Tools for metal ion sorting: *In vitro* evidence for partitioning of zinc and cadmium in *C. elegans* metallothionein isoforms

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Supplementary material

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Experimental details

Expression and purification of C. elegans MT isoforms

The expression and purification of Zn-loaded CeMT-1 and -2 proteins was carried out using the protocol outlined by Zeitoun-Ghandour *et al.*[1] Accurate protein concentrations were determined through simultaneous detection of zinc and sulfur using inductively coupled plasma-optical emission spectroscopy (Perkin Elmer Optima 5300 DV ICP-OES). Concentrations of other metal ions were negligible.

^{19}F NMR spectroscopy of Cd_6Zn-CeMT-1

To determine the affinity of the mixed metal species for Zn and Cd (Table 1), we employed 5F-BAPTA (1,2-bis(2-amino-5-fluoro-phenoxy)ethane-*N,N,N′,N′*-tetraacetate) as competitive metal chelator and ^{19}F NMR spectroscopy using a method developed by Hasler *et al.*[2] An aliquot of purified Zn_7-CeMT-1 in 0.2 M Tris-Cl (pH 7.4) was incubated at room temperature with 6.5 molar equivalents of $CdCl_2$ for 30 minutes. The protein solution was buffer exchanged by ultrafiltration into 10 mM Tris-Cl, pH 8.1, 10% D_2O. This procedure also removed unbound metal ions from the solution. Elemental analysis and electrospray mass spectrometry (ESI-MS) confirmed Cd_6Zn as the major metallospecies in solution. ICP-OES confirmed the Zn:Cd:protein stoichiometry to be 1.5:6.1:1 (error in analysis 10%). Conditions, technical parameters and data evaluation for both mass spectrometry and NMR spectroscopy are described in detail in reference 1. Uncertainties in the binding constants were calculated from the sum of the relative errors in NMR signals (10 times baseline noise) and the errors arising from the determination of concentrations by ICP-OES (estimated at 10% overall).

Competition reaction monitored by mass spectrometry

A mixture of CeMT-1 and CeMT-2 proteins was prepared in 0.2 M Tris-Cl (pH 7.4) in a ratio of equivalent binding sites concentrations, 25 μM and 29 μM, respectively. Accurate concentrations of starting materials were determined by ICP-OES, which also confirmed the Zn:protein stoichiometry of CeMT-1 and CeMT-2 to be 7.3 ±0.7 and 6.1 ±0.6, respectively. The MT mixture was incubated with varying concentrations of $CdCl_2$ at room temperature for 30 minutes. Post incubation, the mixtures were extensively washed by ultrafiltration and buffer exchanged into 10 mM ammonium acetate buffer (pH 7.4). Methanol (10% v/v) was added to each sample immediately prior to mass spectral analysis.

Data were acquired in positive ion mode on a MicrOTOF instrument (Bruker Daltonics, Coventry) using the following MS parameters: source temperature 195 ^{o}C; capillary exit 150 V; skimmer 1 50.0 V; skimmer 2 23.5 V; hexapole RF 550 V; hexapole 1 24.0 V; hexapole 2 20.9 V; transfer time 63.0 µs. Samples were directly infused and spectra recorded for 1.5 minutes (flow rate: 4 μL/min) over the *m/z* range of 500-3000 Th. Data processing and evaluation was carried out using the DataAnalysis^{TM} software (Bruker Daltonics, Coventry). Each dataset was averaged, smoothed and baseline subtracted. Deconvolution of charge state envelopes was not possible due to manifestation of erroneous peaks in reconstructed spectra. Instead, related peaks were identified and subsequent analyses were carried out on the 5+ and 4+ charge states for CeMT-1 and CeMT-2, respectively.

Data analyses of the competition reaction

The following analyses were performed for each isoform separately at every titration point. Direct comparisons between the signal intensities of CeMT-1 and CeMT-2 are not required for this evaluation.

The total signal intensity for a given isoform was calculated by summing the MS intensities of all corresponding metallospecies. The abundance of each metallospecies was then expressed as a percentage of the total isoform signal intensity. These abundance values have been plotted as a function of the number of bound Cd^{2+} ions in Figure 2A. Using these abundance values and the Zn:Cd stoichiometry associated with each metallospecies derived from the observed mass, it is possible to calculate the overall proportion of Zn^{2+} and Cd^{2+} bound to both isoforms, at each titration point. For example, if only Zn_2Cd_5-CeMT-1 was observed, its abundance would be 100%. The proportion of Zn^{2+} bound to this isoform is calculated as a percentage: 100%/7*2 = 28.6%, and that for bound Cd^{2+} as 100%/7*5 = 71.4%. This gives the proportion of bound Zn^{2+} and Cd^{2+} to CeMT-1 as 28.6:71.4 % (see also Table S3). If more than one metallospecies is present, then the method is carried out for all species relating to one isoform and then individually summed for Zn^{2+} and Cd^{2+}. The results of such analyses have been plotted for the individual isoforms in Figure 2B.

