



UNIVERSITÀ
DEGLI STUDI
DI PADOVA

SEDE AMMINISTRATIVA: UNIVERSITÀ DEGLI STUDI DI PADOVA
DIPARTIMENTO DI AGRONOMIA ANIMALI ALIMENTI RISORSE
NATURALI E AMBIENTE - DAFNAE

SCUOLA DI DOTTORATO DI RICERCA IN SCIENZE ANIMALI E
AGROALIMENTARI
INDIRIZZO: PRODUZIONI AGROALIMENTARI
CICLO XXVIII

**Low-molecular-weight thiols: identification of novel thiol
compounds and applications in winemaking processes**

Direttore della Scuola: Ch.ma Prof.ssa Viviana Corich

Coordinatore di Indirizzo: Ch.ma Prof.ssa Viviana Corich

Supervisore: Ch.mo Prof. Antonio Masi

Dottoranda: Marta Fabrega Prats

“To my parents”

ACKNOWLEDGEMENTS

I would like to express my special appreciation and thanks to my supervisor Prof. Antonio Masi and my co-supervisor Prof. Stefano Dall'Acqua for being my tremendous mentors. I would like to thank you for encouraging my research and for allowing me to grow as a research scientist. Your advice on both research as well as on my career have been invaluable.

I would also like to thank to my colleagues and friends Anna Rita Trentin and Sabrina Giaretta for their support either in the good and bad moments during these 3 years and for helping me when it was needed. I would like to thank their advices, availability, help in the lab, and their ears when I needed.

I would also thank to my friends who I met when I reached Italy, you helped me with the Italian language and you were my first friends in an unknown country which was unknown for me. Especially I would like to thank to the other PhD students from Padova University, Paolo Gottardo, Veronica Vendramin and Federica Tinello for their help not only in the statistical analyses, but also in everything I needed.

I gratefully acknowledge to all people I met in Germany, especially Prof. Dr. Doris Rauhut who followed me in this experience. Also special thanks to Roxana Tudorie for being my friend, also to Marga Garcia, Marcus Laier, Jochen Vestner, Heike Semmler and all the people in the Microbiology Laboratory in Hochschule Geisenheim University for helping me in any situation. I would also like to thank to Prof. Ernst Rühl and Dr. Claus-Dieter Patz for the support in my research. And of course, to all the friends and nice people with whom I lived and shared everything during my stage in Geisenheim.

I also acknowledge the funding sources that made my PhD work possible. I was founded by the Cariparo PhD grant (Fondazione Cassa di Risparmio di Padova e Rovigo) during this 3 years. Thanks to this founding support I also met nice people with the same scholarship from other countries and I learnt a lot from them.

My appreciation also extends to Prof. Riccardo Leardi, for his support in the statistics analysis and interpretation. Thanks also go to Purnendu Karmakar for his

corrections in English language and his moral support; and to Gregorio Peron for his support in the MS analyses.

Finally, a special thanks to my family. Words cannot express how grateful I am to my mother and father for all of the sacrifices that you have made on my behalf. Thank you for supporting me for everything. I know I am your only child and you encourage me to leave Spain and to come to Italy to live this experience. I would also to thank to my boyfriend Mauro Lorenzon for being always close to me and for supporting me through this experience. I love you!

Marta Fabrega Prats

Università di Padova

January 2016

CONTENTS

LIST OF ABBREVIATIONS	1
ABSTRACT.....	5
RIASSUNTO	7
CHAPTER 1: GENERAL INTRODUCTION	
“Low-molecular-weight thiols in plants: Functional and analytical implications”	9
Abstract.....	10
1. Introduction.....	10
1.1. Plant sulfur metabolism	11
1.2. Thiol properties	13
1.2.1. Disulfide bonds	13
1.2.2. Enzyme catalytic sites.....	14
1.2.3. Metal coordination	15
1.3. Protein thiolation.....	16
2. LMW thiol separation and detection techniques	17
3. Non-protein LMW thiols in plants.....	26
3.1. Cysteine.....	28
3.2. Homocysteine	30
3.3. Synthesis and degradation: glutathione, γ -glutamylcysteine and cysteinylglycine	31
3.4. Glutathione homologues: homoglutathione and hydroxymethyl-glutathione.....	33
3.5. Cysteamine.....	34
3.6. Phytochelatins	35
3.7. Lipoic acid	36
3.8. Volatile thiols.....	38
3.9. The case of glucosinolates and thioglucose	39
3.10. Diversity of LMW thiol in plants	39
4. Conclusions and future prospects	41
Acknowledgments.....	42
References.....	43
List of Tables	58
List of Figures	59
AIM OF THE STUDY.....	60

CHAPTER II: IDENTIFICATION OF NOVEL COMPOUNDS

“Identification of low-molecular-weight thiols by HPLC-MS/MS analysis of SBD-derivatives”	61
Abstract.....	61
Introduction.....	62
Material and methods.....	63
HPLC analysis from the standards thiols.....	63
HPLC-MS/MS from the standards thiols.....	64
HPLC-MS/MS from the plant extracts	65
Confirmation by qTOF of the unknown molecules	66
Results and discussion	67
HPLC analysis from the standards thiols.....	67
HPLC-MS/MS from the standards thiols.....	69
HPLC-MS/MS from the plant extracts	82
Conclusions.....	92
References.....	93
List of Tables	94
List of Figures	95

CHAPTER III: APPLICATIONS TO WINEMAKING PROCESSES

“Method optimization and quantification of low-molecular-weight thiols in grapes by HPLC-FL detection”	97
Abstract.....	98
Introduction.....	99
Material and methods.....	101
Sampling of the grapes.....	101
Sample preparation	102
Analysis of low-molecular-weight thiols.....	102
Analysis of organic acids and sugars	103
Statistical analysis	103
Results and discussion	104
References.....	112
List of Tables	114
List of Figures	115

CONCLUSIONS.....	116
------------------	-----

LIST OF PUBLICATIONS	117
----------------------------	-----

LIST OF ABBREVIATIONS

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

4-DPS, 4,4'-dithiodipyridine

5-IAF, 5-iodoacetamidofluorescein

6-IAF, 6-iodoacetamidofluorescein

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

AIDS, acquired immune deficiency syndrome

AMS, 4-acetamido-4'-maleimidylstilbene-2,2'-disulfonic acid

ANOVA, Analysis of variance

APR, adenosine-phosphosulfate reductase

ATP, adenosine triphosphate

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

CbL, cystathionine β -lyase

Cgs, cystathionine- γ -synthase

CDNB, 2,4-dinitrochlorobenzene

CMPI, 2-chloro-1-methylpyridinium iodide

CMPT, 2-chloro-4-methoxy-6-(4-(pyren-4-yl)butoxy)-1,3,5-triazine

CMQT, 2-chloro-1-methylquinolinium tetrafluoroborate

Cys, cysteine

Cys-Gly, cysteinylglycine

DBD-F, 4-(N,Ndimethylaminosulfonyl)-7-fluoro-2,1,3-benzoxadiazole

DBPM, N-(p-(2-(6-dimethylamino)benzofuranylphenyl)maleimide)

DHLA, dihydrolipoic acid or reduced lipoic acid

DTNB, 5,5'-Dithiobis-(2-Nitrobenzoic Acid) or Ellman's Reagent

DTT, dithiothreitol

ECG, Glu-Cys-Gly

EDTA, ethylenediaminetetraacetic acid

EFSA, European Food Safety Authority

ESI, electrospray ionization

FL, fluorescence

FM, fluorescein-5-maleimide

γ -Glu-Cys, γ -glutamylcysteine

γ -Glu-Cys-Ser, hydroxymethyl-glutathione

GC, gas chromatography

GC-MS, gas chromatography mass spectrometry

GGCT, γ -glutamylcyclo-transferase

GGT, γ -glutamyl transferase/transpeptidase

GNPs, gold nanoparticles

GRP, grape reaction product

Grx, glutaredoxin

GSH, reduced glutathione

GSH1, γ -Glu-Cys synthetase

GSSG, glutathione disulfide

Hcys, homocysteine

hGSH, homoglutathione or γ -Glu-Cys- β -Ala

HPLC, high performance liquid chromatography

HPLC-MS, high performance liquid chromatography mass spectrometry

IAA, iodoacetic acid

IAM, iodoacetamide

IAB, 3-iodoacetylaminobenzanthrone

LA, lipoic acid or 5-(1,2-dithiolan-3-yl)-pentanoic acid

LC, liquid chromatography

LC-MS, liquid chromatography mass spectrometry

LC-MS/MS, liquid chromatography mass spectrometry

LMW, low-molecular-weight

LOD, limit of detection

LOW, limit of quantification

mBBr, monobromobimane

ME, β -mercaptoethanol

Met, methionine

MIAC, N-(2-acridonyl)maleimide

MIPBO, 5-methyl(2-(m-iodoacetylaminophenyl)benzoxazole

MMTS, S-methyl methanethiosulfonate

MS, mass spectrometry

MW, molecular weight

MWD, variable wavelength detector

NAC, N-acetylcysteine

NAM, N-(9-acridinyl)maleimide

NEM, N-ethylmaleimide

NPR1, nonexpressor of pathogenesis-related protein 1

OPA, ortho-phthalaldehyde

OPHS, O-phosphohomoserine

p-BPB, *p*-bromo phenacyl bromide

PAPS, 3'-Phosphoadenosine-5'-phosphosulfate

PC, phytochelatin

PCA, principal component analysis

PEG-mal, polyethylene glycol maleimides

PPO, polyphenol oxidase

PTM, post-translational modification

QToF, quadrupole time-of-flight mass spectrometer

RID, refractive index detector

ROS, reactive oxygen species

RP-HPLC, reverse phase high performance liquid chromatography

RSD, relative standard deviation

RT, room temperature

RuBisCO, ribulose-1,5-bisphosphate carboxylase/oxygenase

SA, salicylic acid

SAM, S-adenosylmethionine

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

SDS, sodium dodecyl sulfate

SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis

SPE, solid phase extraction

TBP, tributylphosphine

TCEP, tris(2-carboxyethyl)phosphine

TDCI, 1,1'-thiocarbonyldiimidazole

TDDS, turbo data dependent scanning

ThioGloTM3, 9-acetoxy-2-(4-(2,5-dihydro-2,5-dioxo-1H-pyrrol-1-yl)pyeny)-
3-oxo-3H-naphtho[2,1-b]pyran

T&R-MIMS, trap-and-release membrane inlet mass spectrometry

Trx, thioredoxin

UV, ultraviolet

VP, 2-vinylpyridine

ABSTRACT

Thiols are reduced sulphur molecules that occur both in plants and animals with relevant roles. The thiol group is strongly nucleophilic, thus participating in a lot of biological redox processes, as for example the modulation of oxidative stress and participation to enzymatic reactions.

Low-molecular-weight (LMW) thiols are a class of highly reactive compounds mainly involved in the maintenance of the redox homeostasis in the cells, thanks to the reactivity of their nucleophilic sulfur groups. In plants, they are implicated in the responses to almost all stress factors, as well as in the regulation of cell metabolism. Moreover, they can conjugate or make complexes with xenobiotics and toxic compounds (detoxification processes) and deactivate them, and they can post-translationally modify regulatory enzymes. They also have important implications in food quality and safety, as well as in human health. The most studied LMW thiols are glutathione and its biosynthetically related compounds (cysteine, γ -glutamylcysteine and cysteinylglycine). Other LMW thiols are described in the literature, such as cysteamine, homocysteine, and many species-specific volatile thiols but less is known about them. Research shows that exists a huge amount of thiols in plants, but many species-specific and organ-specific thiols remain to be identified. Some of these unknown LMW thiols have light dependence, suggesting that they could be related to the photosynthesis processes.

Recent advances in technology should help in this challenging work, helping to know the physiological and metabolic function in plants. However, their identification remains challenging due to their low concentration in plant tissues.

In order to discover new unidentified thiols, in this work it was carried out a derivatization with SBD-F (ammonium 7-fluoro 2,1,3-benzooxadiazole-4-sulfonate) of the plant extracts, and then, SBD-derivatives were analyzed by HPLC-fluorescence and LC-MS/MS in negative mode using an ion trap (Varian 500 MS), to obtain their fragmentation pattern. Known LMW thiols such as cysteine, homocysteine, glutathione, cysteamine, γ -glutamylcysteine, N-acetylcysteine and cysteinylglycine were used as reference compounds and their fragmentation pattern was first studied in order to highlight a fragmentation rule and molecular markers to systematically identify the

unknown LMW thiols. Also high resolution measurements were obtained on a Xevo G-2 Q-TOF mass spectrometer (Waters).

After the derivatization with the fluorophore, thiols can be easily recognized in fragmentation spectra due to the presence of a clear signal arising from the SBD-S fragment (m/z 231). This fragment corresponds to the fluorophore attached to the sulphur group from the LMW thiol. This signal was then used as a marker to confirm the presence of thiol groups in unknown molecules. In this way, some molecules could be identified and further confirmed by Q-TOF analysis; as for example the presence of thioglucose and glutathione containing derivatives. This protocol now opens the way to the identification of unknown thiol molecules.

In winemaking processes, LMW thiols and specifically GSH, have an antioxidant function, which is present in grapes, must and wine. They help contrasting the oxidative browning by protecting grapes, and also the must during fermentation and the wine during the aging processes. They have a key role in the antioxidant activity by protecting wines, mostly white ones, from the oxidative process during aging. Due to this fact, in this study it was also tried to develop and optimize an easy and fast method to quantify the amount of LMW thiols in several German grape varieties (white and red). These compounds were extracted and analyzed using the fluorescent dye SBD-F and HPLC-FL separation. Also using HPLC, the sugars and organic acids were also quantified.

The results of this quantification show that there is a very good reproducibility either in sampling or in the measurement of these compounds in the grape berries. This method can be also applied in must, wine and yeast. The method allowed not only the quantification of GSH, but also of its related compounds: cysteine, γ -glutamylcysteine and cysteinylglycine in the same chromatogram, showing also the correlation between them. This study on German grape varieties is then showing that GSH is the most important LMW thiol in grapes, whose content is largely depending on the variety. Given their role as an antioxidant and possible beneficial effects during the winemaking processes, GSH and related LMW thiols are an important factor to consider in the evaluation of the grapes used for making wine.

RIASSUNTO

I tioli sono composti ridotti dello zolfo che svolgono importanti funzioni in animali e piante. Il gruppo -SH è fortemente nucleofilo, per tale motivo queste molecole sono spesso coinvolte in processi biologici di ossidoriduzione, come ad esempio la modulazione degli stress ossidativi e la partecipazione a varie reazioni enzimatiche.

I tioli a basso peso molecolare (LMW) sono una classe di composti coinvolti principalmente nel mantenimento dell'omeostasi ossidoriduttiva nella cellula, tale caratteristica si deve alla reattività dei loro gruppi tiolici nucleofili. Nelle piante sono coinvolti nella risposta a quasi tutti i fattori di stress e nella regolazione del metabolismo cellulare. I tioli LMW possono legarsi o creare complessi con composti tossici disattivandoli (detossificazione), inoltre possono modificare, dopo la traduzione, enzimi regolatori. Queste molecole sono implicate nella qualità e salubrità degli alimenti e anche nella salute umana.

I tioli LMW più studiati sono il glutatione e i suoi composti derivati (cisteina, γ -glutamyl-cisteina e cisteinil-glicina). In letteratura sono stati descritti altri tioli LMW come la cisteammina, l'omocisteina e molti altri tioli volatili specie-specifici di cui però poco è conosciuto. In particolare, nelle piante è dimostrata la presenza di moltissimi tioli specie-specifici e organo-specifici molti dei quali però non sono ancora stati identificati. Alcuni di questi tioli LMW sconosciuti sono luce-dipendenti e ciò suggerisce un loro coinvolgimento nel processo della fotosintesi.

I miglioramenti nella tecnologia potrebbero aiutare lo studio e la conoscenza della funzione fisiologica e metabolica di questi composti. Tuttavia la loro identificazione è resa ardua dalla loro bassa concentrazione nei tessuti vegetali.

In questo lavoro, allo scopo di scoprire nuovi tioli non ancora identificati, sono stati derivatizzati con SBD-F degli estratti di piante e in seguito i derivatizzati sono stati sottoposti ad analisi HPLC a fluorescenza e LC-MS/MS in modalità negativa usando una trappola ionica (Varian 500 MS) per ottenere la frammentazione. Sono stati usati come riferimento i tioli LMW già noti come la cisteina, l'omocisteina, il glutatione, la cisteammina, la γ -glutamyl-cisteina, l'N-acetilcisteina e la cisteinil-glicina, i cui modelli di frammentazione sono stati inizialmente studiati per evidenziare la modalità di

frammentazione e i marcatori molecolari che hanno consentito la identificazione sistematica di tioli LMW sconosciuti. Inoltre è stata ottenuta la misurazione ad alta risoluzione su spettrometro di massa Q-ToF (Waters) su Xevo G-2.

Dopo la derivatizzazione con fluoroforo i tioli possono essere facilmente riconosciuti dallo spettro di frammentazione per la presenza di un chiaro segnale dato dal frammento SBD-S (m/z 231). Questo frammento corrisponde al fluoroforo legato al gruppo tiolico, ed è stato usato per marcare e confermare la presenza del gruppo tiolico nelle molecole sconosciute. In questo modo le molecole possono essere identificate poi confermate dall'analisi Q-ToF come nel caso del tioglucosio e di derivati contenenti glutatione. Il protocollo che è stato definito con il presente lavoro permette ora l'identificazione di nuovi composti dello zolfo al momento sconosciuti.

Nel processo di produzione del vino, i tioli LMW e il glutatione in particolare, hanno una importante funzione antiossidante che si manifesta nell'uva, nel mosto e nel vino. I tioli contribuiscono a contrastare l'imbrunimento ossidativo delle proteine nell'uva e soprattutto nel mosto durante la fermentazione e del vino nei diversi processi di lavorazione. I tioli giocano perciò un ruolo chiave nella conservazione del vino con la loro attività antiossidante, in particolar modo nei vini bianchi. Per questa ragione è stato condotto uno studio volto a sviluppare una metodologia semplice e rapida per la quantificazione dei tioli LWM totali. In tale studio sono state poste a confronto diverse varietà tedesche di uva da vino. I composti dello zolfo sono stati estratti ed analizzati utilizzando un colorante fluorescente SBD-F e separazione HPLC-FL. Sono stati quantificati anche gli zuccheri e gli acidi organici tramite HPLC.

I risultati di questa quantificazione mostrano un'elevata riproducibilità tra i campioni e tra le misurazioni di questi composti nelle bacche. Il metodo è utilizzabile anche su mosto, vino e lieviti. Il metodo permette non solo la quantificazione del glutatione ma anche di altri composti relativi nello stesso cromatogramma e mostra una correlazione tra di essi. Il confronto di varietà differenti mostra la presenza di GSH nella maggior parte dei tioli dell'uva, mentre il loro contenuto è molto influenzato dalla varietà. In considerazione del loro ruolo di antiossidanti, il GSH e i tioli LMW possono avere un ruolo favorevole nella vinificazione e sono perciò un fattore fondamentale che deve essere preso in considerazione nelle decisioni che riguardano il processo di produzione del vino.

CHAPTER 1

GENERAL INTRODUCTION

“Low-molecular-weight thiols in plants: Functional and analytical implications”

Micaela Pivato, **Marta Fabrega-Prats**, Antonio Masi*

Archives of Biochemistry and Biophysics 2014 Oct; 560:83-99.

doi: 10.1016/j.abb.2014.07.018

Low-molecular-weight thiols in plants: functional and analytical implications

Micaela Pivato, **Marta Fabrega-Prats** and Antonio Masi*
DAFNAE University of Padua, Viale dell'Università 16, 30520 Legnaro (PD), Italy

Keywords: cysteine; glutathione; redox; sulfur; thiolation; thiols

Abstract

Low-molecular-weight (LMW) thiols are a class of highly reactive compounds massively involved in the maintenance of cellular redox homeostasis. They are implicated in plant responses to almost all stress factors, as well as in the regulation of cellular metabolism. The most studied LMW thiols are glutathione and its biosynthetically related compounds (cysteine, γ -glutamylcysteine, cysteinylglycine, and phytochelatins). Other LMW thiols are described in the literature, such as thiocysteine, cysteamine, homocysteine, lipoic acid, and many species-specific volatile thiols. Here, we review the known LMW thiols in plants, briefly describing their physico-chemical properties, their relevance in post-translational protein modification, and recently-developed thiol detection methods. Current research points to a huge thiol biodiversity in plants and many species-specific and organ-specific thiols remain to be identified. Recent advances in technology should help researchers in this very challenging task, helping us to decipher the roles of thiols in plant metabolism.

1. Introduction

Reduced sulfur is contained in several biomolecules of living organisms, especially in proteins such as methylated in methionine (Met) and as thiol in cysteine (Cys), but there are probably hundreds of non-protein molecules constituting the sulfur metabolome (Gläser *et al.*, 2014). In the past, it was assumed that about 2% of the organic reduced sulfur in plants occurs in the form of non-protein, low-molecular-weight (LMW) thiols (Rennenberg *et al.*, 1993). The thiol moiety is one of the strongest nucleophilic groups in the cell. It is involved in a number of chemical reactions that give thiol-containing molecules a primary role in cellular redox homeostasis, in controlling enzyme activity and detoxifying reactive oxygen/nitrogen species and xenobiotics, as well as in the formation of disulfide bonds needed to define the structural characteristics and

regulatory properties of proteins (Haugaard, 2000). The main cellular LMW thiols are Cys and glutathione (GSH). Cys occupies a key position on numerous metabolic pathways, and it is generally found at low concentrations because it is rapidly converted into other compounds or incorporated in proteins (Pilon-Smits and Pilon, 2006). GSH is the most abundant and best described LMW thiol in both plants and animals because of its importance in redox and regulatory functions.

This review focuses on describing the known LMW thiols in plants, including a number of less well-known compounds that are generally neglected, but play a significant part in plant metabolism. The distinction between plant and animal thiols is necessary for two reasons: first, the metabolic pathways differ considerably, given that animals are unable to assimilate inorganic sulfur and produce Cys from Met, as happens in plants; and second, there are different thiols in the two kingdoms (for example, phytochelatins are a group of LMW thiols peculiar to plants). This work therefore focuses only on plant LMW thiols, although they have many chemical characteristics and functional properties in common with animal thiols.

1.1 Plant sulfur metabolism

Sulfur is an element essential to plant primary metabolism, as a structural component of proteins and lipids, some vitamins and regulatory molecules, antioxidants, metal-binding molecules and cofactors/coenzymes (Pilon-Smits and Pilon, 2006). Plants take up inorganic sulfur mainly from soil in the form of anionic sulfate (SO_3^{2-}), and specific transporters actively carry it to their leaves (Saito, 2004). Anionic sulfate can become a vacuolar sap component as it is, or be fixed to organic molecules after reduction reactions involving a class of ATP sulfurylases and APS reductases (Leustek, 2002). Sulfite (SO_3^-) can be added to an organic molecule by means of the sulfation reaction, or it can be further reduced to sulfide (S^{2-}), and subsequently fixed to Cys, and thus enter a variety of synthesis pathways (Leustek and Saito, 1999). SO_4^{2-} is reduced to sulfide (S^{2-}) in the chloroplast as a result of the addition of eight electrons derived from photosynthesis in a multistep pathway that requires one ATP (Saito, 2004). On the other hand, the sulfation reaction is catalyzed mainly in the cytosol by specific sulfotransferases that covalently add the sulfonate group from 3'-phosphoadenosine 5'-phosphosulfate (known as PAPS) to a hydroxyl group of an organic molecule. Sulfotransferases are involved in

(common in higher plants), and the gaseous plant hormone ethylene (Roj, 2006). SAM is also the methyl donor for a variety of macromolecules, including proteins, nucleic acids and polysaccharides (Brzezinski *et al.*, 2008).

GSH is considered the most important LMW thiol in plants because of its pivotal role in sulfur metabolism as the preferred molecule for storing reduced sulfur. It can move through xylem and phloem fluids, so it is involved in long-distance sulfur transport between organs. It can be rapidly converted into Cys or other compounds and it is used to protect the cell from oxidative stress, detoxify xenobiotics, and regulate protein function. It is also the precursor of phytochelatins (PCs) and other molecules involved in plant signaling and regulation (see sections 3.3 and 3.6).

Met and GSH, together with other sulfate compounds such as sulfolipids, are considered the cell's source of sulfur and, if necessary, they are converted by specific enzymes into Cys, to return part of sulfur metabolism.

1.2. Thiol properties

The thiol group mainly occurs in cells as an amino acid side chain moiety of Cys, the main product of plant sulfur assimilation. In addition to being a component of thiol-containing proteins and LMW thiol compounds, the amino acid Cys is a crucial metabolite for the synthesis of sulfur-containing molecules like Met, some vitamins (e.g. thiamine and biotin), lipoic acid (LA) and coenzyme A.

At physiological pH, Cys residues are protonated, but sub-locally higher pH levels and polar or basic amino acids nearby can reduce Cys pKa, deprotonating the thiol, and the resulting thiolate anion is one of the strongest biological nucleophiles in the cell (Dalle-Donne *et al.*, 2007; Colville and Kranner, 2010). As a result, Cys is more reactive and is involved in a series of redox chemical reactions that enable it to have both structural properties and functional activities as part of the catalytic sites of different classes of enzymes (Jacob *et al.*, 2003).

1.2.1 Disulfide bonds

Disulfide bonds could form both from the thiolate anion, catalyzed by specific enzymes, and spontaneously, generally through an oxidized intermediate (sulfenic acid, -SOH) (Bindoli *et al.*, 2008). Disulfide bond formation is generally induced by

nucleophilic substitution, which can often involve oxidoreductive interchange mechanisms between reducing equivalents of Cys and other compounds (FADH₂, NADPH, GSH or Cys residues of proteins) (Jacob *et al.*, 2003; Bindoli *et al.*, 2008).

Stable disulfide linkages between Cys exert fundamental structural functions in protein folding: intra-molecular disulfide bonds stabilize the protein's tertiary structure, improving rigidity (e.g. loop formation), whereas inter-molecular disulfide bonds between different polypeptide chains support the protein's quaternary structure. For instance, the 11S legumin storage proteins, which include glycinin, are non-covalent hexamers whose monomers consist of two different subunits linked by disulfide bonds (Shewry *et al.*, 1995). Disulfide bonds are also needed for the oligomerization of covalent dimer proteins (homo- or heterodimers), like some heat shock proteins and chaperones, or for higher-level oligomerization (e.g. transcriptional factors important to plant immunity such as the nonexpressor of pathogenesis-related protein 1, NPR1) (Spadaro *et al.*, 2010).

Reversible disulfide linkages on reactive Cys (with a lower pK_a) form the basis of cellular redox maintenance and participate in regulating enzyme activity. LMW thiols spontaneously bind to proteins to protect reactive Cys from reactive oxygen species (ROS) attack under conditions of stress. Protein adducts with GSH and cysteinylglycine (Cys-Gly) could also have regulatory functions (see section 1.3) (Corti *et al.*, 2005; Dalle-Donne *et al.*, 2007).

Two complex families of regulatory enzymes, thioredoxins (Trx) and glutaredoxines (Grx), exert their function by catalyzing Cys thiol/disulfide exchanges. They enable the reduction of protein substrates, and are in turn regenerated exchanging reducing equivalents with NADPH (Trx) and GSH (Grx). A huge number of enzymes provide the substrate for their action (e.g. storage proteins, transcriptional factors, ribonucleotide reductases, etc.), so a number of cellular pathways are regulated via thiol/disulfide mechanisms, including photosynthesis, seed germination, Cys metabolism, and others (Buchanan *et al.*, 1994; Yano, 2014).

1.2.2. Enzyme catalytic sites

Reactive Cys are the catalytic site of a number of enzymes involved in redox reactions, such as oxidases, peroxidases, reductases and dehydrogenases. Jacob and colleagues have thoroughly described the chemical mechanisms defining these reactions

(Jacob *et al.*, 2003). Briefly, depending on the composition of the amino acid environment at reactive sites, Cys can catalyze thiol/disulfide exchanges (as already mentioned for Trx and Grx), and also electron transfer and hydrogen atom transfer reactions. These reactions are implicated in countless processes, ranging from the maintenance of redox homeostasis to energy production.

For example, in the enzyme glyceraldehyde 3-phosphate dehydrogenase, the thiolate anion conducts a nucleophilic attack on the carbonyl group of aldehyde, forming a tetravalent thioether that readily facilitates the progress of hydride transfer. The nucleophilic attack on carbonylic carbons is also the first step in the action of lipases and proteases that contain Cys at their active site. In plants, different classes of Cys proteases are involved in biotic and abiotic stress responses, programmed cell death, and storage protein deposition and degradation (Grudkowska and Zagdańska, 2004). The same catalytic reactions are also employed by acyl-acyl carrier thioesterases involved in the synthesis of fatty acids in plants (of primary interest because they are related to seed lipid production) (Yuan *et al.*, 1995).

1.2.3. Metal coordination

As a thiolate anion, Cys coordinates protein-metal binding with a number of physiological metal ions, such as Fe, Zn, Cu, as well as xenobiotic Co, Ag, Cd and Hg, but it is unable to interact with group 1 and 2 metal ions (Jacob *et al.*, 2003). The relevance of this capability should be glaring, given that one in two known proteins are believed to contain metal cofactors (Dudev, 2014), and the processes that involve metalloproteins include photosynthesis, respiration, signal transduction, epigenetic processes and many others. Cys-metal ion interaction can have both structural (e.g. zinc finger proteins) and functional roles (e.g. metalloproteases).

To give some examples, ferredoxins are iron-sulfur proteins that mediate electron transfer in a variety of reactions involved in a number of molecular pathways (photosynthesis, chlorophyll synthesis, and others) (Hanke and Mulo, 2013). Metallothioneins form a family of Cys-rich proteins involved in metal transport, storage and detoxification of non-essential metals or excessive amounts of essential metals (Hassinen *et al.*, 2010). Together with PCs (see section 3.6), they chelate cytosolic metals in cases of excessive heavy metal load.

1.3. Protein thiolation

PTMs on Cys residues represent the major and most significant redox alterations in plant cells. The physiological relevance of PTMs is underscored by their variety and by the reversibility of most of the chemical reactions involved. As discussed above, the reactivity of the thiol group means that Cys can undergo a number of different redox reactions and result in disulfide bonds, sulfenic acids (S-OH) and further states of oxidation to sulfinic and sulfonic acids (-SO₂H and -SO₃H), sulfhydryl moieties (S-SH), nitrosothiols (S-NO), and, less commonly, S-sulphenyl-amides (S-N) and thiosulfates (SO-S) (Couturier *et al.*, 2013).

One of the Cys redox PTMs involves the formation of disulfide bonds with LMW thiols, known as protein thiolation. The term has often been used inappropriately as a synonym for protein glutathionylation (the formation of protein-GSH adducts) - the best-documented protein-LMW thiol linkage - but binding to Cys-Gly, called protein cysteinylglycylation, and to free Cys (protein cysteinylation) have also been reported and belong to the thiolation PTMs.

Glutathionylation occurs as a defense mechanism in response to oxidative stress, enabling the cell to protect the Cys residues particularly prone to oxidation (i.e. the “reactive” Cys, with low pK_a) from being irreversibly oxidized. It also occurs in physiological conditions as a regulatory mechanism and signaling process (Dalle-Donne *et al.*, 2007; Jozefczak *et al.*, 2012). Glutathionylation modulates cellular life cycle processes (division, differentiation, programmed cell death), energy metabolism and glycolysis, protein folding and degradation, pathogen resistance, certain stages of plant development (rhizobia symbiosis, seed maturation, desiccation), and many other processes (Rhaza *et al.*, 2003; Ogawa, 2005; Dalle-Donne *et al.*, 2009; Pastore and Piemonte, 2012; Frendo *et al.*, 2013). Protein thiol-GSH adducts form spontaneously, but can be catalyzed by Grx as well, which also has the capacity to revert the reaction to GSH and reduced Cys.

