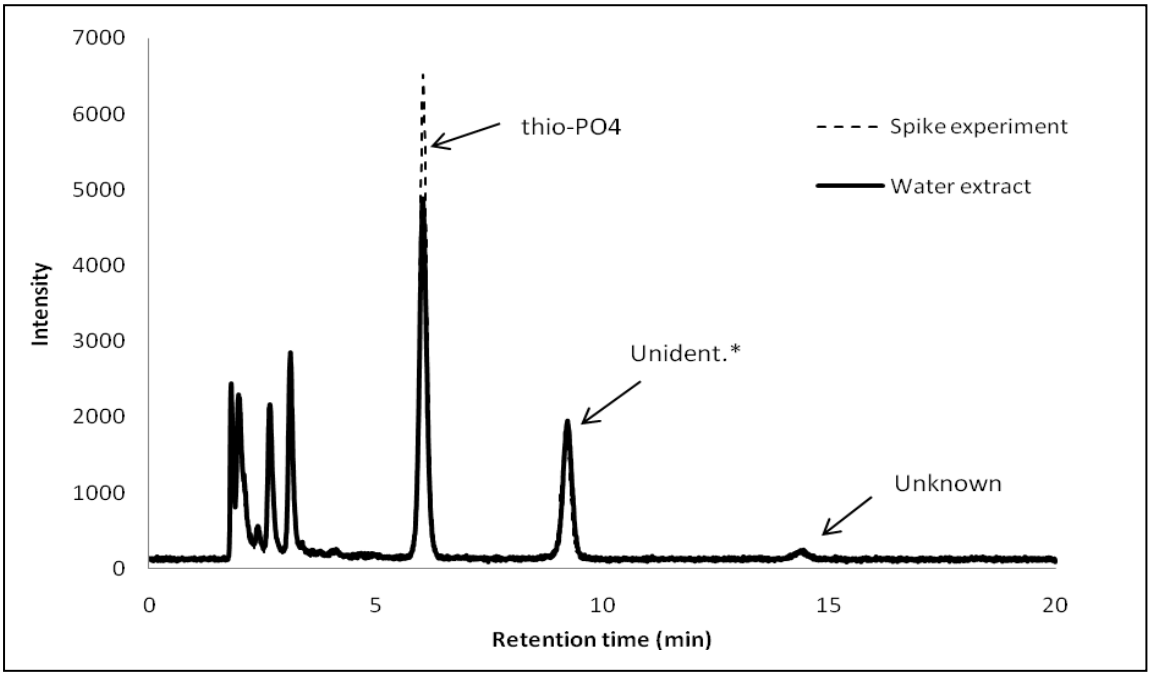


Figure 27. Reversed-phase HPLC/ICPMS chromatogram of mussel extract-column recovery 81%.
Conditions: Atlantis®dC18 column (4.6 x 150 mm; Waters, Massachusetss, USA)
mobile phase 20 mM $NH_4H_2PO_4$ pH 3; flow rate 1.5 ml/min, injection volume 20 µl, column temperature 30°C
*Unidentified peak possibly thio-glycerol

Summary

- The total arsenic concentration was 8.35 µg As/g
- The obtained extraction yield was 54%
- The dominant arsenic species in mussel extract was thio-arsenosugar-phosphate 1.23 µg As/g
- Inorganic arsenic As^{III} + As^{V} represent 0.57 µg As/g
- DMA 0.05 µg As/g
- Oxo- PO_4 0.29 µg As/g
- AB 0.31 µg As/g
- Only about 30% of the total arsenic in the mussel has been identified

The presence of considerable quantities of thio-arsenosugars phosphate is probably connected with anaerobic metabolism in deep water organisms. The role of the thio arsenicals in the biotransformation of arsenic in marine organisms remains unknown.

5.4. DETERMINATION OF WATER SOLUBLE AND LIPID SOLUBLE ARSENICALS IN ESCOLAR

Aim of the study:

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.

Total arsenic analysis

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 convential ICPMS method described above (4.4.2). The determined concentration in fish tissue was 1.8 ± 0.1 µg As/g. (mean ± SD, n=3).

Fractionation of polar and non polar arsenicls

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 9. Percentage of extractable As

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

Examination of water/methanol fraction (water soluble arsenic)

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 20mM 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, 5µm 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.

