CHARLES UNIVERSITY
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SYNTHESIS AND SEPARATION OF ARSENIC-GLUTATHIONE COMPLEXES

Syntéza a separace arzenoglutationových komplexů

Bachelor's thesis

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V Praze dne 15.5.2017

Abstract

The arsenic-glutathione complexes are very unstable in solution and tend to decompose during separation in liquid chromatography. The aim of this work was to develop a relatively fast method of the synthesis and storage conditions for the arsenic-glutathione complexes.

The thesis is focused on synthesis, stability in-solution and separation of arsenicglutathione complexes. The synthesis was carried out in solution of 2 mM TCEP (tris(2carboxyethyl)phosphine) in water and with excess of the glutathione. Solutions of 20 ppb arsenic-complexes were consecutively measured after 1h, 2h, 3h, 4h and 24 hours of synthesis. The results confirmed stability of the arsenic-complexes in the reaction mixtures over 24 h.

The arsenic-glutathione complexes were separated using a reversed phase high performance liquid chromatography (RP-HPLC) coupled with inductively coupled plasma - mass spectrometry (ICP-MS). The chromatographic method was developed using Aeris widepore 3.6u XB-C18 250x2.10mm column. Isocratic and gradient elutions were compared using several compositions of mobile phases and time of the separation. Methods were tested using samples of synthesized arsenic-glutathione complex (DMAs(GS)). An application of the isocratic elution enabled elimination of time needed for the separation and conditioning of the column and the influence of organic solvent on the intensity of arsenic signals in ICP-MS.

Key words: Arsenic, glutathione, arsenoglutathione complexes, isocratic elution, high performance liquid chromatography, inductively coupled plasma mass spectrometry

Abstrakt

Arzenoglutationové komplexy jsou v roztoku velmi nestabilní a mají tendenci degradovat během separace pomocí kapalinové chromatografie. Tato práce je zaměřena na vývin rychlé syntézy a nalezení vhodných podmínek na skladování arzenoglutationových komplexů. Cílem práce je syntéza, stabilita v roztoku a separace arzenoglutationových komplexů.

Syntéza probíhala v roztoku 2 mM TCEP (tris(2-karboxyetyl)fosfán) s vodou a glutationem. Roztoky arzenokomplexů o koncentraci 20 ppb byli postupně měřené po 1h, 2h, 3h, 4h a 24h syntézy. Výsledky potvrdily stabilitu arzenokomplexů v reakční směsi po dobu 24 hodin.

Arzenoglutatiónové komplexy byli separované pomocí vysokoúčinné kapalinové chromatografie na reverzních fázích (RP-HPLC) spojené s hmotnostní spektrometrií s indukčně vázanou plazmou. (ICP-MS). Chromatografie probíhala za použití kolony Aeris widepore 3.6u XB-C18 250x2.10mm. Izokratická eluce byla srovnávána s gradientovou elucí při použití různých složení mobilních fází a rozdílného času separace. Metody byli aplikované na vzorek arzenoglutationového komplexu DMAs(GS). Z opakovaných experimentů bylo zjištěno, že izokratická eluce je výhodnější z důvodu snížení času potřebného na separaci komplexů a malého vlivu rozpouštědla na signál arzénu v ICP-MS.

Klíčová slova:

Arzén, glutatión, arzenoglutationové komplexy, izokratická eluce, vysokoúčinná kapalinová chromatografie, hmotnostní spektrometrie s indukčně vázanou plazmou

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Content

List of Abbreviations

ACN	acetonitrile
As	arsenic
$As(GS)_3$	arsenic triglutathione
DIW	deionized water
$DMAs^{III}$	dimethylarsinous acid
$DMAs^{V}$	dimethylarsonic acid
DMAs(GS)	dimethylarsinous glutathione
FA	formic acid
GSH	glutathione
HPLC	high-performance liquid chromatography
iAs^{III}	arsenite
iAs^{V}	arsenate
ICP - MS	inductively coupled plasma mass spectrometer
IPC	ion-pair chromatography
MAs^{III}	monomethylarsonous acid
MAs^{V}	monomethylarsonic acid
$MAs(GS)_2$	methylarsonous diglutathione
T	temperature
t	time
t_R	retention time
TCEP	tris(2-carboxyethyl)phosphine
$TMAO^{V}$	trimethylarsenic oxide
TMA^{III}	trimethylarsinic acid

1. INTRODUCTION

Arsenic is a metalloid occurring naturally, mostly everywhere, being the 20th most abundant element not only in the earth's crust, but also a component of more than 245 minerals.[1]

As one of the most spread elements it is present in both organic and inorganic forms. Inorganic species of arsenic are commonly found in any kind of water and therefore people across the globe mainly in area of Bangladesh and West Bengal, India are in danger because of extremely high toxicity of this element.[2] It is a well-established human carcinogen. Ingestion of As-contaminated water has led to serious arsenicosis in humans. Diseases like hypertension, keratosis, skin pigmentation, diabetes, cardiovascular disorders and cancer of the bladder, lung and skin have been reported after As exposure.[3] Its toxicity mainly depends on a compound and oxidation stress.[2]

Arsenic is metabolized in living organisms through oxidation-reduction and methylation reactions. Thiol-containing glutathione has been reported to take part in it acting as a reducing agent.[3]

One of the aims of this work was to establish conditions for fast synthesis and chromatographic separation of the following arsenic-glutathione complexes: *Arsenic triglutathion* ($As(GS)_3$), *Methylarsonous diglutathione* ($MAs(GS)_2$) and *Dimethylarsinous glutathione* (DMAs(GS)). As known from literature, [4] the arsenic complexes are not stable in low concentrations and tend to decompose during separation in a HPLC column. Therefore, an additional aim was to establish the most stable conditions for maintaining the arsenic-glutathione complexes in solution.

2. THEORETICAL PART

2.1 Arsenic

2.1.1 Occurrence and use of arsenic

In the past, inorganic arsenic compounds were used as pesticides, primarily on cotton fields, although they were taken off the line and can no longer be used in agriculture. However, organic arsenic compounds, namely cacodylic acid, disodium methylarsenate (DSMA), and monosodium methylarsenate (MSMA), are still used as pesticides. Some of them are used as additives in animal feed.[5] Small quantities of elemental arsenic are used as additives to other metals to form metal mixtures.[6] The greatest use of arsenic in alloys is in lead-acid batteries for automobiles. Also, another important use of arsenic compounds is in semiconductors and light-emitting diodes.[7]

2.1.2 Toxicity of arsenic in human body

The toxicity and bioavailability of arsenic depends on its chemical form. Most chronic arsenic poisoning cases are caused by the consumption of inorganic arsenic, arsenite (iAs^{III}) and arsenate (iAs^{V}), in well water [8] all around the world, especially in some particular parts mentioned before.

Overall analysis of published data implies that arsenic exposure induces various types of cancer, cardiovascular diseases, developmental abnormalities, neurologic and neuroverbal disorders, diabetes, hearing loss and last but not least hematologic disorders.[9] Although exposure may occur via the dermal, and parenteral routes, the main pathways of exposure include ingestion, and inhalation.[10]

Organic methylated arsenic compounds such as MAs^{III}, MAs^{V}, $DMAs^{III}$ and $DMAs^{V}$ as well iAs^{III} and iAs^{V} were found in human's urine of people, that were chronically exposed to inorganic arsenic for a long period of time. Structure formulas of deprotonated forms of arsenospecies are shown in Fig. 2.1.

