

The metabolism of arsenic in humans:

Bioaccessibility in the gastrointestinal tract, diffusion across lipid membranes and biotransformations in liver cells

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*To all the brave women before me who fought
for my right to get an education in science*

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ABSTRACT

Arsenic is ubiquitous in the environment and widely available to humans through their diet. Arsenic toxicity strongly depends on the chemical species absorbed and metabolised once inside an organism. The current regulations to prevent overexposure to arsenic are primarily based on inorganic arsenic species, disregarding more than 30 arsenic species also present in food. There are many studies on the effects of inorganic arsenic on human health, however, only recently the focus has shifted towards the consequences of both endogenic and exogenic complex organic arsenic species. Although little is known about general organic arsenic metabolism, recent publications have shown that some of these species are toxic to humans. Even so, health and environmental organisations still require more information to update the evidence based policies that control arsenic permitted levels in food.

The aim of this thesis was to provide new insights into the poorly understood metabolic pathways of these arsenic species in humans.

Arsenic transformations in the gastrointestinal tract determine the nature of the arsenic species available for metabolism in cells. The studies in *Chapters 2 and 3* investigated the bioaccessibility and degradation of the organic arsenic species commonly found food. Physiological based extraction tests of rice, seaweed, fish and krill oil were performed, as well as of the pure standards of the major arsenic species present in these foodstuffs (arsenic glutathione complexes, arsenosugars, arsено-fatty acids and arsено-hydrocarbons), to assess the effect of the food matrix on arsenic bioaccessibility. It was found that around 80 % of arsenic in these foods is bioaccessible after gastrointestinal digestion. Hydrolysis and demethylation of arsenic glutathione complexes and arsenosugars standards was observed, however, no transformations occurred to arsenosugars present in seaweed. Arsено-fatty acids and arsено-hydrocarbons in krill oil were also found to be bioaccessible. Demethylation of methylarsonic acid and dimethylarsinic acid from rice occurs increasing the amount of inorganic arsenic species available for metabolism in cells.

Arsenic metabolism depends on the ability of different arsenic species to traverse biological membranes. Simple diffusion provides an alternative route to mediated

transport mechanisms that can increase the amount of arsenic available for metabolism in cells. In *Chapter 4*, octanol-water and liposome-water partition coefficients were used to investigate the ability of arsenous acid, arsenate, methylarsonate, dimethylarsinate, thiomethylarsonate, thio-dimethylarsinic acid, arsenotriglutathione and monomethylarsonic diglutathione to diffuse through the lipid bilayer of cell membranes. Molecular modelling of arsenic species aided in the interpretation of results. All arsenic species with the exception of arsenate, methylarsonate and thiomethylarsonate were able to diffuse through the lipid bilayer of liposomes, with liposome-water partition coefficients between 0.04 and 0.13. The highest partition coefficients corresponded to trivalent and thio-arsenic species, which are known to exert higher toxicities than oxo-pentavalent arsenic species in humans.

Arsenous acid and arsenic acid are known carcinogens and their metabolism in mammals has been extensively studied. They are known to undergo methylation in humans, although the existing proposed pathways to explain the process are still under debate. The study in *Chapter 5* focused on the metabolism of arsenate and arsenous acid in HepG2 cells after 24 h exposure. Particular attention was paid to the species produced and their distribution within the different cellular organelles. It was found that microsomes and mitochondria are the main sites for the metabolism of these arsenic species in liver cells.

Arseno-fatty acids and arsено-hydrocarbons are common in seaweed, fish and crustaceans and have been reported to be toxic to human cells. As they were found to survive the physiological conditions of the gastrointestinal tract, *Chapter 6* investigated the biotransformations of arsено-fatty acids and arsено-hydrocarbons in HepG2 cells. Arsenic speciation of subcellular fractions after 24 h exposure showed that both arsenic species are degraded to dimethylarsinoyl propionic acid in mitochondria and peroxisomes.

A synthesis of the outcomes from this thesis is presented in *Chapter 7*. The key arsenic species for future research in human health studies are discussed. The work presented within this thesis will aid toxicologists and epidemiologists to approach arsenic related topics more effectively.

Contents

Certificate of authorship	i
Acknowledgements	iii
Abstract	vii
List of publications	xv
List of figures	xvii
Chapter 1.....	xvii
Chapter 2.....	xviii
Chapter 3.....	xix
Chapter 4.....	xx
Chapter 5.....	xxi
Chapter 6.....	xxi
Chapter 7.....	xxii
List of tables	xxiii
Chapter 1.....	xxiii
Chapter 3.....	xxiii
Chapter 4.....	xxiii
Chapter 5.....	xxiii
Chapter 6.....	xxiii
Chapter 1 – General introduction	1
Arsenic in the environment	1
Human exposure to arsenic	6
Arsenic toxicity.....	8
Arsenic metabolism	12
Inorganic arsenic species	15

Organic arsenic species	22
Scope of this research.....	30
Chapter 2 – Bioaccessibility and degradation of naturally occurring arsenic species from food in the human gastrointestinal tract	35
Introduction	35
Materials and methods.....	38
Arsenic species standards	38
Arsenic-containing foodstuffs	38
Physiologically based extraction test.....	39
Total arsenic concentration in foodstuffs samples.....	40
Arsenic speciation analysis.....	40
Results	41
Arsenic glutathione complexes and rice	41
Arsenosugars and seaweed	43
Arsenic in fish.....	46
Discussion	48
Arsenic glutathione complexes and rice	48
Arsenosugars and seaweed	50
Arsenic in fish.....	52
Concluding remarks.....	53
Chapter 3 – Degradation of arsenic containing hydrocarbons and fatty acids from krill oil and hijiki seaweed in the human gastrointestinal tract	55
Introduction	55
Materials and methods.....	58
Synthesis of arsenolipid standards	58
Arsenolipid-containing foodstuffs	59
Physiologically based extraction test.....	60

Total arsenic concentration analysis	61
Arsenic speciation analysis.....	61
Identification of arsenolipids	63
Results	64
Arsenolipid standards	64
Arsenic species in hijiki seaweed	65
Arsenic species in krill oil.....	70
Discussion	75
Concluding remarks.....	78
Chapter 4 – Evaluation of the ability of arsenic species to passively diffuse across cell membranes using octanol-water and liposome-water partition coefficients	81
Introduction	81
Materials and methods.....	87
Arsenic species standards	87
Octanol-water partition coefficients	88
Fabrication and characterization of liposomes	88
Liposome-water partition coefficients	89
Total arsenic concentration analysis	90
Statistical analysis	91
Molecular modelling of arsenic species	91
Results and discussion	91
Octanol-water partition coefficients	91
Liposomes-water partition coefficients.....	97
Concluding remarks.....	100
Chapter 5 – Subcellular distribution of the metabolites produced from arsenate and arsenous acid biotransformations in HepG2 cells.....	101
Introduction	101

Materials and methods.....	106
Arsenic standards	106
Cell culture reagents preparation	106
Cell culture and incubation	106
Cell seeding, harvesting and subcellular fractionation	107
Total protein concentration	109
Total arsenic analysis	109
Arsenic speciation.....	110
Results	111
Protein concentration in subcellular fractions.....	111
Cellular growth and viability	111
As(III) exposed HepG2 cells	112
As(V) exposed HepG2 cells.....	115
Discussion	118
Cellular growth and viability	118
Arsenic uptake.....	118
Arsenic distribution and metabolism in As(III) exposed HepG2 cells.....	119
Arsenic distribution and metabolism in As(V) exposed HepG2 cells	121
Concluding remarks.....	123
Chapter 6 – Biotransformations of dimethylarsinoyl decane and dimethylarsinoyl undecanoic acid in HepG2 cells.....	125
Introduction	125
Materials and methods.....	127
Arsenic standards	127
Cell culture reagents preparation	127
Cell culture and incubation	127

Cell seeding, harvesting and subcellular fractionation	127
Total protein concentration	128
Total arsenic analysis	128
Arsenic speciation.....	129
Identification of unknown arsenic species	130
Results	131
Protein concentration in subcellular fractions.....	131
Cellular growth and viability	131
AsFA-C11 exposed HepG2 cells	132
AsHC-C10 exposed HepG2 cells	135
Discussion	138
Cellular growth and viability	138
Arsenic uptake.....	138
Arsenic distribution and metabolism in AsFA-C11 exposed HepG2 cells.....	138
Arsenic distribution and metabolism in AsHC-C10 exposed HepG2 cells	143
Concluding remarks.....	146
Chapter 7 – General conclusions and further directions.....	147
References	155
Appendices	189
Chapter 3.....	189
Chapter 4.....	215
Chapter 6.....	216

List of publications

Chapter 2

Teresa Chávez-Capilla, Mona Beshai, William Maher, Tamsin Kelly, Simon Foster, Bioaccessibility and degradation of naturally occurring arsenic species from food in the human gastrointestinal tract, Food Chemistry, 2016, 212, 189-197.

Chapter 4

Teresa Chávez-Capilla, William Maher, Tamsin Kelly, Simon Foster, Evaluation of the ability of arsenic species to passively diffuse across cell membranes using octanol-water and liposome-water partition coefficients, Journal of Environmental Sciences, 2016, 49, 222-232.

List of figures

Chapter 1

Figure 1-1. Major inorganic (highlighted in blue) and organic (highlighted in orange) arsenic species found in nature. The moiety –SG corresponds to a molecule of glutathione.....	5
Figure 1-2. Arsenic species present in drinking water and food. Photo credit: Pexels and Ken Hawkins (CC-BY-2.0).....	8
Figure 1-3. General metabolic pathway for xenobiotics in humans, adapted from (Goodman and Gilman, 2006, Casarett and Doull, 2008, Rang et al., 2012).....	15
Figure 1-4. Suggested metabolic pathways by Challenger (A), Hayakawa (B) and Naranmandura (C) for inorganic arsenic in mammals, adapted from (Kobayashi, 2010, Rehman and Naranmandura, 2012). Abbreviations: <i>AS3MT</i> (enzyme arsenic III methyltransferase), GSH (glutathione molecule), SAM (<i>S</i> -adenosyl-L-methionine), –SG (glutathione moiety).....	16
Figure 1-5. Proposed pathways for arsenate reduction in mammalian cells, adapted from (Foster, 2007, Aposhian et al., 2004, Caspi et al., 2006). Abbreviations: <i>GSTO1-1</i> (glutathione- <i>S</i> -transferase omega class 1-1), <i>PNP</i> (purine nucleoside phosphorylase)....	17
Figure 1-6. Proposed mechanisms for oxidative methylation (A) and reductive methylation through nucleophilic attack to carbon (B), adapted from (Naranmandura et al., 2006). Abbreviations: <i>AS3MT</i> (enzyme arsenic III methyltransferase), GSH (glutathione molecule), –R (thiol moiety of the cysteine residue in a protein), –SG (glutathione moiety), SAM (<i>S</i> -adenosyl-L-methionine)	18
Figure 1-7. Proposed metabolism of thio-arsenic species in red blood cells, adapted from (Hua Naranmandura, 2008). Abbreviations: ECF (extracellular fluid), GSH (glutathione molecule), Hb– (haemoglobin), ICF (intracellular fluid).....	21
Figure 1-8. Metabolic pathway suggested for the degradation of phosphatidylarsenocholine in mice, adapted from (Fukuda et al., 2011)	25
Figure 1-9. Suggested metabolic pathways of arsenolipid formation from DMA(III), adapted from (de Bettencourt et al., 2011).....	25

Figure 1-10. Suggested pathway for arenosugar formation from DMA, adapted from (Edmonds and Francesconi, 1987a).....	26
Figure 1-11. Suggested biogenesis of arseno-fatty acids and arseno-hydrocarbons, adapted from (Rumpler et al., 2008, Taleshi et al., 2008).....	28
Figure 1-12. Overview of proposed pathways for arsenic metabolism from inorganic to complex organic arsenic species.	29

Chapter 2

Figure 2-1. Structures of the arsenic species commonly encountered in food and water relevant to this study. The moiety –SG in ATG, MADG and DMAG corresponds to a molecule of glutathione, in which a sulfur atom is bound to arsenic.....	36
Figure 2-2. Arsenic species (%) identified after the PBET (stomach (s) and small intestine (i) phases) of pure arsenic glutathione complex standards: a) arsenotriglutathione (ATG), b) monomethylarsonic diglutathione (MADG) and c) dimethylarsoglutathione (DMAG).	42
Figure 2-3. Chromatograms of rice samples (CRM-T07151QC) before (a) and after (b) PBET. Arsenic concentrations (%) in the stomach (s) and small intestine (i) phases are shown for each of the arsenic species identified.....	43
Figure 2-4. Arsenic species (%) identified after the PBET (stomach (s) and small intestine (i) phases) of pure arenosugar standards: a) arenosugar glycerol (O-Gly) b) arenosugar-sulfate (O-SO ₄), c) arenosugar-sulfonate (O-SO ₃), and d) arenosugar-phosphate (O-PO ₄).	44
Figure 2-5. Chromatograms of the arenosugar-phosphate (O-PO ₄) standard after the PBET: a) stomach and small intestine phases under anion exchange conditions, b) stomach and small intestine phases under anion exchange conditions coupled with hydride generation and c) small intestine phase under cation exchange conditions.....	45
Figure 2-6. Chromatograms of macroalgae samples (NIES CRM Sargasso N.9) before (a) and after (b) the PBET. Arsenic concentrations (%) in the stomach (s) and small intestine (i) phases are shown for each of the arsenic species identified.	47
Figure 2-7. Chromatograms of the dimethylarsinoyl propionic acid (DMAP) standard before and after the PBET.	47

Figure 2-8. Chromatograms of fish samples (CRM DORM-3) before and after the PBET. Arsenic concentrations (%) in controls (c), and the stomach (s) and small intestine (i) phases are shown for each of the arsenic species identified.	48
Figure 2-9. Proposed mechanism for the degradation of arsenic glutathione complexes (a) and arsenic species in rice (b) during PBET.....	50
Figure 2-10. Proposed mechanism for the degradation of arenosugars standards during PBET.	52

Chapter 3

Figure 3-1. Chemical structures of the types of arsenolipids known to date.	56
Figure 3-2. Chemical structures of dimethylarsinoyl undecanoic acid and dimethylarsinoyl decane.	59
Figure 3-3. Chromatograms of dimethylarsinoyl undecanoic acid (AsFA-C11) samples before (c) and after stomach (s) and small intestine (i) digestions.	65
Figure 3-4. Chromatograms of dimethylarsinoyl decane (AsHC-C10) samples before (c) and after stomach (s) and small intestine (i) digestions.	65
Figure 3-5. Chromatograms of water soluble (a) and lipid soluble (b) arsenic species in hijiki seaweed samples before (c) and after stomach (s) and small intestine (i) digestions. The percentages of lipid soluble arsenic species are calculated relative to the total arsenic concentration in the corresponding fraction.	67
Figure 3-6. Proposed structures for the arsenolipids identified in hijiki seaweed. The structures of AsHC 332, AsHC 360, GlyAsL 1012, GlyAsL 1014, GlyAsL 1042, AsPC 985 and AsPE 1035 are adapted from (Garcia-Salgado et al., 2012, Viczek et al., 2016).69	
Figure 3-7. Anion (A) and cation (B) exchange chromatograms of water soluble arsenic species in krill oil before (c) and after the stomach (s) and small intestine (i) digestions. C) Reverse-phase chromatograms of lipid soluble arsenic species in krill oil before (c) and after the stomach (s) and small intestine (i) digestions. The percentages of lipid soluble arsenic species are calculated relative to the total arsenic concentration in the corresponding fraction.	72
Figure 3-8. Proposed structures for the arsenolipids identified in krill oil samples. The structures of AsHC 330 and AsFA 302 have been adapted from (Lischka et al., 2013,	

Amayo et al., 2013). Double bonds in aliphatic carbon chains are arbitrary as the structures need to be confirmed by high resolution LC-MS.....74

Chapter 4

Figure 4-1. Structures of the arsenic species of interest for this chapter. The moiety –SG in ATG and MADG corresponds to a molecule of glutathione, in which a sulfur atom is bound to arsenic.82

Figure 4-2. Octanol-water partition coefficients (P_{OW}) of arsenic species (mean \pm s.d, n=4). Arsenic recovery \pm s.d is given in parentheses.93

Figure 4-3. Modelled molecular structures and electrostatic potential maps for the arsenic species: arsenous acid (a), arsenate (b), methylarsonate (c), dimethylarsinate (d), thio-methylarsonate (e), thio-dimethylarsinic acid (f), monomethylarsonic diglutathione (g) and arsenotriglutathione (h). The scale next to each molecule shows the values of the electrostatic potential for each colour. The red and dark blue regions correspond to the most negative and most positive regions in the molecule, respectively.96

Figure 4-4. Modelled molecular structure and electrostatic potential map of 1-octanol. The scale next to the molecule shows the values of the electrostatic potential for each colour. The red and dark blue regions correspond to the most negative and most positive regions in the molecule, respectively.....97

Figure 4-5. Modelled molecular structures and electrostatic potential maps for dithio-methylarsonic acid (a), trithio-methylarsonic acid (b) and dithio-dimethylarsinic acid (c). The scale next to each molecule shows the values of the electrostatic potential for each colour. The red and dark blue regions correspond to the most negative and most positive regions in the molecule, respectively.....97

Figure 4-6. Structures of phosphatidylcholine (left) and phosphatidylethanolamine (right) and modelled molecular structures and electrostatic potential maps for their polar heads. The scale next to each molecule shows the values of the electrostatic potential for each colour. The red and dark blue regions correspond to the most negative and most positive regions in the molecule, respectively.....98

Figure 4-7. Liposome-water partition coefficients (P_{LW}) of arsenic species (mean \pm s.d, n=3). Arsenic recovery \pm s.d is given in parentheses.100

Chapter 5

Figure 5-1. Proposed metabolic pathways by Challenger (A), Hayakawa (B), Naranmandura (C) and Wang (D) for inorganic arsenic in mammals, adapted from (Kobayashi, 2010, Rehman and Naranmandura, 2012, Wang et al., 2012). Abbreviations: AS3MT (enzyme arsenic III methyltransferase), ATC (arsenotricysteine), DMAC (dimethylarsocysteine), GSH (glutathione molecule), MADC (monomethylarsonic dicysteine), SAM (<i>S</i> -adenosyl-L-methionine), -SG (glutathione moiety).....	105
Figure 5-2. Schematic representation of the protocol for subcellular fractionation.	109
Figure 5-3. Cell growth and viabilities of control and As(III) and As(V) exposed HepG2 cells.	112
Figure 5-4. Arsenic concentrations and species distribution in subcellular fractions of HepG2 cells after 24 h exposure to As(III). S2, nuclei and cell membranes; P2, granules; P3, mitochondria; S4, cytosol; P4, lysosomes, peroxisomes and microsomes.	113
Figure 5-5. Arsenic concentrations and species distribution in subcellular fractions of HepG2 cells after 24 h exposure to As(V). S2, nuclei and cell membranes; P2, granules; P3, mitochondria; S4, cytosol; P4, lysosomes, peroxisomes and microsomes.	116
Figure 5-6. Proposed sites for As(III) biotransformations in HepG2 cells.	121
Figure 5-7. Proposed sites for As(V) biotransformations in HepG2 cells.	123

Chapter 6

Figure 6-1. Chemical structures of dimethylarsinoyl undecanoic acid and dimethylarsinoyl decane.	127
Figure 6-2. Cell growth and viabilities of control and AsFA-C11 and AsH-C10 exposed HepG2 cells.	131
Figure 6-3. Arsenic concentrations and species distribution in subcellular fractions of HepG2 cells after 24 h exposure to AsFA-C11. S2, nuclei and cell membranes; P2, granules; P3, mitochondria; S4, cytosol; P4, lysosomes, peroxisomes and microsomes.	133
Figure 6-4. Arsenic concentrations and species distribution in subcellular fractions of HepG2 cells after 24 h exposure to AsHC-C10. S2, nuclei and cell membranes; P2,	

granules; P3, mitochondria; S4, cytosol; P4, lysosomes, peroxisomes and microsomes..... 136

Figure 6-5. Proposed pathway for AsFA-C11 biotransformations in HepG2 cells..... 142

Figure 6-6. Proposed pathways for AsHC-C10 biotransformations in HepG2 cells.... 145

Chapter 7

Figure 7-1. General metabolic pathway for xenobiotics in humans, adapted from (Goodman and Gilman, 2006, Casarett and Doull, 2008, Rang et al., 2012). The processes represented by the arrows filled in red involve transport through cell membranes..... 147

Figure 7-2. Suggested activation of DMA by citrate..... 149

Figure 7-3. Increasing order (left to right) of the ability of arsenic species to passively diffuse across cell membranes..... 151

List of tables

Chapter 1

Table 1-1. LC ₅₀ and LC ₇₀ values of different arsenic species.	11
---	----

Chapter 3

Table 3-1. Lipid soluble arsenic species detected in hijiki seaweed by LC-QQQ analysis.	68
---	----

Table 3-2. Lipid soluble arsenic species detected in krill oil by LC-QQQ analysis.	73
--	----

Chapter 4

Table 4-1. Arsenic uptake systems in cells, adapted from (Zangi and Filella, 2012).....	83
--	----

Table 4-2. Arsenic efflux systems in cells, adapted from (Zangi and Filella, 2012).	85
---	----

Table 4-3. Molecular properties computed for the arsenic species of interest. The steric volume includes the electron cloud into the molecule volume.	94
---	----

Chapter 5

Table 5-1. Arsenic concentrations and percentages of arsenic species in the subcellular fractions of HepG2 cells after 24 h exposure to As(III).	114
--	-----

Table 5-2. Arsenic concentrations and percentages of arsenic species in the subcellular fractions of HepG2 cells after 24 h exposure to As(V).....	117
---	-----

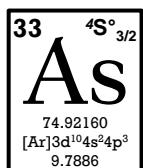
Chapter 6

Table 6-1. Arsenic concentrations and percentages of arsenic species in the subcellular fractions of HepG2 cells after 24 h exposure to AsFA-C11.	134
---	-----

Table 6-2. Arsenic concentrations and percentages of arsenic species in the subcellular fractions of HepG2 cells after 24 h exposure to AsHC-C10.....	137
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CHAPTER 1 – General introduction

Arsenic in the environment



with many other elements, arsenic has a biogeochemical cycle that goes from naturally occurring rocks to high complexity living organisms. In the Earth's crust, arsenic ranks somewhere between the 46th and the 54th most abundant element (Tyson, 2013), with a total amount of approximately 4.01×10^{16} kg (Matschullat, 2000). Pure elemental arsenic is rarely found in the environment, instead, it exists in a number of minerals usually associated with sulfur; or adsorbed onto iron and aluminium oxides, clays, or iron sulfides (Ravenscroft et al., 2009a, Matschullat, 2000). The most common primary arsenic-bearing minerals are orpiment (As_2S_3), realgar (AsS), arsenopyrite (FeAsS_2), mispickel (FeAsS), scorodite ($\text{FeAsO}_4 \cdot 2\text{H}_2\text{O}$), enargite (Cu_3AsS_4), tennantite ($\text{Cu}_{12}\text{As}_4\text{S}_{13}$), cobaltite (CoAsS), loellingite (FeAs_2), niccolite (NiAs), arsenolite (As_2O_3) and claudetite (As_2O_3) (Ravenscroft et al., 2009a, Matschullat, 2000). Meteorological phenomena and geological activity promote weathering and erosion of arsenic-containing rocks, while geochemical and biological activity mobilise and leach arsenic from minerals as arsenate (As(V)) and arsenous acid (As(III)) (Mandal and Suzuki, 2002, Roy and Saha, 2002, Stolz et al., 2006). Arsenate and As(III) are initially taken up by bacteria, fungi, plants and algae (Lunde, 1977, Cullen and Reimer, 1989). Whilst the first three classes of organisms only oxidise/reduce, thiolate and methylate inorganic arsenic (Tamaki and Frankenberger, 1992, Challenger, 1947, Dc.Rubin et al., 2014, Finnegan and Chen, 2012); algal metabolism yields more complex organic forms, such as arenosugars and arsenolipids (Lunde, 1973, Francesconi, 2010, Garcia-Salgado et al., 2012, Raab et al., 2013, Morita and Shibata, 1988, Duncan et al., 2015). Animals are able to incorporate these arsenic species through their diet, which are subsequently converted into different methylated and thio-methylated arsenic species or excreted unchanged (Edmonds and Francesconi, 1987a, Francesconi, 2010).

Figure 1-1 shows the chemical structures of the major arsenic species found in nature, a compilation of inorganic and organic species in which arsenic exists with oxidation states +5 (predominant under normal environmental conditions), +4, +3 or -3. Inorganic arsenic species are not only limited to arsenic-bearing minerals, As(V) and As(III) (Figure 1-1). Mono-, di-, tri- and tetra-thioarsenates ($\text{AsO}_3\text{S}^{3-}$, $\text{AsO}_2\text{S}_2^{3-}$, AsOS_3^{3-} and AsS_4^{3-}) (Figure 1-1) have been reported in sulfidic waters. They originate from the thiolation and oxidation of As(III) (Wallschläger and Stadey, 2007, Maher et al., 2013b). Arsine (AsH_3) (Figure 1-1), produced in soils by bacteria and fungi under anaerobic conditions (Tamaki and Frankenberger, 1992, Cheng and Focht, 1979), was extremely relevant in the development of the detection methods that have made possible the identification of all the other arsenic species known today (Marsh, 1836).

Organic arsenic species are characterised by the presence of one to four methyl groups covalently bound to arsenic. Mono-, di- or trimethylated arsenic species can also bind to oxygen (oxo-methylated arsenic species) or sulfur (thio-methylated arsenic species). The methylated arsenic species most commonly found in the environment are methylarsonous acid (MA(III)), dimethylarsinous acid (DMA(III)), methylarsonic acid (MA) and dimethylarsinic acid (DMA) (Figure 1-1). These arsenic species are metabolites in practically every organism (Li et al., 2016), with few exceptions such as chimpanzees, marmoset monkeys and guinea pigs (Vahter et al., 1995, Zakharyan et al., 1996, Healy et al., 1997). Other important methylated arsenic species include trimethylarsine (TMA) (Figure 1-1), which is specific to microorganisms (Cullen and Bentley, 2005, Wang et al., 2014); dimethylarsinoyl acetic acid (DMAA), trimethylarsinoyl propionic acid (TMAP) and tetramethylarsonium ion (TETRA) (Figure 1-1), found marine organisms (Francesconi et al., 2000, Geiszinger et al., 2002, Larsen, 1995); and trimethylarsine oxide (TMAO) and dimethylarsenoethanol (DMAE) (Figure 1-1) that have been reported in seaweed, shellfish and some mammals (Edmonds et al., 1982, Edmonds and Francesconi, 1987b, Francesconi et al., 2002, Marafante et al., 1987). Arsenobetaine (AB) and arsenocholine (AC) resemble glycine betaine and choline, where nitrogen is replaced by arsenic (Figure 1-1). Arsenobetaine was the first complex organic arsenic species identified in marine organisms (Edmonds et al., 1977) and both AB and AC are widely present in fish, molluscs and crustaceans (Norin and Christakopoulos, 1982, Edmonds et al., 1982, Lawrence et al., 1986, Norin et al., 1983,

Norum et al., 2005). Accumulation of AB at high concentrations in mushrooms has also been reported (Byrne et al., 1995, Nearing et al., 2014). Thio-methylated arsenic species include thio-methylarsonic acid (thio-MA), thio-dimethylarsinic acid (thio-DMA), dithio-dimethylarsinic acid (dithio-DMA), thio-dimethylarsenoethanol (thio-DMAE) and thio-dimethylarsinoyl acetic acid (thio-DMAA) (Figure 1-1). They are formed in microorganisms (Dc.Rubin et al., 2014, Diaz-Bone et al., 2009), marine organisms (Maher et al., 2013b) and mammals (Naranmandura et al., 2007, Naranmandura and Suzuki, 2008, Raml et al., 2006, Hansen et al., 2004a). The arsenic glutathione complexes dimethylarsoglutathione (DMAG) and monomethylarsonic glutathione (MADG) (Figure 1-1) can also be classified as thio-methylated arsenic species, in which the sulfur atom bound to arsenic comes from the cysteine residue of a glutathione molecule (GSH). In arsenotriglutathione (ATG) (Figure 1-1), arsenic is not bound to any carbon atom and cannot be considered an organic arsenic species as per definition. Nevertheless, arsenic in ATG is linked to three glutathione molecules, thus being often grouped with DMAG and MADG. Thio-methylated arsenic species, including arsenic glutathione complexes, are probably derived from arsenic-containing proteins (Raab et al., 2007, Rehman and Naranmandura, 2012) and are considered to be intermediates of arsenic metabolism to finally yield oxo-methylated species (Wang et al., 2015, Hayakawa et al., 2004).

Large organic arsenic species such as arenosugars and arsenolipids predominantly occur in marine ecosystems (Francesconi, 2010, Francesconi and Schwerdtle, 2016). Arenosugars and thio-arnenosugars are metabolic products found in earthworms, cyanobacteria, phytoplankton, algae and molluscs (Foster, 2007, Duncan et al., 2013, Francesconi, 2010, Geiszinger et al., 1998, Miyashita et al., 2012, Kahn et al., 2005, Soeroes et al., 2005, Nischwitz and Pergantis, 2006, Schmeisser et al., 2004). Their basic structure is dimethylarsinoylribose in which arsenic can be either bound to oxygen or to sulfur (Figure 1-1). The four main arenosugars are arenosugar-glycerol (O-Gly), arenosugar-sulfate (O-SO₄), arenosugar-sulfonate (O-SO₃) and arenosugar-phosphate (O-PO₄), which differ from each other on the –R group attached to the ribose ring (Figure 1-1). Trimethyl-arnenosugars are found in algae (Figure 1-1) (Edmonds, 2000, Shibata and Morita, 1988, McSheehy et al., 2002). Arsenic-containing lipids, commonly called arsenolipids, were first proposed in marine oils (Sadolin, 1928) and comprise a number of

lipid soluble arsenic species that can be classified in seven different groups. Arseno-fatty acids (AsFAs) resemble biologically important fatty acids (Rumpler et al., 2008), with a carboxylic acid on one side and a dimethylarsinoyl on the other (Figure 1-1). As for arenosugars and methylated arsenic species, the oxygen linked to arsenic in AsFAs can be substituted by sulfur, leading to thio-arseno fatty acids (thio-AsFAs) (Schmeisser et al., 2005). Arseno-hydrocarbons (AsHCs) are long chain alkanes with a dimethylarsinoyl group covalently bound to one of the ends of the carbon backbone (Figure 1-1) (Taleshi et al., 2008). Both AsFAs and AsHCs exist saturated and unsaturated and have been found in fish (Lischka et al., 2013, Taleshi et al., 2010), fish oil (Rumpler et al., 2008, Amayo et al., 2011, Taleshi et al., 2008) and seaweed (Raab et al., 2013, Garcia-Salgado et al., 2012). Cationic trimethylarsonio fatty alcohols (TMAsFOHs), identified in fish oil (Amayo et al., 2013), consist of long fatty alcohols with a trimethylarsonio group at the other end of the chain (Figure 1-1). Arseno-phospholipids (AsPLs), arsenic-containing phosphatidylcholines (AsPCs) and arsenic-containing phosphatidylethanolamines (AsPEs) have been found only in fish (Francesconi et al., 1990, Edmonds et al., 1992, Hanaoka et al., 1999, Ebisuda et al., 2003, Viczek et al., 2016). These are similar to membrane glycerophospholipids; the former contain arsenocholine instead of the nitrogen based equivalent (Figure 1-1) (Francesconi et al., 1990), and in AsPCs and AsPEs arsenic is found in one of the fatty acids, as an AsFA, instead of in the choline or ethanolamine moiety of the molecule (Figure 1-1) (Viczek et al., 2016). Glycoarsenolipids (GlyAsLs) or arenosugar-phospholipids¹ have the same core structure of arenosugars, in which the ribose ring is connected to a phospholipid (Figure 1-1) (Garcia-Salgado et al., 2012). Glycoarsenolipids are predominant in seaweed (Morita and Shibata, 1988, Garcia-Salgado et al., 2012, Raab et al., 2013).

In addition, there is an unclassified type of arsenic usually referred to as ‘residue’ that includes all the non-extractable arsenic species bound to tissues, all of which release inorganic arsenic or DMA after hydrolysis (Francesconi, 2003).

¹ While the literature often refers to arenosugar-phospholipids (AsPLs), these arsenolipids are referred to as glycoarsenolipids (GlyAsLs) throughout this thesis to avoid confusion with arseno-phospholipids.

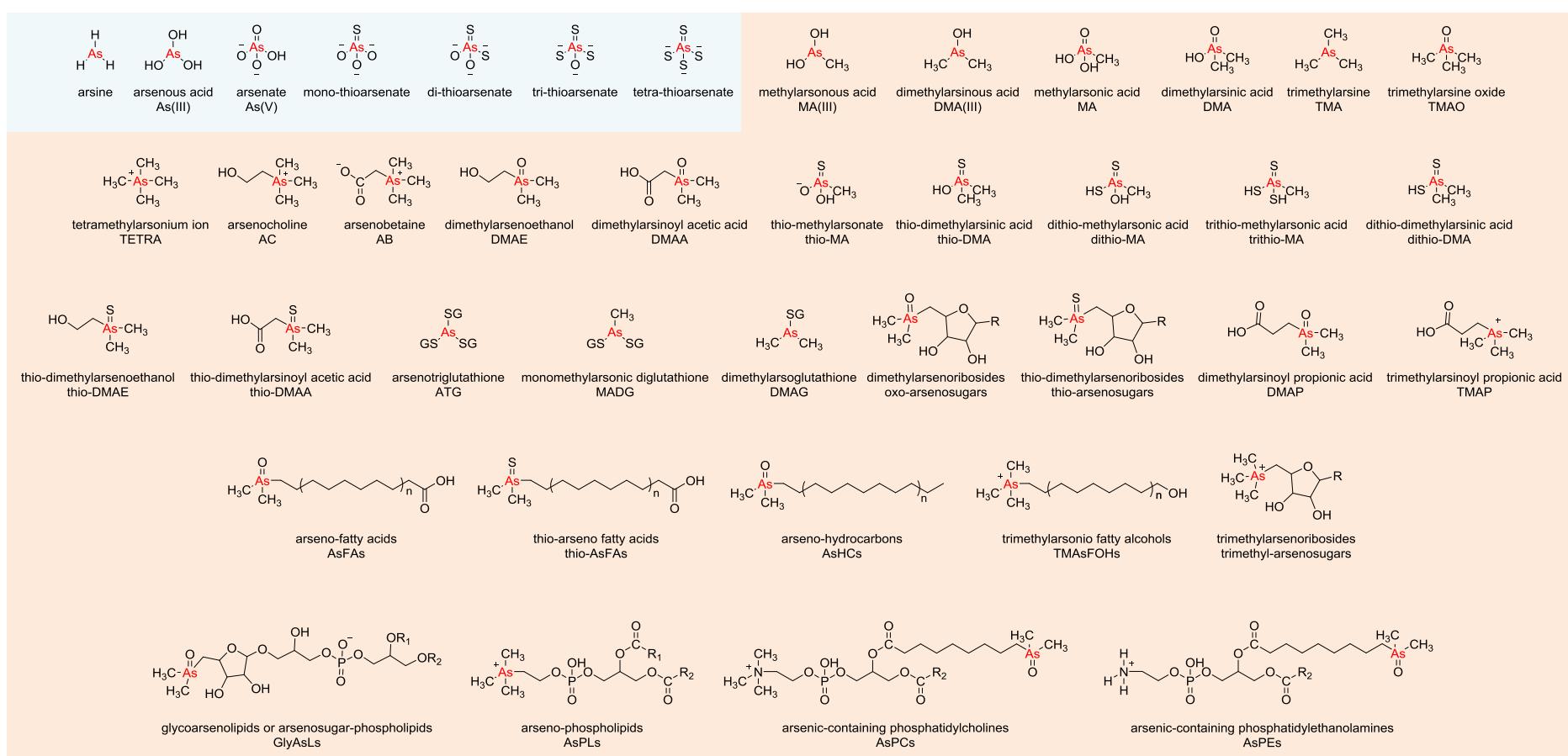


Figure 1-1. Major inorganic (highlighted in blue) and organic (highlighted in orange) arsenic species found in nature. The moiety –SG corresponds to a molecule of glutathione.

Human exposure to arsenic

Humans are not exempt from arsenic exposure. Excluding deliberate poisonings, humans are exposed to arsenic from a wide number of sources of both natural and anthropogenic origin; making arsenic the 31st most abundant element in the human body (Tyson, 2013). Natural emissions of arsenic predominantly arise from terrestrial and submarine volcanic exhalations and eruptions, with an annual release of about 2×10^7 kg of arsenic (Matschullat, 2000). Dissolution of minerals and ores, microbial liberation of volatile arsenic species, exudates from vegetation and wind-blown dusts also contribute to arsenic availability in the environment (Sharma et al., 2014).

In addition, certain industrial activities can increase human exposure to arsenic. These include mining and smelting of copper, lead, cobalt and gold ores; metal treatment and galvanising; high-temperature combustion (fossil fuel burning power plants, waste incineration, cement works); manufacture of glass, semiconductors and sheep dips; hardening and improvement of projectiles; chemical production (e.g. pigments, wood preservatives, pesticides, pyrotechnics, drying agents for cotton, oil and dissolvent recycling) and pharmaceutical industries (e.g. animal feed additives and medicaments) (World Health Organization, 2010, Matschullat, 2000). Industrial activities are responsible for most of the arsenic released into the atmosphere, which is greater in the northern hemisphere (Cullen, 2008). Even so, exposure to arsenic from air is low, with levels of the element up to 920 ng m⁻³ in very polluted countries like China (Hughes et al., 2011, Cullen, 2008).

Contaminated water, however, can increase the intake of both inorganic and organic arsenic species through the diet. Arsenate and As(III) are present in fresh and sea water, with arsenic concentrations ranging from 0.001 to 5 mg L⁻¹ and 0.001 to 0.008 mg L⁻¹, respectively (Mandal and Suzuki, 2002). Due to its similarity to phosphate, As(V) is able to cross membranes in algae (Francesconi, 2010), where arsenobetaine, arenosugars and arsenolipids are formed (Morita and Shibata, 1990). The subsequent ingestion of arsenic species by marine and freshwater animals can lead to formation of other arsenolipids (Edmonds et al., 1992). When humans consume fish, shellfish, crustaceans and seaweed, they incorporate all the accumulated arsenic in marine organisms, where concentrations range between 5 and 100 µg g⁻¹ dry mass (Francesconi, 2010). Likewise,

groundwater represents a major source of arsenic exposure, as it is used for irrigation and to provide drinking water in many countries around the world. Arsenic in groundwater is likely to occur evenly on a global scale (Amini et al., 2008), where concentrations range from 0.5 to 50000 $\mu\text{g L}^{-1}$ (Singh et al., 2015). Lamentably, developing countries often lack of remediation infrastructures and become more susceptible to arsenic exposure from groundwater than developed countries (Ravenscroft et al., 2009b). During the 20th century, several cases of arsenic contamination in groundwater were particularly significant in Argentina, Taiwan, U.S., Canada, northern Chile, West Bengal (India), Inner Mongolia (China), Hungary, Bangladesh, Vietnam and Mexico (Singh et al., 2015, Ravenscroft et al., 2009b).

Arsenic polluted soils primarily affect agricultural crops (Da Sacco et al., 2012). The amount of arsenic found in cultivable soils increases by deposition from contaminated groundwater, mining activity and the use of arsenic-containing pesticides (Rahman et al., 2011). Even though vascular plants are able to take up As(V) through their roots (Wu et al., 2011), crops do not usually exceed 0.05 $\mu\text{g g}^{-1}$ of arsenic in dry mass (Francesconi, 2010). Rice is an exception as it is able to accumulate elevated concentrations of arsenic in shoots and grains, ranging from 0.1 to 0.4 $\mu\text{g g}^{-1}$ (Francesconi, 2010, Carey et al., 2010, Maher et al., 2013a). Livestock grazed on contaminated crops also leads to arsenic consumption in humans (Ravenscroft et al., 2009c). Additionally, arsenic-containing feed additives (roxarsone, carbasone, arsanilic acid and nitarsone) have been used since the 1940s to prevent disease and increase feed conversion efficiency, promoting growth in chickens, turkeys and swine (Cullen, 2008). Consequently, both organic and inorganic arsenic species exist in products derived from these animals, as well as in the crop fertilisers based on poultry manure (Nachman et al., 2016). In 2009, the Centre for Food Safety presented a petition to the Food and Drug Administration (FDA) to withdraw those chemicals from the market. The FDA banned the use of roxarsone, carbasone and arsanilic acid in 2013 (Strom, 2013), whereas nitarsone remained on the market until early 2016 (FDA, 2016).

Among other sources of arsenic exposure to humans, occupational exposure in workers and the intake of arsenic-based pharmaceuticals are worth mentioning. For centuries arsenic has been used to improve human health. The Greeks and Chinese

utilised arsenic-containing compounds in the treatment of skin diseases, alopecia, cancer, breathing problems and regeneration of lost teeth. Indian and other Asian cultures employed arsenic trioxide as a remedy for sexual disorders. During the 16th and 17th centuries, arsenic was an ingredient of magic potions to prevent the bubonic plague; and, in a region of Austria near the actual city of Graz, it was eaten by those who believed in its properties to provide beauty, courage, sexual potency and agility, to aid digestion, and to protect against infections (Cullen, 2008). Many of these practices are nowadays obsolete, however, arsenic trioxide is still used to treat leukaemia in cases where first-line chemotherapy does not work (Cullen, 2008).

In general, the greatest intake of arsenic by humans arises from the consumption of food and beverages, such as drinking water, seafood and rice (Naujokas et al., 2013, Sharma et al., 2014, de la Calle et al., 2012). Figure 1-2 summarises the variety of arsenic species likely to be absorbed through water and diet.

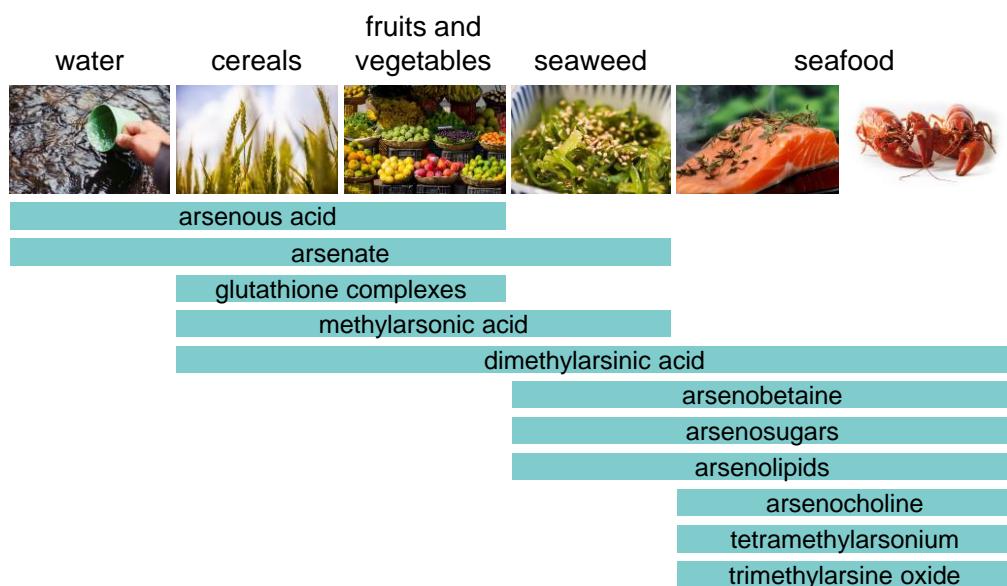


Figure 1-2. Arsenic species present in drinking water and food. Photo credit: Pexels and Ken Hawkins (CC-BY-2.0).

Arsenic toxicity

While acute arsenic poisoning commonly leads to death, chronic exposure, or arsenicosis, has been associated with several major diseases that, if not directly derived from arsenic uptake, can be enhanced in its presence. The major adverse effects

associated with long term arsenic exposure are skin lesions (hyperkeratosis, hypopigmentation and hyperpigmentation), cancer (skin, bladder, lungs) and cardiovascular diseases (Blackfoot, peripheral vascular disease, coronary heart disease, myocardial infarction and stroke) (European Food Safety Authority, 2009). Other health problems include atherosclerosis, hypertension, anaemia, liver disorders, kidney damage and peripheral neuropathy (European Food Safety Authority, 2009). Cancers of kidney, liver and prostate, as well as type II diabetes, may also develop after arsenic exposure, but the evidence of this occurring is still inconclusive (Mead, 2005, European Food Safety Authority, 2009, World Health Organization, 2010).

Arsenic toxicity is intimately related to the chemical form absorbed and produced once inside an organism, and its potential for harm is generally assumed to decrease as follows: arsines > trivalent organic species > trivalent inorganic species > pentavalent inorganic species > pentavalent organic species > arsonium species > elemental arsenic (Petrick et al., 2000, Michalski et al., 2012). The LC₅₀ value (concentration required to kill 50 % of test organisms)² for inhaled arsine (AsH₃) ranges from 16.2 to 145.8 mg m⁻³, making this the most toxic arsenic species (Cullen and Bentley, 2005, World Health Organization, 2002). As it is methylated, arsine's toxicity decreases, with LC₅₀ values for inhaled TMA greater than 64.8 g m⁻³ (Cullen and Bentley, 2005). The toxicity of the inorganic arsenic species As(III) and As(V) has been extensively studied and As(III) is often used as a comparison to assess the toxicity of other arsenic species. After 24 h exposure, the respective LC₅₀ values for As(III) and As(V) are 170 µM and 1530 µM in UROtsa cells; and 130 µM and 500 µM in hepatocytes (Dopp et al., 2008). Within the same experiments, the toxicity of the trivalent organic arsenic species MA(III) and DMA(III) was found to be higher than that of As(III) (Dopp et al., 2008). For MA(III), the LC₅₀ value is 18 µM in UROtsa cells and 12.4 µM in hepatocytes; whereas for DMA(III) these values are 12 µM and 8.6 µM, respectively (Dopp et al., 2008). The pentavalent organic arsenic species MA, DMA and TMAO exert lower toxicity than As(III), with LC₅₀ values higher than 5 mM in UROtsa cells (Dopp et al., 2008). Other studies, however, have shown LC₇₀ values (concentration required to kill 30 % of test organisms) for DMA of 205 µM in UROtsa and of 155 µM in HepG2 after 48 h exposure

² LC₅₀ and LC₇₀ values of arsenic species are expressed in the same units as found in the literature. All values converted to mg L⁻¹ are shown in Table 1-1.

(Leffers et al., 2013a, Meyer et al., 2015a). Thiolated organic arsenic species, including arsenic glutathione complexes, have been shown to be as toxic as trivalent organic species and more toxic than As(III) (Leffers et al., 2013b, Ebert et al., 2013). The LC₇₀ values of DMAG, thio-DMA and As(III) after 24 h exposure in A549 lung adenocarcinoma epithelium cells are 5.8 µM, 20.5 µM and 97 µM, respectively (Leffers et al., 2013b). The potential toxicity of arenosugars and arsenolipids had been previously suggested based on the toxicity of their proposed metabolites (Feldmann and Krupp, 2011, Ebert et al., 2013), but no evidence was published until recently (Ebert et al., 2016, Leffers et al., 2013a, Meyer et al., 2014a, Meyer et al., 2015a, Meyer et al., 2014b); and even so, more work needs to be done to cover the wide range of arenosugars and arsenolipids existing in the environment. Arsenosugars show low toxicity *in vitro*, with no obvious effects at concentrations under 500 µM (Leffers et al., 2013a, Ebert et al., 2016). The main metabolic products of arenosugars are DMA, thio-DMA, DMAA, thio-DMAA, DMAE and thio-DMAE (Francesconi et al., 2002, Ram1 et al., 2005), which, except for DMA and thio-DMA, do not seem to be cytotoxic after 48 h incubation with UROtsa cells (Leffers et al., 2013a). Regarding arsenolipids, only AsHCs and AsFAs have been tested for toxicity in human cells (Meyer et al., 2014a, Meyer et al., 2015a, Meyer et al., 2015b). After 48 h incubation, the LC₇₀ values for cell viability of two different AsFAs are 96 µM and 83 µM in HepG2 cells, compared to 155 µM and 9 µM for DMA and As(III), respectively (Meyer et al., 2015a). Dimethylarsinoyl propionic acid (DMAP) and thio-dimethylarsinoyl propionic acid (thio-DMAP), which are suggested metabolites of AsFAs, exert no toxicity at concentrations under 500 µM (Meyer et al., 2015a). Arseno-hydrocarbons, however, have been shown to be as toxic as As(III) (Meyer et al., 2014b), with LC₅₀ values from 7 to 13.5 µM in UROtsa and from 8 to 21 µM in HepG2 cells (Meyer et al., 2014a). Arsenobetaine and AC, usually excreted unchanged, are considered harmless (Ohta et al., 2004). In human monocyte-derived macrophages, 48 h exposure to AB does not affect cell viability at concentrations below 5 mM (Ohta et al., 2004). Table 1-1 summarizes the lethal concentrations of different arsenic species in humans. These values are mostly based on experiments with cell lines, which is the only ethical way to test arsenic toxicity in humans and obtain data comparable across different arsenic species. Based on these data, the toxicity ranking for arsenic species in humans can be updated as follows: AsH₃ > DMAG > thio-DMA >

DMA(III) > MA(III) > As(III) and AsHCs > AsFAs > As(V) > TMA > MA and DMA > DMAA, thio-DMAA, DMAE, thio-DMAE, DMAP, thio-DMAP, arsenosugars and thio-arsenosugars > AB > TMAO > AC.

Table 1-1. LC₅₀ and LC₇₀ values of different arsenic species.

Arsenic species	LC ₅₀ (mg L ⁻¹ As)	LC ₇₀ (mg L ⁻¹ As)	Cell line	Exposure
arsine	0.016 - 0.146		N/A	inhaled
DMAG		0.4	A549 lung adenocarcinoma	24 h
thio-DMA		0.2	UROtsa	48 h
		1.5	A549 lung adenocarcinoma	24 h
DMA(III)	0.9		UROtsa	24 h
	0.6		human hepatocytes	24 h
MA(III)	1.4		UROtsa	24 h
	0.9		human hepatocytes	24 h
	12.8		UROtsa	24 h
	9.8		human hepatocytes	24 h
As(III)	0.4	0.3	UROtsa	48 h
		7.3	A549 lung adenocarcinoma	24 h
	1.3	0.7	HepG2	48 h
AsHCs	0.5 - 1		UROtsa	48 h
	0.6 - 1.6		HepG2	48 h
AsFAs		6.2 - 7.2	HepG2	48 h
As(V)	114.8		UROtsa	24 h
	37.5		human hepatocytes	24 h
TMA	> 64.8		N/A	inhaled
MA	> 375000		UROtsa, human hepatocytes	24 h
	> 375000		UROtsa, human hepatocytes	24 h
DMA		15.4	UROtsa	48 h
		11.6	HepG2	48 h
DMAA		> 37.5	UROtsa	48 h
thio-DMAA		> 37.5	UROtsa	48 h
DMAE		> 37.5	UROtsa	48 h
thio-DMAE		> 37.5	UROtsa	48 h
DMAP		> 37.5	HepG2	48 h
thio-DMAP		> 37.5	HepG2	48 h
arsenosugars		> 37.5	UROtsa	48 h
thio-arsenosugars		> 37.5	UROtsa, HepG2	48 h
AB	> 375		human monocytes	48 h
TMAO	> 375000		UROtsa, human hepatocytes	24 h

For AsH₃ and TMA lethal concentrations refer to inhaled arsenic whereas for the rest of the compounds they refer to cell viability.

The International Agency for Research on Cancer categorizes As(III) and As(V) as Group 1 carcinogens (i.e. there is sufficient evidence of carcinogenicity in both experimental animals and exposed humans), and MA and DMA as Group 2B carcinogens (i.e. there is enough evidence of carcinogenicity in experimental animals but not in humans) (World Health Organization, 2010). There are no official classifications for other arsenic species despite the increasing scientific evidence demonstrating their toxicity. Likewise, the data on maximum permissible concentrations of arsenic in beverages and foodstuffs is scarce, and the current permitted levels are primarily based on As(III), As(V), MA and DMA, disregarding the presence of other toxic arsenic species. The Joint Food and Agriculture Organization of the United Nations (FAO)/World Health Organization (WHO) Expert Committee on Food Additives (JECFA) established the arsenic tolerable upper intake level in food as $3.0 \mu\text{g kg}^{-1}$ of body weight per day. This value was withdrawn in 2010 for being within the benchmark dose of inorganic arsenic for a 0.5 % increased incidence of lung cancer ($\text{BMDL}_{0.5}$) (World Health Organization, 2011); but no new value was re-established. In the same year, the WHO set the maximum permitted concentration of inorganic arsenic in drinking water as $10 \mu\text{g L}^{-1}$ (World Health Organization, 2010). European and North American guidelines for arsenic in food are centred on rice products, as these present a higher risk than other foodstuffs. The permitted maximum levels of inorganic arsenic in rice go from 0.1 to 0.3 mg kg^{-1} wet weight (Codex Alimentarius Commission, 2012, European Commision, 2015). In Australia and New Zealand, guidelines also include permitted maximum levels of arsenic in seafood, with a limit of 1 mg kg^{-1} for seaweed and molluscs and of 2 mg kg^{-1} for fish and crustaceans (Food Standards Australia and New Zealand, 2017). Although health authorities acknowledge the presence of organic arsenic species in foodstuffs, they consider that more research needs to be done to evaluate the bioaccessibility, bioavailability, biotransformations and toxicity of most organic arsenic species in humans in order to update the current regulations.

Arsenic metabolism

A full assessment of the effects of arsenic accumulated by humans requires an understanding of the metabolic pathways occurring once arsenic species enter the human body. Arsenic will be expected to follow the metabolic pattern of xenobiotics in humans,

that includes absorption, distribution, biotransformations and excretion (Goodman and Gilman, 2006, Casarett and Doull, 2008, Rang et al., 2012).

When ingested, arsenic enters the gastrointestinal tract, where the stomach and the small intestine solubilise the different arsenic species before either being absorbed into the hepatic portal system or excreted in faeces with all the non-absorbed ingesta (Figure 1-3). The physiological and enzymatic conditions of the gastrointestinal tract, together with the presystemic metabolism that takes place across the intestinal wall, modify the arsenic species ingested and determine the nature of the molecules accessing the hepatic portal system (Figure 1-3) (Ioannides, 2002).³ Arsenic species can then enter the systemic circulation and be distributed throughout the body or go into the liver, the main site for storage and detoxification (Figure 1-3) (Goodman and Gilman, 2006, Dworken et al., 2013). The biotransformations occurring in the liver typically turn xenobiotics into more polar, and therefore more easily excretable, species. The process usually takes place in different phases. In Phase I or functionalisation, hydrophilic functional groups ($-\text{OH}$, $-\text{NH}_2$, $-\text{SH}$ or $-\text{COOH}$) are exposed or added to the molecule by means of oxidation, reduction or hydrolysis (Ioannides, 2002, Casarett and Doull, 2008). Often this process can lead to more toxic species than the initial one and generates reactive oxygen species (ROS), inducing oxidative stress in the organism. After functionalisation, xenobiotics can undergo Phase II or conjugation, in which they are attached to an endogenous molecule in order to further increase the hydrophilicity and molecular weight of the initial substrate, as well as to reduce their chemical reactivity (Ioannides, 2002). Conjugation reactions include glucuronidation, sulfonation, acetylation, methylation, conjugation with glutathione and conjugation with amino acids (Casarett and Doull, 2008). Relevant Phase II reactions in arsenic metabolism are methylation and glutathione conjugation (Cullen, 2014, Hayakawa et al., 2004), supposed to respectively deactivate the biological activity and to reduce the electrophilicity of arsenic species. When the products of Phases I and II are not hydrophilic enough for excretion, they enter a Phase III of reactions to prepare the xenobiotic for removal (Ioannides, 2002). The polar species generated in the hepatocytes are readily released to the kidneys and eliminated in urine (Figure 1-3). Lipid soluble species, however, are transported through the bile to the gallbladder where they

³ Chapters 2 (p.35) and 3 (p.55) are centred on the gastrointestinal digestion and bioaccessibility of water and lipid soluble arsenic species in food. The current bibliography on the topic is described in detail in those chapters.

enter the small intestine again, either for biliary excretion or re-absorption in the hepatic portal system (Figure 1-3) (Dworken et al., 2013). Gut microbiota can detach the conjugated molecule from the biliary excreted product of Phase II, thus bringing back the lipophilic character of the xenobiotic that is then re-absorbed into the liver (Ioannides, 2002). Recirculation between hepatocytes and enterocytes is known as enterohepatic circulation and occurs until the molecule is ready for excretion, with the associated risk from a longer exposure to the xenobiotic toxicity (Figure 1-3) (Goodman and Gilman, 2006). Additionally, the biotransformations aiming at detoxification can also generate reactive species that can interact with important biomolecules such as enzymes, DNA or RNA; causing irreversible cellular damage (Ioannides, 2002).

The mechanisms of arsenic toxicity depend, as mentioned above, on the arsenic species of exposure. For instance, arsine produces haemolysis, probably by generation of arsine adducts with haemoglobin that denaturalise the protein (World Health Organization, 2002). In general, arsenic species increase the amount of ROS and reactive nitrogen species (RNS) in cells, which can induce DNA damage and modify the expression of physiologically essential genes (Chen et al., 2003, Ventura-Lima et al., 2011, Ghosh et al., 2008). Trivalent arsenic species can bind covalently to the sulphydryl groups of certain proteins and disturb their biological function leading to cell apoptosis (Ventura-Lima et al., 2011, Shen et al., 2013), while As(V) can interfere with the synthesis of adenosine triphosphate (ATP) by replacing phosphate in phosphorylation reactions (Fattorini and Regoli, 2004).

The following sections review the current understanding of arsenic biotransformations in cells⁴, without discussing the mechanisms of action for arsenic toxicity, as this is outside of the scope of this thesis.

⁴ Membrane permeability is also crucial in arsenic metabolism and it is fully reviewed in *Chapter 4* (p.77).

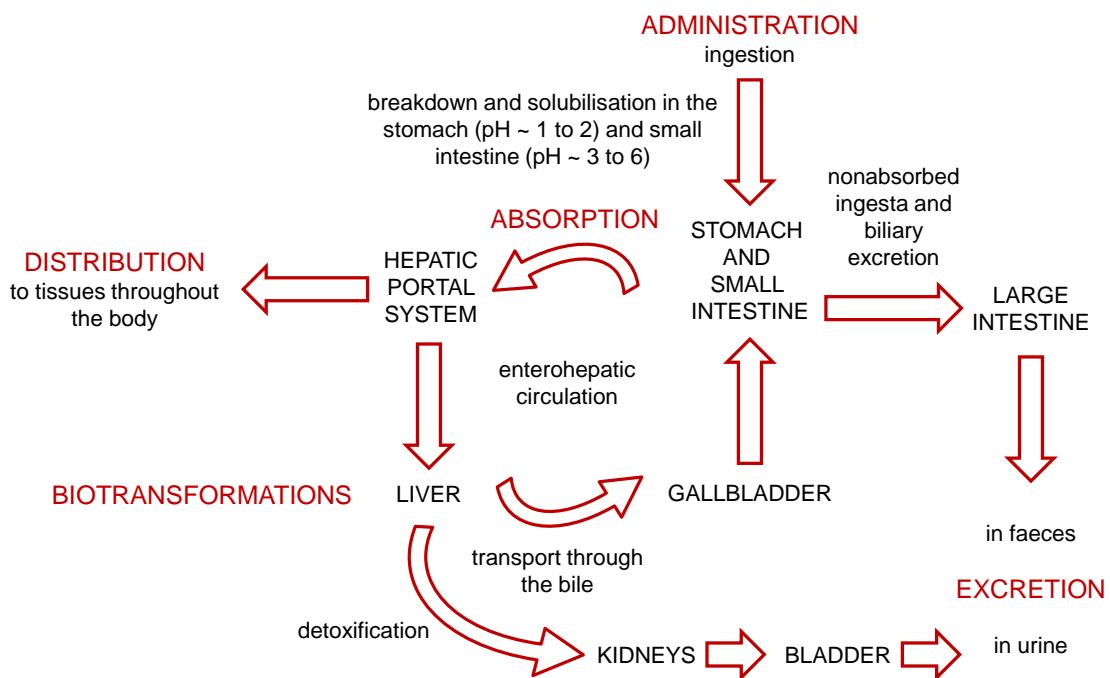


Figure 1-3. General metabolic pathway for xenobiotics in humans, adapted from (Goodman and Gilman, 2006, Casarett and Doull, 2008, Rang et al., 2012).

Inorganic arsenic species

At the end of the 19th century, the identification of trimethylarsine as the volatile microbial product of the inorganic arsenic present in wallpaper stimulated interest in understanding arsenic biotransformations. The work conducted by Bartolomeo Gosio and Frederick Challenger (Challenger, 1947, Cullen and Bentley, 2005) built the foundation of what is now considered the backbone of inorganic arsenic metabolism, a pathway that comprises subsequent reductions and oxidative methylations. Although Challenger's proposed mechanism (Figure 1-4a) was initially based on prokaryotes, it was later demonstrated to be applicable to mammals (Buchet and Lauwerys, 1985). Hayakawa *et al.* (2004) later revisited the model providing new insights into the different arsenic-based intermediates which may cause toxic and carcinogenic effects to humans (Figure 1-4b). A third pathway, proposed by Naranmandura's group in 2012 (Figure 1-4c), seems able to explain some of the limitations of the previous two models, and it is in agreement with most of the experimental evidence so far (Rehman and Naranmandura, 2012).

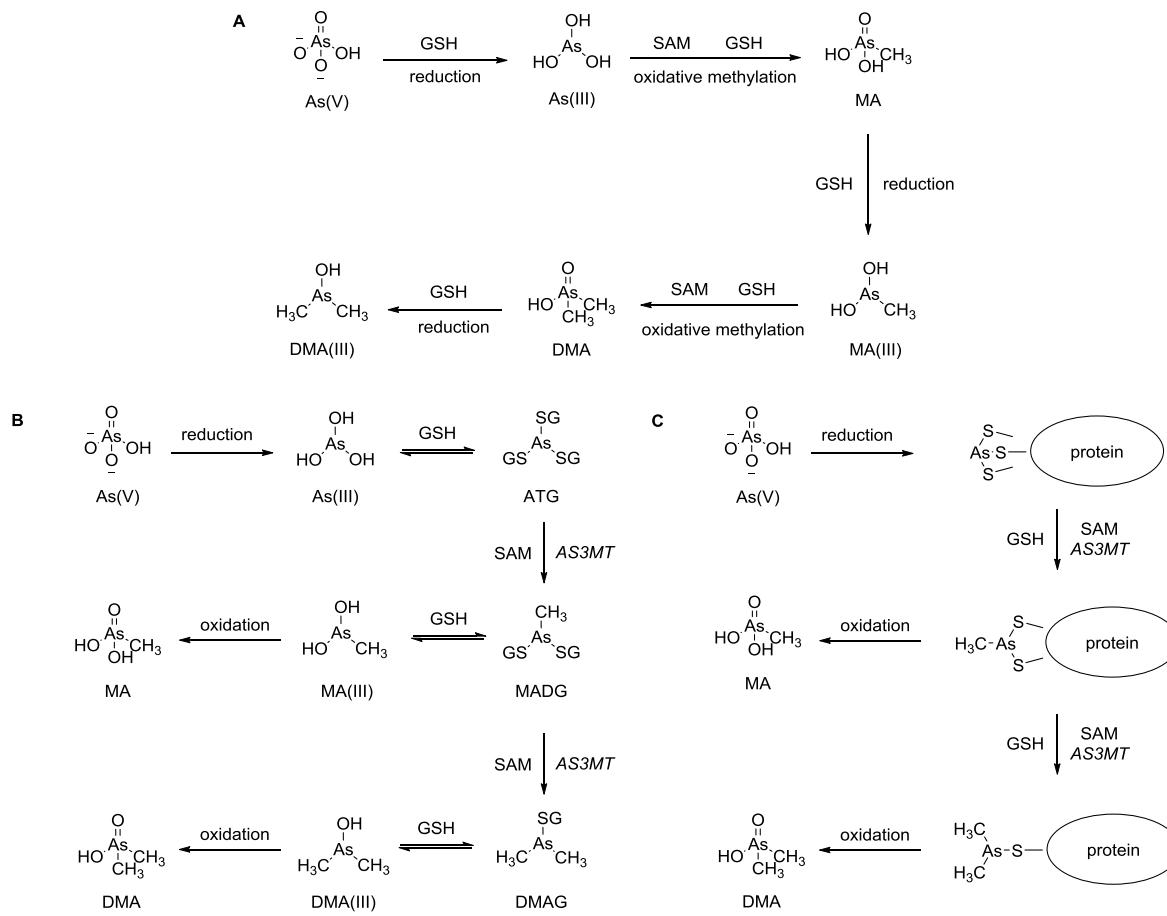


Figure 1-4. Suggested metabolic pathways by Challenger (A), Hayakawa (B) and Naranmandura (C) for inorganic arsenic in mammals, adapted from (Kobayashi, 2010, Rehman and Naranmandura, 2012). Abbreviations: *AS3MT* (enzyme arsenic III methyltransferase), GSH (glutathione molecule), SAM (*S*-adenosyl-L-methionine), -SG (glutathione moiety).

