

University of Crete
Department of Chemistry



Non-targeted arsenic speciation in peptides using ultra-high resolution Mass Spectrometry.

Lefkothea Trasanidou

Supervisor: S.A. Pergantis

Heraklion 2015

Contents

Abbreviations	3
Acknowledgements	4
Abstract	5
1. Introduction	7
1.1 The Arsenic element	7
1.2 Analytical methods for arsenic detection – Mass spectrometry techniques	8
1.3 Scope of thesis	14
2. Materials & Methods	15
2.1 Chemicals	16
2.2 Instrumentation	16
2.3 Other laboratory equipment	16
2.4 Synthesis of As-GSH peptides	16
2.5 Sample preparation for electrospray (ESI-MS) and sonic spray (SSI-MS) mass spectrometry	19
2.6 Electrospray mass spectrometry of As-GSH complex (DMAG)	19
2.7 Electrospray mass spectrometry tandem mass spectrometry	21
2.8 Sonic spray ionization mass spectrometry of As-GSH complexes	22
2.9 Sonic spray mass spectrometry tandem mass spectrometry (SSI-MS/MS) of As-GSH complexes	25

2.10 Matrix-assisted laser desorption/ionization (MALDI- FT-ICR-MS) of As-GSH complexes.....	25
2.11 Extraction of polar arsenicals from the seaweed “hijiki”	27
2.12 Spiking As-GSH complexes into biological extracts.....	27
2.13 High pressure liquid chromatography -electrospray ionization-Fourier transform-ion cyclotron resonance mass spectrometry (HPLC-ESI-FT-ICR-MS) of As-GSH complexes, after spiking into the seaweed.....	28
3. Results and discussion.....	31
3.1 Mass spectrometry & tandem mass spectrometry: mass spectra of As-GSH complexes.....	33
3.2 ESI-MS spectra of DMAG.....	33
3.3 ESI-MS/MS spectrum of DMAG.....	34
3.4 SSI-MS spectra of DMAG, MADG and ATG.....	37
3.5 SSI-MS/MS spectra of DMAG, MADG and ATG.....	40
3.6 MALDI-FT-ICR-MS of DMAG, MADG and ATG.....	47
3.7 HPLC coupled with mass spectrometry: chromatograms & mass spectra of As-GSH complexes (in biological samples).....	51
3.8 HPLC-FT-ICR-MS of DMAG-extract using gradient elution.....	51
3.9 HPLC-FT-ICR-MS of MADG-extract using gradient elution.....	52
3.10 HPLC-FT-ICR-MS of ATG-extract using gradient elution.....	53
3.11 HPLC-FT-ICR-MS of As-GSH mix using gradient elution.....	54
4. Conclusions	56
5. References	58

Abbreviations

A

ATG **Arsenite glutathione**
ACN **Acetonitrile**

C

CHCA **α -cyano-4-hydroxycinnamic acid**

D

DMAG **Dimethylarsinic glutathione**

E

ESI **Electrospray ionization**

F

FT-ICR **Fourier transform ion cyclotron resonance**

G

GSH **Glutathione**
GSSG **Oxidized glutathione**

H

HPLC **High pressure liquid chromatography**

M

MADG **Methylarsonate glutathione**
MALDI **Matrix-assisted laser desorption/ionization**
MS **Mass spectrometry**
MS/MS **Tandem mass spectrometry**

Q

QIT **Quadrupole ion trap**

S

SSI **Sonic spray ionization**
STFA **Sodium trifluoroacetate**

Acknowledgements

I would like to thank my supervisor, Professor Spyros A. Pergantis, for his help and enduring guidance throughout this thesis project, for the support and trust he showed me and the opportunity he gave me to visit UK and work in the University of York.

In addition, I would like to thank Professor Jane Thomas-Oates from the University of York, for her approval to host me in her lovely group and her lab for three months, for her guidance and great kindness during my visit. I would also wish to thank Dr Ed Bergstrom for the education he gave me in new techniques and his valuable guidance in the mass spectrometry lab. It was a great honor and pleasure to have worked with you all.



European Union
European Social Fund



MINISTRY OF EDUCATION, LIFELONG LEARNING AND RELIGIOUS AFFAIRS
MANAGING AUTHORITY

Co- financed by Greece and the European Union



EUROPEAN SOCIAL FUND

Abstract

The scope of this thesis was, to study the interaction of biomolecules and specifically peptides, with arsenic (As), which requires the development of analytical methods for the analysis of trivalent arsenic existing in As-glutathione (As-GSH) complexes without the need for extended sample preparation, using very high resolution mass spectrometry and with low limits of detection. Method optimization was carried out by using a series of arsenic-containing peptides, used as standard compounds. More specifically, a synthetically prepared series of As-complexes, including (α-glutamyl-cysteinyl-glycyl) dimethyl-thio-arsinite or dimethylarsinic glutathione (DMAG), di(glutamyl-cysteinyl-glycyl)methyl- dithio-arsonite or methylarsonate glutathione (MADG), and tri(glutamyl-cysteinyl-glycyl)trithio-arsenite or arsenite glutathione (ATG), were used. To analyze these compounds mass spectrometry with various ionization methods was developed. Electrospray (ESI-MS) and sonic spray ionization mass spectrometry (SSI-MS) as well as tandem mass spectrometry (MS/MS) were used, operated in the selected reaction monitoring (SRM mode), in order to make mass measurements and structural configuration predictions. Electrospray ionization -Fourier transform-ion cyclotron resonance mass spectrometry (ESI- FT-ICR-MS) and also matrix-assisted laser desorption/ionization (MALDI) FT-ICR-MS were used to make accurate mass measurements. Analyte samples were analyzed directly from solution, and also eluting from an HPLC column (Hypersil C18), prior to their detection using their accurate m/z values. The three As-GSH compounds were analyzed using HPLC -ESI-FT-ICR-MS both in aqueous solutions and after spiking into biological extracts, specifically in the seaweed (hijiki) extracts.

Τίτλος εργασίας : “Μη στοχευμένη ειδοταυτοποίηση αρσενικού σε πεπτίδια, με χρήση φασματομετρίας μάζας υψηλής διαχωριστικής ισχύος”.

Περίληψη

Στόχος της παρούσας εργασίας ήταν η μελέτη της αλληλεπίδρασης βιομορίων και συγκεκριμένα πεπτιδίων με το αρσενικό (As), με την ανάπτυξη αναλυτικών μεθόδων για την ανάλυση του τρισθενούς αρσενικού που υπάρχει σε σύμπλοκα As-γλουταθειόνης (As-GSH). Χωρίς ιδιαίτερη παρέμβαση στην προετοιμασία των δειγμάτων, χρησιμοποιώντας φασματομετρία μάζας υψηλής διαχωριστικής ισχύος και εξασφαλίζοντας χαμηλά όρια ανίχνευσης. Η εν λόγω μέθοδος πραγματοποιήθηκε με τη χρήση μιας σειράς από αρσενοπεπτίδια τα οποία χρησιμοποιήθηκαν ως πρότυπες ενώσεις. Πιο συγκεκριμένα, χρησιμοποιήθηκε μια σειρά από συνθετικά σύμπλοκα αρσενικού, συμπεριλαμβανομένων των dimethylarsinic glytathione (DMAG), methylarsonate glutathione (MADG) και arsenite glutathione (ATG). Για την ανάλυση των παραπάνω ενώσεων χρησιμοποιήθηκε φασματομετρία μάζας με εφαρμογή διαφόρων μεθόδων ιοντισμού. Φασματομετρία μάζας με χρήση ηλεκτροψεκασμού (ESI-MS) αλλά και ιοντισμού υπερηχητικής εκνέφωσης (SSI-MS), καθώς επίσης και διαδοχική φασματομετρία μάζας (MS/MS) εφαρμόστηκαν προκειμένου να επιτευχθούν ακριβείς μετρήσεις μάζας και προβλέψεις των δομικών χαρακτηριστικών των ενώσεων. Έγινε χρήση φασματομετρίας μάζας συντονισμού ιόντος κυκλοτρονίου μέσω μετασχηματισμού Fourier (FT-ICR-MS), εφαρμόζοντας τόσο τον ηλεκτροψεκασμό (ESI-FT-ICR-MS) όσο και την μέθοδο εκρόφησης με λέιζερ υποβοηθούμενο από μήτρα (MALDI) FT-ICR-MS, ως μέθοδο ιοντισμού. Η ανάλυση των δειγμάτων πραγματοποιήθηκε απ ευθείας από το διάλυμα καθώς επίσης και μετά από έκλουση από χρωματογραφική στήλη HPLC (Hypersil C18). Τέλος, οι τρεις ενώσεις As-GSH αναλύθηκαν με τη χρήση HPLC -ESI-FT-ICR-MS τόσο σε υδατικά διαλύματα, όσο και μετά από τον εμποτισμό τους σε βιολογικά εκχυλίσματα, συγκεκριμένα σε εκχύλισμα φυκιού “hijiki”.

1. Introduction

1.1 The Arsenic element

Arsenic is the chemical element [1,2] with As symbol, widely spread in nature, with a metallic grey crystal (hexagonal, rhombic) appearance. It occurs in two allotropic forms (forms of an element with different physical and chemical properties). The most common form of arsenic is a shiny, gray, brittle, metallic-looking solid, but there is also the yellow crystalline solid form. Arsenic is a metalloid, as it presents both metallic and non-metallic properties and it belongs to 15th group, p-block and 4th period of the periodic table of elements. Its atomic number is 33, with electron configuration; [Ar] 3d¹⁰4s²4p³, while its relative atomic mass is 74.923 amu. The oxidation states of the element are ± 3 , +5 and ⁷⁵As is its only naturally occurring stable isotope. Below, there is a table of the physical properties of arsenic [3].



Table 1: Physical properties of As

Physical properties	
Molecular Weight	74.92
Valence	3, 5
Boiling Point	612 °C
Melting Point (at 28 atm)	817.0 °C
Vapor Pressure (at 372 °C)	1.0 mm Hg
Density/Specific Gravity (at 14 °C)	5.727

Arsenic is found in the natural environment [4] in some abundance in the Earth's crust and in small quantities in rock, soil, water and air. Humans are exposed mainly through food and water. Food is usually the largest source except in areas where drinking water is naturally contaminated with arsenic.

Inorganic arsenic is found in groundwater and used as drinking-water in several parts of the world.

Organic arsenic compounds, such as arsenobetaine, arsenocholine, tetramethylarsonium salts, arsenosugars and arsenic-containing lipids are mainly found in marine organisms although some of these compounds have also been found in terrestrial organisms

1.2 Analytical methods for arsenic detection – Mass spectrometry techniques

1.2.1. Mass spectrometry (MS) general principles

Mass spectrometry is certainly one of the most powerful tools in the modern analytical laboratory. It is a technique suitable for producing charged gas phase molecules (ions) that originate from a liquid sample, and then separate them, based on their m/z value.

A typical mass spectrometer consists of the following parts; An **inlet system** to introduce the sample into the mass spectrometer, the **ion source** where ions in the gas phase are generated from the sample, the **mass analyzer** where the gaseous ions are separated based on their mass to charge m/z values, and finally the **detector** (usually electron multiplier), which produces a signal proportional to the relative abundance of each ion. There is always a **data system** used to process the mass spectrum provided. A mass spectrum displays the m/z ratio on the x-axis and the relative abundance on the y-axis. It has to be noticed that, all mass analyzers operate under high vacuum in order to minimize collisions between ions and air molecules. Without a high vacuum, the ions produced in the source will not reach the detector.

Tandem mass spectrometry (MS/MS) [5] is a really useful method involving at least two stages of mass analysis. In the most common tandem mass spectrometry experiment a first analyzer is used to isolate a precursor ion, which then undergoes a fragmentation to yield product ions and neutral fragments. Then, a second spectrometer analyses the product ions. It is also possible to increase the number of steps: select ions of a first mass, then select ions of a second mass from the obtained fragments, and finally analyze the fragments of these last selected ions. The number of steps can be increased to yield **MSⁿ** experiments, where n refers to the number of generations of ions being analyzed. MS/MS offers the opportunity to obtain detailed information concerning the compound structure.

Selected reaction monitoring (SRM) mode, used extensively in this study, is a method used in MS/MS where an ion of a particular mass is selected in the first stage of a tandem mass spectrometer and an ion product of a fragmentation reaction of the precursor ion is selected in the second mass spectrometer stage for detection. SRM plots are very simple, usually containing only a single peak for each analyte. This characteristic makes the SRM plot ideal for sensitive and specific quantitation [5, 6].

1.2.2. Ionization methods

The ionization methods used in this study were: Electrospray, Sonic spray and MALDI. The purpose of all these three methods is to ionize the sample prior to analysis in the mass spectrometer.

Electrospray ionization method (ESI), [5,7] is a soft ionization technique as barely no fragmentation of the analyte ions occurs prior to its introduction to the mass spectrometer [Figure 1]. It is produced by applying a strong electric field, under

atmospheric pressure, to a liquid passing through a capillary tube at a low $\mu\text{l}/\text{min}$ flow rate. An electric field induces a charge accumulation at the liquid surface at the end of the capillary, which will break to form charged droplets. A gas injected coaxially at a low flow rate allows the dispersion of the spray to be limited in space. These droplets then pass through a heated capillary to remove the last solvent molecules. When the surface tension of the solvent is broken, the shape of the drop changes to a 'Taylor cone' and the spray appears. The solvent contained in the droplets evaporates, which causes them to shrink. Under the influence of the strong electric field, deformation of the droplet occurs. The droplet elongates under the force resulting from the accumulation of charge, similarly to what occurred at the probe tip, and finally produces a new Taylor cone. These charged droplets will continue to lose solvent, and when the electric field on their surface becomes large enough, desorption of ions from the surface occurs.

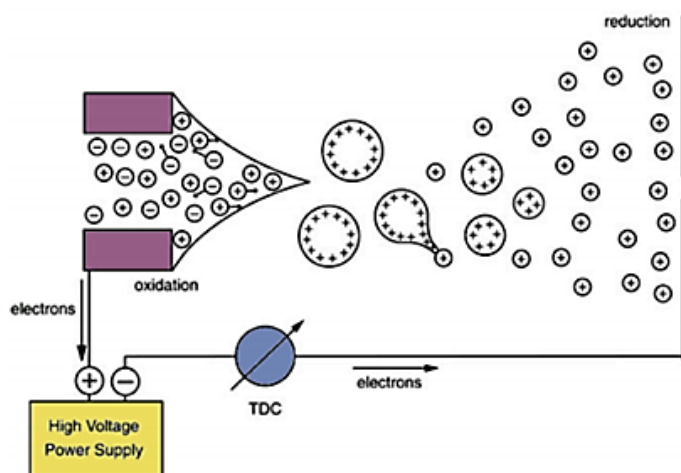


Figure 1 : Electrospray ionization technique [7]

Sonic spray ionization (SSI), is an ambient ionization method for mass spectrometric analysis of different compounds. This method does not require any voltage application. It is relying solely on the pressure of the coaxial nebulizer gas (nitrogen) to induce ionization. SSI was introduced for the first time by Hirabayashi et al. in 1994 [8]. They observed the highest ion intensity when the gas flow reaches to the sonic speed, thus they named this source as sonic spray ionization. However, the

ionization procedure is not yet completely understood. The ion source in SSI consists of pneumatic concentric nebulizer. The nebulizer includes an inner fused-silica capillary, where the gas (N_2) flows, whereas the edge of the nebulizer is just before the mass spectrometer. The sample uptake and spraying is based on the *Venturi effect*, which causes the pressure reduction at the tip of the nebulizer [9].

In **Matrix Assisted Laser Desorption ionization (MALDI)** [Figure 2], there is a mixture of two different substances: the matrix and the analyte. The **matrix** is a substance that surrounds the analyte and assists in the ionization process. The matrix and the analyte are mixed together. The mixture is then added in μL volume, to a metal plate. The solvent is allowed to evaporate for a couple of minutes, and the matrix co-crystallizes with the analyte. The metal plate is inserted in the instrument. A laser is shot at the sample, and ionization of the analyte is promoted. The ions travel through the instrument, and a mass spectrum is recorded. There are a variety of different types of matrix molecules one of the most common matrices is α -cyano-4-hydroxycinnamic acid which was used in this study [5,10].

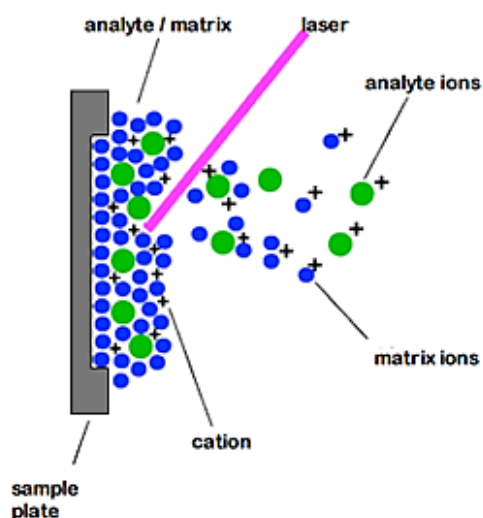


Figure 2 : MALDI ionization technique [7]

1.2.3. Mass analyzers

Two types of mass analyzers were used in this research; the quadrupole ion trap and the FT-ICR analyzer cell [5]. The main characteristics of both of these analyzers are their upper mass limit, their transmission and their resolution.

