Arsenic speciation in biological samples using high performance liquid chromatography (HPLC) coupled with inductively coupled plasma mass spectrometry (ICP-MS) and Investigation of cellular uptake capabilities of arsenicals in different cell lines

Dissertation
zur Erlangung des Grades
"Doktor der Naturwissenschaften"
(Dr. rer. nat.)

am Fachbereich Chemie, Institut für Umweltanalytik
der Universität Duisburg-Essen
Essen, Deutschland

von

Sasan Rabieh, BSc, MSc (Hons)
geboren in Ahvaz, Iran

Essen, März 2007

1.Gutachter: **Prof. Dr. Alfred V. Hirner**

2.Gutachter: **Prof. Dr. Albert W. Rettenmeier**

Vorsitzender des Prüfungsausschusses: **Prof. Dr. R. Zellner**

Tag der Disputation: **08. Mai 2007**

DEDICATION

This thesis is dedicated to:

My supervisor, Prof. Dr. Alfred V. Hirner,

My parents,

&

My wife

Acknowledgements

I wish to express my gratitude to my supervisor, *Prof. Dr. Alfred V. Hirner*, for all his support, friendship, advice, excellent guidance, and valuable discussion throughout the entire projects of studies, particularly during the time of preparing the manuscripts and thesis. Dear Alfred, without your extensive support and dedication I would not be where I am today. Once again, thank you for everything.

I would also like to thank *Prof. Dr. Albert W. Rettenmeier*, my co-referee, for his comments and advice in an attempt to improve the text of my thesis and also for an opportunity to cooperate with his institute.

Thanks are also in order to *Dr. Louise Hartmann*, for all her support, assistance, advice, and motivation during my studies.

I would also like to extend my gratitude to *PD Dr. Elke Dopp* for her excellent cooperation, continuous support, and valuable discussion throughout our projects.

Many thanks to *Prof. Dr. Jörg Matschullat* (University of Mining and Technology, Freiberg, Germany) for providing the Brazilian urine samples.

Special thanks to my colleagues, *Roland Diaz-Bone and Jörg Hippler* for all their help and support since my arrival in Germany. I will never forget your much appreciated kindness and assistance.

I would also like to thank other colleagues, *Joachim*, *Jan*, *Lars*, *Jens*, *Frank*, *Sebastian*, *Marc*, *Maria*, *Martin, Margareta* and all other members of our institute for their support, and help during my studies.

My special thanks to *Dr. Ursula von Recklinghausen*, *Frau Zimmer*, and *Frau Zimmermann* at the Institute of Hygiene and Occupational Medicine, University Hospital of Essen for all their cooperation and assistance throughout our projects, which contributed greatly to their success.

I also have to thank *Prof. Dr. Jörg Feldmann* and his entire group at University of Aberdeen, Scotland while visiting them.

I would also like to take this opportunity to thank *Frau Birgit Wöstefeld* for all her help and assistances that she afforded me.

Financial support from the *Deutsche Forschungsgemeinschaft* (*DFG*), German research foundation, is highly appreciated.

Lastly, I would like to express my sincere thanks to *my parents*, *brothers*, and *sisters* for all their continuous support and encouragement as well as *my wife*, *Mozhgan*, for all her support, encouragement, and love.

CONTENTS

LIST OF FIGURES

LIST OF TABLES

Abbreviations

µL	microlitre
µm	micrometer
µM	micromolar
µmol	micromol
AES	atomic emission spectrometry
Ar	argon gas
As(III)	arsenite
As(V)	arsenate
AsB	arsenobetaine
AsC	arsenocholine
As-GSH	arsenic-glutathione complex
ATG	arsenic triglutathione
ATSDR	agency for toxic substances and disease registry
CE	capillary electrophoresis
CHO-9 cells	chinese hamster ovary cells
CRM	certified reference material
DMAs(III)	dimethylarsinous acid
DMAs(V)	dimethylarsinic acid
DMAsE	dimethylarsinoyl ethanol
DMEM	dulbecco's modified Eagles medium
DMPS	dimercaptopropane sulfonate
ESI	electrospray ionization
esp.	especially
FAAS	flame atomic absorption spectrometry
FCS	fetal calf serum
GC	gas chromatography
GSH	reduced glutathione
HCl	hydrochloric acid
HeLa S3 cells	human cervix adenocarcinoma cells
hGSTO-1	human glutathione-S-transferase-omega
HL-60 cells	human promyelocytic leukaemia cell line
HNO_3	nitric acid

HPLC	high performance liquid chromatography
ICP	inductively coupled plasma
ICP-AES	inductively coupled plasma- atomic emission spectrometry
ICP-MS	inductively coupled plasma-mass spectrometry
IPC	ion pair chromatography
kg	kilogram
LC	liquid chromatography
LD_{50}	amount of a substance given a single does, which causes death of 50% of a group of test animals
MADG	monomethylarsonic diglutathione
MEM	minimum essential medium
MeOH	methanol
mg	milligram
min	minute
m/z	mass to charge ratio
mL	millilitre
mm	millimeter
MMAs(III)	monomethylarsonous acid
MMAs(V)	monomethylarsonic acid
MS	mass spectrometry
MΩ.cm	megaohm×centimeter
ND	not detected
ng	nanogram
NPC	normal phase chromatography
PDH	pyruvate dehydrogenase
ppm	parts per million

Ra Hep	rat hepatocytes
RF	radio frequency
rmp	rotations per minute
RPC	reversed phase chromatography
SAM	S-adenosyl-L-methionine
SD	standard deviation
SEC	size exclusion chromatography
sec	second
SFC	supercritical fluid chromatography
TBAH	tertabutylammonium hydroxide
TMAs(III)	trimethylarsine
TMAsO	trimethylarsine oxide
t_R	retention time
UROtsa	normal human urothelium
V79	chinese hamster cell

List of publications

- Dopp, E., Hartmann, L. M., von Recklinghausen, U., Florea, A. M., Rabieh, S., Zimmermann, U., Yadav, S., Hirner, A. V., Rettenmeier, A. W. (2005). Forced uptake of trivalent and pentavalent methylated and inorganic arsenic and its cyto-/genotoxicity in fibroblasts and hepatoma cells. *Toxicological Sciences,* ***87(1)****, 46-56.*

- Dopp, E., Hartmann, L. M., von Recklinghausen, U., Rabieh, S., Hirner, A. V., Rettenmeier, A. W. (2006). Methylation, oxidation state and membrane permeability determine toxicity of arsenic compounds. *Naunyn-Schmiedebergs Archives of Pharmacology,* ***372****, 115.*

- Dopp, E., Hartmann, L. M., von Recklinghausen, U., Stueckradt, I., Pollok, I., Rabieh, S., Hirner, A. V., Rettenmeier, A. W. (2006).Uptake, subcellular distribution and toxicity of arsenic species in methylating and nonmethylating human cells. *Toxicology Letters*, ***164(Suppl 1****), S260.*

- Dopp, E., Hartmann, L. M., Florea, A. M., von Recklinghausen, U., Rabieh, S., Hirner, A. V., Rettenmeier, A. W. (2006). Trimethylantimony dichloride causes genotoxic effects in China hamster ovary cells after forced uptake. *Toxicology in Vitro,* ***20(6)****, 1060-1065.*

- Rabieh, S., Hirner, A. V., Matschullat, J. (2007). Determination of arsenic species in human urine of an arsenic-affected area in Brazil using high performance liquid chromatography (HPLC) coupled with inductively coupled plasma mass spectrometry (ICP-MS) *(under preparation).*

- Rabieh, S., Diaz-Bone, R. A., Hasenäcker, F., Kösters, J., Hirner, A. V. (2007). Investigation of monomethylarsonous acid [MMAs(III)] in Brazilian human urine by a combined liquid and gas chromatographic approach *(under preparation).*

- von Recklinghausen, U., Hartmann, L. M., Stueckradt, I., Pollok, I., Rabieh, S., Ping, Y., Nüssler, A., Katier, C., Hirner, A. V., Rettenmeier, A. W., Dopp, E. (2007). Subcellular distribution of anorganic and organic arsenic arsenic compounds in human urothelial cells compared to human hepatocytes (*under preparation).*

- Dopp, E., Hartmann, L. M., von Recklinghausen, U., Florea, A. M., Rabieh, S., Hirner, A. V., Obe, G., Rettenmeier, A. W. (2007). The cyto- and genotoxicity of organotin compounds is dependent on the cellular uptake capability. *Toxicology,* ***232(3)****, 226-234.*

In the case where a paper has already been published, then results and/or discussion briefly mentioned in the following text.

1 Introduction

Speciation of arsenic species in biological and environmental samples is important because bioavailability and toxicity of arsenic species strongly dependent on their chemical form (Gong et al., 2002 and Sakai et al., 2001). Thus, studies in the chemical forms of arsenic, their transformation, persistence and bioavailability are important in the understanding of levels of human exposure to arsenic. Arsenic is a human carcinogen that categorized first in a list of hazardous substances by the Agency for Toxic Substances and Disease Registry and the United States Environmental Protection Agency (ATSDR 2005). Chronic ingestion of high levels of arsenic in drinking water has been correlated with skin, bladder, and lung cancer, as well as hypertension and cardiovascular effects. However, mechanisms responsible for arsenic toxicity, metabolism and carcinogenic effects are not well understood. Speciation studies of arsenic in human urine have shown that dimethylarsinic acid (DMAs(V)) and monomethylarsonic acid (MMAs(V)) are the major human metabolites of inorganic arsenic species. These methylated arsenic species are a result of biomethylation, which is the major process of human arsenic metabolism in liver. Biomethylation of arsenic involves a stepwise reduction of pentavalent arsenic to trivalent arsenic followed by oxidative addition of a methyl group to the trivalent arsenic. In humans (Cullen and Reimer 1989) the process stops with the formation of DMAs(V) and probably dimethylarsinous acid (DMAs(III)). In microorganisms, the final product is mainly trimethylarsine oxide (TMAsO) with some trimethylarsine (TMAs(III)). Recently intermediates of monomethylarsonous acid (MMAs(III)) and DMAs(III), have been discovered in human urine that is direct evidence for biomethylation pathway (Aposhian et al., 2000a & 2000b; Sampayo-Reyes et al., 2000; Le et al., 2000a & 2000b; Del Razo et al., 2001; Mandal et al., 1997). Recent studies have also showed that the methylated trivalent arsenic species are as toxic as or even more toxic than the inorganic arsenic species (Styblo et al., 1997; Lin et al., 1999 & 2001; Thomas et al., 2001; Petrick et al., 2000 & 2001; Mass et al., 2001; Vega et al., 2001; Chen et al., 2003; Andrewes et al., 2003). Previously, the methylation of arsenic has been considered as a detoxification process because MMAs(V) (LD_{50} 800–1600 mg kg^{-1}) and DMAs(V) (LD_{50} 800–2600 mg kg^{-1}) are less toxic than the inorganic arsenic species (LD_{50} 10–20 mg kg^{-1}). However, there is a doubt whether arsenic biomethylation is strictly a detoxification process, since toxicity of intermediary arsenic metabolites (MMAs(III) and DMAs(III)) was found more in humans. There is much interest in the determination of MMAs(III) and DMAs(III) in studies of arsenic

metabolism and health effects. Thus an increasing demands for analytical techniques that are capable of speciation the arsenic metabolites in human and animal samples.

In has been considered that determination of total arsenic concentration is insufficient for clinical and environmental considerations. Therefore, for evaluation of the toxic potential to humans and the environment, it is necessary to investigate not only the total arsenic concentrations but also to differentiate each arsenic species.

1.1 Inductively Coupled Plasma Mass Spectrometry in Environmental Analysis

In recent years, most environmental scientists were interested in trace analysis of environmental samples. Currently, various elements, which were not used in past, are consumed in modern industries for production of new materials. New types of environmental pollution must be resulted due to disposal of industrial products. Therefore it requires analytical techniques that are able to rapid measuring of multielement simultaneously such as ICP-MS (Meyers 2000).

ICP-MS is combination of two analytical tools (inductively coupled plasma (ICP) and mass spectrometry (MS). This combination makes an instrument with powerful potential for multielement trace analysis. The main advantages of ICP-MS compared to other analytical methods are increased sensitivity, wide dynamic range, high selectivity, rapid multielement analysis, low background, and the ability to measure specific isotopes. In spite of mentioned advantages, it should be noted that ICP-MS has lower precision compared with inductively coupled plasma-atomic emission spectrometry (ICP-AES) and atomic absorption spectrometry (AAS). Also in ICP-MS, the total dissolved salts should be less than 1000 ppm. Other disadvantages of ICP-MS were included in matrix effects and instrument's cost.

1.1.1 Instrumentation

A schematic of the main components of a typical ICP-MS instrument is shown in Figure 1.1. The sample introduction into ICP was done using a nebulizer combined to a spray chamber. The sample was ionized in ICP and then charged ions were transferred to interface between plasma and mass analyser that consists of two cones named the sampling cone and the skimmer cone. Electrostatic lenses extract the positively charged ions from skimmer cone and focus the ion beam in a suitable form for transmission through a mass filter. The quadrupole mass filter transmits the ions of a particular mass to charge ratio (m/z) to an ion detector.

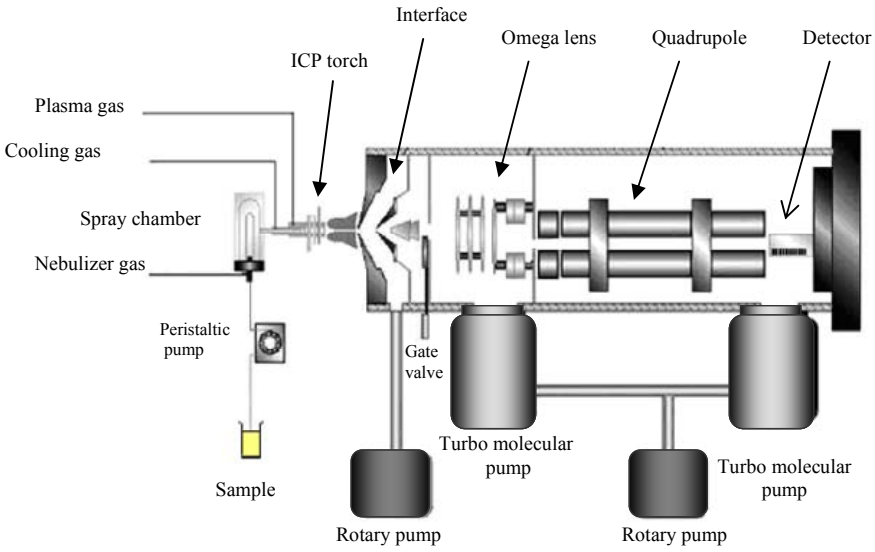


Figure 1.1 Schematic of a typical ICP-MS instrument (modified from the original drawing provided by Agilent Technologies).

The ICP torch (Figure 1.2) is consisting of three concentric tubes and located inside a copper coil that is connected to a radiofrequency (RF) generator. The plasma is generated at the end of the quartz torch via a tesla spark and maintained by using high frequency electromagnetic field. The RF flows in the copper coil and induces oscillating magnetic fields. Electrons and ions flow at rapid acceleration rates while pass through the oscillating electromagnetic field. Then accelerated electrons and ions ionized further due to collision with unionized argon gas (Meyers 2000).

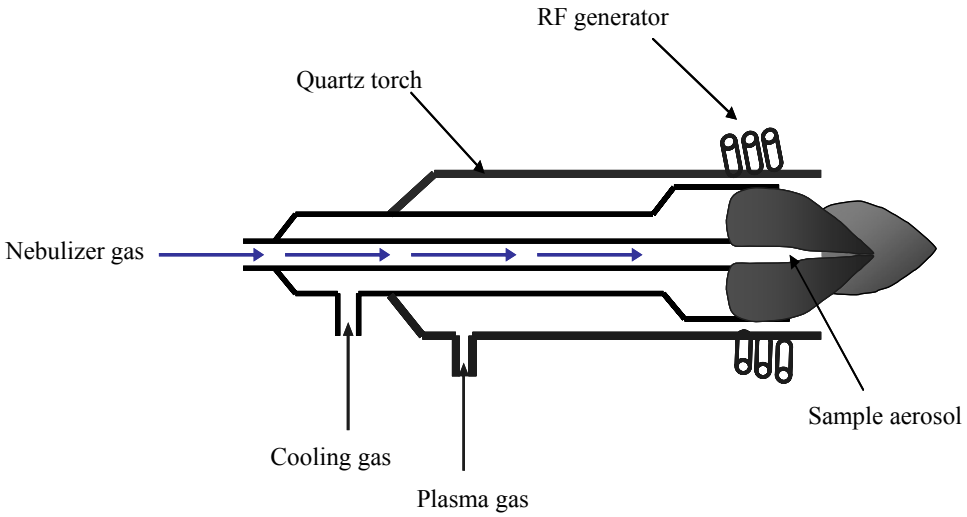


Figure 1.2 Schematic diagram of an ICP system.

1.2 Liquid Chromatography

The basis of liquid chromatography (LC) is a liquid mobile phase. Separation in liquid chromatography are carried out by introducing sample onto a column filled with a solid stationary phase while a liquid mobile phase is pumped through the column continuously. In classic liquid chromatography, introduced by Tswett in 1906, were applied glass columns with inner diameter of 1 to 5 cm and a length of 50 to 500 cm. However the separations were long and attempts by using a vacuums or pumps had not any improvement. Later it was recognized that a higher performance could only be achieved by reduction of the particle size of stationary phase. The practical improvement resulted at the end of the 1960s by introducing particles with diameters of up to 3 or 10 μm (Kellner et al., 1998).

In early LC stationary phases a liquid stationary phase coated onto a solid support, but modern LC stationary phases are usually comprised of chemically modified silica or polymers. The analytes during passing through the column interact with both the stationary and the mobile phases. The time that each analyte resides in the column depends on interaction of the analyte with both phases. Thus, separation is based on different between residual times of analyts in column (Cornelis et al., 2003). LC is applied for separation non-volatile compounds as well as the compounds that decompose at high temperatures. There is a variety of stationary phases are commercially available. Furthermore, separations can be further enhanced by the addition of additives to the mobile phase. Usually, minimum sample preparation is required and LC can readily interface to ICP-MS.

1.2.1 Effecting Variables in Liquid Chromatography Separation

For optimization of LC separation should be considered a variety of variables. The interaction analyte with both stationary and mobile phases are based on dipole forces, electrostatic attractions and hydrophobic interactions. Thus, knowledge of structure of analyte is useful in prediction of its behaviour during the separation. Generally, compounds that have different polarity or chemical structure separate easier. The selection of the stationary phase is usually based on the nature of the analytes for example; anionic species would be separated using an anion exchange column. The analytes may derivative before the separation to make easy separation or detection (Toyo'oka 1999; Liu and Lee 1999). The composition of mobile phase is an important variable in separation. The mobile phase may consist of one component such as methanol, or a mixture of solvents and/or aqueous solutions such as buffers. The sample must be soluble in the mobile phase to prevent the precipitation of sample within the

column. For better separation chiral additives, ion pair reagents, and surfactants may be added to get a better separation. The composition of the mobile phase alters during the separation in gradient separations but in isocratic separations the mobile phase composition is held constant throughout the separation. Gradient separations are often utilized to decrease the time of separation or better resolution. The flow rate of the mobile phase is also a significant variable because of its effects on the time of separation. Increasing the flow rate will decrease the separation time. It should also be considered the method of detection while optimization of the flow rate. Fluctuations in temperature may change retention time of analyte. Thus HPLC columns are frequently housed in water jackets or ovens to control the column temperature. Generally, increases in temperature cause a decrease in analyte retention time due to reduction of mobile phase viscosity and increase the rates of diffusion.

1.2.2 Characteristic Parameters of a Chromatogram

The term retention time (t_r) is defined as the time that analyte take to pass through the column and reach the detector. Separation is based on difference between retention time of sample and standard. The capacity factor, k', represents the normalized retention time for an analyte and is calculated using equation (1.1)

$$k' = \frac{(t_r - t_0)}{t_0} \qquad (1.1)$$

Where t_r is retention time of analyte and t_o is retention time of a analyte that is not retained by the stationary phase.

The selectivity factor shows ability of system in separation of two analytes and is indicated by α in chromatography. The selectivity is the ratio of capacity factor of two analytes.

$$\alpha = \frac{k_1'}{k_2'} \qquad (1.2)$$

The resolution, R_s, shows the efficiency of a separation. The resolution is calculated by dividing the distance between two chromatographic peaks by the average of their widths at the base.

$$R_s = \frac{\Delta t_r}{\frac{1}{2}(w_{t1} + w_{t2})} \qquad (1.3)$$

Analyte peaks in LC separations are Gaussian shape and a mathematical expression for the symmetry of a chromatographic peak is the peak asymmetry factor, A_s. The ideal peak in LC should be narrow and have a peak asymmetry factor of 1.

Two terms that are frequently used in LC are plate height (*H*) and plate count (*N*). These terms relate to a column's chromatographic efficiency.

The plate height is determined by dividing the length of the stationary phase, *L*, by the plate count.

$$H = \frac{L}{N} \qquad (1.4)$$

One method for calculation of *N* is to determine the width peak at half of maximum height, $W_{1/2}$, and replace in this equation (1.5).

$$N = \frac{5.54\, t_r^2}{W_{1/2}^2} \qquad (1.5)$$

1.2.3 Liquid Chromatographic Stationary Phases

1.2.3.1 Normal Phase Chromatography (NPC)

Normal phase chromatographic systems are involving a polar stationary phase and a nonpolar mobile phase such as hexane. In earlier NPC was frequently used unmodified silica or alumina but the obtained peak shapes were often broad and irreproducible retention times. The problems were improved with modified stationary phases that have a polar functional group such as cyano that is chemically bonded to the silica. In spite of NPC allows analytes that are insoluble in polar solvents to be separated but it has limited application in the area of elemental speciation. The major limitation of this technique is the nonpolar mobile phase, which is incompatible with most elemental detectors used in speciation analysis and also the nature of the nonpolar mobile phase, in which many of real samples are not soluble.

1.2.3.2 Reversed Phase Chromatography (RPC)

RPC is one of LC techniques that widely was used in elemental speciation. It is used to separate nonpolar and/or slightly polar species. The polar mobile phases used are typically aqueous or a mixture of water and an organic modifier such as methanol or acetonitrile. The stationary phase is silica with silanol-OH group replaced by alkyl chains. Separation is based on the hydrophobicities of the species. Separations may be influenced by changing variables such as functional group of stationary phase, pH, ionic strength, organic modifiers, and the gradient program. The pH of the mobile phase is an important parameter in RPC which affects the charge of the analyte and its retention on the column. Traditional silica stationary phases

cannot tolerate pHs below 2 or above 7 due to hydrolysis of the stationary phase. Recently, manufacturers have produced silica-based reversed phase stationary phases that can tolerate pHs of 2 to 10. Reversed phase stationary phases may also be made of polymeric materials, but silica-based columns are still the most commonly used.

1.2.3.3 Reversed Phase Ion Pair Chromatography (IPC)

Reversed phase ion pair chromatography has similar stationary phases to RPC but an ion pair reagent is added to the mobile phase. An ion pair reagent is a salt including a cationic or anionic polar head group and a nonpolar tail such as sodium alkyl sulfonate and tetraalkyl ammonium salts. IPC can simultaneously separate anions, cations, and noncharged species. The separation mechanism in IPC has not completely understood. One theory states that an ionic analyte is electrostatically attracted to the charged portion of the ion pair reagent. Then charge neutralization causes the charged analyte to be retained by the nonpolar stationary phase. Another theory is that the hydrophobic portion of the ion pair reagent adsorbs to the stationary phase and a pseudo ion exchange stationary phase is formed. Thus, charged species interact with the pseudo ion exchange stationary phase (Skoog and Leary , 1992).

1.2.3.4 Ion Exchange Chromatography (IEC)

IEC is used to separate free ions and ionisable species. The stationary phase consists of a substrate such as a polystyrene-divinylbenzene polymer or silica which has an ionic functional group such as quaternary ammonium or sulfonate. IEC is commonly divided to cation exchange chromatography and anion exchange chromatography depending on the functional group of stationary phase. Analytes will interact with the stationary phase electrostatically if they have opposite charge to charge of functional group of stationary phase. The retention time of an analyte depends on electrostatic force. Mobile phases used in IEC separations are typically aqueous solutions of inorganic salts. IEC is compatible with element selective detectors such as an ICP-MS. Also IEC can be utilized for the purpose of sample cleanup prior to analysis and sample preconcentration.

1.3 High Performance Liquid Chromatography (HPLC)

HPLC is a widely separation method in analytical chemistry and speciation analysis. Many different HPLC stationary phases are commercially available. In fact HPLC is a subset of liquid chromatography. In HPLC the stationary phase particles have small diameters with order of 3-5 μm. The mobile phase is pumped at high pressure through the stationary phase. Pumping a viscous liquid through the column packed with small very particle produce high pressure. Therefore, HPLC is also called "high pressure liquid chromatography".

Both the mobile and the stationary phases may be modified in order to achieve the desired separation. The most common mobile phases are buffer solutions which mixed with organic solvents like methanol or acetonitrile. The solutions have always to be degassed before experiment to avoid formation of air bubbles in the system. All mentioned variables that are effective in LC separation (pH, temperature, concentration of the mobile phase constituents, and flow rate) must be considered for optimization of HPLC system. HPLC can be easily interfaced with element-specific detectors such as ICP-MS, ICP-AES and FAAS since the sample introduction flow rate of such detectors comparable to those employed for the elution (0.5 – 2.0 $mL.min^{-1}$).

1.3.1 Instrumentation

The components of a *HPLC unit* are included a reservoir containing mobile phase, a pump to pressurize the liquid mobile phase, an injector for injection of a small volume of the sample under high pressure, a separation column containing stationary phase, a detector to detect components that leave the column, and finally recorder for gathering the detector signal. Structure of a HPLC unit is simply shown in Figure 1.3.

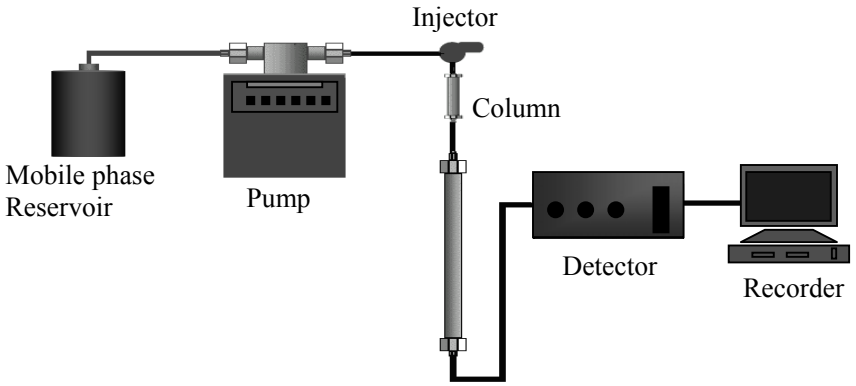


Figure 1.3 Structure of a HPLC unit.

1.4 Hyphenated (Coupling) Techniques

Hyphenated techniques play an important role in online chromatographic separation or enrichment of elements prior to detection. Hyphenated techniques are favourably applied for the determination of elemental species at trace levels. Coupling separations as online compared with offline techniques showed much better repeatability of analytical results, minimized contamination, and they can be run automatically (Heumann 2002). The hyphenated systems that are frequently applied for elemental speciation are coupling of HPLC or GC with an atomic spectrometric method as well as capillary electrophoresis (CE). Inductively coupled plasma atomic emission spectrometry (ICP-AES) and ICP-MS have much attention due to their capability in multi elemental analysis. The coupling of HPLC with ICP-MS offers some advantages such as high sensitivity; capability of multi-element analysis, wide dynamic range, and isotope ratio measurement capability (Gong et al., 2002). A wide range of applications of HPLC-ICP-MS for arsenic speciation has been reported (Mandal et al., 2001; Le et al., 1998; Nakazato et al., 2000; Mattusch and Wennrich 1998; Guerin et al., 2000; Saverwyns et al., 1997; Moldovan et al., 1998; Woller et al., 1998; Saeki et al., 2000). ICP-AES usually allows element detection in range of ng mL^{-1} but ICP-MS is able to detect many elements in range of pg mL^{-1}. The interfacing of HPLC or GC with ICP-AES and ICP-MS detection are relatively simple (e.g. in Rottmann and Heumann 1994; Gallus and Heumann 1996). The most frequent application of hyphenated techniques in elemental analysis is speciation, which is important in environmental sciences, clinical chemistry, toxicology, and nutrition. Figure 1.4 shows the hyphenated techniques using ICP-MS as detection (Szpunar 2005)

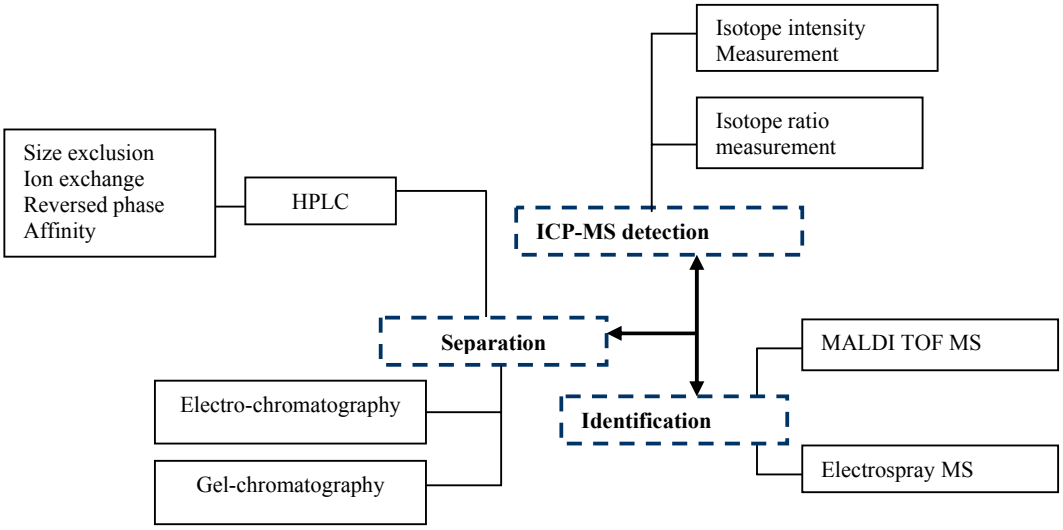


Figure 1.4 Hyphenated systems using ICP-MS detection (modified from the original schematic provided by Szpunar 2005)

1.5 Definition of Metal Speciation

Metal speciation is the different chemical and physical form of metals in the environment. It includes all form of metals such as dissolved metal ions, complexes, colloids, metal into or adsorbed on particle or metal solids which present in the sediment. The environmental chemistry of arsenic is complex because arsenic has different chemical forms in environmental samples and different toxicological effects on the environment (Reeve 1994).

1.5.*1 Sources of Arsenic in the Environment*

Elemental arsenic (As) has an atomic number of 33 and an atomic mass of 74.91. Arsenic is a metalloid widely distributed in the earth's crust. It can exist in four valency states; –3, 0, +3, and +5. Elemental arsenic and arsine (–3) can exist in strongly reducing environments only. Arsenite (+3) may be the main form under moderately reducing conditions but arsenate (+5) is generally the stable in oxygenated environments. Arsenic and its compounds usually occur in trace quantities in all rocks, soil, water and air. Soils may contain arsenic levels between 0.1 and 40 ppm (Yan-Chu 1994; WHO 1981). Arsenical salts have different aqueous solubility depending on the pH and the ionic environment (Gomez-Caminero et al., 2001).

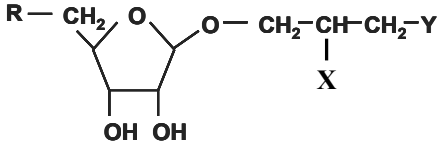
Arsenic is present in more than 200 mineral species, the most common is arsenopyrite. It has been estimated that about one-third of the atmospheric arsenic has natural origin. Volcanic action is the most important natural source of arsenic. Inorganic arsenic of geological origin contained in drinking-water in several parts of the world, for example Bangladesh. Organic arsenic compounds such as arsenobetaine, arsenocholine, tetramethylarsonium salts, arsenosugars and arsenic-containing lipids are mainly found in marine organisms. Some arsenic species present in biological samples are listed in Table 1.1.

Numerous man-made arsenic compounds as used in agriculture gradually accumulated in the soil (Azcue and Nriagu 1994). The use of pesticides containing arsenic and arsenic in the preservation of timber has also led to contamination of the environment. It has been estimated that 70% of the world arsenic production is used in timber treatment as copper chrome arsenate (CCA), 22% in agricultural chemicals, and the rest in glass, pharmaceuticals and non-ferrous alloys. Different sources of human exposure to arsenic and resulted toxicity is shown in Figure 1.5.

Arsenic is detected at a significant concentration [> 1 mg As per litre (1ppm as As)] in several human foods such as fish, shellfish, mushrooms, and algae (Byrne et al., 1995; Cullen and Reimer, 1989; Edmonds and Francesconi, 1993; Francesconi and Edmonds, 1997; Lai et al., 1997; Shibata and Morita, 1992).

