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Speciation of Metallothionein-like Proteins of the Mussel *Mytilus edulis* at Basal Levels by Chromatographic Separations Coupled to Quadrupole and Double-Focusing Magnetic Sector ICPMS

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Characterization and partial purification of metallothionein-like proteins (MLPs) of the mussel *Mytilus edulis* from natural populations of three coastal regions in Spain were performed. Size exclusion chromatography (SEC) with quadrupole (Q-ICPMS) or double-focusing inductively coupled plasma mass spectrometry (DF-ICPMS) detection was used first for speciation of cadmium in such natural samples and those of mussels exposed to 500 mg·L^{-1} Cd in an aquarium tank. SEC results showed always a single Cd−MLP peak (MLP fraction). The contents in Cd, Cu, and Zn of this MLP fraction, of the high molecular weight protein pool (HMW), and of the whole cytosol were then measured by DF-ICPMS. Then, a given aliquot (50 μL) of MLPs with the highest values for UV molecular absorption at 254 nm (also the maximum sulfur and Cd, Cu, or Zn contents) was used to further fractionation. Fast protein liquid chromatography "on line" with Q-ICPMS was used for the purpose. Two Cd-MLP isoforms (MLP-1, MLP-2), with retention times (t_R) of 15.7 and 16.0 min, were then detected in cytosols of the mussel samples of aquarium tank and also of the industrial area and Galicia coast. Conversely, wild coast mussels did not show any Cd-MLP signals at all. Analysis of essential elements copper and zinc in such cytosols by FPLC−Q-ICPMS revealed that these two metals were associated just to MLP-1. These results tend to indicate a different role for the two MLP isoforms detected in mussels (i.e., essential metals' homeostasis role seems to be tied to the MLP-1 isoform only). They illustrate the fact that trace metal speciation of unknown species in biological materials is becoming a challenge and points to the use of several complementary analytical techniques to obtain the required speciation information.

Considerable amounts of heavy metals have and are being discharged in aquatic systems with detrimental effects on the environment and human health. Thus, the knowledge of their fate and the effects on biota have become critical for pollution monitoring, impact assessment, and ecosystem management.[1] In this sense, the need to evaluate the environmental status relative to toxic metals and other xenobiotics has led to the development of biomarkers,[2−4] which could provide long-range overall information on a group of xenobiotics as opposed to individual chemical measurement of every xenobiotic in natural waters.

Metallothioneins (MTs) and metallothionein-like proteins (MLPs) are examples of biomarkers of heavy metal environmental contamination.[5] Both have been utilized as early warning molecular indicators of the biological effects of heavy metals, since heavy metals induce MT/MLP biosynthesis (with Cd the strongest inductor[6]).

MLPs have characteristics similar to mammalian MTs and they have been observed in a wide range of marine invertebrates.[7] It is recognized that such MLPs play a central role in the homeostasis of the essential metals, such as Zn and Cu, and the detoxification of toxic metals, such as Cd and Hg.[8] In fact, an increase in heavy metal concentration in cells stimulates the "de novo" synthesis of apothionein, which can then bind metal cations to produce a nontoxic form.[9] In this vein, measurements of the intracellular content of MLP-bound metal might serve to estimate, indirectly, the level of environmental pollution. The degree of contamination of metal-exposed organisms could be so assessed via MLP-bound cadmium measurements[10] in a most convenient

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[†] University of Oviedo.

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(1) Livingstone, D. R. *Comp. Biochem. Physiol., Part A* 120. **1998**, 43−49.
(2) Livingstone, D. R. *J. Chem. Technol. Biotechnol.* **1993**, 57, 195−211.
(3) Livingstone, D. R.; Forlin, L.; George, S. G. In *Water Quality and Stress Indicators in Marine and Freshwater Ecosystems*, 1st ed.; Sutcliffe, D. W., Ed.; Freshwater Biological Association: Ambleside (Cumbria), England, 1994; pp 154−171.
(4) Solé, M.; Peters, L. D.; Magnussen, K.; Sjolin, A.; Granmo, A.; Livingstone, D. R. *Biomarkers* **1998**, *3*, 46−62.
(5) Viarengo, A.; Ponzano, E.; Dondero, F.; Fabbri, R. *Mar. Environ. Res.* **1997**, *44*, 69−84.
(6) Nordberg, M. *Talanta* **1998**, *46*, 243−254.
(7) Roesijadi, G. *Mar. Environ. Res.* **1981**, *4*, 164−169.
(8) Baudrimont, M.; Metivaud, K.; Maury-Brachet, R.; Ribeyre, F.; Boudou, A. *Environ. Tox. Chem.* **1997**, *16*, 2096−2105.
(9) Viarengo, A.; Nott, J. A. *Comp. Biochem. Physiol.* **1993**, *104C*, 355−372.
(10) Roesijadi, G. *Aquat. Toxicol.* **1992**, *22*, 81−114.

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way due to the specificity of its response and the availability of modern techniques to analyze the metal in biological tissues at extremely low Cd concentrations.

On the other hand, monitoring programs can use "sentinel organisms", such as mussels, because they are widely distributed geographically, sessile and, therefore, easy to collect, and resistant to a wide range of contaminant concentrations.[2] In this work, the sentinel species *Mytilus edulis*[11] was selected because cadmium exposure elicits an unusual response in this organism in terms of MLP induction.[12] In addition, it has been found that MLP turnover rate for molluscs (several months) is generally slow when compared with fish and mammalian species.[13] This particular aspect offers an interesting advantage if an integrated picture of the recent contamination history of a site is required.