Quadrupole ion trap (QIT) mass analyzer [5,10,11] [Figure 3], is made up of a circular electrode, with two spherical caps on the top. Conceptually, an ion trap can be imagined as a quadrupole bent on itself in order to form a closed loop. The inner rod is reduced to a point, the outer rod is the circular electrode and the top and bottom rods make up the caps. That array gives a kind of a 3D quadrupole in which the ions of all masses are trapped on a specific trajectory. Application of a resonant frequency expels the ions of a given mass. Ions with different masses are present together inside the trap, and are expelled according to their masses, finally resulting in a mass spectrum.

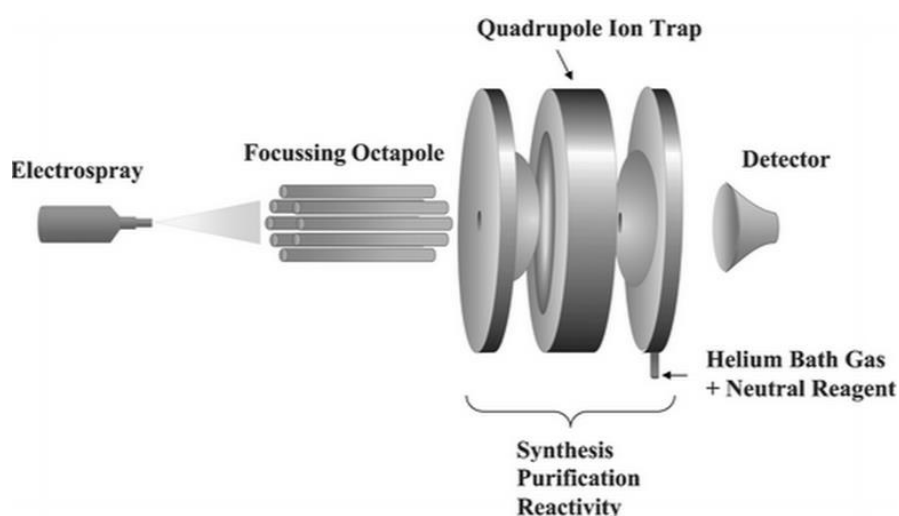


Figure 3 : Quadrupole Ion Trap mass analyzer [12]

1.2.4 Ion Cyclotron Resonance and Fourier Transform Mass Spectrometry

Fourier Transform-Ion Cyclotron Resonance (FT-ICR) [Figure 4], mass spectrometry was first described in the late 1940s by Hipple, Sommer, and Thomas, but it was not until Alan Marshall and Melvin Comisarow invented the FT-ICR mass spectrometer in 1974 [13] that the technique could be used to detect multiple ions simultaneously. For the operation of FTICR-MS, the system is equipped with three main components: a **superconductive magnet**, an **ultrahigh vacuum system**, and an **analyzer cell**. The heart of FT-ICR-MS is its analyzer cell where, cyclotron instruments trap ions electrostatically in a cell using a constant magnetic field. Pulses of RF voltage create orbital ionic motion, and the orbiting ions generate a small signal at the detection plates of the cell (the ion's orbital frequency). The frequency is inversely related to the ions' m/z , and the signal intensity is proportional to the number of ions of the same m/z in the cell. At very low cell pressures, a cyclotron instrument can maintain an ion's orbit for extended periods providing very high resolution measurements [14, 15].

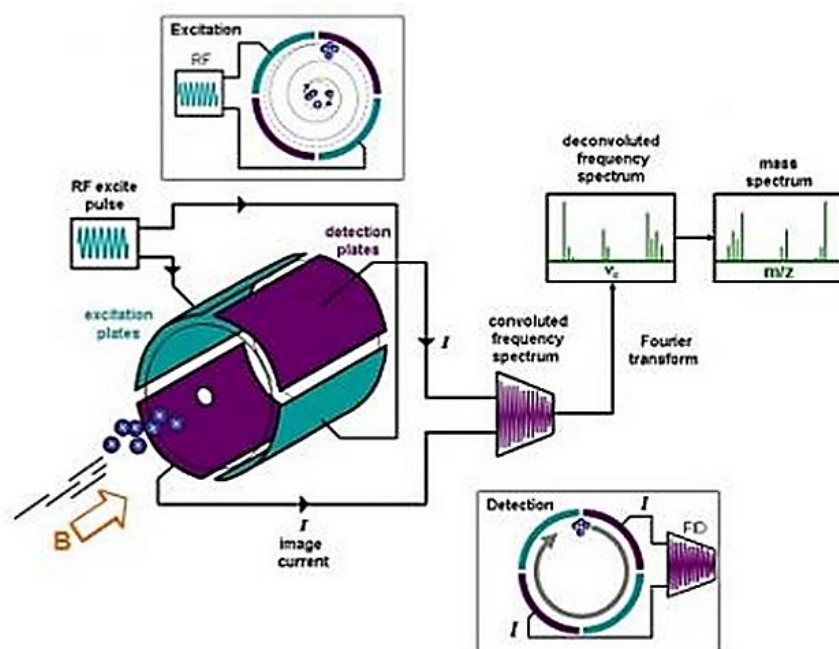


Figure 4 : FT-ICR-MS instrumental configuration [16]

1.3 Scope of thesis

It is known from previous studies that inorganic arsenic species which are considered as human carcinogens are transformed in the human's body by repetitive reduction and oxidative methylation reactions, and they finally form organoarsenic species [17]. Arsenic is an element which has a strong affinity for sulfur groups. These groups (-SH) appear in compounds such as glutathione, the most abundant thiol in cells. According to previously published research [17],[18], arsenic-glutathione complexes play a really important role in inorganic arsenic's metabolic procedure.

The main purpose of this thesis is, to study the interaction of peptides, with both known and unknown arsenic species. The objectives are to develop analytical methods for the non-targeted analysis of trivalent arsenic existing in As-glutathione (As-GSH) complexes without important intervention to the sample, using very high resolution mass spectrometry and with low limits of detection. These objectives will be accomplished following specific steps. In the first place, laboratory synthetic procedures have been carried out, for preparing different As-GSH complexes which will be used as standard compounds. Secondly, mass spectrometric methods will be applied, using and evaluating different ionization techniques. Furthermore, tandem mass spectrometry (MS/MS) will be applied, operated in the tandem MS mode, in order to obtain structural information and study fragmentation pathways for each compound. High resolution mass spectrometry will also be evaluated in this study, with and without coupling with liquid chromatography, to make accurate and precise mass measurements. The synthetically prepared standards will finally be spiked into biological extracts in order to be analyzed not only in aqueous but also in biological samples.

2. Materials and methods

As it was mentioned previously in the introduction section, the purpose of the present study was to detect the arsenic in synthetically prepared arseno-peptides, using ultra-high resolution mass spectrometry, and also to detect the interaction between biomolecules (peptides) with As. Sonic Spray and Electrospray Ionization coupled with mass spectrometry were used, in order to make accurate mass measurements. The synthesis of As-GSH complexes as well as the analysis of these complexes with SSI and ESI-MS took place in the University of Crete under the supervision of Professor Spiros A. Pergantis. These complexes were transferred to the University of York, in order to be further analyzed using HPLC-ESI-FTICR-MS and MALDI-FT-ICR-MS under the supervision of Professor Jane Thomas-Oates and Dr Ed Bergstrom. The analysis included the speciation of As-GSH both in aqueous solutions and after spiking into biological extracts.

2.1 Chemicals

Sodium arsenite (NaAsO_2) was purchased from BDH Ltd., Poole, United Kingdom. Monosodium acid methane arsonate sesquihydrate (purity 99%) was purchased from Supelco (Bellefonte, PA). Cacodylic acid ($\text{C}_2\text{H}_7\text{AsO}_2$, puriss. $\geq 99\%$), L-glutathione reduced ($\text{C}_{10}\text{H}_{17}\text{N}_3\text{O}_6\text{S}$, puriss. $\geq 97\%$) and formic acid (CH_2O_2 , puriss. p.a. ACS; 88%–91%) were purchased from Fluka Chemika, Buchs, Switzerland. Methanol (CH_3OH) was purchased from Sigma (Buchs, Switzerland). CHCA (α -cyano-4-hydroxycinnamic acid) matrix for MALDI, Trifluoroacetic acid ($\text{CF}_3\text{CO}_2\text{H}$, purity 99%), acetonitrile (CH_3CN) and sodium formate solution, (10 mM NaOH in isopropanol/water 1:1 (+0.2% HCOOH), were provided by prof. Jane Thomas-Oates, University of York, UK.

2.2 Instrumentation

- LCQ Advantage Ion Trap Mass Spectrometer with Xcalibur 1.3 software, *Thermo Finnigan*
- Solarix XR FTMS 9.4T with Compass software, *Bruker Daltonics*
- Agilent 1200 High Pressure Liquid Chromatography System with *HyStar* software
- Column BDS HYPERSIL C18, Diameter 150 X 2.1mm, Particle size 5 μ *Thermo Hypersil-Keystone*
- Helium and Nitrogen Gas bottles
- Centrifuge MiniSpin Plus, *Eppendorf*
- Sonic bath, *Elmasonic*
- Analytical balance, resolution: 0.1 mg, linearity: ± 0.2 mg, Kern ABT *Sigma Aldrich*

2.3 Other laboratory equipment

- Flasks
- Magnetic stirrer
- Magnet
- Balloon (used for argon atmosphere)
- Paster & Gilson Pipettes
- Eppendorf tubes
- HPLC vials, closures and inserts, *Agilent*
- Ground steel target for MALDI, *AnchorChip™*

2.4 Synthesis of As-GSH peptides

The synthesis of the three arsenic-glutathione complexes (DMAG, MADG and ATG) was accomplished based on previously published procedures [18].

The exact synthesis procedure is stated below:

Dimethylarsinic glutathione (DMAG): cacodylic acid (0.035g; 0.25mmol) and GSH (0.23g; 0.75mmol) were first mixed in a flask. Then, 2.5 ml of degassed deionized water were added, and the mixture was left at room temperature overnight under an argon atmosphere, with continuous stirring, in order to be dissolved. At the end of the reaction, the product was shared equally into 10 eppendorfs and each one's content was precipitated with 1ml methanol. The precipitate was centrifuged, and the supernatant was removed. Finally, the residue was dried under nitrogen and kept in the fridge (-4°C), until analysis.

Methylarsonate glutathione (MADG): monosodium acid methane arsonate (0.059g; 0.36mmol) and GSH (0.5g; 1.63 mmol) were first mixed in a flask. Then, 2ml of degassed deionized water were added, and the mixture was left at room temperature overnight under an argon atmosphere, with continuous stirring, in order to be dissolved. The rest of the procedure was carried out exactly as described in the previous As-GSH complex.

Arsenite glutathione (ATG): sodium arsenite (0.037 g; 0.28 mmol) and GSH reduced (0.26 g; 0.85 mmol) were mixed in a flask. Then, 2.5 ml of degassed deionized water was added and the mixture was left at room temperature overnight under an argon atmosphere, with continuous stirring, in order to be dissolved. The rest of the procedure was carried out exactly as described in the previous As-GSH complexes.

All the three As-GSH residues were kept in the fridge (-4°C) to avoid decomposition.

L- Glutathione reduced

L- Glutathione reduced (GSH)	
Molecular Formula	C ₁₀ H ₁₇ N ₃ O ₆ S
Monoisotopic mass	307.083801 Da

L-Glutathione reduced [19,20,21] or γ -L-Glutamyl-L-cysteinyl-glycine (**GSH**) is a tripeptide which derives from binding of L-glutamic acid, with glycine and L-cysteine. It is one of the most abundant thiol compounds found in cells and plays a major role in the formation of disulfide bonds in proteins in the endoplasmic reticulum. Glutathione exists in both reduced (GSH) and oxidized (**GSSG**) states [Figure 1]. Oxidized glutathione (GSSG) functions as an oxidant in the formation of disulfide bonds in proteins and reduced glutathione (GSH) functions as a reducing agent that cleaves misbridged disulfide bonds in proteins, resulting in the formation of the thermodynamically stable conformation of proteins *in vivo*. More specifically, in the reduced state, the thiol group of cysteine is able to donate a reducing equivalent ($H^+ + e^-$) to other unstable molecules. In donating an electron, glutathione itself becomes reactive, but readily reacts with another reactive glutathione to form glutathione disulfide (GSSG). Trivalent arsenicals are known to have high affinity for the sulfhydryl groups of biomolecules, such as glutathione, which explains glutathione's use on this study. Furthermore, GSH at the right concentrations plays an important role in preventing hydrolysis of As-GSH complexes and thus, provides stability to these complexes [22].

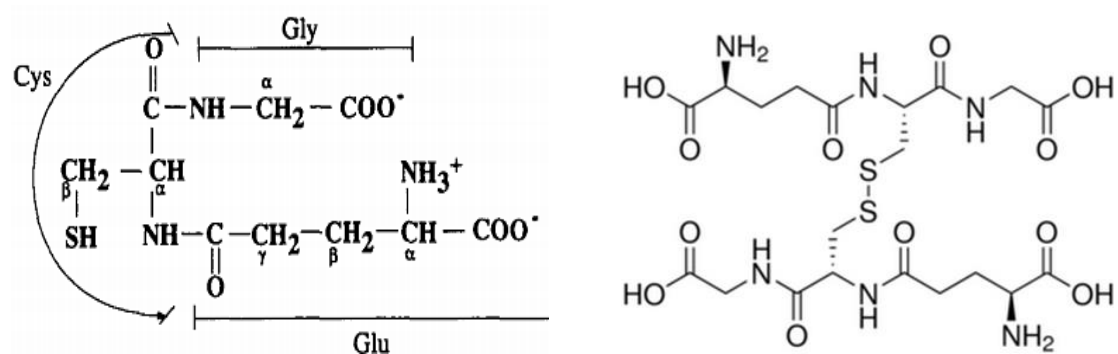


Figure 1 : GSH and GSSG structure [23]

2.5 Sample preparation for electrospray and sonic spray mass spectrometry

Before the analysis with electrospray and sonic spray ionization mass spectrometry the residue of each As-GSH complex, present in each Eppendorf tube, was reconstituted in 1 mL of 0.1% formic acid solution and then diluted further before analysis.

2.6 Electrospray mass spectrometry (ESI-MS) of As-GSH complex (DMAG)

After the synthesis of dimethylarsinic glutathione (DMAG) and its dilution in formic acid, the solution was ready to be analyzed directly by electrospray mass spectrometry.

Every time, before the injection of the sample solution in the spectrometer a first injection with standard sodium trifluoroacetate solution (STFA, pH=3.5), was conducted. This compound gives spectral peaks across a wide mass range in positive and negative ionization modes. So, it is widely used in order to calibrate the instrument's parameters for mass accuracy and also to check and optimize the instrument's sensitivity and resolution. Two injections with water are required before sample analysis, to ensure that the capillary is clean from any contaminants or previously analyzed samples.

The sample was analyzed using the LCQ Advantage Ion Trap Mass Spectrometer, *Thermo Finnigan* [Figure 2].



Figure 2 : Ion Trap mass spectrometer

This mass spectrometer is composed by three basic components, the ion source, the mass analyzer and the detector. An amount of the sample analyte solution (350 μ L) was first infused into the source using a mechanical syringe pump through a needle at low flow rate \sim 10 μ L/min. A high voltage (4-5kV) was applied to the tip of the metal capillary in the **ion source**, relative to the surrounding source sampling cone or heated capillary. This strong electric field caused the dispersion of the sample solution into an aerosol of highly charged droplets. When positive ion mode is applied, a positively charged aerosol is formed and when negative ion mode is applied then a negatively charged aerosol is formed. The positive charges are mostly contributed by protons, and negative charges are contributed by negative ions resulting from the loss of protons. For the specific As-GSH peptides, positive mode was used. Thus, 0.1% formic acid was usually added into the analyte solution to enhance protonation and also increase sensitivity. A coaxial sheath gas (N₂) flew around the capillary for better nebulization. This gas flow also helped to direct the spray emerging from the capillary tip towards the mass spectrometer. The charged droplets were diminished in size by solvent evaporation, assisted by the flow of nitrogen. Below, there is a table (Table 1) representing all the basic ESI ion source parameters. Finally, the charged analytes were released from the droplets, some of which passed through a sampling cone on the orifice of a heated capillary into the mass analyzer, which was held under high vacuum.

Table 1 : ESI source parameters

ESI	
Source spray voltage	5 kV
Sheath gas flow rate	45 arb
Auxiliary gas flow rate	5 arb
Capillary temperature	300 °C
Capillary voltage	5 V
Tube lens voltage	-10V

The **mass analyzer** used to determine the mass-to-charge ratio (m/z), was a **quadrupole ion trap** analyzer. This analyzer contains a ring electrode and two end

cap electrodes. After their production, gas phase ions were collimated by two octapoles. Then, the ions entered the area between the electrodes through one of the end caps. After entry, the electric field in the cavity due to the electrodes caused the ions of certain m/z values to orbit in the space. As the radio frequency voltage was increased, heavier mass ion orbits became more stabilized and the light mass ions became less stabilized, causing them to collide with the wall, and finally being detected by the detector.

The **detector** used in this spectrometer is an **electron multiplier**. The task of the electron multiplier is to detect every ion of the selected mass passed by the mass filter. It consists of many dynodes which have different voltages. By the time a cation strikes on the first dynode with high negative voltage, secondary negatively charged particles are produced. These secondary particles are accelerated after sequential impact upon the dynodes. Thus, more electrons are produced (up to 10^7 electrons from each ion), and they are finally detected as current at the end of the electron multiplier.

The mass spectra of the As-GSH complex (DMAG), was processed by Xcalibur 1.3 software.