A recently-released database dedicated to glutathionylation, dbGSH (Chen *et al.*, 2014), integrates the available datasets on experimentally verified glutathionylation sites (mapped as UniProtKB entries) and provides structural and functional analytical tools and links to the online literature. As at December 2013, the dbGSH counted more than 2200 experimentally verified S-glutathionylated proteins, most of them murine (1128) or

human (1008). Only 12 proteins are reported for *Arabidopsis thaliana* and a few other plant species, despite the plethora of studies asserting the relevance of glutathionylation in plant biology. This is probably attributable to the fact that the database only contains experimentally verified glutathionylated sites and the related peptides, detectable by means of advanced mass spectrometry analyses on PTMs (see section 2), and impossible to identify using indirect methods that detect free LMW thiols after protein reduction. There will presumably be more proteomic studies on thiolation PTMs in plant organisms in the future, and it is reasonable to expect many of the glutathionylated proteins found in mammals to be modified in plants too.

Cysteinylation and cysteinylglycylation have been reported in bacteria and mammals, and the available data strongly suggest their involvement in regulating cell metabolism, like glutathionylation. In *Salmonella typhimurium*, cysteinylation occurs preferentially under infection-like conditions on the same residues where glutathionylation takes place in the basal physiological state (Ansong *et al.*, 2013). In human plasma, linkages to Cys and Cys-Gly seem to be more abundant than glutathionylation as thiolation PTMs in globulins and albumin (Hortin *et al.*, 2006). To our knowledge, these modifications have yet to be reported in plants, though the existence of these PTMs cannot be ruled out.

2. LMW thiol separation and detection techniques

It is important to identify and quantify LMW thiols in plants to shed light on their biological function and metabolism. Several methods have been developed for this purpose, generally based on five main steps: i. extraction; ii. reduction; iii. derivatization; iv. separation; and v. detection. LMW thiols are found in cell as free, soluble thiols that can be reduced and oxidized (e.g. GSH and GSSG), or linked to proteins. The above steps are adapted to suit the aims of a given study, i.e. different experimental flowcharts are used if researchers are interested in profiling the total LMW thiol content, or quantifying the redox state of free thiols, or characterizing thiolation PTMs under specific cellular conditions and the amino acid position where the modifications occur (see schematic overview in Figure 2).

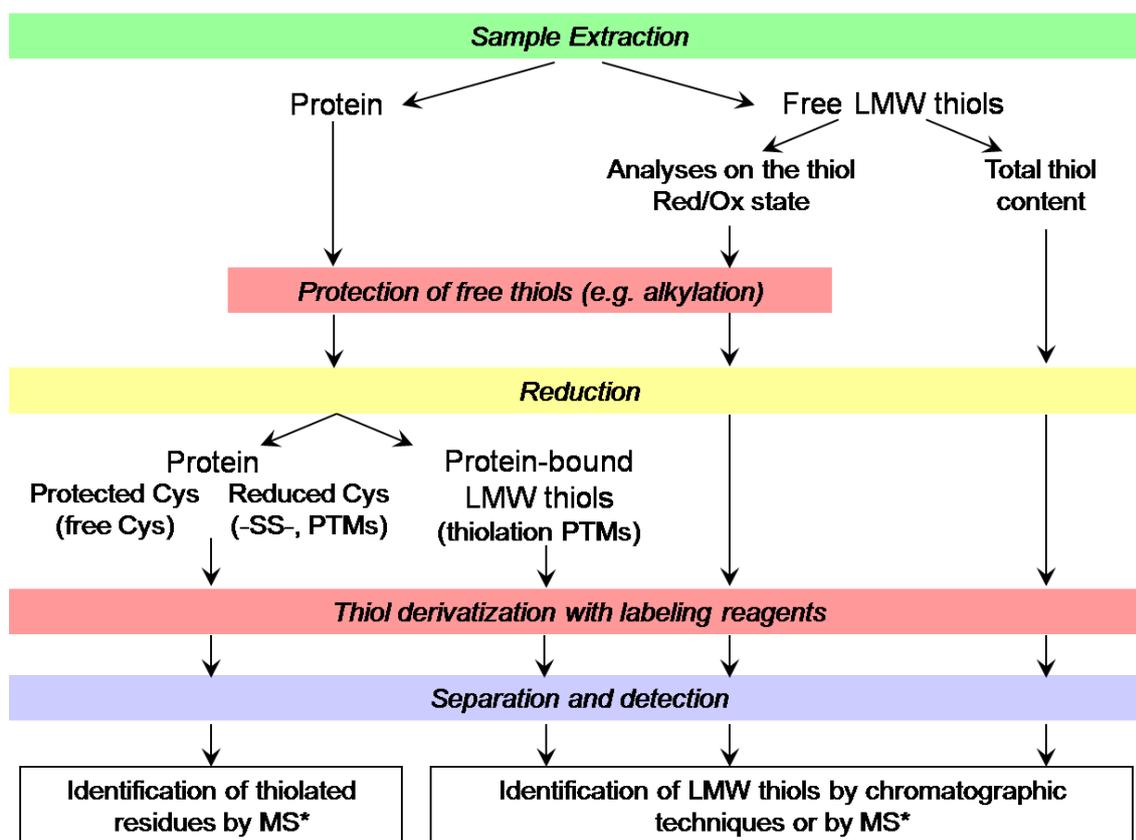


Figure 2. Flow chart of sample preparation and analysis. Given the experimental aims, five main steps are needed to study thiolation PTMs and total thiol content, or to assess the thiol redox state (extraction, reduction, derivatization, separation and detection).

* Free thiols can be identified by MS analysis (both GC- and LC-MS) without any derivatization.

i. Extraction

Free thiols are usually extracted in an acidic environment to protonate the -SH groups. The most often used solutions contain chlorhydric, perchloric, sulfosalicylic or metaphosphoric acid. The acidic conditions induce the precipitation of the proteins, which can then be separated from the free thiols with a simple centrifugation step. To study thiolation, the protein pellet is resuspended with detergents (e.g. SDS, Tween) or other solubilizing agents (e.g. guanidine) for further analysis.

ii. Reduction

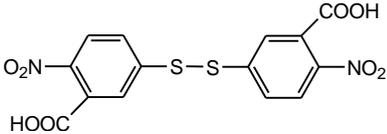
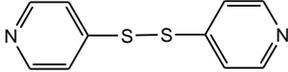
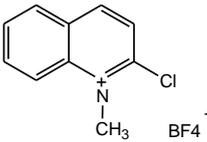
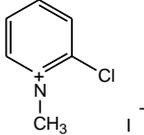
To be detectable, thiols must be reduced prior to any subsequent modification steps. The most frequently used reducing reagents are thiol-containing reductants such as the monothiol β -mercaptoethanol (ME) and the dithiol dithiothreitol (DTT), but they

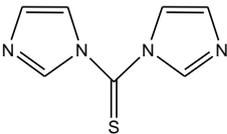
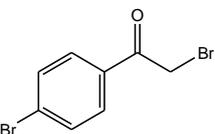
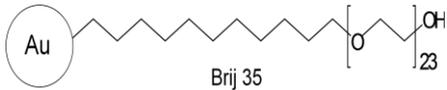
demand an additional removal step (e.g. by gel filtration) to prevent any interference with the derivatization agents. Phosphines are currently preferred in order to avoid this removal step, since they do not usually participate in further reactions. Some examples of phosphines are tris-(2-carboxyethyl)phosphine (TCEP) and tributylphosphine (TBP) (Hansen and Winther, 2009).

iii. Derivatization

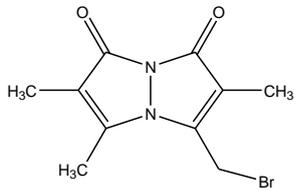
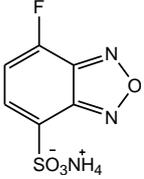
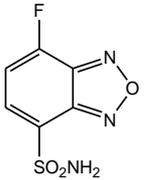
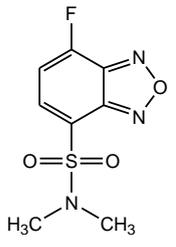
Derivatization consists in the chemical labeling of reduced thiols with compounds that enable their detection. All these reagents prompt an irreversible thiol-disulfide exchange reaction that results in an increase in the thiol molecular mass, and most of them carry a chromogenic or fluorescent moiety for the purpose of detection using spectrophotometric techniques. For mass spectrometry (MS), a labeling step is not strictly necessary, but alkylating reagents are commonly used to distinguish reduced from oxidized thiols. Table 1 lists some commonly-used derivatization reagents.

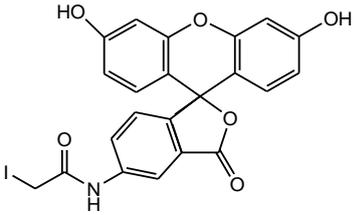
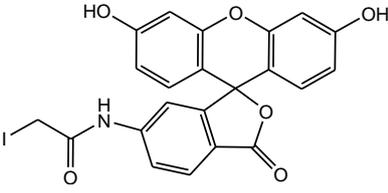
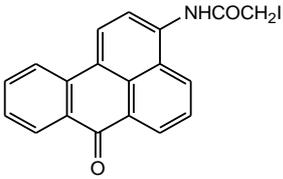
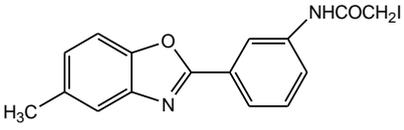
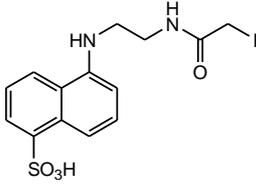
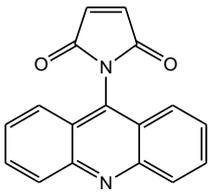
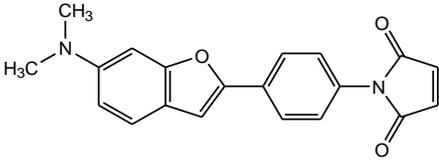
Table 1. Commonly used derivatizing reagents, together with their chemical structure and references.

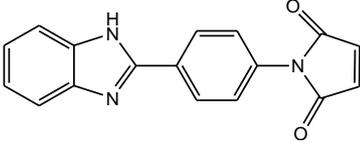
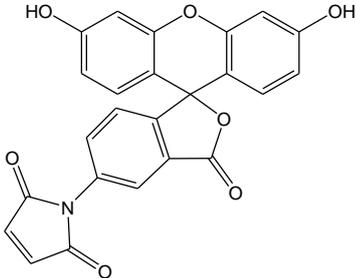
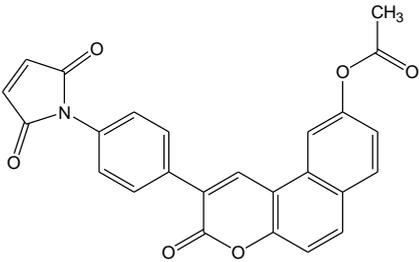
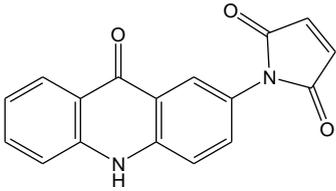
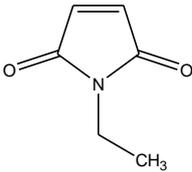
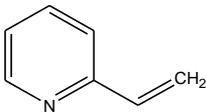
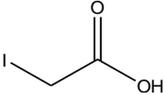
UV Reagents (HPLC-UV)			
Category	Compound	Structure	Reference
Aromatic disulfides	DTNB (5,5'-dithio-bis-(2-nitrobenzoic acid) or Ellman's reagent)		Ellman (1959)
	4-DPS (4,4'-dithiodipyridine)		Grasseti and Murray (1967)
Others	CMQT (2-chloro-1-methyl quinolinium tetrafluoroborate)		Bald and Glowacki (2001)
	CMPI (2-chloro-1-methylpyridinium iodide)		Kaniowska <i>et al.</i> (1998)

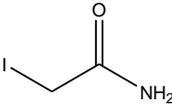
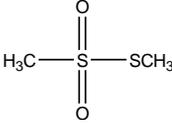
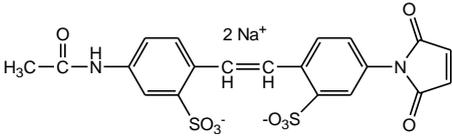
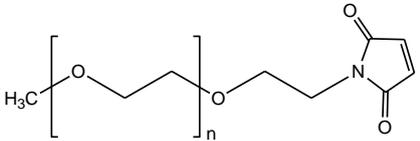
	TDCI (1,1'-thiocarbonyldiimidazole)		Amarnath and Amarnath (2002)
	<i>p</i> -BPB (<i>p</i> -bromo phenacyl bromide)		Huang <i>et al.</i> (2006)
	GNPs (Gold nanoparticles)		Lu <i>et al.</i> (2007)

Fluorescent Reagents (HPLC-FL)

Category	Compound	Structure	Reference
Bimanes	mBBr (monobromobimane)		Fahey and Newton (1987)
Halogeno benzofurazans	SBD-F (ammonium 7-fluoro 2,1,3-benzoxadiazole-4-sulfonate)		Oe <i>et al.</i> (1998)
	ABD-F (4-aminosulfonyl-7-fluoro-2,1,3-benzoxadiazole)		Toyo'oka and Imai (1984)
	DBD-F (4-(<i>N,N</i> dimethylaminosulfonyl)-7-fluoro-2,1,3-benzoxadiazole)		Toyo'oka <i>et al.</i> (1989)

Halides	5-IAF (5-iodoacetamidofluorescein)		Carru <i>et al.</i> (2004)
	6-IAF (6-iodoacetamidofluorescein)		Causse <i>et al.</i> (2000)
	IAB (3-iodoacetylaminobenzanthrone)		Wang <i>et al.</i> (2004)
	MIPBO (5-methyl(2-(m-iodoacetylaminophenyl)benzoxazole)		Liang <i>et al.</i> (2005)
	1,5-I-AEDANS (5-({2-[iodoacetyl]amino}ethyl)aminonaphthalene-1-sulfonic acid)		Clements <i>et al.</i> (2005)
Maleimides	NAM (<i>N</i> -(9-acridinyl)maleimide)		Akasaka <i>et al.</i> (1986)
	DBPM (<i>N</i> -(<i>p</i> -(2-(6-dimethylamino)benzofuranyl)phenyl)maleimide))		Nakashima <i>et al.</i> (1985)

	BIPM (<i>N</i> -[<i>p</i> -(2-benzimidazolyl)phenyl]maleimide)		Kanaoka <i>et al.</i> (1970)
	FM (fluorescein-5-maleimide)		Reddy <i>et al.</i> (1998)
	ThioGlo TM ₃ (9-acetoxy-2-(4-(2,5-dihydro-2,5-dioxo-1H-pyrrol-1-yl)phenyl)-3-oxo-3H-naphtho[2,1-b]pyran)		Yang and Langmuir (1991)
	MIAC (<i>N</i> -(2-acridonyl)maleimide)		Benkova <i>et al.</i> (2008)
Alkylating Reagents			
Compound	Structure	Reference	
NEM (N-ethylmaleimide)		Gregory (1955)	
VP (2-vinylpyridine)		Griffith (1990)	
IAA (iodoacetic acid)		Reed <i>et al.</i> (1980)	

IAM (iodoacetamide)		Maeda <i>et al.</i> (2005)
MMTS (S-methyl methanethiosulfonate)		Laszlo and Mathy (1984)
Heavy maleimide derivatives for SDS-PAGE (shift in mobility)		
Compound	Structure	Reference
AMS (4-acetamido-4'-maleimidylstilbene-2,2'-disulfonic acid) disodium salt		Joly and Swartz (1997)
PEG-mal (polyethylene glycol maleimides): Methoxypolyethylene glycol maleimide		Goodson and Katre (1997)

Examples of labeling reagents for UV detection are DTNB (5,5'-dithio-bis-(2-nitrobenzoic acid)), also known as Ellman's reagent (Ellman, 1959), 4-DPS (a similar reagent), CMPI (2-chloro-1-methylpyridinium iodide), and CMQT (2-chloro-1-methylquinolinium tetrafluoroborate) (Peng *et al.*, 2012). DTNB and 4-DPS are aromatic disulfide compounds, so any reducing reagent must be removed before labeling. A post-column HPLC-UV detection method for detecting LMW thiols has also been developed based on the aggregation of gold nanoparticles functionalized with nonionic surfactant (Lu *et al.*, 2007).

By comparison with UV detection, derivatization with fluorescent dyes is more sensitive and thiol-selective, and it can be done using a variety of reagents (Chen *et al.*, 2010). Monobromobimane (mBBr) has been amply used both for quantifying LMW thiols and for analyzing thiol-containing proteins (Fahey and Newton, 1987; Hansen and Winther, 2009). Other fluorescent derivatives are the benzofurazans SBD-F (ammonium 7-fluoro 2,1,3-benzoxadiazole-4-sulfonate) and ABD-F (4-aminosulfonyl-7-fluoro-2,1,3-benzoxadiazole) (Toyo'oka, 2009). The great advantage of derivatizing with benzofurazans instead of mBBr lies in that they emit light only when linked to thiols,

whereas mBBr has a weak fluorescence of its own that gives rise to system peaks on chromatograms. In addition, with benzofurazans there is no need to remove excess reductants, while mBBr has the drawback of reacting with both phosphines and thiol-based reductants. It is worth noting that SBD-F labeling needs a higher pH and temperature (60°C), and longer incubation times (1h), whereas ABD-F reacts at room temperature and within 10 min at pH 8 (Winther and Thorpe 2014). 5-IAF (5-iodoacetamidofluorescein) is a halide that quickly reacts with thiols at room temperature at pH 12.5. It is used in the capillary electrophoretic analysis of various thiols (Musenga *et al.*, 2007). Other fluorescent derivatives are listed in Table 1.

To analyze the different redox states of LMW thiols, an additional alkylation step is usually needed before reducing and derivatizing the oxidized thiols. Briefly, reduced thiols are irreversibly alkylated with N-ethylmaleimide (NEM) (Gregory, 1955), 2-vinylpyridine (VP) (Lindorff-Larsen and Winther, 2000), or iodoacetic acid (IAA) and iodoacetamide (IAM) (Zander *et al.*, 1998). Excess alkylating agent is then removed to avoid any alkylation of newly-reduced thiols, and this is usually done by phase separation with ethers (e.g. petrol or diethyl ether), or by acid precipitation. The sample containing the oxidized thiol is then reduced and derivatized again using a different labelling strategy. Then the total and oxidized thiols are measured, and the reduced fraction is obtained by subtraction. Alternatively, when studying aminothiols (such as Hcys, GSH and GSH homologs), the oxidized and reduced thiol can also be detected simultaneously, without the reducing step, by using a non-thiol reagent like OPA (ortho-phthalaldehyde), specific for primary amines. For example, when studying GSH and GSSG simultaneously, the sample can be labeled first with NEM (reacting with reduced GSH to prevent further oxidation during manipulation), and then with OPA (reacting with both GSH and GSSG) (Kand'ár *et al.*, 2007; Toyo'oka, 2009).

iv. Separation

Labeled LMW thiols are separated using two different strategies, electrophoresis (capillary and two-dimensional) (Eaton, 2006; Chen *et al.*, 2010) or, more often than not, chromatography. The most commonly-used technique involves high-performance liquid chromatography (HPLC) separation followed by fluorescence detection, due to its high sensitivity. Other chromatographic methods used in this setting include thiol-selective affinity chromatography (e.g. solid phase extraction, SPE) (Huang *et al.*, 2010),

and gas chromatography (GC), which is particularly indicated for detecting volatile thiols (Hinterholzer and Schieberle, 1998; Rafii *et al.*, 2007).

v. Detection

Depending on the derivatizing strategy adopted, UV or fluorescence detectors are coupled to the chromatographic system used for thiol separation. Electrochemical detection requires no derivatizing steps and consists in coupling the HPLC to amperometric or coulometric detectors (Diopan *et al.*, 2010). In all these cases, the thiols are identified by comparing the peak retention times on the chromatograms obtained with the samples with standard ones. The thiols can also be quantified by analyzing the peak areas and using thiol-specific calibration curves.

HPLC and GC can also be coupled with mass spectrometers (MS) for a more accurate identification (LC-MS and GC-MS), where GC-MS is particularly indicated for analyzing volatile thiols (Sass and Endres, 1997; Shinohara *et al.*, 2001; Toyo'oka, 2009). Guan and colleagues developed a method using LC-MS for simultaneously detecting and quantifying GSH, GSSG, Cys, Hcys and their disulfides in biological samples derivatized with Ellman's reagent (Guan *et al.*, 2003); other reagents can be used providing the modification is stable and produces a definite fragmentation pattern. Of course, MS analysis can also be preceded by *ad hoc* separation techniques, e.g. trap-and-release membrane introduction mass spectrometry (T&R-MIMS) (Velasco *et al.*, 2002).

Redox proteomic methods have been developed on MS instrumentation for the purpose of analyzing protein thiolation. As shown in Figure 2, the analysis is generally performed by using two derivatizing steps: the first one protects (i.e. modifies) free, reduced Cys, while the second acts on Cys originally involved in disulfide bridges. Then protein digestion is performed before the peptides are identified using MS (Leichert *et al.*, 2008; McDonagh *et al.*, 2011). The drawbacks of this type of protocol are that Cys linked to a LMW thiol cannot be distinguished from Cys involved in structural disulfide bonds, and different thiolation PTMs cannot be distinguished from one another. Anson and colleagues distinguished between glutathionylation and cysteinylations modifications on the same Cys residue in bacterial samples by skipping the derivatizing and reducing steps, and performing directly LC-MS analyses on digested samples (Ansong *et al.*, 2013).

3. Non-protein LMW thiols in plants

Thiols protect cell components working as redox buffers against a variety of reactive chemical species, such as reactive oxygen and nitrogen species, metals, xenobiotics, and other reactive electrophilic species (Messens *et al.*, 2013). A handful of LMW thiols are known to occur in plant cells, but recent data suggest that there are hundreds of them (Gläser *et al.*, 2014). Most of these LMW thiols derive from Cys or GSH (Figure 1), the latter being the most abundant. Figure 3 lists the structures and molecular masses of the thiols described in the following paragraphs; the concentrations reported in literature are listed in Table 2.

Table 2. Concentrations and localization of LMW thiols in plant tissues reported in literature.

LMW thiols	Localization	Concentration	References
Cys	<i>Zea mays</i> root, cell sap	56 μ M	Miller, 1985
	<i>Zea mays</i> root, exudate	12 \pm 4 μ M	Miller, 1985
	Plants (general)	\leq 10 μ M	Leustek <i>et al.</i> , 2000
	<i>Arabidopsis</i> leaves, cytosol	11 \pm 2 μ M	Yarmolinsky <i>et al.</i> , 2013
Hcys	Wheat (grain/seed)	390 μ g/ 100 g	Ruseva <i>et al.</i> , 2014
GSH	All major cellular compartments (general)	3 -10 mM	Leustek and Saito, 1999
	Chloroplasts (general)	1-4.5 mM	Noctor and Foyer, 1998
	<i>Arabidopsis</i> root, cytoplasm from different cell types	2-3 mM	Fricker <i>et al.</i> , 2000
	Poplar (<i>Populus</i>) leaves, cytosol of both photosynthetic and non-photosynthetic cells	0.2-0.3 mM	Hartmann <i>et al.</i> , 2003
	<i>Arabidopsis</i> leaves	0.45 \pm 0.01 mM	Yarmolinsky <i>et al.</i> , 2013
	<i>Arabidopsis</i> leaves	855.72 \pm 44.61 μ M	Tolin <i>et al.</i> , 2013
	<i>Medicago sativa</i> young leaves	59 \pm 10 μ M	Pasternak <i>et al.</i> , 2014
	<i>Medicago sativa</i> mature leaves	16 \pm 6 μ M	Pasternak <i>et al.</i> , 2014
Cys-Gly	<i>Arabidopsis</i> leaves	5.03 \pm 0.14 μ M	Tolin <i>et al.</i> , 2013
	Barley root	1.1 \pm 0.3 μ M	Ferretti <i>et al.</i> , 2008
	<i>Zea mays</i> leaves	1.2-1.6 μ M	Masi <i>et al.</i> , 2002
γ -Glu-Cys	<i>Zea mays</i> leaves (light hours)	2-3 μ M	Masi <i>et al.</i> , 2002
	<i>Zea mays</i> leaves (dark hours)	7-8 μ M	Masi <i>et al.</i> , 2002
hGSH	<i>Medicago sativa</i> young leaves	0.67 \pm 0.07 mM	Pasternak <i>et al.</i> , 2014
	<i>Medicago sativa</i> mature leaves	1.67 \pm 0.05 mM	Pasternak <i>et al.</i> , 2014
LA	Leaves (15-day-old wheat seedlings)	4.75 \pm 0.24 mM	Sgherri <i>et al.</i> , 2002
	Roots (15-day-old wheat seedlings)	7.22 \pm 0.36 mM	Sgherri <i>et al.</i> , 2002
DHLA	Leaves (15-day-old wheat seedlings)	13.77 \pm 0.69 mM	Sgherri <i>et al.</i> , 2002
	Roots (15-day-old wheat seedlings)	45.37 \pm 2.27 mM	Sgherri <i>et al.</i> , 2002

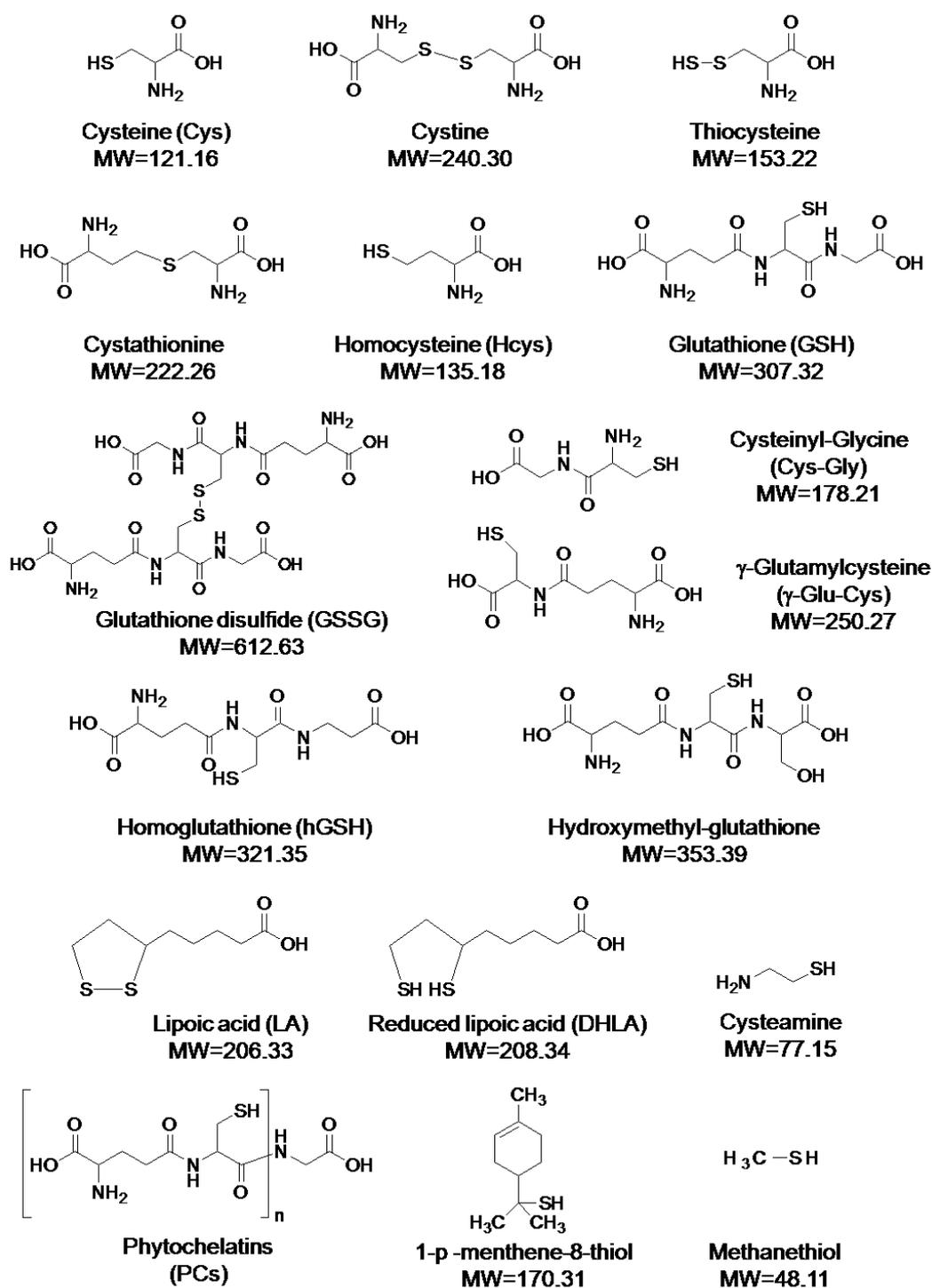


Figure 3. The structure and molecular weight of the best-known LMW thiols in plants.

3.1. Cysteine

Cys is the main product of plant sulfur assimilation. Besides being a component of thiol-containing proteins, in which it has both structural and functional roles (see section 1.2), it is a core metabolite that serves as a sulfur donor for a number of compounds such as Met, vitamins (thiamine and biotin), LA, coenzyme A, GSH, and many others (Figure 1).

Cys is synthesized in two steps: first, an acetyltransferase catalyzes the acetylation of serine from acetyl-CoA, producing O-acetylserine, then an O-acetylserine-(thiol)-lyase adds the reduced sulfur to O-acetylserine by eliminating the acetate moiety and forming Cys (Figure 4). The first step occurs mainly in the mitochondria, the second in the cytosol and chloroplasts (Hell and Wirtz, 2011). As already discussed, Cys concentrations are usually low because it is rapidly incorporated in proteins or converted into other compounds, especially GSH (Pilon-Smits and Pilon, 2006). At high concentrations (above 50 μM), Cys is considered toxic. The mechanisms by which Cys can have toxic effects are an irreversible thiol oxidization that leads to a loss of sulfur, and the formation of complexes with metal ions that can trigger Fenton reactions and the formation of hydroxyl radicals (Meyer and Hell, 2005; Bashir *et al.*, 2013, Zagorchev *et al.*, 2013).

Cys can reversibly dimerize into cystine through a disulfide bond. The metabolism of cystine in plants is still not fully understood: while both cystine transporters and reductases are known in mammals, in plants only a cystine reductase has been described in pea seeds, but its complete characterization is still lacking. It has been hypothesized that cystine can be reduced by GSH, or by enzymes such as Trxs, Grxs or GSH reductases, but several studies have reported that GSH reductases cannot reduce cystine (Romano and Nickerson, 1954; Olm *et al.*, 2009; Zagorchev *et al.*, 2013). In *Arabidopsis*, on the other hand, a cystine lyase reportedly catalyzes the cleavage of cystine's β -carbon-sulfide link, resulting in the release of thiocysteine, pyruvate, and ammonia. Thiocysteine is a LMW thiol compound consisting of a Cys linked to a sulfhydryl moiety through a disulfide bond, and can be further metabolized into cystine or thiocyanate, hydrogen sulfide, iron-sulfur clusters for protein assembly, or elemental sulfur (Jones *et al.*, 2003).