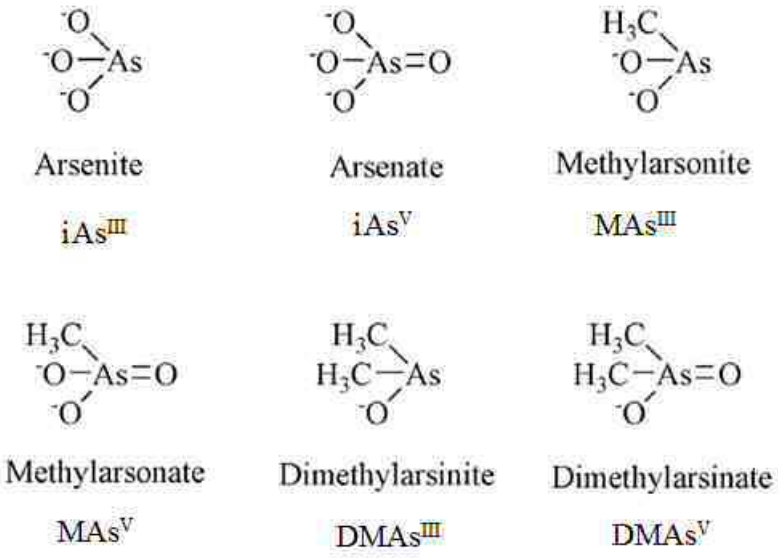


Fig. 2.1 Chemical structures with names and abbreviations for arsenic species [11]

Glutathione, which can readily reduce iAs^{V} to iAs^{III} is likely responsible for the reduction of iAs^{V}, MAs^{V}, and $DMAs^{V}$ to the corresponding trivalent forms. While it is generally accepted that methylation is the principal detoxification pathway, recent studies have suggested that methylated metabolites may be partly responsible for the adverse effects associated with arsenic exposure.[12]

2.1.3 Toxicity of certain arsenospecies

Toxicities of different arsenospecies are dependent on their form. iAs^{III} is more toxic than iAs^{V} methylated trivalent arsenicals are more toxic than their pentavalent counterparts. MAs^{III} and DMA^{V} are also genotoxic.[13] The reported toxicity in human hepatocytes is MAs^{III} > $DMAs^{III}$ = iAs^{III} > iAs^{V} > $DMAs^{V}$ and MAs^{V}.[14] Recent studies have also demonstrated, that $MAs(GS)_2$ and DMAs(GS) are toxic to cells through their dissociation into MAs^{III} and $DMAs^{III}$ in the absence of GSH.[15] These forms are commonly present in urine while an indirect evidence has been provided for the presence of $As(GS)_3$ in human blood cells and plasma.[16] The arsenic-glutathione complexes are shown in Fig. 2.2.

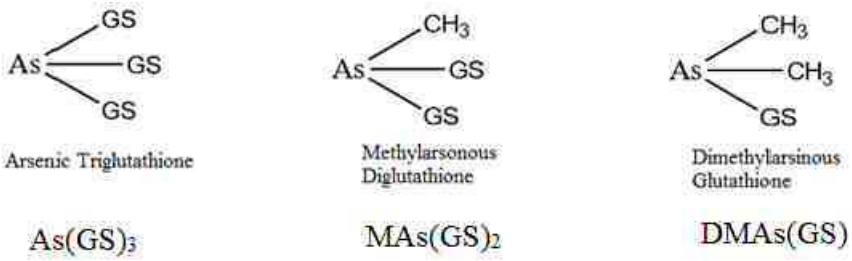


Fig. 2.2 Chemical structures with names and abbreviations for arsenoglutathione species [17]

2.2 Arsenic-glutathione complexes

Glutathione (GSH) in its reduced form is a tripeptide consisting of L-glutamine, L-cysteine, and glycine (Fig. 1.3). It plays an important role in protecting cell components from free radicals, peroxides, lipid peroxides and heavy metals. The molecule is present in every living organism where it acts as a central antioxidant. It has a key role in the cycle of the transport of an acyl group (transport of the amino acids in kidney) and is a reducing agent in some enzymatic processes. [4]

It supports enzymatic methylation of inorganic arsenic (iAs^{III}) to monomethylarsonous acid (MAs^{III}), monomethylarsonic acid (MAs^{V}), dimethylarsinous acid ($DMAs^{III}$), and dimethylarsinic acid ($DMAs^{V}$).[16] Complexes of arsenic compounds and glutathione are very unstable and tend to undergo a decomposition to simple organic-arsenic species and free glutathione during chromatographic separation.[4]

Glutathione (Fig 2.3) has been described just as a defensive reagent against the action of toxic xenobiotics (drugs, pollutants, carcinogens).[18] As a prototype antioxidant, it has been involved in cell protection from the noxious effect of excess oxidant stress, both directly and as a cofactor of glutathione peroxidases.[18]

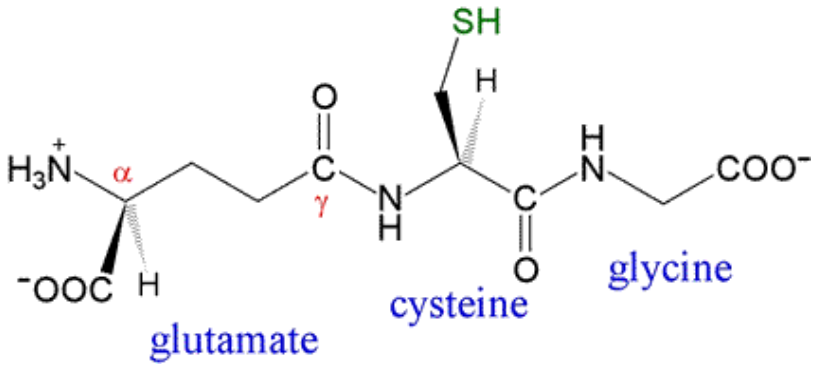


Fig. 2.3 Structure of reduced glutathione; glutamate is linked in an isopeptide bond (*via* its γ-carboxyl group) to cysteine, which in turn forms a peptide linkage with glycine.[19]

The free thiol group in cysteine makes the glutathione a trapper for the arsenic and organic-arsenic compounds. According to Feldmann et al.[4] the complexes are generated during the reaction of trivalent arsenic species: iAs^{III}, MAs^{III} and $DMAs^{III}$ with glutathione. As the pentavalent MAs^{V} and $DMAs^{V}$ are used for the reaction, the excess of the GSH is applied as a reducing agent. (Fig. 2.4)

$$iAs^{III} + 3GSH \rightarrow As^{III}(GS)_3 + 3H_2O$$

$$MAs^{V} + 4GSH \rightarrow GS\text{-}SG + MAs^{III}(GS)_2 + 3H_2O$$

$$DMAs^{V} + 3GSH \rightarrow GS\text{-}SG + DMAs^{III}(GS) + 2H_2O$$

Fig. 2.4 - Formation of arsenic-glutathione complexes

2.3 Metabolic pathways

Nowadays the research is more focused on getting information on exact mechanism of biotransformation of arsenic in human body. The metabolic pathway of arsenic in body described by Challenger in 1945[20] is now innovated of new and more exact alternative mechanisms.

The metabolism of arsenic is generally accepted to proceed by repetitive reduction and oxidative methylation. Arsenite is metabolized as a waste product excreted in urine.[21] Therefore in human urine, the major metabolites of inorganic arsenicals such as arsenite (iAs^{III}) and arsenate (iAs^{V}) are MAs^{V} and $DMAs^{V}$. Previously, methylation of arsenite was considered as a detoxication process,[22] whereas nowadays it is considered more of a bioactivation process, while during combustion of arsenite MAs^{III} and $DMAs^{III}$ are created forms that are way more toxic than inorganic arsenite.

