CAPILLARY LIQUID CHROMATOGRAPHY-MASS SPECTROMETRY AND LASER INDUCED PLASMA BREAKDOWN SPECTROSCOPY FOR ARSENIC SPECIATION AND METABOLITES IN MARINE BIOTA SAMPLES

DISSERTATION

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DEDICATED TO MY FAMILY

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SUMMARY

In modern separation science the popularity of capillary liquid separation technique has been rapidly expanding. Capillary high-performance liquid chromatography (cHPLC) is attractive so as to obtain separations with high resolution and to realise an easy interfacing to mass spectrometers. This study makes use of a coupling of cHPLC to inductively coupled plasma (ICP)- and electrospray (ESI)- mass spectrometry (MS) and deals with their application for trace speciation analysis of arsenic (As) in various marine samples. On-line coupled systems are preferable for speciation analysis as they avoid contamination and/or disintegration or change of the original metal or metalloid species. Two analytical strategies have been evaluated for the determination of As species: cHPLC-MS and a novel laser-induced breakdown spectroscopy method based on plasma generation of mono-disperse microdroplets (LADROP-LIBS). The potential of all these techniques were critically compared for the analysis of marine samples.

Arsenic is an element of high interest, as it occurs in many chemical forms which have different toxicological effects in the environment and the food chain. Many organic arsenic compounds of variable toxicities are found in marine fauna and flora. The organoarsenicals are of lower toxicity or appear to be non-toxic compared to inorganic As [1]. Marine algae appear to accumulate and transform inorganic arsenic mainly to dimethylarsinoylriboside derivatives (arsenosugars) and other methylated arsenic compounds, while in marine animals arsenobetaine and some methylated arsenicals are predominant [51].

cHPLC coupled on-line either with elemental (ICP) or molecular (ESI) MS was used for arsenic speciation analysis in codfish, dogfish, brown and red marine algae. A major aim was development and application of highly effective separation method based on cHPLC for the determination of trace amounts of arsenic species in aquatic samples. The performance of several commercial and in-house packed cHPLC columns was also studied. After a careful optimisation, a strong anion-exchange (SAX) Hamilton PRP-X100 resin, which was packed in-house into a cHPLC column (250 x 0.5 mm, 10 µm particle size) showed to be the optimum for a simultaneous separation of anionic, neutral and amphoteric inorganic and organoarsenic compounds. cHPLC using a strong cation-exchange (SCX) resin was applied as a complementary method, so as to confirm the results obtained under SAX conditions, particularly the presence of the tetramethylarsonium (TMAs) ion in codfish and to realise the separation of As (III), arsenobetaine (AsB), arsenocholine (AC) and arsenosugars, although phosphate-ribose co-eluted to some extent with TMA oxide (TMAO) and As(V).

SAX cHPLC coupled to ICP-MS showed to have a great potential for the determination of different As species in marine biota after liquid/liquid extraction with a water-methanol mixture. Concentrations of As ranging from 0.7 to 24 µg/g of As species were observed in

fish and algae. They were confirmed and identified by on-line SAX cHPLC coupled to ESI-MS/MS. AsB and arsenosugars were shown to be the most abundant forms of As in both algae. Differences in the distribution of different As species in red and brown algae were also found to occur. Although the main species in algae are arsenoribosides and AsB, small traces of dimethylarsinic acid (DMA), As(V) and other As related compounds (e.g. phytochelatins) were detected by cHPLC coupled to ICP-MS and ESI-MS/MS. The newly developed complementary procedures provided higher chromatographic efficiency, resolution and selectivity and a high sensitivity for As speciation in marine algae. The detection limit of the method was at the sub pg level for As species (0.1 pg of AsB). The accuracy of the measurements was tested by analysis of certified reference material (CRM) dogfish DORM-2 and the results obtained were in very good agreement with the certified values.

Six more, so far unidentified compounds could be observed in the SAX chromatograms of cHPLC coupled to ICP-MS of the algae extracts and were further investigated by cHPLC coupled to ESI-MS. The results indicated a presence of As compounds containing thiol groups of glutathione (GSH) and glutathione derived peptides, so called phytochelatins (PCs). Oligomers of two to six sub-units, respectively PC_2, and PC_6, were found in the algae extracts as well as DMAs-PC, As-PC_3 and iso-PC_5-As complexes. cHPLC coupled to ESI-MS/MS, as expected has proved to be a very powerful and valuable tool for the characterisation of bio-ligands synthesised by the algae in response to As exposure. Such compounds are unlikely to be toxicologically significant because of their lower concentrations, but they may offer information on the metabolism and the origin of As compounds in marine algae.

The novel analytical system built on the base of LIBS of plasma generated mono-disperse microdroplets, referred to as LADROP-LIBS, was developed at GKSS Research Centre in Geesthacht, Germany for As speciation and/or elemental analysis. The technique was studied for determinations of Na, Ca, Mg and Cu, because As speciation was not possible. The results showed the potential of the piezoelectric droplet generation principle as a new sample introduction system for element specific detectors coupled to micro scale liquid separation methods, such as cHPLC, especially for applications with sub-µL sample volumes. cHPLC on-line coupled to LADROP-LIBS using the in-house packed SCX Hamilton PRP-X200 column was applied for a separation and determination of Na, Cu, Ca and Mg ions in two seawater CRMs with different salinity - *SLEW-3* and *NASS-5*.

The complementary application of cHPLC-ICP-MS and cHPLC-ESI-MS/MS provided a powerful tool for the determination of As species in complex biological samples, such as marine algae. cHPLC coupled to ICP-MS and LADROP-LIBS were shown to have potential for real-time analyses following the separation of the compounds of interest. They also have multi-element capability and high detection power.

ZUSAMMENFASSUNG

Die Beliebtheit kapillarer Flüßigkeitstrennverfahren ist in der modernen Trennverfahrenstechnik rasch gestiegen. Kapillar-Hochleistungsflüssigkeitschromatographie (cHPLC) ist eine attraktive Methode, um hochauflösende Trennungen mit einfacher Anbindung an Massenspektrometer zu erzielen. In dieser Arbeit wird eine Verbindung von cHPLC mit induktiv gekoppeltem Plasma (ICP), Elektrospray (ESI) und Massenspektrometrie (MS) verwendet. Sie befasst sich mit der Analyse von Zerfallsprodukten des Arsens (As) (oder Arsenverbindungen) in verschiedenen Meerwasser-Proben. Online gekoppelte Systeme werden für derartige Analysen bevorzugt, da sie sowohl eine Verschmutzung als auch den Zerfall oder die Veränderung der ursprünglichen Metall- und Metalloidverbindungen vermeiden. Zwei analytische Strategien sind für die Bestimmung von Arsenverbindungen angewendet worden: cHPLC-MS und die neue Laser-induzierte Zerfalls-Spektroskopie (LIBS), die auf der Plasmaerzeugung aus mono-zerstreuten Mikro-Tröpfchen (LADROP-LIBS) basiert. Beide Techniken und deren Möglichkeiten wurden kritisch für die Analyse der Meerwasser-Proben verglichen.

Arsen ist als Element von großem Interesse, da es in vielen chemischen Formen auftritt, die verschiedene toxikologische Auswirkungen auf die Umwelt, z.B innerhalb einer Nahrungskette haben. Viele organische Arsenverbindungen, mit unterschiedlich toxischen Wirkungen, sind in der Meeresfauna und -flora vorzufinden. Die organischen Arsenverbindungen sind von geringerer Toxizität oder scheinen sogar nicht toxisch zu sein gegenüber As [1]. Meeresalgen sammeln anorganisches Arsen und wandeln dieses in dimethylarsinoylriboside Derivate (Arsenzucker) und andere methylierten Arsenverbindungen um, während bei Meerestieren Arsenobetaine und einige methylierte Arsenikale als Endprodukt überwiegen [51].

Die cHPLC, entweder mit elementarem (ICP) oder molekularem (ESI) MS online gekoppelt, wurde für die Bestimmung arsenhaltiger Verbindungen im Kabeljau, Dornhai sowie braunen und roten Meeresalgen verwendet. Ein Hauptziel war die Entwicklung und die Anwendung einer auf cHPLC basierenden, hochgradig wirkungsvollen Trennverfahrens zur Bestimmung von Arsenverbindungen in den Meeresproben im Spurenbereich. Untersucht wurde weiterhin die Effektivität einiger kommerziell und betriebsintern verpackter cHPLC-Säulen. Nach sorgfältiger Optimierung stellte sich die Hamilton-PRP-X100 Säule, welche aus einem starken Anionenaustauschharz (SAX) (10 µm Partikelgröße) besteht, als optimale Lösung dar, um eine simultane Trennung der anionisch, neutral und amphoter anorganischen und organischen Arsenverbindungen zu erreichen. Die cHPLC, mit einem starken Kationenaustauschharz (SCX) als Säule, wurde als ergänzende Methode angewendet. Ziel war die Bestätigung der Resultate die unter Anionenaustauschbedingungen erzielt wurden, insbesondere das Vorkommen des

Tetramethylarsonium (TMAs) Ion im Kabeljauextrakt. Weiterhin sollte zusätzlich die Trennung von As (III), Arsenobetaine (AsB), Arsenocholine (AC) und Arsenzuckern erreicht werden, obgleich Phosphatribose in gewissen Ausmaßen mit TMA Oxid (TMAO) und As(V) koeluiert.

Die Kopplung von SAX-cHPLC mit ICP-MS zeigte großes Potenzial für die Bestimmung der unterschiedlichen Arsenverbindungen in der Meeresflora und -fauna nach Flüssig/ Flüssig-Extraktion mit einer Wasser-Methanol-Mischung. Arsen-Konzentrationen von 0.7 bis 24 µg/g wurden als Arsenverbindungen bei Fischen und Algen beobachtet. Sie wurden durch die SAX cHPLC, on-line gekoppelt mit dem ESI-MS/MS, bestätigt und identifiziert. AsB und Arsenzucker erwiesen sich als die am häufigsten vorkommenden Arsenverbindungen in beiden Algen. Weiterhin wurden Unterschiede in der Verteilung von As innerhalb verschiedener Arten der roten und braunen Algen festgestellt. Obgleich die Hauptkomponenten in den Algen Arsenoriboside und AsB sind, wurden auch geringe Spuren von Dimethyl-arsenige Säure (DMA), As (V) und andere Arsen-bezogene Verbindungen (z.B. Phytochelatine) mit Hilfe der cHPLC, die mit ICP-MS und ESI-MS/MS gekoppelt war, bestimmt. Die neu-entwickelten, ergänzenden Verfahren ergaben eine höhere chromatographische Effizienz, Auflösung und Selektivität sowie eine hohe Empfindlichkeit für Arsenverbindungen in den Meeresalgen. Die Nachweisgrenze des Verfahrens lag auf dem sub-pg Niveau für Arsenverbindungen bei 0.1 pg AsB. Die Genauigkeit der Messungen wurde durch die Analyse eines zertifizierten Referenzmaterials (CRM) DORM-2 überprüft und die erzielten Resultate stimmten sehr gut mit den zertifizierten Werten überein.

Sechs weitere, bis jetzt nicht identifizierte Verbindungen konnten in den SAX-cHPLC-Chromatogrammen, gekoppelt mit ICP-MS der Algen-Extrakte beobachtet werden. Diese Verbindungen wurden weiter mit Hilfe von cHPLC, gekoppelt mit ESI-MS, untersucht. Die Ergebnisse zeigten das Vorhandensein von As-Komponenten an, die Thiolalkoholgruppen, bestehend aus Glutathion (GSH) und von Glutathion abgeleitete Peptide, sogenannte Phytochelatine (PC), enthalten. Oligomere von zwei bis sechs Untereinheiten, beziehungsweise PC_2 und PC_6, wurden in den Algen-Extrakten gefunden, ebenso wie DMAs-PC, As-PC_3 und iso-PC_5-As Komplexe. Die cHPLC in Verbindung mit ESI-MS/MS erwies sich, wie erwartet, als sehr leistungsfähiges und wertvolles Werkzeug für die Kennzeichnung von Bio-Liganden, die von den Algen als Reaktion auf eine As-Aussetzung synthetisiert worden waren. Solche Komponenten sind aufgrund ihrer geringen Konzentration toxikologisch eher unbedeutend, könnten aber Informationen über den Metabolismus und den Ursprung der Arsenkomponenten in den Meeresalgen liefern.

Das neuartige Analysesystem basierte auf LIBS, welches ein Plasma verwendet, das durch mono-zerstreute Mikro-Tröpfchen generiert wird. Es wird als LADROP-LIBS bezeichnet und wurde im GKSS Forschungszentrum Geesthacht, Deutschland, für die Bestimmung von Arsenverbindungen und/oder Element-Analytik entwickelt. Diese Technik

wurde ursprünglich für die Bestimmungen von Na, Ca, Mg und Cu entwickelt, wobei die Bestimmung von Arsenverbindungen damals nicht möglich war. Die Resultate zeigen das Potenzial des piezoelektrischen Tröpfchen-Generationsprinzips als neues Probensystem für element-spezifische Detektoren, die mit mikro-flüssigen Trennungsmethoden gekoppelt werden – z.B. der cHPLC – und insbesondere für die Anwendungen mit sub-µL-Probenmengen. Die cHPLC wurde online gekoppelt mit dem LADROP-LIBS unter Verwendung einer betriebsintern verpackten SCX-Hamilton-PRP-X200 Säule und für eine Trennung und Bestimmung der Na-, Cu-, Ca- und Magnesium-Ionen in zwei Meerwasser-CRMs mit unterschiedlichem Salzgehalt, *SLEW-3* und *NASS-5*, verwendet. Die ergänzende Anwendung von cHPLC-ICP-MS und von cHPLC-ESI-MS/MS erwies sich als leistungsfähiges Werkzeug zur Bestimmung der Arsenverbindungen in den komplexen biologischen Proben wie z.B. Meeresalgen. Die cHPLC in Verbindung mit ICP-MS und LADROP-LIBS, zeigte ein großes Potenzial für eine Echtzeitbestimmung nach einer Trennung der in Frage kommenden Komponenten. Diese besitzt ein hohes Nachweisvermögen und ist für verschiedene Elemente einsetzbar.

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TECHNICAL ABBREVIATIONS AND ACRONYMS

Abbreviations

AAS	Atomic absorption spectrometry
AC	Arsenocholine
AED	Atomic emission detection
AsB	Arsenobetaine
c	Speed of light
CCD	Charge-coupled device
CE	Capillary electrophoresis
cHPLC	Capillary HPLC
CID	Collision induced dissociation
CRM	Certified reference material
DAD	Diode array detector
DD	Droplet detector (photodiode)
DIHEN	Direct injection high-efficiency nebulizer
DIN	Direct injection nebulizer
DMA	Dimethylarsenic acid
DP	Declustering potential
E	Energy level
ESI	Electrospray ionisation
FIA	Flow injection analysis
GFAA	Graphite furnace atomic absorption
h	Planck constant
HEN	High efficiency nebulizer
HPLC	High performance liquid chromatography
I.D.	Internal diameter
ICP	Inductively coupled plasma
ICP-MS	Inductively coupled plasma-mass spectrometry
ILT	Institute of laser technology
IP	Ion – pair
IR	Infrared
I.S.	Internal standard
IUPAC	International Union of Pure and Applied Chemistry
K	Degrees Kelvin
k	Retention factor

LC	Liquid chromatography
LD	laser diode
LIBS	Laser-induced breakdown spectroscopy
LOD	Limit of detection
MALDI	Matrix assisted laser desorption ionisation
MeC	Measurement chamber
MCI	Multi channel integration
MCN	Microconcentric nebulizer
MeOH	Methanol
MLD	Maximum lethal dose
MMA	Monomethylarsenic acid
MRM	Multiple reaction monitoring
MS	Mass spectrometry
MS/MS	Tandem mass spectrometry
N	Theoretical plate
NMR	Nuclear magnetic resonance
NRC	National Research Centre
O.D.	Outer diameter
ODS	Octadecyl-silica
OES	Optical emission spectroscopy
PC	Phytochelatine
PDG	Pulse (piezoelectric) droplet generator
PE	Polyethylene
PEEK	Polyetheretherketone
PM	Photomultiplier
PP	Polypropylene
PSDVB	Polystyrene-divinylbenzene
PVC	Polyvinyl chloride
QMS	Quadrupole mass spectrometry
RF	Radio frequency
RP	Reversed phase
R_s	Chromatographic resolution
SAX	Strong anion-exchange
SCX	Strong cation-exchange
SEM	Scanning electronic microscopy
SFMS	Sector field mass spectrometry
SI	Stroboscopic illumination

TIC	Total ion chromatogram
TMAO	Trimethylarsenic oxide
TMAP	Trimethylarsoniopropionate
UV	Ultra violet
VIS	Visible
XIC	Extracted ion chromatogram

Units

%	Percentage
℃	Degree Celsius
µg/g	Microgram/gram
µL	Micro litre
µm	Micro meter
Å	Ångström
a.u.	Absorbance units
cm	Centimetre
g	Gram
Hz	Hertz
Da	Daltons
kg	Kilogram
L	Litre
mbar	Millibar units of pressure
meq	Milliequivalent
mg	Milligram
mJ	Millijoule
mL	millilitre
mm	Millimetre
mM	Millimole
m/z	Mass-to-charge-ratio
MΩ	Megaohm
nL	Nanolitre
nm	Nanometre
pL	Pico litre
ppb	Part per billion
Ps	Pico seconds
psig	Pounds per square inch *gauge*

rpm	Revolutions per minute
s (sec)	Seconds
V	Voltage
W	Watt

Symbols

ά	chromatographic separation factor
γ	gamma molecular structure
λ	wavelength
v	frequency

1 INTRODUCTION

Arsenic and some heavy metals are dangerous because they tend to bioaccumulate. For example, marine organisms can consume a particularly dangerous form of mercury called methylmercury [1, 2]. When fish eats these organisms, the methylmercury is not excreted, but retained in their body tissue. The older the fish and the more contaminated organisms it has consumed, the larger is the amount of methylmercury in its tissues. When another fish eats the first fish, the accumulated methylmercury is passed through the food chain, eventually amounting to hundreds or thousands of times its original concentration. An organism at the top of the food chain (humans, polar bears etc.) faces a serious risk of mercury poisoning by eating such fish. This is also the case with a bioaccumulation of As and its species by marine fauna and flora and subsequently a potential risk to human health. Since all heavy metals and arsenic, respectively, become toxic at some concentration level, the accumulation in the body will usually be accompanied by a detoxification of the metal to make it unavailable in the metabolism so as to restrict its potential toxic effects. The role of organic materials in complexing heavy metals remains uncertain, but they may be very valuable [1, 4, 5]. Therefore, the need for element speciation increased more recently, especially in the cases of mercury and arsenic. Indeed, some forms of As have different properties from other forms and this can have a significant impact on the toxicity and treatment efficiency of water and on biological systems requiring research on speciation analysis.

In regard to the terminology, “speciation” refers to determination of a particular kind of atomic nucleus, atom, molecule or ion, or to the fact that a metal ion (or a group of atoms) in solution can potentially exist in a variety of forms (i.e. as a free ion, as a number of complexes and this in different oxidation states). So as to determine the chemical speciation of an element its oxidation state and all forms of that element (or cluster of atoms of different elements) in a given matrix must be identified.

The International Union for Pure and Applied Chemistry (IUPAC) has published guidelines or recommendations for the definition of speciation analysis [6]:

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

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

Speciation of an element: is the distribution of an element over defined chemical species in a system.

To conclude, chemical speciation means the determination of particular chemical forms of an element. Metals/metalloids may be speciated by oxidation state (for example, As(V) vs.

As(III) and Cr (III)/Cr (VI)). From an ecological point of view, it might be more relevant to express the residue level of single elemental species in pollutants than to mention the total element concentration. However, in the case of marine biota information on the chemical forms is also essential for understanding the risk and the role of the present element as well as to learn about its environmental cycle [2].

Chemical speciation in biological samples may also necessitate development of analytical procedures for trace metals and interpretation of analytical findings, since it has to be considered that methods intended to determine the total dissolved concentrations may not be adequate to determine strongly complexed fractions of certain metals. In order to understand the environmental chemistry and the biochemistry of trace element, including the essential and the toxic ones, it would be necessary to characterise in full the fractions and the chemical characteristics of all its forms under the diverse conditions possible in natural systems. Speciation is a rapidly developing field of research, especially in the recent years because of major areas such as separation science, trace element spectrometry and analytical biochemistry. Speciation seeks to characterise at least some of the most important forms of an element in order to understand the transformations between forms, which can occur and must be considered because of environmental consequences.

1.1. Analytical methodology

In modern analytical chemistry, methods and procedures are developed for qualitative and quantitative determination of the components in a complex mixture. To evaluate a single element or a group of elements, the different substances and substance classes have to be separated from each other. Currently, separations in capillary systems are very popular, especially when the need to analyse smaller amounts of liquids sample arises. Capillary high performance liquid chromatography (cHPLC) is an attractive method for these tasks as it enables high resolution separations with flow rates and sample amounts suited for easy interfacing to mass spectrometers. Some problems with the hyphenation of such methods still remain that are predominantly caused by the difficulty of coupling a separation procedure taking place into liquid phase with a detection method that relies on the formation of gas-phase ions. Nevertheless, cHPLC was preferred in this research as it is a powerful separation method offering high chromatographic resolution and efficiency.

In this work an experimental approach for the on-line coupling of cHPLC to atomic and molecular mass spectrometry methods such as inductively coupled plasma mass spectrometry (ICP-MS) and electrospray ionisation mass spectrometry (ESI-MS), respectively, has been undertaken to study naturally occurring small molecular organometallic species of As as well as arsenic-binding biomolecules in marine biota. Unknown arsenic-related species detected by cHPLC-ICP-MS in edible marine algae have

been further investigated and identified using ESI tandem MS (ESI-MS/MS). Further, newly developed analytical system based on laser-induced breakdown spectroscopy (LIBS) with plasma generation of mono dispersed microdroplets, referred to as LADROP-LIBS has been also studied for elemental analysis and on-line coupling with cHPLC was realised. First results of LADROP-LIBS and cHPLC-LADROP-LIBS hereby are reported.

1.2. Hyphenated methods

One of the main reasons for the widespread use of liquid chromatography (LC) coupled to mass spectrometry (MS) lays in the combination of an effective separation method with a highly selective and sensitive detection. Nevertheless, the field of interfacing of liquid-phase separation procedures and mass spectrometers is rapidly developing, both with respect to instrumentation and to the miniaturisation of separation technologies [7]. Different interfaces and ionisation systems have been developed or modified to enable progress here. One approach to overcome the limitations in using micro-scale separations is the coupling to micro flow nebulizers for ICP-MS. Some groups use a direct-injection nebulizer (DIN) or a high-efficiency nebulizer (HEN) for hyphenation, while others utilised a total consumption micro nebulizer with a single-pass low-volume spray chamber [8-10].

In recent years, more and more attention has been paid to the biomonitoring of As species by more sophisticated hyphenated systems, based on a combination of element specific detection methods with separation methods. The term hyphenated methods is often assigned to these analytical methods, of which the best known are gas chromatography - inductively coupled plasma mass spectrometry (GC-ICP-MS), HPLC- inductively coupled plasma mass spectrometry (HPLC-ICP-MS) or HPLC-atomic emission spectrometry (HPLC-AES) and HPLC-atomic absorption spectrometry (HPLC-AAS) [11, 12].

The two most commonly employed ionisation methods for MS in As speciation are inductively coupled plasma (ICP), atmospheric pressure ionisation (API) using electrospray or ionspray techniques and atmospheric pressure chemical ionisation (APCI) [11, 12]. The feature of HPLC-MS for determination of ionic and polar species has been demonstrated in many applications that range from environmental to bioanalytical areas [11-17]. The amount of energy that is transferred to the analytes during the electrospray (ESI) and the APCI processes is relatively small compared to ionisation methods like ICP or EI ionisation. Accordingly, ESI and APCI are called soft ionisation methods. The relatively small amount of energy transferred in ESI and APCI results in mass spectra that are characterised by little fragmentation and base peaks, and include mostly pseudomolecular ions. These pseudomolecular ions are typically formed by protonation in the positive-ion mode and deprotonation in the negative-ion mode but coordination of the analyte with other ions may also be used [18]. This is the reason why ionic and polar substances such as As species are

successfully ionised with good ionisation yields whereas less polar analytes which are not as easily protonated or deprotonated are less accessible to the ESI or APCI processes.

The targets of bioinorganic trace analytical chemistry, i.e. the detection, identification and characterisation of substances and products of a reaction of As with components of living cells and tissues rise many unravelled questions. New analytical approaches are developed and applied to gain more evidence of detoxification and biotransformation of arsenic. A novel laser-induced breakdown spectroscopy (LIBS) of microdroplets has been tested in this work for the application of direct liquid microsampling. There a unique piezoelectric pulse droplet generation sample introduction system, referred to as LADROP, has been used.

1.3. Research aim

It is proved that the distribution, the mobility and the biological availability of the chemical elements, such as As depend not only on their concentration but, significantly, on the forms, i.e. oxidation state in which they occur in natural systems. In the last two decades much attention has been paid to the metalloid arsenic (As) and its species [1, 13, 16]. Unfortunately, speciation analysis of As is not as common as traditional total elemental determinations because speciation data are accepted only by some regulations, as there are no laws or regulations on this matter, except for mercury and methylmercury [19]. It is believed that the lack of species-specific regulations is due to the absence of methods that can reliably determine the analytes of interest at the regulatory levels. For instance, the analytical methods currently available for elements such as As and Cr are either not selective enough or do not provide sufficiently low detection limits.

Most of the information available on As speciation comes from theoretical models rather than from analytical approaches. The methods used to determine the total concentration of the dissolved elements may not include strongly complexed fractions of As and other metals. The development of analytical procedures is also a relevant question for the chemical speciation of traces of As as well as for the interpretation of the analytical findings. In the last decade, there have been considerable improvements in the sampling and analytical procedures for trace metals and metalloids [20]. On-line coupled instrumental techniques are preferred for speciation analysis in order to avoid any contamination, breakdown or change of the original metal/metalloid containing species [12, 14]. Continuing developments and challenges in modern analytical chemistry, in particular in hyphenated systems and in the rationalisation of sample preparation procedures and clean-up, have often made speciation analyses practicable even where analytes are present at ultra trace levels, as it is often the case with As in biological samples.

Typically the body concentrations of As species in marine organisms are at the µg/g or ng/g levels. One problem in bioinorganic analysis by hyphenated methods is lack of extremely low detection limits with respect to the biosamples [8, 17, 21-24]. Clogging or contamination of the standard ICP nebulizers and of the sample orifice is a further problem that arises as a consequence of highly concentrated sample matrixes or mobile phases and buffers in conventional HPLC speciation analysis. Here cHPLC has the big advantage of using only a few nL of sample and much less concentrated buffer solutions as well as mobile phases at µL/min flow rates. cHPLC enables also a high resolution, selectivity and very low detection limits despite the ultra low flow rates (<15 µL/min). Although the coupling of capillary liquid chromatography with ICP-MS is promising for speciation applications, no attempts to use this set-up for As speciation in marine biota samples were reported up to the work for this dissertation [25]. As speciation using separation by capillary liquid chromatography in particular was chosen for investigations with two different types of algae and fish.

The objectives of the research described in this work are to explore and to assess the potential and the suitability of cHPLC coupled on-line with both, elemental and molecular mass spectrometry for the determination of organoarsenic compounds present in marine biota samples and the novel implementation of the LADROP-LIBS system for elemental and/or As analysis.

For that purpose, commercially available and mainly in-house packed cHPLC columns were utilised for a separation of different substances containing arsenic. An in-house modified microconcentric nebulizer and a small-volume spray chamber were used to connect cHPLC and ICP-MS. After tests with standard solutions, the procedures developed for As speciation were applied to real marine biota samples of fish and edible algae. Complementary to this approach other types of separation or detection were considered for full identification and confirmation of the analytical findings for As species present in codfish and brown and red algae. A confirmation of the results of cHPLC coupled to ICP-MS with respect to molecular identification and structural elucidation of target and non-target As species present in marine algae has been performed with triple quadrupole MS coupled on-line to cHPLC (cHPLC-ESI-MS/MS). Codfish and edible algae were chosen as good representative samples of the marine fauna and flora with environmental and human health significance.

Different algae and fish were examined for possible new As species, which may offer information on the origin, the metabolism and the toxicity. However, references As compounds were employed for most of the experiments. As the sample preparation for the As speciation in marine biota including the extraction step had already been extensively reported in the literature [26-73], additional investigations on the sample preparation were considered as out of the scope of the work. Furthermore, it was not the aim to develop fully

validated methods because cHPLC technique is a completely new approach for As speciation in marine biota and the focus of this work is its overall applicability and feasibility. However, accuracy tests of the measurements were performed by analysing dogfish certified reference material DORM-2 for As species.

There is still much to discover about marine life and ecosystems. Indeed, a deeper, multidisciplinary scientific understanding and investigation is what marine bioanalytical chemistry and in particular As speciation at micro scale levels in marine algae and fish here could contribute.

2 THEORETICAL BACKGROUND ON ARSENIC SPECIATION

Arsenic is a ubiquitous element that occurs in the nature in the form of inorganic and organic compounds. This element belongs to the group of the heavy metals. Arsenic has been well known as a poison for centuries, but its effects are highly dependent on its chemical forms, i.e. oxidation state. In addition to differences in toxicity of the different chemical forms of arsenic, also their mobility in the environment may differ. Therefore, new environmental regulations must be developed to specifically monitor individual species of arsenic. Here an overview of As speciation and their analysis reported in the last 15 years is given as As and its speciation in marine biota are the main aim of this investigation.

2.1 Arsenic and arsenic species in marine biota

Arsenic belongs to the category of elements which have no physiological function. However, its toxicity depends primary on the species [1, 3]. In general, it has been found that trivalent As (reduced form) compounds are more toxic than the pentavalent forms. Further, inorganic As is more toxic than the organic forms. Animal studies have shown that methyl- and phenyl-arsenates can produce health effects similar to those produced by inorganic As [2, 59]. Nevertheless, the organic metabolic species of As in marine biota have been found to be less toxic or harmless. However, it must be pointed out that the Maximum Lethal Dose (MLD_{50}), being the dose that is fatal for half a population of experimental animals for As(III) is 14 mg/kg, whereas for arsenobetaine (trimethylarsonioacetate) it is above 10 g/kg [54]. Naturally occurring inorganic As and organoarsenic compounds of environmental significance are listed in Table 7-1 and Table 7-2 in the appendix.

2.1.1 Description, chemical and physical properties

- Name: **Arsenic**
- Symbol: **As**
- Atomic number: **33**
- Atomic weight: **74.92160**
- CAS Registry ID: **7440-38-2**
- Group number: **15**
- Group name: **Pnictogen**
- Period number: **4**
- Classification: **Semi-metallic**
- Availability: As(III), As(V)

Elemental As occurs in two solid modifications: one yellow and one grey or metallic modification with specific gravities of 1.97 and 5.73, respectively. The element is a steel grey, very brittle, crystalline, semimetallic (metalloid) solid. It tarnishes in air and when heated rapidly oxidises to arsenous oxide, which produces a garlic odour. As is the 20th most abundant element in the earth's crust [74] and is a member of group 15 of the periodic table together with nitrogen, phosphorus, antimony and bismuth. It exists in four valency states: -3, 0, +3 and +5. In strongly reducing environments, elemental As and arsine (-3) can exist.

Under moderately reducing conditions, arsenite (+3) may be the dominant form, but arsenate (+5) is generally the stable oxidation state in oxygenated environments.

2.1.2 As compounds in marine ecosystems and their different toxicity

As is not an essential element for the marine fauna and flora and it is generally considered as poisonous. Aquatic plants and animals show different sensitivities to arsenic. Because As tends to bioaccumulate it is dangerous for marine fauna and flora. As is also of interest, because of its presence in food and seafood, which is the main source of human exposure. Morita and Edmonds [75] claim that terrestrial plants (fungi) and freshwater fish contain As at levels of 0.05-0.2 mg/kg and even at higher concentrations when anthropogenic contamination occurred. On the other hand, marine animals and algae contain high concentrations of As which are typically in the range of 1-100 mg/kg. It was already shown that marine organisms contain higher As concentrations than those corresponding to the levels of As found in sea water. Francesconi *et al*. [32] stated that the As level in sea water in most areas of the sea, is generally around 2 µg/L, while in algae it can be 1000 to 50000 times higher.

The plants in the sea are different from the plants on land. Algae are simple plants that mostly grow in water. Many types of fish feed on algae. Some algae are microscopic and monocellular whereas others are macroscopic and also are called seaweed. There are four main groups of algae: green, blue-green, brown and red algae. Most interest focuses on brown and red algae, since they have been an important food item in several human societies for hundreds of years. Such has especially been the case with the Celtic peoples of Europe, the Island nations of Oceania and the countries of East Asia; especially Japan, Korea and China. In the latter countries sea vegetables are used as daily food. More often the spreading of the Japanese and Chinese cuisine and of health food types over the world has drawn the attention on sea vegetables. Certain kelps and seaweeds are harvested for human consumption and used as sources for alginates and other natural products. *Undaria pinnatifida* (Figure 2-1) which is also known as W*akame,* is a popular foodstuff in Japan and other countries. Japanese people eat red *Porphyra* algae (Figure 2-2) that they call *nori*. It is usually sold as dry as paper. As speciation data for both of there algae are rare.

The main way for human exposure to inorganic and organic As is the ingestion of As containing food [74, 76], however, also water may contain As species [11, 77, 78]. Some investigations of seawater showed that small amounts of organoarsenicals such as MMA, DMA also occur, but the route of their presence is not yet fully characterised [21, 77].

Figure 2-1 *Undaria pinnatifida* alga (from www.alga-net.com).

Figure 2-2 *Porphyra* alga (from www.cbsurvey.ucsc.edu).

A rudimentary classification of the representative inorganic and organic arsenic-compounds found in marine biological tissues is given in Figure 7-1, Table 7-1 and Table 7-2 (see appendix). Their molecular structures are given in Figure 7-2 (see in appendix). This global classification is fairly comprehensive and can only serve as a guide. Some elemental species are classified by some authors in one class and they may be classified in another class by other authors.

There are many As related substances found in the marine environment and in the sea food chain as well as unidentified As species in aquatic systems [3, 51, 75, 77-85, 86]. Numerous studies have demonstrated that organoarsenic species generally are predominant in marine organisms, but inorganic forms can be found in small amounts. For example fish, seafood and algae can contain up to hundreds of µg/g of arsenic, but the major species found are arsenobetaine and arsenosugars, which show a very low toxicity [38-45, 47-49, 51-58, 60-64, 66, 70-73, 75]. Strong accumulators of arsenic, such as cirratulid polychaete *Tharyx marioni* (usually exceeding 2 mg/g and being the highest natural level of As recorded in a marine organism) store much of the As in the palps (6-13 mg/g), but its function remains unclear and did not appear to influence predator behaviour [79]. Other anomalously strong accumulators of As are the gastropod molluscs *Hemifusus ternatanus* and *H. tuba* (spindle shells) [80, 89]. A first report on a precise chemical structure of an organoarsenic compound isolated from western rock lobster *Panulirus cygnus* in the crystalline form and identified as arsenobetaine by X-ray crystallographic analysis has been published in 1977 [87]. Since then arsenobetaine is known as the major metabolite isolated from fish and seafood. The most widely known organoarsenic compounds are the quaternary As compounds arsenobetaine and arsenocholine. They are the major organoarsenic metabolites found in marine animals [42-45, 49-51, 53-57, 60-62, 64]. Further arsinoyl ribosides (arsenosugars), which are products of the As metabolism are mainly observed in marine plants and in some bivalves [28-42, 47, 61-63].

It was also discovered that methyl transferase enzymes play an essential role in the methylation of As in marine animals and algae as a main metabolisation (biotransformation)

and detoxification reaction. It yields the less toxic methylarsonic acid (MMAs(V)) and dimethylarsinic acid (DMAs(V)) as well as other related organoarsenicals [5].

However, little is known about As speciation in most of the foods we eat. Therefore, special attention is recently given to the determination of As species in marine plants and animals in order to evaluate more accurately the toxicological risk for human health, especially in countries with considerable seafood consumption [11, 39, 41, 60].

2.1.3 Mechanisms of As biotransformation

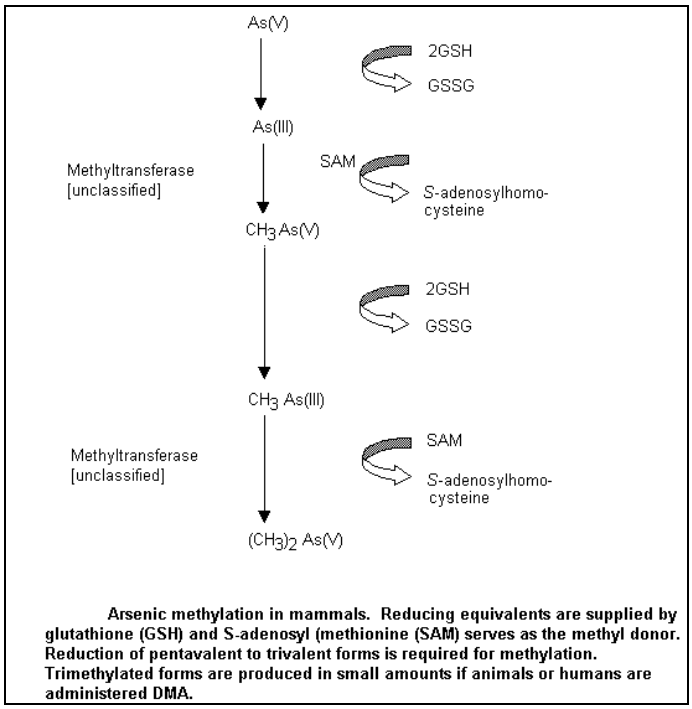
Most environmental transformations of As seem to occur in soil, in sediments, in plants and animals and in the oceans. As can undergo a complex series of transformations, including redox reactions, ligand exchange and biotransformation in the marine ecosystem. The overall cycle is found to be similar to the phosphate cycle, but the regeneration time for As is much slower. Bioconcentration of As under laboratory conditions occurs in aquatic organisms, primary in algae and lower invertebrates. However, biomagnification in aquatic food chains does not appear to be significant [88].

Arsenate can be reduced, either microbially or chemically, to arsenite within the seawater and anoxic sediment and at the same time there is oxidation of arsenite to arsenate. Many factors are affecting the fate processes in seawater including the pH, the temperature, the salinity as well as the distribution and the composition of the biota. Methylation and reduction of arsenate to arsenite and methylarsenic acids happen in the photic zone (see Scheme 2-1). As is taken up by planktonic organisms in the surface waters and transported to deeper waters with biogenic debris. At intermediate levels a regeneration of arsenate occur. Bioutilisation of the element during the warmer months results in the release of dissolved monomethylarsenic and dimethylarsenic compounds. Inorganic arsenite and methylated As species can account for up to 40 and 70% of the dissolved As respectively, but only when the water temperature exceeds 12℃. There is a good correlation between the photosynthetic activity and the concentration of methylated arsenicals. The biological uptake causes changes in the speciation of As resulting in measurable concentrations of reduced and methylated As species. Therefore, biomethylation and bioreduction are probably the most important environmental transformations of arsenic, since they can produce organometallic species that are sufficiently stable to be mobile in air and water. However, the biomethylated forms of As are subject to oxidation and bacterial demethylation back to inorganic forms [5, 77] (see Scheme 2-1).

Three major modes of biotransformation of As have been found to take place in the marine environment:

1) a redox transformation between arsenite and arsenate,
2) a reduction and methylation of arsenic,
3) a biosynthesis of organoarsenic compounds.

Scheme 2-1 As catabolism (from reference [5])



Arsenic methylation in mammals. Reducing equivalents are supplied by glutathione (GSH) and S-adenosyl (methionine (SAM) serves as the methyl donor. Reduction of pentavalent to trivalent forms is required for methylation. Trimethylated forms are produced in small amounts if animals or humans are administered DMA.

2.1.3.1 Oxidation and reduction

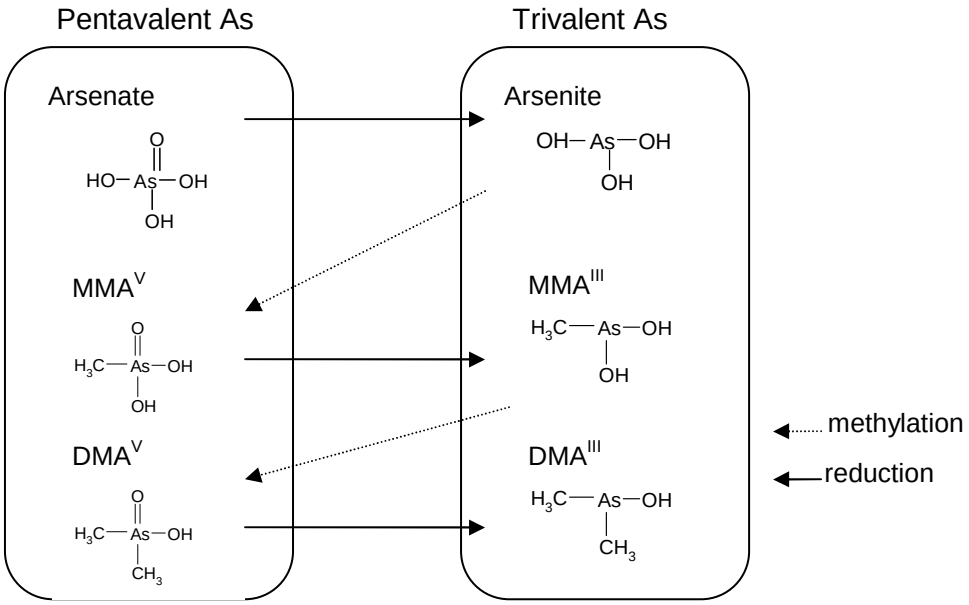
In seawater, which contains free dissolved oxygen, arsenate is the thermodynamically stable form of arsenic. Arsenite is present in amounts exceeding those of arsenate only in reducing, oxygen-free porewaters of sediments. However, arsenite in the surface and deep waters of the oceans may account for up to 10% of the total As and, conversely, some arsenate is still present in anoxic water [81]. Scudlark and Johnson [82] studied the oxidation of arsenite in seawater at low levels. They found that abiotic oxidation takes place at a slow and constant rate, whereas rapid oxidation occurred only in the presence of certain aquatic bacteria. The authors therefore concluded that the oxidation was primary due to microbial activity. Under aerobic conditions the mixed microbial cultures of lake sediments were able to reduce arsenate to arsine and also to oxidise arsenite to arsenate. However, under anaerobic conditions only a reduction was observed [83].

2.1.3.2 As methylation

Biomethylation is primarily restricted to the high-salinity regions of estuaries, where methylated As is predominantly present at lower salinities as a result of the mixing of saline water (containing bioarsenicals) with river water [84]. Several authors have reported on the occurrence of As methylation in macroalgae and other marine organisms [68-73, 85, 90, 91]. The marine algae *Eckonia radiata* and *Polyphysa peniculus* convert methylate arsenate to the dimethylarsenic derivative. It was concluded that methionine or S-adenosylmethionine (SAM) is the source of the methyl groups in this biological alkylation [91, 92]. Maeda *et al.*

[76] found that biomethylation of As gradually increased with the trophic level in a model ecosystem: alga (*Chlorella* sp.) < zooplankton (*Moina* sp.) < goldfish (*Carassius* sp.)

Most marine mammals also metabolise inorganic As by a methylation to methylarsonic acid and dimethylarsinic acid [60, 70, 72, 73]. The exact mechanism has not been revealed yet, however, some assumptions were made that the methylation occurs after a reduction of pentavalent As to trivalent and an addition of the methyl group (see Scheme 2-1). Experimental studies have shown that a major part of the absorbed As(V) is rapidly reduced to As(III). As it is well known that As(III) is more toxic than As(V), this initial step in the As biotransformation could be considered as a bioactivation. Nevertheless, much of the formed As(III) is distributed in the tissues, where it is methylated to MMA and DMA (see Scheme 2-2). On the other hand, in physiological media As(III) mainly is present in the undissociated form (acid dissociation constant pKa 9.2), which facilitates a passage through cellular membranes, while As(V) is in the ionised form. As(III) is probably first bound to a dithiol molecule, of a carrier protein and then the methyl groups are attached [93]. In addition, it was reported that SAM is the main methyl donor in the methylation of arsenic. In experiments with an administration of inorganic As compounds to animals a small range of simple methylated metabolites is produced. Although such experiments are artificial when compared with the natural metabolism of marine organisms, they are helpful for the understanding of the As metabolism in living organisms. Laboratory studies on mice and rabbits have shown up the presence of chemical inhibition of the SAM dependent methylation by periodate-oxidised adenosine, which resulted in a remarkable decrease of the methylation of As [94]. Later, *in vitro* incubations of rat liver preparates with As(III) have confirmed the role of SAM in the formation of MMA and DMA [95].



Scheme 2-2 Reactions for the methylation and the reduction of inorganic arsenic

Some experiments showed that the enzyme methyltransferase is involved in the methylation of arsenic. Arsenite methyltransferase and MMA methyltransferase isolated from liver of rabbit and hamster appear to be present in the same protein with a molecular weight of about 60 kDa [96, 97].

Very little is known about factors which influence the methylation of As in humans. Recently, it has been found that the fraction of various As metabolites in urine are remarkably stable over a period of about a week, indicating that an individual's methylation of inorganic As is stable over a such time [5, 98]. In combination with a large inter-individual variation in As methylation this might indicate that genetic influences are more important than environmental ones. It appeared that MMA and DMA as the final metabolite products of inorganic As react less with tissue constituents and therefore are more easily excreted in urine than inorganic arsenic. Thus Hulle *et al.* [98] concluded that the methylation acts as a detoxification.

2.1.3.3 Biosynthesis of organoarsenic compounds

The hypothesis for a biosynthesis of compound containing an arsenic–carbon bond has been confirmed by the isolation and identification of trimethylarsonioacetate (arsenobetaine) in 1977 [87]. Afterwards it was isolated in a variety of marine organisms including sharks [72], american lobster [44, 52, 66], teleostei fishes [39], crabs [99], shrimps [99], sea cucumber [59], cephalopod molluscs [38, 47] and several species of gastropods and bivalve molluscs [32, 33, 45, 61, 63]. It seems to be nearly ubiquitous in many marine animals of the human food chain and in most animals accounts for the main fraction of the arsenic. The presence of arsenocholine was also reported in scallops [44], fishes from polluted areas [51, 58] and in dogfish reference material [31, 35, 42, 53-55]. Edmonds *et al.* [100] identified arsenocholine-containing lipids as natural products in the digestive gland of the rock lobster (*Panulirus cygnus*). However, Shibata and Morita [101] showed that the trimethylarsonium ion and not arsenocholine was present in dogfish reference material. Trimethylarsine oxide (TMAO) was also identified in estuary catfish [84].

Early studies on marine algae, such as *Fucus sp.* and *Chondrus crista (*Irish *moss)*, traditionally used for pharmaceutical purposes or as food, were reported to contain high concentrations of arsenic. Later it appeared that those species, which usually synthesise many different biological active compounds, also produced organoarsenic compounds [102-104]. These studies on the characterisation of As in algae primarily centered on the ratios of organically bound and inorganic As species and/or the presence of water-soluble and lipid-soluble As compounds.

The distribution of As species in marine brown algae *Hizikia fusiforme* (phylum Sargassaceae) [86, 105] was also studied. Aqueous extracts of *Hizikia* contained methylarsonic acid and dimethylarsinic acid in addition to inorganic As and other unknown

organoarsenic compounds. Recent investigations of *Hizikia* showed that arsenosugars such as glycerol- (**10**), sulfonate- (**9**), sulfate- (**11**) and phosphate-riboside (**17**) and another ammonium-sulfonate riboside (**12**) and a further arsinoylriboside very similar to the hydroxysulfonic acid (**9** in Figure 7-2 of appendix) here occurred [86]. For other members of the Sargassaceae family similar results were found. Studies on *Laminaria japonica* [86] and *Laminaria digitata* [106] indicated that As species such as As(III), As(V), MMA, DMA, AsB, AC, dimethylarsinoylribosides were present as well as some novel As compounds (**12-16**, **18-22** in Figure 7-2 and **23-27** in Figure 7-3 in appendix).

Sargassum lacerifolium is one more representative member of the Sargassaceae family, in which the presence of As compounds was investigated. Arsenomethionine (**27**) and two diastereoisomers of (**28**) (see Figure 7-3) were found as major compounds in *Sargassum lacerifolium* [107]. The polar parts of the compounds (**28** and **28 a,b**) are similar to those of non-phosphorus-containing betaine lipids such as DGTS (diacylglyceryl-O-4'- (*N,N,N*-trimethyl)-homoserine (**29** in Figure 7-4) and DGTA (diacylglyceryl-O-2'-(hydroxylmethyl)-(*N,N,N*-trimethyl)-β-alanine) (**30** in Figure 7-4). These polar betaine lipids are important and are major membrane components of marine and freshwater algae species [108]. Lipids **29** and **30** have a trimethylamino group and **28 a,b** have an As group that carries two methyl and 5'-deoxyribos-5-yl group as substituents. This last group is related to the major dimethylarsinoylribose derivatives of *Sargassum lacerifolium.* The similarities between compounds **28 a,b** and **30** suggest that the biosynthesis is similar (see Figure 7-4) [107]. Edmonds and Francesconi [109] have also identified biosynthesised arsenic-containing ribofuranosides (arsenosugars) and isolated inorganic and methylated As compounds from brown kelp (*Ecklonia radiata*). The authors asserted that these compounds are intermediates in the As cycle and stated that arsenosugars subsequently could be metabolised to arsenobetaine.

Different organoarsenic compounds have also been isolated from other brown algae such as *Sphaerotrichia divaricata, Undaria pinnatifida,* the green alga *Codium fragile* and the red alga *Porphyra tenera* [86], collected from the Japanese and Australian coast.

The synthesis of both lipid and water soluble As compounds has been shown to occur in two green algae *Chlorella ovalis, Ch. Pyrenoides,* cyanobacterium *Oscillatoria rubenscence and* in the two diatoms *Phaeodactylum tricolour* and *Skeletonela costatum* [100].

Much work has been done in the last three decades on the isolation and identification of As compounds so as to better understand the metabolism of inorganic As by marine and terrestrial plants and animals. This gave rise to a number of organic As species which could be considered as naturally occurring compounds. However, this field is open for new developments and further research, since marine organisms and especially their complex metabolism are not yet fully characterised and understood.

2.1.4 Analytical strategies for the speciation of As

There are two main approaches for speciation of environmental and biological As compounds. One is an accurate procedure that has been employed in natural product chemistry and is appropriate for the identification of compounds of previously unknown structure [1]. Here the As species are separated from a large quantity of starting material, purified and isolated and their structures determined by X-ray crystallography, NMR spectroscopy, IR spectroscopy, MS, UV-visible spectroscopy and elemental analysis. The method affords an unequivocal identification, but requires a rather large amount of As compound and, usually, much time and work. This type of approach has been used to provide qualitative data and only limited quantitative data have been obtained.

In the second approach one combines a separation with selective and sensitive detection methods for individual As species. Szpunar *et al*. [12] discussed the use of the analytical approaches for element speciation in biological systems, as shown in Figure 2-3.

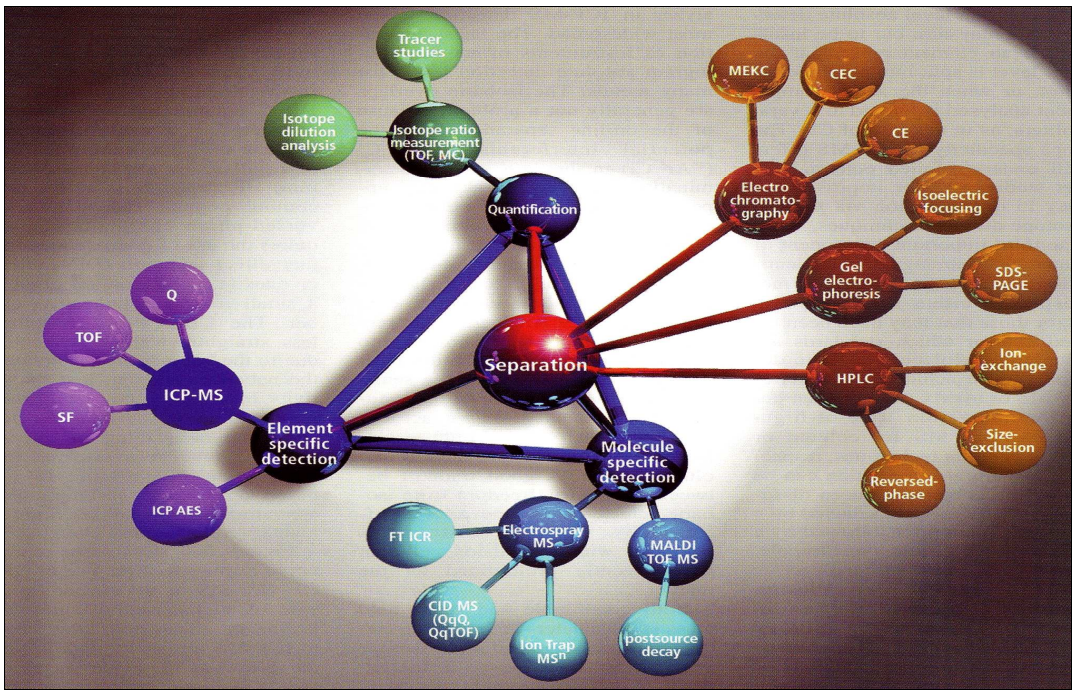


Figure 2-3 Hyphenated techniques for trace element speciation in biological and environmental samples (from Szpunar *et al.* [12]).

The choice of hyphenated systems depends on the research objective. The use of quadrupole ICP-MS detection is most common, but double-focusing sector-field instruments offer higher resolution for interference-free determinations of As and S or of isotope ratios of some elements such as Cr and Fe. On the other side, an increase in resolution often leads to a decrease in power of detection. A good compromise between sensitivity, absence of isobaric interferences and costs may be realised with collision cell ICP-QMS. Both quadrupole and sector field ICP-MS are scanning analysers where *m/z* signals within a time

dependent concentration profile of a transient signal are monitored. As alternative ICP-time-of-flight (TOF)-MS has the ability to produce a complete atomic mass spectrum in less than 50 µs and allows recording of fast transient signals with full mass coverage. This could be very useful for on-line isotope ratio determinations, but a loss in sensitivity of almost one decade with ICP-TOF-MS instruments as compared to the latest ICP-QMS instruments could restrict its application in the speciation analysis of biological samples.

In Figure 2-4 a schematic overview of the most used instrumental set-ups for speciation analysis, which consisted of HPLC or CE coupled on-line with ICP-MS is presented. Barnes comprehensively reviewed the coupling of CE and ICP-MS [111].

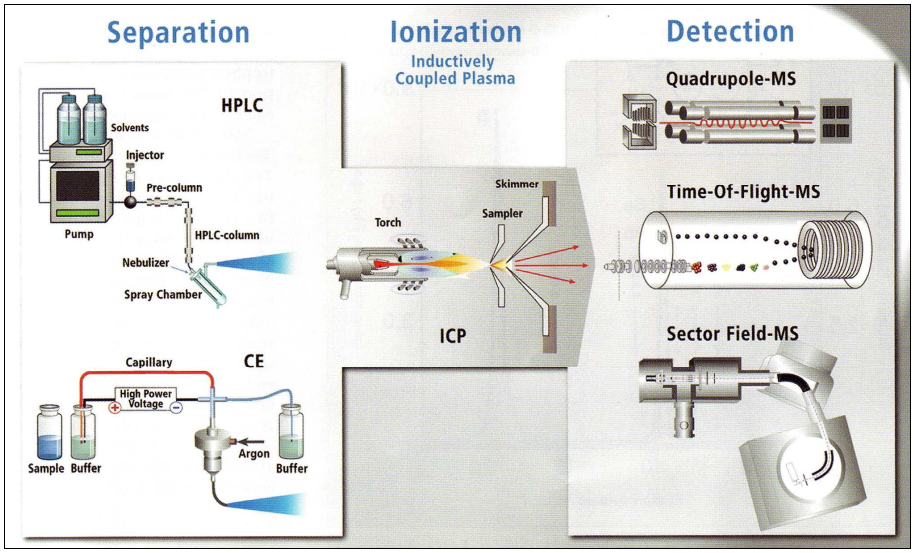


Figure 2-4 Instrumental set-ups for on-line coupling of HPLC or CE with ICP-MS (from Szpunar *et al.* [12]).

The key to a successful on-line coupling of cHPLC or CE to ICP-MS is the interface. In the simplest case, the exit of a standard HPLC column (I.D. 2.1 - 7.0 mm) is connected to a conventional pneumatic concentric or cross-flow nebulizer. The use of capillary or nanobore HPLC columns (I.D. 0.1-1.0 mm) requires the use of micro nebulizers, such as the direct injection nebulizers (e.g. DIN, DIHEN) or microconcentric nebulizers (e.g. Micromist, CETAC MCN-100) together with a small volume nebulization chamber. HPLC-ICP-MS, however, is a robust, sensitive element specific method which is capable to provide a real picture of the elemental species in solution. The elemental response is usually independent of the species and makes it possible to quantitatively determine the species even when their structures remain unknown, provided that the resolution of the HPLC separation and the recovery are high. Nevertheless, the identification is based on the retention time and hence a compound in the sample can currently only be identified by running relevant standard compounds.

In summary, hyphenated techniques based on a coupling of liquid chromatographic separation techniques with ICP-MS detection are now established as the most realistic and

potent analytical tools available for real-life As speciation analysis. On the other hand, there is a concern that the methods of extraction, separation and detection may not be efficient enough or may destroy the original species. Instabilities of the species during the sampling, storage and sample pre-treatment are all very important issues that must be considered. The knowledge of the stability of species examined under different conditions is necessary. When the original distribution of the As species in the sample is changed the result of speciation analysis is questionable. Therefore, a rapid analysis of samples is important so as to prevent species conversion. Further, the separation and the detection methods used in As analysis are only as reliable as the sampling procedure used.

2.1.4.1 Extraction

After sampling the next step is extraction, especially when solid samples are to be analysed. The methods for extraction and clean-up must be efficient and minimise the destruction of the As species present in the solid material. In the case of aqueous samples, pre-concentration and pre-treatment of As species often are not required. But this is not the case for seawater samples, because the chlorides interfere during the separation processes and/or hydride formation and/or following ICP-MS detection. Speciation of As in seawater was reported to be done either by direct analysis or after sample filtration in order to minimise the chloride interference [82-84].

For biological materials, several extraction methods have been developed for As speciation over the past decade, focusing on low solvent volumes for high power of detection and reduced extraction times. Biological tissues with high fat content, such as shellfish or seal may need to be defatted prior to an extraction of water-soluble As species. It is often difficult to extract an analyte from body tissues and fluids. For the extraction from solid samples the techniques used often included shaking, heating or sonication. The latter is most popular, as here the dispersion of the tissue is thought to be maximum. For extractions from biological materials, mixtures of methanol-water or methanol-chloroform [53] are most often used. However, due to the difficulties in handling of chloroform, the water-methanol mixture is widely applied.