2.7 Electrospray mass spectrometry tandem mass spectrometry (ESI-MS/MS) of As-GSH complex (DMAG)

In order to obtain structural information about the specific arseno-peptide and propose a possible fragmentation pathway, tandem mass spectrometry was applied. The first objective is to isolate an ion species designated as the parent ion, and then to determine the m/z ratios of the fragment, or product ions formed by Collision Induced Dissociation (CID) of the parent ions. CID involves the collision of an ion with a neutral atom or molecule in the gas phase (He) and subsequent dissociation of the ion.

In order to acquire the MS/MS data, selected reaction monitoring (SRM) mode, also called multiple reaction monitoring (MRM), was used. SRM delivers a unique fragment ion that can be monitored and quantified. The SRM experiment is accomplished by specifying the parent mass of the compound for MS/MS fragmentation and then specifically monitoring for a single fragment ion [Figure 3]. The results of the fragmentation are presented in the Results and Discussion section.

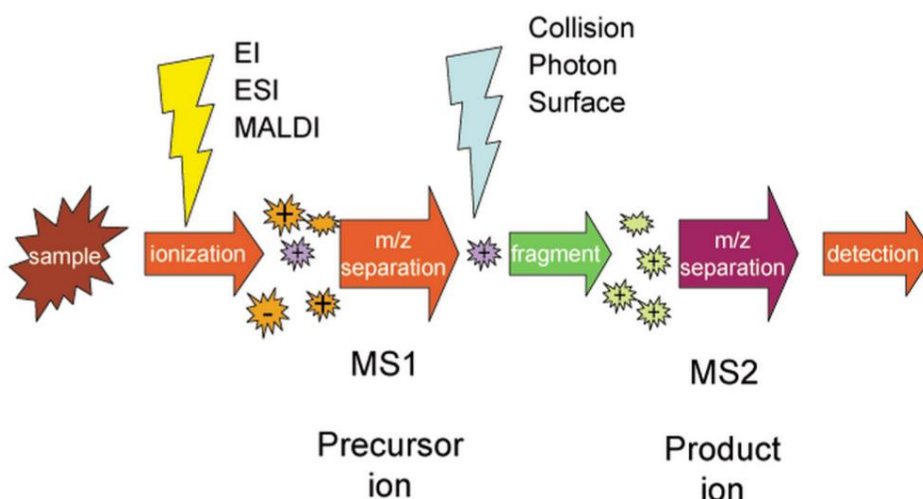


Figure 3 : Selected reaction monitoring: the mass selection stage MS1 selects precursor ions that undergo fragmentation followed by product ion selection [24].

2.8 Sonic spray ionization Mass spectrometry (SSI-MS) of As-GSH complexes

The three As-glutathione complexes were also analysed using sonic spray ionization mass spectrometry. Prior to the injection of the sample solution in the spectrometer, a first injection with standard sodium trifluoroacetate solution (STFA) took place. Furthermore, before the analysis the samples were diluted further in H₂O 1/100, to prevent column blocking.

The samples were analyzed using the LCQ Advantage Ion Trap Mass Spectrometer, after making a few necessary changes on the ion source part. The front cover of the ionization chamber door was removed and a homemade spray source was used [Figure 4].

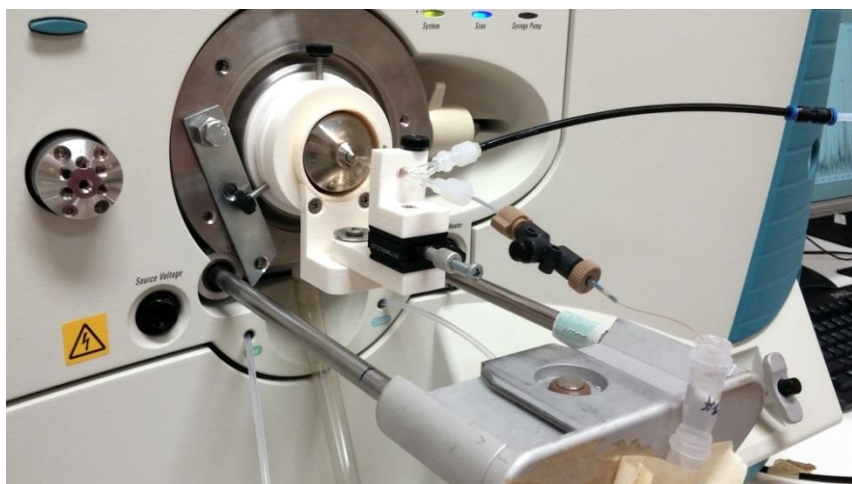


Figure 4 : Ion source of SSI-MS

More specifically, that source consisted of a pneumatic concentric nebulizer made by borosilicate glass (*Meinhard*) [Figure 5]. The nebulizer included an inner fused-silica capillary, where the gas (N_2) flows, whereas the edge of the nebulizer is located a few mm from before the mass spectrometer.

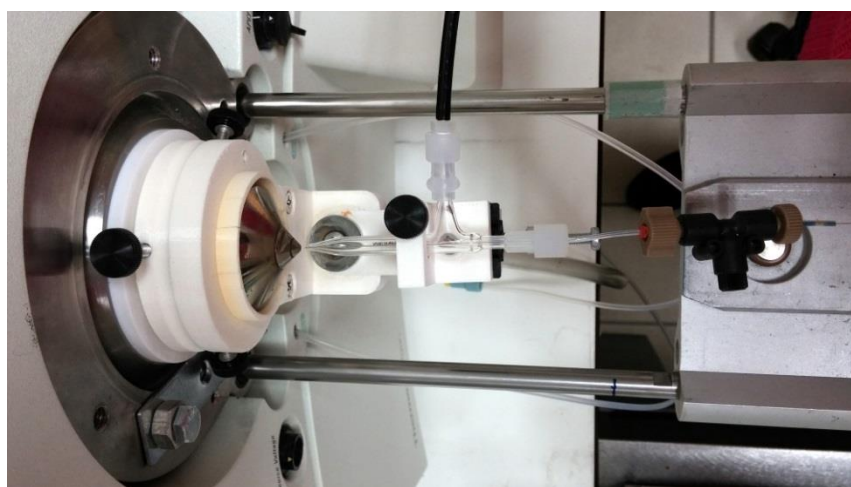


Figure 5 : SSI ion source, view from the top

The whole procedure principle is based on the *Venturi effect* [25], where the pressure reduction on the tip of the nebulizer causes the suction of the sample, leading to the natural aspiration of the sample [Figure 6]. The analyte introduced through the capillary is sprayed under atmospheric pressure with gas flow (N_2) coaxial to the capillary, and when the gas velocity is higher than a certain value, ions

as well as droplets are produced by the spray. These ions are then analyzed by the mass spectrometer.

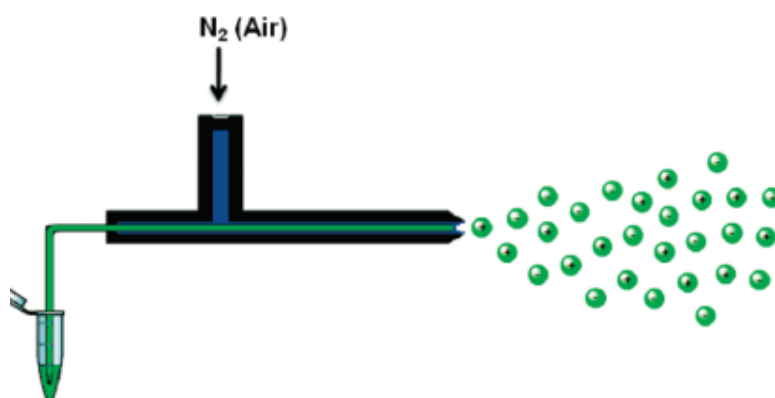


Figure 6: Venturi self-pumping and sonic-spray ionization [25].

Both the nebulizer and the sample Eppendorf are fixed on a mobile platform, by which we can adjust the distance between the nebulizer and the mass spectrometer's entrance. Furthermore, there is a chamber made of plexi glass connected with a fume hood. This system provides protection from possible harmful sprayed sample, and also keeps the ionization source isolated away from the laboratory environment.

Table 2 : SSI source parameters

SSI	
Source spray voltage	0 kV
Sheath gas flow rate	0 arb
Auxiliary gas flow rate	0 arb
Capillary temperature	300 °C
Capillary voltage	20 V
Tube lens voltage	70V

2.9 Sonic spray mass spectrometry tandem mass spectrometry (SSI-MS/MS) of As-GSH complex (DMAG, MADG, ATG)

All three compounds were analyzed further using tandem mass spectrometry, after the production of ions with sonic spray ionization. The followed procedure was stated before, while the results of the fragmentations are presented in the Results and Discussion section.

2.10 Matrix-assisted laser desorption/ionization (MALDI- FT-ICR-MS) of As-GSH complexes

The three As-glutathione compounds were also analyzed using MALDI -FT-ICR mass spectrometry. The *a*-cyano-4-hydroxycinnamic acid (CHCA) was the matrix used for that purpose. The preparation of CHCA included mixing of H₂O/Acetonitrile (ACN) 1:1 with 0.1% trifluoroacetic acid (TFA) [26]. The matrix was kept in the fridge (-4°C) in order to avoid decomposition. The samples solutions were spotted on a steel target, which would be bombarded by a laser beam after entering the mass spectrometer. The spotting was done using the dried droplet method where, after mixing equal quantities of the matrix and the sample solution (1µl of each) and spot them on a certain position on the target, the final solution was left to dry at room temperature for a couple of minutes [Figure 7].

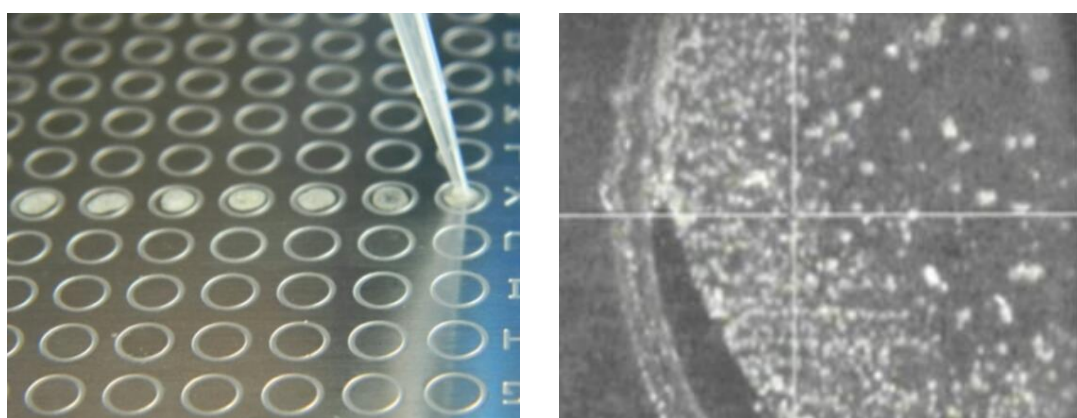


Figure 7 : (left) MALDI spotting on target , (right) CHCA matrix optical image after spotting

After spotting with the mixture of the matrix and the sample, the target was introduced into the mass spectrometer. A Solarix XR FTMS 9.4T, Fourier transform ion cyclotron resonance mass spectrometer was used for that analysis. [Figure 8]



Figure 8: Fourier transform ion cyclotron resonance mass spectrometer (FT-ICR-MS), at university of York

The ionization in MALDI was triggered by a laser beam. The laser is fired at the MALDI plate and is absorbed by the matrix (CHCA). The matrix becomes ionized and transfers charge to the analyte, creating the ions of interest. Then, the ions are guided with a quadrupole ion guide into the ICR cell where the ions cyclotron in a magnetic field. While in the cell, the ions are excited and ion cyclotron frequencies are measured. The frequency, of a charged particle is determined solely by its mass-to-charge ratio. Using Fourier analysis, the frequencies can be resolved into a sum of pure sinusoidal curves with given frequencies and amplitudes. The frequencies correspond to the mass-to-charge ratios and the amplitudes correspond to the concentrations of the compounds in the analyte. All acquisition settings were optimized to reach the sensitivity and resolving power needed for the MALDI-FTICR-MS analysis of the three As-peptide compounds. These settings affect parameters with regard to transient length, ion transfer (in the octapole and in the collision hexapole), transfer optics (hexapole), the ICR cell analyzer and the laser spot size or the number of laser shots, as well.

2.11 Extraction of polar arsenicals from the seaweed “hijiki”

The seaweed used in this study was “hijiki” which was provided by the University of York and Professor Jane Thomas-Oates.

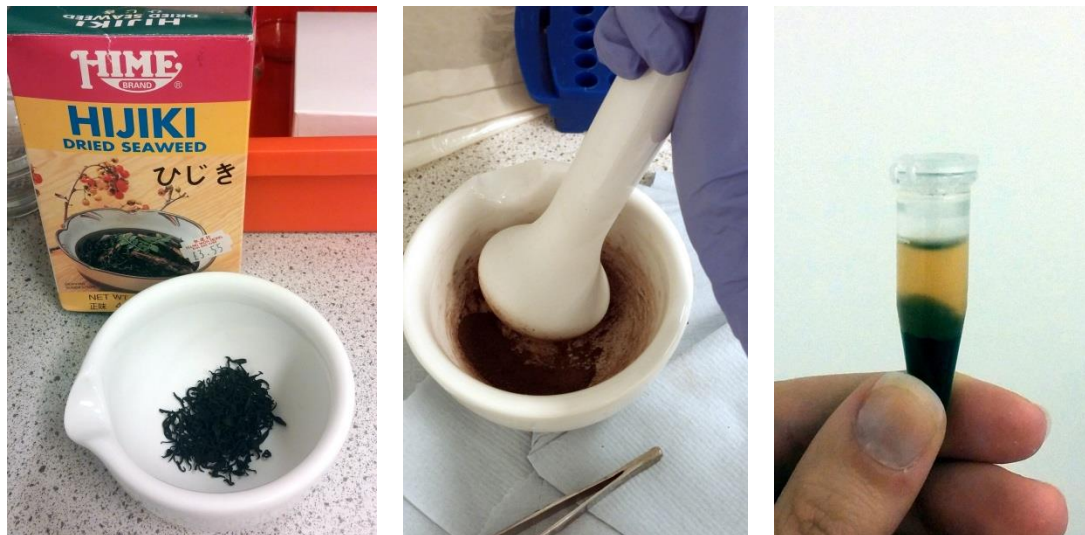


Figure 9: left: “hijiki” seaweed, middle: use of pestle and mortar to grind the seaweed, right: extract (supernatant) after centrifugation

The extraction was carried out by adding 1ml methanol:water (1:1 v:v) to 0.2g of ground and dried seaweed. The mixture was sonicated for 15 minutes and then left overnight at room temperature. The sample was then centrifuged at 2500 rpm for 10 minutes. The pellet was discarded and the supernatant was selected and kept in the fridge (-4°C) until the analysis.

2.12 Spiking As-GSH complexes into biological extracts

Each compound was first diluted in 0.1% formic acid. The spiking took place by mixing equal amounts of each compound’s solution with the seaweed extract. The HPLC-MS analysis took place immediately, after the spiking for better and more precise results.

2.13 High pressure liquid chromatography -electrospray ionization-Fourier transform-ion cyclotron resonance mass spectrometry (HPLC-ESI-FT-ICR-MS) of As-GSH complexes, after spiking into the seaweed extract

The HPLC-ESI-FT-ICR-MS analysis was done for each As-complex separately after spiking into the extract solution and for the mixture of all three As-complexes, as well.

For ease of reference we use

- ✓ **As-GSH mix standard**, for the mixture of three As-GSH complexes (diluted in formic acid)
- ✓ **DMAG-extract**, for the DMAG solution spiked into the seaweed extract.
- ✓ **ATG-extract**, for the ATG solution spiked into the seaweed extract.
- ✓ **MADG-extract**, for the MADG solution spiked into the seaweed extract.

Regarding the first analysis, DMAG, ATG and MADG solutions were inserted into separate vials including conical inserts, which allow for maximum sample recovery and easier sample removal. Equal amounts of extract were added in each vial and the mixture was vortexed for a couple of seconds. Each sample was placed on a specific numbered position in the HPLC auto-sampler tray [Figure 10].



Figure 10: Sample's position on auto-sampler of HPLC

The HPLC system used for the sample analysis was an Agilent 1200 High Pressure Liquid Chromatography System coupled with FT-ICR-MS, while the software used for

data analysis was *HyStar* [Figure 11]. Prior to the beginning of the sample analysis, a sodium formate solution was used as the calibrant in order to obtain highest sensitivity and mass precision of the mass spectrometer.



Figure 11: HPLC-ESI coupled with FT-ICR-MS

Reversed-phase HPLC analysis parameters:

- Mobile phase: 20% MeOH-0.1% formic acid

Steady changes of the mobile phase composition during the chromatographic run, took place based on a *gradient elution* profile. The mobile phase ran from 0%→60% →90% →0%, during the 16 minutes analysis.

- Flow rate: 0.3 ml/min
- Column: BDS HYPERSIL C18, 150 X 2.1mm

The use of a pre-column was crucial to protect the main analytical column from particles, undissolved salts, or dirt that passes from the HPLC pump, as well as sample components that bind irreversibly to the stationary phase used in this study.

- Injection: 5 μ l
- Running time: 16'

After the parameters were settings, the samples were ready to be analyzed and the whole system was set in operation. The HPLC was coupled to the FTICR- mass spectrometer interfaced by an electrospray source. Thus, the eluate from the HPLC column continued to the ESI source, through a capillary, to achieve the ionization of the solution, and finally the ions reach the mass spectrometer, which can evaluate and identify the compound, on the basis of mass-to-charge ratio.

