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Arsenic speciation in freshwater fish: Focus on extraction and mass balance

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ABSTRACT

Arsenic (As) speciation in muscle tissues of freshwater fish was investigated with special emphasis on extraction yields and mass balances. For the quantification of water-soluble As compounds, samples were extracted using a 1:1 (v/v) methanol/water mixture. Various extraction parameters, such as extraction volume, number of additional extractions, intermediate and final volume in sample preconcentration were optimized so as to improve the extraction efficiency. Arsenic compounds were determined by HPLC with online ICP-MS, using both cation and anion exchange separation. The species studied were eel (*Anguilla anguilla* L.), flathead grey mullet (*Mugil cephalus* L.), chub (*Leuciscus cephalus* L.), and carp (*Cyprinus carpio* L.). Mean total As concentrations ranged from 354 μg kg^{-1} dry weight (carp) to 1804 μg kg^{-1} dry weight (mullet). Under optimized conditions, the percentage of total As extracted ranged from 64% for carp, to 82%, 84%, and 89% for grey mullet, eel and chub, respectively. Extraction of lipid-rich eel with *n*-hexane recovered some additional 3% of total As. The sizeable effect of sample matrix on HPLC retention time of some organoarsenicals in gradient elution cation exchange chromatography was exploited to achieve separation of coeluting compounds by analysing the same sample at different dilutions. The recovery of As from chromatographic columns was 101%, 102%, 103% and 104% for carp, chub, mullet and eel, respectively, indicating that no As was retained during chromatography. Arsenobetaine (AB) was the dominating As compound, but several other arsenicals, including arsenous acid (AsIII), arsenic acid (AsV), methylarsonic acid (MMA), dimethylarsinic acid (DMA), trimethylarsine oxide (TMAO), arsenocholine ion (AC), tetramethylarsonium ion (TETRA), oxo-arsenosugar-glycerol (AS1), oxo-arsenosugar-phosphate (AS2), oxo-arsenosugar-sulfate (AS4), thio-arsenosugar-phosphate (ThioAS2), and three unknown As compounds, were found. Arsenic speciation in carp was different compared to the other fish species, and a lower proportion of AB along with a high contribution of AS2 and ThioAS2 was found. DMA and TMAO were noteworthy minor compounds in eel and carp, respectively. Arsenic speciation and the chemical composition of fish muscle both appeared to affect the extraction yield.

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1. Introduction

Arsenic (As) speciation in marine organisms has been widely investigated over the last two decades. Several studies contributed to current understanding about the presence of As compounds in these organisms, in which high concentrations of As are mainly due to virtually nontoxic arsenobetaine (AB) – in fish, crustaceans and molluscs – and arsenosugars – in algae [1–4]. Arsenolipids (e.g., in liver) and occasionally other organoarsenic species can be found as major compounds in marine animals; in some cases they also contain arsenosugars, the source of which being almost certainly algae [5–7]. All marine organisms are characterized by a low content of inorganic As (few exceptions to this general rule have been reported [8,9]), generally higher in shellfish compared to fish. The predominance of organoarsenic compounds in marine organisms has been tentatively ascribed to processes involving biotransformation and detoxification of inorganic arsenic (algae) as well as selective bioaccumulation of organic arsenicals in marine food chains (animals). In marine algae, arsenate (AsV) taken up from seawater – probably because of its structural similarity with the essential phosphate – is detoxified by successive oxidative alkylation steps and accumulated as arsenosugars. The biosynthetic origin of AB remains unknown, even though microbial conversion of arsenosugars into AB *via* dimethylarsinoyl ethanol (DMAE) and *via* either dimethylarsinoylacetic acid (DMAA) or arsenocholine (AC) was put forward [10–12]. Since AB is highly bioaccessible to marine animals, even traces of this compound in seawater might be sufficient to result in the demonstrated ubiquity of AB [5]. As a matter of fact, AB seems to be taken up by marine invertebrates because of its structural similarity to glycine betaine, an osmolyte protecting osmoconforming organisms from changes in the salinity of their ambient water. This hypothesis is supported by experiments which found a positive relationship between As content and salinity in mussels maintained in seawater of varying salinities [13], and a decrease in

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the efficiency of AB uptake from seawater by these mussels with increasing levels of glycine betaine – a result consistent with competitive uptake [14]. The same positive relationship between As content and salinity was found in teleosts of the same species taken from adjacent waters of varying salinities, likely because the higher AB content of the organism on which fish fed in waters with higher salinity causes the increase of As concentration in fish muscle [15].

The limited data on freshwater species make it difficult for a typical pattern of As compounds for these organisms to be defined, even though evidence exists of a more variable As speciation compared to marine organisms, especially in the case of fish [16–25]. It is unquestionable that freshwater animals are characterized by lower concentrations of total As than their marine counterparts, which explains why the investigation of As speciation in these organisms has indeed attracted less interest. This lower As content reflects the limited accumulation of AB in freshwater animals, in accordance with the relationship between salinity and AB bioaccumulation [15]. In turn, this results in relative proportions of As species often different from that of marine species, in which AB almost invariably predominates and makes >90% of the total As present. Of more importance from the perspective of food safety is whether freshwater fishes are able to accumulate toxic inorganic species to a higher extent than their marine counterparts, given that the concentration of inorganic As is more variable in freshwater ecosystems than in seawater because of the greater influence exerted by natural and anthropogenic sources. Studies on As biotransformation in freshwater environments using three-step food chains (alga, shrimp, fish) showed that Asv accumulated from water was not transferred to higher trophic levels but extensively biomethylated [26,27]. Similar results were found for As-contaminated natural environments [28], and only arsenosugars and AB were detected in algae and shellfishes when a species-selective analytical method was used [25]. Arsenosugars were identified as the most abundant arsenicals in algae and mussels in other investigations on freshwater ecosystems; the low AB content of freshwater mussels was a striking difference with their marine counterparts [21,29]. The situation with freshwater fish is less clear. It is apparent that the As speciation is somewhat variable according to the fish species, feeding and living habits, as well as the extent of inorganic As contamination of water. In some cases AB is the major arsenical (e.g., salmonids, farmed fish) [16–18,24], often other compounds are abundant and even predominate [20,21,25], including inorganic species in As-contaminated areas [19,23]. A further obstacle to a better understanding of As speciation in freshwater fish is the low mass balance obtained in many investigations. Almost all of the studies carried out so far have indeed been employing high-performance liquid chromatography–inductively coupled plasma mass spectrometry (HPLC–ICP-MS), but either low extraction efficiencies or low column recoveries – sometimes both – often caused the sum of the detected species to be <30–40% of the total present As. Therefore, apparently abundant species can be, as a matter of fact, relatively minor since those making up the larger portion of the As present remains undetected.