McSheehy *et al.* [40] compared the extracting effectiveness of three solvent systems (water, methanol and methanol/water, 1/1 v/v) for both the species and for the total amount of As in molluscs. No difference between the various solvent systems was found. For the case of marine animals, extraction efficiencies > 90% are commonly obtained [31, 37, 42, 44, 45]. This is expected, as marine animals are rich in AsB and this small molecule is soluble in water, methanol as well as in their mixtures. The extractions from marine algae are more difficult and often less than 80% of the total As is extracted [28, 30, 31, 34]. In terrestrial organisms and plants, extraction efficiencies can be much lower and vary according to the extraction conditions.

Solid phase extraction on cation-exchangers and on C_{18} cartridges was also performed [31, 37, 46, 65], but the extraction efficiency due to the retention of organoarsenicals on the cartridges was not satisfactory.

2.1.4.2 Separation methods

The most popular separation method for As speciation is liquid chromatography, since most of the As compounds are soluble in water and the marine biological tissues or body fluids are also based on aqueous media. On the other hand, HPLC could be easily coupled to element-specific detectors by using an appropriate interface, as it is done for ICP-MS. In general two main types of separations in HPLC have been employed for As speciation. In a first strategy multidimensional LC (size-exclusion, anion-exchange and reversed phase) coupled parallelly to ICP-MS and ESI-MS/MS as a tool for characterisation of As species in a *Laminaria* alga was developed by S. McSheehy *et al.* [40]. In many other applications, reversed phase HPLC is used with the support of ion-pairing (IP) reagents. Either cationic [40], or more commonly, both cationic and anionic reagents, such as a mixture of malonic acid/tetraethyl ammonium hydroxide [28, 29, 40], were used.

The second more favoured method for As speciation is based on ion-exchange separations; including both anion-exchange and cation-exchange chromatography [40-42, 56, 59, 61, 63, 66]. Indeed, the large number of As species (more than 25) with similar physicochemical properties present in marine sample and the insufficient separation efficiency of chromatographic techniques make the co-elution of some species in a single separation procedure practically unavoidable. This problem can be partly alleviated by careful optimisation of the separation conditions [112], or by running the same sample with different separation techniques in parallel [40] or in sequence [31, 38, 55, 63].

2.1.4.2.1 Ion-pair liquid chromatography

In the separation of cations by ion-pair chromatography, the mobile phase usually contains fairly bulky organic ions and counter ions that are different from any of the sample ions. Equilibrium is established in which the eluent ion partitions between the column stationary phase and the liquid mobile phase. The eluent ion and sample ions then undergo a competitive pairing with the charged sites of the stationary phase. Separation of sample cations is based on differences in their affinities for the negative sites on the stationary phase. Examples of cation ion-pair regents include alkyl sulfonates. Anions can be separated in a similar way, but by using an organic anion ion-pair reagent, which is generally an alkyl ammonium salt with a counter anion that is different from the ions in the sample. The retention times of the sample ions are strongly affected by the chemical structure and the concentration of the ion pairing reagent as well as by the ion of the mobile phase.

The retention behaviour of AsB, AC, TMAO, tetramethylarsonium iodide (cationic As compounds), As(III), As(V), MMA and DMA (anionic As compounds) on a Hamilton PRP-1 reversed-phase HPLC column with 10 *mM* solutions of various benzenesulfonic acids as ion-pair reagents in the pH range of 2 to 5 was studied using flame atomic absorption spectrometry as arsenic-specific detector [113].

Freshwater alga *Nostoc sp*. was found to contain a lower concentration of As species when using tetraethyl ammonium hydroxide than when using tetrabutyl ammonium hydroxide under the addition of 4.5 *mM* malonic acid and 0.1% methanol in ion-pair chromatography and ICP-MS detection [28]. Furthermore, As speciation was carried out in the brown alga *Fucus gardneri*, by ion-pair HPLC-ICP-MS with the same mobile phase composition [29]. Tetraethyl ammonium hydroxide and malonic acid were also used as ion-pair reagents for As speciation in sea scallop gonads (*Placopecten magellanicus*) [45].

2.1.4.2.2 Anion-exchange liquid chromatography

Ion-exchange operates on the basis of selective exchange of ions in the sample with counter ions in the stationary phase. The functional ions are permanently bound to the column matrix and to each a counter ion is attached. The sample is retained by replacing the counter ions of the stationary phase with its own ions. The sample is eluted from the column by changing the properties of the mobile phase so that the mobile phase will now displace the sample ions from the stationary phase (i.e. changing the pH).

There are four categories of ion-exchangers: strong and weak cation-exchangers and strong and weak anion-exchangers. The charge of strong ion-exchangers is mostly independent of the pH. Quaternary amines form strong anion-exchangers and sulfonic acids are present in strong cation-exchangers. All these functional groups are available on polymeric packings, primary for separation of large biomolecules. Weak cation-exchangers can also be provided on silica. At the time of this work, ion-exchange cHPLC columns were not commercially available.

Anion-exchange chromatography is very useful for the separation of As species in marine plants, as they mostly contain arsenosugars as it is shown by many studies [37, 40-42].

Four As species, including inorganic As(III), As(V) and methylarsonic acid and dimethylarsinic acid, have been determined in fish by anion-exchange HPLC coupled to ICP-MS [56]. Some or all of the above mentioned species and arsenobetaine have been determined in extracts of dogfish, mussel, shark, white ocean fish and salmon [53], in dogfish [60], dugong [60], oyster [65] as well as together with dimethylarsinoylacetate in microbially enriched seawater [114]. Six As species of interest (As(III), As(V), MMA, DMA, AsB and AC) were determined in the lobster standard reference material TORT-2 and in other lobster organ samples [70].

Since arsenobetaine and arsenocholine are found to be less toxic or harmless, it might be necessary, for toxicological reasons, that the concentrations of arsenobetaine and other arsenicals in food of marine origin must be determined by methods which can be routinely applied. Therefore, quantitative determinations in real samples require the establishment of efficient extraction and clean-up, as well as detection with good precision and accuracy. Only limited information is available on the concentration of As species in marine biological reference materials [66]. Actually, few publications deal with these problems.

2.1.4.2.3 Cation-exchange liquid chromatography

The speciation of As by cation-exchange chromatography is preferred for marine fauna, in which AsB is often the major metabolite. Strongly acidic columns have been employed for jellyfish [71], seals [73], mussel [38], scallops [32, 44, 45], oysters [54, 61, 63, 115], crab [116], shrimps [116], and fish tissues [49, 55, 57, 64, 116] as well as for the analysis of marine algae [31, 33, 41]. As(III), As(V) and MMA have been determined in seawater by cation-exchange HPLC coupled to ICP-MS after clean-up by size-exclusion chromatography and several types of ICP-MS, namely hydride generation-ICP-MS and octapole-reaction cell ICP-MS [89].

2.1.4.2.4 Micro liquid chromatographic separation

Recently, micro-scale separations have increased in popularity for environmental applications, as one tries to improve the separation efficiency with reduced analysis time and reduced sample amount [8, 9]. It is well known, that micro-bore and narrow-bore HPLC columns are more compatible for use with ionisation sources for MS [125]. Some studies have been carried out on reversed phase micro-bore HPLC columns coupled with ICP-MS for the analysis of As animal feed additives [8, 9] and for the determination of inorganic arsenic, MMA and DMA in different wine samples and arsenosugars in kelp powder extract [120].

Capillary electrophoresis has become a possible alternative to ion chromatography with the advantage of having the capability to separate positive, neutral and negative ions in a single run with high separation efficiency and resolution. The separation of five As compounds, namely As(III), As(V), MMA, DMA and AsB by using reversed polarity in a chromate buffer and on-line coupling with ICP-MS via a microconcentric nebulizer and with a self-aspirating make-up electrolyte has been reported [121]. The CE-ICP-MS approach seemed to be troublesome because of peak broadening, high migration time and high peak area RSDs. The limits of detection were in the µg/L range. However, speciation analysis of real samples with this system has not been carried out.

In the majority of cases, a multidimensional approach is applied for the isolation and purification of the As analytes prior to molecular detection, mostly by mass spectrometry

[123, 124], which requires high purity and concentrations higher than those of atomic spectrometry detectors.

2.1.4.3 Detection

2.1.4.3.1 Element-specific detection

Early optical spectrometric methods like atomic absorption spectrometry (AAS) and atomic emission spectrometry (AES) are primarily used for element-specific detection in chromatography to determinate As species [54, 69, 118, 119, 128-130]. These methods are selective and sensitive, when appropriate combinations are made and they are suitable for both quantitative and qualitative analysis, when As standard compounds are available. Such methods can also enable to detect unknown As species by their chromatographic retention data. However, they had limited application due to a lack of sensitivity by which very low concentrations (usually sub pg levels) of elemental species isolated from most biological samples cannot be determined. Accordingly, the more sensitive as well as most element-selective ICP-MS detection coupled to HPLC has become the backbone of speciation analysis over the last 15 years.

In the case of hydride generation (HG) AAS, not all As species form hydrides and decomposition techniques are usually required [131]. The limitations of the above mentioned methods become more evident, since low concentrations of As are involved in speciation of biological tissues and fluids, the more complex the analytical sample is. Since the development of ICP-MS and its commercial availability, it became the most powerful detector in the speciation of metal and metalloids, including arsenic. The advantages of ICP-MS are the capability to discriminate (with isotopic resolution) between metal or heteroelement-containing species and metal-free species even at extremely low concentrations in a HPLC or capillary electrophoresis effluent. Indeed, power of detection is becoming a key issue when capillary separation techniques are used or when the target sample is small (e.g. individual cells).

The use of ICP-MS in speciation analysis has been extensively reviewed [1, 3, 6, 8-10, 14, 39, 116, 132-135]. ICP-MS has been mainly developed by the research groups: of Houk et al. and Gray 25 years ago [137, 138]. The first commercially available ICP-MS instrument was produced by Perkin Elmer Sciex in 1983 [139].

With ICP-MS most of the elements in the periodic table ca be determined with detection limits at or far below the part per trillion (ppt) level.

In ICP-MS one uses some components such as the quadrupole mass spectrometer and vacuum system like in the other types of mass spectrometers as in LC-MS and GC-MS. ICP-MS will not be covered here, as it has been described extensively in the literature [137, 140-143, 146]. The plasma ion source used in ICP-MS is much different from the low-energy

ion sources used in LC-MS and GC-MS instruments. The ICP is highly energetic and is a hard ionisation source. The plasma is operated at atmospheric pressure and typically has temperatures of 5000-10000 K. An aerosol of the sample liquid is introduced into the plasma source where vaporisation, atomisation and ionisation of the analyte occur. The extremely high temperature of the plasma ion source completely breaks down the molecules present in the sample and the elemental ions are passed on into the mass spectrometer. Thus, in ICP-MS one detects only elemental ions, which makes ICP-MS much more comparable with the other element determination methods like AAS and ICP-OES. In addition, the standard and sample preparation procedures, the sample introduction systems and the potential interferences are all very similar to those in AAS and ICP-OES.

The main advantage of ICP-MS over the other methods for elemental determinations is the possibility to determine the individual isotopes of each element. This allows ICP-MS to perform isotope ratio and isotope dilution measurements. Although, As is monoisotopic and has a mass of 75 amu, ICP-MS is an ideal detection system for As in speciation analysis, because of its high sensitivity. As can be determined with rather low interferences under certain conditions. Argon from the plasma and chlorine from the sample matrix, however, may combine to form $^{40}Ar^{35}Cl$ which has the same mass-to-charge ratio as arsenic. Thus, when monitoring the signal at *m/z* 75, it stems from As and argon chloride as interferent. A mathematical correction can be used to correct for the interference. The part of the signal generated by the argon chloride may be calculated and subtracted from the signal at *m/z* 75. Chlorine has two isotopes, ^{35}Cl and ^{37}Cl, so $^{40}Ar^{37}Cl$ should also be formed in the plasma at the isotopic ratio of ^{35}Cl and ^{37}Cl. By monitoring *m*/*z* 77, the part of the signal at *m*/*z* 75 generated from argon chloride may be subtracted allowing for the accurate measurement of the As signal. At certain plasma conditions this interference can be minimised. It has been reported that by providing a reaction cell or a collision cell in front of the quadrupole mass spectrometer some polyatomic interferences can be avoided [141]. In some chromatographic systems, chloride can be separated from As so that its peak occurs before or after the peaks associated with the As species of interest. Kohlmeyer *et al*. [46] and Londesborough *et al.* [39] used a gradient elution anion-exchange with nitric acid as eluent, where the chloride is eluted before the As species.

Interfacing ICP-MS to HPLC is relatively simple. The ICP-MS is very suitable for the analysis of liquid samples. The connection of a HPLC column to an ICP nebulizer, however, delivers no severe problems. The flow rate of an analytical HPLC column is typically 1 mL/min, which matches the flow rate of the commonly used pneumatic nebulizers for ICP-MS. Low flow rate (≤100 µL/min) nebulizers are also commercially available, so that narrow bore and micro-bore HPLC can also be coupled to ICP-MS. However, the main problems in interfacing HPLC to ICP-MS are related to the fundamental nature of the detector. One of the primary rules of HPLC coupled to ICP-MS is that the mobile phase used

must be compatible with the detection by ICP-MS. Na or K phosphate buffers mobile phases are often utilised in reversed-phase HPLC analysis when UV–VIS detectors are used, because these phosphate buffers are UV transparent. However, these buffer mobile phases are not appropriate for ICP-MS detection. Non-volatile buffer salts can form deposits on the lenses and the skimmer cones resulting in signal drift and asking high maintenance involving cleaning of the inner surfaces of the MS detector. This asks for use of volatile buffers or of buffers that have a low residue after exiting the plasma of the ICP-MS. Ammonium salts of organic acids and ammonium bicarbonate are quite acceptable to the ICP-MS because of low residues. Also, the ICP limits the choice of compounds present in the mobile phase. HPLC often makes use of an organic modifier in the mobile phase; and when large volumes of organic solvent reach the ICP resulting in unstable plasma [63]. This problem can be minimised by desolvating the aerosol before it reaches the ICP. A cooled spray chamber can be used to condense a large part of the solvent vapour and to maintain plasma stability. Also a simple flow splitting after the HPLC column, or the use or a small bore or microbore column, can reduce the amount of organic solvent introduced into the ICP. The nature of the organic modifier itself can play a role in plasma stability. Methanol is more widely used than acetonitrile for reversed-phase HPLC-ICP-MS, because it causes less plasma instabilities [63].

There is an extensive literature on the isolation and detection of As species in various samples by HPLC-ICP-MS, but only a few reviews are dealing with analytical methods used for their determination in marine environmental and biological samples, such as publications of Irgolic [112], Morita and Edmonds [78], Guerin *et al.* [141], B'Hymer and Caruso [114], Gong *et al.* [59] and McSheehy *et al.* [55]. McSheehy *et al.* [55] have classified the As species isolated from marine biological tissues into three classes with respect to their chromatographic separations. Simple inorganic and small molecules of mono- and dimethylated arsenicals are one group, whereas complex dimethylarsinoyl-ribosides (arsenosugars), trialkyl-arsenio-ribosides and trimethyl-arsenio phosphate containing chains, which may be polar or neutral, build further groups.

2.1.4.3.2 Molecule specific detection

More recently, molecular mass spectrometry mainly with electrospray ionisation, has also strongly contributed to the field of bioinorganic speciation analysis. In contrast to classical HPLC-MS or CE-MS environmental and biochemical applications with standards available for the most anthropogenic pollutants, the majority of target species in bioinorganic trace analysis have not yet been isolated in sufficient purity and/or concentrations to be commercially available and used as standard samples to determine the retention or migration time. Therefore, it is becoming of great significance to utilise in parallel a molecular mass detection, such as ESI tandem MS, for the identification of the eluted or especially co-eluted

species. For the molecular characterisation and identification of unknown metal bio-complexes or organometalloid compounds the tandem MS (e.g. triple quadrupole or Q-TOF) or MS^n (e.g. ion-trap MS) approach has to be employed (see Figure 2-5). Similarly, the identification of known and perhaps common species in unusual samples or in samples in which they have not yet been reported (e.g. AsB in water or marine plants) could give convincing data. Mass spectrometry techniques, such as fast atom bombardment (FAB) ESI or matrix assisted lased desorption (MALDI)-TOF-MS modes have been used e.g. for organoarsenic compounds detection.

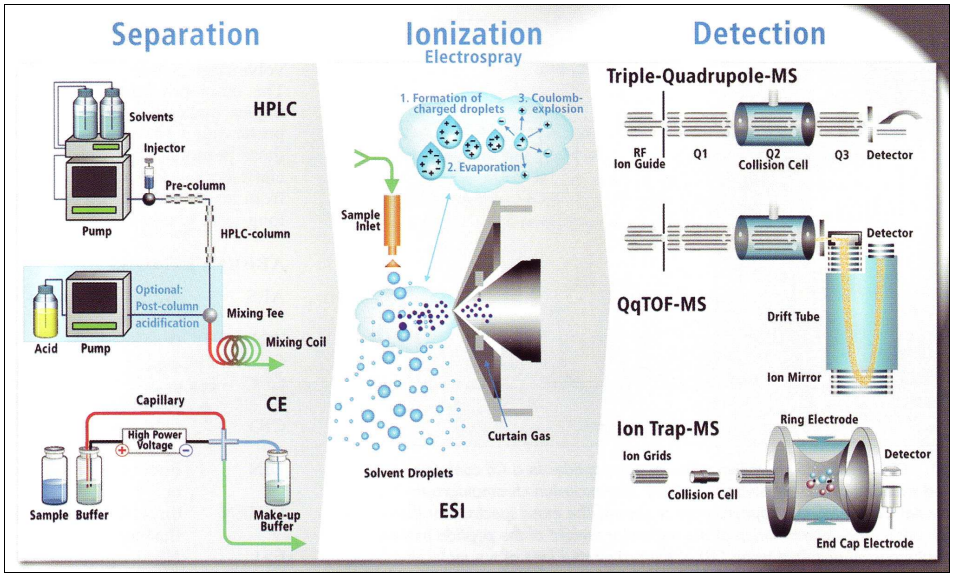


Figure 2-5 Instrumental set-up of HPLC or CE coupled online to electrospray MS and MS/MS detection (from Szpunar *et al.* [12]).

The complementary use of the two mass spectrometric techniques (i.e. elemental and molecular) MS can provide simultaneously data on chemical structures and concentrations of As species at trace levels. Recent reviews comprehensively examine their use in speciation analysis [116, 123, 132, 148, 149]. A such study was first realised in 1996 by Corr and Larsen [115] by connecting HPLC to an ionspray (IS) mass spectrometer for a qualitative verification of the results of speciation analysis performed with HPLC-ICP-MS for organoarsenicals in plaice and for the determination of arsenosugars in extracts from oysters and mussels [144]. This investigation is particularly interesting, since high MS orifice potentials in the ionisation ion source had to be used to generate bare As^+ ions. Thus it was possible to obtain elemental As and molecular As signals on sequential chromatographic runs of one sample. Subsequently, this method was applied by Pedersen and Francesconi [36] for obtaining simultaneous elemental and molecular mass spectra in the positive ion mode of As compounds in the case of HPLC coupled to single quadrupole ESI-MS with variable fragmentor voltage. The system could be used to determine seven organoarsenic compounds: four arsenic-containing carbohydrates (arsenosugars), the quaternary arsonium

compound arsenobetaine, DMA and dimethylarsinoylacetic acid (DMAA). The characteristic common fragment at *m/z* 237 for arsenosugars was also reported and used for the identification and quantification of the major arsenosugars in the crude extract of two brown algae, *Fucus* and *Laminaria.* Further investigations on the characterisation of arsenosugars in extracts of algae by HPLC-ESI-MS were reported [33, 38-41, 145]. Four major dimethylated and minor trimethylated arsenosugars have been found in marine algae and some other marine samples.

For the identification of arsenosugars of biological origin at trace levels by molecular mass spectrometry other ionisation techniques like FAB [146], nanoelectrospray [147] and ionspray ionisation [115] were also applied. FAB-MS was used to generate ions of various arsenosugar compounds [146]. Furthermore, negative-ion collision induced dissociation (CID) tandem MS of $[M-H]^-$ precursor ions resulted in the formation of characteristic ions via charge-remote fragmentation for acidic groups containing of sulfate-, sulfonate- and phosphate-ribosides. Moreover, it was demonstrated by Pergantis *et al*. [146] that positive-ion FAB-MS/MS provides complementary information for the spectral characterisation of the arsenosugars examined. The mass spectrometric investigations were also applied to show up the presence of sulfate-ribose in a partially purified algal *Sargassum lacerifolim* extract.

Negative-ion mode nanoelectrospray was used as an ionisation source for a high resolution quadrupole TOF-MS as well. This system was also employed for the identification of arsenic-containing ribofuranosides (arsenosugars) in the case of trimethylarsonioribosides without using standards [147]. A low energy CID-MS/MS in the negative-ion mode provided ions, which are suitable for unequivocal characterisation and identification of single dimethylated arsenosugar (sulfate-riboside) in S*argassum lacerifolim* algal extract [147]. With a nano-ESI interface the sensitivity of the quadrupole TOF-MS/MS technique for the structural characterisation of arsenosugar compounds, could be significantly improved and requires then only a few picograms of extract material to obtain tandem mass spectra of good quality. In comparison with the high-energy tandem mass spectra of arsenosugars, the low energy CID-MS/MS generally provided more reliable spectra with many structurally significant ions.

In summary, hyphenated techniques are an attractive tool for rapid and sensitive detection as well as for a comprehensive characterisation and quantification of As containing compounds in biological samples. On the other hand, there are still many challenges for the reliable isolation, detection, quantification and accurate characterisation of As species in living systems. In this area, there is still need for method development, interface optimisations and instrumental improvements.

2.1.4.4 Laser-induced breakdown spectroscopy of microdroplets (LADROP-LIBS) as a new analytical approach for As speciation

The miniaturisation of analytical methods for speciation analysis is an on-going trend. A field of growing interest is the development and/or improvement of analytical systems that allow it to obtain maximum detection information with minimum sample consumption. Most environmental, biological, medical and toxicological applications are dealing with liquid samples. Hence, the novel approach of laser-induced breakdown spectroscopy (LIBS) of microdroplets has been tested in this work with the aim to perform direct liquid microsampling. Hereby a unique piezoelectric pulse droplet generation sample introduction system, referred to as LADROP was used.

In this work first analytical results on a monodisperse droplet introduction system for liquid microsamples in spectrochemical elemental analysis are reported. The system is based on the concept of monodisperse microdroplet generation and on the creation of a bright plasma by focusing an intense laser beam on the droplets [148]. With the focused laser beam (or pulse) a single sample droplet is irradiated and the plasma emission detected, as known from laser-induced breakdown spectroscopy [148-150]. A pulse droplet generator (PDG) converts the sample solution into a number of uniformly picolitre droplets. The laser beam is focused on a droplet and a laser plasma is formed. In the high temperature laser spark, the sample is vaporised, atomised, ionised and excited. An atom in its excited state is unstable, remains there for an extremely short time and returns to the ground state spontaneously. This is accompanied by the emission of energy in the form of radiation and this radiation occurs at a wavelength (or wavelengths) which may be calculated from equations (2-1) and (2-2) [151]:

$$\Delta E = E_j - E_0 = h.\nu \tag{2-1}$$

E_j is the energy at the highest level, E_0 is the energy at the lowest level, h - Planck's constant and v is the frequency of the radiation. The wavelength of the radiation is related to its frequency:

$$\lambda = \frac{c}{\nu} \tag{2-2}$$

c is the velocity of the light and λ the wavelength of the radiation.

The emission of radiation does not necessarily occur at a single wavelength. An electron may return to the ground state in a single step or by a series of steps, corresponding to a number of intermediate energy levels. The larger the number of levels involved, the larger the number of spectral lines emitted and the more complex the spectrum.

Each element has a unique set of energy states and therefore, each element also emits a unique set of wavelengths. By measuring the wavelength of a spectral line, it is possible to determine which elements are present in a particular sample. In addition, the amount of radiation emitted is proportional to the number of the emitting atoms. Hence, the radiation emitted by the atoms and ions formed can be used for qualitative and quantitative purposes.

The PDG allows it to control the droplet size and the temporal sequence of the droplets delivery. In addition, the PDG is highly compatible with the very small active volume of the laser spark.

The LADROP-LIBS system used offers several significant advantages:

- extremely low sample amounts (a few nanolitres),
- minimal sample preparation,
- direct transfer of the sample into the plasma state, without the use of additional gas flow,
- less interference, (e.g. from oxide ions in ICP-MS) and lower signal noise,
- elemental composition analysis of complex samples,
- simultaneous multi-element detection, which permits the determination of around 30 different chemical elements at the trace, minor and major concentration levels,
- no sample dilution by the use of a nebulizer (carrier) gas,
- optical emission spectrometry (OES) does not suffer much from chemical interferences; although spectral interferences may be a problem.

Therefore, the LADROP-LIBS system is expected to be of use for both fundamental research and also for analytical applications using individual droplet/particle measurements. Since with LADROP-LIBS OES is performed directly at the small volume sample, the technique can be used for direct coupling to micro flow chromatography, such as cHPLC where only a few microlitres per minute of mobile phase flow are needed. Thus it is expected that very low detection limits can be achieved for elemental speciation analysis, as it is required for the determination of As species in complex biological samples. This would allow it to realise excellent features for different speciation applications using extremely small sample volumes, especially in the environmental, biochemical, toxicological and medical fields.

3 RESULTS AND DISCUSSION – METHOD DEVELOPMENT AND APPLICATION ON MARINE BIOTA

3.1 The use of cHPLC

In general, the separation efficiency and the sensitivity increase with a decreasing diameter of the HPLC columns. This, however, also allows it to work with nanolitre sample volumes, lower mobile phase flow rates and respectively a lower solvent consumption leading to cost effectiveness. As there are needs to miniaturise the instrumentation, the flow rate becomes difficult to reproduce, gradient elution is not as efficient, band broadening and detection volume need to be considered and care must be taken when loading minute sample volumes. For these reasons, the use of cHPLC columns is not yet established as a routine methodology for speciation analysis, nevertheless it has found application in different research areas [25, 12, 114, 117, 150].

One of the reasons for the immense interest in cHPLC is its potential to combine chromatographic selectivity with high efficiency and to work with limited sample amounts. Considering all advantages of cHPLC, it was decided in this work to be used as a main separation technique for the inorganic and organic As compounds. Other important criteria in method development are the chromatographic resolution, the sensitivity, the precision, the accuracy, the limit of detection, the linearity, the specificity and the time of analysis required. In all these respects, the quality of the cHPLC columns play an important role as the peak shape affects all criteria mentioned. Creating a highly effective, sensitive and robust separation in the shortest time possible is a goal in every development of chromatographic procedures.

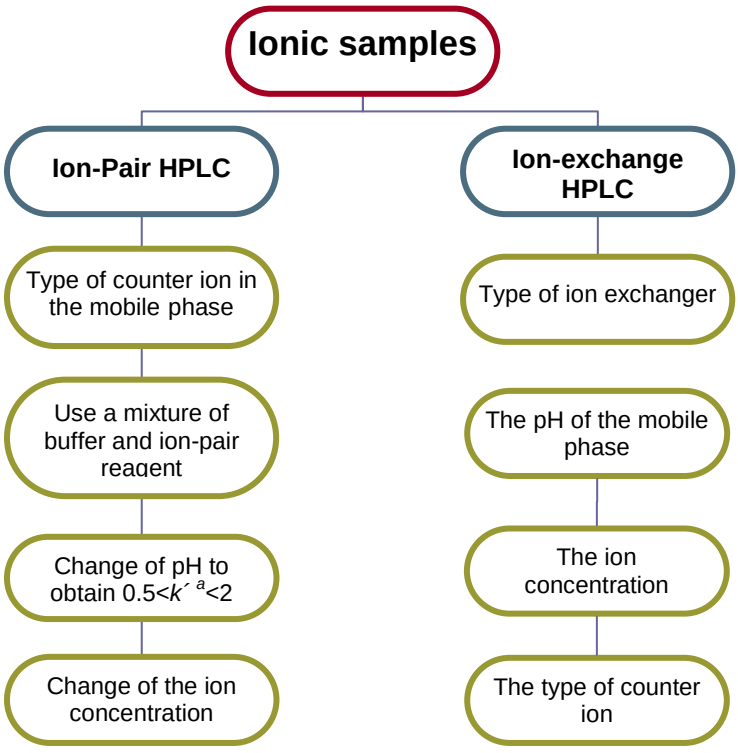
3.1.1 Optimisation of cHPLC for the separation of As containing ionic samples

Element speciation usually involves ionic or easily ionisable compounds. Depending on the pH conditions of the samples, As compounds can be anionic (As(III), As(V), MMA, DMA), cationic (AC, AsB, TMAO), uncharged (TMAO) and amphoteric AsB (which is actually zwitterionic). For the most common ionic species there are at least two HPLC methods which can be used in separation, namely: ion-pair and ion-exchange chromatography as it was already described in Chapter 2. However, the development and use of IP-HPLC separations is complicated. These procedures are subject to additional experimental problems such as artefact from positive and negative peaks for a solvent used as eluent, slow column equilibration, poor peak shape etc.

Ion-exchange chromatography is the most frequently used method for the separation and purification of ionic and ionisable As molecules, as it has already been shown in chapter 2. The reasons for the success of ion-exchange are its widespread applicability, high resolving power, high capacity and the simplicity and controllability of this chromatographic system.

The separation of As species with one chromatographic technique is a big challenge, as they have different physical characteristics. Accordingly, the choice of a good cHPLC column for an application, such as As speciation can be a very difficult process (see Scheme 3-1). The aims of an analytical separation can be realised by optimising the column packing, the column configuration and the elution conditions. The variables of the column packing are the separation mode, the bonded phase, the end-capping, the pore size, the particle size, the carbon load and the surface area. The variables in the column configuration are the length, the internal diameter, and the material of construction (stainless steel, plastic, silica or polyetheretherketone (PEEK)). As elution conditions the eluent composition, the elution profile (isocratic, linear gradient, and step gradient), the flow rate and the temperature are to be optimised. Frequently, the best way to determine the optimal set of variables is the experimental way.

Scheme 3-1 Optimisation of HPLC separations of ionic As samples



[a] - *k´*: retention factor

3.1.2 Behaviour of the As species in aqueous solution and HPLC strategy

When As_2O_3 and As_2O_5 are dissolved in water, they form acids:

$$As_2O_5 + 3H_2O \rightarrow 2H_3AsO_4 \quad (3\text{-}1)$$

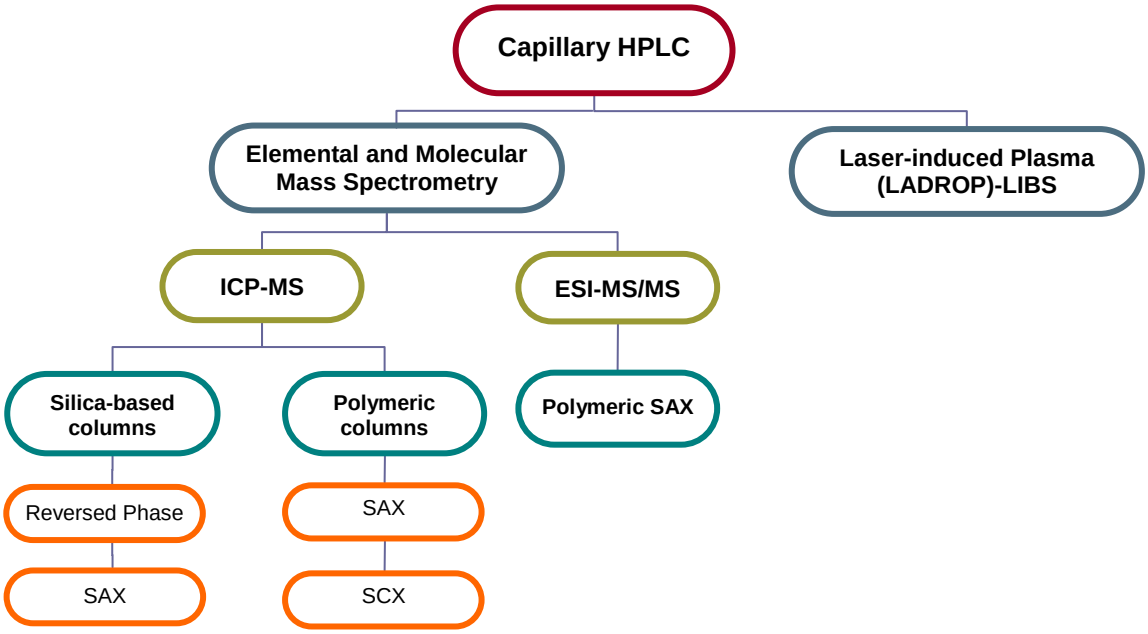
Orthoarsenic acid dissociates to give H^+ ions. Here, As behaves as a non-metal, like phosphorus, and forms *arsenates* with metals. Similarly, As_2O_3 hydrolyses to arsenious acid H_3AsO_3, whose salts are the *arsenites*. In acid conditions *arsenic acid*, H_3AsO_4; is predominant; whereas at weakly acid conditions the dihydrogen arsenate ion, $H_2AsO_4^-$; at weakly basic conditions the hydroarsenate ion $HAsO_4^{2-}$ and at basic conditions the arsenate ion AsO_4^{3-} are found. Most of the naturally occurring As species in the marine environment are listed in Table 3-1.

Table 3-1 As compounds in the marine environment

No. NAME (IUPAC or common)	***Formula***	***pKa*, [50, 123]**
1. Arsenious acid (arsenite- As(III))	H_3AsO_3	9.2; 12.1; 13.4
2. As acid (arsenate- As(V))	H_3AsO_4	2.2; 6.9; 11.5
3. Methylarsonic acid (MMA)	$CH_3AsO(OH)_2$	3.6; 8.2
4. Dimethylarsinic acid (DMA)	$(CH_3)_2AsO(OH)$	1.8; 6.2
5. Trimethylarsine oxide (TMAO)	$(CH_3)_3AsO$	3.6
6. Arsenobetaine (AsB)	$(CH_3)_3As\text{-}CH_2\text{-}COOH$	2.2
7. Arsenocholine (AC)	$(CH_3)_3As^+\text{-}CH_2\text{-}CH_2\text{-}OH$	-

When considering the characteristics of the known naturally occurring As species, the analytical strategy presented in Scheme 3-2 can be applied in the case of cHPLC coupled to MS with elemental (ICP) and molecular (ESI) ion sources.

Scheme 3-2 Development of analytical procedure for As speciation using cHPLC



3.2 Determination of As related compounds by cHPLC separations coupled to ICP-MS

Preliminary results obtained in the separation of As species with a cHPLC-DAD system have been transferred and applied to on-line coupled cHPLC-ICP-MS system. In general, the hyphenation of HPLC and ICP-MS combines a powerful separation method with an element specific detection at trace levels for metal and metalloid species, such as As species [12, 117]. There are many methods developed for the speciation of As on the basis of conventional size HPLC hyphenated to ICP-MS, as reported in the literature. However, none of them provides ultra high sensitive detection for applications in small marine biota samples or even on the cell level. The analytical methods, limitations and applications to various types of samples were reviewed in sections 2.1.2 and 2.1.4.

The analytical methods currently available for As speciation are either not selective enough or they do not provide sufficiently low detection limits especially in the case of short-living species present at ultra trace levels in biological samples. In this context the on-line coupling of highly efficient and sensitive cHPLC and ICP-MS for As speciation is a challenge.

3.2.1 Interferences for As determination by ICP-MS

Generally, the interferences in ICP-MS originating from the matrix, instrumental drift, isobaric overlap of some elemental isotopes and molecular ions formed in the plasma result in a decrease or an increase of the measured ion intensity. The isotopes determined are selected to so as to have a minimal isobaric overlap with signals of other elements and

molecular species that might be present. The production of oxides in the plasma can be minimised by reducing the amount of water vapour reaching the plasma through desolvation. Internal standards are normally used to minimise matrix effects and to correct for instrumental drifts for oxide formation. High concentrations of chloride can react in the argon plasma and form $^{40}Ar^{35}Cl$ with *m/z* 75. Because As is mono-isotopic and is measured at *m/z* 75 (As), chloride ions should be separated from the As species chromatographically in order to resolve the $^{40}Ar^{35}Cl$ signal from the analyte signals.

Rhodium (^{103}Rh), Germanium (^{72}Ge and ^{74}Ge), Yttrium (^{89}Y) or Rhenium (^{187}Re) are often added as internal standards to correct for matrix interferences and instrument instabilities.

3.2.2 Small volume sample introduction systems for ICP-MS

Two approaches were investigated for analysing small volume samples with ICP-MS. The first one makes use of the standard ICP-MS and a micro TEE connection for mixing the cHPLC flow with a microflow of make-up liquid. In second approach an in-house modified microflow concentric nebulizer (MCN) for CE-ICP-MS, based on the CEI-100 from CETAC Technologies, Canada and an in-house made small glass spray chamber without drain are used.

3.2.2.1 Direct coupling of the cHPLC column flow through micro TEE zero dead volume connection with the standard ICP-MS nebulizer

First, the performance of the conventional sample introduction system of the Sciex Elan 5000 ICP-MS at very low sample flow rates has been studied for arsenic. The nebulizer and auxiliary argon flows were optimised prior to the analysis. 2% HNO_3 solution was used as a make-up liquid and mixed with the cHPLC mobile phase flow into a TEE PEEK zero dead volume connection. The cHPLC flow was connected through a PEEK silica capillary to the TEE connection and on the other side the make-up liquid was entered and finally the combined flow was fed into the conventional pneumatic nebulizer. Even at an extremely low flow of only 10 µL/min exiting the cHPLC and a 0.1 µL sample injection, a satisfactory stability of the signal for As with quadrupole ICP-MS was obtained. Furthermore, a mixture of six As standard compounds was analysed with the cHPLC coupled on-line to ICP-MS using a SAX Zorbax capillary column. The larger dead volume of the standard nebulizer and the spray chamber, led to low resolution and to low efficiency of the chromatographic separation. Nevertheless, each species produced one chromatographic peak, confirming the high specificity of the ICP-MS detection. Although the concentration of all As species in the sample solution was equal, for each As species different peak heights were obtained. This has been observed previously and reported as well [35, 42, 53].

Small HPLC columns (internal diameters less than 2 mm) in general offer many advantages, but because of the small peak volumes, special care must be taken to obtain their maximum performance. cHPLC columns and flow rates below 15 µL/min were difficult to handle with the ICP standard concentric nebulizer and a Scott-type spray chamber. Moreover, the silica-based capillary columns gave a high backpressure, which was disturbing the plasma stability and the plasma was sometimes extinguished. However, a determination of the As species at higher concentrations (> 10 mg/L) with this interface was possible.

These results were encouraging so as to further investigate the on-line coupled cHPLC-ICP-MS system and to improve the sample introduction system of the ICP-MS in order to produce better results. Therefore, a micro nebulizer and a small size spray chamber were selected in order to minimise dead volume of the interface and to optimise the performance of the cHPLC column.

3.2.2.2 In-house modified microconcentric nebulizer and micro spray chamber for ICP-MS

This interface is based on a commercial MCN CEI-100 interface [154] for capillary electrophoresis from CETAC Technologies, Canada. Schaumlöffel and Prange [121, 152] developed a new interface for coupling CE to ICP-MS, based on a modified microconcentric nebulizer, able to freely aspirate microflows between 2 and approx. 12 µL/min. Although also here a sheathing liquid flow was employed, they obtained higher sensitivities and nearly 100% sample transport efficiency due to the use of adjustable nebulizer capillary. Later Shaumlöffel *et al*. [10] utilised the interface sheathless for coupling cHPLC and ICP-MS in selenopeptide mapping applications. Recently Pröfrock *et al.* [153] compared this interface with the commercially obtainable PFA 20 concentric and DIHEN nebulizers for coupling cHPLC with collision-cell ICP-MS and demonstrated its advantages at the hand of phosphorylation profiling of tryptic digests. They found that the modified MCN CEI-100 is giving the best performance. Therefore, in all further investigations we used the in-house modified MCN CEI-100 combined with a small volume quartz spray chamber.

3.2.2.3 Performance of the small volume sample introduction system with the Sciex Elan 5000 ICP-MS

The present study focuses on the performance of the in-house modified MCN CEI-100 and the small-volume quartz spray chamber with the Elan 5000 ICP-MS. A stable sample flow could be obtained without addition of a make-up argon gas flow and the Ba^{2+}/Ba^{+} ratio could be kept below 2% when working in the range from 0.5 to 15 µL/min. The optimum sample flow at which no condensation in the spray chamber occurred was between 4 and 8 µL/min (see Figure 3-1).

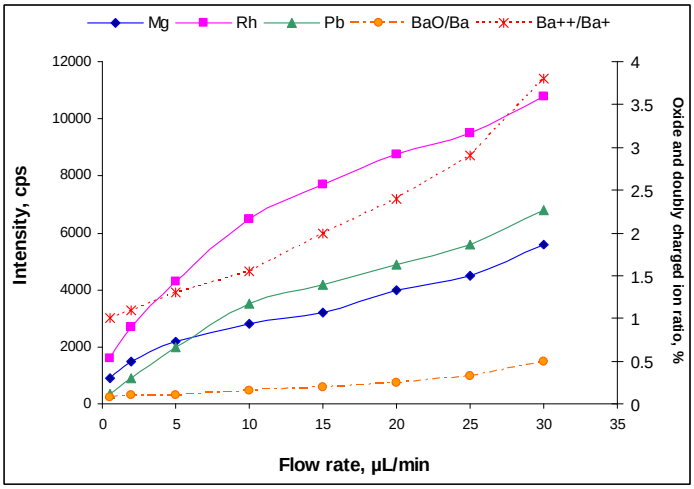


Figure 3-1 Optimisation of the microflow sample introduction system with the Sciex Elan 5000 ICP-MS.

After optimisation of the system, a report was generated at each optimum (nebulizer gas, auxiliary gas and sample flow rate for signal maximum) was used to check the sensitivity, the oxide and doubly charged ion to singly charged ion ratios comparing them to those of the normal set-up (Table 3-2). The results of the experiments are presented in Figure 3-2. The short-term signal stability was very good when rationing the As signal to the one of Rh as internal standard (I.S.) namely 3.3% for ^{75}As and 2.2% after rationing to ^{103}Rh. Although microflow delivery with a peristaltic pump exhibits poor precision even at a low back pressure, the long-term signal stability over eight hours was very good with RDS% below 5%. A negligible instrumental drift occurred, but by using an internal standard high precision of the instrument was obtained. Also, interface clogging did not occur.

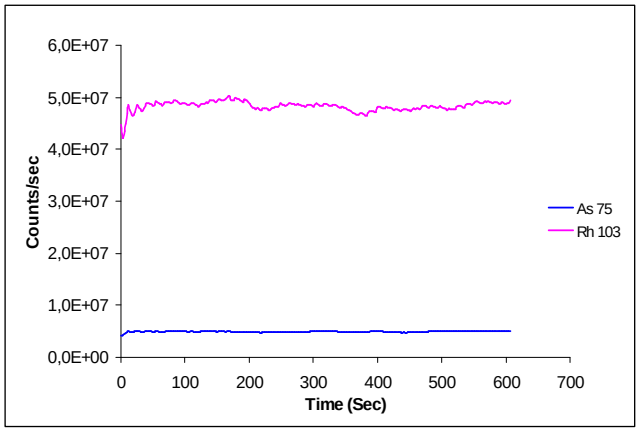


Figure 3-2 Short-term signal stability observed at a 7.5 µL/min sample flow rate when using a peristaltic pump with the Sciex Elan 5000 ICP-MS.

The memory and/or the wash-out times of the small-volume sample interface for ICP-MS were also studied using at same conditions and with a solution of 10 µg/L of As and rhodium. In Figure 3-3 a complete wash-out time for As and rhodium is shown to be at 65 sec

of which around 5 sec item from the wash-out of the micro nebulizer and the spray chamber. The data were collected at the operation conditions listed in Table 4-11 (experimental part). This result also confirms that rhodium is a good internal standard for the determination of arsenic. The wash-out time is namely related to memory effects in the tubing as nearly no condensation could be observed on the walls of the small quartz spray chamber.

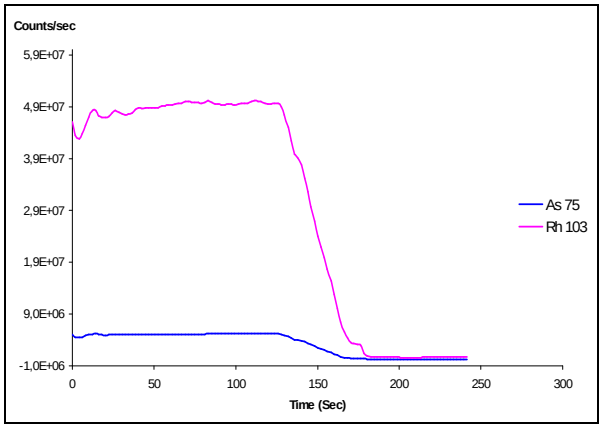


Figure 3-3 Wash-out times for 10 µg/L of As and rhodium as internal standard at concentration. Blank solution: 2% HNO_3, sample flow rate: 7.5 µL/min.

As presented in Table 3-2, the doubly charged and oxide ion interferences were very low. This is due to the small water vapour concentrations in the interface region, as only a sample flow of 7.5 µL/min was used.

Table 3-2 Comparison of the ICP-MS instrument settings and performances with two different sample introduction systems

	Standard set-up (cross-flow nebulizer and Ryton® spray chamber)	**Small volume spray chamber and MCN CEI-100 (at the optimum flow rate and nebulizer gas flow)**
Rf power – W	1030	1035
Plasma gas – L/min	15	15
Nebulizer gas –L/min	1.02	1.03
Auxiliary gas – L/min	1.74	1.74
Pump flow rate -µL/min	1000	**7.5**
$^{24}Mg^{+}$ - counts/second	11000 (3.18% RSD)	2538 (3.85% RSD)
$^{103}Rh^{+}$ – counts/second	39000 (2.66% RSD)	6175 (2.98% RSD)
$^{208}Pb^{+}$ – counts/second	22000 (3.28% RSD)	3595 (4.99% RSD)
$^{138}Ba^{16}O/^{138}Ba^{+}$ - (%)	0.17	0.15
$^{138}Ba^{2+}/^{138}Ba^{+}$ – (%)	1.6	1.55

It has to be pointed out that as a result of the 133 times lower sample flow rate the sensitivity decreased by 84% for Rh and Pb and by 77% for Mg, while the oxide ratio and the doubly charged ions to singly charged ions ratio remain almost the same. The background noise decreased, especially for the oxide ions and the doubly charged ion signals (from

564 ±11 to 62±12 counts). The results for the doubly charged ions and the oxide ions were lower at the instrumental conditions selected than in the results specified for CeO and Ce^{2+} by the manufacturer [154].

These results were encouraging, and were the results of the high efficiency of the small-volume sample introduction system. From basic analytical considerations, the results are consistent and confirm that this instrumental set-up can be used for speciation analysis of arsenic. The small volume spray chamber and the microflow nebulizer are very promising for both the analysis of small sample volumes (100 µL or less) by flow injection analysis (FIA) and for coupling cHPLC with ICP-MS for the speciation of arsenic.

3.2.2.4 On-line coupling of cHPLC with ICP-MS via an in-house modified CEI-100 nebulizer and a micro spray chamber

The flow of the cHPLC pump was directly conducted from the micro autosampler to the CEI-100 nebulizer with the shortest possible connection. These experiments were performed without cHPLC column. Ultrapure water was pumped at flow rate of 7 µL/min to determine the noise level of a blank. The on-line connection was realised with a PEEK silica capillary tubing with an I.D. of 50 µm and a length of 40 cm and zero dead volume fittings. 500 nL samples of a mixture of As and rhodium at concentration of 10 µg/L were injected into the ultrapure water flow and directly introduced into the plasma. The wash-out curve in this case is presented in Figure 3-4. The results ascertain that the memory effect and the wash-out for As and Rh last less then 60 s.

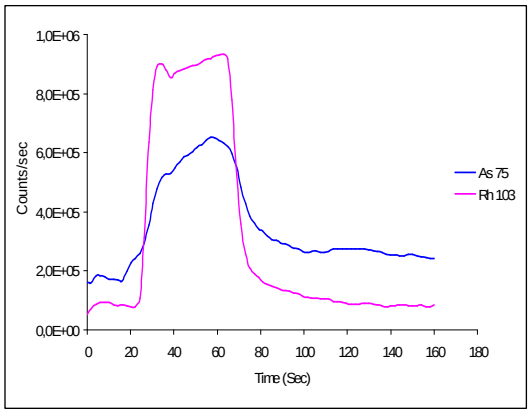


Figure 3-4 Wash-out curves for a test solution containing 10 µg/L As and Rh. Sample injection volume: 500 nL; HPLC flow rate: 7 µL/min of ultrapure water.

The limit of detection of the cHPLC coupled to ICP-MS for As and the I.S. with this instrumental assembly were also determined. The instrumental limit of detection was determined as the concentration corresponding to a signal of 3 times the standard deviation of the blank (SD_{blank}) without capillary column. The signal produced when injecting a 2% nitric acid solution was used to estimate the blank level. With 0.1 picograms extremely low

absolute LODs for both elements were obtained, as it is shown in Table 3-3. This demonstrates the advantages of cHPLC coupled to ICP-MS since up to the time of the experiments performed in this work, no such LODs were published for those elements with an Elan 5000 ICP-MS [133].

Table 3-3 LOD for As and Rh with the capillary HPLC-ICP-MS without column

Element	Counts / sec	LOD*		RSD (%)
		Relative C_L µg/L	Absolute C_L pg	
^{75}As	173.3	10	0.1	7.2
^{103}Rh	426.3	10	0.1	6.6

* LOD= 3 x SD_{blank}
cHPLC flow rate: 7 µL/min, injection volume: 10 nL

This set of instrumental conditions demonstrated several advantages over the standard instrumental conditions, which can be listed as follows:

- analyte transport efficiency into the plasma of nearly 100%,
- reduction of the sample flow rate by over 100 times,
- improvement of the signal to noise ratio,
- low wash-out time / memory effect,
- smaller sample volume are possible,
- easy coupling of the sample introduction system to the plasma torch.

Furthermore, the small sample introduction system may be considered as a total consumption system similar to the direct injection nebulizer (DIN) or to the direct injection high efficiency nebulizer (DIHEN). The main advantage of the set-up used in this work over other systems such as the DIN [130, 155], the high-efficiency nebulizer (HEN) [156] and the DIHEN [153, 157] is the much lower oxide ion to singly charged ion ratio obtained as a results of the lower amount of water vapour being transported into the plasma. An excellent agreement with the findings of Pröfrock *et al.* [153] who used the same interface with collision-cell ICP-MS was obtained. However, some disadvantages such as the lower precision of the small-volume sample introduction system and the reduction of the sensitivity by 77 to 84% remain. The precision of the flow from the cHPLC was also better than obtained with the peristaltic pump. Therefore, the combination of a small-volume spray chamber and a microflow nebulizer is a very suitable interface for cHPLC and capillary electrophoresis to ICP-MS.

As a next step this interface must be used with other ICP-MS systems, such as the Agilent 7500s so as to validate the results obtained. The development of a micro chromatographic separation method with ICP-MS detection for standards containing different

As species was the following step. Also the applicability of the in-house modified MCN CEI-100 and the small volume spray chamber for such an application must be investigated.

3.2.2.5 Performance of the small-volume sample introduction system with the Agilent 7500s ICP-MS

An Agilent 7500s ICP-MS instrument was used for element-specific and highly sensitive MS detection (in comparison with the Elan 5000 ICP-MS) and coupled on-line to the cHPLC. As sample introduction system the in-house modified MCN CEI-100 together with the small volume quartz spray chamber without a drain was used. The ICP-MS Agilent 7500s system with its new Shield Torch interface was developed to virtually eliminate Ar interferences, to reduce ion energy spread, to allow optimum ion focusing and increased sensitivity and to enable determinations of elements, such as mercury, As and Se at the ppt level.

A characterisation of this instrumental set-up was performed by monitoring the doubly charged ions and the oxide ions to singly charged ion ratios of the element ^{140}Ce at different sample flow rates. A blank run without a column in the line was made so as to establish the responses for a tuning solution containing elements with widely differing masses, such as ^{7}Li, ^{89}Y, ^{140}Ce and ^{205}Tl in the case of a the cHPLC pump. Another experiment has been done at identical conditions in order to ascertain the signal response and stability for ^{75}As, ^{103}Rh and ^{72}Ge, which were intended to be used as internal standards.

In Figure 3-5 the characterisation of the interface as a function of the sample flow rate is shown. In both cases, it was found that the intensities increased with the sample flow rate. Doubly charged ions and oxide ions to singly charged ion ratios were higher at very low flow rates and then reached the optimal range (< 2.5%) before they again slightly rise with the flow rate. This profile can be explained with an increase of the water vapour concentration in the plasma. At the flow rates between 8 and 20 µL/min the percentage of the doubly charged ions and oxide ions to singly charged ion ratios found to be below 2%.

The results demonstrate that in the case of the microconcentric nebulizer and a small volume spray chamber stable working conditions are obtained at sample flow rates between 4 and 20 µL/min, regardless of the ICP-MS instrument used. A next step in the method development of As speciation was the optimisation of the separation of the species by cHPLC.

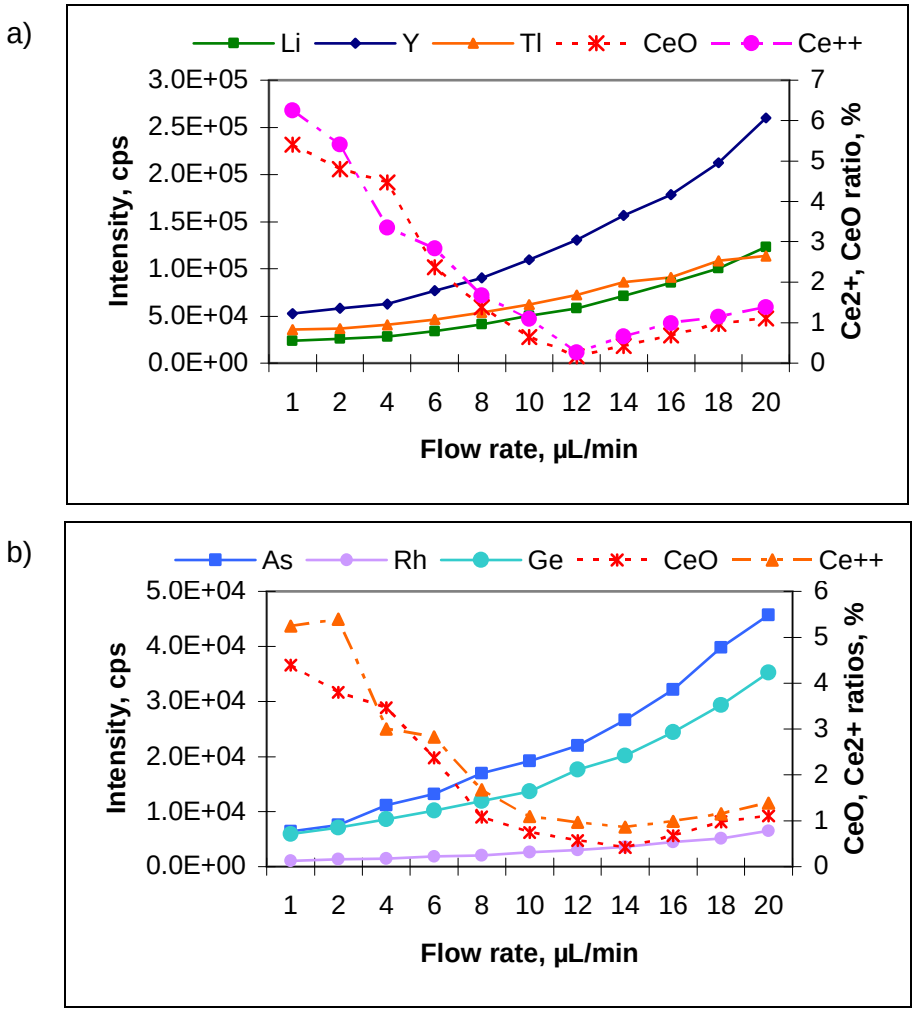


Figure 3-5 Influence of the micro sample flow rate on the element response, a) for Li, Y, Tl, CeO and Ce $^{2+}$ and b) for As, Rh, Ge, CeO and Ce^{2+}.

3.2.3 Chromatographic and detection studies

3.2.3.1 Ion-pair chromatography with a XTerra cHPLC column coupled to ICP-MS with a MCN and a micro spray chamber

For the separation of a mixture of ionic, ionisable and neutral As species, ion–pair chromatography on a bonded reversed phase silica XTerra C_8 cHPLC column has been carried out. It was coupled with ICP-MS detection via an in-house modified MCN and a micro spray chamber. Retention is achieved by two ways of interactions, namely reverse phase (or hydrophobic interaction, which is usually referred to as Van der Waals) and ion-exchange (as a result of electrostatic interaction). For bases the mobile phase must have a pH above 11 so as to guarantee that the compounds are neutral and therefore hydrophobically enough for being retained under reversed-phase conditions. Operation under these conditions requires special phases with hybrid particles that are stable at a high pH. The XTerra C_8 capillary

column contains a hybrid stationary phase and is used for a selective retention of polar As compounds in these investigations.

Based on a theoretical consideration of the resolution (Rs) and the related separation between two components in a mixture are as the chromatographic requirements, as listed in Table 3-4:

Table 3-4 Relation between the chromatographic resolution (Rs) and the peak separation

Rs ≥	Separation, %
1.5	99.7
1.25	99
1	98
0.75	50
0.5	0

- **Separation in the case of weak and strong acids as ion-pair reagents for cationic and amphoteric As species**

To accomplish such separation, a RP ion-pairing cHPLC using different ion-pairing reagents was investigated. The so called weak and strong cation-pair reagents such as malonic acid [28, 29, 40, 44] and tartaric acid, hexanesulfonic acid [114], heptanesulfonic acid [113] and 1,2-benzenedisulfonic acid [35, 42, 50], respectively, were used for the elution of cationic arsenocholine, TMAO, amphoteric AsB and ionisable As(III), As(V) and DMA in acidic media. The chromatographic conditions, the mobile phase composition, the pH and the flow rate were optimised in order to obtain the required resolution with the cHPLC column. The pH of the mobile phase was carefully chosen so as to obtain a maximum ionisation of the As species. Selected results are given in Table 3-5. The concentrations of the ion-pair reagents were preferably kept low so as to prevent salt precipitation in the cHPLC column and to keep the distribution coefficient of the reagent constant.

From these results it can be derived that the cationic and amphoteric As species AC, AsB and TMAO showed a better α and k' (>2). A successful separation of all As compounds investigated was obtained with a mobile phase of 5 *mM* hexanesulfonate at pH 3. In all other cases the addition of ion-pair reagents did not improve the retention and separation. The use of malonic and tartaric acids and the change of the pH did not improve the separation and the selectivity on the XTerra C_8 capillary column, as the pKa values of the analytes are not very similar. As(III) and As(V) are not eluted at the dead volume of the column due to a hydrophobic interaction between the As species molecules and the organic base of the stationary phase. Regardless of the ion-pair reagent and the pH, the preconditioning of the column required a long time (more than 30 min up to 1 hour), which leads to long total analysis times.

