Quantitative Analysis of Proteins via Sulfur Determination by HPLC Coupled to Isotope Dilution ICPMS with a Hexapole Collision Cell

ARTICLE *in* ANALYTICAL CHEMISTRY · JANUARY 2008

Impact Factor: 5.64 · DOI: 10.1021/ac071483t · Source: PubMed

Anal. Chem. **2007,** *79,* 9128−9134

Quantitative Analysis of Proteins via Sulfur Determination by HPLC Coupled to Isotope Dilution ICPMS with a Hexapole Collision Cell

Meng Wang,[†,‡] Weiyue Feng,[*,†] Wenwei Lu,[§] Bai Li,[†] Bing Wang,[†,‡] Motao Zhu,[†,‡] Yun Wang,[†,‡] Hui Yuan,[†] Yuliang Zhao,[†,‡] and Zhifang Chai[†]

Key Laboratory of Nuclear Analytical Techniques and Laboratory for Bio-Environmental Effects of Nanomaterials and Nanosafety, Institute of High Energy Physics, Chinese Academy of Sciences, Beijing, China, Graduate School of the Chinese Academy of Sciences, Beijing, China, and Shanghai Jiao Tong University, Shanghai, China

Quantitative analysis of proteins is an essential part and also constitutes a major challenge in modern proteomics. Quantification of proteins by inductively coupled plasma mass spectrometry (ICPMS) offers an alternative method for quantitative proteomics. In this study, we developed a method of absolute quantification of proteins via sulfur by size exclusion chromatography (SEC) coupled to ICPMS with a collision cell (ICP-CC-MS) and postcolumn isotope dilution. Bovine serum albumin (BSA), superoxide dismutase (SOD), and metallothionein-II (MT-II) served as model proteins. Enriched ^{34}S, ^{65}Cu, and ^{67}Zn isotopic solutions were continuously mixed with the eluate from the SEC. Oxygen was added as a reactive gas into the collision cell where sulfur reacts with oxygen to form sulfur−oxygen ion, the ratio of $^{32}S^{16}O^+/^{34}S^{16}O^+$ thus representing $^{32}S^+/^{34}S^+$. The absolute quantity of proteins could be calculated by the isotopic dilution equation and the content of sulfur in the proteins. The detection limits for BSA, SOD, and MT-II are 8, 31, and 15 pmol, respectively. The relative standard deviations for the proteins are less than 3%. The ratios of S/Cu and S/Zn in the proteins were also determined. The quantitative method was validated by comparing with gravimetric results.

Proteomics, the systematic analysis of proteins in a cell or an organism, has become an active field of the life sciences in the postgenomics era.[1] Most of the initial efforts in proteomics were focused on methods to effectively identify large numbers of proteins. However, unlike the relatively static genome, the proteome is extremely dynamic and constantly changing. An understanding of biological functions of proteins will not only rely on protein identification but also on its quantification, including both the quantity of proteins at various physiological time points and during the physiological time course. Thus, quantitative protein profiling is an essential part and also constitutes a major challenge in modern proteomics.[2]

Currently, molecular mass spectrometry (electrospray ionization mass spectrometry, ESI-MS, or matrix-assisted laser desorption ionization mass spectrometry, MALDI-MS) has undoubtedly acted as the key analytical tool for proteomics. However, one of the possible disadvantages in quantification by molecular mass spectrometry is that in both ESI-MS and MALDI-MS, the relationship between the quantity of proteins or peptides and the obtained signal intensity is complex and ambiguous due to the diverse ionization efficiencies of different proteins or peptides. Therefore, molecular mass spectrometers are inherently poor quantitative devices.[2] In the view of proteomics, it is impossible to obtain all protein standards. Without external standardization, relative quantification can be achieved by methods such as stable isotope tagging of proteins and peptides.[3−5] The isotope can be introduced in vivo (such as yeast cells grown in ^{15}N-enriched media[3]) or in vitro (such as iTRAQ[4] and isotope-coded affinity tag, ICAT[5]). After being labeled with different isotopes, e.g., in ICAT, the same biological system in two different physiological states labeled with a heavy isotope or a light isotope, respectively, the proteins or peptides can be quantitatively analyzed by molecular mass spectrometry. Many successful applications have been reported in the literature.[6]

Remarkably, inductively coupled plasma mass spectrometry (ICPMS), the widely used elemental mass spectrometry, has been applied as an attractive complement to molecular mass spectrometry in recent literature.[7−12] Scientists anticipate that the emerging

* Corresponding author. E-mail: fengwy@mail.ihep.ac.cn.
† Institute of High Energy Physics, Chinese Academy of Sciences.
‡ Graduate School of the Chinese Academy of Sciences.
§ Shanghai Jiao Tong University.