The aim of this present work was to develop an analytical approach for the investigation of As speciation in freshwater fish by HPLC–ICP-MS, with special emphasis on extraction yields and overall recovery. In order to obtain sound mass balances, extraction parameters were optimized for maximum yield and the recovery of As from chromatographic columns was characterized. Attention was paid in developing a method with a suitable detection power since, on the one hand, the concentrations of the analytes are much lower than in the case of marine fishes and, on the other hand, the understanding of As fate in freshwater ecosystems necessitates the determination of minor compounds along with the most abundant ones. Therefore, the procedures for sample preconcentration as well as the instrumental conditions were carefully selected and tailored to the intended application. The determination of As compounds was performed by both cation and anion exchange chromatography in order to achieve a possibly complete picture of the As species present. The analytical method was applied to As speciation in muscle tissue of four edible freshwater teleosts, namely, eel (*Anguilla anguilla* L., Anguillidae), flathead grey mullet (*Mugil cephalus* L., Mugilidae), chub (*Leuciscus cephalus* L., Cyprinidae), and carp (*Cyprinus carpio* L., Cyprinidae).

2. Experimental

2.1. Chemicals and standards

All of the chemicals used during the analytical procedure were of ultrapure grade. Deionized water employed in all solutions was obtained by a Milli-Q Element System (Millipore, Molsheim, France). Calibrants and the internal standard solution (rhodium) used for total element measurements were obtained from standard solutions with a certified content of 1 mg mL^{-1} (High-Purity Standards, Charleston, SC), which were diluted with acidified (HNO_3) deionized water as necessary.

The standard solutions used for the identification and quantification of As compounds were prepared in deionized water with a concentration of 200–1000 mg L^{-1} as As. Arsenic trioxide (Asiii), di-sodium hydrogen arsenate heptahydrate (Asv), and dimethylarsinic acid (DMA) were obtained from Fluka (Dorset, UK). Methylarsonic acid (MMA), trimethylarsine oxide (TMAO), tetramethylarsonium iodide (TETRA), and arsenocholine bromide (AC) were obtained from Tri Chemical Laboratories Inc. (Yamanashi, Japan). Dimethylarsinoylacetic acid (DMAA), dimethylarsinoyl propionate (DMAP), trimethylarsoniopropionate (TMAP), oxo-arsenosugar-glycerol (AS1), and the algal extract of *Fucus serratus* containing the four major oxo-arsenosurgars, i.e., AS1, oxo-arsenosugar-phosphate (AS2), oxo-arsenosugar-sulfonate (AS3), and oxo-arsenosugar-sulfate (AS4) [30] were obtained by Professor K.A. Francesconi (Karl-Franzens University, Graz, Austria). A certified standard solution was used for AB (BCR CRM 626, IRMM, Geel, Belgium). The exact As content of each standard was determined by ICP-MS using the method of standard additions.

2.2. Sample preparation

Specimens of eel, flathead grey mullet, chub, and carp were collected from the Tiber river (Latium, Italy). Four individuals were pooled to obtain the analytical samples. The average length of the specimens was *ca.* 35 cm for eel, 40 cm for mullet, 40 cm for chub, and 50 cm for carp. All sample manipulations in the laboratory were carried out in clean room conditions under a laminar flow box (Spetec GmbH, Erding, Germany). The muscle tissues were removed with titanium and tungsten carbide instruments (Fine Science Tools, Heidelberg, Germany), frozen, and subsequently freeze-dried and homogenized.

For the determination of total As, aliquots of *ca.* 250 mg of freeze-dried muscle tissues were placed in high-pressure Teflon containers with the addition of 3 mL of HNO_3, 2 mL of H_2O_2 and 3 mL of deionized water, and then mineralized by microwave assisted digestion (Milestone Ethos Pro microwave labstation, FKV, Bergamo, Italy). A predigestion step was performed before microwave irradiation with temperature control and automatic continuous adjustment of power output. The temperature profile of the irradiation process was: 5 min ramp to 120 °C, 3 min at 120 °C, 6 min ramp to 190 °C, 15 min at 190 °C. After cooling, the sample solutions were quantitatively transferred into decontaminated disposable tubes and diluted to a volume of 15 mL with deionized water.

For the quantification of water-soluble As compounds, muscle tissue samples were extracted using a 1:1 (v/v) methanol/water mixture under mechanical agitation. Approximately 500 mg of each freeze-dried sample were weighed into 50 mL polyethylene test tubes. Various extraction parameters, such as extraction volume, number of additional extractions, intermediate and final volume in either evaporation or freeze-drying, were optimized so as to improve the extraction efficiency for each fish species under investigation. In details, a first overnight extraction (15 h) was performed using 10 or 20 mL of a methanol/water mixture followed by centrifugation (10 min, 4000 rpm) and half of the sample extracts was preconcentrated by either evaporation, using a Zymark TurboVap II Concentrator Workstation (FKV, Bergamo, Italy), or freeze-drying by means of a Lyolab 3000 (Heto-Holten A/S, Allerød, Denmark). Samples were preconcentrated either to an intermediate volume of 1 mL or to dryness. After preconcentration, sample extracts were made up to a final volume of 2.5 mL with aqueous methanol (MeOH 3%, v/v) and filtered through a 0.45 μm filter. The other half of the samples was further extracted twice with 3 mL methanol/water and the supernatants obtained by centrifugation were combined with that from the first extraction and preconcentrated. For eel, before of the methanol/water addition, an extraction with *n*-hexane was carried out. An aliquot of each sample extract was mineralized by microwave assisted digestion using 3 mL of HNO_3, 2 mL of H_2O_2 and 3 mL of deionized water. At the end of the irradiation, the digested samples were diluted to a volume of 15 mL with deionized water.

