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Simplified and miniaturized procedure based on ultrasound-assisted cytosol preparation for the determination of Cd and Cu bound to metallothioneins in mussel tissue by ICP-MS

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ABSTRACT

A simplified and miniaturized procedure for the determination of Cd and Cu bound to metallothioneins (MTs) by ICP-MS in mussel tissue has been developed. Cytosol preparation was based on the indirect sonication of slurries containing the lyophilized sample dispersed in 1 mL of extractant by means of a sonoreactor Cup-Horn. Rabbit liver MTs (Apo-MT-I, Apo-MT-II and Cd_7-MT-II) and a conventional cytosol preparation procedure were used for validation purposes. The usual heating step and additional centrifugations of the conventional procedure for cytosol preparation can be omitted when using ultrasound treatment. The possible effect of denaturation on MTs and its effect on the metal bound to MTs were evaluated. Variables influencing the ultrasound-assisted cytosol preparation procedure were carefully optimized for simultaneous determination of both metals. Chromatographic conditions to separate the MT fraction from other proteins present in cytosols were also studied. Six samples can be processed within 3 min of sonication. An acid ultrasound-assisted extraction procedure with diluted acid was also proposed for determining total Cd and Cu. Finally, Cd and Cu bound to MTs as well as total Cd and Cu were determined in mussels from Pontevedra and Ares-Betanzos coastal inlets (Galicia, Spain).

1. Introduction

Metallothioneins (MTs) in aquatic organisms are considered to be attractive biomarkers for the assessment of metal pollution in the marine environment. MT biosynthesis is induced under elevated concentration of both, essential and toxic metals. Although not all functions of MTs are fully understood, nowadays it is recognized that these cytosolic proteins are involved in metal homeostasis and detoxification processes [1,2].

Multiple MT isoforms (in special MT-1, MT-2, MT-3 and MT-4) have been identified in animals, though polymorphism is particularly important in invertebrates compared to mammals [2]. Only MT-1 and MT-2 are clearly induced by metals, whereas MT-3 and MT-4 are relatively impassive to metal concentration. Variations in molecular mass owing to the presence of monomeric and dimeric forms have also been suggested [3]. Determination of metal bound to MTs and total metal in biomarkers is an efficient approach for environmental assessment of metal pollution [4,5]. Filter feeding bivalves such as mussels are able to synthesize a high level of MTs in response to elevated concentrations of metals under laboratory and environmental conditions, hence representing excellent biological species for assessing marine contamination [6].

Determination of metals bound to MTs is not easy in real samples, methodologies with high sensitivity and selectivity being necessary. In general, reported procedures entail three stages: cytosol preparation, MT separation and measurement of metal in the corresponding fraction. Most of the studies have been focused on the use of different hyphenated techniques in order to accomplish MT-isolation and detection of metal concentration, e.g. LC–ICP-MS (liquid chromatography interfaced with inductively coupled plasma mass spectrometry), LC–ESI-MS (liquid chromatography interfaced with electrospray ionization mass spectrometry) and CZE-ICP-MS (capillary zone electrophoresis interfaced with inductively coupled plasma mass spectrometry) [7–10]. On the contrary, cytosol preparation has received scanty attention, despite this stage is generally long and tedious. Therefore, determination of metal bound to MTs requires simpler and fast methodologies if their use as biomarkers is to be implemented on a routine basis [11]. These improvements can be reached introducing changes in the stage of cytosol preparation.

In general, preparation of cytosol includes the cutting/homogenization of soft tissue with an extracting buffer (usually Tris–HCl at pH 7.4–8.6 with small concentrations of 2-mercaptoethanol and phenylmethanesulfonylfluoride in order to avoid the oxidation and hydrolysis of MTs) [7,12,13]. Next,

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the mixture is centrifuged and then, it is usual to include a thermal treatment (at 60–70 °C) and further centrifugation in order to eliminate proteins of high molecular weight that suffer denaturalization and precipitate [7].

Besides many steps, some problems from the different devices and conditions used for cutting/homogenization of samples (e.g. cutting mixers or rotating pestles) can be emphasized: (i) contact with air during the process can increase tissue oxidation; (ii) high risk of contamination due to the direct contact with the tissue; (iii) difficulties when small sample amounts are available. To overcome these problems, different alternatives such as sonification with a beaker resonator in argon gas atmosphere [14], pressurized liquid extraction [15] or freeze–thaw cycles in liquid nitrogen [16] have been proposed.

