Talanta 110 (2013) 144–152

Contents lists available at SciVerse ScienceDirect

Talanta

journal homepage: www.elsevier.com/locate/talanta

The high diversity of arsenolipids in herring fillet (*Clupea harengus*)

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ARTICLE INFO

Article history:
Received 30 November 2012
Received in revised form
6 February 2013
Accepted 13 February 2013
Available online 6 March 2013

Keywords:
Arsenic species
Arsenolipids
Fish
Herring
ESI-MS/MS
ICP-TOF-MS

ABSTRACT

Arsenolipids represent a relevant step in the biosynthesis of organoarsenicals from inorganic arsenic compounds. Their fate after human consumption is still uncertain.

By means of a HPLC-ICP-MS/ESI-Q-TOF-MS method, 16 lipid soluble arsenic compounds, including seven formerly unknown organoarsenicals, have been identified in commercial herring fillet.

The structural assignment was done by exact mass and high resolution MS/MS data. This is the first identification of arsenolipids in herring (*Clupea harengus*). They contribute with (3.6 ± 0.2) mg kg^{-1} arsenic to 62.3% of the total arsenic content of (5.7 ± 0.3) mg of arsenic per kg dry mass. Current studies indicate that a metabolization by humans to cancerous dimethylarsinic acid is very likely. The presented results are highly relevant as herring is a very popular food fish species in Europe. Moreover, the screening of different fish species revealed that arsenolipids are more widespread than previously assumed.

1. Introduction

Due to the variety, differences in toxicity and mobility of arsenic species, there is immense academic interest in the species-specific analysis of arsenic. In 2009 the risk of arsenic consumed with food to human health was assessed by the European Food Safety Agency (EFSA). 98% of the more than 100,000 collected data sets were concerned with the total arsenic content [1]. Arsenosugars, arsenolipids, monomethylarsonic acid (MMA) and dimethylarsinic acid (DMA), the latter two are carcinogenic, were not considered by the European Commission because of the lack of data. As a result, more species-specific studies for different foods as well as more toxicological and metabolism studies were recommended [2–4]. Therefore, we focused on the analysis of commercial fish fillets to assess the structures of the arsenic-containing fatty acids (As-FA) and hydrocarbons (As-HC) which they contain. We investigated Atlantic herring (*Clupea harengus*), wolffish (*Anharhichas sp.*), plaice (*Pleuronectes platessa*), pilchard (*Sardina pilchardus*), mackerel (*Scomber scombrus*), salmon (*Onhcorhynchus keta*) and cape hake (*Merluccius capensis*) extracts with HPLC-ICP-MS in this study.

These fish species were chosen because of their commercial availability and their fat content of $> 1\%$ wet mass. Especially the Atlantic herring (*Clupea harengus*) with a total catch in 2010 of 72,852 mt (according to the National Marine Fisheries Service (NMFS)) in the US and 2247 mt in Europe (information from the International Council for the Exploration of the Sea (ICES)) has about 16% fat in wet muscle tissue [5,6]. In 2010 herring accounted for 20.0% and salmon for 12.8% of the fish consumed in Germany (according to Fisch-Informationszentrum e.V.—FIZ) [7]. Already in 1968 Lunde suggested that oil-rich fish like herring (*Clupea harengus*) may contain lipid soluble arsenic in their muscle tissue [8]. Although marine samples have been extensively investigated, little work has been done to identify the structure of arsenic species in their lipid-soluble fraction. In the late 1960s Lunde was the first to determine the arsenic content of oils from fish and algae [9,10]. He found concentrations ranging from 8 to 20 mg of arsenic per kg of fish oil. Since Lunde analyzed the structure of lipid-soluble arsenic, three classes of arsenolipids have been characterized: As-HC [11], As-FA [12] and the phospholipid-like arsenosugar-phospholipids (As-SugPL) [8,13,14]. All three classes have been comprehensively reviewed by Dembitsky and Levitsky in 2004 as well as by Sele et al. [15,16].

As-HC can be found in cod liver (*Gadus morhua*) [17], tuna [18], cod (*Gadus morhua*) [19], capelin (*Mallotus villosus*) [11,19], fish muscle [20] and, together with As-FA, in fish oils [11,12,20]. Structural precursors of arsenolipids are presumed to be in algae [14]. Through the consumption of algae by fish, these are incorporated and further metabolized.

Beside the majority of even-numbered fatty acids, fish oil can contain fatty acids with branched or odd-numbered carbon chains [21,22]. Therefore, the As-FA and the As-HC are found with both odd and even chain lengths [11,19]. There are several theories

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http://dx.doi.org/10.1016/j.talanta.2013.02.051

concerning how As-HC are formed [23–25] and little is known about the toxicity of arsenolipids. The major metabolite of arsenolipids, ingested with cod liver and cod liver oil, in the urine of two volunteers was the cancerous DMA with 41% and 73% of the total urinary arsenic content [26]. Elevated DMA levels reported after consumption of fatty fish like herring or mackerel [27] were found not to be caused by the metabolism of inorganic arsenic or ingestion of DMA [28]. Arsenobetaine was excluded as a potential DMA precursor. This is supported by existing metabolization data on arsenobetaine [29,30]. Consequently, the DMA must result from the transformation of other arsenic species in herring. This and the fact that considerable amounts of structurally unidentified arsenic are contained in the fillet of fatty fish clarify the need for further investigations.

The aim of our work was to elucidate the structures of lipid soluble arsenic species in herring to provide further insight into possible metabolization pathways and to explore the variety of lipophilic arsenic species in marine fish. We wanted to provide data on the different arsenic species in herring to improve the toxicological information available.

2. Materials and methods

All analyzed fish materials were commercial available. We investigated Atlantic herring (*Clupea harengus*), wolffish (*Anharhichas sp.*), plaice (*Pleuronectes platessa*), pilchard (*Sardina pilchardus*), mackerel (*Scomber scombrus*), salmon (*Onhcorhynchus keta*) and cape hake (*Merluccius capensis*). For each species, about 500 g of frozen fillets were bought in a shop and pooled to form a single sample. According to the suppliers, the salmon was caught in the Pacific Ocean, the cape hake in the southeast Atlantic (Food and Agriculture Organization of the United Nations major fishing area 47; in short, FAO47) and the rest of the fish in the northeast Atlantic (FAO27). Mackerel, pilchard and herring were filleted by hand. Their characteristics are summarized in Table 1. All samples were immediately lyophilized with a GAMMA 1–16 LSC (Martin Christ Gefriertrocknungsanlagen GmbH, Osterode, Germany). For the grinding and homogenization of the fish fillets, a centrifugal mill type ZM 1000 (Retsch GmbH, Haan, Germany) was used. During the milling, all samples were cooled using liquid nitrogen. The samples were then lyophilized again and the remaining sample moisture content was determined by a Karl-Fischer-Coulometer 756 (Metrohm, Herisau, Switzerland). During this study, all lyophilized fish samples were stored for six to twelve months at −21 °C under argon to avoid sample degradation. As rotary evaporator, a Laborota4000 Efficient with HB digital heating bath (both Heidolph, Schwabach, Germany) and a CVC 2 vacuum pump (Vacuumbrand, Wertheim, Germany) were employed. The ultra pure water used during all analysis steps was from Milli-Q (18.2 MOhm).

