

Method development and analysis of arsenolipids in  
marine oils



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Dissertation of the degree of Philosophiae Doctor (PhD)

at the University of Bergen

February 2014



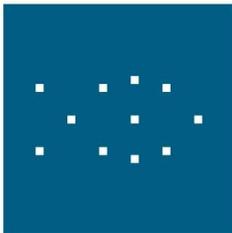
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## Scientific environment

The present work was completed at the National Institute of Nutrition and Seafood Research (NIFES) in Bergen, Norway. Part of the method development was conducted at the National Food Institute at the Technical University of Denmark (DTU). The work was administered through Institute of Biology, University of Bergen (UiB), Norway.

Supervisor was Dr. Heidi Amlund (NIFES). Co-supervisors were Dr. Jens Jørgen Sloth (DTU) and Prof. Anne-Katrine Lundebye (NIFES/UiB).

This work was funded by the Research Council of Norway (RCN) through the project: Arsenolipids in fish oil, fish feed, fish and other seafood (project no. 184986).



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## Acknowledgements

I would like to thank my supervisors. Dr. Heidi Amlund for always having her door open, for the many discussions, advices and encouragement during this work. Dr. Jens J. Sloth for sharing his knowledge on trace element analysis and for the nice discussions, advices and constructive criticism. Prof. Anne-Katrine Lundebye for her patient readthrough of manuscript and text.

I would like to specially thank Øyvind Lie, Bente Torstensen and Robin Ørnsrud for providing excellent work facilities and for letting me complete my thesis at NIFES. Thanks to the Safe feed group for the scientific and non-scientific discussion. Thanks to Marc Berntssen for sharing samples of fish oil, and for sharing his knowledge on refining processes. To the colleagues at the loft; thank you, for the positive and including atmosphere. I would also like to thank the people at grunnstoff lab, especially Siri Bargård and Berit Solli, for all practical help and enthusiastic discussions. Furthermore, thanks to Bjarte Holmelid (UiB) and Stig Valdernes for their expertise help with organic synthesis and mass spectrometry, and for showing great go-ahead spirit. A special thank to Annette Bernhard, Eli Aadland, Eva Lucena Toro and Shalini Jayashankar for nice coffee breaks and many laughs. All in all, I would like to thank all students and colleagues, at NIFES for making it a very nice and educational place to work.

I would also like to thank Prof. Erik Larsen and the M&M group at DTU food for the excellent work facilities, practical help and great hospitality during the work at DTU. Thanks to Birgitte Herbst and Kasper Skov for their expertise help with instrumentation and analysis. Specially thanks to Mesi and Katrin for their great hospitality and friendship.

I would like to thank my friends and family for help and support. I am looking forward to spend more time with you all. A special thank to my mum and dad, and Hildegunn and Øyvind for always helping us with the logistics when needed. Thanks to my little Sofie, for your energy, fantasy and ability to distract me. Finally and most importantly, thanks to my dear Joakim for taking care of everything at home, for your patience, encouragement and support.

-Veronika-

## Abstract

Arsenic in marine oils is mainly present in the form of lipid-soluble compounds; collectively called arsenolipids. Although total arsenic concentrations in marine oils typically range from 0.2 to 16 mg kg<sup>-1</sup> [1-3], knowledge regarding the chemical structures and distribution of arsenolipids in oils is limited. The present work describes the development of analytical methods for the determination of arsenolipids, and their application to marine oil, including fish oil and oil of liver of Northeast Arctic cod (*Gadus morhua*).

In the present work gas chromatography coupled to inductively coupled plasma mass spectrometry (GC-ICP-MS) was applied for analysing arsenic-containing hydrocarbons (AsHCs) in fish oil. The AsHCs were extracted into aqueous methanol, and further subjected to solid-phase extraction (SPE) prior to analysis. Reversed-phase HPLC-ICP-MS was applied as an analytical technique for analysis of both AsHCs and arsenic-containing fatty acids (AsFAs) in methanol phase of marine oil. The AsHCs and AsFAs were accurately quantified in reversed-phase HPLC-ICP-MS analysis using dimethylarsinate as calibration standard. The molecular structures of the arsenolipids were identified using mass spectrometry.

The oils included in the present work contained total arsenic concentrations from 1.6 to 12.5 mg kg<sup>-1</sup> oil. Three AsHCs (AsHC-C<sub>15</sub>, AsHC-C<sub>17</sub> and AsHC-C<sub>21</sub>) were identified as major arsenolipids in all marine oil. Also, two AsFAs (AsFA-C<sub>21</sub> and AsFA-C<sub>22</sub>) were identified as minor arsenolipids in some of the oils. In cod liver, three AsHCs (AsHC-C<sub>15</sub>, AsHC-C<sub>17</sub> and AsFA-C<sub>22</sub>) and up to five AsFAs (AsFA-C<sub>15</sub>, AsFA-C<sub>17a</sub>, AsFA-C<sub>17b</sub>, AsFA-C<sub>21</sub> and AsFA-C<sub>22</sub>) were identified.

The present work contributes with data on the presence of arsenolipids, in the form of AsHCs and AsFAs, in a number of marine oils. The developed methods form the basis for future studies on arsenolipids, e.g. the occurrence of arsenolipids in feed and farmed fish.

## Abbreviations

AB	Arsenobetaine
AC	Arsenocholine
ACN	Acetonitrile
amu	Atomic mass unit
AsFAs	Arsenic-containing fatty acids
AsHCs	Arsenic-containing hydrocarbons
As-sugPLs	Arsenosugar phospholipids
CEN	Comité Européen de Normalisation (European Committee for Standardization)
CHOL	Cholesterol
cps	counts per second
CRM	Certified reference material
DMA	Dimethylarsinate
DMAP	Dimethylarsinoyl propionic acid
dw	dry weight
EFSA	The European Food Safety Authority
EI	Electron impact ionisation
ESI	Electrospray ionisation

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ESI-MS/MS(QqQ)	Electrospray ionisation tandem mass spectrometry
FFA	Free fatty acids
GC	Gas chromatography
GC-ICP-MS	Gas chromatography coupled to inductively coupled plasma mass spectrometry
GC-MS/MS	Gas chromatography coupled to tandem mass spectrometry
HPLC	High performance liquid chromatography
HPLC-ICP-MS	High performance liquid chromatography coupled to inductively coupled plasma mass spectrometry
HPLC-qTOF-MS	High performance liquid chromatography coupled to quadrupole time-of flight mass spectrometry
HR-MS	High resolution mass spectrometry
ICP-MS	Inductively coupled plasma mass spectrometry
IUPAC	International Union of Pure and Applied Chemistry
LLE	Liquid liquid extraction
MeOH	Methanol
MRM	Multiple reaction monitoring
MS2	mass spectra obtained using tandem mass spectrometry
m/z	mass to charge ratio
NMR	Nuclear magnetic resonance

PA	Phosphatic acid
PC	Phosphatidyl choline
PE	Phosphatidyl ethanol amine
PI	Phosphatidyl inositol
PLs	Phospholipids
PS	Phosphatidyl serine
SPE	Solid Phase Extraction
TAGs	Triacylglyceroles
TETRA	Tetramethylarsonium ion
TLC	Thin-layer chromatography
TMA <sub>s</sub> FOH <sub>s</sub>	Trimethylarsonium fatty alcohols
TMAO	Trimethylarsine oxide
TOF-MS	time of flight mass spectrometry
ww	wet weight
qTOF-MS	quadrupole time-of flight mass spectrometry

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## List of publications

- Paper I**      Veronika Sele, Heidi Amlund, Marc H.G. Berntssen, Jannicke Berntsen, Kasper Skov and Jens J. Sloth.  
Detection of arsenic-containing hydrocarbons in a range of commercial fish oil by GC-ICPMS analysis.  
*Analytical and Bioanalytical Chemistry*, 2013, **405** (15), 5179-5190.
- Paper II**      Veronika Sele, Jens J. Sloth, Bjarte Holmelid, Stig Valdersnes, Kasper Skov and Heidi Amlund.  
Arsenic-containing fatty acids and hydrocarbons in marine oils – Determination using reversed-phase HPLC–ICP-MS and HPLC–qTOF-MS.  
*Talanta: The International Journal of Pure and Applied Analytical Chemistry*, 2014, **121**, 89-96.
- Paper III**      Veronika Sele, Jens J. Sloth, Kåre Julshamn, Kasper Skov and Heidi Amlund.  
A study of lipid- and water-soluble arsenic species in liver of Northeast Arctic cod (*Gadus morhua*) containing high levels of total arsenic.  
*Manuscript for submission to Metallomics*.

## Introduction

Arsenic is an element found in relative high concentrations in the marine environment, typically in the range of 1 to 100 mg kg<sup>-1</sup> ww [4, 5]. Arsenic has a complex chemistry, and a diverse group of arsenic compounds or arsenic species exist in the marine environment [6]. Over the past 40 years most research has focused on the water-soluble arsenic species, which consist of both inorganic and organic arsenicals. In contrast, less work has been devoted to the lipid-soluble arsenic species, the arsenolipids.

The arsenolipids are predominant arsenic species in fish oil [3, 7]. Arsenic is naturally present in industrial and pelagic fish, such as blue whiting (*Micromesistius poutassou*) and sand eel (*Ammodytes marinus*), and arsenic is transferred to the marine feed ingredients, fish oil and fish meal, that are produced from the fish. Results from the National surveillance program on fish feed in Norway shows that commercial fish oils contain relative high levels of total arsenic, ranging from 4.6 to 16 mg kg<sup>-1</sup> oil [1, 2, 8]. Fish meal also contain relative high levels of arsenic, up to 18.2 mg kg<sup>-1</sup> dry weight [1, 2], and consequently both fish meal and fish oil will contribute with arsenic to the formulated feed, used in farming of fish [9-11]. Fish meal mainly contains the water-soluble species, where arsenobetaine (AB) predominates [12]. Arsenobetaine is a well-characterised, non-toxic arsenic species [13]. In contrast, only limited knowledge exists on the predominant arsenolipids present in fish oil. Basic knowledge, such as chemical structures, concentration levels and potential variations in the distribution of the arsenolipids in commercial fish oils is currently not known.

The toxicity of the water-soluble arsenic species varies greatly [14, 15]. Inorganic arsenic is highly toxic and carcinogenic, while AB is regarded non-toxic [13, 15, 16]. The toxicity of arsenolipids is currently not known. In this respect, it is important to acquire more knowledge regarding the lipid-soluble arsenic species, as noted by the European Food Safety Authority (EFSA) in their scientific opinion on arsenic in food

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[13]. In the opinion it was stated that more data on several arsenic species, including the arsenolipids is needed for future risk assessments of arsenic in food [13].

Marine oils were shown to be concentrated in arsenic already in the 1960s, as oils extracted from marine samples, *e.g.* herring (*Clupea harengus*), mackerel (*Scomber scomber*) and liver of cod (*Gadus morhua*) contained arsenic levels up to 19 mg kg<sup>-1</sup> [17, 18]. However, no chemical structures of the arsenolipids were identified in the early works on arsenic, and since then, research on arsenolipids has been negligible compared to the research on water-soluble arsenic species. This has mainly been explained by methodological challenges, as the preferred instrumentation the inductively coupled plasma mass spectrometer (ICP-MS) is not compatible with organic solvents which are needed for the analysis of lipids [3]. In 2008, after modification of the ICP-MS, the chemical structures of two groups of intact arsenolipids were identified in marine oils [19, 20]. They were characterised as arsenic-containing hydrocarbons (AsHCs) [20] and the arsenic-containing fatty acids (AsFAs) [19]. These studies initiated a larger research focus regarding the arsenolipids.

Analytical methods are a prerequisite for obtaining quantitative and qualitative information on the occurrence of arsenolipids in marine samples, and the development of analytical methods for determination of arsenolipids is therefore an essential first step for further studies of the species. With an analytical method developed for the determination of arsenolipids, marine samples can be studied, and data on the occurrence of the arsenolipids in samples related to both feed and food safety can be acquired.

## Aims of the study

The main aims of this study are:

- Develop a method for extraction of the arsenolipids.
- Develop quantitative methods for the determination of arsenolipids in marine oils.
- Determine the chemical structures of the arsenolipids present in marine oils.
- Characterise the major arsenic specie present in the liver of Atlantic cod (*Gadus morhua*).

# 1. Background

## 1.1 Arsenic

Arsenic has atomic number 33 and belongs to group 15 in the Periodic Table. Arsenic is classified as a semi-metal or metalloid, and occurs as a single isotope with an atomic weight of 74.92 amu. It has three different allotropic forms; yellow, black and grey, where the grey form is the only stable allotropic form at room temperature [21]. Elemental arsenic was first prepared in 1250 by the German scholar and alchemist Albertus Magnus who isolated the element by heating orpiment ( $\text{As}_2\text{S}_3$ ) with soap [22]. To the general public, arsenic is known for its toxicity. This is mainly linked to arsenic trioxide ( $\text{As}_2\text{O}_3$ ), a tasteless and odourless compound of arsenic that was often used as a poison in the past [23]. However, arsenic has also been used in medicine for treatment of *e.g.* syphilis, psoriasis and leukemia, where Fowler's solution (1% potassium arsenite,  $\text{KAsO}_2$ ) is most known [24].

Arsenic is introduced to the environment by both natural and anthropogenic sources [25]. It is ranked as the 20<sup>th</sup> most abundant element in Earth's crust with an average concentration of 3 mg kg<sup>-1</sup> [4]. More than 200 minerals of arsenic exist, where arsenic is often found in conjunction with sulphur minerals, *e.g.* arsenopyrite ( $\text{AsFeS}$ ) and realgar ( $\text{As}_4\text{S}_4$ ) [25]. Weathering of minerals and volcanic activity are major natural sources of arsenic [25, 26]. Major anthropogenic sources of arsenic includes mining, burning of fossil fuel and agricultural practices, such as use of arsenic-containing pesticides, wood preservatives and growth promoters exist [27, 28]. Arsenic is also used in the manufacture of alloys, in semiconductors and electronics, as well as in the production of leather preservatives, pharmaceuticals and dyes [29, 30].

Arsenic is distributed in both the marine and terrestrial environment, where marine samples generally contain higher concentrations of arsenic than terrestrial samples

[31]. Marine organisms such as fish, crustaceans and algae typically contain between 1 and 100 mg As kg<sup>-1</sup> wet weight (ww) [4, 5], whereas samples of terrestrial origin normally contain less than 0.02 mg As kg<sup>-1</sup> ww [32]. Rice is, however, an exception as rice may contain up to 1 mg As kg<sup>-1</sup> [33, 34].

