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Analytical Methods

Determination of inorganic arsenic species (As^{3+} and As^{5+}) in muscle tissues of fish species by electrothermal atomic absorption spectrometry (ETAAS)

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ABSTRACT

Arsenic speciation was carried out in muscle tissues of freshwater fish species. Inorganic arsenic species ($As^{3+}$and As^{5+}) were extracted with chloroform, prior to microwave assisted digestion with concentrated $HClO_4$ and $Fe_2(SO_4)_3$. The extracted As^{3+} and As^{5+} were determined by electrothermal atomic absorption spectrometry (ETAAS). The accuracy of the technique was evaluated by using certified reference material DORM-2. The limit of detection of the method was 0.004 and 0.005 μg/g for As^{3+} and As^{5+}, respectively. The mean relative standard deviation values (RSD) in real sample analysis were 1.90 and 3.92 for As^{3+} and As^{5+}, respectively. The results demonstrated the suitability of the procedure for screening and quantification of As species in biological samples. The mean concentration of As^{3+} and As^{5+} in muscle tissues of studied fish species ranged from 1.19 to 2.05 and 0.17 to 0.46 μg/g, respectively. The contribution of the daily intake of inorganic As, based on the consumption of 250 g fresh fish muscles/body weight/day was found in the range of 1.21–1.91 μg/kg/day.

1. Introduction

Arsenic is a ubiquitous element, introduced to the environment from natural and anthropogenic sources (Sloth, Julshamn, & Lundebye, 2005). Arsenic is known to be one of the most toxic elements and has serious effects on plants, animals and human health (Munoz & Palmero, 2000). This metalloid is present in many chemical forms that differ with regard to their physical, chemical and biological properties and have very different toxicities (Devesa, Velez, & Montoro, 2008). Due to the natural metabolic processes in the biosphere, arsenic occurs as a large number of different inorganic and organic species. To date more than 25 different arsenic species have been identified in the environment (Francesconi & Kuehnelt, 2004). Arsenic can exist in the inorganic forms as arsenite [As^{3+}] and arsenate [As^{5+}], and organic forms such as monomethylarsonic acid (MMA), dimethylarsenic acid (DMA), arsenobetaine (AsB), arsenocholine (AsC) and a series of arsenolipids and arsenosugars (Elci, Divrikli, & Soylak, 2008). Inorganic arsenic As^{3+} and As^{5+} are the most toxic species, while arsenite (As^{3+}) is more toxic than arsenate (As^{5+}), the toxicity of organic arsenical species is lower, and trimethylated species are recognised to be the least toxic (Tuzen, Citak, Mendil, & Soylak, 2009). The toxicity of As^{3+} is thought to be due to their binding to thiol groups of biologically active proteins, their acute toxicity being attributed to inhibition of metabolic enzymes (Munoz, Velez, & Montoro, 1999; Sari & Tuzen, 2009).

According to literature, the arsenic contents of freshwater fish from arsenic-contaminated and non-contaminated sites reported high arsenic concentrations and significant amounts of inorganic arsenic in the edible muscle tissues. The data suggested that freshwater fish may represent a significant source of inorganic arsenic to human populations (Jankong et al., 2007). Freshwater fishes from a polluted river in Spain contained mainly inorganic arsenic and lower amounts of AsB and arsenosugars (Devesa et al., 2002).

The microwave assisted acid digestion offers some advantages in short reaction time, the prevention of vaporisation, and the contamination control, compared to the conventional hot-plate digestion method (Shah et al., 2009). A number of analytical methods, capable of separating and quantifying several arsenic species have been applied, involving separation by liquid chromatography and detection by inductively coupled plasma-atomic emission spectrometry (ICP-AES), inductively coupled plasma-mass spectrometry (ICP-MS), hydride generation-atomic absorption spectrometry (HG-AAS) or hydride generation-atomic fluorescence spectrometry (HG-AFS) (Cava-Montesinos, Nilles, Cervera, & Guardia, 2005; Ghaedi et al., 2008). Korenovska and Suhaj (2005) have been determined As^{3+} and As^{5+} compounds in sea fish products by inorganic

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arsenic extraction into the chloroform and re-extraction into 1 M HCl solution by GFAAS.

The objective of this study was the quantitative determination of total As and inorganic arsenic species (As^{3+} and As^{5+}) in muscle tissues of ten edible fish species, collected from arsenic-contaminated Manchar Lake, Pakistan. In this work the As^{3+} and As^{5+} was determined by ETAAS, prior to microwave assisted acid digestion. The solubilisation of the inorganic arsenic with HCl and subsequent extraction with chloroform was applied for the extraction of As^{3+} and As^{5+}. The analytical characteristics of the proposed methodology (detection limit, precision and accuracy) were established and it was applied to muscle tissues of fish samples. The estimated daily intake (EDI) of inorganic As were also evaluated by the consumption of muscle tissues of fish species for possible human health risks.

