elsevier_ACA_233369

Absolute quantification of superoxide dismutase in cytosol and mitochondria of mice hepatic cells exposed to mercury by a novel metallomic approach

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Abstract

In the last years, the development of new methods for analyzing accurate and precise individual metalloproteins is of increasing importance, since numerous metalloproteins are excellent biomarkers of oxidative stress and diseases. In that way, methods based on the use of post column isotopic dilution analysis (IDA) or enriched protein standards are required to obtain a sufficient degree of accuracy, precision and high limits of detection. This paper reports the identification and absolute quantification of Cu,Zn-superoxide dismutase (Cu,Zn-SOD) in cytosol and mitochondria from mice hepatic cells using a innovative column switching analytical approach. The method consisted of orthogonal chromatographic systems coupled to inductively coupling plasma-mass spectrometry equipped with a octopole reaction systems (ICP-ORS-MS) and UV detectors: size exclusion fractionation (SEC) of the cytosolic and mitochondrial extracts followed by online anion exchange chromatographic (AEC) separation of Cu/Zn containing species. After purification, Cu,Zn-SOD was identified after tryptic digestion by molecular mass spectrometry (MS). The MS/MS spectrum of a doubly charged peptide was used to obtain the sequence of the protein using the MASCOT searching engine. This optimized methodology reduces the time of analysis and avoids the use of sample preconcentration and clean-up procedures, such as cut-off centrifuged filters, solid phase extraction (SPE), precipitation procedures, off-line fractions insolates, etc. In this sense, the method is robust, reliable and fast with typical chromatographic run time less than 20 min. Precision in terms of relative standard deviation ($n = 5$) is of 3–5% and detection limits is 0.21 ng Cu g^{-1}.

The application of the methodology to hepatic cells from mice exposed to inorganic mercury reveals decreased levels of Cu,Zn-SOD in cytosolic and mitochondrial extracts, as a consequence of the oxidative stress caused by this toxic metal. Additionally, the quantification of mitochondrial Cu,Zn-SOD in hepatic cells from *Mus musculus* has been carried out for the first time.

Abbreviations: AD, Alzheimer's disease; AEC, anionic exchange chromatography; DNA, deoxyribonucleic acid; DTT, dithiothreitol; ESI, electrospray ionization; EC, extra cellular; HPLC, high-performance liquid chromatography; ICP-MS, inductively coupling plasma-mass spectrometry; IAA, iodoacetamide; IDA, isotopic dilution analysis; MS, mass spectrometry; ORS, octopole reaction systems; PD, Parkinson disease; PMSF, phenylmethanesulfonyl fluoride; PBS, phosphate buffer solution; ROS, reactive oxygen species; RBCs, red blood cells; SEC, size exclusion chromatography; SPE, solid phase extraction; SUID, species-unspecific isotope dilution mode; SOD, superoxide dismutase; QqQ-TOF, triple quadrupole-time-of-flight; TCEP, *tris*(2-carboxyethyl)phosphine hydrochloride

Keywords: Superoxide dismutase; Isotopic dilution analysis; Metallomic workflow; Mitochondrial extracts; Column switching; ICP-MS

1 Introduction

Quantitative metalloproteins analysis is nowadays one important challenge in analytical and bioanalytical chemistry. The presence of a heteroelement in proteins enables the application of elemental mass spectrometry in the study of metalloproteins and provides a suitable tool in this field [1]. The Cu-Zn SOD is a homodimer enzyme with molecular mass of 32 kDa that contains six histidine and one aspartate bond to Cu and Zn, in which one histidine is shared between the two metals [2]. SOD catalyzes the dismutation of superoxide radicals into oxygen and hydrogen peroxide, which represents one important defense mechanism against oxidative stress [3,4]. For this reason, the interest on accurate and precise determination of metalloproteins, such as Cu,Zn-superoxide dismutase (Cu,Zn-SOD), involved in the redox balance of living cells, is increasing.

Mitochondria is the main intracellular source for oxidizing free radicals (O_2^- and NO). The free radical chain reactions are started by O_2^- and NO and involve a series of reactive oxygen (ROS) and nitrogen species that are capable of damaging mitochondria membranes (mainly lipids), proteins and DNA [5–9]. The superoxide radical is dismutated rapidly by three types of SOD isoenzymes [10], Cu,Zn-SOD, Mn-SOD and extracellular SOD (EC-SOD). When the subcellular fractionation of SOD activity assay was first explored in liver, the cytosol was found to contain a Cu,Zn-SOD and the mitochondrial matrix Mn-SOD [11,12]. Nevertheless, in the last few years, several authors confirm the presence of Cu,Zn-SOD in the mitochondrial fraction [13,14]. The SOD can be induced by exposure to many pollutants, such as toxic metals [15–19]. Moreover, Cu,Zn-SOD has also been identified as a major target of oxidative damage in brain as a consequence of Alzheimers (AD) and Parkinson (PD) diseases [20]. On the other hand, Hg is known to generate reactive oxygen species (ROS) in vivo and in vitro. Therefore, it is likely that redox enzyme activities are altered to scavenge the increase of ROS levels produced as a result of mercury accumulation. These enzymes, such as SOD, play an important role in the protection of cell membranes against oxidative stress [21]. For all these reasons, a method to accurate absolute quantification of Cu,Zn-SOD is mandatory.

