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Arsenic-containing fatty acids and hydrocarbons in marine oils – determination using reversed-phase HPLC–ICP-MS and HPLC–qTOF-MS

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ABSTRACT

Arsenolipids are the major arsenic species present in marine oils. Several structures of arsenolipids have been elucidated the last 5 years, demonstrating the chemical complexity of this trace element in the marine environment. Several commercial fish oils and marine oils, ranging in total arsenic concentrations from 1.6 to 12.5 mg kg^{-1} oil, were analyzed for arsenolipids using reversed-phase high performance liquid chromatography coupled with inductively coupled plasma mass spectrometry (HPLC–ICP-MS). The arsenolipids were quantified using three different arsenic-containing calibration standards; dimethylarsinate (DMA), triphenylarsinoxide (Ph_3AsO) and a synthesized arsenic-containing hydrocarbon (AsHC) (dimethylarsinoyl nonadecane; $C_{21}H_{43}AsO$). The observed variation in signal intensity for arsenic during the gradient elution profile in reversed-phase HPLC was compensated for by determining the time-resolved response factors for the arsenolipids. Isotopes of germanium (^{74}Ge) and indium (^{115}In) were suited as internal standards for arsenic, and were used for verification of the arsenic signal response factors during the gradient elution. Dimethylarsinate was the most suitable calibration standard for the quantification of arsenolipids, with recoveries between 91% and 104% compared to total arsenic measurements in the same extracts. A range of marine oils was investigated, including oils of several fish species, cod liver and seal, as well as three commercial fish oils. The AsHCs – $C_{17}H_{38}AsO$, $C_{19}H_{42}AsO$ and $C_{23}H_{38}AsO$ – were identified as the major arsenolipids in the extracts of all oils by HPLC coupled with quadrupole time-of-flight mass spectrometry (qTOF-MS). Minor amounts of two arsenic-containing fatty acids (AsFAs) ($C_{23}H_{38}AsO_3$ and $C_{24}H_{38}AsO_3$) were also detected in the oils. The sum of the AsHCs and the AsFAs determined in the present study accounted for 17–42% of the total arsenic in the oils.

1. Introduction

Commercial fish oils, e.g. those used in the fish feed industry, are generally produced from pelagic fish such as sand eel, anchovy and blue whiting. The fish oils are rich in long-chain polyunsaturated fatty acids and lipid-soluble vitamins. They can, however, also contain undesirable substances such as dioxins, polychlorinated biphenyls [1–3] and also arsenic. In contrast to other metals arsenic is generally found in high concentrations in fish oil, ranging from 1.6 to 16 mg arsenic kg^{-1} oil [1,4].

Arsenic is a non-essential trace element, which is normally more abundant in the marine environment compared to the terrestrial environment. In seawater, arsenic exists mainly as inorganic arsenic, i.e. arsenate (As(V)) and arsenite (As(III)), typically in concentrations between 0.5 and 2 μg L^{-1} [5]. Marine algae accumulate arsenic from seawater, and biotransform the inorganic arsenic into organic arsenic compounds, i.e. arsenosugars [6]. Marine animals take up arsenic both from ambient seawater and via their diet. Arsenobetaine is the major arsenic species in marine animals, often constituting more than 90% of the total arsenic in marine fish [7]. In addition to inorganic arsenic, arsenosugars and arsenobetaine, a range of lipid-soluble arsenic compounds exist in the marine environment [8] and until now more than 70 natural-occurring arsenic containing compounds have been identified in marine samples, including both water-soluble and lipid-soluble arsenic compounds.

The lipid-soluble arsenic compounds, also known as arsenolipids, currently constitute four classes of compounds, including

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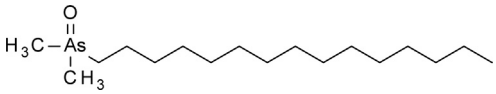
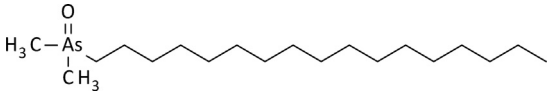
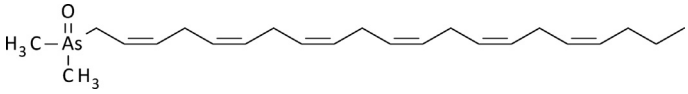
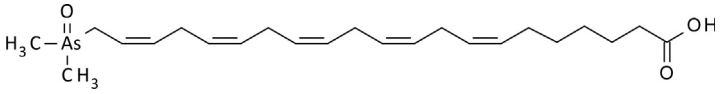
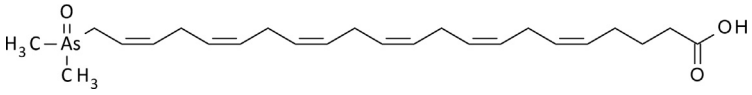
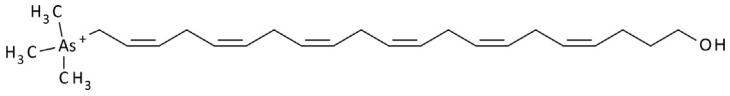
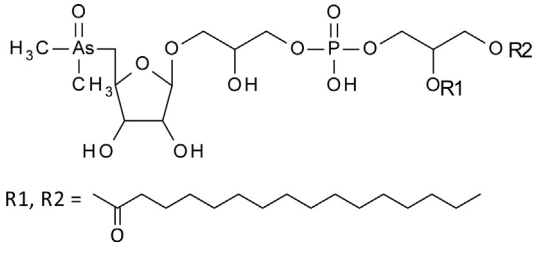
Table 1
Chemical structures of arsenolipids; arsenic-containing hydrocarbons (AsHCs), arsenic-containing fatty acids (AsFAs), cationic trimethylarsenio fatty alcohols (TMAsFOHs) and arsenosugar-phospholipids (As-sugPLs). The As-sugPLs are represented by As-sugPL$_{958}$, where the number is assigned for the molecular weight of the compound. The TMAsFOHs, AsHCs, and AsFAs are assigned with a number representing their carbon chain length. The AsHCs and AsFAs represented are the arsenolipids identified in this work.

