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Journal of Chromatography B, 796 (2003) 379–393
JOURNAL OF CHROMATOGRAPHY B
www.elsevier.com/locate/chromb

Characterization of metallothionein isoforms from rabbit liver by liquid chromatography coupled to electrospray mass spectrometry☆

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Received 26 March 2003; received in revised form 14 August 2003; accepted 18 August 2003

Abstract

Metallothioneins (MTs), a group of low molecular weight proteins found in practically all life forms, are characterized by high sulfur content and an affinity for metal ions. At acidic pH, MTs show metal depletion, leading to apothioneins. In the work described here, in order to optimize the separation of rabbit liver apothioneins using liquid chromatography (LC) with UV detection, the proportion of the organic modifier of the mobile phase was optimized by establishing relationships between Reichardt's E_T^N scale of solvent polarity and the chromatographic retention measured by the capacity factor, *k*. Additionally, such optimum separations were carried out in a LC–electrospray mass spectrometry (ES-MS) coupled system allowing the identification and characterization of the different rabbit liver apo-MT-forms. In this way, electrospray ionization mass spectrometry offers great possibilities aiming at a better understanding of metallothionein polymorphism.

Keywords: Metallothioneins

1. Introduction

Metallothioneins (MTs) are a group of low molecular weight (6–7 kDa) non-enzymatic proteins characterized by high sulfur content and affinity for metals. Their special properties have made these proteins of particular interest to chemical, biological, medical, and toxicological sciences. This active and interdisciplinary work is reflected in a number of meetings, monographies and reviews [1–7]. MTs have been found in practically all life forms and have been isolated from different organs such as liver, kidney and brain. Their high number of cysteines (20 residues in mammals) explains their high capacity of binding metal ions through sulfhydryl groups, forming metal-thiolate complexes. As a consequence, neither disulfide or free sulfhydryl groups are present in the MT structure [8]. Mammalian metallothioneins, the best known, are either 61 or 62 non-aromatic amino

☆ Presented at the Second Meeting of the Spanish Society of Chromatography and Related Techniques, Barcelona, 26–29 November 2002.

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1570-0232/$ – see front matter © 2003 Published by Elsevier B.V.
doi:10.1016/j.jchromb.2003.08.024

acids in length with their N-terminal residues acetylated. Their induced biosynthesis is stimulated by the exposure of living organisms to a wide range of metal ions such as zinc, copper or cadmium, but also by organic compounds, hormones and even stress situations. Thus, the main functions proposed derive from their role in homeostatic control, metabolism, and detoxification of a number of essential (Cu, Zn) and toxic (e.g. Cd, Hg) trace metals [9]. This known fact forms the basis of the use of MTs as possible biomarkers for metal exposure [10].

Metal ions in MTs are distributed in two separated clusters, named α and β domains. The α unit consists of a four-metal cluster binding eleven cysteines and the β domain comprises nine cysteines coordinating three divalent cations [11]. The exposure of metallothioneins to acidic pH values causes metal depletion and unfolding of the protein structure, generating apothioneins, that means metal-free forms. Metallothioneins are heterogeneous proteins, due not only to their different metallic composition but also to an intrinsic polymorphism present in their primary structure. Historically, MTs are classified into two main groups of isoforms according to their elution order in anion-exchange chromatography as MT-1 and MT-2. In mammals, this conventional nomenclature division based on charge differences refers actually to the absence (MT-1) or presence (MT-2) of an acidic amino acid residue in position 10 or 11 of the sequence [12]. Isoforms with minor differences, such as one amino acid residue, were detected as subgroups of the two major isoforms and are termed subisoforms. Since various subisoforms are biosynthesized differentially, they could play a specific biological role, but this aspect still remains unknown. This polymorphism leads to the need for constant development of powerful separation techniques that enable the study and understanding of the importance of individual MT subisoforms.

High performance liquid chromatography (LC) and capillary electrophoresis (CE) are analytical techniques used commonly in MT research [13–15]. Both are capable of separating subisoforms on the basis of the differences between retention times in liquid chromatography or migration times in capillary electrophoresis. Some modalities of liquid chromatography, such as size exclusion chromatography, fast protein liquid chromatography, and reversed phase liquid chromatography, have been developed successfully in many MT studies [16–20] and applied to the analysis of MT isoforms isolated from different animal tissues [21–24]. Within the latter, reversed phase liquid chromatography is the most suitable technique for MT subisoform separations, resolving peptides whose composition differs only in one amino acid.

Mobile phase composition has a primary role in the retention of compounds in LC. One approach for the development of analytical procedures is the application of experimental methods that can predict the chromatographic behavior of substances as a function of the percentage of the organic component in the eluent. To optimize the composition of the mobile phase, the linear solvation energy relationship (LSER) method, which is based on the Kamlet–Taft multiparameter scale, was used in the same way as in previous works [25–30]. The LSER approach applied to chromatographic processes [31–33] correlates retention parameters of solutes with characteristic properties of solutes and both stationary and mobile phases. This correlation can be expressed as follows in a system with a fixed pair of solute and stationary phase (Eq. (1)):

$$\log k = (\log k)_0 + s\pi^* + a\alpha + b\beta \quad (1)$$

where the coefficients $(\log k)_0$, s, a and b depend on the solute and the stationary phase properties and π^*, α, and β are the solvatochromic parameters used to evaluate solvent dipolarity/polarizability, hydrogen-bond acidity, and hydrogen-bond basicity, respectively [34]. Taking into account that the values of β are constant over most of the acetonitrile composition range [34,35] and the parameter E_T^N [34], π^* and α correlate according to $E_T^N = 0.009 + 0.415\pi^* + 0.465\alpha$ [36], Eq. (1) can be reduced to Eq. (2) as follows:

$$\log k = C + eE_T^N \quad (2)$$

This relation has been applied in previous studies for predicting the retention of analytes with very different structural characteristics [25–30]. Therefore, the method of linear solvation energy relationships can predict the chromatographic retention of metallothionein subisoforms to achieve a fast optimization of the separation process.

In most cases, UV absorption has been the detection system of choice in metallothionein chromatographic analysis. However, these approaches often lead to contradictory and speculative data because of the impos-

sibility of knowing what species have been detected. Furthermore, metallothionein standards of sufficient purity are not available; hence, the unambiguous identification of MT species in an LC system ends up being non-viable without the coupling with a specific detector. As an alternative to UV detection, different coupled techniques with LC have been developed. The combination with atomic absorption (AAS) [37,38] inductively coupled plasma emission (ICP-AES) [39] or inductively coupled plasma-mass spectrometry (ICP-MS) [40–42] provides information about metallic content in speciation of MTs, but requires neutral pH values in the chromatographic system. The introduction of electrospray mass spectrometry (ES-MS) detection for the determination of metallothionein subisoforms allows the study of these proteins at acidic and neutral pH values, and the identification and characterization of each chromatographic peak.