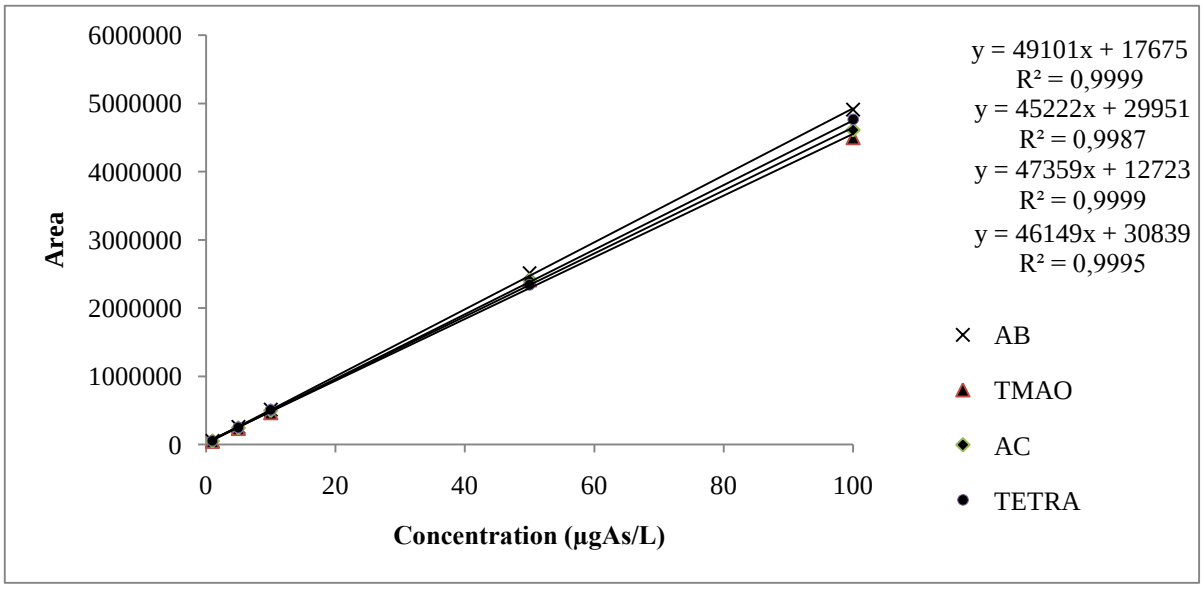
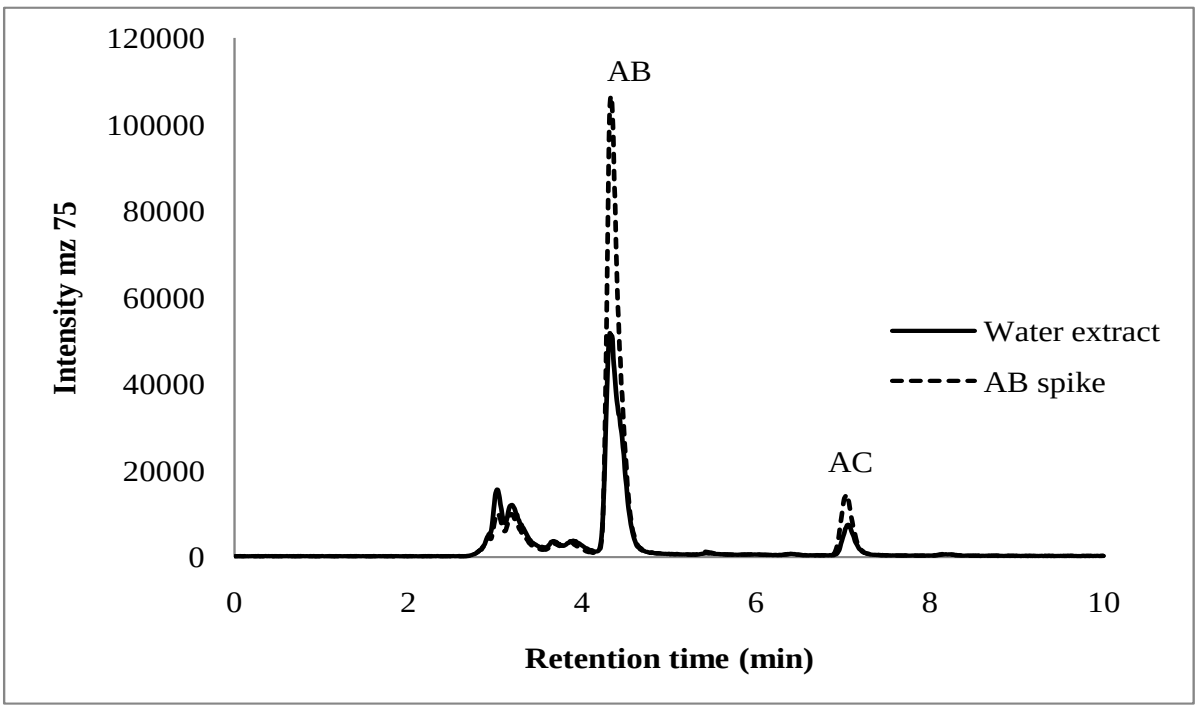