(1) Tyers, M.; Mann, M. *Nature* **2003**, *422*, 193−197.
(2) Aebersold, R.; Mann, M. *Nature* **2003**, *422*, 198−207.
(3) Zhong, H. Y.; Marcus, S. L.; Li, L. *J. Proteome Res.* **2004**, *3*, 1155−1163.
(4) Chong, P. K.; Gan, C. S.; Pham, T. K.; Wright, P. C. *J. Proteome Res.* **2006**, *5*, 1232−1240.
(5) Gygi, S. P.; Rist, B.; Gerber, S. A.; Turecek, F.; Gelb, M. H.; Aebersold, R. *Nat. Biotechnol.* **1999**, *17*, 994−999.
(6) Righetti, P. G.; Campostrini, N.; Pascali, J.; Hamdan, M.; Astner, H. *Eur. J. Mass Spectrom.* **2004**, *10*, 335−348.
(7) Wind, M.; Lehmann, W. D. *J. Anal. At. Spectrom.* **2004**, *19*, 20−25.
(8) Hann, S.; Obinger, C.; Stingeder, G.; Paumann, M.; Furtmuller, P. G.; Koellensperger, G. *J. Anal. At. Spectrom.* **2006**, *21*, 1224−1231.
(9) Busto, M. E. D.; Montes-Bayon, M.; Blanco-Gonzalez, E.; Meija, J.; Sanz-Medel, A. *Anal. Chem.* **2005**, *77*, 5615−5621.
(10) Moberg, M.; Nilsson, E. M.; Holmstrom, S. J. M.; Lundstrom, U. S.; Pettersson, J.; Markides, K. E. *Anal. Chem.* **2004**, *76*, 2618−2622.

10.1021/ac071483t CCC: $37.00
Published on Web 10/24/2007

combination of elemental and molecular mass spectrometry as a synergistic way will open new possibilities for identification and quantification of unknown proteins.[7] Over the past few years several studies have reported applications of the two-combination mass spectrometry in metalloproteins,[8−10] selenium-containing proteins,[11] phosphoproteins,[12] etc.

ICPMS has many outstanding properties in elemental analysis, including fast determination, very low detection limits for most elements, weak matrix effects, simple mass spectra, large dynamic range, obtainable information of isotope ratios, and simple coupling to a high-performance liquid chromatograph (HPLC). In contrast to soft ion sources, such as ESI and MALDI, the ICP operates at a high temperature of about 7000 K. Thus, in the plasma, all chemical bonds in proteins are readily broken, and then the atoms are mainly ionized into ions with a positive charge. In comparison with compound-dependent techniques, the specific response of an element in ICPMS is independent of the molecular environment of the element, meaning that the quantitative analysis of an element in a protein can use any species of the element as a calibrated standard. When proteins have been identified or the elemental stoichiometric ratios of proteins are known, it is possible that after being isolated by a separation method (e.g., HPLC), proteins can be quantified absolutely via an element by ICPMS. Furthermore, ICPMS-detectable tags can be introduced into proteins like the method of MeCAT.[13] Alternatively, quantification can be achieved by the ICPMS-detectable elements which naturally exist in proteins, such as sulfur, phosphorus, selenium, and metals. Among these elements, sulfur is preferable as an internal standard for the goal of quantitative analysis of proteins because of its stability in a covalent form and high abundance in proteins.[14] Quantification of sulfur in proteins by HPLC coupled to ICPMS offers an alternative method for quantitative proteomics.[14,15]

However, there are still two obstacles in the determination of sulfur by HPLC−ICPMS. First, severe polyatomic interference exists in the sulfur analysis by ICPMS, so a high-resolution ICPMS is often required. The coupling of capillary liquid chromatography to a sector field ICPMS (ICP-SF-MS) at a medium resolution of 4000 has been introduced for quantifying peptides and proteins.[14] However, the expensive and complicated method of ICP-SF-MS limits the application in a wide range. Recently, with the development of the dynamic reaction cell (DRC) or collision cell (CC), the quadrupole ICP-CC-MS[15,16] or ICP-DRC-MS[17−19] has been successfully used to determine sulfur. Xenon was first introduced as a collision gas into the collision cell to eliminate the interference to S^+.[15,16] Another method is based on the oxidation of $^{32}S^+$ to $^{32}S^{16}O^+$ with oxygen gas added in the DRC due to less interference existing on $m/z = 48$.[17−19] Hann et al.[17] determined the ratios of Fe/S and Mn/S in metalloproteins by both size exclusion chromatography (SEC) coupled to ICP-DRC-MS and SEC coupled to ICP-SF-MS, suggesting that the ratios obtained by the two methods were in accord with each other. Sturup et al.[19] simultaneously analyzed selenium and sulfur in the selenotrisulfides selenocysteineglutathione (Cys−Se−SG) and selenodiglutathione (GS−Se−SG) by reversed-phase high-performance liquid chromatography (RP-HPLC) coupled to ICP-DRC-MS. However, to our knowledge, there is no successful application of quantitative analysis of proteins via sulfur by ICP-CC-MS in which oxygen is used as a reactive gas in the literature.

Another obstacle for accurate quantitative analysis by HPLC−ICPMS is the instabilities of the ICPMS during the HPLC process, such as signal shifts and matrix effects. In contrast to other calibrated methods, isotope dilution analysis, as one of the most reliable analytical methods, is based on the measurement of the isotope ratio. Since the isotopic signals of an element are affected in the same way, the isotope ratios are free from the instabilities of the ICPMS, and thus the instabilities of the instrument can be overcome by isotope dilution analysis. The application of isotope dilution analysis to HPLC−ICPMS can be divided into two different modes: the species-specific mode and species-unspecific mode (also called postcolumn isotope dilution).[20] One of the most important advantages of the latter mode is that it is possible to quantitatively analyze species whose structures or compositions are unknown.[20] Since the postcolumn isotope dilution was first applied for the quantitative analysis by Rottmann and Heumann,[21] the method has mainly been used in metal species[20] or metalloproteins study.[22] It is still seldom that the isotope dilution method is applied to quantitatively analyze sulfur in proteins by HPLC−ICPMS, except for the pioneering work where Schaumloffel et al.[15] determined the sulfur-containing peptides by precolumn isotope dilution analysis.