ES-MS has been widely employed in rabbit liver metallothionein analysis for the study of metal complexes in native and reconstituted MTs by direct infusion [43,44], while an exhaustive study of their polymorphism can be achieved in terms of the coupling with liquid chromatography LC–ES-MS [45,46]. Most works are focused on the characterization of MT complexes at neutral pH values, but the spectra obtained with these conditions present lack of sensitivity, poor signal-to-noise ratio, and numerous artifacts [47]. Van Vyncht et al. [48] solve these difficulties using acidic pH values in the mobile phase and Chassaigne and Lobinski [49] propose post-column acidification after the chromatographic separation, both with the aim of characterizing rabbit liver apothioneins.

The goal of the present work was two-fold: first, in order to optimize the separation of rabbit liver apothioneins using liquid chromatography with UV detection, the proportion of the organic modifier of the mobile phase was optimized by establishing relationships between Reichardt's E_T^N scale of solvent polarity and the chromatographic retention measured by the capacity factor, k; second, such optima separations were carried out in a LC–ES-MS coupled system, allowing the identification and characterization of the different rabbit liver apo-MT-forms. In this way, electrospray ionization mass spectrometry offers great possibilities aiming at a better understanding of metallothionein polymorphism.

2. Experimental

2.1. Chemical and reagents

Acetonitrile (MeCN) (Merck, Darmstadt, Germany) was of LC grade. Water with a conductivity lower than 0.05 μS/cm, obtained using a Milli-Q water purification system (Millipore, Molsheim, France), was employed. Trifluoroacetic acid (TFA) and potassium bromide were analytical grade, obtained from Merck and potassium hydrogenphthalate (dried at 110 °C before use) was a certified standard from Fluka (Buchs, Switzerland). All eluents were passed through a 0.22 μm nylon filter (MSI, Westboro, MA, USA).

Metallothionein preparations MT-1 (lot no. 80K7012), MT-2 (lot no. 80K7013) and MT (lot no. 20K7000) from rabbit liver were purchased from Sigma–Aldrich (St. Louis, MO, USA). Stock solutions of metallothioneins were prepared by dissolving 1 or 2 mg of protein in 1 ml of water. They were stored in a freezer at −18 °C in the dark when not in use.

2.2. Apparatus

For the LC–UV experiments, a 1100 series Agilent Technologies (Waldbronn, Germany) instrument equipped with a quaternary pump, a degasser, a diode array detector and a Rheodyne injection valve with a 20 μl sample loop were used. The chromatographic system was controlled by Chemstation LC 3D Software (Agilent Technologies). A 5 μm LiChrospher 100 RP-18 column (250 mm × 4 mm i.d.) with a 5 μm LiChrospher 100 RP-18 precolumn (4 mm × 4 mm i.d.) from Merck (Darmstadt, Germany) were used at room temperature. The electromotive force (emf) values used to obtain the pH of the mobile phase where measured with a Model 2002 potentiometer (±0.1 mV) (Crison Instruments, Barcelona, Spain) using a Orion 8102 Ross Combination pH electrode (Orion Research, Boston, MA, USA). The potentiometric system was calibrated with a standard reference solution of 0.05 mol/kg potassium hydrogenphthalate [25], whose reference pH values in the acetonitrile–water mixtures studied were previously assigned [50,51], in accordance with IUPAC rules [52].

LC–ES-MS experiments were performed using a Waters Alliance 2690 quaternary solvent delivery system supplied with a degasser and an automatic injection unit and coupled to a VG Platform II single quadrupole mass spectrometer from Micromass (Manchester, UK), equipped with a nebulizer-assisted electrospray source. The high-flow nebulizer was operated in a standard mode with N_2 as nebulizing (20 l/h) and drying (400 l/h) gas. Separation was carried out on a 5 μm Merck LiChrospher 100 RP-18 column (250 mm × 4 mm i.d.) with a 5 μm Merck LiChrospher 100 RP-18 precolumn (4 mm × 4 mm i.d.). The total flow rate was split 10- or 20-fold with a T-piece to allow a suitable flow value in the electrospray source. A Shimadzu SPD-10A UV-Vis detector operating at 200 nm was placed between the column and the T. Post-column flow was added by means of two Phoenix 20 syringe pumps (Carlo Erba Instruments, Milan, Italy) and was mixed with the chromatographic effluent via another T-piece. Instrument control and data analysis were performed using Masslynx application software from Micromass.

2.3. Procedures

2.3.1. LC–UV procedure

For the optimization of the mobile phase composition, different acetonitrile–water mixtures containing 0.05% (v/v) TFA were used. The acetonitrile percentage of these mixtures ranged from 25 to 30% (v/v). The flow rate of mobile phase was 1 ml/min. Retention factors were calculated from $k = (t_R - t_0)/t_0$, where t_R is the retention time of each substance and t_0 is the hold-up time, established for every mobile phase composition using a 0.01% (w/v) potassium bromide solution in water [53]. The retention times and the retention factors of proteins were determined from three injections of 1 mg/ml solution of metallothioneins at each mobile phase composition considered and the diode-array detector was set to monitor the signal at 200 nm. To predict the chromatographic behavior of the substances, the linear solvation energy relationship methodology based on the Reichardt's solvatochromic parameter E_T^N was applied. The pH was measured in the mixed mobile phase in which the chromatographic separation took place, and it ranged from 2.20 and 2.24, taking into account the reference pH values of primary standard buffer solutions for the standardization of potentiometric sensors in MeCN–water mixtures [50,51]. For the MT-1 and MT samples, LC elution gradients were optimized using (A) 0.05% TFA in Milli-Q water and (B) 0.05% of TFA in acetonitrile, in order to minimize analysis times and improve resolution. The remaining experimental conditions were the same described for the isocratic separations.

2.3.2. LC–ES-MS procedure

2.3.2.1. Optimization of the source and analyzer parameters. The source and analyzer parameters were optimized in order to obtain the best signal stability and the highest sensitivity. Optimization was made by means of injections of a MT-2 standard solution (1 mg/ml) without chromatographic column, introducing in the ES source a 10-fold split of the 1 ml/min total flow, and monitoring the peak of the main subisoform of MT-2. Optimum conditions were as follows: capillary voltage, 3750 V; sample cone voltage, 50 V; counter electrode voltage, 300 V; source temperature, 100 °C; ion energy, 4 V; flow rate, 100 μl/min. Fragmentation was negligible under this working conditions, which allowed correct identification of the molecular identities. In order to improve the sensitivity of spectra, post-column additions of several solvents at different flow rates were made. The best results were obtained using acetonitrile at 50 μl/min. For this reason, the final split ratio was 1/20 in order to keep the flow rate value supplied to the ES source at 100 μl/min.

