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Towards the characterization of metal binding proteins in metal enriched yeast

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Abstract

This paper presents size exclusion chromatography data with on-line coupling to UV and inductively coupled plasma mass spectrometry (ICP-MS) of water soluble metal-binding compounds present in zinc, copper, chromium and iodine enriched yeast nutritional supplements. Molecular weight estimates of the extracted metal-containing compounds are given and are shown to vary substantially from 1.2 kDa to larger than 668 kDa. Seven proteins suspected of containing chromium were identified from one of the chromium-containing fractions. Four of these identified proteins are known to form complexes with other metal ions. The metal chromatographic profiles of zinc, copper and chromium-enriched yeasts were compared to their respective native metal profiles in non-enriched yeast samples. The chromium profiles are shown to be markedly different while those of zinc and copper are qualitatively similar. Only iodide ions or weakly bound, non-aromatic, low molecular weight (~1.2 kDa) iodine species were observed in the iodine-enriched yeast samples.

Keywords: ICP-MS; ESI-MS; Yeast; Metal; Size Exclusion

1. Introduction

The use of nutritional supplements is becoming increasingly common and is growing at a rapid pace. Nutritional supplements adhere to far fewer and significantly less stringent regulations than prescription drugs. Despite this, mineral supplements, in particular, are often recommended to ensure the sufficiency of endogenous and essential trace elements that may be missing or deficient in the modern diet. These trace elements, usually metals and metalloid species, are naturally found in living organisms as a consequence of the biochemical evolution and bio-induction of metal-binding ligands. Imbalances in the concentrations of these metals often represent significant health risks [1].

Typically two forms of mineral supplements are commercially available; inorganic metal salts and metal species that are bound to organic substrates. The latter form has the metal attached to a molecule such as a vitamin (e.g., niacin in polynicotinate versions) or an amino acid derivative (e.g., picolinic acid, a derivative of tryptophan in picolinate versions). This attachment is often claimed to make the metal more efficiently absorbed by the body [2]. It is also suggested that organically bound metals have greater biological activity [2] and are generally less toxic at higher concentrations compared to their respective inorganic salts [3]. Due to its ability to incorporate metals within its cells; yeast biomass is increasingly being used as a delivery vehicle in over-the-counter metal supplements. The bioavailability, desired activity and toxicity of such supplements are functions of the metal concentration, its oxidation state and the chemical forms of its complexes [4,5]. Metal enrichment in yeast is obtained by the inclusion of an inorganic salt of the desired metal in the cultivation medium followed by a heat treatment process to stop yeast growth and inhibit enzymatic activity. The metal is incorporated into the yeast in a variety of ways, including the production of metal-binding proteins (including metallothioneins) [6–8], mineralization [9–11] and sequestration to vacuoles [12–14].

While the vast majority of studies in the area of elemental speciation in nutritional supplements have been devoted to selenium, recently fractionation of soluble species of P, Mn, Fe,

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Co, Ni, Cu, Zn, Se, and Mo in pea and lentil seeds with size exclusion chromatography followed by inductively coupled plasma mass spectrometry detection were reported [15]. Here we present the results of some initial and exploratory experiments on commercial yeast supplements enriched with chromium, zinc, copper and iodine.

2. Experimental

2.1. Reagents

A NANOpure mixedbed ion-exchange system (Barnstead Thermolyne, Dubuque, IA) was used to obtain deionized water at a resistivity of 18.2 MΩ cm^{-1}. All analytical grade reagents were used as purchased without any further purification. Sodium dodecyl sulfate (SDS) was purchased from Fluka (Oakville, ON, Canada). Tris(hydrosymethyl)amino-methane (Tris–HCl), ammonium bicarbonate, acetonitrile, formic acid, proteomics grade trypsin (T6567) and rabbit liver metallothionine (MT) were purchased from Sigma-Aldrich (Oakville, ON, Canada). Homogenized yeast powders were provided by Lallemand-Institut Rosell (Montreal, QC, Canada).

2.2. Protein extraction procedure

Samples of ca. 0.5 g of enriched yeast were used in each extraction. After the addition of 10 ml of extraction solvent, the samples were placed in a sonicating bath (Model 3510, Branson, Danbury, CT, USA) for 30 min then centrifuged (Thermo IEC, USA) at 7200 rpm for 60 min. The solid residue was collected for further analysis and to 5 ml of the resulting supernatant solution an equal volume of cold (4 °C) acetonitrile was added to induce the precipitation of water soluble proteins. Samples were then re-centrifuged at 4800 rpm for 10 minutes. The final precipitate was then re-dissolved in 1 ml of water for analysis.

2.3. Microwave digestion for total chromium determination

The solid residues obtained after the two stages of centrifugation and the remaining supernatant solution from the extraction procedure described previously were quantitatively transferred into individual pre-cleaned Teflon digestion vessels. Five milliliter of high purity nitric acid and 0.2 ml of H_2O_2 were then added to each vessel. The vessels were then sealed and heated in a CEM MDS-2100 microwave oven according to the conditions shown in Scheme 1.

After cooling, the caps were removed and rinsed while keeping the rinse solution in the vessels. The contents of the vessels were then evaporated on a hot plate to a volume of about 0.5 ml and subsequently quantitatively made up to 10 ml in water. The samples were finally serial diluted by 100-fold for analysis by ICP-MS.

2.4. Size-exclusion chromatography ICP-MS

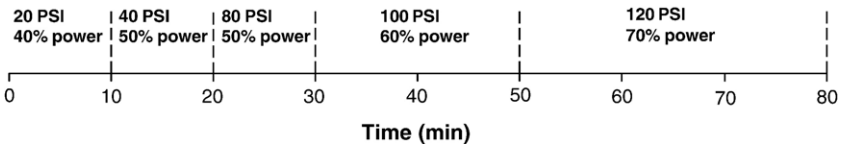
Initial separations were accomplished by an Agilent 1100 HPLC (Agilent Technologies, Palo Alto, CA, USA) employing a Superdex G200 (Amersham Pharmacia Biotech AB, Uppsala, Sweden) size exclusion column. Elemental detection was achieved using an ELAN 6000 (SCIEX, ON, Canada) ICP-MS equipped with a Ryton spray chamber and cross-flow nebulizer. The ICP-MS parameters were optimized daily but typical operating conditions were 0.7 L min^{-1} for nebulizer gas flow, 1100 W for ICP RF power and 10 V for the lens voltage. The injection size was 20 μL and the elution was accomplished using 30 mM of Tris–HCl adjusted to a pH of 7.5 as mobile phase at a flow rate of 0.6 ml min^{-1}. The effluent was directed into a UV detector set at 254 nm coupled on-line to the ICP-MS for elemental detection. Fractions from several runs were collected based on the retention times of the metal-containing species and added together to be concentrated through freeze-drying for further analysis.