The trueness of the determination of total As, AB, DMA, and TETRA was checked by analysis of the certified reference materials (CRMs) DORM-2 Dogfish Muscle (National Research Council of Canada) and BCR627 Tuna Fish Tissue (IRMM). The CRMs were extracted using 20 + 3 + 3 mL of 1:1 (v/v) methanol/water mixture on *ca.* 250 mg of samples. Extracts were evaporated and the residue diluted to a final volume of 5 mL.

2.3. Analytical determinations

Quantification of total As in muscle tissues was performed by ICP-MS using an Elan DRC II spectrometer (PerkinElmer, Norwalk, CT). The sample introduction systems consisted of a peristaltic pump, a Meinhard quartz concentric nebulizer and a cyclonic quartz spray chamber. In order to determine the efficiency of the extraction process, the concentration of total As was determined in extracts of fish and CRMs.

Arsenic compounds were determined by HPLC–ICP-MS. A PerkinElmer 200 series quaternary pump equipped with an autosampler and a column thermostat was used as the chromatographic system. The outlet of the HPLC column was directly connected via PEEK capillary tubing to the nebulizer of the ICP-MS instrument, which served as the As specific detector. A PC^3 Peltier-cooled quartz cyclonic spray chamber (Elemental Scientific Inc., Omaha, NE) set at 2 °C was used for sample introduction. Separations were carried out by a cation exchange column (Chrompack IonoSpher-5C, 100 mm × 3.0 mm i.d., 5 μm particles; Varian, Middelburg, The Netherlands) using aqueous pyridine formate at pH 2.7 with gradient elution and by an anion exchange column (ICSep ION-120, 120 mm × 4.6 mm i.d., 10 μm particles; Transgenomics, San Jose, CA) with either 10 or 20 mM ammonium carbonate at pH 10.3 as mobile phase. The instrumental conditions are given in Table 1.

The response factor of As compounds in HPLC–ICP-MS was studied using the instrumental set-up and the chromatographic conditions adopted for samples. Three determinations of each standard were carried out and the quantitative calculations were based on peak areas. These determinations also enabled to check the presence of other arsenicals as impurities in standard solutions.

Arsenic compounds in sample extracts were identified by matching retention times with those of standard compounds. Spiking experiments were carried out for both qualitative and quantitative analysis. Quantification of As compounds was also carried out by using an external AB calibration curve for comparison purposes.

Gradient elution cation exchange separation was carried out using different dilutions for each of the sample extracts in order to accurately determine both major and minor arsenicals. This also overcame coelution of some compounds because of retention time shifts caused by matrix effects.

In order to obtain sound mass balances, the recovery of As from chromatographic columns was studied as well.

2.4. Statistical analysis

The existence of significant differences in extraction yields on account of the different extraction procedures was checked by analysis of variance for each fish species. ANOVA was significant for all fish species ($p = 0.05$) and thus a multiple comparison test (LSD) was carried out to identify which type of treatment caused significant variations.

3. Results and discussion

3.1. Extraction yield of As compounds

The mean total As concentration in muscle tissue (dry weight basis) ranged from 354 $\mu g\,kg^{-1}$ for carp, to 1091, 1147, and 1804 $\mu g\,kg^{-1}$ for chub, eel and mullet, respectively. These concentrations were comparable with previous data reported for the same fish species collected from unpolluted areas [20,31–33].

The yields obtained by varying the extraction parameters are shown in Table 2 (each value is the mean of 3 experimental replicates). The extraction efficiency did not differ statistically depending on the type of preconcentration procedure (evaporation or freeze-drying). However, preconcentration to dryness gave more reproducible results than when using an intermediate volume of 1 mL (data not shown) and was therefore preferred. Statistical analysis of extraction yield in relation to extraction volume and number of additional extractions gave different results depending on the fish species (Table 2). For eel and mullet, extraction with 10 mL of methanol/water followed by additional extractions with 3 mL of solvent did not differ from extraction with 20 mL of solvent, followed or not by subsequent extractions (20 + 3 + 3 mL); the three procedures yielded better results than extraction with 10 mL of solvent only. It is to be noted that for eel a previous extraction with *n*-hexane was carried out (see below). In the case of chub, extraction with greater volumes of solvent (20 or 20 + 3 + 3 mL) did give higher yields than extraction with 10 + 3 + 3 mL. On the contrary, in the case of carp, performing additional extractions was the key factor in obtaining higher yields and there was no difference if either 10 + 3 + 3 mL or 20 + 3 + 3 mL were used.

Under optimized conditions, the percentage of total As extracted ranged from 82% for mullet, to 84% and 89% for eel and chub, respectively. However, only 64% of total As in carp could be extracted. Taking into account the low As concentration in the samples, these yields represents an improvement when compared to the extraction efficiencies of the studies on freshwater fish carried out so far. However, as observed by other authors in previous studies [20,21], the extraction yield actually depended upon the arsenic species present and was high (80–90%) in fish where AB predominated, that is, mullet, eel, and chub (see below). For carp, it is clear that further efforts in the field of sample extraction are needed in order to achieve results close to the other species. From the toxicological

Table 1
Instrumental conditions for As speciation analysis.

