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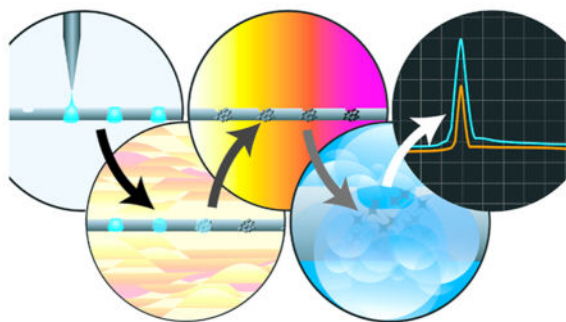
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Directly Coupled High-Performance Liquid Chromatography-Accelerator Mass Spectrometry Measurement of Chemically Modified Protein and Peptides

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Abstract



Quantitation of low-abundance protein modifications involves significant analytical challenges, especially in biologically important applications, such as studying the role of post-translational modifications in biology and measurement of the effects of reactive drug metabolites. ^{14}C labeling combined with accelerator mass spectrometry (AMS) provides exquisite sensitivity for such experiments. Here, we demonstrate real-time ^{14}C quantitation of high-performance liquid chromatography (HPLC) separations by liquid sample accelerator mass spectrometry (LS-AMS). By enabling direct HPLC-AMS coupling, LS-AMS overcomes several major limitations of conventional HPLC-AMS, where individual HPLC fractions must be collected and converted to graphite before measurement. To demonstrate LS-AMS and compare the new technology to traditional solid sample AMS (SS-AMS), reduced and native bovine serum albumin (BSA) was modified by ^{14}C -iodoacetamide, with and without glutathione present, producing adducts on the order of 1 modification in every 10^6 to 10^8 proteins. ^{14}C incorporated into modified BSA was measured by solid carbon AMS and LS-AMS. BSA peptides were generated by tryptic digestion. Analysis of HPLC-separated peptides was performed in parallel by LS-AMS, fraction collection combined with SS-AMS, and (for peptide identification) electrospray ionization and tandem mass

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spectrometry (ESI-MS/MS). LS-AMS enabled ^{14}C quantitation from ng sample sizes and was 100 times more sensitive to ^{14}C incorporated in HPLC-separated peptides than SS-AMS, resulting in a lower limit of quantitation of 50 zmol ^{14}C /peak. Additionally, LS-AMS turnaround times were minutes instead of days, and HPLC trace analyses required 1/6th the AMS instrument time required for analysis of graphite fractions by SS-AMS.

Carbon-14 accelerator mass spectrometry (^{14}C -AMS) has been used for a wide variety of biological experiments including measurement of drugs and their metabolites,^{1,2} bioavailability determination of dietary components,³ and detection of protein⁴⁻⁶ and DNA adducts^{7,8} formed by reactive compounds. Experiments using ^{14}C -AMS have proven useful in predicting pharmacokinetic properties of several drugs.⁹⁻¹¹ In addition, measurement of protein therapeutics in experimental animals using ^{14}C -AMS has recently been reported.^{12,13} ^{14}C -AMS is an attractive approach for quantitation of therapeutic proteins or low-abundance post-translational modifications displayed on proteins because its sensitivity allows the use of doses that impose negligible chemical and radioactive risk to humans, while providing robust quantitative data.^{14,15}

Historically, ^{14}C -AMS has required that all samples be processed into solid carbon in the form of graphite before measurement.¹⁶ Quantitation of ^{14}C by AMS in HPLC separations has required individual fractions to be collected and processed to graphite prior to AMS analysis. This process can be time-consuming and typically involves processing 30 to 60 fractions for each HPLC run. Since the carbon contained in an individual HPLC fraction is less than that required for successful conversion to graphite, ^{14}C -depleted carrier carbon must be carefully added to provide sufficient material for AMS analysis. Small amounts of ^{14}C in the carrier carbon decrease sensitivity and, in some instances, potentially compromise ^{14}C quantitation.

As an alternative to solid carbon sample preparation, we have developed a real-time liquid sample AMS method (LS-AMS). LS-AMS enables the measurement of liquid samples through an ultra high-efficiency moving wire interface (MWD)¹⁷ coupled to an AMS instrument through a CO_2 gas-accepting ion source.¹⁸ LS-AMS accepts samples of nonvolatile material dissolved or suspended in a solvent or buffer solution, evaporates the solvent or buffer, combusts the remaining analyte, and directs the resulting CO_2 to a gas-accepting ion source, generating C^- ions and enabling normal AMS ^{14}C quantitation of the sample (Figure 1). As many materials analyzed by BioAMS laboratories are already suspended in a compatible solvent, very little sample handling is required. Such a sample may be measured in minutes, with results generated in real time. LS-AMS is also capable of accepting a continuous flow of fluid directly from an HPLC instrument, which can lead to increased sensitivity, better temporal peak resolution, and substantial reduction of AMS instrument time. Other approaches to couple biochemical separation instrumentation for the analysis of nonvolatile compounds to AMS have been developed, including systems that use a chemical agent¹⁹ or a laser^{20,21} to oxidize the sample. However, these systems are either more restrictive or not compatible with direct coupling of HPLC to AMS, either due to mobile phase limitations or an off-line solvent removal step.