2.5. In-solution enzymatic hydrolysis

The freeze-dried metal-containing protein fractions collected from size exclusion chromatography were re-dissolved in 100 μL of 100 mM ammonium bicarbonate. A sample of 20 μg of proteomics grade trypsin was dissolved in 20 μL of 1 mM HCl. The re-dissolved metal-containing protein fractions were then added to the 20 μL trypsin solution and were incubated at 37 °C for 24 h.

2.6. Reverse phase chromatography ES-MS/MS

Injections of 20 μL were made on a 5 μm, 150×2.0 mm, Luna C18 column (Phenomenex, Canada). The mobile phases A (95:4.9:0.1) H_2O/ACN/formic acid and B (4.9:95:0.1) H_2O/ACN/formic acid were used in a gradient from 0% to 60% B over 70 min at a constant flow rate of 0.15 ml min^{-1}. Identification and detection was accomplished using a TSQ Quantum triple quadrupole and a DecaXP ion trap mass spectrometers (ThermoFinnigan, San Jose, CA) both employing electrospray ionization. In the case of the triple quadrupole instrument, about ten scans were averaged at unit resolution (FWHM=0.7) to produce each mass spectrum. Precursor ion spectra were taken using the following optimized conditions: electrospray voltage at 4000 V, collision energy at 10 V, collision cell pressure at 0.5 mTorr of argon. The ion trap generated data dependent MS/MS spectra were collected at the following conditions: isolation width set at 2 amu, activation Q



Scheme 1. Experimental conditions of the CEM MDS-2100 microwave oven.

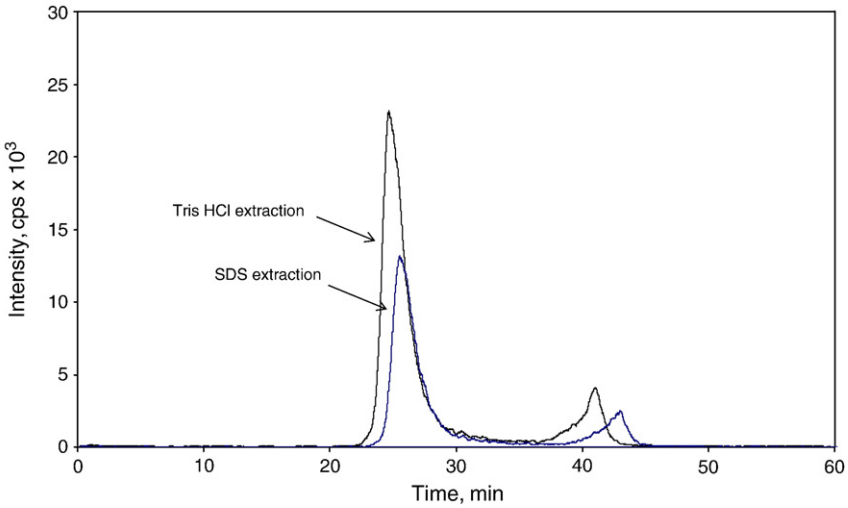


Fig. 1. ICP-MS traces of ^{112}Cd from commercial rabbit liver metallotionine extracted using a 30 mM Tris–HCl buffer and a 1% SDS solution.

set at 0.25, activation time set at 30 ms and collision energy normalized at 35%.

3. Results and discussion

A critical step in such speciation analysis is the extraction of the metal containing compounds from the solid. Among the most common choice of extraction media used is a 10–50 mM Tris–HCl buffer at pH values ranging from 7 to 9. This medium is used to extract metal binding proteins from a variety of matrices including biological fluids, animal tissues, plant material and food stuffs [16,17]. Such soft water or methanol based extraction media typically lead to only 10–20% recoveries while extraction media containing sodium dodecyl

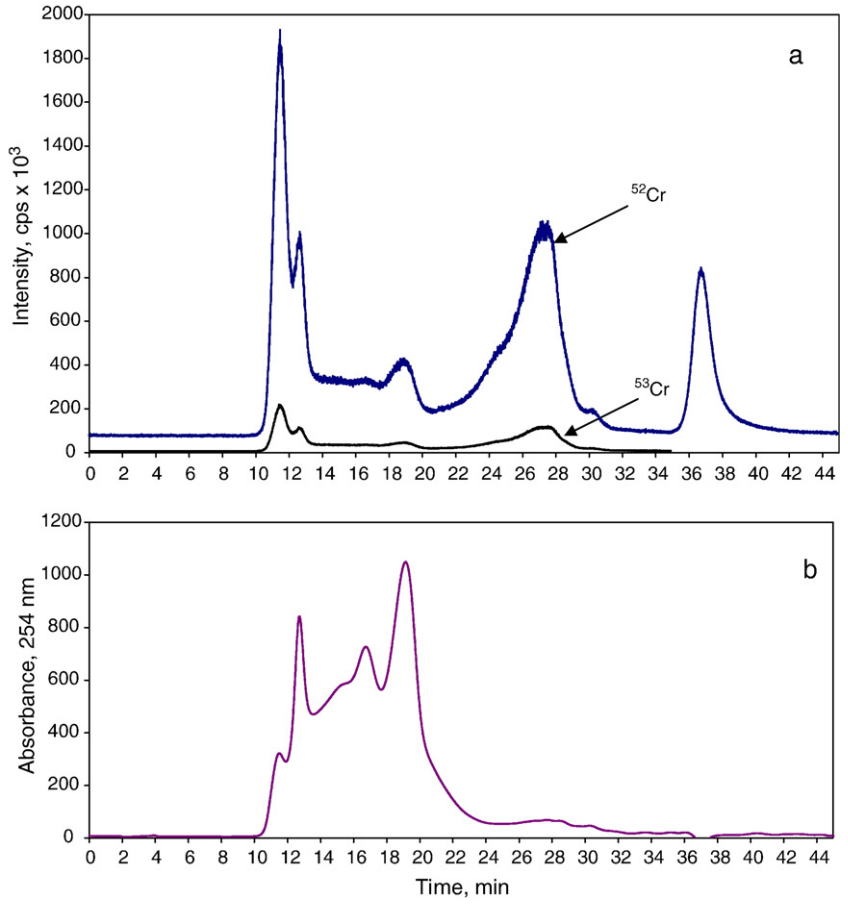


Fig. 2. ICP-MS chromatogram (panel a) and corresponding UV data (panel b) obtained from chromium-enriched yeast extracted using a 1% SDS solution in water.

