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Metal speciation of metallothionein in white sea catfish, *Netuma barba*, and pearl cichlid, *Geophagus brasiliensis*, by orthogonal liquid chromatography coupled to ICP-MS detection

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Abstract

Metal speciation analysis in MTs was carried out in two tropical fish species of Brazil, the freshwater fish pearl cichlid (*Geophagus brasiliensis*) and the marine fish white sea catfish (*Netuma barba*), that are presently used to monitor the effects of heavy metal pollution in aquatic ecosystems in Brazil. In order to obtain the MT fraction, liver cytosols from both fish species where subjected to size exclusion fractionation, monitoring on-line the metal signal (Cd, Cu and Zn) by ICP-MS while protein elution was followed by on-line UV detection. That MT fraction was then separated by anion-exchange (AE)-FPLC, whose optimal chromatographic conditions were optimized for the separation of the different hepatic MT isoforms existing in both fish species. Specific detection of separated metalloforms was carried out again by the hyphenation of the AE chromatographic system with the ICP-MS instrument. The analytical results showed that MTs of these fish species, unknown so far, exhibited unique characteristics in comparison with standard MTs and other fish liver MTs. In fact, MT isoforms of *N. barba* turned out to be very anionic, as indicated by their high retention in the Mono Q column and the strong ionic strength required to separate them. As for *G. brasiliensis*, cadmium was exclusively present in only one of the peaks of the MT isoforms showing a unique metal-binding behavior for MT in this fish species. The differences between the MTs among these species and the different association of metals in particular MT isoforms display the importance of the metal speciation analysis in these proteins prior to its use as bioindicators.

Keywords: Metallothioneins; Metal speciation; Inductively coupled plasma mass spectrometry; *Netuma barba*; *Geophagus brasiliensis*

1. Introduction

Aquatic ecosystems are major recipients of pollutants, whose deleterious effects at the population or ecosystem level can reach a no-return point to take effective countermeasures. Such deleterious effects, however, are preceded by sub-lethal changes at the molecular and cellular level in organisms inhabiting these ecosystems. Detection of such subtle markers in aquatic organisms can serve as an early indication of contaminant impact and possible consequences of prolonged or continued exposure. In this context, the induction of metallothioneins (MTs) has been proposed by many authors as a sub-lethal biological indicator of metal pollution [1–4].

MTs are low-molecular-mass, cysteine-rich metal-binding proteins [5]. The binding of MTs to toxic metals represents a sequester function associated with the cellular protection against metal toxicity [4]. As a general model, MTs synthesis is thought to be induced under conditions of elevated metal concentration, providing more binding sites for metal ions and limiting latent damage [6]. All vertebrates examined so far contain two or more distinct MT isoforms designated MT-1 through MT-4 [7]. The high metal-inducibility of the MT-1/MT-2 isoforms has

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been linked with their role in heavy-metal detoxification [8]. In contrast, MT-3 and MT-4 are tissue specific isoforms, relatively unresponsive to metal inducers [9]. The fact that MT isoforms differ among them with respect to their molecular and biochemical features (they can even exhibit distinct induction patterns in response to the metals to which they bind [10]) complicates the understanding of their biological role and their use as biomarkers [11]. Therefore, accurate determination of different MT isoforms seems mandatory to assess their real and specific potential as biomarkers. One approach to such measurement is an adequate speciation of the metals bound to MT isoforms. However, taking into account that life is only compatible at low toxic metal concentrations, this approach is often difficult since it requires analytical methods with high resolution and sensitivity. Trace metal speciation analysis of MTs has developed the on-line coupling of a powerful separation technique, e.g. HPLC, with an element-specific detector, e.g. inductively coupled plasma-mass spectrometer (ICP-MS) to tackle these limitations [12–15]. However, most of the metal speciation studies in MTs carried out so far are based on the use of hyphenated techniques for the analysis of commercially available samples (rabbit liver MTs). Concerning the presence of these proteins in real samples, most of the research is focused mainly on laboratory animals [16–21] and to a lesser extent on wild animals [22–25]. Moreover, among these latter works only a few employed two or more different separation techniques [22,25].