Here the LS-AMS method is combined with liquid chromatography-mass spectrometry (LC-MS/MS), to quantify ^{14}C chemical adducts of bovine serum albumin (BSA) and characterize peptide modification patterns under several reaction conditions. This model system was chosen to explore the application of LS-AMS for quantitation of low-abundance protein modifications by real-time measurement of bulk protein samples and of HPLC-separated peptides.

EXPERIMENTAL SECTION

Materials

BSA, dithiothreitol (DTT), iodoacetamide (IAC), HPLC-grade acetonitrile, HPLC-grade water, formic acid, acetic acid, and proteomics-grade trypsin were purchased from Sigma (St. Louis, MO). Iodoacetamide [$1\text{-}^{14}\text{C}$] with a specific activity of 56 mCi/mmol was purchased from American Radiolabeled Chemicals, Inc. (St. Louis, MO).

^{14}C -Iodoacetamide Adduct Quantitation and Competitive Binding Experiments

Reaction of ^{14}C -iodoacetamide with BSA was performed under several experimental conditions.

Initial range-finding experiments were performed with the goal of generating protein adducts measuring between 2000 and 20000 amol ^{14}C /mg protein. These experiments used a range of concentrations of native and reduced BSA and ^{14}C -iodoacetamide. A selection of these samples were measured by the solid sample (carbon)-AMS (SS-AMS) method for comparison with LS-AMS.

For competitive binding experiments, 2 mg/mL BSA was suspended in a solution of 100 mM ammonium bicarbonate with 5% acetonitrile. Solutions of 50 mM dithiothreitol, 500 mM reduced glutathione (GSH), and 100 mM unlabeled iodoacetamide were prepared in water. For labeling experiments, a dosing solution of 100 pCi/mL of [$1\text{-}^{14}\text{C}$] iodoacetamide was prepared in water. Activity of the source material was verified by liquid scintillation counting. Each reaction was performed in triplicate. For control (no label) experiments, BSA disulfide bonds were reduced by incubation with 5 mM DTT for 5 min at 65 °C in the dark. The reaction was cooled, and unlabeled iodoacetamide was added to a final concentration of 10 mM and cysteine alkylation was allowed to proceed for 30 min at 30 °C in the dark. For native albumin ^{14}C -iodoacetamide binding experiments, 1 pCi of ^{14}C -iodoacetamide was added to each 1 mL of BSA and reacted at 30 °C for 30 min in the dark. Disulfides were then reduced, and the remaining free cysteine residues were alkylated with unlabeled iodoacetamide as described for the control samples. For reduced albumin experiments, albumin disulfides were reduced with DTT as in the control experiments, followed by reaction with 1 pCi of ^{14}C -iodoacetamide and subsequent alkylation of the remaining free cysteine residues with 10 mM of unlabeled iodoacetamide. For competitive binding experiments, GSH was added to native or reduced BSA to a final concentration of 5 mM, after which BSA was reacted with 1 pCi of ^{14}C -iodoacetamide, with subsequent alkylation of free cysteine (Cys) with unlabeled iodoacetamide or reduction of disulfides followed by alkylation with unlabeled cysteine, as described for the control samples. Following protein modification, all samples were desalted by filtration through 50 kDa molecular weight cutoff

spin tubes. Protein was washed three times with 0.5 mL of 100 mM ammonium bicarbonate with 5% acetonitrile by spinning for 30 min at 14000 rpm. Washed protein was collected in 1 mL of 100 mM ammonium bicarbonate with 5% acetonitrile and stored at -80°C until analysis.

Adducted Peptide Analysis Experiments

For characterization of modified peptides, unlabeled BSA was reduced and alkylated with unlabeled iodoacetamide as above, except that the BSA concentration was 5 mg/mL and reactions were performed in quadruplicate. Alkylation of native and reduced BSA was likewise performed using 5 mg/mL BSA. Reactions used 4 mL of BSA solution per reaction, and labeling experiments used 4 pCi of ^{14}C -iodoacetamide per reaction. After reactions were completed, protein was precipitated by addition of 6 mL of ice-cold methanol and centrifugation for 10 min at 4400 rpm in a swinging bucket centrifuge. The precipitating solution was collected and stored at -80°C for subsequent analysis. Precipitated protein was washed four times with 10 mL of methanol, after which the protein was resuspended in 4 mL of 100 mM ammonium bicarbonate with 5% acetonitrile. Samples were stored at -80°C until analysis or digestion.

