Development of a LC-MS method for analysis of thiol ratios as an indicator of oxidative stress

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Abstract

Reactive oxygen species are free radicals capable of damaging the cellular components in a process called oxidative stress. Among the different biomarkers that are used to determine level of oxidative stress is the ratio between reduced and oxidized thiols, such as glutathione and oxidized glutathione. The use of glutathione ratio as a biomarker of oxidative stress is possible because the thiols are responsible for reducing the oxidizing species in a process that oxidizes the thiols into their disulfides. Under normal conditions, the cells can regenerate the reduced thiols by the action of reductases, which keeps the ratio constant. However, under oxidative stress, the cell cannot regenerate the reduced thiols rapidly enough. This in turn increases the concentration of the disulfide, and the ratio decreases. The ratio can also be inadvertently altered during sample manipulation because thiols can autoxidize. Therefore, for their accurate determination, thiols should be derivatized prior to analysis. The existing protocols using liquid chromatography-mass spectrometry (LC-MS) for thiol analysis largely focus on urine or plasma analysis, and do not consider exposure to oxidation during sample handling, while the few studies on intracellular thiol concentrations employ derivatization after cell lysis. The main objective of this thesis was to develop a LC-MS method to accurately measure individual thiols and disulfides, and their ratios in Jurkat cells.

To achieve this goal, the selectivity and efficiency of two different derivatizing agents that are able to permeate the cell membrane were first compared in detail: N-ethyl maleimide (NEM) and N-phenyl ethyl maleimide (NPEM). They were compared in terms of their derivatization efficiency, electrospray ionization enhancement, stability and selectivity/side product formation with focus on four abundant intracellular thiols: cysteine (CYS), homocysteine (HCY), N-acetyl cysteine (NAC), glutathione (GSH) and their corresponding disulfides. While NPEM provided greater ionization efficiency than NEM (NPEM/NEM varies from 2.1x for GSH to 5.7x for CYS), it was also more unstable, forming more side-products. The instability of its maleimide ring led to reaction with amines, as well as double derivatization and cyclization reactions, which corresponded to about 10% of the signal of CYS. NEM showed only minor contribution of side reactions (about 1.5% of the signal of CYS), so it was chosen as the derivatizing reagent for the protocol. The derivatizing conditions with NEM were further optimized to minimize side product formation, and pH 7.0 was selected for further assay development while being compatible with cell handling.

In the next step, a full cell extraction protocol was developed to quantify the thiol ratios in Jurkat T cells. Briefly, the optimized protocol required 1×10^6 cells and combined NEM derivatization prior to cell lysis, cell lysis and extraction using 20% methanol (v/v) and protein precipitation by methanol. The thiols were then chromatographically separated using a biphenyl, reversed-phase, separation in combination with Quadrupole Time of Flight Mass Spectrometry (QToF-MS) analysis. Protocol optimization included evaluation of different lysis solvents, recovery, matrix effects, and evaluation of the number of washes required to ensure as complete removal of extracellular metabolites as possible without compromising cellular integrity. The final method was tested for its capacity to evaluate oxidative stress in cells stimulated by hydrogen peroxide, a known inducer of oxidative damage. The results show that the method was capable of differentiating between the control, mild and intense oxidative stress conditions.

To the best of my knowledge, this is the first cellular protocol that combines NEM derivatization prior to cell lysis with LC-MS determination of individual thiol ratios. An innovative aspect of this procedure is the protection of reduced thiols prior to lysis, which minimizes changes in the ratio caused by sample manipulation, as opposed to the typical procedure which has the derivatization after extraction. This work is also the first systematic comparison of NEM versus NPEM derivatization for LC-MS analysis and shows clearly the propensity of NPEM for side-product formation under conditions commonly used for maleimide derivatization. In summary, this research contributes towards more accurate measurement of thiol ratios as readouts of oxidative stress.

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Contribution of Authors

Chapter 2

This chapter entitled “Comparison of N-ethyl maleimide and N-(1-phenylethyl) maleimide for derivatization of biological thiols using LC-MS” authored by Mariana de Sá Tavares Russo, Alexandra Paquet and Dajana Vuckovic is the first draft of a manuscript in preparation that will be submitted for publication in Summer 2017.

M. R. and D. V. designed all experiments, interpreted results and co-wrote the draft of the manuscript. The evaluation of derivatization efficiency of NEM and NPEM was executed by M. R. and A. P. All other experiments were executed by M. R.

All authors reviewed and revised the manuscript.

Chapter 3

This chapter entitled “Development of a LC-MS method for analysis of thiol ratios as an indicator of oxidative stress in immune cells” authored by Mariana de Sá Tavares Russo, Catalina Carvajal, Peter John Darlington, Bo Jin and Dajana Vuckovic is the first draft of a manuscript in preparation that will be submitted for publication in the Fall of 2017.

Jurkat cells were cultured by C. C.. Extraction protocols and experiments were designed by M. R. and D. V. and executed by M. R.. The experiment of hydrogen peroxide stimulation of Jurkat cells was designed and executed by P. D., and cells were extracted and analyzed by M. R.. B. J. will design, execute and interpret experiments described in future work (Chapter 4).

M. R. and D. V. interpreted results and co-wrote the draft of the manuscript. All authors will review and revise the final version of the manuscript.

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List of Abbreviations

α-MEM – Minimal essential medium, alpha modification

1,5-I-AEDANS – (5-({2-[iodoacetyl]amino}ethyl}amino)naphthalene-1-sulfonic acid)

2-VP – 2-vinylpyridine

4-DPS – 4,4'-dithiodipyridine

5-AIQC – 5-aminoisoquinolyl-N-hydroxysuccinimidylcarbamate

5iPF2α-VI – 5,9α,11α-trihydroxy-(8β)-prosta-6E,14Z-dien-1-oic acid

8-isoPGF$_{2\alpha}$-III – 9α,11α,15S-trihydroxy-(8β)-prosta-5Z,13E-dien-1-oic acid, 8-iso-15(S)-Prostaglandin F2α

8,12-iso-iPF$_{2\alpha}$-VI – (±)5,9α-trihydroxy-12α-prosta-6E,14Z-dien-1-oic acid

γ-Glu-Glu – Glutamic acid dipeptide with γ-glutamyl bond

ABD-F – 4-(aminosulfonyl)-7-fluoro-2,1,3-benzoxadiazole

ACN – Acetonitrile

AD – Alzheimer's Disease

ALA – Alanine

ALS - Amyotrophic Lateral Sclerosis

APC – N-acetyl(phenyl)-L-cysteine

BIPM – N-[p-(2-benzimidazolyl)phenyl]maleimide

CE – Capillary Electrophoresis

CMQT – 2-chloro-1-methylquinolinium tetrafluoroborate

CYS – Cysteine

CYSS – Cystine

DAC – N,S-diacetylcysteine

DBPM – N-(p-(2-(6-dimethylamino)benzofuranyl)phenyl)maleimide, Akiyama's Reagent

DINAC – N,N'-diacetylcystine

DTNB – 5,5'-dithio-(bis-2-nitrobenzoic acid), Ellman's Reagent

DTT – Dithiothreitol

ESI – Electrospray Ionization

ESI-MS – Electrospray Ionization-Mass Spectrometry

FDNB – 1-fluoro-2,4-dinitrobenzene, Sanger's Reagent

FEM – N-2-(ferroceneethyl)maleimide

FM – Fluorescein-5-maleimide

FMEA – Ferrocenecarboxylic acid (2-maleimidoyl) ethylamide

GC – Gas Chromatography

GC-MS – Gas Chromatography-Mass Spectrometry

GlyCys – Glycyl-cysteine

GSH – Glutathione

GR – Glutathione reductase

GSSG – Oxidized glutathione / glutathione disulfide

HCY – Homocysteine

HCYSS – Homocystine

HILIC – Hydrophilic Interaction Liquid Chromatography

HPLC – High-Performance Liquid Chromatography

IAA – Iodoacetic acid

IAM – Iodoacetamide

IL-2 – Interleukin-2

LC – Liquid Chromatography

LC-FL – Liquid Chromatography-Fluorescence

LC-MS – Liquid Chromatography-Mass Spectrometry

LC-MS/MS - Liquid Chromatography-Tandem Mass Spectrometry

LLE – Liquid-Liquid Extraction

LOD – Limit of detection

LOQ – Limit of quantification

LYS – Lysine

M2VP – 1-methyl-2-vinylpyridinium trifluoromethane sulfonate

M4VP – 1-methyl-4-vinylpyridinium trifluoromethane sulfonate

MAP – Mitogen-activated protein

mBrB – Monobromobimane

MDA – Malondialdehyde

MeOH – Methanol

MET – Methionine

MIAC – N-(2-acridonyl)maleimide

MPPTAB – 1-[3-(4-maleimidylphenoxy)propyl]trimethylammonium bromide

MRM – Multiple Reaction Monitoring

MS – Mass Spectrometry

MS/MS – Tandem Mass Spectrometry

mTOR – Mechanistic Target of Rapamycin

NAC – N-acetylcysteine

NADPH – Nicotinamide adenine dinucleotide phosphate reduced

$NADP^+$ – Nicotinamide adenine dinucleotide phosphate

NAM – N-(9-acridinyl)maleimide

NEM – N-ethylmaleimide

NPEM – (R)-(+)-N-(1-phenylethyl) maleimide

NPM – N-(1-pyrenyl)-maleimide

NTAM – N-[2-(Trimethylammonium)-ethyl]maleimide chloride

OPA – Ortho-phthalaldehyde

PBS – Phosphate-buffered saline

PFP – Pentafluorophenyl

PHE – Phenylalanine

QToF – Quadrupole Time of Flight

RBC – Red blood cells

ROS – Reactive Oxygen Species

RPLC – Reversed-phase Liquid Chromatography

RPM – Revolutions per minute

RT – Retention time

SAC – S-aceamidomethyl-L-cysteine

SAH – S-(5'-Adenosyl)-L-homocysteine

SBD-F – Ammonium-7-fluoro-2,1,3-benzoxadiazole-4-sulfonate

SER – Serine

SIM – Selected Ion Monitoring

SMC – S-methyl-L-cysteine

SPE – Solid Phase Extraction

tBBT – 4-tert-butylbenzenethiol

TBP – Tributylphosphine

TCA – Trichloroacetic acid

TCDI – 1,1'-thiocarbonyldiimidazole

TCEP – Tris(2-carboxyethyl)phosphine

tGSH – Total glutathione

ThioGlo™3 – 9-acetoxy-2-(4-(2,5-dihydro-2,5-dioxo-1H-pyrrol-1-yl)-phenyl)-3-oxo-3H-naphtho[2,1-*b*]pyran

TNB – 2-nitro-5-thiobenzoate

UV – Ultraviolet

UV-Vis – Ultraviolet-Visible

1. Introduction

1.1. Oxidative stress

1.1.1. Definition

During normal metabolism, some biological reactions generate reactive oxygen species (ROS), which are free radicals that can react with cellular components and cause damage to membranes, proteins and metabolites.[1] This is known as oxidative damage or stress[2]. It has been related to aging and many diseases such as Alzheimer's, Parkinson's, Amyotrophic Lateral Sclerosis (ALS), Huntington's, Down's and other neurodegenerative diseases[3], asthma and other problems involving airway function[4], heart diseases such as ischemic stroke, atherosclerosis[5], heart failure[6] and myocardial infarction[7], obesity and diabetes mellitus.[5,8]

Some of the ROS include: superoxide ($O_2^{\bullet -}$), hydroxyl ($OH^{\bullet}$), peroxyl ($RO_2^{\bullet}$), alkoxyl ($RO^{\bullet}$), hydroperoxyl ($HO_2^{\bullet}$), hydrogen peroxide (H_2O_2), hypochlorous acid ($HOC\ell$), ozone (O_3), singlet oxygen ($^1O_2$) and peroxynitrite ($ONOO^-$). They are difficult to measure due to their instability and low concentrations[9]; therefore, measurements of oxidative stress must be carried out by observation of markers, such as lipid peroxidation side products or the depletion of antioxidants.[2]

1.1.2. Biomarkers

Biomarkers are substances that can be measured quantitatively, accurately and reproducibly to evaluate the biological changes in an organism due to a variation that can be caused by a disease or pathogen, its pharmacological treatment, or exposure to a specific environment.[10] They can also be used to predict the outcome of diseases. In order to be useful as a predictor, however, the variability of the substance in the population must be low, and its concentration should be independent of diet, stable after storage and its measurement should be performed using robust techniques.[10,11] No ideal biomarker for oxidative stress has been identified and validated so far[6,11], but due to the interest in oxidative stress, many different biomarkers have been studied. Some of the biomarkers most studied are mentioned below.

Malondialdehyde (MDA), is the product of peroxidation of unsaturated lipids such as arachidonic acid.[6] It is usually measured by the thiobarbituric assay. However, this assay has been found to be of low specificity, since MDA is only formed during decomposition of certain lipid peroxidation products which can lead to false results.[3,9] Increases in this biomarker[4], have been associated with a large variety of diseases, such as Alzheimer's Disease (AD), ALS, asthma, atherosclerosis, cutaneous leishmaniasis, diabetes mellitus, and preeclampsia.[6]

F2 isoprostanes are a class of lipid peroxidation products that have been considered the "gold standard" for measurement of oxidative damage.[12] This is because their concentration in plasma and urine provides a well-recognized and established measurement of oxidative stress. They originate from the lipid peroxidation of arachidonic acid and due to the existence of four double bonds, with eight theoretical diastereoisomeric forms, there are 64 theoretical isomers in this group.[12] Out of these, 9α,11α,15S-trihydroxy-(8β)-prosta-5Z,13E-dien-1-oic acid (8-iso$PGF_{2\alpha}$-III), 5,9α,11α-trihydroxy-(8β)-prosta-6E,14Z-dien-1-oic acid (5iPF2α-VI) and (±)5,9α-trihydroxy-12α-prosta-6E,14Z-dien-1-oic acid (8,12-iso-iPF2α-VI) were increased in HepG2 cells after treatment with copper, and decreased with use of a known terminator of lipid peroxidation, butylated hydroxytoluene.[12] Increases in the concentration of this class of biomarker are associated with over thirty different diseases, from Down Syndrome to multiple sclerosis, obesity and sickle cell disease, to name a few[6], and have been measured by Gas Chromatography-Mass Spectrometry (GC-MS), Liquid Chromatography-Mass Spectrometry (LC-MS) and immunoassays.[13]

During protein oxidation, the side chains of arginine, proline, lysine and threonine are carbonylated.[14] This modification is stable, and has been one of the most common methods used to measure oxidation. However, since it can also occur due to other processes, for instance, the Michael addition of α,β-unsaturated aldehydes to lysine, histidine and cysteine, the source of carbonylation must be verified before it can be directly associated to **protein oxidation**. This biomarker has been associated with asthma, diabetes, meningitis, and other conditions.[6]

Peroxiredoxins are a class of peroxidases that degrade hydroperoxides to water. During this process, they are accumulated because their reductant, thioredoxin, is present in lower concentrations. In addition, the rate of oxidation is faster than that of reduction. This biomarker has been measured by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and mass spectrometry.[15]

The concentrations of **glutathione (GSH) and its disulfide (GSSG)** are considered indicative of the oxidative status of the whole body. Under oxidizing conditions, the concentration of GSH decreases as the disulfide is formed. The ratio of GSH over GSSG has been widely studied in different biological samples, such as blood, serum, plasma, tissues and cells.[9]

If this ratio is low and GSSG accumulates, **protein glutathionylation**, a process in which glutathione forms a disulfide bond with a protein cysteine group, can happen.[16] This process is reversible by the actions of other disulfides, but is less likely to be reduced by glutathione

reductase (GR). Since this happens due to excess GSSG, measurement of glutathionylation has been considered a marker for oxidative stress. The biological consequences of this post-translation modification include changes in protein function due to the charge alteration caused by the glutamic acid present in GSH[17], and the protection of proteins from oxidation.[18] The increase of protein glutathionylation has been observed in diabetes mellitus, hyperlipidemia, Friedrich ataxia and chronic renal failure, and is usually measured by Electrospray Ionization-Mass Spectrometry (ESI-MS), but protocols for Liquid Chromatography-Fluorescence (LC-FL) have also been developed.[16]

1.2. Thiols

1.2.1. Definition and biological significance

Thiols are organic compounds that contain a sulphydryl group (-SH). Also known as mercaptans, these compounds are a part of the antioxidant defense system and help control the oxidative status of cells.[19] The thiol group is very reactive due to the anion thiolate which is one of the strongest biological nucleophiles, so it participates in several reactions.[20] Disulfides (-S-S-) are less reactive than the reduced thiols, but are also important, as they contribute to protein tertiary and quaternary structure.[21,22] In proteins, they are also involved in stability and can be responsible for activity, signal transduction, protein folding and assembly of multi-protein complexes.[20,23] Due to their importance, highly conserved cysteine residues are conserved in proteins more so than any other amino acid, which indicates a pattern of maintaining these residues when they are of structural importance and eliminating them when they are not[24]. The formation of protein disulfide bonds is possible due to thiol/disulfide exchange and condensation of a sulfenic acid and a thiol[25,26].

Under oxidative stress, thiols are oxidized into their disulfides in order to protect the cells from the damage caused by ROS. This changes the redox potential of the cells, making them more oxidative. To avoid this, the disulfides can either be extracted from the cells, be (re-)reduced by the action of reductases and nicotinamide adenine dinucleotide phosphate and its reduced form ($NADP^+$/NADPH)[17] or form mixed disulfides with the thiols of proteins[25,27]. These mixed disulfides with proteins can be responsible for regulation of enzymes and play a part in signaling processes[24].

The oxidation of protein thiols with low pK_a can lead to the formation of sulfenic acid (R-SOH), which is highly reactive and will combine with a thiol to form a disulfide[24,28]. Alternatively, it can also react with amines and amides, forming sulfenamides (R-S-N-R'_2). Additionally, the sulfenic

acid molecules can suffer further irreversible oxidation, resulting in the formation of sulfinic acid ($R\text{-}SO_2H$) and sulfonic acid ($R\text{-}SO_3H$)[24]. In this work, only disulfides are considered as products of thiol oxidation because the other oxidation states are rarely detected in the free thiols, and are more common in protein thiols[26].

The most commonly studied low molecular mass thiols are cysteine, glutathione, homocysteine and N-acetylcysteine, and their corresponding disulfides, are shown in **Figure 1-1**. These compounds are important parts of the transsulfuration pathway, shown in **Figure 1-2**.

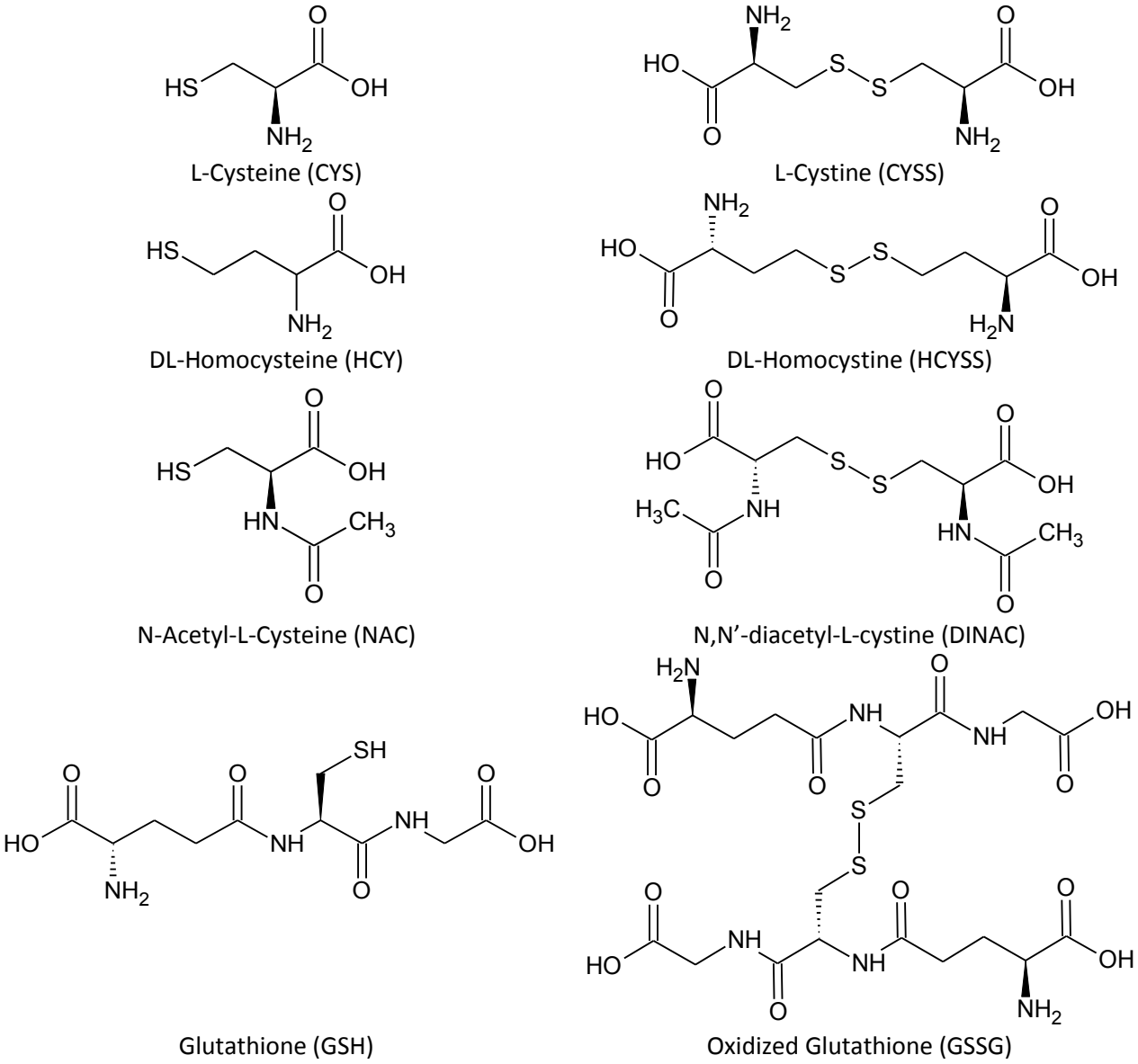


Figure 1-1 – Main biological thiols and their disulfides.

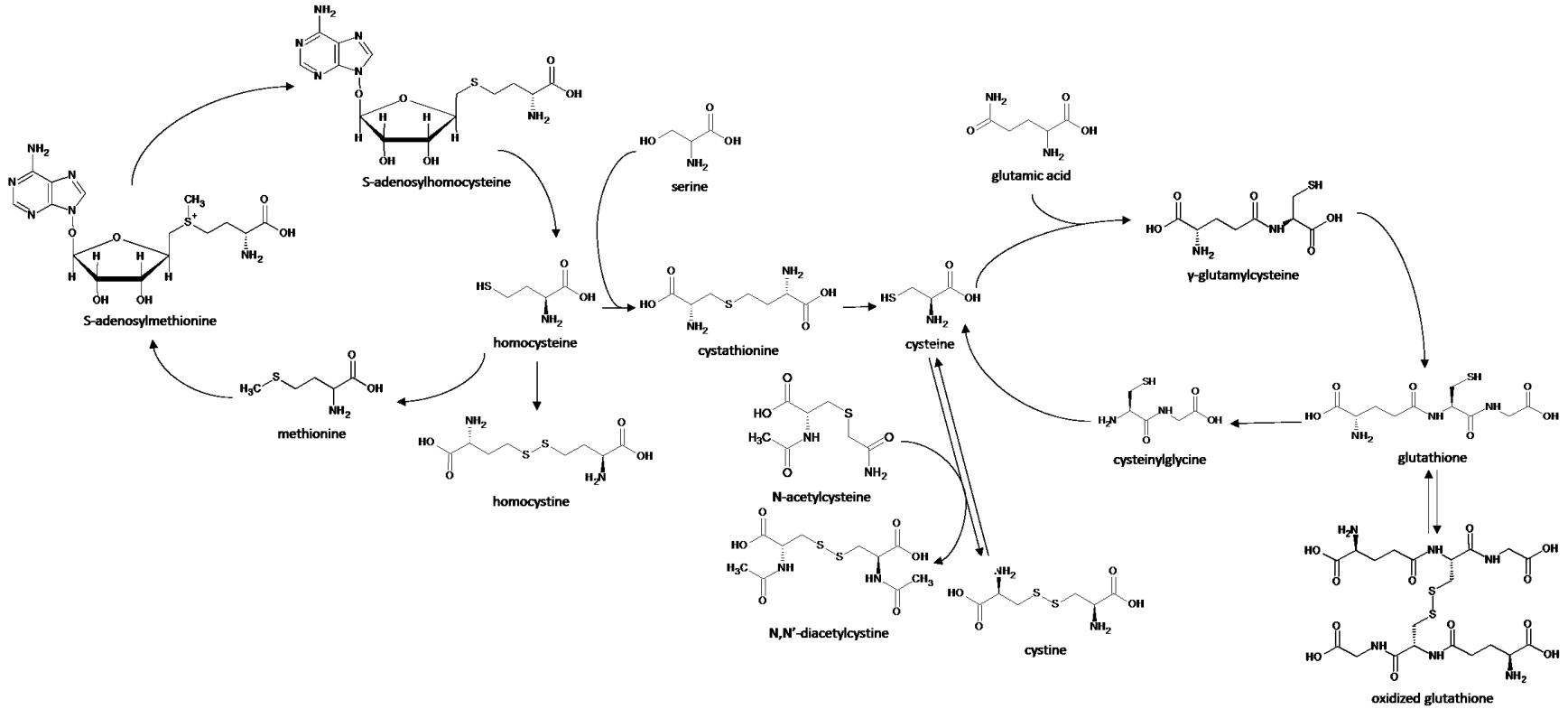


Figure 1-2 – Transsulfuration pathway.[29–32]

1.2.2. Cysteine

Cysteine (CYS) is one of the essential amino acids, and can be synthesized in the liver. It is used for protein synthesis, detoxification and other metabolic processes, including the synthesis of glutathione.[19] Its concentration in human red blood cells (RBC) is around 5 μM (~600 ng/mL)[33]. In plasma it is mostly present as its disulfide, cystine (CYSS)(150-360 μM), whereas free cysteine concentrations correspond to about 10 μM.[33] Intracellular concentration of cysteine ranges from 30-250 μM.[34] While elevated levels of cysteine are related to neurotoxicity, low levels are associated with liver damage, skin lesions and weakness, among other health problems.[34,35] Cystine is poorly soluble and can crystalize in cells, causing damage in patients with nephropathic cystinosis.[19]

1.2.3. Glutathione

Glutathione (GSH) is a tripeptide composed of the amino acids cysteine, glutamic acid and glycine, with a characteristic γ-glutamyl bond that makes it a trapping agent. It is the main non-protein thiol present in cells, existing at millimolar (0.5 – 10 mM, 0.15 – 0.3 g/L) intracellular concentrations, of which 10-15% is found in the mitochondria.[36] Glutathione exists mainly in its reduced form, and is oxidized during oxidative stress, forming the disulfide (GSSG). The disulfide can, in turn, be reduced by glutathione reductase in the presence of NADPH, reforming the GSH.

The thiol moiety allows glutathione to participate in various reducing reactions in the cell, including reduction of hydrogen peroxide, dehydroascorbate to ascorbate and α-tocopherol (vitamin E).[36] It is also important to maintain the free thiols of proteins, allowing them to function normally by scavenging free radicals.[18,36]

Oxidized glutathione is normally present at less than 5% of total glutathione, and the excess is exported from the cells.[1] Ratios (GSH/GSSG) have been reported to decrease from higher than 100 in resting cells to lower than 10 in oxidative stress models.[36]

1.2.4. Homocysteine

Homocysteine (HCY) is formed by demethylation of methionine, and is a precursor of cysteine. As such, it can be produced in large quantities, but is usually kept in low concentrations of around 5-15 μM in plasma.[19] Additionally, only around 2% of homocysteine is found in its reduced form, with most of it oxidized in its disulfide (homocystine - HCYSS), mixed disulfides with other thiols such as cysteine, and bound to proteins.[37] The increase of total homocysteine in plasma,

hyperhomocysteinemia, is considered a marker for oxidative stress due to association with circulation diseases, such as stroke, thrombosis and occlusive vascular disease[9], as well as increased risk of Alzheimer's disease, dementia, inflammatory bowel disease, complications during pregnancy and osteoporosis.[35,37,38]

1.2.5. N-Acetylcysteine

This acetylated version of cysteine, N-Acetylcysteine (NAC), is a common mucolytic agent, administered orally or intravenously to loosen secretions and treat cystic fibrosis, chronic obstructive pulmonary disease, diabetes mellitus and AIDS.[19,39] While it is present in small concentrations in plasma (around 3 μM), it can also be used to increase the intracellular concentration of cysteine and glutathione, by reducing the plasma cystine to cysteine through thiol-disulfide exchange, which can then be used to synthesize glutathione in the cells.[33,40]

1.2.6. Analysis of biological thiols

There are several challenges to the analysis of thiols in biological matrices. The first is their lack of chromophores and fluorophores, which makes derivatization crucial unless analysis is done using mass spectrometry or electrochemical methods.[36] Secondly, there is a large difference – orders of magnitude (nM to low mM) – in the concentration of different thiols and their disulfides, and developing an analytical method that is reliable (robust, sensitive) across various orders of magnitude can be problematic. This wide concentration range also poses a challenge since these compounds must be effectively extracted from the matrix. A third challenge is the instability of the reduced thiols and their tendency to oxidize during sample manipulation[21]. The autoxidation of thiols occurs because the cells maintain a reducing environment to keep the thiols – especially GSH – in their reduced form. During preparation of the samples, the thiols are exposed to more oxidative conditions, which causes them to form disulfides. This can lead to errors in interpretation of the results[40], since the autoxidation leads to an increase in the concentration of disulfides, with a decrease in the concentration of the reduced thiols.

To overcome these challenges, various strategies have been developed over the years. The common approach for quantifying thiols and their disulfides involves preparation and analysis of two samples (**Figure 1-3**). In the first sample, reduced thiols react with a reagent that allows for their detection and the sample is analyzed. The second sample is initially treated to protect the reduced thiols. The disulfides are then reduced forming new thiols, which are reacted to allow their detection. It is important that the reagent used to protect the thiol group react faster than

the disulfide bond can be formed, to avoid autoxidation, and that it is removed prior to the reduction, so that it does not react with the reduced disulfide groups.[20]

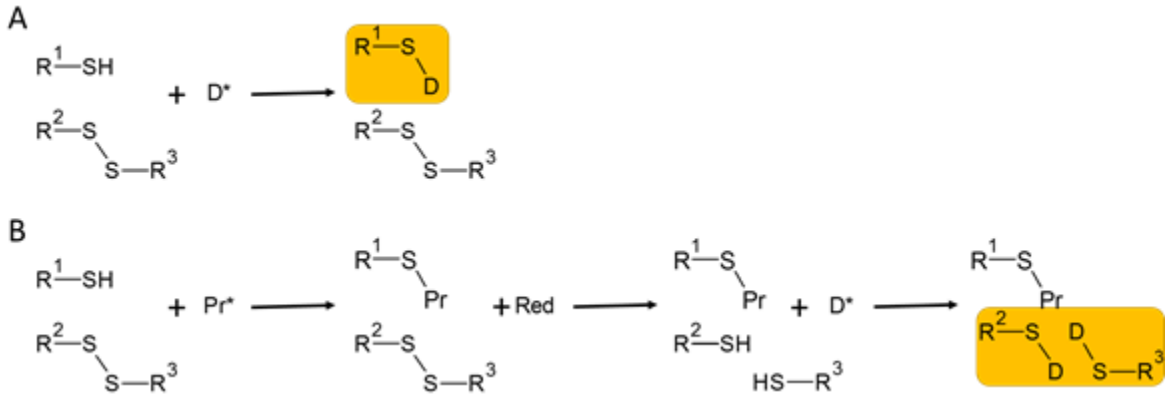


Figure 1-3 – The common strategy for analyzing thiols (A) and their disulfides (B) typically involves three reagents: a derivatizing reagent (D*) makes the thiol-derivatizing agent product detectable by the technique of interest, while a protecting reagent (Pr*) allows safe reduction (Red) of the disulfide prior to derivatization.

Many reagents are available for thiol analysis. Below is an overview of the most common reagents used for thiol detection. By far, the most common reagents react with the thiol or amine moiety present in the biological thiols to allow detection by Ultraviolet/Visible (UV-Vis) or fluorescence spectroscopy. Mass spectrometry and electrochemical analysis have also been used.

1.2.6.1. UV-Vis

Out of the reagents that are used to add chromophores to biological thiols, **Ellman's reagent (5,5'-dithio-(bis-2-nitrobenzoic acid), DTNB, Figure 1-4A)** is the most common.[20]. The reaction mechanism of **4,4'-dithiodipyridine (4-DPS, Figure 1-4C)** is similar to that of DTNB in the sense that both of them have a highly oxidizing disulfide bond that is displaced by the reduced thiol of interest in a thiol-disulfide exchange reaction favored by the stability of the leaving groups. Quantification of the thiols is possible because the leaving group absorbs light (λ = 412 nm for DTNB[20] and 324 nm for 4-DPS[41]) with large extinction coefficients, although other wavelengths have also been reported for DTNB: λ = 280 nm[42] and 330 nm.[43]

DTNB and 4-DPS are specific towards thiols, however, they will also react with reducing agents employed for the reduction of the double bonds[20] and with sulfites, which causes overestimation of thiol concentrations.[44] In choosing between these two reagents one should consider the intended application: DTNB is preferred if the sample has high absorbance at lower wavelengths

and if the analysis is done at neutral pH, since 4-DPS is less soluble in water. 4-DPS is preferred at lower pH values and if membrane permeability is desired.[44] Additionally, 4-DPS has a higher extinction coefficient than DTNB, making it more sensitive to thiols. The main disadvantage of 4-DPS is its short absorption wavelength, which increases the potential for background interference, which can be separated from the analytes of interest by using HPLC.

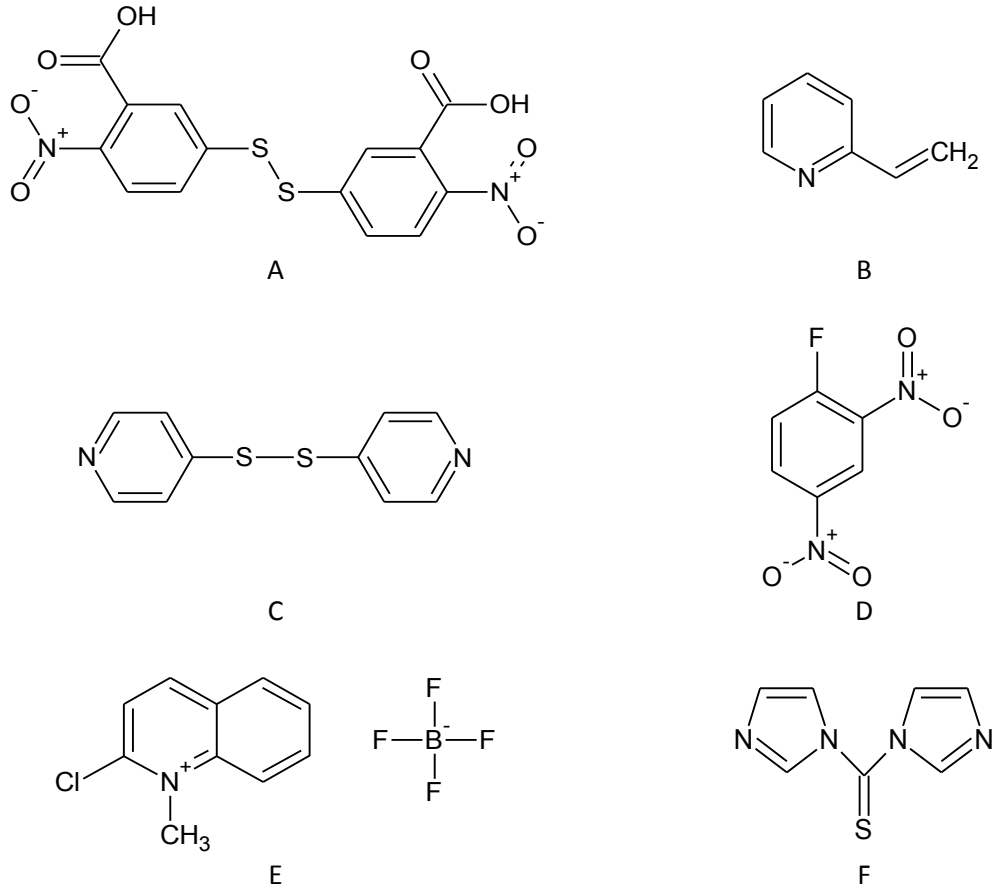


Figure 1-4 – Reagents for analyzing thiols by UV-Vis. A: 5,5'-dithio-(bis-2-nitrobenzoic acid), B: 2-vinylpyridine, C: 4,4'-dithiodipyridine, D: 1-fluoro-2,4-dinitrobenzene, E: 2-chloro-1-methylquinolinium tetrafluoroborate, F: 1,1'-thiocarbonyldiimidazole.