2.3.2.2. Identification of metallothionein subisoforms. For the identification of the subisoforms of the metallothionein samples, 2 mg/ml standard solutions of MT, MT-1 and MT-2 were injected into the LC–ES-MS system, using the optimal chromatographic conditions obtained for each sample with UV detection. The mass spectrometer worked in full-scan and continuous mode from $m/z = 1200$ to 2000, with a quadrupole mass resolution of 1 Da and using a scan and inter-scan time of 2 and 0.2 s, respectively. Positive ion mode was selected, and the +5 and +4 multiply charged ions series for the different metallothionein apo-MT-forms were monitored.

3. Results and discussion

3.1. Optimization of the chromatographic separation of metallothionein samples

In this work, three standards of rabbit liver metallothionein were studied: MT, isolated by size-exclusion chromatography, and the fractions of isoforms MT-1 and MT-2, obtained from the further purification of MT by anion-exchange chromatography by the manufacturer. This purification is allowed because MT-1 and MT-2 have a single charge difference at neutral pH. To simplify the analytical problem, a mobile phase with 0.05% TFA (pH 2.24) was used. Thus, the coordinated metals of the proteins were removed, and the peaks obtained should be apothioneins, subisoforms without metal content. Chromatograms of MT and MT-1 samples yielded seven main peaks, whereas MT-2 isoform has an intense predominant peak and three minor compounds. Peaks observed in chromatograms of these metallothionein samples can be attributed to potential subisoforms, although it is not possible to carry out their identification by UV detection alone.

The retention factor values, k, and their logarithm, $\log k$, were obtained for each component of the three metallothionein samples at different percentages of acetonitrile from 25 to 30%. The contents of acetonitrile assayed in the mobile phases were 25, 27, 29 and 30%. Chromatographic retention was correlated with properties of the hydro-organic mixtures used as mobile phases; that is, the solvatochromic Reichardt's E_T^N parameter whose values for the acetonitrile–water mixtures were obtained from the literature [25] (Table 1). It has been demonstrated in previous studies [25–30] for solutes with very different structural characteristics that this correlation is linear, in a system with a fixed pair of solute and stationary phase, by Eq. (2). The $\log k$ values of the substances studied here were plotted versus E_T^N values of acetonitrile–water systems and good linearity, greater than 0.99 was observed (Table 1). This linearity provides a good tool for predicting chromatographic retention of rabbit liver metallothioneins and hence optimization of eluent composition for best separation. Once linearity between $\log k$ and E_T^N has been proved, only retention data at two mobile phase compositions for each compound are needed to predict k values and therefore selectivity (α) values at any acetonitrile–water composition into the working range.

Table 1
Linear expressions of $\log k$ vs. E_T^N for each chromatographic peak of MT-1, MT-2 and MT samples, and the E_T^N parameter values at the percentages of acetonitrile assayed in the mobile phase

Peak number	Linear relationships, $\log k$ vs. E_T^N	Regression coefficient
MT-1		
1	$\log k = -10.9 + 12.7E_T^N$	0.991
2	$\log k = -12.2 + 14.4E_T^N$	0.991
3	$\log k = -14.1 + 16.7E_T^N$	0.997
4	$\log k = -15.6 + 18.7E_T^N$	0.985
5	$\log k = -22.2 + 26.3E_T^N$	0.999
6	$\log k = -23.1 + 27.4E_T^N$	0.998
7	$\log k = -33.5 + 39.8E_T^N$	0.999
MT-2		
1	$\log k = -14.4 + 16.8E_T^N$	0.992
2	$\log k = -27.0 + 31.8E_T^N$	0.999
3	$\log k = -29.1 + 34.2E_T^N$	0.999
4	$\log k = -26.8 + 31.7E_T^N$	0.998
MT		
1	$\log k = -10.1 + 11.6E_T^N$	0.978
2	$\log k = -17.0 + 19.9E_T^N$	0.999
3	$\log k = -19.3 + 22.5E_T^N$	0.998
4	$\log k = -22.1 + 26.2E_T^N$	0.996
5	$\log k = -25.6 + 30.2E_T^N$	0.997
6	$\log k = -29.3 + 34.5E_T^N$	0.998
7	$\log k = -35.0 + 41.5E_T^N$	0.997

25% (v/v) MeCN: $E_T^N = 0.877$; 27% (v/v) MeCN: $E_T^N = 0.869$; 29% (v/v) MeCN: $E_T^N = 0.862$; 30% (v/v) MeCN: $E_T^N = 0.859$.

In order to examine the accuracy of retention predictions and the quality of metallothionein separations, the selectivity for adjacent peaks was calculated from the predicted k values, obtained from equations of Table 1. Plots of α are shown in Fig. 1, and they conclude that an acetonitrile content of 25% (v/v) provides good chromatographic separations for the three rabbit liver metallothionein samples.

Under these conditions, the analysis of MT-1 sample led to a long retention time for the last peak (appeared at 40 min) while the initial species showed acceptable resolution. For this reason, an additional adjustment of the chromatographic profile was required to minimize the analysis time. Thus, the initial isocratic elution with 25% acetonitrile–75% water (0.05% TFA) for 17 min was followed by a linear gradient—starting from elution of the sixth