ICP-MS settings		
RF power	1500 W	
Plasma gas flow	15.5 L min^{-1}	
Auxiliary gas flow	1.20 L min^{-1}	
Carrier gas flow	1.05 L min^{-1}	
Nebulizer	Meinhard quartz concentric	
Spray chamber	Peltier-cooled cyclonic quartz spray chamber (2 °C)	
Interface	Pt sampler and skimmer cones	
Analytical mass	^{75}As	
Mass for interference assessment	^{40}Ar^{37}Cl (anion exchange chromatography)	
HPLC conditions		
Anion exchange chromatography		
Column	ION-120	
Column temperature	23 °C	
Injection volume	100 μL	
Mobile phase	10–20 mM $(NH_4)_2CO_3$ in 3% (v/v) MeOH, adjusted to pH 10.3 with NH_3	
Elution mode	Isocratic	
Flow rate	0.7 mL min^{-1}	
Cation exchange chromatography		
Column	Chrompack IonoSpher-5C	
Column temperature	35 °C	
Injection volume	100 μL	
Mobile phase	(A) 3% (v/v) MeOH (B) 50 mM pyridinium formate in 3% (v/v) MeOH, pH 2.7	
Flow rate	1.0 mL min^{-1} (unless otherwise stated)	
Gradient programme	0–3 min	99% A
	3–4 min	90% A–10% B
	4–15 min	90% A–10% B
	15–16 min	60% A–40% B
	16–20 min	60% A–40% B
	20–25 min	99% A (2.0 mL min^{-1})

point of view, it is essential to find out whether the unrecovered As is in the form of inorganic species. If carp is able to accumulate As at higher levels than in this study and, at least under certain environmental conditions, in the form of toxic arsenic, this is of course a relevant food safety issue since this fish happens to be a widely consumed species in many countries.

Eel muscle tissue may contain ≥20% of lipids and it was thus investigated whether any As was present in the lipid fraction and could be extracted with a non-polar solvent. In effect, extraction of eel samples with *n*-hexane recovered some additional 3% of total As. Even more interesting is the fact that *n*-hexane extraction enhanced the yield of the extraction subsequently performed with methanol/water. It appears that the partial removal of lipids made it easier to extract polar arsenicals from fish muscle. This is in agreement with the fact that chub, the species with the lower lipid content (2.8%), is also the one with the higher extraction yield of water-soluble As species. Mullet and carp contain higher amounts of lipid in their muscle tissue and did show lower extraction yields; in turn, the sizeable difference in extraction yield between these two species, notwithstanding their similar lipid content (6.8% and 7.1%, respectively), is a likely consequence of the different speciation (i.e., relative abundance of AB and other water-soluble arsenicals). Chemical composition is therefore another factor that possibly plays a role in the extraction of As species in fish and its

Table 2
Extraction yields obtained by varying extraction parameters for each of the fish species ($n = 3$).

Sample#	Steps	Extractant (mL)	Extraction efficiency[a]
Eel[b]			
Sample 1	1	10	76% a
Sample 2	3	10 + 3 + 3	84% b
Sample 3	1	20	80% a,b
Sample 4	3	20 + 3 + 3	81% a,b
Flathead grey mullet			
Sample 1	1	10	67% a
Sample 2	3	10 + 3 + 3	79% b
Sample 3	1	20	74% b,c
Sample 4	3	20 + 3 + 3	82% b
Chub			
Sample 1	1	10	72% a
Sample 2	3	10 + 3 + 3	81% b
Sample 3	1	20	86% c
Sample 4	3	20 + 3 + 3	89% c
Carp			
Sample 1	1	10	53% a
Sample 2	3	10 + 3 + 3	64% b
Sample 3	1	20	47% c
Sample 4	3	20 + 3 + 3	62% b

[a] Treatments with the same letter do not differ statistically ($p = 0.05$).
[b] A previous extraction with *n*-hexane was carried out for eel samples.

interplay with As speciation is something that has to be considered in future studies focusing on the currently non-extractable As in freshwater fish. Since the lipid content of fish species is quite variable, it would be not surprising to find out that the wide differences observed in the amount of polar arsenicals that can be extracted from freshwater fish depended on muscle chemical composition along with As speciation [34].

3.2. Development of the anion and cation exchange HPLC–ICP-MS method

Gradient elution cation exchange HPLC–ICP-MS has been showed to enable the selective determination of a large number of naturally occurring organoarsenic compounds in aquatic organisms [4]. However, it cannot detect species which are not retained on a cation exchange column such as inorganic As (AsIII and AsV) and MMA. Therefore, both anion and cation exchange HPLC–ICP-MS were used as complementary techniques with the aim to get a full picture of the As species present in sample extracts, minimising at the same time the risk of species coelution. Figs. 1 and 2 show anion and cation exchange HPLC–ICP-MS chromatograms for extracts of eel and carp, respectively.

Anion exchange chromatography in the isocratic mode allowed the separation of AsIII, AS3, MMA, AS4, and AsV. DMA coeluted with AS2 (Fig. 2a), but it was accurately determined by cation exchange chromatography (Fig. 2c), thus enabling the indirect determination of AS2. Since the thio-analogues of AS1 and AS2 have been reported in freshwater fish [20,21] a mixture containing these species was obtained by adding a saturated aqueous solution of H_2S to an aliquot of the algal extract of *F. serratus* containing AS1 and AS2. In the experimental conditions used (isocratic mode, 20 mM ammonium carbonate as the mobile phase), ThioAS1 coeluted with AsIII whereas ThioAS2 eluted close to AS4 and MMA. However the identity of the two thio-arsenosugars was easily checked by addition of H_2O_2, which caused their quantitative oxidation to the oxo-analogues [35]. In this way, the identification of ThioAS2 in extracts was accomplished (Fig. 3). Identifications were checked by using different chromatographic conditions, i.e., 10 mM ammonium carbonate as mobile phase, which allowed ThioAS2 to be completely separated from AS4 and MMA. On the other hand, the use of 20 mM ammonium carbonate as the mobile phase enabled the sensitive detection of AS4, MMA and AsV within 15 min (Fig. 2b).

