Bioinorganic Chemistry

Zn– and Cd–Metallothionein Recombinant Species from the Most Diverse Phyla May Contain Sulfide (S^{2-}) Ligands**

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After much multidisciplinary research into metallothioneins (MTs), the ubiquitous metal-binding proteins first described by Vallee and Margoshes[1] in 1957, there is still little information on the structures and functions[2,3] of the biological metal–MT complexes. There are two main obstacles to studying the physiological features of MTs. First, although MTs are present in all living organisms except Eubacteria, most of the existing data refers to mammalian MTs, which precludes any homology-driven structural, functional, or evolutionary inference because of the extreme sequence heterogeneity of this family of metalloproteins (see http://www.biochem.unizh.ch/mtpage/MT.html). Second, the difficulties encountered when trying to obtain homogeneous native preparations have led to the common utilization of in vitro reconstituted metal–MT complexes, based on the assumption that they represent genuine structural and functional native MT species. Thus, most of the data available to date, especially referring to MT structure, comes from nonbiological characterization of metal–MT complexes.[4] Following the MT discovery, another class of eukaryote metal-coordinating molecules was reported in plants and fungi: the enzymatically synthesized γ-glutamyl (γ-EC) peptides, also called phytochelatins and cadystins, which have always been considered as providing a very different mechanism of metal detoxification compared to the gene-encoded MTs, sharing few, if any, structural and functional features with them. From the chemical point of view, MTs bind a variety of metal ions giving rise to individual polynuclear clusters that are linked exclusively to cysteine residues through thiolate bonds. In contrast, γ-EC peptides create oligomeric clusters with a variable number of units that, most significantly, include acid-labile sulfide (S^{2-}) ions as additional ligands which induce the clusters to evolve to peptide-coated particles, so-called crystallites.[5]

Herein, we report the first definite evidence that sulfide ions are also present in nearly all the recovered Zn^{II}–MT and Cd^{II}–MT complexes, but never in the Cu^{I}–MT species of a wide range of recombinant metal–MT aggregates, thus sulfide ions are found in species formed in vivo, that is, in a physiological, although heterologous, environment. We have determined the presence of the acid-labile S^{2-} ligands both qualitatively and quantitatively by analytical, spectroscopic, and spectrometric techniques, and it is clear that the features of the recovered Zn^{II}–MT and Cd^{II}–MT complexes correlate well with those reported for plant and yeast Zn– or Cd–γ-glutamyl peptides,[5] therefore bridging the behavior gap between both types of metal-binding molecules.

Recombinant expression in *E. coli* has permitted the routine synthesis of a large number of proteins that are difficult or even impossible to obtain in their native forms. MTs stand out among them because of their extreme complexity and heterogeneity. Nearly ten years ago we developed an *E. coli* expression system that allows the biosynthesis of intact Zn^{II}–, Cd^{II}–, and Cu^{I}–MT complexes, isolated domains, and mutant variants, in sufficient quantity and purity for analytical, spectrometric, and spectroscopic characterization. As a first characterization on the mouse Zn–MT1 system[6,7] fully validated the correspondence between native and recombinant complexes, research was expanded to mammalian MT isoforms (MT1[8–10] and MT4[11]), the crustacean MTH,[12] *Drosophila* MTs (MTN[13] and MTO[14]), and the plant *Quercus suber* QsMT.[15]

We are now focused on studying MT species from the most diverse taxa (Table 1) and in the course of this work we found that many experimental results from analyzing Zn–MT, and particularly Cd–MT complexes, made little sense, as shown in Table 1. First, the stoichiometry of the Cd–MT complexes determined by inductively coupled plasma atomic emission spectroscopy (ICP-AES) was unusually low in comparison with the values expected from the corresponding Zn–MT complexes and the number of cysteine residues available for metal coordination. The metal-binding stoichiometries are assessed directly from the total sulfur and metal content of the sample provided by ICP-AES,[16] assuming that the total sulfur in the preparation arises from the summation of cysteine and methionine residues within the polypeptide. The unusually low stoichiometries calculated first suggested a partial oxidation of thiol groups and consequent loss of MT metal-binding capacity, but the Ellman reaction[17] quantification of the thiol groups in the samples did not support this prediction. Furthermore, ESI-MS analyses of most of the Cd–MT isolates revealed, besides the expected species, the presence of complexes whose mass did not match any