Figure 28. Calibrations curves for four cationic standards

a)



b)

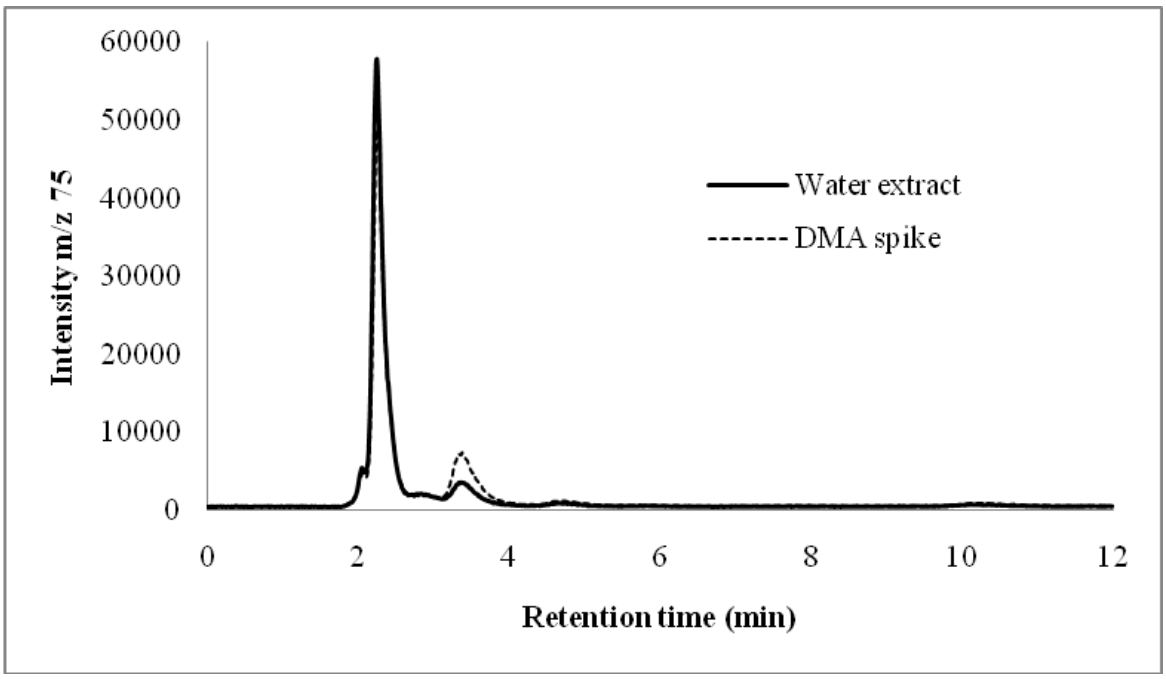


Figure 29. Overlayed HPLC/ICPMS chromatograms of spiked water extracts

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%

Examination of hexane fraction (fat soluble arsenic)

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.