Trypsin Digestion of Modified BSA

Modified protein samples were vortex-mixed and 100 μL aliquots were collected for trypsin digestion. Trypsin was resuspended to a concentration of 1 mg/mL in 1 mM HCl. Reconstituted trypsin was then added to each protein at a 1:20 trypsin:protein ratio (mg/mL). Digestion was allowed to proceed overnight with incubation at 37°C . Following digestion, the reaction was stopped by addition of 10 μL of 0.5% acetic acid.

Analysis of Intact Protein Adducts by AMS

Intact proteins were analyzed for ^{14}C -iodoacetamide incorporation by LS-AMS and SS-AMS analysis. For LS-AMS analysis, 1 μL (2 $\mu\text{g}/\mu\text{L}$) samples were directly applied to the MWI as droplets at 1 min intervals by pipet. Each sample was measured three times. For comparison, standards of known isotopic ratio and carbon content were applied before and after each series of replicates. The design of the MWI was as detailed previously,¹⁷ with the addition that the exhaust of the drying oven is now collected by a sample pump equipped with carbon traps. The instrument configuration is shown in Figure 1. The wire was set to move at 2.5 cm/s with indentations every 0.6 cm. The cleaning furnace was held at 900°C under atmosphere. The drying oven was held at 140°C with a helium flow rate of 400 mL/min. The combustion oven was held at 750°C with an atmosphere of 97.5% helium and 2.5% oxygen. The capillary from the combustion oven to the gas-accepting ion source was 180 μm in diameter and 4.5 m long. From the ion beam generated by the gas accepting ion source, the AMS instrument directed ^{12}C ions to a Faraday cup and ^{14}C ions to a solid-state particle detector. The charge collected by the Faraday cup and number of ^{14}C atoms measured by the particle detector were summed over 1 s intervals. Isotopic ratios of samples were determined by the background subtracted integral of the resulting ^{14}C and ^{12}C signals from the combusted samples in comparison to that of the standards.

For SS-AMS analysis, 300 μL (600 μg C) of each sample was converted to graphite and measured by AMS, following standard biochemical AMS sample preparation procedures.¹⁶

HPLC Analysis of Peptide Adducts

Prior to HPLC analysis, tubes containing labeled peptides were centrifuged at 14000g for 5 min and the supernatant was loaded into HPLC analysis vials. HPLC separation of peptides: an Agilent 300 Extend C18 3.5 μm 2.1 \times 100 mm column; mobile phases: A, water and B, acetonitrile; flow rate = 100 $\mu\text{L}/\text{minute}$; time: 0, 5% B; 25 min, 30% B; 35 min, 60% B; 35.1 min, 90% B; 38 min, 90% B; 38.1 min, 5% B; stop time: 45 min, 5% B. Experiments were initially performed with mobile phases containing 0.1% formic acid to improve peak shape and enhance peptide ionization for MS/MS, but an elevated ^{12}C background attributable to the formic acid was observed. Formic acid was therefore omitted from the HPLC mobile phases without a significant reduction in the quality of peptide separations. LC-MS/MS analysis was performed on a Thermo LTQ XL linear ion-trap instrument. Files were converted to profile.mzXML files using the MassMatrix file conversion utility. Peptides were identified using the MassMatrix search server.^{22,23} In addition to LC-MS/MS analysis, digests were separated using identical chromatographic conditions and fractions were collected to allow for comparison of peptide adduction quantitation by directly coupled liquid chromatography LS-AMS (LC-LS-AMS) and SS-AMS. SS-AMS HPLC fractions were prepared for graphite analysis by the standard biochemical AMS protocol.¹⁶ The LC-LS-AMS and SS-AMS traces were aligned with the total ion chromatograms generated by LC-MS/MS analysis to identify ^{14}C -modified peptides.

RESULTS AND DISCUSSION

Cys residues in proteins play important roles in maintaining protein structure and function.²⁴ Numerous biological modifications of protein Cys residues are known to modulate protein activity. In addition, Cys residues are important sites of modification by reactive chemicals, potentially resulting in impairment of protein function.^{25,26} Bovine serum albumin is a well-characterized protein containing 35 cysteine residues, 34 of which participate in disulfide bonds. Iodoacetamide readily alkylates free Cys residues to form carbamidomethylated cysteine in proteins and peptides but cannot react with disulfide cysteines. Because the reaction of iodoacetamide with BSA is predictable, this reaction was selected to explore the utility of LS-AMS for absolute quantitation of protein and peptide modifications. In principle, this method of protein analysis can be extended to measure protein therapeutics or other proteins synthesized to contain a ^{14}C label.