2.1. Total arsenic determination by TXRF

The total digestion of samples was achieved with an Ultra-CLAVE III from MLS MikrowellenLaborSysteme (Leutkirch, Germany). 200–300 mg of fish fillet were weighed into Teflon cells for microwave assisted digestion. To achieve complete disintegration of the sample, 25 μL ultra pure water, 2 mL sub-boiled nitric acid, 4 mL H_2O_2 pa. and 200 μL HF (conc., suprapur), all from Merck (Darmstadt, Germany), were added. The mixture was allowed to react overnight before the microwave assisted digestion in a high pressure reactor was carried out at 1 kW. The samples were quantitatively transferred into cleaned plastic bottles and diluted to 10–15 mL with ultra pure water after addition of 400 μL gallium (Ga) solution with a concentration of 50 mg L^{-1} (20 μg absolute) as internal standard and 200 μL HCl (subb.) for stabilization purposes. The Ga solution was prepared from the ICP-Merck standard (Darmstadt, Germany) by dilution with ultra pure water. The low concentrated samples (salmon and mackerel) were diluted to 12 mL after the addition of 30 μL Ga solution (c=10 mg L^{-1}) and 200 μL HCL (subb.). Both sample solutions were concentrated to 400 μL using a sand bath. The X-ray fluorescence measurements were performed with a TXRF 8030C (Atomika Instruments GmbH, Oberschleißheim, Germany). The system was equipped with a 2.5 kW X-ray tube with tungsten molybdenum mixed anode, double-multilayer monochromator and an 80 mm^2 SiLi detector (Oxford Instruments, Oxfordshire, UK). Due to the HF content, the sample solutions were measured on commercially available sapphire TXRF sample carriers (diameter 30 mm, thickness 3 mm). 10–20 μL of the sample solution were pipetted into the middle of the sample carrier and were evaporated by heating on a hot plate for 10 min at 70–80 °C. For the measurements, the X-ray tube was operated with 50 kV and 47 mA. The molybdenum K-line (17.48 keV) was used for excitation and filtered by a Zr20 filter. The lifetime was 1000 s. The amount of arsenic was calculated with reference to the dry weight and using gallium as internal standard. The accuracy was verified by analysis of the tuna fish reference material BCR-627 (IRMM, Geel, Belgium) with each of the two digestion cycles which resulted in (5.06 ± 0.06) mg kg^{-1} and (4.6 ± 0.1) mg kg^{-1} total arsenic (cert. value (4.8 ± 0.3) mg kg^{-1}). The limit of determination for the acquisition parameters used was calculated according to Klockenkämper to 16 pg arsenic absolute per sample carrier [31].

Table 1
Fishing area, water, protein and fat content referring to wet weight; total arsenic concentration and percentage of arsenic extractable with methanol, 50% methanol and acetone (n=3, determined by TXRF based on dry weight), concentrations of As III, As V and DMA were measured by HPLC-ICP-MS and the amount of arsenic originating from AsB was determined by HCPL-ESI-MS/MS, all referring to dry weight; extractable arsenic was given as the sum of arsenic contained in 50% methanol and acetone extraction; the data for protein and fat content was given by the distributor, water content was cited from Fisch-Informationszentrum e.V. (FIZ, Große Elbstraße 133, 22,767 Hamburg). nd.=below limit of detection.

Fish species	Fishing area	Water (%)	Protein (%)	Fat (%)	c(As_{total}) (mg kg^{-1})	c(As III) (mg kg^{-1})	c(As V) (mg kg^{-1})	c(As_{DMA}) (mg kg^{-1})	AsB as As (%)	MeOH As (%)	50% MeOH As (%)	Acetone As (%)	Extractable As (%)
Herring	FAO 27	63	22	16	5.7 ± 0.3	0.021 ± 0.004	< 0.09	0.103 ± 0.007	11.0 ± 0.9	68 ± 5	30 ± 1	62 ± 4	93 ± 5
Salmon	Pacific	66	20	14	0.84 ± 0.08	0.020 ± 0.008	< 0.09	< 0.02	47 ± 6	102 ± 11	73 ± 9	35 ± 5	108 ± 14
Mackerel	FAO 27	68	19	12	3.4 ± 0.3	0.020 ± 0.005	< 0.09	< 0.02	50 ± 5	85 ± 10	68 ± 10	26 ± 4	94 ± 14
Pilchard	FAO 27	74	20.8	3.9	10.4 ± 0.2	0.013 ± 0.006	nd.	0.098 ± 0.002	63 ± 4	90 ± 4	69 ± 4	28.2 ± 0.8	97 ± 5
Wolffish	FAO 27	78	19.1	3	88 ± 2	0.131 ± 0.009	< 0.09	nd.	87 ± 5	94 ± 4	100 ± 4	1.9 ± 0.4	102 ± 4
Cape hake	FAO 47	80.5	17.6	1.7	5.5 ± 0.3	0.007 ± 0.001	< 0.09	0.063 ± 0.005	81 ± 5	90 ± 10	88 ± 6	1.4 ± 0.2	90 ± 6
Plaice	FAO 27	79	14	1.3	80.9 ± 0.2	0.039 ± 0.005	nd.	nd.	78 ± 8	99.1 ± 0.9	99 ± 2	1.7 ± 0.1	101 ± 2

2.2. Determination of 50% methanol, methanol and acetone extractable arsenic by TXRF

500 mg of fish sample was extracted based on the work of Cao et al. using an accelerated solvent extraction ASE 200 (Dionex Corporation, Sunnyvale, USA) and picograde acetone (LGC, Wesel Germany) as extracting agent for lipophilic organoarsenicals, and methanol:water (50:50, v/v) for hydrophilic compounds, respectively [32]. Additionally, methanol was used as extracting solvent for another subsample. To avoid adsorption of arsenic species, 250 μL sub-boiled nitric acid was pipetted into the ASE extraction vials ahead of the extraction. The Ga standard was added as solution in ultra pure water for the extraction of polar organoarsenicals. For the determination of the nonpolar compounds, the Ga stock solution was diluted in the corresponding extraction solvent used. After first tests, samples were spiked and concentrated under a nitrogen stream at 40 °C to give a solution containing approximately 1 mg kg^{-1} arsenic and gallium. Sample solutions were measured on regular, commercially available quartz TXRF sample carriers (diameter 30 mm, thickness 3 mm), which were covered with a silicon layer by drying a silicon solution in isopropyl alcohol (SERVA, Heidelberg, Germany). Further sample preparation of the gallium-spiked sample solutions were carried out as described above in Section 2.1. The accuracy of the extraction procedures was verified by calculating the total extractable amount of arsenic, which resulted in extraction efficiencies between 89.8% and 107.8% (see Table 1).

