Blending Procedure for the Cytosolic Preparation of Mussel Samples for AAS Determination of Cd, Cr, Cu, Pb, and Zn Bound to Low Molecular Weight Compounds

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ABSTRACT

Simple methods for the determination of Cd, Cr, Cu, Pb, and Zn fractions bound to low molecular weight compounds (presumably metallothionein-like proteins) in mussels (Mytilus edulis) have been developed. The methods involve the novel application of using a blending (Stomacher) rather than a cutting mixer device to perform the homogenization between the fresh mussel tissue and the extractant solution [10 mM TRIS–HCl buffer at pH 7.4 with 5 mM 2–mercaptoethanol (2–MCE), 0.01 mM phenylmethylsulfonyl fluoride (PMSF), and 25 mM sodium chloride]. Optimum conditions for the cytosolic preparation (after an experimental design approach) were a ratio of sample weight : extracting volume of 0.2 g/mL and a blending time of 5 min. The Cu and Zn concentrations bound to low molecular weight compounds (LMW proteins) were measured by flame atomic absorption spectrometry using a high performance nebulizer, while Cd, Cr, and Pb levels in the cytosolic solutions were assessed by electrothermal atomic absorption spectrometry. A microwave-assisted acid digestion procedure was also applied to determine the total metal concentration. The limits of detection (LOD) for Cd, Cr, and Pb were 0.7, 3.0, and 6.6 ng g^{-1}, respectively, and for Cu and Zn they were 0.05 and 0.01 µg g^{-1}, respectively. The methods were applied to 16 mussel samples from the Ría de Arousa estuary.

INTRODUCTION

Metallothionein-like proteins (MT-like proteins) are low molecular weight proteins that have a high cystein content (around ±30% of the residues) and a strong affinity for heavy metals (1). These proteins play an important role in the metabolism and kinetics of Cd and Cu, and regulate the toxicity of various metals and trace elements. Metallothioneins (MTs) and MT-like proteins have been described for various foodstuffs, mainly fish and mollusks (1). It appears that in fish, MTs should be considered as a kind of stress protein that is particularly responsive to heavy metals. In mollusks, MTs seem more involved in responses to heavy metals and they can be considered as biomarkers of exposure to heavy metal contamination (2).

From the studies performed on MTs in mollusks and the results available in the literature (3), it can be said that several physiological and biochemical parameters might affect the concentration and isolation of MTs from mollusk tissue. Significant variations in isolation and quantification of MTs might depend on the purification and storage protocol and, as described by Isani et al. (4), the development of a standard protocol for MT isolation and quantification is still an important goal to be achieved. Although there is no standard method, it is well known that denaturation of the samples at low temperatures, instead of the solvent precipitation process, allows the isolation of MTs from other high molecular weight proteins without MT alteration (5,6). However, some works have reported important changes on metal distribution in MT-like protein isoforms due to heating treatments (7). Regarding the cytosol isolation process, Wolf et al. (8) have compared several homogenization procedures (cutting mixers, rotating pestles, and sonication devices) in terms of functionality. These authors concluded that sonication in comparison to pestles and cutting devices [Ultra–Turrax (9,10)] offers the best performance because there is no risk of contamination or formation of foam.

As recently reported by Prange and Schaumlöffel (3), several speciation studies have been performed to determine trace elements in MTs from rabbit (11,12), rat (13), and human (7) livers, as well as from mollusks (9,10,13,14,15). Hyphenated techniques with inductively coupled plasma mass spectrometry (ICP–MS) as a multi-element detector are used throughout these studies. Therefore, different modalities of high performance chromatography (HPLC), such as size exclusion chromatography (SEC) and capillary electrophoresis (3) were used to isolate different MTs and even different MT isoforms. These hyphenated techniques are, most of the time, expensive and not available in many laboratories.