On the other hand, an effective use of molecular biomarkers of metal exposure demands a good "sentinel organism" selection. In this work we study metal speciation of MTs in two fish species, *Geophagus brasiliensis*, as a freshwater fish, and *Netuma barba,* as a marine fish. Both are presently used to monitor the effects of heavy metal pollution in two major aquatic ecosystems in Brazil. *G. brasiliensis* is the chosen organism to monitor Paraıba do Sul River, a medium-sized river, 1145 km long, which is the main water supply for more than 11,000,000 people at Rio de Janeiro city, but it is also used as waste disposal for a very large number of industries along its course that are major sources of heavy metal pollution [26]. On the other hand, the catfish *N. barba* would be used to monitor the heavily polluted Guanabara Bay, a 384 km^2 eutrophic waterbody, which receives inputs from untreated domestic and industrial sewage from around 10,000 plants, two harbours, shipyards and oil terminals [27]. Both selected fish species are native tropical organisms, which inhabit those ecosystems and have the physiological and ecological characteristics required to be used as a sentinel species. Clearly, habitat and feeding preferences make these fish species vulnerable to the type of contamination, which occurs in their respective ecosystems. Moreover, as relatively stationary, territorial and commercially important organisms, *G. brasiliensis* and *N. barba* are most adequate sentinel candidates for our studies. However, prior to their use as sentinel organisms of heavy metal pollution it would be necessary to characterize their MTs and metal association to them via metal speciation analysis. To achieve this objective, speciation of Cd, Cu and Zn in different liver MT isoforms of *N. barba* and *G. brasiliensis* was carried out by means of hyphenated techniques, in particular size exclusion-fast protein liquid chromatography (SE-FPLC) followed by anion-exchange (AE)-FPLC, both coupled with the ICP-(Q)MS detection.

Table 1
Operating conditions for ICP-(Q)MS instrument

Plasma conditions	
Instrument	HP4500
Rf power	1300 W
Plasma gas flow rate	15.0 L min^{-1}
Carrier gas flow rate	1.08 L min^{-1}
Nebulizer	Meinhard
Spray chamber	Double pass/Peltier (4 °C)
Data acquisition parameters	
Spectrum mode	
Monitored masses	63, 64, 65, 67, 71, 111, 114, 115, 200, 202, 205
Points per peak	3
Integration time	0.3 s per peak
Time resolved analysis mode	
Monitored masses	63, 64, 65, 67, 111, 114
Points per peak	1
Integration time	1 s per peak

2. Experimental

2.1. Instrumentation

A quadrupole ICP-MS Hewlett Packard model 4500 instrument (Yokogawa Analytical, Tokyo, Japan) was utilised for ICP-MS measurements equipped with a conventional Meinhard nebulizer and a double-pass spray-chamber. Instrumental operating conditions for the ICP-MS detector coupled "on-line" with the exit of the FPLC columns are summarised in Table 1. These conditions were tuned daily by using a multielemental aqueous standard solution containing Li, Y, Tl and Ce (at the 10 ng mL^{-1} level in each element).

An Ultra-turrax T-25 (IKA Labortechnik, Staufen, Germany) system was used for tissue homogenization. Liver cytosol was obtained by ultracentrifugation using a Optima L-90k Beckman ultracentrifuge (Beckman Coulter, Fullerton, CA, USA). A Superdex$^{\text{TM}}$ Peptide HR 10/30 (Pharmacia, Biotech., Uppsala, Sweden), with a separation range of 0.1–20 kDa, was used to perform size-exclusion (SE)-FPLC. Anion-exchange FPLC column was a Mono Q HR 5/5 (50 mm × 5 mm i.d., Pharmacia Biotech.). Shimadzu LC-10Avp HPLC pumps were used as the solvents delivery system and injections were made using a Rheodyne (model 7125) sample injector valve (Berkeley, CA, USA) with a 100 μL loop. A scavenger column (25 cm × 0.5 cm i.d.) was placed between the pumps and the injection valve in order to remove exogenous metal ions from the mobile phases used. This column was packed with Kelex-100 impregnated silica C_{18} material and prepared as described previously [28]. A LKB model 2151 variable wavelength detector, provided with a 10 μL flow cell, was used for spectrophotometric measurements while a Shizadzu C-R6A recording integrator (Shimadzu, Kyoto, Japan) was employed for signal processing and readout.

