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PhD Dissertation 06/2010

Optimization of Analytical Methods for Simultaneous Separation of Hydrophilic and Hydrophobic Arsenic Species with Hydrophilic Interaction Chromatography (HILIC) with Parallel ESI-MS and ICPMS Detection; Mechanisms and Applications

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ISSN 1860-0387

Optimization of Analytical Methods for Simultaneous Separation of Hydrophilic and Hydrophobic Arsenic Species with Hydrophilic Interaction Chromatography (HILIC) with Parallel ESI-MS and ICP-MS Detection; Mechanisms and Applications

by the Faculty of

Chemistry and Physics

of

Technische Universität Bergakademie Freiberg

approved THESIS

to attain the Academic Degree of

doctor rerum naturalium

(Dr. rer. nat.)

submitted

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Defense date: 26th March, 2010 in Freiberg

Abstract

Arsenic occurs in the environment in a multiplicity of different inorganic and organic species, and numerous (until their detection) 'unexpected' organoarsenicals particularly in living organisms were proven in the recent years. Because of the different ecotoxicological and toxicological risk of the different arsenic species the determination of arsenic compounds has progressed with the development of hyphenated analytical methods, especially the coupling of HPLC with ICP-MS. However exist to today large problems with the simultaneous determination of polar and nonpolar arsenic species. Therefore, hydrophilic interaction chromatography (HILIC) - a novel separation technique - is used in this work, in order to eliminate these difficulties. The separation of various polar organoarsenicals in HILIC with different HILIC materials (zwitterionic and neutral) is optimized with ESI-MS and ICP-MS detection.

Separation mechanisms of arsenic species are explained by investigation of chromatographic parameters using zwitterionic HILIC (ZIC®-HILIC). According to chemical structure of arsenic species (methylated and aromatic) both hydrophilic partitioning and hydrogen bonding act on HILIC separation in a different degree obey good linear relationships between 'polarity vs. retention factor (k)' and 'hydrogen bonding vs. k', respectively.

Retention behaviour of arsenic species on ZIC®-HILIC is highly depending on buffer pH, due to electrostatic interactions between bifunctionalities and charged analytes. Buffer salts comprehensively impact on ion-pair formations with bifunctionalities on stationary phase and chemical interactions with counter analyte ions in eluent, leading to changes in retention of arsenic compounds in stationary phase and in mobile phase.

Additionally, by comparison of arsenic retention in zwitterionic HILIC (ZIC®-HILIC) and two neutral HILIC columns (Ascentis®-HILIC and Luna®-HILIC), retention behaviour of arsenic species in individual HILIC column is better understood. Hydrophilic partitioning is the predominated HILIC separation mechanism in all columns investigated. Functionalities on HILIC stationary phase created by different materials lead to extra separation functions. Additionally to the hydrophilic partitioning considerable electrostatic interactions occur in ZIC®-HILIC. A size exclusion effect is caused by a porous silica layer on fused-core stationary phase in Ascentis®-HILIC. The diol modification of the stationary phase in Luna®-HILIC leads to hydrophilic partitioning as dominating separation mechanism.

Compared with conventional analytical methods, arsenic species with a broad range of polarity and toxicity can simultaneously be analyzed using the novel HILIC separation method. The developed HILIC methodologies were applied to analyse arsenic species in marine tissues and algae. Hydrophobic arsenic species and arsenosugars were successfully separated and partially identified. Besides that, long chain arsenic lipids in marine tissues were also separated by this HILIC method.

Acknowledgement

This work was done under supervision of Prof. Dr. Matthias Otto (Technische Universität - Bergakademie Freiberg), Dr. habil. Jürgen Mattusch and Dr. Rainer Wennrich (both Department of Analytical Chemistry, Helmholtz Centre for Environmental Research – UFZ)

I would like to thank all colleagues in the Department of Analytical Chemistry, Helmholtz Centre for Environmental Research - UFZ, for their support and encouragement not only to my work but also to my life in Leipzig.

I appreciate all advice and guide for mathematical modeling from Dr. Albrecht Paschke and Mr. Dominik Wondrousch (Department of Ecological Chemistry, UFZ).

I also thank DAAD (German Academic Exchange Service) and Helmholtz Centre for Environmental Research-UFZ, Leipzig for my scholarship and for financial support.

Finally, to give my indefinite love and appreciation to my family, I want to dedicate the dissertation to my parents (Li Wang and Chaoming Xie) and my husband (Songquan Han) for their continuous love, support and encouragement.

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List of Abbreviations

Abbreviations	Name
AsB	trimethylarsonioacetate (arsenobetaine)
AsC	trimethylarsonioethanol (arsenocholine)
APCI	atmospheric pressure chemical ionization
CAD	charged aerosol detection
CE	capillary electrophoresis
Conc.	concentration
DMA	dimethylarsinate
DMF	dimethylformamide
DORM-2	dogfish muscle
ERLIC	electrostatic repulsion HILIC
ESI	electrospray ionization
GC	gas chromatography
HAc	acetic acid
HCOOH	formic acid
HILIC	hydrophilic interaction chromatography
HNO_3	nitric acid
H_2O_2	hydrogen peroxide
HPLC	high performance liquid chromatography
IC	ion chromatography
ICP	inductively coupled plasma

ICP-AES	ICP-atomic emission spectrometry
LD_{50}	lethal dose for mice, 50%
LOD	limit of detection
MeCN	acetonitrile
MeOH	methanol
MMA	methylarsonate
MS	mass spectrometry
MSD	mass selective detector
MW	molecular weight
m/z	mass-to-charge ratio
NH_4Ac	ammonium acetate
NH_4COOH	ammonium formate
NH_4OH	ammonium hydroxide
NMR	nuclear magnetic resonance
NP-C	normal phase chromatography
PAA	phenylarsonic acid
PAO	phenylarsine oxide
p-ASA	p-arsanilic acid
Rox	3-nitro-4-hydroxyphenylarsonic acid (Roxarsone)
RP-C	reversed-phase chromatography
RT	retention time
SIM	single (selected) ion monitoring
TFA	trifluoroacetic acid
THF	tetrahydrofuran

TIC	total ion counting
TMA	trimethylarsine
TMAO	trimethylarsine oxide
$TMAs^+$	tetramethylarsonium ion
TOF-MS	time-of-fight-MS
UV	ultraviolet
ZIC	zwitterionic

List of Symbols

Symbols	Meaning
C_o	concentration of analytes in octanol phase
C_w	concentration of analytes in aqueous phase
k	retention factor of analytes
pK_a ; pK_b	acid / base dissociation constants
$P_{o/w}$	partition coefficient between octanol phase and water phase
R^2	coefficient of determination
t_0	time of dead volume
t_R	retention time of analytes

1 Introduction

Speciation analysis of metals and metalloids belong to analytical, bioanalytical and environmental chemistry as well as toxicology of high significance. Due to the important role of different species of the elements in biological and geological compartments, analytical methods have to be developed to quantify and identify metal and metalloid species in their real existing forms, since the effects of the species of the elements can significantly differ. Because of the well-known toxicity of arsenic and the evidence of high differences in toxicity of its species, arsenic is in focus of analytical investigation for more than 20 years.

Inorganic and organic arsenic species can be found especially often in different environmental compartments, including biota [1]. Marine organisms are well-known to metabolize the more toxic inorganic arsenic (e.g. As (III), As (V)) to organic arsenic compounds (e.g. arsenobetaine (AsB), dimethylarsinate (DMA)), which are less toxic than the inorganic ones. In contrast, seaweed (algae) metabolizes DMA to arsenosugars and many other arsenic-containing compounds of unknown toxicity. A typical Japanese diet including 20-30 g of seaweed can provide 100-500 µg arsenic per day [2, 3]. Some of arsenic species are excreted without any changes; other ones remain in the human body in various forms with totally different toxicities due to further metabolism. It is undoubtedly true that the consumption of seafood can represent a certain risk in daily human life. Hence, more and more attention is being paid to the indirect influence of arsenic species on human health. Efficient analytical methods to separate and to determine arsenic species in marine organisms are necessary.

The identification of arsenic compounds in marine organisms has progressed in parallel with the development of analytical methods, especially the coupling of high-

performance liquid chromatography (HPLC) with inductively coupled plasma mass spectrometry (ICP-MS), which is characterized by a high sensitivity and selectivity. Most arsenic species were separated by anion or cation exchange chromatographic methods [4]. However, the combined technique was mainly applied for determination of arsenic compounds in hydrophilic matrices. Hydrophobic arsenic species in fish liver oil were analyzed by reversed-phase chromatography containing high methanol (90%) content in the mobile phase [5]. After that, until now analysis of hydrophobic arsenic species by HPLC parallel with element detection has not been processed. This leads to an underdeveloped knowledge of hydrophobic arsenic species in environment and biota.

Since hydrophilic interaction chromatography (HILIC) was first mentioned by Alpert in 1990 [6], it has emerged as an alternative choice to HPLC for applications dealing with hydrophilic and hydrophobic analytes. The new separation technique allows us to simultaneously analyze various arsenic species because of different advantages of HILIC separation mechanisms [7]. Of course, the simultaneous separation of hydrophilic and hydrophobic analytes on HILIC will be limited to analytes which are soluble in the water enriched layer of the stationary phase.

In essence, the separation on HILIC is strongly depending on partitioning of analytes between water-enriched interstitial pores of stationary phase and mobile phase containing a high percentage of organic solvents, such as acetonitrile (MeCN) or methanol. Besides this, minor separation functions can occur if silica material on HILIC stationary phase is modified by various functionalities. For example, on stationary phase of ZIC®-HILIC that is modified by bifunctionalities comprising ammonium group and sulfoalkyl group, electrostatic interactions between analytes and functionalities on stationary phase act on separation. Therefore, ZIC®-HILIC column makes possible the simultaneous separation of various polar compounds, charged analytes and uncharged analytes.

The neutral stationary phase of Luna®-HILIC column containing hydroxide functionalities cannot interact electrostatically with the analytes. Therefore, the retention is mainly performed by partitioning separation mechanisms [6, 8-16].

An additional size exclusion effect is caused by a porous silica layer on fused-core stationary phase in Ascentis®-HILIC.

With these different modifications of HILIC this method should be predestined for an efficient and fast separation of hydrophilic and hydrophobic arsenic compounds, such as organic arsenic species with a broad range of polarity and diverse chemical properties.

For element detection by ESI-MS and ICP-MS, the mobile phase composition in HILIC with a high content of organic solvent is a important parameter because the eluents used benefits an improved ESI-MS detection. In contrast, the ICP-MS detection is strongly disturbed by a high content of organic solvents, meaning that some modifications of the chromatography and the detection are necessary to reduce the influence of organic solvents, as illustrated by Hemström et al. [11] and Nygren et al. [17].

So far, HILIC separation mechanism is not completely explained because of the different effects which take place to the retention: partitioning, or hydrogen bonding, or both [16]. In the majority of reports [6, 12, 16] it is supposed that the primary retention mechanism in HILIC is hydrophilic partitioning of analytes between the eluent, which usually contains a high percentage of organic solvent, and the hydrophilic environment on the stationary phase, a water-enriched layer. Contrarily, Yoshida [18] suggested hydrogen bonding as the principal interaction mode in HILIC. Between two theories, Berthod *et al.* [19] agreed with a combination function of partitioning and hydrogen bonding. Recently, the research group of Yanagida [20] used linear regression of log k vs. log $P_{o/w}$, and log k vs. number of hydroxyl groups, respectively, to explain HILIC separation mechanisms. They found out that HILIC

separation is governed by a combined mode of hydrophilic partitioning and hydrogen bonding, and the ratio of hydrogen bonding is higher than hydrophilic partitioning.

Aim of this study was to separate simultaneously diverse polar organoarsenicals using different HILIC column coupled with detection by ESI-MS and ICP-MS. For this both the separation and the detection parameters were optimized.

Separation mechanisms of arsenic species are explained by investigation of chromatographic parameters in ZIC®-HILIC [7]. Additionally, two other HILIC columns with different properties were inserted in the investigations. By comparison of retention behaviour of arsenic species in zwitterionic HILIC (ZIC®-HILIC) and in the two neutral HILIC columns (Ascentis®-HILIC and Luna®-HILIC), the separation of arsenic species in individual HILIC column should be better understood. The optimized HILIC method should be applied for identification and quantification of arsenic species in hydrophilic and hydrophobic marine tissues and algae.

Successful separation of hydrophilic and hydrophobic arsenic species in HILIC coupled with simultaneous detection by ESI-MS and ICP-MS detectors could be an example and guide for further applications in analysis of environmental contaminations, especially hydrophobic substances.

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2 Theoretical Background

2.1 Arsenic

2.1.1 Chemical properties

Arsenic is a group 15 element on the periodic table along with nitrogen (N), phosphorus (P), antimony (Sb) and bismuth (Bi). The atomic mass of arsenic is 74.921 atomic mass units and its atomic number is 33 [1:pp.9-10]. Arsenic is considered as a metalloid due to intermediate chemical properties between metals and non-metals, as similar as boron (B), silicon (Si), germanium (Ge) antimony (Sb), tellurium (Te) and polonium (Po).

The most common oxidation states of arsenic are -3 (arsines), 0 (element), +3 (arsenites) and +5 (arsenates) [1:p.10, p.238; 2]. In environment, arsenic species mainly occur as +3 and +5. The common arsenic compounds in environment are inorganic forms, arsenite (As (III)), arsenate (As (V)), and organic forms (e.g. arsenobetaine (AsB), dimethylarsinate (DMA)) [3]. The chemical features of main arsenic species in environment are illustrated in Fig 2.1. In biota, these arsenic species can be metabolized from one form to another form. The transport pathways are displayed in Fig 2.1.

Organoarsenicals (chemical features are shown in Fig 2.2) are applied as animal feed additives, especially 3-nitro 4-hydroxphenylarsonic acid (roxarsone, Rox) [4], whilst some other organoarsenicals have been developed as chemical weapon [1:p. 25].

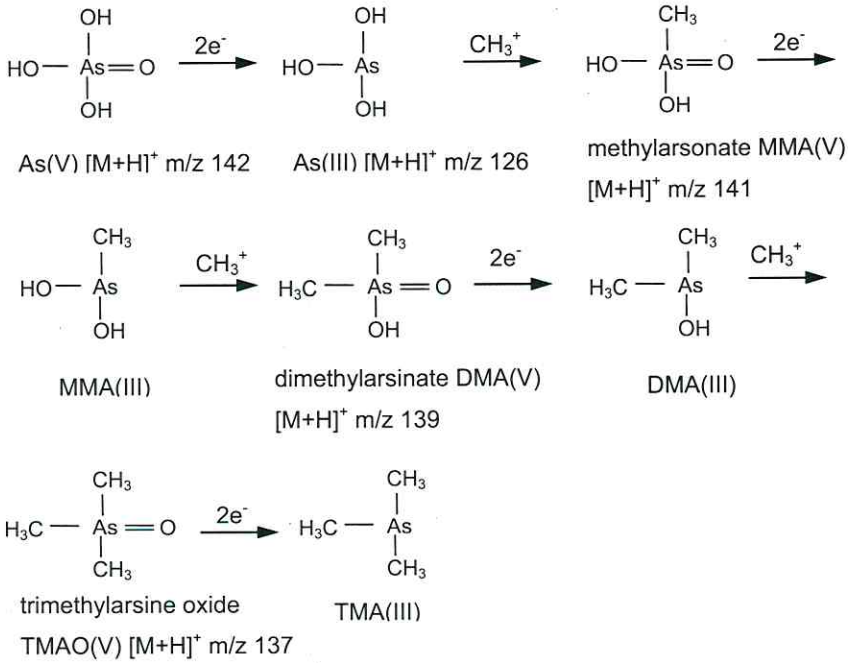


Fig 2.1 Arsenic chemical features and pathways of arsenic metabolism in human body [1: pp. 28-30, pp. 247-248]

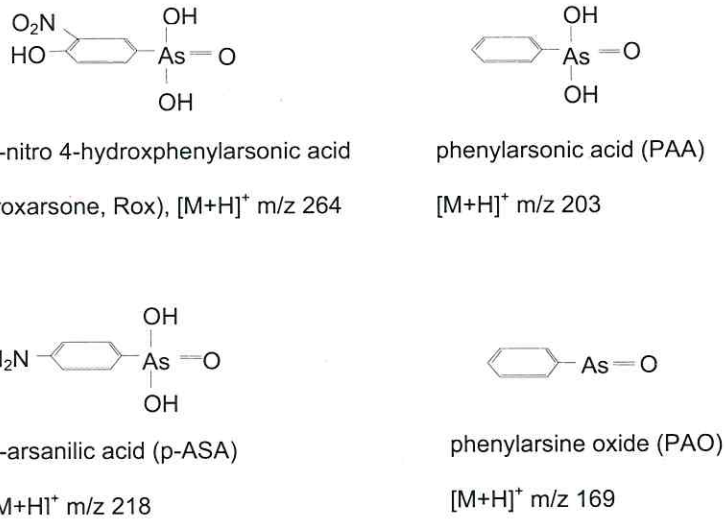
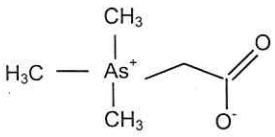


Fig 2.2 Chemical features of selected aromatic arsenic compounds

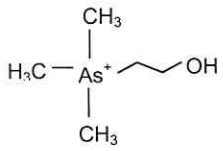
2.1.2 Arsenic in marine organisms

For most people, excluding sources of arsenic pollution and drinking water contamination, the diet is the major source of arsenic (~50 μg/day) [1: p.238; 5]. Seafood, such as fish and shellfish, tend to have the highest level of arsenic, primarily in organic forms (e.g. AsB and AsC), while arsenic is bond in sugars in algae. In algae, the main four arsenosugars are detected, glycerol sugar, phosphate sugar, sulfonate sugar and sulfate sugar [6: pp.238-239]. The chemical properties of arsenic species in marine organisms are presented in Fig 2.3(a) and (c). More recently, some new discoveries about arsenic containing hydrocarbons in marine tissues were reported by the group of K. A. Francesconi [7-10]. Long chain arsenolipids in fish oil samples were analysed. The chemical features of long chain arsenolipids are shown in Fig 2.3(b) as well.



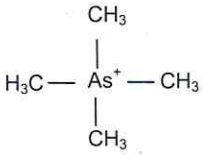
trimethylarsonioacetate

(Arsenobetaine, AsB) $[M+H]^+$ m/z 179



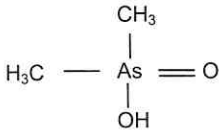
trimethylarsonioethanol

(Arsenocholine, AsC) $[M+H]^+$ m/z 165



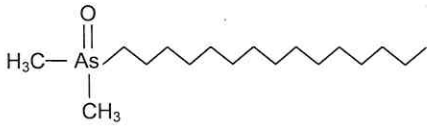
tetramethylarsonium ion ($TMAs^+$)

$[M+H]^+$ m/z 135

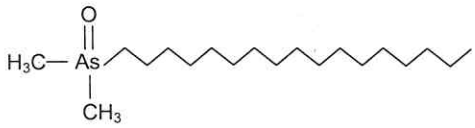


DMA $[M+H]^+$ m/z 139

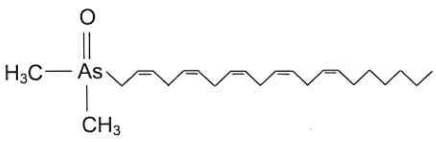
(a): Organoarsenicals in marine tissues



A: $[M+H]^+$ m/z 333

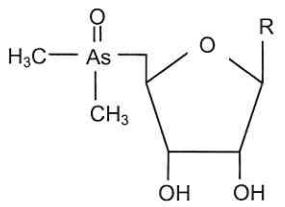


B: $[M+H]^+$ m/z 361



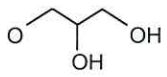
C: $[M+H]^+$ m/z 405

(b): Long chain arsenic species in marine tissues



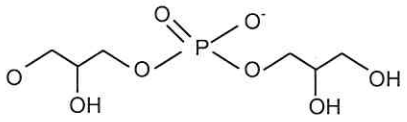
MW 237

R = 1; 2; 3; 4



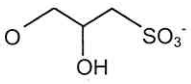
1: glycerol sugar

$[M+H]^+$ m/z 329



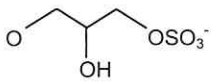
2: phosphate sugar

$[M+H]^+$ m/z 483



3: sulfonate sugar

$[M+H]^+$ m/z 393



4: sulfate sugar

$[M+H]^+$ m/z 409

(c): Arsenosugars in algae

Fig 2.3 Arsenic species extracted from marine organism

2.1.3 Arsenic toxicity

Generally, inorganic arsenic species are more toxic than organic arsenic species. For example, arsenate (LD_{50} (lethal dose for mice, 50%) = 20 mg kg^{-1}) and arsenite (LD_{50} = 14 mg kg^{-1}) are more poisonous than organic arsenic compounds (e.g. DMA (LD_{50} = 1200 mg kg^{-1}) and MMA (LD_{50} = 1800 mg kg^{-1})). Trivalent forms of arsenic, both inorganic and organic, are

more toxic than the pentavalent forms [1: p. 238]. LD_{50} in Table 2.1 shows inorganic arsenite (As(III)) is the most toxic form among arsenic species in environment. In contrast, so far, arsenosugars are not regarded as toxic due to unknown toxicity. Seaweed (algae) metabolizes DMA to arsenosugars and many other arsenic-containing compounds with unknown toxicity. Under acute exposure of arsenic, the lethal dose of ingested inorganic arsenic in adult humans is estimated to be 1-3 mg As kg^{-1} [1: p. 253; 11]. But many organ systems in the human body can be affected by chronic exposure to organic arsenic [1: p. 254]. It is significant and necessary to specify arsenic species, instead of determination of total arsenic concentration in environment, according to their varying toxicity. However, the analytical specification faces a broad range polarity of arsenic.

Table 2.1 Toxicity of arsenic species

Substances	LD_{50} [mg kg^{-1}, lethal dose for mice, 50%]
As (III)	14 [12]
As (V)	20 [12]
$TMAs^{+}$	890 [13]
DMA	1200 [13]
MMA	1800 [13]
TMAO	10600 [13]
AsC	>6500 [13]
AsB	>10000 [13]
Arsenosugars	unknown toxicity

2.1.4 Arsenic polarity

In environment, organic arsenic species exist as methylated forms, such as MMA and DMA. Aromatic arsenic species (e.g. Rox) are used as feed additives. Some organoarsenicals are the ingredients of chemical weapons. Marine tissues contain high level of organic arsenic (AsB, DMA). Algae contain an amount of arsenosugars. Generally, diverse forms of arsenic species in environment have varying polarity.

By means of commercial software *Physical Properties Pro*, the polarity of the most common arsenic species in environment was calculated and shown in Table 2.2. The calculation shows the various polarities of arsenic species under investigation. Due to overflow calculation of the large molecules, the relevant parameters of phosphate sugar, sulfonate sugar and sulfate sugar cannot be calculated by *Physical Properties Pro*.

Table 2.2 Molecular polarity of arsenic species

Substances	Molecular Polarity *
TMAO	0
PAO	1.09
AsC	3.32
AsB	4.12
DMA	6.00
PAA	6.35
p-ASA	7.76
MMA	8.35
Glycerol sugar	9.40
Rox	15.64

* Calculation by *Physical Properties Pro*.

2.2 Arsenic analysis

2.2.1 High Performance Liquid Chromatography (HPLC)

HPLC is an established analytical technique for several decades. The separation principles are based on five mechanisms: adsorption, distribution, ion exchange, exclusion and affinity [6: p.185]. The components of a liquid chromatograph are an eluent medium reservoir containing the mobile phase, a sample injection system, a separation column and different detectors [6: p.186]. The pump in HPLC setup transports the mobile phase with high pressure. The component of a HPLC unit is presented in Fig 2.4.

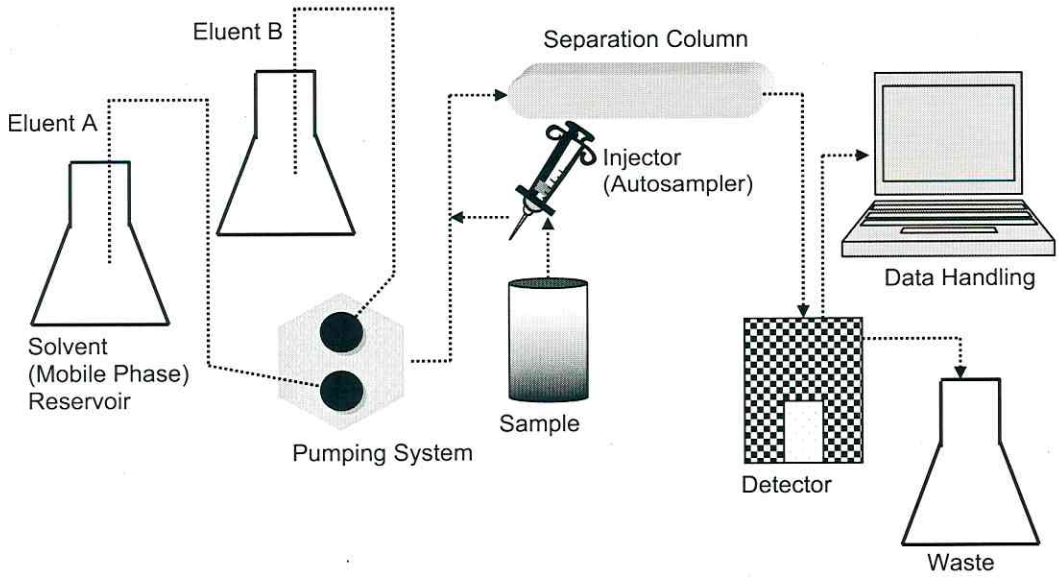


Fig 2.4 A schematic diagram of HPLC setup

2.2.1.1 Eluent

The solvents used for the eluent as mobile phase are stored in reservoirs. Dissolved gas such as nitrogen and oxygen, which can lead to the formation of bubbles, and thus to interference in the detector, as well as suspended matter, must be removed [6: p. 187]. Analytes can be separated through isocratic or gradient elution. In the isocratic method, separation is accomplished with eluent of constant composition, for example, 70% volume acetonitrile (MeCN) and 30% volume water. Better separation is often achieved by gradient elution.

