Speciation of Manganese-binding to biomolecules in pine nuts (*Pinus pinea*) by two dimensional liquid chromatography coupled to UV and ICP-MS detectors followed by ESI-MS identification

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Advances in analytical methodology for speciation of manganese in pine nuts were presented in this work. The approach was based on the use of orthogonal chromatographic systems namely size exclusion chromatography of the extracts and strong anion exchange of the fractions collected by the first column. In both columns, manganese elution was first monitored by a quadrupole ICP-MS equipped with an octopole reaction cell and an UV detector. Size exclusion chromatography were performed by using two columns covering the molecular weight range from < 10 to 70 kDa that allowed an initial screening of the molecular weight of the Mn species. The highest resolution capability of the low molecular range column was the reason to use this later for further experiments. The fraction from SEC-ICP-MS in which Mn was present at highest concentration was submitted to IEC-ICP-MS allowing Mn-citrate and $MnCl_2$ identification by retention time matching with standards. The concentration of these species was estimated to be 75 and 125 µg Kg^{-1} (as Mn) respectively in the pine nuts samples and the presence of Mn-citrate was confirmed by nESI-QqTOF. In the same fraction, a third Mn containing peak was detected in the IEC-UV-ICP-MS chromatogram. This peak corresponds to a protein containing Mn that was latter submitted to a tryptic digestion and analysed by nESI-QqTOF. The MS/MS data of a doubly charged peptide were used to obtain the sequence of the protein with the Mascot searching engine. The peak resulted to be isocitrate dehydrogenase, a protein commonly associated with Mn.

Introduction

Manganese (Mn) is a relatively abundant element, comprising approximately 0.085 % of the Earth´s crust[1]. This element exhibits very low toxicity although chronic overdose causes "manganism"[2]. On the other hand, Mn is an essential element in the human diet, because it activates at trace level many enzymes involved in metabolic processes, for example, the glial-specific enzyme glutamine systhetase, which accounts for 80 % of the brain concentration of Mn[3]. In addition, it is also needed for protein and fat metabolism, nervous and immune systems health, as well as for blood sugar regulation[4]. This element is crucial for enzymes involved in energy production and increases the anti-oxidative protection, with enzymes such as the mitochondrial Mn-superoxide[5]. Moreover, the manganese is involved in the use of vitamin B1 and vitamin E and it is required for normal bone growth and to avoid clotting defects[6]. Therefore, the content of manganese in plants is important for various reasons, such us, nutritional value, toxicity, pollution, geographic origin, etc [7, 8, 9]. From the nutritional point of view, the presence of elements as manganese is of a great concern for adequate nutrition of other organisms of higher in the food chain [10].

Manganese supplements have increased in popularity and nowadays it is present in many of the foods we eat. It has been found that Mn levels in pine nuts (*Pinus pinea*) vary from 26 to 559 µg g^{-1} depending on the area of origin [11]. Mn concentration in a sample of pine nuts has been reported to be 478 µg g^{-1} by other authors [10]. Other foods also contain Mn in this range of concentration namely, black walnut (576.2 µg g^{-1}), peacan (143 µg g^{-1}), sunflower (179 µg g^{-1}), white walnut (68 µg g^{-1}) [10], soybean flour (31.2 µg g^{-1}) [12] or (41.7 µg g^{-1}) [13] or lower as common bean (18.2 µg g^{-1}) [13], brazil nut (12 µg g^{-1}) and cashew (9 µg g^{-1}) [10]. Mn concentrations in a healthy human are typically about 0.5 µg l^{-1} in serum and 1 µg l^{-1} in urine[2], from 2 to10µg l^{-1} in human milk[6] and about 1.5 mg kg^{-1} in human liver [14]. Too much higher concentrations have been found in almond (4780 µg g^{-1}) and peanut (2110 µg g^{-1})[10]. Other elements have also been studied along with Mn and a multielemental (Mn, Zn, Ni and Cu) fractionation in pine nuts (*Pinus pinea*) from different geographic origins by size-exclusion chromatography (SEC) with UV and inductively coupled plasma mass spectrometry (ICP-MS) detection has been performed and described elsewhere[11].

Pine nuts are widely produced in forestry areas from Spain, Portugal, Italy, Greece, Albania and Turkey where it is one of the most important ingredients of the Mediterranean diet[15]. Pine nuts, raw or roasted, are included as ingredients in a great variety of traditional dishes, such as breads, candies, sauces and cakes, as well as in vegetable and meat dishes. It is an edible nut with an exquisite flavour and high protein content which makes them a good source of nutrients. These nuts have a complex chemical composition (5.6% moisture, 31.1% protein, 47.4% fat, 10.7% carbohydrate and 4.3% ash)[16], in addition, they contain vitamins, particularly B1 (thiamine) and minerals [15].

