UNIVERSITY OF SOUTHAMPTON

Faculty of Medicine

Clinical and Experimental Sciences

Capturing the intra- and extracellular redox metabolome by mass spectrometry - Significance of sample processing and methodology

by

Thomas Rupert Sutton

Thesis for the degree of

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ABSTRACT

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Oxidative stress has recently become understood as a disruption of redox signalling and control, and is strongly associated with a wide number of physiological conditions and diseases. Redox metabolism plays a key role in the defence of cells against oxidative damage. Attempts to produce better outcomes in these conditions by correcting the redox imbalance with antioxidants have produced mixed results at best. Therefore, there is an increasing need to better understand redox metabolism and how the many redox pathways involved are connected.

In particular, hydrogen sulfide (H_2S) has emerged as a key signalling molecule in redox regulation, along with its associated metabolites and redox pathways. Whilst there are existing analytical methods available for the analysis of H_2S and its metabolites, they are often limited in scope, and neglect detailed investigation of important steps in the analysis including sample processing. The ascorbate metabolome comprises a separate redox pathway and has close links to the sulfide metabolome. Some aspects of the ascorbate metabolome, such as ascorbic acid-2-sulfate, however have not been widely analysed in humans.

In order to better understand the sulfide redox metabolome, an ultra-high pressure liquid chromatography mass spectrometry (UHPLC-MS) based method was developed to quantify sulfide and other key metabolites. Important aspects of sample processing including collection, preparation and storage, were investigated to determine their impact on the measured metabolites. *N*-Ethylmaleimide was used to trap the sulfide and prevent oxidation and degradation of the other metabolites. The same method was then utilised to analyse persulfides and polysulfides, which are additional redox relevant sulfide related metabolites. The kinetics of the trapping reactions were also investigated in more detail. Additionally, a UHPLC-MS method for the simultaneous analysis of the two key components of ascorbate metabolism, ascorbic acid and dehydroascorbic acid along with ascorbic acid-2sulfate was developed, and specific aspects of sample preparation for this analysis investigated.

The developed method for the analysis of the sulfide metabolome was successfully applied to the analysis of plasma from a cohort of ten healthy volunteers as a proof of concept study. This robust and versatile method could be easily applied to both existing sample cohorts and new studies, and provide new insights into sulfur redox metabolism. It could also be used in conjunction with stable isotope tracing and established methodologies for the analysis of other redox metabolomes. The analysis of polysulfides provided new insights into previously unknown sulfur related metabolic activities of the antioxidant enzyme superoxide dismutase. The method for the analysis of the ascorbate metabolome requires further work due to factors involving sample processing.

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DECLARATION OF AUTHORSHIP

I, **Thomas R. Sutton** .....

declare that this thesis and the work presented in it are my own and has been generated by me as the result of my own original research.

.....

.....

I confirm that:

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3. Where I have consulted the published work of others, this is always clearly attributed;
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    - van der Graaf AM, Paauw ND, Toering TJ, et al. Impaired sodium-dependent adaptation of arterial stiffness in formerly preeclamptic women: the RETAP-vascular study. *Am J Physiol Heart Circ Physiol* 2016;310(11):H1827-33.
    - Olson KR, Gao Y, Arif F, et al. Metabolism of hydrogen sulfide (H2S) and Production of Reactive Sulfur Species (RSS) by superoxide dismutase. *Redox Biology* 2017;15:74-85.
    - Sutton TR, Minnion M, Barbarino F, et al. A robust and versatile mass spectrometry platform for comprehensive assessment of the thiol redox metabolome. *Redox Biology* 2018;16:359-80.
    - Diederich L, Suvorava T, Sansone R, et al. On the Effects of Reactive Oxygen Species and Nitric Oxide on Red Blood Cell Deformability. Frontiers in Physiology 2018;9.

- Erkens R, Suvorava T, Sutton TR, et al. Nrf2 Deficiency Unmasks the Significance of Nitric Oxide Synthase Activity for Cardioprotection. *Oxidative Medicine and Cellular Longevity* 2018.
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Definitions and Abbreviations

- 3MST: 3-mercaptopyruvate sulfurtransferase
- AAS: Ascorbic acid-2-sulfate (Ascorbyl sulfate)
- ACN: Acetonitrile
- ADMA: Asymmetric-dimethylarginine
- AFR: Ascorbate free radical
- AGAT: L-arginine-glycine amidinotransferase
- Arg: Arginine
- AS: Arginosuccinate
- Asc: Ascorbic acid
- CBS: Cystathionine β-synthase
- CDO: Cysteine dioxygenase
- Cit: Citrulline
- CO_2: Carbon dioxide
- CSE: Cystathionine γ-lyase
- Cys: Reduced cysteine
- CysGly: Cysteinylglycine
- CySS: Oxidised cysteine (cystine)
- CysSSH: Cysteine persulfide
- DHA: Dehydroascorbic acid
- DKGA: Diketogulonic acid
- DTPA: Diethylenetriaminepentaacetic acid
- DTT: Dithiothreitol
- EDRF: Endothelium-derived relaxing factor
- EDTA: Ethylenediamine tetraacetic acid
- eNOS: Endothelial nitric oxide synthase
- ESI: Electrospray ionisation
- GluCys: Glutamylcysteine
- Gly: Glycine
- GSH: Reduced glutathione
- GSSG: Oxidised glutathione
- GSSH: Glutathione persulfide
- H_2O: Water
- H_2O_2: Hydrogen peroxide

- H_2S: Hydrogen sulfide
- Hcys: Reduced homocysteine
- HcySS: Oxidised homocysteine (homocystine)
- HILIC: Hydrophobic interaction liquid chromatography
- HPE-IAM: β-(4-hydroxyphenyl)ethyl iodoacetamide
- HSA: Human serum albumin
- IAM: Iodoacetamide
- IEC: Ion exchange chromatography
- iNOS: Inducible nitric oxide synthase
- LC: Liquid chromatography
- LMW: Low Molecular Weight
- *m/z*: Mass to charge ratio
- MBB: Monobromobimane
- MeOH: Methanol
- Met: Methionine
- mM: Millimolar
- MMC: Mixed mode chromatography
- Molar: Moles per litre
- MPA: Metaphosphoric acid
- MRM: Multiple reaction monitoring
- MS/MS: Triple quadrupole (tandem) mass spectrometry
- MS: Mass spectrometry
- MT: Methanethiol
- NEM: *N*-Ethylmaleimide
- nM: Nanomolar
- NMWL: Nominal molecular weight limit
- nNOS: Neuronal nitric oxide synthase
- NO: Nitric oxide
- NO_2^-: Nitrite
- NO_3^-: Nitrate
- Orn: Ornithine
- OxCysGly: Oxidised cysteinylglycine
- OxGluCys: Oxidised glutamylcysteine
- PFP: Pentafluorophenyl
- RBCs: Red blood cells

- RNS: Reactive nitrogen species
- ROS: Reactive oxygen species
- RPC: Reversed phase chromatography
- RSS: Reactive sulfur species
- SAH: S-adenosylhomocysteine
- SAM: S-adenosylmethionine
- SDMA: Symmetric-dimethylarginine
- Ser: Serine
- SFC: Supercritical fluid chromatography
- SIR: Selected ion recording
- SOD: Superoxide dismutase
- SQR: Sulfide quinone reductase
- SSA: Sulfosalicylic acid
- tHcys: Total homocysteine
- ToF-MS: Time of flight mass spectrometry
- TRD: Thioredoxin reductase
- UHPLC: Ultra-high performance liquid chromatography
- UHPSFC: Ultra-high performance supercritical fluid chromatography
- UPC^2: Ultra-performance convergence chromatography
- UV: Ultraviolet
- UV-Vis: Ultraviolet-visible spectroscopy
- μM: Micromolar

Chapter 1: Introduction

Nitric oxide (NO) and hydrogen sulfide (H_2S) are small, low molecular weight molecules that are endogenously generated in mammalian systems via regulated enzymatic catalysis. They play critical roles in numerous biological processes, including regulation of enzyme activity, protein structure and function, and cellular defence. The importance of NO and H_2S in biological systems can be traced back to the evolution of life, before the development of an oxygen rich atmosphere[1,2] with these early pathways being incorporated into oxygen based metabolism and other processes[3]. Along with carbon monoxide (CO) and dioxygen (O_2) they are part of an expanding class of small signalling species, known as gasotransmitters[4], with important biological functions[5] that are only recently becoming better understood. NO is a well-established vasorelaxant, with H_2S being more recently established as also having vasodilation effects[6,7] and is able to facilitate smooth muscle cell relaxation via a different, but not mutually dependant, mechanism to NO[8]. H_2S not only has roles in the cardiovascular system, but also in the nervous and immune systems with the ability to provide protection against oxidative stress. Whilst the concentration of free H_2S is considerably lower than that of other antioxidants such as glutathione it is able to increase the activity of redox regulatory enzymes to help restore antioxidant thiol levels and increasing the availability of rate limiting substrates[9].

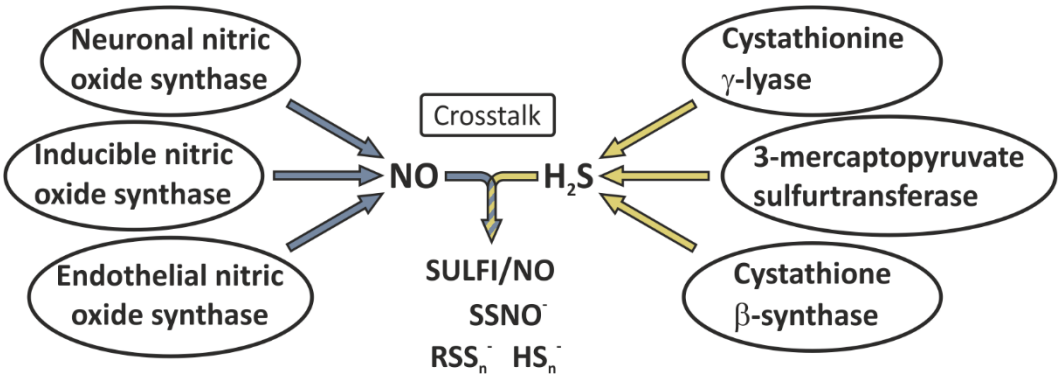


Figure 1: The major products of the direct crosstalk between nitric oxide and hydrogen sulfide at physiological conditions. **SULFI/NO:** *N*-nitrosohydroxylamine-*N*-sulfonate, **SSNO$^-$:** nitrosopersulfide, **RSSn$^-$** and **HSn$^-$:** polysulfides[10].

The NO and H_2S pathways do not operate in isolation, but show significant interactions (crosstalk, **Figure 1**) between the two pathways exhibiting similar and often interdependent biological actions within the cardiovascular system and elsewhere[11]. It has been demonstrated that there is a rich network of coupled chemical reactions between NO and H_2S/sulfide, which suggests that the bioactivity of either transmitter is governed by formation of polysulfides and anionic S/N-hybrid species[10,12,13].

There is a growing body of evidence for the significance of these small anionic species[14-16], however, currently little is known about their uptake into cells, their transport between bodily tissues and their excretion or metabolism. Current methodology for tracing the flux and pathways of these compounds is limited in either specificity, sensitivity or scope[17]. Reported levels of free sulfide range from nanomolar to hundreds of micromolar concentrations[14] depending on the method used for analysis. This wide range in reported concentrations may be due to the liberation of sulfide from carrier molecules such as persulfides, polysulfides and thiosulfate[18,19] during sample preparation, with the higher range of concentrations unlikely to be free sulfide in blood[20,21]. The currently available analytical techniques used to characterise the NO/Arginine pathway and its related metabolites are likewise not entirely satisfactory. The reasons for this include pre-analytical, analytical, and compound-specific difficulties arising mainly from chemical instability and ubiquity, and in part from the relatively low concentrations of some L-Arg/NO family members (e.g., in the low nanomolar range) in biological systems[22]. For these reasons, the development of new methods is important for the exploration of H_2S and NO and their biological functions.

1.1 Oxidative Stress and Redox Regulation

Oxidative stress has been implicated in a wide number of physiological conditions and diseases, including cardiovascular disease[23], pulmonary disease, diabetes[24], cancer[25], obesity[26] and smoking[27]. Overall redox status, related to oxidative stress, is linked to cell survival[28,29] highlighting the importance of redox status in relation to oxidative stress in these conditions. Until recently, oxidative stress has been defined as an imbalance of pro-oxidants and antioxidants. However, this is an oversimplification as shown by the effect, or lack of effect, of treatment of oxidative stress related disorders by antioxidants[30]. The nature of oxidative stress is now becoming better understood as a disruption of redox signalling and control, reflecting the complexities of the wide reaching redox regulatory system. This complex nature is highlighted by the fact that the redox balance of plasma reduced and oxidised glutathione is not always reflected by the redox balance of the more abundant plasma thiol pair reduced and oxidised cysteine[31].

The glutathione and cysteine redox ratios provide useful markers of oxidative stress[32,33] but the lack of equilibration between these thiol redox pairs in either cells or plasma shows that there is clearly no one global measure or balance for oxidative stress[34]. Recognising that there are a wide number of discreet redox regulatory pathways is important in order to increase our understanding of oxidative stress, to help develop a more meaningful biochemical understanding of a powerful clinical problem and to pave the way for more effective therapies to combat oxidative stress related disease. It has been proposed that there is a redox code along the lines of

the genetic code, a set of principles that define the regulatory elements on which organisms rely in order to function. This encompasses the redox organization of cells, tissues, and organisms not just in mammals but ultimately in all living matter[35]. Redox medicine is an emerging area following on from these observations.

1.2 Redox: The H_2S and NO Metabolomes

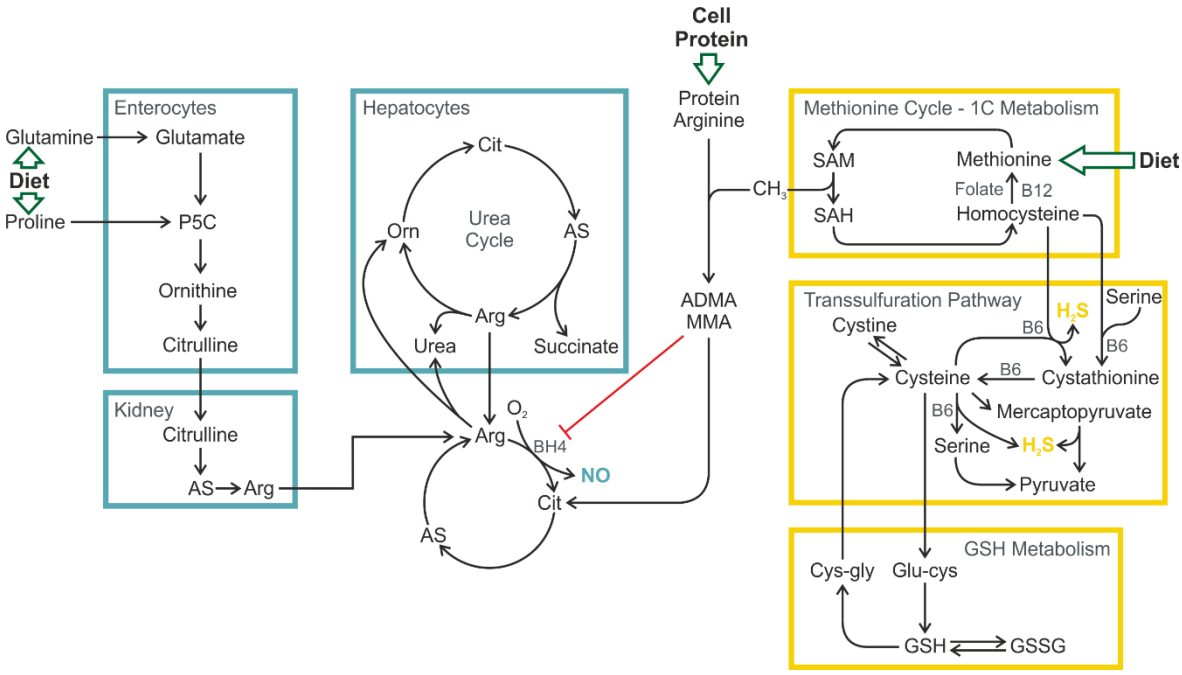


Figure 2: Overview of the pathways involved in the NO and H_2S metabolomes. **Arg**: arginine, **Cit**: citrulline, **Orn**: ornithine, **AS**: arginosuccinate, **P5C**: pyrroline-5-carboxylate, **ADMA**: asymmetric dimethylarginine, **MMA**: monomethylarginine, **GSH**: reduced glutathione, **GSSG**: oxidised glutathione, **Cys-gly**: cysteinylglycine, **Glu-cys**: glutamylcysteine, **SAM**: S-adenosylmethionine, **SAH**: S-adenosylhomocysteine, **B6 and B12:** Vitamins B6 and B12, **BH4:** (6R)-5,6,7,8- tetrahydrobiopterin. For more detailed pathways see the specific schemes discussed later. (Adapted from [36]).

The redox system is essential for the maintenance of cellular homeostasis and redox balance is maintained by a series of complex interlinked and discreet redox pathways. These pathways not only involve reactive oxygen species (ROS) such as superoxide (O_2^-) and hydroxyl radicals (HO^-), but also include reactive nitrogen species (RNS) and reactive sulfur species (RSS) such as organic and inorganic polysulfides[29]. RNS can be formed through the reaction of NO produced from metabolism of arginine (**Figure 2**) with ROS. These interactions can produce RNS, which have stronger oxidative abilities than either of the precursors and have their own oxidative signalling properties[37]. Reactions of ROS and RNS with cysteine can produce analogous RSS that have recently generated interest as the biological functions of these species have emerged. The redox

regulation system is no longer seen as a largely oxygen based system, but rather an integrated system utilising oxygen, nitrogen and sulfur based species[36].

The chemistries of sulfur containing compounds such as organic and inorganic polysulfides (RSS), thiols, and sulfide are largely interlinked, with disturbances to one part of the metabolome having impacts throughout. Organic and inorganic polysulfides are compounds containing chains of two (persulfides) or more (polysulfides) sulfurs and are extremely powerful antioxidants that are actively involved in oxidative stress regulation and redox signalling[38]. Thiols are incredibly versatile in redox processes being able to undergo both one and two electron oxidation. Thiyl radical species that can be formed in these reactions are highly reactive sulfur species that are able to undergo further reactions forming other highly reactive sulfur radical species[39]. Thiols also have important antioxidant effects both outside of and within cells with their transport and metabolism having important effects on the redox system[40]. Homocysteine provides a crucial link between methionine and cysteine, as well as being a key part of the methionine cycle and one carbon metabolism (**Figure 2**), changes to the supply of methionine can not only impact transsulfuration but also transmethylation therefore impacting both the NO and H_2S metabolomes.

In the NO/Arg pathway the synthesis and transport of arginine are clearly important factors and for this reason attracted a lot of initial interest. However, the recognition that products of arginine metabolism also have important biological chemistries themselves shifted the focus towards a pathway wide view instead of simply arginine synthesis[41]. The NO/Arg metabolome comprises of a series of complex interlinked pathways that include significant crossovers with the H_2S metabolome, often referred to as crosstalk. The recognition that these pathways do not operate in isolation, and that the status of one of the pathways does not necessarily reflect the wider redox status has been key to driving the idea that in order to understand redox biology, and related diseases and disorders. There needs to be a systems level approach instead of focusing on individual parts of the redox network.

1.3 The Sulfur Redox Metabolome – Sulfide and Thiols

Low molecular weight thiols and their disulfides such as reduced and oxidised glutathione, cysteine/cystine (reduced/oxidised) and homocysteine/homocysteine (reduced/oxidised) all play important roles in redox processes. The redox pairs of reduced and oxidised glutathione with cysteine and cystine are commonly used as markers for oxidative stress[31,34,42]. Homocysteine, particularly the total levels (free plus bound), is a clinically important marker in cardiovascular and neurological disease[43] as well as a key regulatory intermediate in the methionine cycle (**Figure 2**).

Hydrogen sulfide has recently emerged as an important signalling molecule with numerous cellular functions[17]. The majority of biological sulfide is produced by metabolism of cysteine as discussed in section 1.3.1. The small thiols also have key biological actions including redox regulation and signalling. These small thiols are typically found in micromolar concentrations in plasma and serum[44] with oxidised glutathione being the least abundant of these thiols in plasma but the most abundant in cells. There is emerging evidence for the importance of these small thiol redox pairs with apparent strong links between thiol levels and mortality[45]. Total levels of free thiols are strongly associated with other markers of heart failure severity and prognosis. Lower levels of free thiols are clearly associated with a worse outcome in heart failure[46] and potentially in other oxidative stress related diseases as lower free thiol levels have been recorded in these diseases[47]. These are only the overall changes in all free thiols. In order to better understand how the levels of free thiols are related to outcomes in conditions such as heart failure a more detailed picture of the levels of the individual free thiols is required.

1.3.1 Sulfide

Hydrogen sulfide (H_2S), which had previously been considered only to be a toxic gas, is in fact an important small endogenous gasotransmitter that has recently been shown to have a range of biological actions. H_2S has some similar functions as NO; they are both endothelium-derived relaxing factors (EDRF), a group of compounds that are able to regulate the dilation of blood vessels[6]. H_2S is now known to be an important factor in cardiovascular regulation and disease[7], as well as inflammation[48], neurodegenerative diseases[49] and metabolic disorders such as obesity and diabetes[50,51]. Extracellular H_2S helps to protect against oxidative stress by enhancing the production of the redox regulator glutathione, acting against oxidative glutamate toxicity. Despite the concentration of free H_2S being much lower than that of other antioxidants such as glutathione it is able to modulate the production of GSH by increasing the activity of γ-glutamylcysteine synthetase, which increases glutathione production and replenishes levels depleted by oxidative stress. It is also able to increase the rate of cystine transport into cells, a rate limiting molecule in glutathione production that has a relatively low cellular concentration[9].

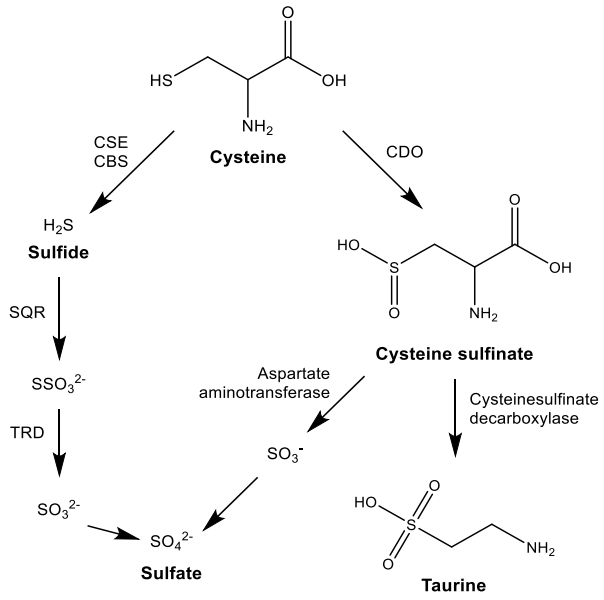
H_2S is synthesised in vivo from cysteine by three different enzymes, cystathionine γ-lyase (CSE)[7], cystathionine β-synthase (CBS)[52] and 3-mercaptopyruvate by 3-mercaptopyruvate sulfurtransferase (3MST)[53] (**Figure 2**, **Scheme 3**). Not only is sulfide production dependent on the redox status as a whole, with levels of the two thiols involved, cysteine and homocysteine tied directly to redox regulation but the redox status is also tied to the levels of free sulfide. Crosstalk between H_2S and NO plays another important role in cellular signalling, H_2S is able to stimulate the NO producing enzyme endothelial NO synthase (eNOS) and increase the bioavailability of NO

resulting in cytoprotective effects when faced with oxidative stress related injuries[54]. This crosstalk is also responsible for the production of species such as polysulfides, persulfides, $SSNO^-$ and nitropersulfides[55] (**Figure 1**).

Sulfide does not only exist freely, but also as bound forms. The bound sulfide is defined as the pool of sulfide that can be liberated from proteins and other biomolecules under reducing conditions. Acid labile sulfide makes up a further pool and is defined as the concentration of sulfide that is liberated from binding to proteins or sulfane sulfur compounds following the treatment of a biological sample with mineral acid (e.g. HCl)[16,18,56]. This form of sulfide is thought to largely originate from the decomposition of inorganic and organic persulfides and polysulfides. These compounds act as stores of sulfide for redox regulation, but also have important biological effects themselves. These inorganic and organic polysulfides can also be formed from sulfide, and the corresponding thiols (for the organic polysulfides) modifying the properties of the thiols and sulfide. For more details on polysulfides see section 1.5. The reducible and acid labile sulfide pools may have some overlap, but the two pools have been demonstrated to be chemically distinct[57]. These different pools of sulfide are likely one of the causes for the large discrepancies in measured free sulfide concentrations, as different methodologies could liberate different levels of sulfide from these pools[18].

1.3.2 Sulfate

Sulfate contributes to numerous processes in physiology and plays important roles during development[56]. A large proportion, approximately one third of requirements, of sulfate is obtained through the diet for adults and children though this can vary largely depending on diet and fluid intake[57]. One of the major ways in which sulfate expresses its biological activity is through sulfonation, which plays an important role in the biotransformation of endogenous compounds such as hormones and neurotransmitters. The sulfonation pathway has also been observed to have important roles in disease, blood clotting and formation of connective tissues[58]. Sulfate as a product of sulfide oxidation in microbes and plants is well known, but been found more recently that this same pathway operates in mammalian biology[58]. Sulfate can be produced in human metabolism by two main pathways (**Scheme 1**), the first utilises cysteine, which can be oxidised to sulfate by the enzyme cysteine dioxygenase (CDO)[59]. The second utilises sulfide produced from cysteine by CSE and CBS, sulfide is oxidised to thiosulfate by sulfide quinone reductase (SQR) which can then be oxidised by thioredoxin reductase (TRD) to produce sulfate.



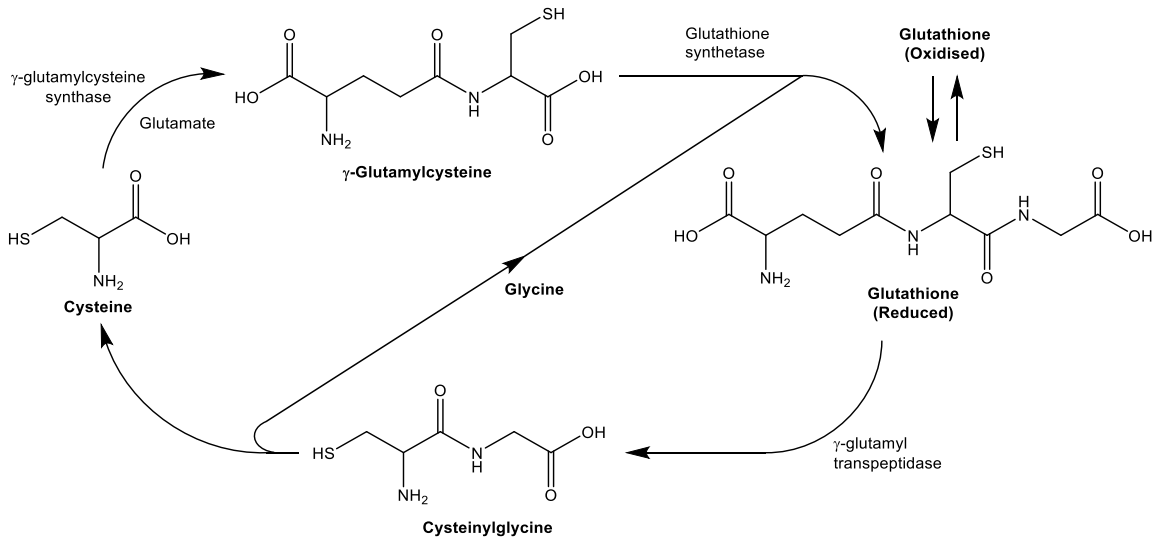
Scheme 1: Pathways for the synthesis of sulfate from cysteine via sulfide and cysteine sulfinate[59], **CSE**: Cystathionine γ-lyase, **CBS**: Cystathionine β-synthase, **CDO**: Cysteine dioxygenase, **SQR**: sulfide quinone reductase, **TRD**: thioredoxin reductase

Sulfate can be considered as the endpoint to sulfide metabolism, where it is either excreted or utilised in sulfonation. However, there are recycling pathways during sulfate metabolism, thiosulfate can be regenerated to sulfide by the enzyme 3MST[58]. Sulfate reducing bacteria form part of the normal intestinal microbiota in healthy humans as well as in saliva. These bacteria can metabolise sulfate to produce sulfide which can then renter the metabolic pathways[60], complicating the tracing of sulfide and sulfate metabolism by monitoring excreted sulfate.

1.3.3 Glutathione

Glutathione (GSH) is a tripeptide formed from cysteine, glutamate and glycine (full name, γ-L-glutamyl-L-cysteinylglycine)[61]. It is biosynthesised from cysteine and glutamate with a regulatory salvage pathway back to cysteine (

Scheme 2) which helps to maintain short-term GSH levels[62]. The availability of cysteine is often a rate-limiting factor for the synthesis of glutathione[63]. Along with its disulfide pair (GSSG) glutathione forms one of the most abundant redox regulation systems in cells where the concentration of GSH is in millimolar levels[64] with lower amounts found outside of cells[44]. GSH forms a key part of the defence against cell damage by reactive oxygen species (ROS) protecting against oxidative stress. The thiol group of the cysteine residue forms the reactive site on GSH with its high nucleophilicity allowing GSH to function as a free radical scavenger and facilitating it's interactions with other thiols and proteins such as human serum albumin (HSA)[39].



Scheme 2: Synthesis and recycling of glutathione, the major controlling metabolic pathway for GSH levels.

Not only is GSH a significant antioxidant itself, but it is also responsible for the regulation and regeneration of other antioxidants such as ascorbic acid (vitamin C)[65,66]. GSH also acts as a cofactor and substrate for several enzymes with antioxidant effects such as glutathione peroxidase and glutathione *S*-transferase thereby providing another indirect mechanism by which GSH can regulate oxidative stress[67]. The glutathione redox system makes up a complex defence mechanism against the effects of oxidative stress and the ratio of reduced to oxidised glutathione and their total levels can be perturbed by a number of disease, nutritional and toxicological conditions[68,69]. GSH is an important storage form of cysteine, which is susceptible to oxidation extracellularly rapidly forming cystine in a process that can form potentially toxic reactive oxygen species. This makes up an important source of cysteine with GSH being transported outside of the cell in order to help regulate the extracellular levels of cysteine[70]. Glutathione plays important biological roles both on its own and as part of the wider redox and regulatory systems.

1.3.4 Glutathione Cycle Thiols

Cysteinylglycine (CysGly) is an intermediate in the conversion of glutathione to cysteine (

Scheme 2). In healthy individuals plasma CysGly has a concentration of ~1-10 μM[71] and slightly higher concentrations in cells[72]. Since in plasma the Cys redox pair is more abundant, and in cells the most abundant redox couple is GSH and GSSG, CysGly is unlikely to be a major antioxidant. However, its contribution to redox regulation should not be underestimated or ignored, as it is a key intermediate in GSH and Cys regulation. CysGly has been shown to be involved in the regulation of GSH interactions with protein thiol groups[73].

γ-Glutamylcysteine (GluCys) is the second intermediate thiol in the Cys-GSH cycle in the synthesis of GSH from Cys[61] (

Scheme 2). GluCys can be transported in and out of cells much more efficiently than GSH facilitating the synthesis of extracellular cysteine from cellular glutathione. There is some evidence to suggest that GluCys itself can act as an antioxidant as well as being a substrate for the synthesis of GSH[74]. GluCys is an important, if minor, thiol to consider when looking at the redox pathways and the flux through them due to its importance as an intermediate.

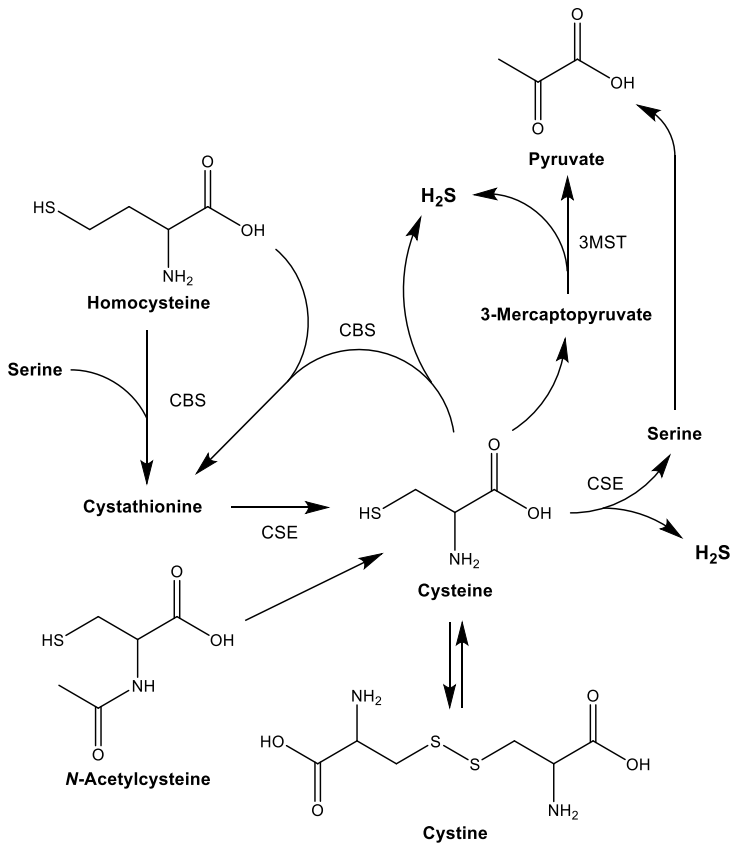
1.3.5 Cysteine

Cysteine and cystine (reduced, Cys and oxidised, CySS) are another important redox couple similar to GSH/GSSG. There is a close link between cysteine and glutathione as it is one of the component peptides and required for the synthesis of GSH and can be a rate-limiting factor in this synthesis (

Scheme 2). Cysteine is synthesised from homocysteine via the transsulfuration pathway by the actions of cystathionine γ-lyase (CSE) and cystathionine β-synthase (CBS) (**Scheme 3**)[75] providing a link between homocysteine and glutathione as part of the complex redox regulatory system[76]. The cysteine redox couple is the most abundant thiol pair in plasma, with oxidised cystine levels in healthy individuals being typically five times greater than reduced levels[31,77]. Levels of cysteine greater than the low micromolar amounts normally found in plasma are in fact toxic[78], Cys is rapidly oxidised compared to GSH and safe, non-toxic levels of Cys are maintained by interconversion to and from GSH (

Scheme 2).

Whilst glutathione is the major redox regulator in cells levels are much lower in plasma, cysteine is the major small thiol redox regulator in plasma with conversely much lower levels than GSH in cells. This has made the status of free Cys and CySS in human plasma an attractive marker for oxidative stress in clinical settings. The Cys/CySS ratio becomes more oxidised in response to oxidative stress factors such as increasing age[77], age related diseases, exercise[79], alcohol consumption[42] and smoking[27]. Since Cys is a rate limiting substrate for the synthesis of GSH and the levels of Cys/CySS are relatively low in cells compared to in plasma, CySS is actively transported into cells in order to facilitate the production of GSH. Cystine uptake into cells is not only important for GSH synthesis but also has protective effects against oxidative stress on its own and so cannot be considered as only part of the glutathione protective system but as a discrete system its self[80].



Scheme 3: Metabolic pathways of cysteine including synthesis from homocysteine and *N*-acetylcysteine, and the production of H_2S by cystathionine γ-lyase (CSE), cystathionine β-synthase (CBS) and 3-mercaptopyruvate sulfurtransferase (3MST). For the pathways involving glutathione, see **Scheme 2**.

Cysteine is a major source of H_2S production[16,81] being a substrate for cystathionine γ-lyase (CSE)[7], cystathionine β-synthase (CBS)[52] and a precursor for the production of H_2S during the synthesis of pyruvate from 3-mercaptopyruvate by 3-mercaptopyruvate sulfurtransferase (3MST)[53] (**Scheme 3**). In a similar manner to glutathione the cysteine oxidised/reduced pair plays an important role in redox regulation, both on its own and as part of the larger interlinked system. Cys also provides a major source for H_2S production and therefore links into the crosstalk between the NO and H_2S metabolomes (**Figure 1**). Cysteine is also a source of taurine which is the most abundant sulfur containing amino acid, it is however not a thiol due to the lack of a free sulfhydryl group. Taurine is semi-essential, it can be synthesised in vivo from cysteine by cysteine dioxygenase[59], but the main source of taurine comes from the diet. Taurine potentially has beneficial effects on the cardiovascular system involving regulation of the NO pathway[82] as well as oxidative stress regulation[83]. Taurine can also increase the expression of the H_2S producing enzymes CBS and CSE providing another mechanism by which it can help regulate the cardiovascular system and redox pathways[84].

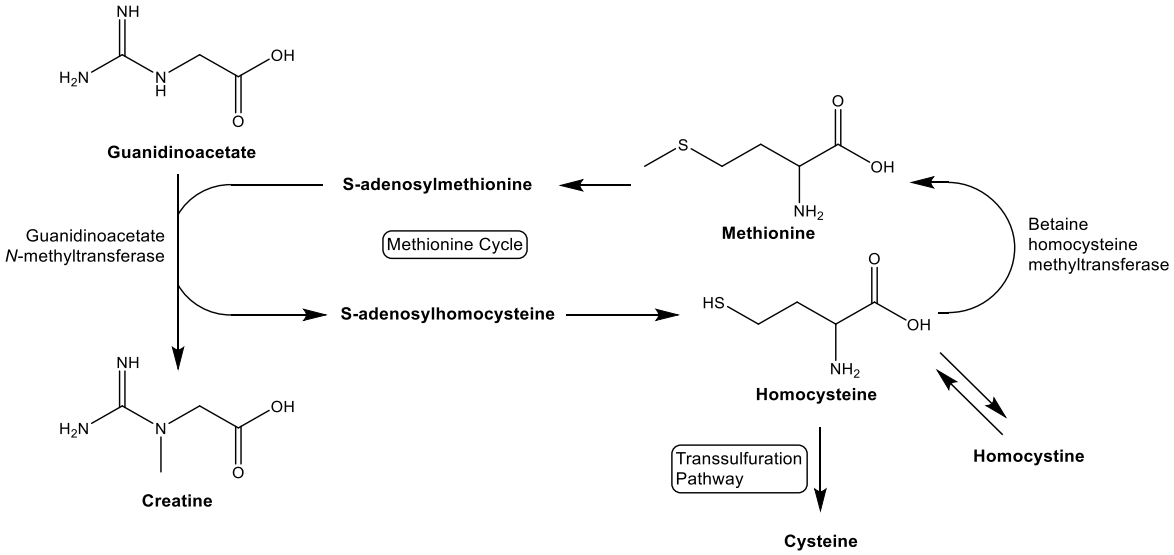
1.3.6 *N*-Acetylcysteine

N-Acetylcysteine (NAC), the acetylated precursor of cysteine (**Scheme 3**), is an important drug as well as being an endogenous aminothiol present both in human plasma and in urine[85]. As a drug, it assumes particular importance as a mucolytic agent for the treatment of chronic bronchitis and other pulmonary diseases and is widely used to treat paracetamol intoxication[86]. The physiological functions of NAC are largely associated with the regulation of intracellular GSH. NAC can undergo redox exchange with cystine, and cysteine-proteins in the plasma producing NAC-cysteine, NAC-NAC and cysteine. NAC provides intracellular cysteine, which can increase the synthesis of reduced glutathione in cells[87]. In turn, GSH has many important effects, and feeds into the wider thiol redox metabolic cycle (section 1.3.3). For this reason, it can be difficult to distinguish the direct physiological effects of NAC from those of GSH.

NAC itself has some antioxidant activities and been shown to interact with various metabolic pathways including, but not limited to, regulation of cell cycle and apoptosis; carcinogenesis and tumour progression; mutagenesis; gene expression and signal transduction; immune-modulation; cytoskeleton and trafficking; and mitochondrial functions[85]. The exact GSH-independent mechanisms behind the many of these biological effects of NAC however are still only partially understood.

Elucidation of these mechanisms is complicated by the fact that NAC interacts with many biological pathways, as well as acting as a radical scavenger and reacting with heavy metals. The endogenous levels of NAC, as well as well as its application as an antioxidant or mucolytic drug means that bioavailability monitoring and pharmokinetic studies require sensitive and selective methodology to ensure accurate and precise measurement of physiological levels. Improved methodologies will also be important in the investigation of its many biological effects and the determination of the biological mechanisms behind them[88,89].

1.3.7 Homocysteine



Scheme 4: Key biological reactions of homocysteine in the methionine cycle and transsulfuration pathway (see also scheme 2)

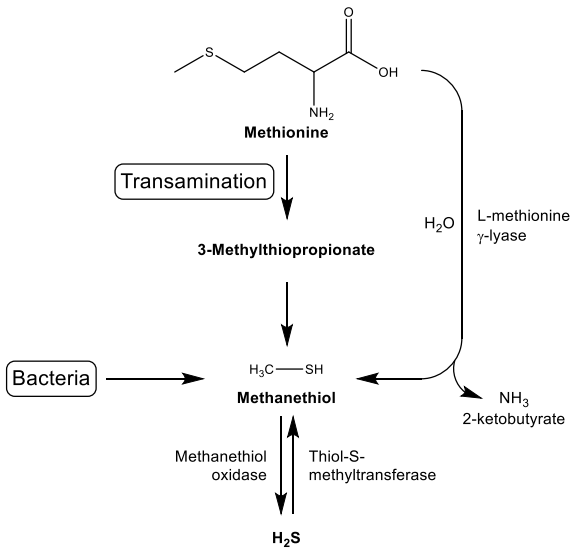
Homocysteine and homocystine (reduced, Hcys, and oxidised, HcySS) make up the third thiol pair of interest. Homocysteine is a key thiol in the sulfide metabolome being an intermediate in both the metabolism of methionine and the transmethylation pathway[76] (**Scheme 4**). This forms another key link between arginine metabolism (NO metabolome) and thiol metabolism (H_2S metabolome). Homocysteine is irreversibly removed from the methionine cycle by conversion to cysteine, this is a one-way reaction and homocysteine cannot be synthesised from cysteine[90]. This is an important link to consider as any perturbation of the cysteine and glutathione systems can feed back through the metabolic pathway and have a significant impact on the transmethylation pathway and methionine cycle. Tissue levels of Hcys are typically maintained at low concentrations with high concentrations exhibiting toxic effects[43], the levels of homocysteine are regulated by the aforementioned transmethylation pathway as well as the transsulfuration pathway (**Scheme 3**).

Disturbances in homocysteine levels can be caused by two different mechanisms, a reduction in the rate of remethylation to methionine or by impaired removal of Hcys by transsulfuration to cysteine. Reduced remethylation can be caused by deficiencies of some vitamins such as folate and vitamin B_{12}[91] as well as inborn errors affecting methionine synthase. Reduced transsulfuration can be caused by deficiencies in vitamin B_6[92] as well as inborn errors in CBS[93]. The redox status of homocysteine is less well defined than for glutathione and cysteine with the majority of studies looking at the total homocysteine levels (reduced, oxidised and bound) but it forms another potentially important redox couple. In plasma, the majority of free Hcys exists in its oxidised form HcySS, but this ratio can be perturbed in disease states. Total homocysteine (tHcys) is used

clinically as an important marker for cardiovascular disease with abnormally raised levels of homocysteine being termed hyperhomocysteinemia[94].

Elevated levels of Hcys in plasma are not only a risk factor for cardiovascular disease, Hcys is also linked to deficiencies in the regulating vitamins such as B_{12} and folate[91] linked with mental disorders[95], genetic disorders[93] as well as Alzheimer's and dementia[96]. However tHcys is a relatively crude measure, as it does not distinguish between the free Hcys which makes up ~2%, free HcySS, and protein bound or mixed disulfides containing Hcys which make up ~98% of the Hcys pool. The redox status of Hcys both affects and is linked to the redox status of the other thiol pairs[97] therefore it is important to try and understand the changes in the levels of the different Hcys species as opposed to looking at the total levels of Hcys.

1.3.8 Methanethiol



Scheme 5: Synthesis pathways of methanethiol from methionine and bacterial sources, and recycling of methanethiol to sulfide, which can feed back into the wider sulfur redox metabolome.

Methanethiol (CH_3SH, MT), also known as methyl mercaptan, like H_2S is a volatile sulfur compound. Methanethiol can be synthesised in mammals from methionine by the enzyme L-methionine γ-lyase as well as via transamination of methionine in mammalian cells. It can also be generated by bacteria found in the gut as well as through the methylation of sulfide by the enzyme thiol S-methyltransferase[98-100]. Conversely, it has been found that MT can be converted back into sulfide by the enzyme methanethiol oxidase[101,102] (**Scheme 5**).

Despite the fact that MT is clearly synthesised, metabolised and recycled the biological relevance of MT is not yet well understood, with few extensive studies performed so far[103]. It has been

found that as well as free MT it also exists in both acid labile forms and DTT labile forms. DTT labile MT has been found to be a reliable measure of exposure to MT and may serve as an important marker for the potential role of MT in both normal metabolism and in disease states[104]. Free MT exists in its highly volatile dissociated form at physiological pH due to its high pK_a. In much of the same manner as free sulfide if it is to be measured in biological samples, care must be taken to avoid the volatilisation, and therefore loss, of MT. However, it has been reported that in the blood and tissues of healthy men and rats there is no measurable MT, and when added to blood MT was rapidly oxidised to thiosulfate and sulfate[104]. Free MT has however been reported in urine, especially in various disease states such as isolated persistent hypermethioninemia where levels have been found to be elevated as well as after eating asparagus[103].

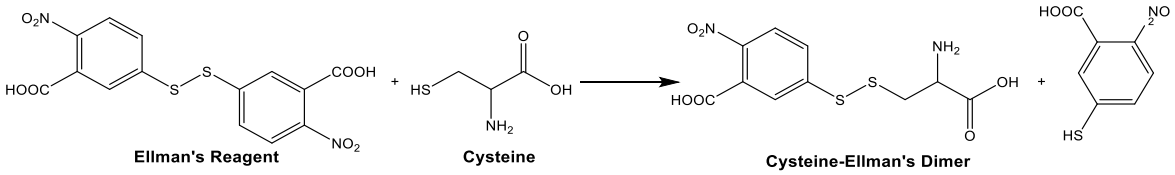
Both acid and DTT labile MT have been reported in serum in normal persons as well in elevated levels some disease states. In a similar manner to the free MT acid and DTT labile MT have also been found in urine in greater concentrations than blood/serum/plasma and with much larger elevations associated with abnormal metabolism[103]. It is clear that methanethiol has biological relevance even if it is not yet fully understood. The importance of all three forms of MT in different biological matrices requires further investigation. This relies on the development of new robust methodology that can be used to gain an accurate picture of the levels of MT and changes in order to gain a better insight into the physiological role of MT in its various forms.

1.4 Analysis of Low Molecular Weight Thiols and Sulfide

Accurately quantifying the levels of free thiols can be difficult[68], in part due to the rapid oxidation of the reduced thiols that can occur during and after sample collection. The plasma concentration of reduced glutathione can be 100x greater than that of oxidised glutathione; any oxidation that occurs will significantly increase the concentration of oxidised glutathione and affect the measured redox ratio[44]. Enzymatic activity will still continue after sample collection if not blocked and enzymes such as glutathione reductase can lead to losses of thiols or changes in their distribution[33,105]. Whilst the differences in concentration between the reduced and oxidised forms of cysteine and homocysteine are not as great, oxidation is still an important effect to consider. These small thiols are not only susceptible to oxidation but both the reduced and oxidised forms can be taken up by proteins as well as forming numerous mixed thiol pairs[44]. Sulfide being a small volatile molecule is susceptible to loss from samples and especially sensitive to changes in pH, derivatisation is important to trap sulfide and prevent this loss. As discussed levels of free sulfide have recently been found to be much lower than previously thought[21]. This is likely due to the accidental liberation of sulfide from labile stores such as polysulfides, persulfides and thiosulfate during sample collection, preparation or analysis.

Whilst there is no shortage of methods for the analysis of the H_2S metabolome and its related pathways, many of those that exist are limited in some fashion. There are currently no methods that encompass the full range of relevant compounds with the required specificity and sensitivity. For these reasons, a comprehensive, sensitive and specific method is needed in order to analyse the key components involved in sulfide production and thiol metabolism. The most extensively used methods generally use an alkylating agent such as monobromobimane, methylene blue and *N*-Ethylmaleimide (NEM) to both enable the detection and analysis of thiols or sulfide as well as to prevent artificial oxidation of samples after collection. The most widely used methods are discussed below.

1.4.1 Ellman's Reagent



Scheme 6: Example of the reaction of Ellman's reagent with a reduced thiol (Cys).

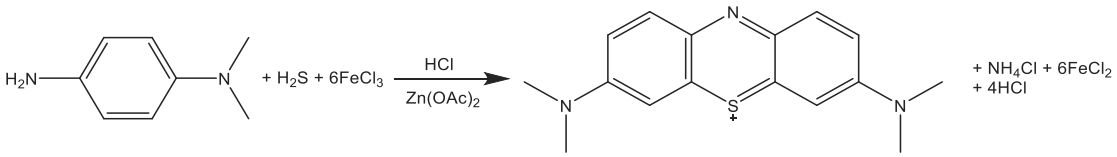
Ellman's reagent (5,5'-dithiobis-(2-nitrobenzoic acid) or DTNB) can be used to measure the levels of thiols by monitoring the absorbance of visible light spectrophotometrically. The reaction of Ellman's reagent with free thiol groups (**Scheme 6**) produces a free chromophore, which absorbs strongly at 412 nm within the UV-vis range[106]. This provides a quick, simple and cheap method of analysis. However as it reacts with all free thiol groups and uses a detection method that is completely non-specific without any form of separation so whilst it can easily measure total free thiol levels it does not provide any further details into the thiol compositions. Ellman's reagent is also unstable over pH 8, but for the reaction to proceed optimally it requires more basic conditions with a compromise needed either for stability or reaction speed[107]. Ellman's reagent has also been utilised for the analysis of polysulfides in wastewater[108] but suffers from the same issues as for the thiols, particularly the non-specificity, in biological matrices.

1.4.1 Sulfur Selective Electrode

A sulfide selective electrode can be used for the measurement of free sulfide down to low μM concentrations[109] though the limit of detection can be affected by the pH of the solution being measured. An advantage of this method is that it measures the free sulfide ions directly, without need for derivatisation, and has a fast response time allowing for dynamic measurements. However, other thiols present may interfere with the electrode, a major disadvantage for

measuring physiological free sulfide in biological systems. This method is also not capable of distinguishing between the different thiol species.

1.4.2 Methylene Blue



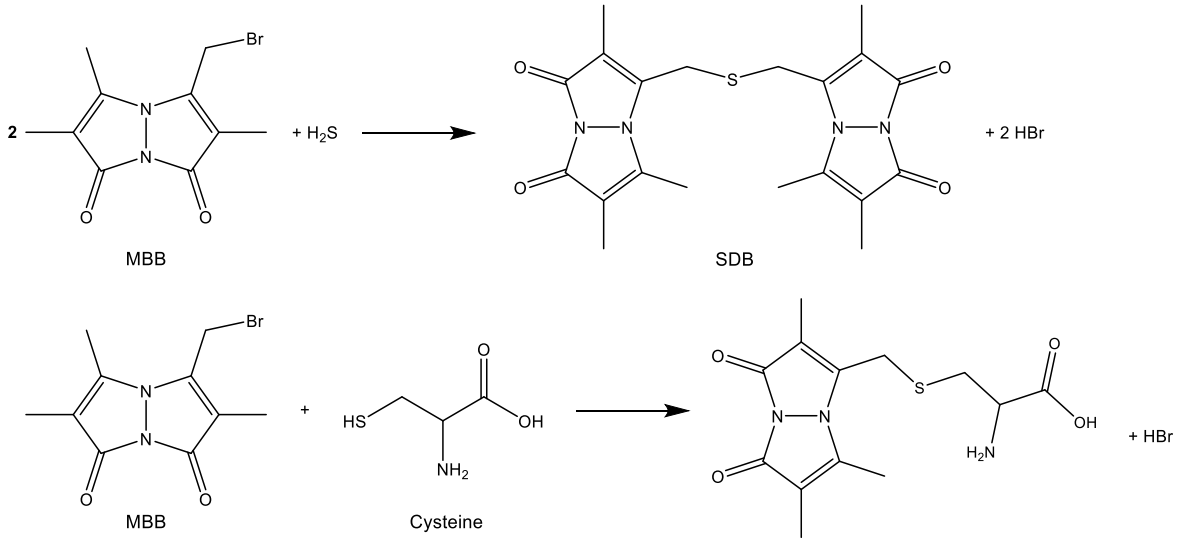
Scheme 7: Reaction of *N*,*N*-dimethyl-*p*-phenylenediamine with H_2S to form methylene blue[18].

The methylene blue method is one of the most commonly used methods for the determination of sulfide. It is based on the reaction of sulfide with *N*,*N*-dimethyl-*p*-phenylenediamine, in a ferric chloride catalysed reaction forming methylene blue, which has a blue colour (**Scheme 7**) with detection by spectrophotometry[110]. There are a few issues with this method, the lower limit of detection by this method is around 1 μM which is above some of the reported levels of physiological free sulfide. There is a potential issue of other thiols reacting with the *N*,*N*-dimethyl-*p*-phenylenediamine producing chromophores which will produce erroneous signal for free sulfide. One way to try and avoid erroneous signals is by precipitating with Zn-Acetate and then re-dissolving the ZnS precipitate under highly acidic conditions in the presence of the *N*,*N*-dimethyl-*p*-phenylenediamine. However, this acidification step means the measurements are not made at physiological pH and there are a large number of acid labile thiols that may still be present and would contribute to the levels of free sulfide measured[111].

1.4.3 Monobromobimane

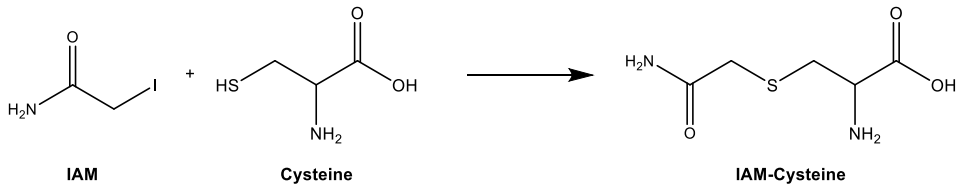
The monobromobimane (MBB) method utilises the alkylation of free RSH groups on thiols and sulfide with MBB to produce highly fluorescent sulfide dibimane (SDB) or thiol-bimane adduct (**Scheme 8**). One advantage of this method is the stabilisation of the sulfide and the other thiols preventing oxidation and the ability to measure other thiols and the mild reaction conditions that should not perturb other sulfide storage forms. SDB can be separated from the remaining MBB and other thiols chromatographically and detected by fluorescence[112] with excitation and emission wavelengths of 390 and 475 nm, respectively. The limit of detection of this method can be as low as in the nM range, however in biological samples with an excess of MBB levels lower than 1 μM can be hard to detect. In order to accurately quantify lower levels of sulfide the SDB first has to be extracted into ethyl acetate in order to remove interference from the remaining MBB[113]. Another disadvantage is the fact that MBB itself fluoresces as well as its hydrolysis

products which can give rise to additional peaks which may interfere with thiol determination[114] though they can normally be separated by reversed phase chromatography.



Scheme 8: Reaction of monobromobimane (MBB) with sulfide to form sulfur dibimane (SDB)[18] and with cysteine (as an example reduced thiol) forming a thiol-bimane adduct.

1.4.4 Iodoacetamide



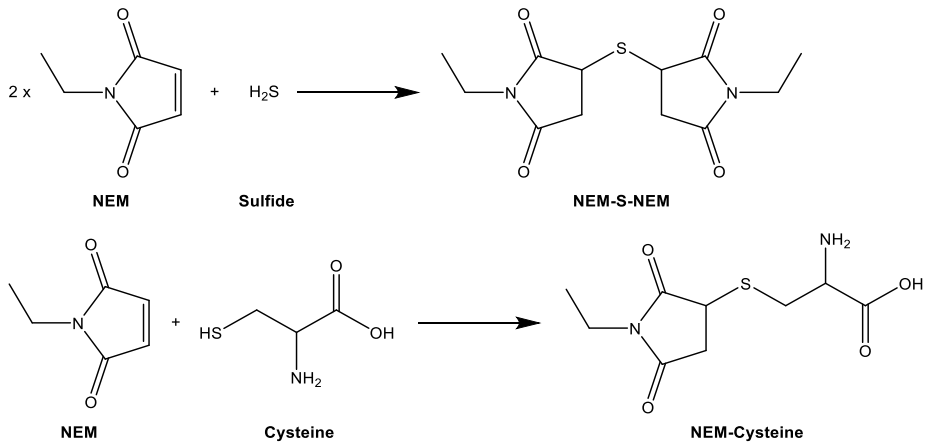
Scheme 9: Reaction of iodoacetamide (IAM) with the thiol cysteine forming the carboxamidomethyl cysteine derivative.

Iodoacetamide (IAM) is a commonly used alkylating agent which irreversibly reacts with thiols and other reactive sulfur species through a nucleophilic substitution (S_N2) reaction mechanism[115]. IAM is less reactive than both MBB and NEM and is considered a milder electrophile[105,116]. For this reason it has been used in the analysis of inorganic and organic polysulfides where it is considered to be more suitable for the analysis of longer chain polysulfides[117]. IAM is membrane permeable and so can freely enter cells without lysis. This offers a big advantage for the analysis of intracellular thiols and sulfide as they can be alkylated without the risk of intracellular thiol levels being disturbed during lysis.

IAM derivatised thiols can be separated using liquid chromatography and detected using a variety of techniques including mass spectrometry and UV absorbance detection[118,119]. However, it has been found that IAM is not as well suited for the alkylation of thiols at physiological pH as some

other alkylation agents, and requires at least a pH of 8 in order to react effectively. It has also been noted that IAM is capable of producing several unwanted reaction products[118], though these can be potentially separated chromatographically and distinguished by mass spectrometry they may pose issues for other less specific methods of detection. IAM has a relatively slow rate of reaction compared to other alkylating agents (see Chapter 5: for a more in depth discussion) which could potentially lead to the artifactual oxidation and loss of thiols as well as the volatilisation of sulfide, make IAM less suited for the analysis of biological thiols and sulfide[120].

1.4.5 *N*-Ethylmaleimide



Scheme 10: Alkylation of the reduced thiol cysteine and sulfide by *N*-Ethylmaleimide (NEM), note that reduced thiols such as cysteine react with a 1:1 molar ratio whilst sulfide reacts with a 1:2 molar ratio with NEM.

N-Ethylmaleimide (NEM) reacts similarly to monobromobimane, rapidly alkylating H_2S and other reduced thiols[121] (**Scheme 10**), both stabilising them[122] and enabling efficient analysis by enhancing the ionisation efficiency in mass spectrometry[123]. NEM is currently used to stabilise *S*-nitrosothiols, products of NO metabolism and potential NO stores[124], and so is already used routinely in the analysis of the NO metabolome. This offers the advantage of being able to use the same sample collection methodology for both nitrosothiol and sulfide/thiol analysis. In a similar manner to IAM, NEM is membrane permeable and so is well suited for the analysis of intracellular thiols and sulfide as it will trap the intracellular levels without the need for lysis[105]. NEM has been used previously for the analysis of sulfide[125] and biological thiols[126] and provides an advantage for the separation of analytes by improving retention on reversed phase chromatographic columns[127].

The derivatisation reaction occurs rapidly, over around five minutes at room temperature at physiological pH making it a rapid reaction that will quickly prevent the volatilisation and therefore loss of free sulfide[120], and should not significantly release sulfide from its storage forms

present in biological samples. The derivatised sulfide and thiols can be easily separated by reversed phase chromatography[128] and detected by tandem mass spectrometry (MS/MS). NEM derivatisation should help prevent artificial oxidation, block enzymatic activity, improve the separation and sensitivity of measurement and stabilise the volatile sulfide. These advantages will help provide a sensitive and selective method allowing the identification and quantification of free sulfide, polysulfides and other thiols[129]. NEM derivatisation is well positioned to deliver a comprehensive and robust method for the analysis of the key small thiols and sulfide, which is superior to existing methodologies.

1.5 Reactive Sulfur Species: Persulfides and Polysulfides

Reactive sulfur species (RSS) are a family of sulfur containing molecules found in biological systems that have numerous biological activities and are key players in a large number of biological processes[130-132]. Among the RSS are persulfides, molecules containing two linked sulfurs, and polysulfides, molecules containing more than two linked sulfurs, which have only come to attention relatively recently and their exact biological mechanisms are still not well understood. Persulfides and polysulfides broadly fall into two different classes, organic and inorganic. Inorganic polysulfides consist of only H_2S_n where $n \geq 2$ (though in solution will be disassociated); organic polysulfides consist of multiple sulfurs joined to the sulfur of a thiol such as cysteine or glutathione (RS_nH, $n \geq 2$). Organic polysulfides can also exist on the cysteine residues of proteins[117,133].

1.5.1 Biochemistry of Polysulfides

The biological importance of inorganic polysulfides has recently become apparent following the discovery of inorganic polysulfides in the brain. They have also been found to be produced from multiple biological pathways including during sulfur containing amino acid metabolism[13] and by the enzyme 3-mercaptopyruvate sulfurtransferase (3-MST)[134]. 3-MST utilises 3-mercaptopyruvate (3MP) as a substrate, which is produced from the metabolism of cysteine and has long been known to be a source of H_2S[134] (**Scheme 3**). Cysteine and glutathione per- and polysulfides can be produced from cystine and oxidised glutathione respectively by the enzymes cystathione β-synthase (CBS) and cystathionine γ-lysase (CSE)[135]. However there must be other biological pathways for the formation of these reactive sulfur species as per- and polysulfides have been measured in appreciable levels in mice lacking both CSE and CBS[136]. It has more recently become apparent that some enzymes thought to be exclusively involved in the metabolism of reactive oxygen species (ROS), such as catalase and superoxide dismutase (SOD), are also able to metabolise sulfide and produce polysulfides[137,138]. Organic persulfides can also be formed by the

direct reaction of H_2S with a disulfide such as GSSG with an equilibrium formed between the corresponding persulfide and reduced thiol species[139].

Per- and polysulfides are reactive molecules that possess both nucleophilic and electrophilic properties and have greater reactivity than their single sulfide equivalents. Organic per- and polysulfides are more nucleophilic than corresponding thiols, which is due to additional sulfur electrons enhancing the nucleophilicity of the external sulfur atom (α-effect) as well as the lower pK_a of persulfides. The increased stability of the persulfide radical form compared to thiol leads to their increased reducing ability as well as contributing to their increased nucleophilicity[36,130,140]. Reactive per- and polysulfides have been proven to be extremely powerful antioxidants which are involved in oxidative stress regulation as well as redox signalling. The antioxidant ability of GSSH and CysSSH is approximately 10-100 times greater than their thiol equivalents GSH and Cys. Much of the presumed utility of persulfides has been based on their unique chemistry, which is distinct from that of thiols, however the mechanisms by which persulfides exert their biological functions have not yet been determined. For example, whilst GSH does not readily react with O_2 at a significant rate, GSSH is able to react much more rapidly, which demonstrates the increased reactivity of the persulfide and opening up additional chemistry by which it may have some biological effects[38].

Free sulfide levels are now understood to be in the low nanomolar range[21] as opposed to the previously reported micromolar ranges[141]; it is possible that these differences are due to the existence of labile stores of sulfide that may be liberated by some analytical methods. Per- and polysulfides have been suggested as making up some of these potential stores of sulfide and acting as mediators of its biological effects. Some of the signalling effects previously attributed to H_2S are now being recognised as being attributed to the more reactive inorganic polysulfides (H_2S_n)[55]. Much of the biological activities currently ascribed to polysulfides are thought to occur through *S*-persulfidation, the formation of protein persulfides (P-S-SH) on protein cysteine residues through reaction with H_2S_n[42,132]. Cellular regulatory mechanisms that have previously been thought to be targets of H_2S have been found to respond much greater to H_2S_n. For example, TRPA1 channels which are involved in the regulation of inflammation, pain, cardiovascular system, gastrointestinal tract, and in cancer. Polysulfides have also been found to upregulate the release of nuclear factor erythroid 2-related factor 2 (Nrf2), which in turn has important antioxidant activities. It is clear that the role of polysulfides in signalling is greater than previously appreciated[142].

Despite these well-known and important physiological activities of both inorganic and organic persulfide and polysulfides there are still many aspects that remain unclear. The precise details of

their mechanism of formation, their prevalence and speciation in biological systems as well as their interaction with NO are only just starting to emerge[10]. It has been found that persulfides and polysulfides are direct intermediates in the interaction between H_2S and NO (crosstalk, section 1.8), and persulfides react with nitrite producing NO via intermediate polysulfide species (**Scheme 15**)[13]. Polysulfides have proven to be challenging to analyse. Many methods make use of an electrophile in order to attempt to trap the physiological distribution of the polysulfide species. However, if the polysulfide species are in a dynamic redox equilibrium, then the levels of the various alkylated species will be a function of the relative rates of electrophilic trapping versus the redox equilibrium interconversions of the trappable species. This will depend on a number of different parameters including concentrations of the electrophile and polysulfides, the pH and the temperature[143]. In order to understand the biochemistry of polysulfides accurate and precise methods must be developed with a proper understanding of the chemistries involved in order to obtain a true picture of the physiological distribution of these important species.

