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Arsenolipids Detected in the Milk of Nursing Mothers

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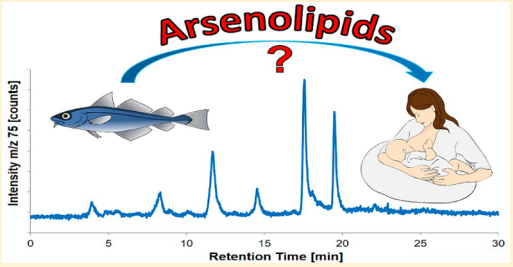
Supporting Information

ABSTRACT: Arsenic-containing lipids (arsenolipids), common constituents of fish, are currently being studied with regard to human health because of recent research that showed that some of the compounds are highly toxic to human cells and that they have the potential to cross the blood−brain barrier. As part of a study of the role of early exposure to environmental toxicants, we determined the arsenic content of milk from nursing mothers. Although the original intention of the study was to focus on inorganic arsenic, we discovered in an initial testing of 10 samples that a significant portion of the arsenic in the milk was lipid-soluble. We then investigated in detail this lipid-soluble arsenic in five of the samples by purifying the major compounds and using high-performance liquid chromatography coupled to both elemental and molecular mass spectrometry to identify them as arsenic hydrocarbons and arsenic fatty acids. This study is the first to report the presence of arsenolipids in human milk. The concentrations of arsenolipids in the milk were low (combined total of approximately 0.5 μg of As/kg) compared to the current recommended maximum for arsenic in water (10 μg/L), but of potential concern when one considers the possibility of the lipids crossing the blood−brain barrier and the critical stage of brain development in the newborn child.



INTRODUCTION

Arsenic is a major global environmental contaminant, and legislation is in place in many countries to limit human exposure to arsenic from water and food. The major arsenic species of concern is inorganic arsenic (iAs), a known toxic substance,[1,2] that is present in drinking water and some foods.[3,4] The organic forms of arsenic such as arsenobetaine and arsenosugars, which can occur at high concentrations in seafoods,[5] have been perceived as nontoxic on the basis of results of toxicity testing of just a few organoarsenicals.[6,7] Those toxicity studies, however, were all performed with water-soluble organoarsenic compounds.

This simplified view of arsenic and arsenic species in food has recently undergone a dramatic change following the discovery of lipid-soluble organoarsenicals in fish[8,9] and other seafoods,[10,11] and investigations demonstrating considerable cytotoxicity of one arsenolipid group, namely arsenic hydrocarbons,[12] and their potential to cross the blood−brain barrier.[13] The first evidence that arsenic hydrocarbons could cross the blood-barrier has come from an experiment with the fruit fly *Melanogaster drosophila*[14] and has raised the question of possible neurological effects in humans resulting from arsenolipids in food.[15] Such concerns with the trace contaminant methylmercury, a lipid-soluble neurotoxic mercury species present in fish, have focused on the effects on brain development, and the early results showing a link between methylmercury in mothers and impaired intellectual development in their children have been incorporated into risk assessments of mercury in fish.[16]

The Norwegian HUMIS-NoMIC study[17,18] is a prospective population-based birth cohort consisting of mother−child pairs established for the purpose of studying environmental toxicants in human milk and their relation to child health. As part of this study, we recently analyzed samples of human milk using a method specifically developed for quantifying iAs and other water-soluble arsenicals in human milk[19] but were surprised to find that a significant portion of the total arsenic was actually lipid-soluble. We report an investigation, using high-performance liquid chromatography (HPLC) coupled to both elemental and molecular mass spectrometry, of the lipid-soluble arsenic constituents of human milk.

MATERIALS AND METHODS

Samples. Milk samples from nursing mothers were collected at a median one month after delivery by study personnel as part of the Norwegian Microbiota study (HUMIS-NoMIC).[17,18] The samples were immediately frozen and stored at −20 °C; they were sent to Graz on dry ice where they were stored at −80 °C before being analyzed for arsenic species. Milk samples from 10 mothers were used in this study.