Sonication of lyophilized tissues could be advantageous in order to simplify cytosol preparation. The cavitation phenomenon promotes cellular disruption and then, cytosol can be easily obtained. The use of a cup-horn sonoreactor displays several advantages over sonication using sonication baths and probes, namely: (i) it is not in contact with the sample (indirect sonication), thus diminishing the risk of contamination; (ii) it allows the simultaneous treatment of six closed Eppendorf vials; (iii) it allows working with low amounts of samples and reagents [17].

In this work, a simplified and miniaturized method for the determination of Cd and Cu bound to MTs by ICP-MS in mussel tissue is presented. A new cytosol preparation based on indirect sonication by means of a cup-horn sonoreactor is developed. Total Cd and Cu concentrations can be also determined using ultrasound-assisted acid extraction.

2. Experimental

2.1. Instrumentation

A quadrupole-based ICP mass spectrometer Thermo Elemental X7 Series (Thermo Fisher Scientific, Bremen, Germany) equipped with impact bead spray chamber (Peltier cooled at 3 °C), PolyPro ST nebulizer (ESI, Elemental Scientific, Inc., Omaha, USA) with a flow rate of 400 μL min^{-1} and PlasmaLab Software was used for ICP-MS measurements. A solution containing 2 μg L^{-1} of Be, Co, In and U in 2% (v/v) HNO_3 was used to optimize the instrument daily in terms of sensitivity, stability and oxide formation rates. Instrumental parameters for determining Cd and Cu are shown in Table 1. The measured isotopes were ^{65}Cu and ^{111}Cd, selected on the basis of their natural abundance and the absence of isobaric interferences and minimum theoretical polyatomic interferences caused by the plasma gas and sample matrix.

A chromatographic system Jasco LC-NetII/ADC (Tokyo, Japan) equipped with an isocratic regime Bioteck model 520 pump (Vermont, USA), a Jasco UV-2075 Plus detector, a ChromNAV Control Center software for data acquisition and a ProteinPak DEAE-5PW anion exchange 8 mm × 75 mm glass column (Waters, Milford, MA, USA) was used for MT separation. Chromatographic conditions are shown in Table 1.

A 200 W, 24 kHz ultrasonic reactor UTR200® (Dr. Hielscher Company, Germany) was employed for ultrasound-assisted cytosol preparation (metal bound to MTs) and ultrasound-assisted acid extraction (total metals). It consists of a cup-horn shaped sonotrode that can accommodate up to six Eppendorf vials.

2.2. Reagents and samples

Ultrapure water was obtained from a Milli Q water system (18.3 MΩ cm^{-1}) (Millipore, San Quentin, France). 69% (w/v) HNO_3 and 37% (w/v) HCl were acquired from Merck (Darmstadt, Germany). Rabbit liver Apo-MT-I/II (metal-free metallothioneins) and Cd_7-MT-II were purchased from Bestenbalt LLC (Tallin, Estonia). Stock standard solution of Cd and Cu (1000 mg L^{-1}) were obtained by dissolving the appropriate amount of $Cd(NO_3)_2$ (Panreac, Barcelona, Spain) and pure metal (Probus, Badalona, Spain), respectively. Stock solutions of ^{72}Ge and ^{115}In containing 100 μg L^{-1} were used as internal standards (Merck). The standard working solutions were prepared daily by suitable dilution with 2% (v/v) HNO_3. Tris–hydroxymethyl-aminomethane (Sigma–Aldrich, Steinheim, Germany), 2-mercaptoethanol (2-MEC) (Sigma–Aldrich), phenylmethylsulfonyl fluoride (PMSF) (Fluka, Sigma–Aldrich) and sodium chloride (Sigma–Aldrich) were used for cytosol preparation.

Eighteen mussel samples from Galicia (NW Spain) were analyzed. Each sample consisted of a pool of 50 specimens (about 2 kg) and a size larger than 6 cm. Tissues were lyophilized, homogenized and stored in closed polyethylene vessels at 4 °C before analysis. CRM ERM CE-278 (mussel tissue) purchased from the Institute for Reference Materials and Measurements (IRMM, Geel, Belgium) and SRM 2976 from the National Institute of Standards and Technology (NIST, Gaithersburg, USA) were employed for validation of total metal concentrations.