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Therefore, the aim of this work was to develop simple atomic absorption spectrometric methods to determine the fraction of metals (Cd, Cr, Cu, Pb, and Zn) bound to MT–like proteins in mussels (*Mytilus Edulis*). Since a prior chromatographic stage is omitted, the term low molecular weight (LMW) compounds will be used rather than MT-like proteins. Additionally, a novel procedure for the homogenization of the mussel samples has been optimized by the use of a blending system (Stomacher) where the samples are contained within sterile metal-free disposable bags, thus eliminating instrument contamination, and achieving homogenization by blending.

EXPERIMENTAL

Instrumentation

A PerkinElmer Model 1100B atomic absorption spectrometer (PerkinElmer Life and Analytical Sciences, Shelton, CT, USA), equipped with an HGA®-400 graphite furnace and an AS-40 autosampler. Deuterium lamp background correction was used for Cd, Cr, and Pb measurements. A PerkinElmer Model 3100 atomic absorption/emission spectrometer with a high-performance nebulizer equipped with ceramic impact beads (PerkinElmer) was used for the Cu and Zn determinations. Background correction was found unnecessary for FAAS determinations.

Cd and Zn (PerkinElmer) and Cr and Cu (Cathadeon, Cambridge, U.K.) hollow cathode lamps, and a Pb electrodeless discharge lamp (PerkinElmer) connected to a power supply (PerkinElmer) were used as the radiation sources. The lamps were operated at the lamp currents/lamp voltages and with slit widths and wavelengths as recommended by the manufacturer. A Stomacher Lab Blender 400 (Seward Med. Ltd., London, UK) was used to blend and homogenize the fresh mussel samples using Stomacher sample bags (Part No. 6041/CLR). Centrifuge Centromix (Selecta, Barcelona, Spain), refrigerated centrifuge Laborzentrifugen 2K15 (SIGMA, Osterode, Germany), and ORION 720A plus pH meter with a glass calomel electrode (ORION, Cambridge, UK) were also used. A Samsung domestic microwave oven (Seoul, Korea), programmable for time and microwave power, was used for total sample digestion. The poly(tetra–fluoroethylene) (PTFE) bombs were laboratory-made with hermetic seals (suitable for work at low pressures). The chemometrics packages used were UNSCRAMBLER, 1998 (CAMO ASA, Trondheim, Norway) and Statgraphics Plus V 5.0 for Windows® OS, 1994-1999 (Manugistics Inc., Rockville, MD, USA).

Reagents and Standards

The chemicals were of ultrapure grade, using ultrapure water, resistance 18 MΩ cm (Millipore Co., Bedford, MA, USA). Stock standard solutions (1000 g L^{-1}) were obtained from Merck, Poole, Dorset, UK. Palladium nitrate stock standard solution, 10,000 g L^{-1} (from PerkinElmer). Magnesium nitrate stock standard solution was prepared from magnesium nitrate (from BDH, Poole, Dorset, UK). Hydrogen peroxide 33% (from Panreac, Barcelona, Spain). Nitric acid 70.0% and hydrochloric acid 37% (from J.T. Baker B.V., Deventer, Holland). The extracting solution was prepared with TRIS–hydroxymethyl–aminomethane (Riedel de Haën, Sigma-Aldrich, Stemheim, Switzerland), 2–mercaptoethanol (2–MCE) (Fluka, Sigma-Aldrich), phenylmethylsulfonyl fluoride (PMSF) (Fluka, Sigma-Aldrich), and sodium chloride (Merck, Darmstadt, Germany).

Mussel Samples

Fresh mussel samples were collected from mussel farms at Ría de Arousa estuary (Galicia, Northwest Spain). The mussels were analyzed as wet samples for total metal and fractions bound to low molecular weight compounds. All studies were performed using all mussel tissue (muscle and gill) by preparing a mussel pool mixture with all the mussels from each mussel farm.