In this study, we developed a method of absolute quantitative analysis of proteins via sulfur element by means of SEC coupled to ICP-CC-MS and postcolumn isotope dilution analysis. Bovine serum albumin (BSA), superoxide dismutase (SOD), and metallothionein-II (MT-II) served as model proteins. The enriched ^{34}S, ^{65}Cu, and ^{67}Zn isotopic solution were used as a spike to continuously mix with the eluate from the HPLC via a three-way connection. Oxygen was added as a reactive gas into the hexapole collision cell where S^+ reacted with oxygen to form SO^+; thus, the ratio of $^{32}S^{16}O/^{34}S^{16}O$ representing $^{32}S/^{34}S$ was measured. Then the absolute quantity of proteins in each chromatographic fraction could be calculated by the isotopic dilution equation and the known numbers of sulfur atoms in the model proteins. The copper and zinc in proteins were also determined by postcolumn isotope

(11) Dernovics, M.; Giusti, P.; Lobinski, R. *J. Anal. At. Spectrom.* **2007**, *22*, 41−50.

(12) Navaza, A. P.; Encinar, J. R.; Sanz-Medel, A. *Angew. Chem., Int. Ed.* **2007**, *46*, 569−571.

(13) Ahrends, R.; Pieper, S.; Kühn, A.; Weisshoff, H.; Hamester, M.; Lindemann, T.; Scheler, C.; Lehmann, K.; Taubner, K.; Linscheid, M. W. *Mol. Cell. Proteomics* [Online early access]. DOI: 10.1074/mcp.M700152-MCP200. http://www.mcponline.org/cgi/content/abstract/M700152-MCP200v1. Published Online: Jul 11, 2007.

(14) Wind, M.; Wegener, A.; Eisenmenger, A.; Kellner, R.; Lehmann, W. D. *Angew. Chem., Int. Ed.* **2003**, *42*, 3425−3427.

(15) Schaumloffel, D.; Giusti, P.; Preud'Homme, H.; Szpunar, J.; Lobinski, R. *Anal. Chem.* **2007**, *79*, 2859−2868.

(16) Mason, P. R. D.; Kaspers, K.; van Bergen, M. J. *J. Anal. At. Spectrom.* **1999**, *14*, 1067−1074.

(17) Hann, S.; Koellensperger, G.; Binger, C.; Furtmuller, P. G.; Stingeder, G. *J. Anal. At. Spectrom.* **2004**, *19*, 74−79.

(18) Bandura, D. R.; Baranov, V. I.; Tanner, S. D. *Anal. Chem.* **2002**, *74*, 1497−1502.

(19) Sturup, S.; Bendahl, L.; Gammelgaard, B. *J. Anal. At. Spectrom.* **2006**, *21*, 201−203.

(20) Rodriguez-Gonzalez, P.; Marchante-Gayon, J. M.; Alonso, J. I. G.; Sanz-Medel, A. *Spectrochim. Acta, Part B* **2005**, *60*, 151−207.

(21) Rottmann, L.; Heumann, K. G. *Fresenius' J. Anal. Chem.* **1994**, *350*, 221−227.

(22) Shi, J. W.; Feng, W. Y.; Wang, M.; Zhang, F.; Li, B.; Wang, B.; Zhu, M. T.; Chai, Z. F. *Anal. Chim. Acta* **2007**, *583*, 84−91.

Table 1. Instrumental and Operational Parameters of the ICPMS

spray chamber	quartz impact bead
nebulizer	glass concentric
interface	Xi cones
chamber temperature	2 °C
forward power (W)	1350
plasma gas (L min^{-1})	13.5
auxiliary gas (L min^{-1})	0.70
nebulizer gas (L min^{-1})	0.82
collision cell gas (mL min^{-1}, oxygen)	0.10
quadrupole bias (V)	1.0
hexapole bias (V)	7.0
focus	20.0
acquired mode	continuous[a] or time-resolved analysis[b]
isotopes monitored	$^{32}S^{16}O$, $^{34}S^{16}O$, ^{63}Cu, ^{65}Cu, ^{66}Zn, ^{67}Zn
dwell time per points (ms)	10,[a] 100[b]
replicates	6,[a] 1[b]
total analytical time (s)	196,[a] 1800[b]

[a] Determined by ICPMS. [b] Determined by SEC−ICPMS.

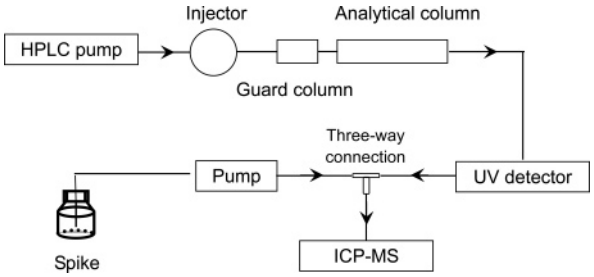

Figure 1. Schematic diagram of the system of HPLC coupled to isotope dilution ICPMS.

dilution. The ratios of S/Cu and S/Zn in proteins were calculated. The method was validated by comparing with gravimetric results.

