Sebastian Tassoti BSc

Determination and Enzymatic Hydrolysis of Arsenic-Containing Phosphatidylcholines in Food Supplements

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Univ.-Prof. Mag. Dr.rer.nat. Kevin A. Francesconi

Institute of Chemistry

8010 Graz, Universitätsplatz 1/I (Analytische Chemie)

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DANKE!

To my friends and family

Abstract

Arsenic occurs in marine waters, primarily as the inorganic species arsenate. Marine animals are able to accumulate arsenic, ingested via the food chain, and contain high levels of arsenic, possibly posing a health risk to consumers of seafood. Arsenic in marine organisms is mainly present as organoarsenicals, i. e. arsenic bound covalently to carbon in an organic molecule. Although water-soluble organoarsenicals are often major arsenic species in marine organisms, research over the last ten years has focused on lipid-soluble compounds. Recently, arsenic-containing phosphatidylcholines (AsPCs) were identified in a sample of herring caviar. The aim of this thesis was to develop an analytical method for determination of the position of the arsenic-containing fatty acids in the AsPCs. Four samples including food (Kombu) and food supplements (krill oil capsules, blue whiting oil, and herring caviar capsules) were studied. The method included a hexane/methanol partitioning of the samples (after a preliminary extraction step in the case of Kombu) and treatment of the aqueous methanol phase with phospholipases A_1, A_2, C and D. The samples as well as the hydrolysates were analyzed by HPLC/ICPMS and HPLC/ESI-MS. With this method, it was possible to identify 26 known arsenic species in the samples, including ten arsenic-containing fatty acids, five arsenic-containing hydrocarbons, five arsenosugar phospholipids, five arsenic-containing phosphatidylcholines and one arsenic-containing phosphatidylethanolamine. Furthermore, 23 new AsPCs were identified and characterized. The hydrolysis experiments showed that the AsFAs incorporated into AsPCs are exclusively bound in *sn*-2-position.

List of Abbreviations – general

ESI-MS	Electrospray Ionisation – Mass Spectrometry
HPLC	High Performance Liquid Chromatography
ICPMS	Inductively Coupled Plasma Mass Spectrometry
LOD	Limit of detection
LOQ	Limit of quantification
RT	Retention Time
CRM	Certified reference material
CID	Collision induced dissociation

List of Abbreviations – compounds

AB	Arsenobetaine
AsFA (number)	Arsenic-containing Fatty Acid (molecular mass)
AsHC (number)	Arsenic-containing Hydrocarbon (molecular mass)
AsPC (number)	Arsenic-containing Phosphatidylcholine (molecular mass)
AsPE (number)	Arsenic-containing Phosphatidylethanolamine (molecular mass)
AsPL (number)	Arsenosugar Phospholipid (molecular mass)
PC	Phosphatidylcholine
lysoPC	Lysophosphatidylcholine

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1 Introduction and Theoretical Background

1.1 Arsenic

Arsenic is a ubiquitous element that occurs in many chemical forms in our environment. It is widely distributed and naturally found in air, soil, water, plants and animals. The concentration of arsenic in the earth's crust is about 2.5 µg g^{-1} (bulk crust concentration).[1] It has two common naturally occurring oxidation states, arsenite (As^{3+}) and arsenate (As^{5+}), whereas elemental arsenic rarely occurs naturally.[2,3] The most abundant of the over 200 known arsenic-containing minerals is arsenopyrite (FeAsS).[3]

The ubiquitous nature of arsenic is partially due to its release from rock and sediment, which is caused by a series of natural processes like weathering and biological activity. Human actions like mining, combustion of fossil fuels and the use of arsenical pesticides, herbicides and wood preservatives further influence arsenic's distribution in the environment.[4] Because of this widespread natural occurrence and its toxicity, arsenic is considered a major global environmental contaminant.[5–7]

The main human hazard linked to arsenic's ubiquity and toxicity arises from arsenic in drinking water.[1] Research regarding arsenic focused on this area for a long time. Arsenic levels in natural waters can range from less than 0.5 µg L^{-1} up to 5000 µg L^{-1}, greatly exceeding the limits for 'affected waters' (50 µg L^{-1}). Ground-water shows the highest arsenic concentrations due to water-rock interactions.[3] Arsenic concentrations in rivers usually range from 0.1 to 2 µg L^{-1}, while lake water often shows similar or lower concentrations.[3] In oceans, arsenic concentrations are fairly constant at around 1-2 µg L^{-1}. (ref. 8)

Arsenic is found in many forms in water and marine organisms. For example, arsenite (As^{3+}) and arsenate (As^{5+}) are major arsenic species in fresh and marine waters, but they usually occur only in low concentrations in marine organisms.[9] However, there are also exceptions. The highest reported level of inorganic arsenic in an organism was in *Hizikia fusiforme*, a brown alga that can contain about 60 % of its total arsenic as inorganic arsenic.[10–12]

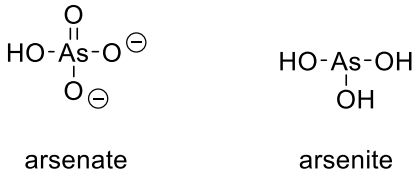

Figure 1. Structures of the inorganic species arsenate and arsenite, drawn in their most abundant form at seawater pH (ca 8).

Organic arsenic compounds are divided into water-soluble and lipid-soluble compounds[13] (see later). Over the past 40 years, research has focused on the water-soluble organoarsenicals, during which time more than 50 water-soluble organic arsenic compounds have been discovered.[14] The most significant constituents of this group are discussed in the following.

Arsenobetaine was first identified by Edmonds et al. in 1977.[15] Since then, it has been shown to represent more than 80 % of the total arsenic[8] in most marine organisms, including fish, mollusks and crustaceans.[16,17] Methylated arsenic species like methylarsonate and dimethylarsinate, common metabolites of inorganic arsenic in human urine,[18] are also found as arsenicals in a wide range of marine organisms.[13] Arsenosugars, the most common ones being dimethylarsinoyl ribosides, are dominant arsenic species in algae.[8]

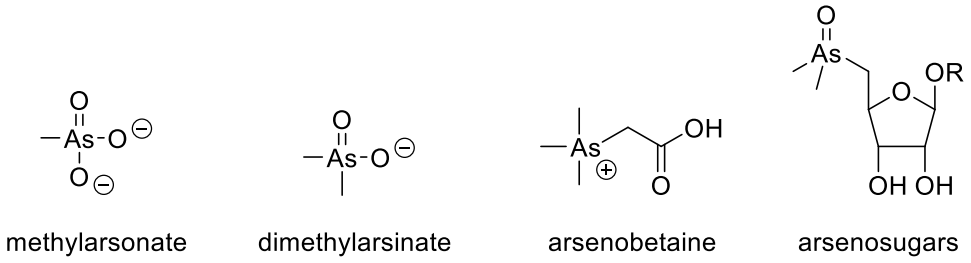

Figure 2. Structures of some water-soluble organic arsenic species commonly found in seafood.

1.2 Arsenolipids

1.2.1 Arsenic-containing fatty acids

Although marine samples were extensively studied in the past, the structure of arsenolipids remained mostly unknown until the early 2000s. In 2008, Rumpler et al.[19] reported the isolation of six arsenolipids from cod-liver oil. They identified the novel class of compounds as both saturated and unsaturated arsenic-containing fatty acids, which occurred in the oil alongside common (non-arsenic) fatty acids. The compounds were derivatives of long-chain fatty acids, where the methyl group was replaced with a dimethylarsinoyl group ($Me_2As(O)$-).

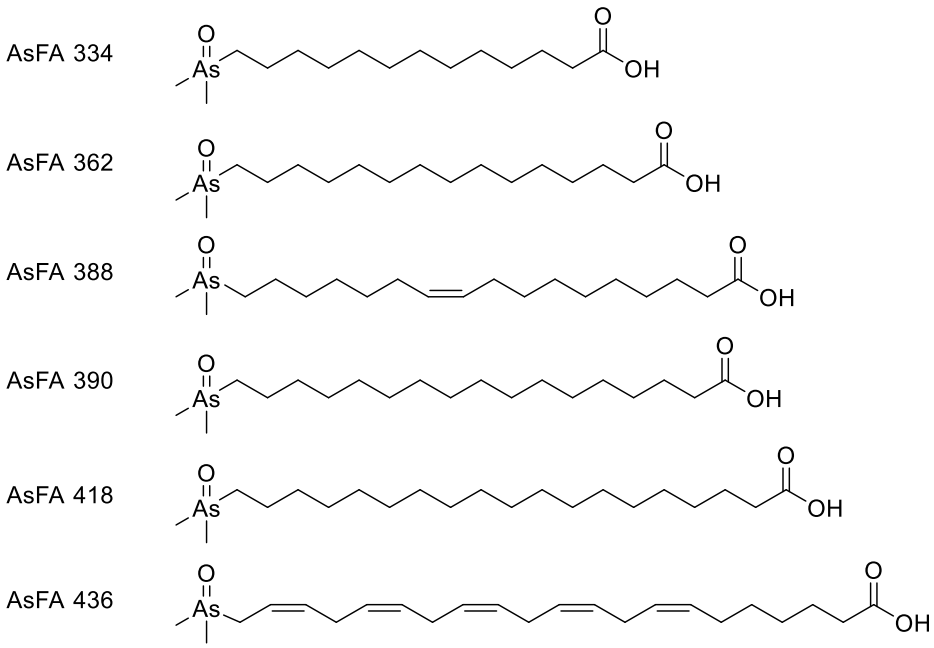

Figure 3. Arsenic-containing fatty acids discovered in cod liver oil by Rumpler et al. Position and geometry of the double bonds in unsaturated AsFAs were not determined. They were assigned by analogy to common fatty acids in cod-liver oil. AsFA is short for arsenic-containing fatty acid, the number that follows corresponds to the nominal molecular mass of the molecule.

The compounds were identified by RP-HPLC/ICPMS and RP-HPLC/ESI-MS, two methods that are described later in this thesis (see Chapter 1.3). The saturated AsFAs were analogues of myristic (C14:0, AsFA 334), palmitic (C16:0, AsFA 362), stearic (C18:0, AsFA 390) and arachidic acid (C20:0, AsFA 418). The unsaturated fatty acids are thought to be analogues of oleic acid (C18:1 n-9, AsFA 388) and docosapentaenoic acid (C22:5 *n*-3, AsFA 436).

1.2.2 Arsenic-containing hydrocarbons

The second main group of naturally occurring arsenolipids was discovered also in 2008 by Taleshi et al.[20] who identified the arsenic-containing hydrocarbons in oil from the plankton-feeding fish capelin. Interestingly, the AsHCs contain the equivalent of an even number of carbons in the carbon chain. Because naturally occurring hydrocarbons are usually biosynthesized via decarboxylation of fatty acids,[21] and as common fatty acids predominantly contain an even number of carbon atoms, odd-numbered carbon chains are usually found for hydrocarbons. Taleshi et al.[20] suggested that no carbon atoms are lost in the biosynthesis of AsHCs, and proposed a mechanism involving the reduction of the corresponding fatty acids to hydrocarbons *via* alcohols.

Figure 4. Arsenic-containing hydrocarbons observed in capelin oil by Taleshi et al.[20] Position and geometry of the double bonds in AsHC 404 were not determined. They were assigned by analogy to docosahexaenoic acid.

1.2.3 Arsenosugar phospholipids

The first arsenolipid structure elucidated was that of the arsenosugar phospholipid AsPL 958, which was found in 1988 by Morita and Shibata[22] in a species of brown alga. Since then, several related compounds were identified in several species of brown algae[23–25] and cyanobacteria.[26] Arsenosugar phospholipids all comprise the same basic structure and differ only in their fatty acid composition (Figure 5). Common long chain fatty acids like myristic, palmitic, stearic, oleic and arachidic acid were found to be constituents of these arsenolipids. A method for quantification of AsPLs in Hijiki, an edible brown alga was developed by Glabonjat et al.[27]

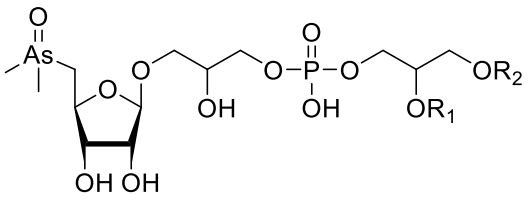

Figure 5. General structure of arsenosugar phospholipids. R_1 and R_2 are long chain fatty acids.

1.2.4 Arsenic-containing phosphatidylcholines and phosphatidylethanolamines

In 2016 Viczek et al.[28] reported a novel group of arsenolipids in the roe of herring. High resolution MS showed that the compounds were arsenic-containing phosphatidylcholines (AsPC). Salmon caviar, analyzed in the same study, did not reveal any AsPCs, but rather it contained arsenic bound into a phosphatidylethanolamine (AsPE), which represent another novel group of arsenolipids.

Arsenic-containing phosphatidylcholines contain three main building blocks: the choline headgroup, esterified with phosphoryl glycerol, which in turn is esterified with two fatty acids, one of which is an AsFA. AsPEs are similar in structure, the headgroup being a phosphatidylethanolamine.

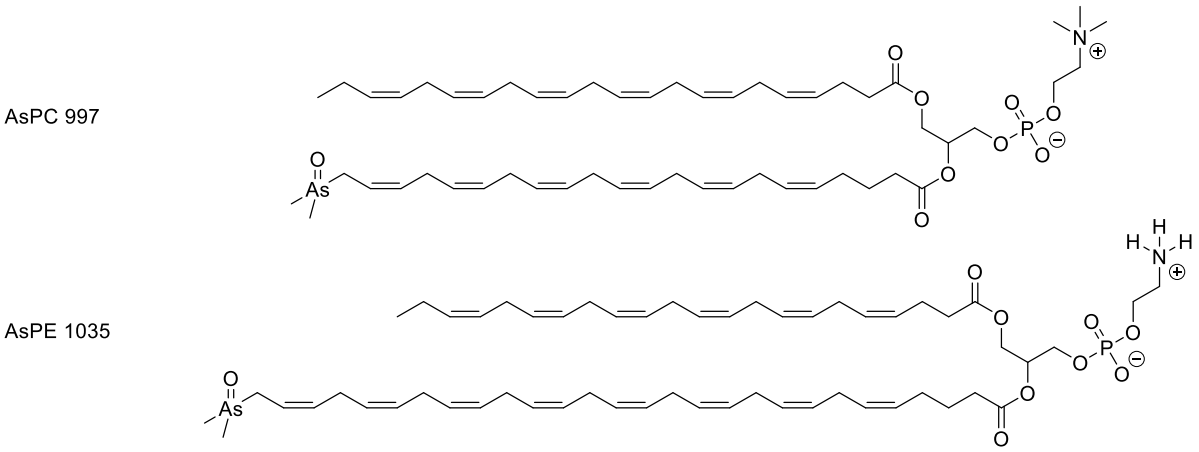

Figure 6. AsPC 997 and AsPE 1035, two examples of the new arsenolipid classes proposed by Viczek et al.[28]

Because the arsenic is bound in a dimethylarsinoyl group at the end of one fatty acid chain, the compounds reported here are different from phosphatidylarsenocholines, where the choline is replaced by arsenocholine, meaning that the nitrogen atom is replaced by an arsenic atom. Phosphatidylarsenocholines were reported in the early 1990s by Francesconi et al.[29] in yellow eye mullet, following the oral administration of arsenocholine, and by Edmonds et al.[30] in the digestive gland of rock lobster.

1.2.5 Biosynthesis of arsenolipids

Biosynthesis of arsenolipids is not well understood. One view is that a biosynthetic infidelity causes the accidental incorporation of arsenic into arsenolipids. For example, common fatty acids are synthesized by addition of two-carbon units, derived from acetyl coenzyme A, to the carbon chain. [31] If AsFAs are synthesized via the same biosynthetic pathway, the arsenic compound serving as a precursor would have to begin with an odd numbered chain. Therefore, dimethylarsinoylacetic acid might be excluded as precursor, whereas dimethylarsionoylpropanoic acid would be favored, as proposed by Edmonds.[19]

It is difficult, however, to ignore the likelihood that arsenolipid biosynthesis involves dimethylarsinate, which is formed from arsenate by addition of two methyl groups.[32] The methylation process is thought to be a possible way for lowering the toxicity of arsenate, which can

mistakenly be taken up by algae from seawater due to its similar chemical properties to phosphate.

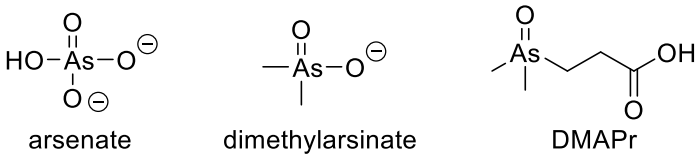

Figure 7. Dimethylarsinate (produced by methylation of arsenate) and DMAPr, two possible precursors for arsenolipids.

In 2014, a study on the biosynthesis of arsenolipids in a cyanobacterium showed that As^{5+} exposure of *Synechocystis* sp. PCC 6803 results in production of arsenosugar phospholipids. It was discovered that this biosynthesis requires arsenic(III) *S*-adenosylmethionine methyltransferase.[26] However, it remains unknown how and why arsenolipids are biosynthesized.

1.2.6 Toxicity of arsenolipids

In 2015, Meyer et al. conducted a study on the toxicological properties of AsFAs.[33] Three compounds of the group were synthesized[34] and tested with human liver cells. It was shown that although AsFAs are less cytotoxic than arsenite, they still have significant detrimental effects on liver cells, even at concentrations of µmol L^{-1}.

Meyer et al.[35] also studied the effects of AsHCs. Three AsHCs exerted toxic effects similar to arsenite on the fruit fly *Drosophila melanogaster*. The compounds also affected the hatching process of the flies at a concentration of 150 µmol L^{-1}. Meyer et al.[36] furthermore conducted an *in vitro* study on the cellular toxicity of AsHCs in cultured human bladder and liver cells. The cytotoxicity of AsHCs is similar to that of arsenite, resulting in a decrease in cellular energy level.

Studies with the Caco-2 intestinal barrier model showed that AsHCs and AsFAs both are intestinally bioavailable. AsFAs were effectively biotransformed while passing the *in vitro* barrier, but still showed a higher permeability than did arsenobetaine and arsenosugars. AsHCs are thought to be transported unchanged via passive diffusion and are likely to be highly intestinally bioavailable to humans.[37]

To sum up, both AsHCs and AsFAs show toxicological properties and bioavailability. AsHCs show higher impacts and higher bioavailability. Both compound types are found in many food sources like fish oil,[19,20,38–41] tuna[42] and cod[43–46] as well as in edible algae[23,24,27,47,48] and fish meal.[49] The facts that no limits on arsenolipids in food have yet been established and that a full

toxicological profile of these compounds is not yet available highlight the urgent need for further toxicological characterization of all arsenolipids.

1.3 Instrumental methods

The main methods for arsenolipid determination are achieved by coupling high performance liquid chromatography with ICPMS or ESI-MS.[50] These methods and their field of application are briefly discussed in the following.

1.3.1 High Performance Liquid Chromatography (HPLC)

HPLC is needed to separate the analytes of the complex samples for further analysis with mass spectrometry. In this work, reversed-phase (RP-) HPLC was used (herein HPLC means RP-HPLC if not stated otherwise). In RP-HPLC, the column contains hydrophobic material with which non-polar compounds strongly interact. The mobile phase is often a mixture of water and methanol run under gradient elution conditions with increasing methanol content whereby the compounds elute in decreasing order of polarity. The technique is capable of not only separating the various types of arsenolipids (e.g. AsFAs from AsHCs), but also compounds within each arsenolipid group (e.g. AsFAs of different molecular masses). In our system, however, compound resolution was lower in the less polar range so that there was considerable overlap between some of the arsenolipid types, e.g. the AsPLs and AsHCs.

1.3.2 Inductively Coupled Plasma Mass Spectrometry (ICPMS)

ICPMS employs a hard ionization technique in the form of a high temperature plasma which decomposes all compounds to their individual elements predominantly as singly ionized species, in combination with a mass analyzer, which can be used to identify the elements and provide quantitative data. Although high resolution mass analyzers are used in ICPMS, usually for accurate isotope measurements, a quadrupole mass analyzer is most commonly employed for ICPMS analyses. The method is robust and sensitive, and readily provides quantitative multi-element measurements making it the method of choice for elemental analyses. ICPMS can also be used as a detector for HPLC to provide quantitative data of various species of a particular element (see below).

1.3.3 Electrospray Ionisation – Mass Spectrometry (ESI-MS)

ESI-MS employs a soft ionization technique to gently transfer analytes, by means of a metal capillary with a voltage applied at its tip, from solution to the gas phase in charged form. The ionized compounds retain their structural information and are observed as quasi-molecular ions, formed by addition of hydrogen or sodium ions (for positive mode) or by loss of hydrogen (negative mode). Addition of multiple nuclei ($[M+nH]^{n+}$) is often observed and characteristic for ESI-MS, allowing the analysis of high mass molecules with instruments of limited mass range. With typical mass analyzers like ion trap, quadrupole and time of flight, structural information is often limited, because mainly the parent ion is produced and there are few product ions. This disadvantage is overcome by coupling ESI with tandem mass spectrometry (ESI-MS/MS). A common tandem MS detector is the triple quadrupole (QqQ), where quadrupoles 1 and 3 act as mass analyzers, and quadrupole 2 serves as a collision-induced dissociation cell, allowing for different scan modes like precursor ion scan or product ion scan. ESI-MS is very dependent on the matrix and therefore not considered as a robust technique. It generally has a higher limit of detection than ICPMS, but can provide structural data.

1.3.4 Coupling of HPLC with ICPMS and ESI-MS

HPLC/ICPMS is a method that can be used for speciation as it can give an overview of all elemental species of one (or various) element(s) in the sample. Instead of complex chromatograms, simpler chromatograms of only (e.g.) arsenic species are obtained thanks to the element (arsenic) selectivity of ICPMS. Unknown arsenic species can be quantified due to the fact that different arsenic species do not differ in arsenic signal, because the number of arsenic ions produced is the same for different arsenic-containing compounds. However, identification of arsenic species requires a standard and can only be achieved by co-elution of said standard and the compound in the chromatogram. The risk of misidentification is still high due to matrix effects and co-eluting elemental species. Also, no structural information is obtained from such chromatograms. HPLC/ICPMS is still not commonly applied, but it is a great complementary technique for the very common HPLC/ESI-MS, which is suitable for identification and structural elucidation of compounds. Because arsenic molecular ions as well as their fragments can be observed with electrospray ionization, arsenolipids can selectively be identified by monitoring m/z = 105 ($C_2H_6As^+$) and m/z = 123 ($C_2H_8AsO^+$), simultaneously obtaining information about the molecular ion of a compound and its fragments. Both techniques can be used complementarily as detector, whereby a chromatogram of arsenic species can be obtained by ICPMS, with structural information for the species provided by ESI-MS.

The coupling of ICPMS with RP-HPLC, however, poses some technical challenges, largely because the high carbon content of mobile phases used in RP-HPLC interferes with the plasma by lowering its temperature, which would eventually collapse the plasma. To reduce the amount of carbon reaching the plasma, only part of the HPLC effluent flow is directed to the ICPMS, and the flow is augmented by the addition of an auxiliary solution containing an internal standard via a T-piece.[51] Another problem with the speciation of arsenolipids is that they require gradient elution, whereby the carbon content of the mobile phase changes during the chromatography, which can greatly affect the arsenic signal (carbon enhancement and other effects).[52,53] This problem is overcome by direct introduction of an organic solvent into the spray chamber, saturating the vapor with organic compounds and thereby reducing the variation in arsenic response.[53]

In this work, HPLC/ICPMS and HPLC/ESI-MS are applied in parallel. To achieve coupling to the HPLC of both detectors, a flow splitter is used after the column, splitting 10 % of the flow to the ICPMS and 90 % to the ESI-MS. This setup allows simultaneous recording of HPLC/ICPMS and HPLC/ESI-MS chromatograms, which can be correlated with each other, and hence provide molecular mass data on the arsenic-containing species observed selectively in the HPLC-ICPMS chromatogram.

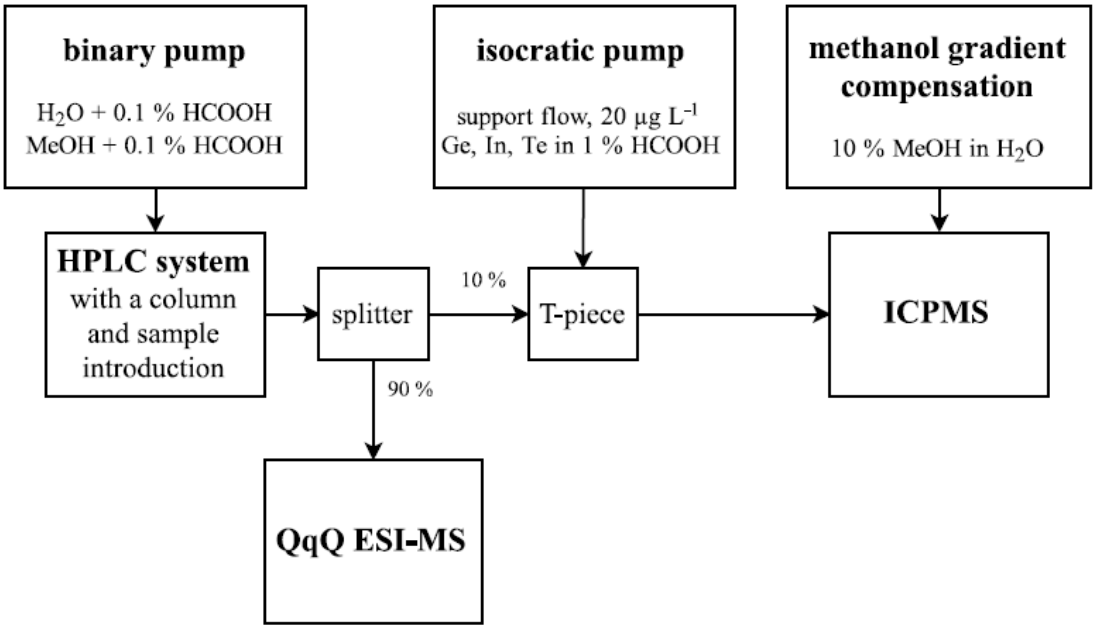

Figure 8. Schematic overview of the coupling of RP-HPLC with ICPMS and ESI-MS.