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Table 1: Analytical characterization of several purified metal–MT aggregates, corresponding to organisms of the most diverse taxa.[a]

Metal–MT	[MT][b] [$\times10^{-4}$ M]	[MT][c] [$\times10^{-4}$ M]	Metal/MT[d]	Metal/MT[e]	$AgNO_3$ test	S^{2-}/MT[f] (GC-FPD)	ESI-MS major species
Zn-QsMT	2.62	2.18	2.3	3.9	+	1.90	Zn_4-QsMT[15]
Cd-QsMT	1.49	0.58	2.5	6.5	+	3.44	Cd_6S_5-QsMT[15]
Cu-QsMT	0.29	0.26	5.8[g]	7.2[g]	–	n.d.	M_8-QsMT[15]
Zn-CRS5	1.72	1.67	5.7	5.8	–	0.44	Zn_6-CRS5[h]
Cd-CRS5	6.01	0.92	3.4	8.5	+	1.85	Cd_8-CRS5[h]
Cu-CRS5	3.22	3.16	11.3	11.4	–	n.d.	Cu_{11}-CRS5[h]
Zn-MTN	0.40		3.5				Zn_4-MTN[13]
Cd-MTN	1.80	0.80	1.7	3.8	+	2.34	Cd_4-MTN[h]
Cu-MTN	0.42		7.0		–		Cu_8-MTN[13]
Zn-MTO	0.78	0.63	3.2	3.7		0.90	Zn_4-MTO[14]
Cd-MTO	0.50	0.30	3.2	4.1	+	2.63	Cd_4-MTO[h]
Cu-MTO	0.37		8.9		–		Cu_9-MTO[14]
Zn-SpMTA	1.40	1.22	5.2	5.6	+	2.33	Zn_8/Zn_7S_2-SpMTA[h]
Cd-SpMTA	0.81	0.62	6.3	7.2	+	3.62	Cd_7-SpMTA[h]
Cu-SpMTA	0.41	0.39	7.1[g]	7.1[g]	–	n.d.	M_8-SpMTA[h]
Zn-ckMT	1.51	1.09	5.5	7.5		3.75	Zn_7-ckMT[h]
Cd-ckMT	2.29	0.63	3.7	8.8	+	4.70	Cd_7-ckMT[h]
Cu-ckMT	0.39		9.9[g]		–	n.d.	M_{10}-ckMT[h]
Zn-MT1	1.38	1.13	6.0	7.3		1.50	Zn_7-MT1[7]
Cd-MT1	1.90	1.13	3.7	6.4	+	3.17	Cd_7-MT1[11]
Cu-MT1	0.13		9.6[g]		–	n.d.	M_{10}-MT1[10]
Zn-MT4	2.18	1.18	6.7	7.0		3.5	Zn_7-MT4[11]
Cd-MT4	0.19	0.11	5.5[g]	6.9[g]	+	14	M_7-MT4[11]
Cu-MT4	0.36		9.4		–	n.d.	Cu_{10}-MT4[11]

[a] QsMT = *Quercus suber* MT; CRS5 = a *S. cerevisiae* MT; MTN and MTO are *Drosophila* MT forms; SpMTA = *S. purpuratus* type A MT; ckMT = chicken MT; MT1 = mammalian isoform 1 MT; MT4 = mammalian isoform 4 MT. Equivalent analyses with *T. pyriformis* MT1, *C. elegans* MT2, and *M. edulis* MT10IV yielded comparable results with regard to the presence of sulfide in their metal–MT complexes (data not shown). [b] Protein concentration calculated from conventional ICP-AES results. [c] Protein concentration calculated from acid ICP-AES results. Amino acid analysis led to identical values ±0.01. [d] Metal/MT ratio deduced from the metal content measured by conventional ICP-AES and results in (b). [e] Metal/MT ratio deduced from the metal content measured by ICP-AES and the acid ICP-AES and the results in (c). [f] n.d. = not detectable. [g] In this case, the metal/MT ratio represents the mean metal content per MT of preparations which had heterometallic composition (Zn and Cu or Zn and Cd). [h] This work. The corresponding manuscripts including the full MT characterization are in preparation.

possible Cd^{II} metallated complexes. These unknown mass peaks did not arise from the presence of other metal ions in putative heterometallic MT species, as Cd was the only metal detected by ICP-AES. Finally, circular dichroism (CD) spectroscopy of the Cd–MT samples showed features in the 260–280 nm range that clearly were not attributable to the known tetrahedral $Cd(SCys)_4$ chromophores that absorb at approximately 250 nm, which were also present in the spectra (Figure 1).