All glassware and polyethylene containers were washed and kept for 24 h in nitric acid 10% (v/v), then were rinsed at least three times with ultrapure water.

Table 1
Operating conditions for ICP-MS measurements and anion exchange chromatographic separation.

ICP-MS	
Forward power (W)	1350
Sample flow rate (mL min^{-1})	0.4
Argon flow rates (L min^{-1})	Plasma, 13.0; auxiliary, 0.8; nebulizer, 0.9
Nebulizer	Polypropylene micro-flow
Torch	Quartz vessel, 1.5 mm injector torch
Spray chamber	Quartz impact bead
Spray chamber temperature	Peltier cooled to 3 °C
Interface	Ni sampler and skimmer cones
Scanning mode	Peak hop
Channels per mass	3
Dwell time (ms)	10
Sweeps per peak	100
Replicates	3
Anion exchange chromatographic separation	
Column	Waters ProteinPak DEAE-5PW 8 × 75 mm
Mobile phase	75 mM Tris–HCl, pH 7.4
Elution mode	Isocratic
Injected volume (μL)	250
Flow rate (mL min^{-1})	0.8
Data acquisition	UV detection
Wavelength (nm)	254

2.3. Ultrasound-assisted cytosol preparation procedure

About 20 mg of lyophilized mussel tissue were weighed into an Eppendorf vial (1.5 mL capacity) and then, 1 mL of extracting solution (Tris–HCl buffer 10 mM pH 7.4, NaCl 25 mM, PMSF 0.01 mM and 2-MEC 5 mM) was added. Next, the suspension was sonicated for 3 min at 60% ultrasound amplitude. Six samples were simultaneously processed. The supernatant liquid was separated by centrifugation for 5 min at 9000 rpm. Mussel extracts containing cytosols were filtered through a 0.45 μm filter (MS® PTFE syringe filters, Membrane Solutions, USA). Blanks were treated in the same way.

2.4. Cytosol preparation procedure used for comparative purposes

This procedure was based on cytosol preparation procedures described elsewhere [12,18,19]. About 0.5 g of lyophilized mussel soft tissue was weighed into a centrifuge tube of 50 mL capacity and then 25 mL of extractant (Tris–HCl buffer 10 mM pH 7.4, PMSF 0.01 mM and 2-MEC 5 mM) were added. The mixture was gently shaken and then maintained at 4 °C about 2 h for hydration. After centrifugation, the supernatant was transferred into a second centrifuge tube and warmed in a water-bath at 60 °C for 15 min. A second centrifugation was carried out. Extracts containing cytosols were obtained by decantation. Blanks were treated in the same way.

2.5. MT separation procedure by anion-exchange LC

The metallothionein fraction was separated from the other protein fractions by LC under isocratic conditions (0.8 mL min^{-1}) using a weak anion-exchange column and 75 mM Tris–HCl pH 7.4 buffer solution as mobile phase. The mobile phase was filtered and degassed for 15 min in an ultrasonic water-bath prior to use. The MTs fraction (including all isoforms) was collected at a retention time between 3.2 and 5.5 min (total eluted volume: 1.8 mL). The internal standards were added to the MTs fraction prior to ICP-MS measurements. External calibration with aqueous standards was used.

2.6. Ultrasound-assisted acid extraction procedure for total Cd and Cu determination

An ultrasound-assisted acid extraction procedure was based on studies reported elsewhere [17,20]. About 20 mg of lyophilized mussel soft tissue were weighed into an Eppendorf vial (1.5 mL capacity) and then 1 mL of 3% (v/v) HNO_3 was added. The suspension was sonicated for 3 min at 60% ultrasound amplitude. The internal standards were added to extracts prior to sonication. Six vials were processed at a time. The supernatant liquid was separated by centrifugation and syringe filtration (MS® PTFE syringe filters with pore size <0.45 μm). An additional clean up of acid extracts (Hipersep IC-RP sample preparation cartridge, Metrohm, Herisau, Switzerland) was carried out prior to their introduction into the ICP-MS instrument. External calibration with aqueous standards was applied. Blanks were treated in the same way.