Table 1.1 List of some arsenic species present in biological samples

Name	Abbreviation	Chemical formula
Arsenite, Arsenous acid	As(III)	$As(OH)_3$
Arsenate, Arsenic acid	As(V)	$AsO(OH)_3$
Monomethylarsonic acid	MMAs(V)	$CH_3AsO(OH)_2$
Monomethylarsonous acid	MMAs(III)	$CH_3As(OH)_2$
Dimethylarsinic acid	DMAs(V)	$(CH_3)_2AsO(OH)$
Dimethylarsinous acid	DMAs(III)	$(CH_3)_2AsOH$
Trimethylarsine oxide	TMAsO	$(CH_3)_3AsO$
Trimethylarsine	TMAs(III)	$(CH_3)_3As$
Arsenobetaine	AsB	$(CH_3)_3As^+CH_2COO^-$
Arsenochline	AsC	$(CH_3)_3As^+CH_2CH_2OH$
Tetramethylarsonium ion	Me_4As^+	$(CH_3)_4As^+$
Dimethylarsinoyl ethanol	DMAsE	$(CH_3)_2AsOCH_2CH_2OH$
Arsenosugars	X, XI, XII, XIII, XIV, XV	see structure below



	R	X	Y
X	$(CH_3)_2As(O)$-	-OH	-OH
XI	$(CH_3)_2As(O)$-	-OH	$-OPO_3HCH_2CH(OH)CH_2OH$
XII	$(CH_3)_2As(O)$-	-OH	$-SO_3H$
XIII	$(CH_3)_2As(O)$-	-OH	$-OSO_3H$
XIV	$(CH_3)_2As(O)$-	$-NH_2$	$-SO_3H$
XV	$(CH_3)_2As^+$-	-OH	$-OSO_3H$

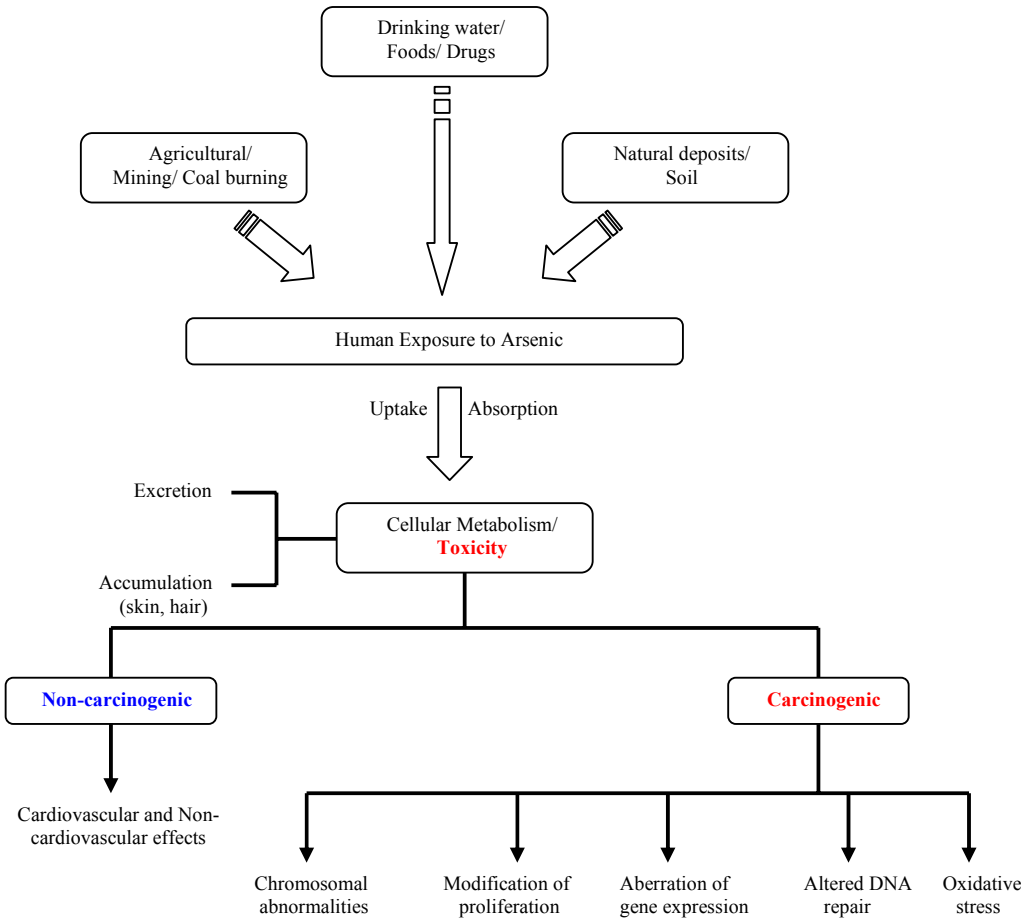


Figure 1.5 Sources of human exposure to arsenic and resulted toxicity (modified from Roy and Saha 2002)

1.5.2 Biomethylation of Arsenic

The term biomethylation describes the formation of both volatile and nonvolatile methylated compounds of metals (Bentley and Chasteen 2002). The major volatile arsenic compounds formed by biomethylation have the structure $(CH_3)_nAsH_{3-n}$; for n =1, 2, and 3, the products are mono-, di-, and trimethylarsine, respectively. The major nonvolatile arsenic compounds are methylarsonate and dimethylarsinate. Biomethylation of arsenic happens not only in microorganisms but also in algae, plants, animals, and humans.

Biomethylation of arsenic is the major metabolic pathway for the metabolism of inorganic arsenic humans and other mammals (Lu et al., 2003). The full pathway as seen for many fungi involves the reduction of a pentavalent arsenic species to a trivalent arsenic species followed by the addition of a methyl group to the trivalent arsenic (Challenger 1945; Cullen et al., 1984 & 1989). Specifically, as shown in Figure 1.6, first inorganic arsenate

As(V) is reduced to arsenite As(III) and then As(III) is oxidatively methylated to monomethylarsonic acid (MMAs(V)). MMAs(V) is then reduced to monomethylarsonous acid (MMAs(III)) and further methylated to dimethylarsinic acid (DMAs(V)). Similarly, further steps produce dimethylarsinous acid (DMAs(III)) and trimethylarsine oxide (TMAsO), which can be reduced to trimethylarsine (TMAs(III)).

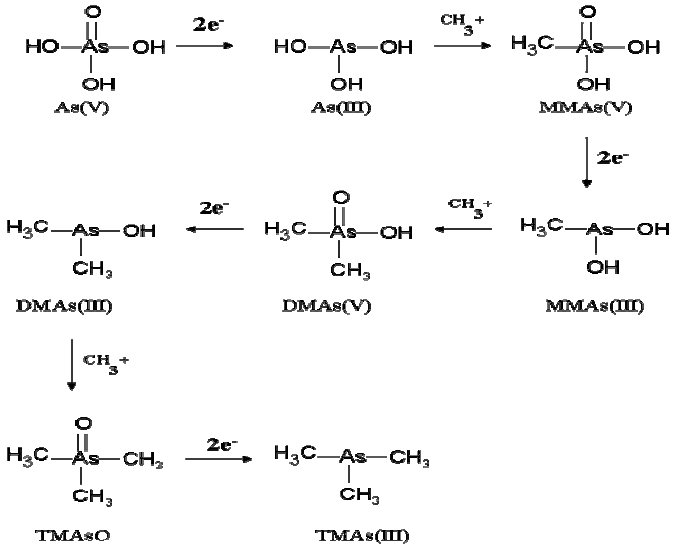


Figure 1.6 Pathway of arsenic methylation, showing alternate steps of two-electron reduction ($2e^-$) and oxidative addition of a methyl group (CH_3^+).

In past has been considered that the metabolic pathway did not usually continue beyond than the dimethylarsenic species in humans and most animals (Cullen and Reimer 1989). Thus biomethylation of arsenic has previously been considered a detoxification process. However, this suggestion was changed with finding trivalent methylation metabolites, MMAs(III) and DMAs(III) in human urine (Aposhian et al., 2000a & 2000b; Sampayo-Reyes et al., 2000; Le et al., 2000a & 2000b; Del Razo et al., 2001; Mandal et al., 1997). The numerous studies showed these metabolites have higher toxicity than the inorganic arsenic species (Styblo et al., 1997; Lin et al., 1999 & 2001; Thomas et al., 2001; Petrick et al., 2000 & 2001; Mass et al., 2001; Vega et al., 2001; Chen et al., 2003; Andrewes et al., 2003). It is now believed that the methylation of inorganic arsenic may not be a detoxification mechanism and could be an activation process (Kitchin 2001; Hughes 2002; Styblo 2002). As a consequence, much attention has been paid to studies of the toxic effects of these metabolites.

Recently it has been suggested that arsenic-glutathione (As-GSH) complexes are directly involved in the methylation of inorganic arsenic metabolites (Hayakawa et al., 2005).

Therefore, a new metabolic pathway has been presented (Figure 1.7), in which trivalent metabolites are converted to the less toxic pentavalent species. This new pathway is in agreement with the concept that oxidation of arsenic is detoxification. In brief, in the new pathway, both arsenic triglutathione (ATG) and monomethylarsonic diglutathione (MADG) are substrates for human arsenic methyltransferase Cyt19. Arsenite is metabolized and converted to DMAs(V) by Cyt19 via formation of As-GSH complexes.

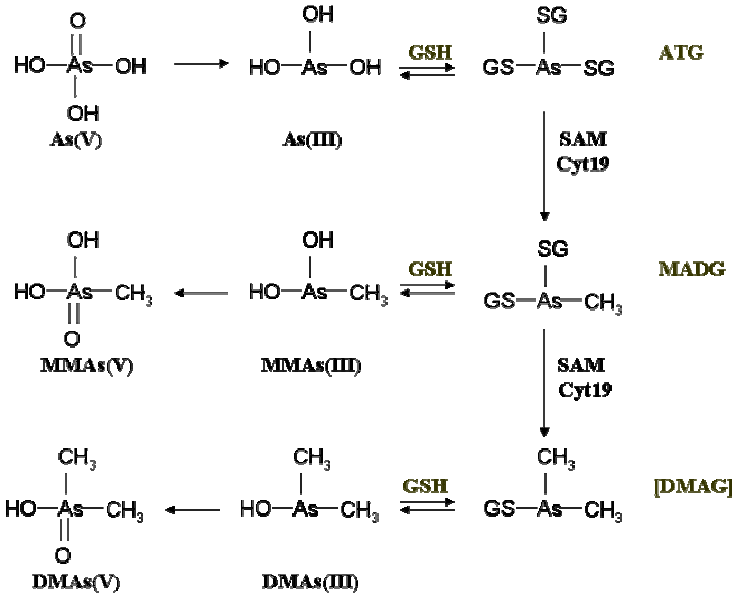


Figure 1.7 A new metabolic pathway of inorganic arsenic via arsenic–GSH complexes (presented by Hayakawa et al., 2005)

1.5.3 Cytotoxicity and Toxicity

The toxicity of arsenic is dependent on its chemical species. Recently has shown that trivalent methylated arsenicals are potent cytotoxins in cultured primary rat hepatocytes. In these cells, both methylarsine oxide (MeAs(III)O) and dimethylarsinous iodide ($Me_2As(III)I$) were significantly more cytotoxic than are arsenite, arsenate, methylarsonic acid and dimethylarsinic acid (Styblo et al., 1999b, 2000). Similarly (Table 1.2), in primary human hepatocytes and in cell lines that are derived from tissues (skin, lung, and urinary bladder), both methylarsine oxide and dimethylarsinous iodide are potentially cytotoxic (Styblo et al., 1999b, 2000).

Table 1.2 Cytotoxicity in rat and human cells of arsenicals containing trivalent arsenic[a]

	Estimated LD_{50} (μM)		
Cell type	$NaAs(III)O_2$	MeAs(III)O	($Me_2As(III)$)-GS
Primary rat hepatocytes	~ 10	2.8	14.5
Primary human hepatocytes	> 20	5.5	> 20
Human epidermal keratinocytes	> 20	2.6	8.5
Human bronchial epithelial cells	3.2	2.7	6.8
Urotsa cells	17.8	0.8	14.2

[a] Estimated LD_{50} (concentration of an arsenical that results in 50% decrease in cell viability) as determined by formazan dehydrogenase (MTT) assay after a 24-h exposure to these arsenicals. Estimates based on cell survival assays in which triplicate or quadruplicate assays of cell viability were performed for each concentration of arsenical tested. The Urotsa cell line is derived from SV40-transformed human bladder epithelial cells. All data are taken from Styblo *et al.* (2000).

In a human liver cell line (Chang cells), MMAs(III) showed more potent cytotoxin than arsenite, arsenate, methylarsonic acid, or dimethylarsinic acid (Petrick *et al.*, 2000). In an in vitro assay contains rat liver cytosol showed that both MMAs(III) and DMAs(III) were associated with specific cytosolic proteins. While, pentavalent methylated arsenicals ((MMA_S(V) and DMAs(V)) were not bound to specific cytosolic proteins (Styblo and Thomas, 1997a). It suggests that generated MMAs(III) and DMAs(III) during the arsenic metabolism in the cell interact with cytosolic proteins and may produce toxic effects.

Modes of cell death have been examined in mouse macrophages exposed to arsenate, arsenite, methylarsonic acid, dimethylarsinic acid, or trimethylarsine oxide ($(CH_3)_3AsO$) (Sakurai et al., 1998). Both arsenate and arsenite were relatively potent cytotoxins with 50% inhibitor concentrations (LD_{50}) of 500 and 5 μM, respectively). In contrast, the methylated arsenicals were much less potent cytotoxins ($LD_{50} \geq 5$ mM for all species). In a human promyelocytic leukemia cell line (HL-60 cells), exposure to 1-5 mM dimethylarsinic acid induced apoptotic but exposure to up to 50 mM methylarsonic acid has not any effect (Ochi et al., 1996). Apoptosis was also induced in this cell line by arsenate (0.1 mM) and by arsenite (0.02 and 0.05 mM). In comparison, As_2O_3 induces differentiation and apoptotic death in various leukemic cell lines (Zhu et al., 1999) and is now used therapeutically in acute promyelocytic leukaemia (APL). It is possible that either methylarsonous acid or dimethylarsinous acid could be more efficacious in the treatment of APL and their effects on the viability, proliferation and differentiation of leukemic cells are now under investigation.

Aposhian and coworkers (Petrick et al., 2001) have compared the acute toxicity of sodium arsenite and methylarsonous acid in hamsters. The estimated LD_{50} for arsenite was 112 μmol per kg; for methylarsonous acid 29.3 μmol per kg. This report that methylated arsenicals containing As(III) are more toxic than arsenite in animal.

1.5.4 Methylated Metabolites of Arsenic

As mentioned before, it is believed that the methylation of arsenic in human may be a toxification pathway because finding trivalent methylation metabolites in human urine that have more toxicity than inorganic arsenicals. Thus current interest has been centred on studies of the toxic effects of the trivalent methylated species of arsenic MMAs(III), DMAs(III). TMAs(III) may be formed by reductive processes from pentavalent arsenicals, exist in higher concentrations in rat tissues than in human tissues.

1.5.4.1 MMAs(III)

Recently, MMAs(III) has been found in urine of humans exposed to arsenic without (Aposhian *et al.*, 2000a) and with concomitant treatment with chelators (Aposhian et al., 2000b). MMAs(III) is an excellent choice as arsenic carcinogenesis because humans excrete much more MMAs than other species. It could be the reason that humans are unusually sensitive to arsenic-induced carcinogenesis. Some of the biological activities MMAs(III) are inhibition of GSH reductase (Styblo et al., 1997), thioredoxin reductase (Lin et al.,1999) cell toxicity (Petrick et al., 2000), and genotoxicity (Mass et al., 2001). This certainly is an impressive list of biological activities for a methylated arsenic metabolite that formerly thought to be a "detoxified" form of arsenic.

1.5.4.2 DMAs(III)

The presence of DMAs(III) and MMAs(III) has been demonstrated in human urine of arsenic-exposed humans administered 2,3- dimercapto-1-propane sulfonate (a chelator) (Le et al., 2000). In a study of hamsters given arsenate (Sampayo-Reyes et al., 2000), substantial hepatic concentrations of trivalent MMAs and DMAs were found in addition to the expected pentavalent MMAs and DMAs. The capacity to separate the trivalent forms of the methylated arsenic species from the pentavalent forms has recently been developed (Del Razo et al., 2000; Sampayo-Reyes *et al.*, 2000; Le *et al.*, 2000). Some of the biological activities that

DMAs(III) is known to possess in various experimental systems are similar to MMAs(III). At physiological pH, DNA is negatively charged and many arsenic chemical forms have also negative charge. The negatively charged arsenic forms would not be expected to easily interact with DNA because of electrostatic repulsion. When arsenic is successively methylated, the ionisable hydroxy group are replaced by uncharged methyl group. Thus, a molecule of DMAs(III) may be able to directly interact with DNA more easily than trivalent arsenite.

1.5.4.3 TMAs(III)

TMAs(III) may be produced from TMAsO by reduction. It has been suggested that only rats may have relatively high levels of TMAsO in their urine. Rats are also the most responsive animal model for arsenic carcinogenesis. A molecule of TMAs(III) possesses no ionizable hydroxyl group to limit the ability of this trivalent arsenic species to interact with DNA.

1.5.5 Arsenic and Metabolites in Urine

Since arsenic is rapidly metabolized and excreted into the urine, total arsenic, inorganic arsenic and the sum of arsenic metabolites (inorganic arsenic + MMAs + DMAs) in urine have all been used as biomarkers of recent arsenic exposure. In common with other biomarkers of arsenic exposure, levels of arsenicals in urine may be a consequence of inhalation exposure or ingestion of arsenic from drinking-water, beverages, soil or foodstuffs (NRC, 1999). However, in the case of exposure to arsenic compounds of low solubility, e.g. GaAs, urinary arsenic will reflect the absorbed dose, but not the inhaled amount (Yamauchi et al., 1989). In many older studies, total urinary arsenic was used as a biomarker of recent arsenic exposure. However, this is increasingly uncommon because organoarsenicals present in substantial amounts in certain foodstuffs are also excreted in urine. For example, the practically non-toxic compound arsenobetaine is present in mg/kg levels in seafood and excreted mainly unchanged in the urine (Kaise and Fukui 1992; Le et al., 1993, 1994). In controlled experiments (e.g. Arbouine and Wilson 1992; Buchet et al., 1994 & 1996), it has been found that consumption of seafood (e.g. marine fishes, crustaceans, bivalves, seaweeds) by human volunteers is associated with increased total urinary arsenic excretion. Under these conditions, assessment of inorganic arsenic exposure using total urinary arsenic would result in overestimation of inorganic arsenic exposure. To avoid the potential for overestimation of

inorganic arsenic exposure inherent in using total urinary arsenic, most studies now measure speciated metabolites in urine and use either inorganic arsenic or the sum of arsenic metabolites (inorganic arsenic + MMAs + DMAs) as an index of arsenic exposure. Relatively recently it has been found that adding all arsenic metabolites together can give misleading results unless a careful diet history is taken and/or seafood consumption is prohibited for 2–3 days before urine collection (Buchet et al., 1996). There are two reasons for this: First, some seafood, especially bivalves, contain the arsenic metabolites MMAs and DMAs, particularly DMAs, in fairly high amounts (Velez et al., 1996). Secondly, arsenosugars present in seaweeds and some bivalves are extensively metabolized (either by the body itself or by the gut microbiota) to DMAs, which is then excreted in urine (Le et al., 1994; Ma and Le, 1998). The issue of the extent to which consumption of seafoods and other foods can compromise the estimation of inorganic arsenic exposure by the measurement of arsenic and its metabolites in urine remains an active area of investigation (Gomez-Caminero et al., 2001).

1.6 Cell Biology

The cell is the structural and functional unit of all living organisms. Some organisms such as bacteria have only a single cell. Other organisms such as humans have about 100,000 billion cells. The cell theory, first developed in the 19th century, states that all organisms are composed of one or more cells and all fundamental functions of an organism occur within cells. Also cells contain the necessary genetic information for regulating cell functions.

1.6.1 Cell Membrane

All cells have a plasma membrane, which is a bag that holds the contents of cell. It is a bilayer of lipids and proteins that covered by carbohydrates. Thus it separates interior of cell from its surroundings. In some cells, outer surface of the plasma membrane are surrounded by cell walls such as bacteria, blue-green alga, fungi, and plants. Thus they prevent dehydration and survives cell in hypotonic environments. Cell walls contain three dimensional structure of cross linked polymers with high molecular weight (Avers, 1978; Thorpr, 1984).

1.6.2 Organelles

Cells have a set of little organs called organelles that carry out special functions. Some of organelles are introduced as follows:

1.6.2.1 Cell Nucleus

The nucleus is the control center of the cell. In fact DNA transcription and RNA synthesis take places in the nucleus. The components of a nucleus are one or more the nucleoli, the nuclear envelope and the chromosomes. Chromosomes act as the bodies which house the genes and regulate gene action. The nuclear envelope is a double membrane that separates the nuclear interior from surrounding cytoplasm (Avers, 1978; Thorpr, 1984).

1.6.2.2 Ribosomes

Ribosomes are the sites of protein synthesis, where RNA transcripts are decoded and proteins are synthesized according the coded information. Ribosomes may occur free in the cytoplasm or may be bonded to various cellular membranes. There are a large number of ribosomes in cell since protein synthesis is important for cell.

1.6.2.3 Mitochondria

Mitochondria are organelles with different size and shape that are found in large number in plant and animal cells. Mitochondria are centers for synthesis of high energy ATP (adenosine triphosphate) during food breakdown. This process involves a number of metabolic pathways including the reactions of the tricarboxylic acid cycle, the oxidation of fatty acids and oxidative phosphorylation. Thus mitochondria have a critical role in generating energy in the cell.

1.6.2.4 Lysosomes

Lysosomes are vesicles that contain a number of hydrolytic enzymes and called intracellular digestive systems. Many different acid hydrolases have been localized in the lysosomes that give the cell digesting ability for all the biologically important molecules.

1.6.3 Movements of Substances across Membranes

Substances move through membranes selectively in the cell. There are different ways which substances cross the membrane barrier. Hydrophobic and small molecules freely diffuse trough the membranes along a concentration gradient. Their diffusion is proportional to their solubility in lipid layer of membrane. Other substances those are insoluble in lipid

(polar and ionic molecules) move across the membrane by specific carriers that are important proteins in membrane cell. These carriers have specific binding site which will bind to molecules and transport them as passive or active. In passive transport, the substances move in the direction of a concentration gradient but in active transport the molecules move against their concentration gradient. The active transport is a process that is entropically unfavourable and consumes energy to move the substance against concentration gradient (Avers, 1978).

1.7 Objective of the Present Work

The aims of this work divided to two majors studies. (**i**): Study arsenic metabolites, specially trivalent methylated arsenic species (MMAs(III) and DMAs(III)) in urine samples collected from Brazilian school children exposed to arsenic-rich drinking water by high performance liquid chromatography coupled to inductively coupled plasma mass spectrometry (HPLC-ICP-MS) technique while was a joint study with Professor Jörg Matschullat (Interdisciplinary Environmental Research Centre, Freiberg University of Mining and Technology, Freiberg, Germany) providing the urine samples. The effects of many chromatographic parameters such as pH, concentration of ion-pair reagent and buffer, flow rate of mobile phase, percentage of added methanol, and also the column temperature were investigated to achieve the best separation for arsenic species in the shortest time. (**ii**) The cellular uptake of inorganic arsenic [arsenate, As(V); arsenite, As(III)] and the methylated arsenic species monomethylarsonic acid [[MMAs(V)], monomethylarsonous acid [MMAs(III)], dimethylarsinic acid [DMAs(V)], dimethylarsinous acid [DMAs(III)], trimethylarsenic oxide (TMAsO) were investigated in Chinese hamster ovary (CHO-9) cells, human UROtsa (normal human urothelium) cells, HeLa S3 cells, Hep G2 cells, and rat hepatocytes (Ra Hep) to determine how arsenic species were taken up by above mentioned cell lines. Since the liver is the primary site of arsenic metabolism within the body and is a target organ for arsenic carcinogenicity, these type of cells were chosen (Dopp et al., 2005). It has been investigated whether the arsenic compounds are bound to membranes or whether they are present in the cytosol. For these purposes, the cell membranes were removed by osmotic lysis and subsequent centrifugation before the intracellular arsenic concentration was measured by ICP-MS. The chemicals were applied at different concentrations (0.1 μM to 10 mM). Intracellular arsenic concentrations were determined by ICP-MS techniques. In other experiment, association and distribution of arsenic substrates with intracellular organelles (nucleus, mitochondria, plasma membrane, ribosomes and cytosol) in UROtsa cells has been

studied while was investigated in the course of a project (DFG Forschergruppe 415) together with a research team of Professor A. W. Rettenmeier/ PD Dr. E. Dopp (Institute of Hygiene and Occupational Medicine, University Hospital of Essen) performing the cell exposure experiments, and their toxicological evaluation.

2 Material and Methods

Caution: Inorganic arsenic compounds have been established as human carcinogens (National Research Council Report 1999). Ingestion of inorganic arsenic may cause cancer of the skin, urinary bladder, kidney, lung, and liver as well as with disorders of the circulatory and nervous system.

2.1 Chemicals and Reagents

All reagents used were of the highest purity available and of at least analytical-reagent grade. Ultra-pure laboratory water of 18.2 MΩ.cm quality (Seralpur Pro 90 CN, Elga, Germany) was used for the preparation and dilution of all reagents, samples and standards.
Solutions of arsenite (As(III)) and arsenate (As(V)) were prepared from sodium arsenite($AsNaO_2$) and sodium arsenate ($AsHNa_2O_4.7H_2O$), respectively. Sodium arsenite was obtained from Fluka (Seelze, Germany) while sodium arsenate ($AsHNa_2O_4.7H_2O$) was purchased from Sigma (Taufkirchen, Germany). Monomethylarsonic acid ($CH_3AsO(OH)_2$) and trimethylarsine oxide ($(CH_3)_3AsO$) were purchased from Tri-Chemical Laboratories Inc. (Yamanashi, Japan), and dimethylarsinic acid ($(CH_3)_2AsO(OH)$) was from Strem (Kehl, Germany). Dimethyliodoarsine ($(CH_3)_2AsI$) and monomethyldiiodoarsine (CH_3AsI_2) were obtained from Argus Chemicals (Vernio, Italy). Preparation of dilute solutions of these iodide arsenicals results in the formation of the related acids, monomethylarsonous acid ($CH_3As(OH)_2$) and dimethylarsinous acid ($(CH_3)_2AsOH$) (Gong et al., 2001; Millar et al., 1960). Working standard solutions of 0-50 $ng.mL^{-1}$ were prepared daily from the stock solutions by appropriate dilution. However, based on high instability of DMAs(III), solutions of this arsenic species were always prepared before each experiment. The reagents used in HPLC mobile phase for the RP chromatography were tetrabutylammonium hydroxide from Sigma-Aldrich (Steinheim, Germany), malonic acid from Merck (Hohenbrunn, Germany), and nitric acid (65%) was obtained from J. T. Baker (USA). HPLC-grade methanol was from Merck (Darmstadt, Germany). The reagents used in mobile phase for the cation-exchange chromatography were pyridine from sds (France) and formic acid from Fluka (Switzerland).

2.2 Urine Samples

At 3 public schools (communities of Galo and Mingu within the township Nova Lima, and the village Brumal in the Santa Barbara district), spontaneous urine was sampled from

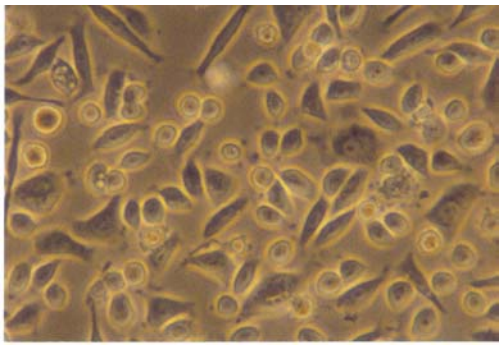
total of 126 children. The children were 7-14 years old (mean 9.8 ± 1.12 years). Several days prior to sampling, a coded questionnaire was completed for each child, providing detailed information on age, gender, place of birth and period of residence in the observed community, breast-feeding, nutrition habits, and health status including previous medical operations and dentistry. During sampling, the answers were randomly cross-checked for accuracy. All materials were handled consistently with disposable non-powdered latex rubber gloves only. An aliquot of fresh urine was kept in acid washed (Nitric acid) PE bottles. All urine samples were stored in a cooler while in transport and frozen within 12 h (at -18 ^{o}C). This procedure complies with standards of the health organisations. Samples were obtained kindly from Professor Jörg Matschullat (Interdisciplinary Environmental Research Centre, Freiberg University of Mining and Technology, Freiberg, Germany). More information about the area where the samples were obtained, and also the map of district, is mentioned on section 3.2.1. Urine samples were frozen by placing them in a freezer at our institute, and were stored at -80 ^{o}C. The frozen human urine samples were defrosted at room temperature. Human urine samples for analysis were undiluted, and filtered through a 0.45µm pore size filter (Nalgene, USA). 20-100 µL of each was injected in to the HPLC column for analysis.

2.3 Cell Lines and Cell Culture Conditions

Preparation of all cell samples listed below, was performed by the research group of PD Dr. E. Dopp at the Institute of Hygiene and Occupational Medicine (under direction of Prof. Dr. A. W. Rettenmeier), University Hospital of Essen, and analyzed at Institute for Environmental Analysis, University of Duisburg-Essen. Original pictures of the cell lines and their information were kindly provided by the research group of PD Dr. E. Dopp.

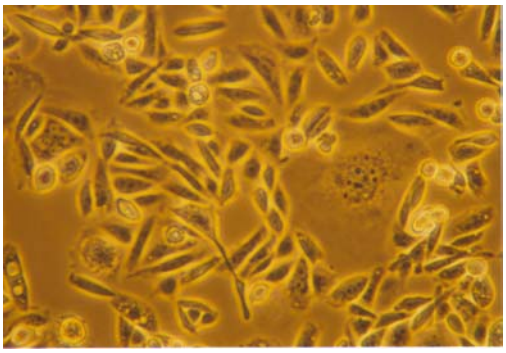
2.3.1 Human UROtsa Cells

The UROtsa cell line was isolated from a primary culture of normal human urothelium through immortalization with a construct containing the SV-40 large T-antigen. These cells which, kindly provided by Dr. Scott H. Garret, Department of Pathology, School of Medicine and Health Sciences, University of North Dakota, USA, were cultured in Dulbecco's modified Eagles medium (DMEM) supplemented with 10% fetal calf serum (FCS), glutamine (20 mg/l) and penicillin/streptomycin (100µg/ml) at 37 °C and 5 % CO_2. UROtsa cells are adherent cell lines that grow as monolayer. Cells were subcultured using trypsin (0.25%) for three minutes.



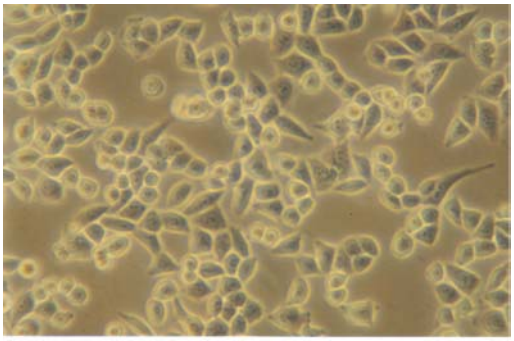
2.3.2 CHO-9 Cells

Chinese hamster ovary (CHO-9) cells (obtained from A.T. Natarajan, Leiden, The Netherlands) were grown in 25 cm² culture flasks (Greiner) with McCoys 5A medium (Gibco) supplemented with 10 % fetal calf serum (Gibco) in the presence of 100 IU/ml penicillin and 100 µg/ml streptomycin (Gibco) at 37 °C and 5 % CO_2.



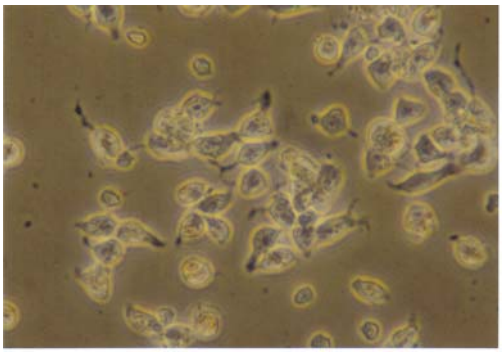
2.3.3 HeLa S3 Cells

HeLa S3 cells were obtained from American Type Culture Collection (ATCC CCL-2.2). These cells are derived from human cervix adenocarcinoma. For cell culture, they were maintained in Ham's F12K medium supplemented with 10% fetal calf serum (Gibco) in the presence of 100 IU/ml penicillin and 100 µg/ml streptomycin (Gibco) at 37 °C and 5 % CO_2.



2.3.4. Hep G2 Cells

Hep G2 cells (ATCC, HB 8065) were maintained at 37°C and 5% CO_2 in Minimum Essential Medium (MEM) with Earle's BSS and sodium-bicarbonate (CC PRO, Germany) supplemented with 10 % heat-inactivated FCS (Gibco), non essential amino acids (0.1 mM), sodium-pyruvate (1 mM) and 100 IU/ml penicillin/streptomycin (CC PRO, Germany).



2.4 Cellular Uptake of Arsenic by Different Cell Lines

In order to investigate and compare the membrane permeability of different cell lines against all different arsenic species applied in this study, a wide range of concentration of these arsenic substrates from 0.1 μM to 10 mM for an exposure time of 1 h and 24 h were used. After incubation, cells were rinsed with phosphate-buffered saline (PBS) and then resuspended in 10 ml fresh culture medium. Following cell counting, the cell suspension was centrifuged for 5 min at 190 × g and the pellet was resuspended in 10 ml distilled water for at least 30 min to lyse the cells. A microscope was used to control the absence of intact cells. From this obtained cell solutions two different cell samples were prepared: (a) whole-cell extract with membranes and proteins present, and (b) cell-free (membrane removed) extract, obtained by osmotic lysis of the whole-cell extract with subsequent centrifugation for 15 min

at 1700 × g to remove the membranes. The samples were kept frozen at -80 ^{o}C until ICP-MS analysis. Cellular uptake process is shown in Figure 2.1.