To determine appropriate labeling procedures and to enable comparison of measurements made by LS-AMS analysis with solid sample AMS analysis, several alkylation experiments involving modification of BSA by ^{14}C -iodoacetamide were performed with native BSA or BSA with chemically reduced disulfide bonds. Aliquots of each sample were measured by LS-AMS and by graphitization followed by AMS. Results are given in Table 1 and a typical LS-AMS signal is depicted in Figure 2. Quantification of binding is computed by the difference between ^{14}C incorporation in the control and the sample. To enable routine solid carbon AMS measurement, samples for this comparison were designed to yield ^{14}C concentrations less than 5000 amol/mg protein.

Comparison of SS-AMS and LS-AMS analysis of selected controls and samples modified with ^{14}C -iodoacetamide. Native and reduced albumin were prepared at 5 mg/mL and alkylated with various preparations of ^{14}C -iodoacetamide to achieve ^{14}C incorporation in the optimal range of SS-AMS. The control was prepared by alkylation of reduced BSA with unlabeled iodoacetamide. ^{14}C incorporation of each sample was computed from the mean and standard deviation of the mean of 4 replicate reactions.

The LS-AMS measurements produced equivalent values to the SS-AMS measurements, albeit with 2–4% precision instead of 1%, using only 1 to 2 μg of material for each measurement (Table 1). For routine SS-AMS quantitation of biomedical materials, a sample carbon mass of 500 μg ^{12}C (~1000 μg protein) is required.¹⁶ ^{14}C -labeled biological samples containing as little as 50 μg ^{12}C are possible to measure by SS-AMS but require substantially more time and effort to measure.²⁷

A typical peak resulting from LS-AMS measurement of unlabeled BSA is depicted in Figure 2. The precision of individual peak $^{14}\text{C}/\text{C}$ quantitation is primarily limited by ^{14}C content, requiring ~40 zmol ^{14}C for 10% precision. Uncertainties of ^{12}C peak integrations indicate that such samples could contain as little as 10 ng ^{12}C with little change to precision.

After appropriate labeling procedures were determined, competitive binding experiments were performed with BSA and ^{14}C -iodoacetamide in the presence or absence of the thiol-containing peptide glutathione (GSH). The molar ratio of albumin to ^{14}C -iodoacetamide in these alkylation experiments was approximately 1.6 million to 1. As expected, measurements of label incorporation demonstrated increased binding of ^{14}C -iodoacetamide to reduced BSA (6468 ± 85 amol/mg protein, indicating 81% of added label bound to BSA) compared to native BSA (4100 ± 200 amol/mg protein, indicating 51% of added label bound to BSA) due to the availability of additional free Cys residues (Figure 3). Competitive binding experiments with GSH show decreased binding of ^{14}C -iodoacetamide to both native (623 ± 25 amol/mg protein, indicating 7.8% of added label bound to BSA) and reduced BSA (3780 ± 75 amol/mg protein, indicating 47% of added label bound to BSA) compared to native and reduced BSA in the absence of glutathione (Figure 3). In all cases, measurement of intact protein allowed absolute quantitation of protein modification by ^{14}C -iodoacetamide.

In order to determine specific sites of BSA modification, quadruplicates of ^{14}C -iodoacetamide-modified native and reduced BSA (respectively ^{14}C -IAC-native and ^{14}C -IAC-reduced) were subjected to tryptic digestion and analysis. Peptide identification was accomplished by LC-MS/MS, and the ^{14}C tracer was detected by LC-LS-AMS. Additionally, fractions collected from identical separations were measured by solid carbon AMS. Representative traces from these separations, including peptide total ion chromatograms and peptide ^{14}C and ^{12}C traces are depicted in Figures 4 and 5.

For LS-AMS analysis, effluent from the HPLC was applied directly to the interface. For solid carbon AMS, as the HPLC separations contained only ~15 μg C, it was necessary to add 1 μL of tributyrin carrier (containing 0.61 mg C with an isotopic ratio of 11.2 ± 0.3 amol $^{14}\text{C}/\text{mg}$ ^{12}C) to each fraction, to provide sufficient carbon for graphitization and AMS

analysis. Under this regime, the amount of ^{12}C from the carrier dominates the ^{12}C from the sample and 1 μL of tributyrin carrier adds 6.8 ± 0.4 amol ^{14}C to each fraction. ^{14}C from the HPLC eluate in each fraction was determined by subtracting the measured isotopic ratio of the carrier from the measured isotope ratio of each fraction and then multiplying by the carbon mass of the carrier.²⁸ This process adds a significant uncertainty to the ^{14}C measurement. As a result, the uncertainty of individual solid carbon AMS fraction measurements is approximately ± 1 amol ^{14}C /fraction, while the lower limit of quantitation (LLOQ) is 10 amol ^{14}C /fraction. In contrast, LC-LS-AMS ^{12}C backgrounds were between 2 to 4 ng/second and the ^{14}C background was less than 10^{-3} amol ^{14}C /second with a LLOQ of 50 zmol ^{14}C /peak.

