Direct determination of Cd in NaCl containing metallothionein fractions of different red mullet tissues by GF-AAS

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Determination of metal concentration in the same fraction of fish tissues in which metallothioneins (MTs) are measured (heat-treated cytosol, S50) is of great importance for correlating MT level with levels of metals that induce it (Zn, Cu, Cd). Separation of MTs from other proteins comprises tenfold dilution of S50 cytosol with 0.9% NaCl solution, and the subsequent heat-treatment. Presence of salt interferes with Cd measurement by graphite furnace AAS. EDTA was, thus, used as a modifier that allows atomisation of Cd at lower temperatures, and consequently efficiently separates Cd signal from NaCl background signal. Method employing atomisation temperature of 900°C, and addition of EDTA solution adjusted to pH ~7.0 was validated, showing that reliable Cd measurements are performed in both Cd standard solutions containing 0.9% NaCl, and in the heat-treated cytosol of red mullet tissues, despite the fact that these samples contain a substantial amount of MTs.

Introduction

Metallothioneins (MTs) are cytosolic proteins inducible by Cd, and used as biomarkers of Cd exposure[1]. Isolation of heat-stable MTs from other cytosolic proteins comprises a step of heat-treatment of S50 cytosol diluted 10 times with 0.9% NaCl solution[2]. In order to assess the biological effect of Cd by means of MTs in aquatic organisms it is necessary to measure the concentrations of Cd and MTs in the same heat-treated cytosolic fraction. In the heat-treated cytosol of fish tissues cadmium concentration is below the detection limit of flame AAS (3.0 μg L^{-1}), while the use of NaCl represents an obstacle in Cd measurement by GF-AAS. The problems (difficulty of removing the salt matrix prior the atomisation step[3]) associated with the determination of volatile elements (Cd, Pb) in seawater with even higher content of salt (3.5% NaCl) were solved with matrix modifiers[4,5,6,7,8].

The additional problem of the heat-treated cytosol of fish tissues is the complex organic matrix. Thus, the aim of our investigation was to apply a novel approach for low-level Cd determination in the heat-treated cytosol of different tissues of *Mullus barbatus* (red mullet) with minimum sample preparation. For that purpose, a method for Cd determination by GF-AAS in seawater was modified and validated.

Materials and methods

Cadmium concentration was determined by GF-AAS on Varian SpectrAA 220, equipped with graphite tube atomiser Varian GTA 100, multielement lamp (Ag/Cd/Pb/Zn), deuterium lamp for the baseline correction and the partitioned pyrolytically coated graphite tubes. The heating program and instrumental parameters are presented in Table 1. Argon (99.99% purity) was a sheath gas.

Table 1 Heating program and instrumental parameters for Cd determination by GF-AAS

Heating program			
Stage	**T (°C)**	**Time (s)**	**Gas flow (L min^{-1})**
Hot injection	95	-	-
Dry	95	60.0	3.0
Ash	300	20.0	3.0
Ash	300	5.0	3.0
Atomisation	900	3.0	0.0
Atomisation	900	1.0	0.0
Clean	2800	3.0	3.0
Clean	2800	2.0	3.0

Instrumental parameters	
Sampling mode	Automix
Measurement mode	Peak area
Calibration mode	Concentration
Wavelength	228.8 nm
Slit Width	0.5 nm
Lamp Current	5.0 mA
Total Volume	40 μL
Sample Volume	20 μL
Modifier Volume	5 μL
Modifier Mode	Co inject

Reagents used were: Cd certified standard solution (1000 mg L^{-1}) (Merck), NaCl Suprapur (Merck), Titriplex® III (Merck), NaOH p.a. (Kemika), and Milli-Q water.

The heat-treated cytosol from fresh tissues (kidney, liver, intestine and brain) of red mullet (*Mullus barbatus*) was prepared according to a method previously described in the literature[2,9,10]. Only for the intestine, the homogenisation buffer additionally contained inhibitors of proteolitic activity, phenylmethylsulphonyl fluoride and leupeptin.