Many analytical methods have been proposed for manganese determination, including spectrophotometry [17], polarography [18], neutron activation analysis (NAA) [19], atomic absorption spectrometry (AAS) [20] and inductively coupled plasma-atomic emission spectrometry (ICP-AES) [21]. For Manganese speciation several methods have been applied such as electrochemical stripping analysis, anodic stripping voltammetry (ASV), differential pulse anodic stripping voltammetry (DPASV)[2], generally used for water samples analysis. Other methods have also been proposed like capillary electrophoresis-ICP-MS [14] in porcine liver, HPLC-ICP-MS in cereal flour samples analysis [22] and flame atomic absorption spectrometry after cloud point extraction in water [23]. Mn speciation has also been performed in milk samples by using SEC combined with ion exchange chromatography (IEC) and ICP-MS[6]. However, under our knowledge manganese-binding biomolecules have not been

analysed by molecular mass spectrometry for a better understanding of manganese species in the fractions obtained by SEC.

In this work, a metallomics analytical approach has been applied for the first time to the study of manganese species in pine nuts with the aim of identifying known and unknown Mn-binding biomolecules in this food. For this purpose, a two-dimensional chromatographic separation combining size exclusion and strong anion exchange chromatography was used to separate the Mn species that were detected by an ICP-MS, equipped with an octopole reaction cell (ORC). This approach reduces the sample complexity prior to tandem mass spectrometry analysis [24,25]. A rigorous quality control has been additionally applied to the ICP-(ORC)MS coupling. Commercial Mn standards were injected in IEC-ICP-MS to match the retention times with the compounds in the samples. The presence of Mn species was confirmed by nano-electrospray (n-ESI) with direct infusion-quadrupole time-of-flight (DI-QqTOF) mass spectrometry. In addition, other Mn compound not correlated with the available standards was sequenced and identified by mass spectrometry.

Experimental

Standard solutions and reagents

Standards used to calibrate the size exclusion chromatographic columns were: Bovine Serum Albumin (67000 Da), Metallothionein I (7000 Da), Gastrin rat I (2126 Da), Vitamin B12 (1352 Da) and Gly6 (360 Da) for the low molecular weight (LMW) column, and Bovine Serum Albumin (67000 Da), Ovalbumin (43000 Da), Chymotrypsinogen A (25000 Da) and Ribonuclease A (13700 Da) for the high molecular weight (HMW) column. The void volume was determined in the LMW and HMW columns using bovine serum albumin (67 kDa) and Blue Dextran 2000 (2000 kDa), respectively. All these reagents were purchased from Sigma-Aldrich (Steinheim, Germany). The mobile phase solution used in SEC was 0.05 M of tris(hydroxymethylaminomethane) (Tris) at pH 8.0, daily prepared from Trizma base and Trizma hydrochloride (Sigma-Aldrich).

The mobile phase components for IEC, NaOH and ammonium acetate (each Suprapure grade) were purchased from Merck (Darmstadt, Germany). The trypsine (EC 3.4.21.4) TPCK was obtained from Sigma-Aldrich (Steinheim, Germany) and the urea, iodoacetamide and dithiotreithol from Bio-Rad (Madrid, Spain). Poros™ R2 50 µm beads from Applied Biosystems (Foster City, CA, USA) was also used in the study.

The Mn standards: arginase, citric acid and $MnCl_2$ were obtained from Sigma-Aldrich (Steinheim, Germany). Working solutions were daily prepared by further dilution with ultrapure water (18 MΩ cm) from a Milli-Q System (Millipore, Watford, UK). Commercial chemicals were of analytical reagent grade and were used without any further purification. Mn-protein stock standard solution used for IEC (arginase) was prepared by weighting 100 mg of each compound to be dissolved in 10 ml Milli-Q water. Mn-citrate stock solution was prepared by mixing a solution of 100 mg L^{-1} of citric acid with $MnCl_2$ aqueous solution (10 mg of Mn L^{-1}). Stock solutions were stored in the dark at -20ºC. The stability of standard compounds was observed under these conditions.

Instrumentation

The element detection for these speciation analyses was carried out using an ICP-MS Agilent, Model 7500 ce (Agilent Technologies, Tokyo, Japan), consisting of an ICP source with a plasma shielded torch, an enclosed octopole ion guide operated in rf mode and a quadrupole mass analyzer with secondary electron multiplier operating in dual mode (pulse counting or analogue mode) This instrument was fitted with a microconcentric nebulizer Micromist with minicyclonic spray chamber. The spray chamber was cooled to 2 ºC.
Mass spectra measurements were performed on a nano-electrospray ionization tandem mass spectrometer API QSTAR® XL Hybrid system (Applied Biosystems, Foster City, CA, USA). The sensitivity of the ICP-MS was optimised by using a 2% HNO_3 solution containing ^{59}Co, ^{89}Y and ^{205}Tl (1 µg L^{-1} each). The background was tested by using ultrapure water while monitoring the 56 *m/z* (ArO^+) until the signal was below 1800 cps.

Size-exclusion chromatography was carried out by using a Hiload 26/60 Superdex 30 Prep for separation range <10 kDa (low molecular weight-LMW) and a Superdex 75 Prep for separation range 3-70 kDa (high molecular weight-HWM) (all from Amersham Biosciences, Uppsala, Sweden). An AKTA-Prime system (pump and UV detector at 280 nm) (Amersham, Biosciences, Uppsala, Sweden) was used as the eluant delivery system, equipped with a 2 ml sample loop. The IEC column (Dionex AS11-HC, Dionex, Indstein, Germany) used for the separation of Mn-containing compounds was mounted in an Agilent 1100 liquid chromatograph with a 75 µl sample loop.

