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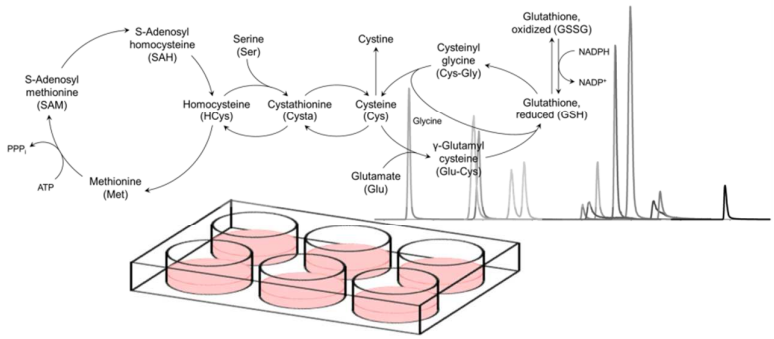
Graphical and Textual Abstract

The developed integrated thiol protection and sample preparation strategy prevents unwanted oxidation and allows accurate profiling of sulfur pathway intermediates in metabolomics applications.



An integrated metabolomics workflow for the quantification of sulfur pathway intermediates employing thiol protection with N-ethyl maleimide and hydrophilic interaction liquid chromatography tandem mass spectrometry.

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Abstract

The sulfur metabolic pathway is involved in basic modes of cellular metabolism, including methylation, cell division, respiratory oscillations and stress responses. The hence implicated high reactivity of the sulfur pathway intermediates entails challenges for their quantitative analysis. Especially the unwanted oxidation of the thiol group-containing metabolites glutathione, cysteine, homocysteine, γ-glutamyl cysteine and cysteinyl glycine must be prevented in order to obtain accurate snapshots of this important part of cellular metabolism. Suitable analytical methodologies are therefore needed to support studies of drug metabolism and metabolic engineering. In this work, a novel sample preparation strategy targeting thiolic metabolites was established by implementing thiol group protection with N-ethyl maleimide into a cold methanol metabolite extraction procedure. It was shown that N-ethyl maleimide derivatization is compatible with typical metabolite extraction procedures and also allowed for the stabilization of the instable thiolic metabolites in a fully ^{13}C-labeled yeast cell extract. The stable isotope labeled metabolite analogs could be used for internal standardization to achieve metabolite quantification with high precision. Furthermore, a dedicated hydrophilic interaction liquid chromatography tandem mass spectrometry method for the separation of sulfur metabolic pathway intermediates using a sub-2 µm particle size stationary phase was developed. Coupled to tandem mass spectrometry, the presented methodology proved to be robust, and sensitive (absolute detection limits in the low femtomol range), and allowed for the quantification of cysteine, cysteinyl glycine, cystathionine, cystine, glutamic acid, glutamyl cysteine, reduced glutathione, glutathione disulfide, homocysteine, methionine, S-adenosyl homocysteine and serine in a human ovarian carcinoma cell model.

Introduction

Metabolites containing sulfur atoms have a distinct relevance for cells of any organism, in that they are involved in a number of vital cellular functions such as redox homeostasis and oxidative stress response[1–4], methylation[5–8] and even cell division[9]. As a result, a variety of cellular processes are linked to one or more intermediates of the sulfur metabolic pathway shown in Figure 1. Particularly well-connected is S-adenosyl methionine (SAM), a sulfonium compound derived from methionine and adenosine triphosphate (ATP). SAM is probably the most important methyl group donor and hence is involved a myriad of intracellular methylation processes[10,11]. Reduced glutathione (GSH) and its corresponding disulfide (GSSG), on the other hand, represent the intracellular redox buffer[12]. As such, their ratio is compartment-specific and tightly regulated by multiple mechanisms[3,13].

The high reactivity of these metabolites issues from the sulfhydryl (-SH) group and its redox and metal binding activity[14] or, as in the case of S-adenosyl methionine (SAM), the methyl group bound to the sulfonium center[15]. The same fact also makes these compounds a challenging target for metabolomic analysis, particularly because enzymatic activity and oxidation processes interfere with the accurate analysis of the thiol group-containing primary metabolites cysteine (Cys), cysteinyl glycine (Cys-Gly), γ-glutamyl cysteine (Glu-Cys), glutathione (GSH) and homocysteine (HCys). Such unwanted oxidation processes can simulate a shift in the cell's redox state and are hence detrimental to accurate metabolic profiling[16]. Unfortunately, oxidation is favored in commonly applied conditions during sample preparation for metabolomics. The accurate determination of these important primary metabolites hence demands for a tailored sample preparation procedure ensuring that the sample content remains unaltered from the moment of sampling until analysis[17].