In gradient elution, the composition of the eluent is constantly altered according to a particular scheme, for example, proportion of MeCN in Fig 2.5 is decreased from 80% (v/v) to 70% (v/v). With the gradient program, eluent polarity is usually varying [6: p. 187]. The achievement of gradient scheme is dependent on the pumping system.

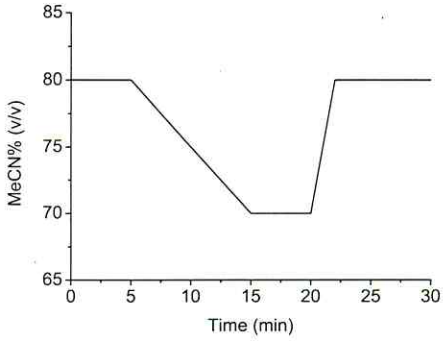


Fig 2.5 An example of gradient mobile phase elution

2.2.1.2 Pumping system

Today reciprocating pumps are primarily used in HPLC. Reciprocating pumps are operated as double piston pumps, which work with a phase shift of 180° to suppress

pulsation. Gradients can be produced on the low pressure or high pressure gradient. High pressure gradients provide more precisely composed gradients than low pressure gradients [6: p. 188].

2.2.1.3 Injection system

The sample injection system must allow volumes in the range of 5 to 500 µL to be introduced. Additionally, pressure has to be kept in the injection system. Thus, the injection system in HPLC is usually a sample loop for sample introduction [6: p. 188]. The sample can be injected into HPLC automatically or manually. Most of modern HPLC adopt autosamplers.

2.2.1.4 Separation column

Separations of arsenic species by HPLC mainly base on adsorption, ion-pair formations and size exclusion.

Adsorption chromatography is including normal phase chromatography and reversed-phase chromatography. Normal phase chromatography has polar stationary phase and non-polar mobile phase, while reversed-phase chromatography is on the opposite, i.e. non-polar stationary phase and polar mobile phase. Today chromatography on reversed-phase is accounted for about 75% of all applications of HPLC [6: p. 192].

Reversed-phase chromatography usually contains a silica material on stationary phase. The silica material is allowed to react with alkyl chlorosilanes in order to become hydrophilic, and receives siloxanes (Si-O-Si groups) as chemically bonded phases. The alkyl groups most frequently used in practice are C_{18} (n-octadecyl) and C_8 (n-octyl). The long chain hydrocarbon groups are aligned parallel to one another and perpendicular to the particle surface. The polar mobile phase of reserve phase chromatography is usually water and methanol (MeOH) or acetonitrile (MeCN). Only in pH range of 2 to 8 of the mobile phase the

siloxane phases are stable. Above pH 8, degradation or reorganization of packing material on stationary phase occurs [6: p. 193].

Normal phase chromatography contains polar functional groups based on silica, alumina or resin ion-exchanger carrier material on stationary phase. The polar functionalities can be diol, cyano, amino, dimethylamino and diamino groups. Less-polar mobile phase can be for example diethyl ether modified with n-hexane.

Ion chromatography serves to separate analytes using ion-exchangers [6: p. 198]. Ion chromatography contains an ion exchanger based on the surface of nonporous glass or polymer beads. The ion exchanger on stationary phase can be designed for varying analytes. A cation exchanger can be used to separate cations, while an anion exchanger is able to retain anions. For example, anion exchange chromatography was used with priority for the separation of MMA and DMA whilst cation exchange chromatography was preferentially applied for the separation of AsB, AsC and TMAO [14].

Size exclusion chromatography is based on the separation of molecules due to their differing size. All molecules over a particular size are excluded on silica material or polymer beads of a defined pore size. Size exclusion chromatography is significant for separation of high molecular species, such as proteins or polymers [6: p. 201].

In partition chromatography, separation of analytes is based on the distribution of analytes in two phases endowed with different polarity. Generally, eluent order of analytes is following the polarity, which is the main separation mechanism in HILIC (discussed in Chapter 2.2.5).

2.2.2 Electrospray ionization mass spectrometry (ESI-MS)

Mass spectrometry (MS) is based on the generation of gaseous ions from analyte molecules, the subsequent separation of these ions according to their mass-to-charge (m/z) ratio, and the detection of these ions. The resulting mass spectrum is a plot of the relative abundance of the ions produced as a function of the m/z ratio. MS consists of five parts, sample introduction, analyte ionization, mass analysis, ion detection, and data handling [6: p. 601].

2.2.2.1 Sample introduction systems

Sample introduction systems used in MS are generally insertion probes and on-line combinations with chromatography. The most important and versatile method for sample introduction is via chromatography. In the last decades, LC-MS and ionization techniques developed in relation to it have revolutionized MS and its applications [6: pp. 618-619].

2.2.2.2 Analyte ionization

An important distinction of analyte ionization technique applied in MS is made between hard and soft ionization. Most of ionization techniques in LC-MS are soft ionization techniques, generally resulting in little fragmentation and thus providing molecular mass information. In soft ionization methods primarily protonated (positive mode) and secondarily deprotonated (negative mode) ions are generated. In a hard technique a considerable amount of energy is transferred to the analyte ion during the ionization process, most likely resulting in subsequent unimolecular dissociation reactions. Electrospray ionization (ESI) is the typical example of a soft ionization technique compared to inductively coupled plasma (ICP) [6: pp. 610-611].

ESI transfers analyte molecules to ions, shown in Fig 2.6. The process of electrospray ionization includes the nebulization, the liquid stream into an aerosol of highly charged droplets, and the ionization of the analyte molecules after the desolvation of the charged droplets. ESI forms multiply charged droplets containing the analytes by applying a strong electric field and usually produces multiply charged ions of large molecules after solvent evaporation and Coulombic explosion. Briefly, the mechanism of ion formation is explained that the aerosol is charged to such an extent that the droplets virtually explode by Coulomb repulsion into sample droplets which eventually evaporate to yield highly charged molecules [6: p. 848].

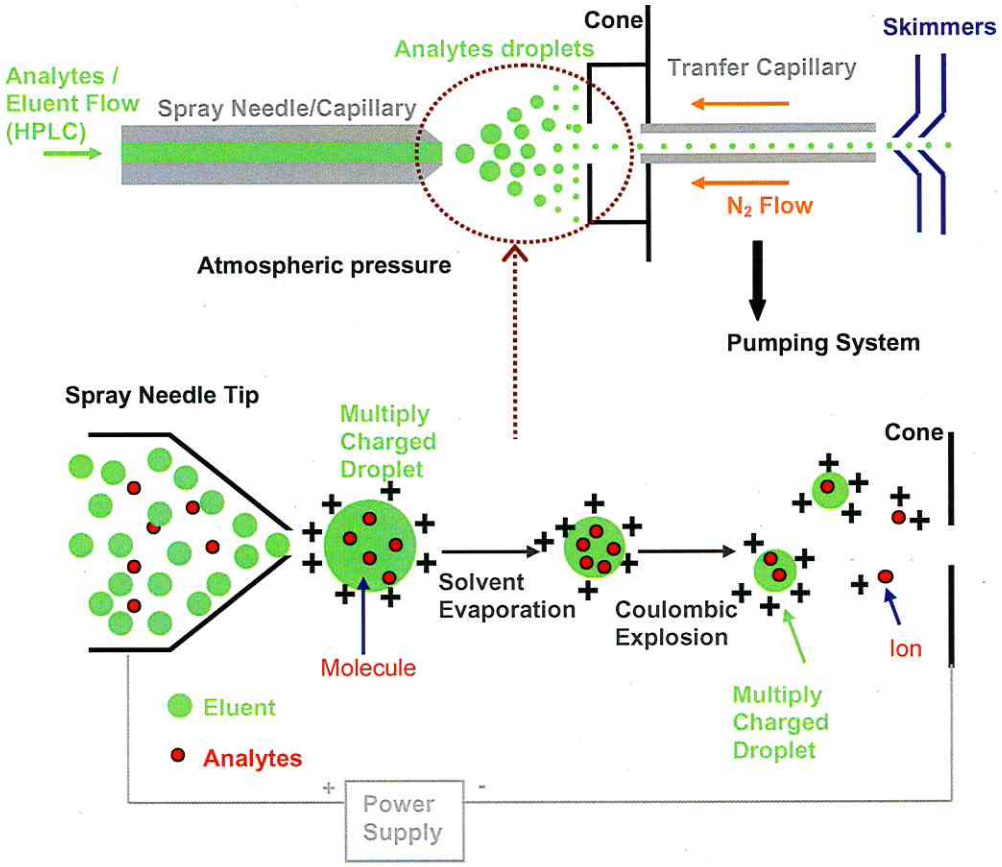


Fig 2.6 A schematic diagram of ESI-MS

2.2.2.3 Mass analysis

Different principles of mass analysis are applied in MS instruments: a combination of magnetic and electric sectors, quadrupole filters, iontraps, time-of-flight measurements, and ion-cyclotron resonance systems [6: p. 615].

Quadrupole mass analysis is primarily applied in our applications dealing with determination of arsenic species. A quadrupole mass filter is a device consisting of four stainless-steel hyperbolic or circular rods that are accurately positioned parallel in a radial array. Desolvated ions in ESI are introduced into a quadrupole mass filter for mass analysis by means of up to 20 V accelerating potential. The ions start to oscillate in a plane perpendicular to the rod length as they traverse through the quadrupole filter. The ions with stable m/z ratio (resonant ions) are transmitted towards the detector, while the ions with unstable m/z ratio (non-resonant ions) do not pass the mass filter [6: p. 607]. The schematic diagram is stated in Fig 2.7.

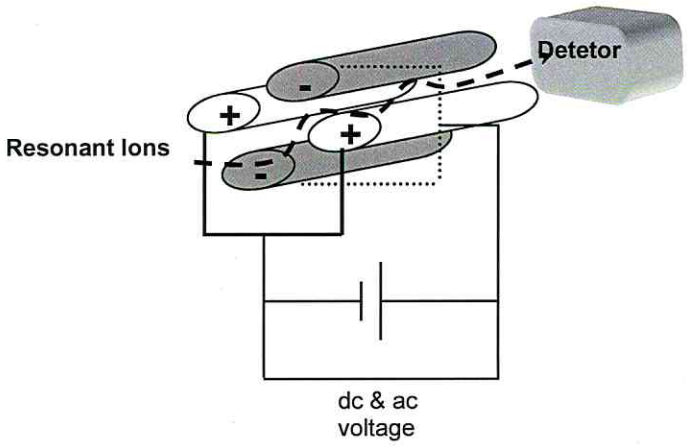


Fig 2.7 A schematic diagram of quadrupole filters

2.2.2.4 Ion detection

The most commonly used detectors in ESI-MS setup are the electron multiplier (continuous or discrete dynode type) [6: p. 523; p. 609]. The detection of ions by means of electron multiplier is based on the emission of secondary electrons, resulting from the collision of energetic particles at a suitable surface. The secondary electrons can be multiplied by consecutively striking subsequent surfaces. A discrete dynode multiplier comprise of 12-20 beryllium-copper dynodes, electrically connected through a resistive network. In modern ESI-MS system, the continuous dynode multipliers have more application. The continuous dynode or channel multiplier consists of a curved lead-doped funnel-shaped tube. The voltage applied between the ends of the tube creates a uniform field along the tube length. Secondary electrons are accelerated further into the tube to cause subsequent collisions with inner wall. The schematic diagram of channel electron multiplier is displayed in Fig 2.8. The current from the electron multiplier is collected by the data system [6: p. 609].

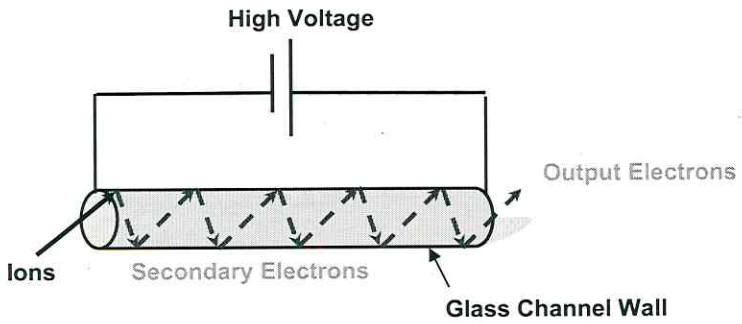


Fig 2.8 A schematic diagram of channel electron multiplier

2.2.2.5 Data handling

Data collection is mostly controlled by computer. Main data output is a chromatogram based on selected ion monitoring (SIM), total ion counting (TIC) or scanning a defined mass range, so that analyte can be identified and quantified [6: pp. 609-610]. TIC is used for quantification of molecules according to summation of the abundances of all m/z ratios in mass spectrum, while mass chromatogram (m/z of ions) is applied in the identification [6: p. 834]. Under scanning mode, mass spectrum is adopted to obtain molecular fragment information. Under selected ion monitoring (SIM) mode, detection sensitivity can be improved by increasing the dwell time on m/z particularly.

2.2.3 Inductively coupled plasma mass spectrometry (ICP-MS)

Inductively coupled plasma (ICP) is one kind of radiation sources in atomic emission spectrometry, which is based on the production and detection of line spectra emitted by de-excitation of electrons from the outer shells of atoms [6: p. 436; p. 441; p. 518].

Plasma that is an ionized gas having the same number of positive particles (ions) and negative particles (electrons) is usually generated in a torch by argon (Ar) [6: p. 441; pp. 518-519]. The torch contains three concentric tubes: an outer tube to confine and insulate the plasma, an intermediate tube which serves to accelerate the plasma gas which is introduced between the outer and intermediate tubes, and the injector tube for sample introduction [6: pp. 441-442].

Samples are usually introduced into the ICP in the form of liquid aerosols produced by pneumatic nebulizer. As the average droplet diameter is too large to ensure complete

volatilization in the plasma, a spray chamber is added to retain the large droplets. The overall sample introduction efficiency is a few % using such a type of nebulizer [6: p. 442].

ICP connected with low resolution quadrupole MS faces a crucial point that the ICP is operated both at atmospheric pressure and high temperature, whereas the MS requires high vacuum conditions and an ambient temperature. Therefore, an interface is necessary for reducing both pressure and the temperature. The interface includes two parts, sampler and skimmer. The tip of the first sampler cone must be located in the central channel of the ICP. The pressure is reduced between the sampler and the skimmer by means of a rotary pump. A molecular supersonic beam is obtained behind the sampler, which ends in a Mach disk. The tip of the second skimmer cone is located in the axis of the supersonic beam, slightly ahead of the Mach disk. The distance of two tips is less than 10 mm. The supersonic beam reduces significantly the temperature of ions because of the expansion of plasma [6: p. 519]. The schematic explanation of ICP-MS is indicated in Fig 2.9.

However, ICP-MS has disadvantages under the following phenomena: the signal overlap occurrence of isotopes of the same mass that belong to different elements, the formation of polyatomic species from analytes and argon. The sensitivity of ICP-MS and stability of plasma can be influenced strongly by the presence of organic solvent in mobile phase.

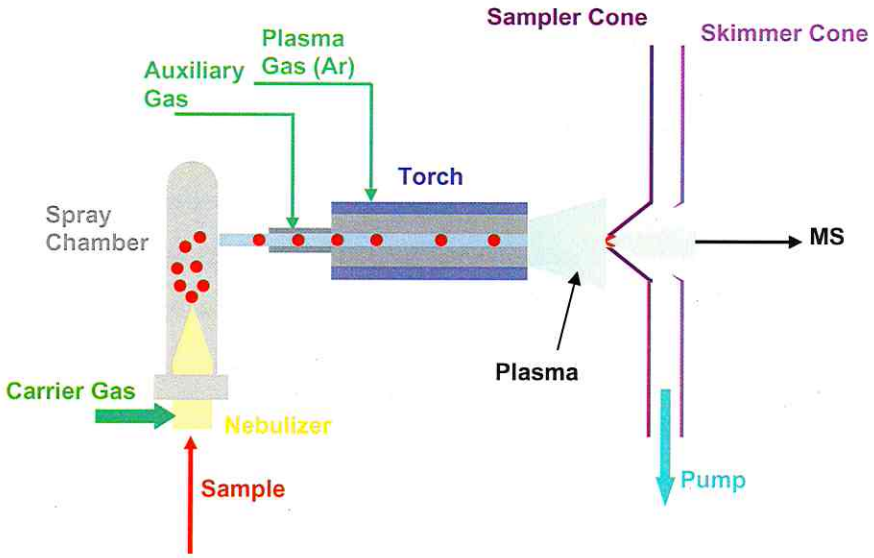


Fig 2.9 A schematic diagram of ICP-MS

2.2.4 Hyphenated techniques for identification of arsenic

Hyphenated techniques couple a separation technique with a spectroscopic detection technique. The advantage of a hyphenated technique is the additional dimensionality of the data obtained [6: p. 827].

Recently, the identification of arsenic compounds in marine organisms has progressed by the coupling of liquid chromatography (LC) with mass spectrometry (MS), especially the coupling of HPLC with ICP-MS, which is characterized by a high sensitivity and selectivity. HPLC-ESI-MS is increasingly used to confirm identification of arsenic species, and to provide structural information on novel arsenic compounds [15]. However, the hyphenated technique

was mainly applied in determination of arsenic compounds in hydrophilic matrices, e.g. plant extracts.

Most arsenic species in hydrophilic matrices were separated by anion or cation exchange chromatographic methods. Anion exchange chromatography was used with priority for the separation of MMA and DMA whilst cation exchange chromatography was preferentially applied for the separation of AsB, AsC and TMAO [14]. Different hydrophobic arsenic species in fish liver oil were analyzed by reversed-phase chromatography containing high methanol (90%) content in the mobile phase [16].

By combination of gas chromatography (GC) with MS hydrophobic arsenic species can also be determined [7-10]. GC-MS provides excellent separation and detection for volatile arsenicals. However, most of naturally-occurring arsenic species are non-volatile, leading to few applications of GC-MS in identification of arsenic species in environment. Combination of capillary zone electrophoresis with mass spectrometry (CE-MS) provides high selectivity but poor limit of detection (LOD). Thus, CE-MS is not suitable for analysis of arsenic species in natural samples [15].

With popularity of liquid chromatography, arsenic species were predominantly separated by reversed-phase, ion-exchange or by mix-mode of reversed-phase and ion-exchange chromatography [15, 17]. Separation of hydrophobic arsenic species is principally feasible in normal phase chromatography, whose hydrophobic mobile phase is appropriate for the retention of hydrophobic arsenic compounds [18]. In addition, organic solvent in mobile phase interferes the ICP-MS detection.

2.2.5 Hydrophilic Interaction Chromatography (HILIC)

The novel HILIC technique appeared firstly in Alpert's investigation in 1990 [19]. HILIC is a revision of normal phase chromatography. HILIC is characterized by a water-rich layer on polar stationary phase and a non-polar mobile phase. The water-rich layer on stationary phase is good for the retention of hydrophilic arsenic species. The typical property of HILIC is high content of organic modifier in the eluent [19]. This point makes the separation of hydrophobic arsenic species possible. A suitable gradient program leads to a shift of the hydrophobic properties of mobile phase to a more hydrophilic one. Hydrophilic species that maintained in water-rich layer on stationary phase can be eluted. Therefore, diverse polar arsenic species can be separated on HILIC. The property and separation mechanism of HILIC are displayed schematically in Fig 2.10.

HILIC stationary phase can be modified by different functionalities. Zwitterionic hydrophilic interaction chromatography (ZIC®-HILIC) in Fig 2.11(a) consists of bifunctionalities on stationary phase. On zwitterionic stationary phase, electrostatic interactions between bifunctionalities and oppositely charged analytes can occur. Thus, anions and cations can be separated on ZIC®-HILIC as well, besides separation of various polar analyte molecules. Neutral stationary phase of HILIC (Luna®-HILIC and Ascentis®-HILIC) has relatively simple separation action. For example, stationary phase of Luna®-HILIC in Fig 2.11(b) is modified by material containing hydroxide groups. Thus electrostatic interactions are minimized.

Although HILIC provides a lot of advantages for separation of arsenic species with a broad range of polarity, the high percentage of organic solvent in mobile phase is not beneficial for the stability of plasma in ICP-MS. In order to reduce the influence of organic solvent to plasma, the application of a special torch and the addition of oxygen to the aerosol are necessary. The special torch with a smaller inner diameter is normally applied for organic

solvents. Two detectors, ESI-MS and ICP-MS, ensure precise and synchronous element detection. ICP-MS is applied to 'hard' element detection. ESI-MS is used to confirm the structure of arsenic species by means of 'soft' element detection. The splitting of the flow into two detectors by a T-piece can also reduce the risk of instability of plasma.

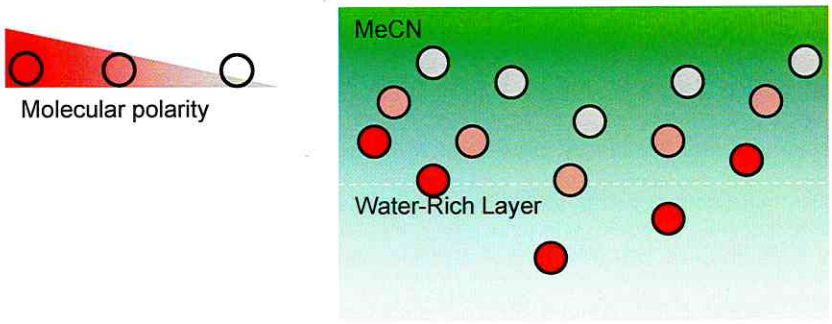
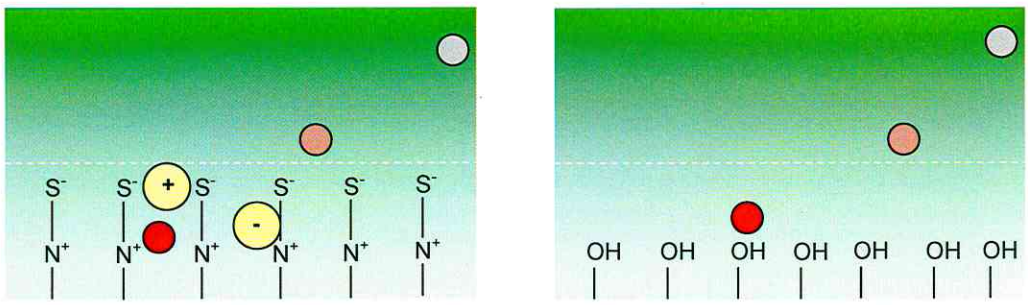


Fig 2.10 Features and separation mechanism of HILIC column



(a): ZIC®-HILIC (b) Luna®-HILIC

Fig 2.11 Stationary phase and separation mechanism of HILIC columns

(S^- means R-SO_3 groups; N^+ is quarternary ammonium group)

2.2.6 HILIC separation mechanisms

So far, the knowledge about separation motivation in HILIC is not definitely concluded. The separation mechanism could be dominated by different effects, by partitioning, or hydrogen bonding, or both of these [18].

As mentioned in Chapter 1 of this work (Introduction), majority reports [19-21] stated that the primary retention mechanism in HILIC is hydrophilic partitioning of analytes between the eluent, which usually contains a high percentage of organic solvent, and the hydrophilic environment on the stationary phase, a water-enriched layer. Contrarily, Yoshida [22] suggested hydrogen bonding as the principal interaction mode in HILIC. Between two theories, Berthod *et al.* [23] proposed a combination of partitioning and hydrogen bonding for retention. Recently, Yanagida *et al.* [24] correlates log k vs. log $P_{o/w}$, and log k vs. number of hydroxyl groups (k is retention factor of analytes, $P_{o/w}$ is octanol-water partition coefficient of analytes) to descibe HILIC separation mechanisms. For the analytes under study they found that the retention was attributed to a complex mode of hydrophilic partitioning and hydrogen bonding, whereas hydrogen bonding was stronger than hydrophilic partitioning.

However, those researches only considered the HILIC separation mechanisms in pure aqueous phase but neglected the influence of the eluent containing high content of organic modifier (>70%). Considering effects from high content of organic solvent on chemical features of analytes and buffer system in eluent, separation mechanisms of analytes on HILIC column should be optimized in hydro-organic phase rather than by simple description in pure aqueous phase. Proton ions of buffer solution in hydro-organic phase differ from active hydrogen ions in aqueous phase, change of some chemical features of analytes as well, such as pK_a. Therefore, mathematic models for optimization of proton capability of buffer solution,

pK_a and dissociation rate of analytes, which are used to investigate separation mechanisms in chromatography containing organic solvent eluent, should be considered [25-31].

2.2.7 HILIC applications

Since the introduction of HILIC in chromatography the research and publications are growing up, particularly in the last 5 years [18]. One aspect is the current challenge in environmental and biochemical research. Another aspect is that more types of HILIC columns are commercially available providing more research interests [18], e.g. ZIC®-HILIC, Luna®-HILIC and Ascentis®-HILIC. Until 2006, plenty of HILIC applications focus on biology, pharmacy and life science [18]. The separation of metalloid and metal species by HILIC in environmental samples, especially marine environment, was not performed up to 2006. Relevant literature published since this time is referred to in Table 2.3.