Despite the ability of both As(III) and As(V) to enter the cell, the latter is rapidly reduced once inside. In 2002, it was reported that the enzyme purine nucleoside phosphorylase (*PNP*) could catalyse As(V) reduction *in vitro* (Figure 1-5b) (Radabaugh et al., 2002). The same reaction tested in human erythrocytes and living rats found a poor correlation between *PNP* and As(V) reduction *in vivo* (Nemeti et al., 2003). Regardless of the uncertainty around this route, it was also observed that glutathione-*S*-transferase omega class 1-1 (*GSTO1-1*), a cytosolic protein which contributes to xenobiotic metabolism, was able to assist in this reduction step in human hepatocytes using glutaredoxin or glutathione (GSH) as a reducing agent (Figure 1-5a) (Zakharyan et al., 2001). The significance of GSH in arsenic reduction relates to the affinity of arsenic for sulfur, regarded by some authors as the basis of its biochemical behaviour (Styblo and

Thomas, 1997), as well as to the ability of thiols to reduce arsenic species (Cullen et al., 1984b).

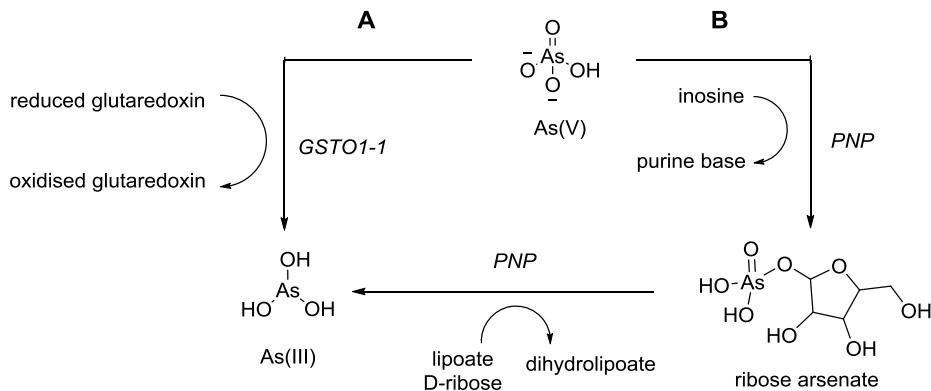
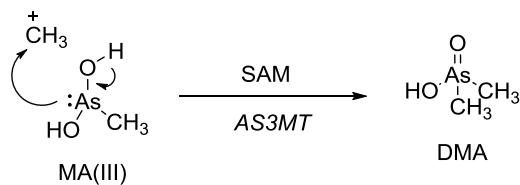


Figure 1-5. Proposed pathways for arsenate reduction in mammalian cells, adapted from (Foster, 2007, Aposhian et al., 2004, Caspi et al., 2006). Abbreviations: *GSTO1-1* (glutathione-S-transferase omega class 1-1), *PNP* (purine nucleoside phosphorylase).

Arsenous acid is then methylated with the methyl donor group of *S*-adenosyl-L-methionine (SAM). The reaction is catalysed by a methyltransferase that requires a thiol group, such as GSH or thioredoxin, to operate (Rossman, 2003). Whereas the relevance of both SAM and GSH in arsenic metabolism was soon confirmed in mammals after studying rat liver cells (Buchet and Lauwerys, 1985); the nature of the enzyme in charge of methylation took longer to be established. Styblo and co-workers were the pioneers in detecting its activity in human hepatocytes (Styblo et al., 1999), and later in identifying the gene encoding the protein (Lin et al., 2002). Originally known as cytochrome 19 (*cyt19*) in humans and mice, the name of this gene was changed according to its newly revealed function to arsenic III methyltransferase (*As3mt*) (Walton et al., 2003). Over many years, different interpretations on arsenic methylation have impeded a formal scientific consensus on how the process takes place. According to Challenger's model (Figure 1-4a), an oxidative methylation is followed by a reduction (Figure 1-6a), meaning that the oxidised methylated arsenic species are formed before their reduced equivalents. In Hayakawa's model it occurs in reverse (Figure 1-4b), in what is defined as a reductive methylation (Figure 1-6b) (Naranmandura et al., 2006). This reaction is also supported by Naranmandura's model of arsenic methylation (Figure 1-4c) (Rehman and Naranmandura, 2012). The feasibility of reductive methylation of arsenic species has been questioned based on the assumption that the mechanism involves the release of a negatively charged methyl group (CH_3^-) from the positively charged SAM molecule

(Cullen, 2014). Reductive methylation, however, does not suggest the transference of CH_3^- to arsenic but a nucleophilic attack of the lone pair of trivalent arsenic to a positively charged methyl group (CH_3^+) (Figure 1-6b).

A) oxidative methylation



B) reductive methylation through nucleophilic attack to carbon

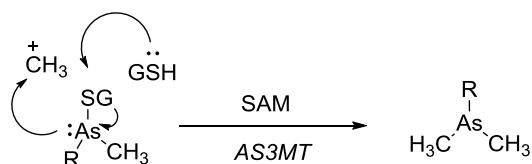


Figure 1-6. Proposed mechanisms for oxidative methylation (A) and reductive methylation through nucleophilic attack to carbon (B), adapted from (Naranmandura et al., 2006). Abbreviations: *AS3MT* (enzyme arsenic III methyltransferase), GSH (glutathione molecule), $-R$ (thiol moiety of the cysteine residue in a protein), $-SG$ (glutathione moiety), SAM (*S*-adenosyl-L-methionine).

Since trivalent arsenic is the preferred substrate of *AS3MT* (Hayakawa et al., 2004), Challenger's model would require the reduction of MA produced after the first oxidative methylation. This reaction can be assisted by the same protein involved in As(III) reduction, *GSTO1-1* (Zakharyan et al., 2001); although the knockout of this enzyme in mice does not stop MA(III) formation, suggesting the existence of other enzymes that are able to progress the reaction (Chowdhury et al., 2006). In the alternative pathway suggested by Hayakawa, the intermediates taken up by *AS3MT* are arsenic glutathione complexes, and MA(III) is derived from MADG (Figure 1-4b) (Hayakawa et al., 2004). Indeed, *AS3MT* is likely to methylate the glutathione-conjugate of MA(III) rather than its free form (Hayakawa et al., 2004). The identification of arsenic glutathione complexes in body fluids also supports this view: ATG and MADG have been detected in bile (Suzuki et al., 2002), where they are known to arrive from the hepatocytes through the *MRP2/cMOAT* protein system (Kala et al., 2000). In contrast, DMAG has not been found in bile, probably due to its instability and immediate decomposition to DMA (Hayakawa et al., 2004), although it is thought to be released into the bloodstream

(Suzuki, 2005). Arsenic glutathione complexes, however, are likely to be derived from arsenic-containing proteins (Raab et al., 2007, Rehman and Naranmandura, 2012) and trivalent arsenic species only exist bound to proteins in liver and kidneys, the major sites of arsenic methylation in mammals (Naranmandura et al., 2006); thus also reinforcing Naramandura's argument (Figure 1-4c) (Rehman and Naranmandura, 2012).

Another important discrepancy between the different proposed pathways is the nature of the metabolic products. Challenger's pathway regards only DMA(III) as the final metabolite (Figure 1-4a); whereas in the new models, trivalent methylated arsenic species are oxidised by perhaps hydrogen peroxide (H_2O_2) (Aposhian and Aposhian, 2006) yielding MA and DMA as products (Figure 1-4b and 1-4c). In 1977, the first report on the different arsenic species excreted in human urine after the ingestion of wine, water and seafood was published (Crecelius, 1977). The results derived from water and wine, where arsenic is present as As(III) and As(V); agree with the methylation pattern of Hayakawa's scheme (Figure 1-4b), with MA and DMA as main metabolic products. Moreover, part of the arsenic was excreted unchanged, indicating the feasibility of direct absorption of As(III) and As(V) into the blood and removal through the kidneys. Further publications have shown ratios of 10 – 30 % of inorganic species, 10 – 20 % of MA (III and V) and 60 – 70 % of DMA (III and V) in human urine (Vahter and Concha, 2001). In some of those studies, the limitations in the identification techniques did not take into account the excretion of trivalent methylated arsenic species, which could have been readily oxidised under the storage and analysis conditions used, giving misleading concentrations of pentavalent organic species (Thomas et al., 2001, Nakayama et al., 2006).

Trivalent methylated arsenic species were not detected in human urine until the beginning of the 21st century (Le et al., 2000). At that time it was unclear whether they were the final products of inorganic arsenic metabolism, or came from the reversible reduction of their pentavalent counterparts. In 2003, it was proposed that the transformation between the two main arsenic oxidation states in inorganic and methylated species could be catalysed by the mammalian xanthine oxidoreductases, which are the major source of free radicals in cells (Aposhian et al., 2003). Although the study was not performed *in vivo*, it provided an explanation for the presence of trivalent

species in urine, as well as for the increment of ROS in arsenic exposed cells. Soon after, it was demonstrated that thio-DMA could have been misidentified as DMA(III) as a result of their similar chromatographic retention times and that, due to DMA(III) instability in aqueous media, it was unlikely to be excreted as such in urine (Hansen et al., 2004b). The misidentification of thio-MA as MA(III) was later verified (Naranmandura et al., 2007), strengthening the consistency on the theory behind Hayakawa's and Naranmandura's models.

Because of these studies, thio-methylated arsenic species, previously unnoticed due to the restrictions of the methods and techniques available, were introduced as important elements of inorganic arsenic metabolism. Thio-dimethylarsinic acid has been detected in the urine of hamsters and rats (Naranmandura et al., 2007), as well as of pregnant women who had consumed contaminated drinking water (Raml et al., 2006). Likewise, dithio-dimethylarsinic acid (dithio-DMA) has been identified in hamsters; and thio-MA in both hamsters and rats (Naranmandura et al., 2007). In addition to the studies showing the formation of thio-arsenic species by the gastrointestinal flora in mammals (Van de Wiele et al., 2010, Yoshida et al., 2003, Pinyayev et al., 2011), experiments conducted in rat and human red blood cells (RBCs) also suggest that thio-DMA is produced in RBCs from the thiolation of the DMA(III) formed in the hepatocytes (Naranmandura et al., 2008, Naranmandura and Suzuki, 2008). Thio-dimethylarsinic acid can be enzymatically hydrolysed to produce DMA and a sulfide ion, which reacts with another thio-DMA molecule to yield dithio-DMA (Naranmandura et al., 2008). In the presence of GSH, DMA is reduced to DMA(III) which subsequently binds to haemoglobin, or oxidised back to DMA with the consequent release of ROS (Figure 1-7) (Naranmandura et al., 2008). Methylarsonous acid, excreted from the liver into the gallbladder, seems to be thiolated *via* enterohepatic circulation (Bu et al., 2011). After entering the gallbladder in bile as MADG, MA(III) is recirculated to the small intestine, where thio-MA and thio-DMA are synthesised by the intestinal microbiota (Bu et al., 2011). On the other hand, it has been suggested that thio-arsenic species could come from a side reaction of the mercapturic acid pathway known as β -lyase shunt (Rumpler, 2010), a phase III metabolic route responsible for the urinary excretion of xenobiotics conjugated with GSH. The expected final product would be a methyl-thiolated xenobiotic in which the sulfur atom comes from the cysteine residue of GSH and the

methyl moiety is bound to sulfur. Given that the final structure of the thio-arsenic species detected to date differs from this one, as well as the lack of scientific evidence to support it, this view remains a theory.

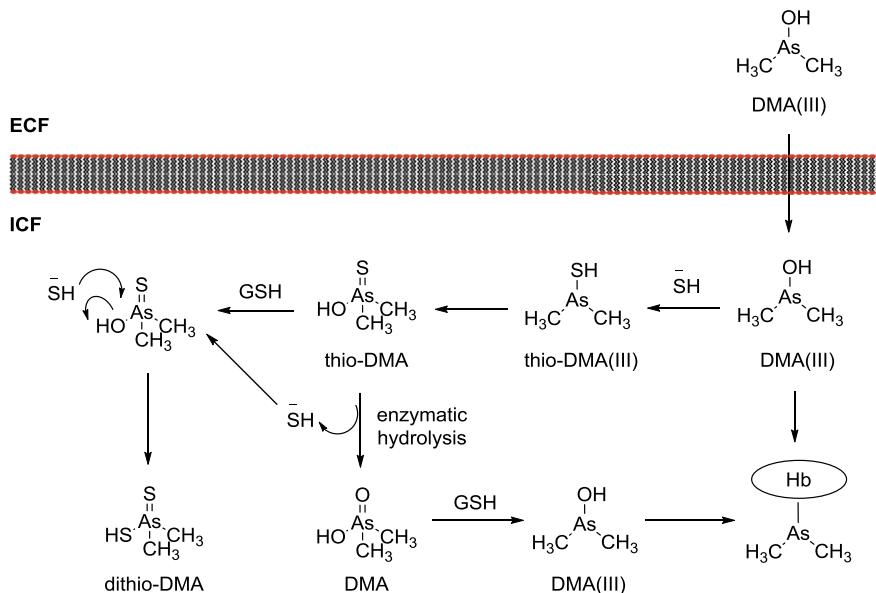


Figure 1-7. Proposed metabolism of thio-arsenic species in red blood cells, adapted from (Hua Naranmandura, 2008). Abbreviations: ECF (extracellular fluid), GSH (glutathione molecule), Hb—(haemoglobin), ICF (intracellular fluid).

For many years, arsenic methylation had been considered a detoxification process (Yamaguchi and Fowler, 1994), but during the last decades enough evidence has arisen to change this view such that it is now thought to be an activation pathway (Thomas et al., 2001). Chimpanzees, marmoset monkeys and guinea pigs lack methyltransferase activity but they do not seem to retain arsenic in their bodies in greater amounts than other animals. Given the higher toxicity of MA(III) and DMA(III) in comparison to As(III) (Petrick et al., 2000), some authors regard the difference as an advantage that protects those mammals from the risk associated with MA(III) exposure (Chowdhury et al., 2006). From the experiments conducted in rats and hamsters (Naranmandura et al., 2007), it could be inferred that methylation does not mean either detoxification or activation but both. Rats methylate arsenic faster than hamsters and form very stable complexes of DMA(III) with haemoglobin (Lu et al., 2004). This limits the availability of inorganic and mono-methylated arsenic species inside the organism, since they are readily methylated to DMA(III) which is accumulated until excreted as TMAO (Naranmandura et al., 2007). Hence, rats seem to be more tolerant to arsenic than

hamsters, whose poor methylation capacity leads to the enterohepatic circulation of MA(III) and accompanying toxicity (Naranmandura et al., 2007). Unlike in rats, arsenic methylation in humans is only to DMA, with both MA and DMA largely eliminated in urine (Vahter, 1994).

Furthermore, methylated species are able to bind to biomolecules (Suzuki et al., 2002, Vahter, 2002, Raab et al., 2007), and, in some cases, accumulate in tissues. Arsenic in hair and nails is a well-known parameter to assess both chronic and acute exposure, and some studies have shown that the arsenic species present in these tissues are analogous to those excreted in urine (Yanez et al., 2005). Both contain keratin, a protein predominantly formed by cysteine and therefore, sulfur centres with high affinity for arsenic. Once hair and nail cells die, their metabolic activity ends and all the arsenic bound to the cysteine residues remains unchanged. A similar interaction has also been observed in active tissues, such as rabbit and rat livers (Bogdan et al., 1994, Styblo and Thomas, 1997). While the arsenic species bound to the cytosolic proteins in rabbits could not be specified, As(III), MA(III) and DMA(III) were found in rats. The strong affinity of these species for sulfur-containing biomolecules has also been observed in rat and human blood (Lu et al., 2004), where the degree of binding to haemoglobin is related to the availability of cysteine residues. This binding efficiency increased from As(III) to MA(III) to DMA(III); implying that hydrophobicity may play an important role in accessing the binding site. The interaction of arsenic with proteins is the basis of Naranmandura's pathway (Figure 1-4c), in which successive methylation of arsenic species occurs in arsenic bound to proteins instead of bound to GSH (Rehman and Naranmandura, 2012). This mechanism is supported by the observation that arsenic species form much more stable bonds with sulphhydryl functional groups in proteins than with GSH (Styblo and Thomas, 1997, Vahter et al., 1983, Bogdan et al., 1994, Suzuki et al., 2002).

Organic arsenic species

Small sized methylated arsenic species have been studied more extensively in terms of endogenous metabolism and there is little information about their fate after ingestion. Only the *in vitro* gastrointestinal digestion of As(III), As(V), MA and DMA in vegetables and seaweed has been reported (Calatayud et al., 2013, Zhao et al., 2014). Arsenate, MA and DMA in vegetables are transformed into MA(III), DMA(III), thio-MA and thio-

DMA (Calatayud et al., 2013), whereas MA and DMA in seaweed are demethylated to As(III) (Zhao et al., 2014).⁵ Arsenobetaine and AC ingested in food do not undergo any transformation in humans and are eliminated unchanged in urine (Crecelius, 1977, Schmeisser et al., 2006b).

Arsenosugar consumption by humans has also been investigated, with particular focus on the arsenic metabolites excreted in urine (Ma and Le, 1998, Francesconi et al., 2002, Raml et al., 2005). Arsenosugar-glycerol, for instance, is metabolised to DMA (Ma and Le, 1998, Francesconi et al., 2002), DMAE, TMAO (Francesconi et al., 2002), thio-DMAA, thio-DMAE and the equivalent thio-arsenosugar, among other unidentified compounds (Raml et al., 2005). In marine organisms, trimethyl-arsenosugars are thought to be anaerobically decomposed to AC (Francesconi et al., 1992) and subsequently oxidised to AB. The proposed pathway for AB biogenesis from arenosugar degradation involves DMAE and DMAA as intermediates (Edmonds et al., 1992), which could explain the metabolites identified in human urine after arenosugar consumption. Since AB was not observed in urine, the final metabolic product would be DMA instead. Thio-arsenosugars reported in animals are likely to have originated from the metabolism of ingested oxo- counterparts (Kahn et al., 2005), possibly due to gut microbiota (Conklin et al., 2006).

The first investigation of arsenolipids in human urine showed DMA as the main metabolite; and thio- and oxo- dimethylarsinoyl propionic and butyric acids in smaller quantities (Schmeisser et al., 2006b). Despite the lack of success in identifying the lipids originally present in cod liver, no evidence was found matching them with thio-arsenic species, again suggesting thiolation as a metabolic process in the body. It was proposed that the arsenolipids in cod were firstly transformed to short chain fatty acids and then to DMA (Schmeisser et al., 2006b). The transformation of AsFAs into DMA has also been reported after studying the ability of AsHCs and AsFAs to cross the intestinal barrier using Caco-2 cells (Meyer et al., 2015b). Both types of arsenolipids transfer across the intestinal barrier, with higher permeability of AsHCs than of AsFAs. While AsFAs are presystemically metabolised after intestinal absorption, AsHCs remain almost unaffected (Meyer et al., 2015b). Another study focused on the fate of phosphatidylarsenocholine in

⁵ These publications are more extensively discussed in *Chapter 2* (p.35).

mice urine and faeces (Figure 1-8) (Fukuda et al., 2011). Elimination was predominantly *via* urine, where AB was identified as the major arsenic species. The excretion rate was longer than that for other arsenic species. This is in agreement with the general metabolism of glycerophospholipids, which are accumulated in the intestinal epithelial cells as lipoproteins and therefore, eliminated slower. The authors explained AB formation as derived from AC, which is initially released from the phospholipid (Figure 1-8). In mice faeces they detected phosphatidylarsenocholine together with AC (Figure 1-8).

Dimethylarsinic acid is a degradation product of both arsanosugars and arsenolipids and similarly, it could also be the starting point in their formation. Most of the arsenic species ingested by humans yield methylated species that could undergo further metabolic transformations and enter metabolic routes of biological importance to produce arsanosugars and arsenolipids, among other arsenic species, as already suggested by a few authors (Foster, 2007, de Bettencourt et al., 2011). *In vitro* experiments have demonstrated how DMA is able to act as an electrophile susceptible to be attacked by the sulfur centre of GSH and react to yield DMAG and dimethylarsonio adenosine glutathione (DMAAG) (Figure 1-9) (de Bettencourt et al., 2011), the latter being a potential precursor of arsanosugars and, possibly, arsenolipids. Supporting these results, it has been shown that knocking out the enzymes responsible for arsenic methylation in cyanobacteria inhibited the formation of arsanosugars and arsenolipids (Xue et al., 2014, Xue et al., 2017). S-adenosyl-methionine is not only involved in the methylation of arsenic but also of phosphatidylethanolamine (Vahter, 2007), indicating that both processes could be intersected. Hence, GlyAsLs could be synthesized from DMA(III), SAM and phosphatidylethanolamine, justifying the findings of Xue and co-workers (Xue et al., 2014).

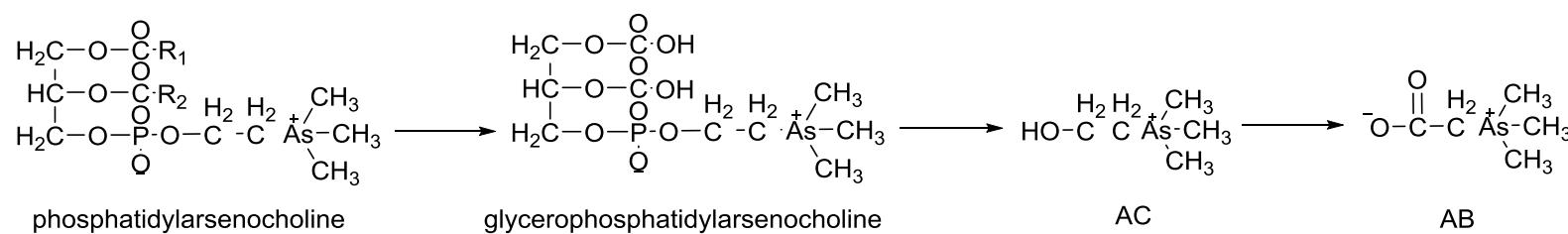


Figure 1-8. Metabolic pathway suggested for the degradation of phosphatidylarsenocholine in mice, adapted from (Fukuda et al., 2011).

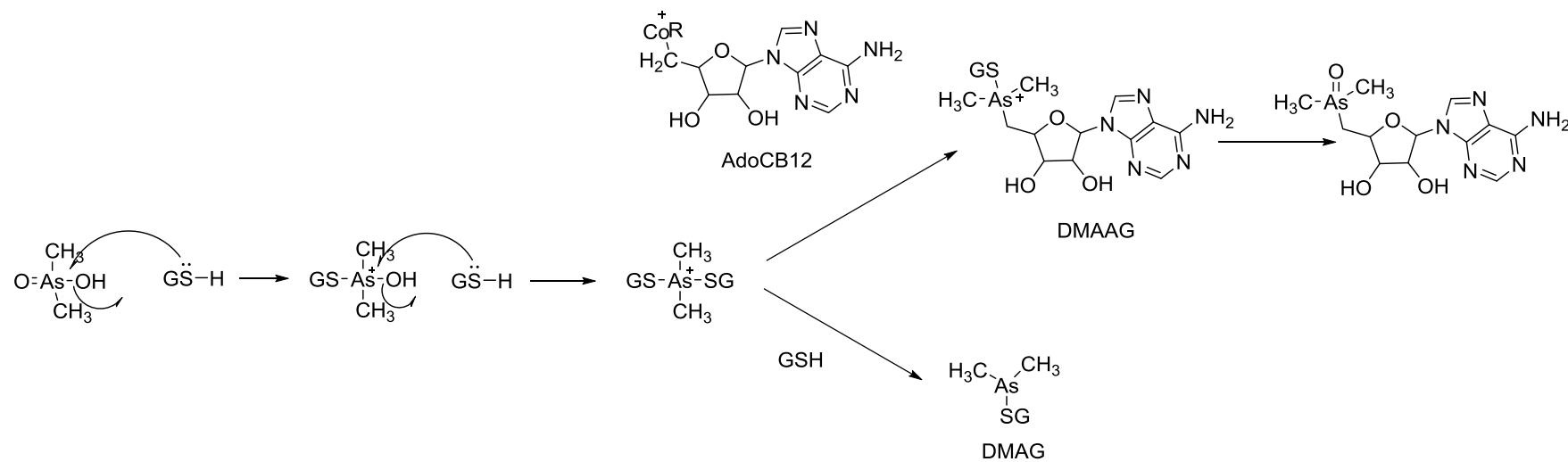


Figure 1-9. Suggested metabolic pathways of arsenolipid formation from DMA(III), adapted from (de Bettencourt et al., 2011).

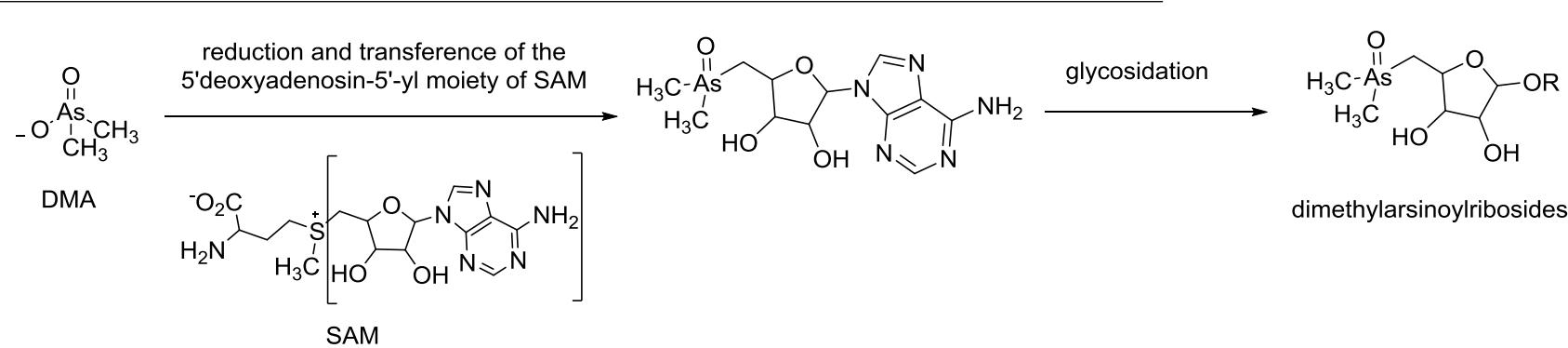


Figure 1-10. Suggested pathway for arsanosugar formation from DMA, adapted from (Edmonds and Francesconi, 1987a).

Whether or not the biosynthesis of arenosugars and arsenolipids has a biological function is a topic under debate among the scientific community. Arenosugars, discovered in 1981 (Edmonds and Francesconi, 1981), were suggested to come from the transfer of 5' deoxyadenosin-5'-yl of SAM to DMA and subsequent glycosylation to remove the adenine moiety (Figure 1-10) (Edmonds and Francesconi, 1987a). The formation of arenosugars from DMA has been confirmed in cyanobacteria and it could respond to a detoxification process in both algae and cyanobacteria to get rid of the toxin (Xue et al., 2017). Nonetheless, GlyAsLs could also be the precursor of arenosugars (Garcia-Salgado et al., 2012), therefore following a different pathway than the one mentioned before, although not necessarily excluding it. The formation of GlyAsLs has been discussed in terms of being a non-selective metabolism. This is supported by a study reporting the ability of algae to substitute phosphorus with sulfur or nitrogen in the biosynthesis of membrane lipids when phosphorus is scarce (Van Mooy et al., 2009). Sustained by this discovery, in 2011 NASA presented enough evidence to state that As(V) could replace phosphate in biologically important molecules (Wolfe-Simon et al., 2011). Soon after, this work was questioned (Tawfik and Viola, 2011, Parke, 2013); and later it was demonstrated that the authors had misinterpreted their results (Hayden, 2012). Indeed, in GlyAsLs, arsenic does not replace phosphorus, as the molar ratio of both elements is 1:1; and their fatty acid composition differs from the one in abundant phospholipids, implying another biosynthetic pathway aside from the non-selective metabolic route (Raab et al., 2013). Arseno-phospholipids were initially reported in 1978 (Cooney et al., 1978) and even though the arsenic species described at that time was incorrect, the phosphatidyl arsenic-based structure has been identified as common to all AsPLs (Francesconi et al., 1990, Hanaoka et al., 1999, Hanaoka et al., 2001a, Devalla and Feldmann, 2003). Some authors have compared these species to lecithin (Francesconi et al., 1990) and sphingomyelin (Hanaoka et al., 2001a), both phospholipids found in animal cell membranes. Arseno-fatty acids and AsHCs do not contain phosphorus, and they are thought to come from the transamination like reaction (arsenylation) between DMA(III) and oxaloacetate, leading to DMAP which is subsequently introduced in the fatty acid biosynthesis *via* acetyl coenzyme A (acetyl-CoA) (Figure 1-11) (Rumpler et al., 2008). Their β -oxidation would yield DMA as a product. Arseno-hydrocarbon biosynthesis could be the same as for AsFAs with an

additional reduction step at the end to remove the carboxylic acid moiety (Figure 1-11) (Taleshi et al., 2008). The following interaction of AsFAs with membrane phospholipids could yield AsPCs. Arsenolipid existence could therefore be a detoxification process in which arsenic is incorporated into lipid soluble structures so as not to disrupt cell's vital functions. Alternatively, the possibility of having a role as membrane components has not been discarded (Taleshi et al., 2008, Francesconi and Schwerdtle, 2016), for which their formation could come from non-selective metabolism or evolutionary adaptation to take advantage of an excess of arsenic in the organism. Given that the essentiality of arsenic has been previously suggested (Uthus, 1992, Uthus, 2003), this theory would not be totally illogical. Either way, arsenolipids have not been found in humans to date, but it does not necessarily mean that they cannot be produced. For so many years the lack of proper methods for the extraction and analysis of the lipid soluble components of tissues, has hindered the focus on these types of arsenic species.

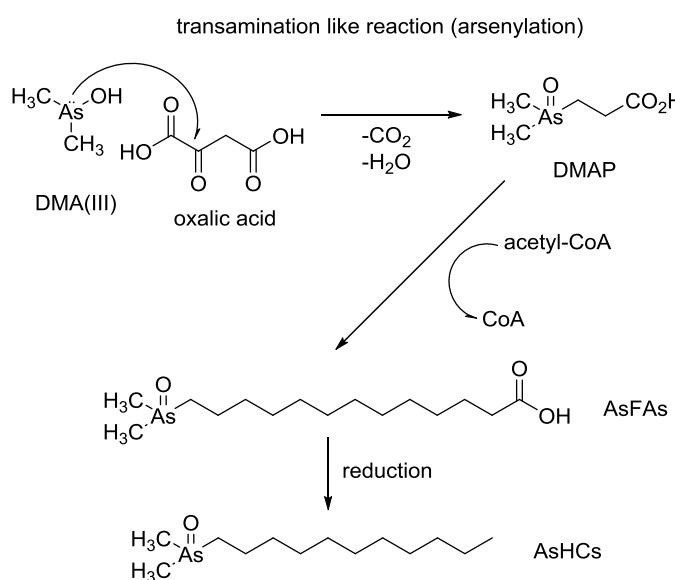


Figure 1-11. Suggested biogenesis of arsено-fatty acids and arsено-hydrocarbons, adapted from (Rumpler et al., 2008, Taleshi et al., 2008).

By considering and collating the selected metabolic routes included in this chapter, a general pathway for arsenic can be proposed (Figure 1-12), encompassing inorganic arsenic species to complex arsenolipids.

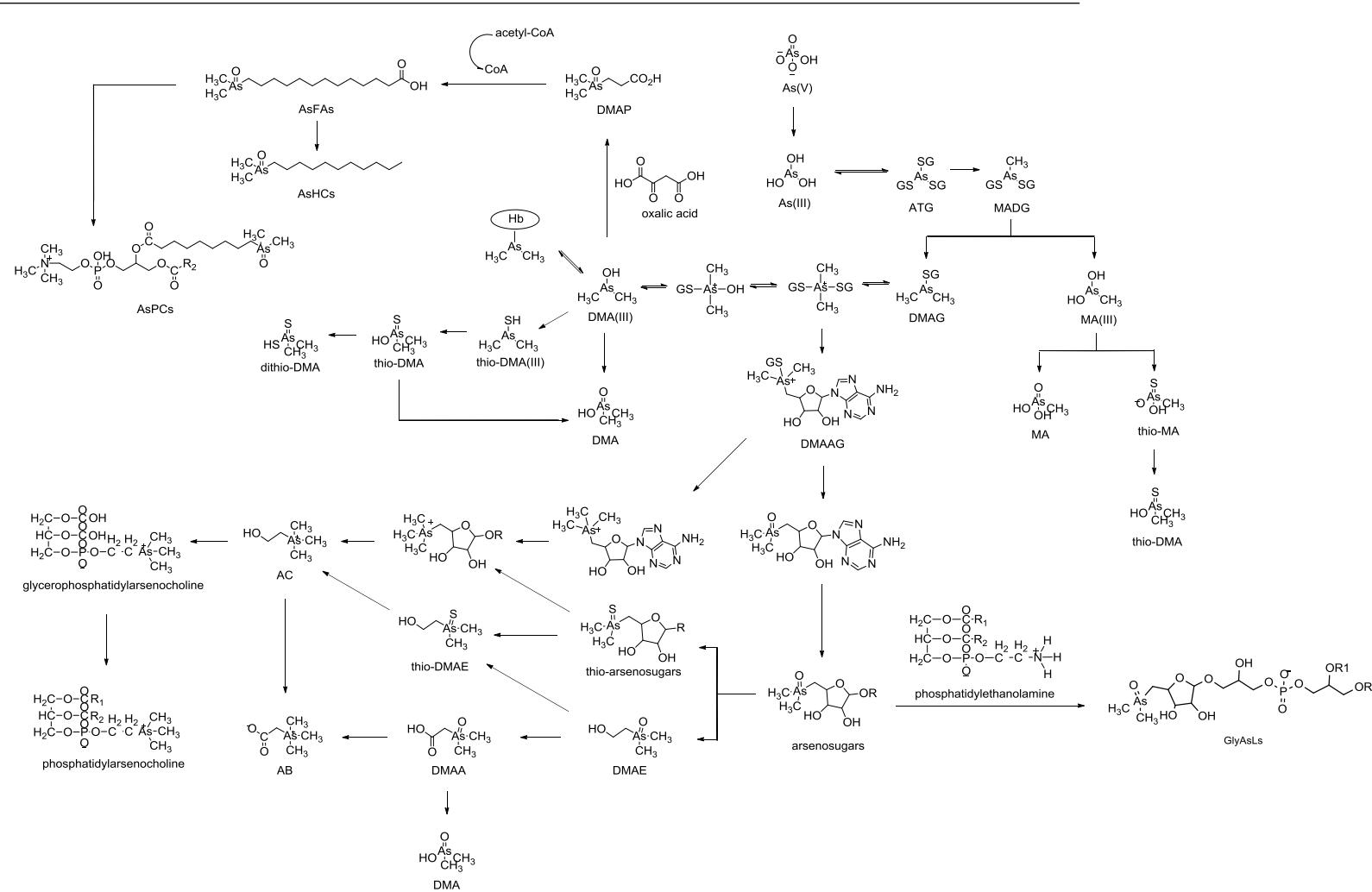


Figure 1-12. Overview of proposed pathways for arsenic metabolism from inorganic to complex organic arsenic species.

Scope of this research

There are many studies on the effects of inorganic arsenic on human health, however, only recently the focus has shifted towards the consequences of both endogenic and exogenic complex organic arsenic species, such as thio-arsenic species, arsenic glutathione complexes, arsenosugars and arsenolipids. The metabolism of these arsenic species is yet to be investigated to properly evaluate the potential risk of exposure to humans. Additionally, the nature of the transformations of inorganic arsenic in mammals is still controversial. A better understanding of the metabolism of arsenate and arsenous acid in cells can help to identify the specific modes of toxic action of these species.

This thesis contributes to the field by providing new and valuable knowledge about the uptake and metabolism of arsenic in humans. The specific research questions were:

1. Do complex organic arsenic species survive the gastrointestinal tract?
2. Can arsenic species diffuse across biological membranes?
3. How are inorganic arsenic species metabolised in human liver cells?
4. What are the biotransformations of arsenolipids in human liver cells?

To answer these questions, this thesis is divided in three main sections that cover the following topics:

1. Bioaccessibility and degradation of arsenic species from foodstuffs.

Chapters 2 and 3 present a comprehensive investigation on the degradation of arsenic glutathione complexes, arsenosugars and arsenolipids from rice, seaweed, fish and krill oil during gastrointestinal digestion; in order to determine which species would enter the liver to be further metabolised.

2. Membrane permeability of arsenic species through simple diffusion.

Chapter 4 focuses on the ability of inorganic arsenic species, pentavalent methylated and thio-methylated arsenic species, and arsenic glutathione complexes to traverse cell membranes by passive diffusion.

3. Biotransformations in human liver cells for arsenic detoxification.

Chapters 5 and 6 study the chemical reactions that inorganic arsenic species, arseno-fatty acids and arsено-hydrocarbons undergo in human liver cells.

Chapters 2, 3, 4, 5 and 6 are aimed for publication and are therefore intended to be self-contained chapters. Thus, the information in the introductory sections of each of these chapters may be repeated to provide a justification to the reader for the experimental work using the pertinent literature and highlighting gaps in knowledge.

The work conducted within this research project is purely fundamental and strictly *in vitro*. While *in vivo* studies can offer accurate results; *in vitro* experiments are more time and cost-effective, as well as better comply with ethical responsibilities and legal regulations.

The final outcome of this thesis aims to give insights into poorly understood pathways in arsenic metabolism and to narrow the number of arsenic species of interest for epidemiologists and toxicologists. Hence, the findings presented herein have prospective implications in environmental and public health.

DECLARATION OF CO-AUTHORED PUBLICATION CHAPTER

For use in theses which include publications. This declaration must be completed for each co-authored publication and to be placed at the start of the thesis chapter in which the publication appears.

Declaration for Thesis Chapter 2

Declaration by candidate

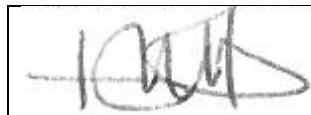
In the case of *Chapter 2*, the nature and extent of my contribution to the work was the following:

Nature of contribution	Extent of contribution (%)
I performed the PBET of arenosugar standards, DMAP and foodstuffs, prepared samples for analysis and performed the total concentration and speciation analyses. I interpreted the results and I am the primary author of the publication.	80

The following co-authors contributed to the work.

Name	Nature of contribution	Contributor is also a student at UC Y/N
Mona Beshai	Performed the PBET of arsenic glutathione complexes	N
William Maher	Contributed ideas and help editing the manuscript	N
Tamsin Kelly	Contributed ideas and help editing the manuscript	N
Simon Foster	Contributed ideas, helped editing the manuscript, provided standards, assisted with the speciation analysis and supervised all the experiments.	N

**Candidate's
Signature**



Date
18/7/17

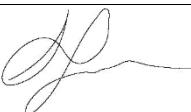
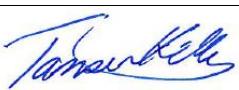
Declaration by co-authors

The undersigned hereby certify that:

- (1) the above declaration correctly reflects the nature and extent of the candidate's contribution to this work, and the nature of the contribution of each of the co-authors.
- (2) they meet the criteria for authorship in that they have participated in the conception, execution, or interpretation, of at least that part of the publication in their field of expertise;
- (3) they take public responsibility for their part of the publication, except for the responsible author who accepts overall responsibility for the publication;
- (4) there are no other authors of the publication according to these criteria;
- (5) potential conflicts of interest have been disclosed to (a) granting bodies, (b) the editor or publisher of journals or other publications, and (c) the head of the responsible academic unit; and
- (6) the original data are stored at the following location(s) and will be held for at least five years from the date indicated below:

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Signature 2		18/7/17
Signature 3		18/7/17
Signature 4		18/7/17

CHAPTER 2 – Bioaccessibility and degradation of naturally occurring arsenic species from food in the human gastrointestinal tract

Introduction

Arsenic is widely distributed in the environment and naturally present at high concentrations in seafood and some terrestrial crops (International Agency for Research on Cancer, 2012). Even though it has been shown that humans are able to adapt to arsenic-rich habitats (Schlebusch et al., 2015), chronic exposure to inorganic arsenic is still associated with the development of diseases that, if not directly derived from arsenic uptake, can be enhanced in its presence (International Agency for Research on Cancer, 2012). Currently, the highest intake of arsenic by humans arises from the consumption of contaminated edibles and beverages, especially drinking water, seafood and rice (International Agency for Research on Cancer, 2012). In drinking water, arsenic exists mainly as the inorganic species arsenate (As(V)) and arsenous acid (As(III)) (Figure 2-1) (International Agency for Research on Cancer, 2012). In rice it can also be found as methylarsonic acid (MA), dimethylarsinic acid (DMA) and the arsenic glutathione complexes arsenotriglutathione (ATG), monomethylarsonic diglutathione (MADG) and dimethylarsoglutathione (DMAG) (Figure 2-1) (Duan et al., 2011). Seaweeds are rich in arenosugars, such as arenosugar-glycerol (O-Gly), arenosugar-sulfate (O-SO₄), arenosugar-sulfonate (O-SO₃) and arenosugar-phosphate (O-PO₄) (Tukai et al., 2002). In fish, arsenobetaine (AB) is the major arsenic species found (Maher et al., 2009) and arsenolipids comprise a 10 – 30 % of the total arsenic content (Figure 2-1) (Sele et al., 2013). Arsenic toxicity depends on the chemical form absorbed and metabolised once inside an organism (Michalski et al., 2012); hence the way each arsenic species affects human health is different. For example, As(III) and As(V) are categorised as Group 1 carcinogens, while MA and DMA are possibly carcinogenic or Group 2B carcinogens (International Agency for Research on Cancer, 2012). Arsenolipids have been shown to be toxic to human hepatocytes (Meyer et al., 2014a) and arenosugars toxicity has been suggested in terms of the arsenic species produced after metabolism in cells (Leffers et al., 2013a). In contrast, arsenocholine and arsenobetaine are non-toxic to mammals (International Agency for Research on Cancer, 2012).

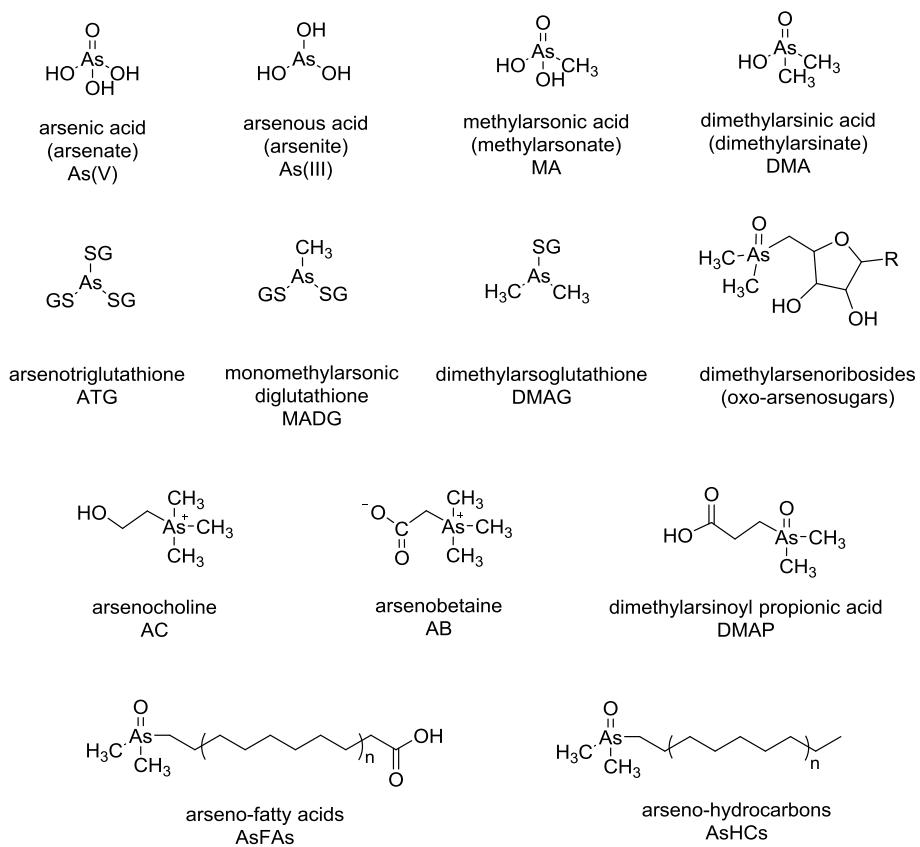


Figure 2-1. Structures of the arsenic species commonly encountered in food and water relevant to this study. The moiety –SG in ATG, MADG and DMAG corresponds to a molecule of glutathione, in which a sulfur atom is bound to arsenic.

To evaluate the effects of arsenic in human health, it is important to understand what happens when specific arsenic species enter the body. To date, most studies have focused on the metabolism of inorganic arsenic in mammalian cells (Hayakawa et al., 2004, Rehman and Naranmandura, 2012), as well as the identification of arsenic metabolites in human urine (Crecelius, 1977, Le et al., 2000, Raml et al., 2005, Schmeisser et al., 2006a). Little attention, however, has been paid to the initial processes after ingestion, which are the transformations occurring in the stomach and small intestine. These are key in understanding the bioaccessible fraction of arsenic, that is the species potentially reaching the bloodstream after enteric absorption (Ruby et al., 1999); it is this fraction that needs to be accounted for when investigating metabolic processes in cells. To study the human digestion process of trace elements, both *in vivo* and *in vitro* approaches have been developed (Ruby et al., 1999). The limitations of *in vivo* experiments are economics, ethics and their complexity, and, furthermore, it has been shown that *in vitro* studies can be correlated to the results obtained after *in vivo*

experiments (Ruby et al., 1999). Nowadays, the most reliable *in vitro* model is known as physiologically based extraction test (PBET), which involves two extractions that mimic the gastric and small intestinal stages of digestion (Karadaş and Kara, 2011).

Few studies have been published on arsenic bioaccessibility (Ruby et al., 1996, Gamble et al., 2002, Laparra et al., 2003, Almela et al., 2005, Calatayud et al., 2013, Zhao et al., 2014). Ruby *et al.* (1996) and Laparra *et al.* (2003) focused on inorganic arsenic, which is already known to enter metabolic pathways in cells; whereas Gamble *et al.* (2002) conducted experiments on the *in vitro* gastrointestinal digestion of four arenosugar standards (O-Gly, O-SO₄, O-SO₃ and O-PO₄) in simulated gastric juices and reported their degradation to dimethylarsinoylribose (O-Ribose). These experiments followed only the chemical effects of the stomach pH (~ 2) and pepsin on the arsenic species tested. Almela *et al.* (2005) considered the effect of both the stomach and small intestine conditions on arenosugar-containing seaweeds. Their findings contradicted Gamble *et al.* (2002) results, as arenosugars in seaweed were intact after digestion. Calatayud *et al.* (2013) reported the *in vitro* gastrointestinal digestion of As(V), MA and DMA added to vegetables. They observed that, after exposure to the stomach and intestine conditions, the water soluble arsenic fraction contained methylarsonous acid (MA(III)), dimethylarsinous acid (DMA(III)), thio-methylarsonic acid (thio-MA) and thio-dimethylarsinic acid (thio-DMA). The digestion of As(V), MA and DMA standards in the absence of vegetables did not produce these species, suggesting that sulfur-containing components in the vegetables, together with the cooking procedure, had important effects on arsenic metabolism. Zhao *et al.* (2014) reported the transformations of As(III), As(V), MA and DMA in various seaweeds after *in vitro* gastrointestinal digestion. It was shown that, for some seaweed, digestion of DMA and MA produced As(III); however, they did not conduct a comprehensive analysis of arsenic species due to the lack of arenosugar standards. The ingestion of arenosugars and arsenolipids has also been investigated regarding their excretion products in human urine (Schmeisser et al., 2006a, Raml et al., 2005, Francesconi et al., 2002). Arsenobetaine, in contrast, was shown to be stable and excreted unchanged after human ingestion (Schmeisser et al., 2006a).

In this study PBETs are used to evaluate the bioaccessibility and degradation of arsenic glutathione complexes, arenosugars and dimethylarsinoyl propionic acid (DMAP) as a model compound of arsono-fatty acids. The foodstuffs known to contain these arsenic species were also examined. Given the wide variety of gut flora among different human populations and individuals (Clemente et al., 2012), only the enzymatic and chemical conditions of the human gastrointestinal tract have been considered, excluding the microbiota existing in the stomach and small intestine.

Materials and methods

Arsenic species standards

Arsenous acid (As(III)), arsenate (As(V)), methylarsonic acid (MA) and dimethylarsinic acid (DMA) standard solutions were purchased from Sigma-Aldrich (Australia). Arsenosugar-sulfate ($O\text{-SO}_4$), arenosugar-sulfonate ($O\text{-SO}_3$) and arenosugar-phosphate ($O\text{-PO}_4$) standards were isolated from marine seaweed (*Fucus sp.*, *Sargassum sp.* and *Ecklonia radiata*) via preparative chromatography as described elsewhere (Foster, 2007). Synthetic arenosugar-glycerol ($O\text{-Gly}$) was kindly donated by Professor KA. Francesconi, University of Graz. The arsenic glutathione complexes arsenotriglutathione (ATG), monomethylarsonic diglutathione (MADG) and dimethylarsoglutathione (DMAG); as well as the short chain arsono-fatty acid dimethylarsinoyl propionic acid (DMAP), were synthesised in house following published methods (Raab et al., 2004, Rumpler, 2010).

Arsenic-containing foodstuffs

Certified reference materials of rice (CRM-T07151QC, FAPAS, Graham B. Jackson PTY LTD, Australia), seaweed (NIES CRM *Sargasso* No.9, Environment Agency, Yatabe-Machi, Tsukura, IBARAKI, 305, Japan), and fish (CRM DORM-3 dogfish protein, National Research Council Canada, NRC-CNRC) were used as arsenic-containing foodstuffs. Control samples were analysed for total arsenic concentration and arsenic speciation. In CRM-T07151QC, NIES CRM *Sargasso* No.9 and CRM DORM-3 dogfish protein, arsenic species were extracted with a mixture of methanol: water 1:1 v/v via end over end rotation at room temperature for 12 h. CRM-T07151QC rice was also extracted using 0.02 M trifluoroacetic acid (99 % w/w, Sigma-Aldrich, Australia) and 1

% v/v hydrogen peroxide (30 % w/w, UNIVAR, US) in a water bath at 100°C for 1 h (Raber et al., 2012). This method is more efficient in extracting arsenic species from rice, although oxidation of As(III) to As(V) occurs (Raber et al., 2012). Control samples for total arsenic analysis were prepared following the procedure developed by Baldwin *et al.* (1994). Certified reference materials were weighted (0.07 g) into 6 mL polytetrafluoroethylene (PTFE) vessels (CEM, US) with 1 mL of concentrated nitric acid (Aristar, BDH, US) and heated in a microwave oven (MARS, CEM, US) using different stages (500 W, 2 min; 0 W, 2 min; 250 W, 45 min).

Physiologically based extraction test

Physiologically based extraction tests of arsenic glutathione complexes, arsanosugars and DMAP, as well as of the foodstuffs containing these arsenic species, were performed following the procedure described in the literature (Ruby et al., 1996) with modifications. A simulated gastric juice stock solution (100 mL) was prepared in deionised water (18.2 MΩ cm, Sartorius, Australia) with 0.125 g of pepsin (1:3000, PB0411-100G, Biochemicals, Australia), 0.050 g of DL-malic acid (MB0333-250G, Biochemicals, Australia), 0.050 g of sodium citrate (Biotech, CB0035-500G, Biochemicals, Australia), lactic acid to a final concentration of 6×10^{-3} M (ACS grade, AMRESCO, US), acetic acid to a final concentration of 9×10^{-3} M (glacial, ACS grade, AMRESCO, US) and adjusted to pH 2 with hydrochloric acid (32 % w/w, analytical grade, Chem Supply, Australia). Plastic tubes containing simulated gastric juice solution with the arsenic species to test were placed in a hybridisation oven (XTRON HI 2001, Bartelt Instruments, US) at 37°C; after 1 h incubation, aliquots were taken for total arsenic and speciation analyses. The conditions in the tubes were changed to an intestinal phase by the addition of bile salts (sodium cholate and sodium deoxycholate, BB0225-25G, Biochemicals, Australia) to a final concentration of 175 mg L⁻¹, pancreatin (8.0, PB0681-100G, high purity grade, Biochemicals, Australia) to a final concentration 50 mg L⁻¹ and saturated sodium bicarbonate (Ajax Finechem, Australia) to attain pH 7. The samples were incubated in a hybridisation oven at 37°C for 4 h before aliquots were taken for total arsenic and speciation analysis.

Total arsenic concentration in foodstuffs samples

The control samples after nitric acid digestion, together with the stomach and intestinal phases, were analysed for total arsenic concentration (m/z 75) by inductively coupled plasma-mass spectrometry (ICP-MS) using a Perkin Elmer NexIon 300D ICP-MS (Waltham, MA, US) with an ASX-520 Autosampler. The plasma power was 1300 W and the nebuliser gas (argon) flow 0.9 L min^{-1} . Calibration standards were prepared from a multi element calibration standard (Accu Trace, Accu-Standards, 10 mg L^{-1}) in $1\% \text{ v/v}$ nitric acid. Blanks of the stomach and small intestine phases were also analysed and their arsenic concentrations subtracted from that in the samples. Calibration standards were run every 30 samples. Total arsenic recoveries for the certified reference materials used were 104 % for rice T07151QC, 91 % for seaweed *Sargasso* No.9 and 81 % for DORM-3 dogfish protein; all within the accepted range for general recoveries according to the IUPAC.

Arsenic speciation analysis

The speciation analysis of control samples, stomach and intestinal phases was performed by high performance liquid chromatography-inductively coupled plasma-mass spectrometry (HPLC-ICP-MS) using a Flexar 10 HPLC coupled to a NexIon 300D ICP-MS (both Perkin Elmer, Waltham, MA, US). Samples matrices from stomach and small intestine phases had higher concentrations of chloride ions, which form adducts with argon ($^{40}\text{Ar}^{35}\text{Cl}$) that can interfere with the signal for ^{75}As . Chloride also forms an adduct with oxygen ($^{35}\text{Cl}^{16}\text{O}$) with the same m/z as ^{51}V . Vanadium was therefore monitored to discard any peak eluting as ^{75}As that may have come from the presence of chloride based salts in samples. Calibration standards using As(III), As(V), MA, DMA and AB were prepared in methanol: water 1:1 v/v at arsenic concentrations ranging from 100 to $1 \mu\text{g L}^{-1}$. Calibration verification standards at the top, mid and low points in the calibration were run to assess the calibration. Arsenic glutathione complexes and arenosugar standards were only used for species identification. Calibration and species identification standards were run every 20 samples. Blanks for stomach and small intestine phases were also analysed to ensure the lack of interferences from both matrices in the identification and concentration measurement of arsenic species. Column recoveries were between 80 – 120 %. Separation of arsenic species was achieved using a Hamilton PRP-X100 anion

exchange column (250 mm × 4.1 mm, 10 µm particle size, Phenomenex, US) with a 10 mM malonic acid (UNILAB, Ajax Chemicals, Australia) mobile phase (pH 5.6 adjusted with aqueous ammonia (Sigma-Aldrich, Australia)). Gradient elution using malonic acid was held at 2.5 mM for 2 min, increased to 10 mM over 6 min and held for 2 min, and then decreased back to 2.5 mM over 2 min. Arsenic species were confirmed by spiking with standards, and the identity of As(V) and As(III) was verified by high performance liquid chromatography-hydride generation-inductively coupled plasma-mass spectrometry (HPLC-HG-ICP-MS) following a modified procedure of Kirby *et al.* (2004). Hydride generation was achieved post column using 3 % v/v hydrochloric acid (Sigma-Aldrich, Australia) and 2 % w/v L-cysteine (Sigma-Aldrich, Australia) to reduce As(V) to As(III); and 1 % w/v sodium borohydride (Sigma-Aldrich, Australia) to generate the hydrides. Arsenosugar standards were run to ensure that no hydrides were formed under the conditions applied. Further confirmation of arsenic species was made using a Zorbax 300-SCX cation exchange column (150 mm × 4.6 mm, 5 µm particle size, Agilent Technologies, US) with a 2.5 mM pyridine (Merck, Australia) mobile phase (pH 2.6 adjusted with formic acid (UNIVAR, Australia)) under isocratic conditions.

Results

Arsenic glutathione complexes and rice

Simulated gastrointestinal digestion of arsenic glutathione complex standards led to the hydrolysis of the glutathione moieties and following demethylation of arsenic (Figure 2-2). In the stomach phase, all ATG was hydrolysed to As(III), from which 36 % was oxidised to As(V) in the small intestine phase (Figure 2-2a). Initially, 56 % of MADG was transformed to MA in the stomach and subsequently demethylated to As(V) in the intestine, as indicated by the 52 % increase in As(V) concentration from the former to the latter (Figure 2-2b). A peak in the chromatogram of both phases of MADG appeared at the same retention time as DMA (2.3 min). Samples were spiked with DMA and thio-MA and the unknown arsenic species was confirmed to be DMA, accounting for 16 % in the stomach and 26 % in the small intestine (Figure 2-2b). Of the initial MADG, 12 % was transformed into an unknown species retained at 5.8 min. Dimethylarsoglutathione was completely hydrolysed to DMA in the stomach, from which 16 % was demethylated to As(V) in the intestine (Figure 2-2c).

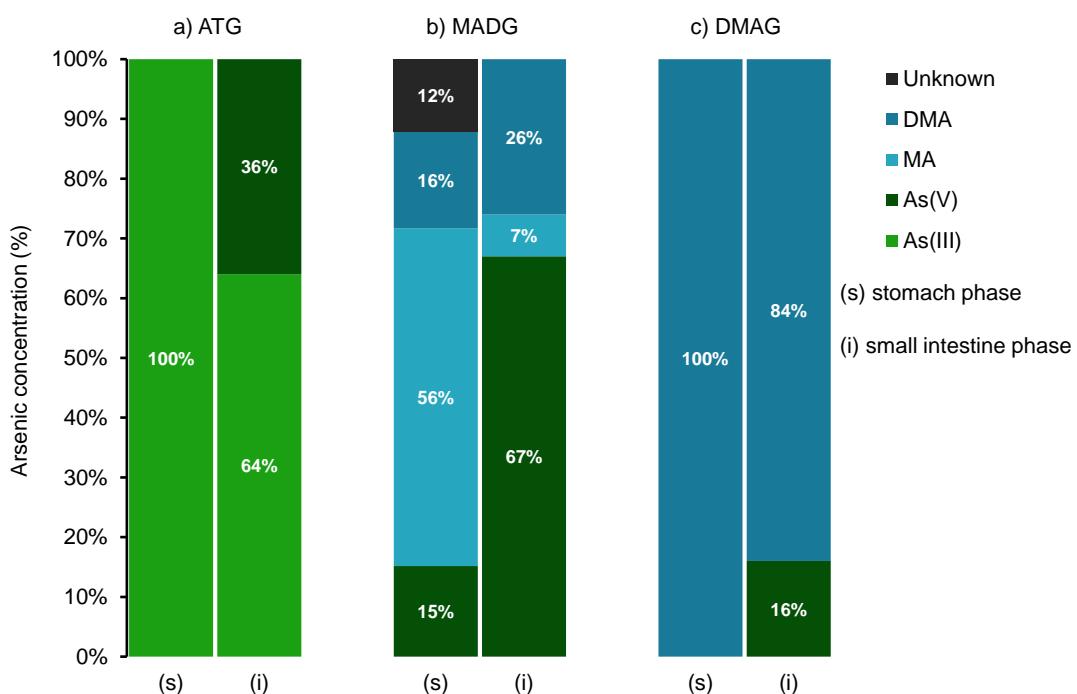


Figure 2-2. Arsenic species (%) identified after the PBET (stomach (s) and small intestine (i) phases) of pure arsenic glutathione complex standards: a) arsenotriglutathione (ATG), b) monomethylarsinic diglutathione (MADG) and c) dimethylarsoglutathione (DMAG).

The chromatograms for the rice samples CRM-T0715QC are shown in Figure 2-3. Rice control samples after extraction with trifluoroacetic acid and hydrogen peroxide contained As(III) (35 %), DMA (28 %), MA (1 %) and As(V) (36 %) (Figure 2-3a). Rice control samples after extraction with methanol: water showed no As(V), indicating that the As(V) observed in Figure 2-3a corresponds to As(III) oxidised to As(V) during the extraction. Therefore, rice control samples contained As(III) (71 %), DMA (28 %) and MA (1 %). After the PBET (Figure 2-3b), the concentrations of As(III) and DMA decreased (35 % and 22 %, respectively), together with the appearance of As(V) (43 %) and the disappearance of MA, implying that oxidation of As(III) and demethylation of DMA and MA occurred. Of the total arsenic present in rice ($0.7 \pm 0.1 \mu\text{g g}^{-1}$), $0.35 \pm 0.05 \mu\text{g g}^{-1}$ was extracted in the stomach, with a further $0.25 \pm 0.11 \mu\text{g g}^{-1}$ in the small intestine, thus, $80 \pm 1 \%$ of arsenic in rice is potentially available.

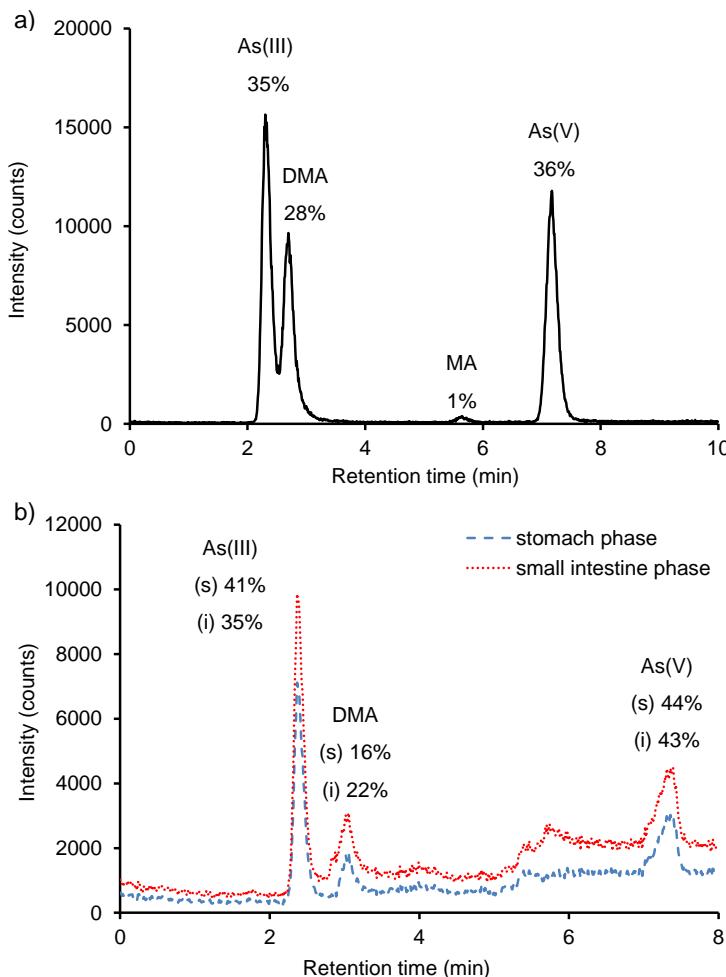


Figure 2-3. Chromatograms of rice samples (CRM-T07151QC) before (a) and after (b) PBET. Arsenic concentrations (%) in the stomach (s) and small intestine (i) phases are shown for each of the arsenic species identified.