Ellman's reagent is also employed in a popular spectrophotometric assay that allows quantification of total glutathione discovered by Owens[45] and developed by Tietze.[46] In this assay, glutathione reductase is used to reduce oxidized glutathione, which is then quantified with DTNB, yielding the total glutathione content. By protecting the existing reduced glutathione prior to the reduction, it is possible to quantify oxidized glutathione. From these two analyses, the thiol ratio can be measured.[36] The main issue with this assay is that reduced thiols (RSH) present in the sample other than GSH will also be reacted. In the step that reduces GSSG and evaluates total

GSH, the reagent also reacts with other thiols (CYS, HCY, NAC…), therefore, this step is measuring GSSG+GSH+RSH. In the step that quantifies only GSH, GSH+RSH is being measured. Therefore, the concentration of GSH and GSSG is being overestimated in these steps unless chromatography is used to separate them from the other thiols present in the sample.[42] In addition, if mixed disulfides are formed (e.g. GSH-CYS), glutathione reductase is unable to reduce the disulfide, resulting in loss of signal.[47] Glutathione reductase is specific for the reduction of oxidized glutathione, so for an assay that allows the reduction of all disulfides, dithiothreitol (DTT) has been used.[42]

To protect the reduced thiols, N-ethyl maleimide (NEM) is commonly used, however, it inhibits the action of GR.[48] An alternative to it was presented by Griffith *et al.*: **2-vinylpyridine (2-VP, Figure 1-4B),** which reacts with the thiols by addition to the double bond and does not inhibit GR. However, the reaction constant is very low, in the range of 0.02-0.05 $M^{-1}s^{-1}$.[48] The studies that compared both reagents found GSSG levels up to 12 times higher for 2-VP because 2-VP allows artefactual oxidation of GSH during sample preparation.[48]

Sanger's Reagent (1-fluoro-2,4-dinitrobenzene, FDNB, Figure 1-4D) reacts with the amine groups of the thiols, after protection of the free thiol with an alkylating reagent to prevent autoxidation. Since it reacts with amines, it allows simultaneous detection of both reduced and oxidized thiols.[40] Detection of the thiols is possible by Ultraviolet absorption (UV, λ = 365 nm). Reaction times are long, requiring 4 hours in the dark[49], so it is important to protect the thiols prior to derivatization.

Other UV-tagging reagents that have been used include: **2-chloro-1-methylquinolinium tetrafluoroborate (CMQT, Figure 1-4E)**, which has been used to detect cysteamine, cysteine and glutathione in urine after reaction with the thiols under mild conditions and analysis at 355 nm[50]; **1,1'-thiocarbonyldiimidazole (TCDI, Figure 1-4F)**, which requires reaction with both amine and thiol to form a cyclic derivative that can be detected at wavelengths between 250 and 300 nm.[19]

1.2.6.2. Fluorescence

An ideal reagent for thiol analysis by fluorescence is one that is non-fluorescent, allows analysis at low concentrations, reacts specifically with thiols and is fast. Currently, there are no reagents that fulfill all of these requirements.[36] Despite this, many reagents have been used to quantify thiols.

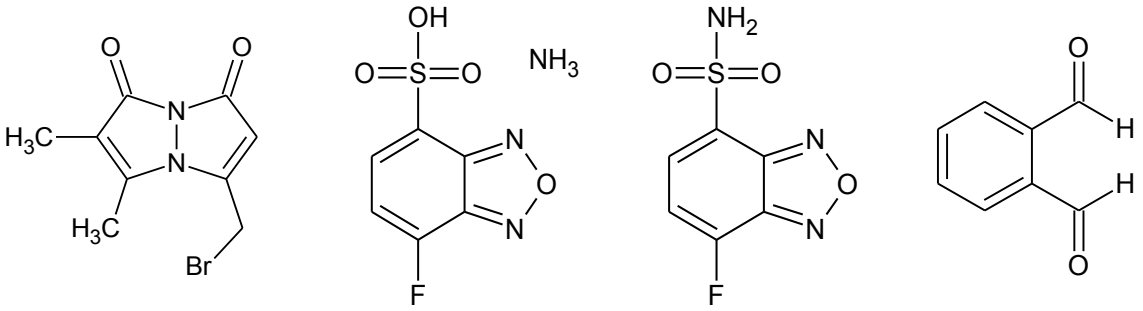


Figure 1-5 – Reagents for analyzing thiols by fluorescence. A: Monobromobimane (mBrB), B: Ammonium-7-fluoro-2,1,3-benzoxadiazole-4-sulfonate (SBD-F), C: 4-(aminosulfonyl)-7-fluoro-2,1,3-benzoxadiazole (ABD-F), D: Ortho-phthalaldehyde (OPA)

Bimanes and **halogenobenzofurazans** are reagents that react with the thiol moiety, but require basic pH for complete derivatization, which causes autoxidation of the thiols. They are also photosensitive, requiring derivatization and require storage in the dark.[20]

The product of reaction of **monobromobimane (mBrB, Figure 1-5A)** with a thiol can be analyzed by fluorescence ($\lambda_{excitation}$ = 400 nm, $\lambda_{emission}$ = 475 nm)[51], but the reagent itself is fluorescent.[36] The halogenobenzofurazan reagents **ammonium-7-fluoro-2,1,3-benzoxadiazole-4-sulfonate (SBD-F, Figure** 1-5**B)** and **4-(aminosulfonyl)-7-fluoro-2,1,3-benzoxadiazole (ABD-F, Figure 1-5C)** are not fluorescent[36], but form adducts that are (SBD-F: $\lambda_{excitation}$ = 380 nm, $\lambda_{emission}$ = 515 nm – values can be altered according to the thiol being analyzed[50], ABD-F: ($\lambda_{excitation}$ = 380 nm, $\lambda_{emission}$ = 515 nm). In a study with SBD-F that used cystamine dihydrochloride as an internal standard, limit of detection (LOD) of 5 pmol and limit of quantification (LOQ) of 15 pmol was obtained, which was declared more sensitive than Ellman's Reagent.[52] ABD-F has been used to detect cysteine residues in proteins.[50]

Neither class is able to react with disulfides[35,53,54], which require reduction. Sodium borohydride ($NaBH_4$) has been used for mBrB analysis, while tributylphosphine (TBP)[50] or Tris(2-carboxyethyl)phosphine (TCEP)[52] are mentioned for SBD-F, as the thiol group in Dithiothreitol (DTT) is known to also react with the reagent.[50]

mBrB adducts are unstable, undergoing hydrolysis and forming degradation products that are also fluorescent.[35,53] Other disadvantages of this reagent are its instability at room temperature and in water, as well as impurities.[35] A disadvantage of ABD-F is that it has been reported to not react specifically with thiol groups, but also with -OH and -NH_3 of tyrosine.[55]

A five-step protocol was proposed by Svardal *et al.*[51] to quantify reduced (GSH), oxidized (GSSG or mixed disulfides – GSSR) and protein-bound glutathione (ProSSG) in plasma with mBrB. Total glutathione (GSH, GSSG, GSSR, ProSSG) was determined after reduction with sodium borohydride ($NaBH_4$) and reaction with mBrB. For quantification of free oxidized thiols (GSSG, GSSR, ProSSG), reduced glutathione was reacted with NEM for protection prior to the reduction of oxidized glutathione with $NaBH_4$ and derivatization with mBrB. Excess NEM in this fraction is neutralized by $NaBH_4$, ensuring it does not react with reduced thiols. Total free GSH (not in protein – GSH, GSSG, GSSR) was determined by reaction with mBrB. Protein bound glutathione (ProSSG) was determined by derivatization of only protein pellet fraction with mBrB, while free GSH (GSH) was determined by analysis of protein-free supernatant with mBrB.

Ortho-phthalaldehyde (OPA, Figure 1-5D) reacts with thiols in the presence of amines to form a highly fluorescent product ($\lambda_{excitation}$ = 338 nm, $\lambda_{emission}$ = 458 nm).[56] It requires mild derivatization conditions, but basic pH (9.5 – 12) is commonly used[47] since it provides maximum fluorescence yield. However, at these pH values, autoxidation may occur. In order to allow analysis of the disulfides, they can be reduced with DTT[56] or another thiol (usually 2-mercaptoethanol) can be added.[36] However, CYS and NAC have also been used as a thiol additive to allow quantification of amines with OPA[57], which leads to the conclusion that the reagent can react with different thiols. To avoid this problem, this reagent is more suited to post-column derivatization.[58] The stability of the GSH-OPA derivative is controversial, with some reports declaring it to be stable[47,56], while others report it is unstable[57,58].

Noctor *et al.*[47] compared OPA to mBrB and DTNB for the analysis of thiols in leaves. They report that the thiol-specific reagents (mBrB and DTNB) suffer from signal instability over time, while GSH-OPA derivatives were found to be stable. They also state OPA has the added advantage of requiring no prior stabilization of the thiol groups, since the reaction requires both thiol and amine moieties. However, in the assay reported, it is not possible to measure thiol ratios, since the reaction with OPA involves a reduction step.

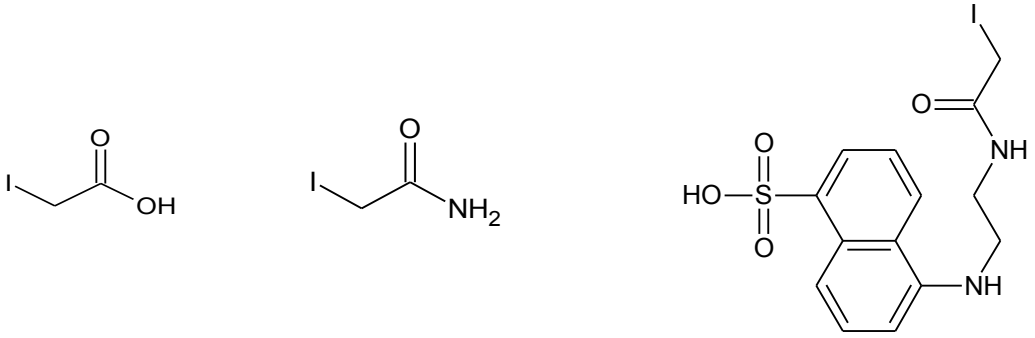
The main disadvantage of UV-Vis based techniques is the possibility of interference by other compounds that absorb the wavelength used in the assay. For instance, this has been observed by Willig *et al.*[59] in the assay with DTNB. Fluorescence, on the other hand, tends to be more selective, since it will detect only a sample that both absorbs and emits at the specific wavelengths being

used, which limits the number of interferents. Most fluorescent tags, however, tend to require higher pH for reaction, which can lead to autoxidation of the thiols.

In conclusion, both UV-Vis and fluorescence reagents have their advantages and disadvantages, and great care must be taken when choosing one of these reagents for thiol quantification. Out of the reagents covered above, the reaction with FDNB seems to be the least problematic, with no disadvantage other than slow reaction times, which can be accepted if the thiol moiety is protected with a fast-reacting alkylating reagent, such as NEM.

1.2.6.3. Derivatization with alkylating reagents

Alkylating reagents are typically used with the goal of protecting the thiol moiety during reduction of the disulfide bonds for quantification of oxidized thiols[20] (**Figure 1-3** – Pr*). They do not allow detection by UV or Fluorescence as they do not have a chromophore. However, with the use of mass spectrometry as a detection technique, thiol derivatization is needed only to prevent autoxidation, allowing the use of these reagents.



A B C

Figure 1-6 – Iodine-based derivatizing reagents. A: iodoacetic acid, B: iodoacetamide, C: 1,5-I-AEDANS

The iodine-based reagents **iodoacetic acid (IAA, Figure 1-6A)** and **iodoacetamide (IAM, Figure 1-6B),** are commonly used to protect the thiols from autoxidation by forming the carboxymethyl and carboxyamidomethyl derivatives, respectively. The reaction of IAA with thiols requires 15 min to 1 hour[1] at room temperature for maximum yield[49], and due to the reagents' sensitivity to light, the reaction must be carried out in the dark.[20] At physiological pH, IAA has a negative charge, which is not the case for IAM. This implies that only IAM can penetrate cellular membranes[20], allowing it to react with intracellular thiols prior to their extraction and therefore protecting them from oxidation.

The reaction of IAA and IAM with thiols is a nucleophilic reaction, therefore, it depends on the deprotonation of the thiols to form the thiolate anion. For this reason, the pH required for the reaction is more basic, such as 8.0. At neutral pH, these reagents are reported to have reacted with the hydroxyl, amine and imidazole groups of tyrosine, lysine and histidine, respectively, indicating inadequate selectivity.[20]

Some analogues of iodoacetamide have been synthesized for fluorescence-based detection of thiols in proteins, of which **1,5-I-AEDANS (5-({2-[iodoacetyl]amino}ethyl}amino)naphthalene-1-sulfonic acid**, **Figure 1-6C**) is one of the most popular since it has the advantage of being soluble in water and being selective for cysteine residues, which helps in quantification and peptide sequencing.[19]

Derivatization with **maleimides (Figure 1-7A)** is a Michael addition of the thiol to the double bond of the maleimide. The reaction progress can be monitored by absorption at 302 nm, where the enone function of the maleimide absorbs. As the reagent is consumed, it no longer absorbs and neither does the derivatized thiol. The reaction of N-ethyl maleimide (**Figure 1-7**B), the most common of maleimide reagents, with thiols is faster than iodoacetamide or iodoacetic acid.[20] One of the advantages of using this reagent is that it can permeate cell membranes[20], which allows derivatization of the thiols before extraction, decreasing autoxidation occurring due to sample manipulation.

If the thiol has a chiral carbon, which is usually the case with amino acids, reaction with a maleimide will result in the formation of a new chiral center, and therefore, the possibility of two diastereoisomers.[60] These peaks are usually separated by chromatography, which implies an additional data processing step, as the areas of the two peaks should be added for quantification.[60]

This reaction is also dependent on the thiolate anion[20], which means that at higher pHs it will be faster. However, reaction at high pH is not recommended with maleimide reagents, since they undergo hydrolysis forming an acid and decreasing the reactivity with thiols. Another problem of using higher pH is that the maleimide will react with deprotonated amines.[40] The hydrolysis of the maleimide ring can also occur after derivatization, with the cleavage occurring at either of the N-C ring bonds[61]. When this occurs, the carboxylic acid can react with the α-amine in an intramolecular transamidation, forming a new cyclic compound.[62] This reaction has been observed in cysteine

(formation of a 6-member ring), but not in acetylcysteine or glutathione, due to the protection of the amine by the acetyl group and inaccessibility of amine, respectively.[62]

NEM is known to inhibit the action of glutathione reductase[63], which is why it is removed by solvent extraction or solid-phase extraction[40] prior to addition of GR in the assays where NEM is used to protect thiols before reduction by GR. However, the inhibition of GR can also be viewed as an advantage, since it prevents artefactual reduction of GSSG, which would result in higher ratios.

Sentellas *et al.* compared IAA, NEM and DTNB for thiol protection using Liquid Chromatography-Tandem Mass Spectrometry (LC-MS/MS).[1] They preferred IAA due to poor chromatographic performance of the DTNB derivative, and the separation of the NEM derivative peak into two peaks due to separation of the diastereoisomers. However, this comparison did not evaluate the reagents after reaction for the same time and at the same conditions (light/dark and temperature), instead using conditions as described in literature. Additionally, the authors report GSH autoxidation of 8.7%, but the GSSG values reported fall within this window, meaning they could be due to autoxidation instead of actually being present in the sample.

The high reactivity of maleimides with thiols led to the use of maleimide reagents that make the thiol groups fluorescent after reaction. Examples of this type of maleimides are[8,19,40,64]: (R)-(+)-N-(1-Phenylethyl)maleimide (NPEM, **Figure 1-7C**), N-(9-acridinyl)maleimide (NAM, **Figure 1-7D**), N-(p-(2-(6-dimethylamino)benzofuranylphenyl)maleimide (Akiyama's reagent, DBPM, **Figure 1-7E**), N-[p-(2-benzimidazolyl)phenyl]maleimide (BIPM, **Figure 1-7F**), Fluorescein-5-maleimide (FM, **Figure 1-7G**), 9-acetoxy-2-(4-(2,5-dihydro-2,5-dioxo-1H-pyrrol-1-yl)-phenyl)-3-oxo-3H-naphtho[2,1-*b*]pyran (ThioGlo™3, **Figure 1-7H**), N-(1-pyrenyl)-maleimide (NPM, **Figure 1-7I**), N-(2-acridonyl)maleimide (MIAC, **Figure 1-7J**), 2-(4-N-maleimidophenyl)-6-methoxybenzofuran, **Figure 1-7K**, 1-[3-(4-maleimidylphenoxy)propyl]trimethylammonium bromide (MPPTAB, **Figure 1-7L**).

Despite the interest in the measurement of the thiol/disulfide ratio, there is still no consensus as to the protocol for their analysis. A direct consequence of this problem is the large variation in the values reported in literature. For example, Giustarini et al.[48] compared the GSSG values reported over time in samples that were treated with NEM or 2-VP during different steps of the analysis protocol. They showed that lack of treatment, treatment after lysis or during sample acidification leads to the differences in the concentrations reported. Additionally, when NEM is used prior to acidification, GSSG values were consistently low throughout the years. This approach, however, seems to have been abandoned in most recent works resulting in erroneously high GSSG values.

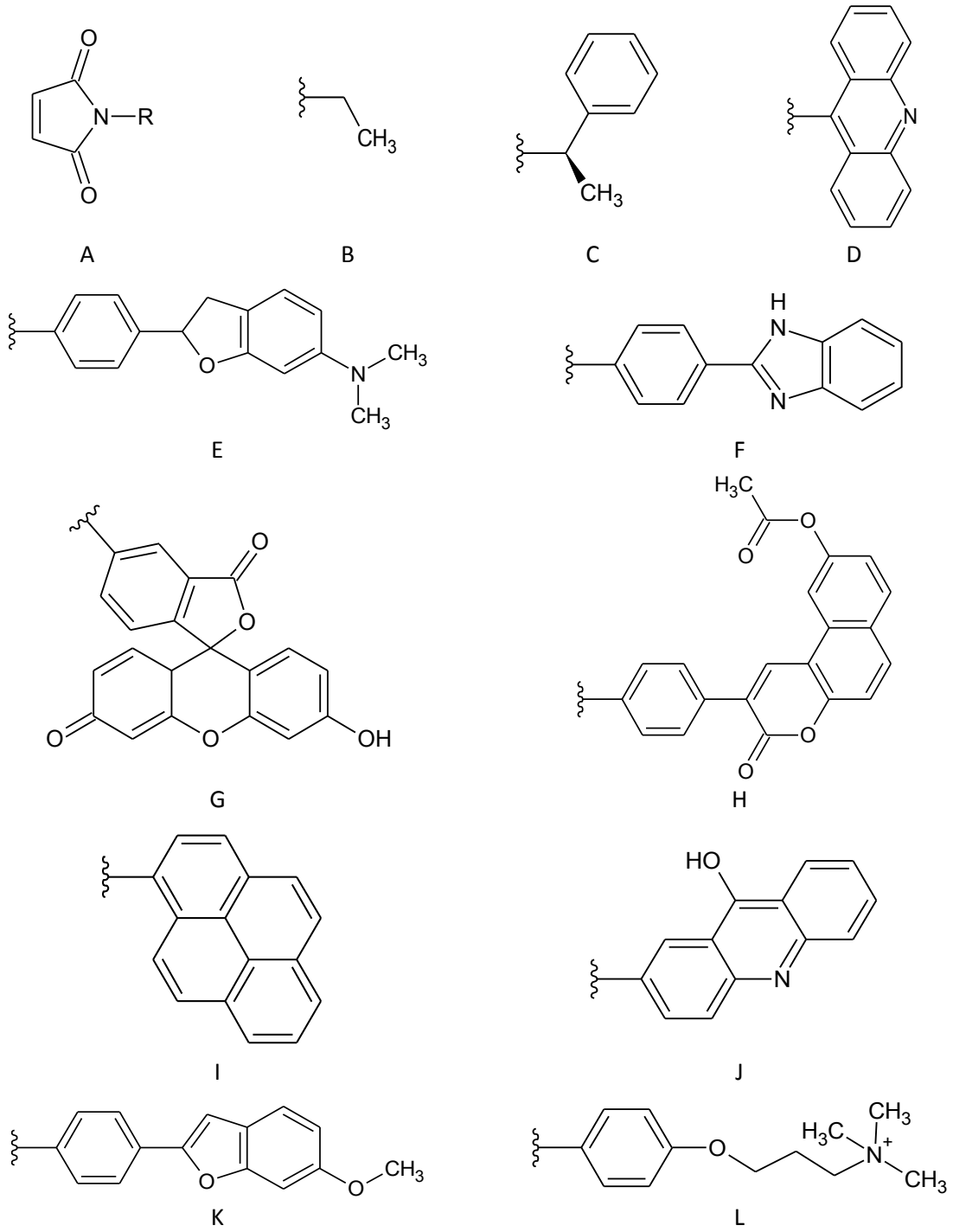


Figure 1-7 – A: Maleimide backbone, with different substitutions at the N-. The R groups correspond to B: N-ethyl maleimide, C: (R)-(+)-N-(1-Phenylethyl)maleimide, D: N-(9-acridinyl)maleimide, E: N-(p-(2-(6-dimethylamino)benzofuranylphenyl)maleimide, F: N-[p-(2-benzimidazolyl)phenyl] maleimide, G: Fluorescein-5-maleimide, H: ThioGlo™3 – 9-acetoxy-2-(4-(2,5-dihydro-2,5-dioxo-1H-pyrrol-1-yl)-phenyl)-3-oxo-3H-naphtho[2,1-b]pyran, I: N-(1-pyrenyl)-

maleimide, J: N-(2-acridonyl)maleimide, K: 2-(4-N-maleimidophenyl)-6-methoxybenzofuran, L: 1-[3-(4-maleimidyl phenoxy)propyl]trimethylammonium bromide.

1.2.6.4. Summary of derivatization reagents

As shown in the sections above, there are many reagents used for derivatization and analysis of thiols by UV and Fluorescence. The most common reagents and some of their properties, such as permeability of the cellular membrane, selectivity for thiols, light sensitivity and derivative stability, are summarized in **Table 1-1.** There is no reagent that fulfills all the criteria mentioned, so the choice of reagent should balance the advantages and disadvantages for intended application as much as possible. Additionally, the knowledge of reagent drawbacks should be used to minimize the negative impact on the analysis. **Table 1-2** contains a summary of the reagents used mainly to protect the thiols from autoxidation, but that do not provide them with the moieties that allow their detection by UV-Vis or Fluorescence. These reagents are commonly used in combination with the ones mentioned in **Table 1-1** to protect the thiols as described in **Figure 1-3.**

1.2.1. Mass spectrometry studies for thiol analysis

Tandem mass spectrometric methods have the advantages of having high selectivity, sensitivity and speed.[40] Selectivity arises from (mass) selection of the parent ion, which is then fragmented and specific daughter fragments are (mass) analyzed by the detector, in multiple reaction monitoring (MRM).[37] When used in combination with chromatographic separations, mass spectrometry becomes even more powerful, since it provides an additional "dimension" of separation between the many compounds present in a biological sample.

One of the main advantages of mass spectrometry as a detector is the ability to identify unknowns based on their parent ion and fragmentation patterns. In Hammermeister *et al.*[65], glutathione, cysteine and their disulfides were studied in rainbow trout liver by derivatization of amine groups with dansyl chloride (dansylation). The authors detected a peak that coeluted with GSSG or the internal standard (glutathione ethyl ester) that they could not identify initially, but using tandem mass spectrometry, it was identified as being due to derivatization of a single amine of oxidized glutathione (monodansylation), instead of the expected reaction with both amine groups.

Table 1-1 – Summary of derivatization reagents used for thiol analysis by UV-Vis and Fluorescence. Orange cells correspond to characteristics that are undesirable in the reagent, while green cells correspond to desirable characteristics.

Reagent	Analysis by	Reaction	Reaction conditions	Cell permeability	Selective for thiols?	Sensitive to light?	Derivative stable?
DTNB	UV-Vis	Thiol-disulfide exchange	Neutral pH	No	Yes	No	No
4-DPS	UV-Vis		Acidic pH (4.5)	Yes	Yes	No	No
FDNB	UV-Vis	Reacts with amine	Dark		No	Yes	
Bimane	FL	Nucleophilic substitution	Basic pH (>8)	Yes	No	Yes	No
OPA	FL	Reacts with amine in presence of thiol	Basic pH (9.5-12)	Yes	No	No	Conflicting reports
ABD-F, SBD-F	FL	Nucleophilic substitution	Basic pH, 60°C		Yes	Yes	1 week

Table 1-2 - Summary of derivatization reagents used to protect thiols prior to analysis. Orange cells correspond to characteristics that are undesirable in the reagent, while green cells correspond to desirable characteristics.

Reagent	Reaction	Reaction conditions	Cell permeability	Selective for thiols?	Sensitive to light?	Derivative stable?
2-vinylpyridine	Addition to double bond	long reaction times	No	Yes	Yes	
N-substituted maleimides	Addition to double bond	Neutral pH (6-7)	Yes (depends on substitution)	No*	No	CYS forms cyclic Varies with substitution
Iodoacetic acid	Nucleophilic substitution	Basic (>8), long reaction times	No	No	Yes	
Iodoacetamide	Nucleophilic substitution	Basic (>8), long reaction times	Yes	No	Yes	

* Selectivity of maleimide reagents depends on pH of the reaction – at neutral pH, the reaction is selective, while at basic pH they will also react with amines.

The detection limits obtained by MS have improved over the years, as instrumentation has improved. One of the first multi-thiol studies performed using mass spectrometry was performed by Bouligand *et al.* with MRM in a Triple Quadrupole MS with ESI (electrospray ionization).[66] They used IAA to protect the reduced thiols extracted from mice liver samples and reversed-phase liquid chromatography (RPLC). They report limit of quantification (LOQ) as 100 ng/mL for all standards, except for cystine (CYSS) and homocystine (HCYSS), which were 200 ng/mL, which were not detected in their samples. One of the shortcomings of this methodology is the internal standard used, glutathione ethyl ester, which hydrolyzes under acidic conditions to form glutathione, which is one of the target molecules.[67] Using dansylation, Hammermeister *et al.*[65] obtained detection limits in fluorescence of about 1 pmoles (S/N = 3) for GSH, CYS and their disulfides, while using mass spectrometry they exceeded 0.5 pmoles (S/N = 10).[65]

Iwasaki *et al.* analyzed thiols in saliva after separation using hydrophilic interaction liquid chromatography (HILIC)[67], since they are better retained in this type of chromatography than in reversed phase. Mass analysis and detection was done with a triple quadrupole mass spectrometer from Shimadzu in selected ion monitoring (SIM). The reduced thiols were protected by reaction with NEM (30 min, room temperature), and solid phase extraction (SPE) was performed with a mixed phase mode (Oasis MAX) to concentrate the analytes. Their method had recoveries of 87.3(±10.4)% for GSH, and 91.7(±10.3)% for GSSG when spiking 50 µM, and LOQ of 0.1 µM for glutathione and 1.0 µM for its disulfide (equivalent to 30.7 ng/mL and 612 ng/mL, respectively).[67]

Thiols from hepatocytes were derivatized with IAA and, after chromatographic separation using a C18 column, were analyzed in a hybrid triple quadrupole/linear ion trap mass spectrometer[1], which provided a LOQ for GSH of 0.05 µM (15.4 ng/mL) and 0.1 µM (61.3 ng/mL) for GSSG. Using a LTQ-Orbitrap, another study obtained limits of detection of 1 nM (or 0.31 ng/mL) for glutathione in standard solutions for analysis of thiols in the sulfur pathway in yeast.[32]

A different approach by Seiwert *et al.*, employed two ferrocene-based maleimides they developed to differentially label reduced and oxidized thiols in urine.[61] Samples were treated with N-2-(ferroceneethyl)maleimide (FEM), which reacted with the free reduced thiols. Excess reagent was eliminated with 4-acetamidothiophenol. Disulfides were then reduced with tris(2-carboxyethyl)phosphine hydrochloride and treated with ferrocenecarboxylic acid (2-maleimidoyl) ethylamide (FMEA). This way, thiol-FEM adducts originated from reduced thiols, while thiol-FMEA

adducts came from the oxidized thiols. For both reagents, derivatization was considered quantitative after 3 min of reaction.

Similar to this approach, Wang *et al*.[68] developed a method for analyzing 124 amine-containing metabolites, including glutathione, cysteine and their disulfides. They reacted reduced thiols with NEM, which was trapped with 4-tert-butylbenzenethiol (tBBT). TCEP was used to reduce disulfides, which were then reacted with 5-aminoisoquinolyl-N-hydroxysuccinimidylcarbamate (5-AIQC) within 10 min. Therefore, reduced thiols formed the 5-AIQC-thiol-NEM adduct, while disulfides formed the 5-AIQC-thiol adducts. The different adducts had different LODs, but they were all in the fmol range for GSH, CYS and HCY. The main disadvantage of differentially labeling reduced and oxidized thiols is the additional sample preparation steps for quenching of the first label, reduction of the disulfides and derivatization of the newly (re-)formed reduced thiols. Additionally, there are two reagents whose stability and side reactions should be considered and evaluated, instead of one. Finally, it is indispensable that the first label be completely quenched, otherwise it will react with the reduced disulfides, introducing systematic error. In protocols where the sample is separated into two aliquots and treated for quantification of reduced and oxidized thiols, this issue is minimized.

The efficiency of seven different N-substituted maleimides was studied by D'Agostino *et al*.[8] with the goal of optimizing the detection of reduced thiols using CE-MS. The authors reported that NTAM (N-[2-(Trimethylammonium)-ethyl]maleimide chloride) provided the best signal enhancement for detection in ESI-MS, while NPEM ((R)-(+)-N-(1-Phenylethyl)maleimide) obtained similar results. Both outperformed NEM and the other maleimides included in that study by about two-fold. The authors chose NTAM because its thiol adducts are divalent, which enhanced mobility in CE (capillary electrophoresis), although this reagent required two additional quenching steps. LOQs of 0.02 µM (6.1 ng/mL), 0.05 µM (6.1 ng/mL) and 0.025 µM (3.4 ng/mL), for GSH, CYS and HCY, respectively, were obtained. In that work, disulfides were analyzed with no pre-treatment, however, the authors do not report reduced/oxidized thiol ratios.

The main advantage of mass spectrometry is that it allows simultaneous quantitation of both reduced and oxidized thiols. The protocols that involve differential labelling have the advantage of improving the retention of oxidized thiols in reversed phase chromatography, but must add sample preparation steps for reduction and labelling of disulfides. The alternative protocols require the analysis of two separate samples: one for determination of total thiol content, and

another for determination of oxidized thiols, which has the advantage of less sample preparation, but requires more analysis time to process the additional samples and increases the error/uncertainty of the determination because of the need for two analyses.

1.3. Immune cells

There are several mechanisms used by eukaryotic organisms, such as animals, plants, fungi and algae, to protect themselves from pathogens. The combination of these mechanisms forms the immune system, which can be separated into two main parts: the innate and the adaptive immune systems.

The innate immune system provides a first line of defense and can be divided into four mechanisms: anatomic, physiological, inflammatory and phagocytic barriers.[69] Examples of anatomic barriers are the skin and mucous membranes, which can physically hinder entry of microbes and pathogens. The temperature and pH of the body destroy the integrity of some pathogens and are examples of physiological barriers. In an inflammatory barrier, the infected tissues leak vascular fluid which contains proteins that have antibacterial properties. Finally, phagocytic barriers are provided by the specialized cells that can internalize the pathogens and neutralize their threat.[69]

The adaptive immune system is the organism's response to specific threats, and it has four characteristics: antigenic specificity, immunologic memory, diversity and self/nonself recognition.[69] Antigenic specificity is the capacity of antibodies to differentiate between different antigens. After recognition of the threat and responding to it, if exposed again to the threat, the system can respond with increased activity due to the immunologic memory it obtained after the first exposure. In a properly operating immune system, it can recognize various antigens and respond to the threats appropriately – it is capable of detecting whether the antigen is foreign or if it belongs to the organism, and will only eliminate the former.[69]

All of the cells of the immune system come from the hematopoietic stem cells, which are self-renewing cells that can be differentiated through cytokines into the different cell types needed by the immune system. The cell types are: natural killer cells, Th and Tc cells, B cells, dendritic cells, monocytes and macrophages, neutrophils, eosinophils, basophils, platelets and erythrocytes.[69] Jurkat T cells are a non-adherent human cell line commonly used to study immune responses[69] such as signaling and inflammation due to their ability to imitate healthy and inflammatory T-

cells.[70] This cell line excretes interleukin-2 (IL-2), which has been recognized as essential for growing T-cells *in vitro*, and explains why they are easy to cultivate.[69] Due to these characteristics of the Jurkat cells, they were selected for the evaluation and optimization of the thiol assay.

1.4. Extraction protocols

To extract the metabolites from the cells, different procedures have been proposed in the literature. Typical sample preparation for metabolomics has the goal of maximizing extraction, while still being simple, fast and reproducible.[71] It is also important that the enzyme activity be quenched to preserve the metabolites as they are at the moment of extraction. The main steps for sample preparation of intracellular metabolites are: cell pellet collection, cell culture medium removal and pellet wash, cell lysis and extraction, and protein precipitation.

Collecting the cell pellet is straightforward for non-adherent cells: the cells are counted, usually using the trypan blue method, and then enough volume (cells) is centrifuged to pellet the number of cells desired. For adherent cells, they must first be trypsinized or scraped, suspended, counted, sampled and then they are centrifuged.[22,72]

It is important to remove the cell culture medium prior to lysis because it contains metabolites, including thiols, which would artificially increase the amount detected in the analysis. To remove the media, fast filtration with aid of vacuum can be performed on adherent cells[73]. Manual removal of the medium after pelleting is performed in non-adherent cells. Following removal of the media, the cells pellets are washed – usually in triplicate[18,29] – with phosphate-buffered saline (PBS)[22,74,75] or saline[18,49,76]; isotonic solutions are preferred to minimize perturbation of the cells and avoid leakage.[71] The effectiveness of the wash step and the occurrence of leakage can be evaluated by comparing the cell media composition to that of the wash and the cellular extract: if a certain metabolite is detected in the media and the extract, but not in the wash, the wash steps were sufficient and there was no leakage; if it is detected in all three samples, or just in the wash and extract, the wash step was either incomplete or there was leakage, in which case the wash step should be re-evaluated.

A wide range of extraction techniques are employed in the literature to extract metabolites from cells. To lyse the cells, most protocols will use a mixture of solvents like water[29,77–79], methanol[25,73-75], ethanol[32,80] and chloroform[79], but methods that induce shock to the cells, like freeze-thaw cycles[81], can also be used. Since it is common to combine the lysis and extraction

steps[71], the polarity of the metabolites of interest should be taken into account when defining which solvents should be used and in which proportion. A protocol that focuses on polar metabolites, for example, does not need to use chloroform, but might use liquid-liquid extraction (LLE) with methanol: water: chloroform (2.7:1:1.4, v/v) to remove the lipids from the sample.[79] Another factor that should be considered is the degradation of the analytes caused by a harsh extraction procedure. Rao *et al*[32], for example, found lower thiol/disulfide ratios after extraction by hot water, indicating that the thiols were autoxidized. This has led some groups to add a derivatization reagent to the lysis solvent[29,82], but at this step, the thiols are already exposed to oxidation.[48]

To inactivate proteins, cold solvents are preferred, and protein precipitation with trichloroacetic acid (TCA)[22], metaphosphoric acid[81], methanol[73] or acetonitrile[82] are typically done using these reagents at cold (4°C) or freezing temperatures (-80°C).

If the extracts are analyzed by LC-MS, it is common to include a step to reduce the amount of organic solvent to match the initial chromatographic conditions, such as dilution[29,82] or solvent evaporation under nitrogen[77,78] or vacuum[83,84] followed by reconstitution with a more adequate solvent.

1.4.1. Evaluation of cell extraction protocols

After defining the protocol used for sample preparation, it is important to consider the efficiency of the recovery of the metabolites of interest. This is done by analyzing three samples: the first is a sample that will provide the endogenous signal for the analyte; the second is a sample to which a known concentration of the analyte(s) of interest are added, also known as spiked, before the extraction protocol (spike before extraction or pre-extraction spike) is applied, and the third is a sample spiked with the same concentration as the previous sample, but the spike is done immediately before the samples are analyzed (spike before analysis or post-extraction spike).[85] The concentrations chosen for the spike should be close to the expected endogenous levels to ensure that the instrument will be able to differentiate between the spiked and non-spiked samples. A range of concentrations can be used to ensure that the method performs well across a wide range of concentrations.[85] To ensure that the spiking procedure will not change the matrix, it should be performed using a small volume of a concentrated standard. The ratio of the samples spiked before extraction and before analysis is then measured after subtracting the signal due to the endogenous analyte, as shown in **Equation 1-1**. This calculation of recovery removes the

matrix effect, giving only extraction recovery. Ideally, the recovery will be consistent across days and analysts, and is as close to 100% as possible.

$$\boldsymbol{Recovery}\ (\%) = \frac{\boldsymbol{Signal\ of\ sample\ spiked\ before\ extraction - endogenous\ signal}}{\boldsymbol{Signal\ of\ sample\ spiked\ before\ analysis - endogenous\ signal}} \cdot \mathbf{100}\%$$

Equation 1-1 – Calculation of extraction recovery

If the signal of pre-extraction spike sample is compared to the same concentration of standard prepared in solvent, then the "process efficiency" is calculated. This parameter includes both matrix effect and recovery[85], as shown in **Equation 1-2**.

$$Process\ efficiency\ (\%) = \frac{Signal\ of\ sample\ spiked\ before\ extraction - endogenous\ signal}{Signal\ of\ spiked\ standard\ in\ pure\ solvent} \cdot 100\%$$

Equation 1-2 – Calculation of process efficiency of a LC-MS assay for a given analyte

Also, important to consider is the matrix effect, the change of the signal caused by the presence of another metabolite or matrix component with the analyte of interest. In mass spectrometry, these compounds compete for the limited amount of charge in the ionization source and can cause ionization suppression or enhancement.[85] To quantify if this occurs, the signal of the analyte in presence and absence of matrix is compared, as shown in **Equation 1-3**. If the calculated values lie within 80-120%, then the sample is considered free of matrix effect.[85] Matrix effects due to sample preparation can also occur, but these are considered and evaluated in the calculation of recovery and process efficiency.

$$Ionization\ suppression\ (\%) = \frac{Signal\ of\ sample\ spiked\ before\ analysis - endogenous\ signal}{Signal\ of\ spiked\ standard\ in\ pure\ solvent} \cdot 100\%$$

Equation 1-3 – Calculation of ionization suppression or enhancement of a compound

To minimize or eliminate ionization suppression, a method providing improved sample clean-up could be used, or longer/different chromatographic conditions can be employed in order to minimize co-elution of analytes of interest with interfering matrix species. Internal standards can also be used to compensate for interferences, especially in targeted measurements.[85] For this approach to perform well, the internal standard must co-elute with the analyte of interest so that any changes in ionization of the analyte will also affect the internal standard. They should also have similar chemistry to the analyte, to ensure that they are affected by the matrix in a similar fashion. In mass spectrometry, isotopically labeled internal standards are ideal for this purpose

because they have the same physico-chemical properties as the analyte, only with a heavier isotope atom(s) in their structure. Therefore, they will co-elute with analyte and will suffer the same matrix effects during sample preparation and ionization, however, they are differentiated by the mass analyzer, allowing their use for correction of the matrix effect.