EXPERIMENTAL SECTION

Instrumentation. The HPLC system consisted of a Waters metal-free 626 gradient pump, a Rheodyne 9725 injector with a 20 μL loop, and a Waters 2487 detector. A TSK-GEL G3000SWxl column (7.8 mm × 300 mm) and a TSK SWxl guard column (6 mm × 40 mm) with a mass range of 10−500 kDa were used for protein separation. The wavelength of 254 nm was chosen to monitor the protein fractions. The flow rate of the SEC mobile phase was 0.5 mL min^{-1}, and the chromatographic time was 30 min.

A Thermo X7 ICPMS (Thermo Electron Corp., U.S.A.) with a hexapole collision cell and Xi interface was used throughout the experiment. High-purity oxygen (>99.995%) was used as a reaction gas in order to eliminate the interferences of polyatomic ions on the determination of sulfur. The instrumental and operational parameters of the ICPMS are given in Table 1.

The spike was continuously added by a peristaltic pump into the eluate from the HPLC column via a "T"-formed three-way connection, and then the isotope-diluted fractions were introduced into the nebulizer of the ICPMS. PEEK tubing (Φ = 0.13 mm) was employed for the connection in the experiment. The schematic diagram of the analytical system is shown in Figure 1.

A Sartorius 4503 electronic microbalance with the readability of 1 μg (Sartorius Company, Germany) was used to accurately measure the mass of proteins.

Reagents, Proteins, and Standards. Tris(hydroxymethyl)-aminomethane was purchased from Roche, and the analytical grade acetic acid was from Beijing Chemical Reagents Company. Ultrapure water (18.2 MΩ cm) from a Milli-Q water purification system was used. The HPLC mobile phase consisted of 0.1 mol L^{-1} Tris−acetate buffer (pH = 7.3).

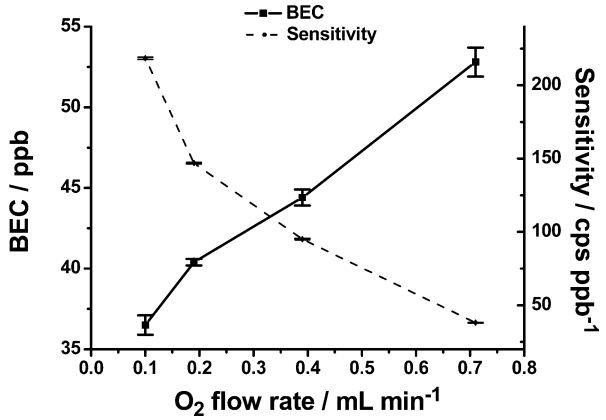

Figure 2. Blank equivalent concentration (BEC) and the sensitivity of $^{32}S^{16}O$ as a function of oxygen flow rate for 1 mg L^{-1} sulfur standard (GBW 04415).

Bovine serum albumin (BSA) and bovine erythrocytes CuZn−superoxide dismutase (SOD) were purchased from Sigma-Aldrich (U.S.A.) and Fluka (Switzerland), respectively. Rabbit liver Zn−metallothionein-II (Zn−MT-II) was bought from Lugu Biotechnology Company (Hunan, China). The proteins were accurately weighed by a Sartorius 4503 electronic microbalance and then dissolved and mixed in the HPLC mobile phase. The protein information is listed in Table 2.

The enriched ^{65}CuO and ^{67}ZnO were supplied from the China Institute of Atomic Energy (Beijing, China), and the elemental ^{34}S (99.90%) was purchased from Isoflex (San Francisco, CA). The stock solution of each enriched isotope was prepared by closed-vessel digestion with concentrated nitric acid at 140 °C for about 4 h. The isotopic compositions in enriched Cu and Zn were determined by the multiple collector ICPMS (Isoprobe, GV Instruments) in the National Research Center for Certified Reference Materials (Beijing, China). The ratios of $^{63}Cu/^{65}Cu$ and $^{66}Zn/^{67}Zn$ in enriched stable isotopes were 0.005225 ± 0.000004 and 0.06398 ± 0.00004, respectively. The accurate concentration of each enriched isotope was calculated by the reverse isotope dilution.

Sulfur isotopic reference material (GBW 04415, Ag_2S) was bought from the Chinese Academy of Geological Sciences (Beijing, China) and digested in concentrated nitric acid. The $\delta^{34}S_{V-CDT}$ value and the absolute $^{32}S/^{34}S$ ratio in GBW 04415 are 22.15 ± 0.14 and 22.1460 ± 0.0013, respectively.[23,24]

RESULTS AND DISCUSSION

Optimization of the ICP-CC-MS. Before being connected to the HPLC system, the ICPMS was first optimized using a tuning solution of 1 μg L^{-1} Be, Co, In, and U in the HPLC mobile phase (0.1 mol L^{-1} Tris−HAc, pH = 7.3). Some parameters (including sampling depth, the flow rate of the nebulizer gas, etc.) were tuned

(23) Ding, T. P.; Bai, R. M.; Li, Y. H.; Wan, D. F. *Sci. China, Ser. D: Earth Sci.* **1999**, *42*, 45−51.
(24) Ding, T. P.; Vaikiers, S.; Wan, D. F.; Bai, R. M.; Zou, X. Q.; Li, Y. H.; Zhang, Q. L.; Bievre, P. D. *Bull. Mineral., Petrol. Geochem.* **2001**, *20*, 425−427.