Historically, the occurrence of MLPs has been extensively reported in different molluscs including mussels, oysters, clams, limpets, and snails.[10,14] However, most of these studies are based on laboratory assays in which exposure conditions can differ greatly from those encountered in natural environments. Furthermore, metal concentrations used to induce MLP synthesis in laboratory experiments are often several orders of magnitude higher than those found even in the most polluted aquatic systems.[14] In contrast, MLPs have rarely been determined in natural populations[15] because of the comparatively low basal level of these proteins and their complexed metals (e.g., 0.3 μg of Cd/g of wet mussel tissue, as measured in mussels grown in wild coast) and also due to the difficulty to differentiate between xenobiotic-induced changes and natural variability.[16] Moreover, it appears that MLPs would be more difficult to isolate from molluscan tissues than MT from mammalian organs.[17] Finally, it should be pointed out that metals entering living organisms are metabolized and so transferred into metal biomolecules of the most varied nature and chemical complexity. This possible complexity of metal speciation in biological materials renders it, very often, extremely difficult to get a correct interpretation of results from a single hybrid technique.[18] In such intances, where unknown metal biomolecules appear, the knowledge, judicious choice, and adequate application to the same sample of several different-principle-based separation techniques (coupled on line with different atomic detectors) appears to be an effective approach to reliable species identification:[19] speciation information obtained in terms of retention times can be validated by a good matching of speciation results obtained by the different, though complementary techniques employed for examination of the same sample.

The aim of the present work is 3-fold: first, to isolate by SEC and identify by their metal content the "basal" mussel MLPs fraction; second, the development of the most sensitive MLP−Cd speciation technique based on complementary separations coupled to ICPMS (following previous work in our group[20,21]); and finally, the application of the array of such complementary techniques to study MLP−Cd speciation in real samples and to estimate relative levels of contamination in three different coastal regions in Spain using mussels as "sentinel organisms".

EXPERIMENTAL SECTION

Instrumentation. Protein lyophilization was carried out using a Virtis lyophilizer (Virtis Co., Inc., Gardiner, NY). Tissue homogenization (under N_2 atmosphere), centrifugation, and fraction collection were carried out using an Ultra-turrax T-25 (Ika Labortechnik, Staufen, Germany), a Centrikon T-1180 (Kontron Instruments, Milan, Italy), and a Frac-100 Pharmacia LKB (Bouwman, Sweden), respectively. Details of the rest of the instrumentation used (HPLC pumps, UV−visible detector, injector valve, Mono Q anionic exchange, scavenger and SEC columns) were given in a previous work.[22]

For ICPMS measurements, a quadrupole Q-ICPMS Hewlet Packard model 4500 instrument (Yokogawa Analytical, Tokyo, Japan) and a double-focusing DF-ICPMS element (Finnigan MAT, Bremen, Germany) were used, as required. Both of them were equipped with a conventional Meinhard nebulizer and a double-pass spray chamber. Figure 1 shows a schematic diagram of the whole strategy and setup used for metal-binding proteins fractionation and Cd speciation. Instrumental operating conditions for both ICPMS detectors coupled on-line with the exit of the Mono Q column are summarized in Table 1. These conditions were tuned daily by using a standard aqueous solution containing a mixture of Li, Be, Na, Sc, Co, Y, Rh, In, Tb, Tl, and Th (at the 10 $\mu g \cdot g^{-1}$ level in each element).

Reagents and Materials. Single-element standard solutions for ICPMS containing 1000 $\mu g \cdot mL^{-1}$ Cu, Cd, Zn, S, and In were obtained from Merck (Darmstadt, Germany) while those of Ga and Sc were from J. T. Baker (Phillipsburg, NJ). Each standard solution was certified at 99.999% purity, and the concentrations of trace metallic impurities were given.

Multielement stock solutions were prepared from sub-boiled (65%) nitric acid (Suprapur Quality, Merck) and Milli-Q water. 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 the experiments were previously treated with diluted (5%) sub-boiled nitric acid and rinsed several times with Milli-Q water.

Details about commercial source and/or preparation of the protein standards, reagents, and SEC/FPLC mobile phases employed have been given elsewhere.[22]

Natural Mussel Samples. Adult mussel specimens were collected from three different coastal regions in northern Spain: 4−5-cm shell length (SHL) mussels from the Avilés estuary (an industrialized area in the Asturias region, suspected to be contaminated by heavy metals); 6−7-cm SHL mussels from the

(11) Livingstone, D. R.; Goldfarb, P. S. In *Environmental. Biomonitoring: The Biotechnology Ecotoxicology. Interface*, 1st ed.; Lynch, J. M., Wiseman, A., Eds.; Cambridge University Press: Cambridge, 1998; pp 101−129.
(12) Frazier, J. M.; George, S. S.; Overnell, J.; Combs, T. L.; Kagi, K. *Comp. Biochem. Physiol.* **1985**, *80C*, 2, 257−262.
(13) Bebianno, M. J.; Langston, W. J. *Talanta* **1998**, *46*, 301−313.
(14) Couillard, Y.; Campbell, P. G.; Tessier, A. *Limnol. Oceanogr.* **1993**, *38*, 299−313.
(15) Bordin, G.; McCourt, J.; Cordeiro Raposo, F.; Rodriguez, A. R. *Mar. Biol.* **1997**, *129*, 453−463.
(16) Engel, D. W. *Water Resour. Bull.* **1988**, *24*, 981−987.
(17) High, K. A.; Methven, B. A.; McLaren, J. W.; Siu, K. W. M.; Wang, J.; Klaverkamp, J. F.; Blais, J. S. *Fresenius J. Anal. Chem.* **1995**, *351*, 393−402.
(18) Sanz-Medel, A. *Spectrochim. Acta, Part B* **1998**, *53*, 197−211.
(19) Sanz-Medel, A. *Analusis* **1998**, *6*, M76−M80.
(20) Soldado Cabezuelo, A. B.; Montes Bayón, M.; Blanco González, E.; García Alonso, J. I.; Sanz-Medel, A. *Analyst* **1998**, *123*, 865−869.
(21) Montes Bayón, M.; Soldado Cabezuelo, A. B.; Blanco González, E. García Alonso, J. I.; Sanz-Medel, A. *J. Anal. At. Spectrom.* **1999**, *14*, 947−951.
(22) Ferrarello, C. N.; Fernández de la Campa, M. R.; Goenaga Infante, H.; Fernández Sánchez, M. L.; Sanz-Medel, A. *Analusis* **2000**, *28*, 351−357.