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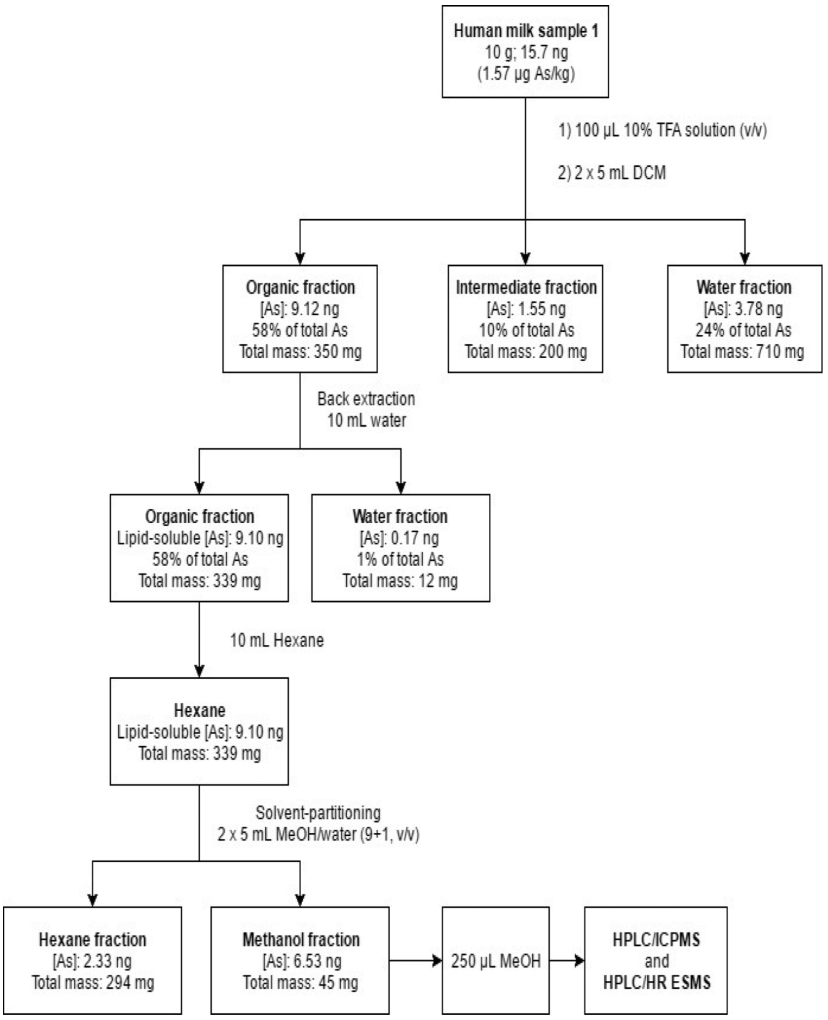
Scheme 1. Purification of Lipid-Soluble Arsenic Species in Human Milk[a]



[a]An example of a typical fractionation. The partitioning[22] between hexane and aqueous methanol separates the polar arsenolipids (methanol-soluble) from the bulk of the normal lipids found in milk. The less polar arsenolipids remain in the hexane layer, but currently available techniques cannot measure these compounds in the high-lipid matrix.

Chemicals, Reagents, and Standards. Water (18 MΩ cm) used throughout the investigation was from a Milli-Q Academic water purification system (Millipore GmbH, Vienna, Austria). The following chemicals, all of analytical grade, were used in the study: nitric acid [68%, p.a., further purified by using an MLS duoPUR sub-boiling unit (MLS GmbH, Leutkirch, Germany)], formic acid (≥99.5%, p.a.), trifluoroacetic acid (≥99.9%), and *n*-hexane (≥99.8%), all purchased from Carl Roth GmbH & Co. KG (Karlsruhe, Germany); dibromomethane (99%), purchased from Sigma-Aldrich (Vienna, Austria); dichloromethane (99.8%, p.a.) and absolute ethanol, both purchased from Chem-Lab NV (Zedelgem, Belgium); HPLC grade methanol, obtained from VWR Chemicals (Vienna, Austria); and acetic acid (≥99.8%), purchased from Merck (Darmstadt, Germany).

For the determination of total arsenic in human milk samples, a single-element standard containing 1000 ± 3 mg of As/L in 2% nitric acid from CPI International (Santa Rosa, CA) was used. A single-element standard at a concentration of 1000 mg of germanium/L from Carl Roth GmbH & Co. KG was used as an internal standard.