Arsenosugars and seaweed

After the gastrointestinal digestion of the arvensosugar standards, their side chains were probably hydrolysed to O-Ribose (Figure 2-4), as previously reported by Gamble *et al.* (2002). Arvensosugar-glycerol and O-Ribose have similar retention times under both the anion and cation exchange chromatography conditions used, however, the presence of the same peak at 2.2 min in the stomach and small intestine phases of all four arvensosugars and not only in O-Gly samples (Figure 2-4), supports the assertion that the arvensosugars were hydrolysed to O-Ribose. For O-Gly, hydrolysis occurred only in the intestine, whereas for O-SO₄, O-SO₃ and O-PO₄, 7 – 10 % was hydrolysed in the stomach (Figure 2-4). The production of As(V) was observed for all arvensosugars during the

intestinal digestion (Figure 2-4). The formation of As(V) accounted for 65 % of the total arsenic concentration for O-Gly, 29 % for O-SO₄, 32 % for O-PO₄, and 10 % for O-SO₃.

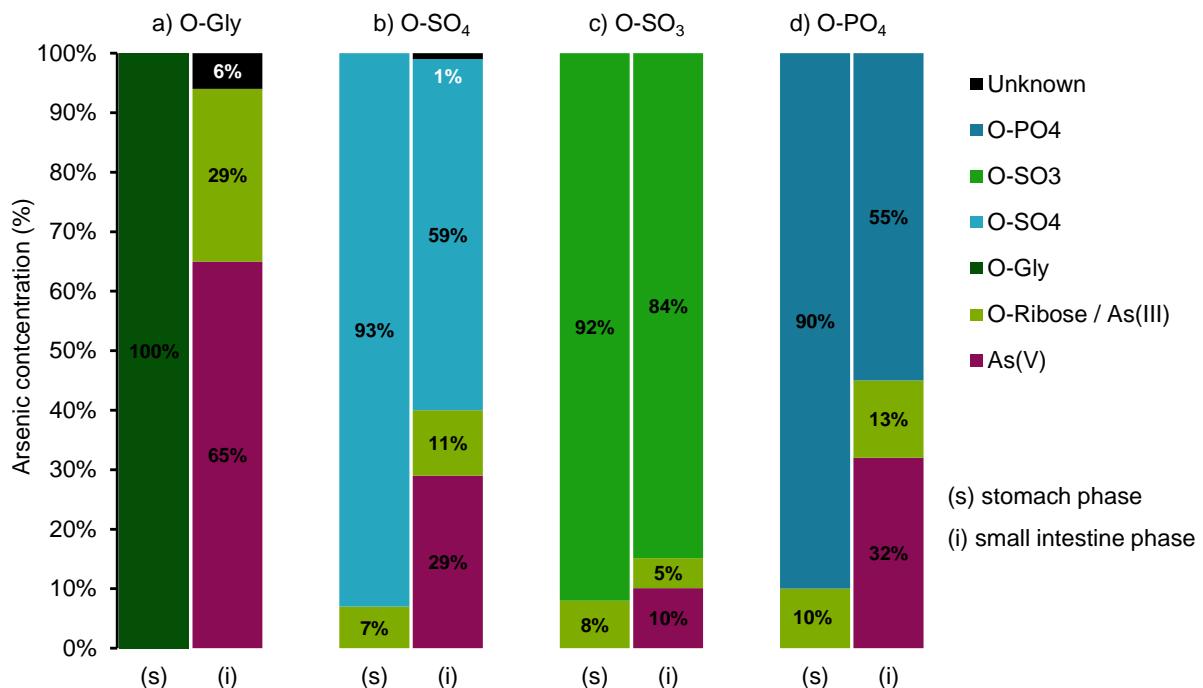


Figure 2-4. Arsenic species (%) identified after the PBET (stomach (s) and small intestine (i) phases) of pure arenosugars standards: a) arenosugar glycerol (O-Gly) b) arenosugar-sulfate (O-SO₄), c) arenosugar-sulfonate (O-SO₃), and d) arenosugar-phosphate (O-PO₄).

Spiking of samples with arsenic standards and analysis by HPLC-HG-ICP-MS confirmed the assignment of the peak corresponding to As(V) (Figure 2-5a and 2-5b). The chromatograms obtained using hydride generation also showed a peak around 2.4 min corresponding to As(III) (Figure 2-5b). This peak was confirmed by cation exchange chromatography, followed by spiking with an As(III) standard (Figure 2-5b). Dimethylarsinoylribose was also identified after cation exchange chromatography, as As(III) and O-Ribose co-elute under the anion exchange chromatography conditions used but can be separated by cation exchange (Figure 2-5a and 2-5c). Therefore, for O-SO₄, O-SO₃ and O-PO₄, hydrolysis to O-Ribose was accompanied by demethylation to As(III) (Figure 2-4 and Figure 2-5).

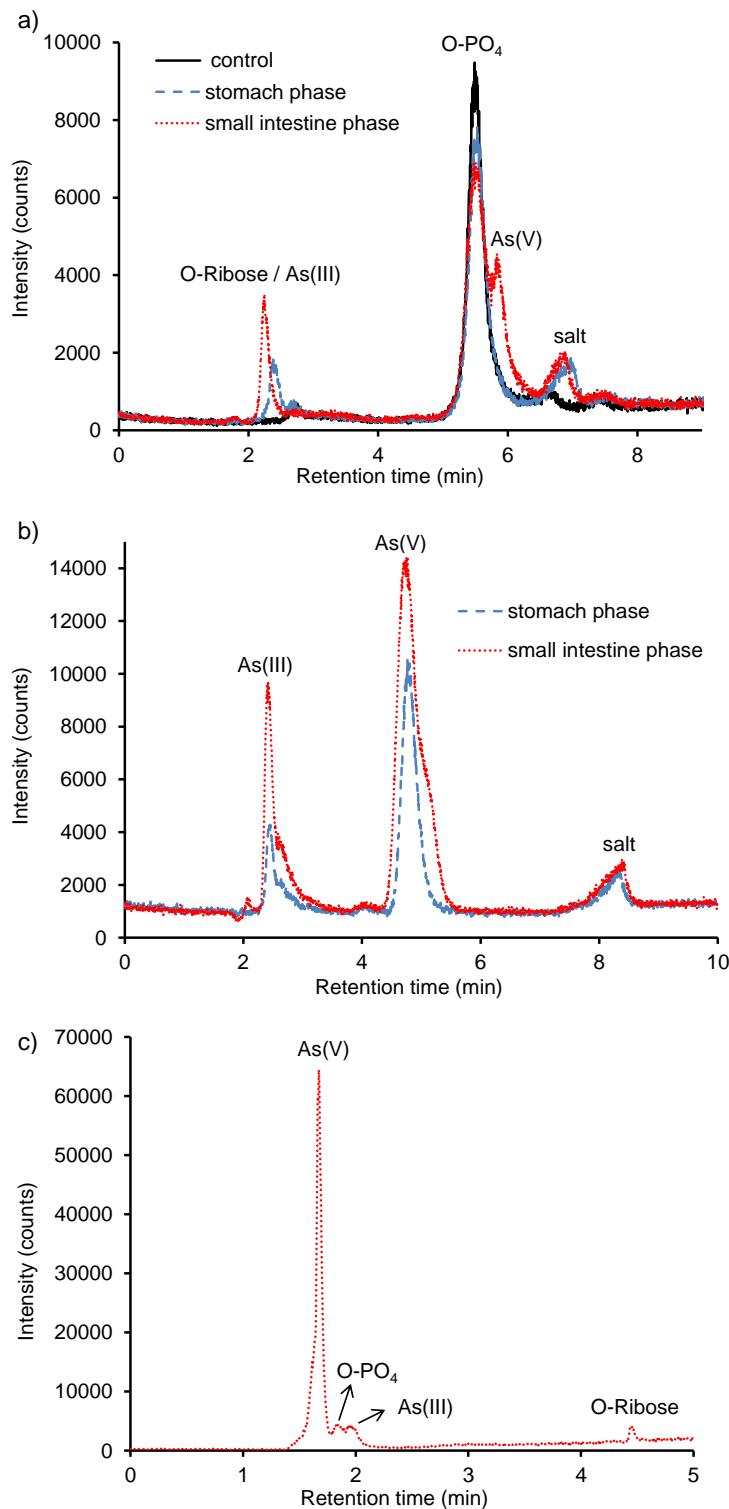


Figure 2-5. Chromatograms of the arsenosugar-phosphate ($O\text{-PO}_4$) standard after the PBET: a) stomach and small intestine phases under anion exchange conditions, b) stomach and small intestine phases under anion exchange conditions coupled with hydride generation and c) small intestine phase under cation exchange conditions.

In NIES CRM *Sargasso* No.9, no hydrolysis of the arenosugars side chains was observed (Figure 2-6). Samples were spiked with O-PO₄ and As(V) to determine the nature of the double peak at around 6 min in the control samples (Figure 2-6a) and at 5 min in the stomach and small intestine phases (Figure 2-6b), which were confirmed as O-PO₄. The peak at 7.7 min in the control sample could not be identified. Dimethylarsinic acid was present in the controls (5 %), as well as in the stomach (3 %) and small intestine (4 %) phases. Due to the similar retention time with O-Gly, DMA was spiked into the samples and the peak at 2.8 min was confirmed as DMA. No demethylation of arenosugars occurred. Of the total arsenic in seaweed ($75 \pm 6 \mu\text{g g}^{-1}$), $53 \pm 2 \mu\text{g g}^{-1}$ was extracted in the stomach and $61 \pm 7 \mu\text{g g}^{-1}$ after gastrointestinal digestion, indicating that in the intestine no significant additional arsenic is solubilised. Thus, $80 \pm 2 \%$ of the arsenic is potentially available from seaweed.

Arsenic in fish

Dimethylarsinoyl propionic acid was not degraded in either of the two stages of simulated gastrointestinal digestion (Figure 2-7). The arsenic species identified in fish (Figure 2-8) were AB (77 %), As(III) (1 %), As(V) (4 %), MA (2 %) and DMA (15 %). None of them were significantly transformed after the PBET. The determination of arsenic concentration in fish indicated that of the total arsenic ($5.6 \pm 0.2 \mu\text{g g}^{-1}$), $5.14 \pm 0.04 \mu\text{g g}^{-1}$ were extracted in the stomach and $4.9 \pm 0.2 \mu\text{g g}^{-1}$ after gastrointestinal digestion, indicating that in the intestinal phase no additional arsenic is solubilised. Thus, $87 \pm 1 \%$ of arsenic is potentially bioavailable from dogfish.

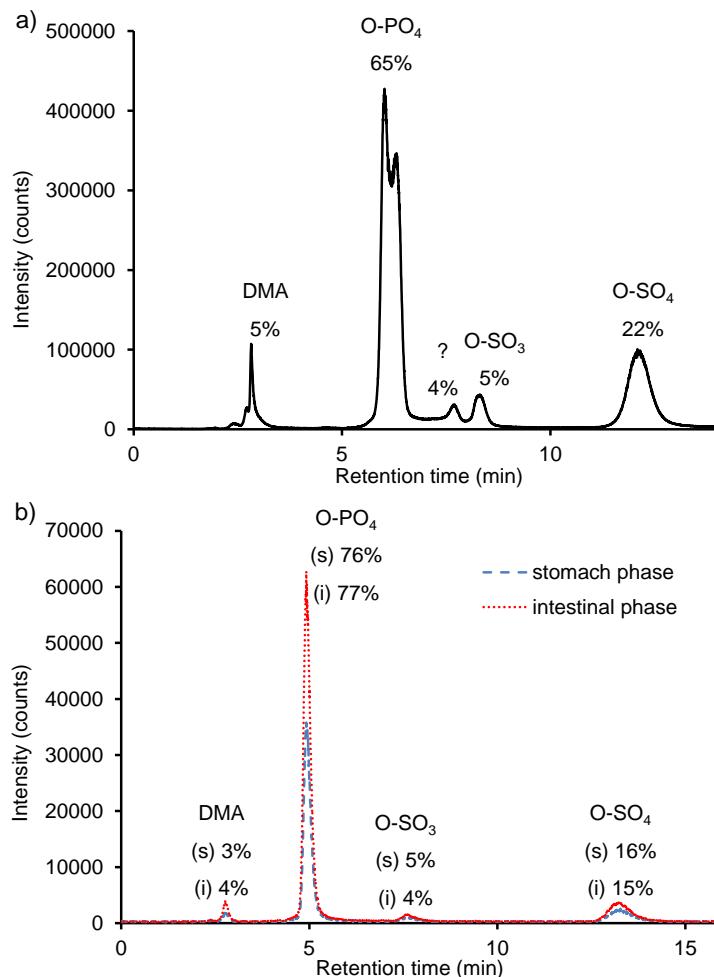


Figure 2-6. Chromatograms of macroalgae samples (NIES CRM Sargasso N.9) before (a) and after (b) the PBET. Arsenic concentrations (%) in the stomach (s) and small intestine (i) phases are shown for each of the arsenic species identified.

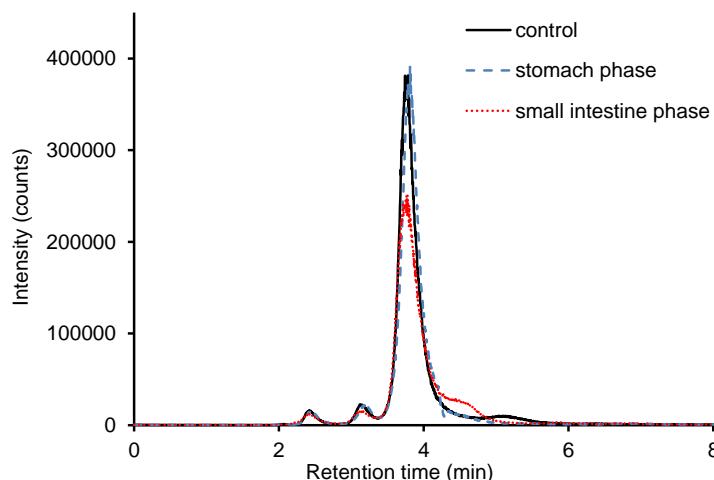


Figure 2-7. Chromatograms of the dimethylarsinoyl propionic acid (DMAP) standard before and after the PBET.

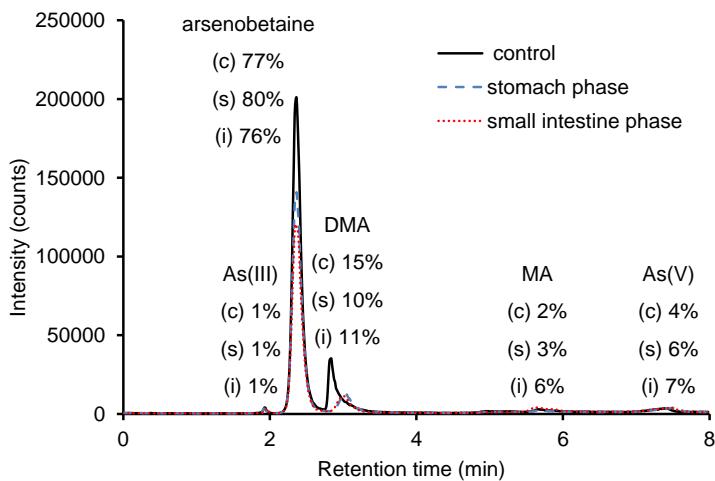


Figure 2-8. Chromatograms of fish samples (CRM DORM-3) before and after the PBET. Arsenic concentrations (%) in controls (c), and the stomach (s) and small intestine (i) phases are shown for each of the arsenic species identified.

Discussion

Arsenic glutathione complexes and rice

Arsenotriglutathione, MADG and DMAG lose their glutathione moieties after hydrolysis during the stomach digestion producing As(III), MA and DMA, respectively (Figure 2-9). Under acidic conditions (pH 2), hydrolysis of arsenic glutathione complexes will occur in agreement with Hayakawa's pathway (Hayakawa et al., 2004), where the arsenic trivalent species formed will be readily oxidised to their pentavalent counterparts (Figure 2-9a). The rapid oxidation of MA(III) and DMA(III) in aqueous solution (Francesconi, 2003) makes only MA and DMA detectable in the stomach phase; whereas As(III) produced from ATG hydrolysis is oxidised later in the small intestine (Figure 2-9a). In rice, free arsenic glutathione complexes do not usually exist, they are found as part of proteins in which arsenic is bound to the sulfur atom of the cysteine residue of glutathione (Raab et al., 2007). Thus, only As(III), MA and DMA were found, as reported in other studies with similar acidic extraction methods (Maher et al., 2013a). What is unusual are the transformations taking place during the gastrointestinal digestion: As(V) is produced from demethylation of MA and DMA (Figure 2-9b). Demethylation of MA and DMA was also observed for MADG and DMAG, where As(V) appears in the intestinal phase of both glutathione complexes (Figure 2-2). Given no As(V) was detected in the control samples, the possibility of contamination was

discarded. Spiking the small intestine samples with As(V) also confirmed the assignment of the peak. It seems that the demethylation of arsenic species starts in the stomach and continues in the small intestine (Figure 2-9). Zhao *et al.* (2014) have also shown that demethylation of DMA occurs after the gastrointestinal digestion of arsenic-containing seaweeds (*L. japonica*, *P. yezoensis*, *U. pinnatifida*, *H. fusiformis* and *E. prolifera*). Microorganisms in soils and marine sediments readily demethylate arsenic (Lehr *et al.*, 2003) but the mechanisms and the enzymes involved still remain unclear. Given that inorganic arsenic species are more toxic than pentavalent organic arsenic species (Michalski *et al.*, 2012); the formation of inorganic arsenic species from the gastrointestinal digestion of MA and DMA will increase the likelihood of arsenic toxicity in the liver after the absorption of arsenic from rice. Regarding the bioaccessibility of arsenic from rice, it has been reported that it is not affected by cooking with non-contaminated water (Rahman *et al.*, 2011). The experiments in this study were performed without cooking the rice and the arsenic bioaccessibility obtained was 80 %. Trenary *et al.* (2012) reported the arsenic bioaccessibility of 17 different rice samples, with values ranging from 45 % to 95 %, thus, showing that bioaccessibility depends on the type of rice consumed. The remaining arsenic, not extracted after the gastrointestinal digestion, accounts for 21 % of the total arsenic in rice. Most probably, this is arsenic bound to proteins through cysteine and other sulfur complexes that could not be extracted in the mild conditions of the stomach and small intestine.

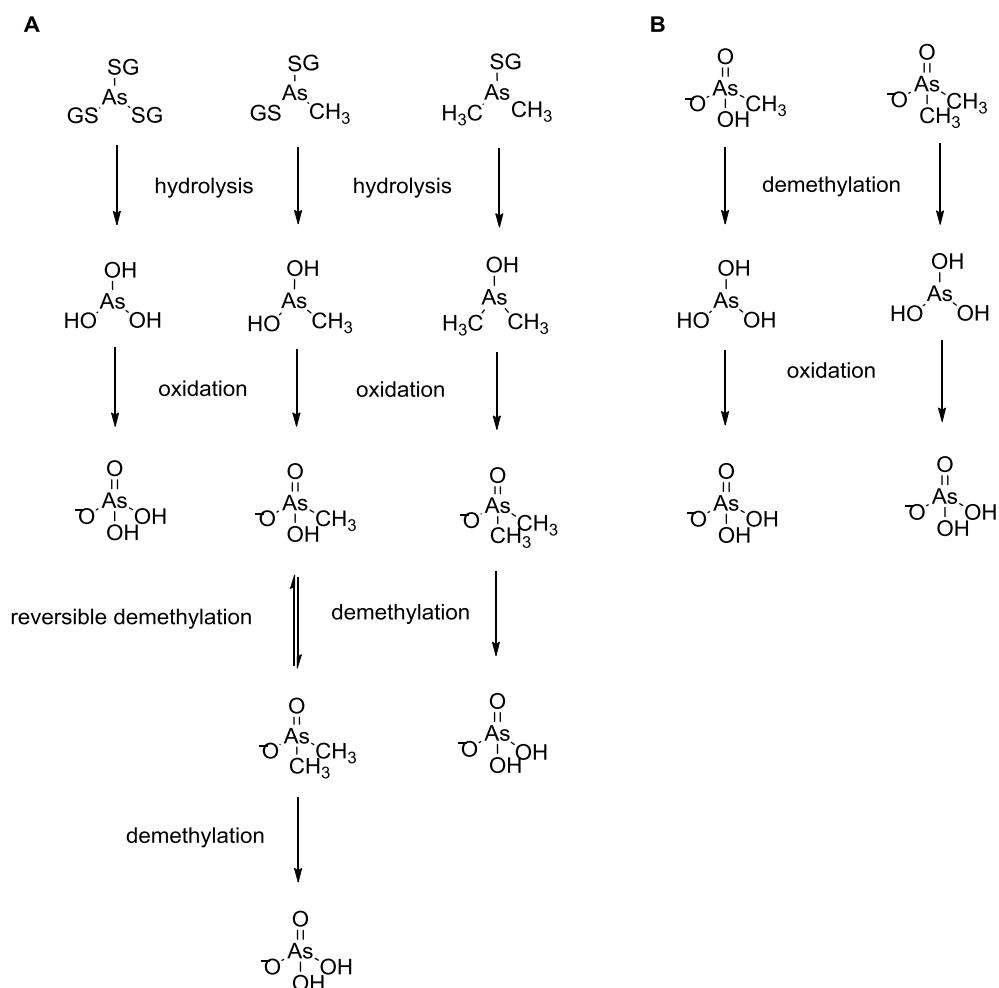


Figure 2-9. Proposed mechanism for the degradation of arsenic glutathione complexes (a) and arsenic species in rice (b) during PBET.

Arsenosugars and seaweed

While the four arvensosugar standards lose their side chains during PBET (Figure 2-10), no hydrolysis was observed for naturally occurring arvensosugars in seaweed. These results are in agreement with the literature (Gamble et al., 2002, Almela et al., 2005). Gamble *et al.* (2002) reported the hydrolysis of arvensosugars only under the stomach conditions but the findings described here suggest that the loss of the side chain is more likely to occur in the small intestine, although it begins in the stomach (Figure 2-4). Additionally, the presence of the arvensosugars in the intestinal phase indicates that hydrolysis is not complete after the whole gastrointestinal digestion and they would be absorbed into the bloodstream together with their hydrolysed form (O-Ribose) (Figure 2-4). Unexpectedly, all four arvensosugars were partially degraded to inorganic arsenic. As

shown in Figure 2-5, this process starts in the stomach with the formation of As(III), which is increased in the small intestine and then oxidised to As(V). A possible mechanism would consist of the ribose ring opening after hydrolysis of the side chain, then tautomerisation of the carbonyl group followed by a retro adol like reaction leading to 1,3-dihydrolxyacetone and dimethylasinoacetaldehyde (Figure 2-10). Given that the electronegativity of arsenic is lower than the electronegativity of carbon, the dimethylarsinoyl moiety of dimethylasinoacetaldehyde would be more electrophilic than the carbonyl group, and thus more susceptible to be attacked by a nucleophile. A nucleophilic substitution could remove the methanal residue from the molecule to produce DMA (Figure 2-10). Subsequent demethylation of DMA to As(III) and further oxidation would lead to As(V) as final product (Figure 2-10). Dimethylarsinic acid would be an intermediate in the process, but the absence of DMA in the chromatograms obtained indicates that either DMA is rapidly demethylated or a different mechanism operates.

In *Sargasso* No.9, DMA and three arenosugars (O-PO_4 , O-SO_3 and O-SO_4) are present (Figure 2-6a). Degradation and demethylation of arenosugars in seaweed was not observed (Figure 2-6b), in agreement to previous work (Almela et al., 2005), implying that arenosugars are absorbed into the hepatic portal system as such when ingested in an algae matrix. Hence, the food matrix plays an important role in arsenic bioaccessibility from seaweed. Different arsenic species have been identified in human urine after the consumption of O-Gly, including DMA, dimethylarsenoethanol (DMAE), trimethylarsine oxide (TMAO) (Francesconi et al., 2002), thio-dimethylarsinoyl acetic acid (thio-DMAA), thio-dimethylarsenoethanol (thio-DMAE), thio-dimethylarsinic acid (thio-DMA) and the equivalent thio-arsenosugar (Raml et al., 2005). None of those metabolites were identified in the present study. The unknown arsenic species reported (Figure 2-4a and 2-4b) could correspond to these arsenic species but in very low concentrations. Thus, the formation of those metabolites occurs either after enteric absorption or, if in the gastrointestinal tract, due to the microbiota present in the stomach and small intestine. The former is supported by the work conducted by Conklin et al. (2006), which showed the transformation of an arenosugar into its thio- analogue by the anaerobic microflora of mouse cecum without additional change. As for rice, cooking of

seaweed does not cause arenosugar degradation and the bioaccessibility of arsenic will depend on the type of seaweed consumed (Almela et al., 2005).

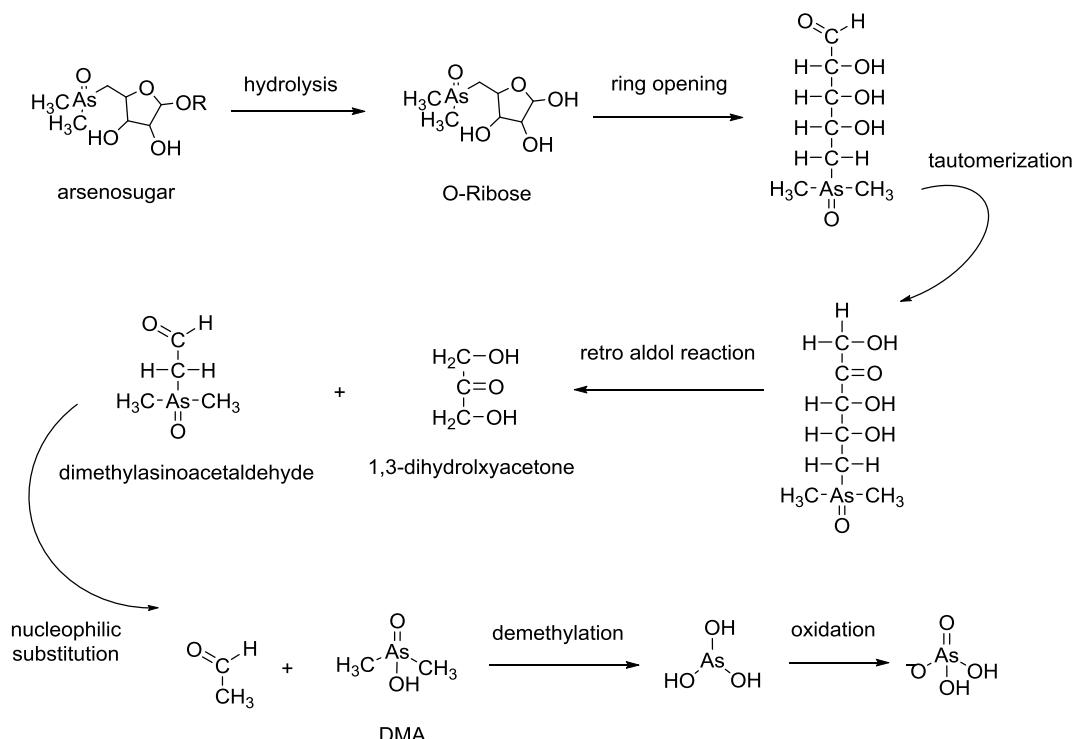


Figure 2-10. Proposed mechanism for the degradation of arenosugars standards during PBET.

Arsenic in fish

Dimethylarsinoyl propionic acid, a short chain arsено-fatty acid (Figure 2-1), was not altered under the conditions of the stomach and small intestine (Figure 2-7). Given that the DMA and carboxylic acid moieties of DMAP are more reactive than the hydrocarbon chain, long chain arsено-fatty acids and hydrocarbons would also be expected to be stable during gastrointestinal digestion. Under the chromatographic conditions used in this study, only As(III), As(V), MA, DMA and AB could be identified in dogfish (Figure 2-8). All of these species were not changed in the gastrointestinal tract and therefore, could be absorbed into the hepatic portal system. In terms of bioaccessibility, 87 % of arsenic in dogfish can potentially reach the bloodstream after digestion. This value falls within those already reported for clams (77 – 92 %) (Koch et al., 2007) and various seafood (53 – 117 %) (Leufroy et al., 2012); but is not in concordance with the results obtained by Leufroy et al. (2012) for DORM-3, where only

55 % of arsenic was bioaccessible. They used different experimental and analytical methods, which could explain the discrepancy.

Concluding remarks

This work shows that the stomach plays an important role in hydrolysing and solubilising the organic arsenic species that become available to humans after ingestion. Regarding arsenic concentration, the bioaccessible fraction does not increase after intestinal digestion. The chemical degradation that occurs, however, is crucial in determining the nature of the arsenic species to be absorbed into the bloodstream. The demethylation of MA, DMA, arsenic glutathione complexes and arsenosugars raises questions regarding arsenic metabolic pathways in humans. In rice, demethylation increases the toxicity of the arsenic species absorbed into the hepatic portal system. For arsenosugars, this process seems to occur only for standards but not when ingested within the corresponding foodstuff, confirming the relevance of the food matrix in arsenic metabolism. Arsenosugars ingested from seaweed are likely to be absorbed into the liver unchanged.

CHAPTER 3 – Degradation of arsenic containing hydrocarbons and fatty acids from krill oil and hijiki seaweed in the human gastrointestinal tract

Introduction

With the increasing global consumption of seafood and fish oil supplements (Food and Agriculture Organization of the United Nations, 2014); humans are exposed to significant levels of arsenic. In marine organisms, arsenic exists in concentrations between 5 and 100 µg g⁻¹ dry mass (Francesconi, 2010) and is present as a wide variety of chemical species ranging from arsenate (As(V)) to arsenic-containing lipids, also referred to as arsenolipids. Arsenolipids comprise around 10 – 30 % w/w of the total arsenic concentration in marine organisms (Sele et al., 2012); and are the main arsenic species in commercial fish oils, with concentrations ranging from 0.2 to 16 mg kg⁻¹ oil (Sele et al., 2012).

Arsenolipids were first detected in 1920s (Sadolin, 1928) and since then, seven categories of these arsenic species have been identified: arsено-fatty acids (AsFAs), arsено-hydrocarbons (AsHCs), arsено-phospholipids (AsPLs), glycoarsenolipids (GlyAsLs) or arenosugar-phospholipids, arsenic-containing phosphatidylcholines (AsPCs), arsenic-containing phosphatidylethanolamines (AsPEs) and cationic trimethylarsonio fatty alcohols (TMAsFOHs). Arseno-fatty acids have been found in cod liver oil (*Gadus morhua*) (Rumpler et al., 2008), capelin oil (*Mallotus villosus*) (Amayo et al., 2011), herring fillet (*Clupea harengus*) (Lischka et al., 2013) and in the brown alga *Saccharina latissima* (Raab et al., 2013). Their structure resembles that of natural fatty acids, with a carboxylic acid on one end and a dimethylarsinoyl on the other (Figure 3-1). Arseno-hydrocarbons, that have been identified in sashimi tuna (*Thunnus sp*) (Taleshi et al., 2010), capelin oil (*Mallotus villosus*) (Taleshi et al., 2008), and in the brown algae *Undaria pinnatifida*, *Hizikia fusiformis* (Garcia-Salgado et al., 2012) and *Saccharina latissima* (Raab et al., 2013); are long chain alkanes with a dimethylarsinoyl group covalently bound to one of the ends of the carbon backbone (Figure 3-1). Arseno-phospholipids, found in yelloweye mullet (*Aldrichetta forsteri*) (Francesconi et al., 1990), western rock lobster (*Panulirus cygnus*) (Edmonds et al., 1992), starspotted shark (*Mustelus manazo*) (Hanaoka et al., 1999) and ringed seal (*Pusa hispida*) (Ebisuda et al., 2003); are similar to

membrane glycerophospholipids, such as lecithines, but contain arsenocholine instead of the nitrogen based choline equivalent (Figure 3-1). In GlyAsLs the dimethylarsinoyl moiety is bound to a ribose, which in turn forms a bond with a phospholipid (Figure 3-1). Glycoarsenolipids are predominant in algae, such as *Undaria pinnatifida* (Morita and Shibata, 1988, Garcia-Salgado et al., 2012), *Hizikia fusiformis* (Garcia-Salgado et al., 2012) and *Saccharina latissima* (Raab et al., 2013). Arsenic-containing phosphatidylcholines and phosphatidylethanolamines have been recently identified in herring caviar (*Clupea harengus*) (Viczek et al., 2016). Their structure resembles AsPLs, as it also corresponds to a phosphatidylcholine or phosphatidylethanolamine; but in this case arsenic is found in one of the fatty acids, as an AsFA, instead of in the choline moiety (Figure 3-1). Trimethylarsonio fatty alcohols, identified in capelin oil (*Mallotus villosus*) (Amayo et al., 2013), consist of a long fatty alcohol with a trimethylarsonio group at the other end of the chain (Figure 3-1).

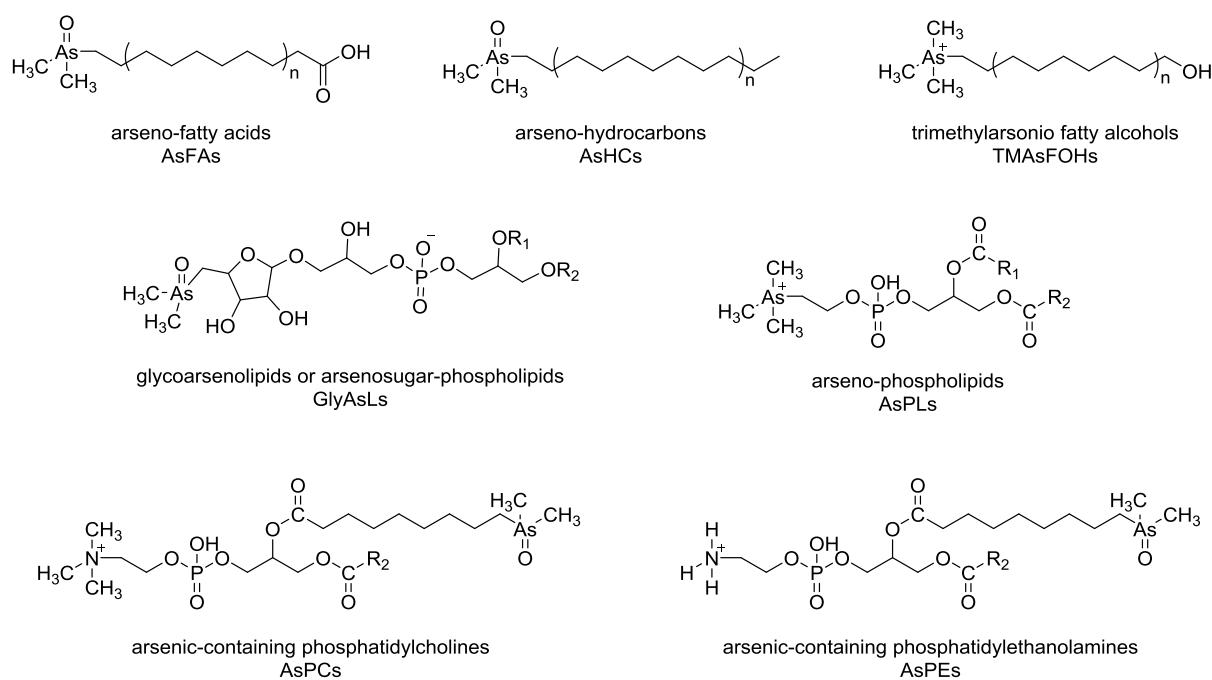


Figure 3-1. Chemical structures of the types of arsenolipids known to date.

The toxicity of inorganic arsenic species and of a few organic arsenic species has been extensively studied and, for example, it is currently known that arsenous acid (As(III)), As(V), methylarsonic acid (MA) and dimethylarsinic acid (DMA) are carcinogenic (International Agency for Research on Cancer, 2012), whereas

arsenobetaine (AB) is relatively harmless (International Agency for Research on Cancer, 2012, Ohta et al., 2004). The focus on arsenolipid toxicity is more recent, and it has already been shown that AsHCs can be as toxic as As(III) (Meyer et al., 2014a, Meyer et al., 2014b). Still, more data is needed for food authorities to establish appropriate policies regarding the safe consumption of arsenolipid-containing oils and foods. Not only are toxicity tests required but also metabolic studies investigating the transformations and metabolites of arsenolipids in humans.

Only a few publications have reported the metabolism of AsFAs and AsHCs in humans (Schmeisser et al., 2006a, Schmeisser et al., 2006b, Meyer et al., 2015b). The main excretion products identified in human urine after the ingestion of cod liver were DMA and AB; with thio-dimethylarsinoyl propionic acid (thio-DMAP), thio-dimethylarsinoyl butyric acid (thio-DMAB), dimethylarsinoyl propionic acid (DMAP) and dimethylarsinoyl butyric acid (DMAB) in smaller concentrations (Schmeisser et al., 2006a, Schmeisser et al., 2006b). Even though the structure of the ingested lipids could not be resolved, the authors did not find any evidence of thio-arsenic species among them, suggesting thiolation as a metabolic process in humans. Arsenobetaine was also present in cod liver but was excreted unchanged. Another study investigated the transfer across the intestinal barrier and presystemic metabolism of AsFAs and AsHCs using Caco-2 cells (Meyer et al., 2015b). It was found that both types of arsenolipids are able cross the intestinal barrier, with the permeability of AsHCs being higher. In contrast, AsFAs are readily presystemically metabolised to their trivalent counterparts and to water soluble arsenic species including DMA; whereas AsHCs remain mostly intact after intestinal absorption (Meyer et al., 2015b). Both studies demonstrate how arsenolipids can be potentially bioavailable to humans (i.e. absorbed into the hepatic portal system). What they did not consider, however, is the transformations arsenolipids could undergo in the gastrointestinal tract after ingestion within a food matrix. Gastrointestinal digestion of arsenolipids ultimately determines the arsenic species bioavailable, which would not necessarily correspond to the original arsenolipids ingested. Additionally, before becoming bioavailable arsenolipids need to become bioaccessible, which means that they have to be released from the food matrix during gastrointestinal digestion.

Bioaccessibility of trace elements can be assessed using both *in vivo* and *in vitro* experiments (Ruby et al., 1999). Whereas the former are more expensive, complex, and involve ethical concerns; *in vitro* experiments provide a reliable and accurate alternative to investigate the fate of metals in the human gastrointestinal tract (Ruby et al., 1993). Different *in vitro* approaches have been used to study the bioaccessibility of As(III) and As(V) (Ruby et al., 1996, Laparra et al., 2003, Trenary et al., 2012, Koch et al., 2007); MA and DMA (Calatayud et al., 2013, Zhao et al., 2014, Koch et al., 2007); and arsenosugars (Gamble et al., 2002, Almela et al., 2005), either as pure standards or components of vegetables and seafood. There are no publications on arsenolipid bioaccessibility and degradation. In a previous study (Chávez-Capilla et al., 2016a), a physiologically based extraction test (PBET) was used to evaluate the gastrointestinal digestion of different organic arsenic species in rice, seaweed and fish. It was shown that DMAP, which is a short chain fatty acid, did not suffer any transformation, suggesting that longer chain AsFAs would also remain intact in the gastrointestinal tract (Chávez-Capilla et al., 2016a). This study uses the same procedure to qualitative assess the stability of longer chain AsHCs and AsFAs in the human gastrointestinal tract. Synthetic standards of both types of arsenolipids were examined, as well as hijiki seaweed (*Hizikia fusiformis*) and krill oil (*Euphausia superba*) as arsenolipid-containing foodstuffs.

Materials and methods

Synthesis of arsenolipid standards

Long chain AsHC and AsFA standards were synthesized by reacting bis(dimethylarsine) oxide with the corresponding bromo hydrocarbon or fatty acid precursor (Taleshi et al., 2014). Dimethylarsinoyl decane (AsHC-C10, Figure 3-2) was prepared from bromodecane (Sigma-Aldrich, Australia) and dimethylarsinoyl undecanoic acid (AsFA-C11, Figure 3-2) from bromoundecanoic acid (Sigma-Aldrich, Australia). Bis(dimethylarsine) oxide was prepared *in situ* by adding iododimethylarsine (1 mL, 10 mmol) that had been synthesised following published methods (Burrows and Turner, 1920) to a cooled (2°C) solution of 10 M sodium hydroxide (Sigma-Aldrich, Australia) (1 mL, 10 mmol) and stirring under a nitrogen atmosphere for 30 min. The top layer, corresponding to bis(dimethylarsine) oxide, was then transferred to a clean rounded bottom flask and covered with sodium hydroxide (Sigma-Aldrich, Australia) (1

mL, 10 mmol). The bromo compound (1.7 mmol) in 2 mL of ethanol (Sigma-Aldrich, Australia) was added and the reaction was heated to 78°C and left mixing overnight. After cooling, diluting with water and washing with ether, the aqueous layer was neutralized (for AsHC-C10) or acidified to pH 3 (for AsFA-C11) with hydrochloric acid (32 % w/w, analytical grade, Chem Supply, Australia). The final arsenic species was extracted from the aqueous layer with chloroform (Sigma-Aldrich, Australia), washed with water and the solvent evaporated in vacuo, obtaining an orange-brownish residue as either wax or oil. Arsenolipids were further purified by resuspension in acetone (Scharlau, Australia) with 1 % v/v formic acid (APS Specialty Chemicals, Australia) and loading onto a silica gel chromatographic column (200 – 400 mesh, 60Å pore size). Three fractions were eluted using methanol (Scharlau, Australia) in 1 % v/v formic acid (APS Specialty Chemicals, Australia); methanol; and methanol in 1 % v/v ammonia (30 % v/v, Chem Supply, Australia). The third fraction was evaporated and the residue, containing the arsenolipids, redissolved in methanol 1 % v/v formic acid. The identity of AsFA-C11 and AsHC-C10 was confirmed by liquid chromatography-mass spectrometry (LC-QQQ, Agilent 6410, Australia) in positive ion MS₂ scan with fragmentor voltages of 125 V and 111 V, and subsequently performing a product ion scan with collision energies of 24 au for AsFA-C11 and 20 au for AsHC-C10 (*Appendix Chapter 3*). Control samples for analysis of AsFA-C11 and AsHC-C10 were prepared in deionised water (18.2 MΩ cm, Sartorius, Australia) at a final arsenic concentration of 1 mg L⁻¹.

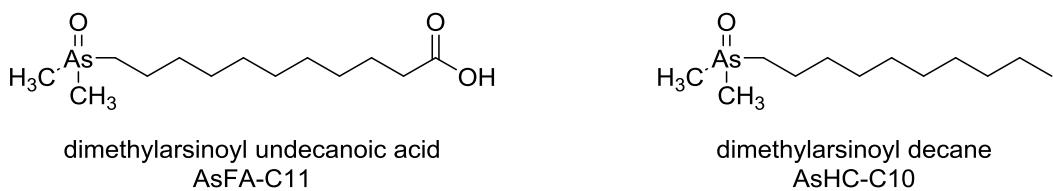


Figure 3-2. Chemical structures of dimethylarsinoyl undecanoic acid and dimethylarsinoyl decane.

Arsenolipid-containing foodstuffs

Commercial krill oil capsules (Macro, Woolworths, Australia) and hijiki seaweed (dried powder, Chinese shop, Australia) were used as source of dietary arsenolipids. Control samples to identify the initial arsenic species in both foodstuffs were prepared as described elsewhere with few modifications (Raber et al., 2009). Samples of krill oil (one capsule) and hijiki powder (ca. 0.2 g) were suspended in chloroform: methanol 1:2 v/v

and left mixing end over end rotation at room temperature for 48 h. Deionised water was added and samples were centrifuged at $180 \times g$ for 20 min (Centrifuge 5804 R, Eppendorf, Australia). The aqueous phase, containing inorganic and water soluble organic arsenic species, was kept at -4°C until analysed. The chloroform phase, containing lipid soluble arsenic species, was washed with saturated sodium bicarbonate (Ajax Finechem, Australia) and the solvent evaporated. The residue was resuspended in chloroform: acetone 1:1 v/v with 1 % v/v formic acid and separation *via* chromatography was performed by collecting three different fractions with methanol in 1 % v/v formic acid, methanol and methanol in 1 % v/v ammonia, respectively. The solvent in all fractions was evaporated and the residue resuspended in methanol with 1 % v/v formic acid. Samples were kept at -4°C until analysed.

Physiologically based extraction test

Physiologically based extraction tests of arsenolipids standards (1 mg L⁻¹), krill oil (one capsule) and hijiki seaweed (ca. 0.2 g) were performed as described in Chávez-Capilla *et al.* (2016a). Two sets of three replicates per standard and foodstuff were done to allow enough samples for total arsenic and arsenic speciation analyses. In all samples, arsenic species were separated into water and lipid soluble fractions as follows: AsHC-C10 and AsFA-C11 samples were extracted with chloroform. The water phase was kept for analysis and, from the chloroform phase, an aliquot was taken, the solvent evaporated and the residue resuspended in methanol with 1 % v/v formic acid. That fraction was labelled as *crude lipid soluble fraction* and kept aside for analysis. The remaining chloroform phase was evaporated and the residue resuspended in chloroform: acetone 1:1 v/v with 1 % v/v formic acid, loaded into a silica column and eluted with methanol in 1 % v/v formic acid, methanol and methanol in 1 % v/v ammonia. The three fractions were kept for analysis. In hijiki samples, the pellet and supernatant were separated by centrifugation at $180 \times g$ for 20 min. The arsenic not extracted by PBET was extracted from the pellet for total arsenic analysis with 1 % v/v nitric acid (Aristar, BDH, US) in a water bath at 100°C for 2 h, followed by the addition of 1% v/v hydrogen peroxide (30 % w/w, UNIVAR, US) and incubation in a water bath at 100°C for another 2 h. To the supernatant samples, the same procedure as for AsHCs and AsFAs samples was applied in order to purify the different arsenic species present. Krill oil samples were extracted

with methanol: hexane (Sigma-Aldrich, Australia) 1:1 v/v. The methanol extract, containing water soluble arsenic species, was washed with hexane to remove any polar lipid soluble arsenic species left and kept aside for analysis. The solvent in the hexane extract was evaporated and the residue resuspended in chloroform: acetone 1:1 v/v with 1 % v/v formic acid, loaded into a silica column and eluted with methanol in 1 % v/v formic acid, methanol and methanol in 1 % v/v ammonia. The three fractions were kept at -4°C until analysed. The lack of arsenolipid-containing certified reference materials made difficult to assess the extraction efficiency of arsenolipids from the different matrices in samples. Thus, the assessment of lipid soluble arsenic species within this study is solely qualitative.

Total arsenic concentration analysis

Aliquots of all the water and lipid soluble fractions from controls, stomach and intestinal phases of AsHC-C10, AsFA-C11, krill oil and hijiki seaweed were prepared for total arsenic analysis by extraction with 1 % v/v nitric acid in a water bath at 100°C for 2 h, followed by the addition of 1 % v/v hydrogen peroxide (30 % w/w) and incubation in a water bath at 100°C for another 2 h. Samples were analysed by graphite furnace atomic absorption spectroscopy using a Perkin Elmer Analyst 600 atomic absorption spectrometer (Australia) with an arsenic lamp ($\lambda = 193.7$ nm) at 38 A, and a THGA graphite tube (Perkin Elmer, Australia) as per Deaker and Maher (1999). Calibration standards were prepared from a multi element calibration standard (Accu Trace, Accu Standards, 10 mg L⁻¹) at arsenic concentrations of 100, 50, 25, 12.5, 6.25 and 3.125 µg L⁻¹ in 1 % v/v nitric acid. Blanks of the stomach and small intestine phases in 1 % v/v nitric acid were also analysed. A matrix modifier of palladium and magnesium nitrate was used.

Arsenic speciation analysis

The speciation analysis of control samples and all the fractions extracted from both the gastric and intestinal phases was performed by high performance liquid chromatography-inductively coupled plasma-mass spectrometry (HPLC-ICP-MS) with a Flexar 10 HPLC coupled to a NexIon 300D ICP-MS (both Perkin Elmer, Waltham, MA, USA). The plasma power was 1300 W and the nebuliser gas (argon) flow 0.9 L

min^{-1} . Besides arsenic (m/z 75), vanadium (m/z 51) and chromium (m/z 52) were monitored to detect salt and carbon in samples. Chloride ions form adducts with argon ($^{40}\text{Ar}^{35}\text{Cl}$) that can interfere with the signal for ^{75}As . Chloride also forms an adduct with oxygen ($^{35}\text{Cl}^{16}\text{O}$) with the same m/z as ^{51}V . Vanadium was therefore monitored to discard any peak eluting as ^{75}As that may have come from the presence of chloride based salts in samples. The presence of carbon in samples and in the mobile phase was monitored by measuring chromium (^{52}Cr), as it has the same m/z as the adduct that carbon forms with argon ($^{40}\text{Ar}^{12}\text{C}$). Water soluble arsenic species were separated by anion exchange chromatography (Hamilton PRP-X100 column, 250 mm \times 4.1 mm, 10 μm particle size, Phenomenex, US) through gradient elution with 10 mM malonic acid (UNILAB, Ajax Chemicals, Australia) as mobile phase (pH 5.6 adjusted with aqueous ammonia (Sigma-Aldrich, Australia)). Malonic acid was held at 3 mM for 2 min, increased to 10 mM over 8 min, held for 4 min, and then decreased back to 3 mM over 2 min. Calibration standards using As(III), As(V), MA, DMA and AB were prepared in methanol: water 1:1 v/v at arsenic concentrations ranging from 100 to 1 $\mu\text{g L}^{-1}$. Arsenosugar standards were only used for species identification. Calibration verification standards at the top, mid and low points in the calibration were run to assess the calibration. Calibration and species identification standards were run every 20 samples. Blanks for stomach and small intestine phases were also analysed to ensure the lack of interferences from both matrices in the identification and concentration measurement of arsenic species. The column recoveries were 120 % for hijiki and 88 % for krill oil. Arsenic species were confirmed by cation exchange chromatography (Zorbax 300-SCX column, 150 mm \times 4.6 mm, 5 μm particle size, Agilent Technologies, US) with 2.5 mM pyridine (Merck, Australia) as mobile phase (pH 2.6 adjusted with formic acid (UNIVAR, Australia)) under isocratic conditions. Calibration standards using As(III), As(V), DMA, AB, tetramethylarsonium ion (TETRA), trimethylarsinoyl propionic acid (TMAP) were prepared as for speciation by anion exchange chromatography. The separation of lipid soluble arsenic species was achieved in an Eclipse XDB-C18 reverse-phase column (150 mm \times 2.1 mm, 5 μm particle size, Eclipse, Agilent, Australia) using deionised water with 0.1 % v/v formic acid as eluent A and methanol with 0.1 % v/v formic acid as eluent B. Elution at 0.2 mL min^{-1} with 75 % v/v A was applied for 1 min, changed to 10 % v/v A over 25 min and held for 2 min, and then back to 75 % v/v A over 8 min. A solution of deionised water

with 1 % v/v formic acid was infused post column at 0.8 mL min⁻¹ to dilute the amount of carbon getting into the plasma. A flow of 0.08 L min⁻¹ of 10 % v/v oxygen in argon was added to the argon flow (0.72 L min⁻¹) in the nebuliser to control the carbon built up in the plasma. Given the lack of certified arsenolipid standards for calibration, the speciation analysis of lipid soluble arsenic species is qualitative. Arsenolipid standards (AsFA-C11 and AsHC-C10) in methanol with 1 % v/v formic acid were only used for species identification.

Identification of arsenolipids

The identification of arsenolipids in hijiki seaweed and krill oil was performed by liquid chromatography- mass spectrometry (LC-QQQ, Agilent 6410, Australia). Separation of arsenic species was achieved with a SB-C18 reverse-phase column (50 mm × 2.1 mm, 1.8 µm particle size, Agilent, Australia) using deionised water with 0.1 % v/v formic acid as eluent A and methanol with 0.1 % v/v formic acid as eluent B. Elution at 0.2 mL min⁻¹ with 75 % v/v A was held for 2 min, changed to 5 % v/v A over 13 min and held for 20 min, back to 75 % v/v A over 1 min and held for 4 min. Post-column ionisation was achieved by ESI in positive mode. The capillary and cell accelerator voltages were 4500 V and 7 V, respectively. The nebuliser pressure was set to 40 psi; and the gas temperature and flow to 325°C and 8 L min⁻¹. The fragmentor voltages and collision energies were optimised using both AsFA-C11 and AsHC-C10 standards. Precursor ion chromatograms were obtained scanning from *m/z* 150 to 1100 with a fragmentor voltage 114 V and collision energies of 32 au for *m/z* 105 and 107; and 20 au for *m/z* 123. Subsequently, product ion scans were performed by flow injection of samples and for each precursor ion of interest. Fragmentor voltages and collision energies were modified between injections in order to obtain different fragmentation patterns and elucidate the possible structure of the precursor ions.

Results

Arsenolipid standards

The reverse-phase chromatograms of AsFA-C11 samples before and after gastrointestinal digestion are shown in Figure 3-3. A main peak at 23.5 min corresponds to AsFA-C11 (Figure 3-3), which in control samples accounted for $92 \pm 5\%$ of the total lipid soluble arsenic species in the standard. The amount of AsFA-C11 did not change significantly after stomach ($86 \pm 10\%$) or small intestine ($90 \pm 3\%$) digestions (Figure 3-3). Two other minor peaks labelled as unknown 01 (UK 01) and unknown 02 (UK 02) were observed at 21.5 min and 25.7 min, respectively (Figure 3-3). Their fluctuating concentrations at the different stages of the PBET are likely to represent protonated and deprotonated forms of the main arsenolipid, due to the different pH in control (pH 6.6), stomach (pH 2) and small intestine (pH 7) phases. Figure 3-4 shows the chromatograms of AsHC-C10 samples before and after the PBET. Dimethylarsinoyl decane eluted at 23.6 min and did not change significantly after gastrointestinal digestion as the percentage concentrations relative to total arsenic in the lipid soluble fraction of controls, stomach and small intestine phases were $69 \pm 1\%$, $51 \pm 11\%$ and $60 \pm 10\%$, respectively (Figure 3-4). The arsenic species labelled as unknown 03 (UK 03) at 22.9 min could, again, correspond to a protonated/deprotonated forms of the main arsenic species that varied with pH. The chromatogram shows another minor peak at 25 min, labelled as unknown 04 (UK 04), and that remained unchanged after all treatments (Figure 3-4).

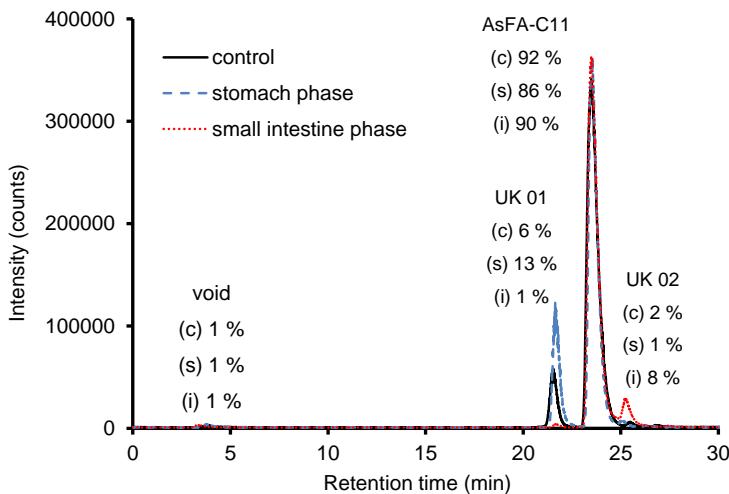


Figure 3-3. Chromatograms of dimethylarsinoyl undecanoic acid (AsFA-C11) samples before (c) and after stomach (s) and small intestine (i) digestions.

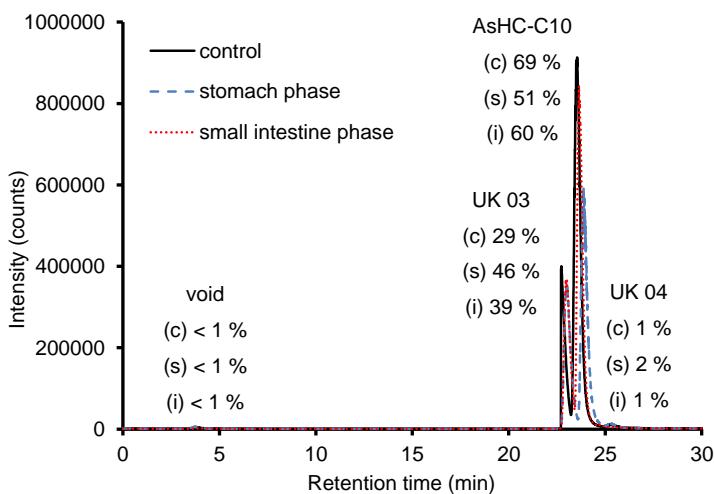


Figure 3-4. Chromatograms of dimethylarsinoyl decane (AsHC-C10) samples before (c) and after stomach (s) and small intestine (i) digestions.

Arsenic species in hijiki seaweed

The total arsenic concentration measured in hijiki seaweed control samples was $92 \pm 9 \mu\text{g g}^{-1}$, of which $79 \pm 9 \%$ were water soluble species and $21 \pm 9 \%$ lipid soluble species. The arsenic species identified in the water soluble extract were As(III) ($2.7 \pm 0.2 \%$), As(V) ($9.07 \pm 0.05 \%$), MA ($2.03 \pm 0.04 \%$), DMA ($56.4 \pm 0.2 \%$), as well as four arsenosugars: arenosugar-glycerol (O-Gly, $6.1 \pm 0.3 \%$), arenosugar-phosphate (O-PO₄, $3.55 \pm 0.02 \%$), arenosugar-sulfonate (O-SO₃, $4.5 \pm 0.2 \%$) and arenosugar sulfate (O-SO₄, $15.7 \pm 0.1 \%$) (Figure 3-5a). In the lipid soluble fraction, three main groups of arsenic species were detected eluting at 20.3 min (A, $1.9 \pm 0.4 \%$), 25 min (B, $4 \pm 1 \%$)

and 29.2 – 32 min (C, $85 \pm 11\%$) (Figure 3-5b). At least nine arsenolipids were identified by LC-QQQ in this fraction (Table 3-1, Figure 3-6). The arsено-fatty acid AsFA 220 and the arsено-hydrocarbons AsHC 332 and AsHC 360, reported before (Garcia-Salgado et al., 2012, Glabonjat et al., 2014), were confirmed by precursor ion scan for m/z 105 and 123 and by product ion scan (*Appendix Chapter 3*). The product ion scan for the precursor ion with m/z 296.6 suggests that it might correspond to an AsHC (*Appendix Chapter 3*), but further analysis by high resolution LC-MS needs to be done to confirm its structure. Additionally, the precursor ion scan for m/z 105 and 123 showed precursor ions with m/z 1036.4, 986.5, 1013.8, 1015.5 and 1042.8 (Table 3-1) that match those of the formerly reported AsPE 1035, AsPC 985, GlyAsL 1012, GlyAsL 1014 and GlyAsL 1042 (Garcia-Salgado et al., 2012, Viczek et al., 2016). Due to the lack of standards to optimise the fragmentor voltages and collision energies for AsPCs, AsPEs and GlyAsLs, these arsenic species could not be confirmed.

Given the similar conditions used to analyse the lipid soluble fraction of hijiki control samples by HPLC-ICP-MS and LC-QQQ, the three peak groups in Figure 3-5b may correspond to AsPE 1035 and AsPC 985 (A); AsFA 220 and AsHC 296 (B); and AsHC 332, AsHC 360, GlyAsL 1012, GlyAsL 1014 and GlyAsL 1042 (C).

The total arsenic concentration extracted from hijiki after digestion under the stomach conditions was $66 \pm 4 \mu\text{g g}^{-1}$. The concentration of lipid soluble arsenic species decreased and that of water soluble species increased, as shown by the percentages of arsenic in each fraction ($99.3 \pm 0.3\%$ and $0.7 \pm 0.3\%$, respectively). The small intestine digestion did not produce further changes, as the measured total arsenic concentration in this phase was $64.7 \pm 0.3 \mu\text{g g}^{-1}$, with $99.7 \pm 0.3\%$ of water soluble species and $0.3 \pm 0.3\%$ of lipid soluble arsenic species. Figure 3-5b shows that the amount of arsenolipids in fraction C decreased after the PBET, while those in fractions A and B remained relatively unchanged. Simultaneously, the only water soluble arsenic species that exhibited a considerable increase after the PBET was As(V) (Figure 3-5a), suggesting degradation of some of the arsenolipids in fraction C to As(V). Spiking of samples with As(V) and O- PO_4 arenosugar standards confirmed the peak at 6.7 min as As(V). The diminution of DMA from $56.4 \pm 0.2\%$ in control samples to $8.17 \pm 0.08\%$ and $7.3 \pm 0.1\%$ in the

stomach and small intestine phases, respectively, indicates that demethylation of DMA to As(V) also occurred (Figure 3-5a).

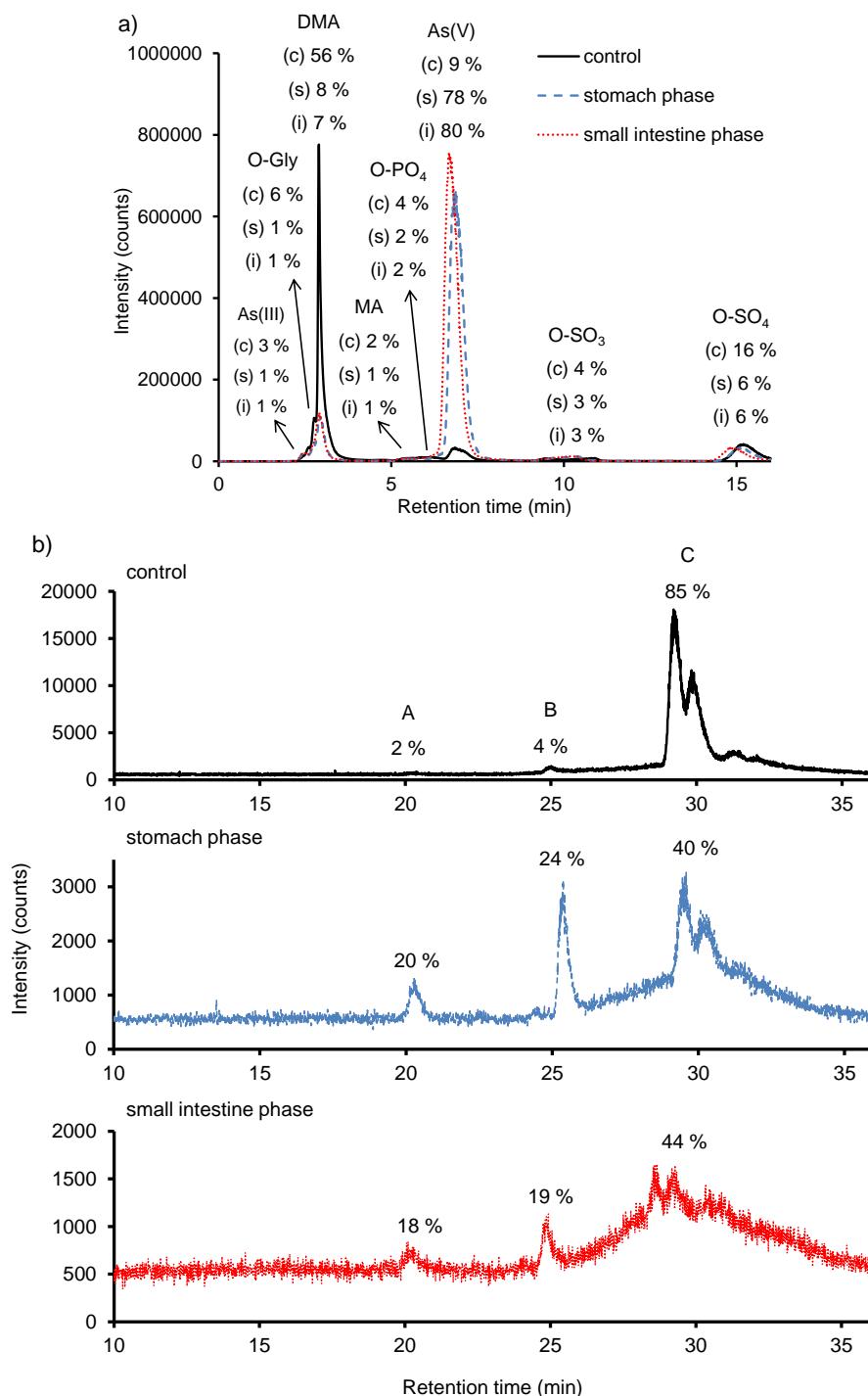


Figure 3-5. Chromatograms of water soluble (a) and lipid soluble (b) arsenic species in hijiki seaweed samples before (c) and after stomach (s) and small intestine (i) digestions. The percentages of lipid soluble arsenic species are calculated relative to the total arsenic concentration in the corresponding fraction.