Although the magnitude of the ^{12}C signal measured by LC-LS-AMS was sufficient for quantitation, peptide coelution made it impossible to assign measured ^{12}C signals to individual peptides (Figures 4C and 5C), thus preventing measurement of an isotope ratio that would easily enable absolute quantitation of each modified peptide. The ^{14}C content in the separation was estimated by comparison to a standard of known ^{14}C content measured before and after the separation. With improved separations, it should be possible to obtain ^{12}C quantitation of individual peptides.

The HPLC traces in Figures 4 and 5 indicate that LC-LS-AMS analysis is more sensitive for quantifying ^{14}C -containing peaks than conventional solid carbon HPLC-AMS. Figure 4C reveals 3 distinct ^{14}C peaks from separation of the digested ^{14}C -IAC-native by LC-LS-AMS. The ^{14}C peak eluting at 33.2 min and containing ~75% (17 amol) of ^{14}C incorporated into the ^{14}C -IAC-native is consistent with the elution time of the peptide containing Cys 58, the only free Cys residue in BSA, as detected by LC-MS/MS (Figure 4C). The other ^{14}C peaks in Figure 4C eluting at 3.8 and 20.3 min, respectively, contained 1.9 and 2.9 amol ^{14}C and correspond to a number of Cys-containing peptides. Conversely, with solid carbon AMS, the only ^{14}C peak detected eluted at fraction 65, corresponding to an elution time of 34 min and was near the practical limit of quantitation for solid carbon AMS (Figure 4B). Additionally, graphitization of 3 fractions, including fraction 66, failed. Without repeating the experiment, it cannot be known if some portion of the ^{14}C elevation detected by fraction 65 carried over to fraction 66.

From separation of the digested ^{14}C -IAC-reduced, LC-LS-AMS detected 13 distinct ^{14}C peaks, corresponding to additional Cys-containing peptides (Figure 5C). ^{14}C peaks 1, 8, and 9 have elution times within a few seconds of ^{14}C peaks 1, 2, and 3 of the digested ^{14}C -IAC-native separation shown in Figure 4C. Peak overlap limits precise quantitation of individual peaks in the ^{14}C trace in Figure 5C. Conversely, no substantially elevated fractions were detected by solid carbon AMS from the ^{14}C -IAC-reduced separation in Figure 5B. Failure to detect elevated fractions from the ^{14}C -IAC-reduced separation is due to a lack of sensitivity, arising from the combination of extremely low-level labeling of numerous peptides and the presence of ^{14}C from the tributyrin present in all fractions. Overall, the data in Figures 4 and 5 suggest that LC-LS-AMS is 100 times more sensitive in quantifying ^{14}C incorporated in HPLC-separated peptides than SS-AMS.

LC-LS-AMS also provides superior temporal resolution compared to fraction collection combined with solid carbon AMS. For LC-LS-AMS, the integrated ^{12}C charge and ^{14}C counts are recorded at 1 s intervals. Peak maximum locations could be determined to the nearest second and varied by less than 5 s between replicates. Typical peak widths, measured by full width at half-maximum, were in the range of 35 s and varied by less than 2 s between replicates. Without employing complex peak fitting algorithms, the temporal resolution of solid carbon AMS analysis is limited by the fraction collection time. Increasing the number of fractions can improve temporal resolution, but this would require more carrier carbon and thus decrease sensitivity.

In addition to improved sensitivity, improved temporal resolution and reduced sample preparation, traces measured by LC-LS-AMS took significantly less AMS instrument time to measure: ~1 h per trace for Figures 4C and 5C. In contrast, the low ^{14}C content and dilution by carrier carbon of the solid carbon fractions (resulting in an average isotopic ratio of ~15 amol $^{14}\text{C}/\text{mg C}$) required ~6 h of AMS instrument time per one separation to measure the 75 fractions collected from each of the two separations shown in Figures 4B and 5B.

CONCLUSIONS AND FUTURE DIRECTIONS

Recent advances in the application of proteins as therapeutics have revealed an urgent need for improved methods for tracking and quantifying proteins and protein modifications in a variety of chemical and biological matrices.^{29,30} Sensitive, specific methods for protein quantitation are essential for determining the biological fates and pharmacokinetic characteristics of protein therapeutics in experimental animals and in humans.³¹ Measurement of protein therapeutics in animals and humans is difficult, as analytical methods such as immunoassays frequently lack sensitivity and are subject to interferences.^{12,30} Analysis is further complicated by the fact that many protein drugs are nearly indistinguishable from proteins that occur naturally in the body.^{12,31}

Detection of a modified protein is difficult using conventional methods, due to the low frequency of occurrence of the modification relative to the total amount of a given protein present (i.e., only a small fraction of the target protein is expected to be modified). In contrast, the approach described here enables sensitive, rapid characterization of minimally modified proteins (in the range of 1 modification for every 10^6 – 10^8 proteins) using a ^{14}C -labeled modifying agent. We envision that application of this technology can be extended to a variety of classes of biological molecules of interest and to HPLC separations of greater complexity.

