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Characterization of denatured metallothioneins by reversed phase coupled with on-line chemical vapour generation and atomic fluorescence spectrometric detection

Emilia Bramanti[a,*], Cristina Lomonte[a], Alvaro Galli[b], Massimo Onor[a], Roberto Zamboni[c], Giorgio Raspi[a], Alessandro D'Ulivo[a]

[a] *Laboratory of Instrumental Analytical Chemistry, Italian National Research Council-Istituto per i Processi Chimico-Fisici, Via G. Moruzzi 1, 56124 Pisa, Italy*
[b] *Laboratory of Gene and Molecular Therapy, Italian National Research Council-Istituto di Fisiologia Clinica, Via G. Moruzzi 1, 56124 Pisa, Italy*
[c] *Department of Chemistry and Industrial Chemistry, University of Pisa, Via Risorgimento 35, 56126 Pisa, Italy*

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Abstract

A new analytical hyphenated technique is proposed for determination and characterization of thiolic proteins, based on reverse phase chromatography (RPC) coupled on-line with cold vapour generation atomic fluorescence spectrometry (CVGAFS). Proteins are pre-column simultaneously denatured and derivatized in phosphate buffer solution containing 8.0 mol l^{-1} urea and *p*-hydroxymercurybenzoate (PHMB). The derivatized proteins are separated on a C4 Vydac Reverse Phase column. Post-column on-line reaction of derivatized denatured proteins with bromine, generated in situ by $KBr/KBrO_3$ in HCl medium, allowed the fast conversion of both the uncomplexed PHMB and of the PHMB bound to proteins to inorganic mercury, also in the presence of methanol in the RPC eluent phase. Hg(II) is selectively detected by AFS in a Ar/H_2 miniaturized flame after sodium borohydride reduction to Hg°. Under optimized conditions, on-line bromine treatment gives a 98 ± 2% recovery of both free and protein-complexed PHMB. The effect of methanol on the sensitivity of Hg(II) detection was studied and controlled. RPC-CVGAFS system has been applied to the analysis of metallothioneins from rabbit liver (MT_{RL}) standard solutions, and their commercial isoforms MT-1 and MT-2. The analysis of denatured, PHMB-complexed MTs allowed the determination of the number of thiolic groups complexed by PHMB. It was found that MTs from rabbit liver have 10.0 ± 0.3 (MT-1) and 6.7 ± 0.3 (MT-2 and MT_{RL}) –SH groups complexed by PHMB. The detection limit (LODc) for PHMB in 95% methanol in the optimized conditions was about 9.3×10^{-9} mol l^{-1} and for the denatured MTs LODc was about 8.6×10^{-10} mol l^{-1}, taking into account an approximate complexating ratio PHMB:MTs of 7:1.

Keywords: Metallothioneins; Atomic fluorescence spectrometry; *p*-Hydroxymercurybenzoate

1. Introduction

The importance placed upon the determination of compounds possessing thiol functionalities has increased dramatically in recent years as their role in various physiological processes has become more evident. From the analytical point of view the specific reactivity of –SH groups of proteins toward metals could represent a useful property which avoids their oxidation and it allows their selective detection/determination in complex matrices. Metals bound to proteins are easily displaced with metals of higher affinities. In particular, mercurial probes (organic RHg^{+} and inorganic mercury Hg^{2+}) interact with –SH groups with high affinity and specificity, and can be revealed by sensitive instrumental techniques [1].

In previous papers we proposed a new hyphenated technique for the determination and characterization and speciation of native and denatured thiolic proteins, based on hydrophobic interaction chromatography (HIC) coupled on-line with chemical vapour generation atomic fluorescence spectrometry (CVGAFS) [2,3]. Thiolic groups of proteins

* Corresponding author. Tel.: +39 050 3152293; fax: +39 050 3152555.
E-mail address: emilia@ipcf.cnr.it (E. Bramanti).

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were derivatized pre-column by *p*-hydroxymercurybenzoate (PHMB), a mono-functional organic mercurial probe, which interacts with high affinity and specificity at room temperature with reactive –SH groups of proteins, giving stable, soluble complexes. The derivatized proteins were separated by HIC and detected by CVGAFS after on-line digestion of the protein-complexed PHMB. This last step, realized by post-column on-line reaction of derivatized proteins with bromine, generated in situ by $KBr/KBrO_3$ in HCl medium, allowed the fast, on-line conversion of PHMB to inorganic Hg(II).

