Analytical Methods

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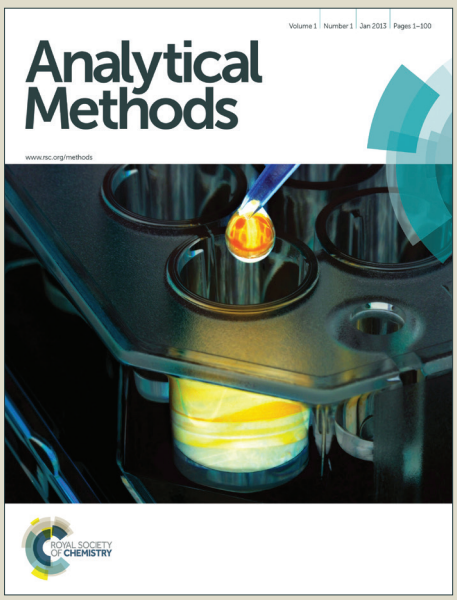

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Quantitation of Thiol Metabolites from Mammalian Cells using Fluorous Tagging and HILIC-MS

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Abstract

Oxidative stress diminishes reduced thiols by decreasing their production or by forming disulfide bonds. This instigates an equilibrium shift in further downstream metabolic pathways. The goal of this work is to develop a rapid and selective fluorous labeling strategy for improved sensitivity in LC-MS analysis of reduced thiols in biological samples. Fluorous tagging is used to augment the MS signal and has limited stationary phase interactions. Thiol standards were reacted with a fluorous maleimide at a ratio of 100:1 in an 80% acetonitrile buffer containing 5mM ammonium formate at pH 8. The reaction time and temperature were optimized. The mixture of thiol metabolites was then separated using a cyano HILIC column with a linear gradient from 90% to 70% acetonitrile. Signal intensity from fluorous-tagged thiols was improved over the untagged thiols by at least four fold. This demonstrates a quick method that can be used to compare levels of reduced thiols in diabetic and normal mammalian endothelial cells. Human tissue was also analyzed using this tagging method. This method will further elucidate the impact of oxidative stress on thiol metabolism and therefore help identify therapeutic targets for the treatment of diabetic complications.

Introduction

Metabolomics is the study of small organic molecules involved in cellular processes.[1] Untargeted or global metabolomics has been used in discovery based analyses to yield novel pathway activation in disease states. Alternately, analyzing a specific group of compounds, called targeted metabolomics allows for hypothesis based investigations of disease progression. These approaches to understanding metabolite changes as they relate to disease pathogenesis hold the promise of uncovering novel pathways through global metabolomics and specific therapeutic targets of these pathways by targeted metabolomics.

Diabetes is a metabolic disease affecting 387 million people worldwide with an expected increase to 592 million by 2035.[2] As a disease, diabetes is characterized by chronic high blood glucose levels, defined as >140 mg/dL fasting blood sugar.[2-3] Along with the high blood glucose levels, vascular and neuronal complications are common. These complications arise from micro as well as macrovascular damage and include high blood pressure, heart disease, stroke, blindness, kidney disease, and neuropathy.

Increases in oxidative stress are associated with diabetes. [3a, 4] Such increases in oxidative stress could provide a mechanism for the vascular damage.[5] One method to measure the oxidative stress is to target the reduced thiols present in the cell. Increases in oxidative stress cause the reduced thiols to be oxidized and form disulfide bonds.[6] Therefore, increases in oxidative stress would correlate with decreased amounts of reduced thiols being present in the cell.

There have been several assays developed to look at levels of reduced thiols. Thiols can be tagged with a fluorescent marker and analyzed by fluorescence detection.[7]

This type of detection gives low limits of detection and when coupled with liquid chromatography provides an easy system for examining thiols. Liquid chromatography-fluorescence detection also provides thiol identification based on retention time. Fluorescent tags are not always thiol specific and occasionally have difficult requirements for reactions to occur. Isobaric tagging coupled with LC/MS has also been used to look at free thiols in biologic samples.[8] This method has provided a picture of the thiols within the cell and coupled with mass spectrometry allows for a way to confirm the identification of thiol metabolites. This method requires reaction times of 12 hours and complicated sample prep that is not ideal for high throughput studies.