A model Sigma 4-10 centrifuge and a constant orbital shaker operating at 105 rpm (Heidolph, Unimax 1010, Germany) were used to accelerate the phase separation process in the extraction of the compounds. The collected fractions were freeze-dried using a benchtop lyophilizer (Hucoa-Erlöss, Spain).

Procedures

Sample and sample preparation. Samples of pine nuts (*Pinus pinea*) were supplied by Frutos Secos Puig, (Tarragona, Spain). All samples were washed with ultrapure water, freeze-dried and finally ground using a conventional grinder Moulinette (Moulinex, Spain). First of all, lipids were eliminated from 5 g of freeze-dried pine nuts with 25 ml of chloroform/methanol (2:1) mixture for 30 min in a constant orbital shaking followed of centrifugation at 10000 rpm for 20 min. Afterwards, lipid-free pine nut samples (approximately 0.2 g) were accurately weighted in PTFE centrifuge tubes to extract the elemental species with 4 ml of 0.1 M sodium hydroxide in a constant orbital shaker for 10 min and centrifugation at 10000 rpm for 20 min [11].

Determination of total Mn concentration in the digested samples. Samples were digested for metal analysis by ICP-MS following a procedure previously described[11]. This can briefly described as follows: Aliquots of 0.2 g of defatted residues with a chloroform/methanol mixture, exactly weighted, were digested in closed PTFE bombs with 10 ml of nitric acid (65 % w/v) using a domestic microwave oven. Three decreasing steps were successively used: heating at 800 W (3 min), 400 W (3 min) and 100 W (3 min) for sample decomposition and cooling at room temperature for 10 min between each step to avoid overpressure. The final solution was filtered through a 0.20 µm surfactant-free cellulose acetate filter.

First chromatographic dimension. Fractionation by size-exclusion LC. The SEC-UV-ICP-(ORC)MS coupling was performed connecting the outlet of the UV detector to the nebulizer inlet of the ICP-MS. Hydrogen was introduced into the octopole cell as the reaction gas. The gas is introduced into cell through a brass line using a mass flow controller. The operating conditions were optimized and they are summarized in Table 1. Elemental fractionation profiles of the pine nut samples were performed with both columns described in the Section "Instrumentation". A solution of 0.002 M EDTA was used

during the washing programme of the chromatographic column to avoid problems related to metal contamination. An additional cleaning with NaOH assures the elimination of the remaining proteins retained in the column 11.

To standardize the molecular weight range of the chromatographic separation, the standards, described in Section "sample and sample preparation", were used. These standard compounds were dissolved in the mobile phase and their chromatographic profiles were monitored by means of the UV detector.

Second chromatographic dimension. Fractionation by strong anion-exchange LC. Tryptic digestion of unknown fractions. The different molecular weight fractions of Mn were spotted by SEC-UV/ICP-MS and latterly purified by using a similar instrumental arrangement based on anion exchange chromatography (IEC-UV/ICP-MS). The instrumental operating conditions are given in Table 1. The elution in the system IEC-UV/ICP-MS, was carried out with 10 mM NH_4-acetate/acetic acid, pH 6.3, serving as eluent A, 0.8 mM NaOH as eluent B and 10 mM NaOH as eluent C. The gradient was performed with 100% eluent A (3 min), 100% eluent B (3-14 min) and 100% eluent C (14-22 min).

Identification of peaks from IEC chromatograms was accomplished by retention time matching with standards. The unknown peaks were collected, freeze-dried and stored at -20 ºC, for no longer than two weeks, for further identification by mass spectrometry and after tryptic digestion when required.

An aliquot of the lyophilizate from IEC (100 mg) was dissolved in 200µl of 8M urea-1M ammonium bicarbonate (pH 8.3), followed by the addition of 5 µl of DTT (180 mM), and incubated at 37 ºC for 1 hour. After that, the sample was maintained in the dark at room temperature for 1 hour after addition of 5µl of iodoacetamide (400 mM). Finally, the sample was dissolved in 800 µl of 50 mM ammonium bicarbonate aqueous solution and 4 µl of the trypsin solution (1 µg μl^{-1}) and digested at 37 ºC for at least 12 hours in a water bath. The reaction was stopped by bringing the pH down to 5 by addition of 0.1% aqueous formic acid. Sample desalting was performed before the mass spectrometric analysis using the ProxeonTM purification capillaries from Applied Biosystems (Foster City, CA,USA). Slurry with the poros R2 material (100 µl) was prepared in 50% MeOH (5 ml), then 5 µl was placed onto the capillary. The packed bed should be no more than halfway up the tapered part. The end of the capillary was slightly opened and centrifuged for 10-20 sec. The stationary phase was cleaned-up by applying 5-10 µl of 3 % formic acid/70% MeOH with centrifugation. Then 5-10 µl of 3% formic acid/5% MeOH was applied twice for preparing the Poros material for peptide adsorption. The sample was applied, re-dissolved in 3% formic acid/5% MeOH and the contaminants were desorbed by applying twice 5-10 µl of 3% formic acid/5% MeOH. Finally, peptides were eluted onto a nanospray capillary by passing 2 µl of 3% formic acid/70% MeOH using centrifugation until the stationary phase was completely dry. The peptide mixture was introduced in the nanospray source.