Table 2. Protein Information

proteins	molecular weight/kDa	sulfur atoms per molecule	metal atoms per molecule	purity	gravimetric concn (mg/mL)
albumin (BSA, from bovine serum)	66.4	40		≥96%	1.842 ± 0.002
superoxide dismutase (CuZn−SOD, from bovine erythrocytes)	31.4	10	2 Cu 2 Zn	unknown	1.203 ± 0.002
metallothionein-II (Zn−MT-II, from rabbit liver)	6.2	21	7 Zn	≥90%	0.581 ± 0.001

daily to obtain higher signal intensity in low-mass isotopes (e.g., Be and Co) for the sake of sulfur analysis. Then, the oxygen was added as a reactive gas into the collision cell, and the ICPMS was optimized for a compromise of the sensitivity and stability on $m/z = 48$ ($^{32}S^{16}O^+$) using a 1 mg L^{-1} sulfur isotopic standard solution (GBW 04415). Since the rf-only hexapole cannot distinguish the interference from the analyte by mass, kinetic energy discrimination is necessary on most occasions. The discrimination is often achieved by setting the collision cell potential slightly more negative than the potential of a mass filter, which signifies that the collision-product ions in the hexapole are rejected, while the ions of the analyte produced in the plasma are transmitted to the detector. In this study, however, the analyzed SO^+ ions were generated in the hexapole. Therefore, kinetic energy discrimination could not be used. The potential of the collision cell (hexapole bias) should set more positive than the potential of the mass filter (quadrupole bias) so that the collision-product ions (SO^+) could transmit more efficiently. Our following results show that the hexapole has little collision-induced interference in sulfur analysis by adding oxygen.

Figure 2 shows the blank equivalent concentration (BEC) and sensitivity of $^{32}S^{16}O^+$ versus oxygen gas flow for 1 mg L^{-1} sulfur standard (GBW 04415) in the HPLC mobile phase. With the increase of the oxygen gas flow, the intensity of $^{32}S^{16}O^+$ decreases while the BEC increases. Therefore, the oxygen gas flow is chosen as 0.10 mL min^{-1}. The optimal conditions of the ICPMS are shown in Table 1.

Under the above conditions, the oxidation reactions of Cu^+ and Zn^+ in the hexapole are thermodynamically prohibited (calculation of enthalpy changes by the available thermochemical data[25]) and the oxides of Cu^+ and Zn^+ can be negligible. Therefore, the isotope ratios of $^{63}Cu/^{65}Cu$ and $^{66}Zn/^{67}Zn$ in proteins can be directly determined. Because the sensitivity of either Cu or Zn in ICPMS is far higher than that of S, the instrumental conditions can be optimized only by the sulfur signal, regardless of the copper or zinc.

Before the introduction of the CC or DRC to ICPMS, Menegario et al.[26] had adjusted the quadrupole ICPMS at a condition of a high yield of oxides and determined the $^{32}S/^{34}S$ isotope ratio in plant samples using measurements at m/z 48 ($^{32}S^{16}O^+$) and 50 ($^{34}S^{16}O^+$). Comparatively, the modern ICP-CC-MS (or ICP-DRC-MS) can perform more efficient ion−molecule reactions (e.g., oxidation of S^+ to SO^+) and thus achieve a lower detection limit.

Accuracy and Precision for Sulfur Isotope Ratio by ICP-CC-MS. In isotope dilution analysis, an isotope ratio is the only parameter determined. Several factors can affect the analytical accuracy of sulfur isotope ratio by ICP-CC-MS, including spectral interference, the dead time of the detector, and mass bias.

Spectral Interference. Spectral interference is a potential source of errors for isotope dilution analysis. In this case of $^{32}S^{16}O^+$ and $^{34}S^{16}O^+$ determination, the potential interference ions mainly include $^{16}O_3^+$, $^{48}Ti^+$, $^{48}Ca^+$ and $^{50}Ti^+$, ^{50}V, ^{50}Cr, and $^{32}S^{18}O^+$, respectively. It is reported that under thermal conditions the endothermic reaction from O_2^+ to O_3^+ cannot occur and Ti^+ can be transformed to its oxide by the reaction with oxygen.[18] Although the interfered metal ions can be separated from proteins by HPLC, the contribution of these ions to the measurement of the sulfur isotope ratio must be carefully considered in the analysis of metal-containing proteins. In the experiment, the counts of Ti, V, Ca, and Cr in the proteins are at the same magnitude as in ultrapure water; thus, the interference of these metals can be neglected. The effect of $^{32}S^{18}O^+$ can be eliminated by mathematical calculation based on the natural ratio of $^{16}O/^{18}O$ and the intensity of $^{32}S^{16}O^+$ in the samples.

Dead Time of the Detector. The channel electron multiplier used in a commercial ICPMS often suffers from a dead time effect. At a high counting rate, the given counts are lower than those actually arriving at the detector in the pulse-counting mode. Therefore, isotope ratios of sulfur isotopic standards at different concentrations are determined to calculate the dead time of the detector.[20] In this case, the dead time of the detector was calculated as 35 ns. Then the dead time was introduced in the software of the ICPMS for automatic correction.