Maleimides have been shown to react quickly and selectively with thiol metabolites.[9] Maleimides therefore make for a good tag for thiol metabolites. Tagged thiols can then be separated based either on the tag or on the thiol moiety. Previous work has used a fixed charge to increase MS signal.[10] While this has benefits, ESI-MS signal can also be augmented by increasing hydrophobicity.[11]

This work has used a fluorous maleimide to tag reduced thiol metabolites. Metabolites were then separated based on the thiol using a hydrophilic interaction liquid chromatography (HILIC) column. Metabolites were identified by using targeted MS/MS scans and comparing fragmentation to standards. Reduced thiol levels for cells grown in high and low glucose were compared. This method is also applied to thiol analysis of human tissue.

Materials and Methods

Reagents and materials

Glutathione and ammonium formate were purchased from Alfa Aesar (Ward Hill, MA). Captopril, N-acetyl-L-cysteine, and cysteine were bought from Acros Organic (Geel, Belgium). Homocysteine, penacillamine, Cys-Gly, and Glu-Cys were obtained from Sigma Aldrich (Saint Louis, MO). The fluorous maleimide tag, 3-(perfluorohexyl)propyl-1-maleimide) was from Fluorous Technologies (discontinued, Ambridge, PA). Formic acid was obtained from Fisher Scientific (Pittsburgh, PA). LC-MS grade water and acetonitrile were purchased from Honeywell Burdick and Jackson (Muskegon, MI).

Direct Infusion

- *Reaction of thiol standards with fluorous maleimide tag*

Mixtures containing 25 μM cysteine, homocysteine, penacillamine, Cys-Gly, captopril, Glu-Cys, and glutathione were reacted with 2.5 mM excess fluorous maleimide tag at a ratio of 100:1. Reaction occurred in %70, %80, or %90 acetonitrile/water (v/v) containing 5mM ammonium formate. pH was adjusted to 5.0, 7.0, or 8.0 using formic acid. Both sample and tag were composed of the same sample composition and pH. Reactions were performed at room temperature for one hour. The reaction of the reduced thiols with the fluorous maleimide tag was analyzed by direct infusion ESI-MS.

- *Direct Infusion analysis*

Reaction optimization experiments were run on a Shimadzu LCMS-2010EV single quadrupole liquid chromatography mass spectrometry system. Injections were made using a six port valve outfitted with a 5μL sample loop. The mass spectrometer used an electrospray ionization (ESI) probe. Spray voltage was 2.5kV, nebulizing gas flowed at

1.5L/min, and the temperature was set to 250°C. Flow rate was set to 0.2mL/min and contained 50% acetonitrile/water (v/v) with 0.1% formic acid in the aqueous phase.

Thiol standards were reacted with the fluorous maleimide tag to optimize signal intensity. All tagged thiols were monitored in positive electrospray mode. Selective ion monitoring was used to monitor signal intensities. The software used for instrument control, data acquisition, and data analysis was LCMS Solution (Shimadzu, Version 3).

Reaction kinetics experiments were run on a Finnigan LCQ Advantage Max ion trap mass spectrometer. Thiol standards were mixed with the fluorous maleimide and then immediately directly infused into the mass spectrometer using a Fusion 100 syringe pump from Chemyx (Strafford, TX). Flow from the syringe pump was set at 3μL/min. Spray voltage 4.5kV, and capillary temperature was 250°C.

Thiol standards were prepared containing cysteine, homocysteine, penacillamine, N-acetyl-L-cysteine, Cys-Gly, captoplril, Glu-Cys, and glutathione at a concentration of 25μM each and fluorous maleimide tag at a concentration of 2mM. Upon mixing solution was immediately directly infused into the mass spectrometer and selective ion monitoring was used to monitor signal intensities. Instrument control, data acquisition, and data analysis were done using Xcalibur software (Thermo Scientific, version 2.5 SP2).

Sample preparation

- *Cell culture conditions*

Bovine aortic endothelial cells (BAEC) were purchased from Cell Applications (San Diego, CA) and maintained in Dulbeco's Modified Eagle's Medium (DMEM) containing 10% fetal bovine serum (FBS). Cells were plated in a 5 cm dish and grown

for five days with the media changed daily. For cells grown in high glucose additional glucose was added to the DMEM at a concentration of 30mM.

- Human Tissue

Fresh human muscle was obtained from human cardiac operations. During sternotomy, a small portion of the sternothyroideus muscle is divided to expose the left innominate vein. Muscle was rapidly flash frozen in liquid nitrogen at the operation location. Sample was subsequently lyophilized to dryness to maintain metabolic integrity. Following freeze drying, samples were ground to powder using frozen mortar and pestle. 2 mg of dry muscle was reconstituted in 80:20 ACN:H_2O pH 8 with 2 mM fluorous maleimide tag. Use of human tissue was reviewed and approved by the Institutional Review Board of Saint Louis University.