## 1.2 Arsenic in the marine environment

In open seawater, the arsenic concentrations is usually low and uniform, from 0.5 to 2 µg As L<sup>-1</sup> [4, 35], while the arsenic concentrations in rivers and lakes may vary considerably, dependent on source, availability and geochemistry [27, 36]. Marine organisms accumulate arsenic through diet and from water, soil and particles [36, 37]. Marine algae may contain high arsenic concentrations, generally from 0.1 to 179 mg kg<sup>-1</sup> dry weight (dw) [13, 32], whereas most marine fish typically contain arsenic concentrations below than 5 mg kg<sup>-1</sup> ww in their tissue samples [38, 39]. Large variation in arsenic concentrations may be observed within same species, as well as between different species [31], e.g. the arsenic concentrations in Northeast Arctic cod (*Gadus morhua*) [40] and Greenland Halibut (*Reinhardtius hippoglossoides*) [41] with range from 1 to 170 mg kg<sup>-1</sup> ww and from 2 to 40 mg kg<sup>-1</sup> ww, respectively.

## 1.3 Arsenic species

The last 30 years there has been a shift in the way of determining elements; from analysis of total elemental concentration to determine the species of element [42]. In the year 2000, the International Union of Pure and Applied Chemistry (IUPAC) defined the terms related fractionation and chemical speciation of elements [43]:

- **Chemical species:** Specific form of an element defined as to isotopic composition electronic or oxidation state, and/or complex or molecular structure.
- **Speciation analysis:** Analytical chemistry: analytical activities or identifying and/or measuring the quantities of one or more individual chemical species in a sample.
- **Speciation of an element:** speciation: distribution of an element amongst defined chemical species in a system.
- **Fractionation:** Process of classification of an analyte or a group of analytes from a certain sample according to physical (*e.g.* size, solubility) or chemical (*e.g.* bonding, reactivity) properties.

In biological samples arsenic occurs mainly in the oxidation state +III and +V, and over 70 naturally occurring arsenic-containing compounds has so far been identified in the marine environment [6, 7]. The arsenic species are based on their chemical properties and hence their solubility in water or in oil, categorised as water-soluble and lipid-soluble arsenic species, respectively. The lipid-soluble arsenicals are a group of species also referred to as arsenolipids. In the present work arsenolipid will be used as a term for lipid-soluble arsenic species, *i.e.* the fraction of arsenic that partitions into an organic solvent phase.

## 1.4 Water-soluble arsenic in marine samples

More than 50 naturally occurring water-soluble arsenic species have so far been identified in the marine environment [6]. The chemical structures and acronyms of some of the most important species are represented in Table 1.1. The acronyms for the water-soluble arsenic species are obtained from the review of Francesconi & Kuehnelt [31]. Of the water-soluble arsenicals, arsenobetaine (AB) is the major arsenic species in most marine organisms [44-46]. Arsenobetaine was first identified in the rock lobster (*Panulirus cygnus*) in 1977 [47]. Since then, AB has been found to

often account for over 90% of the total arsenic present in marine organisms, such as fish, bivalves and crustaceans [48-51].

Simple methylated organic arsenicals, such as methylarsonate (MA) and dimethylarsinate (DMA) are minor arsenic species in marine organisms, such as fish and bivalves [4]. Other organic arsenicals, *e.g.* arsenocholine (AC), trimethylarsine oxide (TMAO) and tetramethylarsonium ion (TETRA) are also minor arsenic species in marine organisms [4]. Some exceptions exist, *e.g.* AC is a major arsenic species in the turtle *Dermochelys coriacea* [52] and in the fish species *Kyphosus syndeyanus* TMAO is a predominant arsenical [53].

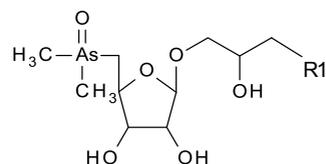
Over 15 chemical forms of arsenosugars have been identified [54]. Arsenosugars are the main arsenicals in marine algae, but are also found in herbivorous molluscs and gastropods [37, 55]. Most arsenosugars have a dimethylarsinoyl moiety bound to the ribofuranoside sugar. The four most common arsenosugars are shown in Table 1.1. Also trimethylarsonium forms [49], and thio-arsenosugars, where oxygen is replaced by sulphur do exist [56, 57].

Inorganic arsenic is the major arsenic species in sea and freshwater [4]. In marine organisms, such as fish, inorganic arsenic typically represents less than 1% of the total arsenic [10, 39]. Some exceptions do exist, *e.g.* brown algae *Hizikia fusiforme*, which can contain up to 50% of the total arsenic as inorganic arsenic [58]. Also, in and blue mussels (*Mytilus edulis*), a large proportion, up to 42%, of the total arsenic has been found as inorganic arsenic [59].

Table 1.1 Acronyms, names and formulas of selected water-soluble arsenic species. Acronyms for the water-soluble arsenic species was proposed by Francesconi & Kuehnelt [31].

Acronym	Arsenic species	Formula
As(V)	Arsenate	$O=As(O^-)_3$
As(III)	Arsenite	$As(O^-)_3$
MA(V)	Methylarsonate	$As(O^-)_3$
DMA(V)	Dimethylarsinate	$(CH_3)_2AsO(O^-)$
AB	Arsenobetaine	$(CH_3)_3As^+CH_2COO^-$
AC	Arsenocholine	$(CH_3)_3AsO$
TMAO	Trimethylarsonium oxide	$(CH_3)_3As^+CH_2CH_2OH$
TETRA	Tetramethylarsonium ion	$(CH_3)_4As^+$

Arsenosugar 1 (glycerol sugar):  $R= OH$   
 Arsenosugar 2 (phosphate sugar):  
 $R = OP(O)(OH)OCH_2CH(OH)CH_2OH$   
 Arsenosugar 3 (sulphonate sugar):  $R=SO_3^-$   
 Arsenosugar 4 (sulphate sugar):  $R =OSO_3^-$



## 1.5 Lipid-soluble arsenic in marine samples

In the present work marine oils are referred to as oils extracted from marine organisms, or of tissues of marine organisms. The presence of arsenic in marine oils were first observed in the early 20<sup>th</sup> century when oil of cod liver was shown to contain arsenic concentrations from 1.4 to 5.1 mg kg<sup>-1</sup> oil [60, 61]. In the 1960s Guldbrand Lunde [18, 62] reported that marine fish and other marine organisms typically contain arsenic levels between 1 and 50 mg kg<sup>-1</sup> in their lipid fractions.

Lipid-soluble arsenic was suggested to account for 10% to 30% of the total arsenic present in marine organisms [62]. However, higher relative proportions of lipid-soluble arsenic have been reported, *e.g.* for tuna (*Thunnus* sp.), containing 87% of the total arsenic as arsenolipids [63] and blubber of ringed seal (*Phoco hispida*), with 90% of the total arsenic in the lipid fraction [64]. Also, herring fillet (*Clupea harengus*) was recently reported to contain 62% of total arsenic as arsenolipids [65].

Marine oils typically contain arsenic concentrations between 0.2 and 19.3 mg kg<sup>-1</sup> oil, as reviewed by Sele and co-workers [7]. Arsenic levels in oils of, *e.g.* herring range from 3.1 to 19.3 mg kg<sup>-1</sup> [17, 65-68], capelin (*Mallotus villosus*) from 6.3 to 13.2 mg kg<sup>-1</sup> [17, 20, 66, 67, 69], mackerel (*Scomber scomber*) from 4.1 to 13.0 mg kg<sup>-1</sup> oil [17, 65-67] and cod liver oil from 0.2 to 10 mg kg<sup>-1</sup> [17, 19, 66, 70-74]. In commercial fish oil that is generally produced from pelagic fatty fish arsenic concentrations from 0.2 to 16 mg kg<sup>-1</sup> oil are typically seen [1-3, 75-78].

Also, oils of marine invertebrates, *e.g.* molluscs and crustaceans, have been reported to contain high levels of arsenic, ranging from 4.6 to 84 mg kg<sup>-1</sup> [66, 79]. In marine algae, various proportion of lipid-soluble arsenic have been reported [55]. Lipid-soluble arsenic accounted for 25% of the total arsenic present in the brown algae *Undaria pinnatifida* [55], while in *H. fusiformis* the lipid-fraction accounted for 1.6% of the total arsenic present [80].

## 1.6 Arsenolipids in marine oils

Four groups of arsenolipids are so far identified in marine samples; the arsenosugar phospholipids (As-sugPLs) [80-82], the arsenic-containing hydrocarbons (AsHCs) [20, 80, 82, 83], the arsenic-containing fatty acids (AsFAs) [19, 65, 84, 85] and the trimethylated arsenic fatty alcohols (TMAsFOHs) [83]. The chemical structures of the respective groups of arsenolipids can be seen in Table 1.2.

### ***Arsenosugar phospholipids***

The As-sugPLs have only been identified in marine brown algae [80-82]. The chemical structure of an arsenolipid, the dipalmitoylglycerophospho-2-hydroxypropyl-5-deoxy-5-(dimethylarsinoyl)-b-ribofuranoside, AsSug-PL<sub>958</sub> (Table 1.2), was first identified by Morita and Shibata [81] in the brown algae *Undaria pinnatifida*. Currently, 15 chemical compounds belonging to AsSug-PLs have been identified in marine brown algae [80-82]. The AsSug-PLs have been reported as major arsenolipids in the brown algae *U. pinnatifida* (67% of the total arsenic) and *Saccharina latissima* (>70% of the total arsenic) [80, 82].

### ***Arsenic-containing fatty acids***

The AsFAs were first identified in 2008 by Rumpler and co-workers who studied oil of canned cod liver [19]. Four AsFAs were identified as saturated fatty acids with a dimethylarsinoyl group (CH<sub>3</sub>)<sub>2</sub>As(O)<sup>-</sup>, replacing the methyl group in myristic (C:14), palmitic (C16:0), stearic (C18:0) and arachidic acid (C20:0), respectively [19]. Also, two unsaturated AsFAs were identified, and proposed to be analogues to the unsaturated fatty acids oleic acid (18:1, *n*-9) and 7,10,13,16,19-docosapentaenoic acid (DHA, 22:5, *n*-3) [19], which are fatty acids commonly found in cod liver oil [70]. The AsFAs accounted for approximately 20% of the total arsenic present in the cod liver oil [19]. Over 20 structures of AsFAs, with carbon chains from C<sub>8</sub> to C<sub>24</sub>, are now identified in marine samples, including fish meal of capelin [85], in liver of cod [84, 86, 87], fillet of herring [65], as well as in brown algae [82].

Table 1.2 Acronyms, names and formulas of the groups of arsenolipids identified, where one identified species represent each group. The numbers in lower-subscript for the AsHC, AsFA and TMAAsFOH refers to the length of the carbon chain. The acronyms are used throughout this thesis.

Acronym	Arsenic species	Formula
AsHC-C <sub>17</sub> <sup>a</sup>	Arsenic-containing hydrocarbon	
AsFA-C <sub>22</sub> <sup>b</sup>	Arsenic-containing fatty acid	
TMAAsFOH-C <sub>21</sub> <sup>c</sup>	Trimethylated arsenic-containing fatty alcohol	
As-sugPL <sub>958</sub> <sup>d,e</sup>	Arsenosugar-phospholipid	

<sup>a)</sup> Identified in [20].

<sup>b)</sup> Identified in [85].

<sup>c)</sup> Identified in [83].

<sup>d)</sup> Identified in [81].

<sup>e)</sup> The number in lower-subscript refers to the molecular weight of the compound.

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### ***Arsenic-containing hydrocarbons***

Three structures of AsHCs, comprising of homologous pair of two dimethylarsinoyl-alkanes with carbon chain lengths of C<sub>15</sub> and C<sub>17</sub>, and one dimethylarsinoyl-alkane with carbon chain length of C<sub>21</sub>, were identified as major arsenolipids in capelin oil [20]. The AsHCs accounted for approximately 70% of the total arsenic present. Also, tuna (*Thunnus sp.*) was observed to contain the same three AsHCs, which accounted for 40% of the total arsenic present in the fish [63]. Similar to the AsFAs, an increased number of chemical structures of the AsHCs have been identified, now counting over 10 different species [20, 80, 82, 83]. The AsHCs are usually found in same sample types as the AsFAs, such as fish meal [85], cod liver [84, 86, 87], fillet or oil of fish [65, 83] and marine algae [80, 82].

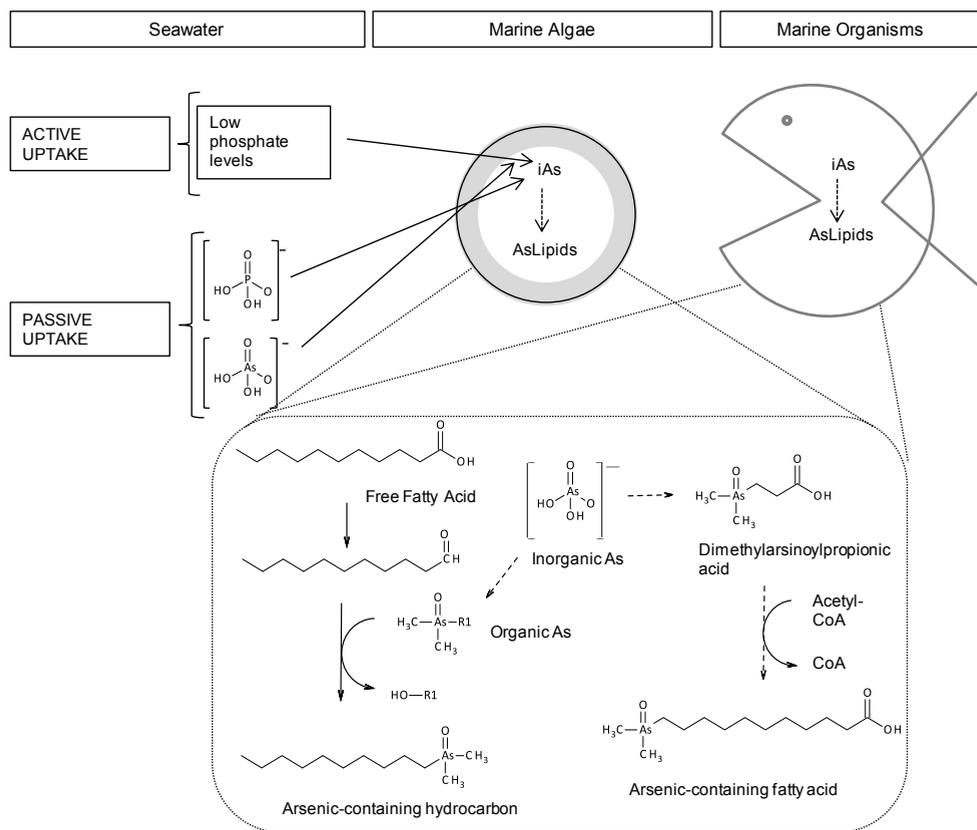
### ***Cationic trimethylated arsenic fatty alcohols***

Two chemical structures of cationic TMAFOHs, comprising of fatty alcohols with a positively charged terminal trimethylarsonium group were recently identified in capelin oil [83]. The arsenolipids were noted as minor arsenic species compared to the AsHCs and AsFAs present in the oil [83].