2.3. Determination of arsenobetaine by HPLC-ESI-MS/MS

The arsenobetaine determination was based on a work by Lischka et al. [33]. Extraction was carried out after addition of twofold ^{13}C-labeled arsenobetaine[1] by ASE (Dionex Corporation, Sunnyvale, USA) with methanol:water (50:50, v/v) as extracting agent. The raw extracts were collected in 60 mL screw cap vials and 20 mL dichloromethane (LGC, Wesel, Germany) was added. After 10 min of shaking using a HS 501 digital horizontal shaker with 300 rpm (IKA, Staufen, Germany) to remove nonpolar matrix compounds samples were allowed to stand until phase separation was complete. The upper aqueous layer was filtered with 0.2 μm PTFE filters (Phenomenex, Aschaffenburg, Germany) and diluted 1:10 with ultra pure water into 2 mL polypropylene vials (Chromophor, Füssen, Germany). In case of reference material samples (BCR-627, IRMM, Geel, Belgium) filtered extracts were diluted 1:100 with ultra pure water. Arsenobetaine was determined by HPLC-ESI-MS/MS on a Supelcosil LC-SCX (250 mm × 3.0 mm, 5 μm, Supelco, Bellefonte, Pennsylvania, USA) with twofold ^{13}C-labeled arsenobetaine as internal standard. The equipment used were a HPLC 1100 (Agilent Technologies, Waldbronn, Germany) coupled to an ESI-MS API 4000™Q TRAP™LC-MS/MS (AB SCIEX, Darmstadt, Germany). The moisture assessment was carried out simultaneously with the analysis. The calibrant used was the NMIJ CRM 7901-a (NMIJ, Tsubaka, Japan) diluted in ultra pure water. Eluent A consisted of 1% acetonitrile in ultra pure water. Eluent B is a 40 mM ammonium formate buffer (reagent grade, Sigma Aldrich, Steinheim, Germany) containing 1% acetonitrile in ultra pure water at pH 3.1. The injection volume employed was 20 μL and the gradient programme used was 3 min with 82.5% A and a linear gradient from 3–7 min to 0% A. The analyte was detected in continuous MRM mode (multiple reaction monitoring; *m/z*179/120 as qualifier and 179/105 as quantifier for arsenobetaine, *m/z*181/120 as qualifier and 181/105 as quantifier for internal standard) using positive ionization at 2000 V and 450 °C, 50 psi curtain gas, 50 psi - gas1 and 75 psi gas2. The results were corrected by the remaining water content in the samples. The accuracy was verified by analysis of the tuna fish reference material BCR-627 (IRMM, Geel, Belgium), which resulted in(8.5 ± 0.7) mg kg^{-1} arsenobetaine (cert. value (9.3 ± 0.5) mg kg^{-1}).

[1] Synthesis described by Lischka et al. [33].

2.4. Species-specific analysis by HPLC-ICP-MS

The extraction was done as described for the determination of arsenobetaine but without the use of a labeled internal standard. The upper aqueous layer was filtered with 0.2 μm PTFE filters (Phenomenex, Aschaffenburg, Germany) into 2 mL polypropylene vials (Chromophor, Füssen, Germany). The samples were analyzed without further dilution using a HPLC Series 1100 (Agilent Technologies, Waldbronn, Germany) coupled to a 7500cs ICP-MS (Agilent Technologies, Waldbronn, Germany). The AsB reference material NMIJ CRM 7901-a, As(V) reference material NMIJ CRM 7912-a, DMA reference material NMIJ CRM 7913-a (all NMIJ, Ibaraki, Japan), mono sodium acid methane arsonate sesquihydrate (98%, Supelco, Bellefonte, Pennsylvania, USA) and arsenic trioxide (99.999%, Heraeus, Hanau, Germany) were used as calibrants. The chromatographic conditions used were an injection volume of 5 μL, a column temperature of 40 °C and a flow rate of 0.3 mL min^{-1} using a IonPac AG7 precolumn (20 mm × 2.0 mm 10 μm, Dionex Corporation, Sunnyvale, USA) and a IonPac AS7 analytical column (250 mm × 2.0 mm 10 μm, Dionex Corporation, Sunnyvale, USA). Eluent A consisted of 0.5 mM nitric acid, 0.05 mM benzene-1,2-disulfonic acid dipotassium salt and 2% methanol in ultra pure water. Eluent B is a solution of 50 mM nitric acid, 0.05 mM benzene-1,2-disulfonic acid dipotassium salt and 2% methanol in ultra pure water. The gradient program consisted of 4 min with 100% A, a linear gradient from 4–18 min to 50% A followed by an equilibration with 100% A for 10 min. The internal standard solution containing 1 μg g^{-1} Se (6.5% nitric acid v/v) was infused post column with 24 μL min^{-1} by peristaltic pump. The samples were measured by Quadrupole ICP-MS with collision cell in He-mode using a MicroMist concentric nebulizer. In the method, the following isotopes were included: the element of interest ^{75}As, the internal standard element ^{82}Se, and ^{37}Cl and ^{35}Cl as isobaric interference. Samples were measured with 1530 W RF power, 9.4 mm sample depth, 15 L min^{-1} plasma gas flow rate, 0.76 L min^{-1} carrier gas flow rate, 0.19 L min^{-1} make up gas flow rate, 4.7 mL min^{-1} collision cell gas (He), 7.5% oxygen as optional gas, and platinum sample cone and skimmer cone. One point per mass and one second as integration time for *m/z* 75 (As) and 82 (Se, internal standard) and 0.5 s integration time for *m/z* 35, 37 (Cl), respectively were used. All chromatograms were corrected point to point with the internal standard Se. The accuracy was verified by analysis of the tuna fish reference material BCR-627 (IRMM, Geel, Belgium), which resulted in (9.4 ± 0.2) mg kg^{-1} arsenobetaine (cert. value (9.3 ± 0.5) mg kg^{-1}) and (0.24 ± 0.02) mg kg^{-1} DMA (cert. value (0.28 ± 0.04) mg kg^{-1}). The limits of quantification (LOQ) were determined by the calculation of nine times the standard deviation of the blank. The LOQ resulted for As(III) in 0.0015 mg kg^{-1}, for As(V) in 0.09 mg kg^{-1}, for MMA in 0.007 mg kg^{-1} and for DMA in 0.017 mg kg^{-1}.