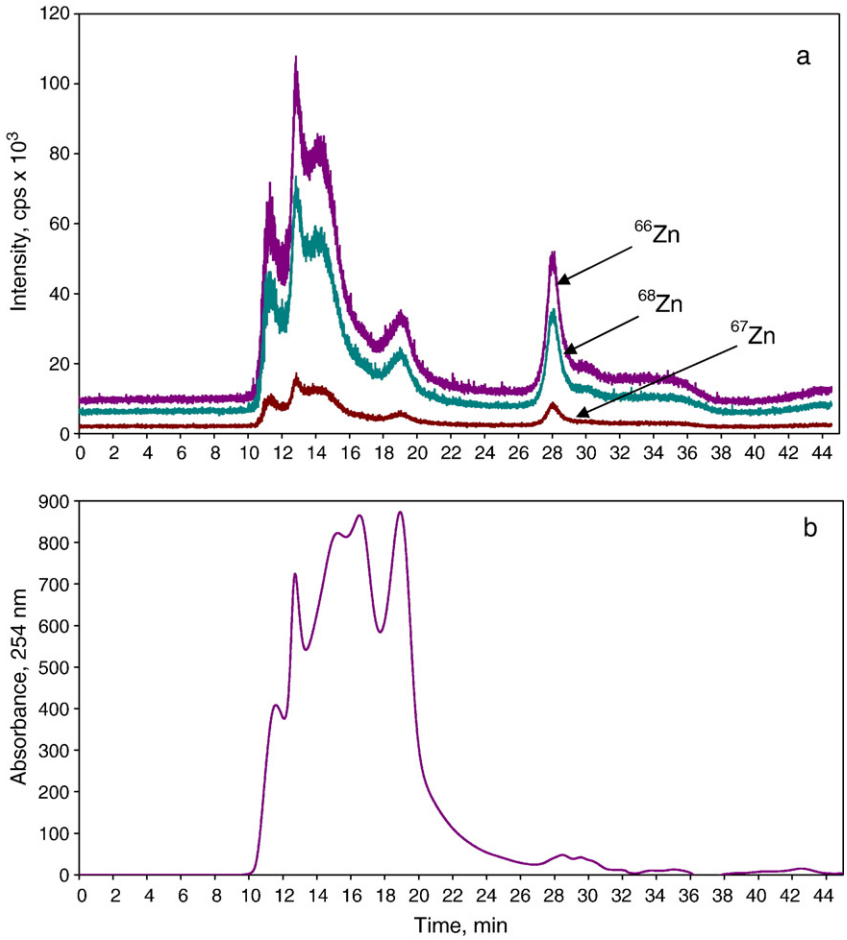


Fig. 3. ICP-MS chromatogram (panel a) and corresponding UV data (panel b) obtained from zinc-enriched yeast extracted using a 1% SDS solution in water.

sulfate (SDS) or protoelytic enzymes lead to recoveries of 40–80% [18,19]. Recently, solutions of SDS were successfully employed to extract selenium-containing proteins from selenium enriched yeasts [20]. However, an excess of SDS (>5%) or the presence of thiol reagents, when heated at 100 °C, denatures protein mixtures. This is typically done to dissociate proteins into individual polypeptide subunits [21]. To establish a suitable extraction medium for this work several test extractions were preformed using 30 mM Tris–HCl (pH 7.5) and a 1% SDS solution in water. All test extractions were conducted on commercial rabbit liver metallothionine (MT) containing 3.7% cadmium. Fig. 1 shows the cadmium metal chromatographic profiles of MT's extracted using the 1% SDS solution and the Tris–HCl buffer. The extraction of MT's using Tris–HCl buffers at the same concentration employed here is known not to cause denaturation of the proteins. Extensive denaturation of the MT's would result in increased signal intensity for ionic cadmium and associated increased signal intensities at the lower molecular weight regions. These are not observed in the extractions employing the 1% SDS solution (see Fig. 1) which suggests that these extractions do not result in extensive denaturation of the proteins.

The preservation of the metal–protein interactions during the extraction as evidenced by the lack of protein denaturation as seen in Fig. 1 makes it possible to estimate the extraction efficiency of the metal-binding proteins by evaluating the total metal content in the extracted protein fractions. To this end the extraction efficiency of both media on the matrix of interest was examined by means of total chromium analysis in multiple water soluble protein fractions extracted from several chromium enriched yeast samples. The extractions which employed the procedure described earlier were followed by microwave digestion and determination of total chromium content by ICP-MS. The chromium content in the protein fractions extracted by the 1% SDS media was determined to be 8% of the total chromium contained in the intact yeast biomass sample used while those extracted by the Tris–HCl averaged about 5% in their chromium content. This slightly higher extraction efficiency for the 1% SDS media coupled with the preservation of the metal–protein interactions justifies its use in this work.

Figs. 2–4 show the ICP-MS and corresponding UV traces of the metal-containing water soluble species extracted from chromium, zinc and copper enriched yeast samples. The ICP-MS traces indicate the presence of several distinct metal-containing species. These species with the exception of those constituting the last eluting peak in the chromium signal correspond to features at matching retention times on their

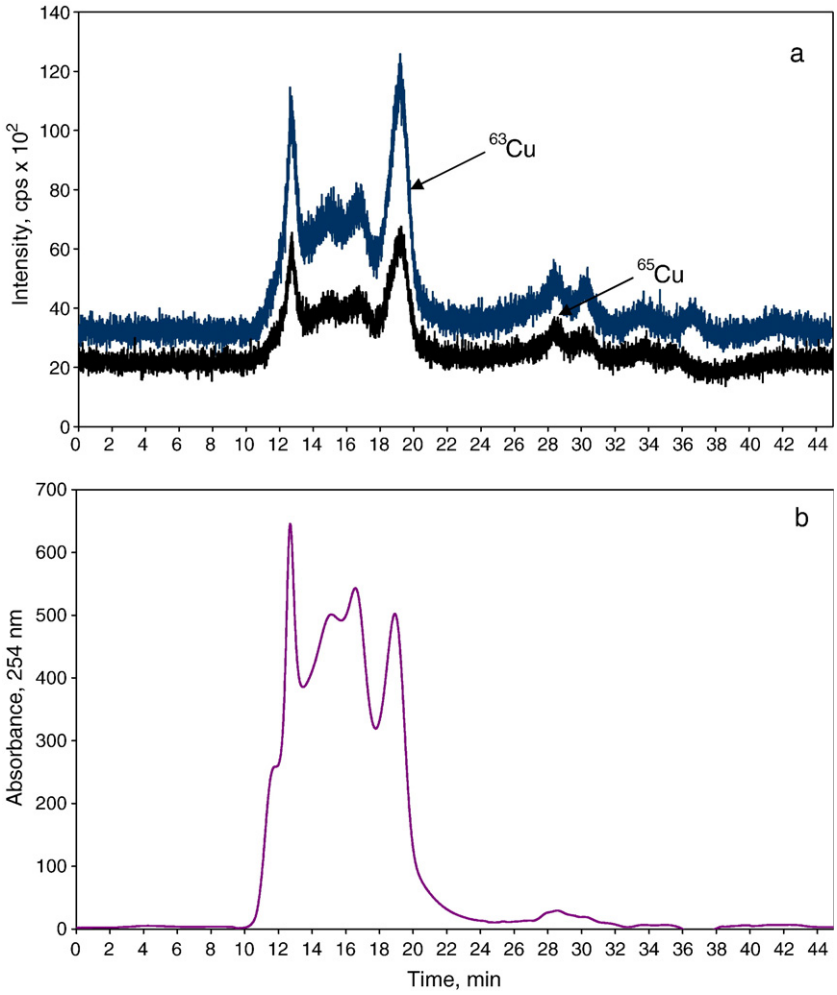


Fig. 4. ICP-MS chromatogram (panel a) and corresponding UV data (panel b) obtained from copper-enriched yeast extracted using a 1% SDS solution in water.