For use as standards for HPLC/mass spectrometry, six arsenolipids found in seafoods, namely, AsFA 362 (15-dimethylarsinoyl-pentadecanoic acid), AsFA 388 (17-dimethylarsinoyl-9-heptadecenoic acid), AsFA 418 (19-dimethylarsinoyl-nonadecanoic acid), AsHC 332 (1-dimethylarsinoyl-pentadecane), AsHC 360 (1-dimethylarsinoyl-heptadecane), and AsHC 444 (1-dimethylarsinoyl-tricosane), were synthesized according to the method described by Taleshi et al.[20]

Instrumentation. Microwave-assisted acid digestions were performed with an Ultraclave III microwave system (MLS). Total As measurements were performed with an Agilent 7900 inductively coupled plasma mass spectrometer (ICPMS, Agilent Technologies, Waldbronn, Germany) equipped with a Scott type spray chamber and a Micro Mist concentric glass nebulizer (Glass Expansion, West Melbourne, Australia). For HPLC/ICPMS measurements of lipid-soluble arsenic species, an Agilent Series 1260 Infinity II HPLC system (Agilent Technologies) was connected to the Agilent ICPMS 7900 instrument with 0.125 mm PEEK (polyetheretherketone) tubing (Upchurch Scientific, Oak Harbor, WA). A high-speed refrigerated microcentrifuge (SCILOGEX, Rocky Hill, CT) and a Hettich Rotina 420 R centrifuge (Andreas Hettich GmbH &

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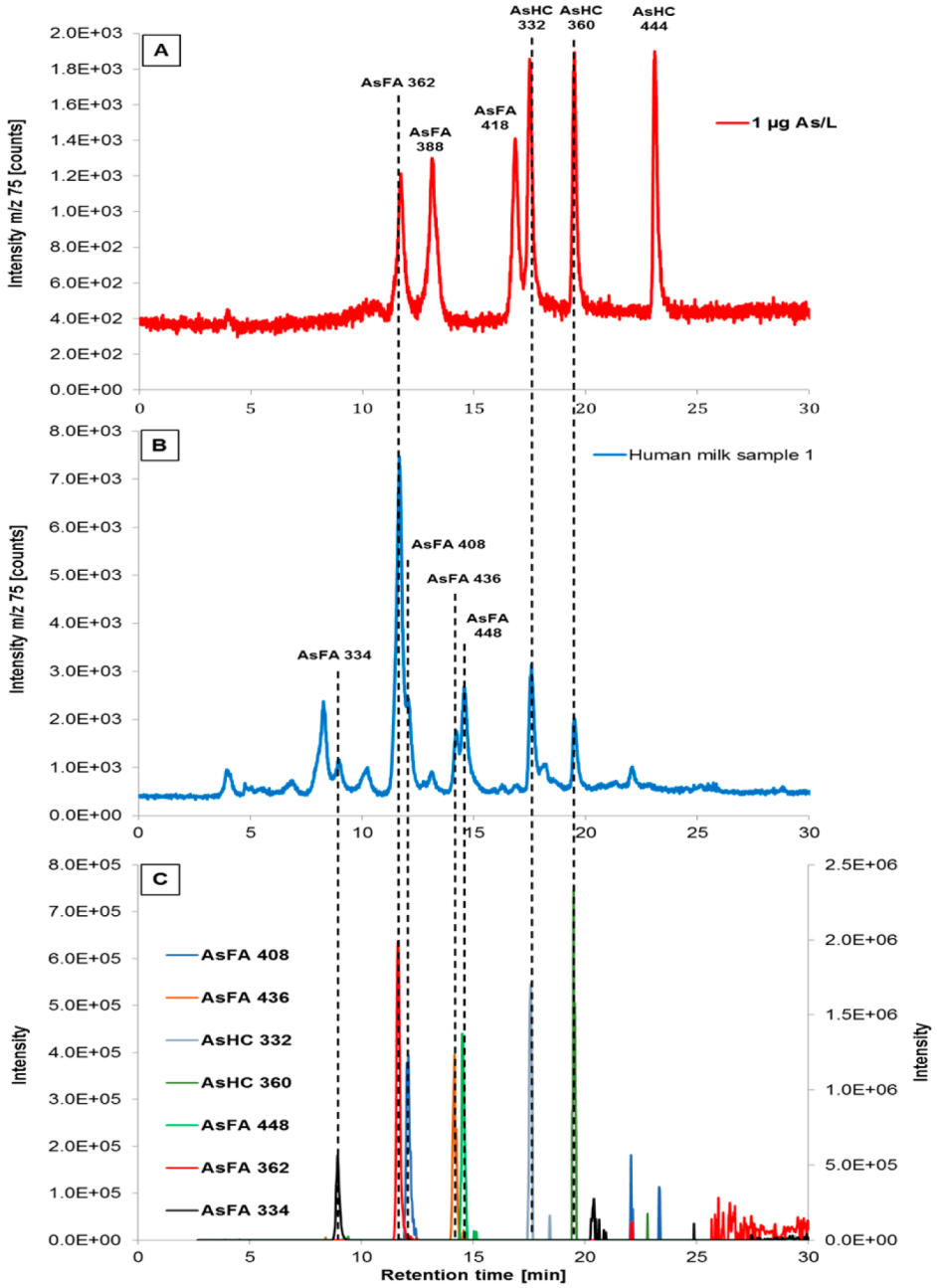