2.5. Exact mass determination by HPLC-ICP-MS/ESI-Q-TOF-MS

For the exact mass determination, 10 g of homogenized freeze dried fish fillet (prepared as described in Section 2) was ultrasonic-assisted extracted with 100 mL HPLC-grade methanol (Labscan, Gliwice, Poland). After centrifugation for 5 min with a Rotafix 32A (Hettich, Tuttlingen, Germany) at 13,400 rpm, the extract was concentrated to 1 mL. It was fractionated by preparative column chromatography on silica gel (0.063–0.200 mm)

Table 2
Parameters for LC-ICP-MS/ESI-MS.

	Conditions	
LC		
Column	Atlantis dC_{18} (5 μm, 4.6 × 150 mm, Waters)	
Flow rate	1 mL min^{-1}	
Mobile phase	Eluent A	0.1% formic acid in water
	Eluent B	0.1% formic acid in methanol
Gradient	0–45 min	0 to 100% B (linear)
	45–60 min	100% B
ESI-Q-TOF-MS		
Ionization	Positive mode	
Fragmentor voltage	175 V	
Capillary voltage	3500 V	
Mass range	100–900 amu	(TOF-MS mode)
	50–600 amu	(Q-TOF-MS mode)
Gas temperature	350 °C	
Drying gas	10 L min^{-1}	
Nebulizer pressure	20 psi	
Sheath gas temperature	400 °C	
Sheath gas flow	12 L min^{-1}	
Collision energy	From 20 to 30 eV	(Q-TOF-MS mode)
ICP-MS		
RF power	1600 W	
Plasma gas flow rate	Ar 15 L min^{-1}	
Optional gas	12% (20% O_2 in Argon)	
Carrier gas flow	0.6–0.7 L min^{-1}	
Sample depth	8–9 mm	
Element monitored	*m/z*75 (As)	

with a gradient from 100% ethyl acetate to 100% methanol (HPLC grade, Merck, Darmstadt, Germany). The single fractions were evaporated to dryness, re-dissolved in methanol and centrifuged. The supernatant was analyzed. For arsenic species analysis, the single fractions were analyzed using an HPLC-ICP-MS/ESI-Q-TOF-MS system consisting of a UPLC Series Infinity 1290 (Degasser, binary pump, thermostated autosampler) coupled with an ICP-MS 7500ce and Accurate Mass Q-TOF LC-MS 6530 in parallel (all Agilent Technologies, Santa Clara, USA) by splitting the mobile phase 1:5 with a flow splitter (Analytical Scientific Instruments, Inc., California, USA). The mobile phase was prepared employing HPLC grade methanol (Merck, Darmstadt, Germany) and formic acid (Fluka, Buchs, Switzerland). The injection volume used was 20 μL. For the detection of the arsenic compounds, the ICP-MS peaks at *m/z* 75 (As) were compared with the peaks obtained by the ESI-Q-TOF-MS detector after their separation by means of reversed-phase chromatography. The conditions for the separation and detection are listed in Table 2. After optimization for compounds **10** and **13**, a collision energy of 30 eV was chosen. Arsenolipids **8**, **9**, **11**, **12**, **15** and **16** were fragmented with 25 eV. For the others, 20 eV was used.

3. Results and discussion

In this study, the fillets of seven different fish species were investigated with respect to their arsenolipid composition. For a better comparability to other studies, their total arsenic content in dry weight and the percentage of polar arsenicals extractable by 50% methanol and nonpolar organoarsenicals extracted with acetone were determined by TXRF (Table 1). The measurement uncertainty was calculated taking into account the uncertainty of the measurement and the uncertainty of the total arsenic determination. A loss of arsenic species during lyophilization could be ruled out by assessing the total arsenic content of milled herring fillet before and after this process.

Compared to other studies, the total arsenic content of the investigated fish samples were in the normal range for their species [34,35]. The two fish species with more than 80 mg kg^{-1} arsenic based on dry mass (plaice and wolffish) were both benthic feeders. This is in agreement with Luten et al., who observed a total arsenic content in plaice fillets of 5.3–135 mg kg^{-1} wet weight [36] and Sloth et al., who found high arsenic concentrations in wolffish [34]. In a recent ecosystem study, benthic feeders had generally incorporated higher amounts of arsenic than pelagic feeders [37]. Comparing sardines originating in the Yellow Sea and pilchards caught in the North Sea, not 4.6% as previously published, but 28.2% of the total amount of arsenic existed in the form of nonpolar arsenic species [32].

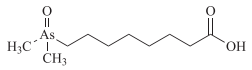
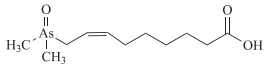
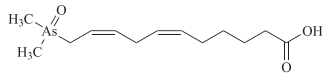
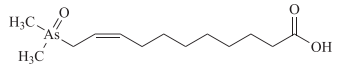
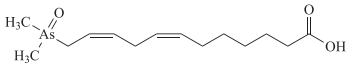
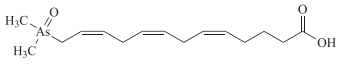
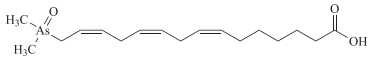
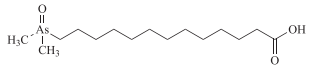
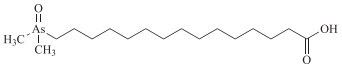
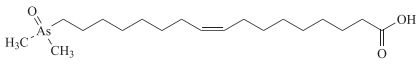
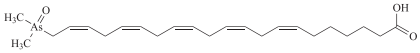
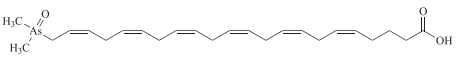
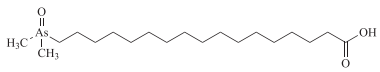
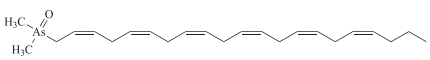
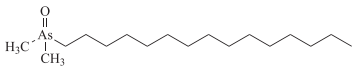
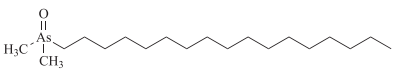
By comparing fat and lipid soluble arsenic content our data support the assumption of Sele et al. that the amount of lipid soluble arsenic rises in proportion to the total lipid content of the fish species [16]. The amount of arsenic in the form of arsenolipids corresponds in a good approximation to the arsenic extractable with acetone. The methanolic extracts are not applicable to determine the arsenolipid amount because they can additionally contain arsenobetaine, arsenic acid, MMA and DMA. Considering the measurement uncertainties, the sum of acetone-extractable arsenic and arsenobetaine (the main polar arsenic species) equals the amount of methanol-extractable arsenic. The seven fish species tested in this study showed a good correlation between total fat content and percentage of acetone-extractable arsenic. The Pearson coefficient was calculated to be 0.88.