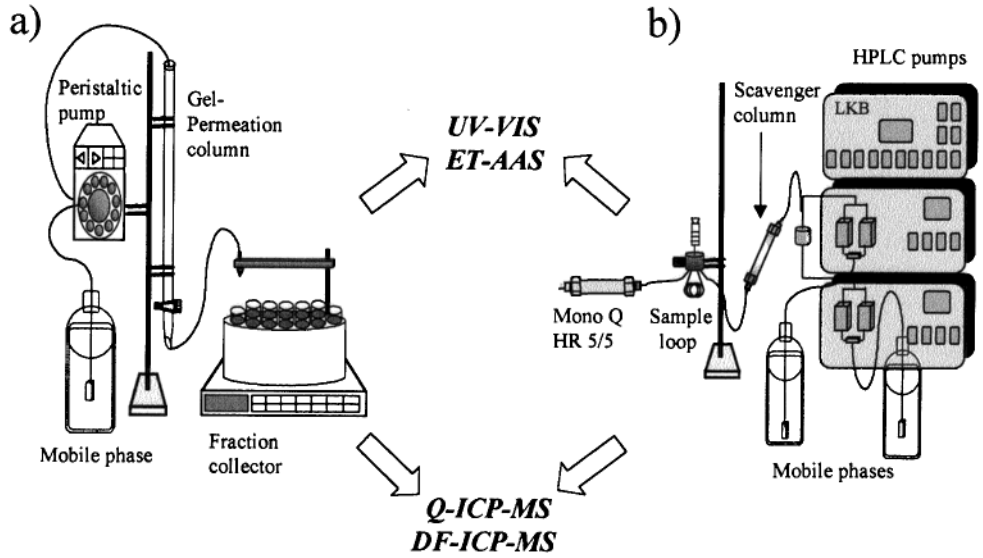

Figure 1. Schematic diagram of (a) the size exclusion chromatography system (Sephadex G-75) and (b) the FPLC system (Mono Q HR 5/5) used for speciation of metal-binding proteins.

Table 1. Optimum Operating Conditions for Q-ICPMS and DF-ICPMS Instruments

Quadrupole ICPMS	
instrument	HP 4500
spray chamber	double-pass/Peltier cooled (4 °C)
rf power	1300 W
carrier gas flow rate	1.2 L min^{-1}
intermediate gas flow rate	1.0 L min^{-1}
outer gas flow rate	15.0 L min^{-1}
sampling depth	5.9 mm
isotopes monitored	^{64}Zn, ^{65}Cu, ^{111}Cd
time-resolved analysis mode	
points per peak	1
integration time	1.5 s/peak
spectrum mode	
points per peak	3
integration time	0.3 s/peak
Double-Focusing ICPMS	
instrument	element
spray chamber	double pass-room temp
rf power	1300 W
carrier gas flow rate	1.15 L·min^{-1}
intermediate gas flow rate	1.05 L·min^{-1}
outer gas flow rate	15.0 L·min^{-1}
sampling depth	fixed
low-resolution parameters (^{111}Cd)	
acquisition mode	E-scan
number of scans	20
mass window	100%
integration window	80%
samples per peak	20
dwell time	0.01 s
medium-resolution parameters (^{34}S, ^{65}Cu, ^{64}Zn)	
acquisition mode	E-scan
number of scans	20
mass window	100%
integration window	80%
samples per peak	40
dwell time	0.005 s

Villaviciosa estuary (a wild unpolluted coast in Asturias), and 7−8-cm SHL commercial mussels (bought in the market and coming from Galicia's coast). They were immediately frozen on dry ice and transferred to the laboratory where the mussel's hepatopancreas were dissected to be used for cytosol preparation.

Aquarium Mussel Samples. Fifty adult specimens of mussel *M. edulis* were collected from a wild coast in Asturias. Fifteen of those mussels (control group) were directly subjected to cytosol preparation. The remaining mussels were transferred to an aquarium tank containing 50 L of aerated seawater. They were not acclimatized and were maintained unfed. Seawater was maintained at 15 °C and changed every day. A spike of Cd ($CdCl_2$; Merck) to achieve a final Cd concentration of 500 $\mu g \cdot L^{-1}$ was added daily. Six days later, 15 specimens were removed from the aquarium, rinsed thoroughly with Milli-Q water, and subjected to cytosol preparation and analysis. The same procedure was repeated to analyze the other 20 mussels, remaining in the aquarium five days later.

Procedures. *Cytosol Preparation.* Mussel cytosolic extracts were obtained based on a modified protocol of the methods of Roesijadi and Fowler [23] and Lobinski et al.[24] as described elsewhere.[22] Oxidation due to prolonged storage of cytosol was observed to generate a complex ion-exchange chromatogram with numerous overlapping peaks.[25] Thus, freshly obtained cytosols were always used in our study.