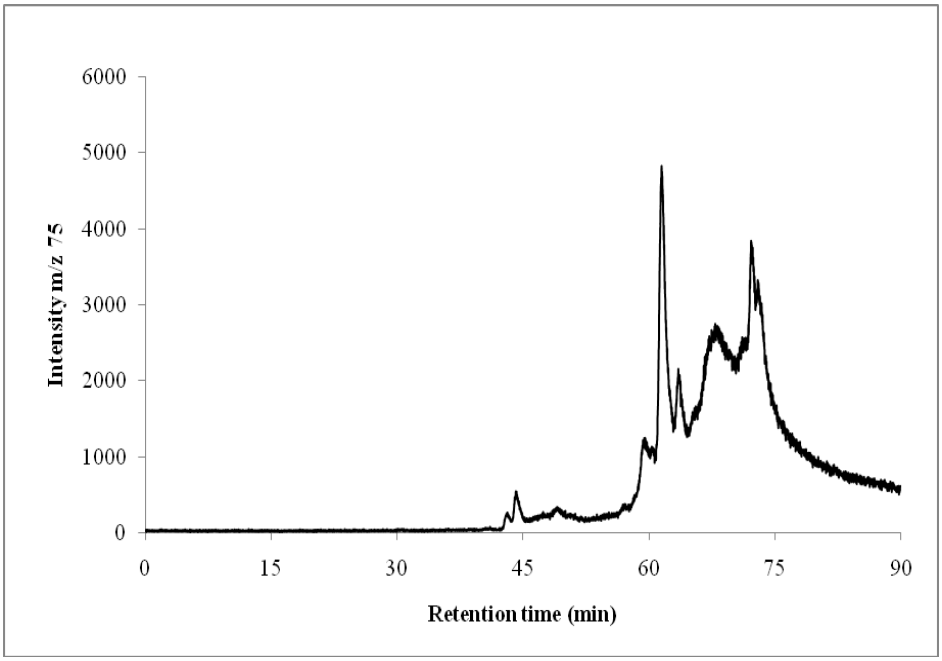
Following ICPMS conditions were used: radio frequency (RF) power 1600 W, the spray chamber was set at -5°C to limit the amount of organic solvent organic solvent reaching the plasma, because it can cause the problems of plasma instability, torch with a narrow i.d. (1.5 mm). The ICPMS was tuned with a 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 10. 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)

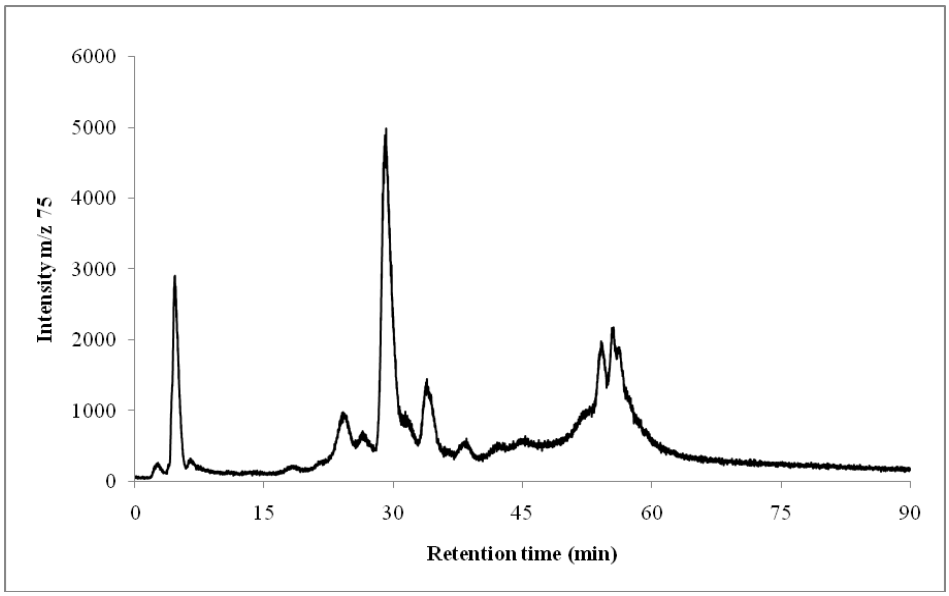


Figure 30. Reversed-phase HPLC/ICPMS chromatogram of the nonpolar arsenolipids (hexane extract) from escolar fish.
a) conditions: Atlantis dC18 (150 x 1.0 mm, 5 µm) at 30°C , mobile phase 20 mM NH_4OAc pH 6 and etanol at a flow rate of 100 l/min. The chromatography was performed with linear gradient 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 1.0 mm, 5 µm) 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.

Summary

The determined total arsenic concentration in the fish muscle tissue with ICPMS was 1.8 ± 0.1 μg As/g (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.

5.5. ARSENIC SPECIATION IN AMPHIBIANS

Aim of the study

The goal of this study was to determine the arsenic species distribution in frogs. Frogs are essentially freshwater or terrestrial animals, but some species are salt tolerant. According to the paper published in 2006 by Schaeffer one frog sample was analyzed and showed very interesting species distribution pattern; arsenite (30%) and TETRA (35%) were major species (Schaeffer, 2006). Laboratory experiment recently published showed the simillar picture, the major arsenic specie was TETRA (Bryszewska, 2011).

Two different batches of samples were analyzed for total arsenic content and for arsenic species ditribution: frog legs from Slovenia and frogs from Thailand.