Table 3-1. Lipid soluble arsenic species detected in hijiki seaweed by LC-QQQ analysis.

Retention time (min)	[M+H] ⁺ experimental	[M+2H] ²⁺ experimental	[M+H] ⁺ calculated	[C ₂ H ₆ As] ⁺	[C ₂ H ₈ OAs] ⁺	[OAs] ⁺	[M-H ₂ O+H] ⁺	Formula	Abbreviation
15.3 - 15.4	1036.4	518.4	1036.5					C ₅₇ H ₈₇ O ₉ NAsP	AsPE 1035
16.8 - 16.9	986.5	493.7	986.5					C ₅₃ H ₈₅ O ₉ NAsP	AsPC 985
18.5 - 18.8	220.9	n/a	221.0	105.2	123.1	90.8	202.9	C ₇ H ₁₃ AsO ₃	AsFA 220
19.6 - 19.7	296.6	n/a	-	105.6	122.7	90.8		?	AsHC 296
20.4 - 20.8	1013.8	506.5	1013.6					C ₄₉ H ₉₄ O ₁₄ AsP	GlyAsL 1012
20.7 - 21.3	333.2	n/a	333.2	104.9	123			C ₁₇ H ₃₇ AsO	AsHC 332
23.9 - 24.8	1015.5	507.8	1015.6					C ₄₉ H ₉₆ O ₁₄ AsP	GlyAsL 1014
24.9 - 25.8	360.8	n/a	361.2	105.1	122.6			C ₁₉ H ₄₁ AsO	AsHC 360
27.4 - 28.9	1042.8	521.9	1043.6					C ₅₁ H ₁₀₀ O ₁₄ AsP	GlyAsL 1042

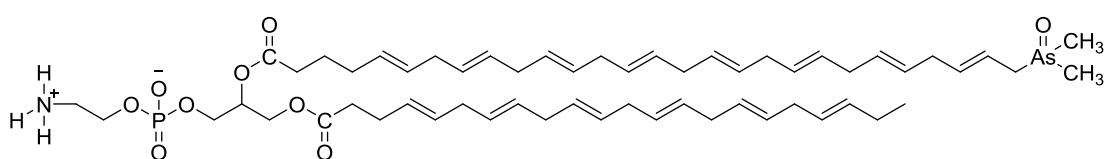
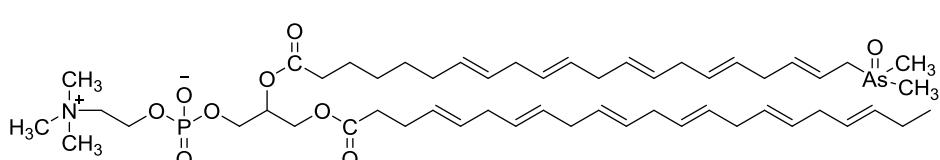
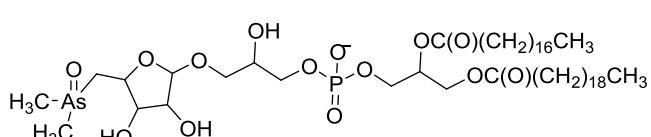
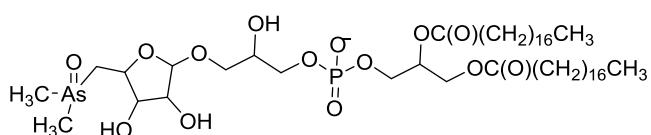
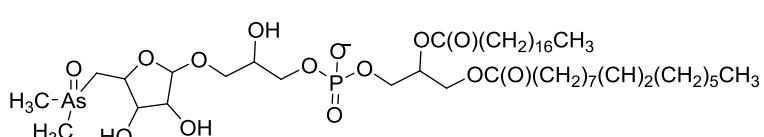
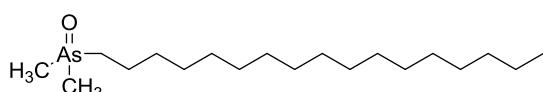
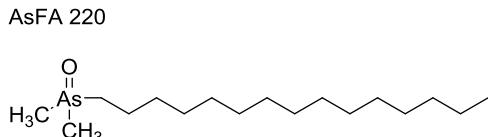
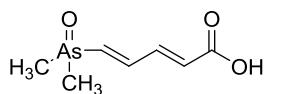
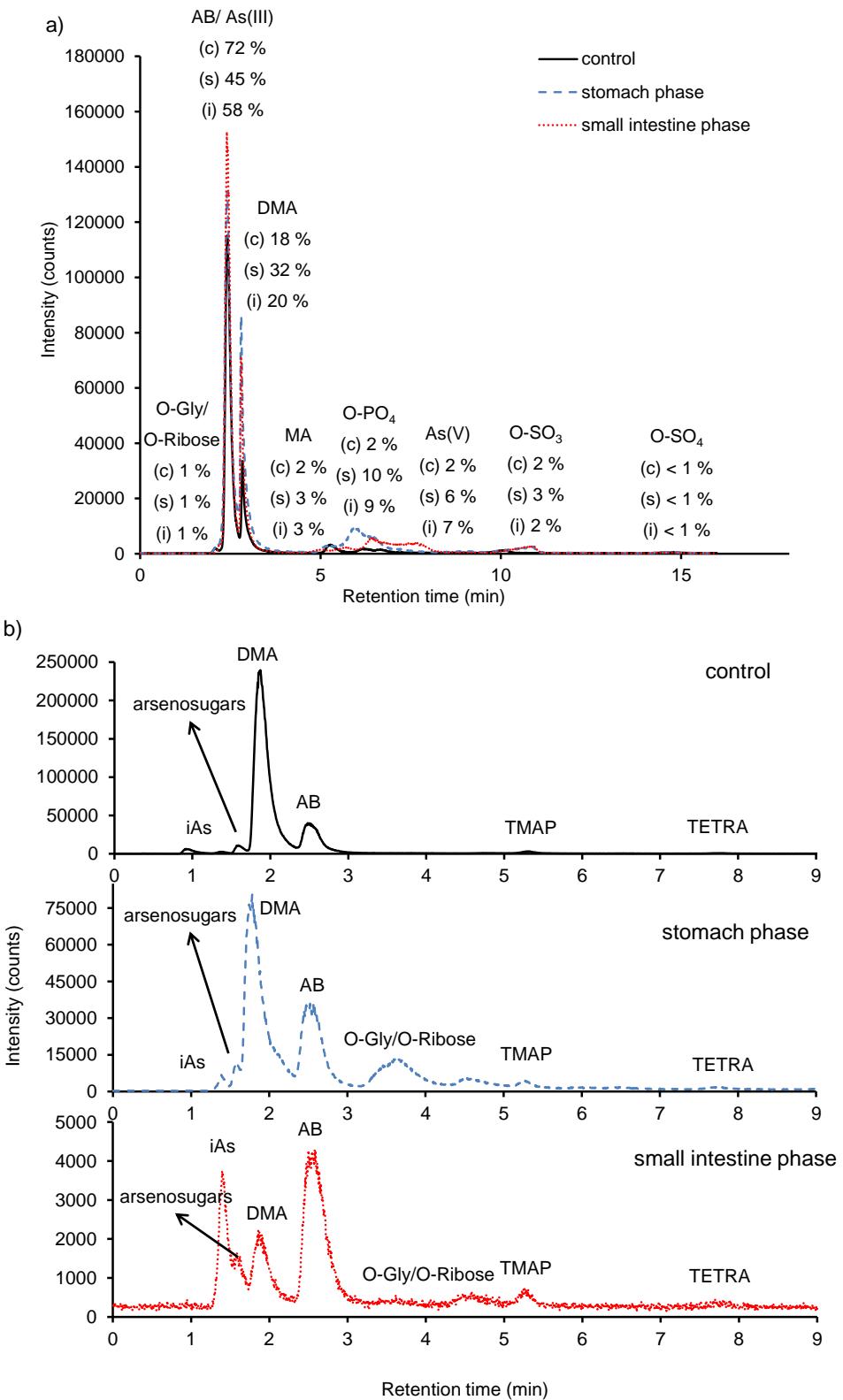


Figure 3-6. Proposed structures for the arsenolipids identified in hijiki seaweed. The structures of AsHC 332, AsHC 360, GlyAsL 1012, GlyAsL 1014, GlyAsL 1042, AsPC 985 and AsPE 1035 are adapted from (Garcia-Salgado et al., 2012, Viczek et al., 2016).

Arsenic species in krill oil

Arsenic in krill oil samples ($3.0 \pm 0.6 \text{ } \mu\text{g g}^{-1}$) before gastrointestinal digestion consisted of $23 \pm 11 \%$ of water soluble species and $77 \pm 11 \%$ of lipid soluble arsenic species. The arsenic species extracted in the water soluble fraction were As(III), As(V), MA, DMA, AB, TETRA, TMAP and the arenosugars: O-Ribose, O-Gly, O-PO₄, O-SO₃ and O-SO₄ (Figure 3-7a and 3-7b). Spiking with O-PO₄ arenosugar and As(V) standards confirmed the elution of both arsenic species at 6.2 min and 6.7 min, respectively (Figure 3-7a). The presence of AB was confirmed by cation exchange chromatography, as co-elution of As(III) and AB occurred at 2.4 min under the anion exchange chromatographic conditions used (Figure 3-7b). The chromatogram of the lipid soluble fraction of krill oil samples shows at least eleven peaks labelled from D to N (Figure 3-7c). Eleven possible arsenic species were consequently identified in this fraction after analysis by LC-QQQ (Table 3-2, Figure 3-8). These include AB (*m/z* 179.2), three AsFAs (*m/z* 303.2, 311.1 and 403.3), three AsHCs (*m/z* 328.9, 331.1 and 459.3) and four TMAsFOHs (*m/z* 305.1, 311.1, 356.9 and 388.9). Additionally, the chemical composition of the precursor ions with *m/z* 308.9, 320.7, 329.9, 346.2, 357.9, 362.4, 374 and 414.2 could not be elucidated although their product ion mass spectra suggest that they might be arsenolipids (*Appendix Chapter 3*). Further analysis needs to be performed to confirm their chemical structures.

After the stomach digestion of krill oil samples, the concentration percentages of arsenic in the water and lipid soluble fractions remained practically unchanged ($18 \pm 3 \%$ water soluble and $82 \pm 3 \%$ lipid soluble). During the small intestine digestion, there was a slight increase of arsenic in the water soluble fraction ($27 \pm 2 \%$), which was accompanied by a decrease of arsenic in the lipid soluble fraction ($73 \pm 2 \%$). The water soluble arsenic species that exhibited changes after the PBET are O-PO₄ (from $2 \pm 1 \%$ to $9 \pm 2 \%$), As(V) (from $2.0 \pm 0.3 \%$ to $7 \pm 1 \%$) and As(III)/AB (from $72 \pm 2 \%$ to $58 \pm 3 \%$).



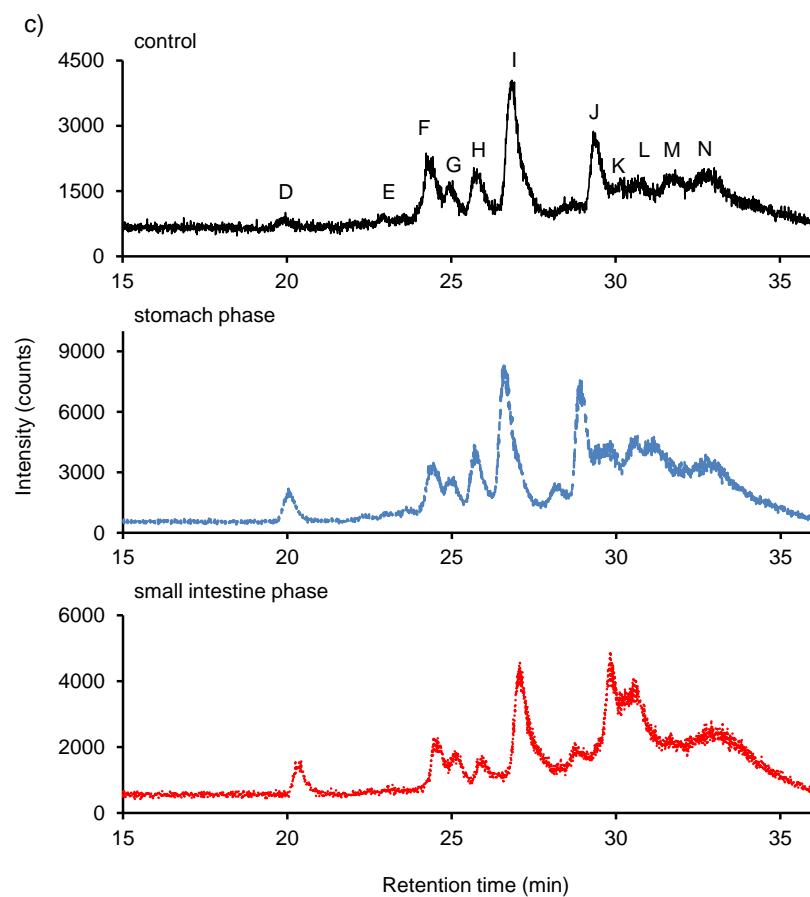


Figure 3-7. Anion (A) and cation (B) exchange chromatograms of water soluble arsenic species in krill oil before (c) and after the stomach (s) and small intestine (i) digestions. C) Reverse-phase chromatograms of lipid soluble arsenic species in krill oil before (c) and after the stomach (s) and small intestine (i) digestions. The percentages of lipid soluble arsenic species are calculated relative to the total arsenic concentration in the corresponding fraction.

Table 3-2. Lipid soluble arsenic species detected in krill oil by LC-QQQ analysis.

Retention time (min)	[M+H] ⁺ / [M] ⁺ experimental	[M+H] ⁺ / [M] ⁺ calculated	[C ₂ H ₆ As] ⁺	[AsS] ⁺	[C ₂ H ₈ OAs] ⁺	[OAs] ⁺	[M-H ₂ O+H] ⁺	[C ₃ H ₁₀ As] ⁺	[M-OH] ⁺	Formula	Abbreviation
20.4 - 20.8	320.7	-	105.3		122.8					?	n/a
20.8 - 21.3	179.2	179.0053	104.9				160.9			C ₅ H ₁₂ AsO ₂	AB
21.4 - 21.7	362.4	-	105.1		123.9	90.6				?	n/a
21.4 - 21.7	459.3	459.4	104.9							C ₂₆ H ₅₅ AsO	AsHC 458
21.7 - 22.1	388.9	389.2765	104.8					120.9		C ₂₁ H ₄₆ AsO	TMAsFOH 387
22.1 - 22.6	414.2	-	104.9		123.3					?	n/a
23.7 - 23.9	346.2	-	104.8		123.3					?	n/a
24.2 - 24.9	403.3	403.2193	105		122.8					C ₂₀ H ₃₉ AsO ₃	AsFA 402
24.9 - 25.3	303.2	303.1	105.2		122.8	91	285.1			C ₁₃ H ₂₃ AsO ₃	AsFA 302
24.9 - 25.3	329.9	-	104.8		122.8					?	n/a
25.6 - 26.1	328.9	329.2	105.2		122.9					C ₁₇ H ₃₃ AsO	AsHC 328
25.6 - 26.1	356.9	357.2139	104.9				121.1			C ₁₉ H ₃₈ AsO	TMAsFOH 357
26.7 - 27.6	305.1	305.2	105					120.9	287.5	C ₁₅ H ₃₄ AsO	TMAsFOH 305
27.6 - 28.7	331.1	331.2	105	107/109	122.8	90.9				C ₁₇ H ₃₅ AsO	AsHC 330
28.6 - 29.8	357.9	-	105	107/109						?	n/a
28.6 - 29.8	374	-	104.8	107/109						?	n/a
29.8 - 32.1	308.9	-	104.8		123.3		290.4			?	n/a
32.1 - 33.1	311.1	311.1	104.8				292.7			C ₁₄ H ₁₉ AsO ₃	AsFA 310
32.1 - 33.1	311.1	311.1356	104.8					120.7		C ₁₆ H ₂₈ AsO	TMAsFOH 311

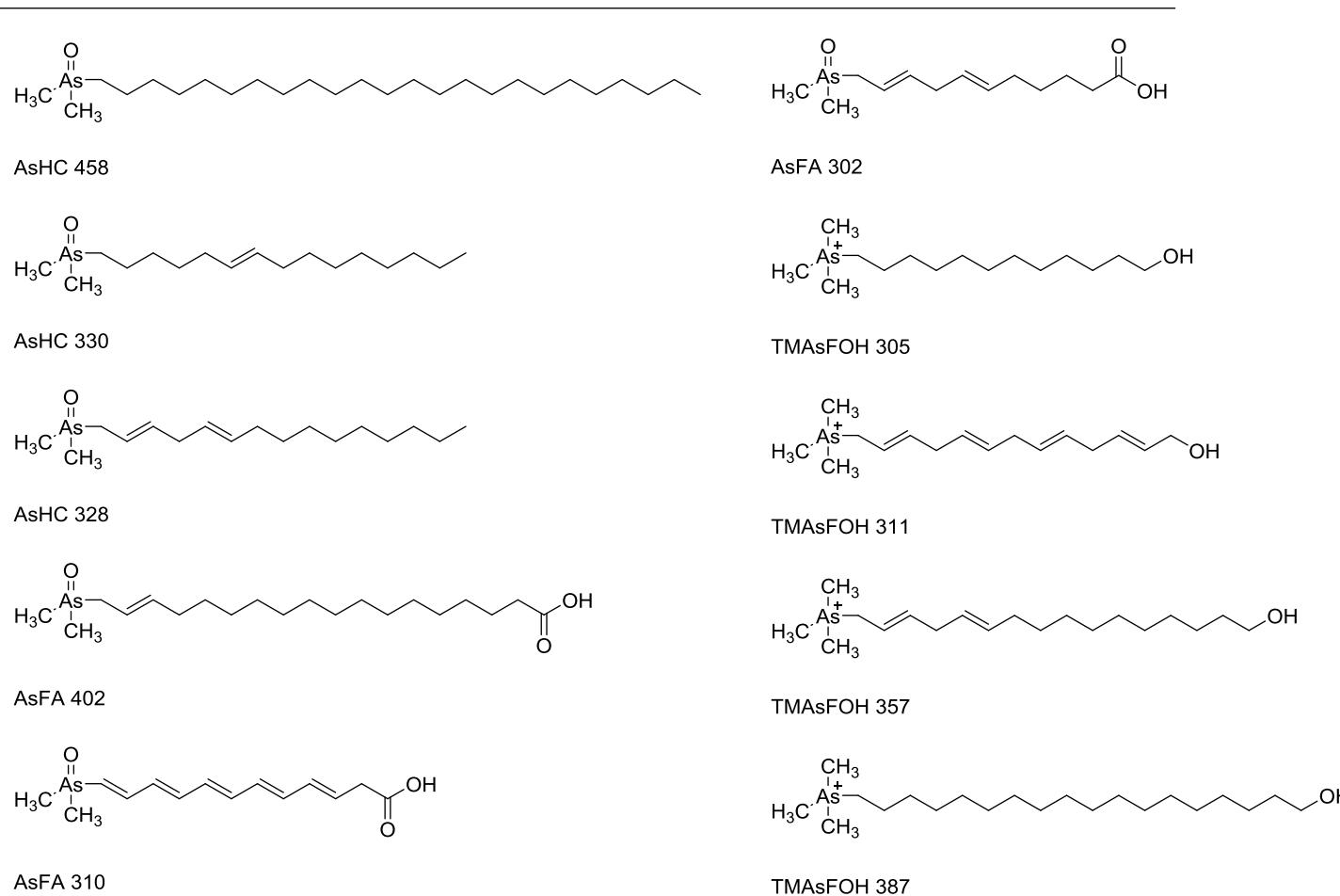


Figure 3-8. Proposed structures for the arsenolipids identified in krill oil samples. The structures of AsHC 330 and AsFA 302 have been adapted from (Lischka et al., 2013, Amayo et al., 2013). Double bonds in aliphatic carbon chains are arbitrary as the structures need to be confirmed by high resolution LC-MS.

Discussion

This study is an assessment of the degradation of the arsenolipids present in hijiki seaweed and krill oil after exposure to the physicochemical conditions of the human gastrointestinal tract.

The experiments with the synthesised standards AsFA-C11 and AsHC-C10 showed that both AsFAs and AsHCs are able to survive the stomach and small intestine and therefore become bioaccessible after gastrointestinal digestion. Arseno-hydrocarbons and AsFAs are stable in solution, although AsFAs can undergo esterification after more than 24 h under acidic conditions (Khan and Francesconi, 2016). While the stomach is an acidic environment (pH 2), the duration of the gastric digestion is not long enough to result in the complete esterification of AsFAs.

In hijiki seaweed, the arsenolipids identified include not only AsFAs and AsHCs (AsFA 220, AsHC 332 and AsHC 360) but also AsPE 1035, AsPC 985, GlyAsL 1012, GlyAsL 1014 and GlyAsL 1042 (Table 3-1, *Appendix Chapter 3*). Although further analysis is needed to confirm these results, the previous identification of AsHC 332, AsHC 360, GlyAsL 1014 and GlyAsL 1042 in hijiki seaweed supports some of these structures (Garcia-Salgado et al., 2012, Glabonjat et al., 2014).

As for AsFA-C11 and AsHC-C10, AsFAs and AsHCs in hijiki seaweed are likely to survive gastrointestinal digestion, as indicated by peak B (assigned to AsFA 220 and AsHC 296) not changing after the PBET (Figure 3-5b). The co-elution of AsHCs with GlyAsLs in fraction C (Figure 3-5b), however, hinders a proper interpretation of the fate of the remaining AsHCs in hijiki seaweed. Given the greater stability of AsHCs (Khan and Francesconi, 2016) compared to GlyAsLs (Glabonjat et al., 2014), the decrease in the amount of arsenolipids in fraction C probably comes from the loss of GlyAsLs. The significant reduction in the percentage of lipid soluble arsenic species in hijiki seaweed after the PBET (from $21 \pm 9\%$ to $0.3 \pm 0.3\%$) may be due to the retention of some arsenolipids within the hijiki sample matrix. Conversely, the accompanying increase of water soluble species supports the degradation of GlyAsLs. A previous study on the quantification of arsenolipids in a certified reference material (CRM) of hijiki showed that GlyAsLs account for the highest arsenic concentration in this seaweed (Glabonjat et

al., 2014). Although our experiments were not conducted using this CRM, they show that the arsenic species disappearing after the PBET are those that initially accounted for $85 \pm 11\%$ of the total arsenolipid concentration in hijiki control samples. Thus, these arsenic species might mostly correspond to GlyAsLs. Degradation of GlyAsLs would be expected to occur through the hydrolysis of the phospholipid attached to the arenosugar moiety. This initially will yield O-PO₄ and then dimethylarsinoylribose (O-Ribose). The chromatograms in Figure 3-5a did not show any increment of the arenosugars in the samples to support this assumption. What can be observed, however, is a significant increase in the peak corresponding to As(V). This could mean that GlyAsLs are degraded to As(V) during gastrointestinal digestion. The transformation of arenosugars to As(V) after the PBET has been formerly reported (Chávez-Capilla et al., 2016a), but only in arenosugar standards and not when they were present in an organic matrix. In addition, the decrease in the peak for DMA in Figure 3-5a suggests that As(V) might also be derived from degradation of DMA. Partial demethylation of DMA to As(V) has been observed in rice but not in seaweed (Chávez-Capilla et al., 2016a). The seaweed used in previous experiments was *Sargasso* and not hijiki (Chávez-Capilla et al., 2016a), therefore, the transformations of arsenic within the algal matrix may differ. It was not possible to quantify arsenolipids with the analytical methods used in this study thus, further experiments need to be conducted to confirm the degradation pathways. It can only be concluded that GlyAsLs do not survive gastrointestinal digestion, probably because they are degraded to As(V).

The lipid soluble arsenic species assigned to peak A in Figure 3-5b were AsPC 985 and AsPE 1035 (Table 3-1). In previous studies on hijiki seaweed there were a few arsenolipids that could not be identified, also eluting before AsHCs and GlyAsLs (Garcia-Salgado et al., 2012). Hence, these could correspond to AsPCs and AsPEs. After the PBET these arsenolipids did not suffer significant variations (Figure 3-5b), therefore suggesting that AsPC 985 and AsPE 1035 in hijiki seaweed may be bioaccessible to humans. Given the complex structure of these arsenic species, their stability under the physiological conditions of the stomach was surprising. Instead, they were expected to hydrolyse to yield AsFAs (Viczek et al., 2016). Since additional experiments are needed to confirm the identity of AsPC 985 and AsPE 1035, the proposed stability of these arsenic species after gastrointestinal digestion is still unclear.

In krill oil, the arsenic species identified were AB, AsFA 302, AsFA 310, AsFA 402, AsHC 328, AsHC 330, AsHC 458, TMAsFOH 305, TMAsFOH 311, TMAsFOH 357 and TMAsFOH 387 (Table 3-2, *Appendix Chapter 3*). Although there is no literature on the composition of arsenolipids in krill oil, the species AsFA 302 and AsHC 330 match previously reported arsenolipids in fish (Lischka et al., 2013, Amayo et al., 2013). The arsenic species suggested as TMAsFOH 305, TMAsFOH 375, TMAsFOH 387, AsHC 328 and AsFA 402 have precursor ions with similar *m/z* values to different arsenolipids formerly identified in seaweed and fish (Table 3-2) (Amayo et al., 2013, Lischka et al., 2013, Rumpler et al., 2008, Raab et al., 2013). The product ion mass spectra obtained, however, showed fragments that could fit better with the arsenic species proposed in this study (Table 3-2, *Appendix Chapter 3*). The precursor ion *m/z* 311.1 fits both AsFA 310 and TMAsFOH 311 (Table 3-2) and therefore additional analyses of samples are needed to confirm either arsenic species. As TMAsFOH 305, TMAsFOH 311, TMAsFOH 375, TMAsFOH 387, AsHC 328, AsFA 402 and AsFA 310 are newly reported arsenolipids, their structures also need to be confirmed by high resolution LC-MS.

The identification of AB in the lipid soluble fraction of krill oil samples (Table 3-2) means that part of this arsenic species can be extracted into the lipid soluble fraction and therefore may justify the loss of AB observed in Figure 3-7b. Additionally, As(III) might decrease after being oxidised to As(V), explaining the increase of As(V) after the PBET; and in agreement with the findings reported in Chávez-Capilla *et al.* (2016a). The 7 % growth of O-PO₄ in the small intestine phase could be explained by the degradation of GlyAsLs (Figure 3-7a). This type of arsenolipids was not detected in krill oil samples, although their presence is not discarded. Instead, krill oil was found to be rich in AsFAs and AsHCs (Table 3-2). The lack of significant changes in samples after the PBET (Figure 3-7) indicates that these arsenolipids are not degraded during gastrointestinal digestion and that they are likely to be bioaccessible.

Additionally, four TMAsFOHs were detected in krill oil. The molecular masses for these arsenic species could also correspond to AsFAs and AsHCs, although there was no evidence supporting the presence of the dimethylarsinoyl moiety in their corresponding product ion mass spectra (*Appendix Chapter 3*). Instead, a fragment with *m/z* 120.9 from

the trimethylarsine cation $[C_3H_{10}As]^+$ was common to all TMAsFOHs, as well as the loss of either the alcohol ($-OH$) or the methanol ($-CH_2OH$) groups attached to the aliphatic carbon chain (*Appendix Chapter 3*). These arsenolipids may not undergo degradation after gastrointestinal digestion and may be bioaccessible to humans, as shown by the lack of significant changes in krill oil samples after the PBET.

Concluding remarks

This study shows that AsFAs, AsHCs and TMAsFOHs can survive the physiological conditions of the human gastrointestinal tract. These findings, together with the reported ability of AsFAs and AsHCs to cross the intestinal barrier (Meyer et al., 2015b), indicate that these arsenolipids can enter liver cells unchanged. Since both AsHCs and AsFAs are toxic to human cells (Meyer et al., 2015a, Meyer et al., 2014a), the availability of these arsenic species in foodstuffs needs to be better monitored by food authorities, especially the consumption of fish oils that often impart a higher exposure to AsHCs and AsFAs. The toxicity of TMAsFOHs also needs to be investigated as they may potentially have harmful consequences to human health. Exposure to GlyAsLs mostly occurs through ingestion of seaweed (Morita and Shibata, 1988, Garcia-Salgado et al., 2012, Raab et al., 2013). These arsenolipids do not seem to be bioaccessible as such and, if their degradation to As(V) is confirmed, the consequences of exposure to GlyAsLs will need to be assessed as inorganic arsenic species are known human carcinogens (International Agency for Research on Cancer, 2012). The questions generated by this research regarding the fate of GlyAsLs, AsPCs and AsPEs after gastrointestinal digestion need to be further investigated as they might provide information about the potential risk of these arsenic species to humans.

DECLARATION OF CO-AUTHORED PUBLICATION CHAPTER

For use in theses which include publications. This declaration must be completed for each co-authored publication and to be placed at the start of the thesis chapter in which the publication appears.

Declaration for Thesis Chapter 4

Declaration by candidate

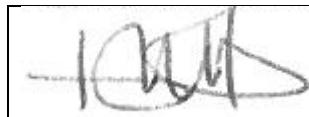
In the case of *Chapter 4*, the nature and extent of my contribution to the work was the following:

Nature of contribution	Extent of contribution (%)
I performed all the experimental work, analyses of samples, molecular modelling and interpretation of results. I am the primary author in the publication.	90

The following co-authors contributed to the work.

Name	Nature of contribution	Contributor is also a student at UC Y/N
William Maher	Contributed ideas and help editing the manuscript	N
Tamsin Kelly	Contributed ideas and help editing the manuscript	N
Simon Foster	Contributed ideas, helped editing the manuscript, assisted with the statistical analysis and supervised all the experiments.	N

**Candidate's
Signature**



Date
18/7/17

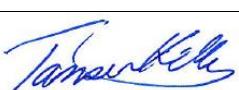
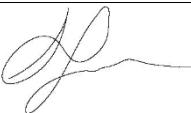
Declaration by co-authors

The undersigned hereby certify that:

- (1) the above declaration correctly reflects the nature and extent of the candidate's contribution to this work, and the nature of the contribution of each of the co-authors.
- (2) they meet the criteria for authorship in that they have participated in the conception, execution, or interpretation, of at least that part of the publication in their field of expertise;
- (3) they take public responsibility for their part of the publication, except for the responsible author who accepts overall responsibility for the publication;
- (4) there are no other authors of the publication according to these criteria;
- (5) potential conflicts of interest have been disclosed to (a) granting bodies, (b) the editor or publisher of journals or other publications, and (c) the head of the responsible academic unit; and
- (6) the original data are stored at the following location(s) and will be held for at least five years from the date indicated below:

Location(s)	Institute for Applied Ecology, University of Canberra, Canberra, Australia
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[Please note that the location(s) must be institutional in nature, and should be indicated here as a department, centre or institute, with specific campus identification where relevant.]

	Date
Signature 1	 18/7/17
Signature 2	 18/7/17
Signature 3	 18/7/17

CHAPTER 4 – Evaluation of the ability of arsenic species to passively diffuse across cell membranes using octanol-water and liposome-water partition coefficients

Introduction

Arsenic is a well-known environmental contaminant, readily available to living organisms from both natural and anthropogenic sources (Mandal and Suzuki, 2002). In humans, exposure to arsenic can result in major health problems such as skin lesions, cancer and cardiovascular diseases (International Agency for Research on Cancer, 2012). Arsenic toxicity not only depends on the concentration of exposure, but also on the chemical form of arsenic (Cullen and Reimer, 1989, Le et al., 2004) and their specific metabolic pathways in living organisms; some of which require transport across biological membranes, i.e. absorption, distribution and excretion (Styblo et al., 2000, Charoensuk et al., 2009, Vega et al., 2001).

Biological membranes consist of a lipid bilayer with embedded proteins and cholesterol, according to the so-called “fluid mosaic model” (Elliot and Elliot, 2005). The bilayer is formed by glycerophospholipids and glycosphingolipids (glycolipids) arranged with their polar heads and non-polar tails pointing outwards and inwards, respectively (Elliot and Elliot, 2005). Membrane proteins can be both integral and peripheral to the bilayer, and their role is to favour selective transport; whereas cholesterol controls the fluidity of the membrane and restricts the permeability of small molecules (Elliot and Elliot, 2005). Since one of the main functions of cell membranes is to limit the movement of undesirable substances, transportation across the lipid bilayer needs to be controlled. When movement occurs in the same direction as the concentration gradient, passive transport can take place with or without assistance. Non-mediated passive transport (simple diffusion) is governed by Fick’s first law of diffusion, in which molecules randomly collide against the bilayer and traverse it proportionally to the concentration on each side of the membrane. Simple diffusion depends on the lipophilicity of the molecule, i.e. size, shape and charge; along with the viscosity of the lipid bilayer (Mathews et al., 2013). When faster speeds are required, passive transport can be mediated by channels or pores, carrier molecules and permeases (facilitated diffusion) (Mathews et al., 2013).

Active transport occurs when molecules are pumped against their concentration gradient, with the consumption of energy in the form of adenosine triphosphate (Mathews et al., 2013). Despite the prevailing selectivity of most transport mechanisms, there are a number of undesired substances that can still be mistaken for the target species in terms of their size, structure and charge; and make use of the existing intrusion and extrusion systems in biological membranes. This is the case for metalloids, such as arsenic, which was initially thought to enter cells by simple diffusion, but then shown to use integral membrane proteins (Zangi and Filella, 2012). For this reason, the study of arsenic transport mechanisms in cells has predominantly been restricted to facilitated diffusion and active transport, with little attention given to the potential for simple diffusion.

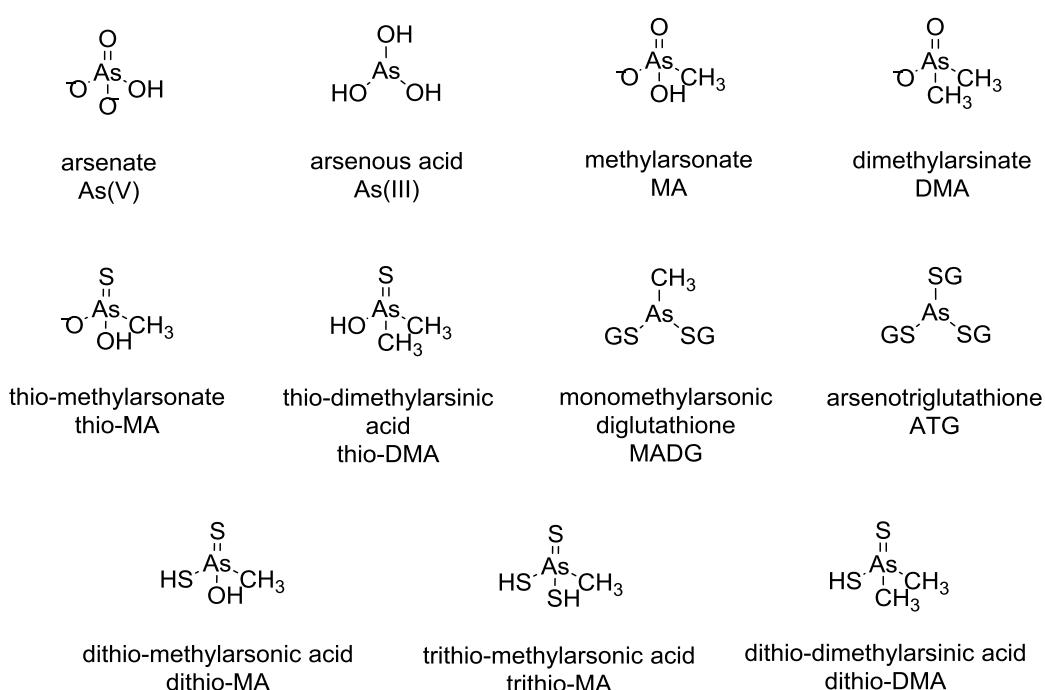


Figure 4-1. Structures of the arsenic species of interest for this chapter. The moiety –SG in ATG and MADG corresponds to a molecule of glutathione, in which a sulfur atom is bound to arsenic.

Table 4-1. Arsenic uptake systems in cells, adapted from (Zangi and Filella, 2012).

ARSENIC SPECIES	TRANSPORTER NAME	ORGANISM
	Facilitated diffusion	Active transport
As(III)	MIP: <i>GlpF</i> (glycerol channel)	<i>E. Coli</i> (prokaryote)
As(III)	MIP: <i>AqpS</i>	<i>S. Meliloti</i> (prokaryote)
As(III)	MIP: <i>Fps1, Rgc1, Rgc2</i> (glycerol channels)	<i>S. Cerevisiae</i> (eukaryotic)
As(III)	Hexose permeases	Yeast
As(III)	MIP: <i>TbAQP2</i>	<i>T. Brucei</i> (eukaryotic)
As(III)	MIP: <i>LmAQP1</i>	<i>Leishmania</i> (eukaryotic)
As(III)	MIP: <i>NIP7;1</i>	<i>Arabidopsis</i> (plants)
As(III)	MIP: <i>NIP2;1, NIP5;1, NIP6;1, NIP3;2</i>	<i>A. thaliana, L. japonicus</i> and <i>O. sativa</i> (plants)
As(III), MA and DMA	MIP: <i>Lsi1</i>	<i>O. sativa</i> (rice) roots
As(III)	MIP: <i>HvLsi1, ZmNIP2-1, ZmNIP2-2, ZmNIP2-3</i>	Plants
As(III) and MA(III)	MIP: <i>Aqp9a, Aqp9b, Aqp3, Aqp3I</i> and <i>Aqp10</i>	Zebrafish
MA and DMA	MIP: human AQP9	<i>X. laevis</i>
As(III) and MA(III)	MIP: <i>AQP7 and AQP9</i> Glucose transporter: <i>GLUT1</i>	Mammals (including humans)
As(III)	MIP: <i>AQP3 and AQP9</i>	Human cancer cells
As(V)	Phosphate transporters: <i>PiT-1, PiT2</i> (sodium-coupled)	<i>E. Coli</i> (prokaryote)
As(V)	Phosphate transporters: <i>Pho84, Pho87</i> (proton-coupled)	<i>S. Cerevisiae</i> (eukaryotic)
As(V)	Phosphate transporters: <i>NaPillb</i> (sodium-coupled)	Mammals

MIP: Major Intrinsic Protein family.

The different arsenic uptake systems in both prokaryotic and eukaryotic cells are summarised in Table 4-1. Under physiological conditions, arsenous acid (As(III)) is a neutral hydroxide ($\text{As}(\text{OH})_3$) (Figure 4-1) that is able to traverse lipid membranes using aquaporins and aquaglyceroporins (Liu et al., 2002, Rosen, 2002). These bidirectional channels belong to the Major Intrinsic Protein family (MIP) and are responsible for assisting the transport of small uncharged molecules across a concentration gradient. Aquaporins are specific for water; whereas aquaglyceroporins transport a range of small molecules, such as glycerol, carbon dioxide, ammonia, carbamides, polyols, purines and

urea (Liu et al., 2002). Since As(III) is physically similar to glycerol, it can easily use certain MIPs to traverse membranes (Zangi and Filella, 2012). In humans, the aquaglyceroporins that facilitate As(III) uptake are *AQP7* and *AQP9* (Liu et al., 2002). The first exists in kidney, testis and adipose tissue (Liu et al., 2006b) and the second in lung, liver and leukocyte tissues (Liu et al., 2002); but their level of expression depends on the age, gender and nutritional status of the individual (Liu et al., 2002). Human *AQP9* expressed in *Xenopus laevis* oocytes also facilitates the uptake of the pentavalent organic arsenic species methylarsonate (MA) and dimethylarsinate (DMA) (McDermott et al., 2010). In mammalian epithelial and blood cells, the influx of As(III) and methylarsonous acid (MA(III)) is assisted by the glucose transporter *GLUT1* (Liu et al., 2006b). Arsenate (As(V)) is an anion (HAsO_4^{2-}) (Figure 4-1) at physiological pH (~7.4) and thus, the way it enters the cell is different from As(III). Given its comparable volume and electronic configuration to orthophosphate, As(V) uptake had been suggested to be achieved by phosphate carriers (Rosen, 2002, Zhang et al., 2000); but this was not demonstrated until 2010 (Villa-Bellosta and Sorribas, 2010), when As(V) was shown to be captured by the Type II phosphate-sodium pump *NaPiIIb*, which usually exists in intestine, liver and lung cells (Villa-Bellosta and Sorribas, 2010).

Regarding arsenic efflux systems (Table 4-2), most of the research conducted on mammals has only reported transport mechanisms for As(III) and MA(III) (Liu et al., 2002, Liu et al., 2006a), as well as for the glutathione complexes arsenotriglutathione (ATG) and monomethylarsonic diglutathione (MADG) (Kala et al., 2000, Carew et al., 2011, Xu et al., 2013, Roggenbeck et al., 2015, Banerjee et al., 2014). Arsenous acid is extruded from cells via facilitated diffusion using MIPs. In humans, *AQP7* and *AQP9* promote As(III) entrance over removal (Liu et al., 2002); whereas for MA(III), *AQP9* only assists its elimination from hepatocytes (Liu et al., 2006a). Efflux of ATG, MADG and DMA in eukaryotes is conferred by the Multidrug Resistance Protein group (MRP), which are xenobiotic transporters from the ATP-binding cassette (ABC) family (Rosen, 2002). The biliary excretion of ATG and MADG is mediated by the protein *MRP2/cMOAT* in rats (Kala et al., 2000) and by *MRP1/ABCC1* in humans (Xu et al., 2013, Carew et al., 2011). Also in humans, the protein *MRP4/ABCC4* is responsible for the hepatic basolateral efflux of MADG and DMA (Roggenbeck et al., 2015, Banerjee et al., 2014).

Table 4-2. Arsenic efflux systems in cells, adapted from (Zangi and Filella, 2012).

ARSENIC SPECIES	TRANSPORTER NAME		ORGANISM
	Facilitated diffusion	Active transport	
As(III)	MIP: <i>Aqps</i>		<i>S. Meliloti</i> (prokaryotic)
As(III)	MIP: <i>Strop634</i>		<i>S. Tropica</i> (prokaryotic)
As(III)	MIP: <i>NIP2;1, NIP5;1, NIP6;1, NIP3;2</i>		<i>A. thaliana, L. japonicus</i> and <i>O. sativa</i> (plants)
As(III)	MIP: <i>Lsi2</i>		Rice
MA(III)	MIP: <i>AQP9</i>		Mammals
assumed As(III)		<i>Acr3p</i> (proton coupled)	<i>S. Cerevisiae</i> (eukaryotic)
trivalent arsenic species		<i>ArsB</i> (proton coupled)	Bacteria and Archaea
assumed As(III)		<i>Lsi2</i>	Plants
trivalent arsenic thiolated complexes		ABC transporters: <i>Ycf1p</i>	<i>S. Cerevisiae</i> (eukaryotic)
MADG and ATG		ABC transporters: <i>PGPA</i>	<i>Leishmania</i> (eukaryotic)
MADG and ATG		ABC transporters: <i>MRP2</i>	Mammals
MADG and ATG		ABC transporters: <i>MRP1</i>	Human cancer cells
DMA and MADG		ABC transporters: <i>MRP4</i>	Human hepatocytes
ATG		unknown ABC transporters	Human hepatocytes
trivalent arsenic thiolated complexes		ABC transporters: 190kDa MRP and P-glycoprotein	Human cancer cells

MIP: Major Intrinsic Protein family; **MRP:** Multidrug Resistance Protein group.

Few studies explain how As(V), DMA(III), DMA and MA can be effluxed from cells. Arsenate has been shown to be reduced to As(III) once inside cells (Nemeti et al., 2003) and DMA(III) is thought to be oxidised to DMA before elimination (Banerjee et al., 2014); but there is no evidence to prove that they cannot be removed from cells as As(V) and DMA(III). Even though the facilitated diffusion and active transport of MA and DMA have been reported (McDermott et al., 2010, Banerjee et al., 2014), the process is pH-dependent and more likely to occur at pH 5.5, when both arsenic species are uncharged (McDermott et al., 2010, Banerjee et al., 2014). At physiological pH (~7.4) MA and DMA are monoanions and their uptake is not as efficient (McDermott et al., 2010, Banerjee et al., 2014). There is little information on the transport of thiolated arsenic species, which together with methylated arsenic species and arsenic glutathione

complexes, are important metabolites of inorganic arsenic in living organisms (Hayakawa et al., 2004, Naranmandura et al., 2007). In a similar way, simple diffusion of arsenic species has been largely ignored; when it could provide an alternative route for arsenic uptake and removal if assisted transport is not possible.

Transport across biological membranes by simple diffusion strongly depends on the hydrocarbon permeability of the molecule involved (Cullen and Nelson, 1992), which can be estimated using partition coefficients. The term partition or distribution coefficient refers to the equilibrium distribution or difference in solubility of the molecule of interest between two immiscible phases (Leo et al., 1971). For many years, octanol has been considered the best organic solvent to represent the physicochemical environment that a foreign substance encounters in a living tissue (Sangster, 1989). The low cost of octanol and the amount of data available correlating octanol-water partitioning (P_{OW}) with lipophilicity have made this the preferred system to evaluate a number of compounds (Sangster, 1989). Still, octanol is far from the ordered and anisotropic phospholipid structure existing in cells and its ability to simulate real membranes has also been questioned (Hartmann and Schmitt, 2004, Matos et al., 2012). For that reason, alternative approaches have been developed including partitioning between water and liposomes (P_{LW}) (Hartmann and Schmitt, 2004), which constitutes a better method to study the permeability of drugs in human tissues (Balon et al., 1999). Liposomes are artificial spherical vesicles formed by the association of phospholipids in mono-, bi- or multilayers with an internal aqueous compartment (Sessa and Weissmann, 1968). Although they do not include proteins in their structure, liposome composition can be easily modelled to be as close as possible to real membranes, thus making P_{LW} an improved approximation to cells for assessing simple diffusion. Only a few publications make use of P_{OW} and liposomes to evaluate the ability of arsenic species to diffuse across membranes (Cullen and Nelson, 1992, Cullen et al., 1994). After inferring that the yeast *Candida Humicola* was able to take up As(III), DMA and MA by simple diffusion (Cullen et al., 1990); the authors tested the mobility of DMA and MA across lipid membranes by determining their P_{OW} , as well as their diffusion rates out of artificial unilamellar liposomes (Cullen and Nelson, 1992).

This study evaluates the ability of different arsenic species, commonly found in the environment (International Agency for Research on Cancer, 2012, Maher et al., 2013a, Mandal and Suzuki, 2002), to traverse biological membranes through simple diffusion. The P_{OW} of As(III), As(V), DMA, MA, thio-dimethylarsinic acid (thio-DMA), thiomethylarsonic acid (thio-MA), ATG and MADG (Figure 4-1) were determined and used as reference to assess the partitioning of the same arsenic species through liposomes. These arsenic species are small enough to participate in simple diffusion, and exist in many organisms due to the metabolism of inorganic arsenic in cells (Suzuki et al., 2002, Hayakawa et al., 2004, Naranmandura et al., 2007, Cullen, 2014, Chen et al., 2011, Wang et al., 2014). For the liposome experiments, phosphatidylcholine (PC) and phosphatidylethanolamine (PE) were chosen as model membrane phospholipids, since they are present in all eukaryotic cells (Van Meer et al., 2008, Mathews et al., 2013). Cholesterol was also added in order to confer structural stability. Given that lipophilicity is intimately related to the combination of the size, shape and charge of a molecule; for each arsenic species these properties were estimated by calculating their volume (size), dipole moment (shape) and electrostatic potential energy (charge) using molecular mechanics. Those properties were used to explain the experimental partition coefficients obtained.

Materials and methods

Arsenic species standards

Arsenous acid (As(III)), arsenate (As(V)), methylarsonic acid (MA) and dimethylarsinic acid (DMA) standard solutions were purchased from Sigma-Aldrich (Australia). Thio-methylarsonic acid (thio-MA) and thio-dimethylarsinic acid (thio-DMA) were synthesised by bubbling hydrogen sulfide, prepared by reacting ferrous sulfide (Sigma-Aldrich, Australia) with hydrochloric acid (Merck, Australia) in a Kipp generator, into a solution of MA and DMA, respectively. Whereas thio-DMA is generally pure, thio-MA coexists in solution with dithio-methylarsonic acid (dithio-MA). Both thio-MA and thio-DMA in solution are oxidised back to MA and DMA over time at a pH dependant rate (Hug et al., 2017). For that reason, they were prepared at the time of conducting the experiments. The arsenic glutathione complexes arsenotriglutathione

(ATG) and monomethylarsonic diglutathione (MADG) were synthesised in house following published methods (Raab et al., 2004).

Octanol-water partition coefficients

Octanol-water partition coefficients (P_{OW}) of arsenic species were determined following the method described in the OECD Guidelines for the Testing of Chemicals 107 (OECD, 1995) with some modifications. For each arsenic species, a buffer solution with 1 mg L^{-1} of arsenic in deionised water ($18.2 \text{ M}\Omega \text{ cm}$, Sartorius, Australia) saturated with octanol (Sigma-Aldrich, Australia) was prepared with 0.0125 M of ammonium dihydrogen phosphate ($(\text{NH}_4)\text{H}_2\text{PO}_4$, Merck, Australia) and 0.0075 M of potassium hydrogen phosphate trihydrate ($\text{K}_2\text{HPO}_4 \cdot 3\text{H}_2\text{O}$, Sigma-Aldrich, Australia) to attain pH 7.4. Plastic tubes (10 mL, Sarstedt, Australia) containing the corresponding arsenic species in the buffer solution and octanol 1:1 v/v (5 mL) were mixed *via* end over end rotation at room temperature for 24 h. Octanol and water fractions were separated by centrifugation at $180 \times g$ for 5 min (Centrifuge 5804 R, Eppendorf, Australia) and aliquots (100 μL) from each phase were taken for total arsenic concentration measurements. Samples were diluted to 1 mL with 1 % v/v nitric acid (Aristar, Merck, Australia). Control samples with 1 mg L^{-1} of arsenic were also analysed to assess the recovery of arsenic. Partition coefficients calculated using the below.

Equation 4-1. Octanol-water partition coefficient.

$$P_{OW} = \frac{C_{As} \text{ in octanol}}{C_{As} \text{ in water}}$$

Fabrication and characterization of liposomes

Liposomes were fabricated as described by MacDonald *et al.* (1991) with modifications. A lipid stock solution in chloroform (Sigma-Aldrich, Australia) was prepared with 60 % w/w of L- α -phosphatidylcholine (PC, from soybean, type IV-S, ≥ 30 % w/w [enzymatic] P7443, Sigma-Aldrich, Australia), 30 % w/w of 1,2-dipalmitoyl-sn-glycer-3-phosphoethanolamine (PE, ≥ 97 % w/w P1348, Sigma-Aldrich, Australia) and 10 % w/w cholesterol (CL, ≥ 99 % w/w C8667, Sigma-Aldrich, Australia); with a final lipid concentration of 8 mg L^{-1} . In a 50 mL round bottom flask, an aliquot of lipid stock solution was diluted with an equal volume of chloroform and, after evaporating the

solvent under vacuum (IKA, RV 10 control V-C, Australia), the residual lipid film was rehydrated with buffer solution (0.0125 M $(\text{NH}_4)\text{H}_2\text{PO}_4$ and 0.0075 M $\text{K}_2\text{HPO}_4 \cdot 3\text{H}_2\text{O}$ in deionised water, pH 7.4). The mixture was frozen-thawed with five cycles of alternating immersions in liquid nitrogen (Coregas, Australia) and boiling water to favour the formation of unilamellar bilayers. Aliquots of the final liposome suspension were extruded eleven times with a mini-extruder (Avanti Polar Lipids, Auspep, Australia) through 100 nm pore size polycarbonate membranes (Avanti Polar Lipids, Auspep, Australia) to ensure a homogeneous size of 100 nm in most liposomes. These liposomes were named PE-PC-CL (30-60-10). A suspension of liposomes with composition 100 % w/w L- α -phosphatidylcholine was also prepared to be used as reference for characterisation, and named PC100. Both types of liposomes were characterised with a Zetasizer Nano ZS (Malvern, UK). Size measurements were performed at 25°C using a DTS0012 disposable sizing cuvette (Malvern, UK) and a detection angle of 173°. Z-potential values were determined at pH 7.4 and at 25°C using a DTS1060C disposable zeta cell (Malvern, UK) and applying the Smoluchowski model. For both size and Z-potential measurements, the viscosity and refractive index of the dispersant were taken as 0.8872 cP and 1,330; while the refractive index and absorption of the lipid material were 1.460 and 0.001, respectively.

Liposome-water partition coefficients

Liposome-water partition coefficients (P_{LW}) were determined as for octanol-water. For each arsenic compound, the extruded liposome suspension PE-PC-CL (30-60-10) was dosed with the solution of the corresponding arsenic species in buffer solution (0.0125 M $(\text{NH}_4)\text{H}_2\text{PO}_4$ and 0.0075 M $\text{K}_2\text{HPO}_4 \cdot 3\text{H}_2\text{O}$ in deionised water, pH 7.4) to a final arsenic concentration of 1 mg L⁻¹. Samples were mixed *via* end over end rotation at room temperature for 24 h. Liposomes were separated by size exclusion chromatography (SEC) (Laouini et al., 2012) using a Sephadex-G 50 column (Amersham Biosciences, location) and deionised water as eluent. For each sample, a total of 70 fractions of 2.5 mL were collected. Elution of liposomes containing arsenic was confirmed in fractions from 25 to 35 using an evaporative light scattering detector (ELSD, Grace, Alltech ELSD 3300, Australia) coupled to a Flexar 10 high performance liquid chromatograph (Perkin Elmer, Waltham, MA, US). Arsenic in solution eluted after fraction number 40. All

fractions were diluted 1:1 v/v and brought to 10 % v/v nitric acid for total arsenic concentration analysis. Control samples with 1 mg L⁻¹ As were also analysed to assess the recovery of arsenic. Partition coefficients calculated using the equation below.

Equation 4-2. Liposome-water partition coefficient.

$$P_{LW} = \frac{C_{As} \text{ in liposomes}}{C_{As} \text{ in water}}$$

Total arsenic concentration analysis

Samples from octanol-water partitioning experiments were analysed by high performance liquid chromatography-inductively coupled plasma-mass spectrometry (HPLC-ICP-MS) using a Flexar 10 HPLC coupled to a Nexion 300D ICP-MS (both Perkin Elmer, Waltham, MA, US). Calibration standards were prepared from a multi element calibration standard (Accu Trace, Accu-Standards, 10 mg L⁻¹) in deionised water with 1 % v/v nitric acid at arsenic concentrations of 400, 40, 20 and 4 µg L⁻¹. Blanks of buffer solution and buffer solution saturated in octanol, as well as control samples, were prepared at the same acid concentration as samples. Standards, blanks and samples were run for 4 min under isocratic conditions using a Hamilton PRP-X100 anion exchange column (150 mm × 4.1 mm, 10 µm particle size, Phenomenex, Australia) and a mobile phase of 10 mM malonic acid (UNILAB, Ajax Chemicals, Australia) adjusted to pH 5.6 with aqueous ammonia (Sigma-Aldrich, Australia). Previous attempts to analyse these samples with graphite furnace atomic absorption spectrometry, flow injection-inductively coupled plasma-mass spectrometry and ICP-MS confirmed HPLC-ICP-MS as the most reliable technique to analyse octanol samples.

Samples from liposome-water partitioning experiments were analysed by ICP-MS using a Nexion 300D ICP-MS with an ASX-520 Autosampler (both Perkin Elmer, Waltham, MA, US) as described by Maher *et al.* (2012). Calibration standards were prepared from a multi element calibration standard in deionised water with 10 % v/v nitric acid at arsenic concentrations of 500, 100, 10 and 5 µg L⁻¹. Blanks of buffer solution and buffer solution with liposomes in suspension, as well as control samples, were prepared at same acid concentration as samples. The plasma power was 1300 W and the nebuliser gas (argon) flow 0.9 L min⁻¹. The elution of liposomes was monitored

by measuring chromium (^{52}Cr), as it has the same m/z as the adduct that carbon forms with argon ($^{40}\text{Ar}^{12}\text{C}$). A solution of 100 $\mu\text{g L}^{-1}$ tellurium (certified standard, Merck, Australia) in 1 % v/v nitric acid and 2 % v/v isopropanol (Scharlau, Chem Supply, Australia) was used as the internal standard.

Statistical analysis

Statistical analysis of P_{OW} and P_{LW} results was conducted using Excel and the software package PAST (Hammer et al., 2001). Analysis of Variance (ANOVA) with post hoc testing using Mann-Whitney pairwise comparisons was conducted to test for significant differences within and between octanol-water and liposome-water partitioning experiments.

Molecular modelling of arsenic species

Arsenous acid, As(V), MA, DMA, thio-MA, thio-DMA, MADG, ATG, dithio-MA, trithio-methylarsonic acid (trithio-MA) and dithio-dimethylarsinic acid (dithio-DMA) were modelled using the software Spartan'08 (Wavefunction, US). The dipole moments, volumes and electrostatic potentials of molecules were calculated after optimisation of their equilibrium geometries at ground state using the Hartree-Fock method with a basis set 3-21G. The chemical structure modelled for each arsenic species is the one most likely to occur at pH 7.4 (Larsen, 1993, Suner et al., 2001). Octanol and the moieties corresponding to the polar heads of both PE and PC were also modelled.

Results and discussion

Octanol-water partition coefficients

Trivalent arsenic species (MADG, As(III) and ATG) exhibit higher P_{OW} values than most pentavalent arsenic species with the exception of thio-MA, with a P_{OW} closer to those of trivalent arsenic species (Figure 4-2). The P_{OW} values of the arsenic species that partition into octanol are separated into two significantly different groups [$F = 9.85$, $df = 5$, $p = < 0.05$ ($p = 1.06 \times 10^{-6}$)]. The P_{OW} of DMA and thio-DMA are similar to each other and different from the group containing ATG, thio-MA, As(III) and MADG. The computed values of volume, dipole moment and electrostatic potential energy for the arsenic species tested are shown in Table 4-3. The electrostatic potential density (EPD),

calculated by dividing the electrostatic potential energy by the total volume, and the ratio between the EPD and the dipole moment (DM) are also displayed. A relationship between the ratio EPD/DM and the P_{OW} is evident, as the arsenic species with a higher absolute value of the EPD/DM ratio have a low P_{OW} or do not partition at all. This can be explained by the distribution of charge in each arsenic species that creates an electrostatic potential in the surrounding space (Politzer et al., 1985). That potential is the basis for the electrostatic interaction of the molecule with the nearby environment and its orientation is determined by the dipole moment, which is linked to the molecular geometry. In that way, a molecule with a highly negative electrostatic potential evenly distributed around its total volume will have a lower dipole moment than a molecule with a more positive electrostatic potential asymmetrically distributed. When approaching the octanol polar heads, the molecule will readjust its electron cloud to minimise the electrostatic repulsion with the electrical double layer at the octanol-water interface. This will be more effective in molecules with a higher dipole moment. Once at the interface, the electrostatic potential density of the molecule, which is related to the magnitude of its charge, will determine the extent of the penetration into the lipophilic medium (Levine, 2002, Tipler and Mosca, 2003). The electrostatic interaction between the molecule and the octanol non-polar tails needs to be strong enough to induce temporal dipoles along the hydrocarbon chains but weak enough as to allow the movement across them. Hence, molecules with a higher DM and a lower EPD will diffuse better into the octanol phase.

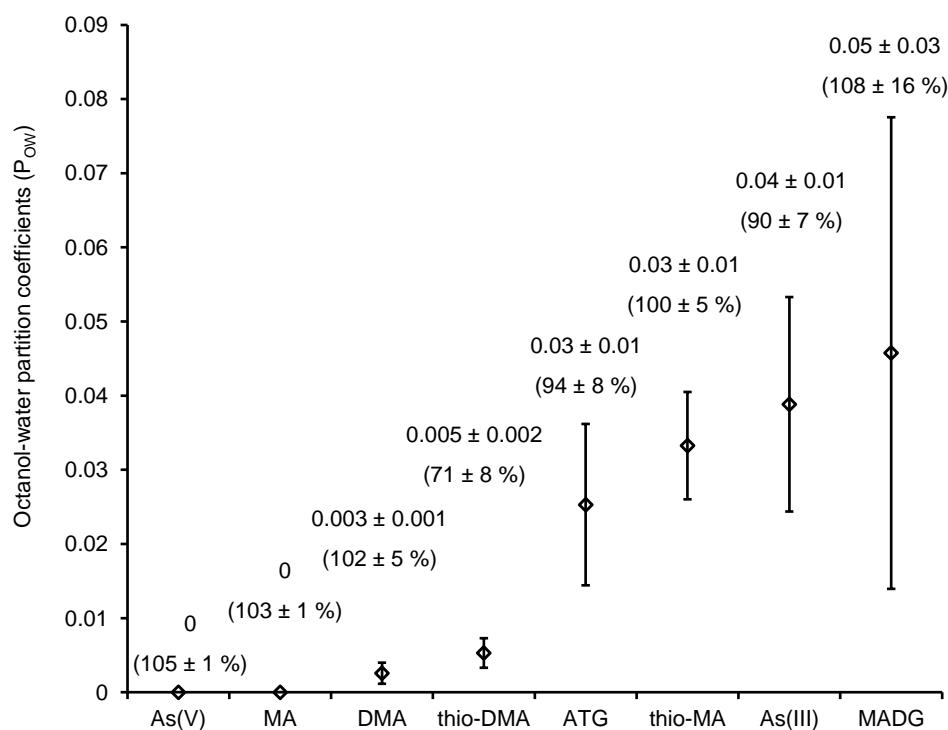


Figure 4-2. Octanol-water partition coefficients (P_{OW}) of arsenic species (mean \pm s.d, n=4). Arsenic recovery \pm s.d is given in parentheses.

Table 4-3. Molecular properties computed for the arsenic species of interest. The steric volume includes the electron cloud into the molecule volume.

Arsenic species	Volume (Å ³)	Steric volume (Å ³)	Dipole moment (Debye)	Electrostatic potential energy (kJ mol ⁻¹)	Electrostatic potential density (kJ mol ⁻¹ Å ⁻³)	EPD/DM
dithio-DMA	115.21	133.99	4.38	18.81	0.14	0.03
trithio-MA	113.6	139.67	2.66	23.09	0.17	0.06
ATG	815.89	861.22	12.54	-975.13	-1.13	-0.09
thio-DMA	104.23	115.84	4.11	62.03	0.54	0.13
MADG	575.80	608.15	7.8	-742.26	-1.22	-0.16
As(III)	62.01	65.16	2.26	35.37	0.54	0.24
dithio-MA	102.66	121.67	2.43	85.88	0.71	0.29
thio-MA	89.69	104.98	4.04	-397.26	-3.78	-0.94
DMA	91.51	97.58	4.82	-492.17	-5.04	-1.05
MA	79.09	84.70	3.18	-421.69	-4.98	-1.57
As(V)	64.51	71.03	2.46	-868.63	-12.23	-4.97

EPD: Electrostatic potential density; **DM:** Dipole moment.