Figure 1. (A) HPLC/ICPMS chromatogram of an arsenolipid mixed standard solution of AsFA 362, AsFA 388, AsFA 418, AsHC 332, AsHC 360, and AsHC 444 (each 1 μg of As/L). (B) HPLC/ICPMS chromatogram of a human milk sample. (C) HPLC/HR ESMS chromatogram of the same milk sample. The HPLC/HR ESMS chromatogram shows the extracted $[M + H]^+$ ions of AsFA 334 (t_R = 9.0 min), AsFA 362 (t_R = 11.7 min), AsFA 408 (t_R = 12.1 min), AsFA 436 (t_R = 14.2 min), AsFA 390 (t_R = 14.4 min), AsFA 448 (t_R = 14.7 min), AsHC 332 (t_R = 17.4 min), and AsHC 360 (t_R = 19.5 min). The retention time shift between HPLC/ICPMS and HPLC/HR ESMS measurements was normalized by comparison of authentic arsenolipid standards, and the HR ESMS chromatogram was adjusted accordingly. Chromatographic conditions: Shodex Asahipak ODP-50 4D C18 column; gradient elution, beginning at 50% methanol and increasing to 100% methanol after 20 min, holding for 10 min at 100% methanol followed by equilibration for 10 min at 50% methanol; injection volumes, 50 μL (HPLC/ICPMS) and 10 μL (HPLC/HR ESMS); flow rate, 0.5 mL/min at 40 °C.

Co. KG, Tuttlingen, Germnay) were used for the fractionation of arsenic species in human milk samples. Solvents were removed from the human milk fractions using a Christ RVC 2-33 CDplus vacuum lyophilizator (Martin Christ Gefriertrocknungsanlagen GmbH, Osterode am Harz, Germany). For HPLC/high-resolution electrospray mass spectrometry (HR ESMS) measurements, a Dionex Ultimate 3000 series instrument HPLC system (Thermo Fischer Scientific, Erlangen, Germany) was connected to a high-resolution mass spectrometer (Q-Exactive Hybrid Quadrupole-Orbitrap MS from Thermo Fischer) equipped with an electrospray ionization source.

Determination of the Total Arsenic Content. The arsenic content was determined in the milk samples and in the various fractions of the purification scheme by ICPMS following a microwave-assisted acid mineralization step; the method was validated by analysis of reference material

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ClinChek-Control from RECIPE GmbH (Munich, Germany) (see the Supporting Information for full details).

Fractionation and Purification of the Lipid-Soluble Arsenicals in Human Milk. For fractionation of arsenic species, a portion (1.00 g) of human milk was fractionated according to a method developed to defat the milk sample prior to its being analyzed for water-soluble arsenic species.[19] For the purification of lipid-soluble arsenic species, a portion (10.0 g) of human milk was transferred to a 50 mL polypropylene tube, and 100 μL of a 10% aqueous TFA solution was added to precipitate the proteins. After 1 h, the lipid-soluble arsenic was extracted with 2 × 5.0 mL of dichloromethane (DCM); the DCM layers were combined, washed with water, and evaporated to dryness to yield a pale yellow oil. This oil was dissolved in 10 mL of hexane and the mixture extracted with a methanol (MeOH)/water mixture [9 + 1 (v/v), 2 × 5.0 mL]. The combined aqueous MeOH fraction was evaporated to dryness and the residue redissolved in 250 μL of MeOH prior to analysis first by HPLC/ICPMS and then by HPLC/HR ESMS. At each stage of the purification procedure, a portion of the fraction was removed for measurement of total arsenic and dry mass (see Scheme 1).