- *Metabolite extraction*

Cells were extracted as described in the literature with slight modifications.[12] Cells were rinsed by warm PBS. Cells were then quenched by adding 500μL of 80:20 acetonitrile:water (v/v) buffered with 5mM ammonium formate and at pH 8 containing 2mM fluorous maleimide tag and 25μM penacillamine to act as an internal standard. Cells were placed in a dry ice/ethanol bath for ten minutes followed by scraping and sonication (Mixonix XL-2000, Qsonica, CT) with ten 1s bursts. Cell lysates were then centrifuged at 14,000rpm for 5 minutes to remove precipitated protein and cell debris. Supernatants were collected and allowed to sit at room temperature for thirty minutes to ensure complete reaction of thiols with the maleimide. Supernatants were then injected into the mass spectrometer for analysis.

- *Liquid chromatography (LC)/ mass spectrometery (MS)*

LC/MS analyses were performed on an Thermo Scientific LTQ ion trap mass spectrometer (Waltham, MA) in positive mode using an ESI interface coupled with an Agilent 1260 Infinity pump (Palo Alto, CA). The flow rate from the Agilent pump was 0.6mL/min. Separations were done on a Zorbax SB-CN column that was 4.6mm in diameter and 15cm long with a particle size of 5 microns. Mobile phase A was water with 0.1% formic acid and mobile phase B was acetonitrile. Analytes were eluted with a gradient from 90-77% over five minutes.

For detection of analytes, the mass spectrometer was run in positive mode with a spray voltage of 4.5kV, the capillary temperature was set to 275°C, and sheath gas was run at 20 arb. MS/MS scans were performed on the mass of each of the analytes of interest using the normalized collision energy of 35%.

Results and Discussion

Reaction Optimization

The goal of this research was to identify and quantify thiol metabolites from cells/tissue from diabetic and non-diabetic conditions. To do this maleimide with an extended perfluoro-alkyl chain was used as a tag to increase signal intensity by increasing hydrophobicity (log P > 5.0). The maleimide reacted with thiols optimized at pH8 in a 80% acetonitrile solution containing 5mM ammonium formate. (Figure 1)

The addition of the tag increased the hydrophobicity of the analytes and therefore increased the signal observed. Using direct infusion, untagged thiol metabolites were analyzed. Only glutathione, captopril and Glu-Cys were observed. After tagging, signal was observed from all standards and signal from glutathione, captopril and Glu-Cys increased by 4-18 fold. In addition to increasing the hydrophobicity, the tag increased

the total analyte mass by >470 Da. This resulted in an increase to the signal to noise ration by shifting the mass of the analytes to a higher mass, less noisy part of the spectrum.

Reaction kinetics were monitored by directly infusing a mixture containing eight thiols, each present at a concentration of 25μM, into the mass spectrometer. (Figure 2) Immediately before infusion, the thiols were mixed with a 100 fold excess of fluorous maleimide. Masses corresponding to the mass of the thiol plus the mass of the tag were then monitored and plotted vs. time in Figure 2. When signal intensities for these masses had reached and maintained a maximum the reaction was considered to be complete. The slight decrease in signal at 2 minutes is due to an air bubble which resulted from connecting the syringe to the infusion tubing. All eight thiols reacted to completion within 20 minutes. For the remainder of experiments reaction time is 30 minutes to ensure that reactions are complete. These results show a rapid reaction with high signal intensity and low noise for tagged thiol analysis.

Metabolite Separation

Thiol metabolites were separated using HILIC-MS. Because of the hydrophobic nature of the fluorous tag, the analytes separated based on the analyte polarity and not the tag hydrophobicity (Figure 3A). A gradient was run starting at 90% acetonitrile and going to 77% acetonitrile over 5 minutes. This was sufficient to elute the reduced thiols that had been tagged with the fluorous chain. . Reasonable resolution was achieved for all targeted analytes (Figure 3A). Separation of the standards allowed for retention times to be used in conjunction with the m/z for the identification of metabolites from endothelial cells. Glutathione and Glutamyl-cysteine both show small peaks which elute

before the major peaks. This may be a result of differential chromatographic interactions of peptide diasteriomers from the standards or impurities. Both samples contain the Glu-Cys moiety while the other analytes do not, suggesting that this group is the source of the split peaks.