Acronyms	Chemical structures	References
AsHC-C_{15}		[15]
AsHC-C_{17}		[15]
AsHC-C_{21}		[15]
AsFA-C_{21}		[14]
AsFA-C_{22}		[18]
TMAsFOH-C_{21}		[9]
As-sugPL$_{958}$		[10]

arsenic-containing fatty acids (AsFAs), arsenic-containing hydrocarbons (AsHCs), arsenosugar-phospholipids (As-SugPLs) [4] and cationic trimethylarsenio fatty alcohols (TMAsFOHs) [9] (Table 1). The first structural identification of an arsenolipid compound was the identification of an As-SugPL in a brown macroalgae (*Undaria pinnatifida*) [10]. The identification procedure was work intensive and included isolation by several chromatographic clean-up steps, saponification, and analysis of the products by gas chromatography (GC) coupled to mass spectrometry (MS) combined with proton-nuclear magnetic resonance spectroscopy (^{1}H-NMR) in addition to atomic emission spectrometry (AES) which was used as an element specific detector [10]. Further work on the arsenolipids focused on analysis of hydrolysis products of the lipids [11–13], until the first identification of intact arsenolipids was reported on the AsFAs in canned cod liver oil [14]. Six AsFAs compounds were identified using preparative chromatographic sample clean-up and analysis by reversed phase high pressure liquid chromatography coupled with inductively coupled plasma mass spectrometry (HPLC–ICP-MS). The structures of the compounds were identified using HPLC coupled with electrospray ionization mass spectrometry (ESI-MS) and high resolution mass spectrometry (HR-MS) [14]. Three AsHCs were subsequently identified in oil of capelin (*Mallotus villosus*) using a similar analytical approach [15].

Several arsenolipids (15 As-SugPLs [10,16,17], 10 AsHCs [9,15–17], over 20 AsFAs [14,17–20] and two TMAsFOHs [9]) have, so far, been identified in various marine samples highlighting the complexity of arsenic compounds in the lipid phases of marine samples. The As-SugPLs have been identified in marine algae [10,16,17] together with AsHCs [16,17] and AsFAs [17]. The AsFAs and AsHCs have also been identified in oils of cod liver [14,20,21], in herring (*Clupea harengus*) filet [19] and in fish meal of capelin [18], while the AsHCs have been identified in tuna [22] and some commercial fish oils [23]. The AsFAs and AsHCs are abundant in fish oils; however, a large proportion of the arsenic present in marine oils is of a more non-polar character compared to the relative polar AsFAs and the AsHCs [15,23]. Between 55% and 75% of arsenic in fish oils was recently found in the non-polar hexane phase of the oils [23]. However, the non-polar phase of fish oil is high in lipid matrix, such as triacylglyceroles, making it difficult to analyze and to identify the arsenolipids present.

ICP-MS is the preferred instrumentation for the analysis of arsenic as it is highly selective and sensitive. A major challenge when using the ICP-MS in analysis of lipid-soluble compounds is, however, the destabilization of the argon plasma when organic solvents are introduced. The challenge can be overcome by modification of the ICP-MS by for e.g. the use of low solvent flow and addition of oxygen to the plasma, and hence make it possible to analyze intact arsenolipids by normal-phase HPLC–ICP-MS using 100% organic mobile phases [24]. The introduction of organic solvents into the ICP-MS additionally affects the ionization of arsenic as the carbon load in the

argon plasma increases the ionization of arsenic [25,26]. Hence, the signal sensitivity for arsenic increases along an organic gradient and causes changes in the signal sensitivities for compounds eluting at different times in the gradient. This affects the quantification of arsenic species if species-specific arsenic calibrants are not available [27]. Various approaches have been suggested to compensate for the changes in signal response for elements in the ICP-MS, such as mathematical compensation [28], addition of organic solvents to the ICP-MS [27,29], isotope dilution [30] and post-column addition of an internal standard [31]. The use of internal standardization and dimethylarsinate (DMA) as calibration standard has been described for analysis of arsenolipids [18]. The signal variation was compensated for using time-resolved response factors for arsenic, which were determined by introduction of an internal standard solution containing isotopes of germanium (^{74}Ge) and arsenic (^{75}As) while simultaneously analyzing a blank sample with the gradient elution program.

Gas Chromatography (GC) coupled with ICP-MS has also been used for the analysis of arsenolipids, e.g. the AsHCs were efficiently separated on GC columns [21,23,32]. An additional advantage of using GC in the analysis of AsHCs is the increased stability of the ICP-MS compared to the use of organic solvents in a HPLC separation method. However, so far, GC–ICP-MS has only been reported for the analysis of AsHCs, in contrast to when using HPLC–ICP-MS, where it is possible to analyze different groups of arsenolipids, independently of the compounds volatility. It was demonstrated that the AsHCs, the AsFAs and the AsSugPLs, as well as several unidentified arsenolipids, can be separated using HPLC in the same run [33].

In a recent study we looked at the presence of AsHCs in a range of fish oils using GC–ICP-MS [23]. In this study the AsHCs and AsFAs in the polar methanol phase of a range of fish oils and marine oils were analyzed by reversed-phase HPLC–ICP-MS and quantified using different arsenic-containing calibration standards. Furthermore, attempts to increase the extraction efficiency of arsenic from the non-polar phase of the fish oils by sequential liquid–liquid extraction procedures were carried out. The extracts were further analyzed by HPLC-HR-MS to obtain complementary structural information.

2. Experimental

2.1. Chemicals and reagents

All chemicals used were analytical grade quality or better. Milli-Q water (EMD Millipore Corporation, Billerica, MA, USA) (18.2 MΩ cm) was used for sample preparation and total arsenic analysis. Methanol, *n*-heptane, acetonitrile, formic acid (99% w/w), hydrogen peroxide (H_2O_2, Emsure ACS, ISO, 32% w/w) and indium (ICP standard solution) were obtained from Merck (Darmstadt, Germany). Nitric acid (HNO_3, *trace select*, ≥69.0% w/w) was obtained from Fluka (Buchs, Switzerland). Dimethylarsinic (dimethylarsinate; DMA(V)) was obtained from Supelco Analytical (Bellefonte, PA, USA). A multielement standard solution and certified stock solutions of rhodium and germanium, respectively, were obtained from Spectrascan TeknoLab (Drøbak, Norway). Triphenylarsenoxide (Ph_3AsO) was obtained from Strem Chemical Inc. (Newbury Port, MA, USA). Cocadylic acid (dimethylarsinic acid), potassium iodide (KI), sodium bisulfitt ($NaHSO_3$), hydrochloric acid (HCl), 1-bromononadecane ($C_{19}H_{39}Br$), methanol HPLC grade, formic acid HPLC grade, metallic sodium, dry calcium chloride ($CaCl_2$) and tetrahydrofuran (anhydrous) (THF) were obtained from Sigma Aldrich (St. Louis, MO, USA).