Methods

Procedure for Cytosol Preparation

Cytosolic extracts were obtained based on a modified protocol of the methods reported by Roesijadi and Fowler (10) and Lobinski et al. (14). The extracting solution was prepared with TRIS-HCl at 10 mM (pH 7.4), 0.01 mM phenylmethylsulfonyl fluoride (PMSF), 5 mM 2–mercaptoethanol (2–MEC), and 25 mM NaCl. The homogenization and blending procedure was as follows: fresh mussel samples were accurately weighed (~5 g) into the Stomacher bag and 25 mL of extracting solution was added. The mixture was subjected to blending at high speed for 5 min, and was then transferred to a centrifuge tube and centrifuged at 3500 rpm for 30 min. The solid precipitated was separated by decantation and the liquid extract was transferred into a second centrifuge tube and warmed in a water bath at 60°C for 15 min. Posterior centrifugation at 4°C and at 19,890 g (15,300 rpm) for 30 min was carried out to separate the tertiary structure proteins which suffered denaturization at high temperature. Cytosols, obtained by decantation, were diluted to 25 mL with ultrapure water.

Microwave Acid Digestion

Acid digestion was carried out in a domestic microwave oven with laboratory-made low pressure PTFE bombs. The optimum conditions for the digestion of wet mussel

samples were based on a modified method for dry mussels (16), and it can be summarized as follows: Approximately 0.7 g of fresh mussel sample was weighed into the PTFE bombs and 2 mL of concentrated HNO_3 was added. The samples were subjected to two microwave heatings (100 W) for 2 min with a cooling stage (ice–bath for 10 min) in between. The low microwave power was required to avoid losses because low pressure PTFE bombs were used. Then, 1 mL of 33% (m/v) H_2O_2 was added and the samples were again subjected twice to microwave irradiation at 200 W for 2 min, also inserting a cooling stage. Finally, the acid digests were made up to 10 mL with ultrapure water and stored in polyethylene bottles at 4°C. This methodology was validated after analyzing different certified reference materials (DORM–1, DOLT–1 and TORT–1) as reported in References 16–18.

ETAAS and FAAS Measurements

Cu and Zn were determined by FAAS (oxidizing air/acetylene flame) while Cd, Cr, and Pb determinations were carried out by electrothermal atomic absorption spectrometry (ETAAS) under optimum conditions (see Table I). Chemical modification was necessary for Cd and Pb determinations and magnesium nitrate / palladium nitrate of 0.6/0.6 µg and 0.9/2.2 µg were found to be optimum for Cd and Pb, respectively. These chemical reagents were previously mixed with the samples and standard solutions before ETAAS measurements. Aqueous calibration was possible only for Cr determination by ETAAS. The use of the standard addition technique was required for Cu and Zn measurements by FAAS and for Cd and Pb determination by ETAAS.

TABLE I
Operating Conditions and Graphite Furnace Temperature Programs[a] for the ETAAS Determination of Cd, Cr, and Pb Bound to LMW Proteins

	Cd	Cr	Pb
Wavelength (nm)	228.8	357.9	283.3
Slit width (nm)	0.7	0.7	0.7
Lamp current (mA)	5	12	10[b]
Injection volume (µL)	20	20	20

	Step	Temp. (°C)	Ramp (s)	Hold (s)	Ar Flow Rrate (mL min^{-1})
Cd[c]	Drying	150	20	20	300
	Pyrolysis	500	15	25	300
	Atomization	1600	0	4	0 (Read)
	Cleaning	2200	1	3	300
Cr	Drying	150	20	20	300
	Pyrolysis	1400	10	15	300
	Atomization	2400	0	5	0 (Read)
	Cleaning	2650	1	2	300
Pb[c]	Drying	150	15	20	300
	Pyrolysis	800	20	10	300
	Atomization	1400	0	3	0 (Read)
	Cleaning	2200	2	3	300

[a] Pyrolytically coated graphite tubes with L'vov platforms, injection volume of 20 µL, integrated absorbance measurement.
[b] Power supply, 10 W.
[c] $Mg(NO_3)_2$ / $Pd(NO_3)_2$ as chemical modifier (0.6/0.6 µg for Cd and 0.9/2.2 µg for Pb).