Size-Exclusion Chromatography (SEC) Procedure. Preparative-scale SEC using a Sephadex G-75 column was carried out with 10 mM Tris-HCl, (pH 7.4), 5 mM 2-mercaptoethanol (2-MCE), 0.1 mM phenylmethylsulfonyl fluoride (PMSF), and 25 mM NaCl (degassed with Ar) pumped at a flow rate of 0.2 mL·min^{-1} with a peristaltic pump P-1 Pharmacia LKB (Brouman, Sweden). PMSF, 2-MCE, and NaCl were added to prevent (or retard) proteinase action, oxidation of cysteinyl residues, and silanophilic effects previously reported by SEC,[26] respectively. Temperature was maintained to 2 ± 1 °C. Metal impurities were removed from the mobile phase by passing them twice through a metal scavenger

(23) Roesijadi, G.; Fowler, B. A. *Methods Enzymol.* **1991**, *205*, 263−273.
(24) Lobinski, R.; Chassaigne, H.; Szpunar, J. *Talanta* **1998**, *46*, 271−289.
(25) Ang, S. G.; Chong, P. S. *Talanta* **1998**, *45*, 693−701.
(26) Micallef, S.; Couillard, Y.; Campbell, P. G.; Tessier, A. *Talanta* **1992**, *9*, 1073−1079.

Table 2. Chromatographic Conditions for MT Speciation by Using FPLC−ICPMS

column	Mono Q HR 5/5
injection loop	50 μL
flow rate	1 mL·min^{-1}
eluents	(A) 4 mM Tris-HCl (pH 7.4) (B) 250 mM ammonium acetate + 10 mM Tris-HCl (pH 7.4)

Gradient Elution

time (min)	B (%)
0	0
1.5	2
2	3
5.5	4
6	10
10	17
11	18
12	99
19	100
23	0

column packed with Kelex-100.[27]

The size exclusion molecular mass column used was calibrated for molecular mass (MM, kDa) by separate injections of L-cysteine (MM <1), rabbit liver MT, mixture of isoforms 1 and 2 (MM 6−10), cytochrome *c* (MM 12.4), lysozyme (MM 14.4), carbonic anhydrase (MM 29), albumin (MM 66), transferrin (MM 80), alcohol dehydrogenase (MM 150), immunoglobulin G (MM 160), β-amylase (MM 200), and dextran blue (MM 2000).

A 1-mL aliquot of cytosol was applied directly to the SEC column and elution carried out in such a way that 40 consecutive 3-mL fractions were collected. In each fraction, several measurements were made: (a) metal−thiolate bonds (monitored by UV−visible absorption spectrophotometry at 220, 254, and 280 nm); (b) Cd, Zn, and Cu levels (semiquantitative analysis by off-line Q-ICPMS detection); (c) S level at mass 34 (higher resolving power, $R = 3000$, is required and so a DF-ICPMS was used).

Fast Protein Liquid Chromatography Procedure. (1) Model Rabbit Liver MTs. Standard mussel MLPs are not commercially available. Thus, standard MTs of rabbit liver were used for preliminary calibration of the Mono Q HR 5/5 AE column used: 50 μL of the commercial Cd/Zn rabbit liver MT-1 and MT-2 mixture were injected into the FPLC column, following the procedure given elsewhere[22] (see Table 2 for details of the chromatographic separation). The effluent from the analytical column was separately connected on-line to the UV−visible spectrophotometer, the Q-ICPMS (Cd, Cu, Zn), or the DF-ICPMS (for S). Thus, the corresponding on-line chromatograms were recorded. All assays were made in triplicate.

(2) Hepatopancreas MLPs of Mussel. Once the MLP fraction was isolated by SEC, it was subjected to the FPLC procedure with on-line Q-ICPMS detection in order to perform metal−MLP speciation. To do so, 2 mL of the most representative MLP fraction separated (having the highest values for UV−visible absorbance and metal contents) was lyophilized. This lyophilizate was then reconstituted for speciation with 60 μL of buffer A and injected into the Mono Q column. The eluate was monitored on-line at 254 and 280 nm for protein identification, while metal detection (Cd, Cu, Zn) was carried out on-line by Q-ICPMS.

ICPMS Analysis. Possible spectral and nonspectral interferences caused by the matrix (on the signal of each considered mass) were investigated by using DF-ICPMS to analyze the mussel cytosol fractions obtained by SEC. Thus, by measuring each element at different resolving powers, we verified that using sample dilution 1 + 1.5 (with Milli-Q water) and internal standardization all matrix effects were satisfactorily minimized (the ratio between standard addition and aqueous calibration slopes was close to 1). ^{34}Sulfur required $R = 3000$ and was measured by DF-ICPMS, while semiquantitative analysis of the sought metals (by Q-ICPMS) was carried out using 1 + 1.5 dilution and the following internal standards (IS): ^{45}Sc, ^{71}Ga, and ^{115}In to correct the signals of ^{34}S, ^{64}Zn, ^{65}Cu, and ^{111}Cd. Quantitative elemental analysis of each SEC fraction was also tried using DF-ICPMS at $R = 3000$ (Zn, Cu) and $R = 300$ (Cd) and the conditions mentioned above. The internal standards were added to sample, calibration, and blank solutions at the same concentration level.

To avoid salt deposits on the ICPMS cones during quantitative analysis of each SEC fraction, sub-boiled 2.5% nitric acid in Milli-Q water was passed between every fraction analysis. In that way, no drift of the DF-ICPMS analytical signal was observed throughout speciation analysis for, at least, 2 h.

Detection limits for Cd, Cu, and Zn were calculated as 3 times the standard deviation of 10 measurements of the blank solution, constituted by the mobile phase used for SEC (diluted 1 + 1.5), sub-boiling nitric acid (0.2%), and the internal standards at their operational concentrations.

