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Human Plasma Copper Proteins Speciation by Size Exclusion Chromatography Coupled to Inductively Coupled Plasma Mass Spectrometry. Solutions for Columns Calibration by Sulfur Detection

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Among the hyphenated techniques used to probe and identify metalloproteins, size exclusion chromatography coupled to inductively coupled plasma mass spectrometry (SEC-ICP-MS) has shown to have a central place. However, the calibration of SEC columns reveals to be tedious and always involves UV detection prior to ICP-MS. The presence of sulfur in 98% of proteins allows their detection by quadrupole ICP-MS, despite the isobaric interference ($^{16}O^{16}O$) on S, by monitoring $^{32}S^{16}O$ at mass to charge ratio (*m/z*) 48. The formation of SO occurs spontaneously in the argon plasma but can be optimized by the introduction of oxygen gas into a reaction cell (RC) to achieve nM levels. In this article, sulfur detection was discussed upon instrumental conditions and S detection was then optimized by applying O_2 as a reaction gas. SO formation was used to calibrate SEC columns without UV detection. This simple SEC-ICP-MS method was used for plasma copper proteins in plasma healthy subjects (HS) and an untreated Wilson disease (WD) patient. Copper proteins identified in healthy subjects were transcuprein, ceruloplasmin (Cp) and albumin. The method led to results in good agreement with other methods of determination. Copper bound to Cp in the WD patient was lowered with regard to the HS, and the exchangeable Cu was highly increased.

In the postgenomics era, proteomics has become a central branch in life sciences. Information from proteomics (and peptidomics) studies may reveal alterations in gene expression due to a disease and facilitate the understanding of the metabolic pathways such as copper metabolism in Wilson and Menkes diseases.[1] Since one-third of all proteins are metalloproteins, a new field recently defined by Haraguchi, namely "metallomics", has been introduced. This topic attracted a fast growing interest during the past decade. It integrates different analytical approaches that focus on metalloproteins and metal-containing biomolecules. Metal ions in metalloproteins have a regulatory and structural role allowing them to fulfill their different functions. The biocatalysis of some specific enzymatic reactions including gene DNA synthesis, metabolism, antioxidation, and detoxification are examples of these functions.[2,3]

Since the separation, identification, and quantification of metalloproteins are mandatory in our understanding of their complex role in biological systems, a growing number of studies are covering these fields. Usually, authors employ hyphenated techniques combining a separation method followed by mass spectrometry analysis. Matrix-assisted laser desorption/ionization (MALDI) MS, electrospray ionization (ESI) MS, two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) laser ablation (LA) ICP-MS and high performance liquid chromatography (HPLC) ICP-MS with different modes of separation have been proposed. More recently, capillary electrophoresis (CE) ICP-MS and Fourier transform ion cyclotron resonance mass spectrometry (FT-ICR-MS) have been used for this purpose.[3,4]

The overwhelming majority of recent applications concerning probing and quantification of metalloproteins were developed using ICP-MS coupled to size-exclusion chromatography (SEC).[5−9] ICP-MS is the most widely used mode of detection because of its extremely low limits of detection (LOD), a wide dynamic range,

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the multielement capabilities, the continuous spectra signals survey and a high sample throughput.[10] In addition, SEC has the advantage, in theory, to preserve metalloproteins from solid phase/analytes interactions. Maintaining the integrity of the probed proteins is actually of a critical importance when metal ions simply coordinate to organic ligands.

Nevertheless, the calibration of SEC columns presents some drawbacks rending SEC-ICP coupling laborious because of (i) a tedious installation of a UV detector in parallel to the ICP-MS, (ii) the use of an additional software for the integration of chromatographic peaks, and (iii) the increase of dead volume. This way of SEC calibration involves the injection of high concentrations of proteins. These are expensive and can irreversibly bound to columns leading to columns clogging. It is noteworthy that other detection techniques have been proposed to calibrate SEC columns such as IR, NMR, MS, evaporative light scattering, and viscometers[11] but their coupling to ICP has not yet been reported.

The capability of ICP-MS to detect in a single run the metal ions of interest and hetero nonmetallic elements present in proteins such as S and P offers great opportunities. It allows a specific detection, molecular characterization, and even quantification of some metalloproteins. For instance, when the amino acid (AA) sequence is known for a given protein, and hence the number of AA containing S, the determination of S provides an accurate value of molar protein concentration.[12,13]