2.2. Reagents and materials

MT-1 and MT-2 standards from rabbit liver were purchased from Sigma (St. Louis, MO, USA) and solved (500 $\mu g\,L^{-1}$ total cadmium, determined by ICP-MS) in 10 mM Tris–HCl buffer (pH 7.4).

Single-element standard stock solutions for ICP-MS analysis containing 1000 $\mu g\,mL^{-1}$ were from Merck (Darmstadt, Germany). Multielement stock solutions were prepared from sub-boiled (65%) nitric acid (Suprapur Quality, Merck) and Milli-Q water (Millipore, Molsheim, France). All dilutions were performed by mass to 0.1 mg using a Precisa, model 180 balance (Zurich, Switzerland) and prepared daily.

All polypropylene containers used during experiments were previously treated with diluted (5%) sub-boiled nitric acid and rinsed several times with Milli-Q water.

All mineral acids and metal salts used were of analytical reagent grade and Milli-Q purified water was used throughout.

2.3. Fish sampling

G. brasiliensis, 15 specimens, were caught at the Paraiba do Sul River and *N. barba*, 12 specimens, were sampled at the Guanabara Bay. The animals were sacrificed by decapitation and livers were dissected out, weighed and stored at −20 °C until analysis.

3. Procedures

3.1. Cytosol preparation and metals quantitative analysis

Liver cytosolic extracts were obtained as described in a previous work [29]: livers of the same group were pooled and homogenized in four volumes (w/v) of buffer (10 mM Tris/HCl, pH 7.4; 1 mM dithiothreitol; 0.1 mM phenylmethylsulphonyl fluoride (PMSF) (Sigma)). The homogenate was centrifuged at 1000 g for 10 min and then the decanted supernatant at 105,000 g for 60 min. All preparative steps were performed at 4 °C. The supernatant liquid was heated at 65 °C for 15 min and then recentrifuged again for 30 min in the same way. The supernatant (cytosol) was collected and stored at −20 °C until analysis.

Cytosols were diluted 1:5 (w/w) with sub-boiled 0.5% nitric acid and total content of Cd, Cu, Zn and Hg was measured using a quadrupole ICP-MS instrument. The internal standards ^{71}Ga (for ^{64}Zn, ^{67}Zn, ^{63}Cu and ^{65}Cu), ^{115}In (for ^{111}Cd and ^{114}Cd) and ^{205}Tl (for ^{200}Hg and ^{202}Hg) were added to sample, calibration and blank solutions, always at the same concentration levels. Table 1 resumes the operating conditions used here for quantitative multielemental analysis by ICP-MS.

3.2. Isolation of liver MTs pool by size exclusion FPLC

Fractionation of *G. brasiliensis* and *N. barba* liver cytosols in the SE-FPLC column was achieved using a mobile phase of 10 mM Tris/HCl, pH 7.4 at a flow rate of 0.8 $mL\,min^{-1}$. The standards used for calibration of the column were cytochrome *C* (12.5 kDa); rabbit liver MT (6–7 kDa), gastrin (2 kDa) and L-glutathione (0.3 kDa). The elution process of the MTs pool from the SE-FPLC column was followed on-line by monitoring the absorbance at 254 nm (metal–thiolate bond absorption) by UV/vis spectrophotometry and metal detection by on-line ICP-MS. Both UV/vis and metal profiles were compared with those observed for the elution of standard rabbit liver MT (which eluted from 600 to 700 s in the SE-FPLC column). The Cd–Cu–Zn–cytosolic fraction, eluting at the same retention time of the standard MT, was collected at the outlet of the SE-FPLC column after five consecutive injections (100 µL of cytosol sample each) and chromatographic runs. Quantitative metal analysis of this cytosolic fraction (MT fraction) was carried out by ICP-MS using 1 + 1.5 dilution (w/w) with sub-boiled 0.5% nitric acid. Internal standards and operating ICP-MS conditions were the same as described above (Table 2).