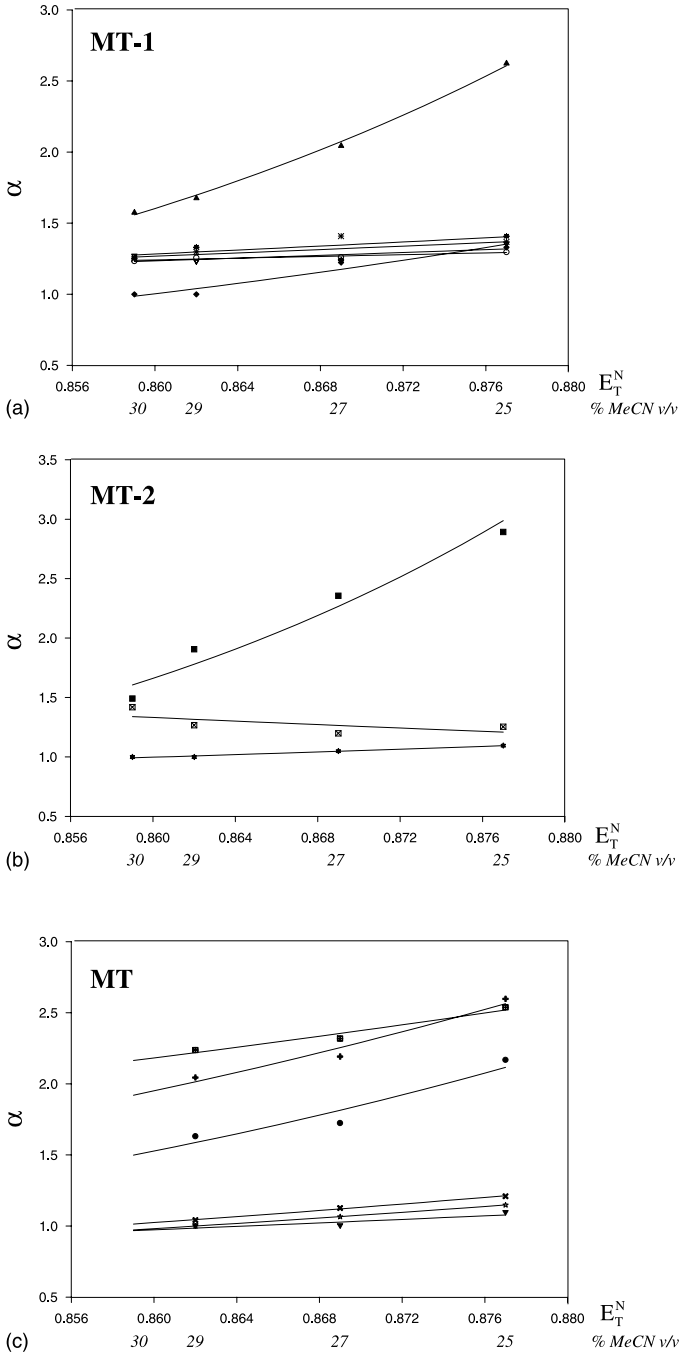

Fig. 1. Plots of selectivity between adjacent peaks vs. E_T^N parameters of the mobile phase. Solid lines indicate theoretical values of selectivity and points indicate the experimental values of selectivity of (a) MT-1 sample peaks: 1/2 (▽), 2/3 (✳), 3/4 (✱), 4/5 (◆), 5/6 (○), 6/7 (▲); (b) MT-2 peaks: 1/2 (■), 2/3 (★), 3/4 (⊠); (c) MT peaks: 1/2 (●), 2/3 (▼), 3/4 (⊞), 4/5 (☆), 5/6 (×), 6/7 (+).

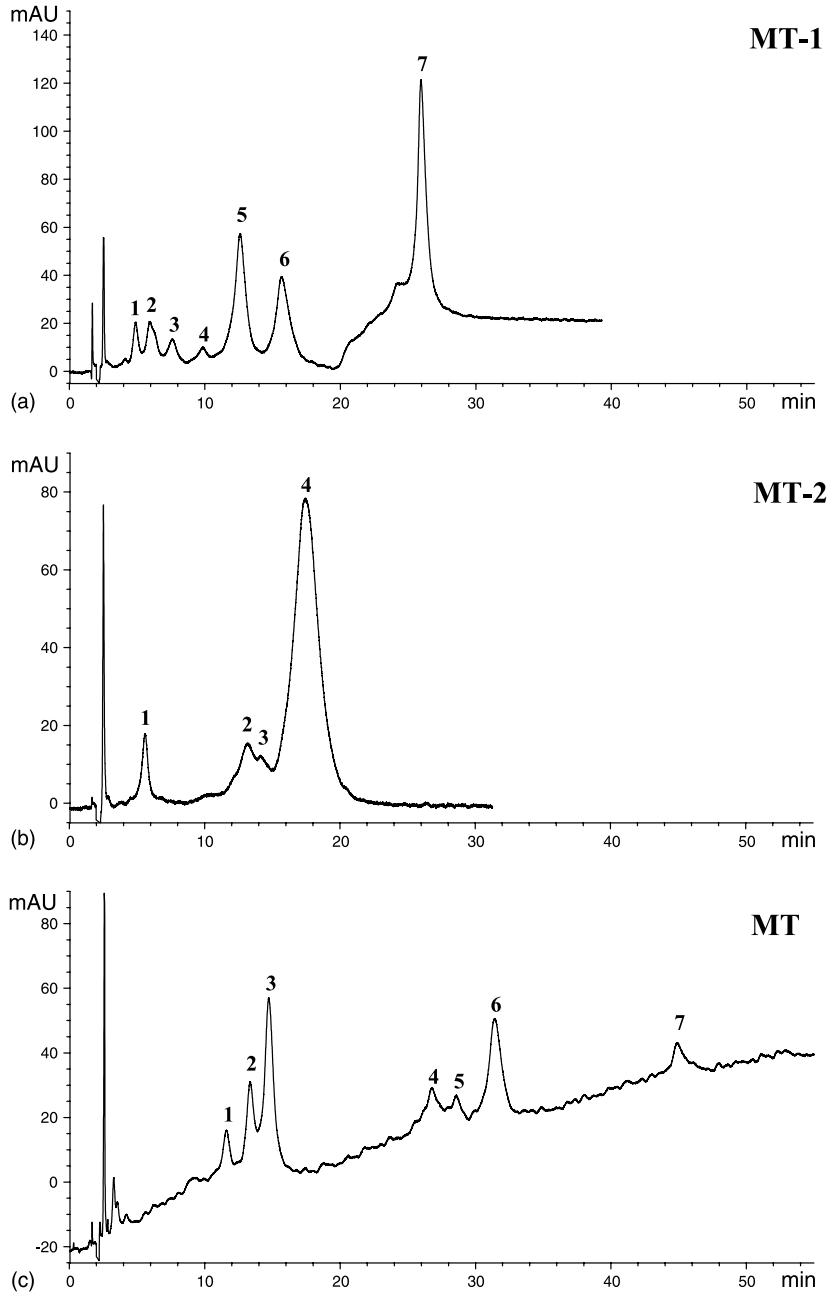

Fig. 2. Chromatograms of rabbit liver metallothioneins obtained by LC with UV detection ($\lambda = 200$ nm) at the optimized conditions: (a) MT-1 1000 ppm sample, gradient elution: 0–17 min, 25% B, 17–22 min, 25–32% B; (b) MT-2 1000 ppm sample, isocratic elution 25% B/75% A; (c) MT 1000 ppm sample, gradient elution: 0–50 min, 22–30% B. The mobile phases are phase A: 0.05% TFA in Milli-Q water and phase B: 0.05% TFA in MeCN.

compound—rising from 25 to 32% acetonitrile (0.05% TFA) over 5 min (Fig. 2(a)). The isocratic separation with a 25% acetonitrile content was chosen for the MT-2 isoform because in this case it provides good resolution. The analysis time was closed to 20 min, as shown in Fig. 2(b). The MT elution carried out at the same percentage of organic modifier (25% acetonitrile) revealed poor resolution within the initial peaks interval and therefore, a composition gradient was applied. To establish the initial step of this composition gradient the relationships between $\log k$ and E_T^N obtained previously were considered. Finally, the MT sample was eluted with a linear gradient from 22 to 30% acetonitrile with 0.05% TFA (v/v) in 50 min (Fig. 2(c)). The analysis time of this gradient profile is similar to that obtained applying the 25% isocratic separation; however, the global resolution was improved. Chromatograms of rabbit liver metallothionein MT-1, MT-2 and MT samples using UV detection at optimal conditions are shown in Fig. 2.