In this paper, the CVGAFS detection system has been optimized for the hyphenation with reverse phase chromatography (RPC) in the presence of methanol in the eluent phase and for the on-line digestion of complexed and uncomplexed PHMB. The effect of methanol on the sensitivity and detection limit of Hg(II) detection has been evaluated by flow injection analysis coupled to CVGAFS (FIA-CVGAFS). The on line digestion procedure of PHMB by in situ generation of bromine in the presence of methanol has been optimized. Finally, the RPC-CVGAFS system has been applied to the analysis and characterization of commercial standard solutions of rabbit liver metallothioneins (MT_{RL}), and its major isoforms MT-1 and MT-2, a class of low molecular weight (6–7 kDa), cysteine-rich proteins, involved in homeostatic control of metals, in the detoxification of toxic elements, free radicals scavenging and other not yet well defined pathophysiological phenomena [4]. In the most studied mammalian MT-1/MT-2 isoforms (61 amino acids), the 20 Cys residues are highly conserved and involved, in the native form, in binding of seven divalent metal ions and up to 12 monovalent ions [5]. The free sulfhydryl (–SH) groups present in MTs are extremely reactive, being via an auto-oxidative process readily converted to disulfides. Therefore, it is very important in order to perform reliable analysis of these molecules, to stabilize their –SH groups in biosamples to guarantee the stability of stock and working solutions as much as possible.

MTs were by us pre-column denatured and complexed with PHMB. Denaturing conditions have been chosen in the derivatization step in order to increases the number of –SH groups reactive with PHMB, making them accessible to the mercurial probe, thus improving the analytical sensitivity and the detection limit of the method [3]. The simultaneous denaturation and derivatization procedure of MTs with PHMB was performed in order to block –SH groups, controlling the possible formation of intra- and intermolecular disulfide bonds. Although no data are reported on PHMB-MTs complexation, it is known that that inorganic mercury replaces endogenous MT cadmium in a 1:1 manner, although there is still uncertainty with regard to the stoichiometry of the Hg:protein binding (Hg_7-MT and Hg_{18}-MT, depending on the coordination geometry adopted by Hg^{2+}) [6]. The results on the stability of denatured PHMB-complexed MT stock solutions, a sticky, not investigated field of MT analysis, are reported and discussed.

2. Experimental

The preparation of stock solution of 4-(hydroxymercuric)benzoic acid (PHMB), sodium salt (CAS No. 138-85-2, $HOHgC_6H_4CO_2Na$, Sigma-Aldrich, Chemical Co., St. Louis, MO, USA), inorganic Hg(II) (Merck Laboratory Supplies, Poole, Dorset, UK), pH 7.2, 0.1 mol l^{-1} phosphate buffer solution (PBS) (NaH_2PO_4 monohydrate, anhydrous Na_2HPO_4, BDH, Merck), urea (SigmaUltra), $NaBH_4$ (Merck, pellets, reagent for AAS, minimum assay >96%), HCl and Br^-/BrO_3 was previously described [3] Working solutions were prepared by dilutions of the stock solution, just before use.

Trifluoracetic acid and methanol for RPC were purchased from Carlo Erba (Rodano, MI, Italy). MT-1 (M-5267), MT-2 (M-5392) and MT_{RL} (M-7641) from rabbit liver were purchased from Sigma, where MT_{RL} is a commercial rabbit liver MT preparation not purified by anion-exchange chromatography. The 24–26% hydrazine standard solution (53847) was purchased from Fluka Chemie and the optimized concentration added to $NaBH_4$ solution containing 0.3% m/v of NaOH.

The optimized operating conditions adopted for on line digestion and determination of PHMB in the presence of methanol resulted analogous to those reported for the presence of urea in the eluent phase and they have been previously described [3].

Water deionized with a Milli-Q system (Millipore, Bedford, MA, USA) was used throughout.

Denatured MT stock solutions (1 mg ml^{-1}) were prepared by dissolving lyophilized powder in PBS, 8.0 mol l^{-1} urea (1 mg ml^{-1}) containing about a 20 molar excess of PHMB, at 25 °C. After a reaction time of 50 min at room temperature (20 ± 1 °C) [3], the stock solution was stored at 4 °C in the dark and diluted in 0.1 PBS, pH 7.2, 8.0 mol l^{-1} urea before use. In denatured conditions the PHMB-complexed MT stock solutions were stable within 3 days from preparation.

All the solutions were filtered before injection by a 0.45 μm cellulose acetate filter (Millipore).

A narrowbore HPLC gradient pump (P4000, ThermoQuest) equipped with a mechanical degassing system (SC1000, ThermoQuest) and with a Rheodyne 7125 injector (Rheodyne, Cotati, CA, USA) and a 85 μL injection loop was used. A diode array detector (DAD, UV6000, ThermoQuest) equipped with a flow cell with a 5 cm path length was employed at the end of the column, just before and in series with the CVGAFS detection system. This detection system (DAD-CVGAFS) allowed the simultaneous acquisition of UV–vis absorbance and mercury-specific chromatograms.

