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Thiol metabolomics of endothelial cells using capillary liquid chromatography mass spectrometry with isotope coded affinity tags

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ABSTRACT

Thiol and disulfide levels are critical to maintaining the redox potential of a cell. Perturbations of these levels are important in disease pathogenesis. To improve endogenous mammalian metabolome quantitation, thiol specific tagging, extraction and relative quantitation were undertaken. Reduced and oxidized thiol (disulfide) metabolites from endothelial cells were tagged and extracted using cleavable isotope coded affinity tags (cICAT). Extracted cICAT labeled thiols were analyzed using capillary reverse phase liquid chromatography coupled to mass spectrometry (capLC–MS) with positive mode electrospray ionization. Reactions between thiol metabolite standards and the reactive group of cICAT indicate completion by 8 h at pH 9 with no apparent disulfide formation. cICAT labeled reduced thiols from endothelial cells showed 1–5% RSD using ratiometric quantitation of isotopes and 6–17% RSD based on signal intensity alone. Sample injection was optimized to 16 pmol. Using high mass accuracy MS, 75 putative thiol metabolites were detected in all experimental samples. Treatment of endothelial cells with 2,3-dimethoxy-5-methyl-1,4-benzoquinone (BQ) shows decreased levels in 28 putative reduced thiols and increased levels of 27 putative disulfides. Treatment of endothelial cells with 30 mM glucose resulted in 22 putative reduced thiols with decreased levels and 7 putative disulfides with increased concentration. Thiols were identified based on accurate mass within 3 ppm and analysis of fragmentation patterns. Using higher collision induced dissociation (HCD), shared product ions between different thiols led to the analysis of thiols from the cysteine–glutathione (Cys–GSH) pathway. Specific reduced thiols and disulfides in this pathway revealed changes different from the overall trends of thiols/disulfides. This suggests varying regulation of the Cys–GSH pathway distinct from other thiol-containing pathways and dependence on the type of environmental stimulus. These results indicate the utility of analyzing reduced thiols and disulfides in eukaryotic samples.

1. Introduction

Wide scale analyses of endogenous biological small molecules offer the potential to discover new metabolites, uncover novel pathway connections and reveal therapeutic targets. To accomplish these goals, both targeted and untargeted MS based methods have been developed. Untargeted metabolomic methods using MS offer complex data sets which often suffer from low sensitivity or poor quantitation [1]. When separations are coupled to MS, competing ionization arising from sample complexity and matrix effects are reduced. Taking into account the size, structural heterogeneity, and dynamic range of the metabolome, single dimension separations are insufficient to resolve biological samples [2]. To overcome these deficiencies, semi-targeted approaches have been utilized to extract classes of metabolites based on functional groups [3–5]. Extractions were then analyzed by LC–ESI-MS or MALDI-MS. These methods have proven beneficial, though quantitation is still difficult in MS. Quantitation of metabolites using isotope labeling tags has emerged as an alternate approach to extracting metabolites [6–8]. While isotope ratio methods have achieved relative quantitation by tagging metabolites, sample complexity was not alleviated.

Proteomics has improved quantitation and reduced sample complexity by using various forms of isotope labels combined with affinity tags [9,10]. In particular, cleavable isotope coded affinity tags (cICAT) have been used to label the thiol groups on cysteine residues of peptides and extract these peptides based on biotin–avidin interactions. Further work has shown the ability to quantify protein oxidation states by tagging and comparing disulfide bonds after reduction [11–13]. The ability to extract and quantitate both thiol and disulfide (oxidized thiol) moieties

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indicates that analyzing the entire class of thiol metabolites and oxidation states is feasible.

Changes in the cellular oxidation state are often a direct result of oxidative stress. Oxidative stress is a naturally occurring biological event which results from inefficiencies in the electron transport chain. Electrons react with molecular oxygen to form superoxide which is then metabolized to H_2O_2 [14–16]. These reactive oxygen species then proceed to further damage cellular processes and compromise integrity. To minimize oxidative stress, the reduced form of glutathione (GSH) is produced as a natural oxidative scavenger. Oxidation of GSH forms GSSG. Many disease states such as Alzheimers and Parkinsons disease, aging and diabetes have root causes in oxidative stress [16–19]. In particular, levels of GSH and GSSG are perturbed in diabetes [20–22]. Taken together with known work on oxidative stress, this suggests an important role of the redox potential in the pathogenesis of diabetic complications. Many studies on the effects of oxidative stress have been undertaken to analyze oxidized nucleic acids, proteins and lipids [23]. Little work has been performed on oxidative stress of metabolites with the exception of GSH.

Previous work on the quantitative analysis of thiols has largely focused on metabolite profiling of cysteine (Cys) and GSH in the reduced form. Electrochemistry has quantified GSH in red blood cells [24]. In addition, both the reduced and disulfide forms of Cys and GSH have been analyzed using a dual electrode system [25]. In a parallel method, isotope dilution LC–MS/MS was used to quantify GSH and GSSG from macrophages [26]. Targeted analysis of GSH, Cys and homocysteine in both reduced and disulfide forms were performed using the Ellman derivitization agent with LC–MS, though competing ionization left some targeted compounds undetected [27]. By tagging the thiol moiety with a maleimide-ferrocene reporter, five metabolites were analyzed by neutral loss LC–MS/MS [28]. Though targeting of thiols without extraction has allowed for quantitation of specific biological metabolites, quantitative approaches for a wide range of thiols has not been undertaken [29–32]. Work by Lafaye analyzed sulfur containing metabolites using ^{15}N enriched media. This allowed for quantitation of multiple metabolites, but was limited to prokaryotic organisms which could be completely labeled on an isotope enriched minimal media [33]. Despite these advances in thiol analyses, a system which extracts and quantitates thiols from eukaryotic tissue is absent.

In this work, endogenous thiol metabolites from endothelial cells were tagged with isotope labels, extracted from the metabolome and quantified using capillary LC–orbitrap MS. The use of cICAT diminishes sample complexity and ameliorates MS quantitation limits. In addition, thiols from the Cys–GSH pathway were identified and quantified using MS/MS. These results show that semi-targeted analyses of mammalian thiol metabolites can quantify major portions of a metabolic pathway and thereby reveal biological complexities

2. Experimental

2.1. Reagents and materials

GSH was purchased from Alfa Aesar (Ward Hill, MA). GSSG was from Applichem (Germany). Cys, N-acetyl cysteine (NAC), ammonium bicarbonate, and iodoacetamide were obtained from Acros Organic (Geel, Belgium). Tris (2-carboxyethyl) phosphine (TCEP) was from Thermo Scientific (Waltham, MA). Formic acid was from EMD chemicals (Gibbstown, NJ). 2,3-dimethoxy-5-methyl-1,4-benzoquinone (BQ) was from MP biomedicals (Solon, OH). All reagents for cICAT labeling were from Applied Biosystems (Foster City, CA). Monomeric avidin kit was purchased from Pierce (Rockford, IL). HPLC-grade solvents were from Honeywell Burdick & Jackson (Muskegon, MI). All other reagents were purchased from Fisher Scientific (Pittsburgh, PA).