Nano-electrospray Q-TOF analysis. The mass spectrometer was calibrated immediately prior to sample analysis, using two MS/MS fragments of glu-fibrinopeptide B [M+H]+=175.1190 and 1285.5444 Da. ESI-TOF-MS data acquisition was performed in positive ion mode with a mass range of 400-1700 m/z. Q1 was fixed at unit mass resolution and the values for ion spray voltage, curtain gas, ion source gas, CAD gas, and electron multiplier voltage were set to: 700-900, 20, 25, 6 and 2300 V, respectively. Data analysis was performed using Analyst QS software (Applied Biosystems).

After the TOF-MS spectra, doubly charged peptide ions were selected, and MS/MS spectra were analyzed by increasing the collision energy. From the MS/MS spectra, peptide sequences were identified by manual peptide sequencing using BioanalystTM (Applied Biosystems, Foster city, CA, USA).

Peptide sequences were then searched by database (NCBI) using Mascot searching engine (http://www.matrixscience.com).

Results and discussion

Extraction of Mn species from pine nuts

The use of NaOH has been proved to be the best extractant for high and low molecular weigh species in comparison with HCl and hot water. HCl solutions mainly extract LMW compounds due to the lower solubility of protonated compounds such as proteins and hot water is used in a less extent [10, 26, 27]. On the basis of these findings, we used NaOH in order to avoid losses of information.

Size-exclusion fractionation profiles of manganese compounds in pine nut extracts

As previously commented, extracts of pine nuts were analyzed by SEC on both columns Hiload 26/60 Superdex 30 and Superdex 75. Eluate from SEC columns was passed trough the UV and the chromatograms registered. The absorbance was initially studied at several wavelength values in the range of 200-500 nm but optimum response was obtained at 232 nm. The combined use of both columns allowed a good separation of compounds in the range of 360-70000 Da. The coupling of the ICP-MS detector with SEC enabled manganese specific detection of eluted compounds. Chromatograms obtained by in series UV and ICP-MS detection are shown in Figure 1. Separation obtained by both columns was similar for HMW region in which they overlap, but for LMW region the Hiload 26/60 Superdex 30 column allowed a better peak resolution. Since the HMW column does not give additional information to the LMW one, this later was chosen for the further experiments.

The manganese fractionation profile obtained with the LMW column (Fig.1) shows three peaks using the ICP-(ORC)MS detection, which are associated to the molecular weight ranges: MW (10-7 KDa), (7000-2126Da) and (2126-1352 Da) according to mass calibration. This seems to indicate Mn bound to biomolecules. However, the confirmation of specific Mn species is not possible with only a SEC separation[6], and a complementary IEC chromatographic method was applied for further purification of the Mn SEC fractions.

Manganese fractionation and quantification by IEC-UV/ICP-MS

The highest intensity Mn peak in the SEC-ICP-MS chromatogram correspond to the fraction in the MW range 10-7 KDa. This fraction was collected and latterly submitted to the second chromatographic orthogonal separation based on anion exchange with Mn elution monitoring by ICP-MS. Results are shown in Fig 2.

The IEC chromatogram showed three peaks with retention times at 1.9, 4.4 and 31.8 min. The first two peaks were identified by retention time matching with standards and resulted to be $MnCl_2$ and Mn-citrate complex, respectively. The peak corresponding with $MnCl_2$ eluted in the void volume of the column, but when ^{55}Mn and ^{35}Cl was on-line monitored by ICP-MS the chromatogram showed the presence of both elements. The third

chromatographic peak does not match with the Mn standards available at the laboratory, so it was collected and further identified by molecular mass spectrometry. As established previously, the chromatogram baseline is noisy that can be related to the presence of different amino acids from the complex matrix of pine nuts [28].

Quantification of Mn compounds identified by retention time matching with the available standards in the IEC-ICP-MS chromatograms was performed by plotting integrated peak areas against the absolute amount of the analytes. The instrumental detection limits were calculated as three times the standard deviation of the calibration curve divided by the slope and they are given in Table 2. Linear calibrations curves were obtained from the quantification limits to 1000 µg L^{-1} for all the analytes. The repeatability of the retention times was estimated from six successive chromatographic runs and estimated to be less than 1.95 % for all the Mn species.

Mass balance monitoring of Mn

In order to carry out a quality control of the analytical procedure, the Mn concentration was measured in each step of the proposed scheme. The total Mn concentration in the studied sample was 65 µg g^{-1} (15.24 µg Mn). The aqueous extract of the sample contained 71% of total Mn of the original nut. The SEC column efficiency was estimated to be 53% (Mn eluted/Mn injected). The major Mn-containing fraction (Fig. 1) represents 25 % of the Mn injected onto the SEC column. The efficiency of the IEC column was 86 % and the final peaks isolated from the IEC at retention times 4.4 and 31.8 min contain 0.6256 and 0.3215 µg of Mn, respectively.