2. Experimental

2.1. Reagents and glassware

Ultrapure water (resistance 0.05 μs/cm) obtained from an ELGA laboratory water system (Bucks, UK) was used throughout the experiment. Concentrated HCl (purity 37%, Sp.gr: 1.19), HNO_3 (purity 65%, Sp.gr: 1.41), H_2O_2 (purity 30%, Sp.gr: 1.11) and $CHCl_3$ were of analytical reagent-grade obtained from Merck (Darmstadt, Germany). HBr (purity 48%) and hydrazine Sulphate was obtained from fishes scientific (Bishop, UK). Iron (III) sulphate hydrate obtained from Scharlau Chemie SA (Made in European Union). Calibration for total As was prepared for each analytical session using certified stock standard solution of As 1000 mg/l, Fluka Kamica (Bushs, Switzerland). Calibrations for As^{3+} and As^{5+} were performed from standard solution of both As species prepared by analytical grade of $As(NO_3)_3$ and As_2O_5, obtained from (Merck). Appropriate dilutions were made from these solutions with 1.0 M HCl. The chemical modifiers, stock standard solution (2000 mg/l) was prepared from $Mg(NO_3)_2$ (Merck), and Pd stock standard solution, 3000 mg/l was prepared from Pd 99.999% Aldrich (Milwaukee, WI, USA). The methods were validated by certified reference material, DORM-2 (Dogfish Muscle) from the National Research Council of Canada (Ottawa, Ontario Canada). All glass wares and polyethylene bottles were thoroughly washed then soaked overnight in 5 M HNO_3 and was thoroughly rinsed with distilled and de-ionised water before use.

2.2. Instrumentation

Fish muscles tissues samples were freeze-dried in Benchtop Freeze Zone, freeze-drying system Make (Labconco, USA). The dried samples were ground in vibrational agate ball mill for 5 min using a power of 60%. The powdered samples were sieved through a nylon sieve to obtain a particle size [ø] of 30–65 μm. The ultrasonic extractions were carried out with an ultrasonic bath Sonicor, Model No. SC-121TH, Sonicor Instrument Corporation (Copiague, NY, USA) with technical specifications; programmable for temperature ranging from 0 to 90 °C, timer 0–30 min, 118 n, 220 V, 119 50–60 Hz, intensification frequency 35 kHz for the ultrasound energy. Digestions for total and inorganic arsenic determinations were performed with a Milestone Microwave System (Bergamo, Italy).

Total arsenic and inorganic arsenic species (As^{3+} and As^{5+}) analyses were carried out by a double beam Perkin–Elmer model A Analyst 700 (Norwalk, CT, USA) atomic absorption spectrophotometer, equipped with a graphite furnace HGA-400, auto-sampler AS-800 and deuterium lamp for background correction. Single element hollow cathode lamp of As operated at 7.5 mA was used as energy source. The arsenic signal at 193.7 nm was isolated with a spectral bandwidth of 0.7 nm and atomization was achieved in a pyrocoated graphite tube with integrated platform. The graphite furnace heating program was set for total As and inorganic As (As^{3+} and As^{5+}) analysis as: drying, ashing, atomization and cleaning as temperature range °C/time (s) (80–120/15, 300–600/15, 2000–2100/5 and 2100–2400/2), respectively (Baig et al., 2009a, 2009b). For all determinations, argon (200 ml/min) was used as a purge gas except during the atomization step. Portions of both, standard or sample and modifier were transferred into auto-sampler cups, and 20 μl [standard or sample volume 10 μl + 10 μl modifier (5 μg Mg $(NO_3)_2$ + 3 μg Pd)] were injected to electrothermal graphite atomizer.

2.3. Sample collection

Fish samples were collected from Manchar Lake, Pakistan (26°3′ N: 67°6′ E). All the fish samples were wrapped in plastic bags, placed in polyethylene bags, held in an ice box, transported to laboratory for further treatment. In first step, the fishes were dissected and muscles were removed from the bone. The muscles tissues were freeze-dried for 20 h at a chamber pressure of 0.225 torr. The lyophilised samples were crushed and homogenised to a fine powder in an agate ball mill. The resulting powder was stored in polyethylene bottles at −20 °C till further preparation and analysis.