HPLC/ICPMS Measurements. The lipid-soluble arsenic species were assessed by reversed-phase HPLC using a Shodex Asahipak ODP-50 4D C18 column (150 mm × 4.6 mm, 5 μm) with guard column ODP-50G 4A (10 mm × 4.6 mm) obtained from Showa Denko Europe GmbH (Munich, Germany). Gradient elution was performed with water containing 0.1% (v/v) formic acid and MeOH containing 0.1% formic acid (v/v) as mobile phases (see the chromatographic conditions in the legend of Figure 1). Just 10% of the HPLC flow was directed to the ICPMS instrument; to maintain stable plasma conditions, and to introduce the internal standard, the weak flow to the ICPMS instrument was supported with a makeup flow of water containing 1% (v/v) acetic acid and 10 μg of Ge/L at a flow rate of 0.45 mL/min provided by an isocratic pump. To maintain a constant carbon load to the plasma, a 10% (v/v) MeOH solution in water was used for carbon compensation and introduced with the peristaltic pump of the ICPMS instrument into the spray chamber.[21] The ICPMS instrument was operated in no gas mode and time-resolved analysis mode using sample and skimmer cones made of nickel. Monitored masses (integration time/point) were m/z 53 (0.01 s), m/z 74 (0.01 s), m/z 75 (0.3 s), and m/z 77 (0.3 s). Mixed calibration standards of three arsenic fatty acids (AsFA 362, AsFA 388, and AsFA 418) and three arsenic hydrocarbons (AsHC 332, AsHC 360, and AsHC 444) were prepared in methanol in the calibration range of 0.25−10 μg of As/L; quantification was based on peak areas. The limit of detection (50 μL injection volume, 3σ of blank noise) of arsenolipids in the methanol fraction, based on extraction blanks after solvent partitioning, was 0.1 μg of As/L in solution.

HPLC/ESI-HR MS Measurements. For HPLC/HR ESMS measurements, the same chromatographic conditions that were applied for HPLC/ICPMS measurements (see the chromatographic conditions in the legend of Figure 1) were used. The HR mass spectrometer was operated in positive mode with nitrogen as the drying gas (450 °C), a capillary voltage of 3200 V, and a capillary temperature of 320 °C. The following instrumental settings were used for the assessment of arsenolipids in human milk samples: full scan at a resolution of 140000 full width at half-maximum (fwhm) within a scan range of m/z 250−600, with automatic gain control set to 10^6, and a maximal injection time of 200 ms. The following settings were used for the data-dependent MS/MS measurements: isolation window set to m/z 0.4, resolution of 17500 fwhm, and automatic gain control set to 5×10^5. A maximal injection time of 300 ms and a loop count of 5 were used. The intensity threshold was set to 6.7×10^3, and normalized collision energies of 40 or 50 eV were used. The limits of detection of arsenolipids, taking matrix effects into consideration, in HPLC/ESI-HR MS measurements were comparable to those used for HPLC/ICPMS.

RESULTS AND DISCUSSION

We first fractionated 10 human milk samples, with a total arsenic content ranging from 0.30 to 4.46 μg of As/kg (median, 1.05 μg of As/kg), on a small scale (1 g of milk) according to a validated analytical method[19] specifically developed to determine water-soluble arsenic species in milk. After protein precipitation and solvent partitioning, however, the aqueous fraction contained only 23−87% of the initial arsenic, with the remainder being associated with the precipitated protein layer and the lipid phase. The level of lipid-soluble arsenic varied notably between samples, ranging from 0.05 to 0.98 μg of As/kg corresponding to approximately 2−61% of the total arsenic.