1.5. Outline and scope of the thesis

Given the extent to which oxidative stress can change the metabolism, it is no surprise the number of publications dedicated to studying it: over 3 thousand articles and reviews were published in the United States in 2016[86]. One of the pathways that is altered during the damage caused by reactive oxygen species is glutathione pathway, due to this thiol's importance in maintaining the ideal intracellular redox conditions and protecting the cell, it is often linked to oxidative stress studies, being mentioned in a third of the published literature[87]. The ratio between reduced and oxidized glutathione has been considered a biomarker for oxidative stress, however, there are many methods in literature describing how to measure it with little consensus among them. In particular, the manner in which the samples are treated to prevent autoxidation varies between studies, with a negative impact on the results obtained.[48]

Recognizing the importance of developing a method for accurate determination of the thiol ratios in cells, this thesis focuses on the optimization of the reaction conditions, as well as on the development of an extraction protocol that minimizes the metabolite changes during sample preparation.

In Chapter 2, the derivatization of thiols is studied with the goal of identifying, out of a suite of reagents that can penetrate cell membranes, one that can react completely with intracellular biological thiols. The criteria used to compare the reagents is their reaction efficiency, selectivity and derivative stability, followed by the signal enhancement in electrospray ionization, which helps to increase the sensitivity of the method.

In Chapter 3, based on the results obtained from derivatization of thiol standards, a protocol that allows derivatization of intracellular thiols in Jurkat cells was developed and optimized. The main feature of this protocol is the derivatization prior to lysis, with the goal of minimizing autoxidation caused by exposure of the thiols to an oxidative environment post-lysis. Additionally, it will improve the accuracy of the thiol/disulfide ratio measurement. This is innovative, since most protocols perform derivatization during lysis at the earliest. Other factors evaluated were the

removal of extracellular metabolites, with the aim of preventing contamination of the samples, the amount of derivatizing reagent added, and the effectiveness of the lysis/extraction solvent.

1.6. Objectives of the thesis

The goal of our method is to allow analysis of the thiol/disulfide ratio using a simple protocol that can be applied to immune cells. Independent of the method used for quantitation of the disulfide, protection of the reduced thiol should be done as early as possible, before sample manipulation leads to their oxidation. For this reason, fast reacting derivatizing reagents that can penetrate the cell membrane will be considered.

The overall objectives of the thesis are:

I. The selection of a cell-permeable derivatization reagent capable of protecting reduced thiols from autoxidation;
II. The optimization of reaction conditions to minimize any potential side reactions of the reagent chosen;
III. The development and optimization of the protocol for analysis of thiol/disulfide ratio as a readout of oxidative stress in immune cells.

2. Comparison of N-ethyl maleimide and N-(1-phenylethyl) maleimide for derivatization of biological thiols using LC-MS.

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2.1. Abstract

During oxidative stress, the reduced thiols present in the cell act as scavengers and reduce reactive oxygen species to protect the cells, while being oxidized in the process. For this reason, the ratio between reduced and oxidized thiols, mainly glutathione and oxidized glutathione, is one of the biomarkers used to evaluate oxidative stress. However, the measurement of this ratio is challenging because reduced thiols are easily oxidized during sample manipulation thereby altering the ratio. Derivatization is used to protect thiols from oxidation. The objectives of this work are to compare two cell permeable derivatizing agents: N-ethyl maleimide (NEM) and (R)-(+)-N-(1-Phenylethyl)maleimide) (NPEM) and to evaluate derivatization conditions to use in combination with a liquid chromatography – mass spectrometry assay for intracellular thiol ratio determination. Four thiols: cysteine, homocysteine, N-acetylcysteine and glutathione, were used to evaluate membrane-permeable derivatization reagents that would allow derivatization of intracellular thiols prior to extraction. The derivatized thiols were separated using PFP and biphenyl columns and analysis by MS allowed detailed evaluation not only of the derivatization efficiency and stability, but also formation of side products.

Using 1:10 ratio (thiol:derivatizing agent), 100% derivatization efficiency was obtained within 30 min for both agents tested with the exception of CYS-NEM, where 97% was obtained. NPEM provided better ionization of the thiols than NEM, with enhancement ranging from of 2.1X for GSH to 5.7X for CYS. However, in further evaluations of side-product formation, NPEM proved to be more unstable, leading to the formation of various side-products which hinder the accurate measurement of the thiol concentration. Double derivatization and ring opening were observed with NPEM, while with NEM side product formation was minimized. Both reagents show poor stability of CYS derivative due to time-related build-up of cyclic cysteine, with complete depletion of the CYS-maleimide peaks within 24h. Taking into account all evaluation criteria, NEM was selected as a more suitable reagent for thiol protection. Finally, the derivatization conditions using NEM were optimized in terms of pH, addition of ascorbic acid, and temperature with the goal to

further minimize the side reactions that can occur with this class of reagents. A reaction pH of 7.0 was found to minimize side reactions. This work emphasizes the need to derivatize the thiols prior to extraction, defining optimal reaction conditions for the reaction of derivatization reagents that are able to penetrate cellular membranes while also evaluating the side reactions they undergo.

2.2. Introduction

Organic thiols such as glutathione, cysteine, homocysteine and N-acetylcysteine are of biological interest due to the fact that the ratio between reduced thiols and their disulfides can be indicative of the oxidative status of the cell.[21] Glutathione (GSH) is the most abundant low molecular mass thiol, present in concentrations of up to 12 mM.[19,36] It is a tripeptide composed of glutamic acid, cysteine and glycine, with γ-linkage between glutamic acid and cysteine. It is involved in various cellular functions, such as amino acid transport, maintenance of active forms of enzymes and detoxification of hydrogen peroxide and other free radicals.[36] L-cysteine (CYS) is one of the 20 essential amino acids and can be synthesized by the liver. It is an important precursor in protein synthesis due to its participation in forming disulfide bonds. The concentration of cysteine in plasma has been reported as 240 – 450 μM.[19] The accumulation of cysteine's insoluble disulfide, cystine (CYSS), is related to formation of stones in the kidney, ureter and bladder. Homocysteine (HCY) is synthesized from methionine in the transulfuration pathway.[29] From there, it can either be re-converted into methionine by methylation, or react with serine to form cysteine and glycine.[29] Due to its participation in the synthesis of both amino acids, it is produced in large quantities of 15-20 mMoles per day, but it is present in low concentrations: 5-15 μM in plasma.[19] N-acetyl-L-cysteine (NAC) is formed by the acetylation of cysteine in the kidney, and can be consumed as cysteine source for glutathione synthesis. It is present in concentrations of 4.25-5.0 μM in plasma. **Figure 1-1** summarizes the structures of reduced and oxidized thiols evaluated in this work.

The above reduced thiols, especially GSH, intercept oxidizing species that might interfere or damage the cell's normal operation and are converted to the disulfide in the process. The resulting disulfides can be reduced by reductases present in the cell, therefore maintaining a constant thiol/disulfide ratio under normal conditions.[21,66] However, under oxidative stress, the cell cannot regenerate the reduced thiol at an appropriate rate, and its concentration decreases, therefore decreasing the ratio.[1]

There is no consensus in the literature in regards to the values of the ratios for healthy patients, cells or biofluids.[48] Most studies compare healthy patients to sick or treated patients, and if the

ratios obtained for the different groups are statistically different they consider the protocol successful. Iwasaki *et al.*[88] reviewed nine articles that measured and compared the GSH/GSSG ratio in blood samples of the healthy control group and patients under various oxidizing conditions such as exercise and diseases such as diabetes and atherosclerosis. Out of the fifteen conditions analyzed in these papers, the ratios of 12 are up to 40 times lower in the "sick" group than in the control group; however, in three of them, the difference between the ratios is less than 25%. In the other three, the ratios are higher in the controls, but again, the differences are small. In addition, the values for control subjects in the different studies vary from 2.8 to 511.1 (μM GSH/μM GSSG), while under various diseases and conditions, ratios are reported from 0.4 to 200 (μM GSH/μM GSSG). Theoretically, the ratio for all control groups should be in the same narrow interval, and oxidative stress should decrease the ratio proportionally to the oxidation suffered. The fact that such wide ranges have been observed reinforces the necessity of evaluating in detail various protocols employed in this analysis to establish and minimize possible sources of errors and increase the accuracy of this important determination.

Typical protocols for determining thiol ratio involve two samples.[20] The first sample is reduced for total reduced thiol quantification. The second sample is used for quantification of the disulfide, by protection of the free reduced thiols, followed by reduction of the disulfides and quantification of the newly formed thiols.[63] There are two main challenges in thiol analysis: the first is the absence of chromophores and fluorophores, and the second is their high reactivity, and tendency to autoxidize forming the disulfide, which complicates the accurate measurement of thiol ratios.[89] To overcome these challenges, thiols are commonly derivatized to allow detection using typical analytical techniques, such as UV-Vis and fluorescence. There are more than fifty different reagents that are commercially available to quantify thiols[35], and reagents that derivatize the thiol moiety are preferred, as they can also act as a protective group to avoid autoxidation.[64] These reagents will react with all thiols, so unless chromatographic separation is performed, the measured signal corresponds to total thiol concentration rather than individual thiol concentrations.

An ideal derivatizing reagent will react quickly and specifically with the thiol, and its reaction requirements should be compatible with the extraction method being used.[29] The most common reagent for thiol analysis is 5,5'-dithio-(bis-2-nitrobenzoic acid), known as Ellman's reagent (or DTNB).[63] It is a disulfide with a highly oxidizing thiol bond that reacts with reduced thiols to form a

mixed disulfide and the leaving group TNB which has a large extinction coefficient at 412 nm. The classic assay uses this reagent in a two-step analysis: first total thiols are determined after reduction of oxidized glutathione (GSSG) with glutathione reductase (GR), then oxidized glutathione is determined by protection of GSH with NEM, followed by removal of excess NEM, reduction of GSSG with GR and reaction of the newly formed GSH with DTNB.[63] The main flaw of this protocol is the fact that DTNB will react with all free thiols, so the signal measured is not due only to glutathione. A second disadvantage is the need to remove unreacted NEM after protection step, which is necessary because it inhibits GR activity.[63] Finally, this reagent is used after the sample has been extracted, exposing the thiols to oxidation.[38,74]

Bimanes, such as monobromobimane have been used due to their fast reaction with thiols with formation of a fluorescent adduct.[36] However, they are unstable and both the reagent and the degradation products are also fluorescent, increasing the background. Sanger's reagent (1-fluoro-2,4-dinitrobenzene) is another reagent that allows thiols to be analyzed by fluorescence, however, it reacts with the amine groups of the thiols, and therefore, requires that the reduced thiols be protected previously to prevent autoxidation. Another caveat is the long reaction times needed: four hours in the dark.[49] Two halogenosulfonylbenzofurazans have also been used, but these reagents require high temperature and pH for derivatization, both of which can cause thiol autoxidation.[50] Finally, ortho-phthalaldehyde (OPA) can react with thiols to form a highly fluorescent derivative under mild conditions, however, it can also react with amines.[90]

The use of mass spectrometry for the determination of thiols is attractive as it allows detection of individual thiols and disulfides by their mass to charge ratio in a single analysis. It also allows detection of thiols present in low abundance, thus providing a more comprehensive picture of thiol status in cells. For derivatization in combination with MS detection, it is desirable to use derivatizing agents which will increase electrospray (ESI) ionization efficiency in addition to protecting thiol moiety. There is a variety of derivatizing reagents used in combination with ESI MS such as halogenated alkylating agents like iodoacetic acid (IAA), and iodoacetamide (IAM) to N-substituted maleimides.[91] The reaction with the halogenated reagents is often done at basic pH for faster reaction times, but at this pH, autoxidation is likely.[64] Amongst the maleimides, NEM (N-ethylmaleimide) is the most commonly[61] used due to its fast reaction time (less than 1 minute)[91] and the fact that it can permeate cells and thus protect the thiols before cell lysis[63].

A recent study by D'Agostino *et al*.[8] compared the efficiency of seven N-substituted maleimides for the derivatization of aminothiols prior to CE-MS analysis. Their evaluation criteria was signal enhancement in ESI – measured by relative response factor against underivatized thiols – and migration times. NTAM (N-[2-(Trimethylammonium)-ethyl]maleimide chloride) was found to provide best signal enhancement in ESI, and also allowed shorter analysis times since mobility is increased for cationic compounds. Another reagent tested, NPEM ((R)-(+)-N-(1-Phenylethyl)maleimide)), provided similar ionization results without the need of additional quenching steps. Both NTAM and NPEM performed approximately 2x better than NEM (N-ethylmaleimide) in terms of signal enhancement.[8] However, there are no studies to date that directly compare the performance of NEM and NPEM in LC-MS.

Seiwert *et al.*[61] developed a protocol for analyzing thiols and their disulfides by reaction of the free reduced thiols with a modified N-substituted maleimide (N-(2-ferroceneethyl)maleimide – FEM). The excess reagent is eliminated by reaction with 4-acetamidothiophenol, followed by reduction of the disulfides with tris(2-carboxyethyl)phosphine hydrochloride (TCEP), and their reaction with a different maleimide (ferrocenecarboxylic acid (2-maleimidoyl) ethylamide – FMEA). In this protocol, therefore, thiol-FEM adducts originated from reduced thiols, while thiol-FMEA adducts originated from disulfides.[61] Another protocol based on differential labelling of thiols and disulfides utilized 5-aminoisoquinolyl-N-hydroxysuccnimidylcarbamate (5-AIQC) derivatization.[68] Reduced thiols were first reacted with NEM, and excess NEM was trapped with 4-tert-butylbenzenethiol. TCEP was used to reduce disulfides, and all reacted with 5-AIQC. Therefore, reduced thiols formed the 5-AIQC-thiol-NEM adduct, while disulfides formed the 5-AIQC-thiol adducts. Both of these protocols allow the simultaneous analysis of thiols and disulfides in the same sample, a major advantage over traditional protocols. However, they require the additional steps of binding excess NEM and reduction of the disulfides.[68]

While side reactions of biological thiols and NEM have been reported in the literature since the 1960's[92], the time-dependent formation of the cyclic-CYS product have not been studied or considered in any recent studies using this or other maleimide type-products. The other side products observed can be expected to occur with all other maleimide-type reagents, and this class of reagent is widely used to quantify thiols with no observation of instability.[8,61,89,93]

In order to obtain an accurate measurement of the thiol/disulfide ratios, the reduced thiol must be adequately protected. The objective of this work was to perform a detailed evaluation of NEM and

NPEM derivatizing agents in order to select an appropriate derivatizing agent for the LC-MS assay. These two reagents were selected for this comparison on the basis of their (i) commercial availability, (ii) cell permeability to allow thiol protection prior to cell lysis, (iii) fast reaction rates under mild physiological reaction conditions, (iv) ability to enhance ESI signal and improve limits of detection and (iv) introduce a hydrophobic group to improve retention in reversed-phase chromatography. The two reagents were compared with respect to their (i) derivatization efficiency, (ii) ESI signal enhancement, (iii) generation of side products at different reaction conditions and (iv) derivative stability. This work identifies the optimal reaction conditions to minimize side product formation and provides first comprehensive side product characterization using MS.

2.3. Experimental

2.3.1. Materials

N,N'-diacetylcystine was purchased from Cayman Chemicals (Ann Arbor, MI, USA). Glutathione (GSH), LC-MS grade water, methanol and acetonitrile were purchased from Fisher Scientific Inc. (Ottawa, ON, Canada). All other chemicals were purchased as L-enantiomer from Millipore Sigma (Oakville, ON, Canada), except for homocysteine, which was obtained as the mixture of L and D enantiomers.

Stock solutions of all thiol and disulfide standards were prepared at 1 mg/mL in 20% methanol (v/v) with 10 mM ammonium acetate/acetic acid (9.5:0.5 mM Am. Ac./Ac. Ac. v/v) buffer at pH 6.0 and stored at -80°C. Working solutions were prepared by diluting the stock solution in water.

NEM and IAM were prepared in water at 1 mg/mL and diluted to 20 µg/mL. NPEM was prepared in methanol at 1 mg/mL and diluted to 20 µg/mL. Reagents were kept at -80°C until use. PBS buffer was prepared by weighing 8.0 g of NaCl, 0.2 g of KCl, 1.44 g of Na_2HPO_4 and 0.24 g of KH_2PO_4 to the nearest 0.1 mg. 800 mL of water was then added, and the pH of the modified PBS buffer was adjusted to 7.0 using HCl. The final volume was then adjusted to 1000 mL.

Calibration curves of the derivatized thiol standards were prepared by sequential two-fold dilution of 200 ng/mL standards until 0.781 ng/mL, and analyzed from the lowest concentration to highest. Dilution solvent was water or buffer, as needed to match final sample composition for LC-MS injection. Calibration curves for NEM and NPEM thiol derivatives were obtained by derivatizing the 200 ng/mL standard as described in Section 2.3.2 and performing the dilutions after 30 min when

reaction was deemed complete. Calibration curves were processed using the TOF quantitative analysis software (version B.07.00 SP1, build 7.0.457.0, Agilent), with 1/x weighting.

2.3.2. Derivatization

Standards at 100 ng/mL were derivatized with 1000 ng/mL of derivatizing agent (1:10 w/w) in ammonium acetate/ammonium hydroxide buffer (1:10 v/v) at pH 7.0. The mixture was mixed for 1 min using a vortex, and reacted for 30 min at room temperature, unless otherwise stated. Whenever mixtures of standards were used, the derivatizing agent was adjusted to maintain 1:10 w/w ratio of analyte to derivatizing agent. No quenching was performed. Blanks were buffer samples subjected to derivatization procedure.

For pH 3.0, 50 mM ammonium formate/formic acid buffer was used (1:5 v/v). For pH 5.0, 50 mM ammonium formate/formic acid buffer was used (9.5:0.5 v/v). For pH 9.0, 50 mM ammonium hydroxide/ammonium acetate (1:1.5 v/v) was used. pH was verified using pH meter Accumet AB150 from Fisher Scientific. For the experiment with narrower pH range described in 2.4.5, the pH 5.0 buffer was prepared and ammonium hydroxide was added to obtain the other pHs: 5.5, 6.5, 7.0, 7.2 and 7.4.

2.3.2.1. *Final optimized conditions for NEM derivatization*

NEM derivatization of thiols produced the least amount of side products after derivatization at pH 7.0 in PBS with 500 ng/mL ascorbic acid for 30 min on ice. Higher pHs are to be avoided, since they increase the hydrolysis of the maleimide ring.

2.3.3. HPLC-ESI-QTOF

All the analyses were performed on an Agilent 1290 UHPLC – 6550 iFunnel QTOF from Agilent Technologies (Santa Clara, CA, USA) in positive ESI mode. The mass range analyzed was 50-1100 m/z range, at 2 scans/s. Drying gas temperature was 250°C and drying gas flow was 15 L/min. Fragmentor voltage was set at 250 V, capillary voltage was set at 3500 V, and nozzle voltage was set at 400 V. Injection volume was 10 µL for all analyses.

During derivatization optimization, Kinetex PFP column (50 mm x 2.1 mm x 2.6 µm, 100 Å) from Phenomenex (Torrence, CA, USA) with guard column (UHPLC PFP for 3.0 mm ID columns from Phenomenex) was used for analyte separation. The mobile phase used was water with 0.1% formic acid (A) and acetonitrile with 0.1% formic acid (B), at 0.3 mL/min. The gradient used was: 0-4 min at 0% B, 4-8 min linear increase from 0 to 4% B, 8-20 min linear increase from 4 to 40% B, 20-25

min linear increase from 40 to 95% B, 25-27 min at 100% B, 27-27.1 min linear decrease from 95 to 0% B and 27.1-31 min at 0% B to recondition the column.

The final recommended method for NEM-derivatized thiols uses a Biphenyl column (100 mm x 2.1 mm x 1.7 µm, 100 Å) from Phenomenex with guard column (UHPLC Biphenyl for 2.1 mm ID columns from Phenomenex). The mobile phase used was water with 0.1% formic acid (A) and acetonitrile with 0.1% formic acid (B), at 0.3 mL/min. The gradient used was: 0-4 min at 3% B, 4-12 min linear increase from 3 to 21% B, 12-13 min linear increase from 21 to 90% B, 13-16 min at 100% B, 16-16.1 min linear decrease from 90 to 3% B and 16.1-21 min at 3% B to recondition the column

Data was acquired using the LC/MS Data Acquisition Software for 6200 series TOF/6500 series QTOF (version B.06.01, build 6.01.6157, Agilent). Internal mass calibration was performed throughout all sample analyses by introduction of calibrant using Agilent 1260 isocratic pump and dual Agilent Jet Stream-ESI source. The calibrant exact masses were 121.050873 from purine and 922.009798 from HP0921.

Extracted ion chromatograms of $[M+H]^+$ ion were processed either using TOF Qualitative Analysis software (version B.07.00, build 7.0.7024.29) or TOF quantitative analysis software (version B.07.00 SP1, build 7.0.457.0, Agilent), using a 15 ppm extraction window. The masses of the NEM and NPEM derivatives, underivatized thiols and disulfides are summarized in in **Table A2**. When double peaks of diastereoisomers were observed, the areas of both peaks were integrated and summed together for quantification. ANOVA was performed using the Data Analysis package from Microsoft Excel 2016 (Redmont, WA, USA).

2.4. Results and discussion

Accurate measurement of reduced thiols and their disulfides is difficult and can benefit from MS detection to allow analysis of individual thiols and their disulfides in a single analysis. However, none of the LC-MS studies reported to date meet all the criteria desired for this application. In this study, we investigated in detail the performance of NEM and NPEM to select the best derivatizing agent prior to LC-MS analysis.

2.4.1. Derivatization efficiency and evaluation of ESI signal enhancement

In order to protect the thiol moiety, three different reagents were compared in initial experiments: NEM, NPEM and IAM. The main criteria for the comparison were the derivatization

efficiency, followed by signal enhancement in ESI. With regards to the efficiency of the derivatization, NPEM was the best reagent, with no underivatized thiols detected for any of the four analytes tested (GSH, CYS, HCY, NAC). NEM was unable to completely derivatize cysteine, with the derivatization efficiency of 97%, verified by comparing the area of the peak of the underivatized thiol left over after derivatization to the area of the thiol in a sample of known concentration to which no derivatizing reagent was added. Iodoacetamide derivatization efficiencies ranged from 1.0% for GSH to 6.8% for NAC. This reagent was therefore excluded from subsequent experiments due to slow reaction kinetics. Previous studies have already indicated that the rate of reaction for IAM is considerably slower than for NEM[44], but considering that IAM is commonly used for thiol protection in proteomics as well as one of the existing LC-MS assays for thiol determination[1], we included it in this preliminary comparison. Although IAM derivatization efficiencies were extremely low in the current study, other studies have shown that IAM derivatization can proceed to completion when a large excess of derivatizing agent (1000x) and longer reaction times are used.[20] However, since the ultimate goal is to improve the accuracy of intracellular thiol assays, cell permeable reagents with fast reaction times are strongly preferred and longer derivatization times were not further investigated for this application.

In addition to protection of thiols, derivatization also provides a second advantage since it can enhance the ESI signal if hydrophobic and surface-active ionisable group(s) are added during derivatization process.[8] Maleimide reagents meet all of these criteria. As expected, NPEM provided better ionization efficiency than NEM, with observed enhancement ranging from 2.1X for GSH and 5.7X for CYS against NEM, determined by the ratio between the slopes of calibration curves shown in **Figure A1**. This enhancement is comparable, or slightly better than, to what was observed in recent comparison of seven maleimide reagents using CE-MS for the derivatization of five different aminothiols[8], with NPEM providing 1.8X (glycyl-cysteine – GlyCys) – 2.4X (GSH) against NEM. The differences in the two studies may be due to different separation mechanisms in LC-MS versus CE-MS and elution of NPEM derivatives in high percent organic (retention time 13 to 17 min on PFP column) which is known to facilitate more efficient ESI due to more facile evaporation of organic solvent versus water-based background electrolytes. The ionization enhancement of derivatized versus underivatized thiols was not measured because the underivatized thiols were not sufficiently retained under the chromatographic conditions used in this analysis.

In summary, based on derivatization efficiency, ESI signal intensity and better chromatographic retention and separation, NPEM outperforms NEM.

2.4.2. Effect of derivatization time on NEM reaction efficiency

The reaction of maleimides with GSH has been cited to be as fast as 1 min[91], which is of interest since it would minimize the autoxidation of the thiol. To ensure if this is seen for all thiols of interest, different reaction times were tested: 1, 5, 15 and 30 min. The results shown in **Figure 2-1** indicate that there is no increase in CYS derivatization over time, but for GSH, HCY and NAC, there seems to be a time-dependent increase. To evaluate if there is a statistical difference between the timepoints, the results shown were evaluated using ANOVA. Only for NAC-NEM did the test show there was a statistical significance between the timepoints ($p_{0.05}$ 1.94×10^{-5}). Although there is no difference between 15 and 30 min for NAC-NEM, 30-minute derivatization was used for all analyses because the ultimate goal was to use the reagent to derivatize intracellular thiols, and the additional time would allow the reagent to penetrate the cellular membrane and react.

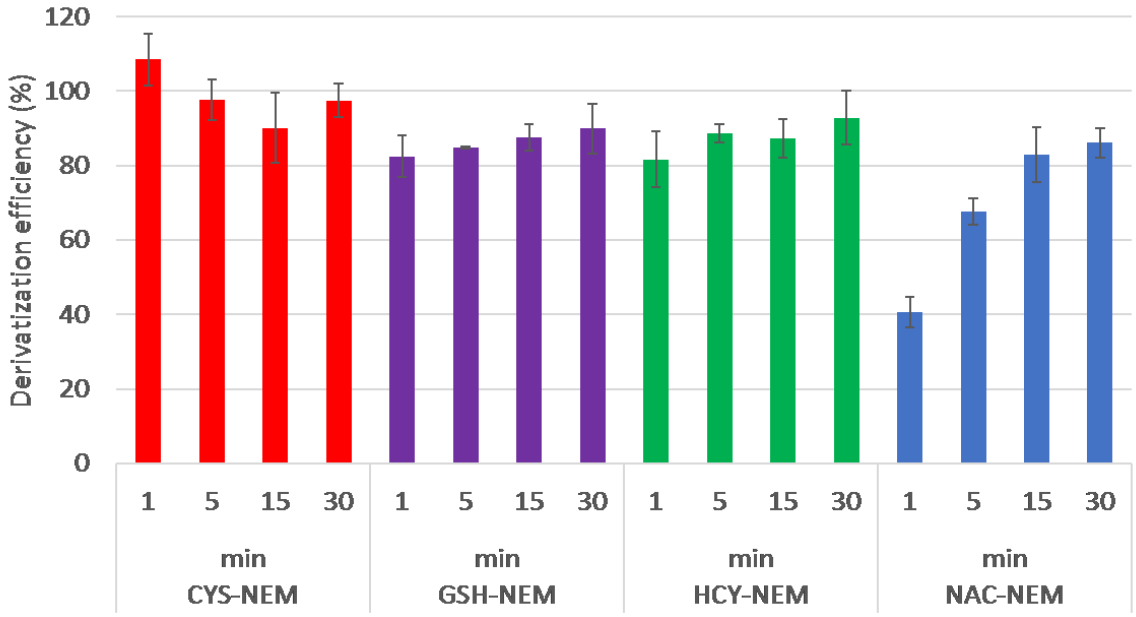

Figure 2-1 – Concentration of thiols derivatized by NEM at different time points: 1, 5, 15 and 30 min. Derivatization of 100 ng/mL mixture of thiols was performed at pH 7.0, samples were kept on ice during derivatization, and transferred to the instrument 1 min prior to analysis n=3. ANOVA analysis shows no significant difference in average derivatization efficiency of CYS, GSH and HCY. For NAC, derivatization efficiency increased significantly with increasing reaction time, which indicates that short reaction times are not recommended for this compound.

2.4.3. Effect of pH on the derivatization of thiols

The addition of the thiol to the maleimide double bond is a Michael addition[19,60], which depends on the existence of a nucleophilic moiety – the thiolate anion – to attack the bond. The reaction is said to be optimal at pH 6 – 7, because the thiolate anion is available for the reaction to occur. For instance, at pH below 5, less than 0.05% of the thiol groups in cysteine are deprotonated, while at pH 7, thiolate anion increases to 4.5%. At low pH the reactivity of the maleimide is low and at more basic pH, it is unstable.[94] To investigate pH dependence, NEM or NPEM derivatization was performed at pH 3, 5, 7 and 9. To obtain calibration curves at different pH values, the calibration standards were derivatized at pH 7.0 and then diluted with the respective buffers before LC-MS analysis to match the sample composition. The results obtained are shown in **Table 2-1**, and show that NPEM is greatly affected by the pH of the reaction, being much less efficient than NEM at pH 5. At low pH, the maleimide has low reactivity[94], which would be further decreased by the protonation of the thiol groups. Indeed, the efficiency of both reagents at pH 3.0 is very low. At high pH, even though the thiolate anion is more available, the maleimide reagents are unstable and prone to hydrolysis of the maleimide ring, which explains the lower efficiencies observed. For both reagents, there was a significant difference in the reaction efficiency, with decreasing derivatization yield as pH decreases.

After HPLC separation, the derivatized thiols are detected as double peaks (**Figure 2-2** for NEM, and **Figure A2** for NPEM). Due to the presence of a chiral center on the amino acids, their reaction with the maleimides yields diastereoisomers due to the creation of a new chiral carbon[19,60–62]. Depending on the HPLC conditions used, these can elute in one or two peaks. In latter case, either additional time in data treatment is required[60], or only one peak is considered for quantification.[95] Additional peaks have also been attributed to cis/trans isomers[1] and decomposition of the NEM derivative. [91,96,97] The MS^2 spectra of the individual peaks shows no difference between them (**Figures A3 – A10**), indicating that they are the diastereoisomers. In all results shown, the area of both peaks is summed together for quantitation.

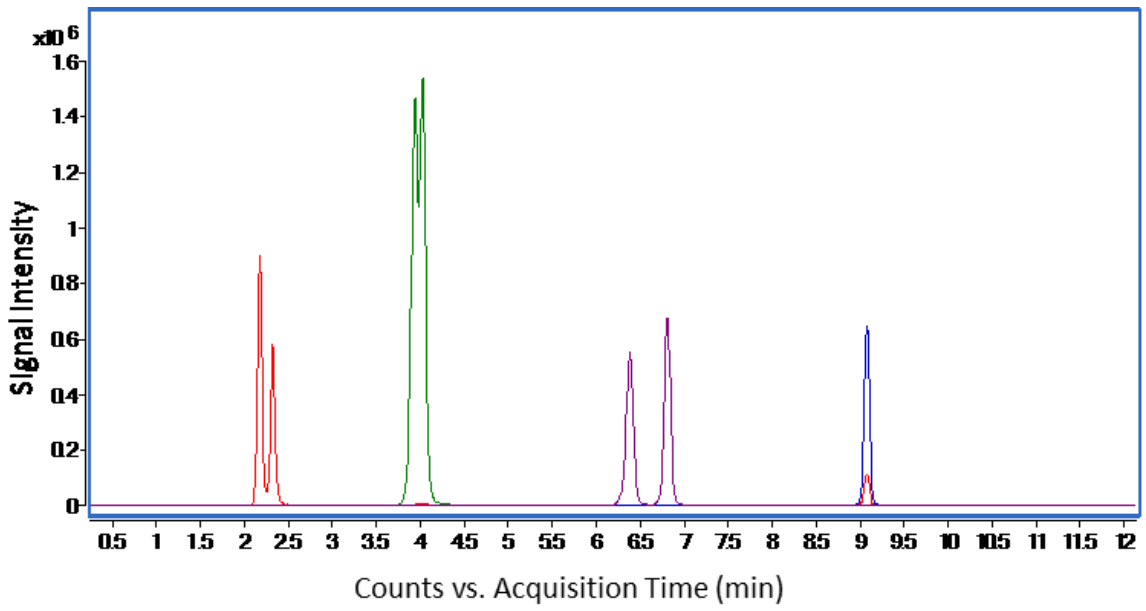

Figure 2-2 – Chromatographic separation of a 100 ng/mL standard mix of CYS-NEM (red, m/z 247.0753), HCY-NEM (green, m/z 261.0909), GSH-NEM (purple, m/z 433.1393) and NAC-NEM (blue, m/z 289.0858) using biphenyl column. CYS-NEM peak observed coeluting with NAC-NEM corresponds to in-source fragmentation of NAC-NEM. Total run time was 21 min.

Table 2-1 – Effect of pH on derivatization efficiency of four aminothiols (CYS, HCY, NAC and GSH) using NEM and NPEM. Percentages indicate comparison to the area of the sum of the peaks of the diastereoisomers at pH 7.0. 100 ng/mL of thiol mix (n = 3) was derivatized with 10-fold excess of derivatizing agent using buffers at different pHs as described in Section 2.3.2.

NEM derivatization				
pH	CYS-NEM	HCY-NEM	NAC-NEM	GSH-NEM
3.0	57 ± 11%	36 ± 7%	25 ± 6%	23 ± 5%
5.0	112 ± 1%	98 ± 3%	105 ± 2%	94 ± 3%
7.0	**100** ± 4%	**100** ± 3%	**100** ± 3%	**100** ± 4%
9.0	ND	72 ± 4%	50 ± 3%	79 ± 3%
NPEM derivatization				
pH	CYS-NPEM	HCY-NPEM	NAC-NPEM	GSH-NPEM
3.0	9 ± 5%	2 ± 1%	1 ± 0%	2 ± 1%
5.0	66 ± 20%	38 ± 16%	43 ± 19%	16 ± 9%
7.0	**100** ± 3%	**100** ± 3%	**100** ± 2%	**100** ± 1%
9.0	ND	23 ± 3%	24 ± 3%	13 ± 1%

2.4.4. Selectivity of derivatization for primary thiol versus amine groups

2.4.4.1. Double-derivatization through thiol and amine groups

Table A3 summarizes the pK_a values for the four thiols studied, according to HMDB. At pH 7, where the reaction occurs[94], the α-amine of the thiol amino acid is deprotonated (pK_a of CYS, HCY, and GSH are 9.05, 9.41 and 9.22, which means 0.89%, 0.39 and 0.60% are deprotonated, respectively), and can react with the maleimide double bond.[92] The mass of the derivative would be the same, and diastereoisomers would also be seen in this case. Moreover, when this happens, the thiol would no longer be protected from oxidation. The unprotected thiol could then subsequently react with another thiol (side product not detected) or derivatizing agent to form a double derivative. No double derivatization is observed for NAC because the amine is protected by the acetyl group, but it was observed for cysteine, homocysteine and glutathione when derivatized with NPEM at pH 7.0, corresponding to 1.3 to 4.4% of the area of the standard derivatized only once (Table 2-2). The amount of double derivatization observed with NPEM increased with the pH, which correlates to the increase in availability of deprotonated amine. Double derivatization was not observed with NEM derivatization at any pH tested. There is no evidence of the formation of N-derivatives based on the MS2 data collected for each of the double peaks, however, the formation of the 105.0704 fragment is indicative of NPEM derivatization (loss of the N-substitution: ethylphenyl).

Table 2-2 – Summary of double derivatization seen for CYS, HCY and GSH after derivatization with NEM or NPEM at the pHs indicated. Retention times indicated are for elution on biphenyl column. Percentages are normalized to the area of the peaks for the compound derivatized only once at pH 7.0 and assumes that the ionization efficiencies are the same. 100 ng/mL standard mix derivatized in buffers as described in Section 2.3.2. pH 9.0 was not used in this experiment since double derivatization seen at this pH is more likely to be caused by reaction of the open maleimide ring with the amine, as opposed to reaction of amine to the double bond of the maleimide. ND = not detected.

Standard	Deriv. agent	$[M+H]^+$	RT (min)	pH 3.0	pH 5.0	pH 7.0
CYS	NEM	372.1230	ND	ND	ND	ND
	NPEM	524.1855	21.4	ND	0.05%	1.8%
HCY	NEM	386.1386	ND	ND	ND	ND
	NPEM	538.2011	21.3	ND	0.03%	4.4%
GSH	NEM	558.1870	ND	ND	ND	ND
	NPEM	710.2495	15.8	ND	0.2%	1.3%

These results indicate that N-derivatization can occur depending on the pK_a of the analyte of interest and reaction pH. To further investigate this, derivatization of compounds that have (i) a free amine but no thiol and (ii) free amine but with a protected thiol moiety was also tested.