Table 3-5 Retention factors (*k'*), separation factors (α) and resolution (Rs) for different anion-pair eluents in the case of XTerra C_8 column

Mobile phase		As(III)	As(V)	DMA	TMAO	AB	AC
4 *mM* Malonic Acid, pH 2.7	*k'* [a]	0.1	0	-	2.7	1.2	2.7
	α [b]	-	-	-	23.4	11	23.6
	Rs [c]	0.4	-	-	10.1	4.7	22.0
4 *mM* Tartaric Acid, pH 4.4	*k'* [a]	0.2	0.1	-	3.1	0.0	0.30
	α [b]	0.8	1.2	-	9.8	n.d.	2.1
	Rs [c]	0.2	0.5	-	7.5		1.1
5 *mM* Hexanesulfonate, pH 3.0 (glacial CH_3COOH): 1% ACN	*k'* [a]	0.7	0.5	0.7	0	2.4	0.5
	α [b]	**8.2**	**6**	**7.3**	**n.d.** [d]	**4.5**	**5.9**
	Rs [c]	1.6	1.2	1.7	-	3.4	1.7
5 *mM* Hexanesulfonate, Citric acid, pH=4.5: 0.1% ACN	*k'* [a]	0.6	0.5	0.07	1.7	0.0	0.04
	α [b]	1.3	11.6	1.7	38.5	n.d	n.d
	Rs [c]	1.2	0.8	0.1	8.1		0.1

[a] $k' = (t_R - t_0) / t_0$, t_R: the retention time of the analyte, t_0: the time of unretained compound, here defined as the retention time of As(V) and AsB, respectively,
[b] $\alpha = k'_2/k'_1$
[c] $Rs = 1.18 \times (t_{R2} - t_{R1}) / (w_{1/2\,2} + w_{1/2\,1})$, where $w_{1/2}$ is the peak width at half height
[d] n.d.: not defined, because $k_1 = 0$

Generally, quaternary ammonium salts in an alkaline medium are used to form ion pairs with strong and weak acids. Above pH=7 As(III), As(V), MMA and DMA became anions and therefore tetrabuthylammonuim hydroxide (TBAH) [9, 28, 29] can be used as an anion-pair reagent for their separation.

- **Separation of the anionic As species with the aid of TBAH**

A chromatographic experiment has been carried out at pH=10. Here the four arsenicals AsB, As(V), MMA and DMA could be well separated in 18 min (1080 sec), as it is shown in Figure 3-6. Arsenobetaine was first eluted from the column and was not well separated from As(III). Unfortunately, incomplete separation of DMA and As(III), as well as non perfect baseline-to-baseline separation of MMA and As(V) was achieved. This could be due to inadequate selectivity of the mobile phase or to a high dead volume of the on-line coupled system, since all peaks had tails.

To further explore the possible use of anion-pair chromatography for the speciation of As, the influence of the mobile phase was investigated. Less efficient separations occurred in the presence of even small amount of methanol (< 0.5%). Also the flow rate of the mobile phase had an influence on the separation efficiency. The best separation was obtained at the low flow rate of 4 µL/min. The influence of the concentration of the TBAH anion pair reagent on the resolution of anionic As compounds is illustrated in Figure 3-7. The best separation was obtained with a mobile phase of 10 *mM* TBAH and at pH 10. All polar or neutral arsenicals were eluted at the front of the chromatogram and were well separated from the anionic As species.

Chromatographic conditions:

Column:	XTerra 150 x 0.32 mm, 3.5 µm
Mobile phase:	10 *mM* Tetrabuthylammonuim hydroxide, pH 10
Flow rate:	4 µL/min
Injection:	0.01 µL
Sample:	20 mg/L standard mix solution
Detection:	Sciex Elan 5000 ICP-MS with MCN and micro spray chamber

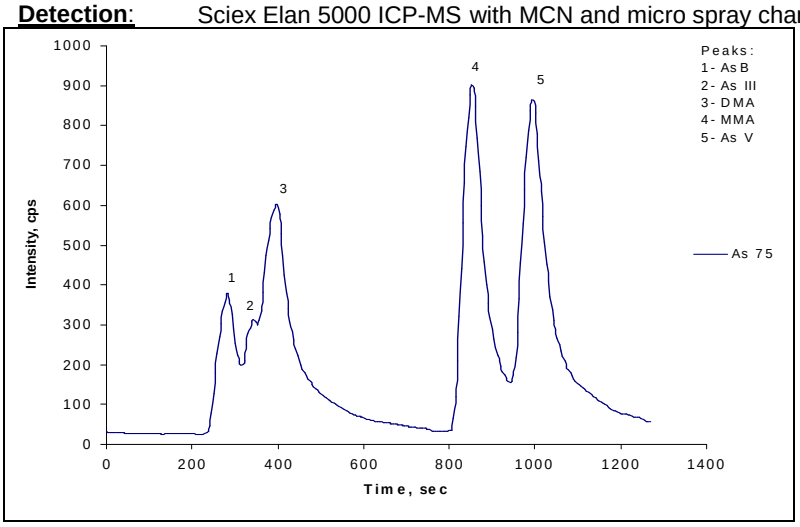
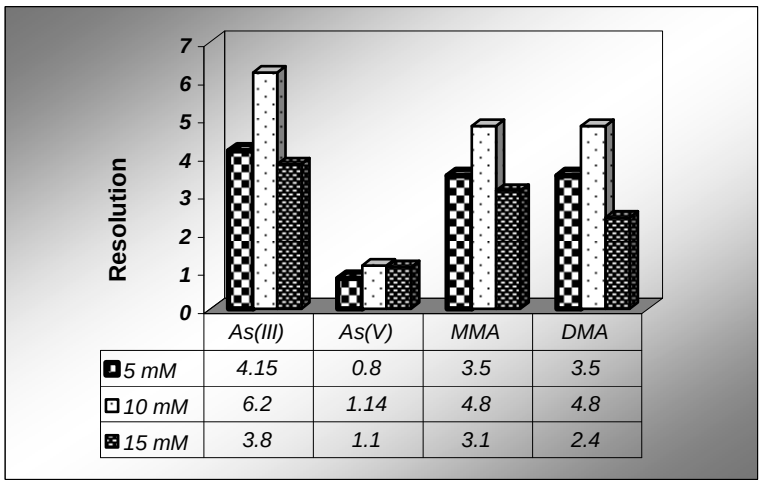


Figure 3-6 Anion-pair chromatogram for As species obtained with anion-pair capillary chromatography coupled to ICP-MS using a small volume interface.

TBAH as ion-pair reagent yields a better separation and a higher selectivity than organic acids and alkylsulfonates for a mixture of all different As species on an XTerra C_8 capillary column. However, the anionic inorganic and organic As species AsB, DMA, MMA and As(V) were retained. This can be explained with the hybrid nature of the stationary phase of XTerra, which was especially developed for use in a wider pH range and for improvements in the separation of basic analytes.



	As(III)	As(V)	MMA	DMA
5 mM	4.15	0.8	3.5	3.5
10 mM	6.2	1.14	4.8	4.8
15 mM	3.8	1.1	3.1	2.4

Figure 3-7 Influence of the concentration of TBAH as ion-pair reagent on the separation of As species.

Ionic samples can also be separated by ion-exchange chromatography. Unfortunately, ion-exchange cHPLC columns were not commercially available. However, silica based capillary SAX column was kindly produced by the column centre of Agilent Technologies in Germany. This Zorbax SAX capillary column is having a limited pH stability up to pH 6.5. Thereafter, polymeric SAX cHPLC columns were packed in-house. Silica and polymeric SAX cHPLC columns were on-line connected to the microflow nebulizer and a separation of the As species was further carried out with this set-up.

3.2.3.2 Determination of As species by silica-based SAX cHPLC coupled to ICP-MS with the aid of small-volume sample introduction system

The analytical system developed was used to separate the naturally occurring organic and inorganic As compounds on a silica-based Zorbax SAX cHPLC column. As species are ionic or easily ionisable in solution depending on the pH. As they occur as anions, cations and neutral molecules in solution, the separation of arsenicals in one chromatographic run is a challenge. The experiments were started with a buffered mobile phase at the pH near the pKa value of As(V) and a good peak shape without tailing for As(V) was achieved. As in some cases the separation was inadequate, the following adjustments were made to the operating conditions:

- change of the counter ions in the mobile phase,
- use of a mixture of buffer and ion-pair reagent,
- adjustment of the percentage of organic solvent in the eluent or gradient range,
- change of the pH and ionic concentration of the mobile phase.

Seven As compounds, the structures of which are shown in Table 3-1, could be separated on this column. Separation of the very toxic inorganic arsenic, the moderately toxic methylated As compounds and the organic species of lower toxicity is required. The retention obtained on the Zorbax SAX cHPLC column was evaluated by analysing mixtures of standard As solutions with different mobile phase buffers.

A first experiment was carried out with ammonium dihydrogen phosphate buffer at a pH of 5.5. As expected, the cationic arsenocholine and the amphoteric arsenobetaine were first eluted, followed by the neutral TMAO. The anionic MMA, DMA and As(V) are more retained on the SAX capillary column. The efficiency and the selectivity of the retention of As(III) and arsenobetaine was not satisfactory as no separation down to the baseline has been achieved. Thus in a next attempt ammonium hydrogen carbonate buffer was tested.

3.2.3.2.1 Ammonium dihydrogen phosphate buffer

With ammonium dihydrogen phosphate buffer (also known as mono ammonium orthophosphate) a good separation for some of the As species was obtained, as it is shown

in Figure 3-8. Apparently, ion-exchange and molecular interaction mechanisms influenced the retention.

Chromatographic conditions:
Column: Zorbax SAX 250 x 0.5 mm, 5 µm and guard 35 x 0.5 mm
Mobile phase: 2 *mM* Ammonium dihydrogen phosphate, pH 5.5
Flow rate: 10 µL/min
Injection: 0.05 µL
Sample: 20 mg/L standard mix solution
Detection: Sciex Elan 5000 ICP-MS with MCN

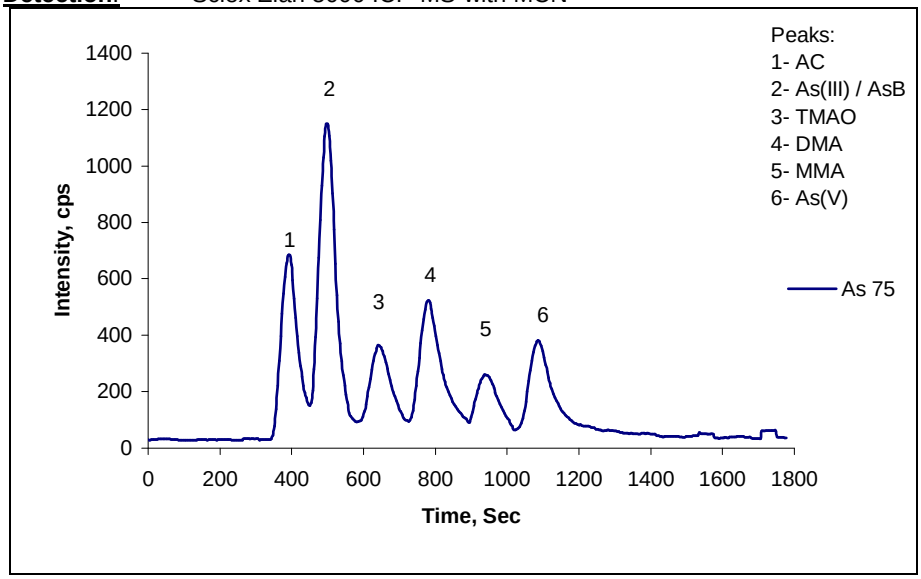


Figure 3-8 Chromatogram of As species in the case of a Zorbax SAX cHPLC column and ICP-MS using small-volume interface (Ammonium dihydrogen phosphate mobile phase).

One drawback of this buffer was an easy blocking of the microconcentric nebulizer, even when working at the very low concentration of 2 *mM*. This is a well-known phenomenon in HPLC and therefore ammonium phosphate buffer was not used in further applications.

3.2.3.2.2 Ammonium hydrogen carbonate buffer

A 0.05 µL of a mixture of As species sample was separated on the strong anion-exchange capillary column, according to the protocol outlined in Table 4-13. The chromatogram of the As compounds obtained with the Zorbax SAX cHPLC column and detection with ICP-MS via the in-house modified MCN and micro spray chamber is shown in Figure 3-9.

The amphoteric AsB and cationic AC were eluted first, followed by As(III) and the neutral TMAO. The anionic MMA, DMA and As(V) are more retained on the SAX capillary column. The separation of AC, AsB and As (III) is more satisfactory with respect to the baseline separation achieved.

Although the concentration of the buffer was lower, blocking of the microconcentric nebulizer also occurred. However, the column provided chromatographic peaks for all inorganic and organic As species studied.

Chromatographic conditions:

Column:	Zorbax SAX 250 x 0.5 mm, 5 µm and guard 35 x 0.5 mm, 5 µm
Mobile phase:	1 m*M* Ammonium hydrogen carbonate, pH 6
Flow rate:	10 µL/min
Injection:	50 nL
Sample:	20 mg/L standard solution
Interface:	direct coupling with MCN-100 and micro spray chamber
Detection:	Sciex Elan 5000 ICP-MS

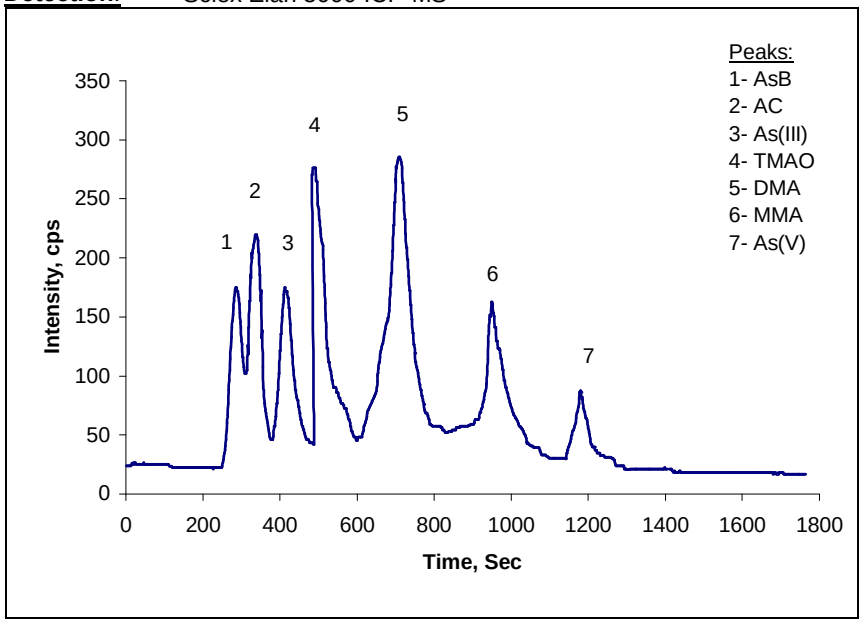


Figure 3-9 Chromatogram of As species in the case of a Zorbax SAX cHPLC column and the Sciex Elan 5000 ICP-MS with a small-volume sample interface (Ammonium hydrogen carbonate mobile phase).

Charged As compounds can form strong ionic bonds with the functional group of the stationary phase, whereas other As compounds are bound by hydrophobic interaction of with silica. This dual interaction requires a high ion-exchange capacity to improve the retention of ionisable compounds. The hybrid XTerra C_8 cHPLC column provided the critical efficiency to separate the more polar basic and zwitterionic compounds from the basic non-polar fraction of organoarsenicals. Anion-exchange chromatography showed to be more efficient, since not only the ion-exchange retention mechanism is involved in the separation of the ionic and neutral As compounds. However, the selectivity and the efficiency of the silica based Zorbax SAX capillary column were not effective enough to provide successful separation of all As species studied down to the baseline. Hence the use of polymeric SAX capillary columns may be of further help. Polymer resins namely may offer several advantages over silica-based materials such as robustness within a larger pH range and inertness to various organic and inorganic eluents. Therefore, polymer cHPLC columns have been packed in-house and used for the chromatographic separation of inorganic and organic As compounds.

3.2.4 Polymer-based cHPLC columns coupled on-line with ICP-MS via microflow concentric nebulizer and micro spray chamber

Two different types of resins were used to pack cHPLC columns for As speciation (See experimental part section 4.3.2). The first type has a PSDVB matrix with a strong anion- or strong cation-exchange modifier. The polystyrene-divinylbenzene (PSDVB) strong anion-exchanger could separate inorganic and organic mono and doubly charged ions. The second type of resin was a latex matrix with a strong anion-exchanger, especially developed for the separation of polyvalent ions such as As speciation.

3.2.4.1 Separation of As compounds with the aid of Hamilton PRP-X100 cHPLC column and ICP-MS detection

For the different ionisable and neutral As compounds up to now no common pH could be found where all the different molecules are retained on the column. Accordingly, it is difficult to analyse samples containing acidic, basic, and zwitterionic compounds within a single type of HPLC procedure.

A separation of the ionic, polar and neutral As species was performed with a cHPLC column 250x0.5 mm packed with the SAX 10 µm Hamilton PRP-X100 resin. The optimised experimental conditions are given in Table 4-12 and Table 4-14 in chapter 4 of the experimental part.

Initially, a simple isocratic elution was attempted. Since the isocratic elution did not deliver an adequate separation for the As species, gradient elution was used as well.

3.2.4.1.1 Gradient elution with different buffer systems

Experiments with mobile phases of different pH were performed in order to obtain a good separation of the As species.

- **Gradient elution with a mobile phase at pH ~ 6**

In Figure 3-10 it is shown that an excellent resolution and low retention times for the anionic arsenicals and some dimethylated arsenoribosides are obtained at pH 5.8. These strongly ionic compounds were capable of interacting with the surface active basic part of the stationary phase through anionic interactions, resulting in symmetrical peaks.

Chromatographic conditions:

Column:	Hamilton PRP-X100, 250 x 0.5 mm, 10 µm
Mobile phase:	0.1 and 20 *mM* Ammonium nitrate, pH 5.8 + 10 µg/L Re
Flow rate:	12 µL/min
Injection:	0.01 and 0.08 µL As species standard solution
Sample:	10 mg/L methylated arsenicals and 0.5 mg/L arsenoribosides
Detection:	Sciex Elan 5000 ICP-MS with MCN-100 and micro spray chamber

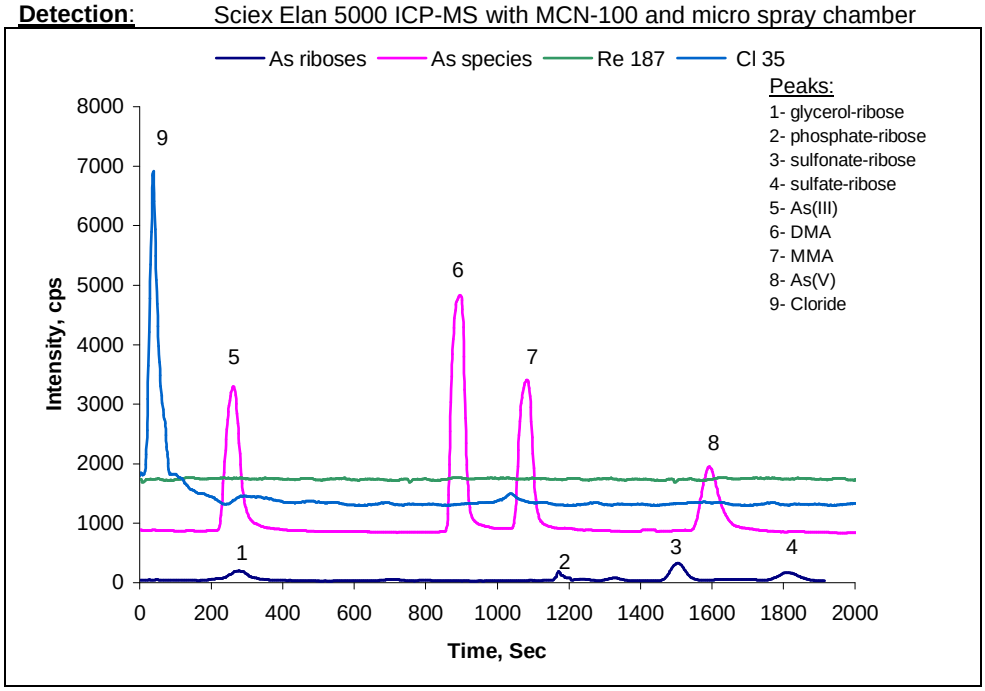


Figure 3-10 Determination of As species by separation on a Hamilton PRP-X100 cHPLC column with gradient elution at pH 5.8 coupled to the Sciex Elan 5000 ICP-MS.

Apart from the alkylarsenicals, the other group of arsenosugars found also as naturally occurring in marine biota need to be separated as well. They are further along in the next investigations, since one objective of this work was to develop a method for a simultaneous determination of as many As species as possible.

- **Gradient elution at pH 8.5**

In Figure 3-11 the separation of As species using a Hamilton PRP-X100 cHPLC column coupled on-line with ICP-MS detection via the in-house modified MCN and a micro spray chamber under gradient elution at basic media is shown. The combination of separation on the Hamilton PRP-X100 cHPLC column and ICP-MS detection with the Sciex Elan 5000 provided a sensitive and selective method for the determination of all As compounds studied. Gradient elution with an ammonium nitrate solution gave an efficient and selective separation by anion-exchange and polar interaction for the 10 inorganic and organic As species studied here.

Chromatographic conditions:

Column:	Hamilton PRP-X100, 250 x 0.5 mm, 10 µm
Mobile phase:	0.2 and 20 *mM* Ammonium nitrate, pH 8.5 + 10 µg/L Re
Flow rate:	12 µL/min
Injection:	0.01 µL
Sample:	10 mg/L methylated species and 0.5 mg/L arsenoribosides solution
Detection:	Sciex Elan 5000 ICP-MS: MCN-100

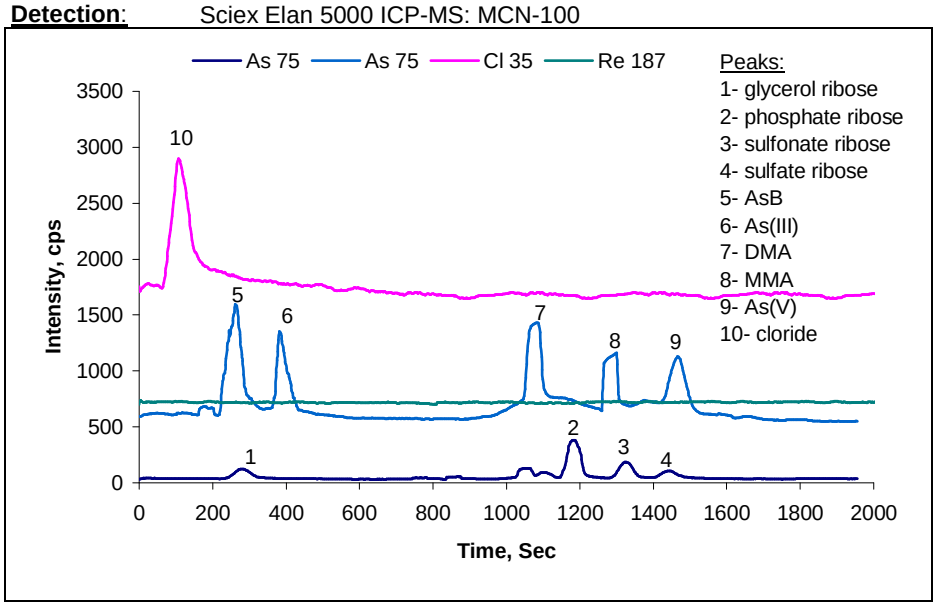


Figure 3-11 Determination of As species by separation on a Hamilton PRP-X100 capillary column with ammonium nitrate gradient elution at pH 8.5 coupled on-line with ICP-MS using the Sciex Elan 5000.

Also chloride, which generally interferes in the determination of As by ICP-MS, can be separated from the As species by anion-exchange chromatography, although the cationic As compounds (AC and TMAO) cannot be separated from arsenobetaine with this method. As(III) can be separated from glycerol-ribose, however, glycerol-ribose is co-eluted to some extent with AsB. The peak shape for As(V), As(III) and some arsenoribosides is asymmetrical and the peak widths are broader than in the chromatograms obtained with cHPLC which are found in the literature [9, 10, 25, 155, 156]. This could be due to the low ionic strength of the 0.2 *mM* ammonium nitrate buffers used. Another reason may be larger dead volume of the hardware of the Sciex Elan 5000 ICP-MS injector liner.

There are many factors that play a role in the peak broadening in the liquid chromatography, especially in the case of separation of polyvalent ionic As species with a complicated on-line coupled system. The peak broadening and the deterioration of the peak shapes may be related to the non uniform size of the stationary phase particles to the eddy-diffusion, to the flow distribution and to the poor mass transfer between the mobile and the stationary phases. A further reason, may be the poor packing of the cHPLC column, however, the column efficiency test did not support this (see Table 3-7). A good column should comply with the rule that "*the packing should be composed of particles with as narrow a size distribution as possible"* [139]. This practically means that the ratio between the diameters of the largest and the smallest particle should not exceed 2. The particle diameter

distribution of the Hamilton PRP-X100 resin was also determined by scanning electron microscopy (SEM). As it can be seen from the results in Figure 4-1 in the experimental part, there are particles with diameter as small as 1.8 µm, whereas the largest have 11.6 µm diameters. Accordingly for a Hamilton PRP-X100 a particle diameter size ratio of 6.4 is obtained which means that the column packing significantly contributed to the broadening of the peaks for the As species.

3.2.4.2 As speciation with the aid of a SAX Hamilton PRP-X100 cHPLC column and an Agilent 7500s ICP-MS

Further experiments were performed with another ICP-MS, namely the Agilent 7500s. Here the SAX polymeric Hamilton PRP-X100 capillary column was coupled on-line to the MCN in the Shield Torch Agilent 7500s ICP-MS. The separation of the As species was carried out at the identical mobile phase and column conditions as previously used with the Sciex Elan 5000 instrument.

In Figure 3-12 the chromatogram for nine inorganic and organic As compounds is shown. In this case, the arsenosugars are well separated from the other arsenicals, but the identification of the peaks for As(III) and glycerol-ribose remained difficult. AC and TMAO were not retained under the conditions selected and co-eluted with AsB. AsB produced a sharper peak on the PSBVD polymeric material at weakly basic conditions as compared to the peak obtained on the latex polymer resin in acidic conditions (see Figure 3-12). This is not the case with the peaks for As(V), sulfate-ribose and particularly for DMA, which are broad and asymmetrical at front.

The peak for chloride is found at the beginning of the chromatogram and within the dead volume of the column as chloride is not retained. Accordingly, interfering cluster ion peaks at *m/z* 75 and 77 did not occur. A 20% increase of the signal for Cl isotope was found in the middle of the chromatogram while the signals for Ge and As remained constant. This indicates a possible contamination of the eluent of the second mobile phase. This is suggested by the similarity in the gradient slope. The element Ge was, added to the mobile phase as an internal standard and its signal was monitored at *m/z* 72 together with the signals for the Cl and As isotopes. Throughout the entire chromatographic run its signal remained constant suggesting stable plasma conditions throughout the gradient elution.

It must be noted that this procedure for As speciation should be used with caution, as the presence of critical substances as resulting in peaks like 2 and 3 (see Figure 3-12) can occur in applications to real biological samples, where many As species can be present. For this and several other reasons, complementary methods must be applied to enable a successful final identification of the As compounds. Additional information could be provided by cation-exchange chromatography such as in the case of the cationic arsenocholine, the zwitterionic arsenobetaine and positive ionisable organoarsenic compounds. In order to

unambiguously determine these compounds other detection methods, such as different types of MS should be made use of enabling molecule specific detection.

Chromatographic conditions:

Column:	Hamilton PRP-X100, 250 x 0.5 mm, 10 µm
Mobile phase:	A- 1 *mM* Ammonium nitrate, pH 8.5 + 10 µg/L Ge
	B- 40 *mM* Ammonium nitrate, pH 8.5 + 10 µg/L Ge
Flow rate:	12 µL/min
Injection:	0.05 µL
Sample:	100 µg/L As species standard solution and 50 µg/L arsenosugars
Interface:	Modified MCN-100 and small volume spray chamber
Detection:	Agilent 7500s ICP-MS: *m/z* 35, 72 and 75

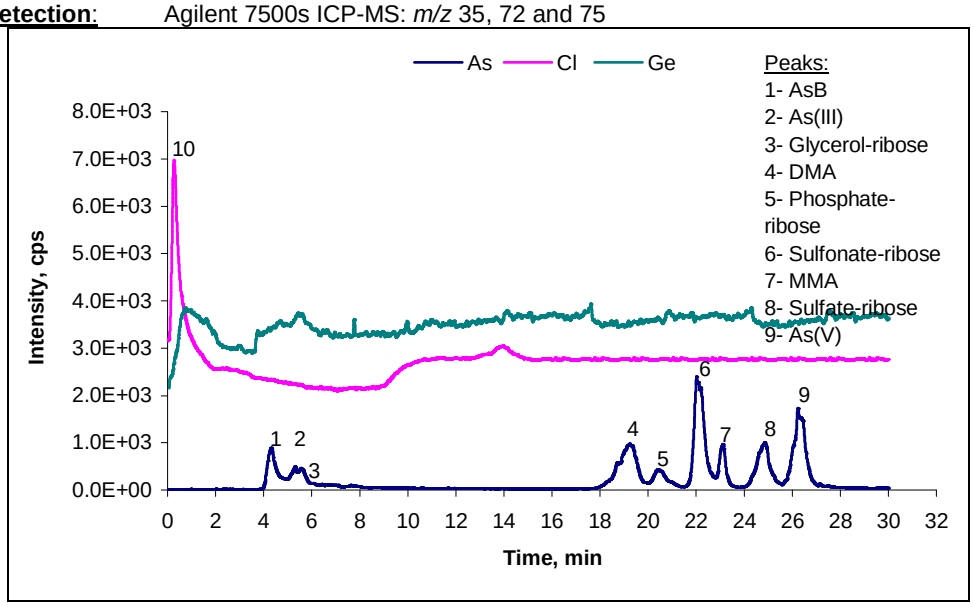


Figure 3-12 Determination of As species by separation on a Hamilton PRP-X100 cHPLC column coupled on-line to ICP-MS using the Agilent 7500s.

3.2.4.3 Anion-exchange cHPLC column with a latex polymeric packing

According to the specifications of Dionex Corp., the IonPac AS7 material is a hydrophobic SAX polymer resin with a chemistry that totally differs from the previously used materials for separation of As species. The IonPac AS7 material enables a separation of ionic and polar compounds [161]. In Figure 3-13 a picture of the IonPac AS7 packing particles is shown. There are three different regions in the IonPac ion-exchange packing, namely:

- inert, non-porous, chemically and mechanically stable core,
- a sulfonated surface region completely covering the core surface,
- an outer layer of attached submicron anion-exchange Micro Beads™.

The latex anion-exchange layer has high loading capacity with very short diffusion paths. These features enable both high efficiency and minimal matrix effects.

Several applications such as polyphosphates and As compounds require both the anion and the cation retention of the IonPac AS7 column.

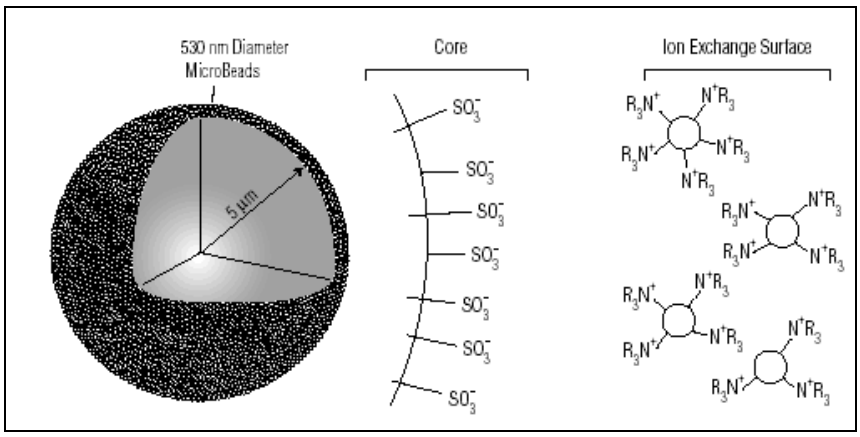


Figure 3-13 Structure of IonPac AS7 packing from [162].

3.2.4.3.1 cHPLC with the latex polymer SAX IonPac AS7 column coupled on-line to ICP-MS with the Agilent 7500s

Gradient elution of the As species with nitric acid was applied. The chromatograms obtained are presented in Figure 3-14. By using this system for As speciation, sharper and more intensive peaks for the analytes were obtained. Interferences of the Cl at the beginning and at the end of the gradient elution do not disturb the retention of the As compounds. The zwitterionic arsenobetaine, the cationic arsenocholine and the ionisable TMAO gave broad peaks which indicate a complex retention mechanism comprising anionic, cationic and molecule adsorption-desorption behaviour in parallel. Sulfonate- and sulfate-ribosides are co-eluted with MMA, while phosphate-ribose is first eluted from the column together with As(III). All anionic, cationic and polar As species are eluted within 18 min. The multi-mode chemistry of the IonPac AS7 cHPLC column did not provide sufficient efficiency for enabling a determination of the As compounds with ICP-MS detection, where the identification of the species is based on matching of the retention times.

Chromatographic conditions:

Column:	**IonPac AS7, 250 x 0.5 mm, 10 µm**
Mobile phase:	A- 0.5 *mM* Nitric acid, 0.05 *mM* BDSA, pH 3.6
	B- 20 *mM* Nitric acid, 0.05 *mM* BDSA, pH 1.8
Flow rate:	12 µL/min
Injection:	0.05 µL
Sample:	100 µg/L As species standard solution and 50 µg/L arsenosugars
Interface:	Modified MCN-100 and small volume spray chamber
Detection:	**Agilent 7500s ICP-MS: *m/z* 35, 89 and 75**

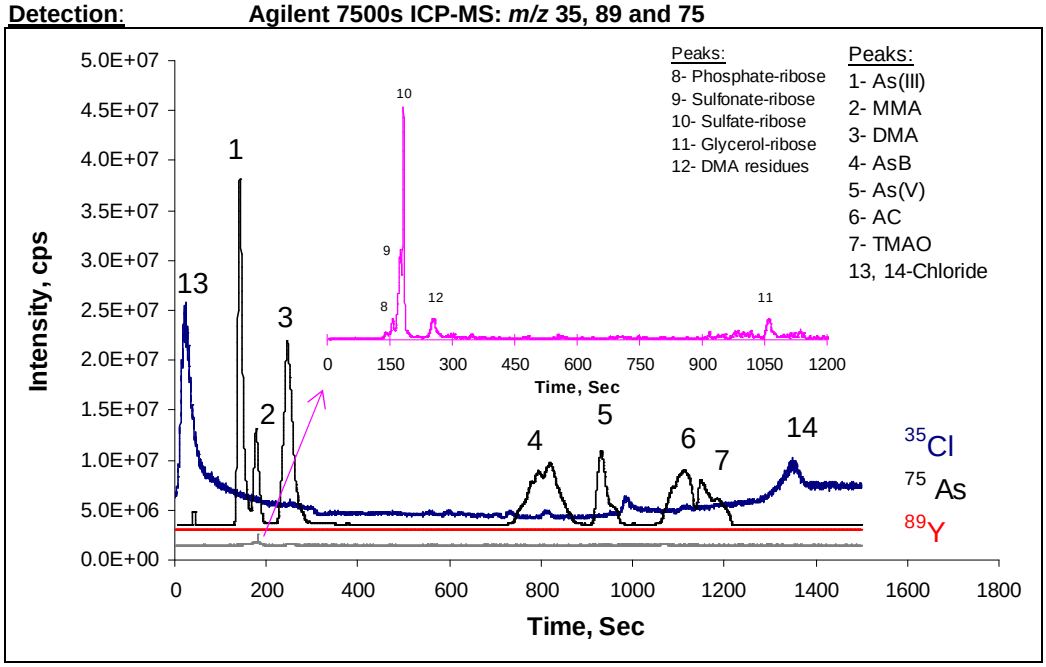


Figure 3-14 Determination of As species by separation on an IonPac AS7 column coupled on-line to ICP-MS the Agilent 7500s with a micro nebulizer.

3.2.4.4 As speciation by cation-exchange capillary chromatography coupled on-line to ICP-MS using the Agilent 7500s

Many investigations have shown that the major As species in biological tissues have a tetraalkylarsonium structure (R_4As^+) or a trialkylarsine oxide structure (R_3AsO) [12, 44, 51, 59]. Therefore, the application of cation-exchange chromatography can provide additional information to enable an identification of As species for which no resolved signals are obtained with anion-exchange liquid chromatography.

The development of SCX cHPLC columns was motivated by the desire to solve at least some of the problems associated with As speciation. A polymer resin was chosen because of the many advantages exhibited over the silica-based materials. Indeed, the PSDVB polymeric support is far more robust to extreme pHs than silica-based materials. This enables to perform chromatography under a more diverse variety of conditions. The following points were regarded as essential.

The PSBVD support should:

- be highly inert to all reasonable eluents (0-100% aqueous or organic; pH 1 to 13),
- be manufactured as spherical particles with a narrow particle-size distribution,

- have a mean particle size in the range of 5 – 20 µm,
- enable to separate inorganic and organic mono and divalent cations,
- be available as a bulk material,
- be compatible with any existing HPLC hardware.

3.2.4.4.1 Structure of strong cation-exchange resin

All requirements stated above were fulfilled by the Hamilton PRP-X200 material. It is a SCX polymeric packing developed to easily separate inorganic and organic cations at concentrations from 20 ng/mL to 20 mg/mL. Its main specifications are listed in Table 4-10 (see experimental part). SEM pictures of this resin are given in Figure 3-15 and Figure 3-16. The material has a narrow particle size distribution in the range 7.1 to 12.4 µm and accordingly a particle diameter ratio of 1.7, which can be accepted as a very good. As compared to the commercially available PRP-X200 columns from Hamilton Corp. applied for the separation of monovalent and divalent cations in the case of conductivity or UV detection with different mobile phases, it can be considered as an appropriate stationary phase for As speciation.

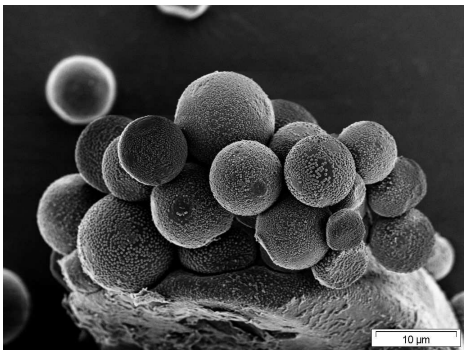


Figure 3-15 Size and form of the Hamilton PRP- X200 resin particles.

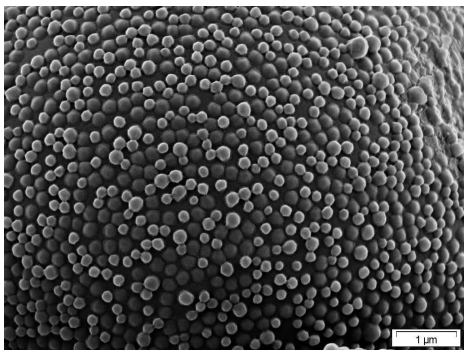


Figure 3-16 Porous structure of a single particle of a Hamilton PRP- X200 resin.

3.2.4.4.2 Retention time studies on the Hamilton PRP-X200 cHPLC column

The retention of closely related As compounds for different compositions and concentrations of the mobile phases was investigated. Small variations in the retention times were observed with different mobile phases, whereas the order of the elution remained the same. The best result was obtained with 0.5 mmol/L pyridine at pH 2.7 adjusted with acetic acid and a flow rate of 10 µL/min (see Figure 3-17).

Arsenite seems not to be retained on the column and elutes first. TMAO becomes protonated at low pH and is retained on the capillary cation-exchange column. AC is a cationic species and is also well retained on the column. The four arsenosugars are

effectively separated on the SCX cHPLC column. DMA appeared to co-elute to a certain degree with glycerol-ribose and AC at the working conditions used.

The separation on the SCX cHPLC column is a complementary method and can be used to confirm some of the results obtained with the SAX cHPLC column. Particularly the presence of the tetramethylarsonium ion, arsenocholine and some arsenosugars, can be shown up, although phosphate-ribose co-elutes to some extent with TMAO and As(V).

Chromatographic conditions:

Column:	Hamilton PRP-X200, 250 x 0.5 mm, 10 µm
Mobile phase:	0.5 *mM* Pyridine, pH 2.7 (with CH_3COOH) + 10 µg/L Ge
Flow rate:	10 µL/min
Injection:	0.5 µL
Sample:	Standard solution of 100 µg/L arsenicals and 50 µg/L arsenosugars
Interface:	Modified MCN-100 and small volume spray chamber
Detection:	Agilent 7500s ICP-MS: *m/z* 35, 72 and 75

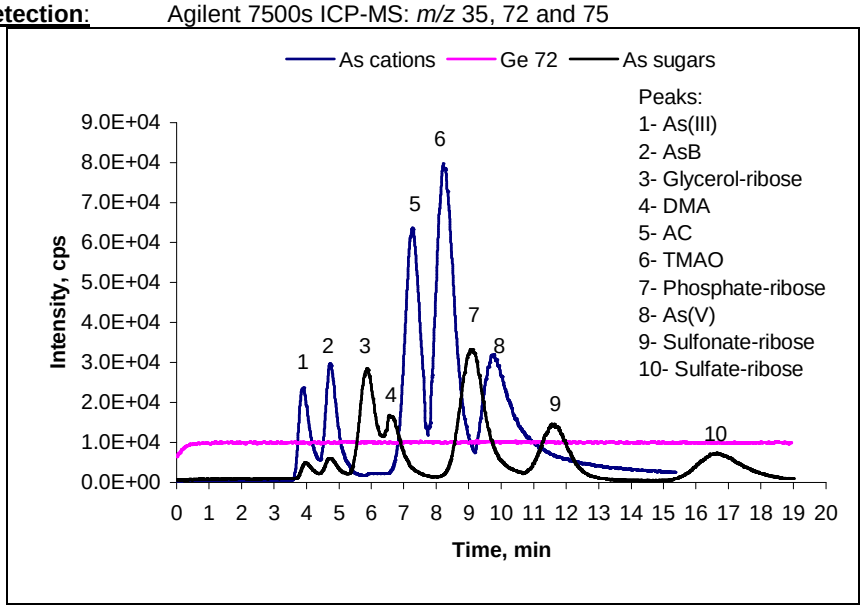


Figure 3-17 As species by separation on a cation-exchange cHPLC column and ICP-MS.

These data are in agreement with those of studies on the separation of arsenosugars, AsB, TMAO, AC, TMAs and DMA [31-33, 40, 115], as well as results on the separation of arsine sulphide arsenosugar [47], AsB and trimethylarsoniopropionate (TMAP) [39] or DMAE [41] by means of SCX chromatography.

It can be concluded that the separation of As species could better be performed with the in-house packed Hamilton PRP-X100 SAX cHPLC column at basic conditions than with the other RP and SAX cHPLC columns coupled on-line to ICP-MS. Accordingly, this method will be applied to the identification of As containing and/or As relating compounds in marine biological samples.

3.3 Characterisation and comparison of the chromatographic separations of As species and ICP-MS detection

Here the information obtained with the different tests of As compounds on similar cHPLC columns will be compared. The objective is to find out to what extent these tests provide similar or contradicting information. The study was performed on different HPLC phases and columns, namely strong anion-exchange and strong cation-exchange, silica-based and polymer-based stationary phases as well as commercial and in-house packed cHPLC columns. In most cases, the available information however was found to be not sufficient to objectively select the optimum column for a particular separation. Furthermore, since manufactures use different tests and evaluation parameters for their columns, their product claims are difficult to compare. Column classification and selection for a specific application such as arsenic speciation can better be done by comparing their physicochemical and chromatographic properties [158-160]. For these tests, a few As compounds were chosen to evaluate the differences or similarities of the chromatographic properties of the cHPLC columns with different stationary phases at almost identical working conditions.

3.3.1 Summary of column tests and their performance

The factors describing a separation are divided into chemical factors, such as selectivity (α) and capacity (*k'*) and physical factor, such as efficiency, or theoretical plate number (N). The data obtained for an optimum separation for As species are summarised below.

3.3.1.1 The chemical factors – selectivity (α) and capacity factor (*k'*)

The selectivity ($\alpha = k'_2/k'_1$) of a HPLC column is a function of the column packing and the elution conditions. The column packing and the elution conditions also affect the capacity factor ($k'=(t_R-t_0) / t_0$). The α and *k'* are the most important for obtainable resolution. A higher capacity factor results in a better resolution. This is at the expense of the analysis time. Typically, α and *k'* between 2 and 5 represent a good balance between analysis time and resolution.

In Table 3-6 the α and k' factors and the resolution for three different SAX cHPLC columns are given. This empirical evaluation shows that there are apparently differences in the chromatographic properties between the SAX-phases studied here. Zorbax SAX is a silica-based phase and gave higher values for the resolution and for *k' as* compared to the polymer-based SAX cHPLC columns. Hamilton PRP-X100 is a polymer-based material having PSDVB resin with trimethyl ammonium exchanger, whereas Dionex IonPac AS7 is latex cross-linking polymer with alkylated quaternary ammonium exchanger.

Table 3-6 Selectivity (α), capacity factor (*k'*) and resolution (Rs) for the tested capillary SAX columns

Capillary SAX column		As(III)	As(V)	MMA	DMA	TMAO	AsB	AC
Agilent Zorbax SAX	*k'*	2.6	2.6	2.7	1.4	2.7	2.66	2.67
	α	1	7.8	1.0	0.1	1	1	1
	Rs	9.9	8.9	10.2	0	7.7	10.1	8.8
Dionex IonPac AS7	*k'*	0	3.4	0.7	1.3	7	4.8	-
	α	n.d.	5.1	0	1.9	1.5	1.4	-
	Rs	n.d.	4.3	2.3	1.9	4	8.9	-
Hamilton PRP-X100	*k'*	0.4	5.1	3.8	3.1	x	n.d.	-
	α	0	7.7	1.2	12.8	x	0	-
	Rs	1.6	7.3	4.2	10.6	x	2.1	-

In general, the all three columns, gave good selectivity and capacity factors for the inorganic and organic As compounds. The resolution is also excellent with values between 1.6 (99.9% separation) and 10.6. The best resolution for these As species was achieved on Zorbax SAX column, however, the selectivity for all As species (including the arsenosugars) suffered.

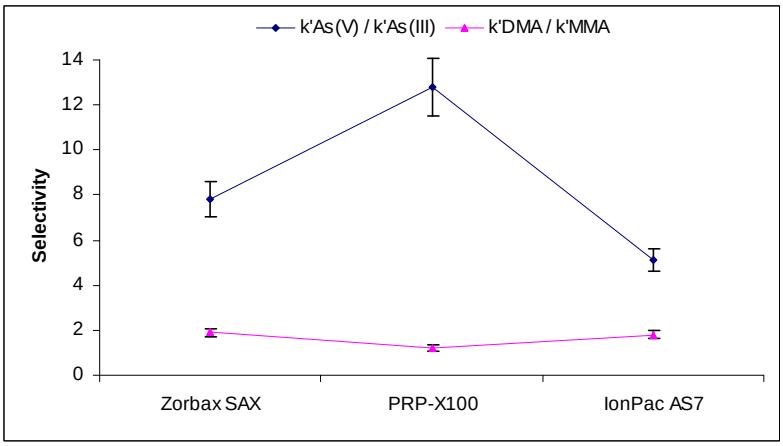


Figure 3-18 Selectivity for cHPLC SAX columns (calculated as $k'_{As(V)}$ / $k'_{As(III)}$).

In Figure 3-18 the selectivities for inorganic and organic compounds in the case of the three capillary columns are plotted. The methylated arsenicals resulted in relatively similar selectivities, suggesting a constant ionic behaviour. In the case of the inorganic As the curve obtained is much different. It could be that the ionic type interactions and the different type of stationary phase are responsible for the high value found for the Hamilton PRP-X100 column. In the silanol groups of Zorbax SAX column exists several types of interaction, of

which ion-ion interactions and hydrogen bonding are probably the most important in the SAX HPLC columns of As species.

Furthermore, the selectivities with similar exchanger ligands at similar conditions differ mostly with the stationary phase basic material, resulting in different penetration of the test substances in between the surface ligands. In addition, the phase ratio values are largely determined by the surface area of the base support material and the carbon content of the stationary phase. Dionex [161] claims that the IonPac AS7 latex anion-exchange layer has a high loading capacity with very short diffusion paths. The Hamilton PRP-X100 PSDVB material has a low capacity with some longer diffusion paths. Both features result in high efficiency and minimal matrix effects.

3.3.1.2 The physical factor efficiency on the number of theoretical plates (N)

To obtain more insight into the (dis)similarities of the different RP- and SAX-cHPLC columns for As species, they were subjected to column efficiency tests using the first eluting As compound. The results of all column efficiency calculations are given in Table 3-7.

Table 3-7 Capillary column efficiency test

Capillary column	Column dimensions	Test Peak	N / column	N /meter
XTERRA	150 x 0.32 mm, 3.5 µm	MMA	13977	55908
Zorbax SAX	250 x 0.5 mm, 5 µm	AC	11620	46480
Hamilton PRP-X100	250 x 0.5 mm, ~ 10 µm	As(III)	14534	58136
Dionex IonPac AS7	250 x 0.5 mm, 10 µm	As(III)	7270	29080

From Table 3-7 it can be concluded that the efficiency rankings of the different columns according to the various tests are in good agreement within certain bandwidths. To facilitate the comparison all values were normalised to the absolute column efficiency (N/meter). The results obtained on different silica-based and polymeric capillary columns from several manufactures and an in-house packed column show that all have a relatively similar number of plates for As compounds. By comparing the data from Table 3-6 and Table 3-7, it can also be seen that there is relationship between the chemical and physical factors of the column, as suggested earlier in this section.

The Dionex's IonPac AS7 cHPLC column has the lowest efficiency for As species. This could be due to the column hardware, particularly the PEEK capillary inner surface. The particle diameter of IonPac AS7 latex resin is 10 µm, which might be too large for obtaining a good packing into capillaries with 0.5 mm I.D. This may explain the poor separation and the bad peak shapes for As compounds observed with this phase. When comparing a XTerra cHPLC column, packed by the manufacturer and the in-house packed Hamilton PRP-X100 cHPLC column no significant difference in the column efficiency could be found. It is worth to

mention that with the polymeric material Hamilton PRP-X100 column the highest column efficiency for As compounds could be achieved. This column also had an excellent efficiency. According to this experience and the results from Table 3-6, Table 3-7 and Figure 3-18, it can be concluded that the Hamilton PRP-X100 column is the best cHPLC column for As speciation.

3.3.1.3 Limit of detection and linearity range for As species

In Table 3-8, the cHPLC columns are ordered depending on their method limit of detection for As compounds, obtained with different modes of capillary liquid chromatography coupled on-line to ICP-MS with the Sciex Elan 5000. The limit of detection according to IUPAC [138] the linearity for each cHPLC column were estimated from calibration curves obtained by analysing aqueous standard solutions with 10 As species at increasing concentrations including a blank of ultrapure water. Each calibration point (including the blank) was the average of three (3) replicative measurements, which were used to obtain the RSD% for a particular As compound.

Table 3-8 Limit of detection and linearity of the determination of As species by cHPLC coupled to ICP-MS

Column name	Injection volume	As species	Linearity range (nM)	Limit of detection Relative, C (mg/L)	Limit of detection Absolute, (pg of Element)	RSD, % n=10
XTerra® RP_8, Waters Corporation	0.03 µL	As(III)	20-800	0.7	21	4.3
		MMA	15-750	0.53	16	1.3
		DMA	10-500	0.35	10.5	5.8
		As(V)	20-800	0.6	18	5.0
Zorbax SAX, Agilent Technologies	0.01 µL	As(III)	5-1000	0.3	3	4.3
		As(V)	5-1000	0.5	5	5.0
		AsB	5-1000	0.5	5	6.7
		MMA	10-1000	0.8	8	8.3
		AC	5-1000	0.4	4	5.8
Dionex IonPac AS7, In-house packed	0.1µL	As(III)	5-1000	0.02	2	1.3
		MMA	5-1000	0.05	5	0.9
		DMA	5-1000	0.03	3	1.6
		AsB	20-800	0.15	15	3.2
		As(V)	10-800	0.10	10	4.6
		As sugars	20-800	0.15	15	3.9
Hamilton PRP-X100, In-house packed	0.05 µL	AsB	5-1000	0.05	2.5	2.4
		As(III)	1-1000	0.032	1.6	1.6
		DMA	10-900	0.12	6	2.0
		MMA	5-800	0.06	3	1.8
		As(V)	5-1000	0.10	5	2.5
		As sugars	10-800	0.10	5	3.4

Obviously, with the SAX capillary columns slightly lower limits of detection and larger linear concentration ranges were obtained for As compounds than with the RP cHPLC

column. The reproducibility for both types of cHPLC columns ranged from 0.9% to 8.3%. The limits of detection with the silica-based cHPLC column coupled to ICP-MS for the inorganic and the organic As derivatives were comparable to those obtained with the polymer-based cHPLC columns coupled to ICP-MS.

Because of the extremely high power of detection of the micro-scale separations coupled to ICP-MS, the chromatographic methods described are especially suitable for the determination of As species in marine biota samples, particularly for analysis of limited sample volumes.

Although the attractive features of the cHPLC coupled on-line to ICP-MS included high power of detection and minimal sample pre-treatment (for example sample extraction, clean-up or dilution), some restrictions were also present. Limitations such as the possible co-elution of As compounds, which may lead to misidentifications, their retention on the column, probable As interferences in the case of real marine samples, inability to identify compounds for which reference compounds are not available, etc., can be avoided by using in addition molecular mass spectrometry, such as tandem mass spectrometry. This especially applies for metabolic, intermediate and transformation studies, in which species identification and structures need to be characterised. Since the best procedure in this study for As speciation is the one with the Hamilton PRP-X100 cHPLC column using ammonium nitrate buffer mobile phases, it was also coupled to molecular mass spectrometry using a triple quadrupole ESI-MS for molecular identification.

3.4 As speciation by cHPLC coupled with electrospray tandem MS

MS experiments can be well used to characterise the structure of a variety of molecules. However, state of the art mass spectrometers also increasingly offer high sensitivity, specificity and reliability for trace analysis, together with high resolution in mass/charge. Therefore, molecular mass spectrometry is a distinct detection method in the modern speciation analysis. This section focuses on the combination of cHPLC with the best separation method developed with triple quadrupole mass spectrometry for the identification and determination of As compounds in marine biota, and especially in algae.

The performance of ESI depends on the following factors [162]:

- sprayer position,
- nebulizer tip position in relation to the nebulizer jet,
- nebulizer gas,
- solvent composition.

All these parameters were experimentally optimised for flow injection analysis (FIA) with AsB standard solution before any other experiments. The optimal conditions were then used for ESI-MS and capillary HPLC-ESI-MS/MS analyses of all organoarsenic compounds.

3.4.1 Characterisation of organoarsenic standard compounds using ESI-MS/MS

Two modes of detection (fully intact molecular and MS/MS) were performed when introducing each standard solution containing MMA, DMA, AsB, AC, TMAO and four dimethylated arsinoylriboside derivatives. In Figure 3-19, Figure 3-20, Figure 3-21 and Figure 3-22, the scans in the positive-ion mode show protonated AsB and AC molecules at *m/z* 179 and 165, respectively. At a declustering potential (DP) of 10 and 80 V sufficiently energetic collisions in the differentially pumped region occurred to eliminate clusters with alkyl As molecule, and resulted in almost no fragmentation of the target analytes (see Figure 3-20 and Figure 3-21).

3.4.2 ESI-MS of alkyl-As derivatives

For most organoarsenic species observation of the simple, yet non fragmented molecules occurred only at a very narrow range of orifice potentials, as it can be seen from Figure 3-19, Figure 3-20 and Figure 3-21. Optimisation of the molecular ion signal includes adjustment of the declustering potential (DP), the curtain gas flow, the ESI potential, the nebulizer gas flow, and ion optics up to Q1**.** The background intensity across the full scan mass range is considerable because of the soft ionisation and detection used.

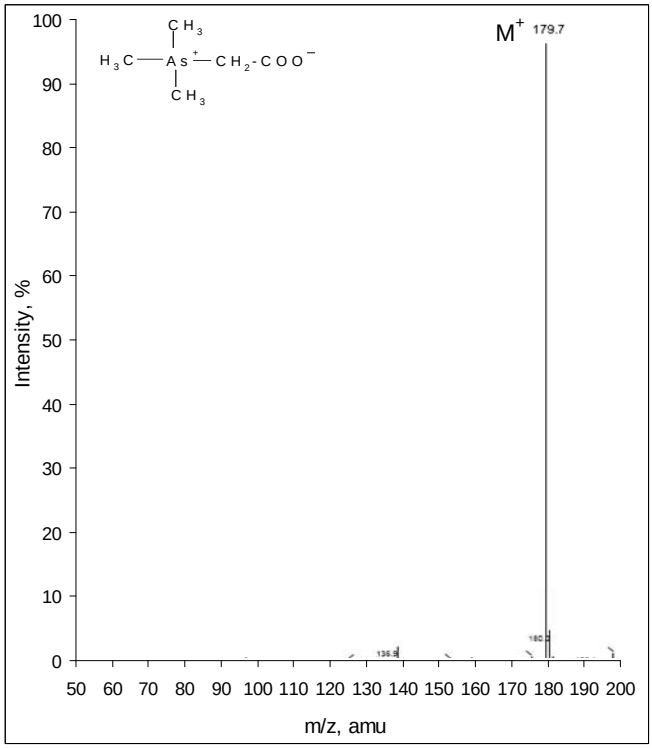


Figure 3-19 ESI-MS spectra of arsenobetaine (1 mg/L), at DP 10 V.

While it was possible to optimise the system for maximum signal of $[AsB-H]^+$ for instance in Figure 3-19, it was not possible to obtain at the same time a selective

fragmentation of other molecules, which then contribute to the background mass spectrum (see Figure 3-20).

3.4.2.1 Positive-ion ESI tandem MS analysis of alkyl-As compounds

In Figure 3-20 the dependency of the mass spectra of arsenobetaine on the DP and the formation of the protonated dimmer at elevated DP is shown. However, the lower DP is efficient enough to provide fully intact pseudomolecular ions ($[AsB+H]^+$ and $[AsB\text{-}AsB+H]^+$) without any further fragmentation of the arsenobetaine molecule. The spectra obtained strongly resemble the ESI-MS spectra reported in the literature [163, 164].