RESULTS AND DISCUSSION

Size-Exclusion Chromatography: Fractionation and Characterization. Gel filtration elution profiles obtained showing the distribution of Cd, Cu, Zn, and S in the heat-treated cytosol of mussel hepatopancreas are illustrated in Figure 2a, while Figure 2b depicts the UV−visible absorbance of the eluate measured at 220, 254, and 280 nm, as a guide to the appearance of proteins during separation.

Vertical lines A, B, etc., in Figure 2 indicate the peak maximum for each model protein (at 220 nm) injected in the column used to calibrate molecular size. The peak corresponding to a standard rabbit liver MT-1 and MT-2 mixture (indicated with letter F) overlapped to the mussel cytosolic fraction containing major amounts of Zn, Cu, Cd, S, and also higher absorbance at 254 nm. These results indicate that fractions 22−28 in Figure 2 should probably contain MLPs (with a molecular mass of 8−10 kDa).

Different workers have reported the presence in mussels of a major Cd-binding compound, which produced two SEC overlapping peaks.[12,28−32] These were designated as Cd−BP_{10} (or Cd−MT_{10}) and Cd−BP_{20} (or Cd−MT_{20}); the Cd−BP_{20} peak was always present for mussels exposed to cadmium. For uncontaminated

(27) López García, A.; Blanco González, E.; Sanz-Medel, A. *Mikrochim. Acta* **1993**, *112*, 19−29.

(28) George, S. G.; Carpene, E.; Coombs, T. L.; Overnell, J.; Youngsen, A. *Biochem. Biophys. Acta* **1979**, *580*, 225−233.

(29) Kohler, K.; Riisgard, H. U. *Mar. Biol.* **1982**, *66*, 53−58.

(30) Veldhuizen-Tsoerkan, M. B.; Holwerda, D. A.; Zandee, D. I. *Arch. Environ. Contam. Toxicol.* **1991**, *20*, 259−265.

(31) Isani, G.; Serra, R.; Cattani, O.; Cortesi, P.; Carpene, E. *J. Mar. Biol. Assoc. U.K.* **1997**, *77*, 1187−1197.

(32) Mackay, E. A.; Overnell, J.; Dunbar, B.; Davidson, I.; Hunziker, P. E.; Kagi, J.; Fotherhill, J. E. *Eur. J. Biochem.* **1993**, *218*, 183−194.

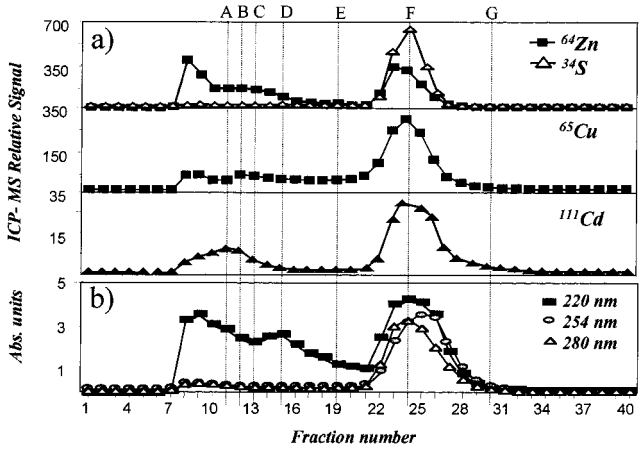

Figure 2. Representative chromatograms of SEC analysis of commercial *M. edulis* hepatopancreas cytosol: (a) qualitative sulfur detection (DF-ICPMS) and semiquantitative (Q-ICPMS) analysis of sought metals in each SEC fraction; (b) molecular absorbance of SEC fractions at three different wavelengths. Calibrating standard proteins: (A) dextran blue (2000 kDa), β-amylase (200 kDa); (B) immunoglobulin G (160 kDa), alcohol dehydrogenase (150 kDa); (C) transferrin (80 kDa), albumin (66 kDa); (D) carbonic anhydrase (29 kDa); (E) lysozyme (14.4 kDa), cytochrome *c* (12.4 kDa); (F) rabbit liver MT isoforms 1 + 2 (8−10 kDa); (G) L-cysteine (<1 kDa).

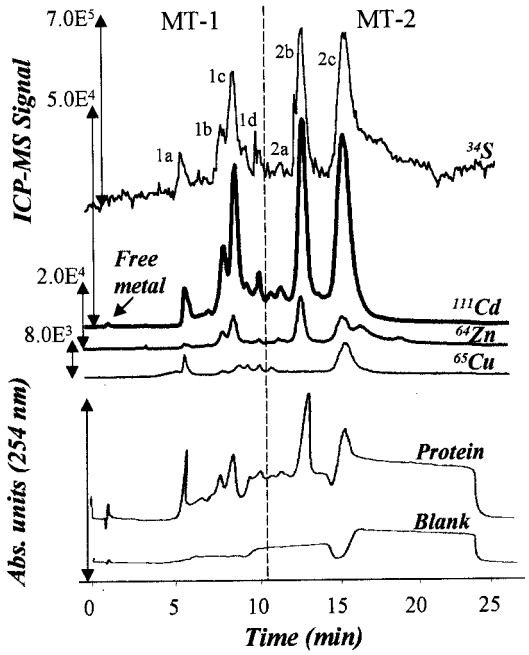

Figure 3. Chromatographic profiles obtained for 50 μL of MT-1 and MT-2 rabbit liver isoform mixture (150 $\mu g \cdot L^{-1}$ total cadmium) by FPLC coupled on-line to DF-ICPMS (^{34}S), Q-ICPMS (metals), and UV−visible spectrophotometry (protein). Letters a, b, c, etc., represent the successive fractions corresponding to each separated isoform.