RESULTS AND DISCUSSION

Optimization of the Cytosol Preparation

Optimization of the LMW protein extraction from mussel samples using the Stomacher blending device was carried out by using an experimental design approach. Variables such as pH and 2–mercaptoethanol (MEC) and phenylmethylsulfonyl fluoride (PMSF) concentrations were not considered in the study. A pH of 7.4 was required to extract MT-like proteins and 2-MEC and PMSF were used only to prevent MT-like protein degradation. Therefore, the variables under study were blending (extracting) time (T) and the ratio of sample weight : extracting volume (w/v). Optimization of these two variables results in reduced time and sample weight required for the analysis. A central composite design, 2^2 + star, involving 18 experiments was used for optimization. Blending time ranged from 7.5 to 25 min and the ratio of sample weight : extracting volume varied from 0.5 to 2.0. The response variables were the metal concentrations (Cd, Cr, Cu, Pb, and Zn) in each extract, determined by using an aqueous calibration or a standard addition graph and under optimum conditions. Details of the central composite design matrix (cube and star experiments) are given in Table II, as well as the response variables (concentrations of metal associated to MTs) for

TABLE II. 2^2 + Star Central Composite Design Matrix

Run	Time (min)	W/V (g mL^{-1})	Response Variables Cd (µg g^{-1})	Cr (µg g^{-1})	Cu (µg g^{-1})	Pb (µg g^{-1})	Zn (µg g^{-1})
1	3.88	1.25	0.029	0.031	0.171	0.009	1.69
2	3.88	1.25	0.023	0.029	0.173	0.010	2.27
3	28.62	1.25	0.031	0.027	0.143	0.010	5.64
4	28.62	1.25	0.033	0.029	0.117	0.009	2.87
5	16.25	0.19	0.057	0.035	0.348	0.047	3.99
6	16.25	0.19	0.069	0.035	0.348	0.044	2.86
7	16.25	2.31	0.015	0.015	0.086	0.007	1.27
8	16.25	2.31	0.014	0.022	0.085	0.009	0.83
9	7.50	0.50	0.035	0.031	0.289	0.023	6.25
10	7.50	0.50	0.037	0.034	0.258	0.018	2.96
11	25.00	0.50	0.031	0.028	0.200	0.007	2.66
12	25.00	0.50	0.030	0.034	0.200	0.008	3.24
13	7.50	2.00	0.024	0.028	0.114	0.004	2.39
14	7.50	2.00	0.017	0.019	0.086	0.006	1.55
15	25.00	2.00	0.030	0.018	0.087	0.006	2.00
16	25.00	2.00	0.032	0.017	0.087	0.006	1.85
17	16.25	1.25	0.031	0.020	0.115	0.006	2.83
18	16.25	1.25	0.036	0.018	0.116	0.006	2.15

TABLE III. Typical ANOVA Table for Cr Obtained by Response Surface Analysis

	Sum of Squares	Degree of Freedom	Main Squares	F–Ratio	p–Value
Summary					
Model	0.667	5	0.134	15.86	0.0001 [a]
Error	0.101	12	0.008		
Adjusted total	0.768	17	0.0452		
Variables					
Intercept	0.739	1	0.739	87.78	0.000 [a]
Time (A)	0.0203	1	0.0203	2.41	0.146
W/V (B)	0.522	1	0.522	61.98	0.000 [a]
AB	0.009	1	0.009	1.076	0.320
AA	0.114	1	0.114	13.51	0.003 [a]
BB	0.065	1	0.065	7.671	0.017 [a]
Model Check					
Main	0.542	2	0.271		
Interaction	0.009	1	0.009	1.076	0.302
Interaction + squares	0.116	2	0.058	6.908	0.010 [a]
Squares	0.116	2	0.058	6.098	0.010 [a]
Error	0.101	12	0.008		
Lack of Fit					
Lack of fit	0.0124	3	0.004	0.421	0.743
Pure error	0.089	9	0.010		
Total error	0.101	12	0.008		

Multiple correlation: 0.932 R–square: 0.869

[a] Statistically significant at a confidence level of 95.0%.

each experiment. It must be noted that after each experiment, the same procedure described before was performed (two centrifugation steps and a heating period in between).