Table 2

Metal concentrations (ng g^{-1} wet tissue) found on *G. brasiliensis* and *N. barba* liver cytosol and the corresponding MT fraction by ICP-MS

	Cytosol[a]		MT fraction[a]	
	G. brasiliensis	*N. barba*	*G. brasiliensis*	*N. barba*
Cu	367 ± 6	4348 ± 31	107 ± 2	2716 ± 15
Zn	3363 ± 96	7506 ± 217	419 ± 3	1880 ± 28
Cd	280 ± 6	1394 ± 27	97 ± 3	899 ± 18
Hg	<L.O.D.	<L.O.D.	<L.O.D.	<L.O.D.

[a] Mean ± S.D. (*n* = 3).

3.3. Anion exchange FPLC procedure

The antioxidant 2-mercaptoethanol was added (final concentration 5 mM) to the MT fraction previously isolated by SE-FPLC, which was pre-concentrated 10 times by ultrafiltration (Centricon YM-3 of 3000 MW cut-off, Millipore, Bebford, MA, USA). In order to separate the different MT isoforms, aliquots of these concentrates (100 µL each) were then subjected to AE-FPLC. A 20 mM Tris–HCl buffer (pH 7.4) and a 20 mM Tris–HCl, 0.4 M ammonium acetate buffer (pH 7.4) were used as mobile phase A and B, respectively. The gradient elution conditions, which are summarized in Table 3, were optimized for

Table 3

Gradient elution conditions for the separation of *G. brasiliensis* and *N. barba* hepatic MTs by anion-exchange fast protein liquid chromatography

G. brasiliensis		*N. barba*	
Time (min)	B (%)	Time (min)	B (%)
0	0	0	0
4	1	4	5
7	2	6	20
9	2.5	8	20
12	5	10	35
15	10	11	35
20	20	12	43
23	0	14	50
		16	50
		18	0

Mobile phase A: 20 mM Tris–HCl (pH 7.4); Mobile phase B: A + 400 mM ammonium acetate (pH 7.4); Flow rate: 1 $mL\,min^{-1}$.

the separation of both *G. brasiliensis* and *N. barba* liver MTs. The detection of the separated MT isoforms was carried out by coupling the AE-FPLC system to the specific metal detection provided by ICP-MS with on-line monitorization of Cd, Cu, Zn and Hg associated to those proteins.

4. Results and discussion

4.1. Isolation and detection of MT fraction by SE-FPLC–ICP-MS

For adequate metal speciation, the chromatographic column should not alter the metal–biomolecule association. It has been shown that the coupling of SE-FPLC with ICP-MS, when operating in a continuous mode, is a powerful approach for the on-line, real-time speciation of trace metals, associated with different molecular weight fractions in cytosolic extracts [15,16,23,30]. Thus, cytosols of liver of *G. brasiliensis* and *N. barba* were applied first to the size exclusion column and metals were monitored on-line using ICP-MS (see procedures).

SE-FPLC elution profiles of liver cytosolic extract, using UV absorbance at 254 nm, for both fish species are shown in Fig. 1a (*G. brasiliensis*) and b (*N. barba*). As can be observed, absorbance profiles at 254 nm were very similar for both fish species with a profile containing at least 15 peaks, yet both fish species showed a peak occurring at a retention time (t_R) of 10.9 min ± 0.3 (mean ± S.D.), which matches the retention time of commercial rabbit liver MT (see Fig. 1c). The main difference between both samples is the presence of a broad peak between 12 and 15 min in the sample of *N. barba*. The number of peaks observed in the fractionation of both *N. barba* and *G. brasiliensis* hepatic cytosols indicates the complexity of these samples, being UV detection unselective to indicate the nature of the individual compounds separated in the size exclusion column.