natural levels, however, only one peak was reported by High et al. in different mussel species.[33−35] However, the same authors reported a second SEC Cd peak in the cytosol of *Dreissena polymorpha* mussel after 10 days of Cd exposure.[35]

On the other hand, Frazier and George [36] found that the largest amounts of cytosolic Cu, Zn, and Cd in polluted specimens of *Ostrea edulis* are always associated with a very low molecular weight (VLMW) protein pool. The authors suggested that such a VLMW metal complex may play an important role for metal accumulation. However, they found that other species of molluscs, i.e., *M. edulis*, do not exhibit such a VLMW complex. Our studies confirm this last finding. Negligible amounts of metals (Cu, Zn, Cd), along with low absorbance values (at 220, 254, and 280 nm), were always associated with the cytosolic VLMW pool (fractions 29−35) measured (Figure 2a). Interestingly, we found significant concentrations of Zn in fractions 7−15 (>30 kDa) corresponding to the high molecular weight (HMW) protein pool. Cd and Cu were also present there at lower concentration levels. Moreover, high values of molecular absorbance at 220 nm (Figure 2b) observed in those fractions tend to indicate that a fraction of those metals could also be sequestered by HMW proteins.

Mammalian MT Speciation by FPLC−ICPMS. Previous Cd speciation studies on a mixture of standard rabbit liver MT 1 and 2, using FPLC with off-line ET-AAS Cd detection, indicated that Cd^{2+} does not seem to be absorbed by the Mono Q column[22] used in this work. Following the experimental separation conditions detailed there, the profiles of sulfur (DF-ICPMS), the sought metals (Q-ICPMS), and molecular UV−visible absorbances (254 nm) for the MT-1 and MT-2 "model" mixture were worked out: Figure 3 shows that FPLC separation was able to distinguish seven different rabbit liver MTs fractions, with narrow peaks, in a relatively short time (<16 min). Table 3 summarizes the retention times observed for the seven fractions obtained (a, b, c, etc., were simply named after their elution order).

Table 3. Retention Times of Rabbit Liver MT Fractions Obtained by Using FPLC Coupled to Q-ICPMS

MT isoform	fraction	t_R (min)[a]
MT-1	1a	5.8 ± 0.09
	1b	8.1 ± 0.07
	1c	8.8 ± 0.08
	1d	10.3 ± 0.05
MT-2	2a	11.5 ± 0.06
	2b	12.8 ± 0.04
	2c	15.2 ± 0.03

[a] Precisions were calculated for three independent chromatograms.

FPLC−ICPMS Speciation of Real Samples. Subsamples of 15 Cd-exposed mussels and control unpolluted mussels were opportunely processed to obtain their cytosols (see protocol described above). Separate 1-mL aliquots of each cytosolic extract were injected first into the SEC column and the metals were analyzed by ICPMS. A single broad peak of cadmium for Cd-exposed mussels was always observed between fractions 22 and 28 (data not shown). We identified this peak as the MLP band. Subsequently, a 50-μL aliquot of the fraction in the maximum of this MLP band was used for trace elemental speciation by FPLC followed by on-line UV−visible spectrophotometric and Q-ICPMS detection.

As shown in Figure 4a, two sharp peaks were observed, at retention times (t_R) of 15.6 ± 0.03 and 16.1 ± 0.02 min, and their

(33) High, K. A.; Azani, R.; Fazekas, A. F.; Chee, Z. A.; Blais, J. S. *Anal. Chem.* **1992**, *64*, 3197−3201.
(34) High, K. A.; Blais, J. S.; Bradley, A.; Methven, J.; McLaren, J. *Analyst* **1995**, *120*, 629−634.
(35) High, K. A.; Barthet, V.; Mc Laren, J.; Blais, J. S. *Environ. Toxicol. Chem.* **1997**, *16*, 1111−1118.
(36) Frazier, J. M.; George, S. G. *Mar. Biol.* **1983**, *76*, 55−61.

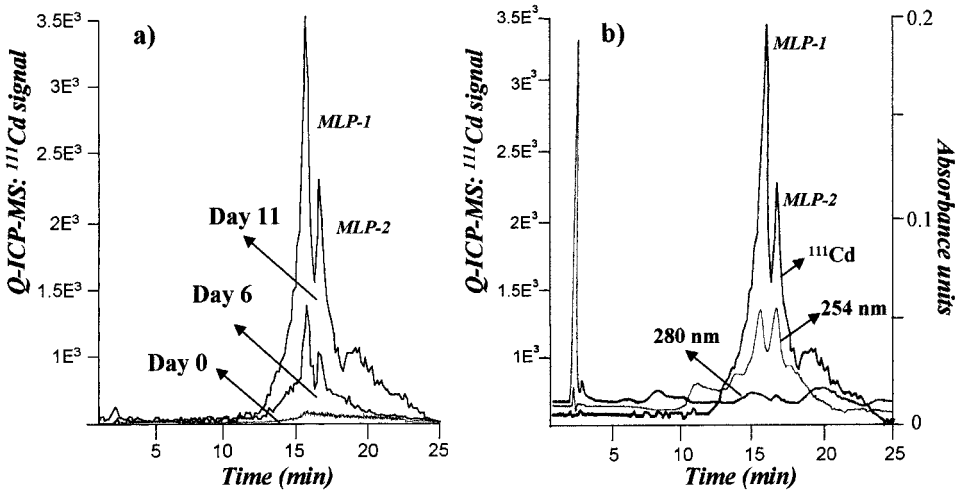

Figure 4. MLP profiles for 50 μL of the SEC fraction containing the MLP peak (of mussels exposed to 500 $\mu g \cdot L^{-1}$ cadmium in an aquarium tank) by "on-line" FPLC-Q-ICPMS or UV−visible spectrophotometry. (a) Cd−MLP profiles at basal levels (day 0) and after 6 and 11 days of "in vivo" Cd exposure; (b) superimposed Cd−MLP and UV absorbance (254 and 280 nm) profiles for those mussels with 11 days of Cd exposure in the aquarium.