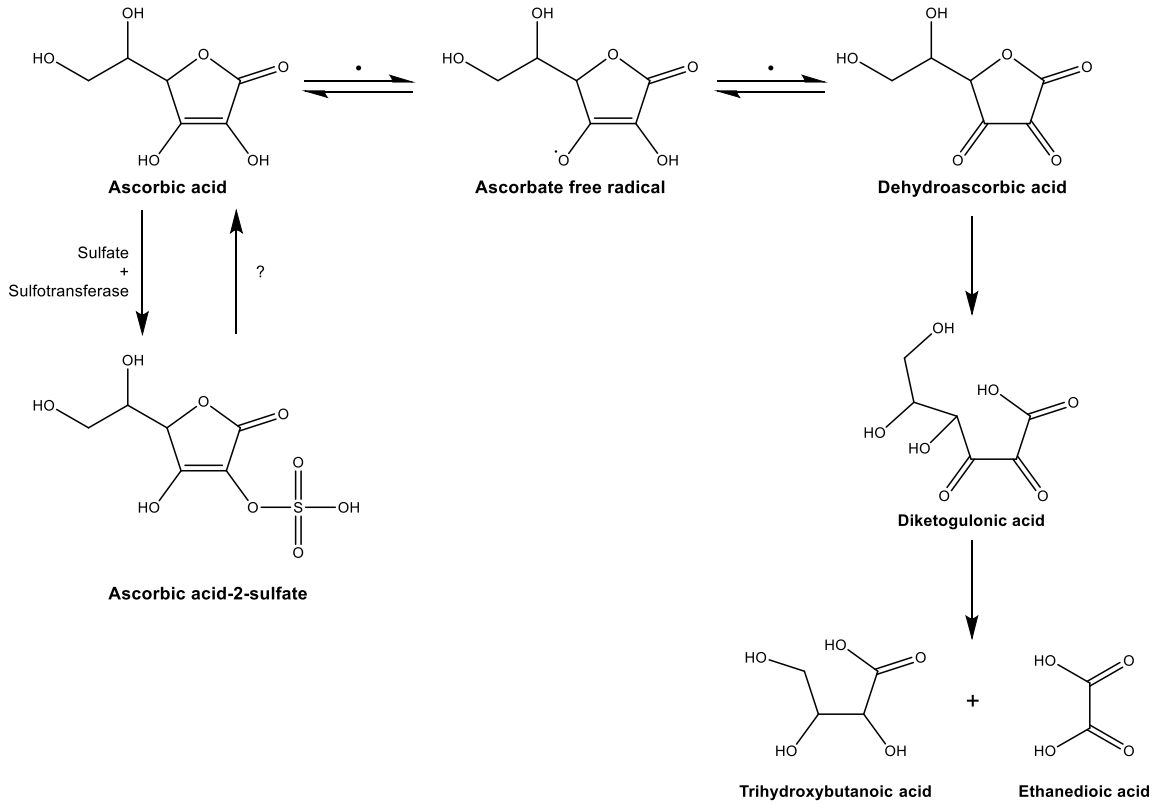
1.5.2 Analysis of Polysulfides

Before the biological importance of polysulfides became apparent[134], a large number of methods for the detection of polysulfides existed focusing on sample matrices such as plants[144], and water sources including, potable water[145], wastewater[108] and natural bodies of water[146]. Whilst these methods may be sufficient for these matrices, they may not be appropriate for analysis of complex human biological samples such as plasma or tissues. The majority of these methods offer no distinction between the polysulfide species, or in some cases not even between any species with a free SH group such as reduced thiols. Polysulfide specific fluorescent probes such as SSP4[137,138] and HSip-1[147] have been used to detect the presence of polysulfides in samples. Although these probes specifically react with sulfane sulfurs they only provide a measure of total polysulfide levels and do not give any information on the speciation of the polysulfide species. Monobromobimane (MBB) has also been employed for fluorescence detection of polysulfides[134,148]. MBB reacts less specifically than the other probes (see section 1.4.3) and will react with any free SH groups present including reduced thiols and albumin potentially confusing the analysis. UV detection has been used for the more specific determination of polysulfide species but has led to varying and conflicting results from different research groups. The UV spectra of aqueous polysulfides only shows at most two peaks within the UV range and therefore requires deconvolution of the spectra complicating the analysis and leading to the varying results[149]. Even when combined with a separation technique such as HPLC, UV detection requires derivatisation (with MBB for example) and complex extraction[146,150], though it does provide a

more specific measure of the polysulfide species and enables quantification of the species present[132].

Electrospray ionisation (ESI) mass spectrometry has been used with and without separation techniques for the specific analysis of polysulfide species. ESI is a soft ionisation technique that should not alter the polysulfide speciation (for more information on ESI see section 1.9). ESI-MS analysis provided more consistent analysis of underivatised polysulfides on two different platforms compared to UV analysis, and looks to be a promising technique for the identification of polysulfide speciation[149]. When coupled to liquid chromatography (LC) an electrophile is typically used to alkylate the reactive end sulfurs of the polysulfides to attempt to trap the distribution of the species and prevent changes during the chromatographic run. LC will be essential for the analysis of polysulfides in biological matrices due to the complex nature of the samples. Many different electrophiles have been used including MBB[151], IAM[152], and NEM[117,153], for more details on these electrophiles see section 1.4. When alkylated the polysulfides are well retained and separated on a reversed phase column using a simple gradient elution[151]. However, there are questions over the suitability of the different electrophiles used as the alkylation reaction could potentially alter the polysulfide speciation and provide a measure of the reactivity of the polysulfide species instead.

1.6 Ascorbate Metabolome



Scheme 11: The ascorbate redox pathway: The redox pair ascorbic acid (Asc) and dehydroascorbic acid (DHA) with the sulfated metabolite of Asc, ascorbic acid-2-sulfate (AAS). The degradation pathway of dehydroascorbic acid to diketogulonic acid can account for loss of ascorbic acid and dehydroascorbic acid from this redox system. Crucially the oxidation of ascorbic acid is reversible but the degradation of dehydroascrobic acid to diketogulonic acid is irreversible.

1.6.1 Ascorbic acid

Ascorbic acid (Asc), or as it is commonly known, vitamin C, is a dietary antioxidant that cannot be synthesised by humans. Since its first isolation in 1928 it has been the focus of a wide range of research due to its importance as a constituent of the human diet[154-157]. Asc is an electron donor and acts as a reducing agent, which is key to its antioxidant properties and biological effects. Asc helps to neutralise damaging free radicals, protecting cells, proteins, and DNA from oxidative damage. Asc also acts as an electron donor for at least eight different enzymes for a variety of functions including the addition of hydroxyl groups to proline or lysine in collagen and other biochemical synthetic pathways[154]. An increased intake of Asc along with elevated plasma levels is associated with lower risk in a variety of diseases including cancer, cataract and heart disease. It is

however not yet clear if this effect is a direct action of Asc or if it is associated with corresponding protective agent[158].

It is one of the most widely used vitamin/antioxidant supplements taken worldwide on top of being used as an antioxidant in cosmetics and the food industry[159]. It is an essential dietary constituent that can be found in many fruit and vegetables, notably citrus fruits[160]. Many fruit juices are fortified with Asc, and the content found naturally in fruit and vegetables can vary depending on factors including the time of harvest, transport, storage and preparation. Asc supplements (referred to as vitamin C in this form usually) are available in many forms including tablets and powders and it is often included in multi-vitamin formulations. At high doses of vitamin C Asc in the plasma is not as easily absorbed as lower doses and there is a maximum level that can be absorbed, excess Asc is excreted through the kidneys and lost in the urine. In cosmetics Asc is used to protect the skin from UV damage in conjunction with vitamin E. As Asc is required to enable the synthesis of collagen, it is also added to anti-aging formulas to boost collagen production in skin[161].

Due to the importance of Asc reliable detection and quantification in a wide range of complex sample types is of great interest. The Asc content of fruit and vegetables is used as a marker of quality[162] and with the popularity of Asc as an antioxidant additive and fortifier, determination of accurate levels is of importance. As with food and drinks the Asc content of cosmetics is of interest to analyse especially concerning the stability of Asc in various formulations[161]. Physiological samples such as plasma and tissues provide a challenging area for the analysis of Asc, though are of great interest, due to their complex nature and the inherent instability of Asc after sample collection. With circulating Asc levels strongly correlated to health and outcomes along with the closely interlinked redox metabolome this area is only increasing in interest.

1.6.2 Dehydroascorbic acid

Dehydroascorbic acid (DHA) is the oxidised form of Asc and makes up the second half of the ascorbate redox pair (**Scheme 11**). Since DHA is the oxidised form of Asc is not as effective an antioxidant, but it does have some distinct protective properties of its own via irreversible oxidation to diketogulonic acid. It is able to chelate metal ions and in fact is better at protecting low-density lipoproteins from damage from copper ions than Asc in some cases[163]. In fruits, the total vitamin C content is assumed to be the sum of the Asc and DHA and the ratio of Asc to DHA is also used as a marker of quality[162]. Key to being a redox couple, DHA is recycled back to Asc. DHA is transported across cellular membranes more efficiently than Asc and is almost immediately reduced to Asc once it enters the cell by a variety of processes including

enzymatically. This process helps maintain cellular Asc levels for defence against oxidative stress[164]. The glutathione dependant enzymes, such as glutathione dependant dehydroascorbate reductase, are the most important recycling enzymes in this regard[165,166]. There are also NADPH dependant enzymes including thioredoxin reductase and NADPH-dependent dehydroascorbate reductase[167,168]. The mechanism by which DHA is reduced to Asc is largely dependent on the availability of GSH, when GSH levels are depleted NADPH dependant reduction is favoured, whilst when GSH is readily available then GSH dependant is favoured[164]. Direct, non-enzyme mediated, interaction of DHA with GSH can also reduce DHA back to Asc whilst GSH is oxidised to GSSG, which itself can be recycled back to GSH by other mechanisms.[65,169].These mechanisms provide redundancy that ensures that cellular Asc concentrations are maintained in a variety of conditions. The ascorbate free radical (AFR) is the intermediary species in this redox couple (**Scheme 11**) and is relatively stable. The AFR is directly detectable in most biological samples by electron paramagnetic resonance and potentially has important biological effects of its own[170].

DHA is highly unstable in solution, even more so than Asc, and is extremely sensitive to factors such as oxygen, high temperature, light, metal ions and pHs neutral and above[171]. These recycling pathways are key for the maintenance of cellular Asc as well as a recycling mechanism for DHA, though they are not 100% effective. Any DHA which is not recycled back to Asc is rapidly and irreversibly hydrolysed to diketogulonic acid (DKGA) which has no apparent biological function and is excreted[172] (**Scheme 11**) leading to the loss of Asc which can only be regained through the diet. The hydrolysis of DHA to DKGA occurs more rapidly under higher oxidative stress conditions and likely occurs to remove harmful oxygen species as a last line of defence against oxidative stress[163].

It has been established that DHA can trigger diabetes[173] and elevated circulating levels are found in the blood of diabetic patients. Elevated levels of DHA could be used as markers for early detection of diabetes or as a measure of risk[174]. Treatment of diabetic patients by Asc supplementation did not significantly lower their blood glucose despite increasing the circulating Asc levels[175]. This, as with the sulfur related redox metabolome, points towards the complex nature of redox regulation. Whilst there is a strong correlation of decreased Asc and increased DHA, pointing towards redox imbalance and oxidative stress, the answer is not as simple as administering an antioxidant to try and correct one point of redox imbalance. The complex interlinked nature of the whole redox metabolome will require a systems level approach rather than a focus on any one pathway.

1.6.3 Ascorbic acid-2-sulfate

Ascorbic acid-2-sulfate (AAS), or ascorbyl-2-sulfate, is found in the diet as well as being a metabolite of Asc, formed by the enzymatic reaction of cellular Asc with sulfate (**Scheme 11**), a metabolic product of H_2S[155] (section 1.3.2). This interaction makes up a further point of crosstalk in the redox interactome linking ascorbate redox regulation and sulfide metabolism. This metabolic activity has not been investigated as a point of crosstalk to my knowledge, and there has been little investigation into the biochemistry of AAS in humans since 2000, and particularly not since the complex interlinked nature of the redox metabolome has become apparent[35,36]. AAS has been detected in human plasma and is rapidly filtered from the blood by the kidneys and excreted in urine[176].

AAS has been shown to have radical scavenging properties[177,178]. Unlike Asc, AAS does not scavenge radicals rapidly but instead more slowly and continuously in a similar manner to GSH. This longer lasting radical scavenging ability is physiologically relevant as slow reacting radical scavengers can effectively inhibit lipid peroxidation and can work in conjunction with the more rapidly reacting cellular defences to provide both rapid short term defence with longer lasting protection against oxidative damage[178]. AAS has also been found to be able to sulfate cholesterol in rats, though the mechanism by which this occurs has not been determined[179]. Beyond this not much is known about its functions in humans or mammals with most literature, particularly recently, focusing on its biochemistry in fish[180,181].

AAS is far more stable in solution than both Asc and DHA and has been detected in urine samples over two years old[176]. This stability could point towards its biological functions as a storage form of Asc, being more resilient to oxidation, or perhaps as a stable metabolic end product. However it has been demonstrated that orally administered AAS does not increase the excreted levels of Asc suggesting that AAS is not able to be metabolised to Asc in humans raising questions about whether it can be a storage form of Asc[182]. On the other hand human arylsulfatase A, which breaks down sulfatides[183,184], is able to hydrolyse AAS to Asc and sulfate and cultured human fibroblasts were demonstrated to have this activity[184]. These apparently conflicting observations only raise further questions about the biochemistry of AAS in humans. Despite it being known that AAS can both synthesised and metabolised in humans, and that it is unlikely this occurs for no reason, it is unclear whether it forms part of a buffer system, acts as a specific cofactor for an enzyme or some other unknown biological function.

1.6.4 Analysis of the Ascorbate Metabolome

Many analytical techniques have been utilised for the analysis of Asc including titrimetric, spectrophometric, flurometric and amperometric methods[159,185] but many of these methods do not directly quantify DHA. The use of a separation technique is regarded as advantageous, high pressure liquid chromatography (HPLC) and ultrahigh pressure liquid chromatography (UHPLC) are the separation techniques most widely used for the analysis of Asc and DHA[159,186-188].

Chromatographic methods are more accurate and selective than other techniques and generally avoid the need for sample derivatisation or other intensive sample preparation. This reduces the analysis time of samples and helps prevent losses of analytes during sample preparation. Since Asc and DHA are both small polar molecules, they can be difficult to retain using reversed phase columns, which are the most widely used type. This is particularly an issue for complex biological samples due to the large number of matrix components interfering with detection if the Asc and DHA are not sufficiently retained. Reversed phase approaches have been utilised but tend to offer limited separation of the two analytes[188]. In order to obtain any retention they often have to utilise a high percentage of aqueous mobile phase, even up to 100%[189,190], which accelerates column degradation (stationary phase collapse) and can cause loss of peak shape over long sample runs[191]. The addition of an ion pairing agent such as trifluoroacetic acid[192] can improve the retention and separation of Asc and DHA on reversed phase columns and has been utilised widely for the analysis of Asc[193,194]. Ion pairing agents unfortunately can cause multiple issues, particularly with MS detetction, including signal suppression, more difficult gradient elution, and can contaminate the LC and MS systems used affecting other analysis and so are generally avoided on instruments used for different types of analysis[186,192]. Hydrophilic interaction liquid chromatography (HILIC) has also been widely utilised offering polar separation characteristics, which better retain and separate Asc and DHA[186]. HILIC utilises a normal phase stationary phase such as silica with a mainly organic mobile. Despite the better polar separation, HILIC has disadvantages over reversed phase chromatography. These include poor stability requiring long column equilibration times before, during and after analysis, drifting peak times and poor peak shape[195].

A variety of detection techniques have been coupled to LC including ultraviolet (UV), fluorescence detection, electrochemical detection, and mass spectrometry (MS). A large number of existing methods for analysing the ascorbate redox pair do so making use of a subtraction technique, particularly those using UV, electrochemical or MS detection. Each sample is analysed twice, once to measure the levels of free Asc, and then a second time following a reduction step to measure the total Asc with the difference being taken as the levels of free DHA[185,196]. Determining the

levels of DHA in this manner will double the analysis time required and require additional sample preparation leading to potential degradation of samples and inaccuracies in the measured ascorbate redox ratio. The imprecision associated with this method for determining DHA concentrations has led to negative values being measured in some cases[197].

The most common detector type for analysis of Asc and DHA is UV detection[159,185,187,198,199] with a UV absorption maximum for Asc in the range 244-265 nm depending on the mobile phase. However, it is difficult to analyse Asc and DHA simultaneously in a single run using UV detection as DHA has a very low UV absorbance[163]. DHA absorbs UV light at 185 nm, but it has a little absorbance above 220 nm[163], direct analysis of DHA by UV requires derivatisation in order to have sufficient UV absorption. UV detection can lack adequate sensitivity and selectivity in biological matrices for both Asc and DHA due to the presence of many organic molecules in these matrices with similar UV absorbances.

Asc and DHA have no fluorescence properties themselves. Therefore, in order to utilise fluorescence detection DHA has to be derivatised with a fluorophore and Asc first has to be oxidised to DHA and then likewise derivatised[200]. This is a complex multi-step, time-consuming process and introduces potential losses of both DHA and Asc through the irreversible oxidation of DHA to DKGA (**Scheme 11**) and is not widely utilised. Simultaneous determination of AA and DHA using fluorescence detection can be accomplished by post-column on-line derivatisation, for example with O-phenyldiamine[201].

HPLC methods utilising electrochemical detection provide high sensitivity, selectivity and specificity for the analysis of Asc. Asc is relatively reactive and therefore well suited for analysis by electrochemical detection[193,202], DHA on the other hand cannot be directly determined without using the subtraction method discussed earlier as it is electrochemically inactive[188]. Electrochemical detection provides some advantages over UV detection for Asc as it is more selective and sensitive. Unfortunately, it can require very long column equilibration times and conditions can be sensitive to minor changes, DHA cannot be directly analysed either.

Mass spectrometry has been coupled with liquid chromatography in order to overcome these issues but only in a very few methods[172,186,189]. However, simultaneous analysis of Asc and DHA can still be challenging due to matrix effects and the relatively low abundance of DHA. DHA is prone to hydrolyisis and can have a complicated mass spectra depending on the sample conditions[203]. The few methods that have utilised mass spectrometry have done so for fruit and vegetables which can have relatively higher concentrations of DHA than samples such as human plasma[204]. MS detection is not very suitable when ion pairing agents and/or non-volatile buffers have been used in the chromatographic conditions. These components can cause signal

suppression, interference and even block the inlet to the instrument. Due to the difficulties in detecting DHA by MS many of the methods make use of the subtraction method as discussed earlier.

To my knowledge, AAS has never been incorporated into a method for the simultaneous analysis of Asc and DHA only having been measured alongside Asc[182,205] thereby missing out on the important information contained is the ascorbate redox ratio. The majority of methods for the analysis of AAS have made use of mass spectrometry[181], UV[155] or electrochemical detection[206] coupled to liquid chromatography, radioactive labelling has also been utilised for the tracing and detection of AAS[176,183]. These methods however are relatively old and many advances have been made in the selectivity and sensitivity of the separation and detection techniques, especially mass spectrometry. It is likely that the application of modern analytical equipment will be beneficial for the detection and provide detailed analysis of AAS to help increase our understanding of its biological importance.

Asc is highly unstable and readily oxidises to DHA particularly when exposed to oxygen, light, high temperatures, metal ions, alkaline pHs or as a consequence of enzymatic activity[158,207-209]. DHA is even more unstable than Asc[171] and is degraded irreversibly to DKGA. In physiology DHA can be recycled back to Asc but this is not a 100% efficient process and some DHA is inevitably lost irreversibly through oxidation to DKGA (**Scheme 11**, see also section 1.6.2). Even when stored at -80^{o}C Asc is irreversibly lost over a matter of weeks if not otherwise stabilised[210,211]. Many different methods have been utilised to try to prevent this loss of Asc and DHA after sample collection, including the addition of meta-phosphoric acid (MPA) to precipitate the proteins and acidify the samples, addition of various metal chelators to block metal ion catalysed oxidation and storage at a variety of different temperatures. Whilst there is agreement in the literature that acidification with MPA is the best method for protein removal and Asc stability there is less agreement on whether further steps are required or not. The type of blood tube use for sample collection is under debate with some groups reporting that EDTA (ethylenediamine tetraacetic acid) tubes are the best for the collection of whole blood[159,204,207] whilst other groups report that heparin tubes are more appropriate[158,171]. Some papers state that use of MPA is sufficient for Asc/DHA stability, whilst others propose that addition of a metal chelator such as EDTA or DTPA is required[204].

1.7 The NO Metabolome – Arginine and Related Metabolites

Small polar metabolites such as arginine, citrulline, ornithine, methionine, proline and glycine all play an important role in the wide reaching, interlinked NO metabolome. Arginine is the major source of NO production by conversion to citrulline by three enzymes, neuronal, inducible and

endothelial nitric oxide synthase (nNOS, iNOS and eNOS)[212]. Arginine is the key component in these cycles and undergoes many other metabolic fates, it is involved in the urea cycle[213] as well as being utilised in peptide and protein synthesis, and as a precursor to ornithine and homoarginine[214] (**Figure 2**). To illustrate the complex nature of this metabolome it should be highlighted that citrulline is also produced from arginine as part of the urea cycle via ornithine with the release of urea instead of NO. This reaction can be inhibited by homoarginine, which is also produced from arginine by arginine-glycine amidinotransferase (**Scheme 12**). Tracing the fluxes through these pathways is challenging due to the wide number of metabolites involved and the multiple linked pathways.

Unlike for the sulfide metabolome there is a much wider range of methods available for the analysis of arginine and its related metabolites in the NO metabolome[22,215-220]. Although there are some limitations in current methodologies[220] the field is much more mature, and whilst further development would be beneficial, analytical development for the sulfide metabolome will be of greater impact. This is one of the reasons why the initial development was focused on the sulfide metabolome including on areas of crosstalk with NO/arginine metabolism. New developments in the analysis of sulfide metabolism can be combined with the existing NO/arginine methodologies and stable isotope tracing to help elucidate the interactions between these two key redox systems in addition to the direction and flux of metabolites though these pathways.

1.7.1 Nitric oxide

Nitric oxide (NO) is a small gaseous molecule which is produced endogenously as a signalling agent[221] (gasotransmitter). The production of NO is largely from the metabolism of arginine by nitric oxide synthase (NOS) enzymes (**Scheme 12**), which can be inhibited by methylated arginines produced during protein turnover[222] (**Scheme 13**). It has many important biological effects[113], including cardiovascular regulation[223], neurotransmission[224] and adhesion of platelets and leucocytes[225,226]. NO has been found to be involved in numerous diseases and inflammatory conditions. The biological properties of NO have been the subject of some confusion with seemingly contradictory effects, for example NO is able to render cells susceptible to cytotoxic agents whilst also exhibiting protective effects including protecting cells from damage caused by oxidative stress. NO can act as an antioxidant, being able to react with and neutralise reactive oxygen species that can cause oxidative stress, whilst also being able to react with oxygen and generate a variety of reactive intermediates that can in turn cause cellular damage[227,228].

Similarly to H_2S, NO is also able to stimulate the enzyme γ-glutamylcysteine synthetase as well as γ-glutamyl transpeptidase in order to increase the synthesis of glutathione as a response to

glutathione depletion by oxidative stress[229]. The uptake of extracellular cystine into cells can be stimulated by NO leading to an increase in the bioavailability of cellular cysteine used for glutathione and H_2S synthesis[230].

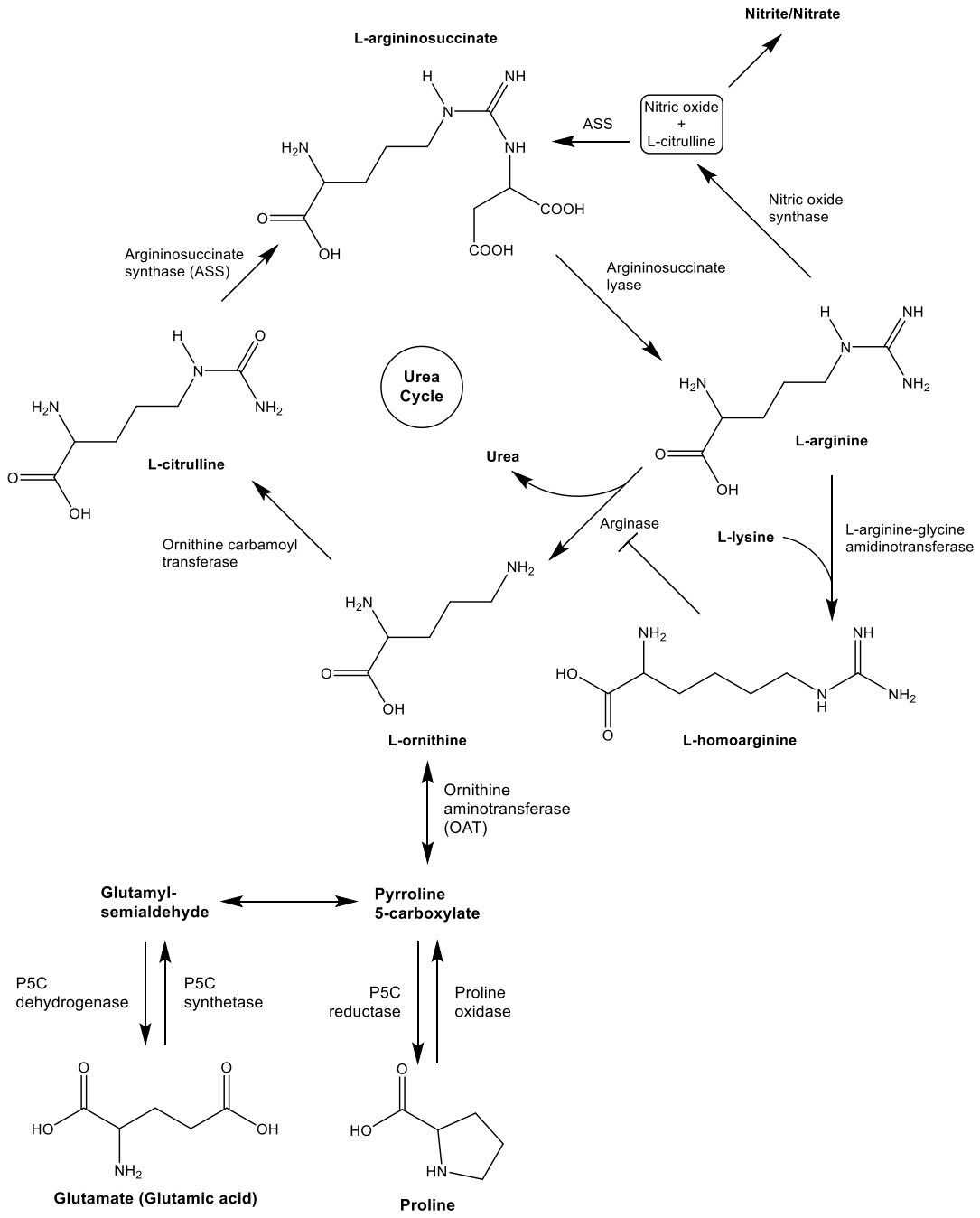
Nitrite (NO_2^-) and nitrate (NO_3^-) form the endpoint of NO metabolism as well as being obtained through the diet. NO is rapidly oxidised to nitrite and nitrate in biological systems, this oxidation can be enhanced in circulation by the multicopper oxidase, ceruloplasmin. The predominant NO oxidation product in plasma is nitrate with micromolar concentrations at least two orders of magnitude higher than nitrite. In circulation, the half-lives of nitrate and nitrite are approximately 6 hours and 20 min respectively, nitrate and nitrite are excreted in urine and can be used as a measure of NO production[231,232]. However nitrate and nitrite can also be reduced and recycled back to NO by several pathways including by haemoglobin acting as a nitrite reductase as wells as NOSs and other enzymes[233]. This provides a pathway by which NO can be produced independent of arginine by reduction of nitrate and nitrite obtained through the diet.

1.7.2 Arginine Synthesis and the Urea Cycle

Arginine is a semi-essential amino acid with additional arginine only being required in the diet during times of maximal growth or illness such as in growth of an infant and recovery from serious injuries[234]. As well as dietary sources, arginine can be biosynthesised from both glutamate and proline[234] (glutamate also being involved in the synthesis of glutathione from cysteine,

Scheme 2) through synthesis into ornithine then into citrulline. The circulating citrulline is taken up by cells and then converted into arginine via arginosuccinate as part of the metabolic pathway of the urea cycle (**Scheme 12**).

The first step of the urea cycle, the conversion of arginine into ornithine with the release of urea can be inhibited by homoarginine. This inhibition is due to the structural similarities between arginine and homoarginine with the only difference between the two being an extra methyl group in the main carbon chain of homoarginine. It is synthesised from the reaction of arginine with lysine by the enzyme L-arginine-glycine amidinotransferase (AGAT) (**Scheme 12**). This reaction is regarded as a side reaction of AGAT with the main activity of the enzyme being the reaction of arginine with glycine in the first step of the formation of creatinine[235]. The ability of homoarginine to inhibit the activity of arginase may play a beneficial role as it increases the bioavailability of arginine for other metabolic processes[216].



Scheme 12: Metabolic pathways of arginine – the urea cycle, NO production, and synthesis of homoarginine.

1.7.3 Production of NO

Arginine is the major source of NO (**Scheme 12**), NO is produced by oxidation of arginine to citrulline and is catalysed by three enzymes; neuronal, inducible and endothelial nitric oxide synthase (nNOS, iNOS and eNOS). nNOS is expressed almost entirely in central and peripheral neurons, it produces NO in these cells in order to effect synaptic plasticity, smooth muscle relaxation, blood pressure regulation and other NO related functions. iNOS is expressed in many cell types and produces NO in response to factors such as oxidative stress[236] and the presence of

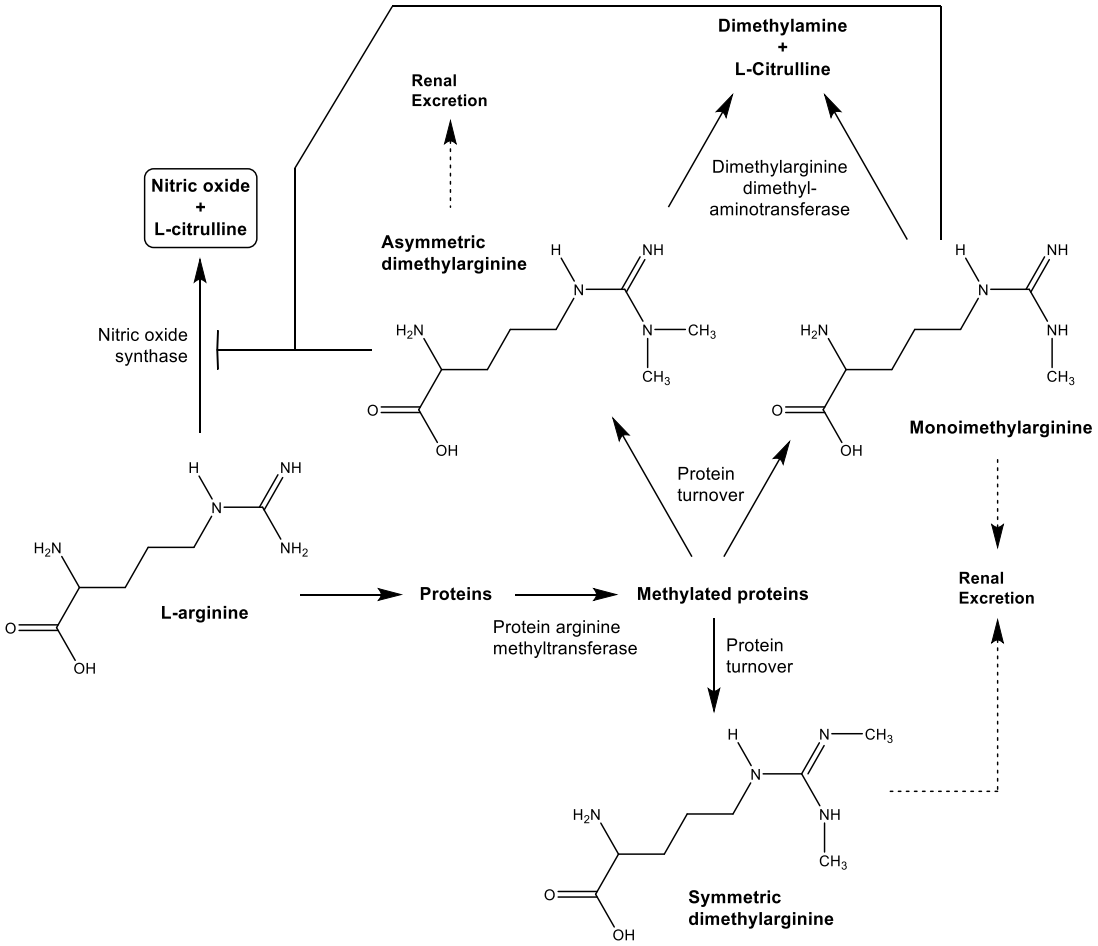
cytokines, iNOS is a major factor in the production of NO in response to disease and inflammation. eNOS is expressed mainly in endothelial cells where it is heavily involved in the production of NO to control blood vessel dilation and blood pressure[237]. However, only a very small amount of arginine is oxidised to produce NO, about 0.2% of endogenous arginine[238] though this is obviously a sufficient amount for normal biological functions. The citrulline produced by NOS can be recycled back to arginine via transamination in a process known as the citrulline/NO or arginine/citrulline cycle, though this is much less efficient than the hepatic urea cycle[41].

Since homoarginine is structurally similar to arginine, it can act as a substrate for NOS instead of arginine for the production of NO. However, despite not being a major source of NO compared to arginine, homoarginine does not appear to have a major impact on NO production as it is much lower in concentration and has a much lower conversion rate into NO by NOS than arginine[239]. In fact, as homoarginine can increase the bioavailability of arginine, lowered levels of homoarginine are associated with increased negative outcomes in cardiovascular disease due to overall decreased NO production[240].

1.7.4 Arginine Methylation

Arginine is a major component in the synthesis of proteins, a proportion of the arginine residues that have been incorporated into proteins are then post-translationally methylated by protein arginine methyltransferase enzymes. This process utilises the transmethylation pathway in the methionine cycle with the conversion of S-adenosyl-methionine to S-adenosylhomocysteine (**Scheme 4**) by SAM methyltransferase providing the required methyl group. During protein turnover these methylated arginine residues are liberated becoming the free methylated arginines; monomethylarginine (MMA), asymmetric-dimethylarginine (ADMA) and symmetric-dimethylarginine (SDMA) (**Scheme 13**).

Asymmetric-dimethylarginine is a competitive inhibitor of NOS[222] therefore interfering with the production of NO from arginine. Due to the reduced production of NO elevated plasma levels of ADMA as well as the Arg/ADMA ratio can be used as strong prognostic markers for many different vascular diseases such as hypertension, atherosclerosis and congestive heart failure[241]. The levels of ADMA are regulated by the dimethylarginine dimethylaminohydrolase family of enzymes that hydrolyse the majority of ADMA (around 80%) into citrulline and dimethylamine (**Scheme 13**) which can feed back into arginine synthesis. ADMA in a lesser extent is also excreted via the kidneys[216].

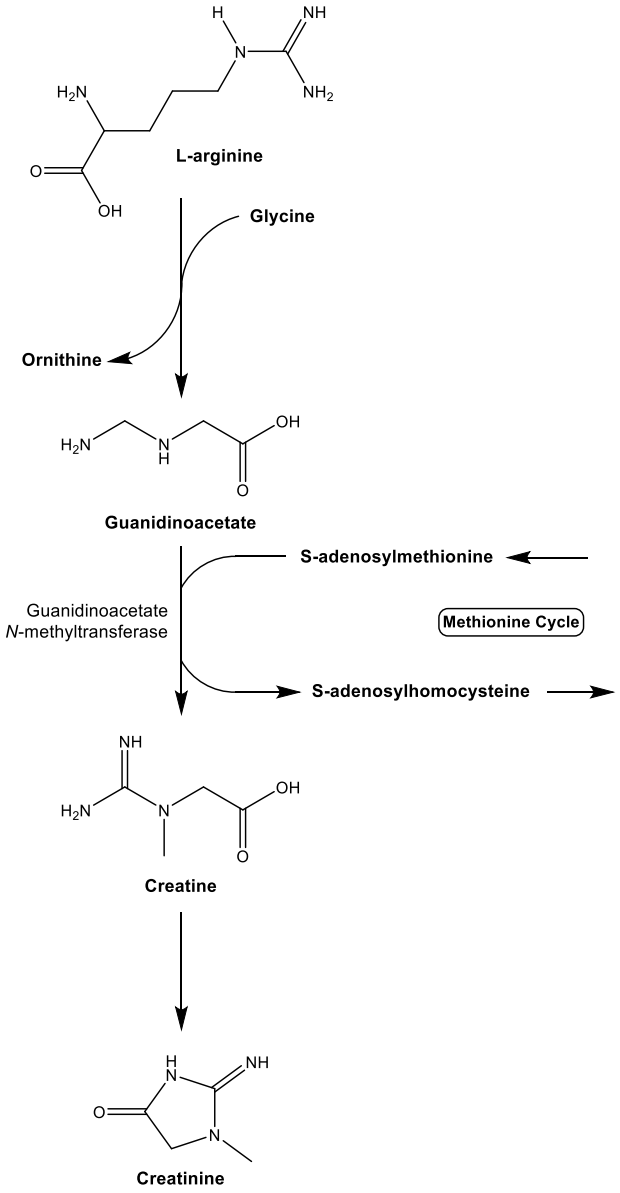


Scheme 13: Methylation of arginine and its further metabolic pathways.

Monomethylarginine (MMA) undergoes many of the same pathways as ADMA, it is also able to inhibit NOS and be metabolised into citrulline and dimethylamine by the same enzymes, however it has a much lower concentration than ADMA, by about 5-10 fold. Therefore, whilst it has many of the same biological functions it is often not used as a cardiovascular marker as it does not show any additional information but is more challenging to analyse than ADMA[242].

Symmetric- dimethylarginine (SDMA) on the other hand does not show any ability to inhibit NOS or the production of NO, neither is it metabolised by the dimethylarginine dimethylamino-hydrolase enzymes. SDMA in fact is mainly excreted unchanged by the kidneys and can be used as a marker for impaired glomerular filtration rate and the early stages of kidney disease[243] but has no direct impact on further arginine metabolism.

1.7.5 Creatine and Creatinine



Scheme 14: The arginine-creatine pathway and its interaction with the methionine cycle (**Scheme 4**).

Large amounts of arginine are metabolised into creatine involving one carbon metabolism and the methionine cycle with the methylation of guanidinoacetate to creatine, (**Scheme 14**). This provides another important link between the NO and H_2S metabolomes (discussed in 1.3.7). In fact the formation of creatine consumes more methyl groups than all other methylation reactions acting as a regulatory step for the availability of methyl groups for other methylation reactions including the synthesis of methionine from homocysteine[244] (**Scheme 4**). Through this mechanism, arginine can indirectly have an impact on one carbon metabolism and even redox regulation by the thiols. The majority of creatine (up to 94%) is found in muscular tissues, creatine synthesis however mainly takes place in the kidneys and liver. Muscle has little or no creatine synthesising

ability so all creatine found in muscle tissue is taken up from the blood after synthesis or dietary intake. Creatine has been shown to have antioxidant functionality itself[245], as well as being able to reduce inflammatory responses[246] and improve glucose tolerance[247]. These benefits have been attributed to the ability of creatine to buffer cellular ATP levels, which if not buffered could decrease and lead to the formation of reactive oxygen species[248].

Muscular creatine is nonenzymatically degraded to creatinine at a steady rate of ~2% of total creatine per day in healthy individuals. Creatinine is widely seen as a metabolically inert substance in humans that diffuses out of the cells and is simply excreted in urine without many further biological actions. However, there is some evidence to suggest creatinine can be recycled back into creatine, arginine, guanidinobutyrate, or guanidinopropionate especially during times of renal insufficiency[249]. Creatinine can be used as a marker of the arginine to creatine pathway due to its high excretion and relatively little recycling. Since creatinine is produced from muscle at a steady rate, and there is little or no reabsorption it can be used as a marker for skeletal muscle mass in healthy individuals as well as individuals with chronic kidney disease[250].

1.8 Crosstalk

There are significant interactions between the NO and H_2S metabolomes, known as crosstalk. These interactions can take the form of direct interaction between NO and H_2S forming S/N-hybrid species (**Figure 1**) as well as less direct interactions such as through methionine and homocysteine and the transmethylation pathway. The function of the key enzyme cysteine-β-synthase (CBS) in the transsulfuration pathway (**Scheme 4**) is in fact regulated by NO, with its expression being enhanced by oxidative stress as well as in response to substrate levels[75]. Conversely H_2S can activate eNOS to facilitate the production of NO with some of the cardioprotective effect of H_2S being mediated by this related NO release[251].

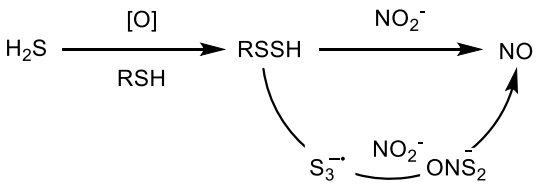
The exact nature of the interactions between NO and H_2S have proven hard to track down due to the highly reactive and unstable nature of some of the interaction products and intermediates such as persulfides and polysulfides. It is clear that both the NO and H_2S metabolomes are closely interwoven presenting both directly linked and discreet pathways. The actions of either H_2S or NO cannot be considered or studied in isolation with the chemistries of either species modulating each other and their interactions producing key hybrid species with unique and distinct bioactivities.

1.8.1 Methylation

The methionine cycle, or transmethylation cycle, provides a point of crosstalk between the two metabolomes. Not only is it responsible for the methylation of protein arginine residues (**Figure 2**) but it also forms a key step in the synthesis of creatine from arginine (**Scheme 14**). The methylation of arginine residues in proteins is another point of crosstalk, relying on the actions of the methionine cycle to provide the methyl groups required. Methylated arginine's have important regulatory actions in the arginine cycle with asymmetric-dimethylarginine (ADMA) competitively inhibiting the production of NO by NOS[222], the actions of the methionine cycle not only affect the cysteine metabolome but also feeds into the regulation of NO production and the bioavailability or arginine. The synthesis of creatine has a direct impact on the bioavailability of both arginine and the methyl groups used for the synthesis of methionine from homocysteine and methylated arginines[244].

1.8.2 Direct NO/H_2S Interaction

NO and H_2S are able to directly interact with sulfide levels directly linked to NO bioavailability[15]. This interaction forms key bioactive reaction products such as persulfides, polysulfides, S/N-hybrid species and nitroxyl (**Figure 1**). These species exhibit distinct chemistries and biological interactions. Nitropersulfide ($SSNO^-$) is resistant to thiols and cyanolysis, and is both a sulfane sulfur and NO donor, as well as potently lowering blood pressure. Dinitrososulfite (SULFI/NO) is also a combined donor but for NO and nitroxyl, releasing mainly N_2O on decomposition, and has less of an impact on blood pressure[10]. The nature of these interactions is still not clear due to the highly reactive and unstable nature of these species with other interaction products such as nitrosodisulfide $[S_2NO]^-$ proposed[252]. It is still not clear if signalling effects involved in vascular control by crosstalk products are due to the actions of $SSNO^-$, NO^- or in fact due to the production of polysulfides[55].



Scheme 15: The production of NO from nitrite via polysulfide formation[13].

Persulfides are able to react with nitrite to generate NO independent of arginine with polysulfides as an intermediary species (**Scheme 15**) with persulfide themselves being generated through the reaction of sulfide with thiols[13]. Persulfides and polysulfides have important chemistries themselves, being potential stores of sulfide and possessing strong antioxidant properties. It has

become apparent that polysulfides are at least in part responsible for many of the biological activities previously ascribed to H_2S, and have greater potency as regulatory molecules. This suggests that the crosstalk between H_2S and NO is multifaceted, with not just the products of this crosstalk playing important physiological roles but also the intermediary compounds.

1.8.1 Antioxidant Enzymes and Reactive Sulfur Species

It has been found that there are a number of similarities between reactive oxygen species (ROS) and reactive sulfur species (RSS)[3,253]. With the importance of RSS becoming more apparent, these similarities are raising questions over the role of RSS compared to ROS and it has been shown that in some cases systems regulated by H_2O_2 have identical responses to polysulfides (H_2S_n, where n = 2-5)[36,254]. In fact, many of the methods currently used to detect ROS such as those based on fluorescent probes or amperometric electrodes are unable to distinguish between ROS and RSS[253]. These findings suggest that RSS may be, at least in part, responsible for some of the functions that have previously been attributed to ROS.

The enzymes catalase and superoxide dismutase (SOD), which were previously thought to solely be involved in the metabolism of ROS have recently been found to have significant biological activities involving RSS. Catalase catalyses peroxide dismutation to oxygen and water as part of cellular defence against oxidative damage but has been shown that it can oxidise both sulfide and polysulfides using oxygen and H_2O_2[137]. SOD is another key antioxidant enzyme which catalyses the dismutation of superoxide to oxygen and peroxide[255]. SOD works in conjunction with catalase as it produces peroxide, which can still have damaging effects, which is then metabolised into oxygen and water by catalase.

These enzymes make up a new area of crosstalk in the reactive species interactome between ROS and RSS. As opposed to direct interaction of the two groups of species, the metabolism of ROS and RSS by the same enzymes makes up a more indirect point of crosstalk, with changes in one of the metabolomes potentially impacting the mediating enzymes and thereby affecting the other metabolome. These links further highlight the complex interlinked nature of the reactive species interactiome[36] and open up new potentially important biological activities of widely known antioxidant enzymes. It is likely that catalase and SOD are not the only antioxidant enzymes that have biological activities involving RSS as well as ROS. These enzymes as well as thioredoxin and peroxiredoxin, all appear to have evolved long before the presence of oxygen in the atmosphere[138], and so before the need to deal with damaging oxygen species. This suggests that they performed important biological functions with substrates other than ROS before adapting to the presence of oxygen.

1.8.2 Ascorbate and Sulfur Metabolism

Reduced glutathione and ascorbic acid are two powerful antioxidants and essential for the maintenance of redox balance. These two antioxidant compounds have separate redox mechanisms whilst also being closely interlinked, both directly and through enzyme mediated interactions. There is a strong correlation between GSH and Asc levels in cells, when one is depleted so is the other and vice versa[256]. As Asc is oxidised to the ascorbate free radical (AFR) and DHA during the process of protecting cells from damage there is a need for a recycling mechanism to regenerate the levels of Asc or it will be irreversibly lost (see section 1.6.2). One of these mechanisms is the direct reduction of AFR and DHA by GSH producing Asc and GSSG. The GSSG can then itself be reduced by other mechanisms to regenerate GSH levels[65,169]. DHA is also recycled back to Asc through a variety of enzymatic processes (see section 1.6.2). The most important enzymes in these processes are the glutathione dependant reductases[163,167] (**Figure 3**). This enzymatic pathway makes up an indirect crosstalk between GSH and Asc, and predominantly functions when GSH levels are normal. If GSH levels become depleted then alternative enzymes become the preferred Asc regeneration pathways[164]. This crosstalk provides regulation of the levels of both of these important antioxidants. Glutathione – ascorbate crosstalk (**Figure 3**) once again illustrates that the different redox cycles cannot be considered as discreet separate mechanisms but must be taken as part of the wider interlinked redox interactome and that in order to gain a full understanding of redox regulation a wider approach to analysis must be taken[36].

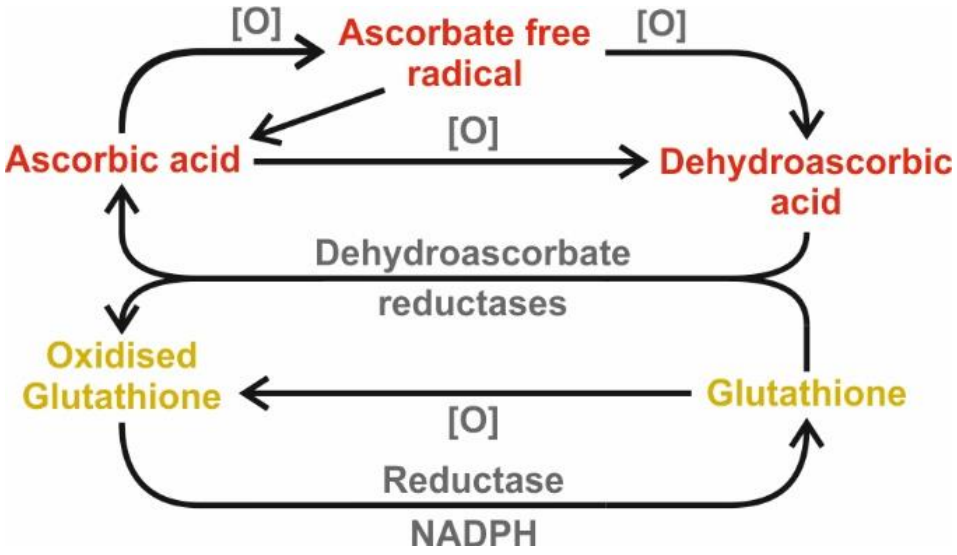


Figure 3: Recycling of DHA by GSH dependant processes resulting in regeneration of Asc and production of GSSG. The GSSG can be recycled back to GSH by other processes including by NADPH dependant reductases. [O] = oxidants, including reactive oxygen, nitrogen, and sulfur species.

There is also crosstalk between the sulfide and ascorbate metabolomes through the interaction of Asc and sulfate. Sulfate can be formed through oxidation of sulfide (**Scheme 1**) and is also obtained through the diet[57,155]. Sulfate can be considered as an endpoint to sulfide metabolism, where it is either excreted or utilised in sulfonation[58], one such reaction being the sulfonation of Asc, producing AAS[155]. As discussed (section 1.6.3), there is not much known about AAS in human physiology, but as it is much more stable than Asc and DHA it could make up a storage form for these antioxidants. It could also be a longer acting radical scavenger[177,178] or even have sulfonation reactions of its own in conjunction with sulfate[179]. It is clear that AAS can have biological activities combining those of Asc and sulfate whilst potentially having distinct redox activities. This crosstalk has not yet been widely investigated but could have wider ramifications within the redox network.

1.9 Liquid Chromatography and Mass Spectrometry

Ultrahigh-performance liquid chromatography tandem mass spectrometry (UHPLC-MS/MS) is a highly versatile and powerful analytical technique coupling separation by liquid chromatography with the mass analysis capabilities of mass spectrometry. UHPLC works on the same principles as high pressure liquid chromatography (HPLC) using a liquid mobile phase with a stationary phase to perform the separation. UHPLC utilises a smaller stationary-phase particle size, typically 1.7 μm, which results in much greater separation efficiency providing a large increase in resolution, speed and sensitivity[257]. In order to analyse complex biological samples such as plasma, urine and red blood cells a separation technique is required to prevent suppression of the analytes of interest by other compounds present in the sample matrix, and allow conclusive identification and quantification.

Electrospray ionisation (ESI) is a popular method of coupling UHPLC to a mass spectrometer. ESI-MS provides sensitive, robust, and reliable tool for studying, at low concentrations in low/limited volume samples, non-volatile and thermally labile bio-molecules that are not amenable to analysis by other conventional techniques[258]. ESI utilises a high voltage applied to a capillary sprayer to aerosolise the mobile phase as it elutes from the column producing charged droplets. These droplets are subjected to a drying gas flow and are continuously reduced in size by solvent evaporation resulting in a concentration of charge leading to Coulombic explosion of the droplets producing many smaller droplets eventually leading to the production of gas phase ions allowing detection in the mass spectrometer[259,260]. ESI is a soft ionisation technique producing very little fragment ions during the ionisation process leading to majority precursor mass +1 (positive ionisation, M+1) or precursor mass -1 (negative ionisation, M-1) ions[261]. This provides little in the way of structural information for the molecules of interest, in order to obtain

more information about the molecules, tandem mass spectrometry (also known as triple quadrupole) is used. By fragmenting the precursor ions, much greater structural information is provided on top of the precursor masses. This allows monitoring of specific precursor-product masses known as multiple reaction monitoring (MRM) providing both a highly selective and sensitive analysis. By coupling the fast and sensitive separation abilities of UHPLC with the specificity and sensitivity of a triple quadrupole mass spectrometer with ESI complex biological mixtures are able to be analysed with minimal sample preparation and analysis time and these techniques are widely utilised in clinical settings[217,258,262].

Supercritical fluid chromatography (SFC) is an emerging separation technique that works on the same principles as UHPLC. Whilst SFC has been practised for 50 years it is only recently that equipment has become robust and reliable enough for routine use, with modern techniques being termed ultra-high performance supercritical fluid chromatography (UHPSFC). The main difference between the two systems lies in the mobile phases used; UHPLC typically makes use of a combination of two liquid mobile phases typically comprised of an aqueous or more polar phase combined with an organic or more apolar phase. UHPSFC uses one mobile phase comprised of a supercritical fluid, typically CO_2, combined with a polar, mainly organic solvent mobile phase such as methanol. Supercritical fluids have the properties of both liquid and gaseous phases; CO_2 is commonly used due to its relatively low critical point, only requiring a pressure of 7.38 MPA and a temperature of 31.1^{o}C to reach its critical point[263]. UHPSFC offers a good alternative to conventional normal phase liquid chromatography, providing more robust and reproducible chromatography. Since the mobile phase can easily switch from apolar CO_2 to increasing amounts of polar co-solvent such as methanol compounds with a wide range of polarities can be efficiently separated on a single column in a single analysis[264]. Whilst UHPSFC can potentially offer a faster, higher resolution and more robust separation than some comparable UHPLC methods it is not a straight upgrade, rather the two techniques can be seen as being complementary, each with their own advantages and disadvantages[265].

1.10 Use of Stable Isotopes

Stable isotope standards are commonly used to enable accurate quantification of analytes measured using mass spectrometry. As the absolute peak areas retuned by UHPLC-MS/MS can vary day to day due to a number of factors including the temperature and pressure of the ion source and the performance of the ion source[266]. The use of internal standards is essential to be able to accurately quantify samples and to allow comparison between different analyses. Internal standards are used to correct for variability in dilutions, evaporation, degradation, recovery, adsorption, derivatisation, and instrumental parameters. In a biological matrix there is also the

potential issue of ion suppression which may cause incomplete ionisation of the analyte of interest and is another uncontrollable variable[267]. Since a stable isotope labelled version of the analyte(s) of interest has the same overall structure but a higher mass it should behave similarly and ionise with the same efficiency and so give an equivalent response whilst being distinguishable by its mass.

Stable isotope tracers have also been widely used in the NO metabolome to measure the direction of travel and rate of substrates, metabolites and products[268] as well as the activity of the enzymes responsible[269]. The first evidence that arginine was a precursor for NO was obtained using ^{15}N-labeled arginine with the labelled guanidine nitrogen found to be incorporated into ^{15}N-labeled nitrate (NO_3^-) in macrophages[270]. ^{15}N is widely used as a stable isotope tracer for the NO metabolome, with ^{13}C and ^{18}O also being used[213,271,272]. Different parts of the arginine-NO-nitrate pathway can be targeted using stable isotope tracing by monitoring the conversion of arginine into either citrulline or NO_3^- (**Scheme 12**)[215]. The production of NO from arginine can be estimated, for example, by using $^{15}N_4$ labelled arginine as a stable isotope tracer and monitoring the levels of $^{15}N_3$-citrulline and $^{15}N_3$-arginine[269]. The use of nitrite and nitrate as NO metabolism endpoints in stable isotope tracing is complicated by dietary nitrite and nitrate as well as the nitrate-nitrite-NO reduction pathway[233].

The methionine cycle (one carbon metabolism) is another metabolic pathway that has been characterised using stable isotope tracing[273], and forms an important link between the NO and H_2S metabolomes (**Figure 2**, **Scheme 4**). The movement of the one-carbon units has numerous important effects in biology tracing the direction and flux of these one-carbon units is important to understand better how numerous biological pathways are interconnected, and how one metabolite can influence the function of numerous interconnected pathways[274]. For example, dual labelled $^{13}C^{15}N$ serine and methionine has been used for the quantification of the one-carbon contribution from extracellular serine and methionine onto DNA and RNA in the crosstalk between cellular metabolism and the epigenome[275].

Stable isotope techniques have not yet been widely applied to the H_2S metabolome as a means to trace the fluxes and enzyme activities throughout its pathways. The fate, direction and overall importance of the different substrates in human biology has not been well established so far[10]. Sulfide metabolism could also make use of ^{15}N, ^{13}C and ^{18}O as well as ^{34}S, which would be a powerful and unique tracer for the fate of sulfur throughout the redox system. As metabolic pathways are often closely interlinked, a single metabolite will often have multiple biological actions. The use of multi-labelled compounds allows the tracing of these different actions, potentially allowing a powerful method that can trace the flux of metabolites through multiple

pathways and how this changes in response to factors such as oxidative stress. Furthermore the interactions between the NO and H_2S pathways have only just started to be explored and would likely benefit from the use of stable isotope tracers. Sulfate (1.3.2) provides a potential target to monitor for stable isotope tracing of the sulfide metabolome. By using a ^{34}S labelled tracer such as methionine or cysteine sulfate would provide an accessible endpoint to sulfide metabolism, as it is readily excreted, and allow the tracing of the flux of sulfur through the sulfide metabolome. However, not only would dietary intake of sulfate have to be considered but also the recycling pathways that would complicate the tracing of ^{34}S, complicating matters.

Using stable isotope tracers the flux through the metabolic pathways will be important to gain a better understanding of how metabolic pathways operate and interact with each other. It is becoming increasingly apparent that these pathways cannot be treated as if they operate in isolation, but are linked to the whole metabolome.

1.11 Summary

There is a large body of evidence for the biological significance of low molecular weight (LMW) thiols and sulfide[93,103,276]. In addition to sulfide, the polysulfides (both organic and inorganic) have emerged as important redox and signalling species[117,132,143,277]. The general biochemistry of these species is well known, their uptake into cells, their transport between bodily tissues and their excretion or metabolism. Yet there is a lack of understanding of how these species are involved in the redox network and their response to oxidative stress. Whilst LMW thiols and sulfide all form part of an interconnected metabolome, the redox status of one thiol pair does not necessarily reflect the redox status of the wider network[34]. There are also close links between glutathione and ascorbic acid, which both form important redox couples each performing different roles but also working in conjunction with each other[65,163,166,256]. There is emerging evidence for the importance of these small thiol redox pairs with strong links between total free thiol levels and mortality[45], with total levels of free thiols strongly associated with other markers of heart failure severity and prognosis. Lower levels of free thiols are clearly associated with a worse outcome in heart failure[46] and potentially in other oxidative stress related diseases, as lower free thiol levels have been recorded in these diseases[47]. However, the methods used to analyse total free thiol levels are non-specific and so questions remain about the contribution of individual thiol redox pairs or species to these diseases[46]. Physiological levels of inorganic and organic polysulfides are poorly characterised, in part due to the difficulty in analysing these species, whilst it is know that they are present and have important biological effects the specifics of these are still unclear[143]. New biochemistries involving polysulfides are emerging including their metabolism by antioxidant enzymes that were previously only associated with reactive oxygen species[137,138]. The biological

importance and activities of Asc and DHA (otherwise known collectively as vitamin C) are well known, but due to their high instability accurate quantification of the physiological concentrations of Asc and DHA has proven to be challenging[171,187,190,196].

Current methods used to analyse these redox networks are often limited in some aspect, in either specificity, sensitivity or scope. Reported levels of LMW free thiols, the ratios between reduced and oxidised thiols and sulfide concentrations can vary depending on the methodology used to quantify them. Reported concentrations of free sulfide can range from nanomolar to hundreds of micromolar[14] depending on the method used for analysis. This wide range in reported concentrations may be due to the liberation of sulfide from carrier molecules such as persulfides, polysulfides and thiosulfate[18,19] during sample preparation, with the higher range of concentrations unlikely to be free sulfide in blood[20,21]. Quantification of free oxidised LMW thiols by reduction[278,279] is flawed as the reduction process also liberates LMW thiols bound to proteins and contained in mixed thiol disulfides. The collection and handling of samples before analysis can also have a drastic impact on the levels of thiols and sulfide measured. Reduced thiols are readily oxidised, skewing the redox ratio of thiol pairs away from the in vivo values whilst oxidised thiols can potentially react with proteins[63,280] or degraded enzymatically[44]. These issues are also present in the analysis of the ascorbate redox pair, with both Asc and DHA readily degrading at physiological pH[171,187,190,196]. Analysis of organic and inorganic polysulfides can also be problematic with existing methods either being limited in the information they provide or potentially altering the speciation of polysulfides during the sample preparation[143].

Treatment of oxidative stress related conditions with antioxidants has so far proven to be largely ineffective. This suggests that the current understanding of these processes is incomplete which could be due in part to the complexity of the redox network[30]. Sulfur redox metabolism does not operate in isolation. There are close links between the sulfur redox metabolome, oxygen redox metabolome, nitrogen redox metabolome, and ascorbate metabolism. Changes to one part of the system can have effects that spread throughout the whole network. On the other hand, the redox status of one pathway is not necessarily reflected by the other pathways. This in part may help explain why so far seemingly obvious methods of treatment have been ineffective.

The aim of the thesis was to develop new methodology that will allow better characterisation of the low molecular weight free thiols involved in redox regulation as well as sulfide, also an important redox regulator, by developing a method that is sensitive, specific and encompasses the full range of LMW free thiols and sulfide involved in redox regulation. This method development includes methods for the analysis of polysulfides, which are closely linked to sulfide and the thiols. I also aim to develop methodology to help characterise the ascorbate metabolome

with a focus on the simultaneous analysis of Asc and DHA and on appropriate sample collection, handling and processing conditions to avoid artefactual losses. I will also be incorporating the analysis of ascorbic acid-2-sulfate, a metabolite of Asc that has not been widely investigated in humans. With a better understanding of how these pathways operate and interact, their role in redox related disorders can be better understood and potentially lead to better therapies to combat redox related disease.

Chapter 2: Thesis Hypothesis and Overview

2.1 Thesis Hypothesis

The primary hypothesis for my project is as follows:

An imbalance between sulfur intake and handling in intermediary metabolism is associated with a disruption of redox mechanisms and unfavourable outcomes in diseases associated with oxidative stress.

Clarification of hypothesis identifying metabolic areas of interest related to the hypothesis:

Sulfur intake occurs mainly through S- containing amino acids as well as inorganic sulfate. The intermediary metabolism of sulfur involves the methionine recycling and transsulfuration pathways with ultimately sulfur being excreted via sulfate, a stable oxidation product of sulfide.

Research question to enable the hypothesis to be answered:

Low molecular weight (LMW) free thiol redox pairs, intermediary thiol metabolites, sulfide and polysulfides can be used as markers of redox status as well as other redox pairs linked to thiol metabolism such as ascorbic acid and dehydroascorbic acid.

2.2 Approach

Initial development was focused on the analysis of the sulfide redox metabolome. This area of metabolism has not yet been fully characterised and lacked the appropriate analytical methods and understanding to do so. Conversely, the NO/arginine metabolome is much better understood and there are a large number of existing methods that can be used for its analysis. Therefore, the initial method development focused on the sulfide metabolome as this is the area in which the biggest impact could be made and any newly developed methods could be used in conjunction with the existing methods for the NO/arginine metabolome to examine the crosstalk between them. Method development was initially performed using standard mixtures before moving on to the more complex biological samples, with the focus being on plasma. The sample collection, processing and handling proved to be just as important, if not more, as the analysis method itself. With differences such as the blood tube used, or the time taken to centrifuge the whole blood after addition of NEM, having large impacts on the measured species. These differences were not only important to understand to establish the best conditions for the samples, but also to understand what effect they would have on the measured species. Optimally prepared samples

may not always be available for analysis, with the sample preparation demands of other analysis for studies having precedence or the desire to analyse existing samples in storage that were not collected with this analysis initially in mind. By understanding the limitations of non-ideal sample collection, processing and handling analysis of these samples can still provide useful information and further insight into thiol and sulfide metabolism.

The analysis of thiols and sulfide lead onto other recently identified biologically relevant sulfur species, the per- and polysulfides. Analysis was performed making use of the methods developed for the analysis of thiols and sulfide and making use of derivatisation using two electrophiles, NEM and IAM. The conditions used to analyse the polysulfides can have a huge impact on the distribution of species that are measured, particularly the choice of electrophile. Despite the difficulty in accurately analysing the polysulfide speciation the methods used can still provide useful information on the biological activities of per- and polysulfides. To this end, the metabolism of sulfide and production of polysulfides by the antioxidant enzyme SOD was measured in detail confirming this unexpected metabolic activity and providing more detailed information.

The use of NEM for the derivatisation of the reduced thiols and sulfide as well as for polysulfides alongside IAM lead to questions on the rates of reaction of each electrophile. The rate of reaction is particularly important as the longer a complete reaction takes the more the concentrations of thiols and sulfide can be altered from physiological levels through oxidation, enzymatic activity and other processes. It will also have an impact on the measured speciation of polysulfides where the rate of interconversion between the species is rapid and so the slower the rate of reaction the more opportunity there will be for the speciation changing from the physiological distribution. MBB was also compared as it is currently the gold standard for analysis of sulfide and has been widely utilised for analysis of thiols.

Ascorbate redox metabolism is closely linked with glutathione metabolism along with potential important crosstalk between ascorbic acid and sulfate. The ascorbate metabolome has less active components to analyse, which should facilitate easier and quicker method development with less components to optimise. Whilst ascorbic acid (Asc) and dehydroascorbic acid (DHA) have been widely measured, ascorbic acid-2-sulfate (AAS) has not widely been analysed in humans using modern analytical techniques. Many existing methods do not directly measure DHA but instead rely on reduction to measure the total Asc. This adds additional steps to the analysis, doubling the amount of measurements that need to be made, increasing sample preparation time and sample volume requirements, and creating extra opportunities for errors to occur. Development of a robust and rapid method for the simultaneous analysis of Asc, DHA and AAS would allow efficient analysis of the strong links between thiol and sulfide metabolism and the ascorbate metabolome.

The inclusion of AAS in the same method would allow the analysis of this less well understood metabolite of Asc. In particular, this would be a powerful combination with stable isotope tracing and separate methodology to measure sulfate levels. Suitable separation and detection conditions were established but the stability of Asc and DHA proved to be problematic. Due to limited time and resources, these issues remain unsolved and require further investigation.

Due to the manner in which the thesis project evolved method development for the NO/arginine metabolome was not investigated. In part, this was due to more time than anticipated being required for method development of the sulfide and thiols method due to unforeseen questions needing to be answered. Likewise, the developed method lead into analysis of polysulfides, which proved to be more challenging and time consuming than expected. Ultimately, the thesis progressed in areas where the biggest contribution could be made in the time available for the project. The existing methods for analysis and tracing of the NO/arginine metabolome could now be combined with the newly developed methodologies and stable isotope labelling in order to trace the crosstalk between these areas of redox metabolism. There is now the potential for further insight into the interlinked nature of these metabolomes; this would not have been as feasible without the development focus used and gives the greatest potential for further work built on the work done in this project.

Chapter 3: Redox Analysis of Thiols and Sulfide

3.1 Introduction

Oxidative stress has previously been associated with an overabundance of reactive oxygen species (ROS) and/or an impaired antioxidant and free radical scavenging capacity resulting in cell death and tissue damage. More recently it has become understood to be more closely linked to a shift in redox balance, affecting both global and regional redox status as well as cysteine-based redox signaling[35,36,281]. At the same time as these developments there has be a resurgence of interest in the biological effects of hydrogen sulfide (H_2S) and related oxidation products such as persulfides and polysulfides[19,55,282]. No specific biological targets have yet been identified for these compounds, and many of their effects appear to be mediated by interaction with either metals, heme proteins or redox interaction with other sulfur species. Additionally sulfide is involved in post-translational protein modification (persulfidation, S-sulfhydration) where reactive cysteine groups involved in redox signaling are modified, resulting in altered chemical reactivity and protein function[283]. There is growing interest in the sulfur related redox metabolome (**Figure 4**) with growing evidence that measurement of these species can be applied in clinical settings to predict morbidity and mortality from redox diseases[36]. Unfavorable outcomes in oxidative stress related conditions may be better managed by an early enough intervention to correct the redox imbalance. It is therefore unsurprising that there is now increased interest in analytical methods that provide a more refined mapping of the associated metabolic changes and enable further study of the underlying mechanisms.