Incubation of Zn_7-CeMT-1 with Cd^{2+} excesses

The Zn_7-CeMT-1 isoform was freshly prepared as outlined in *Expression and purification of C. elegans MT isoforms*. Aliquots of 25 µM Zn_7-CeMT-1 in 0.2M Tris-Cl (pH 7.4) were incubated with (i) 0, (ii) 10 and (iii) 100 molar equivalents, with respect to protein concentration, with Cd^{2+}. Reactions were

allowed to proceed for 20 hours at room temperature. Desalting columns (Sephadex G25 PD10 columns, Amersham Biosciences) were used to remove excess metal and to buffer exchange the mixtures into 10 mM ammonium acetate (pH 7.6). The samples were concentrated by ultrafiltration. For ESI-MS analysis, methanol (10% v/v) was added to the sample prior to infusion. Data were acquired on a HCT-Ultra Ion Trap instrument (Bruker Daltonics, Coventry) in positive mode using the following parameters: Trap drive 151.9; Octopole RF Amplitude 210.4 V; Lens 2 -60V; Capillary Exit 211.0V; Dry Temperature 300°C; HV Capillary -4000V; HV End Plate Offset -500V. Samples were directly infused and spectra recorded for 1.5 minutes (flow rate: 4 μL/min) over the *m/z* range of 800-3000 Th. Data processing and evaluation was carried out using the DataAnalysis™ software (Bruker Daltonics, Coventry). Each dataset was averaged, smoothed and baseline subtracted. The charge state envelope was deconvoluted to give neutral masses.

Incubation of Cd_7-CeMT-1 with Zn^{2+} excesses

Cd_7-CeMT-1 was prepared from Zn_7-CeMT-1 using the protocol outlined in Zeitoun-Ghandour *et al*.[1] Briefly, apo-CeMT-1 was prepared by lowering the pH to 1 and removing metals by gel filtration at pH 1. Cd-loaded CeMT-1 was then reconstituted from this acidified, demetallated solution by adding a small excess of $CdCl_2$, and bringing the pH back to ca. 7.4 with Tris base.

Aliquots of 25 µM Cd_7-CeMT-1 in 0.2M Tris-Cl (pH 7.4) were incubated with (i) 0, (ii) 1 and (iii) 10 molar equivalents, with respect to protein concentration, with Zn^{2+}. These samples were subsequently treated using the same method as outlined above in preparation for mass spectrometry.

Calculation of expected Zn/Cd ratios based on affinity data

The average stability constants, as determined by competition with 5F-BAPTA for Zn_7-CeMT-1, Zn_6-CeMT-2, Cd_7-CeMT-1, Cd_6-CeMT-2, and Cd_6Zn-CeMT-1 (Table 1), were used to calculate expected distributions of Zn^{2+} and Cd^{2+} between CeMT-1 and CeMT-2, using the "Species" subroutine of the IUPAC Stability Constants Database.[3] The calculations were based on the concentrations used in the mass spectrometric experiments, i.e. a total starting concentration of Zn^{2+} of 0.35 mM, and 0.029 mM*6 = 0.175 mM binding site concentration for CeMT-2. For CeMT-1, we used log *K* = 12.0 (for Zn) and 14.2 (for Cd) for six binding sites at a concentration of 0.15 mM (6*0.025 mM), and log *K* = 11.6 (for Zn) or 8.6 (for Cd; the upper limit of our estimate) for one binding site (0.025 mM). Note that if the latter

log *K* value was changed to 6.4 for Cd (the lower value of our estimate of 7.5±1.1), the results were unchanged, because three orders of magnitude between the two constants already led to 100% occupation of the seventh site by Zn^{2+}. The Cd^{2+} concentrations used in the calculations were 0.076 mM, 0.153 mM, 0.264 mM, and 0.525 mM, giving Cd:Zn ratios of 0.22:1, 0.45:1, 0.75:1, and 1.5:1.