3. Results and discussion

3.1. Preliminary studies for Cd and Cu determination by ICP-MS in acid extracts and in MT fraction

First, the suitability of acid extracts obtained following ultrasound-assisted acid extraction for direct measurement of total Cd and Cu by ICP-MS was investigated. Direct introduction of these extracts in the instrument may be complex, since high quantities of co-extracted organic matter, among other components, are present. In order to avoid these drawbacks, an additional clean up step using a C18 cartridge for non-polar solid phase extraction was carried out [21]. Matrix effects from acid extracts of mussel tissue prepared with and without clean up were evaluated by comparing calibration slopes corresponding to external (i.e. aqueous standards) and standard addition calibration methods. The ratio of slopes in terms of percentage of change (aqueous standard/standard addition) was evaluated. When extracts were introduced in the instrument without clean up step a remarkable matrix effect was observed for both elements (percentages of change in slope ratios were 17 ± 2% and 13 ± 5% for Cd and Cu, respectively). On the contrary, these percentages were less than 3% for both elements after clean up. Consequently, this step is mandatory to accurately determine total Cd and Cu by ICP-MS without interferences from co-extracted organic matrix.

In addition, the direct introduction of these acid extracts in the ICP-MS instrument may cause signal drifts due to changes in the nebulization efficiency and salt deposition in the cones during the analysis [21]. Then, ten extracts were measured between an initial calibration and a recalibration. No signal drift was observed and the cones were not affected by salt deposition.

Likewise, suitability of the MT fraction for direct ICP-MS measurement was investigated. After chromatographic separation, a clean fraction is obtained in a 75 mM Tris–HCl buffer. Then, the effect of this medium was investigated. Two calibration curves, one using aqueous standards and other using standards prepared in 75 mM Tris–HCl buffer, were performed for each element. All standards were measured in triplicate and regression coefficients were higher than 0.9997. Matrix effects were evaluated in terms of percentage of change in slope (aqueous standard/buffer standard). Effects from the buffer medium were not observed (the changes were about 0.3% for both, Cd and Cu). A recovery study was also carried out. Obtained fractions were spiked with Cd and Cu prior to ICP-MS measurements. The study was carried out in triplicate. The obtained recoveries were 98.7 ± 1.2% and 97.2 ± 1.5% for Cd and Cu, respectively. Then, calibration with aqueous standards can be carried out in both cases, i.e. for determinations of total metal and metal bound to MTs.

3.2. Working with lyophilized samples

In general, the most usual process of sample preservation for the determination of metal bound to MTs is freezing of soft tissues. However, freezing and later unfreezing of samples do not prevent the appearance of undesirable processes such as mechanical damage to the cell membrane, denaturalization of proteins and sample dehydration [22]. A less controversial process of sample preservation such as lyophilization has been scantily used for this purpose. A lyophilized mouse kidney sample was used by Menegário et al. [19] after rehydration with a buffer Tris/HCl buffer. No problems were appointed by the authors with the use of lyophilized samples for determining Cd-MT.

On the other hand, proteins may freeze-dry with little difficulty [23]; in fact, Apo-MT1 (62 amino acids), Apo-MT2 (63 amino acids) and Cd_7-MT-2A were acquired as lyophilized power.

In addition, lyophilization is advisable in order to miniaturize the extraction procedure for cytosol preparation and to reach suitable limits of detection. Then, lyophilization of mussel samples was performed in this work (−50 °C and 5.8–7.5 × 10^{-2} mB).

3.3. Preliminary studies for chromatography separation

The collection of one MT fraction that includes the different isoforms is desirable in order to determinate Cd and Cu bound to MTs by ICP-MS for environmental monitoring. Thus, chromatographic conditions were optimized to separate the MT fraction for other proteins present in cytosols. Apo-MT-I, Apo-MT-II, Cd_7-MT-II and mussel cytosols were used in this study. Elution profiles (in Tris 75 mM at pH 7.4) are shown in Fig. 1(A–E). As can be observed in Fig. 1(A–C), only one chromatographic fraction is obtained. Fig. 1(D and E) shows the chromatograms from mussel cytosols and mussel cytosols spiked with Cd_7-MT-II. The retention time of the first peak was similar to that obtained with rabbit liver MTs (Fig. 1A–C). Good resolution between mussel MTs and other sample components was obtained. The eluted fraction between 3.2 and 5.5 min (total eluted volume: 1.8 mL) was collected for the off-line determination of Cd and Cu bound to MTs by ICP-MS.