Several reagents are described in literature for the protection of sulfhydryl groups, mostly for studies of cysteines in proteins. Similarly, the study of free thiols requires an efficient strategy to prevent an inter-conversion of oxidized and reduced thiolic metabolites. The major criteria for suitable derivatization reagents are a rapid reaction, selectivity towards thiols and optimum reaction conditions compatible with metabolite extraction. Most protocols and studies reported in literature were performed on protein thiols, where the accessibility of protein-bound thiols buried in hydrophobic cores is an additional point to consider. The different strategies and agents for thiol protection have been reviewed extensively elsewhere[18,19]. Besides the classic chromogenic Ellman's reagent[20], the most frequently used thiol alkylating reagents include iodoacetic acid (IAA)[21,22], iodoacetamide (IAM)[21] and maleimide-based reagents[17,23–25]. While favored over other reagents in some reports[22], IAA and IAM have been associated with a slow reaction and incomplete thiol blocking even at high reagent concentrations and long reaction times[26]. N-ethyl maleimide (NEM), on the other hand, rapidly forms irreversible thioether linkages with the sulfhydryl group[27,28]. As NEM is a small and uncharged molecule, it is not only membrane permeable but also maintains its reactivity towards thiol groups even in hydrophobic environment[18,29].

A limited number of methods for chromatographic separation of sulfur pathway intermediates have been reported in the context of mass spectrometry-based metabolomics. Most liquid chromatography-based analytical platforms employ hydrophilic interaction liquid chromatography (HILIC) with[22] or

without[30] prior thiol group blocking. Following thiol derivatization, other methods make use of reversed-phase liquid chromatography (RPLC)[31,32]. More specialized methods are available, targeting only the critical redox pair of reduced glutathione (GSH) and its disulfide (GSSG)[17,33,34] or S-adenosyl methionine (SAM) and its demethylation product S-adenosyl homocysteine (SAH)[35–38].

The aim of this work was to establish a fast, robust and reliable methodology for the simultaneous analysis of 13 sulfur pathway intermediates for application in LC-MS based metabolomics. The analysis of the oxidation-sensitive and biologically important free thiols cysteine, cysteinyl glycine, γ-glutamyl cysteine, glutathione and homocysteine demands for a strategy for instantaneous thiol group protection upon metabolite extraction. The objective of this work was to accomplish this by a novel combination of metabolite extraction and thiol derivatization by adding N-ethyl maleimide to the extraction solvent. The recovery of five thiols from human ovarian carcinoma cell extracts prepared according to this procedure was investigated in a proof-of-principle study. Seeking to develop a robust and sensitive method with MS detection for the analysis of sulfur pathway intermediates, a novel HILIC method coupled to tandem mass spectrometry was established. A thiol-stabilized ^{13}C-labeled yeast cell extract was also investigated to correct for artifactual alterations to the sample during sample preparation and the analytical process.

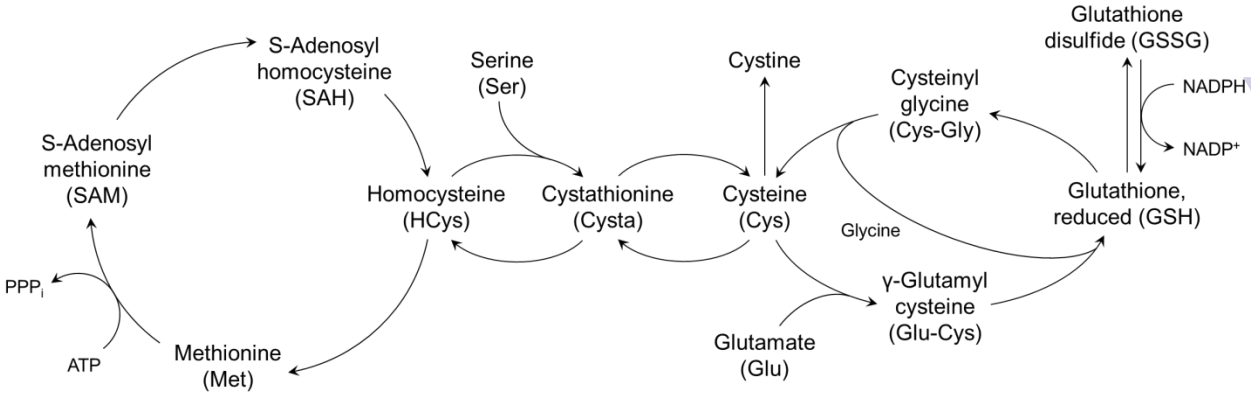

Figure 1. Reactions of the sulfur metabolic pathway intermediates[13,22].

Materials and methods

Metabolite standards. H-Cys-Gly-OH was purchased from Bachem AG (Bubendorf, Switzerland) and L-glutamic acid from Merck (Darmstadt, Germany). L-homocysteine, γ-Glu-Cys, S-(5'-adenosyl)-L-homocysteine, L-cysteine, L-glutathione oxidized, L-glutathione reduced, L-cystathionine, S-(5'-adenosyl)-L-methionine chloride dihydrochloride, L-serine were obtained from Sigma Aldrich (St. Louis, MO, USA).

Solvents and reagents. N-ethyl maleimide, ethanol absolute, ammonium formate and LC-MS grade solvents (water, formic acid, acetonitrile) were purchased from Sigma Aldrich (St. Louis, MO, USA). LC-MS grade methanol was obtained from Fisher Scientific (Vienna, Austria).