2.2. Flow injection

2.2.1. Alkylation of thiol standards by iodoacetamide

GSH, Cys, or NAC (10 μM) was incubated with iodoacetamide (330 μM) in 80% MeOH/H_2O (v/v) solution. Preliminary work using capLC–MS estimated GSH levels in our endothelial cell lysate to be in the low-mid μM range. The pH of the reaction was adjusted by 1 M ammonium bicarbonate and 0.5 M ammonium hydroxide to 8.0, 8.4 or 9.0. The reactions were conducted at room temperature for 0, 1, 3, 5, 8 h or overnight in the dark. At each time point, 100 μL of reaction mixture was collected and the reaction was quenched by adding 2 μL at a time of 2 M formic acid to reach pH 2. Measurements of pH were performed with 1 μL of sample and analyzed using pH indicator paper (Fischer Scientific). The reaction of reduced thiols with iodoacetamide was analyzed by flow injection ESI-MS.

2.2.2. Flow injection analysis

For reaction optimization experiments, flow injection analyses were performed using the divert/inject valve of the Finnigan LTQ Orbitrap discovery ion trap mass spectrometer fitted with a 5 μL sample loop and the Agilent 1200 HPLC. The mass spectrometer was equipped with electrospray ionization (ESI) interface. Spray voltage was 5 kV, sheath gas was 25 psi, auxiliary gas was 5 psi, and capillary temperature was 300 °C. Flow rate was set at 0.15 mL/min using 50% methanol/H_2O (v/v) containing 10 mM formic acid (FA).

Thiol standards were reacted with iodoacetamide (IAM) to optimize S/N. GSH and NAC were monitored at negative electrospray mode while Cys was analyzed in positive electrospray mode. Selective reaction monitoring (SRM) was used for quantitation. The instrument control, data acquisition, and data analysis were performed by Xcalibur software (Thermo Electron Corporation, version 2.0.7 SP1).

2.3. Sample preparation

2.3.1. Cell culture conditions

Human aortic endothelial cells (HAEC) were obtained from Lonza (Walkersville, MD, USA) and maintained in endothelial growth medium (EGM) containing 2% fetal bovine serum (FBS), growth factors (human epidermal growth factor; vascular endothelial growth factor; heparin-binding growth factor 2; R3 insulin-like growth factor-1) and 5 mM glucose. EGM was diluted with endothelial basal medium (EBM) at 1:5 ratio to obtain a growth medium with 0.4% FBS (0.4%EGM). Confluent cells of a 6 cm dish were placed in 0.4%EGM overnight. The next day, cells were then incubated with 0.4%EGM and either 5 mM glucose or 30 mM glucose for 2 h [16]. In a separate experiment, after incubating in 0.4%EGM overnight, the confluent cells were incubated with 10 μM 2,3-dimethoxy-5-methyl-1,4-benzoquinone (BQ) in 0.4%EGM containing 5 mM glucose for 2 h.

2.3.2. Metabolite extraction

Metabolites were extracted as described by Bajad et al. with slight modifications [34]. In brief, cells were rinsed by warm PBS and quenched with 500 μL of ice cold 80:20 methanol–water (v/v) containing 100 mM formic acid and 10 μM of d_4-homocysteine as an internal standard and placed in a dry ice/ethanol bath. After resting in dry ice/ethanol bath for 10 min, cells were scraped and lysed by sonicating (Mixonix XL-2000, Qsonica, CT, USA) on ice with 10 1-s bursts at low power. Cell lysates were then centrifuged at

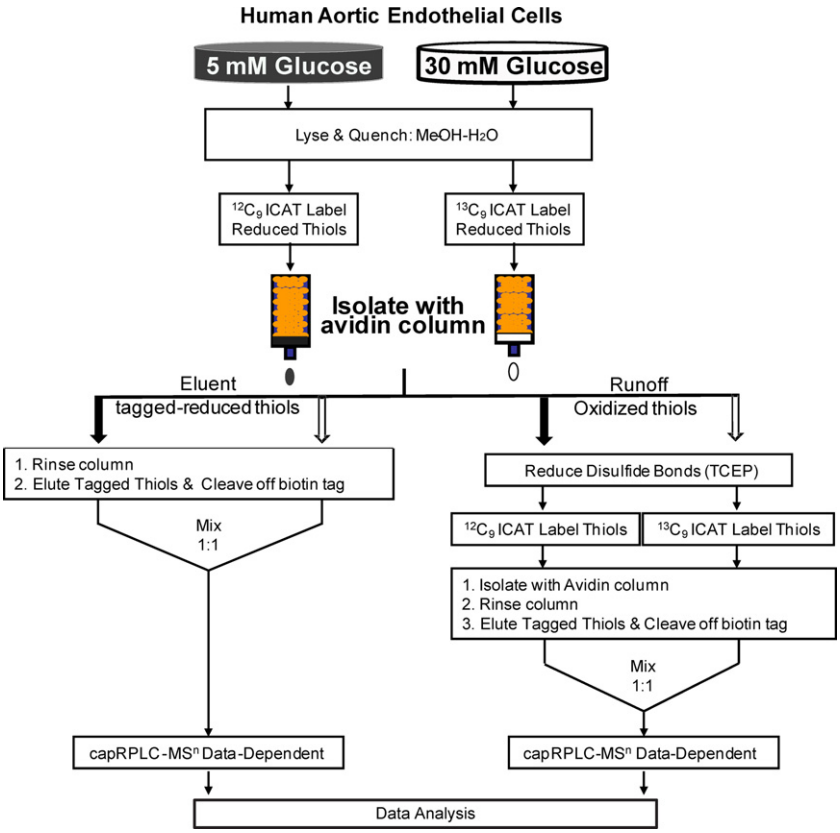

Fig. 1. Schematic of cICAT labeling of thiol and disulfide metabolites in endothelial cells. Overview of experimental workflow for thiol metabolites (reduced and disulfides) cICAT labeling, purification and capLC/MS analysis. Control is 5 mM glucose (dark) and Experimental is 30 mM glucose (light). Colored arrows correspond to either control/dark or experimental/light.

14,000 rpm for 10 min at 4 °C to precipitate proteins and cell debris. Supernatants were collected for cICAT labeling.