Our analyses show that LS-AMS can measure submicrogram protein samples containing tens of zeptomoles of ^{14}C with good accuracy and acceptable precision compared to solid carbon AMS analysis, which requires 100 to 1000 times more ^{12}C and 100 times more ^{14}C per sample. We further demonstrate the utility of LC-LS-AMS for characterization of modified peptides by ^{14}C -labeled compounds of interest. Compared to solid carbon AMS, analysis of HPLC separations by LC-LS-AMS provides around 100 times more ^{14}C sensitivity, the capability to measure HPLC-relevant ^{12}C quantities, superior temporal

resolution, greater reliability, results in real-time instead of days, and a 6-fold reduction in AMS instrument time.

The improvements offered by the LS-AMS approach should find ready application to essential tracer experiments in biology and medicine, especially in studies requiring protein or post-translational modification quantitation, in the development of protein-based drugs, and in the broader field of protein functional characterization.

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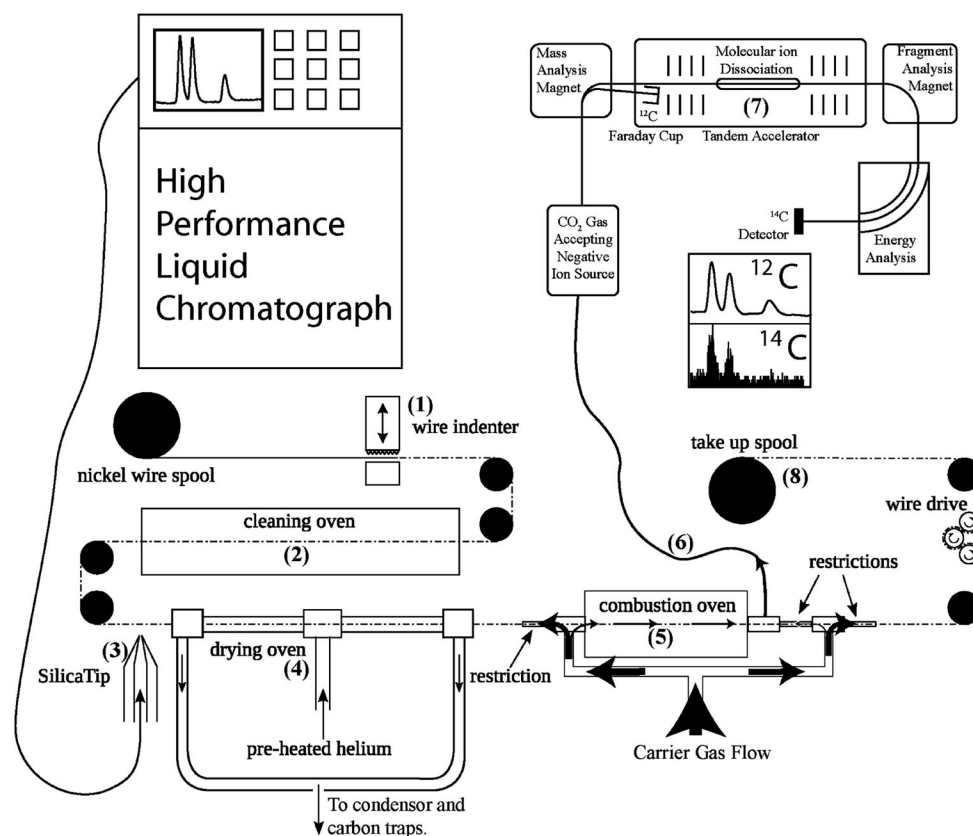


Figure 1. Schematic layout of LS-AMS. (1) Wire indenter generates periodic indentations on the wire. (2) Surface carbon is removed and the wire is oxidized at high temperature. (3) A stream of effluent or single droplet is placed on the wire. (4) Solvent is evaporated at elevated temperature in an atmosphere of helium. (5) Nonvolatile analyte is combusted at high temperature in a helium and oxygen atmosphere. (6) The flow of gas through the combustion oven directs all combustion products to the gas accepting ion source through a narrow i.d. fused silica capillary. (7) The ^{14}C and ^{12}C content of the CO_2 is measured using a gas accepting cesium sputter ion source followed by AMS analysis. (8) Used wire is collected on a separate spool for disposal, preventing cross contamination.