1.4 Phospholipases

Phospholipases are a class of enzymes diverse in structure and function that possess the common property of being able to catalyze the hydrolysis of phospholipids. The class of phospholipases comprises two sub-classes: acyl hydrolases and phosphodiesterases. Enzymes in each of these sets are named according to the ester bond they specifically cleave. Phospholipase A_1 (PLA_1), phospholipase A_2 (PLA_2), phospholipase B (PLB), and lysophospholipase $A_{1/2}$ ($lysoPLA_{1/2}$) are ascribed to the class of acyl hydrolases, whereas phospholipase C (PLC) and phospholipase D (PLD) represent the phosphodiesterases.[54] In this study, phospholipases A_1, A_2, C and D were used to hydrolyze the phosphatidylcholines of interest.

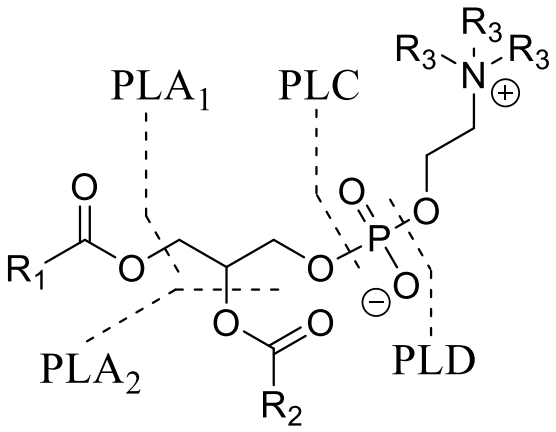

Figure 9. Sites of cleavage for PLA_1, PLA_2, PLC and PLD. R_1 and R_2 are alkyl moieties. R_3 is either -H (phosphatidylethanolamines) or $-CH_3$ (for phosphatidylcholines).

Phospholipases A_1 and A_2 catalyze the cleavage of the ester bond at the carbon atoms in *sn*-1 and *sn*-2 position. Cleavage of the phosphodiester bond at hydrogen attached to the carbon atom in *sn*-3 position is achieved by PLC catalysis. The other phosphodiester bond is hydrolyzed by PLD.

1.4.1 Phospholipase A_1

Phospholipase A_1 (EC 3.1.1.32) produces free fatty acids and lysophospholipids from phospholipids by hydrolysis of *sn*-1 acyl esters. Many enzymes that show PLA_1 activity also exhibit lysoPLA and neutral lipase activity, although to a much lower extent. PLA_1 is found in many different types of organisms including mammals (e.g. guinea-pig, cow), plants, and bacteria.[54]

As phospholipases are useful tools in the analysis of phospholipids, the demand for commercially available products is high. Because there were difficulties in producing and purifying the enzyme, there was for a long time no commercial source for PLA_1. About 10 years ago, a PLA_1

from *Thermomyces lanuginosus*, expressed in *Aspergillus*, was commercialized as Lecitase Ultra® by Novozymes, Denmark.[55] First applications included the synthesis of structured phosphatidylcholines[56] and in enantioselective reactions,[57] but generally a wide field of applications, also in biotechnology, is open to PLA_1.

In this work, PLA_1 is used as a catalyst in several enzymatic reactions performed for positional analysis. A long-known[58] major drawback of PLA_1 is that the produced *sn*-1-lysophospholipids are not stable under acidic or basic conditions. Acid or base catalysis both result in acyl migration of the fatty acid in *sn*-2-position to yield a more stable *sn*-2-lysophospholipid (Figure 10).[59] Acyl migration is favored at alkaline pH,[60] where an equilibrium mixture contains about 90 % of the *sn*-1-acyl-isomer and around 10 % of the *sn*-2-acyl-isomer of the lysophosphatidylcholine.[61] Due to the broad lipase activity of PLA_1, which is also expressed in lysophospholipase A_1 activity, the produced *sn*-1-acyl-lysophospholipids are thought to be readily hydrolyzed to yield free fatty acids and glycerophosphatidylcholine.[62]

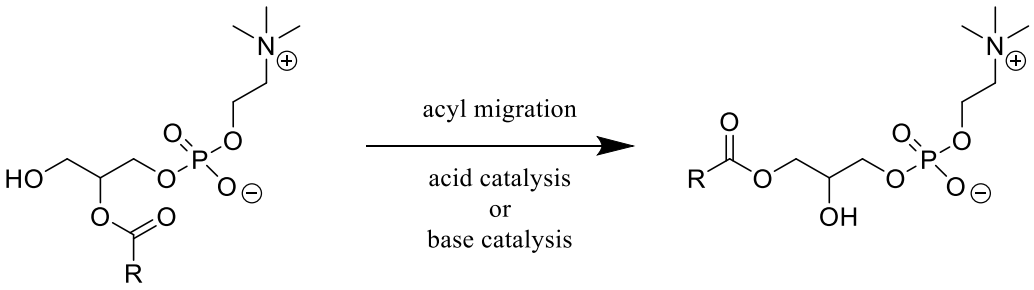

Figure 10. Acid and base catalyzed acyl migration of lysophosphocholines.

1.4.2 Phospholipase A_2

Phospholipase A_2 (EC 3.1.1.4) is a biocatalyst that selectively cleaves *sn*-2 acyl ester bonds of glycerophospholipids. It is a central part of a multitude of cellular processes, e.g. digestion and metabolism of phospholipids, host defense and signal transduction.[63]

In contrast to PLA_1, there is more insight about phospholipases of type A_2, mainly due to the fact that in the early 1950s, there already were studies on PLA_2 from cobra venom and pancreatic juice.[64] Elegant efforts in cloning, expression, purification, sequencing and structure determination have been made for many phospholipases since then.[65] Phospholipases of type A_2 are a diverse class of enzymes not only in function, but also in mechanism, sequence, structure and many more aspects. Several PLA_2s are well studied and knowledge about non-human PLA_2s is equally applicable to human forms of PLA_2.[66]

The vast diversity of this enzyme class becomes apparent when looking at the major groups of different characteristics. PLA_2s can be ascribed to one of at least nine groups according to localization of the individual member (secreted versus cytosolic), sequence homology, cation requirement and molecular mass.[65] The reason for such a diversity in enzyme classes is that many mammalian tissues and cells often contain more than one PLA_2. Mammals possess differently regulated pathways for fatty acid metabolism, requiring PLA_2s to play crucial roles in cellular responses as diverse as phospholipid digestion and turnover, membrane remodeling, host defense, signal transduction and the production of inflammatory mediators.[66]

The phospholipase A_2 used throughout this study is derived from bee venom. It is a member of the group of secretory PLA_2s, which are part of a wide range of mammalian tissues and venoms of different organisms. The toxic activities displayed by some of the secretory PLA_2s are often additional to their primary functions. Both mechanisms, catalytic and toxic, were studied by Fuji et al.[67] in 1998. Secretory PLA_2s furthermore require Ca^{2+} as a cofactor and show a specificity for phospholipids with polar headgroups, hydrolyzing not only phosphatidylcholines, but also phosphatidylethanolamines and other phospholipids.[66,68]

The catalytic cycle of secretory PLA_2-type enzymes was described by Verheij et al.[69] and further established by binding studies on different venom PLA_2s and high resolution x-ray structures by Scott et al.[70] Figure 11 shows the active site amino acids of $sPLA_2$s. The general catalytic mechanism described is analogous to that for serine proteases. A water molecule near the catalytic His 48 is thought to perform a nucleophilic attack on the *sn*2-acyl ester of the phospholipid, releasing the free fatty acid and a lyso-phospholipid. The intermediate substrate-enzyme complex is stabilized by the coordination of Ca^{2+} to the phosphoryl group and the carbonyl group of the bound substrate molecule. This mechanism is also supported by the drastic decrease of PLA_2 activity at acidic pH, which is thought to result from protonation of Asp 49, leading to destabilization of the enzyme-substrate complex.

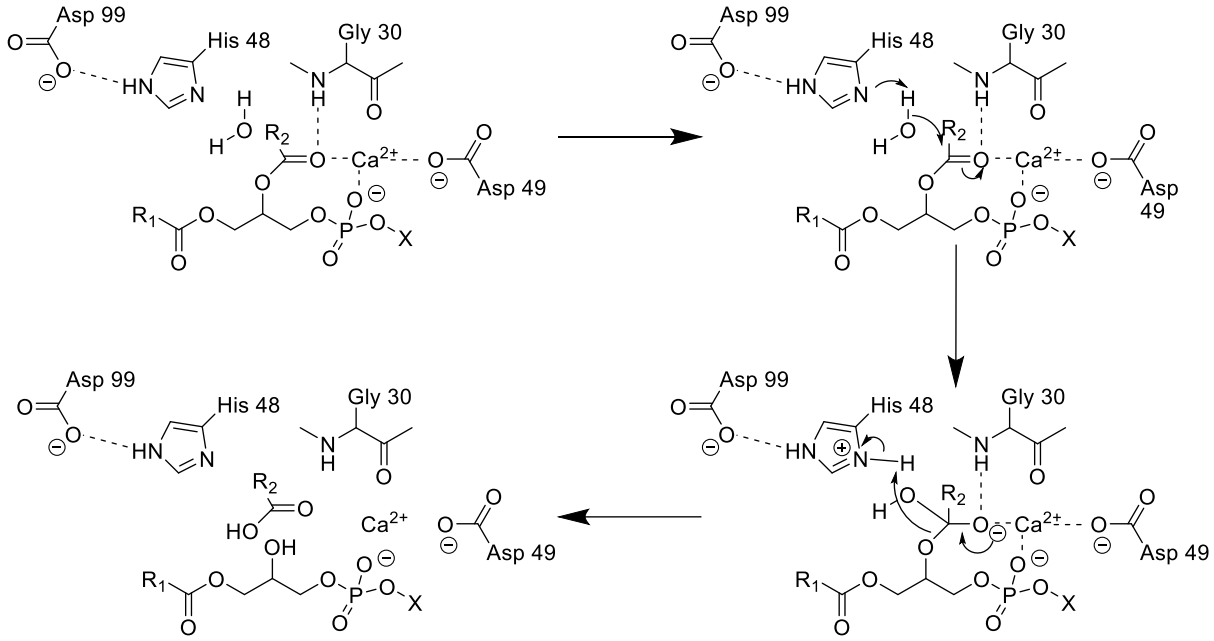

Figure 11. Catalytic mechanism for secretory phospholipases A_2 as described by Verheij et al.[69]

1.4.3 Phospholipase C

Phospholipase C (EC 3.1.4.3) hydrolyzes phosphatidylcholines at the glycerol-phosphate ester bond to yield the phosphocholine headgroup and the 1,2-diacylglyceride. First, enzymes of bacterial origin were studied. They were small exoenzymes (20 000 – 30 000 Da) and their activity was often dependent on the presence of Mg^{2+} or Zn^{2+} ions. Bernheimer[71] reported this in several species of bacteria (e.g. strains of *Bacillus*, *Pseudomonas*, *Staphalococcus*, *Clostridium*, *Streptomyces*, *Acinetobactor*).

Clostridium perfringens type A produces α-Toxin, which was first identified to be a phospholipase C by Macfarlane and Knight[72] who described it as being strongly lethal, necrotizing and hemolytic. α-Toxin is the main virulence factor for gas gangrene in humans. The bacterium uses its lipase activity to generate lipid signals in host eukaryotic cells and to degrade the hosts cell membrane.[73] In this work, *C. perfringens* PLC (CpPLC) was used for enzymatic hydrolysis.

Various attempts have been made to purify *C. perfringens* PLC, with only a few being successful.[74] Pure enzyme in high yield was achieved by affinity chromatography, followed by gel filtration.[75] Purification attempts with CM-Sephadex, DEAE-Sephadex and Sephadex G-100 chromatography showed that a part of the enzyme exists in active polymerized forms.[74] The generally accepted view that *C. perfringens* PLC is only specific for phosphatidylcholines and

sphingomyelins was recently changed. Urbina et al.[73] found that other phospholipids like phosphatidylethanolamines, phosphatidylinositols and phosphatidylglycerol also show favorable interactions with the CpPLC active site.

1.4.4 Phospholipase D

Phospholipase D (EC 3.1.4.4) catalyzes the hydrolysis of phospholipids at the choline-phosphate ester bond of the phosphodiester to yield 1,2-diacylglycero-*sn*-3-phosphate and the hydrophilic head group of phospholipids, but is also known as a useful tool for transphosphatidylation.[76] PLD was first identified in 1947 by Hanahan and Chaikoff[77] in carrot roots and spinach leaves. Since then, it has been reported from several plant,[78–80] microorganism[81] and mammalian sources.[82,83] In this thesis, a phospholipase D from cabbage was used.[84] Several important cellular functions and, in mammals, the control of many hormones, neurotransmitters and growth factors are ascribed to PLD. Its principle hydrolysis product, phosphatidic acid, is widely believed to have a signaling role.[85]

Phospholipases have been used for a number of biocatalytic phospholipid transformations, both on laboratory and industrial scales.[86,87] Interestingly, both PCs and PEs were cleaved by the enzyme.[82] In this work, phospholipase D was used for cleavage of the linkage between the phosphate and the polar headgroup of AsPCs and AsPEs. The expected products were phosphatidic acid (the 1,2-diacylglycero-*sn*-3-phosphate) or its methanol ester, if the reaction went via transphosphatidylation.

1.5 Positional analysis of fatty acids in AsPCs

Properties of phosphatidylcholines depend on their fatty acid composition,[88] and significant effects on both rate and quantity of PC intake have been linked to the structure of the compounds.[89,90] Therefore, the development of methods for positional analysis has received attention. Usually the methods are based on the regiospecific hydrolysis of one acyl ester bond of the PCs. Subsequent chromatographic analysis aims to separate the yielded fatty acids and lysophosphatidylcholines.

Most of the methods developed for positional elucidation involve an enzymatic hydrolysis step. An approach using two different enzymes was made in 2004. Lipozyme was used for ethanolysis of the *sn*-1 ester bond, PLA_2 hydrolyzed the *sn*-2 ester bond. The two different lysoPCs were chemically hydrolyzed and the released FAs were esterified and analyzed by GC.[91] A similar

approach with Lipozyme was reported in 2012.[88] Another multi-enzyme approach used PLA_1 from *T. thermophila* and bee venom PLA_2.[92] Several methods for the selective hydrolysis of the *sn*2-ester bond by phospholipase A_2 were described in the literature. PLA_2 from *Ophiophagus hannah* venom[93] and bee venom[94–96] were used in methods developed previously. A study on the hydrolysis of arsenic-containing phospholipids with bee venom PLA_2 was conducted by Raab et al. [25] They hydrolyzed arsenosugar phospholipids for HPLC/ESI-MS determination of the fatty acid distribution in these compounds.

1.6 Aim

A preliminary study by the Trace Element Metabolism research group at the University of Graz discovered arsenic-containing phosphatidylcholines in herring caviar. Structural elucidation of these compounds, however, was not fully achieved. The primary goal of this thesis was to develop a method using phospholipases to establish the position (*sn*-1 or *sn*-2) of the arsenic fatty acids in arsenic-containing phosphatidylcholines. This work also aims to characterize the arsenic species in four types of food and food supplements and to determine the distribution of arsenic during sample preparation.

2 Experimental

2.1 Chemicals and standards

Table 1. Solvents, reagents and other chemicals used in the experimental part of this work.

Chemical	Company
Acetone (> 99.8 %)	Carl Roth GmbH, Karlsruhe, Germany
Ammonia (25 %)	Carl Roth GmbH, Karlsruhe, Germany
Argon 5.0 (>99.999 %)	Messer, Gumpoldskirchen, Germany
Arsenic acid (1 g As L^{-1}, 99.995 %) in 2 % HNO_3	Carl Roth GmbH, Karlsruhe, Germany
Calcium chloride (≥ 99 %)	Carl Roth GmbH, Karlsruhe, Germany
Dichloromethane (≥ 98 %)	Chem-Lab, Zedelgem, Belgium
Ethanol (≥ 99.9 %)	Chem-Lab, Zedelgem, Belgium
Formic acid (≥ 98 % p. a.)	Carl Roth GmbH, Karlsruhe, Germany
Hexane (≥ 95 %)	Sigma-Aldrich, Vienna, Austria
Hydrochloric acid (37%, then subboiled)	Carl Roth GmbH, Karlsruhe, Germany
Nitric acid (≥ 65 %, then subboiled)	Carl Roth GmbH, Karlsruhe, Germany
Methanol HiPerSolv (LC-MS)	VWR Chemicals, Vienna, Austria
Phospholipase A_1 (from *Thermomyces lanuginosus*)	Sigma-Aldrich, Vienna, Austria
Phospholipase A_2 (from bee (*Apis mellifera*) venom)	Sigma-Aldrich, Vienna, Austria
Phospholipase C (from *Clostridium perfringens*)	Sigma-Aldrich, Vienna, Austria
Phospholipase D (from cabbage)	Sigma-Aldrich, Vienna, Austria
Silica gel 60, 0.063-0.2 mm	Merck, Darmstadt, Germany
Sodium acetate anh. (≥ 99 %)	Lactan, Graz, Austria

Water used throughout this master thesis work was Milli-Q water (18.2 MΩ cm), produced by an Elix® Millipore system (Merck, Darmstadt, Germany). The certified reference material (CRM) was NMIJ CRM 7405-a (Trace Elements and Arsenic Compounds in Seaweed-Hijiki), which was obtained from the National Metrology Institute of Japan (Tsukuba, Japan).

As arsenolipid standards, two arsenic-containing fatty acid standards (AsFA 362 and AsFA 388) as well as 3 arsenic-containing hydrocarbon standards (AsHC 332, AsHC 360 and AsHC 444) were previously synthesized in our lab as reported by Taleshi et al.[34] The compounds (10 µg arsenic) were dissolved in 1 mL of ethanol, followed by dilution to a concentration of about 10 µg L^{-1}.

Quantification of arsenic species was achieved with an AsHC 360 standard. An amount of 250 ± 5 µg of the standard was dissolved in 1 mL of ethanol diluted to appropriate concentrations with aqueous MeOH (1+9 v/v, water/methanol).

2.2 Instrumentation and operating conditions

2.2.1 Sample digestion system

Ultraclave™ IV, MLS GmbH, Leutkirch, Germany; Quartz tubes, Teflon caps; Max pressure: 140 bar, Max temperature: 300 °C

An appropriate portion of sample (ca 100 mg weighed to a precision of 0.1 mg) was weighed directly into 12 mL quartz tubes. Internal standard (1 or 2 mL, 100 µg/L Ge, In and Te in 1 % HNO_3), water (0 or 1 mL) and HNO_3 (2 mL) were added. The tubes were closed with Teflon caps and put into the microwave reactor. An absorption bath (300 g water and 5 g H_2SO_4 conc.) was prepared. The microwave reactor was closed, filled with 40 bar of argon and a heating program was started. Maximum pressure was set to 140 bar. A temperature program (10 min to 60 °C, 15 min to 150 °C, 15 min to 250 °C, hold 30 min at 250 °C, then cool down) was applied. Digested samples (clear and colorless solutions) were transferred to 15 mL polypropylene tubes and diluted with water to the 10 mL mark.

2.2.2 High Performance Liquid Chromatography (HPLC)

HPLC separations were performed with an Agilent 1260 Series HPLC system, which was equipped with a binary pump (G 1312B), isocratic pump (G 1310A), solvent degasser (G 4225A), column oven (G 1316A) and a thermostated auto sampler (G 7167A) with a variable 100 µL injection loop. The second HPLC system used was a Dionex Ultimate 3000 (Thermo Scientific, Waltham, USA) HPLC system consisting of a Rapid Separation (RS) pump, RS autosampler and RS column compartment.

Column	Shodex Asahipak ODP-50 4D RP C18 column (4.6 x 150 mm), Particle size 5 µm, plate number ≥ 9000; Shodex Asahipak ODP-50G 4A guard column (4.6 x 10 mm) (Showa Denko Europe GmbH, Munich, Germany)

HPLC vials	250 µL polypropylene crimp/snap top or 1000 µL polypropylene crimp/snap top with vial insert, 250 µL glass insert, flat bottom; 11 mm aluminum crimp cap, polytetrafluoroethene/rubber TF2 septum (Agilent Technologies, Santa Clara, CA, USA)

Table 2. HPLC conditions used in this work. Flow rate was always 0.5 mL min^{-1}.

Method	Column	Mobile phase	Elution conditions	Comment
Asahipak Method 1	Asahipak ODP-50G 4D	A: water + 0.1 % formic acid B: MeOH + 0.1 % formic acid	0-15 min 50 % B, increasing to 100 % 15-30 min 100 % B 30-40 min 50 % B	used for HPLC/ICPMS-ESI-MS
Asahipak Method 2	Asahipak ODP-50G 4D	A: water + 0.1 % formic acid B: MeOH + 0.1 % formic acid	0-20 min 60 % B, increasing to 100 % 20-30 min 100 % B 30-40 min 60 % B	used for HR-MS

2.2.3 Inductively Coupled Plasma Mass Spectrometry (ICPMS)

A 7500ce Agilent ICP-MS (Agilent Technologies, Waldbronn, Germany) equipped with a PC3 ESI cyclonic spray chamber (Elemental Scientific, Mainz, Germany) was used with argon as plasma gas. The nebulizer was a Burgener Ari Mist HP nebulizer (Burgener Research International, Berkshire, UK). Nickel sampler and skimmer cones from Agilent Technologies were used. The instrument was tuned to give the maximum sensitivity for *m/z* 75 using a 10 µg L^{-1} solution of Li, As, Y and Tl (*m/z* 7, 75, 89, 205). Determination of arsenic in digested samples was done in collision cell mode, which was set up after basic tuning and tuned for optimum gas flow (He, 4.7 – 5 mL min^{-1}). As optional gas, carbon dioxide was introduced to enhance the arsenic signal. Further parameters for typical total arsenic measurements are shown in Table 3.

Table 3. Parameters for measurements of total As on Agilent 7500ce.

ICPMS (total As measurements) Agilent 7500ce

Mode	full quant	Cell Entrance	- 24 V
RF power	1550 W	QP Focus	- 11 V
RF matching	1.61 V	Cell Exit	- 40 V
Sample depth	8 mm	OctP RF	200 V
Torch-H	- 0.1 mm	OctP Bias	- 18 V
Torch-V	1.7 mm	AMU Gain	129
Carrier Gas	0.76 L min^{-1}	AMU Offset	126
Makeup Gas	0 L min^{-1}	Axis Gain	0.9997
Optional gas	12 % CO_2	Axis Offset	- 0.02
Nebulizer pump	0.11 rps	QP Bias	- 16 V
Extract 1	3 V	Discriminator	6.1 mV
Extract 2	- 115 V	Analog HV	2150 V
Omega Bias-ce	- 24 V	Pulse HV	1840 V
Omega Lens-ce	- 1.4 V	Masses recorded	*m/z* 72 (Ge), *m/z* 75 (As), *m/z* 77 ($^{40}Ar^{37}Cl$), *m/z* 115 (In), *m/z* 125 (Te)
Collision mode	4.7 mL min^{-1} He		

2.2.4 Electrospray Ionisation – Mass Spectrometry (ESI-MS)

A triple quadrupole ESI-MS (Agilent 6460, Agilent Technologies, Waldbronn, Germany) was operated in positive ion mode either in full scan mode (*m/z* 200-1200) or with selective reaction monitoring. Chromatographic conditions are described in section 2.2.2 (Asahipak Method 1). Typical source conditions were: Gas temperature: 100 °C, gas flow: 12 L min^{-1}, nebulizer pressure: 45 psi, sheath gas temperature: 350 °C, sheath gas flow: 11 L min^{-1}, capillary voltage: 4500 V, nozzle voltage: 500 V. The gas used was nitrogen.

For selective reaction monitoring, the precursor ions *m/z* selected were the $[M+H]^+$ ions of the arsenolipids. Product ion masses monitored for arsenolipids were at *m/z* = 105 ($C_2H_6As^+$) and *m/z* = 123 ($C_2H_8OAs^+$). Product ion masses derived from more complex molecules, like phosphatidylcholines (*m/z* = 184), were also selected in selective reaction monitoring.

Table 4. Monitored precursor ions, product ions, fragmentor voltages and collision energies for selective reaction monitoring for selected compounds, representative of their group (small parameter adjustments were made for similar compounds).