3.2. Characterization of the metallothionein samples by LC–ES-MS at acidic pH

The three metallothionein samples previously studied by LC–UV, were subjected to electrospray mass spectrometry detection, the aim being to identify and characterize putative subisoforms that can be present at chromatographic peaks. To date, six subisoforms of rabbit liver metallothionein have been reported [22], whose amino acid sequences [54,55] are illustrated in Fig. 3. Subisoforms MT-2a and MT-2c contain 62 amino acids whereas the others reveal a structure with 61 units. In our study, the chromatographic separations use TFA in the mobile phase, and this agent provides an acidic pH responsible of metal depletion. Therefore, identification of molecular masses attributable to apo-MT-forms was expected.

3.2.1. MT-1 sample

A total ion current (TIC) chromatogram of MT-1, carried out under the conditions previously optimized by LC–UV, is shown in Fig. 4. The profile of the TIC chromatogram of MT-1 is very similar to that obtained with UV detection, both showing seven peaks. The averaged mass spectra of each whole chromatographic peak are also displayed in Fig. 4. Two clusters corresponding to the ionization states of +5 and +4 can be

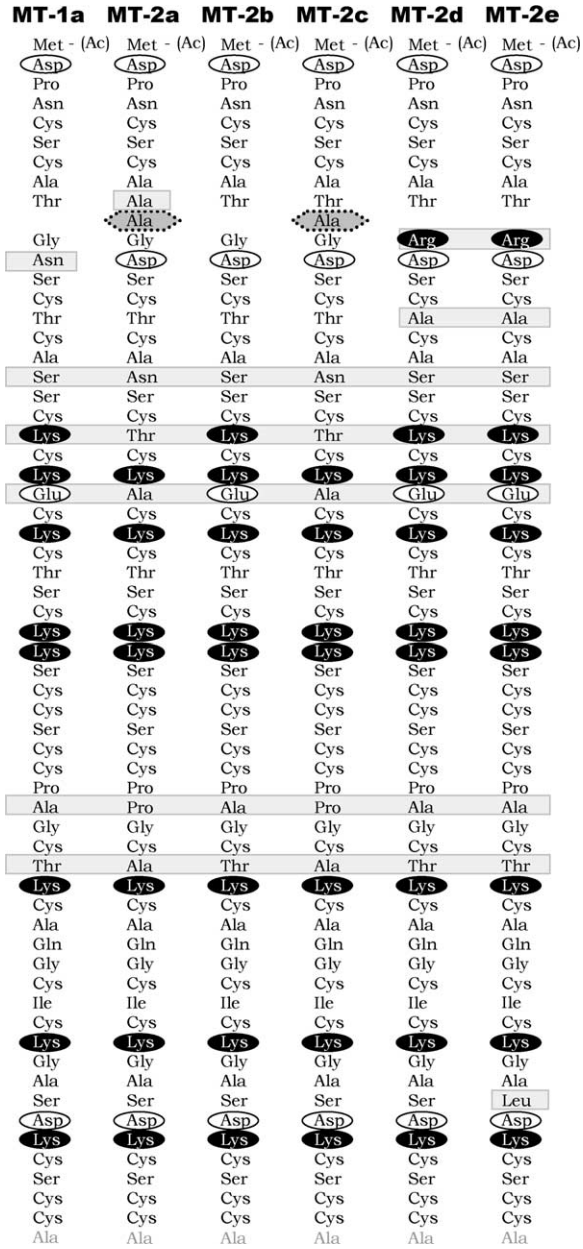

Fig. 3. Primary sequences of the six characterized subisoforms of rabbit liver metallothionein. Amino acids marked with ellipses present charge at neutral pH: ⬭ are negatively charged and ⬬ have positive charge. Differences between primary sequences of subisoforms are enveloped in square sections. The amino acidic compositions of rabbit liver metallothioneins are extracted of Swiss-Prot database [55].