respective UV traces. This confirms the presence of several water soluble metal-binding organic compounds, which may include proteins, in each of the metal enriched yeast samples used. The chromium trace in Fig. 2 clearly shows five peaks at 11.6, 12.7, 19.0, 27.3 and 36.8 min, the zinc trace in Fig. 3 also exhibits five peaks at 11.3, 12.9, 14.5, 19.3 and 28.2 min while the copper trace in Fig. 4 only shows signals at 12.8 and 19.2 min. The higher intensity of the chromium and zinc signals combined with the greater number of peaks observed in those traces relative to the copper trace indicates significantly more incorporation of chromium and zinc into, or their stronger binding capacity with, the extracted yeast proteins than in the case of copper. It is also interesting to note that the zinc chromatographic profile obtained here (see Fig. 3) shows evidence of the presence of significantly more distinct metal-containing species relative to that of the same metal profile obtained from extracts of lentil and pea samples using nearly the same chromatographic setup [15]. This perhaps indicates the presence of more zinc selective binding proteins in the pea and lentil samples than in the zinc-enriched yeast samples used here. In contrast, Fig. 3 indicates more selective zinc binding than the zinc profiles of the cytosolic fraction obtained from mussel tissue exposed to zinc stimulus [22].

Table 1
Molecular weight estimates and observed retention times of the extracted metal-binding species from the enriched yeast samples

	Molecular weight						
Species	>670 kDa	350–670 kDa	170–230 kDa	1.5–50 kda	<1.5 kDa		
Zinc	10–11.5 min	12–13 min	14–16 min	18–20 min	–	27–29 min	–
Copper	–	12–13 min	–	18–20 min	–	–	–
Chromium	10–11.5 min	12–13 min	–	18–20 min	25–29 min	–	36–38 min
Iodine	–	–	–	–	–	–	36–38 min

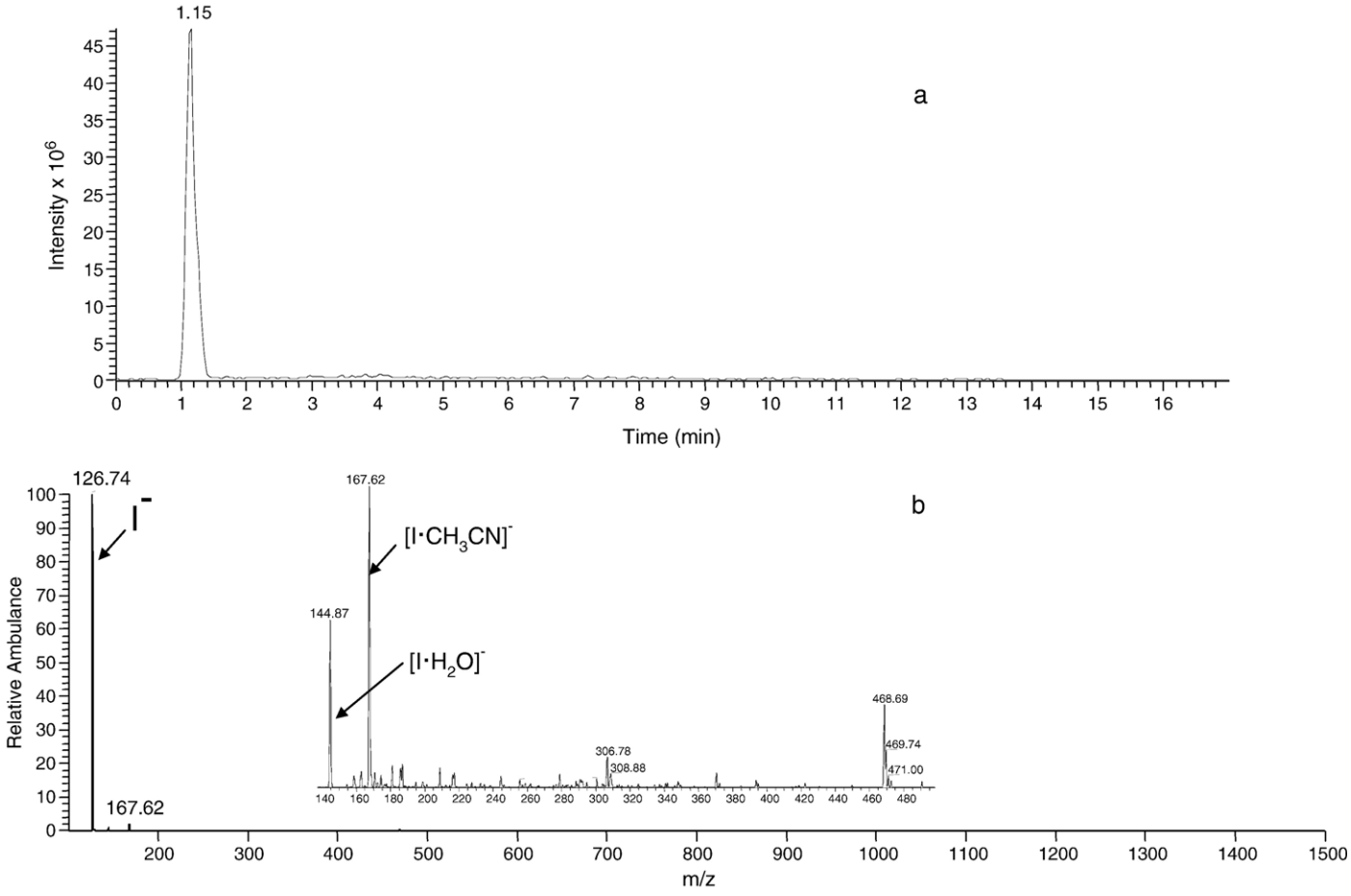


Fig. 5. Negative ion mode chromatogram of the precursors of m/z 127 (panel a) obtained from the extracted iodine-enriched yeast and the corresponding mass spectrum (panel b and insert).