Unfortunately, these heteroelements (i.e., S and P) have high ionization energies resulting in low sensitivities in ICP-MS. Moreover, both S and P are extensively interfered by isobaric polyatomic ions generated in the atmospheric pressure argon plasma ($^{16}O^{16}O$ for ^{32}S and $^{15}N^{16}O$ for ^{31}P). The use of high resolution mass spectrometry systems such as sector field ICP-MS with a resolution higher than 1 800 $m/\Delta m$[14−17] allows distinguishing of these isobaric species. Alternatively, the detection of S remains feasible with lower resolution ($m/\Delta m$ 400) quadrupole ICP-MS by using a reaction cell. This device is a quadrupole filled with a reaction gas (oxygen or xenon) and placed between the ion lenses and the quadrupole mass filter of the ICP-MS instrument. Oxygen used as reacting gas in the reaction cell converts S ions into $^{32}S^{16}O$. Sulfur is then measured as SO at m/z 48 which shows less interferences. This technique was previously used for the determination of some sulfured amino acids.[18,19] In the same way, the measurement of phosphorylation degree of some peptides[20] and the determination of metal−sulfur ratios in some metalloproteins[12] were obtained by detecting S and P as $^{32}S^{16}O^+$ and $^{33}P^{16}O^+$ species (m/z 48 and 49, respectively) together with metals of interest. More recently, Wang used SEC-ICP-MS for a quantitative analysis of proteins via S determination.[21]

Interestingly, by using argon plasma, under atmospheric condition, oxides are formed spontaneously at percentages corresponding to ≈3% of measured elements.[22,23] These percentages are influenced by instrumental parameters of ICP-MS.[24,25] Consequently, in applications involving high concentrations of proteins, such as undiluted or weakly diluted plasma, the amount of produced SO could be sufficient for its detection. Moreover, recommended concentrations of proteins in SEC column calibration kits are around 1−10 $g \cdot L^{-1}$ (i.e., S concentration higher than 50 μM) which is enough to monitor SO without any addition of O_2. In other words, the calibration of SEC columns should be possible by using SEC-ICP-MS in standard mode and without any prior UV detection.

In this study, we present a simple SEC-ICP-MS method for plasma copper proteins speciation. This method was applied to healthy subjects (HS) and an untreated Wilson disease (WD) patient. The distribution of copper in the blood is still an important subject of research. The knowledge of copper distribution contributes to better understand copper metabolism and to better diagnose and monitor related diseases. In WD, a mutation in the gene ATP7B leads to a dysfunction of ceruloplasmin (Cp) which is the major protein binding Cu. This binding is mediated by the protein ATP7B that lacks in WD. Clinically, serum Cp concentration diminishes and the so-called "free Cu" increases and becomes toxic due to Cu deposits in target organs (liver, brain, kidney, and eyes). If not treated, irreversible damages can occur.[26−28]

For the speciation purpose, we demonstrate that SEC column calibration can be conducted with a simple SEC-ICP-MS at moderate m/z resolution and without any UV detection. ICP-MS operational parameters influence on oxides formations in standard mode (without O_2) was studied. The method was then optimized by the application of O_2 as reaction gas in the dynamic reaction cell (DRC). Excellent limits of detection (LODs) for S and Cu were obtained after optimization. Copper proteins identification, stability and quantification are explored.

EXPERIMENTAL SECTION

Reagents and Chemicals. Mobile phase (NH_4NO_3 200 mM) was prepared daily by dissolving 16 g of NH_4NO_3 (Sigma Chemical Co, St-Quentin Fallavier, France) in 1 L of ultrapure water Milli-Q (Millipore, Molsheim, France) and degassed by vacuum filtration on a 0.22 μm Millipore filters.

For SEC column calibration, two SEC marker kits were used. Kit 1 (obtained from Sigma) included: Blue dextran (2000 kDa), thyroglobulin (670 kDa), apoferritin (443 kDa), β-amylase (200

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Table 1. ICP-MS Parameters

nebulizer	glass SeaSpray concentric (nominal flow:1 mL·min^{-1})
spray chamber	glass cyclonic baffled (50 mL)
nebulizer gas flow	0.92 mL min^{-1}
auxiliary gas flow	1.20 mL min^{-1}
plasma gas flow	15 L min^{-1}
ICP RF power	1125 W
O_2flow rate (DRC mode)	0.60 (0.1 − 1 mL·min^{-1})
RPQ	0.25 (0.05−0.7)
pulse stage voltage	800 V
analog stage voltage	−1612.5 V
measured m/z	$^{32}S^{16}O$, ^{34}S ^{16}O,^{63}Cu, ^{65}Cu, ^{70}Ga ^{45}Rh, ^{151}Eu
scan mode	peak hopping
dwell time per isotope	250 ms

kDa), alcohol dehydrogenase (150 kDa), bovine serum albumin (BSA) (66 kDa), carbonic anhydrase (29 kDa). Human serum albumin (HSA) (69 kDa) was needed for void volume (V_0) determination (see results). A second ready-to-use marker kit (Kit 2) (Column Performance Check Std, Aqueous SEC 1, Phenomenex Inc., Le Pecq, France) was used to control SEC calibration containing bovine thyroglobulin (670 kDa), human gamma globulin (150 kDa), ovalbumin (44 kDa), and human myoglobin (17 kDa).

