ANALYSIS OF ARSENIC IN OYSTERS USING REVERSED PHASE HPLC-ICP-MS/ESI-ORBITRAP-MS

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ABSTRACT

The presence of high concentration of arsenic in marine organisms has attracted much attention over the years due to the toxicity of the element. However, only limited investigations have been carried out on the lipid-soluble arsenic species (arsenolipids) relative to the water-soluble compounds. In this study, we investigate the distribution of total arsenic and arsenolipid speciation in samples of Oysters (*Ostreaedulis*) collected from the local market in Aberdeen (OY-A), UK and in Port Harcourt (OY-P), Nigeria. For total arsenic determination, the samples were mineralised by microwave-assisted acid digestion and analysed using ICP-MS. The results showed significant variation in total concentrations of arsenic in Oysters from the different locations. However, analysis of the lipid extract using HPLC-ICP-MS/ESI-Orbitrap-MS showed similarity in arsenolipid profile. The speciation study is significant since toxicity of arsenic depends on the molecular forms of the element. Three arsenolipids were identified with elemental compositions consistent with arsenic fatty acids. This result could also be used to compare the relative arsenic exposures and accumulation patterns of Oysters at the different locations.

Key words: Oyster; Arsenolipid; ICP-MS; HPLC; ESI-MS.

INTRODUCTION

The incidence of arsenic contamination in the environment is widely reported (Allan et al., 2000; Andreae, 1978; Borak and Hosgood, 2007). It is a naturally-occurring element found ubiquitously in the environment. The distribution of arsenic varies geographically and is present in rocks and mineral deposits in combination with other elements such as iron and sulphur. Examples of arsenic-bearing minerals include arsenopyrites (AsFeS), realgar (AsS) and orpiment (As_2S_3). All rocks contain some arsenic, in the range 0.5 – 5 ppm but higher concentrations are mostly found in mineralised rocks with respect to non-mineralised rocks.

Natural arsenic poisoning of groundwater has been detected in many parts of the world including Bangladesh, West Bengal, India, and Midwestern United States. The interaction of ground water with arsenic-bearing rocks is a major source of arsenic in the environment. In sea-water, arsenic is usually present at low concentrations typically 1 - 2 μgL^{-1}.(Grotti and Frache, 2007; Urgast *et al.*, 2010) On the other hand, since marine organisms are capable of bioaccumulation, high concentrations up to 5–100 ppm dry mass have been reported in marine samples Francesconi *et al.*, 2010).

However, the majority of works have only investigated the water soluble compounds such as arsenobetaein, arsenosugars, dimethylarsinic acid (DMA) etc. Inorganic arsenic comprising arsenite and arsenate, have been found in fish and shellfish, but constitute only a few per cent of the total arsenic content (Larsen *et al.*, 1993). The presence of high concentrations of arsenolipids has been known in marine samples since late 1960s but until recently, the analyses of arsenolipids have been neglected due to lack of suitable analytical techniques. Nevertheless, their distribution in most marine samples is yet to be investigated.

The aim of this study was to provide data concerning arsenic levels and arsenolipids profiles in Oysters collected from Aberdeen and Port Harcourt for comparative analyses of geographical differences in bioaccumulation.

EXPERIMENTAL

Reagents and Chemicals

All chemicals used were of analytical grade. Ultra pure water was used for samples preparations (18 MΩ cm Elga, UK). Formic acid and sodium arsenite were supplied from Sigma Aldrich (UK). Hexane, Hydrogen peroxide (H_2O_2, 32%) and methanol were obtained from Fisher Scientific (UK). Nitric acid (HNO_3, 65%) from Fluka (UK) and the reference material used was DORM-3 from National Research Council Canada (NRCC).

Sample Preparation

Samples of Oyster were purchased from local fish market in Aberdeen, UK, and from Port Harcourt, Nigeria. The samples were thoroughly rinsed with tap water and then with de-ionised water and the edible parts were separated from the shells for analysis. The samples were freeze-dried to a constant weight and then pulverized by grinding in liquid nitrogen with mortar and pestle. Triplicate analyses were carried out for total arsenic concentrations and the result expressed as mean value (X) ± standard deviation (SD). Individual OY-A samples and Pooled OY-P samples (n=10) were analysed.