## **1.7 Origin of arsenolipids**

Arsenolipids are believed to be produced in marine algae [88, 89], and further transferred, via the food chain, to other organisms [62, 89-91] (Figure 1.1) In a study on arsenic transfer in a simple food chain, arsenic from seawater was incorporated in phytoplankton (*Dunaliella marina*), which biotransformed arsenic into lipid-soluble arsenic, and further transferred the arsenolipids to zooplankton (*Artemia salina*) and shrimp (*Lysmata seticaudata*) [91]. Over 90% in the *D. marina* was lipid-soluble arsenic. In fish, arsenolipids were suggested to originate from both diet and from

biotransformation within the fish, or from a combination of these processes [92]. When fed high doses of the water-soluble arsenical AB, lipid-soluble arsenic was detected in yellow-eye mullet (*Aldrichetta forsteri*) [93]. The arsenolipid was therefore suggested to be produced in the fish.



**Figure 1.1** A schematic overview of postulated origins of arsenolipids in marine organisms. With copyright permission from [7].

Arsenic is thought to be incorporated in marine organisms as a result of the similarity in chemical properties to phosphorous, also belonging to group 15 in the periodic table [6]. The phosphate transport systems in marine algae is insufficient in

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differentiating between the similar inorganic arsenate  $[\text{H}_2\text{AsO}_4]^-$  and phosphate  $[\text{H}_2\text{PO}_4]^-$ , both compounds present in seawater, causing an incorporation of arsenic in marine algae [6, 94]. Low levels of phosphate increased the uptake of arsenic by algae [95]. Some unicellular algae have been found to utilize nitrogen and sulphur in the biosynthesis of membrane lipids when the phosphate levels are low [96], and based on this it has been suggested that arsenic may actively be used in membranes of algae in a similar way [6]. This hypothesis was questioned by Raab and colleagues as the phosphate levels were 60 times of the arsenic levels in the brown algae *S. Latissima* [82]. The presence of saturated fatty acid C16:0 in position 2" in the AsSug-PL, which is typical for bacterial fatty acid synthesis [97], pointed instead towards a bacterial origin of the arsenolipids [82].

The biosynthesis of organic arsenicals from the incorporated inorganic arsenate has been suggested to be a detoxification mechanism of inorganic arsenic to less toxic organic arsenic species [98, 99]. The presence of arsenic species has also been explained by the inability of organisms to differentiate between arsenic-containing and the non-arsenic containing components [19]. Based on the similarity in chemical structure between the AsFAs and (non-arsenic containing) fatty acids, the AsFAs were suggested to originate from *de-novo* synthesis similar to the *de-novo* synthesis of fatty acids [19]. Dimethylarsinoylpropionic acid (DMAP;  $(\text{CH}_3)_2\text{As}(\text{O})\text{-CH}_2\text{CH}_2\text{COOH}$ ) is the likely start product for the AsFAs with odd-numbered carbon chains, where DMAP is elongated by two carbon-units by acetyl coenzyme A [19] similar to the elongation of fatty acids [100] (Figure 1.1) The identification of AsFA with even-numbered carbon chains meant that the odd-numbered and even-numbered AsFAs must have different origin [65, 85]. For the AsHCs, fatty acids have been proposed as start products [20]. The fatty acids were suggested to be reduced to the AsHCs via synthesis of fatty alcohols [20] (Figure 1.1), a mechanism based on the bacterial conversion of fatty acids to *n*-alkanes [101].

## 1.8 Human metabolism and toxicity of arsenolipids

Humans are mainly exposed to arsenic through the consumption of fish and other seafood [13, 102, 103]. For people in certain parts of the world elevated arsenic levels in drinking water dominates the arsenic intake [13, 15].

The toxicity of arsenic is highly dependent on the chemical form and the oxidation state of the element [16]. Inorganic arsenic is toxic and carcinogenic, while AB is considered to be non-toxic [16, 104, 105]. Adverse toxicological effects, such as cancer of lungs, urinary bladder and skin have been reported for people chronically exposed to inorganic arsenic through drinking water [13, 15]. In seafood, organic arsenicals are major arsenic species present [14].

The potential toxicity of several of the organic arsenic species, including arsenolipids, is still not fully known with regards to human exposure [6, 13, 15]. In one study of two volunteers consuming cod liver and cod liver oil with total arsenic concentrations of 1.0 to 3.3 mg kg<sup>-1</sup>, Schmeisser and colleagues found DMA(V) as the major metabolite in the urine [73]. Also, minor levels of dimethylated arsenic oxide species and thiolated species were detected in urine, which may also be related to the ingested arsenolipids [73, 74]. From these observations, it was suggested that the arsenolipids are metabolised in the human body [73, 74].

The metabolism of arsenic following seafood consumption, the implication for human health has been discussed [6, 14, 54]. It has been emphasised that humans metabolise organic arsenic species, such as arsenolipids, arsenosugars and inorganic arsenic mainly to the same major arsenic metabolite; DMA(V) [6, 54]. The toxicity of inorganic arsenic is, however, believed to be related to the intermediates, the trivalent DMA(III) and MA(III), produced during the metabolism of inorganic arsenic to DMA(V) [106]. The trivalent DMA and MA have been shown more toxic than inorganic arsenic species [107-111]. Similarly, organic arsenic species, including arsenolipids may produce potentially toxic intermediates during their metabolism into DMA(V) [6, 54].

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## 1.9 Analysis and characterisation of arsenolipids

From the initial studies in the 1960s to current use of hyphenated techniques, a range of analytical techniques and principles have been applied for studying the arsenolipids [7, 112].

### *The early studies of arsenolipids*

Guldbrand Lunde was the first to study arsenic and the lipid-soluble fraction of the element in the 1960s and 1970s [17, 18]. Lunde generated data on the abundance of arsenic, bromine and selenium in a range of marine and terrestrial oils using neutron activation analysis [17, 66, 113]. The chemical properties of the arsenolipids present in fish oils were studied by using conventional techniques for analysis of lipids. Based on the detection of arsenic in the same fractions as the phospholipids on a silica column, the arsenolipids were suggested to be chemical similar to phospholipids [113]. It was also observed that arsenic followed both the fatty acid fraction and the water-soluble fractions when the oils were saponified with potassium hydroxide, and it was concluded that at least two types of arsenic species were present [114].

Morita and Shibata [81] identified the first arsenolipid, the AsSug-PL<sub>958</sub> (Table 1.2) in brown algae *U. pinnatifida*. The arsenolipid was isolated using chloroform and methanol, which was further extracted by preparative chromatography prior to saponification and analysis of the extracts by gas chromatography (GC) coupled to mass spectrometry (MS). The structures were identified using proton-nuclear magnetic resonance (<sup>1</sup>H NMR), and an inductively coupled plasma atomic emission spectrometer (ICP-AES) was used as an element specific detector [81]. From the study it was suggested that arsenolipids present in marine algae were structurally different from those present in marine fish [81].

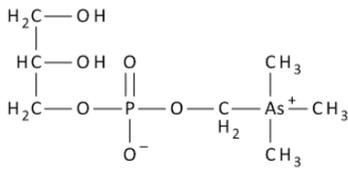
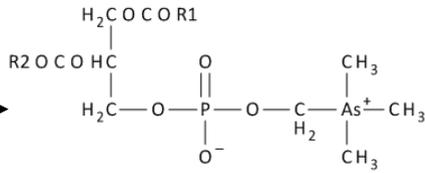
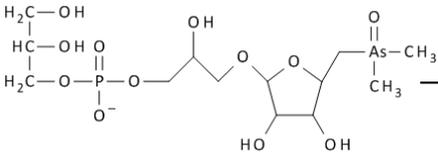
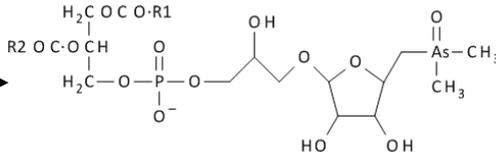
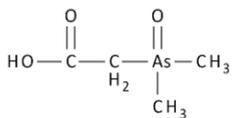
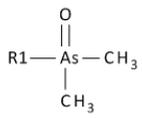
### *Analysis of hydrolysis products*

The inductively coupled plasma mass spectrometry (ICP-MS) is an element specific and a highly sensitive detector that is used in speciation analysis [42]. The ICP-MS has, however, not until recent years been compatible with the use organic solvent, preventing the use of high performance liquid chromatography (HPLC) coupled to ICP-MS for analysis of intact arsenolipids [3]. For a period, from 1990 to 2004, the arsenolipids were characterised based on the chemical structures of the water-soluble arsenicals produced when the lipid-fraction of marine samples were hydrolysed or saponified [93, 112, 115, 116].

Francesconi and co-workers [93] characterised the first arsenolipid in marine fish by analysis of alkaline hydrolysis extracts of yellow-eye mullet. Based on the identification of the glycerylphosphorylarsenocholine, it was suggested that the arsenolipid present in the yellow eye mullet was phosphatidylarsenocholine (Table 1.3). The arsenolipid was verified by NMR spectra ( $^1\text{H}$  and  $^{13}\text{C}$ ) of intact compound and of synthesised phosphatidylarsenocholine. The glycerylphosphorylarsenocholine was also detected in the digestive gland of western rock lobster after alkaline hydrolysis of the ether extracts, indicating the presence of phosphatidylarsenocholine [117]. The additional detection of dimethylriboside-2 in the extracts suggested that lipids with arsenosugar-moieties were present in the lobster [117] (Table 1.3).

Dimethylriboside-2 has also been identified as hydrolysis products in the blubber of ringed seal [64] and seaweed (*Laminara digitata*) [115]. Dimethylated arsenolipids seems to be present in several animals based on the detection of DMA and dimethylarsinoyl acetate in blubber of seal [64], in liver of starspotted shark (*Mustelus manazo*) [118, 120], in Japanese flying squid (*Todarodes pacificus*) [119] and in sheep fed seaweed [115] (Table 1.3). Also, arsenolipids with sphingomyelin structures have been proposed [120]. This was based on the identification of AC and DMA in hydrolysis extracts of starspotted shark, from where it was suggested that AC and DMA replaced the choline moiety in sphingomyelin [120].

Table 1.3 Hydrolysis products of marine oils and the proposed arsenolipid correlating to the products. For more information please refer to text.

Hydrolysis products	Proposed arsenolipids
	
Glycerolphosphorylarsenocholine	Phosphatidylarsenocholine <sup>1</sup>
	
Dimethylarsinoylribose-2	Phosphatidyldimethylarsinylribose
	
Dimethylarsinoyl acetate	Dimethylated arsenolipid(s)

### *Analysis and identification of intact arsenolipids*

The first analysis of intact arsenolipids using HPLC-ICP-MS was reported by Schmeisser and colleagues in 2005 [3]. The ICP-MS was modified based on an approach described for the analysis of phospholipids by HPLC-ICP-MS [121], by adding oxygen to the plasma and by replacing instrumental parts, such as the torch and nebulizer [3, 121]. The modifications stabilised the argon plasma in the ICP-MS to handle organic solvents, and fish oils were analysed using an acetone-based mobile

phase on a normal-phase HPLC-ICP-MS [3]. Three to four predominant arsenolipids, in addition to several minor arsenic-containing peaks were observed in the fish oils. The peaks were not structurally identified in the work by Schmeisser and colleagues [3].

Rumpler and co-workers were the first to identify intact arsenolipids in the oil of cod liver [19]. By partitioning the oil between immiscible solvents of *n*-hexane and aqueous methanol, the aqueous methanol fraction was found more concentrated in arsenic. The aqueous methanol phase was further extracted using preparative size-exclusion and anion-exchange chromatography prior to elemental and structural identifications by HPLC-MS/MS and by high resolution mass spectrometry (HR-MS), respectively [19]. The work resulted in the identification of six chemical structures of long chain fatty acids containing a dimethylarsinoyl moiety instead of the methyl-end; the AsFAs [19]. The AsFAs contained odd-numbered carbon chain lengths from C<sub>15</sub> to C<sub>21</sub> [19].

Using a similar analytical approach three AsHCs, with odd-numbered carbon backbone of C<sub>15</sub>, C<sub>17</sub> and C<sub>21</sub>, were identified in capelin oil [20]. The structural similarities between the AsHCs and AsFAs to fatty acids (non-arsenic containing) were noted, and the positions of the double bonds in the unsaturated AsHCs and unsaturated AsFAs were assigned by analogy to common (non-arsenic containing) fatty acid, *e.g.* docosahexanoic acid (DHA, 22:6, n-3) and docosapentanoic acid (22:5, n-3). The first AsFA with an even-numbered carbon chain length, of C<sub>22</sub>, was identified in the lipid fraction of a fish meal sample of capelin [85]. The AsFA was identified using a hyphenated technique where the outlet of HPLC column was coupled simultaneously to an ICP-MS and an OrbiTrap-MS, which allowed for both elemental and structural information of the arsenic-species to be obtained [85]. A similar analytical approach have been used for analysis of arsenolipids in fish tissues [65], cod liver [84], kelp [82] and capelin oil [83], resulting in the identification of over 30 arsenolipids.

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The AsHCs have also been analysed by GC coupled to ICP-MS and to MS [69, 86]. The three AsHCs (AsHC-C<sub>15</sub>, AsHC-C<sub>17</sub> and AsHC-C<sub>21</sub>) were detected in the extracts of capelin oil when screened by GC-MS [69]. The same AsHCs, in addition to the several unidentified peaks in the low-boiling point (40-300 °C) area of the chromatogram, were detected in extracts of cod liver when analysed by GC-ICP-MS [86].

Arsenosugar phospholipids and AsHCs were identified in extracts of the brown algae *U. pinnatifida* and *H. fusiformis* after extraction on a silica column and analysis by HPLC-ICP-MS and HR-MS [80]. The AsSug-PLs have also been studied using several enzymatic and chemical digestions to provide structural information on the lipid moieties of the AsSug-PLs [82]. Based on enzymatic hydrolysis using phospholipase (PLA2) palmitic acid (C16:0) was observed as the major fatty acid in position 2" of the AsSug-PLs, and it was further shown that the AsFAs were not bound to AsSug-PLs [82].