2.2. Marine oil samples

Commercial fish oils of sand eel (*Ammodytes marinus*) and Atlantic herring (*Clupea harengus*) were obtained from FF Skagen (Fiskernes Fiskeindustri Amba, Skagen, Denmark). A second mixed oil (mixed oil 1: Norway pout (*Trisopterus esmarkii*), blue whiting (*Micromesistius poutassou*), Atlantic herring and sand eel) was obtained from Egersund Fiskefabrikk (Egersund, Norway) and oil of farmed salmon (*Salmo salar*) was obtained from Marine Harvest (Bergen, Norway). Commercial fish oils of blue whiting, anchovy (*Engraulis ringens*), Atlantic herring, a mixed oil (termed mixed oil 2: Atlantic herring, Atlantic cod (*Gadus morhua*) and saithe (*Pollachius virens*)) and three commercial fish oils (termed commercial oil 1–3) were all obtained through a national surveillance program lead by National Institute of Nutrition and Seafood Research (NIFES) on behalf of the Norwegian Food Safety Authority. Oil of cod liver from farmed Atlantic cod and oil of Greenland seal (*Pagophilus groenlandicus*) blubber (inner section) were obtained through a surveillance program led by NIFES on behalf of the Norwegian Food Safety Authority.

2.3. Overview of analytical work

The oils were partitioned into heptane and aqueous methanol (MeOH 1 phase, Fig. 1). The heptane phases of selected oils were further partitioned into methanol (MeOH 2 phase) and heptane, to increase the extraction recovery for arsenic into methanol. The heptane phases were partitioned into acetonitrile (ACN) and heptane, to further increase the extraction recovery of arsenic into a polar solvent. Acetonitrile was selected as third solvent as it is more non-polar compared to methanol and has a different selectivity than methanol [34]. MeOH 1, MeOH 2 and ACN phases were analyzed for total arsenic. The MeOH 1 phase of all fish oils were analyzed by reversed-phase HPLC–ICP-MS, and the arsenolipids were quantified using DMA and Ph_3AsO. The arsenolipids in the MeOH 1 phase of selected fish oils were also quantified using a synthesized AsHC compound (dimethylarsinoyl nonadecane; AsHC–C_{19}). The MeOH 1 phases of selected fish oils were analyzed by HPLC coupled to quadrupole Time-of-Flight Mass Spectrometry (qTOF-MS) for identification of the molecular structures and determination of accurate masses of the arsenolipids present. All MeOH 2 and ACN phases of selected fish oils were analyzed by reversed-phase HPLC–ICP-MS.

2.4. Extraction of the arsenic-containing hydrocarbons

Three replicates of each fish oil (1.0 ± 0.1 g) were partitioned between *n*-heptane (7.5 mL) and methanol/water (9/1, v/v; 2 × 3.75 mL). The MeOH 1 phases were evaporated to dryness using nitrogen and dissolved in 0.5 mL methanol/water (9/1, v/v) and filtered (Acrodisk, Minispike Syringe filter, GHP, 13 mm, 0.45 μm, Waters Corporation, Milford, MA, USA; and Soft-ject Luer, 1 mL, VWR International, West Chester, PA, USA). The samples were further diluted with methanol/water (9/1) prior to analysis

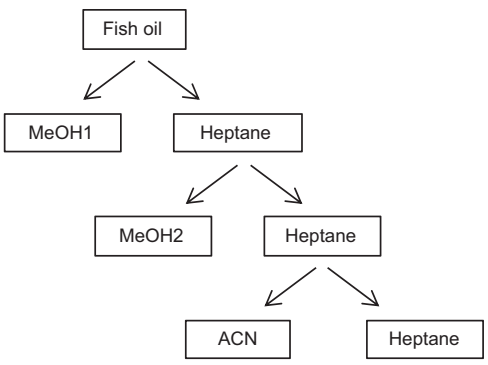


Fig. 1. Overview of the sample preparation work, which included the partitioning of fish oil into *n*-heptane, aqueous methanol (MeOH 1), methanol (MeOH 2) and acetonitrile (ACN) prior to total analysis of arsenic and arsenolipids.

by HPLC–ICP-MS and HPLC–qTOF-MS. The heptane phase of selected fish oils (blue whiting, mixed oil 2 and commercial oils 1–3) were further partitioned with methanol (2×3.75 mL) and acetonitrile (2×3.75 mL) (Fig. 1). The MeOH 2 and ACN phases were evaporated to dryness using nitrogen and dissolved in 0.5 mL methanol. The samples were filtered using 0.45 μm filters prior to analysis.

2.5. Total arsenic determination by ICP-MS

The oils were accurately weighted (0.15 g) in (PTFE) vessels and concentrated HNO_3 (2.00 mL) and H_2O_2 (0.50 mL) were added. The samples were digested in a microwave system (Ethos Pro Milestone, Sorisole, Italy) using the following temperature program: 1 min at 250 W, 1 min at 0 W, 5 min at 250 W, 5 min at 400 W and 5 min at 650 W. The digests were diluted to a final volume of 25 mL with Milli-Q water. For all phases 100 μL were transferred to PTFE vessels. The vessels were left overnight in a fume hood to evaporate the organic solvents. The evaporated extracts were digested by the same procedure described above, and the digests were diluted to a final volume of 10 mL with Milli-Q water.