Cation exchange HPLC with gradient elution was necessary to separate and identify all the other arsenicals investigated in this study, that is, DMA, DMAA, DMAP, AB, AS1, TMAO, TMAP, AC, TETRA. However, as previously noted by others [4], with this method matrix effects are substantial and exert a dramatic influence on the retention times of species like AB and DMAA, whereas other compounds are not affected. Fig. 4 shows the variation in retention times of As compounds in muscle extracts of mullet with three different dilutions. The substantial effect on AB as well as the small or negligible effect on the other peaks is evident. The same variation in retention times was obtained by injecting different volumes of the same sample. The extent of the matrix effect was dependent upon the sample and it was the greatest with mullet and eel, whereas it was smaller with carp and the smallest with chub. This behaviour was exploited in qualitative determinations with the aim to achieve separation of potentially coeluting compounds by analysing the same sample at different dilutions and spiking it with standard substances for peak assignment. For instance, the analysis of undiluted and diluted (1:2, 1:10) extracts of mullet allowed to achieve an effective separation of As compounds and greatly reduced the risk of coelutions. On the other hand, matrix effects did not influence the response factor of As compounds and quantitative results thus agreed among samples analyzed at dif-

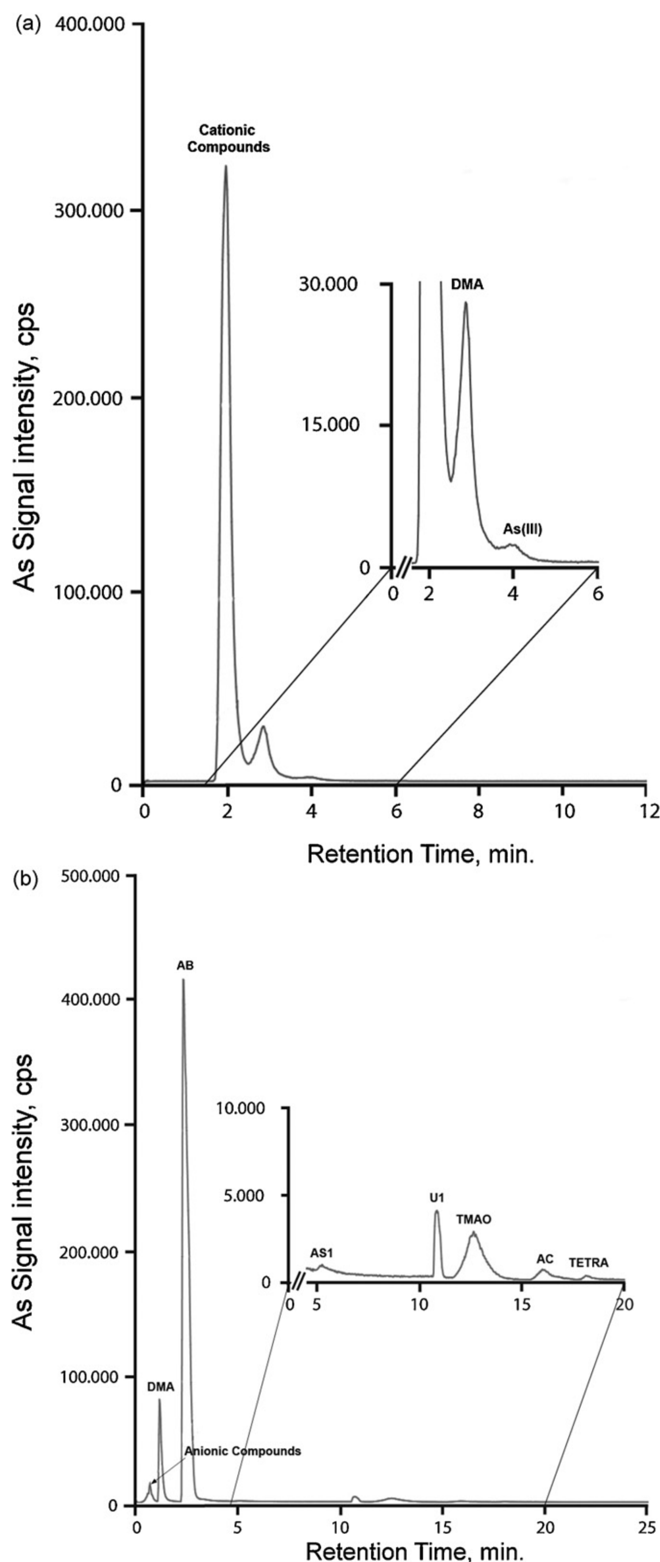

Fig. 1. (a) Anion exchange HPLC–ICP-MS chromatogram of an extract of eel. Experimental conditions: see Table 1. Mobile phase $(NH_4)_2CO_3$ 10 mM, MeOH 3%, pH 10.3. (b) Cation exchange HPLC–ICP-MS chromatogram of an extract of eel. Experimental conditions: see Table 1.