To our knowledge, this is the first study concerning nonpolar arsenic species in herring. Seven new compounds were detected besides the nine already published organoarsenicals (for structures and references, see Table 3). Saturated and unsaturated As-FA and As-HC with odd and even chain numbers were identified. The arsenolipids were measured with exact mass in the methanolic herring extract after silica clean up as described in Section 2.5. For identification purposes, a methanolic raw extract was chosen because it showed a higher extraction efficiency than acetone for the medium polar arsenolipids eluting from 10 to 30 minutes from the Atlantis dC_{18} column. The main amount of arsenolipids was contained in fraction 12 and 13 of the silica clean up. Both fractions eluted with 100% methanol. Fig. 1 shows the HPLC-ICP-MS chromatograms of the corresponding fractions. The peaks of the identified compounds were numbered according to their elution order on a RP column. Hereinafter the same numbers are used to name them (for structures, see Table 3). The compounds eluting from 45 to 60 min are still unidentified due to their low ionization yield in the electrospray. A comparison of the data resulting from the ICP-MS and the ESI-Q-TOF-MS is presented in Fig. 2. For the overlay, the chromatograms of fraction 13 were corrected for the offset time between the two detectors.

The determined arsenolipids with their exact masses, proposed structures, corresponding MS/MS fragments and related literature are listed in Table 3. According to the resulting fragments, the protonation occurs in the electrospray at the dimethylarsinoyl group. All compounds showed a fragment with *m/z*122.9791 for the dimethylarsinoyl and *m/z*104.9685 for the dimethylarsinyl group after fragmentation. These MS/MS fragments can be used to screen for unknown arsenolipids. Only for the compounds **3**, **5** and **12** could further fragments be detected, as outlined in the corresponding fragmentation mechanisms (see Fig. 3). The positions and conformations of the double bonds in the arsenolipids are only postulated and were not experimentally verified.

The arsenolipids found are quite complementary to the common fatty acids in herring fillet [22,38]. Most compounds have shorter carbon chains than the main native FA. Eight of the detected organoarsenicals have no reported analogue in herring

Table 3
Exact mass data and results of MS/MS experiments.

No.	RT (min)	Ion Formula	Proposed structure	m/z_{exp}	m/z_{calc}	$\Delta m/z$ (ppm)	Ref.	Product ions	$\Delta m/z$ (ppm)
1	13.9	$C_{10}H_{22}AsO_3$		265.0772	265.0779	−2.6	In this work	247.0684=$[M\text{-}H_2O+H]^+$	3.2
								122.9786=$(CH_3)_2AsOH_2^+$	0.0
								104.9682=$(CH_3)_2As^+$	1.9
2	15.7	$C_{11}H_{22}AsO_3$		277.0782	277.0779	1.1	In this work	259.0671=$[M\text{-}H_2O+H]^+$	−1.2
								122.9781=$(CH_3)_2AsOH_2^+$	−4.1
								104.9683=$(CH_3)_2As^+$	2.9
3	20.9	$C_{13}H_{24}AsO_3$		303.0937	303.0936	0.3	In this work	285.0822=$[M\text{-}H_2O+H]^+$	−2.8
								267.0703=[285.0819-$H_2O+H]^+$	−8.2
								122.9779=$(CH_3)_2AsOH_2^+$	−5.7
								104.9685=$(CH_3)_2As^+$	4.8
4	22.5	$C_{13}H_{26}AsO_3$		305.1094	305.1092	0.7	In this work	287.0991=$[M\text{-}H_2O+H]^+$	1.4
								122.9781=$(CH_3)_2AsOH_2^+$	−4.1
								104.9685=$(CH_3)_2As^+$	4.8
5	23.3	$C_{14}H_{26}AsO_3$		317.1096	317.1092	3.1	In this work	299.0986=$[M\text{-}H_2O+H]^+$	0.3
								255.1089=$C_{13}H_{24}As^+$	0.4
								122.9780=$(CH_3)_2AsOH_2^+$	−4.9
								104.9684=$(CH_3)_2As^+$	3.8
6	24.3	$C_{15}H_{26}AsO_3$		329.1090	329.1092	−0.6	In this work	311.0997=$[M\text{-}H_2O+H]^+$	3.2
								122.9790=$(CH_3)_2AsOH_2^+$	−6.5
								104.9683=$(CH_3)_2As^+$	2.9
7	28.6	$C_{17}H_{30}AsO_3$		357.1408	357.1405	0.8	In this work	339.1305=$[M\text{-}H_2O+H]^+$	1.5
								122.9790=$(CH_3)_2AsOH_2^+$	−6.5
								104.9683=$(CH_3)_2As^+$	2.9
8	29.3	$C_{15}H_{32}AsO_3$		335.1564	335.1562	0.6	[12,19]	317.1457=$[M\text{-}H_2O+H]^+$	0.3
								122.9785=$(CH_3)_2AsOH_2^+$	−0.8
								104.9685=$(CH_3)_2As^+$	4.8
9	33.2	$C_{17}H_{36}AsO_3$		363.1884	363.1875	2.5	[12,46,18,20,19]	345.1771=$[M\text{-}H_2O+H]^+$	0.6
								122.9786=$(CH_3)_2AsOH_2^+$	0.0
								104.9681=$(CH_3)_2As^+$	0.9
10	34.6	$C_{19}H_{38}AsO_3$		389.2038	389.2031	1.8	[12]	371.1930=$[M\text{-}H_2O+H]^+$	1.1
								122.9780=$(CH_3)_2AsOH_2^+$	−4.9
								104.9685=$(CH_3)_2As^+$	4.8
11	35.5	$C_{23}H_{38}AsO_3$		437.2037	437.2031	1.4	[12,18,20]	419.1933=$[M\text{-}H_2O+H]^+$	1.7
								122.9781=$(CH_3)_2AsOH_2^+$	−4.1
								104.9683=$(CH_3)_2As^+$	2.9
12	35.6	$C_{24}H_{38}AsO_3$		449.2030	449.2031	−0.2	[20]	431.1936=$[M\text{-}H_2O+H]^+$	2.3
								373.1851=$C_{22}H_{34}As^+$	−5.4
								122.9783=$(CH_3)_2AsOH_2^+$	−2.4
								104.9681=$(CH_3)_2As^+$	0.9
13	36.5	$C_{19}H_{40}AsO_3$		391.2193	391.2188	1.3	[12,19]	373.2082=$[M\text{-}H_2O+H]^+$	0.0
								122.9785=$(CH_3)_2AsOH_2^+$	−0.8
								104.9679=$(CH_3)_2As^+$	0.9
14	39.6	$C_{23}H_{38}AsO$		405.2135	405.2133	0.5	[11,46,17,18,20,19]	387.2030=$[M\text{-}H_2O+H]^+$	0.8
								122.9784=$(CH_3)_2AsOH_2^+$	−1.6
								104.9681=$(CH_3)_2As^+$	0.9
15	40.1	$C_{17}H_{38}AsO$		333.2131	333.2133	−0.6	[11,46,17,18,20,19]	315.2021=$[M\text{-}H_2O+H]^+$	−1.9
								122.9786=$(CH_3)_2AsOH_2^+$	0.0
								104.9680=$(CH_3)_2As^+$	0.0
16	42.3	$C_{19}H_{42}AsO$		361.2443	361.2446	−0.8	[11,46,17,18,20,19]	343.2345=$[M\text{-}H_2O+H]^+$	0.6
								122.9786=$(CH_3)_2AsOH_2^+$	0.0
								104.9681=$(CH_3)_2As^+$	0.9