An external calibration curve was made from freshly prepared arsenic standard solutions, diluted from a 1000 mg L^{-1} certified stock solution to appropriate concentrations by 5% (v/v) HNO_3. A diluted solution of 1000 mg L^{-1} rhodium stock solution was added on-line and served as an internal standard to correct for instrumental drift during the analytical run [35]. The samples were analyzed in standard mode (non-cell mode) using an ICP-MS 7500ce (Agilent Technologies, Wilmington, Delaware, USA) equipped with an auto sampler (ASX-500; CETAC Technologies, Omaha, NE, USA). The detector was set at *m/z* 75; the monoisotopic mass of arsenic (^{75}As). The instrumental settings are summarized in Table 2. Oyster Tissue (SRM 1566 b, National Institute of Standards and Technology, Gaithersburg, MD, USA), TORT-2 (Lobster Hepatopancreas, National Research Council Canada (NRC), Ontario, Canada) and DOLT-4 (Dogfish Liver, NRC) were used as reference materials for the total arsenic analysis. The obtained results for total arsenic (average ± SD) 7.6 ± 0.4 mg kg^{-1}; $n=5$ (Oyster Tissue), 20.9 ± 1.0 mg kg^{-1}; $n=6$ (TORT-2) and 9.2 ± 0.5 mg kg^{-1}; $n=6$ (DOLT-4) agreed well with the reference values of 7.65 ± 0.65 mg kg^{-1} (Oyster Tissue), 21.6 ± 1.8 mg kg^{-1} (TORT-2) and 9.66 ± 0.62 mg kg^{-1} (DOLT-4), respectively.

Table 2
Instrumental settings used in this work.

HPLC–ICP-MS	
HPLC	
HPLC column	Waters Acquity BEH C_{18} (2.1 × 100 mm, 1.7 μm)
Mobile phase	A: 0.1% formic acid in water B: 0.1% formic acid in methanol
Gradient program 1	0–2 min: 30–100% B 2–22 min: 100% B 23–35 min: 30% B.
Gradient program 2	0–2 min: 30–100% B 2–32 min: 100% B 33–45 min: 30% B
Flow	0.14 mL min^{-1}
Injection volume	3 μL
Column temperature	30 °C
ICP-MS 7500cx	
RF power	1530 W
Plasma gas flow	15 L min^{-1}
Carrier gas flow	0.22 L min^{-1}
Makeup gas flow	0.46 L min^{-1}
Torch	1.5 mm i.d.
Nebulizer	Micromist, 10–100 μL min^{-1}
Integration time	100 ms
Elements monitored	*m/z* 75 (As), *m/z* 74 (Ge), *m/z* 115 (In), *m/z* 35 (Cl), *m/z* 53
ICP-MS 7500ce	
RF power	1500
Plasma gas flow	15 L min^{-1}
Carrier gas flow	0.9 L min^{-1}
Makeup gas flow	0.16 L min^{-1}
Torch	2.5 mm i.d.
Nebulizer	Babington
Integration time	1000 ms
Elements monitored	*m/z* 75 (As), *m/z* 103 (Rh)
HPLC[a]–qTOF-MS	
qTOF-MS	
Mode	Positive
Ion source temperature	200 °C
Dry gas	10 L min^{-1}
Nebulizer	2 bar
Capillary voltage	4500 V
End plate offset	500 V
Collision energy	20–35 eV

[a] Similar HPLC settings as described for the HPLC–ICP-MS analysis.

2.6. Synthesis of arsenic-containing hydrocarbon AsHC–C_{19}

The AsHC–C_{19} was synthesized using a procedure originally described by Feltham and co-workers [36] and slightly modified by Fricke [37]. The substrate and the intermediates of the reaction were not isolated due to the high toxicity of the compounds. In all synthetic steps the target compound was formed and reacted further in situ. Cocadylic acid (10 g) was dissolved in H_2O (40 mL) and KI (33 g), and $NaHSO_3$ (1 g) were added, followed by rapid addition of concentrated HCl (50 mL) over 1–2 min. The mixture was allowed to stir for 24 h at room temperature with occasional addition of $NaHSO_3$ (4.5 g in total) during the first 4 h. The product of the reaction, the dimethyliodo arsine, occurred as dark brown oil droplets that separated from the reaction mixture as oil and was collected using a pasture pipette. The oil was transferred to a flask containing $CaCl_2$ as drying agent. Sodium (1.0 g) was added to dry THF (50 mL) in a dry 100 mL three-neck round bottom flask equipped with a mechanical stirrer, water condenser and a flux of nitrogen. The flask was cooled using acetone/dry ice (−78 °C). Dimethyliodoarsine (~7 mL) was added to the Na/THF solution by syringe through a rubber frit, while the solution was stirred on cooling bath (−78 °C) for 1 h. The reaction occurred as the mixture became slightly golden. The mixture was allowed to stabilize to room temperature for 15–20 min and then refluxed for 30 min. The product of the reaction was sodium dimethylarsenide. Bromononadecane was added (in large excess) to dry THF (5 mL) in a dry 10 mL glass bottle with rubber frits and a stirrer bar. Flux of nitrogen was added to each bottle, and the bottles were placed on ice-baths. Sodium dimethylarsenide (~5 mL) product was slowly added to each bottle by syringe through the frits. The mixture was left stirring for 2 days. The product of the reaction was the alkylated dimethylarsine $(CH_3)_2AsC_{19}H_{39}$ which was further oxidized adding H_2O_2 (50 μL) to the alkylated arsine (200 μL), mixed for 1 h, and then diluted in THF (1 mL). The product was further diluted in methanol. The original alkylated dimethylarsine and the final oxidized product (dimethylarsinoyl nonadecane) was analyzed by HPLC–ICP-MS (data not shown), and the results indicated a complete oxidization of the product. The identity of the oxidized compound was confirmed by qTOF-MS analysis (data not shown), and the arsenic concentration of the compound was determined by ICP-MS following acid digestion.

2.7. Quantification of arsenolipids by HPLC–ICP-MS

The MeOH 1 phases of the marine oils were analyzed by HPLC (1260 HPLC) coupled with an ICP-MS 7500cx (both Agilent

Technologies) using an Acquity UPLC BEH C_{18} column (2.1 × 100 mm, 1.7 μm, Waters Corporation, Milford, MA, USA). A micromist low flow nebulizer (Glass Expansion, Pocasset, MA, USA), platinum skimmer and sample cones (both Agilent Technologies), and 11% optional gas (20% oxygen in argon, added through a T-connector) were used to prepare the ICPMS for the analysis with organic solvents. The ICP-MS was tuned for optimum sensitivity on m/z 75 using a solution of 100 μg L^{-1} Ph_3AsO in methanol. The instrumental settings for the ICP-MS and the HPLC in analysis mode are listed in Table 2. Gradient program 1 was used when analyzing the MeOH 1 phases.