level increased with increasing Cd exposure. Similarly, Figure 4b shows the corresponding profiles obtained for Cd-MLP (11-days exposure) along with the superimposed trace observed with UV absorbance detection at 254 and 280 nm. The two peaks at 254 nm would probably indicate the presence of mussel MLP-1 and MLP-2 isoforms. In fact, the absorbance profile at 254 nm (tracing Cd−thiolate bonds) was closely coincident with that of Cd−MLP obtained by Q-ICPMS. Moreover, no peaks at 280 nm were noticed at those retention times (Figure 4b), a fact supporting the MT-like identity of the FPLC-separated mussel protein isoforms (such proteins do not seem to have aromatic amino acids).

Natural Mussel Sample Speciation. Lyophilized mussel samples (caught at three different coastal regions in Spain) were examined by following the general speciation procedure developed. Speciation by SEC and FPLC−ICPMS demonstrated the presence of two Cd−MLP isoforms, with retention times identical to those observed for the aquarium samples (Figure 5a), in commercial mussels and those from the industrial area. No Cd signals were detected in the Villaviciosa mussels. As shown by Figure 5a, the sum of both Cd−MLP peak areas was 17 times higher in the mussels caught along the industrial coast than that of mussels bought in the supermarket (Galicia region), confirming that higher MLP contents occur in mussels contaminated by Cd. Meanwhile, wild coast mussels did not show any Cd−MLP at all, indicating a metal contamination-free habitat.

The multielement capability of ICPMS was applied to investigate Cu and Zn simultaneous speciation in the corresponding fraction of Cd. Results have been summarized in Figure 5b and c. As can be seen in Figure 5b, the three different mussel samples showed only a single Cu peak, at t_R= 15.7 min., overlapping that of Cd−MLP-1. Another sharp peak at t_R= 3.7 min contained about 29−32% of the total Cu in the chromatogram. It could probably correspond to Cu^{2+} bound to other proteins, responsible for the molecular absorbance at 280 nm detected in SEC fractions 22−28 (see Figure 2b).

Figure 5c shows the profiles observed for Zn: Zn^{2+} appears to be bound to MLP-1, although an extra Zn-peak appeared at 18.7 min (probably due to Zn^{2+} retention in the column, which would be eventually released when 100% of buffer B is achieved). In summary, data from Figure 5 indicate that Cd was almost totally bound to MLPs (83 ± 1 and 92 ± 2% in Avilés and Galicia, respectively) while Cu and Zn appeared only partially bound to these proteins (33−69%).

Quantitative Analysis of Metals on SEC Fractions by DF-ICPMS. Once the qualitative distribution of the desired metals in the SEC fractions was accomplished, the total content of Cu, Zn, and Cd in the cytosols and in MLPs and HMW proteins (pool) were evaluated. The off-line ICPMS detection limits observed for Zn, Cu, and Cd in the cytosol matrixes were 10.3, 16.1, and 1.7 $ng \cdot L^{-1}$, respectively. Results for the three coastal regions investigated are collected in Table 4. As can be seen, total Cd in cytosols of mussel samples coming from Avilés estuary turned out to be as high as 22.6 $\mu g \cdot g^{-1}$ wet tissue (probably this high Cd level can be associated with an electrolytic zinc factory existing nearby and accounting for the relatively high levels of zinc found in those mussels).

Approximate chromatographic peak areas relationships indicate that the MLP pool of Avilés samples contains ~90 ± 2% of total cytosolic cadmium observed (i.e., MLP could exert their protective role via complexation of the toxic metal). On the other hand, mussel samples of Galicia and Villaviciosa, where Cd contamination did not show up, the fraction of MLP-bound Cd was comparatively lower (~72 ± 2% of total cytosolic Cd).

CONCLUSIONS

Long-term effects of slight contamination by heavy metals in coastal areas are being increasingly studied by using "sentinel organisms". To unveil such long-term detrimental effects, speciation information in the organisms of the trace element chemical forms involved is urgently needed. Several approaches have been developed here for Cd speciation in biological samples including SEC coupled with UV and ICPMS detection, which allowed us to identify and isolate MLPs in naturally harvested mussels. The techniques have been applied also to mussels of three differently polluted coastal regions of Spain. Further direct metal speciation is possible by on-line FPLC−ICPMS of the SEC-isolated fractions