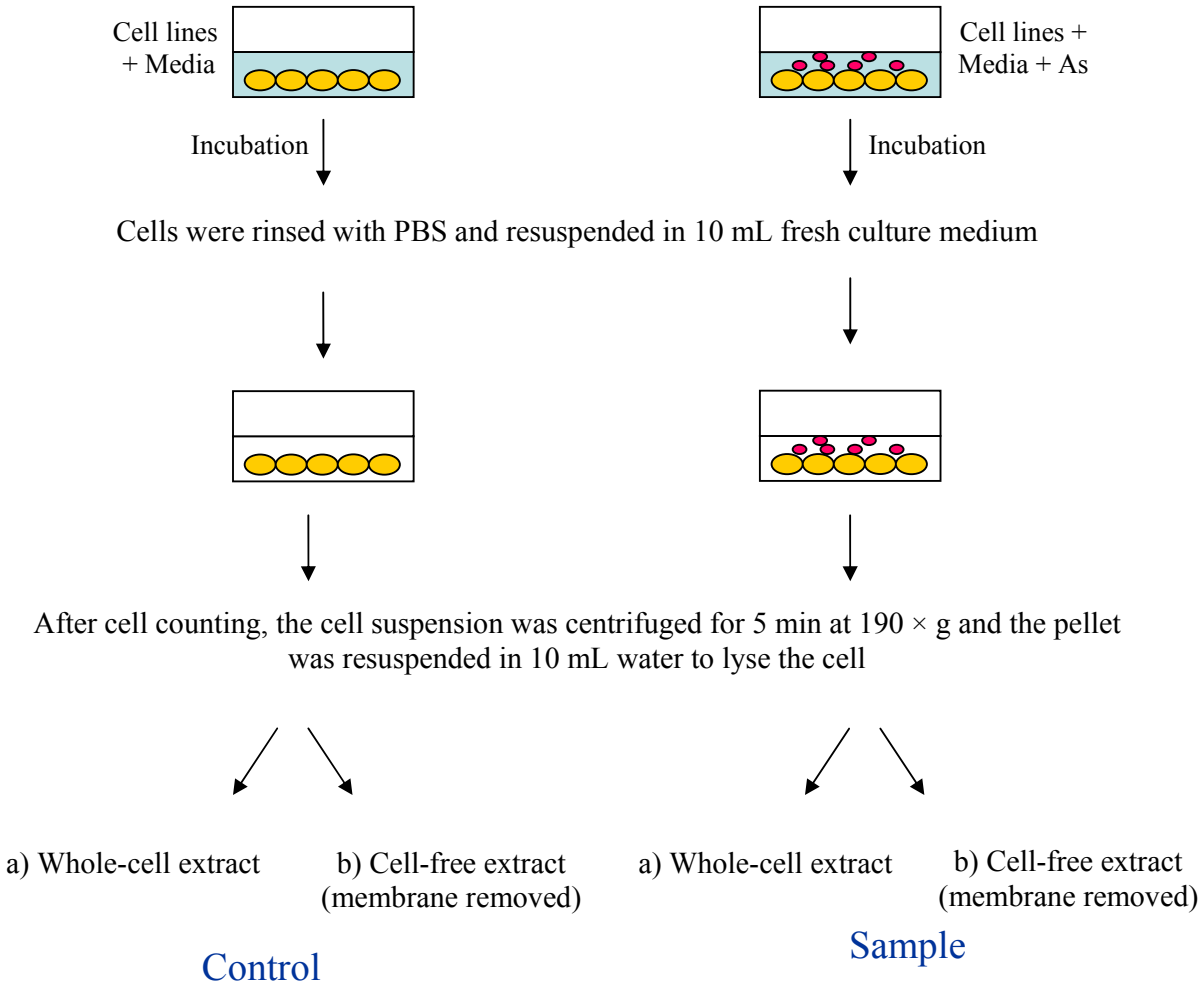


Figure 2.1 Cellular uptake process

2.4.1 Association of Arsenic with Intracellular Organelles in UROtsa Cells

In order to study association of arsenic substrates with intracellular organelles in UROtsa cells, differential centrifugation at five steps was performed. Therefore, six different samples were prepared for analysis (Figure 2.2).10 × 10^{6} UROtsa cells were incubated with the arsenic compounds (5, 500, and 5000 µM) for an exposure time of 1 h and 24 h. Following incubation, cells were rinsed with phosphate-buffered saline (PBS) to remove

unbound test substances, and subsequently resuspended in 10 ml fresh culture medium. The cell suspension was centrifuged for 5 min at 190 × g and the pellet was resuspended in 10 ml distilled water for at least 10 min to lyse the cells. A microscope was used to control the absence of intact cells. Then, filtered homogenate sample (F sample) obtained by filtering to remove clumps of unbroken cells, connective tissue, etc. in this step (Figure 2.2). From this cell solution five kinds of samples were prepared by different rate and time of centrifuging (see Table 2.1). The precipitate was resuspended to final volume of 2 mL with aqua bidest (see Figure 2.2). The samples were stored at -80 ^{o}C until ICP-MS analysis.

Table 2.1 Definition of each sample based on differential centrifugation in UROtsa cells

Sample	Contents
F	Filtered homogenate sample
N	Nuclei
M	Mitochondria, chloroplasts, lysosomes, and peroxisomes
P	Plasma membrane, microsomal fraction, and large polyribosomes
R	Ribosomal subunits, small polyribosomes
C	Cytosol (soluble portion of cytoplasm)

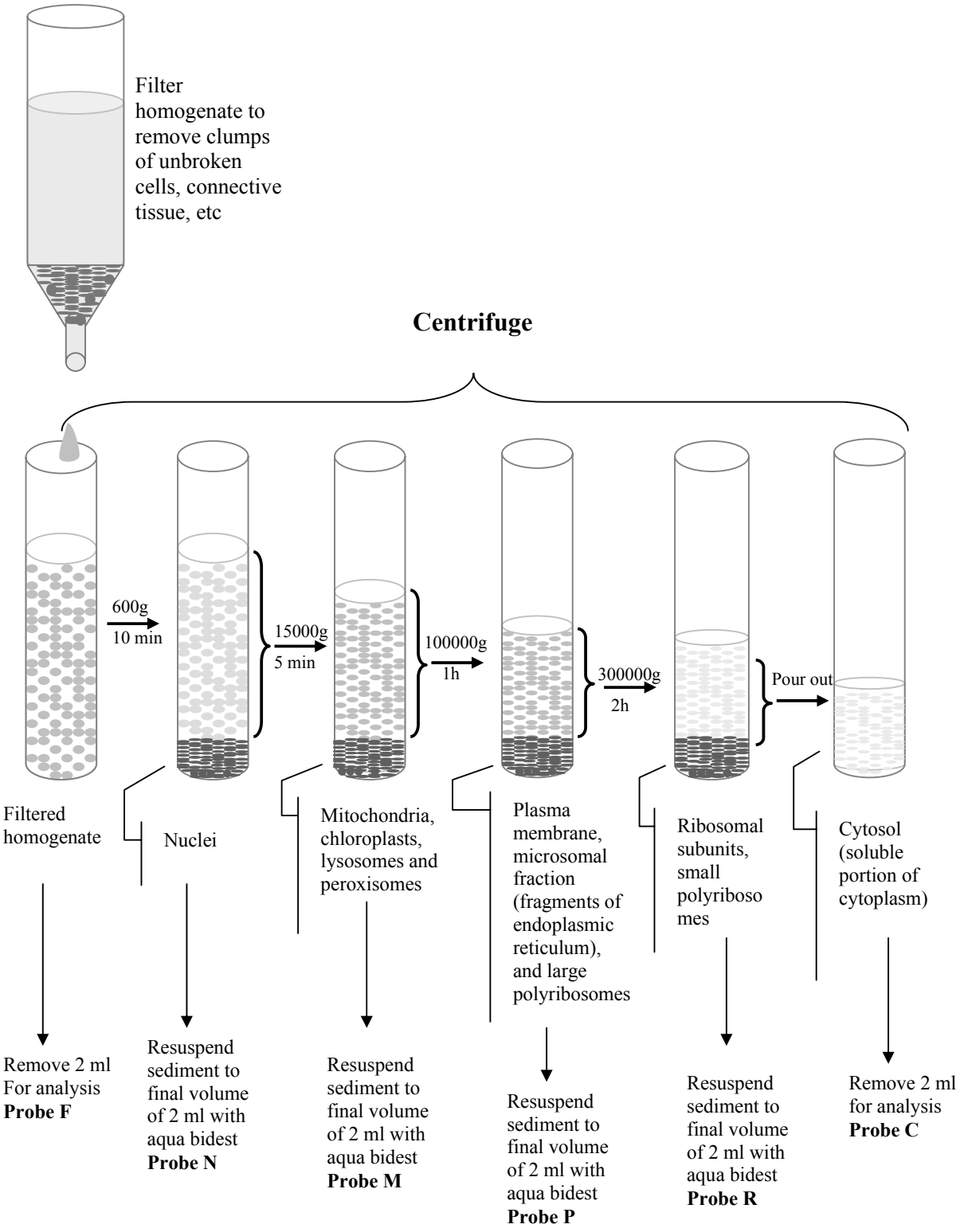


Figure 2.2 Differential centrifugation of UROtsa cell lines

2.5 Instrumentation

Arsenic speciation in urine and cells was quantified by HPLC-ICP-MS based on the description of a previously published method (Gong et al., 2000; Geiszinger et al., 1998) where an HPLC-HG-AFS detection system was applied. In brief, the mobile phase used for a reversed-phase C_{18} column (Prodigy ODS-3, 150 × 4.6 mm, 3 µm particle size, Phenomenex, USA, equipped with a C_{18} a guard column, 2 cm, same material as analytical column) was as follows: 4.7 mM tetrabutylammonium hydroxide + 2mM malonic acid + 4% methanol (pH of the mobile phase was adjusted to 5.95 with 25% nitric acid). Measurements of pH were made with a pH-meter (pH 211 Microprocessor, Hanna Instruments, Italy) using a combined glass electrode. The column was equilibrated with the mobile phase at a flow rate of 0.4 mL/min for at least 1.5 h before any sample injection for detection of arsenic species, a PTFE tubing (150 mm × 0.4 mm) with appropriate fittings was used to connect the outlet of the HPLC column directly to the inlet of the ICP-MS (Agilent 7500a, Agilent Technologies, Germany) nebulizer. The guard column and the column were heated to 30 ^{o}C using a column heater (Therma Sphere ^{TM} T S-130, Phenomenex, USA). The mobile phase used for a cation-exchange column (Supelcosil ^{TM} LC-SCX, 25 cm × 4.6 mm i.d., 5 µm silica based particles with propylsulsonic acid exchange sites, Supelco, USA, equipped with a guard column, 2 cm, same material as analytical column) was simply 20 mM pyridine at pH 3.0 adjusted by the addition of formic acid. The operation conditions for HPLC and ICP-MS are summarized in Table 2.2.

Table 2.2 HPLC-ICP-MS operating conditions

HPLC	
Column	ODS-3, 150 × 4.6 mm, 3 μm particle size
Temperature	30 ^{o}C
Mobile phase	4.7 mM tetrabutylammonium hydroxide + 2mM malonic acid + 4% methanol (pH 5.95)
Flow rate	1.5 mL/min
Injected volume	20 μL
Column	Supelcosil LC-SCX, 150 cm × 4.6 mm, 5 μm particle size
Temperature	30 ^{o}C
Mobile phase	20 mM pyridine (pH 3.0)
Flow rate	1.5 mL/min
Injected volume	20–100 μL
ICP-MS	
Masses	75 (As), 77 (ArCl)
Integration time	0.1 sec
RF power	1580 W
Sample & skimmer cones	Ni
Spray chamber	Double-pass Scott-type
Sample depth	4.8 mm
S/C temperature	2 ^{o}C
Plasma gas (Ar)	15 L/min
Carrier gas (Ar)	0.52 L/min
Make-up gas (Ar)	0.71 L/min
Nebulizer	Microflow

3 Results

3.1 Optimization of the HPLC Parameters

Optimization of the chromatographic parameters for arsenic was carried out with respect to pH, concentration of ion-pair reagent (TBAH), concentration of the buffer (malonic acid), percent of methanol in mobile phase, flow rate of mobile phase, and column temperature to achieve the best separation for arsenic species in the shortest time. The method was then applied to the analysis of biological samples.

3.1.1 Optimization of pH

The separation of arsenic species by HPLC is pH-dependent (Le et al., 1996). For example, at neutral pH, arsenate (pK_{a1} = 2.3), MMAs(V) (pK_{a1} = 3.6), and DMAs(V) (pK_a = 6.2) are present as anions; arsenocholine (AsC) and the tetramethylarsonium ion (Tetra) as cations; arsenobetaine (AsB) as a zwitterion; and arsenous acid (pK_{a1} = 9.3) as an uncharged species. In other words, the pH of the mobile phase should have a remarkable effect on the retention time of analytes (Dong 2006). Therefore, it is necessary to optimize the pH of mobile phase for separating desired arsenic species. It should be considered that at pH<2 the Si-O bonds are subjected to acidic hydrolytic cleavage and at pH>8 the silica structure should be dissolved (Dong 2006). Therefore, pH values ranged 4 to 7 were studied. The results are shown in Figure 3.1.

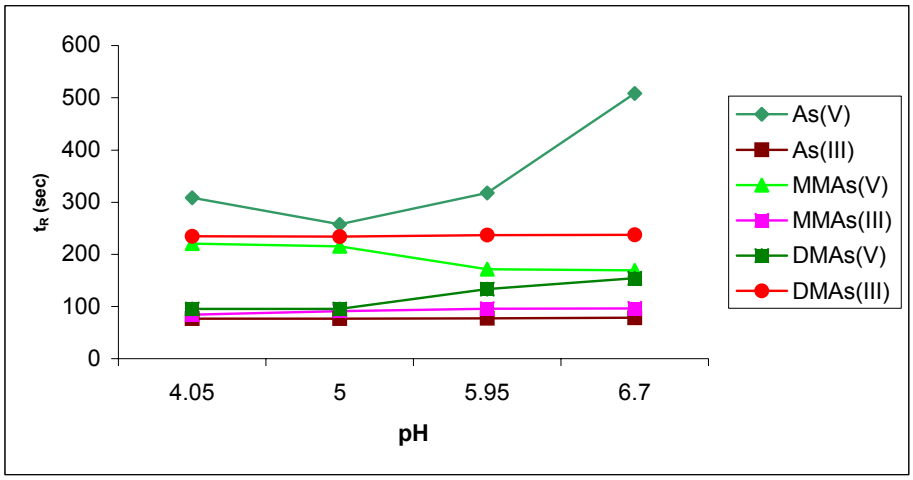


Figure 3.1 Effect of the pH on the retention time of six arsenic species. ODS column was used at 30 °C. The concentration of each species was 10-13.6 ng As/mL. A total volume of 20 µL was injected. Other experimental conditions: 4.7 mM TBAH, 2 mM malonic acid, 4% MeOH, flow rate=1.5 mL/min.

3.1.2 Optimization of the Concentration of TBAH

Tetrabutylammonium hydroxide (TBAH) is used as an ion-pair reagent. Figure 3.2 shows the effects of different concentrations of TBAH. An increase in the TBAH concentration up to 5.5 mM eliminates peak overlap between dimethylarsinic acid (DMAs(V)) and monomethylarsonous acid (MMAs(III)) and also peak overlap between arsenate and dimethylarsinous acid (DMAs(III)). As it can be seen in Figure 3.2, this increase in the TBAH concentration affects the elution time of all species except arsenite, which is independent on changes of TBAH concentration.

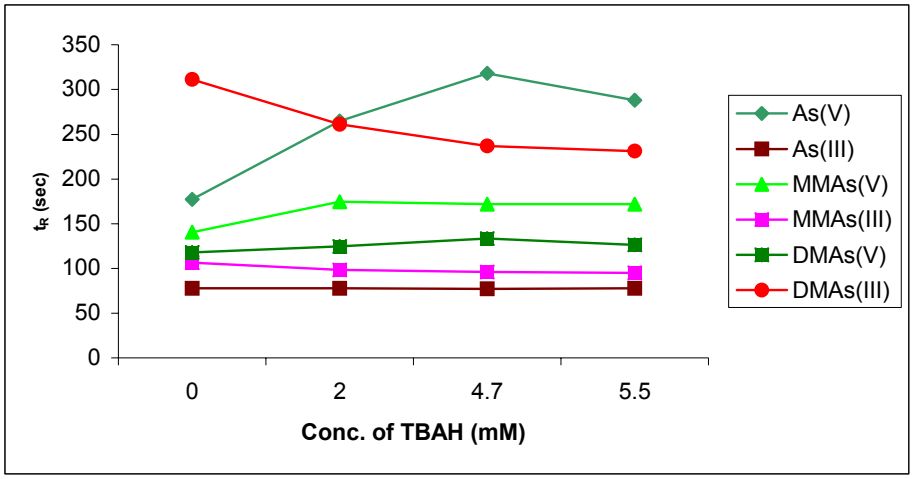


Figure 3.2 Influence of the TBAH concentration on the retention time of six arsenic species; ODS column was used at 30 ^{o}C. The concentration of each species was 10-13.6 ng As/mL. A total volume of 20 µL was injected. Other experimental conditions: pH 5.95, 2 mM malonic acid, 4% MeOH, flow rate=1.5 mL/min.

3.1.3 Optimization of the Concentration of Malonic Acid

In general buffers in the mobile phase are required for controlling the separations of acidic or basic analytes. Buffer concentrations may be used at the different levels. In this study, malonic acid was used as a buffer to get a good separation. Figure 3.3 shows the influence of malonic acid concentration on retention time of each arsenic species. As can be seen in Figure 3.3, an increase in the concentration of malonic acid effectively reduces the elution time of arsenic (V).

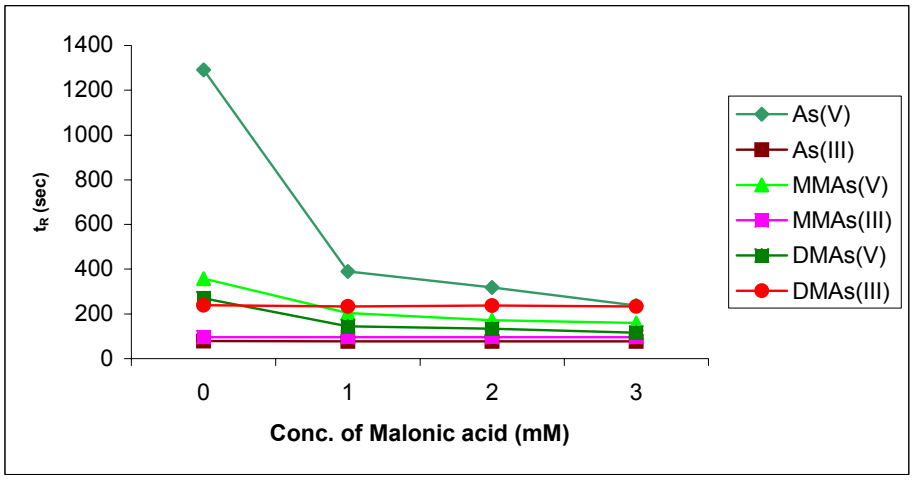


Figure 3.3 Effect of the malonic acid concentration on the retention time of six arsenic species; ODS column was used at 30 ^{o}C. The concentration of each species was 10-13.6 ng As/mL. A total volume of 20 µL was injected. Other experimental conditions: pH 5.95, 4.7 mM TBAH, 4% MeOH, flow rate=1.5 mL/min

3.1.4 Optimization of the Percentage of Methanol

It is known that the addition of carbon as methanol to the mobile phase enhances ICP-MS sensitivity for arsenic (Larsen 1998). Therefore, methanol was added to the aqueous chromatographic mobile phase. The methanol content was varied between 0 and 4% (v/v). With regard to maximum sensitivity, a concentration of 4% (v/v) methanol was found to be optimal amount for separating of investigated arsenic species. Figure 3.4 shows the influence of methanol on the retention time of each arsenic species. As it can be seen, addition of different percent of methanol does not affect significantly the retention time of each arsenic species. But, as it has been mentioned it can affect the signal intensity of each arsenic species, which it has been found that the addition of 4% of methanol is optimized amount of methanol.

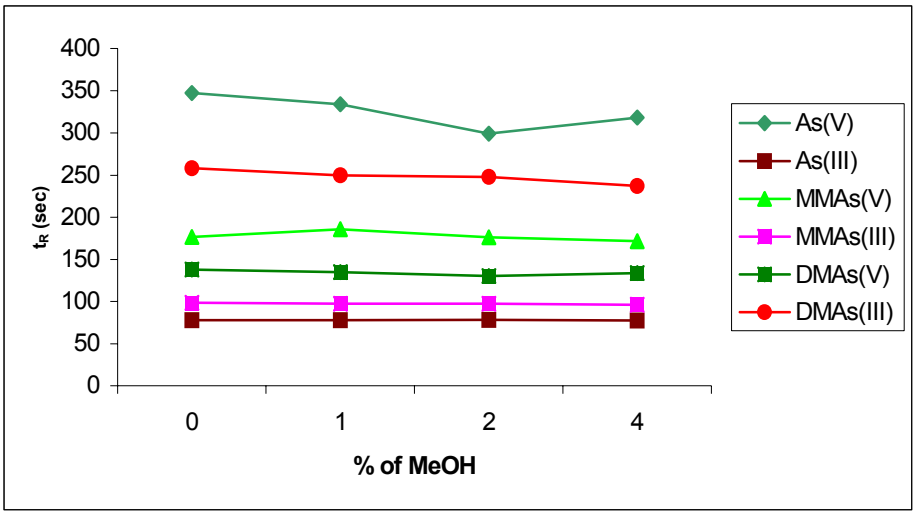


Figure 3.4 Effect of the percent of methanol on the retention time of six arsenic species; ODS column was used at 30 ^{o}C. The concentration of each species was 10-13.6 ng As/mL. A total volume of 20 µL was injected. Other experimental conditions: pH 5.95, 4.7 mM TBAH, 2 mM malonic acid, flow rate=1.5 mL/min

3.1.5 Optimization of Flow Rate and Column Temperature

The effects of mobile phase flow rate with a range from 0.8–1.5 mL/min and also of column temperature from room temperature up to 60 ^{o}C on the retention time of each arsenic species were studied. Based on the elution time and also a good separation of each arsenic species, the values of 1.5 mL/min and 30 ^{o}C for flow rate and the column temperature were selected respectively. Figure 3.5 shows the effects of different applied flow rates. As expected, increasing the flow rate causes decreasing the elution time of each arsenic species. The effect was species dependent, i.e. an increase of flow rate was more effective to decrease in the retention time of As(V), DMAs(III), MMAs(V), and DMAs(V). Therefore, a flow rate of 1.5 mL/min was selected. Figure 3.6 shows the influences of column temperature on retention time of each arsenic species. As it can be seen in Figure 3.6, increasing the column temperature does not affect significantly the retention time of each arsenic species. Therefore, a column temperature of 30 ^{o}C was selected under the optimized conditions.

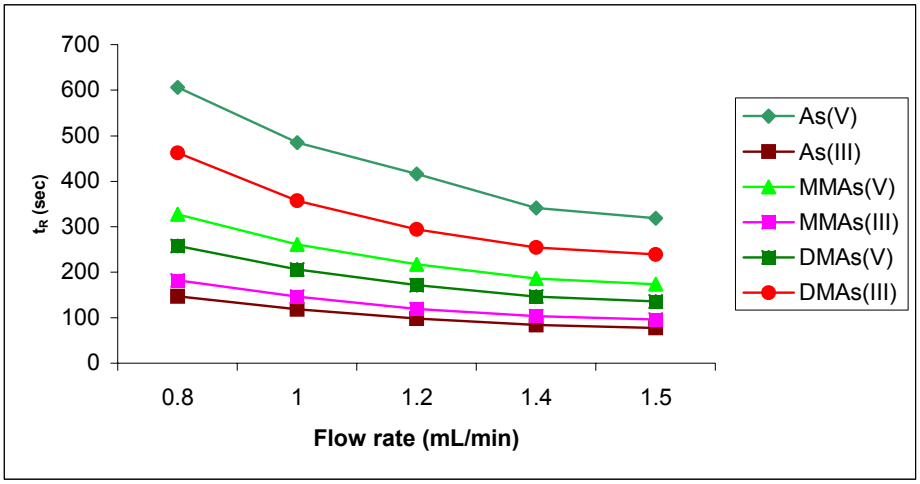


Figure 3.5 Effect of the flow rate on the retention time of six arsenic species; ODS column was used at 30 °C. The concentration of each species was 10-13.6 ng As/mL. A total volume of 20 µL was injected. Other experimental conditions: pH 5.95, 4.7 mM TBAH, 2 mM malonic acid, 4% MeOH

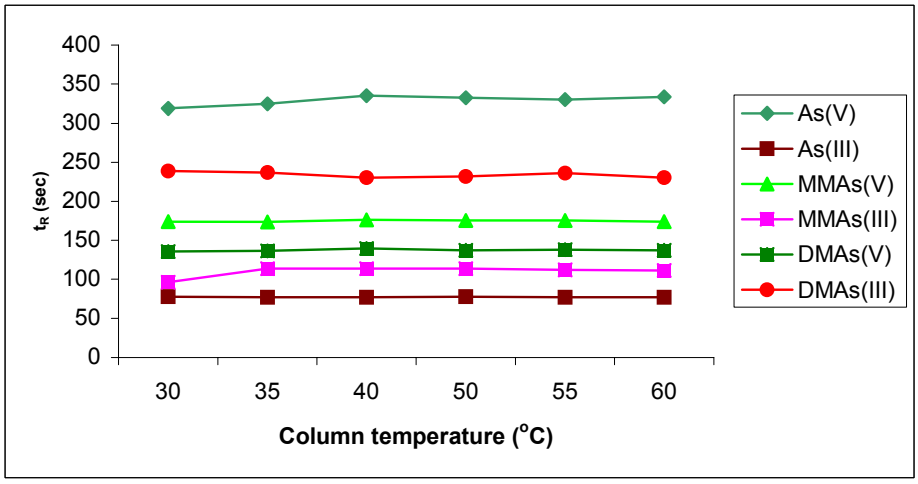


Figure 3.6 Influence of the column temperature on the retention time of six arsenic species; ODS column was used. The concentration of each species was 10-13.6 ng As/mL. A total volume of 20 µL was injected. Other experimental conditions: pH 5.95, 4.7 mM TBAH, 2 mM malonic acid, 4% MeOH, flow rate=1.5 mL/min

The optimum conditions for chromatographic separation and ICP-MS detection of arsenic species are summarized in Table 2.2. A chromatogram obtained with reverse phase ODS 3µ column for standard solutions of six arsenic species by applying the optimum conditions is shown in Figure 3.7. Calibration graphs for each arsenic species are shown in Figure A.1.

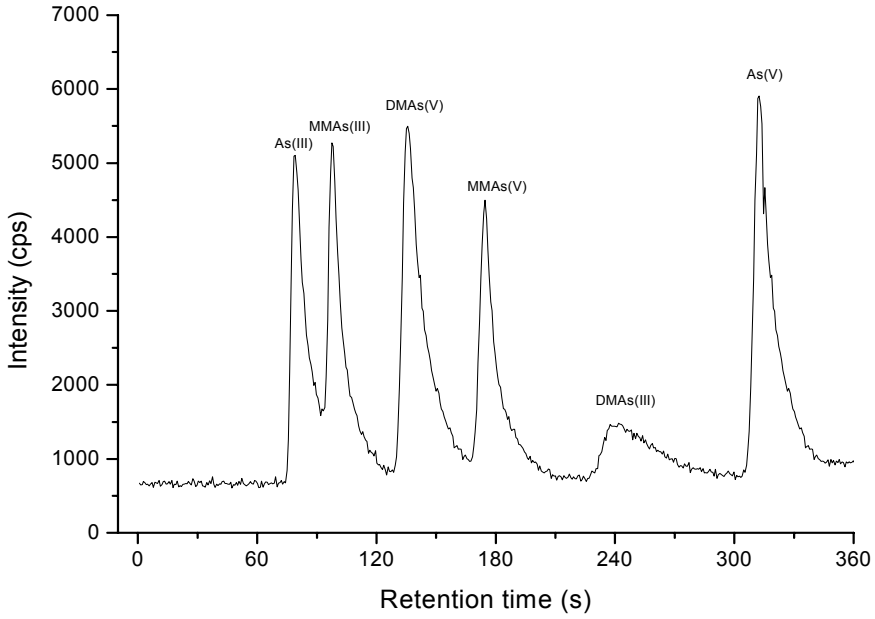


Figure 3.7 A typical HPLC-ICP-MS chromatogram of a mixture of standard solution of six arsenic species as labelled obtained with ODS 3µ column. The concentration of each species was 10-13.6 ng As/mL. A total volume of 20 µL was injected.

3.2 Arsenic Speciation in Urine Samples

3.2.1 Intention of Study

Urine samples from Brazilian children exposed to arsenic-rich drinking water were analyzed using HPLC-ICP-MS technique. The urine samples were subjected to speciation analysis under the optimum conditions. The identification has been made based on chromatographic coelution with standards.

Table 3.1 Total arsenic concentration and the distribution of different arsenic species in urine samples of children from Brazil [#]

Sample	Concentrations[a] of As detected (ng As/mL) ± SD									
	As(V)	**As(III)**	**MMAs(V)**	**MMAs(III)**	**DMAs(V)**	**DMAs(III)**	**AsB**	**Sum[b]**	**Total[c]**	**Recovery%[d]**
Urine 261	2.03 ± 0.21	2.27 ± 0.34	3.49 ± 0.48	nd*	6.85 ± 0.89	nd*	0.64 ± 0.06	15.3 ± 1.8	17.7 ± 0.3	86.3
Urine 254	2.92 ± 0.43	4.86 ± 0.61	4.38 ± 0.43	nd	12.95 ± 1.13	nd	7.99 ± 0.26	33.1 ± 1.8	38.0 ± 0.5	87.1
Urine 212	4.69 ± 0.61	5.28 ± 0.41	16.23 ± 1.97	2.00 ± 0.16	20.45 ± 1.66	nd	nd	48.7 ± 4.3	55.2 ± 0.5	88.1
Urine 265	1.56 ± 0.24	2.31 ± 0.33	1.47 ± 0.26	0.42 ± 0.06	9.16 ± 0.65	nd	nd	14.9 ± 1.3	16.7 ± 0.5	89.3
Urine 314	2.73 ± 0.40	3.79 ± 0.41	2.27 ± 0.17	0.58 ± 0.07	8.37 ± 0.71	nd	nd	17.7 ± 1.7	21.3 ± 0.2	83.3
Urine 263	1.48 ± 0.26	3.64 ± 0.39	5.71 ± 0.61	0.89 ± 0.08	5.28 ± 0.46	nd	0.69 ± 0.07	17.7 ± 1.5	19.2 ± 0.4	92.1
Urine 284	3.06 ± 0.55	5.81 ± 0.42	11.69 ± 0.92	0.74 ± 0.08	6.57 ± 0.56	nd	0.71 ± 0.02	28.6 ± 1.6	30.9 ± 0.4	92.5
Urine 247	2.15 ± 0.39	3.12 ± 0.22	3.25 ± 0.29	0.62 ± 0.06	4.38 ± 0.66	nd	nd	13.5 ± 1.5	16.1 ± 0.3	84.0
Urine 190	1.49 ± 0.21	4.36 ± 0.35	5.01 ± 0.61	0.69 ± 0.06	5.72 ± 0.51	nd	nd	17.3 ± 1.7	18.2 ± 0.3	94.9
Urine 249	2.95 ± 0.42	6.71 ± 0.69	7.36 ± 0.40	1.18 ± 0.13	14.97 ± 0.82	nd	0.76 ± 0.07	33.9 ± 2.4	38.5 ± 0.3	88.1
Urine 258	2.57 ± 0.36	4.75 ± 0.31	nd	0.59 ± 0.08	8.12 ± 0.49	nd	nd	16.0 ± 0.9	19.3 ± 0.2	83.1
Urine 320	2.45 ± 0.46	5.17 ± 0.26	7.27 ± 0.41	0.97 ± 0.06	8.59 ± 0.46	nd	0.47 ± 0.03	24.5 ± 1.3	31.4 ± 0.2	79.4
Urine 318	1.69 ± 0.28	nd	2.56 ± 0.29	nd	10.38 ± 0.87	nd	nd	14.6 ± 1.3	18.9 ± 0.1	77.4
Urine 325	2.26 ± 0.23	4.19 ± 0.34	6.58 ± 0.70	0.74 ± 0.05	7.35 ± 0.41	nd	0.94 ± 0.06	22.1 ± 1.4	26.3 ± 0.2	83.9
Urine 250	3.51 ± 0.36	3.00 ± 0.39	2.86 ± 0.36	0.67 ± 0.07	14.92 ± 0.76	nd	nd	25.0 ± 1.7	27.2 ± 0.6	91.8

[a] The values are given as the average of four repeated injections. [b] The sum of the concentrations for individual arsenic species. [c] The total concentration obtained by ICP-MS (n=10). [d] Recovery % is calculated from [sum(species)×100]/total arsenic. [#] AsC and TMAsO were not detected in any of urine samples. [*] nd=not detected.

3.2.2 Chromatographic Separation of Arsenic Species

Due to the different chemical properties of the investigated arsenic compounds, it is not readily possible to separate all the investigated arsenic compounds within one chromatographic run. A separation technique for anionic arsenic species has been applied using an ion pair reverse phase-high performance liquid chromatography coupled to inductively coupled plasma mass spectrometry (RP-HPLC-ICP-MS). Six arsenic species (arsenite, arsenate, DMAs(V), DMAs(III), MMAs(V), and MMAs(III)) have been separated with isocratic elution within less than six minutes. The condition of separation is summarized in Table 2.2. Furthermore, cation-exchange chromatography was employed to separate the cationic arsenic species. The Supelcosil LC-SCX cation-exchange column allows the separation of arsenobetaine (AsB), trimethylarsine oxide (TMAsO), arsenocholine ion (AsC), and also tetramethylarsonium cation (Me_4As^+ = Tetra) with a 20 mM aqueous pyridine solution (Geiszinger et al., 1998) at pH 3.0 adjusted by addition of formic acid. The identification of the arsenic species present in the urine samples was based on the comparison of the retention times with standards and confirmed by spiking experiments for real samples as matrices are different as standards alone. A chromatogram obtained with cation exchange column for standard solutions of AsB, TMAsO, AsC, and Tetra is shown in Figure 3.8.

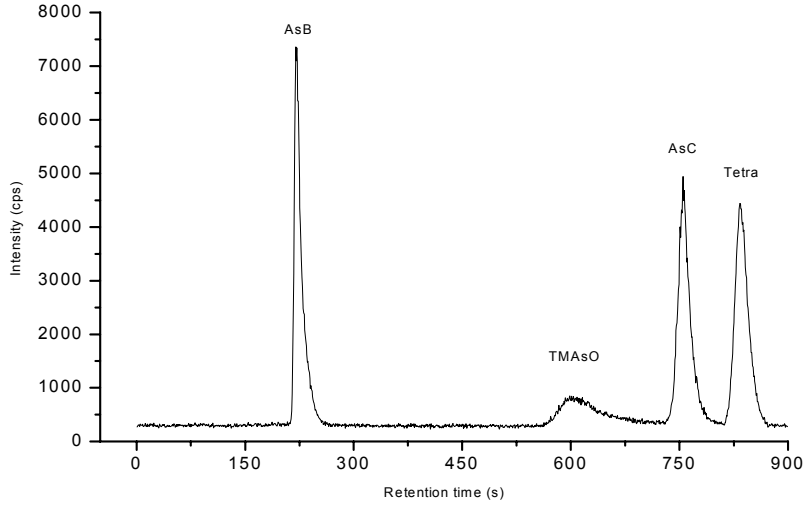


Figure 3.8 A typical HPLC-ICP-MS chromatogram of a mixture of standard solution of arsenic species as labelled obtained with supelcosil LC-SCX cation-exchange column. The concentration of each species was 8.5-14.4 ng As/mL. A total volume of 100 µL was injected.