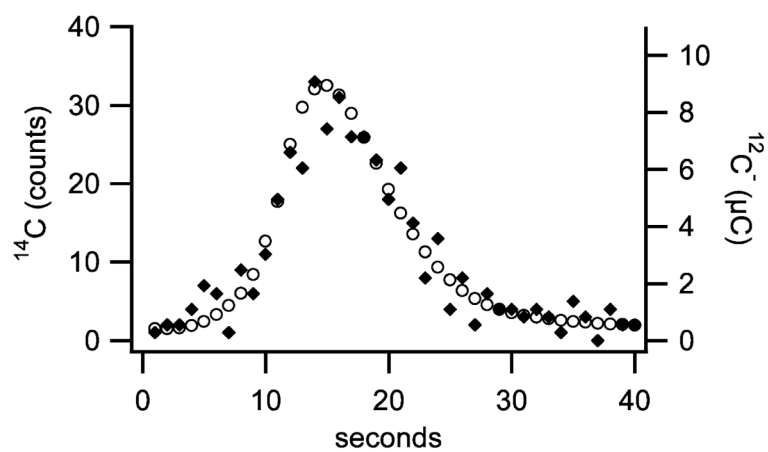


Figure 2.

Typical LS-AMS peak generated from a $1\ \mu\text{L}$ droplet containing $0.7\ \mu\text{g}$ of unlabeled BSA; the symbols \blacklozenge represents ^{14}C and \circ represents ^{12}C , in microCoulombs. Integrated signals from this sample were 94 ± 10 counts ^{14}C and $334.8 \pm 0.1\ \mu\text{C}$ ^{12}C . A comparison to a standard of known isotopic ratio indicated a ^{14}C concentration of $59 \pm 6\ \text{amol } ^{14}\text{C}/\text{mg BSA}$. Precision was limited by the number of ^{14}C counts detected.

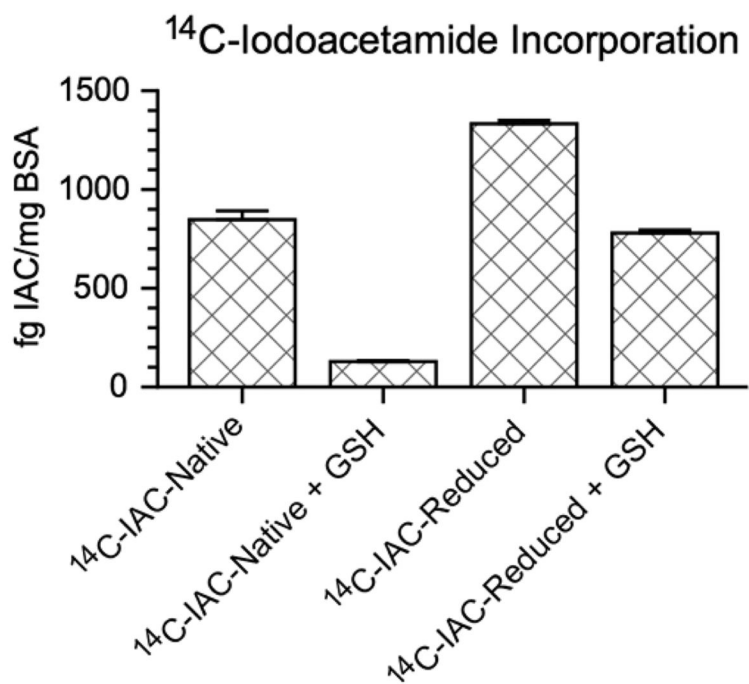


Figure 3.

Absolute quantitation of an albumin ^{14}C -iodoacetamide modification competitive binding experiment. Treated and untreated BSA samples were exposed to 1 pCi ^{14}C -iodoacetamide and described as follows: ^{14}C -iodoacetamide-modified native BSA (^{14}C -IAC-native): native BSA with 1 free Cys residue; ^{14}C -IAC-native + GSH: native BSA and 5 mM GSH; ^{14}C -iodoacetamide-modified reduced BSA (^{14}C -IAC-reduced): disulfide bonds reduced; ^{14}C -IAC-reduced + GSH: disulfide bonds reduced and 5 mM GSH. Results were background subtracted from the control, which showed no excess ^{14}C above the endogenous level expected for a contemporary biological sample. Error bars represent the standard error of the mean of three replicate samples.