Metabolite Fragmentation and Identification

Targeted MS/MS scans of thiol standards were performed by direct infusion so the fragmentation pattern of the thiols of interest could be used in identifying metabolites found in endothelial samples. Using the ion trap MS, a mixture of thiols and maleimide were directly infused. MS/MS scans for each of the thiols was collected using 35% collision energy.

Figure 4 shows that the thiols exhibit unique fragmentation patterns. This indicates that the fragmentation occurred in the thiol moiety and not in the fluorous tag. Analysis of the fragmentation patterns reveals that the major product ions are the result of cleavage across the amide bond. Minor fragments are the result of ammonia or carboxylic neutral losses.

High versus Low Glucose Cell Experiments

We then undertook investigations into the thiol metabolism of mammalian cells. Previous work has indicated that reduced thiols in the cysteine-glutathione pathway (Figure 3B) are present in endothelial cells that are detectable by MS.[12] Calibration curves were performed on these thiols to determine limits of detection of the system. Calibration curves exhibited excellent linearity through the entire range of concentrations tested, up to 25μM (Table). Limits of detection were all below 10 nM.

Cells were cultured for five days in either high or low glucose. Media was changed daily. At the end of five days cells were lysed in 80% acetonitrile containing the fluorous maleimide tag and 25μM penacillamine to act as an internal standard. Samples were then run on the LC/MS system to determine the levels of reduced thiols that were present in each of the samples.

A plate of high glucose cells and a plate of low glucose cells was used to determine the number of cells in the plate. Counting the cells revealed fewer cells in high glucose by a factor of 0.72. To account for differential cell numbers, the signal intensities were normalized to their cell number (0.72 cell number factor).

Penacillamine was added to the cell lysates as an internal standard. Signals were normalized to the internal standard by dividing the signal obtained from the mass spectrometer for each analyte by the signal obtained for penacillamine. Signals that had been normalized to cell number and to internal standard were then divided by the low glucose signal for each analyte. This gave a value of one for each low glucose analyte. All high glucose signals were less than their low glucose counterparts. (Figure 5)

Cells grown in high glucose media had on average only 30% of the reduced thiol metabolites that the low glucose cells had. Previous studies had shown that the levels of reduced thiols began decreasing after only six hours of exposure to high glucose media.[12] Prolonged exposure to the high glucose caused an even more significant decrease in reduced thiols present.

The decrease in reduced thiols is statistically significant for three of the four thiols investigated with p values ranging from 0.03-0.07. (n=5, Table 1) The change in glutathione trended toward but did not achieve statistical significance. These data show

a dramatic drop in reduced thiol levels consistent with oxidative stress found in other models of diabetic complications.

Analysis of Human Tissue

To investigate the utility of our system in more complex samples, human tissue from cardiac surgery was analyzed (Figure 6A). Figure 6B shows the analysis of fluorous tagged Cys, glutathione, Glu-Cys and homocysteine. Previously in the cell culture samples, homocysteine was not detected. In the dried tissue, Cys-Gly was below our detection limits but homocysteine was above the LOD. Homocysteine, which is implicated in cardiovascular disease was confirmed by matching retention time, m/z and MS/MS spectra to a commercially available standard. This tissue was from a non-diabetic human and shows the feasibility of analyzing complex tissue. Future studies for this tissue will focus on comparing diabetic to non-diabetic samples.

Conclusion

This work shows that the use of a tag is beneficial in identifying and separating thiol metabolites. Retention times, mass, and fragmentation patterns were used to identify metabolites in endothelial cell samples. Cells were grown under different conditions to determine thiol metabolite changes. Cells that were grown under high glucose conditions had significantly fewer thiol metabolites and therefore fewer antioxidants to fight oxidative stress. Compared to shorter experiments the decrease in reduced thiol level is even more drastic. This method was also successfully applied to human tissue. Further studies are needed on downstream metabolic process to determine how this drop in reduced thiols affects other processes.

Table 1. Analytical parameters for biologically relevant thiol standards. For p values for thiol metabolite fold changes found between endothelial cells cultured in 30 mM glucose vs. 5 mM glucose. p values correspond to Figure 5.