Confirmation of the presence of Mn-citrate by DI-QqTOF.

The previously identified peak as Mn-citrate by retention time matching with the standard was collected from the IEC column at the corresponding retention time. The obtained fraction was eluted from the IEC in 10 mM NH_4-acetate/acetic acid at pH 6.3, which is in addition suitable for electrospray ionization mass spectrometry[6].

The n-ESI-MS spectrum obtained from the direct infusion of the collected fraction into the mass spectrometer is shown in Figure 3a. As can be observed, the base peak of the spectrum was at m/z 438 that matches with the protonated molecular ion of Mn-citrate complex $[Mn(C_6H_7O_7)_2+H]^+$. The MS/MS of the m/z 438 ion (Figure 3b) gives one abundant fragment at m/z 246 that indicates the loss of 191 units, which can be attributed to one citric acid molecule $[C_6H_7O_7]^+$. The fragment at m/z 246 matches with the 1:1 ratio complex $[Mn(C_6H_7O_7)]^+$.

Identification of the manganese-binding to protein fraction after tryptic digestion

The third Mn containing peak in the IEC-ICP-MS chromatogram was collected and freeze-dried. The collection was repeated several times until a final volume of 1.5 ml. The fraction was submitted to tryptic digestion using the procedure above described and analysed by n-ESI-QqTOF. Figure 4a shows the obtained mass spectrum of the peptides after the tryptic digestion. As can be seen, the sensitivity was not too high due to the complex sample matrix that has also been reported by other authors[29]. For the protein sequencing, the doubly charged peptide ions was located in the range of m/z 530-572 (m/z 537.3, 537.8, 560.8, 560.3, 571.3 and 571.8) and fragmented (Fig.4b). Figure 4c shows MS/MS of m/z 537.3 ion that was selected due to its higher intensity in the mass spectrum than other doubly charged peptide ions.

The data obtained by nano-ESI-MS and nano-ESI-MS/MS were introduced in the MASCOT database for protein identification. The obtained sequences were the following: EATARVGVDR, REAANEGEEK and NGGDGAPQED and gave in each case a single highly significant hit to the same protein, referred as Isocitrate dehydrogenase (SwissProt accession no. Q40658) with a sequence coverage about 51 %. This protein is registered in the database as a protein present in rice (*Oryza sativa*).

This protein, with the molecular mass of about 7204 Da, had not been associated to pine nut (*Pinus pinea*) until now. However, its presence has been stated in chestnut (*Castanea sativa*) [30]. In the present study manganese and isocitrate dehydrogenase have been detected by ICP-MS and ESI-MS, respectively, in the same fraction after two dimensional SEC plus IEC chromatography that indicates some possible interaction of both as stated for other authors [31].

Conclusions

The use of orthogonal chromatographic systems namely, SEC and IEC allowed the purification of Mn species that is mandatory for molecular mass spectrometry identification. A third purification step based in a reversed phase desalting onto a nano capillary was developed to improve the identification of the species. In addition, the use of a sensitive elemental specific detector as ICP-MS for Mn elution monitoring assures the presence of this element in SEC fractions and in the case of octopole reaction system ICP-MS polyatomic interferences are eliminated. Moreover, the use of the nanoelectrospray probe permits low sample consumption allowing the fragmentation of all the peaks in the mass spectra and an accurate mass measurement was attained by means of TOF-MS.

The approach applied in the present work allows the identification of manganese and dehydrogenase, in the same fraction after two dimensional SEC plus IEC chromatography. Quantification data of some Mn containing species in pine nuts are reported for the first time by using IEC-ICP-MS.