Selective reaction monitoring			
Compound	Reaction	Fragmentor voltage (V)	Collision energy (V)
AsHC 332	333.2 → 123	120	20
AsHC 332	333.2 → 105	120	24
AsFA 362	433.2 → 123	135	24
AsFA 362	363.2 → 105	135	30
AsPC 939	940.5 → 940.5	220	0
AsPL 958	959.5 → 409	220	30
AsPL 958	959.5 → 97	220	86

2.2.5 Coupling of HPLC with ICPMS and ESI-MS

For speciation analysis, instead of Ni cones, Pt cones (from Agilent Technologies) were used as sampler and skimmer cones in the ICPMS. The separation was achieved with a gradient elution via reversed-phase HPLC (Asahipak ODP-50 4D C18 column (4.6 x 150 mm)). The injection volume was 20 µL if the flow was split to the ESIMS. A passive splitter split the flow: 90 % of it was directed to an ESIMS or sent to the waste; 10 % was directed to the ICPMS together with 9 parts of an auxiliary solution (20 µg L^{-1} Ge, In, Te in water, 0.1 % formic acid, 0.5 mL min^{-1}) added via a T-piece. For gradient systems used with organic solvents, a compensation solution (10 % MeOH in water) was constantly introduced through the makeup gas inlet with an ISIS pump (0.04 rpm), keeping the carbon signal monitored at *m/z* 53 ($^{40}Ar^{13}C$) constant. Samples were filtered through a 0.2 µm Nylon HPLC syringe filters.

Table 5. Parameters for measurements of As species on Agilent 7500ce.

ICPMS (speciation of organoarsenicals) Agilent 7500ce

Mode	LC	Cell Entrance	- 24 V
RF power	1550 W	QP Focus	3 V
RF matching	1.68 V	Cell Exit	- 40 V
Sample depth	8 mm	OctP RF	200 V
Torch-H	- 0.1 mm	OctP Bias	- 6 V
Torch-V	1.7 mm	AMU Gain	130
Carrier Gas	0.83 L min^{-1}	AMU Offset	125
Makeup Gas	0 L min^{-1}	Axis Gain	0.9997
Optional gas	0 L min^{-1}	Axis Offset	- 0.02
Nebulizer pump	0.11 rps	QP Bias	- 3 V
Extract 1	2 V	Discriminator	6.1 mV
Extract 2	- 90 V	Analog HV	2150 V
Omega Bias-ce	- 18 V	Pulse HV	1840 V
Omega Lens-ce	- 1.4 V	Masses recorded	*m/z* 53 ($^{40}Ar^{13}C$), *m/z* 75 (As), *m/z* 77 ($^{40}Ar^{37}Cl$), *m/z* 82 (Se), *m/z* 115 (In), *m/z* 125 (Te)
Reaction mode	off		

2.2.6 Orbitrap High Resolution Mass Spectrometry (HR MS)

For separation and identification, the Dionex Ultimate 3000 HPLC system was coupled to a Q Exactive Orbitrap Mass Spectrometer (Thermo Scientific, Waltham, USA) equipped with a heated electrospray ionization (HESI) source from the same manufacturer. Chromatographic conditions were the same as described in section 2.2.2 (Asahipak Method 1 and Asahipak Method 2).

A positive polarity of the ionization source was used. Settings were: Gas temperature 440 °C; sheath gas flow 53 arbitrary units and aux gas flow 15 arbitrary units; spray voltage +3600 V and capillary temperature 270°C. Data were obtained as full scan spectra with data-dependent triggering of fragmentation spectra, MSddMS^2. Settings were: Full scan: *m/z*: 300-1100 Thomson; Automatic Gain Control 1x10^6; Maximum injection Time 200 ms; Resolution 70,000 FWHM; MSMS settings: Resolution 17,500 FWHM; Automatic Gain Control 2x10^5; Maximum injection Time 300 ms; isolation window 0.4 Thomson; Normalized Collision Energy 20, 30, 35 (stepped); Minimum Automatic Gain Control for triggering 5x10^2; exclusion time 10 s. Exact masses were calculated using ChemCalc.[97]

2.2.7 Other instruments and equipment

Centrifuge	Hermle Z 200 A (Hermle Labortechnik, Wehingen, Germany)
Scales/Balances	SI-234 (230 g, d = 0.1 mg) (Denver Instrument, Göttingen, Germany) Entris 224i-1S (220 g, d = 0.1 mg) (Sartorius, Göttingen, Germany)
Ultrasonic Bath	Transsonic T 700 IH (Elma Schmidbauer GmbH, Singen, Germany)
pH-meter	pHenomenal pH 1000 L (VWR International, Vienna, Austria)
Vortex	Heidolph Reax top (Heidolph Instruments, Schwabach, Germany)
Subboiling apparatus	MLS GmbH duoPUR apparatus (MLS GmbH, Leutkirch, Germany)
Vacuum centrifuge	Christ Maxi concentrator, RVC 2-33 CO plus (Martin Christ GmbH, Niedersachsen, Germany)
Cooling trap	Christ CT 02-50 SR (Martin Christ GmbH, Niedersachsen, Germany)
Splitter	Passive Splitter (Analytical Scientific Instruments, Richmond, USA)
Pipettes	Acura 825 (10 – 100 µL and 100 – 1000 µL), autoclavable, and Acura 835 (0.5 – 5.0 mL), autoclavable (SOCOREX, Lausanne, Switzerland)
Pasteur pipettes	230 mm long (Poulten & Graf Ltd., Wertheim, Germany)
Tubes	Cellstar, 15 and 50 mL, PP tubes with screw cap, conical bottom, graduated, sterile (Greiner Bio-One, Kremsmünster, Austria)
Vials	Disposable culture tubes with screw cap and seal, GL 18, Glass vials, 16 x 100 mm (Duran group, Mainz, Germany) Rotilabo ® - storage glass vials, 10 mL clear glass, ND 15, 19 x 66 mm, + PP caps with butyl/PTFE sealing pad (Carl Roth, Karlsruhe, Germany)
Syringes	NORM-JECT 2 mL (3 mL) sterile, 2-part disposable syringes (Henke Sass Wolf GmbH, Tuttlingen, Germany)
Syringe filters	25 mm syringe filter with 0.2 µm Nylon Membrane (VWR International, Radnor, PA, USA)

2.3 Quantification by ICPMS

Standard solutions were prepared by dilution of appropriate amounts of stock solutions (1000 mg As L^{-1}, single element standard) in polystyrene tubes. They were filled with water to a volume of 6 or 7 mL and then 1 or 2 mL of an internal standard solution (100 µg L^{-1} Ge, In, Tl, Lu in 1 % HNO_3) were added, always matching the internal standard concentration of the samples investigated. For matrix matching, 2 mL of HNO_3 were added.

2.4 Sample description

Four samples were investigated in this study. The sample ‘herring caviar’ was collected from capsules of a food supplement from Tom Oliver Nutrition (Northamptonshire, United Kingdom). The capsules were marketed under the name ‘MOPL™ the OMEGA 3 herring caviar’. According to the producer, the capsules contained ‘premium marine omega-3 phospholipids’; the eggs were herring caviar, while the fish oil was a mix of herring caviar oil, anchovy oil and sardine oil. In total, 30 of the capsules were opened with a pair of scissors and the contents pressed into a glass vial to give 15 g of a sample comprising brownish eggs in a yellow oil.

The sample ‘krill oil’ was taken from commercially available capsules, a food supplementation (Schiff, MegaRed, Omega-3, LOT-No.: BH261, Reckitt Benckiser, Guglgasse 15, 1110 Vienna, Austria). In total, 24 capsules were opened with a pair of scissors and pressed out into a 40 mL glass vial to yield about 15 g of a deep-orange oil.

The blue whiting oil was from blue whiting fish caught in spring from Iceland (Síldarvinnslan hf, Strandarvegur 1-11, 710 Seyðisfjörður). It had been collected in 2008 and stored frozen or at 4 °C, and more recently at room temperature. The container was shaken for homogenization and 15 g of the oil were transferred to a 40 mL glass vial.

The Kombu sample was obtained in dry form from a Chinese commercial source (Zhousan, China). The alga was washed to remove salt and then freeze-dried and shredded. The freeze-dried, shredded light green powder was weighed into a 40 mL glass vial. A total of 5.6 g of the powder were collected. Photographs of all samples and their containers are presented in Appendix D.

2.5 Solvent partitioning

About 2 g of each sample were separately weighed into a 50 mL separating funnel. The sample was dissolved in hexane (5 mL) and a portion of 200 mg was taken out for digestion and total arsenic analysis. Aqueous MeOH (1+9 v/v water/methanol; 3 mL) was added, and the mixture was shaken. After complete separation of the two layers (ca 20 min) the aqueous MeOH layer was removed, the procedure was repeated, and the two methanol phases were combined. A portion of the hexane fraction and a portion of the MeOH fraction were taken out for digestion. The solvents were then evaporated to dryness *in vacuo* [typical evaporation program: 1 h at 20 °C and 100 mbar, 2 h at 20 °C and 10 mbar, 20 h at 20 °C and 6 mbar (stopped when the

solvent was completely evaporated)]. For the whole procedure, a reagent blank was prepared in the same way as the sample.

For the Kombu sample, a portion (1 g) of the freeze-dried alga powder was transferred to a 15 mL polypropylene tube and extracted with a mixture of DCM and MeOH (2+1 v/v, 5 mL) at room temperature for 2 h, with mixing on a rotary cross. The mixture was centrifuged (6000 rpm, 15 min) and the deep green supernatant was separated from the pellet. A portion (100 mg) of the extract was taken out for digestion, and the remainder was washed with 2 mL of water and then evaporated to dryness overnight to yield 16.5 mg of a green film. This residue was extracted with 5 mL hexane (a portion was taken out for digestion), followed by 3 mL aqueous MeOH (1+9). The two extracts were transferred to a separating funnel, gently mixed, and left until full phase separation was observed (20 min). The aqueous MeOH (deep green solution) phase was collected and the hexane phase (bright green solution) was washed with 3 mL aqueous MeOH (1+9) again. The hexane phase and the MeOH phase were collected. A portion of both phases was removed for digestion, before the extracts were evaporated to dryness overnight.

2.6 Clean-up of the partitioned phases

The clean-up procedure was the same for all 4 samples. A glass pipette (150 x 5 mm, inner diameter 100 x 5 mm) was filled with quartz wool (0.5 cm) and about 5 cm of silica. The column was conditioned with 5 mL of MeOH/acetone 1+1 + 1 % formic acid. Approximately 150 mg of the dry hexane phase were put into an Eppendorf vial. They were suspended (in the case of blue whiting: bright yellow, cloudy suspension) or dissolved (in all other cases) in 1 mL of MeOH/acetone 1+1 + 1 % formic acid. The solution/suspension together with 1 mL of washing from the vial was applied to the column, and the column was washed with (1) 2 mL of MeOH/acetone 1+1 + 1 % formic acid, (2) 2 mL of methanol, and (3) 8 mL of methanol + 1 % ammonia. All fractions were collected in glass tubes. A portion (400 µL) of each fraction was taken out for digestion and measurement of total arsenic. The solvents were evaporated overnight and the dry mass of each fraction was recorded.

For the methanol phase, preparations of the column and solvent mixtures for the fractions as well as all other steps were the same as for the clean-up of the hexane phase. In contrast to the hexane phase, however, the MeOH phase was fully soluble in the solvent mixture for all samples.

2.7 Treatment of the samples with phospholipases

Enzymatic hydrolyses were performed, with small adaptions, based on the method from Sigma[98] and from the work formerly done by Raab et al.[25] Methanol (1 mL) was added to the samples (Table 7), and the mixture was shaken until full dissolution or a uniform suspension was achieved. The hydrolysis experiments were done in flat-bottom 12 mL glass vials with screw cap, equipped with magnetic stirring bars. The solutions were put into the reaction vessels in the following order: buffer, calcium chloride, enzyme and then the sample. Incubation time was 24 h under constant stirring at room temperature. Then, the reaction mixture was transferred to round-bottom 15 mL glass vials with a screw cap. The reaction vessels were washed with methanol and the wash solution was transferred to the new containers. Then, the mixture was evaporated to dryness and the residue was dissolved in 250 µL of aqueous methanol prior to analysis using HPLC/ESI-MS-ICPMS. Pipetted volumes of a typical assay are shown in Table 6. For all hydrolysis reactions, a reference reaction without enzyme was done, leaving all other parameters the same.

Table 6. Conditions for treatment with the four phospholipases.

Solutions	Reference [mL]	A_1 [mL]	A_2 [mL]	C [mL]	D [mL]
TRIS 8.9 (0.1 M)	2.05*	-	1.85	-	-
TRIS 8.5 (0.1 M)	-	1.95	-	-	-
TRIS 7.2 (0.1 M)	-	-	-	1.25	-
NaOAc (1 M)	-	-	-	-	1.45
aq. MeOH phase in MeOH	0.25	0.25	0.25	0.25	0.25
PLA_1 (12 kU mL^{-1})	-	0.1	-	-	-
PLA_2 (60-240 U mL^{-1})	-	-	0.2	-	-
PLC (2.5 U mL^{-1})	-	-	-	0.8	-
PLD (10 U mL^{-1})	-	-	-	-	0.6
$CaCl_2$ (1 M)	0.2	0.2	0.2	0.2	0.2

*Reference reaction was done at the highest pH used.

Following the procedures described above, all reaction mixtures yield the following conditions: total volume of 2.5 mL, 10 % v:v MeOH (from sample), 0.2 mmol of Ca^{2+} (from calcium chloride) and 1.2 kU PLA_1, 12-48 U PLA_2, 2 U PLC or 6 U PLD.

Table 7. Samples (methanol phases) subjected to phospholipase treatments.

Sample	Mass	Comments
herring caviar A	190 mg	cloudy suspension
herring caviar B	507 mg	cloudy suspension, residue
krill oil A	328 mg	slightly cloudy
krill oil B	464 mg	clear solution
blue whiting	189 mg	suspension
kombu alga A	5.4 mg	clear solution
kombu alga B	8.4 mg	clear solution

2.8 Overview of sample treatment

An overview of the experiments performed on the different samples is given in Figure 12. Herring caviar, krill oil and blue whiting oil were each exposed to the partitioning without further sample preparation. For Kombu, the starting point was an extract of the dry algal powder.

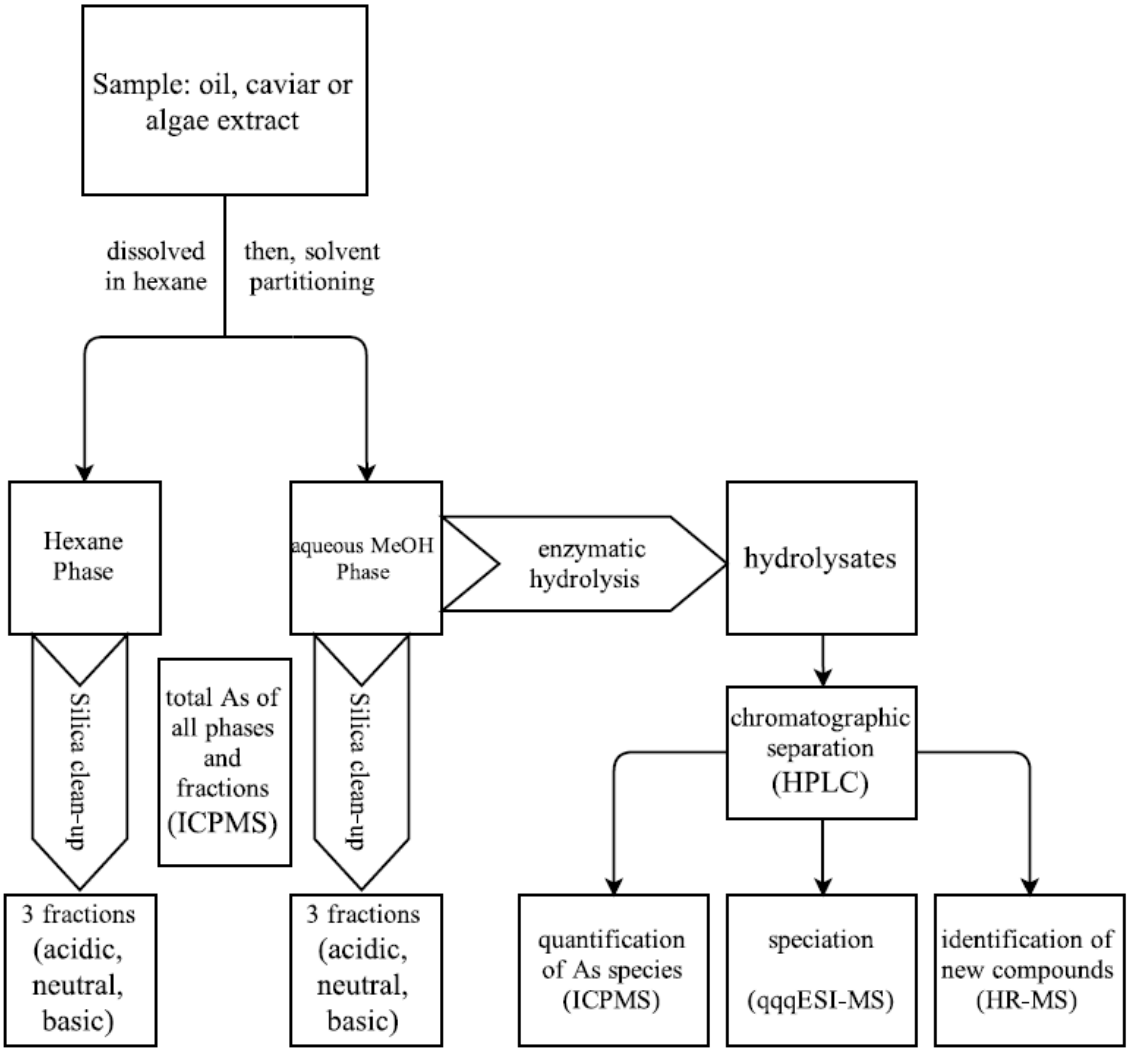

Figure 12. Overview of the sample preparation procedure and determination techniques applied to the 4 samples.

2.9 Assessment of enzymatic activity on arsenolipids

For each sample, a reaction reference chromatogram was obtained from the experiments described in 2.7. The reaction reference was incubated under the same conditions as the hydrolyzed samples, but without enzymes; the hydrolysates were analyzed by using HPLC/ICPMS-ESI-MS.

To determine the chemoselectivity of phospholipases A_2, C and D, hydrolysis of the synthetic arsenic-containing triacylglyceride 2-((15-(dimethylarsinoyl)pentadecanoyl)oxy)propane-1,3-diyl dipalmitate (0.3 mg) was performed under the same conditions as mentioned in 2.7. Phospholipase A_1 was not investigated, because it is known to hydrolyze triacylglycerides.[59] The hydrolysates were analyzed by using HPLC/ICPMS -ESI-MS.

AsPC 839 (2-((15-(dimethylarsinoyl)pentadecanoyl)oxy)-3-(palmitoyloxy)propyl (2-(trimethylammonio)ethyl) phosphate) (1.0 mg) was dissolved in methanol. In order to investigate the influence of arsenic on the hydrolytic behavior of phospholipases A_1, A_2, C and D, the enzymatic assays mentioned in section 2.7 were applied to this synthetic phosphatidylcholine. The hydrolysates were analyzed by using HPLC/ESI-MS.

3 Results and discussion

3.1 Total As content and distribution

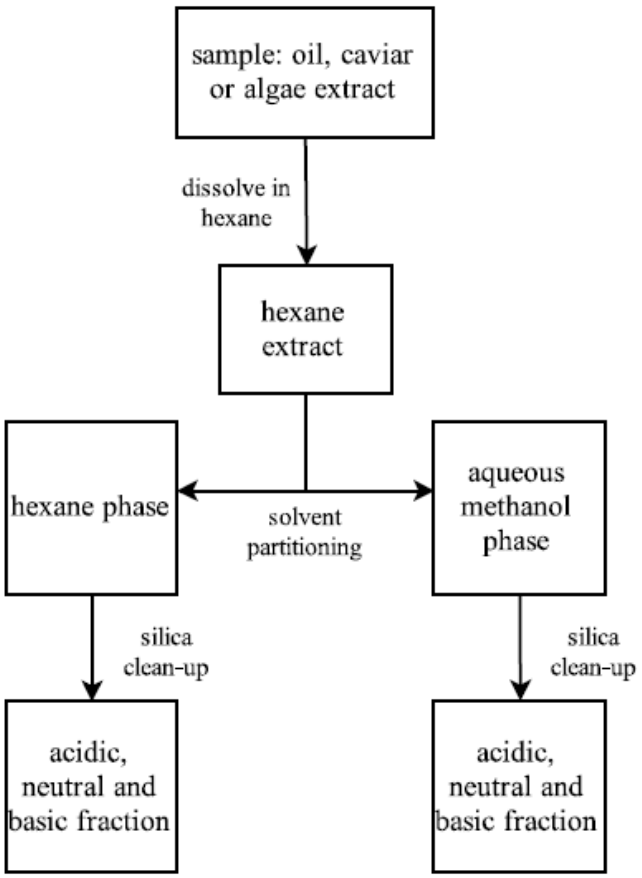

Figure 13. Schematic representation of the steps of the sample preparation.

3.1.1 Solvent partitioning

The sample preparation procedure included a solvent partitioning and a clean-up step (see Figure 13). Arsenic contents of the sample, extract and the partitioned phases were observed by using ICPMS. Also, the mass balance of both the hexane and the methanol phase was recorded. For Kombu, also the preliminary step of extraction was investigated by using ICPMS. The obtained data are listed in Table 8.

Table 8. Total arsenic content and total masses of the samples during the solvent partitioning (as depicted in Figure 13**)**.

Distribution of total As	**Herring caviar (n = 6)**	**Krill oil (n = 6)**	**Blue whiting (n = 3)**	**Kombu alga (n = 3)**
total lipid As (µg As g^{-1})	2.40 ± 0.23	3.81 ± 0.22	1.86 ± 0.02	0.42 ± 0.01
extracted As (µg As g^{-1}) hexane phase	1.01 ± 0.24	1.98 ± 0.12	1.51 ± 0.07	0.010 ± 0.002
extracted As (µg As g^{-1} extract d.m.) hexane phase	1.33 ± 0.32	3.06 ± 0.17	1.79 ± 0.05	0.54 ± 0.05
extracted As (µg As g^{-1}) aq. MeOH phase	1.35 ± 0.08	1.33 ± 0.04	0.33 ± 0.04	0.42 ± 0.13
extracted As (µg As g^{-1} extract d.m.) aq. MeOH phase	6.09 ± 0.33	3.88 ± 0.02	3.95 ± 0.07	18.9 ± 5.0
mass balance (%)	98 ± 2	99 ± 4	93 ± 5	92 ± 14
sum total As (%)	94 ± 1	90 ± 6	98 ± 4	78 ± 18

The total arsenic content of the fish and krill samples was comparable, while Kombu showed a considerably higher concentration (14.70 µg g^{-1} d.m.). These data are in keeping with the arsenic levels found in other seafood, ranging from around 1 to 100 µg g^{-1} dry mass.[13] When subjected to the partitioning, generally the hexane phase showed a higher mass share and less arsenic, yielding a lower concentration. The high lipid content of this phase makes it unattractive for analysis. The methanol phase in turn contains more arsenic and less matrix. Although arsenic concentrations were increased in the methanol phase in every sample, the improvement factor was far below the reported 48-fold improvement reported for other marine samples.[19,39] Also, both mass balance and the arsenic recovery of the procedure were good.

3.1.2 Silica clean-ups

A portion of the hexane and methanol phases of each sample was exposed to silica column chromatography. Each portion contained a distinct amount of arsenic. The distribution of this arsenic content over an acidic, a neutral and a basic fraction is reported in Table 9. Generally, the recovery of arsenic was between 45 % and 100 %. Mass recoveries were between 40 % and 98 %.

Table 9. Results of the silica clean-ups of the hexane and methanol phases of all samples.

	hexane phase		methanol phase	
herring caviar	**µg As**	**mg**	**µg As**	**mg**
initial content	**0.26**	**175.1**	**0.58**	**92.7**
acidic fraction	0.08	124.2	0.09	50.2
neutral fraction	0.03	6.2	0.03	3.0
basic fraction	0.09	27.5	0.14	9.1
sum of the fractions	**0.20**	**157.9**	**0.26**	**62.3**
krill oil				
initial content	**0.46**	**156.9**	**0.61**	**156.3**
acidic fraction	0.30	125.3	0.20	52.5
neutral fraction	0.04	0.1	0.12	5.4
basic fraction	0.09	17.3	0.29	32.4
sum of the fractions	**0.43**	**142.7**	**0.61**	**90.3**
blue whiting	**µg As**	**mg**	**µg As**	**mg**
initial content	**0.30**	**172.9**	**0.62**	**160.1**
acidic fraction	0.15	159.7	0.09	64.8
neutral fraction	0.02	8.9	0.02	0.5
basic fraction	0.09	0.9	0.27	0.1
sum of the fractions	**0.26**	**169.5**	**0.38**	**65.4**
kombu alga				
initial content	no clean-up possible		**0.38**	**5.0**
acidic fraction			0.02	2.1
neutral fraction			0.01	0.2
basic fraction			0.29	0.3
sum of the fractions			**0.32**	**2.6**

On a silica column, owing to their dimethylarsinoyl group, arsenolipids are eluted later than the matrix,[27] as normal lipids are not retained on silica. Therefore, following silica chromatography of both the hexane and methanol phase, the total mass was mostly found in the first fraction whereas most of the arsenic was in the last fraction. It has already been shown that some arsenolipids in fish oil can be changed by the silica column.[39] The AsHCs are unchanged by silica clean-up and for AsFAs recoveries of > 80% were found,[99] but it was suggested that AsPCs

decompose during the silica treatment.[28] Therefore, silica clean-up was not used for the remainder of this work.