Results and Discussion

† Electronic Supplementary Information (ESI) available: [details of any supplementary information available should be included here]. See http://dx.doi.org/10.1039/b000000x/

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When conventional methods with temperature of atomisation set at 1800°C[11] are used for Cd determination in the salt matrix, background absorbance (BG) signal develops simultaneously with Cd signal, leading to a suppression of Cd signal[3]. Among many modifiers, EDTA was a first compound reported as capable of promoting efficient low temperature atomization of Cd from the salt matrix[4]. Outstanding results obtained with direct determination of Cd in seawater by GF-AAS with this modifier were reported by Guevremont *et al*.[12]. Recently, EDTA was applied as a modifier in slurry sampling EV-ICP-MS for determination of Cd in fish[13].

Therefore, we also chose EDTA as a modifier to separate absorption signal of Cd from NaCl signal. In the presence of EDTA, Cd atomisation in seawater begins below 600°C, and is free of intense background absorption. The absorbance signal falls to zero before a significant amount of matrix is vaporized[12]. We have used EDTA stock solution of 6.0 g L^{-1}. By addition of 5 µL of EDTA stock solution to 20 µL of sample, the ratio between EDTA and Cd approximately corresponded to the mass ratio used by Guevremont *et al*.[12] (EDTA/Cd=1.5×10^6), reported to provide an absorption signal with a peak in the 600-800°C range.

Selection of the optimal modifier pH value

The pH value of stock EDTA (Na_2H_2Y) solution was approximately 4.5. Fully protonated EDTA molecule is defined as H_4Y. At pH 4.5 almost 100% of EDTA is present in a form of H_2Y^{2-} anion[14]. Addition of 5 µL of Na_2H_2Y to 20 µL of Cd standard solution (1.5 µg L^{-1}) resulted in absorption signal with two peaks. EDTA complexing capacity was not high enough to completely bind Cd. Increasing the volume ratios of EDTA to Cd, second peak disappeared. However, the addition of EDTA solution with pH adjusted to approximately 7, enabled to obtain Cd signal as a single, sharp peak. At pH 7 more than 80% of EDTA is present in a form of HY^{3-} anion[14], which has higher capacity for binding Cd in the form of chelate. By acidification, the complexing properties of EDTA decrease[13]. As heat-treated cytosolic samples from fish tissues have pH ~7.2, it was optimal to adjust EDTA solution to the similar pH.

After selecting the optimal EDTA pH value, thermal program set by Guevremont *et al*.[12] was applied with some modifications in heating conditions. The atomisation time of 15 seconds without the argon flow caused serious damage to graphite tube after only 100 to 150 firings. Furthermore, after the cleaning step performed at 2500°C, a certain quantity of NaCl still remained in the tube and was carried over to the next measurement. Two modifications of thermal program were, thus, made: a) atomisation time with argon flow interruption was shortened to 4.0 s, with a ramp of 3.0 s, in order to protect the graphite tube; b) cleaning step was set at 2800°C[15], and thereafter problems with NaCl carry over were not observed.

Selection of the optimal atomisation temperature

BG signal rapidly decreased if the atomisation temperature decreased from 1000°C to 900°C. In the temperature range from 900°C to 500°C it stayed nearly constant. Cd signal, on the other hand, increased at lower atomisation temperatures. Similar observations were reported by Liao and Jiang[13]. Guevremont *et al*.[12] reported that very rapid heating does not enhance the signal, but rather shifts it to the region where co-volatilization of the salt matrix occurres. Thus, by decreasing atomisation temperature, while the ramp time remained constant, we have achieved much slower heating rate at 500°C than at 1000°C. Knowing that atomisation of Cd bound to EDTA occurs below 600°C and that slow heating rate enhances the Cd signal, it is not surprising that highest slope of a calibration straight line for Cd measurement was obtained at 500°C. Decrease of atomisation temperature below