2.3.3. *ICAT labeling and labeled metabolite isolation*

The general procedure of cICAT labeling and cICAT labeled metabolites isolation is described in Fig. 1. Briefly, 100 μL of cICAT reagent (1 mM) was added to metabolite lysate immediately after extraction. Ammonium bicarbonate (1 M) and ammoniuim hydroxide (0.5 M) were used to adjust pH to 9 as determined by pH indicator paper. The labeling was conducted at room temperature in the dark for 12 h. After 12 h, the solution was adjusted to pH 4 by formic acid, stopping the reaction. Drying the sample prior to the cICAT tagging resulted in wide intra-sample variability. For this reason, the reaction was conducted immediately after lysis. To eliminate methanol content which diminished biotin–avidin binding, 1 mL of D.I. H_2O was added to the reaction mixture and the volume was reduced to about 0.5 mL by a Savant DNA120 speedvac concentrator at room temperature (Thermo Scientific, Waltham, MA, USA). The volume of each sample was then adjusted to 2 mL using PBS buffer. Tagged thiols were extracted using avidin columns then eluted. Due to the avidin column capacity, 600 μL of the cICAT labeled metabolites (30 nmol) were then isolated by monomeric avidin column (Pierce Scientific, Rockford, IL, USA) according to manufacturer instructions with slight modification. In brief, the pH of the reaction mixture was adjusted to 7.2 before loading on monomeric avidin column. The flow through containing disulfide metabolites was collected for subsequent analysis. After PBS and water washes, the captured cICAT labeled metabolites were eluted from the monomeric avidin column by 30% acetonitrile containing 0.4% TFA. Light and heavy samples were mixed 1:1 (v/v) after affinity purification. The purified cICAT labeled metabolites were then evaporated to dryness by the speedvac concentrator and incubated with cleaving reagents A and B (Applied Biosystems) in a 95:5 ratio (95 μL per sample) per the manufacturer instructions. Cleavage reagents separated the biotin portion of the cICAT from the isotope tagging portion.

The collected flow-through (containing disulfides) was reduced with 5 μL of 10 mM TCEP at pH 8 for 1.5 h at room temperature. After reduction, cICAT labeling and metabolites purification were performed as described above.

2.4. *Fluorescent GSH & GSSG assay*

The glutathione assay kit (#K264-100; Biovision, Mountain View, CA, USA) was used according to manufacturer protocol for GSH and GSSG fluorescence assay. Briefly, HAECs were grown to confluence in a 6 cm dish and treated with glucose or BQ as described in Section 2.3.1. Cells were lysed with 200 μL of assay buffer with 40 μL of perchloric acid (6 M). 100 μL of lysate was neutralized with KOH and centrifuged at 13,000 × *g* for 2 min. 10 μL of OPA probe was added and let react for 1 h. Lysate was transferred to 96 well plate and fluorescence was measured at ex:340 nm and em:420 nm. For GSSG analysis, 100 μL of neutralized lysate was mixed with 10 μL GSH quencher for 10 min followed by 10 μL reduction buffer. Samples were then treated and analyzed the same as GSH.

2.5. *Liquid chromatography (LC)/mass spectrometry (MS)*

LC/MS analyses were performed in the positive ion mode with a Finnigan LTQ Orbitrap discovery ion trap mass spectrometer (San Jose, CA) equipped with nanospray ionization (NSI) interface cou-

pled to an Agilent 1200 HPLC (Palo Alto, CA, USA). The flow rate from the Agilent pump was 0.85 mL/min. A micro cross (Upchurch, Oakharbor, WA, USA) was placed between the Agilent pump and capillary column to reduce the flow rate directed to capillary column at 20–30 nL/min using a 75 μm I.D. fused silica capillary as the flow splitter. Separations were performed on 50 μm I.D. silica capillary (Polymicro Technology, Phoenix, AZ, USA) columns with in-house made frits packed with 3 μm Atlantis T3 C18 aqueous reversed phase particles (Waters, Milford, MA, USA) [35]. In brief, macroporous photopolymerized frits were placed 5 cm from the end of 50 μm I.D. capillaries. Tips were pulled 1 cm downstream of the frit by a P-2000 laser puller. The tips were then etched for 30 s in 49% HF(v/v) and rinsed to create a 2–3 μm opening at the outlet of the column. All columns had ≥20 cm packed bed length. Nanoliter volume samples were injected onto a 50 μm I.D. capRPLC–MS system as previously described [35]. Mobile phase A was 10 mM formic acid in H_2O and mobile phase B was 10 mM formic acid in methanol. Analytes were eluted with a linear gradient of 0–95% solvent B over 30 min.

For the MS, temperature of the heated capillary was reduced to 200 °C for capRPLC–MS. The spray voltage was +1.5 kV. Full scan MS spectra (m/z 150–1200) were acquired in the orbitrap with a resolution of 30,000. When data-dependent acquisition was selected, MS/MS scans were performed on the three most intense peaks in each survey MS scan. Fragmentation was activated by Collision-Induced Dissociation (CID) or Higher Energy Collision Induced Dissociation (HCD). The collision energy for CID was 35% and for HCD was 35%.

2.6. Data processing

Peaks were manually picked by examining pairs of peaks with a mass shift of 9.0302, which is the difference in mass of the two isotopic cICAT labels. The relative abundance of +2 isotopic peaks was verified manually to exclude nonsulfur-containing peaks. Sodium adducts, dimer, repetition due to charges states of same peak and water hydrolysis products from cICAT cleavage were removed. The isotope label of the cICAT tag is cleaved from the biotin portion using acid hydrolysis and yields an amide/terminal amine. A side reaction is water hydrolysis which results in a terminal carboxylic acid. The mass difference of 0.9840 (NH vs. O) and a chromatographic shift of ~0.7 min was not included in our data analysis. Mass Frontier (version 5.0) software was used to generate the fragmentation pattern of cICAT labeled thiols. Peaks were matched against the KEGG-LIGAND (http://www.genome.jp/ligand/), Metacyc (http://metacyc.org) and METLIN (http://masspec.scripps.edu/) databases.

All biological results were from 3 independent experiments. Data were expressed as mean ± SD (standards via flow injection) or mean ± SEM (biological samples via LC–MS) unless otherwise indicated.