3.2 Identification of known arsenolipids

3.2.1 Measurement of arsenolipid standards

One of the strengths of ICPMS as an HPLC detector is that the response (peak area) is dependent only on the amount of arsenic in the compound, and is independent of the nature of the compound. Thus, even unknown arsenic compounds (peaks) can be quantified against the calibration curve of an arsenic standard. This constant response was also shown for the arsenolipids where peak areas for the various arsenolipids at the same concentration were comparable (Figure 14; Table 10). This is not, however, the case with ESI-MS detection where even quite similar compounds can ionize to different degrees, and hence give different peak areas for the same amount of compound. The arsenolipid standards also showed that although the AsFAs showed comparable peak areas, the AsHCs differed by a factor of up to 2.6-fold at the same concentration (Table 10). Therefore, in this study HPLC/ICPMS was used for quantification, and HPLC/ESI-MS was used in a complementary manner to confirm identification.

Table 10. HPLC peaks recorded by ICPMS and ESIMS for 2 AsFAs and 3 AsHCs each at a concentration of 10 µg As L^{-1}.

Retention time	Compound	Area ICPMS	Area ESI-MS
10.5	AsFA 362	12259	60311
11.6	AsFA 388	12011	56569
14.9	AsHC 332	11512	100328
16.3	AsHC 360	11378	122303
18.9	AsHC 444	11785	262878

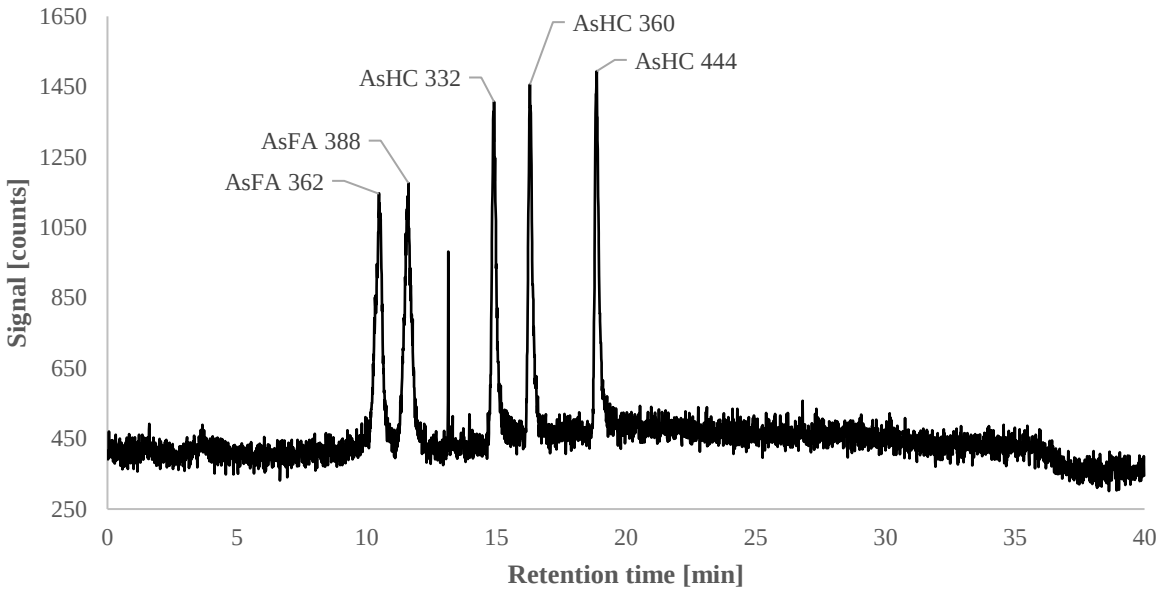

Figure 14. HPLC-ICPMS chromatogram of the standard mixture of 5 arsenolipids, each at 10 µg As L^{-1}. The unlabeled peak is an artifact of the HPLC system.

3.2.2 Column recovery of arsenolipids in extracts of samples

The column recovery was calculated as the sum of arsenic peaks divided by the total amount of arsenic injected onto the column (Table 11).

Table 11. Range and mean value of column recovery with Asahipak Method 1 (n = 4).

sample	column recovery range [%]	column recovery mean [%]
herring caviar	37 – 75	55
krill oil	29 – 66	44
blue whiting oil	25 – 55	48
kombu algae	25 – 77	49

The observed column recoveries indicate that quantification with Asahipak Method 1 is not a viable option. Although the mean recovery is about 50 % for all samples, the range of those values is too big. Even with better recovery values, quantification of individual arsenolipids would not be possible because many of the peaks were unresolved. Consequently, instead of identifying specific arsenolipids, various sections of the chromatogram were assigned as representing particular arsenolipid classes (Figure 15).

3.2.3 ICPMS Chromatograms of the samples

The chromatograms (Figure 15) indicate that many arsenolipids are present in the samples. While the fish and krill samples contained a lot of polar arsenic compounds, only small amounts of polar arsenicals were present in the alga. Krill oil and herring caviar contained many signals at retention times typical for AsFAs and short chain AsHCs, while blue whiting oil only showed small peaks in this area, and Kombu had none. The less polar arsenolipids - long chain AsHCs, AsPEs, AsPCs and AsPLs - are difficult to separate with HPLC/ICPMS; all four samples showed a range of signals in this region. The identification of those species has to be achieved with accurate mass spectrometry.

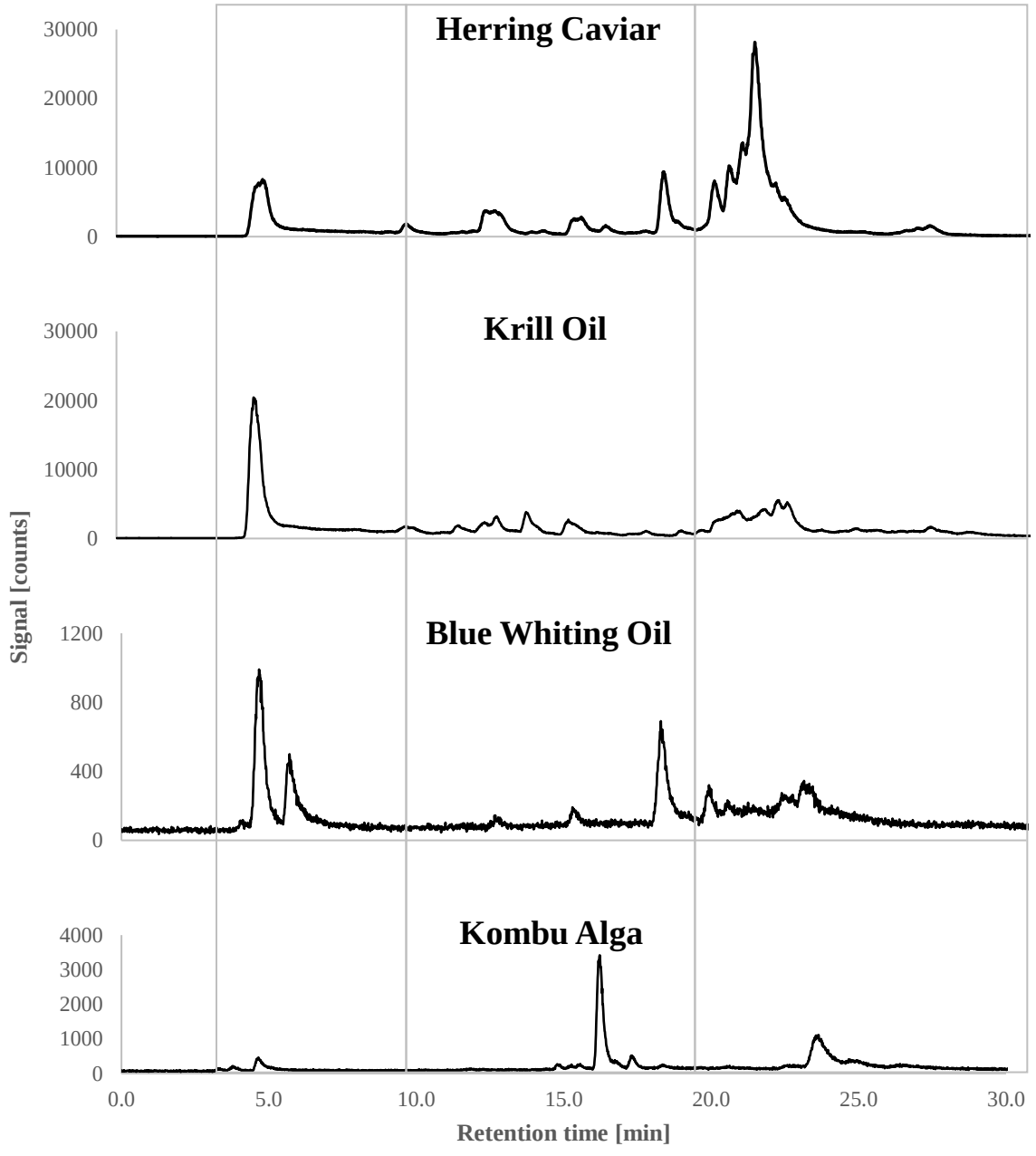

Figure 15. HPLC-ICPMS chromatograms of the four samples, using Asahipak Method 1.

3.2.4 Identification of known arsenolipids

Tandem MS analysis yielded more than 18 000 MS/MS spectra for each chromatographic run representing fragmentation spectra of approximately 700 isolated and fragmented ions. Most of the arsenolipids that have been discovered so far contain a dimethylarsinoyl headgroup, and two very characteristic fragments of this headgroup are found at *m/z* = 105 ($C_2H_6As^+$) and *m/z* = 123 ($C_2H_8AsO^+$). Using accurate mass MS, these fragments can be readily identified, facilitated by their mass defect, and they can thereby serve as indicators for the possible presence of arsenolipids.

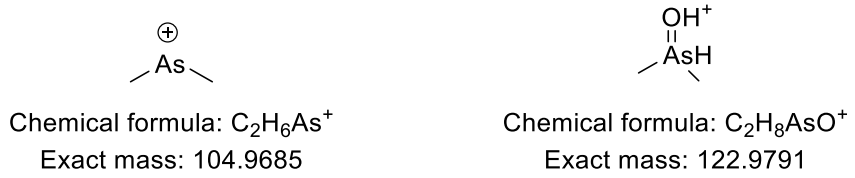

Figure 16. Characteristic fragments of arsenolipids containing a dimethylarsinoyl headgroup.

Because the krill oil and herring caviar sample were selected for their high phosphatidylcholine content, and the main focus of this work revolves around these cholines, the MS/MS spectra were also screened for *m/z* = 184, which represents the free phosphocholine fragment. Occurrence of this fragment at *m/z* = 184 together with one (or both) of the fragments at *m/z* = 105 and *m/z* = 123 suggested the presence of arsenic-containing phosphatidylcholines.

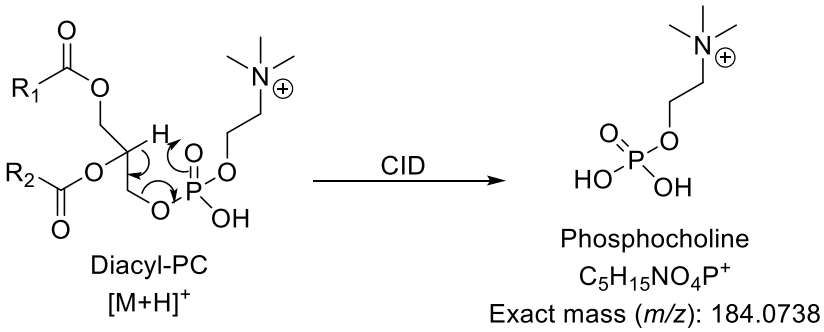

Figure 17. Fragmentation pathway for the phosphocholine fragment from diacyl-PCs as proposed by Murphy et al.[100]

By screening the MS/MS spectra for the fragments at *m/z* 105 and 123, many arsenic-containing compounds were detected in the samples. In total, 49 arsenolipids were identified, including 10 known AsFAs, 5 known AsHCs, and 5 known arsenosugar phospholipids. In addition one AsPE and 28 AsPCs, including 23 new compounds (see section 3.3), were identified.

In herring caviar, three known fatty acids (AsFA 382, 408, 422) and AsPE 1035 were identified that had not previously been found in this type of sample, and four known AsPCs were found

in krill oil. The other compounds found in the samples were already reported for each type of sample. Arsenosugar phospholipids were identified only in the algal sample. Interestingly, krill oil was the only sample that did not contain any AsHCs. AsPCs and AsPEs were not detected in blue whiting nor were they present in Kombu; therefore neither of these samples was used in the hydrolysis experiments.

In Table 12 all of the known compounds identified in this study are listed. Note that for AsPCs, the abbreviated description of the compounds was changed because some of the new AsPCs had the same molecular mass, and hence the existing system did not unambiguously describe the compounds. Thus, the molecular mass of the arsenic-containing fatty acid was added to the abbreviation, giving the general scheme: AsPC molecular mass of the compound – molecular mass of the AsFA.

Table 12. Known arsenolipids identified in the study samples. |***Δm/m***| values are bold and in italics. (*AsPC 939-436 (new compound) in case of krill oil) († compound identified previously, but for the first time in this kind of sample)

Com-pound	Formula $[M+H]^+$	$[M+H]^+$ calcd.	Herring Caviar	Krill Oil	Blue whiting	Kombu Alga	Ref.
AsFA 334	$C_{15}H_{32}AsO_3$	335.1562	335.1569 ***2.1***				19
AsFA 362	$C_{17}H_{36}AsO_3$	363.1875	363.1881 ***1.7***	362.1882 ***1.9***	363.1877 ***0.5***		19
AsFA 382	$C_{19}H_{32}AsO_3$	383.1562	383.1567 ***1.4***†				45
AsFA 388	$C_{19}H_{38}AsO_3$	389.2031	389.2037 ***1.5***				19
AsFA 390	$C_{19}H_{40}AsO_3$	391.2188	391.2191 ***0.9***	391.2201 ***3.3***	391.2174 ***3.5***		19
AsFA 408	$C_{21}H_{34}AsO_3$	409.1718	409.1725 ***1.5***†				45
AsFA 422	$C_{22}H_{36}AsO_3$	423.1875	423.1880 ***1.1***†				44
AsFA 436	$C_{23}H_{38}AsO_3$	437.2031	437.2035 ***0.9***	437.2042 ***2.4***	437.2040 ***2.0***		19
AsFA 448	$C_{24}H_{38}AsO_3$	448.2031	449.2039 ***1.7***	449.2042 ***2.4***	449.2044 ***2.8***		49
AsFA 528	$C_{30}H_{46}AsO_3$	529.2657	529.2673 ***2.8***	529.2654 ***0.7***			28
AsHC 332	$C_{17}H_{38}AsO$	333.2133	333.2137 ***1.3***		333.2141 ***2.2***		20
AsHC 358	$C_{19}H_{40}AsO$	359.2290	359.2293 ***0.8***				41
AsHC 360	$C_{19}H_{42}AsO$	361.2446	361.2452 ***1.7***			361.2454 ***2.1***	20
AsHC 404	$C_{23}H_{38}AsO$	405.2133	405.2138 ***1.2***		405.2143 ***2.3***		20
AsHC 444	$C_{25}H_{54}AsO$	445.3385			445.3400 ***3.4***		39
AsPL 930	$C_{43}H_{85}O_{14}AsP$	931.4887				931.4907 ***2.0***	25
AsPL 958	$C_{45}H_{89}O_{14}AsP$	959.5200				959.5219 ***1.9***	22
AsPL 972	$C_{46}H_{91}O_{14}AsP$	973.5357				973.5383 ***2.8***	25
AsPL 1000	$C_{48}H_{95}O_{14}AsP$	1001.5670				1001.5676 ***0.6***	25
AsPL 1014	$C_{49}H_{97}O_{14}AsP$	1015.5826				1015.5827 ***<0.1***	25
AsPC 885-362	$C_{45}H_{82}AsNO_9P$	886.4938	886.4937 ***0.1***	886.4956 ***2.1***†			28
AsPC 911-362	$C_{47}H_{84}AsNO_9P$	912.5094	912.5101 ***0.7***	912.5100 ***0.6***†			28
AsPC 939-390	$C_{49}H_{88}AsNO_9P$	940.5407	940.5416 ***1.0***	940.542 ***1.6***†*			28
AsPC 985-436	$C_{49}H_{88}AsNO_9P$	986.5251	986.5249 ***0.2***				28
AsPC 997-448	$C_{55}H_{86}AsNO_9P$	998.5251	998.5264 ***1.3***	998.5262 ***1.1***†			28
AsPE 1035-528	$C_{57}H_{87}AsNO_9P$	1036.5407	1036.5405 ***0.2***†				28

3.3 Novel arsenic-containing phosphatidylcholines found in herring caviar

Arsenic-containing phosphatidylcholines elute after the first hydrocarbons, at retention times between 16 and 19 minutes (using Asahipak Method 1, see experimental part for details). The HPLC/ICPMS chromatogram of the sample suggests the presence of a multitude of AsPCs.

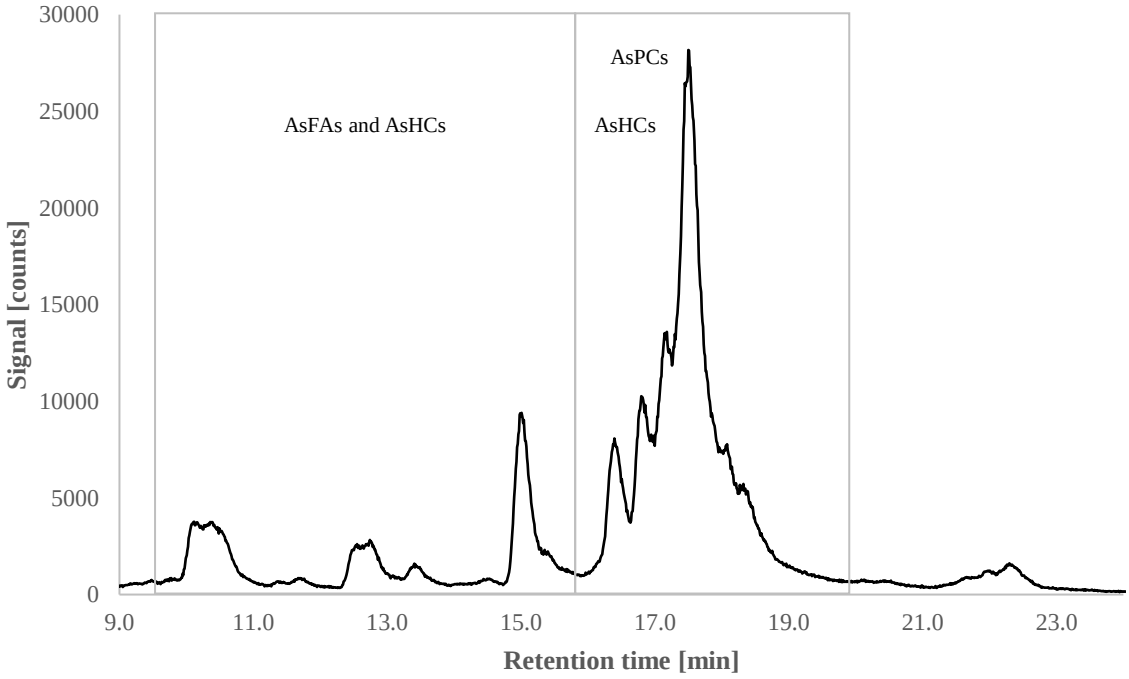

Figure 18. Typical HPLC/ICPMS chromatogram of the herring caviar sample, using Asahipak Method 1 (see also Figure 15).

For characterization of the AsPCs, a multitude of indicators was used. For all new AsPCs, a singly charged $[M+H]^+$ and a doubly charged $[M+2H]^{2+}$ parent species were observed. The fragmentation patterns found were also matching. They included a phosphocholine fragment (*m/z* 184), a vinyl dihydrogen phosphate fragment (*m/z* 125), a choline fragment (*m/z* 104), and a choline fragment without the hydroxyl group (*m/z* 86). Furthermore, a fragment was observed for the AsFA, the AsFA after water loss and a fragment of the molecule after loss of the phosphocholine group.

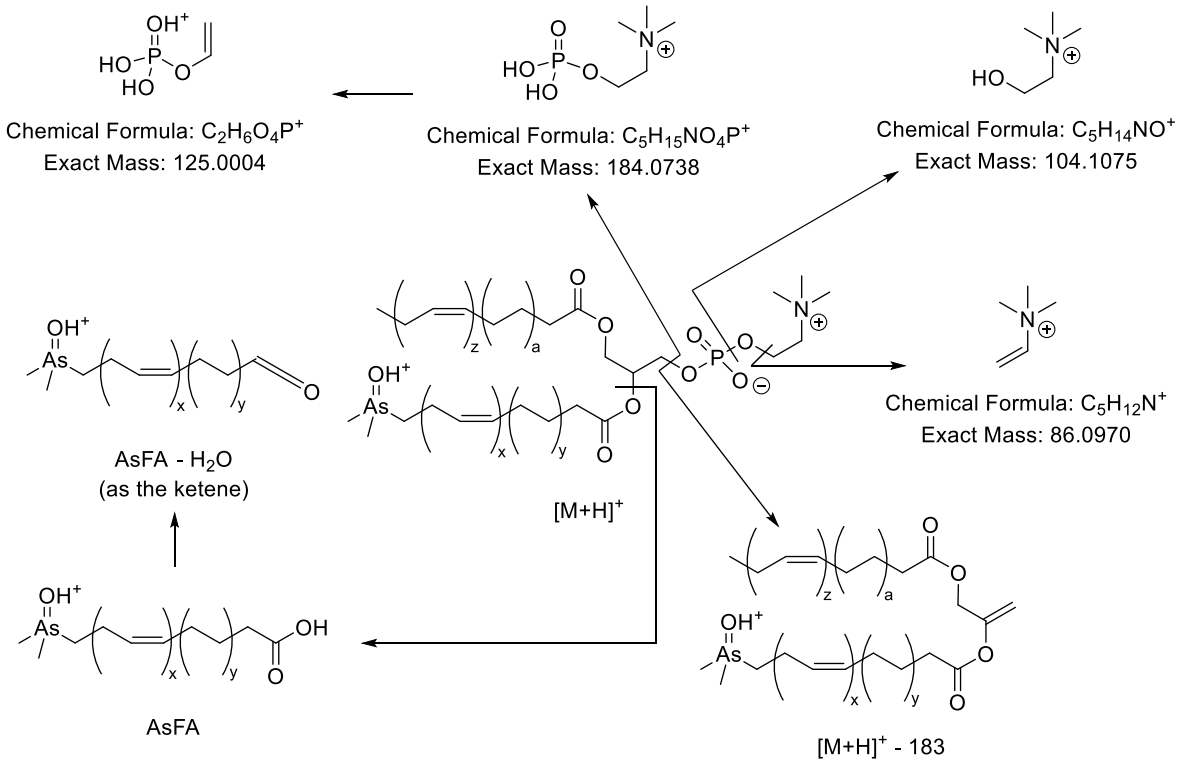

Figure 19. Fragmentation diagram of AsPCs. x, z = 0, 1, 2, … and a, y = 0, 0.5, 1, 1.5, …

Using the fragments shown in Figure 19 (also see Table A 5 in Appendix B – HR-MS data of the new AsPCs) and the singly and doubly charged parent ion (Appendix B), the new AsPCs were identified.

3.3.1 Identification of new AsPCs

In total, 23 new AsPCs were identified in herring caviar. Using AsPC 959-436 as an example, the identification process is described in the following. First, the scans taken from the chromatograms were examined for the presence of either singly charged or doubly charged AsPCs, based on the general formula $C_xH_yO_9NPAs$. For the fitting masses, chromatograms were extracted and it was verified that both the singly and the doubly charged compound had the same retention times (Figure 20).

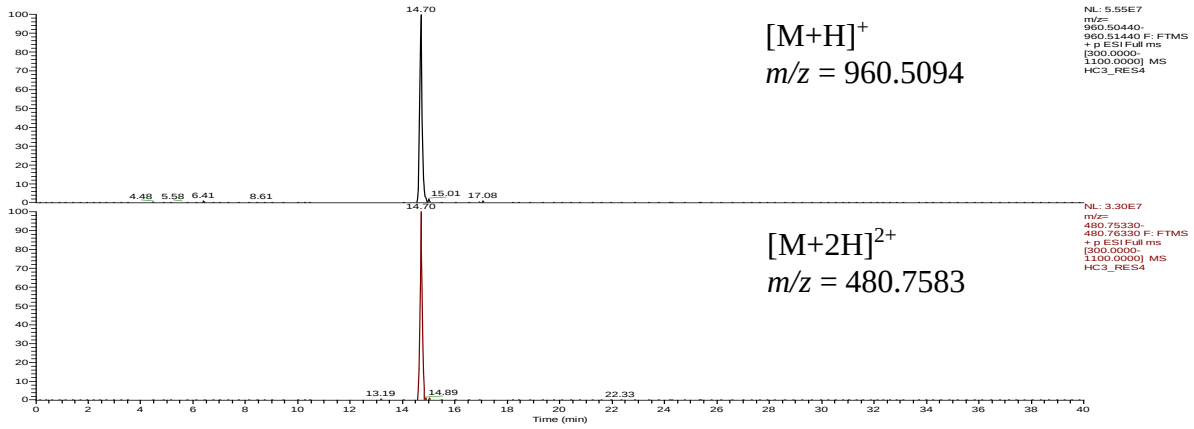

Figure 20. Chromatogram of singly and doubly charged AsPC 959-436.

Then, at the determined retention times, the scan spectrum was compared to a simulated spectrum. If the isotopic patterns of both species (examples in Figure 21) fit well, the fragmentation spectrum of the compound was investigated.