fillet. Tetradecanoic acid (myristic acid), hexadecanoic acid (palmitic acid) and octadecanoic acid (stearic acid) are, in the form of As-FA and the latter also as As-HC, contained in herring (structures **8**, **9**, **13**, **15** and **16**). The only monounsaturated As-FA we detected with a common analogue is compound **10**. If one follows the assumption of Taleshi et al. that compound **14** is

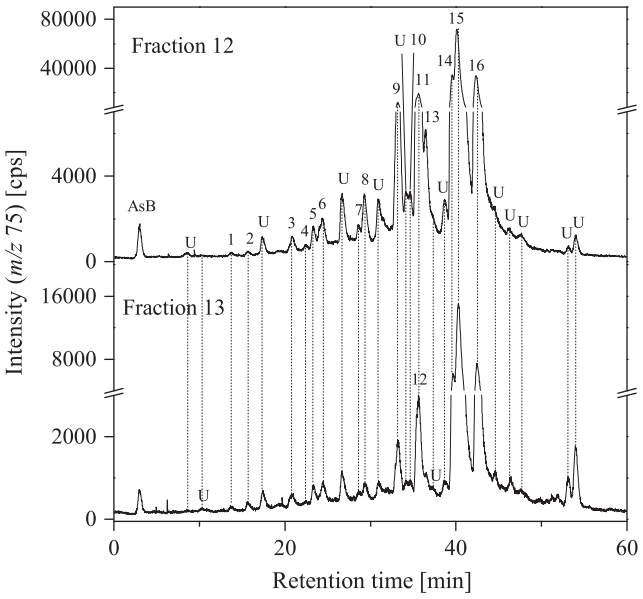


Fig. 1. HPLC-ICP-MS chromatogram on a RP column for the fractions 12 and 13 of methanolic herring extract after silica clean-up. AsB=arsenobetaine; U=unknown; identified compounds were numbered corresponding to their elution order; numbers refer to compounds shown in Table 3.

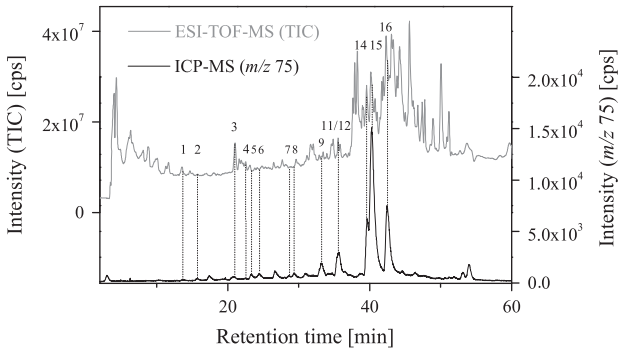


Fig. 2. Comparison of HPLC-ICP-MS chromatogram (m/z75) and HPLC-ESI-Q-TOF-MS chromatogram (TIC) for the fraction 13 of methanolic herring extract after silica clean-up. the HPLC-ESI-Q-TOF-MS chromatogram was corrected for the retention time delay of 0.73 min; identified compounds were numbered corresponding to their elution order.

an analogue to the most abundant polyunsaturated fatty acid in herring 22:6n-3 (all-cis-4,7,10,13,16,19-docosahexaenoic acid) then arsenolipid **11** can be proposed as an analogue to 22:5n-3. This FA has a content below 1% of all FA in herring muscle [38].

Since the amount of common FA and As-FA seems to correlate, a similarity in the biological function of As-FA and As-HC as compared to common FA can be assumed, as previously proposed [12]. The natural mechanism of the fatty acid biosynthesis always involves a chain elongation by two carbon atoms. Consequently, two homologue series of arsenolipids with C_{2n} and $C_{(2n)+1}$ can be identified in herring fillet. Considering the eight analogues to common FA, a metabolization by the natural enzymes and pathways in the human body seems plausible. This raises the question of the degradation pathway and the toxicological relevance of arsenolipids.

Proof of the availability of As-FA to the human metabolic pathways is the occurrence of oxodimethylarsenopropanoic acid and oxodimethylarsenobutanoic acid in urine after arsenolipid ingestion [39]. Urinary DMA is found after consumption of fatty fish like mackerel and herring or of cod liver [27,26,28]. Although the experimental data proved that DMA is a product of the human metabolism, the precursor is still unidentified. We postulate that the arsenolipids described in this paper are most likely responsible for the DMA excretion. As demonstrated by Heinrich-Ramm et al., polar arsenic species can be excluded as explanation for the urinary DMA [28]. Since all volunteers consumed cooked fish, a transformation of arsenolipids into DMA during cooking has to be considered. Although this process cannot be fully excluded, the arsenic species ingested via canned cod liver were mainly present as metabolically inert arsenobetaine and arsenolipids. Without further processing, the cod liver was ingested and DMA excreted in the urine by the volunteers [39]. This supports the assumption that the urinary DMA is formed out of lipid soluble organoarsenicals in human metabolic pathways.