A wide range of separation and detection techniques have previously been utilised for the analysis of small thiols and sulfide. The majority of these methods utilise liquid chromatography as the separation technique coupled with ultraviolet-visible (UV-Vis) spectroscopy, fluorescence detection[107] or mass spectrometry[284,285]. These analytical techniques are often used in conjunction with chemical derivatisation in order to stabilise the thiols, with a number of different electrophiles used including Ellman's reagent, monobromobimane (MBB), *N*-Ethylmaleimide (NEM) and iodoacetamide (IAM) as discussed in section 1.4. There are likewise many different assays for the quantification of sulfide in seawater or simple aqueous buffer systems, though not all are suited for the detection of sulfide in biological material[17,18]. Modifications of the methylene blue assay[286,287] have been used by many research groups to detect sulfide in blood but it suffers from limitations[288]. More recently, number of fluorimetric probes have been developed for the measurement of sulfide[289]. In particular, one of the currently most widely used methods for sulfide quantification in biological material is a HPLC-based method coupled with fluorimetric

detection that utilises the reaction of sulfide with mBB[113,290,291] (**Scheme 8**). Most recently, the same reaction principle was exploited to develop a more specific detection technique for sulfide using liquid chromatography hyphenated to tandem mass spectrometry[292].

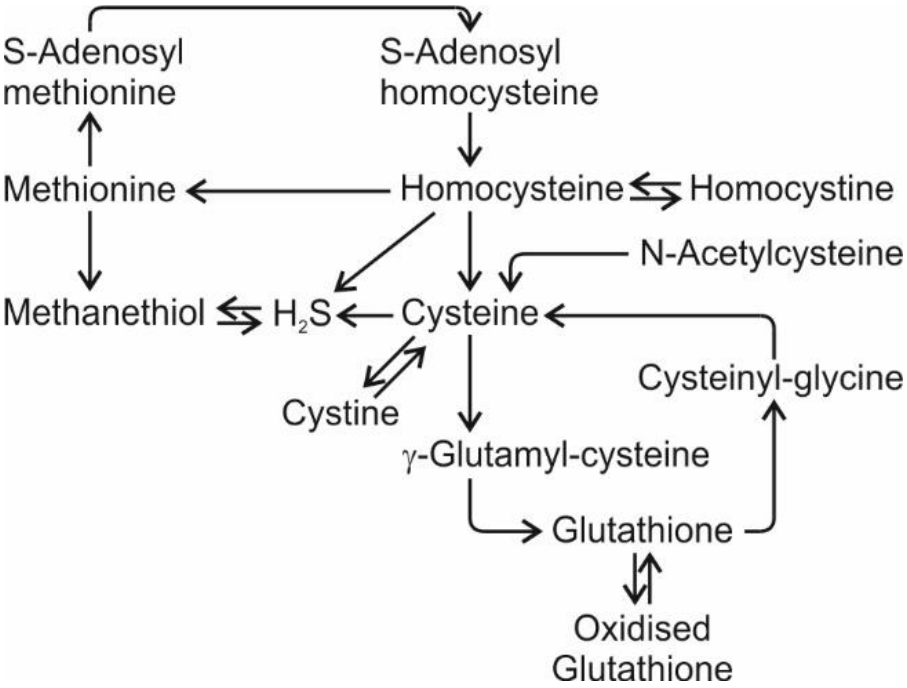


Figure 4: Metabolic pathways defining the thiol redox metabolome[293]. For a more detailed overview of the metabolic pathways involved, see section 1.3.

These methods often have significant drawbacks, UV-Vis and fluorescence detection can be non-specific with no way to distinguish between different reactive sulfur species including the thiols without knowing the retention times and interference by excess of the labelling probe. Mass spectrometry is more specific but can be prone to ion suppression by matrix components and limits the buffers used, as they have to be volatile. Additionally it is not clear how the different steps involved during analysis, from sample collection, processing, handling and storage to the reaction conditions and even the separation and detection method can affect the measured concentrations of the thiols and sulfide. It is apparent from the large variation in reported physiological concentrations of these important compounds that these factors must be having an effect. This is especially noticeable in the reported levels of free sulfide in plasma with reported concentrations varying widely[18,20,290].

One of the aims of the thesis was to develop a reference UHPLC-MS/MS method using NEM derivatisation that is capable of analysing free reduced thiols, oxidised thiols, and sulfide in a single chromatographic run to provide a comprehensive method for the analysis of the small sulfur related metabolites central to redox regulation[276], one carbon metabolism and sulfide metabolism. I also aimed to investigate the many factors that can potentially affect the analysis of

these compounds from the initial sample collection to the quantification of the data after analysis is complete. Ultimately, the results of these investigations will allow stable isotope tracing techniques to be applied to the developed methodology to allow the analysis of the flux and direction of select analytes through the pathways of the sulfur redox metabolome (**Figure 4**) and their further interactions in the reactive species interactome.

3.1.1 Use of Liquid Chromatography Couple with Tandem Mass Spectrometry for the Analysis of Low Molecular Weight Thiols

Despite the many existing methods for low molecular weight (LMW) thiols and sulfide analysis there are still significant blind spots in our knowledge of sulfur redox metabolism. This is largely due to the various limitations of existing methodologies (see section 1.4). Additionally, many existing methods fail to capture the whole range of free thiols and sulfide involved in redox regulation[294] (see section 1.3). Oxidised thiols are often analysed by first, analysing the free reduced thiols, before treating with a reducing agent and taking the increase in reduced thiol concentration as the amount of oxidised thiols present[279]. This approach is flawed, as the increase in reduced thiols does not necessarily represent the physiological levels of the free oxidised thiols, but includes all bound thiols including protein bound and mixed thiol disulfide species. Only by directly measuring the free oxidised thiols simultaneously with the reduced thiols can an accurate picture of the state of the redox balance be determined. Tandem mass spectrometry (MS/MS) provides a powerful tool for this sort of analysis. The ability to monitor specific precursor-product ion pairs for target analytes enables sensitive and specific detection and quantification even in complex mixtures[295].

Chromatographic separation is essential for the analysis of complex mixtures such as biological samples due to the high complexity, with a wide range of matrix components that can interfere with the detection of the compounds of interest. Ideally, the separation will be powerful enough to avoid the need for complex sample preparation whilst being short enough to allow high sample throughput. Reversed phase liquid chromatography utilising a C_{18} stationary phase (silica particles functionalised with C_{18} chains) is a widely used technique due to its robust and reliable nature. The high stability of the column minimises the equilibration time needed, shortening chromatographic runs compared to traditional normal phase chromatography with reproducible results. However traditional reversed phase columns should not be run with a high percentage of the aqueous mobile phase (>80%) for extended periods of time as this can have a negative impact on column stability, retention, and peak shape, which is known as phase collapse[191]. Unfortunately since thiols, especially the oxidised forms, are small polar molecules in order to get sufficient retention on a C_{18} column the starting conditions used generally have a high percentage

of aqueous mobile phase (>80%)[127]. Mixed mode stationary phases combine the stability of reversed phase chromatography with some of the polar separation characteristics normally associated with normal phase chromatography and allows the use of higher percentage of aqueous phase[296]. By utilising mixed mode chromatography the more polar thiols should be better separated and enabling a better analysis.

Reduced thiols and sulfide are all highly reactive compounds and are not stable in solution after samples have been collected[18,44,116]. The reduced thiols are highly susceptible to oxidation resulting in both the loss of reduced thiols and the artifactual increase in the concentration of the corresponding oxidised thiols. Additionally, enzymatic activity continues in biological samples after sample collection which results in additional losses unless the enzyme activity is blocked[297]. Sulfide and its metabolite methanethiol are both highly volatile and easily lost through evaporation unless trapped. Both thiols and sulfide can also potentially be taken up by proteins present in the sample[120,283], form mixed oxidised species and even organic and inorganic polysulfides[143] all of which can contribute to losses after sample collection. For these reasons, many current methods employ an electrophile to alkylate the reactive sulfurs, blocking the loss of these reactive species[105]. One such electrophile is *N*-Ethylmaleimide (NEM) which has long been used for the analysis of hydrogen sulfide[125] and thiols[122] due to its ability to rapidly react with the free –SH group. As well as protecting reduced thiols and sulfide from loss by blocking the reactive sulfur and trapping the sulfide. It also has the added advantage of improving ionisation efficiency in electrospray ionisation sources commonly used in mass spectrometry[123] as well as providing increased retention on reversed phase chromatography columns[127]. These properties make NEM an ideal choice for the analysis of the sulfur redox metabolome using UHPLC-MS/MS.

3.1.2 Opportunities and Challenges in Analysis of Low Molecular Weight Thiols and Sulfide in Biological Matrices

Biological samples present more of a challenge for analysis than buffered standard mixtures due to their complex nature and varying levels of the compounds of interest. Matrix components found in biological samples can potentially cause many issues including degradation of chromatographic separation, ion suppression of analytes and false signal caused by compounds with the same *m/z* or fragments leading to inaccurate quantification. Differences in the abundance of analytes can cause issues, as whilst one analyte might fall in the linear range on the calibration curve for quantification, other analytes at the same dilution may be too low to be quantified reliably or overloaded. For example, red blood cells (RBC) have large amounts of reduced glutathione (GSH) compared to the other thiols[298] which could potentially cause issues when quantifying the less abundant thiols. Conversely, plasma has lower levels of GSH compared

to cells but higher cystine levels. Issues related to matrix components can be alleviated by removing interfering components during sample preparation or by extracting the analytes of interest. Unfortunately, sample purification or extraction adds additional, time-consuming and often expensive steps to the analysis, which makes high throughput analysis difficult. The advantage of using a technique such as UHPLC-MS/MS is that it alleviates the need to use intensive sample preparation for a good analysis and allows the use of simpler techniques saving on both time and cost.

Protein precipitation is a simple method of sample preparation and removes some of the major interfering matrix components[299]. If left in the samples the proteins can precipitate onto the column as well as modify the stationary phase degrading the chromatographic separation. However only removing proteins will leave other small matrix components in the sample that can potentially interfere with the mass spectrometry detection. The simplest method of protein removal involves using organic solvents to precipitate the proteins (at least 1:1 organic solvent to sample) which can then be spun down and the supernatant analysed or stored[300]. However, when the chromatographic gradient starts with a high amount of aqueous mobile phase the relatively high organic solvent content in the prepared sample can lead to a loss of chromatographic retention. Ideally, when a high percentage of aqueous mobile phase used at the beginning of the chromatographic run, the samples must also be mainly aqueous as the sample composition must be close to the chromatographic starting conditions in order to get sufficient separation. Other protein removal methods utilise a strong acid[216] that can potentially disturb the levels of some of the more unstable compounds of interest including sulfide, polysulfides and thiol persulfides. Ultrafiltration uses centrifugal filters with a nominal molecular weight limit (NMWL), for example 10 kDa, which will exclude the majority of proteins found in biological samples without the use of strong acids or organic solvents[301]. Ultrafiltration however will not remove smaller peptides and other matrix components that fall under the molecular weight cut-off potentially resulting in a "dirtier" sample than other protein precipitation methods[302].

3.1.2.1 Plasma

Plasma is collected from whole blood in tubes containing an anti-coagulant such as ethylenediamine tetraacetic acid (EDTA), heparin or sodium citrate; it differs from serum as it contains clotting factors such as fibrinogen. Red blood cells are removed from plasma samples by centrifugation during which care must be taken to avoid lysing the RBCs as this will affect the contents of the plasma. Different anticoagulants can potentially have effects on the contents of plasma samples so the choice of anticoagulant must be carefully considered[303]. Care must also be taken to avoid haemolysis during sample collection and preparation as cellular levels of thiols vary

compared to those in plasma with ~500-times more GSH in RBCs[44] any lysis will therefore cause abnormally high GSH levels to be measured in the plasma.

For the analysis of thiols in plasma, ideally the whole blood should be derivatised on collection or as soon as possible after. This is to avoid any artificial oxidation of the free thiols, loss of sulfide, thiols being taken up by proteins or enzyme activity affecting the thiol makeup. Where this is not possible the plasma should be frozen as soon as possible and then derivatised the first time it is thawed to avoid any possible freeze-thaw effects.

3.1.2.2 Serum

Serum is the liquid fraction of blood excluding cells and clotting factors. It is obtained by letting the collected blood clot, generally for 30-60 min at room temperature, before the sample is spun down and the supernatant collected[303]. The method used to collect serum has some potential issues for thiol analysis, GSH and the other reduced thiols oxidise at room temperature with GSH having a half-life of ~5 minutes[304]. Whilst this might be only a slight decrease in the GSH concentration, it can represent a large increase in the concentration of GSSG and significantly affect the measured redox ratio. Enzyme activity in the sample will also continue during clotting resulting in additional losses. Sulfide being a small volatile molecule is also potentially susceptible to loss from the blood samples whilst they are left to coagulate by irreversibly evaporating or potentially reacting with oxidised thiols forming thiol persulfides[139]. These potential issues may be mitigated by the addition of NEM to the sample as it clots but it is not known how this might affect the clotting procedure or affect the result.

3.1.2.3 Red Blood Cells

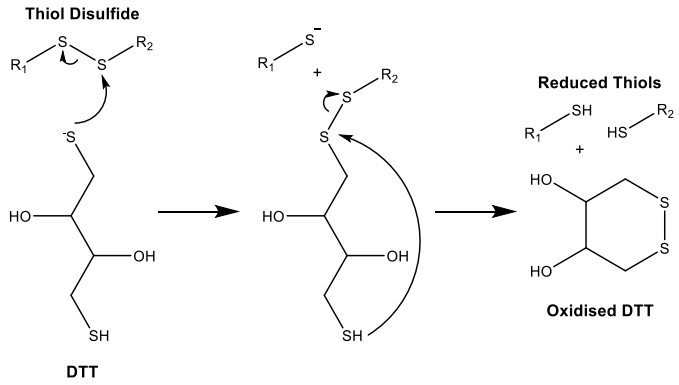
Red blood cells (RBCs) are collected from whole blood using anticoagulant containing tubes in the same way as plasma collection. RBC and plasma collection can be done from the same whole blood sample; after the whole blood is spun down the supernatant (plasma) is removed. The RBCs form a pellet at the bottom of the tube and any remaining supernatant after plasma collection is pipetted off leaving the RBC pellet[305]. Before they can be analysed red blood cells first have to be lysed in order to release the intracellular thiols and sulfide. RBCs can be lysed in 3:1 pure water:RBC, with 10 mM NEM to derivatise the thiols and sulfide as they are released. The cell debris and proteins can then be removed during the protein precipitation or removal step.

The most abundant thiol in RBCs is reduced glutathione, which is ~100 times more abundant than the other thiols[298]. This could potentially cause an issue for RBC analysis, as at the dilutions needed to prevent overloading the GSH, other thiols may be too dilute below the limit of quantification.

3.1.2.4 Urine

Urine makes an attractive sample matrix as collection is non-invasive and often provides a large sample volume and is largely free from interfering proteins or lipids[306]. There are some drawbacks however as urine contains many small metabolic breakdown products and salts that may interfere with analysis[307]. It is especially susceptible to changes in the diet altering the matrix components[308] that may lead to inconsistent ion suppression between samples though the use of good internal standards can alleviate this. Although urine is largely protein and lipid free it is still best to utilise a deproteinisation step before analysis. The pH of urine is also not as consistent as that of plasma or other human sample matrices[309], differing pHs may have an effect on the distribution of some species such as polysulfides or even redox pairs and so buffering of the urine may be required for consistency.

3.1.3 Bound and Total Thiols and Sulfide



Scheme 16: Reduction of a thiol disulfide by dithiothreitol (DTT)[310].

Other methods have utilised reduction of oxidised and bound thiols in biological samples in order to measure the total free and bound thiol content. Total homocysteine (tHcys) is a widely used marker for conditions such as cardiovascular disease[94] and Alzheimer's[96]. The total level of thiols measured in this way provide a relatively crude measure as they often do not distinguish between the free circulating thiols (reduced and oxidised), mixed disulfides, and protein bound thiols[68]. There also exists a further thiol and sulfide component referred to as the acid labile thiols. They are defined as the concentration of LMW thiols that are liberated from binding to proteins or sulfane sulfur compounds following the treatment of a biological sample with mineral acid (e.g. HCl). These three components of the thiol redox metabolome represent 1) the redox status of a tissue/cell, according to the ratio of free reduced/oxidised thiols and 2) the redox reserve (or total thiol status), which is an index of the total reducing capacity of a cell/tissue or organism that defines its resilience to oxidative modification measured by the bound and acid labile components[293].

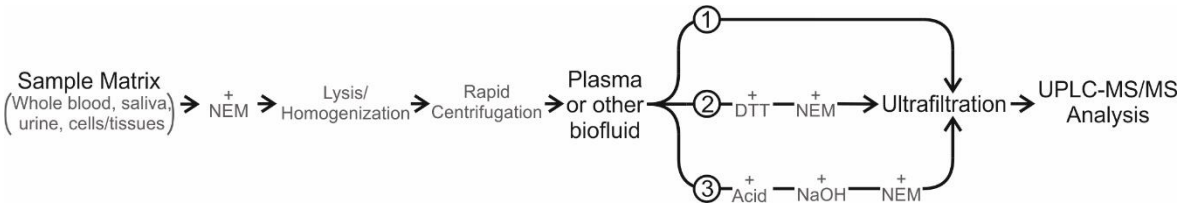


Figure 5: Developed workflow for the efficient use of samples in the determination of free (1), total (2) and acid-labile (3) thiols and sulfide in biological samples such as blood plasma, blood cells and other biofluids.

Dithiothreitol (DTT) is commonly used as a reducing agent for thiol analysis[311], DTT is able to rapidly react with the disulfide bonds in oxidised thiols reducing them and forming a stable six membered DTT ring and leaving the thiols in their reduced form (**Scheme 16**)[310]. An efficient workflow was developed to make the best use of samples (**Figure 5**). First, the free thiols and sulfide in samples can be analysed after NEM derivatisation, the same sample can then either be reduced using DTT in order to measure the total thiols, with the total thiols minus the free thiols giving the levels of bound and mixed disulfide thiols. The same sample could also be treated with acid instead to obtain the acid labile thiol and sulfide component. A single sample split would enable the free thiols and sulfide, bound thiols and sulfide and the acid labile thiols and sulfide to be determined. This powerful combination would make efficient use of samples like plasma that are often limited in volume, and provide a comprehensive picture of physiological thiol and sulfide status. By simultaneously measuring the free reduced and oxidised thiols, followed by reduction and/or treatment with acid to measure the total and acid labile thiols this distinction can be made providing a much more comprehensive picture of thiol status and metabolism.

3.2 Methods

Unless otherwise stated all reagents and materials were of the highest purity available and purchased from Sigma-Aldrich (Gillingham, UK or Munich, Germany). Dual stable isotope (^{13}C, ^{15}N) labelled reduced (GSH) and oxidized glutathione (GSSG) were from Cambridge Isotopes and obtained from CK Isotopes Ltd (Newtown Unthank, UK), N-ethyl-d5-maleimide (d_5-NEM) was from Sigma-Aldrich. Sodium persulfide (Na_2S_2) was provided by Dojindo Europe (Neuss, Germany). HPLC-grade solvents from Fisher Scientific (UK). Argon gas (> 99.99%) was from BOC Group (Guildford, UK). Ultrapure N_2 gas was produced by a nitrogen generator (Parker Balston, UK).

3.2.1 UHPLC-MS/MS Development and Optimisation

3.2.1.1 Mass Spectrometry

The detector used was a Xevo TQ-S triple quadrupole mass spectrometer (Waters) equipped with an electrospray ionisation source (ESI). Optimal mass spectrometry settings were obtained by direct infusion of oxidised thiol standards and NEM derivatised reduced thiol and sulfide standards. All analytes were detected using positive ion mode. To improve the specificity and sensitivity for each analyte specific precursor and product ions (MRMs) were established for each compound (**Table 2**). By monitoring the precursor ion and a specific fragment ion produced by that precursor a highly specific measurement can be made which reduces the noise level and increasing the signal to noise ratio. The reduced thiol standards were prepared to a concentration of 1 mM by addition of the dry powder to pH 7.4 ammonium phosphate buffer containing 10 mM NEM. The standards were then diluted with pure water to bring them down to reduce the concentration to a measureable range (1 nM – 5 μM). The individual standards were infused directly into the ESI source of the mass spectrometer at 8 μl/min and scanned for one minute. To obtain optimal signal for each compound each one-minute scan was performed with manual adjustment of each parameter, adjusting one at a time in order to gain the highest signal intensity for each setting for each compound. The optimised precursor and product ion pairs, cone voltages, and collision voltages used are listed in **Table 2**, further mass spectrometry settings chosen are listed in appendix A.1, **Table 9**. Signals were captured and data processed using MassLynx v.4.0 and Quanlynx v.4.0 software (Waters).

3.2.1.2 Chromatography Development and Optimisation

Different column chemistries and chromatographic gradients were tested with the final gradients and columns used are summarised in **Table 1**. All chromatographic separations were carried out using a Waters Aquity ultrahigh performance liquid chromatography (UPLC) system with a thermostat controlled autosampler (kept at 5°C) with an ultrahigh performance binary pump.

Initial development used an Aquity UPLC CSH C_{18} (1.7 μm), 2.1 x 100 mm column kept at a temperature of 30^{o}C, with a sample injection volume of 5 μl. Following the work performed by Lee-Sun New and Eric C.Y. Chan[127], water (H_2O) and acetonitrile (ACN) were selected as the solvents for the two main mobile phases (A and B respectively). Mobile phase A was H_2O with 5 mM ammonium formate and mobile phase B was 95% acetonitrile with 5% H_2O and 5 mM ammonium formate. The chromatographic gradient (**Table 1**) started at 95% A, decreasing to 40% A over 5 min before returning to 95% A over 1 min and being held at that level for a further 1 min, resulting in a total run time of 7 min at a constant flow rate of 0.2 ml/min. Experimental results

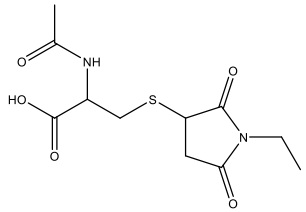
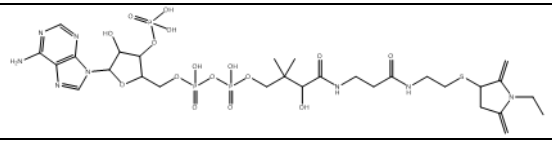
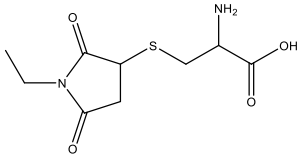
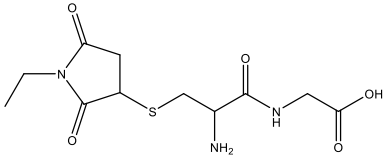
depicted in **Figure 7**, **Figure 12** and **Figure 13** were obtained using these separation conditions, as indicated in the figure legend. For long sample runs due to the high amount of aqueous solvent used a wash step was included every four or five samples. The wash step consisted of a short five minute wash with 100% B followed by a blank run using the gradient in **Table 1** to ensure the column was equilibrated before the next set of samples.

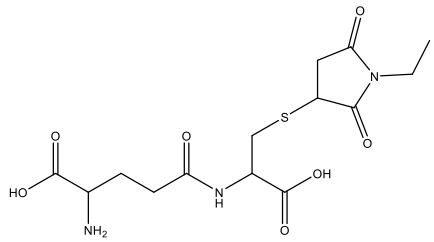
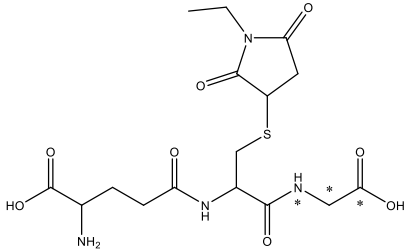
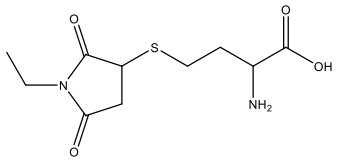
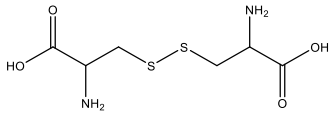
Table 1: Chromatographic conditions for thiol analysis on a reversed phase C_{18} column, A = H_2O + 5 mM ammonium acetate, B = 95% ACN + 5% H_2O + 5 mM ammonium acetate and on a mixed mode Aqua UPLC column, A = H_2O + 0.15 % formic acid and 5 mM ammonium formate, B = 95% acetonitrile + 5% H_2O + 0.15% formic acid and 5 mM ammonium formate. The flow rate for all gradients and columns was 0.2 ml/min.

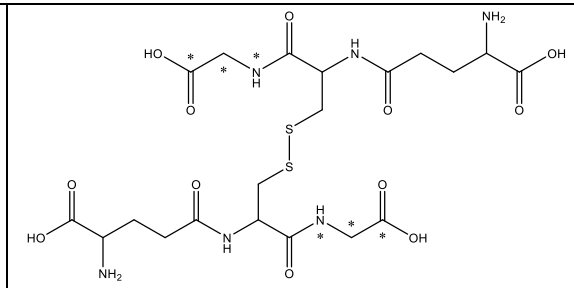
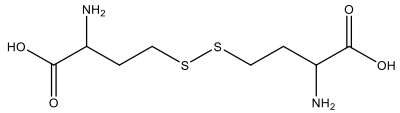
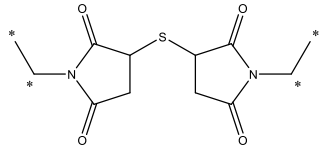
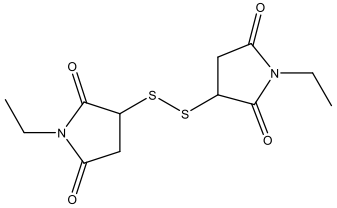
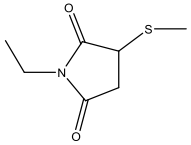
C_{18} Column			Aqua Column		
Time (min)	**% A**	**% B**	**Time (min)**	**% A**	**% B**
0.00	95	5	0.00	99	1
5.00	40	60	4.50	60	40
6.00	95	5	5.00	0	100
7.00	95	5	6.50	0	100
			7.00	99	1
			8.00	99	1

Further development focused on using a 1.6 µm Modus 100 x 2.1 mm Aqua UPLC column (Chromatography Direct, Runcorn, UK) kept at a temperature of 30°C; mobile phase A was H_2O with 0.15 % formic acid and 5 mM ammonium formate and mobile phase B was 95% acetonitrile with 5% H_2O, 0.15% formic acid and 5 mM ammonium formate. The chromatographic gradient (**Table 1**) starts at 99% A, decreasing to 60% A over 4.5 min, before dropping to 0% A over 0.5 min and being held at that level for 1.5 min. The column is brought back up to 99% mobile phase A over 0.5 min and held at 99% for a further 1 min to equilibrate. A flow rate of 0.2 ml/min was used throughout, and total run time including equilibration was 8 min. An injection volume of 5 µl was used, with a wash step every ten injections consisting of 100% mobile phase B for 5 min, followed by a blank using the regular gradient to control for potential carry-over and column equilibration. We found that carry-over was negligible with two needle rinse steps (300 µl methanol, followed by 600 µl H2O /acetonitrile 90/10%, v/v) between injections.

Table 2: Structures, MS parameters, elution times and limits of detection (LOD) and quantification (LOQ) for the 12 analytes and 3 internal standards investigated. (n.d. - not determined)[293]

Analyte/Internal Standard	Structures of Oxidised Thiols, NEM Derivatised Reduced Thiols and Sulfide	Precursor Ion m/z	Product Ion m/z	Cone Energy / V	Collision Energy / V	Elution time (C_{18}) / min	Elution time (Aqua) / min	LOD / nM	LOQ / nM
Reduced Thiols (NEM Derivatised)									
N-Acetylcysteine		289.0	201.0	17	14	n.d.	4.05	0.5	4
Coenzyme A		892.5	389.0	12	31	n.d.	n.d.	n.d.	n.d.
Cysteine		247.1	158.1	8	18	2.18	2.80	8	20
Cysteinyl-glycine		304.0	212.0	8	8	2.30	2.90	20	50

Glutamyl-cysteine		376.0	246.6	5	10	1.80	3.20	0.2	0.5
Glutathione **$^{13}C_2$,^{15}N** Glutathione*		433.1 436.1	304.0 307.0	6	13	2.00	3.26	0.2	0.5
Homocysteine		261.1	56.0	8	16	2.40	3.08	1	4
Oxidised Thiols									
Cystine		241.0	152	8	12	1.15	1.10	60	100

Glutathione (Oxidised) $^{13}C_4$,$^{15}N_2$ Glutathione (Oxidised)*		613.0 619.0	355.0 361.0	10	20	1.00	2.50	0.5	2
Homocystine		269.1	136.1	14	4	1.20	1.50	4	7
Sulfide Related Species (NEM Derivatised)									
Sulfide (D_5-NEM)$_2$-Sulfide*		285.1 295.1	160.1 165.1	8	14	4.85	5.90	4	8
Persulfide		317.0	160.1	8	14	5.50	n.d.	n.d.	n.d.
Methanethiol		173.6	74.6	1	11	n.d.	5.66	50	100

3.2.1.3 Linearity, Precision, Limits of Detection and Reproducibility

The linearity of the standard curves for each analyte was determined using accurately weighed amounts of analytical-grade reduced thiols dissolved in the appropriate volume of NEM-supplemented ammonium phosphate buffer giving a 10-fold molar excess of NEM over thiol. The solutions were allowed to react for 5 min at room temperature. Accurately weighed sulfide was first dissolved in water before addition of a defined volume of this concentrated stock to NEM-containing buffer in a septum-sealed reaction vial under otherwise identical reaction conditions. NEM-derivatised thiol and sulfide solutions were then combined with roughly equimolar concentrations of the oxidised thiols to yield a stock solution of all analytes to be tested. The mixed standard was serially diluted with buffer to yield targeted final concentrations of 10 µM, 5 µM, 1 µM, 500 nM, 100 nM, 50 nM, 10 nM and 5 nM when added to the biological sample (2x more concentrated in the mixed standard). For most compounds this is well within the physiological range, except for cellular/tissue GSH levels (being in the low mM range), which may therefore necessitate appropriate dilution of samples before analysis. To each dilution of the standard calibration set and the biological samples the same volume of internal standard was added yielding final concentrations of 100 nM GSH, 200 nM GSSG, and 100 nM sulfide. This allowed adjustment of peak areas by taking into account the ratios between analyte and internal standard. Limits of detection were determined using measurements of serially diluted standards prepared as previously described. Limits of detection and quantification are defined as signal-to-noise ratio of >3 and >10 respectively. Between-run reproducibility was tested using repeated measurements of a pooled plasma sample over several days following the established method.

3.2.1.4 Quantification

Quantification of the compounds of interest was accomplished using stable isotope labelled internal standards. Stable isotope labelled standards were used as they have different masses than the endogenous thiols and sulfide (NEM reacted) but have the same chromatographic and ionisation properties. Three internal standards were used, one for each of the three classes of compounds analysed; the reduced thiols, oxidise thiols and sulfide. One internal standard for each class is required to account for the differences in ionisation between them as NEM modifies the ionisation (ESI) properties of the molecules it reacts with[123]. For the oxidised thiols, $^{13}C_4{}^{15}N_2$-GSSG was used; for the reduced thiols $^{13}C_2{}^{15}N$-GSH was derivatised with an excess of NEM and used as internal standard. For sulfide, a deuterated stable-isotope labelled standard (^{32}S-(D_5-NEM)$_2$) was prepared by reacting an exact amount of D_5-NEM with a two-fold molar excess of Na_2S in pH 7.4 ammonium phosphate buffer for 30 min at room temperature, and removing excess sulfide by bubbling for 60 min with high-purity argon (see **Table 2** for the structures of the

standards). A mixed internal standard working solution was prepared by combining all three stable-isotope labelled compounds in pH 7.4 ammonium phosphate buffer. Specific MRM conditions were established to monitor each of the internal standards (**Table 2**), and the ratio between the signal intensity of each analyte and its corresponding internal standard was used for quantification. Internal standard was added to all samples and standards to give a final concentration of 100 nM GSH, 200 nM GSSG and 100 nM sulfide. Stock solutions of standards containing each analyte of interest were prepared fresh for each analysis in pH 7.4 ammonium phosphate buffer (except for cystine and homocysteine, which were prepared in 0.1 N HCl and sulfide and methanethiol were first dissolved in water) and serially diluted in buffer to construct a concentration response curve for calibration. Each concentration of standard was spiked with the same final concentration of stable isotope standards.

3.2.2 Derivatisation of thiols and sulfide

Initial experiments carried out with mixed thiol standards at room temperature (RT) and pH 7.4 revealed that the reaction with NEM was complete for all analytes in under 10 min. Those results were confirmed by spectrophotometric monitoring of NEM consumption under the same conditions (302 nm; Cary 60 UV/vis spectrophotometer), demonstrating rapid kinetics for the reactions of NEM with GSH, cysteine (Cys) and homocysteine (Hcys) (at a 1:1 molar ratio, reactions were complete within 12-15 seconds). The reaction between NEM and sulfide (2:1 molar ratio) took longer and required 10 min for running to completion (see Chapter 5: for more details). Optimal reaction pH was assessed either by comparing reactions of NEM with aqueous standards of select thiols (GSH, Cys, Hcys, sulfide and stable-isotope labelled GSH) or by addition of NEM stocks prepared at different pH to human plasma. For those experiments, 100 mM NEM stock solutions were prepared in different ammonium phosphate buffers adjusted to pH 6.0, 7.4, 8.0 and 9.0 and used within 5 min of preparation. Freshly prepared aqueous solutions of thiols were reacted individually with NEM at the different pH values with a final concentration of 10 mM NEM, and after 5 min reaction time aliquots of the NEM-thiol incubates were combined and analysed immediately. Endogenous thiols contained in human plasma (obtained by centrifugation of EDTA blood using the rapid centrifugation protocol as detailed in section 3.2.4.1) were derivatised using freshly prepared NEM stock solutions of different pH at a 1:10 v:v ratio (10 mM NEM final).

3.2.3 Method Validation

The final method was validated for linearity, over a range of biologically relevant concentrations (5 nM – 1 μM for diluted samples), and a preliminary assessment of precision, reproducibility and

limits of detection were carried out using authentic standards, according to standard procedures, and are shown in **Table 2** and **Figure 10**.

3.2.4 Sample Collection, Preparation, Storage and Optimisation

Blood was taken from healthy volunteers of either gender (20-58 years old) with informed consent to participate before enrolment. Procedures were approved by the ethics committees of the University of Southampton (ERGO 30507/31426) and conducted in accordance with the Declaration of Helsinki. Venous blood was collected from healthy human volunteers using a 21-gauge (0.80mmx19mm) butterfly needle and BD VacutainerTM tubes. Following gentle mixing by inversion, blood was processed immediately as described below. Sample aliquots were measured either fresh or transferred into cryovials (Nunc), snap frozen in liquid nitrogen and stored at -80°C until analysis.

3.2.4.1 Plasma and Serum

Each step of the sample preparation was carefully optimised and validated. To compare detection of thiols and sulfide in plasma with that in serum, whole blood was also collected into serum BD vacutainers, incubated for 60 min at room temperature to allow clotting, and then centrifuged at 3000x g according to the manufacturer's protocol. To test the effects of other anticoagulants, whole blood was collected into BD vacutainers containing EDTA, heparin or citrate, diluted 9:1 with NEM (final NEM concentration 10 mM), centrifuged at 800 x g for 10 min at 4°C. Centrifugation speed was optimised by comparing GSH levels in plasma obtained by standard/low-speed centrifugation of whole blood (800 x g, 10 min, 4°C) with short/high-speed centrifugation (1 min 3000x g or 3 min 3000x g at 4°C). Any potential haemolysis was assessed by our collaborators in the Cortese-Krott research group (see acknowledgements) by measuring the plasma free haemoglobin concentration using UV-visible spectroscopy[312]. Sample stability and stabilisation conditions were determined by focusing primarily on the levels of GSH, GSSG and sulfide (although other thiols were assessed in parallel). When comparing the addition of different concentrations of NEM (1, 5, 10, 20 mM final concentration) to whole blood, or when NEM addition was delayed by specified periods of time (30 s; 5, 10, 15 and 20 min). To test the effects of stage of addition of NEM in during sample processing the same blood sample was divided into identical aliquots. NEM was added either before the whole blood sample was spun down or added to the plasma after centrifugation. Additionally to test the effects of freeze/thawing on NEM stabilised samples the same sample was split into aliquots before being subjected to the different freeze/thaw conditions; the aliquots were either analysed without freezing, stored in the autosampler at 5^{o}C overnight, frozen and thawed once, or frozen and thawed twice. The stability

of frozen underivatised samples compared to frozen derivatised samples was also assessed using identical aliquots of plasma that were either, reacted with NEM and analysed without freezing, reacted with NEM after being frozen and then defrosted before analysis, or reacted with NEM before freezing and then analysed after defrosting.

In the final optimised sample collection procedure for human plasma analysis, whole blood was collected directly into EDTA BD vacutainer^{TM} tubes and gently mixed by repeated inversion. 1 volume (vol) of NEM (100 mM stock solution in phosphate buffered saline) was added to 9 vol of blood within 1 min, resulting in a final concentration of 10 mM NEM (1:10 dilution), again followed by gentle mixing. Stabilised, anticoagulated blood was then centrifuged for 1 min at 3000x g to separate plasma from cellular components, snap frozen in liquid nitrogen and kept at -80°C until analysis. Before analysis, NEM-derivatised plasma samples were supplemented with the internal standard mixture (dual stable-isotope labelled GSSG and NEM-derivatised GSH as well as D_5-NEM-derivatised sulfide in ammonium phosphate buffer (see 3.2.1.3), 1:1 v/v; target sample concentrations of 100 nM GSH, 200 nM GSSG, 100 nM sulfide). Proteins were then removed from the samples by ultrafiltration using spin columns with a 10 kD cut-off (Millipore).

3.2.4.2 Red Blood Cells

NEM can freely move across cell membranes and alkylate the cellular thiols before lysis[293,313]. Therefore when NEM is added to whole blood additional NEM is not required during lysis. If the whole blood has not been NEM treated then addition of NEM is required during or before lysis. To release the cellular thiols the red blood cell pellet first has to be lysed in pure water (3:1 H_2O:RBC). In order to do so the RBC pellet was defrosted and 50 µl of RBCs were added to 140 µl ultra-pure H_2O + 10 µl 100 mM NEM solution giving a total volume of 200 µl. The lysing solution was allowed to sit at room temperature for 30 min before proteins and cell debris were removed from the samples by ultrafiltration using spin columns with a 10 kD cut-off (Millipore). The filtrate was decanted and split into two aliquots. One aliquot was diluted 1000 times with pH 7.4 ammonium phosphate buffer for the analysis of GSH and the other 10 times for the other thiols and sulfide, the diluted RBC filtrate was then analysed using the established UHPLC-MS/MS method (see section 3.2.1).

3.2.1 Bound and Total Thiols and Sulfide

Different reaction conditions for the reduction of disulfides by treatment with dithiothreitol (DTT) were tested for each biological matrix to establish optimal reaction conditions. The concentration of DTT needed to reduce the samples completely was assessed using different concentrations of DTT. The reaction time needed for complete reduction was also tested. Samples were reacted

with 10, 25, 50 and 100 mM DTT for 10, 20, 30 and 60 min at RT before NEM was added. After the reduction was finished, the samples were reacted with NEM (in ammonium phosphate buffer) giving an excess of 10 mM over the DTT concentration used (20, 35, 60 and 110 mM respectively). 10 mM excess NEM was used to ensure all of the SH equivalents of DTT were neutralised and to ensure full capture of additionally released thiols (Pathway 2, **Figure 5**). An alternative reaction sequence was tested in which DTT was added to NEM pre-treated human plasma to achieve (following neutralisation of the excess NEM) a similar final DTT concentration. Results obtained with either sequence were not significantly different.

To determine the concentration of total thiols, free thiols and bound-thiols (see section 3.1.3), NEM stabilised plasma was divided into two aliquots: One to assess total thiols (free thiols + bound thiols) one aliquot was treated with 50 mM DTT for 30 min, which reduces LMW disulfides and mixed disulfides with proteins. The other aliquot was used to determine free thiols one aliquot was left untreated. In both aliquots, thiol concentrations were assessed following NEM derivatisation by the same LC-MS/MS method as before. Bound thiols were calculated by subtracting free thiols from total thiols. The same procedure was carried out with the red blood cell pellet.

3.3 Results

3.3.1 UHPLC-MS/MS Development and Optimisation

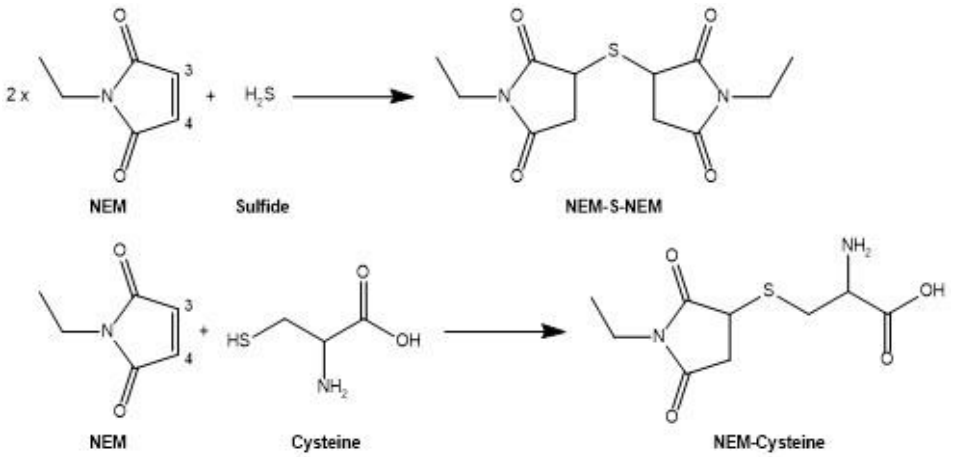


Figure 6: Reaction of NEM with Reaction of NEM with sulfide and reduced aminothiols (using cysteine as an example). The sulfhydryl group can add to either side of the double bond on the maleimide ring (Michael addition reaction, with sulfur adding either to the highlighted C3 or C4 of the maleimide ring) leading to the formation of two distinct diastereomers (with sulfide four different diastereomers can potentially be formed).

3.3.1.1 Initial development on a Reversed Phase C_{18} Column

Initial development focused on key compounds of interest involved in the metabolic pathways summarized in **Figure 4**, including Hcys, homocystine (HcySS), Cys, cystine (CySS), GSH, GSSG, cysteinylglycine (CysGly), glutamylcysteine (GluCys), and sulfide. These compounds were used to establish appropriate separation and detection conditions using UHPLC-MS/MS. During the initial development a reversed phase C_{18} was utilised with a simple chromatographic gradient for analyte separation (**Table 1**), allowing us to make general observations regarding the elution profile of the selected compounds. Generally, the small polar oxidised thiols (GSSG, HcySS and CySS) which do not react with NEM eluted first, they were followed by the reduced thiols (GSH, Hcys, Cys, CysGly and GluCys) with the sulfide which gains two NEM adducts eluting last (**Figure 7**). It is clear that the addition of an NEM adduct increases the retention on the column and the addition of two adducts even more so. Since the oxidised thiols do not react with NEM they are poorly retained and elute close together near to the solvent front. Double or split peaks were observed for a few of the compounds, particularly GSH and Cys. This phenomenon has previously been reported for NEM derivatised thiols[314] and has been attributed to the formation of two diastereomers that differ in position of the sulfur atom in relation to the maleimide nitrogen, depending on where the reduced thiol adds to the double bond of the pyrrole ring (**Figure 6**). The MRM for CysGly also shows two peaks when analysing the mixture of compounds with the additional peak eluting at the same time as the peak for GSH. This additional CysGly peak only occurs when GSH is present at the same time indicating that it is generated by in source fragmentation of GSH. The precursor *m/z* of CysGly is 304 whilst the main fragment ion of GSH has exactly the same *m/z*. The true CysGly peak can only be distinguished from the peak generated from GSH by its elution time.

In order to get suitable retention on the C_{18} column the starting conditions have to be 95% aqueous mobile phase (A) or higher. In large sample numbers using such a high percentage aqueous starting conditions can cause loss of peak shape due to column degradation[191]. This necessitates regular organic solvent wash and re-equilibration steps when running large sample numbers in order to maintain column condition and prevent poor peak shapes developing. The oxidised thiols are not well separated on the C_{18} column due to their small polar nature and elute close to the solvent front. This in particular causes an issue for the analysis of free oxidised thiols in biological samples due to the differences in concentration, with CySS being ~100x more concentrated than GSSG or HcySS leading to ion suppression and other issues.

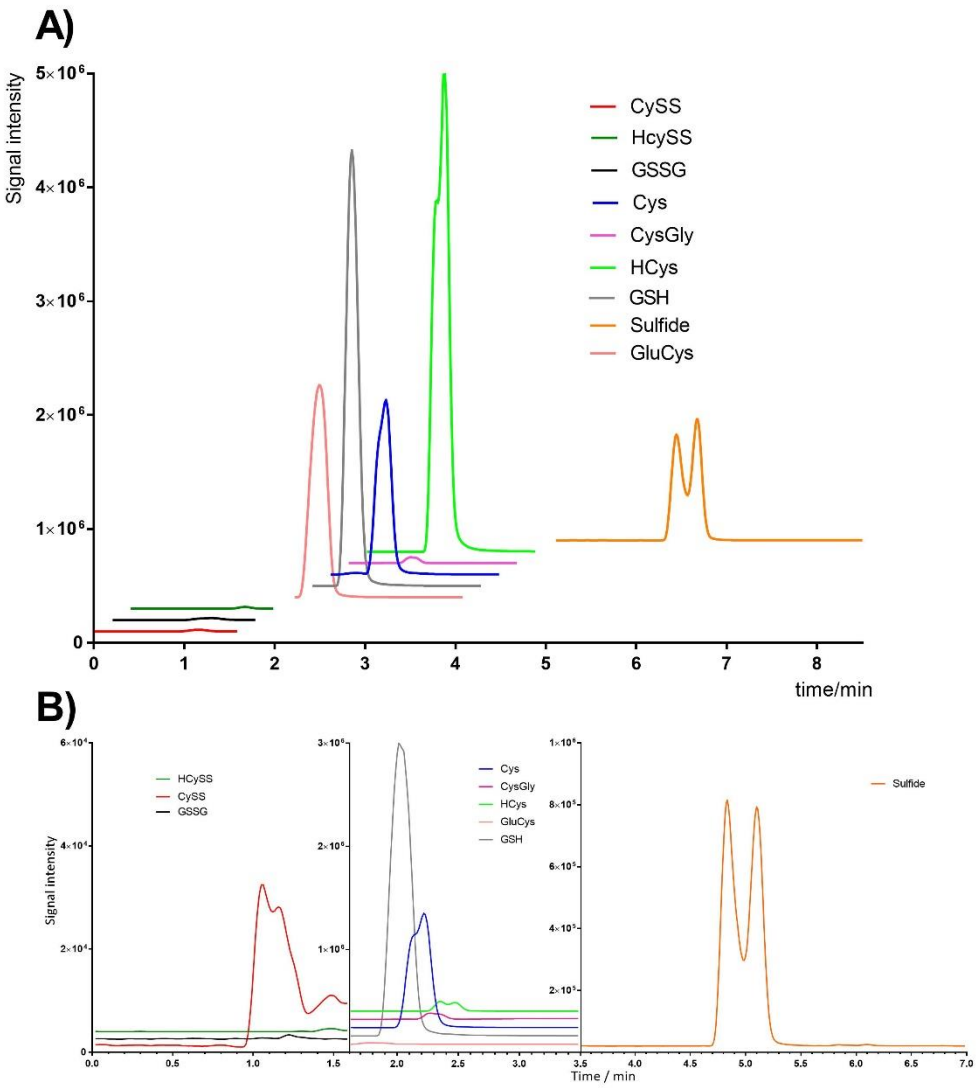


Figure 7: Exemplary chromatographic separations using the developed method on a reversed phase C_{18} column in both a standard mixture (**A**) and in human plasma (**B**). **A)** Chromatographic separation and selective detection by tandem mass spectrometry of authentic stock solutions of all analytes using selected MRM time windows **B)** Representative chromatogram of the same analytes at their natural abundance in human plasma using specific MRM time windows for selected groups of compounds[293]. MRM settings used are listed in **Table 2** and chromatographic conditions in **Table 1**.

3.3.1.2 Further Development Using Mixed Mode Type Columns

Despite the good separation for the reduced thiols and sulfide, the oxidised thiols were not well separated on the reversed phase C_{18} column. It is clear that the addition of an NEM adduct or in the case of sulfide, two adducts, improved the separation but since the oxidised thiols do not react with NEM and are small polar molecules they were poorly retained. Therefore mixed mode

type stationary phases which combine reversed phase type stationary phases with some polar separation characteristics were investigated. The Aqua UHPLC column was found to offer suitable separation properties. The polar separation characteristics of the column allowed for much better separation of the GSSG and HcySS, but unfortunately the retention of CySS was still not ideal and it eluted close to the solvent front with a poor peak shape. The general order of elution remained the same as with the C_{18} column, first the oxidised thiols eluted followed by the NEM derivatised reduced thiols and finally the NEM derivatised sulfide (**Figure 8**). The exact order of elution however did change slightly for the reduced thiols with Cys eluting first followed by CysGly, Hcys, GluCys and the GSH. The same double peak for CysGly is observed as with the C_{18} column due to the signal generated by source fragmentation of the GSH. The true CysGly signal can be determined by its elution time.

Further development of the method included the addition of three further metabolites, N-acetylcysteine (NAC), methanethiol (MT) and coenzyme A (CoA). As with the other metabolites, MRMs were established and detection conditions optimised before testing their chromatographic properties using the developed method. NAC and CoA did not differ much from other thiols in terms of fragmentation properties. Methanethiol, however, was found to be extremely sensitive to in-source fragmentation, requiring a very low cone voltage for optimal detection (**Table 2**). MT was well retained on the column, eluting between the other thiols and sulfide whilst NAC eluted sooner with the other reduced thiols (**Figure 8**). CoA eluted with an extremely long peak tail over many minutes, this is likely due to CoA having multiple strong interactions with the mixed mode column, preventing inclusion of CoA in this method.

In a plasma sample the chromatographic separation remains largely the same, though due to differing endogenous concentrations of the analytes some peaks have different shapes. Specifically CySS which still elutes close to or on the solvent front has poor peak shape with significant tailing and possible ion suppression by matrix components.

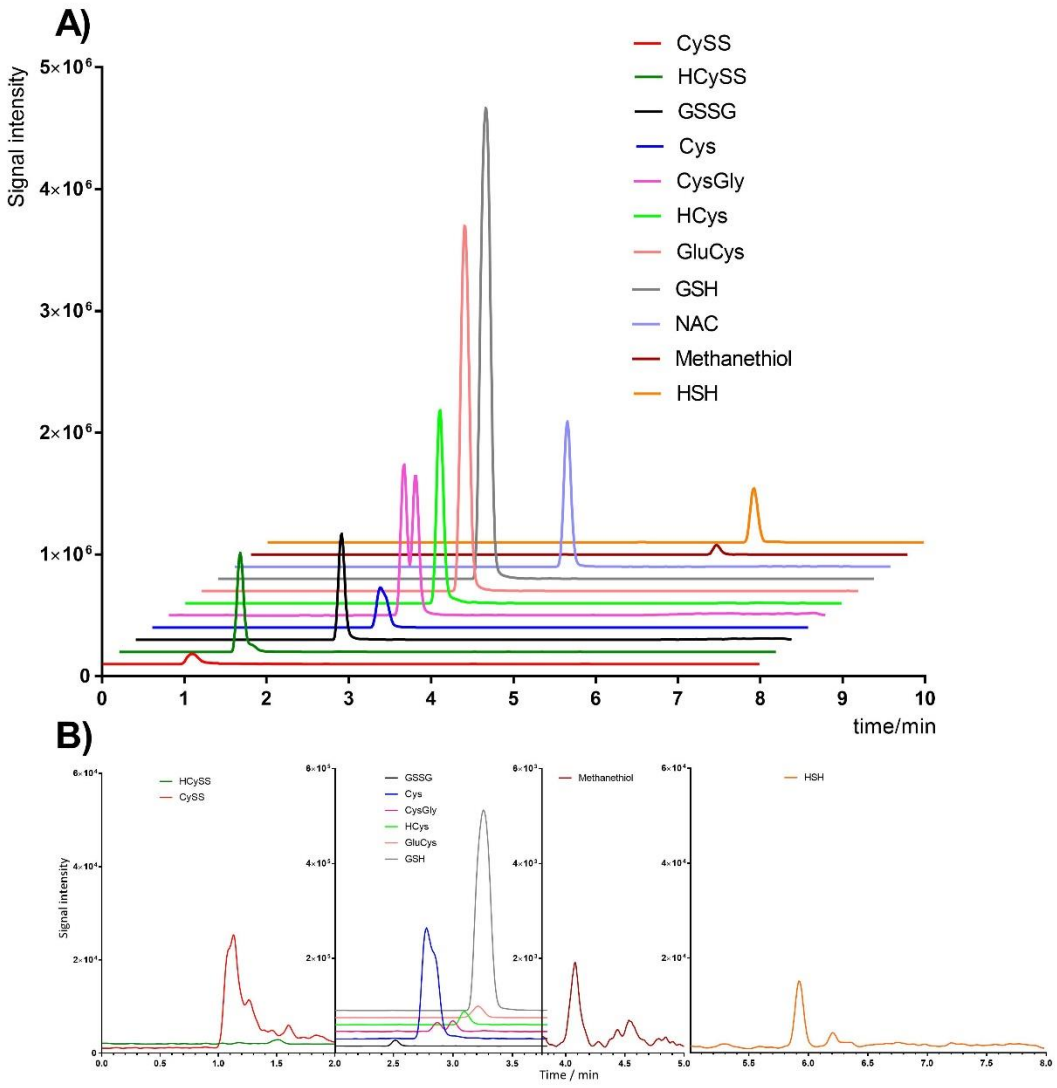


Figure 8: Exemplary chromatographic separations using the final developed method on a mixed mode aqua modus column in both a standard mixture (**A**) and in human plasma (**B**). **A)** Chromatographic separation and selective detection by tandem mass spectrometry of authentic stock solutions of all analytes using full registration for the entire run. **B)** Representative chromatogram of the same analytes at their natural abundance in human plasma using specific MRM time windows for selected groups of compounds[293]. MRM settings used are listed in **Table 2** and chromatographic conditions in **Table 1**.

3.3.1.3 Internal Standards

Three different stable isotope labelled internal standards were required to cover each of the classes of compounds analysed. As NEM enhances ionisation in ESI[123] one stable isotope labelled internal standard was selected to cover the oxidised thiols, which do not react with NEM. A second internal standard was selected to cover the reduced thiols, which all react with one NEM

adduct, and a third internal standard was used to cover the sulfide and related analytes, which react with two NEM adducts. For the oxidised thiols $^{13}C_4{}^{15}N_2$-GSSG was used, and for the reduced thiols $^{13}C_2{}^{15}N$-GSH was reacted with NEM to give $^{13}C_2{}^{15}N$-GSH-NEM for use as the internal standard (see **Figure 9** for structures). On addition of NEM derivatised stable isotope labelled GSH, and GSSG to plasma which had not yet been treated with NEM a rapid loss of internal standard was observed. This is consistent with the known dynamics of glutathione in blood[304]. Treatment of the plasma with NEM before addition of the internal standard prevented this loss. Therefore, the internal standards were only added to plasma and the other matrices analysed after they had been reacted with NEM for at least 10 min consistent with the developed reaction conditions (section 3.2.2). This prevented loss of internal standard even after deproteinisation.

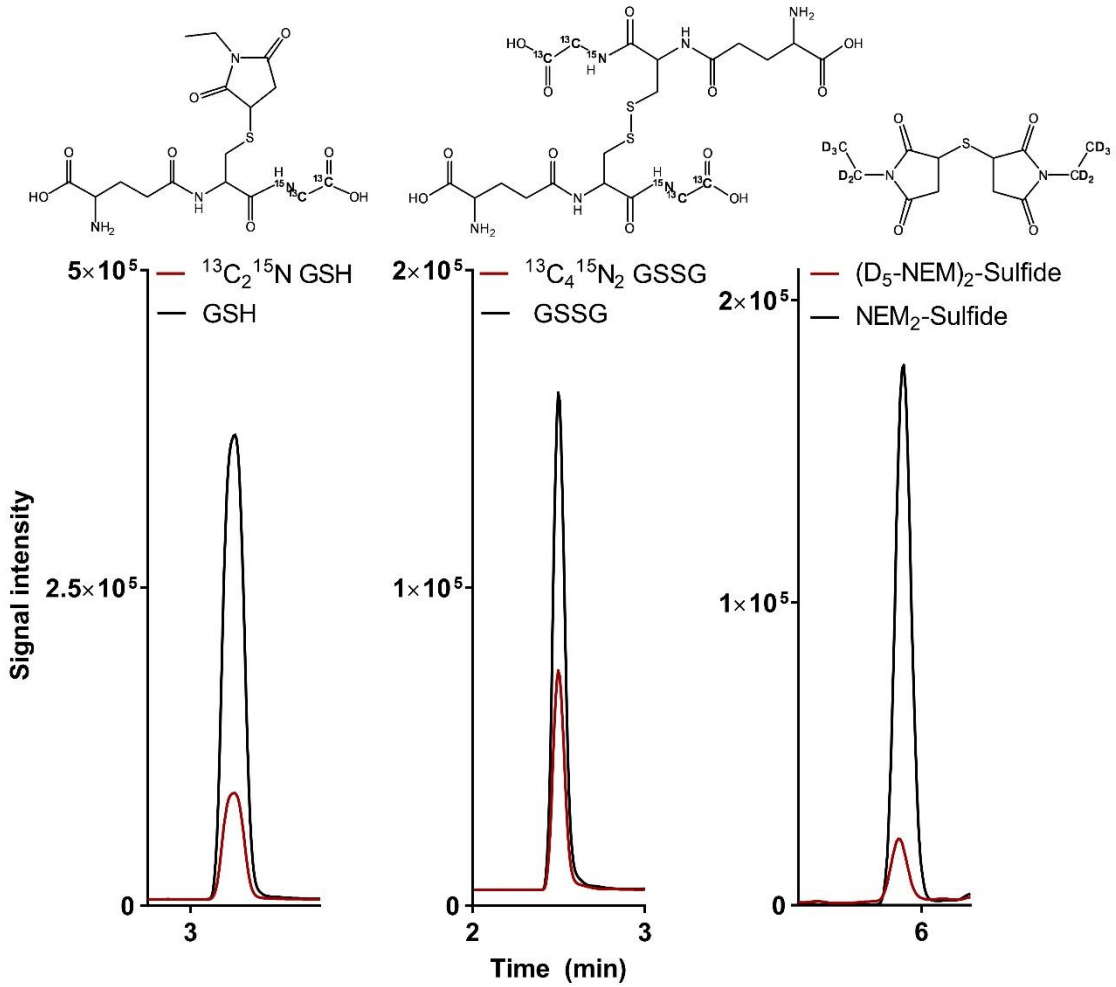


Figure 9: Stable isotope labelled internal standard chromatograms for the three classes of compounds analysed, $^{13}C_2{}^{15}N$-GSH for the reduced thiols, $^{13}C_4{}^{15}N_2$-GSSG for the oxidised thiols and D_{10}-NEM_2-Sulfide for the sulfide. The structure of each standard is shown above the relevant peak. The ratio of the peak area/internal standard peak area is used for quantification of analytes in samples. Final concentrations of the internal standards in samples were 100 nM GSH, 200 nM GSSG and 100 nM sulfide.

Developing a stable isotope labelled standard for the sulfide proved more challenging. The most commonly available stable isotope for sulfide is ^{34}S, which is only different by two *m/z* units from ^{32}S. Initial studies using ^{34}S-$(NEM)_2$ showed that the degree of ion suppression of the sulfide adduct in a variety of biological matrices was different on occasion from that of the GSH internal standard adduct, justifying a separate internal standard for sulfide. However these experiments highlighted the issue with using a stable isotope labelled standard so close in mass to the natural molecule. The natural abundance of the ^{34}S isotope (4.2%) means that high concentrations of endogenous sulfide can lead to interference in measured signal for both the internal standard and the endogenous sulfide. This required the use of cumbersome and inaccurate mathematical correction factors. A more appropriate internal standard was therefore developed, a pentadeuterated NEM analogue, N-ethyl-D5-maleimide (D_5-NEM) was reacted with sulfide (see 3.2.1.3 for details) to produce the stable isotope labelled $(D_5\text{-NEM})_2$-S which has a much larger difference of 10 *m/z* units (see **Figure 9** for the structure). This internal standard coeluted with the regular NEM adduct of sulfide (**Figure 9**) without showing any overlap in signal.

A mixture of $^{13}C_2{}^{15}N$-GSH-NEM, $^{13}C_4{}^{15}N_2$-GSSG and $(D_5\text{-NEM})_2$-S prepared in ammonium phosphate buffer pH 7.4 was used for the internal standard and added to the external standard curve as well as all samples as early on during sample preparation as possible. Specific MRMs were established for each of the internal standards (**Table 2**). A final concentration in the external standard mixture or sample of 100 nM GSH, 200 nM GSSG and 100 nM sulfide was used. The ratio between the signal intensity of each analyte and its corresponding internal standard was used for quantification in the biological matrix.

3.3.1.4 Linearity, Precision, Limits of Detection and Reproducibility

All analytes showed excellent linearity over a wide range of concentrations (>3 orders of magnitude), with overall r^2 values of between 0.994 and 0.999 (**Figure 10**).

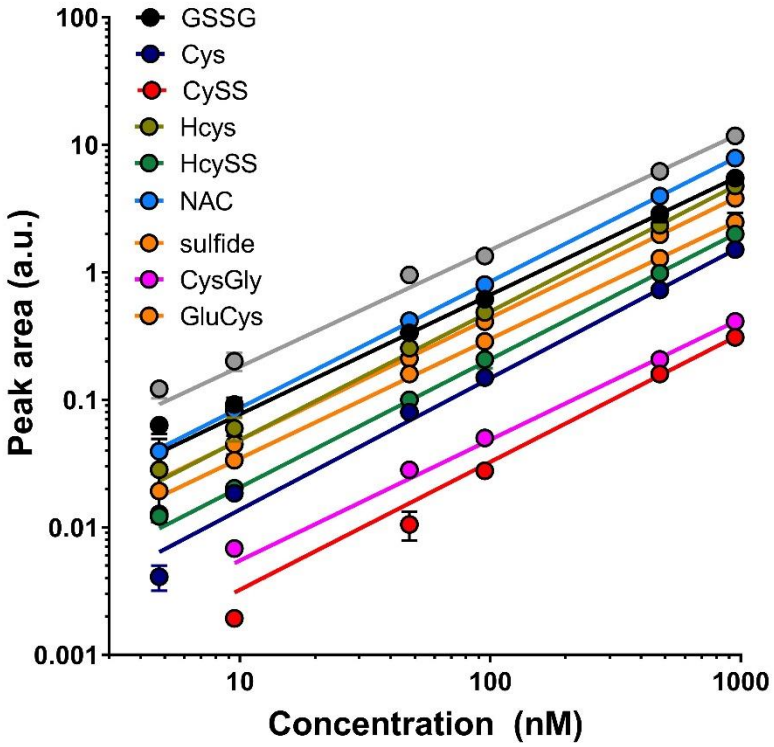


Figure 10: Linearity of detector response for main analytes (n=3) over a range of 5 nM – 1 µM representative of the concentrations of analytes in biological samples after dilution for analysis (see section 3.2.4 for details). All analytes show excellent linearity over the range measured with overall r^2 values of between 0.994 and 0.999[293].

Using an injection volume of 5 µl the sensitivity limits achieved were all in the nanomolar range, differing by class of compound and structure. NEM enhances ionisation efficiency for ESI so it is not surprising that the sulfide with two NEM adducts has the best sensitivity followed by the reduced thiol NEM adducts and the oxidised thiols (no NEM adduct) with the least sensitivity. Limits of detection and quantification, as defined by a signal-to-noise ratio of >3 and >10 respectively, are listed in **Table 2**. The majority of analytes included in the method could be readily detected in fresh human plasma and quantified without problems in each sample measured. Some analytes were not detected in plasma but were found in other sample matrices as detailed later. Between-run reproducibility was tested for a pooled plasma sample over several days and was better than 20% overall.

3.3.2 Optimisation of NEM Derivatisation Reaction Conditions

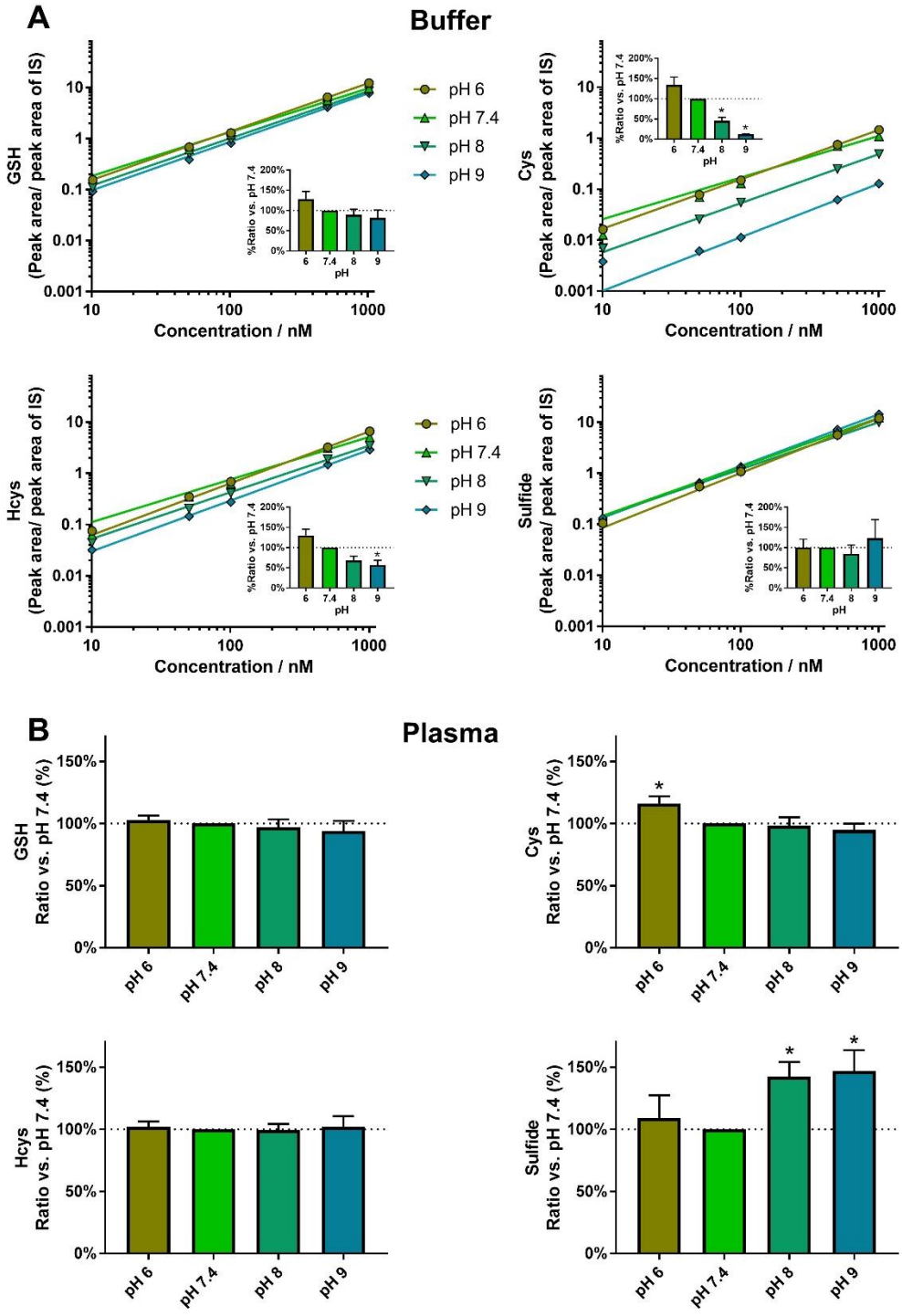


Figure 11: Effects of pH on the measurement of aminothiols and sulfide using NEM[293]. The effect of pH was investigated in a simple aqueous buffer (**A**) and in plasma (**B**). **A)** Standard curves were prepared at four different pHs, 6, 7.4 (physiological), 8, and 9, using NEM in ammonium phosphate buffer at the appropriate pH. In the inset graphs, the percentage change in peak area/internal standard peak area relative to the pH 7.4 buffer is shown for the highest standard, 1 μM, on a linear scale. The

largest impact of pH is seen for Cys with a tenfold decrease in peak area from pH 6 to pH 9, sulfide shows the least difference between the different pHs. Data are from 2 independent measurements. Inset, for GSH and sulfide the difference between groups was not significant. **B)** The percentage changes in peak area/internal standard area is presented relative to those at pH 7.4. Contrary to the analysis in buffer (**A**) sulfide shows the largest difference in measured peak areas with a large increase above pH 7.4 as well as a slight increase at pH 6. Data are from 6 independent samples. For GSH, and HCys the differences among the groups were not significant.