All peaks shown in Figs. 2–4 are not well resolved due to the intrinsically low resolution of size exclusion chromatography. It is therefore likely that each one of these peaks represent multiple species [17,23]. Table 1 clearly shows the nearly exact retention time matching of several peaks in the chromatographic profiles of the zinc, copper and chromium-containing protein extracts of their respective metal-enriched yeasts. This table also lists only one peak in the case of iodine-enriched yeast. This might indicate a similar binding nature of some yeast proteins (or classes of yeast proteins) to the three former transition metals while pointing to a different mode of binding in the case of iodine. Similar observations can also be seen in the SEC-ICP-MS chromatograms of metal stressed pea and lentil extracts, where copper, nickel, zinc and cobalt exhibit one mode of binding while manganese, iron, and selenium are shown to be significantly different [15].

The 1% SDS and Tris–HCl extracts of the iodine enriched yeast samples exhibit a single peak at 37.0 min on their ICP-MS traces (not shown). This single peak, much like the last eluting peak, at 36.8 min, in the chromium trace (Fig. 2a), does not correspond with any features at the same retention time in the UV data obtained (not shown). The presence of these two peaks could be due to iodine and chromium complexes with low molecular weight compounds (<1.5 kDa) which are not strong UV absorbers or to ionic species which could have been produced from the dissociation of very weakly bound complexes.

Further examination of the low molecular weight compound(s) extracted from the iodine-enriched yeast was preformed employing reverse phase LC-MS/MS. Fig. 5 Panel a shows only one peak eluting at around 1 min in the negative precursor ion chromatogram of m/z 127. The mass spectra obtained for this peak were averaged (Fig. 5, panel b) and show a clear signal for iodide ions at m/z 127. Signals are observed due to the adducts of iodide ion with one molecule of water and acetonitrile at m/z 145 and 168, respectively (Fig. 5, panel b and insert); two small very low intensity clusters of ions at m/z 307 and 469 also appear and are not identified. To eliminate the possibility of sample degradation during reverse phase chromatography the extracts of the iodine-enriched yeast were infused at a flow rate of 5 μl min^{-1} directly into the mass spectrometer. This produced a nearly identical averaged mass spectrum. It is still, however, possible that small compounds containing the intrinsically weak C–I bond (average bond strength of about 240 kJ/mol) may undergo in-source fragmentation causing the liberation of iodide ions and resulting in the observed mass spectrum. The signal observed may, on the other hand, indicate that mostly uncomplexed

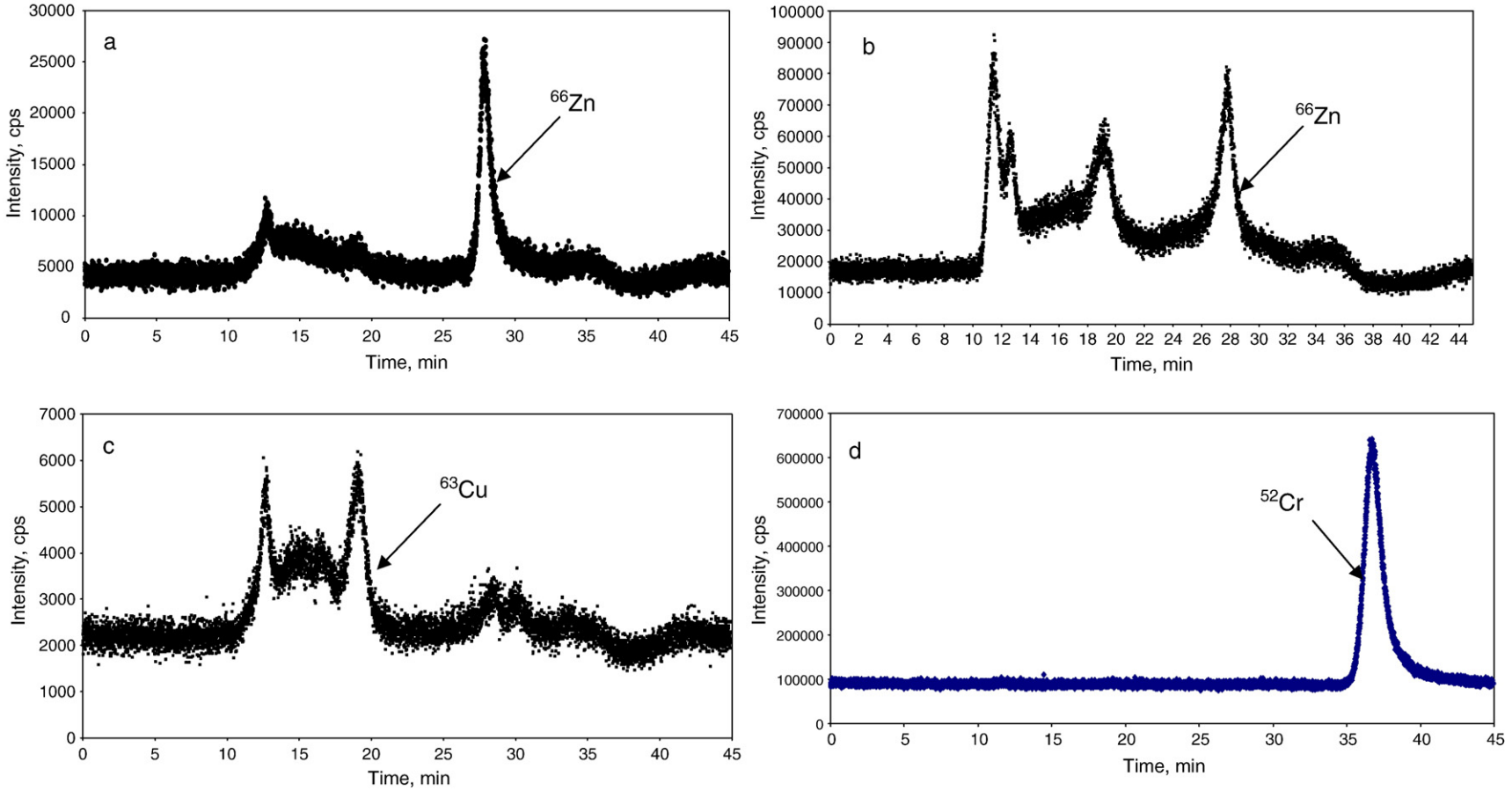


Fig. 6. ICP-MS chromatogram of naturally abundant zinc species (panel a) extracted from copper-enriched yeast and naturally abundant zinc, chromium and copper species extracted from non-enriched yeast (panels b, c, d).