To help visualise this explanation, the modelled structures and potential density maps of the arsenic species studied and of 1-octanol are shown in Figure 4-3 and Figure 4-4, respectively. For example, As(V) is a dianion (HAsO_4^{2-}) with tetrahedral geometry and the charge is evenly distributed around its small volume (Table 4-3 and Figure 4-3b). Arsenate has a small DM in comparison to its EPD, which is the highest of all the arsenic species studied (Table 4-3). When As(V) approaches octanol, it will be electrostatically repulsed by the polar heads (Figure 4-4), hindering further penetration into the non-polar tails of octanol. Indeed, As(V) did not partition into octanol, as its P_{OW} was 0 (Figure 4-2). The same applies for MA ($P_{\text{OW}} = 0$) and DMA ($P_{\text{OW}} = 0.003 \pm 0.001$), which also have negligible P_{OW} and high EPD/DM ratios (Figure 4-2 and Table 4-3). These are consistent with published partition coefficients for MA and DMA (8.4×10^{-3} and 7.4×10^{-3} , respectively) (Cullen and Nelson, 1992). For As(III), despite being an uncharged molecule, the trigonal pyramidal structure leads to an unequal distribution of the electron cloud (Figure 4-3a). Arsenous acid has a positive electrostatic potential energy (Table 4-3) and will be electrostatically attracted to the nucleophilic polar heads of octanol. The small size and low EPD of As(III) will then facilitate its diffusion into the octanol phase, as confirmed by the measured P_{OW} (0.04 ± 0.01 , Figure 4-2). Thiomethylarsonate and thio-DMA could not be synthesised as pure standards without containing a mixture of mono-, di- and tri-thiolated arsenic species (Figure 4-1) (Rumpler, 2010, Maher et al., 2013b). The structures of these species were also modelled (Figure 4-5) and so were their molecular properties (Table 4-3). For thio-MA, the formation of di- and tri-thio-MA in solution had a clear influence on its P_{OW} . Whereas the ratio EPD/DM of thio-MA (Table 4-3) suggests a lower P_{OW} than that measured in this study (Figure 4-2); the presence of di- and tri-thio-MA species would increase the apparent P_{OW} , as indicated by their EPD/DM ratios (Table 4-3). This explains the P_{OW} of thio-MA obtained in these experiments. For thio-DMA, however, the simultaneous formation of dithio-DMA (Figure 4-1), which has also been found in mammals after exposure to inorganic arsenic (Naranmandura et al., 2007, Raml et al., 2006, Mandal et al., 2008, Kubachka et al., 2009), will not substantially modify the P_{OW} . Both arsenic species have similar DM and EPD (Table 4-3), and therefore, should have similar P_{OW} values and higher than the one obtained for thio-DMA in this study (Figure 4-2). The partial oxidation of thio-DMA and dithio-DMA to DMA could explain the decrease in

the P_{OW} observed. For the glutathione complexes MADG and ATG, the stability of the molecule plays an important role in its lipophilicity. It has been reported that MADG is more stable than ATG (Raab et al., 2004), which tends to hydrolyse to As(III). For that reason, even though ATG presents a lower EPD/DM ratio than MADG, partitioning into octanol is hindered by the formation of As(III) in solution, decreasing the P_{OW} value for ATG observed in this study.

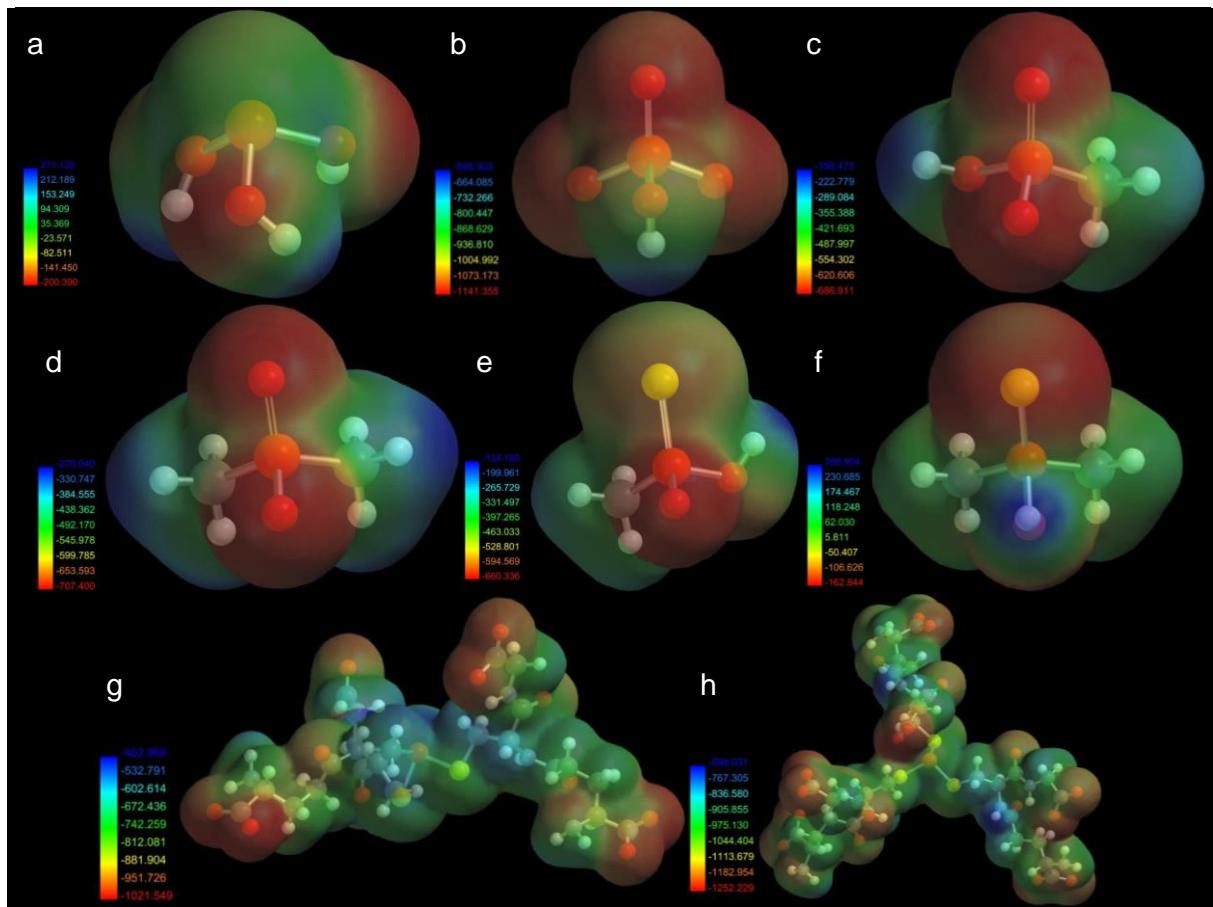


Figure 4-3. Modelled molecular structures and electrostatic potential maps for the arsenic species: arsenous acid (a), arsenate (b), methylarsonate (c), dimethylarsinate (d), thio-methylarsonate (e), thio-dimethylarsinic acid (f), monomethylarsonic diglutathione (g) and arsenotriglutathione (h). The scale next to each molecule shows the values of the electrostatic potential for each colour. The red and dark blue regions correspond to the most negative and most positive regions in the molecule, respectively.

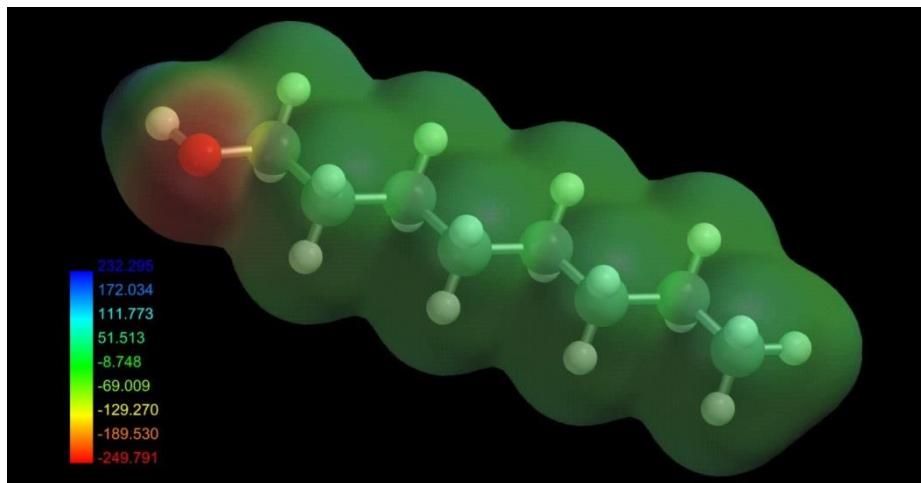


Figure 4-4. Modelled molecular structure and electrostatic potential map of 1-octanol. The scale next to the molecule shows the values of the electrostatic potential for each colour. The red and dark blue regions correspond to the most negative and most positive regions in the molecule, respectively.

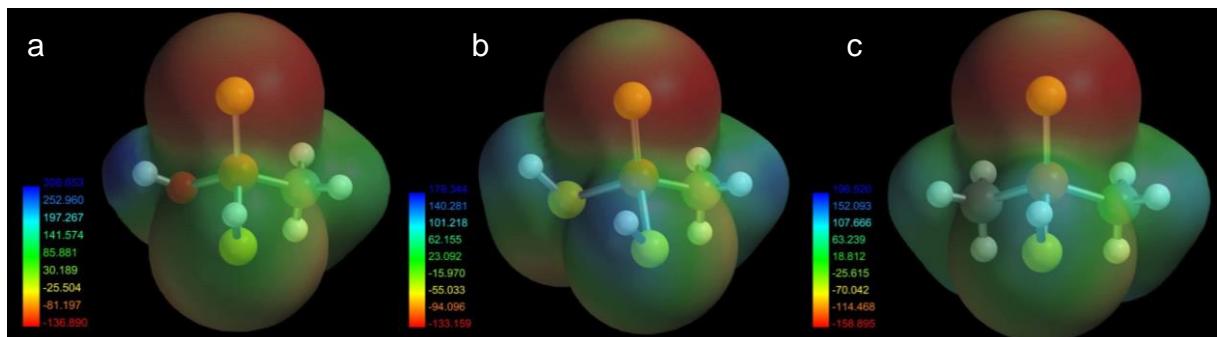


Figure 4-5. Modelled molecular structures and electrostatic potential maps for dithio-methylarsonic acid (a), trithio-methylarsonic acid (b) and dithio-dimethylarsinic acid (c). The scale next to each molecule shows the values of the electrostatic potential for each colour. The red and dark blue regions correspond to the most negative and most positive regions in the molecule, respectively.

Liposomes-water partition coefficients

Whereas P_{OW} gives an approximate indication of the lipophilicity of arsenic species, liposome-water partitioning (P_{LW}) constitutes a more realistic method to assess simple diffusion across the lipid bilayer in cell membranes. By considering the surface potential of octanol polar heads and cell membranes, the difference between both approaches can be understood. Octanol is a non-charged molecule (Figure 4-4) with a much smaller surface potential than that of cell membranes, usually in the range from -40 to -80 mV (Mathews et al., 2013). The cell membrane potential arises from the difference in the electric potential between the inner and outer sides of the bilayer, and it is more negative

when the former negatively charged compared to the latter (Mathews et al., 2013). The measured Z-potentials of the two liposome suspensions prepared for this study are comparable to the surface potentials of cell membranes; -33 ± 6 mV for PC100 and -45 ± 5 mV for PE-PC-CL (30-60-10). The size measurements of both liposome suspensions revealed an average liposome diameter of 100 nm (*Appendix Chapter 4*), indicating that the difference in the Z-potential values is due to the distribution of PC and PE in the outer and inner sides of the bilayer. Both PE and PC are zwitterionic phospholipids at physiological conditions (pH 7.4), as they have a negative and positive charge in their polar head (Figure 4-6). This means that the nett charge in each molecule is zero, but does not necessarily imply a similar electrostatic potential energy, which is slightly more negative in the PC polar head (-76.4 kJ mol $^{-1}$) than in the PE polar head (-45.2 kJ mol $^{-1}$). This, together with the measured Z-potentials, suggests that PE-PC-CL (30-60-10) liposomes have a higher concentration of PC in the inside of the liposome bilayer. Nevertheless, given that the ratio PE: PC is 1:2 w/w, both phospholipids are expected to exist in the outer side of the liposome bilayer and thus, both will have an effect on the P_{LW} of the arsenic species tested.

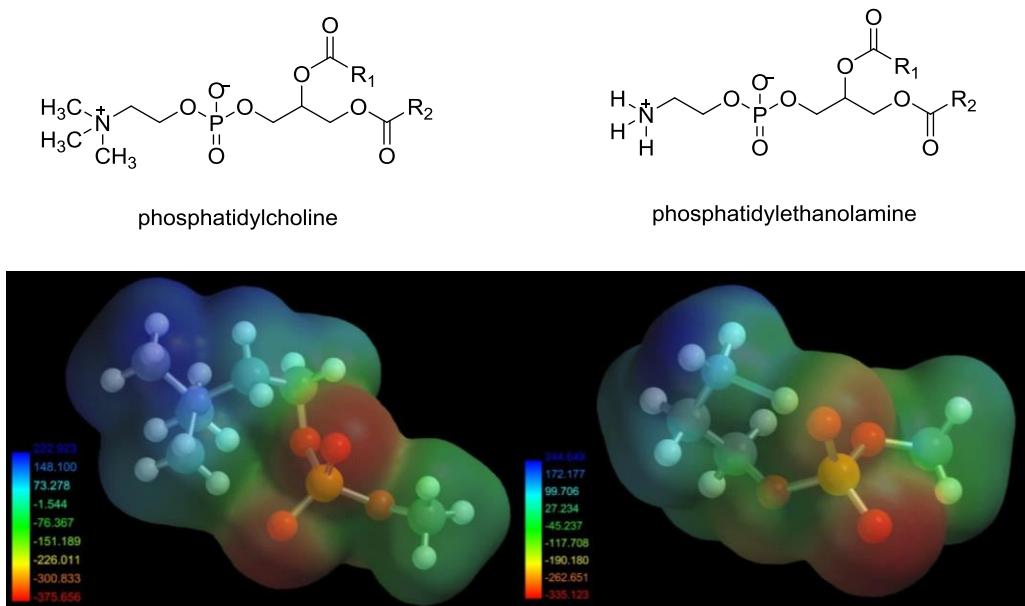


Figure 4-6. Structures of phosphatidylcholine (left) and phosphatidylethanolamine (right) and modelled molecular structures and electrostatic potential maps for their polar heads. The scale next to each molecule shows the values of the electrostatic potential for each colour. The red and dark blue regions correspond to the most negative and most positive regions in the molecule, respectively.

As for octanol, As(V) and MA do not partition into liposomes (Figure 4-7), hence, they will not passively diffuse across cell membranes. The P_{LW} of DMA, thio-DMA and MADG increase compared to P_{OW} ; from 0.003 ± 0.001 to 0.09 ± 0.01 for DMA; 0.005 ± 0.002 to 0.04 ± 0.02 for thio-DMA; and 0.05 ± 0.03 to 0.13 ± 0.03 for MADG. Arsenous acid and ATG partition into liposomes similarly than into octanol, with P_{LW} values of 0.0392 ± 0.0007 and 0.07 ± 0.01 , respectively (Figure 4-7). There were no significant differences between the P_{LW} values for the arsenic species that partition into liposomes [$F = 4.098$, $df = 4$, $p = > 0.05$]. Significant differences occurred between P_{LW} and P_{OW} values for thio-DMA [$F = 85.15$, $df = 1$, $p = < 0.05$], ATG [$F = 27.4$, $df = 1$, $p = < 0.001$], DMA [$F = 262.4$, $df = 1$, $p = < 0.01$] and MADG [$F = 12.94$, $df = 1$, $p = < 0.01$], but not for As(III) [$F = 6.25 \times 10^{-34}$, $df = 1$, $p = 1$]. In contrast to the octanol polar head, which has a negative electron density (Figure 4-4), the polar head of PC and PE present both positive (around nitrogen) and negative (around phosphorus) electron density areas (Figure 4-6). Nitrogen is more exposed than phosphorus, making it easier for molecules with negative electron density areas to approach the outer layer of the liposome. Once at the liposome-water interface, molecules with larger dipole moments will interact better with the zwitterionic head of the phospholipid, as the charges in the molecule will be re-orientated to avoid electrostatic repulsions. This can explain the results obtained for DMA, thio-DMA and MADG, but not why thio-MA does not partition into liposomes (Figure 4-7). The oxidation of thio-MA to MA in solution (Hug et al., 2017) could explain this behaviour, but the results of P_{OW} for thio-MA do not support this occurring. Considering the zwitterionic character of PE and PC, As(III) will not experience the same effect as negatively charged arsenic species and for ATG, hydrolysis into As(III) (Raab et al., 2004) will again influence its lipophilicity. The experimental diffusion coefficients in aqueous solution for As(III), As(V) and MA have been reported as $11.6 \pm 0.1 \times 10^{-6} \text{ cm}^2 \text{ s}^{-1}$, $7.27 \pm 0.03 \times 10^{-6} \text{ cm}^2 \text{ s}^{-1}$ and $7.78 \pm 0.16 \times 10^{-6} \text{ cm}^2 \text{ s}^{-1}$, respectively (Takahashi et al., 2011, Tanaka et al., 2013). These coefficients represent the ability of the arsenic species to diffuse in water and are proportionally related to their partition coefficients in cell membranes (Lodish et al., 2000b). According to this, As(III) should partition better into a lipid membrane than As(V) and MA, not necessarily implying that the two latter should partition at all, which is in agreement with the results in the present study.

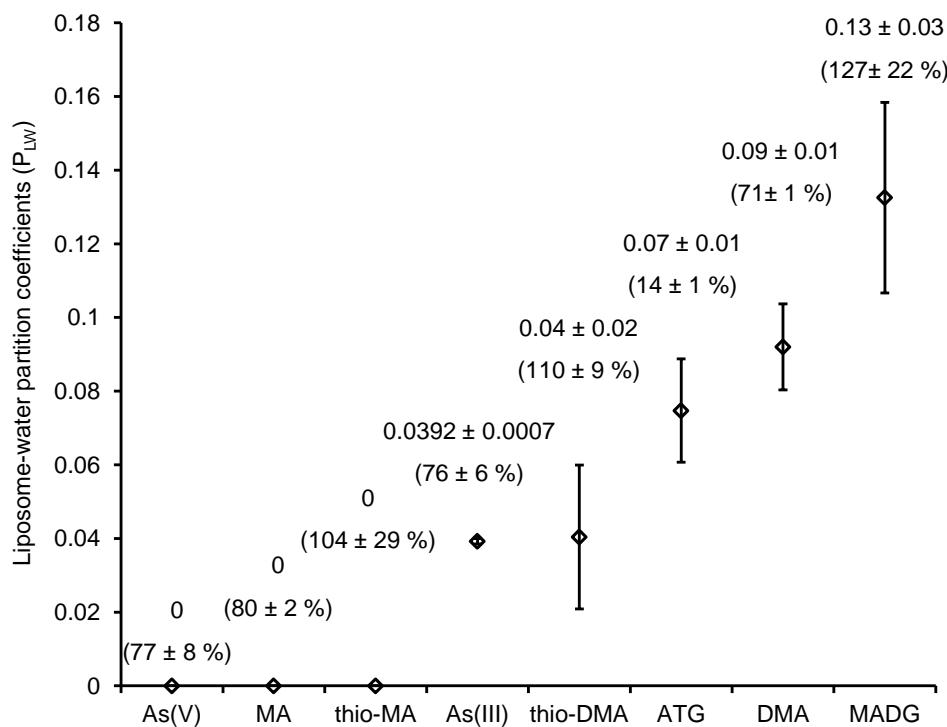


Figure 4-7. Liposome-water partition coefficients (P_{LW}) of arsenic species (mean \pm s.d, n=3). Arsenic recovery \pm s.d is given in parentheses.

Concluding remarks

The ability of arsenic species to passively traverse cell membranes is related to the combination of the electrostatic potential density and the dipole moment of the molecule. Partition coefficients are higher for the trivalent arsenic species, as well as for the thiolated arsenic species. Even though both P_{OW} and P_{LW} display similar trends, the use of liposomes offers a more accurate system to evaluate simple diffusion in real biological systems. It was observed that all the arsenic species tested, except for As(V), MA and thio-MA, are able to diffuse across cell membranes; increasing the possibilities of passive uptake and extrusion by cells. The high toxicity of As(III) and thio-methylated arsenic species (Leffers et al., 2013b) and their potential for bioaccumulation, as shown in this study, increases the risk that the exposure to these arsenic species has in human health.

CHAPTER 5 – Subcellular distribution of the metabolites produced from arsenate and arsenous acid biotransformations in HepG2 cells

Introduction

Arsenic exists as over forty species, with arsenate (As(V)) and arsenous acid (As(III)) being the two inorganic arsenic species most widely distributed in the environment (Mandal and Suzuki, 2002, Roy and Saha, 2002, Stoltz et al., 2006). Although anthropogenic activities can increase the availability of inorganic arsenic to humans (Zhang, 2017, Hughes et al., 2011); exposure to As(III) and As(V) primarily comes from food and drinking water (Sharma et al., 2014). Inorganic arsenic in drinking water is mainly derived from groundwater, where arsenic concentrations range from 0.5 to 50000 µg L⁻¹ (Singh et al., 2015). In foods, As(III) and As(V) come from both water and soil (Da Sacco et al., 2012, Rahman et al., 2011). Inorganic arsenic in animal and plant based terrestrial foods usually do not exceed 0.05 µg g⁻¹ (European Food Safety Authority, 2014, Francesconi, 2010). Rice, however, can accumulate higher arsenic concentrations (0.1 and 0.4 µg g⁻¹), of which 85 – 90 % corresponds to As(III) and As(V) (Mandal and Suzuki, 2002, Francesconi, 2010, Carey et al., 2010, Maher et al., 2013a). Seafood and seafood derived products are rich in organic arsenic species, while As(III) and As(V) concentrations are generally below 0.2 µg g⁻¹ dry mass, nearly 2 % w/w of the total arsenic in seafood (Sharma et al., 2014). Exceptions include the seaweeds *Hizikia fusiforme* and *Durvillea potatorum*, and blue mussels (*Mytilus edulis*); with inorganic arsenic concentrations of 135 µg g⁻¹, 120 µg g⁻¹ and 5.8 µg g⁻¹, respectively (Hanaoka et al., 2001b, Sloth and Julshamn, 2008, Kirby et al., 2004).

Chronic exposure to As(III) and As(V) can produce skin lesions, cardiovascular diseases and skin, bladder and lung cancers; and may also influence the development of kidney, liver and prostate cancers (Mead, 2005, European Food Safety Authority, 2009, World Health Organization, 2010). At a molecular level, the specific modes of toxic action of As(III) and As(V) are not fully understood, although it is known that both arsenic species induce the formation of reactive oxygen species (ROS) and reactive nitrogen species (RNS), which are responsible for genotoxicity in cells (Chen et al., 2003, Ventura-Lima et al., 2011, Ghosh et al., 2008, Faita et al., 2013). Thus, As(III) and As(V)

are classified as Group 1 carcinogens, motivating environmental and health organizations worldwide to regularly assess the permitted levels for these arsenic species in different environmental matrices, drinking water and food (World Health Organization, 2010, European Food Safety Authority, 2014). It is not only necessary to understand the toxic effects of As(III) and As(V) in cells, but also the metabolic pathways they undergo for detoxification, as some intermediates can exert higher toxicity than the initial inorganic arsenic species of exposure.

The study of the metabolism of inorganic arsenic dates back to the first half of the 20th century, when Frederick Challenger observed that inorganic arsenic species were metabolised to methylated arsenic species by fungi (Challenger, 1947). Since then, arsenic methylation has been reported in microorganisms (Cullen et al., 1995), aquatic organisms (Phillips, 1990, Benson et al., 1981), terrestrial plants (Koch et al., 2000, Raab et al., 2005) and mammals (Buchet and Lauwerys, 1985, Aposhian, 1997, Chowdhury and Aposhian, 2008, Klaassen, 1974, Marafante and Vahter, 1987). The enzyme responsible for arsenic methylation in mammals is arsenic III methyltransferase (*AS3MT*), which only operates on trivalent arsenic species, thus requiring the prior reduction of As(V) to As(III) (Styblo et al., 1999, Walton et al., 2003). Arsenic methyltransferase activity has been observed in hamsters, rats, mice, rhesus monkeys, rabbits and humans (Zakharyan et al., 1996, Aposhian, 1997, Chowdhury and Aposhian, 2008, Klaassen, 1974, Marafante and Vahter, 1984); and relies on *S*-adenosyl-L-methionine (SAM) as a methyl donor (Lin et al., 2002). The mechanism for arsenic methylation, however, is still under debate. Challenger proposed a pathway that yields methylarsonous acid (MA(III)) and dimethylarsinous acid (DMA(III)) as final products (Challenger, 1947, Cullen et al., 1984a) (Figure 5-1a). In human urine, arsenic has been found as As(III), As(V), MA(III), methylarsonic acid (MA), DMA(III) and dimethylarsinic acid (DMA) (Vahter and Concha, 2001), although the detection of MA(III) and DMA(III) in some cases could be due to the misidentification of thio-arsenic species (Hansen et al., 2004c), which have been also found in humans, hamsters and rats (Raml et al., 2006, Naranmandura et al., 2007). Even so, MA and DMA are still the most abundant metabolites in human urine (Concha et al., 2002, Shraim et al., 2003), questioning Challenger's pathway.

Reductive methylation has been proposed as a feasible alternative, where methylation produces trivalent species that are later oxidised (Hayakawa et al., 2004, Rehman and Naranmandura, 2012) (Figure 5-1b, 5-1c and 5-1d). Hayakawa and co-workers suggested that trivalent arsenic species form glutathione (GSH) conjugates to be the substrates for *AS3MT* (Hayakawa et al., 2004) (Figure 5-1b). Indeed, *AS3MT* has a higher affinity for arsenic glutathione complexes than for As(III) and MA(III) (Hayakawa et al., 2004, Marapakala et al., 2012); and arsenotriglutathione (ATG) and monomethylarsonic diglutathione (MADG) have been detected in rat bile (Suzuki et al., 2002, Kala et al., 2000). Dimethylarsoglutathione (DMAG) has not been detected *in vivo* (Suzuki et al., 2002, Kala et al., 2000), as it rapidly decomposes to DMA, which is then excreted into the bloodstream (Hayakawa et al., 2004, Suzuki, 2005). This is coherent with arsenic methylation in humans, as DMA is the final metabolic product. Mice and rats, however, are able to methylate arsenic a third time yielding trimethylarsine oxide (TMAO) (Vahter et al., 1984, Yamauchi and Yamamura, 1984, Marafante and Vahter, 1987, Yoshida et al., 1998). In that case, DMAG should also be a substrate of *AS3MT*. Hayakawa and co-workers did not find DMAG in their experiments, neither tested DMAG susceptibility to undergo methylation in the presence of SAM, GSH and *AS3MT* (Hayakawa et al., 2004).

Moreover, As(III), MA(III) and DMA(III) can bind to the cysteine residues of proteins in liver, kidneys and blood (Naranmandura et al., 2006, Styblo and Thomas, 1997, Lu et al., 2004); whereas MA and DMA cannot (Naranmandura et al., 2007). The interaction of trivalent arsenic species with proteins seems to be stronger than with GSH (Suzuki et al., 2004, Bogdan et al., 1994). On the basis of these findings, it was suggested that while the formation of arsenic glutathione complexes may be restricted to the liver, reductive methylation in other organs can occur through arsenic-binding proteins in the presence of *AS3MT*, SAM and GSH (Figure 5-1c) (Naranmandura et al., 2007, Rehman and Naranmandura, 2012, Naranmandura et al., 2006). Further studies have shown the interaction of both trivalent arsenic species and SAM with the active site of *AS3MT*, and suggested that binding to SAM changes the enzyme conformation to allow a better accessibility of arsenic to the active site (Thomas et al., 2004, Marapakala et al., 2012, Song et al., 2009, Song et al., 2011, Wang et al., 2012). Methylation then occurs with both arsenic and SAM bound to the enzyme, producing trivalent arsenic cysteine

complexes that after dissociation and further oxidation yield MA and DMA (Figure 5-1d) (Wang et al., 2012). Despite these experiments not being performed with living organisms, this theory is consistent with Naranmandura's pathway of protein conjugation. Both glutathione conjugation and methylation are well-known mechanisms of xenobiotic metabolism, which aim to increase the hydrophilicity and deactivate the toxicity of the initial substrate (Casarett and Doull, 2008, Ioannides, 2002). These reactions are not complementary or exclusive. Thus, the formation of arsenic glutathione complexes may be independent from arsenic methylation and, based on their reported affinity to *AS3MT* (Hayakawa et al., 2004, Marapakala et al., 2012), their interaction with this enzyme might be unintended but favourable. This means that the pathways in Figure 5-1b, 5-1c and 5-1d could all be possible. As chimpanzees, tamarins, marmosets, squirrel monkeys and guinea pigs lack of methylation capacity but still cope with arsenic exposure, alternative detoxification routes to arsenic methylation might exist (Vahter and Concha, 2001, Aposhian, 1997, Zakharyan et al., 1996).

The pathways in Figure 5-1 have been proposed based on epidemiological studies, *in vivo* experiments using animals, and *in vitro* experiments simulating the environment of mammalian cells. Few studies, however, have focused on the subcellular distribution of arsenic after the metabolism of inorganic arsenic in human cells (Dopp et al., 2008, Dopp et al., 2010). In these studies, human urothelial and liver tissue cultures were dosed with As(III) and As(V) and the different organelles analysed for total arsenic concentration to assess the distribution of these arsenic species (Dopp et al., 2010, Dopp et al., 2008). The metabolic products or potential intermediates, however, were not reported. Investigating where arsenic transformations occur at a subcellular level, as well as elucidating possible intermediates including cysteine or protein conjugates, can provide more evidence supporting any of the current proposed pathways for inorganic arsenic metabolism, as well as a better understanding of the modes of toxic action of inorganic arsenic.

This study examines the metabolism of As(III) and As(V) in hepatocarcinoma cells (HepG2) after 24 h exposure; focusing on the arsenic species produced and their distribution within the different organelles. HepG2 cells were chosen as they constitute an immortal cell line that grows relatively fast and is able to metabolise inorganic arsenic species as effectively as healthy hepatocytes (Chen et al., 2003).

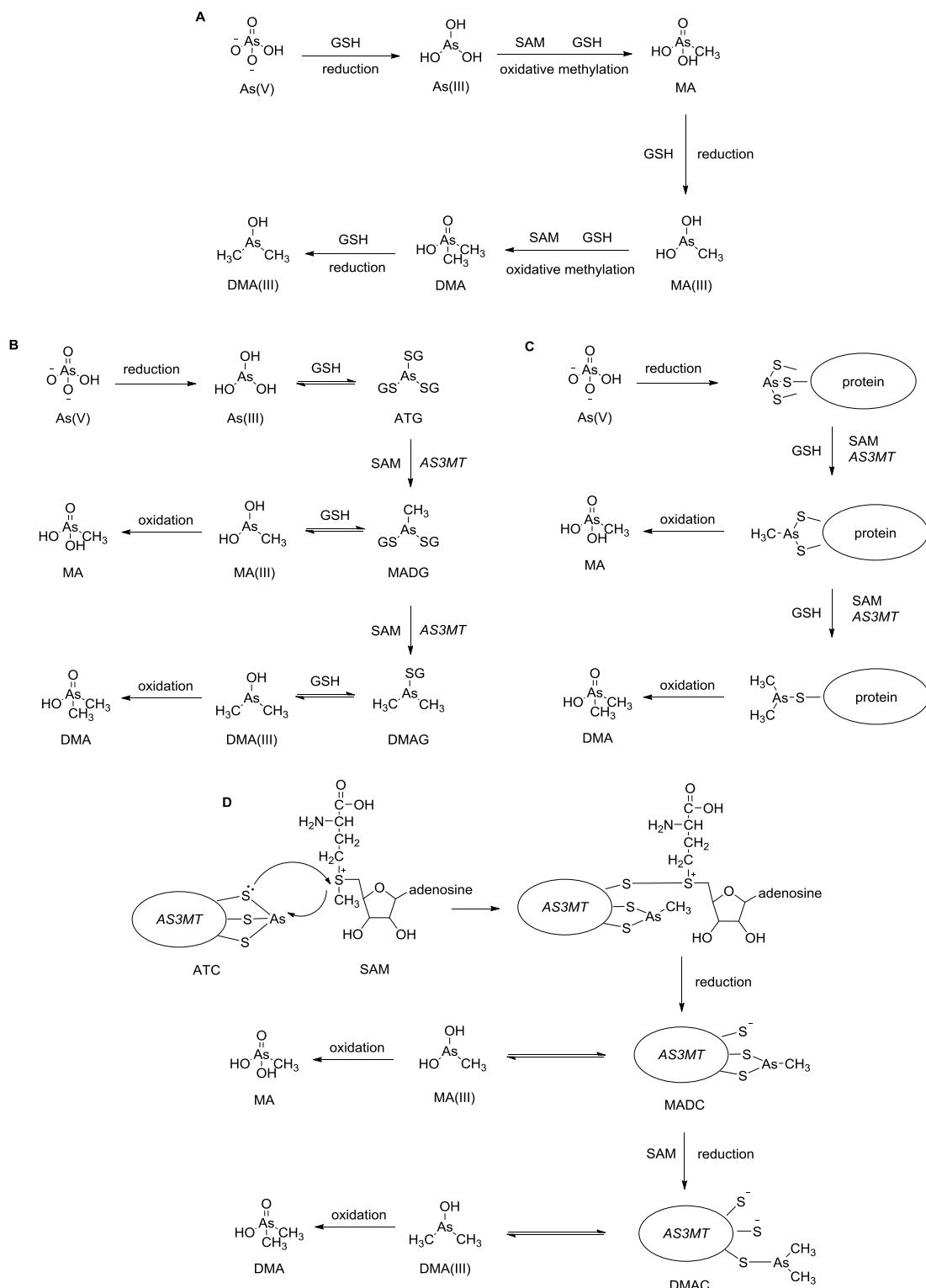


Figure 5-1. Proposed metabolic pathways by Challenger (A), Hayakawa (B), Naranmandura (C) and Wang (D) for inorganic arsenic in mammals, adapted from (Kobayashi, 2010, Rehman and Naranmandura, 2012, Wang et al., 2012). Abbreviations: AS3MT (enzyme arsenic III methyltransferase), ATC (arsenotricysteine), DMAc (dimethylarsocysteine), GSH (glutathione molecule), MADC (monomethylarsonic dicysteine), SAM (*S*-adenosyl-L-methionine), $-\text{SG}$ (glutathione moiety).

Materials and methods

Arsenic standards

Arsenous acid (As(III)), arsenate (As(V)), methylarsonic acid (MA) and dimethylarsinic acid (DMA) standard solutions were purchased from Sigma-Aldrich (Australia). Stock solutions for both As(III) and As(V) were prepared in deionised water (18.2 MΩ cm, Sartorius, Australia) with a final arsenic concentration of 15 mg L⁻¹ and stored at -4°C until used.

Cell culture reagents preparation

Dulbecco's Modified Eagle Medium (DMEM, Gibco, Invitrogen, Australia) was prepared by adding a 10 g DMEM sachet and 3.7 g of sodium bicarbonate (Ajax Finechem, Australia) to 1 L of deionised water previously sterilised by autoclaving (20 min at 121°C); and adjusted to pH 7.2 – 7.4 with hydrochloric acid (Merck, Australia) and sodium hydroxide (Merck, Australia). The final DMEM solution was filtered (500 mL bottle top filter, Sartolab, Sartorius, Australia) under sterile conditions in a laminar flow hood. Phosphate buffer saline 1 × (PBS) was prepared by diluting PBS 10 × (Gibco, Invitrogen, Australia) 1:10 v/v with deionised water. The final PBS 1 × solution was sterilised by autoclaving (20 min at 121°C). Trypsin-EDTA 1 × solution was prepared by diluting trypsin-EDTA 10 × (0.5 % w/v, Gibco, Life Technologies, Australia) to 1:10 v/v with PBS 1 × and adjusted to pH 7.2 – 7.8 with hydrochloric acid and sodium hydroxide.

Cell culture and incubation

A HepG2 cell line donated by Professor Phillip Board (John Curtin Medical School Research, ANU, Australia) and derived from the liver tissue of a fifteen year old male affected with differentiated hepatocellular carcinoma was used. Cells were cultured in 75 cm² vented flasks (Corning, Australia) with DMEM supplemented with 10 % v/v Fetal Bovine Serum (FBS, Gibco, Invitrogen, Australia) and incubated at 37°C with 5 % v/v CO₂ (Coregas, Australia). Cells were passaged every 3 – 4 days at 80 % confluence. After incubation, the spent culture media was discarded and the flask washed with PBS 1 × solution (2 mL per 25 cm² of culture surface area). The wash solution was removed and

pre-warmed (37°C) trypsin-EDTA $1 \times$ was added to the flask (1 mL per 25 cm^2 of culture surface area) and incubated for 5 min at 37°C with 5 % v/v CO_2 , in order to detach the cells from the flask walls. Deactivation of trypsin-EDTA was achieved by adding two volumes of culture media to the flask. A 1 mL aliquot of cell mix was transferred into a sterile centrifuge tube (Sarstedt, Australia) with 1 mL of 0.4 % v/v trypan blue (Sigma Aldrich, Australia) to estimate cell number and viability using an haemocytometer (Hauser scientific, Australia) under an inverted microscope (Nikon Eclipse TS100, Australia) at $10 \times$ magnification. The average number of cells out of three replicates per flask was multiplied by 10^4 to obtain the estimated amount of cells in 1 mL of cell mix. The percentage of viable cells was calculated using the equation below:

Equation 5-1. Calculation of percentage of viable cells.

$$\% \text{ viable cells} = (1 - \frac{\text{blue dyed cells}}{\text{total cells}}) \times 100$$

Cell seeding, harvesting and subcellular fractionation

Five replicates per arsenic species and five negative controls were prepared each week during three consecutive weeks to assess the reproducibility over generations. The experimental flasks (75 cm^2 vented flasks, Corning, Australia) were seeded with 10^6 cells in 20 mL of culture medium and incubated for 24 h at 37°C with 5 % v/v CO_2 to ensure cell attachment to the flask walls (Dopp et al., 2008). The experimental flasks were then dosed with either As(III) or As(V) to a final arsenic concentration of 0.5 μM ($40 \pm 5 \mu\text{g L}^{-1}$ and $37 \pm 6 \mu\text{g L}^{-1}$, respectively). These concentrations were under the toxicity levels reported for both As(III) and As(V) in human hepatocytes (Dopp et al., 2008). After 24 h incubation, the exhausted media was decanted into a sterile centrifuge tube (Sarstedt, Australia) and kept at -20°C for total arsenic and speciation analyses. Cells were washed with 6 mL of PBS $1 \times$ and then with 6 mL of a solution of PBS $1 \times$ with 0.005 % w/v dithiothreitol (DTT, Sigma-Aldrich, Australia) and 5 % v/v glycerol (Astral scientific, Australia). Both washing solutions were decanted into sterile centrifuge tubes and kept at -20°C for total arsenic and arsenic speciation analyses. Pre-warmed trypsin-EDTA $1 \times$ was added to the flasks (3 mL) and incubated for 5 min to detach the cells from the flask walls. Deactivation of trypsin-EDTA was achieved by adding 6 mL of pre-warmed culture media to the flask. The cell mix was transferred to a centrifuge tube (Sarstedt,

Australia) and centrifuged at $150 \times g$ for 10 min (Sigma 2-3E, Australia) after taking an aliquot to estimate the cell number and viability. The supernatant was poured into a sterile centrifuge tube and kept at -20°C for total arsenic and arsenic speciation analyses and the pellet resuspended in 1 mL of deionised water and kept at -80°C for subcellular fractionation (Figure 5-2).

Subcellular fractionation was performed according to published methods (Taylor and Maher, 2012). Cell pellets were frozen-thawed with ten cycles of alternating exposure to -20°C and 37°C and pestled (motor pellet pestle, Sigma-Aldrich, US) to favour the cell membrane lysis. Cell lysates were centrifuged at $1000 \times g$ for 10 min at 4°C (CT15RT versatile refrigerated centrifuge, Techcomp, Australia) (Figure 5-2). The pellet (P1), containing nuclei, granules and cell membranes, was resuspended in 0.5 mL of deionised water and placed in a heat block (Thermoline, Australia) at 100°C for 2 min; and then treated with 0.5 mL of 1N sodium hydroxide (PCCA, Australia) and heated at 60°C for 1 h in order to hydrolyse and precipitate the DNA and lipids (Figure 5-2). The solution was centrifuged at $10000 \times g$ for 10 min at 20°C (Himac CP90WX ultracentrifuge, Hitachi, Australia). The new pellet (P2), containing the granules, and the new supernatant (S2), containing the nuclei and cell membranes, were kept for further analysis (Figure 5-2). The supernatant from the first centrifugation (S1) was again centrifuged at $10000 \times g$ for 30 min at 4°C . The pellet (P3) contained the mitochondria and was kept for analysis and the supernatant (S3) centrifuged at $100000 \times g$ for 60 min at 4°C (Figure 5-2). The pellet (P4) containing the lysosomes, peroxisomes and microsomes; as well as the supernatant (S4) with the cytosol and soluble proteins were kept for further analysis (Figure 5-2). Cell fractions S2, P2, P3, S4 and P4 were resuspended in 200 μL of methanol (Scharlau, Australia): water 1:1 v/v for speciation analysis.

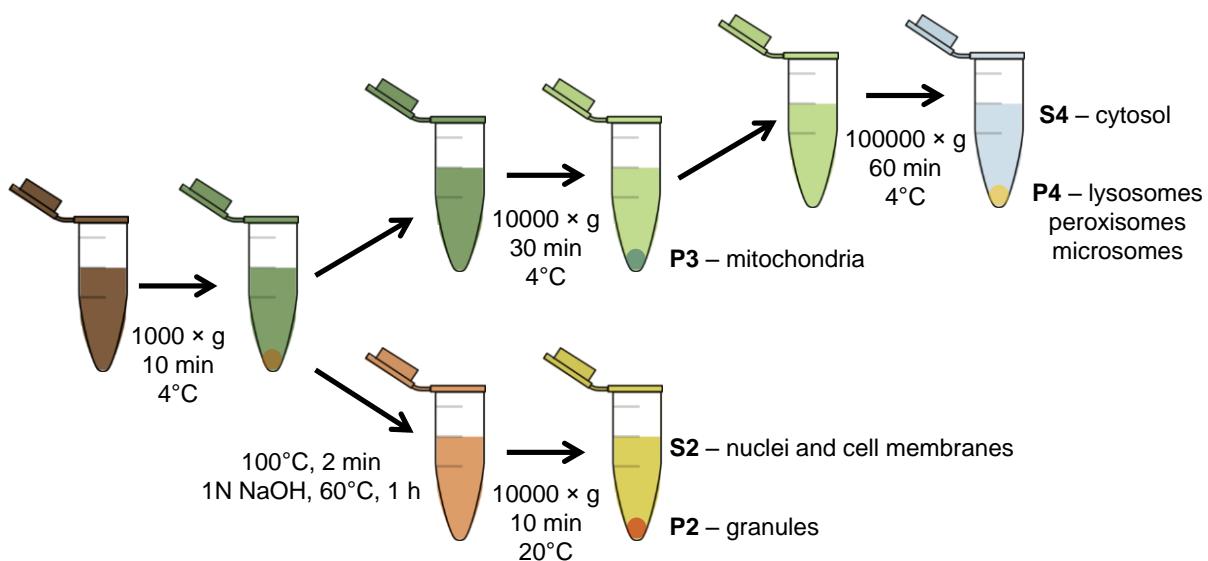


Figure 5-2. Schematic representation of the protocol for subcellular fractionation.

Total protein concentration

Since the mass and volume of cells were difficult to quantify, protein concentrations of subcellular fractions were measured in order to express arsenic concentrations in $\mu\text{g g}^{-1}$ of protein. A high protein amount microplate assay was performed to estimate the total protein concentration in all subcellular fractions using a commercial Bradford protein assay kit (Oz Biosciences, Australia). The cell fractions S2, P2, P3, S4 and P4 were brought up to 200 μL with deionised water and aliquots of 10 μL were mixed with 140 μL of Bradford reagent. Absorbance measurements were made at 595 nm with a BioRad benchmark Plus microplate spectrophotometer (USA) and protein concentrations were calculated using a calibration curve of bovine serum albumin in concentrations from 0 to 1500 mg L^{-1} .

Total arsenic analysis

Samples from exhausted media, PBS and PBS-DTT-glycerol washing solutions, and supernatant media after cell harvest were acidified with nitric acid (Aristar, BDH, US) to a final concentration of 10 % v/v nitric acid and analysed for total arsenic concentration. The richness of salts in the culture media caused interferences when analysing the samples by inductively coupled plasma-mass spectrometry. Thus, they were analysed as per Deaker and Maher (1999) by graphite furnace atomic absorption

spectroscopy using a Perkin Elmer Analyst 600 atomic absorption spectrometer (Australia) with an arsenic lamp ($\lambda = 193.7$ nm) at 38 A, and a THGA graphite tube (Perkin Elmer, Australia). Calibration standards were prepared from a multi element calibration standard (Accu Trace, Accu-Standards, 10 mg L⁻¹) at arsenic concentrations of 100, 50, 25, 12.5, 6.25 and 3.125 µg L⁻¹ in 10 % v/v nitric acid. Blanks of fresh media with FBS and of PBS, and PBS-DTT-glycerol washing solutions, as well as exhausted media control samples and accuracy checks for As(III) and As(V) were also analysed. A matrix modifier of palladium and magnesium nitrate was used. The recoveries of total arsenic for As(III) and As(V) were 106 ± 13 % and 99 ± 17 %, respectively; all within the accepted range for general recoveries according to the IUPAC.

Arsenic speciation

Analysis of samples from exhausted media as well as from subcellular fractionation was performed by high performance liquid chromatography-inductively coupled plasma-mass spectrometry (HPLC-ICP-MS) using a Flexar 10 HPLC coupled to a NexIon 300D ICP-MS (both Perkin Elmer, Waltham, MA, US). The plasma power was 1300 W and the nebuliser gas (argon) flow 0.9 L min⁻¹. Besides arsenic (*m/z* 75), vanadium (*m/z* 51) and chromium (*m/z* 52) were monitored to detect salt and carbon in samples. Chloride ions form adducts with argon (⁴⁰Ar³⁵Cl) that can interfere with the signal for ⁷⁵As. Chloride also forms an adduct with oxygen (³⁵Cl¹⁶O) with the same *m/z* as ⁵¹V. Vanadium was therefore monitored to discard any peak eluting as ⁷⁵As that may have come from the presence of chloride based salts in samples. The presence of carbon in samples was monitored by measuring chromium (⁵²Cr), as it has the same *m/z* as the adduct that carbon forms with argon (⁴⁰Ar¹²C). Calibration standards were prepared in methanol: water 1:1 v/v using DMA at arsenic concentrations of 100, 10, 1 and 0.1 µg L⁻¹. Calibration verification standards of As(III), As(V), MA and DMA were run to assess the calibration and the recoveries found to be in between 80 – 100 %. Blanks of fresh media with FBS and samples from control experimental flasks were also analysed. Separation of arsenic species was achieved using a Hamilton PRP-X100 anion exchange column (250 mm × 4.1 mm, 10 µm particle size, Phenomenex, US) and isocratic elution with a 7.5 mM malonic acid (UNILAB, Ajax Chemicals, Australia) mobile phase (pH 5.6 adjusted with aqueous ammonia (Sigma-Aldrich, Australia)) for 10 min. The

calibration with DMA was used to quantify the concentrations of all arsenic species in samples. Due to the small amount of sample available, total arsenic analysis of cell fractions could not be performed and total arsenic concentrations were calculated as the sum of the concentrations of each arsenic species, under the assumption that no arsenic was lost in the column. For the same reason, the masses of whole cells and the masses of sub-cellular fractions could not be measured. Arsenic concentrations are therefore reported normalised to protein concentration and expressed in $\mu\text{g g}^{-1}$ of protein.

Results

Protein concentration in subcellular fractions

The different fractions in which HepG2 cells were separated comprised the nuclei and cell membranes (S2), secretion granules (P2), mitochondria (P3), cytosol and heat stable soluble proteins (S4) and lysosomes, peroxisomes and microsomes (P4). From those, S4 and S2 exhibited the higher protein concentration ($2.1 \pm 0.6 \text{ mg L}^{-1}$ and $0.6 \pm 0.2 \text{ mg L}^{-1}$, respectively), followed by P4 ($0.11 \pm 0.05 \text{ mg L}^{-1}$), P2 ($0.08 \pm 0.04 \text{ mg L}^{-1}$) and P3 ($0.02 \pm 0.01 \text{ mg L}^{-1}$).

Cellular growth and viability

Figure 5-3 shows the cell growth and viabilities of negative control HepG2 cells, as well as after exposure to As(III) and As(V). Whereas cell growth was slightly higher for control cells (18.05×10^6 cells against 14.99×10^6 cells and 13.43×10^6 cells for As(III) and As(V) exposed cells, respectively), cell viabilities after all treatments did not experiment major changes and were all above 80 % (Figure 5-3).

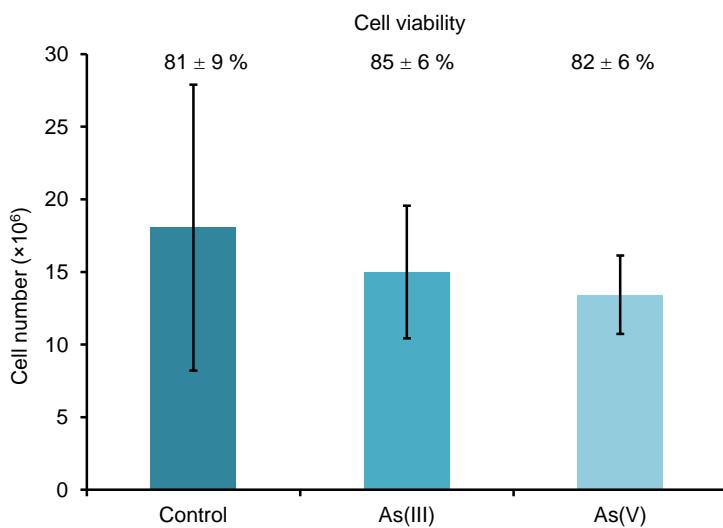


Figure 5-3. Cell growth and viabilities of control and As(III) and As(V) exposed HepG2 cells.

As(III) exposed HepG2 cells

The total arsenic concentration in the exhausted media and wash solutions of HepG2 cell cultures after exposure to As(III) was $36 \pm 7 \mu\text{g L}^{-1}$, of which, $85 \pm 1\%$ was As(III), $8 \pm 1\%$ As(V), $4 \pm 1\%$ MA and $5 \pm 2\%$ DMA. From the initial As(III), 24 % was taken up by HepG2 cells. The total arsenic concentration in cells after exposure was $34 \pm 22 \mu\text{g g}^{-1}$ protein, where $50 \pm 1\%$ was DMA, $25 \pm 13\%$ MA, $14 \pm 1\%$ As(V) and $3 \pm 1\%$ As(III). Additionally, three unknown arsenic species eluted at 4.4 min (UK 01), 3.4 min (UK 02) and 5.7 min (UK 03). Under the chromatographic conditions used, MA eluted at 4 min and As(V) at 5 min, therefore UK 01 did not correspond to either of them. The low concentrations of these unknown arsenic species and the lack of enough sample volume for subsequent HPLC injections did not allow a proper identification. The species UK 02 and UK 03 were not present in samples in a reproducible manner and therefore they were not considered to be representative. The species UK 01, however, existed in all samples although in variable concentrations ($6 \pm 8\%$ from total arsenic in cells).

Arsenic was distributed among all five subcellular fractions with the highest concentrations in granules, lysosomes, peroxisomes and microsomes (Table 5-1). Dimethylarsinic acid was predominant in all subcellular fractions (Table 5-1 and Figure 5-4). Methylarsonic acid was abundant in both the granules and the fraction comprising

lysosomes, peroxisomes and microsomes; and it was also found at lower concentrations in the cytosol and mitochondria (Table 5-1 and Figure 5-4). Arsenous acid and As(V) were distributed in lysosomes, peroxisomes, microsomes and mitochondria. Additionally, As(III) was also detected in granules; and As(V) in the cytosol (Table 5-1 and Figure 5-4). The arsenic species UK 01 was only observed in the fraction containing the nuclei and cell membranes (Table 5-1 and Figure 5-4).

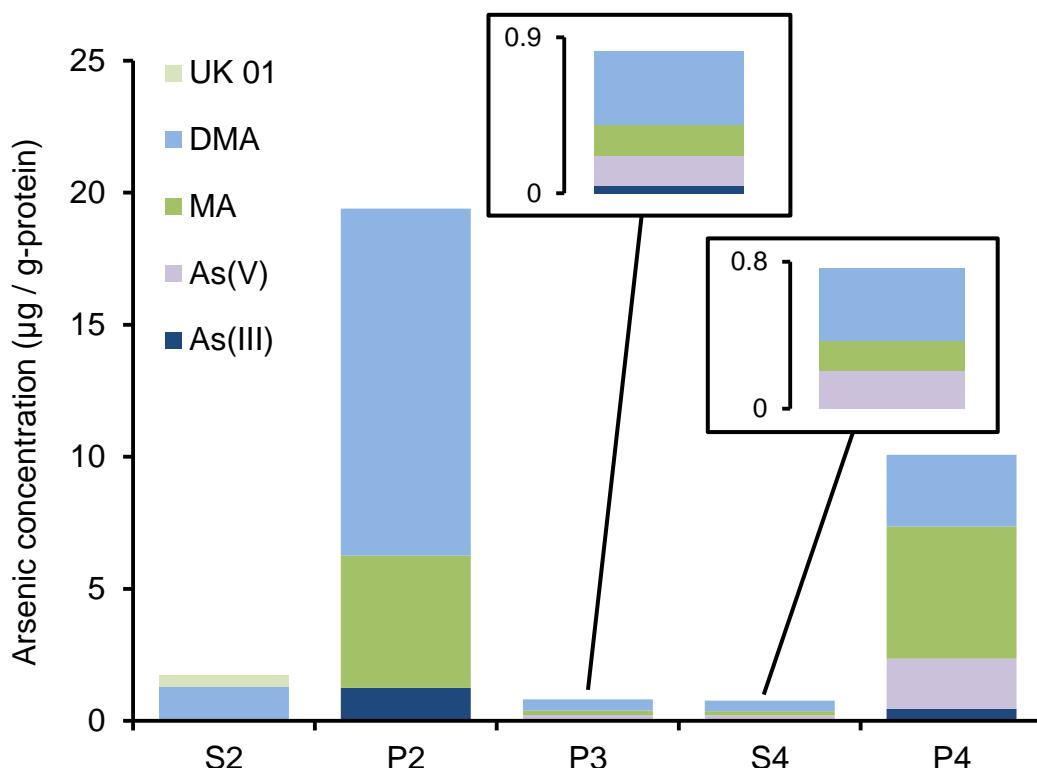


Figure 5-4. Arsenic concentrations and species distribution in subcellular fractions of HepG2 cells after 24 h exposure to As(III). S2, nuclei and cell membranes; P2, granules; P3, mitochondria; S4, cytosol; P4, lysosomes, peroxisomes and microsomes.

Table 5-1. Arsenic concentrations and percentages of arsenic species in the subcellular fractions of HepG2 cells after 24 h exposure to As(III).

Subcellular fraction	Total As concentration ($\mu\text{g/g-protein}$)	% (relative to total arsenic in fraction)			
		As(III)	As(V)	MA	DMA
nuclei and cell membranes	3 ± 2				41 ± 17
granules	20 ± 13	6 ± 2		26 ± 8	65 ± 5
mitochondria	0.8 ± 0.7	6 ± 1	21 ± 1	22 ± 2	51 ± 1
cytosol	0.8 ± 0.2		26 ± 4	22 ± 1	50 ± 5
lysosomes, peroxisomes and microsomes	10 ± 7	8 ± 2	18 ± 4	36 ± 29	41 ± 27

As(V) exposed HepG2 cells

The total arsenic concentration in the exhausted media and wash solutions of HepG2 cell cultures after exposure to As(V) was $35 \pm 6 \text{ } \mu\text{g L}^{-1}$, of which $96 \pm 2 \text{ \%}$ was As(V), $3 \pm 1 \text{ \%}$ As(III), $1.1 \pm 0.6 \text{ \%}$ MA and $1.1 \pm 0.8 \text{ \%}$ DMA. This indicates that 9 % of As(V) was taken up by HepG2 cells. The total arsenic concentration measured in cells after exposure was $45 \pm 27 \text{ } \mu\text{g g}^{-1}$ protein, of which $51 \pm 3 \text{ \%}$ was DMA, $22 \pm 4 \text{ \%}$ MA, $13 \pm 6 \text{ \%}$ As(V), $4 \pm 1 \text{ \%}$ As(III), $8 \pm 8 \text{ \%}$ UK 01 and $3 \pm 3 \text{ \%}$ corresponded to an unknown arsenic species eluting at 6.6 min (UK 04). Again, these unknown arsenic species could not be identified due to the small sample volume and low arsenic concentrations. The presence of UK 01 and UK 04 was consistent in their distribution although their concentrations were very variable. The species UK 02 was also observed, but only in few samples and therefore was not considered to be representative.

The subcellular distribution of arsenic showed higher concentrations in granules and mitochondria than in the remaining organelles (Table 5-2). Both DMA and MA were the only arsenic species detected in all five subcellular fractions, with DMA being the most abundant followed by MA (Table 5-2 and Figure 5-5). Besides DMA and MA, both UK 01 and UK 04 were present in nuclei and cell membranes, where neither As(III) nor As(V) were found (Table 5-2 and Figure 5-5). Most As(V) was detected in mitochondria, with smaller concentrations in the cytosol, lysosomes, peroxisomes and microsomes (Table 5-2 and Figure 5-5). Arsenous acid was found in granules, mitochondria, lysosomes, peroxisomes and microsomes (Table 5-2 and Figure 5-5).

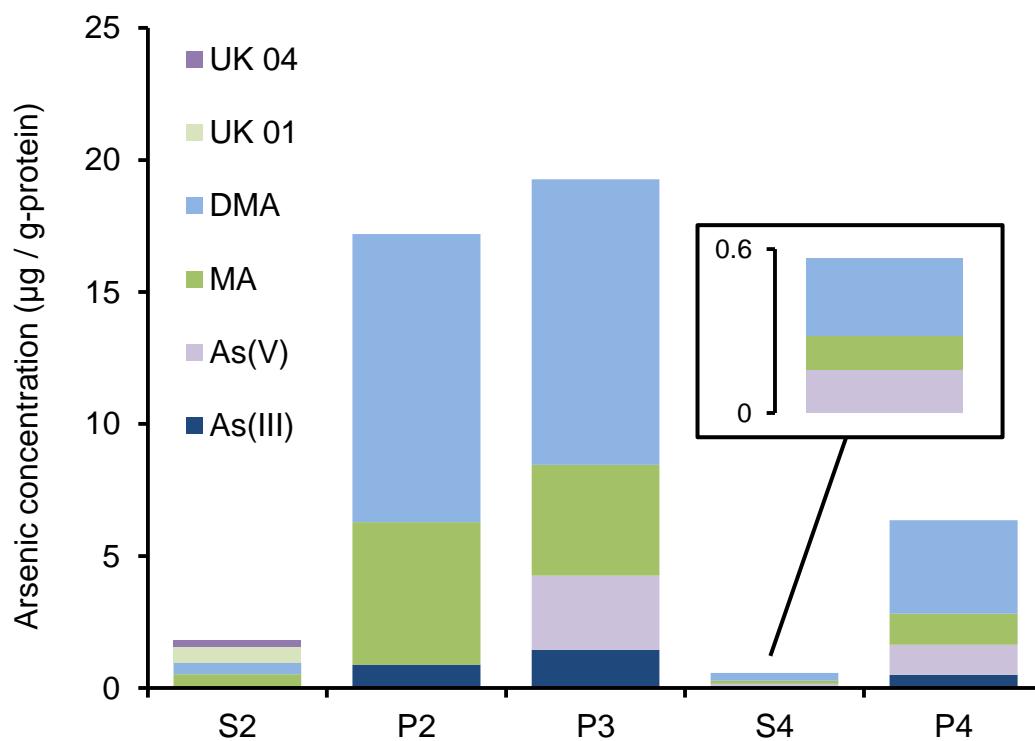


Figure 5-5. Arsenic concentrations and species distribution in subcellular fractions of HepG2 cells after 24 h exposure to As(V). S2, nuclei and cell membranes; P2, granules; P3, mitochondria; S4, cytosol; P4, lysosomes, peroxisomes and microsomes.

Table 5-2. Arsenic concentrations and percentages of arsenic species in the subcellular fractions of HepG2 cells after 24 h exposure to As(V).

Subcellular fraction	Total As concentration ($\mu\text{g/g-protein}$)	% (relative to total arsenic in fraction)					
		As(III)	As(V)	MA	DMA	UK 01	UK 04
nuclei and cell membranes	1.4 ± 0.8			30 ± 24	33 ± 20	38 ± 39	15 ± 14
granules	19 ± 11	5 ± 1		29 ± 11	59 ± 5		
mitochondria	18 ± 11	8 ± 4	18 ± 1	22 ± 5	60 ± 2		
cytosol	0.6 ± 0.3		29 ± 4	21 ± 2	49 ± 6		
lysosomes, peroxisomes and microsomes	6 ± 5	8 ± 1	18 ± 4	18 ± 2	56 ± 4		

Discussion

Cellular growth and viability

This study focuses on better understanding the metabolism of inorganic arsenic species in human liver cells and therefore, the concentrations of exposure needed to stay under the toxicity levels of As(III) and As(V) in hepatocytes (Dopp et al., 2008, Meyer et al., 2015a, Meyer et al., 2014a). The exposure concentrations were $40 \pm 5 \mu\text{g L}^{-1}$ in As(III) and $37 \pm 6 \mu\text{g L}^{-1}$ in As(V). After 24 h, neither As(III) nor As(V) exhibited significant effects in HepG2 cell growth and viability (Figure 5-3), confirming the suitability of these concentrations for the purpose of this work. Additionally, cell viabilities remained higher than 80 %, in agreement with previous studies that used similar As(III) and As(V) concentrations of exposure in human hepatocytes (Dopp et al., 2008).

Arsenic uptake

Both As(III) and As(V) are taken up by HepG2 cells after 24 h exposure, with As(III) uptake being higher than that for As(V). Arsenous acid is known to enter human liver cells by facilitated passive diffusion using the aquaglyceroporins *AQP9* (Liu et al., 2002), as well as to traverse lipid membranes by simple passive diffusion (Chávez-Capilla et al., 2016b). Both mechanisms occur down the concentration gradient and do not consume energy. Arsenate uses the phosphate-sodium pump Type II transporter *NaPiIIb* present in liver cells, an active transport mechanism that requires energy consumption (Villa-Bellosta and Sorribas, 2010). The uptake of As(III), thus, is more favourable than that of As(V), especially in freshly exposed cells, as shown by the uptake percentages of 24 % for As(III) and 9 % for As(V). These values are calculated on the assumption that all As(III) and As(V) detected in the exhausted media and washing solutions of the corresponding experimental flasks came from the initial arsenic of exposure. Inorganic arsenic species can be excreted unchanged (Crecelius, 1977, Vahter and Concha, 2001, Del Razo et al., 2001, Loffredo et al., 2003), as arsenic methylation efficiency depends on the polymorphism of the gene encoding for *AS3MT*, which varies among individuals and populations (Thomas et al., 2007). Even so, levels of excreted inorganic arsenic species

by humans are usually much lower than those measured in the exhausted media and washing solutions in the current experiments.

Arsenic distribution and metabolism in As(III) exposed HepG2 cells

The most abundant arsenic species detected in HepG2 cells after 24 h exposure to As(III) were DMA ($50 \pm 1\%$) and MA ($25 \pm 13\%$). This is in agreement with the final products of arsenic metabolism reported in human urine (Concha et al., 2002, Shraim et al., 2003) that support a reductive methylation pathway (Hayakawa et al., 2004, Rehman and Naranmandura, 2012, Wang et al., 2012) (Figure 5-1b, 5-1c and 5-1d).