To avoid additional sample preparation steps reaction conditions were optimised to perform well under biologically relevant conditions. Human blood plasma does not often vary from a pH of 7.4 under most physiological conditions and is naturally buffered to maintain this pH. At physiological pH NEM reacts rapidly with the thiols and sulfide as confirmed experimentally (see Chapter 5: for details). However, the pH still can have a significant effect on the alkylation of thiols by NEM, and in previous studies a range of pH values from 6.5-7.5 have been used. Selected analytes were used to test the effect of pH on the thiol alkylation reaction from pH 6 to 9 in simple aqueous buffer systems as well as by additional incubations of NEM with human plasma at the same pH values (**Figure 11**).

There are clear differences in the reactions of the different thiols with NEM at different pH's, whilst little difference was observed for the reaction of sulfide and NEM. For GSH only small differences were observed, with larger differences seen for Hcys and the largest differences with Cys. For Cys there was a 10-fold difference in the measured peak areas (representative of concentration) between pH 7.4 and pH 9. When NEM made up in buffers of different pH was added to human plasma, the measured changes in peak area were less pronounced than the changes for the thiols in simple aqueous buffered solution. Nevertheless, a few differences were noted; peak areas decreased slightly with increasing alkalinity for GSH and Cys with a much greater increase for sulfide. A small increase compared to physiological pH was also observed for Cys and sulfide upon acidification. All further experiments were therefore carried out using NEM containing stock solutions kept at pH 7.4. Since the physiological pH of plasma, saliva and cellular samples is almost always pH 7.4 there is no need to add a stabilising buffer for these compartments or to alter the pH. However, the pH of urine may vary from slightly acidic to alkaline, depending on nutritional habits and metabolic status; thus, checking pH after addition of NEM-containing buffer would be advisable.

3.3.3 Optimisation of Sample Preparation

The most commonly used commercially available blood tubes (EDTA, heparin, citrate and serum vacutainers) were tested for their suitability for thiol and sulfide analysis using blood collected from three individuals. The suitability of these tubes were assessed as if this method is to be applied to clinical studies there may not be a choice of blood tube and so the effect of different tubes is important to understand. Plasma from blood collected in citrate tubes had greatly reduced sulfide levels compared to the other anticoagulants. The citrate plasma also had lower levels of GSH, Cys and CysGly than the heparin and EDTA plasma. Blood collected in heparin tubes showed slightly higher levels of GSH and sulfide compared to EDTA, but lower levels of Cys and CysGly. Serum tubes were found to be wholly unsatisfactory for measurement of physiological levels of thiols and sulfide as they showed greatly reduced concentrations of the majority of thiols measured (except GluCys) and sulfide (**Figure 12, A**). Whether this was due to excessive protein thiolation due to the coagulation process remains speculative and was not investigated further. Serum and citrate tubes are clearly unsuitable for thiol redox measurements. EDTA and heparin tubes both gave similar levels of each of the analytes tested, EDTA however has the advantage of being a metal chelator and therefore should reduce oxidation of thiols my metal ions removing the need for addition of a separate metal chelator. All further validation experiments were therefore carried out using EDTA as anticoagulant.

Due to the rapid oxidation of reduced thiols when samples are exposed to the air as well as the volatility of sulfide, it should be apparent that the sooner NEM is added to the sample the better. Therefore, when collecting blood samples for plasma and red blood cell analysis NEM was added immediately after collection before any other sample processing took place. As anticipated, when addition of NEM to whole blood was delayed lower levels of GSH and CysGly were detected with increasing time (**Figure 12, B**). The optimal concentration of NEM to alkylate fully the compounds of interest was investigated. The absolute levels of the thiols and sulfide cannot be known before analysis. Additionally, the most abundant thiol in human plasma is the single free sulfhydryl group of Cys-34 of serum albumin[39], whilst it is not analysed in this method it will still react with NEM and therefore reduce the amount available to react with the analytes of interest. To ensure that all of the low-molecular weight thiols and sulfide are fully alkylated a range of NEM concentrations (1-20 mM final concentrations) were tested (**Figure 12, C**). For the majority of the analytes there was no significant change in the measured concentrations between 1 and 5 mM NEM. Above 5 mM NEM (added to whole blood) however there were large increases in the measured levels of GSH and sulfide, whilst the concentrations of the other thiols were not significantly affected or for Cys and Hcys reduced. Sulfide levels showed the largest variation, tripling between 1 mM NEM and 10 mM but with less of an increase between 10 and 20 mM.

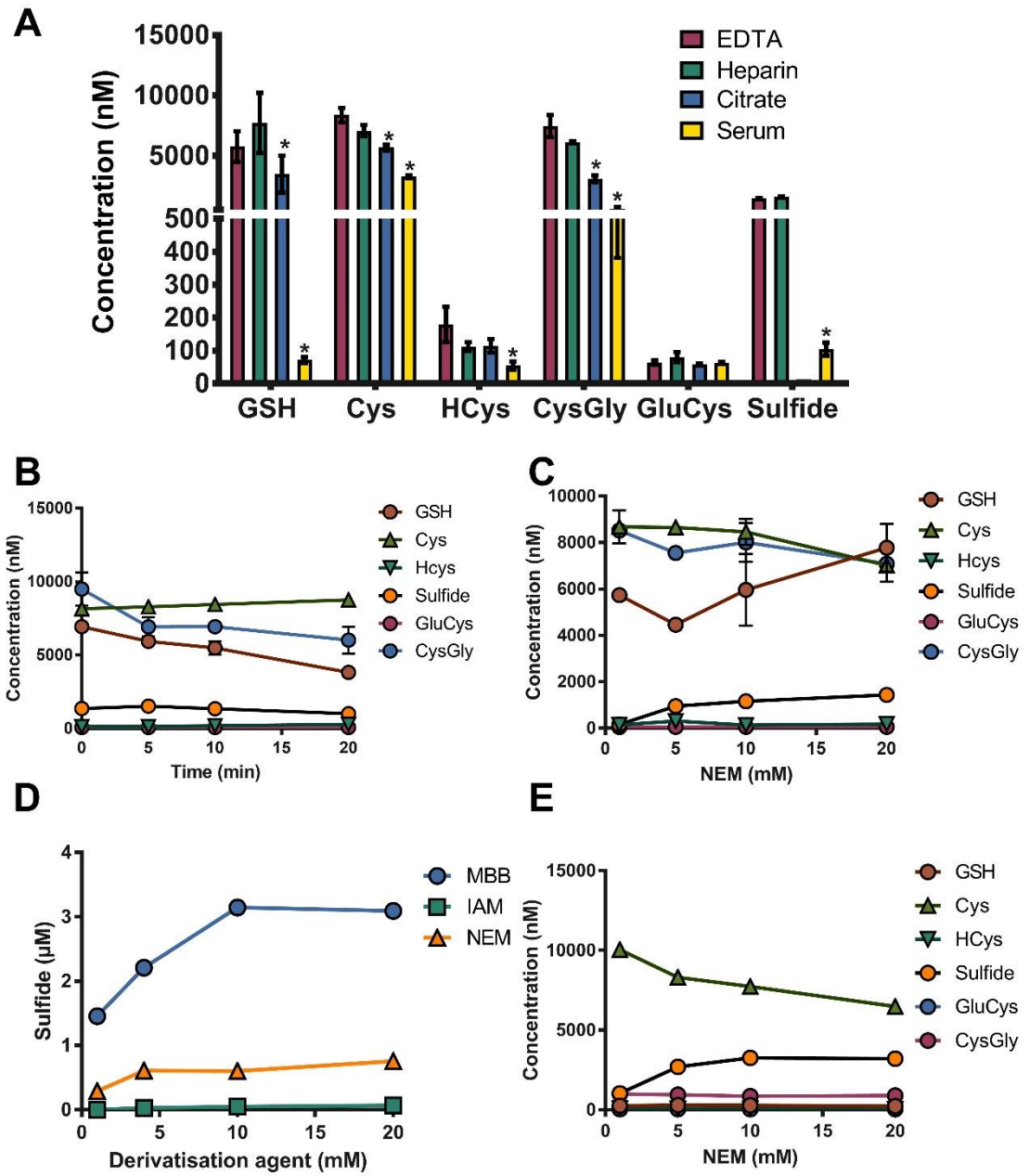


Figure 12: **Optimisation of sample preparation procedure: anticoagulation, and stabilisation with NEM[293].** For all experiments **A-D**, NEM (or other thiol-alkylating agent) was added directly to whole blood after which the sample was centrifuged at 800x g for 10 min at room temperature. In experiment **E** NEM was added to plasma directly after separation from blood cells by the same centrifugation protocol. **A)** The choice of anticoagulant affects the concentrations of thiol measured; because of its metal chelating properties EDTA is the most suitable anticoagulant for assessment of the thiol redox metabolome. **B)** Delay in addition of NEM to whole blood leads to progressive decreases in GSH and CysGly concentrations as compared to time = 0. **C)** The apparent concentrations of GSH and sulfide increase with increasing

concentrations of NEM when added to whole blood. **D)** The concentration of sulfide detected increases with increasing concentrations of alkylating agents added to whole blood**. E)** The NEM concentration-dependent increases of GSH levels were not observed when NEM was added to plasma (although absolute GSH concentrations were considerably lower), suggesting that these increases were largely due to leakage of GSH from blood cells; however, apparent plasma sulfide concentrations still increased with increasing NEM concentrations, suggesting removal of sulfide from bound forms in plasma at elevated concentrations of the alkylating agent. For all panels data were obtained by analysis of two independent biological samples taken from different human individuals (mean ± SD); measurements were carried out at least in duplicate.

Surprisingly, at the two highest concentrations of NEM tested (10 and 20 mM), the measured plasma GSH concentration was much higher than the ranges reported in the literature[44]. When different concentrations of NEM were added to plasma after centrifugation, rather than whole blood, the increase in GSH and sulfide concentrations were much lower (**Figure 12, E**). This difference implies that the increase plasma levels of these two analytes with higher NEM concentrations are an artefact related to the presence of red blood cells during the addition of NEM. This effect was also noticeable for sulfide when utilising alternative alkylating agents such as MBB and IAM (**Figure 12, D**). A comparison of the three alkylating agents showed that IAM gave the lowest measured levels of plasma sulfide whilst MBB gave measured levels of sulfide over five times greater than both IAM and NEM. This shows that the choice of alkylating agent can have significant impacts on the measured levels of sulfide and potentially other analytes.

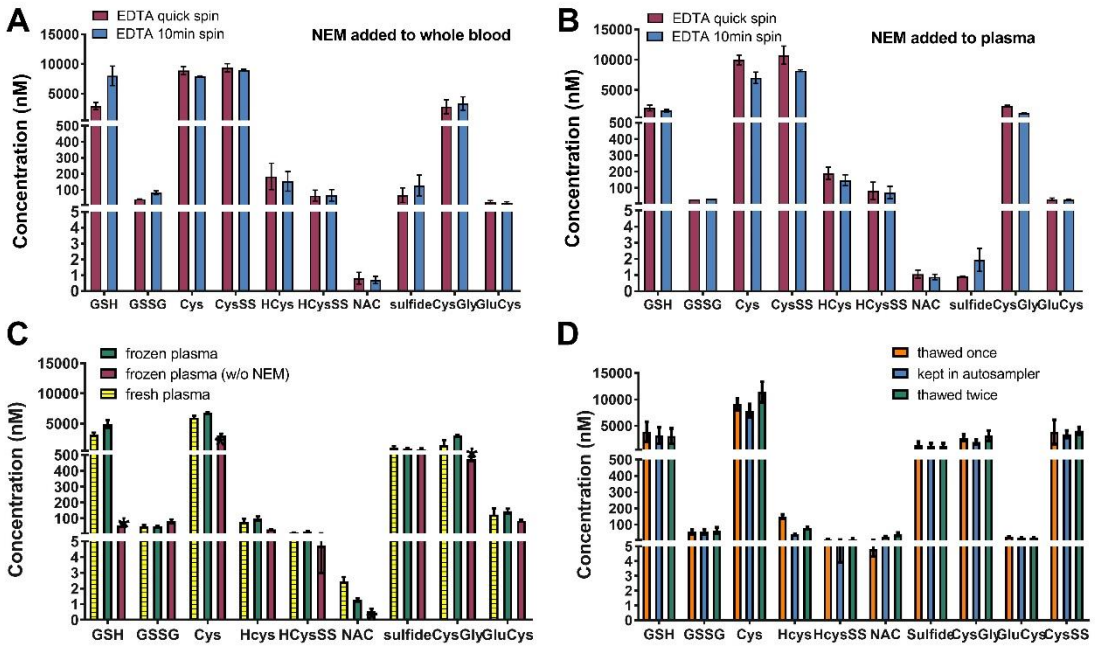


Figure 13: Optimisation of sample preparation procedures: **A**) and **B**) Addition of NEM before or after removal of red blood cells with two centrifugation methods. **C**) and **D**) Effect of freeze thawing and sample storage. **A**) When NEM is added to whole blood the centrifugation time and speed has a drastic effect on some of the measured thiols, particularly GSH and sulfide with the longer time/slower speed giving much higher levels. This is likely due to NEM induced leakage of these compounds from the RBCs. **B**) The same effect not being observed when NEM is added to plasma supporting this hypothesis. The longer centrifugation time and slower speed gives lower levels of thiols due to oxidation and other biological losses. **C**) Freezing of NEM stabilised plasma leads to an increase in GSH, Cys and CysGly concentrations compared to their levels in fresh samples; when samples were frozen without stabilisation by NEM concentrations of GSH dramatically decreased, and GSSG increased (n = 3). **D**) In samples stabilised with NEM, freeze/thawing or storage of samples in the thermostatted autosampler at 5°C did not affect the concentrations of the analytes measured (n = 3, differences among groups are not significant). For all panels 2 or 3 independent biological samples from different individuals were analysed; measurements were carried out in triplicate.

When NEM is added to the plasma, centrifuging the whole blood to separate the red blood cells from the plasma contributes to the delay before addition of NEM resulting in the oxidation and loss of thiols and sulfide (**Figure 12, B**). On the other hand, adding NEM to the whole blood before centrifugation artifactually increases the measured levels of GSH and sulfide (**Figure 12, C**). Therefore, a rapid centrifugation protocol was tested spinning at 3000x g for 1 min (**Figure 13, A**

and **B**). No haemolysis of the RBCs was detected as assessed by UV-vis spectrophotometry of the plasma obtained after centrifugation by our collaborators in the Cortese-Krott research group[293]. It was again seen that when NEM was added to the whole blood before the 10 min centrifugation higher levels of GSH were detected, likely due to leakage from the RBCs. With the shorter centrifugation protocol lower levels of GSH were detected, but these were much more in line with those measured when NEM was added to plasma after centrifugation (compare **Figure 13, A** with **B**). Sulfide levels were also higher when using the quick spin when NEM was added to whole blood versus plasma. There were not many other differences between the two centrifugation methods when looking at whole blood. Nevertheless, with plasma the quicker method generally gave higher levels of the reduced thiols, which is due to the shorter time before addition of NEM after sample collection.

It is unlikely that samples will be able to be analysed soon after collection and generally will need to be stored, especially in the case of large multicentre studies. Samples will therefore be frozen and in the case of whole blood this will be shortly after the red blood cells have been separated from the plasma. For some studies, the stored samples may not even be treated with NEM before freezing. Therefore we investigated the effects of freeze/thawing samples on the concentrations of the different analytes both with and without NEM treatment before freezing (**Figure 13, C** and **D**). When plasma was frozen without NEM there were some significant decreases in the levels many of the analytes, GSH, Cys, Hcys, NAC and CysGly with lesser decreases observed for Hcys, HcySS and GluCys. There was also an increase in the concentration of GSSG after one freeze/thaw. When plasma was frozen with NEM however, some of the analytes in fact increased in concentration after one freeze/thaw including GSH, Cys, and CysGly with the only significant decrease occurring for NAC (**Figure 13, C**). In further comparisons, using NEM stabilised plasma there were no significant changes in analyte concentrations between identical samples that were either stored for an extended time at 5^{o}C in the autosampler of the UHPLC, stored at -80^{o}C and freeze/thawed once or twice (**Figure 13, D**).

Following these results, in order to best preserve the natural speciation of the thiols and sulfide in plasma NEM was added to a concentration of 10 mM as soon as feasibly possible after collection of the blood in EDTA tubes and coupled with the shorter centrifugation protocol. As long as plasma samples have been stabilised with NEM before freezing then they can be stored without significant decreases in analyte concentrations even with multiple freeze/thaw cycles though absolute values may vary slightly from those measured on immediate analysis of samples.

3.3.4 Optimisation of Total and Bound Thiol Analysis with DTT

Total amounts of a specific thiol in a sample can be determined by reduction of all disulfides by DTT before addition of NEM, whilst the corresponding free thiol is assessed in samples untreated with DTT. The bound thiol is then obtained by calculating the difference of total thiol minus free thiol. First, to ensure complete reduction of all disulfides (including sulfide) we determined the minimally concentration of DTT needed for full reduction and the optimum incubation time required to achieve the highest final concentration of thiols and sulfide in the reaction solution. Select key analytes were used to monitor the effect of different DTT reaction conditions (**Figure 14**). Addition of DTT to plasma resulted in much higher measured concentrations for each of the analytes monitored as expected. There is a general increase in analyte concentrations with increasing DTT concentrations from 10 – 50 mM. At 100 mM of DTT sulfide increases in concentration but Cys remains the same whilst GSH and Hcys both decrease in concentration. There is little impact on the concentrations of the analytes when using reaction times between 5 – 20 min. A reaction time of 30 min however results in a drop in both GSH and Cys concentrations.

Therefore, for complete reduction plasma was treated with 50 mM DTT and left to react for 10 min at RT (**Figure 14**). Similar results were obtained by analysing other biofluids, including saliva and urine, whereas human blood cells required 100 mM DTT and 30 min incubation for complete reduction. There were no significant differences in the final concentration of thiols in plasma samples reacted with NEM before reduction compared with samples reduced with excess DTT and then reacted with NEM. This means that any sample collected for measurement of free thiols can also be utilised to measure the total and bound thiols.

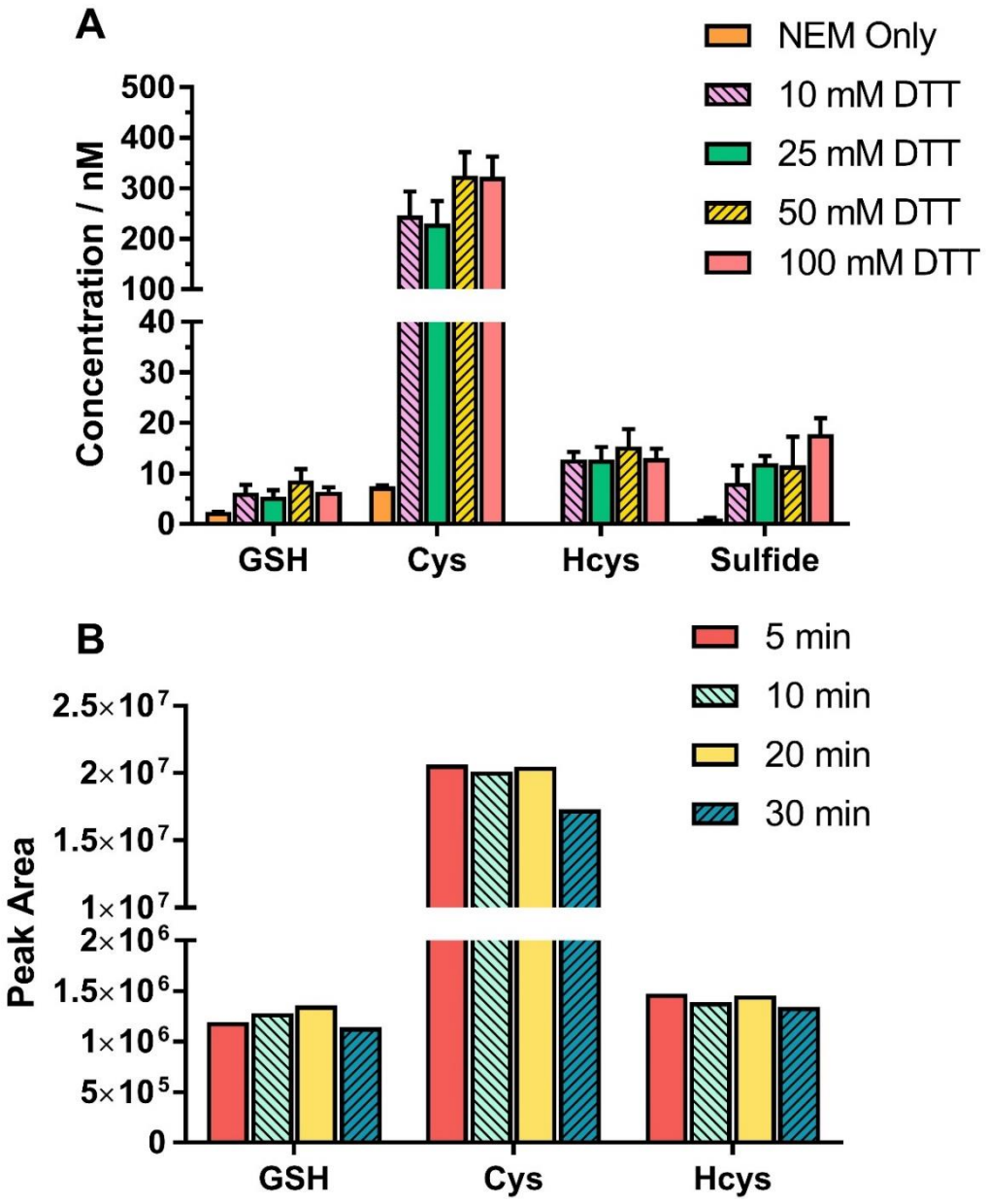


Figure 14: Optimisation of DTT reduction conditions, in each experiment key analytes were chosen to monitor the effect of different reaction conditions. **A)** Optimisation of DTT concentration required to fully reduce a plasma sample using a reaction time of 10 min (Data shown representative of n = 3 samples collected from separate individuals). Increasing the concentration of DTT from 10 – 50 mM results in an increase in all of the analytes, further increasing it to 100 mM only results in an increase in sulfide concentration with the other analytes remaining the same as 50 mM or decreasing. **B)** Optimisation of reaction time required for full reduction of a representative plasma sample using a DTT concentration of 50 mM. Increasing the reaction time from 5 – 20 min does not have much of an impact on the concentrations of the analytes tested. A reaction time of 30 min results in a drop in GSH and Cys concentrations.

3.3.5 Analysis of Low Molecular Weight Thiols and Sulfide in Plasma

As a proof of concept study the steady-state concentrations of free reduced and oxidised thiols as well as the total thiol content in plasma of 10 healthy human volunteers were determined (**Table 3**). As previously established, the difference between total thiol concentrations and the sum of free reduced/oxidised thiols corresponds to the amount of bound thiols[97]. Overall, the measured concentrations in the ten subjects correspond to the previously reported values in the literature[39]. The distribution of the circulating concentrations among healthy human volunteers however shows additional information that is not obvious from just looking at the average values. Firstly, the concentrations of free thiols vary significantly between individuals, sometimes up to 2-3 fold or in some cases as much as 10-fold. Secondly, the ratios between reduced and oxidised thiols (the "redox ratio") vary considerably between glutathione, cysteine and homocysteine. Thirdly, the concentrations of bound thiols are generally higher than the circulating concentrations of free reduced thiols. This is particularly noticeable for GluCys and sulfide, with the bound sulfide exceeding free sulfide by a factor of 100. Neither persulfide nor methanethiol were detected in any of the plasma samples. When NEM treated persulfide (S_2^{2-}) was tested in a standard mixture it was found not to be stable over time, representing a significant limitation that cannot be avoided whilst using NEM as the alkylation agent.

Table 3: Free, total and bound concentrations of the 10 aminothiols and sulfide measured in human plasma from 10 individuals and redox ratios of the key redox pairs. *The measured concentrations of CySS are not physiologically accurate due to poor peak shape and ion suppression. Columns 1–10 show the mean ± SD of 3 technical replicate measures performed on plasma from 10 individuals. Outliers were identified by our collaborators in the Cortese-Krott research group by applying the ROUT test, marked in *italics*, and excluded from descriptive statistics (mean ± SEM)[293].

		1	2	3	4	5	6	7	8	9	10	Mean ± SEM
GSH	Free / μM	6.34 ± 0.04	2.65 ± 0.05	1.40 ± 0.02	3.24 ± 0.01	6.36 ± 0.11	1.60 ± 0.04	2.26 ± 0.04	3.14 ± 0.08	6.25 ± 0.23	5.67 ± 0.18	3.89 ± 0.65
	Total / μM	9.56 ± 0.69	7.33 ± 0.94	5.48 ± 0.22	9.49 ± 0.32	9.26 ± 0.88	5.69 ± 0.38	5.78 ± 0.24	6.75 ± 0.47	8.42 ± 0.37	8.35 ± 0.45	7.61 ± 0.51
	Bound / μM	3.22 ± 0.66	4.68 ± 0.97	4.07 ± 0.21	6.24 ± 0.32	2.90 ± 0.98	4.09 ± 0.35	3.52 ± 0.23	3.61 ± 0.39	2.17 ± 0.18	2.69 ± 0.48	3.72 ± 0.36
GSSG	Free / nM	7.17 ± 0.34	*31.86 ± 2.55*	6.01 ± 0.40	9.76 ± 0.91	6.24 ± 0.89	*26.41 ± 2.24*	6.86 ± 0.81	5.00 ± 0.57	7.89 ± 0.53	7.80 ± 0.59	7.09 ± 0.51
GSH/GSSG	Redox Ratio	884	83	234	332	1020	61	329	628	792	726	509 ± 109
Cys	Free / μM	15.98 ± 0.54	22.3 ± 1.46	10.81 ± 0.47	17.72 ± 0.46	15.14 ± 1.15	12.65 ± 0.71	15.42 ± 0.62	13.50 ± 0.44	9.81 ± 0.34	11.28 ± 0.28	14.46 ± 1.18
	Total / μM	210.01 ± 26.96	198.81 ± 13.25	188.68 ± 1.82	177.75 ± 5.52	195.03 ± 4.37	242.2 ± 8.76	155.08 ± 0.48	215.78 ± 11.31	125.86 ± 12.10	168.66 ± 10.73	187.8 ± 10.43
	Bound / μM	194.03 ± 27.48	178.59 ± 22.71	177.87 ± 1.66	160.02 ± 5.46	179.9 ± 5.45	229.55 ± 8.20	139.66 ± 0.79	202.28 ± 10.88	116.04 ± 11.83	157.39 ± 11.00	173.5 ± 32.26
CySS*	Free / μM	*14.90 ± 0.69	*15.10 ± 0.34	*11.42 ± 0.05	*12.45 ± 0.43	*13.22 ± 0.32	*19.96 ± 5.28	*9.06 ± 0.27	*14.78 ± 0.63	*10.59 ± 0.69	*12.22 ± 0.49	*13.37 ± 0.96
Cys/CySS*	Redox Ratio	*1.07	*1.48	*0.95	*1.42	*1.15	*0.63	*1.70	*0.91	*0.93	*0.92	*1.12 ± 0.10
Hcys	Free / μM	0.15 ± 0.002	0.24 ± 0.004	0.13 ± 0.005	0.17 ± 0.005	0.17 ± 0.005	0.14 ± 0.006	0.14 ± 0.003	0.07 ± 0.001	0.12 ± 0.004	0.13 ± 0.002	0.15 ± 0.01

Hcys	Total / μM	8.62 ± 0.49	5.47 ± 0.46	5.21 ± 0.17	7.81 ± 0.21	5.65 ± 0.21	9.59 ± 0.33	6.02 ± 0.42	6.37 ± 0.09	4.40 ± 0.57	7.95 ± 1.31	6.71 ± 0.53
	Bound / μM	8.47 ± 0.49	5.23 ± 0.45	5.08 ± 0.16	7.64 ± 0.21	5.48 ± 0.21	9.45 ± 0.34	5.88 ± 0.42	6.29 ± 0.09	4.29 ± 0.57	7.82 ± 1.31	6.56 ± 0.53
HcySS	Free / nM	18.95 ± 0.78	15.92 ± 1.44	9.72 ± 3.54	22.08 ± 1.16	11.39 ± 0.74	29.08 ± 11.08	11.7 ± 1.39	5.94 ± 2.32	13.00 ± 2.01	14.22 ± 1.15	15.2 ± 2.12
Hcys/HcySS	Redox Ratio	8.05	15.36	13.06	7.54	14.84	4.64	11.76	12.15	8.95	8.86	10.52 ± 1.09
Sulfide	Free / μM	0.27 ± 0.020	0.29 ± 0.016	0.03 ± 0.001	0.28 ± 0.005	0.05 ± 0.001	0.22 ± 0.017	0.30 ± 0.013	0.06 ± 0.010	0.11 ± 0.011	0.04 ± 0.001	0.17 ± 0.04
	Total / μM	15.82 ± 3.78	15.83 ± 3.34	16.50 ± 2.92	15.45 ± 0.98	12.66 ± 3.73	16.09 ± 1.75	17.82 ± 0.60	16.35 ± 2.30	17.90 ± 1.85	19.55 ± 3.12	16.4 ± 0.58
	Bound / μM	15.55 ± 3.77	15.54 ± 3.35	16.46 ± 2.92	15.16 ± 0.99	12.60 ± 3.73	15.87 ± 1.76	17.52 ± 0.60	16.29 ± 2.29	17.79 ± 1.84	19.51 ± 3.12	16.23 ± 0.58
Cys-gly	Free / μM	1.47 ± 0.08	1.74 ± 0.10	1.12 ± 0.01	1.63 ± 0.06	1.91 ± 0.05	1.65 ± 0.05	1.41 ± 0.02	1.35 ± 0.02	1.64 ± 0.01	1.02 ± 0.05	1.49 ± 0.09
	Total / μM	27.38 ± 0.96	29.75 ± 0.42	24.28 ± 0.37	28.66 ± 0.48	34.13 ± 1.08	41.15 ± 1.18	28.31 ± 1.20	29.11 ± 0.18	19.61 ± 0.54	26.31 ± 0.60	28.87 ± 1.81
	Bound / μM	25.91 ± 0.94	28.01 ± 0.48	23.16 ± 0.37	27.03 ± 0.51	32.22 ± 1.04	39.50 ± 1.22	26.90 ± 1.18	27.77 ± 0.20	17.97 ± 0.53	25.29 ± 0.55	27.38 ± 1.78
Glu-cys	Free / μM	0.07 ± 0.001	0.09 ± 0.003	0.05 ± 0.001	0.09 ± 0.001	0.08 ± 0.002	0.08 ± 0.003	0.07 ± 0.004	0.07 ± 0.001	0.05 ± 0.002	0.08 ± 0.002	0.08 ± 0.004
	Total / μM	3.30 ± 0.21	2.62 ± 0.32	2.01 ± 0.41	3.02 ± 0.60	3.17 ± 0.82	3.18 ± 0.10	2.52 ± 0.11	2.34 ± 0.57	1.33 ± 0.23	2.69 ± 0.29	2.62 ± 0.19
	Bound / μM	3.23 ± 0.21	2.53 ± 0.33	1.96 ± 0.41	2.93 ± 0.61	3.10 ± 0.82	3.10 ± 0.10	2.46 ± 0.12	2.27 ± 0.57	1.28 ± 0.23	2.61 ± 0.29	2.55 ± 0.19
NAC	Free / nM	4.23 ± 0.30	5.48 ± 0.87	5.23 ± 0.15	4.40 ± 0.32	8.50 ± 0.12	4.69 ± 0.38	4.47 ± 0.18	5.12 ± 0.89	9.1 ± 0.07	*95.10 ± 2.42*	5.69 ± 0.61
	Total / nM	*428.94 ± 10.48*	35.04 ± 1.47	34.82 ± 0.79	36.32 ± 1.37	31.34 ± 1.22	34.71 ± 1.79	36.49 ± 2.08	42.77 ± 1.82	36.6 ± 2.69	*512.7 ± 5.27*	36.01 ± 1.14
	Bound / nM	*424.71 ± 10.77*	29.56 ± 0.70	29.59 ± 0.91	31.92 ± 1.07	22.85 ± 1.12	30.02 ± 2.06	32.02 ± 1.93	37.65 ± 2.21	27.51 ± 2.67	*417.6 ± 6.67*	30.14 ± 1.49

3.4 Discussion

A novel specific, highly sensitive and robust mass spectrometry-based approach has been developed for the measurement of the thiol redox metabolome capable of quantifying total and free thiols, and their corresponding disulfides as well and sulfide in complex biological matrices, initially focusing on blood plasma. The method has been carefully validated focusing on 1) the development of the analytical procedure, i.e. suitable detection and separation conditions, quantification strategy (external and internal standards, linearity, and versatility of the method); 2) optimisation of sample preparation, using blood as starting material (choice of anticoagulant, sample stabilisation, centrifugation and storage); 3) versatility of the method with examples of how further compounds of interest can be included for a more comprehensive coverage of the thiol redox metabolome in the future; 4) analysis of the thiol metabolome (including total and free thiols and their corresponding disulfides, as well as hydrogen sulfide) in plasma samples of 10 healthy human volunteers. Altogether, this validation approach has demonstrated that the developed method can be effectively used to characterise the redox metabolome in patient cohorts and animal models of disease. It can therefore be applied in clinical and translational studies in search for novel prognostic and diagnostic strategies for patient stratification; moreover, it may assist in identifying novel interventional approaches for the treatment of redox diseases[36].

3.4.1 UHPLC-MS/MS Method Development

3.4.1.1 Initial development on a Reversed Phase C_{18} Column

Initially, despite the challenges in analysing the small polar reduced and oxidised thiols and sulfide using reversed phase chromatography a workable method was developed. Sufficient retention and separation of the reduced thiols and sulfide was achieved utilising NEM derivatisation that improved retention and stabilised the analytes. This included the separation of CysGly from glutathione, which is particularly important due to the *m/z* 304 fragment produced from glutathione that has the same *m/z* and fragmentation as the precursor ion for CysGly. This causes a false peak for CysGly to be seen eluting at the same time as GSH, this requires sufficient separation to be able to distinguish between the true and false peaks. The addition of an NEM adduct to the reduced thiols improves their retention compared to the oxidised thiols. The addition of two NEM groups to sulfide likewise improves its retention even further eluting much later than the reduced thiols. This gave a rough elution order of oxidised thiols>reduced

thiols>sulfide. The oxidised thiols, which do not react with NEM, were poorly retained on the column due to their small polar nature.

Despite being sufficiently separated in a standard mixture (**Figure 7**, **A**) the oxidised thiols proved to be a challenge when applying the method to more complex biological matrices such as plasma (**Figure 7**, **B**). As they are not well retained and elute at, or close to, the solvent front they are affected by ion suppression and interference by matrix components. Additionally where CySS has a much higher concentration than HcySS and GSSG it can cause interference with the peaks and make quantification difficult. In order to obtain better separation for the majority of the analytes, especially the oxidised thiols, a high starting percentage of the aqueous mobile phase was required (>95%). Unfortunately using such a high amount of aqueous solvent is not ideal for a C_{18} column and can cause degradation of the peak shapes and a loss of retention after only a few samples have been run due to collapse of the stationary phase[191]. A regular column equilibration step and wash with organic solvent is needed for long sample runs to prevent this from happening, increasing the overall time required for sample analysis whilst potentially shortening the column lifetime. This additional step reduces the advantages of the reversed phase column over traditional normal phase chromatography.

3.4.1.1 Further Development Using Mixed Mode Type Columns

Due to the limitations of the reversed phase method alternate stationary phases were investigated. A stationary phase that provides the reversed phase type separation that works well for the NEM derivatised analytes combined with some polar separation characteristics to improve the retention of the oxidised thiols would be ideal. To this end I investigated mixed mode type columns which offer more than one interaction type which can allow separation of polar and apolar components within a single chromatographic run[296]. A Modus Aqua column was selected and used for the final method. This mixed mode column gave a similar separation pattern to the C_{18} column, with the oxidised thiols eluting first followed by the reduced thiols and then the sulfide. This reflects the reversed phase separation characteristics of the column and the effect the NEM derivatisation has on the retention time of the analytes. Additionally there was improved retention and separation of the oxidised thiols due to the polar characteristics of the column (**Figure 8**, **A**), with GSSG in particular eluting later. As well as the improved retention and separation mixed mode columns are far more stable with high amounts of aqueous mobile phase. This enables the chromatographic gradient to start at 99% aqueous, which further improves the chromatography, as well as reducing the need for equilibration and wash steps. This decreases the overall analysis time required for large sample runs. Unfortunately, CySS is still not very well retained on the column and elutes close to the solvent front. Whilst this is sufficient for the

analysis of standard mixtures in more complex samples such as biofluids this leads to interference by matrix components making quantification difficult. In order to improve the analysis of CySS alternate stationary phases will need to be investigated. Potentially coupling two columns in sequence with one being the Aqua Modus column and the other a more polar stationary phase could lead to improved separation of CySS whilst retaining the good chromatography for the other analytes. Coenzyme A was also not suited for analysis on this column, when tested it gave a very poor peak shape spread over multiple minutes. This poor chromatographic resolution is likely due to CoA having a polar head group with a more apolar tail, which is similar to the composition of the mixed mode stationary phase leading to multiple types of interaction. It is likely that a different type of stationary phase such as the reversed phase C_{18} column would be much better suited to analysis of CoA.

3.4.1.2 Internal Standards

The developed method effectively covers three classes of compounds within the thiol redox metabolome. The oxidised thiols which do not react with NEM, the reduced thiols, which react with one NEM adduct and sulfide(s), which react with two NEM adducts (**Figure 6**). Since NEM modifies the ionisation properties of the compounds it reacts with[123] each class of compound analysed will have different ionisation efficiencies, therefore three different stable isotope labelled standards were chosen, one to cover each of the classes of compounds analysed. For the reduced and oxidised thiols, $^{13}C_2{}^{15}N$-GSH-NEM and $^{13}C_4{}^{15}N_2$-GSSG were chosen. Reduced and oxidised glutathione were selected as the representative compounds for their classes as glutathione is one of the major intracellular antioxidants and the GSH/GSSG ratio in plasma and tissues widely regarded as the 'gold standard' for the assessment of systemic or cellular redox status. Initially NEM derivatised ^{34}S was used as the internal standard for sulfide. However, this internal standard was not suitable for quantification of the NEM derivatised sulfide. The natural abundance of the ^{34}S isotope (4.2%) and is only two *m/*z units higher in mass than ^{32}S which led to interference between the endogenous sulfide and the internal standard. A more appropriate internal standard was then developed utilising N-ethyl-D5-maleimide (D_5-NEM) to derivatise sulfide. This produced the decadeuterated $(D_5\text{-NEM})_2$-S as a NEM derivatise sulfide analogue with a difference in mass of ten *m/z* units. This internal standard eluted with the unlabelled sulfide without causing any signal interference.

The internal standards are added to each sample as soon as possible in the preparation process, for example for plasma after removal of the RBCs or after defrosting, to give a final concentration of 100 nM GSH, 200 nM GSSG and 100 nM sulfide standards in the final sample dilution. Exactly the same amount of internal standard is added to each of the concentration points in the

standard curve and the ratio of compound of interest peak area to internal standard peak area in the samples and standard curves is used for quantification. The labelled internal standards elute and should ionise in exactly the same manner as the unlabelled compounds but have a higher *m/z* and so can be distinguished in the mass spectrum. Using stable isotope standards allows correction for a number of factors including variability in dilutions, evaporation, degradation, recovery, adsorption, derivatisation, and instrumental parameters[267]. Since the absolute peak areas retuned by UHPLC-MS/MS can vary day to day due to any number of these factors, the use of internal standards is essential to be able to quantify samples accurately.

3.4.1 Optimisation of NEM Derivatisation Reaction Conditions

Reaction pH is known to be critical for thiol alkylation by NEM for a number of reasons. The pH plays a central role not only for reagent and product stability, but also because it affects the amount of thiolate available along with the pka of the SH group. It is a fortunate coincidence for analytical chemistry that the reaction of NEM with thiols proceeds optimally around the physiological pH, within in the range of 7 to 7.5. At pHs above ~7.5 NEM can potentially react with amines or undergo hydrolysis. Conditions either more acidic or alkaline than the physiological pH were found to affect the yield and/or the stability of the product and/or reaction to a different extent with each of the thiols tested (**Figure 11**). The most dramatic difference in the aqueous buffered mixtures occurs with Cys where an overestimation of up to a factor of 10 could occur. With the fresh plasma the largest difference occurs for the sulfide with increased levels of sulfide becoming available for reaction with NEM outside of pH 7.4. This could occur due to decomposition of metastable per/polysulfides or due to enhanced release of sulfide from non-covalent binding sites but this remains unclear at this stage. The effect of the pH in plasma is likely less so than for the standard mixtures due to the natural buffering capacity of plasma.

Fortunately, the physiological pH of plasma is 7.4 and acts as a buffered system without the need for any additional buffer to be added. At pH 7.4 the rate of reaction of NEM with amine groups is significantly slower than the rate of reaction with sulfhydryl groups[315] and so should not be a major concern at normal physiological pHs. Whilst hydrolysis of NEM will occur at pHs above 6[316] the rate of hydrolysis at pH 7.4 is much lower than the rate of reaction with aminothiols and sulfide (see Chapter 5: for more details on the rate of reaction of NEM). Hydrolysis is therefore not a concern for the quantitative analysis of these compounds using NEM[317]. It should however be noted that not all biological compartments will have a pH of 7.4, such as urine, or the same buffer capacity as plasma. For such samples care should be taken to make sure appropriate buffer is used to ensure the pH does not exceed 7.4 to avoid the issues discussed and ensure consistency in pH across samples as this may effect aminothiol speciation.

3.4.1 Optimisation of Sample Preparation

In order for the developed methodology to be used in translational studies[36] the optimisation for the analysis of thiols and sulfide was focused on biological matrices. This method could easily be applied to any number of different sample matrices but would have to be optimised and validated for each different matrix. Blood is a useful sample matrix to analyse as it is in contact with all tissues and is very likely to play a central role in systemic thiol and sulfide metabolism. Blood samples are also routinely taken meaning that it is a convenient and easily accessible sample matrix. We therefore focused on optimising the sample preparation for blood plasma but these procedures can be easily adapted to analyse other biological matrices[293]. In order to avoid interferences in the chromatographic separation and mass spectrometry, as well as to avoid changes in thiol speciation proteins were removed from samples by ultrafiltration using a centrifugal 10 kDa membrane. This method of protein removal offers the advantages over other protein removal methods of avoiding analyte dilution, pH changes, and addition of organic solvents, which interferes with the chromatographic separation.

It is known that the choice of anticoagulant used for blood collection can have a significant effect on the measured concentrations of the thiols and sulfide. It has previously been highlighted that the presence of metal chelators in the blood tube are important to help reduce artifactual oxidation after the blood has been collected[44]. Initial sample collection and preparation optimisation therefore focused on the type of anticoagulant used (**Figure 12, A**). As the developed method is intended for routine use in clinical studies the comparison of anticoagulants was limited to commercially available vacutainer tubes, heparin, EDTA, citrate and serum. NEM was added immediately to the tube after filling with whole blood, and before centrifugation using the initial 800 x g, 10 min method (see section 3.2.4.1). A final concentration of 10 mM NEM was used (volume of stock solution added depending on the volume of blood collected) which has previously been used successfully by the Feelisch group and others in the context of NO related research[318]. It was unsurprising that the serum tubes were not suitable for the analysis of reduced thiols. In order to collect serum the whole blood is allowed to coagulate at room temperature for 30 min which gives ample time for the reduced thiols to oxidise or be metabolised by enzymes present in the blood. The exact mechanism by which the reduced thiols are lost is not clear at this stage, there was no corresponding increase in the concentrations of the oxidised thiols seen so either the oxidised thiols are also lost by other mechanisms or the reduced thiols are lost by means other than oxidation. It is possible that both reduced and oxidised thiols could be lost in the coagulated component of the blood. As well as symmetrical oxidised species the reduced thiols could also form mixed oxidised species (such as the CysGly or GluCys), only some of which are currently included in the method, and could include the formation of protein-thiol disulfides.

Citrate tubes likewise are also not suitable for the analysis of sulfide and shows decreased levels of most of the thiols tested. It is likely that the low pH of the citrate tubes volatilises the sulfide before it can react with NEM and the lower pH will interfere with the reaction of NEM with the thiols and sulfide (section 3.4.1). There were not any significant differences between the EDTA and heparin tubes so EDTA tubes were selected for use as the metal chelating properties of the EDTA should help with sample stability by preventing metal ion catalysed oxidation.

The choice of protein removal method also required careful consideration. Biological fluids such as plasma are complex matrices, the constituents of which can potentially cause interference with chromatographic separation and analyte detection (for example by lowering ionisation efficiency or causing ion suppression). We opted to remove the majority of the proteins present in plasma by using a 10 kDa ultrafiltration centrifugal column. Many other method utilise a strong acid such as trichloroacetic acid or sulfosalicylic acid (SSA) for the deproteinisation step or metals like zinc sulfate/NaOH[319]. It is clear however that either of these methods will cause drastic changes in the pH which may affect the detection of sulfur species and sulfide[18]. In fact, sulfur species are well known to be particularly labile under acidic conditions though sulfide can also be released from thiols under alkaline conditions[18]. Additionally zinc sulfate is known to trap free sulfide and form a water-insoluble ZnS precipitate. Another widely used protein precipitation method involves the addition of organic solvent, such as MeOH, ACN, or acetone, to the plasma[319]. We observed that using organic solvents for protein precipitation decreases the recovery of GSSG. Furthermore, the high concentration of organic solvent in the sample interferes with the chromatographic separation as the start of the gradient requires a high percentage of aqueous mobile phase. The loss of chromatographic separation results in co-elution of analytes as well as increased interference by matrix components causing issues detecting and quantifying the analytes. Therefore, in order to quantify aminothiols and sulfide using the same sample processing procedure, removing the proteins by ultrafiltration, which neither changes the pH nor add any acid or solvent appears to be best practice to us for matrices such as plasma.

At high concentrations, NEM can also react with the amino groups of peptides/proteins and aminothiols[315]. The specificity of NEM relies heavily on the reaction conditions; the potential side reaction with amines does not pose a major problem as long as the pH of the reaction solution is kept near neutral. However, when reagent concentrations are greater than the levels we determined to be optimal for the majority of the analytes in a specific biological matrix (determined to be 10 mM NEM for plasma in this method) this may lead to lower apparent concentrations of some analytes. Cys in particular has been shown to undergo intramolecular transamidation to form a cyclic reaction product. This reaction can occur under alkaline conditions[315], and may explain the observed progressive decrease in apparent Cys levels when

increasing concentrations of NEM were tested (**Figure 12**, **E**) as well as the drop in reaction yield when the reaction pH was increased from 7.4 to 9 (**Figure 11**, **B**). A direct comparison of mBB, NEM and IAM (**Figure 12**, **D**) revealed that IAM resulted in the lowest sulfide levels detected, demonstrating that the nature of the alkylating agent applied as well as the concentration used can also have a marked influence on the levels of sulfide detected.

One unexpected observation was the large increase in measured plasma GSH and sulfide when using the two highest NEM concentrations (10 and 20 mM) in whole blood. The GSH concentrations were considerably higher than the range typically reported in the literature[44]. By contrast, GSH and sulfide concentrations increased to a much lesser extent when NEM was added to plasma after its separation from RBCs by centrifugation (compare **Figure 13, A** and **B**). This data suggests that the observed elevated plasma concentrations of these two analytes, when higher NEM concentrations were added to whole blood, were an artefact resulting from leakage and/or transport of material from blood cells. This phenomenon was not specific to this particular thiol alkylating agent since even higher levels of sulfide were found when mBB instead of NEM was used as thiol alkylating agent and added to whole blood under otherwise identical conditions. In order to avoid the leeching of GSH and sulfide from the RBCs and improved rapid centrifugation protocol was developed. Instead of centrifuging the whole blood for 10 min at 800 x g it was spun for 1 min at 3000 x g. The faster spin prevents the artifactual elevation of plasma GSH and sulfide when NEM is added to the whole blood before centrifugation (**Figure 13, A** and **B**). It also has an advantage when NEM is not added until after the RBCs have been separated as it reduces the time where reduced thiols can be oxidised or otherwise lost.

For the application of the established methodology in studies, especially when high sample numbers and multiple sites are involved, the stability of samples during storage and the effects of freeze/thawing are key to consider. For this reason, the investigation of the stability of the analytes both before and after derivatisation was key for the development of the method. This includes both storage in the refrigerated autosampler to ensure sample stability during long analysis runs as well as during storage whilst frozen which is essential when collecting large sample numbers over long lengths of time. The effect of multiple freeze/thaw cycles was also considered as repeat analysis of samples can also be important. The only analyte that significantly decreased after freeze/thawing was NAC which dropped by 30-50% after one cycle when compared to unfrozen samples. The other analytes in fact showed an increase in concentration after freeze/thawing. The reasons for this behaviour are not immediately obvious, but clearly the problem does not lie with the stability of the thiol-NEM adduct. One possible explanation for this behaviour may be the presence of other thiol derivatives that we are not currently detecting such as persulfides and polysulfides, if these compounds are unstable during freeze/thaw they could

decompose increase the apparent concentration of the thiols measured. However, levels of sulfide did not change under the same conditions, suggesting that the product of decomposition of these per/polysulfides might be a higher oxidised form not currently captured in this method. When an alternate deproteinisation method, addition of SSA, was used by our collaborators in the Cortese-Krott group as cross validation of the method, the same freeze/thaw effects were not observed[293]. This suggests that an alternative mechanism may be the cause of these observations. In theory, aminothiols may exist not only in free and protein-bound forms (which are DTT-reducible), but a fraction of them could also bind to proteins in a non-covalent fashion. When the protein conformation is changed by precipitation with acid/organic solvents or due to the freeze/thaw process, this non-covalent association is disturbed releasing the aminothiols, which can then react with excess NEM present and increase the measured concentrations. This could also explain the apparent differences in absolute concentration of analytes (which were independent of the detection method) when plasma is processed by ultrafiltration versus acid precipitation[293]. The idea of non-covalent protein binding of thiols is also consistent with the observed concentration dependence of NEM in plasma with greater amounts of NEM pulling aminothiols out from equilibrium with the proteins. It has been reported that H_2S non-covalently binds to albumin and haemoglobin[292], which is consistent with the concentration dependant behaviour of sulfide in our experiments. This hypothesis requires further investigations and potentially offers much greater insight into so far unrecognised thiol redox regulation by modulation of protein association.

3.4.2 Optimisation of Total and Bound Thiol Analysis with DTT

Not only can thiols exist as oxidised symmetric disulfides such as GSSG, CySS and HcySS (in vivo and formed artificially during sample handling), they can also form asymmetric mixed disulfides resulting in both low molecular weight and protein mixed disulfides. It has been proposed that these protein bound thiols represent either posttranslational modifications or that they can be involved in inter-organ thiol transport[36]. It is important to recognise that the formation of these asymmetric disulfides may mask the detection of a particular thiol when only assessing the concentrations of free thiols, especially when these asymmetric disulfides are formed after sample collection. The total (bound + free) concentrations of particular thiols may therefore be of interest for diagnostic or prognostic purposes, such as for total Hcys which is a widely used measure for cardiovascular disease risk factor and a stronger measure than the free Hcys on its own[114]. The total thiol concentrations are determined following the reduction of the covalent bond between the two components of the oxidised thiols. In order to obtain the total thiol concentrations in the different biological matrices tested (plasma, blood cells, urine, and saliva)

the samples were subjected to reduction with DTT. The minimal effective concentration of DTT to ensure complete reduction of all bound thiols and sulfide was determined as well as the incubation time required. Since NEM also reacts with DTT, any excess remaining after the reduction will reduce the amount of NEM available to react with the reduced thiols, which will be in higher concentrations than in un-reduced samples. It was therefore key to ensure that the concentration of DTT used was not so high as to reduce the effectiveness of the NEM derivatisation. For plasma, the final concentration of DTT used was 50 mM and incubation time was 10 min, for RBCs 100 mM DTT was used with an incubation time of 30 min. This highlights the importance of validating each step of the analytical procedure for the different matrices being analysed. For the analysis of plasma, the DTT was added to plasma that had already been treated with NEM. There was no significant difference observed in the final concentrations of the total thiols in samples treated in this manner when compared to plasma that was first reduced with DTT and then alkylated with NEM after reduction was complete. This demonstrates that in contrast to alkalinisation which leads to hydrolysis and ring opening of the thioether[320], the thiol-maleimide adducts formed are stable under acidic conditions.

3.4.3 Analysis of Low Molecular Weight Thiols and Sulfide in Plasma

The developed method was successfully applied in the analysis of free, total and bound low molecular weight thiols and sulfide in human plasma taken from ten healthy volunteers. Whilst the average measured values correspond generally with the values reported in the literature[39] looking at the distribution of the individual values reveals further information which would otherwise be hidden (**Table 3**). The levels of free thiols can vary significantly between individuals, with differences in a particular thiol not necessarily being reflected by the other thiols. This is also true when comparing the redox ratios (reduced/oxidised) of glutathione, cysteine and homocysteine, as within individuals the ratios can vary significantly. It was also found that the concentrations of bound thiols are generally higher than the circulating concentrations of free reduced thiols. These observations highlight the importance of looking at the wider redox metabolome rather than taking a smaller section as representative of the wider network. Although the redox metabolome is closely interlinked, changes in the individual components of the network are not necessarily reflected by the wider network. Likewise, if only the free or total thiols and sulfide are analysed then the major redox relevant component of the bounds thiols would be missed. The bound thiols may even provide further insight into the resilience of the redox network and provide a store for the thiols and sulfide thereby explaining why changes in one component of the network are not more widely reflected. These components of the thiol redox metabolome represent; 1) the redox status of a tissue/cell, according to the ratio of free

reduced/oxidised thiols and 2) the redox reserve (or total thiol status), which is a measure of the total reducing capacity of a cell/tissue or organism that defines its resilience to oxidative modification measured by the bound and acid labile components[293].

It is important to note that sulfide levels assessed here by NEM derivatisation are higher compared to methods using other protocols employing mBB[113,291].We found that the absolute "free" sulfide levels are strictly dependent on the alkylating agent used as well as the concentration, and this dependence may be linked to the chemistry of the reaction of the alkylating agent. Very different concentrations in measured sulfide concentrations were found with aliquots of the same sample using NEM, IAM or mBB (**Figure 12**, **D**). The reason for these differences is not yet clear and requires further investigation. Importantly, other reports have demonstrated that bromobimanes can extract sulfur from other sources[321], and that NEM reacts differently with polysulfides compared to IAM or IAA[143,322] (see Chapter 4: for more details). We also found that the levels of sulfide measured with MBB can vary significantly depending on the concentration of MBB used (**Figure 12**, **D**) suggesting that similar validation experiments for MBB as we have performed for NEM may be useful for these methods.

3.5 Conclusion

Many methods currently exist for the analysis of thiols and/or sulfide with many utilising a chromatographic separation coupled with mass spectrometry for detection[294,302]. Yet, many pitfalls still exist in the analysis of thiols and sulfide in cells and biofluids (some of which have been known for decades) and often appear to have been neglected. Many existing methods are somewhat limited in scope, some relying on reduction to measure the oxidised thiols (as the difference between total and reduced) but this does not reflect the true free oxidised thiols. Likewise, many methods do not cover the wider range of the thiol metabolome, especially sulfide and so do not provide a full picture of thiol redox status. These issues remain to be better characterised in order to avoid the enormous variations in GSH/GSSH ratios reported in the literature[297]. Even sophisticated, validated and robust methods for the simultaneous determination of glutathione and cysteine redox state[68] would appear to leave room for improvement as the pre-analytical sample handling is somewhat cumbersome and time-consuming, involving multiple steps, and the total assay time is rather long by today's standards. Many of the issues leading to the variation in reported concentrations may well be linked to poor sample handling and preparation procedures. The measured concentrations of thiols and sulfide cannot only be artificially decreased through oxidation or degradation (e.g. by enzymes) but can also be increased through alkylating agent induced leakage from cells or inadvertently liberated from storage forms. The platform technology we present here for the measurement of reduced

and oxidised thiols in combination with sulfide related metabolites is robust, sensitive and versatile. Its strengths include the possibility to measure the thiol redox metabolome including total and free thiols and their corresponding disulfides as well as sulfide in complex biological matrices.

A novel specific, highly sensitive and robust mass spectrometry-based approach has been developed for the measurement of the thiol redox metabolome capable of quantifying total and free thiols, and their corresponding disulfides as well and sulfide in complex biological matrices such as blood, saliva and urine. The developed method has been carefully validated at every step starting with the development of the analytical procedure (separation and detection) and quantification of the analytes using stable isotope labelled internal standards. The sample collection, preparation and storage procedures have likewise been carefully optimised using blood as the example matrix (including choice of anticoagulant, sample stabilisation, centrifugation and protein removal). The versatility of the method has been demonstrated through the incorporation of addition analytes such as methane thiol and the analysis of addition biological matrices such as urine and saliva. The developed method was then demonstrated using plasma samples taken from a small cohort of ten healthy volunteers successfully quantifying the total, bound and free thiols as well as their corresponding disulfides as well as hydrogen sulfide. The method can also be easily applied to the analysis of free, total and bound thiols and sulfide in red blood cells, saliva and urine as well as the acid labile components as demonstrated by other members of the Feelisch group[293]. The method can easily be combined with other compatible analytical procedures for detection of NO metabolites and nitrosospecies, using other established methods[10,318], utilising NEM for stabilisation of thiols.

It was important during method optimisation to ensure that workflows were simplified, from sample collection through to preparation and storage, realising there will be a need to translate critical steps into easy-to-follow standard operating procedures. This is important in particular when measurements are to be carried out in large numbers, human studies are run in a multi-centre fashion and/or biobanked samples are used. Lack of reproducibility and robustness or a very particular sample handling requiring specialist training could easily make a particular method wrongly appear unsuitable for use in clinical studies, or worse perhaps, lead clinicians towards incorrect treatment decisions. This method could easily be applied for stable isotope tracing studies to measure the flux and direction of metabolites through the thiol redox metabolome requiring only the addition of specific MRM scans for the labelled compounds or a more general mass spec measurement. Stable isotope labelled compounds could be monitored at any point of the metabolic pathways from the bloodstream through to excretion in urine and saliva. The flux of the isotope labels could likewise be monitored at any point of the metabolic pathway (**Figure 4**) as

intermediary metabolites, in oxidised protein bound and free disulfide storage forms, and as metabolic end products such as sulfide and methanethiol. All of the data taken together demonstrates that the approach developed here can be used to characterise the redox metabolome in patient cohorts and animal models of disease. It can therefore be applied in clinical and translational studies in search for novel prognostic and diagnostic strategies for patient stratification. Additionally it may assist in identifying novel interventional approaches for the treatment of redox diseases that have so far been overlooked and may help provide a deeper understanding on the systemic thiol redox status within the reactive species interactome[36].

Chapter 4: Pitfalls in the Analysis of Biological Polysulfides

4.1 Introduction

Further to my project hypothesis; Reactive sulfur species such as inorganic and organic polysulfides play an important role in redox regulation as well involvement in crosstalk in the reactive species interactome.

Polysulfides are highly reactive species that exist in an equilibrium in solution. Analysis of the polysulfides will disturb this equilibrium meaning that the results will reflect the nature of the analysis rather than the physiological distribution of the polysulfides.

In order to understand the biological relevance of these species first it has to be understood how, or if, analytical approaches alter polysulfide speciation. By doing so the role of organic and inorganic polysulfides in redox biology can be better investigated and understood.

It is only relatively recently that the biological importance of inorganic and organic polysulfides has been realised. Free inorganic polysulfides have been identified as biologically relevant signalling molecules[55,277], with H_2S_3 identified as being produced in the brain by 3-mercaptopyruvate sulfurtransferase (3MST)[148]. Other polysulfide species have also been identified, including H_2S_2 and H_2S_5, with their production potentially being controlled by other metabolic pathways[134]. Polysulfides have also been recognised as important mediators in sulfide based redox biology[10,40,283,323]. With these emerging biological effects it has become increasingly important to be able to accurately determine the speciation of these polysulfides which describes the distribution of the individual polysulfide species in solution. Only by being able to form an accurate picture of their biological distributions will we be able to better understand their biological actions and how the different polysulfide species may have different physiological effects. In fact, many mechanistic details of how polysulfides elicit or mediate these effects remain elusive, in part due to reliable analysis of these compounds continuing to prove a challenge.

The identification and quantification of per- and polysulfide species and their flux through biological systems is challenging in part due to the high reactivity of these compounds as well as the ability of polysulfide species to rapidly interconvert, existing in an easily disturbed equilibrium in aqueous systems. Many of the widely used techniques for the analysis of polysulfides now rely

on an electrophile in order to alkylate the polysulfides and make inferences on their distribution in biology and physiology. These electrophiles include *N*-ethylmaleimide (NEM), iodoacetamide (IAM) and monobromobimane (MBB)[133,322]. However, with an understanding of the chemistry involved in these reactions it is clear that different electrophiles could have differing effects on the measured speciation of polysulfides. If these species exist in an equilibrium system, a reactive electrophile may well disturb this system, NEM which reacts via Michael addition[324] could effect the equilibrium in a different manner to IAM or MBB which react via a S_N2 mechanism[107,325]. This would make it difficult to be certain that the true biological distribution has been trapped or if it has been altered by the alkylation reaction.

In order to assess and accurately quantify the physiological distribution of polysulfide species there is also a need for readily available standards that can be accurately prepared. Accurate standards are required not only for quantification but also essential to assess whether the chosen methodology is altering the measured species away from their physiological distribution. The most commonly used polysulfide standards are prepared by reaction of Na_2S or K_2S with elemental sulfur in either aqueous solution or sodium/potassium hydroxide[326,327], producing polysulfides of mixed chain length as well as other sulfur species such as thiosulfate. The unknown composition of these standards makes them unsuitable for the quantification of biological polysulfides. Pure sodium disulfide, trisulfide and tetrasulfide standards allow a known molar amount of polysulfide to be weighed out of a known chain length. However to be used as an accurate standard for quantification they need to be made up in aqueous solution and alkylated. These standards were tested to establish their suitability for use of identification and quantification of polysulfides in biological samples.

Using the findings from the experiments carried out, the crosstalk between reactive oxygen species metabolism and reactive sulfur species metabolism was investigated, examining the activity of the antioxidant enzyme superoxide dismutase around sulfide and polysulfides.

4.1.1 Direct Infusion Analysis of Aqueous Polysulfides

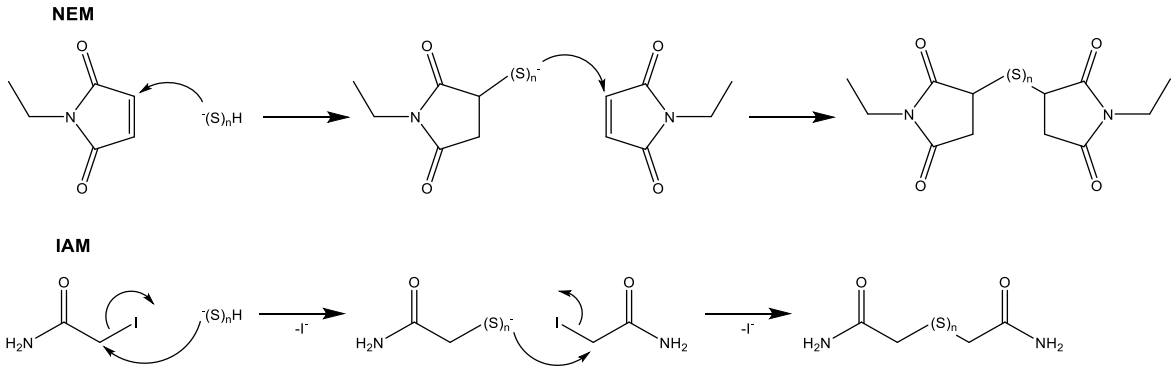
To investigate the speciation of inorganic polysulfides in solution both underivatised and derivatised aqueous inorganic polysulfide solutions were analysed by direct infusion into a triple quadrupole mass spectrometer (TQD-MS). By analysing the polysulfide species distribution before and after derivatisation the effect of the alkylation on this distribution can be observed. Pure sodium trisulfide, tetrasulfide and mixed potassium polysulfide were investigated to assess their suitability as standards for quantification and identification purposes as well as to determine if there was any observable difference in the speciation pattern in solution before and after

alkylation. The underivatised polysulfides were found to have *m/z* values closer to radical anion species rather than the expected hydropolysulfide anions. Each of the standards tested showed the same speciation pattern after dissolution both with and without alkylation. Alkylation of the polysulfides changes the measured speciation pattern, though it is unclear if this is due to the alkylation reaction or a difference in the ionisation between alkylated and unalkylated species.