D-penicillamine (D-pen) (purity >97%), chosen as a source of S, was obtained from Sigma. Cu, Ga, Eu, and Rh 1 g·L^{-1} standard solutions (Inorganic Ventures, distributed by Analab, Bischeim, France) were used to prepare working solutions by appropriate dilution in the mobile phase. Ga, Rh, and Eu were tested as internal standards (IS). All reagents were tested for copper contamination before use. Milli-Q water was used to make intermediate dilutions.

Instrumentation. Separation of proteins was performed via SEC using a BioSep-SEC-S 2000 and Biosep-SEC-S 3000 (300 × 7.8 mm both) columns from Phenomenex. A Series 200 Perkin-Elmer chromatography system, composed of an injector and a high pressure pump equipped of PEEK tubing, (Perkin-Elmer, Courtaboeuf, France) was operating at a flow rate of 1 mL·min^{-1}. The injection volume was 100 μL. The column effluent was directly coupled to a 1 mL·min^{-1} glass SeaSpray concentric nebulizer (Perkin-Elmer) and a 50 mL cyclonic Baffled spray chamber from Perkin-Elmer. Oxygen (purity N48, Air Liquide Santé, Puteaux, France) was used as the reaction gas. Detection and quantification of elements was made by an ICP-MS Elan-DRCe (Perkin-Elmer) and the Chromera software (Perkin-Elmer) was used for the HPLC signal monitoring. Signal intensity was given as counts per seconde (Cps). The ICP-MS operation parameters are given in Table 1.

The two isotopes of Cu were used for copper detection and quantification in all experiments because of the polyatomic interference $^{40}Ar^{23}Na$ that was observed on ^{63}Cu. The ion peak at m/z 63 was attributed to pure Cu when ($^{63}Cu/^{65}Cu$) isotopes ratio was 0.45 ± 5%.

DRC and Reaction Gas Flow Optimization. A 10 μg·L^{-1} solution of D-pen (source of S) was used for the optimization of the oxygen reaction gas flow in the DRC. The optimal flow was obtained by varying the O_2 flow (from 0.1 to 1.0 mL·min^{-1}) as so to attain the highest signal to background ratio (S/BKG). However, the effect of O_2 on the Cu signal (Cps) had to be estimated. Therefore, a solution of D-pen and Cu at 200 and 0.5 μM, respectively, was used to measure SO and Cu signals by varying the oxygen gas flow in the DRC.

SEC Column Calibration. Proteins were dissolved in the mobile phase and 100 μL of the proteins solutions of Kit 1 were injected on columns: apoferritin, β-amylase, alcohol dehydrogenase, BSA and carbonic anhydrase at 10, 5, 4, 5, and 10 g·L^{-1}, respectively. These concentrations are recommended by the manufacturer and are used for a UV detection to give $A_{280\ nm} \approx 1$ on a 90 × 1.6 cm column. In our study, the proteins were detected by their sulfur content (i.e., $^{32}S^{16}O^+$ referred to as ^{48}SO hereafter) by ICP-MS without any reaction gas in the DRC. A calibration curve was calculated between of the known molecular masses of proteins and their respective volume of elution divided by the dead volume (V_e/V_0). To determine the void volume, a 1 g·L^{-1} solution of HSA was injected separately, vortex mixed with blue dextran (Bdx at 3 g·L^{-1}) and injected again (see results for explanation).

After a 100 fold dilution of the previous proteins solutions in the mobile phase, the same volume was injected while the optimal flow of oxygen gas in the DRC was applied. The calibration curve was calculated again with the new data. At last, to confirm the proteins separation, two mixtures were prepared and injected: (1) the ready-to-use mixture and (2) a mixture of apoferritin, alcohol dehydrogenase, BSA and carbonic anhydrase at 1, 15, 6, 15 g·L^{-1}, respectively.

Copper and Sulfur Calibration Curves. External calibrations for Cu and S were performed by using five solutions containing increasing amounts of Cu and D-pen. Concentrations were chosen on the basis of expected amounts of metalloproteins in the plasma samples (0.1−5.0 μM for Cu and 100−5000 μM for S). Ga was added as IS (100 μg·L^{-1}) to all calibration solutions. External calibrations were, first, carried out by flow injection (FI) without any column connected to the HPLC system. The calibrations were operated in both standard (STD) and DRC modes. The peaks' areas of measured elements reported to IS areas were tested for linearity. The same experiments were conducted under HPLC condition by injecting the calibration points on the Precolumn Biosep 2000.

Species Unspecific Calibration. For Cu and S quantification in metalloproteins, the external calibration previously obtained by FI was used for the quantification of all Cu and S peaks in the chromatograms by using the peak areas. This is referred to as the "species unspecific" mode of calibration.[12,13]

Limits of Detection (LODs). LODs were calculated for each measured elements in both modes (STD and DRC) using FI first and the precolumn afterward. Sulfur and Cu LODs were calculated as 3-fold the standard deviation (SD) of the baseline variations for each elements on three blank injections.