Extraction Procedures

Sequential extraction was carried out using Hexane, methanol/DCM (1:2) and water. About 0.2 - 0.3g of sample was dissolved in 10 mL of hexane. The mixture was shaken overnight at room temperature and then centrifuged at 3500 rpm for 15 minutes. The hexane layer was recovered and the residue further extracted using methanol/DCM (1:2) and lastly with water. Total concentrations of arsenic in hexane, methanol/DCM, and water fractions and in the residue were determined using ICP-MS. All the extracts were stored at 4 ^{O}C till analysis.

Determination of Total Arsenic by ICP-MS

Total arsenic in the freeze-dried tissue samples and in the extracts were determined by Agilent ICP-MS 7500c (Agilent Technologies, California, USA) following microwave-assisted acid digestion. About 0.1-0.5 g of the samples were measured in 50 mL plastic vials and 1 mL of conc. HNO_3 added and left overnight, 2 mL of H_2O_2 was then added and placed in the microwave (Mars-5, CEM, UK.) for 40 minutes with temperature ramped up to 90^{o}C. After cooling the digests were diluted to 10 mL and the total arsenic determined after correction for the change in density. ^{74}Ge was used as internal standard and quantification was carried out against standard solutions of sodium arsenite. The accuracy of the measurement was assessed by the analysis of the certified reference materials (CRM); fish muscle, DORM-3 and DORM 2 from National Research Council, Canada.

Speciation Analysis

Speciation analysis was carried out by reversed phase HPLC on line with ICP-MS and ESI on-line-Orbitrap-MS as previously described (Amayo et al., 2011). The arsenic species were separated using a gradient of 0.5% formic acid in water and 0.5% formic acid in methanol in a reversed phase column , Agilent Eclipse XBD-C18(4.8 X 150 mm) with a flow rate of 1 mL/min, injection volume of 100 uL and column temperature of 30°C. The eluent flow was split post column with 25% to the high resolution ICP-MS (Element 2, Thermo Fisher) and 75% to Electrospray Ionization Orbitrap Mass Spectrometer (ESI-Orbitrap-MS). The Orbitrap was used in positive scan mode. HR-ICP-MS was used in organic mode with platinum cones and 20% of oxygen with micro-concentric nebulizer. The ICP-MS signal was optimized to give a maximum response of As signal at m/z 75. ^{74}Ge was used as internal standard to monitor instability in the plasma.

Table 1: HPLC-ICP-MS-ESI-MS parameters

HPLC	**Thermo Scientific**
Column	Agilent Eclipse, XBD-C18; 4.8 mm × 150 mm
Column Temperature	30°C
Injection Volume	100μL
Buffer A	0.1% formic acid in water
Buffer B	0.1% Formic acid in methanol
Splitter ratio	1:3
Flow rate	1mL/min
Gradient	0-25 min: 0-100%, 10 min 100% B
ICPMS	**Element 2 (Thermo Scientific)**
Mode	Organic mode
HF	1570 W
Nebulizer	Micro-concentric
Nebulizer gas	0.86 L/min
Optional gas	20 mL/min O2
Plasma gas	0.89 L/min
Coolant gas	14.9 L/min
ESI-MS	**LTQ Orbitrap Discovery; Thermo Scientific**
Mode	Positive
Spray voltage	4.5 kV
Normalized collision energy	35%

RESULTS AND DISCUSSION

Total Arsenic Concentrations

The results for total concentration of arsenic in the freeze dried tissue samples and in the sample extracts are shown in Tables 2 and 3 respectively. The analytical method was validated by the measurement of certified materials DORM-3 and DORM-2. The total arsenic concentrations obtained for DORM-3 and DORM-2 were 6.85 ± 0.22 μg As / g and 17.66 ± 0.14 μg As / g respectively, which values were consistent with certified values of 6.88 ± 0.30 μg As / g and 18.00 ± 1.1μg As / g respectively. The total arsenic determined in the pooled samples (n=10) of OY-P is 2.88 ± 0.25μgAs/g. Generally, high concentrations of arsenic were found in OY-A species (OYA1 – OYA6) ranging from 18.95 ± 0.36 to 34.30 ± 0.45 μg As / g with an average of 27.82 ± 5.28 μg As / g.