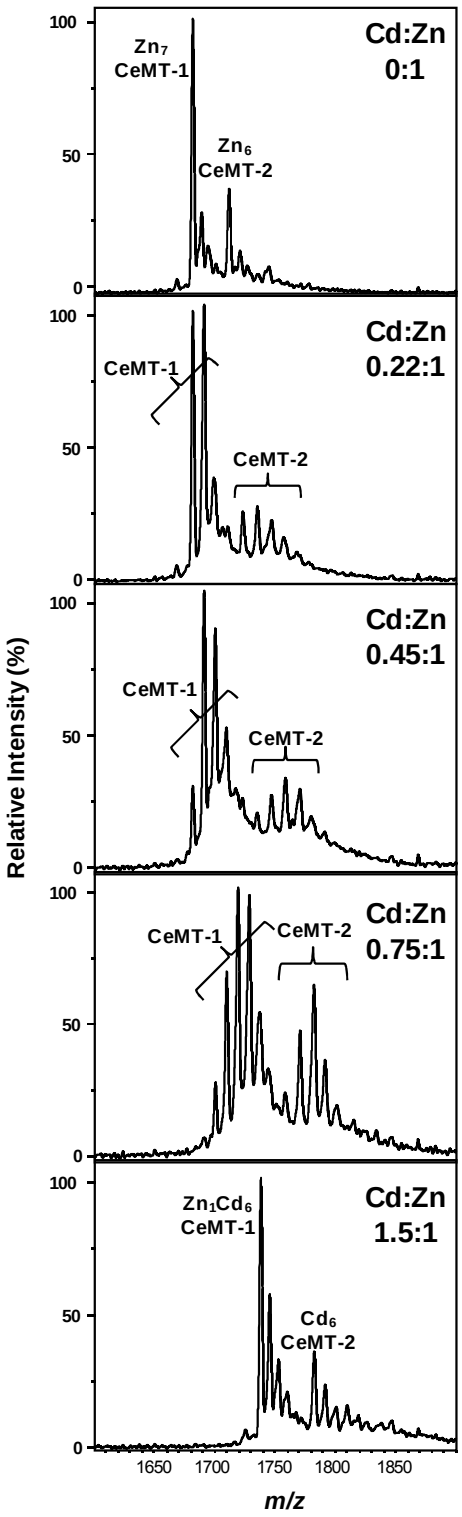

Figure S1. ESI mass spectra following the uptake of Cd^{2+} ions between *C. elegans* MT isoforms. +5 (CeMT-1) and +4 (CeMT-2) charge state signals, showing various Zn/Cd metallospecies on titration with increasing quantities of Cd^{2+} ions. These charge states were selected for data evaluation since the best resolution of signals was observed in this *m/z* region; thus facilitating unambiguous analysis of individual mixed-metal species. The metallospecies present in each mass spectrum are summarised in Tables S1 and S2.

Table S1. Summary of isoform-specific metallospecies observed at different Cd:Zn ratios. The most abundant species in the mass spectra are highlighted in bold.

	Metalloforms observed	
Cd:Zn	**CeMT-1**	**CeMT-2**
0:1	Zn_6; **Zn_7**	**Zn_6**
0.22:1	Zn_6; Zn_7; **$CdZn_6$**	$CdZn_5$; **Cd_2Zn_4**; Cd_3Zn_3
0.45:1	$CdZn_5$; Zn_7; **$CdZn_6$**; Cd_2Zn_5; Cd_3Zn_4	Cd_2Zn_4; Cd_3Zn_3; **Cd_4Zn_2**; Cd_5Zn
0.75:1	$CdZn_6$; **Cd_2Zn_5**; Cd_3Zn_4; Cd_4Zn_3; Cd_5Zn_2	Cd_4Zn_2; Cd_5Zn; **Cd_6**
1.5:1	Cd_6; **Cd_6Zn**	**Cd_6**; Cd_7

Table S2. Summary of speciation of metallospecies observed during titration. The *m/z* values and corresponding original masses together with theoretical masses are provided. The original and theoretical masses are given in their neutral form. Masses marked with an asterisk denote signals taken from other charge states, whose intensities were used to correct those signals which could not be resolved. In all cases this correction had a negligible bearing on subsequent analysis.