Sample Preparation. Blood samples were collected on a Li-heparin Vacutainer tubes (ref. 365952, Becton- Dickinson, Le Pont de Claix, France) centrifuged for 10 min at 2000*g*, diluted (1:3) in a mobile phase solution containing 100 μg·L^{-1} of IS and injected on the column within 15 min. For exchangeable Cu study, determined and discussed in a previous work,[27] plasmas were diluted (1:1) in a 3 g·L^{-1} EDTA solution, kept at room temperature for one hour precisely and injected on the column after a final dilution (1:2) in the mobile phase.

RESULTS AND DISCUSSION

Sulfur Detection by SO Signal in Standard Mode. Sulfur is present in two naturally occurring amino acids, cysteine, and methionine, together exhibiting a global abundance of about 5% of all amino acids in eukaryotic proteins. Therefore sulfur is present in the vast majority of proteins (>98%) and its direct detection by ICP-MS constitutes an almost general screening method for peptides and proteins.[14,15]

The high first ionization potential (10.36 eV) of S^{15} makes the ionization efficiency in argon plasma to be less than 15% leading to low S sensitivity in ICP-MS. However, when Sulfur is present at concentrations sufficiently elevated its survey as ^{48}SO in standard mode is still possible due to a spontaneous oxide formation corresponding to ≈3% of the total element (i.e., SO formation in normal atmospheric pressure argon plasma).

Attempts to increase SO signal in the standard mode by varying RF power or nebulizer gas flow (not shown) in order to facilitate oxides formation did not lead to satisfying results. This is in disagreement with Divjak et al. who's results found that cold plasma increases CeO formation and expected that S would behave the same.[22] Indeed, cold plasma and high nebulizer gas flow were favorable for the formation of CeO in our experiments as well. However, increasing gas flow and decreasing RF was deleterious for both Cu and SO signals. The reaction between Ce and O_2 is highly exothermic and was selected by some manufacturers as an example of a bimolecular reaction whose rate is inversely proportional to temperature.[29] Unfortunately, this is not the case of the reaction between S and O_2 that seems to be endothermic under atmospheric pressure.

SEC Column Calibration. *Standard Mode.* The separation of proteins on a SEC column and their detection by ^{48}SO offer the possibility to calibrate the column on the basis of their apparent molar mass (Mr) vs their volume of elution. This strategy permits to overcome the tedious installation of a UV detector in parallel to the ICP-MS and to use only one data integration software instead of two needed in general.

The proteins of the marker kit 1 at the recommended concentrations were used to calibrate the Biosep 3000 column. Respective V_e of reference proteins are shown in Figure 1. To calculate SEC Calibration curve V_0 is needed to plot molar masses vs V_e/V_0. However, V_0 could not be determined by Bdx alone because it does not have any measurable element by ICP-MS. This obstacle could be sidestepped as follows: giving that HSA easily binds to Bdx[30,31] we tried to produce what manufacturer advice against (i.e., mixing Bdx with other proteins of the marker kit). In fact, the injection of 100 μL of Bdx at 2 g·L^{-1} did not differ from baseline for all measured elements even for ^{12}C. On the other hand, HSA eluted at 11.3 min (namely V_e = 11.3 mL as shown in Figure 2). HSA was then dissolved directly in a 3 g·L^{-1} Bdx solution (HSA = 1 g·L^{-1}), incubated for one hour and injected on the column. We noticed that HSA ^{48}SO peak disappeared while a new peak at 7.31 min (namely V_e = 7.31 mL) appeared. This peak results from the binding of HSA to Bdx and allows measuring the void volume V_0. In such a way, all data needed to calculate the calibration curve are obtained (Figure 3).