Dimethylarsinic acid and MA were particularly significant in granules, lysosomes, peroxisomes and microsomes, which are also the cellular compartments with higher concentration of total arsenic (Table 5-1 and Figure 5-4). Granules are enclosed vesicles often generated to limit the bioavailability of toxic species in cells (Lodish et al., 2000a). The DMA and MA produced after cellular metabolism is probably encapsulated in these vesicles to be isolated and excreted. Dimethylarsinic acid can be eliminated from human hepatocytes by the protein group *MRP4/ABCC4*, an energy dependant transporter (Roggenbeck et al., 2015, Banerjee et al., 2014); and is able to diffuse through lipid membranes (Chávez-Capilla et al., 2016b). Methylarsonic acid is thought to leave cells as MADG, as no other extrusion mechanisms have been identified (Carew et al., 2011). Accumulation of DMA and MA in granules could imply an additional detoxification pathway for both arsenic species. Arsenous acid was also present in granules, probably from the passive diffusion of As(III) through the lipid membrane of these vesicles.

After the granules, the next cellular fraction with the highest arsenic concentration comprised lysosomes, peroxisomes and microsomes, and contained DMA, MA, As(V) and As(III) (Table 5-1 and Figure 5-4). These organelles are the main sites for xenobiotic detoxification and are abundant in liver and kidney (Lodish et al., 2000a). Lysosomes degrade cellular debris and unwanted metabolic products and are rich in acid hydrolases that break down polymers into monomers (Lodish et al., 2000a). Peroxisomes contain oxidases and catalases that transform toxic compounds into harmless products (Lodish et al., 2000a); and microsomes are small vesicles in the endoplasmic reticulum, where the enzymes involved in oxidative metabolism are located (Lodish et al., 2000a, Casarett and

Doull, 2008). Most As(V) was found in this fraction (Table 5-1 and Figure 5-4), suggesting that oxidation of As(III) to As(V) primarily occurs in peroxisomes and microsomes. The enzymes controlling glutathione conjugation and methylation of xenobiotics exist both in the microsomes and the cytosol (Casarett and Doull, 2008). The greater concentrations of DMA and MA in fraction P4 (lysosomes, peroxisomes and microsomes) than in fraction S4 (cytosol) (Figure 5-4), indicate that microsomes may be the first site for As(III) metabolism.

Arsenic in the cytosol was found as DMA, MA and As(V) (Table 5-1 and Figure 5-4). The cytosol in human liver and kidney cells is rich in soluble and heat stable proteins known as metallothioneins (Dziegiej et al., 2016). These proteins have also been reported in mitochondria and lysosomes, although they are mostly found in the Golgi apparatus and the cytoplasm (Banerjee et al., 1982, Baird et al., 2006). Metallothioneins can confer cellular resistance to arsenic, which becomes unavailable after binding to the cysteine residues in these proteins (Shen et al., 2013). Arsenic has been reported to bind to metallothioneins as As(III), MA(III) and DMA(III) (Toyama et al., 2002, Ngu and Stillman, 2006, Ngu et al., 2008, Jiang et al., 2003), forming very stable complexes that release As(V), MA and DMA under strong basic or oxidising conditions (Shen et al., 2013). Although these pentavalent arsenic species were identified in the cytosolic fraction of HepG2 cells (Table 5-1 and Figure 5-4); it is unlikely that they originated from the hydrolysis of arsenic-containing metallothioneins under the experimental and analytical conditions used.

Arsenous acid, As(V), MA and DMA were detected in mitochondria in very low concentrations, suggesting that some metabolism may also occur in this organelle. In nuclei and cell membranes, only DMA and UK 01 were observed (Table 5-1 and Figure 5-4). The large variability associated to the quantification of UK 01 indicates that this arsenic species might be highly unstable and readily degraded to DMA, the only other arsenic species in this fraction. Metallothioneins exist in the nuclei of hepatocytes (Cherian and Apostolova, 2000, Werynska et al., 2013), suggesting that UK 01 possibly is a DMA(III)-containing metallothionein or DMAG, which gets hydrolysed to DMA after the treatment with sodium hydroxide during subcellular fractionation (Figure 5-2). Conversely, DMA and UK 01 in this fraction could also be associated to the lipoproteins

in the membrane. Figure 5-6 summarises the biotransformations occurring in HepG2 cells after exposure to As(III).

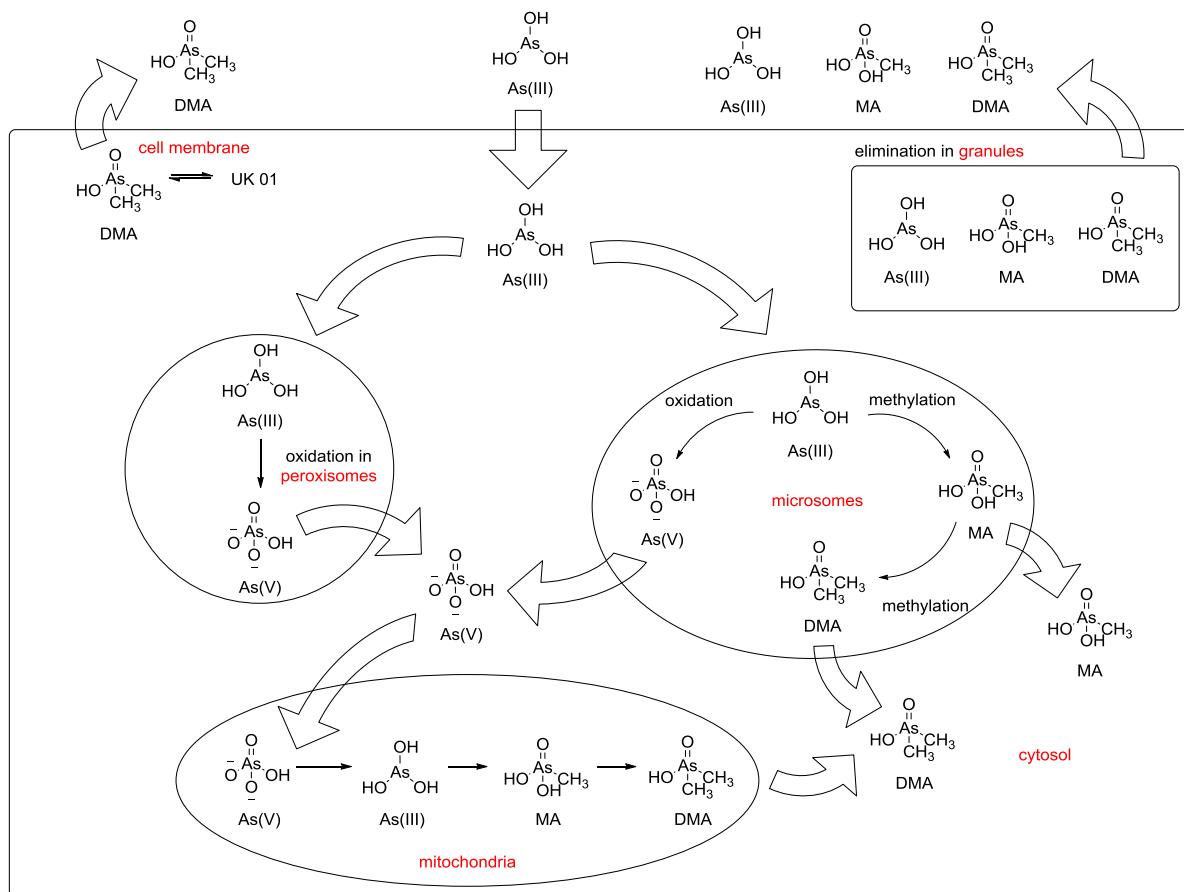


Figure 5-6. Proposed sites for As(III) biotransformations in HepG2 cells.

Arsenic distribution and metabolism in As(V) exposed HepG2 cells

As for As(III), the most abundant arsenic species detected in HepG2 cells after 24 h exposure to As(V) were DMA ($51 \pm 3\%$) and MA ($22 \pm 4\%$). These species were predominant in granules and mitochondria, the subcellular fractions with the highest total arsenic concentrations (Table 5-2). The accumulation of DMA and MA in granules probably aims again the isolation and elimination of these metabolic products; and the traces of As(III) could come from passive diffusion across the granules lipid membranes.

Mitochondria in As(V) exposed HepG2 cells showed a significantly greater arsenic concentration than in As(III) exposed cells (Table 5-1 and Table 5-2), which is in agreement with previous studies (Dopp et al., 2008). The arsenic species identified in this fraction were DMA, MA, As(V) and As(III) (Table 5-2 and Figure 5-5). Mitochondria

control the production of energy from cellular respiration and are widely present in hepatocytes (Lodish et al., 2000a). These organelles are delimited from the cytosol by two membranes separated by an intermembrane space. It is in the inner membrane where most mitochondrial transport systems occur, including the protein phosphate translocase that allows phosphate ($H_2PO_4^-$) into the mitochondrial matrix (Mathews et al., 2013). Arsenate has a similar chemical structure to phosphate and can cross cell membranes using phosphate carriers (Villa-Bellosta and Sorribas, 2010), as well as enter the mitochondria through phosphate translocases (Chan et al., 1969, Nemeti and Gregus, 2002), which may explain the high arsenic concentrations in this fraction. The presence of As(III) in mitochondria confirms that As(V) is reduced before methylation. Previous studies on rat liver showed similar results, although the enzymes responsible for As(V) reduction in mitochondria were not isolated (Nemeti and Gregus, 2002). Arsenate reduction can be catalysed by glutathione-S-transferase ω class 1-1 (*GSTO1-1*), glutathione-S-transferase μ -M1 (*GSTM1*) or purine nucleoside phosphorylase (*PNP*) (Zakharyan et al., 2001, Radabaugh et al., 2002, Chiou et al., 1997), which are predominantly located in the cytosol and microsomes (Casarett and Doull, 2008, Gregus and Nemeti, 2002). The current study shows As(III) in the fraction comprising lysosomes, peroxisomes and microsomes but not in the cytosol (Table 5-2 and Figure 5-5). Hence, the enzymatic reduction of As(V) probably occurs primarily in microsomes.

In mitochondria, lysosomes, peroxisomes and microsomes, inorganic arsenic species coexisted with DMA and MA. Again, methylation is likely to occur in microsomes, due to the presence of the enzymes that facilitate methylation and glutathione conjugation of xenobiotics (Casarett and Doull, 2008). In mitochondria these enzymes exist in a considerably lesser extent (Casarett and Doull, 2008). Since the concentrations of DMA and MA are greater in mitochondria than in fraction P4 (lysosomes, peroxisomes and microsomes), methylation in mitochondria might occur differently.

In nuclei and cell membranes arsenic was found as DMA ($33 \pm 20\%$), MA ($30 \pm 24\%$), UK 01 ($38 \pm 39\%$) and UK 04 ($15 \pm 14\%$) (Table 5-2). The high variability in the concentration of these arsenic species suggests that UK 01 and UK 04 are unstable. Given that the same fraction in As(III) cells showed DMA and UK 01, but not MA or

UK 04; it is possible that DMA comes from UK 01 degradation and MA from UK 04. Here UK 04 probably is either a MA(III)-containing metallothionein or MADG, hydrolysed to MA after the treatment with sodium hydroxide during subcellular fractionation (Figure 5-2). Figure 5-7 summarises the main locations of arsenic biotransformations in HepG2 cells after exposure to As(V).

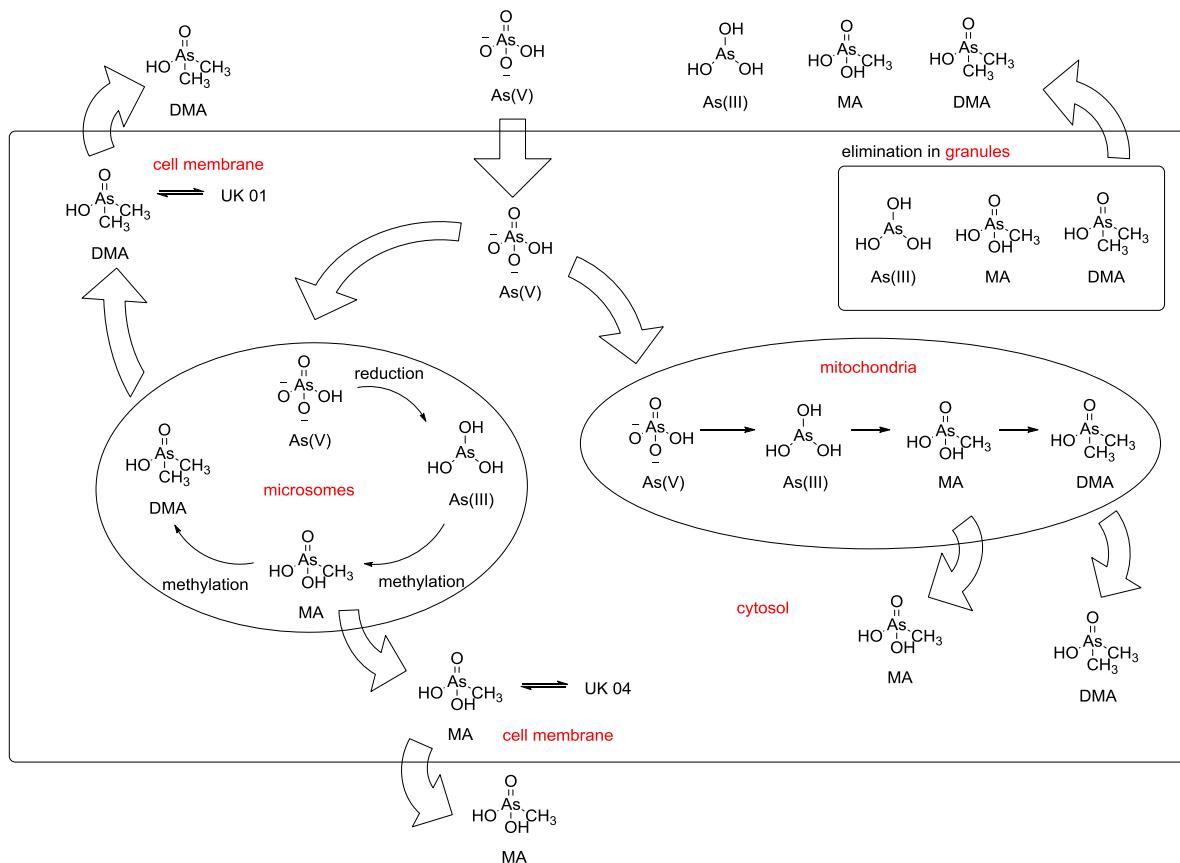


Figure 5-7. Proposed sites for As(V) biotransformations in HepG2 cells.

Concluding remarks

The results in this work support reductive methylation as a detoxification pathway for inorganic arsenic in cells, as MA and DMA were identified as the final metabolic products for both As(III) and As(V). Microsomes constitute the main location for arsenic methylation and mitochondria seems to be important in As(V) metabolism; thus, the consequences of arsenic accumulation in both organelles needs further investigation. The identity of UK 01 and UK 04 could not be elucidated due to the low concentrations of these arsenic species in samples. As they coexisted with DMA and MA, it is not discarded that they might be a MA(III)-containing metallothionein, a DMA(III)-

containing metallothionein, MADG or DMAG. Additional experiments and analyses need to be done to confirm their identity. The full interpretation of the results in this study is therefore subjected to the identification of the unknown arsenic species found.

CHAPTER 6 – Biotransformations of dimethylarsinoyl decane and dimethylarsinoyl undecanoic acid in HepG2 cells

Introduction

Arsenolipids are organic arsenic species with chemical structures similar to those of biologically important lipids (Sele et al., 2012). There are seven types of arsenolipids known to date: arsено-fatty acids (AsFAs) (Rumpler et al., 2008), arsено-hydrocarbons (AsHCs) (Taleshi et al., 2008), arsено-phospholipids (AsPLs) (Francesconi et al., 1990), glycoarsenolipids (GlyAsLs) (Garcia-Salgado et al., 2012), arsenic-containing phosphatidylcholines (AsPCs), arsenic-containing phosphatidylethanolamines (AsPEs) (Viczek et al., 2016) and cationic trimethylarsonio fatty alcohols (TMAsFOHs) (Amayo et al., 2013). Arsenolipids are commonly found in seaweed (Raab et al., 2013, Garcia-Salgado et al., 2012), fish (Lischka et al., 2013, Taleshi et al., 2010, Rumpler et al., 2008, Amayo et al., 2011, Ebisuda et al., 2003), shellfish and crustaceans (Edmonds et al., 1992); and are thought to originate from the detoxification of inorganic arsenic species, or by the accidental introduction of methylated arsenic species in lipid biosynthetic routes (Francesconi and Schwerdtle, 2016).

Humans are exposed to these arsenic species through the diet and are therefore subjected to their potentially adverse health effects (Francesconi, 2010). To date, only the toxicity of AsHCs and AsFAs has been tested in the fruit fly *Drosophila melanogaster* (Meyer et al., 2014b) and in human liver, urothelial and brain tissue cultures (Witt et al., 2017, Meyer et al., 2015b, Meyer et al., 2015a, Meyer et al., 2014a). Both AsHCs and AsFAs exert some degree of toxicity (Meyer et al., 2015a, Meyer et al., 2014a), with AsHCs being as toxic as arsenous acid (As(III)), a known carcinogen to humans (Meyer et al., 2014b, International Agency for Research on Cancer, 2012, Witt et al., 2017).

After ingestion, arsenolipids have to enter the gastrointestinal tract and cross the intestinal barrier before they can be absorbed into the liver (Goodman and Gilman, 2006, Dworken et al., 2013). If liver cells metabolise these lipid soluble arsenic species into water soluble species, they will be excreted in urine; otherwise arsenolipids will be redirected to the small intestine through the bile for further metabolism (Dworken et al., 2013, Ioannides, 2002, Casarett and Doull, 2008).

Both AsHCs and AsFAs have been reported to survive the physicochemical conditions of the human gastrointestinal tract (*Chapter 3* p.55) and to cross the intestinal barrier (Meyer et al., 2015b). Metabolism of arsenolipids in human liver, however, has not yet been studied. In Caco-2 cells, presystemic metabolism has shown that AsHCs are slightly degraded to their trivalent and thiolated counterparts, whereas AsFAs are extensively metabolised to their trivalent counterparts, dimethylarsinic acid (DMA) and other unidentified water soluble arsenic species (Meyer et al., 2015b). Still, more than 50 % of AsHCs and up to 13 % of AsFAs were found to remain unchanged after intestinal absorption (Meyer et al., 2015b), meaning that these arsenic species are readily available for further detoxification in the liver. The current hypotheses on arsenolipid biotransformations in liver cells are based on the metabolites excreted in urine after human consumption of cod liver, which is rich in AsFAs (Rumpler et al., 2008). These are DMA, dimethylarsinoyl propionic acid (DMAP), dimethylarsinoyl butyric acid (DMAB), thio-dimethylarsinoyl propionic acid (thio-DMAP) and thio-dimethylarsinoyl butyric acid (thio-DMAB) (Schmeisser et al., 2006b, Schmeisser et al., 2006a). It has been proposed that the ingested arsenolipids are first metabolised to the short chain fatty acids DMAP and DMAB, thiolated to thio-DMAP and thio-DMAB, and finally degraded to the major urinary metabolite: DMA (Schmeisser et al., 2006b, Schmeisser et al., 2006a). Whereas thio-AsHCs are formed in Caco-2 cells, thio-AsFAs are not (Meyer et al., 2015b), suggesting that the presence of short chain thio-AsFAs in human urine might be due to either gastrointestinal microbiota or liver metabolism. The lack of studies reporting the urinary metabolites of AsHCs and the arsenic species produced from the biotransformations of AsFAs or AsHCs in liver cells; limits the understanding of the metabolism of these arsenolipids in humans. Investigating these processes can provide valuable information about how these arsenic species are degraded, as well as about possible intermediates that might exert different toxicities than the initial species of exposure.

This study uses human hepatocarcinoma cells (HepG2) to investigate the metabolism of an AsFA and an AsHC and after 24 h exposure (Figure 6-1). HepG2 cells were chosen as they constitute an immortal cell line that grows relatively fast and is able to metabolise other arsenic species as effectively as healthy hepatocytes (Chen et al., 2003). After exposure, the concentration and distribution of the arsenic species produced

were analysed by HPLC-ICP-MS and LC-QQQ to elucidate possible metabolic pathways for AsFAs and AsHCs in humans.

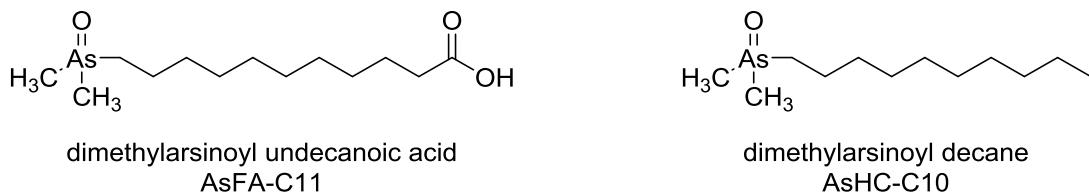


Figure 6-1. Chemical structures of dimethylarsinoyl undecanoic acid and dimethylarsinoyl decane.

Materials and methods

Arsenic standards

Dimethylarsinoyl decane (AsHC-C10) and dimethylarsinoyl undecanoic acid (AsFA-C11) were synthesised following published methods (Taleshi et al., 2014) as explained in *Chapter 3* (p.58). Stock solutions of both arsenolipids were prepared in ethanol (Chem Supply, Australia) at a final arsenic concentration of 30 mg L⁻¹ and kept at -4°C until used. Arsenous acid (As(III)), arsenate (As(V)), methylarsonic acid (MA) and dimethylarsinic acid (DMA) standard solutions were purchased from Sigma-Aldrich (Australia).

Cell culture reagents preparation

Cell culture media and reagents were prepared as explained in *Chapter 5* (p.106).

Cell culture and incubation

Cell culture and incubation was performed following the same procedure as in *Chapter 5* (p.106).

Cell seeding, harvesting and subcellular fractionation

Five replicates per arsenic species and five negative controls were prepared each week during three consecutive weeks to assess the reproducibility over generations. Experimental flasks (75 cm² vented flasks, Corning, Australia) were seeded with 10⁶ cells in 20 mL of culture medium and incubated for 24 h at 37°C with 5 % v/v CO₂ to ensure cell attachment to the flask walls (Dopp et al., 2008). The experimental flasks were then

dosed with either AsFA-C11 or AsHC-C10 to a final arsenic concentration of $50 \pm 2 \mu\text{g L}^{-1}$ ($0.66 \mu\text{M}$) and $48 \pm 3 \mu\text{g L}^{-1}$ ($0.64 \mu\text{M}$), respectively. Both arsenolipid standards contained impurities of DMA, thus the actual concentrations of exposure of the species AsFA-C11 and AsHC-C10 were $29 \pm 4 \mu\text{g L}^{-1}$ and $45 \pm 3 \mu\text{g L}^{-1}$. The final ethanol concentration in the culture media was under 1 % v/v. The concentrations of exposure chosen were below the toxicity levels previously reported for AsHCs and AsFAs in HepG2 cells (Meyer et al., 2015a, Meyer et al., 2014a). After 24 h incubation, cell harvesting and subcellular fractionation were performed as explained in full in *Chapter 5* (p.107). The extraction of arsenolipids from the exhausted media and washing solutions was done by adding 2 mL of chloroform (Sigma-Aldrich, Australia) to a 2 mL aliquot of sample and mixing end-over-end for 15 min. Centrifugation at $4500 \times g$ for 10 min (Centrifuge 5804 R, Eppendorf, Australia) was used to separate different phases. The chloroform phase was transferred into a 2 mL Eppendorf tube and evaporated. The pellet was resuspended in 1.5 mL of methanol (Scharlau, Australia) with 1 % v/v formic acid (APS Specialty Chemicals, Australia) and kept at -4°C until analysed. Cell pellets to be analysed by reverse-phase chromatography were resuspended in 100 μL of deionised water ($18.2 \text{ M}\Omega \text{ cm}$, Sartorius, Australia) and extracted with 1 mL of chloroform: methanol 1:2 v/v. Samples were centrifuged at $2880 \times g$ for 10 min to separate both phases. The solvent in the chloroform phase was evaporated and the pellet resuspended in 200 μL of methanol with 1 % v/v formic acid and kept at -4°C until analysed.

Total protein concentration

Total protein concentrations in subcellular fractions were measured as explained in *Chapter 5* (p.109).

Total arsenic analysis

Samples from exhausted media, PBS and PBS-DTT-Glycerol washing solutions, and supernatant media after cell harvest were acidified with nitric acid (Aristar, BDH, US) to a final concentration of 10 % v/v nitric acid and analysed for total arsenic concentration. The richness of salts in the culture media caused interferences when analysing the samples by inductively coupled plasma-mass spectrometry. Thus, they were analysed as per Deaker and Maher (1999) by graphite furnace atomic absorption

spectroscopy using a Perkin Elmer Analyst 600 atomic absorption spectrometer (Australia) with an arsenic lamp ($\lambda = 193.7$ nm) at 38 A, and a THGA graphite tube (Perkin Elmer, Australia). Calibration standards were prepared from a multi element calibration standard (Accu Trace, Accu-Standards, 10 mg L⁻¹) at arsenic concentrations of 100, 50, 25, 12.5, 6.25 and 3.125 µg L⁻¹ in 10 % v/v nitric acid. Blanks of fresh media with FBS and of PBS, and PBS-DTT-glycerol washing solutions, as well as exhausted media control samples and accuracy checks for AsFA-C11 and AsHC-C10 were also analysed. A matrix modifier of palladium and magnesium nitrate was used. The recoveries of total arsenic for AsFA-C11 and AsHC-C10 were 66 ± 17 % and 64 ± 17 %, respectively.

Arsenic speciation

Analysis of samples from exhausted media as well as from subcellular fractionation was performed by high performance liquid chromatography-inductively coupled plasma-mass spectrometry (HPLC-ICP-MS) using a Flexar 10 HPLC coupled to a NexIon 300D ICP-MS (both Perkin Elmer, Waltham, MA, US). The plasma power was 1300 W and the nebuliser gas (argon) flow 0.9 L min⁻¹. Besides arsenic (*m/z* 75), vanadium (*m/z* 51) and chromium (*m/z* 52) were monitored to detect salt and carbon in samples. Chloride ions form adducts with argon (⁴⁰Ar³⁵Cl) that can interfere with the signal for ⁷⁵As. Chloride also forms an adduct with oxygen (³⁵Cl¹⁶O) with the same *m/z* as ⁵¹V. Vanadium was therefore monitored to discard any peak eluting as ⁷⁵As that may have come from the presence of chloride based salts in samples. Carbon from samples and mobile phases was monitored by measuring chromium (⁵²Cr), as it has the same *m/z* as the adduct that carbon forms with argon (⁴⁰Ar¹²C). Separation of water soluble arsenic species was achieved using a Hamilton PRP-X100 anion exchange column (250 mm × 4.1 mm, 10 µm particle size, Phenomenex, US) an isocratic elution with a 7.5 mM malonic acid (UNILAB, Ajax Chemicals, Australia) mobile phase (pH 5.6 adjusted with aqueous ammonia (Sigma-Aldrich, Australia)) for 10 min. Calibration standards were prepared in methanol: water 1:1 v/v using DMA at arsenic concentrations of 100, 10, 1 and 0.1 µg L⁻¹. Calibration verification standards of As(III), As(V), MA and DMA were run to assess the calibration and the recoveries found to be in between 80 – 100 %. Blanks of fresh media with FBS and samples from control experimental flasks were also

analysed. The separation of lipid soluble arsenic species was achieved in an Eclipse XDB-C8 reverse-phase column (150 mm × 4.6 mm, 5 µm particle size, Eclipse, Agilent, Australia) using deionised water with 0.1 % v/v formic acid as eluent A and ethanol with 0.1 % v/v formic acid as eluent B. Elution at 0.8 mL min⁻¹ with 75 % v/v A was applied for 1 min, changed to 10 % v/v A over 25 min and held for 1 min, and then back to 75 % v/v A over 3 min. The flow was split post column, with 1 mL min⁻¹ going to waste and 0.4 mL min⁻¹ to the ICP-MS. A solution of deionised water with 1 % v/v formic acid was infused to the sample after the splitter at 0.8 mL min⁻¹ to dilute the amount of carbon getting into the plasma. A flow of 0.04 L min⁻¹ of 20 % v/v oxygen in argon (Coregas, Australia) was added to the argon flow (0.74 L min⁻¹) in the nebuliser to control the carbon built up in the plasma. Calibration standards were prepared in methanol with 1 % v/v of formic acid using DMA at arsenic concentrations of 100, 10, 1 and 0.1 µg L⁻¹. Standards with AsFA-C11 and AsHC-C10 were run to confirm or discard the presence of these arsenic species in samples. Blanks of fresh media with FBS and samples from control experimental flasks were also analysed. Calibrations with DMA were used to quantify the concentrations of all arsenic species in samples. Due to the small amount of sample available, total arsenic analysis of cell fractions could not be performed and total arsenic concentrations were calculated as the sum of the concentrations of each arsenic species, under the assumption that no arsenic was lost in the column. For the same reason, the masses of whole cells and the masses of sub-cellular fractions could not be measured. Arsenic concentrations are therefore reported normalised to protein concentration and expressed in µg g⁻¹ of protein.

Identification of unknown arsenic species

The identification of unknown arsenic species was performed by liquid chromatography- mass spectrometry (LC-QQQ, Agilent 6410, Australia). Separation of arsenic species was achieved with a SB-C18 reverse-phase column (50 mm × 2.1 mm, 1.8 µm particle size, Agilent, Australia) using deionised water with 0.1 % v/v formic acid as eluent A and methanol with 0.1 % v/v formic acid as eluent B. Elution at 0.2 mL min⁻¹ with 75 % A was held for 2 min, changed to 5 % A over 13 min and held for 18 min, back to 75 % A over 1 min and held for 4 min. Post column ionisation was achieved by ESI in positive mode. Ionisation conditions, fragmentor voltages and collision

energies were optimised using both AsFA-C11 and AsHC-C10 standards, as well as trimethylarsinoyl propionic acid (TMAP). Precursor ion chromatograms were obtained scanning from m/z 150 to 1100 with a fragmentor voltage of 119 V and collision energies of 32 au for m/z 105 and 107; and 20 au for m/z 123. Subsequently, a product ion scan was performed for the precursor ions of interest to confirm their identity.

Results

Protein concentration in subcellular fractions

The different fractions in which HepG2 cells were separated comprised the nuclei and cell membranes (S2), secretion granules (P2), mitochondria (P3), cytosol and heat stable soluble proteins (S4) and lysosomes, peroxisomes and microsomes (P4). From those, S4 and S2 exhibited the higher protein concentration ($2.1 \pm 0.6 \text{ mg L}^{-1}$ and $0.6 \pm 0.2 \text{ mg L}^{-1}$, respectively), followed by P4 ($0.11 \pm 0.05 \text{ mg L}^{-1}$), P2 ($0.08 \pm 0.04 \text{ mg L}^{-1}$) and P3 ($0.02 \pm 0.01 \text{ mg L}^{-1}$).

Cellular growth and viability

The growth and viabilities of HepG2 cells exposed to AsFA-C11 and AsHC-C10, as well as of the negative controls, are shown in Figure 6-2. Neither cell growth nor viability significantly changed in AsFA-C11 (13.51×10^6 cell count and $87 \pm 5\%$ viability) and AsHC-C10 (14.62×10^6 cell count and $86 \pm 2\%$ viability) exposed cells compared to the negative controls (14.82×10^6 cell count and $87 \pm 3\%$ viability).

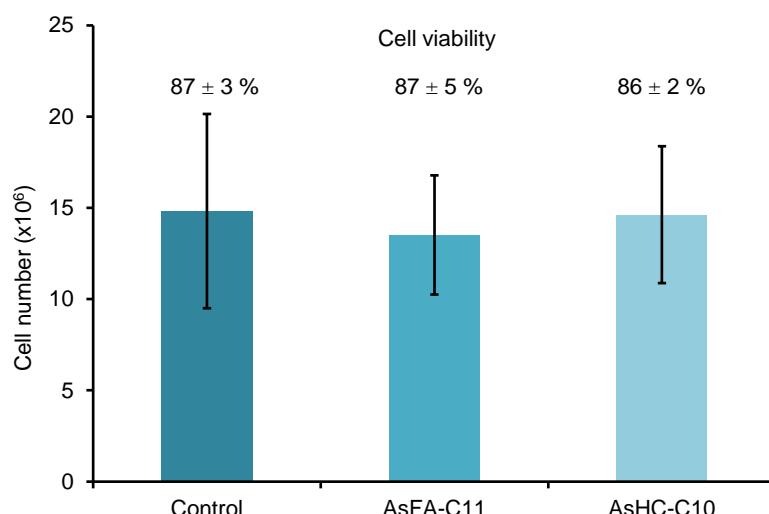


Figure 6-2. Cell growth and viabilities of control and AsFA-C11 and AsH-C10 exposed HepG2 cells.

AsFA-C11 exposed HepG2 cells

After exposure, the total arsenic concentration in the exhausted media and washing solutions was $43 \pm 1 \mu\text{g L}^{-1}$, of which $1.91 \pm 0.02 \mu\text{g L}^{-1}$ was As(III), $16 \pm 1 \mu\text{g L}^{-1}$ DMA and $26 \pm 1 \mu\text{g L}^{-1}$ AsFA-C11. Assuming that these $26 \pm 1 \mu\text{g L}^{-1}$ of AsFA-C11 is what had not been taken up by cells, then around 11 % of AsFA-C11 appears to be bioavailable to HepG2 cells after 24 h exposure. The total arsenic concentration measured in cells after exposure was $15 \pm 3 \mu\text{g g}^{-1}$ protein, of which $11.7 \pm 0.1 \%$ was DMA, $2 \pm 1 \%$ MA, $1 \pm 1 \%$ As(V) and $1.23 \pm 0.01 \%$ As(III). The majority of arsenic was present as an unknown arsenic species eluting at 12.2 min by reverse-phase chromatography ($70 \pm 8 \%$), which was identified as DMAP by LC-QQQ (*Appendix Chapter 6*). Four additional unknown arsenic species were detected: two of them eluting at 4.4 min (UK 01, $8.96 \pm 0.01 \%$) and 6.6 min (UK 02, $2 \pm 1 \%$) by anion exchange chromatography; and two eluting at 16 min (UK 03, $17 \pm 4 \%$) and 19 min (UK 04, $1.4 \pm 0.5 \%$) by reverse-phase chromatography. The retention times of MA and As(V) were 4 min and 5 min, respectively, therefore UK 01 is neither of them. The retention time of AsFA-C11 under the conditions used is 8.9 min, thus none of the lipid soluble unknowns corresponds to the initial compound. The low concentrations of UK 01, UK 02, UK 03 and UK 04 and the lack of enough sample volume for subsequent HPLC injections did not allow a proper identification.

Arsenic was distributed among all five subcellular fractions with the highest concentration in mitochondria (P3); followed by lysosomes, peroxisomes and microsomes (P4), granules (P2), nuclei and cell membranes (S2) and the cytosol (S4), which had the lowest arsenic concentration (Table 6-1). Arsenous acid and As(V) were present in granules, cytosol and lysosomes, microsomes and peroxisomes in very low concentrations (Table 6-1 and Figure 6-3). Although MA was detected in cells, its subcellular distribution was not consistent among fractions, thus MA is not included in Table 6-1 and Figure 6-3. Dimethylarsinic acid was found in all subcellular fractions except for mitochondria, with a higher abundance in granules (Table 6-1 and Figure 6-3). All unknown arsenic species were found in nuclei and cell membranes. While UK 01, UK 02 and UK 04 were only in this subcellular fraction; UK 03 also existed in the lysosomes (Table 6-1 and Figure 6-3). Dimethylarsinoyl propionic acid occurred in all

five subcellular fractions and was also the most abundant arsenic species in HepG2 cells after exposure to AsFA-C11 (Table 6-1 and Figure 6-3).

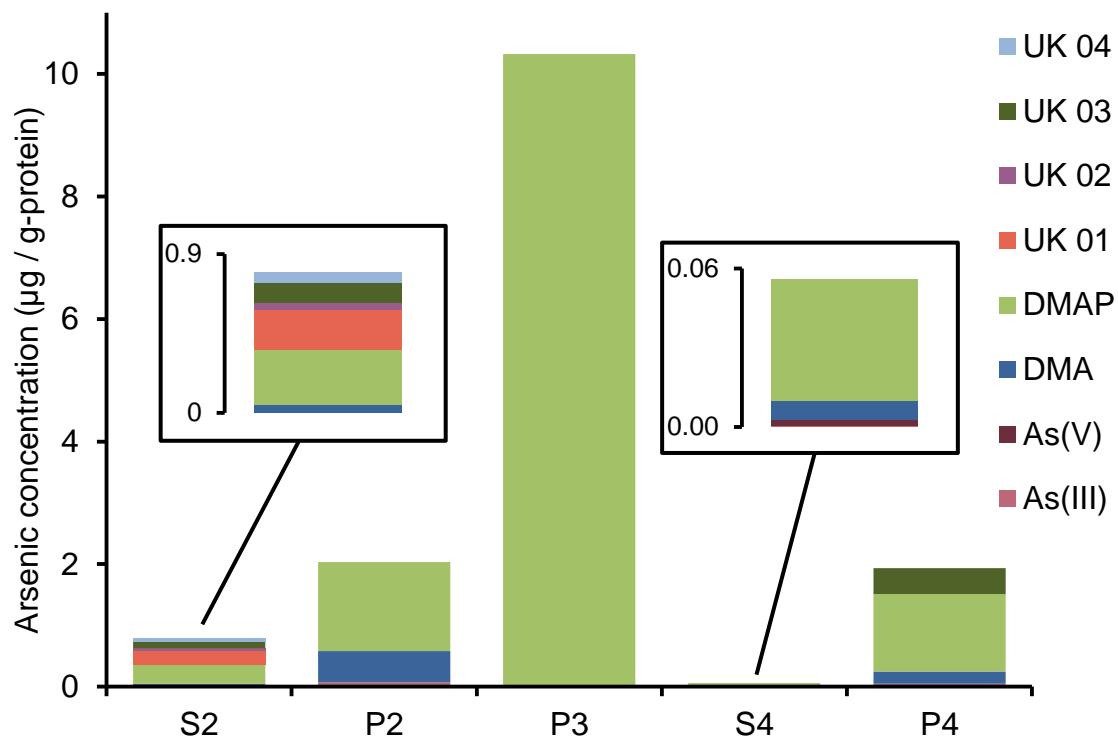


Figure 6-3. Arsenic concentrations and species distribution in subcellular fractions of HepG2 cells after 24 h exposure to AsFA-C11. S2, nuclei and cell membranes; P2, granules; P3, mitochondria; S4, cytosol; P4, lysosomes, peroxisomes and microsomes.

Table 6-1. Arsenic concentrations and percentages of arsenic species in the subcellular fractions of HepG2 cells after 24 h exposure to AsFA-C11.

Subcellular fraction	Total As concentration ($\mu\text{g/g-protein}$)	% (relative to total arsenic in fraction)							
		As(III)	As(V)	DMA	DMAP	UK 01	UK 02	UK 03	UK 04
nuclei and cell membranes	0.7 ± 0.3			9 ± 9	40 ± 21	45 ± 1	9 ± 6	12 ± 11	7 ± 2
granules	2 ± 1	2 ± 4		16 ± 28	69 ± 28				
mitochondria	10 ± 3				100				
cytosol	0.07 ± 0.03	1 ± 1	2 ± 3	8 ± 13	75 ± 22				
lysosomes, peroxisomes and microsomes	2 ± 1	1 ± 2		6 ± 11	65 ± 7			34 ± 9	

AsHC-C10 exposed HepG2 cells

After exposure, the total arsenic concentration in the exhausted media and wash solutions was $46 \pm 11 \mu\text{g L}^{-1}$, of which $3.3 \pm 0.3 \mu\text{g L}^{-1}$ was DMA and $34 \pm 10 \mu\text{g L}^{-1}$ AsHC-C10, indicating that 23 % of AsHC-C10 was bioavailable to HepG2 cells after 24 h exposure. The total arsenic concentration measured in cells after exposure was $19 \pm 10 \mu\text{g g}^{-1}$ protein, of which $66 \pm 19 \%$ was DMAP, $19 \pm 1 \%$ MA, $10 \pm 11 \%$ AsHC-C10, $10 \pm 9 \%$ DMA, $1.0 \pm 0.5 \%$ UK 04, $0.919 \pm 0.001 \%$ As(V), $0.8 \pm 0.3 \%$ UK 02 and $0.41 \pm 0.06 \%$ As(III).

Arsenic concentrations were higher in lysosomes and mitochondria, than in nuclei, granules and cytosol (Table 6-2 and Figure 6-4). Arsenous acid, As(V) and DMA were only present in the cytosol, while MA occurred in the cytosol, nuclei and cell membranes (Table 6-2 and Figure 6-4). Although AsHC-C10 was detected in most subcellular fractions; its presence was solely reproducible in the cytosol and hence, uniquely reported in this fraction in both Table 6-2 and Figure 6-4. The arsenic species UK 02 was found in nuclei and cell membranes and UK 04 in different subcellular fractions in a non-reproducible manner, thus UK 04 is not included in Table 6-2 and Figure 6-4. Dimethylarsinoyl propionic acid was present in all subcellular fractions, being the only arsenic species detected in granules, mitochondria, lysosomes, peroxisomes and microsomes (Table 6-2 and Figure 6-4).

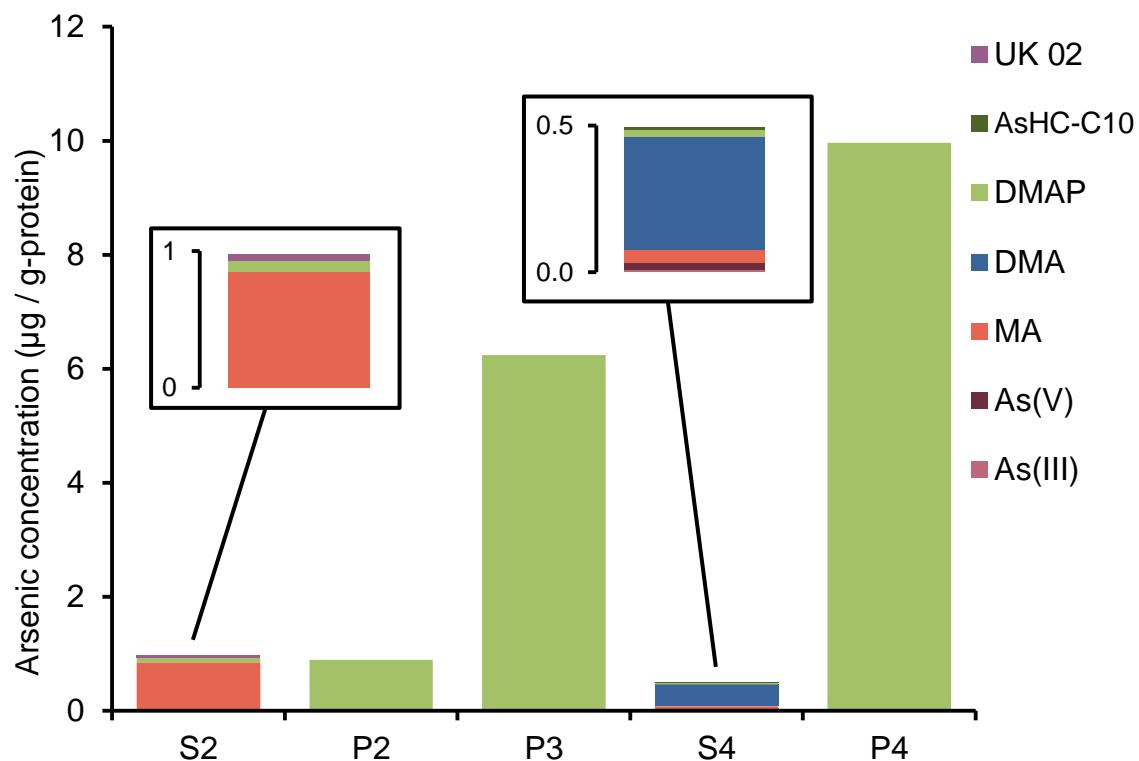


Figure 6-4. Arsenic concentrations and species distribution in subcellular fractions of HepG2 cells after 24 h exposure to AsHC-C10. S2, nuclei and cell membranes; P2, granules; P3, mitochondria; S4, cytosol; P4, lysosomes, peroxisomes and microsomes.

Table 6-2. Arsenic concentrations and percentages of arsenic species in the subcellular fractions of HepG2 cells after 24 h exposure to AsHC-C10.

Subcellular fraction	Total As concentration ($\mu\text{g/g-protein}$)	% (relative to total arsenic in fraction)						AsHC-C10	UK 02
		As(III)	As(V)	MA	DMA	DMAP			
nuclei and cell membranes	1 \pm 0.5			87 \pm 3			8 \pm 3		4 \pm 1
granules	0.9 \pm 0.8						100		
mitochondria	7 \pm 7						83 \pm 29		
cytosol	0.3 \pm 0.5	2 \pm 1	5 \pm 1	9 \pm 1	51 \pm 44	49 \pm 50		6 \pm 7	
lysosomes, peroxisomes and microsomes	10 \pm 6						88 \pm 20		

Discussion

Cellular growth and viability

Total arsenic concentrations of exposure were measured as $50 \pm 2 \mu\text{g L}^{-1}$ for AsFA-C11 and $48 \pm 3 \mu\text{g L}^{-1}$ for AsHC-C10. In both standards there were impurities of DMA from the synthesis, with $29 \pm 4 \mu\text{g L}^{-1}$ being the actual concentration of AsFA-C11 and $45 \pm 3 \mu\text{g L}^{-1}$ of AsHC-C10. The concentrations of these arsenic species in samples were below their toxicity levels in human hepatocytes (Dopp et al., 2008, Meyer et al., 2015a, Meyer et al., 2014a). After 24 h, neither AsFA-C11 nor AsHC-C10 exhibit significant effects on HepG2 cell growth and viability (Figure 6-2), confirming the suitability of the arsenic concentrations used in this study.

Arsenic uptake

Both AsFA-C11 and AsHC-C10 are partially bioavailable to HepG2 cells as shown by the estimated uptake percentages of 11 % for AsFA-C11 and 23 % for AsHC-C10. This is in agreement with previous studies that have shown that AsHCs and AsFAs are bioavailable to HepG2 cells after 48 h (Meyer et al., 2014a, Meyer et al., 2015a); and that AsHCs are more permeable to cell membranes than AsFAs (Meyer et al., 2015b). The higher uptake of AsHC-C10 might be due to the higher hydrophobicity of AsHCs compared to AsFAs, as previously suggested (Meyer et al., 2014a).

Arsenic distribution and metabolism in AsFA-C11 exposed HepG2 cells

Biologically relevant and xenobiotic fatty acids are primarily metabolised in the mitochondria and peroxisomes (Casarett and Doull, 2008, van der Sluis and Erasmus, 2016). This study showed that the highest arsenic concentrations after HepG2 exposure to AsFA-C11 were in the fractions that contain these two organelles (Table 6-1 and Figure 6-3), suggesting that AsFAs might be degraded there.

To traverse the inner mitochondrial membrane, fatty acids are conjugated with coenzyme A (CoA) in the cytosol. The fatty acyl-CoA formed can then enter the mitochondria where β -oxidation occurs (Mathews et al., 2013). This oxidation pathway occurs in several cycles, depending on the number of carbons in the initial fatty acid, in which the fatty acyl chain is shortened by two carbons at a time. These two carbons are

released as acetyl-CoA which will then enter the citric acid cycle, also in mitochondria (Mathews et al., 2013). The only arsenic species present in mitochondria of HepG2 cells after exposure to AsFA-C11 was identified as DMAP, a three carbon chain AsFA; confirming that β -oxidation of AsFA-C11 might occur (Figure 6-3). A proposed pathway would involve four cycles of β -oxidation reactions per AsFA-C11 molecule to produce four molecules of acetyl-CoA and one of dimethylarsinoyl propionyl-CoA (DMAP-CoA), together with 16 molecules of adenosine triphosphate (ATP). The four molecules of acetyl-CoA would be further oxidised in the citric acid cycle yielding another 40 more molecules of ATP (Figure 6-5). Dimethylarsinoyl propionyl-CoA could either undergo another β -oxidation to produce acetyl-CoA, DMA, CO₂ and 14 ATP; or be degraded to DMA and succinyl-CoA and yield 10 molecules of ATP. Previous studies have suggested that DMA in urine after consumption of AsFAs could come from the β -oxidation of these arsenic species (Schmeisser et al., 2006b, Schmeisser et al., 2006a, Rumpler et al., 2008). Even though the results reported here support β -oxidation of AsFAs occurring in mitochondria, DMAP was the only metabolite detected in this organelle (Table 6-1 and Figure 6-3), indicating that DMA might be produced somewhere else.

In peroxisomes, β -oxidation of fatty acids only goes to C4 or C6 acyl-CoA and it is not coupled to ATP production but to hydrogen peroxide (H₂O₂) formation, which is catalysed into H₂O and O₂ (Mathews et al., 2013, Lodish et al., 2000a). Dimethylarsinoyl butyric acid has been reported as a urine metabolite of AsFAs (Schmeisser et al., 2006b, Schmeisser et al., 2006a). This arsenic species has a four carbon chain supporting β -oxidation also occurring in peroxisomes. Since AsFA-C11 has an odd number of carbons, its β -oxidation in peroxisomes would not yield DMAB but dimethylarsinoyl pentanoyl-CoA, which can then enter mitochondria for further oxidation into DMAP (Figure 6-5). The identity of UK 03 could be dimethylarsinoyl pentanoyl-CoA or dimethylarsinoyl pentanoic acid (AsFA-C5), as it was the second most abundant arsenic species in the fraction containing the peroxisomes (Table 6-1). This could not be, however, confirmed due to the low concentrations in samples. Additionally, UK 03 eluted later than DMAP under the reverse-phase chromatographic conditions used, indicating that it is more hydrophobic, as is AsFA-C5. Further identification of UK 03 is needed to support this hypothesis.

The detection of DMA in the fraction comprising lysosomes, peroxisomes and microsomes could be associated with the microsomes. These are small vesicles located in the endoplasmic reticulum and are rich in cytochrome P450, a versatile enzyme involved in xenobiotic biotransformations (Lodish et al., 2000a, Casarett and Doull, 2008). Cytochrome P450 is particularly abundant in liver cells and is able to catalyse the ω -aliphatic hydroxylation of fatty acids, which can be further oxidised to dicarboxylic acids (Casarett and Doull, 2008). In that way, cytochrome P450 could transform DMAP into dimethylarsinoyl keto-propionic acid that after decarboxylation would yield dimethylarsinoyl ketone. Subsequently, a nucleophilic attack on the arsenic atom could cleave ethanol and produce DMA (Figure 6-5). This would explain why both DMAP and DMA coexisted in this subcellular fraction (Figure 6-3). The presence of DMA in samples, however, could also come from the impurities in the AsFA-C11 standard, meaning that DMAP may not be metabolised to DMA in HepG2 cells. The exposure of Caco-2 cells to AsFAs has shown DMA as a metabolic product but not DMAP (Meyer et al., 2015b); thus the mechanism for AsFAs degradation in the liver and intestine tissues could be different.

A small percentage of arsenic in lysosomes, peroxisomes and microsomes was also found as As(III) (Table 6-1), indicating that DMA demethylation may occur. Demethylation of arsenic species has been previously reported, although it is known to happen only in microbial communities (Lehr et al., 2003), and has not yet been observed in human cells. The presence of MA in some samples suggests that the reaction might occur in two steps; though the mechanism for that reaction in human cells needs further investigation. The only other subcellular fraction where As(III) was detected was the granules, where DMAP and DMA were also found (Table 6-1 and Figure 6-3). The granules constitute enclosed vesicles that eliminate xenobiotics from cells by exocytosis (Lodish et al., 2000a) and therefore, the presence of DMAP, DMA and As(III) in these organelles might be associated with this detoxification mechanism. The detection of these arsenic species in the exhausted media of HepG2 cell cultures also supports that they are the excreted from cells.

The cytosol had the lowest arsenic concentration, present as DMAP, DMA, As(III) and As(V) (Table 6-1 and Figure 6-3). While DMAP, DMA and As(III) might be

transitioning between organelles or on the way to be excreted; As(V) production might come from As(III) oxidation by the xanthine oxidases in the cytosol (Aposhian et al., 2003, Casarett and Doull, 2008) (Figure 6-5).

After the cytosol, the nuclei and cell membranes exhibited the lowest arsenic concentrations, which was found as DMAP, DMA, UK 01, UK 02, UK 03 and UK 04 (Table 6-1 and Figure 6-3). The presence of DMAP and DMA in this fraction might be associated with the membrane, and possibly to the proteins responsible for extruding these arsenic species from cells. In human hepatocytes, DMA is eliminated through the *MRP4/ABCC4* transporters (Roggenbeck et al., 2015, Banerjee et al., 2014). Dimethylarsinoyl propionic acid, however, has not been studied in terms of transport mechanisms across cell membranes; although it is excreted in human urine after arsenolipid consumption (Schmeisser et al., 2006b), hence, it has to be eliminated from hepatocytes by some mechanism.

Both UK 01 and UK 02 eluted at similar retention times than two of the unknown arsenic species detected in the same fraction (nuclei and cell membranes) of HepG2 cells exposed to As(III) and As(V) (*Chapter 5 p.101*). Given that xenobiotic metabolism in the nuclei is rare (Casarett and Doull, 2008), these arsenic species probably come from arsenic bound to proteins in the lipid membrane of cells. The unknown arsenic species UK 04 eluted after all the other lipid soluble arsenic species detected in HepG2 cells after exposure to AsFA-C11, indicating that UK 04 is highly hydrophobic. Thiolation or reduction of AsFA-C11 to the equivalent thio-AsFA or AsHC, respectively, could explain these results. Thiolation of AsHCs has been observed in Caco-2 cells, but not for AsFAs (Meyer et al., 2015b); and the thio-AsFAs previously detected in human urine are thio-DMAP and thio-DMAB, both short chain products from AsFAs metabolism (Schmeisser et al., 2006b, Schmeisser et al., 2006a). Reduction of AsFAs to AsHCs would be improbable, given that aliphatic hydrocarbons are usually oxidised to their corresponding fatty acids in hepatocytes (Patzelt, 2007), but not impossible. Again, further analysis needs to be done to confirm the identity of UK 04.

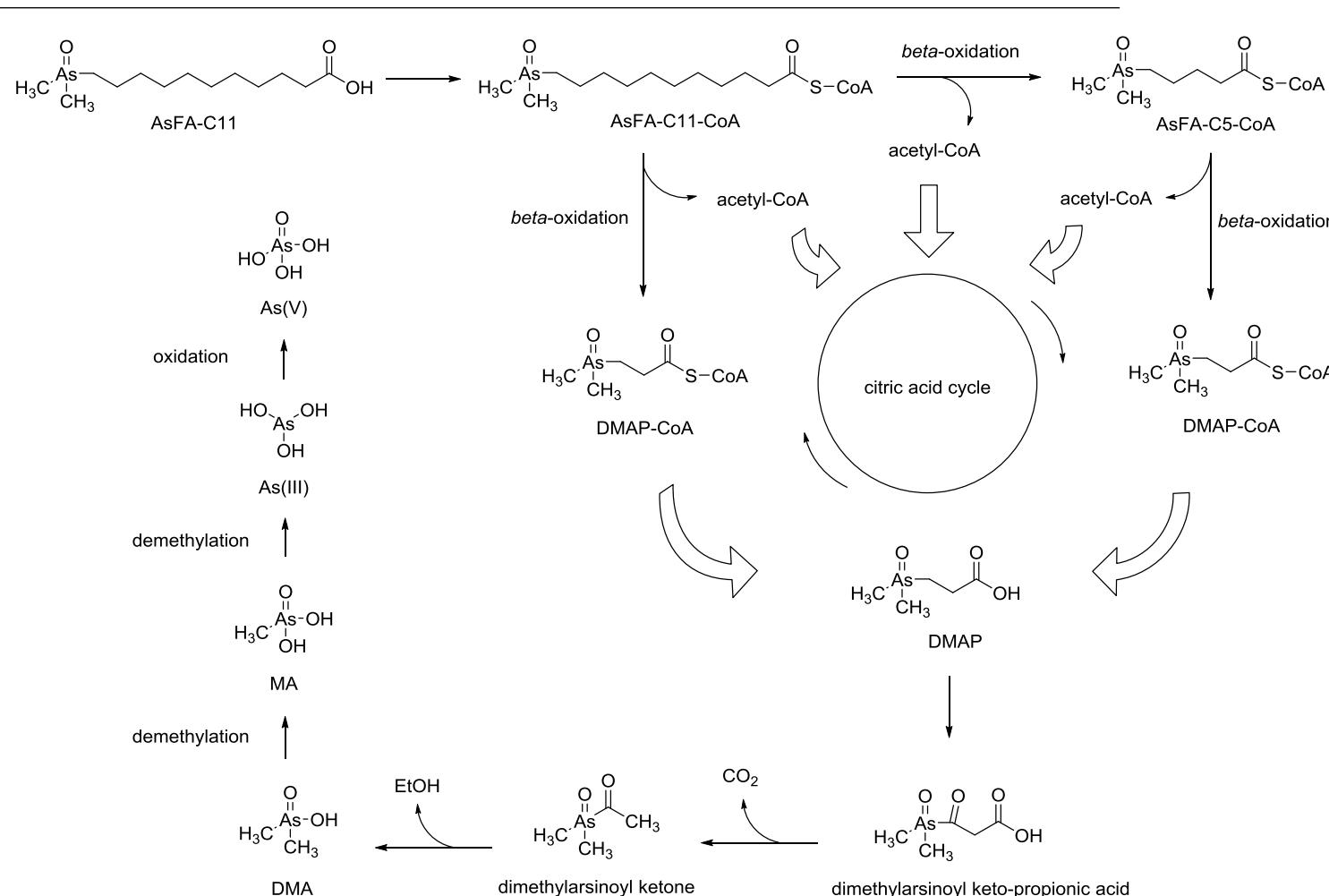


Figure 6-5. Proposed pathway for AsFA-C11 biotransformations in HepG2 cells.

Arsenic distribution and metabolism in AsHC-C10 exposed HepG2 cells

After exposure to AsHC-C10, HepG2 cells exhibited the highest percentage of arsenic as DMAP ($66 \pm 19\%$). This arsenic species is the only one found in the fractions containing granules, mitochondria, lysosomes, peroxisomes and microsomes (Table 6-2 and Figure 6-4). Hence, it can be concluded that DMAP is the final product from AsHC-C10 metabolism. In this case, however, the highest concentration of this arsenic species is not located in mitochondria but in the fraction comprising lysosomes, peroxisomes and microsomes (Table 6-2 and Figure 6-4), suggesting that AsHC-C10 might primarily be metabolised in one of those three organelles.

Aliphatic hydrocarbons are xenobiotics to humans and they are first oxidised into fatty acids before undergoing β -oxidation (Patzelt, 2007). This oxidation can be catalysed by cytochrome P450 in the microsomes, where hydroxylation occurs at the terminal carbons of the aliphatic chain (Casarett and Doull, 2008, Patzelt, 2007). For AsHC-C10, one hydroxylation reaction will take place on the carbon at the other end of the dimethylarsinoyl moiety, and the subsequent oxidation will yield dimethylarsinoyl decanoic acid (AsFA-C10) that will then be degraded *via* β -oxidation in peroxisomes and mitochondria (Figure 6-6a). Alternatively, hydroxylation of the penultimate carbon of the aliphatic chain and further oxidations can produce an ester that is hydrolysed into dimethylarsinoyl octanol and acetyl-CoA (Casarett and Doull, 2008). After the oxidation of the alcohol, dimethylarsinoyl octanoic acid (AsFA-C8) will undergo β -oxidation (Patzelt, 2007). Both AsFA-C8 and AsFA-C10 have an even number of carbons and β -oxidation would produce DMAB or dimethylarsinoyl acetic acid (DMAA) (Figure 6-6b). The former has been reported in human urine after arsenolipid consumption (Schmeisser et al., 2006b, Schmeisser et al., 2006a), whereas DMAA has been found only in marine organisms (Edmonds et al., 1997). In this study, neither DMAB nor DMAA were detected in the mitochondrial fraction of HepG2 cells exposed to AsHC-C10, where only DMAP was found. This could mean that DMAB is degraded to DMAP, although this is unlikely to happen with butyric acid and propionic acid in cells (Mathews et al., 2013). Another possibility is that hydroxylation and subsequent oxidations might take place on the third aliphatic carbon after the dimethylarsinoyl moiety of AsHC-C10; producing DMAP and heptanoic acid (Figure 6-6c). Heptanoic acid will then undergo β -oxidation

and enter the citric acid cycle. This could explain the findings of the present study. Dimethylarsinoyl propionic acid was also the only arsenic species detected in granules (Table 6-2 and Figure 6-4); supporting that this is probably the final product of AsHC-C10 that is accumulated in these organelles for elimination.

Dimethylarsinic acid was found solely in the cytosol (Table 6-2 and Figure 6-4). The AsHC-C10 standard contained traces of DMA although in much lower concentrations than for AsFA-C11. After exposure, DMA concentration in the culture media decreased about 6 %, thus indicating that DMA was also taken up by cells. Hence, it is likely that DMA in the cytosol of AsHC-C10 exposed cells comes only from the exposure media. Demethylation in the cytosol to MA and As(III), and further oxidation to As(V) can explain why these arsenic species are also found here.

A small percentage of the total arsenic in the cytosol corresponds to AsHC-C10 (Table 6-2 and Figure 6-4), in contrast to AsFA-C11 exposed cells, where none of the initial arsenolipid was detected after exposure (Table 6-1 and Figure 6-3). This is likely a consequence of the higher cellular uptake of AsHC-C10 than that of AsFA-C11.

In nuclei and cell membranes, the arsenic species identified were MA, DMAP and UK 02. As the final product of AsHC-C10 metabolism, DMAP here might be associated with membrane proteins to be eliminated from cells. The arsenic species UK 02 was found in this fraction in As(V) (*Chapter 6* p.115), AsFA-C11 (Table 6-1 and Figure 6-3) and AsHC-C10 exposed cells (Table 6-2 and Figure 6-4), and seems to be accompanied by either MA or DMA; which supports the possibility of UK 02 being an intermediate between both arsenic species (e.g. methylarsonous acid, dimethylarsinous acid or monomethylarsonic diglutathione).

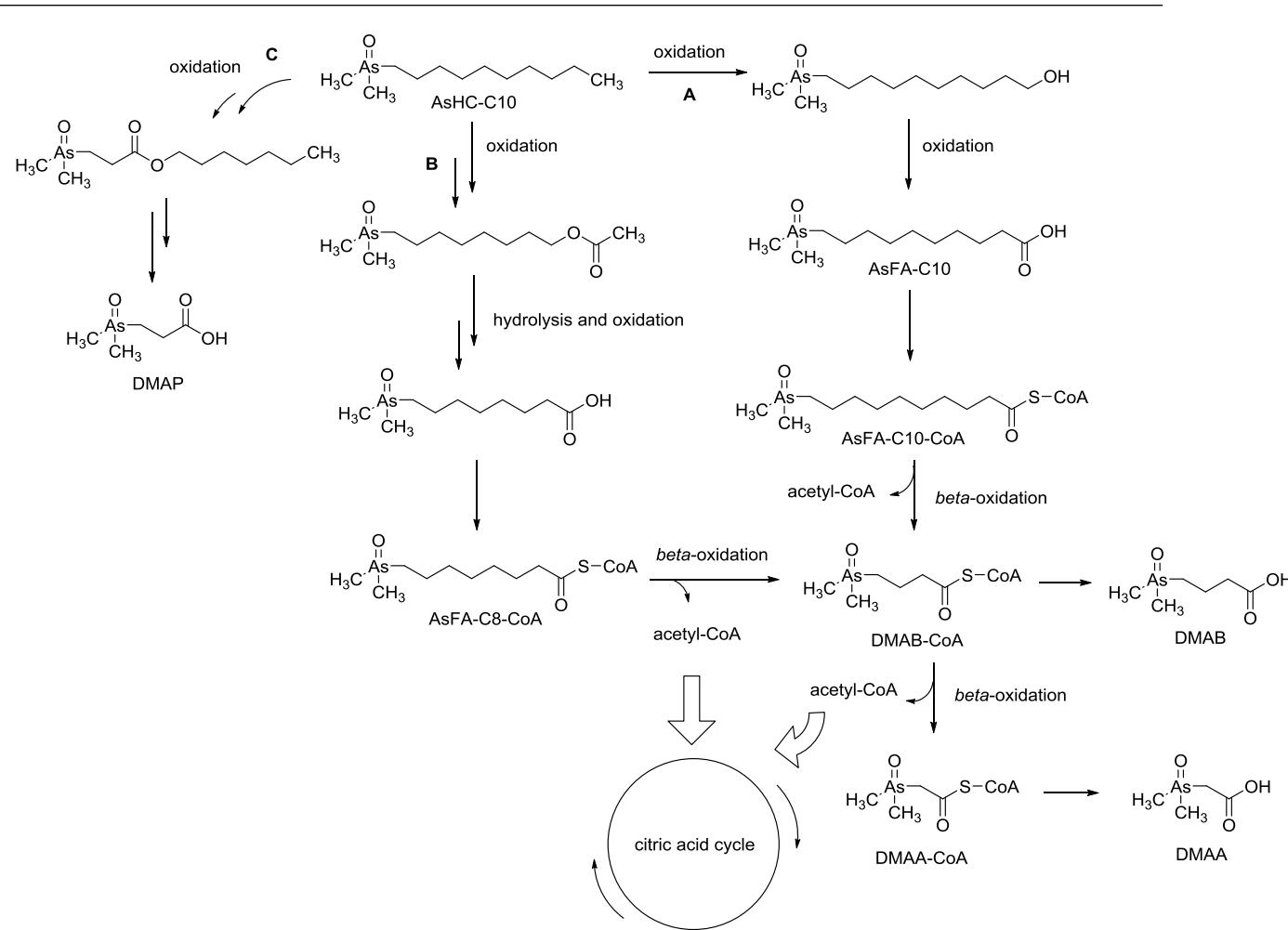


Figure 6-6. Proposed pathways for AsHC-C10 biotransformations in HepG2 cells.