For the determination of arsenic species characterized by a broad range of hydrophilicity (hydrophobicity) the hyphenated technique of HILIC separation with simultaneous ESI-MS and ICP-MS detection seems to be a suitable methodology. ESI-MS is utilized for structure confirmation and element detection (m/z 75 (As), m/z 91 (AsO) under high fragmentor voltage (400 V), while ICP-MS is used for arsenic ions determination (m/z 75 (As)). The analysis of arsenic species by hyphenated techniques is schematically displayed in Fig 2.12. However, the determination of arsenic species in HILIC column faces some technical issues. Organic solvent in eluent can influence the stability of plasma in ICP-MS. Thus, μ-HPLC with low flow rate is needed. That means that most of experiments were performed with flow rates between 50 and 500 μL min^{-1}. The splitter was used to adjust the flow between ESI-MS and ICP-MS, in order to reach the goal of simultaneous detection and reduce organic solvent introduced in the ICP-MS. Additional optional gas (oxygen) and a

special torch with smaller inner diameter that is applied typically in organic solvents in ICP-MS has to be used to minimize the instability risk of plasma in the presence of organic solvent. A disadvantage is that oxygen negatively affects the sensitivity of ICP-MS due to reduced plasma energy.

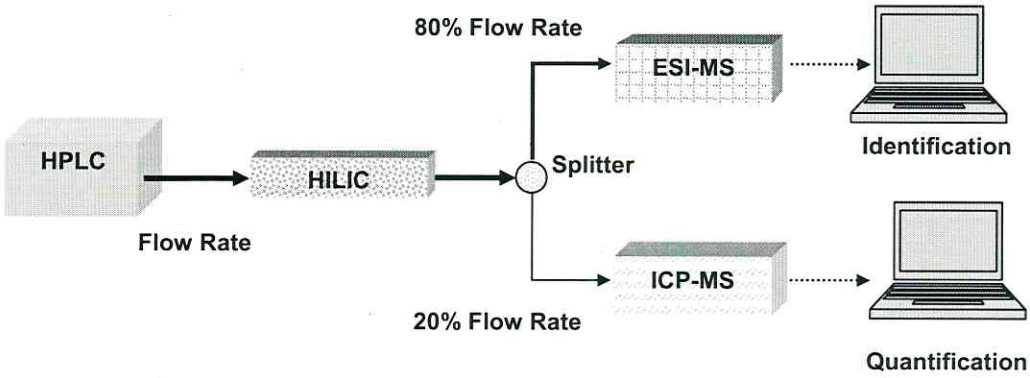


Fig 2.12 Hyphenated technique for analysis of arsenic species in the investigation

Table 2.3 Selected HILIC applications from 2006 to 2009

Authors	Year	HILIC columns	Stationary phase	Mobile phase	Detectors	Applications
M. Q. T. Tran *et al.* [32]	2010	ZIC®-HILIC (150*2.1 mm id, particle size 5 µm, SeQuant, Sweden)	Zwitterionic stationary phase	70% dimethylformamide (DMF and NH_4Ac 20 mM	ICP-MS	Platinum species in human cells
L. Nováková *et al.* [33]	2009	Obelisk N and R (100*3.2 mm, particle size 5 µm, Sielc, IL, USA); Luna HILIC (100*3.0 mm, particle size 3 µm, Phenomenex, Prague, Czech Republic)	Obelisk N and R: zwitterionic; Luna HILIC: diol	MeCN: NH_4Ac (75 mM, pH 4.2) =15:85 (v/v)	Charged aerosol detection (CAD)	Ascorbic acid / dehydroascorbic acid
J. Krenkova *et al.* [34]	2009	PolyGlycoplex (100 mm*150 µm I. D., Columbia, MD, USA)	Polysuccinimide coated silica	Formic acid (HCOOH): H_2O or MeCN= 0.1: 99.9 (v/v)	ESI TOF-MS	Glycans
J. Chen *et al.* [35]	2009	ACQUITY HILIC (2.1*50 mm, particle size 1.7 µm, Waters, Ireland)	Unmodified silica gel	Gradient: A: 100 mM NH_4COOH + 0.1% HCOOH; B: MeCN	TOF-MS	urinary metabolites

Y. Z. Yang *et al.* [36]	2009	TSK gel Amide-80 column (50*2.0 mm, particle size 3 µm, Tosoh, Japan); Zorbax NH_2 (150*0.3 mm, particle size 5 µm sorbent, Agilent Technologies); Zorbax C18 (150*0.3 mm, particle size 3.5 µm sorbent, Agilent Technologies, Wilmington, DE, USA).	amide phase/ charged amino phase	Gradient: A: 90% MeCN with 5 mM or 10 mM NH_4COOH / 100% MeCN; B: 5-20 mM NH_4COOH	ESI-MS	Peptides / protein digests
Y. Wang *et al.* [37]	2008	TSK gel Amide-80 (150*2.0 mm, particle size 3 µm; 10*2.0 mm., particle size 3 µm, Tosoh, Japan)	amide phase	Gradient: A: MeCN; B: 1% HCOOH	TOF-MS	Saponins
N. H. Schebb *et al.* [38]	2008	ZIC®-HILIC (150*2.1 mm id, particle size 3.5 µm, SeQuant, Sweden)	Zwitterionic stationary phase	Isocratic: MeCN: 10 mM NH_4COOH= 75:35 (v/v)	ESI-MS	cell lysate acetylcholine
Y. Wang *et al.* [39]	2008	Atlantis HILIC Silica, (150*2.1 mm; 10*2.1 mm particle size 3 µm, Waters, MA, USA)	Silica	Gradient: A: MeCN, B: 100 mM NH_4COOH	UV/ ESI-MS	Nucleotides, pharmacy, organic chemical compounds

M. Dernovics *et al.* [40]	2008	TSK gel Amide-80 (250*4.6 mm, particle size 5 µm, Tosoh Biosciences, Germany)	amide phase	Gradient: trifluoroacetic acid (TFA) in MeCN	ESI-MS/ICP-MS	Selenium species
Y. Nygren *et al.* [41]	2008	ZIC®-HILIC(150*2.1 mm id, particle size 3.5 µm, SeQuant, Sweden)	Zwitterionic stationary phase	Gradient: 70/30 DMF or MeCN/20 mM NH_4Ac	ICP-MS/ESI-MS	Platinum species
D. Nezirevic *et al.* [42]	2007	ZIC®-HILIC (150*2.1 mm id, particle size 3.5 µm SeQuant, Sweden)	Zwitterionic stationary phase	Isocratic: MeCN: NH_4Ac (0.1 M, pH 4.5)= 82:18 (v/v)	ESI-MS	Aminohydroxyphenylalanines (AHP)
M. Godejohann [43]	2007	Supersphere Si (250*4 mm, particle size 4 µm, Trentec, Gerlingen, Germany)	Silica	100 mM NH_4OH: MeCN with 0.2% HCOOH = 10: 90 (v/v)	Nuclear magnetic resonance (NMR)–MS	Urine samples
P. Ptácek *et al.* [44]	2007	Discovery HS F5 (50*4 mm, particle size 3 µm, Supelco, Bellefonte, PA, USA)	pentafluorophenylpropylsilica	methanol (MeOH): 10 mM NH_4Ac =90:10 (v/v)	ESI-MS	phenylephrine

K. M. Peru *et al.* [45]	2006	Altima™ HP hydrophilic interaction column (150*2.1 mm, particle size 3 µm, Alltech Associates, Deerfield, IL, USA)	Spherical coated Silica	Isocratic: 65% A: 90/10 (v/v) MeCN/water + 0.1% HCOOH; 35% B: 90/10 (v/v) water/MeCN + 0.1% HCOOH	ESI/ (atmospheric pressure chemical ionization) APCI-MS	spectinomycin / lincomycin
W. Jiang *et al.* [46]	2006	KSpolyMPC HILIC (150*4.6 mm, Hopedale, MA,USA)	Zwitterionic stationary phase	Isocratic: MeCN: NH_4Ac = 60:40 (v/v)	UV	Peptides
Q. Song *et al.* [47]	2006	BETASIL silica column (50*3.0 mm, particle size 5 µm, Keystone Scientific, Bellefonte, PA, USA)	Bare silica	Gradient: MeCN: water: HCOOH= 95:5:0.1	ESI-MS	Omeprazole in Human plasma
Y. Xuan *et al.* [21]	2006	ZIC®-HILIC (150*1.0 mm id, particle size 3.5 µm SeQuant, Sweden)	Zwitterionic stationary phase	Gradient: MeCN: NH_4Ac (10 mM pH 7.3)= 90:10	ICP-MS/ESI-MS	Phytosiderophores and their metal complexes

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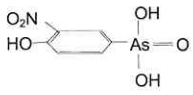
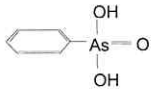
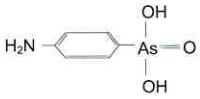
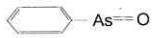
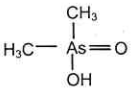
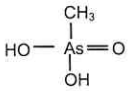
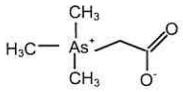
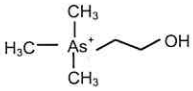
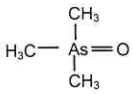
3 Experimental

3.1 Reagents and chemicals

All solutions were prepared with de-ionized water (Milli-Q, Millipore). Organoarsenical standard solutions (3-nitro-4-hydroxyphenylarsonic acid (Roxarsone, Rox, ICN Biomedicals Inc., Aurora, Ohio, USA); phenylarsonic acid (PAA, TCI Europe nv, Zwijindrecht, Belgium); p-arsanilic acid (p-ASA, SIGMA); phenylarsine oxide (PAO, SIGMA); dimethylarsinate (DMA, SIGMA); methylarsonate (MMA, Luxembourg Industries (PAMOL) LTD, Tel-Aviv, Israel); trimethylarsonioacetate (Arsenobetaine, AsB, BCR 626®, Fluka); trimethylarsonioethanol (Arsenocholine, AsC) and trimethylarsine oxide (TMAO) - kindly delivered by the group of Kevin. A. Francesconi, Karl-Franzens University Graz, Austria - were diluted by the mobile phase consisting of 80-90% acetonitrile (MeCN) and 20-10% ammonium acetate (NH_4Ac) 125 mM pH 8.3 (v/v) or ammonium formate (NH_4COOH) 100 mM pH 8 (v/v). The structure and concentration of each arsenic species are shown in Table 3.1.

The chemical reagents (analytical grade p.a.) were used during the study as follows: NH_4Ac (MERCK), MeCN (MERCK), ammonium hydroxide (NH_4OH; MERCK), acetic acid (HAc; MERCK), NH_4COOH (MERCK), formic acid (HCOOH; Fluka), hydrogen peroxide (H_2O_2; MERCK) and nitric acid (HNO_3; MERCK). The pH value was adjusted using NH_4OH, HAc or HCOOH. Standard buffer solution (pH 6.865 and pH 9.180) for pH meter calibration was purchased from Riedel-de Haen®, Germany.

Table 3.1 Names, structures, molecular weights (MW) and concentrations of the investigated standard solutions of organoarsenicals

Name	Structure	MW	Concentration of standard solutions (μg L^{-1})
3-nitro-4-hydroxyphenylarsonic acid (Roxarsone, Rox)		263	2000
phenylarsonic acid (PAA)		202	1000
p-arsanilic acid (p-ASA)		217	1000
phenylarsine oxide (PAO)		168	1000
dimethylarsinate (DMA)		138	2000
methylarsonate (MMA)		140	2000
trimethylarsonioacetate (Arsenobetaine, AsB)		179	10
trimethylarsonioethanol (Arsenocholine, AsC)		165	10
trimethylarsine oxide (TMAO)		136	10

3.2 Instruments

A μ-flow liquid chromatographic system was used, consisting of a degasser, an autosampler, a binary gradient pump and an ESI MSD (G1946 B and 6130) (Agilent Technologies). The mass selective detector (MSD) was used in the positive ionization mode. The scanning mode is used to identify fragments of the compounds, and single ion monitoring (SIM) was applied to improve the sensitivity. The analytical HILIC columns, ZIC®-HILIC (50*2.1 mm id, particle size 3.5 μm; 150*2.1 mm id, particle size 3.5 μm, SeQuant,), Ascentis®-HILIC (100*2.1 mm id, particle size 2.7 μm, Sigma Aldrich), Luna®-HILIC (150*2.0 mm id, particle size 3.5 μm, Phenomenex) were applied. An ICP-MS 7500ce (Agilent Technologies, USA) was performed for arsenic selective detection at m/z 75. A flow splitter (series 600, ASI company, USA), which separated flow rate by a ratio of 1:4, connected the LC with the two detectors ICP-MS and ESI-MS simultaneously. The optimized parameters of HPLC, ESI-MS and ICP-MS are listed in Table 3.2.

Microwave system (Multiwave, Perkin Elmer) was used for sample digestions. ICP-MS (ELAN® DRC-e, Perkin Elmer) or ICP-atomic emission spectrometry (ICP-AES, CIROS, SPECTRO A.I., Germany) were performed for determination of total arsenic concentration in samples. The pH measurement (pH 539, WTW, Germany) was accomplished by microprocessor pH meter.

Table 3.2 HPLC & ESI-MS & ICP-MS parameters

Chromatography parameters	
Columns	ZIC®-HILIC (SeQuant), 50*2.1 mm id, 3.5 µm; 150*2.1 mm id, 3.5 µm Ascentis®-HILIC (Sigma Aldrich), 100*2.1 mm id, 2.7 µm Luna®-HILIC (Phenomenex), 150*2.0 mm id, 3.5 µm
Mobile phases	Isocratic or gradient: 50%-90% (v/v) MeCN and appropriate NH_4Ac or NH_4COOH with varied concentration and pH. Time table of mobile phase is described in detail in each chromatography
Flow rate	50-500 µL min^{-1}
Injection volume	2-8 µL
ESI-MS parameters	
Drying gas flow	10.0 L min^{-1}
Nebulizer gas pressure	30 psig
Drying gas temperature	350°C
Capillary voltage	Positive 1500 V -2500 V
Fragmentor voltage	70 V to 400 V (SIM, Scanning)
ICP-MS parameters	
RF power	1600 W
Carrier gas	0.4 L min^{-1}
Makeup gas	0.11 L min^{-1}
Optional gas Ar/O_2(20%)	9.0 %
Nebulizer	Microconcentric + Scott-type spray chamber

3.3 Mass spectra of arsenic species

Mass spectra of arsenic species are directly analyzed by flow injection analysis (FIA) of ESI-MS positive ionization mode. And then, each single arsenic compound is separated by HILIC column with detection by ESI-MS under selected ion monitoring (SIM) positive ionization mode. Mass spectra of arsenic species present AsB (m/z 179, $[M+H]^+$), AsC (m/z 165 $[M+H]^+$), MMA (m/z 323, $[2M+MeCN+2H]^+$), DMA (m/z 139, $[M+H]^+$), TMAO (m/z 137, $[M+H]^+$), Rox (m/z 264, $[M+H]^+$), PAO (m/z 263, $[M+MeCN+3H_2O]^+$), PAA (m/z 203 $[M+H]^+$), p-ASA (m/z 218 $[M+H]^+$).

3.4 Samples

Dogfish muscle (DORM-2; NRC – CNRC) was used as certified standard reference material for recovery studies and optimization of HILIC separation. Aliquots of 0.2 g of the material were transferred into polyethylene tubes to which each 5 mL of mobile phase consisting of 90%-80% MeCN and 10%-20% NH_4COOH (100 mM, pH 8) or NH_4Ac (125 mM, pH 8.3) were added. Afterwards the samples were shaken over night at room temperature. The homogenate was centrifuged (5000rpm, 5 min). The supernatant (8 µL) was injected into the chromatographic system without any further sample preparation.

Different marine food products were purchased from a local supermarket. Cod eggs food and cod liver oil (aliquots of 1.0 g) were directly used for further sample preparation. Cod liver canned food was lyophilized (LOC-1M, ALPHA, CHRIST, Germany). Aliquots of 0.3 g of the lyophilized material were dissolved in mobile phase (5 mL) ultrasonically assisted (Bandelin Sonopuls GM200, Germany).

Standards of arsenosugars (glycerol sugar, phosphate sugar, sulfonate sugar and sulfate sugar kindly delivered by Kevin A. Francesconi (Karl-Franzens University Graz, Austria)) with a concentration of 200 µg L^{-1} were used as reference. Aliquots (0.2 g) of the different algae materials, e.g. *Hijiki* algae (kindly provided by T. Kaise, Tokyo University of Pharmacy and Life Science), were dissolved in 5 mL mobile phase by shaking overnight. A volume of 8 µL of the supernatant was directly used for analysis.

The total concentration of arsenic was determined by ICP mass spectrometry after microwave assisted digestion as follows: about 0.3 g of the material was weighted in 25 mL quartz vessels; afterwards 1.5 mL H_2O_2 (30 % v/v) and 4 mL HNO_3 (60 % v/v) were added and reacted overnight. The digestion was performed using a microwave device (Multiwave, Perkin Elmer) employing the following program: 0 min at 100 W; 5 min at 600 W; 10 min at 1000 W; 15 min cooling down. The resulting solution was diluted to 15 mL with deionized water (Direct-Q™ 5; Millipore) and afterwards centrifuged (5000 rpm, 5 min). Every sample preparation was repeated twice.

3.5 Equations

The retention factor (k) of each analyte in HILIC column is calculated in accordance with the following Eq. (2.1):

$$k = \frac{(t_R - t_0)}{t_0} \tag{2.1}$$

where t_R is the retention time (min) of analytes and t_0 is the time (min) of dead volume.

The partition coefficient *P* is defined as the ratio of the equilibrium concentrations of a dissolved substance in a two-phase system consisting of two largely immiscible solvents [16]. In the case of octanol and water:

$$P_{o/w} = \frac{C_o}{C_w} \tag{2.2}$$

Here C_o is the concentration of analytes in octanol phase; C_w is the concentration of analytes in aqueous phase (water).

3.6 Software for calculation

The partitioning coefficient ($\log P_{o/w}$) of arsenic species was from *PhysProp* database (Physical Properties database of chemical compounds, Synopsys Scientific Systems Ltd, USA). Hydrogen bonding, polarity parameters, molecular volume and other chemical parameters used in the investigation were calculated by *Physical Properties Pro* (ChemSW®, USA), which are listed in the chromatographic parameters of Table 3.3. The detailed chemical parameters are displayed in each chapter. The inter- and inner-molecular interactions of arsenic are schematically expressed by *ChemBio3D Ultra 11.0* (CambridgeSoft®).

Table 3.3 Chemical parameters of arsenic species in the investigation

Compounds	Hydrogen bonding	Hydrogen bond acceptor	Hydrogen bond donor	Polarity	$\log P_{o/w}$	pK_a
PAO	2.39	0.59	0.03	1.09	2.44	none
PAA	17.921	1.40	0.54	6.35	0.06	3.4, 8.2 [1]
TMAO	0	0.61	0	0	2.32	(3.6) [2]
AsC	11.92	0.37	0.23	3.32	1.89	none
AsB	5.47	1.17	0	4.12	2.12	2.2 [2-4]
p-ASA	20.07	1.99	1.05	7.73	-0.88	2, 4.0, 8.9 [5]
MMA	23.27	1.44	0.51	8.35	1.23	3.6, 8.2 [2, 3]
DMA	16.18	1.04	0.25	6.00	1.77	6.2 [2-4]
Rox	27.18	3.02	1.76	15.64	-0.05	3.4, 6.4, 9.7 [6]

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4 Separation of organoarsenicals by means of zwitterionic hydrophilic interaction chromatography (ZIC®-HILIC) and parallel ICP - MS / ESI - MS detection

Publication: D. Xie, J. Mattusch, R. Wennrich. *Eng. Life Sci.* 8 (2008) 582-588

Abstract

An analytical scheme was developed for the separation and detection of organoarsenicals using a zwitterionic stationary phase of hydrophilic interaction chromatography (ZIC®-HILIC) coupled parallel with electrospray ionization mass spectrometry (ESI-MS) and inductively coupled plasma mass spectroscopy (ICP-MS). The optimization of separation and detection for organoarsenicals was mainly focused on the influence of the percentage of acetonitrile (MeCN) used as a major component of the mobile phase. Isocratic and gradient elution were applied by varying the MeCN percentage from 78% to 70% MeCN and 22% to 30% of an aqueous solution of ammonium acetate (125 mM NH_4Ac; pH 8.3) on ZIC®-HILIC column (150*2.1 mm id, 3.5 µm), to separate and successfully detect 9 organoarsenicals (3-nitro-4-hydroxyphenylarsonic acid (roxarsone, Rox), phenylarsonic acid (PAA), p-arsanilic acid (p-ASA), phenylarsine oxide (PAO), dimethylarsinate (DMA), methylarsonate (MMA), arsenobetaine (AsB), arsenocholine (AsC) and trimethylarsine oxide (TMAO)) within 45 min. All analyte solutions were prepared in the mobile phase. The flow rate of the mobile phase, the splitting ratio between ICP-MS and ESI-MS detection, and oxygen addition were adapted to ensure there was a stably burning inductively coupled plasma. Furthermore, the analytical method was evaluated by the identification and quantification of AsB in the reference material

DORM-2 (dogfish muscle) resulting in 95% recovery with respect to the AsB concentration in the extract.

Keywords: hydrophilic interaction chromatography, zwitterionic stationary phase, organoarsenicals, ICP-MS, ESI-MS

4.1 Introduction

Metal and metalloid speciation belongs to the areas of analytical / bioanalytical chemistry of high significance. Due to their important role in biological and geological compartments analytical methods have to be developed to quantify and identify metal and metalloid species in their real existing form.

Inorganic and organic arsenic species can be found especially often in different environmental compartments [1]. Marine organisms are known to metabolize the more toxic inorganic arsenic (e.g. As (III), As (V)) to organic arsenic compounds (e.g. AsB, DMA), which are less toxic than the inorganic ones. In contrast seaweed (algae) metabolizes dimethylarsinate to arsenosugars and many other arsenic-containing compounds of unknown toxicity. A typical Japanese diet including 20-30 g of seaweed can provide 100-500 µg of arsenic per day [2, 3]. Some of the arsenic species are excreted without any changes; other ones remain in the human body as various forms with totally different toxicities due to continuous metabolism. It is undoubted true that the consumption of seafood can represent a certain risk in daily human life. Hence, more and more attention is being paid to the indirect influence of arsenic species on human health. Therefore, analytical methods to separate and to determine arsenic species in marine organisms are necessary.

The identification of arsenic compounds in marine organisms has progressed in parallel with the development of analytical methods, especially the coupling of high-performance liquid chromatography (HPLC) with inductively coupled plasma mass spectrometry (ICP-MS), which is characterized by a high sensitivity and selectivity.

Most arsenic species were separated by anion or cation exchange chromatographic methods. Anion exchange chromatography was used with priority for the separation of MMA and DMA whilst cation exchange chromatography was preferentially applied for the separation of AsB, AsC and TMAO [4]. The first attempts to separate and detect hydrophobic arsenic species in fish liver oil by means of reversed-phase chromatography were undertaken using high methanol (90%) content in the mobile phase [5].

Since hydrophilic interaction chromatography (HILIC) was first mentioned by Alpert in 1990 [6], it has emerged as an alternative choice to HPLC for applications dealing with hydrophilic and hydrophobic analytes, especially in the last few years. In the field of arsenic speciation, HILIC chromatography can close a gap between reverse-phase and normal-phase chromatography. Because of the broad polarity range of arsenic species, the application of zwitterionic stationary phases (ZIC®-HILIC) should have some advantages for the separation of As species in comparison with conventional chromatographic methods. The main advantage of using this separation principle is that it should be possible to analyse both hydrophilic and hydrophobic analytes in one run. Of course, the simultaneous separation of hydrophilic and hydrophobic analytes on ZIC®-HILIC will be limited by analytes with a high degree of hydrophobic properties that are not retained on the stationary phase.

In essence, the separation on HILIC is based on the hydrophilic partitioning of the analytes between water-enriched interstitial pores of stationary phase and a mobile phase containing a high percentage of organic solvents such as acetonitrile or methanol. From the theory, the separation should be independent of pH, and low electrostatic interactions should

occur in the stationary phase. In the stationary phase of the ZIC®-HILIC column, electrostatic interactions become weak because of bifunctional groups which lead analytes to be attracted and repelled simultaneously once they reach the zwitterionic stationary phase. Therefore, hydrophilic partitioning dominates the retention behaviour of analytes on ZIC®-HILIC, which operates with a high content of organic solvent in the mobile phase and water as the eluting solvent. Meanwhile, because there is less buffer solution in the mobile phase and a decrease in the dissociation of organic arsenic species investigated, the electrostatic interactions with buffer ions are diminished during the change of the pH value of the buffer, so the separation could be independent of pH value [6-14]. Therefore, this method should be predestined for an efficient and fast separation of hydrophilic and hydrophobic organic compounds. Organoarsenicals of interest that show a broad range of hydrophilicity and quite different pK_a values and charges are separated very well by this means. However, for the HILIC separation of peptide mixtures a dependence of retention behaviour and pH in the range of 3 to 8 was found [15]. The mobile phase composition in HILIC with a high content of organic solvent benefits from an improved ESI-MS detection. In contrast, the ICP-MS detection is strongly disturbed by the high organic content of the mobile phase used by HILIC, meaning that some modifications of the chromatography and the detection are necessary to reduce the influence of organic solvents, as illustrated by Nygren et al. [9] and Hemström et al. [16].