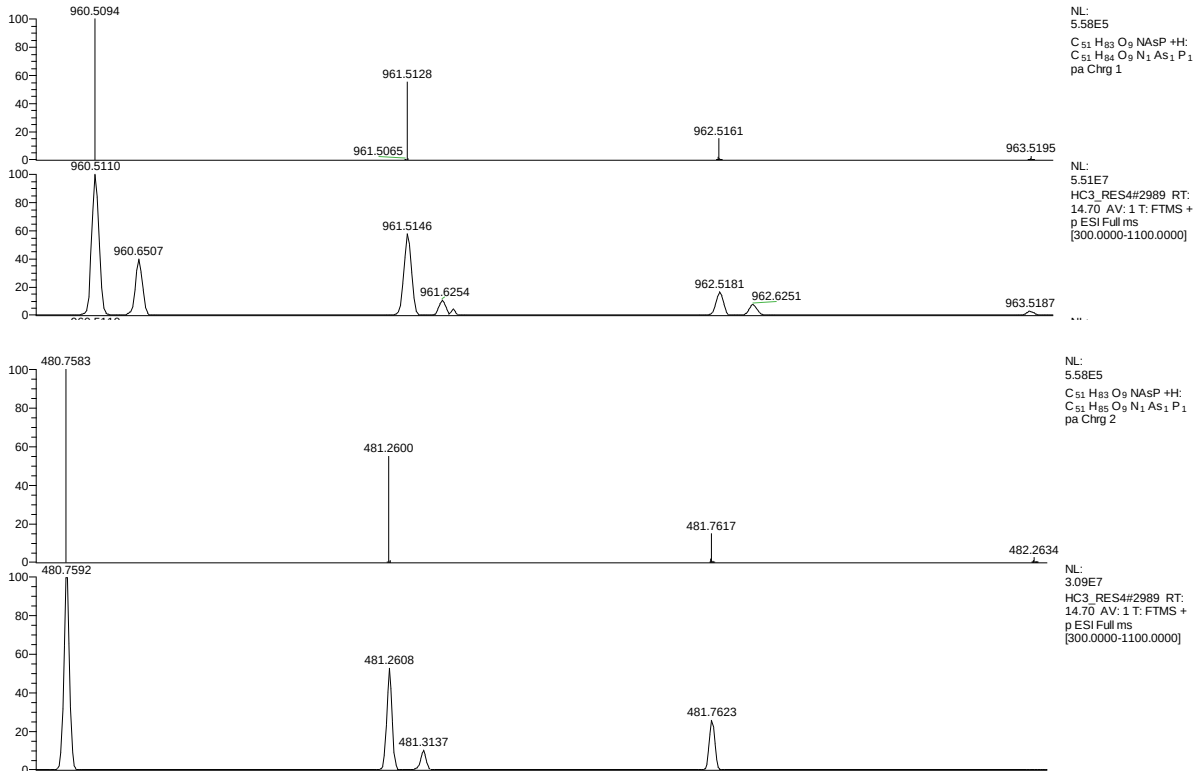

Figure 21. Simulated and measured isotopic pattern of singly and doubly charged AsPC 959-436.

Fragmentation spectra of the new compounds were diverse, but some general trends were observed. Fragmentation spectra of the doubly charged species always contained fewer fragments since fragmentation conditions were not as harsh as those used for singly charged species (for a comparison see Figure 22). Also, saturated AsFAs gave more intense $[M+H]^+$ and $[M+H]^+$-H_2O signals, probably because they are fragmented more easily, which in turn gives a higher

intensity of fragments in the C_xH_y region for unsaturated AsFAs (compare Figure 22 (for unsaturated) and Figure 23 (for saturated AsFAs)).

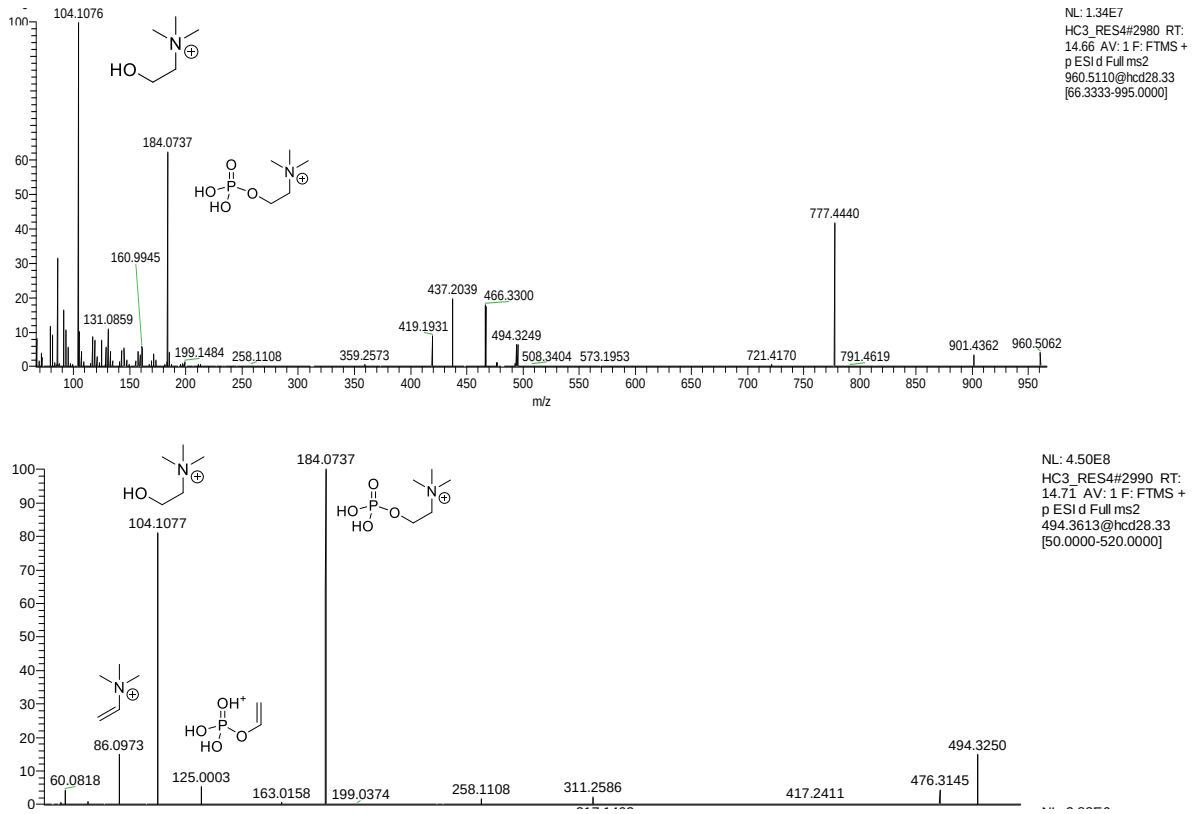

Figure 22. Fragmentation spectrum of $[M+H]^+$ and $[M+2H]^{2+}$ of AsPC 959-436.

Furthermore, it was observed that compounds of the same molecular mass showed different retention time behavior. For example, it was observed that AsPC 887-362 and AsPC 887-390 co-eluted, resulting in the presence of both AsFAs in the same fragmentation spectrum. Co-elution in this case was not surprising because the two AsPCs differ only slightly in chain length of their AsFA and their carbon fatty acid. For other cases, like AsPCs 885, we observed that differences of retention times could be up to a minute (using Asahipak Method 2) between the first and the last observed AsPC of that mass. The retention times, however, were not governed by the AsFA. For example, the free arsenic-containing fatty acids elute in the order AsFA 382, AsFA 362, AsFA 408, AsFA 436, whereas the phosphatidylcholines elute in the order AsPC 885-362 followed by AsPC 885-436, AsPC 885-382 and AsPC 885-408. This indicates that retention time of the molecules does not only depend on chain length and unsaturation of the contained fatty acids.

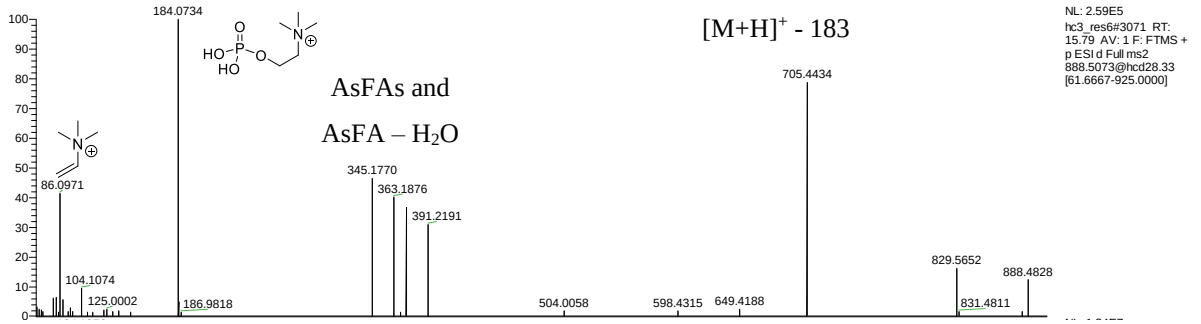

Figure 23. Fragmentation spectrum of AsPC 887, showing two different AsFAs. This is due to co-elution of two AsPCs 887.

3.3.2 Structure and fatty acid constituents

It is noteworthy that all new arsenic-containing phosphatidylcholines contain only those AsFAs that are also found in free form (Figure 24), as was observed previously.[28] In addition to the five AsPCs found previously, which contained four AsFAs (AsFA 362, AsFA 390, AsFA 436, AsFA 448), we found AsPCs containing three additional known AsFAs (AsFA 334, AsFA 382, AsFA 408).

Previously, AsPCs and AsPE 1035-528 were found to contain two highly unsaturated omega-3 fatty acids, namely eicosapentanoic acid (C20:5, EPA) and docosahexaenoic acid (C22:6, DHA). Both these compounds are claimed (by the manufacturer) to be very rich in the herring caviar capsules. Additionally, nine other known non-arsenic fatty acids were found in the new AsPCs.

Marine fish typically contain a multitude of saturated, unsaturated and polyunsaturated fatty acids,[101] and hence the large variety of fatty acids incorporated in the AsPCs is not surprising. Studies on herring have shown the presence of the two saturated, the two monounsaturated fatty acids and an unusual odd-chain penta-unsaturated fatty acid,[102] found also in our study, as well as the occurence of the di-unsaturated linolenic acid and tetra-unsaturated stearidonic and arachidonic acid.[102,103] Another odd-chain, hexa-unsaturated fatty acid was found in the AsPCs. The origin of the free fatty acid is thought to be dinoflaggelates,[104] planktonic species at the base of the food chain.

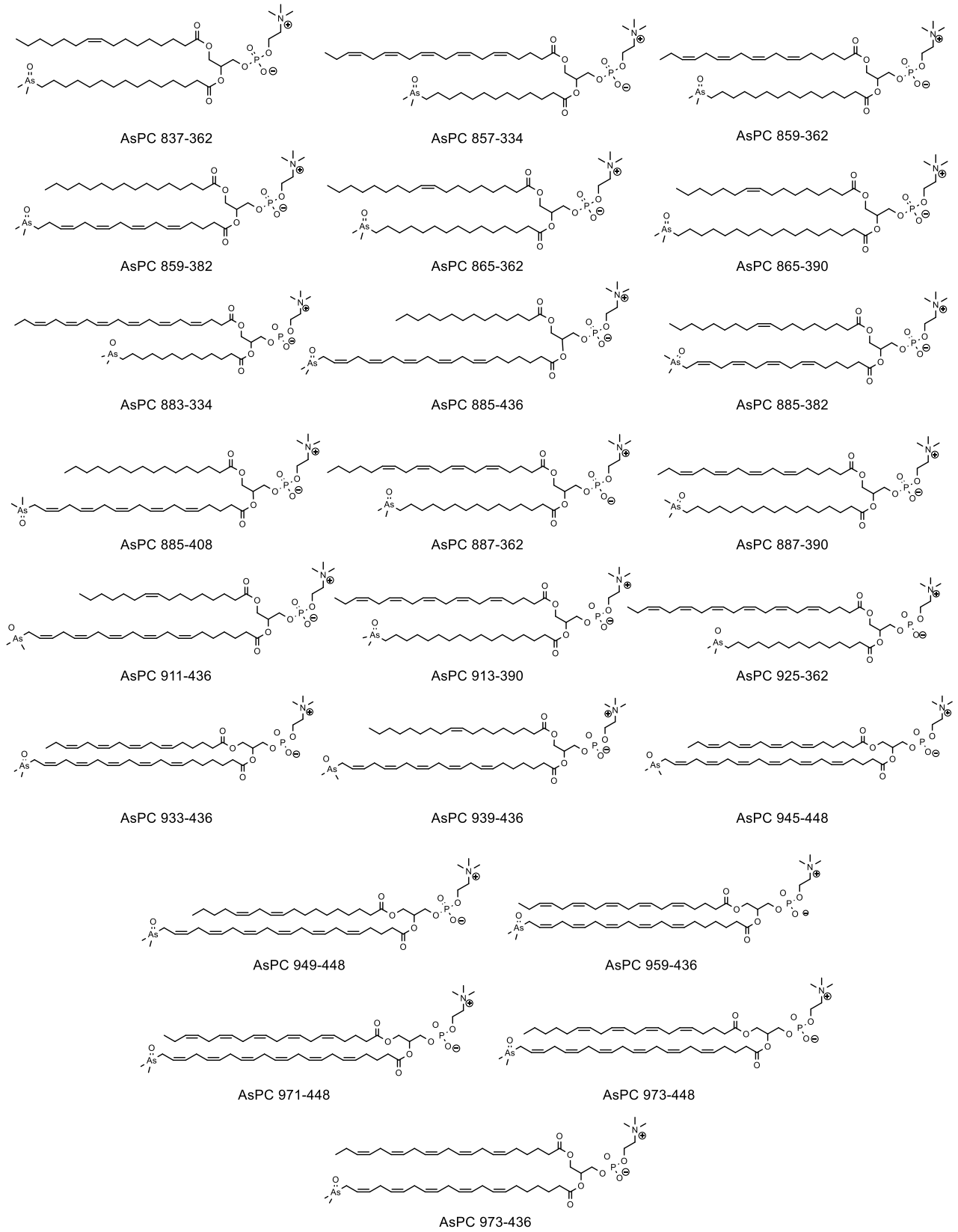

Figure 24. Structures of all 23 new AsPCs identified in this work.

3.3.3 Relative amounts of the AsPCs

Quantification of arsenic-containing compounds with no standard available is usually done by HPLC/ICPMS. In the case of AsPCs, the retention times of the compounds are similar with the method used in this work, with 30 (and likely more) arsenic-containing phosphatidylcholines and phosphatidylethanolamines eluting within two minutes. The AsPCs can, however, be individually measured by ESI-MS, but the signal of each compound might be dependent on both the compound and the matrix. Although the signal intensities might vary a lot even for very similar compounds, they could still be useful as a first indication of the relative abundance of the AsPCs. For HPLC/ESI-MS chromatograms of the compounds, see Figure A 1 to Figure A 7 in Appendix C.

Generally, the ion count of the previously known AsPCs was higher than the ion count of the novel compounds. Overall, the highest ion count was 220 times higher than the lowest one. This can only partly be a consequence of different ionization behavior, as the carbon containing fatty acids should not alter the ionization of the compound, and all AsFAs (which can influence the ionization behavior) are found in AsPCs of all different signal intensities. This observation suggests that the newly identified compounds are also possible constituents of the herring samples in which the first members of the AsPC group were found. However, with the sample preparation used in this work they are likely present in higher concentrations, because the partitioning was directly done with the crude sample instead of an extract.

Table 13. AsPCs found in herring caviar, listed in ascending order of their retention time (using Asahipak Method 2).

Compound	RT [min]	Counts	Compound	RT [min]	Counts
AsPC 857-334	13.25	$1.3 \cdot 10^6$	**AsPC 865-390**	14.70	$9.6 \cdot 10^6$
AsPC 859-362	13.55	$3.2 \cdot 10^6$	**AsPC 973-436**	14.70	$2.2 \cdot 10^6$
AsPC 885-362	13.95	$5.5 \cdot 10^7$	**AsPC 971-448**	14.75	$5.0 \cdot 10^7$
AsPC 883-334	13.70	$8.9 \cdot 10^6$	**AsPC 865-362**	14.75	$7.9 \cdot 10^6$
AsPC 837-362	14.00	$3.1 \cdot 10^6$	**AsPC 885-382**	14.75	$1.7 \cdot 10^6$
AsPC 887-362	14.30	$5.5 \cdot 10^6$	**AsPC 911-436**	14.80	$7.1 \cdot 10^5$
AsPC 887-390	14.30	$5.5 \cdot 10^6$	**AsPC 939-390**	14.95	$6.2 \cdot 10^7$
AsPC 911-362	14.35	$1.0 \cdot 10^8$	**AsPC 985-436**	15.05	$1.5 \cdot 10^8$
AsPC 933-436	14.35	$5.8 \cdot 10^6$	**AsPC 973-448**	15.05	$2.0 \cdot 10^6$
AsPC 945-448	14.45	$2.4 \cdot 10^6$	**AsPC 885-408**	15.05	$1.4 \cdot 10^6$
AsPC 913-390	14.60	$2.1 \cdot 10^7$	**AsPC 997-448**	15.10	$1.5 \cdot 10^7$
AsPC 885-436	14.60	$2.5 \cdot 10^6$	**AsPC 949-448**	15.10	$7.2 \cdot 10^5$
AsPC 859-382	14.65	$3.7 \cdot 10^6$	**AsPC 925-362**	15.40	$4.9 \cdot 10^6$
AsPC 959-436	14.70	$5.5 \cdot 10^7$	**AsPC 939-436**	15.40	$1.4 \cdot 10^6$

Although the ion counts cannot be translated into concentrations, they likely at least give an idea of the relative abundance of the various compounds within the AsPC class. It seems that the concentration of the AsPCs is only partly influenced by the concentration of the free AsFAs, as there are compounds of high and low abundance, both containing AsFA 436. Also, the influence of the second (non-arsenic) fatty acid is not clear. For example, AsPC 925-362 is moderately abundant in herring caviar even though it contains C23:6, a very unusual fatty acid of low natural abundance. The controlling factor here is possibly the AsFA, of which AsFA 362 is the most abundant AsFA in the herring caviar sample. In general, it is thought that the relative abundance of the AsPCs is governed by both the abundance of the AsFA and the abundance of the non-arsenic FA, but there are a few examples that do not fit with this concept.

3.4 Assessment of enzymatic activity on arsenolipids

3.4.1 Effect of the reaction conditions

The herring caviar extract changed little when it was incubated under reaction conditions, but without enzymes (Figure 25). Although small increases were apparent in the AsFA region, which might be at least partly due to partial AsPC hydrolysis, the AsPCs were not significantly affected. These results indicated that any changes observed in the planned enzyme experiments could be ascribed to the enzyme.

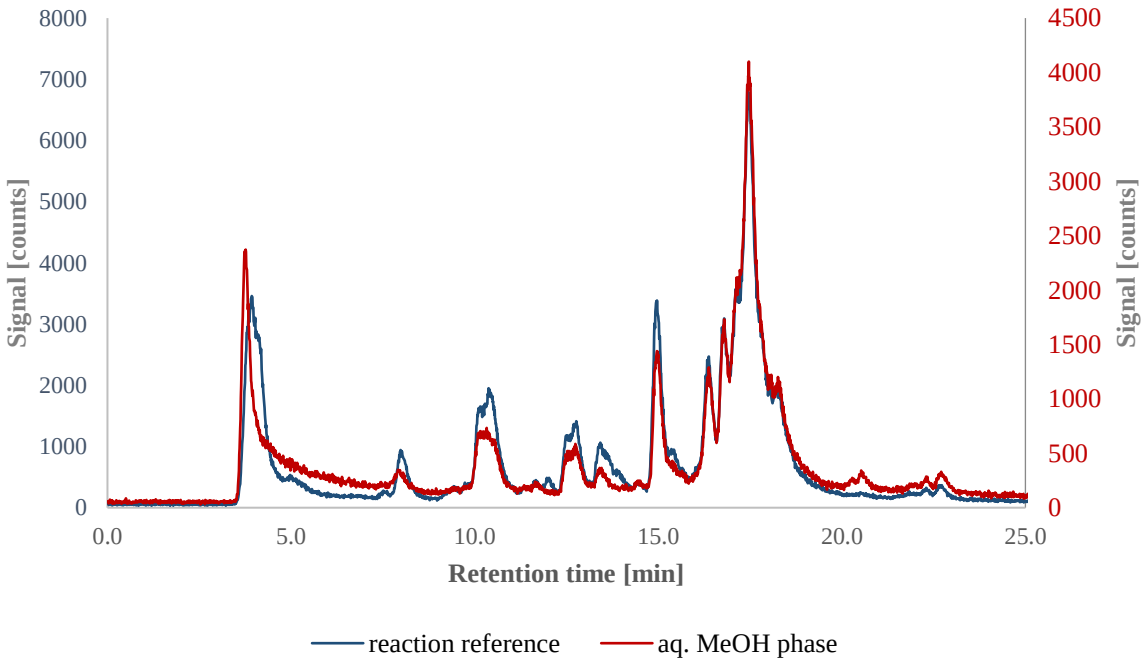

Figure 25. HPLC-ICPMS chromatogram of the MeOH phase reference and the reaction reference, which was incubated under the same conditions as the samples, but without enzymes.

3.4.2 Chemoselectivity of the enzyme

To test the chemoselectivity towards phospholipids of phospholipases A_2, C and D, control reactions were performed with a synthetically available[105] triacylglyceride containing two palmitic acid ester linkages at positions *sn*-1 and *sn*-3, and AsFA 362 in the *sn*-2-position. It is already known that PLA_1 also exhibits lipase activity and readily hydrolyzes triacylglycerides.[59] The starting material was stable under all reaction conditions, with over 99 % of it staying intact in each case. Two trace degradation products were observed at RT = 12 min and RT = 18 min (Figure 26). They were identified by ESI-MS as the methyl ester of AsFA 362 and the diacylglyceride after loss of one palmitic acid molecule, both resulting from nucleophilic attack of methanol at the ester bonds of the triacylglyceride molecule (Figure 27).

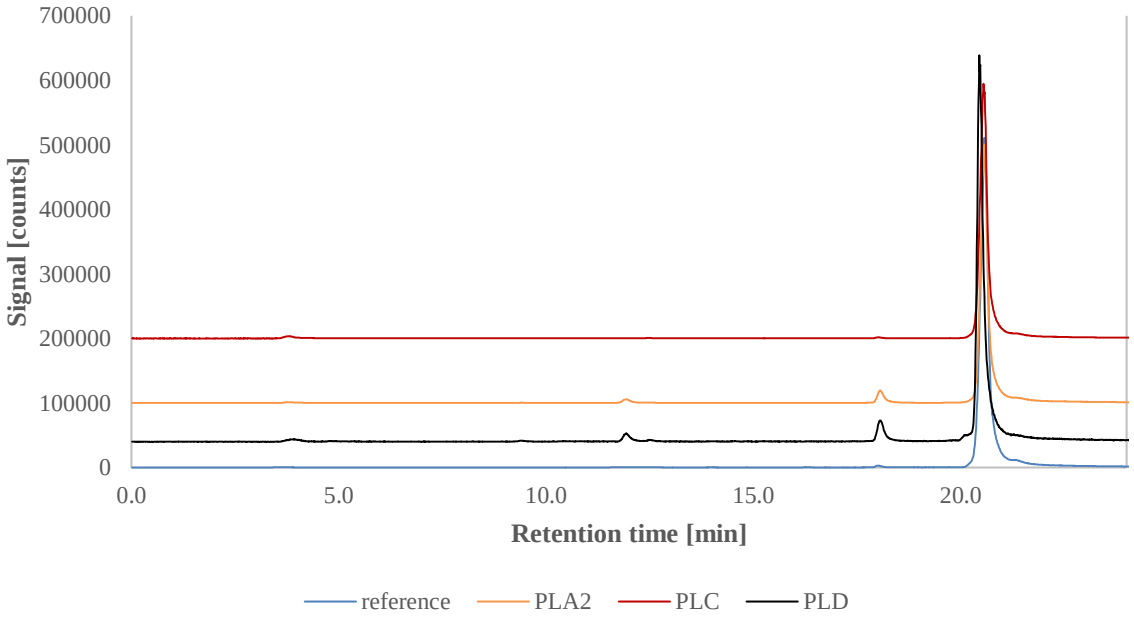

Figure 26. HPLC/ICPMS chromatograms of the arsenic-containing triacylglyceride and its hydrolysates. Chromatograms have been offset for clarity.

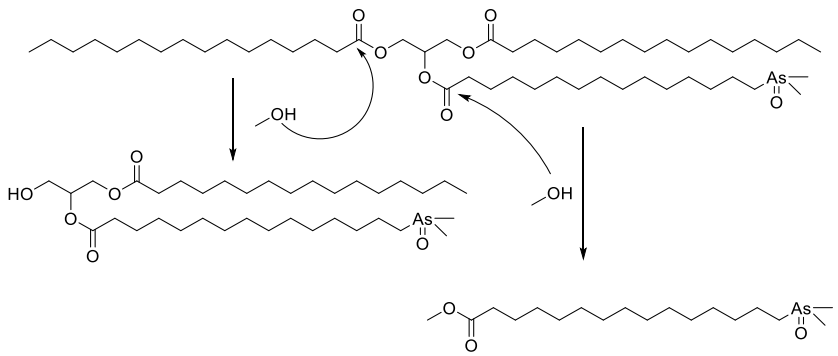

Figure 27. 2-((15-(dimethylarsinoyl)pentadecanoyl)oxy)propane-1,3-diyl dipalmitate and its degradation products as identified by ESI-MS.

