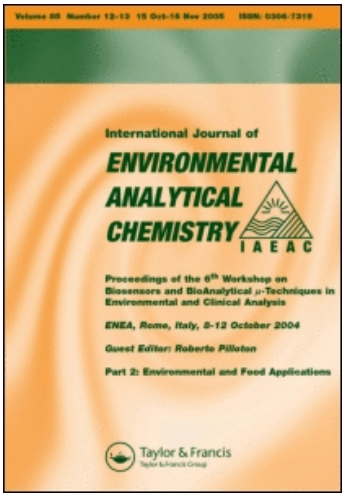

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COMPARISON OF TWO SH-BASED METHODS FOR ESTIMATION OF METALLOTHIONEIN LEVEL IN THE DIGESTIVE GLAND OF NATURALLY OCCURRING MUSSELS, *MYTILUS GALLOPROVINCIALIS*

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Two methods for the evaluation of metallothionein (MT) level in *Mytilus galloprovincialis* digestive gland homogenate extract, polarographic and spectrophotometric, were compared in combination with sample subjected to heat treatment (at 70 and 85°C) as well as to solvent precipitation. Spectrophotometric measurements ranging within 0.081–0.144 mg MTg^{-1} were at least one order of magnitude lower in comparison with the polarographic ones (2.21–2.88 mg MTg^{-1}) depending on the particular sample treatment as well as two specific reagents and reference standards applied.

Sephadex G-75 column chromatography of the differently treated samples and subsequent polarographic analysis in the pooled elution fractions indicated that a level of nonMT interfering thiolic component in homogenate extract may be accounted for more than 50% of the total polarographic signal, irrespective of a method of sample treatment applied. Consequently, the large discrepancy between methods could not be attributed only to the sample treatment procedures but more likely to certain not entirely explained analytical problems, particularly calibration with appropriate reference standard.

Keywords: Metallothionein; MT quantification; *Mytilus galloprovincialis*; Biomarker; Biomonitoring

INTRODUCTION

Bivalve molluscs of the genus *Mytilus* are commonly used indicator organisms in marine pollution control by determination of tissue metallothionein (MT) level as a relevant biomarker of environmental metal exposure. A biomarker such as MT needs to be validated in terms of baseline concentrations so that contaminant-induced stress may be distinguished from natural variability. Therefore, methods used for MT quantification in biomonitoring programmes must be not only relatively simple

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and rapid but also fairly accurate. Among several useful methods available for MT detection [1], those ones based on the determination of sulphydryl level such as differential pulse polarography and spectrophotometric method using Ellman's reagent, seems to be the most promising techniques for routine evaluation of MTs in environmental samples.

However, by comparing literature data available on MT quantification provided by polarographic and spectrophotometric methods in *Mytilus sp*. (Table I), it can be noted that there is a disagreement in determination of MT level between these methods. In general, MT concentrations determined by the electrochemical method were several times higher than those determined by the spectrophotometric method [4,8,11]. Since each of these two SH-based methods requires the specific pretreatment of the sample that may change the sample composition and therefore contribute to the difference in MT level estimation, procedural differences presumably may account for much of the disagreement. To date, the effect of two different centrifugation speeds combined with two isolation procedures upon determination of metal and MT level in gills and digestive gland tissues of *M. edulis* has been demonstrated by Geret *et al*. [4] in order to check possible differences. However, the authors did not clearly explain why such a pronounced difference of MT concentrations in gills occurred following the application of the corresponding isolation protocols.

The main objective of this study has been to find out whether possible changes in the sample composition occurred presumably as the consequence of the particular sample treatment applied which may affect the quantitative determination of MT level in the

TABLE I Survey of results reported quantitative determination of MT in selected tissues of *Mytilus sp*. performed by polarographic (DPP) and spectrophotometric (DTNB) method. Samples of homogenate tissue extracts were subjected prior to analysis either to heat treatment utilizing different temperatures ($70-85^{\circ}C$) or to the treatment by ethanol–chloroform mixture (E–CT)