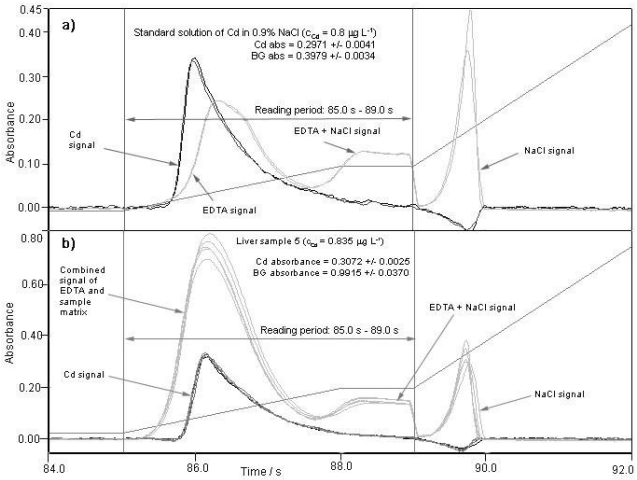

Fig. 1 Cd and BG signals obtained for: a) Cd standard solution in 0.9% NaCl (c_{Cd} = 0.8 µg L^{-1}); b) heat-treated cytosol of liver sample (c_{Cd} = 0.835 µg L^{-1}). Addition of EDTA (pH 7) and heating program from Table 1 were applied

500°C caused the rapid decline of both Cd and BG signal. Both signals were still present, but temperature of 400°C was too low to produce complete Cd atomisation in only 4.0 s of atomisation time.

Cd concentration was, then, measured using all tested atomisation temperatures in three types of samples: Cd standard solution in 0.9% NaCl (0.5 µg L^{-1}), heat-treated cytosol of red mullet kidney, and of intestine. The recovery of Cd in standard solution was excellent for the entire temperature range, even at 400°C when recovery was still above 90%. That could be explained by the fact that composition of this standard solution was identical as calibration solutions, and the atomisation rate of Cd was the same as in the calibration process. Contrary to this, Cd atomisation rate in the heat-treated cytosol of red mullet tissues is slower. Thus, incomplete Cd atomisation at temperatures below 500°C lead to inability to compare Cd absorbance measured in the heat-treated cytosol with Cd absorbance obtained during calibration at the same temperature.

The facts that Cd could be atomised completely at 900°C and that BG signal produced by NaCl is acceptably low at this temperature were the reasons why we chose temperature of 900°C for Cd atomisation, although the slope of calibration straight line was not the highest at this temperature.

Validation of Cd determination in 0.9% NaCl

The BG signals of standard solutions comprised of three peaks (Fig. 1a). To elucidate their origin, signals were separately recorded

for 0.9% NaCl and EDTA solution (pH ~7.0). Based on the obtained signals it could be concluded that the absorbance of the first BG peak is attributed to EDTA. The second peak is produced by both NaCl and EDTA. Certain percentage of EDTA needs a higher temperature for its vaporisation, probably due to specific EDTA chemical form. NaCl, on the other hand, mainly vaporises above 900°C, but starts to evaporate already at temperatures above 800°C. The third peak corresponds to the absorbance of NaCl, and falls out of the reading frame.

Validation was performed according to International Recommendation R100[16]. Calibration straight line in 0.9% NaCl was created for Cd concentration range up to 1.2 μg L^{-1} with the addition of EDTA and applying the heating program from Table 1. Calibration was performed at 11 separate occasions to examine its reproducibility. Relative standard deviation (RSD) of the slope was 3.2%, indicating to high reproducibility of calibration under given conditions. Determination coefficient (R^2) was 0.9995±0.0003, confirming the linearity of the selected working range.

The limit of detection (LOD), calculated on the basis of 3 standard deviations of the blank, was 0.010 μg L^{-1}. The limit of quantitation (LOQ), defined as 10 standard deviations of the blank[17], was 0.033 μg L^{-1}.