Cd:Zn	MT	Charge state	Obs. *m/z* (Th)	Original Mass (Da)	Theoretical Mass (Da)	Metallospecies
0:1	CeMT-1	5+	1668.2	8336.0	8339.2¥	Zn_6
			1681.4	8402.0	8402.6	Zn_7
			1688.8	8439.0	8440.1	$Zn_7 +K^+$
			1693.8	8464.0	8462.1	$Zn_7 +K +Na^+$
			1700.9	8499.5	8500.2	$Zn_7 +2K +Na^+$
	CeMT-2	4+	1356.2*	6776.0	6779.7	Zn_5
			1711.4	6841.6	6843.1	Zn_6
			1716.6	6862.4	6865.1	$Zn_6 +Na^+$
			1720.5	6878.0	6881.2	$Zn_6 +K^+$
			1726.6	6902.4	6903.2	$Zn_6 +K^+ +Na^+$
			1744.3	6973.2	6974.3	Zn_6 +Met
0.22:1	CeMT-1	5+	1668.0	8335.0	8339.2¥	Zn_6
			1681.4	8402.0	8402.6	Zn_7
			1690.7	8448.5	8449.7	$CdZn_6$
			1698.8	8489.0	8487.8	$CdZn_6 +K^+$
			1706.0	8525.0	8525.9	$CdZn_6 +2K^+$
			1710.3	8546.5	8547.9	$CdZn_6 +2K^+ +Na^+$
	CeMT-2	4+	1355.9*	6774.5	6779.7	Zn_5
			1369.1*	6840.5	6843.1	Zn_6
			1722.9	6887.6	6890.1	$CdZn_5$
			1734.9	6935.6	6937.2	Cd_2Zn_4
			1767.6	7066.4	7068.3	Cd_2Zn_4 +Met
			1746.7	6982.8	6984.2	Cd_3Zn_3
			1756.7	7022.8	7031.2	Cd_4Zn_2
			1770.5	7078.0	7078.2	Cd_5Zn
0.45:1	MTL-1	5+	1677.1	8380.5	8386.3¥	$CdZn_5$
			1681.3	8401.5	8402.6	Zn_7
			1690.7	8448.5	8449.7	$CdZn_6$
			1700.0	8495.0	8496.7	Cd_2Zn_5
			1709.2	8541.0	8543.7	Cd_3Zn_4
			1716.8	8579.0	8581.8	$Cd_3Zn_4 +K^+$
			1722.4	8607.0	8603.9	$Cd_3Zn_4 +K^+ +Na^+$
	MTL-2	4+	1365.4*	6822.0	6826.8	$Cd\ Zn_4$
			1734.8	6935.2	6937.2	Cd_2Zn_4
			1746.5	6982.0	6984.2	Cd_3Zn_3
			1779.4	7113.6	7115.7	Cd_3Zn_3 +Met
			1758.2	7028.8	7031.2	Cd_4Zn_2
			1791.0	7160.0	7162.4	Cd_4Zn_2 +Met
			1770.1	7076.4	7078.2	Cd_5Zn
0.75:1	MTL-1	5+	1690.8	8449.0	8449.7	$CdZn_6$
			1700.1	8495.5	8496.7	Cd_2Zn_5
			1709.5	8542.5	8543.7	Cd_3Zn_4
			1718.9	8589.5	8590.8	Cd_4Zn_3
			1728.2	8636.0	8637.8	Cd_5Zn_2
			1736.8	8679.0	8675.9	$Cd_5Zn_2 +K^+$
			1743.7	8713.5	8714.0	$Cd_5Zn_2 +2K^+$
			1750.7	8748.5	8752.1	$Cd_5Zn_2 +3K^+$
	MTL-2	4+	1384.0*	6915.0	6920.8	Cd_3Zn_2
			1397.7*	6983.5	6984.2	Cd_3Zn_3
			1757.9	7027.6	7031.2	Cd_4Zn_2
			1770.3	7077.2	7078.2	Cd_5Zn
			1781.9	7123.6	7125.3	Cd_6
			1791.0	7160.0	7163.4	$Cd_6 +K^+$
			1800.9	7199.6	7201.5	$Cd_6 +2K^+$
			1815.2	7256.8	7256.6	Cd_6 +Met
1.5:1	MTL-1	5+	1725.1	8620.5	8621.5	Cd_6
			1737.9	8684.5	8684.9	Cd_6Zn
			1745.2	8721.0	8723.0	$Cd_6Zn +K^+$
			1752.4	8757.0	8761.1	$Cd_6Zn +2K^+$
			1759.8	8794.0	8799.2	$Cd_6Zn +3K^+$
			1767.1	8830.5	8837.3	$Cd_6Zn +4K^+$
	MTL-2	4+	1782.1	7124.4	7125.3	Cd_6
			1791.1	7160.4	7163.4	$Cd_6 +K^+$
			1800.3	7197.2	7201.5	$Cd_6 +2K^+$
			1815.0	7256.0	7256.6	Cd_6 +Met
			1809.4	7233.6	7235.6	Cd_7
			1818.8	7271.2	7273.7	$Cd_7 +K^+$

¥These signals have a very low intensity in the mass spectra; hence, their masses are greatly influenced by background noise.