Concluding remarks

This study shows that both AsFA-C11 and AsHC-C10 are metabolised in human liver cells, although the main location where this takes places is different for each arsenolipid. Whereas AsFA-C11 can directly enter mitochondria and undergo β -oxidation, AsHC-C10 may be oxidised first to AsFAs in the microsomes. Since AsHCs exert higher toxicity than AsFAs (Meyer et al., 2015a, Meyer et al., 2014a), this oxidation serves as a detoxification mechanism to inactivate AsHCs. The identification of DMAP as the main metabolic product for AsFA-C11 and AsHC-C10 supports that the aim of arsenic biotransformations in hepatocytes is detoxification, as DMAP is much less toxic than AsFAs and AsHCs (Meyer et al., 2015a). In addition, the metabolism of both AsFAs and AsHCs can yield acetyl-CoA that enters the citric acid cycle and produces ATP (Figure 6-5 and Figure 6-6). Hence, the detoxification of AsFAs and AsHCs could be a source of energy in cells, while also limits the availability of CoA and fatty acid CoA ligases for other metabolic purposes. This study did not show evidence of thiolation of AsFAs and AsHCs occurring in HepG2 cells. Although the identification of the unknown arsenic species found may prove otherwise, this could mean that thiolation of arsenolipids does not happen in the liver but in the intestine (Meyer et al., 2015b) or due to the microbial activity in the gut, as observed for other arsenic species (Conklin et al., 2006, Alava et al., 2015, Dc.Rubin et al., 2014, Van de Wiele et al., 2010).

This is the first report on the metabolism of arsenolipids in human liver cells. While the current findings can help to understand the consequences of a high intake of AsFAs and AsHCs with the diet, further experiments are needed to answer the questions generated from this study.

CHAPTER 7 – General conclusions and further directions

The main aim of this thesis was to provide new insights into the poorly understood metabolic pathways of arsenic in humans. Of special interest was to investigate complex organic arsenic species, such as arsenolipids, as they are readily available to humans through the diet, and little research had been conducted before on their metabolism in humans.

Arsenic is a xenobiotic and therefore, it is expected to follow the metabolic pathway shown in Figure 7-1.

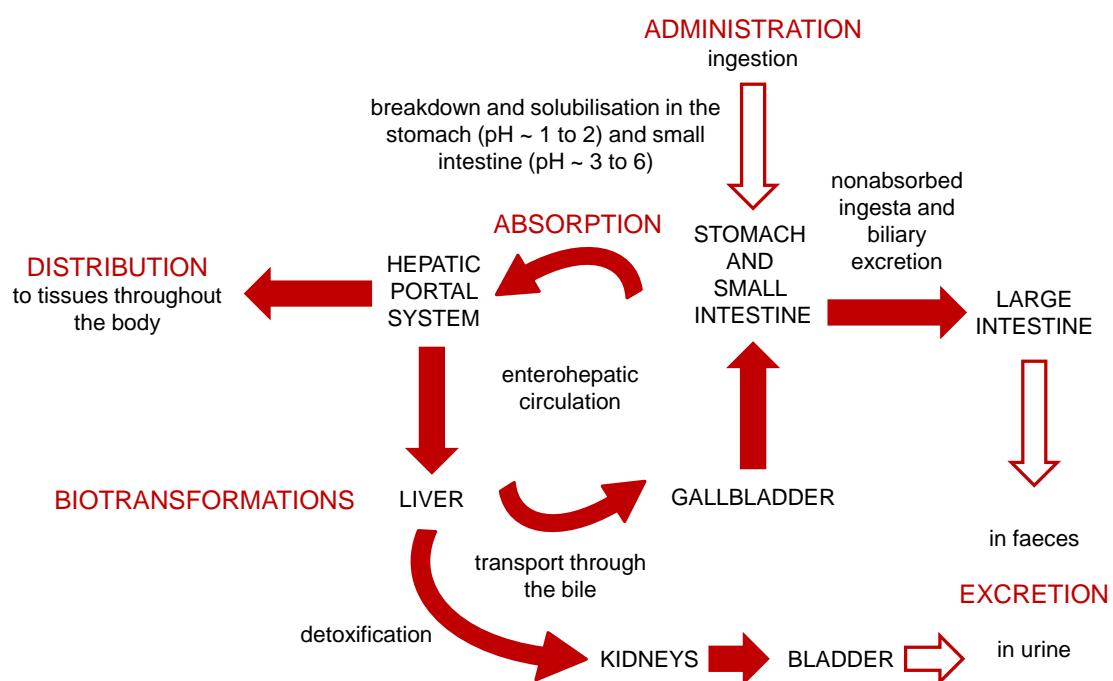


Figure 7-1. General metabolic pathway for xenobiotics in humans, adapted from (Goodman and Gilman, 2006, Casarett and Doull, 2008, Rang et al., 2012). The processes represented by the arrows filled in red involve transport through cell membranes.

After ingestion, arsenic species can be transformed in the stomach and small intestine, from where they are either absorbed into the hepatic portal system or excreted in faeces. For this reason, the bioaccessibility and degradation of complex organic arsenic species in the human gastrointestinal tract was first investigated (*Chapter 2 p.35* and *Chapter 3 p.55*). The specific question was:

1. Do complex organic arsenic species survive the gastrointestinal tract?

The research described in *Chapters 2* and *3* showed that:

A. Arsenosugars can be released from food matrices unchanged and therefore become bioaccessible to humans.

The intestinal bioavailability of these arsenic species has previously been reported (Leffers et al., 2013c), meaning that arseenosugars can be absorbed into the hepatic portal system unchanged. Arseenosugars are practically harmless in cells, and so are most of their metabolites in human urine; with the exception of dimethylarsinic acid (DMA) (Leffers et al., 2013a, Ebert et al., 2016), which is a known carcinogenic to humans (International Agency for Research on Cancer, 2012). The findings from *Chapter 2* suggest that arseenosugar degradation to DMA primarily occurs in the liver, thus, future research on arseenosugar metabolism and toxicity should be focused on this tissue.

B. Arseno-fatty acids (AsFAs) and arseeno-hydrocarbons (AsHCs) are bioaccessible to humans.

These arsenolipids are able to cross the intestinal barrier (Meyer et al., 2015b) and therefore be absorbed into the hepatic portal system. The recently detection of AsFAs and AsHCs in human milk also suggests that these species can be readily distributed through the body (Stiboller et al., 2017). Since AsFAs and AsHCs are as toxic as arsenous acid (As(III)) in human cells (Meyer et al., 2014a, Meyer et al., 2015a, Meyer et al., 2015b), the cellular metabolism of these arsenolipids needs to be understood, thus justifying the research conducted in *Chapter 6* (p.125).

C. Trimethylarsonio fatty alcohols (TMA_nFOH) may be bioaccessible to humans.

This type of arsenolipids was identified in krill oil, although their chemical structures need to be confirmed. The results in *Chapter 3* showed some evidence of TMA_nFOHs ability to survive gastrointestinal digestion. The lack of TMA_nFOHs standards, however, did not allow the confirmation of the stability of these arsenic species. Future research may need to investigate this further, as well as the potential toxicity of these arsenolipids in cells.

Additionally, the research described in *Chapter 2* demonstrated the relevance of food matrix in arsenic metabolism. This means that future studies on bioaccessibility of

arsenic species need to consider environmental matrices as they might affect the chemical transformations occurring.

Some of the findings from *Chapters 2 and 3* generated further questions as demethylation of methylarsonic acid (MA), DMA and possibly glycoarsenolipids (GlyAsLs) was observed. Whether or not GlyAsLs are really demethylated to arsenate (As(V)) needs additional confirmation. Demethylation of MA and DMA in rice samples, however, occurs during gastrointestinal digestion. This increases the bioaccessibility and bioavailability of toxic arsenic species, as As(III) and As(V) are classified as Group 1 carcinogens (International Agency for Research on Cancer, 2012). While further research is needed, a possible explanation for the demethylation of MA and DMA could be the activation of the arsenic atom by the citrate and malate molecules existing in the stomach phase (Figure 7-2). Citrate is used as a quelating agent to remove arsenic species from environmental matrices (Flora and Pachauri, 2010). It forms a coordination complex that could potentially increase the susceptibility of arsenic to be attacked by a nucleophile, in this case water. Arsenic demethylation started in the stomach and continued in the small intestine, where it was predominantly observed. Under the pH conditions in the small intestine (pH 7), citric acid is deprotonated, and therefore, more citrate is available to coordinate with methylated arsenic species.

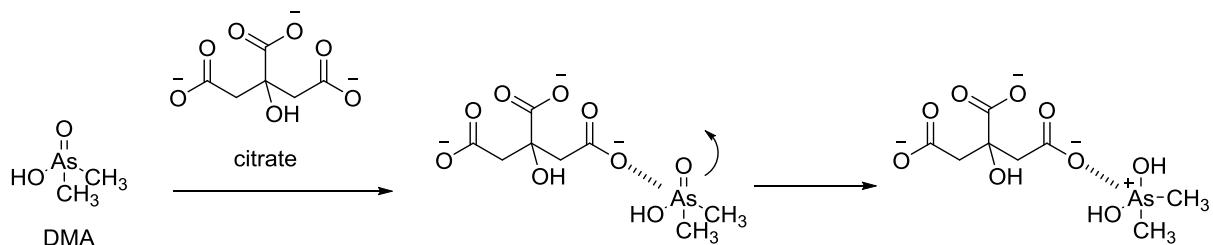


Figure 7-2. Suggested activation of DMA by citrate.

The research conducted in *Chapters 2 and 3* only considered the enzymatic and chemical conditions of the human gastrointestinal tract. Nowadays, improved standardised *in vitro* methods are available (Minekus et al., 2014). These are more realistic approaches to human gastrointestinal digestion but still lack of the biotic component involved in the process. This study addressed only the abiotic transformations of arsenic species as these need to be understood before the microbial biotransformations occurring in the stomach and small intestine are investigated. With the emerging interest

on the field of the human microbiome, few groups have already reported the role that human microbiota may play on inorganic arsenic metabolism (Van de Wiele et al., 2015, Van de Wiele et al., 2010, Alava et al., 2013, Lu et al., 2014). Further work on this topic will require focusing on complex organic arsenic species, such as arsenosugars, AsFAs and AsHCs, as they are stable under the physiological conditions in the gastrointestinal tract. Additionally, xenobiotics can have an influence on the gene expression of the human gut microbiome (Maurice et al., 2013). In the end, this could also affect the gene expression for the enzymes involved in xenobiotic detoxification in humans, which might explain why some populations are able to adapt to arsenic-rich environments better than others (Schlebusch et al., 2015). This opens an interesting field of research as understanding the interaction host-microbiome in the context of arsenic exposure can determine how different diets could prevent or reduce the harmful consequences of different arsenic species when exposure to them is unavoidable.

The next topic this thesis focused on was the ability of arsenic species to passively diffuse through lipid membranes (*Chapter 4* p.81). Membrane permeability not only relates to the potential for absorption into the hepatic system but also to the transport across different body organs (indicated by the arrows filled in red in Figure 7-1). In this case, small pentavalent methylated and thio-methylated arsenic species were studied, as well as two arsenic glutathione complexes. These arsenic species are known metabolites from inorganic arsenic in mammals and the transport mechanisms for some of them are still unclear. The specific question was:

2. Can arsenic species diffuse across biological membranes?

The research described in *Chapter 4* showed that:

- A. Arsenous acid, thio-dimethylarsinic acid (thio-DMA), arsenotriglutathione (ATG), DMA and monomethylarsonic diglutathione (MADG) can passively diffuse across the lipid bilayer of cell membranes**

Arsenous acid and thio-methylated arsenic species, including arsenic glutathione complexes, are highly toxic to humans (Leffers et al., 2013b, Ebert et al., 2013). The ability of these arsenic species to traverse cell membranes by passive diffusion means that they can readily bioaccumulate in cells, therefore increasing the risk

associated with human exposure to these arsenic species. For thio-DMA, ATG and MADG, the results of this study may also explain how these arsenic species can enter cells.

B. Arsenate, MA and thio-methylarsonic acid (thio-MA) are not able to passively diffuse across the lipid bilayer of cell membranes.

To date, there are no known uptake and extrusion mechanisms for thio-MA in cells. This arsenic species may be as toxic as As(III) (Leffers et al., 2013b) and further investigation on the mechanisms thio-MA uses to enter cells seems necessary.

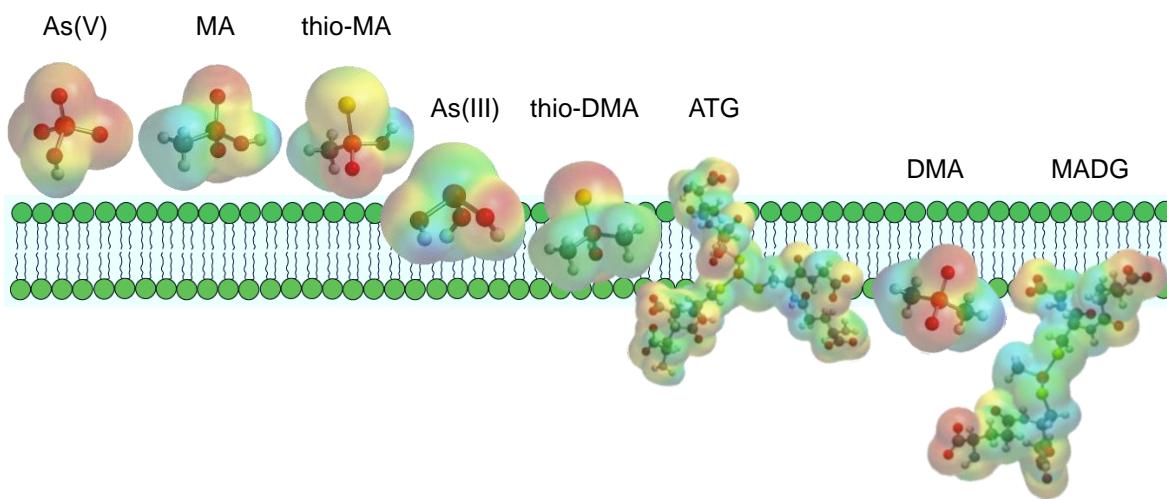


Figure 7-3. Increasing order (left to right) of the ability of arsenic species to passively diffuse across cell membranes.

Additionally, the research described in *Chapter 4* showed that simple passive diffusion of arsenic species can be effectively assessed using molecular modelling and liposome-water partition coefficients. These two methods combined can provide valuable information about the ability of xenobiotics and pharmaceuticals to traverse cell membranes by passive diffusion.

The main findings from *Chapters 2* and *3* determined the last research topic of this thesis, where the biotransformations in liver cells of As(III), As(V), AsFAs and AsHCs were investigated (*Chapter 5* p.101 and *Chapter 6* p.125).

Demethylation of arsenic species in the gastrointestinal tract implies that As(III) and As(V) are also bioaccessible from the exposure to organic arsenic species. Inorganic arsenic species are carcinogenic and have been extensively studied in terms of their metabolism in mammals. To date, four proposed pathways exist to describe the biotransformations of As(III) and As(V) in living organisms (Challenger, 1947, Hayakawa et al., 2004, Rehman and Naranmandura, 2012, Wang et al., 2012). Little work has been done, however, to identify the distribution of inorganic arsenic metabolites at a subcellular level. The specific research question was:

3. How are inorganic arsenic species metabolised in human liver cells?

The research described in *Chapter 5* showed that:

A. Inorganic arsenic species are metabolised to MA and DMA in human liver cells.

This supports reductive methylation as the detoxification pathway for As(III) and As(V). At least four unknowns were detected but at too low concentrations as to allow for a proper identification. The next steps to elucidate their structures will be oxidation with hydrogen peroxide, spiking with glutathione complexes and analysis by high performance liquid chromatography-mass spectrometry. In that way, more evidence will be provided to support any of the current existing pathways.

B. The main locations for As(III) and As(V) metabolism in HepG2 cells are the microsomes and the mitochondria.

The modes of toxic action of inorganic arsenic species in humans have not been fully elucidated yet. The findings reported in *Chapter 5* provide a reason to investigate the toxic effects of arsenic accumulation in microsomes and mitochondria.

Chapter 3 reported that AsFAs and AsHCs are bioaccessible to humans. These arsenic species have been shown to be bioavailable, as they can cross the intestinal barrier (Meyer et al., 2015b). The presystemic metabolism of AsFAs and AsHCs has shown that, while AsFAs are transformed into their thiolated and trivalent counterparts, AsHC remains practically unchanged (Meyer et al., 2015b). After consumption of foodstuffs rich in AsFAs and AsHCs, the main metabolites found in human urine are DMA, dimethylarsinoyl propionic acid (DMAP), thio-dimethylarsinoyl propionic acid (thio-

DMAP), dimethylarsinoyl butyric acid (DMAB) and thio-dimethylarsinoyl butyric acid (thio-DMAB) (Schmeisser et al., 2006b, Schmeisser et al., 2006a). Therefore, the biotransformations of AsFAs and AsHCs into those arsenic species need to happen somewhere between the small intestine and the bladder. Since the liver constitutes a crucial site in xenobiotic metabolism, *Chapter 6* focused on the biotransformations of AsFAs and AsHCs in human hepatocytes. The specific research question was:

4. What are the biotransformations of arsenolipids in human liver cells?

The research described in *Chapter 6* showed that:

A. Both AsFA-C11 and AsHC-C10 are degraded in human hepatocytes to DMAP.

Since DMAP exerts lower toxicity than AsFAs and AsHCs in cells (Meyer et al., 2015a), the findings of *Chapter 6* support that detoxification from these arsenolipids occurs in the liver. The process may involve β -oxidation and production of acetyl-CoA, therefore disrupting the metabolic routes for synthesis and degradation of biologically relevant lipids in humans. The consequences of this need further investigation in order to understand the toxic modes of action of AsFAs and AsHCs. A few unknown arsenic species were also detected and more analyses will be done to elucidate their structure.

Additionally, the findings from *Chapter 6* suggest that the metabolism of AsFAs and AsHCs varies across tissues. The metabolites observed in HepG2 cells are different to those previously reported in Caco-2 cells (Meyer et al., 2015a). Arseno-fatty acids and AsHCs are highly permeable to cell membranes and are able to be distributed through the body (Meyer et al., 2015b, Niehoff et al., 2016, Stiboller et al., 2017). Further research on these arsenic species will require the investigation of their biotransformations in different tissue cultures.

This thesis successfully integrates a series of experiments aimed to provide a better insight into the metabolism of arsenic species in humans, particularly that of complex organic arsenic species. The findings reported within this work highlight the importance of further investigating arsenolipids and their toxicological effects in humans, as they need to be considered by food authorities when updating arsenic exposure regulations.

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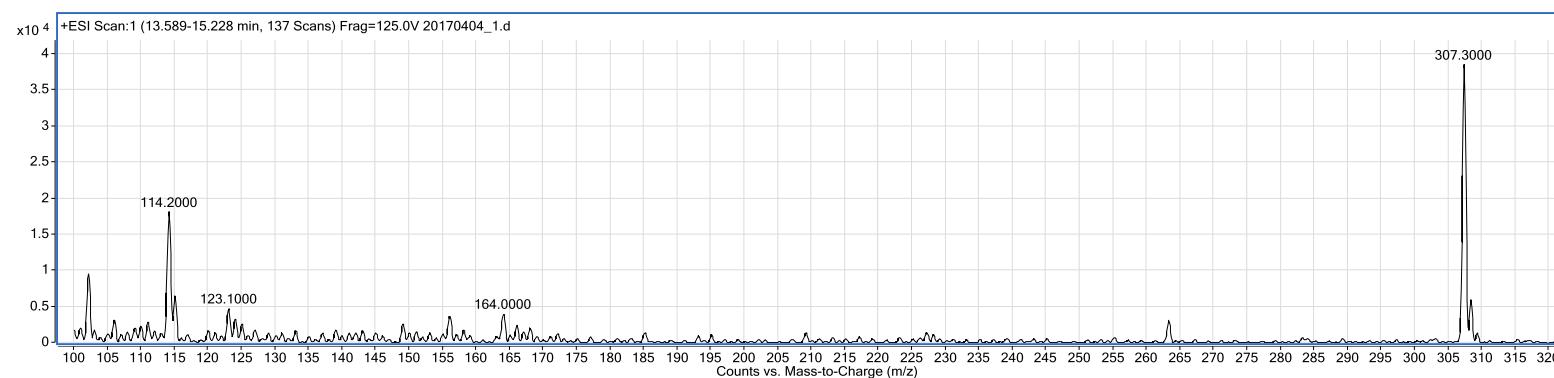
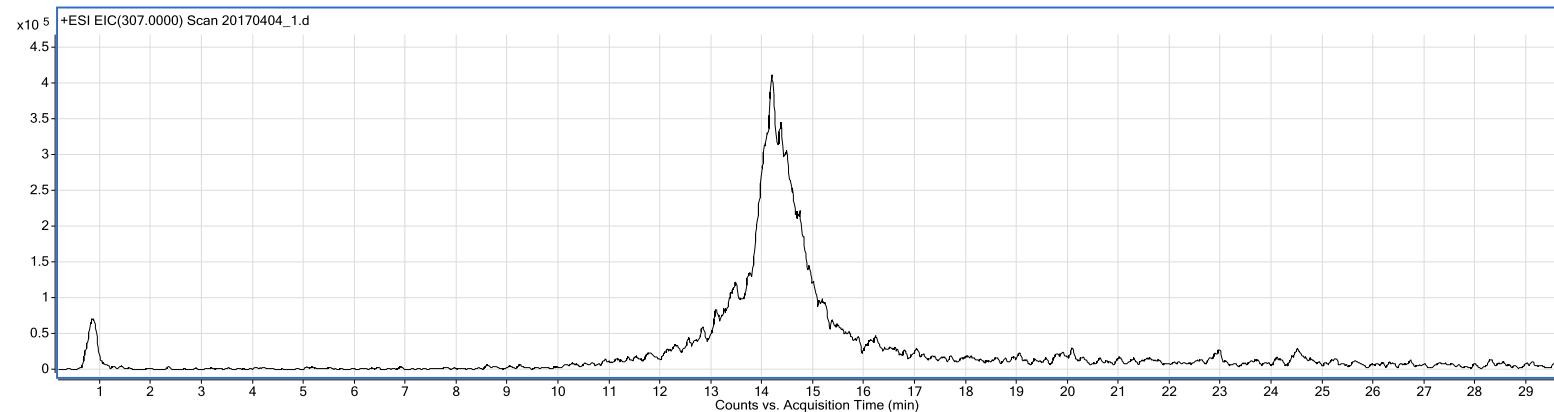
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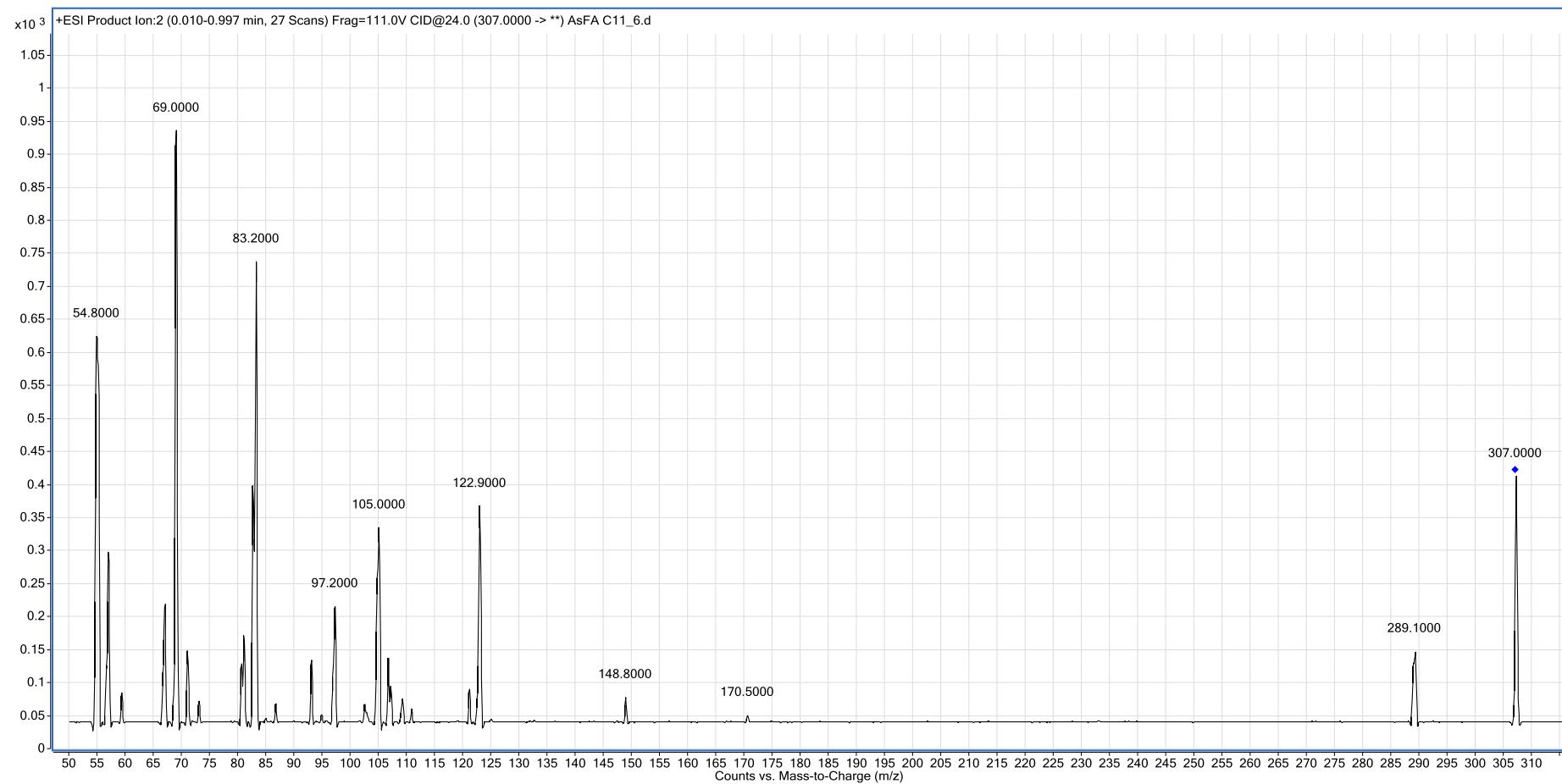
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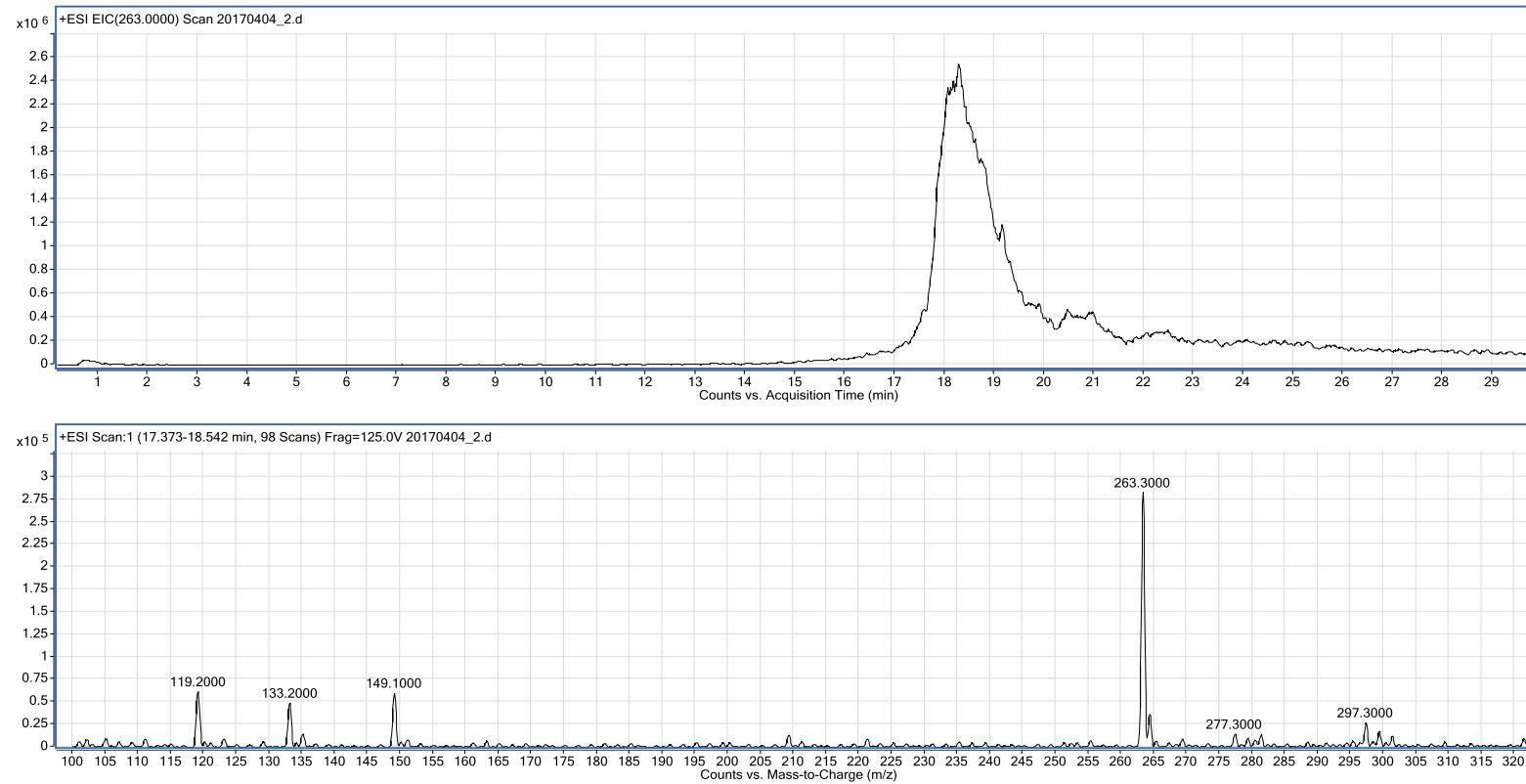
Appendices

Chapter 3

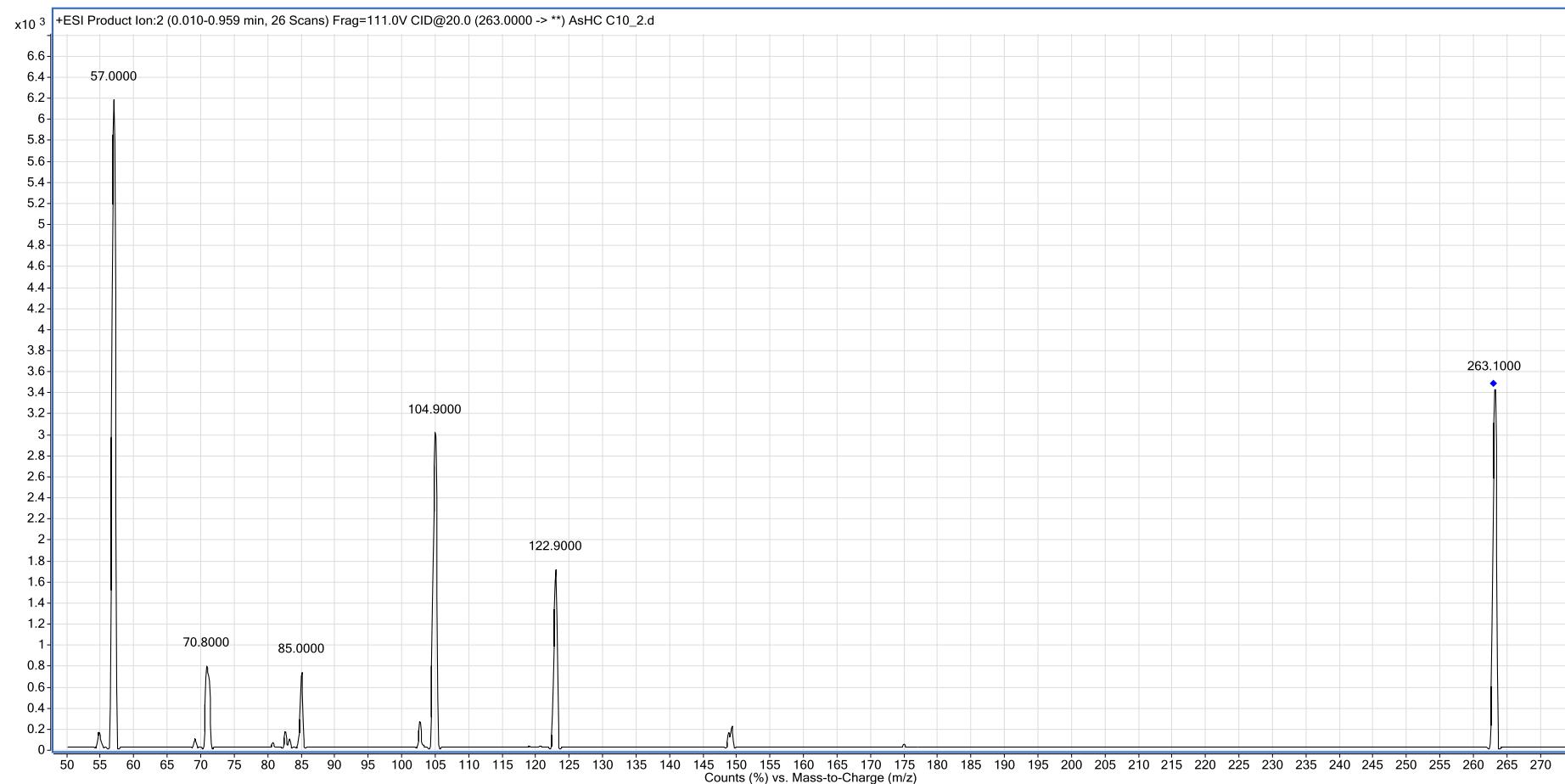
A. Extracted ion chromatogram and mass spectrum of the synthesised AsFA-C11 standard



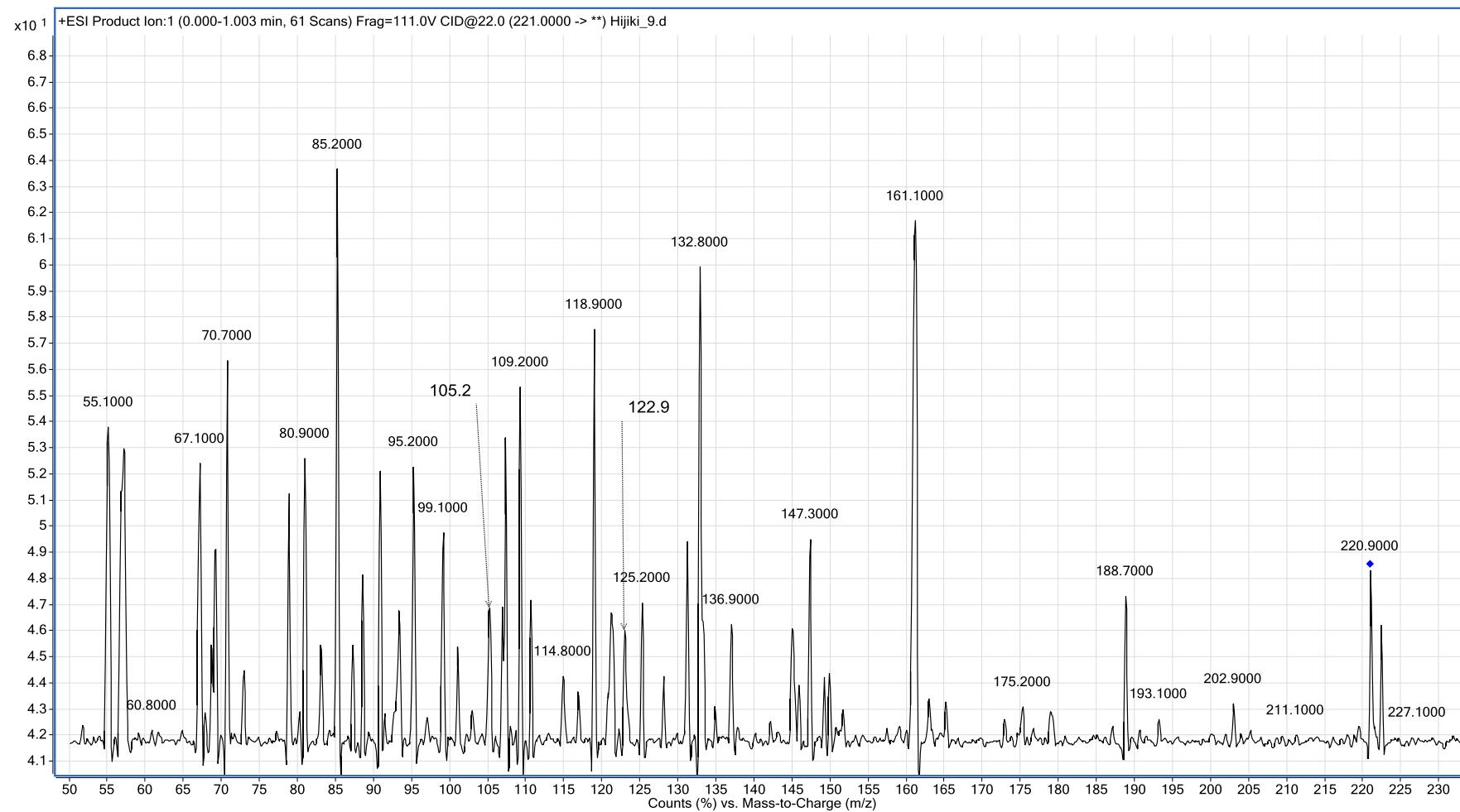
B. Product ion mass spectrum of the synthesised AsFA-C11 standard

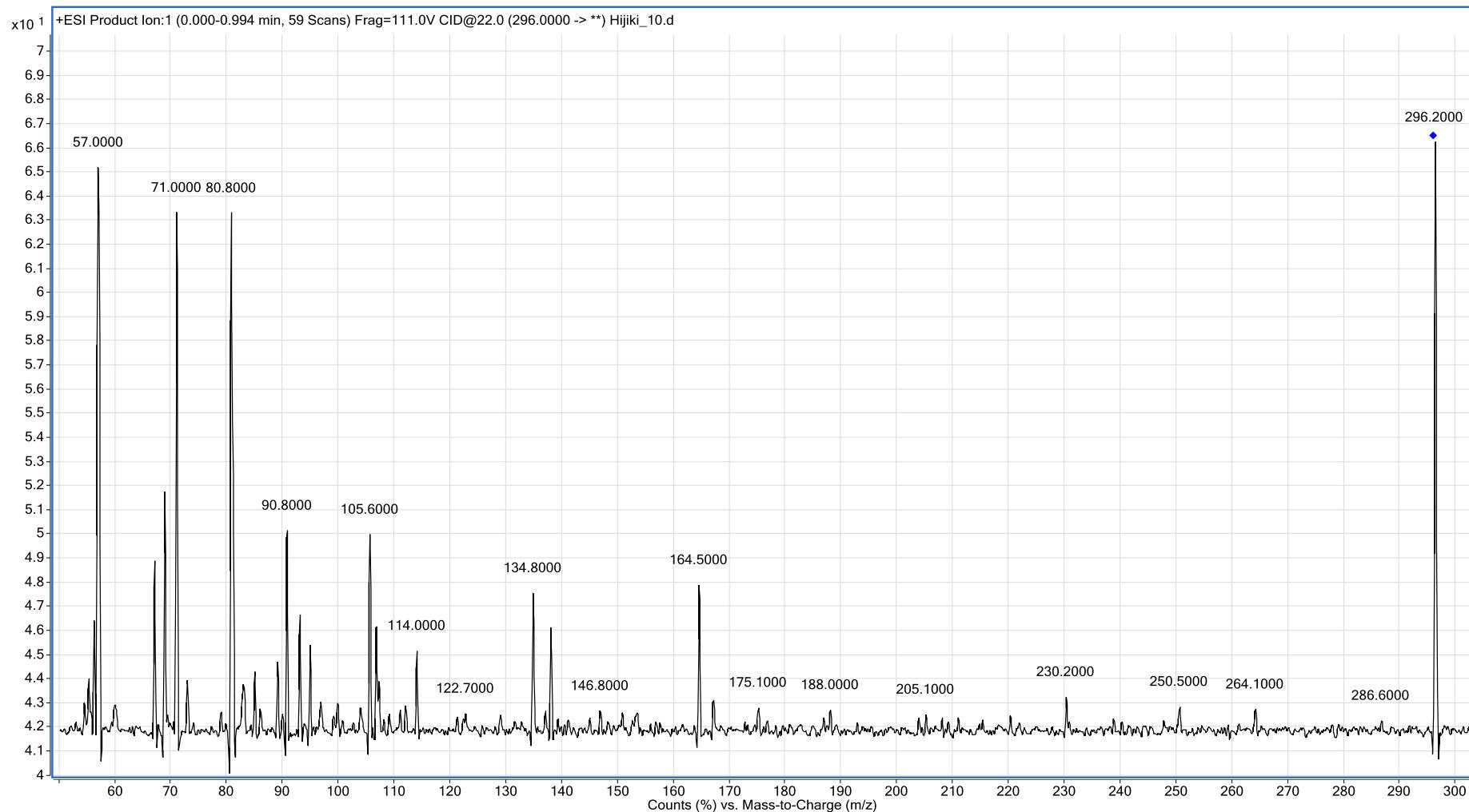
C. Extracted ion chromatogram and mass spectrum of the synthesised AsHC-C10 standard

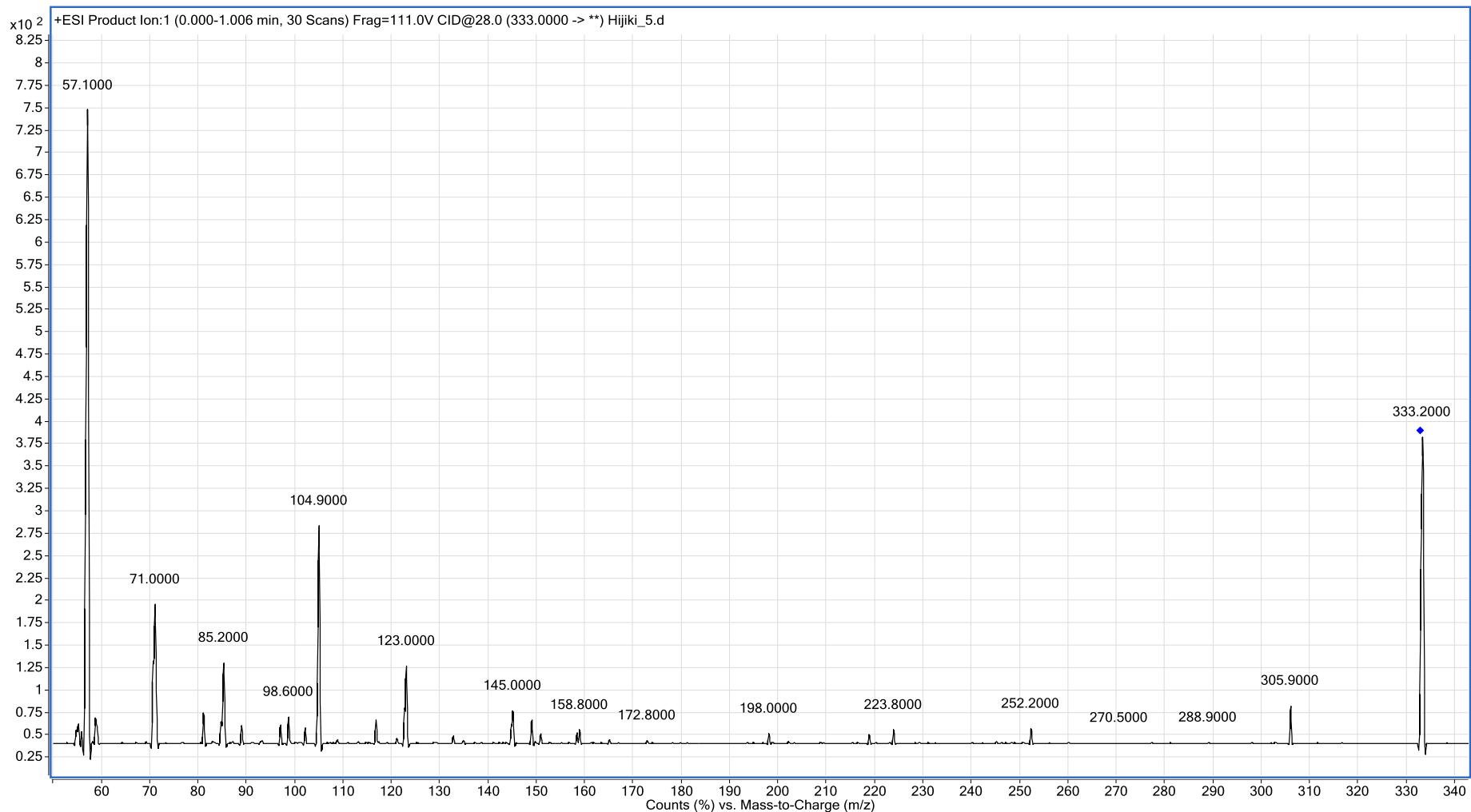
D. Product ion mass spectrum of the synthesised AsHC-C10 standard

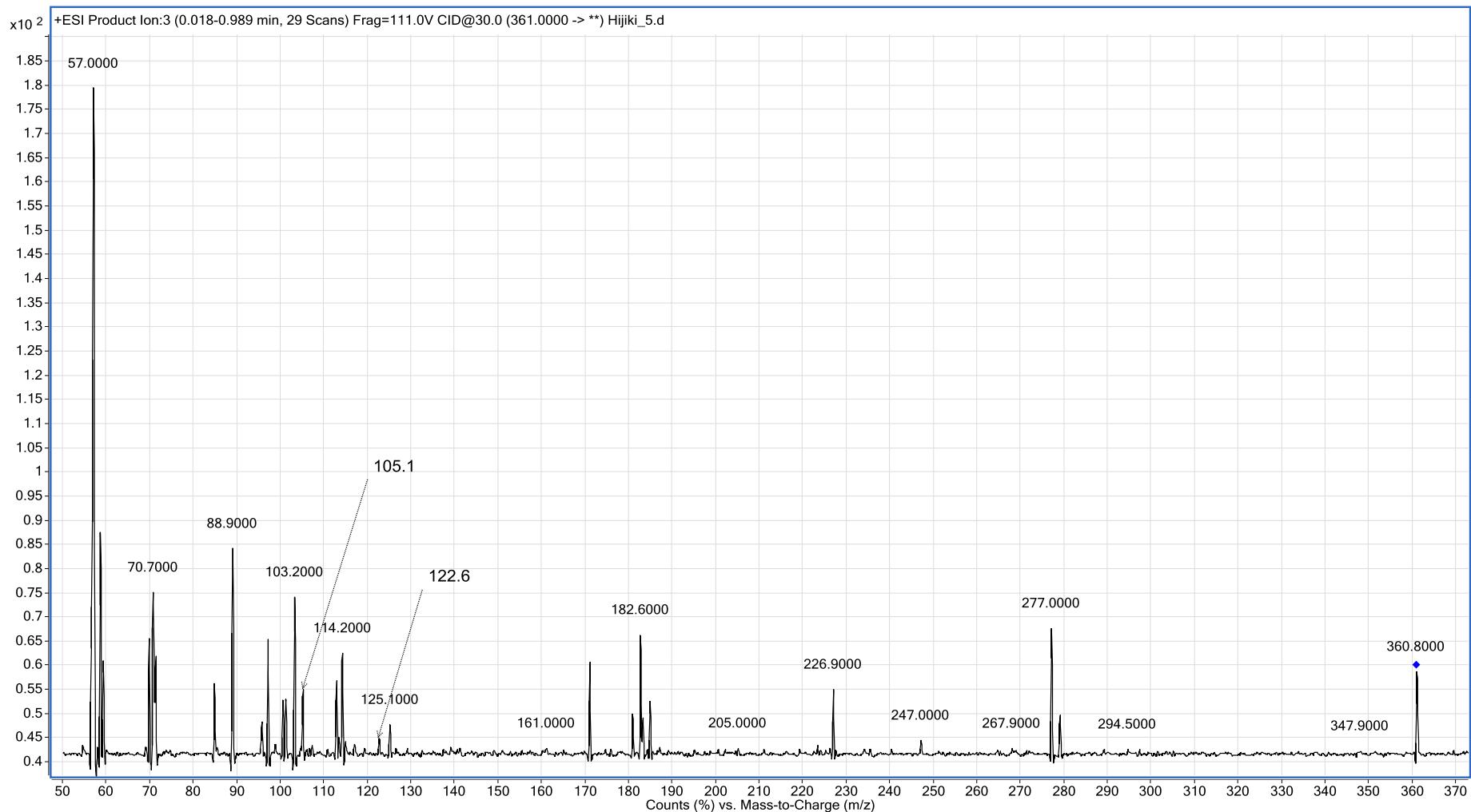


E. Product ion mass spectra of the precursor ions of interest in hijiki seaweed

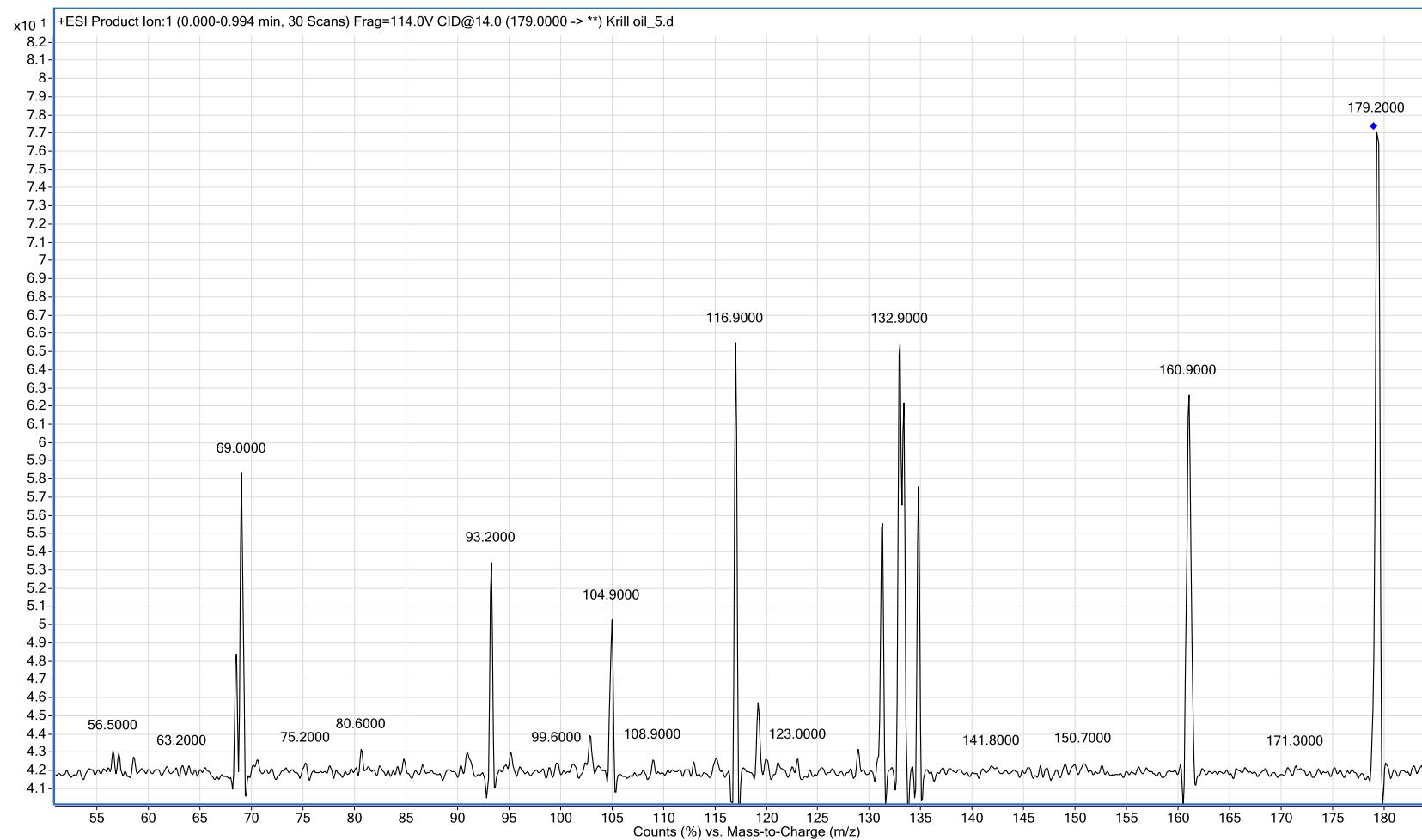


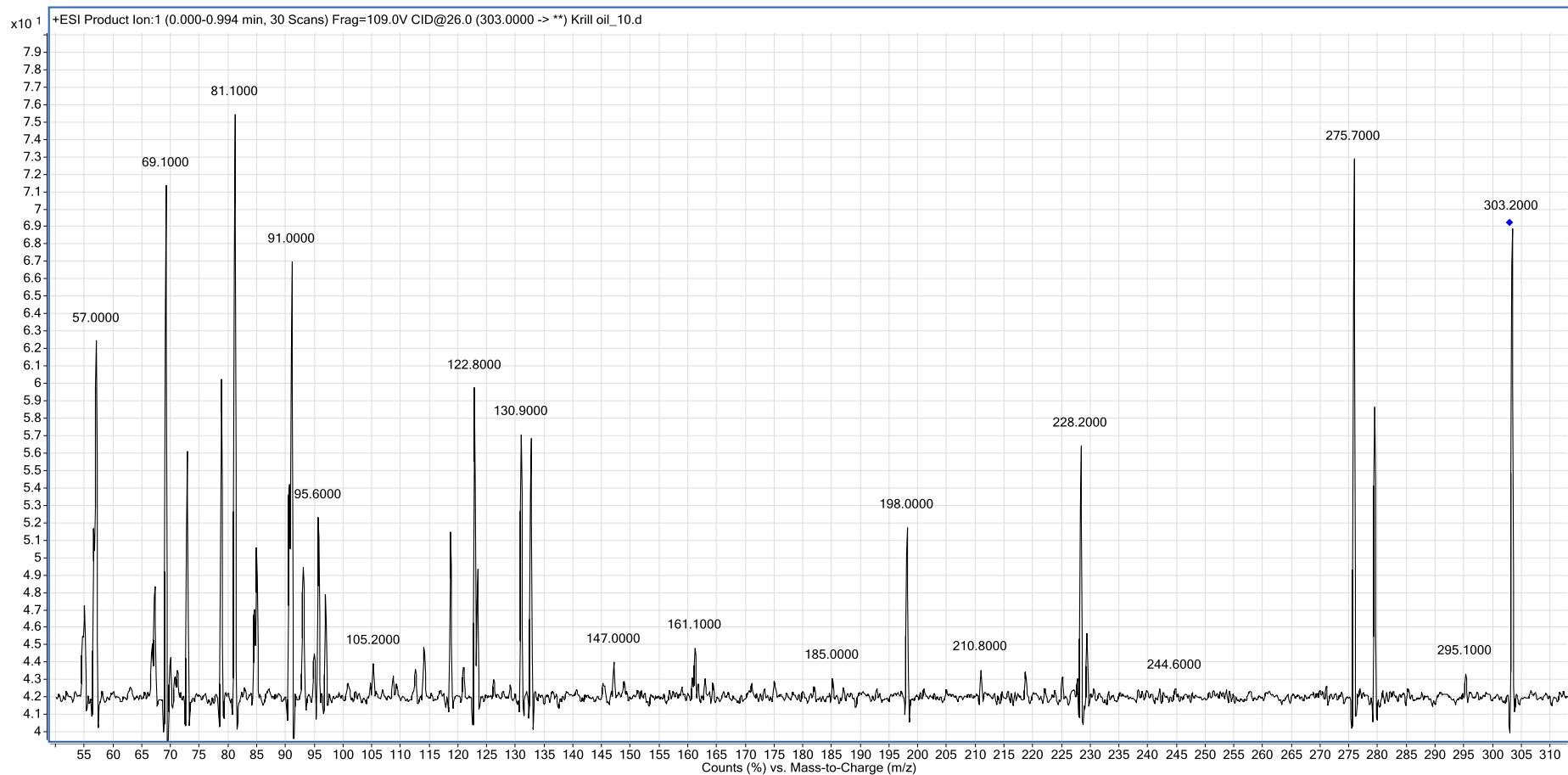


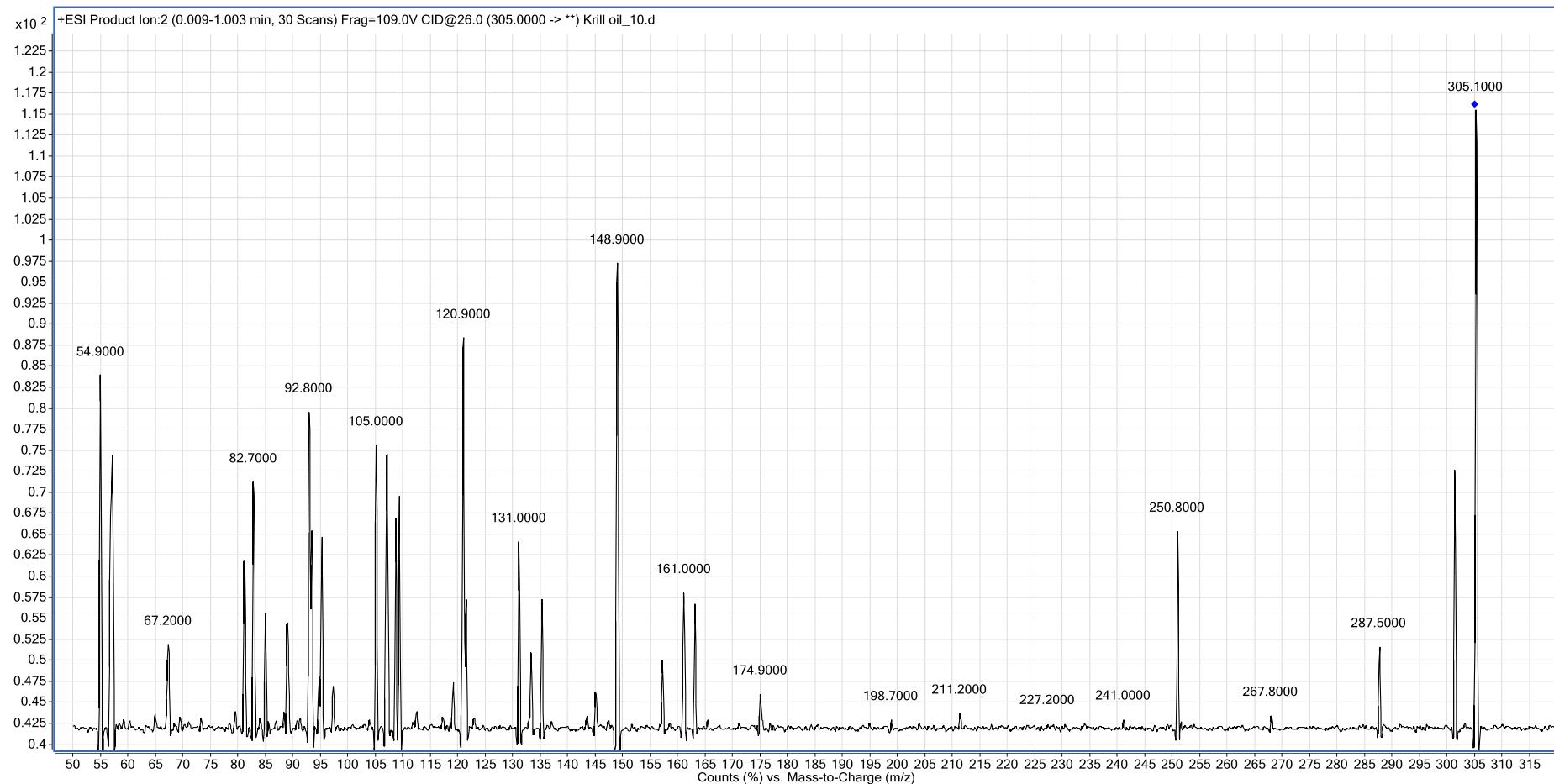


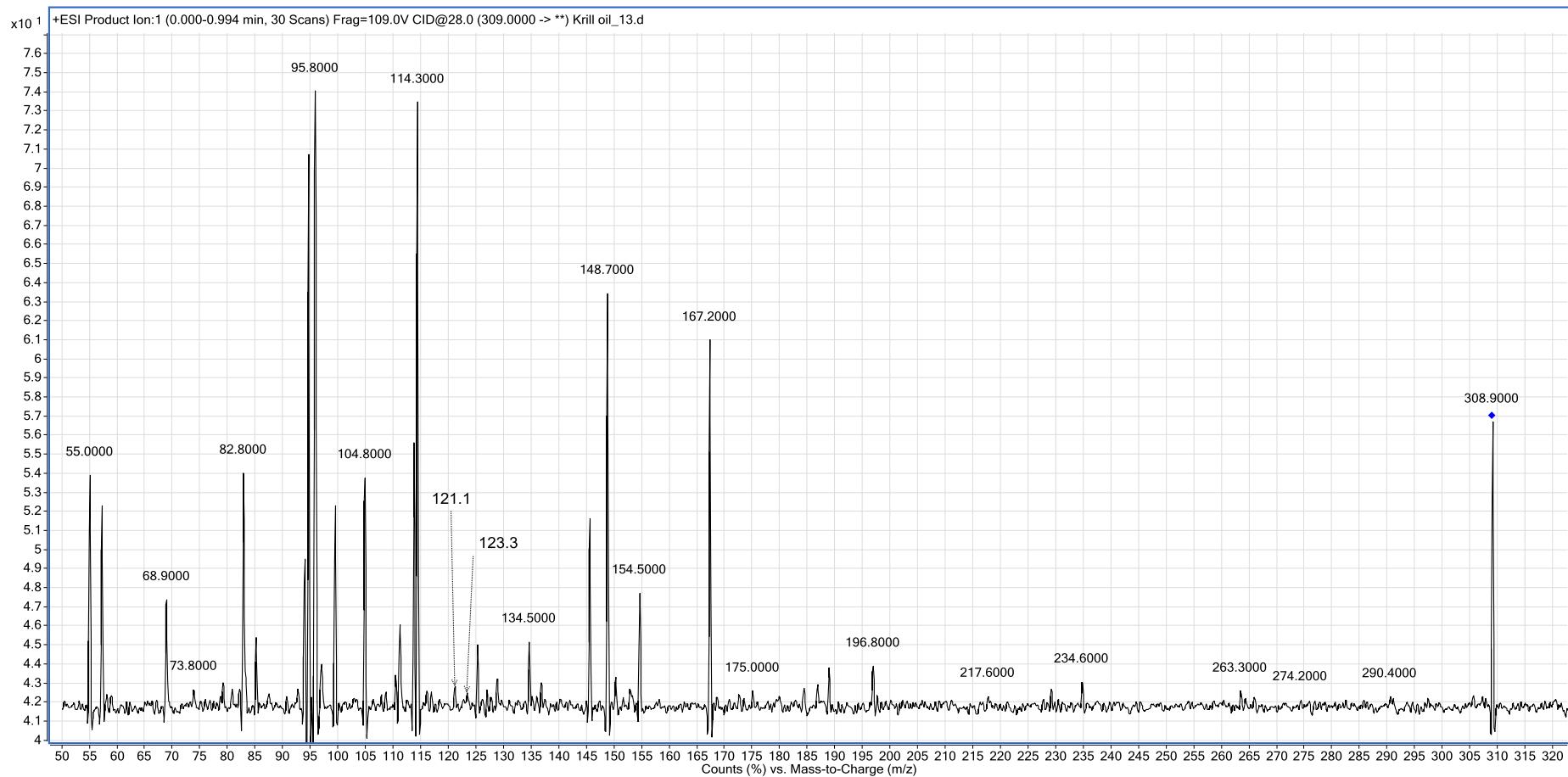


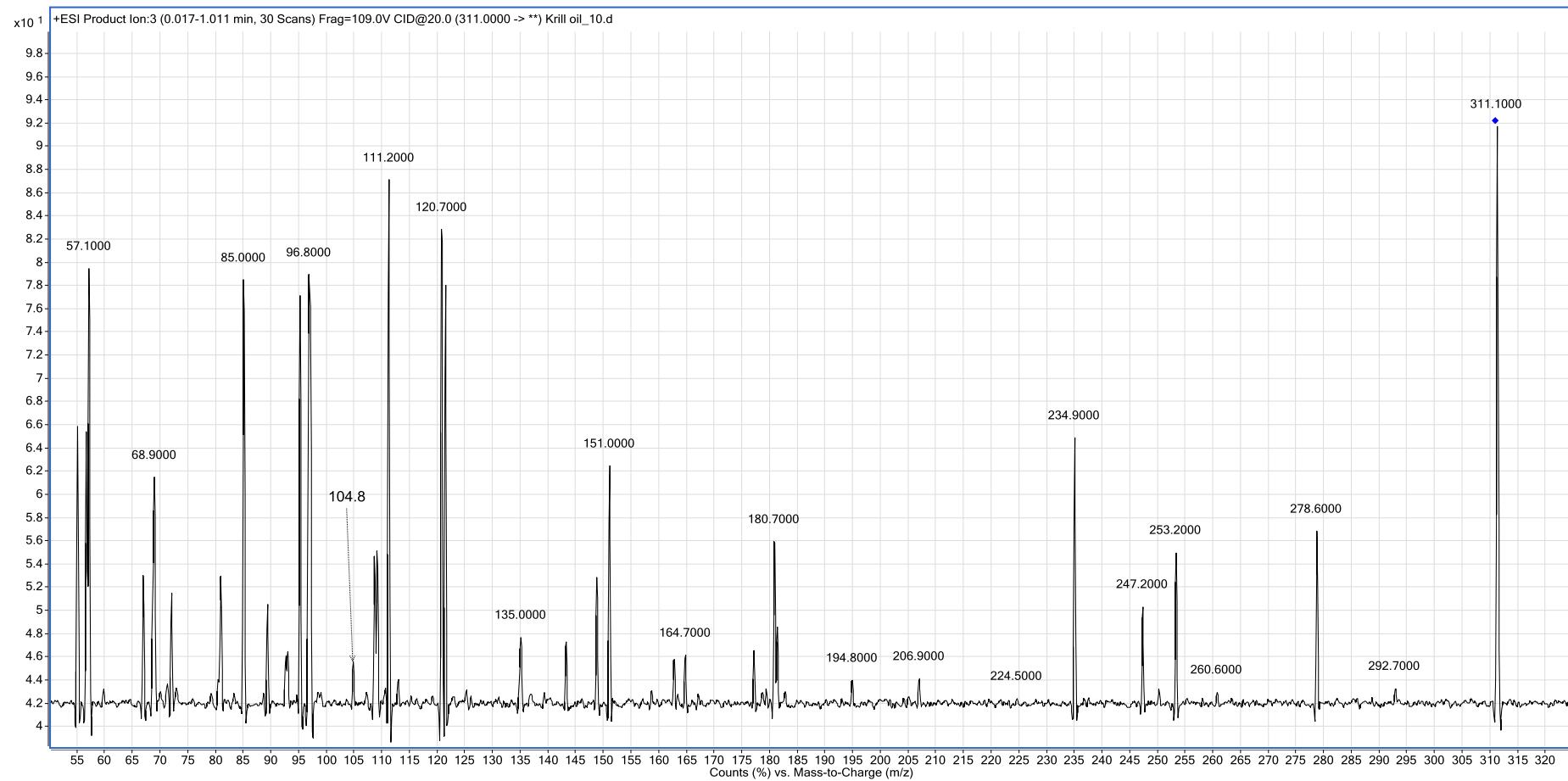
F. Product ion mass spectra of the precursor ions of interest in krill oil