To identify the arsenolipids present in the milk, we selected five samples with the highest lipid-arsenic content, but because of the complex lipid-rich matrix in milk, and the generally poor detection limits for determining arsenolipids by HPLC/mass spectrometry, we needed to partially purify the compounds. Thus, beginning with 10 g of each milk sample, containing 7.1−21 ng of arsenic, and following the purification procedure outlined in Scheme 1, we produced five individual samples containing 1.7−7.4 ng of As in 18−69 mg of total lipid.

HPLC/ICPMS analysis, which provides arsenic-selective detection of eluting compounds, showed the presence of several arsenic-containing peaks or bands, and further analysis by high-resolution molecular mass spectrometry confirmed the presence of three known arsenolipids: two arsenic hydrocarbons, AsHC 332 and AsHC 360, and one arsenic fatty acid, AsFA 362 (Figures 1 and 2 and Figure S1). Furthermore, the data from the accurate mass measurements suggested the presence of four additional arsenic fatty acids in the milk extracts. The relative quantities of the seven identified arsenolipids in the extracts of the milk samples are shown in Figure 3.

It is possible that the fatty acids identified in our purified extracts of the milk samples were artifacts of the purification procedure because acidic conditions used during the procedure could catalyze the hydrolysis of fatty acid esters. A previous study with fish oil showed that arsenic fatty acids were generated by acid hydrolysis of more complex arsenolipids, presumed to be derivatives of triacylglycerides,[23] and arsenic phosphatidyl compounds recently shown to be present in caviar[11] could also produce arsenic fatty acids by acid hydrolysis. We were, however, unable to identify these more complex arsenolipids in the unprocessed milk samples. It should also be noted that a significant percentage of the original lipid arsenic in milk remained in the hexane layer (Scheme 1) and could not be measured by current analytical techniques for arsenolipids.[24]

Although the number of samples examined here is small, we may speculate that fish or other seafood is the origin of the arsenolipids in the milk of Norwegian mothers. In a study[25] of arsenic species in the milk of mothers in Bangladesh, where the

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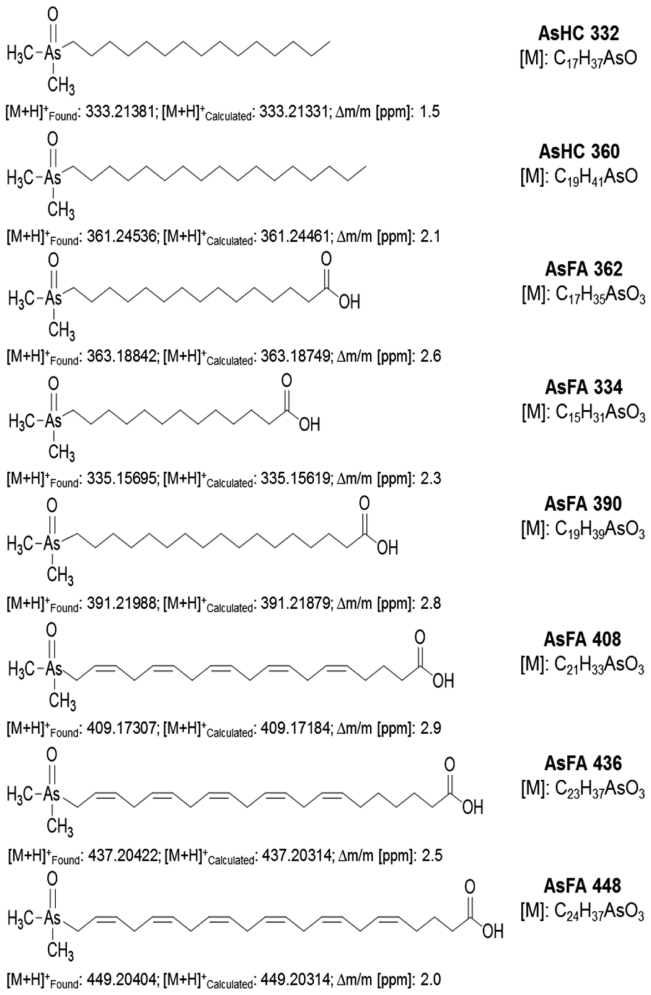