3. Results and discussion

3.1. Reaction optimization

The aim of this research was to physically isolate thiol containing analytes from the metabolomic milieu and quantitate their changes in response to environmental stimuli based on relative isotope ratios. To accomplish this aim, cICAT which is common to proteomics research was used as it isolates based on biotin–avidin affinity, allows for relative quantitation based on $^{12}C/^{13}C$ isotope tags and reacts primarily with thiols based on iodo-nucleophilic substitution reactions. To determine the feasibility using cICAT for thiol metabolomics, GSH, Cys and NAC standards were reacted with

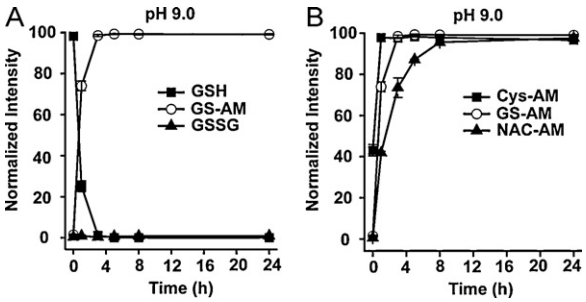

Fig. 2. Reaction of thiols with iodoacetamide using flow injection analysis. Time course of thiols alkylation by iodoacetamide (-AM). The peak areas were determined from reconstituted ion chromatograms of GSH, m/z 306.1 → 272.1; GSSG, m/z 611.2 → 306.1; GS-AM, m/z 363.1 → 272.1; Cys-AM, m/z 179.1 → 161.9; NAC-AM, m/z 219.1 → 89.8. Results are mean ± SD from 3 independent experiments. (A) Time course of GSH reduction and GS-AM formation during alkylation; (B) time course of thiols iodoacetamide products formation.

the functional group of cICAT, iodoacetamide. Using flow injection analysis coupled to ESI-MS, kinetics for this reaction were investigated. Fig. 2A indicates that over 50% of GSH is reacted with IAM (GS–AM) at pH 9 within 1 h and all of the GSH is reacted by 5 h. To ensure that these alkaline reaction conditions did not oxidize thiols, the major oxidized form of GSH, GSSG was also targeted for analysis. GSSG content was below 1% for the duration of the experiments. This level is consistent with the purity of the GSH standard (99%) used. Further analyses indicated that pH 9 yielded the most rapid reaction (Supplementary material S1). Above pH 9, GSSG was being formed and therefore not used.

Though GSH/IAM reacted by 5 h, additional thiol metabolites were investigated to ensure the reaction conditions went to completion for all thiols. IAM reactions of GSH, Cys, and NAC indicate that completion was achieved after 8 h (Fig. 2B), with NAC being the slowest. As IAM is smaller than cICAT and therefore possibly renders faster reaction kinetics, all subsequent experiments used 12 h reaction times to ensure completion of the reaction.

3.2. Reproducibility and loading

To investigate quantitation and loading ability of our capLC–MS system on cICAT labeled thiols, analytical parameters were evaluated. Human aortic endothelial cells were treated for 2 h with control or experimental (BQ or high glucose) as environmental stimuli. Endothelial cells were lysed with MeOH spiked with d_4-homocysteine (10 μM) as an internal standard and reacted with either light ($^{12}C_9$) or heavy ($^{13}C_9$) cICAT (Fig. 1). In brief, the reduced thiols of the control groups were labeled with light cICAT while those of the treatment groups were labeled with heavy cICAT. After TCEP reduction, heavy cICAT was used to label disulfide forms of thiols from the control groups and light cICAT were used for those from treatment groups. The light reduced thiol eluate was split into two samples: (1) analyzed alone and (2) mixed at a ratio of 1:1 with the heavy sample, then analyzed. Light sample analyzed alone yielded 6–17% RSDs for endogenous GSH, Cys and spiked d_4-homocysteine (HCY; Fig. 3A). Samples containing both light and heavy cICAT yielded 1–5%RSD based on ratiometric analysis of peak height. Variance in the isotope ratios was generally related to the signal intensity of the analytes (i.e. higher intensity peaks yielded lower %RSDs). No endogenous homocysteine or NAC were detected in our endothelial cells. cICAT tagging indicates linearity within range of physiological concentrations used in this study (see Supplementary material S3) and has been previously shown to exhibit excellent quantitation at a variety of ratios [36–38].

To optimize detection capabilities of the system, the amount of sample which could be loaded on column without inducing excess

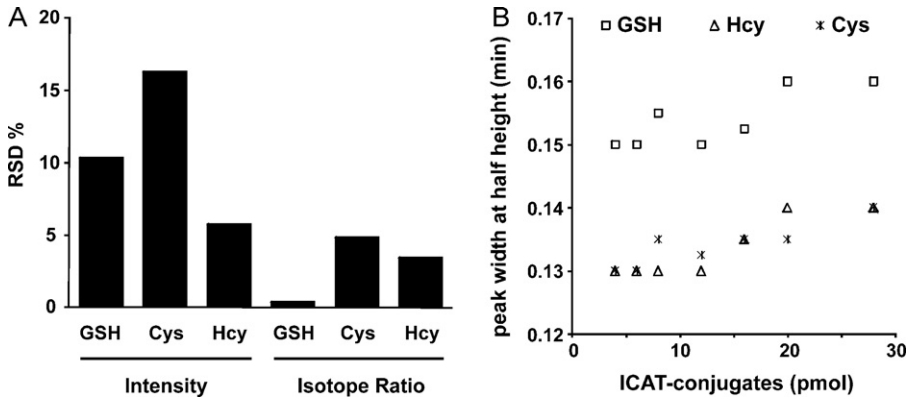

Fig. 3. Variance and loading of cICAT endothelial samples in capLC–MS. cICAT labeling improves repeatability and loading capacity in thiol metabolites quantitation. (A) Comparison of relative standard deviation of peak intensity of cICAT labeled thiols from endothelial cells with their corresponding isotope ratio; (B) plot of peak width at half height of cICAT labeled thiols to the amount of loaded thiols.

band broadening was evaluated. Using peak width as the determining parameter, varying amounts of cICAT tagged sample was injected on column. Sample amount injected was estimated using 30 nmol cICAT in total sample, known volume after reconstitution and flow rate/time of injection. Fig. 3B shows the sample loading plot of tagged GSH, Cys and Hcy. As 20 pmol yielded an increase in peak width, all subsequent injections were performed using 16 pmol.