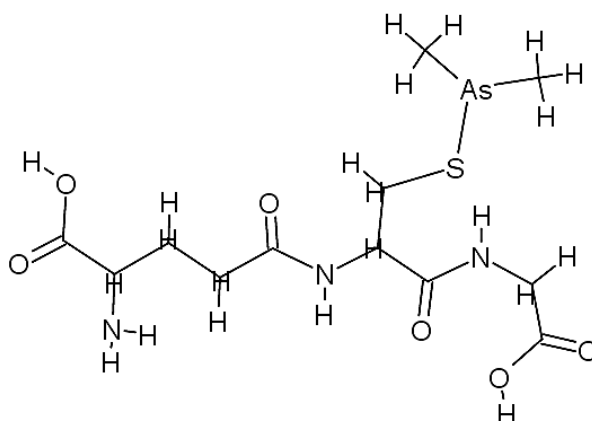
For the second analysis, the As-GSH mix standard was inserted in the vial with the conical insert, and then the extract was spiked into that vial. The sample was placed in its specific location on the auto-sampler and then analyzed, as described before.

3. Results and Discussion

In this study, three arsenopeptides were synthesized and analyzed using various MS techniques. Below, the structure of each As-complex¹ [27, 28], is presented, as long with some other chemical characteristics, which will help us explain the features present in the obtained mass spectra.

- **Dimethylarsinic glutathione (DMAG)**

Dimethylarsinic glutathione (DMAG)	
Molecular Formula	C ₁₂ N ₃ O ₆ AsH ₂₂ S
Monoisotopic mass²	411.0439 Da

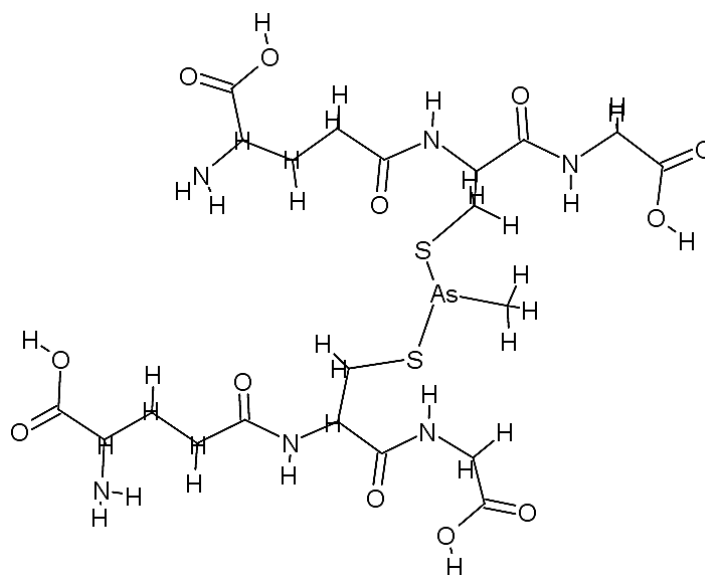


- **Methylarsonate glutathione (MADG)**

Methylarsonate glutathione (MADG)	
Molecular Formula	C ₂₁ N ₆ O ₁₂ AsH ₃₅ S ₂
Monoisotopic mass	702.0964 Da

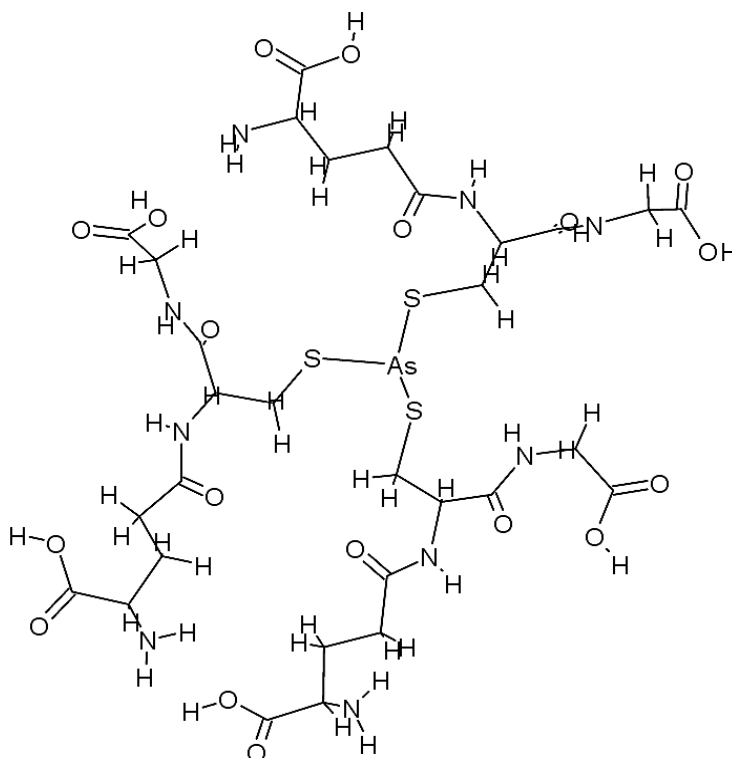
¹ The chemical structure of the three compounds was drawn using the “chemsketch” software

² Mass Data received from <https://pubchem.ncbi.nlm.nih.gov/search/>



- Arsenite glutathione (ATG)

Arsenite glutathione (ATG)	
Molecular Formula	C ₃₀ N ₉ O ₁₈ AsH ₄₈ S ₃
Monoisotopic mass	993.1489 Da



3.1 Mass spectrometry and tandem mass spectrometry: mass spectra of As-GSH complexes

3.2 ESI-MS spectrum of DMAG

LT01_p10_n01 #39 RT: 1.25 AV: 1 NL: 1.12E6
T: + p ESI sid=18.00 Full ms [100.00-2000.00]

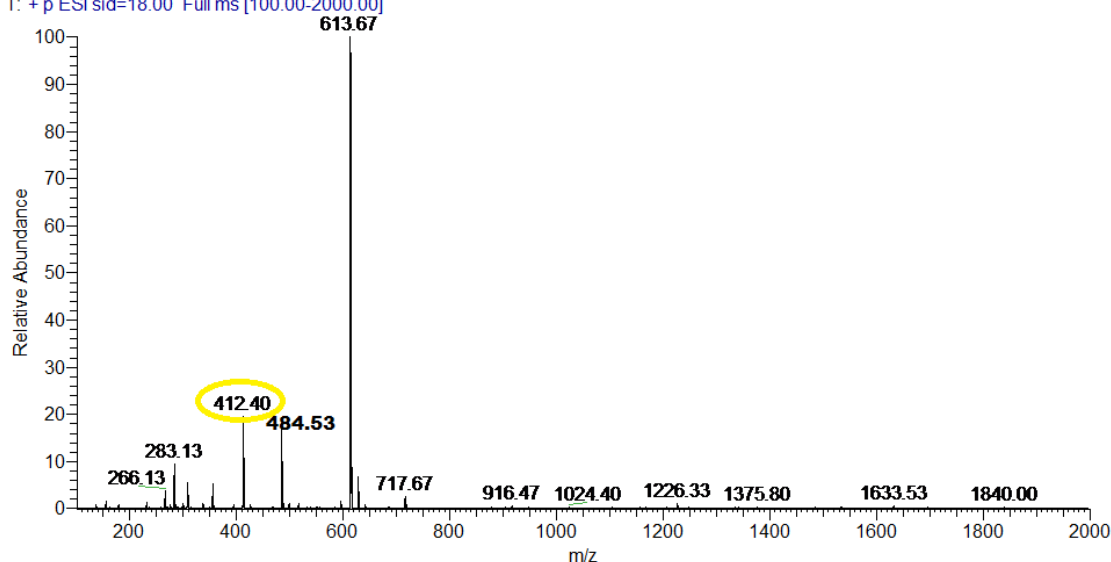


Figure 1 : ESI-MS spectrum of dimethylarsinic glutathione (DMAG) in formic acid 0.1%

The above spectrum represents the full mass spectrum of dimethylarsinic glutathione, using electrospray as an ionization method with positive ionization mode. The yellow circled m/z 412.40 corresponds to the protonated DMAG compound, with approximately 20% abundance. The peak at the 613.67 m/z with 100% abundance corresponds to the oxidized glutathione. There is also another peak at 484.53 m/z with significant relative abundance. This peak could probably represent the oxidized fragment product of GSH that resulted after breaking of the amide bond of glutamic acid [Figure 1].

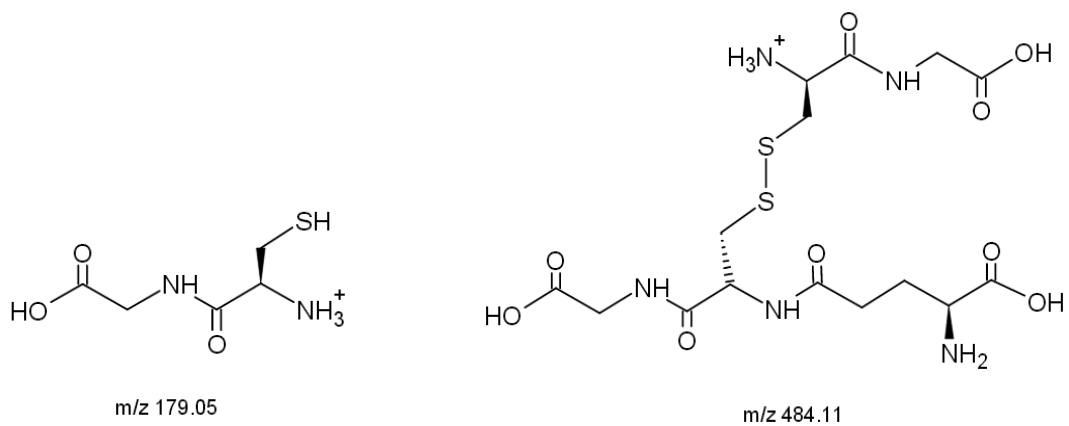


Figure 2 : Molecular structure of fragment m/z 179.05 and oxidized fragment product with GSH

3.3 ESI-MS/MS spectrum of DMAG

Tandem mass spectrometry of the protonated parent molecule of DMAG was done, as well as repeating fragmentation on the product ion (**MSⁿ**), to obtain detailed information concerning the analyte's structure [Figure 3].

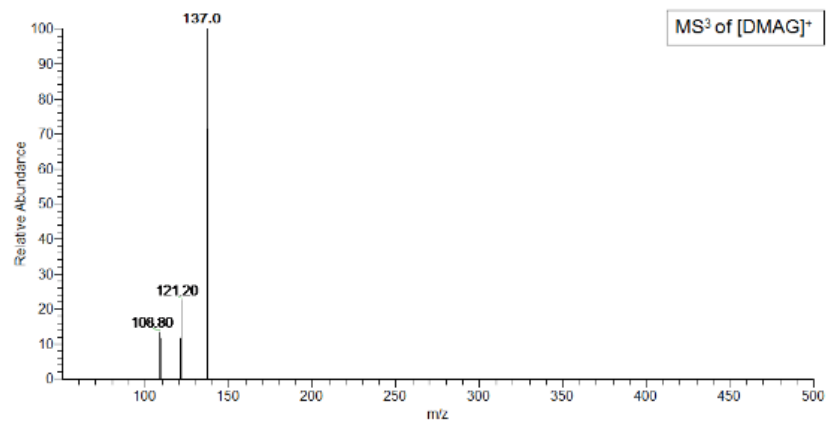
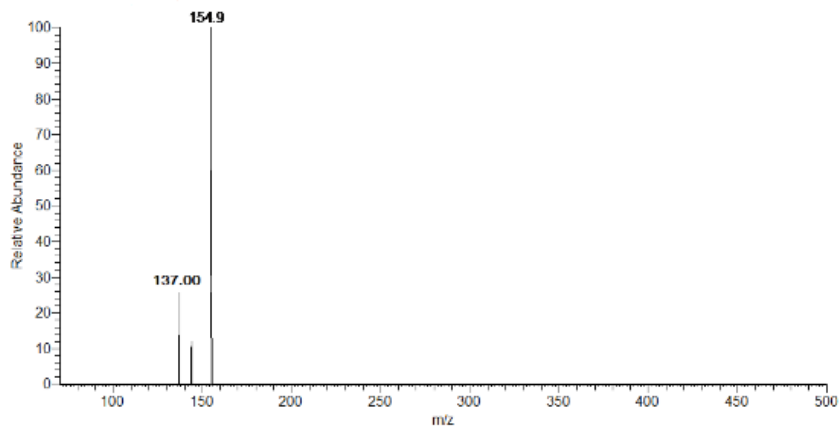
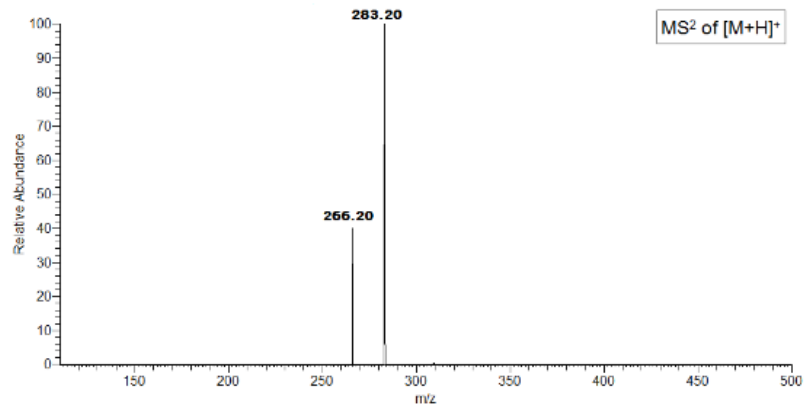
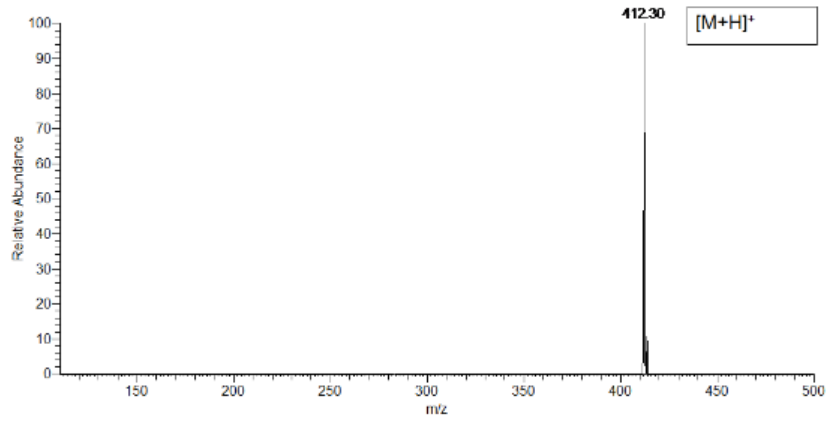
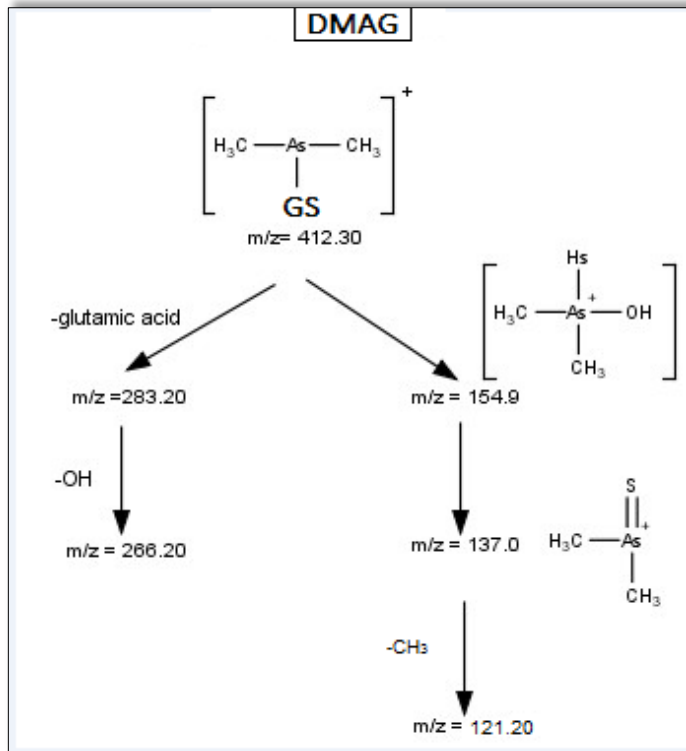


Figure 3 : MSⁿ of the precursor ion $[DMAG]^+$ (ESI-MS)

Firstly, the precursor ion at m/z 412.30 was isolated. Fragmentation of the precursor ion generated two fragment ions, one at m/z 283.20 and the other at m/z 154.9. MS^3 was applied, and 283 fragmented to generate ion at m/z 266.20. MS^3 of 154.9 generated the fragment at m/z 137. Finally, the m/z 137 was selected and fragmented to produce the ion at m/z 121.20.

All these fragmentation steps generate fragment ions that give us the opportunity to obtain detailed information concerning the analyte's characteristic structure. As it is shown above, the precursor ion of specific m/z is selected by the first MS then is directed into a collision chamber, subjected to resonant excitation and finally analyzed further by a second MS. Tandem mass spectrometry is a useful technique that allows for efficient structure elucidation of our As-GSH compounds, provides the selective detection of any target compound and gives the opportunity to study the ion-molecule reactions.

Thus, based on the fragment ions received from the parent ion $[DMAG]^+$, here is a suggested fragmentation pathway, showing briefly the fragmentation steps [Scheme 1].