Frog legs are a delicacy mostly associated with French cousine but they are also eaten in many other counries e.g. Slovenia. The origin of Thai frogs was esturine water. The definition of an estuary is "An estuary is a semi-enclosed coastal body of water which has a free connection with the open sea and within which sea water is measurably diluted with fresh water derived from land drainage" (Cameron, 1963). It was supposed that Thai frogs must be salt tolerant and would show different arsenic species pattern.

Sample preparation

Frogs legs were purchased from local market in Slovenia and transported frozen to laboratory in Graz. The origin of the samples was Indonesia. Indonesia is one of the biggest exporter of frog legs. 6 pieces of frog legs were taken for the analysis. 9 frogs from Thailand were obtained dried and dissected. All samples were homogenate with a mortar and pestle using liquid nitrogen to fine powder.

Total arsenic analysis

0.1 g of sample was weighted for digestion + 2 ml HNO_3 and the digested sample solutions were quantitatively transferred to 15 ml polypropylene tubes and diluted to 10 mL with Milli-Q water. Arsenic concentrations in the digest solutions were determined by ICPMS. In total 6 samples of frog legs and 9 samples of Thai frogs were analysed. Samples were analysed in duplicates.

The accuracy of the total measurement was tested by the analysis of the reference material DORM-2 which has a certified value of 18.0 ± 1.1 mg As/kg dry mass. The obtained value was 19.1 ± 0.5 mg As/kg dry mass (mean ± SD, n=3).

Extraction procedure

In case of frog legs from Slovenia a portion of the frogs tissue (ca 0.2 g) was transferred together with water (3 ml) to 15 ml polypropylene tubes and the mixture was shaken overnight at room temperature. The extracts were treated with 3 ml of acetonitrile in order to remove proteins which can cause problems during HPLC analysis. The mixture was centrifuged (3000 g x 15 min) and the supernantant was transfered to round bottom flask and evaporated to dryness. The residue was redissolved in 1ml of water.

For analysis of Thai frogs a portion of the frogs tissue (ca 0.1 g) was transferred together with water (2 ml) to 15 ml polypropylene tubes and the mixture was shaken overnight at room temperature. The extracts were centrifuged (3000 g x 15 min) and filtrated through 0.22 μm filter prior HPLC. The total arsenic in the extracts was determined directly without digestion by FIA in order to calculate extraction efficiency.

Speciation analysis

Arsenic speciation analysis was performed on the extracts by HPLC/ICPMS under anion- and cation- exchange described above (4.4.3).

The procedure was checked by analysis of DORM-2 certified for AB 16.1 ±1.1 mg As/kg and TETRA 0.248 ± 0.054 mg As/kg; the obtained value agreed well with certified values AB 16.03 ± 0.4 mg As/kg, TETRA 0.256 ± 0.030 mg As/kg (mean ± SD, n=3).

Summary

The total arsenic concentrations found in the frog legs samples ranged between 0.15-0.46 mgAs/kg. Moisture content was 76% and the extraction efficiency was about 31 %. The major extractable arsenic species were As^{V} accounting 36% and DMA 16% from water soluble arsenicals.

Surprisingly in case of Thai frogs from estuarine water was found, more than 90% of the water soluble arsenic was present in form of AB. Traces of AC and DMA were also detected. Estuarine frogs showed species distribution comparable found in marine fish. The extraction efficiency was 97%. Mean total arsenic concentration in muscle tissue of Thai frogs was 1.51 μgAs/g.

The study showed a possible effect of water arsenic concentrations on frogs arsenic concentrations and therefore on arsenic species pattern. In both cases TETRA was not detected.

6 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.

The overall objective of this thesis was to obtain 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 with the relative amounts depending on the extent of contact with air. Moreover the results showed that the amount of insoluble or recalcitrant arsenic dramatically increased in the decaying algae, these findings demand further investigation that will clarify the fate of arsenic cycling in marine systems. Two analytical tools were available to identify the unknown compound found in TFA fraction: HPLC/ICPMS and ESI/MS, however, this techniques were not powerfull enough without an appropriate clean up step.

Rice samples from Czech market were analysed in term of the risk assesment associated with 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.

In the investigation of deep vent mussel tissue were found the dominant arsenic species thio-arsenosugar-phosphate and iAs.

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 discussed in terms of iAs however organic arsenic may represent a potential risk and it should be noted that the metabolism of arsenolipids in human is little understood. The work indicate that a reasonable amount of arsenic in fish escolar is lipid bound.