Separations were carried out by a C_4 Vydac (code 214TP5415) 150 mm × 4.6 mm RP column (silica particle size 5 μm, porosity 300 Å). The pump flow was 1.0 mL/min. Samples were eluted with a 25 min linear gradient from 100% A (95% of a 0.1% TFA solution, 5% MeOH) to 50% B (95% MeOH, 5% of a 0.1% TFA solution. Ten minutes of isocratic elution in 100% B was applied after each run in order to remove the residual PHMB adsorbed to the stationary phase,

followed by a re-equilibration step in 100% A solution. All the solutions were filtered by a 0.45 μm cellulose acetate filter (Millipore).

The schematic diagram of continuous flow (CF) mercury chemical vapour generator modified for on line digestion of organic mercury in a miniaturized Ar/H_2 flame and the laboratory assembled NDAF detector have been previously reported [2,3,7].

3. Results and discussion

3.1. Study of the effect of methanol on the on line reduction of Hg(II) by FIA-CVGAFS experiments and optimization of operating parameters for the on line digestion of PHMB with bromine in medium containing methanol

Methanol and acetonitrile are the most widely used mobile phases in RPC. However, in the case of CVGAFS detection we found in preliminary experiments that the employment of methanol gives a S/N ratio about 10 times better than acetonitrile. For this reason methanol was selected for RPC-CVGAFS experiments.

Experiments were performed by FIA-CVGAFS apparatus in order to study the effect of methanol on the sensitivity of the determination of inorganic mercury and on detection limit. It has been observed that methanol concentrations $\leq$50% slightly affect the Hg(II) reduction, giving an AF signal $\geq$85% with respect to the value obtained in the absence of methanol. For higher percentages the signal decreases up to about the 55% when the methanol concentration is 100%. The detection limit (LOD_C) for the determination of inorganic mercury in PBS without and with 30 and 50% of methanol was 6.3, 9.0 and 1099×10^{-10}, respectively, and the coefficient of variation (CV%) was 1.1, 5.5 and 6.8%, respectively. The higher LODc found in the presence of 50% of methanol is due to the worst baseline noise and sensitivity. The factor contributing to sensitivity loss could be due to the quenching effect arising from the flame composition generated by methanol combustion.

In order to optimize the reductant concentration, Hg(II) standard solutions were analysed without and with 30 and 50% (curve C) methanol, by varying the $NaBH_4$ concentration in the 10^{-11} to 10^{-1} mol l^{-1} range (*reduction curve*) [8] (data not shown for brevity). We observed that while the C_{50} values (concentration of reducing agent needed to obtain the 50% of the total mercury reduction) in the absence and in the presence of 30% of methanol are not significantly different ($C_{50,PBS} = 2.9 \pm 0.1 \times 10^{-9}$, $C_{50,MeOH\,30\%} = 3.4 \pm 0.8 \times 10^{-9}$), the C_{50} in the presence of 50% of methanol is significantly higher ($C_{50,MeOH\,50\%} = 3.3 \pm 0.4 \times 10^{-8}$), indicating a poorer effectiveness of $NaBH_4$ in reducting Hg(II) in the presence of the organic solvent. It cannot be excluded that methanol varies the yield of mercury reduction. Indeed, it is known that the methanolysis of $NaBH_4$ in 100% methanol is one order of magnitude faster than hydrolysis [9].

A concentration of 2.6×10^{-4} mol l^{-1} (0.01 gr l^{-1}) $NaBH_4$ was chosen for the calibration experiments.

The slopes of linear fitting of calibration data of Hg(II) in PBS, without and with 50% methanol were 0.265 ± 0.003 ($N = 5$, $R = 0.999$) and 0.228 ± 0.002 ($N = 5$, $R = 0.995$), respectively, showing about a 15% of sensitivity lost.

On line digestion of PHMB with bromine in medium containing methanol was optimized by performing experiments with standard solutions containing inorganic Hg(II) and uncomplexed PHMB (25 nmol l^{-1}) dissolved in PBS without and containing 50% methanol.

As observed in the case of a matrix containing 8.0 mol l^{-1} urea [3], the employment of 3.5 mol l^{-1} HCl and 4.5×10^{-2} mol l^{-1} Br_2 allowed a PHMB recovery equal to 98 $\pm$ 2%. These conditions were chosen for on line digestion. By using these conditions a quantitative recovery was also verified and obtained for PHMB complexed to proteins. 0.1 mol l^{-1} N_2H_4 were added to $NaBH_4$ solution in order to reduce the excess of Br_2.