Owing the fact that the binding of Cd, Cu and Zn by a cytosolic heat-stable protein of about 6–7 kDa indicates the presence of MTs [31], metal profile obtained by the hyphenation of SE-FPLC with ICP-MS detection turned out to be decisive to ascertain the fraction where *G. brasiliensis* and *N. barba* liver MTs elute (and, of course, also to establish differences between the two species under study). As shown in Fig. 2a (*G. brasiliensis*) and b (*N. barba*), considerable amounts of Cd, Cu and Zn eluted at the t_R of the standard rabbit liver MTs (see Fig. 2c). In both fish species, Zn, Cu and Cd also eluted at t_R earlier than MT, that is, associated to higher molecular weight (HMW) fractions. Essential metals and Cd bound to HMW proteins have been already reported in cytosols of some fish species [16,23,29]. Our results show also the presence of these metals in such fractions in both fish species.

In contrast with *N. barba*, Cd and Cu are also found in one additional peak at t_R = 20 min (not MTs) in *G. brasiliensis*. This difference in the Cd profile obtained by SE-FPLC confirms that metal speciation analysis in liver cytosols should be carried out prior to consider these fish species as sentinel organism for Cd contamination.

4.2. Quantitative analysis of essential (Cu and Zn) and toxic (Cd and Hg) metals

The concentrations of Cd, Cu, Zn and Hg determined in the whole cytosols and also in the separated MT corresponding fraction are shown in Table 2. As can be seen, relatively large concentrations of Cd, Cu and Zn are found in the cytosol and bound to the MT fraction in these two fish species. While most of cytosolic Cu and Cd are bound to the MT fraction, Zn is preferably found in other cytosolic fractions, specially in the HMW fraction, as seen in Fig. 2a and b also. On the other hand, the two species differ in the amount and the ratio in which these metals are present in the cytosols and the corresponding MT fraction. Differences in metal content among fish

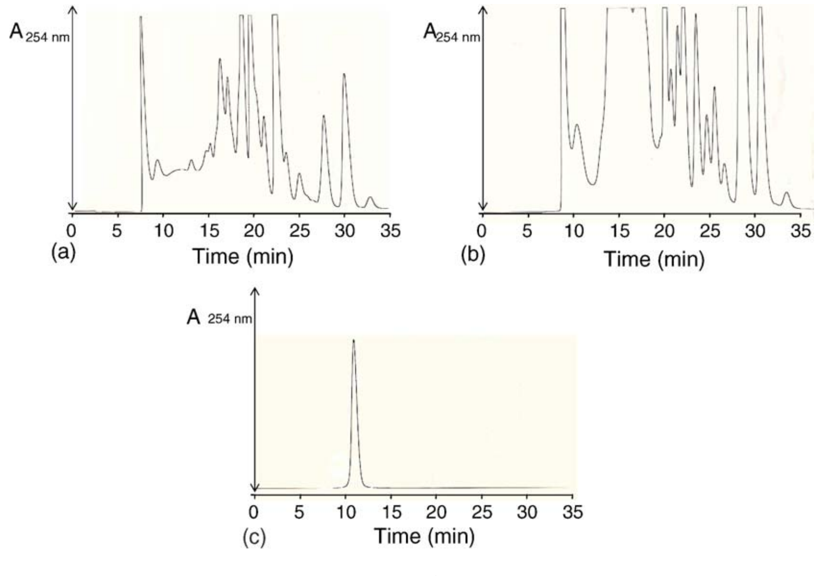

Fig. 1. SE-FPLC profiles obtained by UV/vis spectrophotometric detection (λ = 254 nm) for 100 μL of *G. brasiliensis* (a) and *N. barba* (b) liver cytosol. These chromatographic profiles are compared with the elution profile (c) of standard rabbit liver MTs (6–7 kDa MW) in the SE column.