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Single Column Figure/Scheme

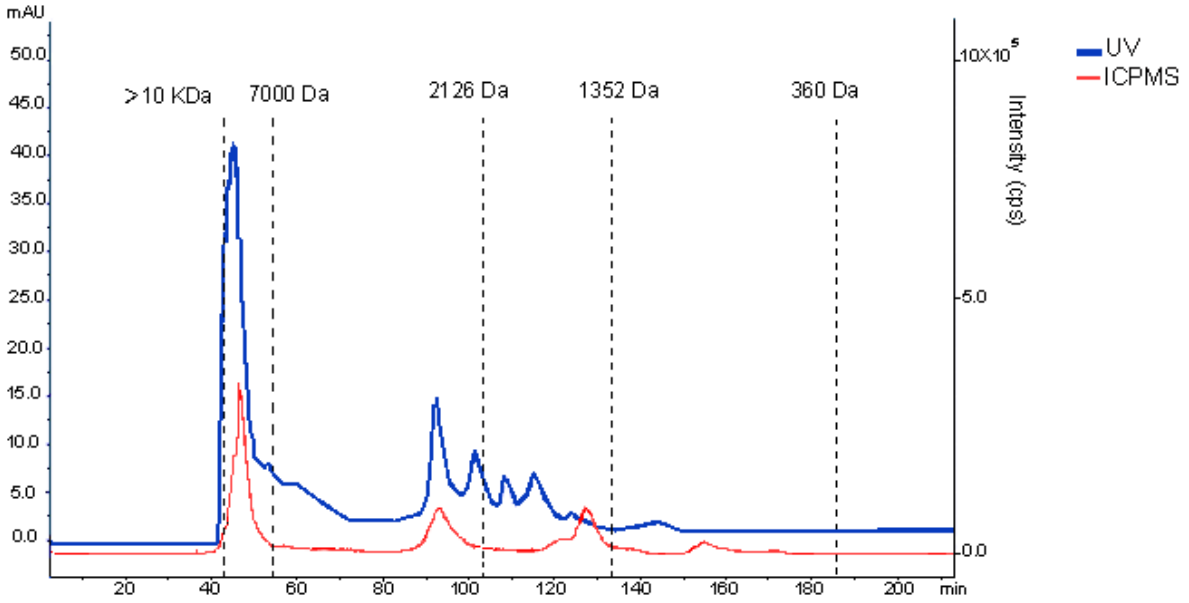


Figure 1. Separation of manganese compounds by size-exclusion chromatography with UV-ICP(ORC)MS detection. Thick line, UV (232 nm) detection; thin line, ^{55}Mn ICPMS detection

Figure 2. IEC-UV-ICPMS chromatogram of Mn-containing fraction collected by SEC. The inset shows SEC fraction collected (MW 10-7 KDa).

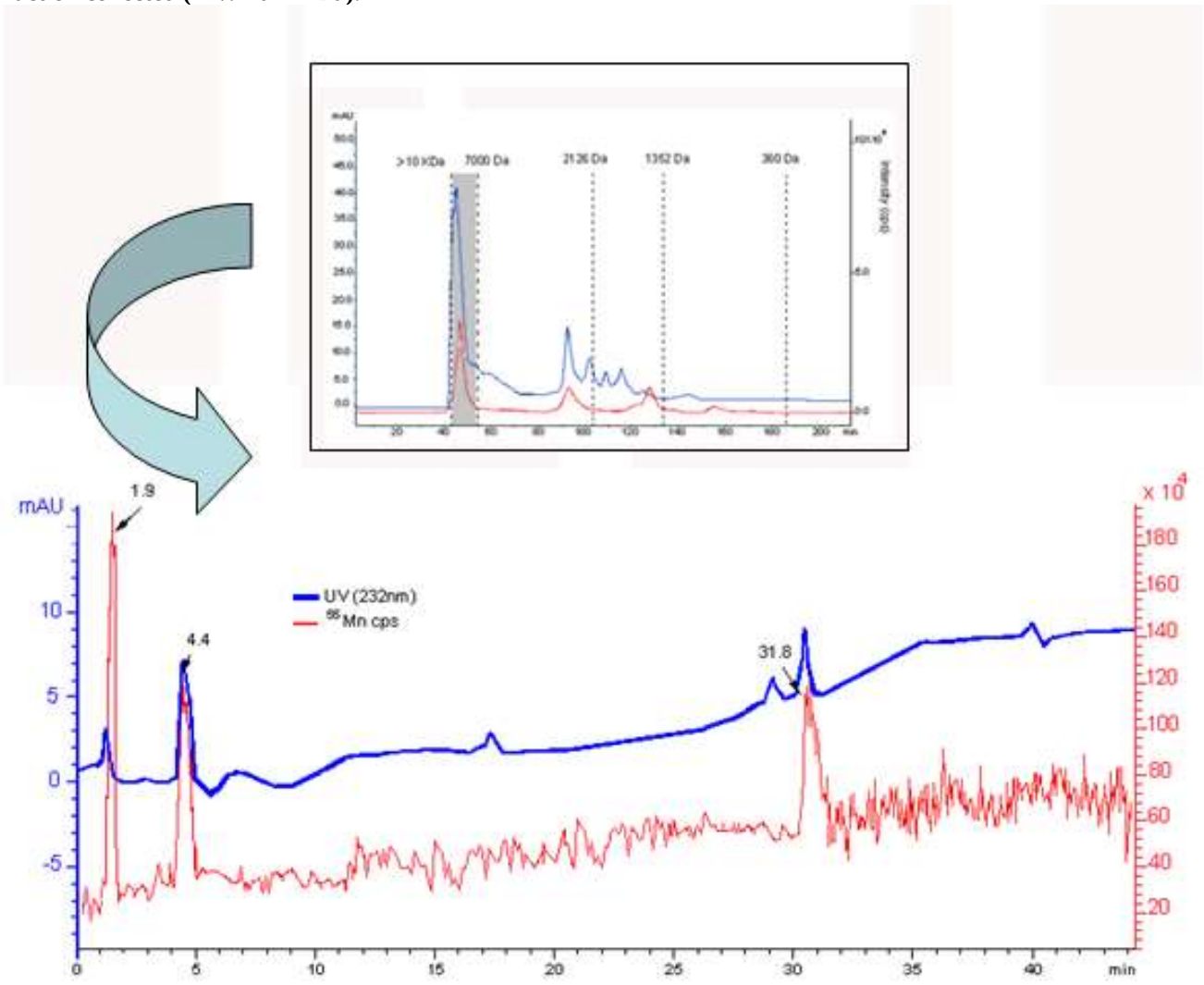


Figure 3. (a) nano-ESI-MS mass spectra of the Mn peak collected from IEC separation at 4.43 min; (b) MS/MS of the m/z 438 ion (Mn-citrate complex)