Mass Bias. The effect is due to the fact that light ions are liable to have lower transmission than heavy ions in ICPMS. The mass bias changes with instrumental settings, including ion focusing parameters, extraction potential, dc pole offset, and parameters of the collision cell. Unlike the mass bias in thermal ionization mass spectrometry (TIMS), the change of the mass bias with time in ICPMS is considered much slower under stable instrumental parameters. Thus, it can be corrected by an isotopic reference material using the following equation:

$$(A/B)_{corr} = (A/B)_{obs}(1 + f\Delta m) \quad (1)$$

where $(A/B)_{corr}$ is the corrected ratio of isotope A ($^{32}S^{16}O^+$) to isotope B ($^{34}S^{16}O^+$), $(A/B)_{obs}$ is the measured ratio of isotope A ($^{32}S^{16}O^+$) to isotope B ($^{34}S^{16}O^+$), and f is the bias per mass unit (mass bias factor).

Δm is the mass difference between the two isotopes. In this case, $\Delta m = 2$.

In the experiment, a series of sulfur isotopic standards (GBW 04415) at different concentrations have been used to calculate the

(25) Lias, S. G.; Bartmess, J. E.; Liebman, J. F.; Holms, J. L.; Levin, R. D.; Mallard, W. G. *J. Phys. Chem. Ref. Data* **1988**, *17*, 1−861.

(26) Menegario, A. A.; Gine, M. F.; Bendassolli, J. A.; Bellato, A. C. S.; Trivelin, P. C. O. *J. Anal. At. Spectrom.* **1998**, *13*, 1065−1067.

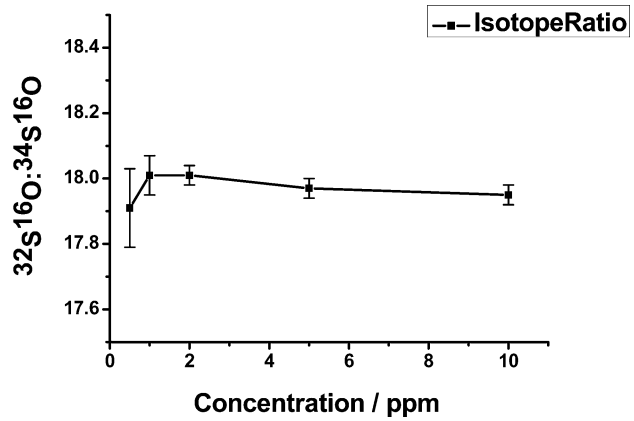

Figure 3. Sulfur isotope ratios at different concentrations (GBW 04415).

mass bias factor. As shown in Figure 3, the average isotope ratio of $^{32}S^{16}O^+/^{34}S^{16}O^+$ is 17.97 ± 0.04 and the mass bias is 11.5% per mass unit, which is higher than the typical bias (1−5% per mass unit) for mass numbers between 10 and 120 in a quadrupole ICPMS without a collision cell.[20] Previous studies have shown that use of a hexapole collision cell results in collisional focusing of heavy ions while light ions can be scattered, and thus the mass bias is altered by the addition of collision gas.[27,28] The isotope ratios of $^{63}Cu/^{65}Cu$ and $^{66}Zn/^{67}Zn$ in natural compositions have also measured under the same condition. Similarly, compared to the results by ICPMS without introduction of O_2 as a collision gas, the mass biases of Cu and Zn isotope ratios increase when using the collision cell (see Table 3).

The precision of the isotope ratio is mainly dependent on the statistics of the ion-counting system. Generally, the precision can be improved by prolonging the integral time. For transient signals in HPLC coupled to ICPMS, the integral time should be set to obtain accurate peak profiles or to avoid the "spectral skew" effect, though the spectral skew effect in HPLC−ICPMS is less serious than in GC−ICPMS.[20]

Quantitative Analysis of Proteins via Sulfur by Postcolumn Isotope Dilution. Three proteins (BSA, SOD, and MT-II) are separated by SEC and determined on-line by ICP-CC-MS. As shown in Figure 4, BSA, SOD, and MT-II are completely separated and elute at 17.9, 20.7, and 22.3 min, respectively. The intensity of a protein which was monitored by UV at 254 nm depends on its molecular absorption. Therefore, the quantification of a protein is difficult by the UV detector without calibration by the standard proteins. The enriched ^{34}S, ^{65}Cu, and ^{67}Zn isotopes are continuously mixed with the eluate from the HPLC via a three-way connection by a high-precision peristaltic pump. Three isotope ratios, including $^{32}S^{16}O/^{34}S^{16}O$, $^{63}Cu/^{65}Cu$, and $^{66}Zn/^{67}Zn$, are measured by ICP-CC-MS using O_2 as a reactive gas. The contents of sulfur, copper, and zinc in each protein are calculated by postcolumn isotope dilution.

The method of postcolumn isotope dilution analysis for element speciation study is well summarized in the literature.[20,29] In brief,

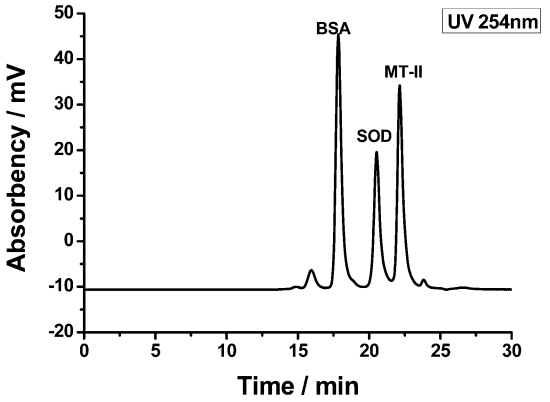

Figure 4. SEC chromatogram of the mixed proteins. BSA, SOD, and MT-II are eluted at 17.9, 20.7, and 22.3 min, respectively.