2.4. Samples preparation for total arsenic

Samples digestion for total arsenic analysis was conducted by a microwave assisted acid digestion procedure. About six replicates samples of CRM (0.2 g) and triplicate samples lyophilised fish muscle tissues (0.2 g) of each fish species were directly weighed into Teflon PTFE flasks. Two ml of a freshly prepared mixture of concentrated HNO_3 and H_2O_2 (2:1, v/v) was added to each flask and was kept for 10 min at room temperature. The flasks were then placed in a covered PTFE container. It was then heated following a one-stage digestion programmed at 80% of total power (900 W) for 2–3 min, to completely oxidise the organic matrices of fish muscle tissues. The digestion flasks were cooled and diluted to 10.0 ml with 0.1 M HCl as stock sample solution. The quality controls included blank, duplicates and certified reference material (CRM) were made. To determine the detection limit, analytical blanks were prepared in a similar manner without the insertion of a sample. The concentration of total As and inorganic arsenic species (As^{3+} and As^{5+}) in each sample were calculated from the corresponding regression lines.

2.5. Moisture content

Moisture content was determined by drying samples to a constant weight in freeze-drying system (Labconco, USA) at −50 °C and was calculated as percent of water loss. The moisture contents in fish muscle tissues were 78.0 ± 2.5%.

2.6. Sample preparation for As^{3+} and As^{5+}

2.6.1. Step 1

Weighed 0.5 g of replicate six sample of CRM (DORM-2) and duplicate sample of lyophilised fish muscles were taken directly in Teflon flasks. Then added 5.0 ml concentrated $HClO_4$ and 50 mg of $Fe_2(SO_4)_3$, digested in microwave oven at one stage heating program involved 80% of total power for 3 min. The resulted digest was evaporated to remove extra $HClO_4$, and then dilute up to 25 ml with 1 M HCl and kept as stock sample solution.

2.6.2. Step 2

2.6.2.1. Determination of As^{3+}. Six replicate of digested samples (5 ml) were taken in separating funnel, added 10 ml of 1 M HCl and placed in ultrasonic bath for 5 min. Added 10 ml of chloroform and placed in ultrasonic bath for 1 min (twicely), kept at room temperature to separate two phase. The chloroform fractions was poured to the second separating funnel, and As^{3+} was extracted with 1 M HCl and determined by ETAAS.

2.6.3. Step 3

2.6.3.1. Determination of As^{5+}. The residual aqueous fractions obtained from step 2, added 1 ml of HBr and 15 mg hydrazine sulphate for reduction of As^{5+} to As^{3+} (Korenovska & Suhaj, 2005). After subjecting to ultrasonic bath for 5 min at 80 °C, the content of the flasks was treated with 10 ml chloroform and placed in ultrasonic bath for 1 min (twicely). The chloroform phase was taken in separating funnel and added 10 ml of 1 M HCl to extract As^{+5} and determined by ETAAS.

3. Results and discussion

In the framework of a broad survey of arsenic contamination, 10 fish species of freshwater were collected from polluted Manchar Lake, Pakistan. The water and sediment samples of Manchar lake have high level of total As, ranged (60.4–101.8 μg/l) and (13.2–17.7 μg/g), respectively. The concentration of total As in lake water is 6.0–10 times greater than permissible limit of As for drinking water (Arain, Kazi, Jamali, Afridi, & Jalbani, 2008). Total As and toxic inorganic arsenic As^{3+} and As^{5+} were determined by the electrothermal atomic absorption spectrometry (ETAAS). The method was assured by the analysis of triplicate samples, reagent blank, certified reference material DORM-2 (dogfish muscles) and standard addition method. The Dorm-2 has certified value of total arsenic, while recoveries of As species were compared with literature reported data. The percentage recoveries of total As and different species (As^{3+} and As^{5+}) were calculated by the equation:

$$\%\text{Recovery} = \frac{\text{Certified/literature values}}{\text{Found value}} \times 100$$

Application of paired *t*-test also showed that there is no difference between found values (obtained from standard addition method)/literature values. The paired *t*-test was calculated for $n - 1 = 5$ degrees of freedom, t_{exp} (0.146) was less than the t_{crit} (2.57) at a confidence interval of 95% ($P = 0.05$) (Table 1). For further assessing the method performance, the expanded uncertainties (U_Δ) for total As, As^{3+} and As^{5+} were calculated as: 1.11, 0.011 and 0.082, respectively, which were higher than Δ_m (i.e. the differences between the certified or literature values and measured values). The measured values are therefore not significantly different from the certified value. The validation data confirm that applied method could be used as a routine procedure for the determination of total As, As^{3+} and As^{5+}contents in biological samples.

