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Detection and Quantification of Arsenolipids in Human Breast Milk by HPLC-ICP-MS and HPLC-ESI-MS - Method Optimization to Enable Quantification of Six Arsenolipids at Low Concentration Levels

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Abstract

Recent research has shown that some arsenic containing lipids, common components of seafood, are highly cytotoxic and have the potential to cross the blood-brain barrier. Infants, in particular, are especially sensitive to exposure due to the critical stage of development of the brain and nervous system. Thus, the aim was to optimize a method to enable identification and quantification of arsenic containing lipids in 21 breast milk samples selected from the Norwegian Human Milk Study (HUMIS- NoMIC). Initial testing of 10 milk samples had shown that a significant proportion of the total arsenic occurred in lipid-soluble forms, and the major arsenolipids occurring in five of those samples were identified as arsenic hydrocarbons and arsenic fatty acids.

In the present work, the extraction solvent used, volumes used of sample material, solvents and reagents, and ruggedness in the sample purification procedure was tested in an attempt of optimizing the method before 21 additional breast milk samples were processed. By performing several spike recovery tests, it was confirmed that the intermediate precision was satisfactory. However, to obtain comparability and traceability it is essential to improve the reproducibility and find a way to obtain information about the accuracy.

The samples were analysed with high performance liquid chromatography coupled with a splitter to elemental and molecular mass spectrometers to determine both molecular structures and concentrations of the arsenolipids. The concentrations of arsenolipids in the human breast milk samples were low (<1.0 µg As/L), and fell below the limit of quantification (LOQ) in most of the samples. However, the presence of four out of six arsenolipids were confirmed. Additional method optimization is required to obtain reliable information on the concentrations of these compounds, by finding the conditions that provides quantitative measurements with an acceptable uncertainty at a sufficient low LOQ.

As a new approach to obtain recovery information, preparation of an arsenolipid internal standard by methylation of standard, purified milk samples, and raw milk samples were attempted. This approach showed some promising results with deuterium labelled methylated internal standards, but further work is essential to develop a reliable internal standard for the speciation of arsenic containing lipids.

Sammendrag

Nyere forskning har vist at noen arsenholdige lipider, som er naturlig forekommende forbindelser i fisk og sjømat, er svært cytotoksiske og kan potensielt krysse blod-hjerne barrieren. Spedbarn er spesielt sensitive for eksponering for disse forbindelsene da utvikling av hjerne og nervesystem er på et kritisk stadie så tidlig i livet. Målet med arbeidet var derfor å optimalisere en metode for å muliggjøre identifikasjon og kvantifikasjon av arsenolipider i 21 morsmelkprøver fra den norske morsmelkstudien, HUMIS-NoMIC. Et tidligere forsøk med 10 melkeprøver viste at en signifikant andel av total konsentrasjon av arsen er fettløselig, og ble identifisert som arsenholdige hydrokarboner og fettsyrer i fem av disse prøvene.

I dette arbeidet ble bruk av ekstraksjonsmiddel, volum benyttet for prøver, reagenser og løsninger, samt prøveprepareringens robusthet testet i et forsøk på å optimalisere den nyutviklede metoden. Ytterligere 21 melkeprøver ble behandlet og analysert. Som et valideringsverktøy ble flere spike-recovery forsøk gjennomført hvor presisjon mellom replikater var tilfredsstillende. For å oppnå sammenlignbarhet og sporbarhet, er det essensielt å forbedre reproduserbarheten og å finne en måte å bestemme nøyaktighet.

Prøvene ble analysert ved bruk av HPLC koblet med en splitter til både elementær og molekylær spektroskopi for bestemmelse av konsentrasjon og molekylær struktur av arsenolipidene. Konsentrasjonen av arsenolipidene i morsmelken var lave (<1,0 µg As/L) og falt under kvantifiseringsgrensen (LOQ) for de fleste arsenolipidene av interesse i de fleste prøvene. Tilstedeværelse av fire av seks arsenolipider ble likevel bekreftet. Videre optimalisering av metoden er nødvendig for å kunne oppnå pålitelig informasjon om arsenolipidenes konsentrasjoner, noe som lar seg gjøre ved å finne betingelser som gir kvantitative målinger med akseptabel usikkerhet ved en tilstrekkelig lav kvantifiseringsgrense.

Som en ny fremgangsmåte for å bestemme gjenfinning, ble det forsøkt å lage en internstandard for disse arsenolipidene ved å metylere syntetiserte standarder, renset melk og urenset melk. Forsøkene med deuterium merkede, metylerte internstandarder var lovende, men videre arbeid er essensielt for å utvikle en pålitelig internstandard for bruk i forsøk som omhandler spesiering av arsenholdige lipider.

Preface

All of the experimental work was performed at the institute of analytical chemistry at the University of Graz, Austria. When working in Graz, I was a part of a research group called TEM group (Trace Element Metabolomics), and most of my work were performed in the TEM lab. Metabolomics is the study of chemical processes in biological systems by investigating the metabolic profiles of organisms. The TEM group focus on trace element metabolomics with methods based on HPLC and GC separations coupled with elemental and molecular mass spectrometry to provide quantitative metabolic profiling of compounds containing essential and toxic trace elements (e.g. Se, As, Cd, Hg). In the experiments using the Q-Exactive HR mass spectrometer, the Central Lab were used. The Central Lab is a joint venture between research groups at the Technical University Graz and the University of Graz. More information about the research groups and their research can be found at the university website: https://chemie.uni-graz.at/en/analytical-chemistry/.

Kevin A. Fransesconi was my supervisor in Graz, and helped with everything from settling in, planning and guiding me through the experiments and interpret the results. Kevin A. Fransesconi is a Professor in Analytical Chemistry at the Institute of Analytical Chemistry at the University of Graz. His research is focused on the development and application of analytical and chemical methods for studying fundamental processes of transformation of metals in biological systems.

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Ås, may, 2018

Abbreviations and definitions

NMBU	Norges miljø- og biovitenskapelige universitet/ Norwegian university of life sciences
FHI/NIPH	Folkehelseinstituttet/ Norwegian Institute of Public Health
As	Arsenic
iAs	Inorganic arsenic
AsFA	Arsenic containing fatty acid
AsHC	Arsenic containing hydrocarbon
AsPC	Arsenic containing phosphocholine
DMA	Dimethyl-arsenic-acid
TFA	Trifluoroacetic acid
HNO3	Nitric acid
H2SO4	Sulfuric acid
MTBE	Methyl-tert-butyl-ether
DCM	Dichloromethane
MeOH	Methanol
EtOH	Ethanol
CH3-I	Iodomethane
CD3-I	Deuterium-labeled iodomethane
HPLC	High Performance Liquid Chromatography
ICP-MS	Inductively Coupled Plasma – Mass Spectrometer
Single Q	Single Quadrupole
QQQ	Triple Quadrupole
ESI-MS	Electrospray ionization - Mass spectrometer
HR-ESI-MS	High Resolution ESI-MS
LOD	Limit of Detection
LOQ	Limit of Quantification
SD	Standard Deviation
RM	Reference Material
CRM	Certified Reference Material
Internal standard	Known compound with known concentration added to the sample in the beginning of sample preparation. The compound can only be

naturally present at negligible concentrations in the sample matrix

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1 Introduction

1.1 Background

Arsenic (As) is a naturally occurring element and is a common constituent in the earth's crust in minerals such as arsenopyrite and realgar (Cullen & Reimer, 1989). Arsenic is a global contaminant from anthropogenic sources such as coal combustion and runoff from mine tailings (Oremland & Stolz, 2003) and from natural sources such as volcanic activity and weathering of minerals (Mandal & Suzuki, 2002). The major concern is inorganic arsenic (iAs), which has known toxicity and is present in some foods, especially rice (Molin et al., 2015), and drinking water, where the greatest range and highest concentrations are found in ground water (IARC, 2004). The lipid-soluble arsenic-containing compounds, mainly found in fish and seafood (Molin et al., 2015) has lately become a field of interest due to recent investigations demonstrating cytotoxicity and potential to cross the blood-brain barrier of some of these compounds (Meyer et al., 2014b; Niehoff et al., 2016). Due to the critical stages of development of brain and nervous system in infants, knowledge about these lipid-soluble compounds and their effects are of great interest in risk assessment of arsenic.

The Norwegian HUMIS-NoMIC study is a prospective population-based birth cohort consisting of mother-child pairs established with the purpose of studying environmental toxicants in human milk and their relation to children's health (FHI, 2017). A recent study by Stiboller and co-workers (2017) reveal the presence of arsenolipids in the Norwegian breast milk.

1.2 Objectives for this thesis

The work by Stiboller et. al (2017) is so far the only work published on speciation of arsenic containing lipids in human breast milk. A series of arsenolipids were detected in the breast milk collected from the Norwegian mothers. Of the detected arsenolipids, the hydrocarbon AsHC 332 (figure 2.1) is especially considered to be toxic after a recent study showed accumulation of this compound in the brain of the *Drosophila melanogaster* (Niehoff et al., 2016), the species known generally as the common fruit fly or vinegar fly. To be able to give the best recommendation of diets, it would be valuable to obtain information about the amount of arsenolipids excreted through breast milk and their effects in infants. To achieve information like this, it is necessary to

have a method developed that enables quantification of arsenic-containing lipids at very low concentrations in a complex matrix rich on lipids. Finding the right parameters for sample preparation and the right conditions on the instrumental analysis is thus important to achieve satisfactory sensitivity and accordingly concentrations levels above the limit of quantification (LOQ). Using the work of Stiboller et al. (2017b) as a basis, the present work aimed on testing different parameters for the sample preparation in an attempt to optimize the method to enable quantification of arsenolipids in human breast milk.

The milk was purified and subsequently analyzed for identification and quantification of six different arsenolipids by means of HPLC coupled to ICPMS and ESI-MS. The attempt of improvements in the sample purification procedure were planned sought by testing and comparing changes of volumes used of sample material, reagents and solvents and change of extraction solvent. The ruggedness of more complex arsenolipids was also planned tested to determine the effect of different concentrations of trifluoroacetic acid (TFA) on degeneration of complex arsenolipids. In addition, a new approach for method validation was attempted by preparing a suitable internal standard of the arsenolipids using methylation experiments. As a method validation tool, several spike-recovery experiments were planned in order to obtain information on analyte recovery and precision. The ultimate goal was to obtain a reliable method, technically appropriate for the intended purpose, to be able to detect, identify and quantify arsenic-containing lipids at very low concentrations in human breast milk.

2 Theory

2.1 Arsenic

Arsenic (As) is element number 33 in the periodic table and is characterized as a metalloid. Arsenic can exist in four oxidation states -III, 0, III and V, where V is the most dominant under normal, oxygenated environmental conditions. In addition, the majority of As species found in organisms and foods are present in oxidation state V. The chemistry of arsenic resembles the chemistry of the other elements in group 15, such as nitrogen and phosphorus. These similarities results in chemical species of arsenic being structurally similar to chemical species of e.g. phosphorus. For example, arsenate (AO_4^{3-}) and phosphate (PO_4^{3-}) are structurally indistinguishable to marine algae and results in uptake of arsenate that is processed and brought in to the food chain. Arsenobetaine is structurally similar to glycine betaine, which is used by aquatic organisms to obtain osmotic balance under conditions where the salinity is varying (Molin et al., 2015).

2.1.1 Effect and toxicity of arsenic

The effect and toxicity of arsenic depends on its chemical species, thus the oxidation state and its chemical binding form is crucial for the toxicity of arsenic. The inorganic arsenites and arsenates are known to be highly toxic and are related with carcinogenic diseases. These compounds are also associated with other diseases such as skin lesions, neurotoxicity, diabetes and disturbance of development of fetuses and infants (IARC, 2012). Inorganic arsenic (iAs) as well as methylarsonate (MA) and dimethylarsinate (DMA) have been shown to inhibit mitochondrial respiration, forming reactive oxygen species, which may cause DNA mutations. Thus, it may have an impact on cancer development and cell death (Molin et al., 2015). Inorganic arsenic is methylated during metabolism, and this pathway has earlier been regarded as the detoxification pathway. Recent studies have suggested that the metabolites MA(III) and DMA(III) are reactive and highly toxic and thus may play a role in the mechanism of toxicity rather than being a detoxification pathway (Molin et al., 2015). The pentavalent DMA has shown genotoxic effect on cultured mammalian cells and studies in animals has reported that DMA(V) promotes carcinogenic effects in the bladder, kidney, liver ad thyroid gland. DMA(V) and MA(V) has been classified as possible carcinogenic to humans by IARC (2012).

Some organic bound forms of arsenic found in fish, such as arsenobetaine is classified as non-toxic and is not metabolized in the human body and excreted through urine (Kaise et al., 1985; Newcombe et al., 2010). In addition to the water-soluble organic arsenicals, fat-soluble arsenic species is also found in fish and seafood, and the study of their effect and toxicity in the human body has recently become a field of interest, due to fish being an important part of the diet for many people around the world. These compounds, referred to as arsenolipids, were recently found to be cytotoxic in some forms and their presence are confirmed in several marine samples such as fish oils (Amayo et al., 2013; Taleshi et al., 2008), cod (Arroyo-Abad et al., 2010; Arroyo-Abad et al., 2013), brown algae (García-Salgado et al., 2012), herring (Lischka et al., 2013), and tuna (Taleshi et al., 2010). The main compounds of concern are the arsenic containing hydrocarbons, AsHC's. This group of compounds is found to be cytotoxic with comparable effects with arsenite in an in vitro study with cultured human liver and bladder cells performed by Meyer et al. (2014a), where the toxicity of three hydrocarbons (AsHC 332, AsHC 360, and AsHC 444) were characterized. Another study performed by Meyer et al. (2014b), an *in vivo* study of the three arsenic containing hydrocarbons in the fruit fly *Drosophila melanogaster* revealed a toxicity of the hydrocarbons in a concentration range similar of arsenite. A study performed by Niehoff et al. (2016) also revealed the uptake and accumulation of arsenolipid AsHC 332 in the brain of *D. melanogaster.* This raises a concern of the possible neurodegenerative effect of this compound and thus the importance of researching these compounds.