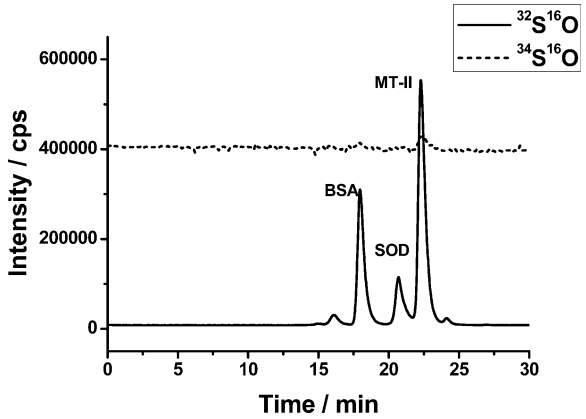

Figure 5. SEC−ICPMS chromatogram of the original intensity.

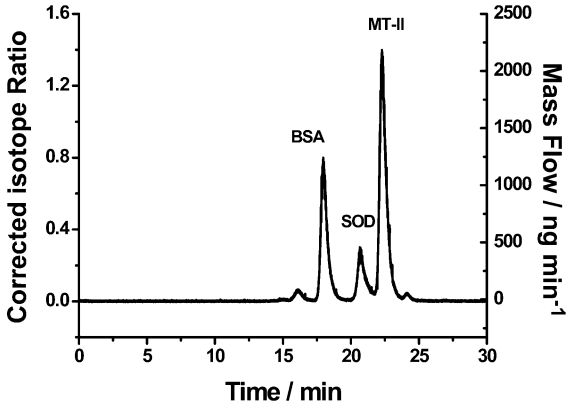

Figure 6. SEC−ICPMS chromatogram of corrected isotope ratio and mass flow of sulfur.

isotope ratios are monitored during the whole chromatographic procedure and corrected by the isobaric interference, dead time, and mass bias. Then, the chromatogram of isotope ratios is transformed into the chromatogram of mass flow (mass vs time) using the following isotope dilution equation:

$$\mathrm{MF_s} = c_{sp} d_{sp} f_{sp} \frac{M_s}{M_{sp}} \frac{A_{sp}^b}{A_s^b} \frac{(R_m - R_{sp})}{(R_s - R_m)} \qquad (2)$$

where MF_s is the mass flow of the sample eluting from the column (ng min^{-1}), c_{sp} is the concentration of the element in the spike (ng g^{-1}), d_{sp} is the density of the spike (g mL^{-1}), f_{sp} is the flow

(27) Boulyga, S. F.; Becker, J. S. *J. Anal. At. Spectrom.* **2002**, *17*, 965−966.
(28) Vanhaecke, F.; Balcaen, L.; Deconinck, I.; De Schrijver, I.; Almeida, C. M.; Moens, L. *J. Anal. At. Spectrom.* **2003**, *18*, 1060−1065.
(29) Heumann, K. G.; Gallus, S. M.; Radlinger, G.; Vogl, J. *Spectrochim. Acta, Part B* **1998**, *53*, 273−287.

Table 3. Mass Bias Determined by ICPMS in Standard and Collision Cell Mode[a]

natural isotopic composition	reference values	determined in standard mode		determined in collision cell mode	
		ratios	mass bias % u^{-1}	ratios	mass bias % u^{-1}
$^{63}Cu/^{65}Cu$ (100 ppb)	2.244 ± 0.002	2.115 ± 0.001	3.0	2.060 ± 0.002	4.5
$^{66}Zn/^{67}Zn$ (400 ppb)	6.820 ± 0.026	6.687 ± 0.002	2.0	6.601 ± 0.009	3.3
$^{32}S^{16}O/^{34}S^{16}O$ (1000ppb, GBW 04415)	22.1460 ± 0.0013			17.97 ± 0.04	11.5

[a] Data are mean ± SD (n = 6).

Table 4. Quantitative Analysis of Proteins[a]

proteins	absolute quantity of sulfur (ng)	protein concentration		S/Zn ratio calcd (theor)	S/Cu ratio calcd (theor)
		by isotope dilution analysis (mg/mL)	by gravimetric method (mg/mL)[b]		
BSA	620 ± 13	1.61 ± 0.03	1.842 ± 0.002		
CuZn−SOD	214 ± 7	1.18 ± 0.04	1.203 ± 0.002	4.03 ± 0.15 (5)	5.52 ± 0.10 (5)
Zn−MT-II	1189 ± 22	0.54 ± 0.01	0.581 ± 0.001	3.12 ± 0.18 (3)	

[a] Data are mean ± SD (n = 3). [b] It is assumed that the purities of the proteins are 100%.

rate of the spike (mL min^{-1}), M_s and M_{sp} are the atomic weights of the element in the sample and spike, respectively, and A^b_{sp} and A^b_s are the abundance of isotope b in the spike and sample, respectively.

R_m, R_s, and R_{sp} are the isotope ratios (isotope a/isotope b) in the mixture, sample, and spike, respectively.

The absolute amount of the element in different species can be obtained by the integration of the corresponding peaks in the chromatogram of mass flow.