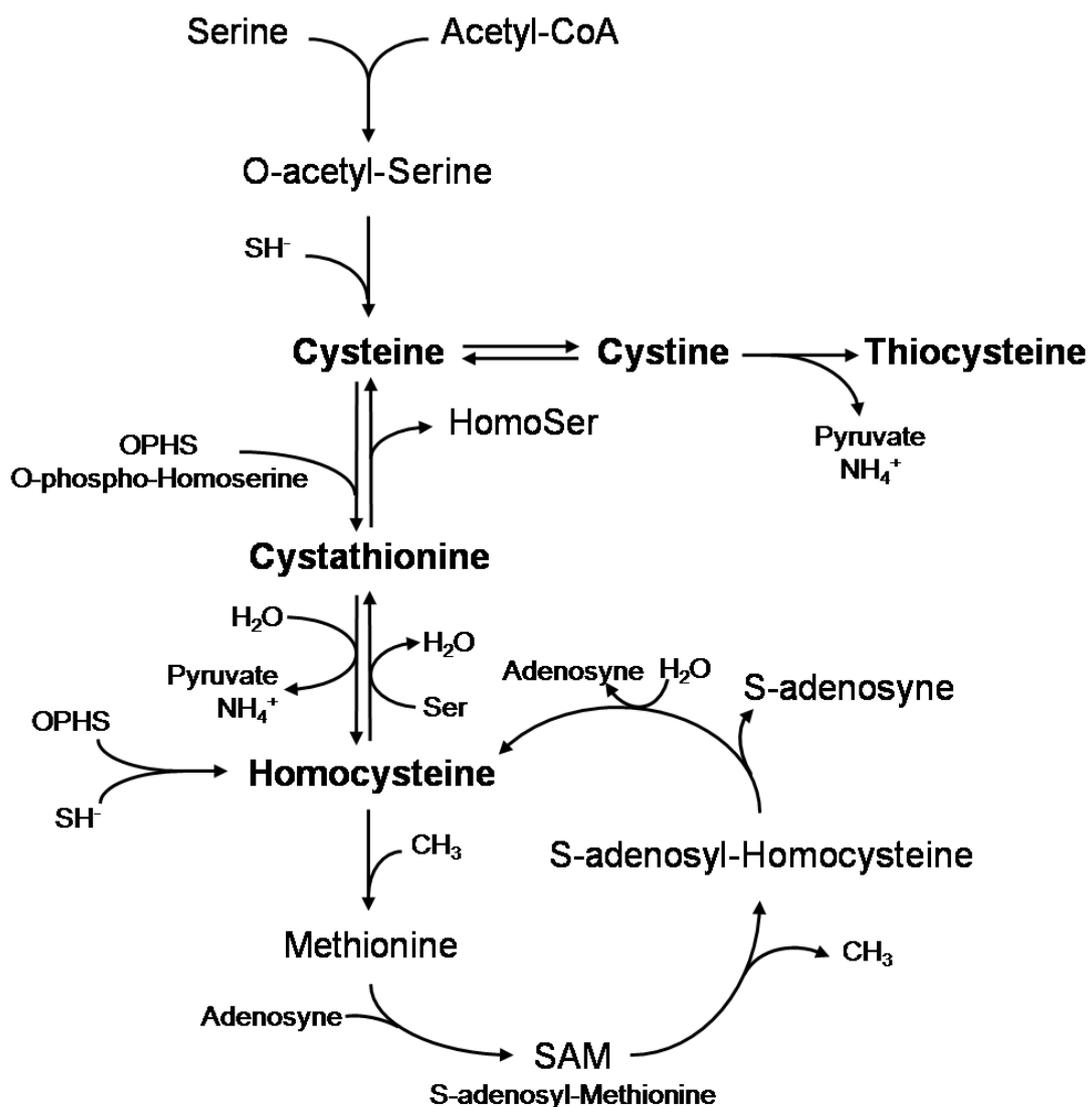


Figure 4. Cys biosynthesis pathway, adapted from the *Arabidopsis* KEGG “Cysteine and Methionine Metabolism” and “Sulfur metabolism” available online http://www.genome.jp/kegg-bin/show_pathway?ath00270 and http://www.genome.jp/kegg-bin/show_pathway?ath00920.

The acetylation of Cys to form N-acetylcysteine (NAC) has yet to be described in plants, but this compound has been reported in several vegetables, including garlic, onion, peppers, and asparagus (Demirkol *et al.*, 2004; Hsu *et al.*, 2004). Synthetic NAC is currently used as a nutritional supplement and drug in humans for its antioxidant properties.

Finally, we should mention that although they are not thiols – there are several non-protein alkyl-Cys and alkyl-Cys-sulfoxides in plants (especially in *Amaryllidaceae*,

Brassicaceae, and *Leguminosae*) that may act as precursors for the release of volatile thiols. Much of the characteristic odor associated with most of these plants is due to the degradation of Cys derivatives by specific lyases (Hamamoto and Mazelis, 1985). Other volatile thiol compounds derived from Cys are discussed in section 3.10.

3.2. Homocysteine

In plants, Hcys is an intermediate in the biosynthetic pathway of Met. Cys is the sulfur donor, which is transferred to Met via a three-step mechanism. First of all, Cys and O-phosphohomoserine (OPHS) are coupled to form the thioether cystathionine, which is rapidly converted into Hcys with the concomitant formation of pyruvate and ammonia (Figure 4) (Datko *et al.*, 1974; Pilon-Smits and Pilon, 2006). The enzymes required for the first and second steps are cystathionine- γ -synthase (Cgs) and cystathionine β -lyase (CbL), respectively, which are believed to share the same ancestral origin (Belfaiza *et al.*, 1986; Ravanel *et al.*, 1998). These reactions occur in plastids and then Hcys is transported to the cytosol via an unknown mechanism (Pilon-Smits and Pilon, 2006). The Met-synthase enzyme methylates Hcys to form Met by using N5-methyltetrahydrofolate as a methyl group donor. Then Met can be transported into the plastids again or remain in the cytosol, where it is involved in other pathways, such as protein synthesis or conversion to SAM, which serves as a methyl donor for a number of molecules (Hesse and Hoefgen, 2003; Pilon-Smits and Pilon, 2006). The synthesis of Met (and its intermediate, Hcys) is controlled by the competition between Cgs and threonine synthase, since they need the same OPHS substrate to form cystathionine or threonine, respectively (Ravanel *et al.*, 1998). When Met is not used for protein synthesis, it can be regenerated through the SAM cycle: specific methylases use SAM as a methyl donor group to produce methylated molecules and S-adenosylhomocysteine, which is then enzymatically hydrolyzed to adenosine and Hcys (Brzezinski *et al.*, 2008).

There is an alternative biosynthetic pathway for the direct formation of Hcys from OPHS. Instead of Cys, the sulfur donor is the sulfide, which is added to OPHS directly by an enzyme with a sulfhydrylase activity. This pathway only takes part in 3% of all Hcys synthesis, however, and is physiologically insignificant (Datko *et al.*, 1977; Macnicol *et al.*, 1981; Hesse *et al.*, 2004). In humans, Hcys has received considerable attention because high levels in plasma represent a risk factor for cardiovascular diseases such as atherosclerosis and venous thrombosis (Guan *et al.*, 2003).

3.3. Synthesis and degradation: glutathione, γ -glutamylcysteine and cysteinylglycine

Glutathione (GSH; γ -glutamyl-cysteine-glycine,) is a key molecule with an essential role in cellular homeostasis. Its properties and regulation have attracted the attention of scientists worldwide, as documented by the vast body of literature on the topic (Tausz *et al.*, 2004; Rouhier *et al.*, 2008; Noctor *et al.*, 2011; Noctor *et al.*, 2012). In plant cells, GSH is thought to occur at concentrations between 3 and 10 mM, and it is found in the major cellular compartments (Leustek *et al.*, 1999).

It is the main non-protein LMW thiol in plants, containing a gamma peptide linkage between the amine group of cysteine and the carboxyl group of the glutamate side-chain; as such, it cannot be a substrate for proteases. This molecule exposes the -SH group of the free cysteine, which can be oxidized to form a dimer (GSSG) held by a disulfide bond between two identical molecules. The ratio between GSH and GSSG is an important indicator of the cell's redox state. Under physiological conditions, the intracellular glutathione pool is kept in its reduced form, but GSSG can accumulate under conditions of oxidative stress.

GSH being the most abundant thiol controlling the redox potential of the major cellular components, the GSH redox state in turn modulates the reduction state of the thiol groups of susceptible enzymes via thiol/disulfide exchange reactions (Kunert *et al.*, 1993).

In plant physiology, GSH is involved in regulating cellular metabolism, with an important role in protecting against oxidative stress, as an antioxidant, preventing damage caused by bioreactive oxygen species. It also participates in xenobiotic and heavy metal detoxification, plant-pathogen interactions, and plant growth. As a component of sulfur metabolism, it serves as a molecule for the storage of reduced sulfur and its long-distance transport between different organs. It is a cofactor of adenosine-phosphosulfate reductase (APR) in the biosynthesis of Cys, and a precursor in the biosynthesis of PCs and glucosinolates (Noctor *et al.*, 2011), and it is needed for the maturation of iron-sulfur proteins (Sipos *et al.*, 2002). The GSH biosynthesis pathway in plants is essentially similar to the one described in other organisms (Figure 5) (Rennenberg and Filner, 1982; Meister, 1988; Noctor *et al.*, 2002). Two ATP-dependent enzymes (GSH1 and GSH2) produce GSH sequentially from Glu, Cys and Gly. In the first step, the intermediate γ -

glutamylcysteine (γ -Glu-Cys) is synthesized in the plastid; following γ -Glu-Cys export across the chloroplast envelope, the addition of glycine can occur in both chloroplasts and cytosol. In its active form, the γ -Glu-Cys synthetase (GSH1) enzyme forms a homodimer linked by two disulfide bonds (Hothorn *et al.*, 2006), which are probably implicated in the up-regulation of GSH synthesis in response to oxidative stress.

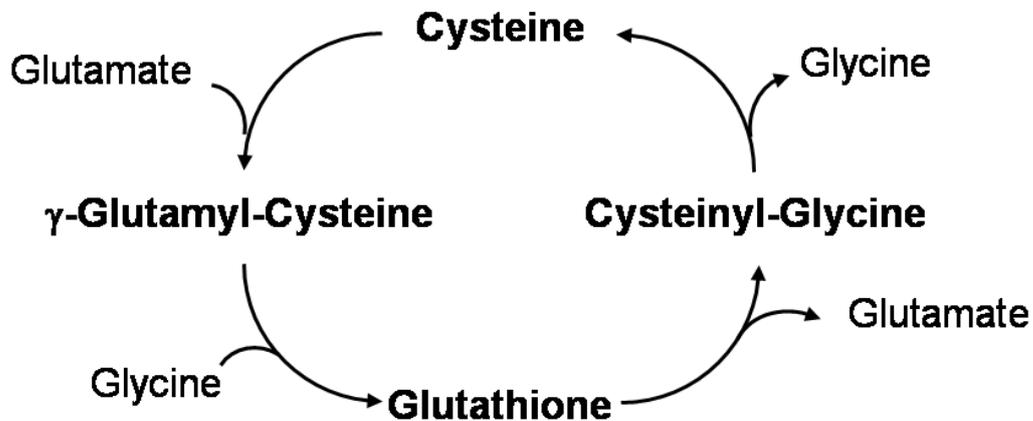


Figure 5. GSH biosynthesis pathway adapted from the *Arabidopsis* KEGG “Cysteine and Methionine Metabolism” available online http://www.genome.jp/kegg-bin/show_pathway?ath00270.

Functional genomic studies using *Arabidopsis thaliana* mutants indicate that a reduced GSH content results in: failure to develop a root apical meristem; a disrupted auxin transport and metabolism; loss of apical dominance and reduced secondary root production; greater sensitivity to cadmium; a decreased camalexin content; and an enhanced sensitivity to pathogens. Oxidative stress conditions and an increased H_2O_2 availability are also known to induce glutathione accumulation in several plant species, whereas *gsh1* and *gsh2* genes respond to jasmonic acid, heavy metals and light treatments, and to conditions of stress such as drought and pathogens (Noctor *et al.*, 2011).

GSH in plant cells is degraded by GGT (γ -glutamyl transferase/transpeptidase) and GGCT (γ -glutamyl cyclo-transferase) activity. These two alternative degradation pathways are located in different compartments. GGTs are extra-cytosolic; in

Arabidopsis, there are two known apoplastic isoforms, GGT1 and GGT2, the former located in the cell wall and the latter attached to the plasma membrane, that take part in the γ -glutamyl cycle involving GSH extrusion to the apoplastic space, degradation to its constituent amino acids, and their re-uptake by aminoacid transporters, followed by GSH resynthesis. Another isoform, GGT4, is vacuolar and assists in degrading GSH conjugates. GGTs catalyze the removal of the γ -glutamyl bond, thus releasing Glu (or γ -glutamyl-peptides) and the thiol Cys-Gly.

Rising levels of Cys-Gly in biological samples can be seen as an indicator of a greater degradation of GSH by GGT, a situation observed experimentally under conditions of stress (Masi *et al.*, 2002). Further confirmation of the role of GGT in response to environmental stress comes from the finding that its activity is also enhanced in *Ceratophyllum demersum* following arsenate treatment (Mishra *et al.*, 2008). Consistently with the location of GGT, Cys-Gly is mainly extracellular; and in *Arabidopsis* mutants lacking a functional *ggt1* gene, Cys-Gly is almost abolished in the apoplast. Proteomic analyses in *ggt1* mutant leaves point to the upregulation of defense and stress response genes, giving the impression that the γ -glutamyl cycle in plants is implicated in redox sensing and redox homeostasis (Tolin *et al.*, 2013).

Some degradation of GSH to Cys-Gly may also occur spontaneously (authors' personal observations). Cys-Gly is a strong nucleophile and may thiolate proteins in animal systems (see section 1.3). The same is probably true of plants, but evidence of this is lacking for the time being.

3.4 Glutathione homologs: homoglutathione and hydroxymethyl-glutathione

There have been reports of GSH analogs (Figure 3) occurring in the plant kingdom that are not reported in any other organisms, such as homoglutathione (hGSH, γ -Glu-Cys- β -Ala) in several members of the *Fabales* order (Klapheck *et al.*, 1988), and hydroxymethyl-glutathione (γ -Glu-Cys-Ser), which is widespread in the *Poaceae* family (Klapheck *et al.*, 1992). Their functions are similar to those of GSH (Bergmann and Rennenberg, 1993), and their occurrence in phloem sap demonstrates that they both serve as major reduced sulfur transporters in whole plants.

Recent literature points to hGSH also having a role in establishing the host-parasite/symbiont relationship. hGSH has been shown to enhance the expression of

salicylic acid (SA), and to induce changes in water transport and salicylic acid signaling pathways, thus interfering with the proper development of the symbiotic interaction between *Medicago truncatula* and *Sinorhizobium meliloti* (Pucciariello *et al.*, 2009). Together with GSH, hGSH has a critical role in the nodulation process (Frendo *et al.*, 2013), in nitrogen fixation in *Medicago truncatula* nodules (El Msehli *et al.*, 2011), and in root-knot nematode development (Baldacci-Cresp *et al.*, 2012).

The conjugation of hGSH to the herbicide fomesan confers tolerance in soybean. The expression of a hGSH synthetase together with a hGSH-preferring GST from soybean was used to confer resistance in tobacco, which is sensitive to fomesan (Skipsey *et al.*, 2005).

3.5. Cysteamine

Cysteamine (also called mercaptamine or β -mercaptoethylamine) is the simplest aminothiols and it is produced by two alternative biosynthetic pathways (Figure 6): Cys decarboxylation or coenzyme A degradation, the latter has been described in animals and it is not clear yet whether it occurs also in plant (Toyo'oka, 2009). Coenzyme A is degraded to pantothenic acid, the breakdown of which produces cysteamine, which is rapidly oxidized into hypotaurine by the enzyme cysteamine dioxygenase (Besouw *et al.*, 2013).

Cysteamine reportedly stimulates GSH synthesis, but its main role is in the synthesis of taurine, through the intermediate hypotaurine, which can also be produced by Cys oxidation (Kwon and Stipanuk, 2001; Coloso *et al.*, 2006). Since both Cys and cysteamine are cytotoxic at high concentrations, they are rapidly converted into taurine (Jeitner and Lawrence, 2001). The taurine biosynthetic pathway has been characterized in mammals, but it has also been detected in plants and prokaryotes, in which the mechanisms of synthesis have yet to be thoroughly elucidated (McCoy, 2012).

The role of cysteamine in plants is not entirely clear, but there is *in vitro* evidence to indicate that RuBisCO, an enzyme involved in the Calvin Cycle, can be completely inactivated with cysteamine/cysteamine buffers (cysteamine is the dimeric, oxidized form of cysteamine). In conditions of oxidative stress, it first becomes inactive, and then it becomes sensitive to proteases as a result of several conformational changes affecting cysteines. These processes demonstrated *in vitro* suggest an *in vivo* involvement of cysteamine in oxidative stress and in senescence processes (Moreno *et al.*, 2008).

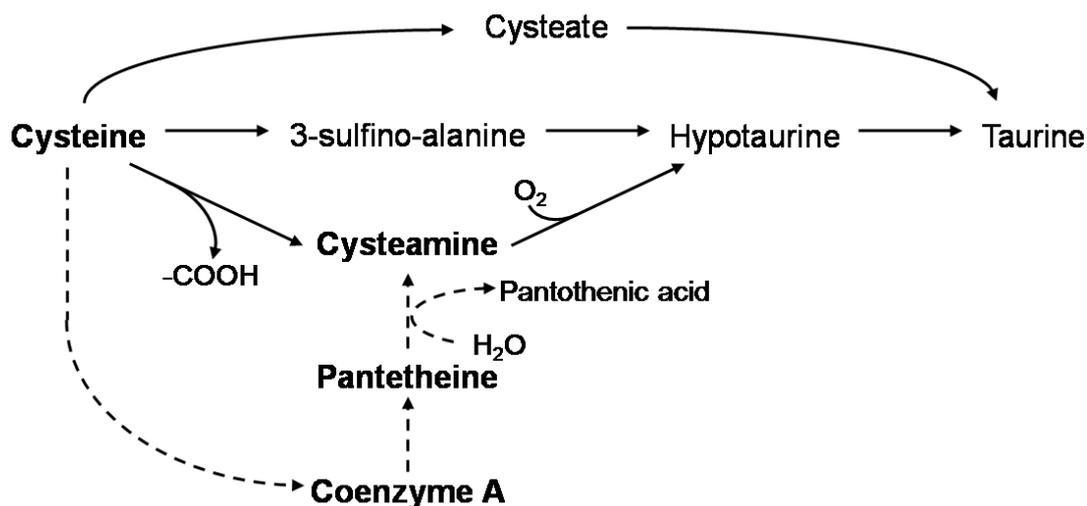


Figure 6. Cysteamine biosynthesis pathway adapted from the *Arabidopsis* KEGG “Taurine and hypotaurine biosynthesis” available online http://www.genome.jp/kegg-bin/show_pathway?ath00430. Dotted arrows refer to a molecular pathway described in mammals by Coloso *et al.*, 2006.

There is still a general shortage of knowledge on this compound, but cysteamine may have regulatory and physiological functions. We have found cysteamine as a major LMW thiol in stored apple skins, more abundant even than GSH (Figure 7). It could be very important to learn more about the process of cysteamine biosynthesis and its metabolism in plants because cysteamine may have been one of the most abundant and stable thiols available on the primitive Earth as demonstrated by Miller and Schlesinger (1993).

3.6. Phytochelatins

PCs are small, Cys-rich polypeptides synthesized from GSH through a PC synthase (γ -glutamylcysteine dipeptidyltranspeptidase) in response to high concentrations of toxic metals in the cell’s cytoplasm as follows (Pal and Rai, 2010; Wood and Feldmann, 2012):



The general structure of PCs is $(\gamma\text{-Glu-Cys})_n\text{-Gly}$, with increasing repetitions of the dipeptide Glu-Cys linked through a γ -carboxylamide bond (Figure 3), where n can range from 2 to 11, but is typically no more than 5 (Grill *et al.*, 1985; Potesil *et al.*, 2005). Based on the repetitions of this dipeptide, PCs are classified as PC2, PC3, PC4, etc. The terminal amino acid is usually Gly, but there are variants in some plant species, such as $(\gamma\text{-Glu-Cys})_n\text{-}\beta\text{-Ala}$, $(\gamma\text{-Glu-Cys})_n\text{-Ser}$, and $(\gamma\text{-Glu-Cys})_n\text{-Glu}$. Irrespective of these differences, all PCs are involved in metal homeostasis and detoxification, i.e. they have the ability to transport heavy metals into the vacuole by means of specialized transporters (Pal and Rai, 2010). They also play an important part in maintaining the ionic homeostasis of the cell (Yadav, 2010).

PCs help with detoxification by forming metal-PC complexes with the Cys thiol group and thus sequestering the heavy metals in the cytosol. The heavy metals can be Cd, Cu, Hg, As and Pb, and each one induces different levels of PC expression (Potesil *et al.*, 2005). They occur in higher plants, marine and freshwater algae, some fungi, lichens and some animal species, responding particularly to Cd (El-Zohri *et al.*, 2005; Petraglia *et al.*, 2014).

3.7. Lipoic acid

LA, or α -LA (5-(1,2-dithiolan-3-yl)-pentanoic acid), is an amphipathic sulfur-containing molecule found in prokaryotic microorganisms, animals and plants (Durrani *et al.*, 2007). It was first isolated by Reed and colleagues from bovine liver in 1951.

Due to its structural properties (Figure 3), LA is soluble in both water and organic solvents, with a preference for the latter. It can neutralize ROS and reduce oxidized forms of other antioxidants, and that is why it is called a super-antioxidant. LA contains a single chiral center and an asymmetric carbon, resulting in two enantiomers, R-LA and S-LA, the first of which is the essential cofactor synthesized in cells. In its reduced form, DHLA, there are two free thiol groups in each molecule and it is the predominant form serving as an antioxidant, by interacting with ROS in conditions of stress and reducing GSH (Navari-Izzo *et al.*, 2002). It can also act as a pro-oxidant, however, by being iron-reducing and generating S-containing radicals that can damage proteins (Goraca *et al.*, 2011; Khan *et al.*, 2014). The oxidized form contains the two S atoms connected by a disulfide bridge. The special position of the two sulfur atoms in the molecule gives LA a marked tendency for reduction. DHLA has vicinal thiols, thus making it more easily

oxidized than monothiols, and making this molecule very active in exchange reactions (Moini *et al.*, 2002).

It is an important cofactor for the activity of several multienzyme complexes such as pyruvate dehydrogenase (responsible of the production of acetyl-CoA), and glycine decarboxylase; complexes involved in the oxidative decarboxylation of α -ketoacids and in the glycine cleavage system (Yasuno and Wada, 1998). In these multienzyme complexes, LA is covalently bound via an amide linkage to the ϵ -amino group of specific lysine residues (which are highly conserved) in the subunit E2 of the multienzymatic complex (Yasuno and Wada, 2002). This function of LA is very important in energy metabolism as part of the complexes regulating carbon flow into the Krebs's cycle and ultimately producing ATP (Sato *et al.*, 2008).

LA is synthesized in mitochondria in both animal and plant cells, but in plants a lipoic acid synthase has been located also in plastids (Yasuno and Wada, 2002). LA biosynthetic pathway has yet to be thoroughly clarified in any organism, and most of collected information comes from bacteria. The direct precursor of LA is octanoic acid (from fatty acid biosynthesis) linked to an acyl carrier protein, while the sulfur donor is less certain, and presumably could be iron-sulfur cluster or SAM (Miller *et al.*, 2000; Kaleta *et al.*, 2010). In *Arabidopsis thaliana* it has been demonstrated that the *LIP1* cDNA encodes a LA synthase, with very similar sequences to those identified in *Escherichia coli* and yeast, but little is known about its mechanism. For sure, LA is first synthesized as linked to an acyl carrier protein and then released/transferred to a target protein (Yasuno and Wada, 1998; Gueguen *et al.*, 2000).

Plant and animal tissues contain small amounts of LA. The most abundant plant sources of LA are spinach, broccoli, tomatoes, brussels sprouts, potatoes, garden peas and rice bran (Goraca *et al.*, 2011). All the properties of LA make it a very useful agent in the treatment of many diseases, including diabetes, atherosclerosis, degenerative processes in neurons, cataract formation, radiation injury, cancer, and acquired immune deficiency syndrome (AIDS). It is also used in anti-age treatments (Bilska and Wlodek, 2005).

3.8. Volatile thiols

Significant amounts of volatile thiols are produced by secondary metabolism in specific plant species and they have an important role as food flavorings. However, most of the sulfur flavoring agents originate not directly from plant biosynthetic pathways, but from fermentation processes or further preparation procedures. To give an idea of the variety of LMW sulfur compounds contained in foods, be they synthetic or derived from natural sources, at least 188 have been classified and tested by the European Food Safety Authority (EFSA, 2010), at least fifty of which are thiol molecules, classified as: *i.* simple thiols with un-oxidized aliphatic or aromatic side-chains; *ii.* thiols with oxidized side chains, in which an alcohol, aldehyde, ketone, ester, or carboxylic acid group is present; and *iii.* dithiols (EFSA, 2010).

The most often studied volatile thiols are those contained in fermented beverages, and especially wine. They are classified as varietal aroma compounds, i.e. molecules synthesized by the plant and occurring in grape fruits in free form or linked to a non-volatile molecule (the cleavage occurs during wine production but the original moiety produced by the plant is preserved), and pre-, post- or fermentation aromas. Odoriferous varietal aromas released by the fermentation process often have a cysteinylated or a glutathionylated odorless precursor synthesized by the grape berry (Roland *et al.*, 2011). In particular, cysteinylated precursors are plentiful in plants, providing an abundant source of aroma for the food industry (Starkenmann *et al.*, 2008). Other compounds do not have a Cys or GSH-precursor, for example 1-p-menthene-8-thiol (Figure 3) occurs in the intact plant (*Vitis vinifera*), and derives from the reaction of limonene with SH₂ (Shung, 1990).

There are also many unpleasant odors associated with volatile thiols, generally deriving from the degradation of Cys, Met or other larger sulfur-containing molecules. Methanethiol (Figure 3) is plentiful in cabbage and other *Brassicaceae* (Attieh *et al.*, 1995), where it is produced from bisulfide by specific methyltransferases, but it is also found in other plant species at lower concentrations (e.g. *Arabidopsis*), produced from Met by a γ -methionine-lyase (Rébeillé *et al.*, 2006). Some volatile alkane thiols have also been characterized in onion (e.g. 3-mercapto-2-methylpentanal and 3-mercapto-2-methylpentan-1-ol).

3.9. The case of glucosinolates and thioglucose

Glucosinolates are a class of sulfur-containing secondary metabolites with a major role in plant defense in the *Brassicaceae* family (Hopkins *et al.*, 2009), and some of them have proven anti-cancer properties in medical treatments (Zhang *et al.*, 1992). They contain one sulfur atom derived from phosphoadenosine phosphosulfate, and another one is obtained from the amino acid Cys, or two if the starting amino acid is Met (Textor *et al.*, 2004). It has now been demonstrated that GSH is a precursor in the synthesis of glucosinolates and also of camalexins, but whether a cytosolic or an extracellular γ -glutamyl-peptidase, or both, are involved is still debated (Geu-Flores *et al.*, 2011; Su *et al.*, 2011; Møldrup *et al.*, 2013; Su *et al.*, 2013). These compounds are substrates for thioglucosidases (myrosinase) but the reaction is prevented because they are restricted to different compartments. On chewing by herbivores, this compartmentalization is lost and hydrolysis occurs, causing a cascade of reactions through thioglucose, and leading to the release of toxic compounds (e.g. thiocyanates, isothiocyanates, indoles) that defend against predators and pathogens. Thioglucose can thus be considered another intermediate thiol in plant metabolism, implicated in plant defense.

Non-enzymatic glucosinolate hydrolysis occurs at alkaline pH also under the analytical conditions imposed for thiol reduction (see section 2), which results in the spontaneous release of thioglucose (Jezek *et al.*, 1999). This molecule is therefore valuable as a means for rapidly estimating total glucosinolate content (Gallaher *et al.*, 2012).

3.10 Diversity of LMW thiols in plants

Gläser and colleagues ascertained that there were about 300 sulfur metabolites in *Arabidopsis* using MS techniques; most of them remain unidentified, and many of these could be LMW thiols (Gläser *et al.*, 2014). Indeed, chromatographic separations performed at our lab revealed the existence of several unknown molecules containing thiols in several plant species, including fruit and vegetables. Figure 7 shows some representative HPLC chromatograms of a series of vegetable and other plant samples after derivatization with SBD-F, some of which show unknown thiols that are specific to some plants, while others exhibit organ/tissue specificity.

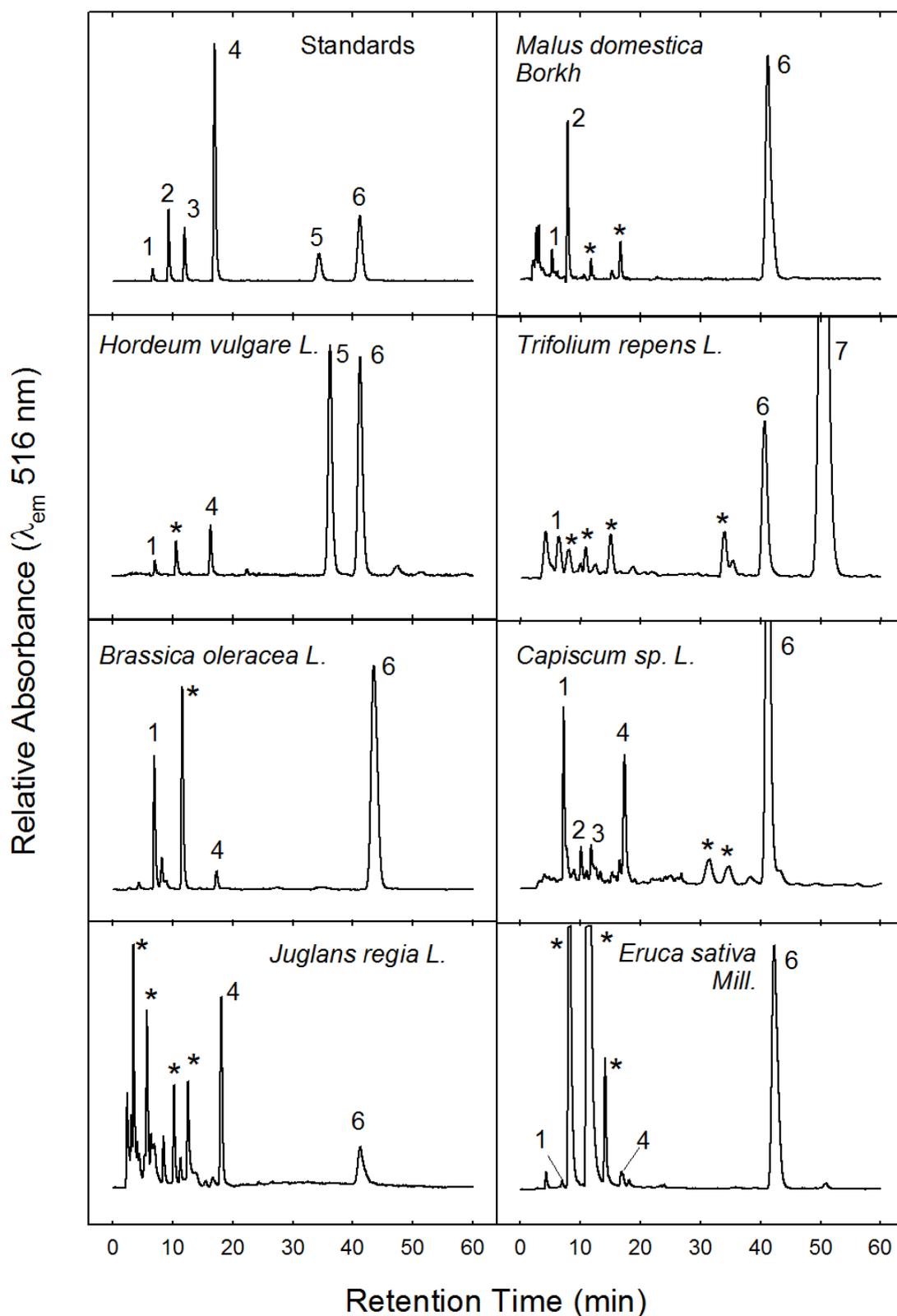


Figure 7. Representative chromatographic separation of LMW thiols from several plant samples, after derivatization with SBD-F: Standards, *Malus domestica Borkh* skin, *Hordeum vulgare L.* root, *Trifolium repens L.*, *Brassica oleracea L.*, *Capsicum sp. L.*, *Juglans regia L.*, *Eruca sativa Mill.*. Numbers refer to: 1 Cys; 2 Cysteamine; 3 Hcys; 4 Cys-Gly; 5 γ -Glu-Cys; 6 GSH; 7 putative hGSH, * unknown LMW thiols.