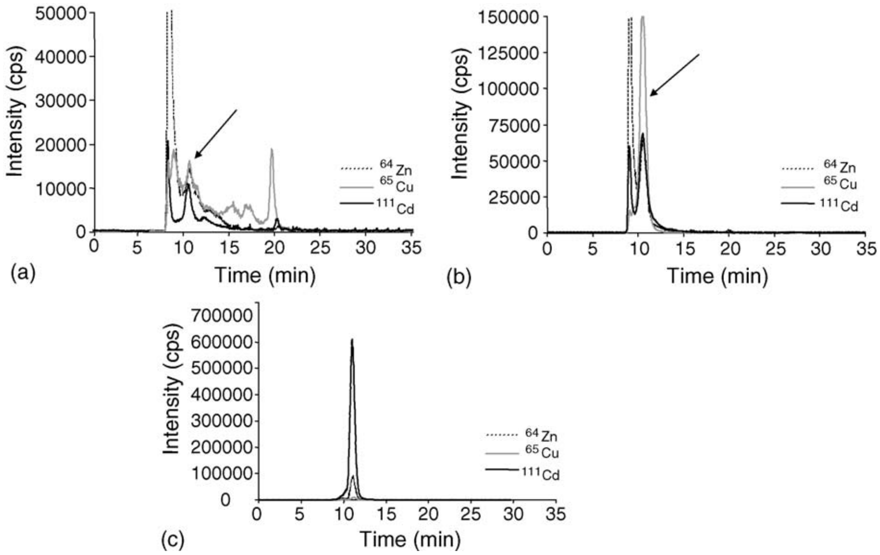

Fig. 2. SE-FPLC–ICP-(Q)MS profiles of Cd, Cu and Zn obtained for 100 μL of *G. brasiliensis* (a), *N. barba* (b) liver cytosol and standard rabbit liver MTs (c). Arrows in (a) and (b) indicate the elution of MT.

species have been previously reported [32] and are attributed to intrinsic characteristics of each fish species; also environmental exposure could influence the metal content of MT [33]. Regarding Cd, the concentration observed was almost five times higher in the hepatic cytosol of *N. barba* than in *G. brasiliensis* and about 10 times higher if the comparison is carried out between the corresponding MT fractions. As earlier introduced, *N. barba* specimens were caught in the heavily polluted Guanabara Bay and the levels of Cd found in *N. barba* could be related to metal contamination (although it would be necessary to establish first the normal levels of Cd in such fish species). Finally, it is known that Hg can also bound to the MT fraction. Curiously enough, the Hg concentration levels found in all samples were similar to those of the blanks, indicating a low contamination in those fishes by mercury.

4.3. Metal profile of different MT isoforms from G. brasiliensis and N. barba by AE-FPLC–ICP-MS

Fractions eluting from the size exclusion column may still contain many biocompounds and also SE-HPLC lacks the selectivity to distinguish between a given protein isoforms [34]. Therefore, a second dimension separation, anion exchange-fast protein liquid chromatography (AE-FPLC) was used, coupled on-line with ICP-MS, for a further separation of the sought MT isoforms [29,22]. The optimum AE conditions worked out for *G. brasiliensis* and for *N. barba* FPLC–ICP-MS speciation are summarized in Table 3.