Quantification method	*Sample treatment*	*Organism*	*Tissue*	*MT* (mg/g)[a]	*References*
Polarographic (DPP)	HT 80°C; 10 min	*M. edulis*	whole soft part digestive gland	2.75 ± 0.99 8.04 (dw)	Bebiano and Langston [2]
Polarographic (DPP)	HT 75°C; 30 min	*M. edulis*	digestive gland	8.8–19.6 (dw)	Amiard-Triquet *et al*. [3]
Polarographic (DPP)	HT 75°C; 15 min	*M. edulis*	digestive gland gills	1.8–1.9 (ww) 0.27–0.29	Geret *et al*. [4]
Polarographic (DPP)	E–CT		digestive gland gills	1.9 0.13	
Polarographic (DPP)	HT 80°C; 10 min	*M. galloprovincialis*	whole soft part	2.81 ± 0.66 (dw)	Bebiano and Langston [5]
Polarographic (DPP)	HT 70°C; 10 min	*M. galloprovincialis*	digestive gland,	2.1 ± 0.4 (ww)	Pavičić *et al*. [6]
Polarographic (DPP)	HT 70°C; 10 min	*M. galloprovincialis*	digestive gland whole soft part gills	2.84–4.23 0.85–1.25 0.45–0.64 (ww)	Raspor *et al*. [7]
Spectrometric (DTNB)	E–CT	*M. galloprovincialis*	digestive gland gills	0.10–0.24 0.08 (ww)	Viarengo *et al*. [8]
Spectrophotometric (DTNB)	E–CT	*M. galloprovincialis*	digestive gland	0.11–0.13 (ww)	Stein *et al*. [9]
Spectrophotometric (DTNB)	E–CT	*M. galloprovincialis*	digestive gland	0.08–0.19(ww)	Pavičić *et al*. [10]

[a]MT concentration referred either on dry weight (dw) or on wet weight (ww).

digestive gland extract of wild mussels, *M. galloprovincialis*. For that purpose the quantification of MT was performed in the aliquots of the same 30 000× g tissue extract:

(a) using heat treatment (at 70 and 85°C) prior to the polarographic analysis
(b) using solvent treatment (E–CT) prior to the each, spectrophotometric and polarographic analysis.

In addition, the supernatant samples treated by (a) and (b) procedures were chromatographically fractionated in order to find out whether a possible redistribution of the proteins (total and SH-containing) and metals (Zn, Cu and Cd) occurred between the selected elution profile components (HMW, MT20, MT10 and LMW).

EXPERIMENTAL

Preparation of the Mussel Tissue Extract Sample

The blue mussels, *M. galloprovincialis*, were collected from unpolluted sites Jadrija and Srima (near Šibenik) at Midlle Adriatic coast, Croatia, in March 1999. Mussel digestive glands were removed and pooled into composite samples, wet weighed, immediately frozen into liquid nitrogen and later kept at −80°C until further treatment. The samples were homogenized in the three volumes of 20 mM Tris–HCl buffer, pH 8.6, containing 0.5 M sucrose, 0.01% β-mercaptoethanol and protease inhibitors (0.006 mM leupeptine and 0.5 mM phenyl-methyl-sulphonilfluoride; PMSF) as described by Viarengo *et al.* [8,12]. They were centrifuged for 20 min at +4°C to obtain the soluble fraction (30000× g supernatant), containing MT. The supernatant was subsequently subjected to different procedures according to the scheme in Fig. 1.

Gel-filtration Chromatography

An aliquot (1.5 mL) of untreated and each of the differently treated supernatants were eluted on a Sephadex G-75 column (0.9 × 60 cm) using a 20 mM Tris-HCl buffer containing 2 mM dithiotreitol (DTT), pH 8.6. The UV-absorbance of the chromatographic fraction was measured at 280 nm. Eluate fractions were pooled according to their molecular weights into four groups: high molecular weight (HMW, > 80 000), metallothionein dimer (MT20, ∼20 000), metallothionein monomer (MT10, ∼10 000) and low molecular weight fraction (LMW, < 6000).

Analytical Methods

The pooled chromatographic fractions were analysed for Cd, Cu and Zn concentration by using a Varian A A-5 model flame atomic absorption spectrometer (FAAS) equipped with an air–acetylene flame and deuterium correction of a nonspecific absorption.

Total protein concentration was determined according to the Bradford's [13] assay using bovine serum albumin as the calibration standard. Quantitative determination of SH-containing compounds was performed by modified Brdička's procedure in the differential pulse polarography (DPP) mode using the calibration straight line obtained by the commercially available rabbit liver MT (I + II), a product of Sigma [14,15].