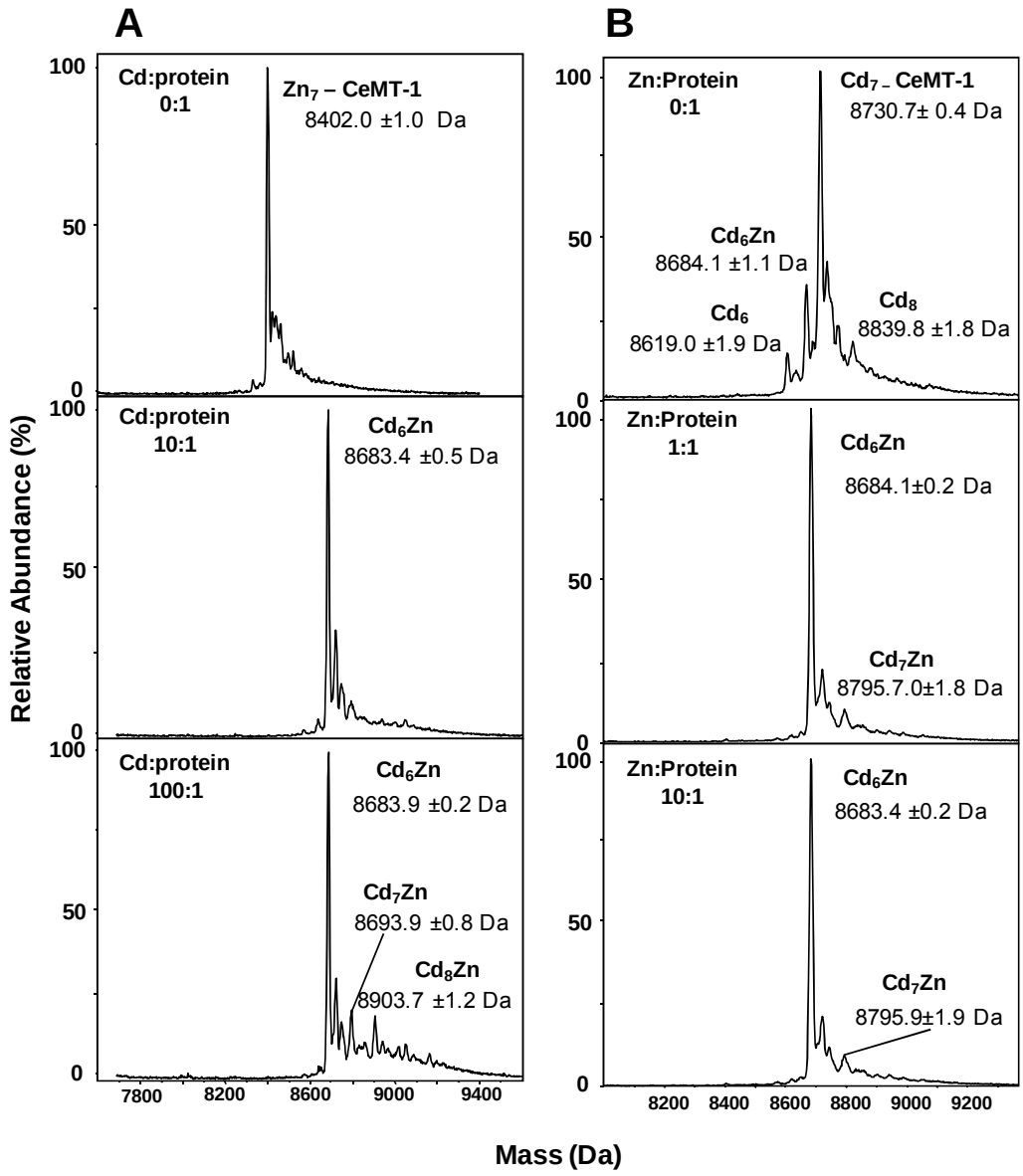

Figure S2: Deconvoluted ESI mass spectra showing CeMT-1 metallospecies present following incubation with excesses of Cd^{2+} and Zn^{2+} after prolonged incubation times (20 h). (A) Incubation of Zn_7-CeMT-1 with 0, 10 and 100 molar equivalents of Cd^{2+} (with respect to protein concentration). (B) Incubation of a preparation of Cd_7-CeMT-1(generated by reconstitution of the apo-protein) with 0, 1 and 10 molar equivalents (with respect to protein concentration) of Zn^{2+}. For experimental conditions, the reader is referred to the experimental section above.