The response factors were determined by injection of a blank sample while adding a solution of arsenic (^{75}As; added as DMA), germanium (^{74}Ge) (both 100 μg L^{-1}) and indium (^{115}In) (50 μg L^{-1}) post-column through a T-split prior to the nebulizer. By determining the time-resolved arsenic response throughout the gradient elution on the HPLC–ICPMS, response factors could be calculated for every arsenic-containing compound, using the approach described by Amayo and colleagues [18]. The arsenic signal at the retention time (r.t.) of the arsenolipids (r.t. 11–16 min) was compared with the arsenic signal at the r.t. of the calibration standards; DMA (0.5 min), Ph_3AsO (10.5 min) and of the synthesized AsHC-C_{19} (17.5 min). A post-column internal standard solution of 100 μg L^{-1} ^{74}Ge and 50 μg L^{-1} ^{115}In was added by the peristaltic pump (0.07 rpm) through the T-split throughout the analysis of samples and calibration standards and was used to correct the signal effect of the organic gradient, as well as for correcting potential matrix effects and instrumental drift in the ICP-MS signal during analysis.

A calibration curve for DMA was produced by flow-injection (column excluded) of three replicates at every concentration level (0–5000 μg As L^{-1}), using the 70/30 mobile phase A/mobile phase B (start of gradient program), flow at 0.14 mL min^{-1}, 3 μL injection volume and 2 min signal detection for every injection. A calibration curve for Ph_3AsO (0–500 μg As L^{-1}), was produced using the same chromatographic settings as used for the samples (Table 2, gradient program 1). The MeOH 1 phases of the oils were diluted by a factor of 3 or 4, depending on concentration, and the arsenolipids were quantified using DMA and Ph_3AsO as calibration standards, respectively. The arsenolipids of selected fish oils, blue whiting, mixed oil 1 and commercial oil 1–3, were additionally quantified using the synthesized AsHC-C_{19}. A calibration curve for the synthesized AsCH–C_{19} (0–600 μg As L^{-1}) was produced using the same chromatographic settings as used for the samples (Table 2, gradient program 1). Minor additional peaks were detected for the synthesized compound when analyzed by HPLC–ICP-MS, accounting for approximately 15% of the total area of the compounds, which was compensated for when used as calibration standard.

The MeOH 2 phases of selected fish oils were analyzed by reversed-phase HPLC–ICP-MS using gradient program 2 and the instrumental settings listed in Table 2.

2.8. Structural identification of arsenolipids by HPLC–qTOF-MS

The MeOH 1 phases of selected oils (commercial oil 1 and 2) were analyzed by HPLC (1260HPLC, Agilent) coupled to qTOF-MS (Bruker Daltonics, Bremen, Germany) for accurate mass determination of the compounds. The instrumental settings are listed in Table 2. Analytes were separated on the Waters Acquity column. Ionization was achieved by ESI in positive mode. The scan range was from m/z 50 to 1000 in positive mode in TOF-MS mode, and from m/z 100 to 1000 in qTOF-MS mode using auto MSMS, with 20 eV at masses of 100 m/z value, 30 eV at 500 m/z value and 40 eV at 1000 m/z. Triphenylarsine oxide was used for qualitative internal control of the retention behavior of the chromatographic system.

A fraction of the MeOH 2 phase of the commercial fish oil 2 was manually collected into a HPLC vial at r.t. 20–30 min. The fraction was collected twice, and the phases (sum approximately 2 mL) were evaporated and dissolved in 100 μL methanol. The phases were subsequently analyzed by HPLC–qTOF-MS for structural identification.

3. Results and discussion

3.1. Total arsenic concentration in marine oils and in phases of the marine oils

The total arsenic concentrations of the oils ranged between 1.6 and 12.5 mg kg^{-1} oil (Table 3). This is in good agreement with previous studies of total arsenic in fish oils, where concentrations ranged between 0.2 and 16 mg As kg^{-1} [1,4]. Between 14% and 42% of the total arsenic in the oils were found in the MeOH 1 phase when the fish oils were partitioned into heptane and methanol (Fig. 1; Table 3). The aqueous methanol is more selective for the arsenolipids than for the other lipid components present in the oils as only 2% of the weight of the oil partitioned into this phase (data not shown) and an up-concentration of arsenic was hence seen for the polar MeOH 1 phase. The high concentration of arsenic in the polar phase compared to the more non-polar phase of fish oils is in accordance with other studies where up to 45% of the total arsenic was found in the polar methanol phase of fish oils [23,32] and of the oily fraction of tuna filet [22].

For all the fish oils analyzed the majority of arsenic partitioned into the more non-polar heptane phase (data not shown). This is in accordance with findings in previous studies of arsenic in the non-polar phases of fish oils [15,23,32]. The heptane phase of selected fish oils were therefore subjected to sequential extractions using methanol (MeOH 2) and acetonitrile (ACN). The additional extractions of the heptane phase increased the extraction efficiency of arsenic with 8–14% for the MeOH 2 phase and 1–4% for the ACN phase (Table 3). The liquid–liquid extraction resulted in a total extraction of arsenic into the polar phases (sum of MeOH 1, MeOH 2 and ACN) ranging from 28% to 45% (Table 3). The majority of arsenic was, however, found in the MeOH 1 phase of the fish oils.

3.2. Quantification of arsenolipids by HPLC–ICP-MS

The arsenic response factor was determined for every arsenic-containing compound found in the MeOH 1 phase using the approach described by Amayo and colleagues [18]. A blank sample was analyzed while a solution of arsenic (^{75}As) and internal standards (^{74}Ge and ^{115}In) were added post-column. The arsenic response increased by a factor of approximately 2 when the gradient increased from 30% methanol (start of analysis) to 100% methanol (r.t. 14 min) (Fig. 3). The enhancement effect of arsenic is often explained by a charge transfer from positively charged carbon ions to arsenic [25,26]. The effect is often an advantage when analyzing water-soluble arsenic compounds, where a constant addition of 3% methanol into the mobile phase can increase the sensitivity for arsenic [25]. However, when performing quantitative analysis using an organic gradient and a calibration standard with different r.t. than the analytes, it is necessary to compensate for the time-resolved variation in the arsenic response along the gradient [18,27]. When analyzing a blank sample with the gradient program and introducing a solution of ^{75}As, ^{74}Ge and ^{115}In post-column, the signal for both ^{74}Ge and ^{115}In was affected in a similar way to ^{75}As by the gradient (Fig. 3). The observed effect on the signal for ^{74}Ge by the organic gradient is in contrast to recent observations by Amayo and co-workers who did not see any specific changes in the ^{74}Ge signal when organic

Table 3
Total arsenic concentrations (mean ± 1SD, $n=3$, mg kg^{-1} oil) in whole oils, the aqueous methanol (MeOH 1) phase, the methanol (MeOH 2) phase and the acetonitrile (ACN) phases of fish oils; and the distribution (%) of arsenic into MeOH 1, MeOH 2 and ACN phases (of As in whole oil), respectively.