Table 1
Validation of the proposed method for determination of total and inorganic As (As^{3+} and As^{5+}) against CRM (DORM-2) (μg/g).

CRM (DORM-2)	Certified/literature values (μg/g)	Found values $\frac{\bar{x} \pm ts}{\sqrt{n}}$[a]	%Recovery[b]	Paired *t*-test[c] $t_{Experimental}$
TAs	18.0 ± 1.1	17.9 ± 0.98	99.4	0.731
As^{3+}	0.05 ± 0.01*	0.053 ± 0.001	102	
As^{5+}	0.05 ± 0.02*	0.051 ± 0.002	106	

* Literature values (Cava-Montesinos et al., 2005).
[a] Average value ± confidence interval ($P = 0.05$).
[b] $\%\text{Recovery} = \frac{[\text{Certified/literature value}]}{[\text{Found values}]} \times 100$.
[c] Paired *t*-test between certified/literature values and found values, degree of freedom $(n - 1) = 5$, $t_{Critical}$ at 95% confidence limit = 2.57.

The detection and quantitation limits for each arsenic species were calculated by $\text{LOD} = 3 \times \frac{s}{m}$ and $\text{LOQ} = 10 \times \frac{s}{m}$, respectively. Where *s* is the standard deviation of 10 measurements of reagent blanks and *m* is the slope of the calibration curve in the best experimental conditions. The LOD of As^{3+} and As^{5+} were reached at 0.004 and 0.005 μg/g, while LOQ were calculated as 0.011 and 0.017 μg/g, respectively. The linear range of the calibration graph ranged from the quantification limit up to 50 μg/l for As^{3+} and As^{5+}.

Arsenic speciation analyses were performed on freeze-dried fish muscle tissues using ETAAS, prior to applying modified reported method (Korenovska & Suhaj, 2005). The accuracy of methodology was checked by CRM DORM-2 for total As, while due to unavailability of certified values of both As species, standard addition method was applied by spiking known amount of As^{3+} and As^{5+} in real sample. Triplicate sub samples of lyophilised muscle tissues (0.2 g) were spiked with standards of both As species at three concentration levels, before treatment. The Quantitative recoveries were obtained for 102 ± 3% and 100 ± 5% for As^{3+} and As^{5+}, respectively (Table 2). These values are evidence for the excellent performance of the methodology used for speciation of inorganic arsenic. The precision of the techniques, expressed as the relative standard deviation (%RSD) of a minimum of six independent analyses of the same real sample, was observed as 1.90 and 3.92 for As^{3+} and As^{5+}, respectively.

The diverse levels of arsenic amongst individual fish made it to some extent difficult to find out significant differences in the contents of total arsenic and different arsenic species. Mean total arsenic in muscles tissues of ten fish species ranged from 6.1 to 11.8 μg/g. The mean concentration of As^{3+} and As^{5+} in the muscle of studied fish species ranged from 1.19 to 2.05 μg/g and 0.17 to 0.46 μg/g, respectively. The sum of inorganic As^{3+} and As^{5+} in fish muscles of ten fish species were found in the range of 17.2–31.9% of total arsenic content (Table 3). The concentration of total As determined in our study was higher than some reported values for fish muscles tissues (Has-Schon, Bogut, & Strelec, 2006; Rosemond, Xie, & Liber 2008), while our results are consistent with reported data (Delgado-Andrade, Navarro, Lopez, & Lopez, 2003).

Differences in accumulation of arsenic in muscle tissues of different fish species may be related to feeding behaviour and specific physiology of fish species. The understudied fish species, *Mrigala*, *Thaila*, *Seenghara*, *Bam* and *Gonia* were omnivorous tend to accumulate more arsenic than fish species belong to herbivorous group (*Calbasu*, *Reba*, *Gullio*, *Dayo*, *Rahu*). It was reported in literature that omnivorous fish tend to have higher body burdens of total arsenic than forage fish (Chen & Folt, 2000; Kirby & Maher, 2002).

The arsenic species in freshwater fish are not extensively studied as compared to marine fish (US, 2003). In present work, we determined the inorganic arsenic species (As^{3+} and As^{5+}) in ten fresh water fish species. The arsenic speciation in the fish muscle tissues revealed marked differences in the concentration of As^{3+}

Table 2
Recovery of inorganic arsenic (As^{3+} and As^{5+}) spiked in muscle tissues of *Labeo calbasu* (μg/g).