At present, most existing methods for Cu,Zn-SOD determination are based on the indirect measurement of its enzymatic activity by using the competition between the enzyme itself and a superoxide scavenger, commonly a colorimetric reagent [22]. However, these spectrophotometric methods are highly susceptible to interferences, such as non-SOD enzyme able to react with superoxide radicals or with colorant reagents. On the other hand, elemental mass spectrometry, mainly ICP-ORS-MS, can be considered as one the of the most important analytical techniques for metals at trace and ultratrace levels in proteins [23,24]. Jointly, separation techniques are usually the key components in the speciation of metalloproteins [25]. Furthermore, ICP-ORS-MS permits element isotope ratios measurement and consequently, IDA for accurate and precise quantification of multi-isotopic elements, particularly Cu containing proteins, which is well documented in the literature [26]. Species-unspecific isotope dilution mode (SUID) is especially useful either when the structure and composition of analyzed species is not exactly known or the corresponding isotopically labeled compounds are not commercially available [27].

For an effective separation and quantification of Cu,Zn-SOD, numerous speciation methods based on chromatographic or electrophoretic separations have been developed [26,28–33], being SEC-ICP-ORS-MS the most widely used as a first dimension due to the high reproducible results and remarkable tolerance to complex matrix such as biological fluids (e.g., serum/plasma) and cytosolic tissue extracts [19,29,33]. Nevertheless, low resolution for copper containing proteins is obtained [19,29,33], and alternatively, a number of methods introduce a second dimension after SEC chromatographic separation in order to improve the resolution, but the time of analysis increases to 100 min, and off-line desalting and preconcentration steps are required [19,29,33]. In this sense, good recoveries were obtained using 1D-AEC-SUID-ICP-ORS-MS, but high molecular mass biomolecules are excluded to the low molecular mass using centrifugal filters, but a cellulose membrane filter with a cutoff of 10 kDa is necessary which leads to longer analysis and off-line steps that could cause protein losses and/or denaturation [26].

The aim of the present study is the identification and absolute quantification by SUID of Cu,Zn-SOD in cytosol and mitochondria of mice hepatic cells exposed to inorganic mercury, using a novel column switching analytical approach. This approach consisted of orthogonal chromatographic systems coupled to ICP-ORS-MS and UV detectors, therefore SEC of the liver extracts samples were followed by AEC separation of Cu and Zn containing biomolecules. The optimized methodology was applied to cytosolic and mitochondrial extracts from mice hepatic cells subjected to controlled inorganic mercury exposure.

2 Materials and methods

2.1 Reagents

Cu,Zn-SOD from bovine liver was purchased from Sigma–Aldrich (St. Louis, MO). Isotopically enriched Cu solution (100 ng g^{-1}) with relative abundances of 99.78% of ^{65}Cu were prepared by dilution of a stock solution (2000 µg g^{-1}) in ultrapure water containing 5% of HNO_3 (Ultra Trace analytical grade, Fisher Scientific, Leicestershire, UK). The ^{65}Cu stock solution was prepared from copper elemental obtained from Cambridge Isotope Laboratories (Andover, MA, USA) dissolved in the minimum volume of nitric acid (Ultra Trace analytical quality). The concentration of this solution was established by reverse isotope dilution analysis as described elsewhere [34].

Standards used for the molecular mass calibration of the size exclusion chromatographic column (SEC) and mobile phase were previously used and described elsewhere using a flow rate of 1.0 mL min^{-1} [19]. Trypsin (EC 3.4.21.4) TPCK was obtained from Sigma–Aldrich (Steinheim, Germany) and urea, iodoacetamide (IAA) and dithiothreitol (DTT) from Bio-Rad (Madrid, Spain). Cu,Zn-SOD from bovine serum was purchased from Sigma–Aldrich (St. Louis, MO). Mitochondria Isolation Kit for Tissue was purchased from Thermo Scientific (Thermo Scientific, Rockford, USA). Phenylmethanesulfonyl fluoride (PMSF) and *tris*(2-carboxyethyl) phosphine hydrochloride (TCEP) (BioUltra grade, >98%) were obtained from Sigma–Aldrich (Steinheim, Germany).

Anion-exchange HPLC separations for the purification of SEC fraction containing Cu–Zn at about 32 kDa was carried out with an AEC column (Protein-Pak DEAE 5PW 7.5 mm × 75 mm, 10 µm, Waters, Milford, MA, USA) using ammonium acetate as an eluent, with a gradient from 2 to 200 mM of this buffer at flow rate of 1.5 mL min^{-1}. The online column switching 2D/SEC-AEC-SUID-ICP-ORS-MS was performed by connecting the outlet of the chromatographic column to the Miramist nebulizer inlet (Burgener, Ontario, Canada) of the ICP-MS by means of a PEEK tube, using post-column SUID of ^{65}Cu enriched solution to quantify Cu,Zn-SOD levels in cytosolic and mitochondrial extracts from mice hepatic cells.

2.2 Instrumentation

A cryogenic homogenizer SPEX SamplePrep (Freezer/Mills 6770, Metuchen, NJ, USA) was used to prepare tissues homogenates. Specific atomic detection was performed using an ICP-ORS-MS model Agilent 7500ce (Agilent Technologies, Tokyo, Japan). Table 1 summarizes the operating conditions of use. On the other hand, chromatographic separations were performed using two HPLC pumps Agilent Model 1100 with detector UV (Agilent, Wilmington, DE, USA) as delivery systems. The ICP-MS measurement conditions (Table 1) for mode He was optimized by using a HNO_3 5% (v/v) aqueous solution of 100 ng g^{-1} of ^{63}Cu. The flow of collision gas in ICP-ORS-MS was fixed at 4.0 mL min^{-1} for He in order to avoid or reduce the interferences.