Scheme 1: Fragmentation behavior of DMAG (ESI ionization)

3.4 SSI-MS spectra of DMAG, MADG and ATG

LT01_p19n02 #14 RT: 0.39 AV: 1 NL: 2.66E5
T: +p SSI sid=20.00 Full ms [150.00-2000.00]

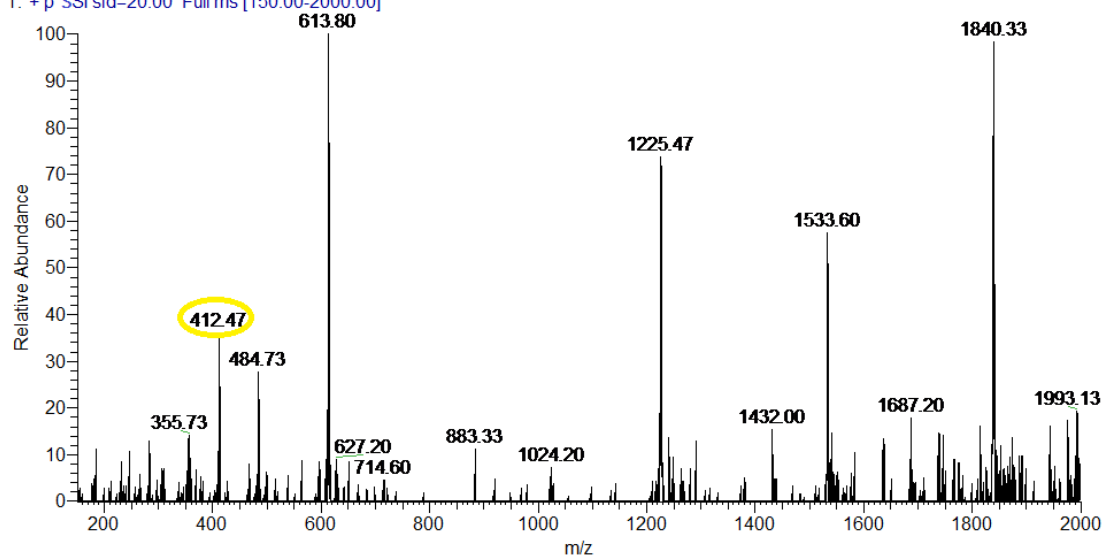


Figure 4 : SSI-MS spectrum of dimethylarsinic glutathione (DMAG) in formic acid 0.1% and 1/100 dilution in H₂O

Figure 4 represents the full mass spectrum of dimethylarsinic glutathione, using sonic spray as an ionization method with positive ionization mode. The yellow bolded peak at m/z 412.4, stands for the protonated DMAG compound and it corresponds to approximately 35% of relative abundance. The peak at the 613.80 m/z with 100% abundance stands for the oxidized glutathione. There is also another peak at 484.73 m/z which as mentioned before, could probably represent the oxidized fragment product of GSH that resulted after breaking of the amide bond of glutamic acid [Figure 2]. There are also other three outstanding peaks at m/z 1225.47 which corresponds to the oxidized GSH dimer (2X613), m/z 1533.60 corresponding to the oxidized GSH dimer plus one GSH molecule and finally, 1840.33 corresponding to the oxidized trimer (3X613).

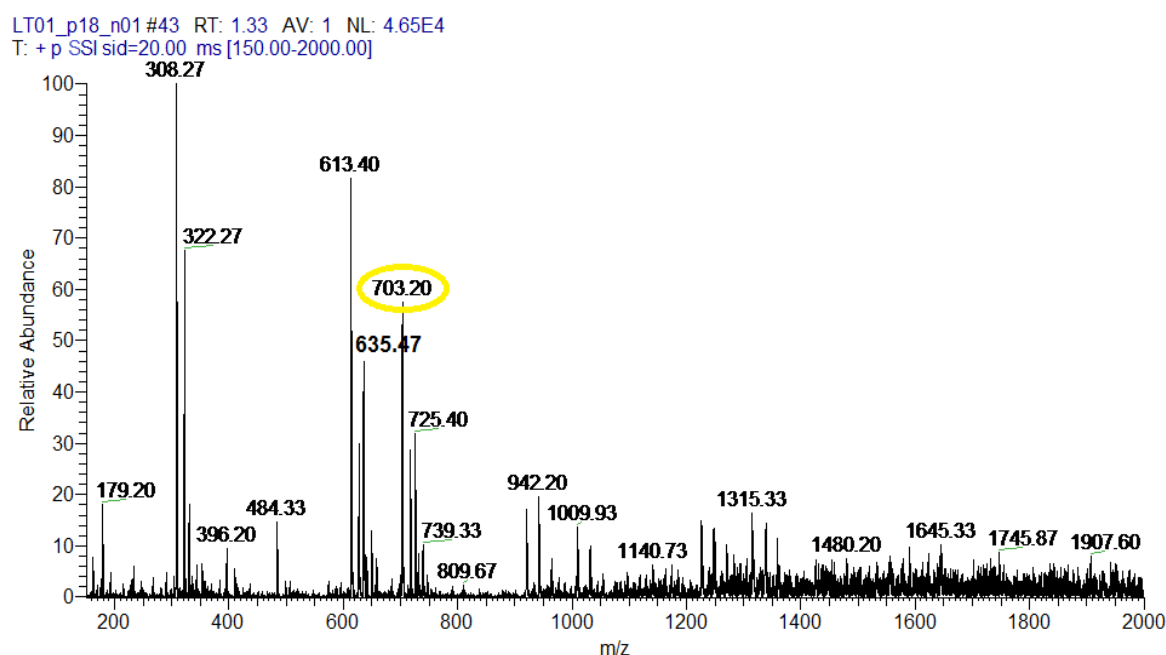


Figure 5 : SSI-MS spectrum of methylarsonate glutathione (MADG) in formic acid 0.1% and 1/100 dilution in H₂O

The above spectrum shows the full MS spectrum of methylarsonate glutathione, using sonic spray as an ionization technique, in positive ionization mode. The peak at m/z 703.20 corresponds to the parent ion [MADG]⁺, with relative abundance at

~60%. The peak with 100% abundance at m/z 308.2, stands for the protonated glutathione, while the lower abundance peak at m/z 322.27 derives from the loss of a nitrogen atom. At m/z value 613.40 there is a peak of oxidized glutathione, while the next peak at m/z 635.47 shows the addition of a sodium atom. The 725.40 m/z value is the $[MADG]^+$ peak plus a sodium atom. Thus, we can conclude that the more the sample solution stays, more and more protons are replaced from Na atoms, which come from monosodium acid methane arsonate ($CH_5AsO_3.Na$), used on the experimental procedure.

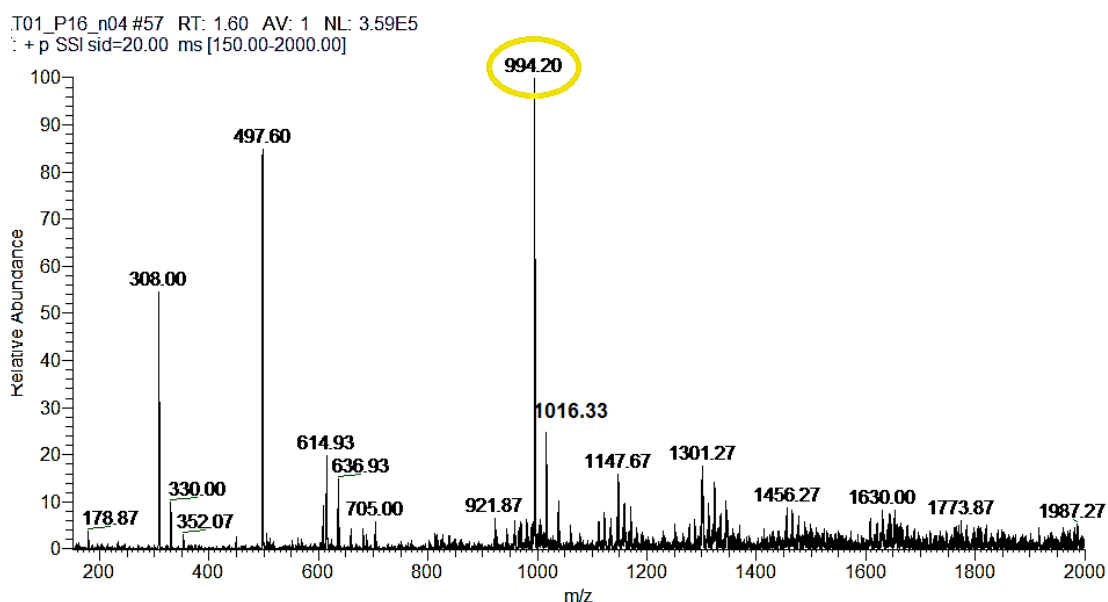
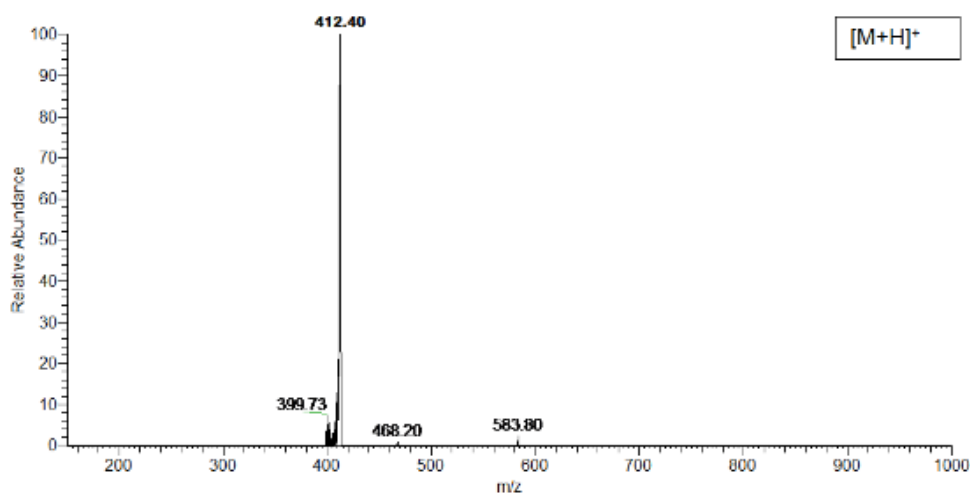


Figure 6 : SSI-MS spectrum of arsenite glutathione (ATG) in formic acid 0.1% and 1/100 dilution in H_2O

On the full MS spectrum of arsenite glutathione, the peak at m/z 994.20 stands for the protonated parent ion of the compound and gives a 100% relative abundance. The presence of protonated glutathione is confirmed by the peak at m/z 308. There is also a peak at 614.93 standing for the oxidized glutathione, while the m/z 636.93 testifies the presence of the sodiated compound. The peaks at m/z 636.93 and 1016.33 represent the sodiated DSSD and $[M+Na]^+$, respectively. Again, the protons are gradually replaced by Na as the time goes by because of the existence of sodium arsenite ($NaAsO_2$) on the solution. Finally, the peak at m/z 497.60 may correspond to the doubly charged ($z=2$) ATG $[(ATG+2H)/2]$.

It is obvious from the previously presented spectra, that there are differences between the two described ionization techniques, ESI and SSI-MS. SSI ionization method does not require any voltage application, which makes it a quite easy and simple technique. It is relying solely on the pressure of the coaxial nebulizer gas (nitrogen) to induce ionization. The relative position of the capillary though the nebulizer, the gas pressure and also the surface tension of the analyte, play a very important role to obtain accurate results and mass values. We can move the nebulizer to a position that gives the best signal and best ionization results. On the SSI-MS spectrum of DMAG, the peak of the protonated parent ion $[M+H]^+$ presents better abundance (35%) compared to ESI-MS spectral peak (20%). Furthermore, SSI spectrum reveals more ion peaks that do not exist on ESI mass spectra, with high abundance values. However, it is obvious that SSI-MS spectrum of the specific compound has a quite higher background noise that may affect the compound's spectrum.

3.5 SSI-MS/MS spectra of DMAG, MADG and ATG



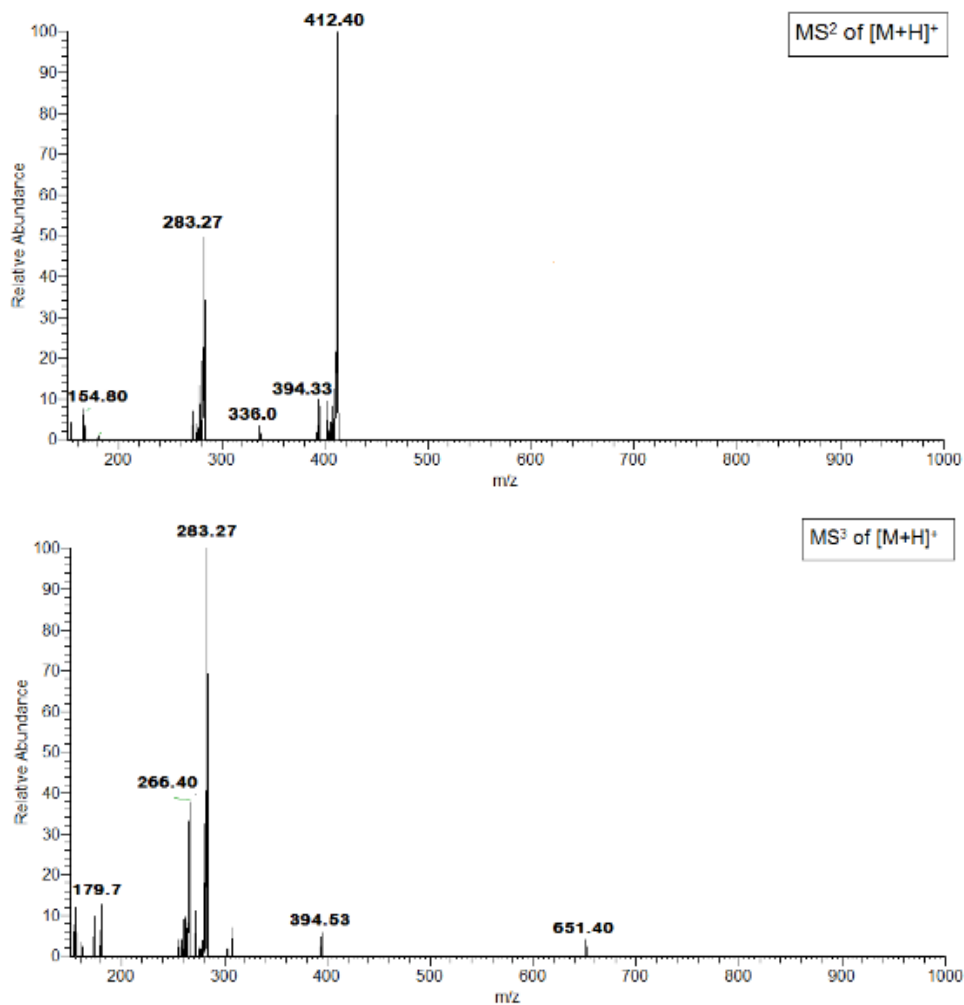
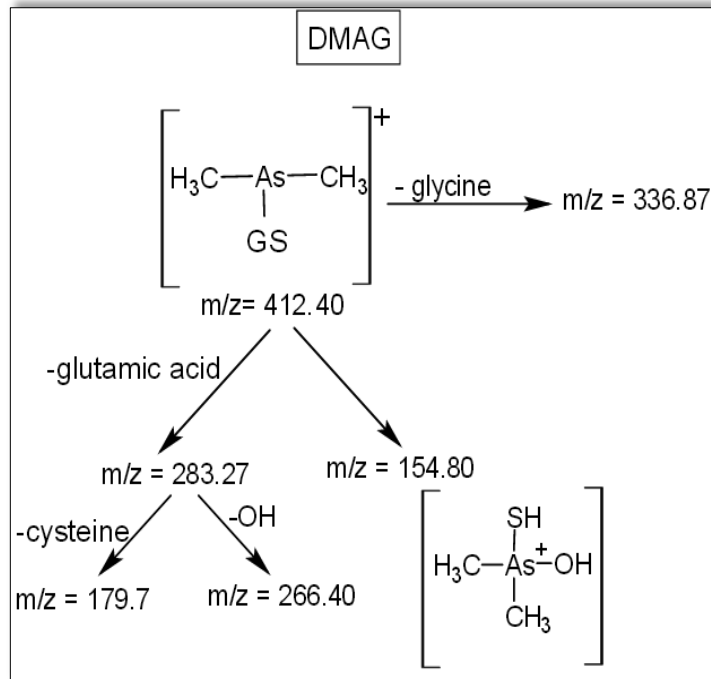


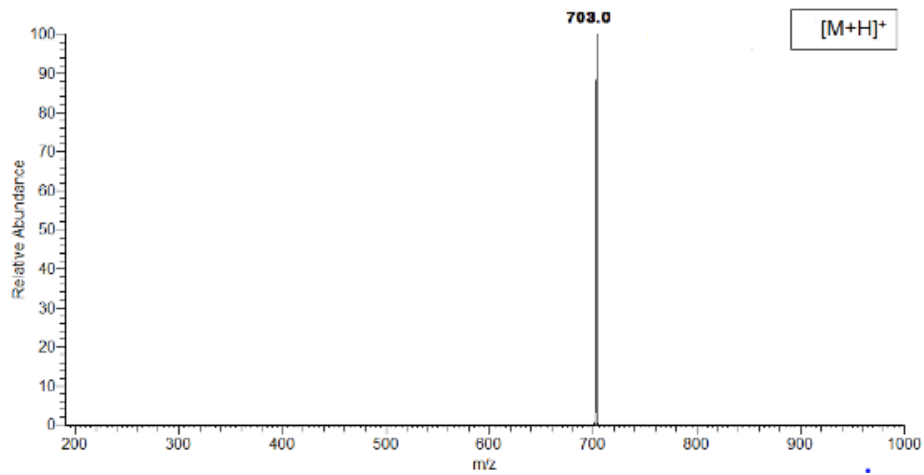
Figure 7 : MSⁿ of of the precursor ion [DMAG]⁺ (SSI-MS)