The ICP-MS chromatographic profiles for ^{111}Cd, ^{65}Cu and ^{64}Zn obtained from liver MTs of *G. brasiliensis* are shown in Fig. 3. As can be seen, such MTs eluted in at least seven metal-containing peaks (the first peak corresponds to unretained free metal ions [29,35]. Both ^{64}Zn and ^{65}Cu can be found associated in three MT forms (peaks numbered in that figure as 3, 5 and 7) while peak at $t_R = 16.2$ min (number 6 in Fig. 3) contains Cu but not Zn. It is also remarkable the fact that ^{111}Cd is only detected in one of the separated MT isoforms (peak 4, at $t_R = 10.5$ min) and that in this peak ^{64}Zn is detected but not ^{65}Cu. This indicate a unique metal-binding behavior for MTs in *G. brasiliensis*. This phenomenon of differential metal-binding by some MT isoforms, which has been reported only in a few organisms [7,36,37], might be related to different roles in the detoxification and regulation of metals by each MT isoform. Our results point out that MTs of *G. brasiliensis* seems to exhibit unique characteristics concerning metal-binding, making them

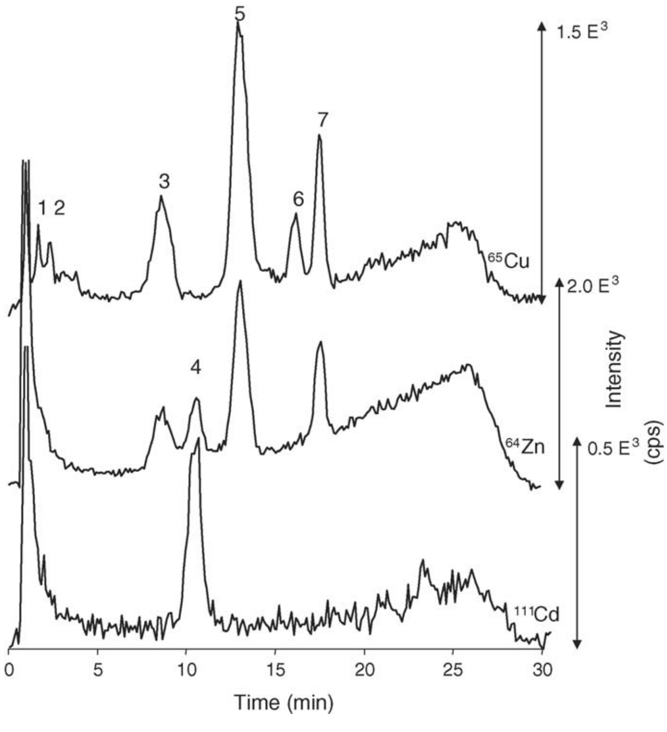

Fig. 3. Chromatographic metal (Cd, Cu and Zn) profiles of *G. brasiliensis* liver MTs by AE-FPLC coupled on line with ICP-(Q)MS detection. Gradient elution conditions are summarized in Table 3.

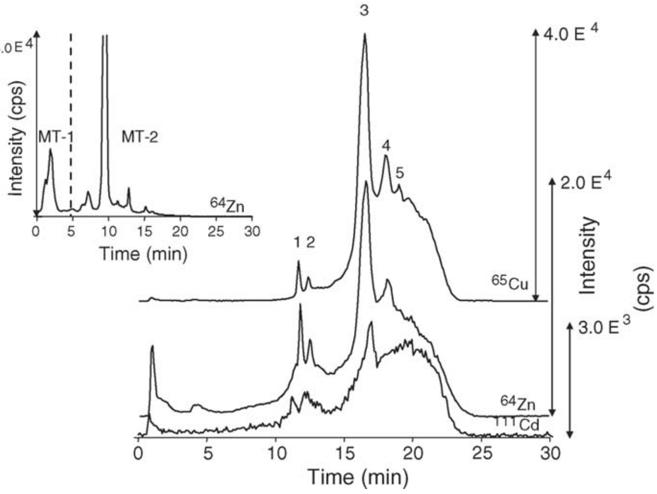

Fig. 4. Chromatographic metal (Cd, Cu and Zn) profiles of *N. barba* liver MTs by AE-FPLC coupled on line with ICP-(Q)MS detection. Inset represents the elution profile (^{64}Zn) of a mixture of rabbit liver MT-1 and MT-2 standards while applying the gradient elution conditions optimised for *N. barba* MTs (see Table 3).

potential candidates for biomarker studies based on the differential, isoform-specific approach [10].