Table 1 Operating conditions for 2D/SEC-UV-AEC-SUID-ICP-ORS-MS.

ICP-MS conditions	
Forward power	1500 W
Plasma gas flow rate	15 L min^{-1}
Auxiliary gas flow rate	1 L min^{-1}
Carrier gas flow rate	0.15 L min^{-1}
Sampling depth	7 mm
Sampling and skimmer cones	Ni
He $_{flow}$	4 mL min^{-1}
Nebuliser	Miramist (Burgener)
Torch	Shield (with long life platinum shield plate)
~~Qoct 18 VQp~~ Q_{oct}	−18 V
Q_p	−16 V
Points per peak	1
Integration time	0.3 per isotope
Replicates	1
Isotopes monitored for SUID	^{63}Cu, ^{65}Cu, ^{64}Zn, ^{66}Zn
Dead time detector	47 ns
PUMP A: SEC conditions	
Column	Superdex^{TM}-75 (10 ~~× 300~~ µm × 300 µm × 13 µm)
Resolution range	3–70 kDa
Mobile phase A	Ammonium acetate 2 mM (pH 7.4)
Flow rate	1.0 mL min^{-1}
Injection volume	100 µL

UV detection	254 nm
PUMP B: AEC conditions	
Sample loop	3.0 mL
Flow rate	1.5 mL min^{-1}
Mobile phase B	2 mM ammonium acetate pH 8.0
Mobile phase C	200 mM ammonium acetate pH 8.0
Gradient	0–12 min 100% A, 12–28 min 100% B, 28–30 min 100% B
8-port valve position	1–9.5 min position A
	9.5–30 min position B

Molecular mass spectrometry measurements were performed on a nano-electrospray ionization tandem mass spectrometer (API Qstar XL Hybrid system; Applied Biosystems, Foster City, CA, USA) using the operational conditions described in a previous work [19].

2.3 Animals and experiment exposure

Mus musculus (inbred BALB/c strain) mice were obtained from Charles River Laboratory (Spain). Mice of 7 weeks of age were fed ad libitum with conventional pellets (rodent global diet Ref. 2014 from Harlan Laboratories Inc., Indianapolis, IN 46250, USA). The animals were allowed to acclimate for 5 days with free access to food and water under controlled condition (temperature 25–30 °C and a 12 h light-dark cycle) prior to start exposure experiment.

Sixteen mice were divided into two groups, one used as control and the other exposed to Hg(**II**) (as $HgCl_2$) by daily subcutaneous injection of 100 µL of saline buffer (0.9% (w/v) NaCl) containing 0.2 mg Hg kg^{-1} of body weight, during 10 days. Controls were subcutaneously injected with 100 µL ultrapure water containing 0.9% (w/v) NaCl per day for 10 days. All mice were sacrificed on the last day of exposure to assess the effect of mercury and the diet.

Mice were individually anesthetized by isoflurane inhalation and exsanguinated by cardiac puncture, dissected using a ceramic scalpel and finally the organs transferred rapidly to dry ice. Individual organs were excised, weighed in Eppendorf vials, cleaned with 0.9% (w/v) NaCl solution, frozen in liquid nitrogen and stored at −80 °C until they were used for extracts preparation. All animals received humane care in compliance with the guidelines of the animal care and use of the European Community. The investigation was performed after approval by the Ethical Committee of the University of Huelva (Spain).

2.4 Preparation of cytosolic and mitochondrial extracts from mice hepatic cells

Hepatic cells from individual mouse of each group (control and mercury exposed mice) were treated separately. The preparation of cytosolic and mitochondrial extracts was carried out following the instructions from the suppliers of Mitochondria Isolation Kit for soft tissue with some modifications. Briefly, individual livers were disrupted by cryogenic homogenization. Then, 200 mg of pulverized livers were washed with 1 mL of cooled phosphate buffered saline solution (PBS), centrifuged at 13,000 × *g* for 3 min and the supernatant discharged. This step was carried out by duplicates. The remaining solid containing hepatic cells was homogenized with 800 µL of PBS using a 2 mL Dounce homogenizer on ice. Sufficient numbers of Dounce strokes were applied to obtain a homogeneous suspension but not to lyse cells; typically 3–5 Dounce strokes are sufficient. After hepatic cells homogenization, the resulting homogenates were centrifuged at 1000 × *g* for 3 min at 4 °C and the supernatant discharged. A Modified BSA/Reagent A Solution was prepared by adding PMSF and TCEP (1 mM) as proteases inhibitor and reductant agent, respectively. After that, the pellet was suspended with 800 µL of Modified BSA/Reagent A Solution, vortexed at medium speed for 5 s and incubated on ice for exactly 2 min (the 2 min of incubation must not be exceeded). Next, 10 µL of Mitochondria Isolation Reagent B was added, vortexed at maximum speed for 5 s and incubated on ice for 5 min, vortexing at maximum speed every minute. Afterwards, 800 µL of Mitochondria Isolation Reagent C was added to the Eppendorf vial and homogenization was performed by inverting the vial several times to mix (without vortex), followed by centrifugation at 700 × *g* for 10 min at 4 °C. At that time, the pellet was discarded and the supernatant transferred to a new 2 mL Eppendorf vial, centrifuged at 3000 × *g* for 15 min at 4 °C and the supernatant (cytosolic extract) separated from the mitochondrial pellet. Finally, 500 µL of wash buffer was added to the mitochondrial pellet and centrifuged at 12,000 × *g* for 5 min discharging the supernatant. For mitochondria lyses, three different procedures were carried out using ultrasound probe, liquid nitrogen cycles and osmotic lyses with sucrose medium. The best results were obtained with osmotic lyses using 0.25 M of sucrose (mitochondrial extract).