Thiol	Limit of Detection (nM)	Sensitivity (Counts-nM^{-1})	R^2	Linear Dynamic Range (μM)	p Value
Cysteine	6	24	0.99988	.018-25	0.03
Cys-Gly	2	29	0.99999	.006-25	0.05
Glu-Cys	1	88	0.99999	.001-25	0.03
Glutathione	8	94	0.99967	.026-25	0.07

Figure Legend

- Figure 1: Reaction of a maleimide with a fluorous chain reacting with a reduced thiol. Reaction occurs at pH8 in 80% acetonitrile.
- Figure 2: A mixture of thiols with each thiol at a concentration of 25uM. 1mM maleimide tag was added and immediately upon adding the tag the mixture was directly infused into the mass spectrometer at a rate of 3μL/min. The masses of the thiols plus the tag were monitored to determine when the reaction had gone to completion by a plateauing of signal intensity. Relative signal intensity for the mass of maleimide tagged analyte is plotted.
- Figure 3: A. Separation of thiol standards using HILIC. Flow: 0.2mL/min Column: Cyano 4.6x150mm 5μm particle Injection: 20μL. Green: Cys-Gly; Red: Penacillamine; Black: Cysteine; Yellow: Glutathione; Blue: Glu-Cys B. Thiol metabolic pathway
- Figure 4: Fragmentation of thiols. A) fragmentation of tagged cysteine B) fragmentation of tagged Gly-Cys C) fragmentation of tagged Glu-Cys D) fragmentation of tagged glutathione.
- Figure 5: Thiol metabolite fold change from endothelial cells after five days in a 30 mM glucose media vs. 5 mM glucose media. * $p<0.05$, error bars are SEM.
- Figure 6: Thiol analysis of human cardiac tissue. A) Human muscle tissue B) HILIC-MS analysis of fluorous tagged thiol metabolites from human cardiac tissue.