$NaBH_4$ concentration was optimized by performing reduction curves analogous to those described for Hg(II) reduction. PHMB reduction curves in absence and in presence of 50% methanol gave a sigmoidal trend analogous to those obtained for inorganic mercury, indicating the complete conversion of PHMB in Hg(II) in the adopted oxidising conditions. A concentration of 2.6×10^{-4} mol l^{-1} (0.01 g l^{-1}) $NaBH_4$ was chosen for all the experiments.

3.2. Analysis of commercial metallothioneins (MTs) from rabbit liver by RPC-DAD-CVGAFS

The optimized on line digestion of PHMB and CVGAFS detection has been applied to determination and characterization of commercial MTs from rabbit liver. The only available commercial MTs are isolated from rabbit liver (MT_{RL}) by size exclusion chromatography and their isoforms MT-1 and MT-2, of typical mammalian origin, are purified further by anion exchange chromatography [10]. Despite this, six rabbit liver MT isoforms have been isolated and sequenced in the literature, characterized by different amino acid composition, different isoelectric points and hydrophobicities [11].

Reversed-phase chromatography was shown to discriminate MT-1 and MT-2 isoforms and several peaks (termed subisoforms) within the MT-1 and MT-2 classes [12]. However, characterization of MT polymorphism by RPC has been based on the retention time and on the intensity of the UV absorption which has led to speculative, confusing and often contradictory data because of the impossibility of knowing what species was detected [13]. The likely reason for this is the presence in the sample of products of MT oxidation (i.e. dimers or polymerized products [14]), of different conformations or different metal composition [13]. Thus, the absence of standards of sufficient and documented purity makes the unambiguous identification of an MT-species in an HPLC

eluate impossible without tedious isolation and off-line sequencing or the employment of powerful, expensive hyphenated instrumentation like liquid chromatography coupled to mass spectrometric (MS) detectors [13]. Despite uncertain purity and identity of the commercial MT_{RL}, MT-1 and MT-2 samples, and the significant batch-to-batch variation, due to the described issues, these samples are, at present, widely used as reference samples [12].

In this framework, because of the importance of characterizing individual MT subisoforms also in real raw samples, the RPC-CVGAFS method has been applied to the analysis of commercial MT_{RL}, MT-1 and MT-2 samples, denatured in 8.0 mol l^{-1} urea and derivatized pre-column by PHMB. This procedure has been choose for several reasons. First, derivatization of MTs with PHMB blocks, in principle, –SH groups avoiding or, at least, controlling their oxidation. Second, the protein denaturation increases the number of –SH groups reactive with PHMB, making them accessible to the mercurial probe, thus improving the analytical sensitivity and the detection limit of the method [3]. Third, the derivatization of denatured MTs with PHMB, should avoid the artefacts due to the differently metallated, or not completely demetallated known isoforms. In fact, because of the high affinity of the PHMB for –SH groups, it displaces and substitutes all the metals originally present (Cd and Zn). Finally, the MT/PHMB complexes are stable at acid pH, thus the problem of the pH-dependent complexation equilibria is also controlled.

It has to be noted that the replacement of endogenous metals by PHMB causes a structural change, likely because of the different hydrophobicity [11]. In the case of MT_{RL} the chromatographic analysis with UV–vis detection of the denatured, *uncomplexed* sample in the same separation conditions adopted in this work are totally eluted in the dead volume of the column likely because of the presence of the chaotropic agent (data not shown for brevity). Thus, no chromatographic peaks are observed in correspondence of the denatured, PHMB-complexed MT peaks.

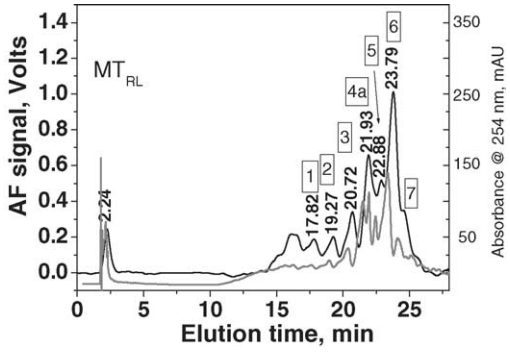

Fig. 1. RPC-DAD-CVGAFS chromatographic profiles of MT_{RL}, denatured and complexed with PHMB (2.8 μg injected): CVGAFS detection (black line, left axis); UV absorption detected at 254 nm (grey line, right axis). Proteins have been treated with a 20 time molar excess of PHMB. Chromatographic conditions: 25 min linear gradient from 100% A (95% of a 0.1% TFA solution, 5% MeOH) to 50% B (95% MeOH, 5% of a 0.1% TFA solution (flow rate = 1 ml/min).