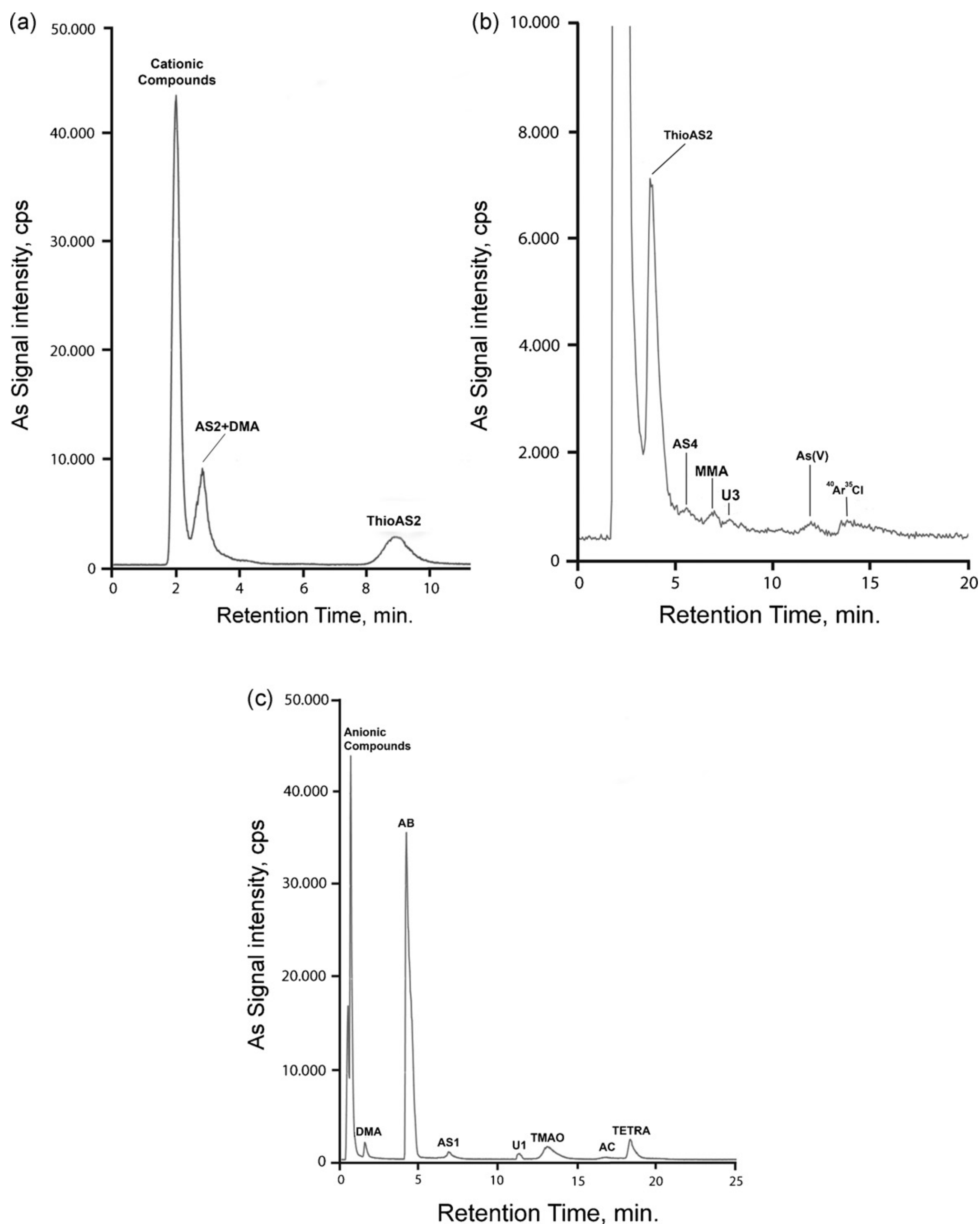

Fig. 2. (a) Anion exchange HPLC–ICP-MS chromatogram of an extract of carp. Experimental conditions: see Table 1. Mobile phase $(NH_4)_2CO_3$ 10 mM, MeOH 3%, pH 10.3. (b) Anion exchange HPLC–ICP-MS chromatogram of an extract of carp. Experimental conditions: see Table 1. Mobile phase $(NH_4)_2CO_3$ 20 mM, MeOH 3%, pH 10.3. (c) Cation exchange HPLC–ICP-MS chromatogram of an extract of carp. Experimental conditions: see Table 1.

ferent dilutions with external calibration using the AB calibration curve, as well as with the method of standard addition. The same was ascertained for anion exchange HPLC and, therefore, unknown compounds were quantified by means of the AB calibration curve in both separation systems. In the case of anion exchange HPLC, however, AsIII showed a significantly lower response factor than the other As compounds. The lower sensitivity found in anion exchange HPLC for AsIII implied that an external AB calibration curve was not appropriate for the quantification of this species and the method of standard addition only was thus used to this purpose.

In order to gain a better insight into the accuracy of HPLC–ICP-MS determinations, the quantitative results of anion exchange chromatography were cross-checked with those of cation exchange chromatography. In other words, the areas of the peaks eluting close to the solvent front in anion exchange chromatography (i.e., neutral and cationic species at the experimental pH) was compared with the sum of the peaks areas in cation exchange chromatography. The same was done for the very early eluting species in cation exchange chromatography, whose peak areas were compared to the sum of peak areas eluted from the anion exchange column. In

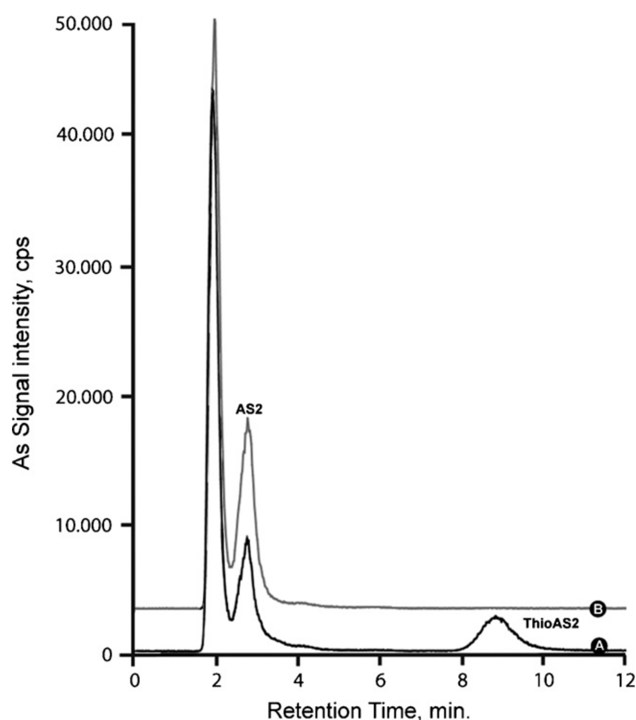

Fig. 3. Anion exchange HPLC–ICP-MS chromatograms of carp extract before and after treatment with H_2O_2. A: chromatogram of carp extracts before H_2O_2 treatment; B: chromatogram of carp extracts after H_2O_2 treatment. Experimental conditions: see Table 1. Mobile phase: $(NH_4)_2CO_3$ 10 mM, MeOH 3%, pH 10.3.