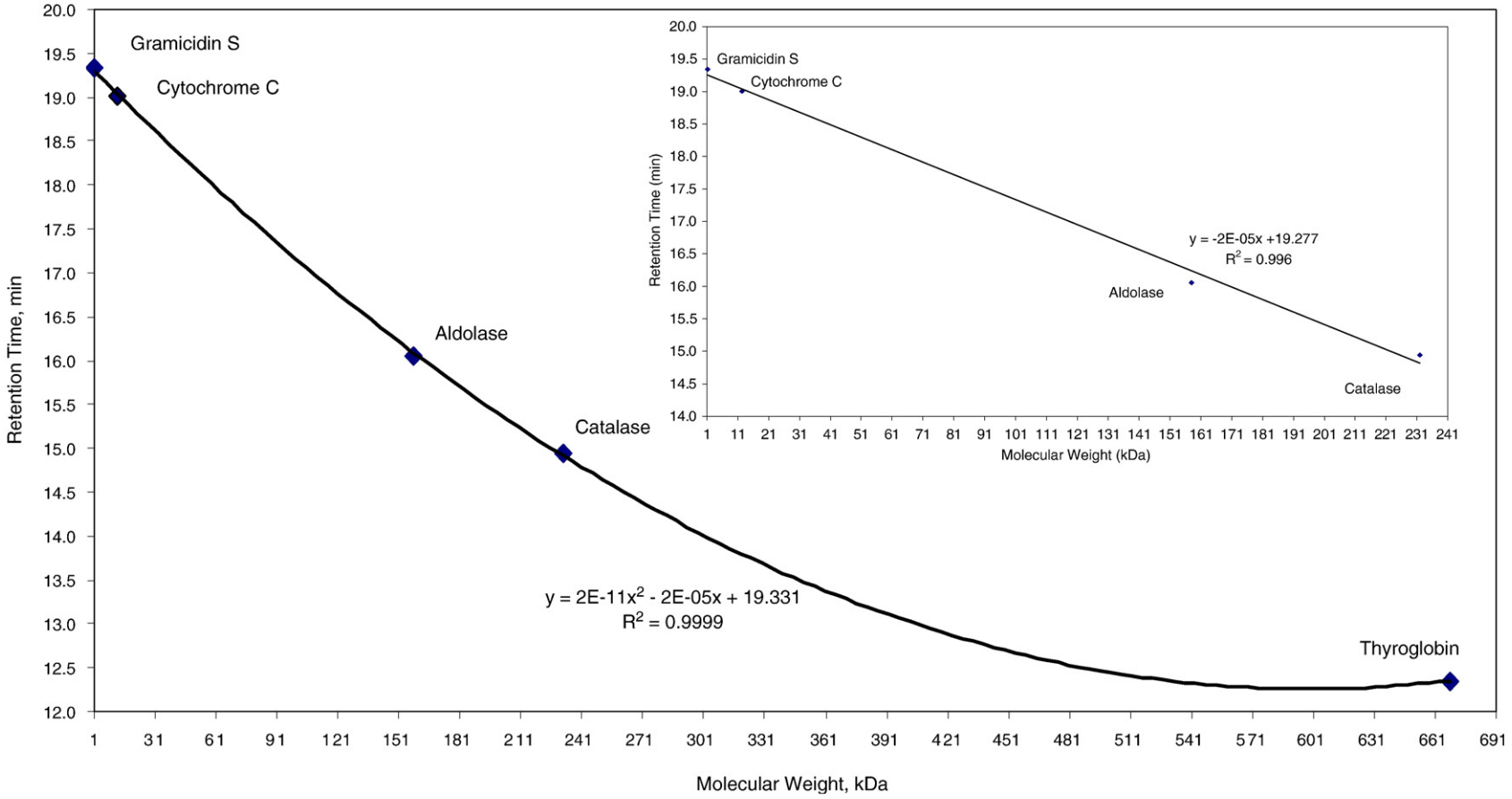


Fig. 7. Correlation plots between molecular weights and observed retention times. The insert shows the linear range of the column used.

Table 2
List of proteins identified from one of the collected chromium containing fractions obtained from the chromium-enriched yeast

Protein	Sequence of peptide identified	Residue number	Biological Process	Cellular component	Known metal activity
TDH 2	YAGEVSHDDK LNKETTYDEIKK	54–63 247–258	Glycolysis, gluconeogenesis	Cell wall, cytoplasm, cytosol, lipid particle	
TDH 3	YAGEVSHDDK LNKETTYDEIKK	54–63 247–258	Glycolysis, gluconeogenesis	Cell wall, cytoplasm, cytosol, lipid particle	
ENO1	GNPTVEVELTTEK VNQIGTLSESIK DDFLISLD	16–23 347–358 81–88	Glycolysis, gluconeogenesis	Cytoplasm, phosphopyruvate hydratase complex	Known to form complexes with magnesium
ENO2	GNPTVEVELTTEK VNQIGTLSESIK DDFLLSLD	16–23 347–358 81–88	Glycolysis, gluconeogenesis	Phosphopyruvate hydratase complex soluble fraction	Known to form complexes with magnesium
APE2	QKIDEIKK	900–907	Peptide metabolism	Cytoplasm, mitochondrion	Zinc–dependent metallopeptidase
CPR1	KVESLGPSGATK	139–151	Protein metabolism, sporulation	Histone deacetylase complex, nucleus	Binds to the drug cyclosporine A
PGK1	YHIEEEGSR	124–132	Glycolysis, gluconeogenesis	Cytoplasm, cytosol	Known to form complexes with ATP, magnesium and manganese

iodide ions are present in the iodine-enriched yeast supplements examined here. This observation compared favorably with recent speciation work on iodine in milk whey where it was determined that iodine in this matrix exists only in the form of iodide or as traces of unidentified low molecular weight (~100 kDa) organic compounds [24,25].

The zinc profile due to zinc-containing proteins found in a copper enriched yeast sample is shown in Fig. 6 (panel a). This