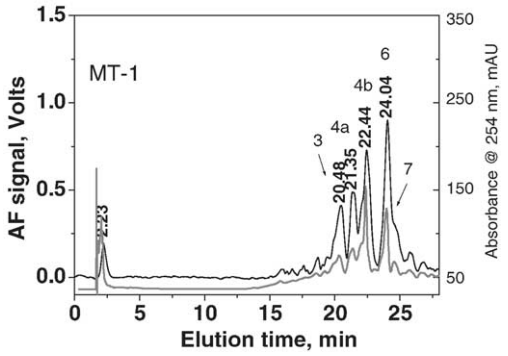

Fig. 2. RPC-DAD-CVGAFS chromatographic profiles of MT-1 isoform, denatured and complexed with PHMB (2.8 μg injected): CVGAFS detection (black line, left axis); UV absorption detected at 254 nm (grey line, right axis). Proteins have been treated with a 20 time molar excess of PHMB. Chromatographic conditions: see Fig. 1 caption.

In Figs. 1–3 the black lines show mercury-specific chromatograms obtained for MT_{RL}, MT-1 and MT-2, respectively, denatured in PBS, 8.0 mol l^{-1} urea and derivatized by PHMB, processed by the RP-DAD-CVGAFS apparatus. RP-DAD-CVGAFS experiments were performed by injecting the protein/PHMB solution onto a C_4 Vydac RP column (2.8 μg of protein injected in each run). In this column the excess of PHMB did not interfere with protein separation/identification, as it is eluted in a broad band between 12 and 30 min, and it has been easily subtracted as baseline in all the CVGAFS chromatograms. Thus, a moderate excess of PHMB can be used without the need of removing the unreacted fraction. In the same figures the grey lines show the UV absorbance chromatograms at 254 nm of the same samples, acquired simultaneously with mercury-specific traces. The CVGAFS chromatogram is delayed of about 30 s with respect to the UV absorbance chromatogram because of the system configuration [3].

CVGAFS chromatogram of MT_{RL} (Fig. 1) shows six fractions (arbitrary marked in the chromatograms 1, 2, 3, 4a, 5, 6) with a shoulder around 24.5 min (marked as 7). Between

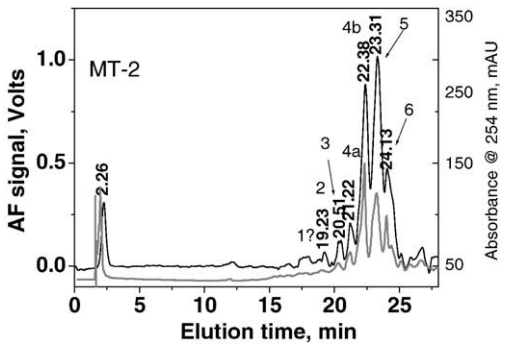

Fig. 3. RPC-DAD-CVGAFS chromatographic profiles of MT-2 isoform, denatured and complexed with PHMB (2.8 μg injected): CVGAFS detection (black line, left axis); UV absorption detected at 254 nm (grey line, right axis). Proteins have been treated with a 20 time molar excess of PHMB. Chromatographic conditions: see Fig. 1 caption.

these, the 4a and 6 peaks are the major peaks in the CVGAFS chromatogram. The morphology of the chromatogram with UV detection is similar to that of AFS chromatogram, although two more peaks identified as 4b and 7, not resolved in the CVGAFS absorbance chromatogram, are present.

Although the number of peaks is similar to that reported by other Authors [15], the chromatografic profile is different, as expected, being the proteins denatured and complexed with the mercurial probe.

MT-1 CVGAFS chromatogram (Fig. 2) shows four major peaks at 20.5, 21.4, 22.4 and 24.0 min and a shoulder around 24.2 min. These major peaks have been named 3, 4a, 4b and 6, respectively, on the basis of their retention time, compared with the peaks observed in MT_{RL} chromatogram. It has to be noted that although the UV absorbance chromatogram has a similar aspect, the proportion between 4b and 6 peaks is not the same. This is an interesting point to discuss. In fact, while the ε_{254} value may depend on the functional group environment and solvent composition, the AF signal depends only on the number of complexed molecules of PHMB.

MT-2 CVGAFS chromatogram (Fig. 3) shows three major peaks at 22.4 (4b), 23.3 (5) and 24.1 min (6) and two minor peaks at 20.5 (3) and 21.2 min (4a). As in the case of MT-1, the CVGAFS and the UV absorbance chromatogram differ in the proportion between peaks, being the peak 5 higher with respect to the other peaks in the CVGAFS chromatogram, but not in the UV absorbance chromatogram.