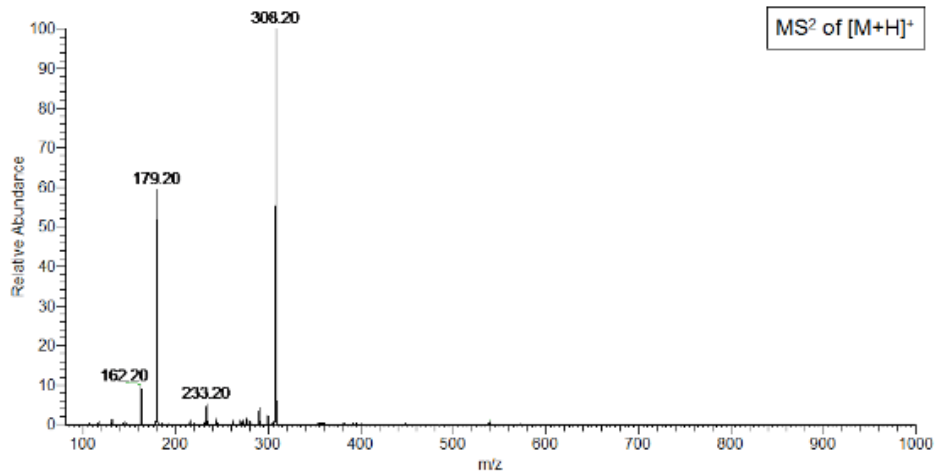
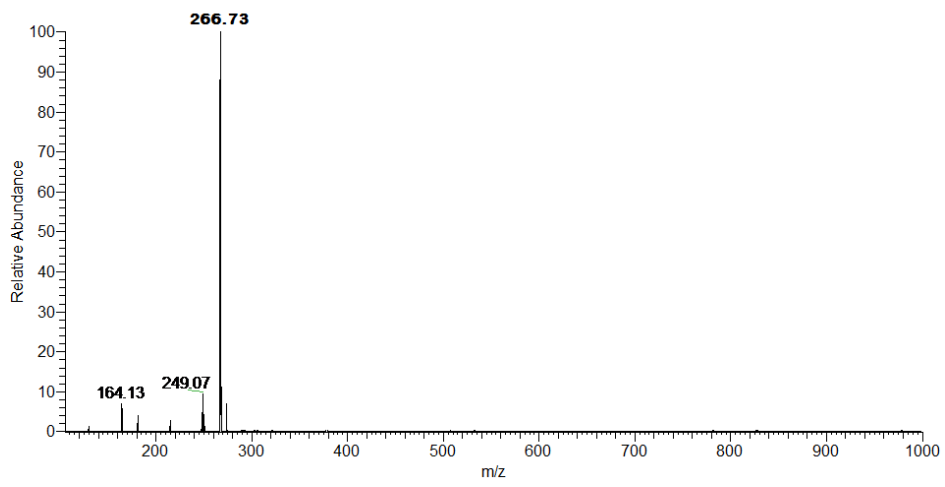
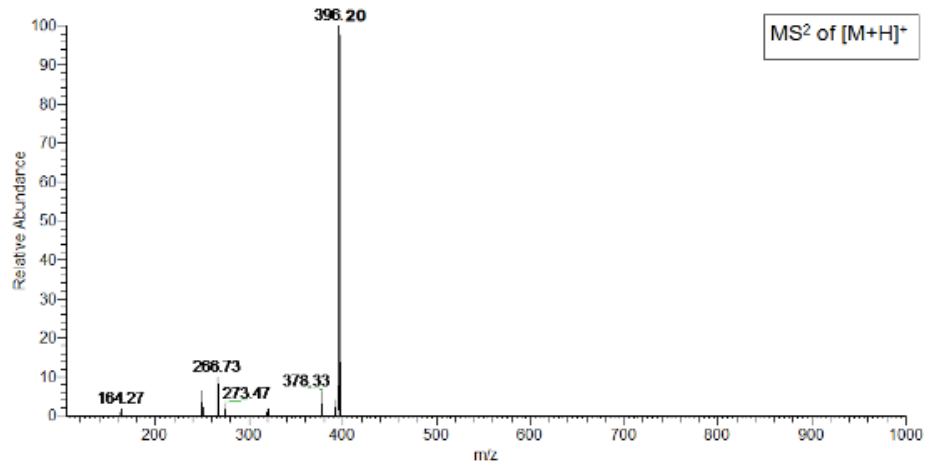
On the first mass spectrum of the above figure the precursor ion at m/z 412.40 was isolated and then fragmented to generate ions at 283.27 and 154.8 m/z value. The ion at m/z 283.27 was then fragmented and generated two fragments at m/z 179.7 and 266.40.

Based on the fragment ions received from the parent ion [DMAG]⁺, here is a suggested fragmentation pathway [scheme 2].



Scheme 2: Fragmentation behavior of DMAG (SSI ionization)





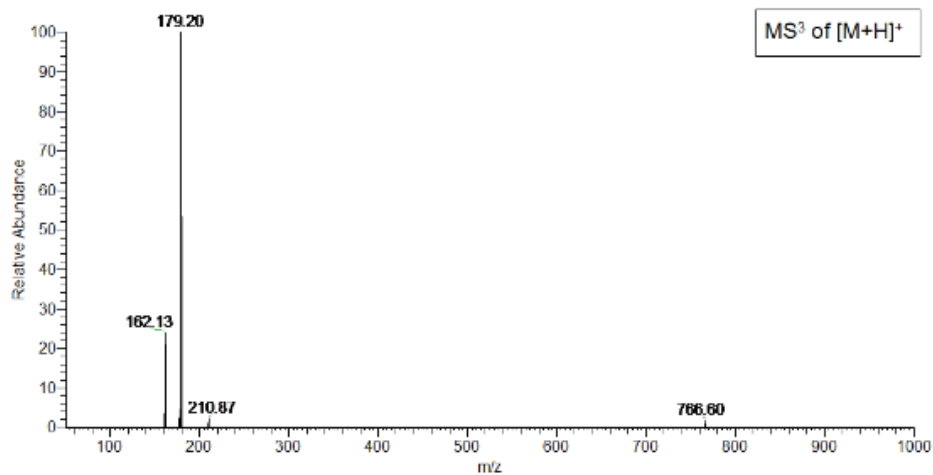
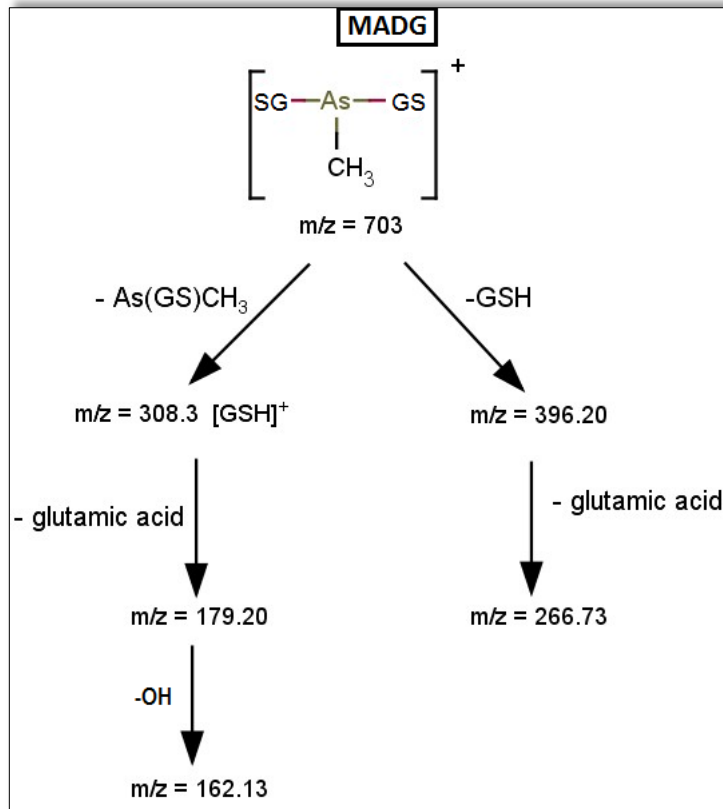


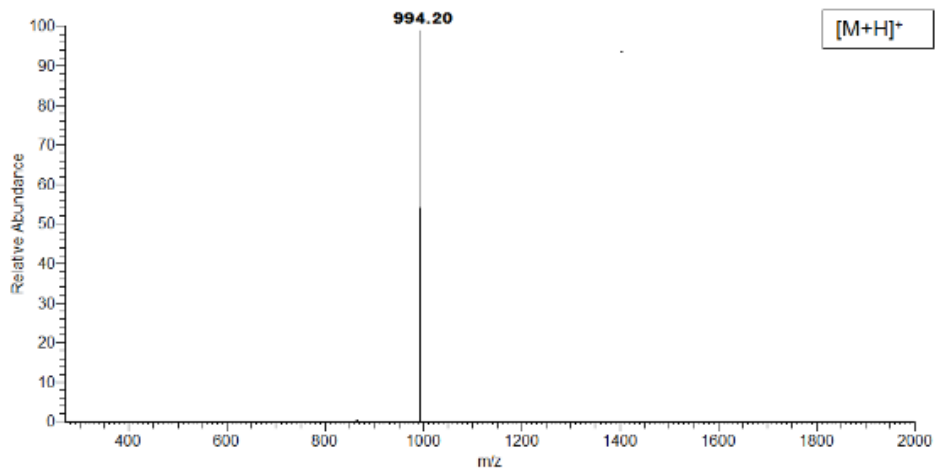
Figure 8 : MSⁿ of of the precursor ion [MADG]⁺ (SSI-MS)

The precursor ion at m/z 703 was first isolated. MS² on that ion generated two main fragment ions at m/z 308 and 396.20. MS² of 703 produces fragment at m/z 396.20 which is then fragmented and generated ions at m/z 266.73 (second and third spectrum). The forth spectrum shows the fragmentation of the selected ion of m/z 308 and the generation of the fragment at m/z 179.20.

Based on the fragment ions received from the parent ion [MADG]⁺, here is a suggested fragmentation pathway [scheme 3].



Scheme 3: Fragmentation behavior of MADG (SSI ionization)



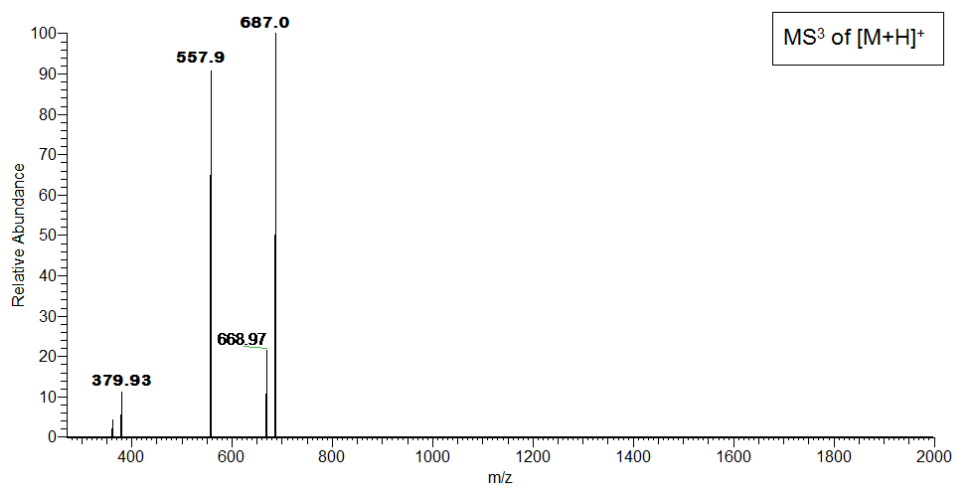
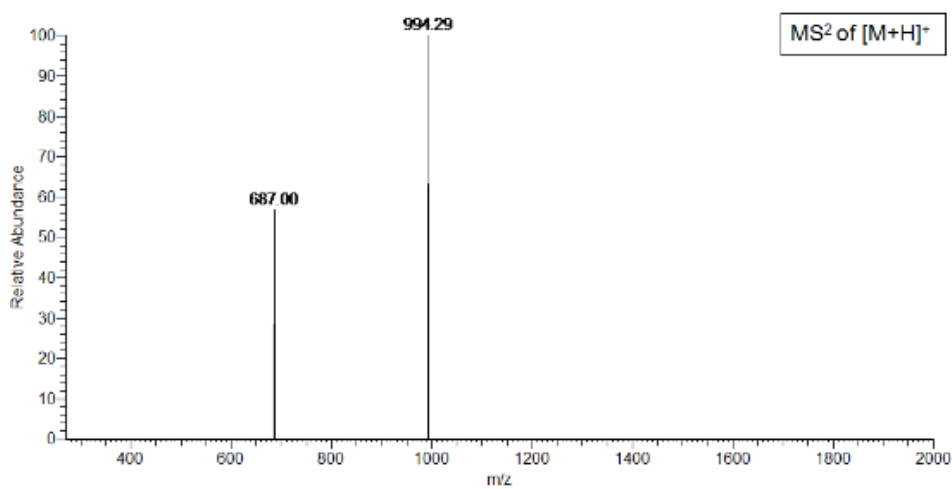
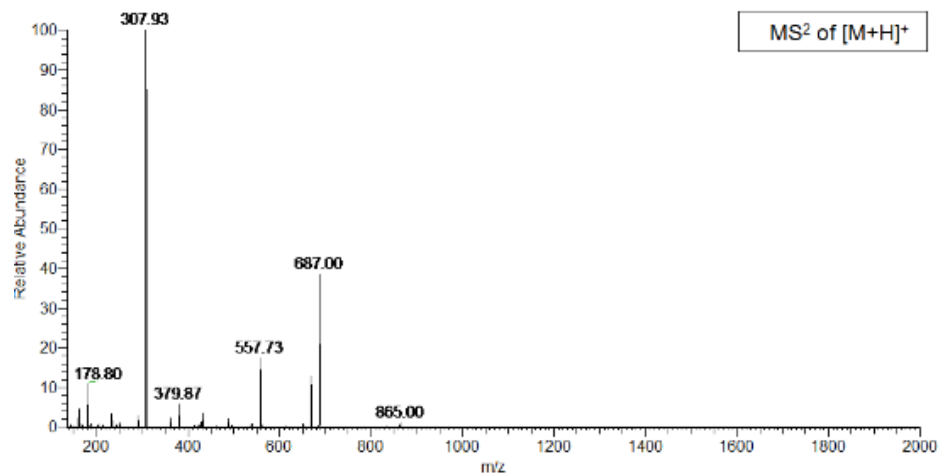
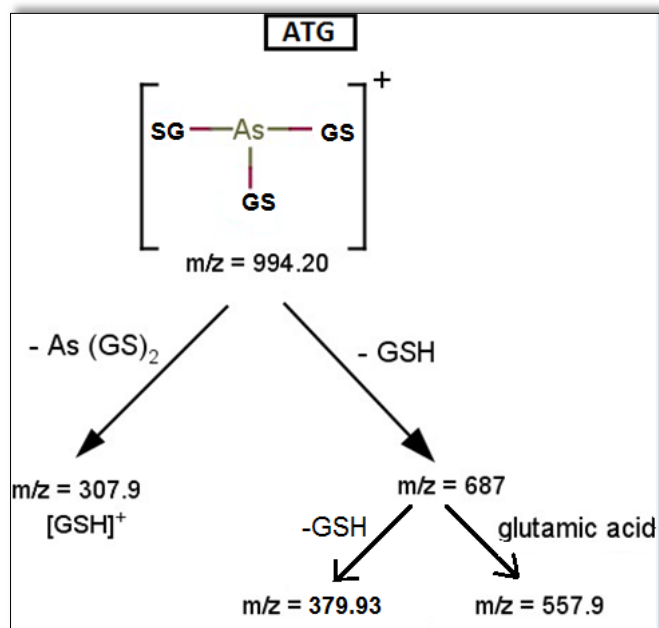


Figure 9 : MSⁿ of of the precursor ion [ATG]⁺ (SSI-MS)

The first mass spectrum shows the peak at m/z 994.20, which was isolated and then fragmented to generate fragment ions with m/z 307.9. MS² of 994.20 also produced

a fragment at 687.0 m/z value (third mass spectrum). Finally, on the last spectrum the fragments of 687.0 are shown at m/z 379.93 and 557.9.

Based on the fragment ions received from the parent ion [ATG]⁺, here is a suggested fragmentation pathway [scheme 4]



Scheme 4: Fragmentation behavior of ATG (SSI ionization)

3.6 MALDI-FT-ICR-MS of DMAG, MADG and ATG

The following figures show the MALDI mass spectrum of the three As-GSH complexes analyzed in positive ionization mode. The As-GSH compounds were diluted just in 0.1% formic acid. The matrix used for the analysis was CHCA (10mg/ml) and the spotting target's material was ground steel.