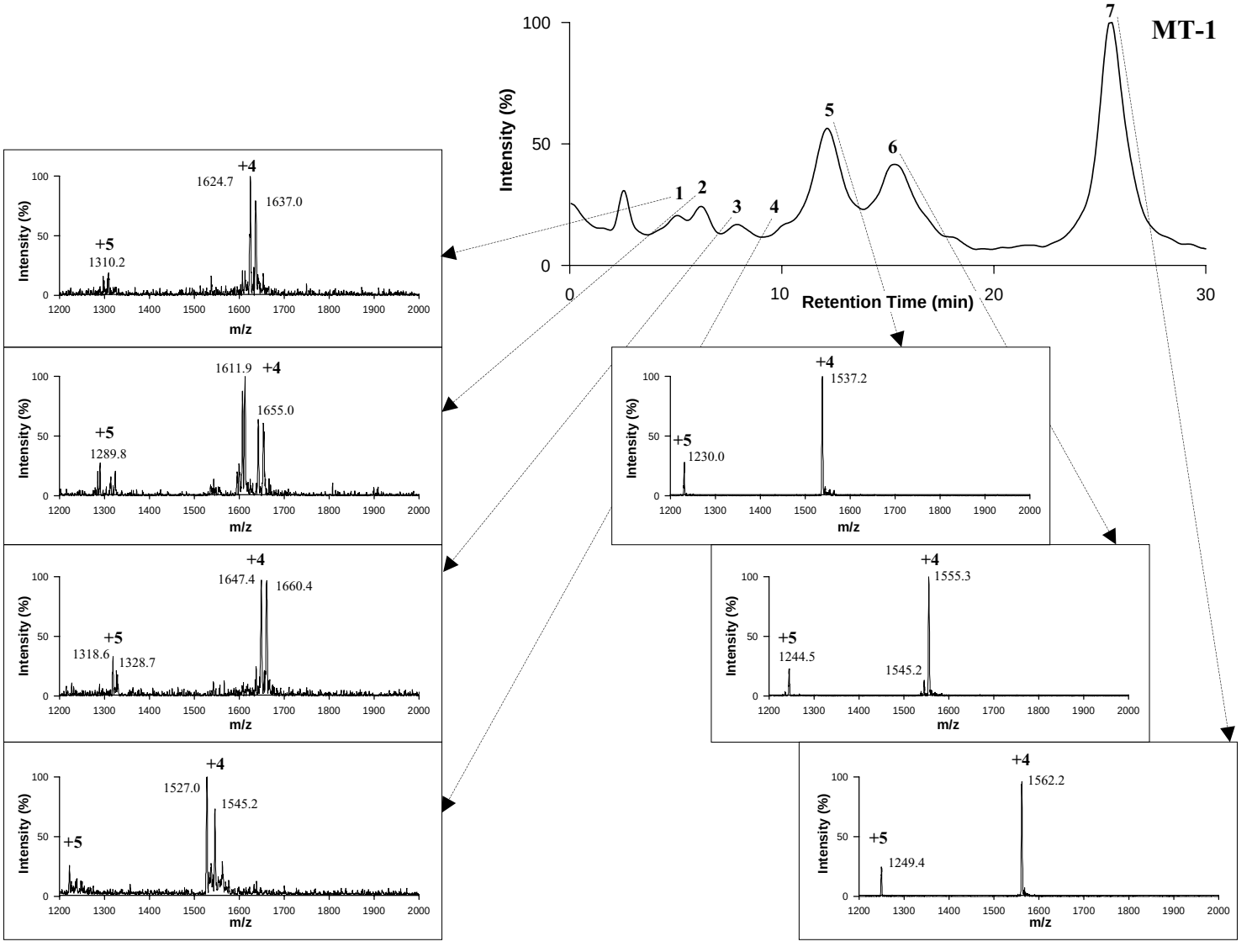

Fig. 4. LC–ES-MS TIC chromatogram of MT-1 carried out at the experimental conditions described in Section 2 and mass spectra registered at each peak.

seen. The molecular masses calculated from the *m*/*z* relationships obtained in the mass spectra of each peak are listed in Table 2 and their probable identification has been included. The uncertainty associated to these calculations ranges between 1 and 5 Da. Some chromatographic peaks can be observed to contain several co-eluting species, especially those that appear at the beginning of the chromatogram. Apo-MT-2d ($m/z = 1555.3$) and apo-MT-2e ($m/z = 1562.2$) are two dominant subisoforms that appear at peaks 6 and 7, respectively. The presence of MT-2 subisoforms in a MT-1 preparation may not be expected because MT-1 and MT-2 fractions should be separated in the anionic exchange purification from MT sample. A more detailed study of MT amino acidic primary sequences shows that MT-2d and MT-2e have the

Table 2
Molecular masses obtained from the *m*/*z* spectra data extracted of each of the peaks present in the TIC chromatogram of MT-1, MT-2 and MT rabbit liver samples and its possible identification

Peak	Retention time (min)	Molecular mass found (Da)	Molecular mass expected (Da)	Identification
MT-1				
1	5.1	6494.8		
		6545.0		
2	6.2	6443.8	6442.0	Non-*N*-acetylated Cu_4MT-2e
		6616.0		
3	7.9	6586.8	6586.9	Cd_4MT-1a
		6638.0	6634.1	Cd_3CuMT-2e
4	10.6	6176.8	6173.4	Non-*N*-acetylated apo-MT-2d
		6104.0	6103.3	Non-*N*-acetylated apo-MT-1a
5	12.1	6144.9	6145.3	Apo-MT-1a
6	15.1	6217.3	6215.3	Apo-MT-2d
		6176.8	6173.4	Non-*N*-acetylated apo-MT-2d
7	25.5	6243.4	6241.4	Apo-MT-2e
MT-2				
1	5.4	6516.6		
		6472.5	6474.0	Non-*N*-acetylated Cd_3CuMT-2a
2	11.8	6147.1	6146.2	Apo-MT-2b
		6082.8	6083.2	Non-*N*-acetylated apo-MT-2a
3	14.4	6124.2	6125.2	Apo-MT-2a
		6154.5	6155.3	Apo-MT-2c
MT				
1	10.5	6516.8		
2	11.83	6441.6	6442.0	Non-*N*-acetylated Cu_4MT-2e
		6392.4		
3	13.4	6419.7		
		6370.5		
4	23.5	6147.4	6146.2/6145.3	Apo-MT-2b/1a
5	27.2	6216.2	6215.3	Apo-MT-2d
		6487.2		
6	30.1	6155.2	6155.2	Apo-MT-2c
		6126.6	6125.2	Apo-MT-2a
7	42.7	6241.9	6241.4	Apo-MT-2e

same global charge as MT-1a. Therefore, these three apo-MT-forms should be eluted at the same fraction, MT-1, during the anion exchange process used by the manufacturer. Moreover, the classification of the mammalian MT subisoforms as the type MT-1 or MT-2 is not based on their global charge [12,22]. Thus, the difference between MT-1 and MT-2 rabbit liver subisoforms is due to the presence of an asparagine residue in the position 11 of the MT-1 amino acidic sequence or an aspartic acid in the same place, in the case of MT-2 subisoforms (see Fig. 3). For this reason, the finding of MT-2 subisoforms in the MT-1 fraction is totally feasible. The significant occurrence of MT-2d and MT-2e forms in this sample has been also observed in different and previous MT-1 Sigma batches by Chassaigne et al. [46] and Van Vyncht et al. [48] by LC–ES-MS, as well as Knudsen et al. [56] by CE–ES-MS. The MT-1 batch used in the present work has been studied recently by Nischwitz et al. [57], and the detection of both apo-MT-2d and apo-MT-2e was also reported. The peak 5 of MT-1 chromatogram shows $m/z = 1537.2$, which very probably corresponds to MT-1a apothionein. This result is in agreement with the majority of authors [46,48,56] but surprisingly, apo-MT-1a was not found by Nischwitz et al. [57]. Furthermore, in MT-1 chromatogram can be noted the presence of non-N-terminal acetylated forms, as non-acetylated apo-MT-2d and apo-MT-1a in peaks 4 and 6. Similar assignments have been described by Van Vyncht et al. [48]. The loss of an acetyl group implies an extra charge in their structures; therefore, these apo-MT-forms elute before the acetylated species, which have a more hydrophobic behavior. The non-*N*-acetylated apo-MT-2d, that appears at peaks 4 and 6, co-elute with its corresponding acetylated form in peak 6, probably due to a deacetylation of the apo-MT-form in the electrospray source, while the presence of non-acetylated apo-MT-2d in peak 4 is assumed by its natural occurrence in the sample. The biological significance of non-acetylated isoforms remains unknown, although an important role in MT degradation has been suggested [56]. On the other hand, in previous works [46,48,56], the MT-2a subisoform was detected as an important compound in MT-1 fraction, but this is not the case of the present study, where no mass attributable to this MT-form has been found. (Nischwitz et al. [57] neither have identified MT-2a at the same MT-1 lot number.) Probably, MT-2a is a remaining contamination of MT-2 in MT-1 and its relative amount can suffer batch to batch variations.