It is interesting to observe that all the MT-1 fractions (3, 4a, 4b and 6) are found in MT_{RL} and MT-2 chromatograms. Fraction 5 and the minor peaks 1 and 2, instead, are characteristic of MT-2 CVGAFS chromatogram. However, in MT_{RL} chromatogram fraction 5 has a significantly different retention time (22.9 instead 23.3 min), probably because of the co-elution with the 4b fraction. It is worth noting that, despite the excess of MT-2 in MT_{RL} preparation (MT_{RL} samples are known to contain a four-fold excess of MT-2 with respect to MT-1 [16]), which should justify a similarity in chromatographic profiles, MT_{RL} and MT-2 chromatograms are different. It is likely to hypothesize that the further purification step by anion exchange chromatography of the commercial MT-2 preparation induces a rearrangement in the MT sub-isoforms. This hypothesis is supported by the following experiment.

Fig. 4A shows the RPC-CVGAFS chromatographic profile of the collected fraction between 18 and 25 min of PHMB-complexed MT_{RL} eluted from a chromatographic experiment analogous to that of Fig. 1 (chromatogram acquired only with the UV absorbance detection), and re-injected in the same conditions (about 80 ng injected, considering the dilution factor). It has to be noted that no signal has been obtained by UV detector, due to its lower sensitivity with respect to CVGAFS detector. The baseline noise in the final part of the chromatogram is due to the methanol in the eluent phase. In Fig. 4A, 4a, 5 and 6 fractions can be well recognized, but globally the chromatographic profile is different from that reported in Fig. 1. In Fig. 4B the normalized CVGAFS chromatogram of the MT_{RL} collected fraction (curve

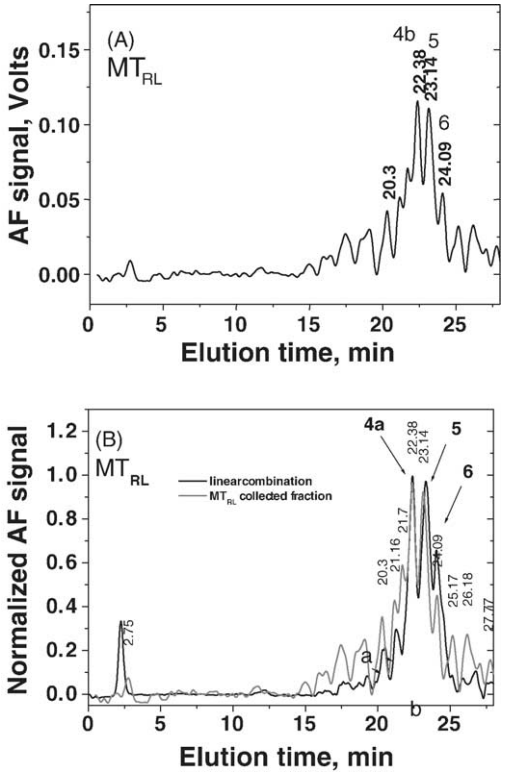

Fig. 4. (A) RPC-CVGAFS chromatographic profile of the collected fraction of PHMB-complexed MT_{RL} eluted from a chromatographic experiment analogous to that of Fig. 4 and re-injected in the same conditions (about 80 ng injected, considering the dilution factor). (B) Comparison of the normalized CVGAFS chromatogram of the MT_{RL} collected fraction (curve a) with the normalized theoretical chromatogram (curve b) resulting from the linear combination of the RPC-CVGAFS profiles of MT-1 and MT-2 samples, taking into account the four-fold excess of MT-2 with respect to MT-1. Chromatographic conditions: see Fig. 1 caption.

a) is compared with the normalized theoretical chromatogram (curve b) resulting from the linear combination of the RPC-CVGAFS profiles of MT-1 and MT-2 samples, taking into account the four-fold excess of MT-2 with respect to MT-1 [16]. The good correspondence between the two profiles demonstrates the effect of the further purification step on the conformation of MT isoforms and sub-isoforms.

3.3. Determination of complexed –SH groups in MT-1, MT-2 and MT_{RL}

Considering that the recovery of inorganic mercury (II) from both uncomplexed and complexed PHMB did not differ significantly, the number of MT –SH groups reactive with PHMB can be easily calculated by the RPC-CVGAFS on the basis of the slope of PHMB calibration curve and MT peak area. In conjunction or alternatively to PHMB calibration curve, the sensitivity factor for PHMB detection can be estimated by injecting at the end of each run in the final eluent buffer a known concentration of PHMB as reference. Both the calibration curve and/or the reference signal of PHMB is injected in 95% methanol (final buffer) in order to obtain a

single peak, for whom the area can be accurately calculated. By adopting this procedure the lost in sensitivity of detection of PHMB in 95% methanol with respect to 25–50% methanol, in which MTs are eluted, has to be taken in account (about 65% less on the basis of the data reported in the previous paragraph).