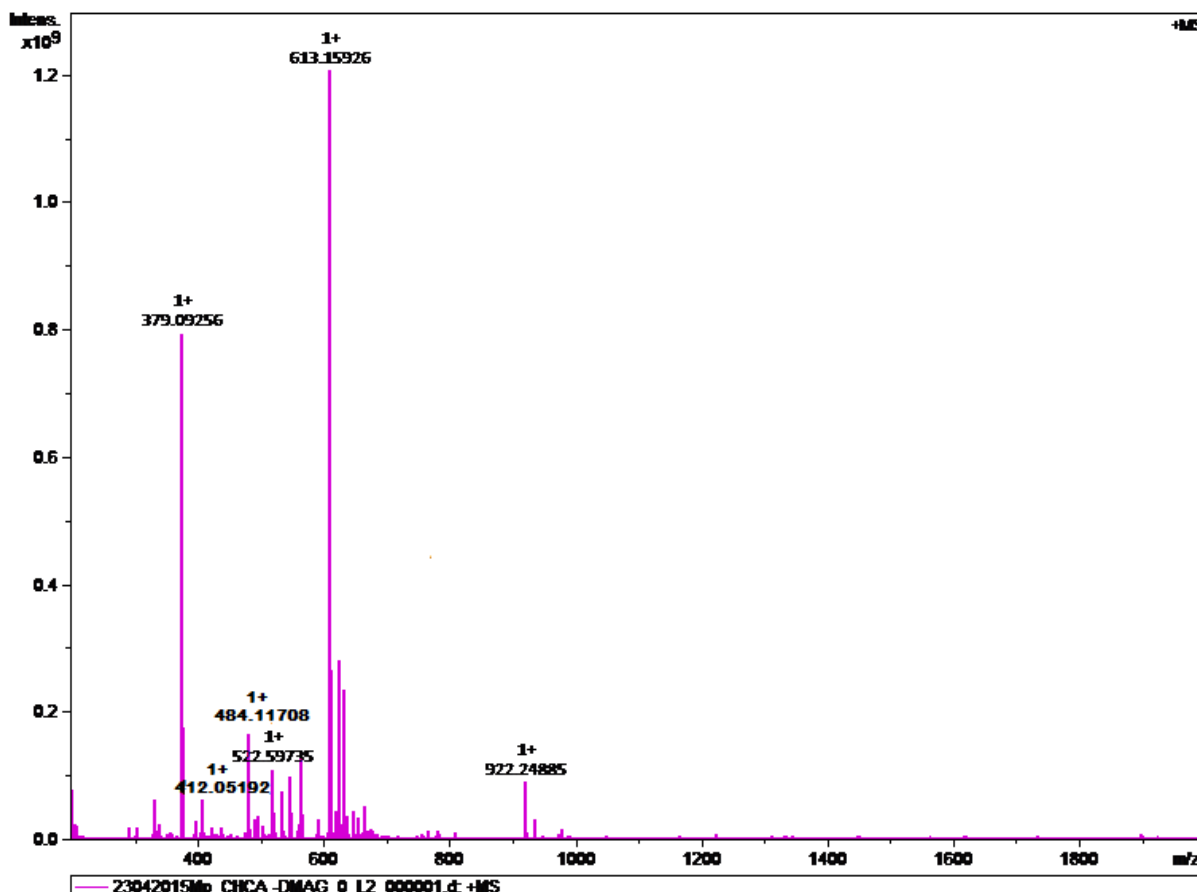


Figure 10 : MALDI-FT-ICR-MS spectrum of dimethylarsinic glutathione (DMAG) in formic acid 0.1%

On the above mass spectrum, the peak at m/z 412.05192 corresponds to the protonated DMAG complex, $[M+H]^+$ with abundance 5.18%. The highest peak 613.15926 with 100% abundance stands for the oxidized GSH. At m/z 484.11708 there is a peak that could probably represent the oxidized fragment product of GSH that resulted after breaking of the amide bond of glutamic acid. There are also three other unknown peaks at m/z 379.09256, 522.59735 and 922.24885.

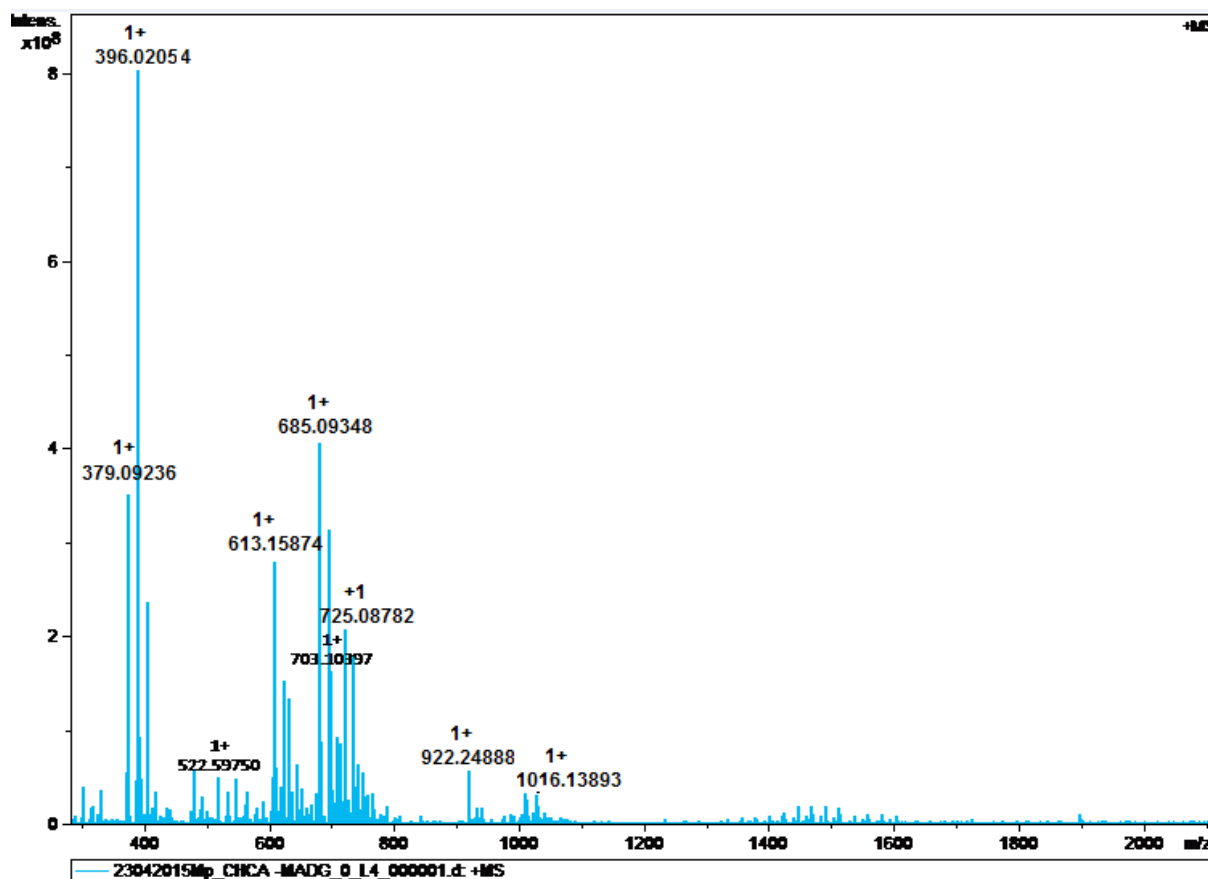


Figure 11 : MALDI-FT-ICR-MS spectrum of methyarsenate glutathione (MADG) in formic acid 0.1%

The above spectrum shows the peak of protonated MADG complex $[M+H]^+$ at m/z value 703.10397, with 19.49% abundance. The m/z 725.08782 value corresponds to $[M+Na]^+$ ion. The most abundant peak (100%) at m/z 396.02054 derives from the loss of a GSH molecule from the protonated MADG. A loss of an $-OH$ leads to the peak of m/z 379.09236. The peak at m/z 613.15874 shows the existence of oxidized glutathione (GSSG). Finally, there are also three other unknown peaks with relatively high abundance at m/z 685.09348, 522.59750 and 922.4888.

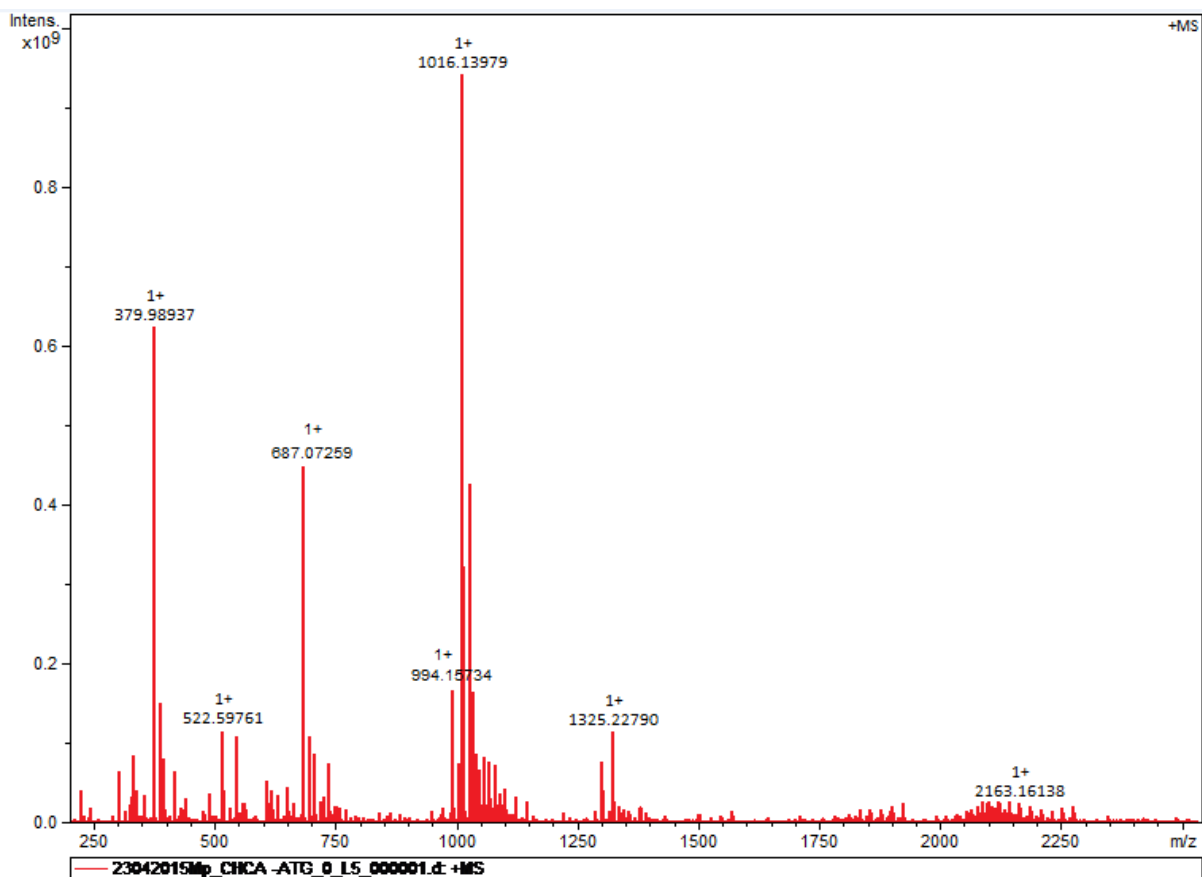


Figure 12 : MALDI-FT-ICR-MS spectrum of arsenite glutathione (ATG) in formic acid 0.1%

On this MALDI-FT-ICR-MS spectrum, the peak at m/z 994.15734 corresponds to the protonated ATG compound, $[M+H]^+$ with abundance 17.6%. The sodiated compound $[M+Na]^+$ appears at m/z 1016.13979 and its abundance reaches the 100%. The peak at m/z 613.15785 corresponds to GSSG (abundance 6.39%), while the monomer of GSH appears at m/z 308.09143 (abundance 4.77%). There is also another peak at m/z 687.07259 (abundance 43.91%), which stands for the protonated ATG after a loss of a GSH molecule. After another loss of GSH we have another ion at m/z 379.98937 and abundance 72.56%. There are also two other unknown peaks at m/z 522.59761 and 1325.2279, respectively.

The greatest observed difference between MALDI and ESI/SSI ionization as far it concerns their mass spectra is that, in MALDI, primarily singly charged ions are formed, while in ESI or SSI, multiply charged ions are formed. MALDI discourages the formation of multiply-charged ions because these, are less easily desorbed from the surface because of electrostatic attraction.

Another crucial point to be noticed is the high abundance of As-GSH complexes in MALDI mass spectra. Responsible for this result is the ultra-high resolution FTICR mass analyzer, with resolving power 1.000.000 (FWHM), while the quadrupole ion trap mass analyzer is limited to resolving power 10.000. The spectral peaks can be seen easily and clearly on the MALDI-FT-ICR-MS spectra with accuracy of five decimal units, and all the peaks that were observed on the previous ionization techniques spectra appear now with a higher abundance value.

3.7 HPLC coupled with mass spectrometry: chromatograms & mass spectra of As-GSH complexes (in biological samples)

3.8 HPLC-FT-ICR-MS of DMAG-extract using gradient elution

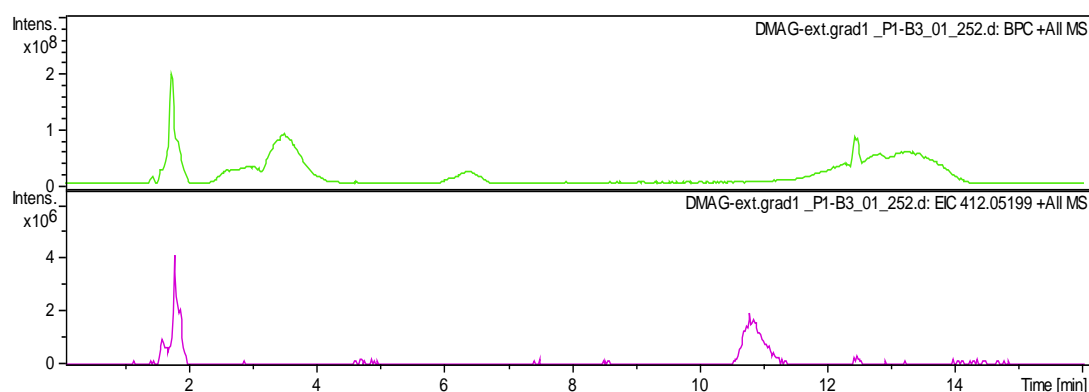


Figure 13: 1st chromatogram; HPLC base peak chromatogram of DMAG -extract (1 μ l in 100 μ l of extract), 2nd chromatogram: extracted ion chromatogram of DMAG (412.05199)

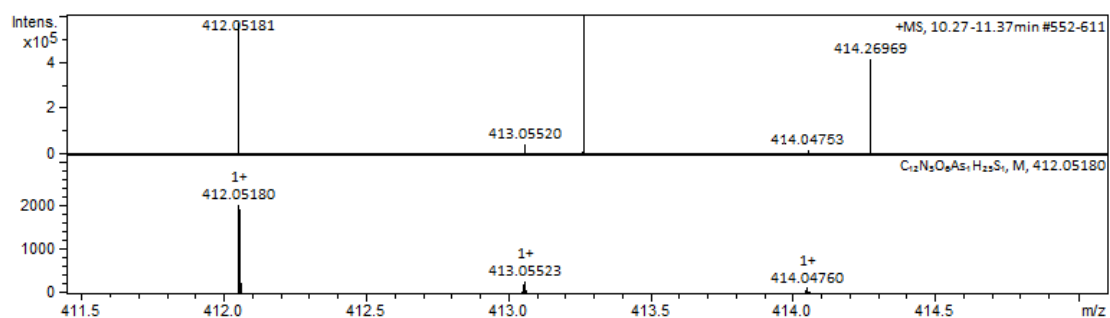


Figure 14: 1st compound spectrum: mass spectrum of DMAG, 2nd compound spectrum: simulated pattern mass spectrum

3.9 HPLC-FT-ICR-MS of MADG-extract using gradient elution

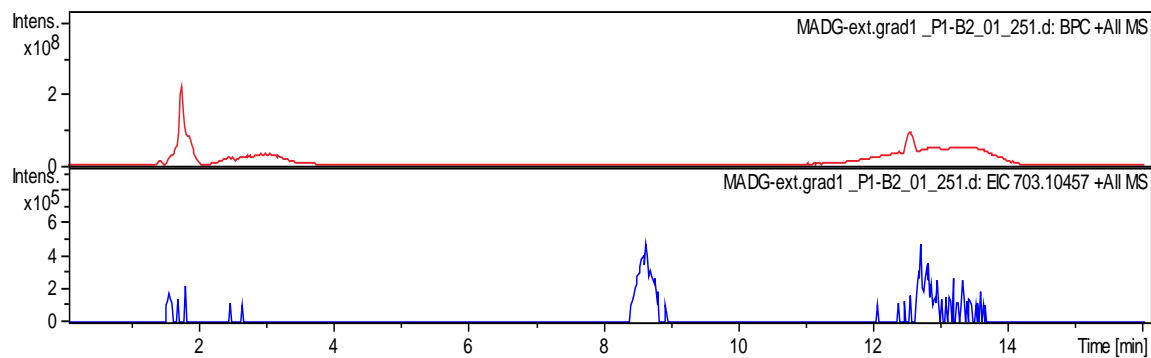


Figure 15: 1st chromatogram: HPLC base peak chromatogram of MADG-extract (5 μ l in 100 μ l of extract), 2nd chromatogram: extracted ion chromatogram of MADG (703.10457)