2.4.4.2. Derivatization of amino acids

The reaction of the deprotonated α-amine with the maleimide is a nucleophilic reaction.[92] As the pH of the buffer used increases, deprotonation of amine increases and therefore, the amine group can become more likely to compete with the thiols for reaction with the maleimide. This was previously observed in **Table 2.2** when considering double derivatization through both amine and thiol groups. To test for N-derivatization, the amino acids without a free thiol moiety and cysteine analogs with protected thiol moiety shown in **Figure 2-3** were reacted with the maleimides. Out of the ten reagents tested, two were cysteines with protected thiol and amine groups and therefore had no moiety that could be derivatized (APC and DAC), four have a free amine group and a thiol that is unavailable for reaction (MET, SAC, SAH and SMC) and the last four were amino acids with no sulfur, therefore, reaction could only occur with the amine group: ALA, LYS, PHE and SER. The derivatization results are summarized in **Table 2-3**. The underivatized amines present in each sample after derivatization are shown in **Table A1**.

As expected, APC and DAC were not derivatized by either of the maleimides. SAC was derivatized at pH 9.0, SAH was not derivatized and SMC and MET were only derivatized by NPEM, at all pHs tested. Except for MET at pH 3, NPEM derivatization was always more evident than NEM derivatization.

ALA was only derivatized at pH 9 by NPEM, and SER was not derivatized by either reagent. However, lysine (LYS) was successfully derivatized by both reagents at pH 7 and 9, as shown in **Table 2-3**. Considering that the pK_a of the NH_2 of lysine is 8.95, at pH 7, 1.1% of the α-NH_2 are deprotonated and at pH 9 it increases to 52.9%. This increase was responsible for a 3-fold and 5-fold increase in NEM and NPEM derivatized lysine respectively. Phenylalanine (PHE) was only derivatized by NEM at pH 9, where 26% of the amine is deprotonated, but it was derivatized by NPEM across all pH values. SMC was only derivatized by NPEM.

In conclusion, while both derivatizing reagents can react with the α-amine of amino acids[92], NEM is less prone to react, and when it does, it reacts less than NPEM. The pH at which the derivatization is done is an important factor that greatly influences the occurrence and extent of the derivatization for both reagents. Furthermore, the results show that for the assays that rely on

NEM or NPEM derivatization without LC separation, amine derivatization of amine-containing metabolites in biological samples can be an important source of inaccuracy.

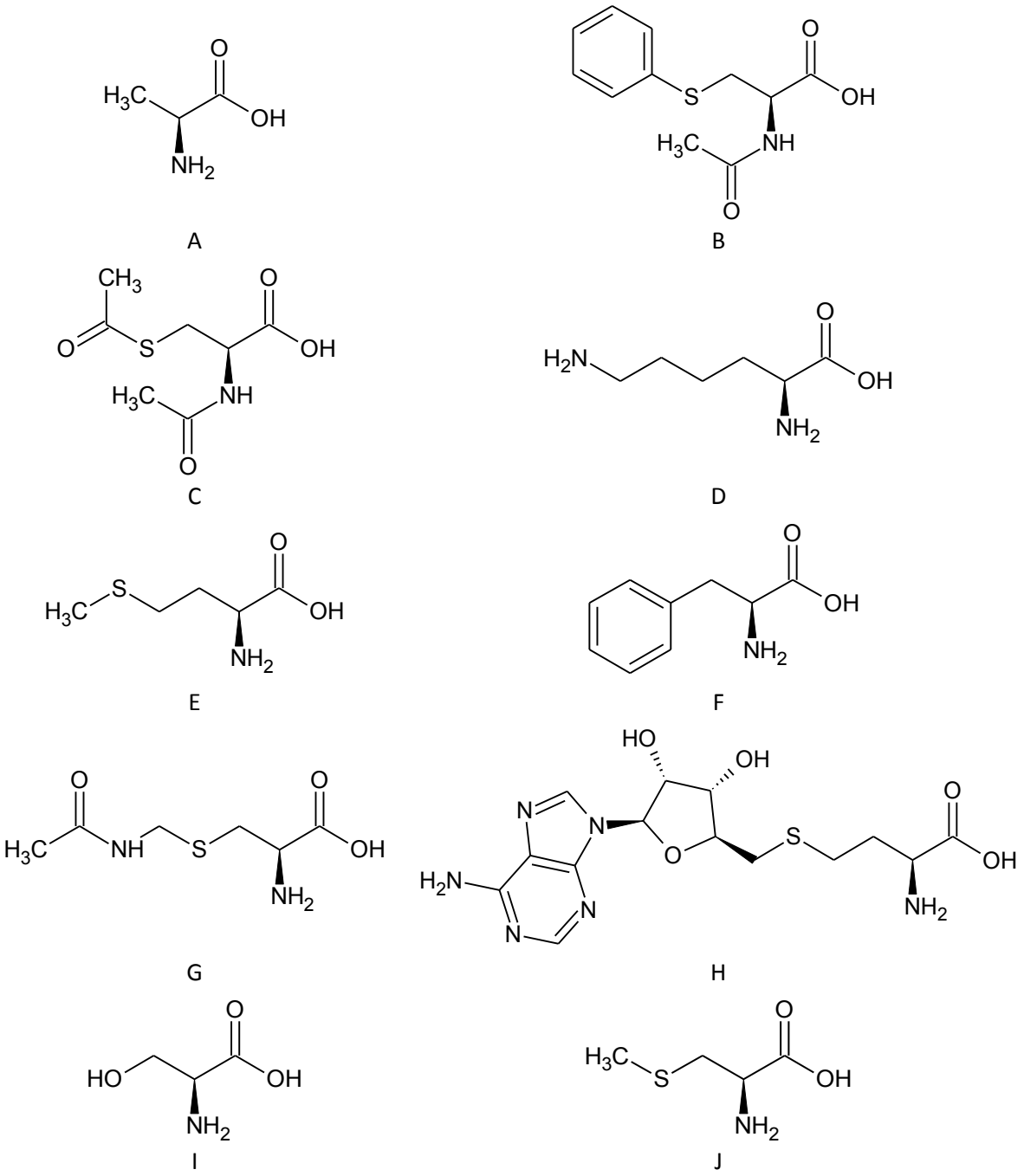

Figure 2-3 – Structures of the amino acids and cysteine analogs used to evaluate the possibility of the derivatization of amines by NEM and NPEM. A: L-alanine (ALA), B: N-acetyl(phenyl)-L-cysteine (APC), C: N,S-diacetylcysteine (DAC), D: lysine (LYS), E: methionine (MET), F: L-phenylalanine (PHE), G: S-acetamidomethyl-L-cysteine (SAC), H: S-(5'-Adenosyl)-L-homocysteine (SAH), I: L-serine (SER), J: S-methyl-L-cysteine (SMC).

Table 2-3 – Summary of the results of derivatization of 100 ng/mL amino acids and thiol-protected cysteines with NEM and NPEM at pH 3.0, 5.0, 7.0 and 9.0, separated in the biphenyl column. Percentages are related to the peak at pH 7 for each substance. (n = 3). No corresponding signal was detected in blanks.

Compound	Deriv. reagent	m/z	RT (min)	pH 3.0			pH 5.0			pH 7.0			pH 9.0		
				AVG	STDEV	% of pH 7.0	AVG	STDEV	% of pH 7.0	AVG	STDEV	% of pH 7.0	AVG	STDEV	% of pH 7.0
ALA	NEM	215.1032	ND	ND			ND			ND			ND		
	NPEM	291.1345	12.0	ND			ND			ND			1.6×10^{4}	7.3×10^{3}	--
APC	NEM	365.1171	13.3	ND			ND			ND			ND		
	NPEM	441.1484	14.1	ND			ND			ND			ND		
DAC	NEM	331.0964	ND	ND			ND			ND			ND		
	NPEM	407.1277	13.9	ND			ND			ND			ND		
LYS	NEM	272.1611	1.2	ND			ND			4.3×10^{3}	1.7×10^{2}	100%	1.3×10^{4}	3.9×10^{2}	314%
	NPEM	368.1920	10.5	ND			ND			9.5×10^{3}	4.8×10^{3}	100%	5.3×10^{4}	1.1×10^{4}	557%
MET	NEM	275.1070	7.6	ND			ND			ND			ND		
	NPEM	351.1379	14.0	2.8×10^{4}	1.2×10^{4}	51%	3.8×10^{4}	2.7×10^{4}	71%	5.4×10^{4}	7.8×10^{3}	100%	1.1×10^{5}	8.6×10^{3}	207%
PHE	NEM	291.1345	11.3	ND			ND			ND			2.8×10^{4}	1.6×10^{3}	--
	NPEM	367.166	14.1	5.9×10^{4}	1.7×10^{4}	72%	3.2×10^{4}	1.8×10^{4}	39%	8.3×10^{4}	3.2×10^{4}	100%	1.8×10^{5}	1.3×10^{4}	217%
SAC	NEM	318.1120	4.7	ND			ND			ND			7.3×10^{3}	3.4×10^{2}	--
	NPEM	394.1437	13.7	ND			ND			ND			1.8×10^{4}	2.3×10^{4}	--
SAH	NEM	510.1766	ND	ND			ND			ND			ND		
	NPEM	586.2079	ND	ND			ND			ND			ND		
SER	NEM	231.0980	ND	ND			ND			ND			ND		
	NPEM	307.1294	ND	ND			ND			ND			ND		
SMC	NEM	261.0909	ND	ND			ND			ND			ND		
	NPEM	337.1222	13.8	ND			ND			ND			ND		

2.4.4.1. *Other side reactions: Ring opening and cyclisation*

The tests described in Sections 2.4.3 and 2.4.4 show that derivatization with maleimides needs stricter pH control in order to prevent derivatization of free amine groups. However, there are also other reactions that may occur depending on the conditions of the system.

At more basic pH, the free amine of cysteine can react with the carbonyl in the maleimide, opening the maleimide ring while forming another ring in an intramolecular transamidation.[94] The mass of this derivative is equal to that of the simple derivative, and may not be seen depending on the LC conditions used.[62] In our LC-MS conditions, a third peak corresponding to the mass of CYS-derivative is observed when either NEM or NPEM is used (**Figure 2-4**). This peak increases in intensity over time, with the simultaneous depletion of the peak corresponding to the diastereoisomers, indicating that cyclisation is occurring in the sample.

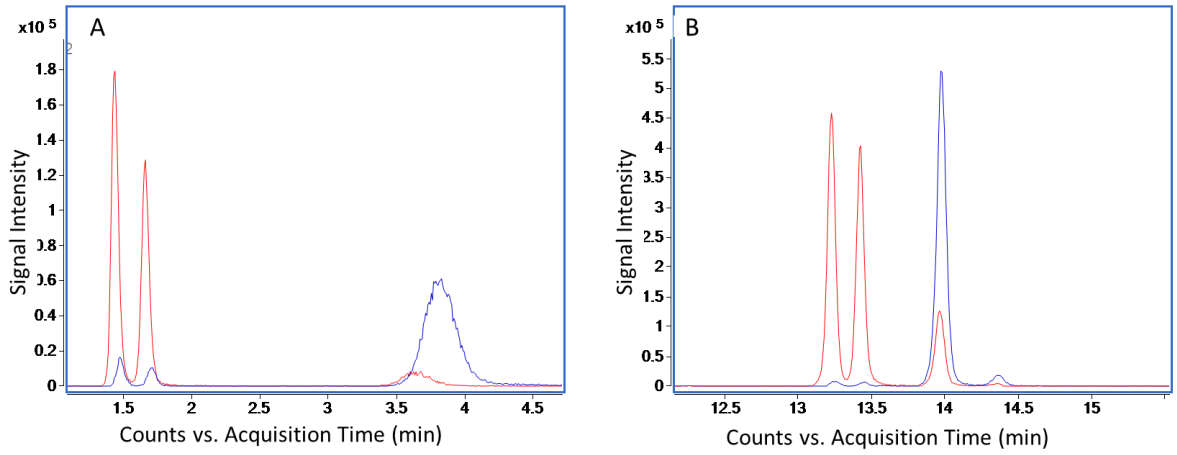

Figure 2-4 – Formation of cyclic CYS-derivative using (A) NEM, extracted m/z 247.0375, and (B) NPEM, extracted m/z 323.1066. Red line corresponds to time 0, and blue line is the same sample analyzed after 24 hours. 100 ng/mL CYS standard was derivatized with 10-fold derivatizing agent, LC separation was performed on PFP column.

As shown in **Figure 2-4**, the conversion of cysteine derivative to new cyclic derivative is time-dependent and very significant, which would cause inaccurate determination of the ratio for this thiol. Therefore, for accurate measurement of cysteine/cystine ratio, the use of a reducing agent (ascorbic acid at 100 ng/mL[8]) and lower temperature (0°C) during derivatization was tested, to evaluate their effect on reducing the formation of the cyclic derivative. There was no difference in the formation of the cyclic products for any of the conditions, as shown in **Figure 2-5**, which means that this precaution did not help to prevent the unwanted side-product.

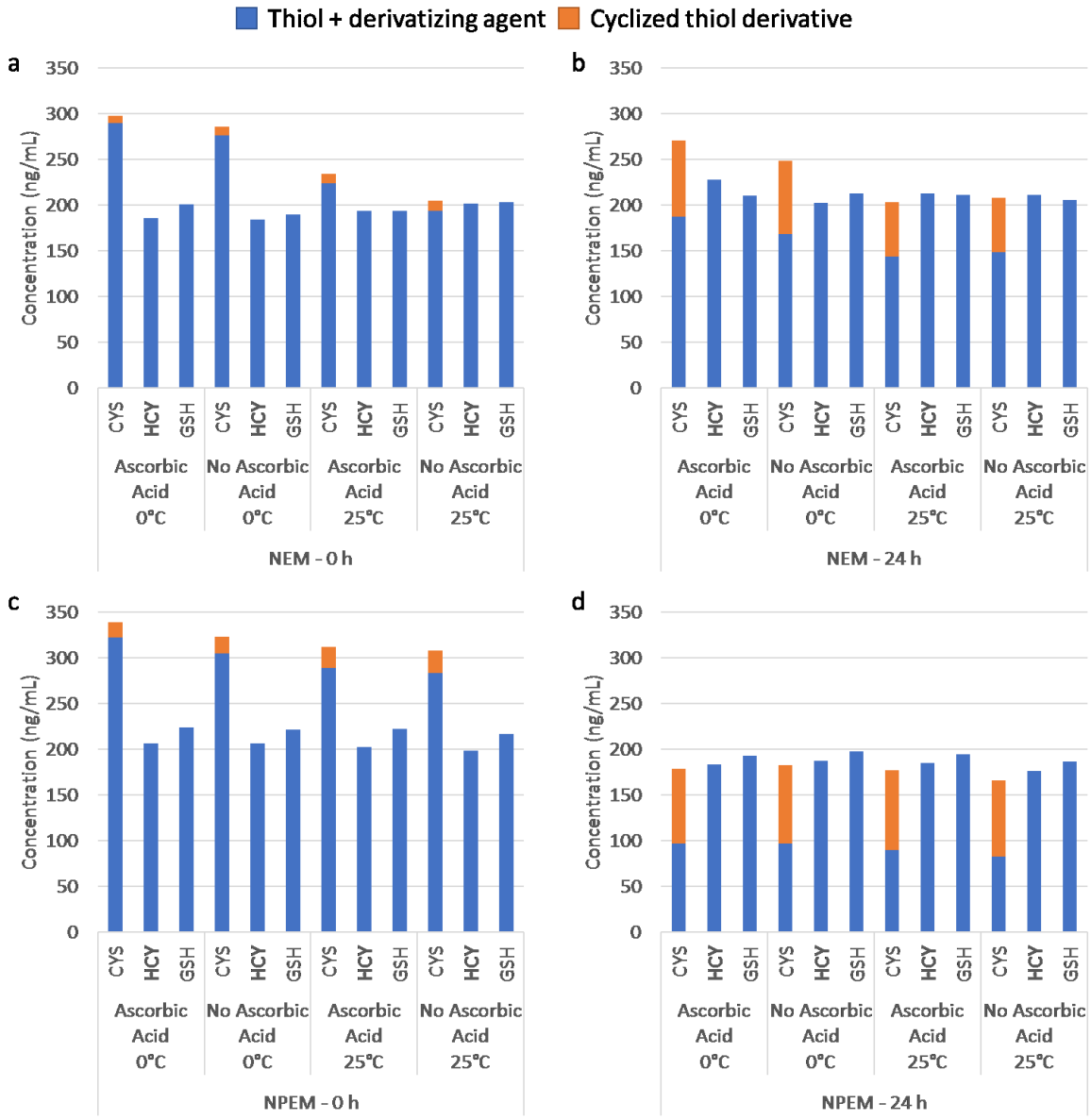

Figure 2-5 – Effect of temperature and ascorbic acid on thiols derivatized by (a) NEM at time 0 h, (b) NEM at time 24 h, (c) NPEM at time 0 h and (d) NPEM at time 24 h. Thiols were present at 200 ng/mL and derivatized with 10-fold (w/w) derivatizing agent. Incubation time was 30 min, and 100 ng/mL of ascorbic acid was used during derivatization. Derivatization was executed either on ice or at room temperature (25°C) (n=1). The concentration of cyclized CYS (orange) was determined with calibration curve of derivatized cysteine, assuming they have the same ionization efficiency. This assumption can underestimate actual concentration in the absence of authentic standard for this side product as it appears based on 24 h results.

Additionally, there is no impact on the formation of the derivative of homocysteine and glutathione, which indicates that these factors do not interfere with the derivatization reaction. N-acetyl cysteine is not shown since it does not have a free amine for cyclization. The same samples

were analyzed after 24 h, and the formation of the cyclic product is responsible for a large decrease in the signal of CYS with either of the derivatization reagents. The presence of ascorbic acid in the sample did not decrease the formation of the cyclic product over time.

The addition of 100 ng/mL of ascorbic acid did not influence the cyclization, so to ensure that this was not caused by limited availability of the reagent, another concentration level was tested: 500 ng/mL. The difference between the treatments is not statistically significant for cysteine (ANOVA $p_{0.05}$ = 0.90675 – **Figure 2-6**).

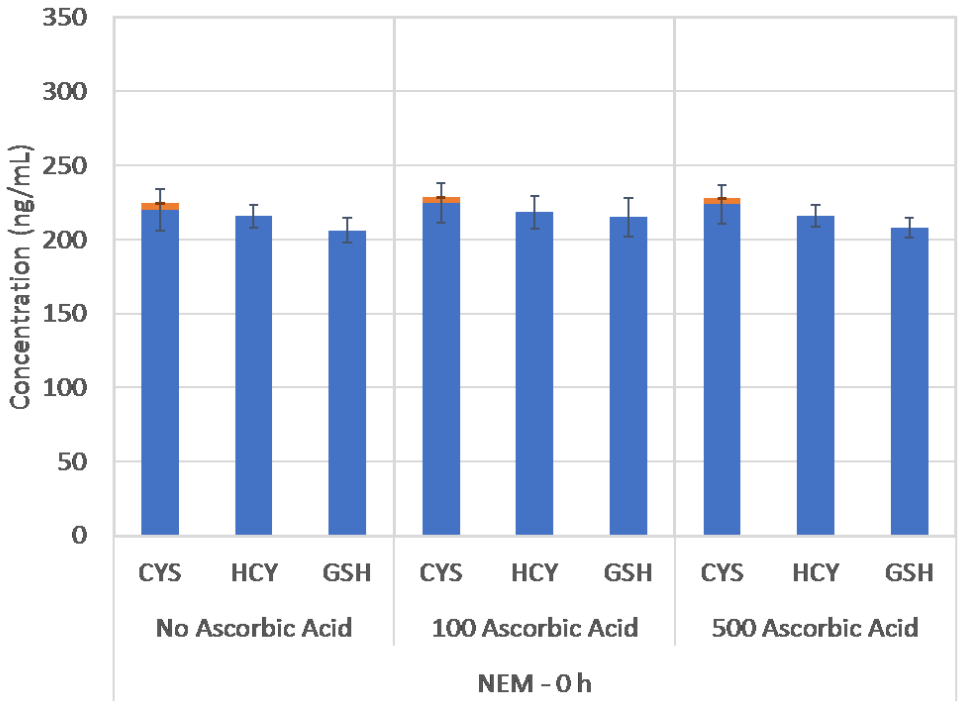

Figure 2-6 – Effect of increasing ascorbic acid concentration on the stability of NEM-derivatized thiols. Thiols were present at 200 ng/mL and derivatized at pH 7.0 with 10-fold (w/w) derivatizing agent. Incubation time was 30 min at 0°C, and 100 ng/mL or 500 ng/mL of ascorbic acid was used (n=3). The samples were analyzed immediately after incubation (t=0). Concentration of cyclized CYS (orange) was determined with calibration curve of derivatized cysteine, assuming they have the same ionization efficiency and can over/underestimate actual concentration in the absence of authentic standard for this side product.

The cysteine cyclisation has extreme consequences in the quantitation of CYS, since this side reaction occurs over time, and during metabolomic studies, samples are often left overnight in the autosampler (6°C). To ensure accurate determination of CYS, there are two possibilities: either

only fresh samples should be analyzed, or the cyclic product must be taken into consideration when measuring CYS. For large batches, the latter is the only viable option.

The cyclisation is only possible due to the cleavage of the maleimide ring, which is also observed at neutral and basic pH[61,62], and characterized by the addition of a water molecule to the mass of the thiol-derivative. The other amino-thiols do not form the cyclic product because the amine is not free for acetylcysteine, and the terminal amino group is not accessible for glutathione.[62] However, the opening of the maleimide ring is observed for NPEM-derivatized cysteine, N-acetylcysteine, homocysteine, glutathione, N,S-diacetylcysteine, S-methyl-L-cysteine and serine.

The opening of the maleimide ring can be tied to the stability of the derivatizing reagent itself. NPEM, which is initially prepared in methanol due to its low solubility in water, is not detected in the samples when it is used (extracted m/z 202.0869); however, it's hydrolysis product it is detected with an open ring (extracted m/z 220.0974 – addition of water). This can explain its reactivity with amines: after the ring is opened, a carboxylic acid is formed, which is likely to react with amines. For NEM (m/z 126.0550), opening is seen (extracted m/z 144.0661), but corresponds to only around 5% conversion, which is mirrored in the observed reactivity with amines.

2.4.5. Fine tuning of optimal pH for NEM derivatization

As shown in 2.4.3, the reaction of thiols with maleimides depends on pH for optimal efficiency. On the thiol side, increasing the pH also increases the availability of the thiolate anion, which is important for the addition reaction. However, it also increases the occurrence of autoxidation, which is unwanted. For the maleimide reaction, pHs lower than 5.0 will decrease its activity, while higher than 7.0 will favor hydrolysis of the maleimide ring. Considering that the end goal is to use this reagent to derivatize biological thiols in cells, it would be ideal if the reaction could be performed at the pH of PBS buffer (7.4) to minimize stress on the cell and simplify the protocol. To define the pH at which reaction is optimized with minimal formation of side products, the effect of pH on the reaction efficiency and selectivity was tested using narrower intervals between pH 5.5 and 7.4, to verify if there were significant differences between these different pHs. For both reagents, pH values higher than 7.2 showed increase in the formation of cyclic-cysteine, as shown in **Figures 2-7 A and B.**

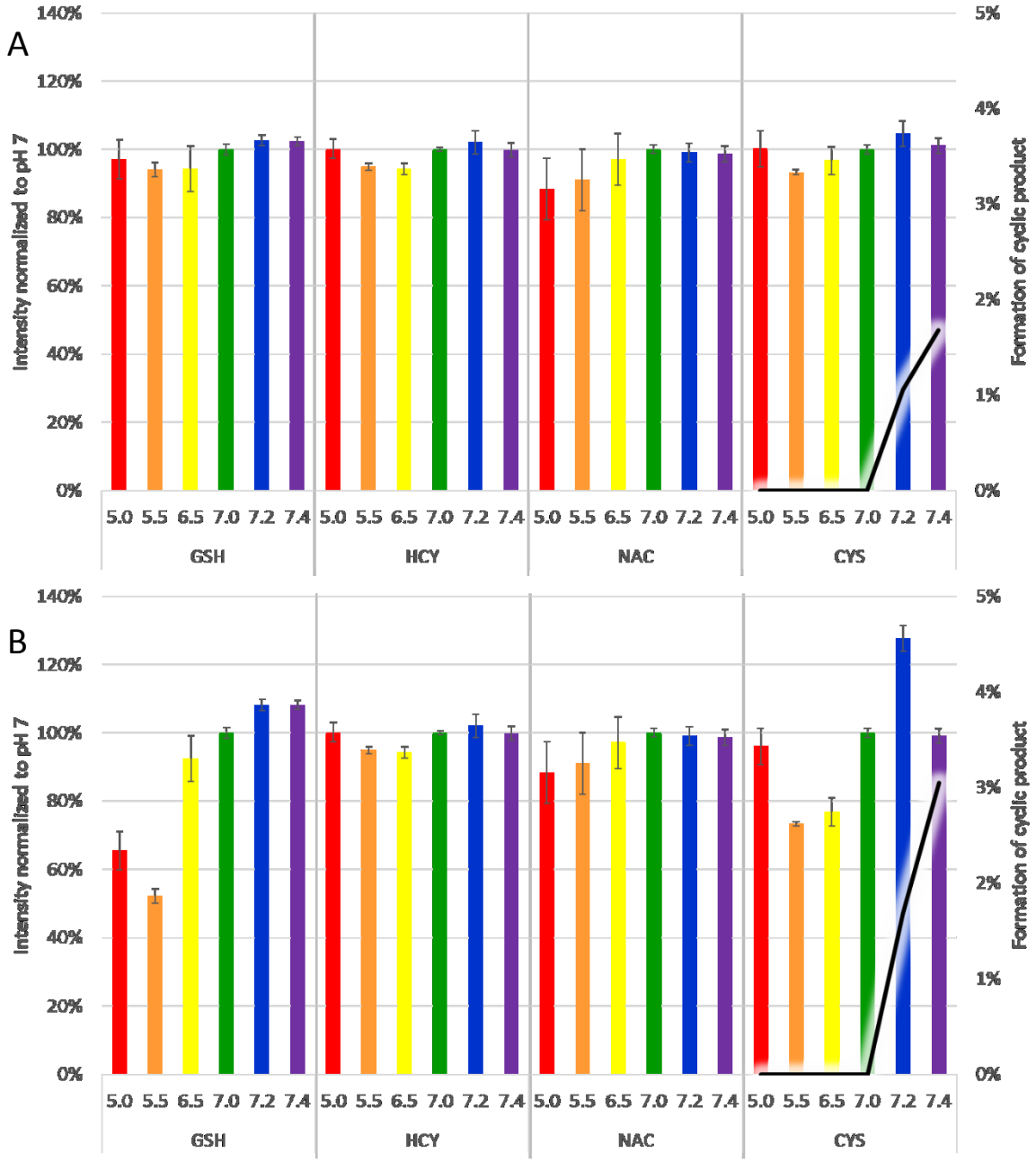

Figure 2-7 – Effect of pH on the formation of side products after derivatization with (A) NEM and (B) NPEM (n=3, t=0h). Concentration of cyclized CYS (black line on secondary axis) was determined with calibration curve of derivatized cysteine, assuming they have the same ionization efficiency and can underestimate actual concentration.

2.5. Conclusions

The literature considers addition to maleimide double bonds to be a specific reaction for thiols, however, without proper control of the reaction, many side products are found, which can

interfere with their quantification. Even though NPEM performs better than NEM in terms of derivatization efficiency and ESI ionization enhancement, it is more prone to undergo the side reactions such as amine derivatization and ring opening, as summarized in **Figure 2-8**, which should discourage its use. NEM, on the other side, provides less signal enhancement, but is more stable, and less secondary products are found when it is used as a derivatizing reagent at pH 5-7 (**Figure 2-9**). NEM is also less sensitive to small changes in pH in the range of 5-7 than NPEM. For NPEM, very strict pH control is required to avoid artefacts and inaccurate quantitation due to side product formation. As pH increases above pH 7.0, for both NEM and NPEM, cyclized cysteine side product formation increases. The formation of this side product also increases with time, i.e. while the samples are in autosampler waiting for LC-MS analysis. This finding has critical consequences for accurate determination of cysteine concentrations, and it is strongly recommended to measure this cyclized product routinely as quality control to evaluate accuracy of CYS concentration measurement. To ensure accurate CYS measurement one of the following three strategies can be employed depending on the application and CYS levels under measurement: (i) samples should be analyzed immediately after preparation to avoid time-course formation of cyclized product (ii) calibration samples should be prepared at the same time as study samples, so that any time-related degradation occurs in both study and calibration samples to similar extent and (iii) cyclized product signal intensity can be summed with the primary product signal intensity and used to build calibration curves and perform quantitation. The last scenario assumes that both products have similar ionization efficiency, which seems to be a more valid approximation for NEM than NPEM based on our results. A caveat for the use of approach (ii) is that degradation of primary product to cyclized derivative occurs almost completely within a 24 hour period as shown in **Figure 2-4**, so this approach may result in no detection of CYS if starting concentrations of CYS were very low.

The goal of this study was to identify a thiol-protecting reagent that is able to penetrate cell membranes to derivatize the intracellular thiols prior to extraction. With this in mind, it is important that the derivatizing conditions be mild, to minimize the impact on the cellular structure and avoid artificial changes in the metabolites. Therefore, because of the nature of this work, NEM is more suitable as a derivatizing reagent than NPEM, as it can react at pH 7.0, with minimal side-reactions. In the literature, many maleimide-type reagents not covered by this study are used to allow analysis and quantification of thiols, and the formation of side products should be investigated for each of them before their use to quantify thiols.

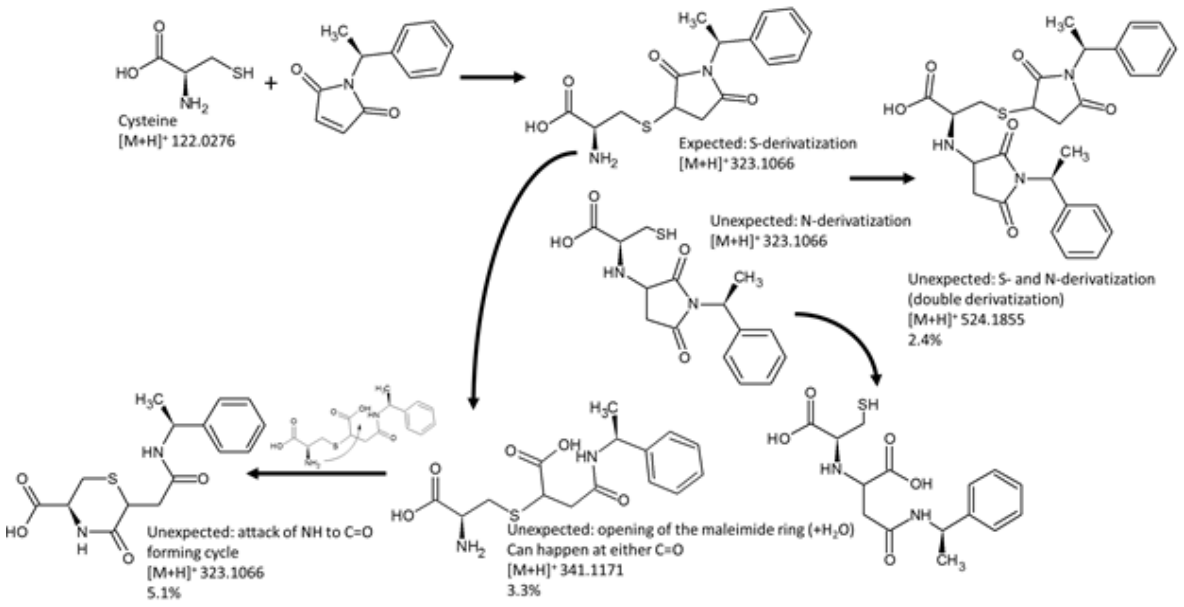

Figure 2-8 – Summary of the reactions observed for cysteine with NPEM. All thiols can undergo double derivatization and maleimide ring opening reactions. Only CYS will react to form cycle after opening of the maleimide ring. Percentages shown correspond to peak area of that side product to sum of peak area of CYS-NPEM diastereoisomers , and assume that the ionization efficiency of all compounds is the same. Data is shown for samples analyzed immediately after 30 min derivatization at pH 7.0. It should be noted that cyclization increases over time, so the example shown above represents the best-case scenario.

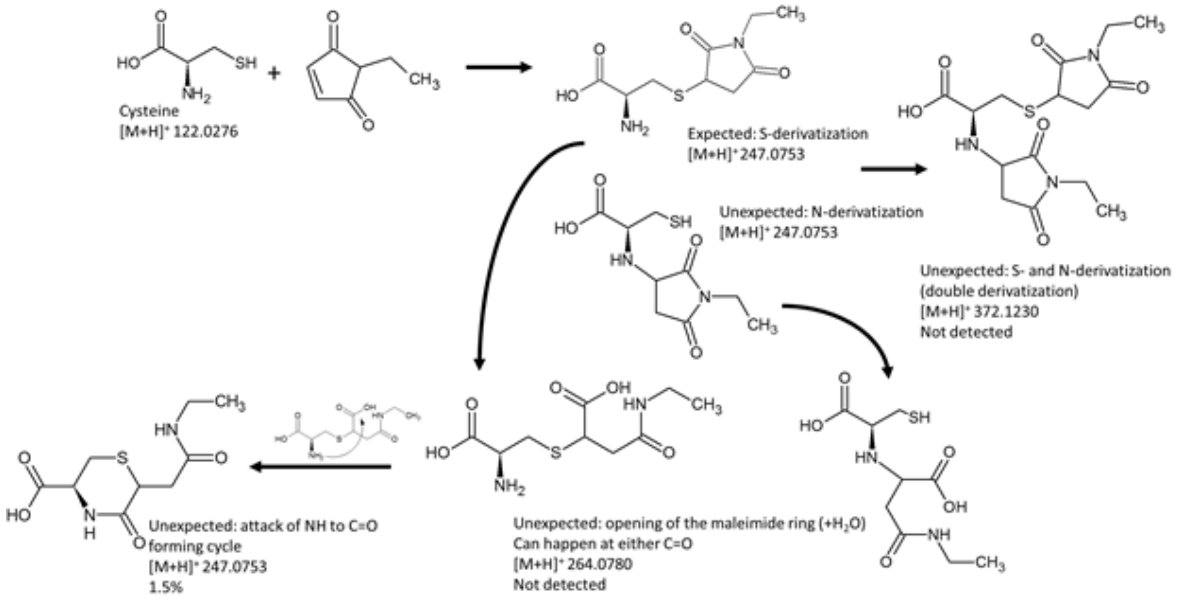

Figure 2-9 – Summary of the reactions observed for cysteine with NEM. All thiols can undergo double derivatization and maleimide ring opening reactions. Only CYS will react to form cycle after opening of the maleimide ring. Percentages shown correspond to peak area of that side product to sum of peak area of CYS-NEM diastereoisomers, and assume that the ionization efficiency of all compounds is the same. Data is shown for samples analyzed immediately after 30 min derivatization at pH 7.0. It should be noted that cyclization increases over time, so the example shown above represents the best-case scenario.

3. Development of a LC-MS method for analysis of thiol ratios as an indicator of oxidative stress in immune cells

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3.1. Abstract

The study of oxidative stress through quantification of the ratio between thiols and their disulfides is possible due to the role of reduced thiols as scavengers of the free radicals that cause intracellular damage. The objective of this research was to incorporate the NEM derivatization optimized in the previous chapter into a full protocol developed to evaluate oxidative stress status of Jurkat cells. The main steps of this protocol include NEM derivatization of free thiols before cell lysis, thus preventing thiol auto-oxidation in order to enable accurate quantification of reduced thiol content. After derivatization, cell lysis and metabolite extraction is performed using 20% methanol (v/v) followed by protein precipitation using pure methanol and evaporation/reconstitution step prior to LC-MS analysis. LC separation of derivatized thiols is achieved by using a biphenyl column followed by quantitation of derivatives using high resolution mass spectrometry.

The cell lysis and extraction solvent, wash conditions, amount of derivatizing agent, side product formation, recovery and matrix effect were optimized and evaluated in the proposed protocol. 20% MeOH was chosen as thecell lysis and extraction solvent due to its ability to extract all species of interest. The wash steps still need further optimization with the goal of minimizing leakage observed. The recovery results indicate that while there is no loss after lysis due to removal of cell debris, there is loss during the subsequent steps, since overall, extraction recoveries for the individual thiols did not surpass 57 ± 6% for GSH, indicating that there is loss in the procedure, which should be reduced if possible. The extract matrix provides enhancement of the signal from 41% (GSH) to 61% (HCY), and must be corrected using isotopically labeled internal standards.