After studying the ANOVA tables for each response variable (i.e., Table III for Cr), both variables (blending time and ratio of sample weight : extracting volume) were found to be independent and the effect of one does not produce variations in the other. In addition, it can be seen that the variable blending time is not statistically significant. Figure 1 shows the response surfaces achieved for each metal. It can be seen that blending time was found to be insignificant as long as it was kept between approximately 4 to 10 min for all elements; 5 min was chosen for further analysis. On the other hand, an optimum ratio of sample weight : extracting volume of 0.19 g mL^{-1} was found for Cd and Pb, and from 0.19 to 0.28 g mL^{-1} for Zn, and from 0.19 to 0.36 g mL^{-1} for Cu and Cr. In order to find compromise extracting conditions, a w/v ratio of 0.2 g mL^{-1} was chosen and was employed for further analysis. This w/v ratio means that 5 g of mussel sample and 25 mL of extracting solution was necessary. The optimum ratio obtained was quite different than previously reported by other authors (9,10,11,14), who used a ratio of 1 g mL^{-1} (10 g of mussel sample and 10 mL of extracting solution).

Comparison to Other Cytosol Preparations

The optimized blending procedure and the procedure described by Lobinski et al. (14), which uses a trituration device under recommended conditions, was applied three times to a mussel sample. Blank controls were performed for both cytosol preparation procedures and the Cd, Cu, Cr, and Zn concentrations were determined under optimum operating condi-