Figure 1

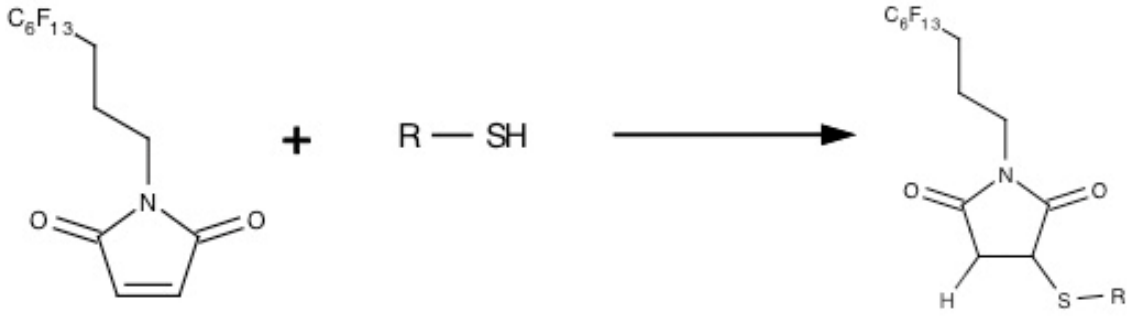


Figure 2

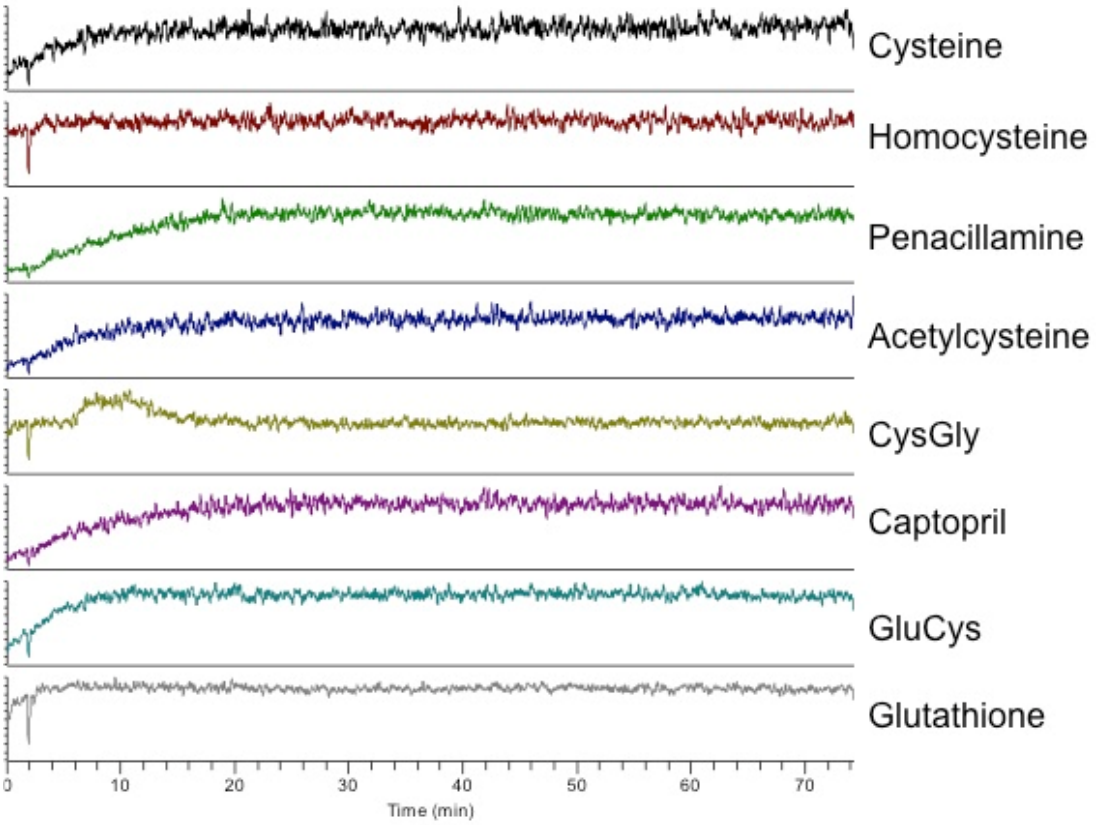
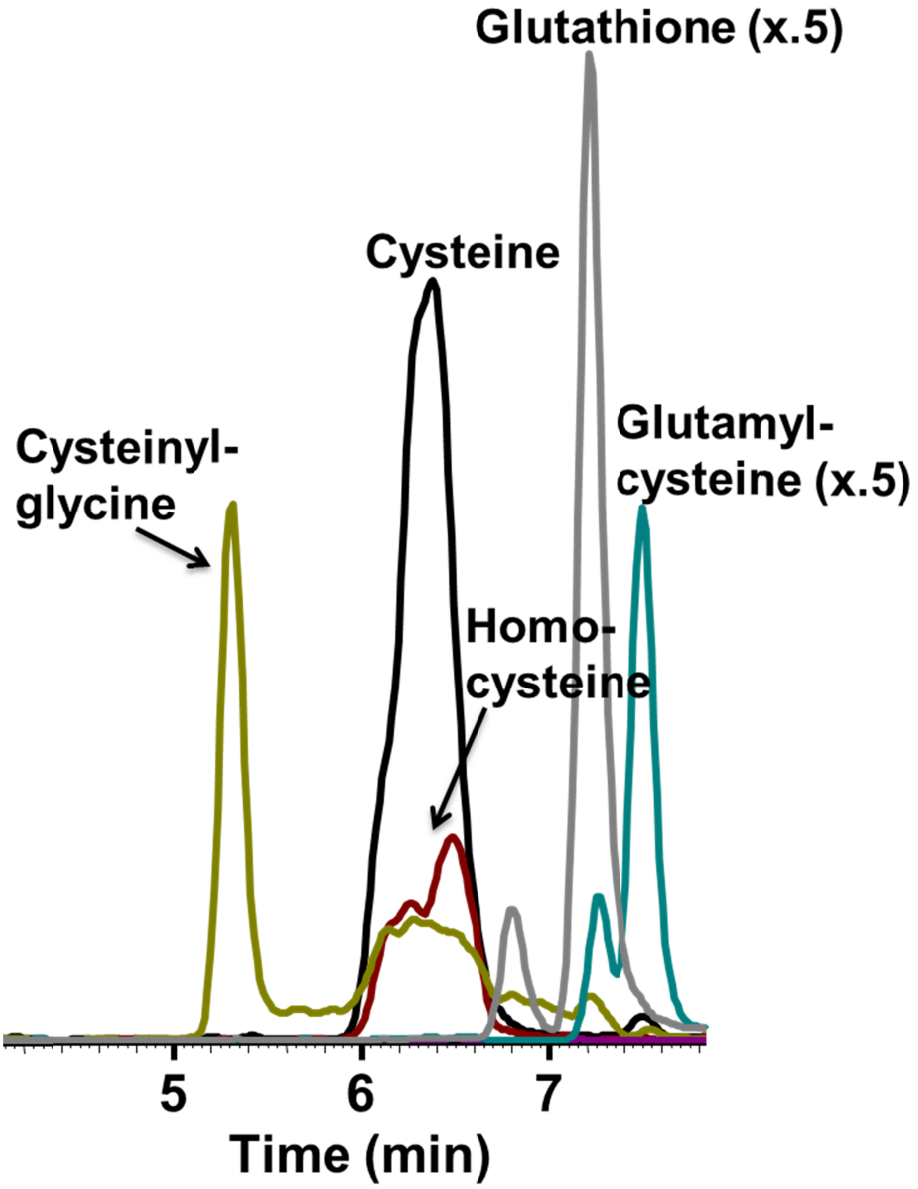


Figure 3

A



B.