3.2.3 Site Description

The Iron Quadrangle in the state of Minas Gerais, Brazil, is one of the richest mineral-bearing regions in the world (Matschullat et al., 2000). Apart from extensive iron ores, hydrothermal Au mineralization can be found in Archean greenstone belt formations (Vieira, 1997; Vieira et al., 1991; Vieira and Simoes, 1992). Active gold mining has been taking place in the Nova Lima area since the early 1700's (Pires et al., 1996). The hydrothermal deposits contain several sulfides such as pyrite (FeS_2), pyrrhotite (FeS), and arsenopyrite (FeAsS) in different proportions. Three major Au deposits can be distinguished within the volcanic sediment of the Nova Lima group: Morro Velho in the town of Nova Lima, Raposos and Cuiaba. These deposits lie within the district of Nova Lima, 10 km to the south east of the city Belo Horizonte, and drain into Rio das Velhas (Figure 3.9A), a tributary to the Rio Sao Francisco. In the Nova Lima area, arsenopyrite occurs with 0.8 to 8% in the fresh ore (Fortes et al., cit. in Rawlins et al., 1997). Arsenic concentrations in the seriticized sections range from several hundred to 2500 mg kg^{-1} (Sichel and Valenca, 1983). Apart from some small-scale mining operations, today most of the mining and smelting operations are carried out with modern facilities. In the 1990's, Au production by Morro Velho was around 6 tons annually with about 1 million tons of ore (information courtesy of Mineracao Morro Velho S.A., 1998; Pires et al., 1996). A control area was chosen within the township of Brumal in the Santa Barbara, 60 km east of Belo Horizonte since the major research field was the area of Nova Lima. The catchment drains into the Rio da Conceicao (Figure 3.9B), a tributary to Rio Doce. On the north west of the Rio da Conceicao, the side opposite to the study catchment, an Au mine (Sao Bento) has workd for the past few years and may have resulted in contamination of the Conceicao river. According to Fortes et al. (cit. in Rawlins et al., 1997), Brumal shows a high potential for a mining related arsenic contamination with 40-70% of arsenopyrite in the vein ore bodies. Arsenic concentrations up to several thousand mg kg^{-1} (Marques de Souza, 1996) reported in stream sediments of the larger Santa Barbara area. However, this area has only recently undergone mineral exploration and data are insufficient. Since extensive amounts of arsenopyrite are reported from both the Nova Lima district (Galo and Mingu) and the Santa Barbara district (Brumal), a large reservoir is available. In the past, an arsenic trioxide factory in the Galo hills may have contributed to the release of arsenic into water and also into the soil. Although serious diseases like skin diseases have been observed in adults that worked in the old factory, but there are no official health studies. Thus there is not available any health authorities which correlate local arsenic enrichment with related public health problems or in particular the child population. Today, arsenic emissions from

the ore processing should be minimum because of modern control facilities. However, due to the economic constraints in the mentioned area, people use surface waters not only for fishing and gardening but in some cases also as their drinking water.

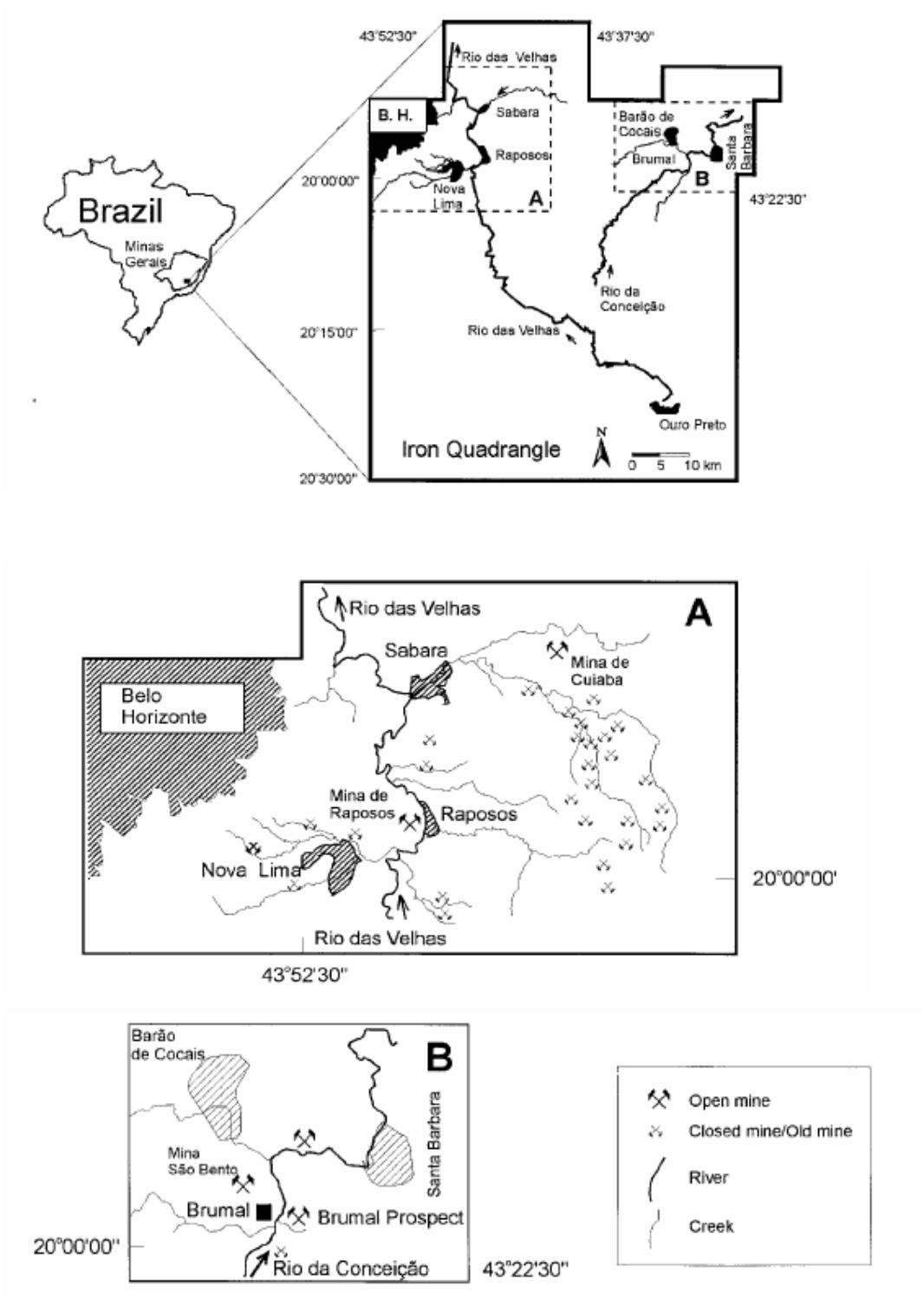


Figure 3.9 The Brazilian State of Mines Gerais and insets showing the area of the Iron Quadangle with the districts of **A** Nova Lima and **B** Santa Barbara, their drainage pattern and the position of abandoned and working mining operations (adapted from Matschullat et al., 2000)

3.2.4 Analyses of Reference Material

In order to validate the method, a certified reference material NIES CRM No.18 human urine (National Institute for Environmental Studies, Tsukuba, Japan), the only CRM available for arsenic species in urine was analyzed. Good agreement was obtained between certified and analyzed values for DMAs(V) and AsB in NIES CRM No.18. Table 3.2 shows the certified and obtained values for AsB, DMAs(V) and also total arsenic concentrations in the CRM No. 18.

Table 3.2 Certified and obtained values for NIES CRM No. 18 human urine (National Institute for Environmental Studies, Tsukuba, Japan)

Element/Species	Unit	Certified values	Obtained values
Total arsenic	µg/L	137 ± 11	134.46 ± 0.64[a]
DMAs(V)	µg/L as As	36 ± 9	38.05 ± 0.76[b]
AsB (Arsenobetaine)	µg/L as As	69 ± 12	71.86 ± 2.08[c]

[a]The total value obtained by ICP-MS. (n=5)
[b]The value is given as the average of four repeated injections using Prodigy ODS 3µ column.
[c]The value is given as the average of four repeated injections using Supelcosil LC-SCX column.

Arsenic species recovery tests were carried out with both human urine and cell samples, by adding standards. The recovery tests for human urine and cell samples were examined by adding standards to a sample to increase the concentration between 5 and 40 ng As/mL. The average of four recovery tests of the six arsenic species, which were 92-102%, is acceptable for the determination of each arsenic species.

The chromatographic recoveries were calculated from [sum(species)×100]/total arsenic.

3.2.5 Summary of Analytical Results

Table 3.1 summarizes all determined arsenic species of children's urine samples from an arsenic-affected area in Brazil. The reported concentrations are based on the average of 4 determinations. To validate the method, a certified reference material NIES CRM No.18 human urine (National Institute for Environmental Studies, Tsukuba, Japan), the only CRM available for arsenic species in urine was analyzed. Good agreement was obtained between certified and analyzed values for DMAs(V) and AsB in NIES CRM No.18. Table 3.2 shows the certified and obtained values for AsB, DMAs(V) and also total arsenic concentrations in the CRM No. 18. Monomethylarsonous acid [MMAs(III)] was detected in twelve of fifteen

samples. Dimethylarsinous acid [DMAs(III)] was not detected at any time, most probably due to volatilisation and some oxidation to DMAs(V) (Rabieh et al., publ. in prep.).

3.2.6 Investigation of Monomethylarsonous Acid (MMAs(III)) in Urine

It has been reported that trivalent methylated arsenic acids, monomethylarsonous acid (MMAs(III)) and dimethylarsinous acid (DMAs(III)), show significantly stronger genotoxic effects than the corresponding pentavalent ones and even inorganic arsenic (Dopp et al., 2004). In one study it was stated that MMAs(III) could supply as an indicator in urine to identify individuals with increased susceptibility to toxic and cancer-promoting effects of arseniasis (Valenzue la et al., 2005). According to analytical principles, in all the cited reports, identification of MMAs(III) and DMAs(III) in urine is based on comparison between HPLC retention time of sample and standard. For example when using the Reay and Asher method for DMAs(III) synthesis, dimethyldithioarsinic and dimethylthioarsinous acid are produced instead of the desired compounds that leading to wrong reference species. Dimethylarsinothioic acid was identified based on mass fragmentograms resulted of HPLC-ESI-MS not only in the Reay and Asher standard, but also as an arsenosugar metabolite in sheep urine (Hansen et al., 2004a,b,c and Martin et al., 2005). However, with regards to these results it cannot be excluded that studies using DMPS (2,3- dimercapto-1-propane sulfonate) as arsenic-complexing agent also produced S-containing analogues of the analytes. To solve this problem, simultaneous HPLC-ICP-MS/ESI-MS analysis will be necessary (Le et al., 2000a,b and Aposhian et al., 2000). However, in most cases the MMAs(III) and DMAs(III) concentrations in urine are too low to be detected by ESI-MS (Hirner 2006). Therefore other approaches have been chosen in this study: **(i)** The retention time interval of MMAs(III) from the HPLC run with urine samples from Brazilian children exposed to arsenic-rich drinking water was cut off, and then by hydride generation at pH 5 volatilized. The GC separation led to clear isolation of $MMAsH_2$ as proven by its mass frangmentogram compared with a library standard. The principle of MMAs(III) identification by 2D chromatography is shown in Figure 3.10.

This states that the analyte is either MMAs(III) (MMAs(V) is separated by HPLC separation and not volatilized under the applied pH conditions) or a compound, which contains a MMAs(III) group that can be cleaved under the reaction conditions applied. Figure 3.11 show the chromatograms after hydride generation at pH 5. **(ii)** Mass of 48 and 50 monitored as sulphur oxide (^{48}SO, ^{50}SO) during arsenic speciation. In this case, ICP-MS with reaction cell (reaction gas: O_2, 0.6 mL min^{-1}) was applied (Figure 3.12). The sulphur amount

within the retention time interval of MMAs(III) in urine sample was not significant on the background of the chromatogram (Rabieh et al., publ. in prep.).

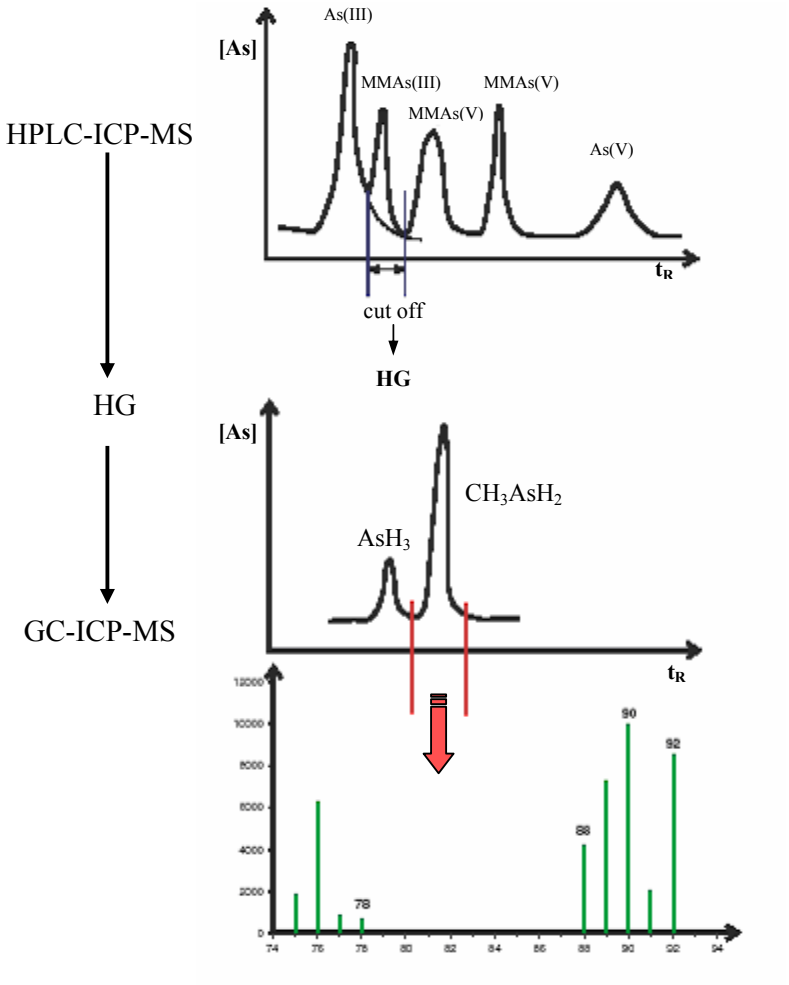


Figure 3.10 Principle of MMAs(III) identification by 2D chromatography and mass fragmentation: GC-ICP-MS/EIMS following HPLC fractionation. AsH_3 and CH_3AsH_2 are hydride generation (HG) products of As(III) and MMAs(III), respectively (adapted from Hirner, 2006)

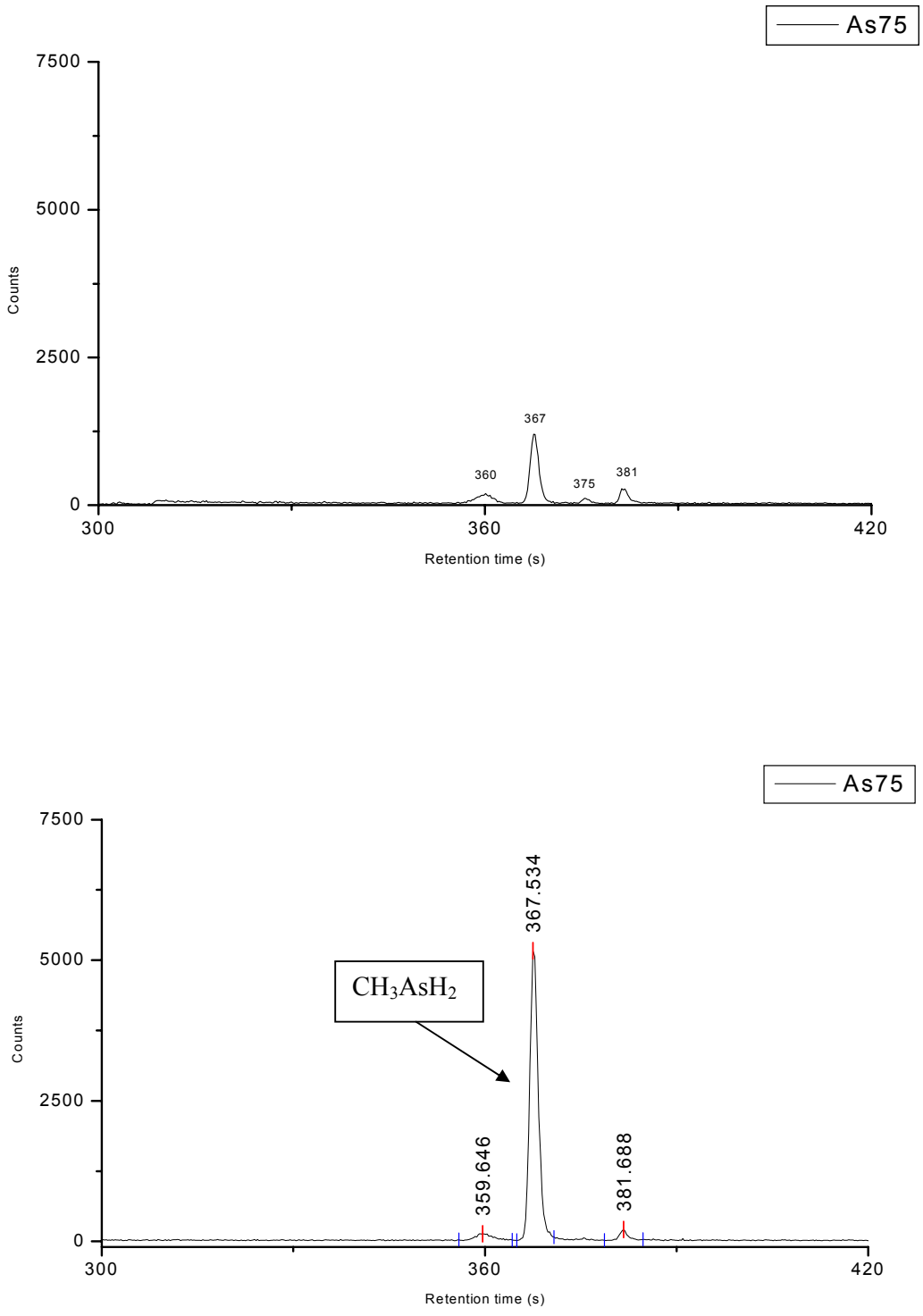


Figure 3.11 ICP-MS chromatogram after hydride generation at pH 5: **A** the retention time interval of MMAs(III) was cut from the HPLC run with urine sample from Brazilian children exposed to arsenic-rich drinking water **B** the retention time interval of MMAs(III) was cut from mix of arsenic species

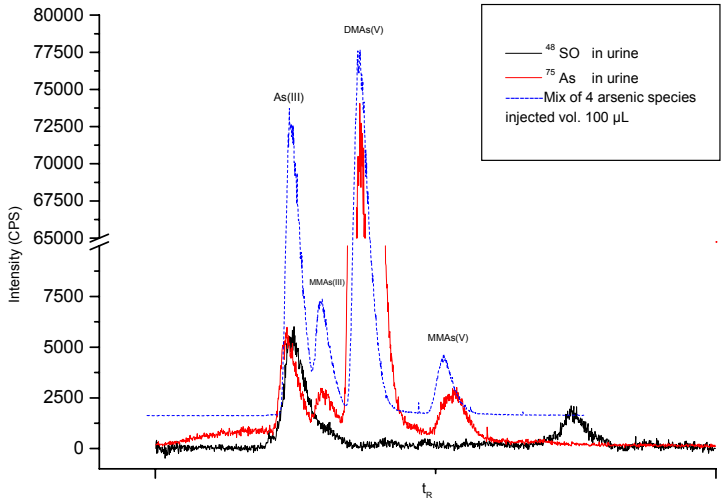


Figure 3.12 Chromatogram of an urine sample, and mix of 4 arsenic standards as labelled above.

3.3 Uptake of Arsenicals by Different Cells

3.3.1 Cellular Uptake of Inorganic and Organic Arsenic Compounds

In the present studies, uptake capabilities of different cell lines (UROtsa, CHO-9, Hep G2, HeLa S3, and Ra Hep) were investigated. In some cases further experiments, e.g. forced uptake by electroporation in CHO cell lines, differential centrifugation in UROtsa cells etc. were performed. Parts of obtained data which have already been published are not presented here or just briefly described.

3.3.1.1 UROtsa Cells

The uptake of the inorganic- and organoarsenic compounds by normal urothelial cell lines (UROtsa cells) was tested using concentration from 0.1 µM to 5mM for 1 h. Up to 13% of the DMAs(III) substrate in the external medium was taken up by UROtsa cells. This highest percentage of DMAs(III) uptake in UROtsa cell was detected at an external concentration of 0.1 µM among other exposed external concentrations (0.1 to 10 µM). These data suggest that UROtsa cells might be susceptible to relatively low concentration of arsenic (0.1 µM). Figure 3.13 shows the percentage of DMAs(III) uptake in UROtsa cell at different external concentration after 1 h exposition. Obtained data show, that the percentage uptake of

the arsenic substrates for pentavalent arsenic compounds (As(V), MMAs(V), DMAs(V) and TMAsO) is negligible, and the percentage range of uptake of other two arsenicals (As(III) and MMAs(III)) ranges from 0 to 0.18 percent. Figure 3. 14 shows the percentage of arsenicals (except of DMAs(III), which is separately shows on Figure 3.13) uptake in UROtsa cell at different external concentration after 1 h exposition. A concentration-dependency was observed in this cell line, which reached maximum at 10µM DMAs(III) (Tables A.1-A.3). Detected arsenic concentrations in the whole-cell extract and in the membrane-removed cell extract indicate no significant differences in the uptake of arsenic species for UROtsa cells (Tables A.1-A.3). Therefore, it is concluded that no arsenic substrates bound to the cell membrane. The amount of arsenic (ng/10^6 cells) as well as the percentage of substrate loading (%) was measured in whole cell extracts and in membrane-removed cell extracts by inductively coupled plasma-mass spectrometry (ICP-MS). Cells were exposed to arsenicals for 1 h. 5.0 × 10^6 cells were analyzed per treatment. Presented values represent means of duplicate incubations and five replicate analyses. ND, not detected (<0.02 ng As per 10^6 cells).

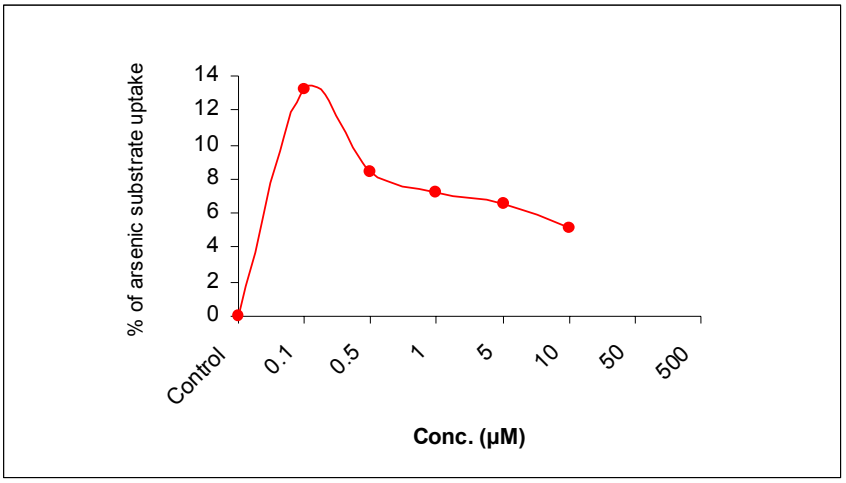


Figure 3.13 Percentage of DMAs(III) uptake in UROtsa cell at different conc. (1 h exposition)

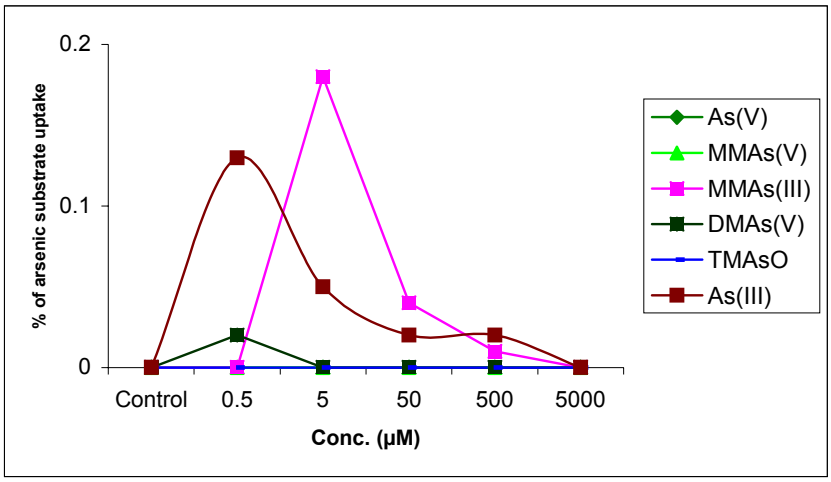


Figure 3.14 Percentage of arsenicals uptake in UROtsa cell at different conc. (1 h exposition)

The uptake of the inorganic and organic arsenic compounds by normal urothelial cell lines (UROtsa cells) summarized in Figure 3.15.

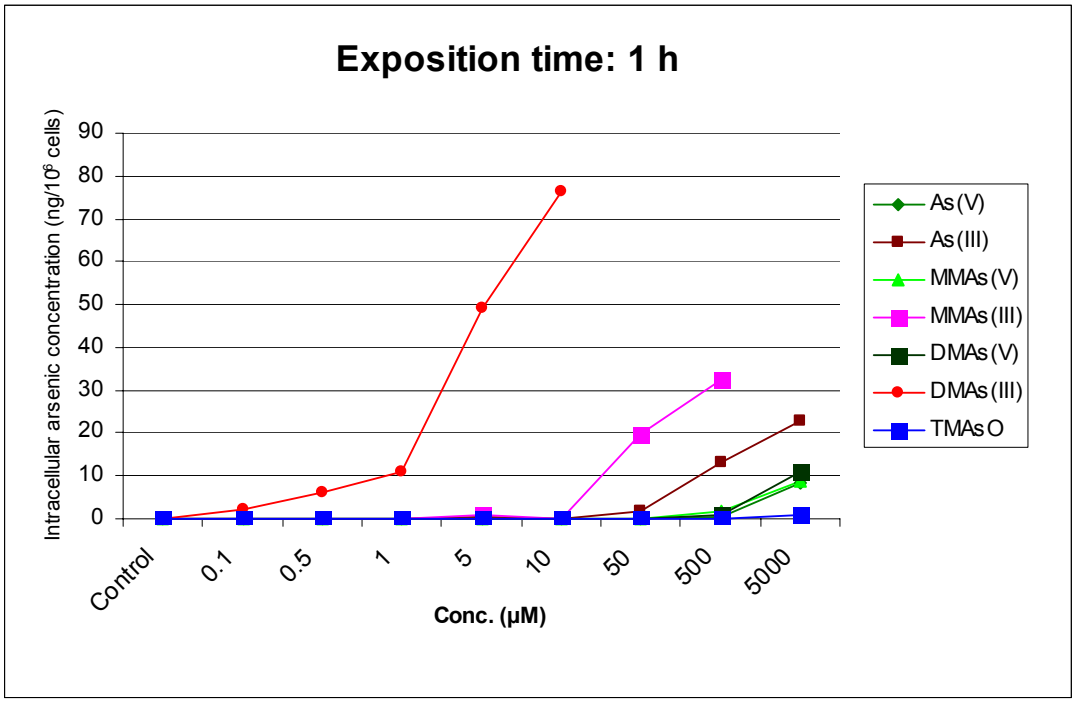


Figure 3.15 Uptake of arsenicals by UROtsa cells

3.3.1.1.1 Association of Arsenic with Intracellular Organelles in UROtsa Cells

In order to study association of arsenic substrates with intracellular organelles in UROtsa cells, differential centrifugation at five steps was performed. Therefore, six different samples were prepared for analysis. Obtained results (Tables A.4-A.10) of differential centrifugation indicate that association of arsenic species with +3 oxidation state is higher than the +5 species.

3.3.1.1.1.1 Nucleus

Percentage of total intracellular As in fraction in the nucleus organelle of UROtsa cell lines for an exposure time of 1 h and 24 h is summarized in Figure 3-16.

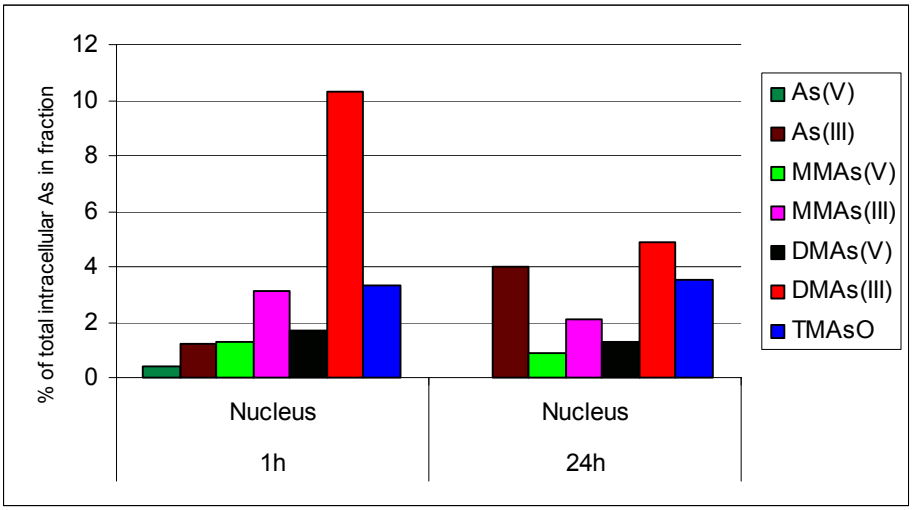


Figure 3.16 Percentage of total intracellular As in fraction in the nucleus of UROtsa cell (exp.time:1h & 24h)

As it can be easily seen in the Figure 3.16, uptake capabilities of all arsenic species (except TMAsO) are dependent on exposure time. Up to 10% of the DMAs(III) substrate in the external medium was taken up by nucleus at 1 h exposition. Results show, that uptake of this compound decrease to 5% for an exposure time of 24h. It's interesting that among the all arsenicals, only behaviour of As(III) is different compared to others. That means increasing of incubation time from 1 h to 24 h increase the uptake capability by the factor of 3.3. The species As(III), MMAs(V), and MMAs(III) were taken up to a slightly lower degree for an

exposure time of 24 h compare to 1 h exposition. A low amount of As(V) was taken up at 1 h exposition, which was not detected as the exposure time increased to 24 h.

Figure 3.17 shows the amount of detected arsenic in ng per 10^6 cells in the nucleus organelle of UROtsa cell lines for an exposure time of 1 h and 24 h. The results show that the detected arsenic concentrations of trivalent species As(III), MMAs(III), and DMAs(III) at the exposure time of 1 h are significantly higher than those at 24 h exposition. Among all tested arsenic species detected DMAs(III) concentration is higher than other species with the amount of 5.6 ng per 10^6 cells for an exposure time of 1 h. Longer exposure time up to 24 h has a different effect on the amount of detected arsenic species. For example, results show that the detected arsenic concentration of pentavalent species MMAs(V), DMAs(V), and TMAsO at the exposure time of 24 h is 2-3 times higher than those at 1 h exposition. In other hand, the detected arsenic concentration of trivalent species As(III), MMAs(III), and DMAs(III) at the exposure time of 1 h is significantly higher than those at 24 h exposition.

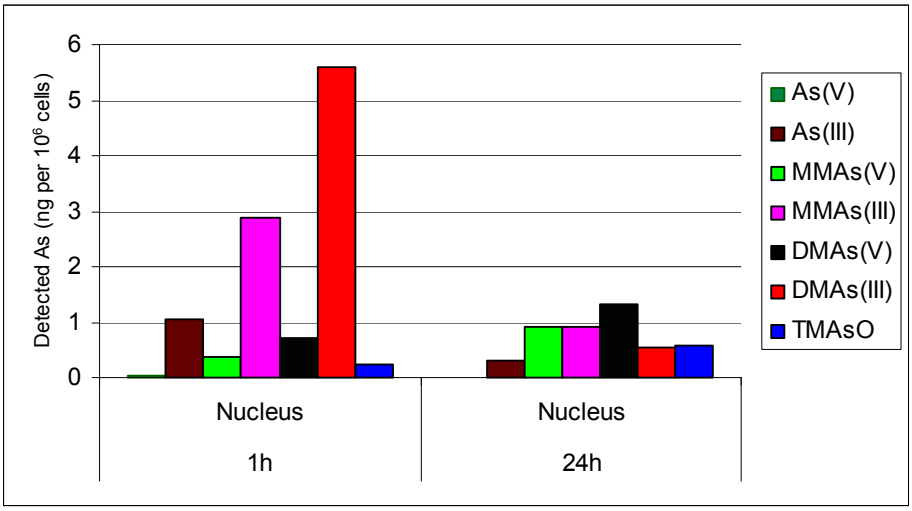


Figure 3.17 Detected arsenic concentration (ng per 10^6 cells) in the nucleus of UROtsa cell (exp.time:1h & 24h)

3.3.1.1.1.2 Mitochondria

Percentage of total intracellular As in fraction in the mitochondria organelle of UROtsa cell lines for an exposure time of 1 h and 24 h is summarized in Figure 3-18.

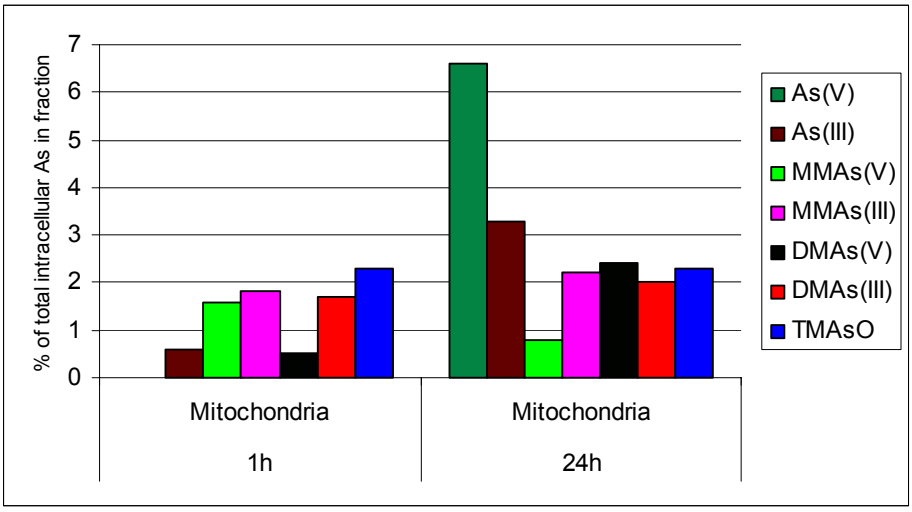


Figure 3.18 Percentage of total intracellular As in fraction in the mitochondria of UROtsa cell (exp.time:1h & 24h)

Results show that the uptake capabilities of all arsenic species (with the exception of TMAsO) are dependent on exposure time. The effect of exposure time for some species is significantly higher than others. Among them, the percentage of As(V) from 0 at exposure time of 1 h becomes 6.6% at 24 h exposition, which is the highest percent of uptake among the other tested arsenic species. The amount of As(III) and DMAs(V) species were taken up for an exposure time of 24 h almost 5 times more than those at 1 h exposition. For MMAs(V) the effect of longer exposition is different i.e. for an exposure time of 24 h the percentage of arsenic was decreased from 1.6% (at 1 h exposition) to 0.8%.