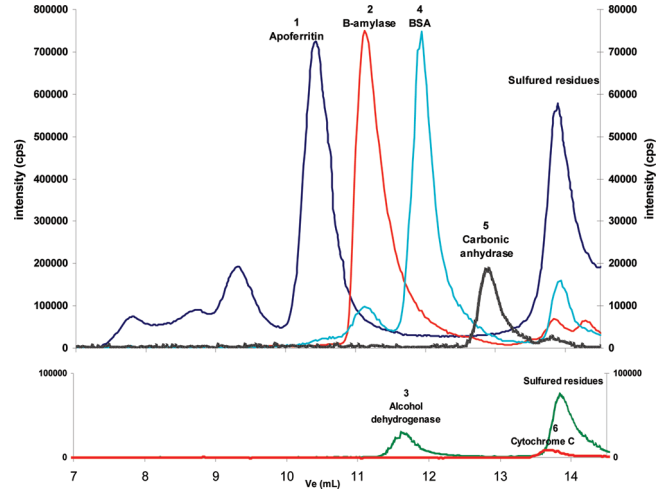

Figure 1. ^{48}SO chromatograms of proteins injected separately. Elution volume of proteins: (1) apoferritin (10 g/L) V_e = 10.45 mL, (2) β-amylase (4 g/L) V_e = 11.32, (3) alcohol dehydrogenase (5 g/L) V_e = 11.64, (4) bovine albumin (10 g/L) 11.94 mL, (5) carbonic anhydrase (30 g/L) V_e = 12.9 mL, (6) cytochrome C (2 g/L) V_e = 13.71 mL. 1, 2, and 4 are on the left scale; 3, 5, and 6 are on the right scale.

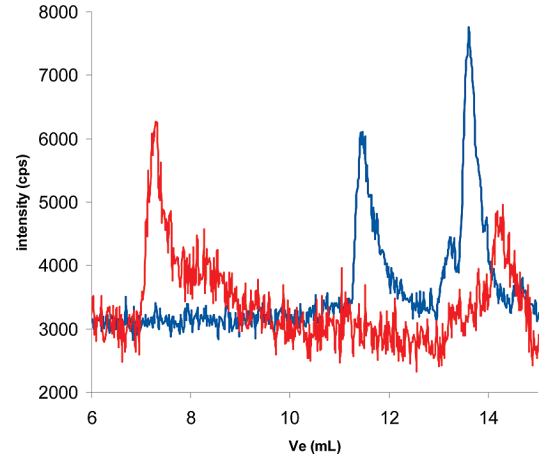

Figure 2. Void volume determination by interaction of Human albumin and Blue dextran. ^{48}SO signal in STD mode for human albumin (1 g/L) (blue line), and Human albumin bound to blue dextran (1 g/L and 3 g/L, respectively) (red line).

The separation of proteins from Kits 1 and 2 was satisfying (not shown). However, It is noteworthy that myoglobin peak is hardly distinguishable from the baseline because of a poor sulfured AA content (only two methionines and one cysteine) and its coelution with low M_r sulfured residues present in all proteins.

Signals Optimization for SO Detection in DRC Mode. The O_2 flow offering the highest S/BKG for SO was found to be 0.6 mL·min^{-1}. At this optimal flow, Cu signal for both isotopes decreases by about 20−30% compared to STD mode. Above 0.6 mL·min^{-1} O_2 all signals decreased.

Sulfured Proteins in DRC Mode. By using O_2 as a reacting gas in the DRC, S detection was markedly improved to achieve nanomolar levels (*see after LOD*). This implies the possibility (1) to inject lower concentrations of proteins on the column and so preserving it from irreversible proteins adsorption, (2) to quantify precisely proteins even those with a low sulfur content, (3) to use minor isotope ^{34}S by following $^{34}S^{16}O$ (^{50}SO)

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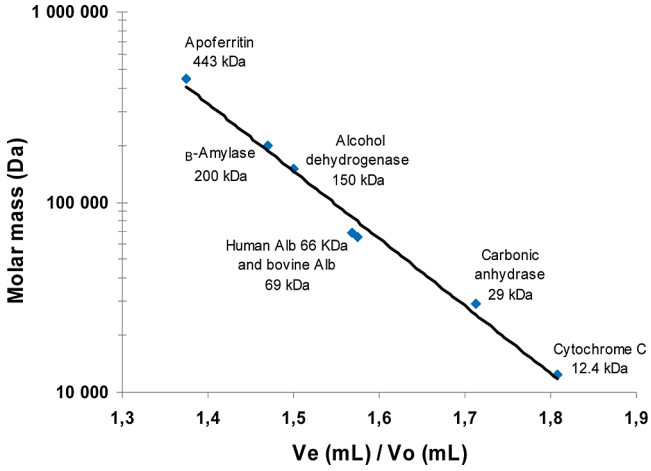

Figure 3. SEC calibration curve between the known molar mass of each protein and their respective volume of elution divided by the dead volume (V_e/V_0).

and finally, (4) to use S/Cu ratios as a specific tool to Cu proteins detection since these ratios are theoretically known (Table 2 adapted from refs 32 and 33). Actually, when S/Cu ratio for a detected peak is higher than the theoretical value, proteins coelution should be suspected.

Proteins injected separately could be identified by their calculated molar masses on the calibration curve confirming the validity of the column calibration. Figure 4 shows an example of chromatograms of cytochrom C (2 $g \cdot L^{-1}$) and B-amylase (4 $g \cdot L^{-1}$). ^{48}SO signals are 1000 fold higher after O_2 introduction and ^{50}SO becomes readily measurable offering a very wide dynamic range for S measurement.