2.5 Optimization of the column switching method 2D-SEC-AEC-HPLC-ICP-ORS-MS

The cytosolic and mitochondrial extracts were filtered through Iso-Disc poly(vinylidene difluoride) (PVDF) filters (25-mm diameter, 0.2-µm pore size) to avoid column overloading or clogging. The quality control of the SEC-ICP-ORS-MS system to

overcome problems related to contamination, loss and stability of species has been described elsewhere [19]. Separation of the analytes was performed with a glass analytical size exclusion column Superdex™-75 (10mm × 300 mm; 13 µm) (GE Healthcare, Uppsala, Sweden) with an exclusion limit of 100 kDa (effective separation range from 3 to 70 kDa). Ammonium acetate 20 mM (pH 7.4) was used as mobile phase, at a flow rate of 1.0 mL min^{-1} and injection volume of 100 µL.

After SEC separation of Cu containing fractions, they were collected into a loop of 3 mL, tracing with ultraviolet detection (Fig. 1, position A). After that, the 8-port valve is changed to inject the content of the loop to the AEC column (Fig. 1, position B). The use of a column switching with a 8-port valve is required by different pressure of the two pumps. When the position of the 8-port valve is changed from position A to B, the fraction containing the 32 kDa Cu-traced peak (at retention time 9.5–12.5 min, see Fig. 2) that has been retained in the 3 mL loop is driven by the pump B through the anion exchange column with ICP-ORS-MS detection. In this way, this SEC fraction is online chromatographically purified and resolved by the AEC column. The effects of phase mobile pH was tested in the range 7–9 (at intervals of 0.5), flow rates between 1.0 and 2.0 and different gradient elution programs were also checked in order to obtain the best resolution efficiency. Separation of Cu,Zn-SOD by 2D/SEC-UV-AEC-SUID-ICP-ORS-MS in cytosolic and mitochondrial extracts was carried out using the operating conditions summarized in Table 1.

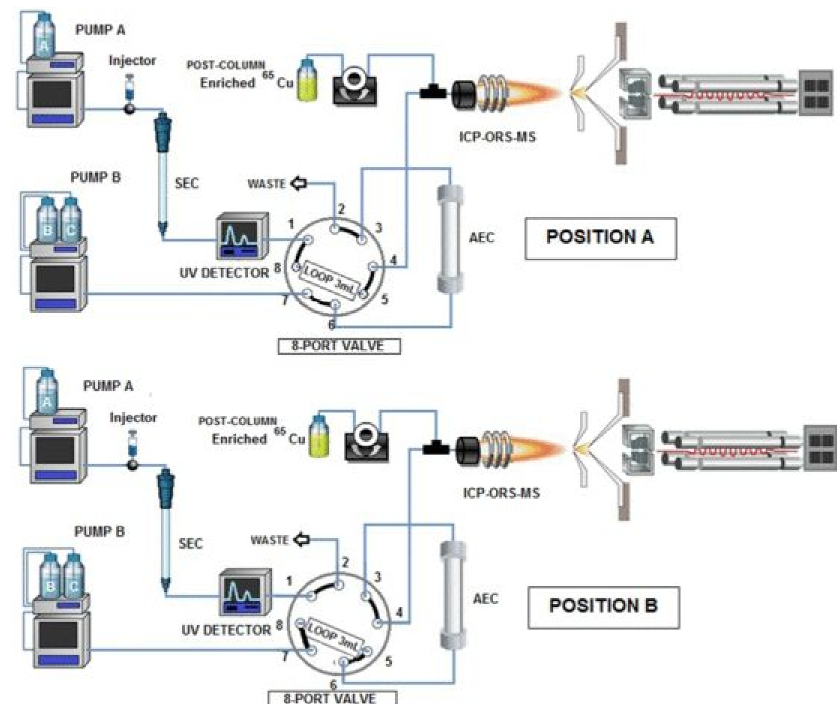


Fig. 1 Schematic diagram of 2D/SEC-UV-AEC-SUID-ICP-ORS-MS arrangement for Cu,Zn-SOD speciation in cytosolic and mitochondrial extracts from mice hepatic cells.

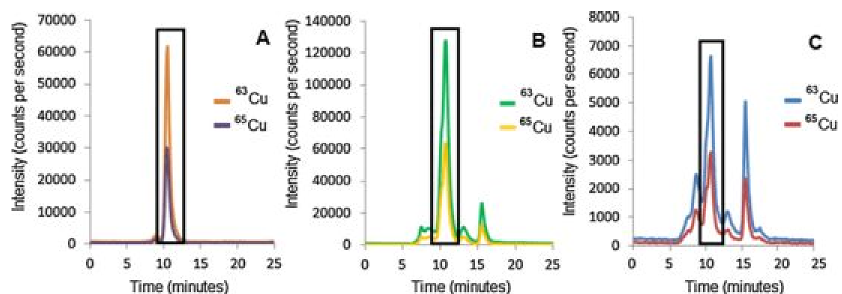


Fig. 2 Copper 1D-SEC-UV-ICP-ORS-MS chromatograms from: (A) Cu,Zn-SOD standard; (B) cytosolic extract from hepatic cell; (C) mitochondrial extract from hepatic cell.