The results presented in chapter "Arsenic speciation in amphibians" demonstrate the possibly influence of arsenic concentration in estuarine waters on arsenic species distribution in frogs. These samples collected in estuarine waters showed speciation distribution similar to marine fish. It is worth mentioning that obtained results differ from those published.

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7. LIST OF ABBREVIATIONS AND SYMBOLS

AAS	atomic absorbtion spectrometry
AB	arsenobetaine
AC	arsenocholine
AES	atomic emission spectrometry
AFS	atomic fluorescence spectrometry
CE	capilary electrophoresis
CRM	Certified Reference Materials
DHA	docosahexaenoic acid
DMA	dimethylarsinate
DMAE	dimethylarsenoethanol
DMPS	sodium 2, 3-dimercapto-1-propanesulfonate
EFSA	European Food Safety Agency
ESI MS	electrospray mass spectrometer
HG	hydride generation
HPLC	high-performance liquid chromatography
iAs	inorganic arsenic
ICPMS	indouctively coupled plasma
MA	methylarsonate
MeOH	methanol
m/z	mass charge to ratio
TETRA	tetramethylarsonium ion
TFA	trifluoric acetic acid
TMAO	trimethylarsine oxide
TLC	thin layer chromatography
TPP	thiamin pyrophosphate
PDH	pyruvate dehydrogenase
WHO	World Health Organization

8. LIST OF FIGURES

9. LIST OF TABLES

10. APENDIX

Summary of chromatographic systems used in this study

Chromatography	Column	Mobile phase
Anion-exchange	Hamilton PRP-X100 (250*4.1 mm)	20 mM $NH_4H_2PO_4$ pH 5.6
Anion-exchange	Hamilton PRP-X100 (250*4.1 mm)	10 mM malonic pH 5.6
Cation-exchange	Zorbax 300-SCX (150*4.6 mm)	10 mM pyridine pH 2.3
Cation-exchange	Chrompack Ionosphere 5C (3*100 mm)	10 mM pyridine pH 2.3
Reversed-phase	Atlantis C18 (150*4.6 mm)	20 mM $NH_4H_2PO_4$ pH 3
Reversed-phase	Atlantis C18 (150*4.6 mm)	*A: 20mM CH_3COONH_4 B: ethanol

* Gradient elution

Curriculum vitae

Education
2007-present: Doctoral study
Brno University of Technology, Faculty of Chemistry, Department of Food Science and Biotechnology
PhD thesis: Arsenic speciation analysis using HPLC-ICPMS
Supervisor: prof. Ing. Peter Simko, DrSc.

2002-2007: Master study
Brno university of Technology, Faculty of Chemistry, Department of Food Science and Biotechnology
Diploma thesis: RFLP-PCR the method for identification of yeast of genus *Saccharomyces*
2007: Year of graduation

1998-2002:
The Secondary Technical School of Chemistry, Analytical chemistry
Secondary-school Professional Activity: The detection of genetically modified soya by PCR

Research visits

April 2011
Granted by specific research grant
Host University: Karl Franzens University of Graz
Project: Metabolism of arsenic in amphibiants
Supervised by prof. Kevin A. Francesconi

Sep-Dec 2010
Scholarship: Ministry of Education Youth and Sports Czech Republic
Host University: Karl Franzens University of Graz
Project: Recalcitrant arsenic
Supervised by prof. Kevin A. Francesconi

Apr-Jun 2010
Granted by specific research grant
Host University: Karl Franzens University of Graz
Project: Arsenosugar degradation
Supervised by prof. Kevin A. Francesconi

Sep-Dec 2009
Scholarship: Ministry of Education Youth and Sports Czech Republic
Host University: Karl Franzens University of Graz
Project: Arsenosugar degradation
Supervised by prof. Kevin A. Francesconi

Oct 2008- Apr 2009
Scholarschip - program AKTION (Austrian Exchange Service)
Host University: Karl Franzens University of Graz
Project: Lipid soluble arsenical compounds and their determination by HPLC-ICPMS
Supervised by prof. Walter Goessler

Awards
2010: 3rd prize "Study on algal decomposition", Student conference, 2010, Brno, Czech Republic

2008: 7 months scholarship awarded from OAD (Austrian Exchange Service)

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) rok ? 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

Navratilova, J., Raber, G., Fisher, S., Francesconi, K.A. Degradation of arsenosugars in decomposing algae determined by HPLC/ICPMS. Poster presentation. European Winter Conference on Plasma Spechtrochemistry, 2011, Zaragoza, Spain.

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Teaching activities
Spring semester 2008: Assistent for Laboratory Practicum "Analytical Chemistry II"