4.1.2 High Resolution Mass Spectrometry of Aqueous Polysulfides

To confirm the tentative assignment of the polysulfide species assigned as radical anions in the low resolution direct infusion experiments using a TQD-MS, high-resolution time of flight mass spectrometry (ToF-MS) was used. The resolution of the ToF-MS[328] (calibrated to 0.5 ppm using sodium formate clusters) used is high enough to be able to distinguish the additional mass of the electron (0.00054 *m/z* units) in a radical ion and confirm the absence of any hydrogen polysulfide anions. These experiments strengthened the assignment of the underivatised polysulfide species as radical anions, although these species may form due to the mechanism of ionisation in the ESI source and exist as different species in solution.

4.1.3 UHPLC-MS/MS Analysis of Inorganic Polysulfide Speciation Following Chemical Derivatisation



Scheme 17: Alkylation of inorganic polysulfides by *N*-ethylmaleimide (NEM) and iodoacetamide (IAM). NEM reacts with nucleophiles such as polysulfides via Michael addition whilst IAM reacts through a nucleophilic substitution (S_N2) reaction[107,325].

Two different electrophiles, *N*-ethylmaleimide (NEM) and iodoacetamide (IAM) were investigated to ascertain their suitability for the analysis of polysulfides. NEM and IAM have different structures (**Scheme 17**), and reaction mechanisms[107,325], with a large difference in rates of reaction[324] that could influence the speciation of the derivatised polysulfides. The mechanism and rate of reaction will likely have a significant impact on polysulfide speciation. If the rate of reaction between the different chain lengths of polysulfide with the electrophile differs, then the

equilibrium between the polysulfide species and speciation could be altered by the derivatisation reaction and would no longer be representative of the true polysulfide speciation in vivo. Different reaction conditions were tested to establish if there was any effect on the polysulfide speciation.

Pure sodium disulfide, trisulfide and tetrasulfide salts, as well as mixed potassium polysulfide salts were tested under a variety of conditions. Important aspects of the alkylation of polysulfides including the electrophile used, the preparation method and the effect of different concentrations of electrophile were investigated in order to determine the impact on the analysis of polysulfides. The UHPLC-MS/MS method developed for thiol analysis (see section 3.2) was used to separate the different polysulfide species improving identification of the individual species as well as avoiding ion suppression and false signal that can be potentially generated by source fragmentation of the higher chain length polysulfides. UHPLC separation will also be important for the analysis of polysulfides in biological samples to avoid matrix effects.

It was found that the choice of alkylating agent can have a significant impact on the measured polysulfide speciation. NEM generally gave shorter chain length polysulfides whilst with IAM longer chain lengths are seen. As with the direct infusion experiments (4.1.1) the choice of starting polysulfide salt used in the reaction did not have an effect on the measured speciation. The preparation methods and reaction conditions were however found to have varying effects on the observed polysulfide speciation with both NEM and IAM. Ultimately, whilst the exact polysulfide standards can be accurately weighed out and prepared, the measured results can vary making their use for quantification tricky. For biological measurements it must be considered that the choice of electrophile, preparation method and reaction conditions can all influence the measured species and may not be representative of the physiological distribution of these species.

4.1.3.1 Impact of NEM on Polysulfide Chains

NEM derivatisation generally results in shorter chain length polysulfides being detected compared to IAM derivatisation for unclear reasons. Key differences between the two electrophiles are; NEM is a stronger electrophile and has a faster rate of reaction than IAM[322] with a different mechanism of reaction, NEM reacts with nucleophiles such as polysulfides via Michael addition[329] whilst IAM reacts through a nucleophilic substitution (S_N2) reaction[115] (**Scheme 17**). Along with these differences in reactivities, there is also the possibility of unexpected secondary reactions between NEM and the polysulfides resulting in the shorter chain lengths.

In order to test the potential impact of NEM a polysulfide standard was derivatised with excess IAM to ensure all of the polysulfide was fully alkylated, NEM was then added to the fully reacted solution. The reactive end sulfurs of the polysulfide chains should be fully alkylated and so unavailable to react with NEM, therefore if any NEM alkylated polysulfide species are observed then they must arise from secondary reactions.

After addition of NEM to the fully reacted IAM polysulfide solution, new alkylated polysulfide species were observed that were either alkylated with one NEM and one IAM or fully alkylated with NEM. Additionally all of the polysulfide species that were detected were of shorter chain lengths than before the addition of NEM. This confirms the hypothesis that NEM is in some way able to react with the mid chain sulfurs and produce shorter chain length polysulfides though the exact mechanism by which this occurs remains unclear.

4.1.4 UHPLC-MS/MS Analysis of Organic Persulfides and Polysulfides

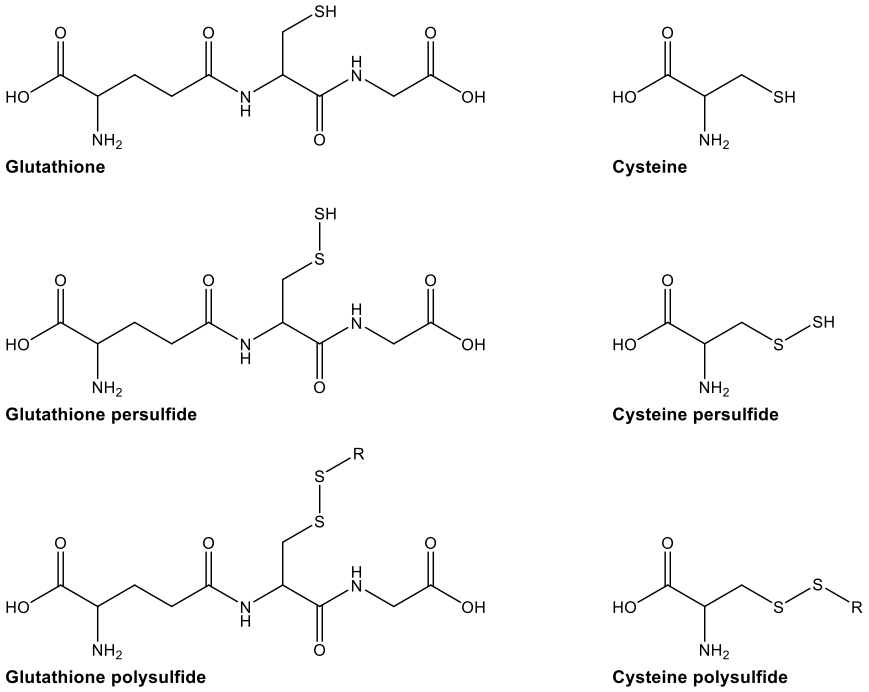


Figure 15: Examples of organic polysulfides: Glutathione and cysteine persulfide and polysulfide (where R = S_nH).

The formation of persulfides and polysulfides on protein cysteine residues is a relatively new concept in redox biology and it has recently been shown that these residues are actually quite abundant in cells and tissues and have important biological functions[131,135,283,322]. The antioxidant capabilities of organic persulfides and polysulfides have also been found to be much greater than their base thiols by approximately 10-110 times[130]. However, the physiological levels of these polysulfides, their speciation and importance of individual species and their biosynthesis are still

unclear, especially in disease states[38]. Methodologies designed to study these species are currently hampered by the fact that there are no readily available accurate standards for thiol persulfides for use in identification and quantification. Coupled to this, the chemistries involved in the gold-standard alkylation based protocols are not well understood, and as we have shown, the conditions used in these methods can have significant impacts on the polysulfide speciation measured.

Since no standards are readily available for organic polysulfides, to investigate the alkylation of these species we followed the method set out by Francoleon N.E. *et al.*[139] in order to generate them (by reaction of sodium sulfide with an oxidised thiol such as GSSG). Two different conditions for generating organic per- and polysulfides were tested, the first using sodium sulfide and the second using sodium trisulfide. The expectation was that using sulfide would generate mainly glutathione persulfide (GSS^-) with the addition of only a single sulfur, whilst using trisulfide would generate longer chain glutathione polysulfides such as glutathione tetrasulfide ($GSSSS^-$) as it would be able to add longer polysulfide chains. As was seen with the inorganic polysulfides electrophiles can significantly alter the measured speciation patterns making it important to use an electrophile that reacts quickly and efficiently, but also preserves the in-vivo speciation pattern. NEM was initially tested due to its rapid rate of reaction, but with the understanding that it will likely be biased towards shorter chain organic polysulfides.

The speciation pattern observed after alkylation of glutathione polysulfides with NEM was similar to that of the inorganic polysulfides with pentasulfide being the highest species seen. Unexpectedly both the reaction with sulfide and trisulfide produced the same speciation pattern showing that organic polysulfides with more than two sulfurs can be synthesised from just sulfide and oxidised thiols. NEM generally produces shorter chain length polysulfides therefore, it is unlikely that the longer organic polysulfides are produced during the alkylation reaction. This reaction could readily occur in biological samples after collection and so caution should be exercised during sample collection and processing to try and avoid the artifactual production of organic polysulfide species.

4.1.5 Investigation of Potential Polysulfide Formation by the Antioxidant Enzyme Superoxide Dismutase

There are a number of key similarities both biological and chemical between reactive oxygen species (ROS) generated from oxygen and reactive sulfur species (RSS) generated from one electron oxidation of hydrogen sulfide[3]. These reactive sulfur species include the thiyl radical ($HS^\bullet$) as well as inorganic persulfides and polysulfides as both hydrated species and radical anions. RSS

have come to attention recently and appear to play a much greater role in redox biology than previously appreciated[55,130,152,330]. In some cases it has been shown that systems regulated by H_2O_2 have identical responses to polysulfides (H_2S_n, where n = 2-5)[36,254]. It has also recently become apparent that many methods used to measure ROS such as those based on fluorescent probes or amperometric electrodes are in fact unable to distinguish between ROS and RSS[253]. This suggests that RSS may in fact be responsible for some, if not many, of the biological effects previously attributed to only ROS.

Catalase, an enzyme that catalyses peroxide dismutation to oxygen and water as part of cellular defence against oxidative damage, has recently been demonstrated to have biological activities involving RSS. It has been shown to be able to oxidise both sulfide and polysulfides using both oxygen and H_2O_2[137]. It is likely that catalase is not the only antioxidant enzyme that has biological activities involving RSS as well as ROS. After all, life evolved in an anoxic and reducing environment, likely depending on RSS to provide the necessary energy. Therefore, there would have been the need to deal with damaging RSS long before ROS. Antioxidant enzymes such as catalase, super oxide dismutase, thioredoxin and peroxiredoxin, all appear to have evolved long before the presence of oxygen in the atmosphere[138] which suggests that they performed important biological functions on substrates other than ROS. Super oxide dismutase (SOD) is a key antioxidant enzyme which catalyses the dismutation of superoxide to oxygen and peroxide[255]. It is closely linked to catalase since it produces peroxide which catalase then dismutases into oxygen and water. Since catalase has recently been demonstrated to have biological activities involving RSS as well as ROS this makes SOD a strong candidate to have similar activities centring on RSS. These antioxidant enzymes may in fact form a new area of crosstalk between ROS metabolism and RSS metabolism in the reactive species interactome[36].

To further explore the hypothesis that antioxidant enzymes were originally involved in RSS metabolism, and indeed still show this biological activity, the potential biological activity of mammalian SOD (Cu/Zn) with sulfide was investigated. The findings from the experiments carried out for this chapter and the developed detection method were applied to the novel investigation into the potential sulfur related metabolic activity of SOD. IAM was used to alkylate and trap any polysulfide species present and the UHPLC-MS/MS used in section 4.2.3 was used for the detection of the polysulfide species. In order to quantify any formed polysulfides pure sodium tetrasulfide alkylated with IAM was used. Although when made up in solution and alkylated the tetrasulfide equilibrates to different polysulfide species since a known molar amount could initially be weighed out the quantity of each polysulfide species was taken as a fraction of the total amount made up. This only provides a rough quantification and so is only used as a guide for

comparison between experiments rather than an accurate measure of the concentration of each polysulfide species.

Inorganic polysulfide species are formed from sulfide in the presence of SOD at a greater rate than autooxidation of sulfide on its own in solution. The polysulfide species appear to be formed by sequential addition of sulfide elongating the chain though the exact mechanism by which this reaction is performed by SOD remains unclear. The radical scavenger DMPO did not have any effect on the formation of polysulfides suggesting that either this reaction does not involve thiyl radicals, the formation of the polysulfides is much more rapid than the scavenging by DMPO or that the catalytic site of SOD is inaccessible to DMPO. This sulfur related activity of SOD forms a further point of crosstalk in what is being termed the "reactive species interactome"[36] with SOD providing a link between ROS and RSS highlighting the complex interlinked nature of redox regulation.

4.2 Methods

Pure sodium disulfide, trisulfide and tetrasulfide standards were kindly provided by by Dojindo Europe (Neuss, Germany). Mixed potassium polysulfide (K_2S_x, where x = 1-5) was obtained from Sigma-Aldrich. All other materials were sourced from Sigma-Aldrich (Gillingham, UK or Munich, Germany) unless otherwise stated. HPLC-grade solvents from Fisher Scientific (UK). Argon gas (> 99.99%) was from BOC Group (Guildford, UK). Ultrapure N_2 gas was produced by a nitrogen generator (Parker Balston, UK).

4.2.1 Direct Infusion Analysis of Aqueous Polysulfides

Polysulfide solutions were made up by addition of three different polysulfide salts as a dry powder, mixed potassium polysulfide (K_2S_x), sodium trisulfide (Na_2S_3) and sodium tetrasulfide (Na_2S_4) to either ultra-pure water or pH 7.4 ammonium phosphate buffer. Solutions were made up to 10 mM and diluted x100 with H_2O before analysis. The polysulfide solutions were directly infused into the ESI source of the triple quadrupole mass spectrometer (ESI-MS/MS) at 8 μl/min and scanned for one minute. Due to the low pK_a of inorganic polysulfides[149], they were detected using negative ion mode.

To investigate the potential effect of the alkylation (**Scheme 17**) on the polysulfide speciation first the underivatised polysulfide solution was analysed by direct infusion in negative ion mode before IAM (100 mM) was added to the remaining solution (volume of IAM added was equal to 1% of remaining polysulfide solution volume). The derivatised polysulfide solution was then analysed by direct infusion using both negative and positive ion mode. Negative ion mode was used to confirm

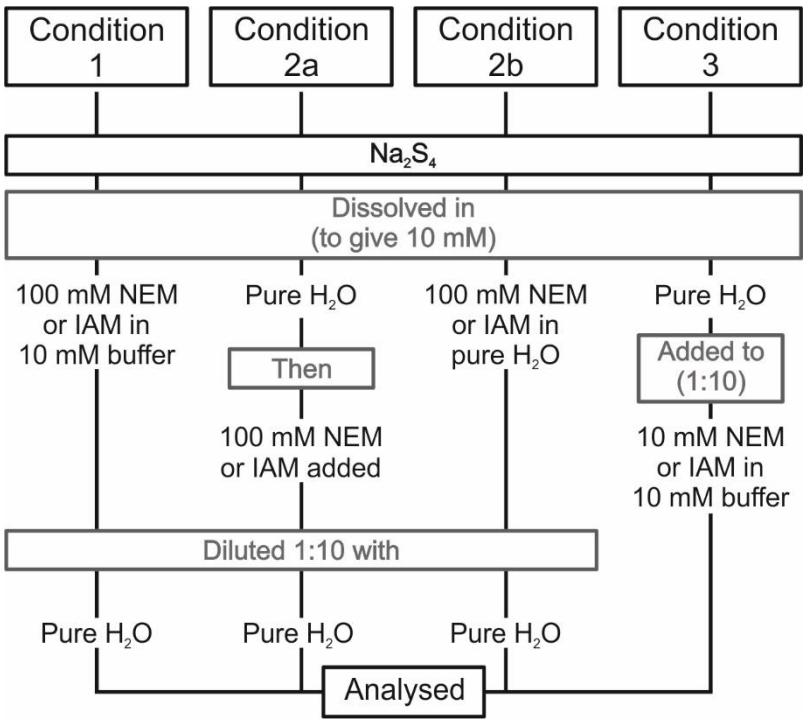
the absence of unreacted polysulfides and positive ion mode was used to confirm the presence of the derivatised polysulfides and analyse their composition. Mass spectrometry settings used are listed in appendix B.1.

4.2.2 High Resolution Mass Spectrometry of Aqueous Polysulfides

Polysulfide solutions were prepared as described in section 4.2.1, mass spectrometry measurements were made by direct infusion of the aqueous polysulfide solution at a rate of 8 μl/min into the ToF-MS ESI source using negative ion mode. The instrument used was a Waters SYNAPT G2-Si ESI-ToF-MS that was calibrated for the low *m/z* range using sodium-formate clusters to a 0.5 ppm resolution using a fifth order polynomial.

4.2.3 UHPLC-MS/MS Analysis of Inorganic Polysulfide Speciation Following Chemical Derivatisation

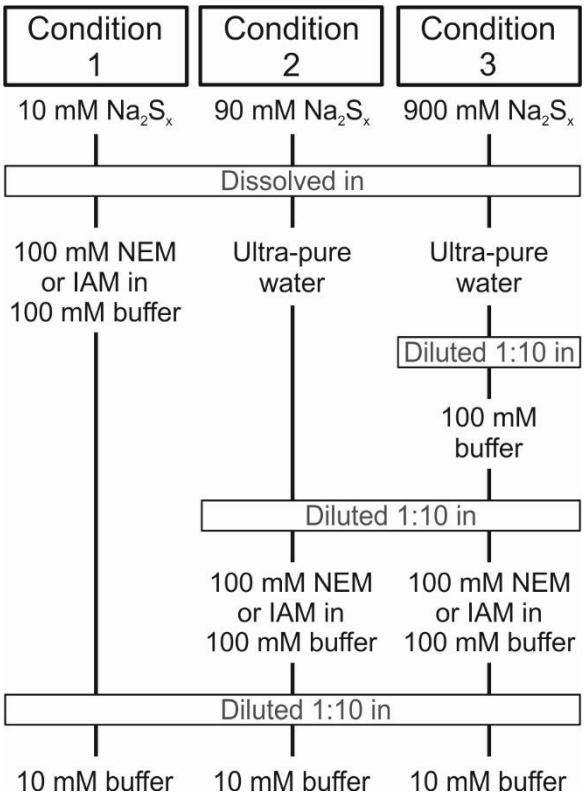
4.2.3.1 Derivatisation with Electrophiles



Scheme 18: Experimental workflow for the first set of polysulfide preparation experiments (**Figure 21**). The buffer used was pH 7.4 ammonium phosphate, in all four conditions the samples were diluted to the same concentration (based on the initial polysulfide concentration) before analysis. All experiments were performed at room temperature.

Stock solutions of 100 mM *N*-ethylmaleimide (NEM) and iodoacetamide (IAM) were prepared in 10 mM pH 7.4 ammonium phosphate buffer. Different concentrations (see appendix B.2 for full list of concentrations used) of each electrophile, ranging from 0.5 mM to 98 mM, were prepared using different dilutions of the stock solutions with buffer to a total volume of 1 ml. Polysulfide solutions were made up by addition of pre-weighed dry powder standards to ultra-pure water using either mixed potassium polysulfide (K_2S_x), sodium trisulfide (Na_2S_3) or sodium tetrasulfide (Na_2S_4) to produce either 10 mM or 1 mM solutions (see appendix B.2 for amounts used in each experiment). 10 µl of the polysulfide solution was added to each of the different reaction solutions containing different electrophile concentrations to give a final (total) polysulfide concentration of 10 or 100 µM. Following the results of the kinetics experiments outlined in Chapter 5: the polysulfides were allowed to react with NEM at room temperature for 10 min before analysis, and IAM was left for 30 min to ensure a complete reaction.

In two separate sets of experiments, different preparation methods were tested to investigate the impact of the starting solution composition on the polysulfide speciation. In the first set of experiments four different preparation methods were tested as shown in **Scheme 18**, briefly; **1)** Na_2S_4 was added to 900 µl buffer with 100 µl NEM/IAM solution. **2a)** Na_2S_4 was added to 900 µl water, then 100 µl NEM/IAM was added after dissolving, **2b)** Na_2S_4 was added to 900 µl water with 100 µl NEM/IAM **3)** Na_2S_4 was added to 1000 µl water, then 100 µl of the solution was added to 800 µl buffer with 100 µl NEM/IAM. Solutions **1)**, **2a)** and **2b)** were diluted 10x in ultra-pure water before analysis.



Scheme 19: Experimental workflow for the second set of polysulfide preparation experiments (**Figure 22**). Na_2S_x, where *x* = 2, 3 or 4, the buffer used was pH 7.4 ammonium phosphate. All experiments were performed at room temperature and repeated three times.

The second set of experiments used the preparation methods detailed **Scheme 19** for both NEM and IAM expanding on the first experiments, briefly; Condition **1**: Na_2S_x dissolved in NEM or IAM containing buffer. Condition **2**: Na_2S_x first dissolved in ultra-pure water, then diluted in NEM or IAM containing buffer. Condition **3**: Na_2S_x dissolved in ultra-pure water, then diluted in buffer, then diluted in NEM or IAM containing buffer. All of these conditions were tested using Na_2S_2, Na_2S_3 and Na_2S_4. All conditions tested used the same final dilution before analysis. As before, in both sets of experiments NEM was allowed 10 min to react and IAM 30 min at room temperature.

Table 4: Chromatographic conditions for NEM or IAM derivatised polysulfide analysis, A = H_2O + 5 mM ammonium acetate, B = 95% ACN + 5% H_2O + 5 mM ammonium acetate.

Time (min)	% A	% B	Flow (ml/min)
0.00	95	5	0.20
5.00	40	60	0.20
6.00	95	5	0.20
7.00	95	5	0.20

Table 5: Precursor and fragment ion pairs used for the analysis of NEM and IAM alkylated polysulfide species.

ESI+	NEM	IAM
S_1	285 > 160	149 > 104
S_2	317 > 160	181 > 91
S_3	349 > 160	213 > 91
S_4	381 > 160	245 > 91
S_5	413 > 160	277 > 91
S_6	445 > 160	309 > 91
S_7	477 > 160	341 > 91

The composition of the derivatised polysulfides was analysed by UHPLC-MS/MS using a Waters Aquity UPLC system with a Xevo TQ-S detector. Mobile phase A was H_2O + 5 mM ammonium acetate; mobile phase B was 95% ACN + 5% H_2O + 5 mM ammonium acetate. An Aquity UPLC CSH C18 (1.7 μm), 2.1 x 100 mm column was used for the separation with the gradient used described in **Table 4**, the injection volume was 5 μl, column temperature 30°C and the MRMs used are listed in **Table 5**. Mass spectrometry settings are listed in appendix B.1.

4.2.3.2 Impact of pH on Polysulfide Speciation After Derivatisation

Stock solutions of 100 mM NEM and IAM were prepared in pH 7.4 ammonium phosphate buffer. Additionally, the pH 7.4 buffer was adjusted to separate solutions of pH 5.0, 6.0, 8.0 and 9.0 using either 1 M HCl or NaOH. The Na_2S_2, Na_2S_3 and Na_2S_4 salts were made up in ultra-pure water to give a concentration of 10 mM assuming all of the polysulfide remained as the starting chain length. Sodium sulfate was made up in ultra-pure water and diluted to a concentration of 10 nM.

For the reaction, to 880 μl of the appropriate pH buffer 10 μl of sulfate (10 nM) and 100 μl of NEM/IAM (100 mM) was added, followed by 10 μl of polysulfide solution (10 mM). The solutions were allowed to react for 5 min for NEM and 20 min for IAM before being analysed using the UHPLC-MS/MS method outlined in the previous section (4.2.3.1). Sulfate was monitored using a selected ion recording (SIR) scan of m/z 97.03 (HSO_4^-) in negative ionisation mode and eluted at 1.10 min.

4.2.3.3 Impact of NEM on Polysulfide Chains

In order to investigate if NEM has any unanticipated effects on polysulfide speciation 0.0019 g sodium tetrasulfide was added to 9 ml water + 1 ml of 100 mM IAM (giving a 1 mM concentration

assuming all the polysulfide remained as tetrasulfide, and 10 mM IAM). The solution was allowed to react at room temperature for 60 min before 100 μl of the solution was added to 800 μl water + 100 μl of 100 mM NEM (10 mM NEM final concentration, 1:10 dilution) and allowed to react at room temperature for a further 30 min. Aliquots were taken for analysis 60 min after IAM addition before the addition of NEM as well as 15 and 30 min after NEM addition. Potential polysulfide species alkylated with one IAM and one NEM were monitored using precursor masses only (selected ion scan) due to unknown fragmentation patterns and a lack of accurate standards. Analysis was performed using UHPLC-MS/MS using the same method as detailed in section 4.2.3.1**Error! Reference source not found.**.

4.2.4 UHPLC-MS/MS Analysis of Organic Persulfides and Polysulfides

Due to a lack of accurate standards glutathione polysulfide species were produced by reaction of oxidised glutathione (GSSG) with either sodium trisulfide or sodium sulfide salts following the method set out by Francoleon N.E. *et al.*[139]. GSSG and either sodium trisulfide or sodium sulfide were mixed with a 1:1 molar ratio (10 mM, assuming for Na_2S_3 that all remained as S_3 after dissolution) in pH 7.4 ammonium phosphate buffer and incubated at 37°C for 15 minutes. The reaction was terminated by addition of excess NEM, which also allowed for analysis of the formed glutathione species. The analysis was performed using UHPLC-MS/MS using the method developed in section 4.2.3.1 and the chromatographic gradient detailed in **Table 4** using specific MRMs for the five detected glutathione species (as well as the remaining unreacted GSSG used in the reaction). The MRMs used were (all NEM derivatised): GSH 433.2>304, GS_2H 465.2>336, GS_3H 497.1>368, GS_4H 529.1>400 and GS_5H 561.1>432. The cone energy was 6 V and collision energy 13 V for all measured species.

4.2.5 Investigation of Potential Polysulfide Formation by the Antioxidant Enzyme Superoxide Dismutase

Formation of inorganic polysulfides was monitored using the UHPLC-MS/MS method described in section 4.2.3.1. In order to provide a rough quantification for any formed polysulfide species pure sodium tetrasulfide was added to a tenfold molar excess of IAM in pH 7.4 ammonium phosphate buffer. The solution was allowed to react at room temperature for 60 min before being diluted serially to give a standard series with the concentrations 100 μM, 10 μM, 5 μM, 1 μM, 500 nM, and 100 nM assuming all of the polysulfide remained as tetrasulfide. Since the tetrasulfide salt actually forms sulfide, disulfide, trisulfide, tetrasulfide and pentasulfide after dissolution the proportion of each of these species is taken as the concentration of that species in relation to the initial concentration of the tetrasulfide as it was weighed out. For example, if the initial

concentration of the tetrasulfide would have been 100 μM if it had all remained as tetrasulfide and the peak for the trisulfide formed represents 10% of the total peak areas for all of the measured polysulfide species then the concentration of the trisulfide is taken as 10 μM.

Different experimental conditions were tested. Initially the ability of mammalian SOD (Cu/Zn, 3700 units/mg) to produce polysulfides from sulfide was investigated using two conditions **1)** 1 μM SOD + 1 mM Na_2S and **2)** 1 mM Na_2S as a control. Both of the reaction mixtures were made up in pH 7.4 ammonium phosphate buffer and left open under normoxic conditions at room temperature. Between 0-90 min 50 μl aliquots of each reaction mixture were taken every ten minutes, and added to 50 μl of 100 mM IAM and well mixed. Two additional aliquots were taken at 150 and 180 min. All aliquots were analysed using the established UHPLC-MS/MS method.

The initial rapid formation of persulfide (within 60 s) and its dependence on the concentration of SOD was investigated following the same method as for the previous experiments. Three different concentrations of SOD were tested, 1 μM, 5 μM and 10 μM with 1 mM Na_2S. Aliquots taken for analysis at 10 s, 30 s, 60 s, 120 s and 300 s after SOD addition, which were added to 50 μl of 100 mM IAM and well mixed before analysis.

To investigate whether the formation of the persulfide occurred by SOD catalysed one electron reduction of two sulfides the radical scavenger 5,5-Dimethyl-1-pyrroline N-oxide (DMPO) was used to attempt to trap any thiyl radicals formed. Four different conditions were used, two controls, **1)** 1 mM Na_2S only, **2)** 1 mM Na_2S + 2 mM DMPO, and two reaction conditions, **3)** 1 mM Na_2S + 1 μM SOD and **4)** 1 mM Na_2S + 1μM SOD + 2 mM DMPO. As before, the initial formation of persulfide was monitored by taking 50 μl aliquots at 10 s, 30 s, 60 s, 120 s and 300 s after SOD addition, which were added to 50 μl of 100 mM IAM and well mixed before analysis.

4.3 Results

4.3.1 Direct Infusion Analysis of Aqueous Polysulfides

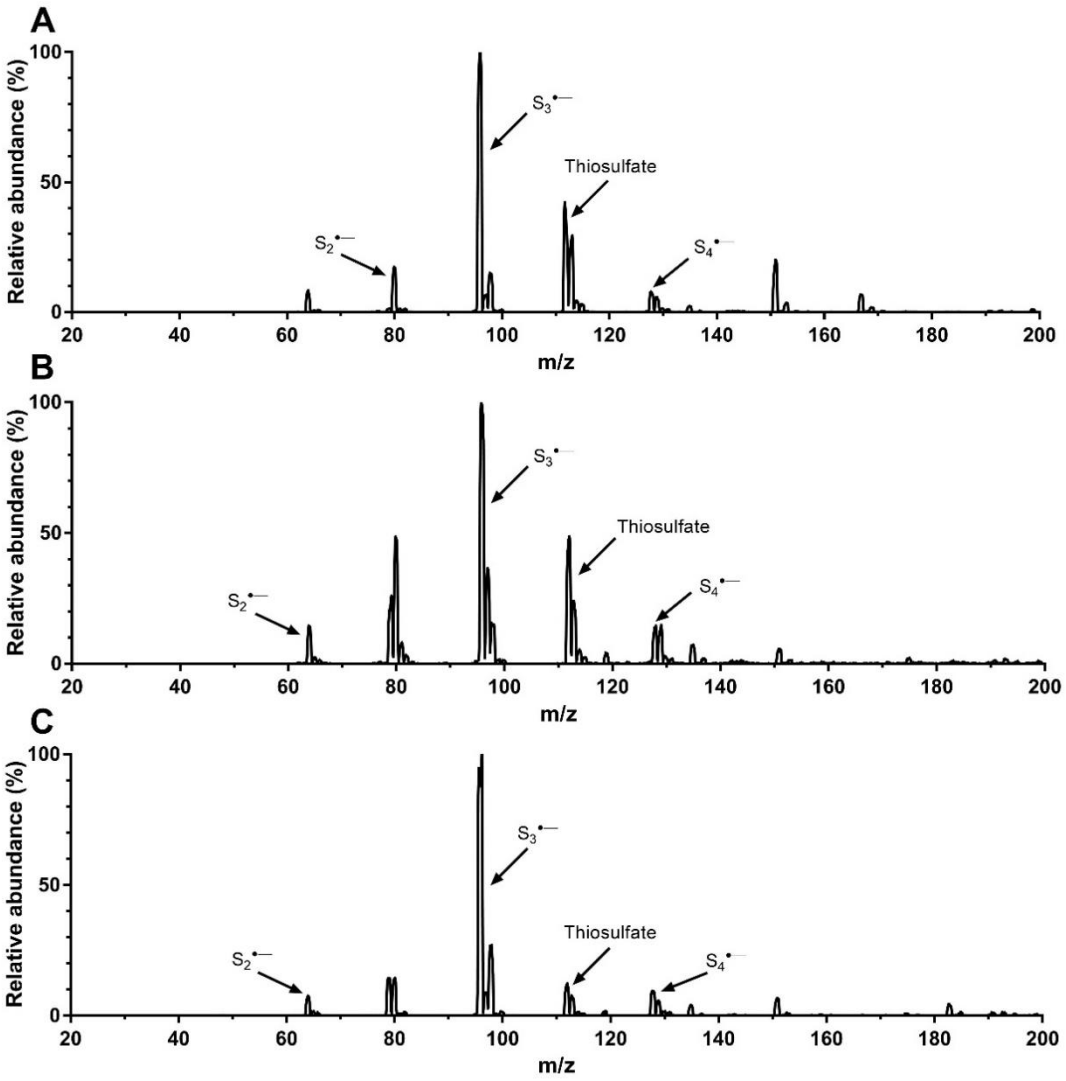


Figure 16: Direct infusion electrospray ionisation MS detection of aqueous inorganic polysulfides. **A)** Mixed potassium polysulfide, **B)** Sodium trisulfide, **C)** Sodium tetrasulfide. The observed polysulfide *m/z* are, $S_2^{\bullet-}$ 63.9, $S_3^{\bullet-}$ 95.9 and $S_4^{\bullet-}$ 127.7, measured *m/z* varies slightly due to the low resolution of the mass spectrometer. Thiosulfate is also visible either occurring as a contaminant in the stock or an oxidation product after dissolution of the polysulfides stock. There is no significant difference in the measured polysulfide speciation regardless of the starting material used.

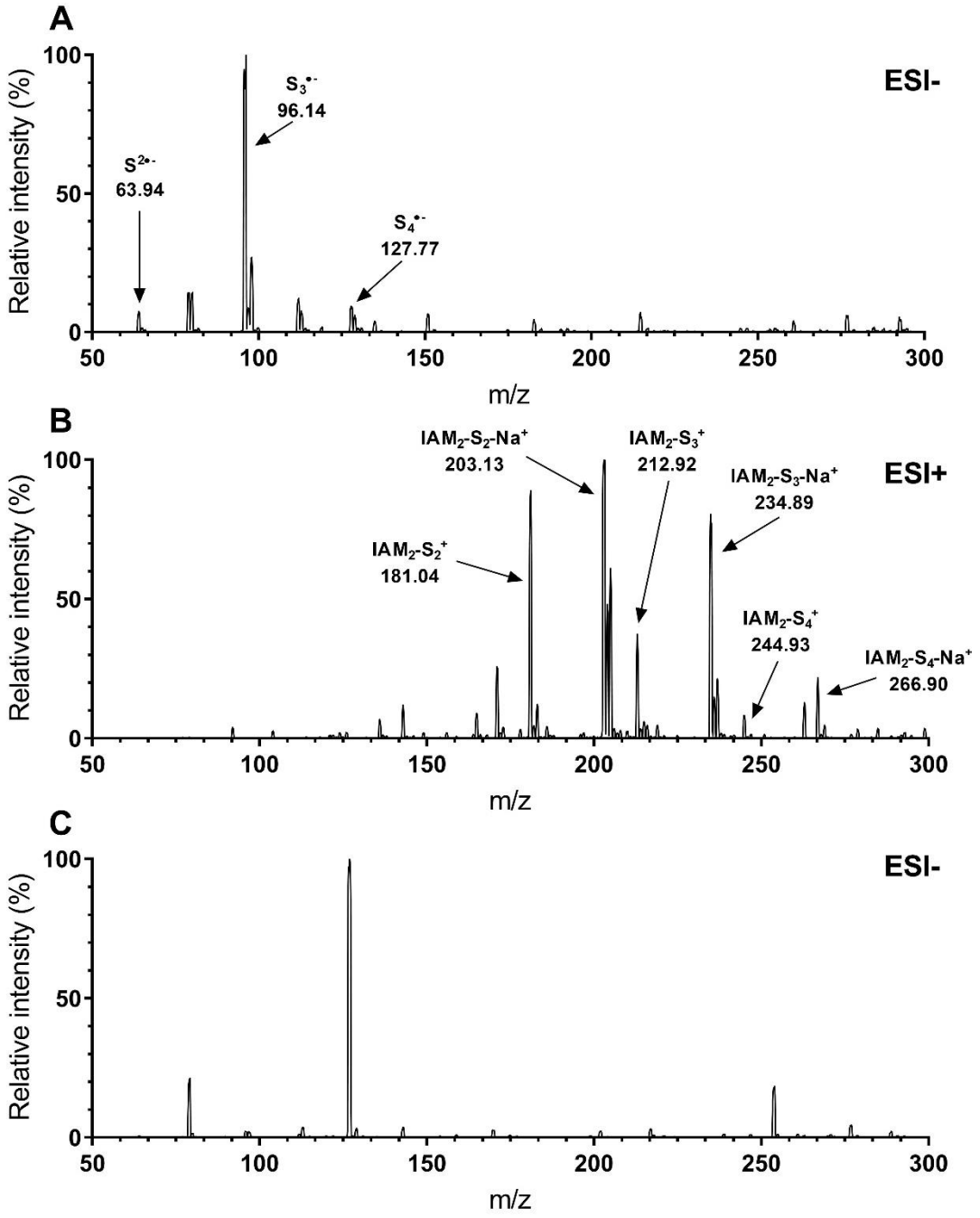


Figure 17: Direct infusion electrospray ionisation MS detection of aqueous sodium tetrasulfide before (**A**) and after (**B** and **C**) addition of IAM. **B** shows the presence of alkylated polysulfide species (and associated sodiated peaks) in positive ionisation mode. **C** shows the absence of the polysulfide peaks in negative ionisation mode observed before IAM addition (**A)**. There appears to be a change in polysulfide speciation after addition of IAM with the most abundant species observed shifting from trisulfide to disulfide.

Sodium trisulfide, tetrasulfide and mixed potassium polysulfide (K_2S_x, where x = 1-5) all show a similar speciation pattern when made up in ultra-pure water (**Figure 16**). Unexpectedly, the

underivatised polysulfide species seen have *m/z* that correspond more closely to radical anion species ($S_n^{\bullet-}$) as opposed to the expected hydropolysulfide anions (HS_n^-) which have been previously reported[149]. The most abundant polysulfide observed has an *m/z* of ~95.9, which corresponds closely to a trisulfide radical anion ($S_3^{\bullet-}$) with *m/z* values corresponding with disulfide (*m/z* ~63.9) and tetrasulfide (*m/z* ~127.8) radical anion species ($S_2^{\bullet-}$ and $S_4^{\bullet-}$) also being observed. The measured *m/z* of the polysulfide species has a slight variance between separate measurements due to the low-resolution nature of the instrument leading to a slight shift in the peak top between measurements.

Upon derivatisation with iodoacetamide (**Figure 17**) the most abundant species measured changes to the disulfide (IAM_2-S_2, *m/z* 181.0) followed by trisulfide (IAM_2-S_3, *m/z* 212.9) and the least abundant being tetrasulfide (IAM_2-S_4, *m/z* 244.9), suggesting that the alkylation reaction has altered the measured speciation pattern. No underivatised polysulfides are observed after the alkylation reaction showing the reaction has completed.

4.3.2 High Resolution Mass Spectrometry of Aqueous Polysulfides

The exact masses for the three potential polysulfide radical ions observed were calculated to be as follows; $S_2^{\bullet-}$ *m/z* 63.9448, $S_3^{\bullet-}$ *m/z* 95.9168 and $S_4^{\bullet-}$ *m/z* 127.8890. The closest in mass is the $S_3^{\bullet-}$ with a mass difference of only 0.0002 *m/z* units. $S_2^{\bullet-}$ and $S_4^{\bullet-}$ have greater differences, 0.0020 *m/z* units and 0.0167 *m/z* units respectively (**Figure 18** and **Table 6**). These larger differences may be due to the lower abundances of these two species giving a lower mass accuracy as well as the low mass of the $S_2^{\bullet-}$ which is close to the lower range of the ToF-MS (*m/z* 50) with a corresponding loss of resolution at that range. This loss of resolution is due to the fact that the lower the *m/z* of the ion, the less time it spends in the ToF region, meaning that less scans can be performed on low mass ions compared to a higher mass ions leading to a loss of mass accuracy[331].

Table 6: Exact and measured accurate masses for polysulfide radical ions, thiosulfate and other observed species in the experiment depicted in **Figure 18**.

	Exact Mass (*m/z* units)	Measured Mass (*m/z* units)	Difference (*m/z* units)	Difference (ppm)
$S_2^{\bullet-}$	63.9448	63.9469	0.0021	33
$S_3^{\bullet-}$	95.9168	95.9170	0.0002	2
$S_4^{\bullet-}$	127.8890	127.9057	0.0167	131
$S_2O_3^{\bullet-}$	111.9294	111.9312	0.0018	16
$HS_2O_3^-$	112.9367	112.9398	0.0031	27
HSO_4^-	96.9601	96.9599	0.0002	2

The isotopic distribution of the observed peaks also provides additional conformation of peak assignment. The most abundant natural stable isotope of sulfur is ^{34}S, meaning that inorganic polysulfide radical anions will only have a significant isotope peak at M+2, and no isotope peak at M+1. The exact difference in atomic mass between ^{34}S and ^{32}S is 1.9958 *m/z* units, which can also be used as further confirmation that these are the correct isotope peaks for the polysulfide species. **Figure 18, B** shows an expanded section of the spectrum showing the $S_3^{\bullet-}$ peak and its +2 isotope peak at *m/z* 97.9128. The difference in mass between the suggested $S_3^{\bullet-}$ and this isotope peak is exactly 1.9958 *m/z* units and it has a relative abundance to the main peak of ~12%. Likewise, **Figure 18, C** shows an expanded section of the spectrum for $S_4^{\bullet-}$ with the +2 isotope peak at *m/z* 129.9001 and a relative abundance of ~16%. This gives a mass difference of 1.9944 *m/z* units, which is close to the theoretical difference between ^{32}S and ^{34}S. No isotope peak was observed for $S_2^{\bullet-}$ (not shown), likely due to its low signal intensity meaning the isotope peak which would be ~8% of the intensity of the main peak would fall into the noise level. There are no M+4 isotope peaks observed which is not unexpected as the abundance of these isotopes would fall into the noise in the spectrum.

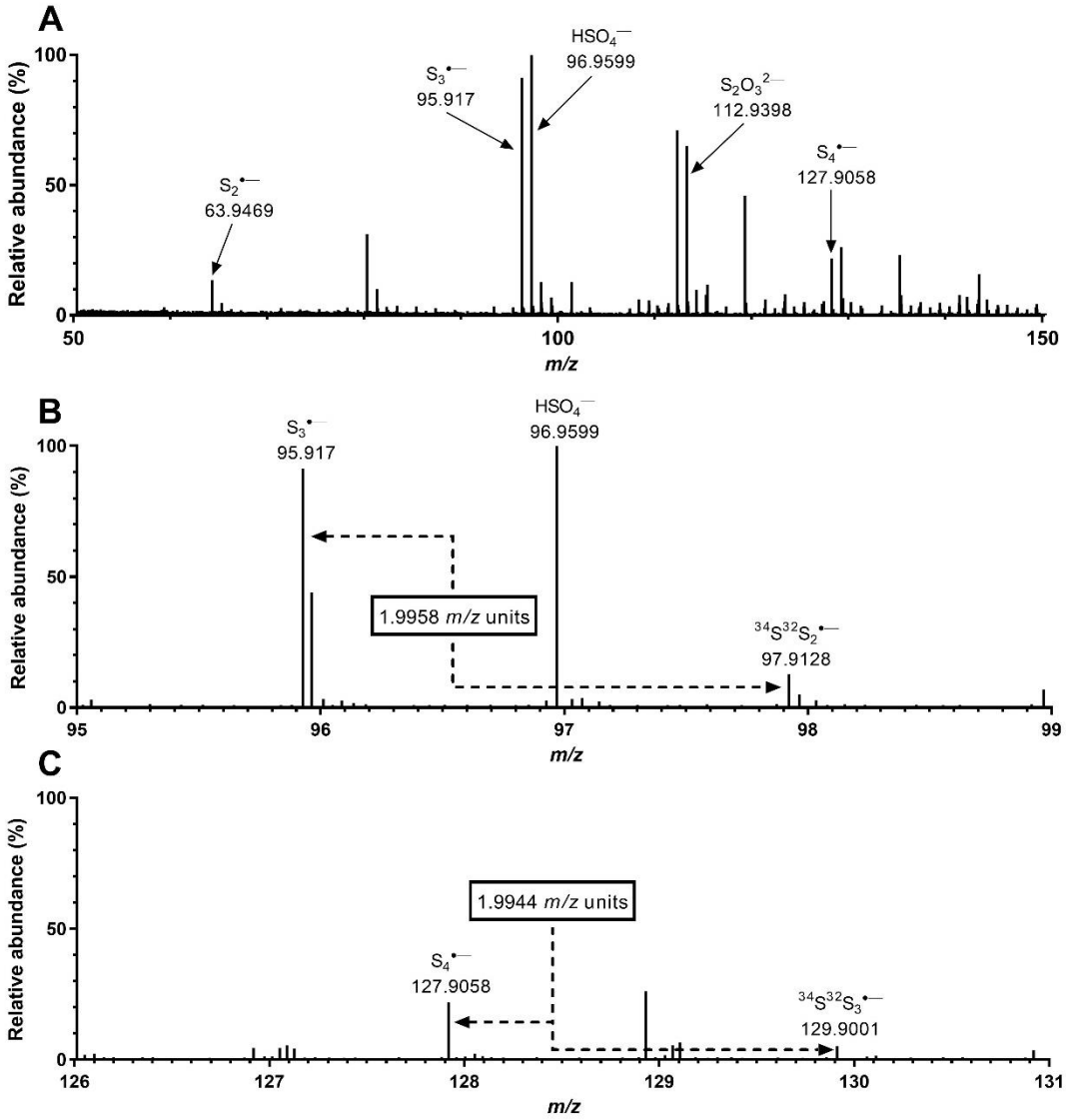


Figure 18: High-resolution direct infusion of aqueous inorganic polysulfides collected using a Waters SYNAPT G2-Si ESI-ToF-MS. All of the spectra are representative of n = 5 experiments with similar results conducted under identical conditions. **A)** Full spectrum showing the observed peaks for disulfide ($S_2^{\bullet-}$), trisulfide ($S_3^{\bullet-}$) and tetrasulfide ($S_4^{\bullet-}$) radical anions as well as thiosulfate and sulfuric acid. **B) + C)** Highlighted sections of the mass spectrum showing the trisulfide (**B**) and tetrasulfide (**C**) radical anion peaks and their M+2 isotope peaks.

4.3.3 UHPLC-MS/MS Analysis of Inorganic Polysulfide Speciation Following Chemical Derivatisation

4.3.3.1 Derivatisation with Electrophiles

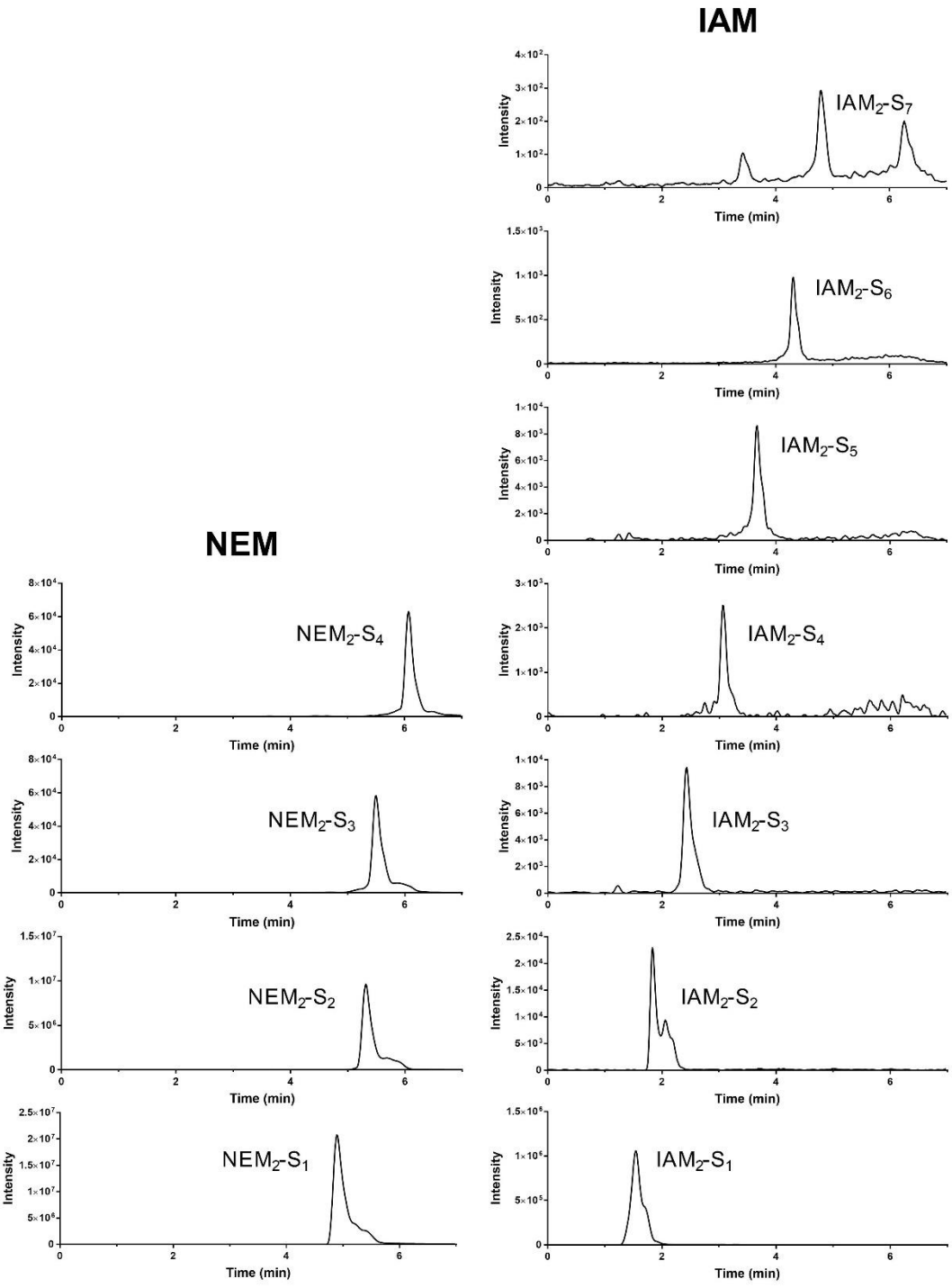


Figure 19: Exemplary chromatograms for NEM and IAM derivatised polysulfides displayed in stacked view using the chromatographic method described in 4.2.3.1. From S_1 at the bottom to S_4 at the top for NEM and S_7 at the top for IAM and showing the increasing elution time with increasing sulfur number.

Using the UHPLC method developed in chapter 3 (see section 3.2.1), NEM alkylated polysulfides elute with increasing number of sulfurs with S_1-S_4 eluting from 4.85 min to 6.45 min (**Figure 19**). NEM derivatised polysulfides higher than S_4 were not observed. IAM derivatised polysulfides likewise elute with increasing sulfur number but a higher range of species is observed than with NEM, with S_1-S_7 eluting from 1.85 min to 4.80 min (**Figure 19**). Unidentified peaks are observed in the chromatogram for IAM_2-S_7 at 3.40 min and 6.25 min, these are likely noise peaks caused by contaminants or from the sample matrix. These unidentified peaks do not follow the expected elution pattern of the derivatised polysulfides on the C_{18} column, with an increasing number of sulfurs the elution time also increases; therefore, I am confident that the peak at 4.80 min is the true peak for IAM_2-S_7.

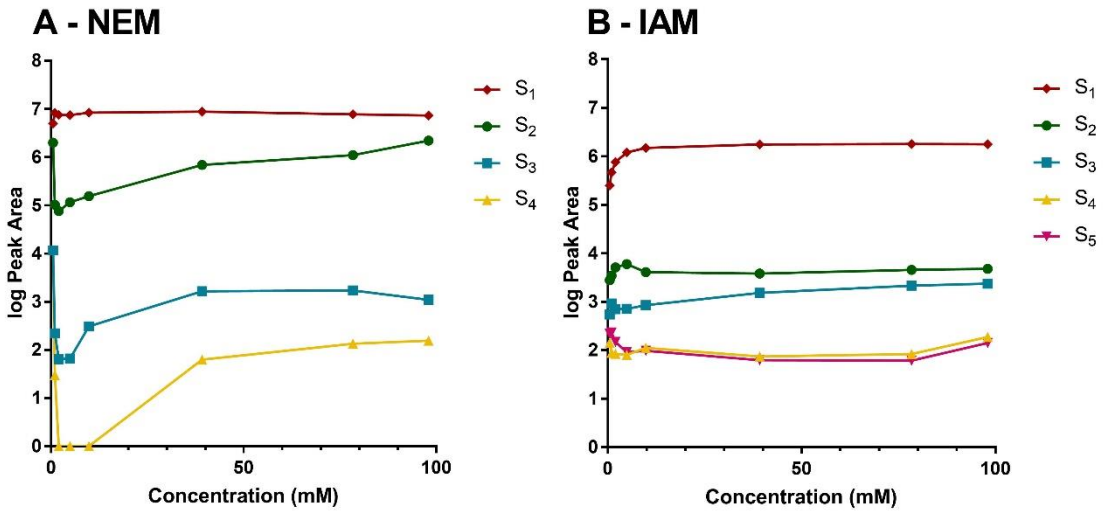


Figure 20: Effect of increasing NEM (**A**) and IAM (**B**) concentrations (0.5-98 mM) on the apparent concentration of individual inorganic polysulfide species. **A:** Increasing NEM concentration from 0.5-20 mM decreases the amount of S_2, S_3 and S_4 above 20 mM the measured amounts then start to increase, apart from S_3, which increases until dropping at 98 mM NEM. Sulfide shows an initial increase from 0.5-20 mM before starting to decrease with increasing NEM. **B:** With increasing IAM concentration sulfide shows an overall increase, S_2 increases from 0.5-5 mM before decreasing up to 40 mM IAM followed by a slight increase. S_3 increases with increasing IAM concentration whilst S_4 and S_5 show a decrease until 98 mM. This data is representative of *n* = 5 experiments all with qualitatively similar results.

As with the underivatised polysulfides (section 4.3.1) the composition of the starting polysulfide (mixed potassium polysulfide or pure sodium tri or tetrasulfide) does not appear to have any significant effect on the measured polysulfide speciation after being made up in solution and

derivatised (**Figure 22**). However, the concentration of NEM or IAM used does have an impact on the speciation (**Figure 20**).

Generally, with NEM the measured levels of S_2, S_3 and S_4 polysulfides decrease from 0.5 to 20 mM NEM before starting to increase. The sulfide however shows an initial increase over the same range before starting to decrease (appendix B.2 and **Figure 20**, **A**). With IAM, the measured sulfide increases with the increasing IAM concentration, though this was not consistent when repeated with a lower concentration of polysulfide (appendix B.1). S_2 and S_3 show an overall increase in measured levels with increasing IAM concentration, though there is a dip in the measured S_3 at 40 mM IAM. Both S_4 and S_5 show a decrease in measured levels until 98 mM where they both show a slight increase (appendix B.2 and **Figure 20**, **B**). Overall increasing NEM and IAM concentrations have significant and varied effects on the speciation pattern of inorganic polysulfides that could be linked to changes in the rates of reaction or possible secondary reactions with the excess alkylating agents.

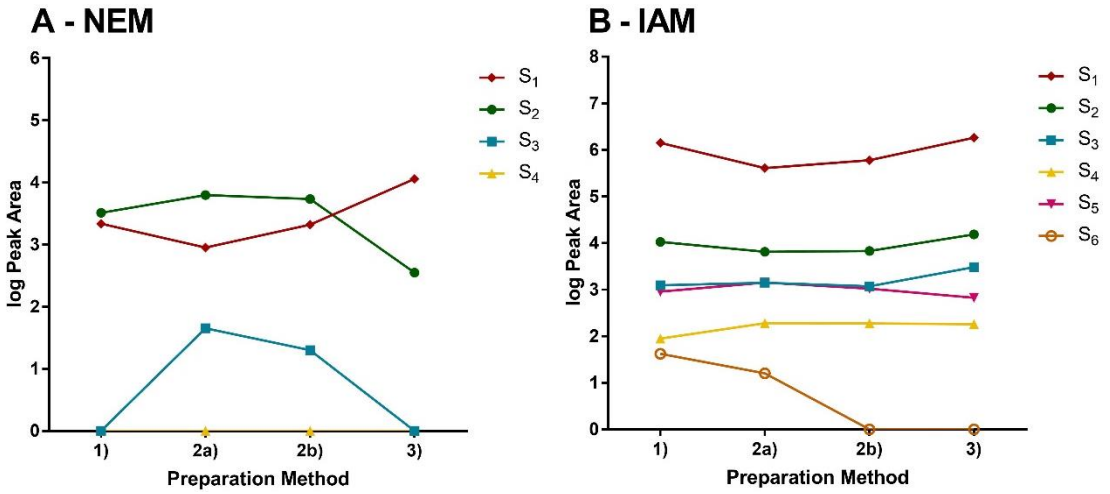


Figure 21: Effects of alkylating conditions on polysulfide speciation using NEM (**A**) and IAM (**B**). Preparation methods: **1)** Na_2S_4 was added to 900 µl buffer with 100 µl NEM/IAM solution. **2a)** Na_2S_4 was added to 900 µl water, then 100 µl NEM/IAM was added after dissolving, **2b)** Na_2S_4 was added to 900 µl water with 100 µl NEM/IAM **3)** Na_2S_4 was added to 1000 µl water, then 100 µl of the solution was added to 800 µl buffer with 100 µl NEM/IAM. Solutions 1), 2a) and 2b) were diluted 10x in ultra-pure water before analysis. **A:** With NEM, in condition 1) only S_2 and S_1 were detected with S_2 being more abundant. Conditions 2a) and 2b) show the same pattern of $S_2>S_1>S_3$ whilst condition 3) shows S_1 as most abundant with only S_2 and no higher-chain polysulfides detected. **B:** With IAM all four of the conditions show the speciation pattern $S_1>S_2>S_3>S_5>S_4>S_6$.

With a lower concentration of polysulfide (1 mM stock solution instead of 10 mM), only S_1 and S_2 are detected across the whole concentration range for both NEM and IAM and with S_3 detectable for IAM only. This is potentially due to the abundance of the larger polysulfides after equilibration dropping below the limit of detection due to the lower starting concentration. The lack of signal for the higher polysulfides may also be due to them being more unstable at lower concentrations and equilibrating to smaller chain lengths more rapidly than the rate of alkylation. Both S_4 and S_5 are detectable at the higher IAM concentrations (appendix B.2).

When using IAM to alkylate polysulfides the preparation method does not appear to have a significant impact on the overall speciation of the polysulfides. In all four starting conditions in the first set of preparation experiments (**Scheme 18**, **Figure 21**) and in all three conditions in the second set (**Figure 22**) the speciation pattern observed is $S_1>S_2>S_3>S_5>S_4>S_6$. However, when using NEM as the alkylation agent the starting conditions do appear to have a more significant effect on the measured polysulfide speciation. Using the same preparation methods as with IAM, in the first set of experiments (**Figure 21**) in condition **1)** only S_2 and S_1 are present with S_2 being the most abundant. In conditions **2a)** and **2b)** S_3 is now observed and both conditions show the same speciation pattern of $S_2>S_1>S_3$ (in terms of abundance). Condition **3)** shows only S_2 and S_1 as with condition **1)** but S_1 is now more abundant than the S_2. These results are consistent with the second set of experiments (**Figure 22**) with generally longer chain polysulfides detected using IAM rather than NEM though there are some differences in the patterns seen.

In the second set of experiments using different preparation methods (**Scheme 19**), when using NEM to alkylate polysulfides the speciation pattern does not change with the three different, polysulfide salts (Na_2S_2, Na_2S_3 and Na_2S_4) in that the lower chain length polysulfides are more abundant. What does change is that longer chain length polysulfides are detectable with the longer starting salts. Na_2S_2 only NEM_2-S_1 and NEM_2-S_2 are observed, with Na_2S_3 NEM_2-S_3 is also detectable and with Na_2S_4 NEM_2-S_4 becomes detectable. Regardless of the starting polysulfide salt however the differences in measured polysulfide speciation are the same with the three different preparation methods. With NEM_2-S_1 and NEM_2-S_2 condition **2** (Na_2S_x first dissolved in ultra-pure water, then diluted in NEM containing buffer) gives the highest abundance followed by a decrease in condition **3** (Na_2S_x dissolved in ultra-pure water, then diluted in buffer, then diluted in NEM containing buffer) and a further decrease with condition **1** (Na_2S_x dissolved in NEM containing buffer). The longer chain polysulfides observed using Na_2S_3 and Na_2S_4 both show the same pattern, with the highest abundance in condition **1** and no detectable signal for either NEM_2-S_3 or NEM_2-S_4 with condition **3**.

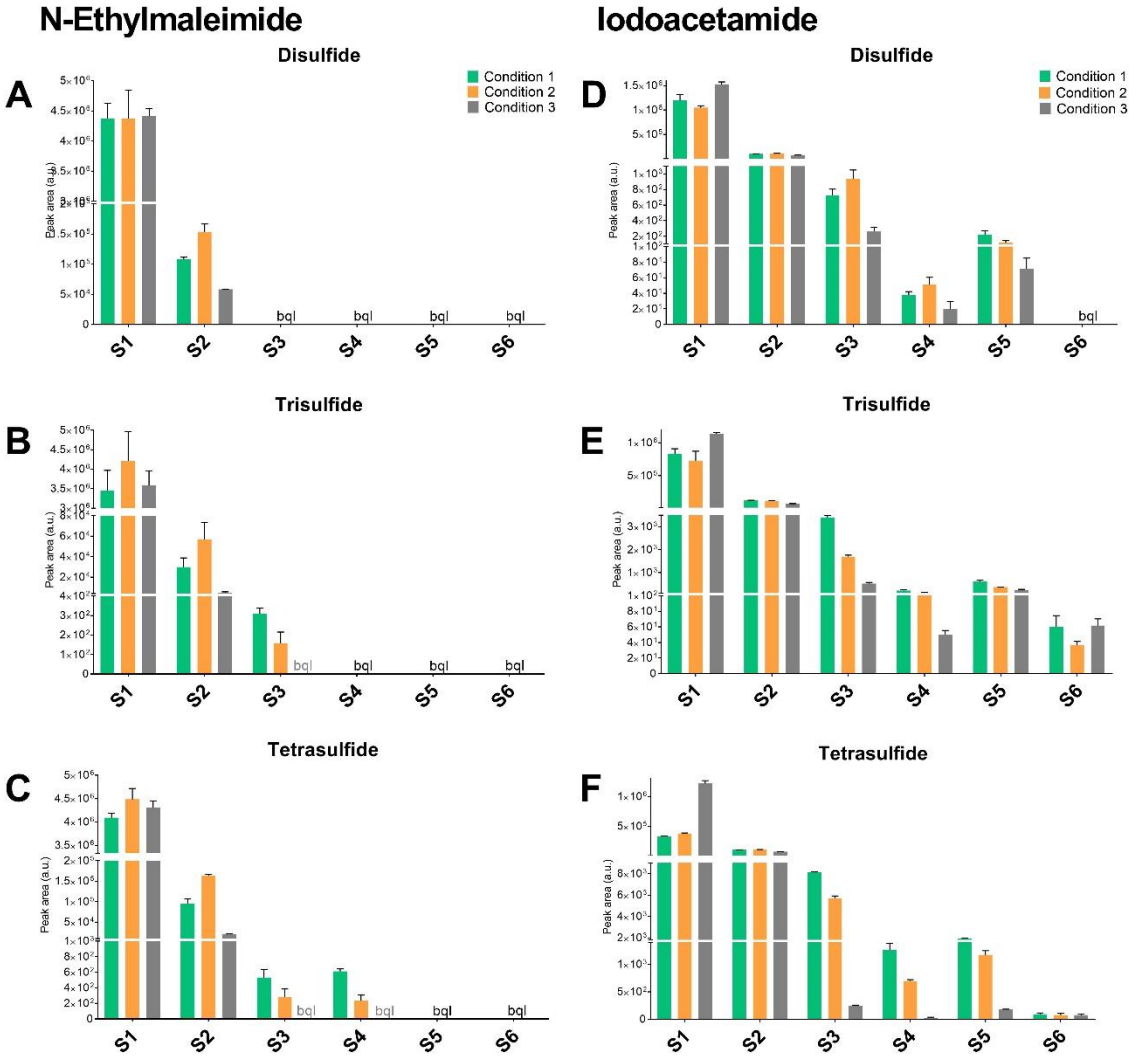


Figure 22: Differences in polysulfide speciation using three different polysulfide salts with three different preparation conditions[143]. **NEM: A)** Na_2S_2, **B)** Na_2S_3 and **C)** Na_2S_4. **IAM: D)** Na_2S_2, **E)** Na_2S_3 and **F)** Na_2S_4. Condition **1**: Na_2S_x dissolved in NEM or IAM containing buffer. Condition **2**: Na_2S_x first dissolved in ultra-pure water, then diluted in NEM or IAM containing buffer. Condition **3**: Na_2S_x dissolved in ultra-pure water, then diluted in buffer, then diluted in NEM or IAM containing buffer. Specific experimental procedures and conditions are described in **Scheme 19**, the shown data is representative of *n* = 3 experiments with similar results. Generally, shorter polysulfide chains were observed and speciation was more affected by the preparation conditions with NEM compared to IAM. These observations are consistent with **Figure 21**.

When using IAM to alkylate the polysulfides the starting polysulfide salt has less of an effect on the number of polysulfide species detected. Using Na_2S_3 and Na_2S_4 IAM alkylated polysulfide species from S_1 to S_6 are all observed. With Na_2S_2 it is only the S_6 that is not detected though with

the Na_2S_4 the S_6 levels are very low so it may be that they have dropped below the limit of detection. The preparation methods used have a varying effect on the alkylated polysulfide speciation depending on the starting salt used. Na_2S_2 shows different changes with different preparation methods than Na_2S_3 and Na_2S_4 which both show similar changes. However with all three salts IAM_2-S_1 has the highest abundance with condition followed by condition **1** then condition **2** (same preparation methods as with NEM). IAM_2-S_2 does not show any differences with any of the preparation methods used. When using Na_2S_3 and Na_2S_4 as the starting salts for the IAM alkylated polysulfides S_3, S_4 and S_5 condition **1** gives the highest abundance followed by condition **2** and then **3**, though the exact decreases vary between different conditions and starting salts (**Figure 22**). For Na_2S_2 with S_3 and S_4 it is condition **2** that has the highest abundance whilst S_5 follows the same pattern as the other two starting salts.

4.3.3.1 Impact of pH on Polysulfide Speciation After Derivatisation

As previously observed (section 4.2.3.1) derivatisation with NEM produces shorter chain length polysulfides than IAM, at all of the pHs tested. Increasing the pH of the reaction mixture for the NEM derivatisation mixture shifts the polysulfide speciation pattern towards the shorter chain length species observed (Figure 23). Above pH 7.4 there is a drop in the relative amounts of S_4, S_3 and S_2 compared to the sulfide. At pH 5.0 there is a relatively higher amount of S_2 and S_4 observed compared to the sulfide. These differences are consistent over the three polysulfide salts tested.

There is however less variation in the speciation pattern with pH for the IAM derivatised polysulfides (Figure 24), with the overall pattern remaining consistent, though some absolute peak areas vary. Slightly lower chain lengths are seen with the disulfide starting salt at pH 7.4 and higher but the overall pattern remains the same. The sulfate peak area did not vary by more than 10% across the whole course of the experiments, with less than 5% variation within each group of pHs and alkylating agents tested.