3.3. Metabolite detection

After determining the reproducibility of our system in mammalian cells, untargeted detection of thiol metabolites from heavy-light mixed endothelial samples was undertaken. Fig. 4A shows a base peak chromatogram of cICAT-labeled samples (top) and the reconstructed ion chromatogram of cICAT tagged GSH (bottom). Triplicate independent biological samples containing both heavy and light cICAT were analyzed via capLC–ESI-Orbitrap-MS. Peaks which were present in all three individual samples were logged for further analysis. Fig. 4B illustrates the resconstructed ion chromatogram of GS-ICAT and Fig. 4C shows the mass spectrum of GSH tagged ICAT. Two isotope based criteria were used to distinguish thiol containing metabolites from non-thiol based signal: (1) appearance of a +9.0303 Da signal which shared identical retention time with the origin mass and (2) the presence of a +2 isotope with a relative intensity ≥4% of the base peak. The use of high resolution (R_s = 30,000)/accurate mass (3 ppm) MS allows for such conditions to be implemented. The first condition indicates that the compound was a nucleophile which was tagged in both samples. The second condition indicated that the compound contained a sulfur atom.

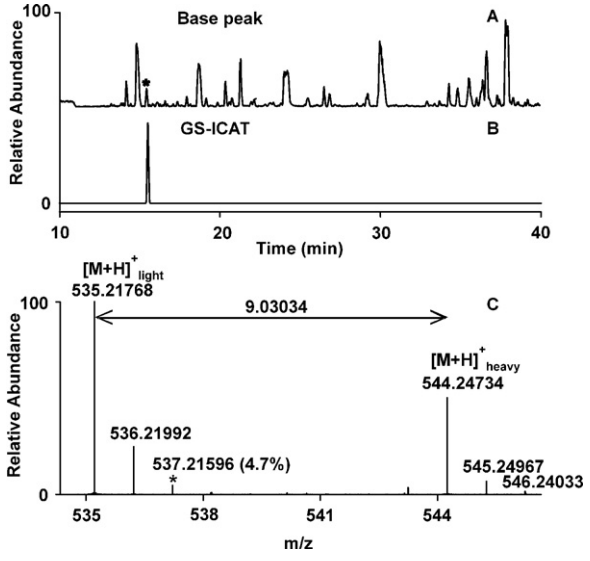

Fig. 4. cICAT labeling of thiol metabolites from endothelial cells using capLC–MS. CapLC/MS analysis of cICAT labeled reduced thiol metabolites extracted from BQ treated HAEC cells. (A) full mass base peak ion chromatogram (*m/z* 300–1200); (B) reconstructed ion chromatogram of cICAT labeled GSH (heavy and light), *m/z* 535.22 and *m/z* 544.25; (C) high resolution mass spectrum of cICAT labeled GSH pairs, 30,000 resolution.

Of the 127 peaks originally detected, the use of the first criteria (+9 isotope) reduced the number down to 83. Using the second criteria (+2 isotope at 5% intensity), 75 tagged thiol metabolites were detected in endothelial cells (Supplementary Table). Amine compounds have the possibility of reacting with iodoacetamide and act as interferents [39]. While our data processing would exclude most amines based on criteria #2, thiol-containing compounds with an amine group would not be excluded. The most likely compounds to act as interferents, taurine and methionine were searched for and not detected. The identity of the eight compounds which passed the first criteria but not the second were not identified though they were not the abundant amines lysine and arginine as would be expected.

Following detection of reduced thiol metabolites, disulfides were analyzed in a similar manner. This system allows for detection by a reduction step of disulfides to free thiols followed by cICAT tagging, extraction and analysis (Fig. 1). Fig. 5 shows a relative quantitation plot of both reduced thiols and their disulfide counterparts relative to control. Detection of disulfides is to be interpreted as disulfides in their reduced form. This is to say that disulfides are analyzed as RSH (e.g. GSH) and can originate from either heterologous (e.g. GSSR) or homologous (e.g. GSSG) species. As a response to oxidative stress, reduced thiols may form disulfides (major product), sulfenic/sulfinic acids or react with electrophiles.

Endothelial cells were treated with two environmental stimuli to elicit distinct changes in thiols levels. Fig. 5 shows changes in reduced thiol and disulfide levels after administration of BQ and high glucose in endothelial cells (corresponding Table in Supplementary material). Fig. 5A shows the effects of BQ treatment on endothelial cells. Some disulfides could not be detected, while their thiol counterparts could. In general, ratios of reduced thiols to their disulfide counterpart are ~30:1 for endothelial cells [40]. This order of magnitude difference in concentration is likely the reason some oxidized thiols were not detected. The cutoff in evaluating thiol changes was set at ±30% to simplify the data sets and ensure that changes are non-transient. Examination of the reduced thiols indicate that 28 (37% of total detected) were decreased by at least 30%

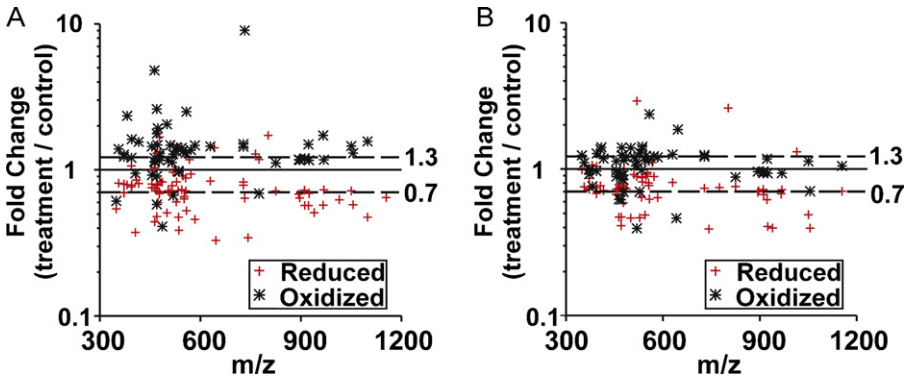

Fig. 5. Changes in Thiol and Disulfide Metabolites from Environmental Stimuli from capLC–MS. Overall fold changes of cICAT labeled thiol metabolites (reduced and disulfides) in 10 μM BQ or 30 mM glucose treated HAEC cells versus control groups. Horizontal lines indicate 0.3-fold higher (1.3) or lower (0.7) of thiol metabolites in treatment groups with respect to controls. (A) HAEC cells treated with 10 μM BQ versus control group; (B) HAEC cells treated with 30 mM glucose versus 5 mM glucose-treated group.

and four were elevated by ≥30%. The large number of thiols which decreased in concentration was expected, as BQ is known to reduce GSH levels [41]. This is believed to result from the addition reaction between BQ and GSH (see Supplementary material S2), similar to the acetaminophen metabolite–GSH reaction [42]. In addition, BQ is known to induce oxidative stress which would increase the number of disulfides. The number of disulfide metabolites which decreased by ≤30% upon BQ administration was five and the number of disulfides which increased concentration by ≥30% was 27 (36% of total detected).