4. Conclusions and future prospects

LMW thiol molecules are biologically relevant due to the intrinsic reactivity of the nucleophilic -SH moiety. By participating in reversible redox reactions, they can modify the redox state of sensitive molecules and the cellular environment. They can conjugate or make complexes with xenobiotics and toxic compounds, and they can deactivate them. They can post-translationally modify regulatory enzymes and control metabolism. They may also be technologically relevant, with implications for food quality and safety, and a possible fallout on human health.

Despite the numerous implications relating to our understanding of LMW thiol metabolism, it is no exaggeration to say that many of them have been neglected so far, and a great deal of work remains to be done in this field. We can outline at least three areas that deserve further investigation.

i. The identification of new compounds. While a few LMW thiols have been described in the literature, a huge and diverse array of unknown thiol molecules clearly exist in plant biology, as evidenced chromatographically by thiol-specific derivatives with fluorescent dyes (Figure 7). Identifying these thiols represents a major challenge, given that in most cases they are hardly abundant - probably in the range of micromolar or sub-micromolar concentrations. A new, upcoming generation of mass spectrometers with a high sensitivity and resolution, combined with the development of thiol purification and concentration protocols, will be of great help in the process of their identification.

ii. The identification of protein residues modified by glutathionylation. It has been demonstrated that glutathionylation regulates a significant number of biological processes, including photosynthesis, germination, seed development and desiccation, but few studies have focused on experimentally verified protein glutathionylation sites. Implementing this knowledge by means of proteomic studies is a necessary step in order to make progress in our understanding of the biological meaning of such modifications, to identify the part played by GSH when linked to the proteins, and how these modifications are regulated.

iii. The investigation of protein thiolation. Glutathionylation is the most widespread and significant thiolation modification, but it has recently been

demonstrated in both mammals and bacteria that other LMW thiols can be bound to proteins, such as Cys-Gly and Cys. In particular, such modifications may occur on the same residues where glutathionylation takes place, modulating different processes depending on which LMW thiol is linked. In the light of these findings, it would be well worth studying disulfide PTMs. MS could be the most appropriate technique for this purpose at present, but new methods need to be developed because standard protocols generally have to include a reducing step.

Acknowledgments

The Authors wish to thank Dr. Dinesh Prasad for critically reading the manuscript and providing advice, Dr. Anna Rita Trentin for technical support, and Frances Coburn for English grammar and style revision. Marta Fabrega-Prats was founded by the Cariparo PhD grant (Fondazione Cassa di Risparmio di Padova e Rovigo), Micaela Pivato by a University of Padova grant.

References

- Akasaka, K., Suzuki, T., Ohrui, H., Meguro, H. (1986). Fluorimetric determination of sulfites in rainwater. *Analytical Sciences*, **2**, 443-446.
- Amarnath, K., Amarnath, K. (2002). Specific determination of cysteine and penicillamine through cyclization to 2-thioxothiazolidine-4-carboxylic acids. *Talanta*, **56**, 745-751.
- Ansong, C., Wu, S., Meng, D., Liu, X., Brewer, H.M., Deatherage Kaiser, B.L., Nakayasu, E.S., Cort, J.R., Pevzner, P., Smith, R.D., Heffron, F., Adkins, J.N., Pasa-Tolic, L. (2013). Top-down proteomics reveals a unique protein S-thiolation switch in *Salmonella typhimurium* in response to infection-like conditions. *Proceedings of the National Academy of Sciences of the United States of America*, **110**, 10153-10158.
- Attieh, J.M., Hanson, A.D., Saini, H.S. (1995). Purification and characterization of a novel methyltransferase responsible for biosynthesis of halomethanes and methanethiol in *Brassica oleracea*. *The Journal of Biological Chemistry*, **270**, 9250-9257.
- Bald, E., Glowacki, R. (2001). 2-Chloro-1-methylquinolinium tetrafluoroborate as an effective and thiol specific UV-tagging reagent for liquid chromatography. *Journal of Liquid Chromatography & Related Technologies*, **24**, 1323-1339.
- Baldacci-Cresp, F., Chang, C., Maucourt, M., Deborde, C., Hopkins, J., Lecomte, P., Bernillon, S., Brouquisse, R., Moing, A., Abad, P., Hérouart, D., Puppo, A., Favery, B., Frendo, P. (2012). (Homo)glutathione deficiency impairs root-knot nematode development in *Medicago truncatula*. *PLoS Pathogens*, **8**, e1002471.
- Bashir, H., Ahmad, J., Bagheri, R., Nauman, M., Qureshi, M.I. (2013). Limited sulfur resource forces *Arabidopsis thaliana* to shift towards non-sulfur tolerance under cadmium stress. *Environmental and Experimental Botany*, **94**, 19–32.
- Belfaiza, J., Parsot, C., Martel, A., de la Tour, C.D., Margarita, D., Cohen, G.N., Saint-Girons, I. (1986). Evolution in biosynthetic pathways: two enzymes catalyzing consecutive steps in methionine biosynthesis originate from a common ancestor and possess a similar regulatory region. *Proceedings of the National Academy of Sciences of the United States of America*, **83**, 867-871.
- Benkova, B., Lozanov, V., Ivanov, I.P., Todorova, A., Milanov, I., Mitev, V. (2008). Determination of plasma aminothiols by high performance liquid chromatography after precolumn derivatization with N-(2-acridonyl)maleimide. *Journal of Chromatography B: Analytical Technologies in the Biomedical and Life Sciences*, **870**, 103-108.
- Bergmann, L., Rennenberg, H. (1993). Glutathione metabolism in plants. In: de Kok, L.J., Stulen, I., Rennenberg, H., Brunold, C., Rauser, W.E. (Eds.), *Sulfur nutrition and sulfur assimilation in higher plants*. SPB Academic, The Hague, The Netherlands, pp. 109–123.
- Besouw, M., Masereeuw, R., Van Den Heuvel, L., Levtchenko, E. (2013). Cysteamine: an old drug with new potential. *Drug Discovery Today*, **18**, 785-792.

- Bilska, A., Wlodek, L. (2005). Lipoic acid - The drug of the future? *Pharmacological Reports*, **57**, 570-577.
- Bindoli, A., Fukuto, J.M., Forman, H.J. (2008). Thiol chemistry in peroxidase catalysis and redox signaling. *Antioxidants & Redox Signaling*, **10**, 1549-1564.
- Brzezinski, K., Bujacz, G., Jaskolski, M. (2008). Purification, crystallization and preliminary crystallographic studies of plant S-adenosyl-L-homocysteine hydrolase (*Lupinus luteus*). *Acta Crystallographica Section F: Structural Biology and Crystallization Communications*, **64**, 671-3.
- Buchanan, B.B., Schürmann, P., Decottignies, P., Lozano, R.M. (1994). Thioredoxin: a multifunctional regulatory protein with a bright future in technology and medicine. *Archives of Biochemistry and Biophysics*, **314**, 257-260.
- Carru, C., Deiana, L., Sotgia, S., Pes, G.M., Zinellu, A. (2004). Plasma thiols redox status by laser-induced fluorescence capillary electrophoresis. *Electrophoresis*, **25**, 882-889.
- Causse, E., Malatray, P., Calaf, R., Charpiot, P., Candito, M., Bayle, C., Valdiguie, P., Salvayre, R., Couderc, F. (2000). Plasma total homocysteine and other thiols analyzed by capillary electrophoresis/laser-induced fluorescence detection: comparison with two other methods. *Electrophoresis*, **21**, 2074-2079.
- Chen, X., Zhou, Y., Peng, X., Yoon, J. (2010). Fluorescent and colorimetric probes for detection of thiols. *Chemical Society Reviews*, **39**, 2120-2135.
- Chen, Y.J., Lu, C.T., Lee, T.Y., Chen, Y.J. (2014). dbGSH: a database of S-glutathionylation. *Bioinformatics*, **30**, 2386-2388.
- Clements, A., Johnston, M.V., Larsen, B.S., McEwen, C.N. (2005). Fluorescence-based peptide labeling and fractionation strategies for analysis of cysteine-containing peptides. *Analytical Chemistry*, **77**, 4495-4502.
- Coloso, R.M., Hirschberger, L., Dominy, J.E., Lee, J., Stipanuk, M.H. (2006). Cysteamine dioxygenase: evidence for the physiological conversion of cysteamine to hypotaurine in rat and mouse tissues. *Advances in Experimental Medicine and Biology*, **583**, 25-36.
- Colville, L., Kranner, I. (2010). Plant growth regulation desiccation tolerant plants as model systems to study redox regulation of protein thiols. *Plant Growth Regulation*, **62**, 241-255.
- Corti, A., Paolicchi, A., Franzini, M., Dominici, S., Casini, A.F., Pompella, A. (2005). The S-thiolating activity of membrane gamma-glutamyltransferase: formation of cysteinyl-glycine mixed disulfides with cellular proteins and in the cell microenvironment. *Antioxidants & Redox Signaling*, **7**, 911-918.

- Couturier, J., Chibani, K., Jacquot, J.P., Rouhier, N. (2013). Cysteine-based redox regulation and signaling in plants. *Frontiers in Plant Science*, **4**, 105.
- Dalle-Donne, I., Rossi, R., Giustarini, D., Colombo, R., Milzani, A. (2007). S-glutathionylation in protein redox regulation. *Free Radical Biology & Medicine*, **43**, 883-898.
- Dalle-Donne, I., Rossi, R., Colombo, G., Giustarini, D., Milzani, A. (2009). Protein S-glutathionylation: a regulatory device from bacteria to humans. *Trends in Biochemical Sciences*, **34**, 85-96.
- Datko, A.H., Giovanelli, J., Mudd, S.H. (1974). Homocysteine biosynthesis in green plants. O-phosphorylhomoserine as the physiological substrate for cystathionine γ -synthase. *The Journal of Biological Chemistry*, **249**, 1139-1155.
- Datko, A.H., Mudd, S.H., Giovanelli, J. (1977). Homocysteine biosynthesis in green plants. Studies of the homocysteine forming sulfhydrylase. *The Journal of Biological Chemistry*, **252**, 3436-3445.
- Demirkol, O., Adams, C., Ercal, N. (2004). Biologically important thiols in various vegetables and fruits. *Journal of Agricultural and Food Chemistry*, **52**, 8151–8154.
- Diopan, V., Zitka, V.S.O., Galiova, M., Adam, V., Kaiser, J., Horna, A., Novotny, K., Liska, M., Havel, L., Zehnalek, J., Kizek, R. (2010). Determination of plant thiols by liquid chromatography coupled with coulometric and amperometric detection in lettuce treated by lead(II) ions. *Electroanalysis*, **22**, 1248-1259.
- Dudev, T. (2014). Modeling metal binding sites in proteins by quantum chemical calculations. *Journal of Computational Chemistry*, **2**, 19-21.
- Durrani, A.I., Schwartz, H., Schmid, W., Sontag, G. (2007). α -lipoic acid in dietary supplements: development and comparison of HPLC-CEAD and HPLC-ESI-MS methods. *Journal of Pharmaceutical and Biomedical Analysis*, **45**, 694-699.
- Eaton, P. (2006). Protein thiol oxidation in health and disease: Techniques for measuring disulfides and related modifications in complex protein mixtures. *Free Radical Biology & Medicine*, **40**, 1889-1899.
- EFSA 2010: Scientific Opinion on Flavouring Group Evaluation 91 (FGE.91): Consideration of simple aliphatic and aromatic sulphides and thiols evaluated by JECFA (53rd and 68th meetings) structurally related to aliphatic and alicyclic mono-, di-, tri-, and polysulphides with or without additional oxygenated functional groups evaluated by EFSA in FGE.08Rev1 (2009). *EFSA Journal* 2010;8(10):1337.
- El Msehli, S., Lambert, A., Baldacci-Cresp, F., Hopkins, J., Boncompagni, E., Smiti, S.A., Hérouart, D., Frendo, P. (2011). Crucial role of (homo)glutathione in nitrogen fixation in *Medicago truncatula* nodules. *New Phytologist*, **192**, 496-506.

- El-Zohri, M.H.A., Cabala, R., Frank, H. (2005). Quantification of phytochelatins in plants by reversed-phase HPLC-ESI-MS-MS. *Analytical and Bioanalytical Chemistry*, **382**, 1871-1876.
- Ellman G.L. (1959). Tissue sulfhydryl groups. *Archives of Biochemistry and Biophysics*, **82**, 70-77.
- Fahey, R.C., Newton, G.L. (1987). Determination of low-molecular-weight thiols using monobromobimane fluorescent labeling and high-performance liquid chromatography. *Methods in Enzymology*, **143**, 85-96.
- Ferretti, M., Destro, T., Tosatto, S.C., La Rocca, N., Rascio, N., Masi, A. (2009). Gamma-glutamyl transferase in the cell wall participates in extracellular glutathione salvage from the root apoplast. *New Phytologist*, **181**, 115-126.
- Frendo, P., Matamoros, M.A., Alloing, G., Becana, M. (2013). Thiol-based redox signaling in the nitrogen-fixing symbiosis. *Frontiers in Plant Science*, **4**, 376.
- Fricker, M.D., May, M., Meyer, A.J., Sheard, N., White, N.S. (2000). Measurement of glutathione levels in intact roots of *Arabidopsis*. *Journal of Microscopy*, **198**, 162-173.
- Gallaher, C.M., Gallaher, D.D., Peterson, S. (2012). Development and validation of a spectrophotometric method for quantification of total glucosinolates in cruciferous vegetables. *Journal of Agricultural and Food Chemistry*, **60**, 1358–1362.
- Geu-Flores, F., Møldrup, M.E., Böttcher, C., Olsen, C.E., Scheel, D., Halkier, B.A. (2011). Cytosolic γ -glutamyl peptidases process glutathione conjugates in the biosynthesis of glucosinolates and camalexin in *Arabidopsis*. *The Plant Cell*, **23**, 2456-69.
- Gläser, K., Kanawati, B., Kubo, T., Schmitt-Kopplin, P., Grill, E. (2014). Exploring the *Arabidopsis* sulfur metabolome. *The Plant Journal*, **77**, 31-45.
- Goodson, R.J., Katre, N.V. (1990). Site-directed pegylation of recombinant interleukin-2 at its glycosylation site. *Biotechnology*, **8**, 343-346.
- Goraca, A., Huk-Kolega, H., Piechota, A., Kleniewska, P., Ciejka, E., Skibska, B. (2011). Lipoic acid - biological activity and therapeutic potential. *Pharmacological Reports*, **63**, 849-858.
- Grassetti, D.R., Murray, J.F. Jr. (1967). Determination of sulfhydryl groups with 2,2'- or 4,4'-dithiodipyridine. *Archives of Biochemistry and Biophysics*, **119**, 41–49.
- Gregory, J.D. (1955). The stability of N-ethylmaleimide and its reaction with sulfhydryl groups. *Journal of the American Chemical Society*, **77**, 3922-3923.
- Griffith, O.W. (1980). Determination of glutathione and glutathione disulfide using glutathione-reductase and 2-vinylpyridine. *Analytical Biochemistry*, **106**, 207–212.

Grill, E., Winnacker, E.L., Zenk, M.H. (1985). Phytochelatins: the principal heavy-metal complexing peptides of higher plants. *Science*, **230**, 674-676.

Grudkowska, M., Zagdańska, B. (2004). Multifunctional role of plant cysteine proteinases. *Acta Biochimica Polonica*, **51**, 609-624.

Guan, X., Hoffman, B., Dwivedi, C., Matthees, D.P. (2003). A simultaneous liquid chromatography/mass spectrometric assay of glutathione, cysteine, homocysteine and their disulfides in biological samples. *Journal of Pharmaceutical and Biomedical Analysis*, **31**, 251-261.

Gueguen, V., Macherel, D., Jaquinod, M., Douce, R., Bourguignoni, J. (2000). Fatty acid and lipoic acid biosynthesis in higher plant mitochondria. *The Journal of Biological Chemistry*, **275**, 5016–5025.

Hamamoto, A., Mazelis, M. (1985). The C-S lyases of higher plants: direct comparison of the physical properties of homogeneous alliin lyase of garlic (*Allium sativum*) and onion (*Allium cepa*). *Plant Physiology*, **80**, 702-706.

Hanke, G., Mulo, P. (2013). Plant type ferredoxins and ferredoxin-dependent metabolism. *Plant Cell & Environment*, **36**, 1071-1084.

Hansen, R.E., Winther, J.R. (2009). An introduction to methods for analyzing thiols and disulfides: reactions, reagents and practical considerations. *Analytical Biochemistry*, **394**, 147-158.

Hartmann, T.N., Fricker, M.D., Rennenberg, H., Mayer, A.J. (2003). Cell-specific measurement of cytosolic glutathione in poplar leaves. *Plant Cell & Environment*, **26**, 965-975.

Hassinen, V.H., Tervahauta, A.I., Schat, H., Kärenlampi, S.O. (2010). Plant metallothioneins – metal chelators with ROS scavenging activity? *Plant Biology*, **13**, 225–232.

Haugaard, N. (2000). Reflections on the role of the thiol group in biology. *Annals of the New York Academy of Sciences*, **899**, 148–158.

Hell, R., Wirtz, M. (2011). Molecular biology, biochemistry and cellular physiology of cysteine metabolism in *Arabidopsis thaliana*. *Arabidopsis Book*, **9**, e0154.

Hesse, H., Hoefgen, R. (2003). Molecular aspects of methionine biosynthesis. *Trends in Plant Science*, **8**, 259-262.

Hesse, H., Kreft, O., Maimann, S., Zeh, M., Hoefgen, R. (2004). Current understanding of the regulation of methionine biosynthesis in plants. *Journal of Experimental Botany*, **55**, 1799-1808.

- Hinterholzer, A., Schieberle, P. (1998). Identification of the most odour-active volatiles in fresh, hand-extracted juice of Valencia late oranges by odour dilution techniques. *Flavour and Fragrance Journal*, **13**, 49-55.
- Hopkins, R.J., van Dam, N.M., van Loon, J.J. (2009). Role of glucosinolates in insect-plant relationships and multitrophic interactions. *Annual Review of Entomology*, **54**, 57-83.
- Hortin, G.L., Seam, N., Hoehn, G.T. (2006). Bound homocysteine, cysteine, and cysteinylglycine distribution between albumin and globulins. *Clinical Chemistry*, **52**, 2258-2264.
- Hothorn, M., Wachter, A., Gromes, R., Stuwe, T., Rausch, T., Scheffzek, K. (2006). Structural basis for the redox control of plant glutamate cysteine ligase. *The Journal of Biological Chemistry*, **281**, 27557-27565.
- Hsu, C.C., Huang, C.N., Hung, Y.C., Yin, M.C. (2004). Five cysteine-containing compounds have antioxidative activity in Balb/cA mice. *Journal of Nutrition*. **134**, 149-152.
- Huang, T.M., Deng, C.H., Yu, Y.J., Zheng, X.W., Duan, G.L. (2006). Determination of tiopronin in human plasma by SPE then reversed-phase HPLC-UV. *Chromatographia*, **63**, 551-556.
- Huang, K.K., Han, C.H., Sun, J.Y., Niu, D.J., Wu, Z.W., Xue, Y.P., Zhang, L.J., Xiong, X.Q. (2010). Sensitive determination of thiols using SPE coupled to LC with fluorescence detection. *Chromatographia*, **72**, 1049-1054.
- Jacob, C., Giles, G.I., Giles, N.M., Sies, H. (2003). Sulfur and selenium: the role of oxidation state in the protein structure and functions. *Angewandte Chemie International Edition in English*, **42**, 4742-58.
- Jeitner, T.M., Lawrence, D.A. (2001). Mechanisms for the toxicity of cysteamine. *Toxicological Sciences*, **63**, 57-64.
- Jezek, J., Haggett, B.G.D., Atkinson, A., Rawson, D.M. (1999). Determination of glucosinolates using their alkaline degradation and reaction with ferricyanide. *Journal of Agricultural and Food Chemistry*, **47**, 4669-4674.
- Joly, J.C., Swartz, J. (1997). *In vitro* and *in vivo* redox states of the *Escherichia coli* periplasmic oxidoreductases DsbA and DsbC. *Biochemistry*, **36**, 10067-10072.
- Jones, P.R., Manabe, T., Awazuhara, M., Saito, K. (2003). A new member of plant CS-lyases. A cystine lyase from *Arabidopsis thaliana*. *The Journal of Biological Chemistry*, **278**, 10291-10296.

- Jozefczak, M., Remans, T., Vangronsveld, J., Cuypers, A. (2012). Glutathione is a key player in metal-induced oxidative stress defenses. *International Journal of Molecular Sciences*, **13**, 3145-3175.
- Kaleta, C., Gohler, A., Schuster, S., Jahreis, K., Guthke, R., Nikolajewa, S. (2010). Integrative inference of gene-regulatory networks in *Escherichia coli* using information theoretic concepts and sequence analysis. *BMC Systems Biology*, **4**, 116.
- Kanaoka, Y., Machida, M., Ando, K., Sekine, T. (1970). Fluorescence and structures of proteins as measured by incorporation of fluorophore. IV. Synthesis and fluorescence characteristics of N-[p-(2-benzimidazolyl)phenyl]maleimide. *Biochimica et Biophysica Acta*, **207**, 269-277.
- Kand'ár, R., Záková, P., Lotková, H., Kucera, O., Cervinková, Z. (2007). Determination of reduced and oxidized glutathione in biological samples using liquid chromatography with fluorimetric detection. *Journal of Pharmaceutical and Biomedical Analysis*, **43**, 1382-1387.
- Kaniowska, E., Chwatko, G., Glowacki, R., Kubalczyk, P., Bald, E. (1998). Urinary excretion measurement of cysteine and homocysteine in the form of their S-pyridinium derivatives by high-performance liquid chromatography with ultraviolet detection. *Journal of Chromatography A*, **798**, 27-35.
- Khan, N.A., Khan, M.I.R., Asgher, M., Fatma, M., Masood, A., Syeed, S. (2014). Salinity tolerance in plants: revisiting the role of sulfur metabolites. *Journal of Plant Biochemistry & Physiology*, **2**, 1.
- Klapheck, S., Zopes, H., Levels, H.G., Bergmann, L. (1988). Properties and localization of the homoglutathione synthetase from *Phaseolus coccineus* leaves. *Physiologia Plantarum*, **74**, 733-739.
- Klapheck, S., Chrost, B., Starke, J., Zimmermann, H. (1992). γ -Glutamylcysteinylserine: a new homologue of glutathione in plants of the family *Poaceae*. *Botanica Acta*, **105**, 174-179.
- Klein, M., Papenbrock, J. (2004). The multi-protein family of *Arabidopsis* sulphotransferases and their relatives in other plant species. *Journal of Experimental Botany*, **55**, 1809-1820.
- Kunert, K.J., Foyer, C.H. (1993). Thiol/disulphide exchange in plants. In: de Kok, L.J., Stulen, I., Rennenberg, H., Brunold, C., Rauser, W.E. (Eds.), *Sulfur nutrition and sulfur assimilation in higher plants. Regulatory, Agricultural and Environmental Aspects*, SPB Academic, The Hague, The Netherlands, pp. 139-151.
- Kwon, Y.H., Stipanuk, M.H. (2001). Cysteine regulates expression of cysteine dioxygenase and gamma-glutamylcysteine synthetase in cultured rat hepatocytes. *American Journal of Physiology. Endocrinology and Metabolism*, **280**, E804-815.

- Laszlo, P., Mathy, A. (1984). A novel, useful, and inexpensive preparation of S-methyl methanesulfonothioate. *The Journal of Organic Chemistry*, **49**, 2281.
- Leichert, L.I., Gehrke, F., Gudiseva, H.V., Blackwell, T., Ilbert, M., Walker, A.K., Stahler, J.R., Andrew, P.C., Jakob, U. (2008). Quantifying changes in the thiol redox proteome upon oxidative stress in vivo. *Proceedings of the National Academy of Sciences of the United States of America*, **105**, 8197-8202.
- Leustek, T., Saito, K. (1999). Sulphate transport and assimilation in plants. *Plant Physiology*, **120**, 637-644.
- Leustek, T., Martin, M.N., Bick, J.A., Davies, P. (2000). Pathways and regulation of sulfur metabolism revealed through molecular and genetics studies. *Annual Review of Plant Physiology and Plant Molecular Biology*, **51**, 141-165.
- Leustek, T. (2002). Sulfate metabolism. *Arabidopsis Book*, **1**, e0017.
- Liang, S.C., Wang, H., Zhang, Z.M., Zhang, H.S. (2005). Determination of thiol by high-performance liquid chromatography and fluorescence detection with 5-methyl-(2-(m-iodoacetylaminophenyl)benzoxazole). *Analytical and Bioanalytical Chemistry*, **381**, 1095-1100.
- Lindorff-Larsen, K., Winther, J.R. (2000). Thiol alkylation below neutral pH. *Analytical Biochemistry*, **286**, 308-310.
- Lu, C., Zu, Y., Yam, V.W. (2007). Nonionic surfactant-capped gold nanoparticles as postcolumn reagents for high-performance liquid chromatography assay of low-molecular-mass biothiols. *Journal of Chromatography A*, **1163**, 328-332.
- Macnicol, P.K., Datko, A.H., Giovanelli, J., Mudd, S.H. (1981). Homocysteine biosynthesis in green plants: physiological importance of the transsulfuration pathway in *Lemna paucicostata*. *Plant Physiology*, **68**, 619-625.
- Maeda, K., Finnie, C., Svensson, B. (2005). Identification of thioredoxin h-reducible disulphides in proteomes by differential labelling of cysteines: Insight into recognition and regulation of proteins in barley seeds by thioredoxin h. *Proteomics*, **5**, 1634-1644.
- Masi, A., Ghisi, R., Ferretti, M. (2002). Measuring low-molecular-weight thiols by detecting the fluorescence of their SBD-derivatives: application to studies of diurnal and UV-B induced changes in *Zea mays* L. *Journal of Plant Physiology*, **159**, 499-507.
- McCoy, E. (2012). Aspects of taurine chemistry in different cell types. In: El Idrissi, A., L'Amoreaux, W. (Eds.), *Taurine in Health and Disease*, Transworld Research Network, Kerala, India, pp. 5-22.
- McDonagh, B., Requejo, R., Fuentes-Almagro, C.A., Ogueta, S., Bàrcena, J.A., Padilla, C.A. (2011). Thiol redox proteomics identifies differential targets of cytosolic and

mitochondrial glutaredoxin-2 isoforms in *Saccharomyces cerevisiae*. Reversible S-glutathionylation of DHBP synthase (RIB3). *Journal of Proteomics*, **74**, 2487-2497.

Meister, A. (1988). Glutathione metabolism and its selective modification. *The Journal of Biological Chemistry*, **263**, 17205-17208.

Messens, J., Rouhier, N., Couillet, J.F. (2013). Redox homeostasis. In: Jakob, U., Reichmann, D. (Eds.), *Oxidative stress and redox modulation*. Brussels, Belgium, Springer, pp. 59-84.

Meyer, A.J., Hell, R. (2005). Glutathione homeostasis and redox regulation by sulfhydryl groups. *Photosynthesis Research*, **6**, 435-457.

Miller, D.M. (1985). Studies of Root Function in *Zea mays*. *Plant Physiology*, **77**, 162-167.

Miller, S.L., Schlesinger, G. (1993). Prebiotic studies of vitamin coenzymes: I. Cysteamine and 2-mercaptoethanesulfonic acid (coenzyme M). *Journal of Molecular Evolution*, **36**, 302-307.

Miller, J.R., Busby, R.W., Jordan, S.W., Cheek, J., Henshaw, T.F., Ashley, G.W., Broderick, J.B., Cronan Jr, J.E., Marletta, M.A. (2000). *Escherichia coli* LipA is a lipoyl synthase: *in vitro* biosynthesis of lipoylated pyruvate dehydrogenase complex from octanoyl-acyl carrier protein. *Biochemistry*, **39**, 15166-15178.

Mishra, S., Srivastava, A., Tripathi, R.D., Trivedi, P.K. (2008). Thiol metabolism and antioxidant systems complement each other during arsenate detoxification in *Ceratophyllum demersum* L. *Aquatic Toxicology*, **86**, 205-215.

Moini, H., Packer, L., Saris, N.E. (2002). Antioxidant and prooxidant activities of α -lipoic acid and dihydrolipoic acid. *Toxicology and Applied Pharmacology*, **182**, 84-90.

Møldrup, M.E., Geu-Flores, F., Halkier, B.A. (2013). Assigning gene function in biosynthetic pathways: camalexin and beyond. *The Plant Cell*, **25**, 360-7.

Moreno, J., García-Murria, M.J., Marín-Navarro, J. (2008). Redox modulation of RuBisCO conformation and activity through its cysteine residues. *Journal of Experimental Botany*, **59**, 1605-1614.

Musenga, A., Mandrioli, R., Bonifazi, P., Kenndler, E., Pompei, A., Raggi, M.A. (2007). Sensitive and selective determination of glutathione in probiotic bacteria by capillary electrophoresis-laser induced fluorescence. *Analytical and Bioanalytical Chemistry*, **387**, 917-924.

Nakashima, K., Akimoto, H., Nishida, K., Nakatsuji, S., Akiyama, S. (1985). A new fluorogenic thiol-selective reagent: N-{p-[2-(6-dimethylamino)benzofuranyl]phenyl}maleimide. *Talanta*, **32**, 167-169.

- Navari-Izzo, F., Quartacci, M.F., Sgherri, C. (2002). Lipoic acid: a unique antioxidant in the detoxification of activated oxygen species. *Plant Physiology and Biochemistry*, **40**, 463-470.
- Noctor, G., Foyer, C.H. (1998). Ascorbate and glutathione: keeping active oxygen under control. *Annual Review of Plant Physiology and Plant Molecular Biology*, **49**, 249-279.
- Noctor, G., Gomez, L., Vanacker, H., Foyer, C.H. (2002). Interactions between biosynthesis, compartmentation and transport in the control of glutathione homeostasis and signalling. *Journal of Experimental Botany*, **53**, 1283-1304.
- Noctor, G., Queval, G., Mhamdi, A., Chaouch, S., Foyer, C.H. (2011). Glutathione. *Arabidopsis Book*, **9**, e0142.
- Noctor, G., Mhamdi, A., Chaouch, S., Han, Y.I., Neukermans, J., Marquez-Garcia, B., Queval, G., Foyer, C.H. (2012). Glutathione in plants: an integrated overview. *Plant Cell & Environment*, **35**, 454-484.
- Oe, T., Ohyagi, T., Naganuma, A. (1998). Determination of γ -glutamylglutathione and other low-molecular-mass biological thiol compounds by isocratic high-performance liquid chromatography with fluorimetric detection. *Journal of Chromatography B: Biomedical Sciences and Applications*, **708**, 285–289.
- Ogawa, K. (2005). Glutathione-associated regulation of plant growth and stress responses. *Antioxidants & Redox Signaling*, **7**, 973–981.
- Olm, E., Fernandes, A.P., Hebert, C., Rundlöf, A.K., Larsen, E.H., Danielsson, O., Björnstedt, M. (2009). Extracellular thiol-assisted selenium uptake dependent on the x(c)-cystine transporter explains the cancer-specific cytotoxicity of selenite. *Proceedings of the National Academy of Sciences of the United States of America*, **106**, 11400-11405.
- Pal, R., Rai, J.P. (2010). Phytochelatins: peptides involved in heavy metal detoxification. *Applied Biochemistry and Biotechnology*, **160**, 945-963.
- Pasternak, T., Asard, H., Potters, G., Jansen, M.A.K. (2014). The thiol compounds glutathione and homoglutathione differentially affect cell development in alfalfa (*Medicago sativa* L.). *Plant Physiology and Biochemistry*, **74**, 16-23.
- Pastore, A., Piemonte, F. (2012). S-Glutathionylation signaling in cell biology: progress and prospects. *European Journal of Pharmaceutical Sciences*, **46**, 279-292.
- Peng, H., Chen, W., Cheng, Y., Hakuna, L., Strongin, R., Wang, B. (2012). Thiol reactive probes and chemosensors. *Sensors (Basel)*, **12**, 15907-15946.
- Petraglia, A., De Benedictis, M., Degola, F., Pastore, G., Calcagno, M., Ruotolo, R., Mengoni, A., Sanità di Toppi, L. (2014). The capability to synthesize phytochelatins and the presence of constitutive and functional phytochelatin synthases are ancestral

(plesiomorphic) character for basal land plants. *Journal of Experimental Botany*, **65**, 1153-1163.