Copper and Sulfur Calibration Curves. By flow injection as well as under HPLC conditions, linearity was found for both Cu isotopes and in both modes (STD and DRC). ^{48}SO in STD mode was linear up to 10 000 μM and only to 2000 μM in DRC mode because of signal saturation. ^{50}SO linearity test was only possible in the DRC mode because it was not detectable in standard mode. The possibility to measure both isotopes permits to choose one or other depending on S concentrations in measured samples. All coefficients of variations (CV) of three injections of each of the calibration points in the same day were less than 3% and the coefficients of correlation (r^2) were higher than 0.99 for calibrated elements. In ICP techniques, internal standard is used to compensate all kind of instrumental drifts. The use of Ga as internal standard improves r^2 to reach 0.999 and higher giving equal slopes for both Cu isotopes in STD and DRC modes. Rh was abandoned as internal standard because it binds to plasma proteins and Eu revealed to be less sensitive to the instrumental drift than Cu and S. None of the tested proteins could be used as source of S for calibration because of the sulfured residues of small molar mass detected in all protein samples. In addition, manufacturer did not mention the purity degree of the used proteins. This explains the lack of accuracy that we observed when we tried to quantify them by the obtained calibration curves.

Limits of Detection. The introduction of O_2 in the DRC and the use of a chromatographic support are sensitive parameters influencing LODs of Cu and S and had to be estimated. LODs of ^{65}Cu, ^{63}Cu, ^{48}SO, and ^{50}SO are given in Table 3. ^{48}SO LOD turned out to be about 27 000 nM in standard mode and about 264 nM in DRC mode. This confirms that the detection of ^{48}SO in standard mode is still possible when S concentrations are sufficiently high. On the other hand, when ^{48}SO saturates in DRC mode (as it is the case of weakly diluted plasmas) ^{50}SO could be used to monitor and measure sulfur despite its high LOD.

Plasma Copper Proteins Analysis. *Detection.* Three healthy subjects plasmas were injected pure or diluted (1:3) on the columns. Neither Biosep 2000 nor Biosep 3000 were able to separate ceruloplasmin copper (Cp−Cu) from albumin copper (Alb−Cu) peaks. Only three peaks were observed in these plasmas: the first one corresponded to transcuprein copper (TC−Cu) (270 kDa), the second to (Cp−Cu) (132 KDa) coeluted with (Alb−Cu) and the last one corresponded to low molar mass Cu containing molecules (LMr). Therefore, the two columns were connected in series to improve the separation. The connected columns were then calibrated as previously described (in DRC mode by monitoring ^{48}SO signal of different proteins at concentrations 10−100 fold lower than recommended). Excellent separation between the Cp−Cu and the Alb−Cu peaks was obtained (Figure 5A). The following peaks were detected: TC−Cu, a major peak of Cu−Cp, Alb−Cu, and one LMr peak. It should be noted that the obtained molar masses are relative to the SEC column calibration and further analysis are needed to clearly identified the detected proteins. Transcuprein, for instance, was recently sequenced and its molar mass determined,[34] but one should be aware that the retention volume is not sufficient to identify this protein.

Manley et al.[32] have been able to detect Cu coagulation factor Va (FVa−Cu), TC−Cu, Cp−Cu, Alb−Cu, and LMr−Cu in freshly sampled rabbit plasma by injecting a large quantity (500 μL) of undiluted plasma. The concentrations of FVa−Cu and Alb−Cu they found were surprisingly abnormal as noted by authors. In order to detect FVa−Cu in human plasma, we sampled blood on a Li heparin tube (LiH 65 UI) to which 50 UI of extra LiH were added (in order to prevent any possible coagulation to which FVa participates). One hundred microliters of pure plasma were then injected immediately after centrifugation (within 10 min). No peak corresponding to the FVa−Cu was detected despite the immediate injection. This is not surprising because of the very low theoretical concentration in human plasma (FVa−Cu = 30 nM approximately). Conversely, in the majority of published studies working on Cu proteins speciation, tris-hydroxyl-methyl-aminomethane (Tris) or acetate buffers were used. We noted that these mobile phases either precipitate ionic Cu or prevent it to be eluted form SEC columns. For instance, injection of $CuNO_3$ or $CuCl_2$ solutions (1−5 μM) on the tested columns was not reproducible at all when Tris or acetate buffers were used. In addition, the injection of EDTA 3 g/L after the injection of plasma or a simple aqueous Cu solution provided an important and variable peak of Cu confirming that SEC columns are able to bound Cu when inappropriate mobile phases are used. This is in concordance with Inagaki et al. and Meng et al. observations.[21,35] To avoid