Table 2: Total Arsenic Concentrations in Oyster Samples, Concentration is Expressed in μg As/ g d.w (X ± SD, n = 3)

N	sample	Origin	Number of pooled samples	Total arsenic (μg/ g)
1	OY-A 1	Aberdeen, UK	1	34.30 ± 0.45
2	OY-A 2	Aberdeen, UK	1	18.95 ± 0.36
3	OY-A 3	Aberdeen, UK	1	25.11 ± 0.42
4	OY-A 4	Aberdeen, UK	1	30.87 ± 0.58
5	OY-A 5	Aberdeen, UK	1	28.87 ± 0.43
6	OY-A 6	Aberdeen, UK	1	28.81 ± 0.81
7	OY-P	P/H Nigeria	10	2.88 ± 0.25

The variation in total arsenic concentrations observed in OY-P and OY-A may be due to the levels of pollution in the marine environment resulting from natural and anthropogenic activities. Anthropogenic causes could also include the use of arsenical pesticides.

Geographical variabilities with arsenic concentrations in marine samples have been reported in previous works. Gerhardt et al. (2002) attributed the relative difference observed in arsenic concentrations between south eastern oysters and oysters elsewhere in the US as partly due to the differences in the habitat of oysters in the two regions. They also found that oysters from South Carolina with average concentrations of arsenic 3.2 times that of oysters from Maryland was similar to enrichments of water (3.4 times), sediment (2.5 times), suspended particles (1.7 times), and pore water (3.1 times) from South Carolina compared to Maryland which suggests that the cause of the apparent arsenic enrichments in the South-eastern oysters is environmental. (Riedel and Valette-Silver, 2002)

Dagma *et al.* (2010) found significant variation of arsenic within the species of whelks by a factor of 3.5. (Urgast *et al.,* 2010). Environmental causes could also include the salinity of surrounding water. Erik *et al.* (2003) observed positive linear relationship between total arsenic content and salinity when three teleost species; herring, cod and founder taken from different locations were analysed.(Larsen *et al.*, 2003).

Another possibility is that OY-A oysters may differ genetically from OY-P oysters in some way which causes them to accumulate high concentrations of arsenic (Sankar et al., 2006). However, both OY-A and OY-P samples exhibited similar accumulation patterns as reflected in their arsenic chromatographic profile (Figure 1) suggesting that the cause of variations in concentrations is environmental.

Sequential Extraction and Mass Balance

Sequential extraction was carried out using hexane, MeOH/ DCM and water. The result of the sequential extraction is shown in Table 3. The extraction efficiencies were 90% and 69% in OY-A and OY-P respectively. The mass balance showed 108% and 101% recoveries of the total arsenic as shown in Table 3.

The extraction procedure was targeted at separating the oil matrix from the polar arsenolipids. The extraction using hexane removed the bulk of the lipid matrix so that subsequent extraction of polar arsenolipids in the methanol phase (Ciardullo et al., 2010; Schmeisser et al., 2005; Taleshi et al., 2010) could be directly analysed by reversed phased HPLC-ICPMS-ESMS without further purification.

The mass balance which accounted for almost 100% of the total arsenic, was evaluated by comparing the sum of arsenic concentrations in different fractions including extracts and residue with the total arsenic concentration in solid sample.

