# Strategy for absolute quantification of proteins: $CH_3Hg^+$ labeling integrated molecular and elemental mass spectrometry†‡

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Currently, molecular mass spectrometry is preferred by many for relative quantification but is not appropriate for "absolute" quantification of proteins. In this article we demonstrate a proof of concept for the absolute quantitative analysis of proteins *via* $CH_3Hg^+$ labeling and integrated application of molecular and elemental mass spectrometry. The smallest size of $CH_3Hg^+$ among monoalkyl mercurials and the specific and covalent interaction with sulfhydryl (–SH) in proteins results in forming a simple complex of $CH_3Hg^+$:–SH = 1:1 when all –SH are exposed, as confirmed by ESI-MS. Based on the known number of –SH per protein, the absolute protein concentration can be obtained *via* Hg determination using ICP-MS, in which $CH_3HgCl$ could be simply used as an external standard. When bovine pancreatic ribonuclease A, lysozyme and insulin, which have an increasing number of various disulfide linkages in their molecules, were taken as model proteins, their corresponding absolute detection limits (3σ) reached 0.6, 1.2 and 0.4 pmol, respectively. These characteristics may be expected to provide an alternative approach for absolute protein quantification, especially specific biomarker determination, in the near future.



## Introduction

Molecular mass spectrometry (MMS) is rapidly maturing as a powerful analytical tool and playing a central role in proteomics research.[1] However, currently molecular mass spectrometry can only provide a very limited quantitative profile of proteomics. Besides being challenged by concomitant matrix effects and variable chemical background, the striking dissimilarity of protein physicochemical behaviors leads to diverse ionization efficiencies on MALDI-MS and/or ESI-MS and results in no strict linear dependence between the amount of analyte present and measured signal intensity.[2] But, quantitative proteomics is important: the quantitative protein profile is expected to provide new functional insights into biological processes, facilitating the identification of diagnostic or prognostic disease markers.[3] Recognition of the fact that protein analysis must "turn quantitative"[4] has boosted the development of more and more sophisticated analytical methods based on MMS in the past few years in order to obtain reliable quantitative results, for example, through stable isotope labeling by amino acids in cell culture and isotope-coded affinity tags.[5–7] Such approaches show certain strengths, but still a number of limitations. These methods are elegant for relative quantification but are not appropriate for the absolute quantification. To add a quantitative dimension to proteomics, elemental mass spectrometry, especially ICPMS, has been introduced for protein analysis. The exceptional abilities of ICPMS including the multi-element (including non-metals such as sulfur, phosphorus, selenium) detection capability, high sensitivity, a wide linear dynamic range, the virtual independence between the signal intensity and the biomolecular structure, and tolerance to matrix as well as the ability to couple with chromatography or electrophoresis (capillary and gel) match the demands of quantitative proteomics pretty well, making ICPMS a valuable complementary technique to ESI-MS and MALDI-MS. More generally, the accurate quantification of peptides and proteins can be accomplished *via* a covalently bound ICPMS detectable heteroatom (any element different from the main constitutes of organic matter: C, H, N or O), either already present (such as sulfur, phosphorus, selenium, iodine, or metals) or labeled as a tag.[8–14] For example, the naturally present sulfur atom has been used for the quantification of proteins[15–17] and phosphor for quantification of post-translational modifications such as phosphorylation.[18–22] Unfortunately, some biologically important elements (mainly S and P) have higher ionization energies and are not as efficiently ionized as metals in the ICP. Moreover, they suffer from a number of polyatomic interferences and are detected with higher detection limits than metals. This makes their detection by ICPMS a feasible but challenging task. The other way to detect and quantify proteins mentioned above is labeling a particular protein with a tag that contains an ICPMS sensitive element. The use of ICPMS in this context was pioneered by Zhang and Baranov who developed a very sensitive immunoassay that used metal tagged antibodies. After reaction with the antibody, the protein of interest was detected *via* the Sm, Eu, and Au signal by ICPMS.[8–10,13] Tags that use the fluorescent properties of Eu, Tb, Dy, or Sm chelates were used to measure