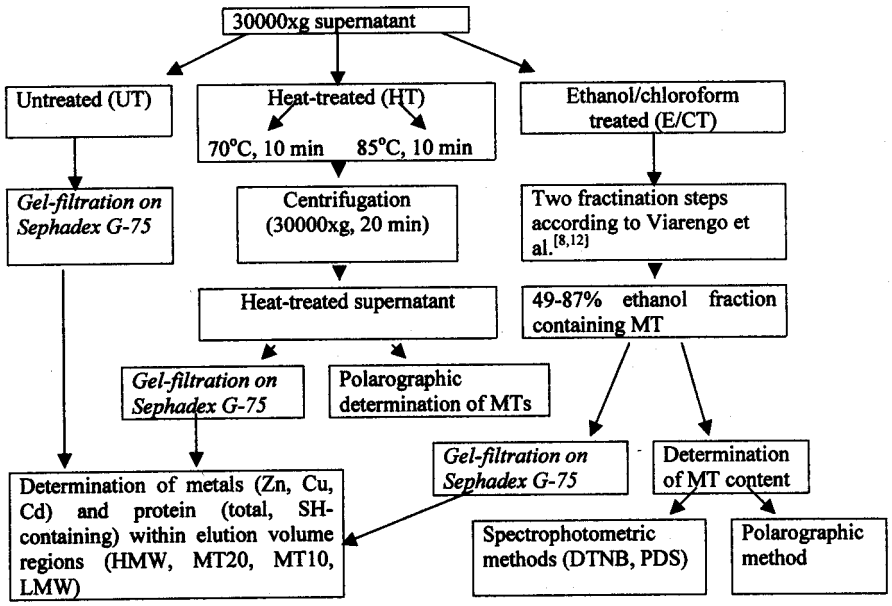

FIGURE 1 The scheme of sample preparation procedures followed by analytical methods performed on *M. galloprovincialis* digestive gland tissue homogenate extract (30 000× g supernatant).

All analytical data were expressed on the tissue wet weight basis.

Quantitative Determinations of MT

Each of two SH-based methods for MT determination were combined with the specific pretreatment of the sample in order to minimize the influence of interfering components.

Electrochemical Method

A differential pulse polarography method for sulphydryl-containing compounds based on the Brdička's reaction [l6], was used for the determination of MT content in supernatant samples which were previously heat-treated at two different temperatures (70 and 85°C) in order to compare their efficiency of removal heat-stable, high-molecular-weight, thiol-containing proteins. This method was also used for estimating the relative thiolic content of pooled fractions after chromatographic separations.

Spectrophotometric Method

Prior to spectrophotometric determination of MT, the 30000× g supernatant was subjected to an ethanol–chloroform treatment (E–CT) comprising of two fractionation steps according to the procedures reported previously [8,12,17,18]. Briefly, cold absolute ethanol and chloroform (−20°C) were added to supernatant sample (final 49% ethanol) in order to precipitate interfering high-molecular weight (HMW) proteins within the first fractionation step. The second step resulted with precipitation

of MT fraction by using markedly higher proportion of ethanol in the sample (87%) required for the removal of low-molecular weight (LMW) soluble thiols. The MT-containing pellet was resuspended in 5 mM Tris–HCl buffer, containing 1 mM EDTA, pH 7. The basic procedure was later slightly modified by addition of RNA, increasing ionic strength and sample acidity in order to improve fractionation and quantification of MT.

For spectrophotometric quantification of MT two alternative analytical methods based on sulphydryl–disulphyde interchange were performed using two different sulphydryl reagents; either 5,5′-dithiobis 2-nitrobenzoic acid (DTNB, Ellman's reagent) in Na-phosphate buffer, pH 8 or 4,4′-dithiodipyridine (4-PDS) [19,20] in Na-acetate buffer, pH 4. Concentrations of MT were quantified by application of two different calibrants (reduced glutathione, GSH and MT from rabbit liver).

In the case that GSH was used as a reference standard, MT concentrations ($mg\,g^{-1}$, wet weight) of *Mytilus sp*. were estimated taking into account number of cysteinyl residuas (21) and molecular weight of mussel MT (8600) [21].