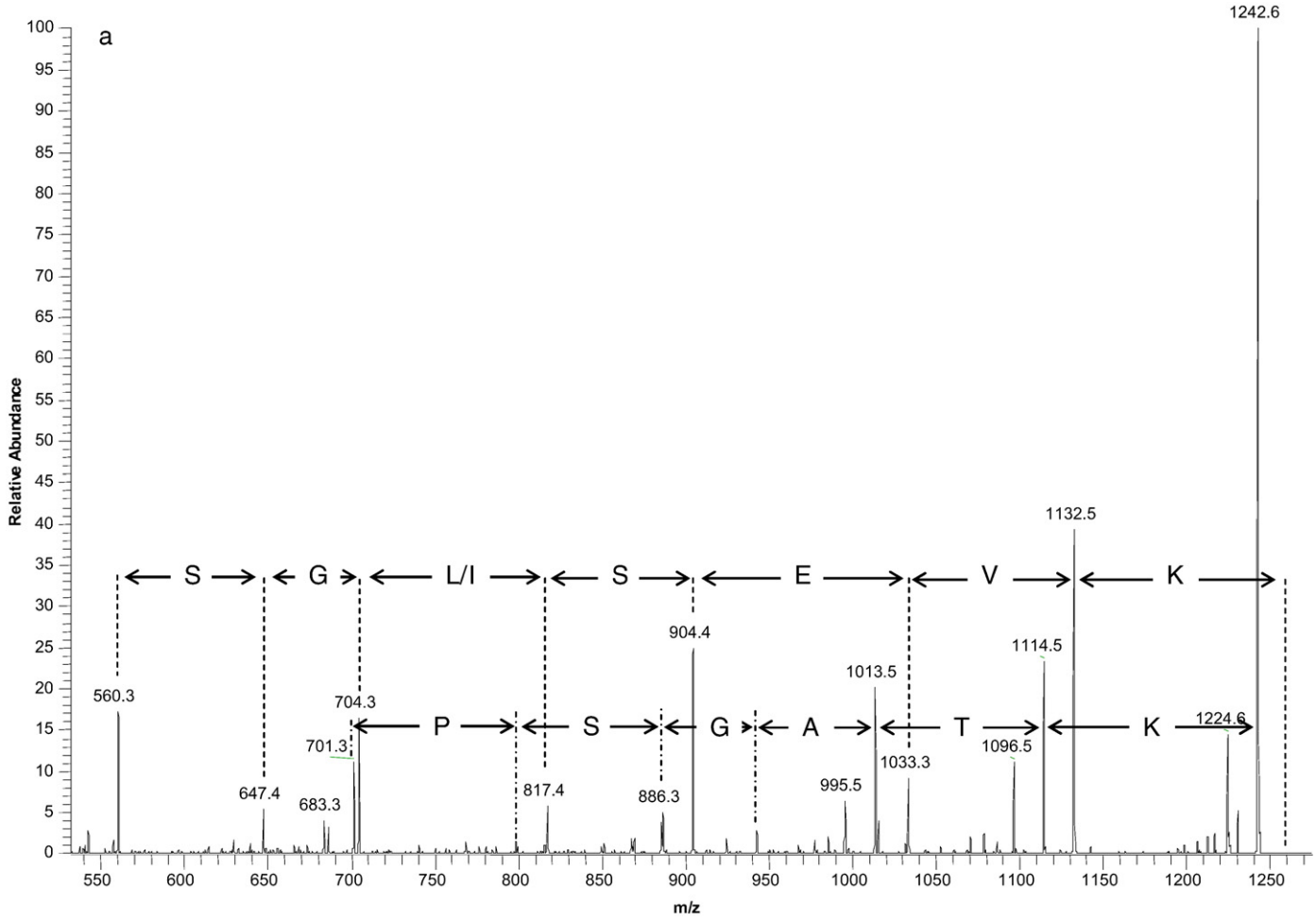


Fig. 8. Examples of positive ion mode ES-MS/MS spectra following reverse phase chromatography on one the collected chromium containing fractions (18–20 min, see Table 1). Cyclophilin pepdyl-propyl *cis–trans* isomerase (panel a), a 17 kDa protein. Phosphoglycerate kinase (panel b), a 44 kDa protein. Enolase 1 or enolase 2 (panel c), a 47 kDa protein.

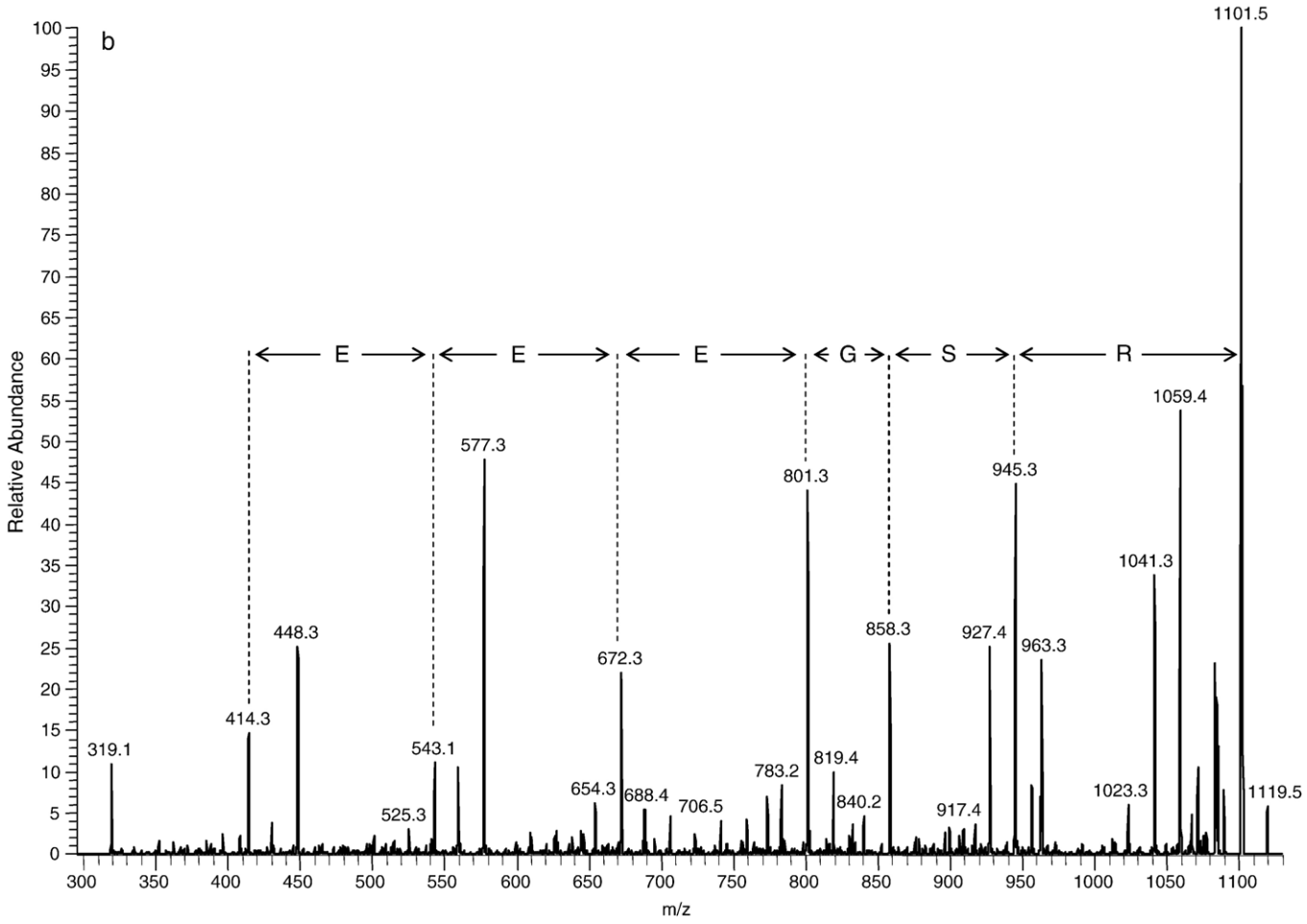


Fig. 8 (*continued*).

profile shows the expected reduction of the absolute signals intensities and the decrease of the higher molecular weight zinc binding proteins relative to those obtained from the zinc enriched yeast (Fig. 3, panel a) and the endogenous zinc profile (see Fig. 6, panel b). This observation agrees with a recent report that noted an inverse correlation between the concentrations of copper-bound metallothionines and their zinc bound counterparts [26]. A similar trend was also observed of zinc being displaced from proteins by other metals that have a higher affinity to –SH groups [22].