If the metabolization of these arsenolipids in humans leads to excretion of DMA in urine, the process has to be slower than the renal elimination of arsenobetaine from the blood stream. We assume therefore that two kinetic constants for the release of inorganic and metabolized arsenic have to exist. In the literature, two different kinetic constants were determined for the urinary arsenic excretion after the consumption of plaice fillet [40]. During the first 10 h a first quick process with a mean half-life of 7.2 h took place. For the next 38 h the process slowed down, having a mean half-life of 62 h. Throughout species specific studies concerning metabolization of arsenic in herring, the main excretion of DMA was observed between the 10th and 23rd hours, with still increased values after 80–88 h [28,27]. This supports our assumption and indicates that the formation of DMA from the arsenolipids is taking place according to the slower excretion with a mean half-life of 62 h.

To estimate the variety of nonpolar organoarsenicals, and therefore the carcinogenic potential in other fish species, wolffish (*Anharhichas sp.*), plaice (*Pleuronectes platessa*), pilchard (*Sardina pilchardus*), mackerel (*Scomber scombrus*), salmon (*Onhcorhynchus keta*) and cape hake (*Merluccius capensis*) extracts were measured with HPLC-ICP-MS. The resulting chromatograms are presented in Fig. 4. They showed a diverse composition. The fatty acid fingerprint of herring (Fig. 1) was similar to the salmon sample whereas the pilchard extract contained only a few discrete fatty acids (arsenolipids **14**, **15**) (Fig. 5).

The first peak (near the void volume) was identified as arsenobetaine. Under the conditions used, it is highly probable that minor amounts of inorganic arsenic, MMA and DMA eluted at the same retention time. In a further experiment, using other separation conditions we were able to detect As(V), MMA and DMA in minor amounts. To determine the concentration of these highly polar arsenic compounds all fish species were extracted with 50% methanol and the extracts were measured with HPLC-ICP-MS as described in Section 2.4. The quantitative results for As(III), As(V) and DMA listed in Table 1 were calculated based on dry mass. MMA was not detected in any of the fish samples. Herring showed, with 2% of the total arsenic concentration, the highest amount of DMA. For pilchard and cape hake, DMA made up 1% of total arsenic. The highest amount of As(III) relative to the total concentration was in salmon with 2%. The highest absolute concentration of (0.131 ± 0.009) mg kg^{-1} was found in wolffish. This value corresponded to 0.15% of the total arsenic amount. All As(V) results were below the LOQ. The arsenobetaine concentrations of the fish materials are presented in the form of percent of total arsenic in Table 1. Herring is the only species investigated with nontoxic arsenobetaine not being the main polar arsenic species. For the other fish, arsenobetaine concentrations range between 64% and 92% of the polar extractable arsenic. The nonpolar arsenic content varies between (0.07 ± 0.01) mg kg^{-1} on dry mass basis for cape hake and (2.93 ± 0.07) mg kg^{-1} dry mass for pilchard.

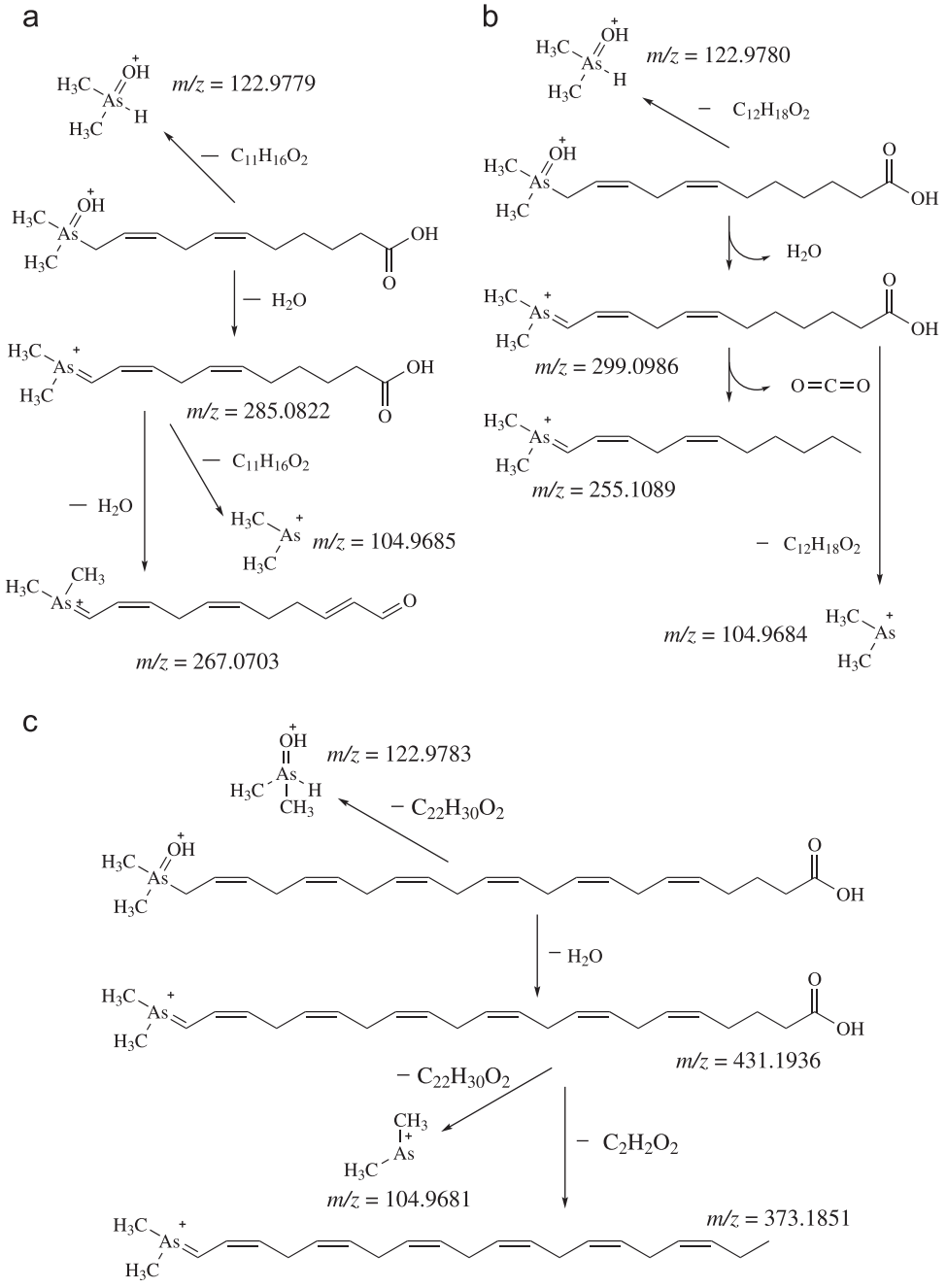


Fig. 3. Postulated fragmentation mechanisms. (a) Arsenolipid **3**, (b) Arsenolipid **5**, (c) Arsenolipid **12**.