Calibration experiments of uncomplexed PHMB and of all the complexes examined (MT-1/PHMB, MT-2/PHMB, MT_{RL}/PHMB), were performed by plotting the areas of PHMB or protein/PHMB peak as a function of PHMB or protein concentration injected (five replicates). The total PHMB complexed to MT isoforms and sub-isoforms has been calculated instead of the PHMB associated to each peak because of the uncertainty in the assignment of each peak to a specific isoform. The slope of calibration curve was 0.11 ± 0.004 (R = 0.9999) for PHMB and 0.74 ± 0.03 (R = 0.9999), 1.1 ± 0.03 (R = 0.9998) and 0.74 ± 0.03 (R = 0.9999) for PHMB/MT-1, PHMB/MT-2 and PHMB/MT_{RL} complexes, respectively.

The number of –SH number of MT-1, MT-2 and MT_{RL} was estimated by the ratio of the slope of MT calibration curve and the slope of PHMB calibration curve. It was found that MT-2 sample has 10.0 ± 0.3 –SH groups titrated by PHMB. 6.7 ± 0.3 –SH groups are complexed by PHMB in MT-1 and MT_{RL} samples. These values are determined by the abundance of each sub-isoform and their complexating ratio with PHMB. At least one of these two data has to be known in order to calculate the other. It is interesting to note that the number of thiolic groups titrated in MT-1 and MT_{RL} samples are the same, despite the presence of a four-fold excess of MT-2 with respect to MT-1 in MT_{RL} samples [16].

The detection limit (LODc) for PHMB in 95% methanol in the optimized conditions was about 9.3×10^{-9} mol l^{-1} and for the denatured MTs LODc was calculated to be about 8.6×10^{-10} mol l^{-1}, taking into account the lost of sensitivity in the determination of PHMB in 95% methanol and an approximate complexating ratio PHMB:MTs of 7:1.

3.4. Stability of denatured, PHMB-complexed MT-1, MT-2 and MT_{RL} stock solutions

Despite of the numerous comments regarding the poor stability of MT solutions, no data are shown in literature on the chromatographic behaviour of aged MT stock solutions. This aspect, in Author opinion, has to be investigated because despite careful precautions against oxidation during sample preparation, stable covalent dimers of native MTs have been detected [14], and the evolution in time of MT stock solutions becomes a fundamental information in the identification/quantitation of MTs in real samples.

A preliminary study on complexation by PHMB of native MT_{RL} solutions did not allow us to obtain reproducible chromatographic data neither during the working day, both in term of number of thiolic group titrated and chromatographic profiles. This can be presumably due to the slow exchange reaction of PHMB with endogenous metals in native MTs and/or to their rearrangement or thiol group oxidation [17], but a focused study of this part it was beyond the aim of this paper.

The RP chromatograms shown in Figs. 1–3, obtained from denatured, PHMB-complexed MT samples were reproducible since about 1 h after preparation of the stock solution and within 3 days from preparation. After this time we found chromatographic evidences that MT stock solution evolves.

Fig. 5A–C show RPC-DAD-CVGAFS chromatographic profiles of denatured and PHMB-complexed MT_{RL}, MT-1 and MT-2 solutions, respectively prepared from a 3-week aged stock solution. Substantial differences are observed both in mercury-specific and UV absorbance chromatographic profiles between "fresh" and aged samples in term of number of peaks and intensity. Essentially, three fraction (4a, 5 and 6) are resolved in MT_{RL}, MT-1 and MT-2 samples. Their reciprocal intensities are similar in UV absorbance chromatograms