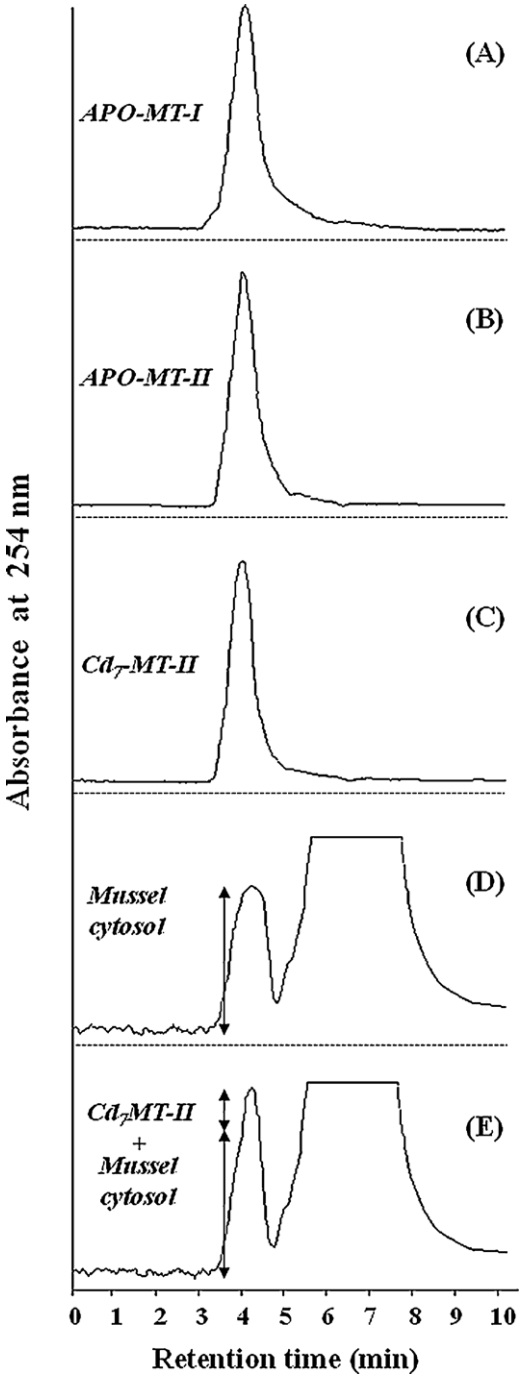

Fig. 1. Chromatographic separation of MTs. Chromatograms of (A) Apo-MT-I; (B) Apo-MT-II; (C) Cd_7-MT-II; (D) mussel cytosol; (E) mussel cytosol spiked with 0.2 μg g^{-1} of Cd_7-MT-II.

3.4. Behavior of MTs under sonication

Though ultrasound energy could potentially cause chemical and conformational changes in proteins, in most cases only conformational changes have been observed. Ultrasound energy can break hydrogen bonding, Van der Waals interactions and it can oxidize the SH-group [24]. The magnitude of these modifications is closely related to protein structure [25] and, to a lesser extent, to molecular weight [26].

Anyway, partial denaturation of proteins can make proteins more susceptible to other factors such as oxidizers and temperature [24]. Therefore, the selected medium for cytosol preparation

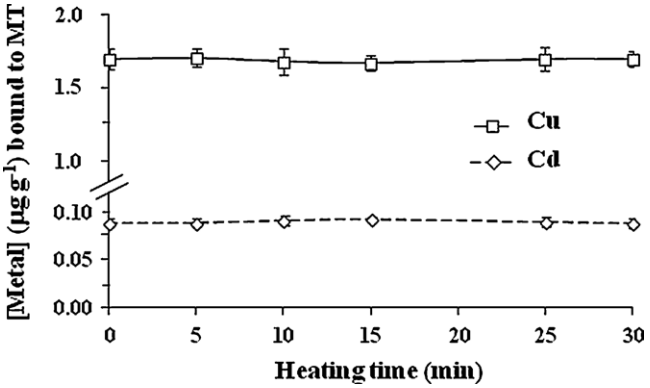

Fig. 2. Effect of heating time on the metal concentration bound to MT.

(including an antioxidant, 2-MEC) and the conditions for sonication (also affecting temperature reached) are considered key features to prevent MTs from alterations.