The finalized protocol requires 1×10^6 cells and was tested on cells that were stimulated with hydrogen peroxide at three levels: 25 μM for 10 min, 200 μM for 10 min and 200 μM for 20 min for induction of oxidative stress. The protocol developed was capable of differentiating between mild and extreme exposure, as indicated by the concentration of GSH: 216.3 ± 3.2 ng/10^6 cells in

control versus 317.1 ± 22.1 ng/10^6 cells for mild exposure ($p_{0.05}$ = 0.016), 13.5 ± 4.6 ng/10^6 cells ($p_{0.05}$ = 1.9 × 10^{-7}) for 10 min of high exposure and 1.2 ± 0.3 ng/10^6 cells for 20 min of high exposure ($p_{0.05}$ = 7.3 × 10^{-5}), showing that the thiol concentration is indeed a biomarker for this condition. The increase of the glutathione concentration after mild exposure to oxidative stress is expected, since the cells will increase production of the thiol in an attempt to prevent damage caused by increased oxidation. To further confirm these findings, the quantification of the disulfides must be performed to allow the measurement of the thiol/disulfide ratio. The procedure for evaluating the disulfides is still under development, but will be achieved by reduction of the disulfides using TCEP, followed by reaction with NEM, allowing quantification of the total thiol content, from which the oxidized concentration can be obtained. This is one of the few methodologies where derivatization is performed prior to cell lysis, a significant improvement over current methods that expose the thiols to autoxidation during sample handling.

3.2. Introduction

Oxidative stress is caused by the reaction of free radicals (known as reactive oxygen species - ROS) with cellular components, leading to damage of metabolites, proteins and membranes.[1] While ROS are formed during normal metabolic processes, their concentration under oxidative stress is increased. This is reflected in the increased modification of intracellular metabolites. By measuring these metabolites, it is possible to estimate the oxidative status of a cell. There are various metabolites that can be measured with this goal: lipid peroxidation markers such as malondialdehyde[6], 4-hydroxynonenal[98] and isoprostanes[12], and low molecular mass molecules, such as thiols.[9] Organic thiols are present in cells and can help to protect them from some of the damage caused by ROS through redox reactions. When they reduce the ROS, the thiols are converted to their oxidized form, i.e. the disulfides. Under normal oxidizing conditions, the ratio between reduced and oxidized thiols is tightly controlled by the action of reductases. However, under oxidative stress, the cells are unable to match the rate of oxidation, and the ratio shifts.[17]

In order to measure the thiol ratio, it is important to protect the reduced thiols from autoxidation, which is an artefactual oxidation that occurs during sample handling. To do so, the free thiol is protected with a derivatizing agent, making it unavailable for further reactions. There are many different reagents that can be used with this purpose, out of which N-ethyl maleimide is one of the most advantageous because it can react with the free thiols fast, completely, and can penetrate cellular membranes, allowing protection before exposure by cell lysis.[20,63]

After defining how to preserve the intracellular thiols in their original redox state, it is important to define how the sample will be treated in order to extract them. The cell lysis, which is the release of metabolites from cells by disruption of the membrane, is often combined with the metabolite extraction step[71], which is the recovery of those metabolites in a solvent that will be analyzed. There are various methods used for this purpose, including: solvent extraction using solvents such as ethanol[32], methanol, chloroform and water[29,32,79,84] at both room temperature and cold temperatures to minimize enzymatic activity, cold trichloroacetic acid (TCA)[22], and freeze-thaw cycles.[99] The ideal extraction procedure will comprehensively extract the metabolome, be simple, and quench the enzyme activity rapidly and completely.[71] This latter requirement is important for thiol analysis due to the presence of the reductases, which may reduce oxidized thiols into their reduced forms, altering the ratio.

There are many human cell lines available for *in vitro* studies. Jurkat T cells are a human lymphoblastic line that are used for studies of immune signaling[70], and are attractive since they can represent cells under both normal and inflammatory conditions.[70] To ensure that the protocol is capable of differentiating between healthy cells and cells under oxidative stress, it is necessary to induce oxidative stress, which can be done using hydrogen peroxide.[70] The cells respond by increasing proliferation, halting cellular growth, inducing apoptosis and activating mitogen-activated protein kinase.[75]

Baty *et al.*[75] used proteomics to study oxidative stress in Jurkat cells induced by hydrogen peroxide. They observed modification of several proteins involved with energy metabolism, cell structure, protein folding and other cellular functions. Out of these, GAPDH (glyceraldehyde 3-phosphate dehydrogenase) and peroxiredoxins were markedly more affected by oxidation, which can be related to their functions, since peroxiredoxins reduce hydrogen peroxide, which would target these proteins upon entering the cell. The authors hypothesize that the inactivation of GADPH has the objective of allowing more formation of NADPH, necessary for reducing processes that can help protect the cell during oxidative stress.[75]

Chkihvishvili *et al*.[70] found that the effect of hydrogen peroxide on Jurkat cells was dose-dependent, with lower doses of 20 μM causing reversible damage, 50 μM and higher inducing apoptosis and even higher (200 and 500 μM) causing necrosis, or cell death. They also studied the effect of extracts from French marigold to protect the Jurkat cells from oxidation and found that

some of the components present in the extract were indeed able to protect the cells, which aligned to the use of this plant in folk medicine.

The existing methods for studying thiols in Jurkat cells derivatized them with monobromobimane after lysis[75,100]. In this study, a protocol that derivatizes thiols with N-ethyl maleimide prior to lysis was developed, with the goal of preventing thiol autoxidation during sample handling, allowing more accurate measurement of the intracellular thiol concentrations. The development of the protocol included optimizing the amount of derivatizing reagent used, choosing the best extraction solvent for the analytes of interest, evaluating the wash step, studying recovery and matrix effects. The method was then tested in Jurkat cells that were exposed to different concentrations of hydrogen peroxide, to evaluate if it was able to differentiate between exposure to mild and severe oxidative stress.

3.3. Experimental

3.3.1. Materials

N,N'-diacetylcystine was purchased from Cayman Chemicals (Ann Arbor, MI, USA). Potassium chloride (KCl) was obtained from BioShop Canada (Burlington, ON, Canada). Glutathione (GSH), LC-MS grade water, methanol and acetonitrile were purchased from Fisher Scientific Inc. (Ottawa, ON, Canada). All other chemicals were purchased as L-enantiomer from Millipore-Sigma (Oakville, ON, Canada), except for homocysteine, which was obtained as the mixture of L and D enantiomers.

Modified PBS buffer was prepared by weighing 8 g of NaCl, 0.2 g of KCl, 1.44 g of Na_2HPO_4 and 0.24 g of KH_2PO_4 to the nearest 0.1 mg. 800 mL of water was added to the salts, and the pH was adjusted to 7.0 using HCl. The volume was then adjusted to 1 L, and ascorbic acid was added to a final concentration of 500 ng/mL. Hydrogen peroxide 3% w/v was purchased from a pharmacy and was diluted in PBS to make 50 μM and 400 μM solutions.

Stock solutions of all standards were prepared at 1 mg/mL in 20% methanol with 10 mM ammonium acetate/acetic acid (95:5 Am. Ac./Ac. Ac. v/v) at pH 6 and stored at -80°C. Working solutions were prepared by diluting the stock solution in water.

NEM was prepared in water at 1 mg/mL and diluted to 20 μg/mL. Reagents were kept at -80°C until use.

3.3.2. Calibration standard preparation

Calibration curves of underivatized thiols and disulfides were prepared by sequential two-fold dilution of 200 ng/mL standards until 0.391 ng/mL, and analyzed from the lowest concentration to highest. Dilution solvent was 3% methanol in water, which matches the solvent composition of the cells prior to analysis. Calibration standards of derivatives were obtained by derivatizing a mixture of the thiols at 200 ng/mL according to procedure below and performing sequential 2-fold dilutions using 3% methanol after 30 min.

Standards at 200 ng/mL were derivatized with 2000 ng/mL of derivatizing agent (1:10 w/w) in modified PBS buffer at pH 7.0, the mixture was mixed for 1 min using a vortex, and reacted for 30 min on ice, unless otherwise stated. Whenever mixtures of standards were used, derivatizing agent was adjusted to maintain 1:10 w/w ratio. No reaction quenching was performed.

3.3.3. Cell cultures

Jurkat T-lymphocyte cells from ATCC (Manassas, VA, USA) were grown in T-75 flasks with RPMI-1640 media with 10% heat-inactivated fetal calf serum supplemented with 1 mM L-glutamine and penicillin-streptomycin. Incubation was done in an incubator with an atmosphere containing 5% CO_2. Cells were passaged every 2-3 days to ensure they are kept alive and under 1×10^6 cells/mL. For experiments, cells were counted using trypan blue. Each biological replicate consisted of 1×10^6 cells.

3.3.4. Cell derivatization and extraction protocol

The final version of the protocol is described below, and main steps of the protocol are summarized in flow chart in **Figure 3-1**. All samples were prepared in a Purefire Logic Plus biological safety cabinet from Labconco (Kansas City, MO, USA). All centrifugation steps were carried out using a Sorvall ST 16R centrifuge from Thermo Fisher (Waltham, MA, USA), while mixing was performed using Fisher Scientific Multi-Platform Shaker.

3.3.4.1. Derivatization and extraction of cells

For extraction of thiols, 1 mL of Jurkat cells (1×10^6 cells/mL) was collected in a centrifuge tube. This was centrifuged at 1600 RPM (300 × g) for 10 min at 4°C, mild conditions to avoid stressing the cells, and both pellet and supernatant were used. The cell pellet obtained was resuspended and derivatized using 200 µL of derivatizing agent in PBS pH 7.0 at 200 µg/mL for 30 min, with mixing at 200 RPM, after which it was centrifuged and washed three times with 200 µL of PBS with 50 µg/mL derivatizing agent for 20 min with mixing at 200 RPM and centrifugation between the

steps. The cell pellet obtained after the final wash step was extracted using 20% methanol for 20 min with mixing at 200 RPM. The cell debris was removed by centrifugation at 25,830 × g, and the extract was transferred into a new centrifuge tube. Proteins were removed by precipitation with 600 µL of cold methanol (-80°C), and after mixing for 20 min, the samples were kept at -80°C for 30 min before centrifugation at 23,314 × g. The supernatant (800 µL) was collected into a new centrifuge tube, and this fraction was called EXTRACT. The samples were evaporated to dryness under vacuum and reconstituted in 3% methanol (800 µL), sonicated and vortexed for 20 min, and centrifuged at 23,314 × g for 10 min. The supernatants were transferred into vials for LC-MS analysis.

For the recovery and matrix effect experiments, after protein precipitation, the samples were diluted 10-fold in water in order to reduce the concentration of methanol. For this set of experiments, the calibration curve was prepared in 8% methanol, to match the final solvent composition of the samples.

For all experiments, a blank sample was extracted using the same protocol using an empty microcentrifuge tube.

3.3.4.2. Optimization of extraction solvent

For metabolite extraction, mixtures of water and methanol (100% H_2O, 20% MeOH, 50% MeOH, 80% MeOH and 100% MeOH, v/v) were tested to evaluate the solvent with the best extraction efficiency. For this set of experiments, NPEM was used as a derivatization reagent as this was performed prior to our experiments proving its derivatives are less stable than NEM.

3.3.4.3. Evaluation of cell culture supernatant

From the supernatant obtained after the very first centrifugation of the cell pellet, 200 µL was collected into a new centrifuge tube, and was derivatized with 200 µL of derivatizing agent in PBS at 200 µg/mL for 30 min. Proteins were removed by adding 600 µL of cold methanol (-80°C), and after mixing the samples for 20 min, they were stored at -80°C for 30 min, followed by centrifugation at 25,830 × g. The supernatant was collected in a new polypropylene HPLC insert and this fraction was called SUPERNATANT. This was analyzed to determine the levels of thiols of interest present in cell culture medium and to evaluate the efficacy of washing steps.

3.3.4.4. Evaluation the effectiveness of the washing procedure

After the second and third wash steps were concluded, the supernatant was collected into a separate test tube, and proteins were removed by cold methanol (-80°C) precipitation, and after mixing the samples for 20 min, they were stored at -80°C for 30 min, followed by centrifugation at 25,830 × g. The supernatant was collected into a new HPLC insert and this fraction was called WASH. These samples were analyzed during protocol development to determine the effectiveness of wash procedure and how many wash steps should be incorporated into final protocol.

3.3.1. Recovery and matrix effect experiments

The quality of the proposed method can be measured by evaluating recovery, which is calculated as shown in **Equation 3-1**. This approach requires the analysis of three samples: one is analyzed to determine endogenous level of thiols, one is spiked at the beginning of the protocol (also known as pre-extraction spike), and one is spiked immediately before LC-MS analysis (also known as post-extraction spike), as shown in **Figure 3-2**. Considering that the composition of pre-extraction and post-extraction spiked samples is similar, other than for the spiked amount of analyte, calculating recovery this way provides pure extraction recovery of the method, without bias of matrix effect, which is calculated separately.

$$Extraction\ recovery\ (\%) = \frac{Signal\ of\ sample\ spiked\ before\ extraction - endogenous\ signal}{Signal\ of\ sample\ spiked\ before\ analysis - endogenous\ signal} \cdot 100\%$$

Equation 3-1 – Calculation of extraction recovery

In this protocol, there is a step for lysis and extraction of the metabolites from the cells followed by removal of the cell debris. To ensure that the metabolites are not being removed with the cell debris at the centrifugation step after the lysis, the recovery of this step was calculated separately from that of the entire procedure. For this calculation, the sample spiked before extraction was

compared to a sample spiked after the lysis and centrifugation steps (post-lysis spike), and the recovery was calculated as shown in **Equation 3-2**.

$$Lysis\ recovery\ (\%) = \frac{Signal\ of\ sample\ spiked\ before\ extraction - endogenous\ signal}{Signal\ of\ sample\ spiked\ after\ lysis - endogenous\ signal} \cdot 100\%$$

Equation 3-2 - Calculation of lysis recovery

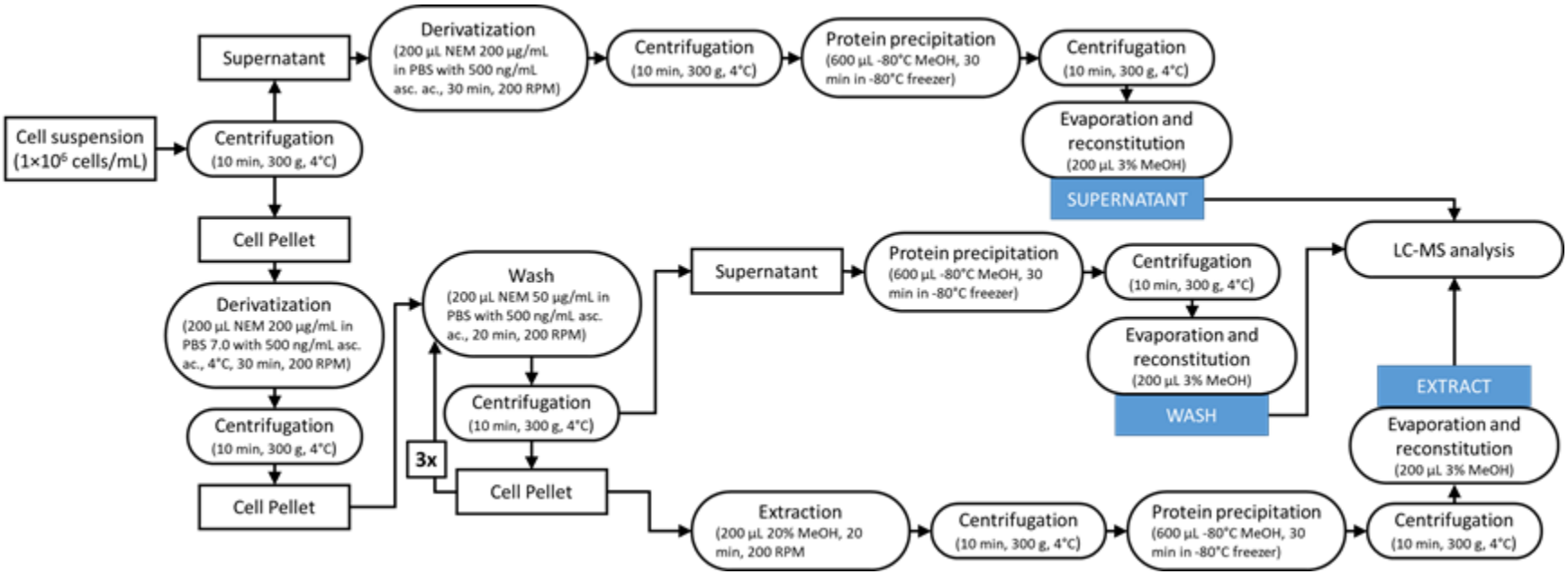

Figure 3-1 – Flowchart of the derivatization and extraction protocol

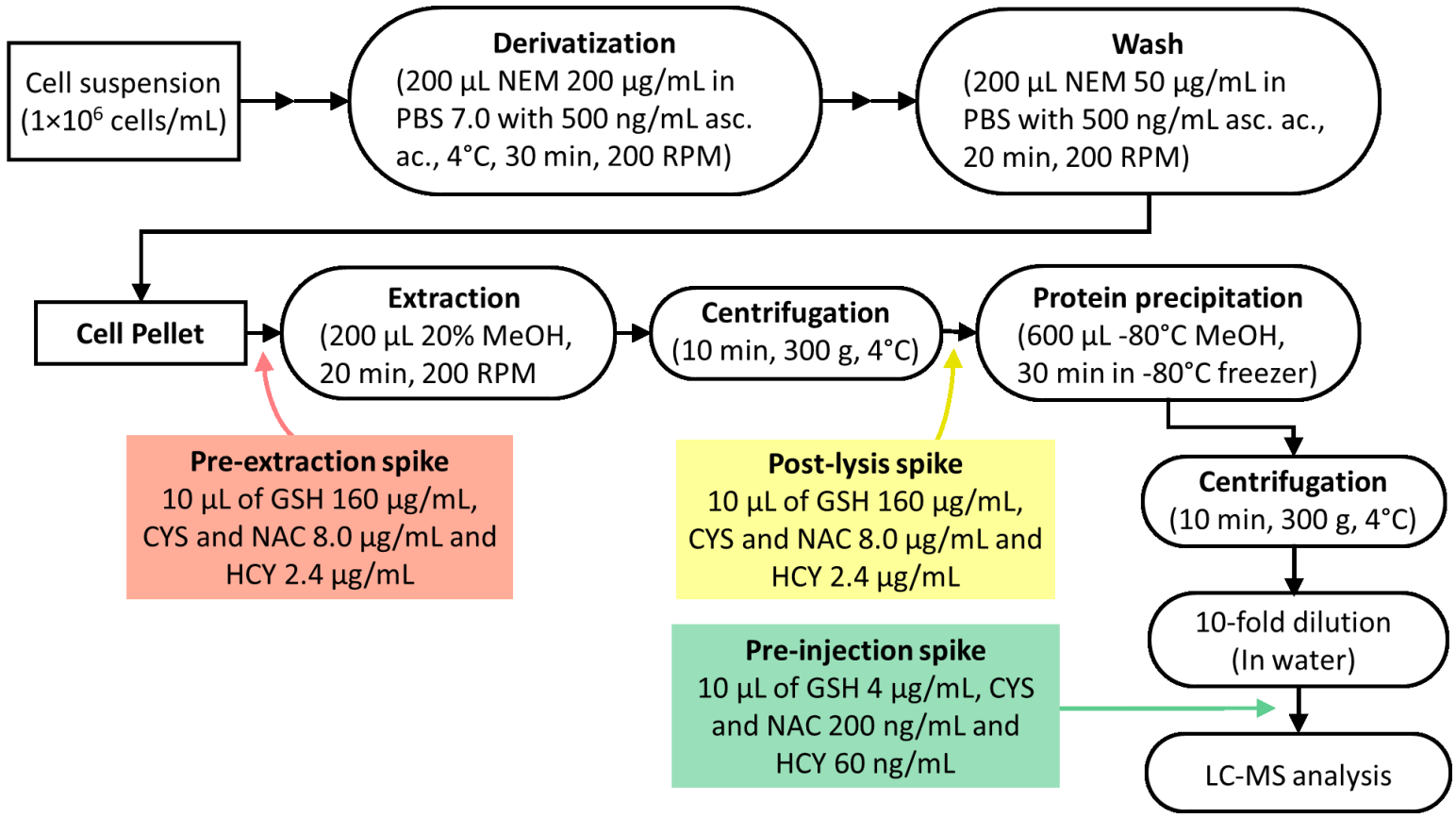

Figure 3-2 – Spiking protocol for calculation of matrix effect and recovery.

The presence of metabolites that co-elute with the analytes of interest can interfere with their ionization in the electrospray, changing their signal. This is known as matrix effect, and can be calculated by comparing the signal of the sample spiked with a known concentration prior to analysis and the signal of the same concentration of analyte in pure solvent, as shown in **Equation 3-3**. The endogenous analyte is subtracted from the sample, to ensure that only the signals of the spikes are being considered.

$$Matrix\ effect\ (\%) = \frac{Signal\ of\ sample\ spiked\ before\ analysis - endogenous\ signal}{Signal\ of\ spiked\ standard\ in\ pure\ solvent} \cdot 100\%$$

Equation 3-3 - Calculation of matrix effects

For these experiments, derivatized thiols were spiked in four biological replicates for each of the necessary samples to obtain the following concentrations: GSH – 200 ng/mL, CYS and NAC – 10 ng/mL, HCY – 3 ng/Ml at the points indicated in **Figure 3-2**. These values were selected based on the concentrations that were detected in previous extractions, except for NAC, which is not typically detected. To obtain these values, the pre-extraction and post-lysis samples were spiked with 10 µL of GSH – 160 µg/mL, CYS and NAC – 8 µg/mL, and HCY – 2.4 µg/mL. The pre-injection samples and solvent were spiked with 10 µL of GSH – 20 µg/mL, CYS and NAC – 0.2 µg/mL, and HCY – 0.06 µg/mL. For post-extraction spike, a calculation error was made and samples were incorrectly spiked. Due to this error, calculation shown in **Equation 3-1** could not be used. Instead, recovery for this set of experiments was calculated by comparing the pre-extraction spike sample minus endogenous content to the area of a spiked blank, as shown in **Equation 3-4**. This calculation results in the procedure recovery, and does not ignore the matrix effect bias.

$$Procedure\ recovery\ (\%) = \frac{Signal\ of\ sample\ spiked\ pre\text{-}extraction\ - endogenous\ signal}{Signal\ of\ blank\ spiked\ pre\text{-}injection} \cdot 100\%$$

Equation 3-4 – Calculation of procedure recovery.

3.3.2. Induction of oxidative stress using hydrogen peroxide

To induce oxidative stress in Jurkat cells, enough cells were collected and centrifuged to make a pellet with 16×10^6 cells, the growth media was removed and discarded. The cells were resuspended in PBS pH 7.4 to a concentration of 2×10^6 cells/mL and 0.50 mL was added to labeled 1.5 mL microcentrifuge tubes. Each test group consisted of four biological replicates. To the control group, 0.50 mL of PBS was added and incubated for 20 min. To two of the groups, 0.50 mL of 400 µM hydrogen peroxide was added, and incubated for total of 10 or 20 min. To the fourth

group, 0.50 mL of 50 µM was added and incubated for 10 min. All groups were centrifuged (8000 RPM for 50 seconds), and put on ice. The supernatant was removed from the tubes, the cell pellet was broken to allow suspension of the cells by lightly scraping it against a serrated surface and 200 µL of NEM 200 µg/mL was added. The rest of the extraction proceeded as described in the final protocol described in 3.3.4.

3.3.3. Liquid Chromatography – Mass Spectrometry analysis

3.3.3.1. HPLC-ESI-QTOF

The analyses were performed on an Agilent 1290 UHPLC – 6550 iFunnel QTOF from Agilent Technologies (Santa Clara, CA, USA). Biphenyl column (100 mm x 2.1 mm x 1.7 µm, 100 Å) from Phenomenex (Torrence, CA, USA) with appropriate guard column was used for analyte separation. The mobile phase used was water with 0.1% formic acid (A) and acetonitrile with 0.1% formic acid (B), at 0.3 mL/min. The gradient used was: 0-4 min at 3% B, 4-12 min linear increase from 3 to 21% B, 12-13 min linear increase from 21 to 90% B, 13-16 min at 100% B, 16-16.1 min linear decrease from 90 to 3% B and 16.1-21 min at 3% B to recondition the column. Injection volume was 10 µL for all analyses. Samples were injected in the following order: System suitability evaluation, QC, calibration curve, randomized samples, QC every tenth run, calibration curve.

During solvent extraction optimization, Kinetex PFP column (50 mm x 2.1 mm x 2.6 µm, 100 Å) from Phenomenex (Torrence, CA, USA) with guard column (UHPLC PFP for 3.0 mm ID columns from Phenomenex) was used for analyte separation. The mobile phase used was water with 0.1% formic acid (A) and acetonitrile with 0.1% formic acid (B), at 0.3 mL/min. The gradient used was: 0-4 min at 0% B, 4-8 min linear increase from 0 to 4% B, 8-20 min linear increase from 4 to 40% B, 20-25 min linear increase from 40 to 95% B, 25-27 min at 100% B, 27-27.1 min linear decrease from 95 to 0% B and 27.1-31 min at 0% B to recondition the column.

Samples were analyzed in positive ESI mode, since the compounds do not ionize well in negative mode. The mass range analyzed was 50-1100 m/z, at 2 scans/s. Drying gas temperature was 250°C, 15 L/min. The nebulizer gas pressure was 35 psig, and sheath gas temperature was 275°C and flow 12 L/min. Fragmentor was set at 250 V, with the octopole voltage at 750 V. ESI capillary voltage was set at 3500 V, and nozzle voltage was set at 400 V.

Data was acquired using the LC/MS Data Acquisition for 6200 series TOF / 6500 series QTOF (version B.06.01, build 6.01.6157, Agilent). Internal mass calibration was performed throughout all

sample analyses by introduction of calibrant using Agilent 1260 Isocratic pump and dual-introduction source. The calibrant exact masses used for mass calibration were 121.050873 from purine and 922.009798 from HP0921, two components of the Agilent calibration mixture.

Data processing and quantification were performed using either TOF Qualitative Analysis software (version B.07.00, build 7.0.7024.29) or TOF Quantitative analysis software (version B.07.00 SP1, build 7.0.457.0, Agilent), with a raw data extraction window of 15 ppm for $[M+H]^+$ ion and calibration curves were calculated using 1/x weighting. All error calculations used error propagation, and statistical analysis was done with Microsoft Excel (Redmond, WA, USA).

3.3.3.2. HPLC-ESI-Orbitrap

The recovery and matrix effect analyses were performed on an Agilent 1100 HPLC from Agilent Technologies (Santa Clara, CA, USA) coupled to a LTQ Velos Orbitrap equipped with HESI electrospray ionization source (Thermo Scientific, San Jose, CA, USA). Biphenyl column (50 mm x 2.1 mm x 2.6 µm, 100 Å) from Phenomenex (Torrence, CA, USA) with appropriate guard column was used for analyte separation and was kept at 30°C. The mobile phase used was water with 0.1% formic acid (A) and acetonitrile with 0.1% formic acid (B), at 0.3 mL/min. The gradient used was: 0-4 min at 3% B, 4-12 min linear increase from 3 to 21% B, 12-13 min linear increase from 21 to 90% B, 13-16 min at 100% B, 16-16.1 min linear decrease from 90 to 3% B and 16.1-25 min at 3% B to recondition the column. Injection volume was 10 µL for all analyses. Samples were injected in the following order: System suitability evaluation, QC, calibration curve, randomized samples, QC every tenth run, calibration curve.

Samples were analyzed in positive ESI mode, since the compounds do not ionize well in negative mode. The mass range analyzed was 150-1100 m/z. The parameters of analysis were: source voltage 4 kV, capillary temperature 275°C, source heater temperature 300°C, sheath gas flow 20 L/min, S-lens RF level 62%, auxiliary gas flow 5 L/min.

Data acquisition and processing were performed using the Xcalibur software (version 2.7 SP1, Thermo Scientific). Raw data extraction window of 10 ppm for $[M+H]^+$ ion was used. Calibration curves were calculated using 1/x weighting. All error calculations used error propagation, and statistical analysis was done with Microsoft Excel (Redmond, WA, USA).

3.4. Results and discussion

3.4.1. Definition of the initial derivatization and extraction protocol

The goal of this protocol is to analyze the individual thiol to disulfide ratios in order to measure the oxidative status of cells. For this reason, it is important to ensure that the thiols are extracted in a way that prevents autoxidation of reduced thiols. A derivatizing agent is used for this purpose. In particular, a derivatizing agent that can penetrate cell membranes and derivatize thiols prior to extraction is preferred because this would prevent autoxidation during cell lysis process. After derivatization, it is important to remove the cell media before cell lysis and extraction, to prevent contamination of the cell extracts with metabolites present in the cell media. During this wash step, it is important that no inadvertent cell lysis takes place, as this would cause inaccurate measurement of thiol and disulfide concentrations. Finally, the cell metabolites should be extracted, and protein precipitation performed prior to analysis.

Three cell-permeating derivatizing reagents were tested previously: N-ethyl maleimide, (R)-(+)-N-(1-Phenylethyl)maleimide) and iodoacetamide. Iodoacetamide was eliminated due to its incomplete reaction with thiols, and NPEM was unstable, forming more undesirable side reactions than NEM. Therefore, NEM was selected as the best derivatizing agent for the development of this protocol. The optimal derivatizing conditions were also tested, and reaction at pH 7.0 and 4°C was defined as ideal with addition of 500 ng/mL ascorbic acid to help prevent side reactions.

3.4.2. Optimization and evaluation of proposed protocol

3.4.2.1. Amount of derivatization reagent

It is very important that enough derivatization reagent is added during the derivatization step to ensure complete derivatization of the thiols, especially because the reagent can also react with free cysteine residues in proteins. Our protocol adds 200 µL of 200 µg/mL (0.317 µmoles) NEM in PBS buffer to the cell samples, which corresponds to 40 µg/10^6 cells, which is more than the 5.6 µg NEM/10^6 cells used by Ortmayr *et al.*[29] to derivatize human ovarian carcinoma cells.

There are two ways that excess NEM was verified: first, the m/z of the reduced (underivatized) thiols was extracted, and if no peaks were detected, derivatization was considered complete for that thiol. Since the underivatized thiols elute at the solvent front, their detection is limited, so the presence of excess NEM was also verified by extraction of its m/z, 126.0555 at the retention time of 4 min. Both conditions were met for all cell samples evaluated, with the cell samples containing

about 60% of the amount of NEM in the blank extraction sample, indicating excess reagent was present during derivatization

After quantification of the thiols in samples that were stressed with hydrogen peroxide, the excess amount of derivatization reagent was calculated, as shown in 3.4.7.

3.4.2.2. Evaluation of wash efficiency

To ensure complete removal of the growth media, multiple sequential washes with PBS containing the derivatizing reagent were performed. The goal is to remove the contamination of the media without disturbing the cells, which would cause leakage of the intracellular metabolites. This evaluation was performed by comparing the concentration of the metabolites detected in the washes to that present in the cell media. As can be seen in **Table 3-1**, GSH is not detected in the cell media, but is detected in the wash steps, which indicates continuous leakage during the wash step, which caused the intracellular levels to be lower. This is undesirable, since it would indicate intracellular metabolites being lost during wash procedure. The same does not occur with CYS: the extracellular concentration is much greater than what is found in the wash and intracellular, where it can be detected but not quantified accurately (below LOQ). These contradictory results do not allow us to conclude whether there is leakage due to the wash steps. In the experiment performed to test extraction solvents, the wash was done with PBS at pH 7.4, and these wash problems did not occur, as shown in **Table 3-2**. This might indicate that the problem lies with using the pH 7.0 modified buffer, however, as the

Table 3-1 – Concentration of thiols detected in the wash solutions in comparison with cell media and intracellular thiol levels. Wash 2 and 3 and endogenous show the average concentration and standard deviation of four replicates. Cell media and extraction blank were analyzed once. Concentrations reported were corrected for dilutions. Limit of quantification for the derivatized thiols is 3.9 ng/mL for wash and endogenous fractions and 18.8 ng/mL for the cell media fraction. Wash was performed using modified PBS pH 7.0.

Sample	CYS-NEM (ng/mL)	HCY-NEM (ng/mL)	NAC-NEM (ng/mL)	GSH-NEM (ng/mL)
Cell media	107.9	ND	ND	< LOQ
Wash 2	< LOQ	< LOQ	ND	637.5 ± 155.8
Wash 3	ND	ND	ND	115.0 ± 14.5
Intracellular	< LOQ	ND	ND	44.7 ± 19.7
Extraction blank	ND	ND	ND	ND

Table 3-2 – Concentration of thiols detected in the wash solutions in comparison with cell media and intracellular thiol levels. Wash 2 and 3 and endogenous show the average concentration and standard deviation of four replicates. Cell media and extraction blank were analyzed once. Concentrations reported were corrected for dilutions. Limit of quantification for the derivatized thiols is 3.9 ng/mL for wash and endogenous fractions and 18.8 ng/mL for the cell media fraction. Wash was performed using PBS 7.4.

Sample	GSH-NPEM (ng/mL)	CYS-NPEM (ng/mL)
Cell media	< LOQ	< LOQ
Wash 2	102 ± 5	< LOQ
Intracellular	2057 ± 45	2.4 ± 0.3

The results obtained indicate that the wash step needs to be further optimized to eliminate leakage. This can be done by testing different volumes of wash solution, different wash times and number of repetitions.

3.4.2.3. Optimization of extraction solvent

An ideal extraction method will result in as complete an extraction as possible. Without knowing how much of each thiol is present in the cells analyzed, the best protocol will be the one that extracts the most. **Figure 3-3** shows the results obtained in the extraction of three thiol-disulfide pairs. NAC/DINAC was not evaluated in this set because at the time we did not have the standard for DINAC to allow quantification.

The different proportions of methanol in the solvent have different extraction capacities for each compound of interest. As shown in **Figure 3-3**, for GSH and HCY, water and then 80% methanol were the best extraction solvents, but water was unable to extract the oxidized thiols GSSG and CYSS, which is an issue when the goal is the measurement of the thiol/disulfide ratio. The only proportions that were able to extract all of the compounds of interest were the 20% and 80% MeOH, although it would be expected that proportions between these two would also provide good results. The choice between the two was made based on how much they were able to extract, and overall, 20% was more efficient than 80%. This was repeated when evaluating extraction of other polar compounds like amino acids, as shown in **Figure 3-4**. Dettmer *et al*.[72] compared several extraction methods for adherent cells and overall, 80% MeOH performed better than 100% MeOH for the extraction of amino acids, however, these were the only proportions tested by the authors.

Based on the results obtained, 20% methanol was defined as the best extraction solvent and was used in all future experiments. In the literature, there is no mention of studies that focus on thiol evaluation in Jurkat cells specifically, but 100% methanol[73], 80% methanol[77], and mixtures of water, methanol and chloroform[79,84] have been used to extract metabolites from this cell line. In other cell lines[72], 80% methanol was considered a good solvent for global metabolomics, and was then adapted for use with thiols[29], but with no further evaluation if other combinations might provide better results. In a study focusing on GSH/GSSG, cold aqueous solution with NEM was used to lyse the cells[82], but no further evaluation was made on the extraction of the metabolites and other thiols.

The differences between the methods could also be caused by differences in ionization suppression, as the calibration curve was prepared in 100% water. This means that any matrix effects caused by the presence of solvent in the sample are not being considered by the calibration curves. To correct for this, calibration curves should be made using each solvent composition tested, and matrix effects caused by the solvents should be evaluated. Using 20% methanol, 2.5 ± 0.1 μg/10^6 cells of GSH, 3.0 ± 0.4 ng/10^6 cells of CYS and 1.2 ± 0.1 ng/10^6 cells of HCY were detected. Baty *et al.*[75] report GSH levels of 0.9 μg/10^6 cells using mBrB and fluorescence, which is lower than our results, but may be the result of their procedure, which derivatized the thiols after lysis. There are no reports on the concentration of the other thiols in Jurkat cells, but CYS was reported in human erythrocytes as 48 ng/10^6 cells, which is lower than our protocol, however, this could be due to variations between the cell types.[100]

The extraction results tested for NPEM were used for NEM without further investigation of whether another composition may be better. Since NPEM is more apolar than NEM, it should prefer more apolar solvents for extraction. Considering this, NEM should be extracted well using more polar solvents such as 20% methanol.

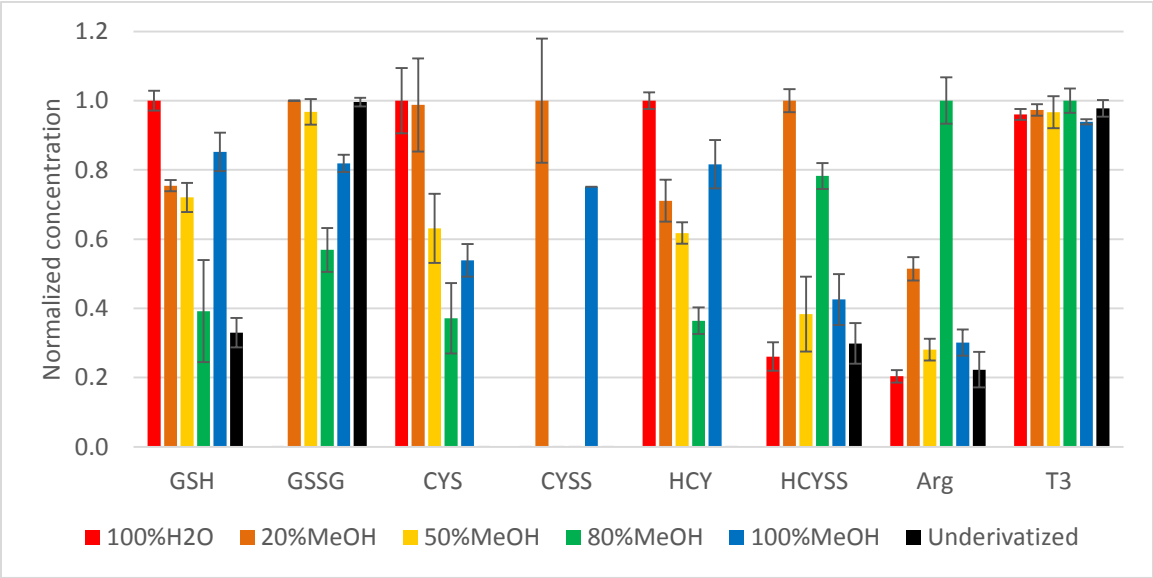

Figure 3-3 – Results obtained for three thiol-disulfide pairs using NPEM for derivatization and different solvents for cell lysis and extraction. LC separation was performed on a PFP column, using the method described in 3.3.3.1. The results show the average of three injections of single biological replicate per condition. Concentrations were calculated using a calibration curve prepared in water and are normalized to the method that provided best results for each compound. Underivatized sample was calculated against a calibration curve of underivatized thiols, and concentration was normalized to the method with highest result for each compound.