Statistical Analysis

Results on quantitative determination of MT were expressed as mean $\pm$ SD of 3–5 replicates of the differently treated supernatant samples. Differences in MT concentration between the sample treatments applied prior to the polarographic analysis as well as between two spectrophotometric methods differing in the specific reagent and reference standard applied were evaluated either by analysis of variance (ANOVA) and post hoc comparisons assessed by the LSD test or by an unpaired Student's *t*-test. These tests were performed using a standard statistical packet (STATISTICA for Windows).

RESULTS AND DISCUSSION

Comparison Between Quantitative Determinations of MT by Polarographic and Spectrophotometric Methods

The results of polarographic and spectrophotometric analysis of MT concentration in differently treated aliquots of 30000$\times$ g supernatant sample are shown in Table II. Metallothionein concentrations determined electrochemically by DPP mode in 30000$\times$ g supernatant previously submitted either to heat denaturation (HT70 and HT85) at two different temperatures or to the ethanol–chloroform fractionation (E–CT), were within the range 2.21–2.84 $mg\,MT\,g^{-1}$, expressed on wet weight basis. Polarographic quantification was performed by utilizing rabbit liver MT as the calibrant. The results obtained do not exhibit statistically significant differences, regardless of the specific sample treatment applied, as evaluated by the analysis of variance (ANOVA). However, further application of the post hoc LSD test indicates that largest difference in MT concentration obtained between samples subjected to heat treatment at 70°C (HT70) and solvent treated samples (E–CT).

Metallothionein concentrations, measured spectrophotometrically by application of either DTNB or 4-PDS assay following ethanol–chloroform fractionation, were within the range 0.081–0.167 $mg\,MT\,g^{-1}$ on the wet weight basis. It is important to note that prior to the spectrophotometric measurements two alternative methods

TABLE II Summarized data on tissue MT concentrations determined by polarographic (a) and spectrophotometric (b) methods expressed as by mean $\pm$ *SD*(*n*) in the *M. galloprovincialis* digestive gland homogenate extract (supernatant 30000$\times$ g). The sample treatment methods, sulphydryl reagents (DTNB, 4-PDS) and calibration standards applied were indicated

Sample treatment	*Sulphydryl reagent* (pH)	*Reference standard*	*Tissue MT concentration* (mg g^{-1}; wet wt.)
(a) Polarographic (DPP) method			
HT70°C		MT	2.84±0.198 (2)
HT85°C		MT	2.40±0.326 (6)
E–CT[a]		MT	2.21±0.320 (3)
(b) Spectrophotometric method			
E–CT[a]	DTNB (8)	GSH	0.084±0.008 (5)
E–CT[a]	DTNB (8)	MT	0.104±0.012 (5)
E–CT[b]	DTNB (8)	GSH	0.081±0.012 (5)
E–CT[b]	DTNB (8)	MT	0.144±0.005 (3)
E–CT[a]	4-PDS[c] (4)	GSH	0.149+0.013 (5)
E–CT[a]	4-PDS[c] (4)	MT	0.167+0.019 (5)

[a]E–CT method based on procedures described by Viarengo *et al.* [12]; [b]E–CT method modified by addition of RNA, increasing sample acidity and ionic strength [8]; [c]4-PDS spectrophotometric assay according to Nielson and Winge [20].

utilizing organic solvents fractionation were performed, the original procedure intended for routine MT evaluation in biomonitoring according to Viarengo *et al.* [12,18] and the other modified by an additional acidification, the increase of ionic strength and the addition of RNA [8].

It has been found very good agreement in spectrophotometric MT determinations (0.081 and 0.084 mg MT g^{-1}) between two alternative E–CT procedures coupled to DTNB assay where reduced glutathione (GSH) was utilized as a calibrant. On the contrary, the difference between MT concentrations (0.10 and 0.14 mg MT g^{-1}) was statistically significant ($p < 0.01$) if quantification referenced to rabbit liver MT. Otherwise, the application of 4-PDS reagent indicates significantly higher MT level in comparison with DTNB (0.15 and 0.17 mg MT g^{-1}) if calibrated using GSH and MT, respectively. The observed difference in MT level was not statistically significant suggesting an additional dissociation of metal-bound-MT amount under the more acidic conditions (pH 4) required for operation of PDS reagent [20,22] opposite to DTNB, being effective under the rather narrow, more alkaline range (pH 7.8–10.4) [19]. The polarographic measurements performed on the aliquots of the same sample, subjected to the different treatments, were approximately 20 times higher than the results of spectrophotometric DTNB assay, when commercially available MT from rabbit liver has been utilized as calibration standard. In case of using reduced glutathione (GSH) instead of MT, as recommended for routine quantification of MT in the sea water control by Viarengo *et al.* [12,18], the results of spectrophotometric assay (0.08 mg MT g^{-1}) were 30 times lower in comparison with the level of polarographic determinations.