The aim of the present study was to attempt the separation and detection of several organoarsenicals as standard solutions by means of the new hydrophilic interaction chromatography (ZIC®-HILIC) coupled parallel with two mass selective detectors: ESI-MS and ICP-MS. These two detectors are necessary to quantify and identify arsenic-containing compounds by the arsenic mass m/z 75 in the "hard ionization" mode with the inductively coupled plasma on one hand and to identify the molecular structure of unknown arsenic species after "soft ionization" by electrospray ionization.

In future, the preliminary results could be an example and guide for further applications in real samples originating from marine organisms, or polluted samples, to check for and protect against arsenic contaminations under eco-toxicological and medical aspects. For this study 9 organoarsenicals were selected as listed in Table 4.1, to estimate the performance of the HILIC separation for this group of analytes. Besides arsenic species which can be detected in marine organisms, phenylarsonic acid derivatives were also involved in this chromatographic separation because of their occurrence as food additives and degradation products of chemical warfare.

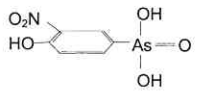
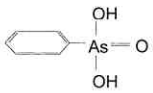
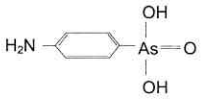
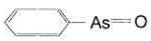
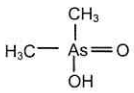
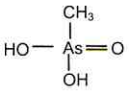
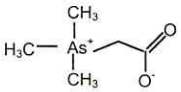
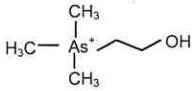
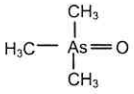
4.2 Experimental

4.2.1 Reagents and chemicals

All solutions were prepared with de-ionized water (Milli-Q, Millipore). Organoarsenical standard solutions (3-nitro-4-hydroxyphenylarsonic acid (Roxarsone, ICN Biomedicals Inc., Aurora, Ohio, USA); phenylarsonic acid (PAA, TCI Europe nv, Zwijindrecht, Belgium); p-arsanilic acid (p-ASA, SIGMA, USA); phenylarsine oxide (PAO, SIGMA, USA); dimethylarsinate (DMA, SIGMA, USA); methylarsonate (MMA, Luxembourg Industries (PAMOL) LTD, Tel-Aviv, Israel); trimethylarsonioacetate (Arsenobetaine, AsB, BCR 626®, Fluka, Switzerland); trimethylarsonioethanol (Arsenocholine, AsC) and trimethylarsine oxide (TMAO) kindly delivered by the group of Kevin. A. Francesconi, Karl-Franzens University Graz, Austria) were diluted by the mobile phase consisting of 80% MeCN and 20% ammonium acetate (NH_4Ac) 125 mM pH 8.3 (v/v). The structure and concentration of each arsenic species are shown in Table 4.1. The chemical reagents (analytical grade p.a.,

MERCK, Germany) were utilized during the study as follows: NH_4Ac, MeCN, ammonia solution (NH_4OH) and acetic acid (HAc). The pH value was adjusted using NH_4OH or HAc.

Table 4.1 Names, structures, molecular weights (MW) and concentrations of the investigated standard solutions of organoarsenicals

Name	Structure	MW	Concentration of standard solutions ($\mu g\ L^{-1}$)
3-nitro-4-hydroxyphenylarsonic acid (Roxarsone, Rox)		263	2000
phenylarsonic acid (PAA)		202	1000
p-arsanilic acid (p-ASA)		217	1000
phenylarsine oxide (PAO)		168	1000
dimethylarsinate (DMA)		138	2000
methylarsonate (MMA)		140	2000
trimethylarsonioacetate (Arsenobetaine, AsB)		179	10
trimethylarsonioethanol (Arsenocholine, AsC)		165	10
trimethylarsine oxide (TMAO)		136	10

4.2.2 Samples

A standard reference material certified for AsB, DORM-2, purchased from the National Research Council of Canada (Institute for Environmental Chemistry, Ottawa, Canada) was used for recovery studies. An aliquot of 0.2 g of this material was weighed and transferred into a polyethylene tube. 2 mL of the mobile phase (80% MeCN, 20% aqueous NH_4Ac solution) was added and afterwards the sample was shaken overnight at room temperature. The homogenate was centrifuged (5000 rpm, 5 min), and 2 µL of the supernatant was injected into the chromatographic system directly without further sample preparation. For comparison, the total arsenic concentration in DORM-2 was determined by ICP-atomic emission spectrometry (CIROS, SPECTRO A.I., Germany).

4.2.3 Instruments

A µ-flow liquid chromatographic system was used, consisting of a degasser, an autosampler, a binary gradient pump and an ESI MSD (G1946 B and 6130) (Agilent Technologies, USA). The mass selective detector (MSD) was used in the positive ionization mode. Single ion monitoring (SIM) was applied to improve the sensitivity and the scanning mode to identify fragments of the compounds. The two analytical HILIC columns (50*2.1 mm id, particle size 3.5 µm; 150*2.1 mm id, particle size 3.5 µm) were purchased from ZIC®-HILIC (SeQuant, Umea, Sweden). An ICP-MS 7500ce (Agilent Technologies, USA) was performed for arsenic selective detection at m/z 75. A flow splitter (series 600, ASI company, USA), which separated flow rate by a ratio of 1:4, connected the LC with the two detectors ICP-MS and

ESI-MS simultaneously. The optimized parameters of HPLC, ESI-MS and ICP-MS are listed in Table 4.2.

Table 4.2 HPLC & ESI-MS & ICP-MS parameters

Chromatography parameters	
Columns	ZIC®-HILIC 50*2.1 mm id, 3.5 µm or 150*2.1 mm id, 3.5 µm
Mobile phases	78 %-70 % MeCN and appropriate NH_4Ac 125 mM pH 8.3 (v/v)
Flow rate	100 µL min^{-1}
Injection volume	2 µL
ESI-MS parameters	
Drying gas flow	10.0 L min^{-1}
Nebulizer gas pressure	30 psig
Drying gas temperature	350°C
Capillary voltage	Positive 1500 V
Fragmentor voltage	70 V to 400 V (SIM)
ICP-MS parameters	
RF power	1600 W
Carrier gas	0.4 L min^{-1}
Makeup gas	0.11 L min^{-1}
Optional gas (Ar/O_2(20%))	9.0 %
Nebulizer	Microconcentric + Scott-type spray chamber

4.3 Results and discussion

In ZIC®-HILIC columns, the separation of analytes is achieved by the hydrophilic partitioning from the mobile phase, which usually contains a high percentage of organic modifier, into the hydrophilic environment on the stationary phase containing a water-enriched liquid layer. Additionally, electrostatic interactions can take place between analytes and stationary phase-covered zwitterionic material with positive and negative charged functional groups [10-14]. This allows hydrophilic, charged analytes, as well as uncharged compounds, to be separated under ZIC®-HILIC conditions. Besides the mobile phase buffer concentration, the most important factor influencing the retention behaviour in ZIC®-HILIC chromatography is the kind and content of the organic solvent in the mobile phase. Thus, in a first trial the effect of the mobile phase composition was studied using acetonitrile as an organic solvent.

4.3.1 Optimization of separation and detection of organoarsenicals by means of MeCN content variation

Preliminary optimization of the separation of four selected organoarsenicals (see below) was carried out based on an isocratic mobile phase composition of 70% MeCN and 30% NH_4Ac (125 mM, pH=8.3) to ensure significant hydrophilic interaction in ZIC®-HILIC columns [6, 10]. The suitability of the ratio between MeCN and NH_4Ac as the eluent was sought in the investigation. Under the condition of the initial separation, phenylarsine oxide (PAO, m/z 169), phenylarsonic acid (PAA, m/z 203), p-arsanilic acid (p-ASA, m/z 218) and roxarsone (Rox, m/z 264) were separated within 8 min and detected by ESI-MS (Fig 4.1A). The degree of hydrophilic interactions with the water-enriched stationary phase depends on the variation of

organic solvent in the mobile phase, and was enhanced by increasing the content of organic solvent and vice versa [11]. With a higher MeCN concentration of 75% in the mobile phase as shown in Fig 4.1B, the retention time of roxarsone was shifted to 17 min. That means that roxarsone is a representative of a hydrophilic compound which remains longer in the water-enriched layer of the stationary phase. Under these conditions the methylated arsenic species methylarsonic acid (MMA, m/z 141) and dimethylarsinic acid (DMA, m/z 139) were also separated and detected by ESI-MS in the retention time window between p-ASA and roxarsone. In general, the retention time of analytes depends strongly on their hydrophilic properties. It must be emphasized that in this study the separation time for roxarsone was influenced acutely by the MeCN rising for more than 10 min when MeCN was increased from 70% to 75% (see Fig 4.1A and Fig 4.1B). It was explained by the chemical structure of roxarsone, characterized by a hydroxyl group on the aromatic ring and a nitro group in ortho-position (see Table 4.1), owing to its relatively stronger hydrophilic properties compared with the amino group of p-ASA. In principle, the retention strength of analytes on the stationary surface of ZIC®-HILIC columns is governed by two main principles. One is the number and kind of polar functionalities, which stands for the hydrophilic properties of compounds; another is the number of charges. And, moreover, electrostatic interactions between the zwitterionic stationary phase and charged analytes actually become weak due to simultaneous electrical attraction and repulsion from the bifunctional modification of the stationary phase when analytes attempt to approach the stationary phase. Under these conditions the hydrophilic partitioning dominates the major retention mechanisms in comparison with electrostatic interactions in ZIC®-HILIC [10-13]. Therefore, the stronger relative hydrophilic properties of analytes are related to stronger retention on the HILIC column. For example, due to the chemical structure stated above, roxarsone has a relatively high polarity than other organoarsenicals under the investigation, so that the influence of the

variation of organic solvent, e.g. MeCN / water ratio in the mobile phase, itself affects the retention the most strongly (shown in Fig 4.1). Thus, the increased concentration of MeCN is regarded as a crucial way to complete the optimization of separation of more organoarsenicals in one run. As can be seen in Fig 4.1C, it was not possible to improve farther the resolution of the analytes using the short (50mm) ZIC-HILIC column and an isocratic mobile phase composition of 77% MeCN.

Therefore, the column length was changed from 50 mm to 150 mm with a constant inner diameter of 2.1 mm. Only with a slight modification of the mobile phase composition (78% MeCN) 9 arsenic species were separated within 60 minutes. Unfortunately, with this isocratic elution it was not possible to base-line separate AsC (peak 8) and AsB (peak 9) or PAA (peak 2) and TMAO (peak 7), as can be seen in Fig 4.2A. The chromatographic separation was shortened by increasing the flow rate of the mobile phase, but was limited by the instability of the inductively coupled plasma under this high organic solvent loading. The application of a special torch with a smaller inner diameter normally applied for organic solvents, as well as the addition of oxygen to the aerosol, did not reduce the organic solvent influence on the plasma stability substantially, but it led to the sensitivity decreasing by a factor of 10. Therefore, the recommended flow rate with respect to column back pressure and ICP-MS stability ranges between 100 and 150 µl min^{-1}. Nevertheless, the example of the separation of 9 sometimes quite different arsenic species shows the great potential of the implementation of HILIC in "element speciation" research [17].

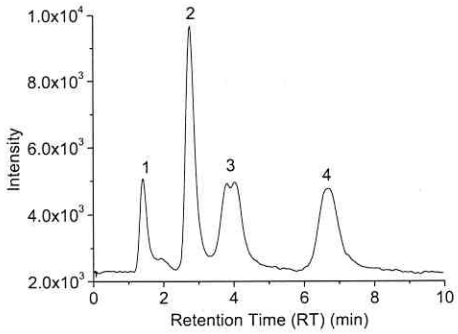
As mentioned above, the retention time was shortened by the decrement of MeCN in the mobile phase. In order to accelerate the separation for 9 mixed organoarsenicals, a MeCN gradient in the mobile phase was applied starting from 78% and finishing with 70%. Under these conditions the separation for these arsenic species still ought to be completed on a basis of 78% MeCN. As can be seen in Fig 4.2A, there is a peak-free interval of about 10

min between 22 min and 32 min which can be used for the separation of additional substances, or the chromatogram can be shortened by further modification of the mobile phase. To accelerate the separation of MMA, DMA and Rox a gradient elution was developed based on the stepwise changing of the concentration of the organic solvent in the mobile phase. The detailed timetable of the gradual changes of MeCN content in the mobile phase is shown in Table 3. With this gradient elution the retention time of roxarsone was reduced to 45 min without any loss of resolution for these compounds (Fig 4.2B).

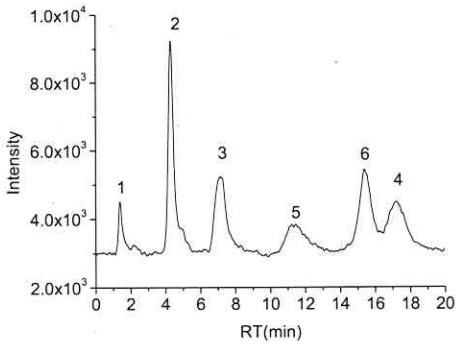
To quantify and identify the standard arsenic compounds two different mass selective detectors operated in parallel to detect firstly the element under investigation and secondly the chemical structure of the elemental species. The first was performed using ICP-MS detection at m/z 75 (As^+) to identify arsenic-containing compounds in the chromatogram. Due to the strong and uniform ionization of arsenic in the plasma this detection mode was favoured for quantification of the element species. The second detection mode (ESIMS) allowed the identification of compounds on the basis of the molecular mass ions $[M+H]^+$ or their arising fragments. The single ion monitoring mode (SIM) was used with fragmentor voltages of 70 V for detection of $[M+H]^+$ (SIM 1) and 400 V for m/z 75 and 91 (AsO^+) (SIM 2).

The limit of detection (LOD) for the ICP-MS detection ranged around 10 µg L^{-1} how to see on the small peaks for TMAO, AsC, and AsB in Fig 4.2A and B. In this case, the sensitivity in the SIM 1 and SIM 2 mode for the three As-species of the ESI-MS detection is substantially enhanced. For the other compounds investigated the ICP-MS peaks are much higher than the appropriate ESI-MS signals such as MMA and Rox. Peaks signed with a question mark represent unknown compounds which could not be identified as arsenic containing compounds in the scan mode of ESI-MS detection, because of its low sensitivity. The elemental specific identification of arsenic species using the detection of m/z 75 and 91

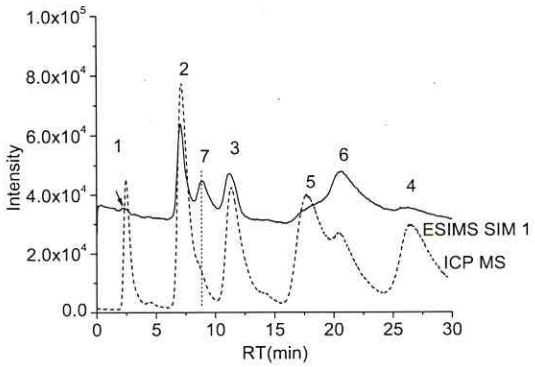
can not guarantee that these masses were created by arsenic / arsenic oxide ions or by other compounds with identical molecular masses or fragment ions.



(a): 4 organoarsenicals with 70 % MeCN as mobile phase



(b): 6 organoarsenicals with 75 % MeCN as mobile phase

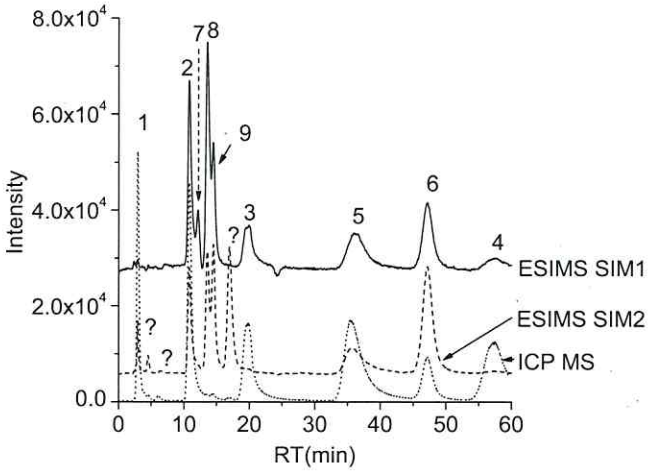


(c): 7 organoarsenicals with 77 % MeCN as mobile phase

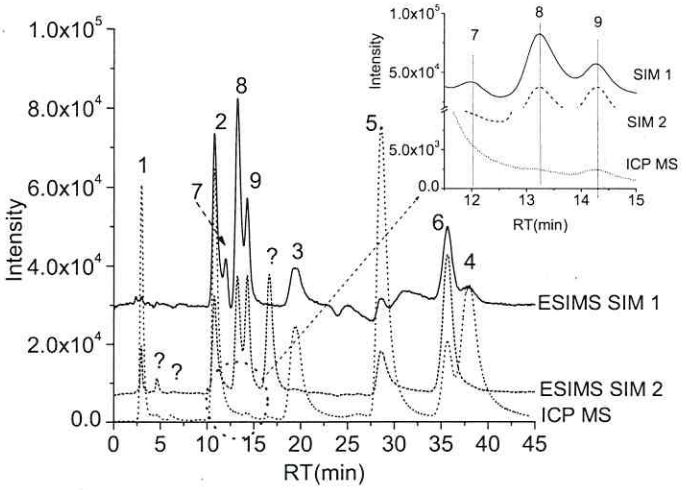
Fig 4.1 Isocratic separation and detection of organoarsenicals by LC-ESI-MS

Column: ZIC®-HILIC (50*2.1 mm id, 3.5 µm)

Detection: ESI-MS SIM1 (m/z) $[M+H]^+$: 1: 169 (PAO), 2: 203 (PAA), 3: 218 (p-ASA), 4: 264 (Rox), 5: 141 (MMA), 6: 139 (DMA), 7: 136 (TMAO), ICP-MS: m/z 75



(a): Isocratic separation of 9 organoarsenicals with 78 % MeCN as mobile phase



(b): Gradient separation and detection of 9 organoarsenicals, timetable for gradient of the mobile phase see Table 4.3

Fig 4.2 Separation and detection of organoarsenicals by LC parallel ESI-MS and ICP-MS Column: ZIC®-HILIC (150*2.1 mm id, 3.5 µm)

Detection: ESI-MS SIM 1 (m/z) $[M+H]^+$: 1: 169 (PAO), 2: 203 (PAA), 3: 218 (p-ASA), 4: 264 (Rox), 5: 141 (MMA), 6: 139 (DMA), 7: 136 (TMAO), 8: 165 (AsC), 9: 179 (AsB); ESI-MS SIM 2 (m/z): 75 (As^+), 91 (AsO^+); ICP-MS: m/z 75

Table 4.3 Timetable of the gradient of the mobile phase used in Fig 4.2(b)

Time (min)	A% (NH_4Ac 125 mM pH 8.3)	B% (MeCN)
0	22	78
15	22	78
15.01	30	70
20	30	70
20.01	25	75
35	25	75
35.01	22	78
45	22	78

4.3.2 Identification and quantification of arsenobetaine (AsB) in DORM-2

The optimized method for the separation and detection of organoarsenicals was applied for the determination of arsenobetaine (as As) in a certified standard reference material, DORM-2, which is the most abundant arsenic species with a certified content of 16.4 ± 1.1 mg As kg^{-1} (91.1% total arsenic concentration) besides tetramethylarsonium ion ($TMAs^+$) with a minor concentration of 0.248 ± 0.054 mg As kg^{-1} which is probably beyond the LOD of the HILIC chromatography with parallel ICP-MS and ESI-MS detection. Obviously, although DORM-2 might be contain other arsenic species such as DMA, As(III) or As(V) which could be

identified in the process of determination of AsB in DORM-2. However, the content of these arsenic species are not certified actually in DORM-2. Therefore, the quantification of $TMAs^+$, DMA, As(III) and As(V) was not accomplished. The calibration plot was carried out based on the addition of different amounts (0.2, 0.4, 0.6 mg L^{-1}) of standard solution of AsB (BCR 626) with concentration of 1.085 mg As L^{-1} into the DORM-2 extracts. The calibration graph ($y= 6.8*10^5+1.2*10^6*x$; peak area vs. added concentration) for AsB standard addition analysis showed a good linearity $R^2 > 0.9993$ when working with ICP-MS detection after HILIC separation. According to the linear equation, the quantitative results (14.3 ± 0.6 mg kg^{-1}) obtained for DORM-2 sample agreed well with the concentration in the extracts (15.1 mg kg^{-1}).

4.4 Conclusions

The optimization of the separation conditions by the variation of the MeCN percentage in the mobile phase yielded a satisfactory result for the determination of organoarsenicals when using ZIC®-HILIC columns. By isocratic separation and by using a gradient with 78% to 70% MeCN, 9 mixed organoarsenicals (Rox, PAA, p-ASA, PAO, DMA, MMA, AsB, AsC and TMAO) were separated and detected within 45 min. Additionally, the identification of AsB and its quantification in DORM-2 sample were evaluated when employing the proposed method with a recovery of about 95%. The advantages of this HILIC method are the direct injection of extracts consisting of 80% MeCN and the good compatibility with the ESI-MS detection. The high organic content in the mobile phase limits the ICP-MS detection. Low flow rates of the mobile phase, the application of micro columns, and the supplement of oxygen can reduce these interferences somewhat, but restrict this dual detection.

Nevertheless, in future this new chromatographic separation principle will allow the determination of hydrophilic and hydrophobic analytes together. This method permits the injection of samples with a high content of organic solvents. Therefore, precipitation of hydrophobic analytes ought not to occur and the extracts obtained from a hydrophobic sample, such as oil, can be used without further treatment such as volatilization of the solvent and re-dissolving in water/methanol mixtures.

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5 Retention behaviour of arsenic species on zwitterionic stationary phase of hydrophilic interaction chromatography (ZIC®-HILIC)

Manuscript: D. Xie, J. Mattusch, R. Wennrich. *J. Separation. Sci.* (under revision)

Abstract

Zwitterionic hydrophilic interaction chromatography had been used for the separation of selected arsenoorganicals. The retention behaviour of nine organic arsenic species on zwitterionic hydrophilic interaction chromatography (ZIC®-HILIC) was investigated in order to elucidate, which is the driving force for their separation; hydrophilic partitioning or hydrogen bonding. For this the retention factor of the compounds *k* were correlated with the octanol-water partition coefficient (*log* $P_{o/w}$) and with 'hydrogen bonding of analytes', respectively. Due to the insufficient coefficients of determination *log k* vs. *log* $P_{o/w}$ and *k*, vs. hydrogen bonding capability, respectively, considering all nine As-species, it was not possible to explain the retention behaviour. If one examine separately according to aliphatic and phenyl compounds, substantially better coefficients were received. This indicates that both phenomena contribute to the separation of these arsenic species on ZIC®-HILIC.

Additionally, the retention behaviour of arsenic species was investigated by varying the separation conditions. The results show that the composition of the eluent has a strong influence on the retention behaviour. It is highly dependent on water/MeCN ratio, pH-value and salt additives. Dissociation degree and polarity of arsenic species, which are varying with pH, regulates the distribution of arsenic species between stationary and mobile phases in HILIC. Increasing the ammonium acetate concentration leads depending on the concentration

to shorten or to prolonged retention of arsenic species. The order of elution of As species on ZIC®-HILIC are not changed by adding ammonium acetate or ammonium formate.

Keywords Arsenic species / HILIC / Hydrophilic partitioning / Retention behaviour / Salts

5.1 Introduction

Arsenic occurs in the environment in a multiplicity of different inorganic and organic species, and numerous (until their detection) 'unexpected' organoarsenicals particularly in living organisms were proven in the recent years [1].

The determination of arsenic compounds has progressed with the development of hyphenated analytical methods, especially the coupling of HPLC with ICP-MS.

As a conventional technique to separate arsenic species in marine organisms ion exchange chromatography is predominant, especially for the determination of hydrophilic arsenic species.

Anion exchange chromatography was used with priority for the separation of As(III), As(V), MMA and DMA whilst cation exchange chromatography was preferentially applied for the separation of AsB, AsC and TMAO. However, the separation of these species within one run was also realized [2, 3]. The retention mechanism of ion exchange chromatography (IC) mainly depends on the chemical interactions of charged analytes with the oppositely charged moieties of the stationary phase. The retention behaviour of analytes highly relies on the pH value and ionic strength of the mobile phase as well as the properties of functional groups on stationary phase [4]. Since HILIC was first mentioned [5], it has emerged as an alternative choice to HPLC for applications dealing with hydrophilic and hydrophobic analytes. As

recently shown this separation technique allows us to simultaneously analyze various arsenic species because of advantage of HILIC separation mechanisms [6].

ZIC®-HILIC, which is endowed with stationary phase modified by bifunctional groups, has a lot of advantages for separation of arsenic species with diverse polarity. The separation mechanisms on ZIC®-HILIC include hydrophilic partitioning, hydrogen bonding and electrostatic interactions [7]. On ZIC®-HILIC, hydrophilic partitioning of analytes seems to be the typical separation principle caused by the high content of organic solvent in eluent. Additionally, bifunctional moieties on stationary phase can have chemical interactions with counter ions. Therefore, uncharged, charged and diverse polar analytes can be separated on ZIC®-HILIC [6].

So far, separation mechanism on HILIC are not clearly enlightened concerning the dominating effects - separation by partitioning, or by hydrogen bonding, or by both phenomena [7]. Based on different studies it was assumed, e.g. [8] that the primary retention mechanism on HILIC is hydrophilic partitioning of analytes between the eluent and the hydrophilic environment on the stationary phase, a so-called water-enriched layer. In contrast, Yoshida [9] suggested hydrogen bonding as the principal interaction mode in HILIC. On the other hand, Berthod *et al.* [10] explained the separation by both partitioning and hydrogen bonding.