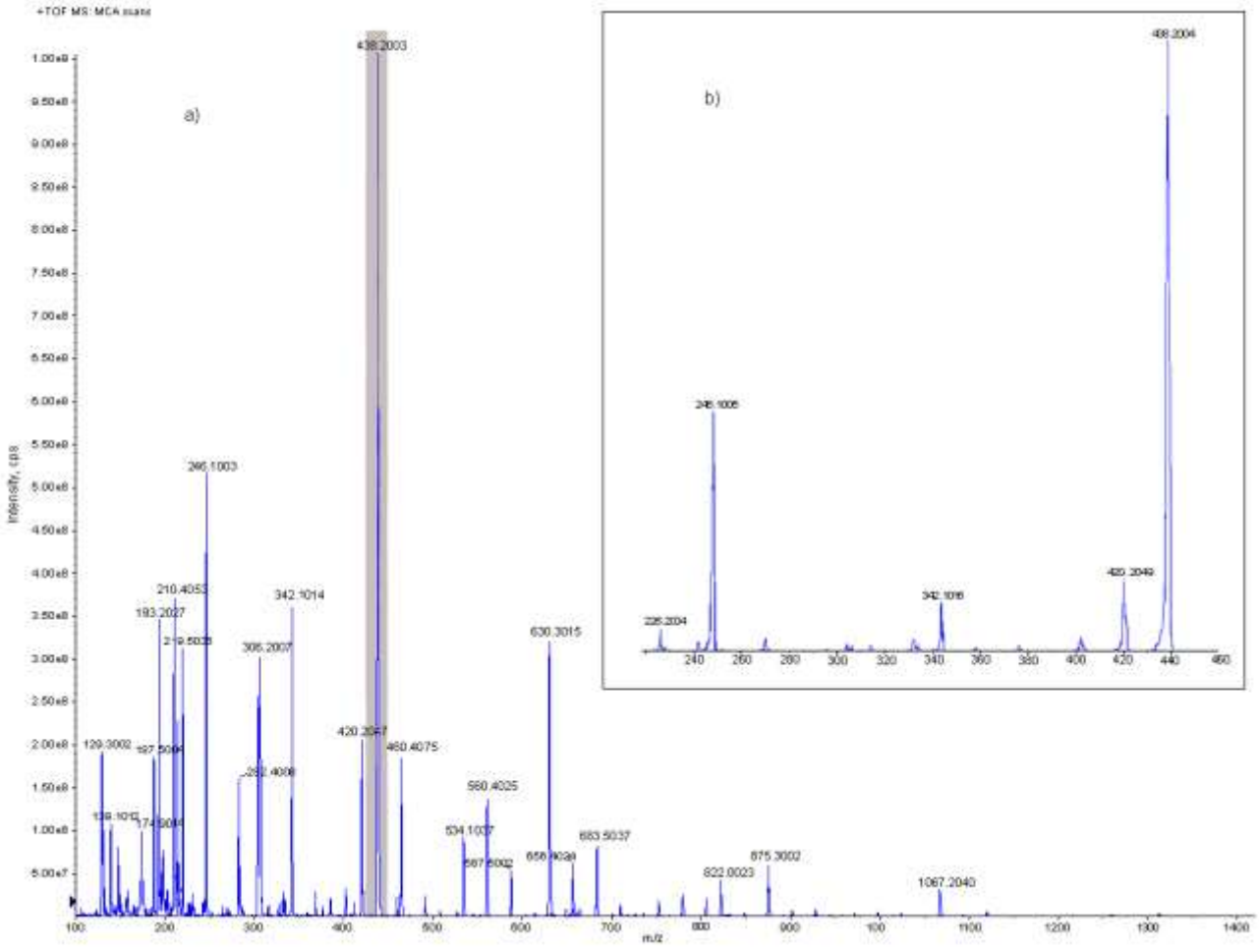
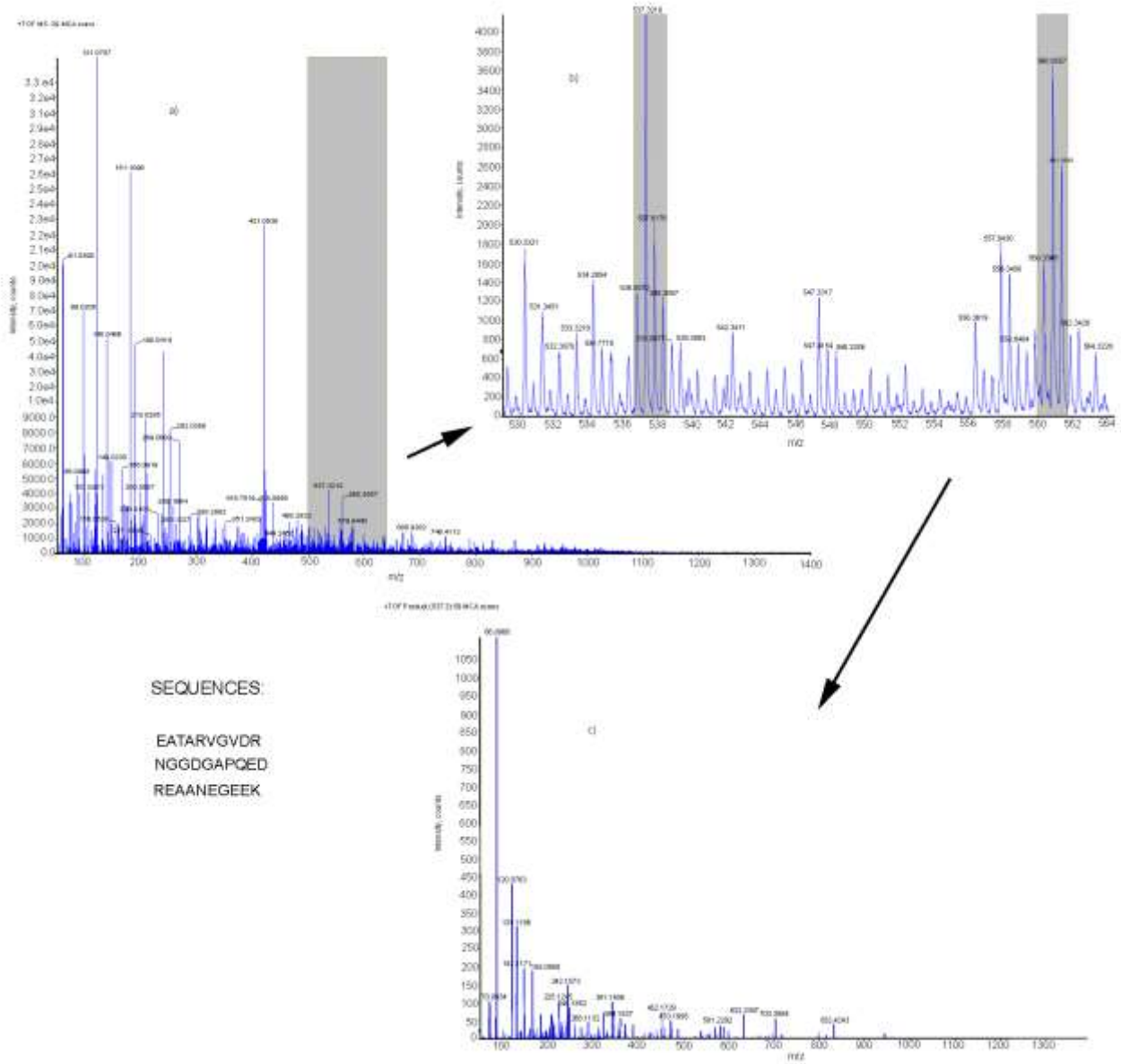