Thiol derivatization. Separate single standard solutions of cysteine, homocysteine, glutathione, cysteinyl glycine and γ-glutamyl cysteine were incubated with a 10-fold excess of N-ethyl maleimide as derivatization reagent for 90 min at room temperature. Following literature recommendations[25,29,39]. NEM was added in a 10 mM ammonium formate (pH 7.0) solution. After initial experiments, the routine NEM derivatization procedure included incubation of thiol-containing solutions with NEM at a final concentration of 25 mM for at least 15 min. For cellular samples, NEM was added to the extraction solvent as described below, allowing for immediate thiol derivatization upon release from the cell and sufficient time for the reaction to proceed during sample treatment.

In vivo synthesis of ^{13}C-labeled yeast cell extract. *Pichia pastoris* was grown in presence of $^{13}C_6$-glucose as single carbon source according to a previously described procedure[40]. The cultivation broth was sampled directly into the 4-fold volume of cold quenching solvent (60% v/v methanol, -30 °C). The quenched cell suspension was kept at -30 °C ± 3 °C, aliquoted and centrifuged (4000 *g*, -20 °C, 10 min). The supernatant was discarded and the cell pellets were stored on dry ice until extraction**.** For metabolite extraction, the cell pellets were resuspended in 2 mL of pre-heated 75% v/v ethanol, 25% v/v 10 mM ammonium formate (pH 7.0) solution with 25 mM NEM, immediately incubated at 85 °C for 3 min with intermediate mixing, cooled on dry ice and centrifuged for 5 min at 4000 *g*. The ethanolic supernatant was used as internal standard as described below or dried by vacuum centrifugation, stored at -80 °C and reconstituted in LC-MS grade H_2O before use.

Mammalian cell culture and metabolite extraction. The human ovarian carcinoma cell model A2780 was purchased from Sigma-Aldrich (St. Louis, MO, USA). Cell cultures were provided by our cooperation partner, the Institute of Cancer Research at the Medical University of Vienna. Aliquots of 1 mL cell medium (RPMI-1640 with 10% FBS) containing 1 x 10^6 A2780 cells were seeded in 6-well plates and allowed to recover for 24 h. 3 h before sampling, 1 mL of fresh cell medium was added. The harvesting and metabolite extraction procedure for adherently growing cells was adapted from Dettmer et al.[41]. At the time point of sampling, the medium was aspirated and the cell layer washed three times with 1 mL of a PBS solution (4 °C). After aspiration of the wash solvent, 200 µL of ^{13}C-labeled yeast cell extract (dried and reconstituted in H_2O) was added per well and cells were scraped in 1.8 mL ice-cold methanol (80% methanol, 20% 10 mM ammonium formate, 25 mM NEM) with a cell scraper. The methanolic cell extracts were transferred to separate sample tubes and kept on ice at all times during further sample preparation. Cell extracts were centrifuged at 4 °C and 20 000 *g* for 5 min. The supernatant was diluted to 90% acetonitrile prior to analysis by HILIC-MS/MS. For analysis by RPLC, aliquots of the methanolic extracts were dried by vacuum centrifugation and reconstituted in LC-MS grade water prior to injection. The pellet, containing precipitated cellular proteins and cell debris, was used for the determination of total protein content using a 2-D Quant Kit (GE Healthcare).

HILIC separation of sulfur metabolites. A silica-based Nucleodur HILIC column (2 x 100 mm, 1.8 µm particle size, Macherey-Nagel, Düren, Germany) was employed for HILIC separations. The column was operated at a flow rate of 300 µL·min^{-1} and a temperature of 40 °C. The injection volume was 5 µL. Mobile phase A was prepared from 98.9% water, 0.1% formic acid and 1% acetonitrile. Mobile phase B consisted of 98.9% acetonitrile, 0.1% formic acid and 1% water. The chromatographic run started at

90% B (1.5 min), followed by a linear gradient from 90% to 55% B in 4.5 min, 1.5 min at 10% B and re-equilibration at 90% B within a total run time of 15 min.

RPLC separation of sulfur metabolites. A silica-based C18 column (Atlantis T3, 2.1 x 150 mm, 3 µm particle size, Waters, Milford, MA, USA) was employed for reversed-phase separation. The flow rate was set to 250 µL·min^{-1} and the column temperature to 40 °C. The injection volume was 5 µL. The chromatographic run started at 100% A (98.9% water, 1% acetonitrile, 0.1% formic acid), maintained for 2 min, followed by a gradient from 0 to 95% B (98.9% acetonitrile, 1% water, 0.1% formic acid) in 13 min. With a cleaning step at 95% B and sufficient column re-equilibration at initial gradient conditions, the overall run time was 20 min.

Mass spectrometry. Initial method development was conducted on an Agilent 6220 TOF LC-MS system with an Agilent 1200 HPLC system. All measurements were performed using electrospray ionization (ESI) in positive ion mode (4000 V capillary voltage, 350 °C drying gas temperature, 12 L·min^{-1} drying gas flow, 45 psig nebulizer pressure, 180 V fragmentor voltage and 60 V skimmer voltage). The TOF detector was operated in 2 GHz extended dynamic range mode (2695 transients per spectrum, 3 spectra per second). Spectral data were recorded in the mass range of 60-1000 m/z. Extracted ion chromatograms (EIC) were obtained using the exact metabolite masses (Table 1, extraction width ± 20 ppm).