The results presented here are in close agreement with those reported in some of our previous studies [6,7,10,23]. They were also comparable with the data reported in the literature for several bivalve mollusc species, which were performed by the respective analytical methods [2,8,9,24]. Polarographic determinations of MT in *Mytilus sp.* digestive gland were within the range 8.04–19.6 mg MTg^{-1} as expressed on the dry weight basis [2,3]. They are comparable with the data expressed on the wet weight basis

ranging within 1.9–4.7 mg MT g^{-1} [4,6,7], taking into account the ratio of five for wet/dry weight conversion. Otherwise, the results of spectrophotometric determinations reported in the literature, exclusively expressed on the wet weight basis [8–10] ranging from 0.08 to 0.24 mg MT g^{-1} were at least one order of magnitude lower than the respective polarographic measurements. Similar disagreement between determination of the Cd-induced MT level was also reported in the fish liver of the sea bass *Dicentrarchus labrax* [11]. They found a good correlation between two methods, although the polarographic data were approximately four times higher in comparison with those quantified by spectrophotometric method.

Chromatographic Separation and Quantification of Total Protein, Thiolic and Metal Content in the Major Components of Differently Treated Samples

Sephadex G-75 chromatography was applied in order to resolve the major components (HMW, MT20, MT10, LMW) of the 30 000× g supernatant samples, both untreated and differently treated, as comparatively presented by the respective elution profiles in Fig. 2, by recording absorbance at 280 nm.

Our results on quantitative determination of MT by DPP, total protein and metals such as Zn, Cu and Cd in the pooled chromatographic fractions within the selected elution profile regions were presented by vertical stacked bars in Figs 3 and 4,

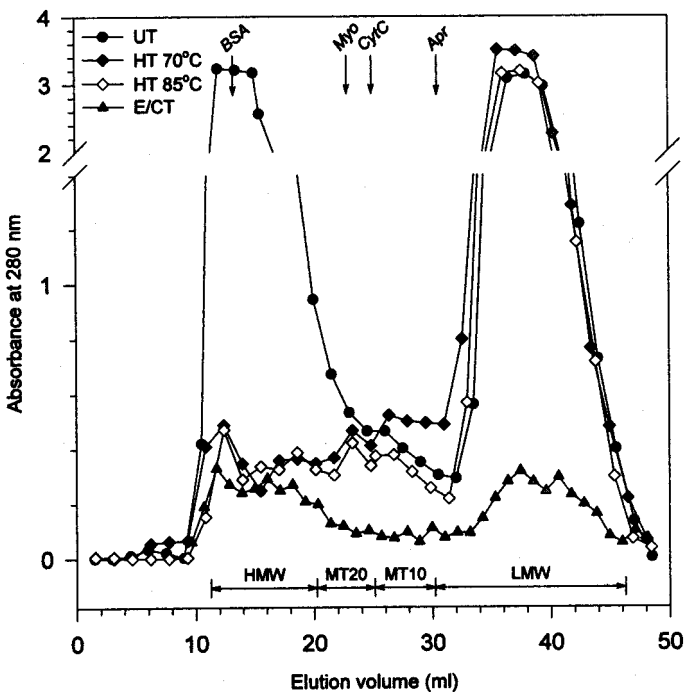

FIGURE 2 Comparative presentation of Sephadex G-75 gel-filtration profiles of the untreated (UT), heat-treated (HT 70°C, HT 85°C) and ethanol–chloroform-treated (E–CT) 30 000× g supernatant obtained from the *M. galloprovincialis* digestive gland tissue homogenate. HMW, MT20, MT10 and LMW indicate the region of pooled fractions for protein, thiol and metal determination. Elution positions of protein molecular mass markers are also presented (*BSA*-bovine serum albumin, *Myo*-myoglobin, *CytC*-cytochrome c, *Apr*-aprotinin).