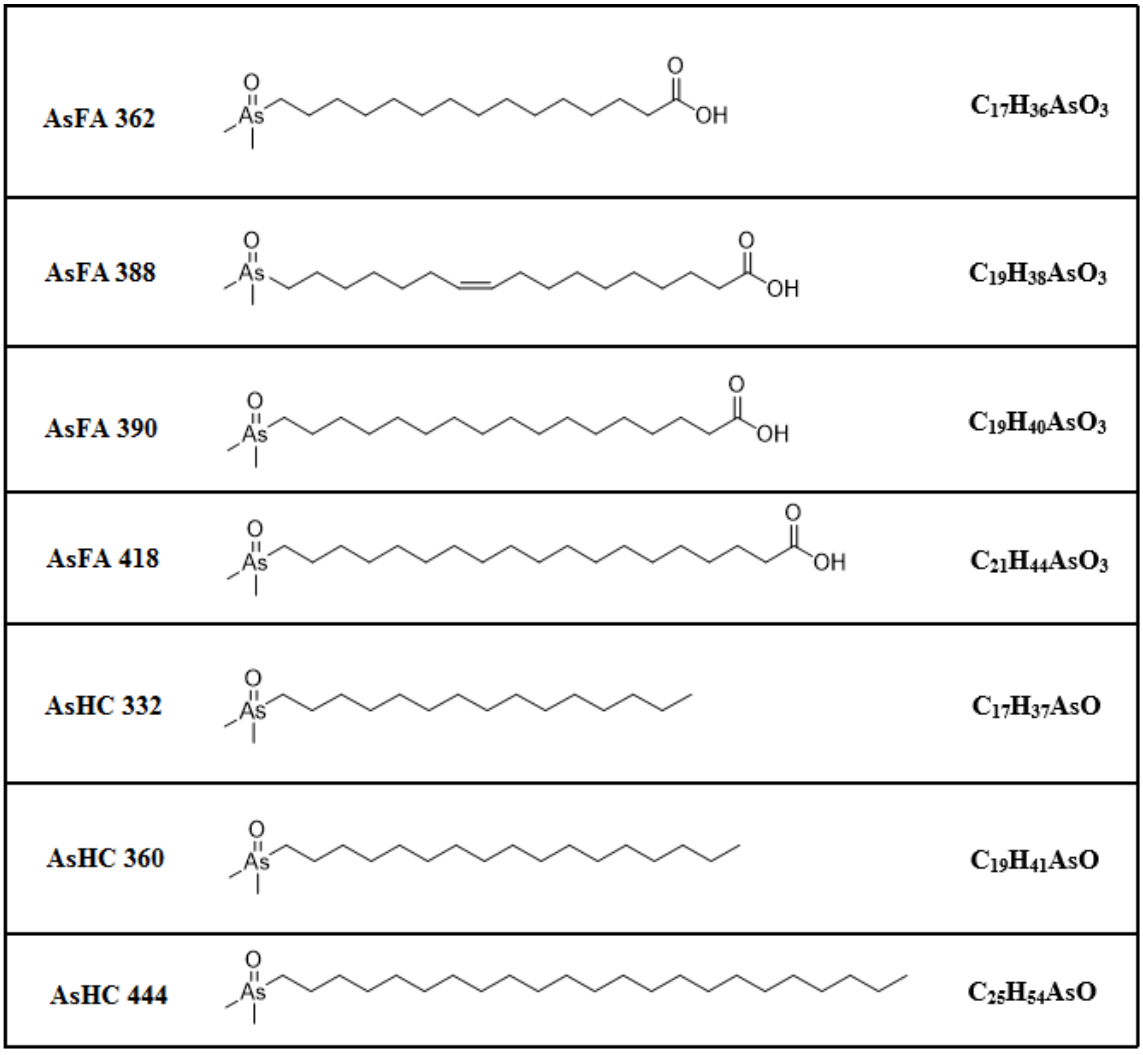

Figure 2.1 presents the six arsenolipids of interest in this study, with the ID used to describe them in this study, their molecular structure and their molecular formula. In addition, the Fatty acids, AsFA 390 is presented, due to difficulties to distinguish between AsFA388 and AsFA 390 with retention time and molecular mass spectrometry.

2.1.2 Sources and exposure of arsenic

Arsenic is both naturally occurring and distributed by anthropogenic sources. With an average concentration of 3 mg/kg in the earth's crust, arsenic is ranked as the 20th most abundant element, with over 200 existing minerals. Arsenic is often found in conjunction with Sulphur in minerals such as arsenopyrite (AsFeS) and realgar (As_4S_4) and weathering of the arsenic containing minerals and volcanic activity are examples of the natural sources (Cullen & Reimer, 1989; Mandal & Suzuki, 2002).

Smelter slag, coal combustion, runoff from mine tailings, hide tanning waste, pigment production for paints and dyes and the processing of pressure-treated wood contributes to the anthropogenic sources of arsenic found in the environment (Oremland & Stolz, 2003). Arsenic is also used in electronics, semiconductors, manufacturing of alloys and production of pharmaceuticals, dyes and leather preservatives (Ishiguro, 1992).

The main sources of exposure of As to humans are through drinking water and some foods. Inorganic arsenic species is the predominant form found in terrestrial food such as rice and in the drinking water. The concentrations of As in groundwater can be as low as <10 µg/L but can reach up to 5000 µg/L in some areas. Surface waters are also used as drinking water but does generally contain less As than groundwater. In the surface waters the conditions are oxygenated and arsenic is mainly found as arsenate, but in the ground water where the conditions are reducing, arsenite could be found as the dominant species (EFSA, 2009). The concentrations of iAs in food are generally low, but recent studies have indicated that exposure to iAs could have an effect on health problems at lower concentrations than previously assumed (Moon et al., 2013) and shows the importance of including exposure to low arsenic levels as a risk factor. Even though the food of terrestrial origin has generally low concentration of total As, there are some exceptions when it comes to plants that take up As from the soil through their roots. These plants, such as rice, and other plants absorbing As from the air through their leaves (e.g. tea) have higher concentrations of iAs. Cereal and rice based products have concentrations around 0.1 – 0.4 mg As/kg dry weight (Molin et al., 2015).
The organic arsenic-containing compounds are mainly found in fish and seafood. According to a review focusing on seafood arsenic, the major contributor to As in the diet is seafood. Most of the arsenic found in seafood are organic arsenic compounds, and the concentrations of iAs are generally low. There are some exceptions of high concentrations of iAs in marine algae (arsenate>60mg/kg) and blue mussels (concentrations ranging between 0,001 – 4.5 mg As/kg) (Molin et al., 2015).

2.1.3 Guidelines and regarding arsenic intake from food

Guidelines are goals set to strive to achieve and should be used where compliance currently is not enforced, whereas standards are achievable targets for water providers to comply with (Meharg & Raab, 2010).

Inorganic arsenic is a so-called ubiquitous, class 1 nonthreshold carcinogen with risk assessment based on linear dose-response. Current assumptions of risk assessment is that all exposure constitutes a risk. Until its proven that dose-response relationships derived from epidemiological studies conducted on highly exposed can be extrapolated to scenarios of low exposure, the linear dose-response model has to be assumed (EFSA, 2009; IARC, 2004)

The JECFA (FAO/WHO, 1983) derived a provisional maximum tolerable limit of 2µg/kg body weight for daily intake of inorganic arsenic. This limit were derived based on dose-response data for arsenic toxicity on a study performed by Grantham and Jones on arsenic-contaminated well-water, and the JECFA concluded that drinking water were the most likely source of exposure for arsenic resulting in health effects (EFSA, 2009). At the 33rd meeting it was presented that, organic arsenic from seafood needed a different consideration than the inorganic arsenic in water based on the findings of low toxicity and rapid metabolism of these compounds. Comparing the nutritious values of fish against the knowledge about presence of organoarsenicals, no recommendations were made to restrict the amount of fish recommended in the daily diet.

The World Health Organization (WHO) has had a focus on health risks of arsenic in drinking water since 1958 and has presented successive editions of international standards for drinking water (1958, 1963, 1971) and guidelines for quality of drinking water (1984,1993, 2004). Due to research showing significant health effects of arsenic, these guidelines and standards were presented with reviews of data leading to lowering of standard or guideline values (EFSA, 2009). In 2009 the guideline value was 10µg/L but this was only provisional due to scientific uncertainties and practical considerations such as limit of detection (LOD) and practicability and costs of removing arsenic from the drinking water. This guideline corresponds to approximately 0.3 µg/kg body weight if a 70kg adult is consuming 2 L water daily.

2.1.4 The development of arsenic speciation analysis: from the 1960's until today

In the 1960's and 70's Gulbrand Lunde studied arsenic and its lipid-soluble compounds and was the first to do it by using neutron activation analysis to generate data on the abundance of arsenic and other elements in marine and terrestrial oils.
Conventional techniques for analysis of lipids were used to study the chemical properties of the arsenolipids and based on these studies arsenolipids were suggested to be chemically similar to phospholipids (Lunde, 1968; Sele, 2014)

The identification of the first arsenolipid were done by Orita and Shibata, where the AsSug-PL958 from brown algae *U. pinnatifida* were isolated by use of chloroform and methanol following a partitioning with hexane/acetonitrile and preparative chromatography prior to analysis with gas chromatography coupled to mass spectrometry (Morita & Shibata, 1988; Sele, 2014).

In 2005 the first analysis of intact arsenolipid were reported by Scmeisser et. al. using a HPLC/ICP-MS system with modifications on the ICP-MS based on the approach described for analysis of phospholipids. The modifications stabilized the plasma and made it possible to handle the organic solvents, and with an acetone-based mobile phase fish oils were analyzed on normal phase HPLC/ICP-MS (Sele, 2014).

By partitioning cod liver oil between immiscible solvents of n-Hexane and aqueous methanol and further extract the methanol using preparative size exclusion and anion exchange chromatography, Rumpler et al. (2008) were the first to identify intact arsenolipids in cod liver oil by elemental and structural analysis on HPLC/MS-MS and HR-MS.

2.2 Instrumental theory

2.2.1 High-Performance Liquid Chromatography, HPLC

An HPLC system is based on chromatographic separation in a column where the carrier medium is a liquid solvent.

HPLC columns use close packing with small particle sizes to achieve sufficient column efficiency. These particles produce a high resistance to fluid flow, and use of high-pressure pumps are necessary to obtain sufficient flow of the mobile phase. The high pressure needed in HPLC is one of the reason for the development of high-performance columns. A basic HPLC system is made of following components: A pumping system, a sample-injection system, a column, temperature control of the column, and a detector system (Skoog et al., 2007).

HPLC systems can be managed on a modular basis in which the user can connect different modules, in example column and detector, depending on the analytical requirements.
When using two or more solvent sources to make the mobile phase it is possible to automatically change the proportions of individual solvents in the carrier medium during the run, this is called gradient elution. Gradient HPLC is very useful for developing new methods as it gives the analyst an additional experimental parameter, namely the solvent concentration, which can be adjusted to obtain optimized separation. However, the reproducibility of a gradient method is not as good as an isocratic method, where an unchanging solvent mixture is delivered, because the retention times may be very sensitive to small variations in the gradient-proportioning profile (Skoog et al., 2007).

The pumping system is required to provide a sufficient pressure to get the fluid through the resistance of the column at a determined flow rate. The flow rate is determined by the physical speed of the pistons used in the pumping system. The pressure caused by the resistance of the column is called the backpressure and the pump must have sufficient power to maintain this pressure when providing the flow. For a column with a low resistance to flow, the back-pressure will be low (Skoog et al., 2007).

The sample is injected between the pump and the column. Most injection-systems use a sampling loop system, where a rotating valve allows a short length of tubing to be switched into the path of the solvent for introducing of the sample. The sample is introduced to this loop while the loop is out of the solvent flow, and injection is done by switching the loop into the solvent path. The injection system is automated with an auto sampler which draws the sample from a set of vials in a rack or a carousel and passes it to the sampling loop (Skoog et al., 2007).

2.2.2 Inductively Coupled Plasma – Mass Spectrometry, ICP-MS

Inductively coupled plasma – mass spectrometry has been the most important technique for elemental analysis since the early 1980's because of its low detection limits, high degree of selectivity and reasonable good precision and accuracy (Skoog et al., 2007).

Using an ICP-MS gaseous, liquid and solid samples could be introduced to the system with different devices, and the sample introduction has an important role in production of ions. Introduction of gaseous solutions could be performed by hydride generation, direct introduction along with an injector or outer gas flow or by chromatographic methods (Gas chromatography or supercritical fluid chromatography). Introduction of solid samples could also be performed by several techniques such as electrochemical evaporizers, Arc and Spark chambers, direct laser ablation and slurry nebulization. The most common method for injection of samples to plasma is however liquid sample introduction, where liquid samples are converted into aerosols and transferred through a spray chamber to condition and remove the larger aerosols before introducing the aerosols small enough to the plasma. The most commonly used sample introduction devices are pneumatic nebulizers (such as cross flow and concentric nebulizers) and ultrasonic nebulizers (Montaser, 1997).

After passing the nebulizer generates aerosols and passes them through the spray chamber, the fine droplets are introduced to the plasma. The plasma is generated in an ICP torch located inside the load coil from the RF generator. Argon gas is flushed through the torch and by application of RF power the plasma immediately ignites (Montaser, 1997). It is in the ICP torch where the fine aerosol droplets are atomized and ionized before they are transferred further into the system through the interface. The interface that couples the ICP torch and the mass spectrometer is a

critical part of the instrument due to the large differences in the pressure from atmospheric pressure in the torch to a pressure less than 10^{-4} torr in the mass spectrometer. This coupling is made possible by a differentially pumped interface coupler consisting of a sampling cone (water-cooled nickel cone with an orifice <1.0mm in the center). A mechanical pump maintains a pressure of 1 torr in the region behind the cone where the hot plasma gas is transmitted and cooled, resulting in expansion of the gas. A fraction of the gas passes through a second cone (called skimmer cone) and into a chamber maintained at the same pressure as the mass spectrometer. A negative voltage separates the positive ions from molecular species and electrons and accelerated and focused by a magnetic ion lens onto the entrance orifice of a quadrupole mass analyzer (Skoog et al., 2007)

Quadrupole-based systems represent around 95% of all ICP-MS used today, and the quadrupole technology is considered to be a very mature, routine, high-throughput trace element technique.

2.2.3 Electrospray Ionization – Mass Spectrometry, ESI-MS

Electrospray ionization – mass spectrometry (ESI-MS) was first described in 1984 and has since become an important technique for analyzing biomolecules and for characterization of inorganic species and synthetic polymers (Skoog et al. 2006). Electrospray ionization is performed under atmospheric pressure and temperatures where a solution is pumped with only a few microliters per minute through a stainless steel capillary needle. A cylindrical electrode that surrounds the capillary needle maintains the needle at several kilovolts that results in a charged spray of fine droplets. These droplets pass through a desolvation capillary where the solvent is evaporated and the analytes gets charged. Due to the evaporation of solvent, the droplets become smaller and the density of the charges increases until the surface tension no longer can support the charge. A coulombic explosion occurs and the droplets are torn apart into smaller droplets and the process is repeated until the output is a stream of positive or negative ions that gets accelerated into the mass analyzer (Skoog et al., 2007).

In this work, an Agilent 6460 ESI-MS was used, which has a triple quadrupole mass spectrometer. The mass analyzer consists of a quadrupole mass filter (Q1) followed by a collision cell and another quadrupole mass filter (Q3). The first quadrupole transfer selected ions

into the collision cell where they are fragmented before they are sent to the second mass filter where isolation and examination of multiple precursor to product ion transitions (MRM).

2.3 Method validation tools

A method should be validated when it is necessary to demonstrate that its performance characteristics are adequate for use for a particular purpose (Magnusson & Örnemark, 2014). The extent of validation depends on the application, the changes made in the method development and under which circumstances the method is to be used. There are several tools, which can be used to validate a method, such as blanks, routine test samples, spike-recovery, measurement standards and statistics.