Accurate quantification of sulfur pathway intermediates was performed via LC-MS/MS analysis on a Thermo TSQ Vantage triple quadrupole mass spectrometer with a Thermo Accela 1250 HPLC system. ESI was operated in positive ion mode (300 °C capillary temperature, 3300 V spray voltage, 40 psig sheath gas pressure and 10 psig auxiliary gas pressure). Data acquisition was performed in selected reaction monitoring (SRM) mode, according to the transitions given in Table 1. The fully ^{13}C-labeled analogs of all analytes were monitored by MS/MS concomitantly with the unlabeled forms, and quantification was based on the signal intensity ratios of each analyte of interest and its ^{13}C-labeled analog. Calibration solutions were prepared as standard mixtures of the metabolites of interest with the addition of an equal aliquot of ^{13}C-labeled yeast cell extract as internal standard.

Table 1. Mass spectrometric detection of 13 sulfur metabolic pathway intermediates in ESI positive ionization mode. The sum of signal intensities obtained for the two product ion masses was used for quantification.

Metabolite	Exact mass, m/z	Precursor ion mass, m/z	Product ion mass, m/z	Collision energy, V	Product ion mass, m/z	Collision energy, V
NEM-Cys	247.0747	247.1	230.1	11	158.0	20
NEM-Cys-Gly	304.0962	304.1	287.1	11	158.0	25
Cysta	223.0747	223.1	88.0	27	134.0	12
Cystine	241.0311	241.0	223.0	6	74.1	41
Glu	148.0604	148.1	84.1	15	130.1	7
Glu-NEM-Cys	376.1173	376.1	247.1	12	230.0	18
GSSG	613.1592	613.2	355.0	20	231.0	37
NEM-GSH	433.1388	433.1	304.1	13	201.1	18
NEM-HCys	261.0904	261.1	56.1	18	215.1	11
Met	150.0583	150.1	133.0	6	56.1	17
SAH	385.1289	385.1	136.1	19	134.0	18
SAM	399.1451	399.1	250.1	14	136.0	25
Ser	106.0499	106.1	60.1	10	88.0	8

Results and discussion

Thiol derivatization. Blocking thiol groups by reaction with N-ethyl maleimide (NEM, Figure 2) is a well-established protocol with many applications in the analysis of free and protein-bound thiols. The reaction pH is of particular importance for this alkylation process. Above pH 7, the maleimide moiety might undergo hydrolysis and ring opening[18], and the risk for unwanted side reactions increases, while the reaction rate decreases rapidly below pH 6[39]. Preliminary experiments within this work confirmed the rapid and efficient derivatization of the thiol group-containing metabolites cysteine (Cys), cysteinyl-glycine (Cys-Gly), γ-glutamyl-cysteine (Glu-Cys), reduced glutathione (GSH) and homocysteine (HCys) at pH 7. NEM derivatives were rapidly formed and remained stable for at least 15 h at 6 °C (Table 2). After 15 min incubation at room temperature, the most abundant ion observed in flow injection TOFMS analysis corresponded to the respective NEM-derivatized thiol, while neither the free thiols nor reaction by-products were present in significant abundance (Figure 2b). Side-reactions with other pathway intermediates resulting in a signal decrease during sample storage were not observed (Table 2). In order to achieve instantaneous thiol quenching and prevent unwanted oxidation of thiol group-containing metabolites in cell extracts, this work aimed at implementing NEM derivatization as early as possible in the metabolite extraction workflow, i.e. during the cell lysis step. As such, the thiolic metabolites are released from the cell directly into the extraction solvent containing the derivatization reagent. A concentration series of standard solutions (0.05 – 50 µM) with the addition of a ^{13}C-labeled yeast cell extract as matrix mimic showed a linear increase in derivative formation with increasing thiol concentration (see coefficients of determination in Table 2). Hence, a linear working range across 3 orders of magnitude could be validated.