(A) The incubation of zinc-loaded MT-1 with 10 molar equivalents of Cd^{2+} with respect to protein concentration yields only one metallospecies, the mass of which corresponds to that of Cd_6Zn-CeMT-1 (Calculated mass: 8684.9 Da). Even at a 100-fold excess after 20 h, the Cd_6Zn-CeMT-1 metallospecies was still the most abundant, but minor species corresponding to Cd_7Zn- and Cd_8Zn-CeMT-1 were also observed. No signals of fully Cd-loaded Cd_7CeMT-1 species were detectable.

(B) The mass spectrum of the Cd_7CeMT-1 preparation shows that the Cd_7 metallospecies (Calculated mass: 8731.9 Da) is the most abundant, but despite rigorous desalting before reconstitution, a residual signal corresponding to the Cd_6Zn-CeMT-1 metallospecies is still present. Over- and under-metallated Cd_6 (8621.5 Da) and Cd_8 (8842.3 Da) are also present simultaneously, indicating that these are not significantly less stable than the Cd_7 species. Therefore, preparation of pure Cd_7 metallospecies is not possible (also observed in ref 1). On incubation with 1 and 10 molar equivalents of Zn^{2+}, the Cd_6Zn metallospecies predominates, with a small trace of Cd_7Zn observed. These experiments indicate that Cd_6Zn is indeed a thermodynamic product, as it is formed quantitatively, even at an only 1:1 ratio of Zn to Cd_7CeMT-1, from the Cd_7 species. They also rule out that the seventh site is kinetically inert, and that slow metal exchange kinetics were the cause for the consistent predominance of this species in the presence of both Zn^{2+} and Cd^{2+} - *in vitro* and *in vivo*.

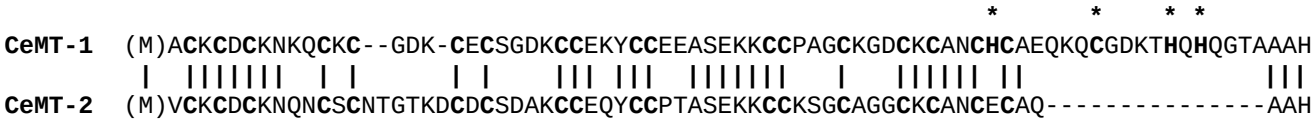

Figure S3. Sequence alignment of *C. elegans* MT isoforms. Residues highlighted with asterisks correspond to additional ligands in the CeMT-1 isoform thought to participate in metal ion coordination.

Table S3. Summary of CeMT-bound Cd:Zn ratios based on metallospecies observed by ESI-MS (Fig. 2B; Obs.) and calculated using conditional average stability constants (from Table 1; Calc.).[1]

Cd:Zn:	% Cd:Zn in individual MT isoform							
	0.22:1		0.45:1		0.75:1		1.5:1	
	Obs.	Calc.	Obs.	Calc.	Obs.	Calc.	Obs.	Calc.
CeMT-1	8.5 : 91.5	7.1 : 92.9	26.4 : 73.6	20.6 : 79.4	52.0 : 42.0	56.6 : 43.4	85.7 : 14.3	84.9 : 15.1
CeMT-2	35.3 : 64.7	36.3 : 63.7:	61.2 : 38.8	66.6 : 33.4	92.8 : 7.2	92.5 : 7.5	100.0 : 0.0	99.8 : 0.2

1. S. Zeitoun-Ghandour, J. M. Charnock, M. E. Hodson, O. I. Leszczyszyn, C. A. Blindauer and S. R. Stürzenbaum, , *FEBS J.,* 2010, **277**, 2531-2542.
2. D. W. Hasler, L. T. Jensen, O. Zerbe, D. R. Winge and M. Vašák, , *Biochemistry*, 2000, 39, 14567-14575.
3. IUPAC Stability Constants Database, Data version 5.5 (compiled by L. D. Pettit and H. K. J. Powell), Academic Software, Timble, Otley, UK, 2005.