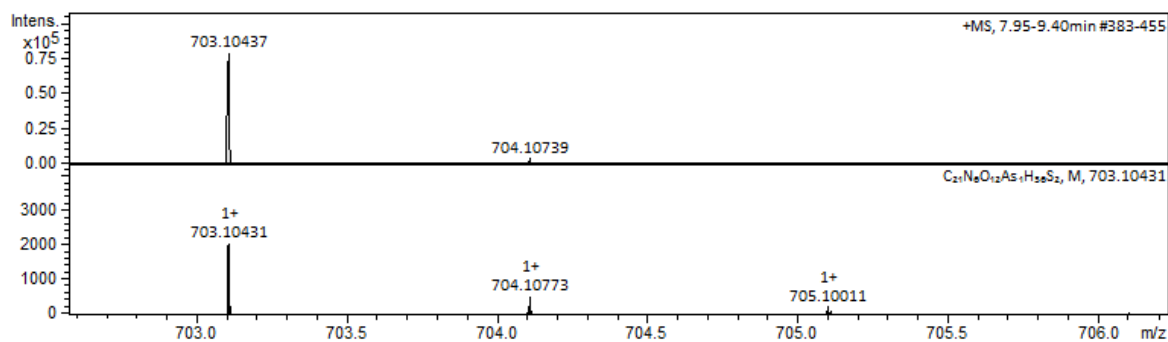


Figure 16: 1st compound spectrum: mass spectrum of MADG, 2nd compound spectrum: simulated pattern mass spectrum

3.10 HPLC-FT-ICR-MS of ATG-extract using gradient elution

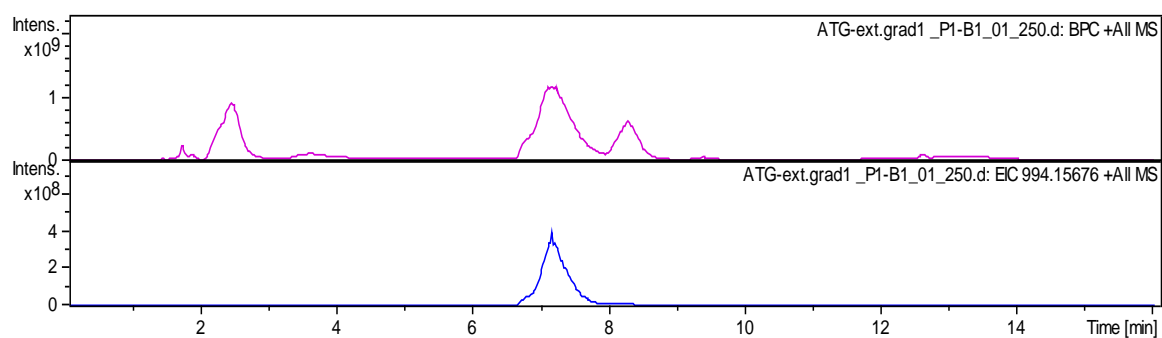


Figure 17: 1st chromatogram; HPLC base peak chromatogram of ATG-extract (1 μ l in 100 μ l of extract with 1/10 dil. in H₂O), 2nd chromatogram: extracted ion chromatogram of ATG (994.15676)

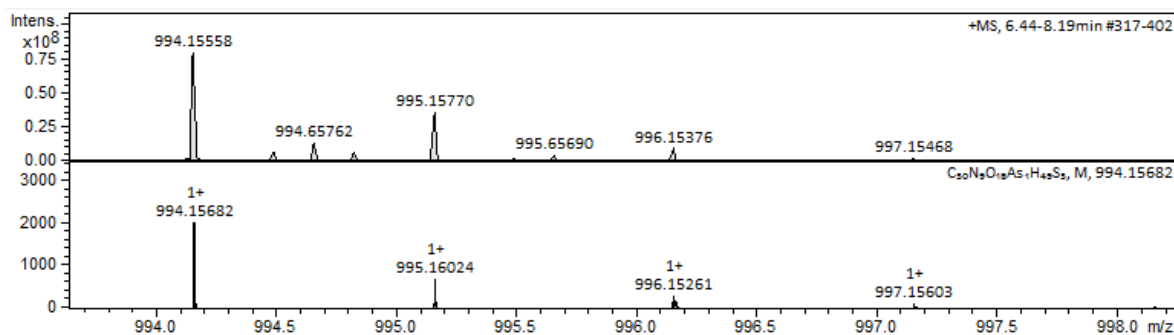


Figure 18: 1st compound spectrum: mass spectrum of ATG, 2nd compound spectrum: simulated pattern mass spectrum

3.11 HPLC-FT-ICR-MS of As-GSH mix using gradient elution

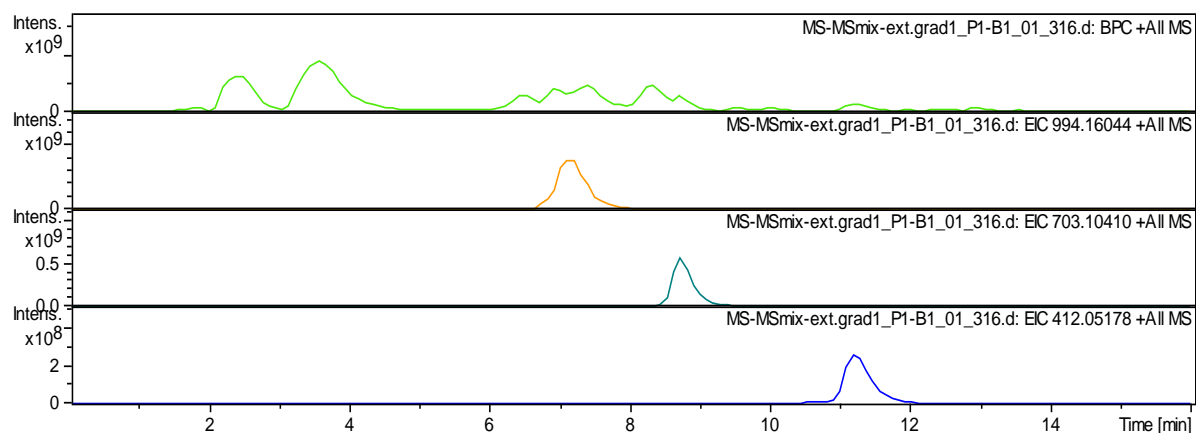


Figure 19: 1st chromatogram: HPLC base peak chromatogram of As-GSH mix-extract, 2nd chromatogram: extracted ion chromatogram of ATG, 3rd chromatogram: extracted ion chromatogram of MADG and 4th chromatogram: extracted ion chromatogram of DMAG

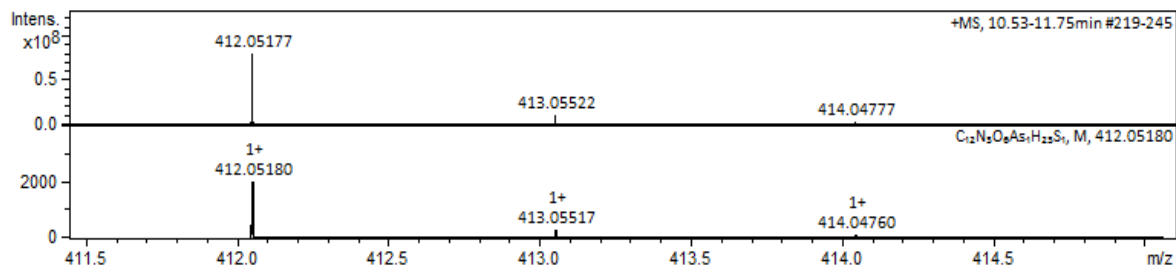


Figure 20: 1st compound spectrum: mass spectrum of DMAG, 2nd compound spectrum: simulated pattern mass spectrum

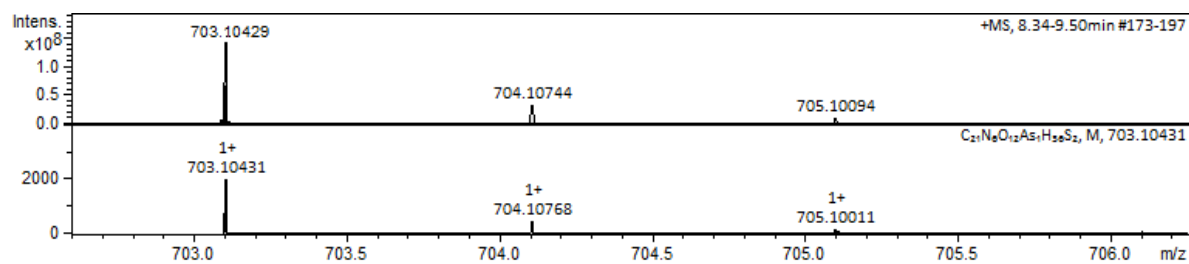


Figure 21: 1st compound spectrum: mass spectrum of MADG, 2nd compound spectrum: simulated pattern mass spectrum

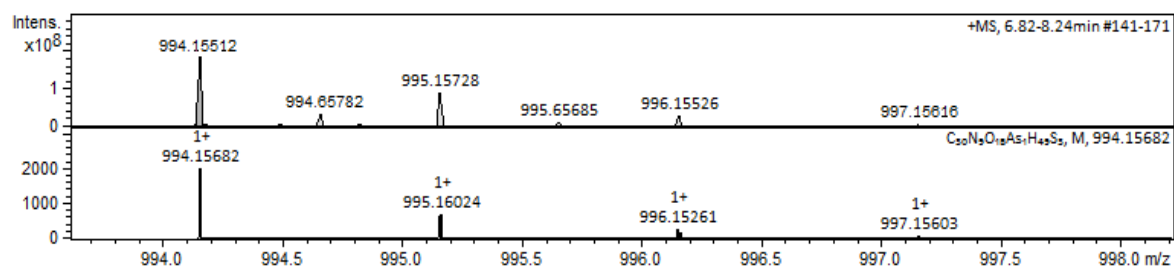


Figure 22: 1st compound spectrum: mass spectrum of ATG, 2nd compound spectrum: simulated pattern mass spectrum

4. Conclusions

- The utility of mass spectrometry for studying the interaction of peptides, with arsenic (As) has been established in the research presented in this thesis. Mass spectrometry has the advantages of providing structural information for molecules with crude fractionation, low detection limits and the ability to identify unknown species.
- In this study we observed how inorganic arsenic can be complexed to form three As-GSH species, dimethylarsinic glutathione (DMAG), monomethylarsonate glutathione (MADG), and arsenite glutathione (ATG). In the first place, dimethylarsinic acid (cacodylic) bound to GSH to form an $\text{As(SG)(CH}_3)_2$ complex. Secondly, monosodium acid methane arsonate bound to GSH to generate an $\text{As(SG)}_2(\text{CH}_3)$. Finally, sodium arsenite, As(III), bound to GSH to form an As(SG)_3 complex. The binding of all the three compounds with glutathione was done through the thiol group.
- Application of tandem mass spectrometry provided us the opportunity for mass-selection of molecular ions and observation of their specific fragment ions. As a consequence, we obtained structural information about the three synthetically prepared As-GSH complexes and proposed possible fragmentation pathways for each.
- Three different types of ionization methods were used to create ions and lead them to the mass spectrometer to be detected analyzed: ESI, SSI and MALDI ionization. SSI seems to be a softer and simpler ionization technique since it does not require any voltage application, which helps to avoid any electrochemical interference such as redox reactions. The SSI ionization source is quite simple as far it concerns the assembling and it is also very simple to the use. Quite a good signal on spectra was obtained as the change of the nebulizer's position was

possible and the generated ions were appeared with higher abundance, compared to ESI spectra. However, SSI-MS spectrum has higher background.

- The intensity of the signal in SSI-MS depends from various parameters such as, nebulizer's position, gas pressure, the solvent were the analyte is solved and the surface intention of the analyte.
- Fourier-transform–ion-cyclotrons mass spectrometry (FT-ICR-MS) was used to analyze the As-GSH complexes. The fact that it is has extremely high resolution 1.000.000 (FWHM), gave us the opportunity to make accurate and precise mass measurements, with good sensitivity and high speed. With this kind of accuracy, it is possible to determine the identity of unknown arsenicals based simply on mass. It has been really well-suited for use with pulsed ionization methods used in this study, such as MALDI and it has been also coupled with HPLC chromatography.

5. References

1. <http://www.lenntech.com/periodic/elements/as.htm>
2. <https://en.wikipedia.org/wiki/Arsenic>
3. <http://environmentalchemistry.com/yogi/periodic/As.html>
4. <http://www.greenfacts.org/en/arsenic/>
5. E. De Hoffman, J Charlette, V. Stroobant. *Mass spectrometry principles and applications*. 3rd edition, p.27,28,51,52,84-88, Wiley
6. <http://www.mrmatlas.org/glossary.php#>
7. E. Uggerud, S. Petrie, D. K. Bohme, F. Turecek, D. Schröder, H. Schwarz, D. Plattner, T. Wyttenbach, M. T. Bowers, P. B. Armentrout, S. A. Truger, T. Junker, G. Suizdak, Mark Brönstrup. *Topics in Current Chemistry: Modern Mass Spectroscopy*, pp. 1-302, 22.
8. Atsumu Hirabayashi, Minoru Sakairi, Hideaki Koizumi. Sonic Spray Ionization Method for Atmospheric Pressure Ionization Mass Spectrometry. *Anal. Chem.* 1994, 66, 4557-4559. DOI: 10.1021/ac00096a025.
9. Abdil Ozdemir, Jung-Lee Lin, Yi Sheng Wang , Chung-Hsuan Chen. A deeper look into sonic spray ionization. *RSC Adv.* 2014, 4, 61290. DOI: 10.1039/C4RA06409G.
10. https://www.chem.tamu.edu/academics/fyp/educator_resources/mass_spec/techniques.php
11. *Quantitative Chemical Analysis*, Daniel C. Harris, Vol B, 7th edition, p.550-560, *W.H. Freeman and Company, 2007*.
12. Richard A. J. O'Hair. The 3D quadrupole ion trap mass spectrometer as a complete chemical laboratory for fundamental gas-phase studies of metal mediated chemistry. *Chemical communications.* 2006, 1469–1481. DOI: 10.1039/B516348J.
13. Comisarow Melvin B., Marshal Alan G. Fourier transform ion cyclotron resonance spectroscopy. *Journal of chemical education.* 1974, 25,15,282–283. DOI: 10.1021/ed052p638.
14. http://fiehnlab.ucdavis.edu/projects/Seven_Golden_Rules/Mass_Resolution

15. Dietmar G. Schmid, Philipp Grosche, Holger Bandel, Gunther Jung. FTICR-Mass Spectrometry for High-Resolution Analysis in Combinatorial Chemistry. *Biotechnology and Bioengineering*. 2001, 71, 149-161. DOI: 10.1002/1097-0290(2000)71:2<149.
16. <http://jlab.chem.yale.edu/research/techniques/fourier-transform-icr>
17. Hayakawa T., Kobayashi Y., Cui X., Hirano S.A. New Metabolic Pathway of Arsenite: Arsenic–Glutathione Complexes are Substrates for Human Arsenic Methyltransferase Cyt19. *Arch. Toxicol.* 2005, 79, 183–191. DOI: 10.1007/s00204-004-0620-x.
18. Katerina Kanaki, Spiros A. Pergantis. Development of Mass Spectrometric Methods for Detecting Arsenic–Glutathione Complexes. *Journal of the American Society for Mass Spectrometry*. 2008, 19, 10, 1559–1567. DOI:10.1016/j.jasms.2008.06.011.
19. Okumura M., Saiki M., Yamaguchi H., Hidaka Y. Acceleration of disulfide-coupled protein folding using glutathione derivatives. *FEBS Journal*. 2011, 1137–1144. DOI: 10.1111/j.1742-4658.2011.08039.
20. Mei-Juan Han, Xiaoguang Meng, Lee Lippincott B. Determination of configuration of arsenite–glutathione complexes using ECSTM. *Toxicol Lett*. 2007, 175, 57–63. DOI: 10.1016/j.toxlet.2007.09.009.
21. Nelson Scott, Kristina M. Hatlelid, Neil E. MacKenzie, Dean E. Carter. Reactions of arsenic(III) and arsenic(V) species with glutathione. *Chem. Res. Toxicol.* 1993, 6, 102–106. DOI: 10.1021/tx00031a016.
22. Kobayashi Y., Cui X., Hirano S. Stability of arsenic metabolites, arsenic triglutathione [As(GS)₃] and methylarsenic diglutathione in rat bile. *Toxicology*. 2005, 211, 115–123. DOI:10.1016/j.tox.2005.03.001.
23. Delnomdedieu M., Basti M.M., Otvos J.D., Thomas D.J. Reduction and binding of arsenate and dimethylarsinate by glutathione: a magnetic resonance study. *Chem. Biol. Interact.* 1994, 90, 139–155. DOI: 10.1016/0009-2797(94)90099-X.
24. Anne H. Payne and Gary I. Glish. Tandem Mass Spectrometry in Quadrupole Ion Trap and Ion Cyclotron Resonance Mass Spectrometers. *Methods in Enzymology*, 402. DOI: 10.1016/S0076-6879(05)02004-5.

- 25.** Vanessa G. Santos, Thaís Regiani, Fernanda F. G. Dias, Wanderson Romao, Jose Luis Paz Jara, Clecio F. Klitzke, Fernando Coelho, and Marcos N. Eberlin. Venturi Easy Ambient Sonic-Spray Ionization. *Anal. Chem.* 2011, 83, 1375–1380. DOI: 10.1021/ac102765z.
- 26.** ProteoChem Inc., “CHCA Protocol and Production Information Sheet”
- 27.** <https://pubchem.ncbi.nlm.nih.gov/search/>
- 28.** <http://www.acdlabs.com/download/>