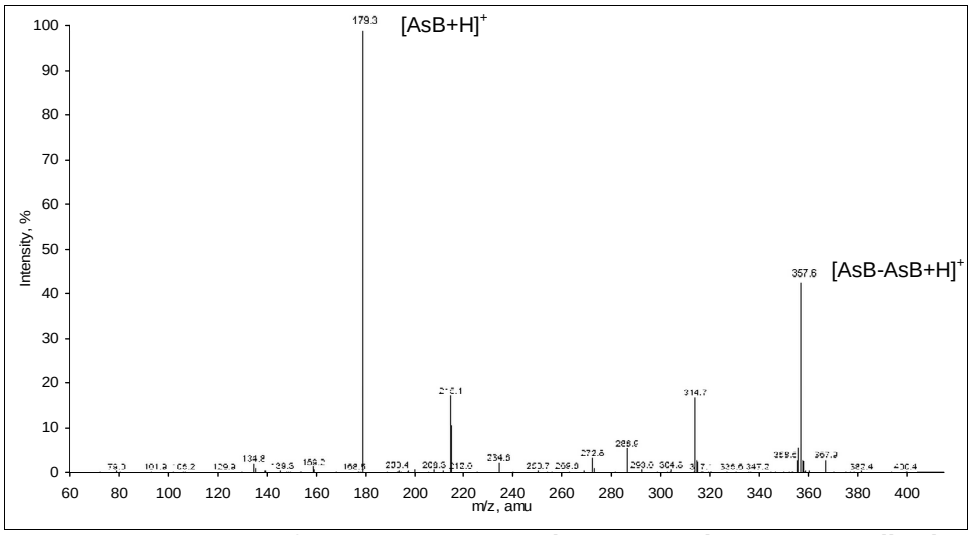


Figure 3-20 ESI mass spectra of AsB, mass range: (50-500 amu), DP: 50 V. All other working parameters were kept constant.

The molecular ion of AC and several fragment ions appeared in the ESI-MS spectrum at a higher DP compared to that used for AsB (see Figure 3-19 and Figure 3-20). Therefore, the DP was kept as low as possible in order to obtain a maximum intensity of the signals for pseudomolecular ions of the As species determined.

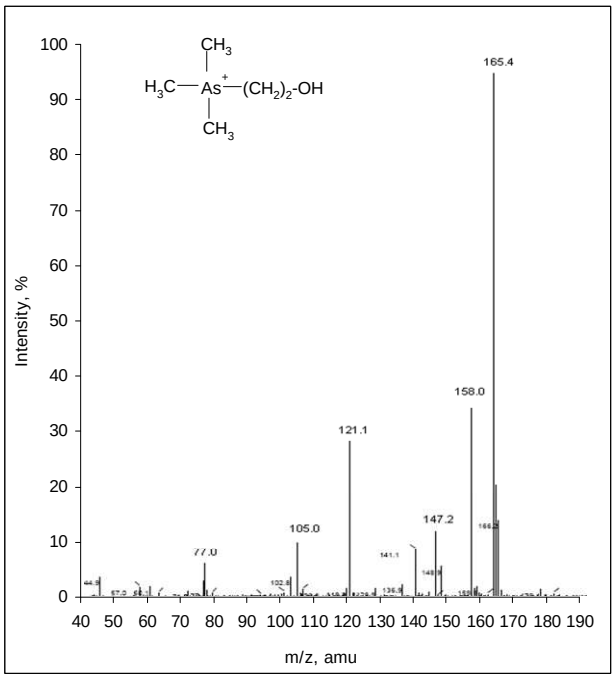


Figure 3-21 Spectra obtained in positive-ion mode ESI-MS for arsenocholine introduced at 10 µL/min sample flow, ESI: 3.5 kV and DP: 80 V.

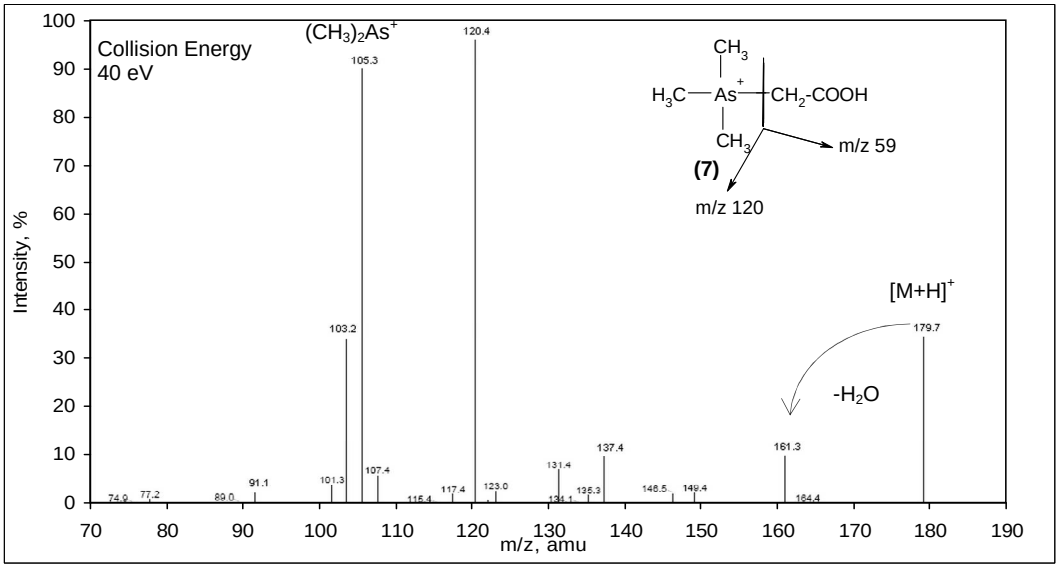


Figure 3-22 Positive-ion mode ESI-MS/MS spectrum of arsenobetaine, acquired at an ESI voltage: 3.0 kV, DP: 10 V, and collision activated dissociation (CAD) at 6 psig with 40 eV collision energy, sample introduction of 1 mg/L arsenobetaine solution at a 5 µL/min flow rate.

In Figure 3-22, Figure 3-23 and Figure 3-24 the ESI-MS/MS spectra are shown and molecular structure information can be obtained. In this case, the Q1 was not scanning, but was operated to allow a selective transmission of the pseudomolecular ions $[M+H]^+$ for each As species. The direct-current (DC) quadrupole offset potential difference between Q1 and Q2 was at least 35 V, providing a collision energy of 35 eV for the singly charged molecules $[M+H]^+$ with the nitrogen gas in Q2 (see scheme of triple quadrupole ESI-MS in

Figure 7-5 in appendix). An important difference between molecular and tandem MS modes of detection is the extremely low background obtained with the ESI-MS/MS method. The selectivity of the MS/MS leads to low background levels, particularly for multiple reaction monitoring (MRM) experiments where the precursor and product ion masses can be preselected. This is one of the most often modes used in the organic mass spectrometry. Small increments in collision energy permit it to get access to further dissociation reactions, spreading the total ion signal over an extended range of fragmentation products. The DP and collision energy can be carefully selected to optimise the production of particular fragment ions, for which MS/MS may then be applied. In such a mode of operation supplementary information on the molecular structure is provided in addition to the higher sensitivity of the MS analysis.

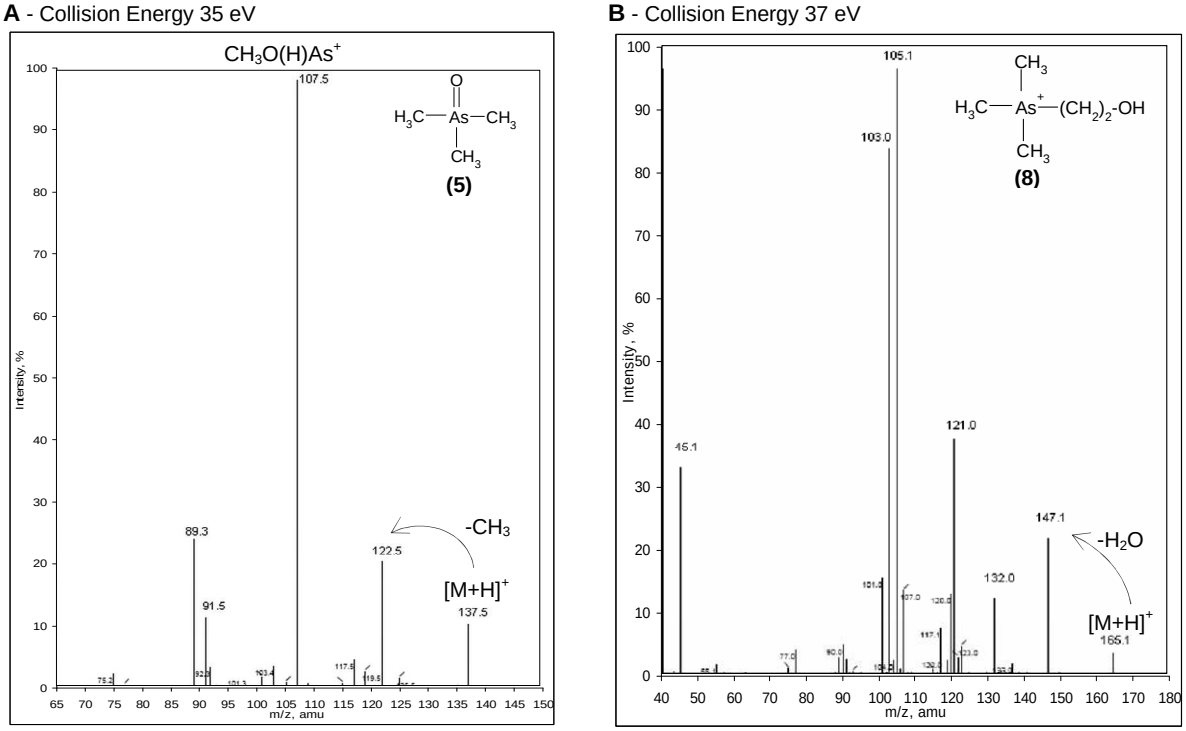


Figure 3-23 Positive-ion ESI-MS/MS spectra of A- TMAO *m/z* 137 and B- AC *m/z* 165, acquired at ESI 3.5 kV, DP 70 V and CAD 6 psig. Sample introduction of TMAO and AC was by infusion at 5 µL/min, respectively.

Interpretation of the negative and positive-ion tandem mass spectra after low-energy CID of alkylarsenicals and arsenosugars was done by considering the mass difference to the precursor or complementary fragments. The spectra have been also examined for other complementary fragments and low mass ions to confirm the molecular sequence.

In Figure 3-22 and Figure 3-23 the positive-ion tandem mass spectrum of AsB, AC and TMAO obtained at 40, 35 and 37 eV collision energies respectively, and the structure of their main daughter ions are shown. The ion signal corresponding to a loss of H_2O molecule, was monitored in all tandem mass spectra obtained. In the spectrum of $[DMA+H]^+$ this is the most

intensive fragment $(CH_3)_2AsO^+$ at *m/z* 121. Although the maximum sensitivity for the main product ions were achieved at a collision energy of 40 eV, a total dissociation of the precursor ion $[M+H]^+$ did not occur. This is a common phenomenon in ESI-MS/MS.

The structure of the $[DMA-H_2O]^+$ fragmentation ions with positive-ion collision induced dissociation (CID) is presented in Figure 3-24 together with the ESI-MS/MS spectrum for MMA. In Scheme 3-3 the fragmentation pathway of DMA dimethylarsinic acid as proposed by Siu *et. al.* [164] is shown. The fragmentation pattern is relatively simple and suggested by the presence of the arsenic-containing ions at *m/z* 91, 105, 107, 109, 120 and 121.

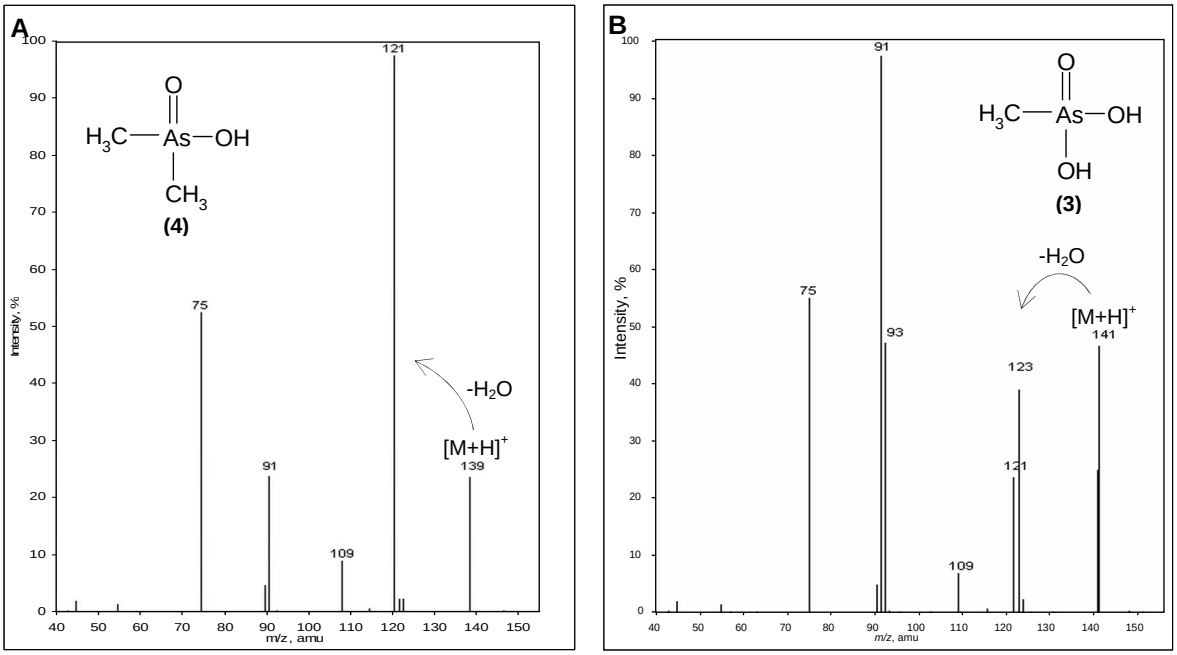
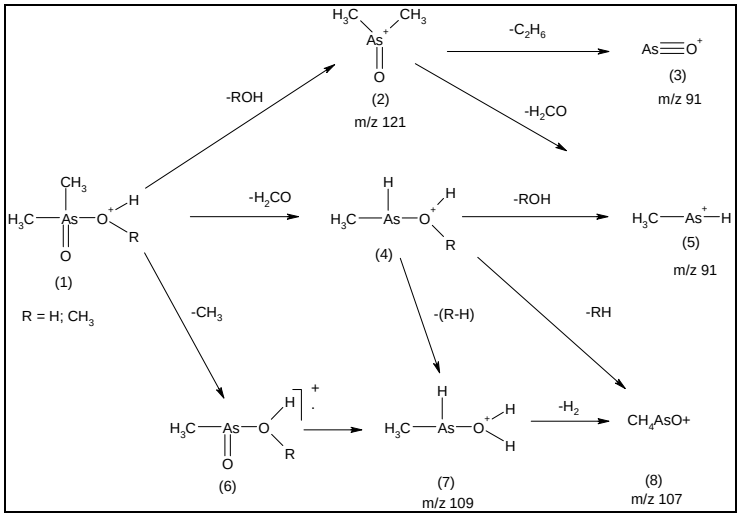


Figure 3-24 Positive-ion mode ESI-MS/MS spectra of A- DMA *m/z* 139 and B- MMA *m/z* 141, ESI voltage: 3.5 kV, DP: 70 V, CAD: 6 psig and collision energy: 35 eV. Sample introduction of MMA and DMA at a flow rate of 10 µL/min, respectively.

Scheme 3-3 MS/MS fragmentation pathway of DMA and its derivative (as proposed in Ref.[164])



3.4.3 Positive-ion ESI-MS and ESI-MS/MS of dimethylarsinoylribosides

Dimethylarsinoylribosides (arsenosugars **9-11** and **17**, see Figure 7-2 in appendix) occur as cations or anions in solution and thus can be detected either in the positive- or the negative-ion mode by ESI-MS and/or ESI-MS/MS. The positive-ion ESI mass spectra obtained from these compounds contain intensive signal for $[M+H]^+$ along with low intensity signals for $[M+2H]^+$ and $[M+Na]^+$ ions. The $[M+H]^+$ ions were selected as precursor ions and further examined under low-energy CID conditions. Tandem mass spectra of the $[M+H]^+$ ions, originating from arsenosugars **9-11** and **17** were obtained. Their examination showed that the product ions observed are likely to be formed as a result of charge induced fragmentation with a positive charge localised on the As atom. In fact, all product ions observed in these tandem mass spectra are thought to contain the As atom.

The tandem mass spectrum obtained from the molecular ion of glycerol-ribose is shown in Figure 3-25. Low intensity signals of the ions present at *m/z* 165 and *m/z* 195 suggest the occurrence of cross-ring cleavage. In general, the features of the low-energy CID mass spectrum of the $[M+H]^+$ ion originating from arsenosugar **10** are similar to those observed under other CID conditions [144].

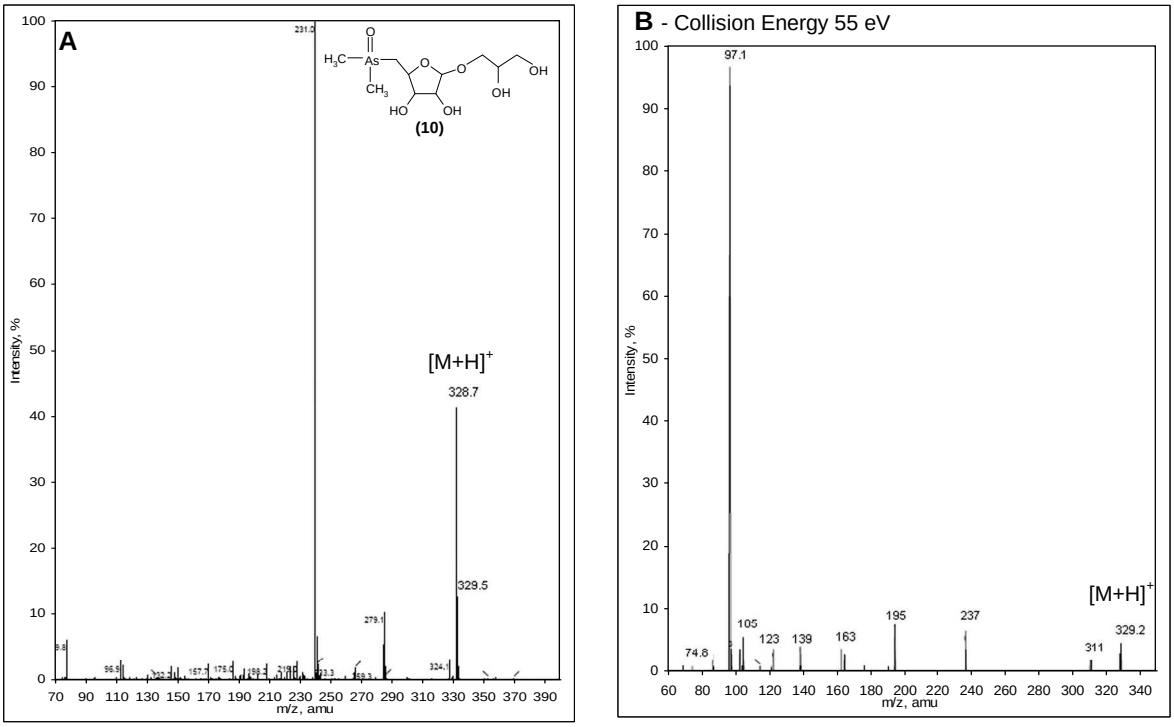


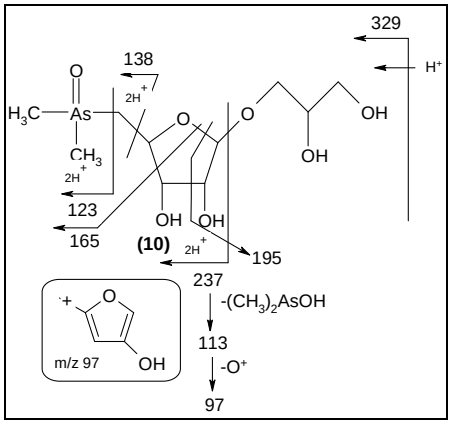
Figure 3-25 Positive-ion ESI mass spectra originating from glycerol-ribose (arsenosugar 10): (A) of the $[M+H]^+$ ion (*m/z* 328) of arsenosugar 10; (B) ESI-MS/MS spectra acquired with the same standard solution. ESI voltage: 3.5 kV, DP voltage: 30 V and CAD: 6 psig.

Of particular importance is the fragmentation ion signal occurring at *m/z* 237, which appeared in all MS spectra of arsenosugars. This product ion, which indicates the presence

of a dimethylarsenoriboside fragment, could be used to screen for the presence of arsenosugars.

In Scheme 3-4 an ion fragmentation pathway for dimethylarsenoglycerolriboside (glycerol-ribose) at positive-ion full scan ESI-MS/MS mode is proposed.

Scheme 3-4 Proposed ion fragmentation pathway for dimethylarsenoglycerolriboside in the case of positive-ion CID



The low-energy CID fragmentation of sulfonate-ribose results in a base peak at *m/z* 163 and another high intensive peak at *m/z* 193 (see Figure 3-26 and Scheme 3-5). Although those peaks have mainly been seen in the negative ion nano ESI-QTOF-MS/MS [147] or in negative-ion mode FAB-MS/MS [146] spectra of the same phosphate-ribose, they gave in the present study of low-energy ESI-MS/MS the most intensive product peaks in the spectrum. Pergantis *et al*. [146, 147] suggests that the fragment ions at *m/z* 193 and *m/z* 163 are formed as a result of charge induced fragmentation.

Charge induced fragmentation is a fragmentation of an ion in which the cleaved bond is not nearby to the apparent charge site. Independent of the charge status, charge induced fragmentations are analogous to gas-phase thermolysis. Under collision activation and with a fixed charge, ions containing long chain or polyring structures undergo charge induced fragmentations, generating fragment ions that are structurally informative. Interpretation of the fragment ion spectra enables it to elucidate molecular structures. Although charge induced fragmentations have been successfully used in the structure elucidation of fatty acids, phospholipids, glycolipids, triglycerides, steroids, peptides, ceramides, and other systems, the energetics and mechanisms of these reactions are still debated because none of the existing mechanisms can explain all the experimental data [18].

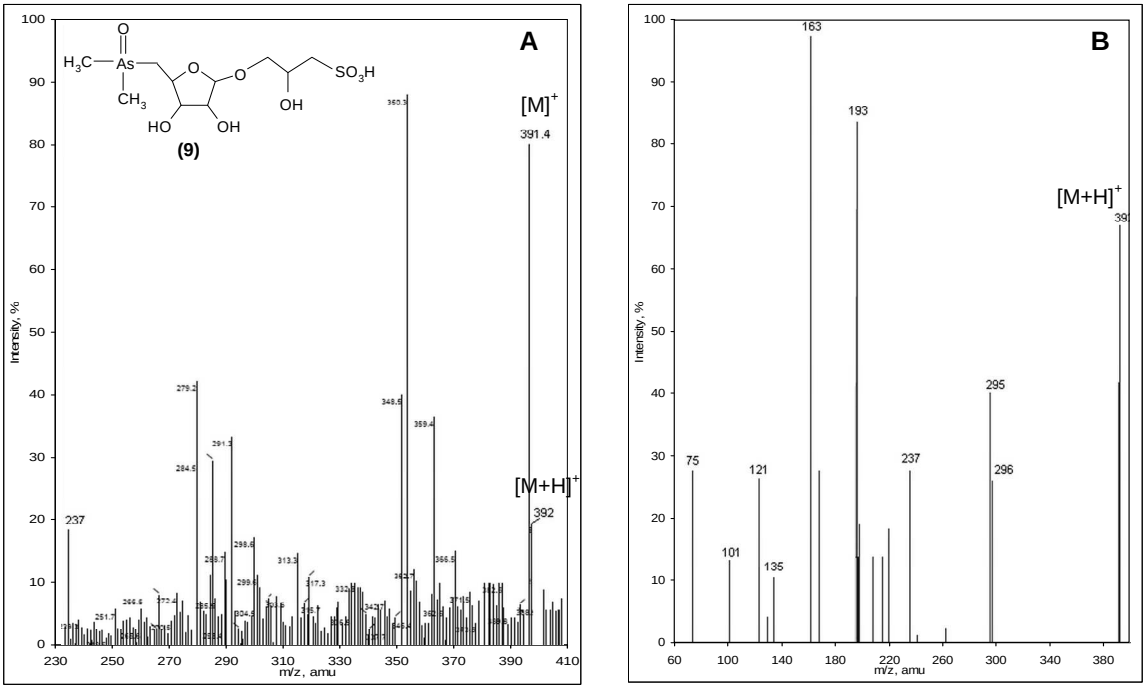
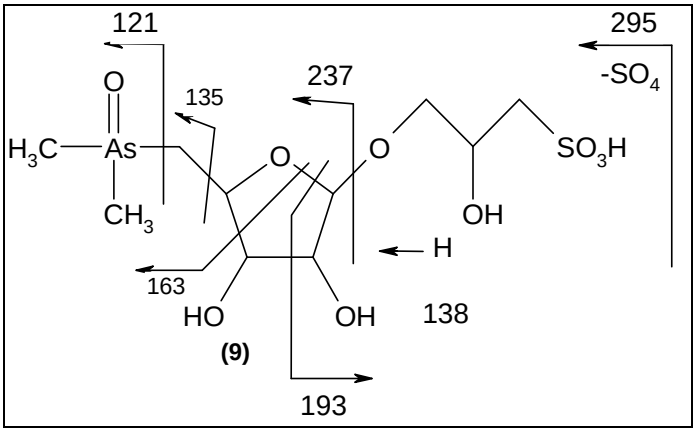


Figure 3-26 Positive-ion mode ESI-MS spectra of sulfonate-ribose. Introduction of approximately 0.1 mg/L of arsenosugar 9. $[M]^+$ (*m/z* 391) (A) and MS/MS spectrum of *m/z* 391 at collision energy 60 eV (B). ESI voltage and DP were set at 3.5 kV and 50 V, respectively.



Scheme 3-5 Possible ion fragmentation of sulfonate-ribose after positive CID

For the $[M+H]^+$ of phosphate-ribose, tandem mass spectra were recorded in the positive ion mode as shown in Figure 3-27. In the positive-ion mode spectrum after CID the most abundant ion is at *m/z* 329 and it is generated by the loss of the phosphate containing part of the molecule. The second intensive daughter ion is the common arsenosugars fragment at *m/z* 237. Other low intensive mass fragments in the tandem MS spectrum of phosphate-ribose are presented in the proposed fragmentation pathway (Scheme 3-5).

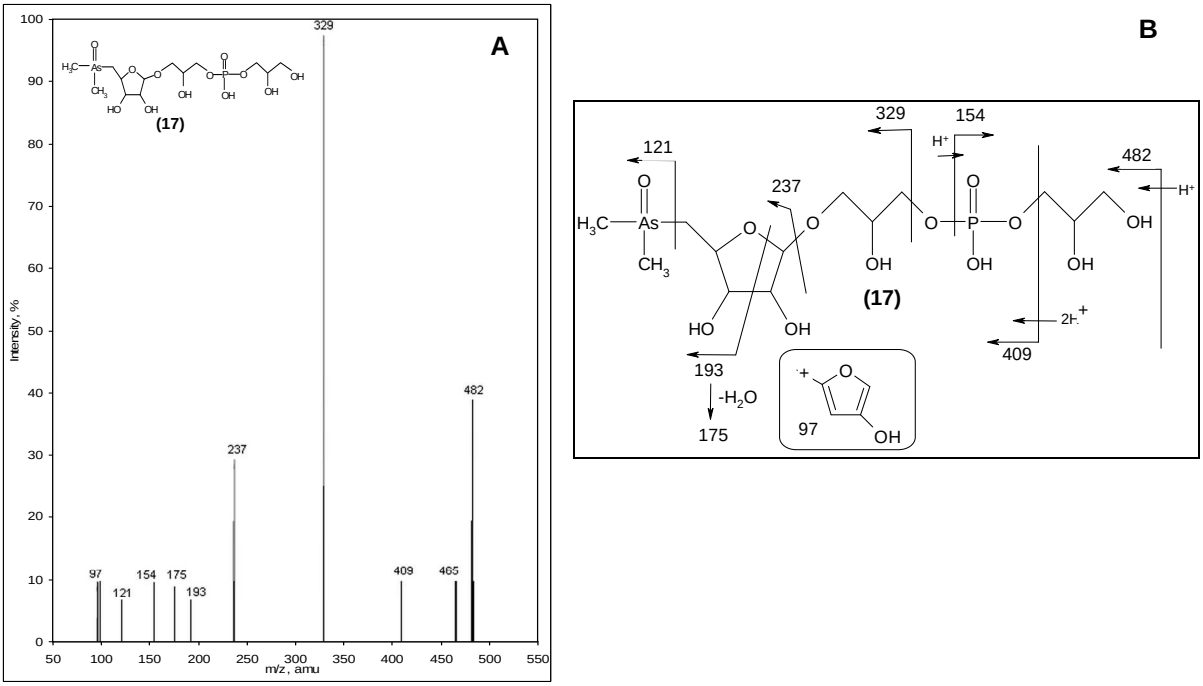


Figure 3-27 Positive-ion mode ESI-MS/MS spectrum of dimethylarsenophosphate-riboside obtained at an ESI voltage: 3.5 kV, DP: 50 V and CAD: 5 psig and a collision energy: 30 eV. Introduction of a solution containing arsenosugar 17 (A). Proposed fragmentation of the $[M+H]^+$ of phosphate-ribose at *m/z* 482 (B).

At a DP of 20 V some ions of sulfate-ribose adducts have been found in the positive-ion mode ESI-MS along with the pseudomolecular ion at *m/z* 409, but no fragmentation in the source seemed to occur (Figure 3-28 (A)). For the $[M+H]^+$ ion originating from arsenosugar **11**, tandem mass spectra were recorded at different collision energies. At a collision energy of 30 eV, the $[M+H]^+$ ions fragmented to a single base ion at *m/z* 330 $[M+H - SO_3]^+$. This is the main daughter ion in the ESI-MS/MS spectrum of sulfate-ribose. At higher collision energy of 55 eV, the *m/z* 330 ion had still relative intensity. However, further fragmentation occurred, resulting in other daughter ions such as the characteristic ones at *m/z* 231 and 97.

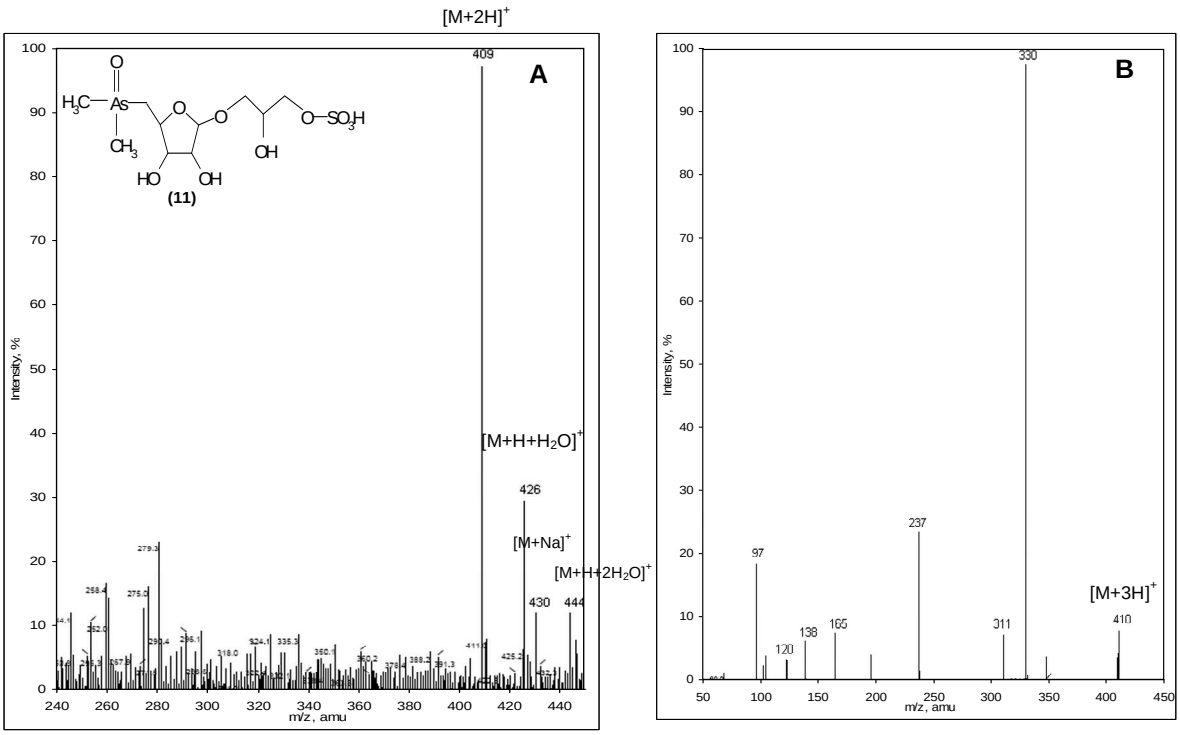
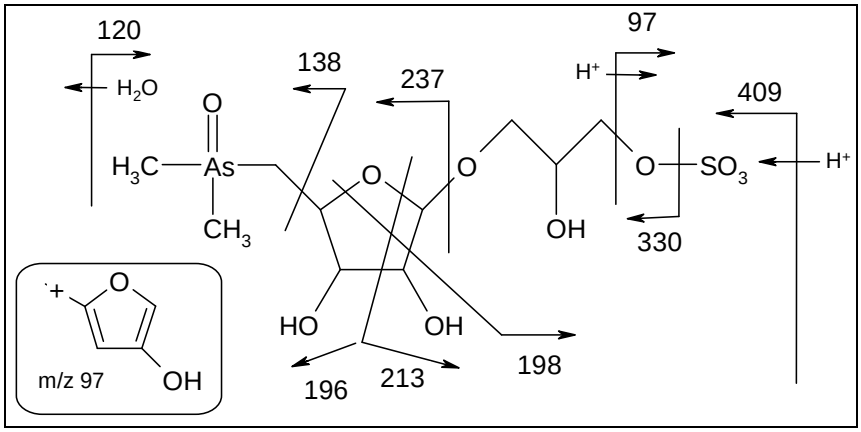


Figure 3-28 Positive-ion mode ESI-MS spectra of dimethylarsenosulfate-ribose (arsenosugar 11). Introduction of arsenosugar 11. $[M+H]^+$ (*m/z* 409) (A), ESI-MS/MS spectrum of *m/z* 409 with collision energy 55 eV (B). ESI and DP voltages were set at 3.5 kV and 20 V, respectively.

For the interpretation of the spectrum a charge induced fragmentation was taken into consideration. Although charge induced fragmentation is mostly observed for high energy CID and then more fragments are produced, the low energy CID in this study provides abundant structural information for the molecular characterisation of sulfate-ribose. Furthermore, the common fragment ions at *m/z* 237 and *m/z* 97 for arsenosugars have also been found in the positive-ion mode ESI tandem mass spectrum of sulfate-ribose. A possible fragmentation pathway for the daughter ions observed in the positive-ion mode ESI-MS/MS spectrum of sulfate-ribose is presented in Scheme 3-6 .



Scheme 3-6 Proposed product ion fragmentation of sulfate-ribose after positive-ion CID

The results presented here show that the main fragments in the positive-ion ESI-MS/MS spectra of phosphate- and sulfate-ribosides are the result of a cleavage of the ester bond. The spectra obtained under positive-ion ESI conditions for all arsenosugars were of good quality. In addition, the positive-ion mode CID spectra of the arsenosugars obtained in this study provide structural information which is to complementary to the information obtained from the negative-ion mode CID spectra.

3.4.4 Negative-ion mode ESI-MS of dimethylarsinoylribosides

Previous research on dimethylated arsenosugars by FAB-MS [146] and nano-ESI-TOF-MS [147] showed that the negative-ion mode is more sensitive than the positive-ion mode when the compounds are dissolved in a basic solution. This can be understood from the presence of acidic functional groups like sulfate, sulfonate and phosphate in the arsenosugar aglycones. Pergantis *et al*. [147] also reported that the negative-ion tandem mass spectra contain a larger number of informative ion signals than the subsequent positive-ion tandem mass spectra.

For standard solutions of the arsenosugars negative-ion mode ESI tandem mass spectrometry was performed under similar conditions as the positive-ion mode. The resulting low energy negative-ion mode CID mass spectra obtained for the respective phosphate-ribose, sulfonate-ribose and sulfate-ribose are presented in Figure 3-29 and Figure 3-30. In the negative mass spectral less structurally informative product ions were observed.

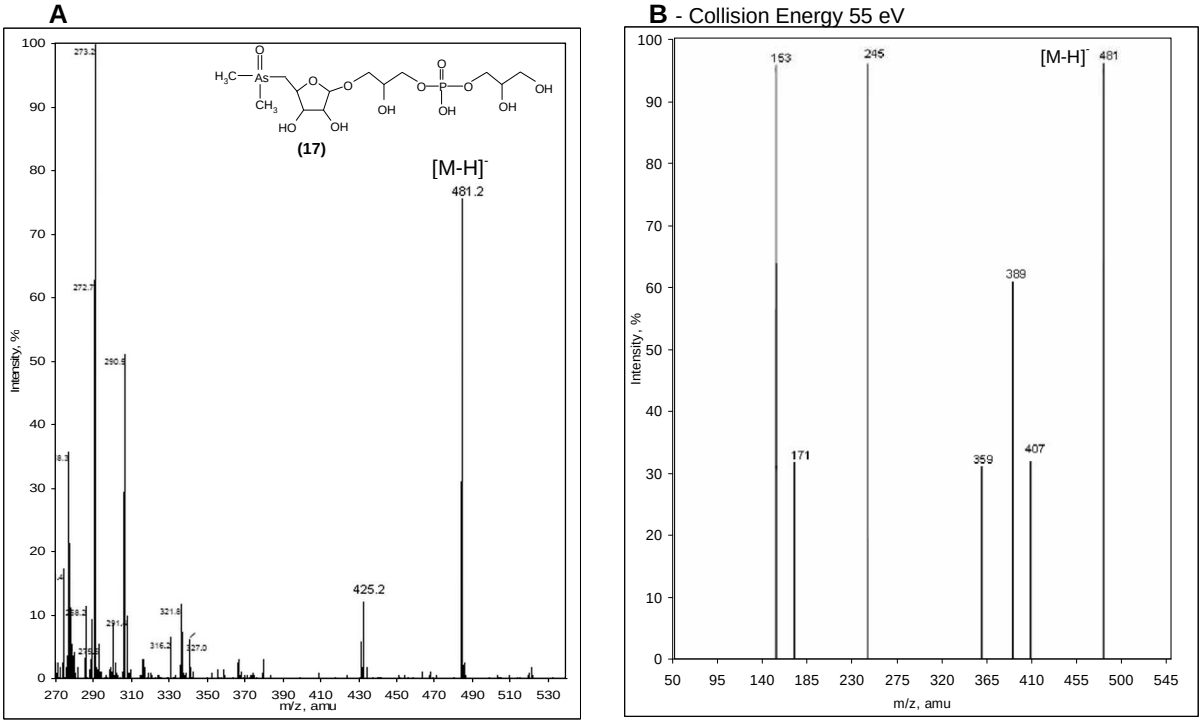
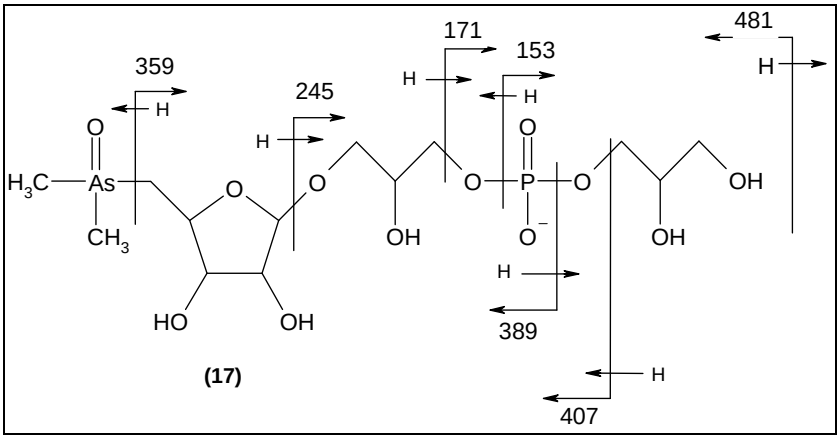


Figure 3-29 Negative-ion mode ESI mass spectra of phosphate-ribose acquired at ESI voltage: 3.5 kV, DP: 20 V and CAD: 6 psig. Introduction of a solution containing approximately 0.1 mg/L of arsenosugar 17, ESI-MS spectrum (A), ESI-MS/MS spectrum with CE: 55 eV (B).

The negative-ion mode tandem ESI mass spectra of phosphate ribose were of a low intensity compared to those obtained in the positive-ion ESI-MS, and accordingly lower number of stable fragment ions were obtained. Maximum signals for the fragment ions were obtained at collision energy of 55 eV. The negative-ion ESI-MS/MS spectra were of good quality and have high signal-to-noise ratios. The interpretation of the tandem mass spectra after CID in the negative-ion mode is shown in Scheme 3-7. The negative-ion mode ESI-MS/MS mass spectra of phosphate-ribose resulted in a nearly the same daughter ions. Most likely the fragment ions resulting from the pseudomolecular $[M-H]^-$ ions are generated by charge induced fragmentation rather then by ring opening fragmentation. The pattern of the negative-ion ESI-MS/MS spectra for arsenosugar **17** provides abundant structural information. Six major fragment ions, all of which can be assigned to structural features of phosphate-ribose, are observed.

Scheme 3-7 Fragmentation of dimethylarsenophosphate-ribose in negative-ion mode ESI-MS/MS



ESI tandem MS spectra of the $[M-H]^-$ ions originating from sulfate- and sulfonate-containing dimethylarsinoylribosides were also recorded as shown in Figure 3-30. Again, maximum ion signals and numbers of structurally informative fragment ions were observed at collision energy of 50 eV and 55 eV, respectively.

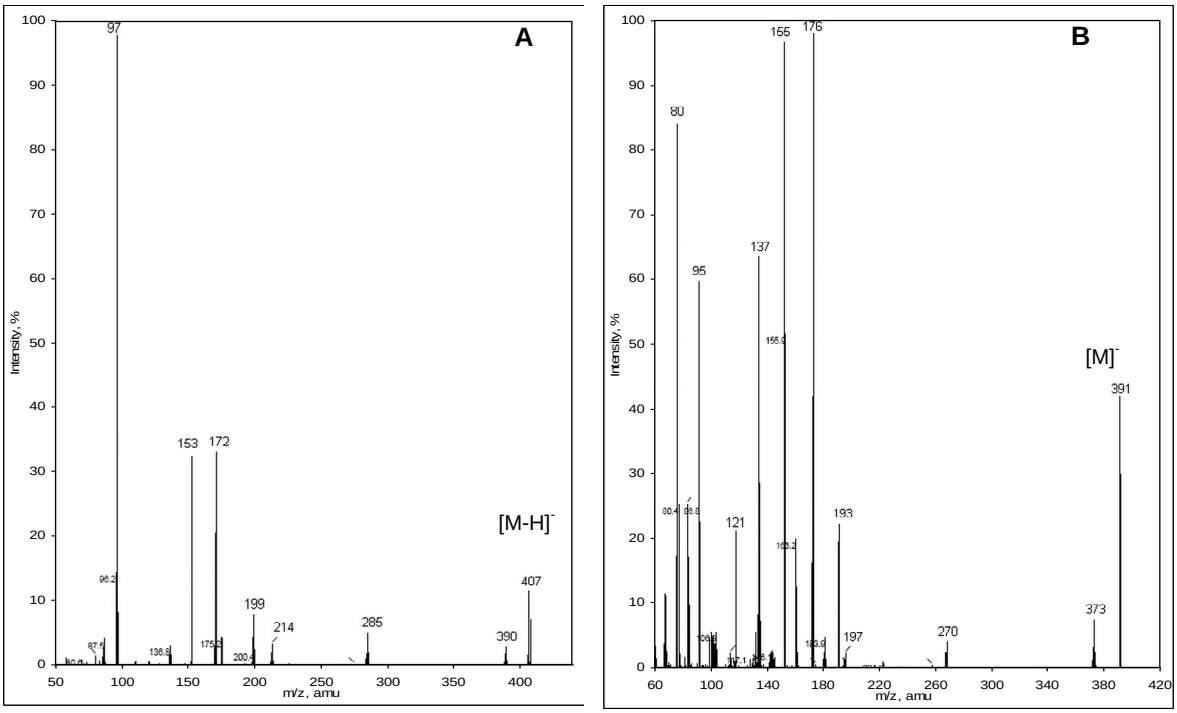
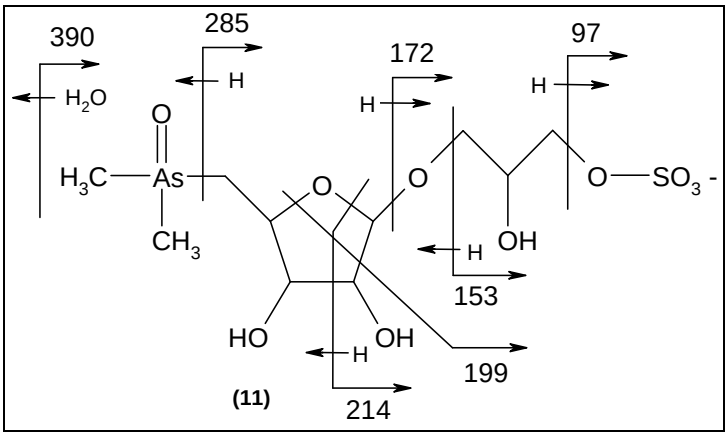


Figure 3-30 Negative-ion ESI mass spectra of sulfonate- and sulfate-ribosides obtained at ESI voltage: 3.5 kV, DP: 50 V and CAD: 6 psig. Introduction of arsenosugars 9 and 11, sulfate-ribose at collision energy 50 eV (A) and sulfonate-ribose with collision energy 55 eV (B).

Proposed fragmentation pathways for various specific and abundant fragment ions from arsenosugar **11** in the negative-ion ESI-MS using CID are presented in Scheme 3-8. In this case daughter ions are formed by charge induced fragmentation. At least eleven product ions, all of which can be traced back to sulfate-ribose, are observed under the conditions of low energy CID. This is a progress as compared to the nine structurally relevant fragment ions obtained from the same compound under high-energy CID FAB-MS by Pergantis *et al.* [146].

In the case of sulfate-ribose, ring opening cleavage of the ribose was observed comparing to the negative-ion ESI-MS/MS spectra of phosphate-ribose, scanned at the same conditions. In fact, two fragment ions resulted from cross-ring cleavage, namely *m/z* 214 and 199. These daughter ions have been also observed in high energy CID FAB-MS, except that they occurred at significantly lower relative intensities. In negative-ion MS using CID ions at *m/z* 153, 172, 285 and 390 provided additional structural information to the results of positive-ion CID mass spectra of the same compound. Thus both ionisation CID techniques are complementary for structural elucidation and for the confirmation of the presence of sulfate-ribose in various biological and environmental samples.

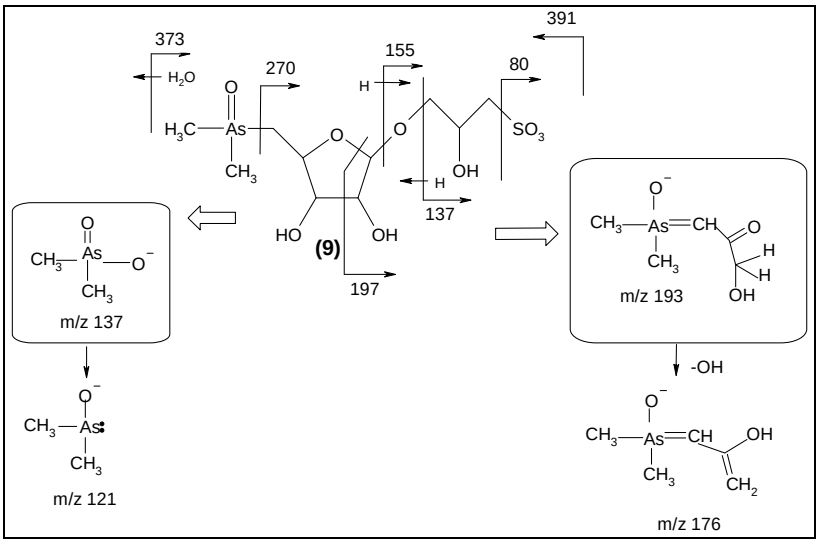
Scheme 3-8 Proposed fragmentation pathway for dimethylarsenosulfate-ribose in negative-ion mode ESI-MS/MS



The ESI tandem mass spectrum of $[M-H]^-$ ions arising from sulfonate-ribose contained a high number of characteristic ions (see Figure 3-30). The spectrum of the sulfonate-ribose has more fragment ions than the spectra obtained for phosphate- and sulfonate-ribosides obtained at the same working conditions. A very similar spectrum of the sulfonate-ribose has been obtained by high energy CID FAB-MS, as described in Ref. [146]. The main fragment ions in the spectrum occur at *m/z* 176, 155, 137 and 80. Dissociation processes from various precursor ions to fragment ions are proposed in Scheme 3-9. Fifteen product ions, all of which can be specified to structural features of the sulfonate-ribose were observed under the conditions of low energy CID. This is a great progress compared to the ten structurally relevant fragment ions were reported under high-energy CID FAB-MS for the same compound [146]. Only a low intensity signal for ions at *m/z* 197, which are supposed to be due to cross-ring cleavage of the ribose fragment similar to that previously described for high energy CID in reference [146], was also detected.

All negative daughter ions of the sulfonate-ribose are well comparable to those observed under negative and positive-ion high energy FAB-MS/MS [146]. Ions in the negative-ion mode ESI-MS and CID at *m/z* 155, 176, 95 and 80 provide additional structural information to the spectra obtained at the positive-ion mode ESI-MS and CID in the case of the sulfonate-ribose. Therefore, when providing sufficient material for both positive and negative-ion ESI tandem mass spectra of arsenosugars a full characterisation can be realised.

Scheme 3-9 Proposed fragmentation of sulfonate-ribose in negative-ion mode ESI-MS/MS



Unfortunately, in this study inorganic arsenate and arsenite did not generate ions at *m/z* 75 in the positive-ion ESI and therefore could not be detected by this soft ionisation technique.

ESI-MS/MS enables improvements for the study of metabolites of arsenic-related compounds. All organoAs species investigated so far at low energy CID have produced clear, abundant, selective and informative negative- and positive-ion electrospray tandem mass spectra. After collecting spectra in ESI-MS and ESI-MS/MS for all nine As species available, separation by SAX capillary chromatography could easily be completed with ESI-MS for an unambiguous compound identification of the As species eluting from the column.

3.4.5 cHPLC-ESI-MS/MS of organoarsenic compounds

cHPLC-ESI-MS/MS provided molecular information about the organoarsenic compounds eluting from the capillary column. Thus, co-elution of some As species did not disturb their identification. SAX cHPLC-ESI-MS/MS was used to verify the elution order for a mixture of arsenosugars and alkyl arsenicals with the best separation method previously developed. With SAX cHPLC-ESI-MS/MS nine As species could be determined in one run and this from a single sample injection. The usual optimisation of the ion source and the interface parameters during the sample elution, however, were also necessary. The values for the DP and ESI voltage of 50 V and 3.5 kV were set up as a compromise and kept constant during all measurements, while the collision energy was varied according to the optimum value found for each organoarsenic compound.

The use of MRM was preferred, because this is the most sensitive and at the same time the most reliable mode of measurement in ESI-MS/MS. For each arsenic-containing compound at least two MRM transitions and their ratios were chosen to be monitored in the positive-ion mode because of the higher fragment stability and intensity found during the

previous experiments. This approach has been applied as a validation tool. A standard solution containing mixture of alkyl arsenicals and arsenosugars have been analysed by SAX cHPLC-ESI-MS/MS in the positive-ion mode with a simultaneous recording of the $[M+H]^+$ and two specific MRM transitions for each species as shown in Table 3-9.

Unfortunately, arsenate and arsenite did not produce ion signals at *m/z* 75 in positive-ion ESI mode. The quality of the ESI-MS results for MMA is compromised by impurities in the mobile phase solvents at the same *m/z* 141 as MMA. Thus MMA could not be further determined by SAX cHPLC-ESI-MS with a good precision and low detection limits. The limits of detection with SAX cHPLC-ESI-MS/MS were determined by analysing a solution of organoarsenic standard compounds when using the two most intensive MRM transitions. The limits of detection for alkylarsenicals and arsenosugars based on the 3σ criterion in SAX cHPLC-ESI-MS/MS together with their relative standard deviation for five replicates are given in Table 3-9. The MRM for the most abundant transition and peak area were used for quantification, whereas the second MRM was used as confirmation. It is obvious that the LODs are much more species- and mass transition-dependent than in cHPLC-ICP-MS. As a major consequence, an accurate quantification is only possible when standards of the analytes are available for calibration. The RSDs for all arsenosugars and methylated arsenicals are below 5% with exception of MMA.

Extracted ion chromatograms (XIC) for a mixture of AsB, MMA, DMA, TMAO, AC and the four dimethylated arsenosugars investigated within one chromatographic run in positive-ion ESI-MS/MS and MRM detection are shown in Figure 3-31. From Figure 3-31 the advantages of the cHPLC-ESI-MS/MS can be seen. Although AC, TMAO, AB and glycerol-ribose are eluting almost at equal retention times, they could be undoubtedly very well differentiated through the specific MRM transitions. This demonstrates the great potential of using ESI-MS/MS for compound identification in As speciation.

Table 3-9 MRM transitions and LOD for As species with SAX cHPLC-ESI-MS/MS

Compound	MRM	Collision Energy	LODs (ng/mL), RSD, (%)
Arsenobetaine	179 → 120 179 → 105	35	0.125 (3.2%)
Arsenocholine	165 → 105 165 → 121	35	1.0 (1.1%)
TMAO	137 → 107 137 → 122	35	0.5 (1.8%)
MMA	141 → 91 141 → 123	25	50 (20%)
DMA	139 → 121 139 → 91	40	0.5 (2.9%)

Compound	MRM	Collision Energy	LODs (ng/mL), RSD, (%)
Dimethylarsinoyl-glycerol-ribose	329 → 97 329 → 237	40	0.25 (4.3%)
Dimethylarsinoyl-phosphate-ribose	483 → 329 483 → 237 483 → 97	40	0.5 (4.9%)
Dimethylarsinoyl-sulfonate-ribose	392 → 163 392 → 193 392 → 237	40	2.0 (3.8%)
Dimethylarsinoyl-sulfate-ribose	409 → 330 409 → 237 409 → 97	40	1.0 (3.4%)

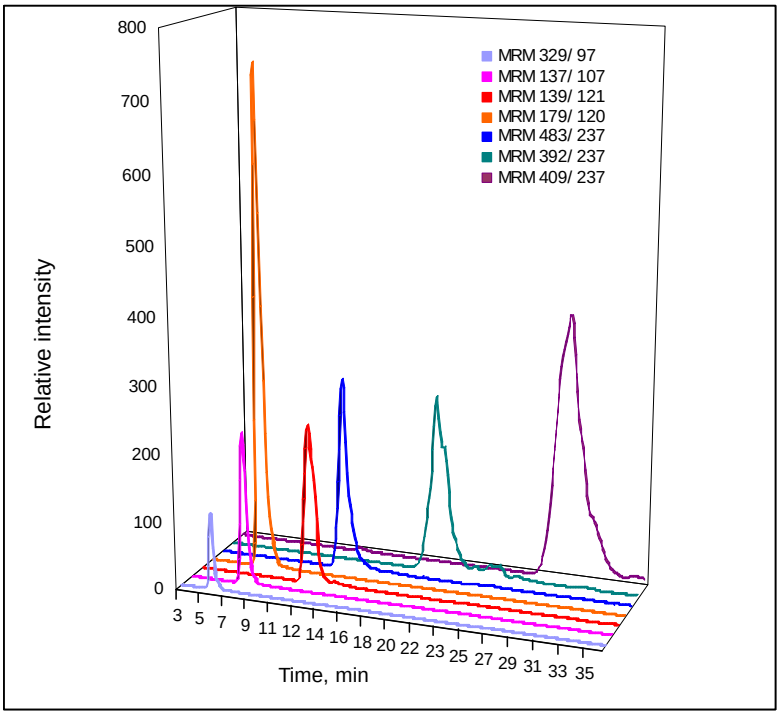


Figure 3-31 Determination of organoarsenic compounds by SAX cHPLC-ESI-MS/MS in positive-ion mode using MRM transitions listed in Table 3-9.

Almost all organoarsenic species generated symmetrical chromatographic peaks even with low intensity MRM transitions, except for phosphate- and sulfate-ribose, for which a higher methanol content of the mobile phase seemed to be required. Phosphate-ribose also undergoes some fragmentation in the source ($[M+H]^+$ = 483 → daughter *m/z* 409) resulting in a small trace peak occurring in the XIC of the MRM 409 → 97 for the parent ion of the sulfate-ribose, but with the same retention time as for the parent ion of the phosphate-ribose monitored at the MRM transition 483 → 97 (Figure 3-31 and Figure 7-6 in appendix).

Sulfate-ribose is the last eluting species which has wider, but symmetrical chromatographic peak. This can be due to the change in the gradient elution at the time of elution of sulfate-ribose and/or partly ionised molecules.

cHPLC-ESI-MS/MS in MRM mode combines high selectivity with high sensitivity for the detection of organoarsenic species. With the MRM, limits of detection of 0.25 ng/mL (AsB) were obtained for alkyl As derivatives. The hyphenation of cHPLC and ESI-MS/MS was successfully accomplished with the advantages of higher selectivity and sensitivity as compared to HPLC-ESI-MS [33, 36]. However, in complex matrixes, such as biological samples there is generally a possibility of artefact peaks coming from matrix compounds which may have MRM transition in common with the target analyte. Moreover, As is monoisotopic and an isotopic pattern cannot be used to check if the signal originates from an arsenic-containing compound. In spite of this limitation, cHPLC-ESI-MS/MS offers several alternative quality control tools, such as:

- matching chromatographic retention times,
- comparing MRM ratios,
- recording full fragment ion spectra in the positive- and negative-ion mode
- determination of accurate masses, to ensure an unambiguous identification of peaks for arsenic-related compound peaks in real samples.

The molecule-selective detection method developed is capable unambiguously identify and quantify all target organoarsenic species in environmental and/or biological applications.

3.5 As speciation in fish and edible marine algae by cHPLC coupled on-line to ICP- and ESI-MS

The improvements of the analytical procedures for the speciation of As and the investigations of marine organisms, which have not yet been analysed continue to be a challenge, especially when it comes to the understanding of the biogeochemistry of arsenic, its toxicity and its metabolism. The new procedures developed using capillary liquid chromatography coupled to ICP- or ESI-MS have been applied for the determination of inorganic and organic As species in the certified reference material (CRM): dogfish muscle, in two codfish muscle and in two types of algae samples. The additional information obtained from the ESI-MS/MS technique was used to enable an unambiguous identification of organoarsenic compounds.