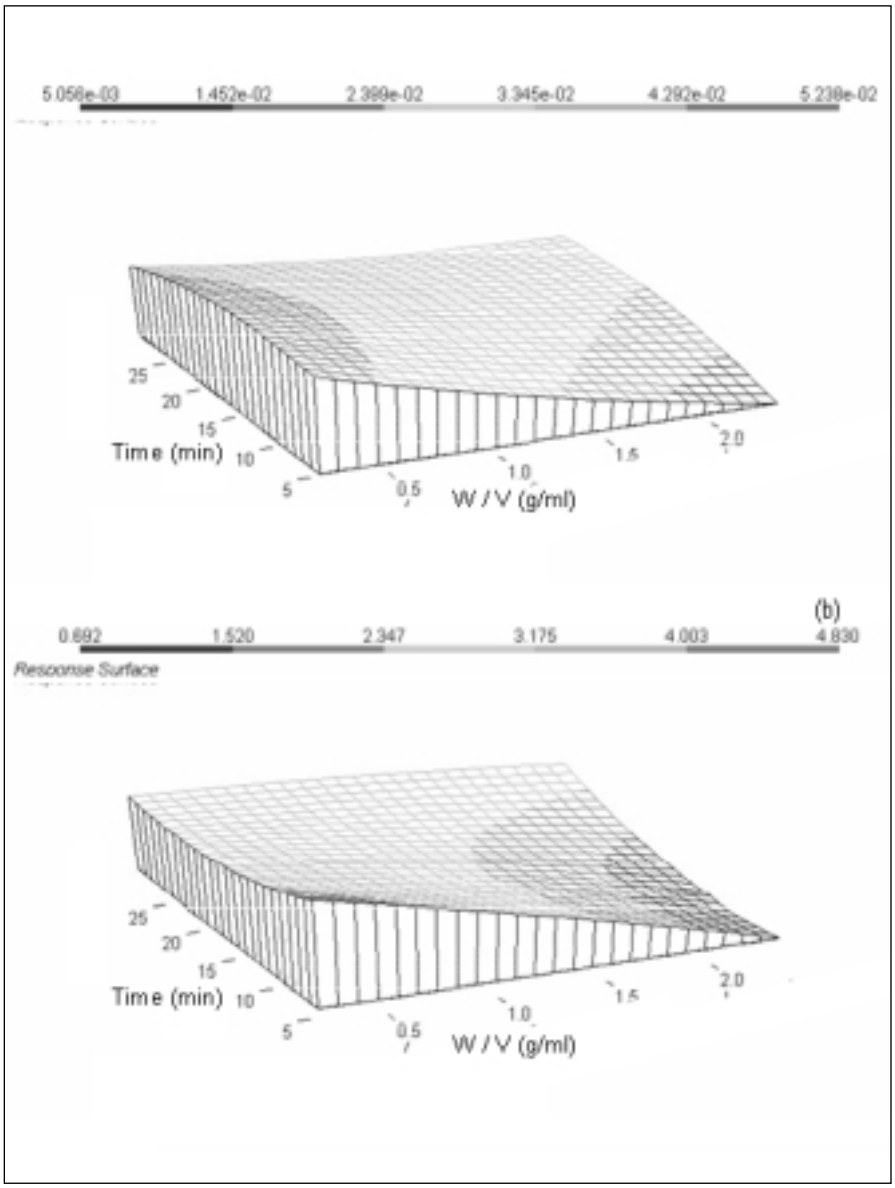

Fig. 1. Some estimated response surfaces from the central composite design in the extraction of LMW-compounds from mussels: (a) cadmium and (b) zinc.

TABLE IV
Results of Cd, Cu, Cr, and Zn Determination After Stomacher Blending and OMNI Mixer Trituration Cytosol Preparation Procedures (µg g^{-1})

Element	Stomacher		OMNI Mixer	
	Mussel	Blank[a]	Mussel	Blank[a]
Cd (µg g^{-1})	0.02 ± 0.01	0.0030 ± 0.0001	0.02 ± 0.01	0.0040 ± 0.0001
Cu (µg g^{-1})	0.10 ± 0.01	0.0010 ± 0.0001	0.12 ± 0.01	0.0010 ± 0.0001
Cr (µg g^{-1})	0.03 ± 0.01	0.0050 ± 0.0002	0.16 ± 0.01	0.071 ± 0.016
Zn (µg g^{-1})	2.73 ± 0.28	0.0080 ± 0.0002	2.58 ± 0.17	0.0070 ± 0.0003

[a] Absorbance units.

tions. Table IV lists the mean and standard deviations obtained for each case. It can be seen that the concentration of metals bound to LMW compounds reached by both procedures is similar, although random Cr contamination was found when using the OMNI Mixer trituration device. This contamination was also found after determining high Cr levels in the blank solutions. After applying an ANOVA test (95% confidence level), it can be concluded that the efficiency of the Stomacher blending procedure for mussel cytosol preparation is comparable to the efficiency obtained using the OMNI Mixer trituration procedure, with the additional advantage of avoiding Cr contamination.

Analytical Performance

A statistical comparison between the slopes of the aqueous calibration and the standard addition graphs was made. Both calibration techniques were performed seven times on different days, and the results are listed in Table V. The differences between the slopes was studied by employing the ANOVA test to assess whether there are statistically significant differences for the standard deviation of each group (aqueous calibration and standard addition graphs) according to the Barlett and Cochran tests (19). The results show that the slopes of the aqueous calibration and standard addition graphs are statistically different for Cd and Pb determination by ETAAS, and for Cu and Zn measurement by FAAS. There were no statistically significant differences in the ETAAS determination of Cr. Therefore, matrix effects are important for Cd, Cu, Pb, and Zn determination, and the standard addition technique was used for further experiments. The aqueous calibration technique will be used for Cr.

The limit of detection (LOD), limit of quantification (LOQ), and

the characteristic mass (20) were established. LOD values of 0.7, 3.0, and 6.6 ng g^{-1} were obtained for Cd, Cr, and Pb, respectively, with characteristic masses of 1.1 ± 0.2, 5.7 ± 0.2, and 34.1 ± 1.6 pg, respectively. The LOD values for Cu and Zn were 0.05 and 0.01 µg g^{-1}, respectively.