In order to make the effects quantifiable, recently, Yanagida *et al.* [11] proposed to correlate stochastically the retention factor k of analytes and physico-chemical data of the substances. As criterion for the hydrophilicity the number of the OH-groups of the appropriate compound was selected. The octanol-water partition coefficient of analytes ($P_{o/w}$) was chosen to indicate their polarity. For a series of oligomeric proanthocyanidins they received linear regression plots for *log* k vs. *log* $P_{o/w}$ and *log* k vs. number of hydroxyl groups, respectively. They concluded that solute hydrophilicity and hydrogen bonding corporately contribute to the

separation of these compounds by HILIC. Because of the improved coefficient of determination (R^2-value) for the regression plot *log k* vs. number of OH-groups (compared with the R^2 value of the regression plot log *k vs.* log $P_{o/w}$), the effect of hydrogen bonding is assumed to be stronger than hydrophilic partitioning.

Besides these separation mechanisms, electrostatic interactions between analytes and moieties of ZIC®-HILIC should not be neglected. Hu [12, 13] believed that an electrical double layer on zwitterionic stationary phase can be formed and influences the separation mechanism. Cook [14] explained the retention mechanism of ions in zwitterionic ion chromatography by the formation of a Donnan membrane. Comprehensively, Xuan [15] stated that the separation of phytosiderophores and their metal complexes on ZIC®-HILIC is achieved by hydrophilic partitioning and weak electrostatic interactions of the bifunctionalities on stationary phase with either positive or negative charges. They found that the interactions between cations with the negative charged sulfonic group is stronger than the interaction between anions with the R_4N^+ group, leading to higher retention time for cations and shorter retention time for anions. In addition, these mechanisms are influenced by eluent conditions, since buffer properties and concentration impact both activities of functionalities on stationary phase and analytes [16].

Since hydrophilic and hydrophobic arsenic species have been separated successfully in one run on ZIC®-HILIC recently published [6], it is essential to explain the retention mechanism of these organoarsenicals on ZIC®-HILIC, which is goal of this work.

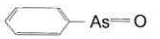
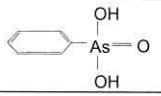
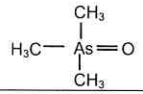
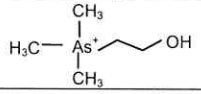
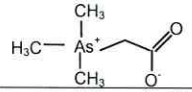
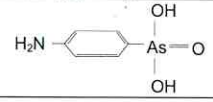
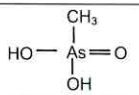
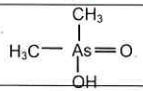
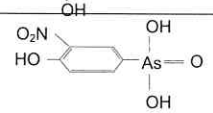
5.2 Experimental

5.2.1 Reagents and chemicals

All solutions were prepared with de-ionized water (Milli-Q, Millipore). Organoarsenical standard solutions: 3-nitro-4-hydroxyphenylarsonic acid, Roxarsone, (ICN Biomedicals Inc.); phenylarsonic acid (TCI Europe nv); p-arsanilic acid, phenylarsine oxide, dimethylarsinate (all SIGMA); methylarsonate (Luxembourg Industries (PAMOL) Ltd); trimethylarsonioacetate (BCR626®, Fluka), trimethylarsonioethanol (AsC) and trimethylarsine oxide (TMAO) – both kindly provided by Kevin A. Francesconi, Karl-Franzens University, Graz - were diluted with the mobile phase applied in the different experiments. The structural parameters of the As species are summarized in Table 5.1.

Different stock solutions were prepared. One solution contained each 2 mg L^{-1} of Rox, DMA, MMA; the other ones each 1 mg L^{-1} (PAA, p-ASA, PAO) and 10µg L^{-1} (AsB, AsC and TMAO), respectively. The chemical reagents (analytical grade p.a.) were utilized during the study as follows: NH_4Ac, MeCN, ammonia, acetic acid, ammonium formate (all MERCK), formic acid (Fluka). The pH value was adjusted using NH_4OH, HAc and HCOOH, respectively.

Table 5.1 The relevant chemical parameters of arsenic species

Eluting order [6]	Compounds	Structural parameters				Chromatographic parameters [a]		Polarity in variable valence [b]				
		Structure	Hydrogen bonding [b]	log $P_{o/w}$	pK_a	k	log k	+1	0	-1	-2	-3
1	phenylarsine oxide (PAO)		2.39	2.44	none	0.08	-1.10		1.09	/ [c]	/	/
2	phenylarsonic acid (PAA)		17.92	0.06	3.4, 8.2 [17]	2.94	0.47		6.35	7.97	7.80	/
3	trimethylarsine oxide (TMAO) [d]		0	2.32	(3.6) [18]	3.38	0.53		(3.6)	/	/	/
4	trimethylarsonioethanol (Arsenocholine, AsC)		11.92	1.89	none	3.84	0.59		3.32	/	/	/
5	trimethylarsonioacetate (Arsenobetaine, AsB)		5.47	2.12	2.2 [18-20]	4.22	0.63		4.12	/	/	/
6	p-arsanilic acid (p-ASA)		20.07	-0.88	2, 4.0, 8.9 [21]	6.08	0.78	6.62	7.84	7.72	9.15	
7	methylarsonate (MMA)		23.27	-1.18	3.6, 8.2 [18, 19]	9.48	0.98		8.25	11.49	10.46	/
8	Dimethylarsinate (DMA)		16.18	0.36	6.2 [18-20]	12.05	1.08		6.00	4.35	/	/
9	3-nitro-4-hydroxyphenylarsonic acid (Roxarsone, Rox)		27.18	-0.05	3.4, 6.4, 9.7 [22]	12.80	1.107		15.64	17.04	16.47	18.73

[a] at pH 8.2; [b] Calculation by *Physical Properties Pro.*; [c] No dissociation; [d] $(CH_3)_3As(OH)_2$ - $(CH_3)_3As{=}O \cdots HOH$ [23];

5.2.2 Instrumentation

A μ-flow HPLC system consisting of degasser, autosampler, binary gradient pump and ESI MSD (G1946 B and 6130) (Agilent) was used. The analytical column (150*2.1 mm id, particle size 3.5 μm) was purchased from ZIC®-HILIC (SeQuant).

The outlet of the HPLC column was connected in parallel by a flow splitter (series 600, ASI Company) with ICP-MS and ESI-MS as detectors. The mass selective detector (MSD) operated in the positive ionization mode. Scanning mode was used to identify fragments of the compounds and single ion monitoring (SIM) was applied to improve the sensitivity. An ICP-MS 7500ce (Agilent) was performed for arsenic selective detection at m/z 75. The optimized parameters of HPLC, ESI-MS and ICP-MS are listed in Table 5.2.

The partition coefficients of arsenic species under study were obtained from *PhysProp* database (Synopsys Scientific Systems Ltd). Polarity and hydrogen bonding parameters of arsenic molecules, which are listed in Table 5.1, were calculated by *Physical Properties Pro* (ChemSW®).

Table 5.2 HPLC & ESI-MS & ICP-MS parameters

Chromatography parameters	
Columns	ZIC®-HILIC (SeQuant, Sweden), 150*2.1 mm id, 3.5 µm
Mobile phases	MeCN and NH_4Ac or NH_4COOH varied concentration and pH
Flow rate	50-500 µL min^{-1}
Injection volume	2-8 µL
ESI-MS parameters	
Drying gas flow	10.0 L min^{-1}
Nebulizer gas pressure	30 psig
Drying gas temperature	350°C
Capillary voltage	Positive 1500 V
Fragmentor voltage	70 V to 400 V (SIM)
ICP-MS parameters	
RF power	1600 W
Carrier gas	0.4 L min^{-1}
Makeup gas	0.11 L min^{-1}
Optional gas (Ar/O_2)	9.0 %
Nebulizer	Microconcentric + Scott-type spray chamber

5.3 Theoretical backgrounds

The retention factor (k) of each analyte in HILIC column is calculated in accordance with the following equation:

$$k = \frac{(t_R - t_0)}{t_0} \tag{5.1}$$

where t_R is the retention time (min) of analytes and t_0 is the time (min) of dead volume.

The partition coefficient P is defined as the ratio of the equilibrium concentrations of a dissolved substance in a two-phase system consisting of two largely immiscible solvents [17]. In the case of octanol and water:

$$P_{o/w} = \frac{C_o}{C_w} \tag{5.2}$$

Here C_o is the concentration of analytes in octanol phase; C_w is the concentration of analytes in aqueous phase (water).

5.4 Results and discussion

5.4.1 Investigations for the separation mechanism of arsenic species

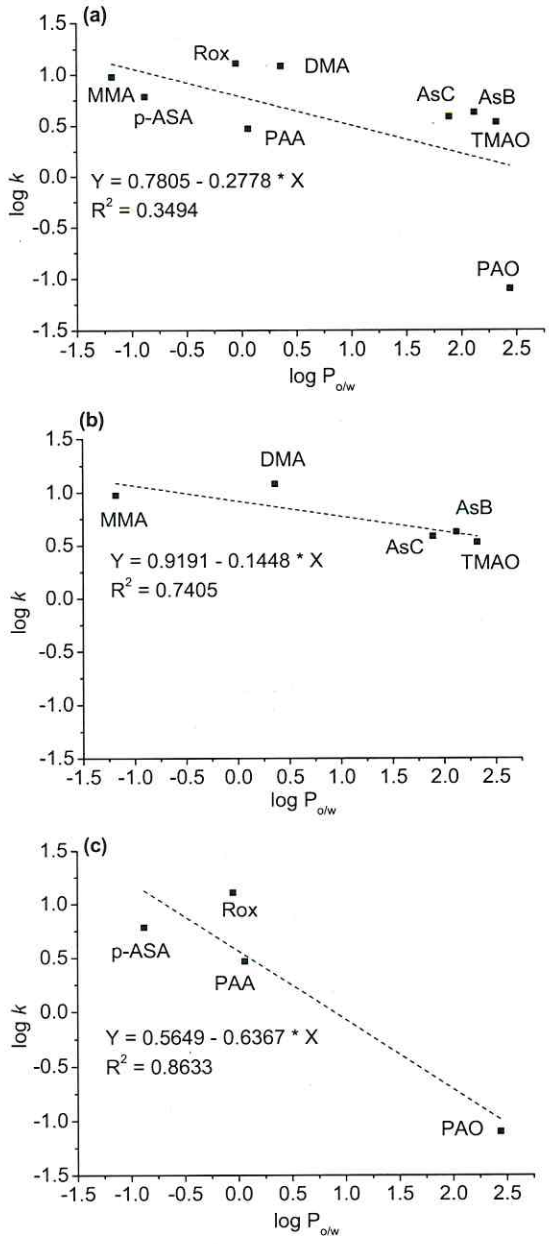
To investigate the separation mechanism of arsenic species on ZIC®-HILIC, the initial step commences with the basic separation theories; hydrophilic partitioning and hydrogen bonding. As had been demonstrated for proanthocyanidins in [11] the retention of the organoarsenicals was regressed versus partitioning and the capabilities of hydrogen bonding, respectively. Partitioning ability determined by polarity of substances and hydrogen bonding parameters, respectively, are listed in Table 5.1. To summarize chemical parameters with

chromatographic parameters from separation in previous research [6], all related parameters are also listed in Table 5.1.

As shown in Fig 5.1A, the coefficient of determination (R^2) for the linear regression of $\log k$ and $\log P_{o/w}$ is 0.349 if all nine compounds are included, i.e. hydrophilic partitioning seems to be not the dominating in separation on ZIC®-HILIC. Thus, the retention factor *k* was regressed against hydrogen bonding (Fig 5.1B). Likewise, the regression plot of *k* versus hydrogen bonding is also not sufficient (R^2= 0.555).

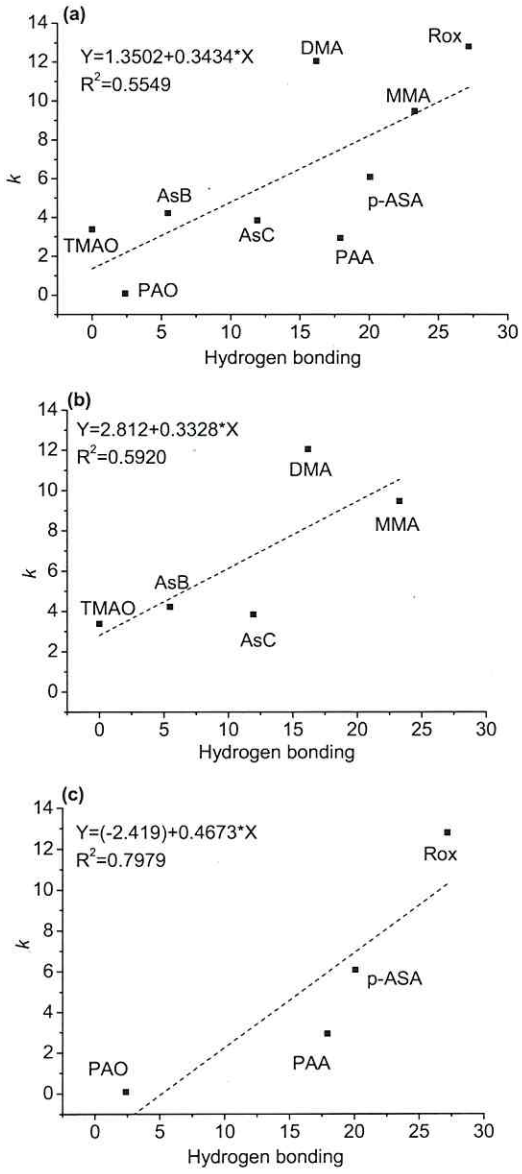
Dividing the arsenic compounds under study with respect to their structure in aliphatic and phenylic compounds a substantially better regression between *log k* versus hydrophilic interaction and *k* versus hydrogen bonding was received. For the phenyl compounds (n=4) thus obtained values increased to R^2=0.863 and R^2=0.798 for hydrophilic partitioning and hydrogen bonding, respectively. Whereas the R^2 for the aliphatic arsenic compounds (n=5) were R^2=0.741 and R^2=0.592 for hydrophilic partitioning and hydrogen bonding, respectively. This indicates that for both the phenylic and the aliphatic arsenic compounds both phenomena can supply a substantial contribution for the separation on ZIC®-HILIC.

However, one has to consider the electrostatic effects. On one hand, electrostatic interactions highly rely on pH condition leading to varying dissociation degree of analytes. On the other hand, the number and property of charged functionalities of the compounds are assignable factors that influence the distribution of analytes between stationary and mobile phases [5, 7, 8]. Analytes are retained on stationary phase, if electrostatic adsorption between charged analytes and oppositely charged moieties on stationary phase is stronger in comparison with repulsion from functionalities with same charges. In contrast, when electrostatic repulsion is stronger than electrostatic adsorption from moieties of the stationary phase, analytes are eluted faster. Therefore, the influence of charged additives (salts) has to be investigated more intensively.



A. Regression: retention factor (log k) versus log $P_{o/w}$

(a) all As species; (b) aliphatic; (c) phenylic



B. Regression of retention factor (*k*) versus hydrogen bonding

(a) all As species; (b) aliphatic; (c) phenylic

Fig 5.1 Linear calibration of retention factor (*k*) and chemical parameters

5.4.2 pH effects

The buffer pH is an important parameter for the retention behaviour of compounds on HILIC as shown for separation of peptide mixtures [24] and ERLIC (electrostatic repulsion HILIC) [25], respectively.

As known, in ZIC®-HILIC the analytes are subject to the simultaneous change between electrostatic adsorption and repulsion if they approximate the stationary phase. The intensity of electrostatic forces governs the distribution of analytes between mobile phase and stationary phase. Additionally, the dissociation degree of analytes in various pH impacts the retention.

Therefore, the influence of pH on the retention was examined in the range of 4.5 to 8, within which most of the organoarsenicals are dissociated (Table 5.1). In these studies $HCOONH_4$ was added to the eluent consisting of MeCN and water. The conditions for the gradient mode are given in Table 5.3.

The data in Fig 5.2 show that the retention behaviour of Rox, DMA, p-ASA, PAA and AsC on ZIC®-HILIC is dependent on the pH-value. Contrary, the retention of AsB, MMA, TMAO and PAO was not influenced by varying the pH. The highest influence was established for DMA and Rox, which are anionic in the pH range under study (pK_a in Table 5.1).

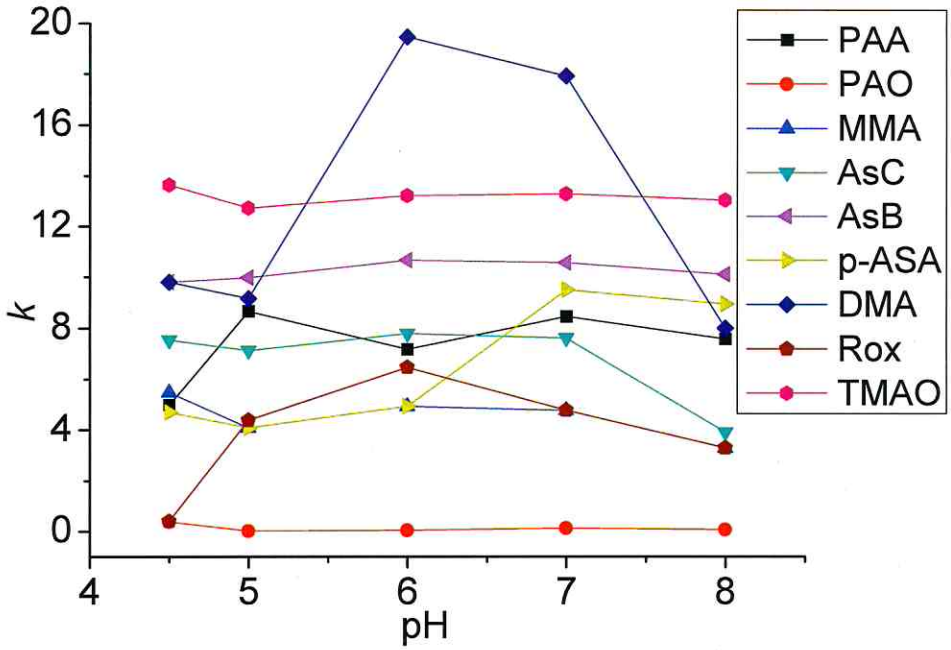


Fig 5.2 Influence of pH on retention of arsenic species

Flow rate: 500 µl min^{-1}, injection volume: 8 µl;

Elution: A_1: 90% MeCN+10% 100 mM $HCOONH_4$

B_1: 50% MeCN+10% 100 mM $HCOONH_4$ +40% H_2O

Gradient mobile phase is described in Table 5.3

Table 5.3 Gradient mobile phase of separation of arsenic substances in Fig 5.2

Time (min)	A%	B%
0	90	10
2	90	10
20	80	20
22	90	10
25	90	10

Roxarsone

The retention behaviour of Rox is depending on the pH the pK_a. As one can see in Fig 5.2 the retention factor (*k*) is increased from 0.4 to 6.5 increasing the pH from 4.5 to 6, which can be attributed to an increase of the bivalent anionic Rox. In this pH region the electrostatic adsorption on the R_4N^+ groups seems to be stronger than the repulsion from alkylsulfonate groups on the stationary phase The strongest retention was found at pH adjusting near to pK_{a2} = 6.4 (equilibrium of univalent and bivalent ions). Higher pH leads to reduced retention time of Rox again. This can be traced back to two different effects. First, there is an increasing fraction of the bivalent anion. Second, the cationic force of the R_4N^+ groups of the stationary phase is decreased with increasing pH. Both effects can contribute to a stronger electrostatic repulsion with rising pH.

Not only charges of analytes (and the moieties of the stationary phase), but also polarities of the analytes are changed with the dissociation degree under different pH-values according to the calculation shown in Table 5.1. Higher than pH 4.5 and below pH 6.4 (pK_{a2}) Rox transfers from a univalent anion to a bivalent one, which is interrelated with an increase of the polarity (17.04). With increasing polarity the residence time of Rox in the water-rich layer on stationary phase is prolonged, so that also longer retention times could be found. Above pH = pK_{a2} = 6.4 the negative bivalent ions dominate and the polarity drops down (polarity: 16.47). In contrast, decrement of polarity causes the remaining of Rox in the mobile phase containing high content of MeCN and therefore the retention time of Rox decreases with increasing pH.

DMA

The retention factor of DMA shows the same course of the plot as Rox. The highest one was observed near the pK_{a2} = 6.2. Below pH 4, DMA exists predominately as neutral molecule. Increasing the pH DMA is more dissociated, and shifts to a univalent anion (DMA^-).

Regarding the influence of the change of polarity on the retention of DMA shows an opposite behaviour to Rox. As listed in Table 5.1, the calculated polarities of the DMA are distinct lower than those for Rox (Table 5.1). The polarity of neutral DMA is also lower than those of its anionic form. That means that the transfer of DMA into the aqueous phase should be inhibited with rising pH value. Consequently, the behaviour of DMA is explainable by the strong electrostatic interactions between the analyte and the moieties of the stationary phase.

MMA, p-ASA, AsB, PAA

How to see in Fig 5.2 there is only a slight influence of the pH on the retention of these compounds, which are anions in the investigated pH range. Only for p-ASA an increase in retention was observed changing the pH from 6 to 7. This correlates very well with an increase in the calculated polarity.

TMAO, PAO, AsC, AsB

No influence of pH on the retention coefficient of these species was found in the pH range 4.5 to 8. PAO and TMAO basically appear as neutral molecules in the separation process while AsC is a cation and AsB is zwitterionic at pH > 4.5.

It is noticed that retention order of PAO (polarity: 1.09), AsC (polarity: 3.32) and AsB (polarity: 4.12) is following the ascending value of polarity, apart from the unexplained phenomenon that retention of AsC is decreased between pH 7 and 8.

In neutral solution water can be bonded to TMAO forming either $(CH_3)_3AsO\cdots HOH$ or $(CH_3)_3As(OH)_2$ [23]. For this reaction pK_a = 3.6 is referred [18]. That means a very high affinity to the aqueous layer of the stationary phase is conceivable resulting in the high retention coefficient and the constant values within the pH range under study.

5.4.3 Influence of salt additives

Using ion-pair chromatographic separation of ionic compounds the mobile phase should contain an ion-pairing reagent. The analyte ion forms together with the counter ion a less polar ion-pair, which is stronger retained on the likewise hydrophobic stationary phase resulting in higher retention. This effect can be also utilized in ZIC®-HILIC. By addition of ion-pairing reagents to the eluent the electrostatic interactions between the analyte ions and the stationary phase can be manipulated to govern the separation process. They can simultaneously participate in ion-pair formation with counter analyte ions and oppositely charged moieties of stationary phase [16]. Certainly, electrostatic interactions still happen between dissociated ions of the salt and functionalities on stationary phase. The intensity of these interactions is depending on the chemical behaviour of the analytes and also on the salts and their ion strength. By selection of a suitable additive (salt and/or buffer) as well as varying its concentration the retention of the analytes on the column is affected. For ZIC®-HILIC the use of several ammonium salts, e.g. acetate, formate, bicarbonate are reported, e.g. [8, 16, 26-28].

The question arose, how salts affect the retention of the different organoarsenic compounds. For this investigation each two methylated (DMA, MMA) and two phenylic (PAA, p-ASA) arsenic species were used. These compounds are characterized by pK_a values of 6.2, 8.2, 8.3 and 8.9, respectively.

To see in Table 5.1 the eluting order of these four compounds is responded neither by their polarity nor by charged functionalities. Additionally, the influence of the pH on the retention was very different, as was shown in the preceding chapter.

Ammonium acetate

NH_4Ac has been widely used as additive due to the relative high solubility in mobile phase containing high concentration of MeCN [8, 26, 27]. In these studies the effects of salt in mobile phase had been investigated at lower concentrations (<20 mM).

In this study the effect of NH_4Ac was investigated at pH 8.2 up to 37.5 mM. As shown in Fig 5.3A distinct differences were obtained depending on the polarity of the arsenic compounds. As discussed above, at pH 8.2 the analytes are dissociated to be univalent or bivalent anions. The retention order follows the polarity values of dissociated ions, MMA^{2-} (10.46), p-ASA^{2-} (9.15) and PAA^{2-} (7.80). However, the retention of DMA^- with a polarity value of 4.35 deviates from this tendency. That could be attributed to the fact that DMA is a univalent anion.

The binding between this univalent anion and the R_4N^+ group should be more stable than by the bivalent anionic arsenic species due to the electrostatic repulsions between the still existing free electronegative charge of the anions and the negatively charged SO_3^- group present at the stationary phase.

It is noticed that eluting order is not influenced by salt concentration, which one can attribute to the fact that no changes in the chemical properties of the analytes occur by variation of the salt concentration.

The plots in Fig 5.3A show that the retention behaviour of the organoarsenicals is similar. It is decreased with increasing NH_4Ac concentrations from 1.25 mM to 12.5 mM. For example, the retention factor of DMA is diminished from k= 9.2 (1.25 mM) to k= 6.2 (12.5 mM).

The retention of PAA is changed from k= 1.8 to k= 0.2 increasing the salt concentration. The tendency agrees with results reported for other anionic compounds [27, 28]. It could be explained in principle by an increase of the eluting power by increasing acetate concentration. Additionally, NH_4Ac is more dissociated in the aqueous layer on the stationary phase and is enriched there yielding in reduced ionic interactions between the moieties of the stationary phase and the analytes.