A mass balance for Cu was also evaluated by measuring the concentration of a Cu,Zn-SOD standard using the couplings 1D/SEC-UV-SUID-ICP-ORS-MS and 2D/SEC-UV-AEC-SUID-ICP-ORS-MS, providing quantitative results in the range of 98 ± 6%

(n = 5). Cu was selected for the recovery experiments due to the Zn lability in this enzyme as has been previously reported by other authors [35,36]. Additionally, several metalloproteins containing Zn as co-factor lead to poorer chromatographic resolution and therefore errors for quantification [26]. In this work, various isotopes of both elements have been monitored, but in the case of Zn as qualitative information, since the Cu,Zn-SOD contains both elements.

2.6 Identification of Cu,Zn-traced peak from cytosolic and mitochondrial hepatic cell extracts

The characterization of Cu,Zn-traced peak was performed as described elsewhere [19]. A total volume of 1 mL (5 injections of 200 µL) of cytosolic extracts was purified and collected by 2D/SEC-UV-AEC-SUID-ICP-ORS-MS, desalted using AMICON filters and lyophilized. The lyophilized fraction was redissolved in 100 µL of 6 M urea and 50 mM ammonium bicarbonate (pH 8.3). An aliquot of 50 µL of this solution was collected and 5 µL of 180 mM DTT was added to reduce disulfide bonds in the proteins. After 30 min at 37 °C, 5 µL of 400 mM iodoacetamide (IAA) was added to the reaction mixture and left it in dark at room temperature for 30 min. Finally, the sample was dissolved in 290 µL of water to reduce the urea concentration to 2 M, to retain the activity of trypsin. Then, 50 µL of trypsin (0.1 µg $µL^{-1}$) was added and the mixture incubated at 37 °C overnight. The reaction was then stopped by addition of 10 µL acetic acid (glacial). After tryptic digestion, the peptides were desalted, preconcentrated and purified using ZipTips C_{18} (Millipore, Massachusetts, USA). The TOF mass analyzer was calibrated immediately prior to sample analysis using glu-fib peptide as standard. Molecular MS data acquisition was performed in positive ion mode and the MS spectra of peptides were acquired in the range of 400–1600 *m/z*. The values for nano ion spray voltage, electron multiplier voltage, curtain gas and declustering potential were set to: 1100 V, 2200 V, 20 psi and 90 V, respectively. Data analyses were performed using the Analyst QS software (Applied Biosystems). After recording the MS spectra, doubly charged peptide ions were selected, and MS/MS spectra obtained with a collision energy of 45 V. Peptide sequences were then searched by database (NCBI) using MASCOT searching engine.

2.7 Absolute quantification of Cu,Zn-SOD using species-unspecific isotope dilution analysis

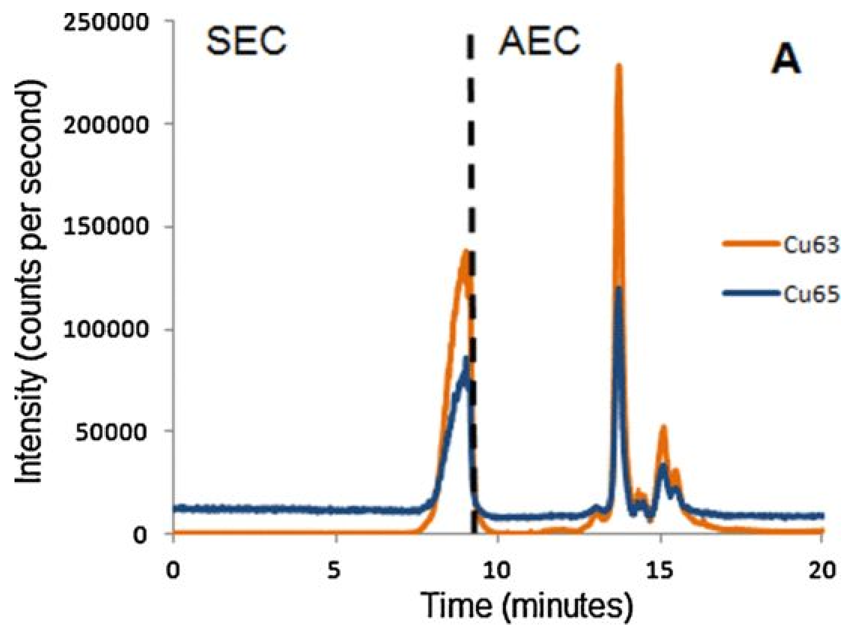
The quantification of copper containing proteins in the different chromatographic peaks was carried out by post-column SUID as described by Nuevo-Ordoñez et al. [26]. Briefly, the intensity of different Cu isotopes and polyatomic interferences were converted to mass flow chromatogram for the quantification of copper species in cytosolic and mitochondrial extracts from mice hepatic cells. Finally, an online dilution equation was applied to each point of the chromatogram and the amount of copper in each peak was calculated using the Origin 8.5.1 software (Microcal Software Inc., Northampton, MA, USA).