3.4.3 Influence of the dimethylarsinoyl group on enzyme activity

The possible influence of the dimethylarsinoyl group on the hydrolysis efficiency of the phospholipases towards arsenic-containing phospholipids was then assessed by using a synthesized AsPC, namely AsPC 839-362, which contains AsFA 362 in the *sn*2-position. PLA_1, PLA_2 and PLC showed high hydrolysis efficiency - no AsPC 839-362 was observed in the hydrolysates, indicating full conversion. Thus, the presence of an unusual fatty acid with an arsenic-containing headgroup did not hinder the activity of these three enzymes The PLD assay, however, only showed unspecific degradation of the compound, suggesting that this enzyme assay does not work with AsPCs.

3.5 Hydrolysis experiments with herring caviar

The hydrolysis of AsPCs with phospholipasese A_1 and A_2 is expected to yield free fatty acids and lysophosphatidylcholines (Figure 28). Both fatty acid residues can either be AsFAs or normal FAs, leaving either a normal lysophosphatidylcholine or an arsenic containing phosphatidylcholine. PLC hydrolysis could yield arsenic containing diacylglycerides, and PLD would be expected to give arsenic containing diacylglyceridephosphates. All of these products should be detectable and distinguishable by HPLC/ICPMS-ESI-MS.

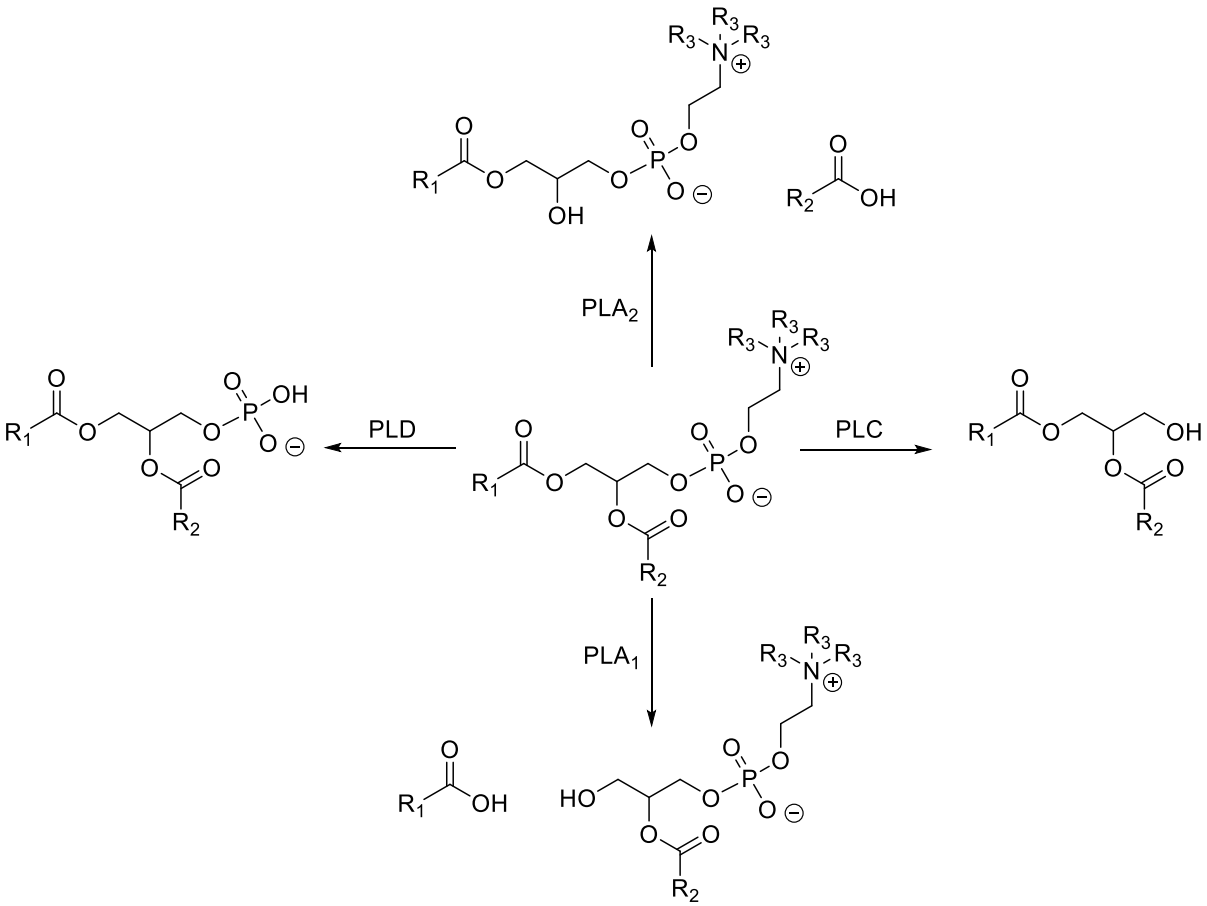

Figure 28. Expected hydrolysis products for the four phospholipases used. R_1, R_2 = fatty acid or AsFA, R_3 = Me for AsPCs and R_3 = H for AsPEs.

3.5.1 Hydrolysis with Phospholipase A_1

Treatment of herring caviar with PLA_1 resulted in complete hydrolysis of all AsPCs with a concomitant increase in AsFAs (Figure 29). The possibility that the hydrolysis products included lysoAsPCs that co-eluted with the free fatty acids was excluded by HPLC/ESIMS analysis of the reaction mixture. The remaining signal that decreased in comparison with the reference corresponds to AsHC 360 that co-elutes with the AsPCs.

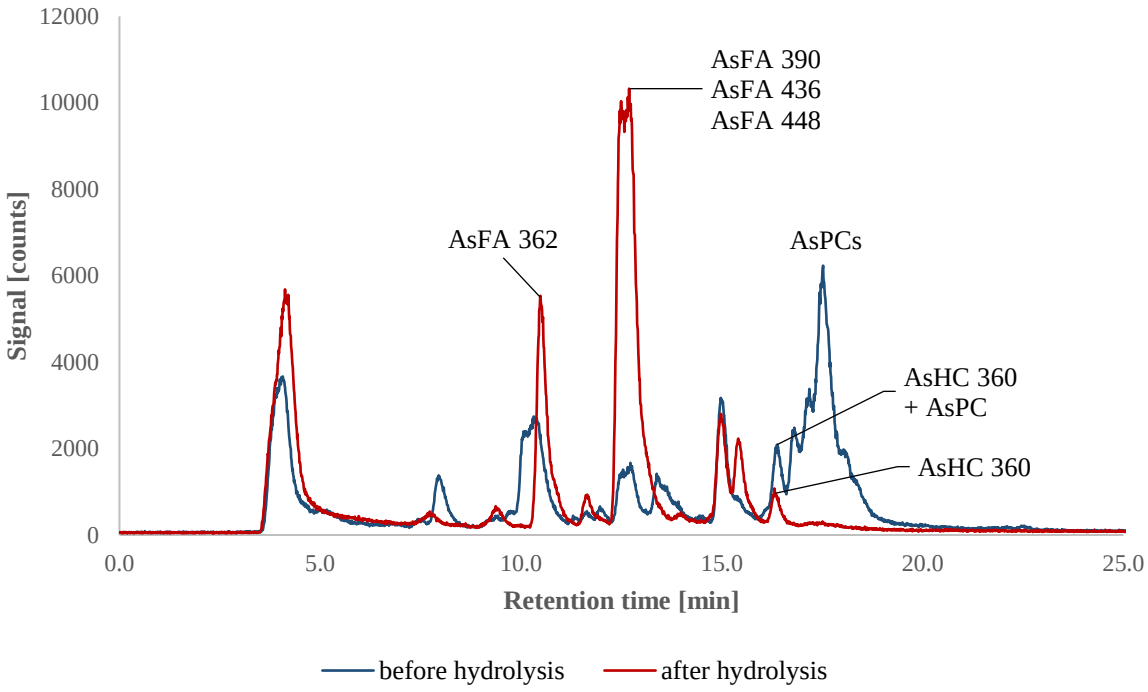

Figure 29. HPLC/ICPMS chromatogram of herring caviar (methanol phase) before and after treatment with PLA_1.

The quantitative cleavage of the ester linkage by PLA_1 would at first glance suggest that AsFAs are exclusively incorporated in the *sn*-1 position of AsPCs. It was reported by Jiang et al.[62] and Yang et al,[106] however, that positional analysis with PLA_1 is not straightforward. First, the produced *sn*-1-lysophospholipids are not stable under basic conditions; acyl migration yields the more stable *sn*-2-lysophospholipid. Second, the enzyme exhibits also lipase activity and lysoPL activity, meaning it also hydrolyses triacylglycerides as well as *sn*2-lysophospholipids. Therefore, it is expected that PLA_1 hydrolyses the AsPCs, and the hydrolysis products undergo acyl migration and subsequent enzymatic hydrolysis, yielding only free fatty acids (Figure 30).

Thus, it is not possible to distinguish whether the increase of the signal of free AsFAs is a consequence of PLA_1 hydrolysis of the AsPCs or of a lyso-AsPC after acyl migration. HPLC/ESI-MS data suggest the latter because no lysophosphatidylcholines, neither arsenic-containing nor standard lysoPCs, were observed. The absence of these compounds indicates that they are also hydrolyzed by PLA_1, meaning that no conclusion about the fatty acid positions can be drawn.

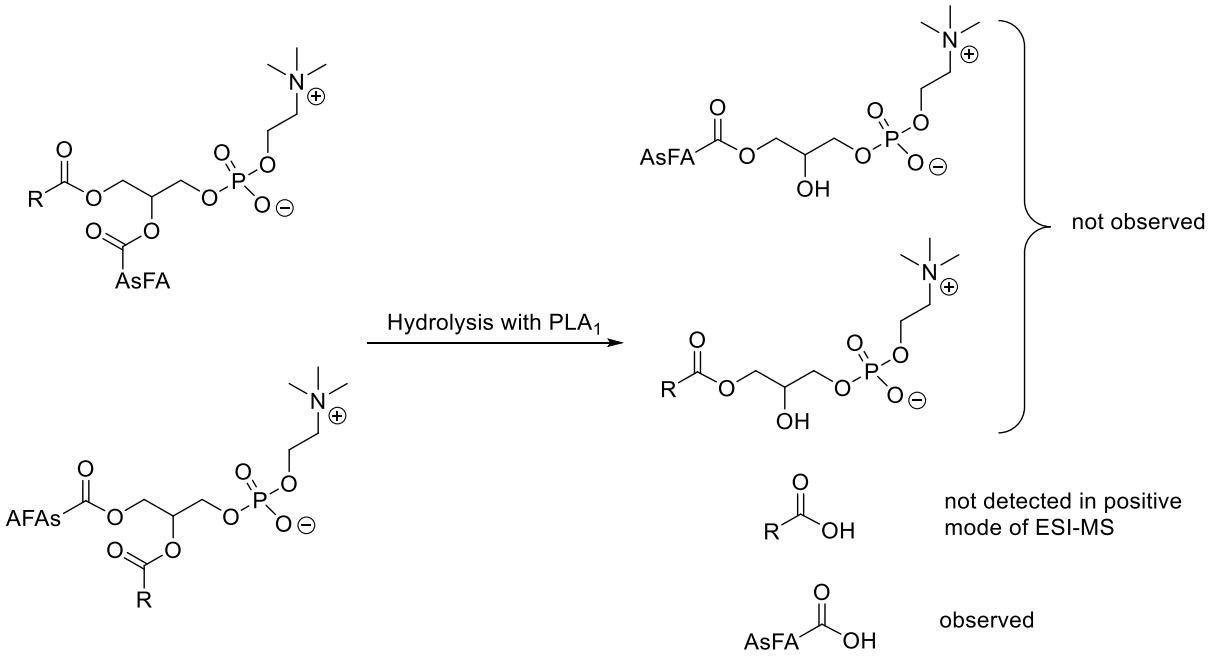

Figure 30. Proposed hydrolysis products of PLA_1. Lysophoposphatidylcholines are formed by hydrolysis and subsequent acyl migration.

3.5.2 Hydrolysis with Phospholipase A_2

The HPLC/ICPMS chromatogram of PLA_2 indicates residual arsenic-containing compounds at retention times similar to the AsPCs (Figure 31). One of these compounds is, as mentioned before, AsHC 360 co-eluting with AsPCs (RT = 16.3 min). The other small peaks in this region indicate either incomplete hydrolysis of the AsPCs or the presence of other arsenic-containing lipids, possibly arsenic-containing triacylglycerides. This interpretation is consistent with the fact that there are no residual peaks after PLA_1 hydrolysis; PLA_1 is known to be unselective and can hydrolyze other lipids including triacylglycerides, whereas PLA_2 selectively catalyzes only the hydrolysis of phosphatidylcholines, as shown in section 3.4.2.

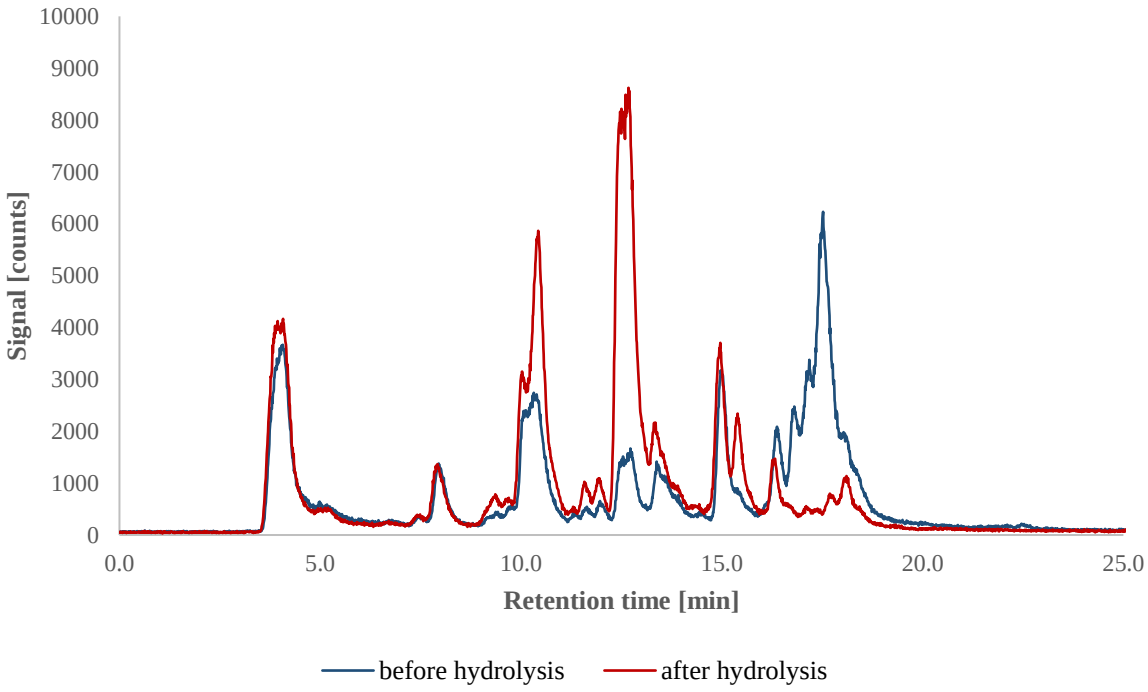

Figure 31. HPLC/ICPMS chromatogram of herring caviar (methanol phase) before and after treatment with PLA_2.

The selectivity of PLA_2 does not only exclude other lipids and phospholipids from hydrolysis, but also prevents the hydrolysis of lysophosphatidylcholines. Consequently, lysoPCs were detected as hydrolysis products of PLA_2, in contrast to the outcome from PLA_1 hydrolysis.

Table 14. Lysophosphatidylcholines found by HPLC/ESI-MS (Orbitrap) after hydrolysis of the herring caviar sample with PLA_2.

Compound	Formula	$[M+H]^+$ calculated	$[M+H]^+$ experimental
C14:0 lysoPC	$C_{22}H_{46}NO_7P$	468.3090	468.3094 (***0.9***)
C16:0 lysoPC	$C_{24}H_{50}NO_7P$	496.3403	496.3406 (***0.6***)
C16:1 lysoPC	$C_{24}H_{48}NO_7P$	494.3247	494.3249(***0.4***)
C18:1 lysoPC	$C_{26}H_{23}NO_7P$	522.3560	522.3566 (***1.2***)
C18:2 lysoPC	$C_{26}H_{50}NO_7P$	520.3403	520.3409 (***1.2***)
C18:4 lysoPC	$C_{26}H_{46}NO_7P$	516.3090	516.3076 (***2.7***)
C20:4 lysoPC	$C_{28}H_{50}NO_7P$	544.3403	544.3386 (***3.1***)
C20:5 lysoPC	$C_{28}H_{48}NO_7P$	542.3247	542.3251 (***0.7***)
C21:5 lysoPC	$C_{29}H_{50}NO_7P$	556.3403	556.3414 (***2.0***)
C22:6 lysoPC	$C_{30}H_{50}NO_7P$	568.3403	568.3405 (***0.4***)
C23:6 lysoPC	$C_{31}H_{52}NO_7P$	582.3560	582.3553 (***1.2***)

HPLC/ICPMS and HPLC/ESI-MS showed that arsenic-containing lysoPCs were not present in the hydrolysis mixture. The increase of all As fatty acid signals as well as the detection of lysoPCs indicate that AsFAs are exclusively bound in *sn*2-position (Figure 32).

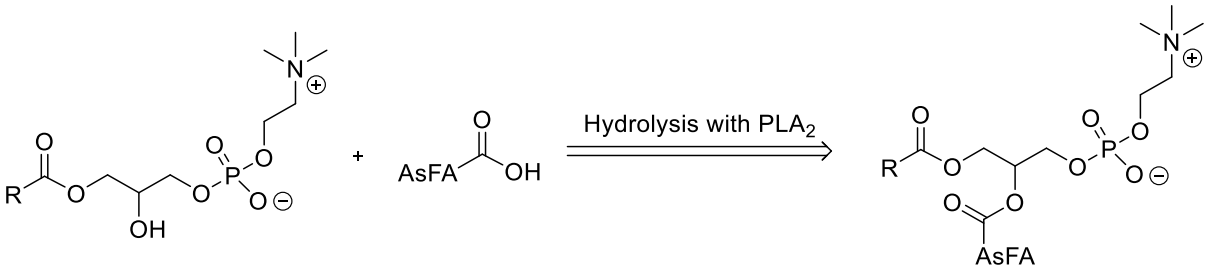

Figure 32. The hydrolysis products of AsPCs indicate the AsFAs in *sn*2-position, as suggested by the retrosynthetic arrow.

3.5.3 Hydrolysis with Phospholipase C

The products of PC hydrolysis with PLC are diacylglycerides, which are expected to be less polar than the substrate owing to the loss of the polar choline headgroup. When the AsPCs were treated with PLC, the group of peaks with retention time ca 16-18 min moved to longer retention times while maintaining the peak shape (Figure 33). This result is consistent with the loss of phosphocholine (Figure 34), and hence it can be concluded that hydrolysis with PLC yields the expected products.

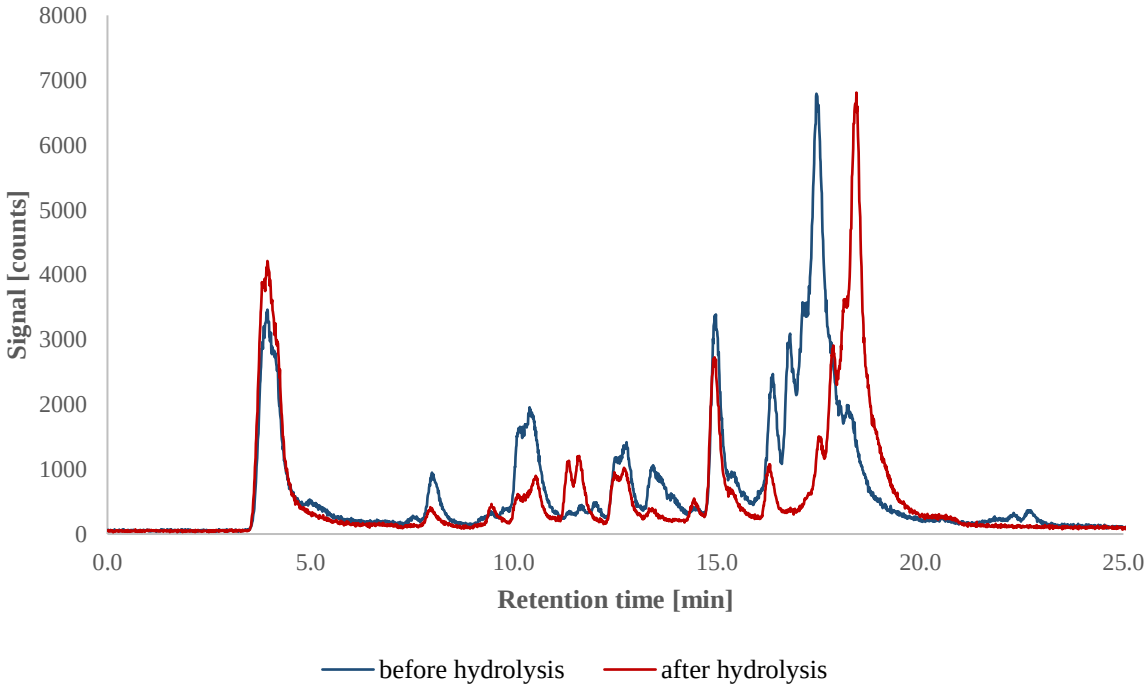

Figure 33. HPLC/ICPMS chromatogram of herring caviar (methanol phase) before and after treatment with PLC.

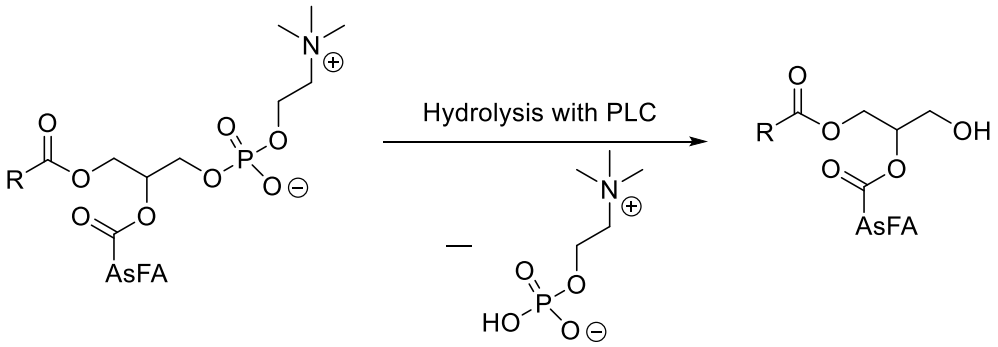

Figure 34. Expected hydrolysis products with PLC.

3.5.4 Hydrolysis with Phospholipase D

No large changes in the arsenic signals were observed for PLD assays. The predicted products of PLD hydrolysis, the diacylglycerolphosphates, were expected to differ in polarity from the starting AsPCs, but, in contrast to the other assays, the AsPC peaks did not change (Figure 35). The small difference between the HPLC/ICPMS before and after the assay points to an ineffective enzymatic hydrolysis, as already mentioned in 3.4.3. HPLC/ESI-MS experiments further supported this interpretation: AsPCs were still detected, even after application of the enzymatic assay, and there were no differences in signal intensity when compared to the reference.

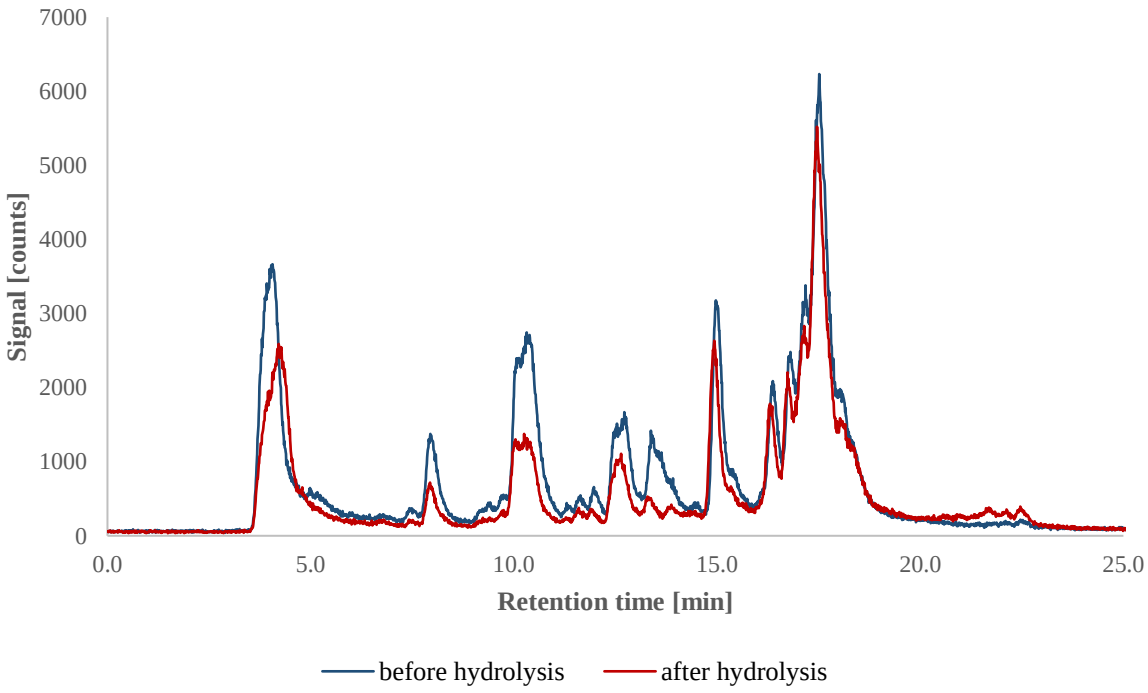

Figure 35. HPLC/ICPMS chromatogram of herring caviar (methanol phase) before and after treatment with PLD.