Two species of TMAsFOHs were recently identified in capelin oil using a derivatization and analysis of extracts by HPLC coupled to both ICP-MS and HR-MS [83]. The structures were identified based on identification of derivatisated compounds, by acetylation and thiolation, and by comparison to the intact compounds [83].

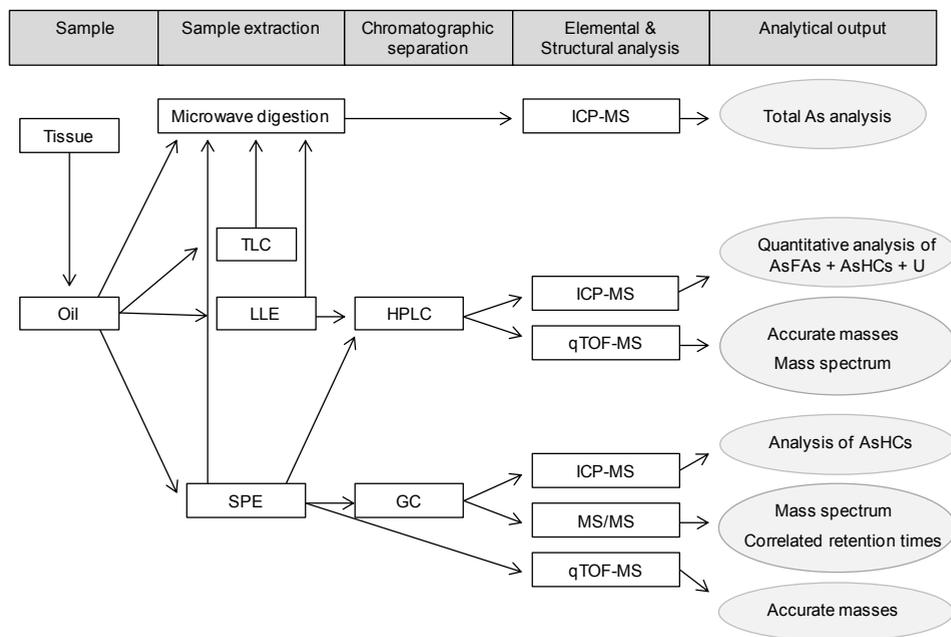
## 2. Method development

A schematic overview of analytical techniques and principles applied in the present work, and the analytical outcome are represented in Figure 2.1. Total arsenic concentrations of oils, and of extracts of oils, were determined by microwave digestion and analysis using inductively coupled plasma mass spectrometry (ICP-MS) (**Paper I – II**). The method is a Nordic and a European standard method [122-124]. For analysis of arsenolipids, including liquid liquid extraction (LLE) (**Paper I-III**), thin layer chromatography (TLC) and solid phase extraction (SPE) (**Paper I**) for the sample extraction. For elemental determination gas chromatography (GC) coupled to ICP-MS (**Paper I**) and high performance liquid chromatography (HPLC) coupled to ICP-MS (**Paper II-III**) were used. Structural analysis was performed by quadrupole time-of-flight mass spectrometry (qTOF-MS) (**Paper I**), GC coupled to tandem mass spectrometry (GC-EI-MS/MS) (**Paper I**) and by HPLC-ESI-qTOF-MS (**Paper II-III**). The instrumental settings for the analysis are listed in **Paper I-III**.

### *General comments on the method development*

One of the major challenges in the development of speciation methods for the characterisation of arsenolipids is the lack of commercially available standards for the analytes. Also, the presence of still unidentified arsenolipids challenges the method development of extraction procedures, as well as the qualitative assignments. The method development was based on:

- ✓ Use of real samples for the development of extraction and chromatographic methods.
- ✓ Use of total arsenic concentrations of sample extracts for the determination of recoveries of extraction methods.
- ✓ Use of fragmentation patterns previously reported arsenolipids in the structural identification by mass spectrometry.



**Figure 2.1** A schematic overview of the analytical techniques and principles applied, and the analytical output from the respective techniques.

## 2.1 Samples

The samples analysed in the present work are shown in Table.2.1. The fish oils were commercially produced for use in fish feed production. The oil of salmon (*Salmo salar*), cod (*Gadus morhua*) liver and seal (*Pagophilus groenlandicus*) were obtained as crude oils. The livers of Northeast Arctic cod (*Gadus morhua*) from the Barents Sea and of Atlantic cod from coastal areas of Norway were from a baseline study on environmental contaminants in cod from Norwegian waters, led by the National Institute of Nutrition and Seafood Research (NIFES), between 2009-2011 [40, 125]. All samples and sample extracts were stored at -20 °C until analysed. Light and heat

exposure of the oils, livers, and extracts were kept to a minimum during analysis to prevent oxidation of lipids, and a potential degradation of analytes.

Table 2.1 The samples analysed in the present work and the corresponding papers.

Sample type	Paper
Sand eel ( <i>Ammodytes marinus</i> ) oil	<b>Paper I-II</b>
Decontaminated sand eel oil	<b>Paper I</b>
Herring ( <i>Clupea harengus</i> ) oil	<b>Paper I-II</b>
Decontaminated herring oil	<b>Paper I</b>
Anchovy ( <i>Engraulis ringens</i> ) oil	<b>Paper I-II</b>
Blue whiting ( <i>Micromesistius poutassou</i> ) oil	<b>Paper I-II</b>
Mixed oil I <sup>1</sup>	<b>Paper II</b>
Mixed oil II <sup>2</sup>	<b>Paper I-II</b>
Salmon ( <i>Salmo salar</i> ) oil	<b>Paper II</b>
Seal oil (blubber of Greenland seal, <i>Pagophilus groenlandicus</i> )	<b>Paper II</b>
Oil of liver of cod ( <i>Gadus morhua</i> )	<b>Paper II</b>
Commercial fish oil I-III	<b>Paper II</b>
Commercial fish oil IV	data not published
Livers of Northeast Arctic cod ( <i>Gadus morhua</i> ) <sup>3</sup>	<b>Paper III</b>
Livers of Atlantic cod ( <i>Gadus morhua</i> ) from coastal Norwegian waters <sup>4</sup>	data not published

<sup>1</sup>) A mixture of oils of Norway pout (*Trisopterus esmarkii*), blue whiting, Atlantic herring and sand eel.

<sup>2</sup>) A mixture of oils of Atlantic herring, Atlantic cod and saithe (*Pollachius virens*).

<sup>3</sup>) From the Barents Sea ( $n = 26$ ).

<sup>4</sup>) From Hardangerfjorden, Balsfjorden and Borgundfjorden ( $n = 6$ ).

## 2.2 Sample extraction

The arsenolipids are minor constituents compared to the major (non-arsenic-containing) lipids of marine oils, *e.g.* the triacylglycerols (TAGs) and phospholipids (PLs) [97, 126]. An important step in the analysis of arsenolipids is therefore the extraction of arsenic species from the lipid matrix, as this improves the separation and robustness in the chromatographic analysis and up-concentrates the analytes.

### *Extraction of arsenolipids from tissues of marine organisms*

Lipids are usually defined by their solubility into organic solvents, *e.g.* “tissue components that are soluble in lipid solvents” [127]. For the extraction of lipids, one or several organic solvents are used to separate the lipid-soluble components from the water-soluble components [128], and the extraction procedures are often based on mixtures of chloroform and methanol [128-130]. An extraction approach based on the Bligh & Dyer approach [129] was used for extracting the arsenolipids from the cod liver tissues in the present work (**Paper III**). Compared to use of solvents, *e.g.* ether [93] and methanol [84, 86], a solvent mixture of chloroform and methanol has been used for the extraction of arsenolipids from marine tissues, *e.g.* cod liver [73], muscle of tuna [63], starspotted shark [118, 120] and ringed seal [64, 116] (Table 2.2).

Table 2.2. Solvent and solvent mixtures used for the extraction of arsenolipids from tissues of marine organisms.

Sample	Organic Solvent	Reference
Cod liver and herring	CHCl <sub>3</sub> /MeOH (2 + 1, v/v)	[113]
Cod liver	Hexane or hexane/ CHCl <sub>3</sub> /MeOH (5 + 4 + 1, v/v)	[3]
Cod liver	MeOH	[84, 86]
Tuna <sup>1</sup>	CHCl <sub>3</sub> /MeOH (2 + 1 v/v)	[63]
Western rock lobster <sup>2</sup>	MeOH (100%)	[117]
Yellow eye mullet	Ether (100%)	[93]
Starspotted shark <sup>3</sup>	CHCl <sub>3</sub> /MeOH (2 + 1 v/v)	[118, 120]
Ringed seal <sup>4</sup>	CHCl <sub>3</sub> /MeOH (2 + 1 v/v)	[64, 116]

<sup>1</sup>) Muscle tissue.

<sup>2</sup>) Digestive gland.

<sup>3</sup>) Muscle, dark muscle, stomach, heart, glass bladder, intestine, skin, spleen, brain, liver, kidney and bone.

<sup>4</sup>) Liver, kidney, muscle, gonad, stomach content and blubber.

### ***Extraction and separation of arsenolipids by thin layer chromatography***

In TLC lipids are separated according to their different affinities to a stationary phase or sorbent, such as silica, in a selected solvent system. Each class of lipids has a characteristic mobility, which is the distance travelled by the compound(s) on the TLC plate, and is typically directly compared with the mobility of a standard [131].

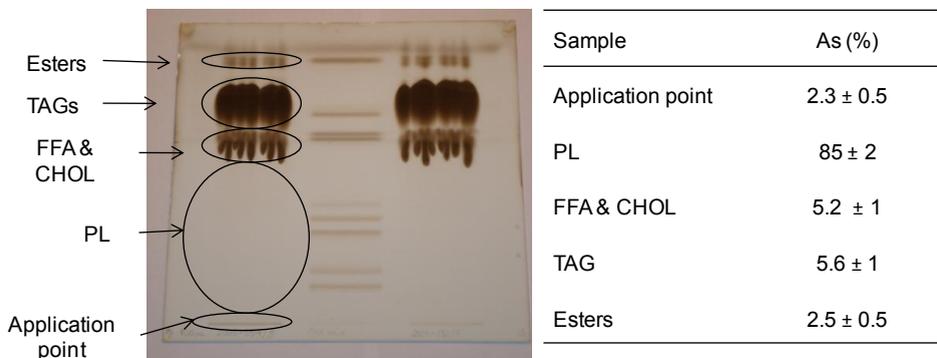
In a preliminary study of arsenolipids in marine oils, the commercial fish oil II and IV were separated on a TLC plate (silicagel, 60G, 200 x 200 mm, VWR International LLC, Radnor, PE, USA) using an approach described by Henderson and colleagues

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[132]. The lipid classes were separated on the TLC by first eluting the analytes by a polar solvent mixture (potassium chloride/chloroform/iso-propanol/methylacetate, 9/10/25/25/25) followed by a non-polar solvent mixture (isohexane/diethylether/acetic acid, 95/12/2) [132]. The sample volume was set to 100  $\mu$ L to ensure detectable levels of arsenic in the separated lipids. The plate with separated lipids was submerged in a solution of 3% copper acetate and left in an oven (160 °C) for 15 min. The lipid classes in the fish oil samples were compared to a standard mixture of PLs (phosphatidyl choline (PC), Sigma Aldrich, St. Louis, MO, USA; phosphatidyl serine (PS), Aventi Technology As, Oslo, Norway; phosphatidyl inositol (PI), Sigma Aldrich; phosphatic acid (PA), Sigma Aldrich; phosphatidyl ethanol amine (PE), Sigma Aldrich), free fatty acids (FFA) (linolenic acid, Sigma Aldrich), cholesterol (CHOL) (cholesterol, Sigma Aldrich), TAGs (trilinolenin, Sigma Aldrich) and esters (linoleyl behenate, Sigma Aldrich).

The major lipid class in both commercial oils was TAGs (Figure 2.2; data not published). Also, FFA, CHOL and esters were detected in the oils, while no PLs were detected (Figure 2.2). Due to the increased application volume of sample, a low resolution in the separation of the lipid classes was observed (Figure 2.2).

Total arsenic was determined in the separated lipid classes of the commercial fish oil IV ( $n = 3$ ) (Figure 2.2). The bands of lipid classes were carefully scraped of the plate into PTFE vessels for total arsenic determination. Due to the low resolution in the lipid class separation, the FFA and CHOL were analysed as one sample in the total arsenic analysis. The total arsenic analysis of lipid classes, showed that 85% (w/w) of the total arsenic was retained in the area of the PLs on the TLC plate (Figure 2.2, data not published). Only minor amounts of arsenic was retained in the areas of the non-polar lipids, *e.g.* TAGs and esters, containing 5.6% (w/w) and 2.5% (w/w) of the total arsenic, respectively. The result shows that the major part of the arsenolipids has polar characteristics, similar to PLs. This is consistent with the work by Lunde [66], who found arsenic mainly in the polar methanol-containing fraction when fractioning oils of marine fish on a silica column.



**Figure 2.2** The separation of commercial fish oil IV (left) and commercial fish oil II (right) on a TLC plate (left picture). A standard mixture consisting of PLs (PC, PS, PI, PA, PE), FFA (linolenic acid), CHOL (cholesterol), TAG (trilinolenin) and ester (linoleyl behenate) was applied in the middle. The amount of arsenic (% (w/w), mean  $\pm$  SD,  $n = 3$ ) in the lipid classes of the commercial fish oil IV (right table).

The preliminary study clearly emphasizes the importance of highly selective analytical techniques that can distinguish between arsenic-containing lipids and (non-arsenic-containing) lipids. Further work on arsenolipids was therefore not performed by use of TLC, but instead it was focused on the use of more sensitive and selective techniques.

### ***Extraction of arsenolipids by Liquid Liquid Extraction***

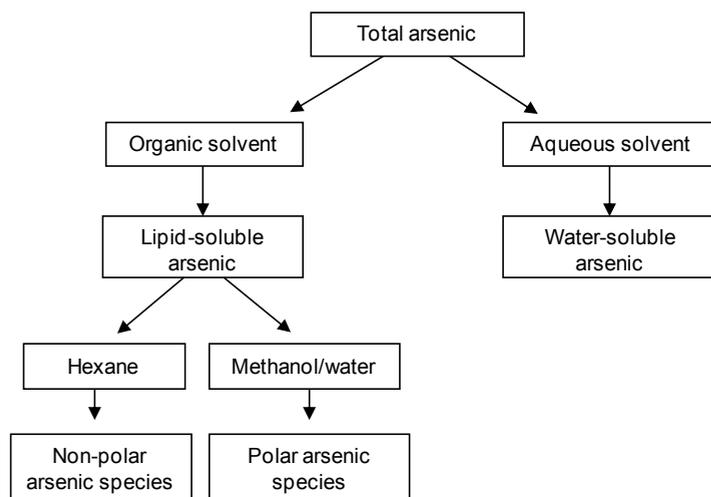
Lipids are generally regarded as hydrophobic compounds that are soluble in organic solvents. However, the solubility of lipids depends on the relative strength of the interactions between solvent and the hydrophobic or hydrophilic part of the compound, where lipids with a low polarity, *e.g.* TAGs, are very soluble in non-polar solvents, such as hexane, and tend to be insoluble in polar solvents [128]. Also, the

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selectivity of solvents may affect the partitioning of compounds into solvents, where *e.g.* methanol, being a proton-donor, is a selective solvent for fatty acids, which are proton-acceptors [133, 134].