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Table 2. Molecular Properties and Concentrations of Cu Proteins and Stoichiometric S/Cu Ratio (Adapted from Refs 31 and 32

protein	molar mass (kDa)	number of Cu bound per protein	number of sulfur containing AA	S/Cu ratio	plasma proteins concentration	maximum Cu concentration (nM)
blood coagulation factor *V*	330	1	17	17	10 mg/L	30.3
transcuprein	190	0.5	56	112	180 μg/L	0.47
ceruloplasmin	132	6	40	6.7	0.2−0.6 g/L	9090-27 272
albumin	66	0−1	42	42	36−53.6 g/L	
Cu, Zn-SOD	32	0−2			0.014-0.021 mg/L	

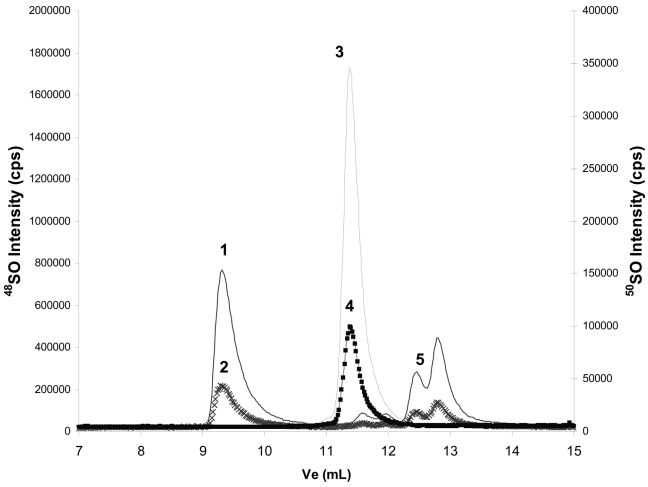

Figure 4. ^{48}SO and ^{50}SO chromatograms of separate injections of β-amylase (4 g/L) and cytochrome C (2 g/L) in DRC mode. (1) ^{48}SO β-amylase; (2) ^{50}SO β-amylase; (3) ^{48}SO cytochrome C; (4) ^{50}SO cytochrome C; (5) sulfured residues.

this, Inagaki choose to pretreat plasmas on a chelating resin before SEC analysis to be sure that loosely bound Cu is captured.

Plasma from the untreated Wilson disease patient was also tested. Despite a retention time shift that was observed, due to columns loss of efficiency, three major peaks were detected: Cp−Cu, Alb−Cu, and low molar mass Cu complexes (Figure 5B). The loss of efficiency, is a well-known phenomena that occurs when SEC column performance deteriorates after a large number of injections or after injection of highly concentrated samples.

Copper Proteins Quantification. In ICP-MS, a simple inorganic compound of an element can calibrate the element in all kind of proteins because the response is independent of the element environment. Therefore, the conventional calibration methods (internal and external standardization and standard additions) can be used for protein quantification but only if the analytes have been identified.[1,4] This is referred to as the species unspecific mode of calibration.[12,13]

Koellensperger et al demonstrated that isotope dilution remains the method of choice for species unspecific quantification in hyphenated ICP-MS techniques although requiring mathematical approximations.[36] They explained, however, that flow injection yielded acceptable uncertainties and could be used for quantification. In our study, no difference was observed between the calibration curves obtained by flow injection and those obtained by injection on the precolumn, allowing the quantification of all species by using the daily flow injection calibrations.

Ceruloplasmin coeluted with other sulfured proteins making its quantification impossible for healthy subjects and WD. Nevertheless, Alb was well separated and could be measured by its ^{50}SO peak and found at 47.3 g·L^{-1} (716 μM) to be compared with 49.7 g·L^{-1} obtained by a routine immunochemical analysis.

Calculated concentrations of Cu species in a healthy subject showed that Transcuprein, Ceruloplasmin, Alb and LMr bound 0.4%, 87.5% 6.0%, and 6.0% of total Cu, respectively, (namely 0.08, 14.7, 1.0, and 1.0 μM) giving a total Cu in good agreement with the concentrations found by atomic absorption spectrometry (AAS) (16.8 vs 15.5 μM). For the WD patient, total Cu was at 4.4 μM by HPLC-ICP-MS vs 3.6 μM by AAS and included: 14.9% Cp−Cu, 12.4% Alb−Cu, and 72.7% of LMr−Cu (namely 0.66, 0.55, and 3.2 μM, respectively).

Unexpectedly, Cu−LMr represents 6% or more of total Cu in the healthy subject plasma. In our previous study,[27] normal values of ultrafilterable Cu (CuUF) were established (obtained by the ultrafiltration of 44 healthy subjects plasmas on a 30 kDa cutoff filter) and we know that this fraction does not exceed 0.15 μM in healthy subjects (less than 1% of total Cu). This means that only 0.15 μM of Cu do not bind proteins having molar mass greater than 30 kDa. We have described in this previous work that loosely bound Cu is the copper fraction complexed to Alb and/or other proteins that can be mobilized in the presence of high copper affinity chelators, such as EDTA. To quantify this exchangeable Cu (CuEXC) fraction the most useful ETDA concentration was 3 g/L with incubation time of one hour (time found necessary to reach exchange equilibration). Quantification of CuEXC in healthy subjects offered normal values ranging from 0.6 to 1.1 μM. Interestingly, the LMr fraction of the healthy subject (1 μM Cu) lies within this reference range and is likely to correspond to CuEXC. Similarly, the Cu−LMr fraction found in the WD patient (3.2 μmol/L) is in good agreement with the CuEXC fraction determined by EDTA-ultrafiltration (2.8 μmol/L).