As^{3+} added	As^{3+} found values	%Recovery
0	1.70	–
1	0.98	98.0
2	2.05	102
4	4.14	104
As^{5+} added	As^{5+} found values	%Recovery
0	0.34	–
1	0.99	99.0
2	2.01	101
4	4.12	103

Table 3
Analytical results for total and inorganic arsenic (As^{3+} and As^{5+}) of muscle tissues of fresh water fish species (μg/g) on dried basis (n = 100).

Fish species	TAs	As^{3+}	As^{5+}	% of iAs
	$\bar{x} \pm s$			
Labeo calbasu	11.8 ± 0.21	1.70 ± 0.15	0.34 ± 0.10	17.3
Cirrhinus mrigala	7.13 ± 0.12	1.19 ± 0.17	0.27 ± 0.08	28.2
Cirrhinus reba	10.2 ± 0.46	1.79 ± 0.17	0.34 ± 0.06	20.8
Mystus gullio	8.11 ± 0.46	1.70 ± 0.23	0.30 ± 0.23	24.6
Catla catla	6.91 ± 0.08	1.38 ± 0.41	0.33 ± 0.15	28.0
Labeo gonius	9.10 ± 0.39	2.00 ± 0.31	0.30 ± 0.07	25.5
Mastacembelus armatus	10.1 ± 0.45	2.05 ± 0.12	0.20 ± 0.10	22.2
Tilapia mossambicus	6.11 ± 0.08	1.65 ± 0.41	0.17 ± 0.09	31.9
Labeo rohita	10.6 ± 0.12	1.79 ± 0.21	0.46 ± 0.04	21.2
Mystus seenghara	8.21 ± 0.19	1.86 ± 0.27	0.18 ± 0.06	24.8

Table 4
Estimation of inorganic arsenic (As^{3+} and As^{5+}) intake by consumption of 250 g of fish muscles/kg body weight/day (wet basis).

Fish species	μg of Inorganic As/kg body weight/day
Labeo calbasu	1.70
Cirrhinus mrigala	1.21
Cirrhinus reba	1.77
Mystus gullio	1.67
Catla catla	1.42
Labeo gonius	1.91
Mastacembelus armatus	1.87
Tilapia mossambicus	1.51
Labeo rohita	1.87
Mystus seenghara	1.71

and As^{5+} (Table 3). It was reported in literature that the toxicity and physiological function of As differed greatly with its chemical forms (Yamauchi & Fowler, 1994). Measured values of As^{3+} and As^{5+} in fish muscles tissues are relatively higher and their sum was found in the range of 17.1–31.9% of the total arsenic measured, which are higher in accordance with published data about inorganic arsenic species in fish tissues (Rosemond et al., 2008). It was observed that in fish species, *Cirrhinus mrigala*, *Catla catla*, *Labeo gonius*, *Mastacembelus armatus*, *Tilapia mossambicus*, and *Mystus seenghara* have higher % of inorganic As species (As^{3+} and As^{5+}), while only one specie (*Labeo calbasu*) have low inorganic arsenic (17.2%) of total arsenic content. The results of present study indicates that the inorganic arsenic species was higher as compared to some other freshwater fish (Chen & Folt, 2000; Holak & Specchio 1991; Munoz & Palmero, 2000), because due to high salinity level of understudy Lake Ecosystem (Arain et al., 2008). Present work was also confirmed the assumption that the major part of the arsenic in fish exists as low-toxic organic arsenic species (Branch, Ebdon, & O'Neill, 1994).

Estimated daily intake of inorganic arsenic based on the consumption of 250 g fresh fish muscles per day ranges from 1.21–1.91 μg/kg body weight/day (Table 4), which are below the FAO/WHO tolerable daily intake of 2.1 μg/kg/day of inorganic arsenic (FAO/WHO, 1983). Estimated daily dietary intake of inorganic arsenic ranges from 8.3 to 14 mg/day in the United States and from 4.8 to 12.7 mg/day in Canada for various age groups, which is corresponding to the 21–40% of total dietary arsenic occurs in inorganic forms (Yost, Schoof, & Aucoin, 1998).

4. Conclusion

The toxicity of arsenic is species dependent; therefore speciation information is mandatory for those fish species living in As contaminated ecosystem. The results obtained demonstrate the suitability of the procedure for screening and quantification of inorganic arsenic species (As^{3+} and As^{5+}) in muscles samples of different fish species. The resulted data indicates s that dietary intakes of inorganic arsenic via understudied fish species are higher than reported values in literature. While the calculated daily intake of inorganic arsenic contents in fish muscles did not exceed the permissible values recommended by FAO/WHO. Future study is also needed to develop methodologies to evaluate the total, inorganic and organic arsenic in different fish species and their bioavailability.

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