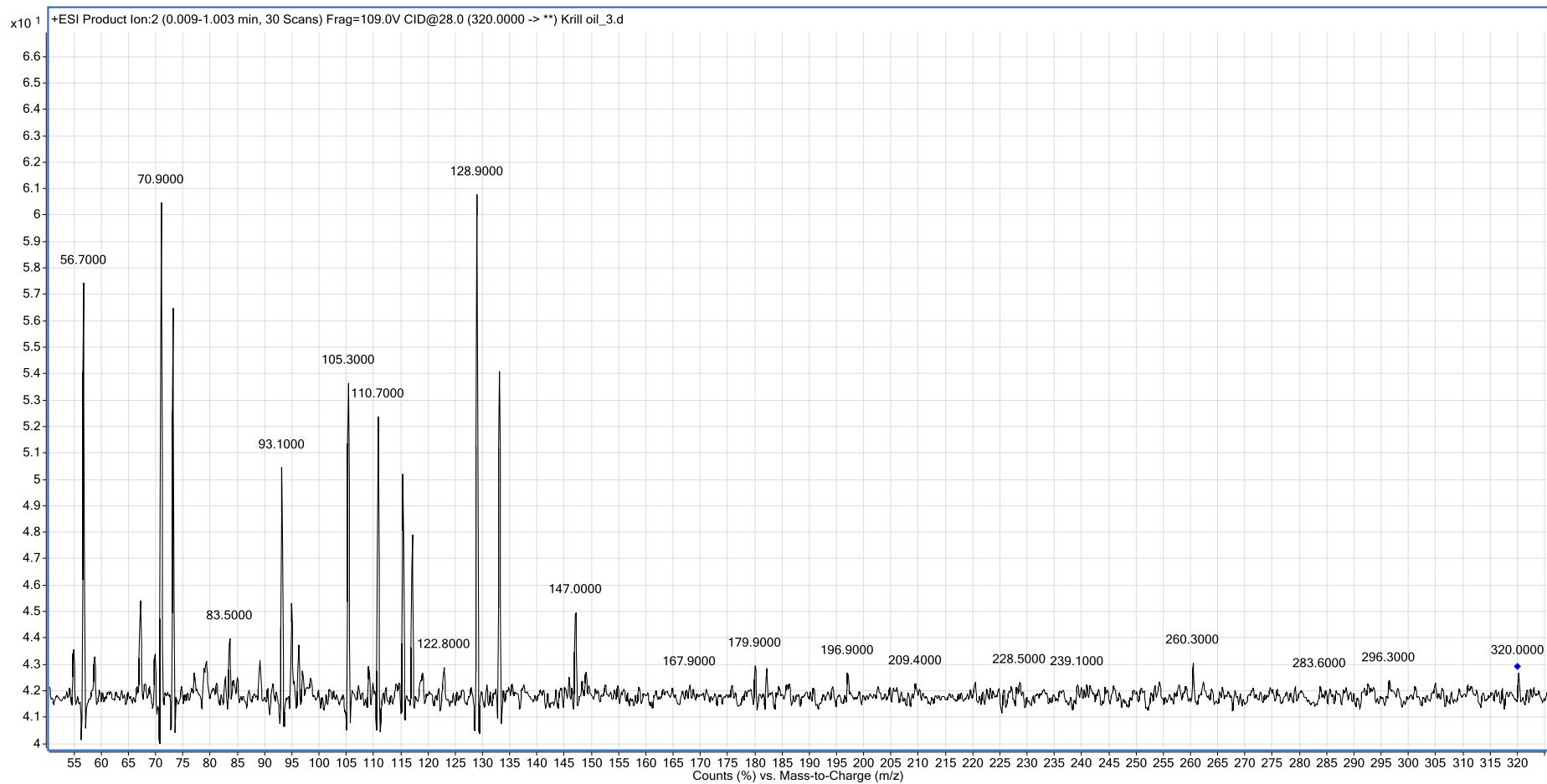


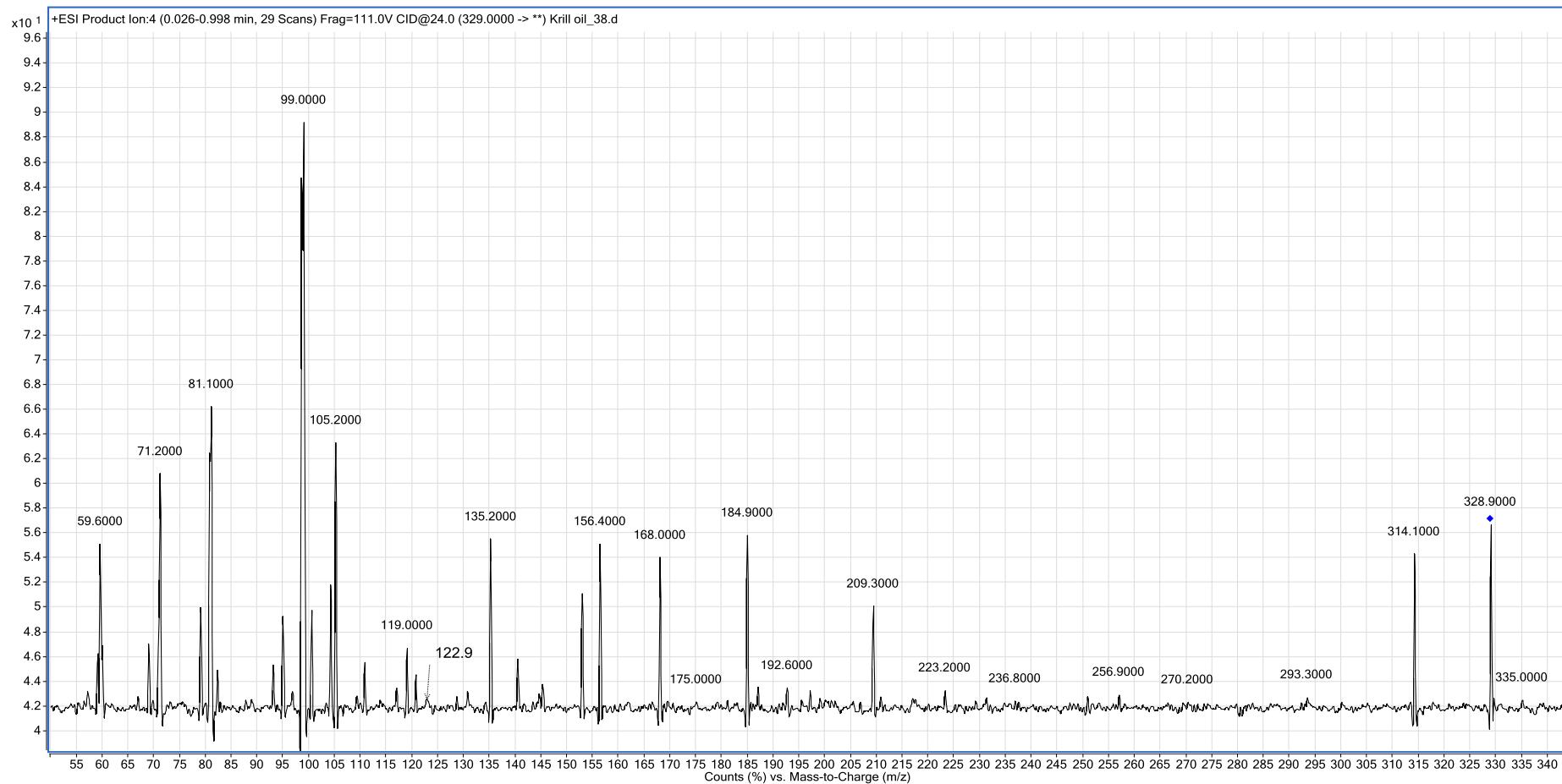


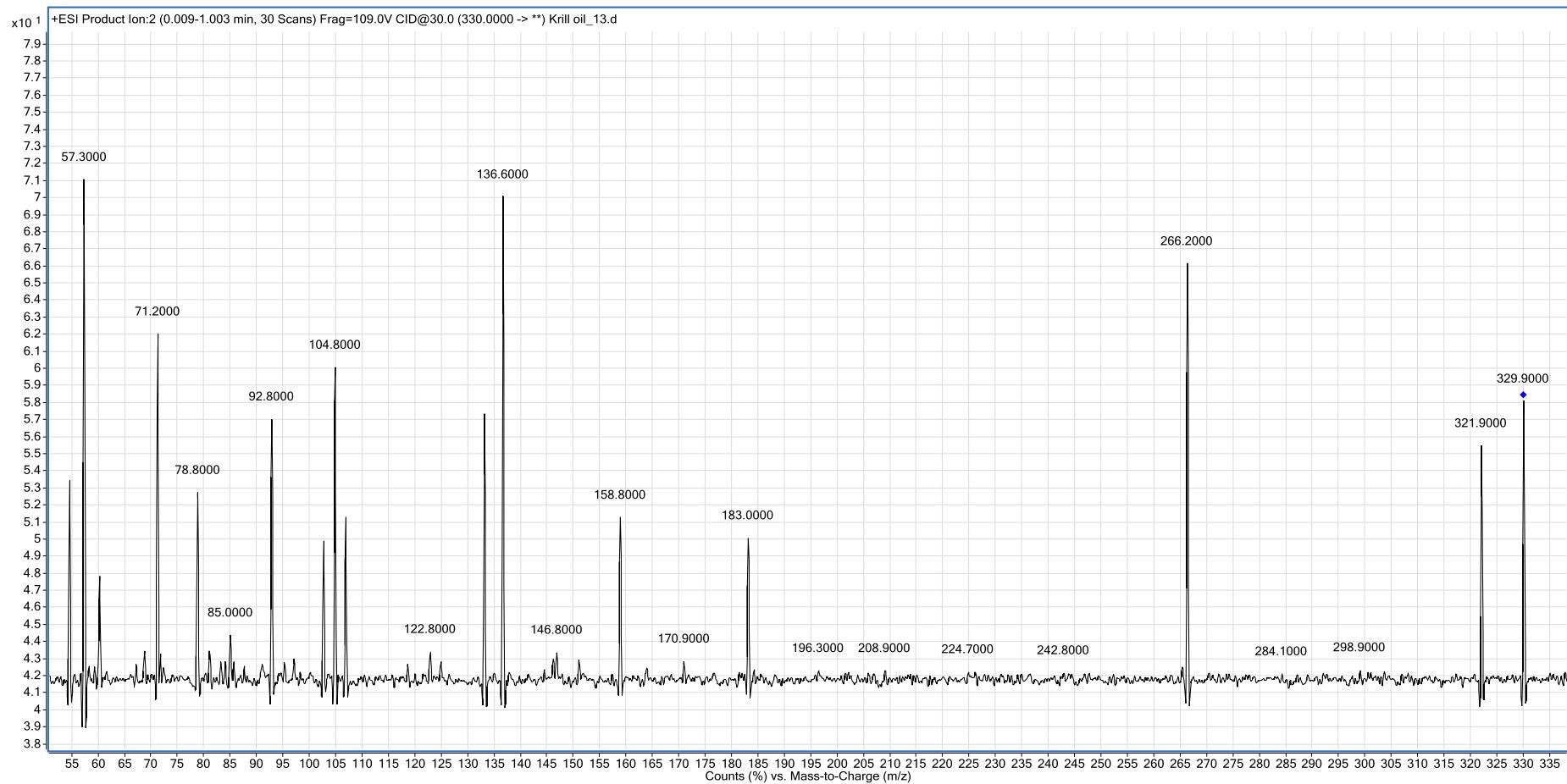


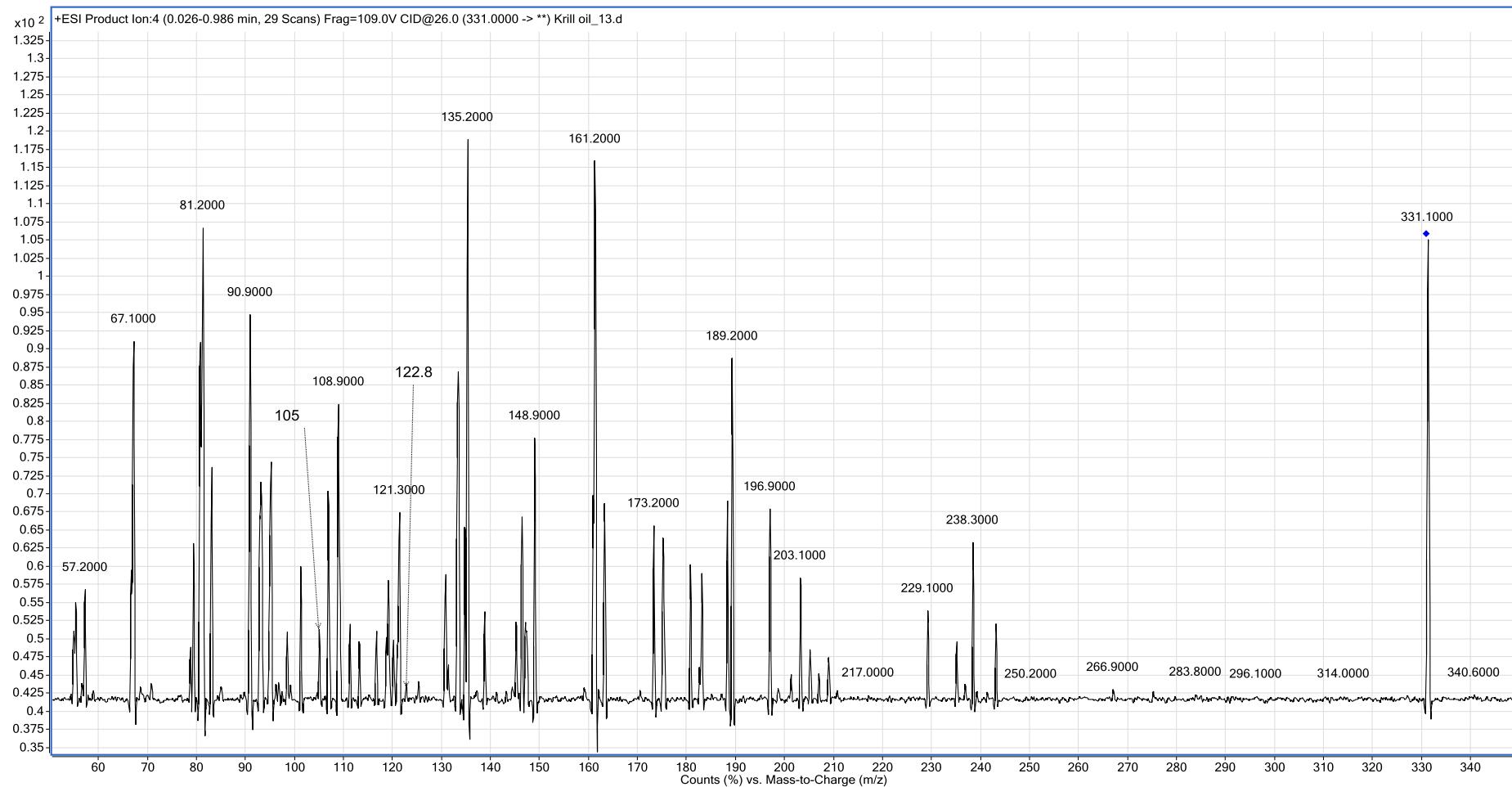


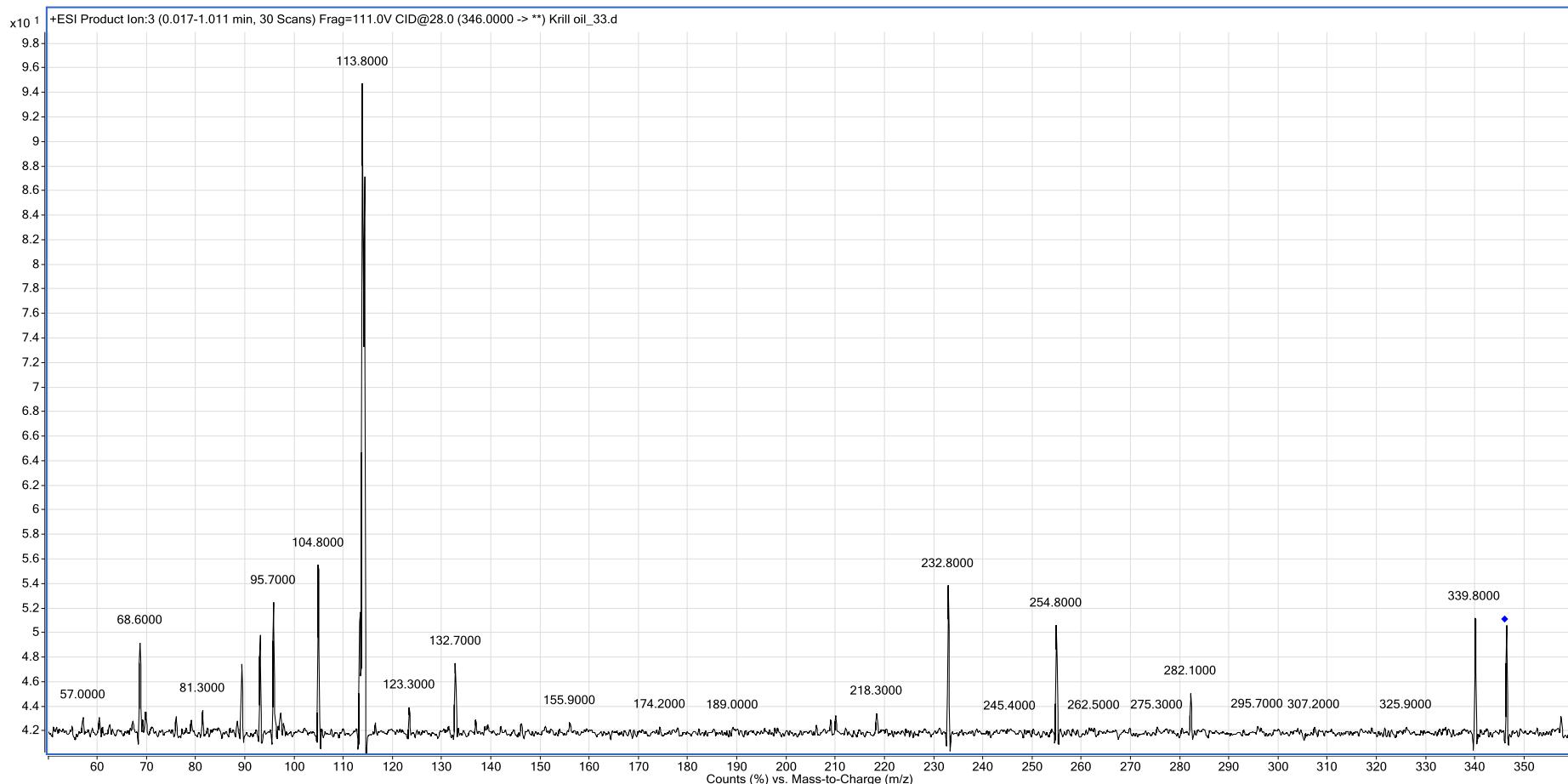


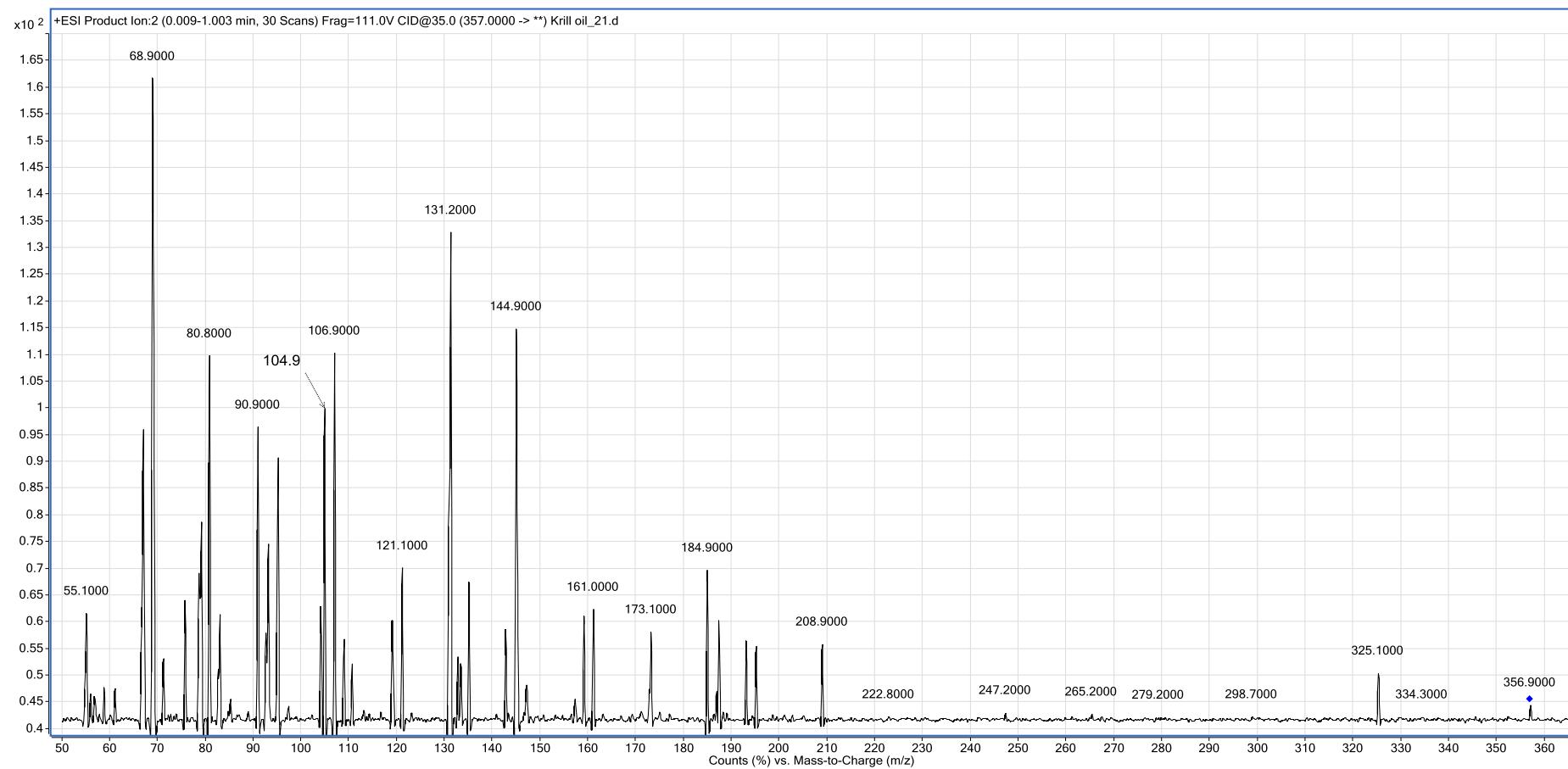


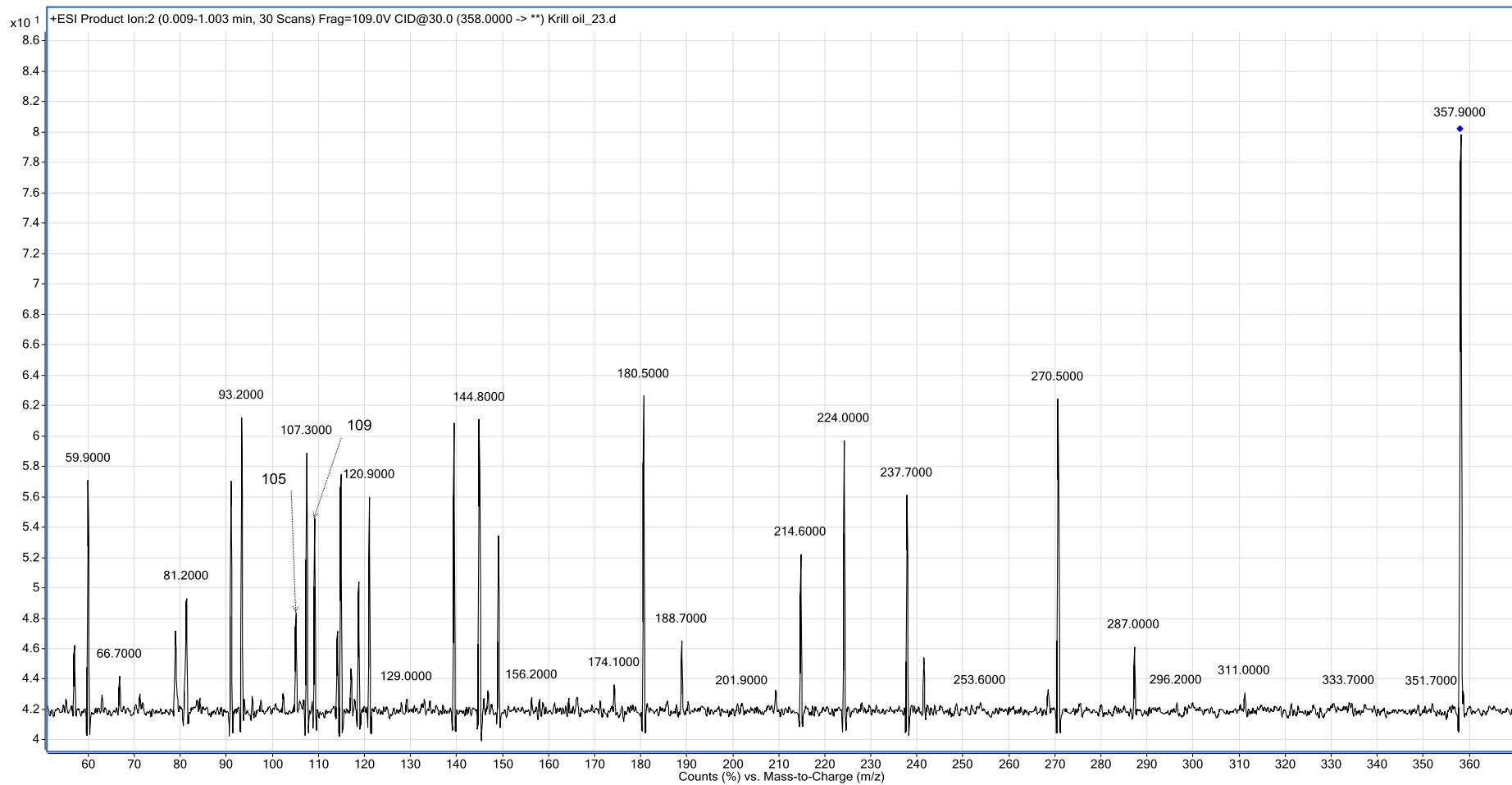


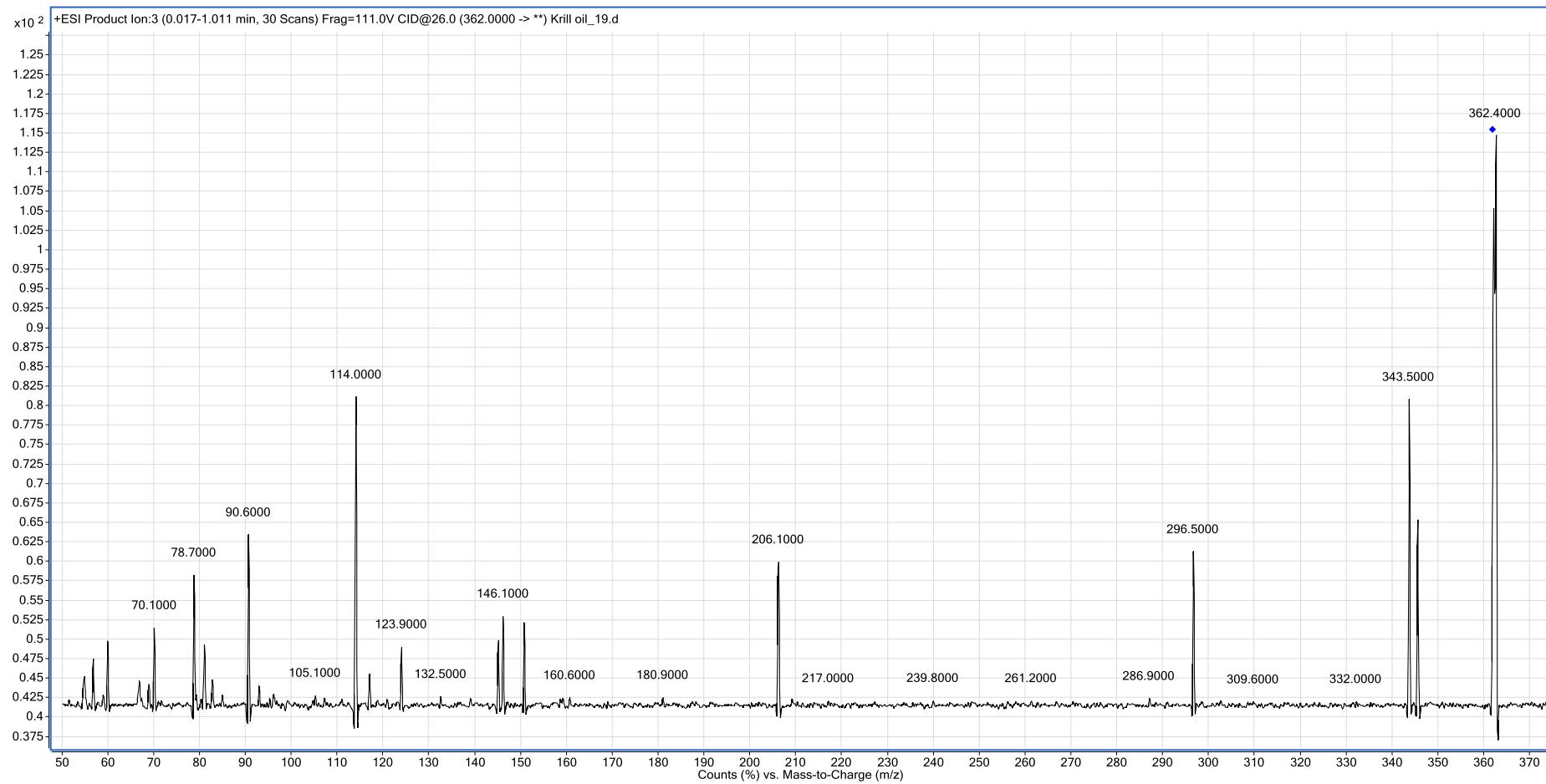


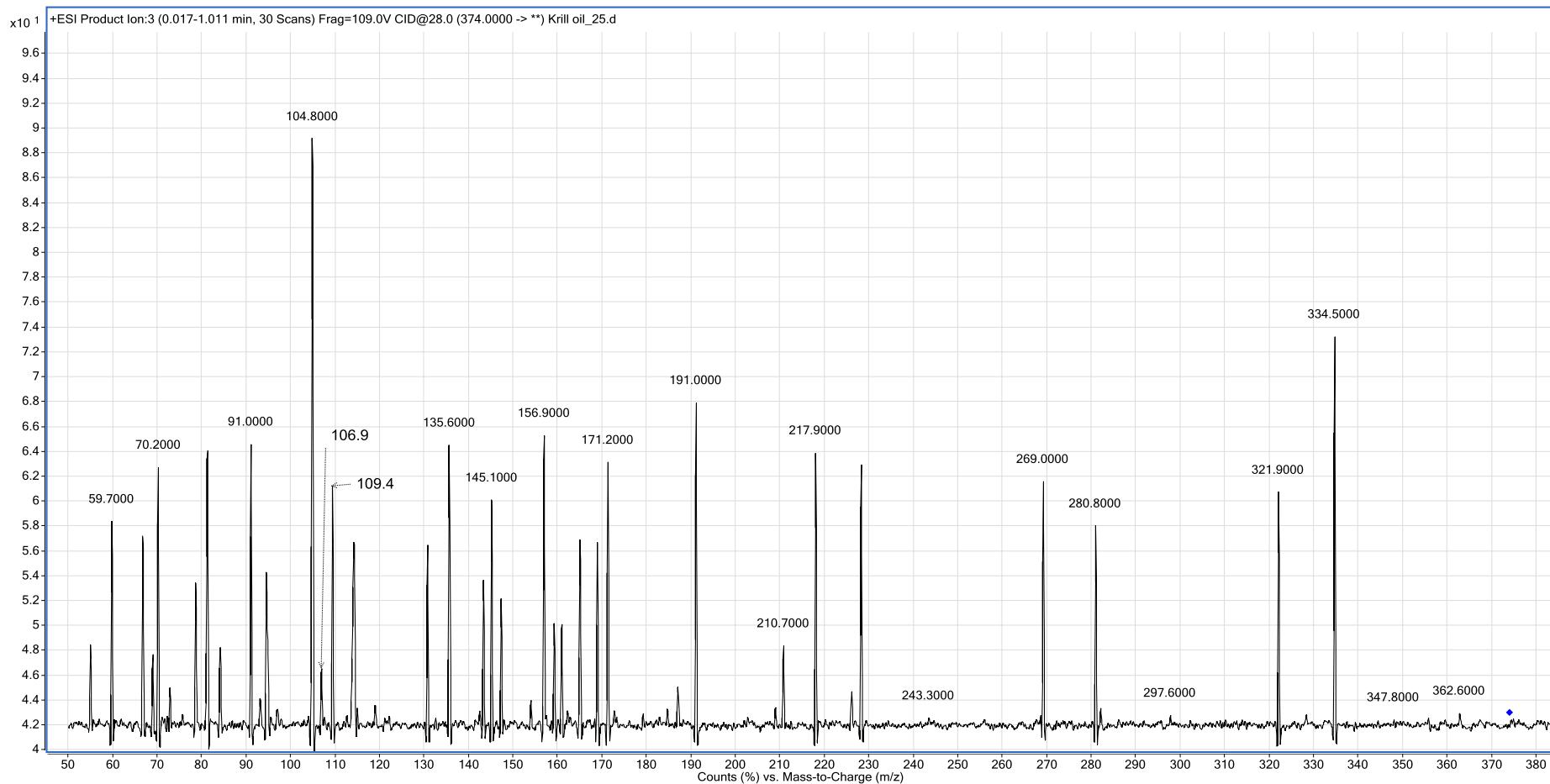


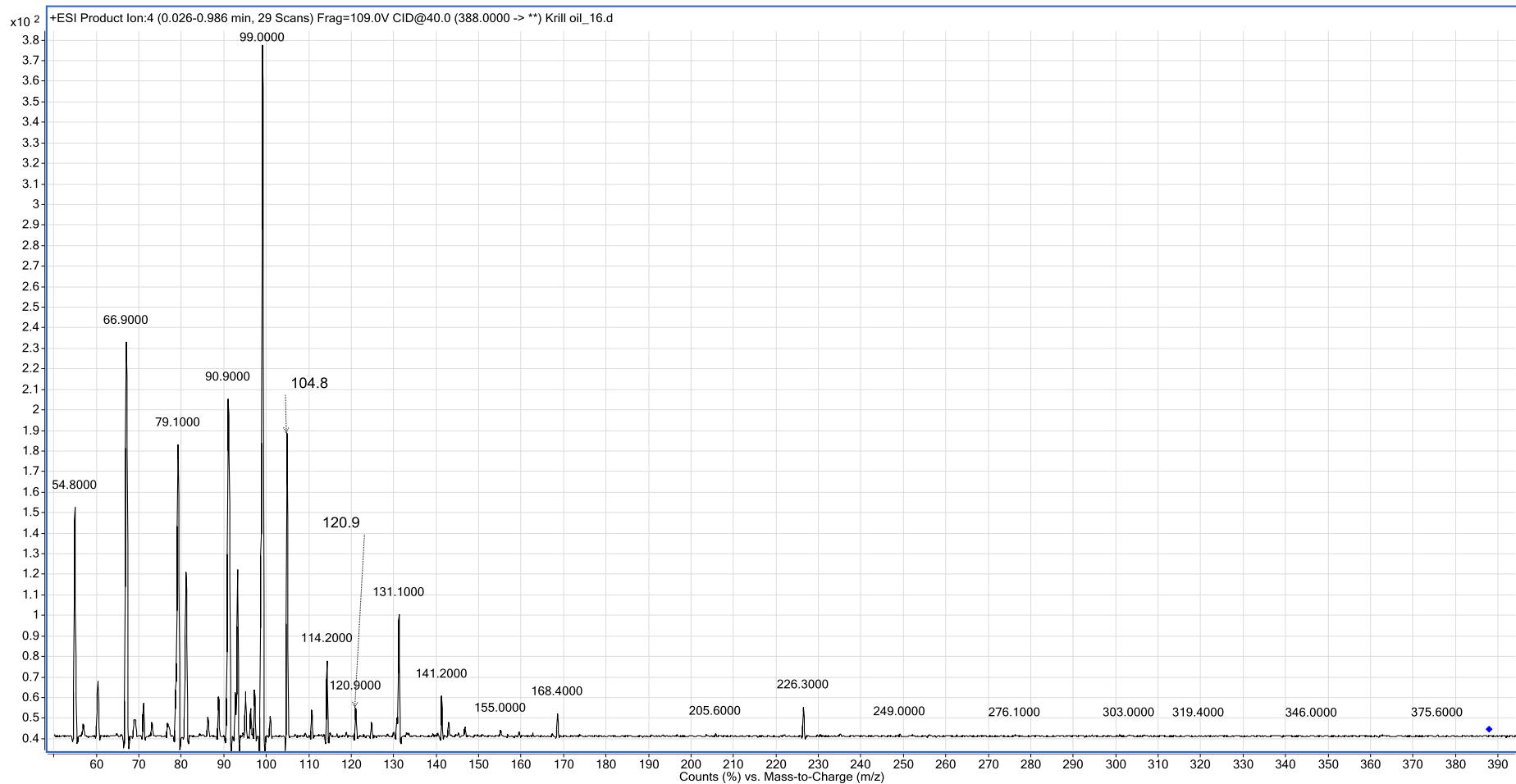


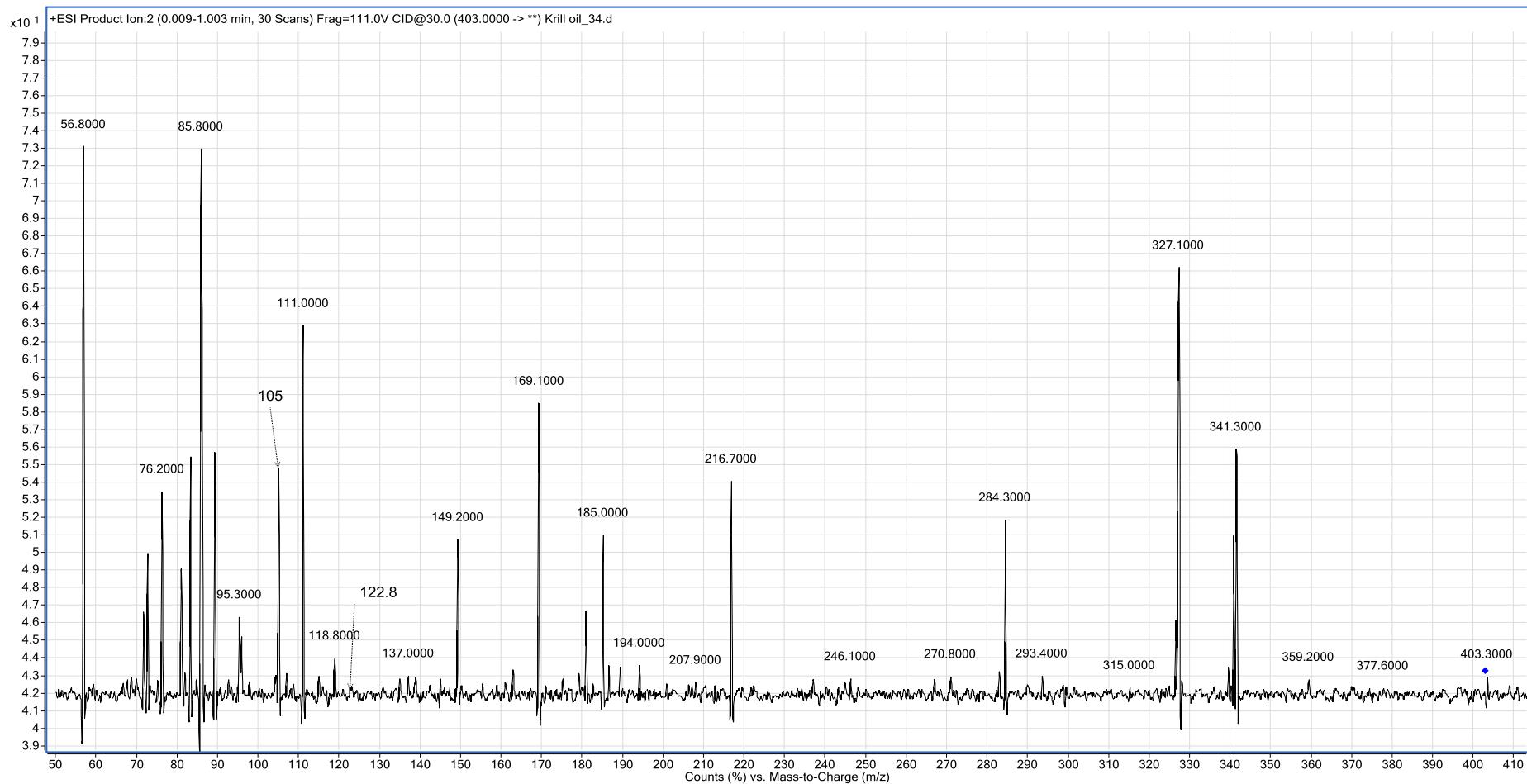


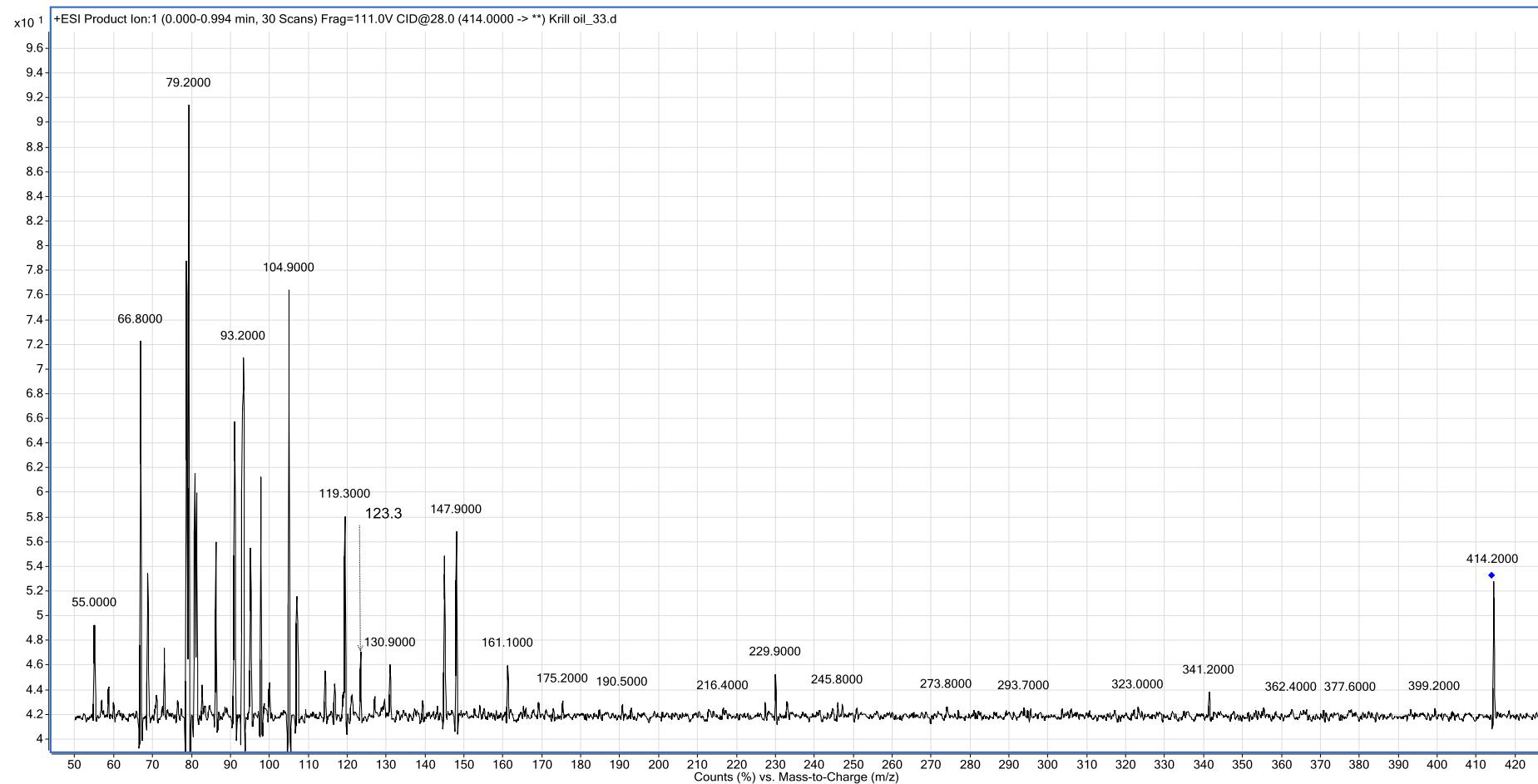


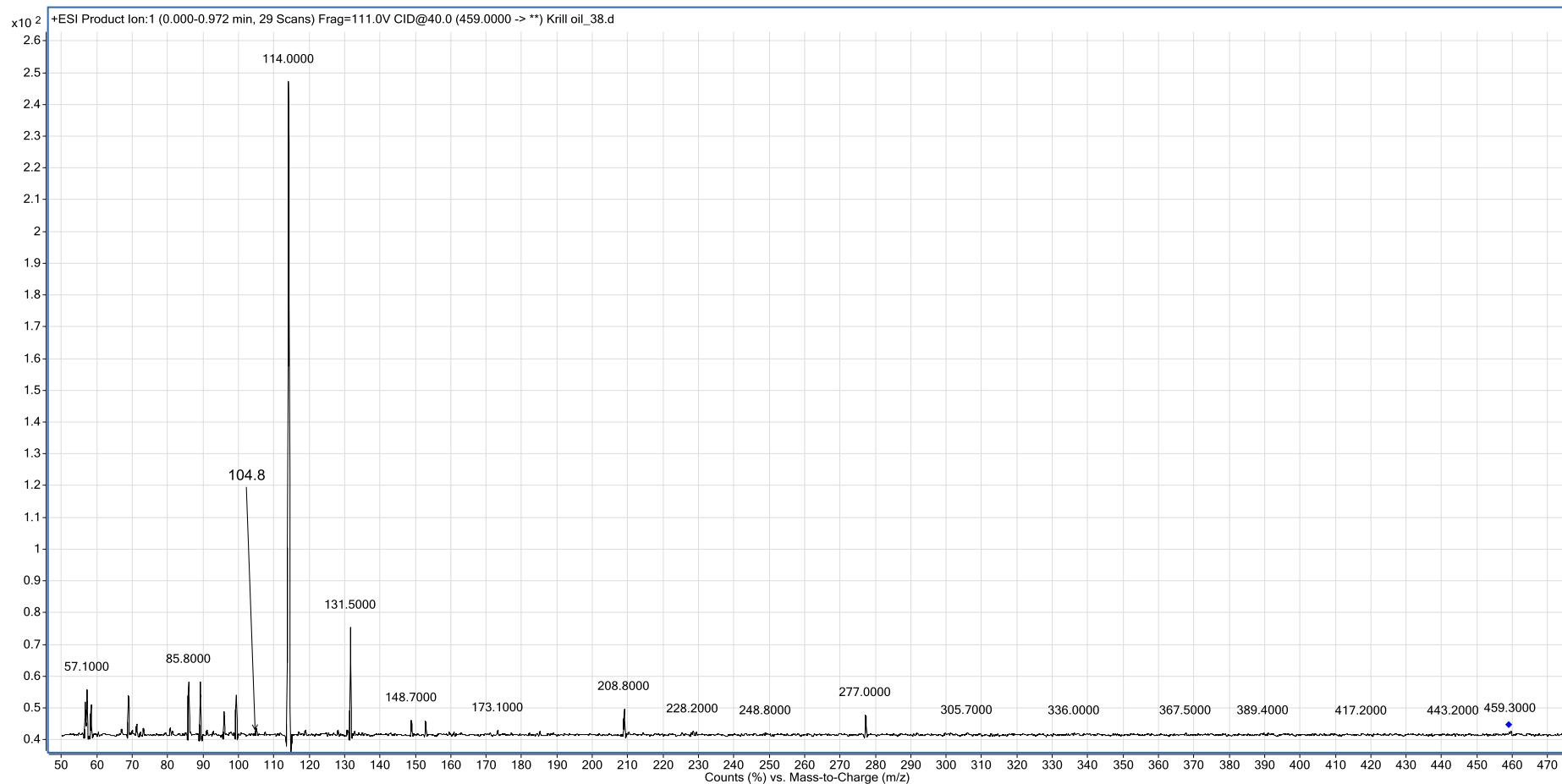


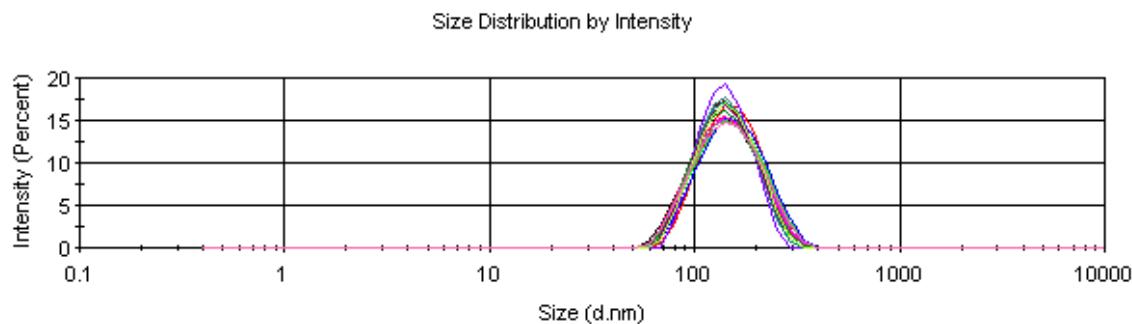
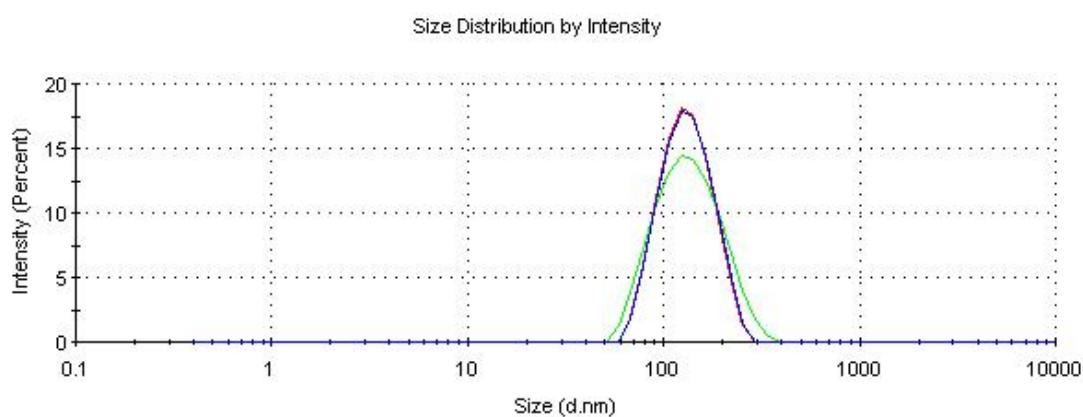






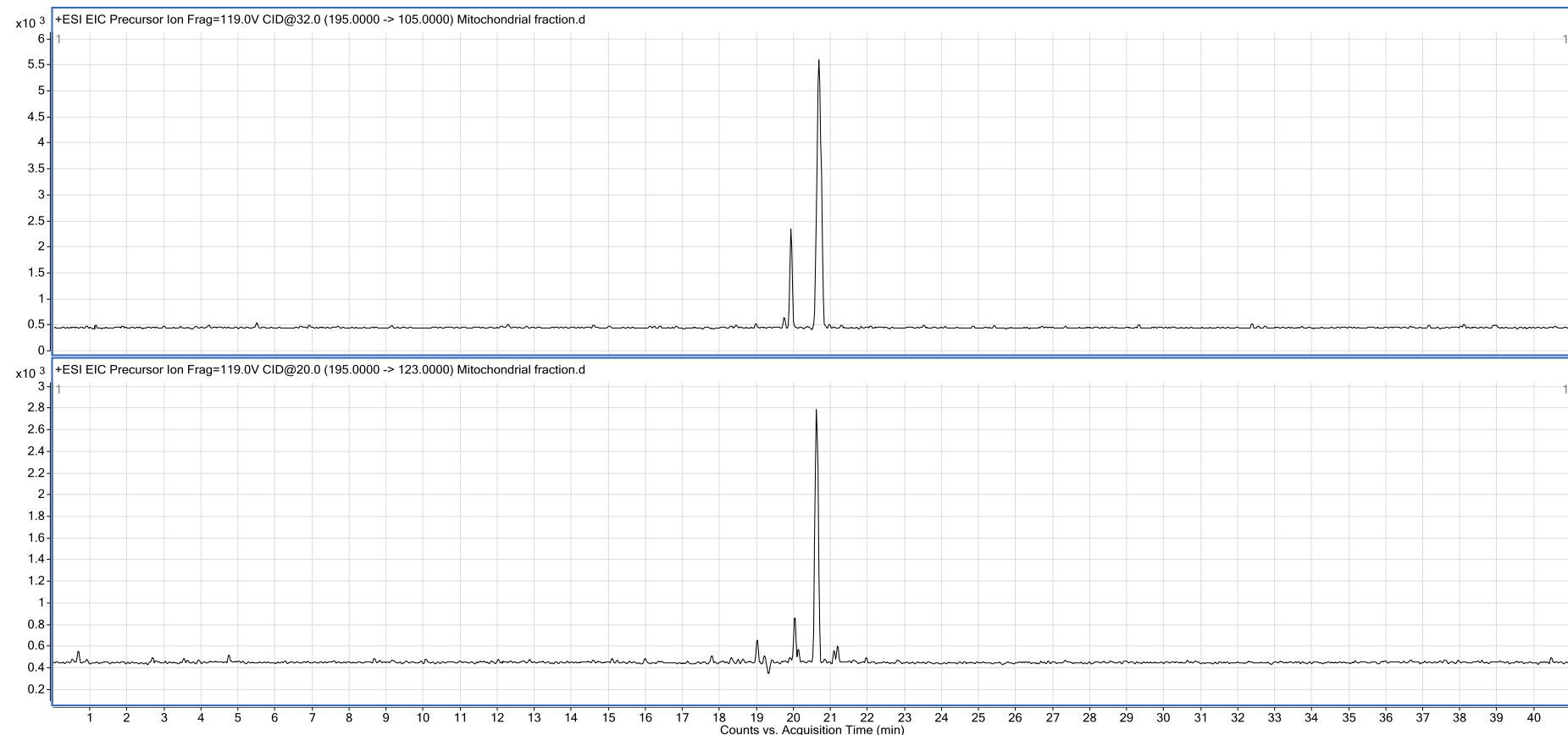


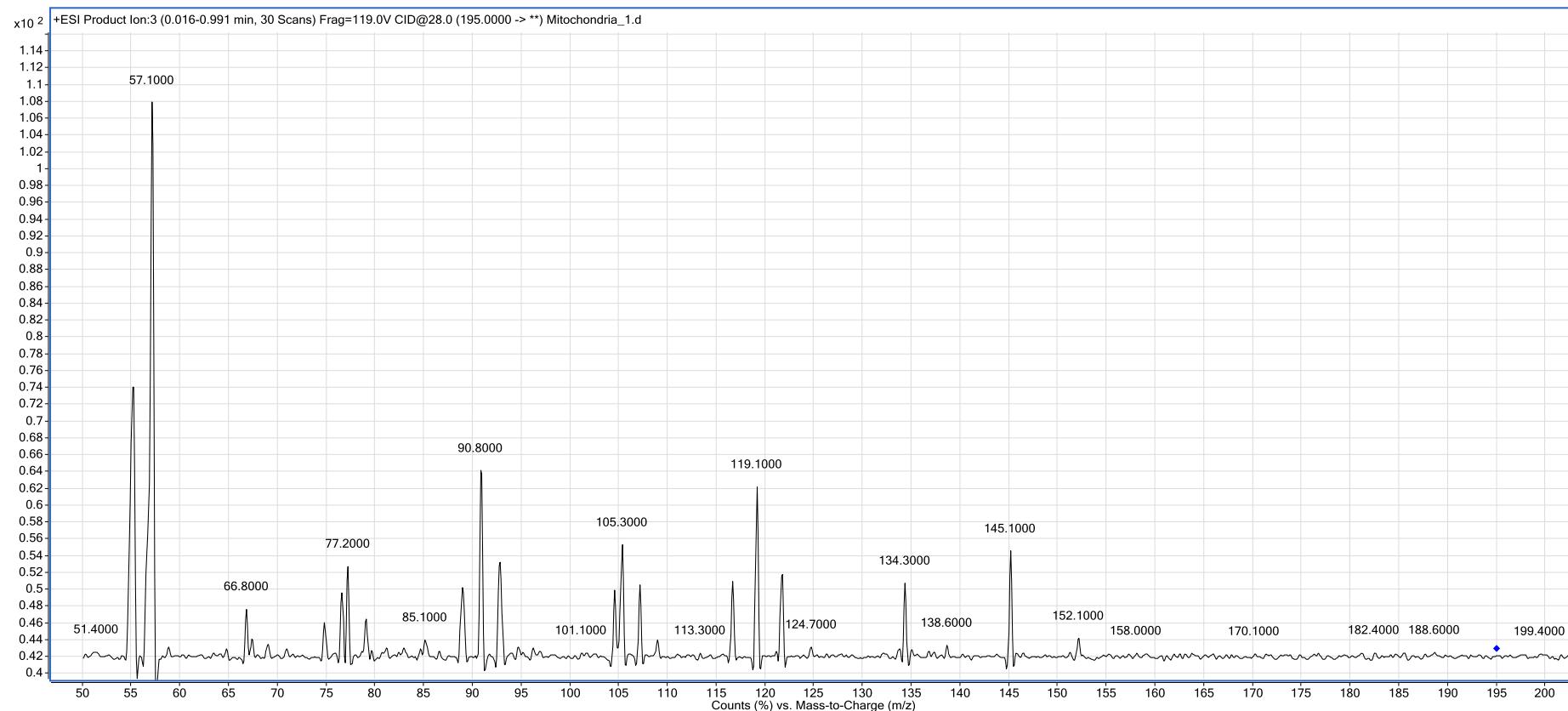


Chapter 4**A. Size distribution of PC100 liposomes****B. Size distribution of PE-PC-CL (30-60-10) liposomes**

Chapter 6

A. Precursor ion mass spectra of the mitochondrial fraction of cells after exposure to AsFA-C11



B. Product ion mass spectra of the precursor ion with m/z 195 in the mitochondrial fraction of cells after exposure to AsFA-C11

C. Reverse-phase chromatograms of the mitochondrial fractions of HepG2 cells after exposure to AsFA-C11 and AsHC-C10.