Sample	Whole oil	MeOH1		MeOH2		ACN		Sum extracted
	(mg kg^{-1})	As (mg kg^{-1})	As (%)	As (mg kg^{-1})	As (%)	As (mg kg^{-1})	As (%)	As (%)
Sand eel	7.96 ± 0.04	1.80 ± 0.06	23					
Herring	7.7 ± 0.1	1.87 ± 0.05	24					
Anchovy	8.3 ± 0.1	3.2 ± 0.2	39					
Salmon	1.61 ± 0.09	0.45 ± 0.05	28					
Cod liver oil	5.5 ± 0.1[a]	2.3 ± 0.2	42					
Seal oil	4.34 ± 0.03[a]	0.75 ± 0.04	17					
Mixed oil 1	7.39 ± 0.03	1.24 ± 0.08	17					
Blue whiting	8.7 ± 0.1	2.09 ± 0.08	24	1.25 ± 0.01	14	0.326 ± 0.004	4	42
Mixed oil 2	8.34 ± 0.01	2.20 ± 0.03	26	0.76 ± 0.09	9	0.23 ± 0.1	3	38
Commercial oil 1	11.0 ± 0.3	2.1 ± 0.1	19	0.90 ± 0.05[a]	8	0.066 ± 0.004	1	28
Commercial oil 2	12.5 ± 0.4[a]	3.07 ± 0.03	25	1.05 ± 0.1	8	0.19 ± 0.02	2	35
Commercial oil 3	4.9 ± 0.4	1.48 ± 0.07	30	0.63 ± 0.01	13	0.107 ± 0.001[a]	2	45

[a] $n=2$.

solvents were introduced [18]. The reason for the different observations may be ascribed to different instrumental settings, such as flow rates, carrier gas, makeup gas and type of nebulizer [18]. The ratio between the signals of arsenic and the internal standards (^{75}As/IS) was constant at approximately 0.9 for ^{75}As/^{74}Ge and 0.5 for ^{75}As/^{115}In throughout the analysis (Fig. 3). Consequently, the effect of the organic gradient and the potential matrix effects on elements were compensated for by continuous post-column introduction of an IS solution of ^{74}Ge and/or ^{115}In during the analysis.

Between four and seven arsenic-containing compounds were seen in the MeOH 1 phase of the fish oils when analyzed by reversed-phase HPLC–ICP-MS (peaks A–G, Fig. 2). The concentrations of the arsenic-containing compounds were determined by using the arsenic response factors for the peaks and the use of the calibration curves established from three different calibration standards, DMA, Ph_3AsO and the synthesized AsHC-C_{19} compound (Table 4). Using DMA as a calibration standard, the sum of the quantified AsFAs and AsHCs in the MeOH 1 phases had a recovery ranging from 91% to 104% when compared to the total arsenic measurements of the extracts (Table 4). Using Ph_3AsO as calibration standard, however, the recovery of arsenic was lower (between 64% and 89% of the total arsenic in the phases). When using the synthesized AsHC-C_{19} compound the recovery ranged from 110% to 122%, which is slightly higher than for the other calibration standards. The Limit of Detection (LOD) was estimated at 0.02 μg kg^{-1} for the arsenolipids with r.t. between 14 and 25 min. The LOD was calculated as 3 times the baseline noise, using DMA as a calibrant and taking the response factors into account. The sand eel oil was used to estimate the between-days precision of the quantification analysis and an RSD of 10% ($n=12$) of the sum of quantified arsenolipids was found using DMA as a calibration standard was obtained.

The results suggest that DMA can be used for quantification of the arsenolipids if compensating for the response factor variation of arsenic at the specific r.t. of the analytes. The use of DMA also reduces the time of analysis as the calibration curve can be made by flow-injection, in contrast to the use of Ph_3AsO and the synthesized AsHC-C_{19} as calibration standards, which demands full chromatographic runs for each standard solution. Quantification of the arsenolipids can also be done using a synthesized AsHC compound. The impurity of the synthesized compound highlights, however, the need for commercial available certified standards of the arsenolipids, which would additionally simplify the qualitative assignments of arsenolipids in HPLC–ICP-MS analysis.

Peaks E–G (Fig. 2) were the major arsenolipids seen in the MeOH 1 phases of all the marine oils, where peak E was the predominant peak in all of the marine oils examined, ranging from 0.178 ± 0.008 mg As kg^{-1} in the salmon oil to 2.69 ± 0.06 mg As kg^{-1} in commercial oil 3 using DMA as calibration standard. The concentrations of the minor peaks were less than 0.07 mg As kg^{-1} oil.

3.3. Structural identification of arsenolipids

In the selected oils, the structures of the four arsenic-containing peaks (compounds C–G, Fig. 2) detected in the MeOH 1 phase by HPLC–ICP-MS were identified by HPLC–qTOF-MS. The arsenic-containing peaks E, F and G (Fig. 2) correspond to the AsHCs; AsHC-C_{21}, AsHC-C_{15}, and AsHC-C_{17} (Table 1), respectively, while peak C correspond to two co-eluting AsFAs; AsFA-C_{21} and AsFA-C_{22}. The results from the HR-MS gave a relative error (Δm) for the calculated and found masses, $[M+H]^+$, within ±2 ppm for the arsenolipids detected. As an example the results for commercial oil 1 are listed here: AsHC-C_{21} ($C_{23}H_{38}AsO$: calculated 405.2133; found 405.2136; $\Delta m=0.7$ ppm), AsHC-C_{15} ($C_{17}H_{38}AsO$: calculated 333.2133; found 333.2135; $\Delta m=0.6$ ppm), AsHC-C_{17} ($C_{19}H_{42}AsO$: calculated 361.2446; found 361.2448; $\Delta m=0.6$ ppm), AsFA-C_{21} ($C_{23}H_{38}AsO_3$: calculated 437.2031; found 437.2032; $\Delta m=0.2$ ppm) and AsFA-C_{22} ($C_{24}H_{38}AsO_3$: calculated 449.2031; found 449.2033; $\Delta m=0.4$ ppm). The obtained MS/MS mass spectra of the compounds correspond well with the mass spectra earlier reported for the compounds, where m/z 104.9685 and m/z 122.9791 were seen as major fragment ions for compound AsHC-C_{15} and AsHC-C_{17} [18,19]. The ions with mass of m/z 104.9685 and m/z 122.9791 corresponded to the fragment ions $(CH_3)_2As^+$ and $(CH_3)_2AsOH_2^+$, and confirmed the presence of a dimethylarsinoyl moiety in the molecule [18,19].