4 Conclusions and Future Studies

In this work, a method for positional analysis for AsPCs in food samples was elaborated. Sample preparation involved the Bligh and Dyer modification of a Folch extraction for solid samples, or direct dissolution in hexane for oil samples. The second step was a solvent partitioning between hexane and aqueous methanol. After the partitioning, the polar aqueous methanol phase was exposed to phospholipases (PLA_1, PLA_2, PLC and PLD) and the hydrolyzed samples were analyzed by RP-HPLC/ICPMS-ESI-MS (QqQ) and RP-HPLC/ESI-MS (Orbitrap). It was discovered that the AsFAs of AsPCs are exclusively linked to the *sn*-2 position of the AsPCs.

Additionally, using HPLC/ESIMS (Orbitrap), more than 40 arsenic species were identified in the samples. Ten AsFAs, five AsHCs and five AsPLs were found in the foods and food supplements, including three known fatty acids and one known AsPE not observed in herring caviar before. Furthermore, 23 new AsPCs were found in herring caviar and one of these new compounds was also detected in the krill oil sample, together with three known AsPCs. These findings demonstrate widespread occurrence of arsenolipids in fish products and, together with recent cytotoxicity tests showing high toxicity for some arsenolipids, indicate the urgent need for full toxicological characterization of these compounds leading to recommendations for maximum allowable levels for arsenolipids in food.

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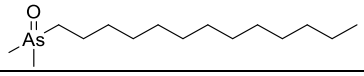
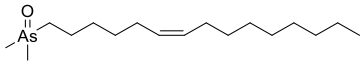
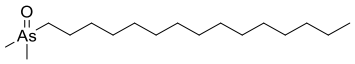
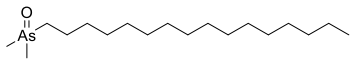
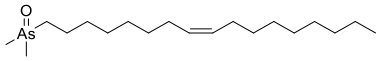
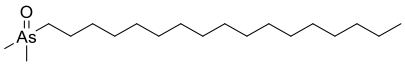
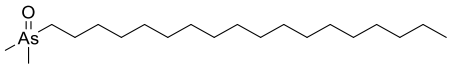
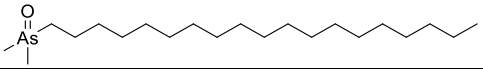
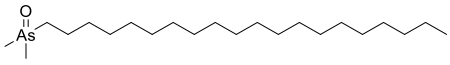
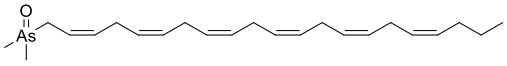
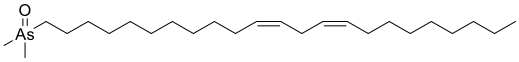
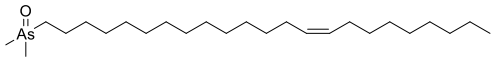
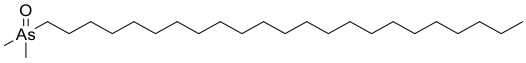
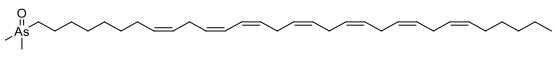
6 Appendices

Table of contents - Appendices

6.1 Appendix A – List of known As-containing lipids (March 2017)

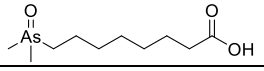
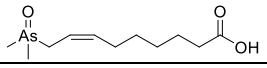
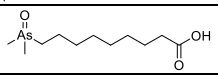
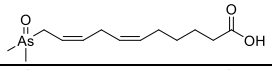
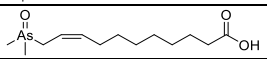
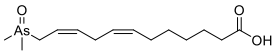
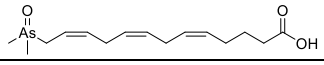
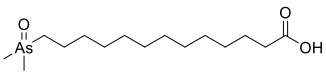
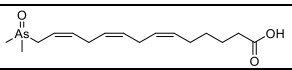
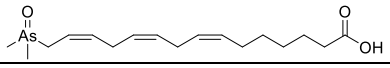
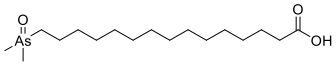
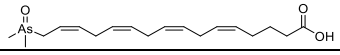
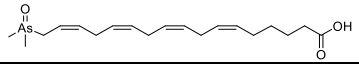
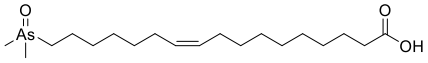
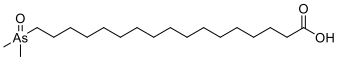
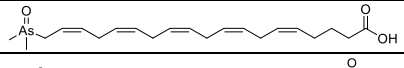
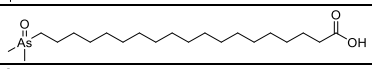
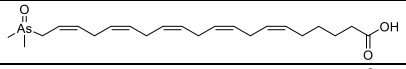
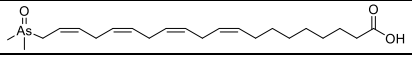
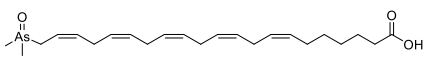
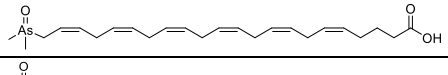
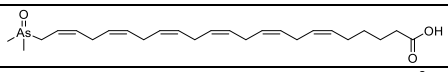
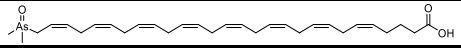
6.1.1 Arsenic-containing hydrocarbons

Table A 1. List of known arsenic-containing hydrocarbons (March 2017).

Compound	Structure	Formula	Sources and References
AsHC 304		$C_{15}H_{33}AsO$	Capelin[41]
AsHC 330		$C_{17}H_{35}AsO$	Capelin[41]
AsHC 332		$C_{17}H_{37}AsO$	*S. latissima*[25] herring[28] capelin[20] tuna[42] capelin[49] Wakame, Hijiki[23] cod liver[44,45]
AsHC 346		$C_{18}H_{40}AsO$	Capelin[41] *S. latissima*[25] herring[28]
AsHC 358		$C_{19}H_{40}AsO$	Capelin[41] herring[28] cod liver[45]
AsHC 360		$C_{19}H_{41}AsO$	*S. latissima*[25] herring[28] capelin[20] tuna[42] capelin[49] Wakame, Hijiki[23] cod liver[44,45]
AsHC 374		$C_{20}H_{44}AsO$	*S. latissima*[25] Capelin oil[41]
AsHC 388		$C_{21}H_{46}AsO$	*S. latissima*[25] Wakame, Hijiki[23]
AsHC 402		$C_{22}H_{48}AsO$	*S. latissima*[25] cod liver[44,45]
AsHC 404		$C_{23}H_{38}AsO$	herring[28] capelin[20] tuna[42] capelin[49] cod liver[44,45]
AsHC 440		$C_{25}H_{50}AsO$	blue whiting[39]
AsHC 442		$C_{25}H_{52}AsO$	blue whiting[39]
AsHC 444		$C_{25}H_{54}AsO$	blue whiting[39]
AsHC 542		$C_{33}H_{56}AsO$	blue whiting[39]

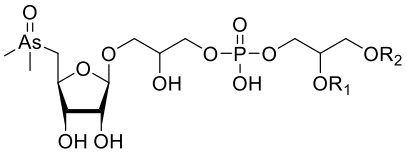
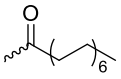
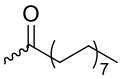
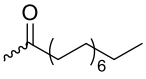
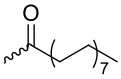
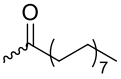
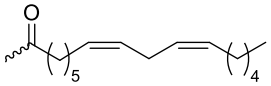
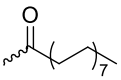
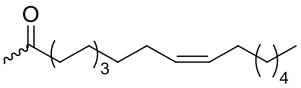
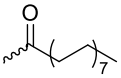
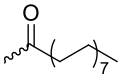
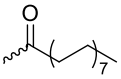
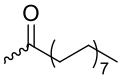
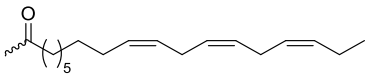
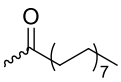
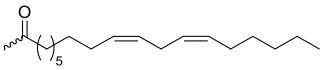
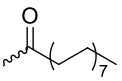
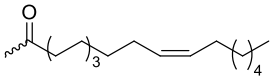
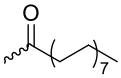
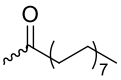
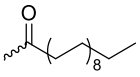
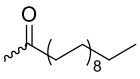
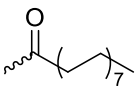
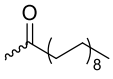
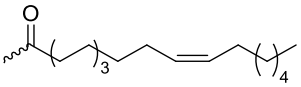
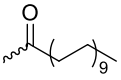
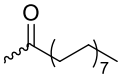
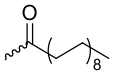
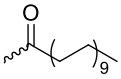
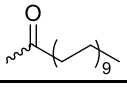
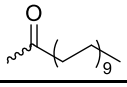
6.1.2 Arsenic-containing fatty acids

Table A 2. Full list of known arsenic-containing fatty acids (March 2017).

Compound	Structure	Formula	Sources and References
AsFA 264		$C_{10}H_{22}AsO_3$	herring fillet[107] cod liver[44]
AsFA 276		$C_{11}H_{22}AsO_3$	herring fillet[107] cod liver[44]
AsFA 278		$C_{11}H_{23}AsO_3$	cod liver[44]
AsFA 302		$C_{13}H_{24}AsO_3$	herring fillet[107] cod liver[44]
AsFA 304		$C_{13}H_{26}AsO_3$	herring fillet[107]
AsFA 316		$C_{14}H_{26}AsO_3$	herring fillet[107] cod liver[44]
AsFA 328		$C_{15}H_{26}AsO_3$	herring fillet[107] cod liver[44]
AsFA 334		$C_{15}H_{32}AsO_3$	herring[28] cod liver oil[19] cod liver[44,45]
AsFA 342		$C_{16}H_{27}AsO_3$	cod liver[44,45]
AsFA 356		$C_{17}H_{30}AsO_3$	herring fillet[107]
AsFA 362		$C_{17}H_{36}AsO_3$	herring[28], tuna[42] capelin[49] cod liver oil[19] cod liver[44,45]
AsFA 368		$C_{18}H_{29}AsO_3$	cod liver[45]
AsFA 382		$C_{19}H_{31}AsO_3$	cod liver[45]
AsFA 388		$C_{19}H_{38}AsO_3$	herring[28] cod liver oil[19] cod liver[44,45]
AsFA 390		$C_{19}H_{40}AsO_3$	herring[28] cod liver oil[19] cod and capelin oil[108] cod liver[44,45]
AsFA 408		$C_{21}H_{33}AsO_3$	cod liver[45]
AsFA 418		$C_{21}H_{44}AsO_3$	cod liver oil[19]
AsFA 422		$C_{22}H_{36}AsO_3$	*S. latissima*[25] cod liver[44,45]
AsFA 424		$C_{22}H_{38}AsO_3$	*S. latissima*[25]
AsFA 436		$C_{23}H_{38}AsO_3$	herring,[28] tuna[42] capelin[49] cod liver oil[19] cod liver[44,45]
AsFA 448		$C_{24}H_{38}AsO_3$	herring,[28] capelin[49] cod liver[44,45]
AsFA 462		$C_{25}H_{39}AsO_3$	cod liver[45]
AsFA 528		$C_{30}H_{46}AsO_3$	herring[28]

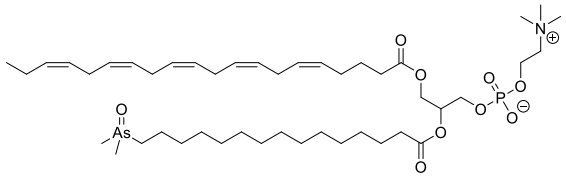
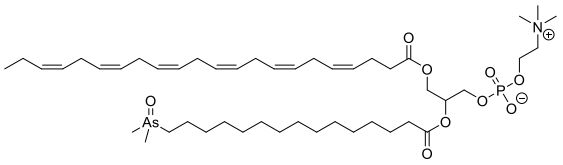
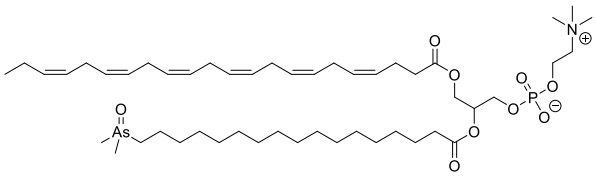
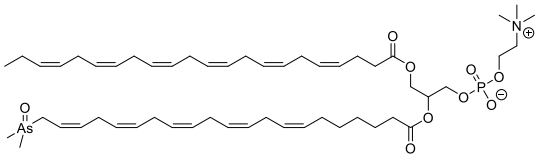
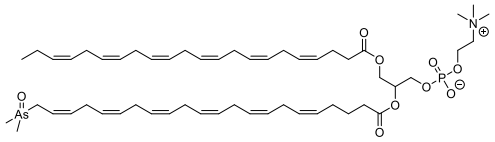
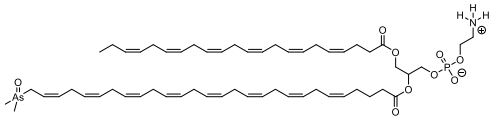
6.1.3 Arsenosugar phospholipids

Table A 3. List of known arsenosugar phospholipids (March 2017). a/b means R_1 and R_2 are interchangeable.

Compound	Structure		Sources and References
General			
	R_1	R_2	
AsPL 930 a/b			Wakame, Hijiki[23]
AsPL 944			Wakame, Hijiki[23]
AsPL 954 a/b			*Saccharina latissima*[25]
AsPL 956 a/b			Wakame, Hijiki[23]
AsPL 958			Wakame, Hijiki[23]
AsPL 972			*Saccharina latissima*[25]
AsPL980 a/b			*Saccharina latissima*[25]
AsPL 982			Wakame, Hijiki[23]
AsPl 984			Wakame, Hijiki[23]
AsPL 986			Wakame, Hijiki[23]
AsPL 1000			*Saccharina latissima*[25]
AsPL 1012			Wakame, Hijiki[23]
AsPL 1014			Wakame, Hijiki[23]
AsPL 1042			Wakame, Hijiki[23]
AsPL 1070			Wakame, Hijiki[23]

6.1.4 Arsenic-containing phosphatidylcholines

Table A 4. Full list of known arsenic-containing phosphatidylcholines (March 2017). For compounds discovered in this work, see Figure 24.

Compound	Structure	Sources and References
AsPC 885-362		herring caviar[28]
AsPC 911-362		herring caviar[28]
AsPC 939-390		herring caviar[28]
AsPC 985-448		herring caviar[28]
AsPC 997-448		herring caviar[28]
AsPE 1035-528		salmon[28]

6.2 Appendix B – HR-MS data of the new AsPCs

Table A 5. Main fragments of the new AsPCs identified in herring caviar. n. d. = not detected.

compound	$C_5H_{12}N^+$ 86.0970	$C_5H_{14}ON^+$ 104.1075	$C_2H_6O_4P^+$ 125.0004	$C_5H_{15}O_4N^+P$ 184.0738	AsFA	AsFA – H_2O	$[M+H]^+$ - 183
AsPC 837-362	86.0971 ***1.2***	104.1076 ***1.0***	125.0003 ***0.8***	184.0735 ***1.6***	363.1874 ***0.3***	345.1770 ***0.3***	655.4279 ***0.2***
AsPC 857-334	86.0971 ***1.2***	104.1075 ***<0.1***	125.0000 ***3.2***	184.0733 ***2.7***	335.1562 ***<0.1***	317.1455 ***0.3***	675.3936 ***4.1***
AsPC 859-362	86.0970 ***<0.1***	104.1074 ***1.0***	125.0000 ***3.2***	184.0733 ***2.7***	363.1870 ***1.4***	345.1766 ***0.8***	677.4113 ***1.1***
AsPC 859-382	86.0970 ***<0.1***	104.1072 ***2.9***	125.0002 ***1.6***	184.0733 ***2.7***	365.1467 ***2.9***	383.1556 ***1.6***	677.4111 ***1.5***
AsPC 865-362	86.0970 ***<0.1***	104.1074 ***1.0***	124.9999 ***4.0***	184.0733 ***2.7***	363.1871 ***1.1***	345.1767 ***0.7***	683.4579 ***1.6***
AsPC 865-390	86.0971 ***1.2***	104.1075 ***<0.1***	124.9999 ***4.0***	184.0735 ***2.2***	391.2188 ***0.2***	373.2083 ***0.3***	683.4586 ***<0.1***
AsPC 883-334	86.0973 ***3.5***	104.1077 ***1.9***	125.0002 ***1.6***	184.0739 ***0.5***	335.1569 ***2.2***	317.1463 ***2.2***	701.4136 ***2.1***
AsPC 885-408	86.0973 ***3.5***	104.1078 ***2.9***	125.0003 ***0.8***	184.0739 ***0.5***	409.1725 ***1.7***	391.1625 ***3.2***	703.4281 ***0.5***
AsPC 885-436	86.0971 ***1.2***	104.1076 ***1.0***	125.0000 ***3.2***	184.0735 ***2.2***	437.2032 ***0.2***	419.1925 ***1.3***	703.4286 ***1.3***
AsPC 885-382	86.0973 ***3.5***	104.1077 ***1.9***	124.9999 ***4.0***	184.0738 ***<0.1***	383.1570 ***2.0***	365.1469 ***3.4***	703.4287 ***1.4***
AsPC 887-362	86.0971 ***1.2***	104.1074 ***1.0***	125.0002 ***1.6***	184.0734 ***2.2***	363.1876 ***0.1***	345.1770 ***0.3***	705.4434 ***1.3***
AsPC 887-390	86.0971 ***1.2***	104.1074 ***1.0***	125.0002 ***1.6***	184.0734 ***2.2***	391.2191 ***0.7***	373.2076 ***1.7***	705.4434 ***<0.1***
AsPC 911-436	86.0973 ***3.5***	104.1077 ***1.9***	125.0004 ***<0.1***	184.0738 ***<0.1***	437.2040 ***2.1***	419.1927 ***0.4***	729.4444 ***1.4***
AsPC 913-448	86.0973 ***3.5***	104.1077 ***1.9***	125.0000 ***1.6***	184.0738 ***<0.1***	449.2042 ***2.3***	431.1930 ***1.0***	731.4599 ***1.2***
AsPC 925-362	86.0972 ***2.3***	104.1074 ***1.0***	125.0001 ***2.4***	184.0734 ***2.2***	363.1875 ***0.1***	345.1771 ***0.4***	743.4581 ***1.3***
AsPC 933-436	86.0971 ***1.2***	104.1075 ***<0.1***	125.0004 ***<0.1***	184.0734 ***2.2***	437.2025 ***1.7***	419.1925 ***0.2***	751.4259 ***2.4***
AsPC 939-436	86.0973 ***3.5***	104.1078 ***2.9***	125.0005 ***0.8***	184.0739 ***0.5***	437.2041 ***2.3***	419.1938 ***3.0***	757.4762 ***2.0***
AsPC 945-448	86.0970 ***<0.1***	104.1075 ***<0.1***	125.0002 ***1.6***	184.0733 ***2.7***	449.2040 ***2.0***	431.1918 ***1.7***	763.4262 ***1.2***
AsPC 949-448	86.0970 ***<0.1***	104.1076 ***1.0***	124.9998 ***4.8***	184.0734 ***2.2***	449.2017 ***3.3***	431.1924 ***0.5***	767.4581 ***1.2***
AsPC 959-436	86.0972 ***2.3***	104.1076 ***1.0***	125.0002 ***1.6***	184.0737 ***0.5***	437.2039 ***1.6***	419.1931 ***1.2***	777.4440 ***0.8***
AsPC 971-448	86.0972 ***2.3***	104.1076 ***1.0***	125.0002 ***1.6***	184.0737 ***0.5***	449.2037 ***1.2***	431.1932 ***1.5***	789.4440 ***0.8***
AsPC 973-436	86.0971 ***1.2***	104.1074 ***1.0***	125.0002 ***1.6***	184.0733 ***2.7***	437.2015 ***3.7***	419.1925 ***0.3***	791.4542 ***4.8***
AsPC 973-448	86.0971 ***1.2***	104.1074 ***1.0***	125.0002 ***1.6***	184.0733 ***2.7***	449.2036 ***0.9***	431.1925 ***0.3***	n. d. n. d.

Table A 6. Singly and doubly charged parent ions of the novel AsPCs in herring caviar.

Compound	Formula (neutral)		$[M+H]^+$	$[M+2H]^{2+}$
AsPC 837-362	$C_{41}H_{81}AsNO_9P$	mass (exp)	838.4929	419.7498
		mass (calc)	838.4938	419.7505
		|Δm/m| [ppm]	***1.1***	***1.7***
AsPC 857-334	$C_{43}H_{77}AsNO_9P$	mass (exp)	858.4609	429.7346
		mass (calc)	858.4625	429.7349
		|Δm/m| [ppm]	***1.9***	***0.7***
AsPC 859-362	$C_{43}H_{79}AsNO_9P$	mass (exp)	860.4794	430.7424
		mass (calc)	860.4781	430.7427
		|Δm/m| [ppm]	***1.5***	***0.7***
AsPC 859-382	$C_{43}H_{79}AsNO_9P$	mass (exp)	860.4792	430.7429
		mass (calc)	860.4781	430.7427
		|Δm/m| [ppm]	***1.3***	***0.4***
AsPC 865-362	$C_{43}H_{85}AsNO_9P$	mass (exp)	866.5262	433.7663
		mass (calc)	866.5251	433.7662
		|Δm/m| [ppm]	***1.3***	***0.2***
AsPC 865-390	$C_{43}H_{85}AsNO_9P$	mass (exp)	866.5262	433.7663
		mass (calc)	866.5251	433.7662
		|Δm/m| [ppm]	***1.3***	***0.2***
AsPC 883-334	$C_{45}H_{79}AsNO_9P$	mass (exp)	884.4800	442.7431
		mass (calc)	884.4781	442.7427
		|Δm/m| [ppm]	***2.1***	***0.9***
AsPC 885-408	$C_{45}H_{81}AsNO_9P$	mass (exp)	886.4938	443.7498
		mass (calc)	886.4938	443.7505
		|Δm/m| [ppm]	***<0.1***	***1.7***
AsPC 885-436	$C_{45}H_{81}AsNO_9P$	mass (exp)	886.4948	443.7503
		mass (calc)	886.4938	443.7505
		|Δm/m| [ppm]	***1.2***	***0.2***
AsPC 885-382	$C_{45}H_{81}AsNO_9P$	mass (exp)	886.4930	443.7515
		mass (calc)	886.4938	443.7505
		|Δm/m| [ppm]	***0.8***	***2.3***
AsPC 887-362	$C_{45}H_{83}AsNO_9P$	mass (exp)	888.5102	444.7581
		mass (calc)	888.5094	444.7583
		|Δm/m| [ppm]	***0.9***	***0.4***
AsPC 887-390	$C_{45}H_{83}AsNO_9P$	mass (exp)	888.5102	444.7581
		mass (calc)	888.5094	444.7583
		|Δm/m| [ppm]	***0.9***	***0.4***
AsPC 911-436	$C_{47}H_{83}AsNO_9P$	mass (exp)	912.5104	456.7586
		mass (calc)	912.5094	456.7583
		|Δm/m| [ppm]	***1.1***	***0.6***
AsPC 913-448	$C_{47}H_{85}AsNO_9P$	mass (exp)	914.5264	457.7669
		mass (calc)	914.5251	457.7662
		|Δm/m| [ppm]	***1.4***	***1.5***
AsPC 925-362	$C_{48}H_{85}AsNO_9P$	mass (exp)	926.5237	463.7661
		mass (calc)	926.5251	463.7662
		|Δm/m| [ppm]	***1.5***	***0.2***
AsPC 933-436	$C_{49}H_{81}AsNO_9P$	mass (exp)	934.4934	467.7503
		mass (calc)	934.4938	467.7505
		|Δm/m| [ppm]	***0.4***	***0.4***
AsPC 939-436	$C_{49}H_{87}AsNO_9P$	mass (exp)	940.5406	470.7745
		mass (calc)	940.5407	470.7740
		|Δm/m| [ppm]	***<0.1***	***1.0***
AsPC 945-448	$C_{50}H_{81}AsNO_9P$	mass (exp)	946.4933	473.7505
		mass (calc)	946.4938	473.7505
		|Δm/m| [ppm]	***0.5***	***<0.1***
AsPC 949-448	$C_{50}H_{85}AsNO_9P$	mass (exp)	950.5254	475.7673
		mass (calc)	950.5251	475.7662
		|Δm/m| [ppm]	***0.3***	***2.3***
AsPC 959-436	$C_{51}H_{83}AsNO_9P$	mass (exp)	960.5109	480.7592
		mass (calc)	960.5094	480.7583
		|Δm/m| [ppm]	***1.6***	***1.9***
AsPC 971-448	$C_{52}H_{83}AsNO_9P$	mass (exp)	972.5079	486.7592
		mass (calc)	972.5094	486.7583
		|Δm/m| [ppm]	***0.9***	***1.8***
AsPC 973-436	$C_{52}H_{85}AsNO_9P$	mass (exp)	974.5242	487.7665
		mass (calc)	974.5251	487.7662
		|Δm/m| [ppm]	***0.9***	***0.6***
AsPC 973-448	$C_{52}H_{85}AsNO_9P$	mass (exp)	974.5242	487.7665
		mass (calc)	974.5251	487.7662
		|Δm/m| [ppm]	***0.9***	***0.6***

6.3 Appendix C – HPLC/ESIMS chromatograms of new AsPCs

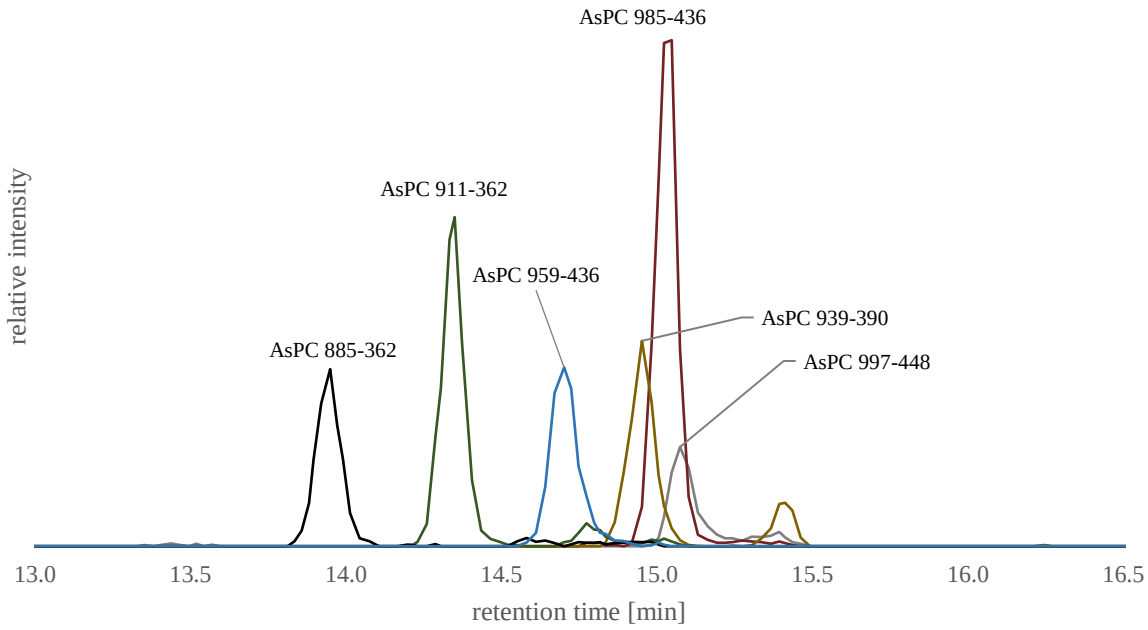


Figure A 1. HPLC/ESI-MS chromatogram of AsPCs with the highest concentration.