To confirm this, we injected the plasmas after one hour incubation with EDTA 3 g/L (1:1) in order to probe the CuEXC. No difference was observed between the chromatograms before and after incubation confirming that loosely bound Cu is released on the column after injection and further elutes in the low M_r range. This loosely bound Cu is originally Alb copper and the

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Table 3. LODs in nM for Measured Elements in Different Operating Conditions (NM: Not Measurable)

measured masses	flow injection STD mode	flow injection DRC mode	precolumn SEC STD mode	precolumn SEC DRC mode
^{63}Cu	4.8	5.8	13.0	20.0
^{65}Cu	6.8	7.6	17.0	27.0
^{48}SO	27 077	264	55 760	747
^{50}SO	NM	1816	NM	5241

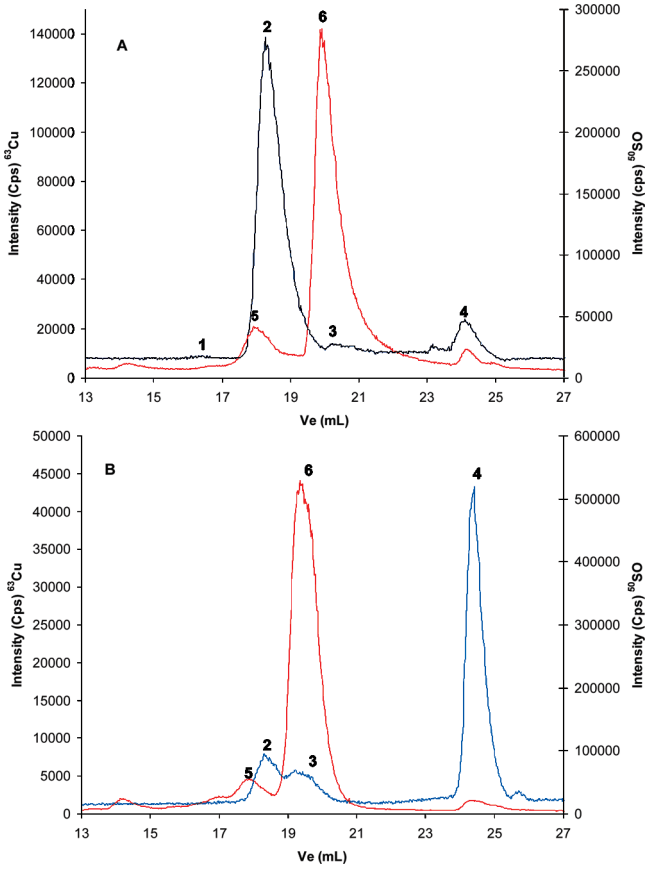

Figure 5. (A) chromatogram of diluted (1:3) healthy subject plasma and (B) Wilson disease untreated patient plasma by using Biosep 3000 and Biosep 2000 connected in series. Cu peak (blue line): (1) TC−Cu; (2) Cp−Cu; (3) Alb−Cu; (4) LMWM−Cu. ^{50}SO peak (red line): (5) unknown protein(s); (6) albumin.

type 1 copper from ceruloplasmin reported to be as labile copper.[37]

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Even if SEC columns are known to be neutral toward the analytes/solid phase interactions, our results show that weakly bound Cu by unspecific coordination with proteins such as albumin and the type 1 Cu of ceruloplasmin can be partially released. SEC columns are neutral toward proteins but not toward metals of the proteins if it is not structurally protected. The copper atoms in our case only coordinate to amino acids of the protein and can readily be captured by remaining silanol groups in this silica based column. Upon the profile obtained from the WD we better understand the deposits of Cu in target organs which is unlikely to happen in healthy subjects because their loosely bound Cu is much lower. We believe that this fraction represents the copper that proteins can deliver and that its quantification is useful in WD.

CONCLUSION

This method is designated to be applied to a larger number of WD patients in order to use it for diagnostic purpose. Moreover, a possible correlation between the loosely bound Cu and the clinical state should be explored in WD. The same approach could be undertaken for other metalloproteins where metal ions are bound by coordination such as zinc proteins. At last, the sulfur monitoring improved tremendously our proteins analysis method. It allowed the SEC column calibration and offered a wide dynamic range for S determination, and hence proteins quantification, by offering the possibility to use both S isotopes.

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