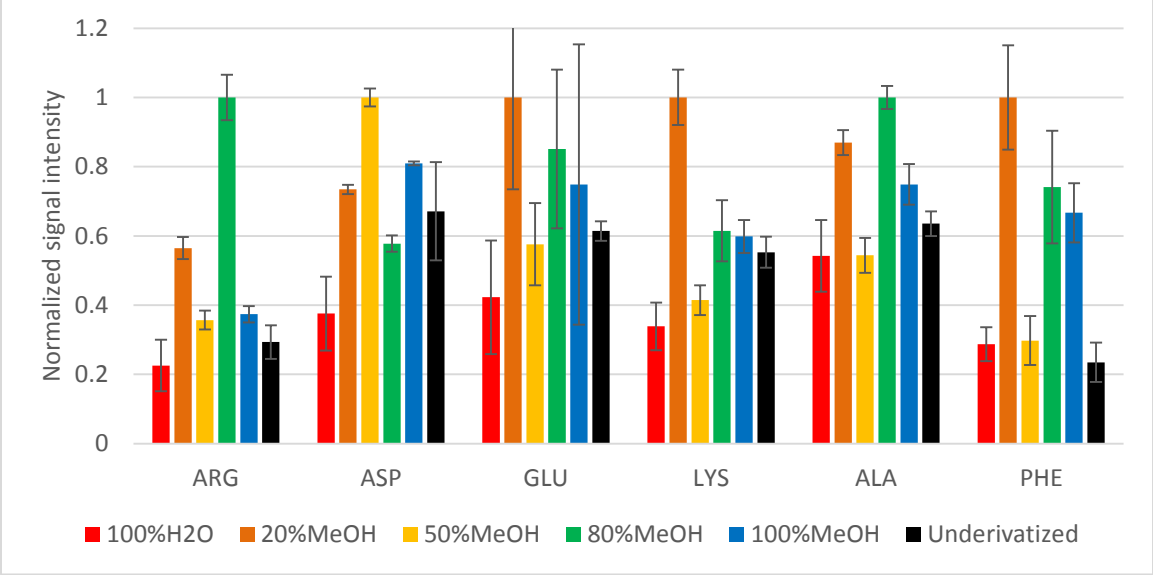

Figure 3-4 – Results obtained for the extraction of several amino acids using the different extraction solvents. LC separation was performed in a PFP column, using the method described in 3.3.3.1. Average of three injections of the same sample. Signals obtained are normalized to the method that provided best results for each compound, and differences between the

methods may be caused by ionization suppression. From left to right, logP values increase (ChemSpider).

3.4.3. NEM side products

One of the disadvantages of reacting thiols with maleimides is the fact that significant concentrations of secondary reactions can be observed if the derivatization conditions are not optimized. The side products of NEM derivatization, namely, double derivatization and open cycle were studied previously in standards, but were not observed in the cell extracts, indicating that the conditions used were successful in preventing them for all four thiols tested. However, it may be that they were not observed in the cell extracts because they correspond to a small proportion of the thiol peaks, and may be present in concentrations lower than the LOD. To ensure this was not the case, further studies will be done to ensure stability of the NEM-derivatized thiols in cell extracts. For example, thiol standards can be spiked to the extract, derivatized and evaluated over time. This way, the influence of the cell extract matrix will be considered in the results.

3.4.4. Recovery

To test the recovery of the method, the sample is spiked at the very beginning of the sample preparation procedure and the concentration of that sample, after subtraction of the endogenous concentration, is compared to a sample spiked immediately before analysis. This ensures that the values obtained are due only to recovery and not matrix effects. In this protocol, however, the cells are grown in media that contains metabolites and they have to be washed before lysis can be done. For this reason, the spike was performed in the first possible step, which was at the cell pellet obtained after the third wash step.

For this set of experiments, a 10-fold dilution was done prior to injection to have injection solvent compatible with the LC method used and to avoid an evaporation and reconstitution step, which could affect the precision. Pre-injection spike was calculated so that the final concentration of all samples would be the same, independent of the dilutions. Additionally, results in 3.4.2.3 supported that 10-fold dilution could be an adequate approach However, the endogenous low abundance thiols CYS, HCY and NAC were detected near the LOD or not detected, so the final protocol was them modified to evaporate the solvent after precipitation (80% MeOH), and reconstitute the samples in 800 µL of 3% MeOH. The recovery and matrix effect will be re-evaluated for this final protocol.

Due to a miscalculation, the pre-injection spike samples were spiked with a lower concentration than the pre-extraction samples, so the two sets were not comparable. Recovery values were therefore calculated for the whole procedure, as described in Section 3.3.1.

The lysis recovery results shown in **Table 3-3** indicate that there is no loss of analyte in the lysis step, which includes lysis, extraction of metabolites from the cells and precipitation of the cellular debris. The estimated extraction recovery results, which is the procedure recovery as calculated by **Equation 3-4** corrected with the matrix effects results shown in **Figure 3-5**, indicate, however, that there is loss of metabolites during the protein precipitation and/or dilution steps. The recovery values are low, especially for HCY, and it is necessary to troubleshoot the method to enhance these numbers. This could be due to intermolecular interactions between the molecules and the proteins, which would cause them to precipitate with the proteins. Additionally, their reproducibility in different trials should be tested, to ensure these results are consistent.

Table 3-3 – Recoveries of four thiols derivatized with NEM in Jurkat cells (n=4). Samples were spiked with derivatized thiols during different steps of extraction procedure to obtain the following concentrations: GSH: 200 ng/mL, CYS and NAC: 10 ng/mL, HCY: 3 ng/mL, as described in Section 3.3.1. Recoveries were calculated according to equations provided in Section 3.3.1.

	CYS-NEM	HCY-NEM	NAC-NEM	GSH-NEM
Lysis recovery	117 ± 35%	123 ± 37%	123 ± 28%	120 ± 35%
Procedure recovery	77 ± 8%	63 ± 7%	84 ± 7%	81 ± 9%
Estimated extraction recovery	52 ± 5%	39 ± 4%	54 ± 5%	57 ± 6%

3.4.5. Matrix Effect

One of the concerns in sample preparation of biological samples is the matrix effect, which is the change in analyte signal due to the presence of other compounds in the sample which can, for example, compete for ionization if they have the same retention time as the analyte of interest[101], change the recovery, cause degradation of the analyte or isobaric interferences with the signal. To measure this, a known concentration of analyte is spiked into the sample prior to analysis, and it is compared to the signal of the same concentration in pure solvent. A method is considered free of matrix effects if the signal obtained in the sample matrix is within 80-120% of signal obtained in pure solvent. As shown in **Figure 3-5**, for all thiols evaluated, there is ionization enhancement due

to the presence of the sample matrix. The results shown are for samples that were diluted 10-fold prior to injection.

To minimize matrix effects, an isotopically labeled internal standard can be used, and a mixture of d_5-NEM-derivatized thiols in a known concentration will, in the future, be added to the samples prior to injection and compared to the areas of the same concentration spiked in pure solvent. Since the d_5-NEM-derivatized thiols have the same chemical structure as the NEM-derivatized thiols, they will elute at similar retention times, and will suffer the same matrix effects. By comparing the area of the peak of the heavy isotope in the matrix and in pure solvent, the matrix effect can be measured, and therefore, can be removed from the actual samples.

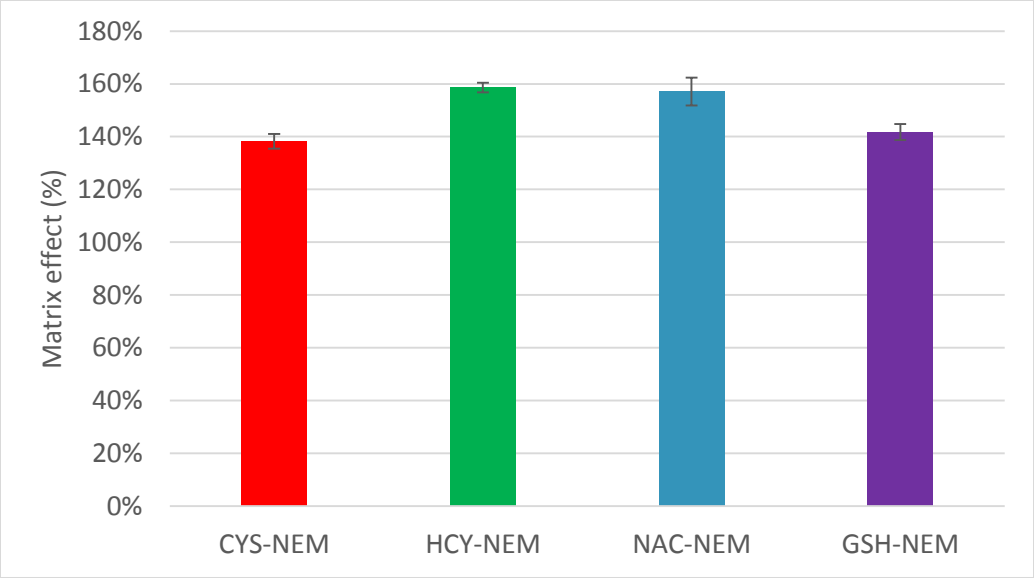

Figure 3-5 – Matrix effect calculated for four thiols derivatized with NEM (n=4). Prior to injection, samples were diluted 10-fold. Samples were spiked with derivatized thiols to obtain the following concentrations at injection: GSH: 40 ng/mL, CYS and NAC: 2 ng/mL, HCY: 0.6 ng/mL.

3.4.6. Determination of thiol/disulfide ratio

To measure the thiol disulfide ratio, ideally, it would be possible to analyze both species in a single run, with no sample treatment other than extracting the analytes from the biological matrix.[102] This, however, is challenging because the reduced thiols autoxidize into their disulfides, requiring a derivatization step to stabilize them.[103] In fact, the analysis of both species in a single run is only possible using mass spectrometry, and there are few examples where this was done.[102] There are three paths that are more reliable to determine the thiol/disulfide ratio, since they involve

protection of reduced thiols, thus stabilizing them. The most common method derivatizes the reduced thiols and analyzes the disulfides in the same experiment.[64,67,80] The difficulty lies in the fact that derivatized thiols and disulfides have different polarities, so simultaneous analysis using reversed-phase chromatography is troublesome. The second path is simpler: the reduced thiols are derivatized, the analytes are extracted from the matrix, and the extract is separated into two fractions, one is analyzed and the other is further treated to reduce the oxidized thiols and measure total thiol content in the sample.[74,95,104] The biggest disadvantage of this procedure is the necessity of analyzing each sample twice. Finally, the third path, with the most sample handling, involves derivatizing the reduced thiols for their protection, then binding of excess reagent, followed by reduction of oxidized thiols and subsequent protection of them with a second derivatizing reagent.[61,68] This path allows simultaneous analysis of reduced and oxidized thiols, but the many processing steps can introduce errors.

The disulfides are present in low concentrations, they have low ionization efficiency (**Figures B5 – B7**) and their polarities make them difficult to retain in RPLC (**Figure B9**), as opposed to the derivatized thiols, which ionize well (**Figures B1 – B4**), and are retained in the biphenyl column used in the final protocol (**Figure B8**). With this and the existing protocol in mind, we opted for using the second approach to measure the thiol/disulfide ratio, to minimize the number of steps added. Many reducing reagents are available for reduction of disulfides such as dithiothreitol (DTT) [105,106], tri-n-butyl phosphine (TBP)[54,107], tris-(2-carboxyethyl) phosphine (TCEP)[108,109] and glutathione reductase (GR).[63,67,110] While DTT has the advantage of being able to permeate cell membranes[32], it is a thiol, so the reagent excess can react with the thiol label[19]. Also, the reagent is not neutralized after the reaction but forms a disulfide that can participate in additional thiol-disulfide reactions.[20] Between TBP and TCEP, the latter is preferred since TBP is insoluble in water and is volatile.[111]

Before proceeding to the biological samples, a mixture of disulfides (GSSG, CYSS, HCYSS and DINAC) was reduced with TCEP and derivatized with NEM to verify the efficiency of reduction. While the literature mentions that the reaction is straightforward and should be completed in a short time at room temperature[112], reduction of the disulfides was not observed even after 4 hours. Better results were obtained after 30 min reaction at 70°C, but they are still not ideal. Therefore, this step of the protocol still needs optimization before it can be applied to the cell extracts.

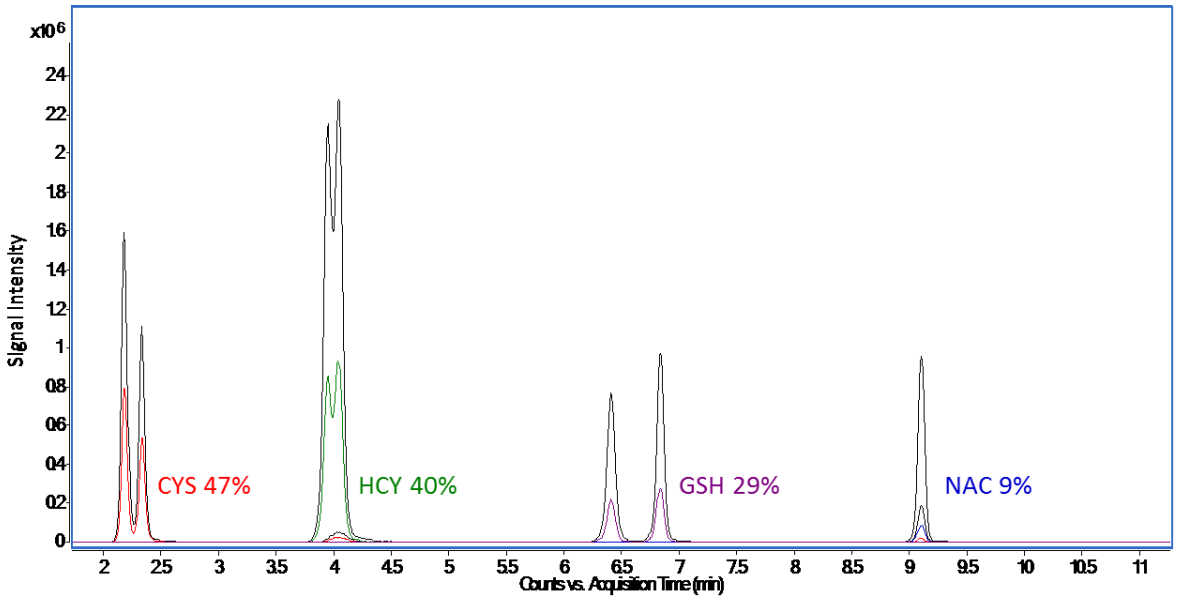

Figure 3-6 - Results of reduction of a mixture of oxidized thiols at 100 ng/mL with TCEP at 70°C for 30 min, followed by derivatization with NEM for 30 min on ice at pH 7.0. Percentages shown compare peak areas obtained after reduction with those of NEM-derivatized reduced thiol standards at 200 ng/mL.

3.4.7. Evaluation of oxidative stress induced by hydrogen peroxide

To test if the developed method can properly quantify oxidative stress, Jurkat cells were stimulated with hydrogen peroxide, a known inducer of oxidative stress, in three conditions: 25 µM for 10 min, 200 µM for 10 and 20 min.[75] As a control, PBS pH 7.4, instead of hydrogen peroxide was used. Thiols were extracted and analyzed as described above, with evaporation and reconstitution of the solvent, since analysis of the 10-fold diluted sample had poor results due to insufficient limits of detection of the method.

No thiols were detected in the most extreme condition tested (200 µM for 20 min), and thiols were found in the other three conditions in the concentrations shown in **Table 3-4**. There is an increase in GSH levels after treatment with low dose of hydrogen peroxide when compared to the control ($p_{0.05}$ = 0.016), and a marked decrease when treated with the higher doses ($p = 3.72 \times 10^{-7}$ and 7.37×10^{-5} for 10 and 20 min, respectively). For CYS, an increase was observed after low dose treatment ($p_{0.05}$ = 0.0362) and not detected in the stronger treatments, as expected.

Baty *et al.*[75] tested cell viability after exposure to hydrogen peroxide, and cells were found viable at the times and concentrations used. The decrease in quantity of thiols is the expected outcome of oxidative stress, but for GSH an increase is observed under mild oxidation. This may be due to

increased synthesis of GSH in the cell after increased oxidation[113], and after a certain point, the cell is unable to revert the oxidation of GSH to GSSG, and then the concentration of GSH decreases. If this is indeed the case, then this protocol allows differentiation of different extents of oxidation. To verify this hypothesis, more oxidation conditions should be tested. Additionally, during oxidative stress, excess oxidized thiols may be exported from the cells[24], which should also be considered in the final protocol by evaluation of the spent culture medium after collection of the cell pellet.

The thiol concentrations found in this experiment are different from the ones found when the wash step was being evaluated (Section 3.4.2.2). To compare the results, the results in **Table 3-4** should be divided by 0.8 [$concentration\left(\frac{ng}{mL}\right) = concentration\left(\frac{ng}{10^6 cells}\right) * \left(\frac{10^6 cells}{800\ \mu L}\right) * \left(\frac{1000\ \mu L}{1\ mL}\right)$], since the samples were reconstituted in 800 µL of 3% MeOH. The difference in the results could be due to the use of different protocols: while the samples for evaluation of the wash step were diluted 10-fold, these were evaporated and reconstituted, which means the matrix effects for the samples are different. Additionally, the experiments were performed using different biological replicates, so the biological variability between the samples and the availability of thiols for the samples could also play a part in the different results. Since this experiment was performed only once, as a proof-of-concept, it should be repeated once the protocol is finalized to verify these results as well as evaluate their repeatability.

Table 3-4 – Concentration of four thiols found in Jurkat cells with and without treatment with hydrogen peroxide at 25 µM and 200 µM. Averages and standard deviations for three replicates.

Treatment with H_2O_2	CYS-NEM (ng/10^6 cells)		HCY-NEM (ng/10^6 cells)		GSH-NEM (ng/10^6 cells)		NAC-NEM (ng/10^6 cells)	
	AVG	STD DEV	AVG	STD DEV	AVG	STD DEV	AVG	STD DEV
No treatment, 20 min (control)	1.36	0.12	<LOQ		173.04	2.54	ND	
25 µM, 10 min	1.11	0.08	< LOQ		253.66	17.70	ND	
200 µM, 10 min	ND		ND		13.5	10.79	3.64	
200 µM, 20 min	ND		ND		1.2	0.97	0.28	

In the 25 µM, 20 min samples, the total thiols detected were 318 ng/10^6 cells. In our method, we add 200 µL of 200 µg/mL NEM, or 40,000 ng/10^6 cells, which corresponds to 126 times the reduced thiols measured. Considering that GSH is the most concentrated low molecular weight

thiol, it is not expected that the other thiol species present would significantly change this proportion. Our previous experiments demonstrate that 10-fold excess ensures complete derivatization after 30 min, so this excess is surely enough to derivatize all the thiols in the samples, assuming that all of it penetrates the cellular membrane.

3.5. Conclusions

A protocol was proposed for quantification of reduced thiols in which they are derivatized with NEM prior to lysis of cells. We verified that derivatization with 200 μL of 200 μg/mL NEM provided enough derivatization reagent for the cells. Performing lysis and extraction with 20% MeOH provides extraction of GSH that is comparable to previous reports in literature, while for the other thiols, values in these cells are not reported. The wash step was observed to be unable to remove the media without causing leakage and needs to be further optimized.

The protocol developed thus far is able to quantify reduced thiols in Jurkat cells, which is important since the goal of this protocol is to evaluate oxidative stress. There was a statistically significant difference between the low dose and high doses with different exposure times ($p_{0.05}$ = 0.016, 3.72×10^{-7} and 7.37×10^{-5}, respectively). This shows the potential of this protocol to not only detect oxidative stress in cells, but also to quantify the extent to which the cells were exposed.

The reduction of disulfides by TCEP followed by derivatization with NEM will be tested on standards. Once the reaction conditions are optimized, the protocol will be amended by adding a step to the protocol, after reconstitution of the cell extracts, in which the extract will be divided into two portions: the first will be analyzed as is, and the second will be reduced and derivatized for quantification of the oxidized thiols. This will allow the analysis to also provide the concentration of the disulfides, which in turn will provide accurate determination of the thiol/disulfide ratio, which can be used a biomarker for the determination of the oxidative status of a cell. Additionally, this procedure will increase the sensitivity of the method for the disulfides, which in turn will improve LOD/LOQ for their determination.

4. Conclusions and future work

4.1. Conclusions

Thiols have been used extensively for determining oxidative stress in cells, but they present a great analytical challenge because they lack chromophores and fluorophores that would make their analysis by UV-Vis and fluorescence possible. They are also polar, which means they have poor retention in the common reversed-phase chromatography stationary phases, eluting at the solvent front, however, their retention using HILIC was also not ideal, with poor peak shapes. Furthermore, and most importantly, they are not stable and tend to autoxidize, thus altering the ratio that is measured during sample manipulation and handling. The concentrations of thiols and their corresponding disulfides also span a wide concentration range, thus requiring methods with wide linear dynamic range.

The first three of the above difficulties can be overcome by the procedure used in preparing the sample for analysis. To avoid autoxidation, the thiols can be protected using a derivatization reagent. Since the goal of the proposed protocol is to ultimately measure the thiol/disulfide ratio in cells, only reagents that would be able to penetrate the cell membrane and react with the thiols prior to cell lysis were considered: NEM and NPEM were compared in detail. The parameters evaluated were derivatization efficiency, signal enhancement in electrospray, side product formation and best derivatization conditions. The results show that NEM completely derivatized three of the four thiols tested, but for CYS (97% efficiency) it was less efficient than NPEM (100% efficiency).

NPEM also provided at least 2x signal enhancement in ESI and better retention/separation on RPLC. However, since these reagents are used to stabilize the signal of the reduced thiols, they should themselves be stable, and NPEM was found to be highly unstable in aqueous solutions, with its maleimide ring opening and allowing side reactions with amines and double derivatization, especially at high pHs. These side reactions corresponded to around 10% of the signal for cysteine for NPEM, while for NEM they corresponded to around 1.5%. The systematic evaluation of the effect of pH on derivatization showed that NPEM was more sensitive to slight pH changes, and requires stricter pH control than NEM. Taking into account all of the above results, NEM, while not ideal, was chosen as a reagent for further studies.

The most surprising observation concerning these reagents was their tendency to form a cyclic product with CYS, due to the attack of the α-amine to the maleimide carboxylic groups. This

reaction was found to almost completely deplete the signal of the original product of CYS-maleimide reaction within a day, and is a serious concern in metabolomics studies, since samples can be kept in the autosampler for more than a day waiting for analysis. When evaluating cell extracts, however, this side reaction was not observed, possibly because the levels of cysteine detected were low, which would mean that if the cyclic cysteine is present, it would be at even lower concentrations, below the LOD of the method. Additionally, the cell samples were analyzed fresh, so there might not have been enough time to form the cyclic product. The stability of the derivatives in extracts is a parameter that should be studied in the future. It should be mentioned that even in the works that mention the formation of side products[61], they are only evaluated at t=0, and no time dependence is investigated, which is a major issue, since our work shows large time-dependent formation of the cyclic cysteine product.

As shown in this thesis, mass spectrometry can play an important role to help characterize selectivity of derivatizing agents and identify the most appropriate derivatizing conditions that balance properly derivatization efficiency and side product formation. Majima *et al.*[62] used LC-UV to evaluate the stability of NEM and another maleimide derivatization reagent: eosin-5-maleimide. Like us, they also observed conversion of the CYS-NEM derivative into the cyclic product. They found that incubation of CYS-NEM, NAC-NEM and GSH-NEM at basic pH for 15h led to depletion of the peaks, which they link to hydrolysis of the maleimide ring, since they were monitoring the enone functional group of the maleimide. They studied the pH-dependence of the ring hydrolysis and concluded that it is proportional to the concentration of the hydroxide anion, therefore, as the pH increases, so does the hydrolysis.

Many studies performed on cells derivatize the thiols at the lysis step, some without considering the compatibility of the lysis conditions and the derivatization reagent.[48] This leads to inappropriate determination of the thiol/disulfide ratio, and incorrect biological conclusions may be reached.[48] The only other protocol that derivatized cells prior to lysis[80] adds NEM (10 µM = 1.25 µg/mL) to the samples before a lysis-extraction-protein precipitation step with cold ethanol, but they do not evaluate if the amount added was enough for complete derivatization. It may be argued that adding the thiol-blocking reagents during cell collection might be even better, and this possibility should be evaluated as well, especially since it may help prevent any oxidative stress caused by the pelleting step. The protocol described in this thesis has the unique advantage of derivatizing the reduced thiols prior to extraction, which ensures proper quantification of reduced

thiols, therefore leading to more accurate results. It may also be used to allow analysis of intracellular thiol content for any cell line. Accurate measurements of thiols and thiol ratios can facilitate improved studies on oxidative stress and increase our knowledge on how organisms respond to various stimuli, from exercise to serious diseases. Additionally, having a standardized testing procedure will allow for better comparisons between different studies, as currently the correct ratios in different cell lines are not available in literature.Different groups obtain different results, and define/use different methods whose accuracy for this analysis may be questionable.

The importance of this cannot be overlooked, since the study of many diseases and physiological alterations often leads to or encompasses the measurement of oxidative stress. The method proposed was able to differentiate between mild and extreme oxidative stress when it was induced in Jurkat cells, showing that the thiol concentrations can be used to evaluate the oxidative status of cells. More importantly, there is the potential for determining a threshold of oxidation after which the damage to the cell is excessive and leads to apoptosis.

4.1. Future Work

Although the developed protocol was able to address many of the problems surrounding current methods for analysis of thiols in cells, there are some concerns that remain to be optimized.

The formation of the cyclic -NEM and -NPEM side product is of concern, and was only observed in standards. In cell extracts, due to low CYS concentrations, method LOQ would not be sufficient to detect cyclization unless longer times were evaluated. The occurrence of this side-reaction in cell extracts treated with NEM will be further investigated by (i) reconstituting the extracts after evaporation in a smaller volume, to increase the concentration of the metabolites and allow detection of side products present in the extract and (ii) spiking extracts with CYS-NEM and performing a time-course evaluation of the progress of the cyclization. Additionally, the ionization efficiency of cyclic CYS-NEM will be evaluated and compared to that of CYS-NEM to test if they are similar, which would allow the area of the peaks to be added for quantitation, eliminating this issue. To test this, a sample of CYS-NEM that was left at room temperature until complete depletion of the CYS-NEM peak will be used to prepare a calibration curve that will be compared to one made with freshly prepared standard. If there is no difference between the slopes, it will indicate that the two compounds have similar ionization efficiencies.

Another concern is the wash step. As performed, the results are ambiguous, since for CYS they indicate no leakage, with the wash performing as expected; while for GSH, leakage was observed. In previous analysis of the cell growth medium, large quantities of GSH and CYS were observed, however, as was shown in **Table 3-1**, it is not always the case. This step needs to be further optimized and evaluated by testing different volumes, duration and number of repetitions of the wash procedure. Additionally, the use of pH 7.0 may lead to some osmotic shock that may rupture the membranes allowing leakage. We will investigate this possibility using reagents to test for lysis during wash, and/or performing washes at pH 7.4.

An important part of the protocol is the analysis of oxidized thiols. Due to their polarity, they elute at the solvent front in the stationary phases used in this project, which affects their quantitation because of increased matrix effect observed in this region. The simplest way to deal with this issue is to add a step in the protocol where a portion of the sample extracts are reduced and then derivatized to measure total thiol content. TCEP was chosen as a reducing agent for this purpose since it has been used alongside maleimide tagging[61,68], but the reaction conditions required for full reduction and subsequent derivatization were not studied yet. A second option would be to separate and quantitate oxidized thiols using a different method, such as HILIC, or CE-MS. The problem with these approaches is that the NEM-derivatized thiols are not well retained in the HILIC column, and CE-MS is not sensitive enough to detect the oxidized thiols, which are present in low concentrations. Therefore, the use of a reducing agent followed by derivatization of the newly formed thiols is the better option.

Finally, since dilution of the cellular extracts after protein precipitation caused some of the thiols to be too dilute for detection, evaporation and reconstitution was tested and provided better results. However, there is still a need for further improvement of the signals and method LOQ. Some strategies to increase the signal include: injecting more sample to the LC-MS, and using more cells per sample. Considering that when NPEM was used to derivatize cells, there were no issues with the detection of the thiols of interest, and considering that NPEM provides signal enhancement of 2-6X versus NEM, ~10x improvement is how much the signals need to be increased. The proposed alterations of the protocol require that the matrix effect and recovery of the method be re-evaluated. Furthermore, the final edition of the protocol will correct ionization matrix effects by spiking samples prior to injection with a mixture of thiols derivatized with d5-NEM.

For method validation, the following parameters should be evaluated: selectivity, linearity, accuracy, precision, stability and LOQ, following FDA guidelines on how to perform validation of bioanalytical methods[114].

Repeatability should be evaluated, which means the extraction protocol should provide similar results independent of the day it is performed. This can be done by assessing the recovery on different days and comparing the results. Once these optimizations are completed, the improved protocol will be able to accurately quantify reduced and total thiols, allowing the measurement of their ratio for oxidative stress studies.

In this work, we focused on CYS, HCY, GSH and NAC, however, there are other thiols present in cells. Their identification is possible by the use of global metabolomics software that allow comparison of samples treated with NEM and samples untreated. After identification, they can be added to the standard list for quantification, and their authentic standards obtained.

The steps taken in this project to evaluate thiol-derivatization reagents can be used in the investigation of other reagents that enhance ESI sensitivity but that are more stable than NPEM.

Appendix A

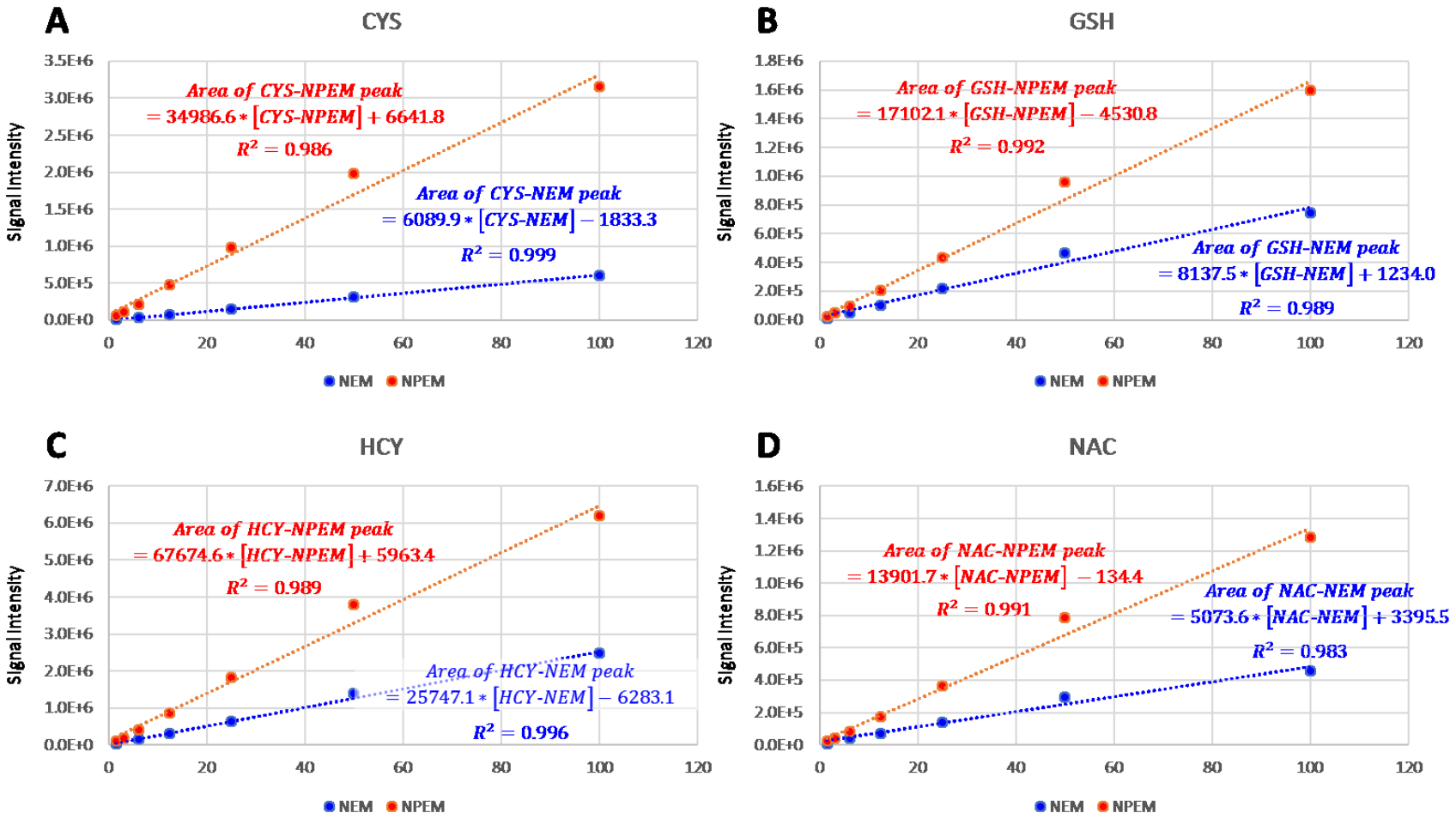

Figure A1 – Ionization enhancement provided by derivatization of thiols (A: CYS, B: GSH, C: HCY, D: NAC) with NEM and NPEM. Curves were calculated using linear regression with 1/x weighting, in the range of 0.39 to 100 ng/mL.

Table A1 – Comparison of the areas of the underivatized amine and thiol-protected cysteines peaks at the different pHs after derivatization at different pHs (n=3) to the area of the peak at pH 7.0 for each substance. Separation was performed on the biphenyl column using the method described in 2.3.3. 100 ng/mL standard mix derivatized in buffer pH 7.0. SER, SMC and ALA are not shown because they were not detected.

Compound	Deriv. reagent	m/z extracted	RT (min)	pH 3.0	pH 5.0	pH 7.0	pH 9.0
APC	NEM	240.0694	13.6	100 ± 3%	100 ± 3%	100 ± 4%	99 ± 7%
	NPEM			101 ± 3%	105 ± 2%	100 ± 4%	97 ± 3%
DAC	NEM	206.0487	4.7	101 ± 5%	89 ± 12%	100 ± 15%	51 ± 16%
	NPEM			105 ± 12%	109 ± 5%	100 ± 12%	61 ± 20%
LYS	NEM	147.1134	0.8	79 ± 1%	128 ± 14%	100 ± 10%	136 ± 15%
	NPEM			92 ± 1%	136 ± 4%	100 ± 35%	169 ± 9%
MET	NEM	150.0590	1.2	126 ± 7%	116 ± 8%	100 ± 3%	86 ± 7%
	NPEM			126 ± 6%	112 ± 4%	100 ± 7%	88 ± 5%
PHE	NEM	166.0868	2.6	102 ± 8%	100 ± 7%	100 ± 6%	92 ± 7%
	NPEM			108 ± 2%	105 ± 4%	100 ± 7%	92 ± 5%
SAC	NEM	193.0650	0.9	111 ± 5%	110 ± 13%	100 ± 6%	91 ± 7%
	NPEM			112 ± 10%	115 ± 10%	100 ± 5%	96 ± 8%
SAH	NEM	385.1289	1.3	97 ± 3%	94 ± 2%	100 ± 3%	102 ± 4%
	NPEM			97 ± 3%	95 ± 4%	100 ± 3%	104 ± 2%

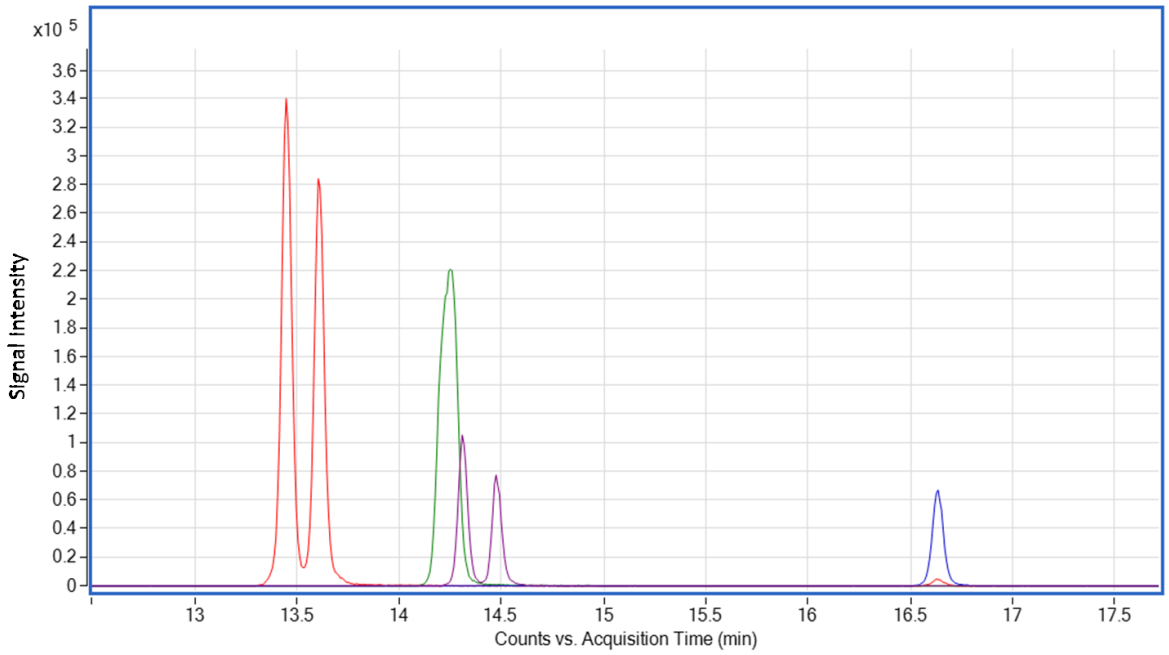

Figure A2 – Chromatographic separation of CYS-NPEM (red, m/z 323.1066), HCY-NPEM (green, m/z 337.1222), GSH-NPEM (purple, m/z 509.1706) and NAC-NPEM (blue, m/z 365.1171) on PFP column, using method described in 2.3.3. CYS-NPEM peak coeluting with NAC-NPEM peak corresponds to in-source fragmentation of NAC-NPEM. Concentration of samples was 100 ng/mL in water. Total run time was 31 min.