2.3.1 Blanks

The use of blank samples makes it possible to estimate how much of the measured signal is caused by the actual analyte and how much signal is received due to noise. There is two types of blanks for this use, reagent blanks, and sample blanks. Reagent blanks undergoes the same analytical procedure as the rest of the samples and are analysed to determine if the reagents used for sample preparation contributes to the signal of the measurement. These blank samples are generally used to determine the methods LOQ. Sample blanks is blank samples of the same matrix as the samples for analysis, but without the analytes of interests present. When performing the analysis, analytical blanks are also measured, which is a sample of the same analytical matrix but without going through the sample preparation, also used as calibration blank (Magnusson & Örnemark, 2014).

2.3.2 Measurement standards

Measurement standards could be any kind of solution in which a particular parameter or property has been characterized to the extent it can serve as a metrological reference. When using the term it is important to clarify whether it is standards used for reference or standards used for calibration. Calibration standards, also known as calibrants or calibrators are standards of known concentration used for calibrating the instrument before analysis. Standards used for reference

(reference materials or certified reference materials) are used to control the method procedure and to control the calibration of the instrument.
It is important to distinguish between reference materials (RM's) and certified reference materials (CRM's) due to the different requirements for the degree of characterisation, metrological traceability, uncertainty and documentation. The requirements for the RM's are not as high as for the CRM's. By using a CRM consisting of the same matrix and with concentration in the same region as the samples which are analysed, it is possible to assess the bias (Magnusson & Örnemark, 2014).

2.3.3 Estimating the loss of analyte: Spike-recovery experiments

When analysing complex matrixes, it is a necessity to perform a clean up to transfer the analytes of interest from the complex matrix to a matrix suitable for the instrumental analysis. This clean up or sample preparation could include several steps of extractions which results in loss of analyte due to a significant proportion of the analyte remaining in the original matrix after extraction. When the transfer of analyte is incomplete, the measurement will give lower values than the true value of the analyte of interest in the samples measured. If these losses aren't corrected, it could be possible that results between laboratories will be significantly discrepant (Thompson et al., 1999). However, the use of recovery information varies among analytical chemists and laboratories, and without general guidelines there will be an additional uncertainty around the use of recovery information if some laboratories compensate for the losses and other do not (Thompson et al., 1999). The most important inconsistency of performing corrections on measurements is that it could eliminate the low bias due to loss of analyte, making the results less reliable.

Recovery information includes reference materials, isotope dilution, internal standards and spiking (Spike-Recovery). Spike recovery, which is a less expensive and a commonly applied approach to obtain recovery information, is to perform a separate or parallel experiment where the analyte is added as a spike. If a matrix blank sample is available, the spiking compound could be added to the sample prior to the preparation procedure. By preparing and analysing this spiked sample the same way as the other samples, the recovery of the analyte could be estimated (Thompson et al., 1999). The spike could also be added to an ordinary test portion as long as

unspiked test portions are analysed alongside the spiked samples. The added analyte may not come to effective equilibrium with the native analyte, this could result in an incorrect bias in a corrected analytical result (Thompson et al., 1999)

3 Materials and methods

3.1 Equipment, reagents, solutions and reference materials

An overview of the equipment used are found in table B.1, an overview of the reagents and solutions used is found in table B.3, and an overview of reference materials and control materials used in this study is shown in table B.2. All tables are found in appendix B.

3.2 Sample material

In addition to the test milk samples used in testing and the attempt of method optimization (Table 3.1), a sample series of 21 human breast milk samples were analyzed. The requirements for selection were a minimum total arsenic concentration of 0.5 µg/L and the milk samples were selected from the HUMIS-NoMIC study.

Table 3.1 shows an overview of the milk samples analyzed, their identification in the HUMIS NoMIC*, identification used in this thesis, and which experiments they were used in. the sample series of 21 milk samples are not presented in this table.*

Sample ID	Referred to as	Used in following tests
118427	Test milk 1	Total analysis Testing concentrations of TFA[a] Testing MTBE as extraction solvent Spike Recovery round 1,2, and 3
*	Test milk 2	Total analysis Spike recovery round 4
104679a	104679a	Testing the changes[c] made in the method
110733	110733	Testing the changes[c] made in the method

* no sample ID, test milk 2 were made by mixing 10 mL of 10 different milk samples from the sample series due to limited amounts of test milk 1. [a] Trifluoroacetic acid, [b] Methyl-tert-butyl-ether, [c] Changes made in the method: volumes used of sample material, reagents and solution and use of extraction solvent.

3.3 Method

3.3.1 Sample transfer

The milk samples were transported from minus 80 °C storage at FHI in Oslo, Norway, to Graz, Austria, in polypropylene vials on dry ice, and stored at minus 80 °C until analysis. Working in a clean room at the Institute of analytical chemistry at the University of Graz, samples were thawed and heated to 37 °C in a shaking water bath. Subsequently, aliquots of homogenized milk samples were transferred from the vials by means of 3.0 mL polyethylene pipettes, and weighed in on an analytical scale with a precision of 0.1 mg. For total As analysis the samples were transferred to 12 mL quartz tubes, and for sample preparation for speciation of As lipids the samples were transferred to 15 mL polypropylene tubes.

3.3.2 Sample preparation for total arsenic analysis

For the determination of total As the test milk 1 and 2 were analyzed. The total arsenic analyses were performed to determine if the test milk samples were homogenous before using them to perform the following experiments.

Six replicates of the milk were weighed accurately to about 1.00 g in 12.0 mL quartz tubes. In each tube 2.00 mL 66 % (w/w) HNO_3 and 1.00 mL 100 µg/L internal standard (IS) were added, giving a final concentration of 10.0 µg/L IS in the diluted samples after digestion. The nitric acid was added with a dispenser, and the internal standard was added with a calibrated 100 – 1000 µL manual pipette. An aliquot of 1.00 ml milliQ-water was weighed three times to check the calibration of the pipette.

In addition to the milk samples, six blank replicates were made by adding only the nitric acid and internal standard to the 12.0 mL quartz tubes. Accuracy was checked by analyzing three replicates of a reference material ClinChek® Serum Controls, lyophilized serum control for trace elements. The serum was weighed accurately to about to 0.1 mg in quartz tubes before the adding nitric acid and internal standard was added as described for the replicates of the milk samples.

A microwave assisted acid mineralization step was applied to the samples using a Milestone UltraClave III. Adding 3.00 mL 98 % (w/w) H_2SO_4 to 300 mL milliQ-water made the load. The program used for digestion was specially made for the sample matrix with a maximum temperature of 250 °C (the temperature profile is shown in figure A1 in appendix A).
The digested samples were diluted to 10.0 mL and analyzed on ICP-MS as described in chapter 3.3.6.

3.3.3 Sample preparation for speciation of arsenolipids in human breast milk

To be able to detect and measure the different arsenolipids in human breast milk, a sample purification was essential to transfer the analytes to a matrix suitable for analysis. A flow chart of the final method of sample preparation is shown in figure 3.1 and the process of purification of this sample preparation is described in detail in the section below. The tests performed to decide the parameters of sample preparation chosen in this work (figure 3.1, flowchart b) is described in the next chapter, 3.3.4.

Purification process:

Replicates of 5.00 g human breast milk were treated with 50.0 µL 10% (v/v) trifluoroacetic acid (TFA) and then attached to a rotating cross for one hour to precipitate the proteins in the milk. Lipid soluble arsenic species were extracted with 2.50 mL methyl-tert-butyl-ether (MTBE), added to the milk with a calibrated 100 – 1000 µL manual pipette. The sample material and MTBE were shaken well for extraction and centrifuged at 4500 rpm for 20 minutes at 20 °C in a Hettich Rotina centrifuge. The organic MTBE fractions were transferred with a calibrated 100 – 1000 µL manual pipette into new weighed 15.0 mL polypropylene tubes. The breast milk samples were extracted with MTBE one more round, giving a final organic fraction of 5.0 mL MTBE.

To wash the organic MTBE fraction, a back extraction with 2.50 mL MilliQ-water was done. The washed MTBE fractions were evaporated to dryness using a Christ RVC 233 CDplus vacuum lyophilizator and the yellow pale oil were dissolved in 5.00 mL n-hexane. Further purification consisted of a solvent partitioning where 2.50 mL MeOH/water (9+1 v/v) were added to the n-hexane fraction. The tubes were shaken well before centrifugation at 4500 rpm for 20 minutes at 20 °C. After centrifugation, the MeOH/water fractions were transferred to new, weighed 15.0 mL polypropylene tubes, before a new round of solvent partitioning were performed on the hexane fractions. The 5.00 mL MeOH/water fractions were evaporated to dryness and dissolved in 200 µL pure MeOH and transferred to 2.00 mL Eppendorf tubes. The samples were centrifuged in a SCILOX micro centrifuge for 20 minutes at 4 °C.

In some rounds of sample preparation, the last evaporation was not successful, and dryness was not achieved. To solve this challenge, 500 µL ethanol was added to the tubes before a new evaporation step was applied.

Sample preparation flow chart:

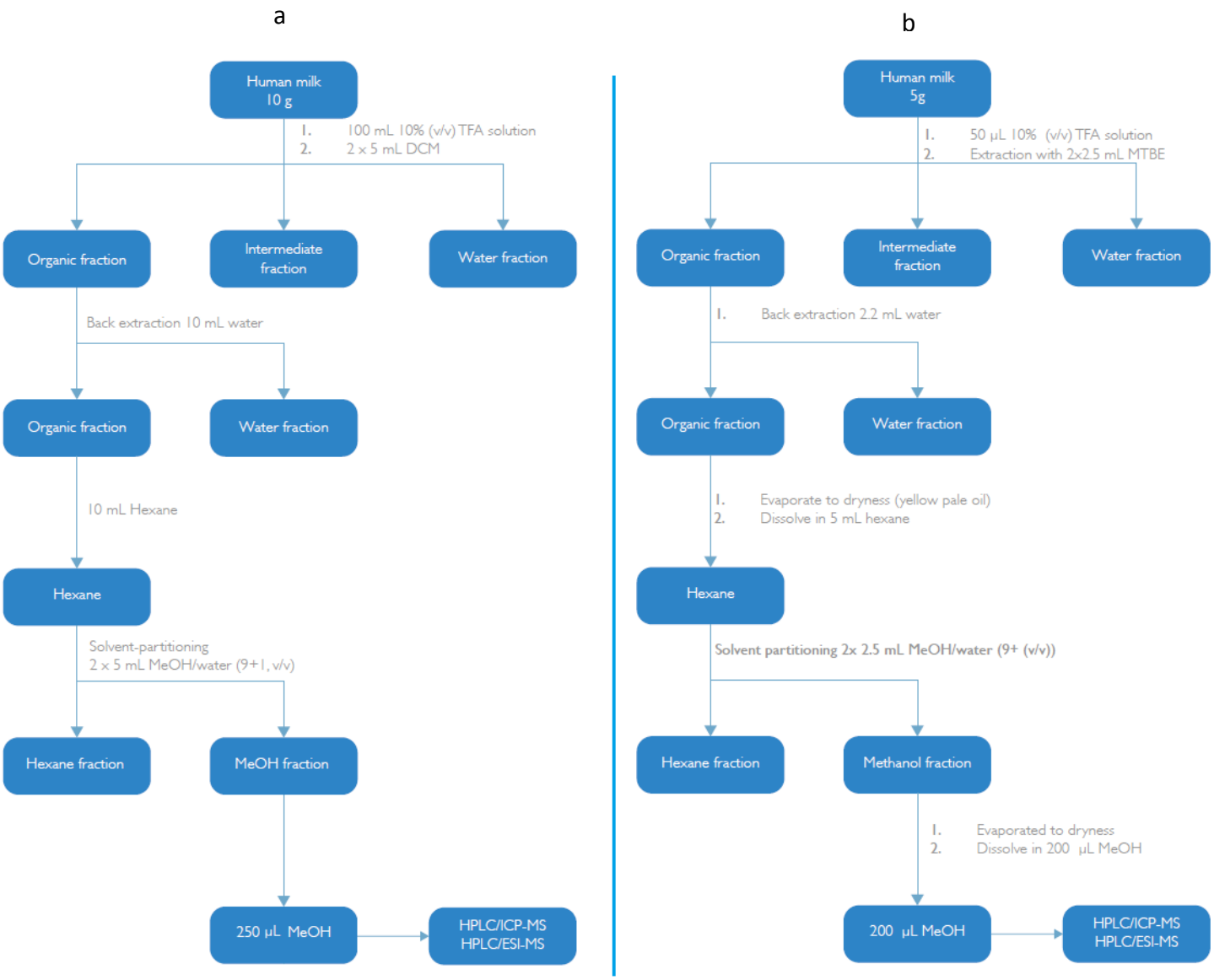

Figure 3.1 shows the sample preparation flow chart for purification of the lipid fraction from the milk. The original method developed by (Stiboller et al., 2017b) is presented on the left[a] and the final modified method is presented on the right[b]. Through the sample preparation with the modified method, a 25-fold increase in concentration is gained, making it possible to determine lower concentrations of As-lipids in the milk.

3.3.4 Testing of parameters in sample preparation method

The preparation flow chart on the right (b) in figure 3.1 is based on the one on the left (a) in the same figure and is the method of sample preparation used in analyses of the sample series of 21 human breast milk samples. Several experiments were performed to test different parts of the sample preparation method to optimize the method. This chapter presents the experimental work done during testing and optimization.

Changing the volume of sample materials, reagents and solvents

Primarily because of limited amounts of test milk available, it was early on decided to halve all volumes of samples, solvents, and reagents used in the sample preparation with one exception, the volume added of MeOH in the final step. In the original method the oil from the evaporated MeOH/water (9+1 v/v) were dissolved in 250 µL. Halving this volume could lead to difficulties of dissolving and analyzing, due to the volume being so low. Using the original volume of 250 µL would result in a lower concentration increase during sample preparation, thus this volume were decided to be 200 µL, giving a 25-fold increase in concentration through the preparation procedure.

Testing different concentrations of TFA, an evaluation of Ruggedness

Trifluoroacetic acid (TFA) was used as the first step in the sample preparation to precipitate the proteins before extraction of the lipid soluble arsenic species. To determine if the concentration of TFA could have an impact on degeneration of complex arsenolipids, which could affect the measured amount of arsenolipids of interest in the milk, experiments were performed with a synthesized arsenophosphocholine (AsPC 840oxo). Solutions of 100 µg/L of the AsPC-standard were treated with 1 % (v/v) TFA (10-fold higher than used in the sample preparation) and analyzed on a time based analysis at t= 0, 30, 60, 90, 120, 150, and 180 minutes on the HPLC/ESI-MS as described in chapter 3.3.7.

In addition to experiments performed with the standard solution of AsPC 840oxo, a set of three replicates of the test milk 1 were treated with 10% (v/v) TFA and heated in a water bath at 60 °C for 12 hours (in comparison to 0.1% (v/v) TFA, with one hour on the rotating cross). These samples were prepared following the sample preparation described in chapter 3.3.3, using

dichloromethane (DCM) as extraction solvent instead of MTBE and analyzed on HPLC/ICP-MS and HPLC/ESI-MS as described in chapter 3.3.7.