Up till now three main mechanisms of As metabolism have been proposed. First was stated by Challenger in 1945[20] (Fig. 2.5), later on improved by Hayakawa in 2005[23] (Fig. 2.6) and then by Suzuki and Naranmandury in 2006.[24]

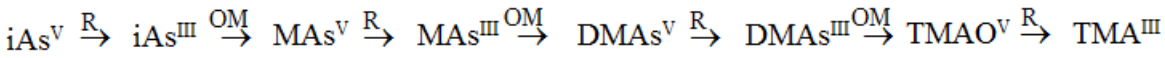


Fig 2.5 - Challenger 's mechanism for arsenic biomethylation. R = reduction, OM = oxidative methylation[20]

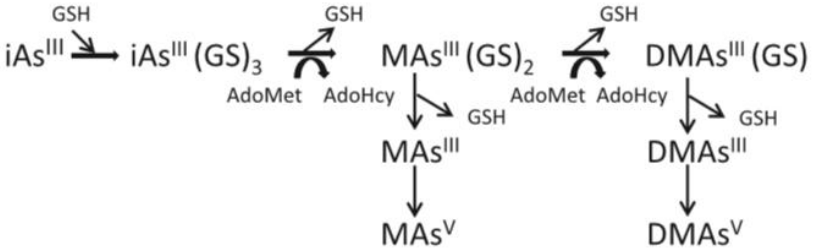


Fig 2.6 - Hayakawa's reductive methylation pathway [4]

2.4 HPLC - ICP -MS

HPLC-ICP-MS is the most common method used for speciation analysis of arsenic. HPLC method in general is highly specific and can separate large spectrum of As species, including ones that originate in foods and are not products of arsenic metabolism in human body.[25] ICP-MS is the most often used detector for elemental speciation analysis, because of its high sensibility, large linear dynamic range and is easily combined with separation techniques [24]. Result of combining HPLC and ICP-MS devices is sensible system with large dynamic range with option of determination of more elements at once. [2]

However, system HPLC-ICP-MS contains some disadvantages in general such as limited use of organic mobile phase as well as no information about the structure which leads to reduced ability to identify new arsenospecies. [11]

2.4.1 The separation of arsenospecies by HPLC

The common method for the determination of inorganic and organic arsenospecies is ion-change chromatography.[4] The principles of this method are electrostatic interactions between ions which are bound on the surface of the stationary phase and charged ions of analyte. [26] This method is only appropriate for ion separation or easily ionized compounds. [27]

Separation is also influenced by solubility of chemical analyte being determined, mobile phase, pH and temperature. Method is divided into two separation modules: anion and cation ion-exchange chromatography. Anion-exchange chromatography is efficient for determination of iAs^{III}, iAs^{V}, MAs^{V} and $DMAs^{V}$ with use of high pH mobile phase. On the other hand arsenospieces such MAs^{III} and $DMAs^{III}$ are efficient to be separated by cation ion-exchange column with low pH mobile phase.[26]

Separation of arsenic-glutathione complexes has been described in several publications.[4,16,28] The results presented by Raab at. al.[4] suggest, that it is impossible to separate arsenic-glutathione species by anion-change chromatography whether using acidic or basic mobile phase. However detection of arsenic-glutathione complexes by cation-exchange chromatography was successful using acidic mobile phase, although complexes were not separated.

Next approach that is being widely used for speciation analysis is called reverse phase chromatography (RPC). The stationary phase is hydrophobic and commonly made out of silica particles with covalently bond alkyl chains. The mobile phase is polar (aqueous). The hydrophobic molecules in the polar mobile phase adsorb to the hydrophobic stationary phase. The hydrophilic molecules in the mobile phase pass through the column and are eluted first. Hydrophobic molecules are eluted from the column by decreasing the polarity of the mobile phase using an organic (non-polar) solvent (acetonitrile, methanol). The more hydrophobic the molecule, the more strongly it is bound to the stationary phase. Higher concentration of the organic solvent enables elution of the molecule.[28]

Separation of arsenic-glutathione complexes using RPC method was also described in paper by Raab et. al.[4], where ODS2 C18 (Waters Spherisorb S5 (250 6 4.6 mm)) column was used with mobile phase in gradient mode. According to the authors, results were successful and complexes were separated. Unfortunately the RPC method is not suitable for the separation of simple arsenospecies,[13] like arsenite or arsenobetaine under standard conditions.[29] However, this problem can be solved by application of an ion-pairing agent such as tetraalkylammonium salts to the mobile phase. Ion-pair chromatography (IPC) mostly uses stationary phase with C18 alkyl chain, water based mobile phase with addition of ion-pairing agent and buffer solution. It is charged on the one end and hydrophobic on the other one. The hydrophobic end can interact with the stationary phase.[27] The most commonly used ion-pairing agent is tetrabutylammonium hydroxide (TBAH). [30] Addition of this agent to the mobile phase is supposed to rise solubility of analyte and enhance wettability of stationary phase wall.[24]

Ion-pair chromatography was described and used for separation of simple arsenospieces in different matrixes by Currier at. el. [25] The authors came to the conclusion, that IPC is an effective method for separation of trivalent and pentavalent standards (iAs^{III}, MAs^{V}, $DMAs^{V}$) in deionized water or TRIS-HCl buffer. However, if this method is used for determination of human samples such as urine or saliva, a loss of MAs^{III} and $DMAs^{III}$ appears on the column. As for the separation of arsenic complexes by ion-pair chromatography, the process has not been described in literature yet.

2.4.2 Detection techniques for separation of arsenospecies

A range of detectors is available for arsenic speciation, although only some of them are able to provide promising results. In Tab. 2.1 is a brief comparison of single atomic spectrometry techniques, showing their advantages and disadvantages when used.

Tab. 2.1 Comparison of atomic spectrometry techniques [31]

Technique	Metals	Approximate DL range	Advantages	Disadvantages
ICP-MS	Most metals and non-metals	ppt	Rapid, sensitive, multi-element, wide dynamic range, good control of interferences	Limited total dissolved solids (TDS) tolerance
ICP-OES	Most metals and some non- metals	Mid ppb to mid ppm	Rapid, multi-elemental, high TDS tolerance	Complex interferences, relatively poor sensitivity
GFAA	Most metals (commonly Pb, Ni, Cd, Co, Cu, As, Se)	ppb	Sensitive, few interferences	Single element technique, limited dynamic range
Hydride AA	Hydride forming elements (As, Se, Tl, Pb, Bi, Sb, Te)	ppt to ppb	Sensitive, few interferences	Single element, complex

Some instrumental approaches for arsenic speciation involve the use of a separation technique, such as HPLC for non volatile species combined directly to a suitable atomic detector.[32] The direct coupling between HPLC and atomic absorption spectrometry (AAS)[33] provides poor detection limits, which are improved using the direct coupling HPLC-ICP-MS.[34] Coupled technique as HPLC-ICP-MS is commonly used for this kind of speciation. It is very efficient for detection of non-volatile metal species in animal and plant biochemistry.

This multielementary method offers very low detection limits,[35] however its sensitivity is limited. For instance, the sample volume is limited by separation method also the analyte zone is augmented during the separation itself.[36]

Performances of two atomic detectors, Inductively Coupled Plasma-Mass Spectrometry (ICP - MS) and Atomic Fluorescence Spectrometry (AFS) have been compared by Gómez-Ariza at. el.[37] The authors came to the conclusion that both AFS and ICP-MS are useful detection techniques for arsenic speciation. Although AFS presents benefits of much lower running costs, easy handling and shorter warm up times prior to the analysis.[37]

3. EXPERIMENTAL PART

3.1 Chemicals

- Deionized water (Ultrapure device, Watrex, USA)
- Mobile phase A (2% ACN, with 0,1% FA in DIW)
- Mobile phase B (80% ACN with 0,1% FA in DIW)
- Tris(2-carboxyethyl)phosphine (TCEP; $C_9H_{15}O_6P$; M_r = 286,60; Sigma-Aldrich, Germany)
- Formic acid, 98% (HCOOH; M_r = 46,03; Penta, Czech Republic)
- Nitric acid, 65% (HNO_3; M_r = 63,01; Merck, Germany)
- Tellurium, 1000 mg/l (M_r = 127,60; BDH Prolabo, Englamd)
- Glutathione (GSH; $C_{10}H_{17}N_3O_6S$; M_r= 307,32; Sigma-Aldrich, Germany)
- Argon, 99,996 % (SIAD S.p.A., Czech Republic)
- Helium, 99,998 % (SIAD S.p.A., Czech Republic)
- Acetonitrile, 99,9% (C_2H_3N; M_r = 41,05; Lach-Ner, s.r.o., Czech Republic)

3.2 Preparation of standards

Stock solution with concentration of 1026 mg/l was prepared from arsenic trioxide (As_2O_3; Mr = 198,00; Lach-Ner, s.r.o, Czech republic) dissolved in 0,5 ml 10% potassium hydroxide (KOH; Mr = 56,11; Lech-Ner, s.r.o, Czech republic) and dissolved with deionized water.