In liquid liquid extraction (LLE) compounds are extracted by their solubility in two immiscible solvents or phases, typically an aqueous and an organic phase [135, 136]. In the present work marine oils were separated using two immiscible solvents of *n*-hexane and aqueous methanol (**Paper I**), and *n*-heptane and aqueous methanol (**Paper II-III**). Hexane and heptane easily dissolves the oils, whereas the addition of aqueous methanol forms a two-phased system of a polar methanol phase and a non-polar hexane phase (Figure 2.3). All oils analysed showed similar partitioning of arsenic where the aqueous methanol phase was more concentrated in arsenic than the hexane or heptane phase (**Paper I-III**). The non-polar hexane and heptane phase, contained lower arsenic concentrations due to the high lipid content in this fraction ( $98 \pm 2\%$  of the total amount of oil) (data not shown). The methanol phase (MeOH 1 phase) was further subjected to analysis by GC-ICP-MS (**Paper I**) and HPLC-ICP-MS (**Paper II-III**).

To further increase the extraction efficiency of arsenic into polar solvents to be analysed by reversed-phase HPLC-ICP-MS, the *n*-heptane phase was subsequently extracted with methanol (MeOH 2 phase), and then with acetonitrile (ACN phase) (**Paper II**). The MeOH 1 phase contained the highest concentrations of arsenic, with 20% to 40% of the total arsenic in the oils (**Paper II**). The sequential extractions increased the extraction efficiency of arsenic into polar solvents by 8-14% and 4-8% of the total arsenic in the oils for the MeOH 2 and ACN phase, respectively (**Paper II**). The hexane phase is more challenging to analyse due to the high lipid (non-arsenic containing) content, lower arsenic concentrations, and also due to the incompatibility of hexane as a solvent in reversed-phase HPLC analysis. The hexane phase was not further analysed in the present work.



**Figure 2.3** A schematic diagram of the categorisation of arsenic into water-soluble and lipid-soluble fractions, and the categorisation of lipid-soluble arsenic as non-polar and polar arsenic species.

High concentrations of arsenic have also been seen in the methanol fraction of other marine oils, *e.g.* fish oils [3, 20, 63] and cod liver oil [19, 73] (Table 2.3). In oils of tuna, 50% of the arsenolipids partitioned into the polar methanol phase, but as only 5% of the (non-arsenic containing) lipids in the oils were found in this fraction, it was more concentrated in arsenic than the hexane phase of the oil [63]. Using a sequential extraction procedure of the hexane phase with aqueous methanol, an extraction recovery of 94% of the total arsenic in capelin oil was recently achieved [83]. A larger sample amount and larger solvent volumes were used in the work by Amayo and colleagues compared to the present work.

Table 2.3 The partitioning of arsenic (%) in non-polar and polar solvents for marine oils.

Sample	Partitioning of arsenolipids	Reference
Cod liver	40% in hexane phase	[73]
Cod liver oil	40% in methanol phase	[19]
Capelin oil	70% in the methanol phase	[20]
Oil of tuna muscle	50% in the methanol phase	[63]
Marine oils	17 – 45% in the methanol	<b>Paper I-II</b>
Oils of cod liver	31 – 60% in the methanol phase	<b>Paper II – III</b>

### ***Solid Phase Extraction***

Chromatographic columns have been used for the extraction of arsenolipids in oils of marine samples [19, 84, 85, 113]. Most of the studies on arsenolipids have, so far, focused on identification of novel species, and hence typically used large volumes of samples and solvents [19, 65, 84, 87]. Few studies have used commercially available SPE columns for the separation of arsenolipids which enables low sample and solvent volumes to be applied for extraction [136, 137], which is beneficial when studying a large number of samples.

In the present work a weak-anion exchange SPE column (1 g, 12 mL, Phenomenex, Torrance, CA, USA) was used for the extraction of AsHCs in an aqueous methanol phase of commercial fish oils (**Paper I**). The approach was based on the extraction procedure described by Taleshi and colleagues [20]. The aqueous methanol phase was applied to the SPE and the AsHCs were extracted by eluting with a solvent mixture consisting of methanol/chloroform/water (60/30/8). The AsHCs were found to account for 55% to 92% when comparing the total arsenic of the SPE extract with the total arsenic in the aqueous methanol phase (**Paper I**). This is consistent with the

work of Taleshi and colleagues [20] who found 65% of the arsenic in the methanol fraction of capelin oil to be the AsHCs, when using a preparative sized column [20].

## 2.3 Organic solvents and ICP-MS

The ICP-MS in its standard set-up is not compatible with the organic solvents that are necessary to use when analysing lipid-soluble compounds [3, 138]. Organic solvents cause instability of the plasma by cooling, furthermore the high load of carbon causes deposition of carbon on the cones of the ICP-MS, which decreases the signal sensitivity [121]. For analysis with organic solvents, the ICP-MS was modified using a low flow nebulizer, a torch with a narrow inner diameter, platinum sample and skimmer cones, and tubings resistant to organic solvents (**Paper II-III**). Oxygen was added as an optional gas (typically 20% oxygen in argon) through a T-piece, located on the torch. The ICP-MS was optimized for arsenic with triphenylarsine oxide ( $\text{Ph}_3\text{AsO}$ ) as standard, dissolved in the organic solvent(s) used in the mobile phase, and introduced by self-aspiration (**Paper II-III**). The typical instrumental settings for the ICP-MS can be seen in Table 2.4.

Table 2.4 Typical instrument settings for the ICP-MS when optimizing for arsenic ( $m/z$  75), using  $100 \mu\text{g L}^{-1}$   $\text{Ph}_3\text{AsO}$  in the respective solvents as tuning solution.

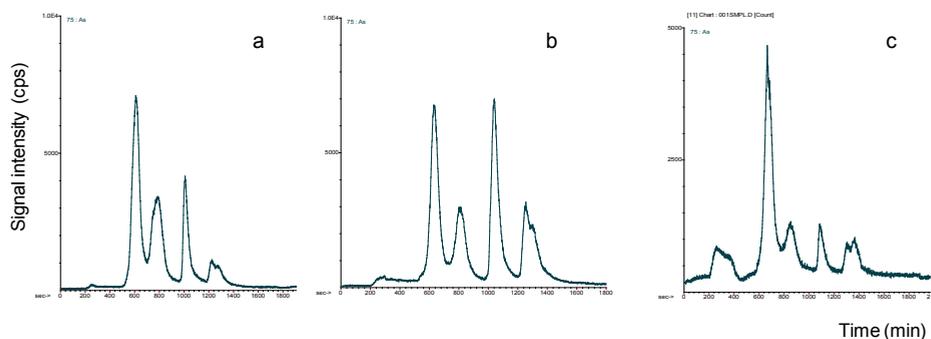
Settings	Normal phase mobile phase <sup>1</sup>	Methanol
Torch (inner diameter, mm)	1	1.5
Carrier gas flow ( $\text{L min}^{-1}$ )	0.23	0.3
Makeup gas flow ( $\text{L min}^{-1}$ )	0.24	0.24
Optional gas flow (% of carrier gas)	23	11
Spray chamber temperature ( $^{\circ}\text{C}$ )	-5	-5

<sup>1</sup>) acetone/hexane/acetic acid/triethylamine (90/7/1.4/0.2).

## 2.4 Analysis by normal phase HPLC-ICP-MS

In normal phase chromatography the analytes are separated based on that their affinity to a stationary phase is of a polar character, *e.g.* silica or diols, while the mobile phase is non-polar [139]. Normal-phase HPLC with silica based columns has been used for separating lipid classes in marine oils [140, 141]. In a preliminary study of the arsenolipids, commercial fish oils ( $n = 3$ ) were analysed by normal-phase HPLC-ICP-MS (Figure 2.4). The fish oils (1.0 g,  $n = 2$ ) were diluted in the mobile phase (1 mL; acetone/hexane/acetic acid/triethylamine (90/7/1.4/0.2)), and the samples (5  $\mu\text{L}$ ) were separated using an isocratic elution with flow of  $80 \mu\text{L min}^{-1}$  on a Hilic column (Kinetex,  $2.1 \times 100 \text{ mm}$ ,  $2.7 \mu\text{m}$ , Phenomenex).

All fish oils contained at least four to six arsenolipids (Figure 2.4). This is consistent with the results of Schmeisser and colleagues who analysed fish oils with normal-phase HPLC-ICP-MS, and found 4-6 arsenolipids in the oils [3]. The levels of the arsenic-containing peaks varied between the fish oils analysed (Figure 2.4) which may suggest biological variations among the species of fish that the oils were extracted from. The peaks were not structurally identified by mass spectrometry.



**Figure 2.4** The chromatograms of long sand eel oil (a), blue whiting (a) and Atlantic herring (a) analysed by normal-phase HPLC-ICP-MS.

The advantage of using a normal-phase separation is the possibility to analyse whole fish oil directly without further sample preparation than dilution, and hence, no arsenolipids are lost during the extraction procedure. However, as a consequence the sample extracts are abundant in other (non-arsenic containing) lipids, *e.g.* TAGs, which challenges the potential identification of the arsenolipids. Furthermore, the retention mechanisms in normal-phase HPLC chromatography are based on the affinity of polar functional groups to the polar stationary phase [139, 142], and the peaks in the normal-phase chromatograms may therefore correspond to more than one arsenolipid if the analytes are structurally different only in their carbon chain lengths. From these results it was concluded that reversed-phase HPLC-ICP-MS or GC-ICP-MS would give better chromatographic resolution in the separation of the arsenolipids.

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## 2.5 Analysis by GC-ICP-MS

Gas Chromatography is normally applied for the analysis of free fatty acids, as the methyl esters are efficiently separated and can be easily identified according to their relative retention times on a GC column [143]. In GC the analytes are separated based on volatility, solubility or affinity to the stationary phase and on the temperature-program used [142]. In the present work several commercial oils were analysed for their content of arsenic-containing hydrocarbons (AsHCs) using GC-ICP-MS (**Paper I**). The oils were extracted into aqueous methanol, and the AsHCs were further extracted by weak-anion exchange SPE columns (**Paper I**). The analytes were separated on a GC (5%-Phenyl)-methylpolysiloxane column (HP-5, 30 m×0.32 mm, 0.25 µm, Agilent Technologies) with helium as the carrier gas.

Three major arsenic peaks were detected in all fish oils analysed by GC-ICP-MS using the instrumental settings listed in **Paper I**. The molecular structures of the compounds were determined by tandem mass spectrometry as AsHC-C<sub>15</sub>, AsHC-C<sub>17</sub> and AsHC-C<sub>21</sub> (Table 2.5, **Paper I**). The structural identification is described in section 4.9. Also, a minor arsenic-containing peak, eluting close to the AsHC-C<sub>17</sub> was detected by the GC-ICP-MS analysis, however, the peak could not be identified by mass spectrometry due to the low signal intensity. The results were consistent with previous work where AsHC-C<sub>15</sub>, AsHC-C<sub>17</sub> and AsHC-C<sub>21</sub> have been shown to be the major arsenolipids in extracts of capelin oil [69] and of cod liver [144] when analysed by GC-ICP-MS.

Gas chromatography is suitable for analysis of volatile compounds. Most naturally occurring arsenicals are non-volatile, and therefore there is few application of GC in arsenic speciation [44, 145]. The AsHCs can be analysed by GC due to the arsine group attached to the alkyl chain of the compounds [69, 86]. Gas chromatography is, however, not suitable for analysis of other groups of arsenolipids. Analysis of the arsenic-containing fatty acids (AsFAs) would require a derivatization step prior to GC analysis [69], an approach similar to that used for analysing (non-arsenic containing) fatty acids by GC [146-149].

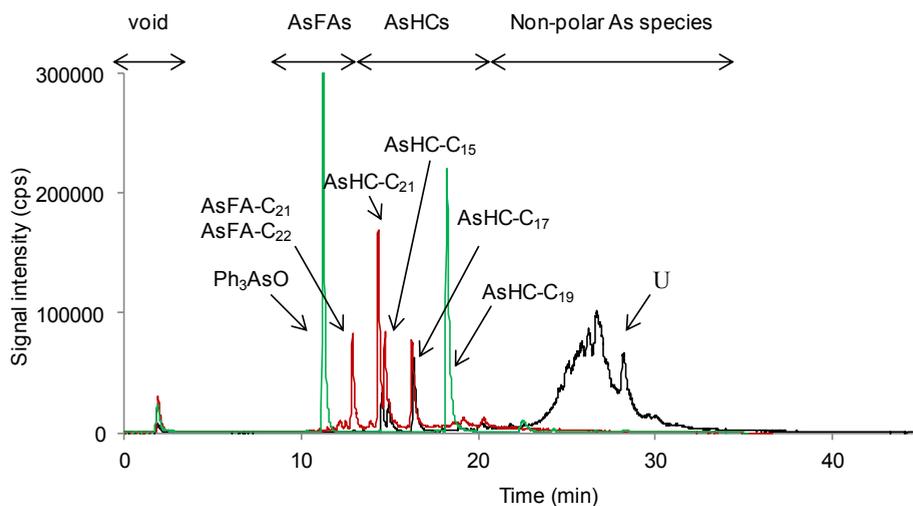
Table 2.5 Acronyms, chemical formula and chemical structures of the arsenic-containing hydrocarbons (AsHCs) and arsenic-containing fatty acids (AsFAs) identified in this work (**Paper I – III**).