Relatively high amount of arsenic was extracted in the methanol/DCM fraction. It is also possible that some of the water-soluble organo arsenicals might also have been taken up in this fraction. This was further confirmed from the speciation result of methanol/DCM showing large amount of

Table 3: Total arsenic concentrations in the extracts (A – C), and residues (D) showing the concentration of arsenic (μg As/ g) in each fraction. The mass balance expressed in **%** recovery was evaluated by comparing the Sum (A – D) with the Total concentration of arsenic determined in solid tissue sample.

sample ID	Hexane extract (A)	Methanol/DCM extract (B)	water extract (C)	Residue (D)	Sum (A-D)	Total	%Recovery (mass bal.)
OY-A	1.64	21.40	4.88	3.25	31.17	28.81	108
OY-P	0.13	1.45	0.44	0.89	2.91	2.88	101

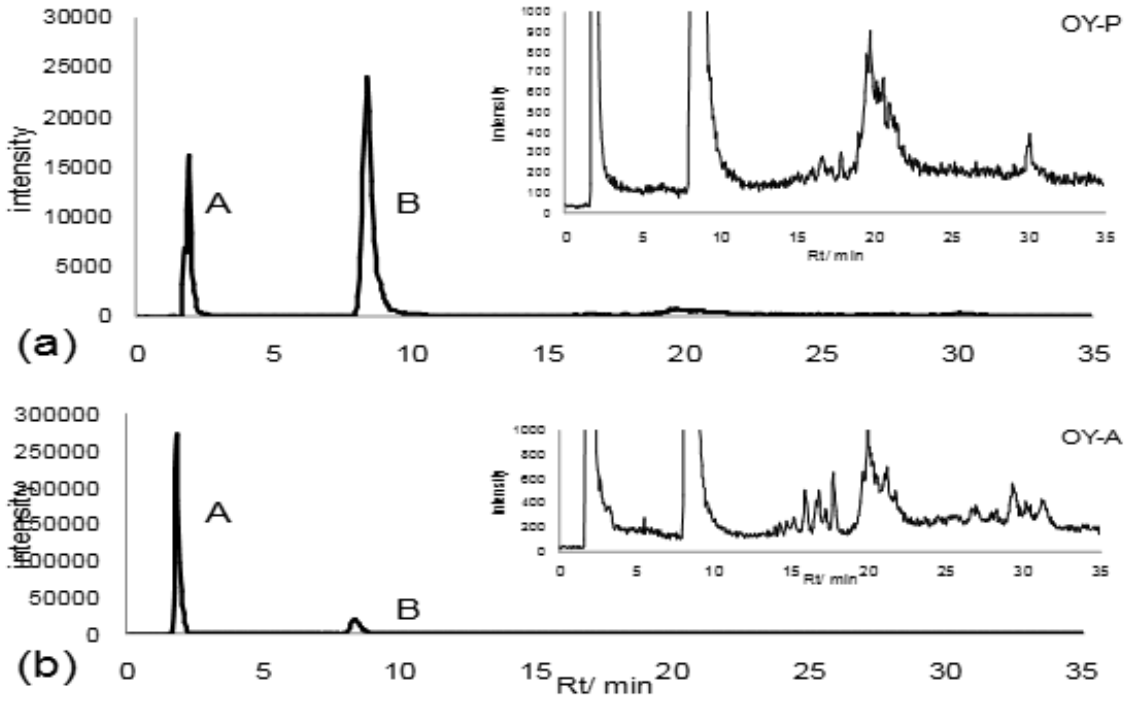

Figure 1**:** Reversed phase HPLC-ICPMS chromatogram of Oyster samples, (a) OY-P from Nigeria and (b) OY-A collected from Aberdeen, UK. (Zoomed in the insets to reveal the arsenic peaks in low intensities).