3 Results and discussion

3.1 Optimization of copper-containing proteins separation by 2D/SEC-UV-AEC-ICP-ORS-MS column switching method

To optimize the conditions of separation by 2D/SEC-UV-AEC-ICP-ORS-MS, a commercial standard of Cu,Zn-SOD from bovine liver was used. First of all, 1D/SEC-UV-ICP-ORS-MS was applied to Cu,Zn-SOD standard, as well as cytosolic and mitochondrial extracts from mice hepatic cells (Fig. 2). During the chromatographic separation, the isotopes ^{63}Cu, ^{65}Cu, ^{64}Zn, and ^{66}Zn were simultaneously monitored online by ICP-ORS-MS. The use of SEC chromatographic column allows a peak at about 10 min corresponding to the fraction of 32 kDa (Fig. 2A).

In order to obtain a better resolution of the 32 kDa fraction, it was purified online using AEC separation. For this purpose, isolation of Cu,Zn-SOD fraction was performed using 1D/SEC-UV-ICP-ORS-MS (Fig. 2) for collection of this metalloprotein in a 3 mL loop inserted in a 8-port column switching valve (Fig. 1, position A), to be later introduced into AEC, changing the valve position to B (Fig. 1, position B). In addition, the 8-port valve compensates the pressure gradient between low and high pressure pumps used in the chromatographic arrangement. The effect of pH of mobile phases B and C on the AEC separation was checked in the range 7–9 (with intervals of 0.5), flow rates between 1.0–2.0 mL min^{-1} and several ammonium acetate concentrations. The optimum conditions for the best separation were pH 8, 2–200 mM of ammonium acetate gradient elution and flow rates of 1.0 mL and 1.5 mL for SEC and AEC, respectively. The results obtained for Cu,Zn-SOD purification from cytosolic and mitochondrial extracts of mice hepatic cells are shown in Fig. 3.



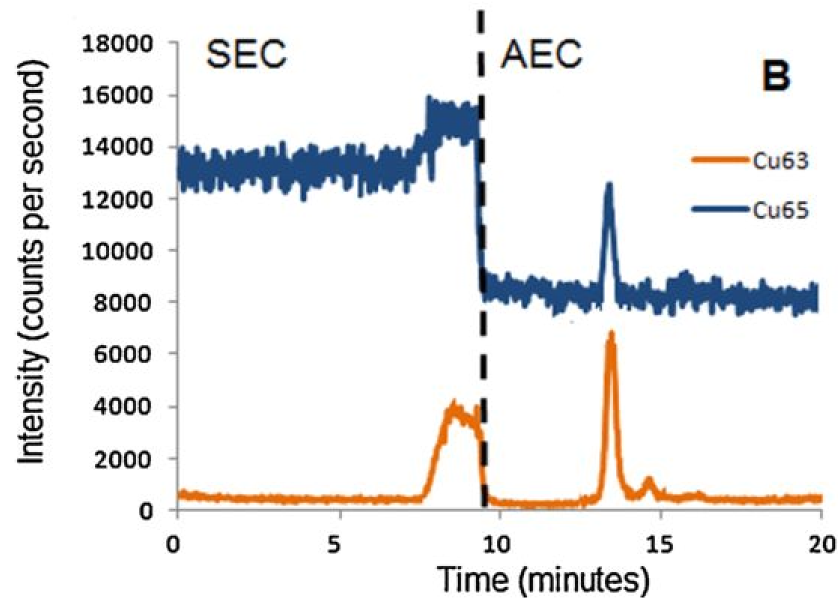


Fig. 3 Copper 2D/SEC-UV-AEC-SUID-ICP-ORS-MS chromatograms from: (A) Cytosolic extract from mice hepatic cells; (B) mitochondrial extract from mice hepatic cells.

3.2 Identification of Cu,Zn-SOD in cytosolic extracts from mice liver by nESI-QqQ-TOF-MS

Further confirmation of the identity of Cu,Zn-SOD specie in the peak at about 14 min (Fig. 4A) was performed by peak collection, purification and analysis by nESI-QqQ-TOF-MS. Fig. 4B shows the mass spectrum of peptides obtained after the tryptic

digestion. For protein sequencing, the doubly charged peptide ions of *m/z* 584.31, 684.38 and 587.78, which were selected by the higher intensities, were fragmented. The data obtained by nESI-MS and nESI-MS/MS were introduced into the MASCOT database for protein identification. The sequences obtained in each case gave a single, highly significant hit to the same protein, referred to as superoxide dismutase (SwissProt accession no. P08228). The nominal mass of the protein is 16,104 Da since it has a subunit structure (homodimer). The retention time obtained by Cu,Zn-SOD purified from cytosolic extracts was the same as that obtained in the mitochondrial extracts, because they present the same protein sequence since both are coded for by the same gene [37] (Fig. 4).