These fragment ions were, however, not detected for the three double-bond containing compounds AsHC-C_{21}, AsFA-C_{21} and AsFA-C_{22}, although the collision energy was increased to 60 eV. Instead, several ions in the low mass area of the mass spectra were observed for these arsenolipids with double bonds. Fragment ions with m/z 119, 131, 145 and 159 were predominant for the arsenolipids with double bonds (data not shown), which are consistent with other studies reporting the MS/MS spectra of AsHCs with doubled bonds and AsFAs with double bonds [17,18]. The m/z ion 119 is dominant in the product ion MS spectra of (both) the AsHCs and AsFAs with double bonds, and is most likely the fragment ion $(CH_3)_2AsCH_2^+$. Several of the same fragment ions, which are observed for the unsaturated AsHCs and the unsaturated AsFAs in this study, were recently also observed for fatty acids (non-arsenic-containing) [20]. The fragments m/z 104.96 and 122.97, which are regarded as

exclusive fragment ions for arsenolipids, are observed with high signal intensities for saturated arsenolipids both in this work and in the work by others [17,20]. The mass spectrums of polyunsaturated arsenolipids reported by others [17,20] may, together with the results in this study, suggest that the m/z 104.96 and 122.97 fragment ions are not so easily produced from unsaturated arsenolipids as they are observed to have low signal intensities compared to the m/z 119, 131, 145 and 159 fragment ions. The double bonds in the structures may affect the fragmentation pattern for the unsaturated compounds, and therefore do not favour the formation of product ions m/z 104.96 and 122.97. The chemical structures of the arsenic-containing peaks B and D in the HPLC–ICP-MS chromatogram (Fig. 2) could not be determined by HPLC–qTOF-MS because of insufficient sensitivity by this technique.

The identification of the AsHCs and AsFAs in marine oils is consistent with previous findings where the AsHCs were identified in capelin oil [32], the AsFAs in oil of canned cod liver [14], and both AsHCs and AsFAs have been identified in fish meal of capelin [18], in cod liver [20] and in capelin oil [9]. The AsHCs and AsFAs consist of a dimethylarsinoyl moiety with a fatty acid carbon chain and hydrocarbon chain, respectively. The locations of the double bonds for compounds AsFA-C_{21}, AsFA-C_{22} and AsHC-C_{21} have not yet been determined. It has been assumed that the positions of the double bonds of unsaturated AsFAs and AsHCs are similar to well-known polyunsaturated fatty acids present in fish oil; e.g. docosehexanoic acid (DHA, 22:6, $n-3$) [14,15]. The first identified AsFAs and AsHCs contained all odd-numbered carbon chains [14,15]. Rumpler and co-workers [14] suggested that the dimethylarsinoyl moiety replaces the terminal CH_3 of fatty acids, resulting in AsFAs with odd-numbered carbon chains. However, the recent identifications of even-numbered AsFAs in fish meal of capelin [18], in herring filet [19] and in brown alga [17], indicate that both odd- and even-numbered AsFAs exists in marine samples, and that these compounds most likely have different biosynthetic starting points [19]. In this study the even-numbered AsFA-C_{22} was detected in commercial fish oils, suggesting that the even-numbered AsFAs are present in oils of marine fish. Further studies need to be conducted to find the origin, as well as the biological role of the AsFAs and AsHCs in marine organisms.

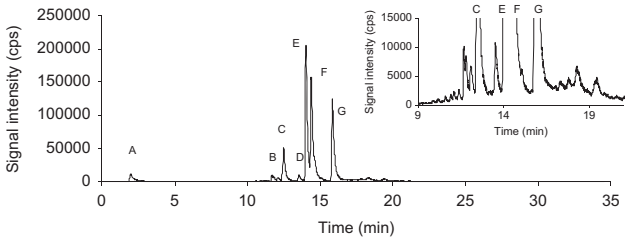


Fig. 2. Chromatograms of the aqueous methanol phase (MeOH 1) of commercial oil 1 analyzed by HPLC–ICPMS.

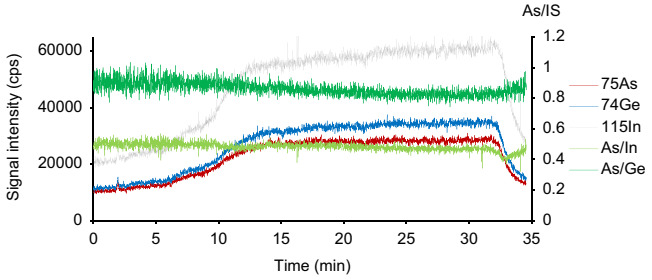


Fig. 3. The effect on signal for arsenic (^{75}As), germanium (^{74}Ge) and indium (^{115}In) when increased amount of methanol (30–100%) is introduced to the ICP-MS. A solution of arsenic (50 ppb), germanium (100 ppb) and indium (50 ppb) is added post-column by the peristaltic pump, and a blind sample is injected onto the column and analyzed with the gradient program 1 given in Table 2.

3.4. Arsenolipid profiles in the oils

The AsHCs (peaks E–G, Fig. 2) were the major arsenolipids identified in the MeOH 1 phases of all the marine oils. The AsHCs constituted for 69–92% of the total arsenic in the MeOH 1 phases of the fish oils (Table 5). These findings are consistent with a previous study of some of the same fish oils; the AsHCs accounted for 78–92% of the total arsenic in the aqueous methanol phase of oils of sand eel, herring, blue whiting, anchovy and mixed oil when analyzing by GC–ICP-MS [23]. The AsHCs have also been found to account for over 90% of the total arsenic in extracts of a fish meal sample of capelin [18]. The distribution of the AsHCs in the MeOH 1 phase of the fish oils correspond well with the distribution previously seen for sand eel oil, herring oil and mixed oil when analyzing by GC–ICP-MS [23]. However, the results for blue whiting and anchovy oil differ somewhat from previous observations by the GC–ICP-MS analysis, as the abundance of AsHC-C_{21} was higher for the HPLC–ICP-MS analysis than observed by the GC–ICP-MS analysis [23]. Variations in the extraction efficiency could account for this difference, in addition to the possible degradation of the arsenolipids in the samples.