Pilon-Smits, E.A.H., Pilon, M. (2006). Sulfur metabolism in plastids. In: Wise, R.R., Hooper, J.K. (Eds.), *The structure and function of plastids*. Springer, AA Dordrecht, The Netherlands, pp. 387–402.

Potesil, D., Petrlova, J., Adam, V., Vacek, J., Klejdus, B., Zehnalek, J., Trnkova, L., Havel, L., Kizek, R. (2005). Simultaneous femtomole determination of cysteine, reduced and oxidized glutathione, and phytochelatin in maize (*Zea mays* L.) kernels using high-performance liquid chromatography with electrochemical detection. *Journal of Chromatography A*, **1084**, 134-144.

Pucciariello, C., Innocenti, G., Van de Velde, W., Lambert, A., Hopkins, J., Clémen, M., Ponchet, M., Pauly, N., Goormachtig, S., Holsters, M., Puppo, A., Frendo, P. (2009). (Homo)glutathione depletion modulates host gene expression during the symbiotic interaction between *Medicago truncatula* and *Sinorhizobium meliloti*. *Plant Physiology*, **151**, 1186-96.

Rafii, M., Elango, R., Courtney-Martin, G., House, J.D., Fisher, L., Pencharz, P.B. (2007). High-throughput and simultaneous measurement of homocysteine and cysteine in human plasma and urine by liquid chromatography-electrospray tandem mass spectrometry. *Analytical Biochemistry*, **371**, 71-81.

Ravanel, S., Gakiere, B., Job, D., Douce, R. (1998). The specific features of methionine biosynthesis and metabolism in plants. *Proceedings of the National Academy of Sciences of the United States of America*, **95**, 7805-7812.

Rébeillé, F., Jabrin, S., Bligny, R., Loizeau, K., Gambonnet, B., Van Wilder, V., Douce, R., Ravanel, S. (2006). Methionine catabolism in *Arabidopsis* cells is initiated by a γ -cleavage process and leads to S-methylcysteine and isoleucine synthesis. *Proceedings of the National Academy of Sciences of the United States of America*, **103**, 15687–15692.

Reed, L.J., DeBusk, B.G., Gunsalus, I.C., Hornberger Jr, C.S. (1951). Crystalline alpha-lipoic acid: a catalytic agent associated with pyruvate dehydrogenase. *Science*, **114**, 93–94.

Reed, D.J., Babson, J.R., Beatty, P.W., Brodie, A.E., Ellis, W.W., Potter, D.W. (1980). High-performance liquid chromatography analysis of nanomole levels of glutathione, glutathione disulfide, and related thiols and disulfides. *Analytical Biochemistry*, **106**, 55-62.

Reddy, P.Y., Kondo, S., Fujita, S., Toru, T. (1998). Efficient synthesis of fluorophore-linked maleimide derivatives. *Synthesis*, **7**, 999-1002.

Rennenberg, H., Filner, P. (1982). Stimulation of h(2)s emission from pumpkin leaves by inhibition of glutathione synthesis. *Plant Physiology*, **69**, 766-770.

- Rennenberg, H., Lamoureux, G.L. (1993). Physiological processes that modulate the concentration of glutathione in plant cells. In: de Kok, L.J., Stulen, I., Rennenberg, H., Brunold, C., Rauser, W.E. (Eds.), Sulfur nutrition and sulfur assimilation in higher plants. SPB Academic, The Hague, The Netherlands, pp. 53–65.
- Rhaza, L., Cazalisa, R., Lemelina, E., Aussenac, T. (2003). Changes in the glutathione thiol–disulfide status during wheat grain development. *Plant Physiology and Biochemistry*, **41**, 895–902.
- Roj, S. (2006). S-Adenosyl-l-methionine: beyond the universal methyl group donor. *Phytochemistry*, **15**, 1686-1689.
- Roland, A., Schneider, R., Razungles, A., Cavelier, F. (2011). Varietal thiols in wine: discovery, analysis and applications. *Chemical Reviews*, **111**, 7355–7376.
- Romano, A.H., Nickerson, W.J. (1954). Cystine reductase of pea seeds and yeasts. *The Journal of Biological Chemistry*, **208**, 409–416.
- Rouhier, N., Lemaire, S.D., Jacquot, J.P. (2008). The role of glutathione in photosynthetic organisms: emerging functions for glutaredoxins and glutathionylation. *Annual Review of Plant Biology*, **59**, 143–166.
- Ruseva, S., Vasileva, A., Aleksandrova, D., Lozanov, V., Mitev, V. (2014). Liquid chromatography orbitrap mass spectrometry method for analysis of homocysteine and related aminothiols in cereal products. *Food Analytical Methods*, **7**, 865-871.
- Saito, K. (2004). Sulfur assimilatory metabolism. The long and smelling road. *Plant Physiology*, **136**, 2443-2450.
- Sass, J.O., Endres, W. (1997) Quantitation of total homocysteine in human plasma by derivatization to its N(O,S)-propoxycarbonyl propyl ester and gas chromatography–mass spectrometry analysis. *Journal of Chromatography A*, **776**, 342–347.
- Satoh, S., Shindoh, M., Min, J.Z., Toyo’oka, T., Fukushima, T., Inagaki, S. (2008). Selective and sensitive determination of lipoyllysine (protein-bound α -lipoic acid) in biological specimens by high-performance liquid chromatography with fluorescence detection. *Analytica Chimica Acta*, **618**, 210–217.
- Sgherri, C., Quartacci, M.F., Izzo, R., Navari-Izzo, F. (2002). Relation between lipoic acid and cell redox status in wheat grown in excess copper. *Plant Physiology and Biochemistry*, **40**, 591-597.
- Shewry, P.R., Napier, J.A., Tatham, A.S. (1995). Seed storage proteins: structures and biosynthesis. *Plant Cell*, **7**, 945-956.
- Shinohara, Y., Hasegawa, H., Tagoku, K., Hashimoto, T. (2001). Simultaneous determination of methionine and total homocysteine in human plasma by gas

chromatography-mass spectrometry. *Journal of Chromatography B: Biomedical Sciences and Applications*, **758**, 283-288.

Shung, E. (1990). Physiological functions and environmental relevance of sulfur-containing secondary metabolites. In: de Kok, L.J., Stulen, I., Rennenberg, H., Brunold, C., Rauser, W.E. (Eds.), *Sulfur nutrition and sulfur assimilation in higher plants*. SPB Academic, The Hague, The Netherlands, pp. 179-190.

Sipos, K., Lange, H., Fekete, Z., Ullmann, P., Lill, R., Kispal, G. (2002). Maturation of cytosolic iron-sulfur proteins requires glutathione. *The Journal of Biological Chemistry*, **277**, 26944-26949.

Skipsey, M., Cummins, I., Andrews, C.J., Jepson, I., Edwards, R. (2005). Manipulation of plant tolerance to herbicides through co-ordinated metabolic engineering of a detoxifying glutathione transferase and thiol cosubstrate. *Plant Biotechnology Journal*, **3**, 409-420.

Spadaro, D., Yun, B.W., Spoel, S.H., Chu, C., Wang, Y.Q., Loake, G.J. (2010). The redox switch: dynamic regulation of protein function by cysteine modifications. *Physiologia Plantarum*, **138**, 360-371.

Starkenmann, C., Troccaz, M., Howell, K. (2008). The role of cysteine and cysteine-S conjugates as odour precursors in the flavour and fragrance industry. *Flavour and Fragrance Journal*, **23**, 369-381.

Su, T., Xu, J., Li, Y., Lei, L., Zhao, L., Yang, H., Feng, J., Liu, G., Ren, D. (2011). Glutathione-Indole-3-Acetonitrile Is Required for Camalexin Biosynthesis in *Arabidopsis thaliana*. *The Plant Cell*, **23**, 364-380.

Su, T., Li, Y., Yang, H., Ren, D. (2013). Reply: complexity in camalexin biosynthesis. *The Plant Cell*, **25**, 367-370.

Tausz, M., Sircelj, H., Grill, D. (2004). The glutathione system as a stress marker in plant ecophysiology: is a stress-response concept valid?. *Journal of Experimental Botany*, **55**, 1955-1962.

Textor, S., Bartram, S., Kroymann, J., Falk, K.L., Hick, A., Pickett, J.A., Gershenzon, J. (2004) Biosynthesis of methionine-derived glucosinolates in *Arabidopsis thaliana*: recombinant expression and characterization of methylthioalkylmalate synthase, the condensing enzyme of the chain-elongation cycle. *Planta*, **218**, 1026-1035.

Tolin, S., Arrigoni, G., Trentin, A.R., Veljovic-Jovanovic, S., Pivato, M., Zechman, B., Masi, A. (2013). Biochemical and quantitative proteomics investigations profiling in *Arabidopsis ggt1* mutant leaves reveal a role for the gamma-glutamyl cycle in plant's adaptation to environment. *Proteomics*, **13**, 2031-2045.

Toyo'oka, T., Imai, K. (1984). New fluorogenic reagent having halogenobenzofurazan structure for thiols: 4-(aminosulfonyl)-7-fluoro-2,1,3-benzoxadiazole. *Analytical Chemistry*, **56**, 2461-2464.

- Toyo'oka, T., Suzuki, T., Saito, Y., Uzu, S., Imai, K. (1989). Fluorogenic reagent for thiols: 4-(N,N-dimethylaminosulphonyl)-7-fluoro-2,1,3-benzoxadiazole. *Analyst*, **114**, 413-419.
- Toyo'oka, T. (2009). Recent advances in separation and detection methods for thiol compounds in biological samples. *Journal of Chromatography B: Analytical Technologies in the Biomedical and Life Sciences*, **877**, 3318-3330.
- Velasco, A.P., Haddad, R., Eberlin, M.N., Hoehr, N.F. (2002). Combined cysteine and homocysteine quantitation in plasma by trap and release membrane introduction mass spectrometry. *Analyst*, **127**, 1050-1053.
- Wang, H., Liang, S.C., Zhang, S.M., Zhang, H.S. (2004). 3-Iodoacetylaminobenzanthrone as a fluorescent derivatizing reagent for thiols in high-performance liquid chromatography. *Analytica Chimica Acta*, **512**, 281–286.
- Winther, J.R., Thorpe, C. (2014). Quantification of thiols and disulfides. *Biochimica et Biophysica Acta*, **1840**, 838-846.
- Wood, B.A., Feldmann, J. (2012). Quantification of phytochelatins and their metal(loid) complexes: critical assessment of current analytical methodology. *Analytical and Bioanalytical Chemistry*, **402**, 3299-3309.
- Yadav, S.K. (2010). Heavy metals toxicity in plants: an overview on the role of glutathione and phytochelatins in heavy metal stress tolerance in plants. *South African Journal of Botany*, **76**, 167-179.
- Yang, J.R., Langmuir, M.E. (1991), Synthesis and properties of a maleimide fluorescent thiol reagent derived from a naphthopyranone. *Journal of Heterocyclic Chemistry*, **28**, 1177–1180.
- Yano, H. (2014). Ongoing applicative studies of plant thioredoxins. *Molecular Plant*, **7**, 4-13.
- Yarmolinsky, D., Brychkova, G., Fluhr, R., Sagi, M. (2013). Sulfite reductase protects plants against sulfite toxicity. *Plant Physiology*, **161**, 725-743.
- Yasuno, R., Wada, H. (1998). Biosynthesis of lipoic acid in *Arabidopsis*: cloning and characterization of the cDNA for lipoic acid synthase. *Plant Physiology*, **118**, 935-943.
- Yasuno, R., Wada, H. (2002). The biosynthetic pathway for lipoic acid is present in plastids and mitochondria in *Arabidopsis thaliana*. *FEBS Letters*, **517**, 110-114.
- Yuan, L., Nelson, B.A., Caryl, G. (1995). The catalytic cysteine and histidine in the plant acyl-acyl carrier protein thioesterases. *The Journal of Biological Chemistry*, **271**, 3417-3419.

Zagorchev, L., Seal, C.E., Kranner, I., Odjakova, M. (2013). A central role for thiols in plant tolerance to abiotic stress. *International Journal of Molecular Sciences*, **14**, 7405-7432.

Zander, T., Phadke, N.D., Bardwell, J.C.A. (1998). Disulfide bond catalysts in *Escherichia coli*. *Methods in Enzymology*, **290**, 59-74.

Zhang, Y., Talalay, P., Cho, C.G., Posner, G.H. (1992). A major inducer of anticarcinogenic protective enzymes from broccoli: isolation and elucidation of structure. *Proceedings of the National Academy of Sciences of the United States of America*, **89**, 2399-2403.

List of Tables

Table 1. Commonly used derivatizing reagents, together with their chemical structure and references.

Table 2. Concentrations and localization of LMW thiols in plant tissues reported in literature.

List of Figures

Figure 1. Central role of Cys and GSH in sulfur-thiol metabolism; in black: thiols, in grey: non-thiol sulfur compounds.

Figure 2. Flow chart of sample preparation and analysis. Given the experimental aims, five main steps are needed to study thiolation PTMs and total thiol content, or to assess the thiol redox state (extraction, reduction, derivatization, separation and detection).

* Free thiols can be identified by MS analysis (both GC- and LC-MS) without any derivatization.

Figure 3. The structure and molecular weight of the best-known LMW thiols in plants.

Figure 4. Cys biosynthesis pathway, adapted from the *Arabidopsis* KEGG “Cysteine and Methionine Metabolism” and “Sulfur metabolism” available online http://www.genome.jp/kegg-bin/show_pathway?ath00270 and http://www.genome.jp/kegg-bin/show_pathway?ath00920

Figure 5. GSH biosynthesis pathway adapted from the *Arabidopsis* KEGG “Cysteine and Methionine Metabolism” available online http://www.genome.jp/kegg-bin/show_pathway?ath00270.

Figure 6. Cysteamine biosynthesis pathway adapted from the *Arabidopsis* KEGG “Taurine and hypotaurine biosynthesis” available online http://www.genome.jp/kegg-bin/show_pathway?ath00430. Dotted arrows refer to a molecular pathway described in mammals by Coloso *et al.*, 2006.

Figure 7. Representative chromatographic separation of LMW thiols from several plant samples, after derivatization with SBD-F: Standards, *Malus domestica* Borkh skin, *Hordeum vulgare* L. root, *Trifolium repens* L., *Brassica oleracea* L., *Capiscum sp.* L., *Juglans regia* L., *Eruca sativa* Mill.. Numbers refer to: 1 Cys; 2 Cysteamine; 3 Hcys; 4 Cys-Gly; 5 γ -Glu-Cys; 6 GSH; 7 putative hGSH, * unknown LMW thiols.

AIM OF THE STUDY

A huge diversity of LMW thiols exists, as evidenced by chromatographic analyses, but many of them are still unknown. Since their concentration is typically very low, their identification is also challenging.

The aim of this work was to identify these LMW thiols by investigating thiol-specific derivatives with the specific fluorescent dye SBD-F. With the help of HPLC coupled with MS, analysis of SBD-derivatives was carried out in search of a distinctive fragmentation pattern in order to establish a protocol that may serve as a tool to enable a systematic identification of LMW thiols in future experiments.

The use of the fluorescent dye for the quantification of LMW thiols was then applied in the winemaking processes with the aim to quantify these compounds in grapes. The method was developed and optimized and it can be applied not only in grape analysis but also in must, wine and yeasts.

CHAPTER II

IDENTIFICATION OF NOVEL THIOL COMPOUNDS

“Identification of low-molecular-weight thiols by HPLC-MS/MS analysis of SBD-derivatives”

Marta Fabrega-Prats¹, Anna Rita Trentin¹, Gregorio Peron², Antonio Masi¹, Stefano Dall’Acqua^{2*}

¹ DAFNAE University of Padua, Viale dell’Università 16, 30520 Legnaro (PD), Italy

² Department of Pharmaceutical and Pharmacological Science, University of Padua, Via Marzolo 5, 35131 Padova (PD), Italy

Abstract

Thiols are reduced sulfur molecules that occur both in plants and animals with relevant roles, as for example the modulation of oxidative stress and participation to enzymatic reactions.

Low-molecular-weight (LMW) thiols are fundamental molecules due to the intrinsic reactivity of their nucleophilic -SH group. They can modify the redox state of sensitive molecules by participating to reversible redox reactions. Moreover, they can conjugate or make complexes with xenobiotics and toxic compounds, and deactivate them; they can post-translationally modify regulatory enzymes and control metabolism. They also have important implications in food quality and safety, as well as in human health. A limited number of LMW thiols are described in the literature, but a huge amount of unknown thiols exists in plants and also in plant derived products. Their identification represents a major challenge since they are at very low concentration in plant tissues.

In order to discover new unidentified thiols, as starting approach, extracts from plants were derivatized with SBD-F (ammonium 7-fluoro 2,1,3-benzoxadiazole-4-sulfonate) and analyzed by HPLC-fluorescence and LC-MS/MS in negative mode using an ion trap (Varian 500 MS), to obtain their fragmentation pattern. Known LMW thiols such as cysteine, homocysteine, glutathione, cysteamine, γ -glutamylcysteine, N-

acetylcysteine, and cysteinylglycine were used as reference compounds. Also high-resolution measurements were obtained on a Xevo G-2 Q-TOF mass spectrometer (Waters).

We have found that after SBD-F derivatization, thiols can be easily recognized in fragmentation spectra due to the presence of the SBD-S fragment (m/z 231), the fluorophore with attached sulfur from the thiol. This signal was then used as a marker to confirm the presence of thiol groups in unknown molecules. In this way, we identified and further confirmed by Q-TOF analysis, the presence of thioglucose and glutathione containing derivatives in *Brassicaceae* and other species such as potato and green chilly.

Furthermore, extracts from different plant species showed distinctive thiol composition, indicating that such compounds are species-specific. In maize leaves, we observed a light dependence of some of these unknown-LMW thiols, suggesting that they could be related to photosynthesis. Given their importance in plant metabolism, and due to the potential health benefits, LMW thiols deserve more attention from the scientific community.

Introduction

Low-molecular-weight (LMW) thiols are a class of highly reactive compounds massively involved in the maintenance of cellular redox homeostasis; they are fundamental molecules due to the intrinsic reactivity of their nucleophilic -SH group. Because of their antioxidant activity, they may have significant implications in food quality and safety, as well as in human health.

Glutathione (GSH) is the most abundant and best described LMW thiol because of its importance in redox and regulatory functions. But there are also a number of less well-known compounds that are generally neglected, playing a significant part in plant metabolism as for example cysteamine and homocysteine (Hcys) (Pivato *et al.*, 2014). Indeed, chromatographic separations by HPLC-fluorescence performed at our lab during this work supported the existence of different unknown thiol-containing molecules at very low concentrations in several plant species, including fruits and vegetables. Some of them are specific to some plants while others have organ/tissue specificity.

Due to their low concentration in plant tissues, their analysis is challenging. The use of HPLC-FL after derivatization allows the quantification and identification of known

thiols. Nevertheless, for the analysis of unknown species, HPLC-MSⁿ and HPLC tandem MS approaches offer the opportunity to study their molecular structure due to the evaluation of fragmentation pattern, as well as exact mass measurements.

To our knowledge, not so many investigations have been published up to now in this field. Some studies referred to the fragmentation pattern of the GSH (Murphy and Fenselau, 1992; Rellán-Álvarez *et al.*, 2006; Xie *et al.*, 2013) where they start showing that this peptide breaks through the peptidic bond either in negative or in positive mode. The fragmentation pattern of GSH and GSSG by LC-ESI-MS/MS was studied showing that these molecules fragment through the peptidic bond (Thakur and Balaram, 2008). Also Feng and colleagues in 2014 performed derivatization of GSH and its isomer GluCysGly (ECG) with CDNB, and after that, they obtained a fragmentation pattern using MS/MS (Feng *et al.*, 2014).

In this project, the idea was to use the derivatization with SBD-F and to follow the MS generated fragments from SBD-derivatives in search of a fragmentation rule, which could be used to assess the presence of unknown LMW thiols in different species. The aim of the study was to identify these thiols by obtaining their LC-MS/MS fragmentation pattern, in order to understand their possible role in plant physiology and metabolism.

Material and methods

HPLC analysis from the standards thiols

A derivatization step was carried out using the method described by Masi *et al.*, (2002) of all the standards (Sigma-Aldrich). It was used a solution at 0.1 mM of every standard diluted with HCl 0.1M. The standards used are GSH, Cys, Hcys, Cys-Gly, NAC, Cysteamine and γ -Glu-Cys. First of all, 1M H₃BO₃ at pH 10.5 was added to provide the solution with alkaline conditions, which are necessary for the following reduction step. Sample reduction was achieved with TBP (tributylphosphine) with the aim to have all –SH groups reduced and afterwards, the fluorophore SBD-F (ammonium 7-fluoro 2,1,3-benzooxadiazole-4-sulfonate) was added, which reacts specifically with the –SH groups. After 1 hour of incubation at 60°C, the reaction was stopped with 4M HCl.

The runs were carried out in a reverse-phase HPLC (RP-HPLC) using the Agilent 1260 Infinity system (Agilent Technologies Inc., Santa Clara, CA) equipped with a spectrofluorimeter (Agilent Technologies Inc., Santa Clara, CA). Aliquots of 5 μ L from

every standard already derivatized were injected. A column Agilent C18 Eclipse Plus (2.1 mm x 150 mm I.D., 3.5 μ m particle size; Agilent Technologies Inc., Santa Clara, CA) was used at a flow rate of 0.220 mL/min at room temperature (RT). Eluents A and B were used for gradient elution. Solution A was 100% methanol and solution B was 75mM NH₄-formiate buffer (pH 2.9). The following gradient was used: 100% B (0 min), 15% B (0-22 min), 15% B (22-24 min), 100% B (24-25 min) and 100% B (25-30 min). Thiols were detected by fluorescence at an excitation wavelength of 386 nm and an emission wavelength of 516 nm. Data were analyzed using the Chemstation software (Agilent Technologies Inc., Santa Clara, CA).

These conditions were used to obtain a good separation of all the standards analyzed, and they were also used for the following analyses.

HPLC-MS/MS from the standards thiols

Once the HPLC conditions were defined, the same standards were injected in the LC-MS system. A Varian 500 LC-ion trap-MS, Prostar model 430 autosampler and two Varian 212-LC pumps (Walnut Creek, CA, USA) were used.

Data acquisition and processing were performed on a Varian MS Workstation Version 6.9.1 (Walnut Creek, CA, USA). For the chromatographic analysis, the same column was used as before. With the same HPLC conditions; but solution B was 1.5 mM NH₄-formiate buffer (pH 2.9) to obtain a good ionization and fragmentation of the compounds in the MS. The compounds were analyzed using electrospray ionization (ESI) in negative mode and drying gas temperature of 350 °C. MS parameters were then optimized by using 5 μ L of each standard already derivatized (Table 1).

Table 1. Parameters and conditions used in the MS.

PARAMETER	CONDITIONS
Ion source	ESI
Capillary voltage	80.0 Volt
Needle voltage	5000 Volt
RF loading	100%
Nebulizing gas	Air
Nebulizing gas pressure	35.0 psi
Drying gas temperature	350 °C
Drying gas pressure	10.0 psi

To obtain fragmentation of unknown species the TDDS mode (Turbo Data Dependent Scanning) was applied, which allowed fragmentation of ions having a sufficient current intensity during the runs. Following, it was done an MSⁿ to fragment the peaks and generate information about the structure of the molecules analyzed. This procedure allowed to see how the standards are fragmented in these conditions and to understand which pattern of fragmentation is needed to follow the unknown and more complex samples.

HPLC-MS/MS from the plant extracts

After having a clear pattern of fragmentation defined by using the standards, the same procedure was used to analyze the plant extracts. In this case, several plants were used, such as wild rocket or arugula (*Diplotaxis tenuifolia*), cultivated arugula (*Eruca sativa*), radish (*Raphanus sativus*) and cauliflower (*Brassica oleracea*); all of them belonging to the same family *Brassicaceae* or also called Cruciferae. Additionally, it was done an analysis using the potato (*Solanum tuberosum* L.) and the green chilly (*Capsicum* L.). Taking 1.5 mg of leaves in the case of the arugula, root in the radish, the head (the white curd) in the cauliflower, and the tuber in the potato plant; i.e. the edible parts of each species; and with the help of liquid nitrogen, they were homogenized with mortar and pestle. Afterwards, 1.5 mL of 0.1 M HCl was added, to have at the end a 1:1 extract. After a centrifugation step, the supernatant was taken and then 50 μ L were derivatized with the procedure explained in the previous section. A volume of 5 μ L of the derivatized product was injected in the machine using the method performed and already explained to the HPLC-MS/MS section.

In all the mass spectra, those ions with a similar pattern of fragmentation of the standards were looked for, speculating that these compounds should be “potential thiols”. After that, the software Varian MS Workstation Version 6.9.1. (Walnut Creek, CA, USA) was used to investigate the pattern of fragmentation of these unknown molecules, in order to assign an identity to the molecules we were looking at. To achieve the molecular structure of these unknown molecules by modifying the chemical groups, it was used the software ChemDraw Ultra 7.0.1. (CambridgeSoft Corporation, Cambridge, USA, <http://www.camsoft.com/>).

Confirmation by qTOF of the unknown molecules

After having the hypothetical molecular structure for these molecules with the help of their pattern of fragmentation found in the ion trap MS, a confirmation of their exact mass was needed.

It was performed an HPLC-MS-qTOF analysis using the Agilent 1290 Infinity system (Agilent Technologies Inc., Santa Clara, CA) coupled with a Xevo[®] G2-S QTOF MS (Waters Corporation, Milford, U.S.A.) to have a good separation of the molecules together with the exact mass (the level of precision of the exact mass determination is 0.001 Da). The ions were produced with the ESI and their detection was achieved using a Time of Flight (ToF) detector. This software also allowed to have the hypothetical molecular formula from the unknown thiols from the database. The same column and method as for the previous analysis were used (Figure 1).

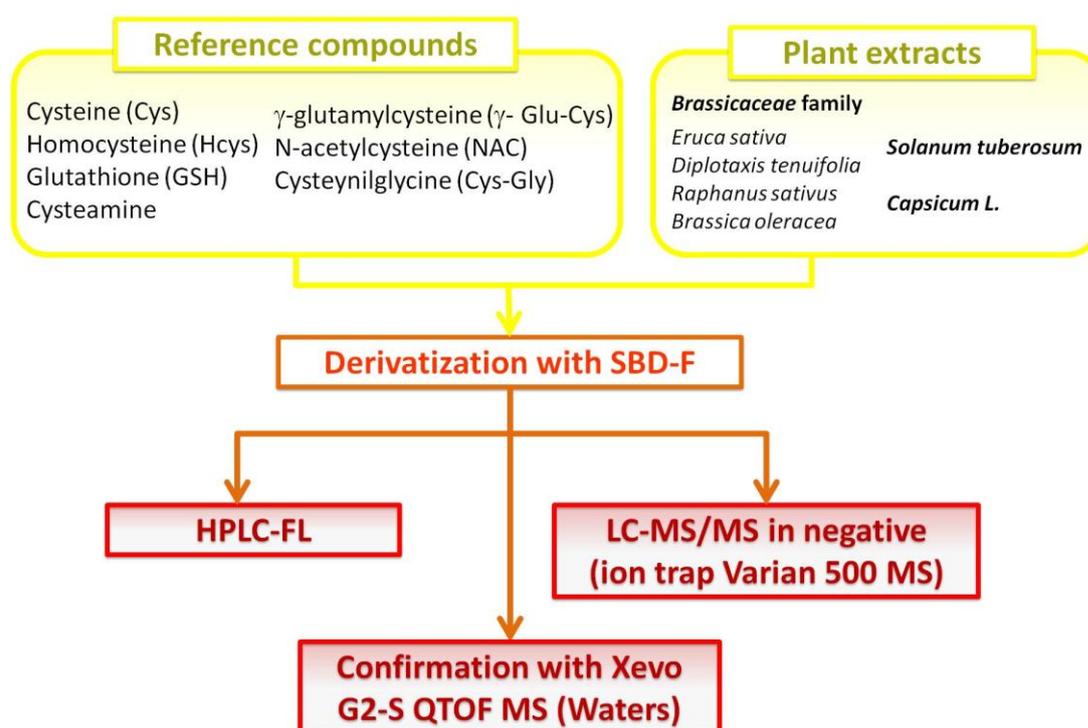


Figure 1. Workflow of the methods used in the study.

Once the exact mass is confirmed, if it is possible and there is the standard in the market, it is needed their run in HPLC-FL and also in HPLC-MS/MS using the same method. If their retention times and the fragmentation pattern of the standard compared

with the hypothetical molecule are the same, it can be totally confirmed the presence of that molecule in the sample and the identification.

Results and discussion

HPLC analysis from the standards thiols

The protocol developed by Masi *et al.*, 2002 was not suitable for the mass spectrometry analysis because it makes use of a nonvolatile buffer (citrate) which has a very high concentration of salt (75 mM). In this case, the aim of the study was not only the good separation of the compounds but also a good fragmentation for studying their fragmentation pattern. Due to this, several buffers were tried together with several columns to find the best for this aim. At a first instance, it was used only the HPLC-FL analysis trying to see the effect using different columns to reach a good chromatographic separation with the less time possible. Then, other buffers which can be also used in MS getting a good ionization of the molecules were also tried like acetic acid or formic acid but in lower concentrations (1-5%).

The first trial was carried out using the column Agilent C8 ZORBAX Eclipse XDB (2.1 mm x 150 mm I.D., 3.5 µm particle size; Agilent Technologies Inc., Santa Clara, CA) with a buffer of 1% of formic acid at a flow rate of 0.200 mL/min at room temperature. The solution A was the buffer and the solution B the methanol. Beginning with 100% of solution A, it was done a gradient in 10 min to 5% of methanol. Then, during 5 min it was going in these conditions and at 16 min of the run went back to 100% solution A till 20 min to get the equilibrium. But in this case, a good separation could not be obtained because all 7 standards were eluted out of the column more or less between 2-3 min without any separation, except NAC which was retained inside the column (results not shown).

After the negative result of the trial, using the same column it was tried to change the flow rate to 0.220 mL/min and till 100% of methanol in the gradient. But also in this trial, the result was the same and the peak shape was worse.

It was then decided to prepare a buffer of 5% of formic acid in water at a flow rate of 0.220 mL/min and with the same gradient till 100% of methanol. But the results were also negative. Due to the chemical structure of the SBD group and also the acid and basic groups from the aminoacids the separation in these cases was not successful.

The change of the column was then decided to an Agilent C18 Eclipse AAA (3 mm x 150 mm I.D., 3.5 μ m particle size; Agilent Technologies Inc., Santa Clara, CA) with 5% of formic acid and the same run conditions but the results were not good in terms of separation. This column is usually used for aminoacids analysis.

After these trials, the conclusion was that it is needed the buffer 75 mM NH₄-formiate buffer (pH 2.9) to have a good separation and change to the column Agilent C18 Eclipse Plus (2.1 mm x 150 mm I.D., 3.5 μ m particle size; Agilent Technologies Inc., Santa Clara, CA). In this way, it was possible to reach the good conditions for the analysis with a good separation of all 7 standards (Table 4) in the HPLC-FL system. Instead of working with isocratic conditions as it is reported in Masi *et al.*, 2002, it was decided to work in gradient with methanol allowing a good separation within only 30 min.

Table 2. Method used in HPLC to have the good separation of the standards.