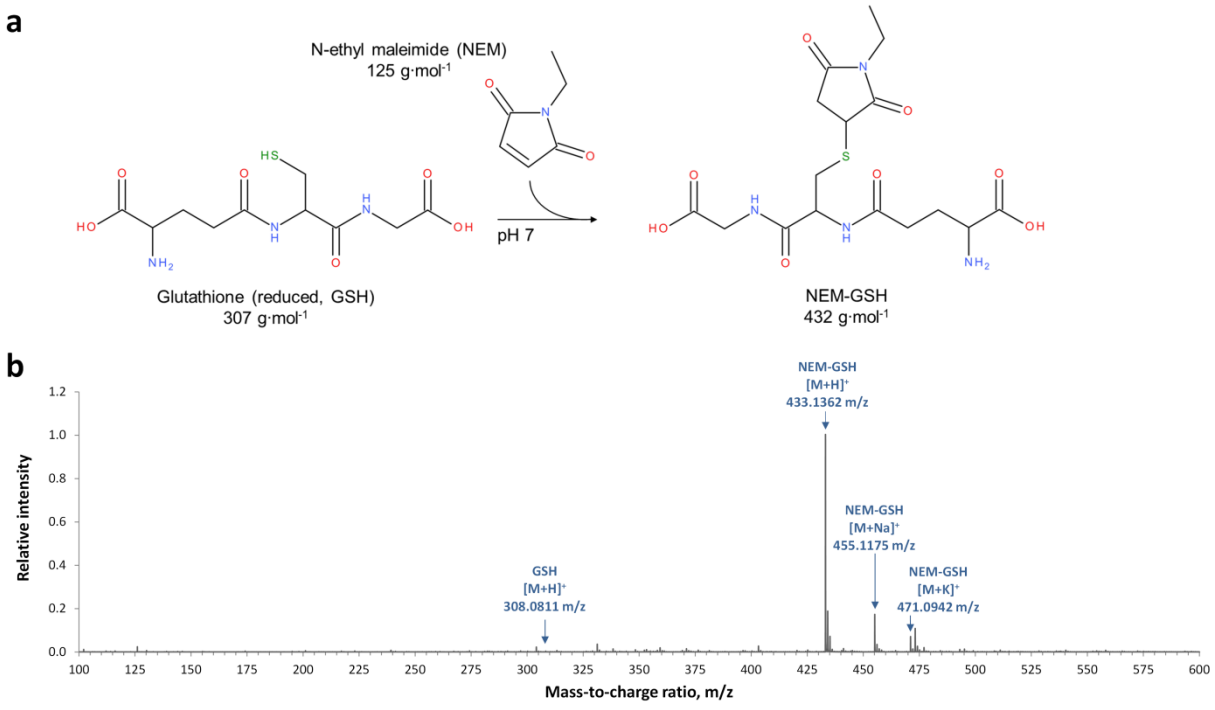

Figure 2. Thiol group derivatization with N-ethyl maleimide on the example of the formation of NEM-GSH from reduced glutathione. a) The double bond of the maleimide reagent forms a stable thioether bond with the sulfhydryl group at pH 7. b) Flow injection-TOFMS analysis of a single standard of glutathione after incubation with a 10-fold excess of NEM for 15 min at room temperature.

HILIC separation of sulfur pathway intermediates. For the sulfur pathway intermediates within the scope of this work (Figure 1), successful chromatographic separation was achieved using hydrophilic interaction liquid chromatography (HILIC) under acidic conditions (pH 2.7). The stationary phase of choice was a silica-based Nucleodur HILIC column with 1.8 µm particle size. It was found to provide high-efficiency separations within relatively short chromatographic run times at low back pressures. The thiol group-containing metabolites cysteine (Cys), homocysteine (HCys), cysteinyl glycine (Cys-Gly), γ-glutamyl cysteine (Glu-Cys) and reduced glutathione (GSH) were analyzed after derivatization with N-ethyl maleimide (NEM). The excess derivatization reagent eluted as a sharp peak near the void volume of the column and can be routinely diverted to the waste or can be used for monitoring purposes. Two consecutive peaks were observed for NEM-Cys (R = 2.0, FWHM), indicating the existence of two isomeric forms of the derivative. Indeed, the formation of diastereomers during NEM derivatization has been reported and studied by Kuninori and Nishiyama[42]. Their findings suggested that such stereoisomers also exist for the NEM derivatives of the other four thiols, but it seems that these are not resolved using the chosen HILIC conditions. S-adenosyl methionine (SAM), a positively charged and highly reactive metabolite, was strongly retained and could not be eluted as a sharp peak. The remaining pathway intermediates, i.e. cystathionine (Cysta), cysteine (as NEM-Cys), cysteinyl glycine (as NEM-Cys-Gly), cystine, glutamic acid (Glu), glutamyl cysteine (as Glu-NEM-Cys), reduced glutathione (as NEM-GSH), glutathione disulfide (GSSG), homocysteine (as NEM-HCys), methionine (Met), S-adenosyl homocysteine (SAH) and serine (Ser), were separated using a linear gradient (Figure 3). Within a total run time of 15 min including sufficient column re-equilibration time, excellent retention time stability and repeatability were achieved (Table 2). The best results were obtained when the acetonitrile content in the sample matched gradient start conditions, i.e. samples were routinely diluted to 90% acetonitrile prior to analysis. Coupled to tandem MS analysis, the method provides detection limits in the low nanomolar range (fmol on-column, calculated based on the observed standard deviation of peak areas in replicate injections of a low-concentration standard).

Table 2. Analytical figures of merit for the analysis of sulfur metabolic pathway intermediates by HILIC-ESI-MS/MS and results for the determination of their intracellular levels in mammalian cells (A2780, human ovarian carcinoma cell line). A standard mix of all sulfur pathway intermediates was incubated with NEM and stored at 6°C for 15 h to evaluate the stability of the metabolites in the mixture and in the presence of NEM. The linearity of the derivatization was assessed via separately derivatized standard solutions in the concentration range 0.05 – 50 µM. Stable-isotope labeled metabolite analogs were recovered from a cell extract of *Pichia pastoris* grown on $^{13}C_6$-glucose and used for internal standardization.