Stock solution of concentration of 1000 mg/l as prepared from disodium methylarsenate ($Na_2CH_3AsO_3.6H_2O$; Mr = 291,99; Chem Service,USA) dissolved in deionized water.

Stock solution with concentration of 1000 mg/l was prepared from dimethylarsinic acid ($(CH3)_2AsO(OH)$); Mr = 137,99; Chem Service, USA) dissolved in deionized water.

Tellurium solution with concentration 200 ppb used as internal standard was prepared from stock solution with concentration 1000 mg/l (Mr = 127,60; BDH Prolabo, England) by dilution with 2% nitric acid.

3.2 Preparation of arsenic-glutathione complexes

The arsenic-glutathione complexes were synthesized according to the literature.[39] The stock solutions of iAs^{III}, MAs^{V} and $DMAs^{V}$ in DIW were used to prepare the stock reaction solutions (100 ppm, 200 ppm and 300 ppm) of each As specie in DIW. The glutathione solution was prepared in 2 mM TCEP in DIW. The concentrations of GSH solutions were twice concentrated to obtain the requested GSH:As ratios after mixing with the stock reaction solutions of As species. The both solutions were mixed in molar ratio 1:1 achieving final concentrations of each As species: 50 ppm, 100 ppm and 150 ppm. The reactions were carried out at 25 °C for 24 hours and then stored at 8 °C in a fridge. The reaction samples were diluted to 2 ppm and then to 20 ppb of each arsenic-glutathione complex prior to the analysis. The dilutions were carried out in mobile phase A (% ACN, with 0,1% FA in DIW), that was kept in ice.

Tab. 3.1 - Concentrations of As specie and ratios or arsenospecies and glutathione used in the experiments

NRM*	As (ppm)	GSH:As**
1	50	1:12
2	50	1:24
3	100	1:73
4	150	1:24

* NRM – number of the reaction.

** The ratio GSH:As reflects the molar ratio of the glutathione to each arsenospecie in the mixture.

3.3 Methods

3.3.1 Instrumentation

HPLC:

- HPLC pump 1200 Series, Quaternary Pump (Agilent Technologies, USA)
- HPLC thermostated autosampler (Agilent Technologies, USA)

- HPLC thermostated column compartment (Agilent Technologies, USA)
- Aeris widepore 3,6 µm XB-C18 precolumn and separation column 250 x 2.10 mm (Phenomenex, USA)

ICP-MS:

- ICP-MS spectrometer 7700x (Agilent Technologies, USA)
- ASX-500 asutosampler (Agilent Technologies, USA)
- Micro-Mist nebulizer (Agilent Technologies, USA)
- High Matrix interface (Agilent Technologies, USA)

OTHERS:

- Analytical balance R160P with accuracy 0,00001g (Sartorius, Germany)
- Automatic pipettes (Biohit, Finland)

3.3.2 Experimental arrangement

Scheme of HPLC ICP-MS system is shown in Fig. 3.1. Mobile phase A (MP A) and B (MP B) were drawn from eluent bottles to the column by HPLC gradient pump with flow 0,25 ml/min. Injection volume of each 20 ppb sample was 20 µl and for each 2 ppm sample 1 µl. 200 ppb Tellurium solution was mixed to the column effluent at ratio 1:2.5.

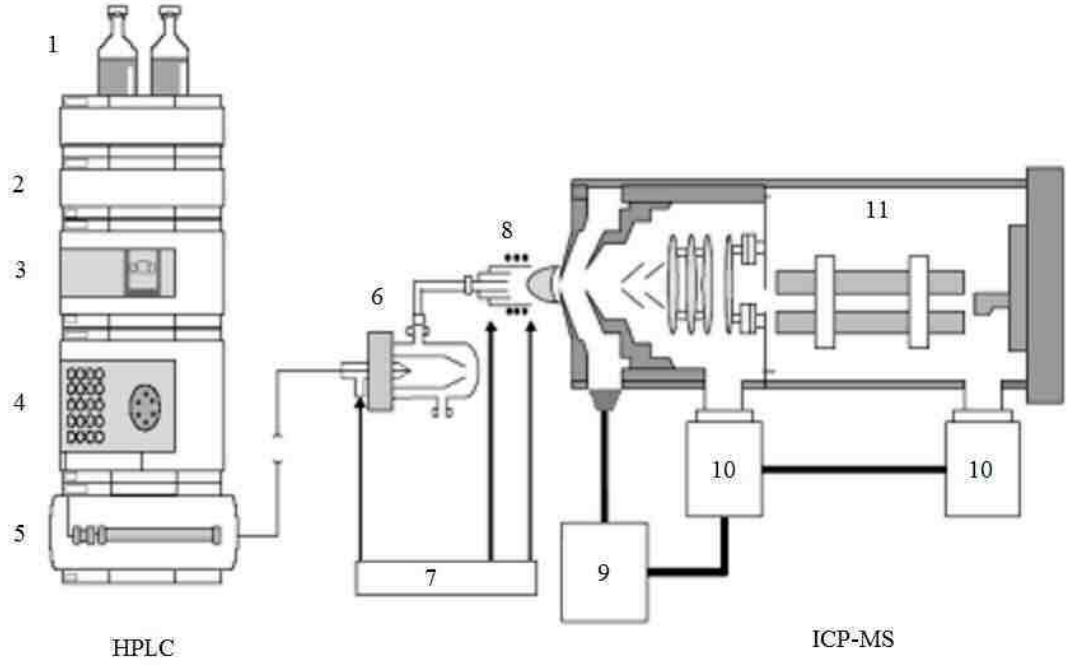


Fig. 3.1 - Scheme of used HPLC ICP-MS system[38] (1 - eluent bottles, 2 - degasser, 3 - pump, 4 - autosampler/injector, 5 - column, 6- nebulizer, 7 - gas controller, 8 - ICP torch, 9 - rotary pump, 10 - turbo pumps, 11 - quadrupole)

3.4 HPLC separation and ICP-MS detection

Tab. 3.2 Measurement conditions for HPLC

HPLC	
Column	Aeris widepore 3,6 µm XB-C18 100 Å, 250 x 2,10 mm (Phenomenex, USA)
Mobile phase A	2% ACN, 0,1% FA in DIW
Mobile phase B	80% ACN with 0,1% FA in DIW
Mobile phase flow	0,25 ml/min
Injected sample volumes	20µl, 1ul
Column compartment temperature	5 °C
Autosampler temperature	5 °C
Isocratic separations	
iso 1	Elution using 20% MP B over 20 minutes
iso 2	Elution using 15% MP B over 20 minutes
iso 3	Elution using 5% MP B over 10 minutes

Gradient separations

Grad1		**Grad2**		**Grad3**	
t (min)	**%B**	**t (min)**	**%B**	**t (min)**	**%B**
0	0	0	0	0	0
5	30	5	0	15	100
10	30	10	30	16	100
12	30	11	30	17	0
13	0	12	100	25	0
20	0	14	100		
		15	0		
		25	0		

Tab. 3.3 - Measurement conditions for ICP-MS

ICP - MS	
Internal standard	200 ppb Tellurium in 2% nitric acid
Diameter of plasma torch (for organic solvent)	1,50 mm
Carrier gas flow	0,60 l/min
Dilution gas flow	0,25 l/min
Collision gas flow	3,50 ml/min
Nebulizer pump	0.2 rps (~ 0.66 ml/min.)
The m/z signal	75 for As

3.5 Data processing

All of the measured signals were recorded, labeled and integrated by MassHunter Workstation software (Agilent Technologies). Collected data were sequentially exported to Microsoft Excel for further processing.