Acronyms	Chemical formula	Chemical Structures	
AsHC-C <sub>15</sub>	C <sub>17</sub> H <sub>39</sub> AsO		Paper I-III
AsHC-C <sub>17</sub>	C <sub>19</sub> H <sub>41</sub> AsO		Paper I-III
AsHC-C <sub>21</sub>	C <sub>23</sub> H <sub>37</sub> AsO		Paper I-III
AsFA-C <sub>21</sub>	C <sub>23</sub> H <sub>37</sub> AsO <sub>3</sub>		Paper II-III
AsFA-C <sub>22</sub>	C <sub>24</sub> H <sub>37</sub> AsO <sub>3</sub>		Paper II-III
AsFA-C <sub>15</sub>	C <sub>17</sub> H <sub>35</sub> AsO <sub>3</sub>		Paper III
AsFA-C <sub>17a</sub>	C <sub>19</sub> H <sub>37</sub> AsO <sub>3</sub>		Paper III
AsFA-C <sub>17b</sub>	C <sub>19</sub> H <sub>35</sub> AsO <sub>3</sub>		Paper III

## 2.6 Analysis by reversed-phase HPLC-ICP-MS

In reversed-phase HPLC compounds are separated with a non-polar stationary phase, *e.g.* C<sub>18</sub> (octadecylsilane) or C<sub>8</sub> (octylsilane), and with a polar mobile phase, *e.g.* water and methanol, often in a gradient [150]. Reversed-phase HPLC-ICP-MS can be used for separating different groups of arsenolipids, where the AsFAs, the AsHCs and more non-polar arsenolipids are separated using a gradient elution comprising of 20% methanol to 100% methanol (Figure 2.5) (**Paper II**). This is consistent with analysis

of arsenolipids by gradient elution in reversed-phase HPLC-ICP-MS analysis, where arsenic-containing species with a range in polarities are separated [151, 152].



**Figure 2.5** Overlay of MeOH 1 phase (red) and MeOH 2 phase (black) of commercial oil II analysed by reversed-phase HPLC-ICP-MS (Instrumental settings are found in **Paper II**). Please note that the dilution factors for the MeOH 1 phase (x3) and MeOH 2 phase (x2) differ. A standard mixture of  $\text{Ph}_3\text{AsO}$  (r.t. 12 min) and synthesised AsHC-C<sub>19</sub> (r.t. 18 min) is overlaid (green). The elution order of the arsenic species is noted above the chromatogram.

The major arsenic species in the methanol phases of all marine oils were the AsHC-C<sub>15</sub>, AsHC-C<sub>17</sub> and AsHC-C<sub>21</sub> (Table 2.5, **Paper II**). In addition two AsFAs; AsFA-C<sub>21</sub> and AsFA-C<sub>22</sub> were detected by the reversed-phase HPLC-ICP-MS analysis (Table 2.5, **Paper II**). The species were identified by high resolution mass spectrometry, which is discussed in the following section. In addition, up to 20 arsenic-containing peaks were observed in the aqueous methanol phases (the MeOH1 phase) and in the methanol phase (the MeOH 2 phase) of marine oils analysed by

gradient elution reverse-phase HPLC-ICP-MS in **Paper II**. These peaks could not be structurally determined by HPLC-qTOF-MS analysis.

### ***Quantitative analysis by reversed-phase HPLC-ICP-MS***

The introduction of organic solvents into the ICP-MS affects the ionization of certain elements, such as arsenic and selenium, and causes increased signal [153, 154]. The effect is explained by a charge transfer from positively charged carbon ions to arsenic, occurring in the plasma of the ICP-MS [153-155]. The effect may be an advantage when analysing water-soluble arsenic species, where a constant addition of 3% organic solvent (methanol) into the mobile phase increases the sensitivity for arsenic [153]. However, when using a gradient elution with organic solvents, the variation in carbon content leads to changes in the response of the detector and challenges the quantification of elemental species [85, 151-153]. Approaches such as isotope dilution [156, 157], mathematical compensation [158] and post-column addition of an internal standard [159] have been described for compensating for the signal effect of selenium and phosphorous.

The variation in signal response for arsenic through the organic gradient was determined by introducing a solution of arsenic ( $^{75}\text{As}$ ) and internal standards, comprising of germanium ( $^{74}\text{Ge}$ ) and indium ( $^{115}\text{In}$ ), into the ICP-MS (**Paper II**). A blank sample was analysed simultaneously with the gradient program (**Paper II**). The procedure was based on the work by Amayo and colleagues [85]. The methanol containing gradient increased the signal sensitivity of arsenic with a factor of approximately 2.5 (from 30% to 100% methanol) (**Paper II**). A time-dependent arsenic-response factor was determined for every arsenolipid by comparing the arsenic response at the retention times of the arsenolipids with the arsenic response at the retention times of the calibration standard (**Paper II**).

To determine the most suited calibration standard for quantitative analysis of the arsenolipids, three different calibration standards; DMA,  $\text{Ph}_3\text{AsO}$  and a synthesised

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arsenic-containing hydrocarbon (AsHC-C<sub>19</sub>) were tested (**Paper II**). The AsHCs, AsFAs and unknown peaks were quantified using the time-dependent response factors and the calibration curves for each calibration standard. (**Paper II**). Dimethylarsinate gave the best recovery in the quantitative results for arsenolipids, from 91% to 104% ( $n = 12$ ) compared to total arsenic measurements in the same extracts. The recoveries in Ph<sub>3</sub>AsO and the synthesised AsHC-C<sub>19</sub> were 64-89% ( $n = 12$ ) and 110-122% ( $n = 5$ ), respectively (**Paper II**). Similarly, Amayo and colleagues found that DMA provided accurate quantitative results for the arsenolipids, with a recovery of 94.3% compared to the total arsenic in a extract of a fish meal sample [85].

## 2.7 Structural identification by mass spectrometry

Since the plasma in the ICP-MS combusts the sample at a temperature of 6,000-10,000 K, and consequently atomises all molecules, the structural assignments of arsenic containing peaks can only be done by matching in retention times and is dependent on available standards of the compounds [160]. For structural information and identification of novel arsenic species softer ionisation techniques, *e.g.* electrospray ionisation (ESI)-MS or electron impact ionisation (EI)-MS is necessary [161]. In the present work the arsenolipids were structurally identified by GC-EI-MS/MS (QqQ) (**Paper I**) and high resolution ESI-MS/MS (qTOF) (**Paper I – III**).

The high selectivity of the ICP-MS was seen from the analysis of AsHCs by GC-MS/MS in MS1 mode compared to the analysis by GC-ICP-MS (**Paper I**). While the AsHCs were selectively detected by GC-ICP-MS, with detector set for mass  $m/z$  75, the AsHCs could not be separated from other components in the extracts by GC-MS1 analysis (**Paper I**). First when using multiple reaction monitoring (MRM) in the GC-MS/MS, with specific precursor and product ions for the AsHCs (Table 2.6), could the AsHCs be detected and identified in the GC-MS/MS (MS2) spectra (**Paper I**). For all AsHCs the product ions  $m/z$  105 and 106 were selected, while for the AsHC-C<sub>21</sub>

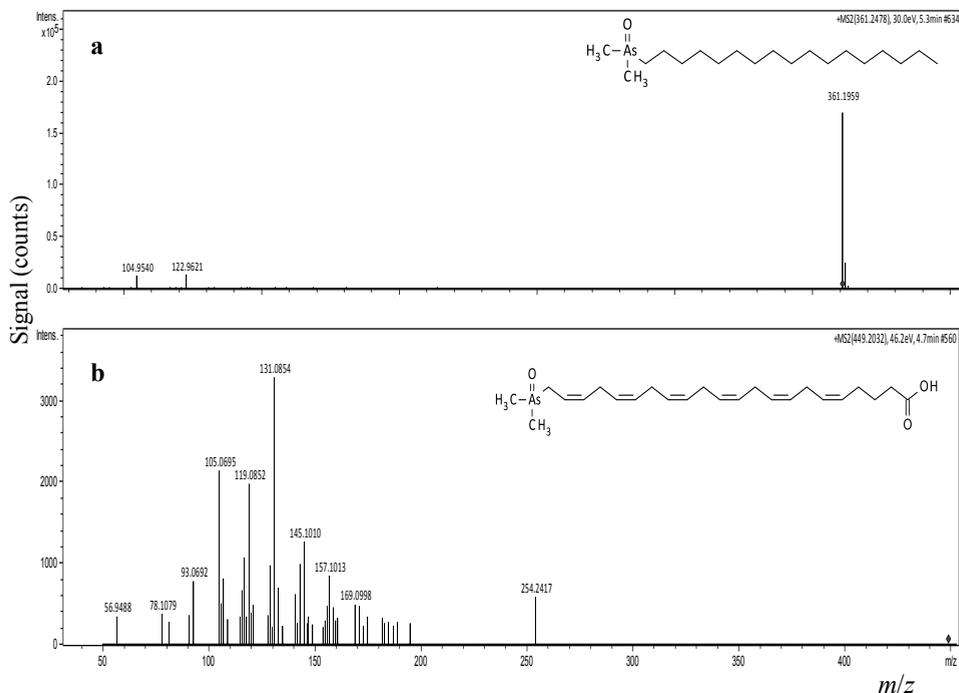
also  $m/z$  159 was selected (Table 2.6, **Paper I**). The precursor and product ions were chosen based on the work by Raber and colleagues [69] who determined the AsHCs by GC-MS.

Table 2.6 The precursor ions ( $m/z$ ) (the molecular ion with loss of oxygen  $[M-16]^+$ ) and the product ions ( $m/z$ ) used in Multiple Reaction Monitor (MRM) in the GC-EI-MS/MS analysis (**Paper I**).

AsHC	Precursor ion $[M-16]^+$ ( $m/z$ )	Product ions ( $m/z$ )
AsHC-C <sub>15</sub>	316	105, 106
AsHC-C <sub>17</sub>	344	105, 106
AsHC-C <sub>21</sub>	388	105, 106, 159

When analysing the arsenolipids by HPLC-qTOF-MS the ions  $m/z$  104.97 and  $m/z$  122.97 were detected as major fragment ions for all saturated AsHCs and AsFAs (Figure 2.6, **Paper II-III**). These ions have similarly been observed as major fragment ions of AsHCs and AsFAs in HR-MS analysis and have been suggested to correspond to the arsenoyl-moiety of the arsenolipids,  $(CH_3)_2As^+$  ( $m/z$  104.97) and  $(CH_3)_2AsOH_2^+$  ( $m/z$  122.97) [65, 85]. For unsaturated AsHCs and unsaturated AsFAs, however, the  $m/z$  104.97 and  $m/z$  122.97 could not be detected as fragment ions (**Paper II-III**). Instead, the  $m/z$  119, 131, 145 and 159 were observed fragment ions for all unsaturated arsenolipids in the qTOF-MS analysis (**Paper II-III**). These fragment ions have also been seen for unsaturated AsHCs and unsaturated AsFAs in the work of others [82, 84]. The fragmentation patterns of the AsFAs were recently shown to be similar to the fragment ions of (non-arsenic-containing) fatty acids, demonstrating that the fragment ions  $m/z$  119, 131, 145 and 159 are not exclusive to the arsenolipids [84].

With the exception of three AsFAs (AsFA-C<sub>15</sub>, AsFA-C<sub>17a</sub> and AsFA-C<sub>17b</sub>) in one of the cod liver samples (**Paper III**) with relative error in mass ( $\Delta m$ ) of  $\pm 12.7$  ppm, all other AsHCs and AsFAs identified in the present work had relative error for calculated and measured accurate mass ( $\Delta m$ ) within  $\pm 5$  ppm (**Paper I-III**). The retention times for the arsenolipids correlated well with the retention times in the HPLC-ICP-MS and GC-ICP-MS analysis, respectively (data not published), supporting the identification of the arsenolipids. A possible explanation for the absence of  $m/z$  104.97 and 122.97 as fragment ions for unsaturated AsHCs and AsFAs may be the influence of the double bonds on the fragmentation mechanisms, as differences in fragmentation patterns for saturated and polyunsaturated arsenolipids are observed in this study (Figure 2.6) and by others [82, 84].

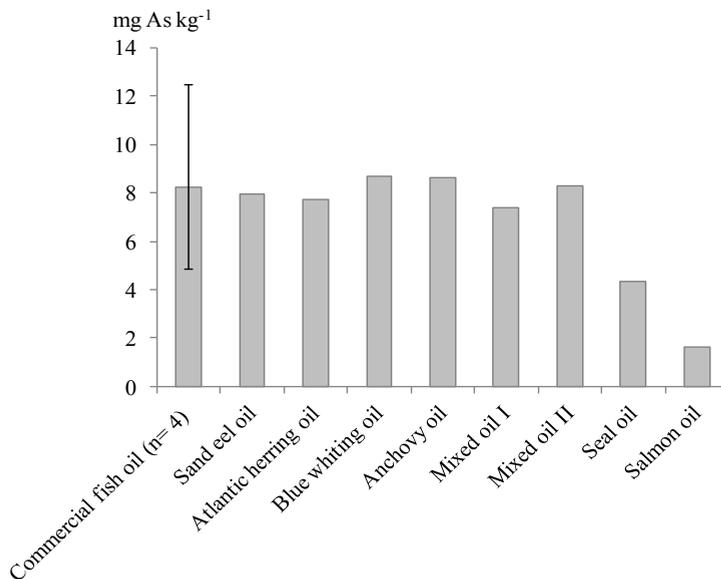


**Figure 2.6** The MS/MS spectra of AsHC-C<sub>17</sub> (a) and AsFA-C<sub>22</sub> (b) when analysed by HPLC-qTOF-MS.

### 3. General discussion: Arsenolipids in marine oils

#### 3.1 Total arsenic in marine oils

The oils in the present work, which included oils of different fish species and mixtures of oils (termed mixed oils and commercial oils), contained arsenic concentrations from 1.6 to 12.5 mg kg<sup>-1</sup> oil (Figure 3.1, **Paper I - II**). The oils of pelagic fish were all within the typical range of 4.6–16 mg As kg<sup>-1</sup> oil, observed for commercial fish oils [1-3].



**Figure 3.1** Total arsenic concentrations (mg kg<sup>-1</sup> oil) in marine oils. The commercial oils are represented by the average arsenic concentration of four oils ( $\pm$  range in concentrations) (**Paper I-II**).

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The diet has been suggested as a factor influencing the levels of arsenic in oils of fish [17, 92]. Also, the total arsenic levels in whole fish have been suggested to be a reflection of the dietary intake of arsenic [38, 40]. The lower arsenic levels in the oil of farmed Atlantic salmon, 1.6 mg kg<sup>-1</sup> oil (**Paper II**), compared to the arsenic levels in oils of pelagic fish (Figure 3.1), may hence be ascribed to differences among fish species and/or by differences in the diet.