Mass spectra of peaks 1–3 could be assigned to partially metallated metallothioneins. The acidic pH value employed in this study originates the removal of metals of metallothioneins, leading gradually to apo-MT-forms, but it is known that apothioneins can coexist with cadmium and copper complexes. Cadmium was reported to be cleaved at pH lower than 2 [43], with copper still remaining coordinated to the protein at this pH value. The amount of copper ion in commercial metallothionein samples is lower than cadmium content and this fact is reflected in the ratio Cd:Cu of the metallic complexes. Interpretation of mass spectrometry data in these first eluted substances is relatively difficult because of the complexity of the observed clusters and the poor signal-to-noise ratio (ionization of metal complexes is lower than apometallothionein ionization) [49]. Therefore, peaks 2–4 have been assigned in a tentatively way while peak 1 remains unidentified (Table 2).

3.2.2. MT-2 sample

Fig. 5 shows a MT-2 rabbit liver TIC chromatogram and the mass spectra obtained as in the case of MT-1 for each chromatographic peak. The separation was carried out at the same conditions of Fig. 2. As can be seen in the chromatographic profile, the fraction MT-2 presents fewer characteristic peaks than MT-1. The total ion current signal draws three peaks whereas before, with UV detection, the same chromatogram showed four peaks. The UV peaks 2 and 3 probably co-elute at the LC–ES-MS conditions. Table 2 shows the molecular masses found from the *m/z* data and their identification. The major compound that appears in the main peak of the chromatogram, peak 3, can be clearly identified as belonging to the apo-MT-2a subisoform, together with low amounts of apo-MT-2c. Peak 2 has been identified as apo-MT-2b co-eluting with a non-acetylated form of apo-MT-2a. The same main subisoforms found in the MT-2 sample have been identified by several authors [46,48,56] in different MT-2 preparations, although they have found also trace amounts of the species MT-2d and MT-2e, which may be impurities of the MT-2 Sigma batch employed. Unfortunately, Nischwitz et al. [57] have not included the study of MT-2 in their work,

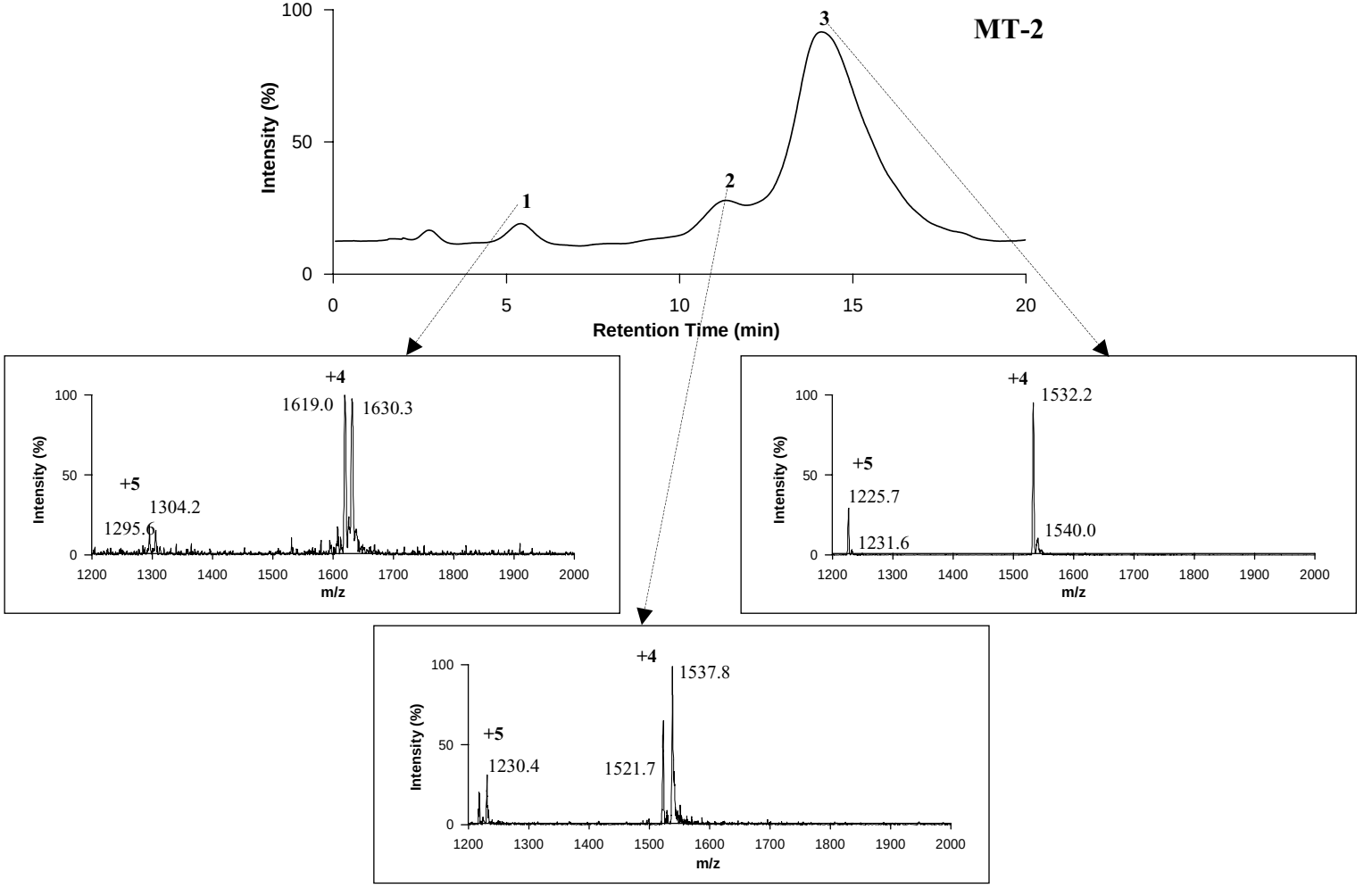

Fig. 5. LC–ES-MS TIC chromatogram of MT-2 carried out at the experimental conditions described in Section 2 and mass spectra registered at each peak.