ZIC®-HILIC possesses a significant negative charge due to weak-positively charged R_4N^+ - group and stronger negatively charged $-SO_3^-$ group. NH_4^+ is available for binding with the SO_3^- groups and ac^- can bind to the R_4N^+ functionalities of the ZIC®-HILIC, respectively. The anionic arsenic compounds can be electrostatically bind to R_4N^+ group and also to NH_4^+ and are repulsed by the $-SO_3^-$ group on the other hand. That means depending on pH value different effects can occur caused by the chemical behaviour of its hydrolysis products (CH_3COOH: pK_a 4.75) and (NH_4OH: pK_b 4.75), respectively.

At pH 8.2 NH_4Ac is dissociated in the aqueous layer of the HILIC material. This excess concentration leads to a competition of acetate and anionic arsenic compounds around the binding sites of the quaternary ammonium moieties of the HILIC. With increasing concentration of acetate the R_4N^+ groups are more blocked and the binding capacity of the analyte anions is reduced resulting in lower retention. On the other hand, the higher concentration of acetate could cause a displacement of the anionic arsenic compounds out of the aqueous layer, which also shorten the retention times. Additionally, the NH_4^+ ions can be bound electrostatically to the $-SO_3^-$ groups of the stationary phase. Both effects lead to a diminishing of the ionic interactions between the analytes and the moieties of the stationary phase. Also, increasing concentration of NH_4^+ leads to neutral anionic arsenic species by ion-pair formation, which are higher soluble in the mobile phase than the charged arsenic compounds. This leads also to decreasing retention.

At NH_4Ac concentrations >7.5 (or >12.5) mM, the retention times for all four compounds rises again with increasing salt concentration, e.g. *k* of DMA is prolonged from 6.2 (12.5 mM) to approximately 8 (37.5 mM). For this unexpected result there are several explanation possibilities. By increasing the salt concentration in the aquatic layer at the surface of the stationary phase, the osmotic pressure becomes stronger, which could lead to an enlargement of the volume of the layer, whereby the retention time of the ions in this one is again extended. It is also conceivable that by further rise of the NH_4^+ concentration the number of free SO_3^- groups is strongly diminished, whereby the repulsion of the anionic arsenic species is also reduced.

Ammonium formate

In order to generalize the effects using NH_4Ac as additive, additional investigations were accomplished using ammonium formate, i.e. changing the anion, (at pH 8.2). Hereby one have to consider the differences compared with NH_4Ac. Formic acid is a stronger acid (pK_a 3.77) than acetic acid, and it is also smaller.

As one can recognize in Fig 5.3B there is no change in the sequence of the retention compared with NH_4Ac. However, the retention times are generally shorter than using NH_4Ac. However, contrary to the behavior in NH_4Ac the retention characteristics was not affected if low concentrations (<12.5 mM) of $HCOONH_4$ were added. That shows the significant influence of the kind of the salt anions on the retention behaviour. It is explainable by a strong electrostatic interaction between the small-sized formate anion with the positively charged R_4-N^+ group, which leads to weak effective positive charges of the stationary phase with low formate concentrations.

At elevated salt concentration similar pattern by adding $HCOONH_4$ and NH_4Ac was found.

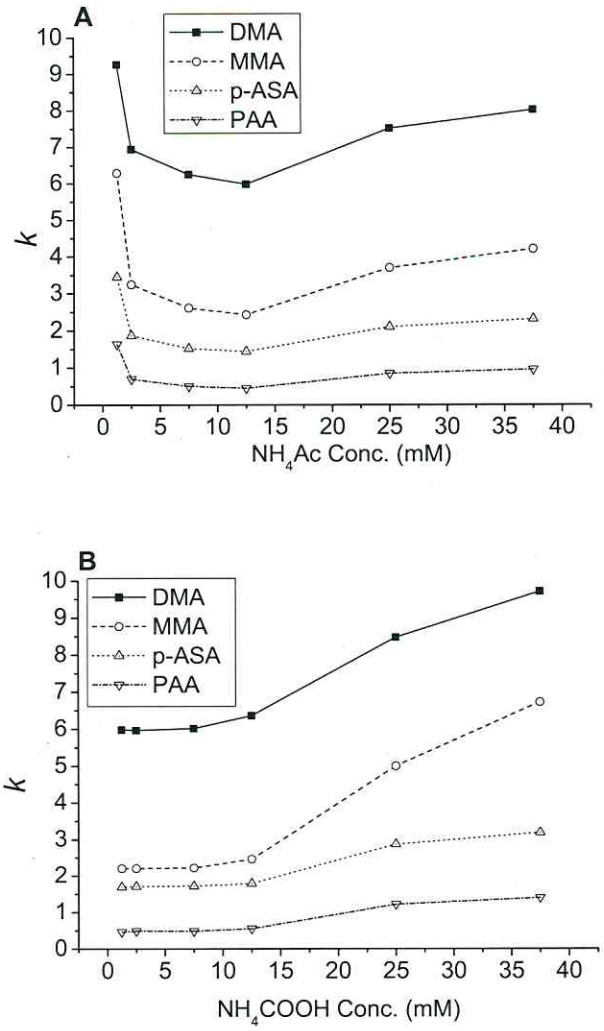


Fig 5.3 Influence of salt additives on the retention of arsenic species

A: CH_3COONH_4 **B:** $HCOONH_4$

(Flow rate: 250 µl min^{-1}, injection volume: 2 µl; isocratic elution: 75% MeCN, 25% aqueous solution with varied concentration of salts (pH 8.2))

5.4.4 Influence of ammonium acetate (NH_4Ac) concentrations at different water contents in eluent on the retention behaviour

In HILIC the water contents in MeCN/water eluents affect the retention behaviour strongly, e.g. [7, 8]. The following investigations were accomplished in MeCN/water 60:40 with changing concentration of NH_4Ac (75:25 in the previous attempts).

Comparing the results shown in Fig 5.4A with those in Fig 5.3A one can recognize clearly that the retention times were substantially shortened with increasing water content in the eluent., which is in accordance to the findings reported in [7, 8]. The retention sequence was not changed, however. That means the sequence of retention is associated with the polarity of the bivalent anionic arsenic species.

There is also a strong decrease in the *k* values with increasing concentrations of NH_4Ac from 1.25 to 2.5 mM. In contrast to the behaviour shown in Fig 5.3A, however, the retention time becomes larger with further increase of the salt concentration (>2 mM) immediately. (Using MeCN/water 75:25 this was observed at salt concentrations >12.5 mM.) That means that the action of NH_4Ac differ depending on the water content in the eluent as comprehensive interaction of the different variables (ionic interaction, hydrophilic interaction and size-exclusion [28]).

As shown in Fig 5.5 that there is a linear regression between log *k* vs. log c (NH_4Ac) in the range from 2.5 to 100 mM, with positive slopes, and R^2 values ≥0.86 are obtained.

In ZIC®-HILIC such behaviour was explained for retention of chloride by reducing the net negative surface charge leading to declined repulsion between the anions and the SO_3^- group [16].

We found however an increase in the slope in the order $MMA^{2+} \approx DMA^{+} < p\text{-}ASA^{2+} < PAA^{2+}$. That is opposite the polarity values of the anions (Table 5.1). One can conclude that hydrophilic interaction with rising salt concentration becomes stronger.

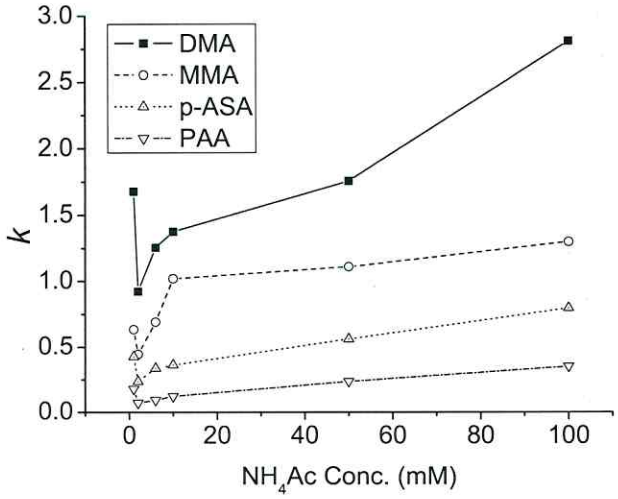


Fig 5.4 Influence of NH_4Ac concentration in mobile phase on the retention of arsenic species using an eluent consisting of 25 % aqueous NH_4Ac (pH 8.2) and 75 % water/MeCN (20:80) with NH_4Ac (pH 8.2); flow rate: 250 µl min^{-1}, injection volume: 2 µl; isocratic elution

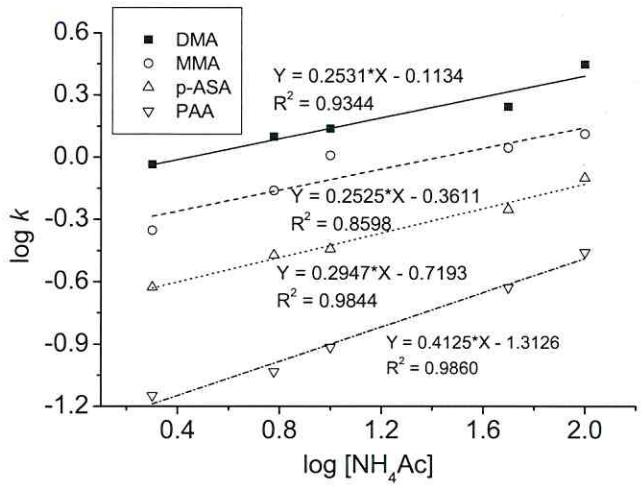


Fig 5.5 Regression: log *k* versus log c (NH_4Ac)

5.5 Conclusions

ZIC®-HILIC is an alternative to ion exchange chromatography for the separation of neutral and anionic organoarsenicals with a broad polarity range. The retention behaviour of nine organoarsenicals was investigated under the aspects hydrophilic partitioning and electrostatic interaction. Retention behaviour of the arsenic species under study on ZIC®-HILIC cannot describe simply by one of these phenomena. A combination of the two basic separation mechanisms becomes possible. The extent to which each mechanism contributes to the retention is dependent on the structure of the arsenic species at the applied separation conditions.

Linear graphs with acceptable R^2 values between retention factors and hydrogen bonding and between log *k* and log $P_{O/W}$, respectively, were received, if one considers the different chemical characteristics of the arsenic species (aliphatic, phenylic).

The results show a clear influence of the pH value on the retention behaviour and the retention sequence of the organoarsenic species on ZIC®-HILIC.

The intensity of electrostatic interactions and polarity of arsenic anions, which are caused by the dissociation degree of arsenic species in variable pH conditions, decide the retention mechanism.

The retention behaviour is also influenced by the composition of the eluent as shown by salt addition to the eluent (MeCN/water). The concentration and type of salts do not affect the eluting order of arsenic species on ZIC®-HILIC.

The addition of small salt concentrations leads to a shortening of the retention time of the anionic arsenic species at pH 8.2, if one uses ammonium acetate, however causes an addition of small concentrations of ammonium formate no change in retention.

With elevated concentration of salt increases the retention of arsenic species on ZIC®-HILIC. This indicates ion-exchange interactions with arsenic species and with the moieties of the stationary phase and also changes in the behaviour of the water-enriched layer on the surface of stationary phase. That proves that not only electrostatic interactions but other primary mechanisms are taken part in the separation of arsenic species on ZIC®-HILIC, such as hydrophilic partitioning.

The authors declared no conflict of interest

Acknowledgements

The authors gratefully acknowledge Dr. Albrecht Paschke and Dominik Wondrousch (Dept. Ecological Chemistry, UFZ) for calculation of the polarity parameters.

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6 Description of separation mechanism of arsenic species by comparison of retention behaviour on different HILIC columns

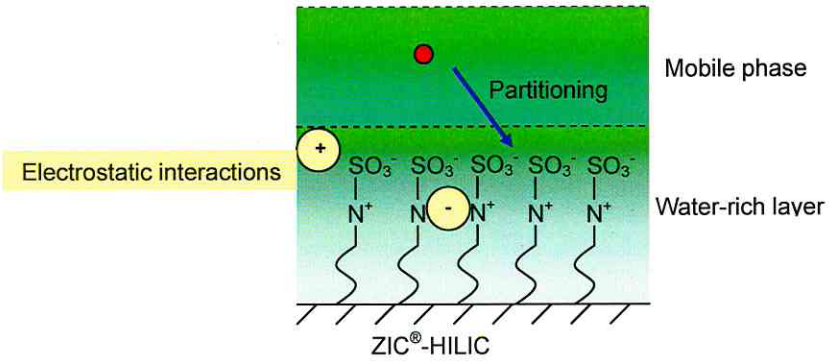
Manuscript: D. Xie *et al., J. Chromatography A.* (in preparation)

6.1 Comparison of separation of arsenic species on ZIC®-HILIC with two neutral HILIC columns

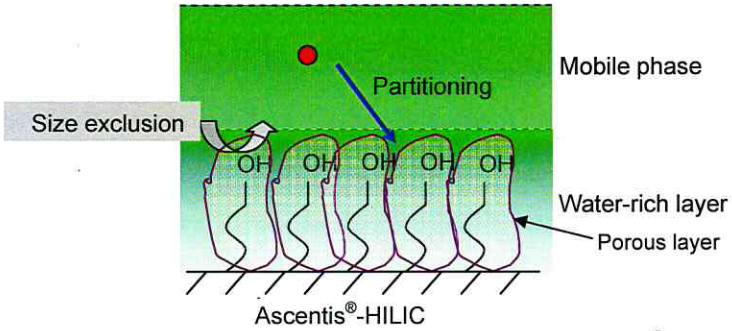
As described before, hydrophilic partitioning of analytes from mobile phase containing high percentage organic solvent into water-rich layer on stationary phase is the primary separation principle on HILIC column. At ZIC®-HILIC the separation can be still forced by electrostatic interactions between charged analytes and bifunctionalities on stationary phase. This is reasoned by zwitterionic functionalities of stationary phase. In order to comprehend and to measure the contribution of electrostatic interactions for separation, the retention behaviour of arsenic species at three different HILIC columns was compared:

- ZIC®-HILIC with zwitterionic stationary phase,
- Luna®-HILIC with neutral stationary phase,
- Ascentis®-HILIC containing a neutral stationary phase on a fused-core material.

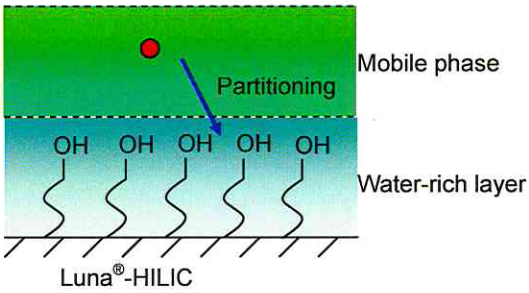
The structural explanation and separation mechanism of three different HILIC columns are schematically described in Fig 6.1.



(a): retention mechanism of analytes on ZIC®-HILIC



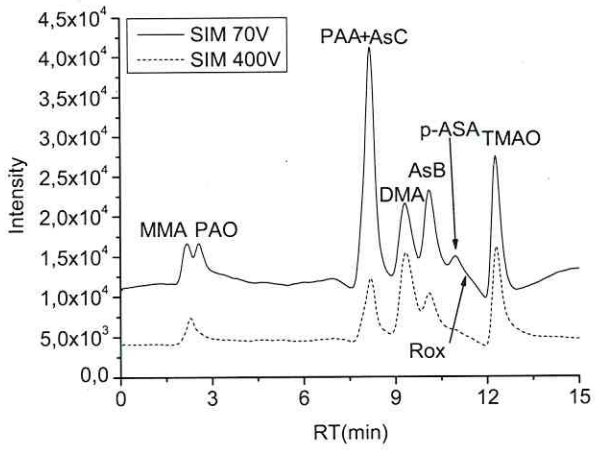
(b): retention mechanism of analytes on Ascentis®-HILIC



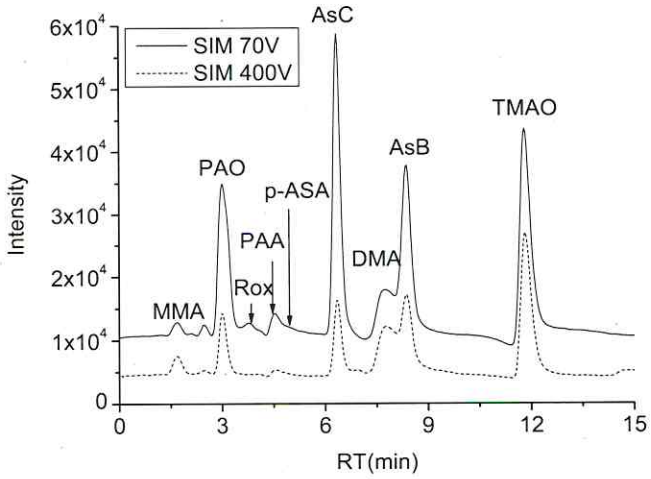
(c): retention mechanism of analytes on Luna®-HILIC

Fig 6.1 Structural explanation and separation mechanisms of three HILIC columns

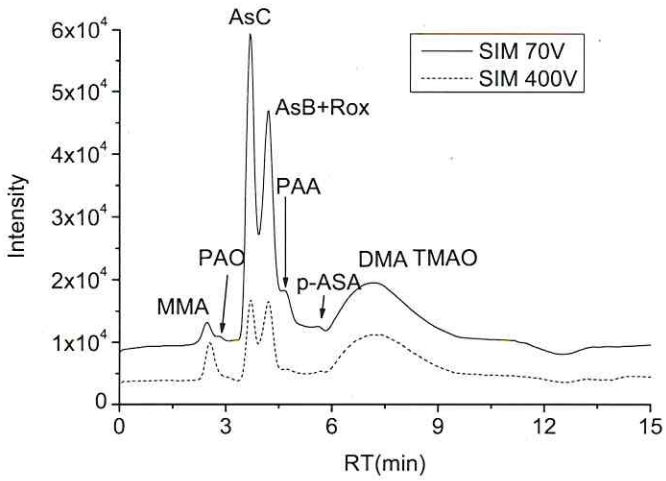
In fact, as shown in Fig 6.2(b) and (c), separation of nine arsenic species on Ascentis®-HILIC and Luna®-HILIC are not totally consistent with their retention on ZIC®-HILIC, illustrated in Fig 6.2(a) and summarized in Fig 6.3, This is valid in particular for Rox and p-ASA at Ascentis®-HILIC as well as for AsC, AsB, Rox and p-ASA at Luna®-HILIC. One can also seen in Fig 6.3, that on ZIC®-HILIC the retention of arsenic compounds is the strongest compared to retention at Luna®-HILIC and Ascentis®-HILIC. The retention strength of analytes on Luna®-HILIC is the weakest under the tested HILIC columns. The behaviour at ZIC®-HILIC can attribute clearly to the additional effect of the electrostatic forces, which acts in addition to the partitioning process. With exception of MMA, generally, it becomes clear that for all other compounds the retention factors are on the average 6 times higher (2 - 8.5) compared with the values obtained at Luna®-HILIC. Comparing the *k* values at ZIC®-HILIC vs. Ascentis®-HILIC the values are on average 3 times higher (1.5 - 7). As example retention factor (k= 9.11) of AsB at ZIC®-HILIC is eight times of retention (k= 1.097) on Luna®-HILIC and twice of separation (k= 4.578) on Ascentis®-HILIC. That shows that electrostatic interactions on ZIC®-HILIC is considerable.



(a): ZIC®-HILIC



(b): Ascentis®-HILIC



(c): Luna®-HILIC

Fig 6.2 Separation of arsenic species on three HILIC columns

Flow rate: 200 µl min^{-1}, injection volume: 8 µl;

Elution: A_1: 90% MeCN+10% 100 mM NH_4COOH pH 4.5

B_1: 50% MeCN+10% 100 mM NH_4COOH pH 4.5+40% H_2O

All chromatographic condition and gradient mobile phase in chapter 6 are same, and gradient mobile phase is described in Table 6.1.

Table 6.1 Gradient mobile phase description for separation of organoarsenicals

Time (min)	A(%): 90% MeCN+10% 100 mM NH_4COOH pH 4.5	B (%): 50% MeCN+10% 100 mM NH_4COOH pH 4.5+40% H_2O
0	90	10
1	90	10
1.1	70	30
5	80	20
5.1	0	100
7.5	0	100
7.6	80	20
15	80	20
16	90	10
20	90	10

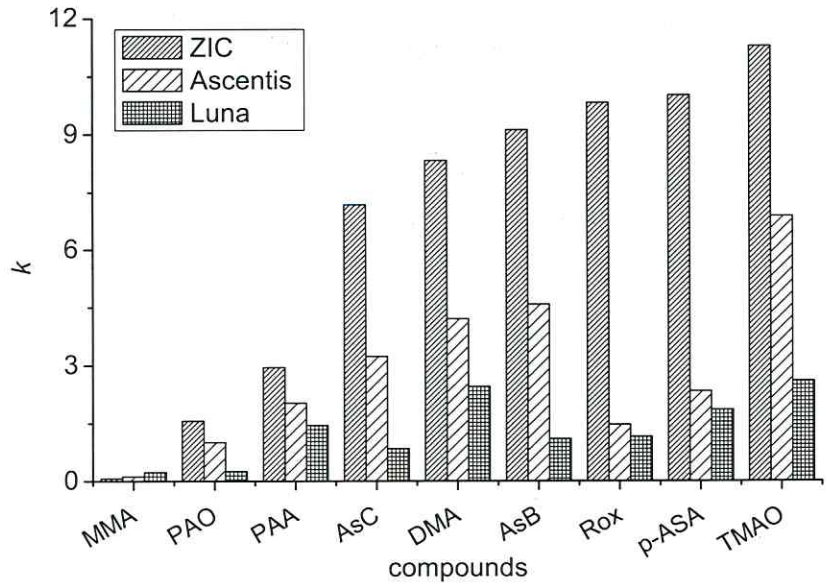


Fig 6.3 Comparison of retention factor (*k*) using three different HILIC columns

6.2 Separation characteristics of arsenic species on two neutral HILIC columns

As discussed, retention of arsenic compounds on ZIC®-HILIC is stronger than those at the two neutral stationary phases. It is believed that retention behaviour of analytes on Luna®-HILIC and Ascentis®-HILIC are variable, even if both HILIC columns are endowed with neutral phase. The results presented show that significant differences between the retention behaviour at the two columns with neutral stationary phases exist. With exception of MMA all retention factors (k) of the arsenic compounds at Luna®-HILIC are obviously smaller than at Ascentis®-HILIC. At neutral stationary phase, hydrophilic partitioning and possibly hydrogen bonding are forces for separation. Linear regression of retention factor (k) vs. polarity can be used to estimate the contribution of hydrophilic partitioning on the separation process. Assuming that all arsenic species are neutral due to the ion-pair formation with the eluent the calculated polarity of each molecule was used as data set of independent variables for regression.

The k values using Luna®-HILIC were used for such a regression. The coefficient of determination (R^2=0.125) for the linear regression of k vs. polarity (data are given in Table 2.2) is insufficient. If one uses however only selected species, that are three aromatic (PAO, p-ASA, PAA) and two methylated (AsB and AsC) compounds, we receive a linear equation with R^2=0.985 (as also shown in Fig 6.4). It is assumed that hydrophilic partitioning on HILIC is the dominating separation mechanisms for these compounds. A possible explanation for the similar behaviour of AsB and AsC with the phenylic species can be the formation of ring-like structures by these compounds. Possible ring formation of AsB and AsC are schematically stated in Fig 6.5 through inter- and inner molecular electrostatic interactions.