Metabolite	HILIC retention time, min	Retention time RSD (n = 10)	Peak area RSD (n = 10)	Signal recovery (15 h, 6 °C)	A2780 extracted amount, pmol·µg^{-1} $_{protein}$ (n = 6) AV	SD	Linearity, Coefficient of determination R^2	*U*^{13}*C-P. pastoris* Signal-to-noise ratio
NEM-Cys-Gly	1.5	1.7%	3%	97%	1.2	0.1	0.9994	-
NEM-HCys	2.8	0.6%	3%	103%	1.4	0.1	0.9999	-
Met	2.9	0.5%	3%	102%	6.8	0.8	0.9996	-
NEM-Cys	3.6	0.4%	3%	99%	5.5	0.6	0.9997	1100
Ser	5.1	0.4%	4%	100%	25	6	0.9998	11000
SAH	5.2	0.7%	4%	96%	-	-	0.9999	300
Glu	5.3	0.4%	2%	97%	241	27	1.0000	20000
NEM-GSH	5.7	0.4%	2%	98%	48	4	1.0000	24000
Glu-NEM-Cys	6.0	0.4%	2%	98%	0.64	0.03	1.0000	-

Cysta	6.4	0.3%	6%	79%	11	1	0.9999	700
Cystine	6.6	0.3%	3%	79%	-	-	0.9997	-
GSSG	7.9	0.3%	5%	66%	-	-	1.0000	70

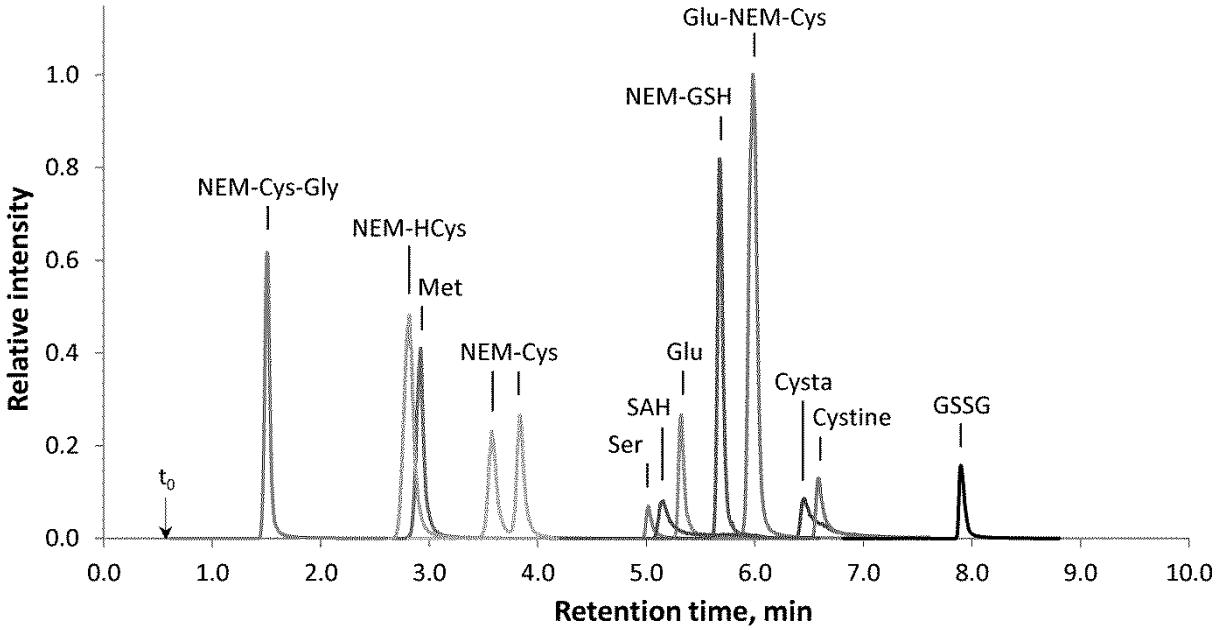

Figure 3. Representative chromatogram from HILIC-ESI-MS/MS analysis of sulfur metabolic pathway intermediates. The sample was prepared from metabolite standards in 90% acetonitrile (1 µM each, except for cystine and GSSG – 5 µM). Data were acquired on a Thermo TSQ Vantage triple quadrupole MS/MS system in SRM mode (Table 1).