At this stage, the most likely explanation for our observations was the presence of an additional ligand in the purified Zn–MT and Cd–MT species, but none of the reported possibilities (chloride participation,[18] phosphate-driven dimerization[19]) were consistent with the analytical and spectral features of the samples. However, transitions between 260 and 300 nm have been reported in the CD spectra of Cd-phytochelatins containing sulfide anions[5,20] and we have already suggested that these ligands could be present in *Drosophila* Cd–MTN species.[13] The presence of extra sulfur in our samples as sulfide ligands would provide a sound explanation for all the bizarre results mentioned above as well as the negative oxidation degrees measured by reactions with the 3,3′-dithiobis(6-nitrobenzoic acid) (DTNB).[21] Therefore, we performed a quick test[22] to assess the presence of S^{2-} ions in our panel of recombinant metal–MTs by determining hydrogen sulfide gas generation after acidification of the samples with HCl. Blackening of a filter paper moistened with a solution of $AgNO_3$ which was capping the sample tube, as a consequence of silver sulfide precipitation, unequivocally identified the presence of sulfide in all the Cd–MT and some Zn–MT samples, but not from the control (sample without MT) or any Cu–MT preparations.

Zn–, Cd–, and Cu–MT species from different taxa were analyzed more thoroughly to determine whether sulfide ion binding was common in the diverse metal–MT complexes. If sulfide ions were present, then the ICP-AES approach for global metal–MT ratio calculation was not valid, as the sulfur quantization would not report exclusively on Cys and Met

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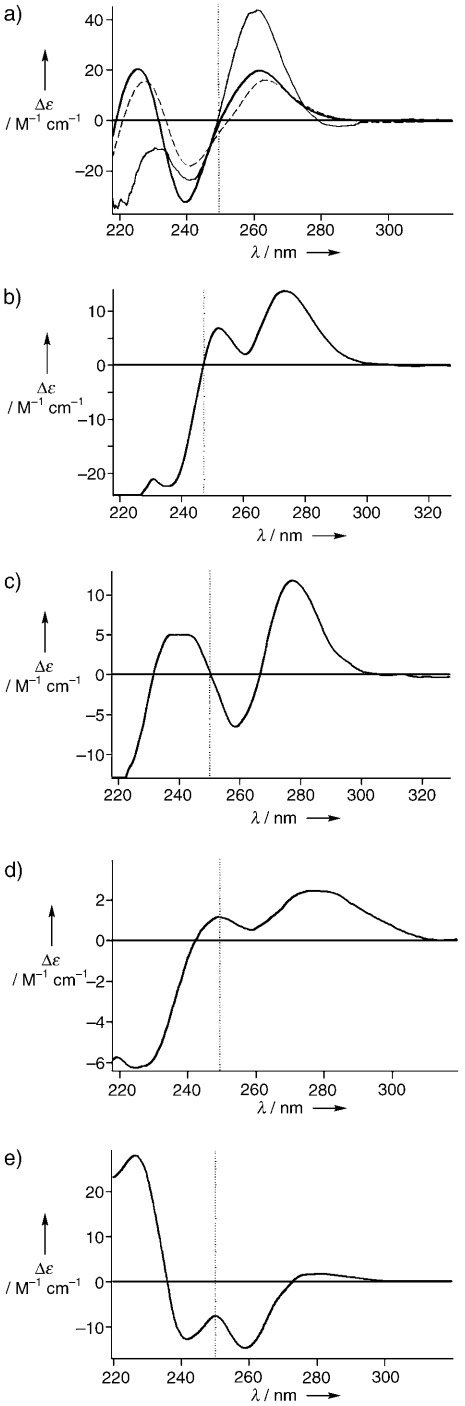