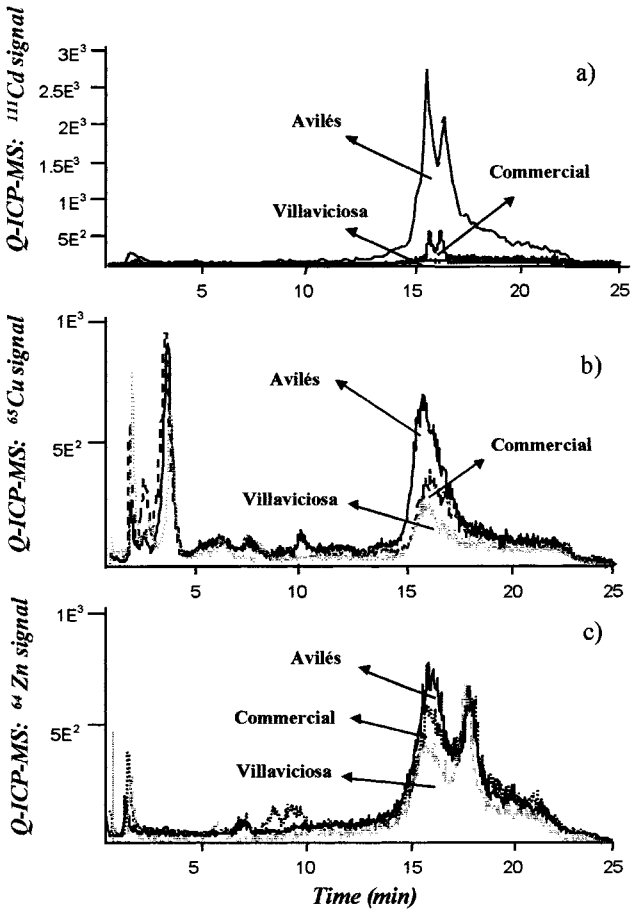

Figure 5. Comparative profiles of metal−MLPs of mussels from three different coastal regions in Spain, obtained by injecting 50 μL of the lyophilized SEC fraction (containing the peak of the MLP band) into the FPLC−Q-ICPMS system. (a) Cd−MLPs profiles of Avilés (83 ± 1%), Galicia (92 ± 2%), and Villaviciosa (nd). (b) Cu−MLPs profiles of Avilés (65 ± 2%), Galicia (33 ± 1%), and Villaviciosa (34 ± 1%). (c) Zn−MLPs profiles of Avilés (69 ± 2%), Galicia (52 ± 2%), and Villaviciosa (51 ± 2%). Values in parentheses correspond to percent of total metal bound to MLPs; nd, not detected.

Table 4. Cu, Zn, and Cd Contents[a] in SEC Fractions of Mussels from the Three Coastal Regions of Spain

		Villaviciosa	Galicia	Avilés
	Cu	2.9 ± 0.1	2.9 ± 0.1	5.3 ± 0.1
HMW pool	Zn	14.4 ± 0.2	23.7 ± 0.3	24.7 ± 0.3
(∑ fractions 815)	Cd	0.067 ± 0.006	0.126 ± 0.007	2.4 ± 0.1
	Cu	14.6 ± 0.2	17.2 ± 0.2	16.8 ± 0.2
MLP	Zn	19.4 ± 0.2	16.7 ± 0.3	72.1 ± 0.7
(∑ fractions 22−28)	Cd	0.24 ± 0.01	0.37 ± 0.01	19.9 ± 0.2
	Cu	22.2 ± 0.3	34.3 ± 0.3	26.3 ± 0.3
cytosol	Zn	42.4 ± 0.4	55.3 ± 0.5	118 ± 1
(∑ fractions 5−35)	Cd	0.31 ± 0.01	0.52 ± 0.01	22.6 ± 0.3

[a] Values are reported in $\mu g \cdot g^{-1}$ wet tissue. Precisions were calculated for three independent measurements. Metal contribution in fractions 1−4 and 36−40 was negligible and so it is not included.

containing MLPs. Application of such techniques to investigate Cd−MLP isoforms in mussels of the Avilés estuary (an industrial area suspected to be polluted by heavy metals) demonstrated a high degree of metal contamination as compared to the other region investigated. Two Cd−MLP peaks (MLP-1 and MLP-2) are clearly identified and are related to the degree of contamination (see Figure 5a). Moreover, it appears that even very low environmental Cd levels (≅0.04 $\mu g \cdot L^{-1}$ in coastal seawater of Galicia) could induce formation of the second isoform of MLP (MLP-2).

On the other hand, it is known that MT synthesis may be induced by a great variety of chemical and physical stress agents[6,37,38] and metals have been shown to be probably the strongest inductors (including metals not bound by MTs [39]). Thus, as no Cd−MLPs were found in the mussels of the Villaviciosa estuary (Figure 5a), this result would indicate a toxic metals-free habitat.

The fact that Zn and Cu are present only in the MLP-1 fraction (for samples of the three regions) could point to speculation that this MLP isoform plays a pivotal role in metal homeostasis, while MLP-2 could be more involved in metal detoxification.

As a general conclusion, it could be said that complementary separation and detection hybrid techniques, and particularly the coupling of FPLC−ICPMS after SEC fractionation, offer a great potential for future MLPs investigations and its possible use as biomarkers for heavy metal pollution. For instance, such complementary techniques have demonstrated that metal speciation in vertebrate animals MTs is quite different from that observed for invertebrate MLPs (see Figures 3 and 5) and it is possible to investigate these problems at extremely low levels of concentration (as measured by the tag metal).

Previous studies related to the synthesis of metallothioneins, as a defense against toxic metals, have been mainly on laboratory assays in which exposure conditions and metal concentration levels differ greatly from those in natural aquatic environments.[5,6] The most novel aspect of this work is the application of several different-principle-based separation schemes (complementary or orthogonal separation mechanisms) to speciate Cd−MLPs at basal levels and the use of such information as an "alarm" technique to detect actual contamination (see Figure 5a) even at apparently metal contamination-free ecosystems.

In brief, the usefulness of resorting to complementary techniques[18,19] to unveil Cd speciation in MLPs of mussels is illustrated here, along with the demonstration of the great analytical potential of FPLC−ICPMS for such purposes. Both aspects, strategic and instrumental, may open important avenues (particularly in the hands of complementary professionals, i.e., biochemists) to tackle the modern challenge of trace and ultratrace metal speciation in biological systems.

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(37) Pedersen, S. N.; Lundebye, A. K.; Depledge, M. H. *Aquat. Toxicol.* **1997**, *37*, 183−200.
(38) Kagi, J. H. R. *Methods Enzymol.* **1991**, *205*, 613−626.
(39) Palmiter, R. D. *Proc. Natl. Sci. U.S.A.* **1994**, *91*, 1219−1223.