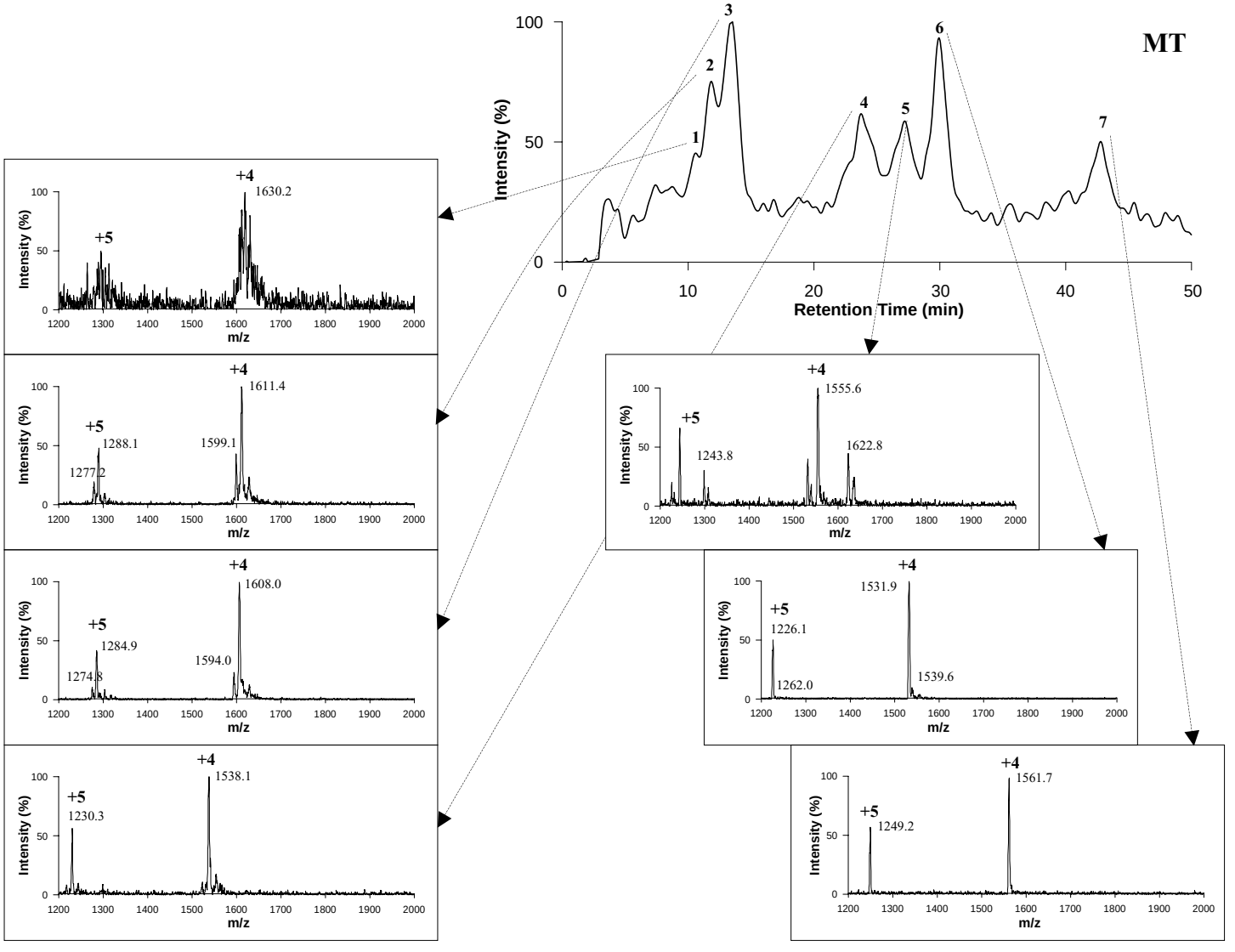

Fig. 6. LC–ES-MS TIC chromatogram of MT carried out at the experimental conditions described in Section 2 and mass spectra registered at each peak.

published in 2003, and the comparison of the MT-2 results of the present work with recent studies that have used the same MT-2 batch is not possible. Peak 1 has been tentatively attributed to a non-acetylated cadmium–copper complex of MT-2a. In the case of MT-2 sample, no chromatographic peak provides a pure mass spectrum corresponding to a single substance, occurring subisoform co-elution. Some compounds such as apo-MT-2a and apo-MT-2c cannot be separated by the method described here because of their similar structures.

3.2.3. MT sample

The gradient profile described in Section 3.1 was used to achieve the separation of MT subisoforms by LC–ES-MS. As in the case of UV-detection, TIC chromatogram of MT (Fig. 6), shows seven characteristic peaks. The mass spectra obtained indicate that MT chromatogram is not a simple overlap of MT-1 and MT-2, and MT-2 subisoforms contribute more than MT-1 in the MT sample. Table 2 summarizes the molecular masses of the rabbit liver MT subisoforms found in this study, together with their identification and the theoretical masses of apo-MT-forms reported in the literature. The six potential subisoforms of rabbit liver apo-MT have been identified: apo-MT-2e ($m/z = 1561.7$) is detected in peak 7, apo-MT-2a and apo-MT-2c ($m/z = 1531.9$ and 1539.6, respectively) co-elute at peak 6, apo-MT-2d appear in 5 and the fourth spectrum with $m/z = 1538.1$ could be attributed to apo-MT-1a or apo-MT-2b (or the sum of both contributions). In this case, typical masses of non-acetylated forms are not present and assignment of metallothionein complexes coordinated partially with cadmium or copper could not be made. The identifications achieved are in agreement with those by Van Vyncht et al. [48] but moreover, these authors have also detected some non-acetylated species in the MT sample. On the other hand, Chassaigne and Lobinski [49] only have found masses corresponding to the apo-MT-forms: apo-MT-2a, apo-MT-2b and apo-MT-2c, and no MT-1 contribution to MT was detected in the preparation that they have investigated. The MT unidentified peaks found in the present work may be attributed to some degradation products or oxidized MT forms. Two of these masses (6516.8 and 6441.6 Da) are detected also in MT-1 (peak 2) and MT-2 (peak 1) fraction samples but the rest (6392.4, 6419.7 and 6370.5 Da) are not found in MT-1 and MT-2 samples. Therefore, it can be assumed that the compounds with these masses are lost during the anion exchange process that MT suffers. On the other hand, some *m/z* relationships that appear in MT-1 and MT-2 preparations are not observed in MT.

4. Conclusions

Liquid chromatography is capable of resolving directly more than the main isoforms MT-1 and MT-2 of a MT sample, without the need to apply a previous anion exchange step. The use of LSER methodologies can help the optimization of chromatographic separations but, a complete resolution of these subisoforms still depends on finding more efficient chromatographic methods, or using capillary electrophoresis procedures. Anyway, the developed methods offer more advantageous conditions than those of other authors [48], obtaining faster separations and providing, in some case, better resolutions.

The combination with the electrospray mass spectrometric detection allows the knowledge of the molecular masses of the different peaks, and therefore, the characterization of apo-MT-forms. The presence of some non-acetylated species and metal complexes has been also detected working at acidic conditions.

The results obtained agree in most cases with those reported by several authors, although the absence of standards of documental purity hinders the identification and the cataloguing of the naturally occurring MT subisoforms. Comparison with literature data, also reveals, the need of systematization of the isoforms nomenclature.

The apo-MT-forms identifications accomplished in this work are a starting point for a better knowledge of the metallothionein polymorphism. Once the apothioneins are identified, their chromatographic and electrophoretic study at different pH values will be able to complete the knowledge about these proteins. On the other hand, the LC–ES-MS analytical methods developed in this work may be applied to real samples allowing the characterization of MTs isolated from other species.

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