Table A2 – Summary of monoisotopic m/z of underivatized thiols, their disulfides, NEM and NPEM derivatives and secondary reaction products

m/z	CYS	NAC	HCY	GSH
Reduced $[M+H]^+$	122.0276	164.0381	136.0432	308.0916
Oxidized $[M+H]^+$	241.0317	325.0527	269.0629	613.1597
NEM derivative $[M+H]^+$	247.0753	289.0858	261.0909	433.1393
Double NEM derivative $[M+H]^+$	372.1230	414.1335	386.1386	558.1870
Open NEM derivative $[M+H]^+$	264.0780	306.0885	278.0936	450.1420
NPEM derivative $[M+H]^+$	323.1066	365.1171	337.1222	509.1706
Double NPEM derivative $[M+H]^+$	524.1855	566.1960	538.2011	710.2495
Open NPEM derivative $[M+H]^+$	341.1171	383.1276	355.1327	527.1811

Table A3 – Experimental pK_a values for the most acidic (COOH), most basic (NH_3) and thiol (SH) groups in the aminothiols of interest.

	CYS	NAC	HCY	GSH
pK_a NH_3	9.05[115]	N/A	9.41[116]	9.22[117]
pK_a COOH	2.35[115]	3.82[118]	2.46[116]	1.94[117]
pK_a SH	8.3[119]	9.5[119]	8.87[120]	8.8[119]
HMDB number	HMDB00574	HMDB01890	HMDB00742	HMDB00125

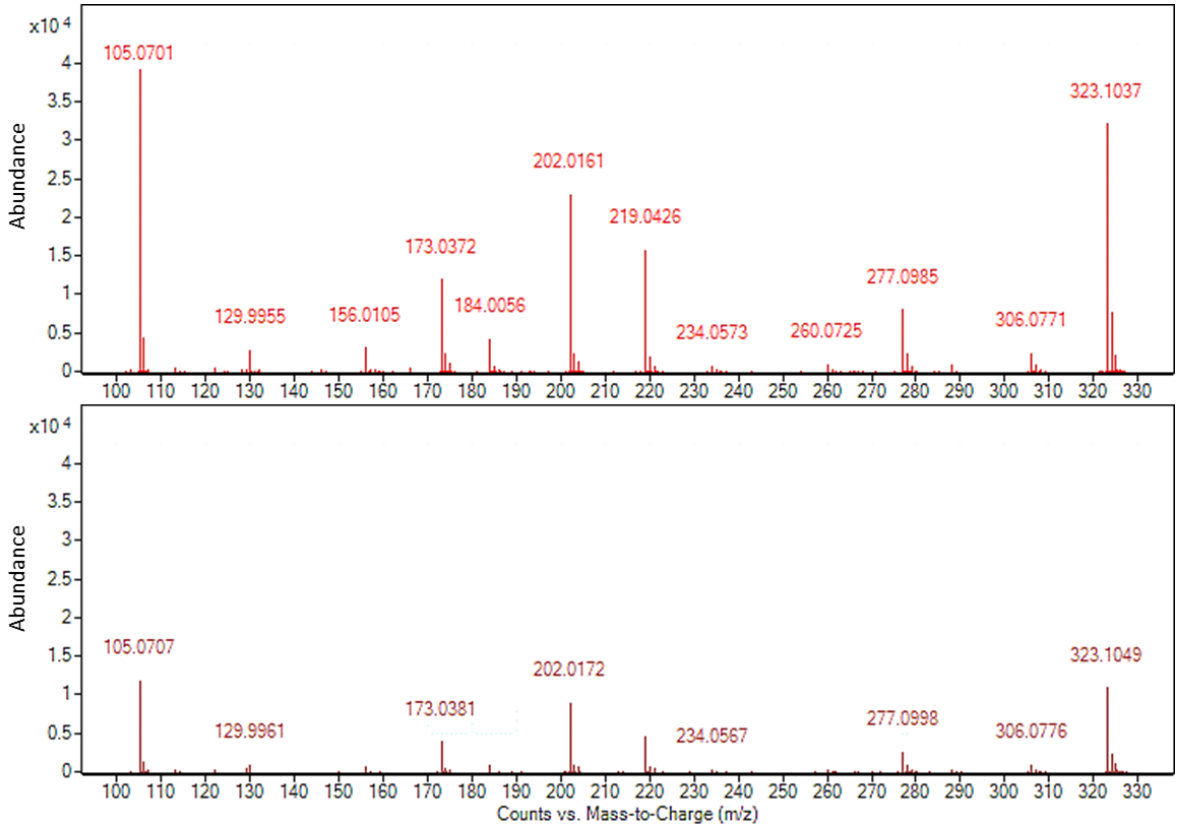

Figure A3 – MS^2 of double peaks of CYS-NPEM at pH 7.0, using 10.0 V as CID energy to fragment the m/z 323.1066 peaks at 13.456 and 13.660 min, eluted in the PFP column.

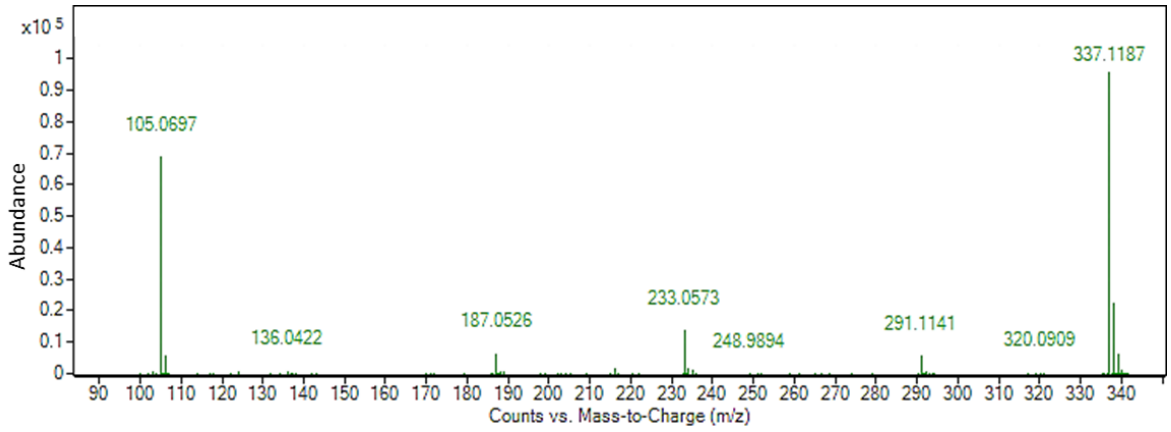

Figure A4 – MS^2 of HCY-NPEM at pH 7.0, using 10.0 V as CID energy to fragment the m/z 337.1222 peak at 14.271 min, eluted in the PFP column.

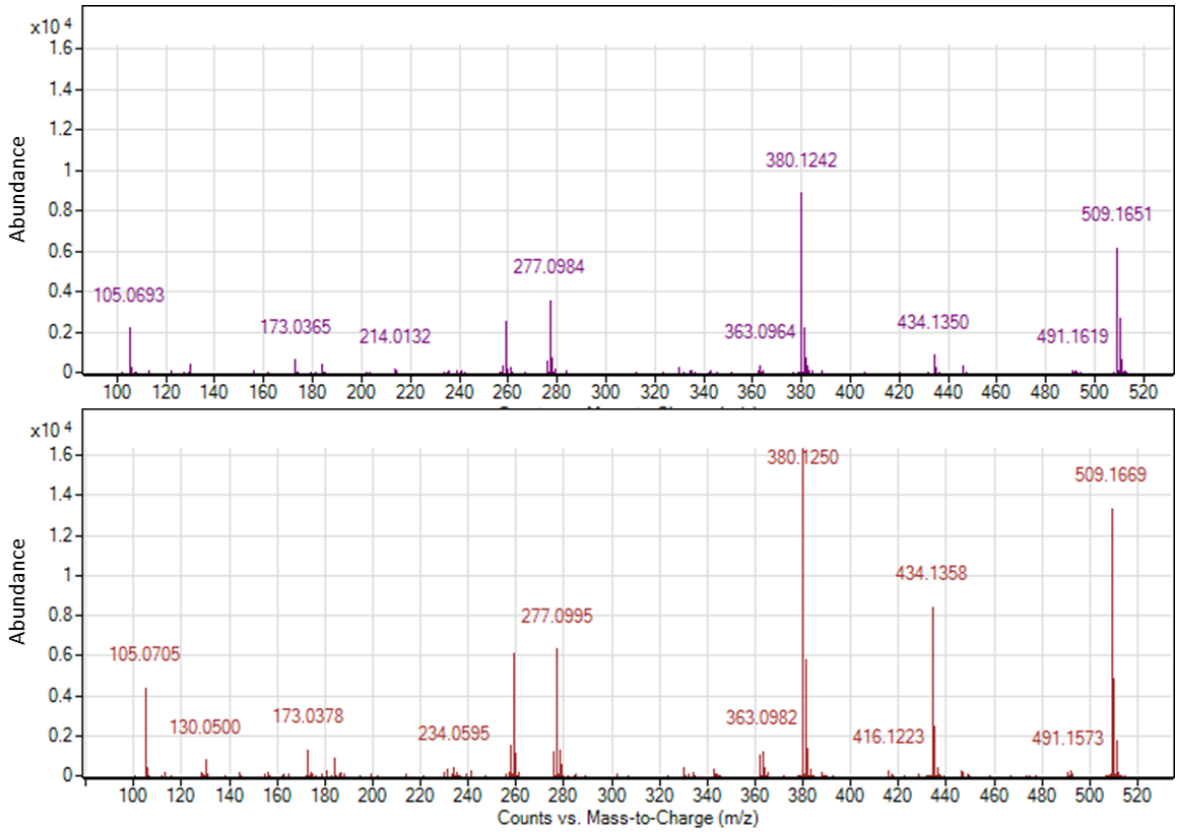

Figure A5 – MS^2 of double peaks of GSH-NPEM at pH 7.0, using 10.0 V as CID energy to fragment the m/z 509.1706 peaks at 14.373 and 14.475 min, eluted in the PFP column.

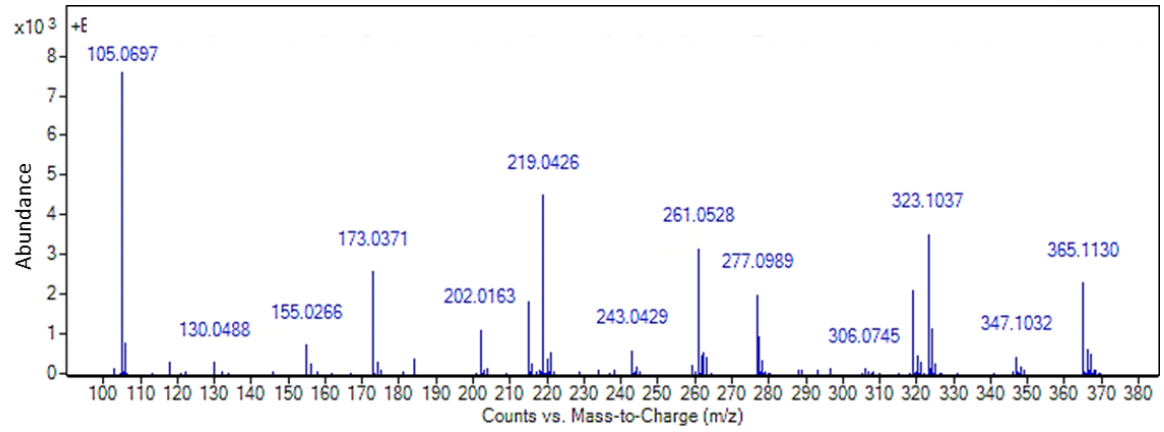

Figure A6 – MS^2 of NAC-NPEM at pH 7.0, using 10.0 V as CID energy to fragment the m/z 365.1171 peak at 16.719 min, eluted in the PFP column.

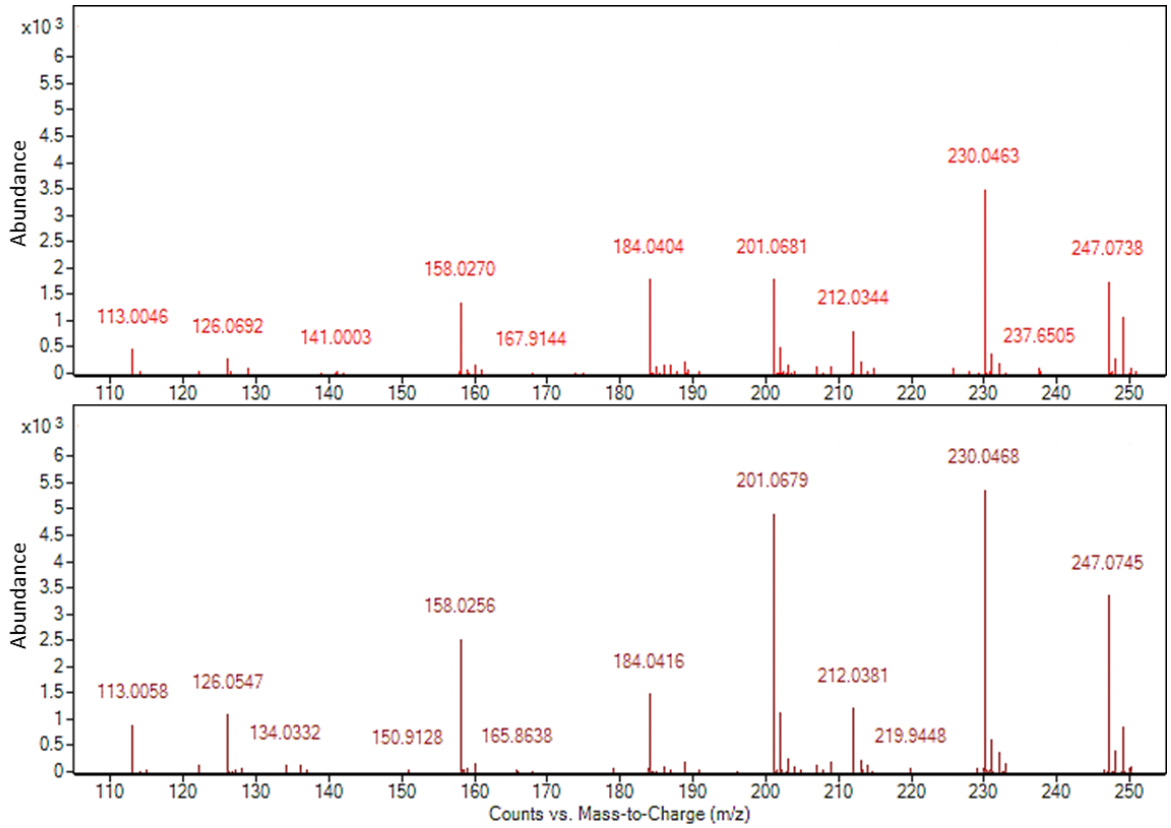

Figure A7 – MS^2 of double peaks of CYS-NEM at pH 7.0, using 10.0 V as CID energy to fragment the m/z 247.0753 peaks at 1.731 and 2.139 min, eluted in the PFP column.

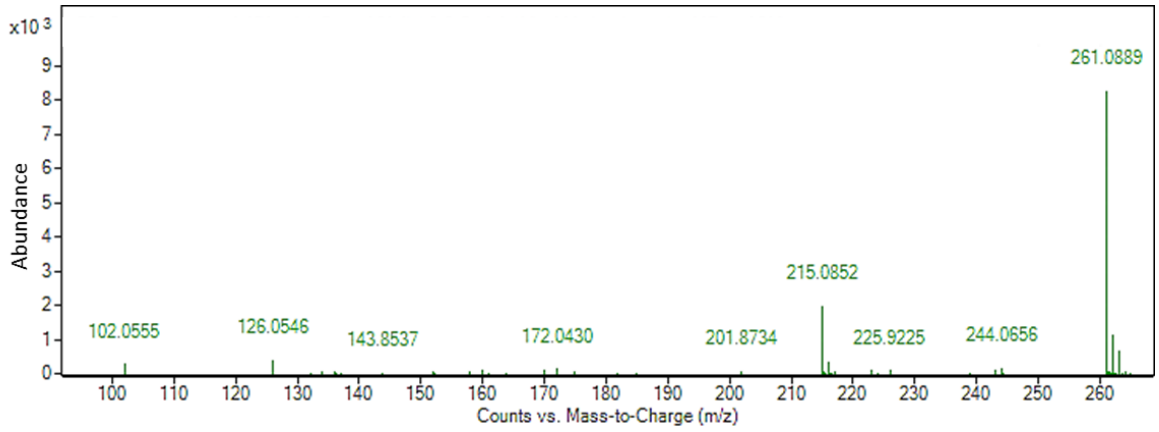

Figure A8 – MS^2 of HCY-NEM at pH 7.0, using 10.0 V as CID energy to fragment the m/z 261.0909 peaks at 3.973 min, eluted in the PFP column.

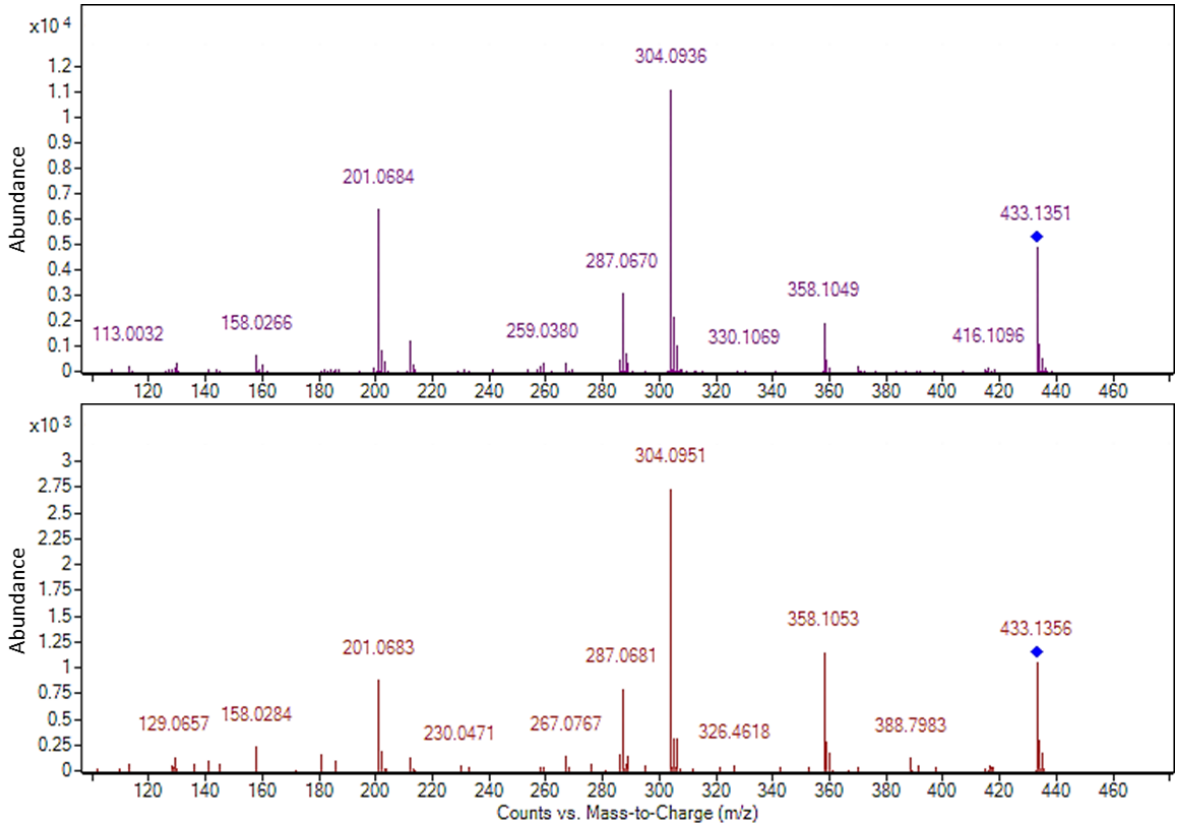

Figure A9 – MS^2 of double peaks of GSH-NEM at pH 7.0, using 10.0 V as CID energy to fragment the m/z 433.1393 peaks at 8.258 and 8.972 min, eluted in the PFP column.

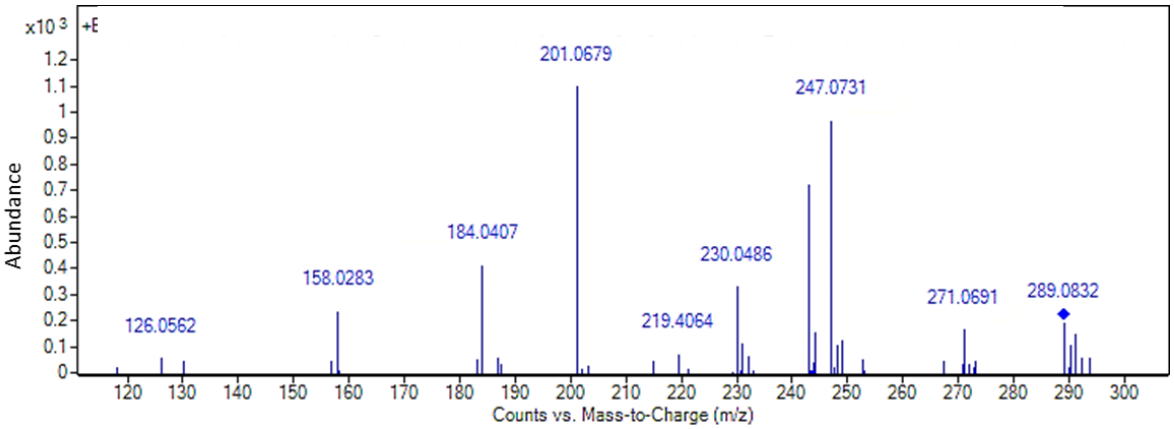

Figure A10 – MS^2 of NAC-NEM at pH 7.0, using 10.0 V as CID energy to fragment the m/z 289.0858 peak at 10.502 min, eluted in the PFP column.

Appendix B

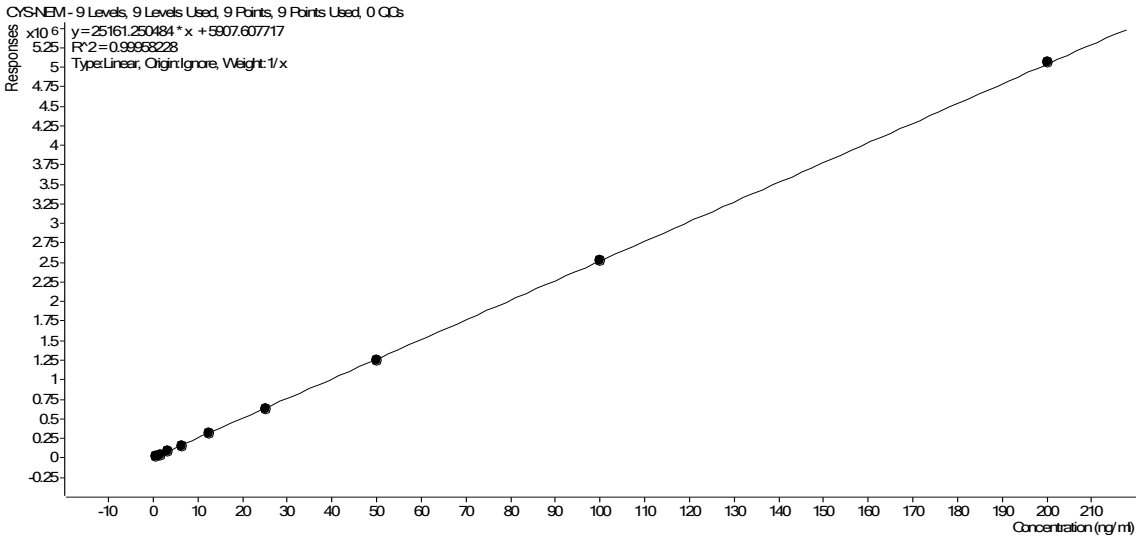

Figure B1 – Calibration curve for CYS-NEM using PFP column. Range of calibration from 0.391 to 200 ng/mL, 1/x weighting used, R^2 0.999. $\boldsymbol{Area\ of\ CYS-NEM\ peak = 25161.2 * concentration\ of\ CYS-NEM + 5907.6.}$

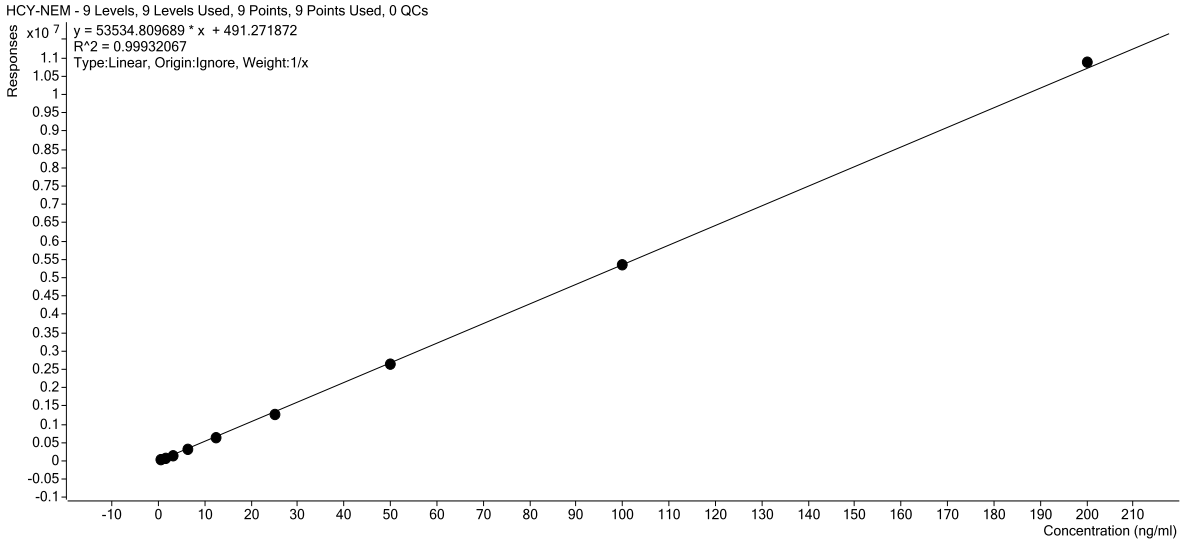

Figure B2 – Calibration curve for HCY-NEM using PFP column. Range of calibration from 0.391 to 200 ng/mL, 1/x weighting used, R^2 0.999. $\boldsymbol{Area\ of\ HCY-NEM\ peak = 53534.8 * [HCY-NEM] + 491.3.}$

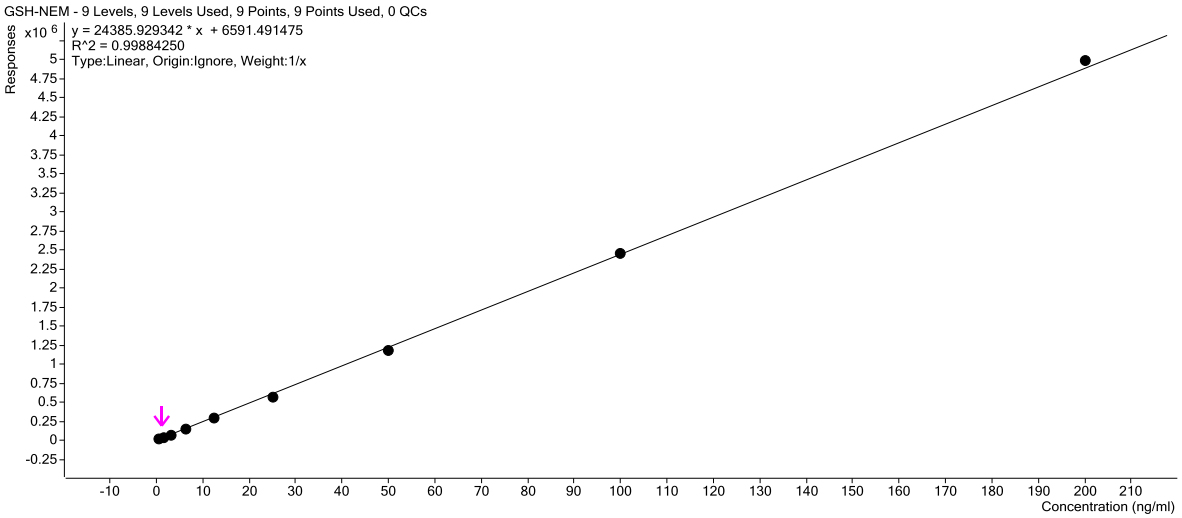

Figure B3 – Calibration curve for GSH-NEM using PFP column. Range of calibration from 0.391 to 200 ng/mL, 1/x weighting used, R^2 0.999. $Area\ of\ GSH-NEM\ peak = 24385.9 * [GSH-NEM] + 6591.5$.

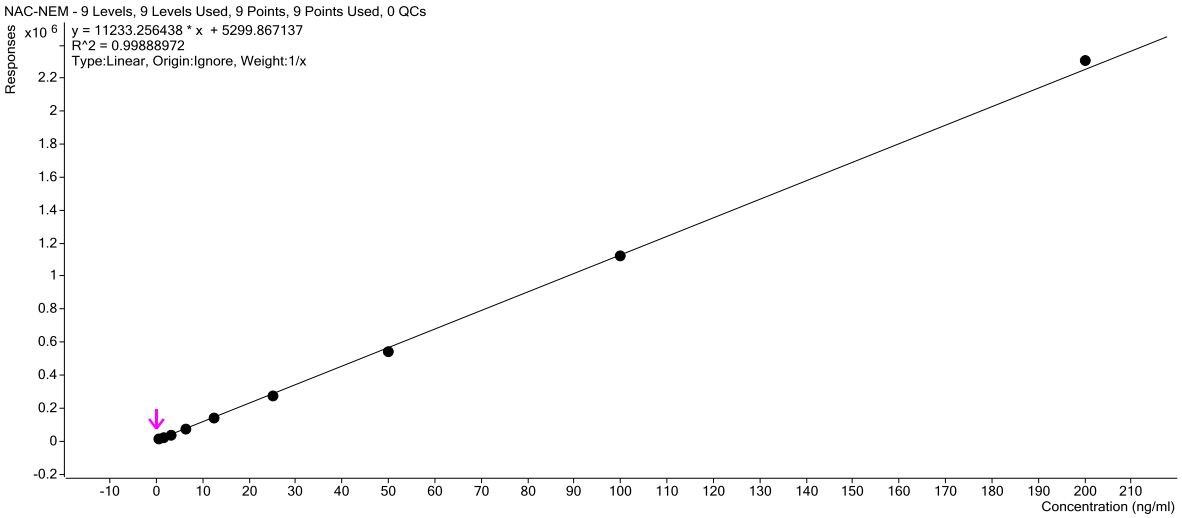

Figure B4 – Calibration curve for NAC-NEM using PFP column. Range of calibration from 0.391 to 200 ng/mL, 1/x weighting used, R^2 0.999. $Area\ of\ NAC-NEM\ peak = 11233.2 * [NAC-NEM] + 5299.9$.

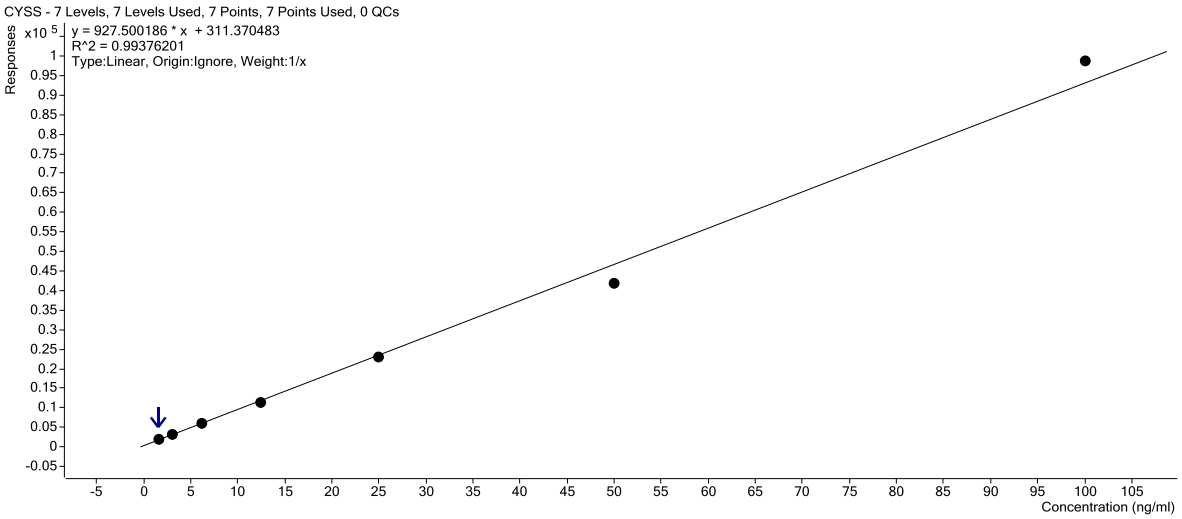

Figure B5 – Calibration curve for CYSS using PFP column. Range of calibration from 1.56 to 100 ng/mL, 1/x weighting used, R^2 0.994. $Area\ of\ CYSS\ peak = 927.5 * [CYSS] + 311.4$.

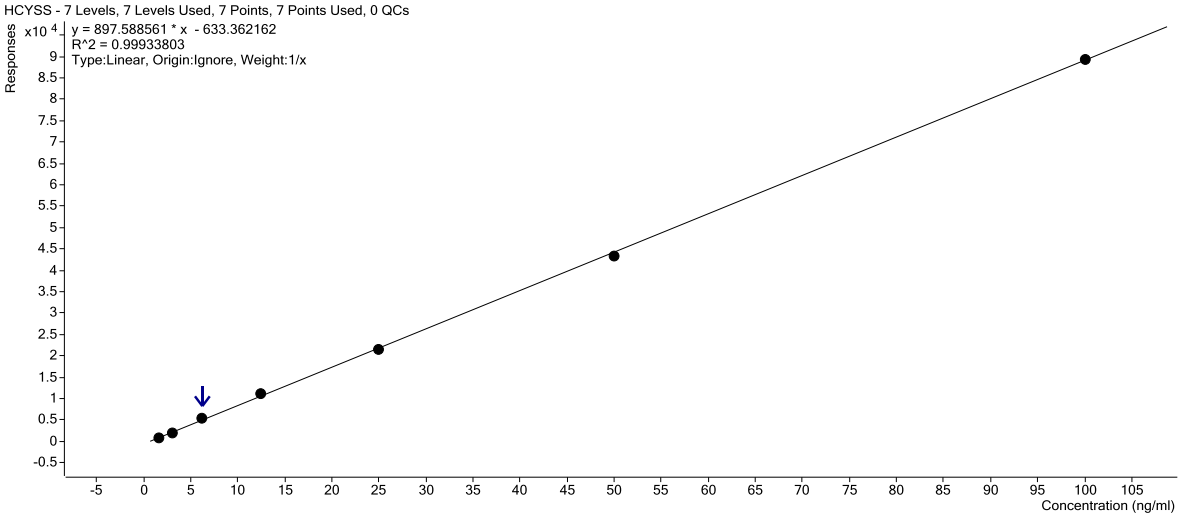

Figure B6 – Calibration curve for HCYSS using PFP column. Range of calibration from 1.56 to 100 ng/mL, 1/x weighting used, R^2 0.999. $Area\ of\ HCYSS\ peak = 897.6 * [HCYSS] - 633.4$.