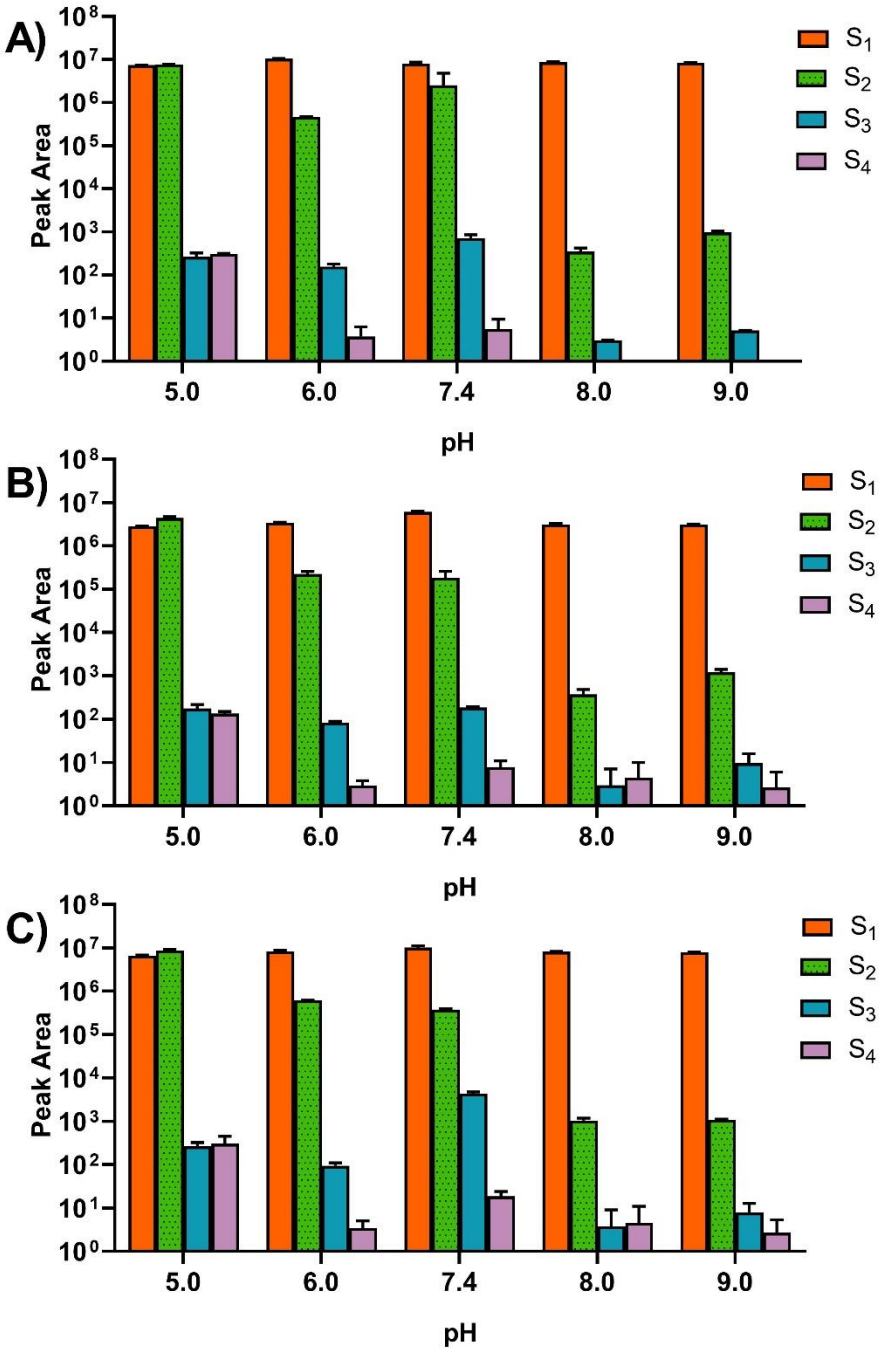


Figure 23: Effect of pH on the measured speciation of polysulfides after derivatisation with NEM. **A)** Disulfide salt, **B)** trisulfide salt and **C)** tetrasulfide salt. Increasing pH leads to the speciation pattern shifting towards lower chain lengths for all three of the tested polysulfide salts.

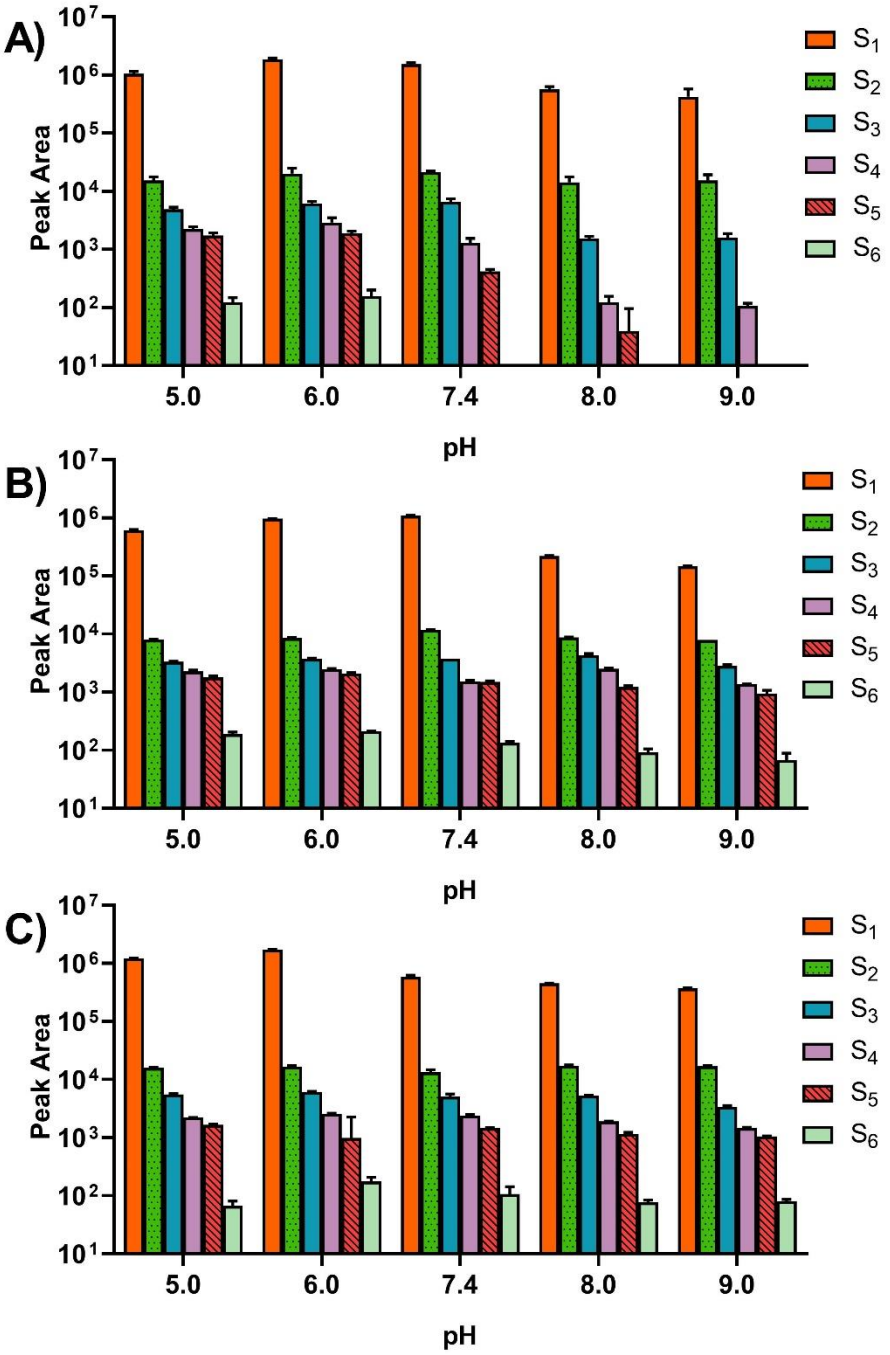


Figure 24: Effect of pH on the measured speciation of polysulfides after derivatisation with IAM. **A)** Disulfide salt, **B)** trisulfide salt and **C)** tetrasulfide salt. Changing the pH of the reaction mixture does not appear to significantly affect speciation pattern for all three of the tested polysulfide salts.

4.3.3.2 Impact of NEM on Polysulfide Chains

Unexpectedly the addition of NEM to polysulfides that have been fully alkylated with IAM results in significant changes to the speciation pattern observed as well as resulting in the appearance of alkylated species other than those derivatised with two IAM groups (**Figure 25**). Before the addition of NEM (60 min after IAM addition) sulfide (S_1), disulfide (S_2), trisulfide (S_3), tetrasulfide (S_4) and pentasulfide (S_5) IAM alkylated species were observed. The lack of higher chain length polysulfide species, which were observed using IAM in earlier experiments (**Figure 19**), may be due concentration of sodium tetrasulfide used as the levels of S_6 and S_7 may be below the limit of detection. In order to confirm this the experiment would have to be repeated using a higher initial concentration of tetrasulfide. No other alkylated polysulfides species were observed.

After the addition of NEM, IAM derivatised polysulfides (S_1, S_2, S_3 S_4 and S_5) are still detected but overall in a lower abundance than before NEM addition (**Figure 25, A**). The most significant decrease occurs in the S_4 and S_5 IAM alkylated species. Also observed after NEM addition are new alkylated polysulfide species which are alkylated with one IAM group and one NEM group, S_1, S_2, S_3 and S_4 mixed alkylated species are all observed (**Figure 25, B**). The S_2 species is initially the most abundant followed by the S_3 then the S_2 with S_4 being the least abundant, mixed species above S_4 are also not observed. Since the polysulfides should have fully reacted with IAM (x10 molar excess) there should have been no end-chain sulfurs left with which to react (**Scheme 23**). This implies that the addition of NEM has caused the polysulfide chains to fragment, either by direct reaction or by another mechanism. A small amount of fully NEM derivatised sulfide and disulfide are also observed after the addition of NEM (**Figure 25, C**).

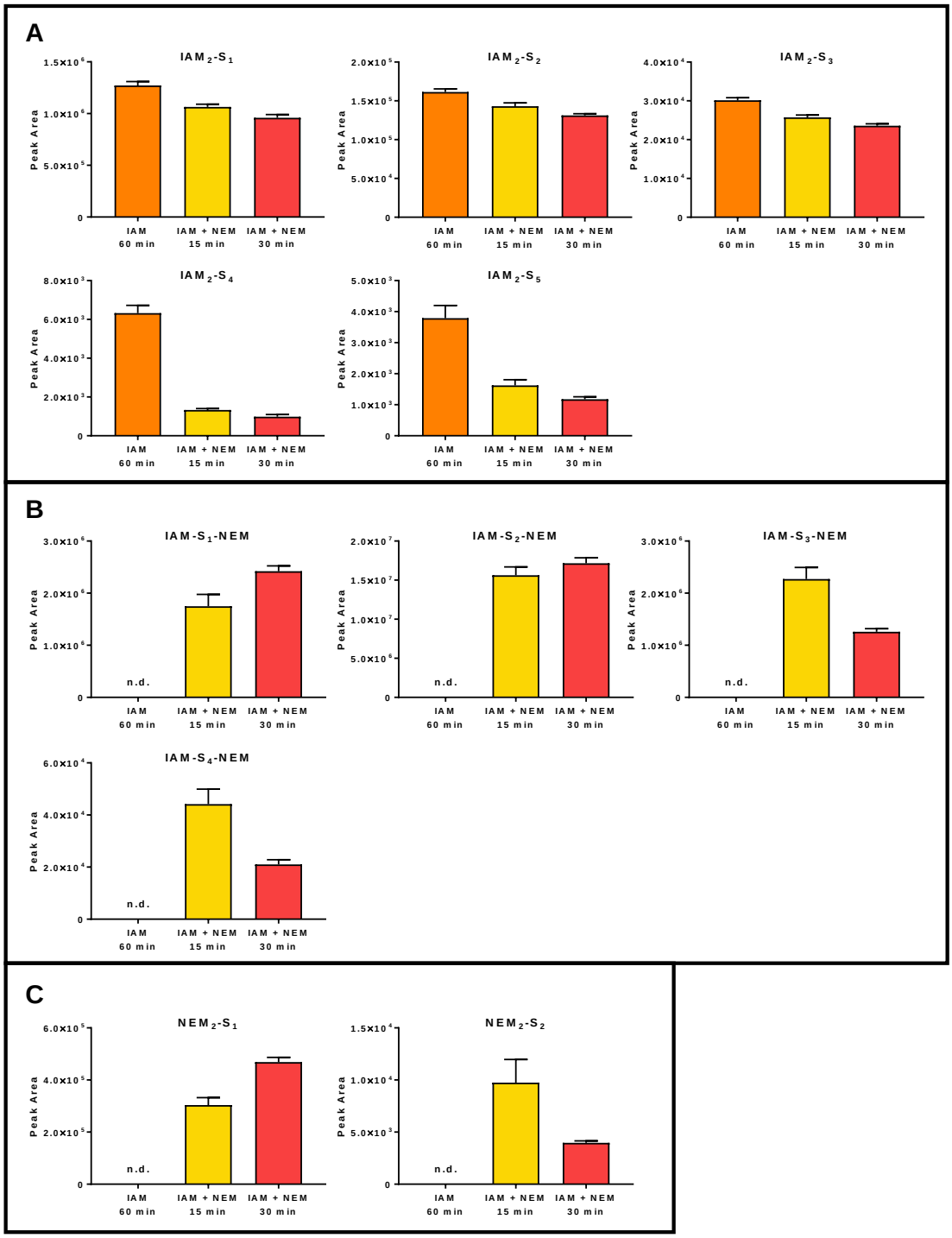


Figure 25: Impact of the addition NEM to fully IAM alkylated polysulfides. **A)** Observed IAM alkylated polysulfide species before and after the addition of NEM. In all species, there is a decrease after the addition of NEM, but it is much greater for tetrasulfide and pentasulfide. **B)** The appearance of sulfide and polysulfide species alkylated with both IAM and NEM. Both tetrasulfide and pentasulfide show a decrease in abundance between 15 and 30 min after NEM addition whilst the sulfide, disulfide and trisulfide show an increase. **C)** The appearance of wholly NEM alkylated sulfide and disulfide after addition of NEM. The disulfide shows a decrease in abundance between 15 and 30 min after NEM addition whilst the sulfide shows an increase.

The time of reaction with NEM also has significant effects on the observed speciation and alkylated polysulfides detected. All of the fully IAM alkylated species show a decrease between 15 and 30 min after NEM addition with the largest decrease being for the S_5 species. For the polysulfide species that have been alkylated with one IAM group and one NEM group the S_1 and S_2 species both increase between 15 and 30 min after NEM addition, with the S_1 increasing the greatest. Conversely the S_3 and S_4 species both decrease with the greatest being for the S_4. The fully NEM alkylated species show the same pattern with the S_1 increasing between 15 and 30 min after NEM addition and the S_2 decreasing. This suggests that even the short chain NEM alkylated polysulfides are vulnerable to chain fragmentation.

Overall, NEM appears to be able to cause fragmentation of polysulfide chains even when fully alkylated and this is likely the reason that generally shorter polysulfide chains are observed using NEM compared to IAM.

4.3.4 UHPLC-MS/MS Analysis of Organic Persulfides and Polysulfides

The reaction of sodium sulfide with oxidised glutathione produces glutathione persulfide as expected. Surprisingly however this reaction also produces glutathione polysulfides, specifically, glutathione trisulfide, glutathione tetrasulfide and glutathione pentasulfide (**Figure 26, A**). The species measured (glutathione - S_1, S_2, S_3, S_4 and S_5) elute in the same manner as the inorganic polysulfides, with the retention time increasing with increasing number of sulfurs, and with decreasing abundances. GSH-NEM elutes at 2.20 min, the persulfide at 2.30 min, trisulfide at 2.60 min, tetrasulfide at 3.20 min and glutathione pentasulfide elutes last at 3.45 min. Equally surprising there were no measured differences in the glutathione per- and polysulfide species produced either in relative abundance or the number of species observed when using sodium trisulfide instead of sulfide in the reaction mixture (**Figure 26, B**). Sulfide is able to generate glutathione polysulfides from oxidised glutathione as easily as inorganic polysulfides can. No inorganic polysulfides were detected in the sodium sulfide solution over the same period of time (not shown) eliminating inorganic polysulfide contamination or production as the source of the longer chain organic polysulfides.

Unknown peaks were observed in the chromatogram for glutathione pentasulfide staring at 2.20 min. These peaks are likely to be contaminants either in the reaction mixture or on the column used or potentially false peaks stemming from the other glutathione species present. Particularly the first unknown peak at 2.20 min is likely false signal originating from the reduced glutathione as the elution times are the same, it has a high abundance compared to glutathione pentasulfide and can produce the same fragment as the pentasulfide. The true pentasulfide peak can be

distinguished by the elution order, the glutathione polysulfides elute with increasing sulfur number and the peak at 3.45 min on the pentasulfide chromatogram falls in the expected elution time following this pattern. This highlights the importance of chromatography when analysing inorganic and organic polysulfides due to the non-specific fragmentation patterns of these species (**Table 5**).

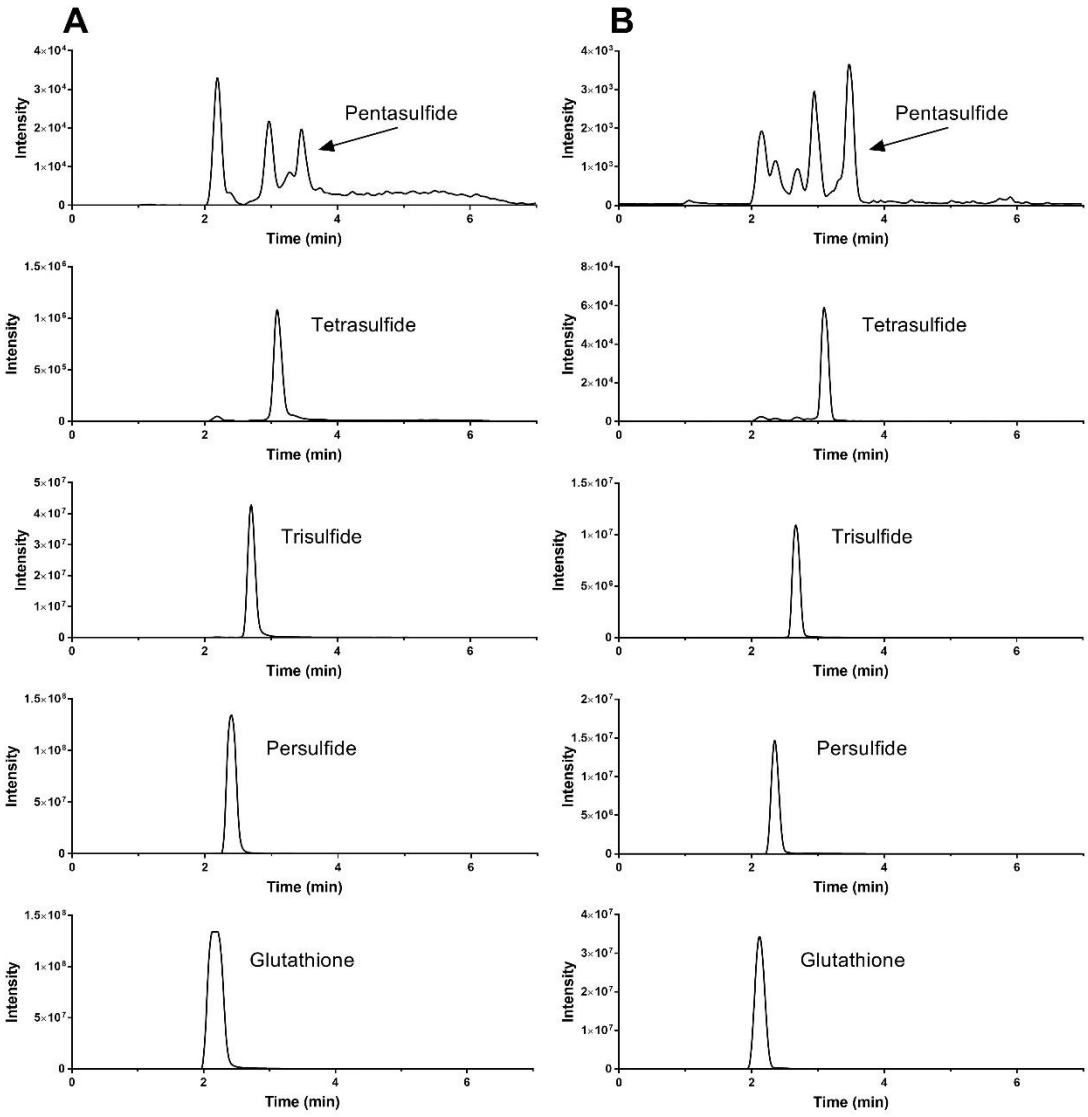


Figure 26: Exemplary chromatograms showing the glutathione polysulfides produced by incubation of GSSG with **A)** Na_2S and **B)** Na_2S_3 (1:1 molar ratio). For both Na_2S and Na_2S_3 the same organic polysulfide species are observed, from bottom to top: Glutathione, glutathione disulfide, glutathione trisulfide, glutathione tetrasulfide and glutathione pentasulfide.

4.3.5 Investigation of Potential Polysulfide Formation by the Antioxidant Enzyme Superoxide Dismutase

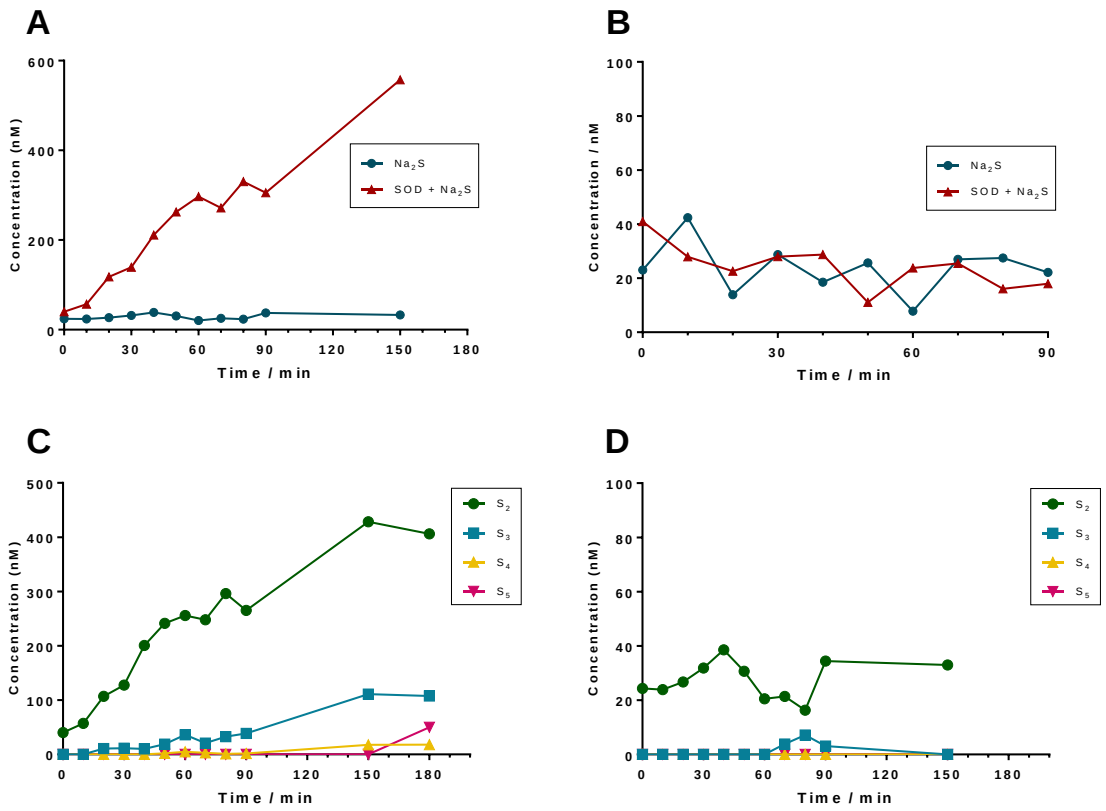


Figure 27: Production of inorganic polysulfides by SOD metabolism of Na_2S. **A)** Total production of polysulfides by 1 μM SOD + 1 mM Na_2S and 1 mM Na_2S only, no significant amounts of polysulfide are produced by only Na_2S. **B)** Lack of production of polysulfides (total) by 1 μM SOD + 1 mM Na_2S under hypoxic conditions, with no significant difference in polysulfide levels in the absence of SOD the polysulfides present are likely contaminants in the stock Na_2S. **C)** Production of individual polysulfide species by 1 μM SOD + 1 mM Na_2S showing sequential formation of polysulfide species ranging from persulfide to tetrasulfide. **D)** Lack of production of polysulfides with 1 mM Na_2S only (note the reduced scale), persulfide is initially present, likely as a contaminant in the stock Na_2S but does not show a significant increase over 150 min.

By using mass spectrometry detection, we were able to identify the specific polysulfide species formed by SOD metabolism of Na_2S in aqueous solution. Under normoxic conditions no polysulfides were produced by 1 mM Na_2S only in solution over 150 min, some persulfide was observed but this did not increase over the 150 min and is likely a contaminant in the Na_2S stock. However, in the presence of 1 μM SOD significant amounts of polysulfides are formed over 180

min (**Figure 27**, **A-D**). Persulfide is formed initially before longer chain polysulfides start to form, trisulfide after 20 min, tetrasulfide after 150 min, and pentasulfide after 180 min. The polysulfides appear to be produced sequentially, with longer chain lengths not being produced until the preceding polysulfide has been formed. In hypoxic conditions when 1 μM SOD is incubated with 1 mM Na_2S there is no production of polysulfides (**Figure 27**, **B**).

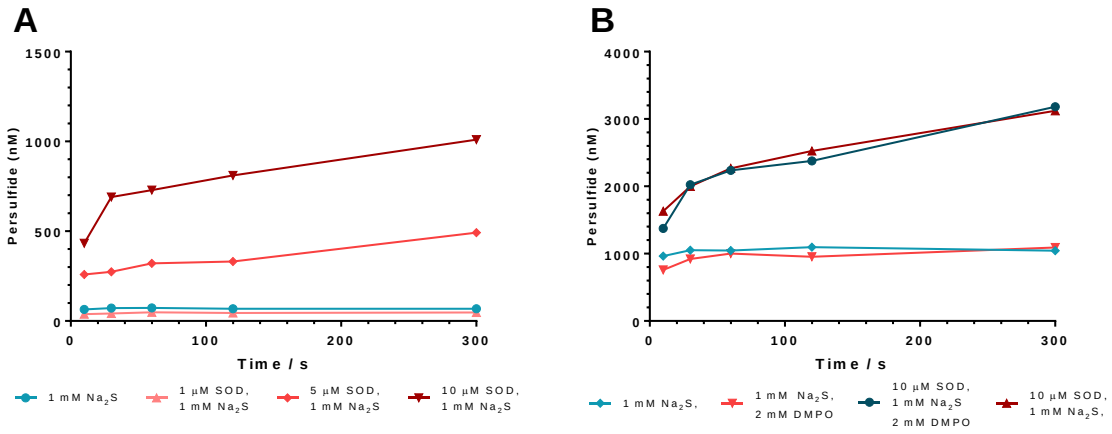


Figure 28: Short-term measurements of the production of persulfide by SOD metabolism of Na_2S. **A)** The impact of SOD concentration on the initial production of persulfide from 1 mM Na_2S. Increasing the concentration of SOD increases the rate of formation of persulfide, at elevated SOD concentrations the persulfide starts to form within 10 seconds. **B)** Addition of the radical scavenger DMPO (2 mM) does not affect the production of persulfide by 10 μM SOD + 1 mM Na_2S.

Persulfide formation occurs within two minutes after addition of 1 μM SOD to 1 mM Na_2S (**Figure 28**, **A**). The rate of persulfide formation increases in a SOD concentration dependant manner with 5 μM and 10 μM SOD showing an initial increase in persulfide formation occurring rapidly within 10 s of SOD addition (faster than samples could be processed) followed by a slower rate of production. Both the rapid and slower rates of formation appeared to be SOD concentration dependant. In order to investigate the potential mechanism of polysulfide production by thiyl radical formation DMPO was added to the SOD/Na_2S reaction mixture in an attempt to trap any thiyl radicals potentially formed. However, the addition of DMPO has no effect on the formation of persulfide by SOD metabolism (**Figure 28**, **B**).

4.4 Discussion

4.4.1 Direct Infusion Analysis of Aqueous Polysulfides

Regardless of the starting polysulfide salt used the measured distribution of aqueous inorganic polysulfide species in pure water are qualitatively the same. The mixed potassium polysulfide (S_1-S_5) shows a very similar species distribution as the pure sodium tri and tetrasulfides. Initially it was expected that hydropolysulfide anions (HS_n^-) would be observed[149], the underivatised polysulfide species seen in these experiments however have an *m/z* that corresponds more closely to that of a radical anion species ($S_n^{\bullet-}$). Due to the low-resolution nature of the TQD detector the exact assignment cannot be made with a high degree of certainty, so higher resolution analysis was needed to confirm the assignment of these polysulfide species in aqueous solution.

Derivatisation with IAM changes the measured speciation pattern; when underivatised the $S_3^{\bullet-}$ is the most abundant polysulfide species observed, after derivatisation however the IAM_2-S_2 species is the most abundant. Therefore, chemical derivatisation with an electrophile most likely alters the distribution of polysulfide species upon reaction. One explanation for this observation is that the rate of equilibration between the different polysulfide species in solution is more rapid than the rate of reaction with the electrophile; if this were the case, the derivatisation reaction would disturb the equilibrium and favour the most reactive polysulfide species. It is also possible that the differences in polysulfide distribution seen are due to the mechanism of ionisation in the ESI source. The underivatised polysulfides are best seen using negative ionisation due to their low pK_a[149], whilst the derivatised species are best observed using positive ionisation mode. It should be considered however that there may be differences in the ionisation efficiencies of the different polysulfide species. Any differences could also potentially be altered after alkylation, therefore it may not be a shift in the abundance of the species after derivatisation but instead a change in the ionisation efficiencies of the different species.

Because the pure polysulfide standards all produce the same speciation pattern as each other and the mixed potassium polysulfide when made up in solution, and this pattern then changes upon derivatisation, these standards cannot be easily used for the quantification of polysulfide species in biological samples. A compromise could be used by making up a known amount of pure polysulfide such as Na_2S_3 or Na_2S_4 (e.g. to 100 mM) and alkylating with the relevant electrophile. The relative proportion of each measured polysulfide in solution could then be taken from the initial concentration as the amount of each species. For example if the IAM_2-S_3 was 40% of the total polysulfides from an initial 100 mM it would be assumed to have a concentration of 40 mM. These changes in polysulfide composition on dissolution and alkylation call into question the

speciation patterns observed in biological samples. If the derivatisation reaction changes the speciation, how can it be certain that the speciation measured is representative of the actual physiological distribution.

4.4.2 High Resolution Mass Spectrometry of Aqueous Polysulfides

The results of the high-resolution analysis strengthen the previous assignment of the aqueous polysulfide species observed using ESI-MS/MS as radical anions. The measured accurate masses of the previously assigned radical anion polysulfide species ($S_2^{\bullet-}$, $S_3^{\bullet-}$ and $S_4^{\bullet-}$) are all in close agreement with the calculated exact masses with the biggest difference of 131 ppm being for the $S_4^{\bullet-}$ species (**Table 6**), which is likely that high due to the low abundance of that species. Key to note is that even with the larger difference between exact and measured mass the measured masses are still much closer to those of radical anions than for hydropolysulfide anions. The isotopic distribution of the observed species provides further confirmation of the assignments. The most abundant natural stable isotope of sulfur, other than ^{32}S is ^{34}S with a natural abundance of 4.22%, ^{33}S has a relatively low natural abundance (0.76%)[332]. The only isotope peak that should be observed for radical anion polysulfide species will be at M+2 with a signal intensity relative in height to the parent peak. This is reflected in the isotope peaks of the measured polysulfide species. No M+1 peaks are observed, but for $S_3^{\bullet-}$ and $S_4^{\bullet-}$ and there are clear M+2 peaks at *m/z* 97.9128 and *m/z* 129.9001 respectively (**Figure 18, B and C**). These isotope peaks also show the expected relative abundance when compared to their parent peaks.

The observed *m/z* for the polysulfide species could potentially be explained by doubly charged anions rather than radical anion species. A doubly charged hexasulfide anion (S_6^{2-}) would give exactly the same *m/z* as the proposed trisulfide radical anion ($S_3^{\bullet-}$) whilst a doubly charged octasulfide anion (S_8^{2-}) would give the same *m/z* as tetrasulfide. These two possibilities can be distinguished by looking at the *m/z* of the isotope peaks and their relative abundances to the parent peak. For $S_3^{\bullet-}$ the isotope peak would fall at the parent *m/z* + ~2 *m/z* units (M+2) and have an intensity of ~12% of the parent peak as ^{34}S has an abundance of 4.22% and there are three potential ^{34}S in the trisulfide. For S_6^{2-} the isotope peak would fall at the parent *m/z* + ~1 *m/z* units (M+1) due to the multiple charge halving the *m/z* of both the parent and isotope peaks, and would have a much higher relative intensity of ~25% of the parent peak. In the measured polysulfide solution there is a clear isotope peak for the *m/z* 95.9168 observed at M+2 (*m/z* 97.9128) with an intensity of ~12% of the parent peak. No isotope peak with ~25% intensity is observed at M+1 (**Figure 18, B**) which suggests that it is in fact a trisulfide radical anion species being observed and not a doubly charged hexasulfide anion. The same argument is equally applicable to the assigned tetrasulfide peak as to why it is not a doubly charged octasulfide anion.

A point of caution however is that the SYNAPT G2-Si ESI-ToF-MS uses the same ESI source as the TQD mass spectrometer used in the previous experiments (section 4.2.1). Therefore, if the polysulfide radical ions originated in the source or were due to the mechanism of ionisation no differences in apparent species would be observed between these two experiments. A different ionisation source could potentially produce hydro-polysulfides instead and/or different polysulfide speciation patterns. Investigating the effect of different ionisation sources would be a useful comparison and help determine if the polysulfide radical anions are generated in solution or generated by the ESI source. It has however been noted that in similar experiments no major changes in the measured speciation were seen upon changing some MS parameters including source temperature, voltage and flow rate[143,149].

4.4.3 UHPLC-MS/MS Analysis of Inorganic Polysulfide Speciation Following Chemical Derivatisation

Even with a rapidly reacting electrophile such as NEM the rate of polysulfide equilibration on dissolution occurs much more rapidly than the rate of reaction with the electrophile (NEM or IAM). As a result the speciation of the derivatised products appears the same regardless of the starting polysulfide salt used (mixed potassium polysulfide, sodium trisulfide and tetrasulfide). This phenomenon is described by the Curtin-Hammett principle[333]; Under Curtin-Hammett control, the ratio of products (the speciation) is not equal to the ratio of the precursors, but instead dependant on the rate of interconversion between the precursors in equilibrium, as the rate of interconversion is much greater than the rate of reaction. In this case it is apparent that the polysulfides are able equilibrate in solution much more rapidly than they react with the electrophile. Under Curtin-Hammett control the reaction with the electrophile can also serve to pull the equilibrium towards the most stable and reactive polysulfide species and away from the true distribution in solution, with the most reactive polysulfide species then being overrepresented in the derivatised products.

The choice of electrophile has a significant impact on the speciation observed after derivatisation; IAM shows a larger range of polysulfide species than NEM, with longer chains being observed (**Figure 19**, **Figure 22**). The two electrophiles have significantly different reactivities as well as different reaction mechanisms; NEM reacts with nucleophiles such as polysulfides via Michael addition whilst IAM reacts through a nucleophilic substitution (S_N2) reaction (**Scheme 17**). The difference in measured species could be due to NEM being a more reactive electrophile than IAM[322] and potentially causing fragmentation of the longer polysulfide chains. The longer observed chain lengths could also be due to the slower rate of reaction of IAM allowing the polysulfides to equilibrate to longer chain lengths before complete alkylation. The differences in

reaction mechanism could also cause the equilibrium between the polysulfide species to shift differently depending on which polysulfide species reacts the quickest with either electrophile. The concentration of the electrophile also has impact on the measured speciation, which could be linked to the rate of reaction. With NEM the average chain length of detected polysulfides tended to decrease with increasing NEM concentration (**Figure 20**, **A**). With IAM however the polysulfide speciation appears to be more stable with changing IAM concentration (**Figure 20**, **B**). It should also be noted that when lower starting concentrations of polysulfide only S_1 and S_2 were detected for all concentrations tested for NEM and S_1, S_2 and S_3 for IAM. This could be due to the abundance of the longer chain polysulfides dropping below the limit of detection due to the overall lower sulfane sulfur concentration. Another explanation could be due to slower disproportionation of the smaller polysulfides as this is a second order reaction meaning the rate depends on the overall concentration.

The methods used to prepare the polysulfide salts were also found to have varying effects on the measured polysulfide speciation depending on the alkylation agent used. The effect of the exact composition and order of mixing of the starting solution was investigated (**Figure 21**) at first using four different conditions, see **Scheme 18** for details. It was found that the preparation method when using IAM did not have a large impact on the speciation pattern observed, though the absolute levels of some of the polysulfide species did vary slightly. However when using NEM there was a more significant effect with the speciation pattern differing depending on the preparation method used. This difference between IAM and NEM is likely due to the polysulfide distribution equilibria shifting more during alkylation with NEM than with IAM under the conditions tested. This assumption is backed up by the second set of experiments looking further at different preparation conditions (**Figure 22**), three further conditions were tested, see **Scheme 19** for details. When using NEM the highest chain length polysulfides observed corresponded to the polysulfide salt used as the starting material whilst with IAM longer chain length polysulfides are also observed. These differences further illustrate the huge complexities associated with measuring the speciation of aqueous inorganic polysulfides and the huge number of factors that can potentially alter the speciation that is measured.

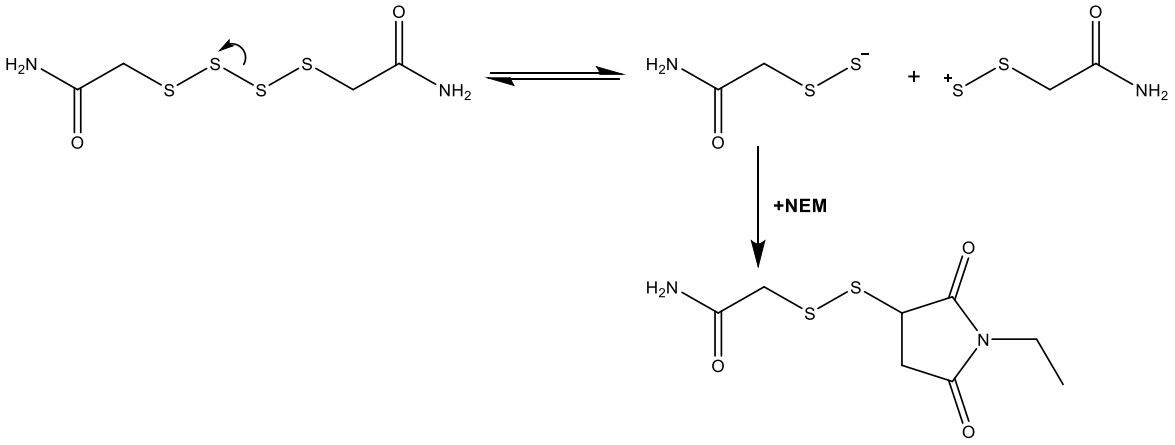
Despite the three polysulfide salts tested having different pK_a values (disulfide pK_{a1} = 5.0, pK_{a2} = 9.7, trisulfide pK_{a1} = 4.2, pK_{a2} = 7.5 and tetrasulfide pK_{a1} = 3.8, pK_{a2} = 6.3)[149], the pH of the reaction mixture does not have a large impact on the measured speciation of the different polysulfide salts with either NEM or IAM. With NEM whilst there is some variation with pH with (Figure 23) these differences are reflected by all three polysulfide salts and therefore not influenced by the different pK_a values. This change in polysulfide speciation with pH was not reflected when using IAM. This is potentially due to the difference in rate of reaction between the two alkylating

agents, with IAM taking significantly longer for a complete reaction (see Chapter 5:. The longer reaction time would allow a longer equilibration time for the polysulfides during the reaction and reduce the effect of different pHs on the speciation pattern.

Initially NEM was considered more appropriate for the trapping of polysulfide speciation than IAM due to its much higher rate of reaction. However, throughout the series of experiments it became apparent that when using NEM consistently shorter polysulfide chains were measured when compared to the equivalent reaction using IAM (**Figure 19** and **Figure 22**). It has also been noted that NEM may be able to cause the decomposition of protein-bound cysteine polysulfides[117] making NEM less suitable for the analysis of longer chain polysulfides. In order to test the hypothesis that NEM is able to attack midchain sulfurs in inorganic polysulfides and cause the chains to fragment, polysulfide solutions were fully reacted with an excess of IAM before NEM was added to the solution. After full reaction with IAM there should be no end-chain sulfurs available for NEM to react with based on the data shown in **Figure 17**. After the addition of NEM to the IAM alkylated polysulfide solution, the measured IAM alkylated species all show a decrease in abundance along with the appearance of polysulfide species that are alkylated with both IAM and NEM (**Figure 25**). This suggests that NEM was able to react with non-terminal sulfurs leading to the polysulfur chain fragmenting. The largest decrease in the IAM alkylated species occurs with S_4 and S_5, it is likely that these species are more vulnerable to attack by the NEM as they have longer polysulfide chains that may give NEM more opportunities to attack the mid chain sulfurs or the mid chain sulfurs may have a greater reactivity. For the mixed alkylated species, the S_2 and S_3 are the most abundant with no S_5 observed either. This supports the hypothesis that the mixed species are forming from chain fragmentation and that the longer chains are more vulnerable to mid chain attack, longer chains will always fragment to produce two shorter chains. This equally applies to the observed S_1 and S_2 NEM alkylated species. In order to produce doubly NEM alkylated species the IAM alkylated polysulfide chains will have to undergo fragmentation twice, greatly shortening the chain lengths observed. These observations are further supported by the continuing changes in the measured polysulfide species between 15 and 30 minutes after addition of NEM (**Figure 25**). An increase in the concentrations of the S_1 and S_2 mixed alkylated species is mirrored by a decrease in the S_3 and S_4 species. Again this suggests that the longer chains, including the mixed polysulfides, are more vulnerable to chain fragmentation by NEM whilst the shorter chains are continuing to be generated from the fully IAM alkylated species.

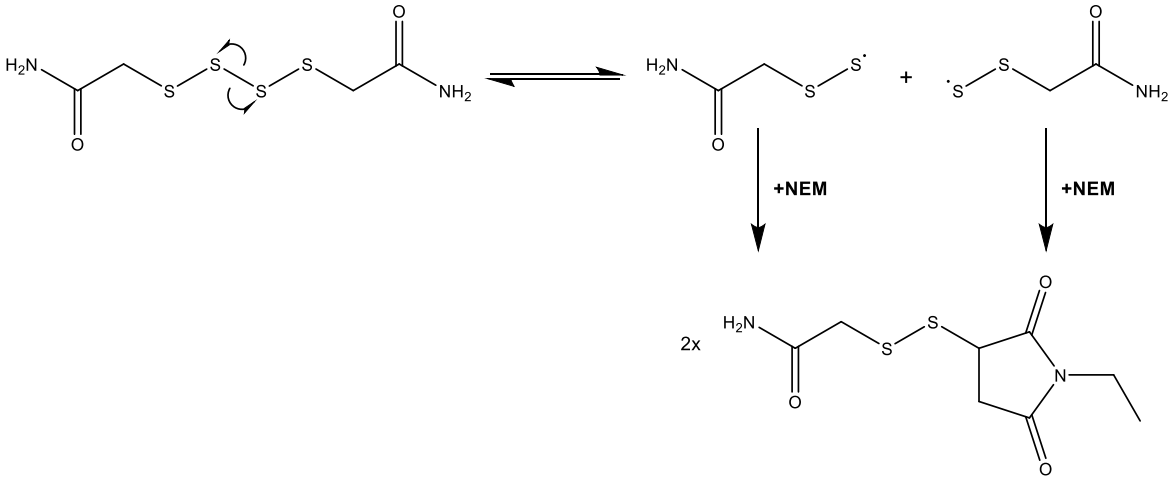
This reaction is somewhat unexpected and the exact mechanism is unclear as it suggests that NEM is able to react with polysulfides by a mechanism other than nucleophilic addition. There are several potential models that could explain these observations[143].

1) Heterolytic cleavage; It is possible that preceding the reaction with NEM there is a heterolytic cleavage of the S-S bond in the fully IAM alkylated polysulfide adduct resulting in the formation of two alkylated polysulfur chains one with a positive charge and one with a negative charge. In a following second step NEM could react with the negatively charged IAM polysulfide to form the hybrid adduct (**Scheme 20**).



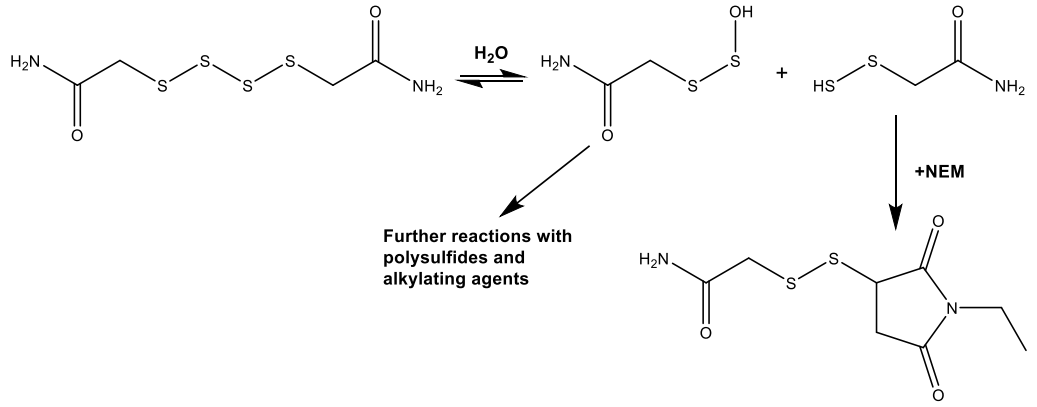
Scheme 20: Example of heterolytic cleavage of IAM alkylated polysulfide and subsequent reaction with NEM to form a mixed alkylated species.

2) Homolytic cleavage; The IAM alkylated polysulfides may have a tendency to cleave homolytically resulting in the formation of IAM-S_x radical species that could then add to the double bond of NEM. This is supported by the observation that the NEM adduct of S_2^- is not stable at pH 7.4[293] suggesting that NEM and IAM derivatised polysulfide adducts are metastable with both NEM-S and IAM-S being good leaving groups (**Scheme 21**).



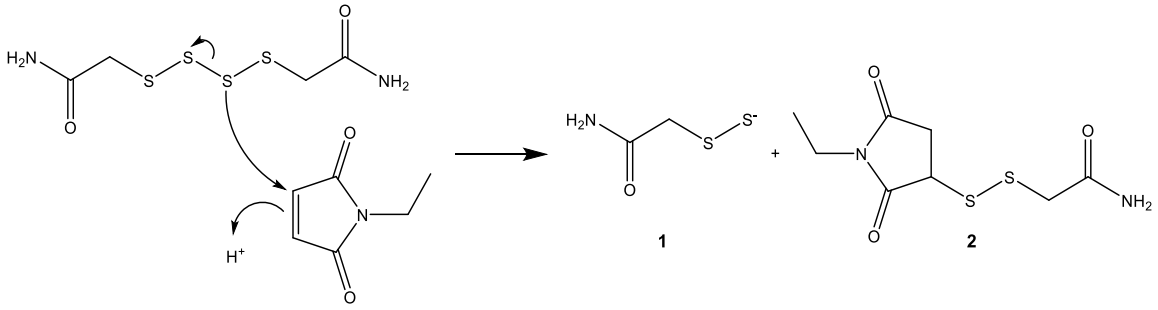
Scheme 21: Example of homolytic cleavage IAM alkylated polysulfide and subsequent reaction with NEM to form two mixed alkylated species.

3) Hydrolysis; It is also possible that the alkylation by NEM is able to shift the hydrolysis of longer chain polysulfide equilibria towards shorter chain lengths. As well as producing shorter chain alkylated species it would also generate alkylated sulfenic acid (**Scheme 22**).



Scheme 22: Example of hydrolysis induced IAM polysulfide fragmentation and subsequent reaction with NEM to form mixed alkylated species.

4) Direct reaction; The alkylating agents could also potentially attack a midchain sulfur bond (**Scheme 23**), producing an IAM alkylated polysulfide anion (**1**) and a mixed NEM and IAM alkylated polysulfide species (**2**). The IAM alkylated polysulfide anion could react further with NEM producing another mixed derivatised species such as **2** or with H_2O to produce an alkylated sulfenic acid. This potential model is supported by the fact that he longer chain (S_4 and S_5) IAM alkylated polysulfides are more vulnerable to fragmentation after addition of NEM (**Figure 25**).



Scheme 23: Suggested direct reaction between IAM derivatised polysulfide and NEM resulting in fragmentation of the polysulfide chain and the formation of a mixed IAM and NEM alkylated species.

Tools and methods for the analysis of polysulfides have been available for some time[133,149,283] and will continue to be developed and refined as the importance of reactive sulfur species becomes better known. However many of these methods have serious caveats as has been demonstrated with alkylation-based methodology shown here. Whilst these methods are more than capable of detecting polysulfides, the measured speciation is likely not representative of the physiological

distribution. NEM based methodology is biased towards shorter chain polysulfides and may even miss the existence of longer polysulfide species when compared to IAM. However, IAM has a much slower reaction time so for the analysis of disulfides NEM will provide a benefit with the faster reaction time preventing artifactual oxidation compared to IAM. Monobromobimane (MBB) is another commonly used electrophile for the analysis of thiols and sulfide (section 1.4.3, **Scheme 8**). The use of MBB is often coupled with fluorescence detection[112], but can also easily be detected using mass spectrometry. The effects of MBB on inorganic polysulfides have not yet been thoroughly investigated, but since IAM and NEM have differing effects on the polysulfide species measured and their distribution, MBB could also potentially cause other changes in the speciation of polysulfides. It has also been demonstrated that MBB has similar effects to NEM on protein cysteine polysulfides[143] and so will be less suited for analysis of longer polysulfide chains. Whilst these alkylation based methods still have a place in the analysis of polysulfides the discussed chemistries should be considered when applying these methods when analysing the chemical biology of polysulfides.

4.4.4 UHPLC-MS/MS Analysis of Organic Persulfides and Polysulfides

Initially it was thought that the reaction of oxidised glutathione (GSSG) with sodium sulfide would produce glutathione persulfide and no other longer chain organic polysulfides, whilst the reaction with sodium trisulfide would produce longer chain polysulfides. The reasoning being that sulfide would only be able to add single sulfurs to the GSSG, producing the persulfide, whist the trisulfide which equilibrates in solution to form multiple different chain length polysulfides (see section 4.3.1) would be able to add multiple sulfurs therefore forming a mix of glutathione polysulfides. Unexpectedly however, the glutathione per- and polysulfide species generated by reaction of sodium sulfide and sodium trisulfide with GSSG show qualitatively the same distribution of species (**Figure 26**). These results have been confirmed by a separate group using both oxidised glutathione and cysteine[143].

This has implications for the measurement of organic persulfides and polysulfides in biological samples. Since free sulfide will be present in the biological matrix per- and polysulfides could be artifactually generated during sample collection and handling by the same reaction employed here. Therefore, it will be important to ensure that sample collection and preparation is done in such a way that minimises the artifactual production of these species. This could potentially be done by keeping samples on ice as much as possible throughout processing to slow down the reaction producing organic polysulfides as well as by adding a rapidly reacting electrophile to trap the organic polysulfide species as quickly as possible after sample collection in order to block any reactive sulfane sulfurs.

However, as discussed in section 4.4.3, NEM causes fragmentation of polysulfide chains by reacting with the mid chain sulfurs. It should therefore be considered that whilst NEM is one of the fastest reacting electrophiles used in this context it will be able to fragment organic polysulfides as well and so will not be as suitable an electrophile for trapping the in-vivo status of organic polysulfides. IAM or other electrophiles may be more suitable for this kind of analysis, in that they will cause less fragmentation of the organic polysulfides, but slower rates of reaction would likely allow the artifactual generation of some organic polysulfide species. Further investigations into the alkylation of organic polysulfides using monobromobimane (MBB) and the IAM based electrophile β-(4-hydroxyphenyl)ethyl iodoacetamide (HPE-IAM) have been performed in conjunction to the work presented here. It was found that MBB has similar effects to NEM and when used it produces shorter polysulfide chains when compared to IAM, with its effectiveness sitting between NEM and HPE-IAM. HPE-IAM was not found to cause any significant fragmentation of the organic polysulfides with little effect on the observed speciation with concentration of HPE-IAM[143] but if the rate of reaction is comparable to IAM or slower, the measured species may still not be representative of the physiological distribution.

Generally, NEM is unsuited for the analysis of long chain organic polysulfides though analysis of organic persulfides could benefit from the faster reaction time of NEM though caution should be used to ensure NEM is not artifactually generating persulfides from polysulfides. Care must also be taken during sample collection, handling and processing to ensure artifactual production of organic polysulfides is kept to a minimum.

4.4.5 Investigation of Potential Polysulfide Formation by the Antioxidant Enzyme Superoxide Dismutase

IAM was chosen as the electrophile to trap the polysulfides as they were produced despite its limitations, such as its slower reaction time and potential to alter the speciation (section 4.4.3). NEM was not used, as the chain fragmentation caused by NEM would lead to a more inaccurate picture of the polysulfide species formed (section 4.4.3). The main aim of the experiment was to confirm the formation of inorganic polysulfides by SOD and IAM is suitable for this aim as polysulfides will only be measured using IAM derivatisation when they are indeed present (see **Figure 27**, **C** and **D**).

Na_2S in ammonium phosphate buffer does not produce any significant polysulfides (**Figure 27**, **A** and **D**), whilst there is some persulfide present this does not significantly increase and is likely a contaminant in the sulfide stock. It is only in the presence of SOD that production of polysulfides from Na_2S is observed (**Figure 27**, **A** and **C**). This confirms that SOD has biological activity involving

RSS as it is able to catalyse the oxidation of sulfide to polysulfides. The polysulfide formation occurs in a sequential fashion, first the persulfide is formed, then the trisulfide followed by tetrasulfide and finally pentasulfide (in the time monitored for these experiments, longer chains may be formed if allowed to react for longer than 180 min). The mechanism of formation of the polysulfides is unclear; one possibility is that SOD generates thiyl radicals ($HS^{\bullet}$) from sulfide, two of which could combine to form the initial persulfide, further thiyl radicals generated could then react with the sulfane sulfurs on persulfide (and other polysulfides) elongating the sulfur chain[138]. Alternatively, the longer polysulfide chains could be a result of normal polysulfide equilibration (see section 4.3.3), which would occur more over the longer reaction times, or possibly through the combination of shorter polysulfide chains, i.e. H_2S_2 and H_2S_3 forming H_2S_5.

The radical scavenger DMPO was used to investigate the potential mechanism by which SOD catalyses the formation of polysulfides from sulfide. If SOD catalyses one electron reduction of sulfide by formation of thiyl radicals ($HS^{\bullet}$), then by adding a radical scavenger any thiyl radicals formed would potentially be trapped by the DMPO, thereby preventing the formation of the initial persulfide and other polysulfides. However adding DMPO to the SOD and Na_2S reaction mixture did not have any effect on the formation of persulfide (**Figure 28**, **B**). There are a number of potential explanations as to why DMPO had no effect; Persulfide formation may not occur through the formation of thiyl radicals but rather via two-electron oxidation of sulfide that could then combine with a second sulfide. The rate of reaction of the thiyl radicals with one another may exceed the rate of reaction of DMPO with the thiyl radicals, the rate of equilibration of polysulfide species has already been observed to be incredibly rapid and so the reaction of the thiyl radicals may well out compete the reaction with DMPO. Alternatively, if the production of persulfide occurs wholly in the catalytic site of SOD then DMPO may have limited access and not be able to scavenge the thiyl radical as it is formed.

What is clear about the mechanism of formation is that it requires the presence of oxygen to proceed. Under normoxic conditions SOD readily produces polysulfides with rapid formation of persulfide within 2 min (1 mM SOD + 1 mM Na_2S). Under hypoxic conditions, there is no significant formation of any polysulfides observed using the same amounts of SOD and Na_2S (**Figure 27**, **B**). The reaction is also SOD concentration dependant, with the initial rate of formation of persulfide increasing with SOD concentration. In fact, increasing SOD from 1 μM to 5 and 10 μM has such a large impact on the initial rapid rate formation of the persulfide that a large increase in the persulfide concentration is seen within the first 10 seconds of reaction, which is faster than the samples can be reasonably processed (**Figure 28, A**). On top of this initial rapid increase in persulfide, there is then a slower but steadier increase in persulfide concentration that is also SOD concentration dependant.

Overall, these experiments help confirm the hypothesis that antioxidant enzymes not only are involved in ROS metabolism but also have potentially important activities around RSS. However, these experiments show that the presence of oxygen is required for the metabolism of sulfide to polysulfides. If these enzymes did originate before the advent of oxygen in the atmosphere by SOD then alternate, and as of yet unidentified, electron acceptors would have been required for this metabolic activity. Indeed in further sets of experiments collaborating groups have shown that H_2O_2 can also act as an electron acceptor in this metabolic activity[138]. In rat hepatocyte cytosol the Cu/ZnSOD concentration is equivalent to 42 μM, and in human red blood cells it is equivalent to 75 μM[334,335]. This is far greater in excess than the 1 μM SOD used for the majority of the experiments and the lower threshold for polysulfide production by SOD was found to be ~0.3 μM in another series of experiments[138]. The Na_2S concentrations used in these experiments (1 mM) are far greater than are thought to exist freely in the cell which are <1 μM. The initial concentrations of persulfide produced when compared to the starting concentration of Na_2S are also relatively low. Several factors might account for these discrepancies. Firstly intracellular concentrations of H_2S are expected to be considerably higher in the vicinity of their production[336], but as of yet these levels have not been determined. It has also been proposed that whilst the free circulating and intracellular levels of H_2S may be relatively low there likely exists readily available storage forms such as the inorganic, organic and protein bound polysulfides[19,277] that could rapidly produce more H_2S as it is consumed. H_2S is very volatile, as evidenced by the obvious odour upon dissolution. It is very likely that the actual concentration of sulfide in solution during the experiments is in fact lower than the initial 1 mM that was made up, and will be decreasing throughout the length of the experiment. Finally, it may also be possible that other sulfur moieties in addition to the polysulfides are produced but are not detected. In order to understand the full physiological relevance of H_2S metabolism by SOD these issues will need to be examined and resolved.

4.5 Conclusion

The analysis of polysulfides using a combination of derivatisation with an electrophile, to stabilise the polysulfides, followed by separation and detection by UHPLC-MS/MS is possible but has significant limitations. NEM or IAM alkylated polysulfides are well separated by the chromatographic method, combined with the selective and sensitive mass spectrometry detection, the individual inorganic and organic polysulfide species are easily resolved providing a rapid and robust analytical method for these key species. However, there are significant caveats to the analysis of polysulfides that are not only relevant to the methodologies investigated here but to other analytical methods.

When different polysulfide salts were made up in solution, they all equilibrated to show the same speciation pattern without alkylation. Even with alkylation, the speciation patterns seen were not dependant on the starting polysulfide salt but more on the choice of electrophile and the reaction conditions used. All of this suggests that the alkylation of polysulfides occurs under Curtin-Hammett control as the rate of interconversion is much greater than the rate of reaction. This means that the ratio of products (the speciation) is not equal to the ratio of the precursors, but instead dependant on the rate of interconversion between the precursors in equilibrium. Additionally, the choice of alkylating agent has a larger impact on the measured polysulfide speciation than the pH and pK_as of the aqueous polysulfides. These factors present an issue for the quantification of polysulfides as without a standard that can be accurately made up quantification proves difficult. The best compromise to enable the better comparison of results between experiments was to alkylate a known molar amount of a pure polysulfide salt. The concentration of each of the species measured was then taken as a ratio of the individual peak area over the total peak area with the total peak area representing the initial molar concentration of the starting polysulfide salt. NEM was found to produce shorter polysulfide chains than IAM, as it is able to cause fragmentation of alkylated polysulfides. IAM would then be assumed to be a more suitable electrophile for this analysis but due to the much slower rate of reaction the polysulfide equilibrium is likely to change during derivatisation under Curtin-Hammett control. Both NEM and IAM will significantly alter the speciation of aqueous polysulfides and will not reflect the physiological distribution. Neither electrophile will generate polysulfides when only sulfide is present making both suitable for detecting the presence of polysulfides, but care must be taken when drawing conclusions based on the individual species measured as they likely will not be representative of the physiological distribution. In order to get a true picture of the physiological distribution of polysulfides using these methods then alternative electrophiles will need to be investigated to determine if there are any with a high enough rate of reaction without fragmenting longer polysulfide chains.

With these caveats in mind, the developed method was successfully applied to the detection of polysulfide species generated from sulfide by SOD. Not only was the previously unknown production of polysulfides by SOD confirmed but we also gave insight into how they are formed. My monitoring the specific species it was found that polysulfides are produced sequentially with higher chain lengths not being formed until the preceding polysulfide had been produced. With the limitations of IAM in mind, this provides further insight into potential mechanisms by which SOD catalyses polysulfide production. The production of per and polysulfides by SOD presents yet another area of crosstalk in the reactive species interactome[36]. Polysulfides are now well known as important signalling species, as well as potential stores for sulfide which is an important

gasotransmitter[55,148,337]. SOD shares this activity with another antioxidant enzyme, catalase[137] which also has metabolic activities around sulfur being able to oxidise sulfide using O_2 or H_2O_2, or in the absence of oxygen regenerate sulfide from other sources. These enzymes could form an important regulatory loop that could potentially involve other antioxidant enzymes such as thioredoxin and peroxiredoxin. Further investigations into the potential activities of these enzymes with reactive sulfur species as well as identifying alternative electron acceptors other than oxygen will help provide further insight into the mechanisms by which the polysulfides are formed and even the evolution of these enzymes in an anoxic environment.

Chapter 5: Kinetics of Thiol Derivatisation

5.1 Introduction

Further to my project hypothesis; Differences in the rates of reaction of alkylating agents commonly used for thiol and sulfide redox analysis may in part be responsible for differing reported results.

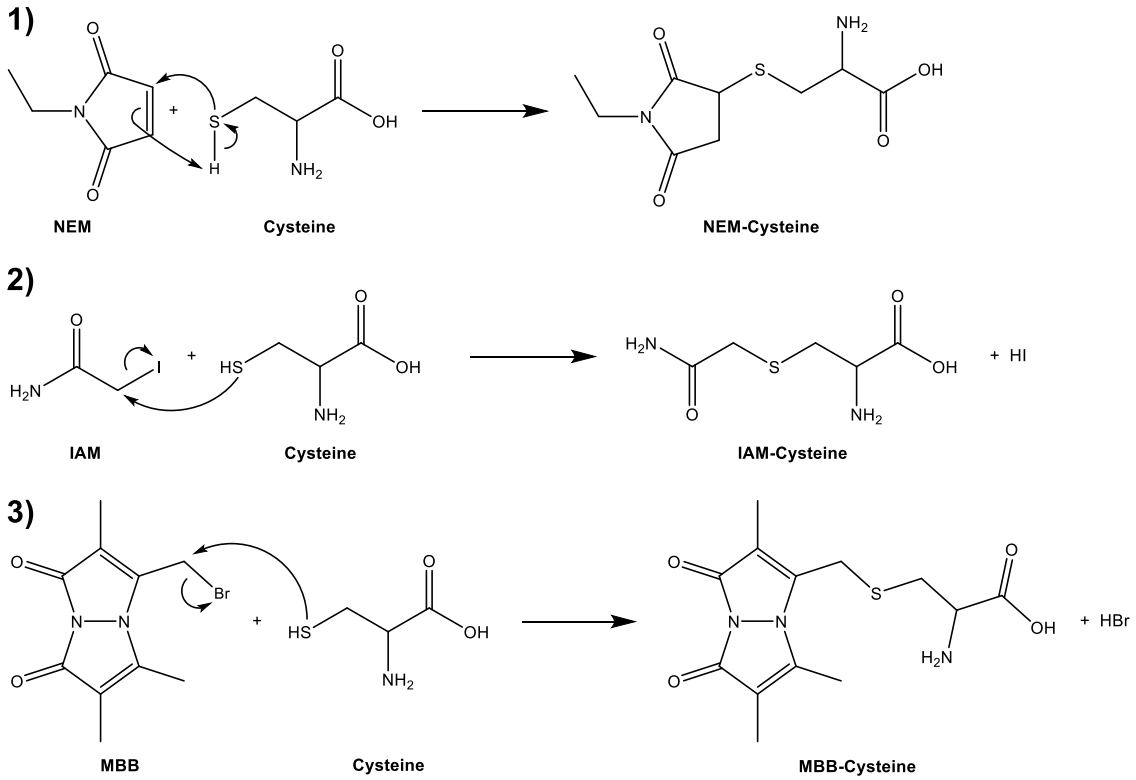
The rates of reaction of some commonly used alkylating agents are often referred to but not backed up by data. These assumptions can lead to inconsistencies in results and make comparisons of data collected using different methods more challenging.

Detailed kinetic analysis can give further insight into the rates of reaction of the commonly used alkylating agents NEM, IAM and MBB and further inform their use for thiol and sulfide redox analysis.

Alkylating agents such as *N*-ethylmaleimide (NEM), iodoacetamide (IAM) and monobromobimane (MBB) have been extensively used for the analysis of thiols and sulfide[112,317,324,338]. Each alkylating agent has a different mechanism of reaction[324], NEM reacts via Michael addition, IAM and MBB by a S_N2 reaction[107,325] (**Scheme 24**). The differences in reaction mechanism will have a significant impact on how the reaction with thiols and sulfide proceeds, the rate of reaction, and can potentially have an effect on the measured results. As discussed in Chapter 4:, there are significant differences in the observed speciation of inorganic polysulfides when using either NEM or IAM to alkylate them before analysis[143]. This could be affected by the difference in reaction mechanism causing less chain fragmentation with IAM, or could be due to a slower rate of reaction giving the polysulfides longer to equilibrate before the reactive end sulfurs are alkylated.

The rate of reaction of an alkylating agent with reduced thiols and sulfide is an important factor to consider in sulfur related redox analysis. Sulfide and its metabolite methanethiol are both highly volatile and the longer the collected sample is left without alkylation the lower the measured concentrations will be. If the alkylation reaction itself is slow, it will also result in lower concentrations being measured even if the alkylating agent is added soon after sample collection. Likewise, where the concentration of reduced glutathione (GSH) can be up to 100x greater than oxidised glutathione (GSSG) in plasma the longer the GSH is left un-alkylated the greater the artifactual oxidation skewing the GSH/GSSG redox ratio. There are also many other biological process that can still occur even after collection and separation of blood and other biological samples. Glutathione reductase, for example, can still function after separation of the plasma

until the GSH has all been alkylated. Therefore, in order to preserve the most accurate picture of the physiological redox ratios and cycle it is best to use an alkylating agent with a raid rate of reaction to avoid these potential issues.