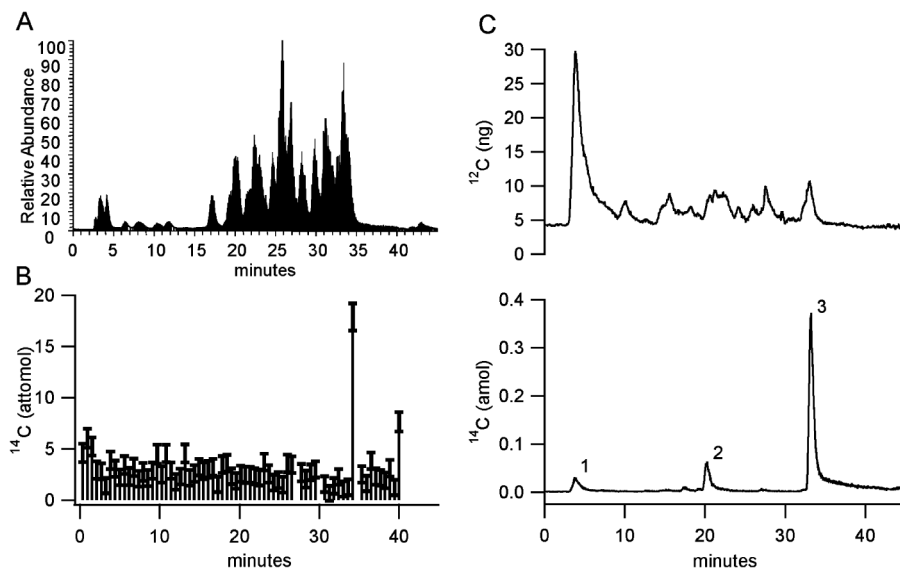


Figure 4. Modification of native bovine serum albumin by ^{14}C -iodoacetamide. (A) MS/MS relative peptide abundance total ion chromatogram. (B) Peptide fraction collection and SS-AMS analysis. (C) LS-AMS ^{12}C trace in units of ng ^{12}C /measurement interval (top) and LS-AMS ^{14}C trace in units of amol ^{14}C /measurement interval (bottom). ^{14}C peak 3 is due to the peptide containing Cys 58, GLVLIAFSQYLQCPFDEHVK. Minor ^{14}C peaks 1 and 2 contain multiple Cys-containing peptides.

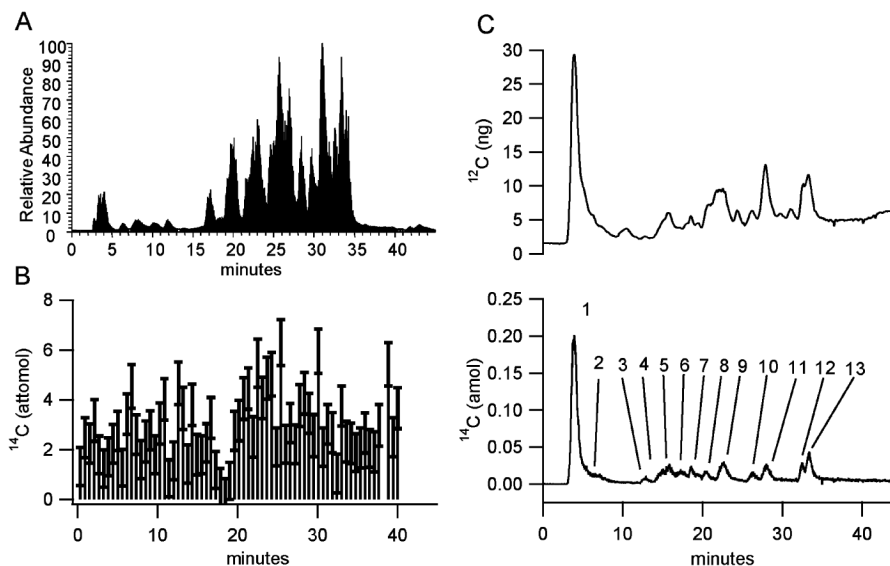


Figure 5.

Modification of reduced bovine serum albumin by ^{14}C -labeled iodoacetamide. (A) MS/MS relative peptide abundance total ion chromatogram. (B) Peptide fraction collection and SS-AMS analysis. (C) ^{12}C (top) and ^{14}C (bottom) traces from LS-AMS. The following numbered ^{14}C peaks were found by MS/MS to correspond to the following Cys containing peptides: 2, ETYGDMADCCEK; 3, TCVADESHAGCEK and QNCDQFEK; 4, ETYGDMADCCEK and SHCIAEVEK; 7, LKPDNTLCDEFK and YNGVFQECCQAEDK; 8, RPCFSALTPDETYVPK and SLHTLFGDELCK; 10, GLVLIAFSQYLQQCPFDEHVK and LFTFHADICTLPDTEK. All other numbered peaks have elution times corresponding to more than two Cys-containing peptides.

Table 1

Comparison of Solid Sample and Liquid Sample AMS

sample	amol ¹⁴ C/mg BSA	
	graphite	LS-AMS
control BSA	57.2 ± 0.8	57.2 ± 1.1
modified native BSA	3074 ± 35	2900 ± 130
modified reduced BSA	2342 ± 21	2313 ± 59