Seal oil (of blubber) contained an arsenic concentration of 4.5 mg kg<sup>-1</sup> (Figure 3.1, **Paper II**). Blubber of harbour seals (*Phoca vitulina*) and ringed seals (*Phoca hispida* and *Pusa hispida*) have been reported to contain between 1.9 and 2.0 mg kg<sup>-1</sup> [162] and 0.6 to 1.76 mg kg<sup>-1</sup> [64, 116], respectively. This is somewhat lower than the arsenic level observed in the oil analysed in the present work (**Paper II**). Blubber is more concentrated than other tissues, e.g. kidneys, muscle, hair, lung and liver of harbour seals [162], and the arsenolipids are the major arsenic species present in blubber, accounting for 90% of total arsenic present [64]. Based on hydrolysis of the blubber and determination of DMA as the major arsenic-containing hydrolysis product, the blubber was suggested to mainly contain dimethylated arsenolipids [64].

## 3.2 Arsenic-containing hydrocarbons and fatty acids in marine oils

The three AsHCs; AsHC-C<sub>15</sub>, AsHC-C<sub>17</sub> and AsHC-C<sub>21</sub> were identified as the major arsenolipids present in the methanol phase of the fish oils (**Paper I-II**). Table 2.5 in the method development section shows the chemical structures of the arsenolipids identified in the present work. The AsHC-C<sub>15</sub>, AsHC-C<sub>17</sub> and AsHC-C<sub>21</sub> have previously also been identified as major arsenic species present in capelin oil [20] and in fillet of tuna [63], accounting for 70% and 40% of the total arsenic present, respectively. Two AsFAs; AsFA-C<sub>21</sub> and AsFA-C<sub>22</sub> were also identified in the fish

oils (**Paper II**). The AsFAs accounted for 5 to 16 % of the total arsenic present in the methanol extracts of the fish oils (**Paper II**). The same AsHCs and AsFAs have also been reported as major arsenolipids in extracts of fish meal of capelin [85], capelin oil [83], herring muscle [65] and cod liver [84].

In addition to the two AsFAs and three AsHCs identified in the fish oils, over 20 unidentified arsenic-containing compounds were detected by the reversed-phase HPLC-ICP-MS analysis (**Paper II**). This was also seen by Rumpler and colleagues when analysing extracts of cod liver oil [19]. In addition to six AsFAs with carbon chain lengths of C<sub>11</sub>, C<sub>13</sub>, C<sub>15</sub>, C<sub>17</sub> and C<sub>21</sub>, up to 15 unidentified arsenic-containing peaks were present [19]. Several AsFAs and AsHCs, with carbon chains ranging from C<sub>11</sub> to C<sub>27</sub>, were recently identified as minor arsenolipids in oils of cod liver, capelin and herring [65, 83, 84, 87].

A similar distribution was observed for the AsHCs and AsFAs in the methanol extracts of the different fish oils, where the AsHC-C<sub>21</sub> was the predominant arsenolipid in most of the oils examined, accounting for 27 to 43% of the total arsenic in the extracts (**Paper II**). Seal oil (blubber) also contained AsHC-C<sub>15</sub>, AsHC-C<sub>17</sub> and AsHC-C<sub>21</sub> and AsFA-C<sub>21</sub> and AsFA-C<sub>22</sub> (**Paper II**). Salmon oil, in contrast, contained only the AsHCs. This may be explained by the lower total arsenic concentration in the salmon oil (**Paper II**). The same AsHCs were recently also identified in muscle of salmon (*Onchorhynchus keta*). Additionally, over 10 minor AsFAs were identified in the salmon muscle [65]. The differences in AsFAs may be explained by analytical or/and biological differences.

### ***Decontamination of fish oils***

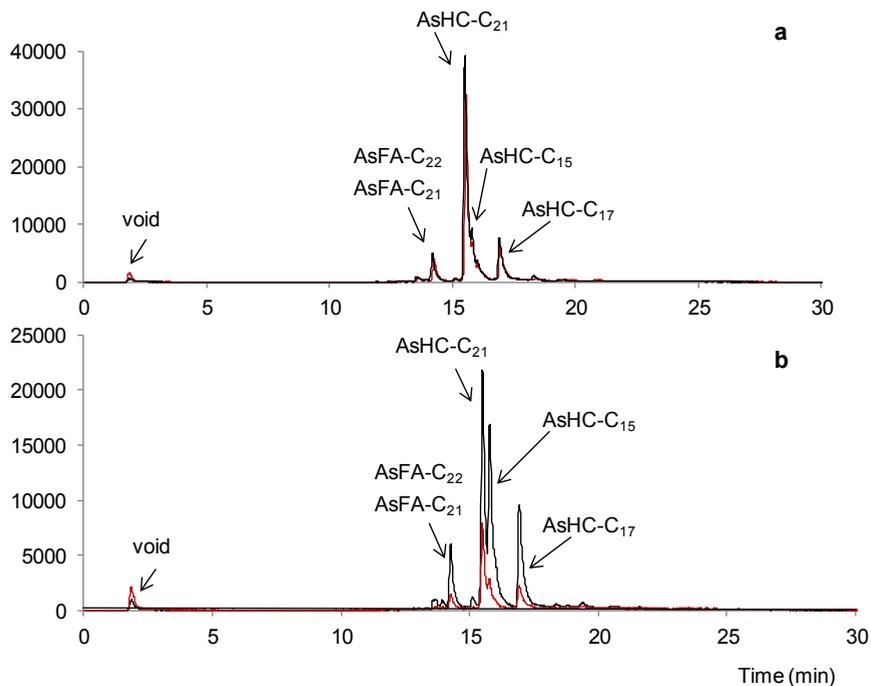
Marine pelagic fish accumulate lipid-soluble contaminants, such as persistent organic pollutants (POPs), in their lipid-rich tissues, and fish oil is therefore a major source of POPs, e.g. polychlorinated biphenyls (PCBs) and dioxins, in formulated feed [11, 163, 164]. Several decontamination techniques, such as active carbon and steam

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deodorization, have been developed to remove POPs from oils used in feed production and for human consumption [165-168]. Two decontaminated fish oils of herring and sand eel were included in this work (**Paper I**). Decontamination by active carbon and steam deodorization [169] reduced the levels of total arsenic by 25% in the herring oil and by 10% in the sand eel oil (**Paper I**).

Analysis of the methanol phase of the original oils and the decontaminated oils by GC-ICP-MS (**Paper I**) and reversed-phase HPLC-ICP-MS (data not published) showed that the level of AsHCs was reduced by the decontamination process. The reduction was observed to be greater for the decontaminated herring oil than for the decontaminated sand eel oil (**Paper I**, Figure 3.2). From the reversed-phase HPLC-ICP-MS analysis there was additionally a reduction in the AsFAs in the decontaminated herring oil (Figure 3.2, data not published).

The differences in reduction of arsenic in the decontaminated sand eel oil and herring oil are likely related to the decontamination procedures as the two oils were decontaminated at different times (**Paper I**). Decontamination processes commonly used for removal of POPs, such as active carbon and steam deodorization, are known to remove other lipid-soluble compounds, *e.g.* oxidative products, sterols, tocopherols and free fatty acids (FFA) [170-172]. A larger removal of FFA was observed in the herring oil compared to the sand eel oil (data not shown), and this may explain the larger removal of arsenolipids in the herring oil, as discussed in **Paper I**. The structural similarity of AsFAs and AsHCs to FFA [19, 20], further suggest that AsHCs and AsFAs could be affected similar to FFA during a decontamination process.



**Figure 3.2** Chromatogram of the aqueous methanol phase of (a) sand eel oil (black) and decontaminated sand eel oil (blue), and (b) herring oil (black) and decontaminated herring oil (blue) analysed by HPLC-ICP-MS (data not published). The instrumental settings are listed in **Paper II**.

### 3.3 Arsenic in cod liver

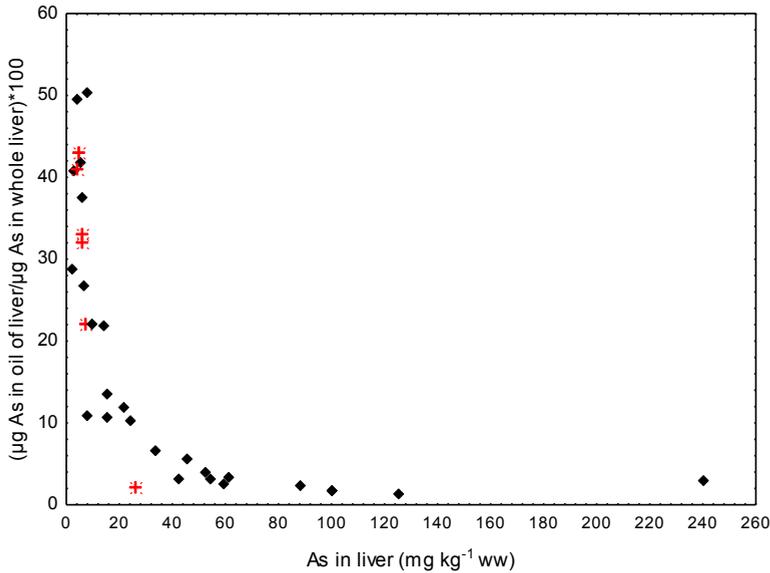
The Northeast Arctic cod is a stock of Atlantic cod that is found in the Barents Sea. It is possibly the largest cod stock in the world [173]. In a baseline study of the level of contaminants in Northeast Arctic cod ( $n = 804$ ), exceptionally high concentrations of arsenic was observed in muscle and liver, ranging from 1 to 170 mg kg<sup>-1</sup> ww and from 1 to 240 mg kg<sup>-1</sup> ww, respectively [40]. Liver samples of Northeast Arctic cod ( $n = 26$ ), with total arsenic concentrations between 2.1 and 240 mg kg<sup>-1</sup> ww, were selected

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for further analysis of total arsenic in the lipid- and water-fractions, and for arsenic speciation analysis of the respective fractions of livers (**Paper III**).

The lipid-fraction of livers contained arsenic concentrations ranging from 1.8 to 16.4 mg kg<sup>-1</sup> oil (**Paper III**). A linear correlation ( $r^2 = 0.80$ ,  $p < 0.001$ ) was observed between the total arsenic concentrations of whole livers and the arsenic concentrations of the oils of the livers (**Paper III**). The arsenic concentration in the lipid-fraction accounted for 3% to 50% of the total arsenic present in the liver from cod (**Paper III**). A trend was observed for the relative proportions of lipid-soluble arsenic and the total arsenic in whole liver, where lower relative proportions of lipid-soluble arsenic (less than 10%) were found for the liver samples with arsenic levels above 33 mg kg<sup>-1</sup> ww compared to the liver samples with arsenic levels below 33 mg kg<sup>-1</sup> ww (up to 50%) (Figure 3.3, **Paper III**).

Atlantic cod from fjords of Norway (Hardangerfjorden, Borgundfjorden and Balsfjorden,  $n = 6$ ) followed the same trend in terms of the relative proportions of lipid-soluble arsenic (data not published, Figure 3.2). The livers contained total arsenic concentrations from 2 to 26 mg kg<sup>-1</sup> ww [125], and between 2.8 and 42% of the total arsenic was found as lipid-soluble arsenic (data not published, Figure 3.3). Large variations in the relative proportion of lipid-soluble arsenic to total arsenic (between 10% and 50%) were observed for all cod livers with total arsenic concentrations below 20 mg kg<sup>-1</sup> ww (Figure 3.3).



**Figure 3.3** The relative proportion of arsenic (% w/w) in the lipid fraction ( $\mu\text{g As in oil}/\mu\text{g As in whole liver} \times 100$ ) in cod livers of Northeast Arctic cod (*Gadus morhua*) (black) and of Atlantic cod (*Gadus morhua*) from coastal areas of Norway (red) (**Paper III**).

Arsenic concentrations from 0.2 to 10 mg kg<sup>-1</sup> oil have been reported oils of cod liver [7, 84, 87] (Table 3.1). Variation in the relative proportions of lipid-soluble arsenic have been seen for canned cod liver, where two livers with total arsenic concentrations of 2.6 and 3.3 mg kg<sup>-1</sup> dw, contained 25% and 77% of the total arsenic as lipid-soluble, respectively [73]. In a liver sample of Atlantic cod, 27% of the total arsenic, at 1.5 mg kg<sup>-1</sup> ww, was found as arsenolipids [84], and hence also follow the same trend in terms of relative proportions of lipid-soluble arsenic as observed in present study (**Paper III**).

Table 3.1 Total arsenic concentrations ( $\text{mg As kg}^{-1}$  ww) in cod liver, and in the lipid-fraction or oil ( $\text{mg As kg}^{-1}$  oil) of the livers; the relative proportion of arsenolipids (%) of total arsenic present in liver.

Type of sample	Total As in liver ( $\text{mg kg}^{-1}$ ww)	As conc ( $\text{mg kg}^{-1}$ oil)	As in oil (%)	<i>n</i>	Reference
Cod liver <sup>1</sup>		0.2-10	25-75 <sup>2</sup>	76	[7] <sup>3</sup> .
Cod liver oil <sup>4</sup>		5.8		1	[87]
Cod liver	1.53		27	1 <sup>5</sup>	[84]
Cod liver oil <sup>4</sup>	-	5.5	-	1	<b>Paper II</b>
Northeast Arctic cod liver	2.1-240	0.5-16.3	3 – 50	28	<b>Paper III</b>

<sup>1</sup>) Canned and fresh cod liver.

<sup>2</sup>)  $n = 2$ .

<sup>3</sup>) And references therein.

<sup>4</sup>) Crude oil produced in factory.

<sup>5</sup>) Pooled sample of 10 liver samples.

The origin of arsenolipids in marine organisms is currently not known [6, 7]. These chemical species have been suggested to be produced in primary producers and transferred to higher animals in the food chain [88, 91]. However, arsenolipids have also been suggested to be produced in marine fish [92]. Rumpler and colleagues suggested that the AsFAs, found present in cod liver, were *de-novo* products, produced by an elongation similar to (non-arsenic containing fatty acids) [19]. It was not specified by Rumpler and colleagues [19] if the process took place in the cod livers or in the primary producer.