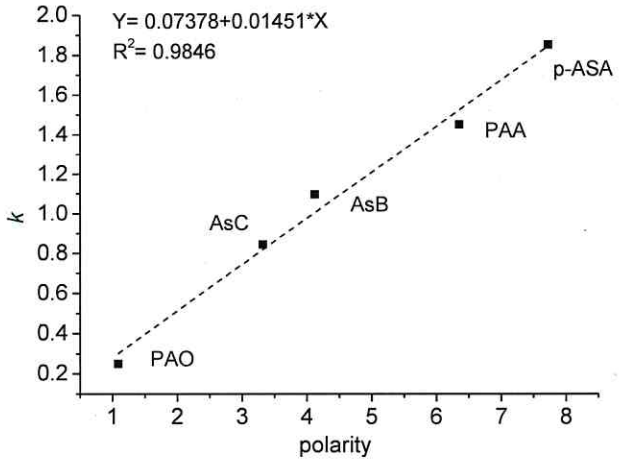
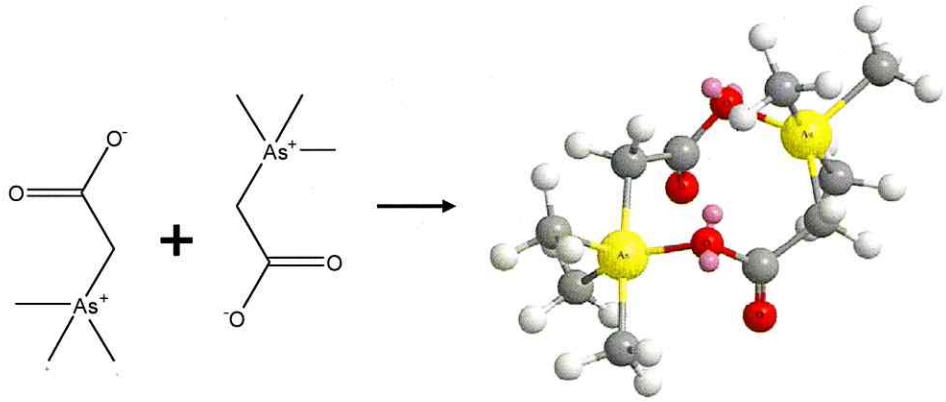
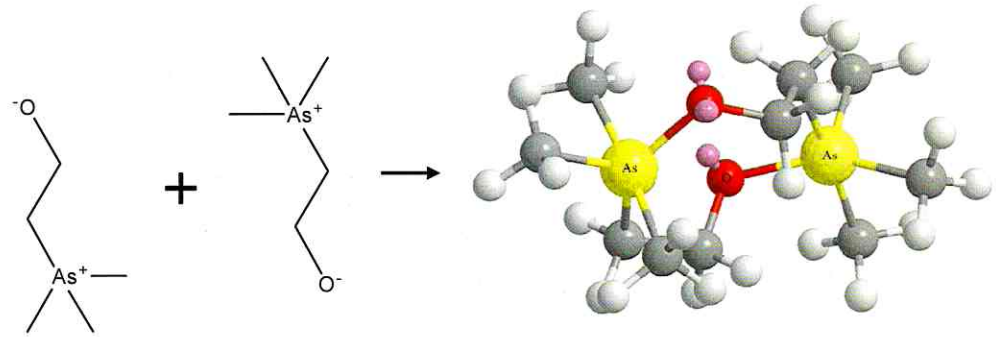


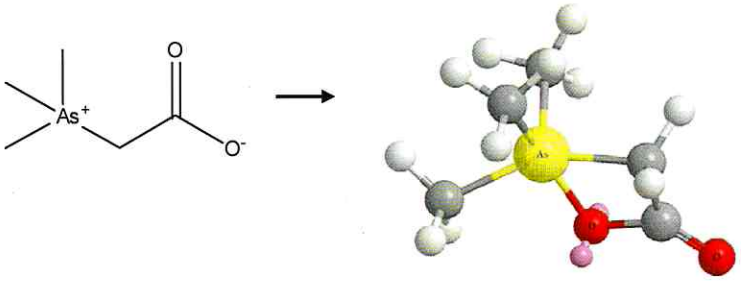
Fig 6.4 Linear regression of retention factor (*k*) and polarity of arsenic species on Luna®-HILIC



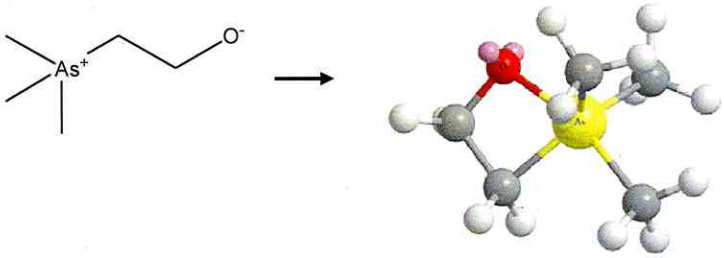
(a): AsB intermolecular interaction



(b): AsC intermolecular interaction



(c): AsB inner molecular interaction



(d): AsC inner molecular interaction

Fig 6.5 Proposals for molecular interactions of arsenobetaine (AsB) and arsenocholin (AsC)

The neutral stationary phase used in Ascentis®-HILIC is slightly different from Luna®-HILIC. Ascentis®-HILIC, produced by Fused-Core™ particle technology [1, 2], consisted of a solid core (a.d. 1.7 µm) and a porous shell of 0.5µm thickness. That means this modification acts additionally on retention behaviour of analytes compared with Luna®-HILIC. As proved in Fig 6.4, partitioning mechanism dominates normally in neutral HILIC. However, with the modification of the silica core by the porous layer new function on separation of analytes on Ascentis®-HILIC can be affected. To rearrange the retention order of arsenic species on Ascentis®-HILIC, it was found out in Fig 6.6 that two groups of compounds can be distinguished according to their molecular size, except for MMA located nearly to the dead volume. Big-sized molecules like aromatic arsenic compounds: PAO, Rox, PAA, p-ASA are eluted speedily relative to methylated arsenic compounds (AsC, DMA, AsB and TMAO). The differentiation might be explained by the effect of porous layer which can induce an additional separation by size exclusion.

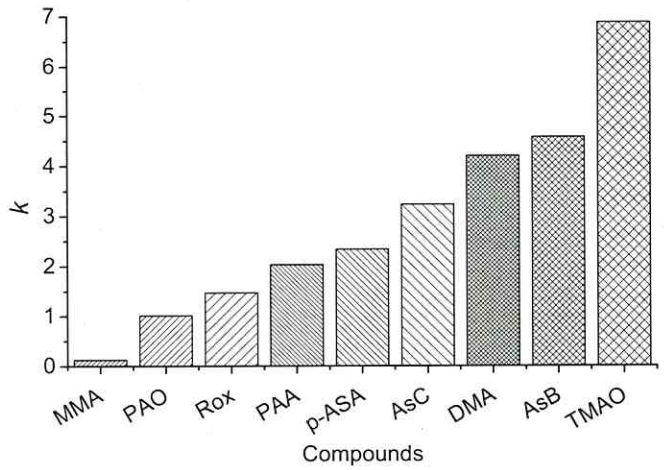


Fig 6.6 Retention factor (*k*) vs. arsenic molecular groups using Ascentis®-HILIC

However, the size exclusion effect cannot be described easily using molecular volume of each single arsenic compound (shown in Table 6.2) which does not exactly follow descending sequence. For example, PAO with the smaller molecular volume (volume: 65.31 cm^3/mol) is related to an initial separation position while AsB with 78.89 cm^3/mol molecular volume is in backend of separation. Hence, size exclusion efficiency of arsenic species (except for MMA) can be generally deduced but not analyzed in details, that aromatic arsenic compounds are separated firstly and methylated arsenic species are retained last, shown in Fig 6.6.

Table 6.2 Steric molecular volume of arsenic compounds

Substances	Volume (cm^3/mol)
MMA	46.960
PAO	65.309
Rox	100.800
PAA	79.754
p-ASA	86.479
AsC	77.414
DMA	51.621
AsB	78.890
TMAO	56.283

So far, the investigations of the separation mechanism of arsenic compounds on HILIC columns confirms that retention behaviour of arsenic compounds on each HILIC column is individually according to modified material on stationary phase. Commonly, hydrophilic partitioning of analytes from mobile phase containing high percentage organic

solvent into water-rich layer on stationary phase is the primary separation principle on HILIC column. On ZIC®-HILIC with zwitterionic stationary phase, the separation is still forced by electrostatic interactions between charged analytes and bifunctionalities on stationary phase, shown in Fig 6.1(a). On Ascentis®-HILIC with neutral stationary phase, size exclusion efficiency came from the porous shell on the fused core of the stationary phase, displayed in Fig 6.1(b). In addition, separation of arsenic compounds on Luna®-HILIC is regarded as the fundamental separation principle of HILIC column, hydrophilic partitioning in Fig 6.1(c).

6.3 Summary

By comparison of retention behaviour of arsenic species on ZIC®-HILIC and two neutral HILIC columns, Ascentis®-HILIC and Luna®-HILIC, separation mechanism of arsenic species is figured out that partitioning of analytes dominates the separation on each HILIC column. With various modified material on stationary phase, electrostatic interactions between charged analytes and zwitterionic modified stationary phase can happen on ZIC®-HILIC while on Ascentis®-HILIC a size exclusion effect from fused-core with porous shell material on stationary phase takes place. The electrostatic interactions on ZIC®-HILIC might be as strong as partitioning. The size exclusion efficiency on Ascentis®-HILIC is only generally deduced but not analyzed in details. Relatively, separation of analytes on Luna®-HILIC is only determined by partitioning.

References

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7 Hydrophilic interaction chromatography (HILIC) coupled to ESI-MS for detection of hydrophilic and hydrophobic arsenic species in marine organisms

Manuscript: D. Xie *et al.*, *J. Separation. Sci.* (in preparation)

Abstract This investigation presents separation of hydrophobic and hydrophilic arsenic species from original samples of marine organisms on hydrophilic interaction chromatography (HILIC) coupled with ESI-MS detection. The sample was extracted by mobile phase containing 90% acetonitrile (MeCN) and 10% ammonium formate (NH_4COOH 100 mM; pH 8) and can injected after centrifugation without any further sample pre-treatment. By comparison of separation of arsenobetaine (AsB) in certified reference material DORM-2 (dogfish muscle) at three HILIC columns e.g. ZIC®-HILIC, Ascentis®-HILIC and Luna®-HILIC combined with high efficient detention by ESI-MS, Ascentis®-HILIC is preferably fit for this investigation. AsB in DORM-2 is identified and quantified with good recovery (more than 97%). The analytical method using Ascentis®-HILIC with ESI-MS detection can be applied in detection of arsenolipids in extracts of cod liver, cod eggs and cod liver oil. Long chain arsenic lipids (m/z 333, 361, 405) could be found out in extracts of canned cod liver, but not in cod eggs and cod liver oil. In *Hijiki* algae, four arsenosugars (glycerol sugar, phosphate sugar, sulfonate sugar and sulfate sugar), which are main forms of arsenic species in algae, are successfully separated on Ascentis®-HILIC with ESI-MS detection as well.

Keywords: Arsenosugars / Hydrophobic arsenic species / Hydrophilic arsenic species / Hydrophilic interaction chromatography / Marine organisms

Abbreviations: HILIC, hydrophilic interactions chromatography; **AsB,** trimethylarsonioacetate, Arsenobetaine; **DMA,** dimethylarsinate; **$TMAs^+$,** tetramethylarsonium ion

7.1 Introduction

Arsenic is a metalloid well-known to be potentially toxic depending of its species. In a wide range of biological samples a lot of different of organoarsenic compounds had been found in the last decades [1-6]. The data concerning the number of un-known species in the last years (connected with improved analytical methods) strongly rose. Beside that for some years examined species, like arsenobetaine, (AsB), arsenocholine (AsC), monomethylarsonate (MMA), dimethylarsinate (DMA) and trimethylarsine oxide (TMAO), arsenosugars became interesting already in the last years [6-9], despite the fact that they were identified more than 40 years ago [10]. Arsenolipids have recently attracted considerable interest [11-13]. Metallothionine and phytochelatin complexes [14-16], respectively, are also in focus of recent studies.

In biologic and environmental samples, the determination of arsenic compounds was predominantly realized with high performance liquid chromatography (HPLC), particularly reversed-phase chromatography (RP-C) and ion chromatography (IC) coupled with inductively coupled plasma mass spectrometry (ICP-MS). However, these combined techniques were mainly employed to determine hydrophilic arsenic compounds. However, about the determination of more hydrophobic arsenic species one reported more rarely.

The direct separation of arsenolipids in fish oil by normal phase chromatography (NP-C) containing mobile phase with high content organic solution was investigated by Schmeisser et al. in 2005 [17]. For identification of hydrophobic arsenic species gas chromatography (GC) combined with mass spectrometric detection had been used [18]. But the determination of such

analytes in real samples by means of GC-MS is sophisticated since most of naturally-occurring arsenic species are non-volatile. Therefore, there is an eager demand to create a high efficient analytical method for the determination of hydrophilic and hydrophobic arsenic species together. Hydrophilic interaction chromatography (HILIC) could be the method of choice to solve this problem.

Since HILIC was first mentioned [19], it has emerged as an alternative to HPLC for applications dealing with hydrophilic and hydrophobic analytes. HILIC combines the characteristics of normal-phase chromatography (NP-C) and reverse-phase chromatography (RP-C). HILIC column consists of polar stationary phase modified by different functionalities and the separation is performed by a gradient generated by non-polar (acetonitrile) to polar (H_2O) mobile phase. The separation mechanisms on ZIC®-HILIC include hydrophilic partitioning, hydrogen bonding and electrostatic interactions [20]. That means different polar species can be analyzed after separation at HILIC column. Furthermore, high content of organic solvent in eluent solves the solubility issue of hydrophobic species.

Combined with parallel ICP-MS and ESI-MS detection the separation and determination of nine hydrophilic and hydrophobic arsenic species was realized [21]. From the fact we concluded that the identification of hydrophilic and hydrophobic arsenic species in biologic or environmental samples is feasible.

Thus, the goal of this work is the application of an optimized HILIC method for the determination of arsenicals in marine samples. HILIC separations combined with molecular selective detection by ESI-MS are utilized for determination of hydrophilic and hydrophobic arsenic species in extracts of samples originating from marine organisms (cod liver, cod eggs and cod liver oil) and algae (Japanese *Hijiki*). The application of HILIC technique for the determination of analytes in more hydrophobic matrices can support investigations in life and environmental sciences.

7.2 Experimental

7.2.1 Reagents and chemicals

All solutions were prepared with de-ionized water (Milli-Q, Millipore). The reagents (analytical grade p.a.) utilized in the study were as following: acetonitrile (MeCN), ammonium hydroxide (NH_4OH), ammonium formate (NH_4COOH), hydrogen peroxide (H_2O_2; suprapur, 30 %v/v) and nitric acid (HNO_3; suprapur 60 %v/v) (all MERCK), formic acid (HCOOH) (Fluka).

Trimethylarsonioacetate (arsenobetaine, AsB, BCR®-626, Fluka) standard solution was diluted by mobile phase (90% MeCN and 10% NH_4COOH (100 mM; pH 8.3)). The pH value was adjusted using NH_4OH and HCOOH.

7.2.2 Instruments

A µ-flow liquid chromatographic system (µ-HPLC) consisting of degasser, autosampler, binary gradient pump and ESI MSD (G1946 B and 6130) (Agilent) was used. The mass selective detector (MSD) in the positive ionization mode was applied. The scanning mode was used to identify fragments of the compounds. And single ion monitoring (SIM) was applied to improve the sensitivity. The analytical columns (ZIC®-HILIC (SeQuant); Ascentis®-HILIC (Sigma Aldrich); Luna®-HILIC, (Phenomenex) were used in these studies. An ICP-MS 7500ce (Agilent Technologies, USA) was performed for arsenic selective detection at m/z 75. A flow splitter (series 600, ASI company, USA), which separated flow rate by a ratio of 1:4, connected the LC with the two detectors ICP-MS and ESI-MS simultaneously. The optimized parameters of HPLC, ESI-MS and ICP-MS are listed in Table 7.1. Samples were digested microwave assisted, Multiwave (Perkin Elmer). The determination of total arsenic concentration in sample extracts was performed by ICP-MS (ELAN® 6000 DRC-e; Perkin Elmer).

Table 7.1 HPLC, ESI-MS and ICP-MS parameters

Chromatography parameters	
Columns	ZIC®-HILIC (SeQuant), 150*2.1 mm id, 3.5 µm Ascentis®-HILIC (Sigma Aldrich), 100*2.1 mm id, 2.7 µm Luna®-HILIC (Phenomenex), 150*2.0 mmid, 3.5 µm
Mobile phases	Gradient or isocratic: A: 90%MeCN and 10% NH_4COOH 100-1000 mM pH 4.5 B: 50%MeCN and 10% NH_4COOH 100-1000 mM pH 4.5
Flow rate	200-300 µL min^{-1}
Injection volume	8 µL
ESI-MS parameters	
Drying gas flow	10.0 L min^{-1}
Nebulizer gas pressure	30 psig
Drying gas temperature	350°C
Capillary voltage	Positive 1500 V-2500 V
Fragmentor voltage	70 V to 400 V (SIM, scanning)
ICP-MS parameters	
RF power	1600 W
Carrier gas	0.4 L min^{-1}
Makeup gas	0.11 L min^{-1}
Optional gas Ar/O_2(20%)	9.0 %
Nebulizer	Microconcentric + Scott-type spray chamber

7.2.3 Samples, sample preparation

DORM-2 (dogfish muscle), was used as certified standard reference material for recovery studies and optimization of HILIC separation. Aliquots of 0.2 g of the material were transferred into polyethylene tubes to which each 5 mL of mobile phase consisting of 90%-80% MeCN and 10%-20% NH_4COOH (100 mM, pH 8) or NH_4Ac (125 mM, pH 8.3) was added. Afterwards the samples were shaken over night at room temperature. The homogenate was centrifuged (5000 rpm, 5 min). The supernatant (8 µL) was injected into the chromatographic system without any further sample preparation.

Different marine food products were purchased from a local supermarket. Cod eggs food and cod liver oil (aliquots of 1.0 g) were directly used for further sample preparation. Cod liver canned food was handled by lyophilization (LOC-1M, ALPHA, CHRIST, Germany). Aliquots of 0.3 g of the lyophilized material were dissolved in mobile phase (5 mL) ultrasonically assisted (Bandelin Sonopuls GM200, Germany).

Standards of arsenosugars (glycerol sugar, phosphate sugar, sulfonate sugar and sulfate sugar kindly delivered by Prof. Kevin A. Francesconi, Karl-Franzens University Graz, Austria) with a concentration of 200 µg L^{-1} were used as reference of separation [22]. *Hijiki* algae (kindly provided by T. Kaise, Tokyo University of Pharmacy and Life Science), aliquot (0.2 g) of the different algae materials was dissolved in 5 mL mobile phase by shaking overnight. A volume of 8 µL of the supernatant was directly used for analysis.

The total concentration of arsenic was determined by ICP mass spectrometry after microwave assisted digestion as following: about 0.3 g of the material was weighted in 25 mL quartz vessels; afterwards 1.5 mL H_2O_2 (30 % v/v) and 4 mL HNO_3 (60 % v/v) were added and reacted overnight. The digestion was performed using a microwave device (Multiwave, Perkin Elmer) employing the following program: 0 min at 100 W; 5 min at 600 W; 10 min at

1000 W; 15 min cooling down. The resulting solution was diluted to 15 mL with deionized water (Direct-Q^{TM} 5; Millipore) and afterwards centrifuged (5000 rpm, 5 min). Every sample preparation was repeated twice.

The value of total arsenic concentration of each sample is listed in Table 7.2.

Table 7.2 Total arsenic concentration of each sample

Samples	Total As concentration (mg kg^{-1})
Cod liver	10.8
Cod eggs	1.38
Cod liver oil	2.48
Japanese *Hijiki* algae	108
DORM-2	18.9±0.5*

*: Reference total arsenic concentration in DORM-2: 18.0 ±1.1 mg kg^{-1}

7.3 Results and discussion

7.3.1 Optimization of HILIC columns

In this investigation, the performance of three different HILIC columns are compared by determination of AsB in certified reference material DORM-2 (dogfish muscle) combined with ESI-MS detection, in order to select the most suitable for determination of arsenic species in extract of marine samples. These HILIC columns are ZIC®-HILIC, containing the zwitterionic stationary phase, Luna®-HILIC and Ascentis®-HILIC that both HILIC columns comprise of the neutral stationary phase.

The chemical structure and chromatographic parameters of the arsenic species under investigation are shown in Fig 2.3.

It is found as shown in Fig 7.1 that AsB extracted from DORM-2 is separated efficiently on the Ascentis®-HILIC column. The ESI-MS detection was performed with respect to the identification of the molecular structure $[M+H]^+$ with m/z 179 with a low fragment voltage of 70 V. The second signal resulted from the detection of m/z 75, 91 (As^+/AsO^+) with high fragmentor voltage of 400 V. Under this condition the chromatogram can be used for element quantification. The comparison of the retention behaviour of arsenic species on three HILIC columns shows, that AsB (BCR 626® standard) is stronger retained in ZIC®-HILIC column compared to Luna®-HILIC and Ascentis®-HILIC, because of the effect of electrostatic interactions between bifunctionalities on stationary phase and charged analytes on ZIC®-HILIC. Additionally, the retention of AsB is different on Luna®-HILIC and Ascentis®-HILIC to see in Fig 7.2A, even if both of HILIC columns are neutral. It is believed that the retention behaviour of AsB on three HILIC columns can be explained by individual HILIC separation mechanisms.

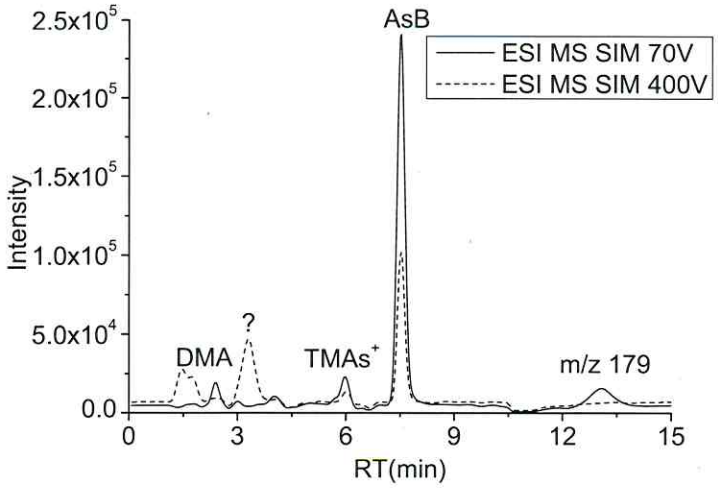


Fig 7.1 Separation of AsB in certified reference material DORM-2 (dogfish muscle) on Ascentis®-HILIC with detection by ESI-MS

Gradient mobile phase: timetable of the gradient mobile phase shown in Table 7.3

Detection: ESI-MS SIM Positive 1, fragmentor voltage 70 V, (m/z) $[M+H]^+$: 136 ($TMAs^+$), 139 (DMA), 179 (AsB)

ESI-MS SIM Positive 2, fragmentor voltage 400 V, (m/z): 75 (As^+), 91 (AsO^+)

Table 7.3 Composition and gradient program for the mobile phase in separation of arsenolipids

Time (min)	A(%): 90 % MeCN and 10 % NH_4COOH 100 mM pH 4.5	B (%): 50 % MeCN and 10 % NH_4COOH 100 mM pH 4.5	MeCN % in mobile phase
0	90	10	86
1	90	10	86
1.1	70	30	78
5	80	20	82
5.1	0	100	50
7.5	0	100	50
7.6	80	20	82
15	80	20	82
16	90	10	86
20	90	10	86

Theoretically, hydrophilic partitioning dominates the primarily separation of analytes on HILIC. Retention behaviour of analysis is decided by the polarity of analytes. With increased polarity of the mobile phase, retention of polar compounds is decreased. AsB is analyzed faster using isocratic elution with decreased MeCN concentrations (from 80% to 50% MeCN), shown in Fig 7.2A. All HILIC columns show the same trend with increasing water content in the eluent which is attributed to the changed conditions for the separation by hydrophilic partitioning. The differences in the retention factors for the HILIC columns under investigation results from the specific HILIC column type:

- ZIC®-HILIC: stationary phase is modified by zwitterionic functionalities - additional electrostatic interaction
- Luna®-HILIC: stationary phase is modified by diol (neutral) functionalities - only hydrophilic partitioning
- Ascentis®-HILIC: stationary phase is modified by diol (neutral) functionalities on a fused-core material with porous silica shell - partitioning with size exclusion effects

Therefore, retention of AsB on ZIC®-HILIC is longer than the separation on two neutral HILIC columns because of double forces. Luna®-HILIC can separate AsB within the shortest time because of relatively simple separation mechanism. On Ascentis®-HILIC, small molecular size AsB is prolonged by size exclusion efficiency relative to retention on Luna®-HILIC. Of course, compared with ZIC®-HILIC (150*2.1 mm id, 3.5 µm) shown in Table 7.1, the length of Ascentis®-HILIC (100*2.1 mm id, 2.7 µm) is also factor shortened retention of AsB. It is believed the Ascentis®-HILIC is better suitable to analyze arsenic species than the other two HILIC columns. Separation on Ascentis®-HILIC is moderately, which is neither as fast as on Luna®-HILIC nor so slow as on ZIC®-HILIC.

The comparison of the intensity and shape of AsB peaks on three chromatographic conditions after HILIC separation shows, that the chromatographic signal of AsB on

Ascentis®-HILIC is the highest and sharpest compared to ZIC®-HILIC and Luna®-HILIC. It is supported that detection efficiency of AsB by ESI-MS totally relies on eluent status, which is indirectly dependent of gradient separation on various HILIC columns. Gradient eluent in retention time is considered as the vital reason of different detection efficiency in ESI-MS. AsB ionization in ESI-MS at different retention time on each single HILIC column is varied with gradient mobile phase containing different composite of MeCN and relevant ratio of buffer solution. The gradient mobile phase contains 86% to 50% MeCN, and details are described in Table 7.3.

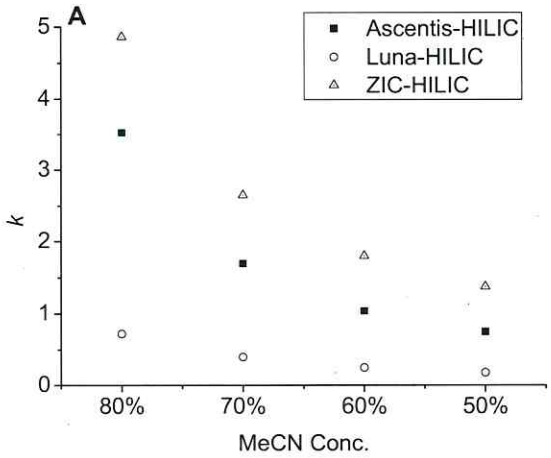
After AsB separation on Ascentis®-HILIC, AsB is detected at 7.5 min by ESI-MS, when mobile phase contains relative lower MeCN concentration (50% MeCN, shown in Table 7.3). ESI-MS detection efficiency, including ionization and nebulization efficiencies, at an eluent composition containing 50% MeCN might be higher in comparison with detection conditions with higher MeCN concentration, leading to high intensive and sharp AsB peak in Fig 7.2.

The influence of MeCN percentage on detection efficiency is investigated for further explanation of MeCN influence. Relative peak area of AsB, which is separated on three HILIC columns with a constant composition of the mobile phase varied between 80% and 50% MeCN, is compared in Fig 7.2B. Fig 7.2B displays that ESI-MS detection efficiency is decreased with raised isocratic MeCN concentration and independent of separation on HILIC columns. This phenomenon argues with the MeCN influence theory stated in previous paragraph.