The above process for sulfur measurement is illustrated in Figures 5 and 6. The same is for copper and zinc. Figure 5 shows the original intensity of $^{32}S^{16}O$ and $^{34}S^{16}O$ in the whole chromatographic procedure. After being corrected by the isobaric interference, dead time, and mass bias, the chromatogram of isotope ratios in the mixture (R_m) can be obtained. Then, the chromatogram of isotope ratios is transformed into the chromatogram of mass flow using eq 2, as shown in Figure 6. In eq 2, c_{sp} is calculated by reverse isotope dilution by ICPMS; the flow ($d_{sp}f_{sp}$) is assessed by gravimetric data; R_s in proteins and R_{sp} in the spike are measured by ICP-CC-MS. The absolute amount of sulfur in each protein can be obtained by integrating the corresponding peak in Figure 6. Therefore, the absolute protein quantity can be calculated by the sulfur stoichiometric ratio of each protein.

Table 4 shows that the protein concentrations calculated via sulfur element in protein by isotope dilution analysis are in good agreement with the concentrations obtained by the gravimetric method. The detection limit for sulfur by SEC−ICP-CC-MS is 10 ng, based on the signal-to-noise (3σ) in the chromatogram of mass flow. The corresponding absolute detection limits for BSA, SOD, and MT-II are 8, 31, and 15 pmol, respectively. The relative standard deviations (RSDs) are less than 3% for each protein. Although the precision of an isotope ratio measured by transient signals is usually worse than ones from continuous introduction (typical RSD is 0.5%), our results show that the precision (RSD < 3%) is perfectly good for protein quantification in comparison with the precision of the ICAT technique, whose RSD is in the range of 4−28%.[30]

The BSA, CuZn−SOD, and Zn−MT-II are proteins that can bind metals. BSA is stabilized by 17 disulfide bridges. It is relatively stable and can bind several cations.[31] CuZn−SODs are metalloenzymes involved in the mechanisms of cellular defense against oxidative damage. Eukaryotic CuZn−SODs are homodimers that contain one atom of zinc and one atom of copper per subunit. An imidazolate-bridged Cu−Zn heterodinuclear metal center undertakes its active site. The ion of Zn is more stable than Cu.[32] Mammalian metallothioneins have been characterized containing a single polypeptide chain of 61 amino acid residues, among them 20 cysteines providing the ligands for seven metal-binding sites. Each metal ion is bound to four thiolate ligands, and each complex is close to a tetrahedron.[33] In our BSA samples, Cu and Zn were not detectable, indicating that the samples contained no Cu and Zn. Only Zn was detected in MT-II. The obtained ratio of S/Zn in MT-II is in accord with the theoretical value. No detectable Cu was found in MT-II. However, for the samples of CuZn−SOD, the ratios of S/Cu and S/Zn in SOD deviate from the theoretical values. One of the explanations is that metal exchanges occur in the analytical process. The binding metal ions can be readily released or substituted by other ions, especially in the pH range that binding metal ions are not stable or in the metalloproteins whose metal ions are weakly coordinated. Thus, many derivatives of CuZn−SOD can be formed by metal

(30) Wu, W. W.; Wang, G. H.; Baek, S. J.; Shen, R. F. *J. Proteome Res.* **2006**, *5*, 651−658.
(31) Peters, T., Jr. *All About Albumin: Biochemistry, Genetics and Medical Applications*; Academic Press: San Diego, CA, 1996.
(32) Tainer, J. A.; Getzoff, E. D.; Richardson, J. S.; Richardson, D. C. *Nature* **1983**, *306*, 284−287.
(33) Kägi, J. H.; Vasák, M.; Lerch, K.; Gilg, D. E.; Hunziker, P.; Bernhard, W. R.; Good, M. *Environ. Health Perspect.* **1984**, *54*, 93−103.

exchanges, which may make the ratios of S/Zn and S/Cu be unequal to the theoretical values.

Therefore, the following two conclusions can be drawn that (1) reliable quantification of proteins via their binding metals, generally, cannot be achieved; however, the characterization of their stoichiometric composition can be determined by HPLC−ICPMS, and (2) sulfur is preferable as an internal standard for the goal of quantitative analysis of proteins because of its high abundance in proteins and stability in a covalent form.

It should be stressed that elemental mass spectrometry (e.g., ICPMS) and molecular mass spectrometry (e.g., ESI-MS) are really complementary techniques for proteomics. Elemental mass spectrometry has a unique quantitative ability and unmatched sensitivity for elemental detection, whereas molecular mass spectrometry can probe the structure, dynamics, and functional properties of proteins.

CONCLUSIONS

A method of absolute quantitative analysis of proteins via sulfur analysis using SEC coupled to ICP-CC-MS and postcolumn isotope dilution analysis has been developed. The method has abilities not only to accurately quantify proteins via sulfur but also to characterize protein stoichiometric composition, which will be helpful for identification of a protein. ICPMS, as an attractive complement to molecular mass spectrometry, will become a potential tool for proteomics study.

ACKNOWLEDGMENT

The authors are grateful to Mr. Hai Lu at the National Research Center for Certified Reference Materials for his help in the analysis of the enriched isotopes. This work is supported by the Foundations of the National Natural Science Foundation of China (10490181, 20475055, and 10525524), the CAS Knowledge Innovation Program, the MOST 973 Program (2006CB705605 and 2007CB935604), and the Key Laboratory of Nuclear Analytical Techniques (K135).

Received for review July 13, 2007. Accepted September 17, 2007.

AC071483T