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the concentrations of various antigens in an automated immunoassay system.[23,24] There is a great potential for such metal chelates being utilized as tags for both identification and quantification using ICPMS after the necessary chemical derivatization procedures, such as the metal-coded affinity tag technique developed by Linscheid *et al.*[25,26] In this case, a macrocyclic metal chelate complex loaded with different lanthanides was the essential part of the tag, the other part of the tag was a reactive group for reaction with amino acids in the proteins to achieve specific labeling and quantitative proteomics.[25]

Our interest focuses on direct labeling of the sulfhydryl(s) in proteins with monofunctional organic mercury ions ($MFOHg^+$) in order to accomplish the absolute quantification of proteins using HPLC-ICPMS. Compared with other –SH specific reactive reagents used for protein labeling, the $MFOHg^+$ as a tag has its own superiority because it directly attaches the $MFOHg^+$ to the –SH(s) in a protein without any additional "bridge" despite its relative high ionization potential and memory effect.[27–30] The reaction between Hg and S belongs to soft–soft interactions and are strongly exothermic. In a final labeled protein obtained Hg is associated to one C atom (in the organic moiety) at an average distance of 2.03 ± 0.02 Å and to one S atom (in the –SH) at an average distance of 2.34 ± 0.03 Å, clearly indicating the formation of an Hg–S covalent bond.[31,32] The standard entropy change is also very favorable for the labeled protein and the two factors combine to produce a very large stability constant as high as $10^{16.3}$ to $10^{16.7}$.[31,33] Such a high stability makes the labeled proteins stable adducts during chromatographic separations, and quantitatively transport into the subsequent mass spectrometers. Actually, our previous study[29] has demonstrated the specific interaction between $MFOHg^+$ (including monomethylmercury chloride, monoethylmercury chloride and p-hydroxymercuribenzoic acid) and –SH for counting the number of free –SH(s) and disulfide bond(s) in peptides and proteins using ESI-MS; and Bettmer *et al.*[30] demonstrated the promising approach for ovalbumin quantification using ESI-MS and ICP-MS with pHMB labeling. In this article, we describe the "proof of concept" of the absolute quantification of proteins labeled by $CH_3Hg^+$ using HPLC coupled to ICPMS with $CH_3HgCl$ as a simple external standard. The use of HPLC/ESI-MS allowed comprehensive characterization of the labeled proteins and unambiguous confirmation of the binding stoichiometry.

## Experimental

$CH_3HgCl$ was obtained from Alfa Aesar (Ward Hill, MA, USA). A $CH_3HgCl$ stock solution at approximately 1 μg $mL^{-1}$ (as Hg) was prepared from solid $CH_3HgCl$ in methanol and kept in a freezer at −20 °C. Acetonitrile, methanol and acetic acid were of HPLC grade (Merck, Darmstadt, Germany). Tris-(2-carboxyethyl)-phosphine (TCEP), bovine pancreatic ribonuclease A (RNase A), lysozyme and insulin were all purchased from Sigma-Aldrich (St. Louis, MO, USA). Ultra pure water (UPW) (18 MΩ) was prepared with a Milli-Q system (Millipore Filter Co., Bedford, MA), and purged with $N_2$ (10 min at 200 mL $min^{-1}$) to get rid of dissolved $O_2$, and used throughout this study. Other chemicals used were at least of analytical reagent grade.

Free –SHs in proteins were labeled directly with $CH_3Hg^+$, while disulfide bonds (–S–S–) in proteins were subjected to reduction by TCEP and the resulting nascent –SHs were labeled immediately with $CH_3Hg^+$. Briefly, the disulfide bonds in lysozyme (50 μL, 10 μM) were reduced with TCEP (10 times in excess compared to the disulfide bonds) at room temperature for 20 min. Derivatization of the nascent free –SHs by $CH_3HgCl$ (30 mM, 2.5 times in excess compared to TCEP) was performed in the dark for 40 min as described previously.[29] RNase A and insulin were treated in the same way.