To the best of our knowledge, no information about MT stability under US treatment has been published. No problems have been mentioned in relation to this subject by other authors [14]. Thus, a careful evaluation of possible denaturation of MTs and its repercussion on the determination of metal bound to MTs was carried out. For this purpose, Apo-MT-I, Apo-MT-II and Cd_7-MT-II solutions prepared in the extraction media (Tris–HCl buffer 10 mM pH 7.4, NaCl 25 mM, PMSF 0.01 mM and 2-MEC 5 mM) were subjected to sonication. Different times (up to 15 min) and extreme conditions of amplitude (100%) were applied. No chromatographic differences were observed for these proteins. In addition, Cd from Cd_7-MT-II was determined by ICP-MS in order to ascertain the strength of the metal-MT binding under sonication. Recovery results (93.3 ± 2.9%) were calculated considering the theoretical content of Cd in the protein.

3.5. Elimination of the heating step

Metallothioneins are thermally stable proteins, so a heating step (usually at 60–70 °C for 15 min) is commonly carried out in conventional procedures in order to eliminate proteins of high molecular mass, which suffer thermal denaturalization [7,27,28].

Propagation of ultrasound energy through a liquid medium causes some heating. Inside the cup-horn sonoreactor, Eppendorf vials are placed in a water bath, which may reach up to 65 °C after short sonication times. Thus, the additional heating step in a water bath after sonication could be removed. To verify it, two procedures were compared: sonication with an additional heating stage (using different heating times) and sonication without heating step. Results for Cu and Cd bound to MTs are shown in Fig. 2. As can be observed, the procedure without heating step can be successfully applied, which in turn allows the removal of centrifugation.

3.6. Optimization of the ultrasound-assisted cytosol preparation procedure

Variables influencing the procedure of ultrasound-assisted cytosol preparation were studied: Tris–HCl buffer concentration, pH, sample mass, sonication time and sonication amplitude.

Concentration of Cu and Cd bound to MTs remained constant for all studied pH values (between 7.4 and 8.2) and from a concentration of Tris–HCl 5 mM in the range 0–100 mM. Thus, a concentration 10 mM of Tris–HCl and a pH 7.4 were selected.

Generally, sample mass is a critical variable in solid–liquid extractions in order to achieve quantitative extractions [29]. In this case, the buffer volume was fixed at 1 mL and the sample mass

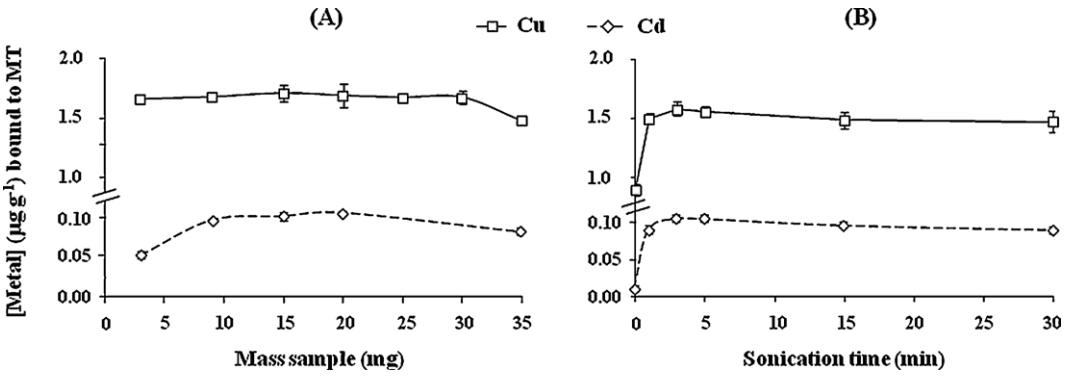

Fig. 3. Optimization of conditions for ultrasound-assisted cytosol preparation: (A) effect of mass sample and (B) effect of sonication time.

was varied from 3 to 35 mg. As can be seen in Fig. 3A, metal concentration bound to MTs remains constant until 30 mg for Cu and 20 mg for Cd. When higher sample amounts are treated, a decrease in metal bound to MTs is observed. A mass of 20 mg was selected as a compromise for simultaneous determination of both metals.