Time (min)	% methanol (A)	% elution buffer (B)	Flow (μ L/min)
0	0	100	220
22	85	15	220
24	85	15	220
25	0	100	220
30	0	100	220

With the conditions used, there is a good separation of all the standards used in the analysis (Figure 2).

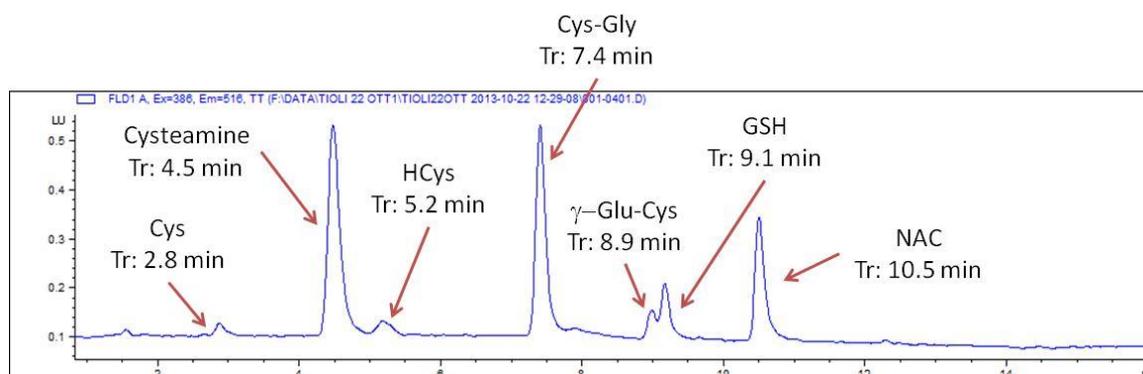


Figure 2. Chromatogram obtained in HPLC-FL, where it is possible to see a good separation of all the standards tried in this study.

HPLC-MS/MS from the standards thiols

After obtaining a good separation of our standards in HPLC-FL, the standards were run in the HPLC-MS. It was needed to change to a lower concentration of formic acid in the buffer. The concentration was 1.5 mM NH₄-formiate buffer (pH 2.9), 50 times more diluted than in the HPLC-FL. In such a way, the retention times of the standards are slightly changed, but the ionization improved significantly.

The first step was to choose the ionization mode of the molecules if it was better in positive or negative ion mode. After running all the standards in both ionizations conditions and looking for the chemical properties of the molecules, it was decided to work in negative mode. Figure 3 and 4 show the case of GSH and the two ionization modes. Even if the area is higher in the positive mode (Figure 4), the background noise is better in the negative one (Figure 3). Also, in the case of positive mode there are not good fragmentations which can be seen due to the molecular structure of the molecules. SBD derivatives do not have good ionization in positive mode, giving no signal.

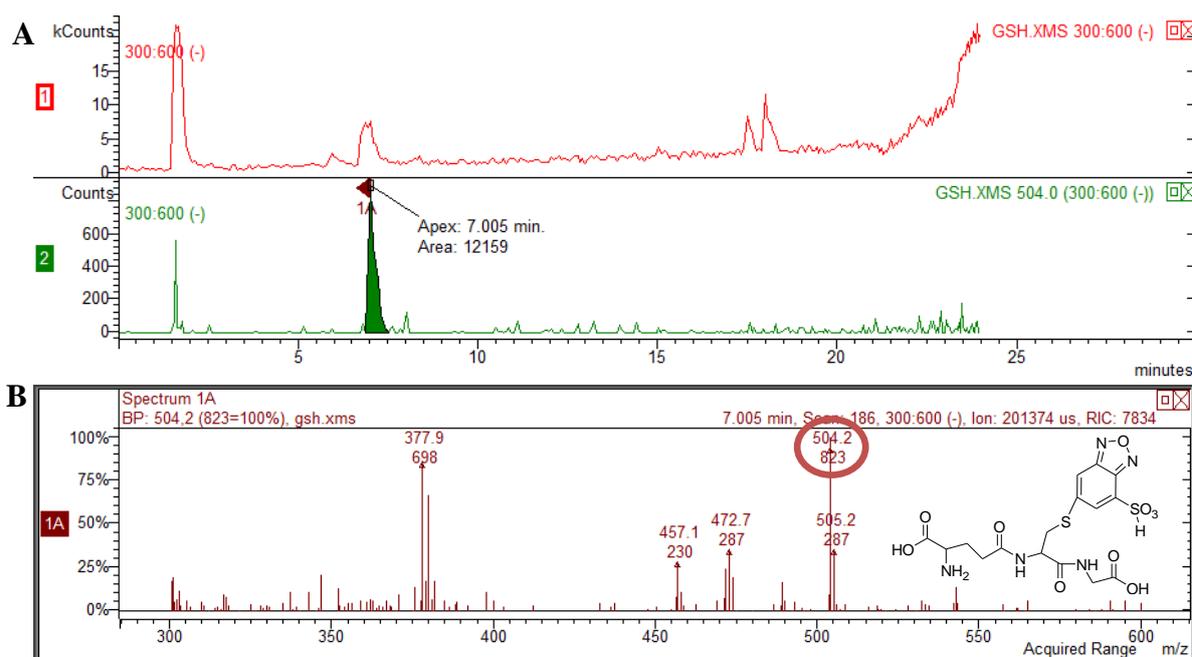


Figure 3. GSH in negative ion mode (m/z 504). (A) HPLC chromatogram, which shows the peak of the molecule obtained. (B) Mass spectra.

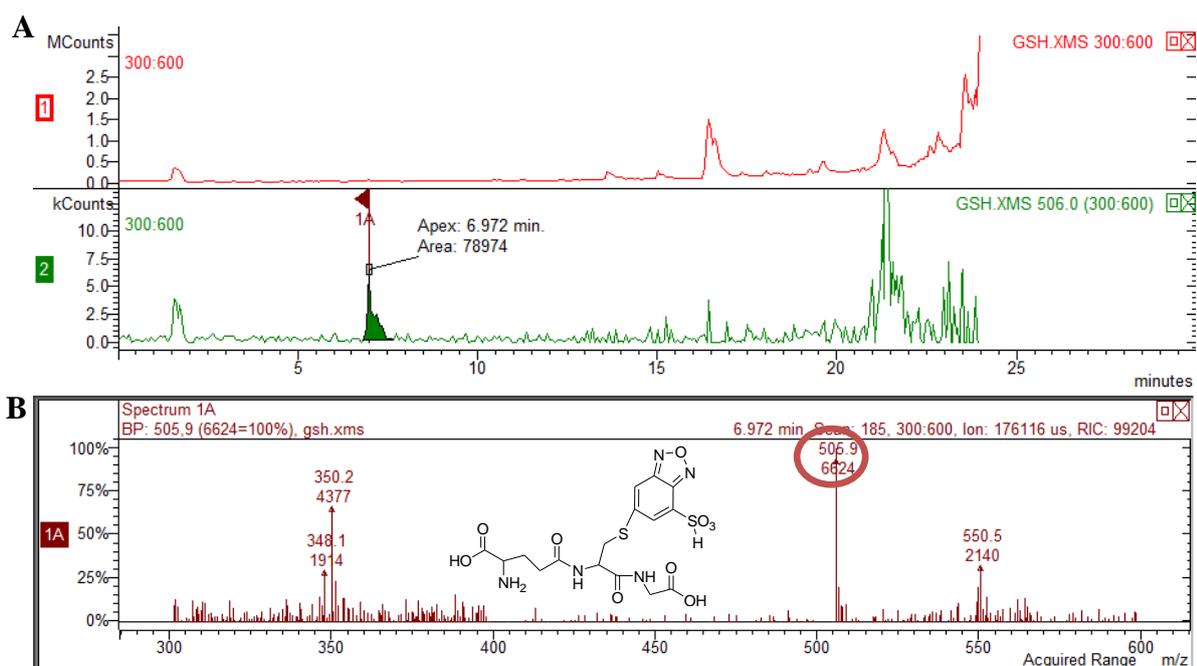


Figure 4. GSH in positive ion mode (m/z 506). (A) HPLC chromatogram, which shows the peak of the molecule obtained. (B) Mass spectra.

All the other standards showed the same behavior as GSH. Figure 5-10 show them in negative ion mode.

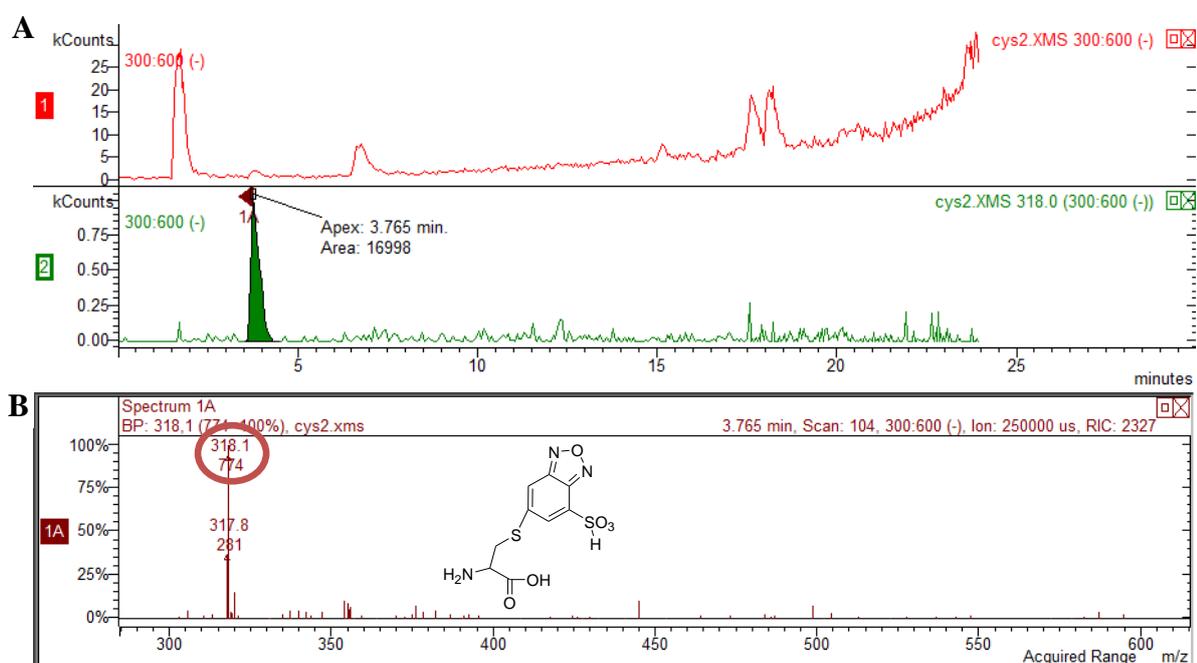


Figure 5. Cys in negative ion mode (m/z 318). (A) HPLC chromatogram, which shows the peak of the molecule obtained. (B) Mass spectra.

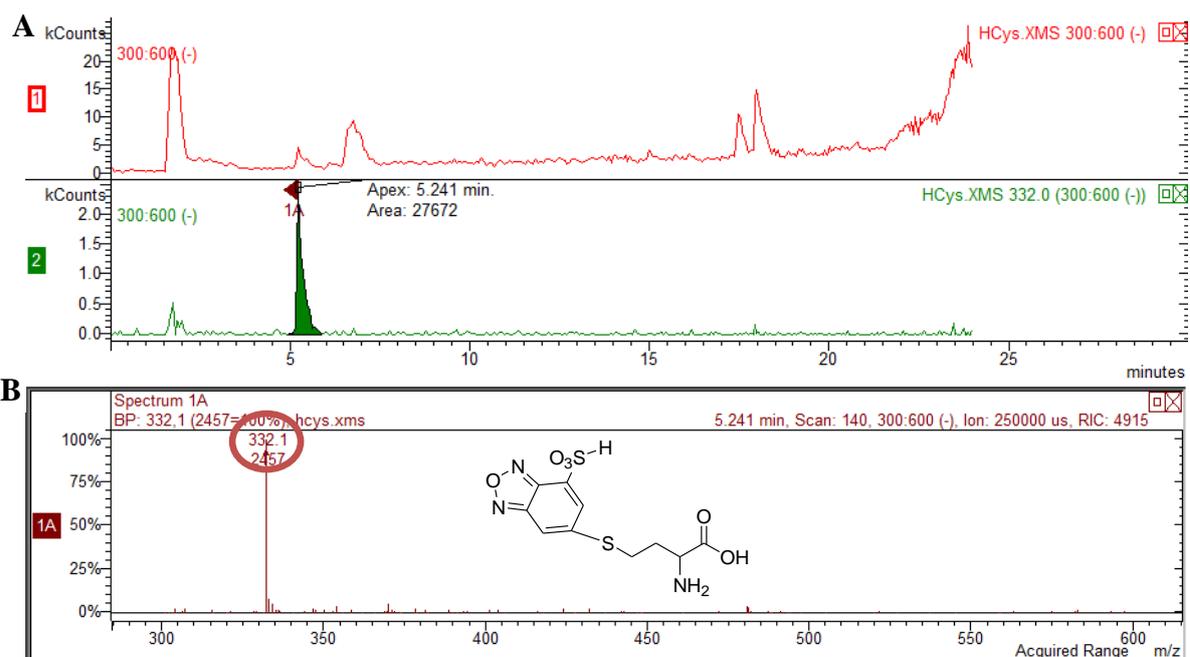


Figure 6. Hcys in negative ion mode (m/z 332). (A) HPLC chromatogram, which shows the peak of the molecule obtained. (B) Mass spectra.

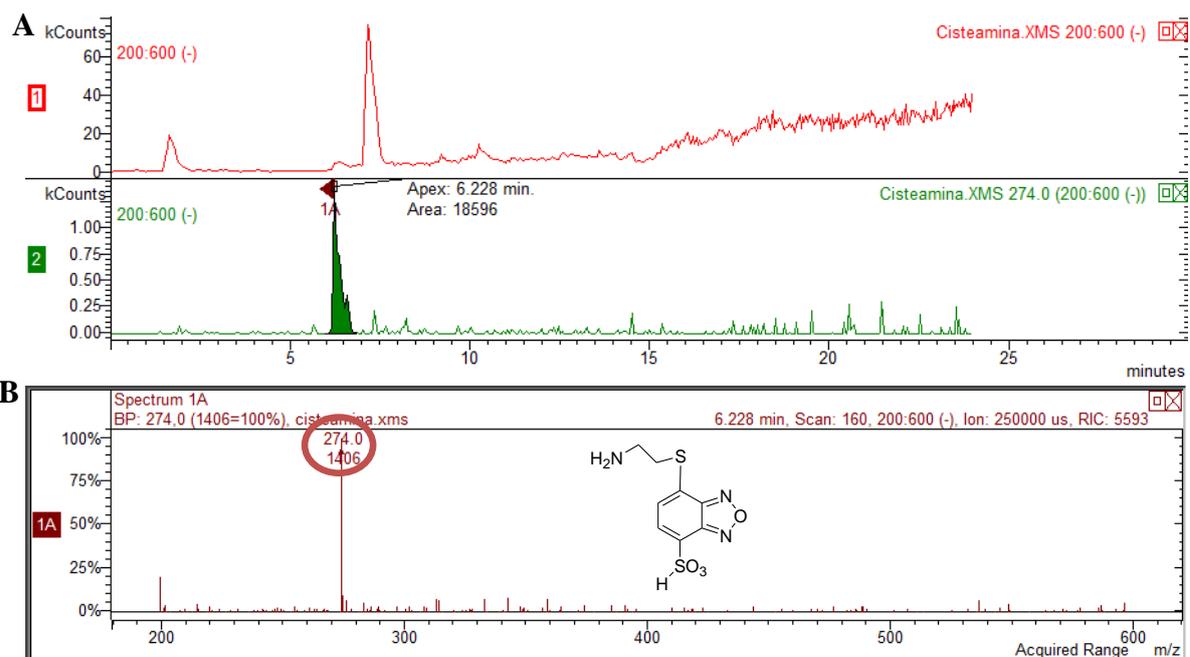


Figure 7. Cysteamine in negative ion mode (m/z 274). (A) HPLC chromatogram, which shows the peak of the molecule obtained. (B) Mass spectra.

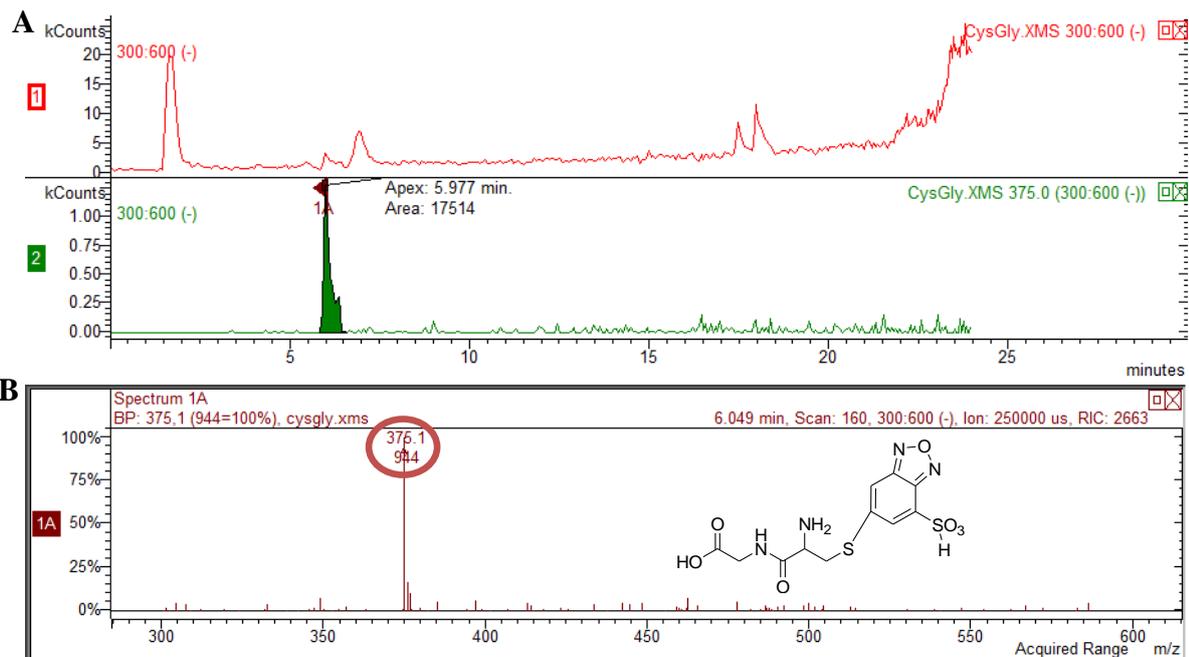


Figure 8. Cys-Gly in negative ion mode (m/z 375). (A) HPLC chromatogram, which shows the peak of the molecule obtained. (B) Mass spectra.

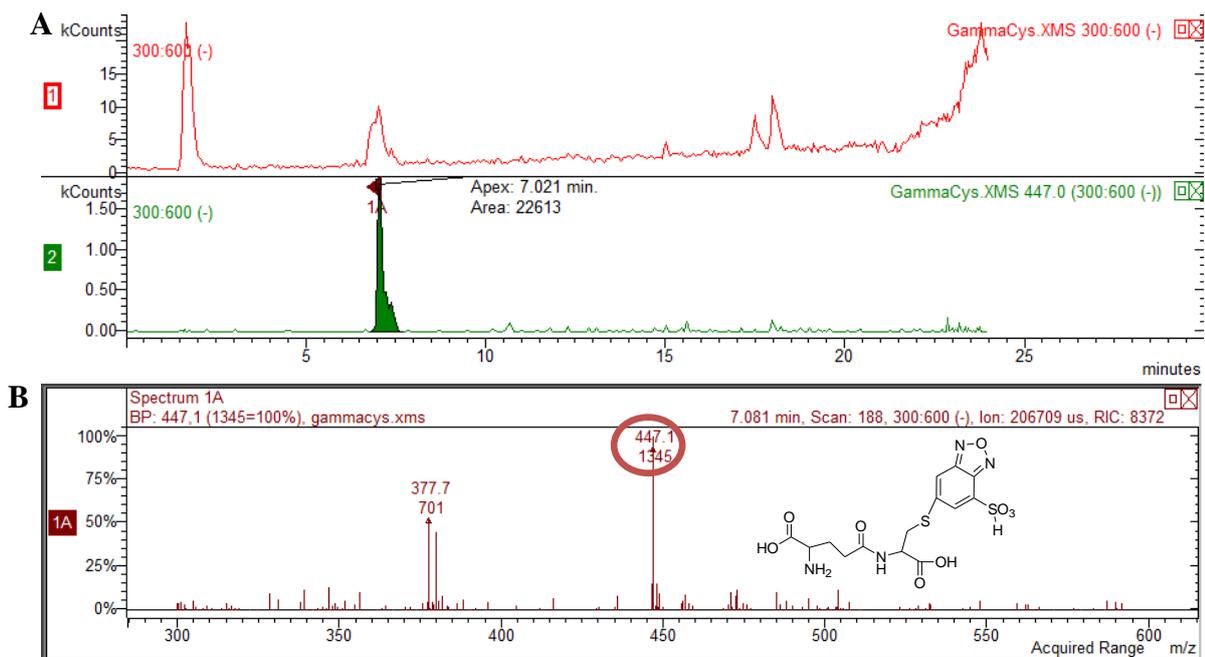


Figure 9. γ -Glu-Cys in negative ion mode (m/z 447). (A) HPLC chromatogram, which shows the peak of the molecule obtained. (B) Mass spectra.

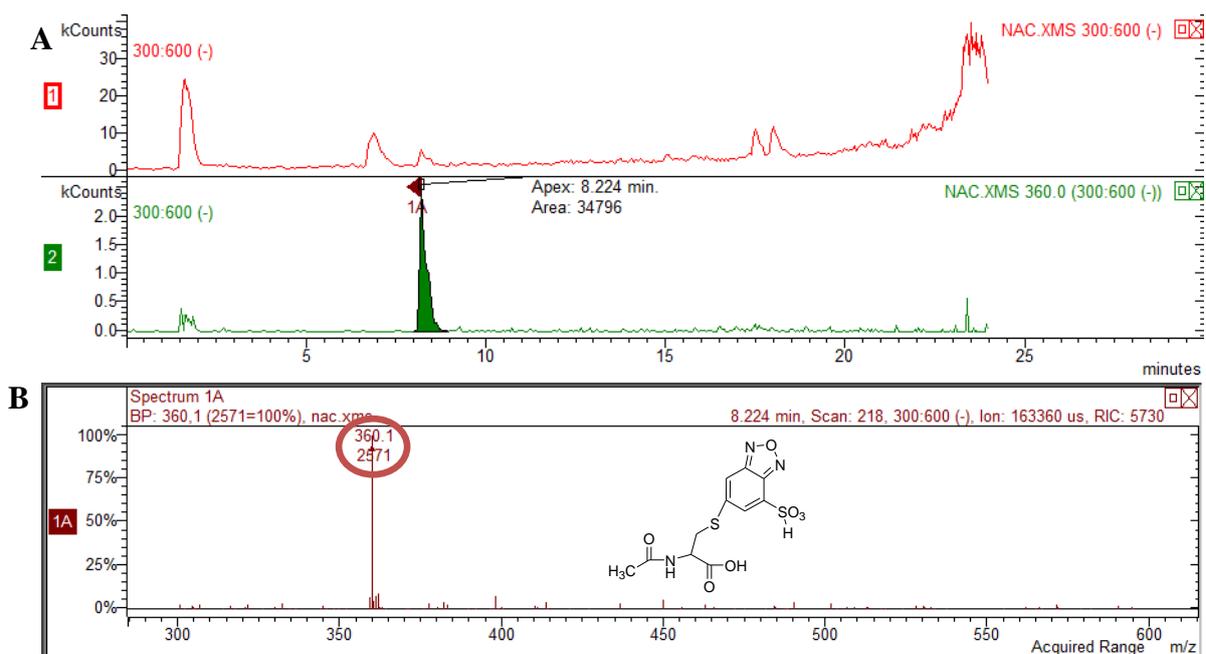


Figure 10. NAC in negative ion mode (m/z 360). (A) HPLC chromatogram, which shows the peak of the molecule obtained. (B) Mass spectra.

Once it has been decided to run the samples in the negative ion mode, the next step was the fragmentations of the standards, to try to understand their pattern of fragmentation. Their mass and corresponding retention times are reported in Table 3.

Table 3. Masses in the negative ion mode from the standard-SBD, and their respective retention times in HPLC-MS, after using the diluted buffer.

Standard	Mass in negative [$M + SBDF - H$] ⁻	Retention Time (min)
Cys	318	3.765
Hcys	332	5.241
Cys-Gly	375	6.049
Cysteamine	274	6.228
GSH	504	7.005
γ -Glu-Cys	447	7.081
NAC	360	8.224

In Figure 11 the pattern of GSH fragmentation is shown. The GSH attached to the fluorophore has a MW of 505, and if the mass spectrum is in negative mode, it is detectable the ion with $[M-H]^-$ 504. After the fragmentation, in the MS^2 it is possible to see the fragments belonging to the decarboxylation (m/z 460), γ -Glu-Cys (m/z 477), also a hypothesis of reorganization of the molecule (m/z 411), loss of glutamic acid (m/z 375), loss of glycine together with HSO_3 from the fluorophore (m/z 367), loss of glutamic acid together with HSO_3 from the fluorophore (m/z 296) and the fluorophore with the SH group from the molecule attached (m/z 231). This fragment with m/z 231 has a very high intensity and it is also possible to see its fragmentation pattern in the MS^3 and MS^4 with the respective fragments from the molecule (Figure 12).

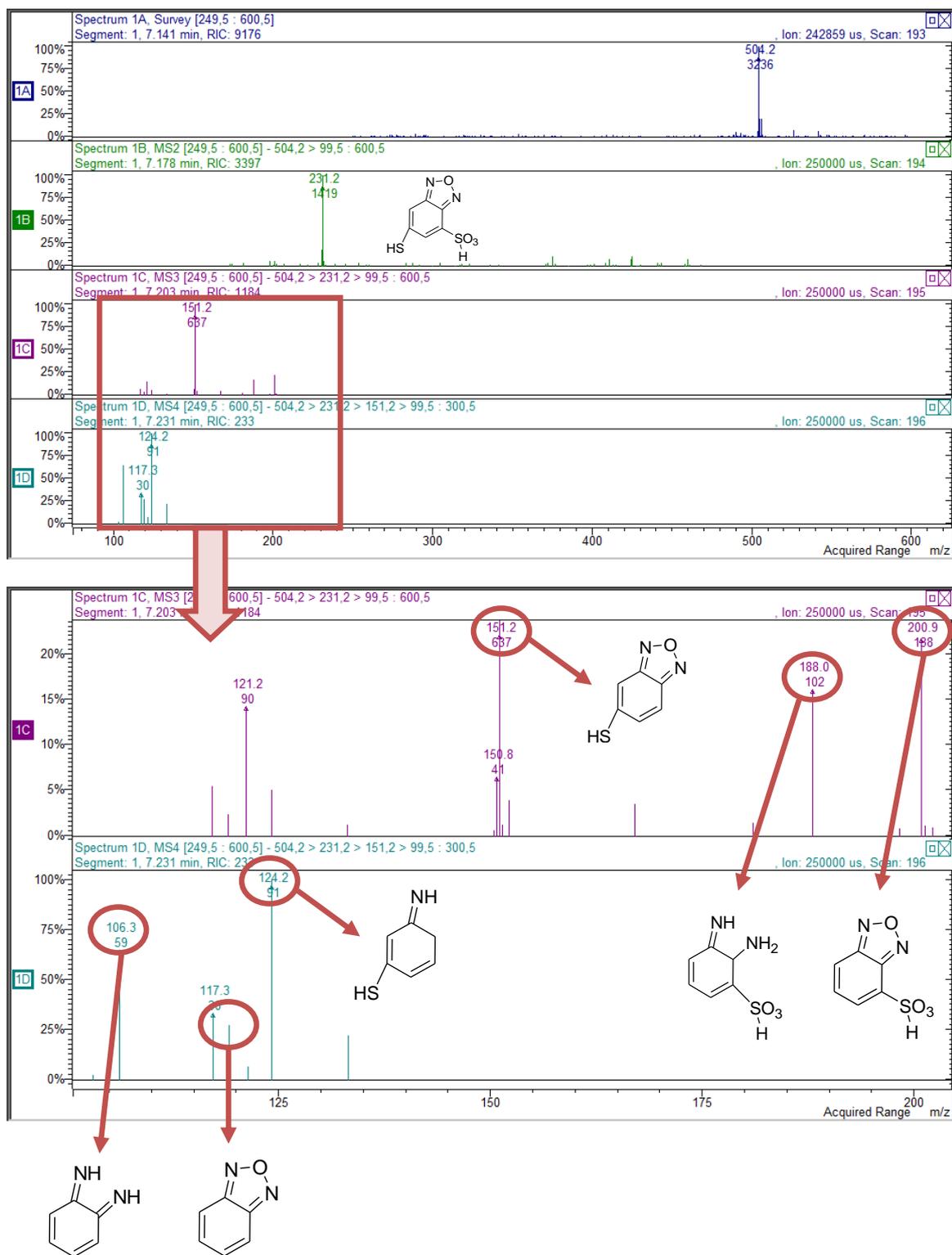


Figure 12. Fragmentation from the fragment m/z 231 corresponding to the SBD-S. MS² in green, MS³ in purple and MS⁴ in blue.

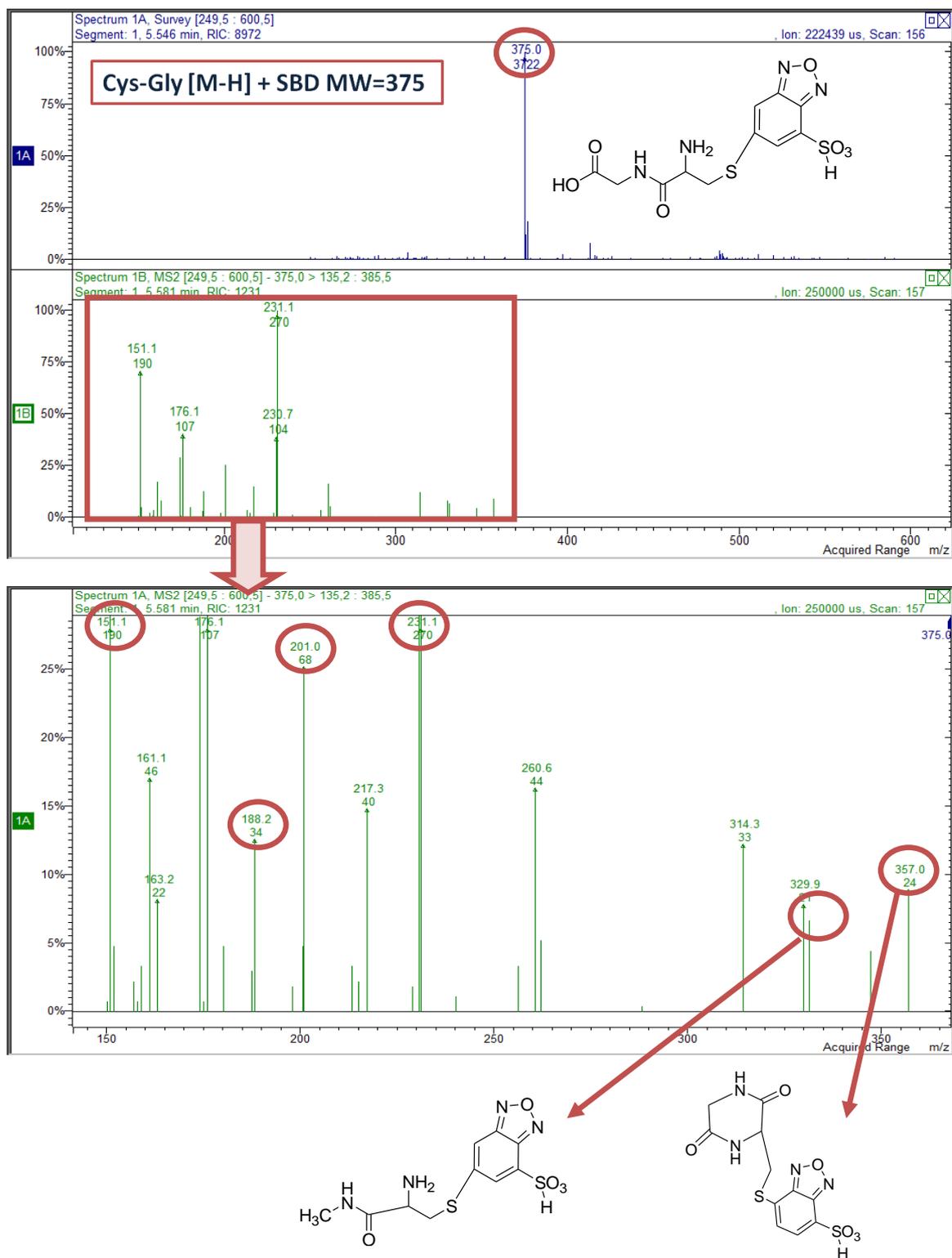


Figure 13. Fragmentation (MS² in green) from the Cys-Gly standard and the fragments obtained.