### HPLC/ESI-MS and HPLC/ICPMS analysis

Isolation of the labeled proteins was carried out on an Agilent 1100 series chromatographic system (Agilent Technologies) using a Zorbax 300SB-C18 column (1.0 I.D. × 150 mm in length, 3.5 μm). A gradient elution program was used to linearly increase the percentage of mobile phase B (0.3% acetic acid in acetonitrile) from 10% to 45% while decreasing the mobile phase A (0.3% acetic acid in UPW) from 90% to 55% with a flow rate of 0.05 mL/min. The HPLC was directly coupled to a Bruker Daltonics Esquire-LC™ ESI ion trap mass spectrometer (Bremen Germany) for structural analysis of the labeled proteins (see ESI‡). The column eluate was introduced on-line into the ELAN-DRC II™ ICP-MS (PerkinElmer, SCIEX, Canada) through a direct injection high efficiency nebulizer (DIHEN-170-AA, Meinhard, USA) for protein quantification. The operational parameters of the ICP-MS were as follows: ICP RF power, 1105W; plasma gas flow, 15 L $min^{-1}$; auxiliary gas flow, 1.2 L $min^{-1}$; isotope monitored, $^{202}Hg$, $^{34}S$ and $^{32}S^{16}O$.

## Results and discussion

### HPLC/ESI-MS for binding stoichiometry

It has been shown in Fig. S1 that the binding stoichiometry is 1:1 for all exposed –SH (see ESI‡).

### Hg signal independence from the chemical structure of labeled-proteins using ICPMS

External standardization relies initially on the assumption that any Hg species provides the same ICPMS intensity. In ICP (about 5500 K) the labeled proteins are completely pyrolyzed.[34] However, the ICP-MS signal response depends not only on the ionization efficiency and ion transmission but also on the sample introduction efficiency. In our case, the use of DIHEN minimized the influence of the sample introduction process. Consequently, a series of equimolar (as Hg) of $CH_3HgCl$, $CH_3Hg$-labeled RNase A and $CH_3Hg$-labeled lysozyme separately and at different concentrations were analyzed by direct infusion to the ICPMS. As shown by the results in Fig. 1, the $^{202}Hg$ intensity signal obtained by ICPMS was not structure-dependent and the relative standard deviation of $^{202}Hg$ intensity obtained was less than 3% among the three Hg-containing compounds tested, suggesting no chemical structure effect. These results clearly demonstrated that, as expected, the element signal provided by ICP-MS is proportional to the total amount of Hg entering the plasma.

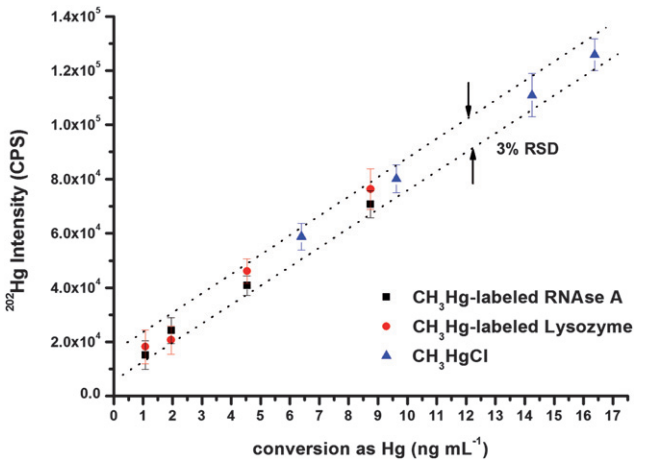


**Fig. 1** Structure-independent $^{202}$Hg signal from different mercury-containing compounds.

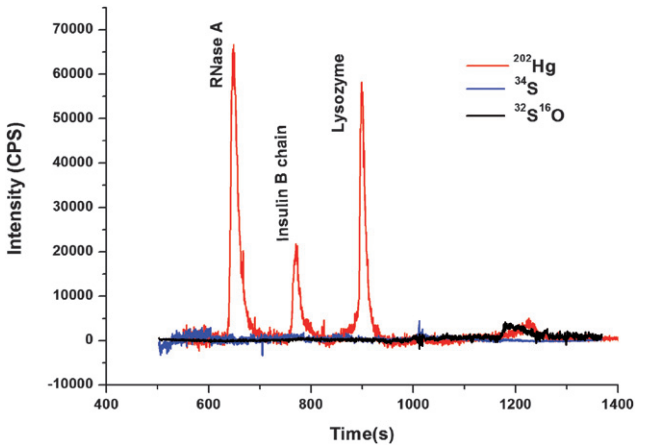


**Fig. 2** Typical HPLC/ICPMS chromatogram for the absolute quantification of RNase A, insulin and lysozyme. HPLC conditions and ICPMS parameters were described in the experimental section. Concentrations of RNase A, insulin and lysozyme are 1.39 ± 0.03, 1.08 ± 0.03 and 1.51 ± 0.03 μg mL$^{-1}$ (n = 5), respectively.