Similarly, the AE-FPLC–ICP-MS profiles obtained from liver MTs of *N. barba* are shown in Fig. 4. We observed at least five metal-containing peaks (numbered in Fig. 4 in their order of elution) with a predominant peak (t_R = 16.5 min), which contains clearly Cd, Cu and Zn. The metal profiles from MTs of *N. barba* also showed that ^{111}Cd, ^{65}Cu and ^{64}Zn co-elute in peaks numbered as 1 and 2 (in contrast to what was found for MTs of *G. brasiliensis*). It is noteworthy that MTs in both fish species, *G. brasiliensis* and *N. barba*, showed relatively high content of copper, a fact previously reported in some other fish species [29,31–33].

Standard MTs used (rabbit liver) and fish MTs can be compared among them taking into account that both belong to the family of vertebrate MTs and accordingly they contain alignable sequences [38]. Our results indicated that *N. barba* MTs seem to be more anionic than standard MTs since strong conditions of ionic strength had to be applied to separate the isoforms by AE-FPLC–ICP-MS (see Table 3). When the gradient elution conditions for AE-FPLC of *N. barba* MTs were applied to standard MTs (see inset in Fig. 4), they eluted at about 20% of mobile phase B while the complete elution of *N. barba* MTs occurred at 50% of B. Conversely, *G. brasiliensis* MTs did not require hard ionic strength conditions (see Table 3) to be eluted in the AE column, which is in concordance with previous studies on MTs from other fish species [29,16,39]. In our opinion, the possibility that the exceptional very anionic character of *N. barba* MTs might have a functional role should be investigated in further studies.

Anyway, it should be pointed out that the elution of *G. brasiliensis*, *N. barba*, and standard MTs keep similarity, not in the retention times of each MT form, but in the chromatographic profile itself. For instance, fish liver MTs studied here contains two minor peaks eluting earlier than a major peak, which reminds the elution of standard rabbit liver MT-1 and MT-2 (see inset in Fig. 4). This fact could suggest that peaks 1 and 2 of Figs. 3 and 4 could correspond with MT-1 while peaks 3–7 of Fig. 3 and 3–5 of Fig. 4 could correspond to *G. brasiliensis* and *N. barba* MT-2, respectively.

5. Conclusions

The orthogonal use of two different principle-based chromatographic separation techniques in combination with the element-specific detection by ICP-MS has provided extensive information about the so far unknown MTs of *G. brasiliensis* and *N. barba*. First, Cd–Cu–Zn speciation analysis in liver cytosols of wild tropical fishes has been successfully carried out by SE-FPLC coupled on-line with ICP-MS, which provided useful information on the metal–biocompounds associations in the hepatic cytosol. These metal profiles turned to be decisive to monitor the elution of the MT fraction and also to establish differences among these fish species.

On the other hand, the metal quantitative results showed that the two fish species under study differ in the amount and the ratio in which Cd, Cu and Zn are present in the cytosols and the corresponding MT fraction. Regarding cadmium, *N. barba* samples contained considerable amounts of this toxic metal, probably reflecting the polluted environment where they inhabited (Guanabara Bay, Brazil).

The application of AE-FPLC–ICP-MS to the MT fraction, previously isolated by SE-FPLC, provided more detailed characterization of *N. barba* and *G. brasiliensis* MTs. The unique characteristics found for MTs in these tropical fish species from eastern South America display the importance of the metal speciation analysis in this group of proteins prior of using individual MT isoforms as specific biomarkers of exposure to heavy metal pollution.

Nevertheless, further studies involving other chromatographic separation techniques (e.g. reversed-phase HPLC) as well as alternative detection systems such as molecule-specific detectors (e.g. electrospray MS/MS) should be used to identity unequivocally the eluted species.

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