As it can be seen from the Figure 3.18, TMAsO is independent of exposure time, and the percent of TMAsO which was taken up, was the same for an exposure time of 1 h and also 24 h.

Figure 3.19 shows the amount of detected arsenic in ng per 10^6 cells in the mitochondria organelle of UROtsa cell lines for an exposure time of 1 h and 24 h. The results show that the detected arsenic concentration of pentavalent species As(V), MMAs(V), DMAs(V), and TMAsO at the exposure time of 24 h is higher than those at 1 h exposition. The highest detected arsenic was obtained for DMAs(V) at the exposure time of 24 h, which was 2.32 ng arsenic per 10^6 cells.

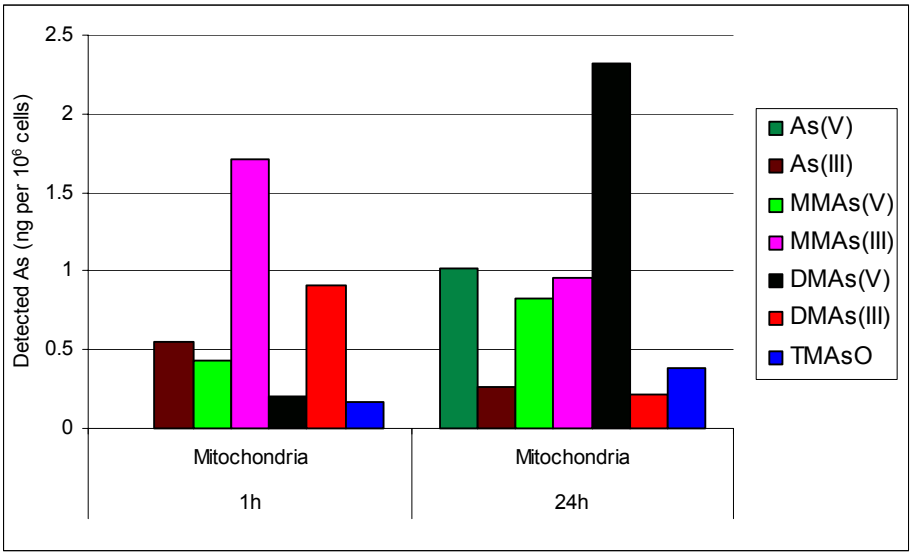


Figure 3.19 Detected arsenic concentration (ng per 10^6 cells) in the mitochondria of UROtsa cell (exp.time:1h & 24h)

Detected arsenic concentration of trivalent species As(III), MMAs(III), and DMAs(III) at the exposure time of 1 h is higher than those at 24 h exposition.

3.3.1.1.1.3 Plasma Membrane

Percentage of total intracellular As in fraction in the plasma membrane of UROtsa cell lines for an exposure time of 1 h and 24 h is summarized in Figure 3-20.

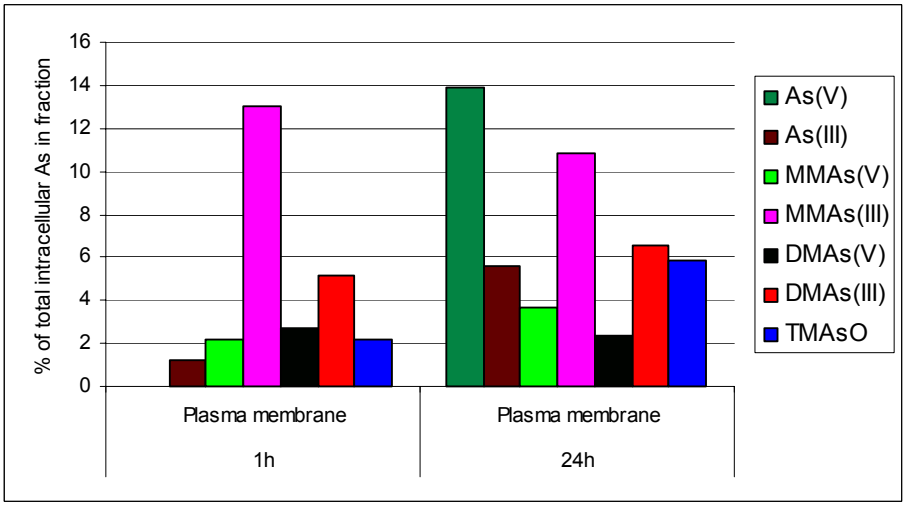


Figure 3.20 Percentage of total intracellular As in fraction in the plasma membrane of UROtsa cell (exp.time:1h & 24h)

As it can be seen in the Figure 3.20, uptake capabilities of all arsenic species are dependent on exposure time. Increasing of incubation time from 1 h to 24 h increase the uptake capability of all tested arsenic compounds with the exception of MMAs(III) and DMAs(V). At the exposure time of 1 h no arsenate substrate was detected, whereas up to 14% of it was detected at the exposure time of 24 h. This amount was the highest amount which was taken up among of all tested arsenic compounds. The amount of As(III) was taken up for an exposure time of 24 h almost 5 times more than that at 1 h exposition. Monomethylarsinic acid and dimethylarsinous acid were taken up to a slightly higher degree for an exposure time of 24 h compare to 1 h exposition, while for DMAs(V) and MMAs(III) were vice versa. The amount of TMAsO was taken up in the plasma membrane for an exposure time of 24 h almost three times more than that at 1 h exposition.

Figure 3.21 shows the amount of detected arsenic in ng per 10^6 cells in the plasma membrane of UROtsa cell lines for an exposure time of 1 h and 24 h. The highest detected arsenic was obtained for MMAs(III) at the exposure time of 1 h, which was 12.06 ng arsenic per 10^6 cells. The results show that the detected arsenic concentrations of pentavalent species As(V), MMAs(V), DMAs(V), and TMAsO at the exposure time of 24 h are higher than those at 1 h exposition, while the detected arsenic concentrations of trivalent species As(III), MMAs(III), and DMAs(III) at the exposure time of 1 h were higher than those at 24 h exposition. Among all tested arsenic species, As(V) was not detected at the exposure time of 1 h.

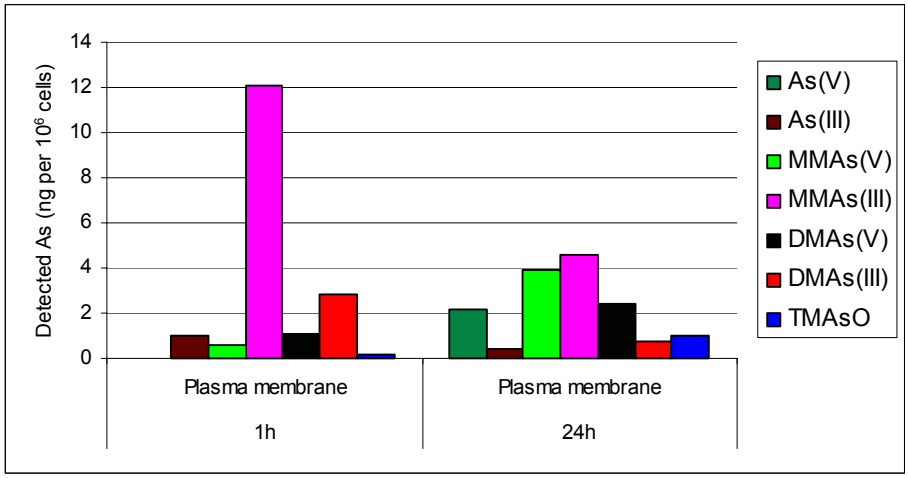


Figure 3.21 Detected arsenic concentration (ng per 10^6 cells) in the plasma membrane of UROtsa cell (exp.time:1h & 24h)

3.3.1.1.1.4 Ribosomes

Percentage of total intracellular As in fraction in the ribosomal subunits of UROtsa cell lines for an exposure time of 1 h and 24 h is summarized in Figure 3.22.

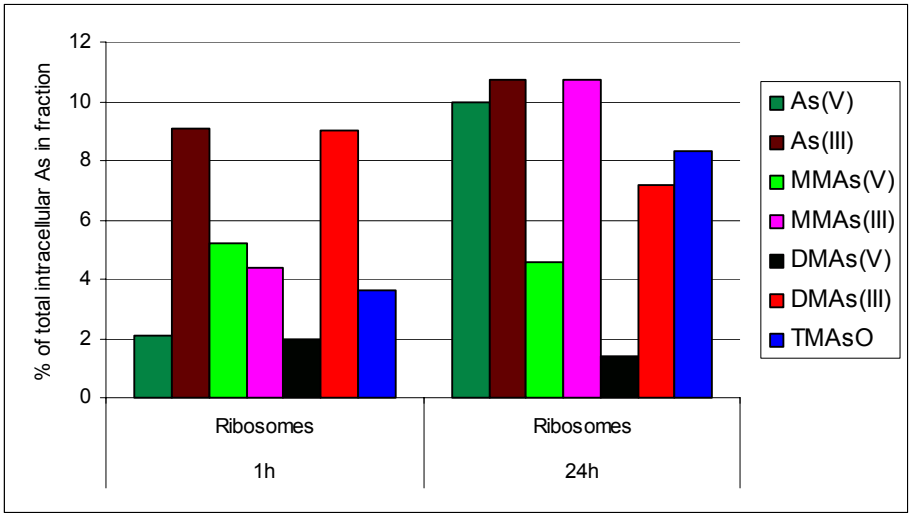


Figure 3.22 Percentage of total intracellular As in fraction in the ribosomal subunits of UROtsa cell (exp.time:1h & 24h)

As it can be seen in the Figure 3.22, uptake capabilities of all arsenic species are dependent on exposure time. 10.7% of As(III) and MMAs(III) species were taken up at the exposure time of 24 h. This amount was the highest percentage of uptake among the all tested arsenic compounds. The amount of As(V) was taken up for an exposure time of 24 h five times more than that at 1 h exposition. As(III) was taken up to a slightly higher degree for an exposure time of 24 h compare to 1 h exposition. But results show that MMAs(V) and DMAs(V) were taken up to a slightly lower degree for an exposure time of 24 h compared to 1 h exposition. Percentage of DMAs(III) uptake at the exposure time of 24 h decreased slightly from 9% to 7.2 percent at 1 h exposition. In other hand, TMAsO was taken up at the exposure time of 24 h more than two times higher than that at 1 h exposition.

Figure 3.23 shows the amount of detected arsenic in ng per 10^6 cells in the ribosomal subunits of UROtsa cell lines for an exposure time of 1 h and 24 h. The highest detected arsenic was obtained for As(III) at the exposure time of 1 h, which was 7.77 ng arsenic per 10^6 cells.

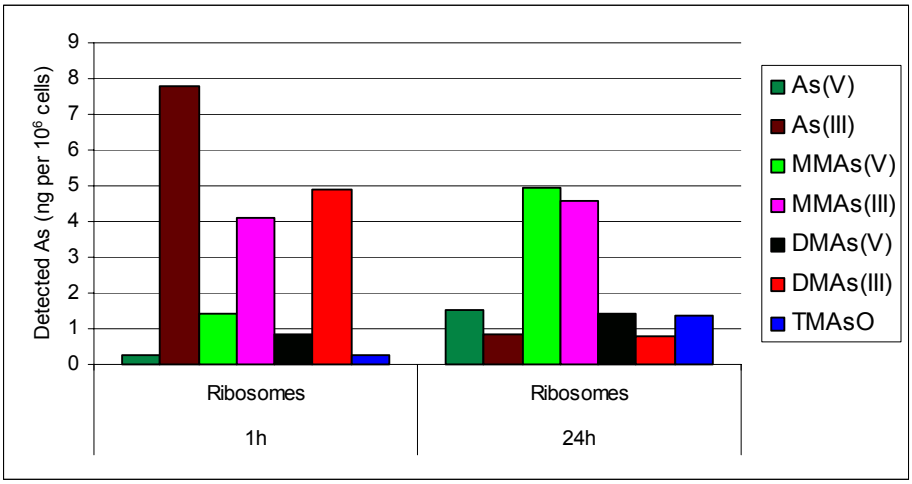


Figure 3.23 Detected arsenic concentration (ng per 10^6 cells) in the ribosomal subunits of UROtsa cell (exp.time:1h & 24h)

The results show that the detected arsenic concentrations of pentavalent species As(V), MMAs(V), DMAs(V), and TMAsO at the exposure time of 24 h are higher than those at 1 h exposition, which the highest detected arsenic was obtained by MMAs(V) with almost 5 ng arsenic per 10^6 cells among the pentavalent species. MMAs(III) was taken up to a slightly higher degree for an exposure time of 24 h compare to 1 h exposition, whereas the uptake of DMAs(III) decreased significantly at the exposure time of 24 h.

Subcellular distribution of different arsenic species in UROtsa cells for an exposure time of 1 h and 24 h are shown in Figures 3.24 and 3.25.

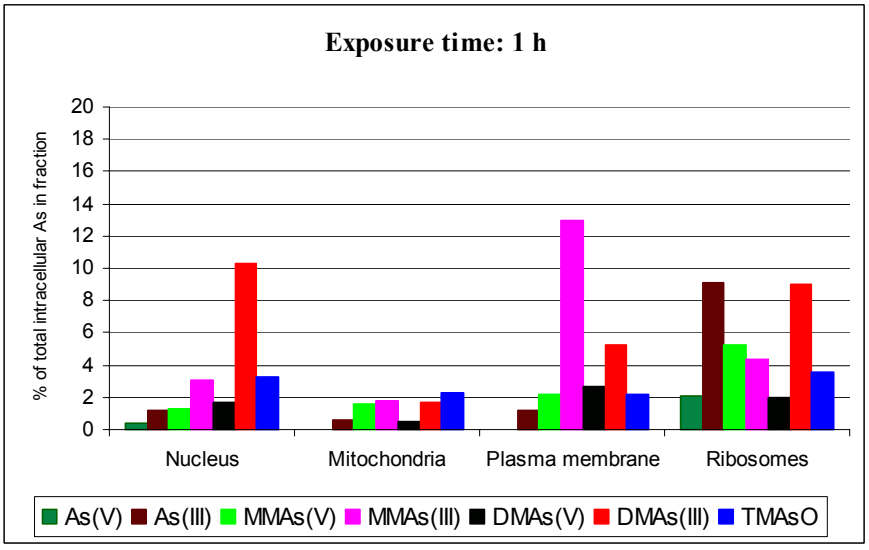


Figure 3.24 Subcellular distribution of different arsenic species in UROtsa cells (exposure time: 1 h)

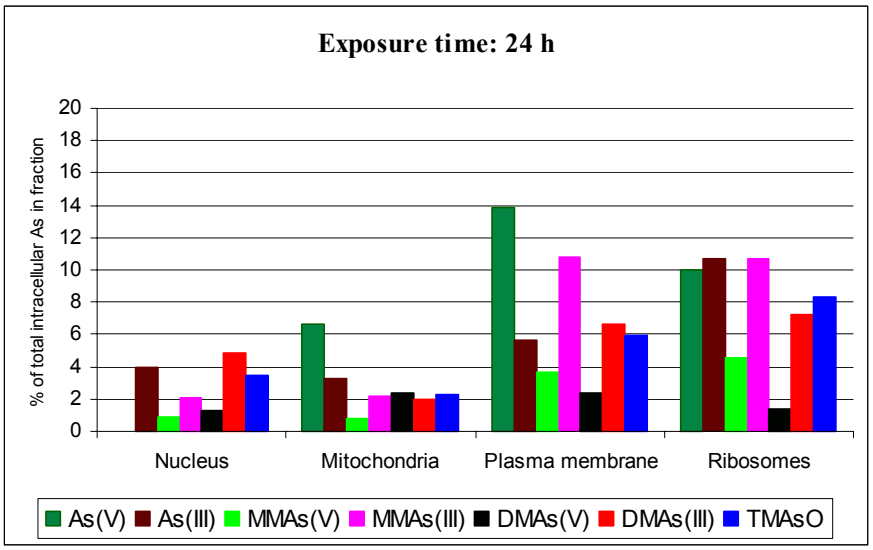


Figure 3.25 Subcellular distribution of different arsenic species in UROtsa cells (exposure time: 24 h)

3.3.1.2 CHO Cells

The uptake of the inorganic and organic arsenic compounds by CHO cell lines was tested using concentrations from 0.1 µM to 1 mM for 1 h. Most of the results from this study has already been published (Dopp et al., 2005). Obtained data is given in appendix (Tables A.11-A.13). The amount of arsenic (ng/10^6 cells) and also the percentage of substrate loading (%) was measured in whole-cell extracts and in cell-free (membrane removed) extracts by inductively coupled plasma-mass spectrometry (ICP-MS). Cells were exposed to arsenic compounds for 1 h. 8.8×10^6 cells were analyzed per treatment. Presented values represent means of duplicate incubations and five replicate analyses (mean %RSD = 3.3%). ND, not detected (<0.02 ng As per 10^6 cells) (Tables A.11-A.13).

Percentage of arsenate/arsenite, methylated pentavalent, and methylated trivalent arsenic species uptake in CHO-9 cell lines for an exposure time of 1 h is summarized in Figures 3.26-3.28.

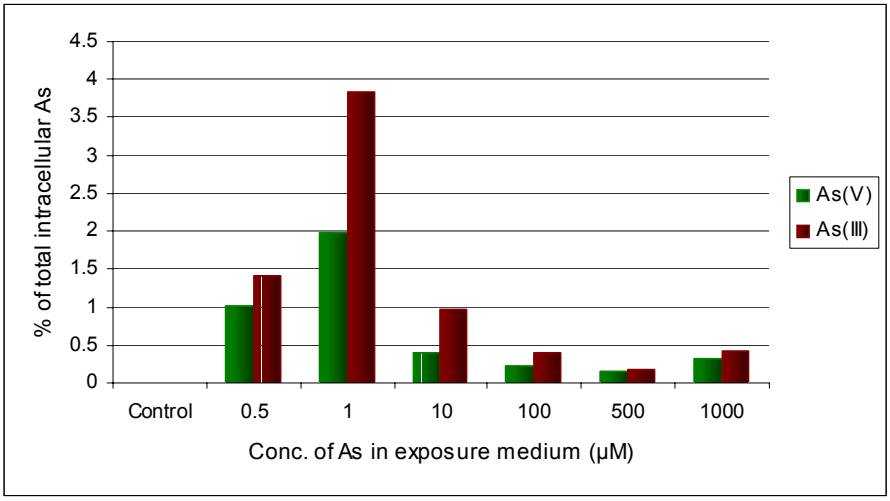


Figure 3.26 Percentage of total intracellular arsenic in the CHO-9 cell lines (exp. Time:1h)

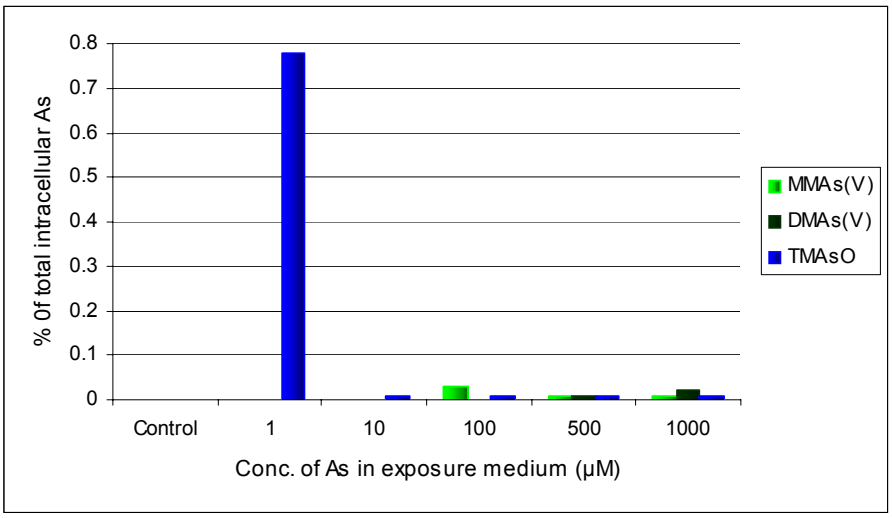


Figure 3.27 Percentage of total intracellular arsenic in the CHO-9 cell lines (exp. Time:1h)

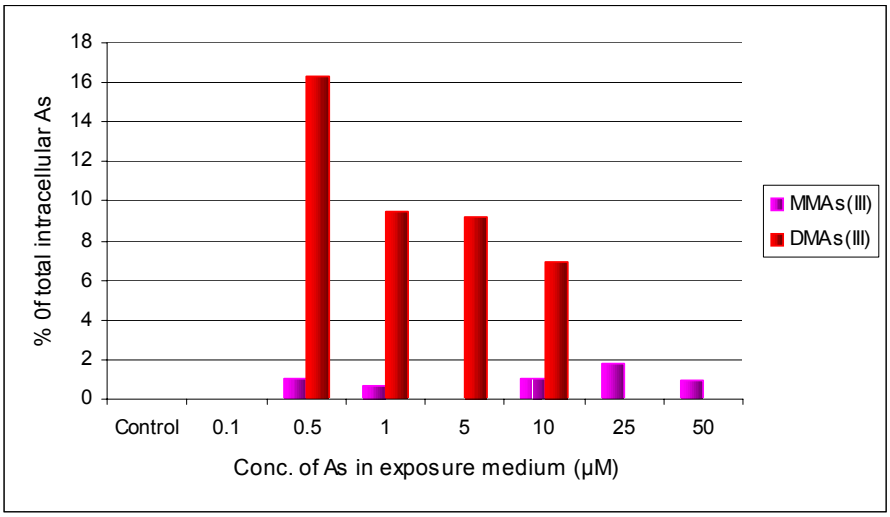


Figure 3.28 Percentage of total intracellular arsenic in the CHO-9 cell lines (exp. Time:1h) (note: external conc. of 0.1 & 5 µM of MMAs(III) and also 25 & 50 µM of DMAs(III) have been not investigated).

3.3.1.3 Hep G2 Cells

The uptake of the inorganic and organic arsenic compounds by Hep G2 cells was tested using concentrations from 0.5 µM to 5 mM for 1 h. Most of the results from this study have already been published (Dopp et al., 2005). Obtained data is given in appendix (Tables A.14-A.16). The amount of arsenic (ng/10^6 cells) and also the percentage of substrate loading (%) was measured in whole-cell extracts and in cell-free (membrane removed) extracts by inductively coupled plasma-mass spectrometry (ICP-MS). Cells were exposed to different arsenic compounds for 1 h. 3.3 × 10^6 cells were analyzed per treatment. Presented values represent means of duplicate incubations and five replicate analyses (mean %RSD = 3.3%). ND, not detected (<0.02 ng As per 10^6 cells) (Tables A14-A16).

Comparison of obtained data between hepatoma cells (Hep G2) and UROtsa cells reveal the following points:

- DMAs(III) was best taken up by both cell types follow by MMAs(III) and As(III)
- A concentration-dependency was observed for all arsenic compounds
- Arsenic compounds did not appear to associate with the cell membrane of both cells
- With the exception of DMAs(III), intracellular arsenic concentrations for As(III), As(V), MMAs(III), and DMAs(V) were 2-8 fold higher in Hep G2 than in UROtsa cells.

The uptake of the inorganic and organic arsenic compounds by Hep G2 Cells summarized in Figure 3.29.

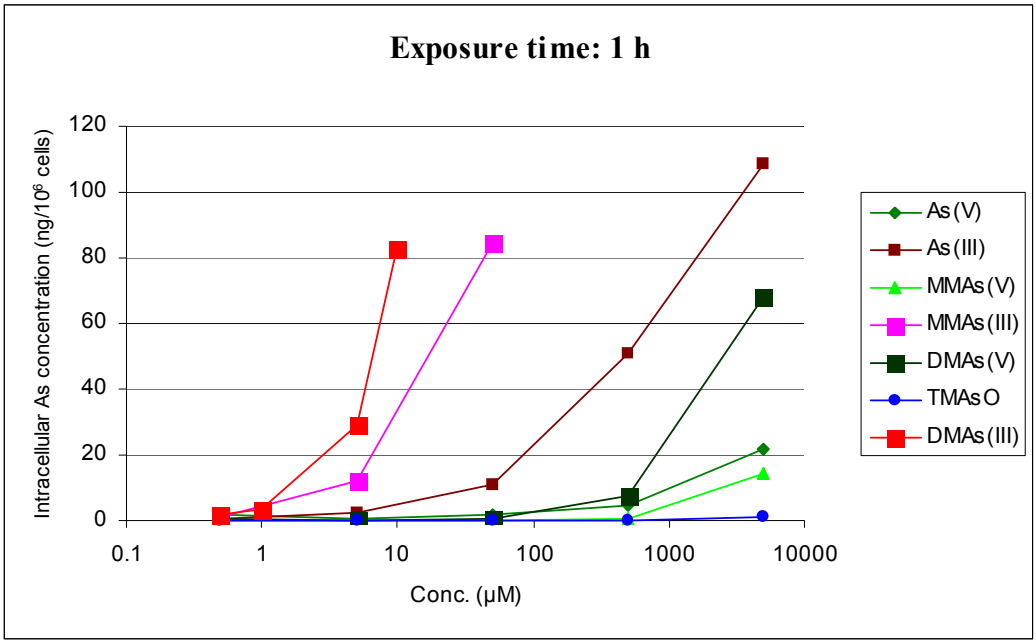


Figure 3.29 Uptake of arsenicals by Hep G2 cells

3.3.1.4 HeLa S3 Cells

HeLa S3 has been selected because it is an established cellular system for testing types of cell death and physiologic distributions. The uptake of the inorganic and organic arsenic compounds by HeLa S3 cells was tested using concentrations from 0.5 µM to 5 mM for 1 h. The obtained results are summarized in Tables A.17–A.18.

As it can be seen from the obtained results, up to 1.2% of the MMAs(III) substrate in the external medium was taken up by HeLa S3 cells. This highest percentage of MMAs(III) uptake in HeLa S3 cell was detected at an external concentration of 0.5 µM among other exposed external concentrations (0.5 to 10 µM). These data suggest that HeLa S3 cells might be susceptible to relatively low concentration of arsenic (0.5 µM). Detected arsenic concentrations in the whole-cell extract and in the cell free (membrane removed) extract indicate no significant differences in the uptake of arsenic species for HeLa S3 cells (Tables A.17-A.18). Therefore, it is concluded that no arsenic substrates bound to the cell membrane. Obtained data indicate that with the exception of MMAs(III) and DMAs(III), the percentage of other tested arsenic substrates in the external medium, which were taken up by HeLa S3

was zero. Almost 0.5% of DMAs(III) substrate in the external medium was taken up by HeLa S3 cells. This amount was almost same for all exposed external concentrations range (0.5 to 10 µM). The uptake of the inorganic and organic arsenic compounds by HeLa S3 cells summarized in Figure 3.30.

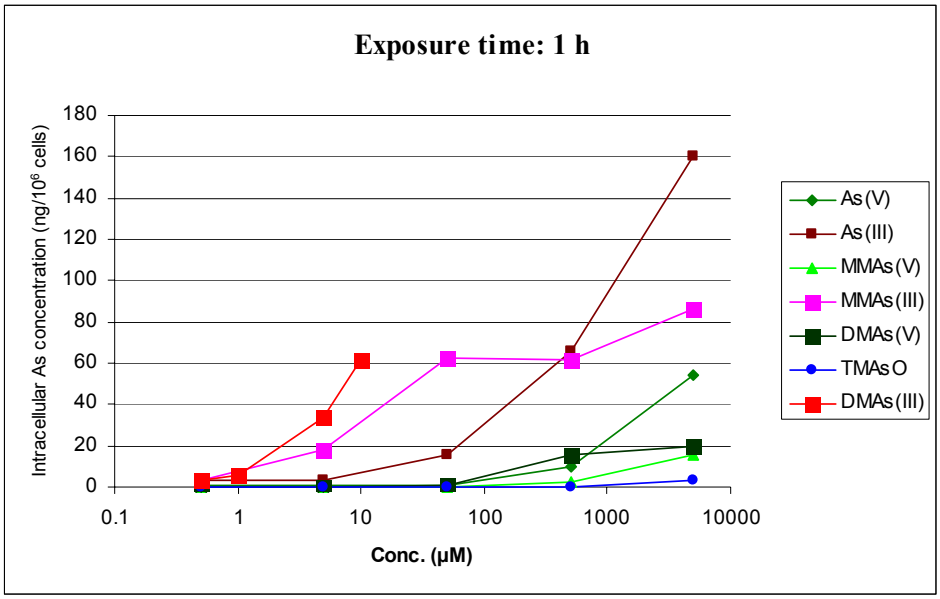


Figure 3.30 Uptake of arsenicals by HeLa S3 cells

3.3.1.5 Ra Hep Cells

Rat hepatocytes (Ra Hep) cell was the last cell type which has been studied. The uptake of the inorganic and organic arsenic compounds by rat hepatocytes (Ra Hep) cells was tested using concentrations from 0.5 µM to 5 mM for 1 h. The obtained results are summarized in Tables A.19–A.20.

As it can be seen from the obtained results, up to 0.75% of the MMAs(III) substrate in the external medium was taken up by rat hepatocytes (Ra Hep) cells. This highest percentage of MMAs(III) uptake in Ra Hep cell was detected at an external concentration of 0.5 µM among other exposed external concentrations (0.5 to 500 µM). These data suggest that Ra Hep cells might be susceptible to relatively low concentration of arsenic (0.5 µM). Detected arsenic concentrations in the whole-cell extract and in the cell free (membrane removed) extract indicate no significant differences in the uptake of arsenic species for Ra Hep cells (Tables A.19-A.20). Therefore, it is concluded that no arsenic substrates bound to the cell membrane. Obtained data indicate that the percentage of pentavalent tested arsenic substrates in the external medium, which were taken up by rat hepatocytes (Ra Hep) was zero. Almost 0.4% of As(III) and DMAs(III) substrates in the external medium was taken up by Ra Hep

cells. The uptake of the inorganic and organic arsenic compounds by rat hepatocytes (Ra Hep) cells summarized in Figure 3.31.

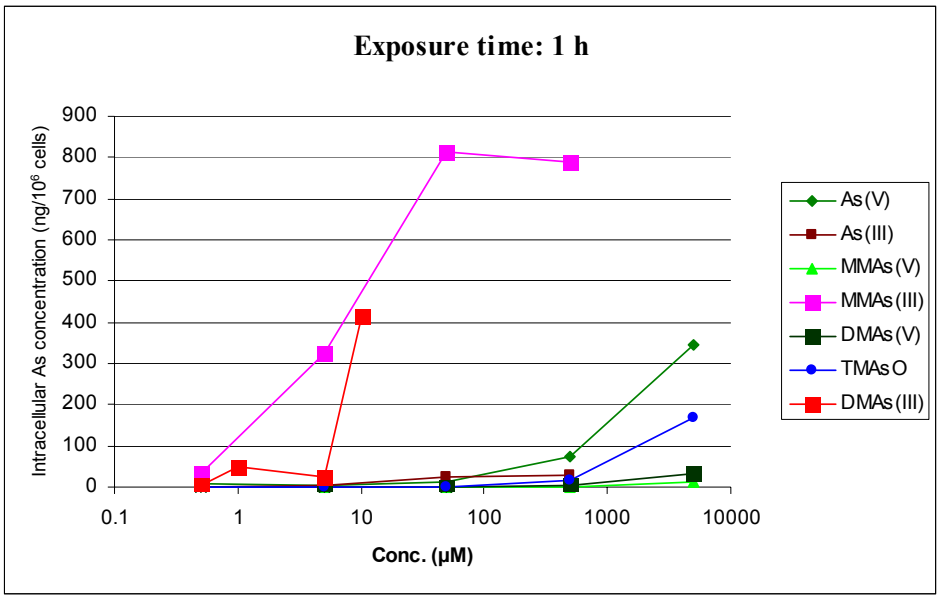


Figure 3.31 Uptake of arsenicals by Ra Hep cells

4 Discussion

Humans are exposed to arsenic and their organic metabolites mostly via food, water, and less via air. Following uptake, inorganic arsenic undergoes biotransformation to mono- and dimethylated metabolites (Dopp et al., 2004). Chronic arsenic exposure increases risk for the development of diabetes, vascular disease, and cancers of the skin, lung, kidney, and bladder (Chiou et al., 1995).Therefore, the following studies were performed:

1. Study arsenic metabolites, specially trivalent methylated arsenic species (MMAs(III) and DMAs(III)) in urine samples collected from Brazilian school children exposed to arsenic-rich drinking water by high performance liquid chromatography coupled to inductively coupled plasma mass spectrometry (HPLC-ICP-MS) technique. The effects of many chromatographic parameters such as pH, concentration of ion-pair reagent and buffer, flow rate of mobile phase, percentage of added methanol, and also the column temperature were investigated to achieve the best separation for arsenic species in the shortest time. In the results section these effects have also been described.

2. The cellular uptake of inorganic arsenic [arsenate, As(V); arsenite, As(III)] and the methylated arsenic species monomethylarsonic acid [MMAs(V)], monomethylarsonous acid [MMAs(III)], dimethylarsinic acid [DMAs(V)], dimethylarsinous acid [DMAs(III)], trimethylarsenic oxide [TMAsO] were investigated in Chinese hamster ovary (CHO-9) cells, human UROtsa (normal human urothelium) cells, HeLa S3 cells, Hep G2 cells, and rat hepatocytes (Ra Hep), to determine how arsenic species were taken up by above mentioned cell lines. Since the liver is the primary site of arsenic metabolism within the body and is a target organ for arsenic carcinogenicity, these type of cells were chosen (Dopp et al., 2005). It has been investigated whether the arsenic compounds are bound to membranes or whether they are present in the cytosol. For these purposes, the cell membranes removed by osmotic lysis and subsequent centrifugation before the intracellular arsenic concentration were measured by ICP-MS. The chemicals were applied at different concentrations (0.1 µM to 10 mM). Intracellular arsenic concentrations were determined by ICP-MS techniques.

3. In the other experiment, association and distribution of arsenic substrates with intracellular organelles (nucleus, mitochondria, plasma membrane, ribosomes and cytosol) in UROtsa cells has been studied. For these investigations, differential centrifugation at five steps is considered. Therefore, six different samples were prepared for analysis (see chapter 2, Figure 2.2).

4.1 Speciation of Arsenic in Urine Samples

The urine samples were subjected to speciation analysis under the optimum conditions. Obtained results show that, in addition to the inorganic arsenic species (arsenite and arsenate), organoarsenic species also were detected. In the following the obtained results will be discussed in the light of the relevant literature (see also Table 4.1).