Figure 4. (a) n-ESI-MS of the tryptic digest from the unknown peak;(b) Enlarged region between m/z 530 and 560; (c) MS/MS of m/z 537.3 selected for protein sequencing



Double Column Table

Table 1 Operating conditions for the ICP-(ORC)MS, SEC and IEC

SEC conditions	
Columns	Hiload 26/60 Superdex 30 Prep; Hiload 26/60 Superdex 75 Prep
Resolution range	Mr< 10 000 Da; 3000-70 000Da
Mobile phase	Tris 50 mmol L^{-1} (pH 8.0)
Flow rate	2 ml min^{-1}
Injection volume	2ml
UV-visible wavelength	232 nm
IEC conditions	
Column	Dionex AS11-HC
Mobile phase	A=10 mM NH_4-acetate/acetic acid (pH 6.3), B=0.5 mM NaOH, C=10mM NaOH
Flow rate	0.5 ml min^{-1}
Injection volume	75 µl
UV-visible wavelength	232 nm
ICP-(ORC)MS conditions	
Forward power	1350W
Plasma gas flow rate	15.0 L min^{-1}
Auxiliary gas flow rate	0.87 L min^{-1}
Carrier gas flow rate	0.975 L min^{-1}
Sampling depth	6 mm
Sampling and skimmer cones	Nickel
H_2 flow rate	4 mL min^{-1}
Q_{oct}	-13 V
Q_p	-11.5V
Dwell Time	0.1 s per isotope
Isotopes monitored	^{55}Mn

[a] Footnote text.

Table 2.- Performance data of the IEC-UV-ICPMS coupling for Mn species

Compounds	**t_r(min)**	**Detection limits/µg Kg^{-1}**	**Identification (retention time matching with standards)**	**Concentration (as Mn) in µg Kg^{-1}**
$MnCl_2$	1.9	26	detected	75
Mn-citrate	4.4	96	detected	125
Arginase	22.2	150	Not detected	—
Peak 3[a]	31.8	—	Unknown peak	65

[a] Peak 3 was latterly identified as isocitrate dehydrogenase by ESI-MS