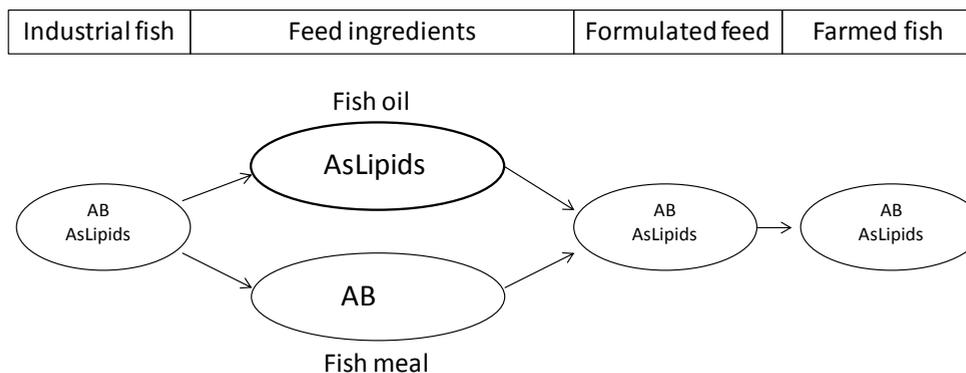
Arsenolipids in the livers of cod may either originate from the diet or be *de-novo* products, as discussed in **Paper III**. An important part of the diet of cod is shrimp, herring and capelin [174, 175]. Shrimp may contain particular high levels of arsenic, up to  $100 \text{ mg kg}^{-1}$  ww [176, 177], and is a dietary source that can contribute with high total arsenic levels in cod [40]. Arsenobetaine is the major arsenic species in shrimp

[4, 50]. Fatty fish, such as herring and capelin contains arsenolipids [20, 65], and may be dietary sources for arsenolipids in cod. Considering a possible *de-novo* synthesis, based on the *de-novo* synthesis of fatty acids, for the arsenolipid, it should be noted that carnivorous fish such as cod, mainly acquire lipids through the diet, and to a limited extent by *de-novo* synthesis [178]. A *de-novo* synthesis of arsenolipids in cod liver seems therefore unlikely, and the arsenolipids present in the liver of cod is suggested to originate from the diet of the fish (**Paper III**).

### 3.4 Arsenolipids in the aquaculture production chain

In formulated fish feeds, fish oil and fish meal are ingredients contributing with lipids and proteins, respectively [179, 180]. The feed ingredients may, however, also contain contaminants, and the presence of certain contaminants, such as POPs, in feed are reflected in fillet of the farmed fish [181-183]. Arsenic is naturally occurring in marine fish, which are a source of arsenic in marine feed ingredients, and further result in the transfer of arsenic into the aquaculture production chain [9, 10] and to the farmed fish [11, 184] (Figure 3.4). Data from the National surveillance programme for Norwegian fish feeds shows high levels of total arsenic in both fish oil and fish meal, ranging from 4.6-15.8 mg kg<sup>-1</sup> and 1.8-18.2 mg kg<sup>-1</sup> dw, respectively [1, 2].

The European Union (EU) has established maximum limits for certain contaminants in feed and food to ensure consumer safety as well as to protect animal health and welfare [185]. In contrast to other trace elements, such as mercury, cadmium and lead, there is currently no agreement on maximum levels for arsenic in foodstuffs (EC, 2006 and amendments) [186]. In the feed legislation the maximum limits for arsenic is 25 mg As kg<sup>-1</sup> for feed ingredients of marine origin, and 10 mg As kg<sup>-1</sup> for complete feed for fish and fur animals (EC, 2002 and amendments) [187, 188]. None of the fish oils analysed in the present work (**Paper I** and **Paper II**) exceeded the current maximum level of 25 mg kg<sup>-1</sup>.



**Figure 3.4** A schematic diagram of the presence, and potential transfer of arsenic species in the whole chain of aquaculture production of farmed fish.

Fish meal mainly contains water-soluble AB [12], while less has been known about the arsenolipids present in fish oil (Figure 3.4). Oils of various fish species, including commercial oils, mixed oils, and oils of fish species such as blue whiting, sand eel, herring and anchovy characterised in the present work contained relative uniform levels of arsenic, ranging from 4.9 to 12.5 mg kg<sup>-1</sup> oil (**Paper II**). This is comparable to the average arsenic concentration of 9.4 mg kg<sup>-1</sup> oil, in fish oils used in feed products [1, 2]. The major arsenolipids identified in the oils all contained the three AsHCs; AsHC-C<sub>15</sub>, AsHC-C<sub>17</sub> and AsHC-C<sub>21</sub> (**Paper I-II**). In addition, two AsFAs; AsFA-C<sub>21</sub> and AsFA-C<sub>22</sub> were identified as minor arsenolipids (**Paper II**). The AsHCs and AsFAs accounted for 17% to 45% of the total arsenic present in the oils (**Paper I-II**).

Although, fish meal mainly contains AB [12], arsenolipids have also been detected in fish meal [85, 189]. Fish meal of mackerel, capelin, herring, anchovy and Norway pout contained 5-10% lipids, the arsenic concentrations in the lipid-fraction ranged from 4.6 to 23.2 mg As kg<sup>-1</sup> oil [189]. Amayo and colleagues [85] found that the arsenolipids accounted for 12% of the total arsenic present in a fish meal of capelin. The AsHCs; AsHC-C<sub>15</sub>, AsHC-C<sub>17</sub> and AsHC-C<sub>21</sub>, and the AsFAs; AsFA-C<sub>21</sub> and

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AsFA-C<sub>22</sub> (**Paper II**) were identified as major arsenicals in the fish meal [85]. Fish meal is hence also a source of arsenolipids in the aquaculture production chain.

Arsenic concentrations from 0.7 to 7.1 mg kg<sup>-1</sup> are seen for fish feed used by the aquaculture industry in the surveillance programme for Norwegian feed (2009-2012) [1, 190]. Of the total arsenic present in feed, more than 95% are organic arsenic species, while low levels (< 2%) inorganic arsenic are found [10, 78, 190]. The presence of arsenolipids in fish feed is currently not documented. Based on the presence of the three AsHCs (AsHC-C<sub>15</sub>, AsHC-C<sub>17</sub> and AsHC-C<sub>21</sub>) and the two AsFAs (AsFA-C<sub>21</sub> and AsFA-C<sub>22</sub>) in commercial fish oils (**Paper I-II**) [20, 83], as well as in fish meal [85], it can be assumed that these specific arsenolipids may also be present in fish feed, and possibly in farmed fish. It should be noted that there is still a relative large proportion of the arsenolipids in the fish oils that is still unidentified. These unidentified, and possibly more non-polar, arsenolipids (**Paper II**), may also be present in the feed and in the fish.

In fillet of farmed Atlantic salmon the total arsenic concentrations range from 0.02 to 3.1 mg kg<sup>-1</sup> (w.w.) [177]. Also, in other farmed marine fish, *e.g.* Atlantic cod and Atlantic halibut (*Hippoglossus hippoglossus*), relative low levels of arsenic are reported in the muscle, with concentrations ranging from 0.54 to 2.0 mg kg<sup>-1</sup> ww and from 0.6 to 3.3 mg kg<sup>-1</sup> ww, respectively [177]. In the present work one oil of farmed salmon was analysed for the content of arsenolipids. The oil, with a total arsenic concentration of 1.6 mg kg<sup>-1</sup>, contained AsHC-C<sub>15</sub>, AsHC-C<sub>17</sub> and AsHC-C<sub>21</sub> (**Paper II**). This is consistent with a recent study of one Chum salmon muscle where the AsHC-C<sub>15</sub>, AsHC-C<sub>17</sub> and AsHC-C<sub>21</sub> were identified, in addition to several AsFAs [65]. However, few data on the presence of arsenolipids in farmed fish, wild fish and other seafood, currently exist. More data on the arsenolipids is needed to gain better understanding of the distribution of the arsenolipids in seafood, and their significance with regards to seafood safety. Larger data sets on the arsenolipids were called up on by EFSA, and noted as important for a future risk assessment of arsenolipids in food [13].

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## 4. Conclusion

The present work describes the development of analytical methods for the determination of arsenolipids, and the application of methods for characterising the arsenolipids in marine oils (**Paper I-II**) and in oils extracted from cod liver (**Paper III**).

The arsenolipids were extracted using LLE, where the oils were partitioned into *n*-hexane/*n*-heptane and aqueous methanol, which was further analysed (**Paper I-III**). The method gave an extraction recovery of maximum 60% of the total arsenic present in the oils (**Paper I-III**). A further clean-up of the methanol phase was conducted by a SPE column, prior to analysis by GC-ICP-MS. The SPE removed some of the lipid matrix in the extracts (**Paper I**). To increase the extraction efficiency of arsenic into polar solvents the heptane phase of the oils was sequentially extracted by methanol, and then acetonitrile (**Paper II**). This increased the recovery of arsenic into polar solvents by 9-18%. This shows that the extraction method still needs to be improved for a complete recovery of the arsenolipids from marine oils.

By using GC-ICP-MS the extracts could be analysed for the AsHCs (**Paper I**). In the methanol extracts of commercial fish oils, three AsHCs; AsHC-C<sub>15</sub>, AsHC-C<sub>17</sub> and AsHC-C<sub>21</sub> were found as major arsenolipids present, accounting for 55 to 95% of the total arsenic present (**Paper I**). A minor arsenic-containing peak was also observed, but not identified, in all oils analysed.

A quantitative method based on reversed-phase HPLC-ICP-MS was developed for determination of both AsHCs and AsFAs in marine oils (**Paper II**). The challenges associated with the quantification of novel arsenic species in a gradient HPLC-ICP-MS analysis was addressed using three arsenic-containing compounds as external calibration standards. Dimethylarsinate was best suited as a calibration standard compared to Ph<sub>3</sub>AsO and the synthesized AsHC-C<sub>19</sub> (**Paper II**). The AsHCs; The AsHC-C<sub>15</sub>, AsHC-C<sub>17</sub> and AsHC-C<sub>21</sub> were the major arsenic species in the methanol phase of all marine oils analysed, accounting for 69-92% of the total arsenic in the

methanol phase. The AsFAs; AsFA-C<sub>21</sub> and AsFA<sub>22</sub> were identified as minor arsenic species in the methanol extracts of the oils, with the exception of the salmon oil (**Paper II**). Furthermore, several unidentified arsenic-containing peaks were detected in the marine oils (**Paper I-II**).

The chemical structures of the AsHCs were identified in commercial fish oils using GC-MS/MS, that provided retention times correlating to the GC-ICP-MS analysis, as well as fragmentation patterns of compounds (Paper I). The accurate masses of the AsHCs were verified using qTOF-MS analysis (**Paper I**). The chemical structures of the AsHCs and AsFAs were identified in marine oils and in oil of cod liver using HPLC-qTOF-MS, which provided retention correlating to the HPLC-ICP-MS analysis, as well as the accurate masses of compounds and the fragmentation patterns of the compounds, which support the identification (**Paper II-III**).

The arsenolipids extracted from the liver of Northeast Arctic cod were partitioned into a lipid- and a water-fraction, and total arsenic was measured in both fractions. A positive correlation was observed between total arsenic concentrations of liver (from 2.1 to 240 mg kg<sup>-1</sup> ww) and of the lipid-fractions (from 1.8 to 16.4 mg kg<sup>-1</sup> oil). The proportion of lipid-soluble arsenic decreased with increasing arsenic levels of whole liver, indicating a threshold for the accumulation of lipid-soluble arsenic (**Paper III**). The lipid-fraction of the cod livers were analysed by the reversed-phase HPLC-ICP-MS and HPLC-qTOF-MS approach (**Paper III**). Up to three AsHCs; AsHC-C<sub>15</sub>, AsHC-C<sub>17</sub> and AsHC-C<sub>21</sub> and five AsFAs; AsFA-C<sub>15</sub>, AsFA-C<sub>17a</sub>, AsFA-C<sub>17b</sub>, AsFA-C<sub>21</sub> and AsFA-C<sub>22</sub>, were identified in the methanol phases of the oils of the cod liver. Of the water-soluble arsenic species, AB was the dominant species, whereas DMA and AC were minor constituents in cod liver (**Paper III**).

## 5. Future perspectives

### *Analytical consideration- Extraction recovery*

The oil matrix is a major challenge in the analysis of arsenolipids. In the present work a maximum extraction recovery of 60% of the total arsenic present in the oils was achieved (**Paper I-III**), which emphasizes the need for more efficient sample extraction methods. A LLE of fish oil, using other solvents than acetonitrile and methanol, may result in a more selective extraction of arsenolipids. Also, a longer solvent interaction time may increase the extraction recovery of arsenolipids in polar solvents. A SPE procedure that extracts the arsenolipids from whole oil will be an approach that typically requires less sample and solvent volumes than LLE, and may also provide a increased extraction recovery and more efficient separation of analytes from interferences. SPE columns with functional groups, *e.g.* alumina-based may prove to be more selective for the arsenolipids than pure silica columns, as they possess different chemical properties. The synthesised AsHC-C<sub>19</sub> standard may be used for optimising a SPE method for the arsenolipids as the standard is structurally similar to naturally occurring AsHCs. The synthesised AsHC-C<sub>19</sub> may also be a potential internal standard in an extraction procedure of arsenolipids, improving the repeatability in a LLE and a SPE procedure.

### *Analytical consideration- GC-ICP-MS*

The quantitative results for the AsHCs when analysed by GC-ICP-MS may be improved using another calibration standard instead of Ph<sub>3</sub>As (**Paper I**). The synthesized AsHC-C<sub>19</sub> is a potential calibration standard for the quantitative analysis of AsHCs in GC-ICP-MS. Another improvement in the GC-ICP-MS method may be achieved by changing the transfer line, linking the GC and the ICP-MS, with a transfer line that tolerates more than 300 °C. By doing so, the GC-ICP-MS can be

used for analysis of AsHCs that are potentially less volatile and sharpen the peak of the late-eluting AsHC-C<sub>21</sub>.

#### ***Analytical consideration- HPLC-ICP-MS***

The chromatographic resolution in reversed-phase HPLC-ICP-MS analysis (**Paper II-III**) of AsFAs and AsHCs may be improved using a different mobile phase constituting of *e.g.* acetonitrile instead of methanol. In the present work, the ICP-MS was, however, the limiting factor in the use of other organic solvents beside methanol.

#### ***Analytical consideration- identification of arsenolipids***

Several more non-polar arsenolipids were detected, but not identified, in the present work (**Paper II-III**). An improved sample extraction procedure for arsenolipids will remove interfering matrix components and concentrate the arsenolipids, which is facilitates a structural identification of novel compounds.

#### ***Analytical consideration- Standards and quality assurance***

In the present work an AsHC standard was synthesised (**Paper II**). Synthesis of several products was limited by safety issues. Commercially available standards of arsenolipids are needed for method development and optimisation of methods, but also for quality assurance. There is also a need for certified reference materials for arsenolipids, as this is essential for the quality assurance of analysis and methods.

#### ***Arsenolipids in the whole chain***

In the present study arsenolipids have been characterised in commercial fish oils. However, little is currently known about the presence of arsenolipids in complete feed. Future work should therefore focus on the presence and distribution of arsenolipids in fish feed. Furthermore, studies of the carry-over of arsenolipids from feed to the farmed fish will provide information on the accumulation, distribution, metabolism and elimination of arsenolipids in fish. Such studies are important in regards to both feed and seafood safety.

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