Using MTBE or DCM as the extraction solvent during sample preparation

A series of six replicates of the test milk 1 were spiked with 1.0 µg/L lipid standard (AsFA 362 and AsHC 332) where three of the replicates were further extracted with DCM and the remaining three were extracted with MTBE during the first step of the sample preparation. As a control, three replicates of the test milk 1 were extracted with DCM without any spike. The samples were analyzed on HPLC/ICP-MS and HPLC/ESI-MS as described in chapter 3.3.7. The recoveries and precision between the replicates extracted with the different solvents were then calculated and compared. Previous work with MTBE and practical benefits were also considered before deciding which extraction solvent to use in further work.

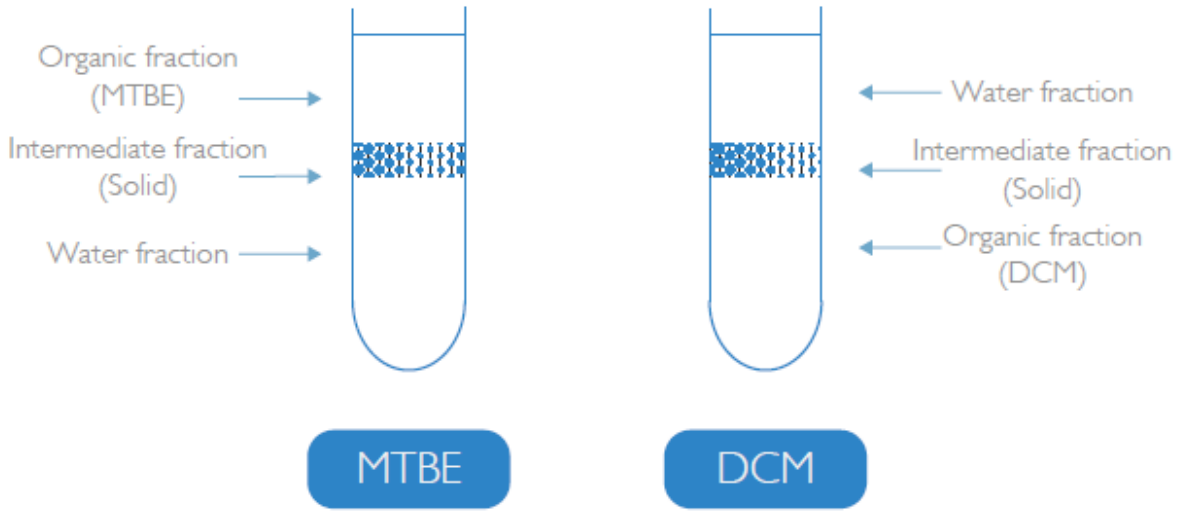

Figure 3.2 shows the difference between extraction with MTBE and extraction with DCM. The drawing is a recreation the phase distribution after the extraction step.

Estimating the loss of analyte during sample preparation with spike-recovery tests

Several spike-recovery experiments were performed to get information about loss of analyte during sample preparation.

As a parallel to the experiment with the samples heated with 10% (v/v) TFA, two sets of three replicates of the test milk 1 were spiked with 1.0 µg/L AsFA 362 and AsHC 332 standards and prepared following the sample preparation scheme, one set extracted with DCM and one set extracted with MTBE as described in the section above. A set of three replicates were prepared without spiking with any standards and used as a control.

After deciding to change extraction phase from DCM to MTBE, three additional spike-recovery experiments were performed. The first two rounds were performed with test milk 1, where sets of three replicates spiked with 1.0 µg/L AsFA 362 and 1.0 µg/L AsHC 332 were analyzed in addition to parallel sets of three unspiked replicates. The third experiment were performed with test milk 2 where three replicates were spiked with 0.5 µg/L of a mixed lipid standard containing AsFA 362, 388, 414 and AsHC 332, 360, 444.

3.3.5 Testing the changes made in the method on two human milk samples

Two of the milk samples with highest total arsenic concentration from the sample series were used to check the precision of the sample preparation method. A set of three replicates from each of the samples 110733 and 104679a were transferred and prepared following the sample preparation scheme b (figure 3.1) and analyzed on HPLC/ICP-MS and HPLC/ESI-MS as described in chapter 3.3.7.

3.3.6 Determination of total arsenic in human breast milk on ICP-MS

The analysis of the replicates of the test milk 1 was performed on an ICP-MS 8800 QQQ with help from laboratory assistant Jaqueline Rieger. An overview describing the preparation of the calibration standards and their respective concentration is found in appendix C
The instrumental parameters after tuning as well as the solutions used for analysis are listed in appendix D. The calibration standards, sample replicates, blank replicates, and certified reference material were analyzed the same day as the sample were prepared. An in-house reference

material made of As-containing water, and a NIST standard reference material 1640A Trace elements in natural water were used to control the accuracy in the calibration.

The second analysis, of the replicates of the test milk 2 was performed on an ICP-MS 7900 single quadrupole with help from Doctor Chan Xiong. An overview describing the making of calibration standards and their concentrations is found in appendix C. The instrumental parameters after tuning as well as solutions used for analysis are listed in appendix D Calibration standards, sample replicates, blank replicates, and certified reference material were analyzed a week after preparation. Reference material for control of calibration were not analyzed this round.

3.3.7 Speciation of arsenolipids on HPLC/ICP-MS, HPLC/ESI-MS and HPLC/ESI-HR-MS

All the speciation analyses were performed on an Agilent 1200 LC binary pump gradient system, connected with a splitter to both an Agilent 7900 ICP-MS and an Agilent 6460 Electrospray-MS with a flow ratio of 100 µL / 900 µL. All of the analyses were performed with help from Doctor Chan Xiong. An overview of the gradient system, mobile phases and column is presented in appendix C. The instrumental parameters and specification are presented in appendix D.

The calibration standards were prepared the same day as the analysis and to control the calibration, a 10.0 µg/L DMA standard was used, as well as a lipid standard. The standards were measured for 25 minutes and the milk samples were analyzed for 35 minutes. The DMA control was analyzed for 6 minutes, knowing that the retention time of this compound is around 2.5 minutes.

3.3.8 Preparation of Internal standard: Methylation of standards, purified and raw milk

Being able to use an internal standard is valuable, considering both that it is time saving and a more precise measure on the loss of analyte during sample preparation. The problem with the analytes of interest in this work, is that it is organic compounds and no internal standards are commercial available. An internal standard should have the same properties as the analyte of interest, at the same time not interfere and affect the results when analyzing the compounds.

An attempt of making internal standards of the AsFA 362 and AsHC 332 were done by experiments with methylation. CD_3-I was used to methylate the lipid standards to make the internal standard. Standards were also methylated with regular methyl-iodide to study the effectiveness of methylation to decide if this could be a possible approach. A series of standards and samples were methylated as described in the sections below and analyzed on an HPLC/ESI-HR-MS system to determine if methylation were effective and if methylation of raw milk before sample preparation was successful. The analysis was performed with help from Doctor Ronald Glabonjat and the instrumental parameters and specifications are listed in table D.7 and D.8 in appendix D.

Methylation of Standards:

A mixed standard of 10.0 µg/L AsHC332 and 10.0 µg/L AsFA362 was made to a volume of 1.00 mL from a stock solution of each compound. A fraction of the standard was transferred to a 1.00 mL HPLC vial for measuring.

A fraction of the standard was methylated by transferring 100 µL standard to a 250 µL HPLC vial and adding 10.0 µL CH_3-I and then 10.0 µL 2-mercaptoethanol. After adding the reactant and reducing agent, the vial was closed with a cap and the sample was heated in an oven at 60 °C for one hour before the sample was ready for analysis

Methylation of Purified milk:

Two replicates of milk sample 104679 and three replicates of milk sample 110733 were already purified as described in chapter 3.3.3 and analyzed on HPLC/ICPMS and HPLC/ESMS. The remaining sample volumes were used to study the effectiveness of methylation of purified milk samples. These samples were methylated as described for the standards above.

Preparation of Internal standard:

The methylated internal standard was made with the same mixed standard, by transferring 500 µL to a 15 mL polypropylene tube and adding 50.0 µL CD_3-I and then 50.0 µL 2-mercaptoethanol. After adding the reactant and reducing agent, the sample was heated in an oven at 60 °C for one hour. In order to remove any remaining reducing agent and reactant, the methylated standard was evaporated and re-dissolved in 500 µL MeOH.

Preparation of non-methylated standard used for spiking:

A mixed standard of 50.0 µg/L AsHC332 and 50.0 µg/L AsFA362 was made to a volume of 1.00 ml from a stock solution of each compound. The standard was prepared in a 15 mL polypropylene tube.

Preparation and methylation of raw milk:

Three replicates of about 5.00 g each of test milk 1, spiked with 1.00 µg/L of the lipid mix and 1.00 µg/L of internal standard were methylated by adding100 µL CH3-I, following 100 µL 2-mercaptoethanol before heating for one hour at 60 °C in an oven. After methylation the samples were prepared following the preparation scheme as normal (figure 3.1).

3.3.9 Calculating LOD, LOQ, and recoveries (%)

The Limit of Detection was calculated as three times the standard error of the intercept of the calibration curves, and the Limit of Quantification were calculated as ten times the standard error of the intercept. The standard error of intercept was calculated by using the LINEST formula in Excel with area as Y and standard concentration as X.

The recoveries (%) were calculated based on the added amount of spiked standard. If the recovery were complete, 100% of the added spike would be measured during analysis. The measured amounts of spiked standards were divided by the expected amount with 100% recovery. The mean recovery and standard deviation were then calculated to get information about the precision between the replicates and about the certainty of adjusting measured values considering loss of analyte during sample preparation.

4 Results and discussion

The small changes done to optimize the method developed by Stiboller et al. (2017b) gave a good precision between replicates in the spike-recovery experiments. This could make it possible to correct the values based on the loss of analyte during sample preparation. The problem however, was that the sensitivity was too low to obtain quantitative measurements of the low concentration levels of the arsenolipids in the human breast milk. In the following sub chapters, the results from test experiments and the attempt of quantification is presented and discussed.

4.1 Quality of analysis

For the total analysis, accuracy is determined by the use of a reference material, ClinCheck® - Control serum (level 1) for trace elements from Recipe ® (the results of the measurements of the RM is presented in table 4.1 in chapter 4.2) and by comparing several replicates measured (n=6) of both blanks and the same milk sample. The accuracy in the calibration of the instrument was controlled by an in-house reference of arsenic containing water and the NIST standard reference material 1640A Trace elements in natural water.

For the speciation analysis, there are no commercial available certified reference materials, which is a challenge for determining the accuracy of the analysis. To solve this challenge, spike-recovery experiments were performed in several of the test experiments in addition to the analysis of the sample series. The spike-recovery experiments provide information about the precision between replicates, and the sample preparation procedure. By performing several spike-recovery experiments, information about the intermediate precision was obtained. In addition, synthesized standards of the arsenolipids of interest were analyzed to confirm the match between the compounds and retention time (an example is presented in figure 4.1). To control the calibration with respect to the instrumental accuracy, a water solution of DMA was analyzed, making it possible to adjust the calibration. The results of the spike recovery experiments are presented and discussed in chapter 4.3.4.

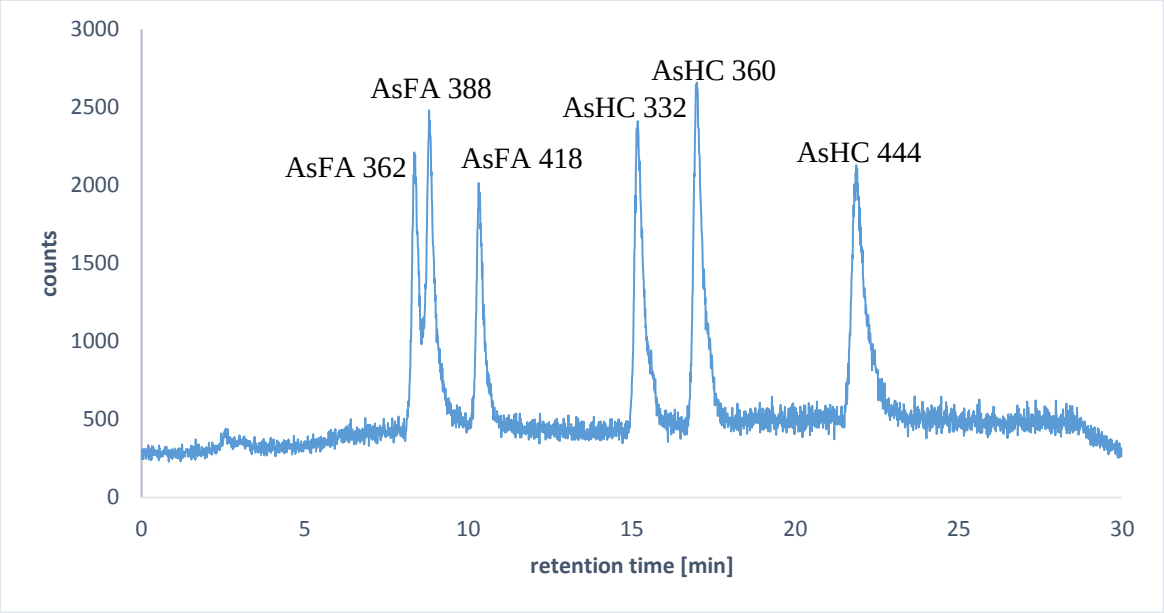

Figure 4.1 presents a chromatogram of a 10 µg/L mixed lipid standard solution used to control and confirm the match between compounds and retention time. The standard was also analyzed to confirm the presence of these compounds in the milk by comparing them in the ESI-MS.

Preparation of several replicates of reagent blanks or several replicates of one sample could give information about systematical or random errors in the method. This was not applied to the method due to time-consuming analysis. Bearing in mind that the speciation of arsenolipids with HPLC/ICP-MS and HPLC/ESI-MS is an analysis with low interferences and the probability of contaminating samples with As-lipids are low when the routine work is done properly and pipette tips and other equipment in contact with the sample matrix and standards are changed between samples. The fact that it is a time-based analysis of arsenic species with both elemental and molecular mass spectrometer, the amount of possible interferences is insignificant.

Analyzing one sample several times could give information of the instrumental precision. the difficulties with this is that the speciation analysis is very time consuming, since one sample is measured for 35 minutes to ensure that all lipids retained to the column are eluted before measuring the next sample.

4.2 Total arsenic in human breast milk

Stiboller et al. (2017b) performed total arsenic analysis in each fraction from the sample preparation (figure 3.1), which were not a focus in this work. The focus was the lipid soluble arsenic compounds obtained in the end of the sample preparation. The total analysis was performed to determine if the test milk 1 and 2 were homogenous and could be used to perform the speciation experiments. The results from the analysis of both test milk samples are presented in table 4.1.