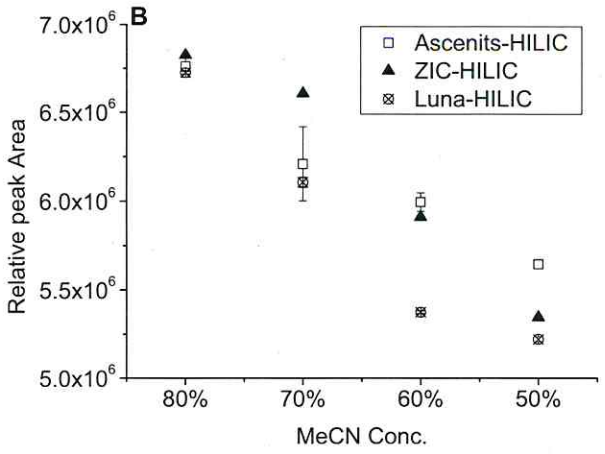
In ESI-MS, complicated gradient mobile phase might lead to acutely changed pressure and MeCN, which isocratic mobile phase cannot reflect the chromatographic change. This is probable reason to have contradictory phenomena between theoretical presumption and experimental data in Fig 7.2B. Thus, classic and relative simple gradient mobile phase with decreased 90%-70% MeCN (0-2 min 90% MeCN, 2-8 min 90%-70% MeCN, 8-12 min

70% MeCN) was investigated to explain MeCN influence to ionization efficiency in ESI-MS in Fig 7.2C. After ZIC®-HILIC separation time 3.5 min, which AsB is detected under mobile phase containing proximately 85% MeCN, relative peak area is the highest. At retention time of AsB at about 9.5 min and 11 min, mobile phase containing about 70% MeCN leads to lower relative peak area. This means that lower MeCN concentration in simple gradient mobile phase causes lower detection efficiency in ESI-MS. Under isocratic and linear gradient mobile phase conditions, ESI-MS detection is more efficient than of lower MeCN content. But under complex gradient mobile phase, ESI-MS detection efficiency is perhaps affected by not only MeCN ratio, but also other chromatographic parameters, such as pressure.

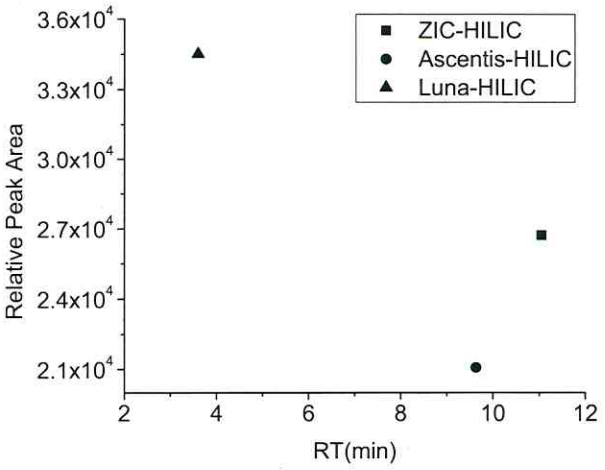
In terms of moderate separation of AsB and efficient detection under gradient mobile containing less MeCN concentration, Ascentis®-HILIC is indeed better fit for analysis of hydrophilic arsenic species.



A: Retention factor (k) of AsB versus MeCN ratio through isocratic elution



B: Relative peak area of AsB versus MeCN ratio through isocratic elution



C: Relative peak area of AsB versus retention factor (*k*) through simple gradient elution

Fig 7.2 The investigation of MeCN Influence on ESI-MS detection efficiency by comparison of separation of standard AsB on three HILIC columns

Detection: ESI-MS SIM Positive 1, fragmentor voltage 70 V, (m/z) $[M+H]^+$: 179 (AsB)

ESI-MS SIM Positive 2, fragmentor voltage 400 V, (m/z): 75 (As^+), 91 (AsO^+)

7.3.2 Identification and quantification of arsenic species in DORM-2

DORM-2 as certified marine reference materials were analyzed following the above procedure to ensure the accuracy and precision of the analytical procedures (mineralization and ICP-MS determination of total arsenic and identification/ quantification of arsenic species on Ascentis®-HILIC). As referred in Table 7.2 the total concentration of arsenic was found within the certified range, indicating the applicability of the extraction procedure. On the other hand for the determination of arsenic species using HILIC separation and ESI-MS detection after appropriate leaching matrix matched calibration functions are required. DORM-2 with a certified content of 16.4 ± 1.1 mg As kg^{-1} was used as basis for standard addition to quantify AsB. By addition of different amounts (0.2, 0.4, 0.6 mg L^{-1}) of standard solution of AsB (BCR®-626) with concentration of 1.085 mg As L^{-1} into the DORM-2 extracts, a regression plot with a good linearity (R^2 = 0.9955) was realized. According to the linear equation ($y= 4.14*10^6+1.5*10^7*x$; peak area vs. added concentration), the quantitative results (17.2 ± 0.4 mg kg^{-1} as As) obtained for AsB in the extracts of DORM-2 agreed well with certified concentration (16.4 ± 1.1 mg kg^{-1}).

Tetramethylarsonium ($TMAs^+$) has a minor concentration of 0.248 ± 0.054 mg As kg^{-1}, which is probably beyond the LOD of the HILIC chromatography with ESI-MS detection. Obviously, although DORM-2 might be contain other arsenic species such as DMA, As(III) or As(V), which could be identified in the process of determination of AsB in DORM-2, the content of these arsenic species are not actually certified in DORM-2. Therefore, the quantification of $TMAs^+$, DMA, As(III) and As(V) was not accomplished.

7.3.3 Identification of arsenolipids in marine tissues

As discussed before, the characteristics of HILIC permit also the analysis of hydrophobic arsenic compounds. On one hand hydrophobic arsenic species are soluble in the mobile phase (with typically high concentrations of organic solvent). On the other hand, the organic solvent can guarantee hydrophilic partitioning of different polar arsenic species. The eluent sequence is following the polarity ascending of analytes as recently shown [21, 23].

Unfortunately one cannot use ICP-MS for the sensitive detection of the species because of the high contained organic solvent, which is used in HILIC. The inductively coupled plasma is unstably even if one transports only very small sample volumes of the extract into the plasma and additionally oxygen as optional gas is added. Therefore, the ESI-MS with two detection modes was used. Single (or selected) ion monitoring (SIM) with positive ionization mode of mass detection of arsenic species $[M+H]^+$ with low fragmentor voltage (SIM Pos 70V) is used for identification, and the detection mode in ESI-MS under high fragmentor voltage (SIM Pos 400V) for arsenic ions detection (As^+/AsO^+, m/z 75, 91) is used for quantification.

Identification of arsenic species focuses on the analysis of AsB, which is the most abundant arsenic species in marine tissues, in addition to compounds of DMA and $TMAs^+$ with low concentrations. As Fig 7.3A and Fig 7.3B shown, in extracts of cod liver and cod eggs, AsB is separated clearly on Ascentis®-HILIC and detected by ESI-MS. Certainly, as shown in Fig 7.3A and Fig 7.3B, some other compounds detectable on SIM m/z 75, 91, which might be contain arsenic but with unknown mass spectra are observed, such as, in Fig 7.3A question marked peaks at approximately 1.5 min, 3 min and 11 min and unknown "arsenic" compounds at about 1.5 min and 10.5 min in Fig 7.3B, respectively. With high fragmentor voltage 400 V, those peaks are detected as arsenic ions (As^+/AsO^+, m/z 75, 91). But, under lower fragmentor voltage 70 V, molecular mass spectra of those arsenic compounds could

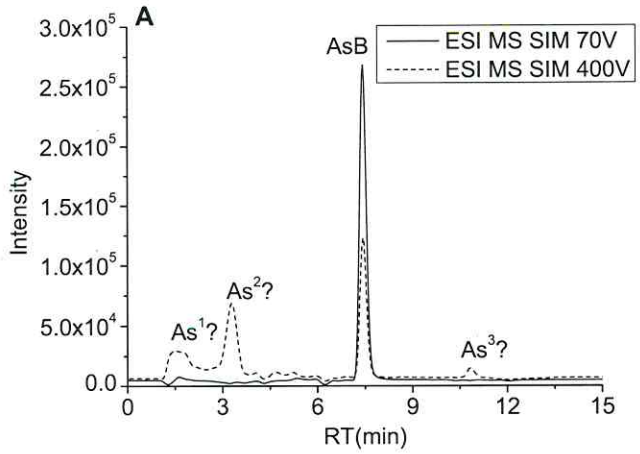
not be found. On the contrary, in Fig 7.3B, some peaks are identified by molecular mass spectra (m/z 179, 139 and 135) without arsenic ions signal, like peaks at retention time 3 min and 12 min. At retention time 6 min in Fig 7.3B, peak with weak As/AsO^+ signal (m/z 75, 91) resembles DMA (m/z 139) molecular detection. As Fig 7.3C shown, AsB (m/z 179) and $TMAs^+$ (m/z 135) are individually decided at about 7.5 min and 4.5 min, while DMA (m/z 139) is not indentified according to uncertain spectrometric signal. However, so far, the HILIC method was mainly used to identify arsenic species in marine tissue, but not further quantification of them.

7.3.4 The discovery of long chain arsenic lipids in marine tissues

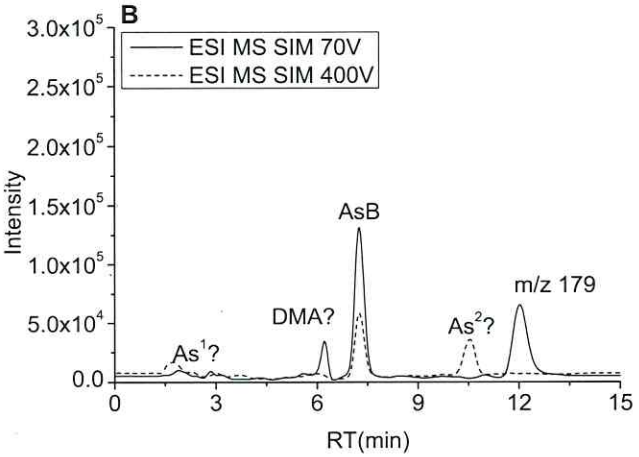
Recently, Francesconi et al. reported on the evidence of the long chain arsenolipids in fish oil samples [12, 17, 18]. Therefore, further identification of long chain arsenolipids in cod liver, cod eggs and cod liver oil is processed in this investigation. As shown in Fig 7.3 arsenic species with 'unknown mass spectra' appeared, such as, peaks at 1.5 min, 3 min and 11 min in Fig 7.3A and 1.5 min and 10.5 min in Fig 7.3B.

Scanning (m/z 300-500) positive ionization mode is used to study molecular mass and fragments of these long chain arsenolipids, whereas high limit of detection (LOD) disrupts further experiments. So, molecular mass spectra with m/z 179, 333, 361, 405 referred in literature [24] (molecular structure shown in Fig 2.3B) are investigated, coupled with fragment arsenic structure detection m/z 106 ($(CH_3)_2AsH^+$) under fragmentor voltage 150V, as well as As^+ and AsO^+ (m/z 75, 91) detection with 400 V fragmentor voltage (see Fig 7.3D). As Fig 7.3D shown, in cod liver, substances with mass spectra (m/z 333, 361, 405) are determined at about 1.5 min nearly the dead volume, shown in the figure in right top of Fig 7.3D. However, mass spectra with m/z 333 and 405 are not arsenic species because of absent

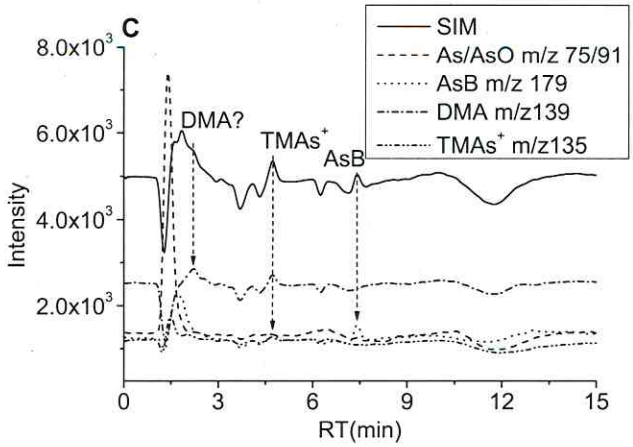
identification of arsenic ions. Thus, peaks with masses 333 and 405 are not certainly ensured to correspond on these long chain arsenic compounds reported in reference [24]. For certainty, long chain arsenic lipid with mass 361 is determined about 2.2 min with identification and quantification by molecular and arsenic ions spectra. Similarly, molecular mass spectra of some arsenic compounds, like peaks at about 3 min (with As/AsO^+ (m/z 75/91) signal) and 6 min (with m/z 106 $(CH_3)_2AsH^+$ detection), are not certainly identified. But, without expected, in cod eggs and cod liver oil these long chain arsenolipids could not be detected under same HILIC chromatographic condition, which data are not shown here.



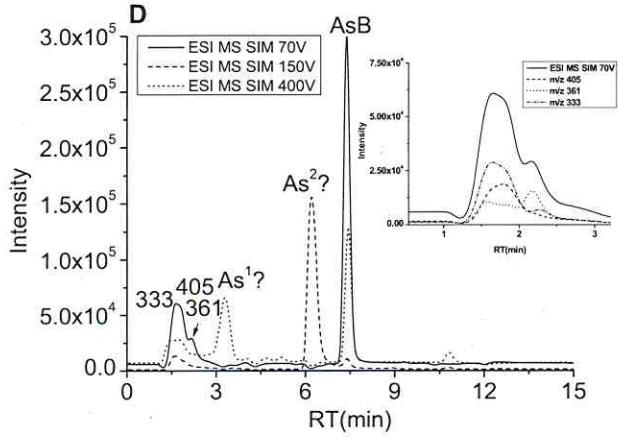
A: cod liver



B: cod eggs



C: cod liver oil
Gradient mobile phase: timetable of the gradient mobile phase shown in Table 7.3
Detection: ESI-MS SIM Positive 1, fragmentor voltage 70 V, (m/z) $[M+H]^+$: 136 ($TMAs^+$), 139 (DMA), 179 (AsB)
ESI-MS SIM Positive 2, fragmentor voltage 400 V, (m/z): 75 (As^+), 91 (AsO^+)



D: Long chain arsenic lipids in cod liver
Detection: ESI-MS SIM Positive 1, fragmentor voltage 70 V, (m/z) $[M+H]^+$: 179 (AsB), 333, 361, 405 (the structure of long chain arsenic lipids are shown in Fig 2.3B)
ESI-MS SIM Positive 2, fragmentor voltage 150 V, (m/z) $[M+H]^+$: 106 ($(CH_3)_2AsH^+$)
ESI-MS SIM Positive 3, fragmentor voltage 400 V, (m/z): 75 (As^+), 91 (AsO^+)

Fig 7.3 Separation of arsenolipids in extracts from marine tissues by Ascentis®-HILIC with detection by ESI-MS

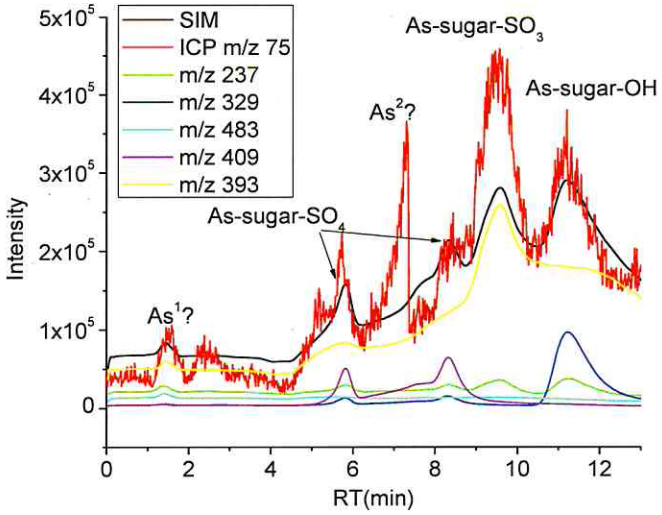
7.3.5 Determination of arsenosugars in algae

In algae, arsenic species mainly appear as arsenosugars (glycerol, phosphate, sulfonate and sulfate sugars) [13, 25]. Chemical structures and chromatographic characteristics of these arsenosugars are shown in Fig 2.3C. After HILIC separation, different operation modes in ESI-MS detection are used to 'softly' detect arsenic species, including arsenic ions and arsenosugars fragment with m/z 237. And fragmentor voltage for identification of As/AsO+ (m/z 75, 91) is 400 V. The typical fragment of arsenosugars m/z 237 is detected by ESI-MS with a fragmentor voltage of 150 V. ICP-MS was applied for element selective detection of arsenic ions As+ (m/z 75). Gradient mobile phase is shown in Table 7.4.

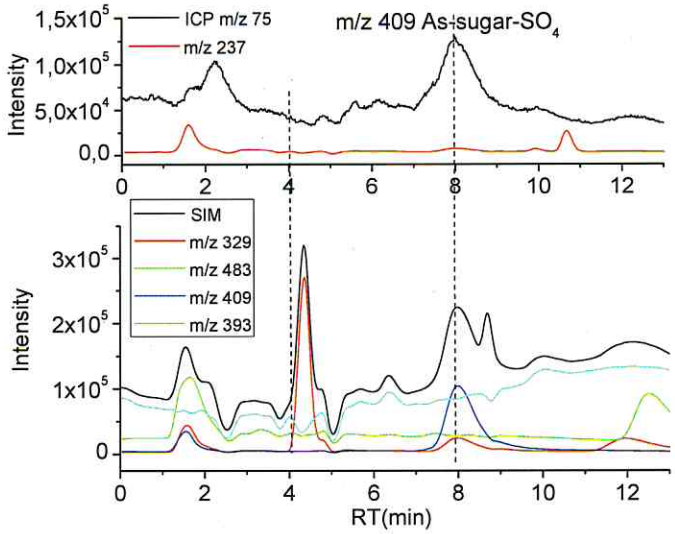
As shown in Fig 7.4A, arsenosugars (glycerol sugar m/z 329, phosphate sugar m/z 483, sulfonate sugar m/z 393, sulfate sugar m/z 409) are analyzed in standard solutions prepared from this four arsenosugars. Actually, glycerol sugar, sulfate sugar and sulfonate sugar are separated through optimized HILIC method combined with arsenic ions detection by ICP-MS and molecular selective ESI-MS shown in Fig 7.4A. But within the analysis time of 12 min, phosphate sugar is not analyzed. Arsenic compounds with unknown mass spectra has appeared at dead volume and retention time 7min, as these two questioned arsenic compounds shown in Fig 7.4A. To use the optimized HILIC method in analysis of arsenic species in *Hijiki* found similarly that only sulfate sugar could be identified clearly by three reasons (Fig 7.4B):

a) arsenic selective ICP-MS signal (m/z 75) at retention time 8 min
b) molecular selective ESI-MS signal for As-sugar sulfate (m/z 409) at retention time 8 min
c) typical As-sugar fragment ESI-MS signal at m/z 237 at retention time 8 min

For glycerol sugar and sulfonate sugar also ESI-MS signals on their molecular mass could be found, but the appropriate ICP-MS and/or the typical fragment ESI-MS signal could not be detected at the same retention time. The possibilities for identification of arsenosugars are also limited by the existence of different peaks with the same m/z. The presence of arsenic in a separated compound can be clearly proved by ICP-MS shown in Fig 7.4. However, the ICP-MS works well only by using a low flow of the eluent. That is why the ICP-MS signal intensity is low and noisy.



A: analysis of standard arsenosugars



B: HILIC separation arsenosugars in *Hijiki* algae after extraction by Eluent A

Fig 7.4 HILIC separation of arsenosugars in a standard solution and algae extract by Ascentis®-HILIC with detection by ICP-MS (m/z 75) and ESI-MS (SIM, m/z: 237, 329, 393, 409, 483)

Gradient mobile phase: timetable of the gradient mobile phase shown in Table 7.4

Detection: ESI-MS SIM Positive 1, fragmentor voltage 70 V, (m/z) $[M+H]^+$: 329, 483, 393, 409 (the structure of arsenosugars are shown in Fig 2.3C)

ESI-MS SIM Positive 2, fragmentor voltage 300 V, (m/z) $[M+H]^+$: 75 (As^+), 91 (AsO^+)

ESI-MS SIM Positive 3, fragmentor voltage 150 V, (m/z) $[M+H]^+$: 237 ($C_7H_{14}O_4As$) (the structure of fragment is shown in Fig 2.3C)

Table 7.4 Composition and gradient program for the mobile phase in separation of arsenosugars

Time (min)	A(%): 90 % MeCN and 10 % NH_4COOH 100 mM pH 4.5	B (%): 50 % MeCN and 10 % NH_4COOH 100 mM pH 4.5	MeCN % in mobile phase
0	90	10	86
2	90	10	86
10	0	100	50
12	0	100	50
12.1	90	10	86
17	90	10	86

7.4 Conclusions

In summary, the application of Ascentis®-HILIC with ESI-MS detection fits better for analysis of arsenic species in marine organisms than the other HILIC columns under investigation. With efficient and successful HILIC method, arsenolipids including long chain arsenic lipids in marine tissues (cod liver, cod eggs, cod liver oil) and arsenosugars in *Hijiki* algae can be identified and quantified. But, so far, the variable detection efficiency, which is caused by gradient eluent and interference of substances in samples, cannot be explained very well.

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8 Conclusions

In this research, analytical methods for simultaneous separation of different polar arsenic species on HILIC combined with ESI-MS and ICP-MS detection by variation of the MeCN content were investigated. By means of optimized HILIC method, 5 methylated and 4 aromatic arsenic species can be separated in one run within 45 minutes by using a gradient of organic solvent (80%-50% MeCN) in mobile phase. The analytes were detected by means of simultaneous parallel mass spectrometric detectors, by ESI-MS and ICP-MS.

Separation mechanisms of arsenic species were explained by investigation of chromatographic parameters on ZIC®-HILIC, at which the stationary phase contains zwitterionic functionalities. Generally, neither '*retention factor (log k) vs. polarity of arsenic species (log $P_{o/w}$)*' nor '*k vs. hydrogen bonding parameter*' yielded the good linear regressions, i.e. hydrophilic partitioning and hydrogen bonding are not the only separation mechanisms of arsenic species on ZIC®-HILIC. But if one divides the arsenic species under this study according to their chemical structure in methylated and aromatic arsenic compounds, both hydrophilic partitioning and hydrogen bonding support the separation mechanism to a different degree.

Separation of arsenic species on ZIC®-HILIC is highly dependent on buffer conditions. On ZIC®-HILIC, buffer pH affects the electrostatic interactions between bifunctionalities on stationary phase and charged arsenic species. The salt additives (ammonium acetate, ammonium formate) in mobile phase comprehensively impact the retention behaviour of arsenic species. This is because salt ions influence the ion-pair formation with bifunctionalities of stationary phase and chemical interactions with analytes as counter

analytes ions in the eluent, resulting in increased or decreased retention of arsenic species on ZIC®-HILIC.

The retention behaviour of arsenic species on different HILIC columns is compared. On one hand, HILIC separation mechanism is dominated by partitioning of analytes between two phases. On the other hand, stationary phase with different modified material leads to additional interactions influencing the retention behaviour of analytes. ZIC®-HILIC is characterized by electrostatic interactions of the analyte ions with the zwitterionic stationary phase. Using Ascentis®-HILIC size exclusion effects take place additionally to the partitioning mechanism, which can be observed on the retention order of aromatic and methylated arsenic species. The aromatic arsenic species with larger molecular size are separated firstly and methylated arsenic species are eluted later, caused by the pores in the surface layer of the fused-core material. Luna®-HILIC column used contain a neutral stationary phase which is characterized by a primary partitioning mechanism as to see by the good linear regression of '*retention factor (k) vs. polarity*'.

Compared with established analytical methods for separation of arsenic species, it is possible to separate arsenic species with a broad range of polarity simultaneously employing HILIC. In practice, the optimized method for analysis of various polar arsenic species is applied successfully for separation and identification of arsenic species in samples originating from marine tissues and algae. Long chain arsenic lipids fractionated from marine fish tissues and arsenosugars extracted from algae samples can be separated using HILIC principles.

9 Outlook

The current HILIC research applied for organic and inorganic arsenic analysis is a challenge and breakthrough of analytical methodology. Analytical methods for separation of various polar arsenic species coupled with detections by ESI-MS and ICP-MS have been optimized continuously. Separation mechanisms of HILIC have been investigated. These mechanistic investigations of HILIC were described by hydrophilic partitioning and hydrogen bonding of analytes based on calculations in aqueous phase, only. In fact, HILIC eluent contains a very high ratio of organic solvent, leading to the shifts in pH, pK_a of analytes and dissociation degree of arsenic species considering the real existing composition of the eluent. In order to better insight into HILIC separation mechanism in water/organic phase, mathematic models for optimization of HILIC mechanisms in eluent should be introduced in future. The application of HILIC methods in the analysis of metalloids and metal species in environmental and biochemical samples should further optimize not only for identifications of analytes, but also for more accurate quantifications.

A disadvantage of the studies was in the strong interferences in ICP-MS detection caused by the kind and the composition of the applied eluents. Further investigations should be focused on modification of the sample introduction systems in ICP-MS and / or on application of other organic solvents, e.g. THF, as eluents in HILIC to overcome these problems.

To improve the separation efficiency of metal organic / metalloid organic species more suitable columns with a higher capacity should be developed and tested for the simultaneous determination of hydrophilic and hydrophobic species.

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Leipzig, 26. März 2010