Short term high glucose administration has been shown to increase oxidative stress in endothelial and neuronal cells as a model of diabetic complications. Fig. 5B shows the results of high glucose administration on reduced thiols and disulfides. Examination of reduced thiols showed three metabolites (4% of total) with an increased levels of ≥30% and 22 metabolites (29% of total) which decreased by at least 30%. Examination of disulfides showed that seven metabolites increased concentration by at least 30% and six which decreased concentration by at least 30%. Examination of the literature shows that certain thiol levels in fact remain unchanged during short term hyperglycemia induced oxidative stress, as discussed below [41].

3.4. Fragmentation and identification

After the detection and relative quantitation of thiols, identification was undertaken using high mass accuracy MS and fragmentation analysis. Despite the use of high mass accuracy (3 ppm), many thiol metabolites did not yield a unique molecular formula. This led to an identification approach which utilized fragmentation patterns. GSH and Cys are high abundance endogenous thiols whose exact masses were detected at 13.3 and 15.5 min. Upon low energy CID of these molecules, similar fragmentation patterns were found. This was expected as Cys is a component of GSH. Fragmentation was also performed under higher energy HCD showing a base peak of 269 *m*/*z* (Fig. 6). CID and HCD provided distinct but complimentary fragments for the tri-peptide GSH. CID yielded high intensity product ions through the cleavage in either of the two amide bonds of GSH (Fig. 6, *m*/*z* 460.2 and *m*/*z* 406.2). HCD resulted in higher intensity fragmentation of *m*/*z* 269.1, 286.1 and 314.1 through cleavage of the Cys–Gly amide bond and production of the z-ion from the Glu–Cys portion of GSH (Fig. 6).

The presence of 269 *m*/*z* as the base peak of both GSH and Cys, suggested the possibility that metabolites within the same pathway may contain similar fragmentation patterns. Based on the idea of the "similarity search" in XCMS^2 fragmentation patterns of homologous molecules were anticipated to produce similar fragmentation patterns [43]. In this case, thiol metabolites within the same pathway were expected to yield similar product ions. After subjecting our samples to data-dependent MS/MS, the MS/MS data was searched for 269 *m*/*z*. Four metabolites (GSH, Cys, glutamyl–cysteine and cystine–glycine) containing the 269 fragment also matched the Metacyc and KEGG-LIGAND databases within 3 ppm. These compounds are all thiol containing members of the Cys–GSH pathway (Fig. 7A). In addition to the four thiol compounds this pathway also contains thiocystine and sulfanyl-glutathione. Thiocystine and sulfanyl-glutathione are identified based on accurate mass (3 ppm) and fragmentation patterns as determined by Mass Frontiers. Thiocystine and sulfanyl-glutathione did not yield the 269 *m*/*z* product ion, likely due to the terminal reactive thiol also being part of a disulfide bond.

Further confirmation was achieved matching exact mass of neutral losses. GSH is well known to yield a neutral loss of pyroglutamic acid with a mass loss of 129.042 Da [44]. GSH, sulfanyl-GSH and glutamyl–cysteine all yielded a loss of 129.042 confirming identity based on exact mass, neutral loss and fragmentation pattern.

Many thiol metabolites were not identified based on matching accurate mass to the databases. Using KEGG, Metlin and Metacyc, only the thiols from the Cys–GSH pathway were identified. Metlin database searching results indicated that only two of the 75 thiol containing metabolites (*m*/*z*: 551.212 at T_r 13.8 and 14.0 min) matched the mass of a cysteine containing peptide. This diminishes the possibility that the unidentified metabolites are small peptides.

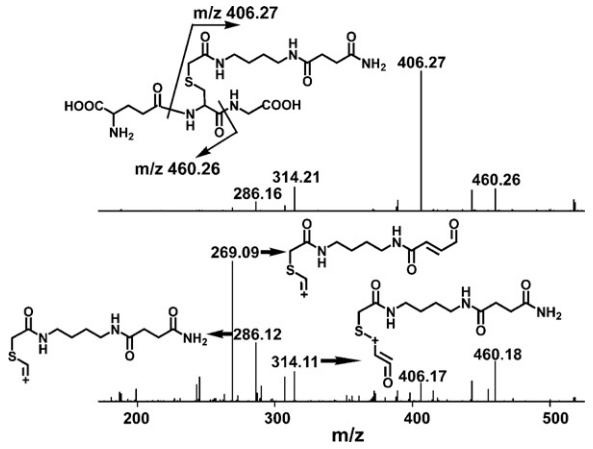

Fig. 6. Fragmentation pattern of thiol metabolite. MS/MS analysis of ^{12}C-cICAT labeled GSH by CID (top) and HCD (bottom). Structure of cICAT labeled GSH is found on top. Fragments and their corresponding structures are found on the bottom.

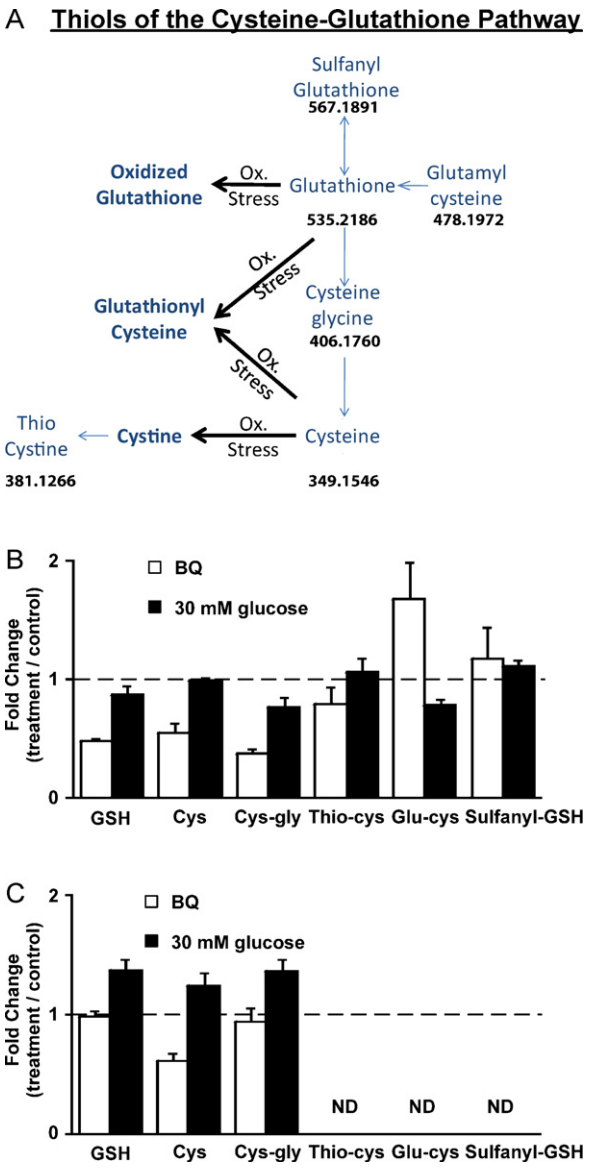

Fig. 7. Effects of stimuli on thiols of the cysteine–glutathione pathway. (A) Thiol metabolites of the cysteine–glutathione pathway. Number next to each compound is the exact ^{12}C-cICAT tagged mass of the compound; (B) fold change of reduced thiol metabolites identified in cysteine–glutathione pathway in 10 μM BQ or 30 mM glucose treated HAEC cells versus control groups; (C) fold change of disulfide metabolites identified in cysteine–glutathione pathway in 10 μM BQ or 30 mM glucose treated HAEC cells versus control groups. Results are mean ± SEM from 3 independent biological experiments. ND: not detected.