4.2 Trivalent Methylated Arsenic Acids

The determination of trivalent arsenic species (MMAs(III) and DMAs(III)) are important because it has been demonstrated that these metabolites exhibit significantly stronger genotoxic effects than the corresponding pentavalent ones and even inorganic arsenic (Hirner 2006).

4.2.1 Dimethylarsinous Acid (DMAs(III))

In a simple experiment, it has been shown that DMAs(III) is very unstable: After running mixture of arsenic species including DMAs(III) through HPLC-ICP-MS (Figure 3.5), it has been kept frozen (-25 ^{o}C) for 24 h. Then, after this period of time, this mixture of arsenic species was analyzed again, which there was no corresponding peak for DMAs(III). It has already been shown from the obtained results (Table 3.1) that DMAs(III) was not detected at any time most probably due to volatilisation and oxidation to DMAs(V). In one study both DMAs(III) and MMAs(III) have been reported in urine of some humans exposed to inorganic arsenic through their drinking water in West Bengal, India (Mandal et al., 2001). The authors, reported the range of 4-21% of DMAs(III) of the total urinary arsenic, where the samples were analyzed ~2 months after collecting. In an other study, Mandal et al. (2004) reported up to 21.9 µg/L DMAs(III) (13%) of the total urinary arsenic, where the sample was analyzed ~2 months after collecting. Thus it seems to be unrealistic to report DMAs(III) in over 2 month old samples from West Bengal (Hirner 2006) when the stability of this species has been reported not to exceed one day (Gong et al., 2001). Other studies reported relatively high amount of DMAs(III) in urine samples from individuals chronically exposed to inorganic arsenic by consumptions of drinking water containing this metalloid, in these cases however, the samples have been analysed within 5-6 h of collection. Del Razo et al. (2001) reported the range of 6-31% of DMAs(III) of the total urinary DMAs. The major metabolite, DMAs(III), represented 49% of total urinary arsenic, reported by Valenzuela et al. (2005) from the urine samples chronically exposed to high inorganic arsenic from an area located in the central part

of Mexico. On the other hand, these studies were also criticized because of not strictly differentiating between free and glutathione-complexed DMAs(III) (Francesconi and Kuehnelt, 2004).

4.2.2 Monomethylarsonous Acid (MMAs(III))

Up to 5% of monomethylarsonous acid (MMAs(III)) of the sum urinary metabolites has been detected in urine samples collected from Brazilian school children exposed to arsenic-rich drinking water (Table 3.1). The percentage of trivalent methylated arsenic metabolites obtained here were typically comparable or lower than those reported in the literature. This might be due to malnutrition of the population that was being studied. MMAs(III) and DMAs(III) are normally bound to proteins and glutathione in the body because the trivalent arsenic species have a high affinity for thiol group in these molecules (Cullen et al., 1989; Zakharyan et al., 1999; Thompson, 1993 and Styblo, et al., 1995). When dimercaptopropane sulfonate (DMPS) is administered, those MMAs(III) and DMAs(III) species presumably were chelated to the excess DMPS and excreted into the urine (Aposhian et al., 1997 and Aposhian, 1983). But here, all urine samples collected without any prechemical treatment. Due to malnutrition, the concerned population lacked proteins and the trimethylated arsenicals were not bound fully to proteins in vivo. Hence, obtained results here might be at the lower side compared to those of Aposhian et al. (2000b). Many studies reported the percentage of MMAs(III) as different ranges in urine. Aposhian et al. (2000b) reported the amount of 11 and 7% for MMAs(III) of the urinary arsenic from the subjects in Romania who have been exposed to 84 or 161 μg of As/L in their drinking water. The range of 4 to 9% of MMAs(III) of the total urinary methyl arsenic has been reported by Del Razo et al. (2001) in the urine samples from individuals chronically exposed to inorganic arsenic by consumptions of drinking water containing this metalloid in the central Mexico. The range of 2 to 5% of MMAs(III) have found in the human urine samples of an area in West Bengal, India (Mandal et al., 2001). Le et al. (2000a) reported a high amount of MMAs(III) (240 μg/L) in human urine of Inner Mongolia.

In all reports, identification of MMAs(III) and DMAs(III) in urine is only based on comparison between HPLC retention time of sample and standard. While using the Reay and Asher method for DMAs(III) synthesis, dimethyldithioarsinic and dimethylthioarsinous acid are produced instead of the desired compounds, that leading to wrong reference species (Hirner, 2006). To try to solve this problem, simultaneous HPLC-ICP-MS/ESI-MS analysis will be necessary. However, obtained results here show very low concentration of MMAs(III)

(maximum concentration was 2 µg/L) in urine samples, and can not to be detected by ESI-MS. Therefore, another approaches (Hirner 2006) has been chosen: (**i**) The retention time interval of MMAs(III) from the HPLC run with urine samples from Brazilian children exposed to arsenic-rich drinking water was cut off and then by hydride generation at pH 5 volatilized (Rabieh et al., publ. in prep.). The GC separation led to obvious isolation of $MMAsH_2$ as proved by its mass fragmentogram compared to a library standard (see Figures 3.8-3.9). **(ii)** Monitoring mass of 48 and 50 as sulphur oxide (^{48}SO, ^{50}SO) during arsenic speciation. In this case, ICP-MS with reaction cell (reaction gas: O_2, 0.6 mL min^{-1}) was applied (Figure 3.10). The amount of sulphur on the background of the chromatogram within the retention time interval of MMAs(III) in urine sample was negligible.

4.2.3 Other Metabolites of Arsenic

As it can be seen from the Table 3.1 different range of other arsenic metabolites have been detected in this work. Percentage of DMAs(V) in the range of 27 – 55% (except one sample which was 21%) was detected in the urine samples in this study, which was comparable to those previously reported in the other studies. This metabolite was the major arsenic compound found in the urine. Mandal et al. (2001) reported the range of 44 – 74% of DMAs(V) of the total urinary arsenic in the urine samples from West Bengal, India. The range of 26 – 58% of DMAs(V) was detected in the urine samples from Promyelocytic Leukemia Patients collected 24 h after the injection of As(III) (Wang et al., 2004). Other studies also reported similar concentrations stating that DMAs(V) was the major arsenic metabolite which is excreted in the urine (Le et al., 2000a,b; Francesconi et al., 2002; Suzuki 2005; Feldmann et al., 2000; Stoeppler and Vahter 1994; Hakala and Pyy 1995; Del Razo et al., 2001; Meza et al., 2004).

Arsenite has been obtained in this study in the range of 9 to 19% (except one sample which has a high amount of 24%). Different range for arsenite has been reported in the literature: Le et al. (2000a) reported relatively high amount of arsenite ranging 17 to 32% of the total arsenic from the urine samples in Inner Mongolia, China. The range of 8-14 % has been reported in one study (Mandal et al., 2001) in the human urine samples of the areas in West Bengal, India. Del Razo et al. (2001) reported the range of 8 to 19% of the total arsenic in urine samples from the central Mexico. The range of 22 – 36% of arsenite was detected in the urine samples from Acute Promyelocytic Leukemia Patients collected 24 h after the injection of arsenic trioxide (Wang et al., 2004). Meza et al. (2004) reported 16 to 30% of arsenite of the total urinary arsenic in the urine samples in Sonora, Mexico. In another study

of a population in northern Argentina, children were found to have a substantially high percentage of inorganic arsenic (50%) in their urine samples (Concha et al., 1998).

Arsenate in the range of 7 to 13% was detected here. Mandal et al. (2001) reported 2-13% of arsenate in their study. The range of 7 to 11% was detected by Del Razo et al. (2001) in the human urine samples from central Mexico.Valenzuela et al. (2005) reported 9% of As(V) of the total arsenic in urine samples from Zimapan in the state of Hidalgo, in the central part of Mexico. MMAs(V) is obtained ranging 8.8 to 29% with the exception of one sample contains 37% of the total arsenic which was comparable (and also for some of the samples higher) to those previously reported in the literature. Le et al. (2000a) reported the range of 11-21% of this metabolite in the urine samples in Inner Mongolia, China. 8 to 11% of MMAs(V) has been reported by Mandal et al. (2001) in the urine samples of areas in West Bengal, India. Del Razo et al. (2001) reported 9 to 22% of this metabolite in the urine samples from Zimapan in central Mexico. The range of 14 to 32% of MMAs(V) was obtained in the urine samples of APL patients collected 24 h after the injection of arsenic trioxide (Wang et al., 2004).

Trimethylarsine oxide (TMAsO) is not detected at any time in this study. TMAsO has been identified as a metabolite in bacterial systems. Little is known about TMAsO in humans. Failure to observe TMAsO suggests that either its concentration in urine is below the detection limit of the method or it is further metabolized to trimethylarsine and subsequently exhaled into the breath as it is a volatile species (Le et al., 2000a). Francesconi et al, (2002) reported a very low amount of this metabolite (0.5%) in human urine after ingestion of an arsenosugar. Sur and Dunemann (2004) reported also a low amount of this species (TMAsO) up to % in human urine after consumption of seafood.

Tetramethylarsenium ion (Tetra) and Arsenocholine (AsC) metabolites have been not detected in any urine samples studied here. Arsenobetaine (AsB) has been detected at low range of 2 to 4% (except one sample with 21%) in some samples. This metabolite is the major constituent in seafood products, which excreted unchanged in the urine (Le et al., 1994).

Table 4.1 Arsenic species analyzed in human urine

Percentage of As detected in human urine samples								
As(V)	As(III)	MMAs(V)	MMAs(III)	DMAs(V)	DMAs(III)	TMAsO	AsB	Ref
2–13	8–14	8–11	2–5	44–74	4–21	–	–	Mandal et al., 2001
10.1	11.3	10.5	6.6	47.5	13	–	1	Mandal et al., 2004
7–11	8–19	9–22	4–9	7–61	6–31	–	–	Del Razo et al., 2001
7–13	9–19	8–29	up to 5	27–55	ND	ND	2–4	This study
9	9	3	7	24	49	–	–	Valenzuela et al., 2005
–	–	13–14	7–11	–	–	–	–	Aposhian et al., 2000b
1–5	22–36	14–32	1–2	26–58	0.1–0.6	–	–	Wang et al., 2004
–	–	–	–	11–21 (μg L^{-1})	–	–	18–137 (μg L^{-1})	Moldovan et al., 1998
1–5 (μg L^{-1})	4–9 (μg L^{-1})	2–4 (μg L^{-1})	–	16–26 (μg L^{-1})	–	–	–	Meza et al., 2005
2–3	16–30	7–14	–	40–71	–	–	–	Meza et al., 2004
ND	17–32	11–21	up to 40	15.3–61.2	up to 12	–	–	Le et al., 2000a
–	–	–	–	67	–	0.5	–	Francesconi et al., 2002
0.3–15	0.4–23	0.2–21	–	1–69	–	–	13–91	Ritsema et al., 1998
0.7–9.3	0.3–12.8	0.2–9.3	–	6–53	–	–	–	Hakala and Pyy, 1995

–: not reported
ND: not detected

Ritsema et al, (1998) reported AsB as the main constituent of the total arsenic present in the urine samples of volunteer who consumed a portion (250 g) of tuna fish, ranging from 88% after 12 h to 58% after 94 h.

4.3 Uptake Experiments

4.3.1 Comparison Between Fibroblasts and Hepatoma Cells

Monomethylarsonous acid (MMAs(III)) and dimethylarsinous acid (DMAs(III)) have been reported to be highly toxic in mammalian cells (Cohen et al., 2002; Styblo et al., 2000, 2002) and genotoxic (Mass et al., 2001; Nesnow et al., 2002). The reason for the high toxicity of methylated trivalent arsenicals has not been adequately explained, except that methylated trivalent arsenicals exert genotoxicity via reactive oxygen species (Nesnow et al., 2002). The trivalent methylated arsenic metabolite DMAs(III) was the most membrane-permeable species whereas up to 16% uptake by CHO-9 cells from the external medium was observed, when the cells were cultured with 0.5 µM DMAs(III), and the uptake of As(III) was lower, amounting to 3.8% of initial substrate loading. As shown in Tables A.11-A.13, the highest arsenic uptake was mostly detectable at relatively low concentrations (DMAs(III): 0.5 µM, As(III): 1.0 µM) and this percentage decreases with increasing arsenic concentrations in the external medium. It might be that a defence mechanism exists i.e. the extrusion of As(III) out of the cells and the prevention of an uptake at higher concentrations.

The uptake capability of DMAs(III) by Hep G2 cells was much lower (~4.5% from the external medium at 10-fold higher concentrations i.e. at 5 µM) compared to CHO-9 cell lines. An intensive comparison between these two cell types has been discussed in a published paper (Dopp et al., 2005).

4.3.2 UROtsa Cell Line

4.3.2.1 Cellular Uptake

The pentavalent arsenic species were not taken up by UROtsa cell lines at different applied concentrations (0.5 µM to 5 mM). The trivalent methylated arsenic derivative DMAs(III) was the most membrane-permeable species whereas up to 13.3% uptake by UROtsa cells from the external medium was shown, when the cells were cultured with 0.1 µM DMAs(III), and the uptake of MMAs(III) and As(III) were much lower, amounting to 0.18% and 0.13%, respectively of initial substrate loading. As shown in Tables A.1-A.3, the highest

arsenic uptake was mostly detectable at relatively low concentrations (DMAs(III): 0.1 µM, MMAs(III): 5 µM, As(III): 0.5 µM) and this percentage decreases with increasing arsenic concentrations in the external medium. It might be that a defence mechanism exists in this case i.e. the extrusion of As(III) out of the cells and the prevention of an uptake at higher concentrations (Wang and Rossman, 1993; Wang et al. 1996). Detected arsenic concentrations in the whole-cell extract and in the cell-free (membrane removed) extract indicate no significant differences in the uptake of arsenic species for UROtsa cell lines. Therefore, it is concluded that no arsenic substrates bound to the cell membranes. The order of cellular uptake for the arsenic compounds in trivalent state was: DMAs(III) >> MMAs(III) > As(III). Similar differences between the uptake and/or retention of methylated trivalent arsenicals and As(III) have been found in primary rat hepatocytes (Styblo et al., 2000) and differentiated 3T3L1 adipocytes (Drobna et al., 2005). A concentration-dependency was obsorved for all arsenic compounds.

The uptake capability of DMAs(III) by UROtsa cells were much higher compared to Hep G2 cell lines, but 3% lower compared to CHO-9 fibroblasts.

4.3.2.2 Subcellular Distribution of Arsenicals in UROtsa Cell Lines

Cellular uptake of arsenicals in UROtsa cells have been studied (see 4.3.2.1 section). Further investigation has been focused on association and distribution of arsenic substrates after 1 and 24 h exposition with intracellular organelles (nucleus, mitochondria, plasma membrane and etc) in UROtsa cells. Results show that after 1 h exposition, the trivalent arsenicals were more effective than pentavalent one. Among the tested arsenicals, the highest percentage of total intracellular arsenic observed for MMAs(III) whereas 13% of this substrate was taken up by plasma membrane, 10.3% of DMAs(III), and 9.1% of As(III) of initial substrate loading, were taken up by nucleus and ribosomes, respectively.

Comparison of the pecentage of uptake by each organelle type after 1 and 24 h exposition is interesting. Results show, that the percentage of As(III) in the ribosomes increased very slightly after 24 h exposition to 10.7%. That means, only 1.6% was the difference of uptake capability by ribosomes. This can be concluded that uptake of arsenite by ribosomes may be occured very fast within 1 h, and as a result of that, the synthesis of proteins may be affected, because the ribosomes are the sites of protein synthesis, where RNA is translated into protein. Pyruvate dehydrogenase (PDH) is a multi subunit complex that requires the cofactor lipoic acid, a dithiol, for enzymatic activity. Arsenite inhibits PDH (Peters, 1955; Szinicz and Forth, 1988; Hu et al., 1998), perhaps by binding to the lipoic acid

moiety. Arsenite causes also enzymes inhibition through chemical reaction with thiol functional group of enzymes (Bredfeldt et al., 2004).

Subcellular distributaion pattern of MMAs(III) is different regards to changing of exposition time. Results show, that after 24 h expostion almost the same percentage of this substrate were taken up by plasma membrane and ribosomes, which compare to 1 h exposition ribosomes had 2.5 times more, and plasma membrane had 2.2% less uptake. In the ribosomes, MMAs(III) may play as an inhibitor for GSH-reductase (Styblo et al., 1997).

Up to 10.3% of total intracellular arsenic as DMAs(III) was taken up by nucleus at the expostion time of 1 h, whereas this amount decreased to 4.9% with inceasing the exposure time to 24 h. Inceasing the exposure time to 24 h, among the organelles, ribosomes has the major uptake capability with 7.2% followed by plasma membrane, and nucleus. Schwerdtle et al. (2003) observed oxidative DNA damage via trivalent methylated arsenicals in PM2 DNA and HeLa cells. DMAs(III) react rirectly with DNA, nicking naked DNA *in vitro* and damaging nuclear DNA in intact human leukocytes (Mass et al., 2001). Styblo et al. (2002) suggested that exposures to methylated trivalent arsenicals associated with a variety of adverse effects that have a profound impact on cell viability or proliferation. The known effects include a) inhibition of several key enzymes, b) demage of DNA structure, and c) activation of AP-1-dependent gene transcription. These authors reported that MMAs(III) and/or DMAs(III) derivatives are more potent than As(III) in producing these effects.

The percentage of arsenate, which has been taken up by plasma membrane and ribosomes were 13.9 and 10.0%, respectively at the exposure time of 24 h. Whereas, at the exposure time of 1 h , no arsenate was detected in plasma membrane, and low percentage of this metabolie was observed in ribosomes (2.1%). It might be, the chemical similarity of arsenate to phosphate explaines the relatively high percentage uptake of this metabolite by plasma membrane and ribosomes at the exposition time of 24 h. Arsenate like arsenite may causes inhibition of PDH enzyme and blocks cellular respiration (Szinicz and Forth, 1988).

In the case of MMAs(V), the percentage of this metabolite at the exposure time of 1 h in the organelles with the exception of plasma membrane is slightly higher compare to 24 h exposition. The highest percentage of detected DMAs(V) was in plasma membrane with 2.7% at the exposure time of 1 h. TMAsO was taken up to 8.3% by the ribosomes at the exposure time of 24 h and in the lower amount at the exposure time of 1 h. However, cytotoxic effects in the urothelial cells are not induced by pentavalent methylated arsenicals (von Recklinghausen et al., publ. in prep.).

4.3.3 HeLa S3 and Ra Hep Cell Lines

The pentavalent arsenic species were neither taken up by HeLa S3 nor by rat hepatocytes (Ra Hep) cell lines at different applied concentrations (0.5 µM to 5 mM). The trivalent methylated arsenic derivative MMAs(III) was the most membrane-permeable species among the tested arsenicals, whereas up to 1.22 and 0.75% uptake by HeLa S3 and Ra Hep cell lines from the external medium were shown, respectively, when the cells were cultured with 0.5 µM MMAs(III). The uptake of DMAs(III) and As(III) were lower amounting to 0.5% and 0.02% for HeLa S3, 0.4% and 0.35% for Ra Hep cell lines, respectively. As shown in Tables A.17-A.20, the highest arsenic uptake was mostly detectable at relatively low concentrations. It might be here like UROtsa cell line a defence mechanism exists (see 4.3.2.1 section). Detected arsenic concentrations in the whole-cell extract and in the cell-free (membrane removed) extract indicate no significant differences in the uptake of arsenic species for these cell lines. Therefore, it is concluded that no arsenic substrates bound to the cell membranes. The order of cellular uptake for the arsenic compounds in trivalent state was: MMAs(III) > DMAs(III) > As(III). The uptake capability of DMAs(III) by these cell lines was much lower compared to other cell lines studied here (UROtsa, CHO-9, and Hep G2), but the uptake capability of MMAs(III) was comparable.

4.3.4 Summary of Uptake Capabilities of Different Cell Lines

Different cell types have different capabilities for the uptake of arsenic compounds. The oxidation state and the degree of methylation of the arsenicals determine the uptake and subsequently the toxicity of the compounds. Table 5.1 (in summary section) shows the comparison of uptake capabilities of different cell types which have been studied here. As it has been shown in Table 5.1 trivalent methylated and inorganic arsenic species are more membrane-permeable than pentavalent arsenic metabolites and are taken up by these cells to a higher degree from the external medium. The present study revealed that trivalent arsenic metabolites [As(III), MMAs(III), and DMAs(III)] were best taken up by CHO cell lines and also in higher degree compared to other cell types which have been studied in this work. The order of cellular uptake of trivalent arsenicals by different cell types was:

As(III): CHO-9 > Hep G2 > Ra Hep > UROtsa > Hela S3

MMAs(III): CHO-9 > Hela S3 > Hep G2 > Ra Hep > UROtsa

DMAs(III): CHO-9 > UROtsa > Hep G2 > Hela S3 > Ra Hep

It is interesting that the pentavalent arsenic species [As(V), MMAs(V), DMAs(V), and TMAsO] were not taken up by UROtsa, HeLa S3, and also by rat hepatocytes (Ra Hep) cell lines at different applied concentrations (0.5 µM to 5 mM).

The order of cellular uptake (intracellular concentration of arsenic) of pentavalent arsenic compounds by CHO-9 and Hep G2 cell lines was as follows:

As(V): CHO-9 > Hep G2

MMAs(V) and DMAs(V): No significant difference was shown

and finally the intracellular concentration of arsenic for TMAsO was higher in CHO-9 compared to Hep G2 cell lines.

Summary

A speciation technique for anionic arsenic species has been applied using an ion pair reverse phase-high performance liquid chromatography coupled to inductively coupled plasma mass spectrometry (RP-HPLC-ICP-MS). Six arsenic species (arsenite, arsenate, dimethylarsinic acid, dimethylarsinous acid, momomethylarsonic acid, and monomethylarsonous acid) have been separated with isocratic elution within less than 6 minutes. Furthermore, a cation exchange column was used for separation of AsB, AsC, tetra, and TMAsO. The chemical form and oxidation state of arsenic is very important with regards to toxicity, therefore analysis of total arsenic is insufficient for complete toxicological and risk assessment evaluation. Thus, arsenic speciation has been studied on some urine samples of the children from an arsenic-affected area in Iron Quadrangle, Brazil. DMAs(V) and MMAs(V) were the major urinary metabolites in these samples which have been detected. The mean value for total arsenic concentration of all urine samples analysed (n=15) is 26.33 ng As/mL with a range from 16.1 to 55.2 ng As/mL. TMAsO and AsC (arsenocholin) were not detected in any urine samples in this study. In the most of these samples, monomethylarsonous acid [MMAs(III)] was detected up to 2.0 ng As/mL. DMAs(III) was not detected at any time, most probably due to volatilisation and some oxidation to DMAs(V). The chromatographic recoveries calculated from [sum(species)×100]/total arsenic in urine samples were from 77.4 to 94.9 percent. To validate the method, a certified reference material NIES CRM No.18 human urine (National Institute for Environmental Studies, Tsukuba, Japan), the only CRM available for arsenic species in urine was analysed. Good agreement was obtained between certified and analyzed values for DMAs(V) and AsB in NIES CRM No.18.

More investigation has been made on identification of monomethylarsonous acid (MMAs(III)): **(i)** The retention time interval of MMAs(III) from the HPLC run with urine samples from Brazilian children exposed to arsenic-rich drinking water was cut off, and then by hydride generation at pH 5 volatilized. The GC separation led to clear isolation of $MMAsH_2$ as proven by its mass frangmentogram compared with a library standard. This shows that the analyte is either MMAs(III) (MMAs(V) is separated by HPLC separation and not volatilized under the applied pH conditions) or a compound, which contains a MMAs(III) group that can be cleaved under the reaction conditions applied. Figure 3.9 show the chromatograms after hydride generation at pH 5. **(ii)** Mass of 48 and 50 monitored as sulphur oxide (^{48}SO, ^{50}SO) during arsenic speciation. The sulphur amount within the retention time

interval of MMAs(III) in urine sample was not significant on the background of the chromatogram.

Different cell types have different capabilities for the uptake of arsenic compounds. The oxidation state and the degree of methylation of the arsenicals determine the uptake and subsequently the toxicity of the compounds. Table 5.1 shows the comparison of uptake capabilities of different cell types which studied here.

Table 5.1 Comparison of uptake capabilities of different cell types

		Arsenic species						
Cell type		As(V)	As(III)	MMAs(V)	MMAs(III)	DMAs(V)	DMAs(III)	TMAsO
CHO-9	HP*	1.99	3.83	0.03	1.78	0.02	16.3	0.78
	C	1	1	100	25	1000	0.5	1
Hep G2	HP	0.97	0.63		1.16	0.63	4.17	
	C	0.5	0.5	ND**	5	0.5	5	ND
UROtsa	HP		0.13		0.18	0.02	13.27	
	C	ND	0.5	ND	5	0.5	0.1	ND
Hela S3	HP		0.02	0.05	1.22		0.46, 0.48	
	C	ND	0.5	0.5	0.5	ND	0.5, (5, 10)	ND
Ra Hep	HP	0.08	0.33		0.75		0.41, 0.46	
	C	0.5	0.5	ND	0.5	ND	1, 10	ND

*HP: Highest Percent (detected As in whole-cell extract) & C: concentration (μM)
**ND: not detected

As it has been shown in Table 5.1 trivalent methylated and inorganic arsenic species are more membrane-permeable than pentavalent arsenic metabolites and are taken up by these cells to a higher degree from the external medium. The present study revealed that trivalent arsenic metabolites [As(III), MMAs(III), and DMAs(III)] were best taken up by CHO cell lines and also in higher degree compared to other cell types which have been studied in this work.

References

Andrews, P., Kitchin, K. T., Wallace, K. (2003). Dimethylarsine and Trimethylarsine Are potent Genotoxins In Vitro. *Chem Res Toxicol* **16**, 994-1003.

Aposhian, H. V. (1993). DMSA and DMPS-water soluble antidotes for heavy metal poisoning. *Annu Rev Pharmacol Toxicol* **23**, 193-215.

Aposhian, H. V., Arroya, A., Cebrian, M. E., Del Razo, L. M., Hurlbut, K. M., Dart, R. C., Gonzalez-Ramirez, D., Kreppel, H., Speisky, H., Smith, A., Gonsebatt, M. E., Ostrosky-Wegman, P., Aposhian, M. M. (1997). DMPS-arsenic challenge test. I. Increased urinary excretion of monomethylarsonic acid in humans given dimercaptopropane sulfonate. *J Pharmacol Exp Ther* **282**, 192-200.

Aposhian, H. V., Gurzau, E. S., Le, X. C., Gurzau, A., Healy, S. M., Lu, X., Ma, M., Yip, L., Zakharyan, R. A., Maiorino, R. M., Dart, R. C., Tirus, M. G., Gonzalez-Ramirez, D., Morgan, D. L., Avram, D., Aposhian, M. M. (2000b). Occurrence of monomethylarsonous acid in urine of humans exposed to inorganic arsenic. *Chem Res Toxicol* **13**, 693-697.

Aposhian, H. V., Zakharyan, R. A., Avram, M. D., Sampayo-Reyes, A., Wollenberg, M. L. (2004). A review of the enzymology of arsenic metabolism and a new potential role of hydrogen peroxide in the detoxification of the trivalent arsenic species. *Toxicol Appl Pharmacol* **198**, 327-335.

Aposhian, H. V., Zheng, B., Aposhian, M. M., Le, X. C., Cebrian, M. E., Cullen, W. R., Zakharyan, R. A., Ma, M., Dart, R. C., Chang, Z., Andrewes, P., Yip, L., O'Malley, G. F., Maiorino, R. M., van Voorhies, W., Healy, S. M., Titcomb, A. (2000a). DMPS-Arsenic Challenge Test II Modulation of Arsenic Species, Including Monomethylarsonous Acid (MMA^{III}), Excreted in Human Urine. *Toxicol Appl Pharmacol* **165**, 74-83.

Arbouine, M. W., Wilson, H. K. (1992). The effect of seafood consumption on the assessment of occupational exposure to arsenic by urinary arsenic speciation. *J Trace Elem Electrolytes Health Dis* **6**, 153–160.

ATSDR(Agency for Toxic Substances and Disease Registry), U.S. Department of Health and Human Services, Case Studies in Environmental Medicine, http://www.atsdr.cdc.gov

Avers, C. J. (1978). Basic cell biology. Litton Educational Publishing, Inc. D. Van Nostrand Company, NY.

Azcue, J. M., Nriagu, J. O. (1994). Arsenic in Environment. Part I: Cycling and Characterization (ed. Nriagu, J. O.), John Wiley & Sons Inc., pp. 1-15.

Bacon, J. R., Ellis, A. T., McMahon, A. W., Potts, P. J., Williams, J. G. (1994). Atomic Spectrometry Update – Atomic Mass Spectrometry and X-ray Fluorescence Spectrometry. *J Anal At Spectrom* **9**, 267R-305R.

Bentley, R., Chasteen, T. G. (2002). Microbial Methylation of Metalloids: Arsenic, Antimony, and Bismuth. *Microbiol Mol Biol Rev* **66**, 250-271.

Bhumbla, D. K., Keefer, R. F. (1994). Arsenic in Environment. Part I: Cycling and Characterization (ed. Nriagu, J. O.), John Wiley & Sons Inc., pp. 51-82.

Bredfeldt, T. G., Kopplin, M. J., Gandolfi, A. J. (2004). Effects of arsenite on UROtsa cells: low level arsenite causes accumulation of ubiquitinated proteins that is enhanced by reduction in cellular glutathione levels. *Toxicol Appl Pharmacol* **198**, 412-418.

Buchet, J.P., Lison, D., Ruggeri, M., Foa, V., Elia, G. (1996). Assessment of exposure to inorganic arsenic, a human carcinogen, due to the consumption of seafood. *Arch Toxicol* **70**, 773–778.

Buchet, J.P., Pauwels, J., Lauwerys, R. (1994). Assessment of exposure to inorganic arsenic following ingestion of marine organisms by volunteers. *Environ Res* **66**, 44–51.

Byrne, A. R., Slejkovec, Z., Stijve, T., Fay, L., Gössler, W., Gailer, J., Irgolic, K. J. (1995). Arsenobetaine and other arsenic species in mushrooms. *Appl Organomet Chem* **9**, 297– 303.

Challenger, F. (1945). Biological methylation. *Chem Rev* **36**, 315-361.

Chen, G. Q., Zhou, L., Styblo, M., Walton, F., Jing, R., Weinberg, R., Chen, Z., Waxman, S. (2003). Methylated Metabolites of Arsenic Trioxide Are More Potent than Arsenic Trioxide as Apoptotic but not Differentiation Inducers in Leukemia and Lymphoma Cells. *Cancer Res* **63**, 1853-1859.

Chiou, H. Y., Hsueh, Y. M., Liaw, K. F., Horng, S. F., Chiang, M. F., Pu, Y. S., Lin, J. S. N., Huang, C. H., Chen, C. J. (1995). Incidence of internal cancers and ingested inorganic arsenic: a seven-year follow-up study in Taiwan. *Cancer Res* **55**, 1296-1300.

Cohen, S. M., Arnold, L. L., Uzvolgyi, E., Cano, M., John, M. S., Yamamoto, S., Lu, X. F., Le, X. C. (2002) Possible role of dimethylarsinous acid in dimethylarsinic acid-induced urothelial toxicity and regeneration in the rat. *Chem Res Toxicol* **15**, 1150-1157.

Cohen, S. M., Yamamoto, S., Cano, M., and Arnold, L. L. (2001). Urothelial cytotoxicity and regeneration induced by dimethylarsinic acid in rats. *Toxicol Sci* **59,** 68–74.

Concha, G., Vogler, G., Lezcano, D., Nermell, B., Vahter, M. (1998). Exposure to inorganic arsenic metabolites during early human development. *Toxicol Sci* **44**, 185-190.

Cornelis, R., Caruso, J., Crews, H., Heumann, K. G. (2003). Handbook of Elemental Speciation, Tchniques and Methodology, John Wiley & Sons, Chichester, UK.

Cullen, W. R., McBride, B. C., Manji, H., Pickett, A. W., Reglinski, J. (1989). The metabolism of methylarsine oxide and sulfide. *Appl Organomet Chem* **3**, 71-78.

Cullen, W. R., McBride, B. C., Reglinski, J. (1984). The reaction of methylarsenicals with thiols: Some biological implications. *J Inorg Biochem* **21**, 179-194.

Cullen, W. R., Reimer, K. J. (1989). Arsenic speciation in the environment. *Chem Rev* **89**, 713– 764.

Del Razo, L. M.; Styblo, M.; Cullen, W. R.; Thomas, D. J. (2001). Determination of Trivalent Methylated Arsenicals in Biological Matrices. *Toxicol Appl Pharmacol* **174**, 282-293.

Dong, M. W. (2006). Modern HPLC for practicing scientists, John Wiley & Sons, Inc., Hoboken, New Jersey, p.31.

Dopp, E., Hartmann, L. M., Florea A. M., von Recklinghausen, U., Pieper, R., Rettenmeier, A. W., Hirner, A. V., Obe, G. (2004). Uptake of inorganic and organic derivatives of arsenic associated with induced cytotoxic and genotoxic effects in Chinese hamster ovary (CHO) cells. *Toxicol Appl Pharmacol* **201**, 156-165.

Dopp, E., Hartmann, L. M., Von Recklinghausen, U., Rabieh, S., Hirner, A. V., Rettenmeier, A. W. et al. (2005). Forced uptake of trivalent and pentavalent methylated and inorganic arsenic and ist cyto-/genotoxicity in fibroblasts and hepatoma cells. *Toxicol Sci* **87**, 46-56.

Drobna, Z., Waters, S. B., Devesa, V., Harmon, A. W., Thomas, D. J., Styblo, M. (2005). Metabolism and toxicity of arsenic in human urothelial cells expressing rat arsenic (+3 oxidation state)-methyltransferase. *Toxicol Appl Pharmacol* **207**, 147-159.

Edmonds, J. S., Francesconi, K. A. (1993). Arsenic in seafoods: human health aspects and regulation. *Mar Pollut Bull* **26**, 665– 674.

Feldmann, J., John, K., Pengpreecha, P. (2000). Arsenic metabolism in seaweed-eating sheep from northern Scotland. *Fresenius J Anal Chem* **368**, 116-121.

Ferguson, J. F., Gavis, J. (1972). A review of the arsenic cycle in natural waters. *Water Res* **6**, 1259-1274.

Francesconi, K. A., Edmonds, J. S. (1997). Arsenic and marine organisms. *Adv Inorg Chem* **44**, 147– 189.

Francesconi, K. A., Kuehnelt, D. (2004). Determination of arsenic-species: A critical review of methods and applications, 2000-2003. *Analyst* **129**, 373-395.

Francesconi, K. A., Tanggaard, R., McKenzie, C. J., Goessler, W. (2002). Arsenic metabolites in human urine after ingestion of an arsenosugar. *Clin Chem* **48**, 92-101.