Figure 1. Representative CD spectra of several Cd–MT species from the most diverse taxa. Spectra have been grouped as a) type A (conventional spectra expected for Cd–MT species): Cd–MT1 (solid black line), Cd–SpMTA (solid gray line), and Cd–ckMT (dashed black line); b) type B: Cd–QsMT; c) type C: Cd–MTN; d) type D: Cd-CRS5; e) type E: Cd–MTO. All spectra show the characteristic absorption features of the $Cd(SCys)_4$ chromophores at approximately 250 nm (vertical dotted line) either as derivative shaped signals of different signs (A and B versus C), Gaussian bands (D) or more complex absorptions mixed with chloride participation[18] in Cd binding (E). The most (B, C, D and E) and least (A) intense absorptions at approximately 280 nm could be attributed to the presence of $Cd–S^{2-}$ bonds.

concentrations. Protein concentrations were then redetermined simultaneously by two methods: acid ICP-AES and amino acid analysis. Acid ICP-AES involves acidification of the sample with 1M HCl prior to the conventional method[16] to favor the loss of the acid-labile ligands such as H_2S and thus ensure that all the sulfur remaining in the sample corresponded to Cys and Met. Acid ICP-AES results gave new metal–MT ratios that were now highly consistent with the expected stoichiometries (Table 1). The amino acid analysis was used to quantify protein levels, and these data nicely matched those obtained by acid ICP-AES. Finally, considering single or multiple 32 Dalton (sulfur atomic weight) additions to the value of the mass of the canonical metal–MT species yielded values that were coincident with the unassigned ESI-MS peaks of the sample.

We attempted quantification of the sulfide content in all the samples, initially using a methylene blue method[23] similar to that applied for Cd–phytochelatin analyses.[20] However, this method has serious drawbacks, as some degree of interference with cadmium ions has been reported[24] and it is not suitable for samples containing copper owing to the insolubility of copper sulfide. As a consequence, we adapted a GC-FPD (flame photometric detector) approach initially reported for solid samples.[25] Both methods gave consistent results and permitted the mean S^{2-} ion content per MT to be determined (Table 1). These data demonstrated that acid-labile sulfide was present in all the recombinant Cd–MT samples and in many Zn–MT isolates, although always at lower ratios than in the corresponding Cd–MT samples. Furthermore, ESI-MS analyses of the Zn– and Cd–MT preparations not only accounted for the presence of S^{2-} ions as additional ligands but also showed coexistence of S^{2-}-containing and S^{2-}-devoid metal–MT species (Figure 2), indicating an equilibrium between the two forms in the original preparations. None of the MT proteins synthesized in Cu-supplemented media gave any evidence of sulfide ligands in their complexes. All of these findings are consistent with the features reported for the presence/absence of sulfide in Zn– or Cd–, and Cu–phytochelatins, respectively.[5,26] As for the spectroscopic analyses, evidence that sulfide in the Cd–MT complexes is associated with absorptions in the 260–280 nm range came from acidification/reneutralization experiments(see Supporting Information).

The data presented herein support the hypothesis that recombinant synthesis in *E. coli* of diverse MTs results in the recovery of sulfide-containing complexes in addition to the expected non-sulfide-containing canonical species. This is the first demonstration that Zn–MT and Cd–MT species have the capacity to form Zn- and Cd-thiolate complexes in which labile sulfide ligands contribute to the Zn^{II} and Cd^{II} binding.

This situation has major practical and theoretical implications. From a practical point of view, it is important that the ever-growing community of MT researchers using recombinant strategies to produce metal–MT complexes evaluates the presence of S^{2-} in their preparations to attain a true interpretation of their analytical, spectroscopic, and spectrometric data. From a theoretical angle, the presence of S^{2-} ions in metal–MT aggregates raises significant questions. Because of the observed metal and protein dependency and the