4. RESULTS AND DISCUSSION

4.1 Determination of the conditions for HPLC separation of the arsenic-glutathione complexes.

Several gradient and isocratic methods have been tested to achieve satisfactory separation of the complexes and relatively short time of the analysis. The experiments were carried out using DMAs(GS) complex. This particular complex was chosen because it elutes as a last one from the column. In the case of the gradient separations the analysis lasts from 20 to 25 minutes. The retention times of the DMAs(GS) are listed in Tab. 4.1. Particular separation modes that were used are described in Tab. 3.2 in previous chapter.

Tab 4.1 - Retention times of DMAs(GS) complexes based on particular conditions used

Gradient separation	t_R (min)
grad 1	12,7
grad 2	13,6
grad 3	13,6
Isocratic separation	**t_R (min)**
iso 1	2,6
iso 2	3,1
iso 3	7,6

All gradient elutions with increasing mobile phase B (Fig. 4.1) resulted in elution of the complex with retention times around 13th minute (Tab. 4.1). That is a reasonable time of the elution, but in this case the column needs to be conditioned for at least 10 minutes to be ready for the next analysis. This extends the time of the separation. During the isocratic separations the DMAs(GS) was eluted within 2,6 min, 3,1min and 7,6 min using 15%, 20% and 5% MP B respectively (Fig. 4.2). As the iso 3 method is finished within just 10 minutes, supposed to give very good separation of all complexes it has been chosen for further experiments. Additional advantages are no need for further conditioning of the column and the same influence of the mobile phase on ionization of all analytes in plasma.

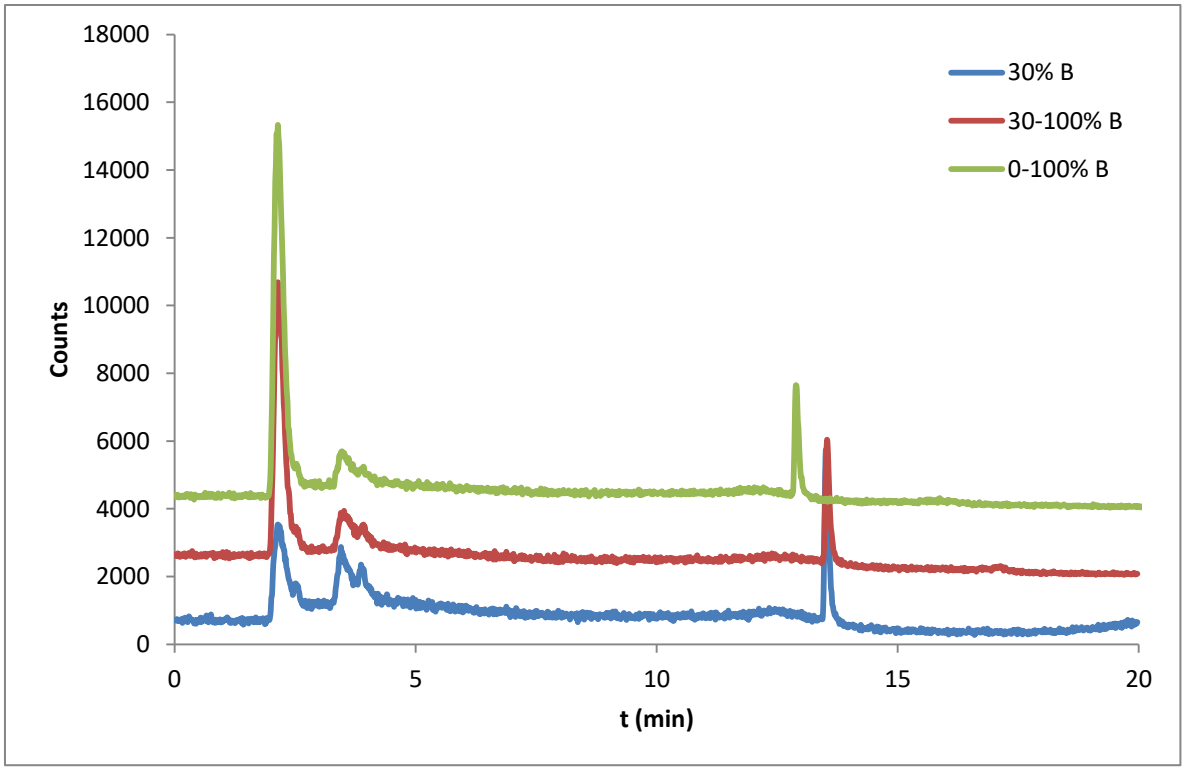


Fig. 4.1 - Separation of DMAs(GS) using: 25 min gradient elution with increasing MP B (80% ACN with 0,1% FA in DIW) from 0-30%, from 30-100% and from 0-100%

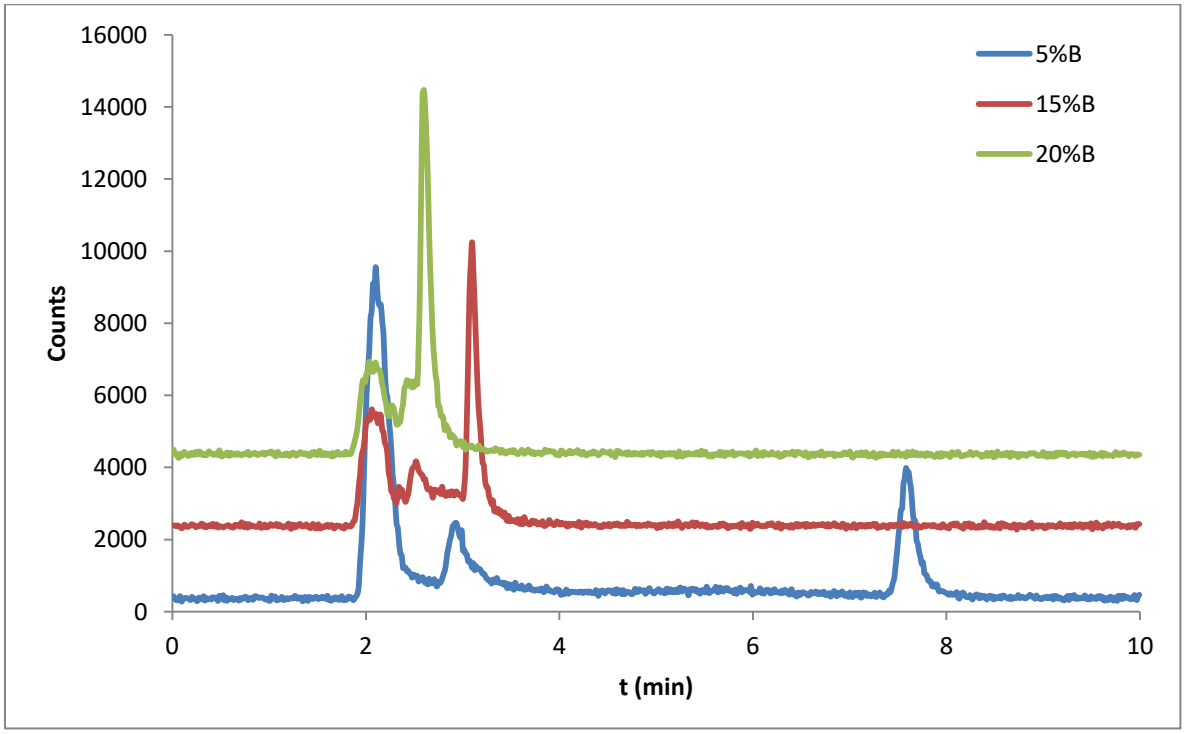


Fig. 4.2 - Separation of DMAs(GS) using: isocratic elution with 5%, 15% and 20% MP B (80% ACN with 0,1% FA in DIW)