both cases the agreement was quantitative. Therefore, the column recovery of As was obtained by combining quantitative data from cation and anion exchange chromatography. The results ranged from 101% for carp, to 102%, 103%, and 104% for chub, mullet, and eel, respectively, indicating that no As was retained during chromatography. For all four studied fish, the overall mass balance was consequently dictated by the yields obtained in sample extraction.

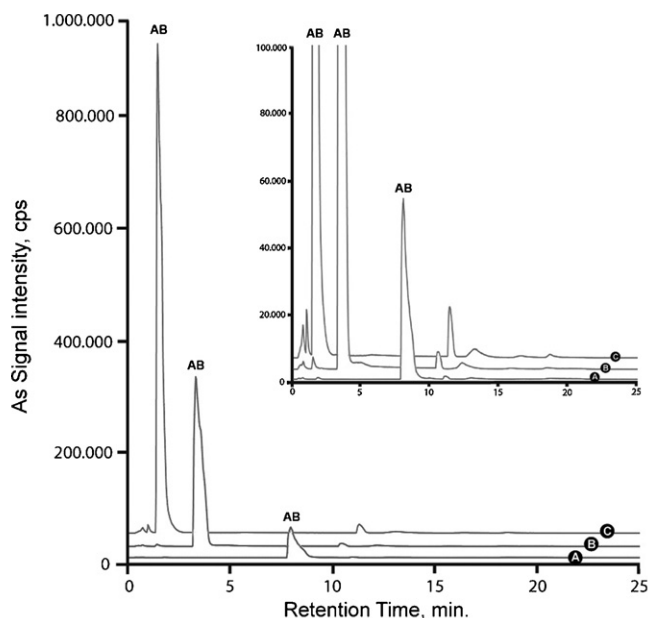

Fig. 4. Shift in cation exchange HPLC retention times of As species in extracts of mullet with different dilutions. A: HPLC–ICP-MS chromatogram of the sample extract diluted 1:10; B: HPLC–ICP-MS chromatogram of the sample extract diluted 1:2; C: HPLC–ICP-MS chromatogram of the undiluted sample extract. Experimental conditions: see Table 1.

Table 3
As compounds in methanol/water extracts of muscle tissue of freshwater fish (%)[a].

As compound	Eel	Mullet	Chub	Carp
AsIII	0.02 (0.00)	0.65 (0.02)	0.19 (0.01)	1.07 (0.03)
AsV	0.10 (0.00)	Traces[b]	Traces[b]	0.34 (0.01)
MMA	0.10 (0.00)	Traces[b]	–	Traces[b]
DMA	7.64 (0.41)	0.22 (0.03)	0.07 (0.00)	1.50 (0.10)
AB	88.89 (3.79)	95.80 (5.12)	95.44 (6.05)	58.35 (5.43)
TMAO	1.84 (0.11)	0.91 (0.19)	2.42 (0.06)	6.00 (0.97)
AC	0.30 (0.02)	0.13 (0.02)	0.05 (0.00)	0.35 (0.06)
TETRA	0.04 (0.00)	0.15 (0.02)	0.34 (0.01)	4.39 (0.50)
AS1	0.10 (0.01)	0.17 (0.01)	0.14 (0.01)	1.54 (0.17)
AS2	–	–	–	13.72 (0.17)
AS4	–	Traces[b]	0.07 (0.00)	Traces[b]
ThioAS2	0.12 (0.00)	0.06 (0.00)	0.43 (0.02)	11.99 (0.14)
U1[c]	0.84 (0.05)	1.90 (0.22)	0.75 (0.03)	0.74 (0.05)
U2[c]	–	–	0.09 (0.01)	–
U3[d]	Traces[b]	Traces[b]	–	Traces[b]

[a] Data expressed as the mean concentration of each species divided by the sum of the concentrations of the species detected × 100. Standard deviation in parentheses.
[b] Concentration values slightly below the LOD.
[c] Cation exchange chromatography: U1 *Tr* = 11.08–11.65 min (range for the different fish species, undiluted sample extracts); U2 *Tr* = 6.78 min (undiluted sample extracts).
[d] Anion exchange chromatography: U3 *Tr* = 7.70–7.81 min (range for the different fish species, undiluted sample extracts; mobile phase $(NH_4)_2CO_3$ 20 mM, MeOH 3%, pH 10.3).

Incomplete recovery of As from the chromatographic column has been reported in previous studies on freshwater fish, notably in the case of carp, for which mass balances as low as 2–29% were obtained [20].

3.2.1. Method validation

Quantitative results for the As speciation analysis of the two CRMs used to check the trueness of the method were in good agreement with the certified values. The found (mean ± SD) and certified values for AB and DMA in BCR627 were 3.9 ± 0.3 μg As g^{-1} *vs.* 3.9 ± 0.2 μg As g^{-1} and 0.139 ± 0.005 μg As g^{-1} *vs.* 0.15 ± 0.01 μg As g^{-1}, respectively. For DORM-2, the found and certified values for AB and TETRA were 16.6 ± 1.0 *vs.* 16.4 ± 1.1 and 0.24 ± 0.03 μg As g^{-1} *vs.* 0.248 ± 0.054 μg As g^{-1}. Moreover, the results obtained for other As compounds such as DMAA, DMAP, TMAO, TMAP and AC were in acceptable agreement with those previously reported by other authors (see [4] and references therein). As discussed above for real samples, injection of sample extracts with different dilutions proved beneficial in order to resolve closely eluting peaks. For instance, 1:10 and 1:20 dilution of BCR627 and DORM-2, respectively, led to the separation of TMAO and TMAP, two species at high risk of coelution [4,36].