4. Conclusion

In summary, we reported the existence of a homologue series of nonpolar organoarsenicals in herring fillet. The screening of six other species revealed similar compounds in each fish fillet investigated. We found (3.6 ± 0.2) mg kg^{-1} nonpolar extractable arsenic calculated based on dry mass in herring. Only 13% of the total arsenic content in herring was quantified in the form of arsenobetaine and DMA. The remaining 87% were potentially toxic medium polar and unpolar organoarsenicals. This was the highest percentage of all fish species investigated.

Interpreting the existing data about the arsenic species in human urine after consumption of fatty fish leads to a strong demand of further toxicological tests and a reevaluation of the risk potential. This includes the identification of the remaining nonpolar arsenic compounds, the production of pure calibration standards and analytical procedures for their exact quantification, as well as the acquisition of toxicological data. Our knowledge in these areas is very limited. The extent of the seasonal variation in the arsenolipid content of various fish species is still unknown. The biological function of the nonpolar organoarsenicals and how they are formed has to be determined. The main problem for further investigations is the non-availability of unsaturated arsenolipids. Being difficult to synthesize, they have to be isolated from fish muscle. The pure compounds are needed for exact quantification and the investigation of their toxic potential and their transformation in the human body. If a sufficient amount of arsenolipids is available, the metabolism could be investigated by hepatocytic cell assays. Acute and subchronic toxicity tests using oral administration estimating the LD_{50} level and the investigation of immunological effects as well as the assessment of nephrotoxicity, teratogenicity and genotoxicity should be done [41]. Ames mutagenicity plate

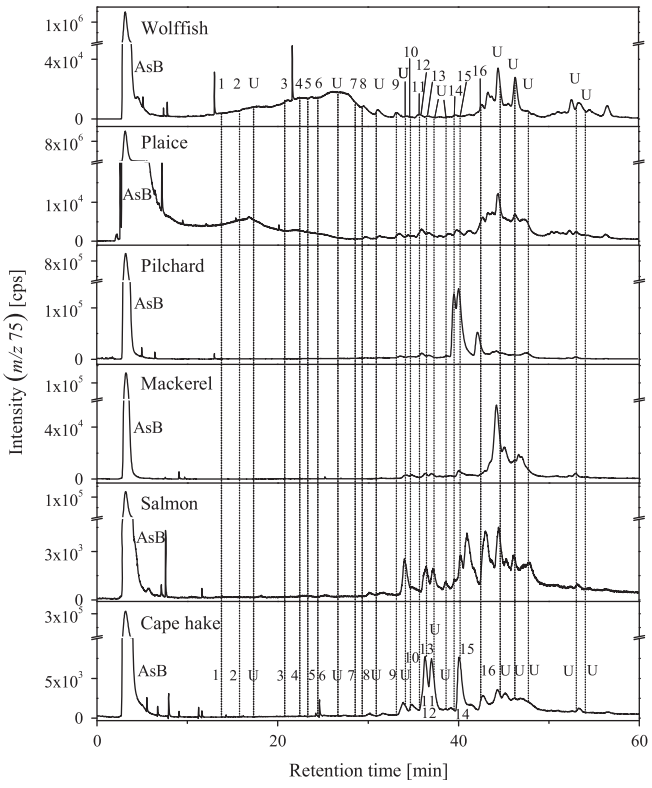


Fig. 4. HPLC-ICP-MS chromatograms for wolffish, plaice, pilchard, mackerel, salmon and cape hake; the labels and dotted lines are added for better comparison and symbolize retention time markers for the positions of the arsenolipids detected in herring extract. AsB=arsenobetaine; U=unknown; methanolic extracts without silica clean-up were measured for each fish under the same conditions applied for herring presented in Fig. 1.

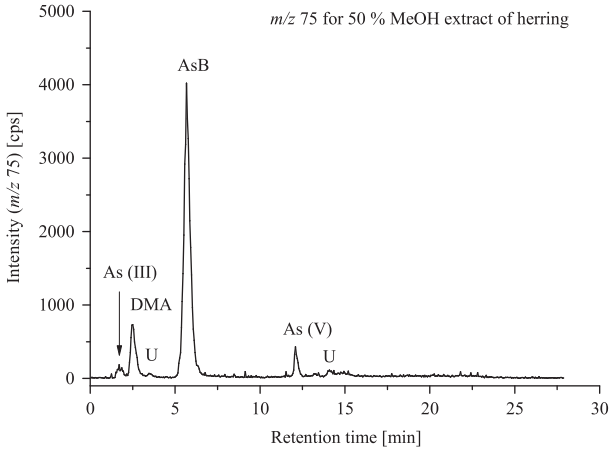


Fig. 5. HPLC-ICP-MS chromatogram of herring extracted with 50% methanol; extract was gained and measured under conditions described in Section 2.4. As(III)=arsenite; DMA=dimethylarsinate; AsB=arsenobetaine; As(V)=arsenate; U=unknown.

incorporation assays, microbial mutation assays with and without metabolic activation using yeast or bacteria, or a comet assay could give further information about mutagenic activity [41]. Finally, it is important to identify the no observed adverse effect level (NOAEL) to determine the acceptable daily intake of arsenolipids.

The existing data is insufficient to generally condemn fatty fish. One also has to consider the benefits from polyunsaturated fatty acids (PUFAs) [42]. While polyunsaturated n-3 fatty acids inhibit breast and prostate cancer in animal experiments, case-control studies show diverse results [43]. Among 12 epidemiologic studies concerning the association between the risk of breast cancer and marine fish consumption and 14 concerning prostate cancer, about 50% showed an inverse relationship [43]. In a Swedish nationwide case-control study concerning endometrial cancer, a weakly inverse effect between the consumption of fatty fish and the cancer risk was found [44]. Thus the promotion of hormone-dependent cancers by fatty fish is unlikely. For most kinds of cancers, both studies showing an increased risk and studies showing a decreased risk after marine fish consumption can be found [45]. Until now no evidence for a significant association between PUFA and cancer incidence exists. Since marine fish contain multiple contaminants and nutrients (e.g. Hg, PCBs, dioxin, pesticides and PBDEs) it is difficult to define a separate reason for the observations made during epidemiologic studies. The second problem is that few studies distinguish between fatty and lean fish when estimating the fish consumption. The majority of research revealed that the benefits of seafood consumption are more relevant than the risks [42]. The future task is to elucidate the toxicity of arsenolipids and identify the main arsenolipid-containing fish species in order to include organoarsenicals in existing risk-benefit analyses for marine fish.

Acknowledgments

U. Arroyo-Abad thanks the National Council on Science and Technology (CONACyT, Mexico) for its postdoctoral fellowship (Grant number 170548).

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