The Oyster samples showed unique arsenic profile as shown in Figure 1. A large arsenic peak A in the void volume and another major unknown early eluting peak B around the retention time of 8 – 9.5 minutes. The unknown arsenic peak B suggests the presence of water soluble organoarsenicals including arsenosugars (Sánchez-Rodas *et al.*, 2002) or short chain (polar) arsenolipids. Several other minor late eluting arsenic peaks were also detected in the Oyster samples. The two samples investigated OY-P and OY-A exhibited similar profiles which suggests some similarity in feeding habits and accumulation pattern by the species. Molecular information was generated by high resolution accurate mass ES-Orbitrap-MS. Though the chromatogram indicated the presence of several arsenolipids in low concentrations, only three arsenolipids could be identified within retention time range 16 – 18 minutes as shown in Table 4. Two of the compounds identified with the protonated molecular formula $C_{11}H_{24}AsO_3$ and $C_{21}H_{36}AsO_3$ have not been reported before and the proposed molecular structures consistent with their elemental compositions are shown in Figure 2. The third compound with protonated molecular formulae $C_{23}H_{38}AsO_3$ was previously reported in Capelin fishmeal. The mass spectra are available in the supporting information S1 – S3.

Table 4**:** Accurate masses of MeOH/ DCM arsenolipids extract from RP-HPLC-Orbitrap-MS analysis. (OY-A and OY-P represent oyster samples from Nigeria and Aberdeen respectively)

Compound	Exp. MH	Cal. MH	□m (ppm)	Rt/ min
OY-A				
$C_{21}H_{36}AsO_3$	411.1875	411.1875	-0.06	17.24
$C_{23}H_{38}AsO_3$	437.2031	437.2031	-0.18	17.77
OY-P				
$C_{11}H_{24}AsO_3$	279.0934	279.0936	-0.86	16.37

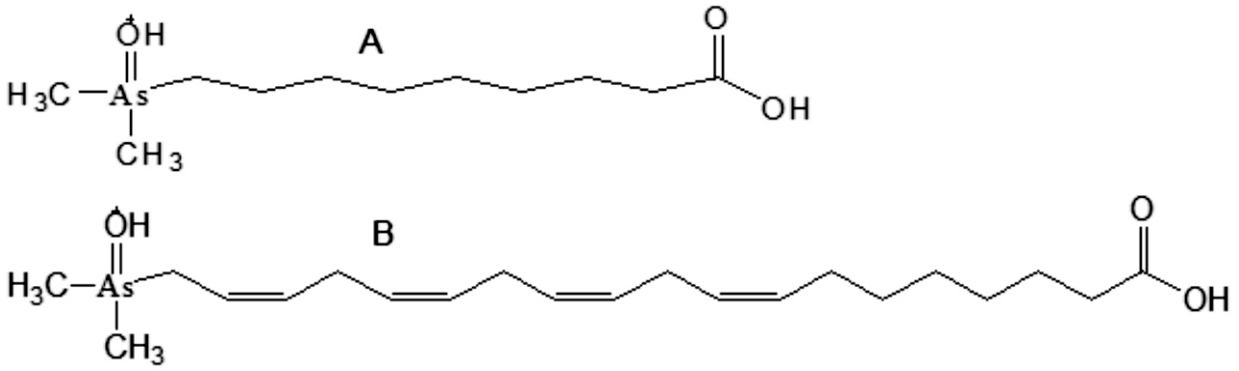

Figure 2: New compounds identified, **A** ($C_{11}H_{24}AsO_3$) and **B** ($C_{21}H_{36}AsO_3$) in Oyster samples from Nigeria and Aberdeen respectively.

CONCLUSION

The concentrations of total arsenic and arsenolipids profiles were investigated in Oysters from Aberdeen and Port Harcourt using ICP-MS and HPLC-ICP-MS/ESI-MS. Extraction method was optimized for the purification and speciation of arsenic in the lipid fraction. The data generated was used for comparative analyses of the geographical difference in bioaccumulation of arsenic which could also be used to compare the relative arsenic exposures and accumulation patterns of Oysters at the different locations. Analysis of the lipids fractions indicated the presence of several arsesenolipids in low concentrations. However, only three arsenolipids were identified including two that have not been reported before. Majority of the arsenic peaks in low intensities could not be identified due to complexity of the matrix.

ASSOCIATED CONTENT

S: Supporting Information, additional information as noted in text is provided

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