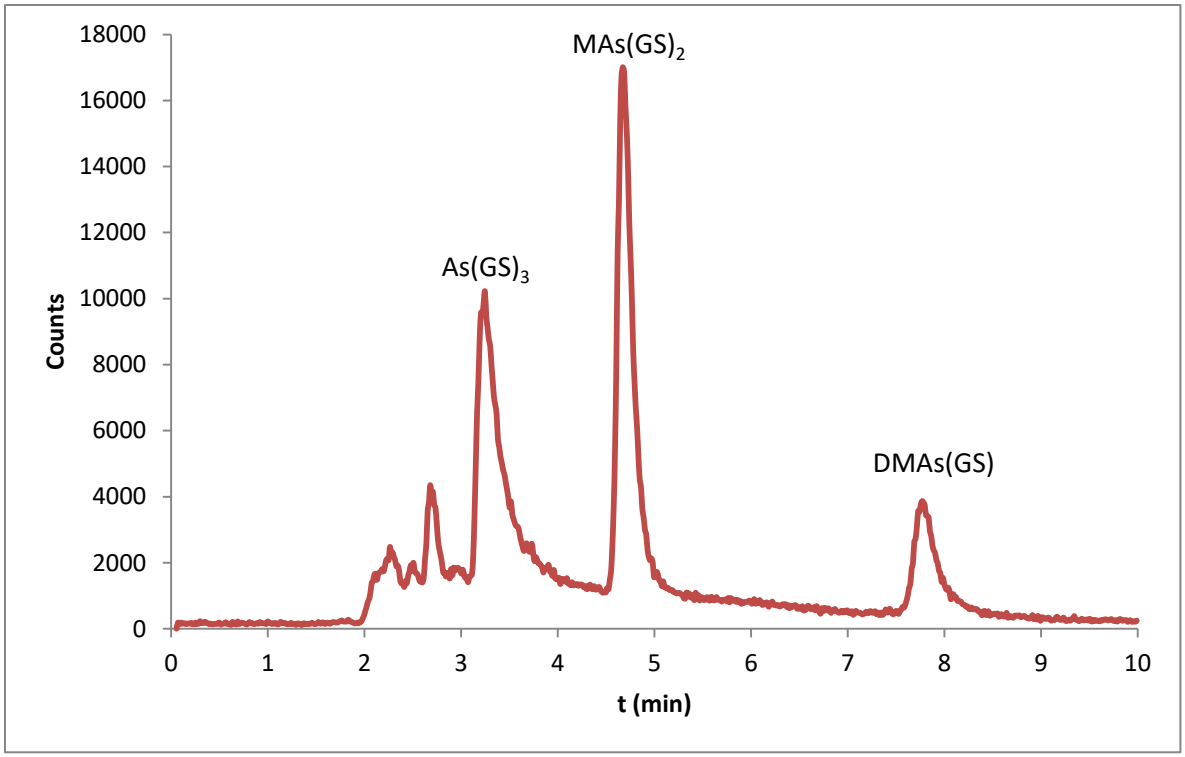


Fig. 4.3 - Chromatographic separation of arsenic-glutathione complexes after dilution to 20 ppb of the reaction mixture after 24 hours of synthesis

Fig 4.3 shows chromatogram of nicely separated arsenic-glutathione complexes, which were measured according to mode iso 3 (Tab 4.1 in previous chapter).

4.2 Synthesis of arsenic-glutathione complexes

The synthesis of the arsenic-glutathione complexes was carried out for 24 and 72 h at room temperature. A portion of the reaction mixture was taken after 1h, 2h, 3h, 4h, 24h and 72h diluted to 2 ppm, which was then diluted to 20 ppb of each arsenic-glutathione complex and immediately analyzed.

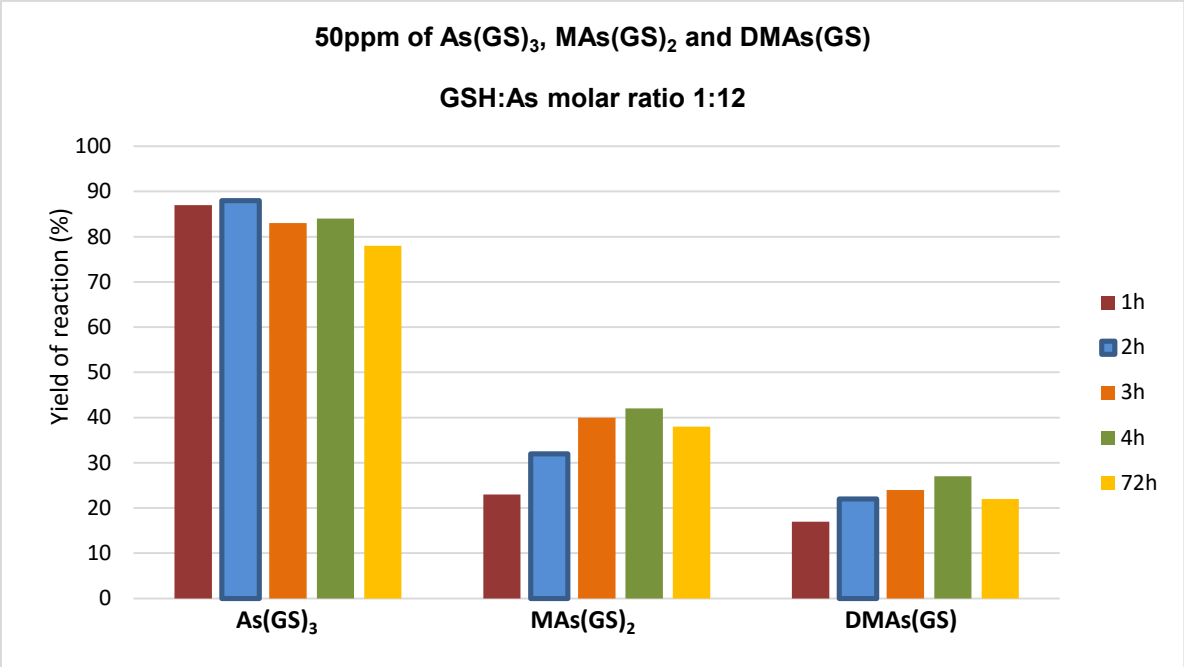
Because of very low stability of the complexes in the diluted solutions and not reported so far yields of the ionization in plasma several assumptions had to be made:

- Each arsenic-glutathione complex ionize the same way in plasma under the same conditions, therefore the same concentrations should result in the same counts in ICP- MS analysis.
- The integration of the whole chromatogram (1-10 min.) reflects the counts of the total arsenic content (300% =100% $As(GS)_3$ + 100% $MAs(GS)_2$ + 100% DMA(GS)) of the injected sample.

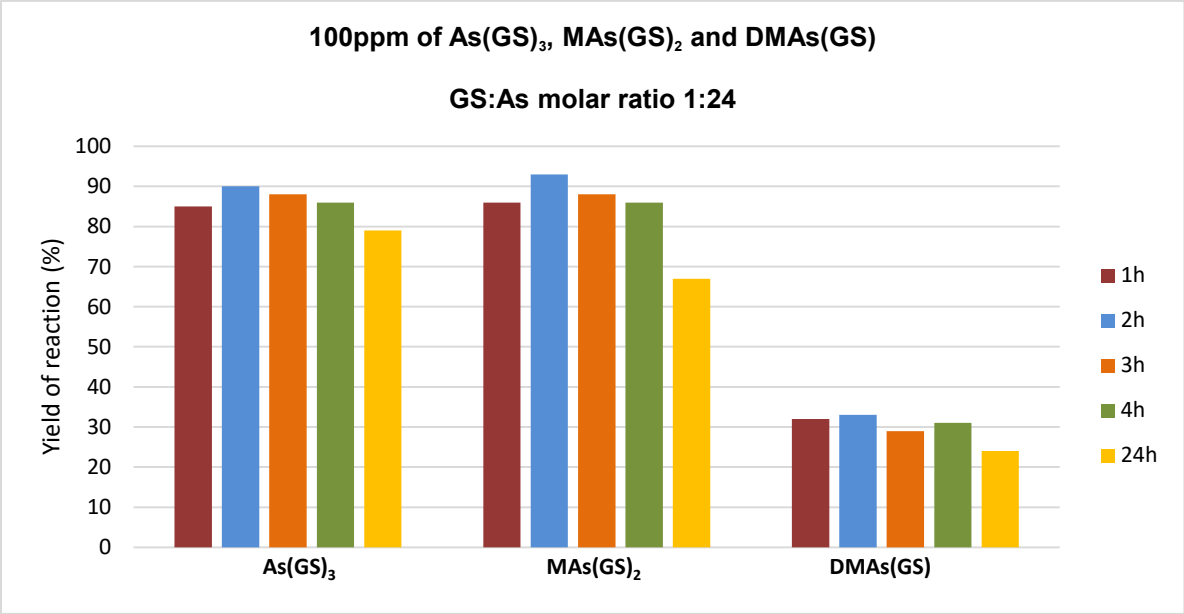
The yield of the reaction was calculated according to equitation:

% yield = counts of a single complex x 300% / total counts

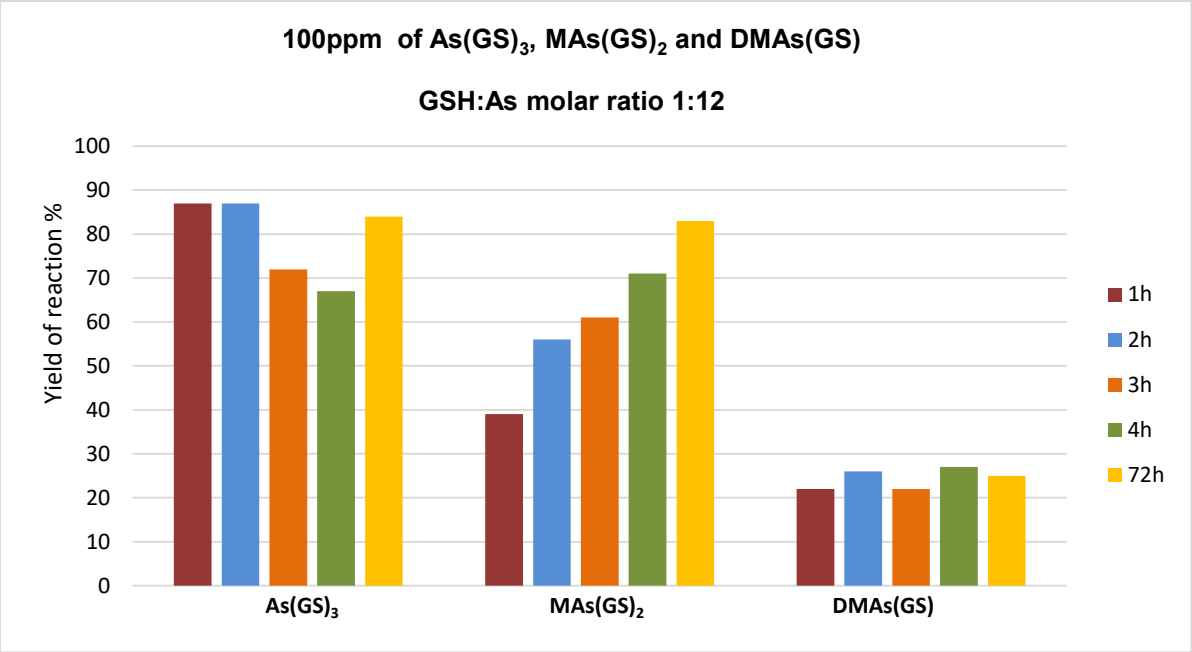
The calculated values of the yields were collected in graphs. Gathered results reveal the stability of the arsenic-glutathione complexes in time.



Graph 4.1 - Yield of the reaction gathered after 1h, 2h, 3h, 4h and 72h



Graph 4.2 - Yield of the reaction gathered after 1h, 2h, 3h, 4h and 24h



Graph 4.3 - Yield of the reaction gathered after 1h, 2h, 3h, 4h and 72h

As shown in graphs 4.1, 4.2 and 4.3, the yield of reaction for $As(GS)_3$ is still around 80% even after 72 hours. Yield of $MAs(GS)_2$ dramatically changes based on the molar ratio of GSH:As. Most satisfactory conditions for this complex were with molar ratio 1:24 (Graph 4.2). In case of DMAs(GS) the yield has not changed much and stayed between 18% - 30% during whole time and also when measured with different molar ratios of GSH:As. Results suggest that with conditions that were used for separation of this complex, it is unable to get better yield and that this complex decomposes really fast.

4.3 Stability of the arsenic-glutathione complexes at selected concentrations

Stability of arsenic-glutathione complexes is influenced by time, temperature and concentration. Graphs 4.1, 4.2 and 4.3 show, how particular complexes tend to undergo degradation during the short period of time. Arsenic-glutathione complexes were also analyzed after 3min, 13min, 23 min and 90 min (Fig. 4.4).

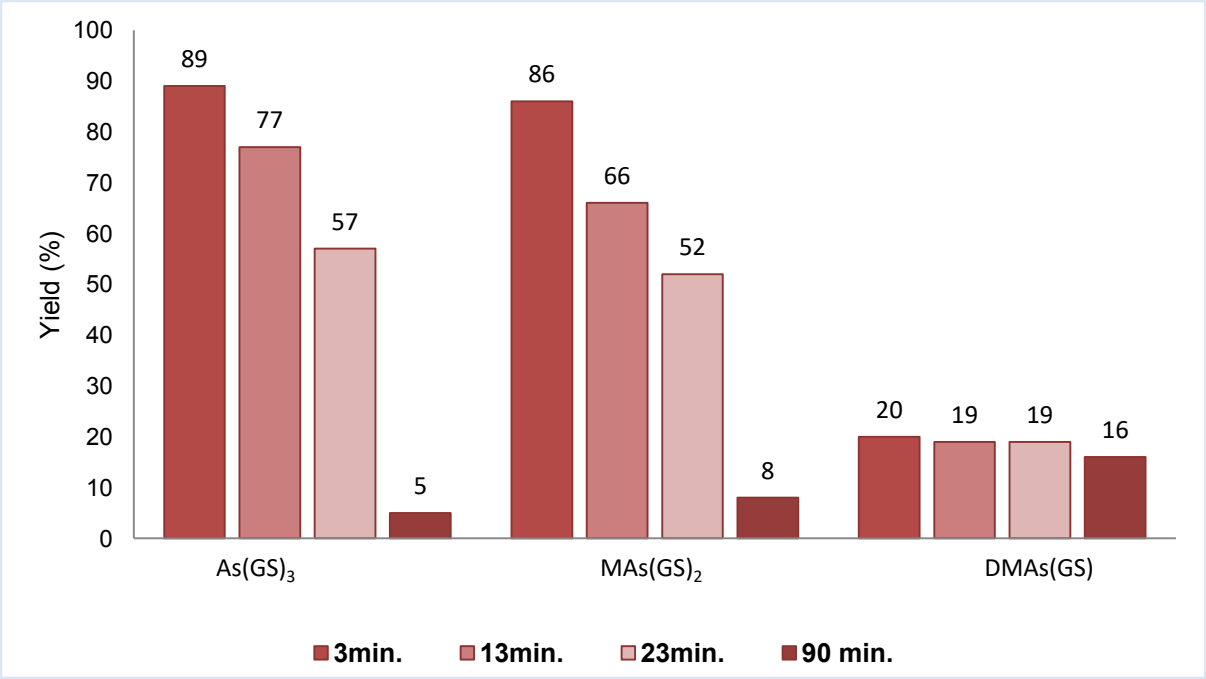


Fig 4.4 - The stability of the arsenic-glutathione complexes in the 20 ppb solution over 90 minutes at 5 °C. Separation was carried out using Aeris widepore 3,6 µm XB-C18 100 Å, 250 x 2,10 mm column

Stability of samples with concentration of 20 ppb was tested while kept in autosampler at 5 °C. Dramatic change in yield was observed for $As(GS)_3$ and $MAs(GS)_2$ complexes during 90 minutes of synthesis. As for DMAs(GS) the change was not radical and yield was around 19% during 90 minutes of synthesis.