Figure 2. Structures of arsenolipids identified in human milk by HPLC/HR ESMS. Arsenic-containing hydrocarbons (AsHCs) and arsenic-containing fatty acids (AsFAs) are referenced by the aforementioned abbreviation followed by the nominal molecular mass of the compound. [M] and $[M + H]^+$ refer to the molecular formula and protonated molecular formula, respectively. AsHC 332, AsHC 360, and AsFA 362 have been assigned by exact matching with synthesized standards; the other arsenolipids have been assigned on the basis of accurate mass measurements, chromatographic properties, and analogy to known non-arsenic fatty acids.

level of fish consumption is low and the rate of inorganic arsenic intake is high as a consequence of drinking water drawn from aquifers contaminated with natural sources of arsenic, inorganic arsenic was the major arsenical and lipid-soluble arsenic was not reported. There are no other published data on arsenic species in human milk.

Implications of Arsenolipids in Human Milk. There is currently a high level of interest in the status of arsenic in food with regard to human toxicology and risk assessment.[4,26,27] So far, the focus has been on iAs, a proven human carcinogen[28] that has also been linked to cardiovascular disease, diabetes, and adverse neurodevelopmental outcomes.[29,30] Preliminary toxicity testing of arsenolipids with human bladder and liver cells, however, has demonstrated high toxicity for the arsenic hydrocarbons, including AsHC 332.[12] Additionally, in the first study[31] using a whole organism, arsenic hydrocarbons were shown to be toxic to the fruit fly *D. melanogaster*, and in a second study,[14] AsHC 332 was shown to accumulate in the brain of the fly.

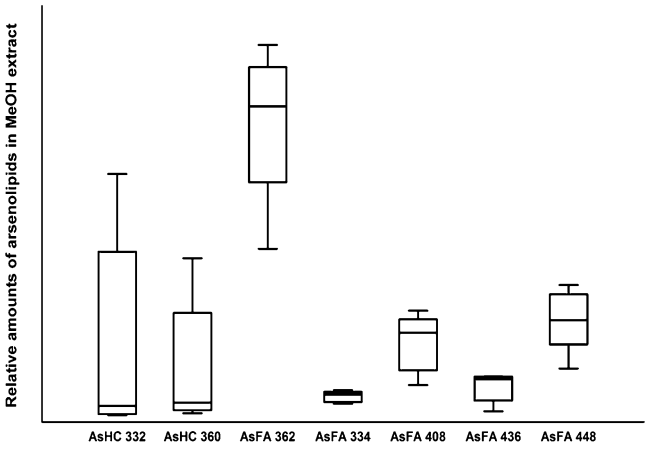

Figure 3. Box-and-whisker plot showing the distribution of arsenolipids in the processed extracts of five human milk samples, as quantified by HPLC/ICPMS. The median, 25th, and 75th percentiles are represented as lines in vertical boxes, with error bars representing the 10th and 90th percentiles. The values represent the relative amounts of the arsenolipids identified in the purified extract and serve as an indication of the relative amounts in the original milk samples. AsFA 390 was detected by HR ESMS but could not be quantified by HPLC/ICPMS and thus is not displayed in this plot.

A recent study[15] with arsenolipids and fully differentiated human brain cells (LUHMES cells) showed that AsHCs massively disturbed the neuronal network, while iAs, as arsenite, showed comparatively weaker effects. The *in vitro* neurotoxic potential of the AsHCs, and the possibility that they could transfer across the physiological barriers of the brain,[14] suggests that these compounds might also display *in vivo* neurotoxicity. The brain of a newborn child is undergoing rapid growth and changes that govern future neurological development and performance. Thus, the presence of lipids that contain arsenic in the milk of nursing mothers is likely to be of toxicological interest and may potentially have adverse health effects.

In summary, we report that the milk from nursing mothers in Norway contains a significant proportion of its total arsenic in lipid-soluble forms, and we identify the major arsenolipids present as two arsenic hydrocarbons and six arsenic fatty acids. Dietary fish is a likely source of the arsenolipids detected in the milk. The sensitivity of the developing brain to toxicants and the neurotoxicological potential of arsenolipids dictate the need for further investigations leading to a risk assessment.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.estlett.7b00181.

Microwave-assisted acid digestion procedure, method for total arsenic measurements, quality control total arsenic measurements, and HPLC/ICPMS chromatograms of human milk samples (PDF)

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Notes

The authors declare no competing financial interest.

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