Scheme 24: Reactions of **1)** NEM, **2)** IAM and **3)** MBB with and an example reduced thiol, cysteine. The different mechanism of reaction are illustrated, Michael addition for NEM and S_N2 reaction for IAM and MBB.

Despite their widespread use, there is limited information on the reaction rates of these alkylating agents. It is widely accepted that NEM has a very rapid rate of reaction, whilst IAM and MBB have a slower rate of reaction. Nevertheless, this is hardly ever backed up by data and is simply taken as a fact. It is important to understand how much slower than NEM that IAM and MBB react with thiols and sulfide. Different thiols will also have different rates of reaction with an alkylating agent, and especially sulfide as it reacts with the nucleophiles with a 1:2 ratio. To this end, studies were undertaken investigating the rates of reaction of the three different alkylating agents using the three major reduced thiols glutathione, cysteine and homocysteine as well as sulfide and its related metabolite methanethiol (MT). MT was used as an analogue to sulfide that reacts with the nucleophiles with a 1:1 molar ratio to examine whether this had a major effect on the reaction rate.

It was found that generally, the reaction rates of NEM, IAM and MBB are largely as reported in the literature, NEM reacts rapidly and both IAM and MBB react much slower. However, the

magnitude of the differences between the different alkylating agents was unexpected. Additionally, whilst NEM reacted rapidly with reduced thiols taking around 30 seconds to complete, the reaction with sulfide took up to twenty times longer to complete. Both IAM and MBB took hours to react completely with differing individual rates with the tested reduced thiols and sulfide. This raises questions about the suitability of both for the analysis of reduced thiols and sulfide as the slow rate of reaction could lead to changes in the physiological distribution of these species before the reactive sulfurs can be fully blocked.

5.1.1 *N*-ethylmaleimide

NEM has a long history of use for the analysis of both thiols and sulfide[279,317,339]. NEM has numerous advantages, it both rapidly reacts with sulfhydryl groups and is relatively stable at physiological pH, can freely pass through cell membranes and alkylate cellular thiols and sulfide without the need for lysis[293,313]. Alkylation with NEM is also beneficial for the analysis of thiols and sulfide either by enhancing the ionisation of the alkylated molecules for analysis by mass spectrometry or through the formation of fluorescent products that can be easily monitored. Whilst it is known to react rapidly, this has not been studied in depth. The rate of reaction is unlikely to be the same for all of the thiols and especially with sulfide since it reacts with a molar ratio of 1:2 (sulfide:NEM) as opposed to reduced thiols which react with a 1:1 ratio (**Scheme 10**). Despite this, the potential difference in rates of reaction with the different species is currently neglected.

5.1.2 Iodoacetamide

Iodoacetamide is less widely used than NEM and MBB for the analysis of reduced thiols and sulfide. It neither improves the ionisation of the thiols it reacts with for analysis my mass spectrometry nor forms a fluorescent product. Another disadvantage is that alkylation with IAM results in less of an improvement in the retention of reduced thiols when using reversed phase chromatography when compared to NEM (section 4.3). IAM does have an advantage when analysing inorganic and organic polysulfides as it causes less chain fragmentation than NEM and MBB (section 4.3)[143]. Like NEM IAM is able to pass easily through cell membranes enabling the alkylation of cellular target molecules without the need for lysis.

The rate of reaction of IAM with both reduced thiols and sulfide is known to be slower than that of NEM. Similarly to NEM, potential differences in the rate of reaction with different thiols, and sulfide especially, are not considered. As IAM reacts slower it is likely that any differences in rates of reaction will be more pronounced than with the NEM, though due to the different mechanism

of reaction these potential differences may not be the same as with NEM. IAM is not as well suited as NEM for the alkylation at physiological pH, requiring at least a pH of 8 in order to react effectively[118]. This could factor into the rates of reaction for redox analysis as it is desirable to keep the pH at physiological levels in order to avoid perturbing the redox ratios.

5.1.3 Monobromobimane

Like NEM, MBB is widely used for the alkylation of both reduced thiols and sulfide[17,112,113]. MBB has many of the same advantages as NEM with the alkylation reaction taking place at physiological pH as well as being cell membrane permeable[340]. One advantage for the analysis of sulfide is that the double nucleophilic substitution reaction product, the sulfur dibimane (**Scheme 8**), is highly fluorescent. Whilst MBB will also react with reduced thiols the single nucleophilic substitution reaction products are not fluorescent allowing the sulfide to be easily determined in biological samples without the need for intensive sample preparation or complex detection methods[18].

The reaction time of MBB is known to be slower than NEM and has been noted to be in the order of hours when used for the detection of very low concentrations of sulfide[18]. Other methods however use shorter reaction times from ten minutes up to one hour[294]. This raises the question; what are the reaction times of MBB with different reduced thiol and sulfur species and how might this affect their analysis?

5.2 Methods

Unless otherwise stated all reagents and materials were of the highest purity available and purchased from Sigma-Aldrich (Gillingham, UK or Munich, Germany). HPLC-grade solvents were from Fisher Scientific (UK).

All measurements were carried out using an Agilent Cary 60 UV-vis spectrophotometer, data processing was performed using Cary WinUV software. Quartz cuvettes were used and all experiments were carried out using 10 mM pH 7.4 ammonium phosphate buffer. Before each analysis, the spectrophotometer was zeroed against an ammonium phosphate buffer blank to account for background absorbance. The reaction temperature was set to 25 ± 0.1^{o}C using a thermostat controlled circulating water bath and the solutions were mixed with a magnetic stirrer. NEM was monitored using a wavelength of 302 nm, IAM with 280 nm and MBB was monitored by scanning from 200 to 500 nm. For MBB the absorbance decrease at the MBB absorbance peak (400 nm) was minimal due to the product peak being close in wavelength (**Figure 31, A**). In order to get a larger decrease in absorbance to obtain clearer kinetics data the

difference spectrum was calculated by $Ab_D = Ab_n - Ab_1$ where Ab_D = the "difference" absorbance, Ab_1 = the initial absorbance in the first scan and Ab_n = the following scans. In the difference scan the largest decrease in absorbance is seen at a wavelength of 250 nm which was then used for subsequent kinetics graphs and calculations.

Stock solutions of each electrophile were prepared using the same 10 mM pH 7.4 ammonium phosphate buffer, NEM and IAM both were made up to a concentration of 100 mM and MBB to 10 mM. A lower concentration of MBB was used, due to its higher absorbance, in order to keep the starting absorbance in the same general level as NEM and MBB. Solutions of each thiol were prepared by accurately weighing out the dry powder and dissolving in ammonium phosphate buffer apart from methanethiol (MT) and sulfide, which were dissolved using ultra-pure water. The reactions were performed for NEM and IAM by adding 20 μl of the stock electrophile to 1960 μl ammonium phosphate buffer in the cuvette giving a final concentration of 1 mM, and allowed to equilibrate in the spectrophotometer at 25^{o}C. A lid was used on the cuvette for MT and sulfide to minimise losses through evaporation. The absorbance measurement was then started, and after 30 seconds 20 μl of either 100 mM of GSH, Cys, Hcys or methanethiol was added or 10 μl of 100 mM NaS giving final concentrations of 1 mM and 0.5 mM respectively. The experiments using MBB were performed following the same overall procedure, using 10 mM stock solutions for all of the reactants. In the same manner as for NEM and IAM 30 μl of MBB was added to 1940 μl of ammonium phosphate buffer, which was allowed to equilibrate at 25^{o}C before either 30 μl of 10 mM GSH, Cys, Hcys or MT was added or 15 μl of 10 mM NaS. The reactions were monitored until beyond completion. All of the NEM reactions were repeated five times, for IAM and MBB the GSH, Cys, Hcys and sulfide reactions were repeated five times, the MT reactions however were only repeated twice due to the long reaction times.

Data analysis was performed using Microsoft Excel, the reaction order was determined by a mathematical fit of the data using either ln[Abs] (1st order) or 1/[Abs] (2nd order). The fit that gives a linear plot determines the order of reaction. The rate constant was determined from the slope of the trendline and averaged over the number of repeats performed.

5.3 Results

5.3.1 *N*-ethylmaleimide

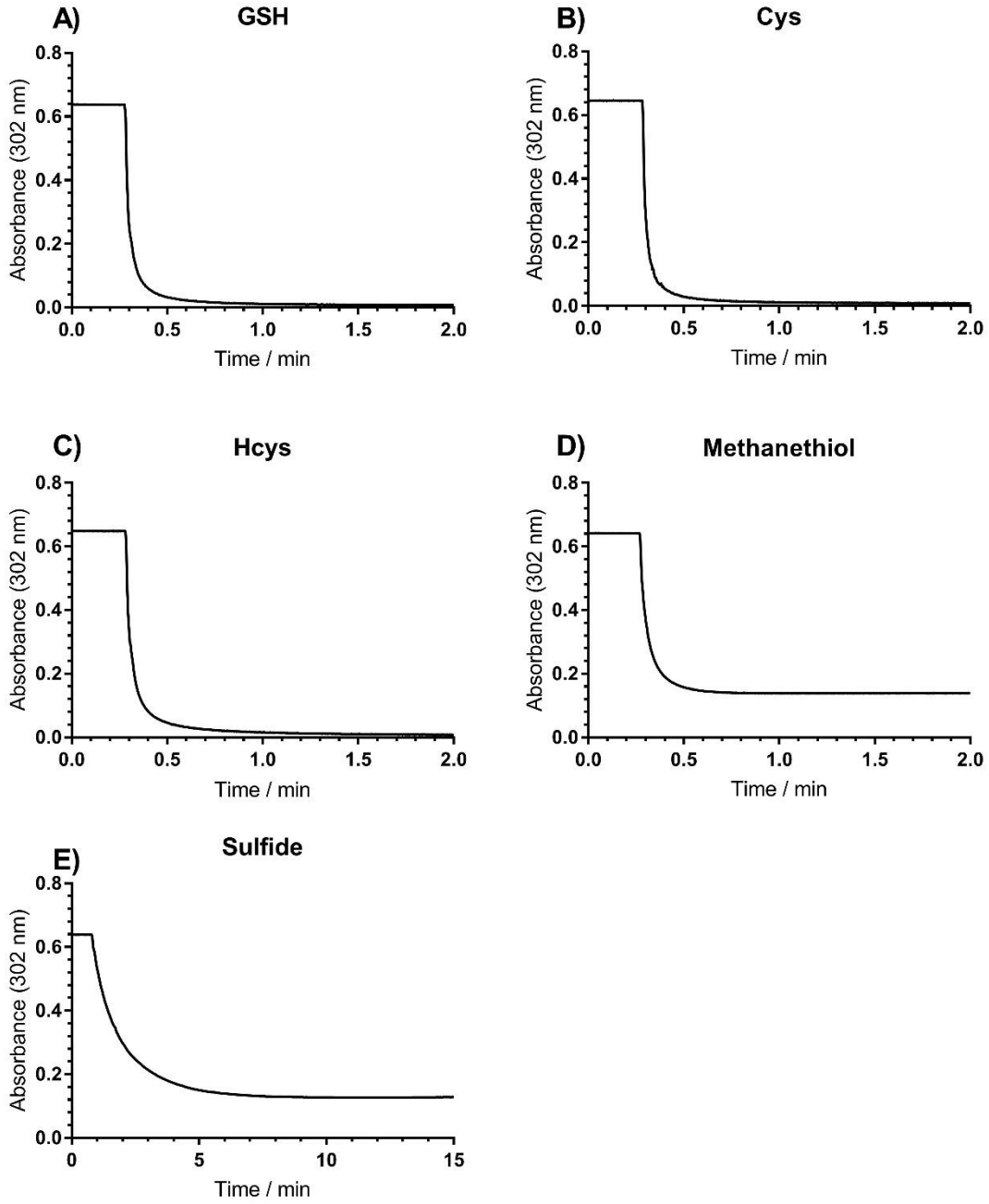


Figure 29: Rates of reaction of NEM with example reduced thiols and sulfide. **A)** GSH with a 1:1 molar ratio, **B)** Cys with a 1:1 molar ratio, **C)** Hcys with a 1:1 molar ratio, **D)** Methanethiol with a 1:1 molar ratio and **E)** sulfide with a 2:1 molar ratio (NEM:sulfide). The reaction of NEM with reduced thiols proceeds with second order reaction kinetics whilst sulfide reacts with first order kinetics.

At physiological pH the rate of reaction of NEM with the three reduced thiols tested, GSH, Cys and Hcys are all very rapid (**Figure 29, A, B and C**). At a 1:1 molar ratio, the reactions are all complete within 30 seconds of addition of NEM. The rate of reaction with sulfide however is much slower in comparison to the reduced thiols. At a 2:1 molar ratio (NEM:sulfide) it takes at least 10 minutes for the reaction to complete (**Figure 29, E**). There does appear to be a slight difference in the rates of reactions between the three reduced thiols tested with Hcys taking slightly longer to complete than GSH and Cys. However, due to the short reaction times these differences are difficult to discriminate with accuracy. Based on the mathematical fits of the data, NEM reacts with the reduced thiols, including methanethiol with second order kinetics. GSH reacts with a rate constant of 182 ± 3 $M^{-1}min^{-1}$, Cys with 192 ± 16 $M^{-1}min^{-1}$, Hcys with 105 ± 3 $M^{-1}min^{-1}$, and MT with a rate constant of 110 ± 4 $M^{-1}min^{-1}$. However, NEM seemingly reacts with sulfide in a first order reaction with a rate constant of 0.551 ± 0.13 min^{-1}.

For both methanethiol and sulfide, the measured absorbance does not drop to close to zero, as with the reduced thiols (**Figure 29**), despite the molar ratios used in the reactions meaning that all of the NEM should react. This is likely due to an incomplete reaction occurring due the loss of sulfide, because of its high volatility, leading to a decreased molar amount of sulfide being available to react with the NEM.

5.3.2 Iodoacetamide

IAM has a much slower rate of reaction than NEM with all of the reduced thiols tested including methanethiol, and sulfide. For GSH and Cys the equimolar reaction takes at least 150 min to complete, with Hcys taking up to 300 min (**Figure 30, A, B and C**). Based on the mathematical fits of the data, IAM reacts with all of the reduced thiols and sulfide with apparent second order kinetics. The differences in the rates of reaction of the three reduced thiols are much more obvious than with NEM, due to the longer reaction times, and follow the same order; IAM reacts with Cys the fastest followed by GSH and then Hcys. Unlike with NEM sulfide does not take longer to react than the reduced thiols. In fact, the reaction of sulfide with IAM completes in under 150 min, which is quicker than for Hcys (**Figure 30, E**). Methanethiol on the other hand appears to take much longer to react with the complete reaction taking up to 600 min (**Figure 30, D**). GSH reacts with a rate constant of 0.187 ± 0.008 $M^{-1}min^{-1}$, Cys with 0.202 ± 0.012 $M^{-1}min^{-1}$, Hcys with 0.101 ± 0.004 $M^{-1}min^{-1}$, MT with 0.116 ± 0.011 $M^{-1}min^{-1}$ and sulfide with a rate constant of 0.185 ± 0.003 $M^{-1}min^{-1}$.

Even when completely reacted there is still some absorbance at 280 nm due to the acteamide-thiol adduct having some absorbance at this wavelength. For both sulfide and methanethiol, the

absorbance does not drop as low upon completion as with the reduced thiols. This is likely due to the volatility of sulfide and methanethiol resulting in a large loss before they can react, leaving unreacted IAM in the solution.

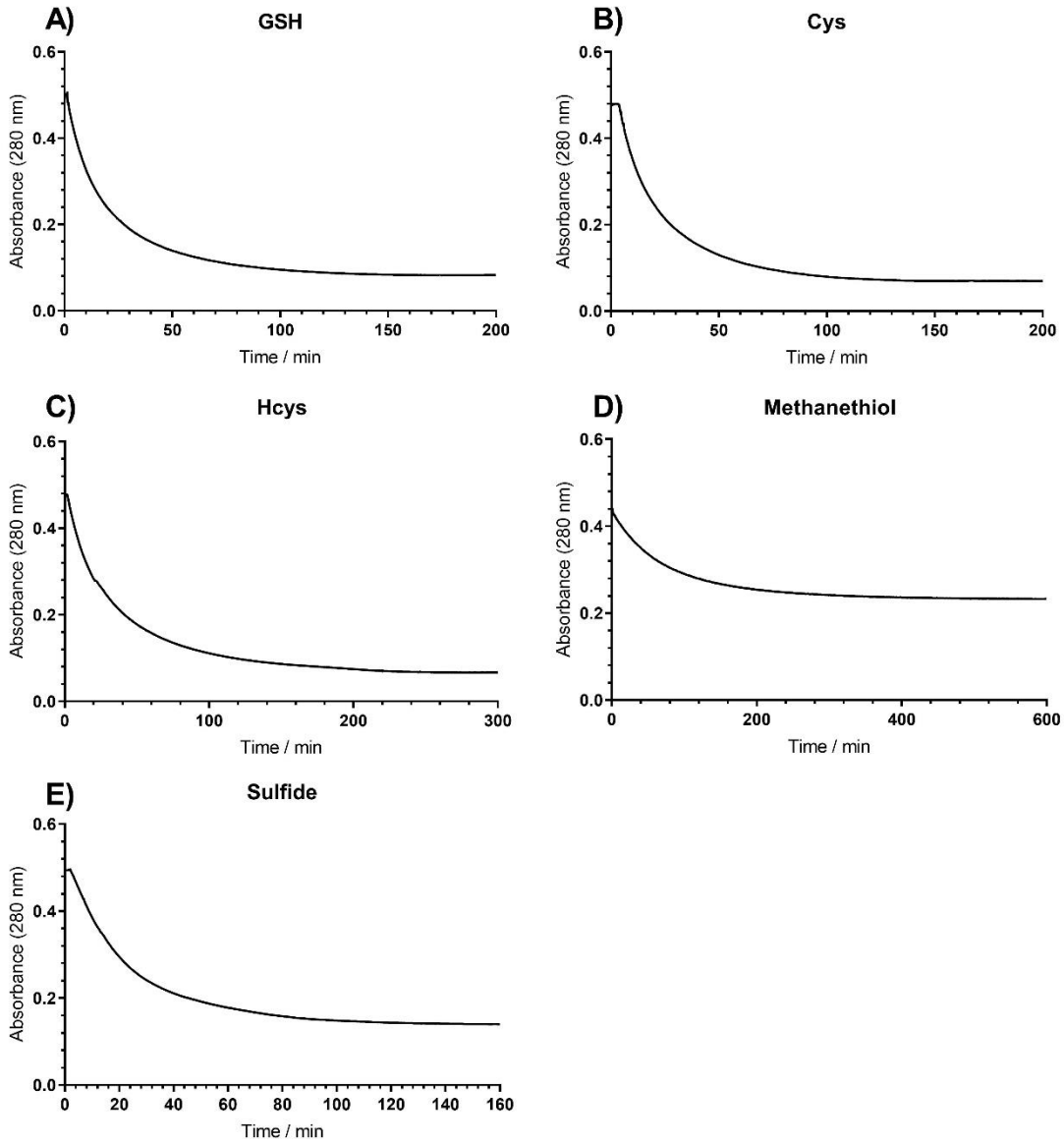


Figure 30: Rates of reaction of IAM with example reduced thiols and sulfide. **A)** GSH with a 1:1 molar ratio, **B)** Cys with a 1:1 molar ratio, **C)** Hcys with a 1:1 molar ratio, **D)** Methanethiol with a 1:1 molar ratio and **E)** sulfide with a 2:1 molar ratio (IAM:sulfide). The reaction of IAM with reduced thiols and sulfide proceeds with first order reaction kinetics.

5.3.3 Monobromobimane

MBB has a similar rate of reaction as IAM, with a complete reaction taking hours rather than minutes for NEM. The absorbance of MBB (400 nm) does not drop by as much as NEM or IAM making following the reaction more difficult. Following the reaction by scanning between a wavelength of 200-500 nm allows the area of maximal decrease in absorption to be observed (**Figure 31**, **A**). In order to make this decrease clearer the difference spectrum is calculated from the initial scan absorbance minus the absorbance of each successive scan (**Figure 31**, **B**). The reaction of MBB with the thiols and sulfide was therefore followed using a wavelength of 250 nm in the difference spectrum to give the maximum decrease and make the reaction easier to follow.

With the reduced thiols, MBB follows a different pattern in rate of reaction to NEM and IAM (**Figure 32**). Cys is still the fastest reacting reduced thiol with a complete reaction taking less than an hour. Hcys is the next fastest taking less than three hours for a complete reaction followed by GSH, which takes over three hours to complete. MT is even slower to react, and after six hours, the reaction had still not completed. As with IAM, sulfide reacted faster than both Hcys and GSH with the reaction finishing in around two hours. Based on the mathematical fits of the data, all of the reactions of MBB with reduced thiols and sulfide take place with apparent second order reaction kinetics. GSH reacts with a rate constant of 0.179 ± 0.02 $M^{-1}min^{-1}$, Cys with 0.307 ± 0.051 $M^{-1}min^{-1}$, Hcys with 0.092 ± 0.006 $M^{-1}min^{-1}$, MT with 0.023 ± 0.008 $M^{-1}min^{-1}$ and sulfide with a rate constant of 0.188 ± 0.052 $M^{-1}min^{-1}$.

As with NEM and particularly, IAM with sulfide and methanethiol, the absorbance does not drop as low upon completion as with the reduced thiols. This is likely due to the volatility of sulfide and methanethiol resulting in a large loss before they can react, leaving unreacted IAM in the solution.

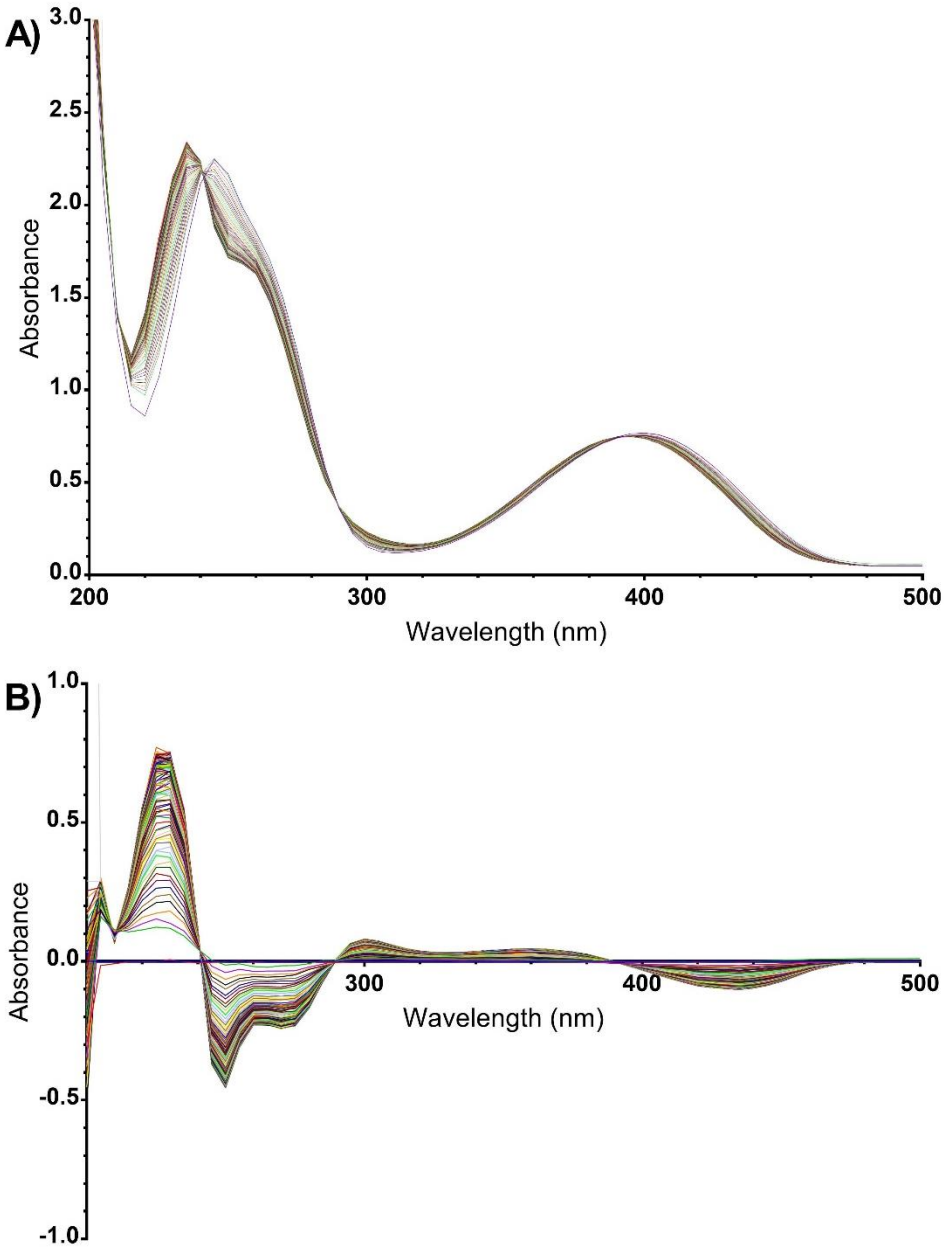


Figure 31: Scanning measurements of MBB + Cys (1:1 molar ratio) over 60 min as an example for the method used to follow MBB reaction kinetics as discussed in section 5.3.3. **A)** Full scan over 200-500 nm, note that at the maximal absorption of MBB at 400 nm there is not much decrease as it reacts with Cys. The absorption maxima in fact appears to shift to a lower wavelength instead. **B)** The difference spectrum of the data shown in **A)** taken as each successive scan minus the initial scan. The largest difference between the first and last scans is seen at 250 nm, therefore the change in absorbance is monitored by using the difference spectrum at this wavelength.

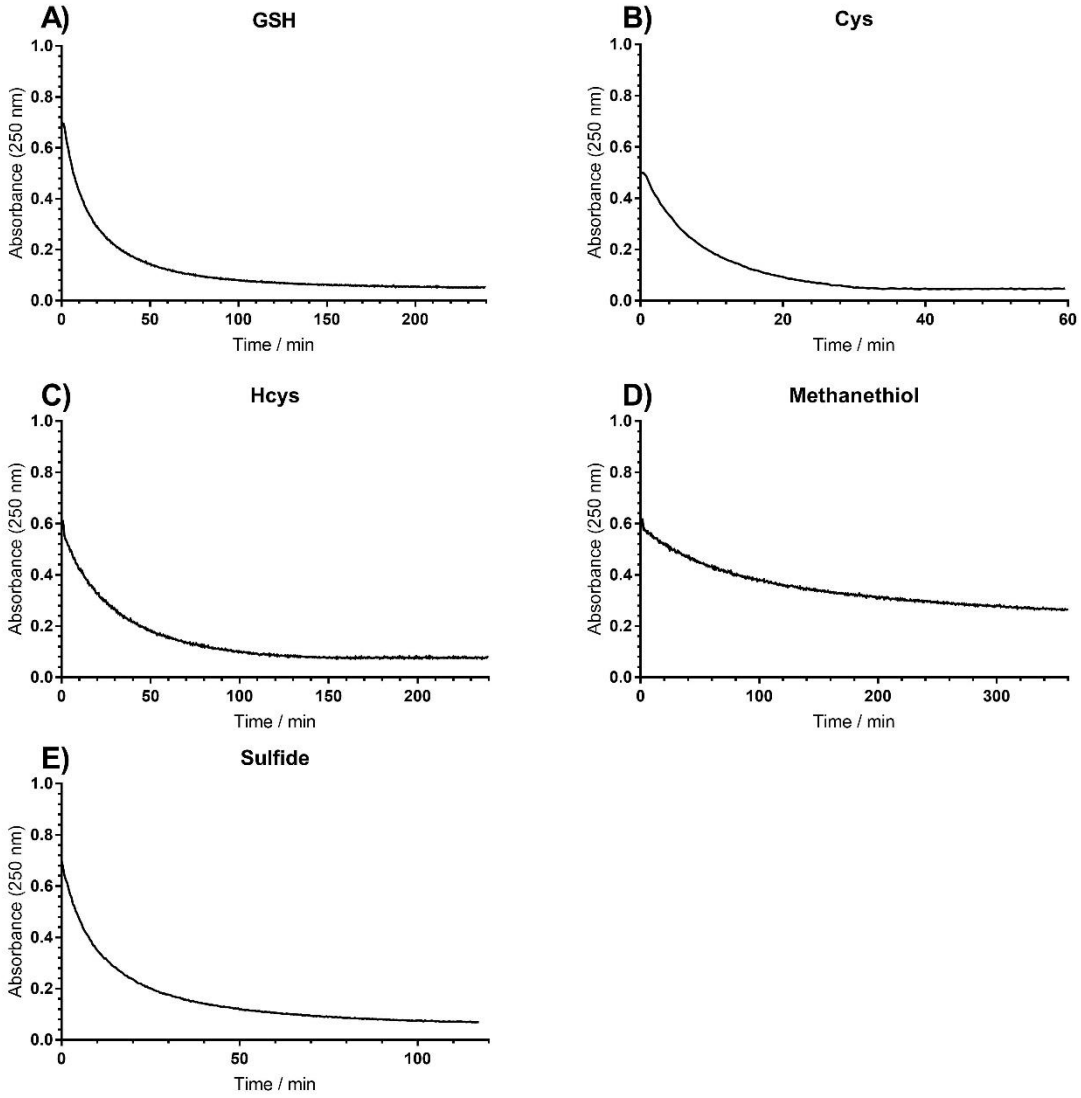


Figure 32: Rates of reaction of MBB with example reduced thiols and sulfide. **A)** GSH with a 1:1 molar ratio, **B)** Cys with a 1:1 molar ratio, **C)** Hcys with a 1:1 molar ratio, **D)** Methanethiol with a 1:1 molar ratio and **E)** sulfide with a 2:1 molar ratio (MBB:sulfide). The reaction of MBB with reduced thiols and sulfide proceeds with first order reaction kinetics.

5.4 Discussion

5.4.1 *N*-ethylmaleimide

As was expected, the rate of reaction of NEM with reduced thiols was rapid with GSH, Cys and Hcys all reacting fully within 30 seconds. All of the reduced thiols, including methanethiol react with apparent second order reaction kinetics where the rate of reaction depends on the concentration of both reactants. There does appear to be slight differences in the rates of

reaction with each of the thiols, with Cys ($k = 192 \pm 16\ M^{-1}min^{-1}$) being the quickest followed by GSH ($k = 182 \pm 3\ M^{-1}min^{-1}$) and Hcys ($k = 105 \pm 3\ M^{-1}min^{-1}$). These differences were consistent throughout the repeats of the experiment, but due to the rapid reaction time the differences are only very small.

It was surprising to find that the rate of reaction of NEM with sulfide is much slower than with the thiols with a complete reaction taking almost 10 minutes ($k = 0.551 \pm 0.13\ min^{-1}$). There are three major differences between the two sets of reactions. Sulfide is much smaller than any other of the reduced thiols, and does not have any functional groups that might modify the reaction speed, though MT only has a methyl group. Based on the mathematical fit of the data, NEM and sulfide appear to react with first order reaction kinetics where the rate of reaction is more dependent on the concentration of one of the reactants rather than both. This could be explained by the fact that each sulfide reacts with two NEMs (unlike the reduced thiols) with each addition potentially having different reaction kinetics. It is unlikely that the addition of two NEMs occurs simultaneously but rather sequentially. The addition of the first NEM adduct to the sulfide not only increases the size of the molecule significantly potentially causing steric effects but could also significantly affect the availability of the electron lone pair and therefore alter the kinetics of the second reaction. It also has to be considered that due to the volatility of sulfide even when accurately weighed out and made up in solution there will inevitably be some loss of sulfide due to the lid only loosely fitting before the reaction with NEM can complete. This explains why the UV absorbance does not drop to close to zero as with the thiols but should not decrease the rate of reaction from under 30 seconds to almost ten minutes.

Methanethiol was used as an analogue for sulfide with only one reactive site to investigate whether the addition of the second NEM adduct was the reason for the much slower rate of reaction. The rate of reaction of MT with NEM occurs at a similar rate as the other reduced thiols, completing in under 30 seconds ($k = 110 \pm 4\ M^{-1}min^{-1}$). Although like the sulfide the UV absorbance does not drop to as close to zero as with the reduced thiols as MT is also very volatile and will rapidly evaporate after the dissolution of the dry salt. As with the sulfide some loss through evaporation of methane thiol can be observed by the relatively high baseline absorbance after the reaction has completed. This will likely have affected the rate of reaction as when the methanethiol is lost the molar ratio will be skewed. As with the reduced thiols, MT reacts with second order kinetics versus the apparent first order reaction of sulfide with NEM. The rapid rate of reaction supports the theory that the second addition of a NEM adduct is the slow step of the reaction between sulfide and NEM and the cause of the slower rate of reaction. Since the reaction kinetics appear to be first order and it is the absorbance (therefore concentration) of NEM being measured this would suggest that it is the concentration of NEM that the rate of reaction is

depending on, not the sulfide which supports the conclusion that it is the addition of the second NEM slowing the rate of reaction. This however will require further investigations in order to confirm. The exact mechanism behind the difference in rate of the first and second addition is still unknown, whether it is a steric or kinetic effect or otherwise. It is possible that the addition of one NEM adduct to the sulfide affects the electron density, reducing the reactivity of the remaining lone pair on the sulfur and therefore slowing the second NEM addition but further investigation would be required to test this.

The order of reactivity of Cys, GSH and Hcys with NEM follows the inverse of their pKa, with Cys having the lowest pKa (8.22)[341], followed by GSH (9.42)[341] and then Hcys (9.99)[342]. Whilst a lower pKa can correspond with a lower nucleophilicity, the lower the pKa, the more available the thiolate will be at physiological pH and therefore more readily available to react with the electrophile[343,344]. The availability of the thiolate however is not the only factor than can influence thiol reactivity, steric factors can also have a significant impact on the reactivity[344]. This could explain why MT, despite having a higher pKa (10.33)[345] and so less thiolate availability, reacts faster than Hcys as the methyl group will offer less steric hindrance. Sulfide also does not follow the same pattern with the pKa, sulfide has a pKa_1 of ~7.02 and a pKa_2 of ~15, the pKa of sulfide however if difficult to determine, particularly the pKa_2 and the values reported vary significantly[18,346]. Nevertheless, at physiological pH there will be very little S^{2-} present in solution. If the first reaction step follows the same pattern as Cys, GSH and Hcys the it will be faster due to the lower pKa_1 and the higher availability of HS^- with very little steric effects. However, the complete reaction will also depend on the reactivity of the electrophile-sulfide adduct (RS^-) for the second step of the reaction as determined by the availability of the thiolate (pKa) and its steric properties. These properties of the initial electrophile-sulfide adduct are unknown and would likely be difficult to determine accurately.

5.4.2 Iodoacetamide

As anticipated, IAM is slower reacting with reduced thiols and sulfide than NEM; it was, however, considerably slower than anticipated. Cys reacts the fastest (0.202 ± 0.012 $M^{-1}min^{-1}$) followed by GSH (0.187 ± 0.008 $M^{-1}min^{-1}$) then Hcys (0.101 ± 0.004 $M^{-1}min^{-1}$), which is almost twice as slow; this follows the slight differences in reaction rate with NEM but is seen more clearly due to the slower reaction times. What differed was the rate of reaction of IAM with sulfide was in fact faster or equal to ($k = 0.185 \pm 0.003$ $M^{-1}min^{-1}$) Hcys and GSH, compared to the increased reaction time of NEM with sulfide versus the reduced thiols. This difference between IAM and NEM is most likely due to the different mechanisms of reaction, with the second addition of IAM to sulfide not taking significantly slower than the first addition. The rate of reaction with methanethiol on the

other hand was slower ($k = 0.116 \pm 0.011\ M^{-1}min^{-1}$) than with sulfide. This may be due to the high volatility of methanethiol resulting in a large loss before it can react, as evidenced by a strong odour before addition of the electrophile. As with the NEM this results in a change in the molar ratios but to a larger extent due to the longer reaction times. This can be seen by the absorbance at 280 nm remaining high and not dropping to the same level as with the reduced thiols after the reaction has completed showing that unreacted IAM remains in the solution. The same effect can be observed with the sulfide but to a lesser extent. Additionally, all of the reduced thiols and sulfide react with second order kinetics. This differs from NEM, which reacted with second order kinetics for the reduced thiols, but not for sulfide. This may explain why with IAM no large increase is observed in reaction time for sulfide compared to the reduced thiols as there was for NEM as there is no apparent difference in reaction order.

The relatively slow rate of reaction of IAM is a concern for the analysis of thiols and volatile sulfur compounds in biological samples. The longer the reaction takes the more reactive compounds such as reduced thiols will oxidise and remain unprotected from enzymatic breakdown and other mechanisms of loss. Of particular concern are the volatile sulfur compounds such as methanethiol and sulfide. In the time it takes for a complete reaction there will have been significant losses due to evaporation which will give an inaccurate picture of the true levels of these compounds. The differences observed in measured polysulfide speciation between NEM and IAM in Chapter 4: may in part be explained by the slower rate of reaction allowing the polysulfides more time to rearrange before the reactive end sulfurs are alkylated but this will require further investigation.

The order of reactivity of Cys, GSH and Hcys with IAM follows the same pattern as with NEM and is the inverse of their pKa, with Cys having the lowest pKa (8.22)[341], followed by GSH (9.42)[341] and then Hcys (9.99)[342]. MT also followed the same pattern as it was faster than Hcys but slower than both Cys and GSH. This is again potentially due to lower steric hindrance from the methyl group compared to Hcys having a greater impact on reaction rate than the availability of the thiolate ion. Whilst sulfide is not the slowest reacting thiol with IAM as it is with NEM, it is still slower than Cys, which does not follow the inverse pattern with the pKa values. Since sulfide both has a low pKa_1 (~7.02[18,346]) and low potential steric hindrance compared to the other thiols the overall rate of reaction will, as with NEM, be determined by the second step of the reaction as well. It is possible that the acetamide-sulfide adduct has lower steric hindrance than the NEM-sulfide adduct and this may potentially explain why the rate of reaction is relatively quick compared to the other IAM thiol reactions in contrast to NEM.

5.4.3 Monobromobimane

Alkylation with monobromobimane is considered one of the gold standard methods for the determination of sulfide in biological samples[113,277,347] as well as other sulfur containing species, such as thiols[325,340 thiols]. Whilst it is known that the rate of reaction is slower than that of NEM it was somewhat surprising how much slower it really is. The measured absorbance levels are generally higher (**Figure 32**) than with IAM (**Figure 30**) and NEM (**Figure 29**) as the main absorbance peak for MBB is seen to shift to the left as it reacts, resulting in a smaller drop in absorbance (**Figure 31, A**). To give a much greater drop in absorbance in order to better visualise the reaction rates, the difference spectrum was calculated and the absorbance monitored at 250 nm as this gave the largest overall decrease (**Figure 31, B**).

The rate of reaction of MBB with the reduced thiols is closer to that of IAM than NEM, with a complete reaction being on the scale of hours rather than minutes. Likewise, both IAM and MBB react with sulfide with second order kinetics whilst NEM appears to react with first order kinetics. Like NEM and IAM, with reduced thiols MBB reacts with Cys the fastest ($k = 0.307 \pm 0.051$ $M^{-1}min^{-1}$) followed by GSH ($k = 0.179 \pm 0.02$ $M^{-1}min^{-1}$) and then Hcys ($k = 0.092 \pm 0.006$ $M^{-1}min^{-1}$). The relative reaction rates of MBB with Cys and GSH also differ; there is a much greater difference between the two for MBB compared to IAM. As with NEM and IAM, the reactions of MBB with sulfide and methanethiol complete with a higher final absorbance than with the reduced thiols, despite exact molar ratios of reactants being used. This is likely due to the high volatility of both sulfide and methanethiol, coupled with the long reaction times, which results in significant amounts being lost by evaporation from the solution before complete reaction. This loss will have an effect on the rate of reaction as it is reducing the concentration of one reactant but not the other, changing the molar ratio. Overall, this change in reaction rate is not a major concern as MBB is clearly an unsuitable electrophile for the trapping of volatile compounds due to the slow rate of reaction. In order to avoid these losses a closed reaction vial would be required with minimal headspace which may complicate sample preparation. It does raise questions over the widespread use of MBB for the analysis of sulfide, are the lower sulfide concentrations reported when using MBB due to the slower reaction rate leading to losses in the sample. The slow rate of reaction is more of a concern for the reduced thiols as in biological samples they will be lost due to oxidation, enzymatic activity and other biological processes before all of the reduced thiols can be alkylated and stabilised.

The order of reactivity of Cys, GSH and Hcys with MBB follows the same pattern as with NEM and IAM, being the inverse of the order of pKa values, with Cys having the lowest pKa (8.22)[341], followed by GSH (9.42)[341] and then Hcys (9.99)[342]. Unlike NEM and IAM, MT is the slowest reacting

thiol which follows the inverse pattern with the pKa values (MT = 10.33)[345]. With MBB, it is possible that steric effects have less of an impact resulting in the pKa having more of an impact on the rate of reaction for MT. The rate of reaction with sulfide follows the same pattern as with IAM. As with NEM and IAM it is hard to make a judgement on how the availability of thiolate ions and steric effects will impact the rate of reaction as the overall rate will depend on both the first and second steps and the pKa values of the electrophile-sulfide adducts are unknown.

5.5 Conclusion

Many methods utilising NEM for the alkylation of reduced thiols take the rate of reaction of NEM as being very rapid and running to completion within five minutes[120,279,317,348]. This matches with our findings here, and is in fact even quicker than anticipated with the reactions of GSH, Cys and Hcys all completing within 30 seconds at a 1:1 molar ratio at 25^{o}C. With an excess of NEM the reduced thiols should be completely alkylated even faster as with second order kinetics doubling the concentration of one of the reactants will double the rate of reaction. What came as a surprise is the difference in reaction times between the reduced thiols and sulfide. On further investigation, this difference in rate of reaction has been mentioned in the literature[122,349] but is often overlooked and not explored in detail.

When used to alkylate biological samples NEM, IAM and MBB are all used in molar excess since the total levels of all reactive species cannot be known in advance. Since the levels of reactive species (including non-target compounds) will vary from sample to sample, the molar ratio of electrophile to the reactive species will vary between samples. For IAM and MBB the slow rates of reaction will mean that from sample to sample there may be significant differences in the time it takes for all of the compounds of interest to alkylate fully. This will also be true for NEM but as the rate of reaction is much faster the absolute differences will be smaller and therefore of less consequence. With MBB and IAM this could mean that differences observed between samples may not be just due to differing endogenous levels but also due to the slow rate of reaction resulting in higher losses in some samples.

Due to the high volatility of sulfide and methanethiol, an electrophile with a high rate of reaction is required to avoid large losses through evaporation. The high volatility can be seen by the higher baseline absorbances after the alkylation reactions have completed than with the reduced thiols. As the reaction is started with exact molar ratios this shows that sulfide or methanethiol must have been lost leaving unreacted electrophile in solution. Out of the three electrophiles tested only NEM has a sufficient rate of reaction to avoid most of these losses. Both IAM and MBB react slowly and therefore may not provide an accurate picture of the concentration of volatile species

in solution, any data on volatile species collected using these electrophiles should be considered with this in mind. However, the slower rate of reaction of NEM with sulfide is still of concern for the same reasons. NEM is still faster than IAM and MBB and with a larger molar excess the rate of reaction should be sufficient to trap the majority of the free sulfide before it is lost through evaporation or oxidation. In Chapter 4: significant differences are observed in the measured polysulfide species when using either NEM or IAM to alkylate them. This was found to be in part due to the ability of NEM to attack the mid chain sulfurs and fragment the chains, but could also be due to the differences in reaction rate. As IAM reacts much slower than NEM the polysulfides, which exist in an equilibrium between different chain lengths in solution, will have longer to rearrange before all of the reactive end sulfurs are alkylated. This could lead to higher chain length polysulfides being generated during the reaction.

These experiments support the use of NEM to alkylate reduced thiols and sulfide over slower reacting alternatives. Questions are raised about the suitability of both IAM and MBB for sulfur related redox analysis as the much longer reaction times required compared to NEM could have significant impact on the species measured. In particular, care needs to be taken with sulfide as it cannot be assumed to react in the same manner as the reduced thiols, as well as other volatile sulfur compounds, which are not well suited for analysis using slower reacting alkylating agents. IAM was chosen as a more suitable alkylating agent for inorganic polysulfides in Chapter 4: due less chain fragmentation being observed. Following these experiments, it is clear that this could also partly be due to the much slower reaction time, leading to artificial shifts in apparent sulfur chain lengths due to incomplete alkylation of both chain ends; this possibility requires further investigation. HPE-IAM (β-(4-hydroxyphenyl)ethyl iodoacetamide) has been used as an alternative electrophile for the analysis of polysulfides with longer chain lengths observed compared to IAM[143,322], the rate of reaction of HPE-IAM compared to IAM has not been investigated in detail but should be considered as a possible factor for these observations.

Chapter 6: Ascorbate Redox Analysis

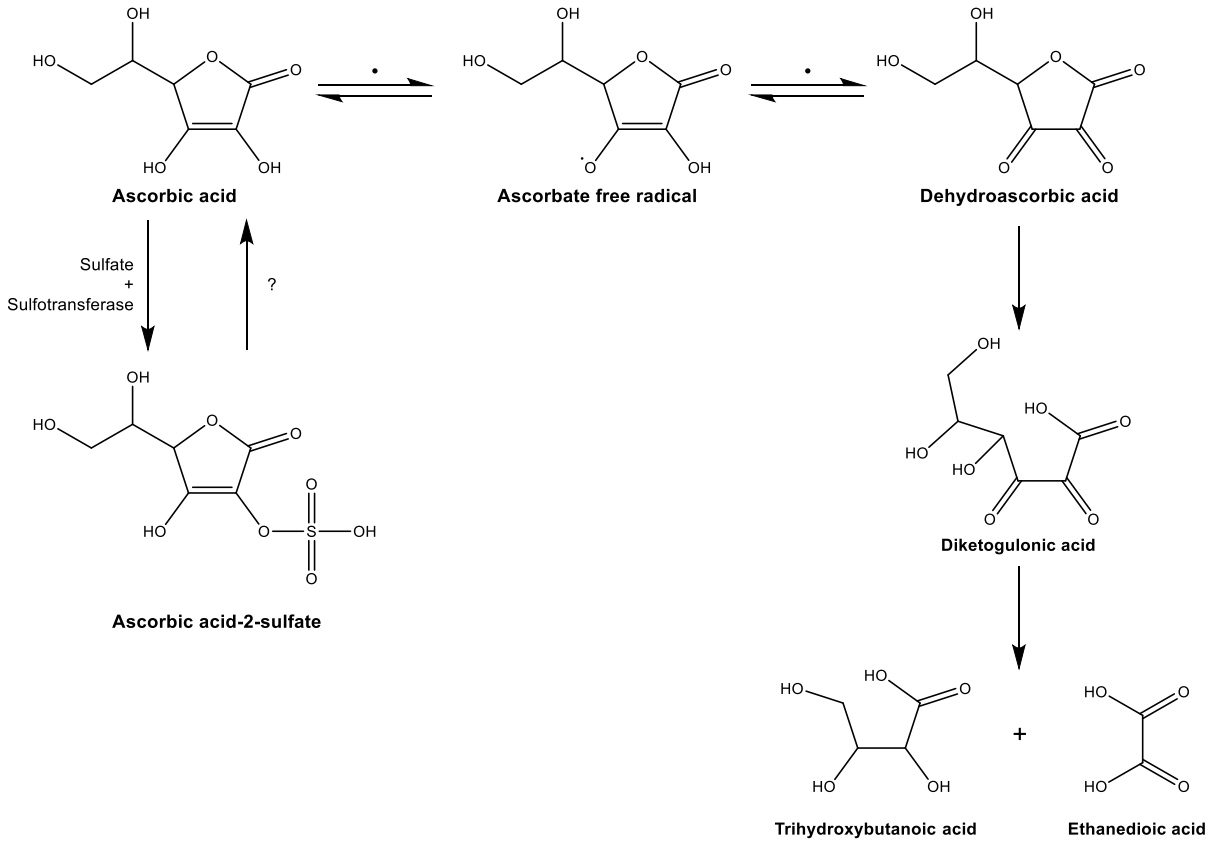
6.1 Introduction

Further to my project hypothesis; An imbalance in the ascorbate redox cycle is indicative of further imbalance in the sulfur redox metabolome as well as being associated with unfavourable outcomes in diseases associated with oxidative stress.

Intake of ascorbic acid occurs entirely through the diet. Ascorbate metabolism is closely linked to sulfur metabolism through the crosstalk between ascorbic acid and glutathione as well as the interaction of sulfate with ascorbic acid forming the metabolite ascorbic acid-2-sulfate.

By measuring the three key metabolites, ascorbic acid, dehydroascorbic acid, and ascorbic acid-2-sulfate further insight into the hierarchy of the redox metabolite and specific roles of ascorbate can be gained.

Ascorbic acid (Asc) and its oxidised form dehydroascorbic acid (DHA) are an important redox regulatory couple (**Scheme 25**) with links to the glutathione redox system[65,154,169]. Asc is an important dietary antioxidant that cannot be synthesised by humans. It is one of the most widely used vitamin/antioxidant supplement taken worldwide on top of being used as an antioxidant in cosmetics and the food industry[159]. Since DHA is the oxidised form of Asc is not as effective an antioxidant, but it does have some distinct protective properties of its own (see section 1.6.2 for details). DHA is also strongly associated with diabetes[173,174]. DHA is readily recycled back to Asc, forming an important pathway for the maintenance of cellular Asc[165,166]. If DHA is not recycled back to Asc then it can irreversibly oxidise to diketogulonic acid (DKGA)[172] (**Scheme 25**) leading to the loss of Asc which can only be regained through the diet. The regeneration of Asc from DHA and the interaction with glutathione makes a point of crosstalk between the ascorbate and sulfur related redox regulation pathways. Ascorbic acid-2-sulfate (AAS) forms a different point of crosstalk between these two metabolomes as it can be formed by the interaction of Asc with sulfate, a metabolic product of H_2S[155] (see section 1.3.2 for details). AAS has been detected in human plasma and is rapidly filtered from the blood by the kidneys and excreted in urine[176] but not much is known about its biological functions in humans (see section 1.6.3 for details). These points of crosstalk once again illustrate that the different redox cycles cannot be considered as discreet separate mechanisms but must be taken as part of the wider interlinked redox interactome[36].



Scheme 25: The ascorbate redox pathway: The redox pair ascorbic acid (Asc) and dehydroascorbic acid (DHA) with the sulfated metabolite of Asc, ascorbic acid-2-sulfate (AAS). The degradation pathway of dehydroascorbic acid to diketogulonic acid can account for loss of ascorbic acid and dehydroascorbic acid from this redox system. Crucially the oxidation of ascorbic acid is reversible but the degradation of dehydroascrobic acid to diketogulonic acid is irreversible.

The analysis of this key redox system is important to both improve our understanding of redox biology as well as for the monitoring of the many products and supplements that make use of Asc and DHA. Most analytical methods focus on Asc, but this does not show the complete picture of redox status linked to the Asc/DHA pair. Additionally DHA has its own distinct biological activities and should not be ignored. Ascrobic acid-2-sulfate (AAS) is a metabolite of Asc found in humans with not much known about its biological activities. The elucidation of AAS's potential activities is important to build on previous literature. Making use of more powerful modern methodologies will hopefully provide further insight into this little known metabolite in humans.

To this end I undertook a feasibility study to determine if either supercritical fluid chromatography (SFC) or ultra-high pressure liquid chromatography (UHPLC) coupled with tandem mass spectrometry (MS/MS) would be suitable for the simultaneous analysis of these three important metabolites. The stability of Asc and DHA in plasma samples during storage and analysis was also investigated to ensure that with the proper collection, handling and storage

procedures the sample analysis would provide the best possible picture of circulatory ascorbate redox status and would be applicable to high throughput analysis.

Initially the SFC based method showed good promise for the separation and simultaneous analysis of Asc and DHA. However a suitable compromise between Asc peak shape and ion suppression of DHA by the mobile phase was not reached. The focus of the chromatographic development was then shifted to UHPLC. Whilst the separation was not as good as with the SFC it was still suitable for our needs. Asc and DHA suffer from significant stability issues which presents a significant challenge for the sample collection processing and storage. It was found that none of the commonly used methods for stabilising Asc and DHA worked adequately to enable high throughput analysis of the Asc/DHA redox couple and requires further investigation. AAS is much more stable than both Asc and DHA but is only present in low levels in human plasma making quantification challenging. It is likely that the analysis of AAS will be better suited to urine and other sample types than plasma. Unfortunately, the time and resources remaining were insufficient to further address this topic and further work remains to understand and resolve the issue of sample stability.

6.1.1 Supercritical Fluid Chromatography

Supercritical fluid chromatography (SFC) is a separation technique that utilises a supercritical fluid, typically CO_2, as the major apolar mobile phase. A supercritical fluid is any substance at a temperature and pressure above its critical point[350]. When a substance becomes a supercritical fluid it has properties of both a gas and a liquid[351] as the distinction between these two phases does not exist above the critical point. Modern ultra-high pressure supercritical fluid chromatography (UHPSFC) offers an alternative to conventional normal phase liquid chromatography, providing faster, more robust and reproducible chromatography. The mobile phase can easily switch from apolar CO_2 to increasing amounts of polar co-solvent such as methanol meaning compounds with a wide range of polarities can be efficiently separated on a single column in a single chromatographic run[264].

Asc, DHA and AAS are all small polar molecules, with the major difference in the properties of the two compounds being their polarity due to the oxidation of the carbonyl groups of Asc or sulfated at the 2-O position (**Scheme 25**). Due to this, they are all not well suited for separation on reversed phase chromatography as was observed with other small polar metabolites such as homocystine and cystine, which are also poorly retained (Chapter 3:). Therefore, SFC was investigated to determine if it could provide a rapid, robust normal phase type separation for Asc,

DHA and AAS in order to provide a high throughput method for the determination of Asc/DHA redox status, levels of AAS and their fluxes.

6.1.2 Ultra-High Pressure Liquid Chromatography

Due to limitations we experienced during the development of the SFC based methodology, it was decided to pursue methodology based on more traditional UHPLC separation. As discussed in (section 3.4.1 and 6.1.1), reversed phase chromatography will most likely be poorly suited for this analysis due to poor retention of small polar molecules such as Asc, DHA and AAS without the use of highly polar mobile phases or ion pairing agents. These can lead to complications of their own and affect the stability of the column or decrease the ionisation of target compounds and affect the chromatography, removing the main advantages of RPLC. HILIC has been used previously for the simultaneous analysis of Asc and DHA[186] but has other limitations such as long equilibration times and inconsistent chromatography including poor peak shapes with drifting retention times which can change with different concentrations of injected compounds[195]. It was therefore decided to investigate mixed mode chromatography (MMC) as an alternative mode of separation, as with the thiol redox analysis (section 3.4.1). MMC combines polar separation characteristics with the stability of reversed phase chromatography providing a more robust separation than HILIC based methods[296].

There is limited previous work on the simultaneous analysis of Asc, DHA and especially AAS using liquid chromatography coupled with mass spectrometry. A large number of methods for Asc redox analysis using chromatographic separation have relied on UV detection[159,187,199], which is not well suited for the detection of DHA due to its low UV absorbance[163]. For this reason, many methods use what is known as a subtraction technique where each sample is analysed twice, once to measure the levels of free Asc, and then a second time after a reduction step to measure the total Asc with the difference being taken as the levels of free DHA[185,196]. This can be somewhat time consuming as each sample needs to be analysed twice and the extra time required for preparation may result in more sample degradation. The use of tandem mass spectrometry coupled with UHPLC mixed mode separation should help avoid these issues by enabling the selective, sensitive and direct analysis of Asc, DHA and AAS within a single rapid chromatographic run.

6.1.3 Sample Analysis, Preparation and Stability

Biological samples such as plasma and urine are complex mixtures containing many additional components other than the analytes of interest from larger proteins to other small polar

metabolites. Whilst a method might be capable of separating and quantifying standard mixtures, it may not be suitable for the analysis of biological samples especially without careful sample preparation. This can be due to the additional complexity of the sample matrix, which can cause numerous effects including suppression of analyte signal, false peaks arising from matrix components and shifts in the chromatographic separation[319]. There may also be biological or physical mechanisms that continue to function after sample collection, such as enzymatic pathways or metal catalysed degradation, which can have a negative impact on analyte stability further complicating the analysis of complex biological samples[196,207,209,352].

To avoid these potential negative effects the chromatographic separation has to be sufficient so the analytes do not co-elute with any interfering matrix components[211]. Not only does the chromatography have to be suitable, but the collection, storage and preparation of the samples also have to be carefully considered[171]. For the analysis of small metabolites in biological samples such as plasma or urine, the most common preparation methods involve the removal of proteins by precipitation, often using an organic solvent or acid[319].

It has been widely noted that ascorbic acid and dehydroascorbic acid are not stable at physiological pH even when stored at -80^{o}C. Numerous different causes have been attributed to this instability including oxidation by metal ions, enzymatic degradation and pH[158,207-209]. For the analysis of ascorbic acid, it is widely agreed that the best method of protein precipitation is acid precipitation[352]. By acidifying the sample, the stability of both Asc and DHA is improved whilst removing a large percentage of the proteins that might otherwise negatively affect the analysis. Metaphosphoric acid is one of the most widely used acids for protein precipitation and has been found to be the best precipitant for the analysis of Asc[159,210,352].

Other than sample acidification, there are differing reports in the literature over the best collection, preparation and storage methods for the analysis of Asc and DHA in plasma. Some groups have reported that EDTA (ethylenediamine tetraacetic acid) tubes are the best for the collection of whole blood[159,204,207] with other groups reporting that heparin tubes are more appropriate[158,171]. Whilst EDTA chelates metal ions and so should block metal ion induced oxidation of Asc it has been reported that the complexes it forms with transition metal ions can remain redox active and therefore facilitate metal ion dependant oxidation of Asc rather than blocking it[171]. There is further confusion over what is required in order to stabilise Asc and DHA in storage. It is clear that at physiological pH even when stored at --80^{o}C both Asc and DHA are not stable, degrading significantly over a matter of weeks[210,211]. Some groups have deemed it sufficient only to acidify samples[352], for example with metaphosphoric acid (MPA). Other groups

have found this to be insufficient to stabilise Asc with additional additives needed such as EDTA or diethylenetriaminepentaacetic acid (DTPA)[204].

Due to the confusion over what is required to stabilise Asc and DHA in biological samples, it was key to investigate the existing methods presented in the literature to establish for ourselves their suitability. There is less information available about the stability of AAS in biological samples but it should be more stable than Asc/DHA[176] but will also require investigation to ensure accurate and robust analysis.

6.2 Methods

Unless otherwise stated all reagents and materials were of the highest purity available and purchased from Sigma-Aldrich (Gillingham, UK or Munich, Germany). HPLC-grade solvents were from Fisher Scientific (UK). Argon gas (> 99.99%) and food grade CO_2 were from BOC Group (Guildford, UK). Ultrapure N_2 gas was produced by a nitrogen generator (Parker Balston, UK).

6.2.1 Supercritical Fluid Chromatography

Mass spectrometry settings were established using Asc, DHA and AAS standards. The standards were made up to a concentration of 1 mM by addition of the dry powder to pH 7.4 ammonium phosphate buffer. They were then diluted with pure water as need to bring them down to a measurable concentration range (1 nM – 5 μM). In order to optimise the mass spectrometry settings the individual standards were infused onto the triple quadrupole (TQD) mass spectrometer at 16 μl/min and scanned for one minute using negative ionisation mode. Optimal precursor and fragment product ions were established for both Asc and DHA, with cone and collision energies were manually adjusted to give the highest signal for each compound (**Table 7**), additional mass spectrometry settings are listed in appendix C.1. Further optimisation was performed after initial SFC conditions had been established as the presence of the supercritical CO_2 phase can alter ionisation properties. Optimisation was carried out by running the established chromatographic method (see later) and adjusting the mass spec settings as required. For the SFC analysis standards were first dissolved in pure water and then diluted down using methanol.

Three different co-solvents (mobile phase B) were initially investigated in order to obtain the best separation and peak shapes. Co-solvent **1** was 100% methanol; Co-solvent **2** was 98% methanol + 2% H_2O and; Co-solvent **3** was 98% methanol + 2% H_2O + 50 mM ammonium acetate. Each of the different co-solvents were run on a torus diol normal phase column (ACQUY UPC^2 Torus diol column, 130 Å, 1.7 μm, 3.0 mm x 100 mm) with a gradient of: 0 min, 10% B to 10 min, 40% B at

1.5 ml/min with a 1 min inlet pre-run at 10% B for column equilibration. The post column make-up solvent was 5 mM ammonium acetate in methanol with a flow rate of 0.2 ml/min.

Table 7: MRM settings for SFC analysis of ascorbic acid (Asc) and dehydroascorbic acid (DHA) using negative ion electrospray ionisation (ESI-).

ESI-	Precursor (*m/z*)	Product (*m/z*)	Cone (V)	Collision (V)
Asc	174.9	86.9	15	17
DHA	172.6	142.5	1	15

Further experiments focused on the DHA peak. The chromatographic method was shortened to 6 min using a compressed version of the longer gradient. The chromatography was started with a 10% mobile phase B pre run followed by a gradient starting with 0 min 10% B going to 40% B at 6 min. For these experiments the make-up solvent was changed to 0.5% ammonia flowing at 0.2 ml/min but other settings remained the same.

6.2.2 Ultra-High Pressure Liquid Chromatography

Due to the ion suppression effects observed when using the ammonia related modifiers such as ammonium acetate and ammonium hydroxide with the SFC analysis (section 6.3.1) it was decided to focus on formic acid as the solvent modifier. Since UHPLC uses a regular liquid mobile phase A as opposed to the supercritical mobile phase used in SFC the ionisation in the ESI source is different. Therefore, the MRM conditions for all three compounds were re-optimised specifically for the UHPLC method (**Table 8**). Mass spectrometry settings were established using Asc, DHA and AAS standards. The Asc and AAS standards were made up to a concentration of 10 mM by addition of the dry powder to pure water, DHA is less soluble in water and so was made up to 1 mM. They were then diluted with pure water as need to bring them down to a measurable concentration range (1 nM – 5 μM). The mass spectrometry settings were optimised using the individual standards as detailed before in section 6.2.1. DHA was optimised using both positive and negative ionisation.

Table 8: Final MRM setting for UHPLC analysis of ascorbic acid (Asc), dehydroascorbic acid (DHA) and ascorbic acid-2-sulfate (AAS) using positive ion (ESI+) and negative ion (ESI-) electrospray ionisation.

	Precursor (*m/z*)	Product (*m/z*)	Cone (V)	Collision (V)
Asc (ESI-)	174.9	86.9	15	17
DHA (ESI+)	174.9	128.6	6	15
DHA (ESI-)	172.6	142.5	6	12
AAS (ESI-)	254.9	174.6	15	17

Mixed mode chromatography (MMC) was used with a 1.6 mm Modus 100 x 2.1 mm Aqua UPLC column. The best separation was obtained using an isocratic method using 100% of mobile phase A (H_2O plus formic acid). Regular washes consisting of 100% MeOH were used to ensure the column remained in good condition. Optimal separation was obtained using a flow rate of 0.1 ml/min with a total run time of 6 min, the column was kept at 30^{o}C throughout the analysis. Three different concentrations of formic acid were tested as the mobile phase modifier, 0.1%, 0.5% and 1.0%.

6.2.3 Sample Analysis, Preparation and Stability

Blood was taken from a healthy male volunteer with informed consent to participate before enrolment. Procedures were approved by the ethics committee of the University of Southampton (ERGO 30507/31426) and conducted in accordance with the Declaration of Helsinki. Venous blood was collected from healthy human volunteers using a 21-gauge (0.80mmx19mm) butterfly needle and EDTA or heparin BD Vacutainer™ tubes. Following gentle mixing by inversion, blood was processed immediately by centrifugation for 1 min at 3000 × g at 4^{o}C to separate plasma from cellular components following the centrifugation method established in chapter 3.2.4[293].

The separated plasma was immediately added to a 1:1 volume of 10% metaphosphoric acid (MPA). The plasma was then immediately spun down to separate out the proteins and then either immediately analysed or frozen at -80^{o}C, as well as being frozen without removing the proteins in order to investigate the impact this had on Asc stability. The plasma was split into aliquots for storage and unless otherwise stated all aliquots were treated identically. Plasma that was frozen without removing the proteins was spun down immediately after defrosting. The effect of the metal chelator DTPA on Asc stability was tested by addition of 10 μl of 10 mM DTPA to 100 μl of fully defrosted MPA extracted heparin plasma to give a final DTPA concentration of 100 μM. Likewise the effect of DTT was tested by addition of 10 μl of 2 M DTT to 100 μl heparin plasma plus 100 μM DTPA giving a final DTT concentration of 20 mM.

All analysis was carried out using the UHPLC-MS/MS method detailed in section 6.1.2 using the established MRM scans. All samples were analysed using duplicate injections and quantified using prepared standard curves.

6.3 Results

6.3.1 Supercritical Fluid Chromatography

When using co-solvent 1 (100% MeOH, **Figure 33**) on the torus diol column DHA elutes first at 2.31 min with some minor tailing, followed by Asc which elutes at 6.61 min with major tailing. The tailing is likely due to the retention on the diol column being too strong[353] with the polarity of the co-solvent not being strong enough to elute Asc off the column efficiently.

The added water to co-solvent 2 (98% MeOH, 2% H_2O, **Figure 33**) increases the polarity of the co-solvent, the elution time of DHA does not change, eluting at 2.31 min with the peak shape largely unaffected though with a lower intensity, Asc however elutes earlier at 6.01 min with a much improved peak shape but still tailing slightly.