The within-run precision for 11 injections of LMW compound extracts and expressed as %RSD was 1.7, 3.1, and 1.5 % for Cd, Cr, and Pb, respectively. The values obtained for Cu and Zn by FAAS with regard to 11 subsequent measurements was 0.2% for both cases. In addition, the repeatability of the overall procedure was assessed by preparing a cytosolic extract seven times from the same mussel sample and analyzing each sample twice. The RSD values of 6.1, 7.0, 9.1, 7.8, and 6.3% were obtained for Cd, Cr, Cu, Pb, and Zn, respectively.

TABLE V
Aqueous Calibration and Standard Addition Slopes
(Expressed as mean ± standard deviation for n = 7)

	Slopes (µg L^{-1})	
	Standard Addition	Aqueous Calibration
Cd	0.053 ± 0.011	0.039 ± 0.001
Cr	0.0190 ± 0.004	0.0201 ± 0.003
Cu[a]	0.151 ± 0.008	0.199 ± 0.002
Pb	0.0043 ± 0.0003	0.0020 ± 0.0003
Zn[a]	0.472 ± 0.026	0.805 ± 0.024

[a] (mg L^{-1}).

TABLE VI
Total Metal and Metal-Bound LMW Compounds
(Concentration ranges in mussel samples.)

	Mussels (16 Samples)		
	Total Concentration Range (µg g^{-1}) Wet Weight of Tissue	Metal-bound LMW Compounds Range (µg g^{-1}) Wet Weight of Tissue	Metal-bound LMW Compounds Range (%)
Cd	0.069 – 0.122	0.010 – 0.036	10 – 39
Cr	0.075 – 0.294	0.010 – 0.029	8 – 24
Cu	0.507 – 0.940	0.179 – 0.370	24 – 56
Pb	0.034 – 0.730	0.020 – 0.075	4 – 56
Zn	7.0 – 15.7	1.5 – 3.4	15 – 46

Application

Sixteen mussel samples from the Ría de Arousa estuary were analyzed for the elements Cd, Cr, Cu, Pb, and Zn bound to LMW compounds. A mixture was made with mussel samples selected from each sampling location. Five g of each mixture was subjected (in duplicate) to the LMW compound extraction procedure, and each cytosolic extract was measured by ETAAS or FAAS. Additionally, mussel mixture from each sampling location were subjected (in duplicate) to a microwave acid digestion procedure to assess the total metal concentrations. Table VI shows the (%) metal concentration ranges for total metal concentration and metals bound to the LMW compound in the 16 mussels. It can be seen that Cd, Cu, and Zn are the metals with high percentages bound to LMW compounds, slightly above 10%. The maximum yield was up to 56% for Cu and Pb, while values of 46%, 39%, and 24% were reached for Zn, Cd, and Cr, respectively. It should be noted that the highest percentages of Cr, Cu, Pb, and Zn bound to LMW compounds were found in mussel samples from sampling location 12, (while the highest Cd percentages of 39% and 3% were for mussel samples from locations 24 and 12, respectively. Supplementary studies about metal concentrations in seawater and marine sediments from each of the sampling locations would be of interest in order to confirm possible metal stress in those areas.

CONCLUSION

The novel use of a blending system (Stomacher) to prepare cytosolic extracts from fresh mussel tissue offers important advantages over classical devices which are based on the cutting process. The proposed form of homogenization avoids metal contamination normally found to be significant with other homogenization systems. The proposed method also allows a fast LMW metal compound extraction from mussel tissue in which the homogenization process is complete within 5 min. The fractions of metals, such as Cd, Cu, Cr, Pb and Zn, associated to LMW compounds in mussels can be measured under the optimized ETAAS and FAAS, resulting in simpler and less expensive methods than those based on hyphenated techniques (such as HPLC-ICP-MS). The limits of detection (LOD) for Cd, Cr, and Pb were 0.7, 3.0, and 6.6 ng g^{-1}, respectively, and for Cu and Zn they were 0.05 and 0.01 µg g^{-1}, respectively. The proposed methods are sensitive and precise (RSDs lower than 10% for repeatability), and they can be applied as a first approach to measure the total concentration of metals bound to LMW compounds.

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