Table 4
Concentrations of arsenolipids (sum of quantified peaks; mean ± 1SD, $n=3$, mg kg^{-1} oil) in MeOH 1 phase of oils using dimethylarsinate (DMA), triphenylarsine oxide (Ph_3AsO) and a synthesized arsenic-containing hydrocarbon (AsHC-C_{19}) as calibration standard in reversed-phase HPLC–ICP-MS. The recovery (%) is determined comparing the concentration of sum arsenolipids to the total As concentration.

Sample	Total As (mg As kg^{-1})	DMA (mg As kg^{-1})	Recovery (%)	Ph_3AsO (mg As kg^{-1})	Recovery (%)	Synthesized AsHC-C_{19} (mg As kg^{-1})	Recovery (%)
Sand eel	1.80 ± 0.06	1.7 ± 0.1[a]	94	1.19 ± 0.05	66		
Herring	1.87 ± 0.05	1.75 ± 0.1	94	1.19 ± 0.2	64		
Anchovy	3.2 ± 0.2	3.1 ± 0.3	97	2.4 ± 0.3	75		
Salmon	0.45 ± 0.05	0.46 ± 0.05[a]	102	0.40 ± 0.04[a]	89		
Cod liver oil	2.3 ± 0.2	2.31 ± 0.07	100	2.0 ± 0.06	87		
Seal oil	0.75 ± 0.04	0.73 ± 0.03	97	0.53 ± 002	71		
Mixed oil 1	1.24 ± 0.08	1.13 ± 0.04	91	0.86 ± 0.03	69		
Blue whiting	2.09 ± 0.08	2.08 ± 0.07	100	1.5 ± 0.05	71	2.40 ± 0.07	115
Mixed oil 2	2.20 ± 0.03	2.12 ± 0.01	96	1.59 ± 0.01	71	2.46 ± 0.01	112
Commercial oil 1	2.1 ± 0.1	2.0 ± 0.2	95	1.6 ± 0.2	76	2.3 ± 0.2	110
Commercial oil 2	3.07 ± 0.03	3.14 ± 0.04	102	2.33 ± 0.03	76	3.63 ± 0.05	118
Commercial oil 3	1.48 ± 0.07	1.54 ± 0.09	104	1.14 ± 0.07	77	1.8 ± 0.1	122

[a] $n=2$.

Table 5
The distribution (%) of arsenic-containing peaks in the MeOH 1 phase of selected fish oils, where A is the void, B is an unknown compound, C is the two co-eluting AsFAs; AsFA-C_{21} ($C_{23}H_{38}AsO_3$) and AsFA-C_{22} ($C_{24}H_{38}AsO_3$), D is an unknown compound, E is AsHC-C_{21} ($C_{23}H_{38}AsO$), F is AsHC-C_{15} ($C_{17}H_{38}AsO$) and G is AsHC-C_{17} ($C_{19}H_{42}AsO$).

Fish oil sample	A (%)	B (%)	C (%)	D (%)	E (%)	F (%)	G (%)
Blue whiting	2	2	7	1	41	28	18
Mixed oil 2	5	1	4	1	27	38	23
Commercial oil 1	10	2	14	1	30	23	19
Commercial oil 2	5	2	4	1	43	23	21
Commercial oil 3	7	4	8	2	31	31	18

Interestingly, several minor peaks were observed in the chromatograms of the MeOH 1 phases, indicating the presence of a number of additional unidentified arsenolipids. An example is given in Fig. 2 (insert) where the chromatogram indicates the presence of more than 20 different arsenolipids in the commercial fish oil 1. However, the concentration of the compounds was too low to identify the structures of the compounds by qTOF-MS. All fish oils, with exception of the salmon oil, contained AsFAs in the MeOH 1 phase analyzed. The absence of the AsFAs in the salmon oil may be explained by the low total arsenic concentration in the salmon oil compared to the other oils. The observed differences may also be related to the origin of the fish; the salmon oil is obtained from farmed salmon, while the other oils are obtained from wild caught fish.

Selected fish oils were subjected to additional extractions using methanol (MeOH 2) and acetonitrile (ACN) (Fig. 1) in order to increase the recovery of arsenic. The MeOH 2 and ACN phases of these fish oils were analyzed by prolonging the gradient in the HPLC program at 100% methanol for further 30 min (HPLC program 2, Table 2). Different profiles were seen for the MeOH 2 and ACN phases compared to the MeOH 1 phase. In addition to the detection of AsHCs in the fish oils, the MeOH 2 phase contained one peak of co-eluting arsenic compounds, with r.t. of 22–30 min. The compounds, having more non-polar characteristics than the AsFAs and the AsHCs, were seen in both the MeOH 2 and the ACN phases, although, in lower abundance in the ACN phases. The MeOH 2 and ACN phases of one of the fish oils (commercial oil 2) were also analyzed by keeping the gradient at 100% methanol for an extra 60 min to allow more non-polar compounds to elute from the column. No additional arsenolipids were detected in the chromatograms. The major peak detected in the MeOH 2 phase of fish oils was collected manually from the HPLC column into HPLC vials, and the phase was analyzed by HPLC–qTOF-MS for structural determination by searching for the precursor ions of the m/z 123 and m/z 105 fragment ions. Arsenic-containing compounds could, however, not be detected by this approach, presumably because of too low sensitivity due to the presence of abundant lipid components in the phase.

4. Conclusion

Several fish oils were analyzed for arsenolipids using reversed-phase HPLC–ICP-MS. By determining the time-resolved response factor for arsenic at different r.t. in the HPLC gradient, the arsenolipids were quantified using different arsenic-containing calibration standards. The best recovery was obtained when using DMA as calibration standard. Three AsHCs and two AsFAs were identified in the aqueous methanol phases of all the marine oils using HPLC–qTOF-MS, with the exception of the salmon oil which only contained the AsHCs. By sequential extraction of the heptane phase using methanol and acetonitrile the extraction efficiency increased by 9–18%; resulting in a total recovery of 28–45%.

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