Table 4.1 shows the average value of ^{75}As measured in the replicates of RM and test milk for both rounds of total As analysis. Round one represents the analysis of test milk 1 on the 8800 ICP-MS QQQ. Round two represents the analysis of the test milk 2 on the 7900 ICP-MS single-Q. The concentrations are presented in µg/L and mg/kg. The concentration of the reference material is only presented in µg/L due to the values being presented with µg/L in the document (RECIPE, 2015)

		Total Arsenic	
	Sample matrix (n*)	Concentration (µg/L)	Concentration (mg/kg)
Round 1	**Milk (6)**	0.56 ± 0.02	0.006
	RM, Serum (3)	9.90 ± 0.25	-
Round 2	**Milk (6)**	0.79 ± 0.04	0.008
	RM, Serum (3)	10.5 ± 0.63	-

*n number of samples

Comparing the measurement of As in the measured RM to the mean value and control range of As in the reference material document shows good correspondence. The values of the reference material, 9.9 ± 0.25 µg/L and 10.5 ± 0.63 µg/L correspond to the reported value of 9.87 µg/L and are within the control range of the RM. The standard deviations of the milk replicates were low and indicated homogenous test milk samples. Combined with good precision, low detection- and quantification limits and values close to zero in the blanks, this indicates that the method sensitivity is satisfactory and the method working range is adequate. The LOD and the LOQ are presented in table F.1 in appendix F.

4.3 Testing of parameters in sample preparation

The results obtained from the experiments presented in the following chapter were essential to determine which changes to make in the attempt of optimizing the method. All the values presented of arsenolipids in human milk in this chapter are uncorrected for the 25-fold concentration increase from sample preparation, as well as the loss of analyte (% recovery).

4.3.1 Changing volumes of sample materials, reagents and solvents

The decision of halving the volumes through the sample preparation were necessary due to limited amounts of test milk, giving the opportunity to perform several more experiments than if the original amount of 10.0 g milk were used for each replicate. If this limitation was not a fact, it could have been a better choice to use 10.0 g and an end volume of 250 µL instead of 5.0 g with 200µL as the end volume, since the first choice would give a 40-fold increase of measured concentration instead of 25-fold. A larger increase of concentration would increase the measured amounts of each lipid, making it possible increase the amount of values above the instrument's limit of quantification. Due to the time consuming analysis, the instrument's LOQ were used instead of the method's LOQ. Calculating the LOQ from the error of intercept in the calibration curve, rather than based on measurement of several blank samples, was a timesaving alternative and thus economic (saving solvents, gas, etc.).

The volume injected (20µL) in the instrument could also have been increased to achieve a higher signal during the measurement. This indicates that the volumes used throughout the experiment, both in sample preparation and during analysis are important when the goal is to be able quantify concentrations at low levels.

4.3.2 Testing different concentrations of TFA, an evaluation of ruggedness

The stability test conducted on a more complex arsenolipid, AsPC 840oxo, using different concentrations of TFA, showed no degeneration of the AsPC over time after treating with 1% (v/v) TFA (time-based analysis is presented in figure 4.2).

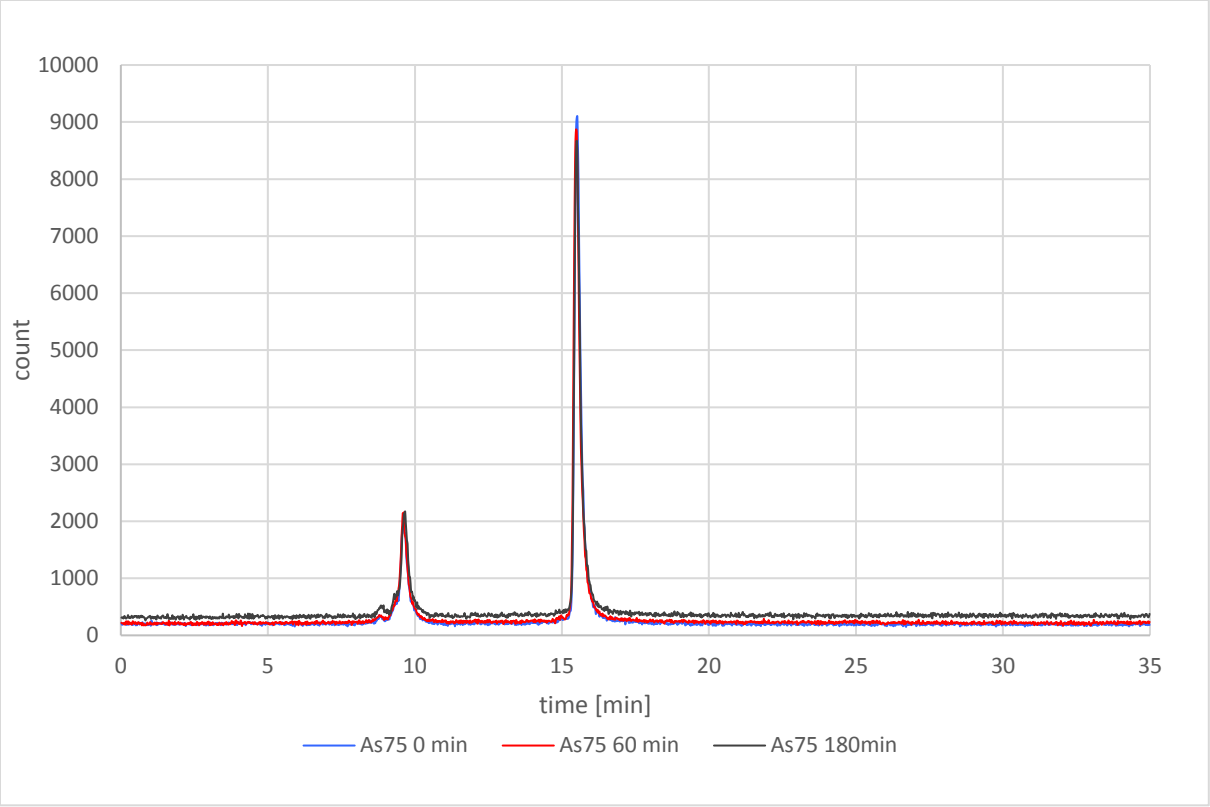

Figure 4.2 shows the results from the time based analysis with 100 µg/L AsPC dissolved in EtOH with 1% (v/v) TFA. At around 9 minutes, there is an unknown contamination. Just before the contamination, it is possible to see traces of the fatty acid generated from the AsPC. The peak from the AsPC is visible at 15.5 min. The blue line presents the results at t=0, the red line presents the results at t= 60 and the grey line presents the results at t=180 minutes. As shown by the chromatograms, 1% (v/v) TFA will not have any significant impact on degenerating the AsPC into less complex lipids.

After seeing no significant changes with 1% (v/v) TFA on degeneration of an AsPC 840oxo standard, three replicates of test milk 1 were treated with 10% (v/v) TFA and heated in a water bath at 60°C for 12 hours before continuing the sample preparation as described in chapter 3.3.3. All results were indecisive, i.e. < LOQ (Table 4.2).

Table 4.2 presents the concentrations (µg/L) of AsFA 362 and AsHC 332 found in the three replicates of test milk 1, treated with 10% (v/v) and 0.1% (v/v) TFA, respectively.

Sample matrix	AsFA 362 (µg/L)	AsHC332 (µg/L)
Milk (10% (v/v) TFA)	< 1,41[a]	< 1,99[b]
Milk (0,1% (v/v) TFA)	< 1,41[a]	< 1,99[b]

[a]= LOQ AsFA [b]= LOQ AsHC

The values of the untreated milk were just above the detection limits while the samples heated with 10% (v/v) TFA had values closer to the LOQ. Due to low concentrations and thus high uncertainty the values below LOQ were not presented, and degeneration of the complex arsenolipid cannot be confirmed.

The focus of performing stability-experiments with AsPC 840oxo standards were to determine the ruggedness of the method, by determining if higher concentrations of acid could degenerate more complex arsenolipids into fatty acids or hydrocarbons of interest in the study. If higher concentrations had an impact, it could also be of concern during digestion of the breast milk in the stomach of infants, bearing the gastric acid in mind. It would also give rise to an uncertainty of the measured values, resulting in an overestimating of the concentrations of the compounds measured in the milk. Giving that the results from the experiments with the synthesized standard did not show significant degeneration of the AsPC 840oxo treated with 1% (v/v) TFA, degeneration of complex lipids would not be of concern when precipitating proteins using 0.1% (v/v) TFA during the sample preparation.
When heating milk samples with 10% (v/v) TFA, the results showed a higher concentration of the compounds of interest, rising a question about degeneration of complex lipids could actually happen when the milk is digested in the stomach by the gastric acid. However, the concentrations were below the quantification limit, and are thus indecisive and cannot be used to conclude whether the acid concentrations have an impact or not. Thus, the method could be considered of satisfactory ruggedness.

4.3.3 Using MTBE or DCM as extraction solvent during sample preparation

MTBE and DCM were compared as extraction solvents for use in the sample preparation and the results are presented in table 4.3 where both the concentrations and the percentage recovery with standard deviation is given. The values presented have a large standard deviation and the extraction with DCM shows better recovery than the extraction with MTBE.

Table 4.3 presents the recovery of AsFA 362 and AsHC 332 added to three portions of milk (n=3) extracted with DCM and MTBE, respectively. Extraction with DCM shows higher recovery and higher uncertainty, while extraction with MTBE shows a lot lower recovery, but also lower uncertainty.

Extraction Solvent	AsFA 362	AsHC 332
	µg/L / % recovery	µg/L / % Recovery
DCM	16 ± 3 / 64 ± 12	14 ± 6 / 56 ± 24
MTBE	5 ± 2 / 20 ± 8	5 ± 2 / 20 ± 8

Even though the results showed better recoveries by use of DCM as extraction solvent, it was decided to change the extraction solvent to MTBE. This choice was made based on practical advantages, as MTBE forms the top layer and DCM is found as the bottom layer in the extraction. Bearing in mind that the precipitated proteins in the milk forms a solid pellet between the organic fraction and water fraction, it was easier to transfer the MTBE without any contamination from the solid intermediate fraction and water fraction (figure 3.2). Based on previous uses of MTBE as extraction solvent with results showing a faster and cleaner recovery of most of the major lipid classes (Matyash et al., 2008), in addition to the practical advances of MTBE (figure 3.2), it was decided to do further work with this solvent. The results from several performed spike-recovery experiments performed with MTBE showed much better recoveries than the first attempt (table 4.3 and 4.4), which strengthened the choice of switching extraction solvent.

4.3.4 Estimating the loss of analyte during sample preparation with spike-recovery tests

Several spike-recovery experiments were performed to verify if the precision between replicates were good enough to use as a measure of loss of analyte in the samples. The results showed good repeatability, but the intermediate precision was not satisfactory. The results of the first spike-recovery experiment is presented in table 4.3 and the results with percentage recovery and standard deviation for the three later experiments are presented in table 4.4

Table 4.4 presents the percentage recoveries of the three experiments described in chapter 3.3.4. Round 2 and 3 were performed with two lipid standards, and the last round was performed with a spike consisting of all the compounds of interest in the analysis of the sample series. All experiments were conducted with three replicates (n=3).

	Recovery ± SD (%)					
	AsFA 362	AsFA 388	AsFA 414	AsHC 332	AsHC 360	AsHC 444
Round 2	42 ± 1,1			48,5 ± 1,0		
Round 3	40 ± 0,12			58 ± 0,12		
Round 4	45 ± 0,61	53 ± 0,17	60 ± 0,7	69 ± 0,46	57 ± 2,4	21 ± 0,75

Looking at the results from the spike-recovery experiments it shows a variation of recovery between each experiment. This variation does not strengthen the reproducibility of the method, but the good precision between the replicates in every experiments makes it possible to use the method as long as one spiked and one unspiked set of replicates is implemented in the preparation of samples for analysis. The question that arises when performing these spike-recovery experiments is if correction of the measured values due to recovery should be done or if the values should be presented uncorrected. Thompson et al. (1999) have presented arguments both for and against correction of values with regard to the recoveries. Correcting the values could be an alternative when the recoveries are good and thus the corrections are significantly small. Uncorrected values due to low concentrations means that results will not be universally comparable or transferable and will therefore be unfit to support mutual recognition (Thompson et al., 1999). However, estimated recoveries could be higher than the corresponding values for the native analytes and the resultant corrected values would have a negative bias. The estimated correction factors could also be of doubtful applicability because they could have variations for

different concentrations of the analyte, and have a high relative uncertainty. The uncorrected values would normally have smaller relative uncertainty associated with volumetric and instrumental measurement alone. The loss of analyte would not be proportional with the concentration, when with low concentrations, a fraction of the analyte could be unrecoverable due to adsorption on surfaces and the loss of analyte would not be the same as estimated. With higher concentrations this would have a lower impact (Thompson et al., 1999). In this thesis, it was decided to leave the values uncorrected, due to the uncertainties around the effect on the trueness of the values from correction.

4.3.5 Testing the changes made in the method on two human milk samples

Milk sample 104679a had a total As content of 2.02 µg/kg and sample 110733 had a total As content of 2.03 µg/kg. These total As values along with the rest in table E.1 (appendix E) were obtained by Stiboller as a part of his work (Stiboller et al., 2017a; Stiboller et al., 2017b). Relatively high LOD and LOQ made it difficult to detect, identify and quantify the six arsenolipids of interest in the samples. The results from the analysis are presented in table 4.5.

Table 4.5 presents the average concentration (µg/L) with standard deviation of the six arsenolipids in three replicates of milk sample 104679a and 110733, respectively. Three of six compounds were detected in the analysis and only the AsFA 362 could be quantified in milk sample 104679a.

Sample (n=3)	AsFA 362 (µg/L)	AsFA 388[a] (µg/L)	AsFA 418 (µg/L)	AsHC 332 (µg/L)	AsHC 360 (µg/L)	AsHC 444 (µg/L)
104679a	5 ± 0,25	< 3,37[b]	< 0,71[c]	< 4,22[d]	< 0,98[e]	< 0,98[e]
110733	< 3,37[b]	< 3,37[b]	< 0,71[c]	< 0,98[e]	< 0,98[e]	< 0,98[e]

[a] Electrospray MS suggested AsFA 390 at the same retention time. [b]LOQ (AsFA) [c]LOD (AsFA) [d]LOQ (AsHC) [e]LOD (AsHC)

The chromatograms from the measured samples are presented in figures 4.3 and 4.4 where the first peak in both chromatograms is confirmed to be the AsFA 362. The electrospray suggested the AsFA 390 at the same retention time as the 388, making it difficult to confirm which of the compounds actually present in the sample.