Closer examination of the databases and our identifications showed that there are a total of 16 human metabolites (not drug or peptide) with a thiol moiety which could be expected to be detected from cICAT LC–MS system. Six of these thiols were detected and all found in the Cys–GSH pathway. Four of the undetected thiols (molybdopterin; molybdopterin AMP; dihydrolipoate; S-acetyldihydrolipoamide) are found primarily in the liver and not in endothelial cells. Cysteamine and mercapto-pyruvate were not detected. Their presence is possible but not reported in HAECs. The absence of CoA and its pantetheine precursors was unexpected as CoA has been detected in capRPLC–MS from HAEC lysate (data not shown). The absence of CoA is likely due to slow reaction kinetics with cICAT from the higher pKa of the thiol moiety (10.4) [45] or from the added hydrophobicity of the cICAT tag resulting in poorly eluted analyte. In spite of these absences, the vast majority of putative thiols detected were not identified from the databases. The lack of database matching indicates a need for structure determination methods beyond accurate mass LC–MS to identify novel compounds and further expand the databases.

3.5. Pathway analysis

Thiols from the Cys–GSH pathway were examined for changes that result from BQ or high glucose administration. The cICAT labeling approach allowed for detection of all thiol metabolites in the Cys–GSH pathway except homocysteine which is toxic to endothelial cells [46] and therefore likely present at extremely low levels. To validate the cICAT tagging approach, lysates from HAECs treated with both BQ and high glucose were analyzed for GSH and GSSG using standard fluorescence assays. Results between cICAT and fluorescence measurements showed good agreements with the same changes in response to external stimuli. The difference between fluorescence and cICAT were not statistically significant (Supplementary Table S1). Levels of reduced thiols of the Cys–GSH pathway were generally lower in BQ treatments, except for glutamyl–cysteine (Fig. 7B). The lower concentrations are likely due to a combination of the BQ-thiol reaction and oxidative stress. The elevation of glutamyl–cysteine which is the precursor to GSH formation, may be a response to GSH depletion to initiate GSH biosynthesis. Treatment of high glucose on endothelial cells had little effect on the level of reduced thiols in the Cys–GSH pathway, though 30% of all reduced thiol detected were decreased. The stability of thiols in response to hyperglycemia induced oxidative stress is previously documented [41].

Examination of the disulfides in the Cys–GSH pathways revealed differences between BQ and high glucose treatments. Fig. 7C shows levels of GSH, Cys and cysteine–glycine in their oxidized form. BQ treatments show no change in oxidized GSH or cys–glycine levels and a reduction in oxidized Cys levels. As many disulfides increased in response to BQ treatment and the three members of the Cys–GSH pathway were found to exhibit either non-change or a decrease in concentration, the possibility exists that tight cellular regulation is responsible. Because the overall levels of disulfides were elevated in BQ treatment, the stability of oxidized GSH and Cys–Gly suggests increased cycling of RSSR back to RSH. After hyperglycemic treatment, GSH, Cys and Cys–Gly all showed elevation in their disulfide forms. This is expected due to the elevation in oxidative stress that results from high glucose treatments in endothelial cells. Taken together with the reduced thiol data, an interesting phenomenon is observed: an elevation in oxidized thiols (disulfides) and no apparent change in reduced thiols. One possibility for this phenomenon is that either biosynthesis of GSH is up-regulated or that GSH is released from bound proteins. The absence of this phenomenon in BQ treatment suggests considerable differences in cellular regulation of the Cys–GSH pathway in response to various environmental stimuli.

Close examination of the Cys–GSH pathway shows trends different from that of the general thiol/disulfide populations. For BQ treatment, the general trend was elevated disulfide levels, but for the Cys–GSH pathway, levels were stable or decreased levels. For high glucose treatment, reduced thiols showed a general trend in decreasing concentration. In the Cys–GSH pathway, thiol levels were maintained. This suggests that thiols and disulfides in the Cys–GSH pathway are tightly regulated such that their changes are not necessarily indicative of the rest of the thiol/disulfide metabolome.

4. Conclusion

This work shows the utility of cICAT and capLC–MS to isolate and quantitate thiol metabolites in eukaryotic samples. By incor-

porating a reduction step after the initial reduced thiol capture and repeating the cICAT tagging, disulfides were also quantified. Reduced and oxidized thiols were quantified based on isotope ratios. Treatment of endothelial cells with pharmaceutical (BQ) and environmental (hyperglycemia) stimuli led to modulations in thiol and disulfide levels. Identifications based on accurate mass and/or database matching resulted in limited matches. This suggests the need for extended databases and novel approaches to dissect the identity of unknown compounds. As more work is undertaken to identify novel metabolites and thereby expand the databases, the probability of identifying metabolites will grow. Work is currently underway to identify such metabolites. Fragmentation patterns led to grouping of compounds based on similar product ions. From this, all thiol and disulfide containing metabolites from the Cys-GSH pathway were quantified in response to external stimuli. cICAT tagging of thiol/disulfide metabolites proves to be an advantageous approach to understanding biochemical changes in eukaryotic cells.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.chroma.2011.02.063.