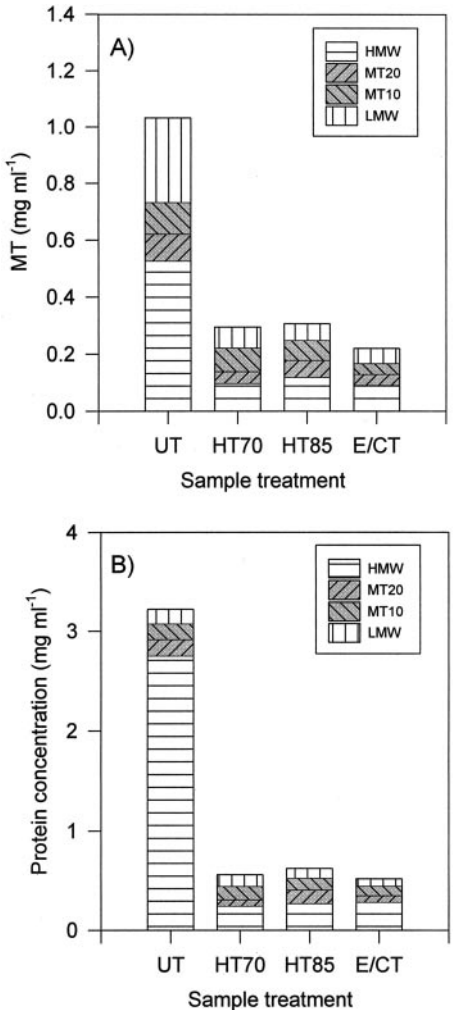

FIGURE 3 The estimation of supernatant mass fraction of (A) SH-responding polarographic activity calibrated using rabbit liver MT (Brdička's assay) and (B) total protein (Bradford's assay) in the resolved components (Sephadex G-75) of untreated and differently treated samples.

representing integrated mass of each component per mL of supernatant, applied to the chromatographic column.

It may be observed that each of the utilized treatment methods produce the marked reduction of SH-responding polarographic activity, expressed as a rabbit liver MT equivalent, in both MT as well as in nonMT components (HMW and LMW) in comparison with the untreated sample (Fig. 3A). Consequently, the remaining polarographic activity of nonMT components in an integral sample may contribute to an overestimation of total MT (MT10 + MT20) with approximately 50%, irrespective of the treatment method applied. It may be also noticed that MT component was largely reduced by etanolic fractionation than by each of temperature treatments applied.

For comparison, the concentrations of total protein determined in the differently treated samples according to the spectrophotometric Bradford assay (Fig. 3B)

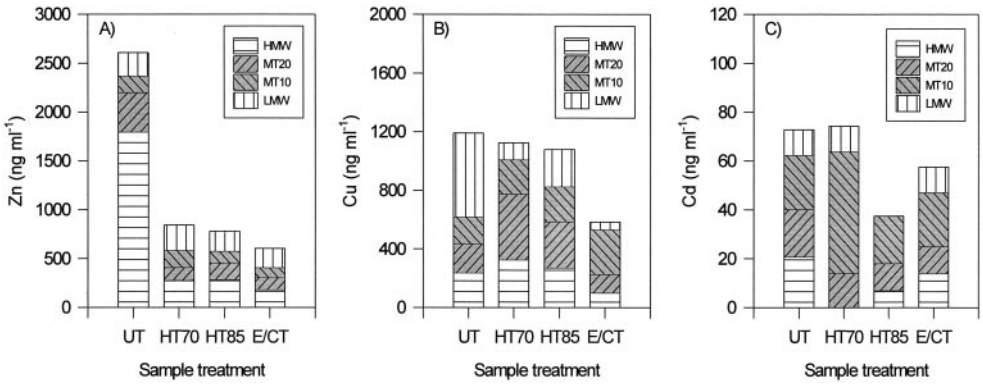

FIGURE 4 The estimation of metal content using flame AAS technique in supernatant mass fraction of (A) Zn, (B) Cu and (C) Cd, based on the resolved components (Sephadex G-75) of untreated and differently treated samples.

were approximately two times higher than MT concentration, but indicating similar proportion of interfering nonMT components in the differently treated samples.

Furthermore, the content of Zn, Cu and Cd associated with MT components was also affected by application of different treatment procedures as been shown in Fig. 4(A,B,C). In the untreated sample Zn was predominantly associated with HMW component which has been drastically reduced following sample treatment utilization. Significant part of Zn associated with MT20 component prior to sample treatment was lost during each procedure.