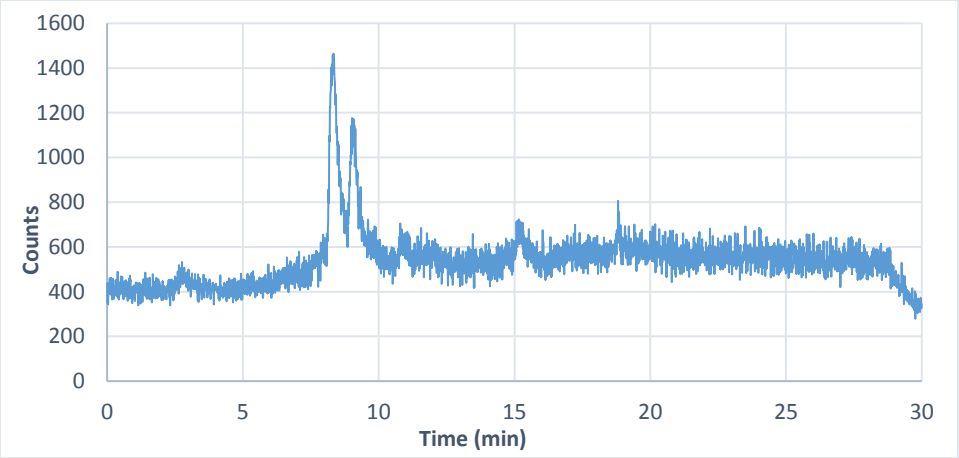

Figure 4.3 presents the chromatogram of one of the replicates from sample 104679a, where the counts are plotted against time of analysis (min). The two remaining replicates were close to identical to the presented chromatogram with no significant differences. Even though the LOQ is relatively high, the chromatogram shows two peaks correlating to the retention time of the two fatty acids AsFA 362 and AsFA 388, which strengthens the identifications and results indicating their presence in the milk.

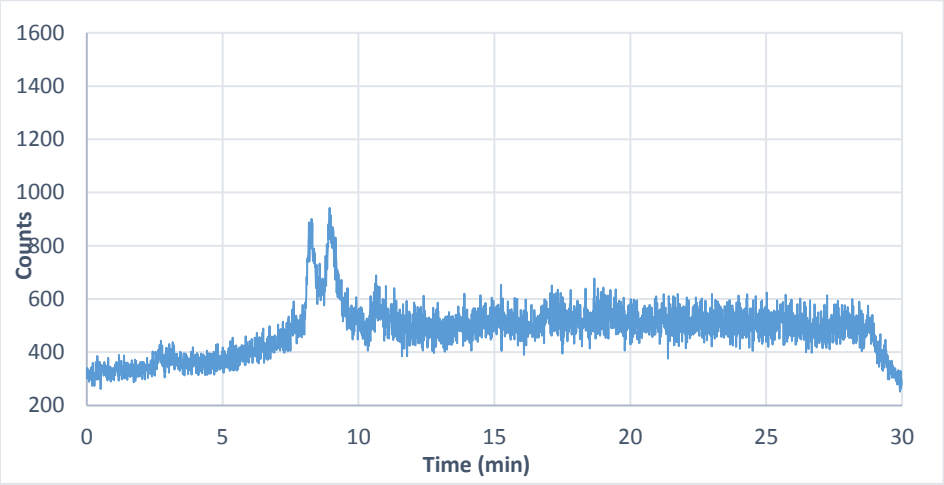

Figure 4.4 presents the chromatogram of one of the replicates from 110733, where the counts are plotted against time of analysis (min). The two remaining replicates were close to identical to the presented chromatogram with no significant differences. Even though the LOQ is relatively high, the chromatogram shows two peaks correlating to the retention time of the two fatty acids AsFA 362 and AsFA 388, which strengthens the identifications and results indicating their presence in the milk.

Looking at the chromatograms, the three replicates of each sample matched well, strengthening the precision between replicates during sample preparation. The main focus of the analysis was to see if the concentrations were quantifiable with the method and if the precision between replicates were satisfactory. Unfortunately, a set of spiked and unspiked replicates were not prepared and analyzed in this experiment, thus is not any information of the recoveries obtained in this test experiment. Bearing in mind that the recovery has varied in all experiments, it would not be correct to use recovery information from the earlier analyses. Thus it is not possible to compare the measured concentrations in this experiment with the measured values of the same samples in the analysis of the sample series. Having a spike-recovery set in this experiment could have given some information of the reproducibility of the method.

The information obtained by this experiment indicated that the method was not fully optimized, due to measured values being below the LOQ. However, without information about the recoveries and the precision, it was decided to continue with the slightly modified sample preparation and perform the analysis on the sample series of human breast milk in parallel to a spike recovery experiment, with a hope of quantifiable values of the arsenolipids.

4.4 Identification and quantification of arsenolipids in 21 human breast milk samples

Due to very low concentrations of arsenolipids in the human breast milk samples, the focus became mainly the detection and identification of the six different arsenolipids in the samples rather than quantification. Bearing in mind that the series only consists of 21 samples and most values were found to be below the LOQ and LOD, it would be of little use to perform statistical calculations due to the high uncertainty with values below LOQ. As with the test experiments, the presented results are the measured values after sample preparation, the 25-fold increase and loss of analyte during sample preparation is not corrected for. It would be incorrect to do the corrections of the values of LOD and LOQ the same way as the sample values because the LOD and LOQ are calculated from the calibration curve and not from blank replicates that went through sample preparation. Thus it was more presentable to use the uncorrected values.

4.4.1 Identification of arsenolipids in human breast milk

The focus was to identify the presence of six arsenolipids, three fatty acids, and three hydrocarbons (figure 2.1). These lipids were available as synthesized standards, and by analyzing a mixed standard containing the six compounds of interest, the retention time could be matched with the samples and with the molecular mass spectrometer the molecular structure of the compounds of interest could be confirmed in the mixed standard and thus in the sample series. In table 4.6 an overview of the detection of the six compounds in the sample series is presented. For each compound, the number of samples with values <LOD, >LOQ and between the limits are presented. The detection is confirmed for all measured values above LOD.

Table 4.6 presents the number of samples out of 21 in total, with values below and above LOD and LOQ for each compound of interest, where the number of samples with values above LOQ, between LOD and LOQ or below LOD is given for each compound.

As lipid	**Number (N) of samples**		
	N > LOQ	LOD < N < LOQ	N < LOD
AsFA 362	4	14	3
AsFA 388/390	2	13	6
AsFA 418	0	0	21
AsHC 332	0	7	14
AsHC 360	0	3	18
AsHC 444	0	0	21

N = number of samples.

The identified samples are shown in table 4.6 as samples with a positive number (N) of samples above LOQ or between LOD and LOQ. Two compounds, AsFA 418 and AsHC 444 could not be detected, due to not having any measured values above the LOD in any of the 21 samples. However, the presence of the 4 remaining compounds were confirmed by the molecular mass spectrometer, ESI-MS

Considering the low concentrations of the arsenolipids of interest in human breast milk, and insufficient method sensitivity, the focus became, as mentioned, to confirm the presence of the

compounds of interest in the 21 human breast milk samples rather than quantify them. Due to the knowledge about the concentration levels, it was known that quantification could be a challenge. After analysis, the presence of four out of six compounds were confirmed by HPLC/ESI-MS and by comparing the retention times of the measured compounds with the retention times of the mixed lipid standard. The two compounds not detected in the samples were the largest most un-polar of the compounds, AsFA 418 and AsHC 444. The detected compounds are more polar lipids with lower retention time in the column.

Confirming the presence of the analytes by HPLC/ESI-MS was done by looking at two measured fractions for each compound at their retention time. If both fractions were detected on the ESI-MS, the presence of the compound could be confirmed. Figure 4.5 shows an ESI-MS chromatogram of a sample spiked with a mixed lipid standard containing all six compounds of interest, where the matching peaks are circled.

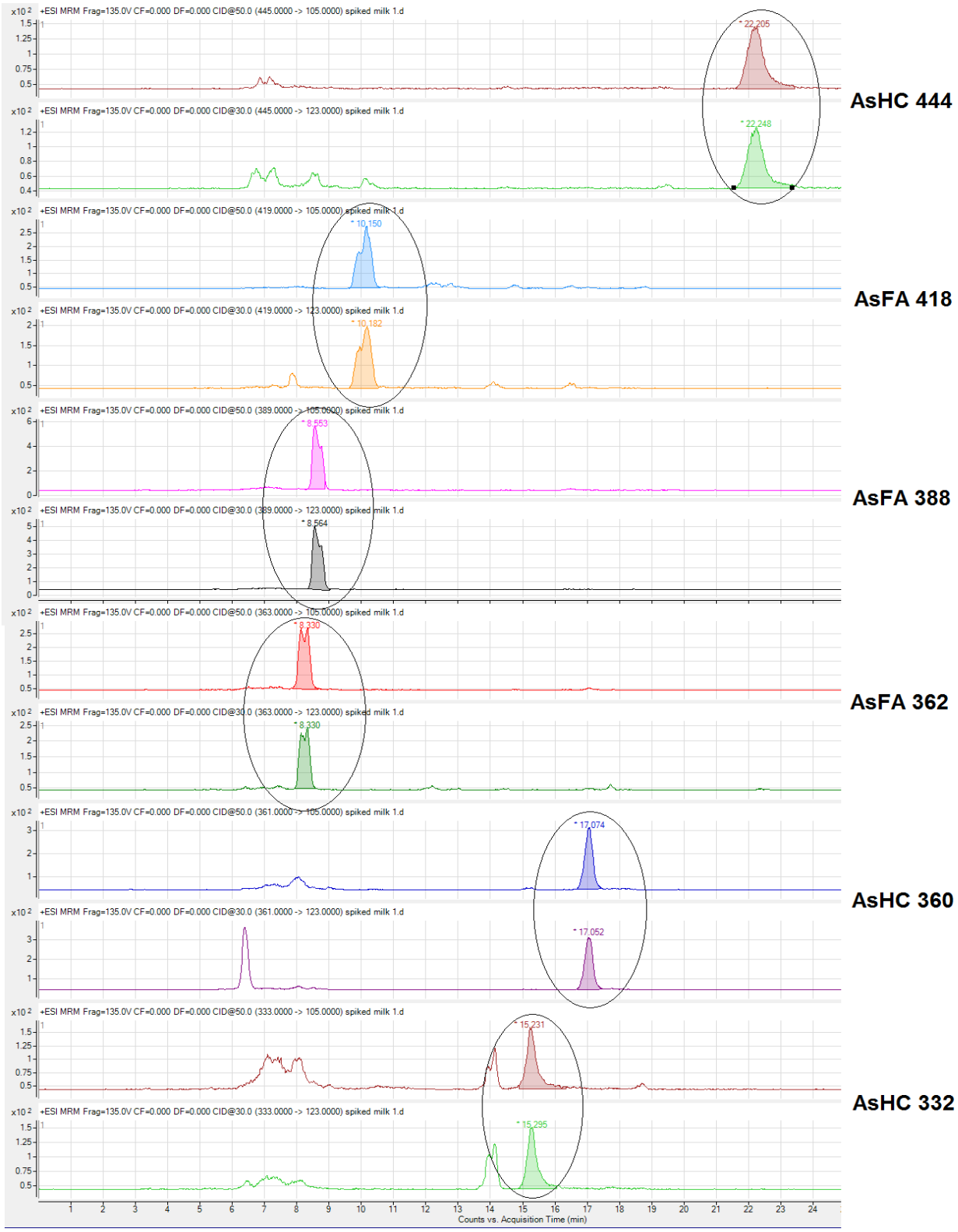

Figure 4.5 shows a fractionation chromatogram from the ESI-MS for one of the samples spiked with the mixed lipid standard containing all six compounds of interest. When matching peaks for both fractions measured of each compounds are obtained, the presence of the compound can be confirmed. This was used to confirm the detection of the analytes of interest in all the samples analyzed by HPLC/ESI-MS.

4.4.2 Quantification of arsenolipids in human breast milk

The results presented in table 4.7 shows that the quantification of the identified arsenolipids was not successful due to poor instrumental sensitivity, resulting in too high LOD and LOQ values to be able to quantify more than two different compounds in a total of four samples.

Table 4.7 presents the results from the attempt to quantify the arsenolipids of interest in the 21 samples of human breast milk. The mean value was below LOQ in all of the compounds and is thus not reported in the table. The median, percentiles and interquartile range is presented.

Compound	Mean (µg/L)	Median (µg/L)	Percentile 25%	Percentile 75%	Percentile 90%	Min. – Max. (IQR) (µg/L)
AsFA 362	-	<1,6[a]	<1,6[a]	<1,6[a]	2,7	<0,46[b] – 4,5
AsFA 388/390	-	<1,6[a]	<0,46[b]	<1,6[a]	1,8	<0,46[b] – 2,7
AsHC 332	-	<0,47[c]	<0,47[c]	<1,5[d]	<0,47[c]	<0,47[c] - <1,5[d]
AsHC 360	-	<0,47[c]	<0,47[c]	<0,47[c]	<0,47[c]	<0,47[c] - <1,5[d]

[a]LOQ (AsFA), [b]LOD (AsFA), [c]LOD (AsHC), [d]LOQ (AsHC)

Four samples had measured values of AsFA 362 above the LOQ and two of the same samples measured levels of AsFA 388/390 above the LOQ. With few measurable values and a sample series consisting of only 21 samples, there is no use in doing any statistical testing. Doing statistical tests with the majority of measured values below LOQ and LOD would give information with high uncertainty and thus it would be wrong to conclude anything with the information obtained from these tests. When having such low concentrations, the most common procedure in environmental chemistry is to substitute some of the fraction of the detection limit. Helsel (2006) concluded that using a fraction of the detection limits to substitute values for nondetects (low-level concentrations with values known only to be somewhere between zero and the laboratory's detection/reporting limits) results in that estimates of correlation coefficients, regression slopes, hypothesis tests and even simple means and standard deviations will be inaccurate and irreproducible.

In order to obtain reliable results, further work is required, with respect to both the sample preparation and the instrumental analysis. Higher sensitivity and better recoveries or a larger increase of concentration during sample preparation is essential to make the method suitable for analysis of breast milk.

To achieve higher sensitivity on the instrument, a lower background measurement is essential, and could be achieved by changing some instrumental parameters or cleaning the system for a time-period before starting analysis. A change of cones (from nickel to platina) was done on the ICP-MS to increase sensitivity. The counts of samples increased, but also the background, resulting in approximately the same signal/noise ratio.

4.5 Preparation of internal standards: methylation of standards, purified and raw milk

When testing the possibilities of making an internal standard, methylation was performed on standard solutions, purified milk samples, and on raw milk. Due to a large drift during the analysis, the concentrations listed in table 4.8 are presented as intervals calculated from calibration standards measured before and after measuring the samples. The calibration curves of each compound are presented as figures in appendix C.

Methylation of purified Milk:

Table 4.8 presents the concentration range of the different compounds analyzed in the purified milk samples. Due to rather large drift in counts during the measurement, the concentration ranges are calculated from the two measures of the standard, in the beginning and at the end of each measurement.