3.5.1 Qualitative studies

3.5.1.1 Fish Samples

Two tissue samples of codfish were analysed by ion-pair cHPLC coupled to ICP-MS and SAX cHPLC coupled to ICP-MS after extraction with methanol/water mixtures. The silica-based Zorbax SAX cHPLC column with strongly basic quaternary ammonium anion-exchange coating was found to be effective for the separations in As speciation (see Figure 3-32). The identification of the species is based on the chromatographic retention

times as compared with those of the available reference substances and on standard addition. For a clear identification and quality check of the measurements for As compounds detected in the fish extracts, a spiking experiment with AsB, DMA, MMA and As(V) at 100 µg/kg was performed. A poor resolution for As species, such as AC, As(III), TMAO, MMA and DMA in the fish extract was observed. Therefore, only a sum parameter can be obtained for these species.

For codfish a variety of organic As compounds was found, whereas inorganic As contributed only with very small part. The chromatogram indicated that three As species, namely TMAO, presumably arsenocholine (eluting at the dead volume of the column) and $TMAs^+$ (coeluting with TMAO) were present. Single compounds (in the case of AB and As(V)) or a group of As compounds were assigned to the different peaks. Arsenobetaine gave the most intensive peak in the chromatogram. This was well expected from reports about arsenobetaine in the literature; it is namely the end product of As metabolism in animals and the major arsenical in a great variety of fish tissues [31, 38, 42, 49, 53, 72]. Several peaks due to coeluting As species were accordingly broad. Additionally, the dead volume of the interface and the hardware of the Sciex Elan 5000 ICP contributed to the peak broadening.

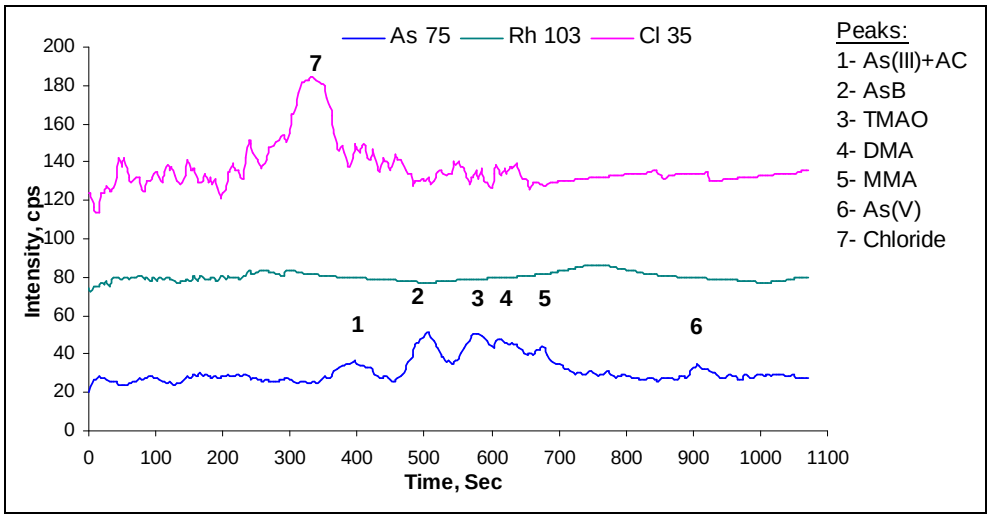


Figure 3-32 Chromatogram of codfish extract obtained by cHPLC coupled to ICP-MS using the Zorbax SAX cHPLC column.

3.5.1.2 Algae samples

Generally the metabolic pathway and the number of As compounds in marine flora are more complex than in marine animals. Indeed more As species were found in algae [29-34, 76]. In order to further investigate the feasibility of the analytical method developed and applied for fish, as well as to study the geogenic effect of As contamination on marine ecosystem two different type algae samples were selected. On the other hand marine animals eat algae and the bioavailability of As and differences in the species in tissue could contribute to the understanding of the uptake and excretion of As in the marine ecosystem.

Literature data on organoarsenic species in the alga *Undaria pinnatifida* are scarce [19]. Some authors have reported the presence of all four dimethylated arsenoribosides in an extract from the brown algae *Laminaria* [37, 40].

The extracts of the brown *Undaria pinnatifida* and the red *Porphyra* seaweed were analysed by anion-exchange cHPLC coupled to ICP-MS using the Agilent 7500s instrument. A strong anion-exchange Hamilton PRP-X100 polymer resin at pH 8.5, where most of the As species investigated may be neutral or anionic was used. A chromatogram obtained for brown alga *Undaria pinnatifida* is shown in Figure 3-33. This chromatogram of the brown algae was recorded in the time resolved analysis mode with the Agilent 7500s ICP-MS in order to be able to detect a large variety of arsenicals at very low concentrations.

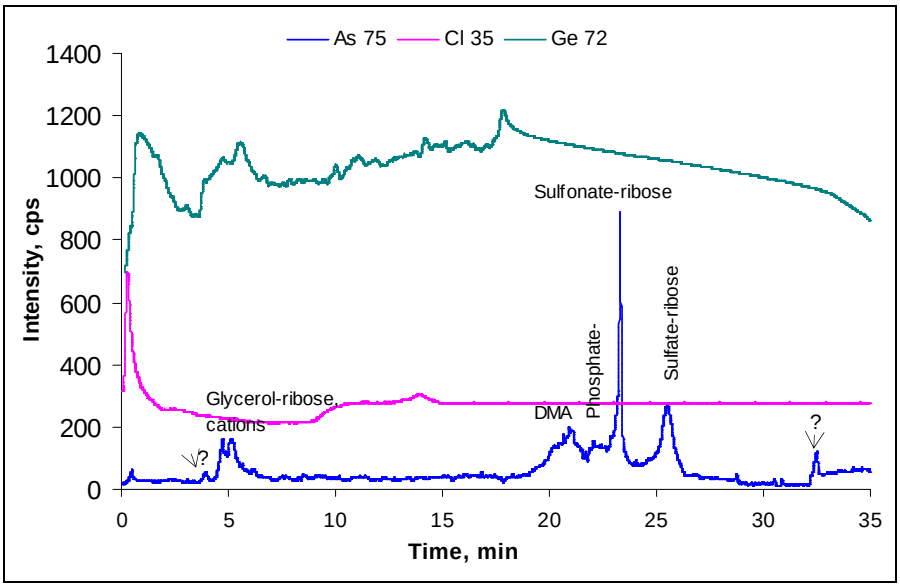


Figure 3-33 Chromatogram of brown alga *Undaria pinnatifida* extract obtained by SAX cHPLC coupled to ICP-MS ("?": unidentified peak).

The quality of the sample extraction and the measurement was assured by analysing the CRM dogfish DORM-2 and by spiking experiments. The detection of the analytes in the algae and the dogfish extracts is based on a matching of the chromatographic retention times with those of the As compounds standards available.

In the algae samples mainly arsenosugar compounds were found. The arsenoribosides mostly are acidic or basic depending on the aglycone. In the dimethylarsinoylglycerol-ribose with the neutral aglycone $–O-CH_2-CH(OH)-CH_2OH$, the dimethylarsine oxide may become protonated at pH<3.0. At a pH of 3.6 to 9.0 the glycerol-ribose should be neutral and accordingly this not retained on the anion-exchange column. The dimethylarsinoyl phosphate-, sulfonate- and sulfate-ribosides have one acidic proton. So far no dissociation constant was published for these compounds. With an acidity constant for dibutyl phosphate of 1.0, the phosphate-ribose is expected to carry a negative charge at pH 8.5 and has been

retained on the Hamilton PRP–X100 capillary column. Butyl sulfonic acid has an acidity constant of pKa 1.5 and therefore sulfonate-riboside can be assumed to be deprotonated and retained on the column. Dissociation constants for monoalkyl sulfates were not found in the literature. Nevertheless, the sulfate-ribose can be assumed to be deprotonated and retained on the anion-exchange column at pH 8.5. The same chromatographic conditions are also suitable for determining AsB, DMA, MMA and arsenate in the presence of arsenoribosides. However arsenite is not well separated from glycerol-ribose and AsB and its determination with this approach is ambiguous. Hence, all four arsenoribosides, DMA, MMA and As(V) can be separated when using the Hamilton PRP-X100 anion-exchange capillary column with ammonium nitrate gradient at pH 8.5 as mobile phase.

In the chromatogram obtained of *Undaria pinnatifida* extract (Figure 3-33) two main peaks are found, which are assigned to sulfonate-ribose and sulfate-ribose. The matrix in this case had a smaller effect on the retention times of glycerol-ribose, arsenite and AsB, as showed by spiking experiments. Nevertheless, an insufficient separation from cationic As species, which are not retained on the anion-exchange column was observed.

In anion-exchange cHPLC chromatogram of the red seaweed algal extract four major and several minor As peaks were present (Figure 3-34). Three of them were assigned to phosphate- ribose and glycerol-ribose As compounds, sulfonate-ribose and one to As(V). The first peak was a front peak and presumably contained cationic and neutral As species together with glycerol-ribose. The algal matrix had a significant effect on the retention time of glycerol-ribose and arsenite. They co-eluted with AsB near the solvent front resulting in a wider chromatographic peak with a tail. This was checked by spiking experiments and by standard addition as shown in Figure 3-35. Small traces of DMA and several unknown species were also present in the red seaweed extract.

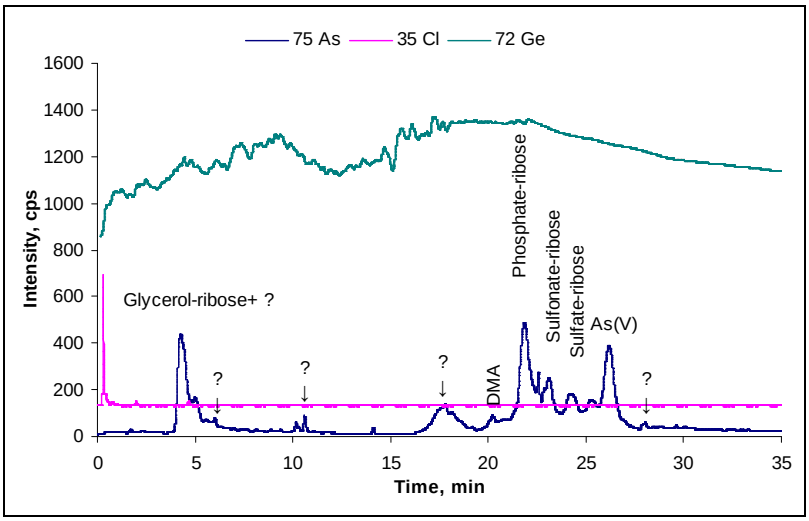


Figure 3-34 Chromatogram of red *Porphyra* seaweed extract obtained by SAX cHPLC coupled to ICP-MS ("?": unidentified peaks).

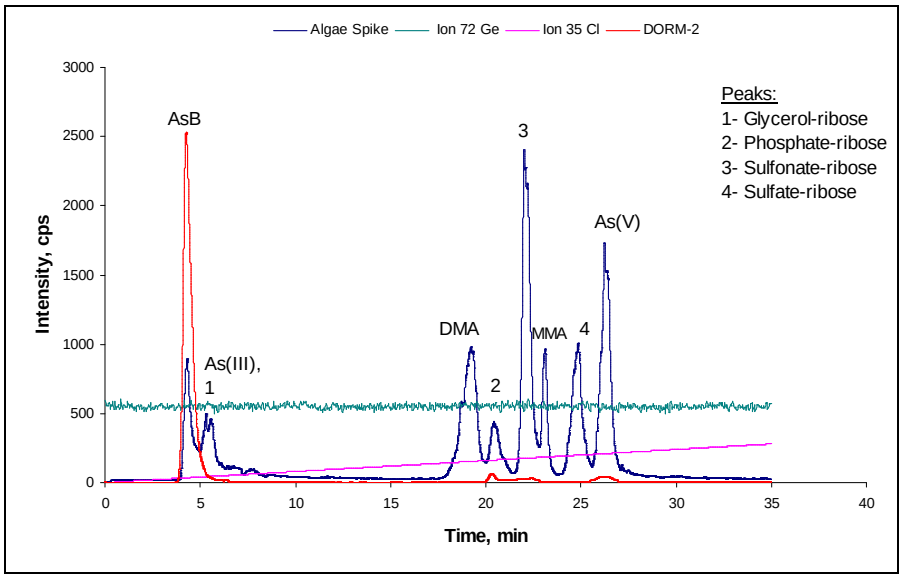


Figure 3-35 Chromatograms of DORM-2 and red seaweed spike extracts obtained by SAX cHPLC coupled to ICP-MS.

As it can be seen from the chromatograms shown in Figure 3-33 and Figure 3-34, the As distribution in algae is more complicated that in codfish and dogfish samples. These results are entirely consistent with early studies [30, 41, 42]. Additional small As peaks eluting during the anion-exchange separation could not be identified. This makes the speciation of As in the seaweeds extracts very difficult and faces one of the limitations of cHPLC coupled to ICP-MS as an element specific, but not molecular specific detection. Complementary cation-exchange capillary liquid chromatography and/or HPLC-ESI-MS/MS would deliver more information for the identification, the structural elucidation and the quantification of the As species present in the analysed algae extracts.

A separation of the most common organoarsenic compounds found in marine biota, however, has been achieved with the strong anion-exchange capillary columns. The HPLC behaviour of the As compounds in the algae extracts is apparently influenced by hydrophobic interactions leading to long retention times on the capillary SAX columns. The cHPLC coupled to ICP-MS methods developed have been used to identify and determine the As species in codfish, dogfish DORM-2 reference material, brown alga *Undaria pinnatifida* and red seaweed *Porphyra*. For the DORM-2 sample similar distribution of the As species was found as the one reported in the literature [31, 35, 42]. The four dimethylated arsenoribosides, AsB, DMA, MMA and As(V) present in marine biota could be well separated in the case of the polymer anion-exchange resin at basic conditions. A co-elution of As(III), AsB and glycerol-ribose was obtained for the *Undaria pinnatifida* extract. Their chromatographic retention times those of *Fucus* extract or of standards, which allowed the identification of the As species. Unfortunately not for all compounds found in the algae extract standards are available. A determination of different As species in marine biota by ICP-MS is generally accepted to be the most powerful method, although it yields only

elemental information [30, 35, 42, 49]. cHPLC coupled to ICP-MS can be concluded to be suitable for a successful determination of As(III), As(V), AB, MMA, DMA and some dimethylated arsenosugars at low trace levels in complex biological samples such as fish and algae tissue.

3.5.2 Analysis of algae by cHPLC coupled to ESI-MS/MS

The purposes of further analyses of the algal extracts by cHPLC and ESI-MS/MS, were a full As speciation characterisation and the evaluation of ESI-MS as a molecule selective detector for ion-exchange capillary liquid chromatography.

3.5.2.1 Analysis of target As compounds

To obtain more information about the eluting As compounds tandem mass spectrometry was employed. The two algae extracts were analysed by cHPLC/(+)ESI-MS/MS. The chromatograms were recorded in the MRM ion mode since this is the most sensitive and reliable way, when using the selected precursor and the most intensive daughter ions as given in Table 3-9 and section 3.4.5. A total ion chromatogram (TIC) for the brown alga *Undaria pinnatifida* extract is shown in Figure 3-36, while for red seaweed extract it is given in Figure 3-37.

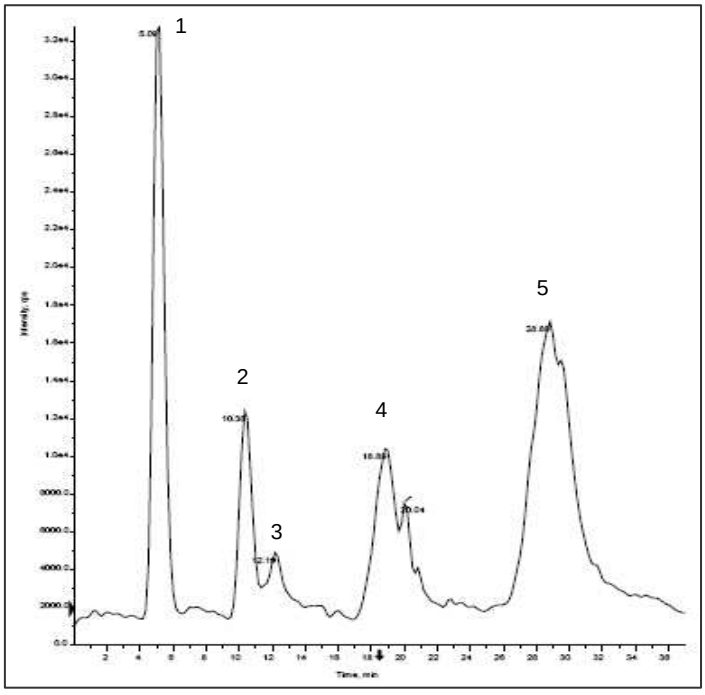


Figure 3-36 Total ion chromatogram for brown alga *Undaria pinnatifida* extract with positive ion MS/MS mode of target As compounds Peaks: (1): Glycerol-ribose, (2): Phosphate-ribose, (3): DMA, (4): Sulfonate-ribose and (5): Sulfate-ribose.

Several As derivatives were detected starting with glycerol-ribose at a retention time of 5.3 min. The arsenosugar that elutes at 18.6 min was identified as a phosphate-ribose. These results are also entirely consistent with those of earlier studies [86].

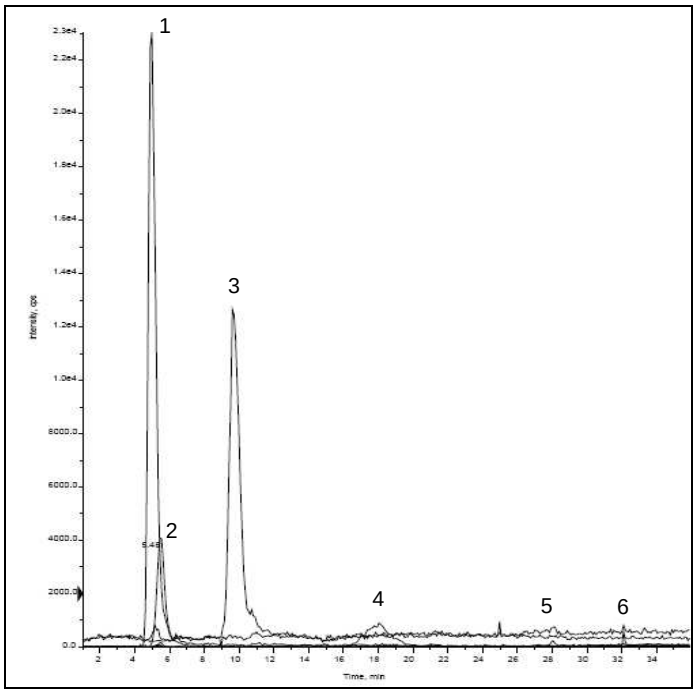


Figure 3-37 Extracted ion chromatogram in positive-ion mode for the determination of target As compounds in red seaweed Porphyra extract Peaks: (1): Glycerol-ribose, (2): AsB, (3): DMA, (4): Phosphate-ribose, (5): Sulfonate-ribose and (6): Sulfate-ribose.

3.5.2.2 Determination of non-target of As derivatives in the algae samples

In order to collect more information on the unknown eluting substances of As related compounds in the algae extracts, a scanning single quadrupole MS and tandem mass spectrometry with positive and negative ionisation were employed. Although As species were isolated at very low concentrations, these experiments offered the possibility to identify other As binding compounds in the algae extracts, of which the chromatographic behaviour did not match with any of the As standards originally available. Selected ion monitoring (SIM) and MRM modes, which usually offer excellent detection limits in ESI-MS were further applied. These chromatographable unknown As species were not reported in the earlier work of Francesconi [106]. In a few studies a full characterisation of As compounds in algae was attempted. Novel trimethylarsonioribosides (compounds (**20**) and (**21**), Figure 7-2 in appendix) were isolated from *Sargassum thunbergii* and *Sargassum lacerifolium* [86, 107] and gastropod [32]. Compound (**21**) is interesting, because the acylated trimethylated nitrogen analogue has been identified as an important membrane lipid in several marine algae [143]. Other novel thio-arsenicals were recently identified by LC-ESI-MS and CID

tandem MS in mollusc [47, 165]. The potential of tandem mass spectrometry and especially HPLC-ESI-MS/MS as a tool for the identification and structure elucidation of novel As compounds in marine biota is shown in several studies up to now [33, 37, 38, 40, 47, 144, 147, 164].

The first unidentified compound found in *U. pinnatifida* extract and quantified by cHPLC coupled to ICP-MS (see Figure 3-33) represents 4.8% of the total As content, whereas the second unidentified compound accounts for 16.3%. The first significant unidentified compound for the red seaweed extract accounts for 1.3%, while the second unidentified compound for only 0.9% of the extractable arsenic.

Most of the arsenic, which has in marine algae typically concentrations between 2 and 40 mg/kg, is present in the form of dimethylarsinoylribosides [107]. The biosynthesis of these compounds probably involves the transfer of two methyl groups from S-adenosylmethionine (SAM) to arsenic, since this is the main biological donor of methyl groups, as shown in Scheme 2-1 [5].

The evidence that As has a particularly strong affinity for sulfur results in a stable chelation of As(III) by a variety of sulfur-rich peptides and proteins, such as glutathione (GSH) and phytochelatines (PCs), as identified in plants that had been exposed to metal stress [72, 166]. These peptides are synthesised by eukaryotes for various cellular functions, such as protection against oxidative stress, metal detoxification and homeostasis [168]. GSH (γ-Glu-Cys-Gly) provides an abundant source of sulfur in biological systems [167]. PCs and GSH are thiol (-SH or sulfhydryl group) containing peptides with the basic formula $(\gamma\text{-Glu-Cys})_n\text{-Gly}$, where n=2-11. Phytochelatin synthase catalyses the transfer of γ-Glu-Cys from GSH to another GSH molecule to form PC_2 or $(\gamma\text{-Glu-Cys})_n\text{-Gly}$ to form PC chain lengths of n+1 [168]. PCs production serves to detoxify intracellular metals by chelation through coordination with the sulfhydryl group in cysteine. Cd-, Pb-, Cu- and Zn-phytochelatin complexes were found in the marine micro alga *Phaeodactylum tricornutum* [167, 169, 170], *T. pseudonata* [169] and green alga *Dunaliella sp.* [169] offer to metal exposure. Arsenotriglutathione and phytochelatines are known compounds [26, 166, 168, 171] and the unidentified chromatographic peaks in the algal extracts could result from a chelation of As by glutathione and/or low molecular weight thiol peptides. GSH-conjugated As and As-PC complexes were previously also eluted as such on an anion-exchange column and simultaneously detected by HPLC-ICP-MS in body fluids and plant [26] as well as by HPLC coupled to ESI-MS/MS in plant [166]. In 2000 Madsen *et al*. [33] reported the presence of an unknown As compound in several species of marine algae using HPLC coupled to ICP-MS. These As species could be isolated, but their structures remained unknown.

- **Structural elucidation of novel As compounds with cHPLC coupled to ESI-MS/MS**

The suspicion that the unidentified chromatographable peaks were As containing peptides led to the application of cHPLC-ESI-MS/MS for an analysis of the algae extracts. Three As related phytochelatines with sulphur bridges were found. The new species are referred to as listed in Table 3-10. The tentatively identified ligands belong to three PCs classes: desGly-PCs, normal PCs and iso-PCs (Ser). Regardless of the class they have amino-acid chains of different length of corresponding to the coefficients n ~ 3, 5 and 6 in the general formula of PCs.

Table 3-10 As-GSH and PC complexes found in the algae extracts by cHPLC coupled to ESI-MS/MS

Undaria pinnatifida			***Porphyra***		
Unknown peak	$[M-H]^+$	Peptide sequence	Unknown peak	$[M-H]^+$	Peptide sequence
Oxidised Glutathione	613	$[\gamma\text{-Glu-Cys-Gly}]_2$	Dimethylarsenic-Glutathione	412	$(CH_3)_2$-As-γGlu-Cys-Gly
desGly-PC_6	1394	[desGly-$(\gamma\text{Glu-Cys})_6$]	As-PC_3	847	$(\gamma\text{-Glu-Cys})_3$-Gly-As
			Cd-As-isoPC$_5$ (Ser)	1453	[Cd-As-isoGly-$(\gamma\text{Glu-Cys})_5$-Ser]

Evidence of the proposed structures was obtained by cHPLC coupled to ESI-MS and tandem MS. In Figure 3-38 the extracted ion chromatogram and ESI mass spectra for the first eluting unidentified As peak in the case of the *U. pinnatifida* extract as found by cHPLC coupled to ICP-MS are shown. The ESI-MS data for oxidised GSH were obtained in the positive-ion mode using identical conditions for capillary anion-exchange chromatography (see section 4.3.5 in experimental part). The ESI-MS/MS spectrum of the precursor ion *m/z* 613 showed significant background noise. However, three major fragment ions could be observed were associated with the protonated molecular ions $[M-H]^+$ at *m/z* 613, at *m/z* 417.8 $[\gamma\text{Glu-Cys-Gly}]_2^+$-Gly-Cys]$^+$ and at *m/z* 324.4 $[\gamma\text{Glu-Cys-Gly}]^+$.

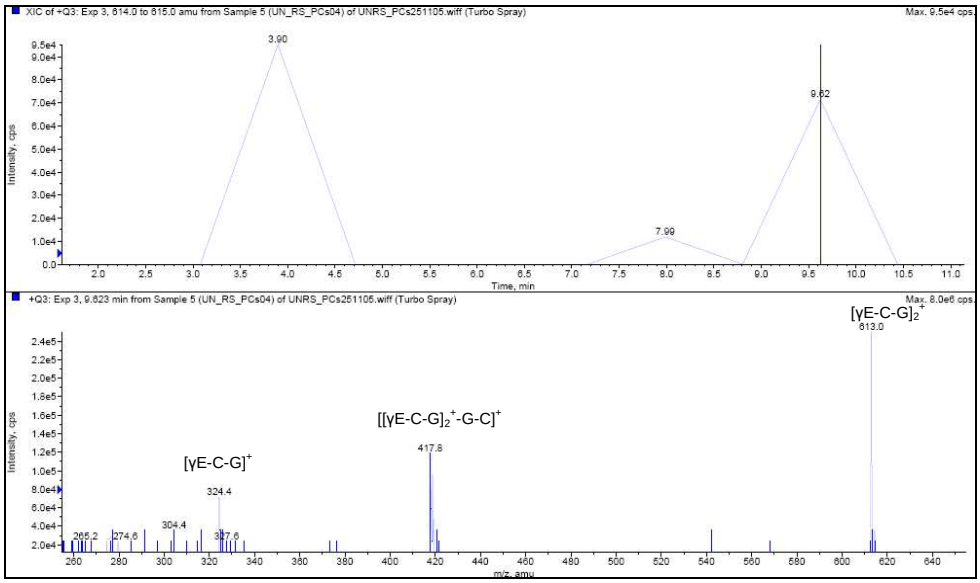


Figure 3-38 XIC chromatogram and MS spectra for $[(\gamma Glu\text{-}Cys\text{-}Gly)_2]^+$.

The chromatogram and the ESI-MS/MS spectrum associated with *m/z* 1394 in the case of the brown alga *Undaria* extract are shown in Figure 3-39. These assignments are made using the full-scan MS/MS spectrum of the precursor ion *m/z* 1394 of desGly-PC_6 $[(\gamma Glu\text{-}Cys)_6+H^+]^+$ with a CE voltage of 25 eV. Additional data supporting the desGly-PC_6 structure are the ions assigned for [desGly-PC_5+H^+]$^+$ at *m/z* 1180 and at *m/z* 1162 ([desGly-PC_5-H_2O]$^+$), at *m/z* 947 [desGly-PC_4]$^+$ and at *m/z* 929 [desGly-PC_4-H_2O] $^+$, at *m/z* 715 [desGly-PC_3]$^+$, at *m/z* 697 [desGly-PC_3-H_2O] $^+$ and at *m/z* 483 [desGly-PC_2]$^+$ and at *m/z* 465 [desGly-PC_2-H_2O]$^+$. Those ion signals were present in the same mass spectra and were also detected in the MRM and SIM modes in the case of the algal extract.

The unidentified As species eluting from the capillary SAX column and monitored by ICP-MS for red *Porphyra* seaweed extract were of very low concentration. However, from the investigations by cHPLC coupled to ESI-MS/MS the signals are believed to be from dimethylarsenic-glutathione (DMAs-GSH or DMAs-PC_1), As-PC_3 and Cd-As-isoPC_5(Ser) at m/z 412, 847 and 1453, respectively. In this case, As-PCs complexes from the algae extract were detected. As the cHPLC coupled to ESI-MS/MS use lower flow rates, the amounts of sample needed for analysis was only 500 nL. This has allowed the performance of series of full MS scan and MS/MS scan in order to obtain structural information from a limited amount of algae samples.

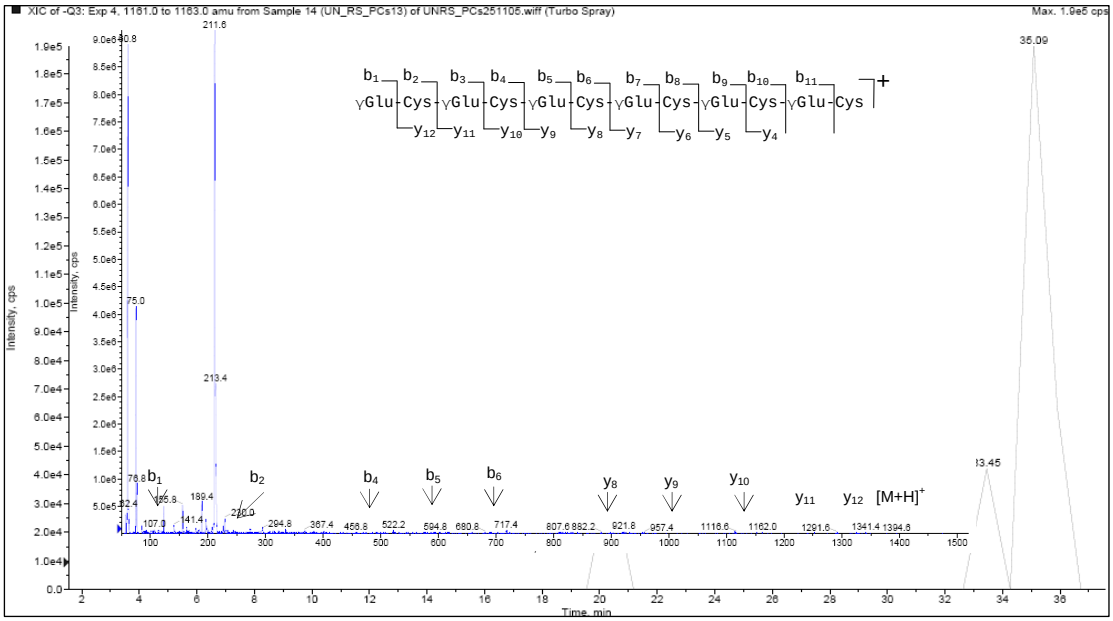


Figure 3-39 Extracted ion chromatogram and mass spectra of $[desGly\text{-}(\gamma Glu\text{-}Cys)_6]^+$.

desGly PC_6	b_1	y_{12}	b_2	y_{11}	b_3	y_{10}	b_4	y_9	b_5	y_8	b_6	y_7
Theoretical	130.0	1353.2	233.1	1250.4	362.1	1121.1	465.1	1018.2	594.5	889.6	697.2	786.2
Found	130.1	1353.3	233.1	1250.2	362.2	1121.2	465.2	1018.4	594.2	889.4	697.1	n.o.

n.o. means that an ion signal should theoretically be present in the spectrum but was not observed in this work

The lower mass peptides DMAs-PC_1 (Figure 3-40) and As-PC_3 (Figure 3-41) were eluting earlier than Cd-As-isoPC$_5$ on the SAX capillary column, which suggests that a hydrophilic chromatographic mechanism may also be involved in the retention. The full scan MS/MS spectra of DMAs-GSH was very noisy, because of the low concentration of the $[M+H]^+$ ion. Using the more sensitive MRM mode resulted in good signals for DMAs-GSH. Nevertheless, additional verification and further investigations and preconcentration are requested for an unambiguous determination of the DMAs-GSH complex in the extract of red seaweed *Porphyra*.

Another As related As-PC_3 complex was detected in the red seaweed extract by ESI-MS/MS scans, because of the lower background noise of the tandem MS. Using the positive-ion scan mode (see Figure 3-41), As(III)$(GS)_3$ showed relatively strong molecular mass peak at *m/z* 848.2 $[M+H]^+$. Three main fragment ion peaks appeared in the ESI-MS/MS spectrum at the lower collision energy of 15 V. The daughter ions generated in cHPLC coupled to ESI-MS/MS of As-PC_3 (precursor ion *m/z* 847.2, as in Figure 3-41) in the case of the red seaweed *Porphyra* extract were associated with $[PC_2]^+$ and $[PC_2\text{-Gly}]^+$ with *m/z* 538 and 462.6, respectively.

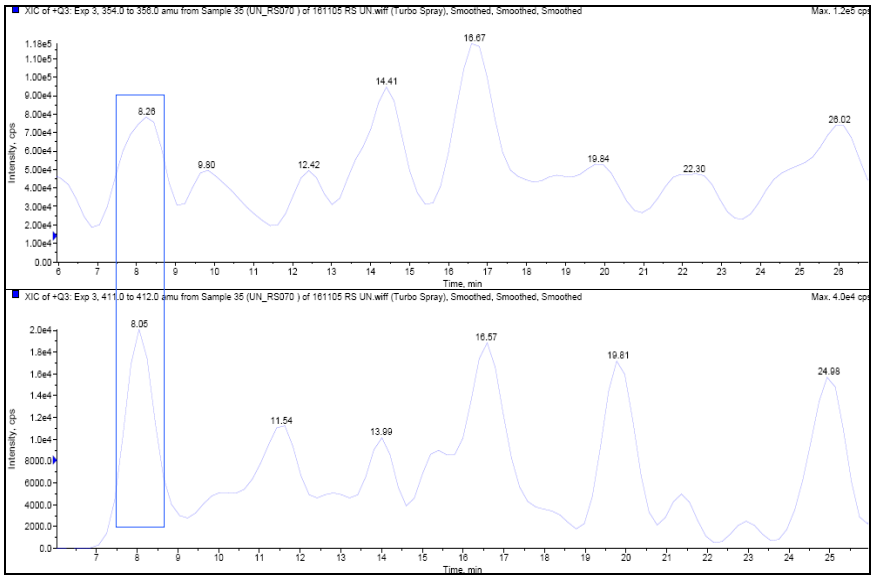


Figure 3-40 MRM chromatograms of $(CH_3)_2$-As-γGlu-Cys-Gly in red *Porphyra* extract.

Analysis of red seaweed extract in the case of separation with a basic mobile phase (pH 9) at higher ionic strength showed the presence of a more complex GSH-conjugated As species. A XIC and ESI-MS/MS spectra of Cd-As-isoPC$_5$-Ser with $[M\text{-}H_2O]^+$ at *m/z* 1435 are shown in Figure 3-42. Cd-PC$_n$ complexes were induced and detected by HPLC in marine algae *P. tricornutum* and other phytoplankton species [140] as the result of metal detoxification mechanism. Cd is associated with higher PCn (n= 4, 5 and 6) complexes (oligomers) in plants exposed to Cd^{2+} and was found by gel filtration chromatography and HPLC [140]. Additionally, PCn complexes with two metal ions (Zn-Cd-PC$_{2\text{-}5}$ and Cu-Zn-PC) were found in marine diatom *P. tricornutum* [170].

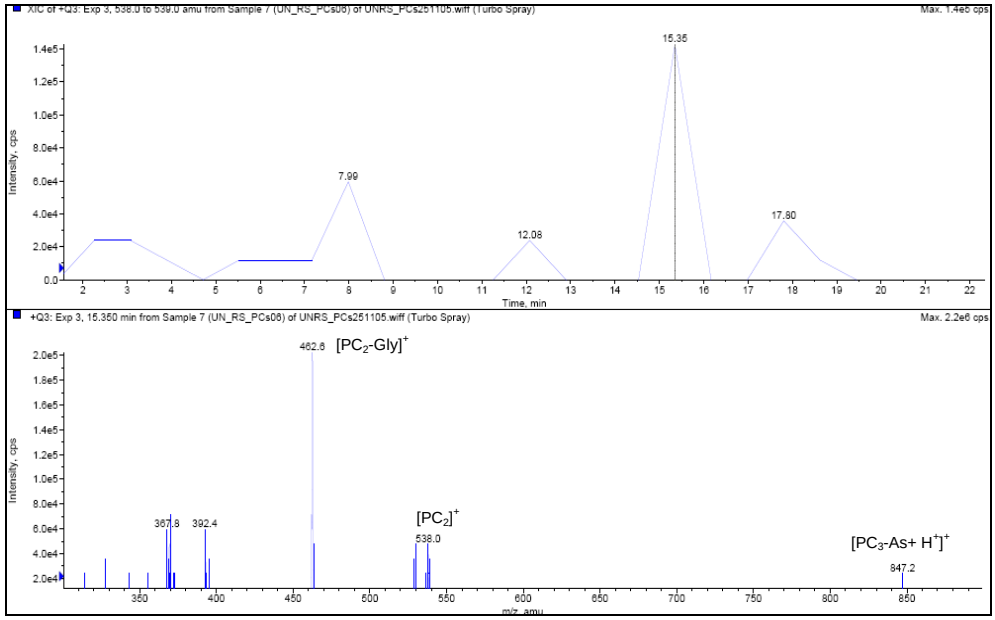


Figure 3-41 Extracted ion chromatogram and ESI-MS/MS spectra of $[(\gamma Glu\text{-}Cys)_3\text{-}Gly\text{-}As{+}H]^+$.

Iso-PCs are usually formed by replacing glycine with β-alanine, serine and/or glutamic acid, as it is demonstrated in the work of Mounicou *et. al* [172] on the determination of phytochelatines in soybean cells exposed to Cd^{2+}.

The extracted ion chromatogram (Figure 3-42) is showing an intensive peak that belongs to the family of iso-PCs. Lack of baseline resolution in the TIC chromatogram is poor, which is due to co-elution of ions other than PCs of much more abundant compounds. The ESI-MS/MS spectra are moderately noisy, however the specific peptide sequence of PC_5-Ser was observed, as it is shown in Figure 3-42. γ-Glutamine was also detected at *m/z* 129. Unfortunately, the elements As^+ and Cd^{2+} were not detected with this soft CID of 35 eV.

In all extracts unbound, reduced glutathione (GSH) was detected at *m/z* 308 $[M+H]^+$ and oxidised glutathione (GSSG) at *m/z* 613 $[M+H]^+$. Furthermore, ESI-MS in the negative ionisation mode obtained a molecular peak of GSH at *m/z* 306 $[M-H]^-$ and GSSG (*m/z* 611 [M-H]-), but with lower intensities and ion stability. Therefore, in the positive-ion mode ESI was used throughout most studies of algae extracts. cHPLC coupled to ESI-MS in the negative-ion mode confirmed the findings of the cHPLC coupled to ESI-MS in positive-ion mode.

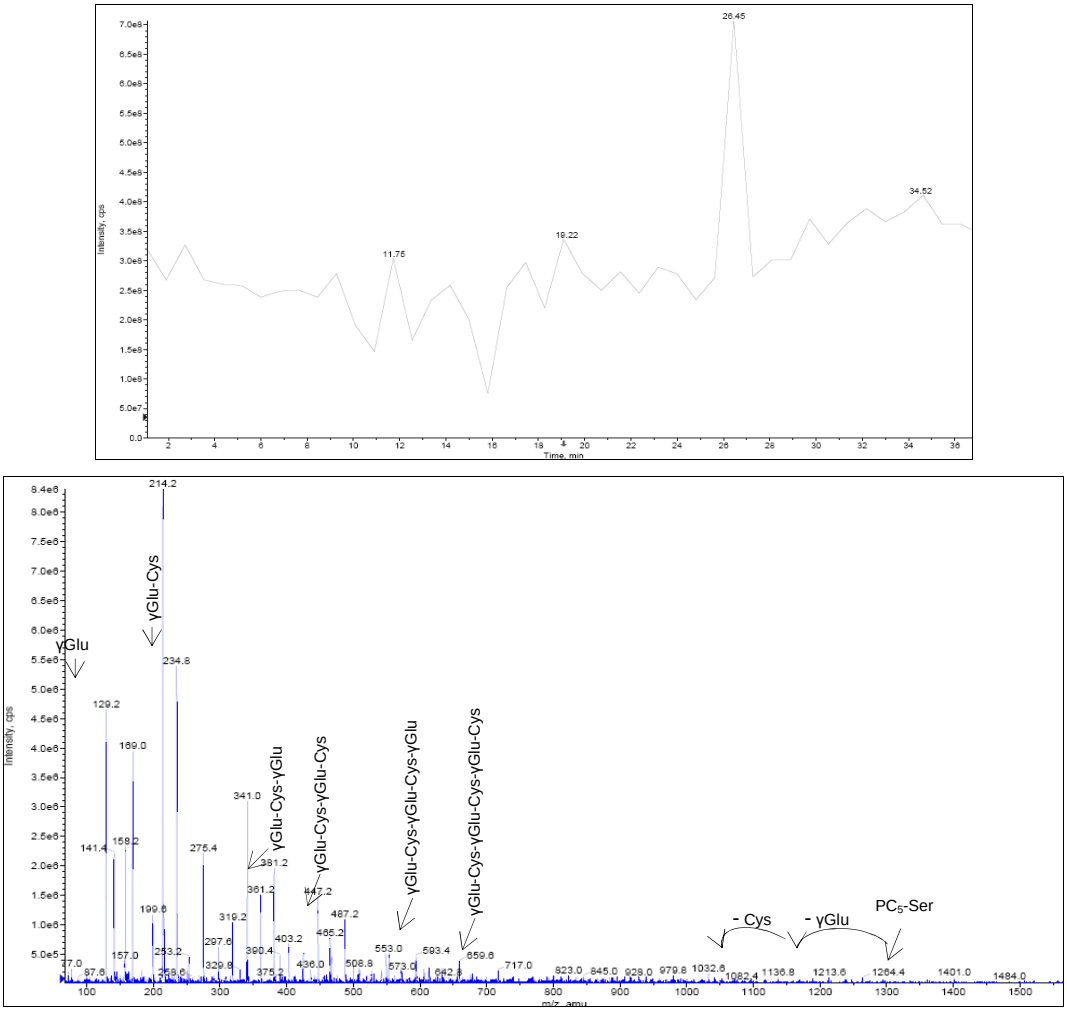


Figure 3-42 XIC and ESI-MS/MS spectra of [Cd-As-isoGly-(γGlu-Cys)$_5$-Ser]$^+$ in *Porphyra* extract.

As(III) and As(V) could be detected by FIA-ESI-MS in the both algae extracts at *m/z* 75, but not with the cHPLC coupled to ESI-MS. To date, this algae analysis has not been done by ESI-MS/MS and this remains an area of active research. The use of ESI-MS/MS and cHPLC coupled to ESI-MS/MS, as expected, has proved to be a very powerful and valuable tool for the characterisation of the bio-ligands synthesised by the algae being exposed to As.

Some other As peptide complexes such as methylarsenic glutathione and As triglutathione were previously determined in rat bile and urine by anion-exchange HPLC-ICP-MS, as reported in two studies [26, 171]. Raab *et al*. [168, 173] reported in 2004 that the As–GSH complexes are stable in solution, during extraction (from a freeze-dried and powdered grass matrix) and during the separation under different chromatographic conditions. Neither the intermediates with GSH attached to MMA (as As(III)) in the form of MMAs(III)GS or two GSH attached to As(III), nor pentavalent As glutathione complexes as proposed by Raab *et al.* [173] and Cullen and co-workers [174] were detected. Raab *et al.* [173] also proposed the hypothesis that As exported from cells as As(III), under the form arsenic-glutathione complexes. Other studies demonstrate that experiments in cell culture using synthesised arsenic-glutathione complexes show that their toxicity is comparable to or higher than inorganic arsenic. Thus, they are potential inhibitors of certain enzymes [173]. Such compounds are unlikely to be toxicologically significant because of their low concentrations, but may offer clues to the metabolism and origin of major As compounds in marine algae. Standard materials must also be available.

All these experiments on the separation and detection of As-glutathione complexes in marine algae show that biological samples that may contain these complexes must be treated very carefully. Many scientific articles have published studies on As speciation in which the integrity of the As species has been tested or assumed [30, 35, 42]. The results presented in this work indicate that many previously published results need to be reinterpreted, especially when unidentified As related compounds were reported, since possible As glutathione complexes have not successfully been detected due to the harsh conditions used in routine analysis of As species in biological extracts.

3.5.3 Quantitative determination of As species in fish and algae using cHPLC coupled to ICP/ESI-MS

So far, no comprehensive determinations of As species in brown alga *Undaria pinnatifida* were performed, but since this alga is widely available in large quantities and edible it can be expected that it will be used for further environmental and toxicological studies in the future. Therefore, it was chosen as a suitable material to test the sensitivity and selectivity of the method developed.

The accuracy of the measurements was tested by analysis of a commercial available CRM DORM-2 dogfish muscle for As speciation from National Research Council of Canada

(NRCC). The DORM-2 sample was analysed by cHPLC coupled to ICP-MS and cHPLC coupled to ESI-MS/MS after applying the sample preparation procedure used for fish and algae. Arsenobetaine, DMA and inorganic As were found in the resulting chromatograms using isotope mass of 75 and MRM, respectively. A quantitative analysis was performed by a calibration with synthetic standards and the results were compared to data obtained by the NRCC using HPLC-ICP-MS, ICP-MS and ionspray MS as well as by Wahlen *et al*. [56], Wrobel *et al.* [49] and Kohlmeyer *et al.* [42, 53] who used HPLC-ICP-MS. The validated methods were then applied for quantitative determinations of the As species in fish and algae extracts.

Since Kohlmeyer *et al*. [42] kindly provided the dimethylarsinoylriboside derivatives isolated from brown alga *Fucus serratus*, the newly developed cHPLC coupled to ICP-MS and cHPLC coupled to ESI-MS/MS procedures [175, 176] could be successfully used to determinate As species in algae samples.

3.5.3.1 Method validation with cHPLC coupled to ICP-MS

Once the analytical method for As speciation has been developed, the next step in this work was to validate it, according to the guidelines of AOAC and IUPAC [138, 177] for reliable and cost effective analytical procedures for each matrix (muscle tissue, algae tissue, etc) and species (fish, algae and etc.) to which the method is to be applied. Validation experiments include assessments of the specificity, the linear range, the limit of detection (LOD), the precision and reproducibility, the accuracy and the recoveries.

3.5.3.1.1 Detection limits

The system for cHPLC coupled to ICP-MS with a small-volume sample introduction interface from Agilent Technologies was found to be very suitable for the determination of As species at low concentrations (≤ 1 µg As/L). cHPLC coupled to ESI-MS was not precisely investigated with respect to its limit of detection (LOD) for As speciation, since this system in general has LODs which are by one order of magnitude lower than in cHPLC coupled to ICP-MS [176]. The absolute LOD of cHPLC coupled to ICP-MS for As was tested by injecting reagent blank samples, MMA, DMA, As(III), As(V), AsB, TMAO and arsenoribosides samples dissolved in a mobile phase containing 1 *mM* ammonium nitrate buffer at pH 8.5. The cHPLC column outlet was connected to the MCN of the ICP-MS via 65 cm x 50 µm I.D PEEK silica capillary.

The calculations of the integrated peak area for the As signal were made using the equation:

$$C_{lod} = k(sd) / m \qquad (3\text{-}2)$$

C_{lod} = smallest detectable concentration of As at signal-to-ratio ≥ 3 [138]

k = 3 (LOD, 99. 86% confidence)
= 10 (LOQ, 99.99% confidence)
sd = Standard deviation for the reagent blank samples
m = Slope of the curve displaying the function calculated by linear regression

In Table 3-11 a comparison of the detection limits for As species with either the capillary strong anion-exchange chromatography in this study or with conventional SAX HPLC and detection with the Agilent 7500s ICP-MS by U. Kohlmeyer *et al*. [42] is given.

Table 3-11 Limit of detections for inorganic and organic As species by HPLC/cHPLC coupled to ICP-MS

As species	Detection Limits (as As)			
	cHPLC-ICP-MS, Agilent 7500s		Conventional HPLC-ICP-MS, Agilent 7500s [42]	
	Relative C_L µg/L	Absolute C_L pg	Relative C_L µg/L	Absolute C_L pg
Arsenite	2.0	1.0	5.0	1.0
MMA	1.0	0.5	5.0	1.0
DMA	5.0	2.5	5.0	1.0
Arsenate	1.0	0.5	10	5.0
Arsenobetaine	1.0	0.5	15	3.0
TMAO	15	7.5	10	5.0
Arsenocholine	10	5.0	10	5.0
Arsenoribosides	5.0	2.5	10	5.0

The highly sensitive Agilent 7500s ICP-MS system and the relatively straightforward on-line coupling to the cHPLC provided a powerful element specific detection for As species.

The limits of detection for alkylarsenicals and DMAs-ribosides in the case of both analytical systems and the same cHPLC column appeared to be at the low µg/L level, as it is shown in Table 3-12. These results allow an analysis of marine biological samples using a Hamilton PRP-X100 capillary column and MS detection.

Table 3-12 Detection limits for organoarsenic compounds with cHPLC coupled to ICP-MS and ESI-MS

Organoarsenic compounds	LODs (µg/L) of cHPLC-ICP-MS (RSD %)	LODs (µg/L) of cHPLC-ESI-MS/MS (RSD %)
MMA	1.0 (1.1%)	50 (20%)
DMA	5.0 (1.9%)	0.5 (2.9%)
AsB	1.0 (0.4%)	0.125 (3.2%)
AC	10 (2.0%)	1.0 (1.1%)
Arseno-glycerol-ribose	5.0 (8.5%)	0.25 (4.3%)
Arseno-phosphate-ribose	8.0 (3.5%)	0.5 (4.9%)
Arseno-sulfonate-ribose	12 (1.6%)	2.0 (3.8%)
Arseno-sulfate-ribose	10 (2.6%)	1.0 (3.4%)

3.5.3.1.2 Linearity relation between the As signal and the concentration

The signal linearly of As relates to the concentration of inorganic or organic As species separated on the SAX cHPLC column, as shown in Figure 3-43. Linear calibration curves were obtained in the range of 1.0 to 100 µg/L of As with a coefficient of correlation R^2>0.990. Higher concentrations were not investigated, since As species concentrations in marine biota samples rarely exceed these levels. However, the linear dynamic range for As is expected to be much wider. Furthermore, one organic As compound could be used for the quantification of all As species in the real samples. This is a great advantage of the analytical procedure developed; since unknown organic As species could be quantified for which standards are not commercially available.

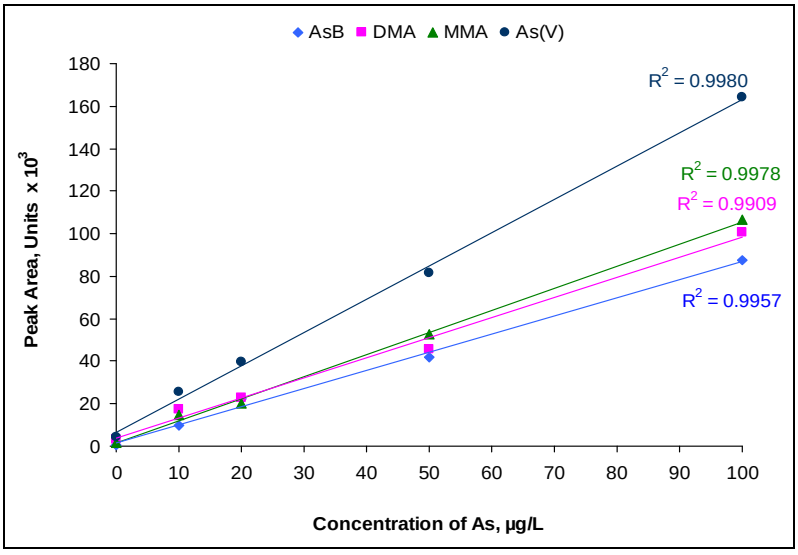


Figure 3-43 Calibration curves for inorganic and organic As species by anion-exchange cHPLC coupled to ICP-MS.

The lower limits of detection and the very good linearity of inorganic and organic As species obtained with SAX cHPLC coupled to ICP-MS allowed quantitative determinations of As species in real marine biota samples.

3.5.3.1.3 Precision

The precision was tested using three fortified samples for each fish and algae matrix containing low (first quartile of the standard linear range), medium (second or third quartile), and high (fourth quartile) concentrations of As species. Furthermore, triplicate aliquots of each spiked sample were extracted and measured at each of seven separate days and the concentrations of the analytes were determined using calibration curves prepared and measured on the day of analysis. The precision of the method was evaluated using the same protocol but in a one day measurement.

The results obtained are summarised in Table 3-14 and Table 3-15.

At a concentration of 10 µg/kg of As (for each compound) the percentage of the relative standard deviations (RSD %) for the retention times, peak area and peak height (at three replicate injections) never exceeded 17%. The RSD% of the retention times for all As species investigated, were lower than 4.5%. This is a good indication for the stability of the cHPLC method developed, with which an unambiguous species identification based on chromatographic retention time match is possible. The RSD percentages of the day-to-day reproducibility did not exceed 24%, with the exception of As(V), for which in the worst case 35% RSD for the peak area was obtained. One possible explanation could be the change in the gradient elution during the retention of arsenate, which results in a wider peak and subsequently inaccurate peak integration. As shown in Table 3-14, the analytical recoveries for As(III), AsB, MMA, DMA and As(V) at concentration levels of 10-250 µg/kg of As were in the ranges of 90-94%, 62-89%, 87-101%, 81-101% and 61-79%, respectively with a mean recovery per analyte in the range of 70-110%, as required in the guidelines [138, 177].

The results indicate that As(III), As(V), MMA, DMA and AsB in fish samples can be successfully determined based on the differences in retention and elution behaviour of the As compounds.

3.5.3.1.4 Accuracy

The accuracy of the cHPLC coupled to ICP-MS procedure for As speciation was tested by analysing CRM DORM-2 dogfish muscle [178] after liquid/liquid extraction. External calibration with AsB standard solutions was applied for the quantification. The recovery for AsB from the CRM dogfish muscle by cHPLC coupled to ICP-MS was 95%.

The mean values for the five samples analysed with the procedure developed in this study were 15.6 ± 0.8 µg/g (as As) arsenobetaine and 17.5 ± 1.1 µg/g total As (see Table 3-13 and Figure 3-44). The certified value for arsenobetaine in the dogfish reference material is 16.4 ± 1.1 µg/g (as As) based on determinations by HPLC-ICP-MS and Ionspray Mass Spectrometry. The results obtained are in good agreement with the certified values and compared well with the data reported in the literature (see Table 3-13 and Table 3-16).

Table 3-13 Arsenobetaine in the extract of CRM dogfish DORM-2

Arsenobetaine certified value (as As), µg/g	Arsenic certified value, µg/g	Arsenobetaine, found (as As), µg/g	Arsenic, found, µg/g	Extraction recovery found, %	cHPLC-ICP-MS recovery found, %
16.4 ± 1.1	18.0 ± 1.1	15.6 ± 0.8	17.5 ± 1.1	96	95

Table 3-14 Analytical results for the determination of As species in fish by cHPLC coupled to ICP-MS. RSD % of the recoveries are used for the precision of spiked experiments at µg/kg of As (triplicate analysis)

		Precision			Day-to-day reproducibility			Spike Recovery (R) [a]	
Sample	**Species**	**Retention time**	**Peak height**	**Peak area**	**Retention time**	**Peak height**	**Peak area**	**Added, µg/kg**	**R ± RSD, %**
Codfish	As(III)	0.35	2.02	8.68	0.88	13.83	17.18	10	88 ± 3.2
								50	92 ± 2.5
								250	95 ± 1.6
	AsB	0.39	0.35	1.58	1.54	8.01	11.28	10	62 ± 4.8
								50	74 ± 4.7
								250	90 ± 1.4
	MMA	3.41	4.59	16.77	4.30	23.99	23.95	10	77 ± 2.2
								50	92 ± 0.9
								250	88 ± 0.4
	DMA	0.98	8.23	11.19	3.54	11.03	11.24	10	103 ± 3.5
								50	89 ± 1.7
								250	81 ± 2.3
	As(V)	0.41	10.76	15.33	0.36	30.61	35.19	10	62 ± 4.4
								50	69 ± 1.6
								250	79 ± 1.4

[a] Mean ± relative standard deviation from triplicate analyses

Table 3-15 Analytical results for the determination of As species in algae by cHPLC coupled to ICP-MS. RSD % of the recoveries are used for the precision of spike experiments at µg/kg of As (triplicate analysis)

Sample	Species	Precision			Day-to-day reproducibility			Spike Recovery (R) [a]	
		Retention time	Peak height	Peak area	Retention time	Peak height	Peak area	Added, µg/kg	R ± RSD, %
Brown alga, *Undaria pinnatifida*	AsB	0.25	2.7	1.0	0.78	12	2.3	20	62 ± 0.7
								50	60 ± 1.0
								100	89 ± 1.8
	MMA	0.02	1.5	2.2	0.34	2.2	4.5	20	66 ± 0.5
								50	75 ± 0.3
								100	87 ± 1.1
	DMA	0.04	10.1	5.6	0.53	11.49	6.8	20	78 ± 0.4
								50	101 ± 6.2
								100	97 ± 2.5
	As(V)	0.29	3.1	3.9	0.52	4.0	5.1	20	61 ± 1.4
								50	73 ± 0.9
								100	75 ± 0.8
Red alga, *Porphyra*	AsB	0.33	3.3	3.1	1.3	4.2	5.4	20	96 ± 0.7
								50	96 ± 0.5
								100	90 ± 0.9
	MMA	0.24	2.6	5.9	0.9	4.3	6.2	20	81 ± 1.2
								50	85 ± 0.8
								100	78 ± 1.3
	DMA	0.23	1.8	2.7	1.1	3.4	10.8	20	66 ± 1.2
								50	76 ± 1.8
								100	69 ± 0.9
	As(V)	0.13	2.7	3.3	0.6	3.6	7.2	20	79 ± 1.1
								50	71 ± 1.1
								100	80 ± 0.7

[a] Mean ± standard deviation from triplicate analyses

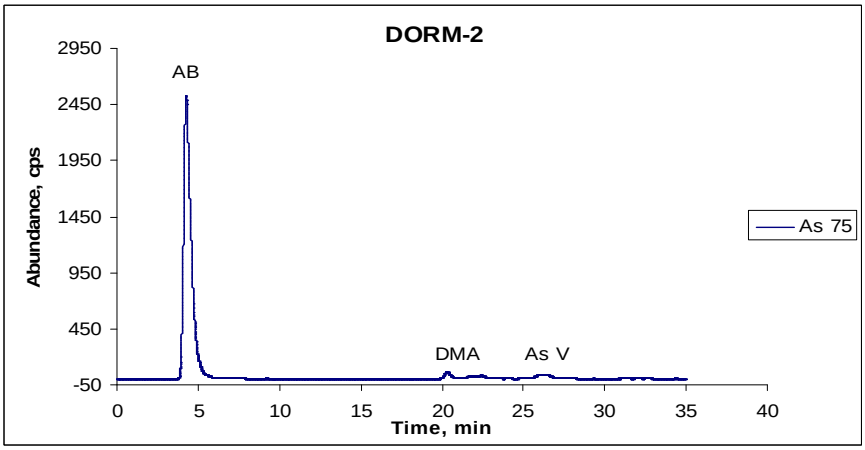


Figure 3-44 Chromatogram of CRM DORM-2 extract obtained by anion-exchange cHPLC coupled to ICP-MS.