Fig. 7 shows a rough correlation between the molecular weights of several proteins and peptides and their observed retention times obtained at the same chromatographic conditions employed for the metal-enriched yeasts. Even though the cross-section that a molecule presents to the column is not solely based on its molecular weight but also depends on other factors including its conformation and charge state, a correlation is clearly seen here. Thus, it is possible to assign estimates of molecular weights for the species observed in the ICP-MS traces of the enriched yeast extracts as listed in Table 1. Immediately evident from that table is the broad molecular weight range of the extracted metal-binding proteins.

The endogenous zinc, copper and chromium profiles of yeast samples that have not been exposed to metal stress during their growth are shown in panels b, c and d of Fig. 6, respectively. The zinc and copper profiles show signals that are significantly lower in intensity but qualitatively similar to those obtained from their respective metal enriched yeasts while the chromium trace (Fig. 6, panel d) shows no evidence for complexation with any proteins. This strongly suggests the over expression of some yeast proteins in response to the chromium metal stimulus. This observation led us to a more detailed investigation of the chromium-enriched yeast.

In order to identify some of the chromium-binding proteins, fractions containing chromium complexes (10–13 min and 18–20 min, see Fig. 2) were collected following size exclusion chromatography of the chromium-enriched yeast extracts. These fractions were subjected to trypsin proteolysis and were subsequently injected on a reverse phase column coupled to an ion trap mass spectrometer using the experimental conditions previously described. Unambiguous data were only obtained from the fraction at the later retention time and are summarized in Table 2. Examples of the positive ion mode ES-MS/MS spectra obtained from this fraction are also presented in Fig. 8. Seven proteins were assigned here; six of them have molecular weights that are within the range estimated using size exclusion chromatography (see Table 1) and four are known to form complexes with metal ions. While this does

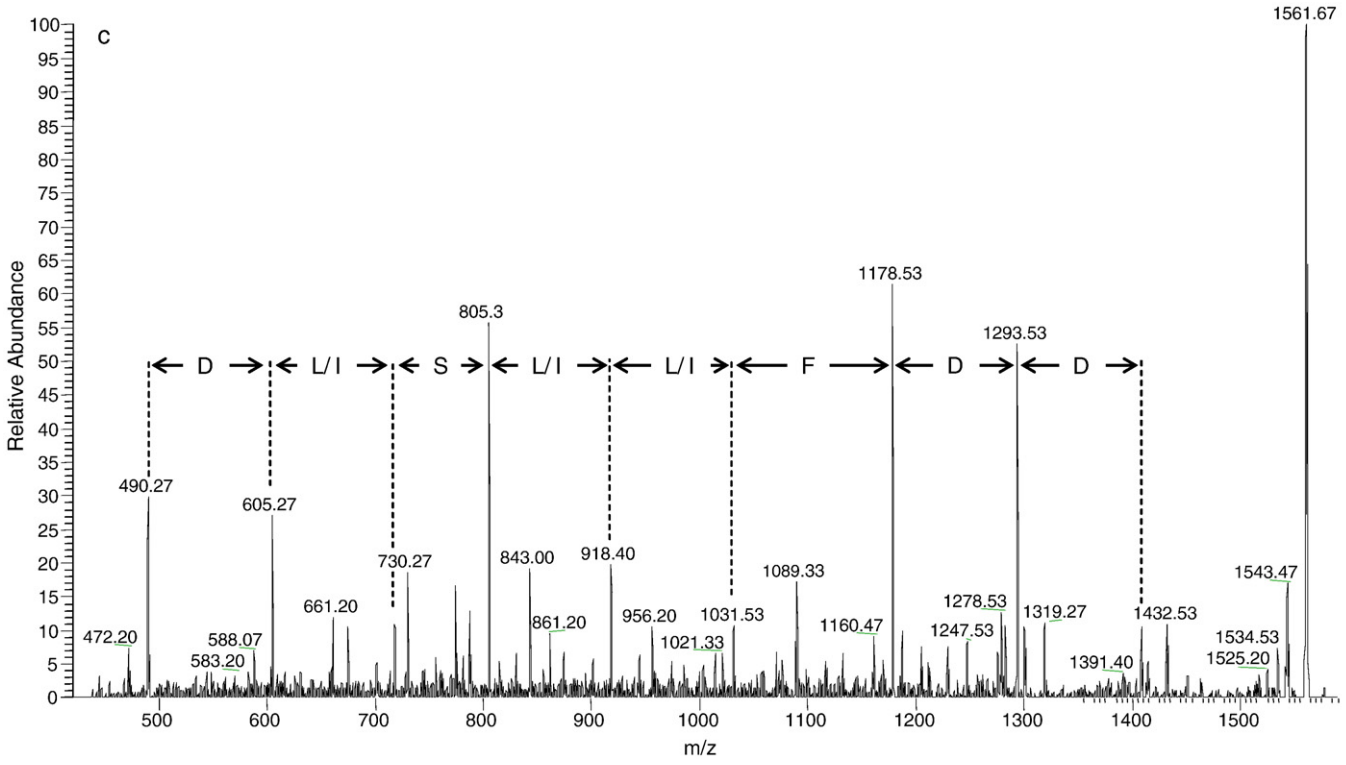


Fig. 8 (*continued*).

not provide conclusive proof that the proteins identified here are chromium binding proteins, however this data increases the confidence in that claim.

4. Conclusions

Data showing the presence of water soluble metal-binding proteins extracted from metal-enriched yeast nutritional supplements were presented. Several of these proteins were shown to have very similar retention times suggesting they are of the same size. The sizes of the extracted metal-binding species were shown to vary substantially. The smallest species observed are suggested to be either low molecular weight (smaller than 1.2 kDa) complexes of chromium and iodine or free chromium and iodide ions. In the case of the chromium-enriched yeast these low molecular weight compounds were shown to represent a major component of the total chromium content while in the case of the iodine-enriched yeast these were the only species observed. Seven proteins were identified from one of the collected chromium-containing fractions obtained from the chromium-enriched yeast extracts. Four of these identified proteins are known to form complexes with metal ions. Further work is required to gain more detailed knowledge of the actual association of the metals with the identified proteins. This should include better purification of the obtained fractions to further isolate the metal-biomolecules of interest allowing for a more in-depth identification by means of MS/MS.

Acknowledgements

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