Stability of samples with concentration of 2 ppm was also tested. They were kept in fridge overnight and measured after 24 hours. Signals from the chromatogram were significant. This suggests that samples with concentration of 2 ppm are good to store if they contain excess glutathione over arsenic.

Under the given conditions it is clear that more concentrated samples of arsenic-glutathione complexes are more stable and decompose at a later time than ones with lower concentration. The most diluted (20 ppb) solutions resulted in fast decomposition of the complexes.

4.4 Recovery of arsenic-glutathione complexes

Several samples were measured to check the recovery of arsenic-glutathione complexes without column. Total counts (1-10 minutes) after manual integration were used to calculate the recovery. Therefore the percentage may vary based on integration that was made particularly for every sample.

The recovery was calculated from following equation.

counts without column . . . . . 100% recovery
counts (1-10 minutes) . . . . . x % recovery

For analyzed samples, the recovery was between 78%-100%.

5. CONCLUSION

The aim of this work was to synthesize and separate arsenic-glutathione complexes: $As(GS)_3$, $MAs(GS)_2$ and DMAs(GS). Separation was carried out by reversed phase high performance liquid chromatography coupled with inductively coupled plasma mass spectrometry using Aeris widepore 3,6 µm XB-C18 precolumn and separation column 250 x 2.10 mm with ICP-MS spectometer 7700x.

Fast and effective conditions for separation of arsenic-glutathione complexes were established during a set of experiments. Compared to the results, where gradient mode was used, the isocratic mode applied here appeared to be more successful. It enabled to separate the complexes, shorten the retention times as well as customize length of the analysis to only 10 minutes.

Yield of the synthesis was tested with several GSH:As molar ratios (1:12, 1:24, 1:73) and As concentrations 50 ppm, 100 ppm and 150 ppm. Received results showed the highest yield of the reaction for $As(GS)_3$, $MAs(GS)_2$ synthesized in 100 ppm of iAs^{III}, MAs^{V} and $DMAs^{V}$ with the ratio GSH:As of 150 ppm. In all cases the yield of synthesis of DMAs(GS) was between 18-32%.

The results also suggest that the stability of the complexes is strongly dependent on the concentrations of the reagents. The experiments prove that samples of concentration at least 2 ppm (with the excess of GSH) still contain arsenic-glutathione and can be stored for 24 hours in the fridge.

6. BIBLIOGRAPHY

[1] Mandal B. K.,Suzuki K. T.,: Arsenic round the world: A review. *Talanta*, 2002, **58**, 201-235

[2] Flora S.J.S., Bhaduria S., Kannan G.M, Singh N., *Journal of Enironmental Biology*, 2007, **28**, 333-347

[3] Ding L., Saunders R.J., Drobná Z., et al.,*Toxicology and applied pharmacology*, 2012, **264**, 121-130

[4] Raab A., Meharg A. A., Jaspars M., Genneyb D. R., Feldmann J., *Anal. At. Spectrom*, 2004, **19**, 83

[5] FDA, *Food and Drug Administration: Arsanilic acid (21CFR558.62)*, 2008, 413–414

[6] IARC, *Monographs on the Evaluation of Carcinogenic Risks to Humans*, 2006, **86**, 1–294

[7] ATSDR: *Agency for Toxic Substances and Disease Registry*, 2007

[8] Bagla P., Kaiser J., *Science*, 1996, **274**,174

[9] Tchounwou P. B., Centeno J.A. and Patlolla A.K., *Mol. Cell. Biochem.,* 2004, **47**, 255

[10] Hostynek J. J., Hinz R. S., Lorence C. R., et al.: Metals and the skin. *Crit Rev Toxicol*, 1993, **23**,171–235

[11] Francesconi K. A., Kuehnelt D., *Analyst*, 2004, **129**, 373-395

[12] Vega L., Styblo M., Patterson R., Cullen W., Wang Ch., Germolec D., *Toxicology and Applied Pharmacology*, 2001, **172**, 225-232

[13] Mass M. J., Tennant A., Roop B. C., Cullen W. R., Stýblo M., Thomas D. J. and Klingerman A.D., *Chem. Res. Toxicol,* 2001, **163**, 355-361

[14] Petrick J. S., Ayala-Fierro F., Cullen, W. R., Carter D. E. and Vasken Aposhian H., *Toxicol. Appl. Phatmacol.,* 2000, **163**, 203-207

[15] Kojima C., Sakurai T., Waalkes M. P. and Himeno S., *Biol. Pharmac. Bull.,* 2005, **28**, 127-1832

[16] Yehiayan L., Pattabiraman M., Kavallieratos K., Wang X., Boise L. H., Cai Y., *J. Anal. At. Specrtom.,* 2009, **24**, 1397-1405

[17] Kala S. V., Nely M. W., Kala G., Prater Ch. I., Atwood D. W., Rice J. s., Liebarman M.W., *The Journal of*

biological chemistry, 2000, **43**, 33404-33408

[18] Pompella A. et al. / *Biochemical Pharmacology*, 2003, **66**,1499–1503

[19] Ziglari T., Allameh A., *The Significance of Glutathione Conjugation in Aflatoxin Metabolism*, 2013, **13**, 270

[20] Challenger F., *Chem. Rev.*, 1945, **36**, 315–361

[21] Sun, H.: *Biological chemismy of Arsenic, Antimony and Bismuth*. Chichester, West Sussex, 2011

[22] Hayakawa T.; Kobayashi Y.; Cui X., *Arch Toxicol*, 2005, **79**, 183-191

[23] Burford N., Carpenter Y., Conrad E., and Saunders C. D. L., *Biological Chemistry of Arsenic, Antimony and Bismuth*, 2011, 153

[24] Rehman, K.; Naranmandura, H.: Arsenic metabolism and thioarsenicals. *Metallomics*, 2012, **4**, 881-892

[25] Currier J.M.; Saunders J.; Ding L.; Bodnar W.; Cable P.; Matoušek T.; Creed J.T.; Stýblo M., *Journal of Analytical Atomic Spectrometry*, 2013, **28**, 843-852

[26] Xie R.; Johnson W.; Spayd S.; Hall G.S.; Buckley B., *Analytica chimica acta*,2006, **578**, 186-194

[27] B´Hymer, C.; Caruso, J.A., *Journal of Chromatography A, 2004*, **1045**, 1-13

[28] Szpunar, J., *The Analyst*, 2000, **125**, 963 -988

[29] Morin P., Amran M. B., Lakkis M. D., Leroy M. J. F. *Chromatographia*, 1992, **33**, 584

[30] Cecchi T., *Critical Reviews in Analytical Chemistry*, 2008, **38**, 161-213

[31] AgilentTechnologies. USA., *ICP-MS Inductively Coupled Plasma Mass Spectrometry A primer*, 2005. Publication Number 5989- 3526EN

[32] Ebdon L., Hill S., Walton A. P., Ward R. W., *Analyst*, 1988, **113** ,1159

[33] Hansen S. H., Larsen E. H., Pritzl G., Cornett C., *J. Anal. At. Spectrom*.,1992, **7**,629

[34] Zhang X., Cornelis R., De Kimpe J., Mees L., J. *Anal. At. Spectrom.*, 1996, **11** 1075

[35] Musil, S.; Matoušek, T., *Spectrochimica Acta Part B*, 2008, **63**, 396 – 406 41

[36] Szpunar, J.; Lobinski, R.: *Hyphenated techniques in speciation analysis*. The Royal society of chemismy, Cambridge, 2003

[37] Gómez-Ariza J. L., Sánchez-Rodas D., Giráldez I., Morales E., *Talanta*, 2000, **51**, 257-268

[38] EVISA, *The European Virtual Institute for Speciation Analysis,* 2007

[39] Scott N., Hatlelid K.M., MacKenzie N. E., Carter D. E., *Chem. Res. Toxikol.,* 1993, **6**, 102-106