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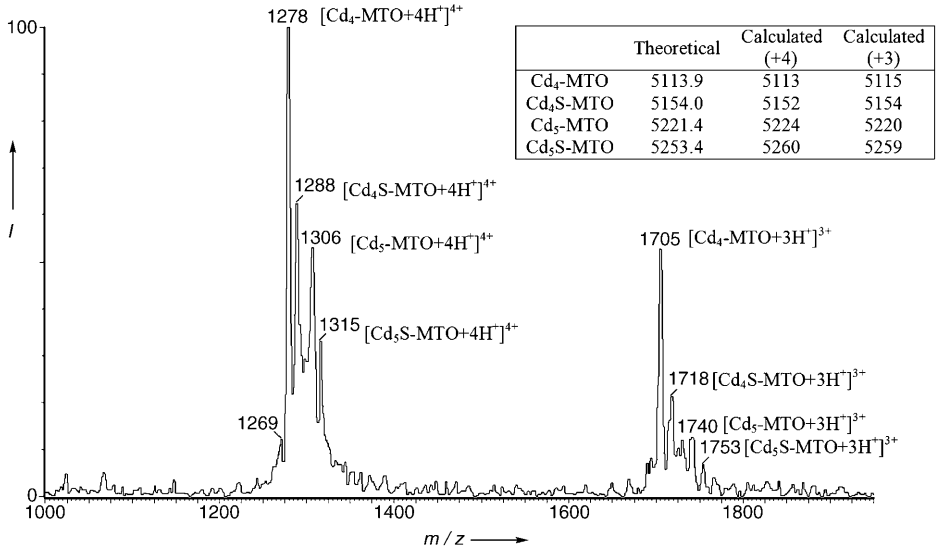

	Theoretical	Calculated (+4)	Calculated (+3)
Cd_4-MTO	5113.9	5113	5115
Cd_4S-MTO	5154.0	5152	5154
Cd_5-MTO	5221.4	5224	5220
Cd_5S-MTO	5253.4	5260	5259

Figure 2. ESI-MS spectrum of the recombinant Cd–MTO preparation. The two sets of peaks corresponding to two charge states (+4 and +3) are shown, to illustrate the coexistence of sulfide-loaded and sulfide-devoid Cd–MTO forms. The table gives the corresponding molecular weights of the species as calculated from the MS data and predicted.

equilibrium between S^{2-}-containing and S^{2-}-devoid forms, it is unlikely that S^{2-} binding is a mere artifact, thus it is pertinent to ask if sulfide incorporation also occurs in endogenous metal–MT complexes, that is, in native forms. Should this be the case, it is intriguing why it has not been previously detected. A likely explanation is that for nearly half a century most of the MT research has been carried out using in vitro reconstituted metal–MT species, which are prepared by adding the corresponding metal ions to apo-MT (metal-devoid MT) polypeptides. These species are obtained after heavy acidification of the purified metal–MT forms and thus, any sulfide ions involved in the initial Zn^{II} or Cd^{II} complexation would be lost. Furthermore, the native isolates of MT complexes from many species contain significant heterogeneity of isoforms and metal compositions.[27,28] Therefore a lot of effort has been devoted to obtaining pure chromatographic fractions, which may involve discarding other minor metal–MT species originally present in the sample and that are assumed to be impurities, mixed-metal species, or simply, non-MT metal-containing aggregates.

The S^{2-} ion is a species not restricted to *E. coli*, but rather a universal cell component. At present, evidence is being gathered that relates the presence of S^{2-} ions with physiological events, which have also been proposed for MT function candidates (redox equilibrium,[29] neurotransmission, and neuromodulation[30]). Consequently, evaluation of the sulfide-binding MT capability in endogenous MT forms is worth considering in further studies of native MT forms, especially Zn–MT, as new data could shed light into the physiological significance of this poorly understood protein in view of the recently revised relationship between the biological and inorganic zinc clusters in nature.[31] But, in any case, knowledge of the capability of divalent metal–MTs to include S^{2-} ions in their aggregates, or in other words, to build metal-MT aggregates that include S^{2-} ions would be highly significant for structural, nanobiological, biotechnological, and biomedical applications in which MTs are involved. In conclusion, a new attribute must be added to the already long list of peculiar features of these versatile proteins.

Experimental Section

Zn–, Cd–, and Cu–MT complexes were synthesized according to a recombinant fusion-protein strategy (GST-MT) fully reported in references [6,7]. This method is based on cultures of *E. coli* cells transformed with the corresponding expression constructs in LB (Luria Bertini) medium supplemented with Zn^{II}, Cd^{II}, or Cu^{II} (300 μM, 300 μM, and 500 μM, respective final concentrations). After cell sonication, all the steps were performed under argon atmosphere. The total protein extract was only submitted to Glutathione-Sepharose affinity chromatography, thrombin digestion, and FPLC (fast performance liquid chromatography) in 50 mM Tris-HCl (Tris = 2-amino-2-(hydroxymethyl)-1,3-propanediol), pH 7.5 buffer. All MT complexes were synthesized and purified under identical conditions.