RPLC separation of sulfur pathway intermediates. Via derivatization with NEM, the alkylated thiols become amenable to analysis by RPLC. Using a silica-based C18 stationary phase under acidic conditions (0.1% formic acid), suitable retention was observed for 8 of the 13 sulfur pathway intermediates. The fact that all five NEM derivatives under investigation eluted within a time window of 1 min and close to the excess derivatization reagent indicates that the retention is mainly mediated by the NEM moiety in the derivatized analytes (Table 3). Except for NEM-HCys, all NEM-derivatized metabolites were characterized by two peaks (R < 1.5, FWHM), corresponding to the aforementioned formation of diastereomers during NEM derivatization. Several other intermediates of the sulfur pathway (including Ser, Glu, Cysta, cystine and SAM), however, showed weak retention on the C18 stationary phase and eluted near the void volume. Depending on the sample origin, this weakly-retained fraction may be fraught with matrix components that cause ion suppression during electrospray ionization. This problem can be alleviated by a straight-forward heart-cutting two-dimensional setup in which the low-retained fraction from RPLC is transferred to an orthogonal separation dimension such as porous graphitized carbon liquid chromatography, as described recently[43] (Table 3, Figure S1). A general drawback of RPLC in the context of metabolomics is its incompatibility with high organic solvent fractions in typical cell extracts. As a consequence, near-complete loss of separation and/or retention is often observed and solvent evaporation steps are routinely employed for solvent exchange and sample pre-concentration. However, solvent evaporation is typically a time-consuming step and can result in

extensive losses of unstable metabolites. If available, stable isotope-labeled internal standards allow correction for these losses, while an adaptation of the metabolite extraction procedure to use aqueous conditions should be considered if a correction strategy is not available and RPLC is a necessary part of the analytical method employed. Overall, HILIC-ESI-MS/MS was the preferred method for metabolite quantification in methanolic cell extracts within this work.

Table 3. Detailed description of RPLC separation of sulfur metabolic pathway intermediates after NEM derivatization. The weak retention of some analytes can be increased using heart-cutting two-dimensional RP-PGC-ESI-MS (2DLC, as previously described[43]).

Analyte	RPLC retention time, min	RPLC retention time RSD (n = 10)	Comment	2DLC retention time, min	2DLC retention time RSD (n = 5)	Comment
Cysta	1.6	0.5%	Weak retention	4.0	0.8%	Eluting from PGC
Cystine	1.6	0.5%	Weak retention	4.3	1.1%	Eluting from PGC
SAM	1.6	1.2%	Weak retention	2.6	0.5%	Eluting from PGC
Ser	1.6	0.6%	Weak retention	3.7	0.4%	Eluting from PGC
Glu	1.7	0.4%	Weak retention	4.5	0.7%	Eluting from PGC
Met	3.2	0.3%	-	3.1	0.8%	RP
GSSG	6.1	0.3%	-	5.4	0.3%	RP
SAH	6.1	0.3%	-	5.4	0.3%	RP
NEM-Cys	6.4	0.3%	Isomer 1	5.8	0.4%	RP, isomer 1
NEM-Cys	6.5	0.2%	Isomer 2	6.0	0.3%	RP, isomer 2
NEM-Cys-Gly	6.5	0.2%	Isomer 1	6.0	0.3%	RP, isomer 1
NEM-Cys-Gly	6.6	0.2%	Isomer 2	6.3	0.6%	RP, isomer 2
NEM-Hcys	6.8	0.2%	No isomeric form observed	6.5	0.4%	RP, no isomeric form observed
NEM-GSH	7.0	0.2%	Isomers co-elute	6.9	0.5%	RP, isomers co-elute
Glu-NEM-Cys	7.1	0.2%	Isomers co-elute	6.9	0.5%	RP, isomers co-elute
NEM	7.9	0.2%	Excess derivatization reagent	7.7	0.5%	RP, excess derivatization reagent

Internal standardization with a ^{13}C-labeled cell extract. Stable isotope-labeled metabolite analogs are ideal internal standards for the analysis of intracellular metabolites in complex matrices such as cell extracts. If added to the sample immediately prior to metabolite extraction, accurate quantification along with reduced overall measurement uncertainty becomes possible, since the internal standardization corrects for compound-specific errors introduced during sample preparation, the injection process and differences in ionization efficiency or ion suppression. The use of stable isotope-labeled cell extracts obtained from cultures grown on fully ^{13}C-labeled glucose ideally provides the full primary metabolome in its uniformly ^{13}C-labeled form. However, thiol group-containing metabolites are prone to a variety of oxidation processes and are hence (in most cases) not recovered in their reduced form. In this work, cell pellets from a *P. pastoris* culture grown on $^{13}C_6$-glucose were extracted using boiling ethanol extraction, a commonly utilized standard protocol for yeast cells. The suitability of boiling ethanol as solvent for the extraction of intracellular metabolites from *P. pastoris* has been thoroughly investigated and verified in a previous study[40]. In fact, the exceptionally robust cell envelope of yeast cells renders most alternative metabolite extraction procedures inefficient. The standard protocol was adapted here in that the aqueous fraction was substituted by a 10 mM ammonium

formate solution (pH 7.0) and NEM was added to the pre-heated solvent at a final concentration of 25 mM, i.e. in large excess with respect to the expected amount of thiol group-containing metabolites. Using this novel combined strategy, the reduced thiolic metabolites GSH and Cys could be stabilized as NEM derivatives. The non-thiolic sulfur pathway intermediates Cysta, Glu, GSSG, SAH and Ser could also be detected at sufficiently high signal intensity to be used for normalization (Table 2). However, the ^{13}C-labeled yeast cell extract faced limitations due to the endogenously low levels of the remaining metabolites of interest in *P. pastoris*, including Cys-Gly, HCys and Glu-Cys, which could not be detected.