Cysteine → GluCys → Glutathione → CysGly

Figure 4

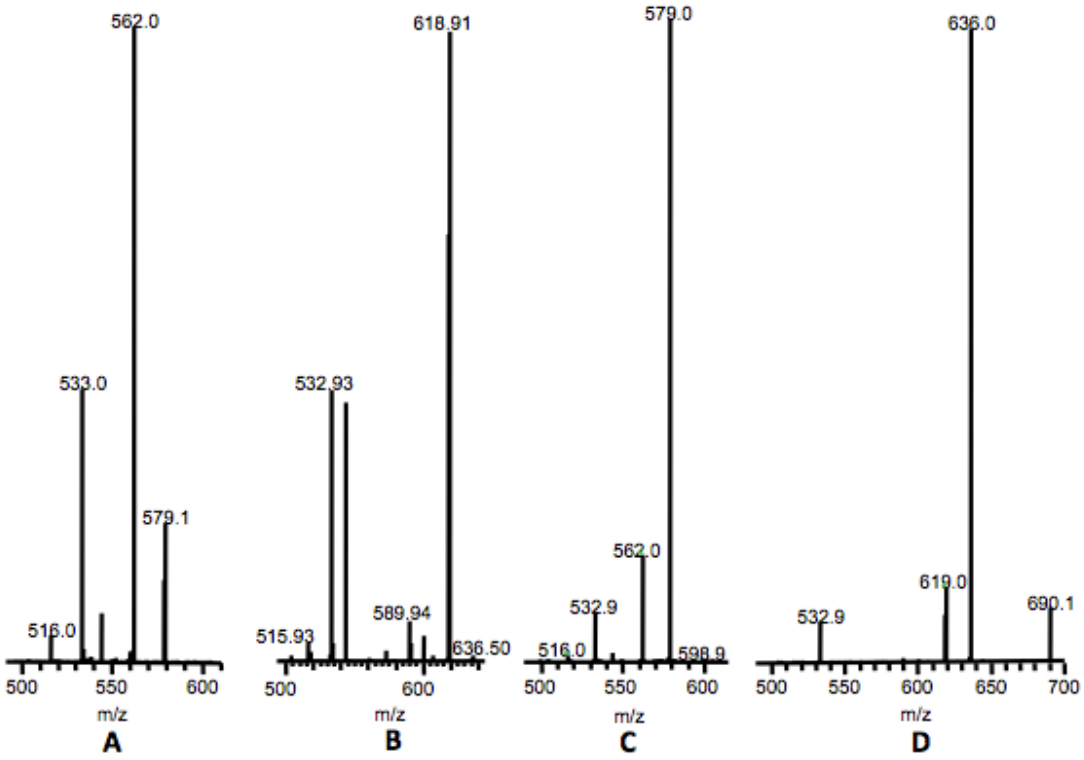


Figure 5

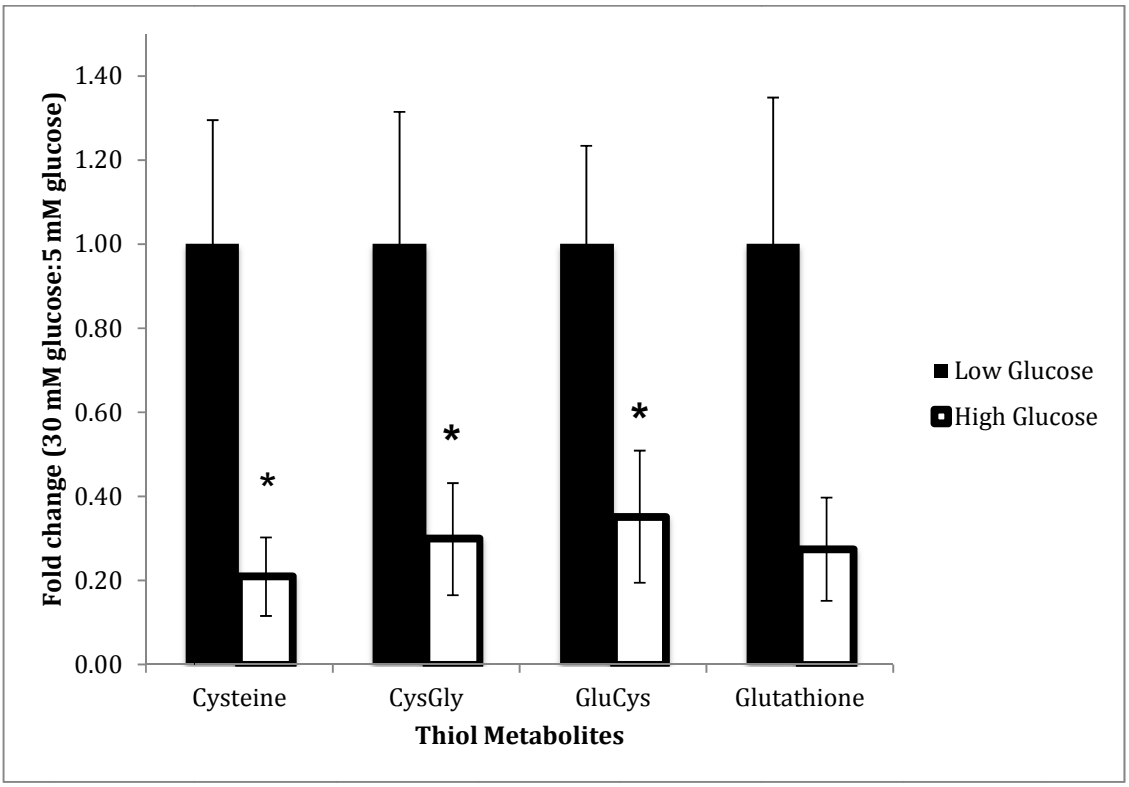
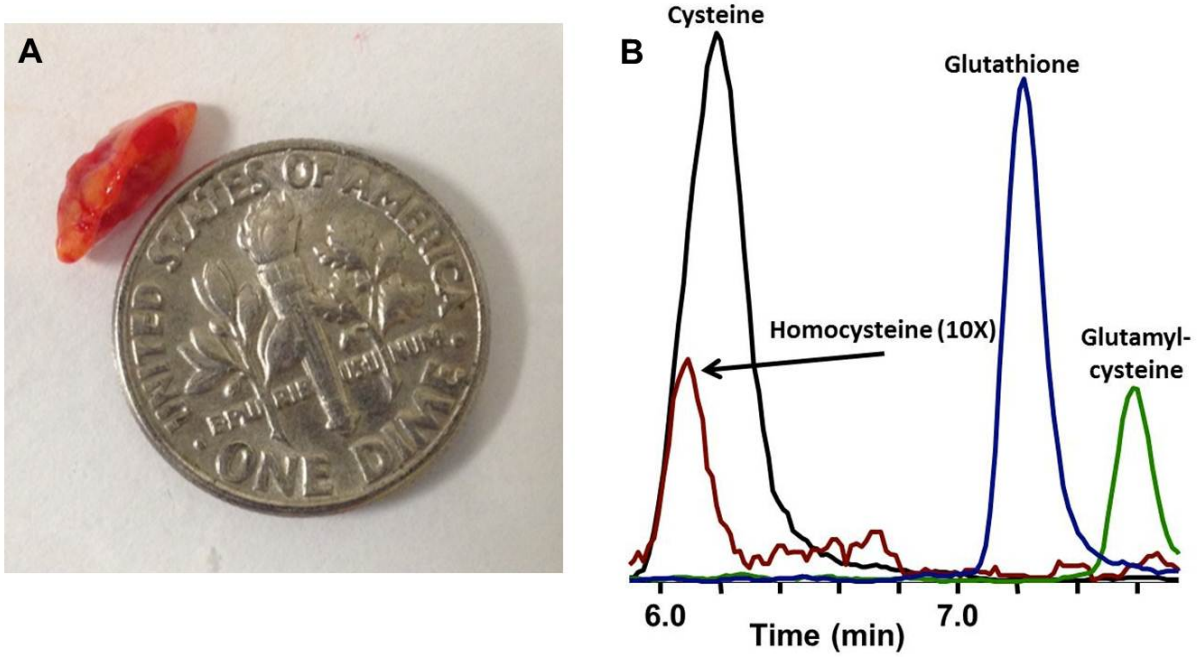


Figure 6



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Supplemental Data

S1 Fragmentation patterns for tagged biological thiols corresponding to Figure 4.