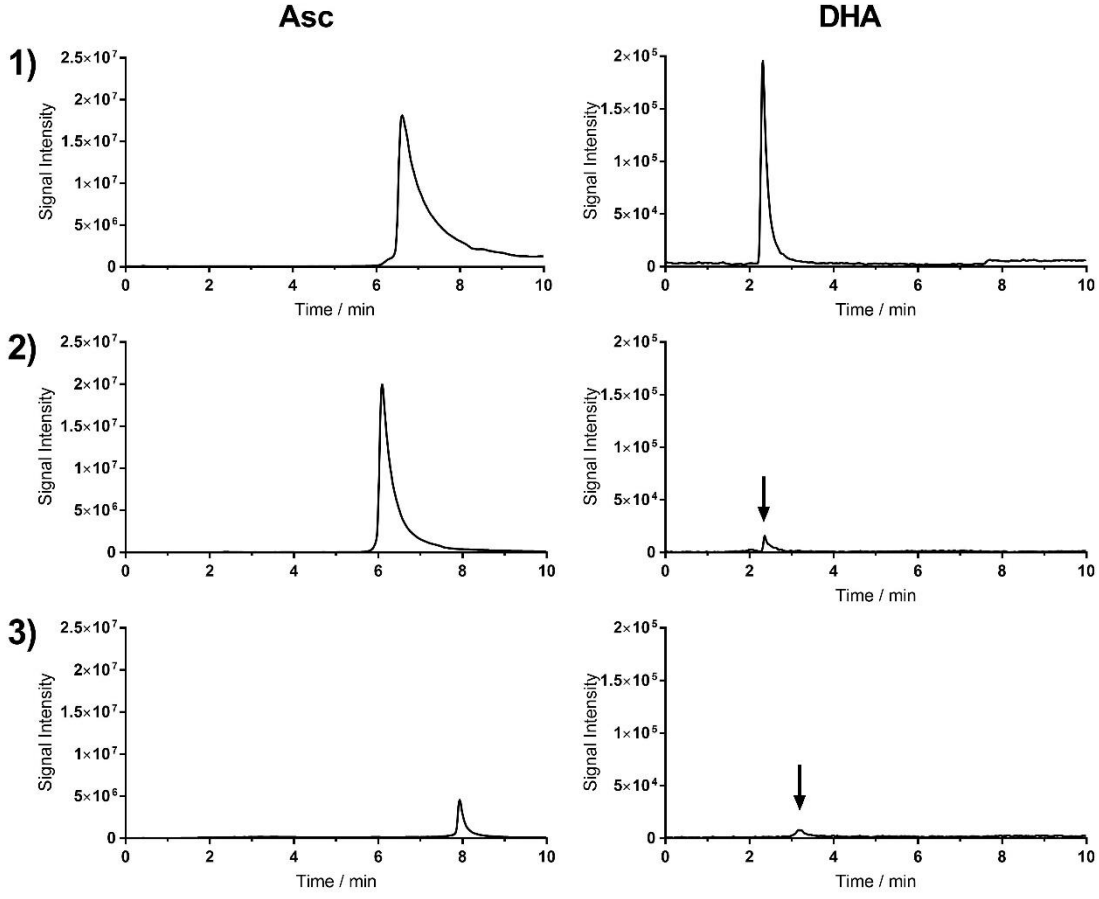


Figure 33: Separation of Asc and DHA with three different co-solvents on a Torus diol column; **1)** 100% MeOH, **2)** 98% MeOH, 2% H_2O and **3)** 98% MeOH, 2% H2O + 50 mM ammonium acetate. All three co-solvents give a very good separation between Asc and DHA. **1)** With pure methanol DHA has a well-shaped peak but Asc shows significant tailing. **2)** The addition of 2% water to the co-solvent improves the peak

shape of Asc whilst moving its elution time slightly forward. DHA however shows a drop in signal intensity. **3)** The addition of ammonium acetate to the co-solvent further improves the peak shape of Asc and moves the elution time later. DHA shows a further reduction in signal intensity and a slightly later elution time.

Co-solvent 3 (98% MeOH, 2% H2O + 50 mM ammonium acetate, **Figure 33**) produces the best chromatography with DHA eluting later at 3.22 min with less tailing than co-solvent 2, Asc also elutes later at 7.93 min with a much improved peak shape with virtually no tailing. Adding a mobile phase modifier can help improve peak shapes by interacting with the stationary phase of the column helping to prevent the excess retention which causes peak tailing as well as also controlling the mobile phase pH which can also have an impact on peak shape[353,354]. However, the peak intensity for DHA is considerably reduced compared to co-solvent 1, seemingly due to the addition of ammonium acetate to the co-solvent. Despite the precursor ions being close in mass (*m/z* 175 and *m/z* 173) there is little or no false signal generated for Asc or DHA by the other peak.

Shortening the chromatographic method from 10 to 6 min does not significantly affect the separation of Asc and DHA, both peaks elute earlier but are still well separated by 2 min, allowing for faster analysis time (**Figure 34, A and B**). Using ammonium formate in the co-solvent shows the same suppression effects as ammonium acetate but does improve the peak shape for Asc (**Figure 34, A and B**).

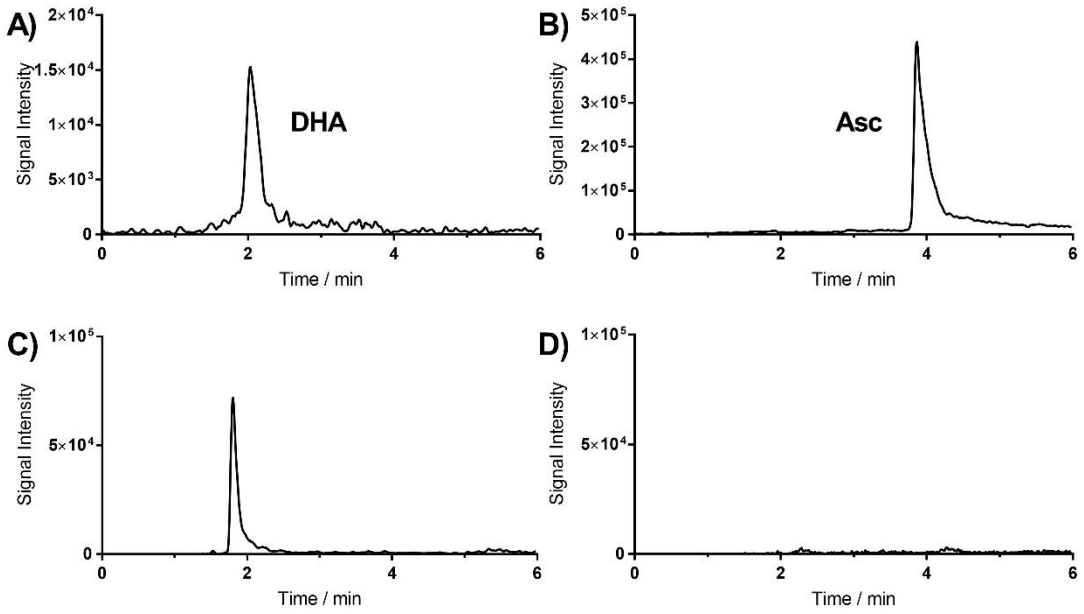


Figure 34: **A** and **B**, faster 6 min elution gradient with 98% MeOH, 2% H_2O and 25 mM ammonium formate co-solvent for the seoaration of Asc and DHA. **C** and **D**, the effect of ammonia in the co-solvent for the analysis of DHA using the shorter 6 min

gradient. **C)** DHA with 100% MeOH co-solvent. **D)** DHA with 98% MeOH, 2% H_2O + 0.5% ammonia co-solvent. The addition of ammonia to the co-solvent significantly effects the peak shape for DHA to the point where no peak is visible.

It was hoped that by using ammonia instead of ammonium acetate or formate as the modifier in the co-solvent the ion suppression of DHA by the acetate[189] could be avoided whilst improving the peak shape for Asc. Focusing on DHA, the addition of 0.5% ammonia to the co-solvent significantly worsens the peak for DHA to the point where there is no longer a true peak visible but one that is spread out from 2 min to 6 min+ (**Figure 34, C and D**). This is entirely unsuitable for the analysis of DHA.

6.3.2 Ultra-High Pressure Liquid Chromatography

Both Asc and DHA have a good peak shape and are well separated by 0.5 min using an isocratic gradient of 100% H_2O plus formic acid. AAS co-elutes with DHA, but has a poor peak shape with significant tailing when using 0.1% formic acid. This peak tailing causes issues with the measured Asc peak as AAS is subject to source fragmentation and generates signal for Asc that interferes with the true Asc peak (**Figure 36, A**). Increasing the concentration of formic acid improves the AAS peak shape, with 1.0% formic acid giving the best peak shape (**Figure 35**) that also leaves a distinct peak for Asc (**Figure 36, B**). DHA appears to have a split peak in the standard mixture but the second peak is likely due to interfering signal from Asc. A DHA standard on its own gives a single peak and the later DHA peak is observed when analysing an Asc standard on its own. The separation between Asc and DHA is sufficient that the true DHA peak can be distinguished from the false peak. AAS and DHA do not interfere with each other so the co-elution of these two compounds does not cause any issues. With the final chromatographic conditions, Asc elutes at 3.34 min, DHA at 2.86 min and AAS at 2.88 min (**Figure 36, B**).

Increasing the formic acid concentration in the mobile phase however does appear to supress the signal for DHA as with the ammonium acetate in the SFC method development. DHA is normally analysed using negative ionisation due to its low pK_a but due to the relatively high concentration of formic acid in the mobile phase it was investigated if positive ionisation was more appropriate for the analysis of DHA. Using positive ionisation does give 10-fold increased signal for DHA (**Figure 37**).

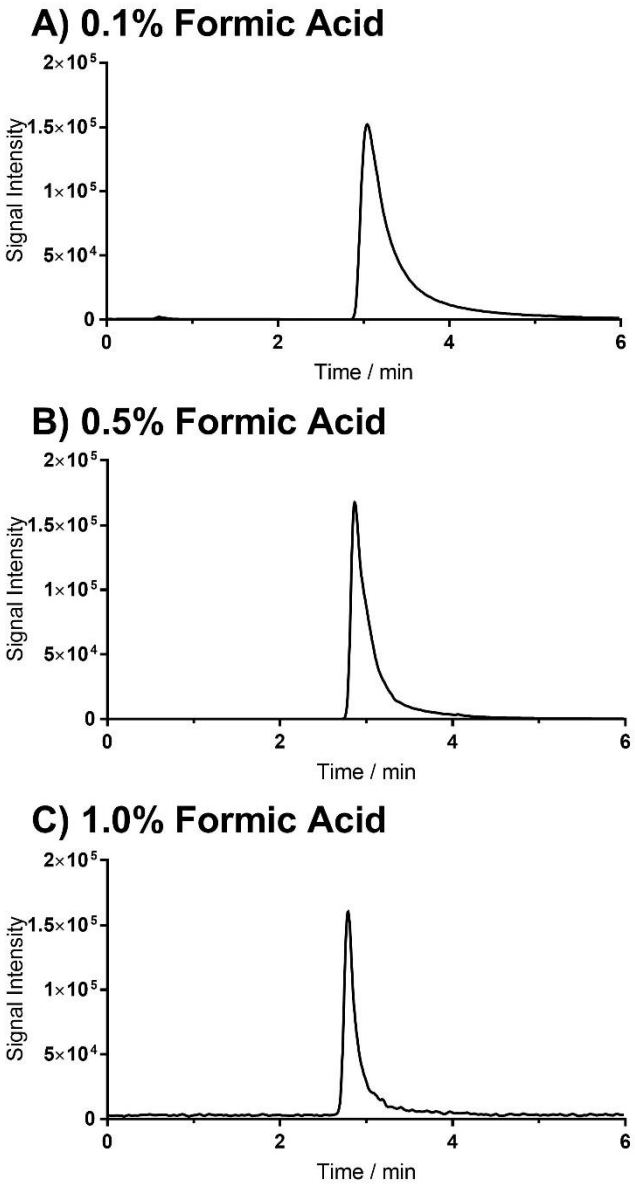


Figure 35: Effect of increasing mobile phase formic acid concentration on the peak shape of ascorbic acid-2-sulfate. The peak shape improves with signifcantly less tailing with increasing formic acid from **A** to **C**.

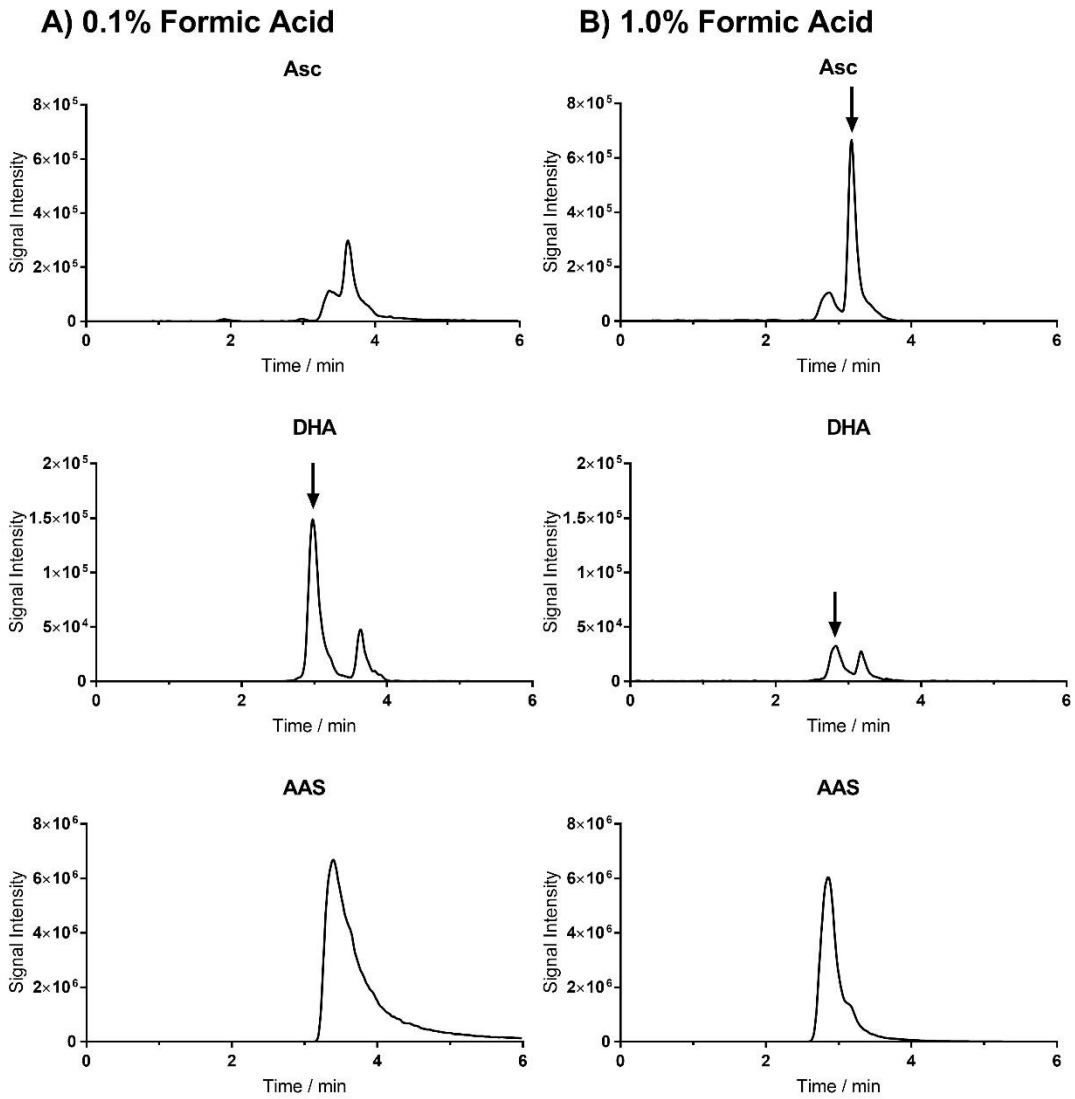


Figure 36: Effect of formic acid concentration on the chromatographic separation of Asc, DHA and AAS. With 0.1% formic acid (**A**) AAS has significant peak tailing which interferes with the measured peak for Asc. With 1.0% formic acid (**B**) the peak shape for AAS is much improved and the Asc becomes more distinct.

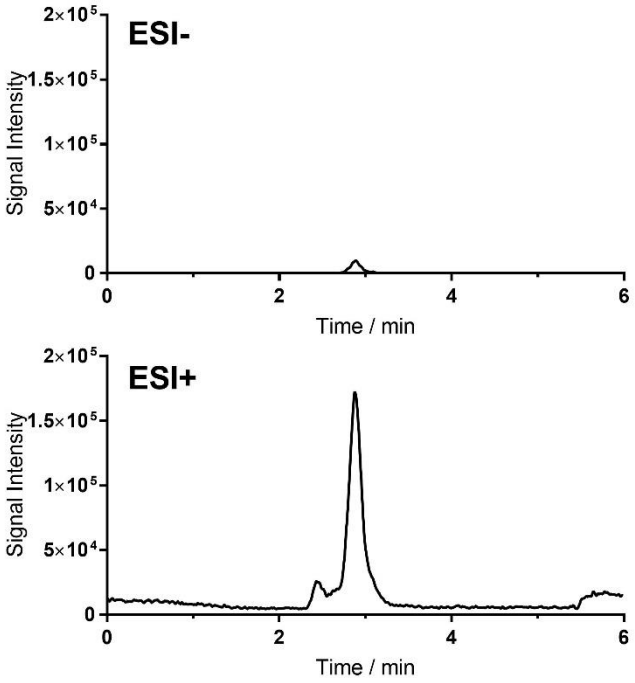


Figure 37: Analysis of DHA using negative ion (ESI-) and positive ion (ESI+) electrospray ionisation. Positive ionisation gives 10-fold higher signal than negative ionisation when measuring the same standard mix.

6.3.3 Sample Analysis, Preparation and Stability

The chromatograms for freshly collected plasma samples looks very different to the standard mixture. This is unsurprising as physiological levels of Asc DHA and AAS will vary from sample to sample, and the levels of each analyte are equal. Asc is much more abundant than DHA and the levels of plasma AAS have not been widely investigated in humans and so remain unclear. **Figure 38** shows an exemplary chromatogram for a freshly collected and extracted plasma sample measured using the UHPLC method detailed in section 6.2.2. A good peak for Asc is clearly visible, but DHA is absent and AAS has a small peak that is difficult to quantify reliably. There are two peaks visible in the DHA chromatogram at 3.60 min and 4.50 min that do not match up with the retention time of DHA (3.00 min, **Figure 36, B**). This suggests that these peaks are originating from unknown matrix components and not from DHA. The physiological levels of DHA are lower than the detection limit of this method, which complicates the ascorbic acid redox analysis.

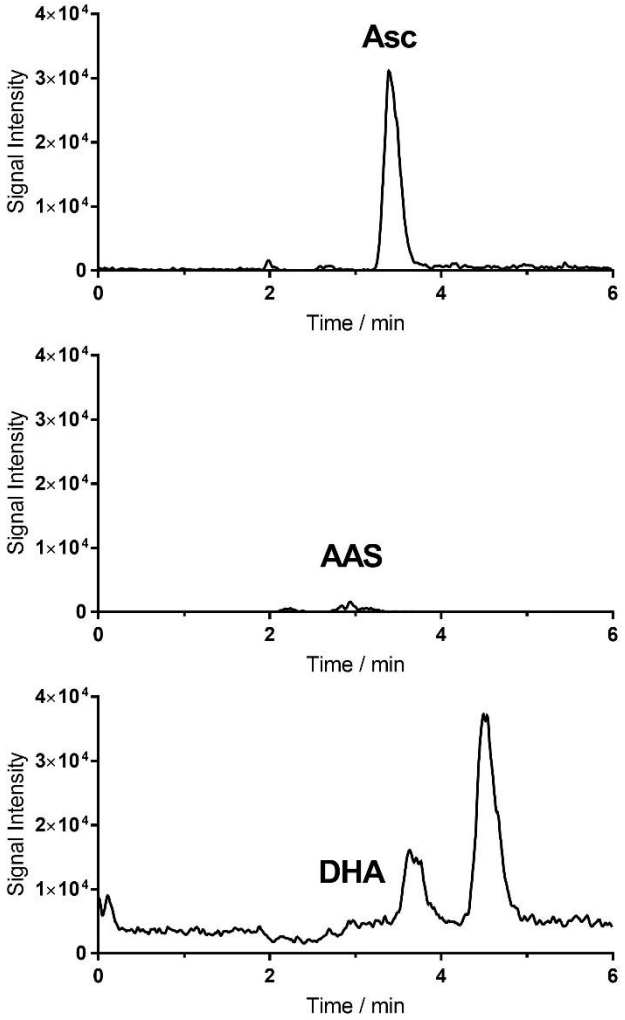


Figure 38: Exemplary chromatogram showing the detected Asc, AAS and DHA in freshly collected EDTA plasma extracted with metaphosphoric acid. There are two visible peaks in the DHA trace but neither of them elute at the correct time for DHA (3.00 min) and so are likely arising from other matrix components. Asc has a strong peak but AAS whilst visible is not a very strong peak.

The stability of Asc in plasma was investigated following different methods reported in literature along with different sample collection conditions. The effect of using both EDTA and heparin blood tubes for blood collection on the stability of Asc was tested as there is no agreement on which of these blood tubes are the most suitable. There is a small difference in Asc concentration observed between the tubes, with EDTA giving the highest concentration of Asc (**Figure 39, A**). Asc is also slightly more stable when collected with an EDTA tube with a smaller decrease in concentration when refrigerated for 72 min than in heparin plasma (**Figure 39, B**). From our experiments, EDTA tubes appear to be the most suitable. When removing the plasma proteins the addition of MPA rapidly precipitates the proteins, it was tested if leaving the precipitated proteins in the plasma before freezing had any effect on the levels of Asc compared to removing them

before. There is a clear drop in Asc levels when the precipitated proteins are left in the plasma when frozen (**Figure 39, D**), which occurs in both heparin and EDTA plasma. For best results, the precipitated proteins should be spun down and the supernatant decanted before freezing.

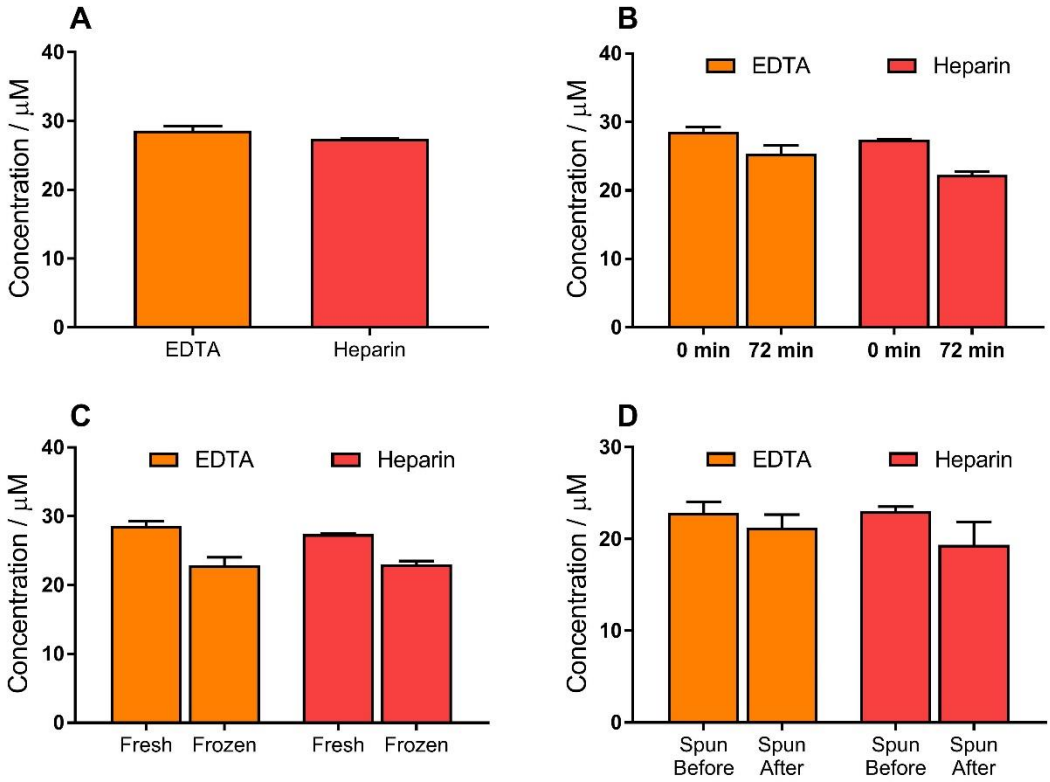


Figure 39: Initial investigations into the stability of Asc in plasma, all blood samples were immediately spun down after collection and the plasma was extracted with metaphosphoric acid. **A)** The difference in measured Asc levels in freshly collected plasma using EDTA and heparin blood tubes. Higher levels of Asc are recorded using EDTA tubes. **B)** Asc stability in plasma collected in both EDTA and heparin blood tubes in the sample manager (set to 4°C) over 72 min. Both plasma samples show significant drops in Asc concentration, more so in heparin than EDTA. **C)** Asc stability in plasma collected using both EDTA and heparin tubes after freezing at -80°C, there is a large drop in the measured Asc concentration after one freeze thaw for both tubes. **D)** Effect of spinning out and removing the proteins after MPA addition before or after freezing. All samples were stored in the same manner at -80°C. Leaving the proteins in the sample during storage decreases the measured levels of Asc.

Asc in both EDTA and heparin acid extracted plasma was found to be unstable when kept in the autosampler set to 4°C. Over 72 min at 4°C there was a significant drop in measured Asc concentration (**Figure 39, B**). Asc in acidified plasma was also found to not only unstable when refrigerated but also when stored frozen. In both heparin and EDTA plasma there is a large

decrease in Asc concentration after only one freeze thaw after storage at -80°C for 12 hours (**Figure 39, C**). DTPA is an alternative metal chelator to EDTA[171], when tested in MPA extracted heparin plasma (kept at 4°C) no stabilising effect was observed. The concentration of Asc decreased at the same rate with and without the addition of DTPA (**Figure 40, A**).

Dithiothreitol (DTT) is also used widely in the analysis of Asc, since it is a strong reducing agent, it should reduce any DHA present in the plasma and continue to do so as Asc oxidises to DHA. The DHA concentration can be calculated from the increase in Asc concentration after the addition of DTT, which is advantageous with the lack of a DHA peak in the chromatogram. The initial concentration of Asc is higher when DTT is added to plasma, but the addition of DTT does not prevent the loss of Asc over time (**Figure 40, B**). In fact, whilst it did start higher the concentration of Asc in the DTT plasma converges with the MPA only plasma suggesting that there is another mechanism other than just oxidation occurring.

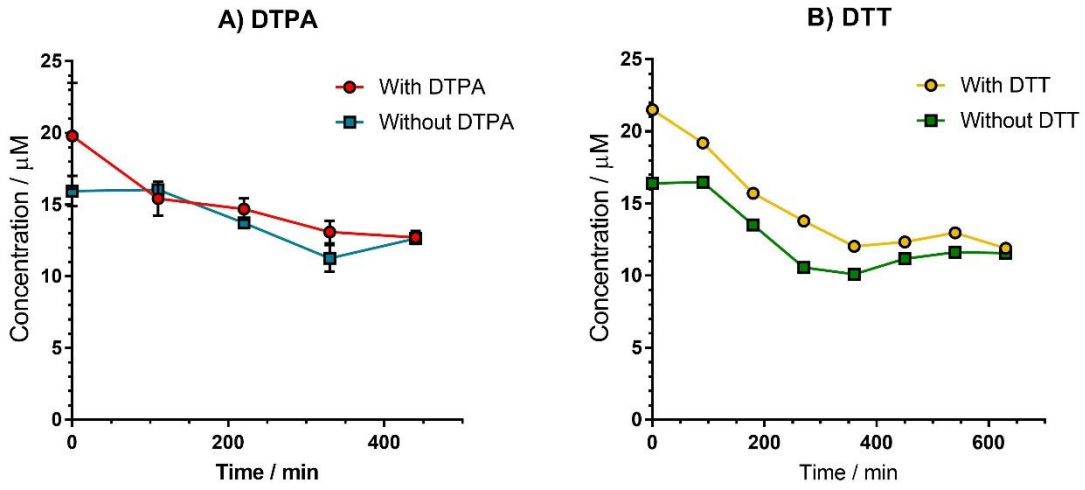


Figure 40: Stability of Asc in heparin plasma over time in the chromatography system sample manager (set to 4°C) with either **A)** the metal chelator DTPA or **B)** the reducing agent DTT. With both DTPA and DTT there is no improvement in Asc stability over the time measured compared to plasma with only MPA. The DTT treated plasma (**B**) starts with a higher Asc concentration as expected but the measured levels converge with the MPA plasma.

6.4 Discussion

6.4.1 Supercritical Fluid Chromatography

Supercritical fluid chromatography is a promising technique for the separation and analysis of Asc and DHA. The initial conditions tested using a torus diol column all had a large separation of 4-5

min between the two compounds using a quick and simple gradient. All of the co-solvents tested gave a good separation with the main differences being the peak shapes and sensitivities. Peak tailing can be caused by too strong an interaction between the analytes and the stationary phase, to improve the peak shape and reduce peak tailing this interaction needs to be weakened by either modifying the mobile phase, modifying the stationary phase or both[353].

The addition of 2% H_2O in co-solvent 2 improves the peak shapes (**Figure 33**) over co-solvent 1 (100% methanol) significantly reducing the tailing for Asc. Adding H_2O increases the polarity of the co-solvent increasing the interaction between the mobile phase and analytes. This decreases the peak tailing by eluting Asc from the column quicker and by decreasing the interaction of Asc and the stationary phase. The peak shapes however are not ideal, as there is still some tailing for Asc. There is also a decrease in DHA signal that may be due to decreased ionisation efficiency because of the added water[355].

Co-solvent 3 gives the best peak shapes (**Figure 33**) with the addition of H_2O and ammonium acetate markedly improving the peak shapes compared to co-solvents 1 and 2 with little tailing for Asc. However compared to co-solvent 2 DHA has a much weaker signal, likely due to ion suppression caused by the addition of ammonium acetate. This effect has been previously reported elsewhere with the acetate found to be responsible for the signal suppression[189]. Since the addition of ammonium acetate to the co-solvent improves the Asc peak shape, ammonia was investigated to identify if it can also give better peak shapes but without supressing the signal for DHA. Ammonium formate was also investigated as another possibility, however due to the structural similarities between acetate and formate it was unsurprising that it was also found to cause suppression of the DHA signal (**Figure 34, A**).

The lower signal intensity for DHA compared to Asc seen with the initial three co-solvents was thought to be potentially due to the use of ammonium acetate in the post column make-up solvent. Even with only MeOH as the co-solvent the signal for DHA is two orders of magnitude lower than for the equivalent concentration of Asc (**Figure 33**). The make-up solvent is required to ensure sufficient ionisation after the CO_2 has evaporated off in the ESI source. Both Asc and DHA ionise best in negative mode due to their low pK_a and ammonium acetate improves the ionisation efficiency in both positive and negative mode. However, since acetate supresses the signal for DHA it should be avoided in the make-up flow as well as the co-solvent, ammonia could potentially be a better choice to assist with the ionisation in negative mode without supressing DHA signal. When the make-up solvent was changed to MeOH with 0.5% ammonia the signal for DHA was still found to be lower than for Asc. Whilst ammonia also appears to supresses the signal for DHA it is likely that DHA does not ionise as efficiently as Asc using negative ion electrospray.

DHA has a lower pKa than Asc[356] and so might be expected to ionise better with negative ion ESI but it has been found that the pKa of a molecule does not necessarily correspond to its ionisation efficiency in negative ion ESI[355].

SFC has proven to be a powerful chromatographic technique for the separation of Asc and DHA using normal phase type separation without suffering from the typical drawbacks associated with HPLC/UHPLC normal phase chromatography. However, despite the good separation of Asc and DHA in order to give Asc a good peak shape a modifier has to be used in the co-solvent. Ammonium acetate, ammonium formate and ammonia, the most commonly used modifiers for negative electrospray ionisation all give good peak shapes for Asc. Nevertheless, both ammonium acetate and formate supress the signal for DHA whilst ammonia results in a very poor peak shape if not a non-existent peak for DHA. These modifiers are all unsuitable for the analysis of DHA, and without any modifier in the co-solvent Asc had a poor peak shape on the Torus Diol SFC column. Due to these issues, time constraints, and instrument availability, it was decided that the best course of action was to investigate alternate stationary phases that would give a better peak shape for Asc without the need for ammonium acetate, ammonium formate or ammonia. To this end the focus of the development was shifted to UHPLC using a mixed mode column following the method development performed in chapter 2.

6.4.2 Ultra-High Pressure Liquid Chromatography

With the developed UHPLC method, the separation between Asc and DHA is not as good with the SFC method. However, the separation of Asc and DHA is sufficient that any false signal for DHA generated by the Asc due to due to their similarities in structure and *m/z* is separated giving a distinct DHA peak. Likewise, AAS generates false signal for Asc elutes at the same time as DHA, 0.5 min before Asc. When using a lower concentration of formic acid in the mobile phase AAS has a large tail that continues past the true Asc peak partly obscuring it and making reliable quantification difficult. By increasing the concentration of formic acid in the mobile phase, the peak shape for AAS was improved with minimal tailing allowing the true Asc peak to be distinguished from the false signal (**Figure 35**).

The increase in formic acid concentration however had a negative impact on the measured signal for DHA, decreasing it by 10-fold (**Figure 36**). DHA is normally measured using negative ionisation, however due to the increased formic acid it was investigated if using positive ionisation would provide a better signal for DHA. By re-optimising the MRM scan for DHA using positive ionisation the measured signal in the standard mixture was improved by 10-fold (**Figure 37**) negating some of the negative impact of the higher formic acid concentration. However, in a more complex

sample matrix such as plasma or urine there will be many more potentially interfering matrix components that may have a negative impact on the measurement of DHA when using positive ionisation.

Overall, the developed UHPLC-MS/MS method is capable of the separation and analysis of ascorbic acid, dehydroascorbic acid and ascorbic acid-2-sulfate in standard mixtures (**Figure 36**). The method could nevertheless run into issues when applied to more complex biological samples.

6.4.3 Sample Preparation, Analysis and Stability

Despite being widely discussed in the literature there is no consensus on the best way to collect, process and store samples for the analysis of Asc and DHA[171,209,211,357,358]. This is further complicated by the lack of methods for the simultaneous analysis of Asc and DHA with many methods focusing on measuring total ascorbic acid. In order to determine the best method for the collection, processing and storage of blood and plasma for ascorbate redox analysis a number of different methodologies reported in the literature were tested to determine their suitability for use with the developed UHPLC-MS/MS method.

Both heparin and EDTA blood tubes have been stated to be the best tubes for the collection of samples for Asc analysis[158,159,171,196,207]. Due to this lack of agreement, we investigated both types of tube to establish for ourselves which type would be the most suitable for our use with the developed UHPLC-MS/MS method. In our experiments EDTA tubes gave the highest levels of Asc but only by a small margin of ~5% (**Figure 39, A**). Asc also appears to be more stable in plasma collected using EDTA tubes rather than heparin. Both types of plasma see a large decrease when kept at 4^{o}C with a decrease of ~10% with EDTA and ~20% for heparin (**Figure 39, B**). Overall, for our analysis EDTA tubes are the most suitable for the collection of whole blood.

The main method for stabilising Asc and DHA in plasma is acidification of the sample preventing the oxidation of Asc and the irreversible degradation of DHA to DKGA[172] (**Scheme 25**), this also conveniently doubles as a protein precipitation method. Metaphosphoric acid has been found to be the best acid for this purpose[171,185,190] and is best added to the plasma as soon as it has been separated from the red blood cells. It was found that after addition of MPA it is best to spin down and remove the proteins before freezing the plasma for storage. When the proteins were precipitated but left in the plasma during freezing there was a significant drop in Asc concentration with both EDTA and heparin plasma (**Figure 39, C**). This suggests that the plasma proteins are at least partly involved in the oxidation and loss of Asc after sample collection. Unfortunately the acidification of plasma is not sufficient to stabilise Asc, even when refrigerated or frozen at -80^{o}C. Contrary to what is reported in the literature when refrigerated, for example

during sample analysis (sample manager set to 4°C), Asc decreases in concentration by up to ~20% over 72 min (**Figure 39, B**). This instability would significantly affect any analysis involving even a small number of samples as with a 6 min chromatographic run ten samples would take upwards of 60 min, not including any standards or blanks. This would result in a substantial decrease in Asc in the last samples compared to the first. Whilst this instability appears to be worse in heparin plasma it is not much better than with EDTA. Even storing the acidified samples at -80°C does not appear to be sufficient to prevent the loss of Asc, with a decrease of ~18% when frozen for less than 12 hours and only defrosted once (**Figure 39, C**).

EDTA blood tubes are suggested to be more appropriate for the collection of blood for Asc analysis as EDTA can chelate metal ions that can otherwise catalyse the oxidation of Asc[171]. Whilst EDTA tubes do give better stability than heparin tubes (**Figure 39, B**) they are clearly not sufficient to block completely the loss of Asc through oxidation or other means. In fact, whilst EDTA is an effective metal chelator it does not completely block the redox activity of some metal ions allowing them to continue to catalyse the oxidation of Asc. The metal chelator DTPA has been suggested as a more appropriate alternative as it more effectively blocks the redox activity of the metal ions[171]. Heparin plasma was tested with and without DTPA, avoiding EDTA plasma in order to avoid EDTA chelating metal ions and potentially blocking the DTPA leaving the metal ions redox active. There however is still a large drop in Asc concentration over time when stored in the refrigerated sample manager regardless of whether DTPA is added or not. There is no clear difference in stability observed between the two samples (**Figure 40, A**). This suggests that there may be a different degradation mechanism occurring as sample acidification should have blocked autoxidation and addition of metal chelators should have neutralised any metal ion catalysed oxidation.

Many methods do not simultaneously monitor Asc and DHA but instead rely on reduction in order to measure the DHA. The free Asc is measured as well the total Asc which is measured after the addition of a reducing agent such as dithiothreitol (DTT). The concentration of free DHA is taken as the difference between the total Asc and free Asc. This adds extra preparation steps, as the sample either has to be split before analysis (requiring larger sample volume) or it has to be treated with DTT after the first measurement has been taken. Either way this doubles the number of measurements that have to be made which is undesirable in large sample sets. The addition of DTT to the sample will not stop the oxidation of Asc, it will however reduce any DHA produced by oxidation and therefore prevent the level of Asc from decreasing in the sample. When DTT was added to a heparin plasma sample, there was a measured increase in the initial Asc concentration as would be expected. Nevertheless, when stored at 4°C in the sample manager the concentration of Asc in the DTT treated sample decreases at a similar rate as the MPA only plasma. In fact, the

concentration of Asc in DTT plasma converges with the Asc in MPA plasma (**Figure 40, B**). In all experiments whilst Asc was decreasing there was no peak observed for DHA that would be expected to start appearing as Asc oxidised. Coupled with the lack of stability even in the presence of DTT this further reinforces the conclusion that there is a different mechanism other than oxidation occurring that is responsible for the loss of Asc in plasma.

6.5 Conclusion

Ascorbic acid is easily detected and quantified in plasma using the established UHPLC-MS/MS method. Unfortunately, however our investigations into the simultaneous analysis of Asc, DHA and AAS in plasma have not been successful in establishing a fully workable method. The developed chromatographic separation and tandem mass spectrometry detection works well in a simple matrix such as a standard mixture but in more complex biological matrices there are significant limitations. Whilst SFC looked promising for the rapid separation of Asc and DHA time constraints, instrument availability and issues with the method prevented further development. This provides an interesting avenue for future method development.

The analysis of Asc in plasma is complicated by its instability with none of the tested stabilisation methods having a significant impact on its stability. The acidification of samples should block the degradation of DHA to diketogulonic acid (DKGA) (**Scheme 25**), and therefore the loss of Asc following this pathway. This should then be reflected by a corresponding increase in DHA, however throughout our experiments there was no significant increase in DHA observed. In fact when treated with the reducing agent DTT, which will convert any oxidised Asc back, there was no improvement in the stability of Asc. Likewise, the addition of metal chelators to block metal ion induced oxidation also had no impact on stability. All of these factors imply that there is a different mechanism of loss occurring other than simple oxidation.

The endogenous levels of DHA present in plasma are below the limit of detection of the method meaning that the ascorbate redox ration cannot be calculated. This is not unexpected as the physiological levels of free DHA have been reported to be very low and methods that have reported higher levels may have been measuring artifactual DHA generated from oxidation of the Asc[171,204]. AAS is detectable in plasma samples but only in very low levels near the limit of detection making it hard to quantify consistently. It is likely that the measurement of AAS will be more applicable to urine samples that contain higher endogenous levels[176]. The method could be used for the tracing of AAS metabolism using a stable isotope AAS the levels in plasma would be increased and potentially more reliably quantifiable and the tracer should be easily monitored as it is excreted in the urine. This could help determine if AAS has any biological roles in human

biology building on the previous works[176,183,359] by using more modern methodologies to help increase our understanding.

The instability of Asc has substantial implications for its analysis, significant drops in Asc concentration when kept in the refrigerated autosampler mean that measurements will be unreliable even over short sample runs. For example, ten samples would take over 60 min to run and the levels of Asc will be significantly lower at the end of the run compared to the beginning. During analysis it was also noticed that an unknown deposit was forming on the sample cone of the mass spectrometer ESI source and column performance was degrading faster than expected. This was likely occurring due to one of the additives used to try to improve sample stability, as it has not been encountered in other plasma analysis such as in Chapter 3:. It will require further investigation to identify the interfering compound and find an ESI compatible alternative. Due to restrictions on time the further development on this method required to solve these issues was not possible.

Ultimately, the established method provides a good starting point but is not currently suitable for the simultaneous analysis of Asc, DHA and AAS in plasma and requires significant further development.

Chapter 7: Further Work

7.1 Outlook

Significant progress has been made on the development of a method for the simultaneous analysis of key components of the sulfide redox metabolome including oxidised and reduced thiols and sulfide. The ability to readily analyse the free thiols and sulfide as well as their bound forms using efficient workflow makes effective use of limited sample volumes whilst providing a large amount of information. Key factors affecting sample collection, processing and storage have also been assessed to not only establish the best conditions for the most accurate analysis currently possible, but also to investigate the effect of each of the different factors. Knowing the limitations of samples collected under non-ideal conditions allows for better use of such samples, which, for example may have been collected for the analysis of different metabolites/pathways but may still contain important information relating to the sulfide metabolome. This allows access to a wider pool of potential samples including those previously collected for studies and currently being stored whilst being able to draw conclusions that are more confident from the analysis. The established method could easily be combined with stable isotope tracing for the sulfide metabolome as well as being used in conjunction with existing stable isotope tracing methods for the NO metabolome.

Further insight into the analysis of per- and polysulfides has been gained. These highly reactive species have proven difficult to accurately analyse and quantify. The measured speciation pattern of pure polysulfide standards depends on the reaction conditions used, in particular the electrophile selected. Whilst NEM reacts rapidly it was found to be able to either react with the mid chain sulfurs or cause chain fragmentation, thereby decreasing the overall chain lengths of the polysulfides detected. When using electrophiles to trap the polysulfide speciation the reaction occurs under Curtin-Hammett conditions where the relative concentrations of the products depend on the reactivities of the individual polysulfide species and the rate of interconversion between them rather than the relative concentrations of the polysulfides in solution. As long as these caveats are known derivatisation with an electrophile can still be a useful tool for the analysis of polysulfides. The developed method does not show or produce polysulfides when none are present in the sample, and therefore can be used to confirm or rule out the presence of polysulfides. To this end the metabolism of sulfide by superoxide dismutase, producing polysulfides was analysed by derivatisation with IAM confirming this unexpected metabolic activity.

Following from the use of NEM for the analysis of thiols and sulfide, as well as IAM for the analysis of polysulfides it was apparent that the kinetics of the derivatisation reaction were important to understand as they can have significant impacts on the measured results. Whilst NEM reacts rapidly with reduced thiols, it was found that the reaction with sulfide takes substantially longer. This difference is not reflected by IAM or MBB and may be due to the difference in reaction mechanism with NEM reacting via Michael addition and IAM and MBB via a S_N2 mechanism. The kinetic investigations highlighted the importance of using rapidly reacting electrophiles, particularly for volatile compounds such as sulfide and methanethiol.

The simultaneous analysis of ascorbic acid (Asc), dehydroascorbic acid (DHA) and ascorbic acid-2-sulfate (AAS) proved more challenging than first anticipated. Super critical fluid chromatography showed promise for the separation of Asc and DHA but due to time constraints and limited access to instrumentation was not completed. A UHPLC based method was then established and is able to separate Asc, DHA and AAS. However, sample stability was found to be a significant challenge. None of the methods to stabilise Asc and DHA that were tried were able to sufficiently stabilise the samples to make accurate analysis possible. Even reduction with an excess of DTT in the sample was insufficient to stabilise Asc. Due to time and resource limitations further investigation into this issue was not possible and still remains to be solved.

7.2 Thiols and Sulfide

The method developed for the analysis of the sulfur redox metabolome is by no means exhaustive. The in depth analysis of this metabolome could benefit from the incorporation of further thiols and sulfide metabolites. These include the oxidised forms of glutamylcysteine (OxGluCys) and cysteinylglycine (OxCysGly) which may form redox pairs of the glutathione cycle intermediate thiols (see section 1.3.4). Taurine, whilst not a thiol, is the most abundant sulfur containing amino acid. It is semi-essential with the main source being through the diet but is also produced in vivo through the metabolism of Cys by cysteine dioxygenase[59]. Taurine potentially has beneficial effects on the cardiovascular system involving regulation of the NO pathway[82] as well as oxidative stress regulation[83]. Taurine can also increase the expression of the H_2S producing enzymes CBS and CSE providing another mechanism by which it can help regulate the cardiovascular system and redox pathways[84]. 3-mercaptopyruvate (3MP) would be another small thiol that would be beneficial to incorporate into the method, as it is a precursor to polysulfide synthesis by the enzyme 3-mercaptopyruvate sulfurtransferase[134]. Incorporation of 3MP, which is a metabolic product of Cys, would help the elucidation of the links between thiol metabolism and the biological activities of polysulfides.

It was also attempted to incorporate coenzyme A (CoA) into the thiols and sulfide method. Unfortunately, CoA is not well suited for analysis using the mixed mode aqua column and had very poor peak shape (section 3.4.1.1). This poor peak shape is likely due to the structure of CoA (polar head group with a more apolar tail, **Table 2**) resulting in multiple modes of interaction with the stationary phase. Alternate column chemistries will be needed for the analysis of CoA, with a standard reversed phase C_{18} column likely providing suitable retention characteristics.

In order to add these compounds into the method appropriate MRM scans will have to be established, and mass spectrometry (MS) settings optimised, for CoA these have already been established. Once the MS settings are established, their chromatographic behaviour in the current method will have to be determined and if necessary, the method adjusted to incorporate these analytes. Most likely OxCysGly, OxGluCys and taurine will all elute close to the other oxidised thiols, as they will all not react with NEM. For CoA different chromatographic conditions will certainly have to be established. With an alternate separation method, the analysis of CoA could be incorporated with analysis of the rest of the thiols/sulfide metabolome by utilising the same samples with the established collection, preparation and storage protocols.

Thiol metabolism does not exist in isolation, a key point of interaction between the NO and H_2S metabolomes exists in the methionine cycle (**Figure 42**, Box 2). Homocysteine is synthesised from methionine and provides an irreversible metabolic link through synthesis of cysteine. A better understanding of the metabolic pathways leading into the H_2S metabolome would help better understand thiol metabolism and the factors that can influence it. A separate method would likely be needed to analyse the key metabolites involved as the lack of a free SH group in these metabolites will mean they will not react with NEM and be poorly retained in the method developed for thiol analysis. Alternate derivatisation or column chemistries will be needed to get sufficient retention for the analysis of this metabolic pathway.

7.3 Polysulfides and Persulfides

Whilst iodoacetamide (IAM) has been shown to produce longer polysulfide chains than NEM when used to alkylate inorganic polysulfides and is considered more suitable for this task (section 4.3.3), there remains some questions about the appropriateness of IAM for polysulfide analysis. It is possible IAM is able to fragment polysulfide chains in the same manner as NEM, though to a lesser extent. This should be investigated utilising the same experimental procedure as for NEM (section **Error! Reference source not found.**); in short the polysulfides should be completely derviatised with an excess of an alternate electrophile (such as MBB) before IAM is added and monitored for the potential formation of any IAM polysuifde adducts. The speciation of inorganic

polysulfides in solution has so far been investigated using NEM and IAM, the effect on polysulfide speciation of alternate electrophiles. Alternate electrophiles include the 'milder' HPE-IAM and the commonly used MBB. MBB in particular is a widely applied for the analysis of thiols and sulfide[112] (section 1.4.3), therefore its impact on polysulfide speciation will be important to investigate for comparison with other electrophiles. β-(4-hydroxyphenyl)ethyl iodoacetamide (HPE-IAM, **Figure 41**) is an IAM derivative which is a milder electrophile than NEM, MBB and IAM[322]. HPE-IAM could provide a more accurate picture of the polysufide speciation but the rate of reaction should also be considered. IAM reacts much slower than NEM (Chapter 5:), so if HPE-IAM reacts even slower than IAM the polysufide speciation will likely change before the reaction has completed. The impact of different electrophiles should be investigated to determine their suitability for the analysis of polysulfide speciation and to attempt to determine the true physiological distributions of polysulfides.

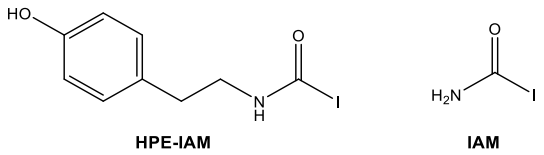


Figure 41: Chemical structure of the electrophile HPE-IAM compared to IAM

It has recently been reported that the enzyme catalase, which is a well-known antioxidant enzyme involved in dismutating H_2O_2 to O_2 and H_2O, is also involved in the metabolism of reactive sulfur species (RSS)[137]. Catalases evolved when metabolism was largely sulfur-based, long before oxygen based metabolism was abundant, so it involvement in RSS metabolism is not a complete surprise. Catalase was shown to be able to catalyse the oxidation of H_2S and H_2S_x under normoxic conditions, but also that it is able to generate H_2S under hypoxic conditions[137]. This forgotten biochemistry of catalase raises questions about similar dismutase enzymes such as superoxide dismutase (SOD)[255]. SOD in fact has since been demonstrated to have functions involving the metabolism of sulfide and polysulfides. In the presence of SOD sulfide is metabolised into polysulfides faster than oxidation without SOD[138] (section 4.4.5). The mechanism by which this metabolism occurs is not yet clear and warrants further investigation. Additionally the biological relevance of this metabolic activity is not yet apparent. Is this a metabolic relic from earlier evolution before the presence of reactive oxygen species (ROS) or is the metabolism of sulfide by SOD still biologically relevant? The activity of what were thought to be solely ROS related enzymes with RSS suggests that some of the biological effects so far attributed to ROS could potentially involve RSS. Further investigations into catalase, SOD and other antioxidant enzymes should shed more light on their roles in sulfur biology and their involvement in the reactive species interactome.

Inorganic polysulfides are intermediates in the synthesis of $SSNO^-$, a key product of the direct interaction of NO with H_2S, and products of its decomposition[10]. The importance of polysulfides in the biological activities of this key species and its interactions with other thiols are not yet fully understood[12,36]. There is potential for collaborative work with other researchers more focused on the properties of the $SSNO^-$ species using the developed methods in Chapter 4: to help characterise the decomposition of $SSNO^-$ and its interactions with other relevant compounds including the small thiols such as cysteine and glutathione.

7.4 Small Polar Metabolites and NO

Supercritical fluid chromatography (SFC) is a promising technique for the analysis of small polar metabolites related to one carbon metabolism and the NO metabolome. Currently only ascorbic acid (Asc) and dehydroascorbic acid (DHA) have been investigated using SFC. Development of a SFC based method would be beneficial for the NO/arginine metabolome as it could provide better separation of the small polar metabolites, whilst likely decreasing the analysis time and even allowing simplified sample preparation techniques to be used. Initial investigations using standard mixtures will need to be undertaken to identify if SFC is suitable for this analysis and to identify an appropriate stationary phase. Currently available SFC specific columns include the Torus series with diol, 2-picolylamine selector, diethylamine selector and aminoanthracene selector stationary phases[360]. Any HPLC or UHPLC column can also be used with SFC including traditional stationary phases such as reversed phase C_{18}, though not specifically designed for the use with SFC. The torus diol column would be a likely candidate for initial method development as this has shown promise for the analysis of Asc and DHA, which are also small polar molecules. If SFC proves to be suitable for the separation and analysis of the mixed metabolite standard the method will then have to be tested in biological samples such as urine, plasma, red blood cells and saliva. Further method validation will also be needed and sample collection and handling procedures established in the same manner as has been undertaken for the thiol and sulfide method (section 3.2.4).

Suitable internal standards for quantification purposes in biological samples will have to be identified. Stable isotope labelled compounds would provide suitable internal standards as long as the mass difference between the labelled and unlabelled compounds is sufficient. It would be unfeasible to have an internal standard for every single compound of interest, especially when using expensive stable isotope labelled compounds. A number of compounds that are representative of the different classes of compounds of interest will have to be selected.

7.5 Ascorbate Redox Analysis

Initial investigations into the analysis of ascorbic acid (Asc) and dehydroascorbic acid (DHA) show good initial separation. Development of the SFC method for the simultaneous analysis of Asc, DHA and AAS was stopped largely due to time constraints on the instrumentation. Whilst a suitable alternative separation method using UHPLC was developed, there would be a benefit in further work into the SFC method to improve the peak shape for DHA as the SFC method provided better separation and faster analysis. Regardless of the separation method used an internal standard would need to be selected for this method for quantification purposes in biological samples, a stable isotope labelled version of either Asc or DHA would be sufficient as long as there is a high enough mass difference between the labelled and unlabelled compounds.

Of a greater concern is the instability of both Asc and DHA. Whilst they are both widely known to oxidise readily at physiological pH after sample collection none of the methods tested to stabilise the samples were successful despite what has been reported in the literature. Additionally it appeared that one or some of the additives tried were causing rapid degradation of the column and so would not be suitable for routine use. Due to these reasons, further investigation into stabilisation of Asc and DHA is required. Since DHA is also unstable, even analysis total ascorbate using a reducing agent such as DTT will not be sufficient (**Figure 40**, **B**), as DHA is irreversibly lost. Derivatisation may be required to stabilise Asc and DHA as with the reduced thiols, which would also likely alter their chromatographic properties as well potentially improving their separation by the UHPLC method. If they are successfully stabilised then method validation will need to be performed to ensure it is suitable for the reliable and accurate analysis of Asc, AAS and DHA. The reproducibility of the method along with the reliability and accuracy of the measurements will have to be investigated. As with the NEM-thiol method, the collection and handling of samples will also have to be considered to ensure the levels of Asc DHA and AAS are not significantly perturbed post sample collection.

7.6 Stable Isotope Tracing

Stable isotope tracing has been widely utilised in tracing NO and arginine metabolism with many well-established methods and does not require significant development (section 1.10). These established protocols can target different parts of the arginine-NO-nitrate pathway by monitoring the conversion of arginine into either citrulline or NO_3^- (**Figure 42**)[215]. One carbon metabolism is another target of stable isotope tracing[273], and forms an important link between the NO and H_2S metabolomes (**Figure 2**, **Scheme 4**). By using a ^{13}C stable isotope label the movement of carbon could be traced through the methionine cycle and identify its direction and flux into and through

the two metabolomes. Creatinine could provide one important metabolic pathway endpoint to monitor the isotope labelling as it considered metabolically inert and is largely excreted in urine (section 1.7.5). Once appropriate methodology has been established stable isotope tracing will then be assessed to determine the most important areas to trace and how best to apply the established method to it.

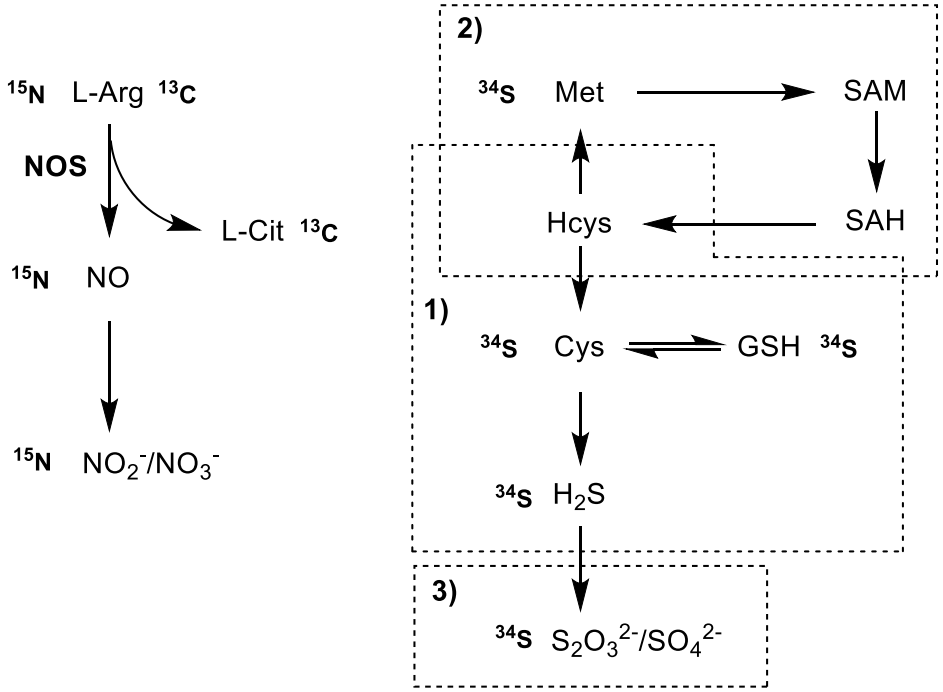


Figure 42: Metabolic pathways for potential targeted stable isotope tracing in the NO and H_2S metabolomes. Arg: arginine, Cit: citrulline, NO: nitric oxide, NO_2^-/NO_3^-: Nitrite/Nitrate, Met: methionine, SAM: S-adenosylmethionine, SAH: S-adenosylhomocysteine, Hcys: Homocysteine, Cys: Cysteine, GSH: Reduced glutathione, H_2S: Hydrogen sulfide, $S_2O_3^{2-}/SO_4^{2-}$: Thiosulfate/Sulfate. Box **1)**: Small thiol metabolism. Box **2)**: The methionine cycle. Box **3)** Thiosulfate/Sulfate, targets for stable isotope tracing using existing methodology.

Stable isotope tracing has not yet been applied to the H_2S metabolome, development of methodology that can make use of stable isotope tracing techniques in sulfide metabolism, similar to those used in the NO metabolome. Stable isotope tracing is needed to fill gaps in the current knowledge of sulfur metabolism including questions relating to the biological activities of reactive sulfur species such as per and polysulfides[36,130]. Using ^{34}S as a tracer for the movement of sulfur through this metabolome would provide an important insight into its importance in biology (**Figure 42**). The use of ^{34}S could also be combined with other stable isotopes such as ^{15}N, ^{13}C and ^{18}O to provide a comprehensive picture of the fluxes through the H_2S metabolome and its crosstalk with NO, reactive oxygen species and the ascorbate metabolome. Sulfate will form an

important endpoint of H_2S metabolism to monitor, as it is one of the final metabolic products of H_2S and is excreted in urine[361] and can be found in saliva[362] providing a good potential point to monitor the endpoint of H_2S metabolism including labelling by ^{34}S.

The stable end products of oxidative sulfide metabolism are thiosulfate ($S_2O_3^{2-}$) and sulfate (SO_4^{2-}) (**Figure 42**, box **3)**). Sulfate is excreted as a metabolic product of sulfide as well as performing some biological functions, and forms a convenient endpoint to monitor sulfur metabolism. Analysis of sulfate could be performed using already established methodology such as ion chromatography mass spectrometry and would not require significant development. The principles of stable isotope tracing techniques, which have been widely utilised in the NO/Arginine metabolome[215], could be applied to the sulfide metabolome. NO metabolism can be traced by using ^{15}N labelled arginine and monitoring either the production of labelled citrulline or the NO oxidation products nitrite and nitrate using existing methods. For sulfide, ^{34}S labelled methionine could be used to trace the metabolism of sulfur through homocysteine to cysteine and glutathione as well as monitoring the production and metabolism of sulfide by analysing the production of labelled sulfate (**Figure 42**) making use of the methodologies developed in this thesis.

Appendix A UHPLC-MS/MS Development

A.1 Mass Spectrometry Settings

Table 9: Additional mass spectrometry settings for the NEM-thiol UHPLC-MS/MS method

Capillary (kV)	2.80
Source Offset (V)	4
Desolvation Temp. (^{o}C)	300
Desolvation Gas Flow (L/h)	800
Cone Gas Flow (L/h)	150
Nebuliser Pressure (Bar)	7.0
Collision Gas Flow (ml/min)	0.14

Appendix B Polysulfides and Persulfides

B.1 Mass Spectrometry Settings

Capillary (kV)	2.80
Source Offset (V)	4
Desolvation Temp. (^{o}C) - **Direct**	100
Desolvation Temp. (^{o}C) - **UHPLC**	300
Desolvation Gas Flow (L/h)	800
Cone Gas Flow (L/h)	150
Nebuliser Pressure (Bar)	7.0
Collision Gas Flow (ml/min)	0.14

B.2 Additional data generated for NEM and IAM concentration series

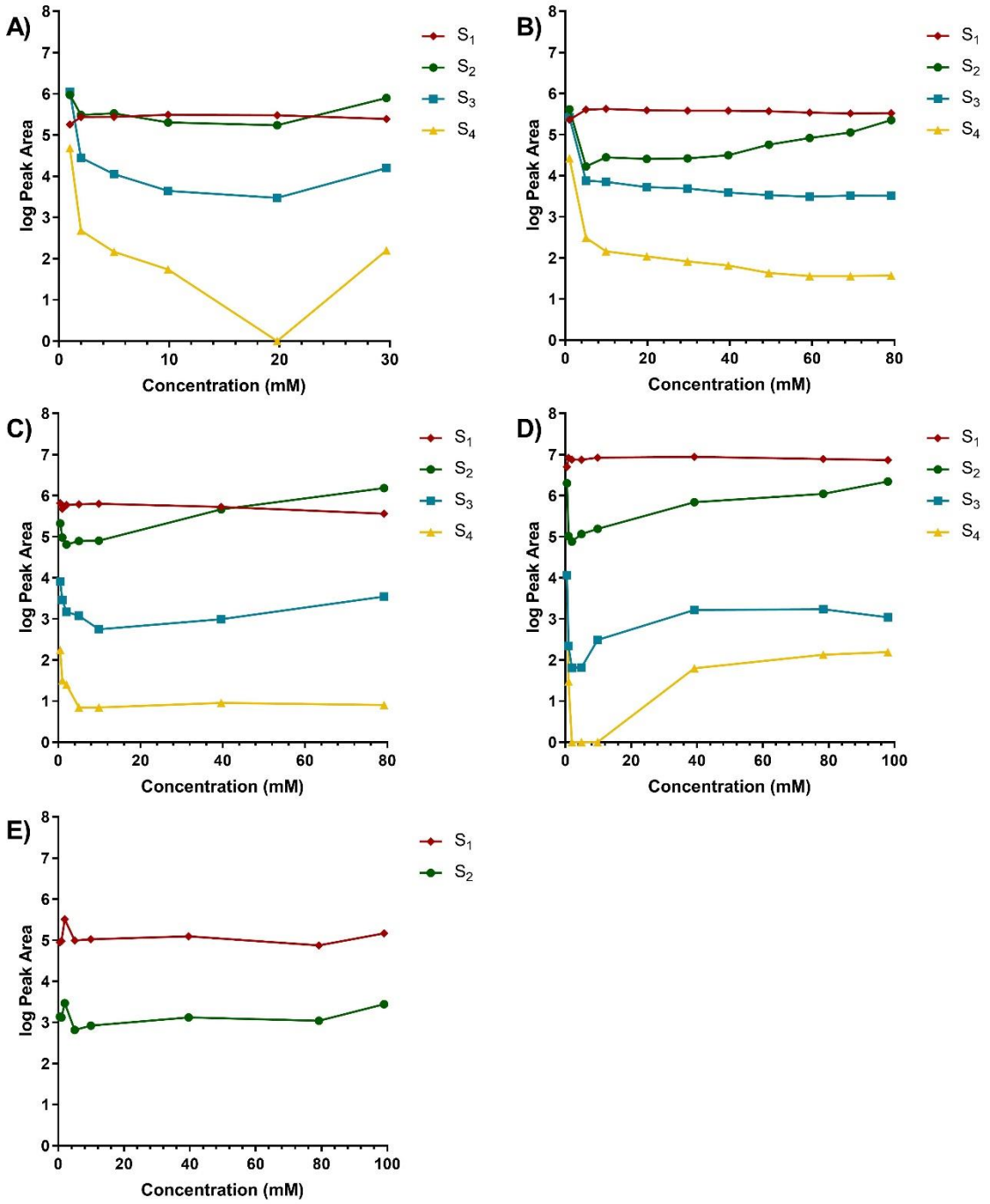


Figure 43: Measured polysulfide speciation with a series of NEM concentrations using different polysulfide salts. **A)** 0.0031 g mixed potassium polysulfide (K_2S_x) in 1 ml H_2O. **B)** 0.0054 g mixed potassium polysulfide (Na_2S_x) in 2 ml H_2O. **C)** 0.0038 g mixed potassium polysulfide (K_2S_x) in 1 ml H_2O. **D)** 0.0018 g sodium trisulfide (Na_2S_3) in 1 ml H_2O (120 µM). **E)** 0.0017 g sodium tetrasulfide (Na_2S_4) in 10 ml H_2O (10 µM).

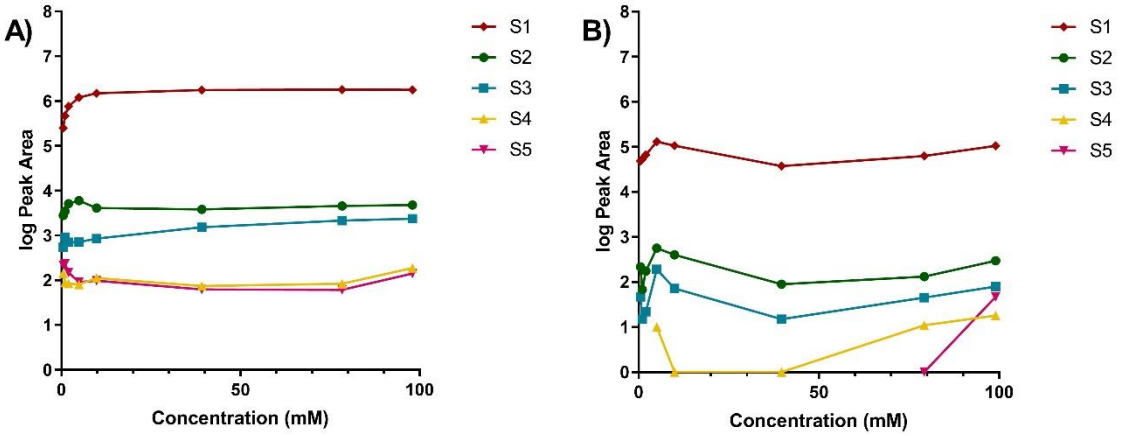


Figure 44: Measured polysulfide speciation with a series of IAM concentrations using different polysulfide salts. **A)** 0.0018 g sodium trisulfide (Na_2S_3) in 1 ml H_2O (120 μM). **B)** 0.0017 g sodium tetrasulfide (Na_2S_4) in 10 ml H_2O (10 μM).

Appendix C Small Polar Metabolites

C.1 UHPSFC Mass Spectrometry Settings

Table 10: Additional mass spectrometry settings for the ascorbic acid UHPSFC-MS/MS method on the Xevo TQD-MS.

Capillary (kV)	3.00
Source Offset (V)	10
Desolvation Temp. (^{o}C)	400
Desolvation Gas Flow (L/h)	800
Cone Gas Flow (L/h)	150
Nebuliser Pressure (Bar)	7.0
Collision Gas Flow (ml/min)	0.14

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