In Figure 13, there is the pattern of fragmentation from Cys-Gly. In the negative ion mode, its ion is detectable with a mass of $[M-H]^-$ 375. After the fragmentation, in the MS^2 , there is not a lot of information. It is also possible to see the same fragmentations from the SBD-S (m/z 231) obtaining the m/z 201, 188 and 151, described in Figure 12. Also in this thiol, the ion with m/z 231 is the most abundant. Additional information which can be extracted from this spectrum is an intramolecular cyclization with the loss of water (m/z 357) and a decarboxylation (m/z 331).

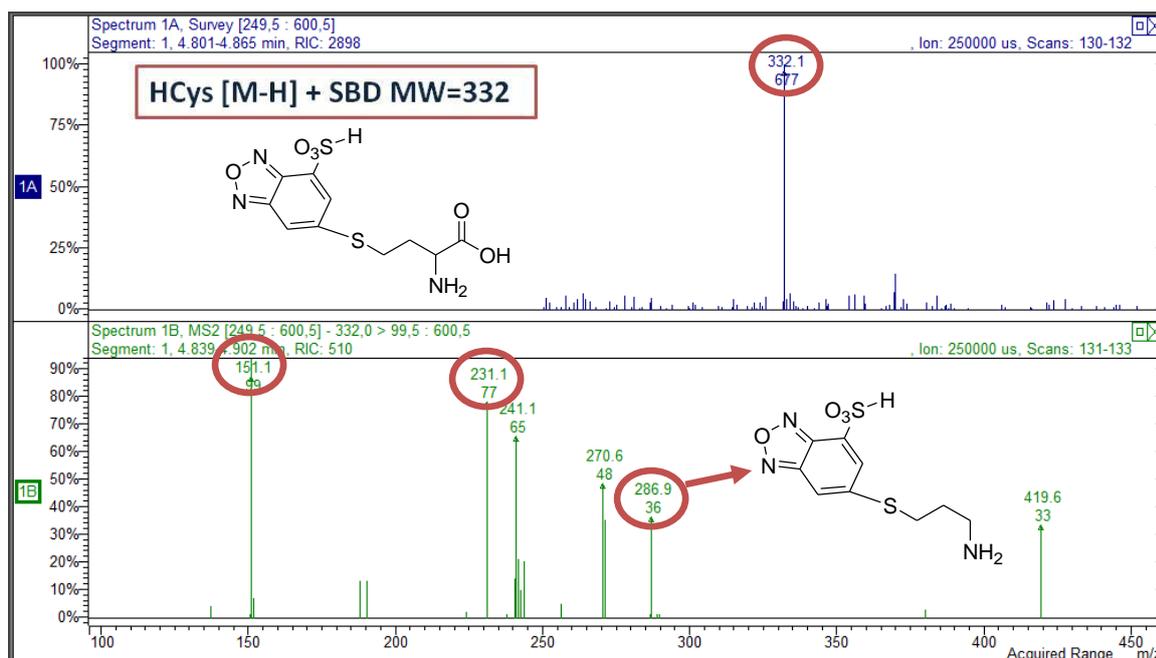


Figure 14. Fragmentation (MS^2 in green) from the Hcys standard and the fragments obtained.

In the case of the Hcys (Figure 14), the thiol molecule attached to the fluorophore has a mass in negative ion mode of $[M-H]^-$ 332. After the fragmentation, MS^2 is not giving any type of information because there are only the same fragmentations from the SBD-S (m/z 231) obtaining the m/z 151; and the decarboxylation (m/z 288).

In Figure 15, there is the pattern of fragmentation from γ -Glu-Cys, which attached to the SBD in negative ion mode has an $[M-H]^-$ 447. After its fragmentation, in the MS^2 the only information which can be extracted is the decarboxylation (m/z 405) and the loss of glutamic acid, and consequently, the Cys attached to the fluorophore with m/z 318. The other fragments belong to the SBD-S (m/z 231) with its fragment (m/z 151).

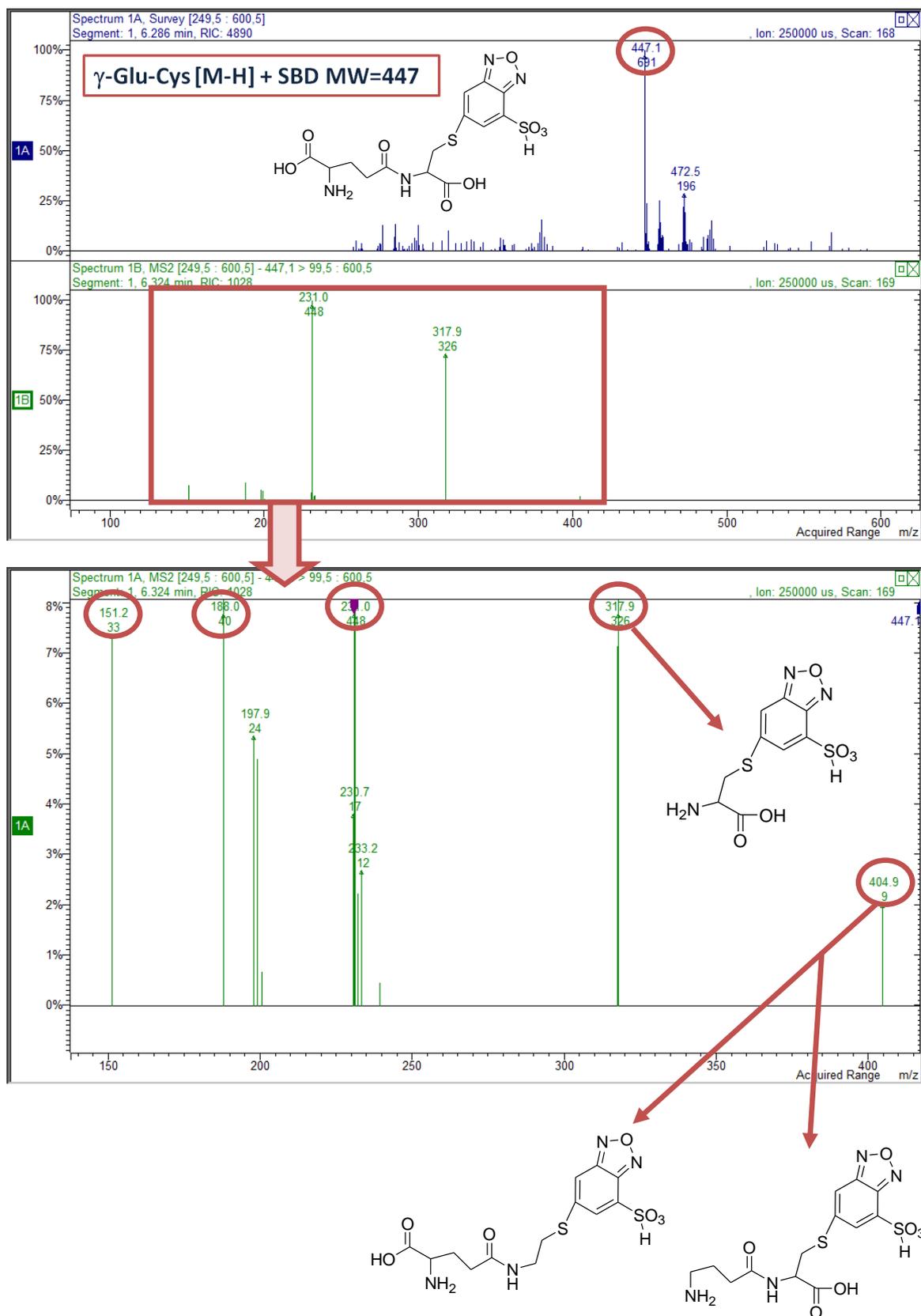


Figure 15. Fragmentation (MS² in green) from the γ -Glu-Cys standard and the fragments obtained.

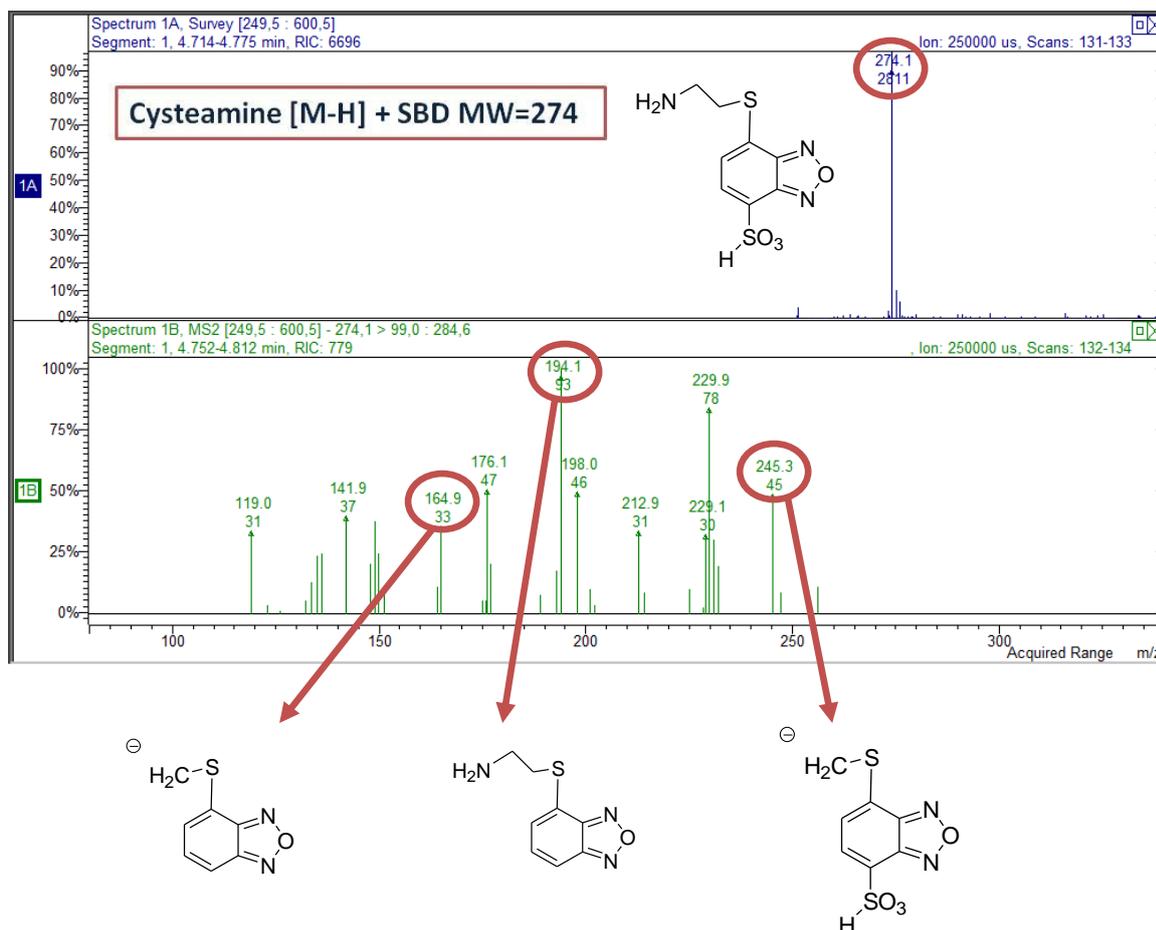


Figure 16. Fragmentation (MS^2 in green) from the cysteamine standard and the fragments obtained.

The fragmentation pattern from cysteamine (Figure 16) with $[M-H]^-$ 274, is characterized for the loss of the aminic group (m/z 245), the loss of SO_3 from the fluorophore (m/z 194) and the loss of the aminic group together with the loss of SO_3 (m/z 165). No more information can be extracted from this fragmentation. Also the fragmentations from Cys $[M-H]^-$ 318 (Figure 17) and NAC $[M-H]^-$ 360 (Figure 18) are not showing any interesting fragment. They only have the fragmentations from the fluorophore (SBD-S), with the fragments m/z 151 in the case of the Cys and 151, 188 and 199 in the case of the NAC.

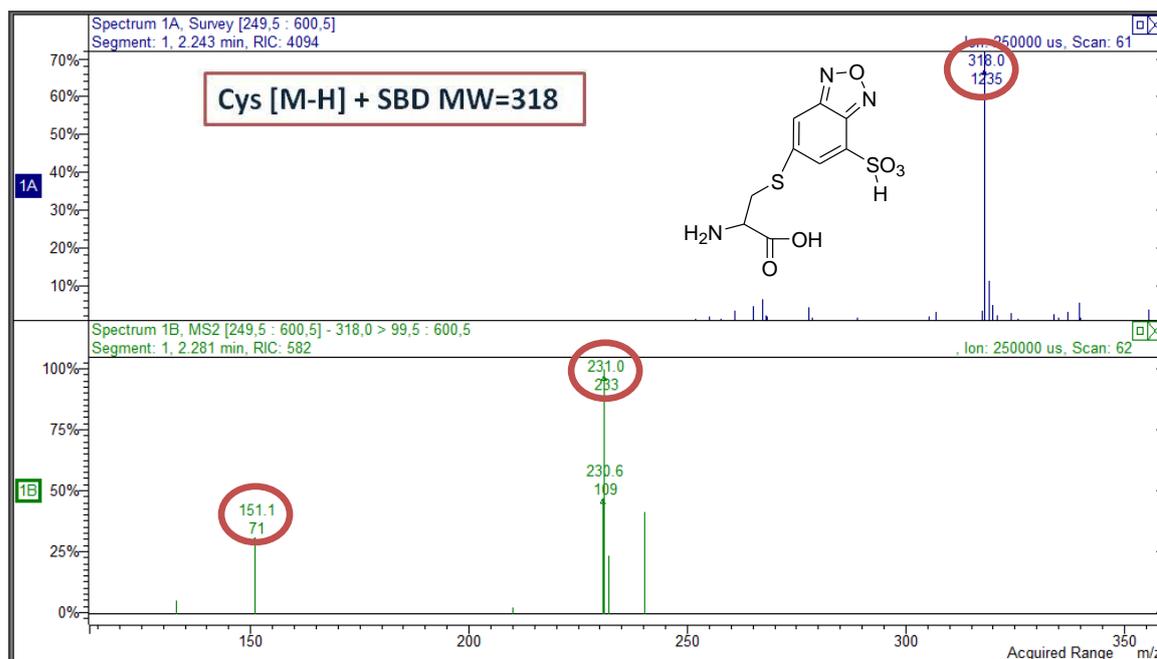


Figure 17. Fragmentation (MS^2 in green) from the Cys standard and the fragments obtained.

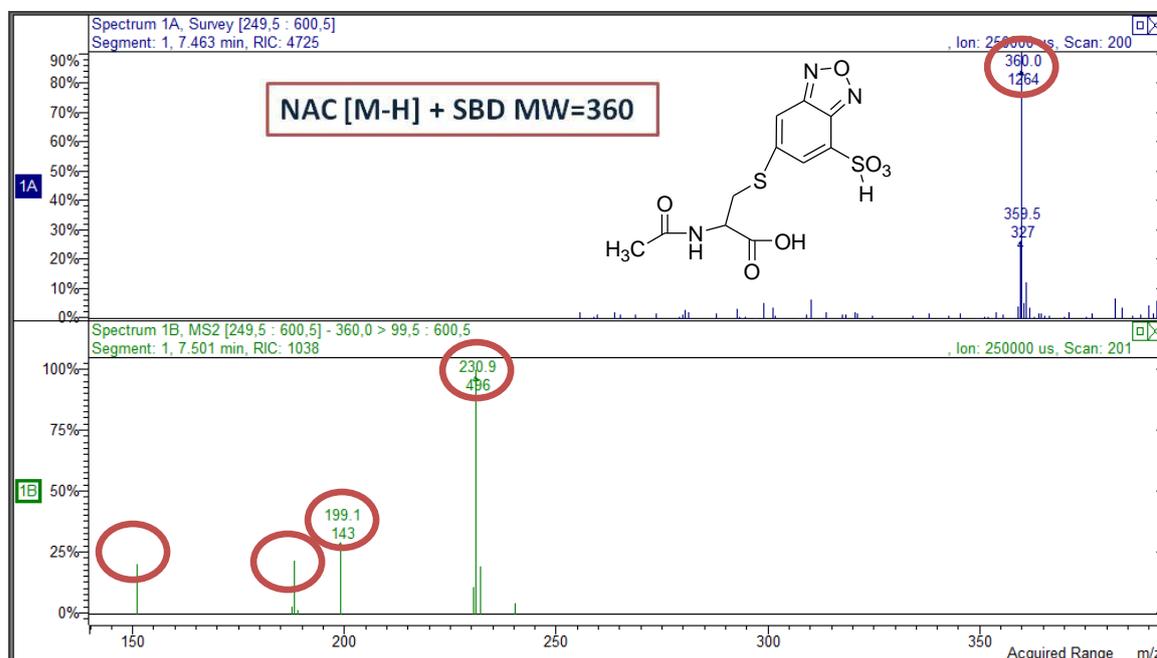


Figure 18. Fragmentation (MS^2 in green) from the NAC standard and the fragments obtained.

From the fragmentation of the standards, it is possible to conclude that after their derivatization with SBD-F, the method developed allows to obtain in the MS^2 the high fragment with m/z 231 corresponding to the SBD with the S from the thiol attached. The fragments obtained (Figure 12) have been studied and relations between the different masses obtained from the fragment m/z 231 are reported in Figure 19.

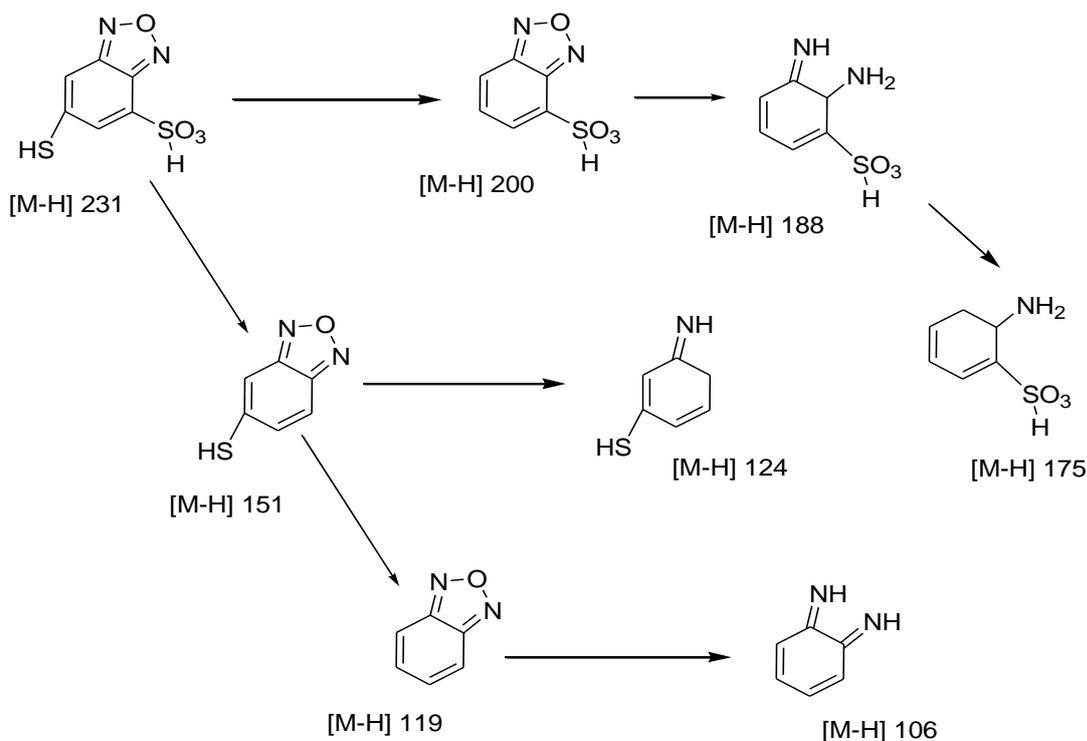


Figure 19. Fragments obtained from the SBD-S (m/z 231).

This means that thiols can be easily recognized in the fragmentation spectra due to the presence of SBD-S fragment (m/z 231). Therefore, this signal was used in the subsequent analyses as a marker to confirm the presence of thiols in more complex samples (for example in the plant extracts). When a sample is fragmented, in the middle of all molecules which will be fragmented, only the LMW will have this high peak 231 corresponding to the SBD-S. In this way, it is possible to do a screening, by selecting only the molecules which have this fragment.

HPLC-MS/MS from the plant extracts

Looking at the fragmented molecules from the plant extracts, LMW thiols were thus assigned to the m/z values which had the peak with m/z 231. In several cases, the fragments reported in the Figure 12 were detected also in MS spectra of plant samples, and this demonstrated that unknown molecules were authentic LMW thiols.

In the case of some species belonging to the *Brassicaceae* family as for example the cauliflower, it was possible to see in all of them a molecule with m/z 393 (Figure 20-22). This molecule had a very high intensity and when the 231 peak was fragmented; its fragments were the ones belonging to the SBD-S already described. This molecule was taken as a possible LMW thiol and its fragmentation pattern was then analyzed.

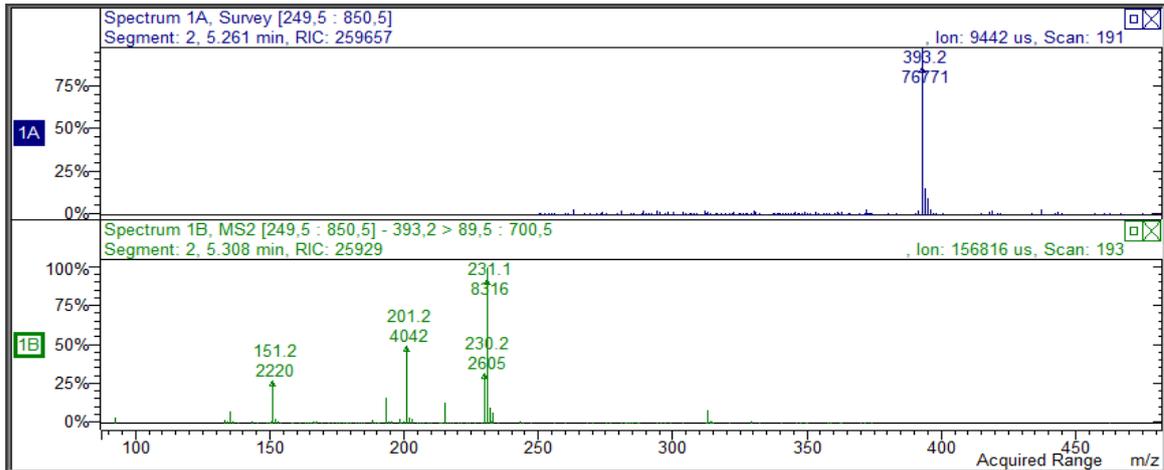


Figure 20. Mass spectra from the molecule m/z 393 in the cauliflower (*Brassica oleracea*).

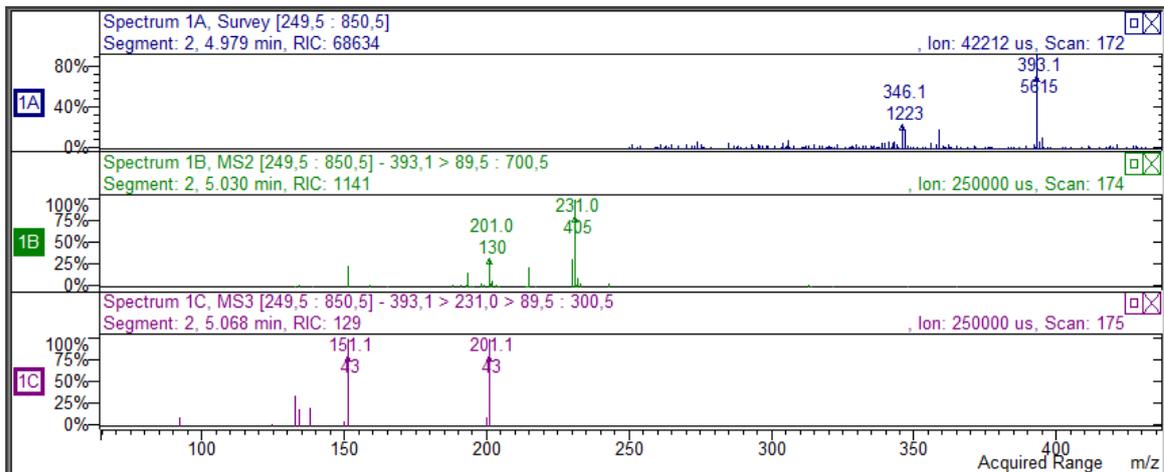


Figure 21. Mass spectra from the molecule m/z 393 in the wild rocket (*Diplotaxis tenuifolia*).

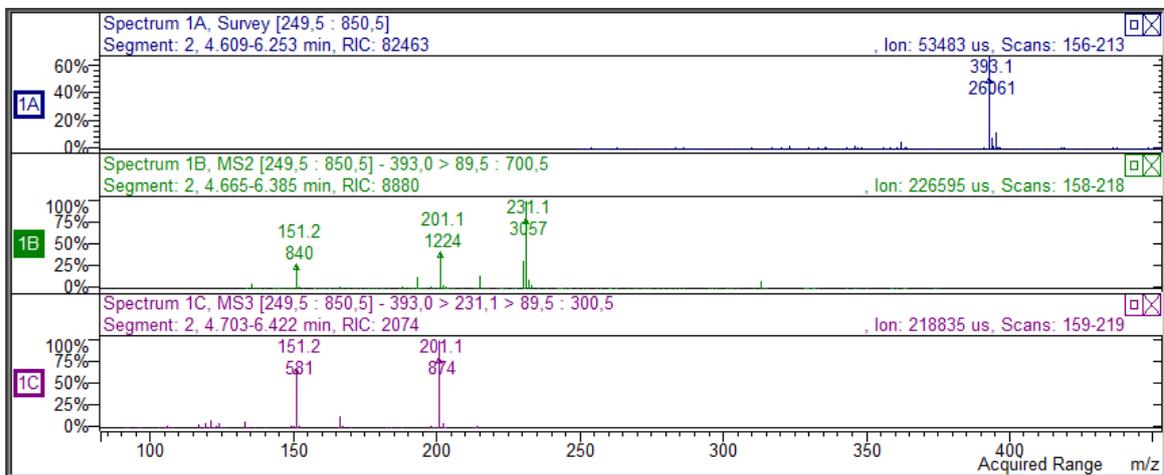


Figure 22. Mass spectra from the molecule m/z 393 in the cultivated rocket (*Eruca sativa*).

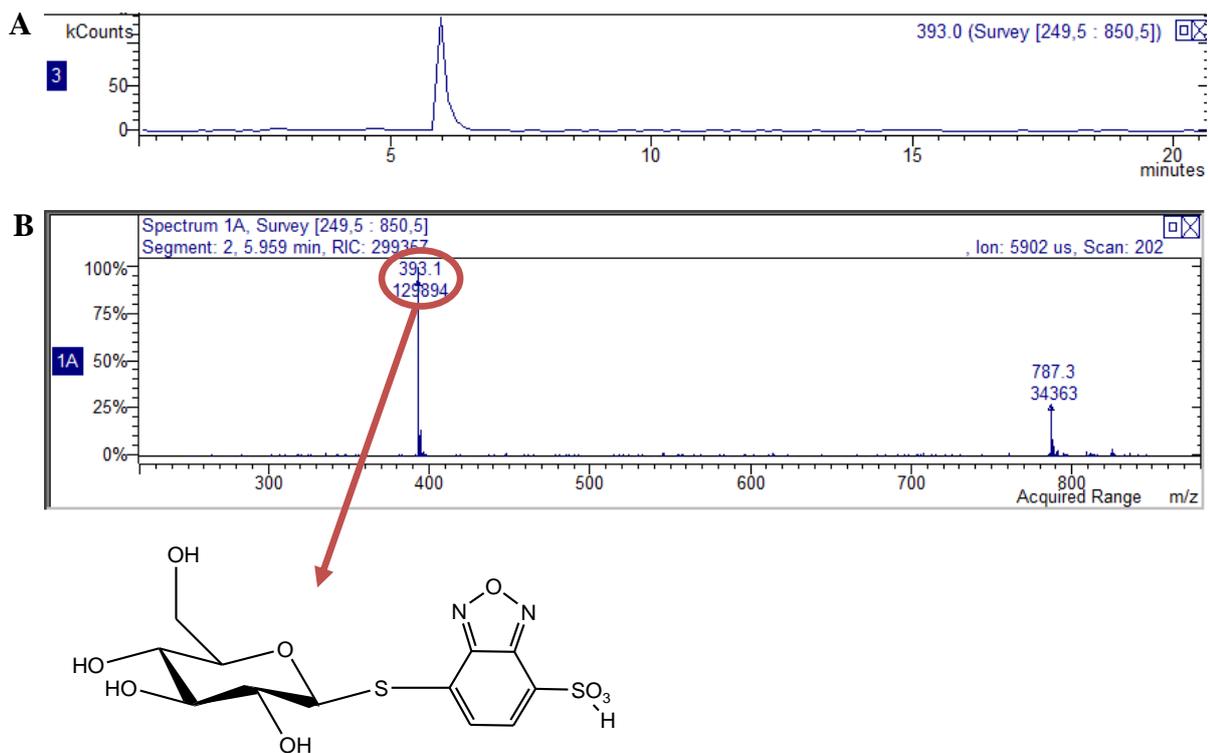


Figure 23. Ion 393 from *Eruca sativa*. (A) HPLC obtained showing the peak corresponding to the ion 393 at 6 min (B) ion $[M-H]^-$ 393 and its dimer $[2M - H]^-$ 787.

The unknown molecule was assigned to the thioglucose. As can be seen in Figure 23, this molecule in the source can be seen as the ion $[M-H]^-$ and also, the ion $[2M - H]^-$ corresponding to a non covalent (hydrogen bonds) dimer which can be detected not only in the ion trap but also in the qToF.

The thioglucose cannot be directly found in plant organisms as it is, but it is part of the glucosinolates. The glucosinolates are secondary metabolites produced in the plant family of *Brassicaceae* (also called *Cruciferae*). Some examples of this family can be the cabbage, arugula, broccoli or cauliflower. They are synthesized in the plant in defense against insects, herbivores, pests, diseases or when it is damaged. In small amounts, they are beneficial for health, but in higher amounts they can be toxic for animals who eat those plants or also for humans who eat that animal. In this case, the thioglucose is an artifact produced by the derivatization procedure used to add the fluorophore to the sample. The first step of the derivatization is the alkalization of the sample (pH 10.5) with the borate buffer. In such a way, the TBP can then work reducing the $-SH$ groups. This alkalization of the sample causes spontaneous degradation of the glucosinolates,

thus leaving the thioglucose free with its –SH group, which will be then attached to the fluorophore (Figure 24) (Jezek *et al.*, 1999; Gallaher *et al.*, 2012). The dimer is formed inside the sample once the thioglucose occurs by the spontaneous degradation from the glucosinolates. The two monomers are bound together through hydrogen bonds.