Geiszinger, A., Goessler, W., and Kosmus, W. (2002). Organoarsenic compounds in plants and soil on top of an ore vein. *Appl Organometal Chem* **16**, 245-249.

Geiszinger, A., Goessler, W., Kuehnelt, D., Francesconi, K., Kosmus, W. (1998). Determination of arsenic compounds in earthworms. *Environ Sci Technol* **32**, 2238-2243.

Goering, P. L., Aposhian, H. V., Mass, M. J., Cebrian, M., Beck, B. D., Waalkes, M. P. (1999). The enigma of arsenic carcinogenesis: Role of metabolism. *Toxicol Sci* **49**, 5-14.

Gomez-Caminero, A., Howe, P., Hughes, M., Kenyon, E., Lewis, D. R., Moore, M., Ng, J., Aitio, A., Becking, G. (2001). Envronmental Health Criteria 224: Arsenic and Arsenic Compounds, United Nations Environment Programme, the International Labour Organisation, and the World Health Organisation.

Gong, Z., Lu, X., Cullen, W. R., Le, X. L. (2001). Unstable trivalent arsenic metabolites, monomethylarsonous acid and dimethylarsinous acid. *J Anal At Spectrom* **16**, 1409-1413.

Gong, Z., Lu, X., Ma, M., Watt, C., Le, X. C. (2002). Arsenic speciation analysis. *Talanta* **58**, 77-96.

Guerin, T., Molenat, N., Astruc, A., Pinel, R. (2000). Arsenic speciation in some environmental samples: a comparative study of HG-GC- QFAAS and HPLC-ICP-MS methods. *Appl Organomet Chem* **14**, 401-410.

Hakala, E., Pyy, L. (1995). Assessment of exposure to inorganic arsenic by determining the arsenic species excreted in urine. *Toxicol Lett* **77**, 249-258.

Hayakawa, T., Kobayashi, Y., Hirano, X. C. S. (2005). A new metabolic pathway of arsenite: arsenic-glutathione complexes are substrates for human arsenic methyltransferase Cyt19. *Arch Toxicol* **79**, 181-191.

Heumann, K. G. (2002). Hyphenated techniques – the most commonly used method for trace elemental speciation analysis. *Anal Bioanal Chem* **373**, 323-324.

Hirano, S., Kobayashi, Y., Cui, X., Kanno, S., Hayakawa, T., Shraim, A. (2004). The accumulation and toxicity of methylated arsenicals in endothelial cells: important roles of thiol compounds. *Toxicol Appl Pharmacol* **198**, 458-467.

Hirano, S., Kobayashi, Y., Cui, X., Kanno, S., Hayakawa, T., Shraim, A. (2003). Difference in uptake and toxicity of trivalent and pentavalent inorganic arsenic in rat heart microvessel endothelial cells. *Arch Toxicol* **77**, 305-312.

Hirner, A. V. (2006). Speciation of alkylated metals and metalloids in the environment. *Anal Bioanal Chem* **385**, 555-567.

Hughes, M. F. (2002). Arsenic toxicity and potential mechanisms of action. *Toxicol Lett* **133**, 1-16.

Hu, Y., Su, L., Snow, E. T. (1998). Arsenic toxicity is enzyme specific and its affects on ligation are not caused by the direct inhibition of DNA repair enzymes. *Mutat Res* **408**, 203-218.

Kaise, T., Fukui, S. (1992). The chemical form and acute toxicity of arsenic compounds in marine organisms. *Appl Organomet Chem* **6**, 155–160.

Kellner, R., Mermer, J. M., Otto, M., Widmer, H. M. (1998). Analytical Chemistry. Part II: Chromatography, Wiley-Vch GmbH.

Kimura, A., Ishida, Y., Wada, T., Yokoyama, H., Mukaida, N., Kondo, T. (2005) MRP-1 expression levels determine strain specific susceptibility to sodium arsenic-induced renal injury between C57BL/6 and BALB/c mice. *Toxicol Appl Pharmacol* **203**, 53-61.

Kitchin, K. T. (2001). Recent Advances in Arsenic Carcinogenesis: Modes of Action, Animal Model Systems, and Methylated Arsenic Metabolites. *Toxicol Appl Pharmacol* **172**, 249-261.

Koppenaal, D. W. (1992). Atomic Mass Spectrometry. *Anal Chem* **64**, 320R-342R.

Larsen, E. H. (1998) Method optimization and quality assurance in speciation analysis using high performance liquid chromatography with detection by inductively coupled plasma mass spectrometry. *Spectrochimica Acta (part B)* **53**, 253-265.

Le, X. C., Cullen, W. R., Reimer, K. J. (1993). Determination of urinary arsenic and impact of dietary arsenic intake. *Talanta* **40**, 185-193.

Le, X. C., Cullen, W. R., Reimer, K. J. (1994). Human urinary arsenic excretion after one-time ingestion of seaweed, crab and shrimp. *Clin Chem* **40**, 617–624.

Le, X. C., Li, X. F., Lai, V., Ma, M. et al. (1998). Simultaneous speciation of selenium and arsenic using elevated temperature liquid chromatography separation with inductively coupled plasma mass spectrometry detection. *Spectrochimica Acta (Part B)* **53**, 899-909.

Le, X. C., Lu, X., Ma, M., Cullen, W. R., Aposhian, H. V., Zheng, B. (2000a). Speciation of key arsenic metabolic intermediates in human urine. *Anal Chem* **72,** 5172–5177.

Le, X. C., Ma, M., Cullen, W. R., Aposhian, H. V., Lu, X., Zheng, B. (2000b). Determination of monomethylarsonous acid, a key arsenic methylation intermediate, in human urine. *Environ Health Perspect* **108,** 1015–1018.

Le, X. C., Ma, M., Wong, N. A. (1996). Speciation of arsenic compounds using high-performance liquid chromatogrphy at elevated temperature and selective hydride generation atomic fluorescence detection. *Anal Chem* **68**, 4501-4506.

Lin, S., Cullen, W. R., Thomas, D. J. (1999). Methylarsenicals and arsinothiols are potent inhibitors of mouse liver thioredoxin reductase. *Chem Res Toxicol* **12,** 924–930.

Lin, S., Del Razo, L. M., Styblo, M., Wang, C., Cullen, W. R., and Thomas, D. J. (2001). Arsenicals inhibit thioredoxin reductase in cultured rat hepatocytes. *Chem Res Toxicol* **14**, 305–311.

Liu, W., Lee, H. K. (1999). Chemical modification of analytes in speciation analysis by capillary electrophoresis, liquid chromatography and gas chromatography. *J Chromatogr A* **834**, 45-63.

Liu, Z., Shen, J., Carbrey, J. M., Mukhopadhyay, R., Agre, P., Rosen, B. P. (2002). Arsenic transport by mammalian aquaglyceroporins AQP7 and AQP9. *Proc Natl Acad Sci U.S.A* **99**, 6053-6058.

Lu, X., Arnold L., L., Cohen, S. M., Cullen, W. R., Le, X. C. (2003). Speciation of Dimethylarsinous Acid and Trimethylarsine Oxide in Urine from Rats Fed with Dimethylarsinic Acid and Dimercaptopropane Sulfonate. *Anal Chem* **75**, 6463-6468.

Ma, M., Le, S. C. (1998). Effect of arsenosugar ingestion on urinary arsenic speciation. *Clin Chem* **44**, 539–550.

Macchioni, A. (2005). Ion Pairing in Transition-Metal Organometallic Chemistry. *Chem Rev* **105**, 2039-2073.

Mandal, B. K., Ogra, Y., Anzai, K., Suzuki, K. T. (2004). Speciation of arsenic in biological samples. *Toxicol Appl Pharmacol* **198**, 307-318.

Mandal, B. K., Ogra, Y., Suzuki, K. T. (2001). Identification of dimethylarsinous and monomethylarsinous acids in human urine of the arsenicaffected areas in West Bengal, India. *Chem Res Toxicol* **14,** 371–378.

Mass, M. J., Tennant, A., Roop, B. C., Cullen, W. R., Styblo, M., Thomas, D. J., Kligerman, A. D. (2001). Methylated trivalent arsenic species are genotoxic. *Chem Res Toxicol* **14**, 355–361.

Matschullat, J., Borba, R.P., Deschamps, E., Figueiredo, B.R., Gabrio, T., Schwenk, M. (2000). Human and environmental contamination in the Iron Quadrangle, Brazil. *Appl Geochem* **15**, 181-190.

Mattusch, J., Wennrich, R. (1998). Determination of Anionic, Neutral, and Cationic Species of Arsenic by Ion Chromatography with ICPMS Detection in Environmental Samples. *Anal Chem* **70**, 3649-3655.

Meyers, R. A. (2000). Encyclopedia of Analytical Chemistry. Volume 3: Inductively Coupled Plasma Mass Spectrometry in Environmental Analysis (ed. Yamasaki, S.I.), John Wiley & Sons Ltd., pp. 2672-2692.

Meza, M. M., Kopplin, M. J., Burgess, J. L., Gandolfi, A. J. (2004). Arsenic drinking water exposure and urinary excretion among adults in the Yaqui Valley, Sonora, Mexico. *Environ Res* **96**, 119-126.

Meza, M. M., Yu, L., Rodriguez, Y. Y., Guild, M., Thompson, D., Gandolfi, A. J., Klimecki, W. T. (2005). Developmentally restricted genetic determinations of human arsenic metabolism: Association between urinary methylated arsenic and CYT19 polymorphisms in children. *Environ Health Perspect* **113**, 775-781.

Moldovan, M., Gomez, M. M., Palacios, M. A., Camara, C. (1998). Arsenic Speciation in Water and Human Urine by HPLC–ICP-MS and HPLC–MO–HG-AAS. *Microchem J* **59**, 89-99.

Murai, T., Iwata, H., Otoshi, T., Endo, G., Horiguchi, S., and Fukushima, S. (1993). Renal lesions induced in F344/DuCrj rats by 4 weeks oral administration of dimethylarsenic acid. *Toxicol Lett* **66,** 53–61.

Nakazato, T., Taniguchi, T., Tao, H., Tominga, M., Miyazaki, A. (2000). Ion-exclusion chromatography combined with ICP-MS and hydride generation-ICP-MS for the determination of arsenic species in biological matrices. *J Anal At Spectrom* **15**, 1546-1552.

National Research Council Report (1999). Arsenic in Drinking water, National Academy Press, Washington, DC.

Nesnow, S., Roop, B. C., Lambert, G., Kadiiska, M., Mason, R. P., Cullen, W. R., Mass, M. J. (2002) DNA damage induced by methylated trivalent arsenicals is mediated by reactive oxygen species. *Chem Res Toxicol* **15**, 1627-1634.

NRC (National Research Council). (1999). Arsenic in drinking water. Subcommittee on Arsenic in Drinking Water, Committee on Toxicology, Board on Environmental Studies and Toxicology Commission on Life Science. Washington, DC, National Academy Press.

Ochi, T., Kaise, T., and Oya-Ohta, Y. (1994). Glutathione plays different roles in the induction of the cytotoxic effects of inorganic and organic arsenic compounds in cultured BALB/c 3t3 cells. *Experientia* **50,** 115–120.

Ochi, T., Nakajima, F., Sakurai, T., Kaise, T., and Oya-Ohta, Y. (1996). Dimethylarsinic acid causes apoptosis in HL-60 cells via interaction with glutathione. *Arch Toxicol* **70,** 815–821.

Peters, R. A. (1955). Biochemistry of some toxic agents. I. Present state of knowledge of biochemical lesions induced by trivalent arsenicals poisoning. *Bull John Hopkins Hospital* **97**, 1-20.

Petrick, J. S., Ayala-Fierro, F., Cullen, W. R., Carter, D. E., and Aposhian, H. V. (2000). Monomethylarsonous acid (MMA(III)) is more toxic than arsenite in Chang human hepatocytes. *Toxicol Appl Pharmacol* **163**, 203–207.

Petrick, J. S., Jagadish, B., Mash, E. A., and Aposhian, H. V. (2001). Mono methylarsonous acid (MMA^{III}) and arsenite: LD50 in hamsters and in vitro inhibition of pyruvate dehydrogenase. *Chem Res Toxicol* **14**, 651-656.

Phillips, D. J. H. (1994). Arsenic in Environment. Part I: Cycling and Characterization (ed. Nriagu, J. O.), John Wiley & Sons Inc., pp. 263-268.

Pires, A. S., Frizzera da Cunha, J.M., de Lima e Fonseca, T.N. (1996). Morro Velho, the story, events and achievements. Mineracao Morro Velho Ltda, Nova Lima.

Rabieh, S., Diaz-Bone, R. A., Hasenäcker, F., Kösters, J., Hirner, A. V., Feldmann, J. (2007). Investigation of monomethylarsonous acid [MMAs(III)] in human urine by a combined liquid and gas chromatographic approach. (*under preparation)*.

Rabieh, S., Hirner, A. V., Matschullat, J. (2007). Determination of arsenic species in human urine of an arsenic-affected area in Brazil using high performance liquid chromatography (HPLC) coupled with inductively coupled plasma mass spectrometry (ICP-MS). (*under preparation)*.

Rawlins, B.G., Williams, T.M., Breward, N., Ferpozzi, L., Figueiredo, B., Borba, R. (1997). Preliminary investigation of mining related arsenic contamination in the provinces of Mendoza and San Juan (Argentina) and Minas Gerais state (Brazil). In: BGS Overseas Geology Series, Technical Report WC/97/60.

Reeve, R. N. (1994). Environmental Analysis; John Wiley & Sons, New York, NY.

Ritsema, R., Dukan, L., Navarro, T. G., van Leeuwen, W., Oliveira, N., Wolfs, P., Lebret, E. (1998). Speciation of arsenic compounds in urine by LC-ICP MS. *Appl Organometal Chem* **12**, 591-599.

Rosen, B. P. (2002) Transport and detroxification systems for transition metals, heavy metals and metalloids in eukaryotic and prokaryotic microbes. *Comparative Biochem Physiol A-Molec Integ Physiol* **133**, 689-693.

Rossi, M. R., Masters, J. R. W., Park, S., Todd, J. H., Garrett, S. H. et al. (2001). The Immortalized UROtsa Cell Line as a Potential Cell Culture Model of Human Urothelium. *Environ Health Perspect* **109**, 801-808.

Roy, P., Saha, A. (2002). Metabolism and toxicity of arsenic: A human carcinogen. *Curr Sci* **82**, 38-45.

Saeki, K., Sakakibara, H., Sakai, H., Kunito, T., Tanabe, S. (2000). Arsenic accumulation in three species of sea turtles. *Biometals* **13**, 241-250.

Sakai, T., Inoue, Y., Date, Y., Aoyama, T., Yoshida, K., and Endo, G. (2001). Simultaneous determination of neutral, anionic and cationic compounds within one chromatographic run using an inductively coupled plasma mass spectrometer as element-specific detector. *Appl Organometal Chem* **15**, 285-290.

Sakurai, T., Kaise, T., and Matsubara, C. (1998). Inorganic and methylated arsenic compounds induce cell death in murine macrophages via different mechanisms. *Chem Res. Toxicol* **11,** 273–283.

Sampayo-Reyes, A., Zakharyan, R. A., Healy, S. M., and Aposhian, H. V. (2000). Monomethylarsonic acid reductase and monomethylarsonous acid in hamster tissue. *Chem Res Toxicol* **13**, 1181–1186.

Sampayo-Reyes, A., Zakharyan, R. A., Healy, S. M., Aposhian, H. V. (2000). *Chem Res Toxicol* **13**, 1181-1186.

Saverwyns, S., Zhang, X., Vanhaecke, F., Cornelis, R., Moens, L., Dams, R. (1997). Speciation of Six Arsenic Compounds Using High-performance Liquid Chromatography–Inductively Coupled Plasma Mass Spectrometry With Sample Introduction by Thermospray Nebulization. *J Anal At Spectrom* **12**, 1047-1052.

Schwerdtle, T., Walter, I., Mackiw, I., Hartwig, A. (2003). Induction of oxidative DNA damage by arsenite and ist trivalent and pentavalent methylated metabolites in cultured human cells and isolated DNA. *Carcinogenesis* **24**, 967-974.

Shiobara, Y., Ogra, Y., Suzuki, K. T. (2001) Animal species difference in the uptake of dimethylarsinous acid (DMA(III)) by red blood cells. *Chem Res Toxicol* **14**, 1446-1452.

Sichel, S.E., Valenca, J.G. (1983). Geologia do Arqueano da regiao de Santa Barbara, Quadrilatero Ferrifero, MG. Anais II. Simp. Geol. MG Bol. 3: 197-208; Belo Horizonte, Brasil, Soc. Geol. Bras.

Skoog, D. A., Leary, J. J. (1992). Priciple of instrumental analysis. Fourth ed., Saunders College Publishing, New York.

Stoeppler, M., Vahter, M. (1994). In: Trace elements in biological specimens, Herber, R. F. M. And Stoeppler, M. (eds), Elsevier, Amsterdam, pp. 291-320.

Stueckradt, I. (2006). Subzelluläre Verteilung von Arsen und dessen methylierten Verbindungen in humanen Harnblasenepithelzellen. Diplomarbeit, Fachhochschule Gelsenkirchen.

Styblo, M., and Thomas, D. J. (1997a). Binding of arsenicals to proteins in an *in vitro* methylation system. *Toxicol Appl Pharmacol* **147,** 1–8.

Styblo, M., Del Razo, L. M., LeCluyse, E. L., Hamilton, G. A., Wang, C., Cullen, W. R., and Thomas, D. J. (1999a). Metabolism of arsenic in primary cultures of human and rat hepatocytes. *Chem Res Toxicol* **12,** 560–565.

Styblo, M., Del Razo, L. M., Vega, L., Germolec, D. R., LeCluyse, E. L., Hamilton, G. A., Wang, C., Cullen, W. R., and Thomas, D. J. (2000). Comparative toxicity of trivalent and pentavalent inorganic and methylated arsenicals in human cells. *Arch Toxicol* **74,** 289–299.

Styblo, M., Delnomdedieu, M., Thomas, D. J. (1995). Biological mechanisms and toxicological consequences of the methylation of arsenic. In *Toxicology of metals-biochemical aspects, handbook of experimental pharmacology* (Goyer, R. A., and Cherian, G., Eds.) pp 407-433, Springer-Verlag, Berlin.

Styblo, M., Drobna, Z., Jaspers, I., Lin, S., Thomas, D. J. (2002). The Role of Biomethylation in Toxicity and Carcinogenicity of Arsenic: A Research Update. *Environ Health Perspect* **110 (Supplement 5)**, 767-771.

Styblo, M., Serves, S. V., Cullen, W. R., and Thomas, D. J. (1997). Comparative inhibition of yeast glutathione reductase by arsenicals and arsenothiols. *Chem Res Toxicol* **10,** 27–33.

Styblo, M., Vega, L., Germolec, D. R., Luster, M. I., Del Razo, L. M., Wang, C., Cullen, W. R., and Thomas, D. J. (1999b). Metabolism and toxicity of arsenicals in cultured cells. In *Arsenic Exposure and Health Effects* (W. R. Chappell, C. O. Abernathy, and R. L. Calderon, Eds.), pp. 311–323. Elsevier, Amsterdam.

Sur, R., Dunemann, L. (2004). Method for the determination of five toxicologically relevant arsenic species in human urine by liquid chromatography-hydride generation atomic absorption spectrometry. *J Chromatography (part B)* **807**, 169-176.

Suzuki, K. T. (2005). Metabolomics of arsenic based on speciation studies. *Anal Chim Acta* **540**, 71-76.

Szinicz, L., Forth, W. (1988). Effects of As_2O_3 on gluconeogenesis. *Arch Toxicol* **61**, 444-449.

Tatum, F. M., Hood, R. D. (1999). Arsenite uptake and metabolism by rat hepatocyte primary cultures in comparison with kidney- and hepatocyte-derived rat cell lines. *Toxicol Sci* **52**, 20-25.

Thomas, D. J., Styblo, M., Lin, S. (2001). The cellular Metabolism and Systemic Toxicity of Arsenic. *Toxicol Appl Pharmacol* **176**, 127-144.

Thompson, D. J. (1993). A chemical hypothesis for arsenic methylation in mammals. *Chem Biol Interact* **88**, 89-114.

Thorpe, N. O. (1984). Cell biology. John Wiley & Sons, Inc.

Toyo'oka, T. (1999). Modern Derivatization Methods for Separation Sciences, John Wiley & Sons, Ltd, Chichester, UK.

Valenzuela, O. L., Borja-Aburto, V. H., Garcia-Vargas, G. G., Cruz-Gonzalez, M. B., Garcia-Montalvo, E. A., Calderon-Aranda, E. S., Del Razo, L. M. (2005). Urinary Trivalent Methylated Arsenic Species in a Population Chronically Exposed to Inorganic Arsenic. *Environ Health Perspect* **113**, 250-254.

Vega, L., Styblo, M., Patterson, R., Cullen, W., Wang, C., and Germolec, D. (2001). Differential Effects of Trivalent and Pentavalent Arsenicals on Cell Proliferation and Cytokine Secretion in Normal Human Epidermal Keratinocytes. *Toxicol Appl Pharmacol* **172**, 225-232.

Velez, D., Ybanez, N., Montoro, R. (1996). Monomethylarsonic and dimethylarsinic acid contents in seafood products. *J Agric Food Chem* **44**, 859–864.

Vieira, F.W.R. (1997). Genese das mineralisacoes auriferas do setor W do greenstone belt Rio das Velhas, M.G. Internal paper of the mapping and petrography group of Mineracao Morro Velho S.A.

Vieira, F.W.R., Biasi, E.E., Lisboa, L.H. (1991). Geology of and excursion to the Morro Velho and Cuiaba mines. In: Fleischer, R., Grossi Sad, J.H., Fuzikawa, K., Ladeira, E.A. (Eds.), Field and mine trip to Quadrilatero Ferrifero, Minas Gerais, Brazil. Brazil Gold '91 Internat. Symp. On the Geology of Gold, Belo Horizonte, pp. 87-99.

Vieira, F.W.R., Simoes, E.J.M. (1992). Geology of the Nova Lima area and excursion to the Raposos mine. Gold deposits modelling course at IUGS UNESCO Brazil.

von Recklinghausen, U., Hartmann, L. M., Stueckradt, I., Pollok, I., Rabieh, S., Ping, Y., Nüssler, A., Katier, C., Hirner, A. V., Rettenmeier, A. W., Dopp, E. (2007). Subcellular distribution of anorganic and organic arsenic arsenic compounds in human urothelial cells compared to human hepatocytes. (*under preparation).*

Wang, Z., Dey, S., Rosen, B. P., Rossman, T. G. (1996). Efflux-mediated resistance to arsenicals in arsenic-resistant and –hypersensitive Chinese hamster cells. *Toxicol Appl Pharmacol* **137**, 112-119.

Wang, Z., Rossman, T. G. (1993). Stable and inducible arsenite resistance in Chinese hamster cells. *Toxicol Appl Pharmacol* **118**, 80-86.

Wang, Z., Zhou, J., Lu, X., Gong, Z., Le, X. C. (2004). Arsenic speciation in urine from Acute Promyelocytic Leukemia Patients undergoing arsenic trioxide treatment. *Chem Res Toxicol* **17**, 95-103.

WHO (1981), Geneva, Report published by joint sponsorship under the United Nation Environmental Programme, International Labour Organization, and World Health Organization, Geneva, Switzerland, Vol. 18.

Woller, A., Garraud, H., Boisson, J., Marie Dorthe, A., Fodor, P., Donard, O. F. X. (1998). Simultaneous speciation of redox species of arsenic and selenium using an anion-exchange microbore column coupled with a microconcentric nebulizer and an inductively coupled plasma mass spectrometer as detector. . *J Anal At Spectrom* **13**, 141-149.

Yamauchi, H., Takahashi, K., Mashiko, M., Yamamura, Y. (1989). Biological monitoring of arsenic exposure of gallium arsenide- and inorganic arsenic-exposed workers by determination of inorganic arsenic and its metabolites in urine and hair. *Am Ind Hyg Assoc J* **50**, 606–612.

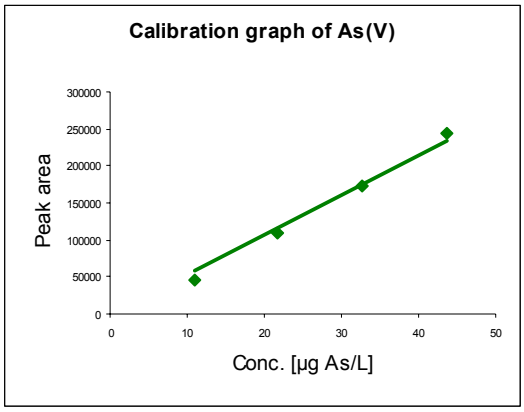
Yan-Chu, H. (1994). Arsenic in Environment. Part I: Cycling and Characterization (ed. Nriagu, J. O.), John Wiley & Sons Inc., pp. 17-49.

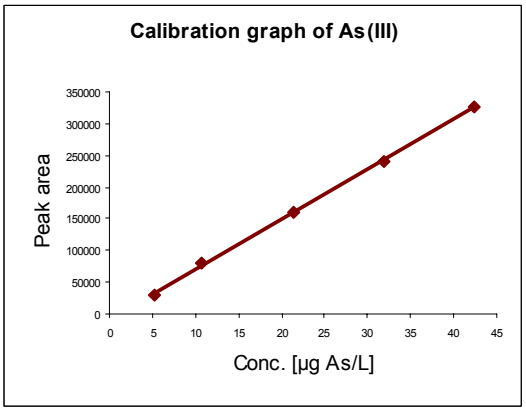
Zakharyan, R. A., Ayala-Fierro, F., Cullen, W. R., Carter, D. M., Aposhian, H. V. (1999). Enzymatic methylation of arsenic compounds. VII. Monomethylarsonous acid (MMA^{III}) is the substrate for MMA methyltransferase of rabbit liver and human hepatocytes. *Toxicol Appl Pharmacol* **158**, 9-15.

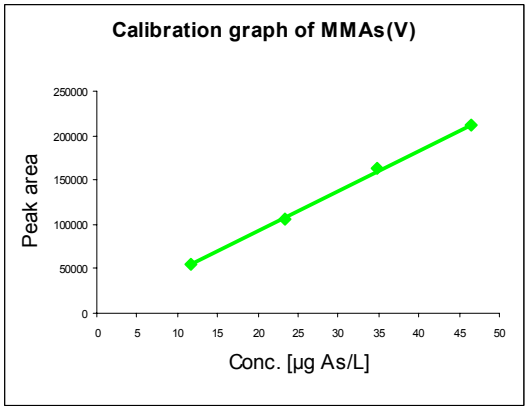
Zhu, X.-H., Shen, Y.-L., Jing, Y.-K., Cai, X., Jia, P.-M., Huang, Y., Tang, W., Shi, G.-Y., Sun, Y.-P., Dai, J., Wang, Z.-Y., Chen, S.-J., Zhang, T.-D., Waxman, S., Chen, Z., and Chen, G.-Q. (1999). Apoptosis and growth inhibition in malignant lymphocytes after treatment with arsenic trioxide at clinically achievable concentrations. *J Nat Cancer Inst* **91**, 772–778.

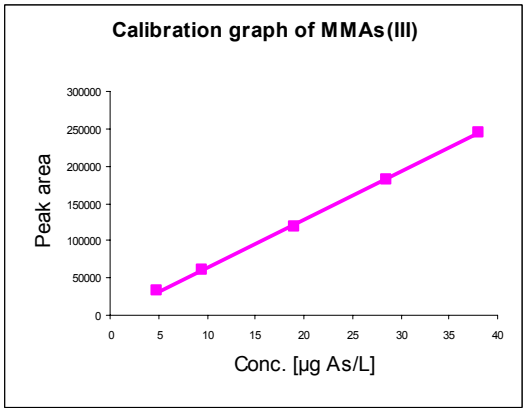
Appendix

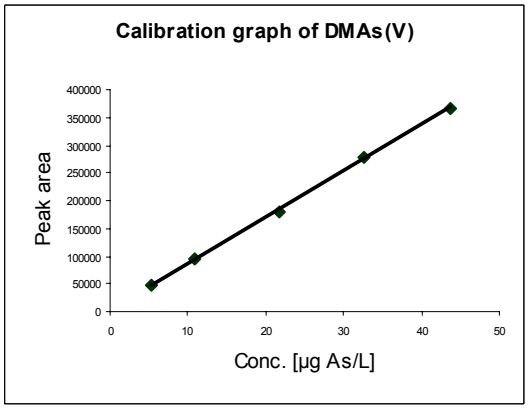
Figure A.1 Calibration graphs of arsenic species

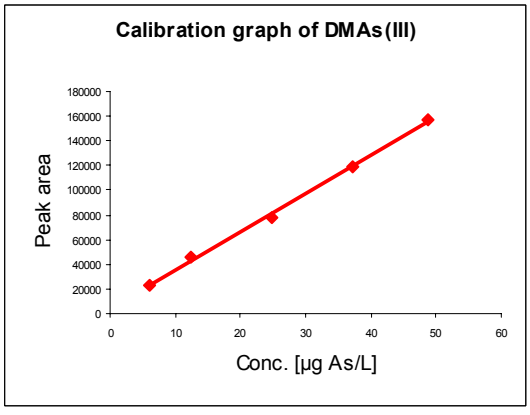


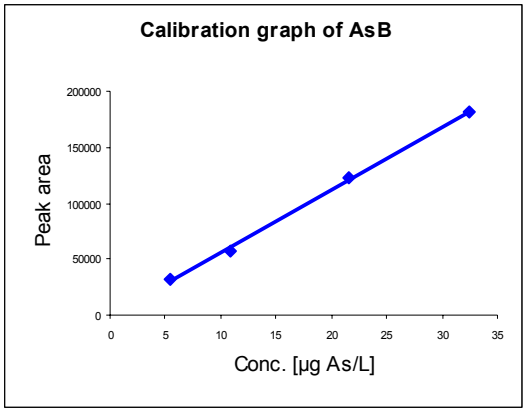


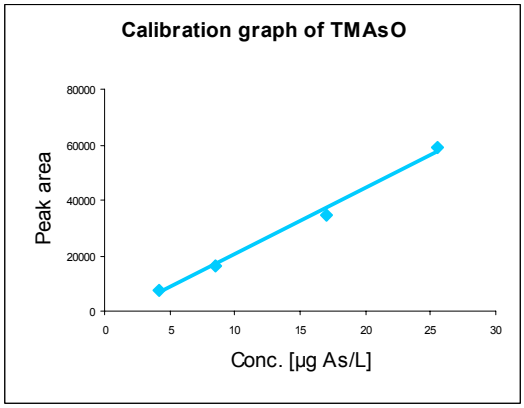


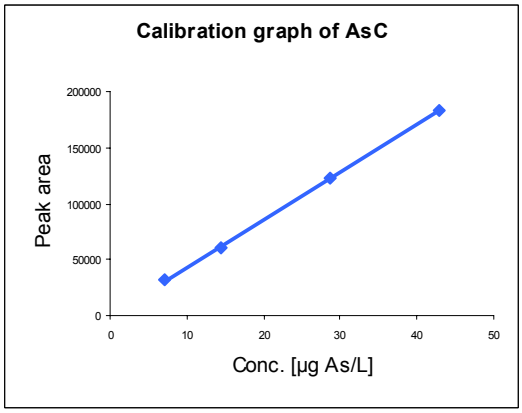







Calibration graph of AsB
Peak area
200000
150000
100000
50000
0
0
5
10
15
20
25
30
35
Conc. [µg As/L]


Calibration graph of TMAsO
Peak area
80000
60000
40000
20000
0
0
5
10
15
20
25
30
Conc. [µg As/L]


Calibration graph of AsC
Peak area
200000
150000
100000
50000
0
0
10
20
30
40
50
Conc. [µg As/L]

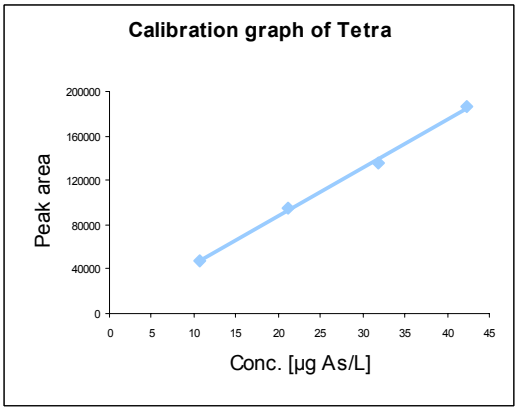

Calibration graph of Tetra
Peak area
200000
160000
120000
80000
40000
0
0
5
10
15
20
25
30
35
40
45
Conc. [µg As/L]

Table A.1 Uptake of sodium arsenite and sodium arsenate by UROtsa cells: whole-cell and cell-free extract.

Conc. of As in exposure medium (μM)	Detected As concentrations ± SD			
	Whole-cell extract		Cell-free (membrane removed) extract	
	ng/10^6 cells	% of arsenic substrate	ng/10^6 cells	% of arsenic substrate
As(III)				
control	0.10 ± 0.10	—	0.10 ± 0.10	—
0.5	0.09 ± 0.05	0.13 ± 0.08	ND	ND
5	0.38 ± 0.04	0.05 ± 0.02	0.24 ± 0.07	0.03 ± 0.02
50	1.93 ± 0.93	0.02 ± 0.02	1.67 ± 0.59	0.02 ± 0.01
500	13.33 ± 0.08	0.02 ± 0.00	10.72 ± 1.20	0.01 ± 0.00
5000	22.79 ± 2.41	ND	19.23 ± 0.66	ND
As(V)				
control	0.13 ± 0.47	—	0.01 ± 0.31	—
0.5	ND	ND	ND	ND
5	ND	ND	ND	ND
50	ND	ND	ND	ND
500	0.50 ± 0.26	ND	0.50 ± 0.21	ND
5000	8.31 ± 1.28	ND	8.10 ± 1.62	ND

Table A.2 Uptake of the trivalent organoarsenic species MMAs(III) and DMAs(III) by UROtsa cells: whole-cell and cell-free extract

Conc. of As in exposure medium (μM)	Detected As concentrations ± SD			
	Whole-cell extract		Cell-free (membrane removed) extract	
	ng/10^6 cells	% of arsenic substrate	ng/10^6 cells	% of arsenic substrate
MMAs(III)				
control	ND	—	ND	—
0.5	ND	ND	ND	0.05 ± 0.17
1	0.09 ± 0.15	0.07 ± 0.11	0.38 ± 0.21	0.21 ± 0.03
5	1.09 ± 0.40	0.18 ± 0.05	1.41 ± 0.69	0.23 ± 0.09
50	19.61 ± 9.94	0.04 ± 0.02	21.96 ± 11.22	0.04 ± 0.02
500	32.64 ± 6.23	0.01 ± 0.00	32.30 ± 9.85	0.01 ± 0.00
DMAs(III)				
control	ND	—	0.48 ± 0.45	—
0.1	1.99 ± 0.23	13.27 ± 1.51	1.96 ± 0.23	13.04 ± 1.56
0.5	6.31 ± 2.18	8.41 ± 2.91	6.28 ± 1.29	8.38 ± 1.72
1	10.82 ± 3.17	7.21 ± 2.12	10.98 ± 2.42	7.32 ± 1.62
5	48.98 ± 11.59	6.53 ± 1.54	50.13 ± 11.47	6.68 ± 1.53
10	76.56 ± 8.75	5.10 ± 0.58	79.20 ± 7.67	5.28 ± 0.51

Table A.3 Uptake of the pentavalent organoarsenic species MMAs(V), DMAs(V), and TMAsO by UROtsa cells: whole-cell and cell-free extract.