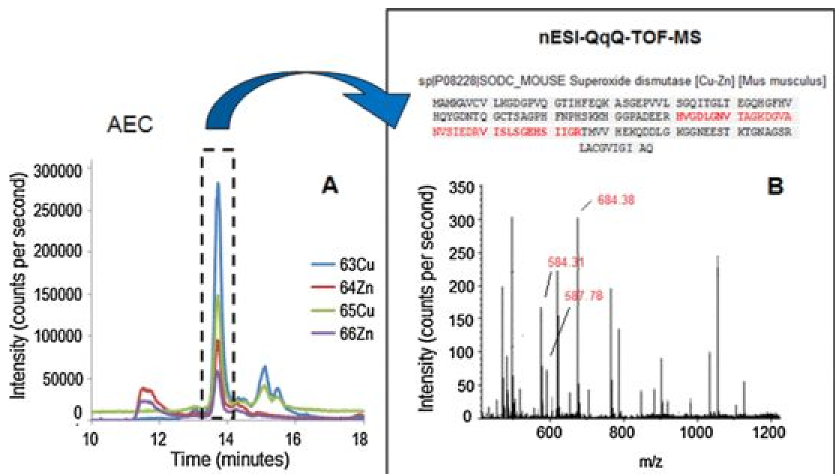


Fig. 4 (A) AEC-SUID-ICP-ORS-MS chromatogram of the Cu and Zn-containing fraction of 32 kDa collected by SEC; (B) mass spectrum obtained by nESI-MS of the Cu and Zn-containing fraction collected by AEC after tryptic digestion.

3.3 Evaluation of the analytical performance of Cu-proteins quantification in not exposed mice cytosol and mitochondria extracts

Quantification of Cu-proteins in mice cytosolic and mitochondrial extracts can be performed after conversion of Cu isotopes intensity and polyatomic interferences to mass flow chromatogram, using mathematical equation corrections. An online dilution equation was applied to each point of the chromatogram and the amount of Cu in each chromatographic peak (Fig. 5). Since the stoichiometry of Cu and Zn in SOD is well-established, the absolute quantification of Cu,Zn-SOD can be carried out by the determination of these metal ions by ICP-MS [26,29], which might serve as an indirect method to quantify the enzyme. Among the different metal quantification methods that can be used by ICP-ORS-MS detection, IDA provides the best capabilities in terms of precision, reproducibility and accuracy [27].

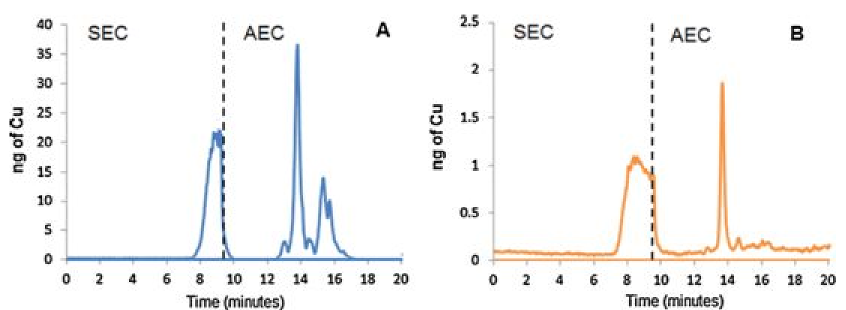


Fig. 5 (A) Typical mass flow chromatogram of copper from cytosolic extract from mice hepatic cells by 2D/SEC-UV-AEC-SUID-ICP-ORS-MS; (B) typical mass flow chromatogram of copper from mitochondrial extract from mice hepatic cells by 2D/SEC-UV-AEC-SUID-ICP-ORS-MS.

Table 2 shows the analytical performance of the method described. The accuracy in terms of relative standard deviation (RSD, %) was calculated for successive injections of 5 replicates from cytosolic and mitochondrial extracts of a pool of the eight control mice livers and detection limit (LD, ng g^{-1}) was evaluated by 5 successive injections of the analytical reagent blank (3x standard deviation of the blank criterion). The relative standard deviation is lower for Cu,Zn-SOD quantification of cytosolic hepatic cells than those of mitochondrial fraction, probably due to the lower concentration of the protein in the later. In addition, it is remarkable that mass balance of Cu-Zn-SOD standard levels by 1D-SEC is in accordance with the concentration obtained by 2D/SEC-UV-AEC-SUID-ICP-ORS-MS (Table 2).

Table 2 Quantification of Cu,Zn-SOD in cytosolic and mitochondrial extracts from mice hepatic cells.

Extract (n = 5)	Hepatic cells			
	Cu,Zn-SOD mean (ng g^{-1} as Cu)	Cu,Zn-SOD mean (µg g^{-1} as SOD)	Relative standard deviation (RSD, %)	Detection limit (LD, ng g^{-1} of Cu)
Cytosolic	1212	306.4	3	0.21
Mitochondrial	98.34	24.86	5	0.21

This optimized metallomic approach for the quantification of Cu,Zn-SOD presents numerous advantages in comparison with other methodological proposals from other authors [26,28–33]. Firstly, online methods prevent the use of off-line clean-up and preconcentration steps between the different chromatographic dimensions (such as cut-off centrifugation filters, solid phase extraction (SPE), precipitation procedures and off-line fractions insolation). Secondly, for electrophoretic and off-line metallomic approaches increased time of analysis are required. In addition, the short time of analysis associated to on-line methodologies ensures the preservation of metalloproteins integrity. Finally, ICP-ORS-MS detection permits element isotope ratios measurement and consequently, isotopic dilution analysis (IDA) for accurate and precise quantification of multi-isotopic elements, particularly Cu containing proteins using species-unspecific isotope dilution mode (SUID), which allows species quantification even when their structure and composition is not exactly known or the corresponding isotopically labeled compounds are not commercially available [23].