References

[1] J.L. Edwards, R.T. Kennedy, Anal. Chem. 77 (2005) 2201.
[2] W.E. Yuan J.L., Bioanalysis 2 (2010) 953.
[3] E.E. Carlson, B.F. Cravatt, J. Am. Chem. Soc. 129 (2007) 15780.
[4] E.E. Carlson, B.F. Cravatt, Nat. Methods 4 (2007) 429.
[5] E.P. Go, W. Uritboonthai, J.V. Apon, S.A. Trauger, A. Nordstrom, G. O'Maille, S.M. Brittain, E.C. Peters, G. Siuzdak, J. Proteome Res. 6 (2007) 1492.
[6] K. Guo, C. Ji, L. Li, Anal. Chem. 79 (2007) 8631.
[7] S.M. Lamos, M.R. Shortreed, B.L. Frey, P.J. Belshaw, L.M. Smith, Anal. Chem. 79 (2007) 5143.
[8] M.R. Shortreed, S.M. Lamos, B.L. Frey, M.F. Phillips, M. Patel, P.J. Belshaw, L.M. Smith, Anal. Chem. 78 (2006) 6398.
[9] J.H. Kim, M. Sedlak, Q. Gao, C.P. Riley, F.E. Regnier, J. Adamec, Omics 14 (2010) 689.
[10] E.C. Yi, X.J. Li, K. Cooke, H. Lee, B. Raught, A. Page, V. Aneliunas, P. Hieter, D.R. Goodlett, R. Aebersold, Proteomics 5 (2005) 380.
[11] P. Hagglund, J. Bunkenborg, K. Maeda, B. Svensson, J. Proteome Res. 7 (2008) 5270.
[12] L.I. Leichert, F. Gehrke, H.V. Gudiseva, T. Blackwell, M. Ilbert, A.K. Walker, J.R. Strahler, P.C. Andrews, U. Jakob, Proc. Natl. Acad. Sci. U. S. A. 105 (2008) 8197.
[13] M. Sethuraman, M.E. McComb, T. Heibeck, C.E. Costello, R.A. Cohen, Mol. Cell. Proteomics 3 (2004) 273.
[14] J.L. Edwards, A.M. Vincent, H.T. Cheng, E.L. Feldman, Pharmacol. Ther. 120 (2008) 1.
[15] G.M. Leinninger, J.L. Edwards, M.J. Lipshaw, E.L. Feldman, Nat. Clin. Pract. Neurol. 2 (2006) 620.
[16] T. Nishikawa, D. Edelstein, X.L. Du, S. Yamagishi, T. Matsumura, Y. Kaneda, M.A. Yorek, D. Beebe, P.J. Oates, H.P. Hammes, I. Giardino, M. Brownlee, Nature 404 (2000) 787.
[17] A. Gella, N. Durany, Cell Adhes. Migrat. 3 (2009) 88.
[18] C. Henchcliffe, M.F. Beal, Nat. Clin. Pract. Neurol. 4 (2008) 600.
[19] V.I. Perez, R. Buffenstein, V. Masamsetti, S. Leonard, A.B. Salmon, J. Mele, B. Andziak, T. Yang, Y. Edrey, B. Friguet, W. Ward, A. Richardson, A. Chaudhuri, Proc. Natl. Acad. Sci. U. S. A. 106 (2009) 3059.
[20] P.S. Samiec, C. Drews-Botsch, E.W. Flagg, J.C. Kurtz, P. Sternberg Jr., R.L. Reed, D.P. Jones, Free Radic. Biol. Med. 24 (1998) 699.
[21] K. Winiarska, J. Drozak, M. Wegrzynowicz, T. Fraczyk, J. Bryla, Mol. Cell. Biochem. 261 (2004) 91.
[22] S.A. Wohaieb, D.V. Godin, Diabetes 36 (1987) 1014.
[23] A.C. Maritim, R.A. Sanders, J.B. Watkins 3rd, J. Biochem. Mol. Toxicol. 17 (2003) 24.
[24] N.G. Batz, R.S. Martin, Analyst 134 (2009) 372.
[25] M. Zhong, S.M. Lunte, Anal. Chem. 71 (1999) 251.
[26] P. Zhu, T. Oe, I.A. Blair, Rapid Commun. Mass Spectrom. 22 (2008) 432.
[27] X. Guan, B. Hoffman, C. Dwivedi, D.P. Matthees, J. Pharm. Biomed. Anal. 31 (2003) 251.
[28] B. Seiwert, U. Karst, Anal. Chem. 79 (2007) 7131.
[29] J.M. Johnson, F.H. Strobel, M. Reed, J. Pohl, D.P. Jones, Clin. Chim. Acta 396 (2008) 43.
[30] A.F. Loughlin, G.L. Skiles, D.W. Alberts, W.H. Schaefer, J. Pharm. Biomed. Anal. 26 (2001) 131.
[31] M.E. McMenamin, J. Himmelfarb, T.D. Nolin, J. Chromatogr. B: Anal. Technol. Biomed. Life Sci. 877 (2009) 3274.
[32] J.H. Suh, R. Kim, B. Yavuz, D. Lee, A. Lal, B.N. Ames, M.K. Shigenaga, J. Chromatogr. B: Anal. Technol. Biomed. Life Sci. 877 (2009) 3418.
[33] A. Lafaye, J. Labarre, J.C. Tabet, E. Ezan, C. Junot, Anal. Chem. 77 (2005) 2026.
[34] S.U. Bajad, W. Lu, E.H. Kimball, J. Yuan, C. Peterson, J.D. Rabinowitz, J. Chromatogr. A 1125 (2006) 76.
[35] J.L. Edwards, R.L. Edwards, K.R. Reid, R.T. Kennedy, J. Chromatogr. A 1172 (2007) 127.
[36] S. Li, D. Zeng, Chem. Commun. (Camb.) (2007) 2181.
[37] F. Turecek, J. Mass Spectrom. 37 (2002) 1.
[38] W. Yu, J. Liu, C. Colangelo, E. Gulcicek, H. Zhao, Comput. Biol. Chem. 31 (2007) 215.
[39] Z. Yang, A.B. Attygalle, J. Mass Spectrom. 42 (2007) 233.
[40] C.G. Kevil, H. Pruitt, T.J. Kavanagh, J. Wilkerson, F. Farin, D. Moellering, V.M. Darley-Usmar, D.C. Bullard, R.P. Patel, Faseb J. 18 (2004) 1321.
[41] C. Quijano, L. Castro, G. Peluffo, V. Valez, R. Radi, Am. J. Physiol. Heart Circ. Physiol. 293 (2007) H3404.
[42] A.B. Reid, R.C. Kurten, S.S. McCullough, R.W. Brock, J.A. Hinson, J. Pharmacol. Exp. Ther. 312 (2005) 509.
[43] H.P. Benton, D.M. Wong, S.A. Trauger, G. Siuzdak, Anal. Chem. 80 (2008) 6382.
[44] Z. Yan, G.W. Caldwell, N. Maher, Anal. Chem. 80 (2008) 6410.
[45] I.H. Pitman, I.J. Morris, Aust. J. Chem. 33 (1980) 1625.
[46] J.C. Chambers, O.A. Obeid, J.S. Kooner, Arterioscler. Thromb. Vasc. Biol. 19 (1999) 2922.