The molecular mass of the metal–MT species was determined by electrospray ionization mass spectrometry (ESI-MS) on a Fisons Platform II Instrument, equipped with MassLynx software and calibrated with horse-heart myoglobin (0.1 mg mL^{-1}). The assay conditions for the Zn- and Cd-containing species were as follows: 20 μL of protein solution injected at 40 μL min^{-1}; the use of an HPLC Uptisphere C_4 33 mm × 2 mm × 5 μm column to separate analytes; capillary counterelectrode voltage, 4.5 kV; lens counterelectrode voltage, 1.0 kV; cone potential, 60 V; source temperature, 120 °C; *m*/*z* range, 850–1950; scanning rate, 3 s scan^{-1}; interscan delay, 0.3 s. The assay conditions for the Cu-containing species were: 20 μL of protein solution injected at 30 μL min^{-1}; capillary counterelectrode voltage, 3.5 kV; lens counterelectrode voltage, 1.0 kV; cone potential, 35 V; source temperature, 160 °C; *m*/*z* range, 850–1950; scanning rate, 3 s scan^{-1}; interscan delay, 0.3 s. In all cases, the running buffer was an appropriate mixture of acetonitrile and 5 mM ammonium acetate/ammonia, pH 7.5.

Electronic absorption measurements were performed on an HP-8453 Diode array UV/Vis spectrophotometer. A Jasco spectropolarimeter (J-715) interfaced to a computer (GRAMS 32 Software)

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was used for CD determinations. The temperature for all measurements was kept at 25 °C by means of a Peltier PTC-351S apparatus.

ICP-AES, using a Polyscan 61E (Thermo Jarrell Ash) spectropolarimeter, allowed determination of S (182.040 nm), Zn (213.856 nm), Cd (228.802 nm), and Cu (324.803 nm) sample contents. Two alternative methods were used: conventional ICP implied no previous sample treatment,[16] while acid ICP included acid incubation of the sample (1 M HCl, 65 °C, 5 min) prior to ICP measurements. In both cases, protein concentration was calculated from the S content, assuming it was only contributed to by the Cys and Met residues of the peptides. In addition, protein concentration was assessed by amino acid analysis (hydrolysis in 6 M HCl at 110 °C for 22 h) on an Alpha Plus Amino Acid Autoanalyzer (Pharmacia LKB). Ser, Lys, and Gly contents were used to extrapolate MT concentrations.

GC-FPD[32] was used as the most suitable methodology for direct (i.e. without sample derivatization) S^{2-} detection at low concentrations, since it presents a quadratic response to compounds including one unique S atom.[25] H_2S was generated by strong acidification (H_2SO_4, pH 0.0) of the sample to ensure disruption of the metal–MT complex and to avoid precipitation of insoluble ZnS and CdS. The calibration curve was determined with dilute standards of S^{2-} at 0, 0.25, 0.5, 1, 1.5, 2.5, and 3 ppm prepared from a stock solution of S^{2-} at 1000 ppm which was obtained by dissolving a single crystal of $Na_2S{\cdot}9H_2O$ in oxygen-free water and standardized by iodine titration using $NaIO_3$.[22] Sample aliquots, as well as the standard solutions, were transferred to airtight 2-mL vials, acidified to a final volume of 0.5 mL, and immediately sealed. Vials were then incubated at 40 °C for 2 h with agitation (250 rpm) to accelerate the evolution of H_2S from the aqueous phase and equilibration of gas phase in the head-space. 500 μL of the head-space gas were subjected to gas chromatography (HP 5890 Serie II coupled to a FPD80 CE Instruments (Thermo Finnigan) detector). The gaseous mixture was carried by a 6.6 mL min^{-1} flux of He through the GC glass column (SPB 608 30 m × 0.25 mm i.d. with 0.5 μm particle size). Both the injection and the detection port were kept at 110 °C while the column was operated at a constant temperature of 35 °C. The H_2S peak generated from MT samples was readily identified by a retention time coincident with that of the standard solutions. All determinations were performed in duplicate with independent samples.

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