3.4 Quantification of Cu,Zn-SOD in cytosolic and mitochondrial extracts from mice hepatic cells under inorganic mercury exposure

In this work, the exposure of mice to mercury ($HgCl_2$) during 10 days causes a decrease in the levels of cytosolic and mitochondrial Cu,Zn-SOD in liver ($p < 0.05$) (Table 3). Similar effect has been observed by other authors in Wistar rats under acute exposure of Hg during 30 days [38]. Our results are also in concordance with other studies showing that the increased lipid peroxidation under mercury exposure [39,40] is associated with significant decreased levels of SOD antioxidant enzyme. In summary, elevation of lipid peroxidation in mice hepatic cells suggests formation of free radicals and induction of oxidative cell injury under the toxic effect of mercury. Decreased levels of SOD in cytosolic and mitochondrial fractions from mice exposed to Hg leads to disruption of pro-antioxidant balance in mammals. Additionally, when about 4 mg Hg kg^{-1} of body weight was injected in Swiss albino male mice by other authors in a single dose, the Cu,Zn-SOD activity was decreased about 45% in cytosolic extract of liver from Hg-treated mice in comparison with control mice [41]. Our results, showed a reduction in Cu,Zn-SOD concentration of 40% (Table 3), and are consistent with the results obtained by Agarwal et al. [41].

Table 3 Quantification of Cu,Zn-SOD cytosolic and mitochondrial extracts of hepatic cells from mice exposed to mercury.

Group of exposure	Cytosolic hepatic cell from *Mus musculus*		Mitochondrial hepatic cell from *Mus musculus*	
	Cu,Zn-SOD mean (ng g^{-1} as Cu)	Relative standard deviation (RSD, %)	Cu,Zn-SOD mean (ng g^{-1} as Cu)	Relative standard deviation (RSD, %)
Control mice (n = 5)	1163	5	87.92	9
Hg exposed mice (n = 5)	754.2	7	72.33	12

4 Conclusions

The proposed metallomic approach based on the use of orthogonal chromatographic systems, namely SEC and AEC, with ICP-ORS-MS detection allowed the separation and quantification of Cu,Zn-SOD present in cytosolic and mitochondrial extracts form mice hepatic cells. This optimized methodology reduces the time of analysis and avoids the use of sample preconcentration and clean-up procedures, such as cutoff centrifuged filters, solid phase extraction (SPE), precipitation procedures, off-line fractions insolates, etc. In this sense, the method is robust, reliable and fast with typical chromatographic run time less than 20 min. Precision in terms of relative standard deviation (n = 5) is of 3–5% and detection limits is of 0.21 ng Cu g^{-1}. The application of this optimized methodology to Hg-exposed mice during 10 days shows a decreased level of Cu/Zn-SOD in cytosolic and mitochondrial fraction from hepatic cells, as a biological response to oxidative stress caused by this toxic metal. Additionally, the quantification of mitochondrial Cu,Zn-SOD in hepatic cells from *Mus musculus* has been carried out for the first time. In summary, this method has been shown to be a useful metallomic workflow for the absolute quantification of a Cu-proteins and could be used to evaluate the EC-SOD status in biological fluids and red blood cells (RBCs) from human for toxicological and clinical studies in the future.

Acknowledgements

This work was supported by the project CTM2012-38720-C03-01 from the Spanish Ministry of Economy and Competitiveness, and P12-FQM-0442 from the Regional Ministry of Economy, Innovation, Science and Employment (Andalusian Government, Spain). Miguel Ángel García Sevillano thanks Ministerio de Educación, Cultura y Deporte for a predoctoral scholarship. We also thank to the group of Prof. Sanz-Medel at the University of Oviedo, especially to Dr. M.L. Fernández-Sánchez and Dr. Nuevo-Ordóñez, for introducing and training in copper isotope dilution analysis.

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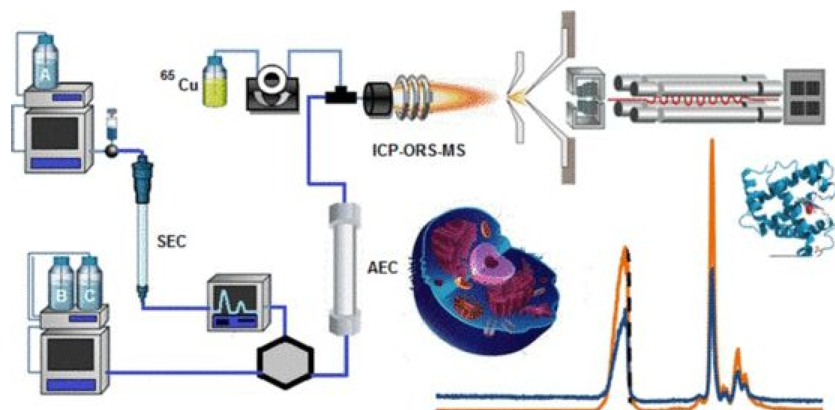

Highlights

- Identification and quantification of Cu,Zn-superoxide dismutase in mice hepatic cells.
- IDA-ICP-MSis applied to obtain a high degree of accuracy, precision and sensibility.
- This methodology reduces the time of analysis and avoids clean-up procedures.
- The application of this method to Hg-exposed mice reveals perturbations in Cu,Zn-SOD.

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