In contrast to Zn, the participation of Cu in total MT fraction following heat treatments was markedly increased. Such effect was particulary expressed in MT20 component producing 1.6–2.2 larger increase of Cu-MT20 fraction in comparison with untreated sample through redistribution of Cu from nonMT components, possibly due to competitive exchange reaction with loosely bound Zn. In the sample subjected to organic solvent procedure the redistribution of Cu from MT20 toward MT10 component may occur.

In comparison with Zn, Cd was predominantly associated with MT fraction in both, untreated and differently treated samples. Each of the sample treatments applied resulted by reduction of considerable part of Cd previously associated with MT20 component. The most significant reduction of Cd was evident following heat treatment at 85°C. Furthermore, a possible redistribution of HMW-bound Cd toward MT10 component may occur as the consequence of HT70 utilization.

Our results on metal-binding composition of *M. galloprovincialis* MT following application of different treatment methods indicate highly expressed stability of MT10 in comparison with MT20 component with respect to competitive metal-exchange reaction.

Before considering critically disadvantages of two sulphydryl-based methods applied in this study it would be important to note that they were established on fundamentally different approaches. The polarographic method assesses the overall MT content [25,26] irrespective of a degree of sulphydryl-metal saturation, in opposite to spectrophotometric methods [8,19] based upon the metal-free thiolic content. Furthermore, different treatment methods applied prior to analysis were selected

in order to minimize the specific interfering substances. The heat treatment which has been proposed as a less time-consuming method satisfying for removal of HMW component prior to analysis, presumed relatively weak polarographic response of the residual LMW compounds. Otherwise, a solvent fractionation method was conventionally utilized prior to spectrophotometric analysis in order to remove both interfering, HMW and LMW SH-containing compounds.

The problem related to the suitability of calibration standard for quantification of MT in the tissues of bivalve mollusc has been already discussed mainly in the context of biomonitoring [6,8]. Keeping in mind that MT from natural populations of mussels beside Zn and Cd contains firmly bound Cu ions [3,27,28], confirmed also in the present study, in contrast to that of rabbit liver, an incomplete SH-metal dissociation of mussel MT in the sample may be expected in comparison with that of reference standard possibly producing certain underestimation of mussel MT level determined by DTNB assay.

Consequently, the degree of metal dissociation may have a much stronger implication on spectrophotometric than on polarographic determination. It is due to the prerequisite of the Ellman's reaction to attain metal-free SH-groups in order to react with DTNB. Having in mind that presence of mercaptide bound would not affect seriously the intensity of polarographic signal, as previously reported [25,29], the degree of metal dissociation may not have such marked influence upon electrochemical quantification of MT as it might be in case of using the spectrophotometric method.

Further disadvantage of spectrophotometric method, associated with certain risk in removal of dimeric or higher polimeric MT form within organic slovent procedure should be prevented by addition of the reductive agent, β-mercaptoethanol within a tissue homogenization step. The satisfactory efficiancy of reductive agent in preventing oxidative MT polimerization has been reported previously by Viarengo *et al.* [8] and confirmed by a high rabbit liver MT recovery (85–90%) obtained following solvent fractionation procedure.

Otherwise, the results dealing with polarographic analysis in the pooled chromatographic fraction within selected fractionation ranges (Fig. 3) indicated that one of the main disadvantages of polarographic MT quantification would be the high retention, more than 50%, of SH-responding compounds (HMW + LMW) other than MT regardless of the specific sample treatment method applied. In some of the previous studies, including those from our laboratory [2,14,30,31] on electrochemical analysis of SH-containing proteins within LMW component of the Sephadex G-75 profile, its contribution upon the whole supernatant thiolic content was generally underestimated. In spite of relatively low LMW thiolic concentration level recorded polarographically in pooled chromatographic fractions, its considerable participation in differently treated samples prepared from the wild mussels' digestive gland may be accounted between 20 and 25% of an integral sample.