For Cd concentrations in the range from 0.150-1.000 μg L^{-1} precision (RSD) was below ±3%. Only one standard solution of 1.150 μg L^{-1} exhibited RSD 6.3%. Recoveries for these solutions were between 99% and 105%. For Cd concentration of 0.050 μg L^{-1} RSD was somewhat higher (13.5%). However, standard deviation (±0.006 μg L^{-1}) was comparable or even lower than for higher Cd concentrations. At this level, recovery was in the range from 82% to 110%, but the difference from defined concentration was actually very low (0.005-0.009 μg L^{-1}).

Validation of Cd determination in the heat-treated cytosol of four red mullet tissues

Cd and MT analyses were performed in the heat-treated cytosol isolated from fresh tissues of red mullet: liver, kidney, brain and intestine[10]. In Fig. 1, for the purpose of comparison, two Cd signals are displayed in parallel. First one, in Fig. 1a, is a Cd signal obtained with Cd standard solution (c=0.8 μg L^{-1}), while Fig. 1b illustrates Cd signal of similar concentration (c=0.835 μg L^{-1}) obtained with the heat-treated cytosol of liver. Although level of Cd absorbance of two samples is similar, the first BG peak inside the reading frame is higher for the heat-treated cytosol of liver, which has a complex organic matrix. The heat-treated cytosol (S50) predominantly contains MTs. BG absorbance highly correlates with MT concentration ($r=0.951$, $p<0.00001$, $n=20$), indicating that the first BG peak is associated with MT level. Thus, BG signal decreased as follows: kidney>intestine>liver>brain, and coincided with the decrease of MT concentration in these organs of *M. barbatus*[10].

In order to evaluate if the protein matrix interferes with Cd analysis, precision of Cd measurement in all four tissues was determined. Precision (RSD) of Cd measurement was mainly better than ±5% in the samples from all four tissues (12 samples). Higher RSD (±9%) was obtained only for one brain sample due to the very low level of Cd (0.068 μg L^{-1}), but the standard deviation was acceptably low (±0.006 μg L^{-1}).

Since the certified reference material corresponding to the heat-treated cytosol of fish tissue is not available, the accuracy of the method was established by determining the recovery in spiked samples. Recovery of Cd in spiked samples of kidney and intestine (3 samples each), as well as in 4 brain samples was in the range from 98%-106%. Recovery of Cd in one brain sample was slightly above this range (111%), but still acceptable considering low concentration of Cd in that sample (0.113 μg L^{-1}). Just one brain sample had the recovery considerably above acceptable limit (147%), and it was also a sample with rather low Cd concentration (0.109 μg L^{-1}). Recoveries in six liver samples were in the range from 87% to 110%. Since liver samples exhibit slightly higher deviation from the expected values, it could be presumed that composition of liver samples affects Cd atomisation. It is possible that liver samples are somewhat more viscous compared to the other tissues. Although physical effects as viscosity and surface tension are less important with graphite furnace than with flame atomic absorption, they still can affect the reproducibility of sample dispensing. The main effect is in the degree to which the sample spreads inside the graphite tube[11]. This slight decline of measurement quality, however, could not be connected with MT level. MT concentration in liver cytosol is approximately two times lower than concentration in kidney and intestine cytosol, and quality of measurement in those samples was excellent, as already discussed.

The problem of sample viscosity was observed in all heat-treated cytosols. It was reflected during the droplet delivery into the graphite tube. Instead of falling from the capillary into the tube, it ascended along the outer capillary wall. This problem was solved by simply wiping the capillary with the wet cloth between two sample deliveries. Increasing number of replicates is also recommended, so that possible deviations in droplet delivery could be noticed on time and excluded from the data treatment.

Our results show that the reliable low-level Cd determination by GF-AAS in the biochemical samples of complex composition (NaCl and proteins), such as the heat-treated cytosol (S50) of the fish tissues, is possible with minimum sample preparation, applying EDTA as a modifier and atomisation temperature of 900°C.

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