Table 3-16 Concentration of As compounds (in µg/g) extracted from DORM-2 as compared to literature data

Extraction mode	Liquid Sonication	Liquid extraction with Ultraturrax [42]	Liquid Sonication [49]	Liquid Sonication [56]	Certified values [178]
AsB	15.6 ± 0.8	16.1 ± 0.7	16.1 ± 0.6	17.64 ± 0.37	16.4 ± 1.1
As(III)	n.d.	0.05 ± 0.01	n.d.	n.d.	
As(V)	0.48 ± 0.08	0.05 ± 0.02	0.05 ± 0.01	0.0063 ± 0.0015	
DMA	0.3 ± 0.1	0.49 ± 0.03	0.29 ± 0.02	0.23 ± 0.03	
MMA	n.d	0.14 ± 0.02	n.d.	0.015 ± 0.004	
TMAs ion	n.i	0.3 ± 0.02	n.i.	0.266 ± 0.033	0.248 ± 0.054
TMAO	n.i	0.3 ± 0.03	n.i.	n.i.	
TMAP	n.i	n.i.	n.i.	0.154 ± 0.059	
Sum of species	16.38 ± 0.4	17.4 ± 0.7	16.44 ± 0.6	18.35 ± 0.37	16. 648
Total As	17.5 ± 1.1	17.4 ± 0.3	n.i.	19.63 ± 0.69	18 ± 1.1
Percentage of total As	90	96	91	102	92

n.i.: not investigated; n.d. not determined

The uncertainty of the measurements by cHPLC coupled to ICP-MS for arsenobetaine and As was also evaluated. Arsenobetaine (15.6 ± 0.8) µg/g was found to contribute the main part of the total As concentration (17.5 ± 1.1) µg/g in Dorm-2.

The validated procedure showed encouraging results and was then applied for quantification of As speciation in tissues from codfish and algae.

3.5.3.2 As speciation analysis of fish extracts

Naturally occurring As compounds have widely differing polarities and toxicities and some biological samples may contain ten or more As species. A specific sample preparation procedure only at ideal conditions allows full extraction of all compounds of interest. In practice, extraction conditions which are optimal for one compound may be quite unsuitable for another. Reliable quantitative analyses accordingly are a challenge to the analytical chemistry because most compounds have a relatively limited stability, low concentrations and ambiguous chromatographic behaviour. Sample treatment for As speciation in marine biota including extraction had already been vastly reported in the literature [26-73]. Therefore attempts to optimise it were considered out of the scope of this work. However, several investigations concerning the sample extraction procedure similar to the one proposed by Shibata and Morita [19, 75] have been undertaken.

3.5.3.2.1 As species concentrations in codfish

Six water-soluble species were considered in the case of As speciation in codfish by SAX cHPLC coupled to ICP-MS. Arsenite, arsenate, MMA, DMA, AsB and AC were separated on the silica-based SAX cHPLC column, Zorbax SAX with 1 *mM* ammonium bicarbonate buffer at pH 6 (see Figure 3-32). Two aliquots per codfish sample were extracted and three replicates were injected on the cHPLC coupled to ICP-MS. A complementary analysis by SCX cHPLC coupled to ICP-MS for the codfish extract was also performed. A typical chromatogram for the As trace compounds is shown in Figure 3-45. The last eluted chromatographic peak remained unidentified at this state of the work due to lack of As cationic reference compounds to match the chromatographic retention time. However, according to literature data [42, 56, 178] and the fact that the compound was well retained on the SCX capillary column it could be considered as a derivative of the TMAs ion.

Matrix effects were assessed by carrying out recovery studies with spiked fish samples. A shift in the retention times of the As species was observed in the codfish samples, but with spike experiments and standard addition an identification of As compounds present in the fish samples could be realised. Under the experimental conditions described, the average of the percentual analytical yield is above 75% as shown in Table 3-19. The recoveries for known amounts of As by this method were very satisfactory (between 75 and 110%). Blank measurements for ultrapure water (Milli Q system) and reagent-solutions showed no measurable peaks. According to these results, external calibration can be used for the determination of the extractable As species from codfish. Calibration was performed with synthetic standard solutions of AsB with increasing concentrations and coefficients of correlation obtained were above 0.99.

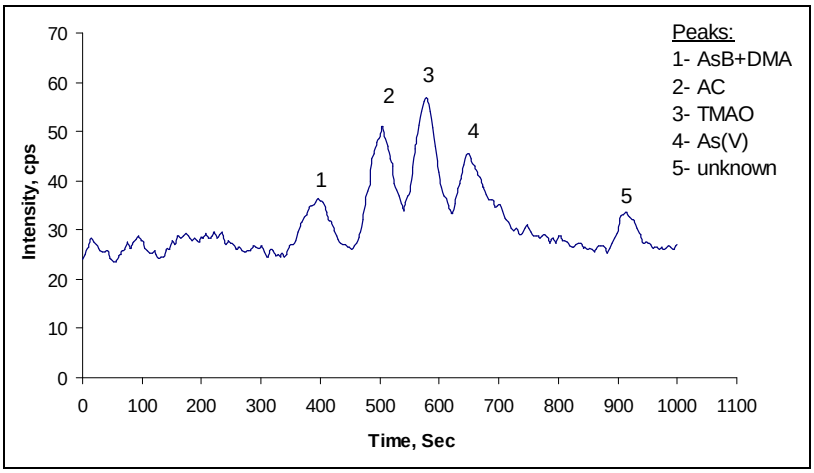


Figure 3-45 Chromatogram for codfish extract obtained by SCX cHPLC coupled to ICP-MS.

The concentrations of the arsenicals in the extract were in the very low µg/g range. Therefore, further investigations of the codfish extract with cHPLC coupled to ESI-MS have not been performed. At these low concentrations, a broad shoulder in the baseline is observed. There are different possible explanations for this observation. The main reason appeared to be the large dead volume of the analytical system developed. However, it could be also related to a baseline dependence on the composition of the mobile phase because the nebulization changes with increasing buffer content. Nevertheless, this would not explain why this effect seems to influence the signals of different As species in a different way. Additionally, only a small effect was observed in the chromatograms of blanks. A second possible cause could be some chemical interferences in the extracted codfish sample.

In Table 3-17 the As compound concentrations found with the developed cHPLC coupled to ICP-MS procedure are listed. The organic matter content ranged from 0.55 to 0.69 µg/g of As. The main part is harmless arsenobetaine (about 60-70% of total As extracted). The total As content determined by ICP-MS was 0.68 and 0.85 µg/g. The toxic As(III) represents only about 5% of the total As content in the samples. No other peaks for As apart from those mentioned in Figure 3-45 were obtained. These results are in a good agreement with those of other studies on As speciation in fish [49, 70, 75, 86, 114].

Table 3-17 As concentration (in µg/g) in codfish as determined by cHPLC coupled to ICP-MS [a] and ICP-MS [b]. Values for MMA, As(III), AC and TMAO are given in italics as their values are uncertain due to the low concentrations and integration problems

Species	Codfish I	Codfish II
AsB	0.38 ± 0.01	0.53 ± 0.07
MMA	*(0.04 ± 0.03)*	*(0.04 ± 0.03)*
DMA	0.10 ± 0.03	0.10 ± 0.08

Species	Codfish I	Codfish II
As(V)	0.04 ± 0.02	0.06 ± 0.03
As(III)	*(0.05 ± 0.02)*	*(0.04 ± 0.03)*
AC	*(0.01 ± 0.03)*	*(0.01 ± 0.02)*
TMAO	*(0.02 ± 0.03)*	*(0.01 ± 0.02)*
Sum of species [a]	**0.64 ± 0.03**	**0.79 ± 0.08**
Total As [b]	**0.68 ± 0.07**	**0.85 ± 0.05**

[a] Mean value ± S.D., n=5
[b] Analysis by ICP-MS

Table 3-18 Spike recoveries and concentrations found for MMA, DMA and As(III) when 10 µg/g are spiked on codfish samples

Species analysis	Spike I Concentration (Recovery % ± RSD)	Spike II Concentration (Recovery % ± RSD)
MMA, µg/g of As	7.78 ± 0.1 (78 ± 3)	7.63 ± 0.38 (76 ± 2)
DMA, µg/g of As	9.89 ± 0.22 (99 ± 2)	10.7 ± 0.25 (107 ± 3)
As(III), µg/g	8.42 ± 0.29 (84 ± 4)	9.07 ± 0.32 (91 ± 3)
Sum of species [a]	22.79 ± 0.39	27.4 ± 0.4
Total As of the extract [b]	28.3 ± 0.13	30.83 ± 0.31
Mean extraction recovery	81 ± 1	89 ± 2

[a] mean value ± S.D., n=3 detected by cHPLC coupled to ICP-MS
[b] analysis by ICP-MS

3.5.3.3 Determination of As in algae

3.5.3.3.1 Concentrations of As species in algae extracts determined by cHPLC coupled to ICP-MS

In the first procedure, the acidic, basic and neutral components in the algal extracts were separated by SAX capillary chromatography and subsequently determined with ICP-MS (see Figure 3-33 and Figure 3-34). Quantification with ICP-MS was performed using an internal standard and synthetic calibration solutions. Additional evidence for the assignment of the As peaks in the chromatograms of extracted species from the algae was obtained by comparing with results of gradient elution on the anion-exchange capillary column coupled on-line to ESI-MS (see Figure 3-36 and Figure 3-37). The concentrations determined for the As species in the algae are listed in Table 3-19.

Table 3-19 As species concentration (µg/g of As in dry mass) in brown *Undaria pinnatifida* and red *Porphyra* seaweed

	Brown alga *U. pinnatifida*		Red seaweed *Porphyra*	
	cHPLC-ICP-MS	cHPLC-ESI-MS/MS	cHPLC-ICP-MS	cHPLC-ESI-MS/MS
Total As content from digestion (by ICP-MS)	**3.23 ± 0.03**		**29.44 ± 0.13**	
Total As content in the extract (by ICP-MS)	**2.42 ± 0.04**		**25.06 ± 0.08**	
Sum of all extracted and chromatogriphied As species	**2.27 ± 0.13**	**2.25 ± 0.12**	**23.7 ± 0.21**	**22.55 ±0.64**
Glycerol-ribose, (cations)	0.14 ± 0.09	0.65 ± 0.05	6.68 ± 0.29	7.65 ± 0.82
AsB [a]		*(0.05 ±0.06)*		*(0.2 ± 0.07)*
DMA	0.19 ± 0.09	0.21 ± 0.03	0.37 ± 0.11	1.7 ± 0.12
Unknown 1	0.11 ± 0.08	-	0.3 ± 0.06	-
As acid (As(V))	n.d.	n.d.	2.05 ± 0.1	n.d.
Unknown 2	0.37 ± 0.03	-	0.22 ± 0.04	-
Phosphate-ribose	0.08 ± 0.04	0.15 ± 0.06	11.96 ±0.23	13.2 ± 0.5
Sulfonate-ribose	0.61 ± 0.26	0.88 ± 0.07	0.34 ± 0.24	n.d.
Sulfate-ribose	0.77 ± 0.18	0.31 ± 0.05	1.78 ± 0.24	n.d.
Chromatographic recovery, % [b]	**94 ± 2.2**	**93 ± 1.21**	**95 ± 2.5**	**90 ± 1.4**
Recovery, % [c]	**75 ± 3.1**	**70 ± 2.1**	**81 ± 2.4**	**77 ± 1.5**

n.d.: not detectable

[a] Value for AsB is given in italics to emphasise the uncertainty due to interferences and integration problems

[b] calculated from the concentration of the total As in the extract (measured by ICP-MS) and the sum of the extractable As compounds (determined by cHPLC-ICP-MS). RSD (%) from three replicates for two samples analysed on different days.

[c] based on the concentration of total As in the dry powder and the total As in the algal extract. RSD (%) from three replicates for two samples analysed on different days

The literature on organoarsenic species in the brown alga *Undaria pinnatifida* (*Wakame* in Japanese) is scarce. Morita and Shibata [19] reported on the isolation and identification of arseno-lipid from the edible brown seaweed *Wakame* and on the presence of dimethylated As ester of palmitic acid and one water-soluble As compound assigned as DMAsphosphate-ribose. The brown alga *U. pinnatifida*, which belongs to the Alariaceae family (order Laminariales) contains all four DMAs-ribosides investigated with higher amounts of DMAs

sulfonate-ribose and DMAs sulfate-ribose than the red *Porphyra* seaweed. On the contrary, the latter contain mainly DMAs phosphate-ribose and DMAs glycerol-ribose. It is observed that sulfonate- and sulfate-ribosides predominate in the brown algae, while the phosphate- and glycerol-ribosides are found mainly in the red algae. This phenomenon has been confirmed by our findings for the brown alga *U. pinnatifida* and red *Porphyra* seaweed. The DMAs phosphate-ribose has been found in various algae including *Ecklonia radiata* [35], which belongs to the same Alariaceae family as *U. pinnatifida*, *Hizikia* from the Sargassaceae family (order Fucales) [36] and *Laminaria japonica* from the Laminariaceae family [42]. Some authors have reported the presence of all four arsenoribosides in extracts from the other brown algae *Laminaria and Fucus* [28-31, 33, 34, 40-42, 105, 104, 109, 145]. These findings agree with our results and indicate that significant differences in the arsenate contents and the As metabolism may occur at the level of the algae family, but not at the level of the algal order, because Alariaceae and Laminariaceae both belong to the order Laminariales.

Peaks with the same retention time as arsenate were detected only for the case of red seaweed. In previous studies the presence of arsenate was reported for brown algae from the Sargassaceae family (*Hizikia fuisiforme*) [42], and the family Laminariaceae family (*Laminaria*) [40, 42], as well as for *Fucus serratus* from the Fucaceae family [33] and red algae but not for species from the red *Porphyra* algae. In both kelp extracts small traces of DMA have been found. The percentage amount of the quantified toxic As species, e.g. As(V) and DMA, with respect to the total extracted As content ranged from only 7.8% for the brown alga *U. pinnatifida* to 9.6% for the red seaweed *Porphyra*. The other 86 and 84% represent many harmless arsenicals found in brown and red algae, respectively. The distribution of the As species in both algae extracts is shown in Figure 3-46. Due to the high toxicity of arsenate and the moderate toxicity of DMA, this is a very important fact to be considered when using marine algae in the food production.

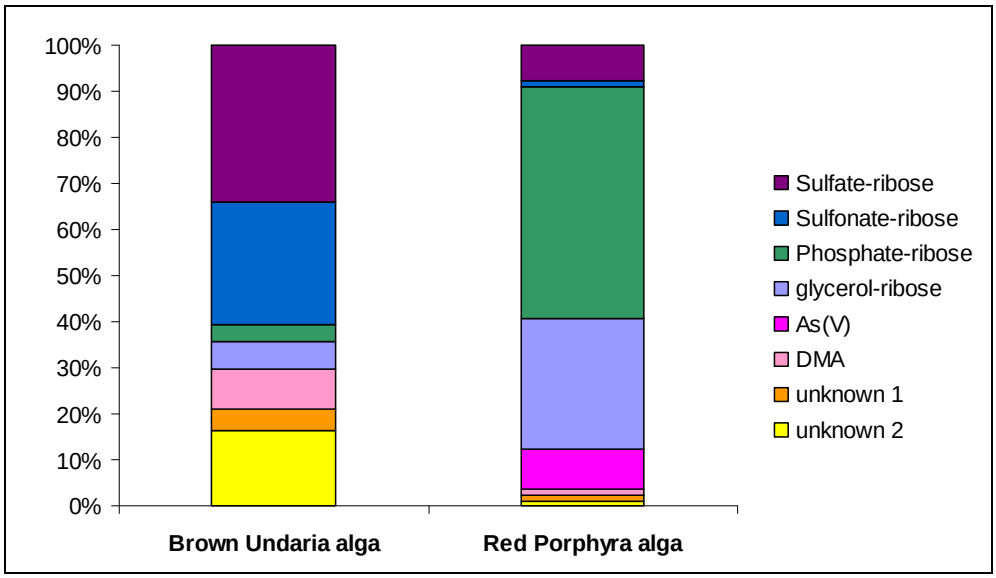


Figure 3-46 Distribution of As species in brown and red algae.

The percentage of the As species found by cHPLC coupled to ICP-MS or cHPLC coupled to ESI-MS with respect to the total As content in the extract, determined by ICP-MS (listed as recovery in Table 3-19) for both algae samples is about 93%. Therefore, the analytical procedures developed and applied in the present study for As speciation in edible marine algae can be accepted to give very satisfactory results.

3.5.3.3.2 Algae spiking experiments

The recoveries and the matrix effects found in the case of real algae were determined by analysing samples of brown and red seaweed powder spiked with 10 and 100 µg/g of As as sulfate-ribose, DMA, As(III) and AsB, respectively. The algae spiked samples and the dogfish extracts were analysed with the same working conditions on the same day by cHPLC coupled to ICP-MS using a Hamilton PRP-X100 capillary column. The results obtained are shown in Table 3-20 and Table 3-21.

The recoveries for known amounts of As species by this procedures were satisfactory. The day-to-day reproducibility for the results has been estimated by analysing three different batches of spiked algae samples for a period of one month under identical working conditions.

Table 3-20 Recoveries and reproducibilities for spiked extracts of brown alga *U. pinnatifida* with As species analysed by cHPLC coupled to ICP-MS

	Spike I- Concentration 10 µg/g; Recovery, %	Reproducibility, RSD %	Spike II- Concentration 100 µg/g; Recovery, %	Reproducibility, RSD %
AsB	9.38 ± 0.6 (94 ± 1.2)	3.1	104.1 ± 1.7 (104 ± 2.4)	3.2
Sulfate-ribose	7.04 ± 1.1 (70 ± 3.1)	7.2	88.6 ± 3.1 (89 ± 2)	4.1
DMA	9.50 ± 0.9 (95 ± 1.2)	5.1	91.4 ± 1.4 (91 ± 2.2)	2.8
As(III)	9.78 ± 0.4 (98 ± 2.2)	4.4	89.2 ± 2.6 (89 ± 3.2)	5.2
Sum of species	38.16 ± 1.2	7.3	376.2 ± 4.1	8.6
Total As of the extract*	49.38 ± 2.1	8.1	407.4 ± 3.6	5.7
Recovery	77 ± 4	6.2	92 ± 4.1	6.7

* Determined by octopole collision cell ICP-MS, n=3

Table 3-21 Recoveries and reproducibilities for spiked extracts of red seaweed *Porphyra* with As species analysed by cHPLC coupled to ICP-MS

	Spike I- 10 µg/g concentration; Recovery, %	Reproducibility, RSD %	Spike II- 100 µg/g concentration; Recovery, %	Reproducibility, RSD %
AsB	9.0 ± 1.3 (90 ± 3.7)	6.1	110.4 ± 3.4 (110 ± 4.5)	7.2
Sulfate-ribose	9.4 ± 3.4 (94 ± 2)	4.2	91.2 ± 2.7 (91 ± 1.7)	5.4
DMA	9.4 ± 1.7 (94 ± 3.8)	5.2	95.4 ± 1.1 (95 ± 1.5)	4.6
As(V)	10.4 ± 1.8 (104 ± 2.5)	3.1	102.7 ± 3.7 (103 ± 3.1)	5.8
Sum of species	60.3 ± 2.3	4.5	438.2 ± 3.8	6.1
Total As of the extract*	69.4 ± 3.2	2.3	473.2 ± 4.3	3.8
Recovery	87 ± 4.5	7.1	93 ± 3.3	6.3

* Determined by octopole collision cell ICP-MS, n=3

3.5.3.3.3 Determination of total As in the algae extracts by octopole reaction / collision cell ICP-MS

Total As in the algae was determined by ICP-MS. In an octopole reaction cell ICP-MS, a set of eight metal poles that bend the ion beam and direct only the sample ions toward the mass spectrometer is used. This process reduces interferences from other ions. In the case of the monoisotopic arsenic, interferences from chloride in the sample matrix may occur as ArCl. The latter, however, is removed by collision induced dissociation.

For each alga type two different portions of sample were digested by microwave-assisted dissolution. The ion signal at *m/z* 75 (As^+) was monitored when using He gas in the collision cell so as to prevent chloride interferences. Calibration with synthetic As standard solutions was used for quantification by ICP-MS. The As contents determined in the brown and red seaweed are shown in Table 3-22.

Table 3-22 Total As content in algae (based on dry mass)

Algae	Total As concentration*, (µg/g) with He collision cell	Total As concentration*, (µg/g) without He collision cell
Undaria pinnatifida	3.23 ± 0.08	2.85 ± 0.9
Porphyra	29.44 ± 0.13	27.8 ± 0.6

* Mean value (n=6)

The comparison between the results of conventional ICP-MS analysis and those of collision cell ICP-MS for As revealed concentration differences of around 12% for *U. pinnatifida* and of 5% for the red *Porphyra* alga. The percentual RSDs for As are lower in the case of reaction cell ICP-MS. However, with both ICP-MS techniques the precision for the As signals was very good (RSDs of 1%).

As a summary, the accurate analyses of the reference material showed that the developed procedure cHPLC coupled to ICP-MS can be applied to real samples although some resolution problems remain. Due to the large number of structural similarities of the alkylarsenicals and the limited separation efficiency of ion liquid chromatography only a sum parameter that includes all organoarsenic species of a particular ion charge can be obtained. This can be a drawback when there is a special interest in a selected organoarsenic species but it can also be an advantage when a quick overview of the overall organic and inorganic As content is required. The results obtained in this study demonstrate that in codfish and algae As is predominantly present in the organic and less toxic forms, as expected. The comparison of the results of both developed procedures with the reported data showed that there are some differences for the brown algae with very low organoarsenic content, but a good agreement for the red algae containing larger concentrations of organic As species. This investigation can provide useful information for the understanding of the metabolic pathways of As in marine organisms and for the assessment of the toxicological risks in the food chain. It is also observed that sulfonate- and sulfate-ribosides predominate in brown algae, while the phosphate- and glycerol-ribosides are found mainly in red algae. This phenomenon has been found to agree with the findings in this work for the brown algae *Undaria pinnatifida* and red *Porphyra* seaweed. It was further found that standard compounds of di- and tri-methylarsinoylriboside derivatives are not readily available and their presence in marine biota may be underestimated. Additionally, the use of cHPLC coupled to

ESI-MS allowed it to determine novel arsenic-containing biocompounds of higher molecular mass (phytochelatines) that normally are not accessible for gas chromatographic analysis.

3.6 The use of LADROP-Laser induced breakdown spectroscopy (LIBS) by generation of mono-dispersed microdroplets in speciation analysis

Questions of chemical speciation in biological samples also ask for design of analytical methods for determination of trace metals. Development of new approaches and instrumental systems is a motor of innovation in the modern analytical chemistry and research. In this sense a novel LADROP-LIBS setup has been built in a co-operation of industrial and academic partners in Germany including GALAB Technologies GmbH in Geesthacht, the Fraunhofer Institute of Laser Technologies in Aachen, OBLF Spectrometry GmbH in Witten and the Department of Operational Systems of the Institute of Coastal Research at GKSS Research Centre in Geesthacht.

In this system, a FIA manifold was used to enter samples into pulse (piezoelectric) droplet generator. Recently, one has focused on the use of very low flow rates for atomic spectroscopy purposes. The results of this research for several elements of time-resolved emission spectra taken with the LADROP-LIBS system are reported. An optimisation of various parameters has been performed in order to obtain the best linearity range and detection limits. The first results of these studies are given bellow [148]. However, As speciation analysis of marine biota with this system was not yet possible, due to the large dead volume of the measuring chamber and the higher detection limit for arsenic.

3.6.1 Laser-induced plasma of microdroplets with LADROP system

3.6.1.1 Optimisation of the measurement parameters

3.6.1.1.1 Dependence of the emission line intensity of the elements on the laser pulse energy

In order to investigate the influence of the laser pulse energy and wavelength on the analytical sensitivity a series of experiments have been performed. A Nd-YAG Laser operated at 266 nm has been used to perform LIBS at microdroplets and the results were compared with those of a laser at 1064 nm. For both wavelengths, calibration curves with different sodium solutions with concentrations between 0 and 10 µg/mL were determined and the limits of detection were compared. With 60 mJ pulses in the cases of the 1064 nm laser wavelength a limit of detection of 0.75 µg/mL was obtained [179]. The same measurements were made with a laser with 25 mJ pulses and a wavelength of 266 nm and a limit of detection of 2 µg/mL obtained. With lasers using a wavelength of 1064 nm and the same pulse energy (25 mJ), the intensities for the Na 589.00 and 589.59 nm lines are approximately twice as high as with a laser operating at 266 nm. Since the laser could not deliver more than 25 mJ per pulse at the UV wavelength the fundamental wavelength has

been applied in all subsequent experiments. The signals show a strong correlation with the laser pulse energy. In Figure 3-47 the rise of the intensities of the Na lines for droplets containing 500 µg/mL of Na with the laser pulse energy is shown. For more than 60 mJ/pulse a saturation of the line intensity is observed. Since the use of very high pulse energies can lead to instabilities in the operation of the piezoelectric droplet generator, most measurements were performed at energies between 60 and 100 mJ/pulse.

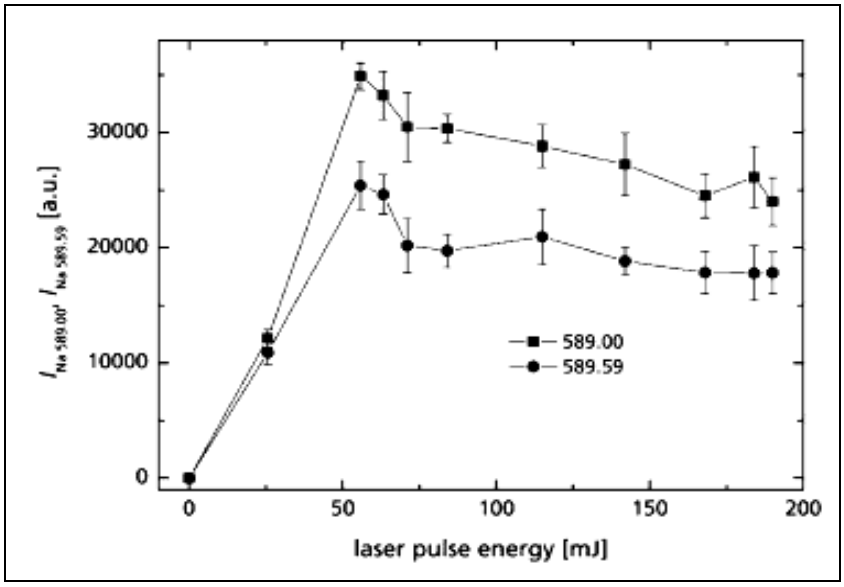


Figure 3-47 Influence of the laser pulse energy on the intensities of Na lines. Signals for 50 laser pulses were accumulated.

3.6.1.2 Formation of the plasma dynamics in LIBS

By hitting the single micro droplets the laser pulse brings the sample into the plasma state. In the case of a water sample, the direction of plasma propagation is not limited to the direction perpendicular to the target. As a result, hereof the plasma looks like a round lobe.

The plasma dynamics were studied with a solution containing 500 µg/mL of Ca which produces an intensive element-specific radiation. The latter was monitored with a high-speed electro-optical camera allowing exposure times down to 200 ps.

In Figure 3-48 a series of images recorded at a 25 ns shutter time after 200 ns, 0.5 µs and 1 µs and are shown. The white spot indicates the initial position of the droplet. The laser beam (50 mJ pulses and with a pulse width of 10 ns) enter from the right side and the focus is a few millimetres behind the droplet.

The first breakdown can be detected at the rear side of the droplet because the beam is focused by the droplet itself. Therefore, the highest laser intensity is obtained at the rear side of the droplet. The intensity distribution for the plasma radiation is asymmetric and it is most intensive at the side facing the laser. The plasma grows within 500 ns to a diameter of approximately 2.5 mm. No fragmentation of the droplets was observed on the images. The

expansion of the luminous plasma front is most interesting [148]. Normally, the plasma front is held back by a shock wave. An analysis of shock waves produced during a LIBS experiment is described by Noll *et al*. [150].

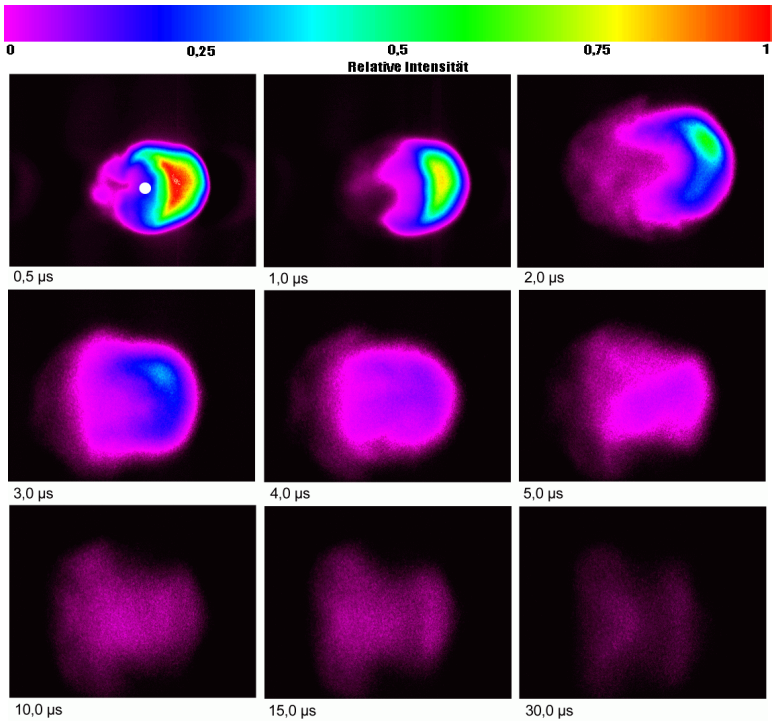


Figure 3-48 Plasma dynamics in the case of a solution of 500 µg/mL Ca. Line: 423 nm; shutter time: 80 ns; laser pulse: 50 mJ, 10 ns; focus: 5 mm behind the droplet; MCP: 800 V [148].

When the plasma is generated in the case of a water sample, water vapour reduces the energy density and the excitation temperature. Some portions of the laser energy are consumed for evaporation of the water. Considering the heat capacity of liquid water (4.18 J/g *K) and the enthalpy of vaporisation (2258 J/g at -100 °C) as well as the heat delivered it takes approximately 0.7 mJ to vaporise 270 pL of liquid water. As the laser pulse has an energy of 50 mJ complete vaporisation can be obtained. From the fast expansion and the homogeneity of the plasma we see no hints for any fragmentation into smaller sub-droplets or for the fact that parts of the droplet would not be transferred to the plasma state [148].

3.6.1.3 Linearity

A highly linear correlation of the signal with the concentration of Ca could be observed, when the average integral intensity for 20 measurements, with each measurement consisting of 100 droplets, is plotted as a function of the concentration (see Figure 3-49). Integrated peak intensities were used and calculated by fitting a Gaussian function to the profiles of the Ca 393.37 nm line and subtracting the background intensity.

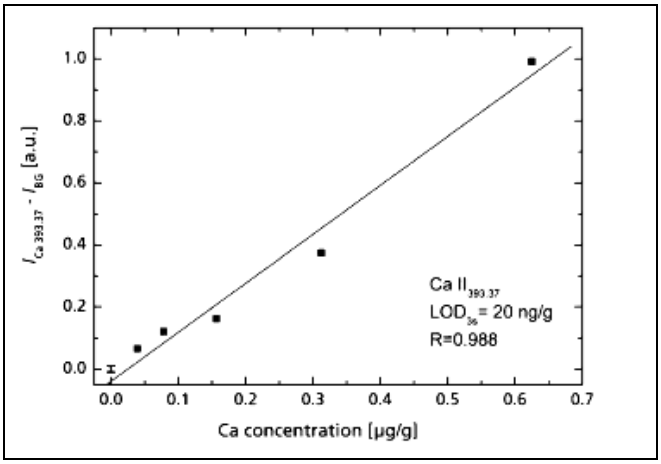


Figure 3-49 Calibration curve for Ca with coefficient of correlation: R^2=0.988. Experimental parameters: delay time: 1.5 µs; integration time: 50 µs; laser pulse energy: 140 mJ and pulse width: 10 ns.

3.6.1.4 Limit of detection

The limit of detection for Ca was determined according to the IUPAC definition and is 20 µg/L. Comparing with data for detection limits for many elements obtained with ICP-MS in the literature, which are at the low ng/L levels, the detection limit for Ca achieved with LADROP-LIBS in the case of an Echelle spectrometer is poor. The absolute power of detection of the laser induced plasma spectroscopy approach, however, is very high and comparable to ICP-MS. This only applies for elements having strong emission intensities. Ca belongs to the group of elements with intensive atomic emission lines leading to very low absolute limit of detection. When comparing the LODs of this approach with those of other similar LIBS methods [151] and commercial state-of-the-art ICP-OES [181], as shown in Table 3-23, it appeared that the relative LODs of LADROP-LIBS setup are high. In the case of Ca, however, the LOD of 20 µg/L in 100 droplets of 280 pL corresponds to an absolute amount of 560 fg of Ca [148], which can be considered satisfactory for many applications.

Table 3-23 Detection limits of elements in LIBS and ICP-OES

Elements	Analytical line, nm	LADROP-LIBS, µg/L [148]	Isolated Droplet Generator–LIBS, mg/L [151]	ICP-OES, Optima 5300 with axial viewing, µg/L [181]
Na	589.00		2.2	0.5
Li	670.78		0.3	0.3
Ca	393.37	20	0.4	0.05
Mg	279.55	*20*	1.9	0.04
Cu	324.75			0.4
As	234.98	100		2

3.6.2 FIA with cHPLC at low flow rates combined to LADROP-LIBS

FIA of Mg solution has been realised with the aid of a cHPLC pump. The droplet generation had to be adjusted to the micro volume flow of the HPLC pump, otherwise the droplet generator may either run dry or the droplet formation may fail, because too much liquid is delivered to the glass capillary. Typical cHPLC flow rates are in the range of 5 to 20 µL/min. In order to match the volume flows of the cHPLC pump to the one required by the droplet generator, an active closed loop was used to control the droplet frequency. With a liquid flow of 13 µL/min a droplet frequency of 800 Hz (droplet volume 270 pL) could be realised. For sample introduction a Rheodyne six-port switching valve was integrated in the flow system in front of the droplet generator. Herewith the injection of small amounts of sample (< 20 µL) was possible without interrupting the droplet stream. When monitoring the signal obtained as a function of time a peak equivalent to those in chromatographic separations was obtained. The dead volumes of the droplet generator and of the connecting tubes lead to peak broadening. In Figure 3-50 the results of a FIA measurement for 20 µL of sample containing 18 µg/mL of Mg is shown. The total amount of Mg used to obtain the signal shown is 4.2 ng because only one out of 85 droplets was analysed due to a mismatching of the droplet generation and the laser pulse frequency.

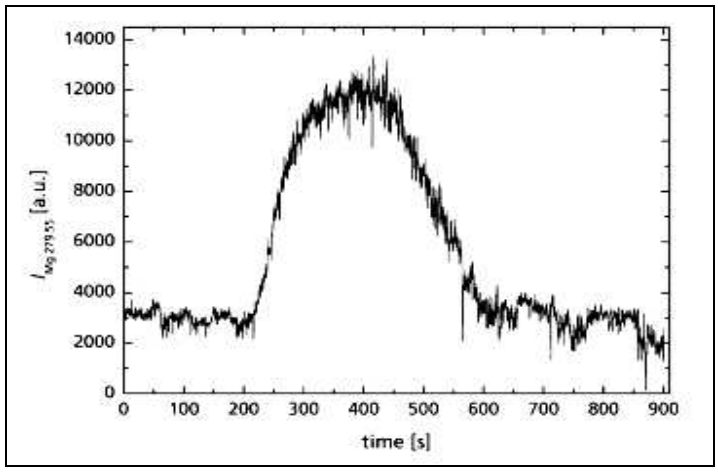


Figure 3-50 Signal obtained by LADROP-LIBS using FIA for a solution containing 18 mg/L of Mg. The sample was injected with a Rheodyne 6-port valve into the continuous water flow of a cHPLC pump. Droplet frequency: 800 Hz; laser frequency: 10 Hz. The analysed amount of sample is 4.2 ng Mg. The data are smoothed with a 10 point moving average function [148].

Nevertheless, LADROP-LIBS with FIA show that low flow rate chromatographic separation techniques here could be successfully used for speciation analysis. The results demonstrate the potential of a piezoelectric droplet generation for the development of a new sample introduction system for element detection in micro scale liquid separation methods, such as cHPLC and/or capillary electrophoresis, working with sub µL sample volumes.

3.6.3 Matrix effects in LADROP-LIBS using FIA

In the case of the system described the matrix effects for Mg and Ca caused by Na chloride have been studied. They are illustrated by analyses of the *SLEW-3* estuarine water reference sample for the three elements using FIA LADROP-LIBS. As it is shown in Figure 3-51 matrix effects occur at the higher concentrations, where the values obtained were lower than expected.

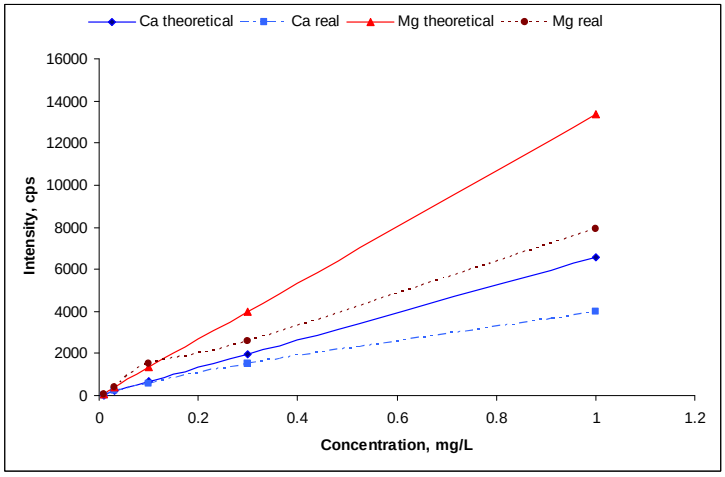


Figure 3-51 Matrix effect in LADROP-LIBS for Ca and Mg as caused by NaCl.

3.6.4 cHPLC-LADROP-LIBS

A cHPLC method for the determination of Ca and Mg has been performed using the in-house packed SCX cHPLC column with Hamilton PRP-X200 PSDVB sulfonate resin. An Agilent 1100 Series cHPLC system with a DAD at 220 nm using indirect UV detection for Ca and Mg was used with a mobile phase of $CuSO_4$ at flow rate of 10 µL/min. In Figure 3-52 is shown the chromatogram for Mg and Ca, where well-separated negative peaks were obtained.

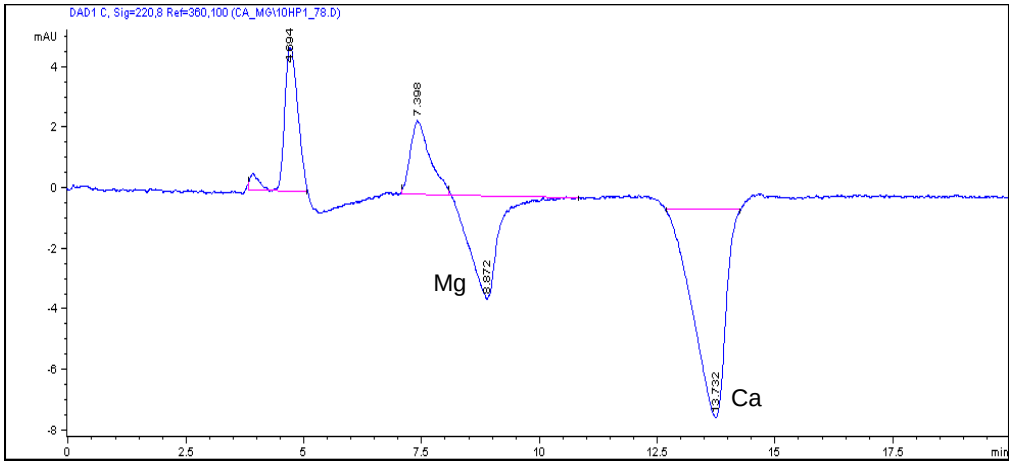


Figure 3-52 Chromatogram obtained by cHPLC coupled to DAD for a solution containing 250 mg/L of Ca and Mg.

The separation of Ca and Mg using the SCX Hamilton PRP-X200 cHPLC column has also been combined and applied to LADROP-LIBS. With this complicated on-line coupled system chromatographically separated laser-induced emission signals could be monitored with a Paschen–Runge spectrometer for Ca and Mg, as shown in Figure 3-53.

The intensity of the Ca emission is higher than for Mg. For both elements very wide chromatographic peaks with peak width ~10 min have been monitored. This is mainly due to the large dead volume of the PDG (5 µL) and the long connection needed between the cHPLC column and the PDG.

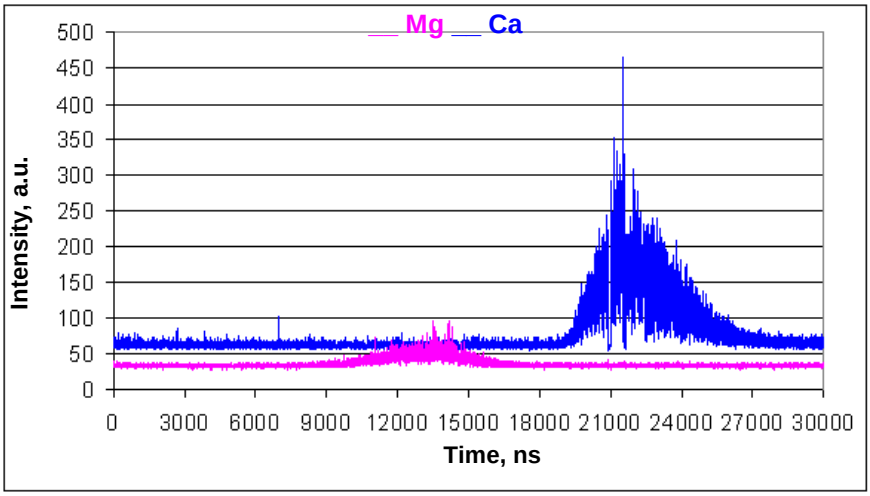


Figure 3-53 Chromatogram obtained by cHPLC coupled to LADROP-LIBS for a solution containing 250 mg/L of $CaSO_4$ (73.6 mg Ca /L) and 750 mg/L of $MgSO_4$ (151.5 mg Mg/L).

Nevertheless, this is a good demonstration that determinations of elements are possible when using cHPLC coupled to LADROP-LIBS. However, the LADROP-LIBS needs further improvements in order to obtain better performance.

3.6.5 Water analysis

Due to the important role of water in our lives, water samples are more frequently analysed than any other type of sample. Mineral water and seawater were chosen here as real samples. They are very convenient samples to analyse by LADROP-LIBS as they require a minimum of sample preparation prior to analysis and contain the elements of interest mentioned above. On the other hand, interferences from major matrix elements, like Ca, Mg, Na etc., are minimal or can easily be corrected. However, the high concentration levels of dissolved solids in seawater and some wastewater can cause problems as a result of blockage in the piezoelectric nozzle. This means that dilution and/or separation from the matrix might be necessary.

3.6.5.1 Mineral water

Na, Ca and Mg have been determined in degassed mineral water by LADROP-LIBS using FIA. In Figure 3-54 the LIBS spectra for Na and Mg are presented. The Ca spectra profile is not shown in Figure 3-54, because of the higher intensities ~ 770 a.u. The profiles for the elements are overlapping very well, which illustrates that the spectrometer exit slits were at the right positions.

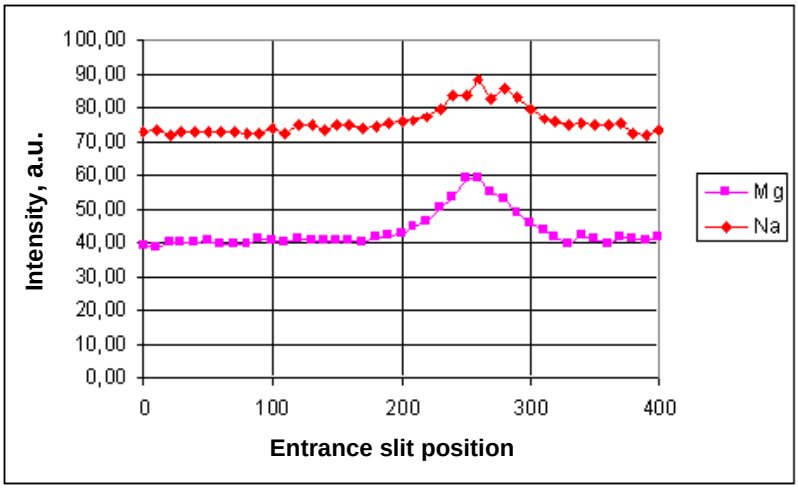


Figure 3-54 Entrance slit scans for Na and Mg in LADROP-LIBS using FIA.

The results of LADROP-LIBS using FIA for mineral water were encouraging to further investigate the use of cHPLC coupled on-line to LADROP-LIBS for the determination of Ca and Mg in seawater.

3.6.5.2 Seawater

Seawater reference materials with different salinity *NASS-5* and *SLEW-3* were chosen as real samples so as to investigate the matrix effect in the case of LADROP-LIBS. A separation of Cu, Ca and Mg has been carried out on the capillary SCX Hamilton PRP-X200 column in the presence of different NaCl concentrations.

In Figure 3-55 a chromatogram of reagent blank of ultra pure water is shown. It presents the background noise for the elements of interest in case of cHPLC coupled to LADROP-LIBS.

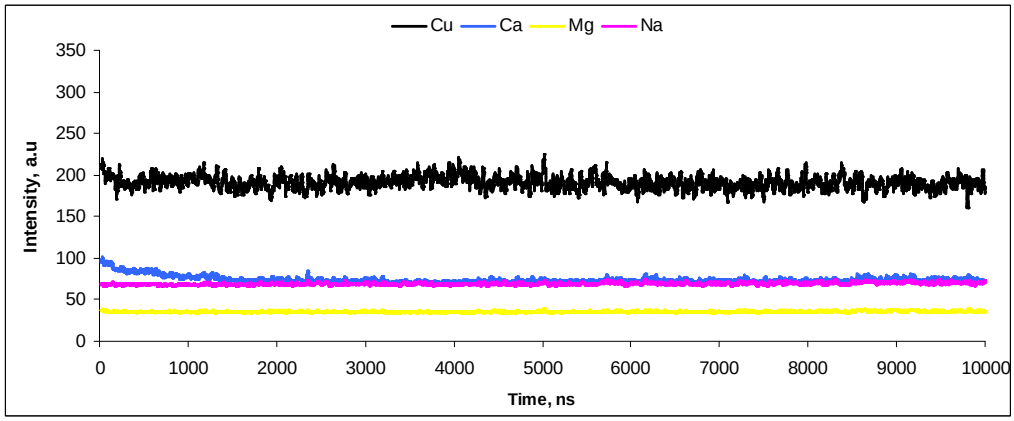


Figure 3-55 A chromatogram showing the reagent blank in the case of SCX cHPLC coupled to LADROP-LIBS.

The ten fold diluted *NASS-5* ocean water was analysed using SCX cHPLC coupled to LADROP-LIBS. The resulting traces of Cu, Ca and Mg were detected by LIBS after chromatographic separation in the presence of 1.5 mol NaCl. In Figure 3-56 the chromatogram obtained for the four elements is shown.

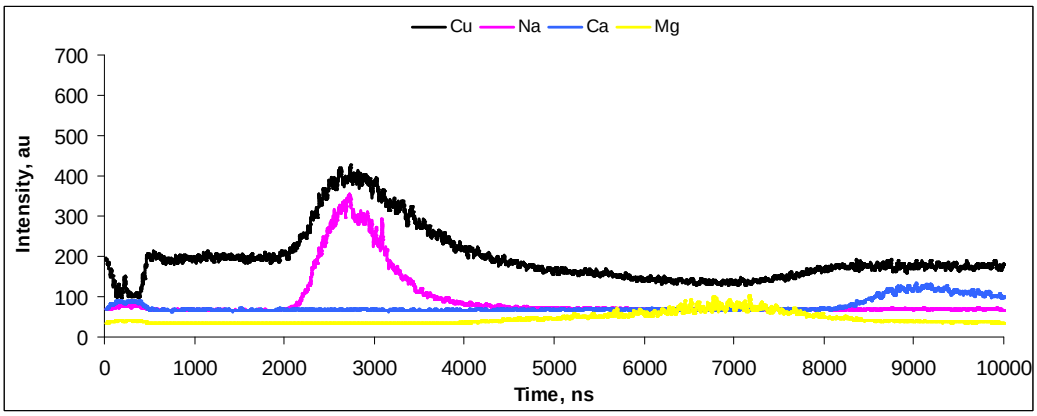


Figure 3-56 A chromatogram of *NASS-5* ocean water in the case of SCX cHPLC coupled to LADROP-LIBS.

The content of Ca and Mg in *NASS-5* was in the low µg/mL range and therefore the chromatographic peaks are of a low intensity. The peaks for all four elements are very wide because of the large dead volume of the system.

In Figure 3-57 a chromatogram obtained for *SLEW-3* estuarine water is shown. In this sample the Ca concentration is higher than for Mg, which resulted in a low intensity signal for Mg that is near the background noise level. On the other hand, the salinity is almost twice as low as in the *NASS-5* seawater and as a result hereof the peak for Na is low. This can also influence the shape of the peak for Cu, which in this case is narrower than in the chromatogram for *NASS-5* but appears at a similar elution time.

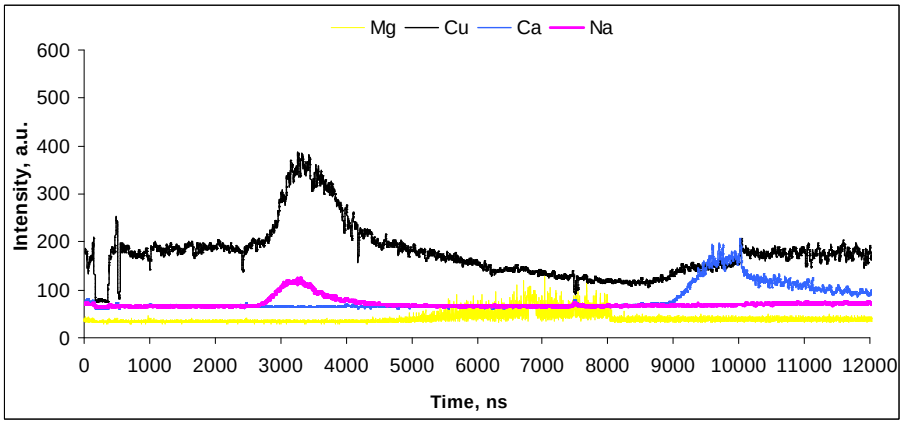


Figure 3-57 Chromatogram for *SLEW-3* estuarine water obtained with SCX cHPLC coupled to LADROP-LIBS.

Nevertheless, all peaks in the chromatograms remain wide, which can be explained with the large dead volume of the system. Due to these chromatographic results and the very high LOD for As with this system, As speciation at this state-of-the-art could not be performed with the LADROP-LIBS detection system.

3.6.6 Conclusions

The combination of PDG and LADROP-LIBS is promising as a new sample introduction system for analysis of small volume liquid samples. It offers a 100% sampling efficiency, since each droplet is hit by the laser beam and produces plasma. The similarity of the time-resolved LIBS emission spectra to those obtained with aerosols provide credence to the hypothesis that the characteristics of the laser plasma are not significantly perturbed by the presence of such small droplets.

The on-line coupling of cHPLC with LADROP-LIBS allow this system to be used with smaller sample volumes (< 1 µL) than in the conventional HPLC or direct liquid sampling nebulizers for atomic spectroscopy. Although the relative LODs are much higher at this point than those obtained by ICP-OES [181], the absolute LODs of the technique are very low (0.5 pg Ca), since low µg/mL concentration levels of elements could be detected in pL droplets. That means that pg and even fg quantities of elements can be detected.

4 EXPERIMENTAL PART

4.1 Materials and apparatus

For the experiments carried out in this work the following laboratory equipment, instruments and materials were employed.

4.1.1 Laboratory equipment and apparatus

In Table 4-1 an overview of the applied laboratory equipment and instruments is given. Common laboratory glassware was also used.

Table 4-1 Laboratory Equipments and instruments

Name	Manufacture
Sartorius Analytical balance, ±0.01g	Sartorius Analytic, Göttingen, Germany
Sartorius Research balance, ±0.0000g	Sartorius Analytic, Göttingen, Germany
Büchi Rotavapor R-200 with vertical water condenser	Büchi, Switzerland
Centrifuge Heraeus	Thermo Electron Corporation, Germany
Clean Bench and Room, Type 100 and 1000	Cryo-Technik, Hamburg, Germany
pH Metter	Mettler-Toledo GmbH Analytical, Schwerzenbach, Switzerland
ICP-MS Sciex Elan 5000	Perkin Elmer SCIEX, Canada
ICP-MS Agilent 7500s	Agilent Technologies, Yokogawa Analytical Systems Inc, Japan
ICP-MS Agilent 7500c	Agilent Technologies, Yokogawa Analytical Systems Inc, Japan
cHPLC Agilent 1100 Series with DAD and 500 nL flow cell	Agilent Technologies, Waldbronn, Germany
ChemStation software for LC 3D (Rev. A. 09.03) 1990-2002	Agilent Technologies, Waldbronn, Germany
Microconcentric nebulizer for CE-ICP-MS CEI-100	Cetac, Omaha, USA
Triple Quadruple Mass Spectrometer, API 4000	Applied Biosystems, USA
Model 11 syringe pump	Harvard Apparatus, Southnatick, (MA), USA
Microwave assisted system for extraction and digestion, MARS 5, Ethos 1600	CEM GmbH, Kamp-Lintfort, Germany MLS, Germany
Vent vessel, 80 mL	CEM GmbH, Kamp-Lintfort, Germany
Ultrasonic bath	Bandelin Sonorex, Bandelin *Electronics*, Germany
Ultraturrax	IKA, Germany
Vortex Reax Top Heidolph	Schwabach, Germany
Millipore Elix 3/Milli-Q Element	Millipore, Milford, USA

4.1.2 Materials

The main materials used in this work are listed in Table 4-2.

Table 4-2 Materials

Name	Manufacture
Argon gas, 5.0 (99.999%)	Air Liquide, Germany
Nitrogen gas, 5.0 (99.999%)	Air Liquide, Germany
Helium gas, 5.0 (99.999%)	Air Liquide, Germany
HPLC Vacuum Filter System and acetate membrane filters, 0.45 and 0.22 µm	Millipore, USA
PEEK coated silica tubing with various I.D., 1/32". O.D. and zero dead volume fittings	Chromatografie Service GmbH, Langerwehe, Germany
Various membrane filters for syringe, 0.22 and 045 µm	VWR International GmbH, Darmstadt, Germany
Variable volume automatic pipettes Eppendorf and ep T.I.P.S.	Eppendorf AG, Hamburg, Germany
PE, PP Tubes 15 mL and 50 mL	SARSTEDT Aktiengesellschaft and Co., Nümbrecht, Germany
Silanised screw top vial w/spot, 2 mL, i.e. Part N. 5183-2071	Agilent Technologies, USA
Blue and Red Screw Caps with septum, Part N. 5182-0717	Agilent Technologies, USA
100 µL insert, polypropylene, Part Number: 5182-0549	Agilent Technologies, USA
PTFE-Magnetic stirrer 8x3 and 3x3 mm Pr. Nr. 2417350070	Schlee Karl GmbH, Germany
pH-Buffer Solution pH 4.01, Order N. 51302040, pH 7.00; Order N. 51302047; pH 9.21, Order N. 51302070	Mettler-Toledo GmbH Analytical, Schwerzenbach, Switzerland
Zorbax® Chromatographic Packing SAX, 5 µm	Agilent Technologies, USA
Ion-Pac As 7 Latex SAX Resin	Dionex Corporation, USA
Hamilton PRP-X100 and PRP-X200 resins	Hamilton Corporation, USA

4.2 Chemical reagents and standards

Chemicals, solvents and deionised water used to prepare standard solutions and eluents were of the highest purity available.

4.2.1 Solvents

The deionised water for the preparation of eluents and standard solutions was Type I Reagent Grade Water with a specific resistance of 17.8 MΩ-cm or greater, obtained from Millipore Elix 3/Milli-Q Element water purification system (Millipore, Milford, USA). Organic solvents used were:

- Methanol LiCHROSOLV® gradient grade, for HPLC, ≥99.9% (GC) Hazard code: F, T; R phase :11-23/24/25-39/2324/25; S phase: 7-16-36/37-45 — Merck KGaA, Darmstadt, Germany
- Acetonitrile LiCHROSOLV® gradient grade Hazard code F, Xn; R phase : 11-20/21/22-36; S phase: 16-36/37 — Merck KGaA, Darmstadt, Germany

4.2.2 Chemicals and their hazard details

The main laboratory chemicals and reagents used in this work are given in Table 4-3.