Since the polarographic method is very sensitive and the linearity range is considerably narrow, special care has to be taken in a way that polarographic activity of an unknown sample has to be in the range of a calibration curve. Preliminary results from our laboratory [32,33] show that an appropriate dilution of the sample before the thermal treatment could increase a reliability in reading the electrochemical catalytic signal. In addition to that, the comprehensive study [34] dealing with biomedical application of Brdička reaction, particularly an increase of a polaro-

TABLE III Summarized results on metal to metallothionein stoichiometry estimated on the basis of the relevant analytical data presented in Table II and in Fig. 4. The results on MT determinations were designated respective to different sample treatments (polarography) in addition to usage of specific reagent and calibrant (spectrophotometry). Determinations of trace metals (Zn, Cu and Cd) were performed by flame AAS technique. All estimations were made on pooled chromatographic fractions except those of spectrophotometric method which was performed on supernatant (E–CT) samples taking into account 55.9% reduction of MT level due to SH-responding nonMT components (HMW + LMW) determined by DPP

Analytical method sample treatment (reagent, calibrant)	*MT* ($\mu M\,mL^{-1}$)	*Zn + Cu + Cd* ($\mu M\,mL^{-1}$)	*Molar ratio Zn + Cu + Cd : MT*
Polarography (DPP)			
HT70°C	0.049	0.0255	0.520
HT85°C	0.052	0.0225	0.433
E–CT[a]	0.035	0.0176	0.503
Spectrophotometry			
E–CT (DTNB)[a]			
GSH	0.00136	0.0176	12.9
MT	0.00169	0.0176	10.4
E–CT (DTNB)[b]			
GSH	0.00132	0.0176	13.4
MT	0.00234	0.0176	7.5
E–CT[a] *(4-PDS)*[c]			
GSH	0.00242	0.0176	7.3
MT	0.00271	0.0176	6.5

[a]E–CT method based on procedures described by Viarengo *et al*. [12]; [b]E–CT method modified by increasing sample acidity and ionic strength [8]; [c]4-PDS spectrophotometric assay according to Nielson and Winge [20].

graphic activity as a consequence of sample denaturation, should be also taken under consideration.

The results on determination of MT concentration by spectrophotometric and polarographic methods displayed similar coefficient of variation, 10 and 13%, respectively.

Summarized results dealing with metal-binding stoichiometry of *M. galloprovincialis* digestive gland MT were presented in Table III. In general, the stoichiometry of metal ions associated to mammalian MT is well defined showing either 7 divalent (Zn + Cd) or 11–12 monovalent Cu metal ions. Coordination geometry and precise number of metal-binding sites in mussel MT has not been reported by now. Evaluations of metal (Zn + Cu + Cd) to MT molar ratio based on different modifications of spectrophotometric method ranging between 6.5 and 13.4, were also in agreement with previously reported data on *M. galloprovincialis* metal-binding stoichiometry [22,35]. For comparison, metal to MT molar ratio (0.43–0.52) evaluated on polarographic analysis would be less reliable, possibly due to considerable overestimation of supernatant MT content.

Assuming the complexity of mussel MTs composed of several multiple forms with specific metal-binding properties [2l,36] differently expressed at basal and induced level, the precise number of metal-binding sites may reflect variation of environmental factors, both biotic and abiotic.

CONCLUSIONS

The results on quantification of MT level in digestive gland of *M. galloprovincialis* by polarographic and spectrophotometric method, performed on different aliquots of the same sample (30000× g supernatant) were comparatively presented and their advantages as well as the observed shortcomings were critically considered. Spectrophotometric determinations of MT obtained in the present study were at least one order of magnitude lower in comparison with the polarographic ones supporting the results previously reported in the literature.

We found that each of the sample treatment applied, either heat treatment or ethanol precipitation, may contribute to a similar extent to an overestimation of MT level by polarographic method due to an incomplete removal of the interfering SH-containing substances. However, the relatively high level of residual thiolic, nonMT polarographic activity, approximatively acounted 50% of the integral sample, could not entirely explain the large discrepancy between the results presented in this study. It may be probably ascribed to certain analytical problems, such as calibration with respective reference standard more closely related in metal-binding properties to mussel MT. This problem was more intensively studied in spectrophotometric analysis, by using two reference standards (GSH and rabbit liver MT) in combination with two disulphyde reagents (DTNB and 4-PDS). In order to minimize observed shortcomings of spectrophotometric quantification, an alternative sulphydryl reagent, 4-PDS was proposed for MT quantification due to its more efficient deliberation of cysteine SH groups. Otherwise, having in mind that polarographic analysis were not markedly affected by a degree of metal dissociation, the more specific problem dealing with improvement of the sample polarographic response linearity has been recently recognized and presently is under investigation in our laboratory.

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