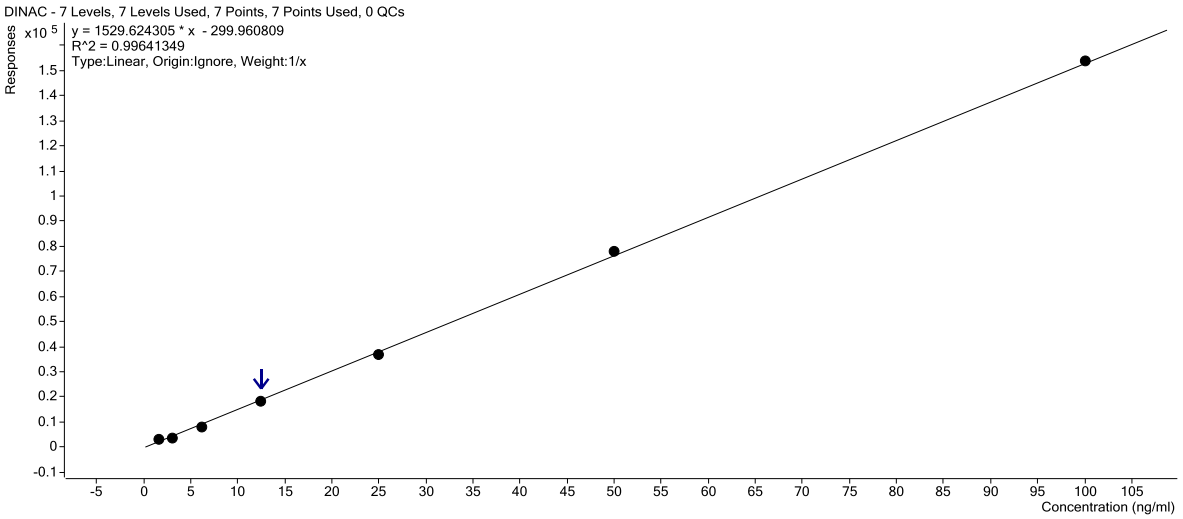

Figure B7 – Calibration curve for DINAC using PFP column. Range of calibration from 1.56 to 100 ng/mL, 1/x weighting used, R^2 0.996. $Area\ of\ DINAC\ peak = 1529.6 * [DINAC] - 300.0$.

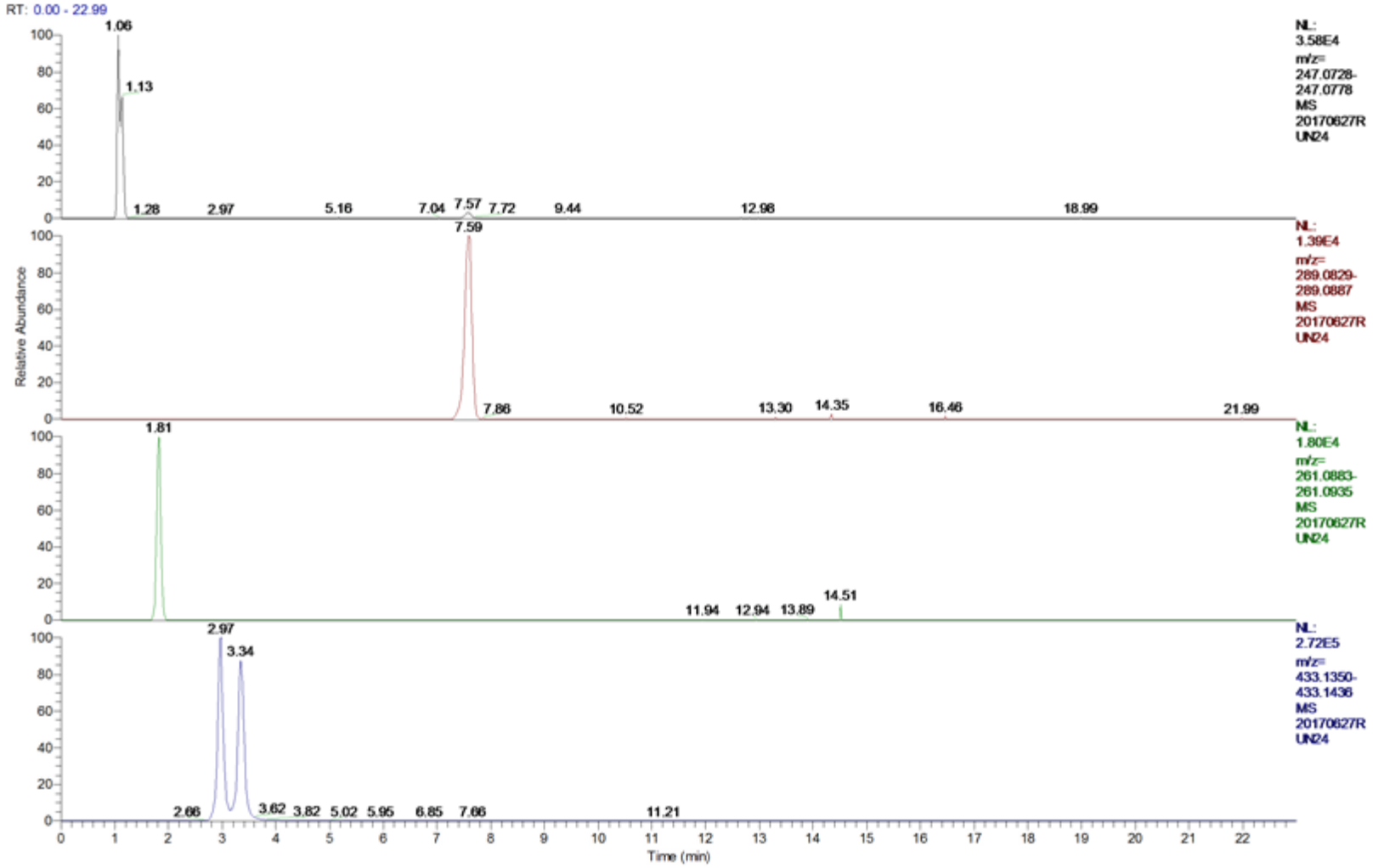

Figure B8 – Extracted ion chromatogram for A: CYS-NEM (m/z 247.0753), B: NAC-NEM (m/z 289.0858), C: HCY-NEM (m/z 261.0909), and D: GSH-NEM (m/z 433.1393) in a Jurkat cell extract on biphenyl column (particle size: 2.6 µm).

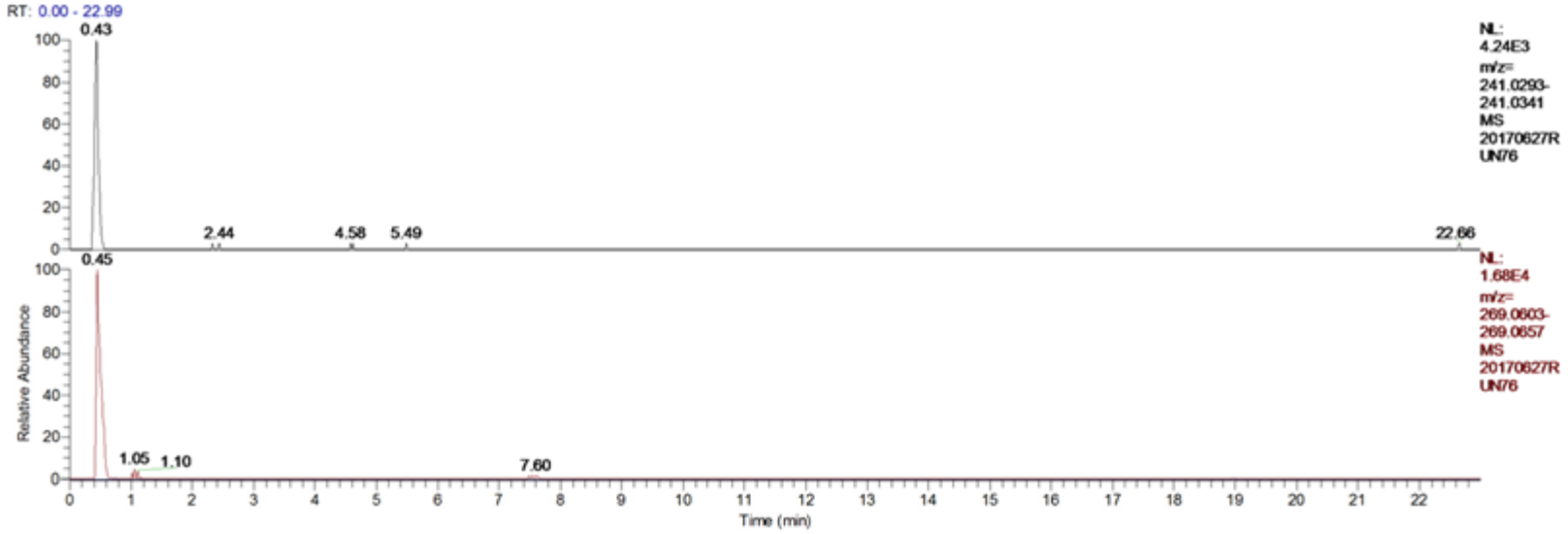

Figure B9 – Extracted ion chromatogram for A: CYSS (m/z 241.0317), B: HCYSS (m/z 269.0629), C: DINAC (m/z 325.0527) and D: GSSG (m/z 613.1597) of a cell extract on biphenyl column.

References

1. Sentellas, S., Morales-Ibanez, O., Zanuy, M. & Albertí, J. J. GSSG/GSH ratios in cryopreserved rat and human hepatocytes as a biomarker for drug induced oxidative stress. *Toxicol. Vitr.* **28,** 1006–1015 (2014).

2. Betteridge, D. J. What is oxidative stress? *Metabolism.* **49,** 3–8 (2000).

3. Pizzimenti, S. *et al.* Interaction of aldehydes derived from lipid peroxidation and membrane proteins. *Front. Physiol.* **4 SEP,** 1–17 (2013).

4. Wood, L. G., Gibson, P. G. & Garg, M. L. Biomarkers of lipid peroxidation, airway inflammation and asthma. *Eur. Respir. J.* **21,** 177–186 (2003).

5. Rossi, R., Dalle-Donne, I., Milzani, A. & Giustarini, D. Oxidized forms of glutathione in peripheral blood as biomarkers of oxidative stress. *Clin. Chem.* **52,** 1406–1414 (2006).

6. Dalle-Donne, I., Rossi, R., Colombo, R., Giustarini, D. & Milzani, A. Biomarkers of oxidative damage in human disease. *Clin. Chem.* **52,** 601–623 (2006).

7. Lokireddy, M. Measurement of Lipid Peroxidation in biology models using gas-chromatography-mass spectrometry. (Eastern Michigan University, 2005).

8. D'Agostino, L. A., Lam, K. P., Lee, R. & Britz-McKibbin, P. Comprehensive plasma thiol redox status determination for metabolomics. *J. Proteome Res.* **10,** 592–603 (2011).

9. Winnik, W. M. & Kitchin, K. T. Measurement of oxidative stress parameters using liquid chromatography-tandem mass spectroscopy (LC-MS/MS). *Toxicol. Appl. Pharmacol.* **233,** 100–106 (2008).

10. Strimbu, K. & Tavel, J. a. What are Biomarkers? *Curr Opin HIV AIDS* **5,** 463–466 (2011).

11. Halliwell, B. & Whiteman, M. Measuring reactive species and oxidative damage *in vivo* and in cell culture: how should you do it and what do the results mean? *Br. J. Pharmacol.* **142,** 231–255 (2004).

12. Labuschagne, C. F., Van Den Broek, N. J. F., Postma, P., Berger, R. & Brenkman, A. B. A protocol for quantifying lipid peroxidation in cellular systems by F2-isoprostane analysis. *PLoS One* **8,** 1–12 (2013).

13. Janicka, M., Kot-Wasik, A., Kot, J. & Namieśnik, J. Isoprostanes-biomarkers of lipid peroxidation: Their utility in evaluating oxidative stress and analysis. *Int. J. Mol. Sci.* **11,** 4631–4659 (2010).

14. Dalle-Donne, I., Rossi, R., Giustarini, D., Milzani, A. & Colombo, R. Protein carbonyl groups as biomarkers of oxidative stress. *Clin. Chim. Acta* **329,** 23–38 (2003).

15. Poynton, R. A. & Hampton, M. B. Peroxiredoxins as biomarkers of oxidative stress. *Biochim. Biophys. Acta - Gen. Subj.* **1840,** 906–912 (2014).

16. Giustarini, D. *et al.* Protein glutathionylation in erythrocytes. *Clin. Chem.* **49,** 327–330 (2003).

17. Giustarini, D. *et al.* Glutathione, glutathione disulfide, and S-glutathionylated proteins in cell cultures. *Free Radic. Biol. Med.* **89,** 972–981 (2015).

18. Mandal, A. K. *et al.* Quantitation and characterization of glutathionyl haemoglobin as an oxidative stress marker in chronic renal failure by mass spectrometry. *Clin. Biochem.* **40,** 986–994 (2007).

19. Toyo'oka, T. Recent advances in separation and detection methods for thiol compounds in biological samples. *J. Chromatogr. B Anal. Technol. Biomed. Life Sci.* **877,** 3318–3330 (2009).

20. Hansen, R. E. & Winther, J. R. An introduction to methods for analyzing thiols and disulfides: Reactions, reagents, and practical considerations. *Anal. Biochem.* **394,** 147–158 (2009).

21. Dalle-Donne, I. & Rossi, R. Analysis of thiols. *J. Chromatogr. B Anal. Technol. Biomed. Life Sci.* **877,** 3271–3273 (2009).

22. Hansen, R. E., Roth, D. & Winther, J. R. Quantifying the global cellular thiol-disulfide status. *Proc. Natl. Acad. Sci.* **106,** 422–427 (2009).

23. Oka, O. B. V & Bulleid, N. J. Forming disulfides in the endoplasmic reticulum. *Biochim. Biophys. Acta - Mol. Cell Res.* **1833,** 2425–2429 (2013).

24. Poole, L. B. The basics of thiols and cysteines in redox biology and chemistry. *Free Radic. Biol. Med.* **80,** 148–157 (2015).

25. Bechtel, T. J. & Weerapana, E. From structure to redox: The diverse functional roles of disulfides and implications in disease. *Proteomics* **17,** 1600391 (2017).

26. Gupta, V. & Carroll, K. S. Sulfenic acid chemistry, detection and cellular lifetime. *Biochim. Biophys. Acta - Gen. Subj.* **1840,** 847–875 (2014).

27. Kurilova, L. S., Krutetskaya, Z. I., Lebedev, O. E. & Antonov, V. G. The effect of oxidized glutathione and its pharmacological analogue glutoxim on intracellular Ca2+ concentration in macrophages Ca2+. *Cell tissue biol.* **2,** 322–332 (2008).

28. Van Laer, K., Hamilton, C. J. & Messens, J. Low-Molecular-Weight Thiols in Thiol-Disulfide Exchange. *Antioxid. Redox Signal.* **18,** 1642–1653 (2013).

29. Ortmayr, K., Schwaiger, M., Hann, S. & Koellensperger, G. 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. *Analyst* **140,** 7687–7695 (2015).

30. Tomaiuolo, M. *et al.* A new method for determination of plasma homocystine by isotope dilution and electrospray tandem mass spectrometry. *J. Chromatogr. B Anal. Technol. Biomed. Life Sci.* **842,** 64–69 (2006).

31. Innis, S. M. & Hasman, D. Evidence of choline depletion and reduced betaine and dimethylglycine with increased homocysteine in plasma of children with cystic fibrosis. *J. Nutr.* **136,** 2226–31 (2006).

32. Rao, Y., McCooeye, M. & Mester, Z. Mapping of sulfur metabolic pathway by LC Orbitrap mass spectrometry. *Anal. Chim. Acta* **721,** 129–136 (2012).

33. Whillier, S., Raftos, J. E., Chapman, B. & Kuchel, P. W. Role of N-acetylcysteine and cystine in glutathione synthesis in human erythrocytes. *Redox Rep.* **14,** 115–124 (2009).

34. Osman, L. P., Mitchell, S. C. & Waring, R. H. Cysteine, its Metabolism and Toxicity. *Sulfur Rep.* **20,** 155–172 (1997).

35. Wang, W. *et al.* Detection of homocysteine and cysteine. *J. Am. Chem. Soc.* **127,** 15949–15958 (2005).

36. Pastore, A., Federici, G., Bertini, E. & Piemonte, F. Analysis of glutathione: Implication in

redox and detoxification. *Clin. Chim. Acta* **333,** 19–39 (2003).

37. Rafii, M. *et al.* Measurement of homocysteine and related metabolites in human plasma and urine by liquid chromatography electrospray tandem mass spectrometry. *J. Chromatogr. B Anal. Technol. Biomed. Life Sci.* **877,** 3282–3291 (2009).

38. Guan, X., Hoffman, B., Dwivedi, C. & Matthees, D. P. A simultaneous liquid chromatography/mass spectrometric assay of glutathione, cysteine, homocysteine and their disulfides in biological samples. *J. Pharm. Biomed. Anal.* **31,** 251–261 (2003).

39. Finamor, I. *et al.* Chronic aspartame intake causes changes in the trans-sulphuration pathway, glutathione depletion and liver damage in mice. *Redox Biol.* **11,** 701–707 (2017).

40. Monostori, P., Wittmann, G., Karg, E. & Túri, S. Determination of glutathione and glutathione disulfide in biological samples: An in-depth review. *J. Chromatogr. B Anal. Technol. Biomed. Life Sci.* **877,** 3331–3346 (2009).

41. Hansen, R. E., Østergaard, H., Nørgaard, P. & Winther, J. R. Quantification of protein thiols and dithiols in the picomolar range using sodium borohydride and 4,4'-dithiodipyridine. *Anal. Biochem.* **363,** 77–82 (2007).

42. Reeve, J., Kuhlenkamp, J. & Kalowitz, N. Estimation of glutathione in rat liver by reversed-phase high-performance liquid chromatography: separation from cysteine and γ-glutamylcysteine. *J. Chromatogr.* **194,** 424–428 (1980).

43. Katrusiak, A. E., Paterson, P. G., Kamencic, H., Shoker, A. & Lyon, A. W. Pre-column derivatization high-performance liquid chromatographic method for determination of cysteine, cysteinyl-glycine, homocysteine and glutathione in plasma and cell extracts. *J. Chromatogr. B Biomed. Sci. Appl.* **758,** 207–212 (2001).

44. Winther, J. R. & Thorpe, C. Quantification of thiols and disulfides. *Biochim. Biophys. Acta - Gen. Subj.* **1840,** 838–846 (2014).

45. Owens, C. W. I. & Belcher, R. V. A Colorimetric Micromethod for the Determination of Glutathione. *Biochem. J.* **94,** 705–711 (1965).

46. Tietze, F. Enzymic Method for Quantitative Determination of Nanogram Amounts of Total and Oxidized Glutathione Applications to Mammalian Blood and Other Tissues. *Anal.*

Biochem. **27,** 502–522 (1969).

47. Noctor, G. & Foyer, C. H. Simultaneous measurement of foliar glutathione, gamma-glutamylcysteine, and amino acids by high-performance liquid chromatography: comparison with two other assay methods for glutathione. *Anal. Biochem.* **264,** 98–110 (1998).

48. Giustarini, D. *et al.* Pitfalls in the analysis of the physiological antioxidant glutathione (GSH) and its disulfide (GSSG) in biological samples: An elephant in the room. *J. Chromatogr. B* **1019,** 21–28 (2016).

49. Reed, D. J. *et al.* High-performance liquid chromatography analysis of nanomole levels of glutathione, glutathione disulfide, and selected thiols and disulfides. *Anal. Biochem.* **106,** 55–62 (1980).

50. Imai, K. & Toyo'oka, T. Fluorometric assay of thiols with fluorobenzoxadiazoles. *Methods Enzymol.* **143,** 67–75 (1987).

51. Svardal, A. M., Mansoor, M. A. & Ueland, P. M. Determination of reduced, oxidized, and protein-bound glutathione in human plasma with precolumn derivation with monobromobimane and liquid chromatography. *Anal. Biochem.* **184,** 338–346 (2000).

52. Abukhalaf, I. K. *et al.* High performance liquid chromatographic assay for the quantitation of total glutathione in plasma. *J. Pharm. Biomed. Anal.* **28,** 637–643 (2002).

53. Baeyens, W., Van Der Weken, G., Ling, B. L. & De Moerloose, P. HPLC Determination of N-Acetylcysteine in Pharmaceutical Preparations After Pre-Column Derivatization With Thiolyte R MB Using Fluorescence Detection. *Anal. Lett.* **21,** 741–757 (1988).

54. Oe, T., Ohyagi, T. & Naganuma, A. Determination of γ-glutamylglutathione and other low-molecular-mass biological thiol compounds by isocratic high-performance liquid chromatography with fluorimetric detection. *J. Chromatogr. B Biomed. Appl.* **708,** 285–289 (1998).

55. Husted, L. B., Sørensen, E. S. & Sottrup-Jensen, L. 4-(Aminosulfonyl)-7-fluoro-2,1,3-benzoxadiazole is not specific for labeling of sulfhydryl groups in proteins as it may also react with phenolic hydroxyl groups and amino groups. *Anal. Biochem.* **314,** 166–168 (2003).

56. Michaelsen, J. T., Dehnert, S., Giustarini, D., Beckmann, B. & Tsikas, D. HPLC analysis of human erythrocytic glutathione forms using OPA and N-acetyl-cysteine ethyl ester: Evidence for nitrite-induced GSH oxidation to GSSG. *J. Chromatogr. B Anal. Technol. Biomed. Life Sci.* **877,** 3405–3417 (2009).

57. Zuman, P. Reactions of orthophthalaldehyde with nucleophiles. *Chem. Rev.* **104,** 3217–3238 (2004).

58. Jacobs, W. A., Leburg, M. W. & Madaj, E. J. Stability of o-phthalaldehyde-derived isoindoles. *Anal. Biochem.* **156,** 334–340 (1986).

59. Willig, S., Hunter, D. L., Dass, P. & Padilla, S. Validation of the use of 6,6'-dithiodinicotinic acid as a chromogen in the Ellman method for cholinesterase determinations. *Vet. Hum. Toxicol.* **38,** 249–53 (1996).

60. Jemal, M. & Hawthorne, D. High performance liquid chromatography/ionspray mass spectrometry of N-ethylmaleimide and acrylic acid ester derivatives for bioanalysis of thiol compounds. *Rapid Commun. Mass Spectrom.* **8,** 854–857 (1994).

61. Seiwert, B. & Karst, U. Simultaneous LC/MS/MS determination of thiols and disulfides in urine samples based on differential labeling with ferrocene-based maleimides. *Anal. Chem.* **79,** 7131–7138 (2007).

62. Majima, E. *et al.* Stabilities of the fluorescent SH-reagent eosin-5-maleimide and its adducts with sulfhydryl compounds. *BBA - Gen. Subj.* **1243,** 336–342 (1995).

63. Giustarini, D., Dalle-Donne, I., Milzani, A., Fanti, P. & Rossi, R. Analysis of GSH and GSSG after derivatization with N-ethylmaleimide. *Nat. Protoc.* **8,** 1660–1669 (2013).

64. Camera, E. & Picardo, M. Analytical methods to investigate glutathione and related compounds in biological and pathological processes. *J. Chromatogr. B* **781,** 181–206 (2002).

65. Hammermeister, D. E., Serrano, J., Schmieder, P. & Kuehl, D. W. Characterization of dansylated glutathione, glutathione disulfide, cysteine and cystine by narrow bore liquid chromatography/electrospray ionization mass spectrometry. *Rapid Commun. Mass Spectrom.* **14,** 503–508 (2000).

66. Bouligand, J., Deroussent, A., Paci, A., Morizet, J. & Vassal, G. Liquid chromatography-

tandem mass spectrometry assay of reduced and oxidized glutathione and main precursors in mice liver. *J. Chromatogr. B Anal. Technol. Biomed. Life Sci.* **832,** 67–74 (2006).

67. Iwasaki, Y., Hoshi, M., Ito, R., Saito, K. & Nakazawa, H. Analysis of glutathione and glutathione disulfide in human saliva using hydrophilic interaction chromatography with mass spectrometry. *J. Chromatogr. B Anal. Technol. Biomed. Life Sci.* **839,** 74–79 (2006).

68. Wang, J. *et al.* Simultaneous Quantification of Amino Metabolites in Multiple Metabolic Pathways Using Ultra-High Performance Liquid Chromatography with Tandem-mass Spectrometry. *Sci. Rep.* **7,** 1423 (2017).

69. Kindt, T. J., Goldsby, R. A., Osborne, B. A. & Kuby, J. *Kuby Immunology*. (W. H. Freeman, 2007).

70. Chkhikvishvili, I. *et al.* Constituents of French Marigold (Tagetes patula L.) Flowers Protect Jurkat T-Cells against Oxidative Stress. *Oxid. Med. Cell. Longev.* **2016,** (2016).

71. Vuckovic, D. Current trends and challenges in sample preparation for global metabolomics using liquid chromatography-mass spectrometry. *Anal. Bioanal. Chem.* **403,** 1523–1548 (2012).

72. Dettmer, K. *et al.* Metabolite extraction from adherently growing mammalian cells for metabolomics studies: Optimization of harvesting and extraction protocols. *Anal. Bioanal. Chem.* **399,** 1127–1139 (2011).

73. Dhakshinamoorthy, S., Dinh, N.-T., Skolnick, J. & Styczynski, M. P. Metabolomics identifies the intersection of phosphoethanolamine with menaquinone-triggered apoptosis in an in vitro model of leukemia. *Mol. BioSyst.* **11,** 2406–2416 (2015).

74. Chen, W., Zhao, Y., Seefeldt, T. & Guan, X. Determination of thiols and disulfides via HPLC quantification of 5-thio-2-nitrobenzoic acid. *J. Pharm. Biomed. Anal.* **48,** 1375–1380 (2008).

75. Baty, J. W., Hampton, M. B. & Winterbourn, C. C. Proteomic detection of hydrogen peroxide-sensitive thiol proteins in Jurkat cells. *Biochem. J.* **389,** 785–795 (2005).

76. Kand'ár, R., Žáková, P., Lotková, H., Kučera, O. & Červinková, Z. Determination of reduced and oxidized glutathione in biological samples using liquid chromatography with fluorimetric detection. *J. Pharm. Biomed. Anal.* **43,** 1382–1387 (2007).

77. Ramanathan, A. & Schreiber, S. L. Direct control of mitochondrial function by mTOR. *Proc. Natl. Acad. Sci. U. S. A.* **106,** 22229–22232 (2009).

78. Lewis, G. D. *et al.* Metabolite profiling of blood from individuals undergoing planned myocardial infarction reveals early markers of myocardial injury. *J. Clin. Invest.* **118,** 3503–3512 (2008).

79. Baumann, S. Starvation of Jurkat T cells causes metabolic switch from glycolysis to lipolysis as revealed by comprehensive GC-qMS. *J. Integr. OMICS* **3,** 70–74 (2013).

80. Harwood, D. T., Kettle, A. J., Brennan, S. & Winterbourn, C. C. Simultaneous determination of reduced glutathione, glutathione disulphide and glutathione sulphonamide in cells and physiological fluids by isotope dilution liquid chromatography-tandem mass spectrometry. *J. Chromatogr. B Anal. Technol. Biomed. Life Sci.* **877,** 3393–3399 (2009).

81. Cereser, C. *et al.* Quantitation of reduced and total glutathione at the femtomole level by high-performance liquid chromatography with fluorescence detection: application to red blood cells and cultured fibroblasts. *J. Chromatogr. B. Biomed. Sci. Appl.* **752,** 123–132 (2001).

82. Camera, E., Rinaldi, M., Briganti, S., Picardo, M. & Fanali, S. Simultaneous determination of reduced and oxidized glutathione in peripheral blood mononuclear cells by liquid chromatography-electrospray mass spectrometry. *J. Chromatogr. B. Biomed. Sci. Appl.* **757,** 69–78 (2001).

83. Sitnikov, D. G. G., Monnin, C. S. S. & Vuckovic, D. Systematic Assessment of Seven Solvent and Solid-Phase Extraction Methods for Metabolomics Analysis of Human Plasma by LC-MS. *Sci. Rep.* **6,** 38885 (2016).

84. Liao, W. *et al.* Combined Metabonomic and Quantitative Real-Time PCR Analyses Reveal Systems Metabolic Changes in Jurkat T ‐ Cells Treated with HIV ‐ 1 Tat Protein. *J. Proteome Res.* **11,** 5109–5123 (2012).

85. Matuszewski, B. K. K., Constanzer, M. L. L. & Chavez-Eng, C. M. M. Strategies for the assessment of matrix effect in quantitative bioanalytical methods based on HPLC-MS/MS. *Anal. Chem.* **75,** 3019–3030 (2003).

86. Scopus. Scopus search for Oxidative Stress articles published in the United States in 2016.

Available at: https://www.scopus.com/results/results.uri?sort=plf-f&src=s&sid=f1f2708d2a781de6e4b73abd3719c558&sot=a&sdt=a&cluster=scoaffilctry%2C%22United+States++%22%2Ct%2Bscopubyr%2C%222016+%22%2Ct&sl=35&s=%28TITLE-ABS-KEY%28%22oxidative+stress%22%29%29&origin=searc. (Accessed: 16th August 2017)

87. Scopus. Scopus search for Oxidative Stress and Glutathione articles published in the United States in 2016. Available at: https://www.scopus.com/results/results.uri?sort=plf-f&src=s&sid=6800a12fb2451e368cd7084ff3d00e89&sot=a&sdt=a&cluster=scoaffilctry%2C%22United+States++%22%2Ct%2Bscopubyr%2C%222016+%22%2Ct&sl=53&s=%28TITLE-ABS-KEY%28%22oxidative+stress%22%29%29+AND+%28gluta. (Accessed: 15th August 2017)

88. Iwasaki, Y. *et al.* Chromatographic and mass spectrometric analysis of glutathione in biological samples. *J. Chromatogr. B Anal. Technol. Biomed. Life Sci.* **877,** 3309–3317 (2009).

89. Winters, R. A., Zukowski, J., Ercal, N., Matthews, R. H. & Spitz, D. R. Analysis of Glutathione, Glutathione Disulfide, Cysteine, Homocysteine, and Other Biological Thiols by High-Performance Liquid Chromatography Following Derivatization by N-(1-Pyrenyl)maleimide. *Analytical Biochemistry* **227,** 14–21 (1995).

90. Paroni, R. *et al.* HPLC with o-phthalaldehyde precolumn derivatization to measure total, oxidized, and protein-bound glutathione in blood, plasma, and tissue. *Clin. Chem.* **41,** 448–454 (1995).

91. Asensi, M., Sastre, J., Pallardo, F. V., Estrela, J. M. & Vina, J. Determination of oxidized glutathione in blood: High-performance liquid chromatography. *Methods Enzymol.* **234,** 367–371 (1994).

92. Smyth, D. G., Nagamatsu, A. & Fruton, J. S. Some Reactions of N-Ethylmaleimide. *J. Am. Chem. Soc.* **82,** 4600–4604 (1960).

93. Ates, B., Ercal, B. C., Manda, K., Abraham, L. & Ercal, N. Determination of glutathione disulfide levels in biological samples using thiol-disulfide exchanging agent, dithiothreitol. *Biomed. Chromatogr.* **23,** 119–123 (2009).

94. Fontana, A. & Toniolo, C. in *The Chemistry of the Thiol Group* (ed. Patai, S.) 294–298 (John Wiley & Sons, Ltd., 1974). doi:10.1002/9780470771310.ch5

95. Giustarini, D., Dalle-Donne, I., Milzani, A. & Rossi, R. Detection of glutathione in whole blood after stabilization with N-ethylmaleimide. *Anal. Biochem.* **415,** 81–83 (2011).

96. Asensi, M. *et al.* A High-Performance Liquid Chromatography Method for Measurement of Oxidized Glutathione in Biological Samples. *Analytical Biochemistry* **217,** 323–328 (1994).

97. Santori, G. *et al.* Different efficacy of iodoacetic acid and N-ethylmaleimide in high-performance liquid chromatographic measurement of liver glutathione. *J. Chromatogr. B Biomed. Appl.* **695,** 427–433 (1997).

98. Halliwell, B. & Chirico, S. Lipid peroxidation: its mechanism, measurement and significance. *Am. J. Clin. Nutr.* **57,** 715S–725S (1993).

99. Carretero, A. *et al.* In vitro/in vivo screening of oxidative homeostasis and damage to DNA, protein, and lipids using UPLC/MS-MS. *Anal. Bioanal. Chem.* **406,** 5465–5476 (2014).

100. Cotgreave, I. A. & Moldéus, P. Methodologies for the application of monobromobimane to the simultaneous analysis of soluble and protein thiol components of biological systems. *J. Biochem. Biophys. Methods* **13,** 231–249 (1986).

101. Suh, J. H. *et al.* Clinical assay of four thiol amino acid redox couples by LC-MS/MS: Utility in thalassemia. *J. Chromatogr. B Anal. Technol. Biomed. Life Sci.* **877,** 3418–3427 (2009).

102. Rellán-Álvarez, R., Hernández, L. E., Abadía, J. & Álvarez-Fernández, A. Direct and simultaneous determination of reduced and oxidized glutathione and homoglutathione by liquid chromatography-electrospray/mass spectrometry in plant tissue extracts. *Anal. Biochem.* **356,** 254–264 (2006).

103. Steghens, J. P., Flourié, F., Arab, K. & Collombel, C. Fast liquid chromatography-mass spectrometry glutathione measurement in whole blood: Micromolar GSSG is a sample preparation artifact. *J. Chromatogr. B Anal. Technol. Biomed. Life Sci.* **798,** 343–349 (2003).

104. Alwael, H., Connolly, D., Barron, L. & Paull, B. Development of a rapid and sensitive method for determination of cysteine/cystine ratio in chemically defined media. *J. Chromatogr. A* **1217,** 3863–3870 (2010).

105. Kosower, N. S. & Kosower, E. M. Thiol labeling with bromobimanes. *Methods Enzymol.* **143,** 76–84 (1987).

106. Bakirdere, S., Bramanti, E., D'ulivo, A., Ataman, O. Y. & Mester, Z. Speciation and determination of thiols in biological samples using high performance liquid chromatography-inductively coupled plasma-mass spectrometry and high performance liquid chromatography-Orbitrap MS. *Anal. Chim. Acta* **680,** 41–47 (2010).

107. Dudman, N. P. B., Guo, X. W., Crooks, R., Xie, L. & Silberberg, J. S. Assay of plasma homocysteine: Light sensitivity of the fluorescent 7- benzo-2-oxa-1,3-diazole-4-sulfonic acid derivative, and use of appropriate calibrators. *Clin. Chem.* **42,** 2028–2032 (1996).

108. Nolin, T. D., McMenamin, M. E. & Himmelfarb, J. Simultaneous determination of total homocysteine, cysteine, cysteinylglycine, and glutathione in human plasma by high-performance liquid chromatography: Application to studies of oxidative stress. *J. Chromatogr. B Anal. Technol. Biomed. Life Sci.* **852,** 554–561 (2007).

109. Nielsen, J. W., Jensen, K. S., Hansen, R. E., Gotfredsen, C. H. & Winther, J. R. A fluorescent probe which allows highly specific thiol labeling at low pH. *Anal. Biochem.* **421,** 115–120 (2012).

110. Brigelius-Flohé, R. Mixed results with mixed disulfides. *Arch. Biochem. Biophys.* **595,** 81–87 (2016).

111. Han, J. C. & Han, G. Y. A Procedure for Quantitative Determination of Tris(2-carboxyethyl)phospine, an Odorless Reducing Agent More Stable and Effective Than Dithiothreitol. *Anal. Biochem.* **220,** 5–10 (1994).

112. Zhang, W. *et al.* Simultaneous determination of glutathione, cysteine, homocysteine, and cysteinylglycine in biological fluids by ion-pairing high-performance liquid chromatography coupled with precolumn derivatization. *J. Agric. Food Chem.* **62,** 5845–5852 (2014).

113. Mosharov, E., Cranford, M. R. & Banerjee, R. The quantitatively important relationship between homocysteine metabolism and glutathione synthesis by the transsulfuration pathway and its regulation by redox changes. *Biochemistry* **39,** 13005–13011 (2000).

114. Food and Drug Administration. *Draft Guidance for Industry: Bioanalytical method validation. U.S. Department of Health and Human Services* (2013).

115. Wishart, D. S., Jewison, T., Guo, A. C., Wilson, M. & Knox, C. L-Cysteine. Available at: http://www.hmdb.ca/metabolites/HMDB00574. (Accessed: 15th July 2017)

116. Wishart, D. S., Jewison, T., Guo, A. C., Wilson, M. & Knox, C. Homocysteine. Available at: http://www.hmdb.ca/metabolites/HMDB00742. (Accessed: 15th July 2017)

117. Wishart, D. S., Jewison, T., Guo, A. C., Wilson, M. & Knox, C. Glutathione. Available at: http://www.hmdb.ca/metabolites/HMDB00125. (Accessed: 15th July 2017)

118. Wishart, D. S., Jewison, T., Guo, A. C., Wilson, M. & Knox, C. Acetylcysteine. Available at: http://www.hmdb.ca/metabolites/HMDB01890. (Accessed: 15th July 2017)

119. Winterbourn, C. C. C. & Hampton, M. B. B. Thiol chemistry and specificity in redox signaling. *Free Radic. Biol. Med.* **45,** 549–561 (2008).

120. Iciek, M., Chwatko, G., Lorenc-Koci, E., Bald, E. & Włodek, L. Plasma levels of total, free and protein bound thiols as well as sulfane sulfur in different age groups of rats. *Acta Biochim. Pol.* **51,** 815–824 (2004).