### HPLC/ICPMS for absolute quantification of the proteins

It is well documented that the total amount of organic modifier in the mobile phase used in an HPLC system being introduced into the ICP has a profound effect on plasma stability and ionization efficiency. Obviously, this change in element sensitivity would result in different detector responses depending on the retention time of the Hg-labeled proteins because of different percentage of acetonitrile at the time eluted from the column and then reaching the plasma. This fact would prevent the use of a $CH_3HgCl$ external standard for the quantification of the different Hg-labeled proteins separated during the reversed-phase gradient. The addition of a postcolumn sheath-flow (only UPW) with a high constant flow rate up to 100 times the column splitted flow rate could be a solution to the above-described problem. We observed that this could ensure that Hg sensitivity remains constant during the gradient elution since the increase in acetonitrile content in the effluent of the column was negligible compared to the total flow.

Moreover, an accurate quantification of the $CH_3Hg$-labeled protein requires the determination of labeling efficiency. In order to check the labeling procedure, defined amounts of the RNase A, insulin and lysozyme were labeled with $CH_3Hg^+$ and analyzed with HPLC/ICPMS. The mercury, quantified in the protein peak, can be correlated with the concentration of the labeled protein. Consequently, comparison with the initially employed protein concentration enabled the determination of protein recovery indicating labeling efficiency as well as sample loss throughout the whole procedures. In five independent labeling experiments the average recoveries of RNase A, insulin and lysozyme were found to be greater than 94.8, 96.5 and 91.3%, respectively. In addition to the already demonstrated high specificity of the labeling reaction by ESI-MS, the conditions for the absolute protein quantification *via* $CH_3Hg^+$ labeling and Hg determination were established.

As shown in Fig. 2, three labeled proteins (RNase A, B chain of insulin, and lysozyme) were completely separated by the HPLC and determined online by ICPMS through the interfacing with DIHEN. The $^{202}$Hg, $^{34}$S and $^{32}S^{16}O$ measured by ICPMS and background corrected are shown in Fig. 2. In this study, even the ICPMS was equipped with the dynamic reaction cell, the measurements of $^{202}$Hg, $^{34}$S and $^{32}S^{16}O$ were performed in a standard mode, severe polyatomic interference existed in the $^{34}$S analysis, and the detection of $^{32}S^{16}O$ was unsatisfactory as expected. Their background corrected sensitivities were much lower than that of $^{202}$Hg, demonstrating that almost no peaks at the retention times of the corresponding proteins, while the absolute amount of the Hg in different proteins could be obtained by the integration of the corresponding peaks in Fig. 2. The number of sulfhydryls in each protein is known and therefore the absolute protein quantity can be determined. The corresponding absolute detection limits (DLs, 3σ) for RNase A, insulin, and lysozyme in the chromatogram of mass flow were 0.6, 1.2, and 0.4 pmol, respectively. The RSDs (n = 5 at 100 pmol) of RNase A, insulin and lysozyme were 2.3, 2.5 and 1.8%. These DLs suggested the method to be a significant improvement over direct determination of S by sector field or collision cell instruments.[15–17]

In summary, a strategy of the absolute quantitative analysis of proteins *via* $CH_3Hg^+$ labeling using ESI-MS and ICP-MS has been developed. It provides an significant approach for the absolute quantification of proteins having known amounts of –SHs and –S–S– considering the importance of quantitative proteomics and the few methods currently available for this task. Furthermore, the mercury element provides greater sensitivity, wider dynamic range and better precision than the direct determination of heteroatoms (such as S). The developed method would be better when a less toxic mercurial, which can dynamically release $CH_3Hg^+$ in solution, is available. Such fundamental studies are ongoing in our laboratory.

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