Conc. of As in exposure medium (µM)	Detected As concentrations ± SD			
	Whole-cell extract		Cell-free (membrane removed) Extract	
	ng/10^6 cells	% of arsenic substrate	ng/10^6 cells	% of arsenic substrate
MMAs(V)				
control	ND	—	ND	—
0.5	ND	ND	ND	ND
5	ND	ND	ND	ND
50	ND	ND	ND	ND
500	1.74 ± 1.55	ND	1.26 ± 1.32	ND
5000	8.86 ± 4.49	ND	6.59 ± 3.27	ND
DMAs(V)				
control	ND	—	ND	—
0.5	0.01 ± 0.00	0.02 ± 0.00	ND	ND
5	ND	ND	ND	ND
50	0.07 ± 0.05	ND	0.08 ± 0.07	ND
500	0.86 ± 0.36	ND	0.89 ± 0.30	ND
5000	10.79 ± 1.65	ND	18.64 ± 13.40	ND
TMAsO				
control	ND	—	ND	—
0.5	ND	ND	0.00 ± 0.00	ND
5	ND	ND	0.00 ± 0.00	ND
50	ND	ND	0.01 ± 0.01	ND
500	0.16 ± 0.03	ND	0.24 ± 0.11	ND
5000	0.98 ± 0.28	ND	1.04 ± 0.28	ND

Table A.4 Uptake of the ***5 mM*** pentavalent organoarsenic species monomethylarsonic acid [MMAs(V)], dimethylarsinic acid [DMAs(V)], and trimethylarsenic oxide [TMAsO] by UROtsa cells, ***1 h*** incubation.

Sample	Detected As concentration	
	Detected As (ng per 10^6 cells) ± SD	% of total intracellular As in fraction
MMAs(V)		
F	24.09 ± 3.55	
N	0.36 ± 0.32	1.3
M	0.43 ± 0.10	1.6
P	0.58 ± 0.43	2.2
R	1.40 ± 0.91	5.2
C	23.97 ± 1.52	89.6
DMAs(V)		
F	43.64 ± 2.11	
N	0.70 ± 0.14	1.7
M	0.20 ± 0.15	0.5
P	1.11 ± 0.35	2.7
R	0.83 ± 0.11	2.0
C	38.06 ± 6.15	93.1
TMAsO		
F	3.62 ± 1.62	
N	0.24 ± 0.32	3.3
M	0.17 ± 0.06	2.3
P	0.16 ± 0.00	2.2
R	0.26 ± 0.07	3.6
C	6.42 ± 3.75	88.6

Table A.5 Uptake of the ***500 µM*** sodium arsenite and ***5 mM*** sodium arsenate by UROtsa cells, ***1 h*** incubation.

Sample	Detected As concentration	
	Detected As (ng per 10^6 cells) ± SD	% of total intracellular As in fraction
As(III)		
F	107.26 ± 28.74	
N	1.04 ± 0.80	1.2
M	0.55 ± 0.21	0.6
P	0.99 ± 0.24	1.2
R	7.77 ± 5.03	9.1
C	75.25 ± 15.94	87.9
As(V)		
F	14.98 ± 0.04	
N	0.04 ± 0.07	0.4
M	ND	—
P	ND	—
R	0.25 ± 0.12	2.1
C	11.75 ± 0.92	98.1

Table A.6 Uptake of the ***500 µM*** monomethylarsonous acid [MMAs(III)] and ***5 µM*** dimethylarsinous acid [DMAs(III)] by UROtsa cells, ***1 h*** incubation.

Sample	Detected As concentration	
	Detected As (ng per 10^6 cells) ± SD	% of total intracellular As in fraction
MMAs(III)		
F	113.09 ± 42.95	
N	2.88 ± 1.69	3.1
M	1.71 ± 0.64	1.8
P	12.06 ± 5.24	13.0
R	4.09 ± 3.26	4.4
C	72.07 ± 19.80	77.7
DMAs(III)		
F	57.70 ± 11.18	
N	5.60 ± 5.19	10.3
M	0.91 ± 0.27	1.7
P	2.84 ± 1.79	5.2
R	4.91 ± 1.08	9.0
C	40.40 ± 7.30	73.9

Table A.7 Uptake of the ***5 mM*** pentavalent organoarsenic species monomethylarsonic acid [MMAs(V)], dimethylarsinic acid [DMAs(V)], and trimethylarsenic oxide [TMAsO] by UROtsa cells, ***24 h*** incubation.

Sample	Detected As concentration	
	Detected As (ng per 10^6 cells) ± SD	% of total intracellular As in fraction
MMAs(V)		
F	119.92 ± 0.78	
N	0.92 ± 0.06	0.9
M	0.83 ± 0.16	0.8
P	3.94 ± 0.05	3.7
R	4.96 ± 0.02	4.6
C	96.92 ± 3.01	90.1
DMAs(V)		
F	114.23 ± 8.56	
N	1.32 ± 0.29	1.3
M	2.32 ± 0.40	2.4
P	2.41 ± 0.94	2.4
R	1.41 ± 0.81	1.4
C	90.99 ± 2.15	92.4
TMAsO		
F	14.86 ± 0.85	
N	0.57 ± 0.12	3.5
M	0.38 ± 0.01	2.3
P	0.96 ± 0.07	5.9
R	1.36 ± 0.13	8.3
C	13.05 ± 0.19	80.0

Table A.8 Uptake of the ***50 μM*** sodium arsenite and ***500 μM*** sodium arsenate by UROtsa cells, ***24 h*** incubation.

Sample	Detected As concentration	
	Detected As (ng per 10^6 cells) ± SD	**% of total intracellular As in fraction**
As(III)		
F	7.08 ± 2.30	
N	0.31 ± 0.10	4.0
M	0.26 ± 0.11	3.3
P	0.43 ± 0.29	5.6
R	0.83 ± 0.59	10.7
C	5.96 ± 3.13	76.5
As(V)		
F	14.08 ± 1.36	
N	ND	—
M	1.02 ± 0.03	6.6
P	2.14 ± 1.54	13.9
R	1.53 ± 0.14	10.0
C	10.81 ± 2.43	70.4

Table A.9 Uptake of the ***500 μM*** monomethylarsonous acid [MMAs(III)] and ***5 μM*** dimethylarsinous acid [DMAs(III)] by UROtsa cells, ***24 h*** incubation.

Sample	Detected As concentration	
	Detected As (ng per 10^6 cells) ± SD	**% of total intracellular As in fraction**
MMAs(III)		
F	35.88 ± 3.03	
N	7.01 ± 1.30	21.6
M	1.16 ± 0.56	3.6
P	2.04 ± 0.25	6.3
R	1.43 ± 0.11	4.4
C	20.77 ± 0.38	64.1
DMAs(III)		
F	11.16 ± 1.33	
N	0.53 ± 0.32	4.9
M	0.22 ± 0.03	2.0
P	0.73 ± 0.06	6.6
R	0.78 ± 0.35	7.2
C	8.69 ± 1.05	79.4

Table A.10 Uptake of the ***5 μM*** monomethylarsonous acid [MMAs(III)] and ***5 μM*** dimethylarsinous acid [DMAs(III)] by UROtsa cells, ***24 h*** incubation.

Sample	Detected As concentration	
	Detected As (ng per 10^6 cells) ± SD	**% of total intracellular As in fraction**
MMAs(III)		
F	46.06 ± 2.64	
N	0.91 ± 0.16	2.1
M	0.96 ± 0.23	2.2
P	4.62 ± 0.30	10.8
R	4.59 ± 0.35	10.7
C	31.84 ± 3.17	74.2
DMAs(III)		
F	11.16 ± 1.33	
N	0.53 ± 0.32	4.9
M	0.22 ± 0.03	2.0
P	0.73 ± 0.06	6.6
R	0.78 ± 0.35	7.2
C	8.69 ± 1.05	79.4

Table A.11 Uptake of the pentavalent organoarsenic species monomethylarsonic acid [MMAs(V)], dimethylarsinic acid [DMAs(V)], and trimethylarsenic oxide [TMAsO] by CHO cells: whole-cell and cell-free extract.

Conc. of As in exposure medium (μM)	Detected As concentrations ± SD			
	Whole-cell extract		Cell-free (membrane removed) extract	
	ng/10^6 cells	% of arsenic substrate	ng/10^6 cells	% of arsenic substrate
MMAs(V)				
control	0.01 ± 0.02	—	0.02 ± 0.03	—
1	<0.01	ND	0.02 ± 0.03	0.01
10	<0.01	ND	<0.01	ND
100	2.92 ± 2.91	0.03	3.40 ± 2.89	0.03
500	8.47 ± 2.00	0.01	8.15 ± 1.28	0.01
1000	15.14 ± 0.02	0.01	15.24 ± 0.27	0.01
DMAs(V)				
control	0.03 ± 0.04	—	0.05 ± 0.07	—
1	<0.01	ND	<0.01	ND
10	<0.01	ND	<0.01	ND
100	0.23 ± 0.33	ND	0.50 ± 0.63	0.01
500	5.61 ± 1.11	0.01	9.73 ± 1.78	0.02
1000	18.68 ± 1.34	0.02	18.47 ± 2.00	0.02
TMAsO				
control	0.22 ± 0.31	—	0.10 ± 0.14	—
1	0.70 ± 0.27	0.78	5.01 ± 3.52	1.84
10	0.07 ± 0.04	0.01	1.33 ± 0.78	0.13
100	0.66 ± 0.93	0.01	0.84 ± 0.89	0.01
500	5.29 ± 0.62	0.01	3.42 ± 1.31	0.01
1000	8.14 ± 0.81	0.01	4.75 ± 5.21	ND

Table A.12 Uptake of sodium arsenite and sodium arsenate by CHO cells: whole-cell and cell-free extract.

Conc. of As in exposure medium (µM)	Detected As concentrations ± SD			
	Whole-cell extract		Cell-free (membrane removed) extract	
	ng/10^6 cells	% of arsenic substrate	ng/10^6 cells	% of arsenic substrate
As(III)				
control	0.13 ± 0.13	—	0.17 ± 0.29	—
0.5	1.01 ± 0.43	1.41	0.71 ± 0.44	0.99
1	5.15 ± 0.61	3.83	0.42 ± 0.42	0.31
10	12.25 ± 1.17	0.97	9.80 ± 1.29	0.78
100	55.65 ± 3.52	0.39	46.58 ± 0.42	0.33
500	123.83 ± 8.09	0.18	105.51 ± 7.94	0.15
1000	56.10 ± 1.64	0.41	57.03 ± 1.67	0.46
As(V)				
control	0.13 ± 0.13	—	0.16 ± 0.28	—
0.5	0.60 ± 0.10	1.02	0.47 ± 0.67	0.80
1	2.03 ± 0.72	1.99	1.27 ± 0.14	1.24
10	4.45 ± 0.47	0.40	3.94 ± 0.51	0.35
100	24.09 ± 0.27	0.22	29.22 ± 0.95	0.26
500	88.06 ± 3.78	0.16	76.03 ± 10.35	0.14
1000	384.80 ± 91.31	0.32	359.89 ± 83.74	0.30

Table A.13 Uptake of the trivalent organoarsenic species monomethylarsonous acid [MMAs(III)] and dimethylarsinous acid [DMAs(III)] by CHO cells: whole-cell and cell-free extract

Conc. of As in exposure medium (µM)	Detected As concentrations ± SD			
	Whole-cell extract		Cell-free (membrane removed) extract	
	ng/10^6 cells	% of arsenic substrate	ng/10^6 cells	% of arsenic substrate
MMAs(III)				
control	0.05 ± 0.07	—	0.07 ± 0.09	—
0.5	0.50 ± 0.70	1.01	0.19 ± 0.27	0.38
1	0.78 ± 0.55	0.70	0.39 ± 0.07	0.35
10	11.70 ± 4.82	1.02	11.20 ± 3.21	0.98
25	55.40 ± 4.36	1.78	55.72 ± 1.93	1.79
50	77.71 ± 14.14	0.91	80.14 ± 10.29	0.94
DMAs(III)				
control	0.03 ± 0.04	—	0.10 ± 0.15	—
0.1	<0.01	ND	0.07 ± 0.11	0.61
0.5	8.35 ± 1.70	16.30	4.64 ± 0.69	9.06
1	10.69 ± 7.50	9.44	6.80 ± 5.15	6.01
5	45.84 ± 8.20	9.18	31.02 ± 1.69	6.21
10	75.24 ± 7.52	6.90	57.15 ± 12.08	5.24

Table A.14 Uptake of sodium arsenite and sodium arsenate by Hep G2 cells: whole-cell and cell-free extract.

Conc. of As in exposure medium (μM)	Detected As concentrations ± SD			
	Whole-cell extract		Cell-free (membrane removed) extract	
	ng/10^6 cells	% of arsenic substrate	ng/10^6 cells	% of arsenic substrate
As(III)				
control	ND	—	ND	—
0.5	0.68 ± 0.03	0.63	0.56 ± 0.02	0.52
5	2.27 ± 0.00	0.27	2.13 ± 0.04	0.25
50	10.71 ± 0.07	0.14	10.31 ± 0.04	0.14
500	50.83 ± 0.24	0.05	47.50 ± 0.20	0.05
5000	108.39 ± 5.00	ND	128.73 ± 1.24	ND
As(V)				
control	0.02 ± 0.02	—	0.02 ± 0.02	—
0.5	1.43 ± 0.03	0.97	1.54 ± 0.06	1.05
5	0.46 ± 0.01	0.03	0.46 ± 0.03	0.03
50	1.54 ± 0.03	0.01	1.51 ± 0.06	0.01
500	4.71 ± 0.04	ND	4.43 ± 0.09	ND
5000	21.86 ± 0.34	ND	20.65 ± 0.13	ND

Table A.15 Uptake of the pentavalent organoarsenic species MMAs(V), DMAs(V), and TMAsO by Hep G2 cells: whole-cell and cell-free extract.

Conc. of As in exposure medium (μM)	Detected As concentrations ± SD			
	Whole-cell extract		Cell-free (membrane removed) extract	
	ng/10^6 cells	% of arsenic substrate	ng/10^6 cells	% of arsenic substrate
MMAs(V)				
control	0.16 ± 0.19	—	0.02 ± 0.01	—
0.5	ND	ND	0.09 ± 0.04	0.07
5	ND	ND	0.11 ± 0.23	0.01
50	ND	ND	0.21 ± 0.02	ND
500	0.56 ± 0.34	ND	2.10 ± 0.03	ND
5000	14.11 ± 2.36	ND	25.15 ± 0.08	ND
DMAs(V)				
control	0.03 ± 0.01	—	0.03 ± 0.00	—
0.5	0.50 ± 0.91	0.63	0.07 ± 0.00	0.09
5	0.17 ± 0.07	0.02	0.09 ± 0.11	0.01
50	0.57 ± 0.12	0.01	0.37 ± 0.12	ND
500	7.15 ± 0.00	0.01	6.67 ± 0.00	0.01
5000	68.21 ± 0.19	0.01	66.19 ± 0.12	0.01
TMAsO				
control	0.25 ± 0.06	—	ND	—
0.5	ND	ND	ND	ND
5	ND	ND	ND	ND
50	ND	ND	ND	ND
500	ND	ND	ND	ND
5000	1.27 ± 0.10	ND	29.93 ± 0.26	ND

Table A.16 Uptake of the trivalent organoarsenic species monomethylarsonous acid [MMAs(III)] and dimethylarsinous acid [DMAs(III)] by Hep G2 cells: whole-cell and cell-free extract

Conc. of As in exposure medium (μM)	Detected As concentrations ± SD			
	Whole-cell extract		Cell-free (membrane removed) extract	
	ng/10^6 cells	% of arsenic substrate	ng/10^6 cells	% of arsenic substrate
MMAs(III)				
control	0.07 ± 0.02	—	ND	—
0.5	0.95 ± 0.01	0.87	1.04 ± 0.01	0.96
5	12.08 ± 0.18	1.16	10.55 ± 0.02	1.01
50	84.74 ± 0.16	0.08	65.14 ± 0.13	0.06
DMAs(III)				
control	0.09 ± 0.08	—	0.47 ± 0.00	—
0.5	1.72 ± 1.80	0.67	0.94 ± 0.00	0.49
1	3.18 ± 0.37	2.33	2.56 ± 0.00	2.06
5	29.10 ± 0.07	4.17	33.46 ± 0.01	4.59
50	82.65 ± 0.00	2.81	48.54 ± 0.00	2.09

Table A.17 Uptake of sodium arsenite and sodium arsenate by HeLa S3 cells: whole-cell and cell-free extract.

Conc. of As in exposure medium (μM)	Detected As concentrations ± SD			
	Whole-cell extract		Cell-free (membrane removed) extract	
	ng/10^6 cells	% of arsenic substrate	ng/10^6 cells	% of arsenic substrate
As(III)				
control	2.20 ± 0.21	—	1.16 ± 0.28	—
0.5	2.59 ± 0.02	0.02 ± 0.00	3.02 ± 0.23	0.02 ± 0.00
5	9.62 ± 0.12	ND	3.26 ± 0.06	ND
50	4.60 ± 0.14	ND	15.60 ± 0.61	ND
500	68.68 ± 0.74	ND	65.82 ± 0.24	ND
5000	160.87 ± 0.01	ND	160.11 ± 1.38	ND
As(V)				
control	0.17 ± 0.13	—	0.17 ± 0.09	—
0.5	0.01 ± 0.04	ND	0.42 ± 0.01	0.08 ± 0.00
5	0.08 ± 0.03	ND	0.70 ± 0.04	0.01 ± 0.00
50	0.73 ± 0.18	ND	1.04 ± 0.15	ND
500	9.35 ± 0.09	ND	9.63 ± 0.09	ND
5000	52.99 ± 0.36	ND	54.43 ± 0.35	ND

Table A.18 Uptake of organoarsenic compounds by HeLa S3 cells: whole-cell and cell-free extract.

Conc. of As in exposure medium (μM)	Detected As concentrations ± SD			
	Whole-cell extract		Cell-free (membrane removed) extract	
	ng/10^6 cells	% of arsenic substrate	ng/10^6 cells	% of arsenic substrate
MMAs(III)				
control	0.08 ± 0.00	—	0.08 ± 0.00	—
0.5	4.20 ± 0.01	1.22 ± 0.00	3.33 ± 0.05	0.96 ± 0.02
5	21.22 ± 0.10	0.62 ± 0.00	18.04 ± 0.02	0.52 ± 0.00
50	112.91 ± 0.82	0.33 ± 0.00	62.46 ± 0.31	0.18 ± 0.00
500	160.19 ± 1.40	0.05 ± 0.00	61.58 ± 1.16	0.01 ± 0.00
5000	190.08 ± 7.85	ND	85.92 ± 0.35	ND
MMAs(V)				
control	0.52 ± 0.51	—	ND	—
0.5	0.43 ± 0.03	0.05 ± 0.00	ND	ND
5	0.32 ± 0.00	ND	0.21 ± 0.01	ND
50	0.06 ± 0.00	ND	0.27 ± 0.17	ND
500	2.46 ± 0.00	ND	2.61 ± 0.00	ND
5000	16.06 ± 0.23	ND	15.29 ± 0.02	ND
DMAs(III)				
control	0.01 ± 0.09	—	0.12 ± 0.02	—
0.5	3.43 ± 0.02	0.46 ± 0.00	3.11 ± 0.00	0.42 ± 0.00
1	6.13 ± 0.00	0.41 ± 0.00	5.75 ± 0.00	0.39 ± 0.00
5	35.28 ± 0.13	0.48 ± 0.00	33.77 ± 0.06	0.45 ± 0.00
10	71.28 ± 0.26	0.48 ± 0.00	61.96 ± 0.12	0.42 ± 0.00
DMAs(V)				
control	0.01 ± 0.02	—	0.02 ± 0.01	—
0.5	ND	ND	0.09 ± 0.01	0.01 ± 0.00
5	ND	ND	0.20 ± 0.01	ND
50	0.50 ± 0.03	ND	0.92 ± 0.08	ND
500	10.84 ± 0.05	ND	15.60 ± 0.21	ND
5000	20.29 ± 0.10	ND	20.11 ± 0.10	ND
TMAsO				
control	ND	—	ND	—
0.5	ND	ND	ND	ND
5	ND	ND	ND	ND
50	ND	ND	ND	ND
500	ND	ND	ND	ND
5000	2.80 ± 0.00	ND	2.97 ± 0.00	ND

Table A.19 Uptake of organoarsenic compounds by rat hepatocytes (Ra Hep) cells: whole-cell and cell-free extract.

Conc. of As in exposure medium (μM)	Detected As concentrations ± SD			
	Whole-cell extract		Cell-free (membrane removed) extract	
	ng/10^6 cells	% of arsenic substrate	ng/10^6 cells	% of arsenic substrate
MMAs(III)				
control	0.99 ± 0.00	—	0.99 ± 0.00	—
0.5	39.79 ±11.77	0.75 ± 0.22	32.79 ± 9.48	0.62 ± 0.18
5	352.52 ± 17.25	0.67 ± 0.04	325.92 ± 0.53	0.62 ± 0.00
50	1055.61 ± 57.70	0.20 ± 0.01	813.54 ± 20.04	0.15 ± 0.00
500	1165.90 ± 16.60	0.02 ± 0.00	788.76 ± 15.14	0.01 ± 0.00
MMAs(V)				
control	0.30 ± 0.00	—	0.30 ± 0.12	—
5	1.55 ± 0.42	ND	1.49 ± 12.65	ND
50	0.19 ± 0.09	ND	0.09 ± 0.99	ND
500	1.41 ± 0.00	ND	0.55 ± 0.82	ND
5000	16.60 ± 0.25	ND	14.17 ± 0.14	ND
DMAs(III)				
control	ND	—	0.04 ± 0.00	—
0.5	4.12 ± 0.22	0.08 ± 0.00	3.13 ± 0.00	0.06 ± 0.00
1	41.43 ± 1.22	0.41 ± 0.01	48.63 ± 0.00	0.48 ± 0.00
5	17.15 ± 0.47	0.03 ± 0.00	24.09 ± 0.16	0.05 ± 0.00
10	466.57 ± 4.27	0.46 ± 0.00	416.37 ± 2.54	0.41 ± 0.00
DMAs(V)				
control	ND	—	0.05 ± 0.00	—
0.5	ND	ND	0.11 ± 0.18	ND
5	0.15 ± 0.00	ND	0.09 ± 0.00	ND
50	0.18 ± 0.04	ND	0.08 ± 0.00	ND
500	4.38 ± 0.06	ND	4.30 ± 0.05	ND
5000	33.13 ± 0.02	ND	32.30 ± 0.16	ND
TMAsO				
control	2.84 ± 0.13	—	2.37 ± 0.26	—
0.5	ND	ND	ND	ND
5	ND	ND	ND	ND
50	0.28 ± 0.00	ND	0.94 ± 0.00	ND
500	13.43 ± 0.05	ND	14.64 ± 0.06	ND
5000	169.73 ± 1.51	ND	169.82 ± 1.70	ND

Table A.20 Uptake of sodium arsenite and sodium arsenate by rat hepatocytes (Ra Hep) cells: whole-cell and cell-free extract.

Conc. of As in exposure medium (μM)	Detected As concentrations ± SD			
	Whole-cell extract		Cell-free (membrane removed) extract	
	ng/10^6 cells	% of arsenic substrate	ng/10^6 cells	% of arsenic substrate
As(III)				
control	0.03 ± 0.01	—	1.47 ± 2.24	—
0.5	2.91 ± 0.08	0.35 ± 0.00	ND	ND
5	10.99 ± 0.08	0.13 ± 0.00	3.90 ± 0.00	0.05 ± 0.00
50	32.51 ± 0.56	0.04 ± 0.00	23.79 ± 0.00	0.03 ± 0.00
500	37.98 ± 0.33	ND	27.48 ± 0.00	ND
As(V)				
control	1.78 ± 0.64	—	0.67 ± 0.00	—
0.5	6.29 ± 0.00	0.08 ± 0.00	7.70 ± 0.02	0.09 ± 0.00
5	ND	ND	2.50 ± 0.38	ND
50	8.59 ± 0.03	ND	10.79 ± 1.07	ND
500	78.79 ± 0.44	ND	75.88 ± 1.38	ND
5000	345.08 ± 0.26	ND	345.84 ± 1.31	ND

Curriculum vitae (CV)

Personal Information:

First name: **Sasan**
Last name: **RABIEH**
Father's name: Abbas
Marital status: Married (no children)
Date and place of birth: 11 August, 1972- Ahvaz
Nationality: Iranian (German resident)
Languages: Persian, English, German, Arabic
Email: sasan.rabieh@uni-due.de
srabieh@yahoo.com

Educational Qualification:

- *10/2002 to 03/2007* **PhD** degree in Environmental Analytical Chemistry- University of Duisburg-Essen (Campus Essen), Germany. Title of the PhD thesis: "Arsenic speciation in biological samples using high performance liquid chromatography coupled to inductively coupled plasma mass spectrometry (HPLC-ICP-MS) and Investigation of cellular uptake capabilities of arsenicals in different cell lines". (*under supervision of Professor Dr. Alfred V. Hirner*)

- *10/1995 to 09/1998* **MSc‡** degree in Analytical Chemistry-Chamran University in Ahvaz, Iran. Title of the MSc thesis: "Study of the effects of chemical modifiers on the determination of Lead and Chromium (IV) in aqueous samples by FAAS". (*under supervision of Professor Dr. Nahid Pourreza*)
  ‡ *First class honours at Chem. Dept.*

- *10/1991 to 06/1995* **BSc*** degree in Chemistry-Chamran University in Ahvaz, Iran.
  * *First class honours at Chem. Dept. and at Sciences Faculty in the year 1995-1996.*

- *10/1988 to 06/1989* **High School Diploma** in Experimental Sciences- Dr. Shariati high school in Ahvaz, Iran.

Visiting Scholar:

- 02/2004 to 07/2004 Chemistry Department of University of Aberdeen, Aberdeen, Scotland. (*under advising of Professor Dr. Jörg Feldmann*)

Professional Experiences and Employments:

- ***February 1996 to July 2001*** Lecturer at Ahvaz Azad University (Part time)
- ***February 1997 to September 1998*** Lecturer at Ahvaz Chamran University (Part time)
- ***September 1998 to July 2001*** Lecturer at Omidieh Azad University (Part time)
- ***February 1999 to July 1999*** Lecturer at Abadan Oil University (Part time)
- ***February 2004 to July 2004*** Visiting scientific researcher at Aberdeen University, Scotland
- ***October 2001 to present*** Scientific researcher at Institute of Environmental Analytical Chemistry, University of Duisburg-Essen (Campus Essen).

Courses Taught (in Iran):

- General Chemistry (I & II) + Laboratory
- Analytical Chemistry (I) + Laboratory
- Instrumental Analysis + Laboratory
- How to use Chem. Sci. Literature

Summary of Research Interests:

- A major area of research interest is on chemical speciation of trace elements. The primary interest is on the arsenic speciation, in light of its significant impact on the environment and human health.
- Developing analytical techniques for the speciation analysis of trace levels of arsenic compounds in biological samples.
- Cellular uptake of arsenic, antimony, bismuth, and tin and their methylated derivatives.
- **Keywords**: HPLC, ICP-MS, AAS, AFS, cellular uptake, toxicity, arsenic, etc.

Publications:

- Dopp, E., Hartmann, L. M., von Recklinghausen, U., Florea, A. M., Rabieh, S., Zimmermann, U., Yadav, S., Hirner, A. V., Rettenmeier, A. W. (2005). Forced uptake of trivalent and pentavalent methylated and inorganic arsenic and its cyto-/genotoxicity in fibroblasts and hepatoma cells. *Toxicological Sciences, **87(1)**, 46-56.*

- Dopp, E., Hartmann, L. M., von Recklinghausen, U., Rabieh, S., Hirner, A. V., Rettenmeier, A. W. (2006). Methylation, oxidation state and membrane permeability determine toxicity of arsenic compounds. *Naunyn-Schmiedebergs Archives of Pharmacology, **372**, 115.*

- Dopp, E., Hartmann, L. M., von Recklinghausen, U., Stueckradt, I., Pollok, I., Rabieh, S., Hirner, A. V., Rettenmeier, A. W. (2006).Uptake, subcellular distribution and toxicity of arsenic species in methylating and nonmethylating human cells. *Toxicology Letters*, ***164(Suppl 1**), S260.*

- Dopp, E., Hartmann, L. M., Florea, A. M., von Recklinghausen, U., Rabieh, S., Hirner, A. V., Rettenmeier, A. W. (2006). Trimethylantimony dichloride causes genotoxic effects in China hamster ovary cells after forced uptake. *Toxicology in Vitro, **20(6)**, 1060-1065.*

- Rabieh, S., Hirner, A. V., Matschullat, J. (2007). Determination of arsenic species in human urine of an arsenic-affected area in Brazil using high performance liquid chromatography (HPLC) coupled with inductively coupled plasma mass spectrometry (ICP-MS). *(under preparation).*

- Rabieh, S., Diaz-Bone, R. A., Hasenäcker, F., Kösters, J., Hirner, A. V. (2007). Investigation of monomethylarsonous acid [MMAs(III)] in Brazilian human urine by a combined liquid and gas chromatographic approach. *(under preparation).*

- von Recklinghausen, U., Hartmann, L. M., Stueckradt, I., Pollok, I., Rabieh, S., Ping, Y., Nüssler, A., Katier, C., Hirner, A. V., Rettenmeier, A. W., Dopp, E. (2007). Subcellular distribution of anorganic and organic arsenic arsenic compounds in human urothelial cells compared to human hepatocytes. (*under preparation).*

- Dopp, E., Hartmann, L. M., von Recklinghausen, U., Florea, A. M., Rabieh, S., Hirner, A. V., Obe, G., Rettenmeier, A. W. (2007). The cyto- and genotoxicity of organotin compounds is dependent on the cellular uptake capability. *Toxicology, **232(3)**, 226-234.*

Presentations in Conferences:

- "Organometalloid compounds in compost materials", *International workshop on Organometallics in the Environment, Essen, Germany 14-16 October 2002.*

- "Study of the effects of chemical modifiers on the determination of Lead in aqueous samples by FAAS", *The 2003 Eastern Analytical Symposium, Somerset, New Jersey, U.S.A, November 17-20, 2003.*

- "Zelluläre Aufnahme, Reaktivität und Cytotoxizität von Arsenverbindungen in humanen Leberzellen", *DGAUM-45 Jaherstagung, Bochum, Germany, 06-09 April, 2005.*

- "Aufnahme und Toxizität von Arsenverbindungen in humanen Leber-und Urothelzellen". *DGAUM-46 Jaherstagung, Hannover, Germany, 22-25 March, 2006.*

- "Die Toxizität von Arsenverbindungen wird durch deren Methylierungsgrad, den Oxidationsstatus und die Membrangängigkeit bestimmt". *47th Spring Meeting Deutsche Gesellschaft für Experimentelle und Klinische Pharmakologie und Toxikologie, Mainz, Germany, 4-6 April, 2006.*

- "Arsenic speciation in human urine of an arsenic-affected area in Brazil using HPLC-ICP-MS", *The 2006 Eastern Analytical Symposium, Somerset, New Jersey, U.S.A, November 13-16, 2006*.

- "Investigation of monomethylarsenous acid [MMAs(III)] in Brazilian human urine by a combined liquid and gas chromatographic approach", *The 2007 European Winter Conference on Plasma Spectrochemistry, Taormina, Italy, February 18-23, 2007.*

References:

- Prof. Dr. Alfred V. Hirner
  Universitaet Duisburg-Essen (Campus Essen)
  Institute of Environmental Analytical Chemistry
  Universitaetsstr. 3-5
  D-45141 Essen
  Germany

  Tel. +49-201-183 3950
  Fax. +49-201-183 3951
  Email: alfred.hirner@uni-due.de

- PD Dr. Elke Dopp
  Universitaetsklinikum
  Institute of Hygiene and Occupational Medicine
  Hufelandstrasse 55
  D-45122 Essen
  Germany

  Tel. +49-201-723 4578
  Fax. +49-201-723 4546
  Email: elke.dopp@uni-due.de

Declaration

I declare that this thesis is my own work and has not been submitted in any form for another degree or diploma at any university or other institution of tertiary education. Information derived from the published or unpublished work of others has been acknowledged in the text and a list of references is given.

Sasan Rabieh Essen, 20 March, 2007

ERKLÄRUNGEN

ERKLÄRUNG

Hiermit erkläre ich, gemäß § 6 Abs. 2, Nr. 7 der Promotionsordnung der Fachbereiche 6 bis 9 zur Erlangung des Dr. rer. nat., dass ich das Arbeitsgebiet, dem das Thema:

"*Arsenic speciation in biological samples using high performance liquid chromatography (HPLC) coupled with inductively coupled plasma mass spectrometry (ICP-MS) and investigation of cellular uptake capabilities of arsenicals in different cell lines*"

zuzuordnen ist, in Forschung und Lehre vertrete und den Antrag von Herrn Sasan Rabieh befürworte.

Essen, 14.03.2007

Prof. Dr. Alfred V. Hirner

ERKLÄRUNG

Hiermit erkläre ich, gemäß § 6 Abs. 2, Nr. 6 der Promotionsordnung der Fachbereiche 6 bis 9 zur Erlangung des Dr. rer. nat., dass ich für die vorliegende Dissertation selbstständig verfasst und mich keiner anderen als der angegebenen Hilfsmittel bedient habe.

Essen, 14.03.2007

Sasan Rabieh, BSc. MSc. (Hons)

ERKLÄRUNG

Hiermit erkläre ich, gemäß § 6 Abs. 2, Nr. 8 der Promotionsordnung der Fachbereiche 6 bis 9 zur Erlangung des Dr. rer. nat., dass ich keine anderen Promotionsversuche in der Vergangenheit durchgeführt habe und dass diese Arbeit von keiner anderen Fakultät abgelehnt worden ist.

Essen, 14.03.2007

Sasan Rabieh, BSc. MSc. (Hons)