Precision as average intra-day repeatability was estimated to be in the range of 1–4% and 4–6% for the different compounds determined by anion and cation exchange chromatography, respectively. The limits of detection (LODs) for individual species were calculated as the mean blank response plus three times the baseline noise (3σ) of 10 method blanks. The LODs in solution were obtained by extrapolation of the AB calibration curve in the low concentration range (except for AsIII, see above). LODs for the samples, calculated from those in solution using the dry sample mass and the final volume of the extract, were 0.18 ng As g^{-1} for MMA and AS4, 0.20 ng As g^{-1} for AsIII, 0.23 ng As g^{-1} for Asv, 0.24 ng As g^{-1} for Asv and AS2, 0.29 ng As g^{-1} for TMAO, AC and TETRA, 0.31 ng As g^{-1} for DMA, and 0.32 ng As g^{-1} for AB. The LODs of the three unknown compounds (see Table 3) were 0.32, 0.30 and 0.18 ng As g^{-1} for U1, U2 and U3, respectively.

3.2.2. As compounds in freshwater fish

Arsenic speciation analysis revealed marked differences among the fish species (Table 3). AB was the dominating As compound in

muscle tissue of mullet, chub and eel. However, it represented only 58% of the detected species in carp, where AS2 (14%) and ThioAS2 (12%) were significant compounds. AsIII and AsV were detected in all fish species at very low concentrations, which are not cause for any toxicological concern. AS1, ThioAS2, DMA, TMAO, AC, TETRA were identified generally at low concentrations in all samples as well. DMA and TMAO were noteworthy minor compounds in eel and carp, respectively. Furthermore, AS4, MMA and three unknown As compounds were found. To the best of our knowledge, the presence of AS4 in fish is described here for the first time.

Altogether, these results are indicative of a freshwater ecosystem not polluted by inorganic arsenic. The arsenic species in fish exposed to inorganic As *via* water or the food chain typically include AsIII and AsV, and the methylated compounds – which are likely detoxification products – MMA, DMA, TMAO; DMA is often present at relatively high levels whereas the content of AB is variable and appears to be mainly linked to the uptake *via* the diet. In the present study, AB has been found to be the major compound in four fish species belonging to three different families (*Anguillidae*, *Mugilidae*, *Cyprinidae*). Particularly interesting is the finding of AB in carp, since a previous report did not detect the arsenical in this species [20]. Similarly, it has to be highlighted the large predominance of AB in chub, something that has never been found for a species of the *Cyprinidae* family in previous studies [18,20]. At a first glance, from the present literature on freshwater fish, one could get to the conclusion that the low abundance of AB is a typical feature of this family, whilst it is likely the result of the fact that many study have been carried out in environments contaminated with inorganic arsenic.

In a study encompassing 9 freshwater fish belonging to 4 different families, cluster analysis suggested that taxonomically related fish exhibit a similar As speciation [18]. This hypothesis seems to be supported by several studies on salmonids, which consistently showed that the representatives of this family have AB as the largely predominating arsenical [16,18,24]. However in the present study, two members of the *Cyprinidae* family, carp and chub, showed a clearly different As speciation suggesting that other factors, such as feeding habits, likely plays a role as well. Carp is an omnivorous species which feeds on algae and plants, whereas adult chubs (such as those collected for this study) are predatory fish [37,38]. The occurrence of oxo- and thio-arsenosugars in muscle tissue of the carp specimens appears to be related to the presence of these As compounds in the fish diet. The oxo-arsenosugars AS1 and AS2 have been previously found in freshwater fish, including carp, with AS2 being usually the most abundant of the two [20,25]. As far as ThioAS2 is concerned, it has been previously reported in white bream and, at trace level, in silver carp [21], whereas this study gives the first evidence of its presence in carp. Another important compound in muscle tissue of carp was TMAO, which might be related to the living habits of this species, that is, the close association with sediments where microbial formation of TMAO takes place [12,39,40].

Eel, a predatory fish [41], showed a distinct speciation, with DMA as the second arsenical, following AB. Miyashita et al. [25] recently found DMA as the dominating water-soluble As compound in *Anguilla japonica* from an As-rich river, followed by AB. A different pattern was found in mullet, since this species showed the highest As content of all of the fish investigated in this study, almost exclusively in the form of AB. This fish feed on organic detritus, microalgae, and plankton, but its living habits, rather than its diet, likely plays a major role in dictating the accumulation of As species in its tissues. Being a euryhaline fish which inhabits estuarine intertidal, freshwater and coastal marine habitats, with widely different salinity, the pattern of As species found in this study (i.e., the large predominance of AB) was not unexpected [42].

4. Conclusions

An analytical approach for the investigation of water-soluble arsenicals in freshwater fish by HPLC–ICP-MS was developed. It allowed the sensitive and selective detection of minor compounds along with the most abundant ones and it is thus suitable for studies on As fate in freshwater ecosystems. By combining cation and anion exchange, 17 arsenicals – for which standards or natural sources were available – could be determined. Previous literature highlighted the difficulty in achieving satisfactory extraction efficiencies for As species in freshwater fish and some of them showed a strikingly wide range of extraction yields (<1–90%). In this study, four fish species with background As levels in their tissues and widely differing As speciation and muscle chemical composition were studied. After optimization of extraction parameters, extraction with a 1:1 (v/v) methanol/water mixture generally gave satisfactory yields, lying in a much narrower range (64–89%). The lowest recovery was obtained for carp, a fish species with a relatively high lipid content and with only 60% of extracted As in the form of AB. HPLC column recovery of injected As was quantitative and led to satisfactory mass balances, which allowed to obtain meaningful speciation data for the investigated fish species.

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