Sample Name	AsHC 332 [µg/L]	AsFA 362 [µg/L]	AsHC 332-Me_3 [µg/L]	AsFA 362-Me_3 [µg/L]
104679a_1	0.0065 – 0.106	1.61-3,20	0	0
104679a_2	0.022 – 0.122	1.42-3.03	0	0
104679a Me_3_1	0	0	0.095-0.285	0.55-1.20
104679a Me_3_2	0	0	0.136-0.409	0.65-1.44
110733_1	0-0.033	0.18-0.55	0	0
110733_2	0	0.19-0.57	0	0
110733_3	0	0.16-0.51	0	0
110733 Me_3_1	0	0	0	0.13-0.29
110733 Me_3_2	0	0	0	0.13-0.29
110733 Me_3_3	0	0	0	0.08-0.17

Methylation of test milk 1:

Table 4.9 presents the ratio between the counts from deuterium labeled internal standard and the methylated compounds in the milk. The ratios for the hydrocarbon are almost one, but the fatty acid had some difficulties due to esterification of the internal standard in methanol, resulting in lower ratio values.

Sample matrix	AsHC332-Me_3 (Smpl/IS)	AsFA362-Me_3-ester (Smpl/IS)
Milk sample 1	0.974	0.306
Milk sample 2	1.033	0.321
Milk sample 3	0.910	0.374

Methylation of the internal standard in methanol resulted in a methyl-ester group on the fatty acid in addition to the methylated group. This was a challenge, but when the fatty acid in the tests with standards and purified milk reacted the same way, an attempt on the raw milk was performed.

The results indicate that the methylation of the hydrocarbon was successful, considering the ratio between the internal standard and the spike is close to one. The results of the methylation of the fatty acid did not look as good due to reactions from the methyl-ester group back to the oxo-compound during sample preparation that gave a mixture of the AsFA 362oxo and the methyl-ester compound. Thus, the methylation of the fatty acid was not successful with this approach.

To make quantification of arsenolipids in human breast milk easier with more reliable results, it would have been ideal to use a labeled synthesized standard as internal standard. Synthesis of these labeled compounds is not easy, but by methylation of an already synthesized arsenolipid standard with a deuterium labeled methyl group, it would be possible. The methylated internal standard would not behave like the un-methylated lipids in the milk, so the milk had to be methylated after adding the deuterium labeled internal standard.

The first tests were performed with standard solutions where one fraction of a mixed standard of AsFA 362 and AsHC 332 were methylated and the remaining fraction were analyzed as it was, to compare methylated and non-methylated standards. The methylation turned out to be effective with close to complete methylation.

The next step was to methylate already purified milk samples, which also turned out to be effective looking at the results in table 4.8. The next step was to test the methylation on raw milk samples. If the arsenolipids of interest could be methylated in the lipid-rich matrix, the labeled internal standard could be added to the milk prior to sample preparation. The results showed that even with the complex milk matrix, the arsenolipids were methylated in the raw milk samples. The spike and the internal standard were added with same concentrations, and the methylation of the hydrocarbon 332 had a ratio close to one (table 4.9). This indicates that the internal standard followed the same path as the hydrocarbon during sample preparation. However, looking at the

results for the fatty acid, the ratio between internal standard and compound of interest was not as good as expected.

Methylation of the fatty acid with methanol as a solvent resulted in an esterification of the acid-group giving a different compound than the methylated fatty acid, thus the internal standard consists of this methyl-ester compound. Working with the standards this was not a problem, because the fatty acid in the standard also went through the esterification. The problem, however, appeared when the raw milk was methylated. Somehow, during the sample preparation, the methyl-ester compound of the spiked fatty acid reacted back to the oxo-form. This gave a mixture of the methylated fatty acid and the methyl-ester compound. The internal standard however, consists only of the methyl-ester compound, resulting in the low ratio values in table 4.9.

It is still a lot of work to do, before this could be an approach in the analysis of arsenolipids, but the experiments showed promising results. Further work with this approach is discussed in chapter 5.

5 Suggestions for further work

As mentioned in the previous chapter, it would be necessary to work further with optimizing the method in order to obtain reliable measurements of the low levels of arsenolipids in the human breast milk. Some possible approaches were discussed about both the sample preparation steps and the instrumental parameters. Small changes could be made in attempt of lowering the LOD and LOQ or increasing the concentration even more. Changing the sample volumes and volumes of reagents and solvents back to the double amount and then dissolve the oil from the solvent partitioning in 200 µL MeOH, would give a 50-fold concentration increase through the sample preparation. Combined with a larger injection volume (e.g. 50uL instead of 25uL) could give a larger signal and thus may rise the measured values above the LOD and LOQ.

The further work with the methylation experiments should first of be to find a way to avoid the esterification of the fatty acid in both the internal standard and the milk. Using a solvent without a methyl-group could do this. An approach with acetonitrile was attempted, without satisfying results due to lower effectiveness of methylation. However, finding a suitable solvent for the internal standard could be of great use. Performing several tests with methylated standards could give reliable information about trends such as effectiveness and intermediate precision.

6 Conclusion

The experiments performed in the attempt of optimizing the method gave results of satisfactory intermediate precision. It would be essential to achieve a more satisfactory reproducibility and a reliable tool to obtain information about the method accuracy. However, it was still challenging to quantify the arsenolipids of interest in the human breast milk samples. The change of extraction solvent from DCM to MTBE had practical advances given that the organic fraction forms as the top layer during extraction, but the recovery were still low. The changes in volumes used of sample material, solvents and reagents resulted in a lower concentration increase during purification and combined with the low recovery the method was not fully optimized.

The concentrations of arsenolipids in the human breast milk samples were low (<1,0 µg As/L), and fell below the limit of quantification (LOQ) in most of the samples. Four out of six arsenolipids of interest were however detected in several of the samples in the series of 21 human breast milk samples, and their presence were confirmed by matching with the mixed standard of synthesized arsenolipids. The method is suitable for detection, but not yet for quantification of arsenolipids in human breast milk.

Looking at the methylation experiment, the approach is promising. The methylation was successful for the hydrocarbons but challenging due to production of methyl-ester in the fatty acids, resulting in incomplete methylation and structural mismatch between the methylated internal standard and the arsenolipids in the milk matrix. The internal standard prepared were thus not currently suitable for use.

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Appendix A – Temperature profile Ultraclave

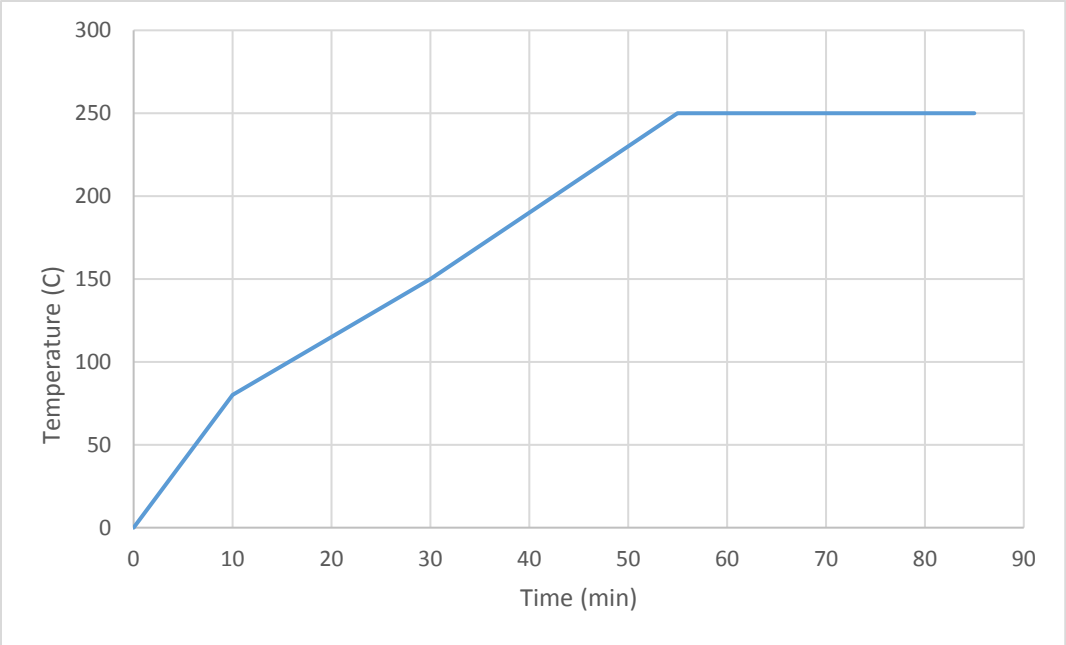


Figure A.1 presents the temperature profile of the program used on the UltraClave to digest the human breast milk samples.

Appendix B – Overview: Equipment, reagents, gases and reference materials

Table B.1 presents the instruments and equipment used in the work with the study. Quartz tubes and Teflon caps were washed with acid in the UltraClave. All the plastic equipment were new.

Instrument/ equipment	Specification	Supplier
HPLC	1260 series infinity II,	Agilent technologies
Column	ACE ultracore 5 super C18 (250 x 4.6 mm)	ACE
HPLC	Dionex Ultimate 3000	Thermo Fischer
Column	Asahipak ODP-50 (150 x 4.6 mm; with 5um particles)	
Splitter	splits flow from HPLC ratio 1/9	-
ICP-MS	7900 Single Quadrupole ICP-MS	Agilent technologies
ICP-MS	8800 triple quadrupole	Agilent technologies
ESI-MS	6460 QQQ ESI	Agilent technologies
HR-ESI-MS	Q- Exactive Hybrid Q-Orbitrap	Thermo Fischer
tubing	0.125 PEEK tubing	Upchurch scientific, Oak Harbour, USA
Vacuum Lyophilizator	Christ RVC 233 CDplus	Martin Christ Gefriertrocknungsanlagen GmbH, Osterode am Harz, Germany
Centrifuge	Hettich Rotina 420R	Andreas Hettich GmbH & Co. Karlsruhe Germany
micro centrifuge	High speed, refrigerated.	SCILOGEX, Rocky hill, CT
Ultraclave	III microwave system	Milestone
40 position containers	Quartz tubes with Teflon caps	Milestone
Weight (Graz)	analytical scale	Sartorius
Automatic pipettes	10-100 µl, 100-1000µl	Socorex Acura
pipette tips	100 µl, 1000µl	Greiner Bio-one
Polypropylene tubes	15 ml	Greiner Bio-one
Polypropylene tubes	50 ml	Greiner Bio-one
Disposable syringes	3 mL	Henke Sass Wolf; NROM-JECT®
Disposable needles	-	Braun; Sterican
Disposable pipettes	3 mL, Polyethylene	Bartelt

Table B.2 presents the reference materials and controls used for total analysis and speciation analysis in this study.

Type	Specification	Material	Supplier
Serum lyophilized	ClinCheck® control serum for trace elements	Reference material	RECIPE®
In-house reference	Arsenic containing water	In-house reference validated against CRM water from NIST (1640a)	University of Graz
1640a	Arsenic containing water	CRM water sample	NIST
Mixed lipid standard	AsFA 362, 388,418 & AsHC 332, 360, 444	Control of retention time and calibration	University of Graz
DMA		Control of calibration	University of Graz

The lyophilized serum control sample was prepared by adding 3.0 mL milli Q-water to the vial containing the dry serum sample.

Table B.3 presents an overview of the reagents and gases used for sample preparation and analysis in both total As and speciation analysis.

Reagent/ Gas	Quality	Concentration	Supplier
Milli Q water	-	-	Millipore
HNO3	Double sub-boiled	66% (w/w)	
H2SO4	Pro analysi	98% (w/w)	Carl Roth GmbH & Co.
Argon gas (Ar)	-	-	Messer Austria
Helium gas (He)	-	-	Messer Austria
Germanium (Ge) (Internal standard)	-	1000 mg/L	Carl Roth GmbH & Co. Karlsruhe Germany
Indium (In) (Internal standard)	-	1000 mg/L	Carl Roth GmbH & Co. Karlsruhe Germany
Lutetium (Lu) (Internal standard)	-	1000 mg/L	Carl Roth GmbH & Co. Karlsruhe Germany
Iridium (Ir) (Internal standard)	-	1000 mg/L	CPI
Tellurium (Te) (Internal standard)	-	1000 mg/L	Carl Roth GmbH & Co. Karlsruhe Germany
TFA	For biochemistry and synthesis	≥99.9%	Carl Roth GmbH & Co. Karlsruhe Germany
MTBE	p.a.	≥99.5%	Carl Roth GmbH & Co.
n-Hexane	For synthesis	≥99.8%	Carl Roth GmbH & Co.
Methanol	HPLC grade	≥99.9%	VWR chemicals Vienna
Ethanol	HPLC grade	≥99.9%	ChemLab
Ammonium acetate	Pro analysi	≥98%	Merck (Darmstadt, Germany)
Acetone (carbon compensation)	HPLC grade	≥99.8%	ChemLab
2-mercaptoethanol	For synthesis	≥99%	Merck (Hohenbrunn Germay)
Iodomethane (CH_3-I)	For synthesis	-	Merck (Hohenbrunn Germay)
Iodomethane deuterated (CD_3-I)	For synthesis	≥99.5% atom % D, ≥99% (CP)	Sigma Aldrich (Steinheim Germany)

Appendix C: Calibration standards

The equipment needed to prepare the calibration standards are listed in table C.1. the elements and compounds were transferred from stock solutions and the standard with the highest concentration were made first. Diluting fractions of the standard with highest concentration made the standards of lower concentrations. The concentrations of the standard series are presented in table C.2 and C.3.

The standards for total analysis were made to have the same acid concentration as the digested samples. The concentrations of stock solution and the supplier of the elements and compounds are listed in table C.4.

Table C.1: equipment used to prepare the calibration standard series.

Equipment	Specification	Supplier
Automatic pipettes	10-100µL 100-1000µL	Socorex Acura
Pipette tips	-	Greiner Bio-one
Polypropylene tubes	15mL	Greiner Bio-one

Table C.2: The concentrations of the standards used to perform the calibration for total As Analysis. Lead (Pb) was applied in the standard series due to problems with high values of lead in some samples.

Element	Calibration blank	Standard 1	Standard 2	Standard 3	Standard 4	Standard 5
	µg/L	µg/L	µg/L	µg/L	µg/L	µg/L
As	0	0.02	0.05	0.1	0.5	1
Pb	0	0.02	0.05	0.1	0.5	1

Table C.3: The concentrations of the standards used to perform the calibration for the speciation analysis of arsenolipids.

Element	Calibration blank	S 1	S 2	S 3	S 4	S 5	S 6
	µg/L	µg/L	µg/L	µg/L	µg/L	µg/L	µg/L
AsFA 362	0	0.1	0.5	1	5	10	20
AsHC 332	0	0.1	0.5	1	5	10	20

S = Standard

Table C.4: Standard solutions used to do calibrations and the supplier.

Element/Product	Concentration stock solution	Supplier
Arsenic (As)	1000mg/L	ROTH
Lead (Pb)	1000mg/L	ROTH
AsFA 362	250mg/L (synthesized std)	University of Graz
AsHC332	250mg/L (synthesized std)	University of Graz

Figure C.1 – C.4 presents the calibration curves made for each of the compounds of interest in the methylation experiment. Figure C.1 and C.2 presents the calibration of the non-methylated standards and C.3 and C.4 presents the calibration of the methylated standards. Each figure contains two calibration curves. Due to large drift from the beginning to the end of the measurement, the calibration standards were measured in the beginning and in the end, and the concentrations measured were calculated as a range between the two calibrations.