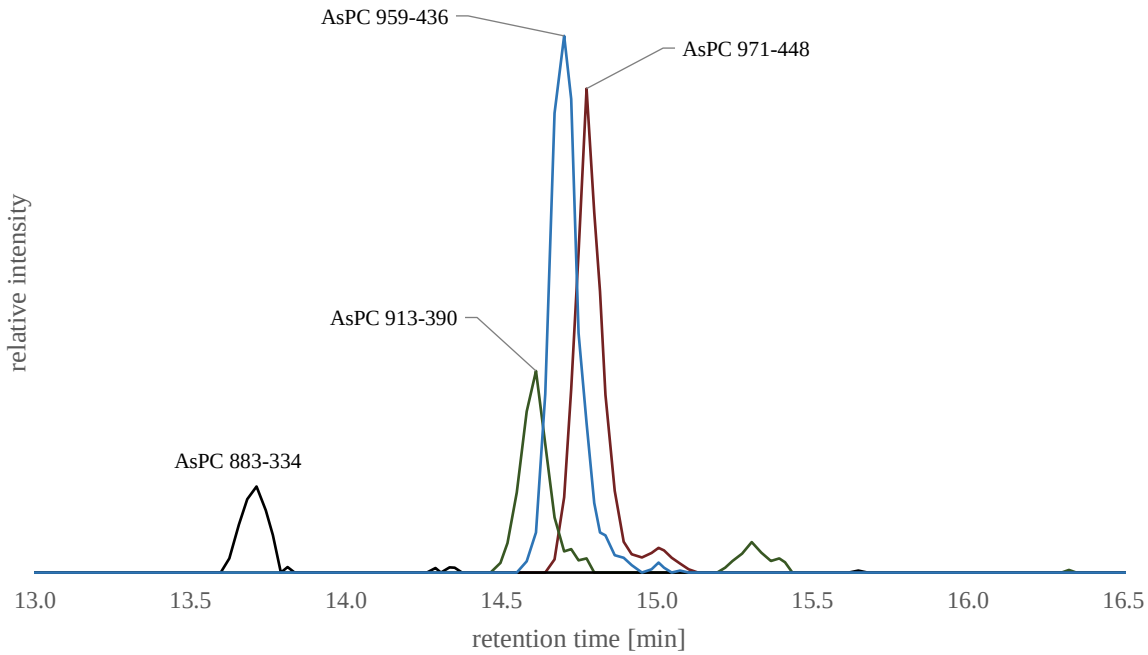


Figure A 2. HPLC/ESI-MS chromatogram of AsPCs with high concentration. AsPC 959-436 is used as a reference for relation to Figure A 1.

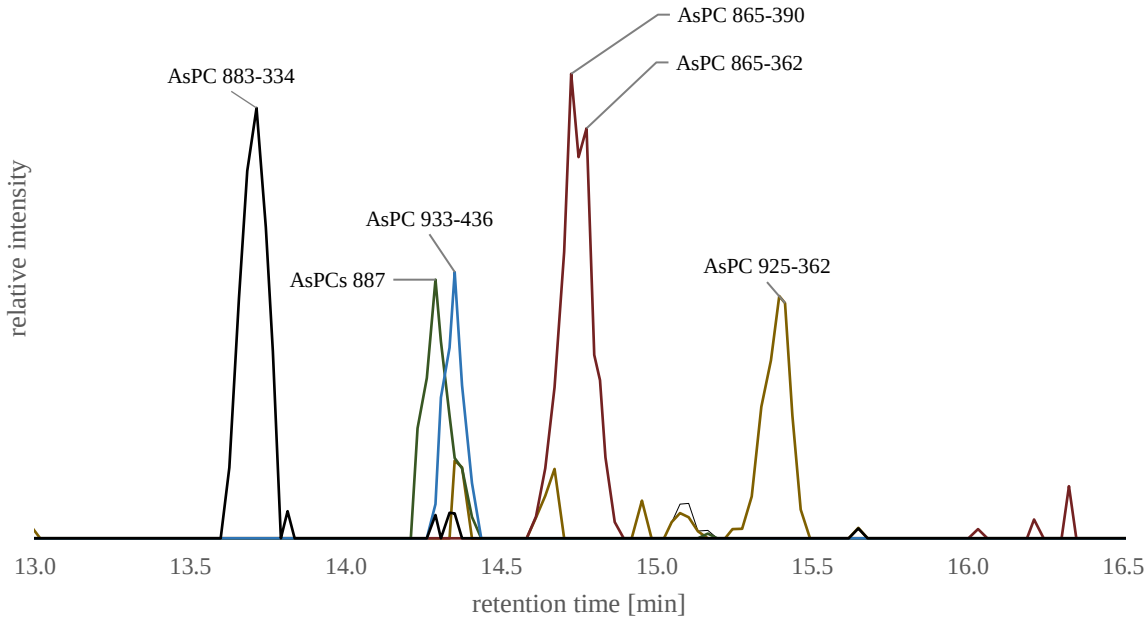


Figure A 3. HPLC/ESI-MS chromatogram of AsPCs with medium concentration. AsPC 883-334 is used as a reference for relation to Figure A 2.

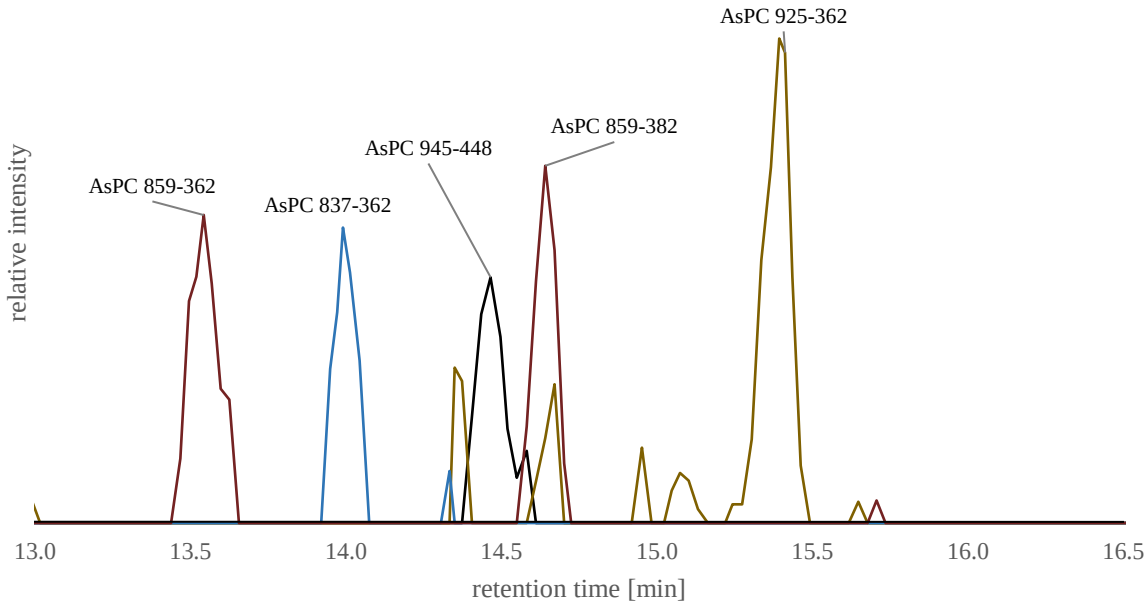


Figure A 4. HPLC/ESI-MS chromatogram of AsPCs with medium-low concentration. AsPC 925-362 is used as a reference for relation to Figure A 3.

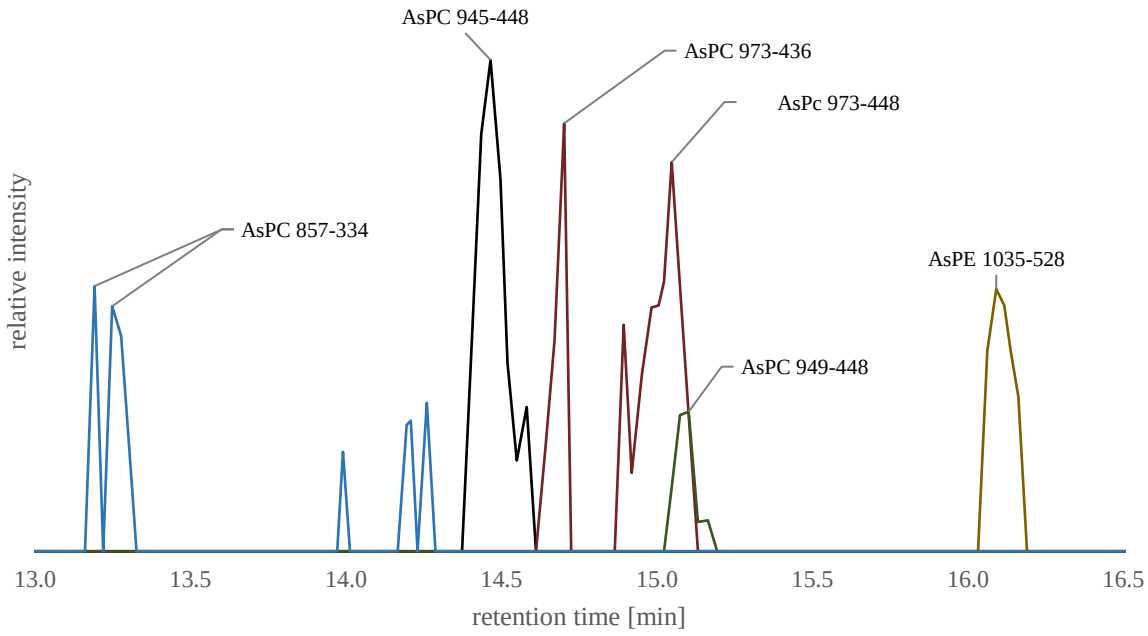


Figure A 5. HPLC/ESI-MS chromatogram of AsPCs with low concentration (and AsPE 1035-528). AsPC 945-448 is used as a reference for relation to Figure A 4.

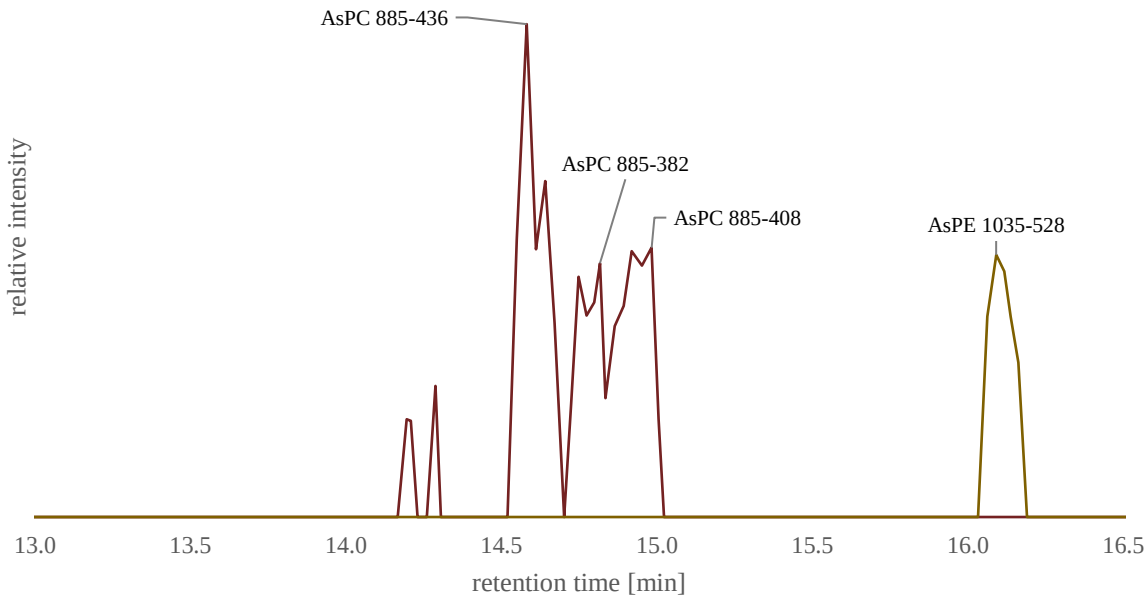


Figure A 6. HPLC/ESI-MS chromatogram of AsPCs with low concentration (and AsPE 1035-528). AsPE 1035-528 is used as a reference for relation to Figure A 4 and Figure A 5.

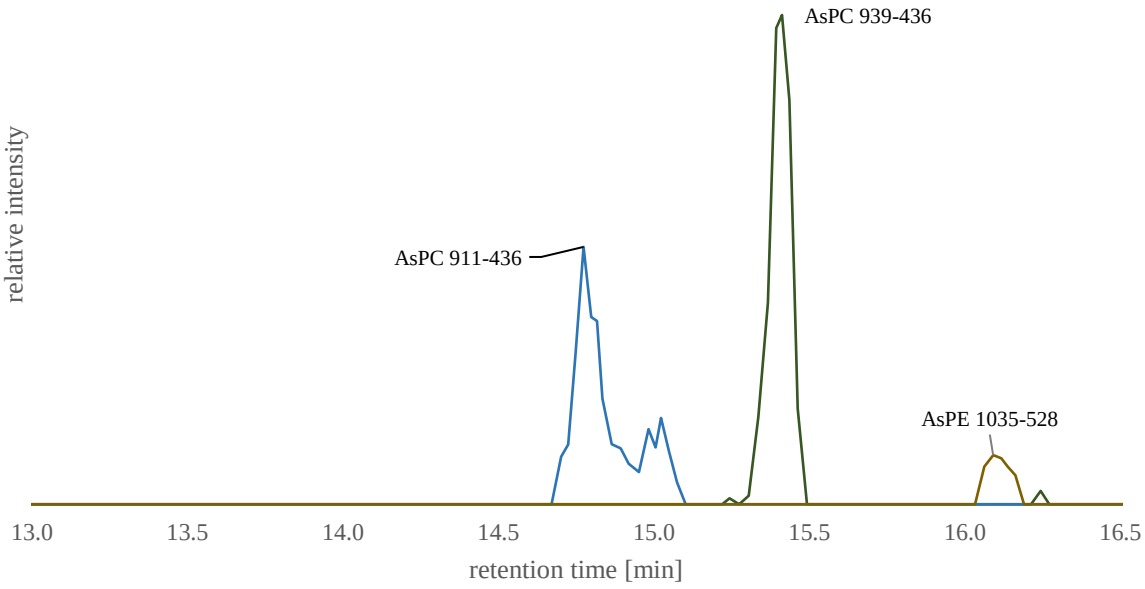


Figure A 7. HPLC/ESI-MS chromatogram of AsPCs with medium-low concentration (and AsPE 1035-528). AsPE 1035-528 is used as a reference for relation to Figure A 4 and Figure A 5.

6.4 Appendix D – samples and their origins

Figure A 8. Container of the Omega-3 herring caviar capsules, the herring caviar capsules and the collected sample from capsules.

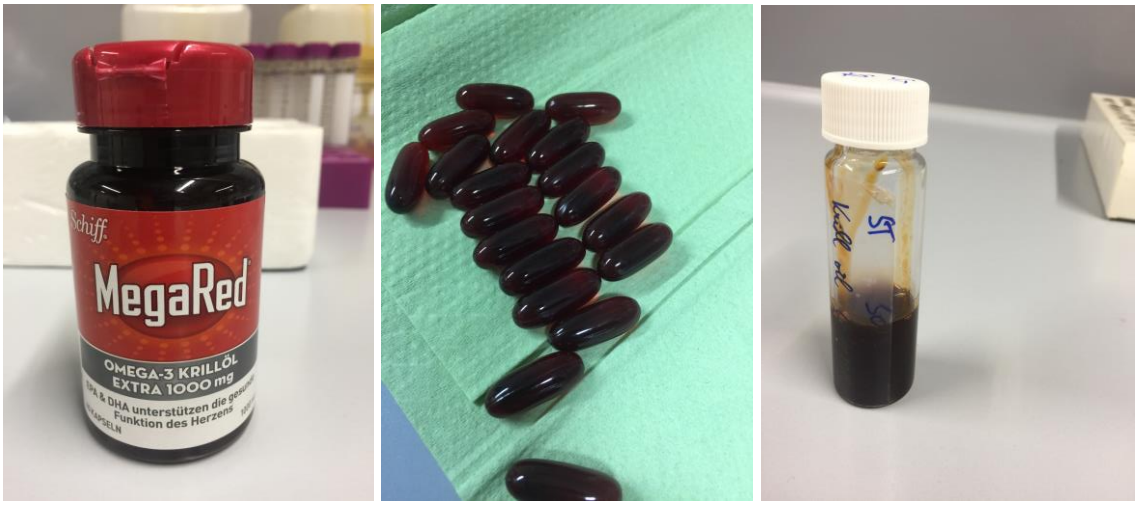


Figure A 9. Container of the Omega-3 krill oil capsules, the krill oil capsules and the collected oil from capsules.

Figure A 10. Container of the blue whiting oil from Iceland, and a sample of the oil transferred to a glass vial.

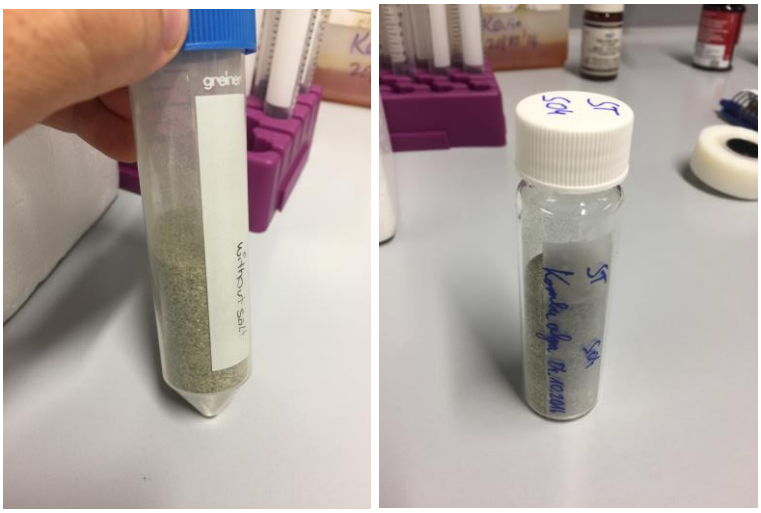


Figure A 11. Container of the freeze dried Kombu algae powder, which was transferred to a glass vial.

6.5 Appendix E – HPLC/ICPMS: calibration chromatograms and calibration curve

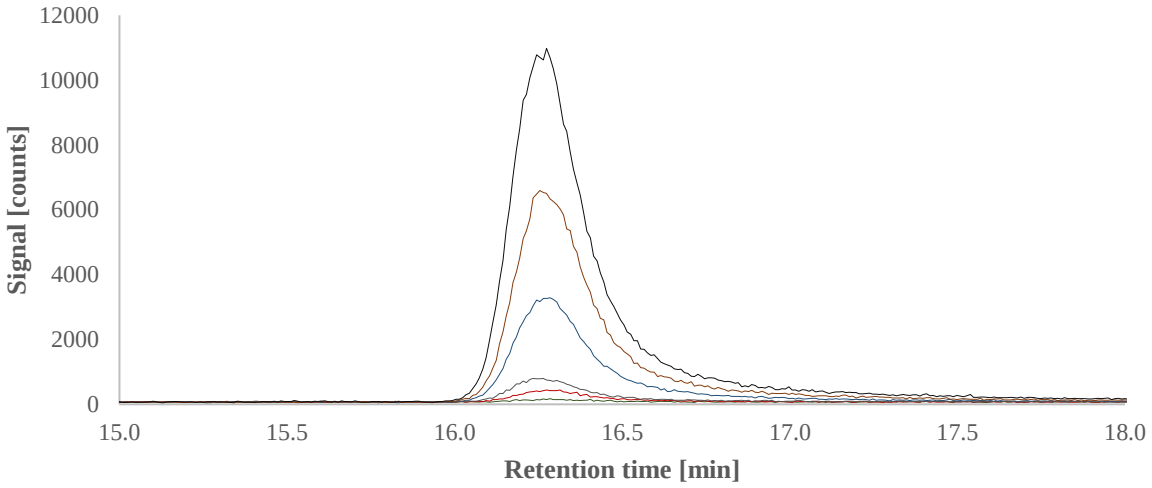


Figure A 12. HPLC-ICPMS chromatograms of a blank and of the calibration standard AsHC 360 at concentrations 1 – 100 µg As L^{-1} (see Table A 7).

Table A 7. Peak areas taken from the chromatograms shown in Figure A 13.

Concentration of AsHC 360 [µg As L^{-1}]	Peak area [ ]
0	0
1	1257
5	5130
10	11340
50	50413
100	101453

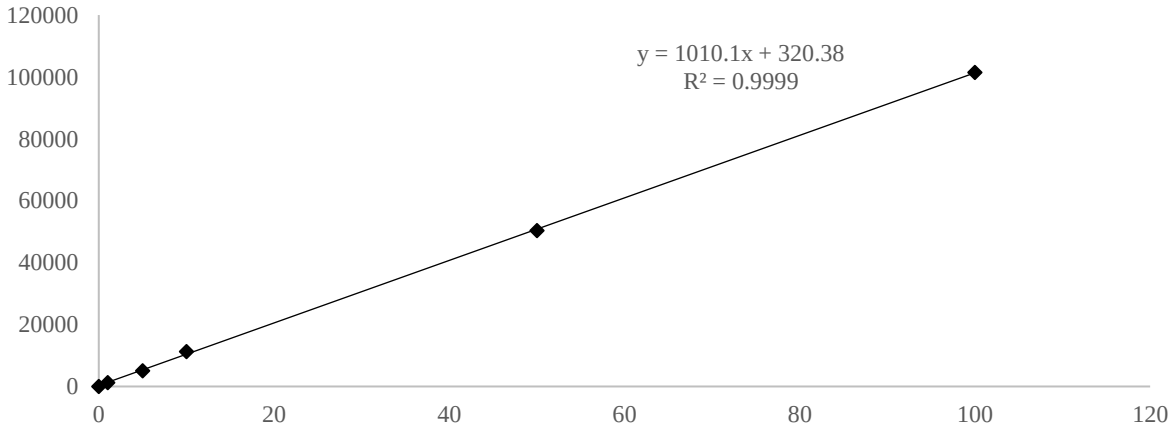


Figure A 13. Example of a calibration curve taken from the HPLC-ICPMS chromatograms showing the corresponding equation.