The concentration of Cu and Cd bound to MTs was significantly increased with the application of ultrasound from 0 to 1 min (Fig. 3B). For higher sonication times, Cu and Cd concentrations remained constant. The selected sonication time was fixed at 3 min. Sonication amplitude had a little influence in the process. A 60% sonication amplitude was selected for both metals.

3.7. Analytical characteristics

Analytical characteristics of the procedure for both the determination of Cd and Cu bound to MTs and total Cd and Cu, are shown in Table 2. Procedural limits of detection (LODs) and quantification (LOQs) were calculated considering instrumental limits and the sample treatment (instrumental limits of detection and quantification were calculated following 3σ and 10σ criteria, respectively). Precision, expressed as relative standard deviation (RSD, %), was evaluated in terms of repeatability.

Reference materials for validation of the proposed method were not available, and therefore, accuracy of ultrasound assisted cytosol preparation procedure for determination of Cd and Cu bound to MTs was tested by means of recovery studies with the commercial protein Cd_7-MT-II and by comparison with the cytosol preparation procedure described in Section 2.4. No significant differences at the 95% confidence level were observed.

Accuracy of the procedure for Cd and Cu total was checked with CRMs ERM CE-278 and SRM 2976. No statistical differences were observed when certified and found values were compared.

The proposed methodology for determining Cu and Cd bound to MTs allows simplifying and shortening the conventional cytosol preparation procedure. Three steps prior to chromatographic separation, i.e. (i) sonication, (ii) centrifugation and (iii) filtration, were enough for this purpose, only a time of 9 min being spent, in contrast to conventional procedures that involve more steps and treatment times of more than 2 h.

3.8. Determination of Cd and Cu bound to MTs and total Cd and Cu in Galician mussels

Cd and Cu bound to MTs and total Cd and Cu were determined in Galician mussels (*Mytilus galloprovincialis*). Galicia is located in the far North-West of Spain and it is one of the largest producers of mussels in the world. These mussels are cultivated in the coastal inlets (called rías) by means of floating rafts. Sampling was carried out in two Galician rías: Pontevedra and Ares-Betanzos. These rías show different geomorphology, lithology, food availability and pollution sources. Pontevedra Ría can be affected by an industrial complex (with a chlor-alkali plant and a paper mill), an important shipyard and some urban and industrial sewage discharges. In the case of Ares-Betanzos, industry activity is scanty and only there are small villages and fishing ports. Higher values of Cd have been found in mussels from the Pontevedra Ría than in those from the Ares-Betanzos Ría [30]. The analytical results are shown in Table 3.

Table 2
Analytical characteristics and validation results.

Procedure/element	LOD ($ng\,g^{-1}$)	LOQ ($ng\,g^{-1}$)	RSD (%)	Sample	Reference value ($\mu g\,g^{-1}$)	Proposed method ($\mu g\,g^{-1}$)[e]	Recovery (%)
Ultrasound-assisted cytosol preparation procedure for determination of metal bound to MTs							
Cd	0.17	0.58	6.5	Mussel tissue	0.21 ± 0.02[a]	0.20 ± 0.02	95.2
				Mussel tissue + Cd_7-MT-II	0.42 ± 0.04[b]	0.39 ± 0.10	92.8
Cu	4.93	16.42	7.3	Mussel tissue	2.50 ± 0.16[a]	2.45 ± 0.12	98.2
Ultrasound-assisted acid extraction procedure for total Cd and Cu determination							
Total Cd	0.10	0.32	5.0	ERM-CE 278	0.348 ± 0.007[c]	0.324 ± 0.009	93.1
				SRM 2976	0.82 ± 0.16[d]	0.780 ± 0.006	94.4
Total Cu	2.74	9.12	6.4	ERM-CE 278	9.45 ± 0.13[c]	8.74 ± 0.12	92.5
				SRM 2976	4.02 ± 0.33[d]	3.75 ± 0.18	93.2

[a] Reference value obtained with the comparative cytosol preparation procedure described in Section 2.4. Experimental results expressed as average value ± standard deviation ($N = 3$) obtained with the proposed method.
[b] Reference value considering the obtained with the comparative procedure and the addition of 0.2 $\mu g\,g^{-1}$ of Cd from Cd_7-MT-II. Experimental results expressed as average value ± standard deviation ($N = 3$) obtained with the proposed method.
[c] Certified values corresponding to ERM-CE 278. Average value ± confidence interval.
[d] Certified values corresponding to SRM 2976. Average value ± confidence interval.
[e] Experimental results expressed as average value ± standard deviation ($N = 3$) obtained with the proposed method.