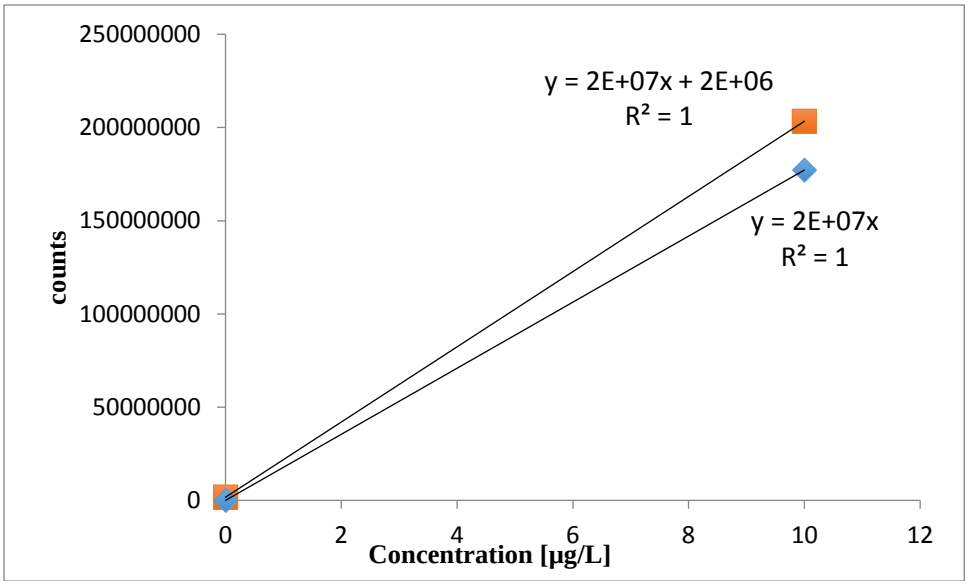


Figure C.1 presents two two-point calibration-curves of AsHC332. Due to drift during the analysis, we had to make calibration curves for both the standard measure before analyzing the samples and after the samples. The equations from the calibration curves were used to calculate a concentration range of which the concentrations of the samples would be found.

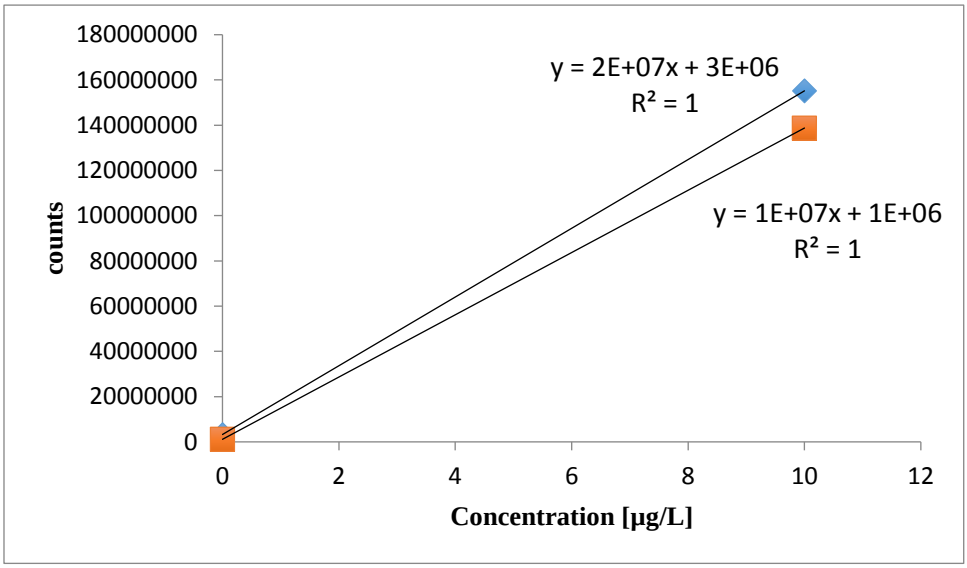


Figure C.2 presents two two-point calibration-curves of AsFA362. Due to drift during the analysis, we had to make calibration curves for both the standard measure before analyzing the samples and after the samples. The equations from the calibration curves were used to calculate a concentration range of which the concentrations of the samples would be found.

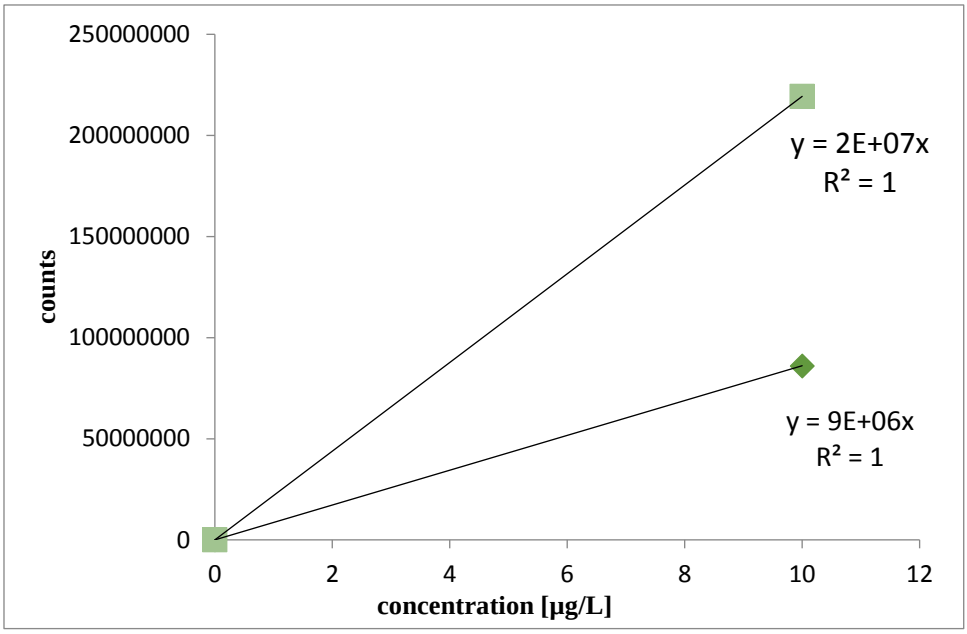


Figure C.3 presents two two-point calibration-curves of AsFA362-Me$_3$-ester, the methylated methyl ester fatty acid 362. Due to drift during the analysis, we had to make calibration curves for both the standard measure before analyzing the samples and after the samples. The equations from the calibration curves were used to calculate a concentration range of which the concentrations of the samples would be found.

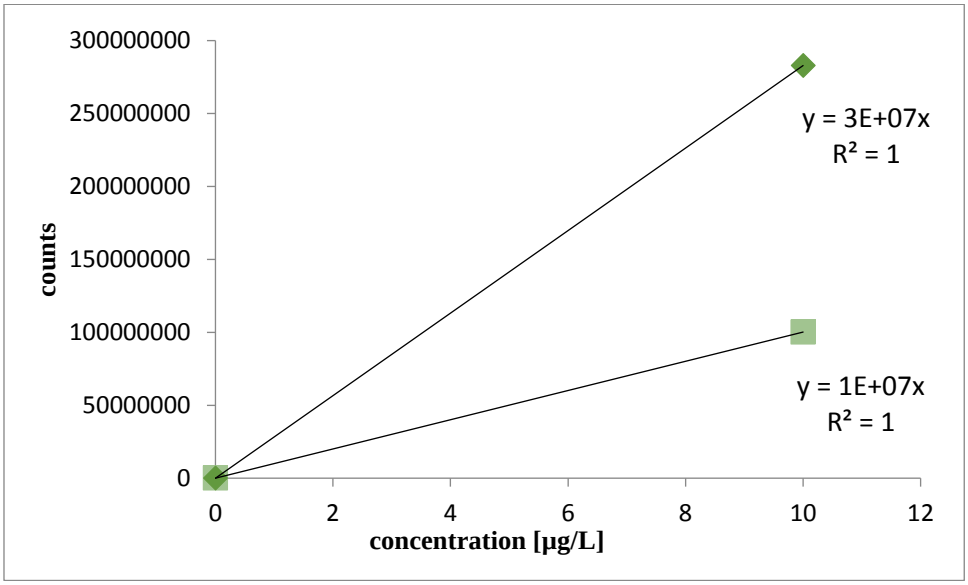


Figure C.4 presents two two-point calibration-curves of AsHC332-Me$_3$, the methylated hydrocarbon 332. Due to drift during the analysis, we had to make calibration curves for both the standard measure before analyzing the samples and after the samples. The equations from the calibration curves were used to calculate a concentration range of which the concentrations of the samples would be found.

Appendix D - Solutions, Instrumental parameters and specifications; Analysis on ICP-MS, HPLC/ESI-MS and HPLC/ICP-MS

Table D.1 presents the instrumental parameters and specifications of the Agilent 7900 ICP-MS single-Q. The sample introduction is a mixture of 0.8 mL/min from the isocratic pump and 0.1 mL/ min from the splitter 9/1 between the HPLC and the ICP-MS and ESI-MS.

ICPMS 7900	
Instrumental parameters	
Model Name	G8403A
Serial number	JP14440511
RF power	1550 W
Carries gas	0.96 l/min
Mode	He & no gas
Helium flow	4.0 mL/min
Nebulizer pump	0.5 rps
Instrumental specification	
Cones	Nickel
Nebulizer	micro mist
Spray chamber	Scott double pass
Sample introduction	Agilent 1200 LC, Binary pump ALS
Sample depth	8 mm
S/C temperature	2 ºC

Table D.2 presents the instrumental parameters and specifications of the Agilent 8800 ICP-MS QQQ.

ICPMS 8800	
Instrumental parameters	
RF power	1550 W
Carries gas	0.90 l/min
Mode	He & No gas
Helium flow	4.0 mL/min
Nebulizer pump	0.1 rps
Instrumental specification	
Cones	Nickel
Nebulizer	micro mist
Spray chamber	Scott double pass
Sample introduction	ISIS
Sample depth	8 mm
S/C temperature	2 ºC

Table D.3 presents the instrumental parameters of the Agilent 1200 HPLC – 6460 ESI-MS system.

HPLC / ES MS 6460	
instrumental parameters	
Multisampler:	
injection volume	20 µL
needle wash	Standard wash
Stoptime	As pump/no limit
posttime	off
Isocratic pump:	
Flow	0,8 mL/min
Pressure limits (min.-max.)	0 - 160 bar
Solvent	0,1% FA in Milli-Q, 20 µg/L Te, In Ge
Stop time	As injector/ no limit
Posttime	Off
Binary pump:	
Flow	1 mL /min
Pressure limits (min.-max.)	0 - 500 bar
Solvent A	Ammonium acetate 25mM in H20 pH 9,2
Solvent B	Ammonium acetate 25mM in MeOH pH 9,2
Stoptime	30.0 min
Posttime	Off
Timetable	Table D.3
Column compartment:	
Temperature left	40 ºC
Temperature right	combined
Stoptime	As pump/injector
QQQ:	
ion source	AJS ESI
Stoptime	30.0 min
Time filtering	Peak width 0,07 min
Time segments	Table D.4
Acquisition, scan segments	Table D.5

Table D.4 presents the timetable of the gradient with solvent A and Solvent B. Stop after 30.0 minutes.

Time (min)	A (%)	B(%)	Flow (mL/min)	Max. Pressure limit (Bar)
0,00	80.0	20.0	1.000	500.00
15,00	0.0	100.0	1.000	500.00
25,00	0.0	100.0	1.000	500.00
25,10	80.0	20.0	1.000	500.00

Table D.5 presents the Time segments in the method editor of the ESI-MS.

Start time	*Scan type*	*Div valve*	*Delta EMV (+)*	*Delta EMV (-)*
0	*MRM*	*To MS*	*0*	*0*

Table D.6 presents the acquisition parameters for fragmentation of the arsenolipids in the ESI-MS. Parameters identical for all were dwell = 50, Fragmentor = 150, Cell accelerator voltage = 4 and positive polarity.

Compound name	Precursor ion	Product ion	Collision energy
AsFA 362	363	123	30
AsFA 362	363	105	50
AsFA 388	389	123	30
AsFA 388	389	105	50
AsFA 418	419	123	30
AsFA 418	419	105	50
AsHC 332	333	123	30
AsHC 332	333	105	50
AsHC 360	361	123	30
AsHC 360	361	105	50
AsHC 444	445	123	30
AsHC 444	445	105	50

Table D.7 presents the instrumental parameters for the HPLC/ESI-HR-MS system.

HPLC Dionex Ultimate 3000	
Column	Asahipak ODP-50 (150 x 4.6 mm; with 5um particles)
Column temperature	40 ºC
Injection volume	10 µL
Flow rate	0.500 ml/min
Mobile phase	a: 0.1 % Formic acid in water
	b: 0.1 % Formic acid in MeOH
Gradient	0 min 20% B, 0-15 min 100%B, 15-20 min 100% B, 20-20.1 min 20%B 20.1-25 20% B
HR-MS Thermo Fisher - Q - Exactive Hybrid Quadropole-Orbitrap	
Mode	Electrospray (ES) ionization in positive SCAN and data dependent MS2 mode
Inclusion	on
Spray voltage	3,5 kV
Capillary temperature	268.75 ºC
Probe heater temperature	437.5 ºC
Resolution	70 000 (FWHM)
SCAN range	m/z 150-2000

Table D.8 presents the inclusion list for the analysis on the HPLC/ESI-HR-MS system used for analysis of the methylation experiments.

INCLUSION LIST		
Mass	Formula species	CS polarity
331.23405	C18H39As	plus H 1 positive
333.21331	C17H37AsO	plus H 1 positive
334.25752	C18H42As	plus H 1 positive
361.20823	C18H37AsO2	plus H 1 positive
363.18749	C17H35AsO3	plus H 1 positive
364.23179	C18H40AsO2	plus H 1 positive
375.22388	C19H39AsO2	plus H 1 positive
378.24735	C19H42AsO2	plus H 1 positive

Appendix E – Results from sample series; total Arsenic of the sample series

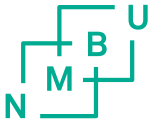
Table E.1 presents the measured results for each of the samples in the sample series, with total analysis of As (values obtained and given by Michael Stiboller).

Sample name	Total As (µg/L)
101553	0,51
103882	1,90
103994	1,06
104321	0,96
104679	2,02
105737	1,55
107291	1,83
110733	2,03
110979	1,32
111071	1,00
111712	0,89
112061	0,61
112779	0,52
113837	0,66
118348	1,05
118888	0,66
119957	1,05
121419	1,42
123941	0,60
128676	0,88
129824	1,27

Appendix F – LOD and LOQ for total arsenic analysis

Table F.1 shows the limit of detection and the limit of quantification of ^{75}As determined in helium mode for both rounds of analysis. The limits of round 1 were calculated based on the values of the 6 blank replicates, and the limits of round 2 were calculated based on the error of the intercept in the calibration curve, as described in chapter 3.3.8

	As (µg/L)	
	Round 1	Round 2
LOD	0,002	0,034
LOQ	0,005	0,094



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