Table 4-3 Chemicals and their hazard details

Name	Hazard codes	R (risk) statements	S (safety) statements	Producer
Nitric Acid, Assay ≥ 99.5%	C	8-35	23.2-26-36/37/39-45	Merck KGaA, Darmstadt, Germany
1 M Hydrochloric acid	C	34-37	26-36/37/39-45	Fluka Chemie GmbH, Buchs, Switzerland
Ortho-phosphoric acid, p.a. 85%	C	34	26-36/37/39-45	Fluka Chemie GmbH, Buchs, Switzerland
Acetic acid (glacial) 100%, p.a.	C	10-35	23-26-45	Merck KGaA, Darmstadt, Germany
Hydrogen peroxide, Assay ≥ 30%	X	34	28.1-36/39-45	Aldrich – Chemie GmbH andCo KG, Steinheim, Germany
Ammonia solution, 30% Suprapure	C, N	10-23-34-50	9-16-26-36/37/39-45-61	Merck KGaA , Darmstadt, Germany
Sodium hydroxide	C	35	26-37/39-45	Sigma- Aldrich, Steinheim, Germany
Malonic acid, Assay ≥ 99.0%	Xn	20/22-41	26-36/39	Merck KGaA, Darmstadt, Germany
L-Tartaric acid, 99.5% purriss analytical grade	Xi	36/37/38	26-36	Aldrich – Chemie GmbH and Co KG, Steinheim, Germany
Ammonium hydrogen carbonate, Assay ≥99.5% (T)	Xn	22-36/37/38	26-36/37/39	Fluka Chemie GmbH, Buchs, Switzerland
Ammonium nitrate	O, Xi	8-36/37/38	17-26-36	Merck KGaA, Darmstadt, Germany
Ammonium dihydrogenphosphate	-	36/37	26-36	Merck KGaA, Darmstadt, Germany
Ammonium hydrogenphosphate	Xi	36/37/38	26-36	Merck KGaA, Darmstadt, Germany
Tetrabuthylammonium hydroxide 30 hydrate	C	34	26-36/37/39-45	Merck KGaA, Darmstadt, Germany
1-Hexanesulfonic acid sodium salt monohydrate, Assay ≥99.0% (T)	-	-	-	Fluka Chemie GmbH, Buchs, Switzerland
Cupper(II) sulfate-pentahydrate, p. A., 99.999%	Xn, N	22-36/38-50/53	22-60/61	Sigma-Aldrich, Steinheim, Germany
Sodium acetate, ≥99.0% anhydrous	-	-	22-24/25	Sigma-Aldrich, Steinheim, Germany
Potassium bromide, ≥99.0%	Xi	36/37/38	26-36	Sigma-Aldrich, Steinheim, Germany
Calcium sulfate dehydrate, ≥99.0%	-	-	22-24/25	Sigma- Aldrich, Steinheim, Germany

Name	Hazard codes	R (risk) statements	S (safety) statements	Producer
Mg sulfate x $7H_2O$, ≥99.5%	Xn	22	-	Fluka Chemie GmbH, Buchs, Switzerland
Pyridine	Xn, F	11–20/21/22	26-28, 1	Merck KGaA, Darmstadt, Germany
1,2-Benzenedisulfonic acid disodium salt	-	-	-	Aldrich-Chemie GmbHand Co KG, Steinheim, Germany
CertiPUR® Rhodium Standard solution, 1000 mg/L	Xi	36/38	26	Merck KGaA , Darmstadt, Germany
CertiPUR® Germanium Standard solution, 1000 mg/L	-	-	-	Merck KGaA , Darmstadt, Germany
CertiPUR® Yttrium Standard solution, 1000 mg/L	Xi	36/38	26	Merck KGaA , Darmstadt, Germany
CertiPUR® Multi-element Standard solution VI	Xi	36/38	26	Merck KGaA , Darmstadt, Germany
Tuning Standard solution, 1 ppb Li, Y, Ce, TL, Co in 2% HNO_3	-	-	-	Agilent Technologies, USA
Tuning Standard solution, 10 µg/L Ba, Mg, Pb in 2% HNO_3	-	-	-	Merck KGaA , Darmstadt, Germany
As (V) ICP Standard solution, 1000 mg/L in 4% HNO_3	T	45-25-36/38-52/53	53-26-45	Merck KGaA , Darmstadt, Germany
As trioxide, As_2O_3, 99.995% puriss	T+, N	45-28-34-50/53	53-45-60-61	Sigma- Aldrich-Chemie GmbH and Co KG, Steinheim, Germany
Monomethylarsonic acid disodium salt	-	-	-	Argus Chemicals, Italy
Dimethylarsinic acid sodium salt trihydrate, >98% puriss; (cocodylic acid)	T, N	23/25-50/53	20/21-28-45-60-61	Sigma-Aldrich, Germany
Arsenobetaine, purum p.a >95% (NMR)	T, N	23/25-50/53	20/21-28-45-60-61	Sigma-Aldrich, Germany
Arsenocholine	-	-	-	Argus Chemicals, Italy
Trimethyl arsine oxide	-	-	-	Argus Chemicals, Italy

The 65% suprapure nitric acid was further purified by sub-boiling distillation in a quartz apparatus and used for the preparation of all dilutions.

4.2.3 As species and non-commercial natural arsenosugar standards

As species standard stock solutions with 1000 µg As/mL were prepared from the following reagents: As trioxide As_2O_3; dimethylarsinic acid Na salt trihydrate; monomethylarsenic acid disodium salt, arsenocholine and trimethyl arsine oxide. Arsenate (As(V)) with 1000 ± 2 µg/mL and arsenobetaine (CRM BCR 626, 1031±6 mg/kg as AsB, Sigma-Aldrich, Germany) were bought as solutions. Arsenite stock solution was prepared in

mixture of 1 *M* HCl and 0.8% NaOH. The dimethylarsinoylriboside derivatives used as standard compounds with a concentration of approx. 10 mg/L of As are natural products isolated from brown alga *Fucus serratus* and were kindly donated by U. Kohlmeyer (GALAB Laboratories GmbH Geesthacht, Germany) [42]. *Fucus* extracts were stored at -18 °C. Suitable aliquots of these water solutions were then used to prepare working and calibration standard solutions containing the four arsenosugars at 10, 50, 100, 250, 500 and 800 µg/L.

For the preparation and dilution of the stock solutions and eluents ultrapure Milli Q water was used. Stock and working solutions of all As compounds were stored at 4°C in the dark. The stability of these standard solutions in terms of the total As content and purity was checked by ICP-MS and HPLC coupled to ICP-MS, respectively. Each stock solution was further diluted to a solution with a concentration of 10 mg As/L, out of which multi-compound working and calibration standard solutions were prepared on the day of analysis.

All standard solutions were prepared under clean room conditions (class 1000) on a clean bench (class 100) to avoid contamination and particles which can block the micro capillaries used.

4.2.4 Preparation of elemental standard solutions

Single element working standard solutions or solutions containing Li, Ba, Mg, Pb, Rh, Ge, Re, As, Y, Tl, Ce and Cl with concentrations of 1 mg/L or 10 µg/L in 2% HNO_3 were prepared, respectively. They have been further diluted to the required concentrations with ultrapure water prior to analysis. All As calibration solutions were freshly prepared and stored at 4°C in the dark.

All standard solution and solutions of standard reference samples were prepared under clean room conditions (class 1000) on a clean bench (class 100) to avoid contamination and particles which can block the micro scale capillaries used.

4.3 Analytical methods and procedures

As speciation analysis was performed using cHPLC coupled to ICP-MS or ESI-MS detection. Marine biota samples were investigated for the presence of As species using liquid/liquid extraction followed by cHPLC coupled online to ICP-MS or ESI-MS. cHPLC columns of different types were used in this study. The chromatographic packing materials differ in particle composition and size as well as in functional groups, which all played a major role in the success of the separation of closely related As compounds.

4.3.1 Silica-based cHPLC columns

4.3.1.1 Reversed phase cHPLC column

The main characteristics of the commercial RP cHPLC column used are summarised in Table 4-4.

Table 4-4 Characteristics of reversed phase capillary column

Column name	XTerra ®
Dimensions	
I.D.	0.32 mm
Length	150 mm
Packing material type	
Particle size	3.5 µm
Pore size / Surface area	126 Å / 182 m^2/g
Functionality	Hybrid organic (polymer) and inorganic (C_8- Silica) particles
pH Range	1 - 12
Manufacturer	Waters Corporation

4.3.1.2 SAX cHPLC column

The separation of As species was performed on a silica-based SAX capillary column, Zorbax SAX 250 x 0.5 mm with 5 µm particles. Agilent Technologies in Waldbronn, Germany, kindly provided this column, and its properties are given in Table 4-5.

Table 4-5 Characteristics of silica-based SAX cHPLC column

Column name	Zorbax SAX
Dimensions	
I.D.	0.5 mm
Length	250 mm
Packing material type	
Particle size	5 µm
Pore size / Surface area	70 Å / 300 m^2/g
Functionality	Organosilane - Quaternary amine
pH Range	2 - 6.5
Manufacturer	Agilent Chromatographic Centre, Germany

4.3.2 In-house prepared polymeric cHPLC columns

4.3.2.1 Hamilton PRP-X 100 SAX capillary column

Hamilton PRP-X100 is a PSDVB polymeric SAX packing for the separation of inorganic and organic anions in concentration ranges from 10 to 500 mg/L. This resin is a macroreticular styrene-divinyl benzene copolymer (Figure 4-1), which is extremely chemically stable and can be used with a variety of organic and inorganic eluents. The Hamilton PRP-X100 material is a low capacity SAX phase. The details of the Hamilton PRP-X100 characteristics are listed in Table 4-6.

Table 4-6 Hamilton PRP-X100 SAX packing specification

Packing name	Hamilton PRP-X100
Support material	PSDVB* with Trimethyl ammonium exchanger
Exchange capacity	0.19 meq/gm
Pore size	100 Å
Temperature limits	pH Dependent**
Mobile phase limits	pH 1-13. 0-100% aqueous and organic
Buffer strength	0.0-0.5N
Max. pressure	350 bar

* PSDVB is poly (styrene-divinylbenzene); ** pH: 1-7.9, Temperature: 5-60℃; pH: 8-13, Temperature: 5-30℃ .

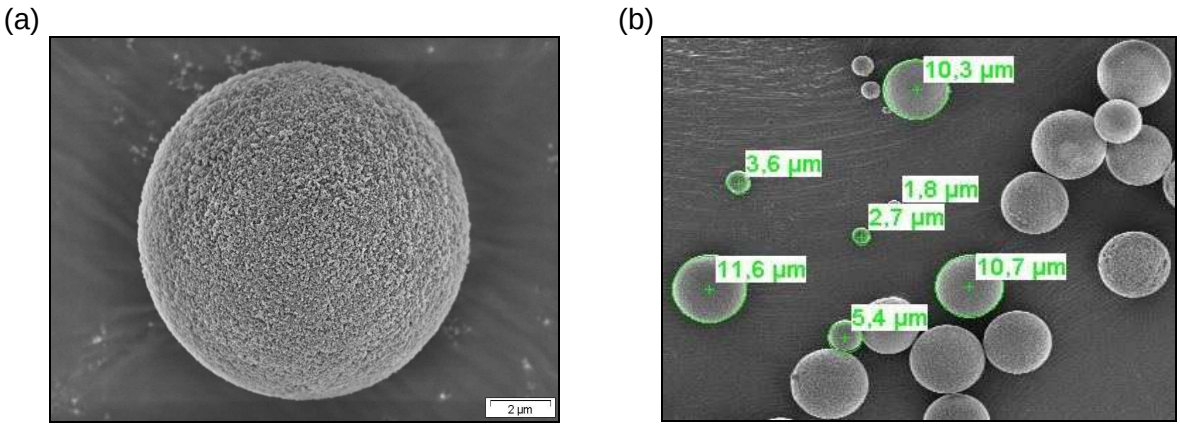


Figure 4-1 Single spherical particle (a) and particle size distribution of Hamilton PRP-X100 resin (b).

4.3.2.1.1 Preparation of Hamilton PRP-X100 cHPLC column

Hereby a PEEK column hardware with internal style 10-32 threaded ferrule-style end-fittings was used. All components are non-metallic, except for the column frits which consisted of 2 µm stainless steel screens. The Hamilton PRP-X100 capillary column has a length of 250 mm and an I.D of 0.5 mm. Several capillary columns were packed and their performance tested at Pfizer Analytical Centre, Ghent University, Belgium. Guard columns with 20 mm length and I.D. 0.5 mm were also packed into PEEK capillaries. The capillary column with the best performance was used for As speciation. The packing procedure, equipment and test conditions are described in Table 4-7.

Table 4-7 Preparation of Hamilton PRP-X100 capillary column

Resin slurry preparation	
Slurry solution:	MeOH : H_2O (1/1, v/v).
Preparation of slurry solution:	Mix 500 mL Methanol with 500 mL deionised (DI) water. Degas for 10 min.
Preparation of resin slurry:	0.05 g resin slurry + 300 µL packing solution.
Sonication time:	10 minutes
Packing equipment:	Haskel pump, restrictor valve with coil, water jacketed tube packer 27 mL, 2 mm adapter, compressed air (shop grade), balance, 100x 2.1 mm, 3 µm ODS Hypersil pre-column filter, open-end wrenches (11/16”, 5/8”, 9/16”, 5/16”, ¼”), electronic pressure transducer.
Packer preparation	
Packing solution:	MeOH:H_2O (1/1, v/v).

Preparation of packing solution:	Mix 500 mL methanol with 500 mL DI water. Degas for 10 min.
Packer preparation:	Disconnect tube adapter, flush tube, replace O-ring, reconnect adapter, pump with DI water. Connect to the packing solution and pump, packing solution for 2 minutes.
Packer temperature:	20º C
Packing	
Pre-column filter:	100 x 2.1 mm, 3 µm ODS Hypersil
Blank column installation:	Install a partially assembled blank PEEK column.
Resin packing technique:	Pour resin slurry into reservoir tube. Fill up with packing solution. Cap the tube and pack. Increase packer regulator at 50 every 5 seconds.
Packing pressure and time:	250 bar for 20 minutes
Post packing:	Leave for 60 min and then open above the pre-column filter to release the backpressure
Washing	
Washing equipment:	Gilson LC pump 307
Washing solution:	DI water
Washing flow rate and time:	20 µL/min
Washing temperature:	30º C
Equilibration	
Equilibration equipment:	Agilent 1100 Series LC pump
Equilibration stock solution:	MEOH/ H_2O (85/15, v/v)
Preparation of equilibration solution:	Degas for 5 minutes each solvent.
Equilibration flow rate and time:	15 µL/min for 2 hours.
Equipment requirements for testing	
Testing equipment:	1) Isocratic cHPLC pump, 2) Mobile phase degasser, 3) Thermostatted column compartment 4) Micro well-plate thermostatted auto sampler 5) DAD Absorbance detector
Data collection:	DAD: 254 nm, 8; Ref: 360, 100 nm
Test standard and elution order	
Standard stock solution:	Stock Solutions: Acetone 1000 mg/L Acenaphtene 1000 mg/L in Dichloromethane
Standard injection solution:	Dilute the following amount of standard stock solutions to 250 µL with MeOH:
Preparation of standard injection solution:	Acetone 2.5 µl Acenaphthene 5.0 µL **Final Concentration:** Acetone 0.25 mg/mL Acenaphthene 0.5 mg/mL

4.3.2.2 Dionex IonPac AS7 capillary column

The IonPac® AS7 material is designed for the determination of a variety of anions including polyvalent anions in matrices with a high ionic strength. The selectivity of the IonPac AS7 has been designed to accommodate a wide variety of applications, including polyphosphates, polyphosphonates, hexavalent chromium, As speciation and iodide. It is compatible with a pH 0-14 and with eluents containing organic solvents up to a concentration of 5%. IonPac AS7 resin is designed to be used with systems using nitric acid as eluent. The main specifications of the material are given in Table 4-8 [161].

Table 4-8 Ion Pac AS7 Packing Specification

Packing name	IonPac AS7
Particle diameter	10.0 µm
Substrate X-linking (DVB)	2%
Column capacity	20 µeq/g
Functional group	Alkyl quaternary ammonium
Latex characteristics	Latex Cross-Linking: 5 % Latex Diameter: 530 nm
Hydro-phobicity	Medium-High
Mobile phase compatibility	pH 0–14

4.3.2.2.1 Preparation of a Dionex IonPac AS7 capillary column

PEEK column hardware with internal style 10-32 threaded ferrule end-fittings was used. All components are non-metallic, except for the column frit, which consists of 2 µm stainless steel screens. IonPac AS7 suspension in NaOH and packing procedure were kindly obtained from Dionex, USA. The IonPac AS7 capillary column has a length of 250 mm and an I.D. of 0.5 mm. Packing and washing equipment used were the same as for Hamilton capillary columns. The packing procedure, equipment and test conditions are described in the Table 4-9.

Table 4-9 Preparation of the Dionex IonPac AS7 capillary column

Resin slurry preparation	
Slurry solution:	500 *mM* NaOH.
Preparation of slurry solution:	Dilute 40 g 50% NaOH to 1 L with DI water.
Preparation of resin slurry:	16g resin slurry + 11g packing solution.
Sonication time:	10 minutes
Equipment requirements for packing	
Packing equipment:	Haskel pump, restrictor valve with coil, water jacketed tube packer 27 mL, 2mm adapter, compressed air (shop grade), balance, open-end wrenches (11/16”, 5/8”, 9/16”, 5/16”, ¼”), electronic pressure transducer.
Packer preparation	
Packing solution:	500 *mM* NaOH.
Preparation of packing solution:	Dilute 40 g 50% NaOH to 1 L with DI water.
Packer preparation:	Disconnect tube adapter, brush tube, replace O-ring, reconnect adapter, pump with DI water to pH neutral. Connect to the packing solution and pump, packing solution for 2 minutes.
Packer temperature:	20º C
Packing	
Blank column installation:	Install a partially assembled blank column.
Resin packing technique:	Pour resin slurry into tube. Fill up with packing solution. Cap the tube and pack. Increase packer regulator at 50 every 5 seconds.
Packing pressure and time:	200 bar for 15 minutes
Washing	
Washing equipment	Gilson LC pump 307
Washing solution:	DI water
Washing flow rate and time	10 µL/min
Washing temperature:	30º C

Equilibration	
Equilibration equipment	Agilent 1100 Series LC pump
Equilibration of stock solution:	1000 *mM* HNO3
Preparation of equilibration stock solution:	Dilute 63 mL of HNO3 Concentrate to 1 L with DI water. Titrate it to ensure the molarity of nitric acid is 1000 *mM*
Equilibration solution:	5 *mM* HNO3
Preparation of equilibration solution:	Dilute 5 mL of 1000 *mM* HNO_3 to 1 L with DI water. Stir and degas for 5 minutes.
Equilibration flow rate and time:	15 µL/min for 5 hours.
Equipment requirements for testing	
Testing equipment	1. Isocratic/gradient cHPLC pump 2. Mobile phase degasser 3. Micro well-plate thermostatic autosampler 4. ICP-MS
Data collection	1. DAD: 254 nm, 8; Ref: 360, 100 nm 2. ICP-MS, Graphic mode PeakHop, RF Power 1300W, Replicate time: 150 ms, Dwell time 15 ms, Sweeps/Reading: 10, Readings/Replicate: 1, Points/Spectral Peak: 5, Resolution: Normal, Polarity: +, Element/ Mass: ^{75}As and ^{35}Cl
Test standard and elution order	
Standard stock solution:	Stock Solutions: Arsenite 1000 mg/L MMA 1000 mg/L KCl 1000 mg/L
Standard injection solution:	Dilute the following amount of 1000 mg/L Standard stock solutions to 100 µL with DI water: Arsenite 10.0 µL, 100 mg/L MMA 10.0 µL, 100 mg/L KCl 1.0 µL, 10 mg/L

4.3.2.3 Hamilton PRP-X200 SCX cHPLC column

Hamilton PRP-X200 is a strong cation-exchange PSDVB polymeric resin for the separation of inorganic and organic cations in concentrations from 10 to 50 mg/L. This resin is chemically very stable and therefore can be used with a variety of organic and inorganic eluents. The PRP-X200 material is a low capacity material and was purchased from Hamilton AG Banduz, GR, Switzerland. The details of the Hamilton PRP-X200 characteristics are listed in Table 4-10.

Table 4-10 Technical specifications of the Hamilton PRP-X200 resin

Packing name	PRP- X200
Support material	PSDVB with sulfonate exchanger
Exchange capacity	35 µeq/g
Pore size	100 Å
Temperature limits	5-60 ℃
Mobile phase limits	pH 1-13; 0-100% aqueous, organic modifier
Buffer strength	0.0-0.5 N
Max. pressure	350 bar

4.3.2.3.1 Preparation of the Hamilton PRP-X200 capillary column

PEEK column hardware with internal style 10-32 threaded ferrule end-fittings was used. All components are non-metallic, except for 2 µm stainless steel screens utilised as column frits. The capillary Hamilton PRP- X200 column has a length of 250 mm and an I.D. of 0.5 mm, with 10 µm particles. Guard columns with the same material were packed into 20 mm length and 0.5 mm I.D. PEEK capillaries. The slurry packing method together with the equipment and conditions were the same as described in section 4.3.2.1.1. The packed capillary columns were tested in combination with ICP-MS. A connection of the column to the ICP-MS nebulizer was made with 20 cm 1/32" O.D and 50 µm I.D. PEEK coated silica tubing.

The mobile phase for As speciation consisted of 0.5 mmol/L pyridine at a pH 2.7 adjusted with acetic acid. The mobile phase was filtered through 0.22 mm filter prior to analysis.

4.3.3 ICP-MS systems

A Perkin-Elmer Sciex Elan 5000 ICP-MS was coupled with cHPLC and consists of the components shown in Figure 4-2.

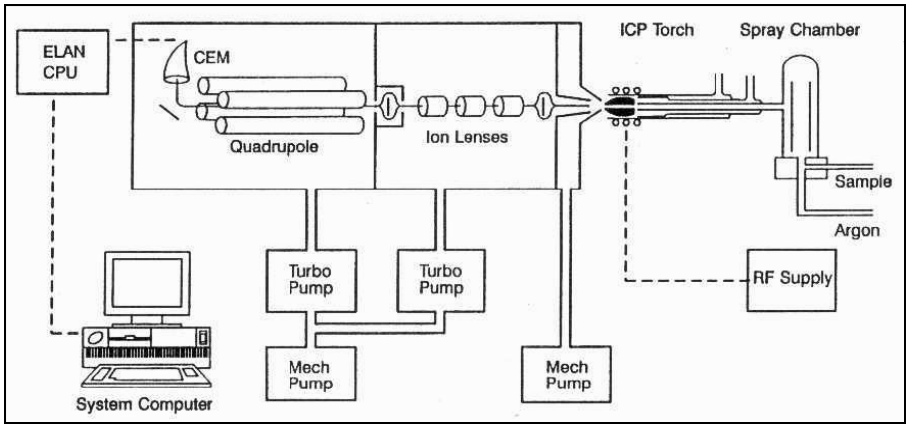


Figure 4-2 Quadrupole Sciex Elan 5000 ICP-MS, according to the Perkin Elmer User Manual (1992) [133]

Alternatively, the Agilent 7500s and octopole reaction cell Agilent 7500c ICP-MS systems (Agilent Technologies, Tokyo, Japan) with a Shield Torch technology were also used for element-specific detection of As speciation or for the determination of the total arsenic.

4.3.3.1 Working conditions for ICP-MS using the Sciex Elan 5000

For tuning of the ICP-MS, the signals for Mg, Rh, Pb, Ba, Ge and As in 2% nitric acid solutions with 10 µg/L were monitored at *m/z* 24, 103, 208, 138, 72 and 75 and the intensities were optimised. The signals for the oxide and doubly charged ions were evaluated by measuring the mass ratios $^{154}BaO^{+}/^{138}Ba^{+}$ and $^{69}Ba^{2+}/^{138}Ba^{+}$ and bringing them to a minimum.

Mass resolution and calibration were optimised by monitoring *m/z* ^{24}Mg, ^{103}Rh and ^{208}Pb. The interference of the As and chloride was checked by monitoring *m/z* ^{35}Cl, ^{37}Cl, ^{77}Se (^{40}Ar^{37}Cl) and ^{75}As (^{40}Ar^{35}Cl). The signals for blanks and rinsing solutions containing 2% of nitric acid were also measured so that any trace impurity signals could be subtracted from the analytical signals.

In Table 4-11 the optimised working parameters for the detection of the As species with the Sciex Elan 5000 ICP-MS are listed.

Table 4-11 Optimised conditions for the determination of As by ICP-MS using the Sciex Elan 5000

ICP – MS parameters	Condition 1 for tuning	Condition 2 for on-line coupling to cHPLC
Plasma RF power, W	1300	1300
Nebulizer flow, L/min	1.040	0.995
Plasma flow, L/min	15.00	
Sampling and Skimmer cones	Nickel, 0.75 mm orifice diameter	
Replicate time	300	150
Dwell time, ms	60	15
Sweeps/reading	5	10
Readings/Replicate	1	1
Points/spectral peak	5	5
Number of replicates	300	1080
Resolution, amu	Normal, 0.7	
Isotope Masses	24, 35, 72, 75, 77, 103, 138, 154, 187, 208	

4.3.3.2 Working conditions for ICP-MS using the Agilent 7500s

The Agilent 7500s ICP-MS (Agilent Technologies, Tokyo, Japan) with Shield Torch technology was also used for element-specific detection. The ions are extracted by a dual extraction omega lens system for increased sensitivity and with an off-axis design which provides for overall low background. The standard daily optimisation procedure suggested by the manufacturer was applied to determine the optimum nebulizer gas flow. For tuning of the ICP-MS the signals for Li, Y, Ce and Tl as well as for As, Ge or Y in 2% nitric acid solutions with 10 µg/L concentrations were used. 10 µg/L of Ge or Y were used as internal standards and added to the cHPLC mobile phases. Instrumental parameters, such as the nebulizer and auxiliary argon flow were optimized in order to match the sample microflow and the cHPLC flow.

Details on the optimised ICP-MS settings are given in Table 4-12.

Table 4-12 Optimised conditions for the Agilent 7500s ICP-MS

Parameter	Value
RF power	1300 W
Carrier Gas flow rate	0.94 L/min
Sampling depth	6 - 7 mm
Cones	Platinum
Extraction lens	-8 V
Quadrupole bias	-6 V
Measured isotopes dwell time	$^{75}As^{+}$ (0.1s), $^{72}Ge^{+}$(0.1s), $^{35}Cl^{+}$ (0.1s) and $^{89}Y^{+}$ (0.1 s), $^{140}Ce^{+}$ (0.1 s), $^{7}Li^{+}$ (0.1 s), $^{204}Tl^{+}$ (0.1 s)

4.3.4 Hyphenated techniques for arsenic speciation

The cHPLC Agilent 1100 series has been used during all investigations as cHPLC coupled to ICP-MS or cHPLC coupled to ESI-MS.

4.3.4.1 cHPLC coupled to ICP-MS using the Perkin-Elmer Sciex Elan 5000

All modules were arranged to achieve the lowest possible dead volume between the cHPLC column and the ICP-MS. Details on the chromatographic conditions are given together with the chromatograms shown in Chapter 3 and the ICP-MS parameters are listed in Table 4-11.

To minimise blank contributions from leached pump materials, the steel tubing, filters, unions and connectors of the cHPLC pump were replaced by PTFE hardware. All connections were made of 1/32 in. O.D., 50 µm I.D. polyetheretherketone (PEEK) coated silica capillaries and PTFE zero dead volume fittings.

4.3.4.1.1 Sample introduction system

cHPLC requires the use of small sample volumes (< 0.5 µL) and of low mobile phase flow rates (2-20 µL/min), which minimise waste and solvent cost. The conventional sample introduction system consists of a cross-flow pneumatic quartz nebulizer for aqueous samples with an uptake rate of 2 mL/min and Scott-type spray chamber. A quartz body and an Elan DRC torch adaptor were used to connect the spray chamber with the quartz injector with 1.8 mm I.D. A copper RF coil was used. The Gylson Minipuls 3 channel peristaltic pump used had PVC tubing and two bridges. The tubes have a length of 152 mm and two different internal diameters:

- Orange / green - 0.38 mm I.D.
- Yellow / orange - 0.51 mm I.D.

A make up liquid containing 2% of nitric acid (HNO_3) was used to adjust the micro sample flow to the conventional sample introduction system. Two approaches were tested to connect the cHPLC column to the nebulizer. The first one involves a microflow mixing TEE union to introduce the cHPLC flow and a make-up liquid to the nebulizer. Instrumental parameters, such as the nebulizer and auxiliary argon flow and the make up liquid flow were selected in order to match the sample micro flow and the cHPLC flow.

In another approach an a in-house modified microflow concentric nebulizer CEI-100 from CETAC Technologies (Omaha, NE, USA) [154] and in-house made small volume glass spray chamber were used for sample introduction, as shown in Figure 4-3. The inner capillary of the commercial MCN was connected with the 20 µm I.D. silica capillary. Details of the interface can be found in the literature [10, 152, 154].

(a) (b)

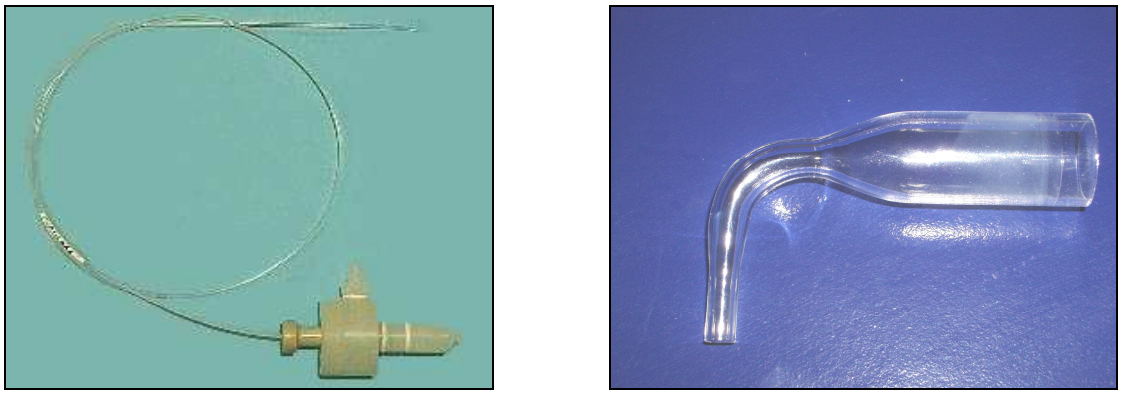


Figure 4-3 CEI-100 modified microflow concentric nebulizer (a) and small volume micro spray chamber (b).

4.3.4.1.2 Working conditions

The cHPLC flow was coupled on-line through PEEK silica tubes to the ICP interface (Figure 4-3). When a parallel on-line coupling with DAD and ICP-MS detectors was employed, a PEEK silica tubing of 70 cm x 75 µm I.D. was used, whereas direct coupling of the cHPLC column to the ICP-MS made use of a PEEK coated silica capillary of 50 or 28 cm x 50 µm I.D. The connection between the end-fitting of the column and the interface was kept as short as possible to reduce peak broadening and loss of ions during transport.

The optimised working parameters for As speciation resulting from a series of chromatographic separations and ICP-MS instrumental tuning and adjustments, are summarised in Table 4-13 and Table 4-14.

Table 4-13 Optimised parameters for the speciation of As by cHPLC coupled to ICP-MS using silica-based columns

Chromatographic conditions **Agilent 1100 cHPLC**	**ICP-MS element detection** **Perkin Elmer SCIEX Elan 5000**
1. Ion-pair chromatography (IPC) *Column*: XTerra® RP_8 (150 x 0.32 mm, 3.5 µm), Waters GmbH, Germany *Mobile phase:* 10 *mM* Tetrabuthyl ammonium hydroxide (TBAH) at pH 10, adjusted with 2% nitric acid *Flow rate*: 4 µL/min *Injected sample volume*: 0.01µL **2. Anion-exchange chromatography (AEC)** *Column:* Zorbax SAX, (250x0.5 mm, 5 µm) Agilent Technologies GmbH, Waldbronn, Germany *Mobile phase:* 1 *mM* ammonium bicarbonate buffer at pH=6 *Flow rate:* 10 µL/min *Injected sample volume*: 0.03µL	**1. ICP parameters** *RF Power*: 1030 W *Nebulizer:* MCN CEI-100 *Spray chamber*: Microspray, quartz glass *Outer gas*: 15 L/min *Auxiliary gas*: 1.74 L/min *Nebulizer gas*: 1.060 L/min **2. Quadrupole Mass Spectrometer parameters** Graphic mode analysis *Dwell time*: 15 ms *Sweeps/reading*: 10 *Scanning mode*: Peak hopping in positive mode *Monitored isotopes*: ^{75}As, ^{77}Se (ArCl), ^{35}Cl and ^{103}Rh

- *Preparation of mobile phases for the determination of As compounds by cHPLC coupled to ICP-MS using the Perkin-Elmer Sciex Elan 5000*

Mobile phases with tetrabutyl ammonium hydroxide (TBAH) 30-Hydrate at different concentration (5, 10 and 15 *mM*) in deionised water were used for ion-pair cHPLC coupled to ICP-MS. The pH of the TBAH eluents was measured with a pH meter and the pH was adjusted to the desired value of pH 10 with 2% HNO_3 or 30% ammonium solution.

Solutions of 2 *mM* $NH_4H_2PO_4$, at pH 5.5 and 1 *mM* NH_4HCO_3, at pH 6 were prepared first with ultrapure water, and then the pH was adjusted with diluted nitric acid or ortho-phosphoric acid. They were further employed for a separation of the As species on Zorbax SAX capillary columns coupled online to ICP-MS with the Elan 5000.

All mobile phases were filtered (0.22 µm Nylon filter) before introducing them into the cHPLC.

A separation of the As species was also carried out on the polymeric Hamilton PRP-X100 capillary column. A gradient elution was used for the separation of the As species and the mobile phases consisted of:

- 0.1 *mM* ammonium nitrate and 20 *mM* ammonium nitrate buffer at pH 5.8

- 0.2 *mM* ammonium nitrate and 20 *mM* ammonium nitrate at pH 8.5 (adjusted with ammonia), and 10 µg/L of Re was added as internal standard.

The optimised flow rate of 12 µL/min was used and with ICP-MS the signals at *m/z* 75 for As and *m/z* 187 for Re were measured. 0.01 µL up to 0.08 µL methylated As compounds (10 or 20 mg/L, each) and arsenoribosides (0.5 mg/L, each) were injected on the column and the retention factors for all peaks were measured.

The optimised working conditions for the separation of As species by gradient elution on the Hamilton PRP-X100 column and of ICP-MS using the Sciex Elan 5000 are given in the Table 4-14.

Table 4-14 Optimised conditions for the determination of As species by gradient elution on the Hamilton PRP-X100 cHPLC column and ICP-MS using the Sciex Elan 5000

Chromatographic conditions:	Gradient elution at pH 5.8	Gradient elution at pH 8.5
Mobile phase:	**A**- 0.1 *mM* NH_4NO_3, pH 5.8 **B**- 20 *mM* NH_4NO_3, pH 5.8 10 µg/L Re were added	**A**- 0.2 *mM* NH_4NO_3, pH 8.5 **B**- 20 *mM* NH_4NO_3, pH 8.5 10 µg/L Re were added
Flow rate:	10 µL/min	10 µL/min
Injection:	0.3 µL	0.3 µL
Sample:	20 mg/L methylated arsenicals and 0.5 mg/L arsenosugars	10 mg/L methylated arsenicals and 0.5 mg/L arsenosugars
Interface	Modified CETAC MCN-100 and small volume glass spray chamber	
Detection:	Sciex Elan 5000 ICP-MS with MCN and small spray chamber	

pH gradient elution was applied for the determination of the As compounds on an IonPac AS7 capillary column coupled to the Sciex Elan 5000 ICP-MS. An optimum separation of the As species was achieved when the eluent first consisted of 0.5 *mM* nitric acid and 0.05 *mM* BDSA, at pH 3.6 and the second eluent of 20 *mM* nitric acid and 0.05 *mM* BDSA at pH 1.8. The pH of the mobile phases was adjusted with 2% HNO_3. The mobile phases again were filtered on a 0.22 µm Nylon filter before introduction to the cHPLC.

4.3.4.2 cHPLC coupled to ICP-MS using the Agilent 7500s ICP-MS

For connecting the outlet of the capillary column to the nebulizer of the Agilent 7500s ICP-MS a 20 cm long, 50 µm I.D. and 1/32". O.D. PEEK coated silica tubing and zero dead volume fittings were used. All modules were arranged so as to achieve the lowest possible dead volume between the cHPLC column and the ICP-MS interface.

4.3.4.2.1 Micro nebulizer interface for cHPLC coupled to ICP-MS for the determination of As compounds with the Agilent 7500s ICP-MS

A modified MCN CEI-100 nebulizer from CETAC (CETAC Technologies, Omaha, NE, USA) and an in-house made quartz injector tube extension with an internal volume of 4 mL

and I.D. 16 mm (Quartzschmelze Geesthacht, Geesthacht, Germany) were used and a direct connection of the nebulizer to the ICP torch realised. This set-up was used as an interface for the on-line coupling of cHPLC and the ICP-MS systems. (see Figure 4-4). Details about this interface can be found elsewhere [182].

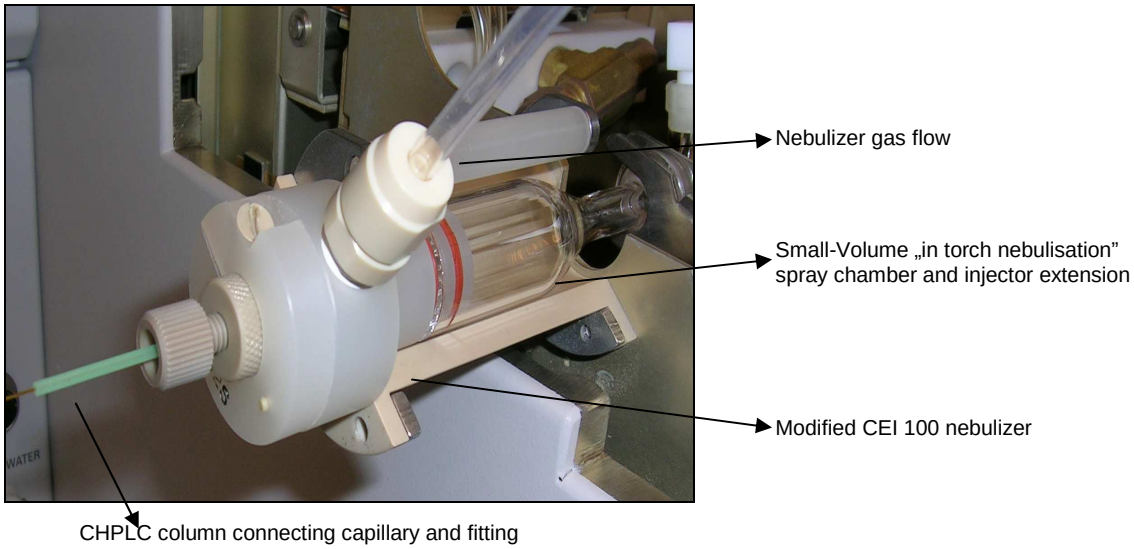


Figure 4-4 Modified CEI-100 nebulizer and spray chamber for direct coupling of cHPLC to the Agilent 7500s ICP-MS.

4.3.4.2.2 Optimised working conditions for the mobile phases for cHPLC coupled to ICP-MS for the determination of As compounds with the Agilent 7500s ICP-MS.

Solutions of 1 *mM* and 40 *mM* ammonium nitrate at pH 8.5 (adjusted with ammonia) were used for the separation of alkylarsenicals and arsenoribosides on the Hamilton PRP-X100 capillary column.

The Dionex IonPac AS7 capillary column was also coupled online to the Agilent 7500s ICP-MS for determination of the As species and the mobile phases in this experiment were the same as given in 4.3.4.1.6.

4.3.5 Experimental details of ESI-MS

4.3.5.1 Mass Spectrometer

A triple quadrupole API 4000 mass spectrometer from Applied Biosystems / MDS SCIEX (Toronto, Canada) utilising a V-type ion spray interface was used for all ESI-MS and ESI-MS/MS analyses. A schematic diagram of the ESI probe and the triple quadrupole mass analyzer is shown in

Figure 7-5 (see appendix). The potential between the orifice and the grounded skimmer is referred to as the declustering potential (DP), and is equivalent to the orifice potential. Although a number of source and interface parameters were adjusted to select a particular

mode of operation, the declustering potential was the most critical one, as it is compound dependent.

There were two forms of molecular operation modes used, namely a detection of fully intact molecular species and ESI-MS/MS. In the fully intact molecular mode, the instrument was operated as a single quadrupole device where only the first quadrupole (Q1) is employed as a mass analyser. In this mode of operation RF potentials were only applied to the second and third quadrupoles Q2 and Q3, respectively, causing them to transport ions, but not to separate them according to mass. For ESI-MS/MS analyses, the Q1 was used to allow only transmission of the molecules of interest, namely the precursor ions, and the Q2 quadrupole was filled with nitrogen gas to create a high-pressure collision cell. Molecules of selected masses then collided with the collision gas with an energy given by the DC potential difference between Q1 and Q2 multiplied by the charge of the precursor ion exiting Q1**.** CID of the precursor ion then occurs, resulting in characteristic fragment or product ions. The third quadrupole (Q3) was operated as a mass analyser for monitoring the specific fragment ions, namely multiple reaction monitoring (MRM) or to scan the full mass range.

The mass spectrometer was operated with a resolution in the full scan mode at which for peaks corresponding to ions with a mass difference of 1 Da the valley between the peaks was less than 10% of the more intense peak. Full widths at half height were less than 0.6 Da. Further details for the operational parameters are given in Table 4-15.

Table 4-15 Working parameters for ESI-MS

Ion Source	
Curtain gas, psig	10
Electrospray, Volt	3000÷4500
Nebulizer gas, psig	10÷15
Declustering potential, Volt	10÷70
Mass analyser	
Mass range, amu	50-2500
Scan Time, sec	1
Resolution	Q1 and/or Q3-high with 0.01 amu or unit with 1 amu
Step size	0.1 amu
Polarity	positive or negative
Detector	
Deflector, Volt	-200
Channel Electron Multiplier	2200

4.3.5.2 Electrospray ionisation (ESI) source

In the ion source the solution-carrying tube protruded approximately 1 mm from the nebulizer tube. The ESI probe was inserted into a V-type source housing. The probe is oriented orthogonal by to the ion inlet orifice of the mass spectrometer, so that the edge of the aerosol cone formed by the ESI device was sampled into the mass spectrometer.

In analyses using the positive ion mode the potentials applied to the ESI probe were in the range of 3.0 kV to 4.5 kV. The curtain gas and the ion source (or nebulizer) gas pressure

were usually kept constant at 10 psig. Ultrahigh purity nitrogen gas (99.999%) was used as curtain gas and ion source gas.

For the flow injection experiments the analyte solution was delivered by a Harvard Apparatus, Model 11 syringe pump at 5 or 10 µL/min. Adjustments of the sample tube in the X and Y direction versus the nebulizer jet when spraying a 1 mg/L arsenobetaine standard solution were performed in order to produce an optimal signal. The optimum position in the X direction was 5.1 mm, while in the Y-direction it was 5.6 mm. These values were kept constant during all ESI-MS and ESI-MS/MS analyses.

4.3.5.3 cHPLC coupled on-line to ESI-MS

For connecting the outlet of the cHPLC column to the ESI interface of the mass spectrometer a 50 cm long, 1/32" O.D. and 50 µm I.D. PEEK coated silica capillary and zero dead volume fittings were used. The system is shown in Figure 4-5.

Figure 4-5 cHPLC coupled on-line to a triple quadrupole ESI-MS system.

4.3.5.4 Working standards of As species for ESI-MS

For the experiments, standard solutions of the individual arsenosugars were prepared at a concentration of 100 µg/L in a 10 mmol/L ammonium bicarbonate buffer/methanol mixture (1/1, v/v). All 1 mg/L working standard solutions of methylated organoarsenic compounds (MMA, DMA, AsB, AC and TMAO) for flow injection ESI-MS/MS experiments were prepared by 1:10 diluting each 10 mg/L stock solutions with the same solvent mixture by 1:10 and arranging for a volume up to 1 mL.

For the cHPLC coupled to ESI-MS/MS experiments aqueous mixtures of these standards were prepared which contained each 50 µg/L species.

4.3.5.5 Chromatographic conditions for the determination of As species by cHPLC coupled to ESI-MS/MS

All separations of organoarsenic compounds were carried out on a Hamilton PRP-X100 (250 mm x 0.5 mm, 5 µm particle size) cHPLC column, packed in-house as described in detail in section 4.3.2.1.1. The analytical column was also equipped with a guard column with the same material and the following dimensions: particle size: 5 µm; pore size: 100 Å; I.D: 0.5 mm and length: 20 mm.

Details of the chromatographic conditions are given in Table 4-16, while the working conditions of the ESI interface and the mass analyzer given in Table 4-15.

Table 4-16 Chromatographic parameters for the determination of As species by cHPLC coupled to ESI-MS/MS

Composition of mobile phase	**A- 10 mmol/L Ammonium hydrogen carbonate, pH 8.5 B- Methanol**							
Gradient elution	t [min]	0	5.5	18	23	28	38	40
	CH_3OH [%]	0	0	50	50	0	0	stop
Flow rate	12 µL/min							
Injected sample volume	0.3 µL							

4.4 Sample preparation for the analysis of fish and edible algae

4.4.1 Marine biota samples

Two codfish muscle tissue and one dogfish powder were kindly provided by U. Kohlmeyer and Dr. E. Jantzen from GALAB Laboratories GmbH Geesthacht (Geesthacht, Germany). The samples were kept at -18 °C in the de ep freezer. Codfish samples were analysed on a fresh weight basis. A dogfish CRM DORM-2 (powdered) from the National Research Council of Canada (NRCC), Ottawa, Ontario (Canada) was used. The concentration of inorganic arsenic, arsenobetaine and TMAs ion in DORM-2 are certified by NRCC . Unfortunately, this is not the case for all As species determined in DORM-2 by other groups. Accordingly, the concentration of MMA, DMA and trimethylarsoniopropionate (TMAP) are given for information purposes although the values are not certified [46, 48, 55].

In Table 4-17 the certified values of inorganic and organoarsenic species established for dogfish (*Squalus acanthias*) reference material are given.

Table 4-17 As speciation in the CRM Dogfish muscle

	Concentration, Uncertainty, mg/kg	Methods of analysis
Arsenic	18.0 ± 1.1	ICP-MS, Graphite furnace AAS, Hydride generation AAS, X-ray fluorescence spectrometry
Arsenobetaine (as As)	16.4 ± 1.1	HPLC coupled to ICP-MS, Ionspray MS
Tetramethylarsonium (as As)	0.248 ± 0.054	HPLC-ICP-MS

The brown alga *Undaria Pinnatifida* and red seaweed *Porphyra* were kindly obtained in dry condition from Dr. E. Dumont from the Department of Analytical Chemistry, University of Ghent, Belgium. These dry samples originated from Japan and China. They were pulverised in a coffee grinder and then kept in a dry place away from direct sunlight.

4.4.2 Sample preparation scheme

Two types of sample treatment have been applied to all types of samples. Codfish samples were analysed on a fresh weight basis, while in the case of dogfish and algae samples dry powders were analysed.

For the determination of the total As content acid digestion of the samples was applied. Soft extracting agents such as water and methanol, with which the chemical integrity of the different As species could be preserved, were employed for the extraction of the respective species from fish and algae. The extraction of the marine biota samples was performed according to a procedures described by Morita and Shibata [19] and by Kohlmeyer *et al*. [42] with small modifications. The sample preparation scheme is described in Figure 4-6.

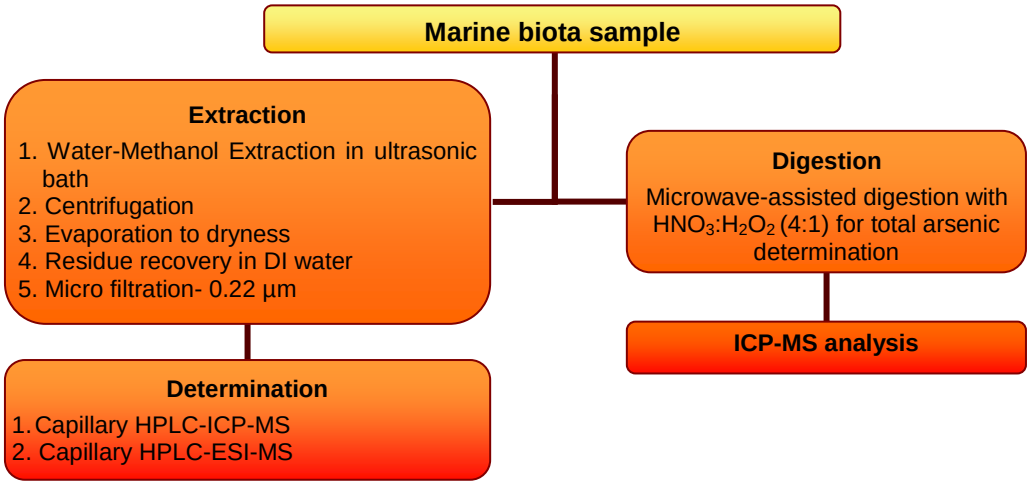


Figure 4-6 Sample preparation of marine biota prior to elemental and species determinations.

4.4.3 Microwave-assisted acid digestion for total As determinations

Suitable amounts i.e. 0.3 g of algal powder, and 0.25 g of DORM-2 dogfish powder and fish muscle were weighed into a PTFE vessel. A mixture of 4 mL subboiled HNO_3, 1 mL H_2O_2 and 1 mL Milli-Q water was added. The algae and fish samples were digested at a microwave power of 1000 W and 180°C with a 25 min temperature program in a microwave oven, as shown in Table 4-18. After cooling, the residue were quantitatively transferred into a PE tube and diluted with Milli-Q water to 15 mL. All samples gave clear digest solutions. The digested sample solutions were diluted 1:10 with ultrapure water prior to the determination of the total As concentration in the sample by ICP-MS. Calibration with synthetic solutions of As at concentrations from 1 to 100 µg/L has been applied. All As concentrations in the samples are reported on a dry mass basis.

Table 4-18 Microwave digestion temperature program for marine biota

Step	**Sample**: Marine Biota
Step 1	
Time	10 min
T_1	75 ℃
T_2	110 ℃
P	1000 W
Step 2	
Time	15 min
T_1	130 ℃
T_2	180 ℃
P	1000 W
Cooling time	25 min
Total time	50 min

4.4.4 Liquid extraction of As species from fish

A 1 g codfish tissue sample was weighted into a 50 mL PP centrifuge tube and 10 mL of a 3:1 methanol-water mixture were added. The sample was homogenised for 3 minutes and blended with an Ultraturrax (11000/N=1). Then the tube was centrifuged for 5 min at 3500 rpm. After centrifugation the extract was transferred into a round-bottom glass flask of 50 mL and subsequently into a PP tube of 50 mL volume for evaporation to dryness in the microwave oven.

The extraction procedure was repeated twice with 5 mL of a 1/1 methanol-water mixture. The tube was vigorously shaken manually and then centrifuged. After centrifugation the washings were added to the first extract with the aid of a Pasteur micro pipette. The extract and the washings were combined and the solvent was removed by using either an evaporation at reduced pressure (at 120 psig) at 40 ℃ in a rotary evaporator or by the use of microwave oven (temperature from 75 to 150 ℃ for 4 0 min). No significant differences in the recoveries of all As species in the codfish samples by both evaporation methods. The dried extracts were kept in the freezer overnight if needed and were analysed by cHPLC coupled to ICP-MS. Before analysis the reminder was redissolved in 1.0 mL mobile phase buffer,

then sonicated for 5 min and transferred with the aid of a Pasteur micro pipette into a 2 mL screw-capped HPLC vial after filtering through a 0.2 µm Nylon filter. The final extract was in parallel analysed by ICP-MS and cHPLC coupled online to ICP-MS for residues of arsenic.

4.4.5 Liquid extraction of As species from algae

Sample amounts of 0.05 g and 0.1 g dry algae powder in the case of red seaweed *Porphyra* and brawn *Undaria pinnatifida*, respectively, were weighed into 15 mL PE centrifugation tubes. 1.0 mL of a 9/1 methanol/water mixture was added. Extraction was carried out for 20 min in an ultrasonic bath at 35 kHz frequency. The suspension was centrifuged for 10 min at 4200 rpm and then the supernatant was transferred into a 20 mL bottom-round glass flask. This procedure was repeated 4 times with 1.0 mL of a 1/1 methanol-water mixture. All fractions were collected into the round bottomed flask and evaporated to dryness. The residue of the extract was stored at -18 °C. On the day of analysis, the dry extracts were redissolved with 1.0 mL Milli-Q water; shaken for 30 sec at 2000 min^{-1} on a mini shaker (Vortex) and at 4 °C centrifuged for 2 min at 10000 rpm. The clear supernatant was transferred with the aid of Pasteur micro pipettes into a 2 mL screw-capped HPLC vial of PE and subjected to As speciation or stored at 4°C prior to analysis.

After the analysis by cHPLC coupled to ICP-MS the rests of the algae extracts were stored at -18 ºC prior to the determination of the total As by collision cell ICP-MS. Then the samples again were kept in the freezer for further studies by ESI-MS.

Three equal portions of brown alga *Undaria pinnatifida* and red seaweed *Porphyra* samples were extracted and the extracts subjected to As speciation by cHPLC coupled to ICP-MS or ESI-MS, for method validation purposes. External calibration with synthetic solutions of AsB and DMA was applied for the quantification.

4.4.6 Liquid extraction of As species from dogfish muscle CRM

Two portions of 0.25 g were in parallel prepared for each sample batch of marine biota. The extraction procedure for CRM DORM-2 dogfish was the same as applied for the codfish muscle or for the algae samples. Solvent evaporation was done in a rotary evaporator. Two equal portions of DORM-2 for every algae sample batch were extracted identically and the extracts were analysed by cHPLC coupled on-line to ICP-MS or ESI-MS/MS.

4.4.7 Sample preparation for spike of algae samples

In a 15 mL PE centrifugation tube 0.1 g dry algae powder of red seaweed *Porphyra* and brown *Undaria pinnatifida*, were weighted. Defined volumes of AsB, DMA, MMA and As(V) water stock solutions were added to the algae sample as listed in Table 4-19 and extraction and washing steps carried out as it is described above. For each algae type and

fortification level also replicate spikes were made. Calibration with synthetic solutions of AsB and DMA was applied as well.

Table 4-19 As species for algae spike samples

Compound	Concentration of spike solution mg/mL	Spike volume taken, µL	Spike concentration µg/g
As(III)	0.1	10 / 100	10 / 100
As(V)	0.1	10 / 100	10 / 100
MMA	0.1	10 / 100	10 / 100
AsB	0.1	10 / 100	10 / 100
DMA	0.142	7.4 / 74	10 / 100

4.4.8 cHPLC conditions for the analysis of marine biota

Fish and algae samples were analysed using several cHPLC columns for the separation of the As species. Different mobile phases have been used as is described below. Multi-compound calibration standards were prepared on the day of analysis by an appropriate dilution of the working solutions with pure water.

4.4.8.1 Fish samples

0.3 µL fish extract was injected onto Zorbax SAX cHPLC analytical (250 x 0.5 mm, 5 µm) and guard (20 x 0.5 mm, 5 µm) columns and As was detected by the Sciex Elan 5000 ICP-MS coupled on-line to the cHPLC. The extracts were analysed by monitoring the isotope signals at *m/z* 75 for As, at *m/z* 77 for Se (ArCl), at *m/z* 35 and 37 for chloride and at *m/z* 103 for Rh. The mobile phase used consisted of 1 *mM* ammonium bicarbonate buffer at pH 6. A flow rate of 10 µL/min was used and the CETAC MCN-100 with a small volume spray chamber selected as interface.

In addition SCX cHPLC coupled to ICP-MS was used for the determination of the As species in the fish extracts. For this aim a Hamilton PRP-X200 (250 x 0.5 mm, 10 µm) cHPLC column and 0.5 *mM* Pyridine, at pH 2.7 and at a 10 µL/min flow rate were used. The sample injection volume was 500 nL.

4.4.8.2 Algae samples

Separations were performed on Hamilton PRP-X100 SAX capillary guard and analytical HPLC columns and the experimental conditions are given in Table 4-20. The column was coupled on-line to the Agilent 7500s ICP-MS via the modified CETAC MCN-100 and a small volume glass spray chamber.

Table 4-20 Chromatographic conditions for the analysis of algae extracts by cHPLC coupled to ICP-MS

cHPLC working conditions	
Column:	Hamilton PRP-X100, 250 x 0.5 mm, 5 µm particle size
Pre-column:	Hamilton PRP-X100, 20 x 0.5 mm, 5 µm particle size
Mobile phase:	A: 2 *mM* NH_4NO_3 buffer at pH=8.5 +10 µg/L Ge (internal standard) B: 40 *mM* NH_4NO_3 buffer at pH=8.5 +10 µg/L Ge (internal standard)
Gradient elution:	0-6 min: 100% A; 6-7 min: 0% A, 100% B; 7-25 min: 100% B; 26 min: 100% A, 0% B; 26-35 min: 100% A
Flow rate:	12 µL/min
Injected sample amount:	500 nL

4.4.9 Instrumental parameters for the analysis of marine biota samples by ICP-MS

To determine the As compounds of interest eluting from the cHPLC columns, the PerkinElmer Sciex Elan 5000 and the Agilent 7500s ICP-MS system with a Shield Torch were used together with in-house modified microconcentric nebulizer CEI-100 and a small volume glass spray chamber. As software the Xenix Version 2.2 and ICP-MS ChemStation, and the ICP-MS Chromatographic Software, Version A.02.00 (Copyright Agilent Technologies 1989-2002) were used.

The ICP-MS signals were optimised with a solution containing 10 µg/L As to achieve a maximum response for the As signal at *m/z* 75 and then on a daily basis with a 10 µg/L Rh solution in the case of the Elan 5000 or Ge for the Agilent 7500s as internal standard.

The ICP-MS parameters used are given in Table 4-21 and they were kept constant during the analyses of the calibration standards, the spiked samples and the fish extracts.

Table 4-21 ICP-MS instrument parameters

1. Elan 5000 ICP-MS
Plasma conditions: RF Power: 1030 W, Outer gas: 15 L/min, Nebulizer gas: 1.06 L/min, Auxiliary gas: 1.74 L/min
Quadrupole Mass Spectrometer: Isotope masses: ^{75}As (0.1 s), ^{35}Cl (0.01 s), ^{37}Cl (0.01 s), ^{77}Se (ArCl) (0.01 s), ^{103}Rh (0.1 s) Graphic analysis mode
2. Agilent 7500s ICP-MS
Plasma conditions: RF Power: 1300 W, Sample depth: 6 mm, Outer gas: 15 L/min, Nebulizar gas: 1.03 L/min
Quadrupole Mass Spectrometer: Isotope masses: ^{75}As (0.1 s), ^{77}Se or ArCl (0.1 s), ^{35}Cl (0.1 s) and ^{72}Ge (0.1 s) Q pole bias: -6 V Time resolved analysis mode

4.4.10 Analysis of algae extracts by cHPLC coupled to ESI-MS/MS

- **Chromatographic conditions**

A Hamilton PRP-X100 cHPLC column and gradient elution with 10 *mM* ammonium bicarbonate buffer at pH 8.5 and methanol were used. An injection volume of 0.3 µL was employed, and the percentages of MeOH at 0.01, 5.5, 18, 23, 24 and 40 min were 0, 0, 50, 50, 0 and 0, respectively.

The column outlet was connected to the ESI interface of the API 4000 Mass spectrometer via a capillary of a length of 50 cm, an I.D of 50 µm, and an O.D. of 1/32 in., respectively, with the aid of zero dead volume fittings.

The chromatographic conditions used were the same for the determination of the target organoarsenic substances, as well as for the determination of the non-target As compounds.

This setup was also used for the quantitative analysis of target organoarsenic compounds in the algae with ESI-MS/MS using two specific MRM for each substance (see Table 4-22). Calibration with synthetic solutions of multi-component standard solutions with increasing concentrations of each organoarsenic compound was applied for the analysis of the algae extracts. All organoarsenic compound concentrations are based on dry mass.

- **ESI-MS working conditions for the analysis of algae extracts**

For all ESI-MS/MS measurements in the positive MRM mode the following parameters were used: ESI voltage: 4.5 kV, DP: 40 V, curtain gas pressure: 10 psi, nebulizer gas pressure: 20 psi and CAD pressure: 8 psi. The collision gas used was nitrogen. In the MRM, scans for the precursor and daughter ion of the target As species (see Table 4-22) were performed at a collision energy of 35 V.