Table 3
Cd and Cu concentrations in mussels from Galician Rías as total and metal bound to MTs ($\mu g\,g^{-1}$, dry weight).

Samples	Total Cd	Cd bound MTs	% of Cd bound MTs[a]	Total Cu	Cu bound MTs	% of Cu bound MTs[b]
Pontevedra Ría						
P1	0.82 ± 0.01	0.019 ± 0.002	2	3.13 ± 0.06	1.4 ± 0.1	45
P2	0.90 ± 0.01	0.063 ± 0.005	7	2.7 ± 0.1	0.8 ± 0.1	30
P3	1.233 ± 0.008	0.029 ± 0.004	2	2.85 ± 0.09	0.9 ± 0.1	32
P4	1.13 ± 0.01	0.057 ± 0.002	5	2.5 ± 0.2	0.8 ± 0.1	32
P5	1.83 ± 0.01	0.124 ± 0.002	7	3.21 ± 0.07	0.9 ± 0.1	28
P6	1.605 ± 0.007	0.074 ± 0.007	4	3.8 ± 0.1	0.8 ± 0.1	21
P7	1.932 ± 0.004	0.153 ± 0.008	8	2.7 ± 0.1	0.8 ± 0.1	30
P8	2.040 ± 0.005	0.134 ± 0.006	6	3.8 ± 0.2	0.9 ± 0.1	24
P9	0.494 ± 0.004	0.035 ± 0.003	7	3.8 ± 0.1	1.0 ± 0.1	26
P10	0.926 ± 0.007	0.088 ± 0.004	9	3.69 ± 0.07	0.8 ± 0.1	22
Ares-Betanzos Ría						
A1	0.316 ± 0.006	<LOD	–	3.85 ± 0.07	1.52 ± 0.08	39
A2	0.343 ± 0.008	<LOD	–	3.6 ± 0.1	1.2 ± 0.1	33
A3	0.351 ± 0.007	0.013 ± 0.002	4	3.8 ± 0.12	1.2 ± 0.1	32
A4	0.23 ± 0.01	<LOD	–	3.7 ± 0.1	1.1 ± 0.1	30
A5	0.232 ± 0.009	<LOD	–	3.3 ± 0.1	1.1 ± 0.1	33
A6	0.297 ± 0.008	<LOD	–	3.5 ± 0.1	1.2 ± 0.1	34
A7	0.205 ± 0.005	0.014 ± 0.002	7	3.3 ± 0.1	1.0 ± 0.1	30
A8	0.213 ± 0.009	0.023 ± 0.003	11	4.1 ± 0.1	1.5 ± 0.1	37

Results are expressed as average values ± standard deviation, $N = 3$.
[a] Percentages of Cd bound to MTs with regard to total Cd present in samples.
[b] Percentages of Cu bound to MTs with regard to total Cu present in samples.

Total Cd and Cu found in mussels reveal no contamination. Cd bound to MTs was lower than 11% of the total Cd present in the samples, and it was not detected in almost all samples from the Ares-Betanzos Ría. Percentages of Cu bound to MTs were from 21 to 43% in relation with the total Cu present in the mussels. Cu bound to MTs, unlike Cd bound to MTs, constituted a significant fraction of the total metal. It can be due to the differences in the utilization of MTs for metabolism of both essential and toxic metals [31]. It is generally considered that MTs can act as essential metal stores to fulfill enzymatic and other metabolic demands [2].

4. Conclusions

The proposed method for the determination of Cd and Cu bound to MTs by ICP-MS in mussel tissue is advantageous in comparison with conventional procedures. The indirect sonication with a cup-horn sonoreactor allows simplifying and miniaturizing the cytosol preparation procedure. In special, it allows eliminating some steps as the heating and some centrifugations included in conventional procedures. In addition, it entails the enhancement of sample throughput since six samples can be simultaneously processed. No problems related with the denaturation of MTs under sonication were observed. Low amounts of reagents and samples are used. The combination of the simplified procedures for determining Cd and Cu bound to MTs and total Cd and Cu concentrations by ICP-MS could encourage many environmental studies for assessment of metal pollution.

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