A comparison with an equivalent cell extract prepared without NEM provided further insight into its ability to prevent thiol oxidation. When NEM was added to a previously NEM-free cell extract, NEM-GSH could not be recovered in significant amounts, while it was detected at high signal intensity in cell extracts prepared in the presence of NEM. The changes in GSSG levels after addition of NEM were insignificant, and signal recoveries of 104% (10% RSD) were observed and confirmed using analytical standards. On the other hand, NEM-free cell extracts contained significantly higher levels of GSSG than cell extracts prepared in the presence of the derivatization reagent, i.e. only 6% of the GSSG signal was found when NEM had been added to the extraction solvent. These findings indicate that, when the thiol group is not protected, GSH oxidation occurs during boiling ethanol extraction. The addition of NEM, however, rapidly and efficiently quenches the thiol group upon cell lysis, which seems to avoid artifactual GSSG formation that would falsify the created metabolic profile of sulfur metabolism.

The importance of implementing internal standardization can be readily seen from the signal recovery data provided in Table 2 (e.g. Cysta, cystine, GSSG). As it can be assumed that stable isotope-labeled metabolites are affected by degradation processes to the same extent as their unlabeled analogs, this type of quantification strategy proves to be extremely valuable for analyzing large numbers of samples.

Determination of sulfur pathway intermediates in human ovarian carcinoma cells. The protocol of choice for metabolite extraction from mammalian cells was simultaneous quenching and cell lysis by addition of an ice-cold methanol/water mixture (-20 °C) and subsequent scraping to detach the adherently growing cells as described by Dettmer et al.[41]. Conveniently, protein precipitation is achieved in these conditions without acidification. As a novel feature, the aim of this work was to implement NEM derivatization into this well-established metabolite extraction procedure by replacing the aqueous fraction of the extraction solvent by 25 mM NEM in a 10 mM ammonium formate solution (pH 7.0). Furthermore, analyte-specific internal standardization was implemented via the addition of the ^{13}C-labeled yeast cell extract prepared as described above.

In a proof-of-principle study on the A2780 human ovarian carcinoma cell model, excellent repeatability precisions (< 10% RSD, n = 6 biological replicates) could be achieved using the presented sample preparation workflow followed by HILIC-ESI-MS/MS analysis of cell extracts. As indicated above, a major contributor to this high precision was the normalization of metabolite intensities by the corresponding signal intensities of the internal standard, as it compensates even for compound-specific errors introduced during sample generation, handling and the analytical process itself. The concentrations of sulfur pathway intermediates in extracts from $1 \cdot 10^6$ cells were found to be in the micromolar range (0.1 – 30 µM). Normalized to the total protein content in the sample, the extractable amount of sulfur metabolites was between 0.64 and 241 pmol·µg^{-1} protein (Table 2). Cystine was not found in cell

extracts, while GSSG and SAH were detected, but could not be quantified. Despite the low detection limit provided by the LC-MS/MS system used for this work, it might be necessary to use a higher number of cells in order to get reliable quantitative information on GSSG and SAH for this particular cell line.

Conclusion

The intermediates of sulfur metabolism are challenging targets for accurate quantification in cell extracts. The aim of this work was therefore to combine metabolite extraction and thiol protection steps, resulting in a novel, dedicated sample preparation workflow that specifically avoids unwanted oxidation. Derivatization with N-ethyl maleimide was verified in this study as suitable strategy to quench unwanted thiol reactivity. Furthermore, its implementation in the initial stages of sample preparation (i.e. metabolite extraction) was shown to be crucial to achieve reliable thiol quantification. The selective and sensitive analysis of 12 sulfur pathway intermediates was successfully achieved using a state-of-the-art LC-MS/MS method employing internal standardization via stable-isotope-labeled metabolite analogs. Hydrophilic interaction liquid chromatography provides excellent selectivity, while the stationary phase in the form of sub-2 µm particles ensured separation and subsequent column re-equilibration within 20 min, resulting in a stable and robust analytical platform. Alternatively, reversed-phase liquid chromatography showed good retention for all NEM derivatives, while the comprehensive analysis of all pathway intermediates necessitates the use of complementary chromatographic techniques.

Comparable methodologies in literature often omit thiol protection and internal standardization, both of which were addressed in this work. A stable isotope-labeled yeast cell extract was shown to contain several sulfur pathway intermediates that were available for intensity correction, but this approach also faced limitations due to the very low intracellular concentrations of certain metabolites in *P. pastoris*, including three thiols. Overall, the thiol protection strategy described here is versatile and compatible with different solvents and sample preparation procedures. This makes this integrated workflow ideally suited for application in metabolomics. Finally, the presented thiol protection and internal standardization strategy was applied to a human ovarian carcinoma cell model used in the investigation of metallodrug metabolism. Excellent repeatability precisions across several biological replicates demonstrated its efficiency, and sulfur metabolites could be successfully quantified in these cell extract samples.

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