Table 4-22 Selected precursor and daughter ions in the MRM experiments

Compound	MRM, *m/z*	Dwell time, sec
Glycerol-ribose	329 → 97	0.25
	329 → 237	0.25
Phosphate- ribose	483 → 97	0.25
	483 → 237	0.25
Sulfonate-ribose	392 → 163	0.25
	392 → 237	0.25
Sulfate-ribose	409 → 237	0.25
	409 → 330	0.25
Arsenobetaine	179 → 120	0.25
	179 → 105	0.25
DMA	139 → 121	0.25
	139 → 91	0.25
TMAO	137 → 107	0.25
	137 → 122	0.25
Arsenocholine	165 → 121	0.25
	165 → 158	0.25
MMA	142 → 91	0.25
	141 → 75	0.25

Compound	MRM, *m/z*	Dwell time, sec
DMAEt	167 → 107	0.25
	167 → 121	0.25
DMAsAC	181 → 121	0.25
	181 → 139	0.25

The monitored mass ranges from 30 to 1000 amu and from 50 to 3000 amu with a step size of 1 or 0.5 amu (for more precise mass scanning) and a dwell time of 0.1 sec or 5 msec, respectively were applied.

Since in MRM the sensitivity is higher than in the single ESI-MS records, this ESI-MS/MS mode was also used to investigate the presence of further unknown As compounds in the algae extracts that were eluting from the SAX cHPLC column.

4.4.11 Sample preparation for the determination of the total As content in the extracts

The frozen stored (-18 °C) algae and fish extracts were brought to room temperature. After tempering 200 µL aliquots were taken and transferred into 15 mL PE tubes. Then 4.8 mL of 1% HNO_3 were added.

The octopole reaction cell Agilent 7500c ICP-MS from Agilent Technologies, Japan with ICP-MS ChemStation software was employed for the determination of the As in the marine biota digests and extracts. The measurement conditions were optimised on a daily basis. The average nebulizer gas flow rate was: 0.84 L/min Argon, the collision reagent gas flow rate was 4.5 mL/min He, the RF power was (1280 W) and the lens voltage was (-12 V).

Traces of As and some other heavy metals were determined in the extracts. Calibration was performed with synthetic solutions prepared from the Merck VI ICP-MS standard solution with different concentrations.

4.5 LADROP-LIBS

4.5.1 Preparation of standard solutions

The Na acetate, $CuSO_4$, KBr, $MgSO_4$ and $CaSO_4$ used were of ultrapure analytical grade.

Merck ICP-MS VI multi-element standard solution, containing 23 elements at a concentration of 1 mg/mL was used for the multi-elemental determinations.

Na acetate solutions with concentrations from 1 to 10 mg/L were prepared by dissolving the appropriate amounts of salts in ultrapure water and the solutions were filtered through a 0.22 µm nylon filter. A stock solution of Ca with a concentration of 100 mg/L was made in 5% HNO_3 and also filtered. Calibration solutions of Ca with a concentration of 0.04 to 0.7 mg/L were prepared by dilution with ultrapure water. Working solutions of different

concentrations were prepared by dissolving the required amounts of reagent in ultrapure water.

4.5.2 Design and performance of the instrument

4.5.2.1 Overall design consideration and function

The principle and the realisation of the LADROP-LIBS coupled to cHPLC system are shown in Figure 4-7 and Figure 4-8. The eluate of the chromatographic separation (CS) is fed into a droplet generator (DG) that produces a continuous stream of droplets (D). The individual droplets are detected by a unit consisting of a laser diode (LD), an achromatic lens and a photodiode for droplet detection (DD). The detection of a droplet triggers a Q-switched Nd-YAG laser (LA), of which the pulses transform single droplets into the plasma state. The radiation is guided into a Paschen–Runge spectrometer (PR) and measured by multi-channel integrator electronics (MCI). Measurements are stored and processed in a computer (PC). A gas guiding system (GG) is used to flush argon into the measurement chamber (MeC) [148].

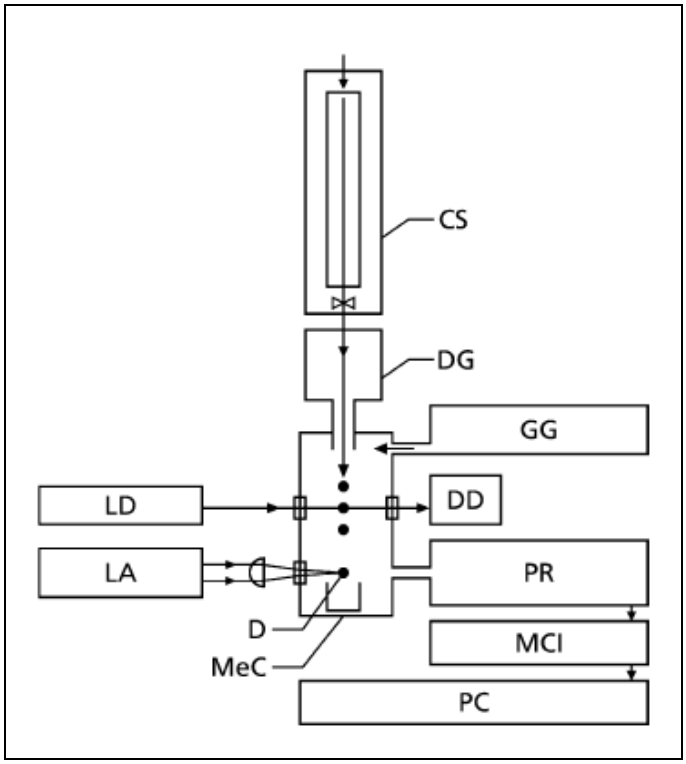


Figure 4-7 Schematic view of the cHPLC-LADROP-LIBS setup (from ref. [148]). Abbreviations are explained in the text above.

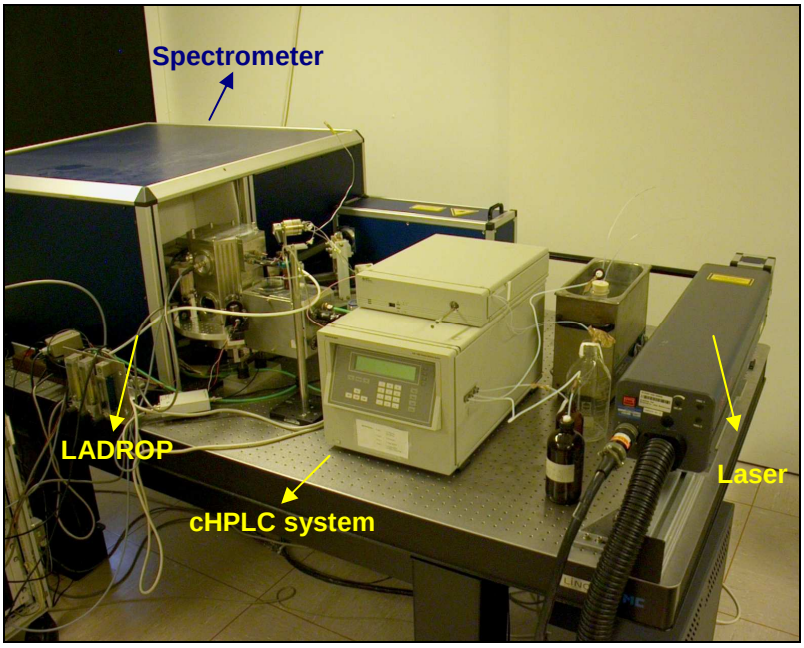


Figure 4-8 Photograph of HPLC coupled to LADROP-LIBS.

In Figure 4-8 a picture of the LADROP-LIBS system and the measurement chamber connected to the HPLC are shown. In Figure 7-7 in the appendix the basic optic layout for the analysis of single picolitre droplets is shown.

The design of the LADROP measurement chamber is given in details in Figure 7-8 in the appendix, while in Figure 7-9 in the appendix a cross section is presented.

4.5.2.2 Measurement chamber

The measurement chamber consists of a piezoelectric droplet generator, diodes for droplets detection, a wide angle lens (macro objective) of the monitoring camera, focusing lenses and stroboscopic illumination. The droplet generator produces a free falling stream of droplets. It is made of a glass capillary with an orifice of 50 µm in diameter (Microdrop GmbH, Norderstedt) on one side, that is enclosed by a tube-shaped piezoelectric element (GeSiM GmbH, Rossendorfer Technological Centre, Großerkmannsdorf, Germany) (See Figure 4-9). The piezoelectric element is activated with a voltage pulse that induces a pressure wave in the liquid causing the formation of a single isolated droplet with a volume between 100 pL and 1 nL, diameters of 50- 100 µm and an initial velocity of 1–2 m/s.

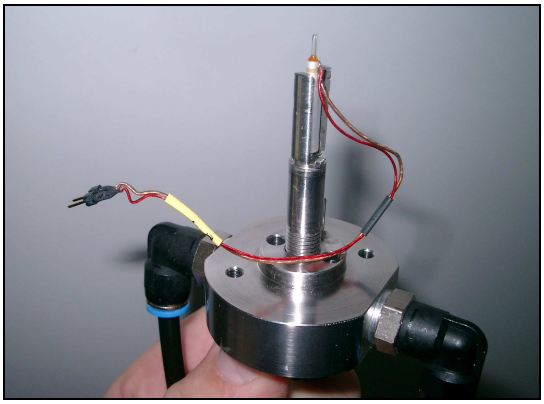


Figure 4-9 Piezoelectric droplet generator.

The PDG is basically a “drop on demand” piezoelectric nozzle that produces single isolated droplets with volumes typically between 100 pL and 1 nL and an initial velocity of 1-2 m/s. The droplet formation process is highly reproducible and the droplet size uniform. After detection of an individual droplet the laser is triggered to emit a single Q-switched pulse. By synchronising the laser pulses with the single droplets, a plasma is generated and its radiation is collected and transported to the spectrometer.

In the case of coupling cHPLC with LADROP-LIBS, the outlet of the HPLC is connected to the droplet generator inlet though zero-dead volume fittings.

A charge-coupled device (CCD) camera with a stroboscopic illumination is used to control and adjust the droplet generation. In Figure 4-10 the droplet generator nozzle and uniform droplets under stroboscopic illumination are shown. The piezoelectric nozzle is capable of delivering up to 2000 droplets per second. However, droplet frequencies as low as 10–20 Hz are also possible. The droplet size can only be varied within a narrow range by adjusting the voltage pulse. A higher peak voltage leads to a higher droplet speed and larger pulse lengths lead to larger droplets. Every individual piezoelectric nozzle has a set of parameters that give the best stability and droplet uniformity and the nozzles show little tolerance to prevent deviations in these parameters. Therefore systematic studies with droplets of different sizes were not made.

An electrooptical high speed camera (Stanford Computer Optics, UK) was used to monitor the plasma dynamics.

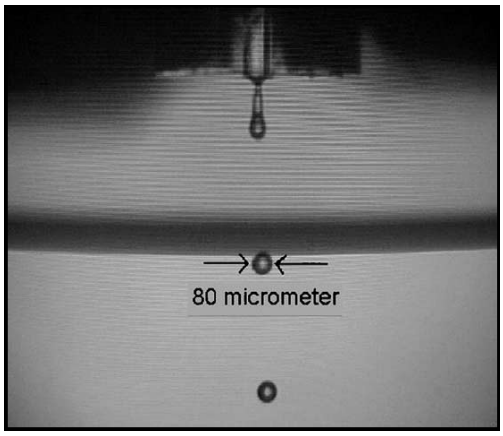


Figure 4-10 Image of 270 pL droplets as photographed with stroboscopic illumination. The droplet formation frequency: ~800 Hz. A part of the glass capillary of the droplet generator can be seen in the upper part of the picture (from reference [148]).

4.5.2.3 Laser

A Q-switched Nd:YAG laser (Lumonics HY 1200, pulse width: 10 ns, repetition rate: 10 Hz) was available from the Fraunhofer Institute of Laser Technology (ILT), Aachen, Germany. The repetition rate of the laser restricts the measurement frequency to 10 Hz, although other components, such as the droplet generator, the spectrometer, the signal integration and the recording would permit measurements at a rate of up to 1000 Hz. The laser pulse energies were attenuated with the aid of a Glan laser-polarizer and a λ / 2-plate to 50–140 mJ. The laser beam is expanded to a diameter of approximately 3 cm and then focused with an achromatic lens having a focal length of 10 cm. For adjustment, the lens can be translated perpendicularly to the laser beam and in the direction of the laser beam. For the timing of the signal detection and the laser trigger relative to the droplet detection a digital delay generator (Stanford DG 535) was used [148].

4.5.2.4 Simultaneous spectrometer

- Paschen–Runge spectrometer

The plasma radiation is resolved spectrally with the aid of a Paschen–Runge spectrometer (type: QSN 750 from OBLF; Witten, Germany) equipped with 33 photomultipliers (type C 932, C 933, C 911 from Perkin Elmer). It enables the simultaneous detection of 31 different elements. The choice of the elements for the Paschen–Runge spectrometer was made to enable a broad spectrum of possible chromatographic applications with the LADROP-LIBS instrument. The lines showing the strongest intensity were chosen. A complete list of all elemental lines detectable with the Paschen–Runge spectrometer is given in the appendix in Table 7-3.

The plasma emitted a strong continuum directly after the laser pulse. After a short delay of few microseconds, only the element-specific line emissions still can be detected. For

a better signal-to-noise-ratio the first part of the plasma emission was excluded from the analytical signal detection. Typical delay times between the laser pulse and the start of the signal integration were 0.5–1.5 µs, the integration width was between 20 and 50 µs.

The photomultiplier signals are processed with the aid of multi-channel integration electronics (MCI, developed by the Fraunhofer-Institute of Laser Technology, Aachen, Germany).The photocurrents from 33 photomultipliers can be integrated simultaneously with individual time gates. A description of the MCI is given by Noll *et al.* [149]. For single pulse evaluation the signals for all channels were transmitted to a computer. The multi-channel integration electronics allows fast measurement frequencies of up to 1000 Hz with single pulse evaluation. In order to achieve higher signal intensities, the plasma emission was collected with a spherical mirror and focused on the entrance slit of the Paschen–Runge spectrometer. The mirror is placed inside the measurement chamber and can be adjusted without opening the chamber [148].

- Echelle spectrometer

As Echelle spectrometer an ESA 3000 (Laserlabor Adlershof, Berlin, Germany) was used. This instrument enables it to select the elemental lines and to study the laser-induced breakdown spectra of droplets between 200 and 780 nm. The experimental setup was similar as described above, except that the spectrometer was connected to the source with fibre optics.

For most experiments, a flow injection analysis (FIA) manifold was used to introduce samples to the PDG.

All instrument parameters could be manually adjusted on a daily basis in order to achieve the maximum signals for the elements determined.

4.5.2.5 cHPLC coupled to LADROP-LIBS

The HPLC ABI 140D-Micro gradient delivery system (Perkin Elmer, Canada) consists of a high pressure capillary binary pump and a thermostatted column compartment with an injector valve Model 112 A. The pump can be operated at flow rates in the range between 1 to 500 µL/min with a maximum pressure of 3500 psi.

A strong cation-exchange capillary column Hamilton PRP-X200 has been packed in-house into a 250 x 0.5 mm PEEK capillary with PSDVB-sulfonate exchanger with a particle size of 10 µm. The technical specifications and the packing procedure for the column are described in details in Sections 4.3.2.3.

This column has been used for the separation of Na and Cu ions, as well as divalent Ca and Mg with a mobile phase that consists of 2 *mM* $CuSO_4$ at pH 5.2 and at a flow rate of 5 µL/min.

An Agilent 1100 Series cHPLC system with 500 nL flow cell DAD (λ=220 nm) was employed for optimization of the Hamilton analytical method for inorganic cations.

4.5.2.6 Preparation of water samples

4.5.2.6.1 Mineral water

Mineral water from the local market has been degassed by treatment in an ultrasonic bath. The sample was then filtrated through 0.2 µm Nucleopore filter. The concentrations of the elements in the water as given by the producer were: 4.1 mg Mg/L, 61.9 mg Ca/L and 10 mg Na/L.

4.5.2.6.2 Sea water

Ocean water and estuarine water reference materials for trace metals were purchased from the Institute for National Measurement Standards, NRC of Canada. These samples have been used for the determination of Cu, Ca, Mg and Na. *NASS-5* is ocean water collected in the North Atlantic at a depth of 10 m, 35 km southeast of Halifax, Canada and has a salinity 30.4 ‰. *SLEW-3* is estuarine water collected in the San Francisco Bay at a depth of 5 m and the salinity is 15 ‰.

The concentration of the metals published by the NRC, Canada are certified values since the concentration was determined with two methods of which every step is based on a different principle. Certified values for Cu were given by NRC [184, 185]. Unfortunately, this is not the case for Ca, Na and Mg in *SLEW-3* and *NASS*-5. The *SLEW-3* and *NASS-5* samples were 1:10 diluted with ultrapure prior to analysis by cHPLC coupled to LADROP-LIBS.

5 CONCLUSIONS AND OUTLOOK

The chemistry of As in marine biota is very interesting. The investigation of its different forms, metabolites and/or the enzymes involved requires speciation and the results are not only scientifically of interest but may also have industrial relevance in biotechnology, environmental remediation, bio-sensors, etc.

Although ICP-MS coupled to liquid chromatography is recently recognised as the best tool for speciation studies, it does not deliver structural information for the organoarsenic compounds and for other species of interest present in marine algae. Additionally, the highly sensitive cHPLC coupled to ESI-MS/MS was required in order to determine and provide a complementary molecular and structural information of target and non-target As compounds that are present at low levels in *Undaria pinnatifida* und *Porphyra* algae samples. cHPLC coupled to ESI-MS/MS is most suitable for As speciation studies and has received increasing attention during the last five-seven years, although it is not as sensitive as ICP-MS.

In light of all these challenges of As speciation analysis in marine biota, several contributions could be realised in the frame of this thesis.

- A new hyphenated method of cHPLC coupled on-line to mass spectrometry was successfully developed for highly sensitive detection of As species in marine biota.
- Theoretical and experimental studies were accomplished to demonstrate that analytical systems (such as cHPLC coupled to ICP(ESI)-MS) have great potential and advantages over the traditional chromatographic methods because of their high resolution and sensitivity achieved here and a diverse range of application possible. The use of cHPLC and the in-house modified microconcentric nebulizer with a small spray chamber for ICP-MS has three major advantages when compared to conventional HPLC coupled to ICP-MS. The sample amounts could be less then 1 µL, which is very valuable for the case of very small samples. Moreover, even without addition of oxygen, the plasma tolerates high concentrations of organic solvents as normally used in cHPLC separations. The background noise of the signal became lower, due to the transport of less water vapour, which significantly improved the absolute power of detection of the cHPLC coupled to ICP-MS.
- The additional selectivity obtained by coupling cHPLC to molecular ESI-MS can be used to decrease the interferences in the determination of As in complex biological samples. As a low flow rate was used with the cHPLC coupled to ESI-MS/MS, the amount of sample required for analysis was dramatically reduced. This has allowed the performance of a series of full scans and ESI-MS/MS scans required to obtain structural information with biological extracts less then 10 µL. cHPLC coupled to tandem ESI-MS with the multiple reaction mode could be successfully accomplished incorporating the advantages of higher selectivity and sensitivity as compared to conventional HPLC coupled to ESI-MS [12, 33, 39-41]. Therefore,

the less toxic organoarsenic compounds and the new As related peptides determined in the brown and red marine algae extracts could be unambiguously identified based on a matching of the retention times and ESI-MS/MS spectra.

- When applying cHPLC coupled to ESI-MS/MS for the determination of the organoarsenic ions in the multiple reaction monitoring (MRM) and following the most intensive mass transitions of the $[M+H]^+$ charged precursor ions into their specific product ions absolute detection limit down to 0.10 pg for AsB and 2 pg for arsenosugars could be obtained.
- As species could be extracted from marine algae and codfish with methanol-water mixture. After clean up the extracts were concentrated and then could be analysed on the in-house packed SAX cHPLC columns coupled to ICP-MS with either the Sciex Elan 5000 or the Agilent 7500s Shield torch. The soft extraction conditions prevented chemical decomposition of the As species and allowed fast and efficient recovery for the species considered. When using the Sciex Elan 5000 ICP-MS for the analysis of fish samples the detection limits for the As species was 5 pg, but for algae samples with the Agilent 7500s ICP-MS it decreased to 0.5 pg. This can be attributed to the differences in ionisation efficiency of the As species as well as differences of the instrumental hardware, especially to the lower dead volume of the online coupled system, and to some ionic suppression.
- Three different marine biota samples and one dogfish certified reference material were analysed and the inorganic and organic As species were found at the concentrations from 0.7 to 24 µg/g. For a clear identification of the detected As compounds in the marine biota samples with the cHPLC coupled to ICP-MS, spiking experiments with AsB, DMA, MMA, As(III) and sulfate-ribose at 10 and 100 µg/g were performed with very good analytical recoveries between 77 and 93%. The results obtained in this study demonstrate that in codfish and marine algae As is predominantly present in its organic, i.e. less toxic forms. In the algae samples arsenosugar compounds are mainly detected, however AsB was also found. This can provide useful information for the understanding of the metabolic pathways of As in marine organisms and on the other hand for the assessment of its toxicological risk in the food chain.
- Initially the As species were extracted from 100 mg algae or 300 mg fish and the residues were analysed with an optimised separation procedure based on cHPLC coupled to ICP-MS using a capillary polymeric SAX column with dimensions of 250 x 0.5 mm packed with Hamilton PRP X-100. The organoarsenic species could be identified and their structure and concentration confirmed by cHPLC coupled to ESI-MS/MS. The complementary results of the newly developed methods were in very good agreement with each other and with literature data, which demonstrates their applicability for marine biota samples with lower As content.

- Novel As related peptides could be detected at low levels and identified in the both algal extracts with cHPLC coupled to ESI-MS/MS due to the high chromatographic resolution, soft extraction and ionisation, and the molecule-specific structural and sequential information of the ESI-MS/MS spectra.
- The PDG and LADROP measurements are promising for the sample introduction of small volume liquid samples for LIBS and for elemental analysis. It offers 100 % sample efficiency, since each droplet is hit by the laser beam and produces plasma. The use of FIA and especially on-line coupling of cHPLC with LADROP-LIBS allows its use with much smaller sample volumes than conventional HPLC or direct liquid sampling nebulizers for multi-elemental analysis. Although the relative LODs are much higher than those obtained by ICP-OES at present, the absolute detection limits of the technique are very low, since low µg/mL levels of elements can be detected in pL droplets. However, As speciation analysis was not possible, due to the large dead volume of the system and the resulting higher detection limits for arsenic.
- The accuracy of the procedures developed was studied by analysing a CRM DORM-2 with very satisfactory results. The uncertainty of the analytical measurements was evaluated for arsenobetaine and was found to be 15.6 ± 0.8 µg/g As and for the total As 17.5 ± 1.1 µg/g. Accordingly, they may be used for many routine applications.
- The easy sample preparation and excellent results, could allow the procedures to be used in the routine quality control of environmental and food samples as the As speciation takes only one hour.

Outlook

The methods developed in this work, could be easily extended to other arsenic-containing compounds in environmental, biological and food samples.

When using suitable internal standards, cHPLC coupled to ESI-MS/MS could be expanded into a quantitative method for the determination of the actual amount of arsenic-glutathione and arsenic-phytochelatin complexes in marine algae samples.

As highlighted in chapter 3, future studies must be carried out so as to enable definitive conclusions on the determination and full identification of As species in the algae, for which reference substances are not available. The chromatographic peaks of the unknown As species eluted from the SAX cHPLC column and monitored by ICP-MS in red *Porphyra* seaweed extract were of a very low concentration. However, from the studies with cHPLC coupled to ESI-MS/MS are believed to be dimethylarsenic-glutathione (DMAs-GSH or DMAs-PC_1), As-PC_3 and Cd-As-isoPC$_5$(Ser) at m/z 412, 847 and 1453. As(III) and As(V) could be found by ESI-MS using FIA in the both extracts at m/z 75, but they could not be detected with cHPLC coupled to ESI-MS under the chosen conditions. Additional

investigations should allow it to gain more evidence for the specific binding of As to PCs and to enable an unambiguous determination of the DMAs-GSH and arsenic-induced PC complexes in the both seaweed extracts. To date, this has not been realised with cHPLC coupled to ESI-MS/MS and remains an area of research.

All experiments on the separation and detection of As-glutathione and arsenic-induced PC complexes show that biological samples that may contain these complexes must be treated very carefully. The samples must be kept permanently cooled in an acidic media and analysed as soon as possible.

Many authors have published studies on As speciation in which the integrity of the As species has been assumed [3, 51, 75, 77, 81-85, 86]. The results presented in this work indicate that many previously published results must be reinterpreted, especially when unidentified As related compounds were reported, since some As glutathione complexes have not successfully been detected due to the too harsh conditions used for the analysis of As species in biological extracts.

LADROP-LIBS needs further improvements, since the large dead volume of the PDG did not allow the best possible performance to be obtained, and As speciation was not yet possible.

In the future it is possible to use hybrid instrumentation that provides ESI-MS spectra as well as elemental ICP-MS spectra in parallel following a microbore or capillary chromatographic liquid separation. This kind of instrumentation for which a prototype has been developed by G.M.Hieftje et al. [190], together with isotope dilution MS protocols, will certainly improve the reliability and accuracy of results in speciation studies in comparison to what is acknowledged today.

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7 APPENDIX - FIGURES AND TABLES

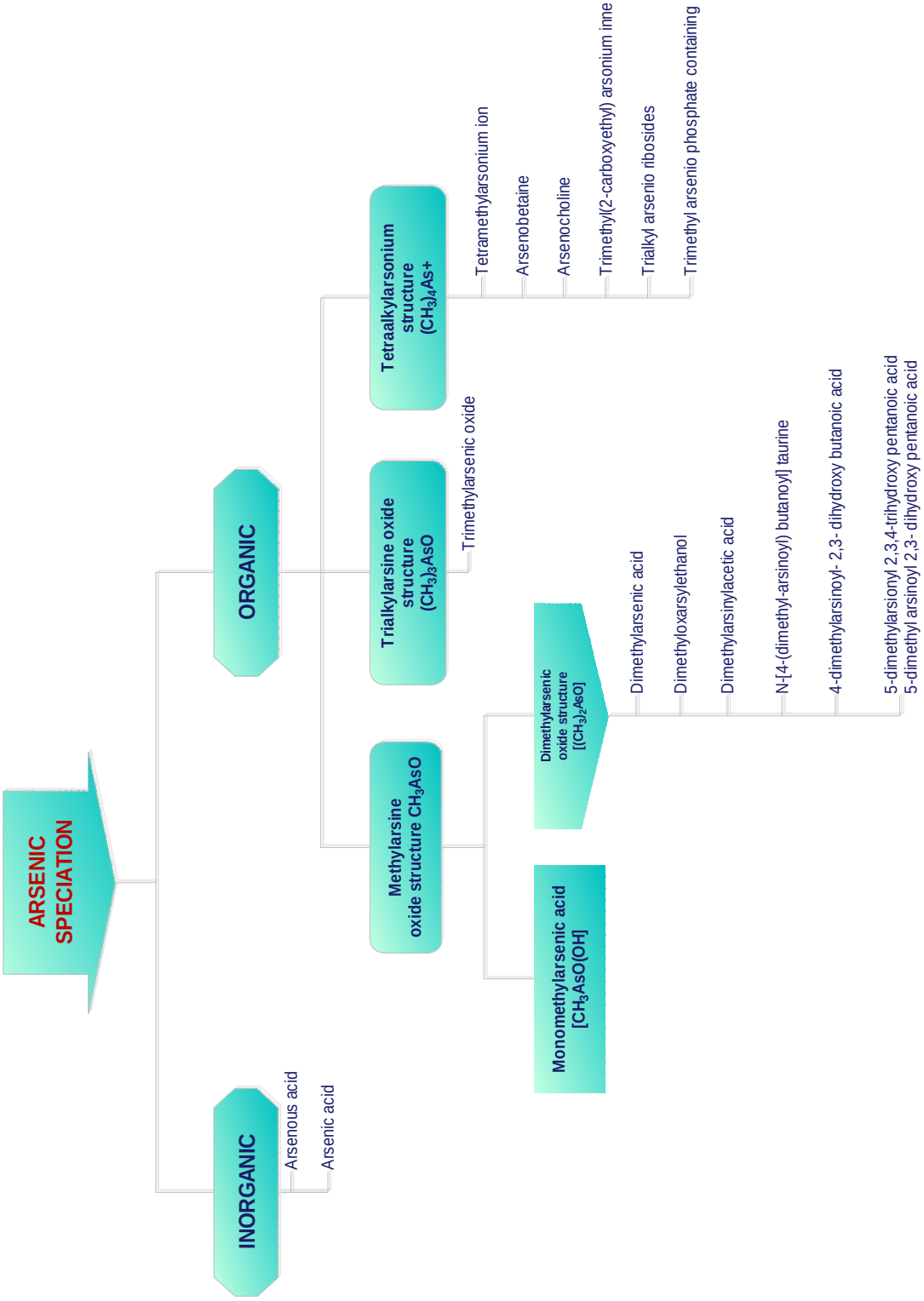
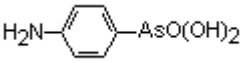
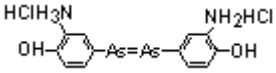
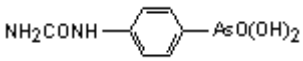
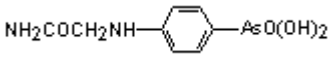
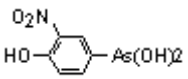
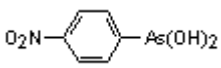


Figure 7-1 Arsenic speciation classification scheme for marine biological tissues.

Table 7-1 Naturally occurring inorganic and organic As species (see Figure 7-2 for the structures [1]–[22])

CAS No.	Name	Synonyms	Structure
	arsenate		[1]
	arsenite		[2]
124-58-3	methylarsonic acid	monomethylarsonic acid, MMA	[3]
75-60-5	dimethylarsinic acid	cacodylic acid, DMA	[4]
4964-14-1	trimethylarsine oxide		[5]
27742-38-7	tetramethylarsonium ion		[6]
64436-13-1	arsenobetaine		[7]
39895-81-3	arsenocholine		[8]
	dimethylarsinoylribosides		[9]–[19]
	trialkylarsonioribosides		[20], [21]
	dimethylarsinoylribitol sulfate		[22]

Table 7-2 Other As compounds of environmental significance

CAS No.	Name	Synonyms	Formula
	Inorganic As, trivalent		
1327-53-3	Arsenic trioxide	As trioxide, arsenous oxide, white As	As_2O_3 (or As_4O_6)
13768-07-5	arsenenous acid	arsenious acid	$HAsO_2$
7784-34-1	Arsenic trichloride	Arsenic chloride, arsenous trichloride	$AsCl_3$
	Inorganic As, pentavalent		
1303-28-2	Arsenic (V) oxide	Arsenic pentoxide	As_2O_5
7778-39-4	arsenic acid	*ortho*-arsenic acid	H_3AsO_4
10102-53-1	arsenic acid	*meta*-arsenic acid	$HAsO_3$
	arsenates, salts of *ortho*-arsenic acid		$H_2AsO_4^-$, $HAsO_4^{2-}$, AsO_4^{3-}
	Organic As		
593-52-2	methylarsine		CH_3AsH_2
593-57-7	dimethylarsine		$(CH_3)_2AsH$
593-88-4	trimethylarsine		$(CH_3)_3As$
98-50-0	(4-aminophenyl)-arsonic acid	arsanilic acid, *p*-aminobenzene-arsonic acid	
139-93-5	4,4-arsenobis(2-aminophenol) dihydrochloride	arsphenamine, salvarsan	
121-59-5	[4-[aminocarbonyl-amino]phenyl] arsonic acid	carbarsone, *N*-carbamoylarsanilic acid	
554-72-3	[4-[2-amino-2-oxoethyl)amino]-phenyl] arsonic acid	tryparsamide	
121-19-7	3-nitro-4-hydroxy-phenylarsonic acid		
98-72-6	4-nitrophenylarsonic acid	*p*-nitrophenyl-arsonic acid	
	dialkylchloroarsine		R_2AsCl
	alkyldichloroarsine		$RAsCl_2$

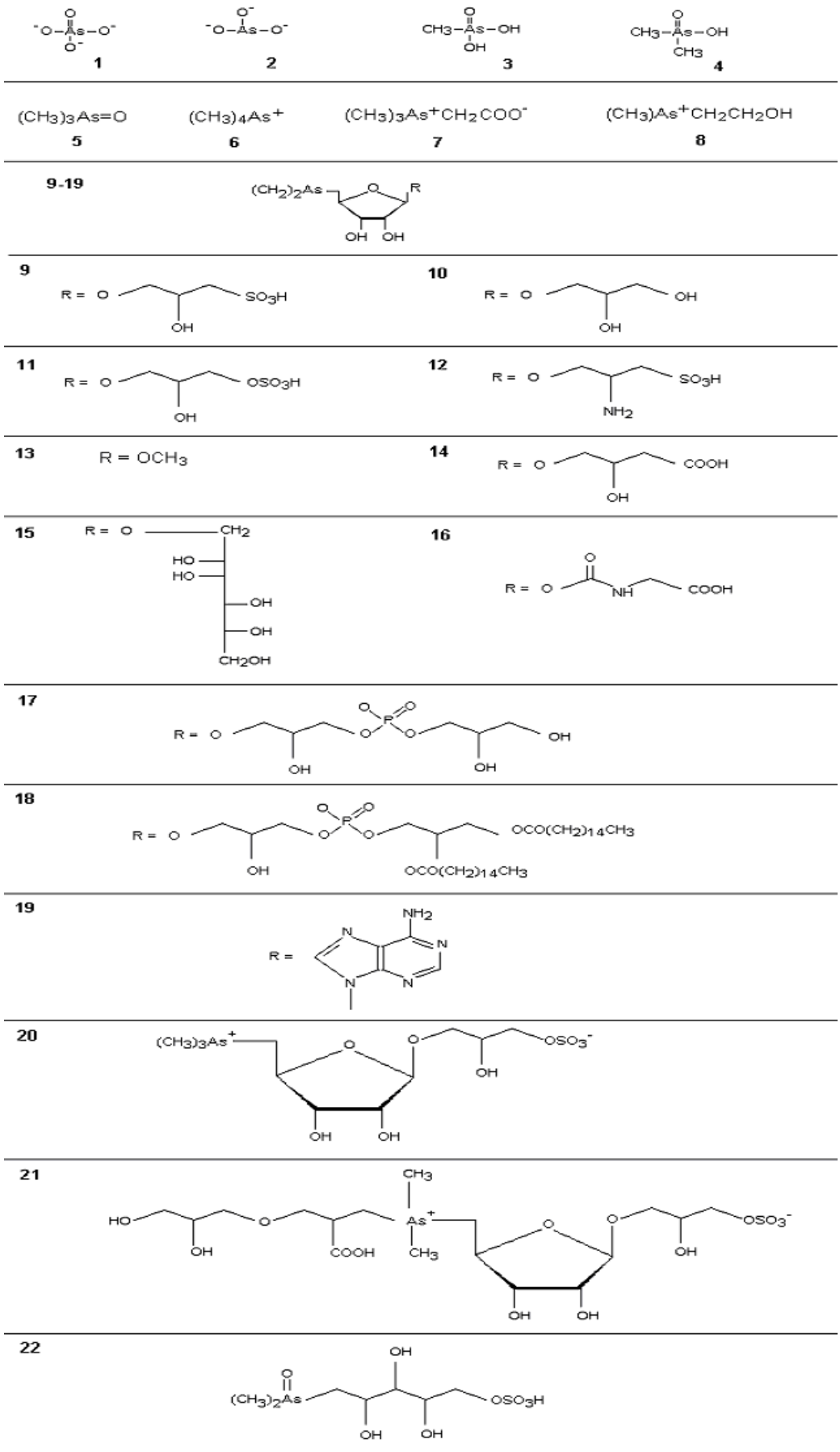


Figure 7-2 Structures of naturally occurring inorganic and organic arsenic species.

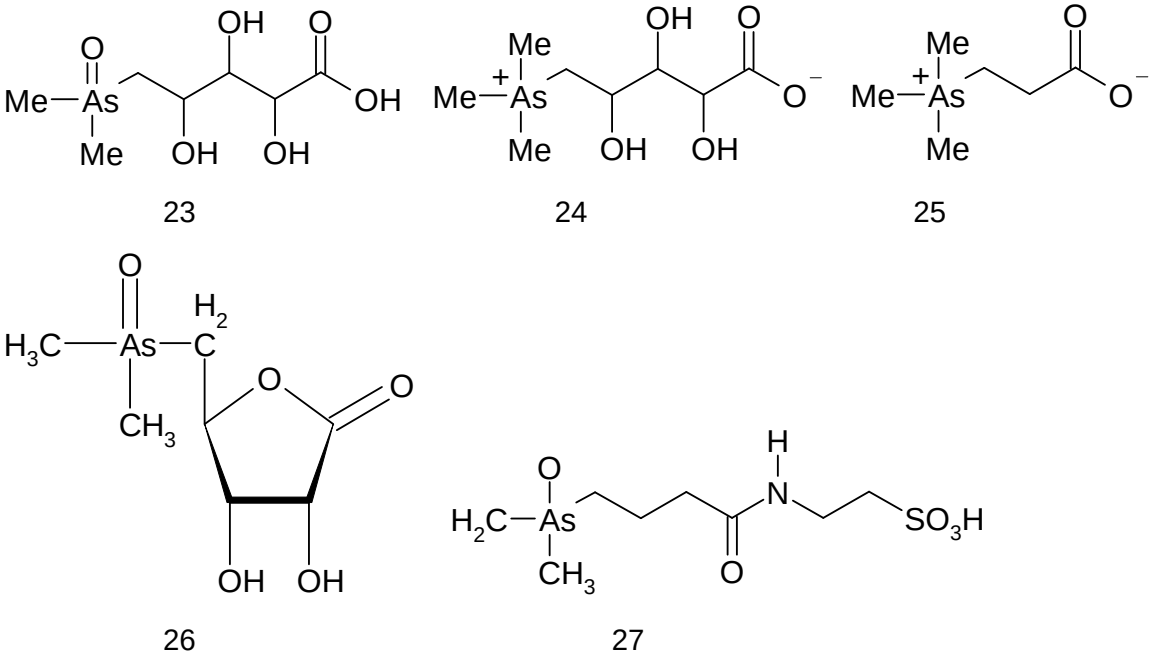


Figure 7-3 Novel arsenolipids found in the Sargassaceae family.

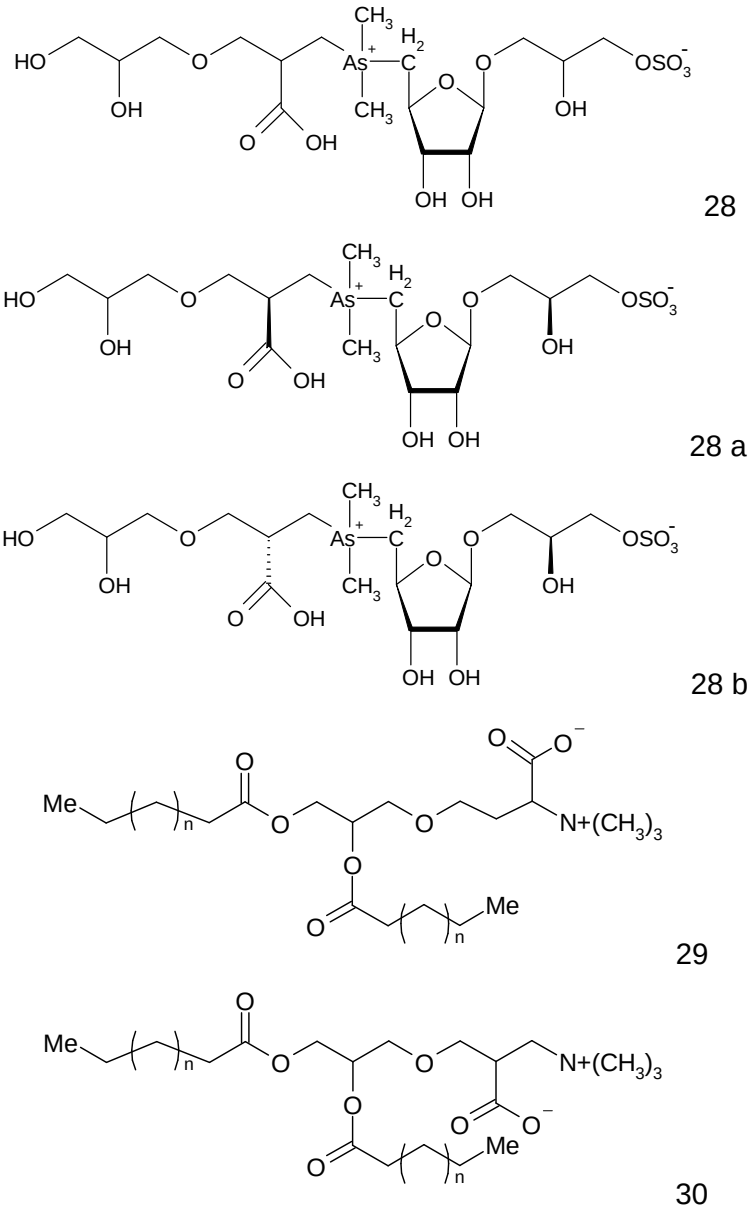


Figure 7-4 Arsenosugars isolated from marine algal species.

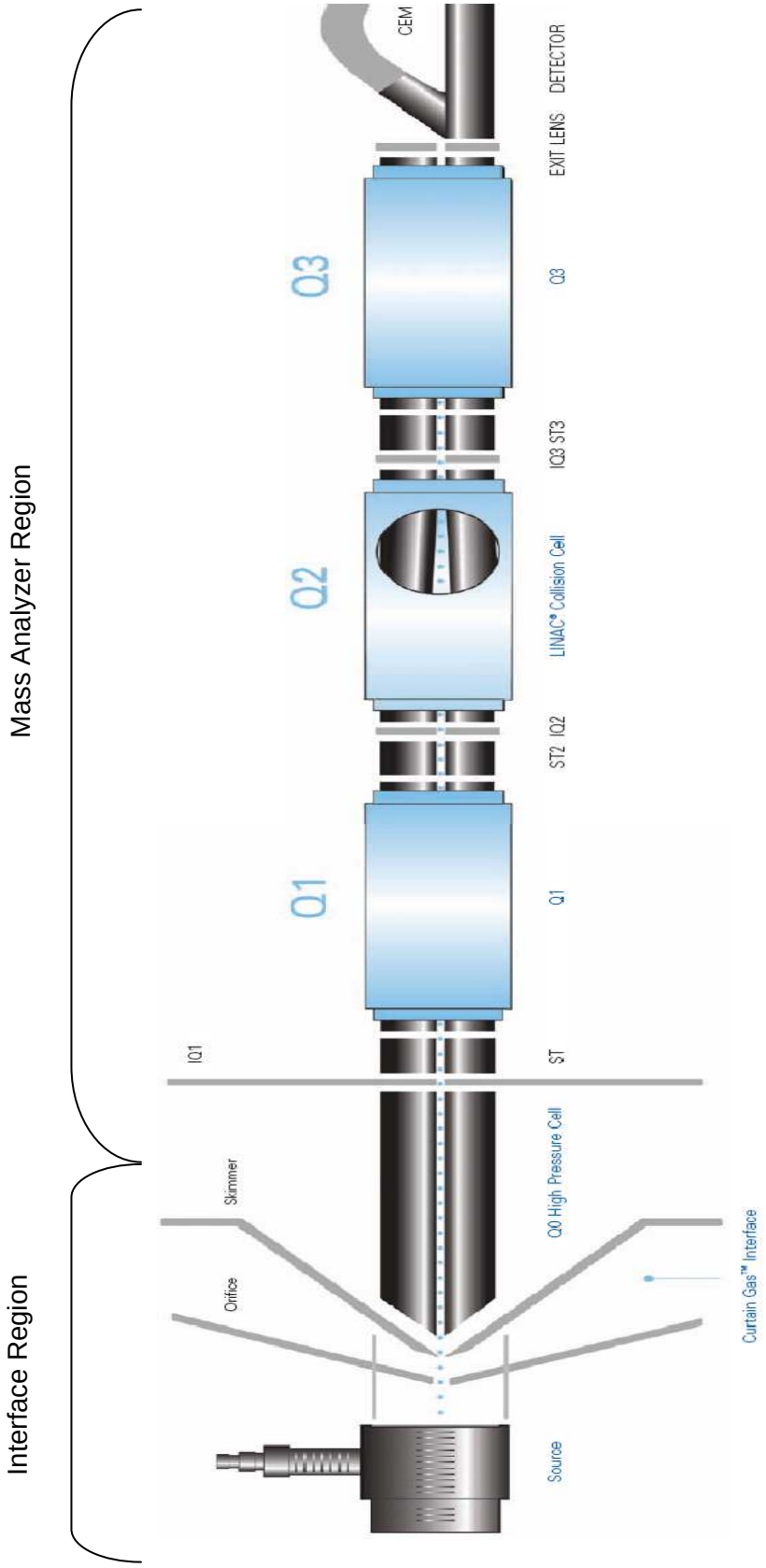


Figure 7-5 Schematic diagram of the ESI and Triple Quadrupole Mass Spectrometer. For MS/MS experiments, nitrogen collision gas was introduced into Q2 and RF voltage applied to Q2 [162].

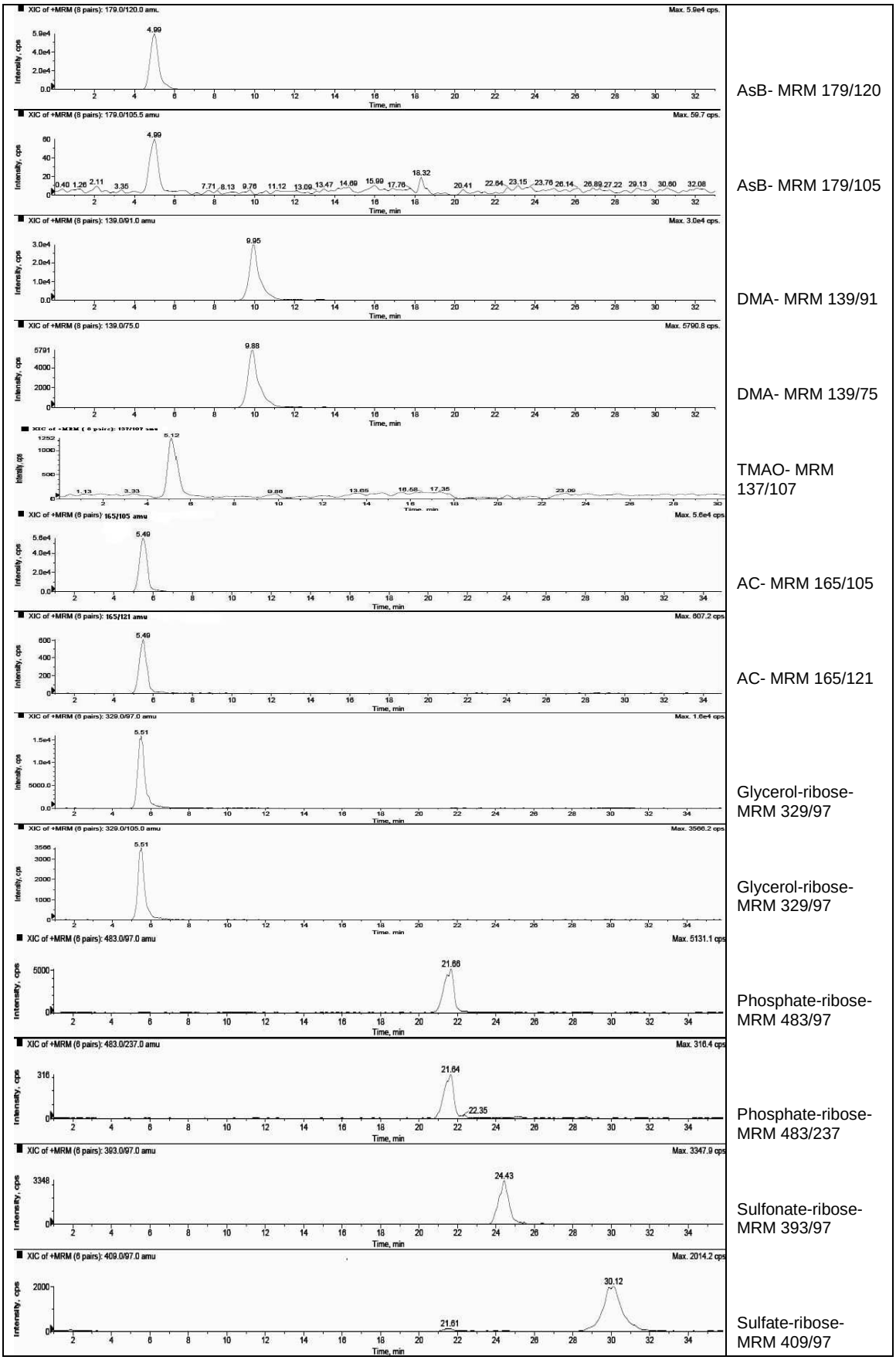


Figure 7-6 Exchanged ion chromatogram of organoarsenic species standard solution obtained by SAX cHPLC coupled to ESI-MS/MS in positive-ion MRM mode. Concentration: 50 ng/mL; ESI: 3.5 kV, DP: 50 V, CAD: 6 and 35 eV collision energy.

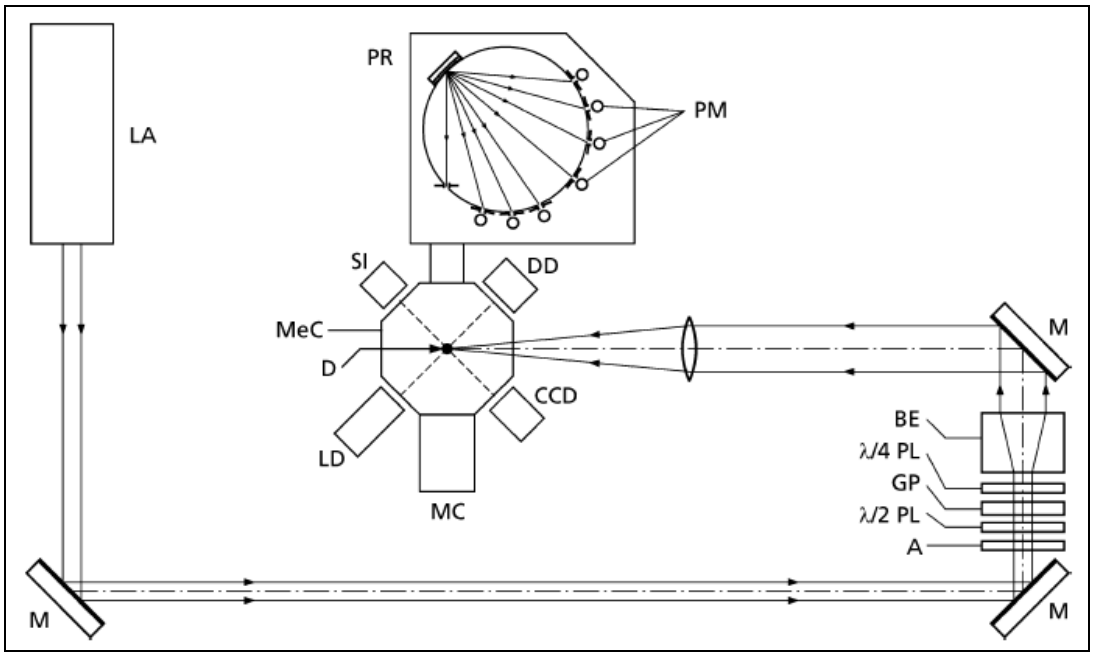


Figure 7-7 Optic mounting for LADROP-LIBS, where M=mirror, LA=laser, A=aperture, k /2 PL=k /2 plate, k /4 PL=k /4 plate, GP=Glan laser-polarizer, BE=beam expander, D=droplet, MC=mirror chamber, MeC=measurement chamber, CCD=CCD-camera for droplet observation, DD=droplet detector (photodiode), SI=stroboscopic illumination, LD=laser diode (part of the droplet detector), PM=photomultiplier (from reference [148]).

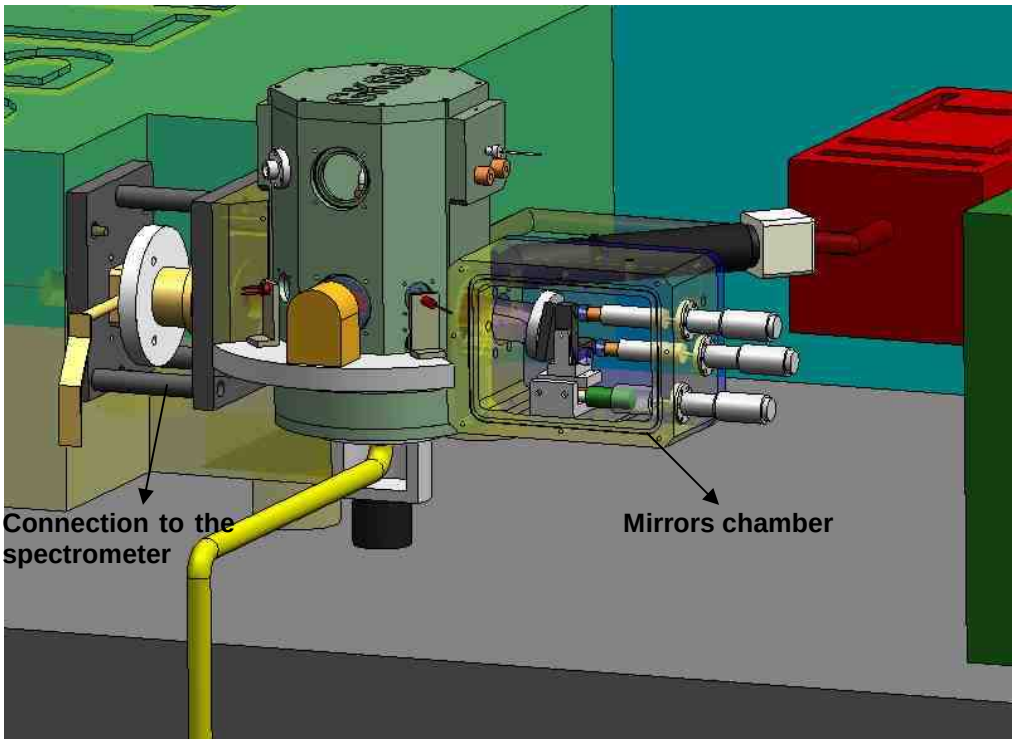


Figure 7-8 LADROP Measurement chamber.

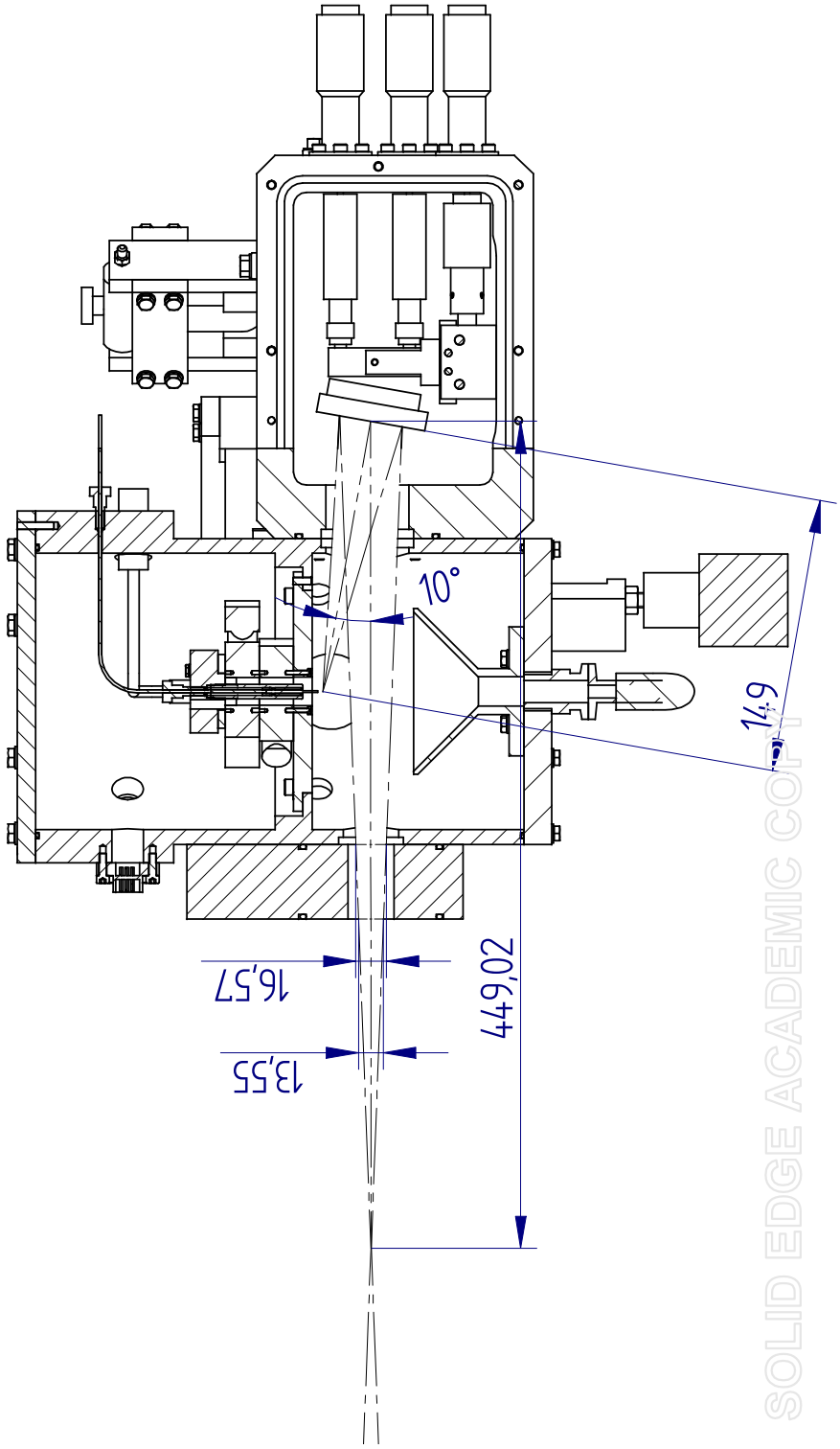


Figure 7-9 Cross section of LADROP measure chamber, (from reference [148]).

Table 7-3 List of all element lines installed in the Paschen–Runge spectrometer for LADROP-LIBS

Element	OBLF-wavelength	Grating order	Theoretical wavelength
P	178.28	1	178.28
S	180.7311	1	180.7311
Se	196.09	1	196.09
Zn	213.86	1	213.86
Cd	228.8018	1	228.8018
As	234.9839	1	234.9839
H	121.513	2	243.026
B	249.678	1	249.678
Si	251.61	1	251.61
Hg	253.6517	1	253.6517
O	130.2168	2	260.4336
Pt	265.945	1	265.945
Mg	279.5528	1	279.5528
N	149.262	2	298.524
Sn	317.502	1	317.502
Cu	324.75	1	324.75
C	165.701	2	331.402
Ti	337.28	1	337.28
Ni	341.476	1	341.476
Co	345.3508	1	345.3508
Cr	357.8684	1	357.8684
Fe	371.9935	1	371.9935
Al	394.4006	1	394.4006
Ca	396.8469	1	396.8469
Pb	405.78	1	405.78
Ar	420.0675	1	420.0675
V	437.92	1	437.92
H	486.1323	1	486.1323
Sb	259.8044	2	519.6088
Na	588.9951	1	588.9951