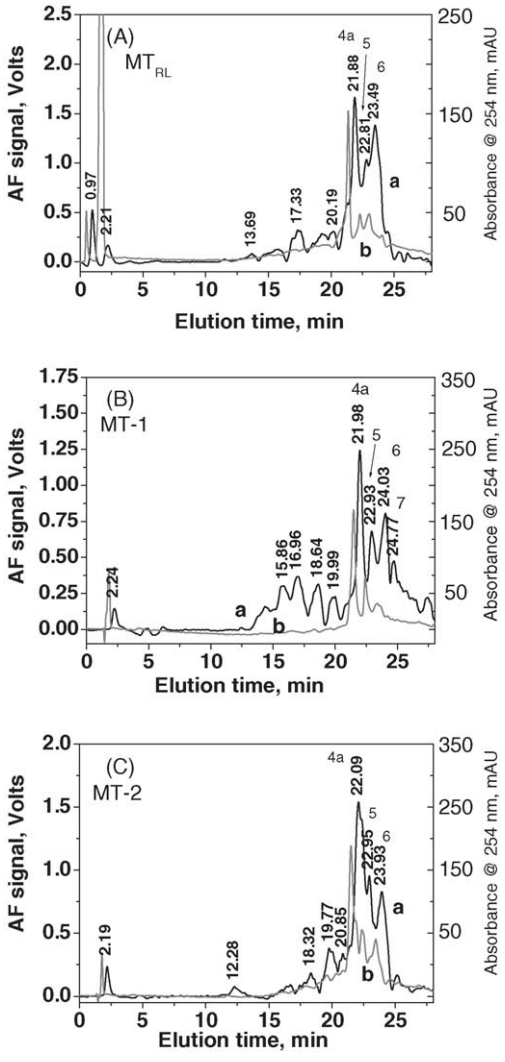

Fig. 5. RPC-DAD-CVGAFS chromatographic profiles of denatured and PHMB-complexed MT_{RL} (A), MT-1 (B) and MT-2 (C) and solutions (2.8 μg injected) prepared from a 3-week aged stock solution. (a) CVGAFS chromatogram; (b) UV absorption chromatogram detected at 254 nm. Proteins have been treated with a 20 time molar excess of PHMB. Chromatographic conditions: see Fig. 1 caption.

(b curves), and significantly different in CVGAFS profiles (a curves). In particular for MT_{RL} (Fig. 5A) and MT-1 samples (Fig. 5B), CVGAFS and UV profiles once again differ: the peaks 5 and 6 are higher in CVGAFS than in UV absorbance chromatogram. It can be hypothesized that aged MT solutions evolve toward three stable sub-isoforms. Between these, the extinction coefficient at 254 nm is different, probably because of the different accessibility of R-S-Hg-R chromofore to the solvent of 5, 6, and 4a fractions.

The number of –SH groups titrated in the aged samples was also estimated. We found 1.8 ± 0.2, 4.6 ± 0.3 and 5.0 ± 0.2 –SH groups complexed with PHMB in MT_{RL}, MT-1 and MT-2 samples, respectively. The decrease with respect to the fresh solutions could be due to a rearrangement of the protein structure in which intra- and inter-molecular reactions between –SH groups could give rise to a release of PHMB and the disulfide bond formation. It is worth noting that the decreases in the number of PHMB-complexed –SH groups is bigger for MT_{RL} than for MT-1 and MT-2 isoforms. An hypothesis could be that the simultaneous presence of MT-1 and MT-2 isoforms in the MT_{RL} sample favourites the exchange reactions of PHMB and thiolic groups, their rearrangement or oxidation. On the other hand, the described and well known instability of MTs may be correlated to their well known physiological role of transferring metals between proteins in cells [18].

Finally it could not be a coincidence that 4a, 5 and 6 fractions are the same found in the CVGAFS chromatogram of the MT_{RL} sample collected and re-injected (Fig. 4A and B), and that the number of titrated –SH groups in the sample of Fig. 4 was 1.7. The isoforms or sub-isoforms corresponding to these fractions could be the same stable fractions found in aged MT solutions.

4. Conclusion and perspectives

Derivatization of –SH groups of MTs with PHMB followed by RPC-CVGAFS analysis has proven to be an original and reliable tool for determination and characterization of denatured MTs commercial samples.

Although the proposed method does not allow the exact peaks assignment (peak authentication) with respect to the different isoforms and sub-isoforms, such as in LC–MS, it allows the selective and sensitive detection of MTs by employing a low cost instrumentation and it is promising for the quantitation of MTs in real raw samples. Nevertheless, the LC–MS analysis of PHMB-derivatized MTs could give fundamental information on the identity and identification of the single peaks.

The employment of denaturing conditions in the sample preparation step extends the applicability of the method to the analysis of MTs in proteic complexes matrices, often not easily solubilized in native conditions, such as raw (e.g. seeds) and processed foods (e.g. flours), destined to human consumption. Indeed, MTs both represent early warning molecular indicators of the biological effects of heavy metals in many matrices [19–22], and they have a role in nutrition (e.g. Zn absorption) [23].

Finally, as seed storage proteins of soy, corn and wheat have a high content of metal complexating amino acids (Cys and Met), the application of this technique coupled with PHMB derivatization procedure can be extended to these samples, informing on transportation of heavy metal pollution in the food chain.

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