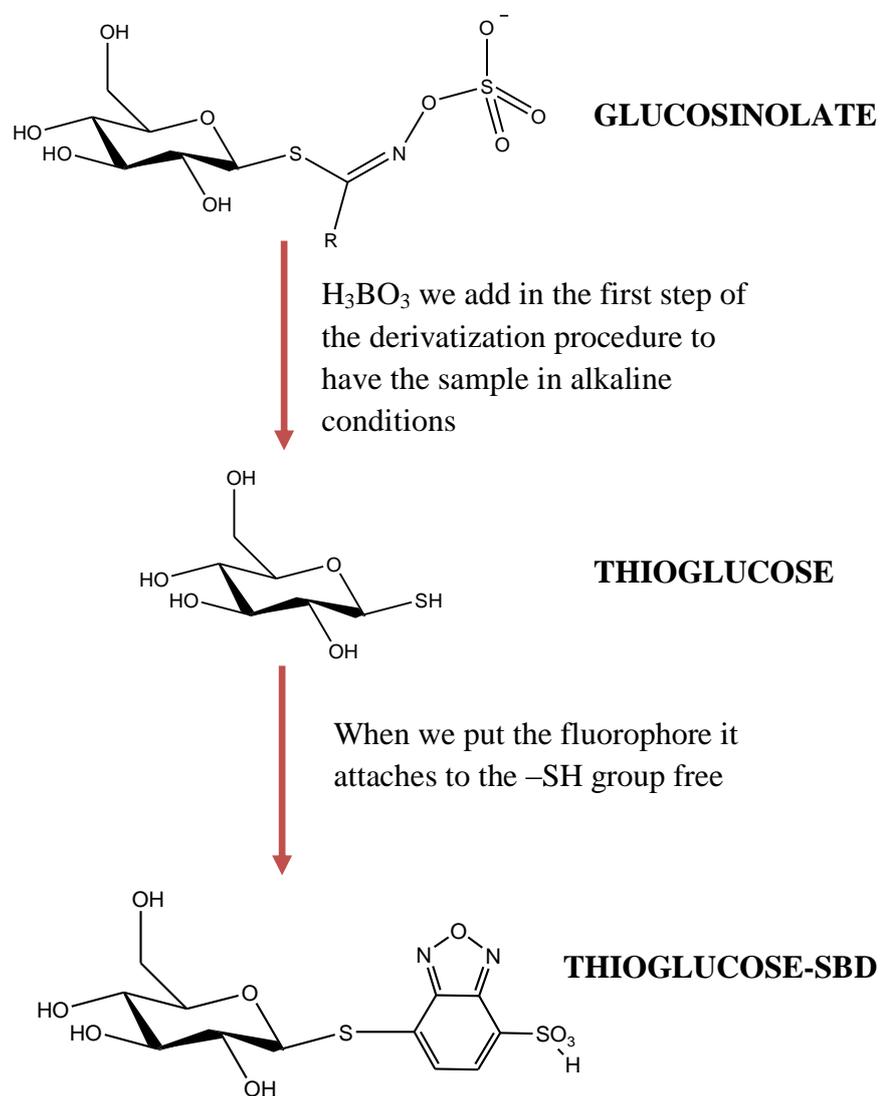


Figure 24. Spontaneous degradation of the glucosinolates in alkaline conditions produces the thioglucose, which is then attached to the fluorophore through the free –SH group.

Further analyses of this molecule in the HPLC-qToF allowed to confirm the exact mass of the thioglucose and to confirm (also with the runs of the standard in HPLC-FL) that this was really that molecule.

Taking advantage from this kind of artifact, some trials trying to measure and quantify it were done in the laboratory. The first step was the run in HPLC-FL from the thioglucose standard and the construction of the calibration curve with it. Once this was done, the next step was the quantification of this compound in *Brassica napus* and in *Diplotaxis tenuifolia*. The idea was that this artifact, occurring after spontaneous degradation of the glucosinolates, can be used as an estimation of total glucosinolate content in *Brassicaceae* using HPLC-FL by the detection and quantification of thioglucose [work in progress].

But this was not the only compound found in the several samples which were run. It was also detected the compound with m/z 762 in the species *Solanum tuberosum*. The same compound with the same spectra can be also seen in *Brassica oleracea*. The peak has a retention time more or less about 7 min and its mass spectrum shows in the MS² that there is a fragment, which can be also seen in the MS, corresponding to the GSH-SBD (m/z 504). This fact suggests us that it should be a molecule with the GSH (Figure 25).

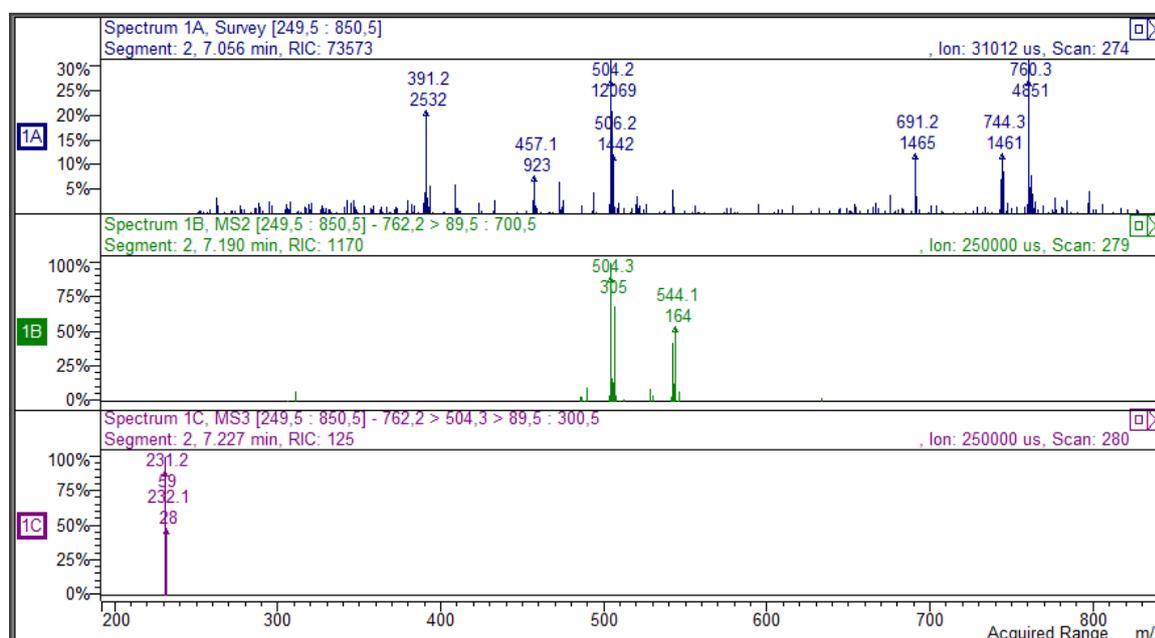


Figure 25. Mass spectra from the ion with m/z 762 from *Solanum tuberosum*. MS² in green and MS³ in purple.

Looking more detailed, there is the peak with m/z 504 corresponding to GSH with high intensity and which is also fragmenting. This confirms that it is GSH because it can be seen the peak with m/z 231, the marker for the compounds of interest (Figure 26).

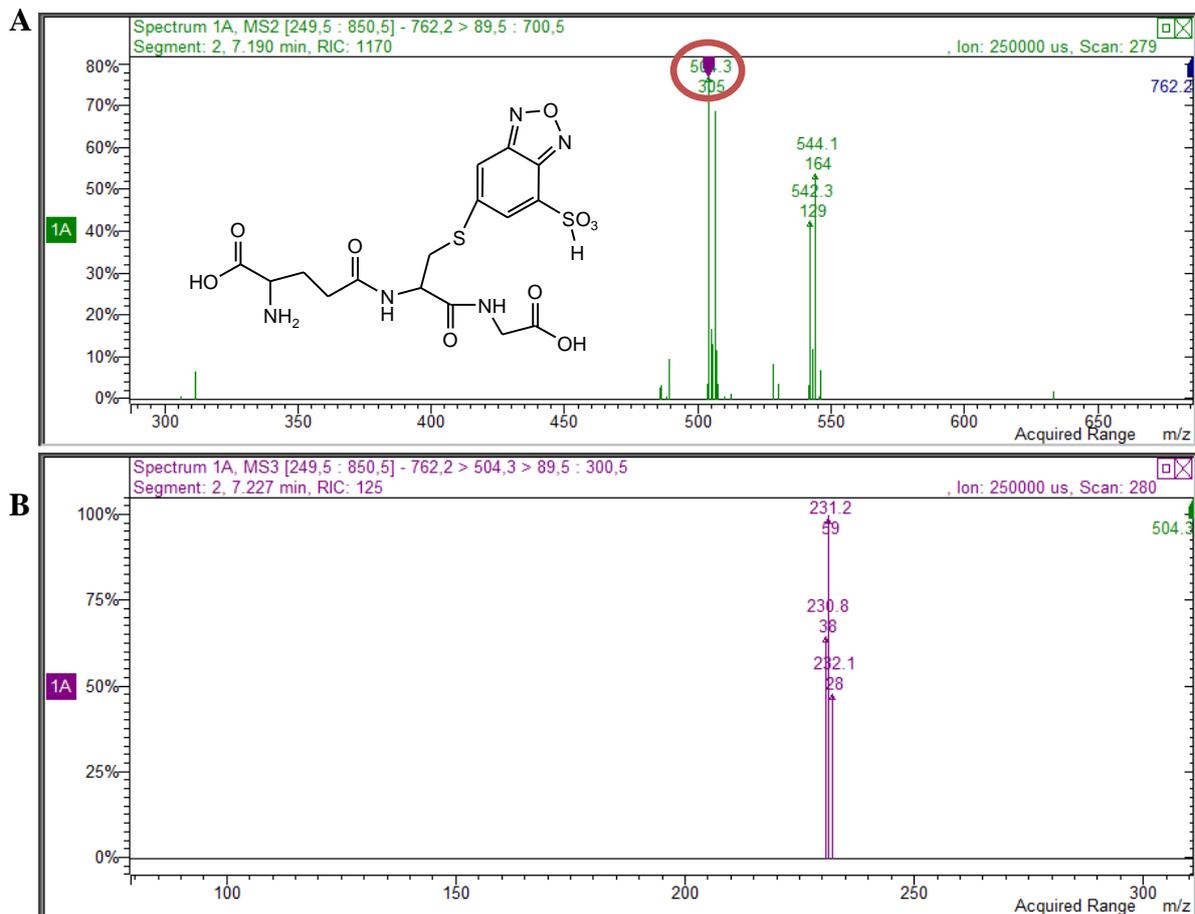


Figure 26. Detail from (A) the ion present in the MS² corresponding to the GSH-SBD (m/z 504) (B) the ion present in MS³ corresponding to the SBD-S (m/z 231).

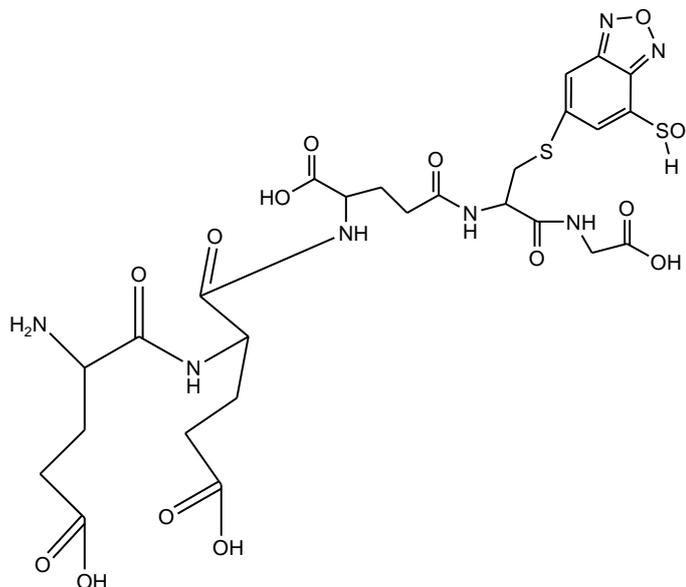


Figure 27. Hypothetical structure from the molecule with m/z 762.

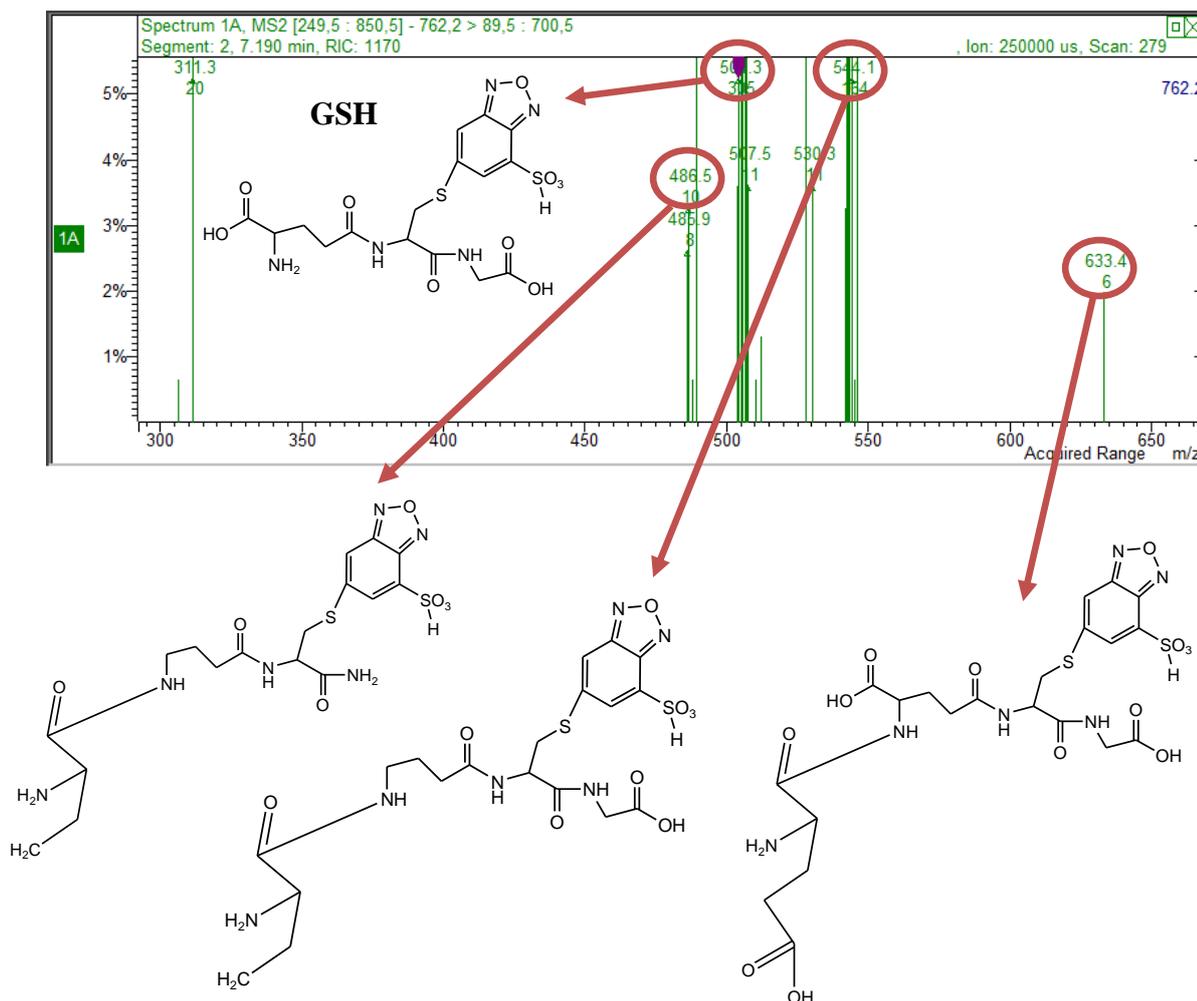


Figure 28. Fragments obtained in the MS² spectra from the molecule with m/z 762.

Looking more exhaustive, the fragments obtained in the fragmentation, it can be seen in a first instance the GSH (m/z 504). But other interesting ones which helped to obtain the final hypothetical structure (Figure 28) for the molecule are present. In the Figure 28 are shown the loss of one glutamic acid (m/z 633), the loss of the glutamic acid together with two decarboxylations (m/z 544); and with three decarboxylations (m/z 487). This information is very important in order to get the hypothetical structure for that molecule (Figure 27).

After that, the same extract was then injected to the qToF MS and it allowed to know the exact mass of this compound and to obtain a hypothetical formula for it, which corresponds with the structure reported in Figure 27. The molecular formula is (Glu)₂GSH.

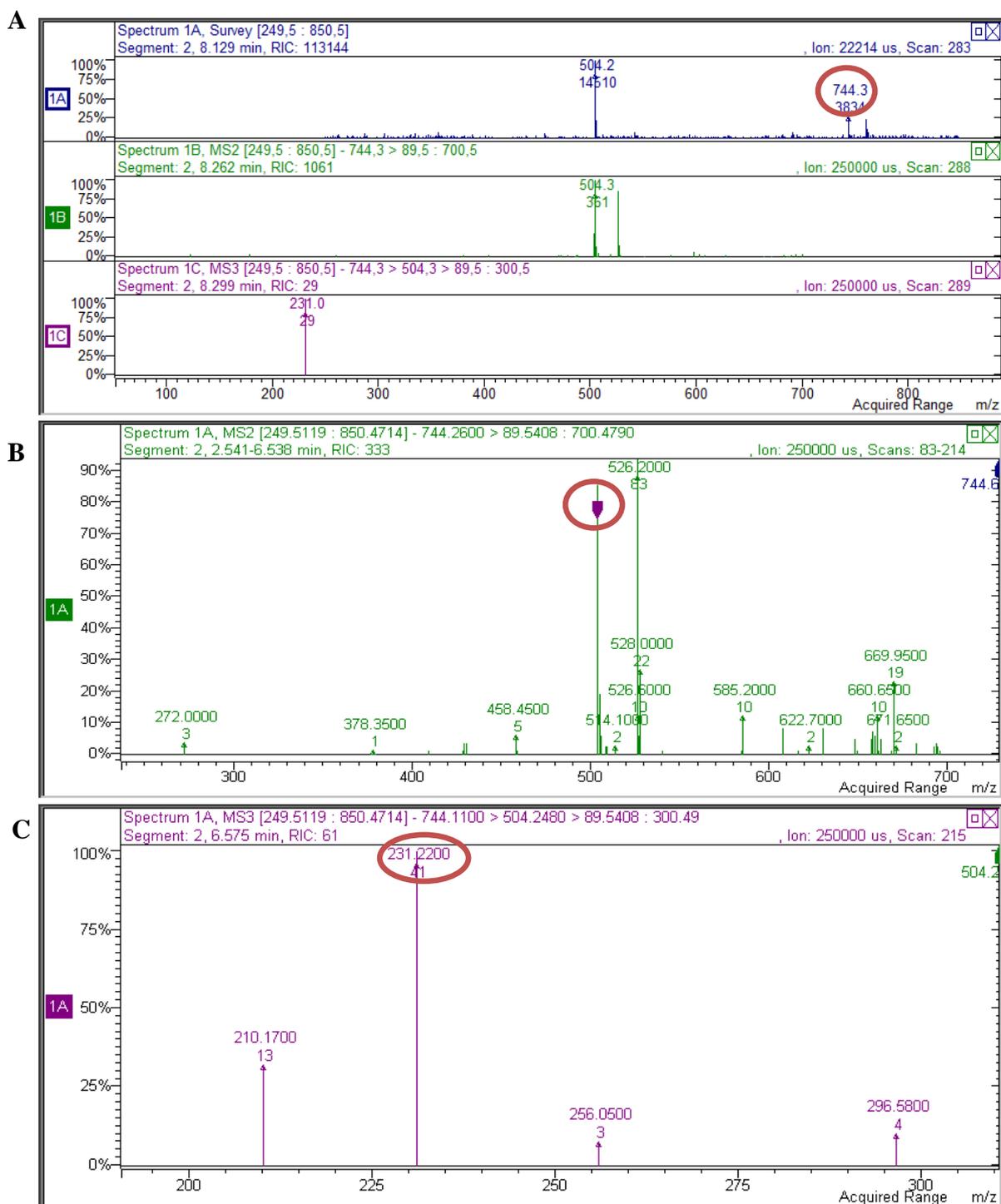


Figure 29. The pattern of fragmentation from the molecule with m/z 744 in green chilly (*Capsicum L.*). (A) The ion path for the ions, MS^2 from 744 in green and MS^3 from 504 in blue. (B) Detail from the MS^2 (C) Details from the MS^3 .

When it was analyzed the green chilly (*Capsicum L.*), it was found a compound with some similarities with the molecule described before. It is also a molecule which has an intense signal corresponding to GSH (m/z 504), then it should be a compound derived of it, but with some differences (Figure 29).

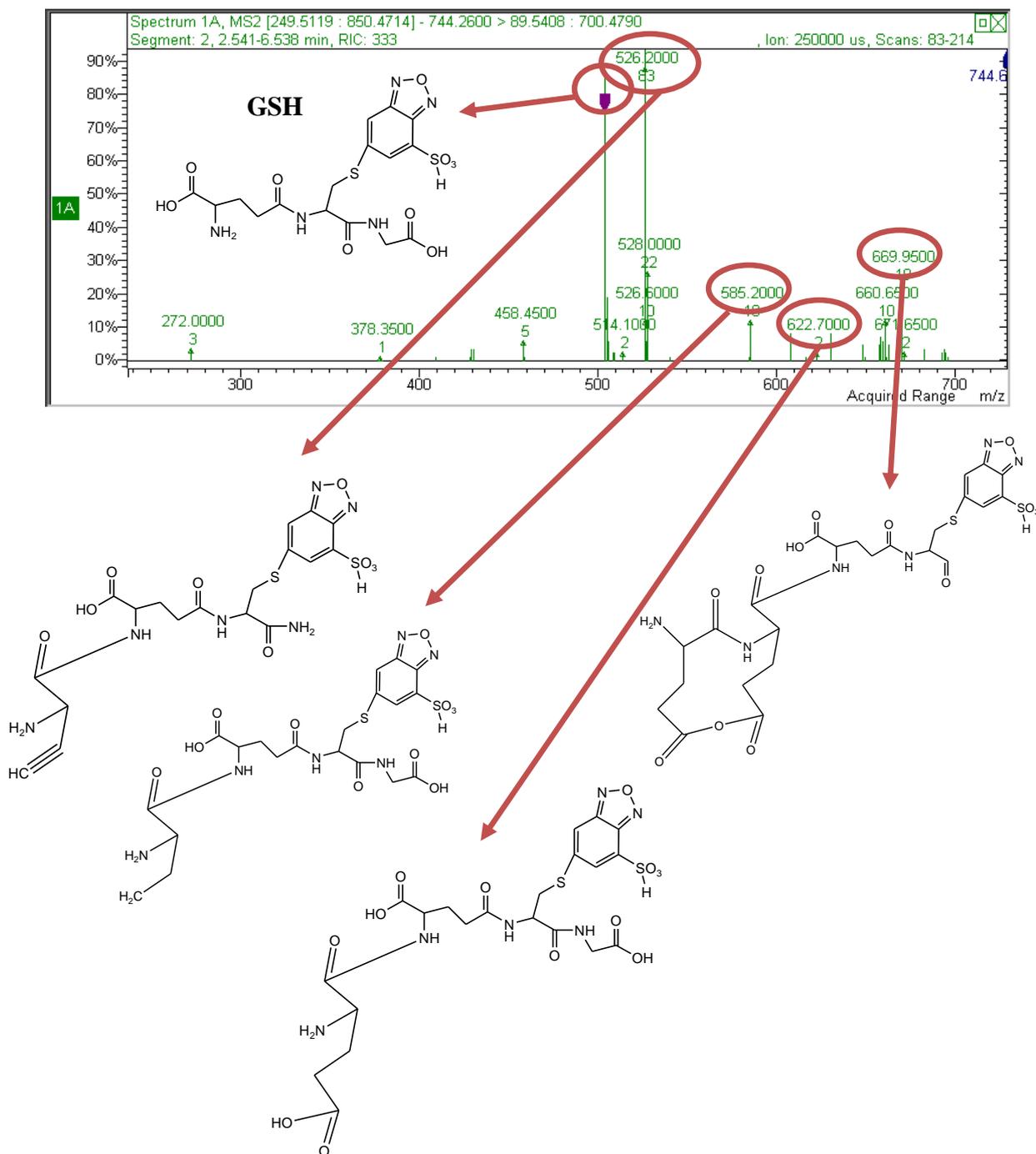


Figure 30. Fragments obtained in the MS2 spectra from the molecule with m/z 744.

This molecule with m/z 744 has in the fragmentation spectra, several interesting fragments. The most important is the GSH (m/z 504), but other ones were also found (Figure 30). There is a loss of the glycine in the GSH (m/z 670), a loss of one glutamic acid (m/z 630), a loss of the glutamic acid plus the anhydride (m/z 585) and the same loss of glutamic acid and anhydride but with a triple bond in the carbons (m/z 526). These

several fragments helped to reach to a hypothetical molecule (Figure 31) also (Glu)₂GSH as the one it was found before, but with the carboxylic residues from the glutamic acids condensed in an anhydride bond. This is in every case a hypothetical structure which should be confirmed by the synthesis of this molecule in the laboratory [work in progress].

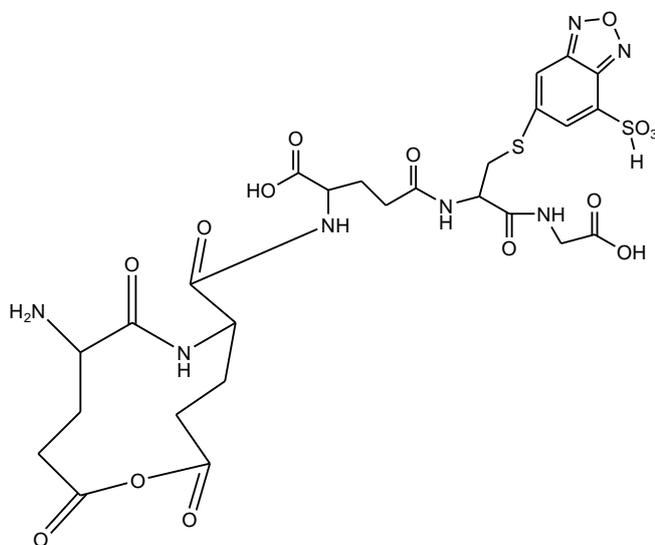


Figure 31. Hypothetical structure from the molecule with m/z 744.

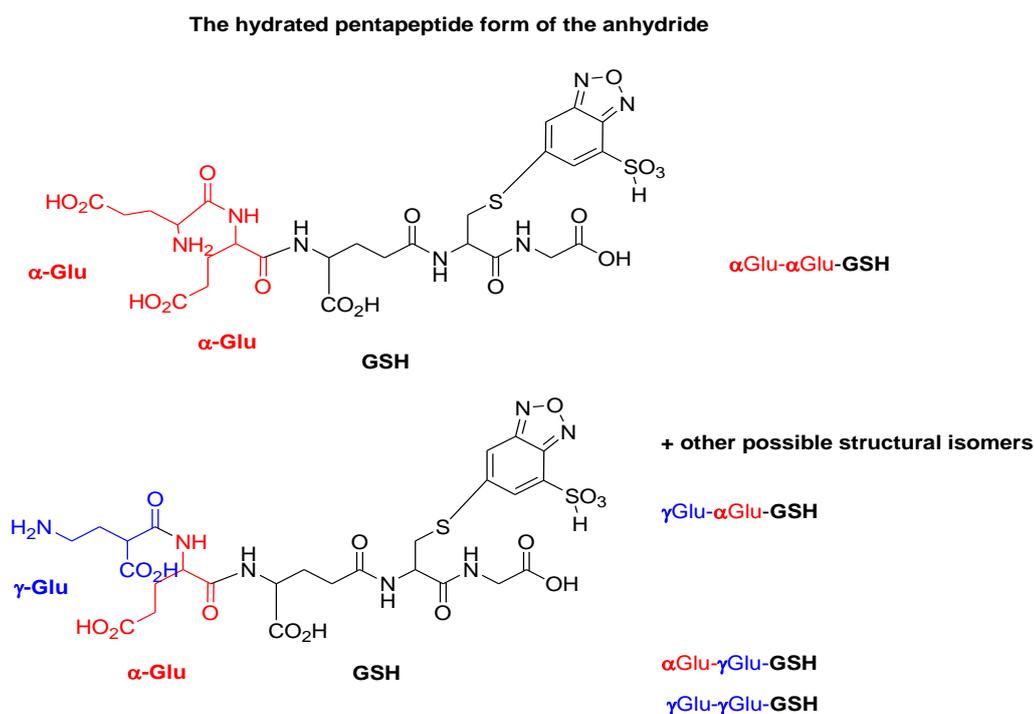


Figure 32. Other possible hypothetical structures from the molecule with m/z 744.

This structure could likely be an artifact in the mass spectrometry analysis (i.e. the tetrapeptide dehydrates to form the anhydride during the MS analysis). Because of that, other possible structures are proposed for this (Glu)₂GSH (Figure 32).

This sample also has been analyzed in qToF MS confirming that it has really that mass. But due to these several structures that can adopt this molecule, it is necessary the synthesis of them and then to run again in HPLC-FL and HPLC-MS/MS to know exactly which one of them it is and to know if it is not really an MS artifact [work in progress].

There are also other molecules, still unknown but present in some extracts (Figure 33). Their identification can bring to a new molecule which can have important attention in plant metabolism and physiology. Due to the lack of information and scientific work done about this field, their identification is challenging [work in progress].

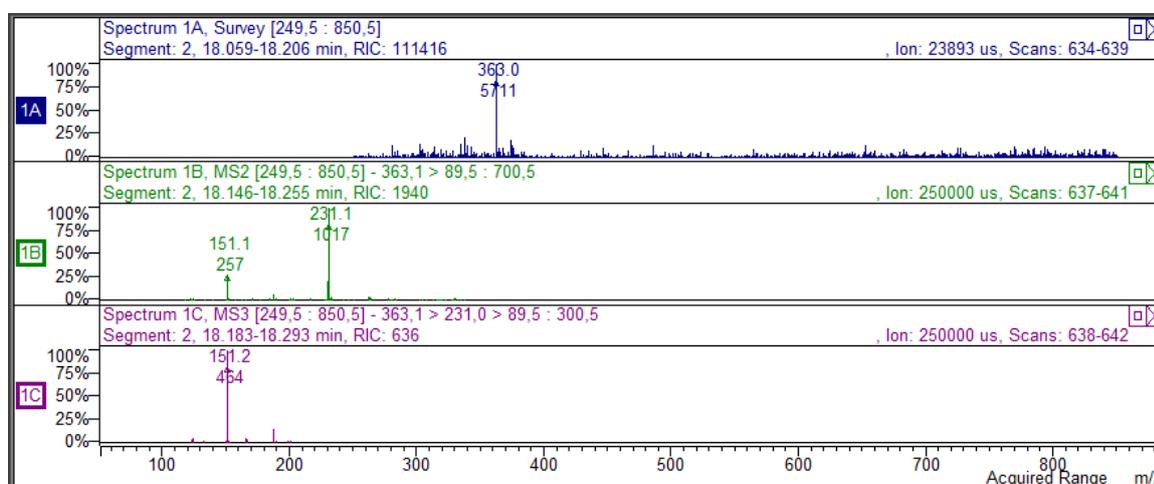


Figure 33. Mass spectra from the molecule m/z 363 in cultivated arugula (*Eruca sativa*).

Conclusions

In this work, it was found a method which can be used to identify LMW thiols by using SBD-derivatives together with HPLC-MS/MS based on the presence of the fragment with m/z 231, which thus represents a marker reporting that a given molecule is an authentic LMW thiol.

This method can be applied in the future to study other species, and will enable identification of unknown LMW thiol molecules. Itself, it can be used not only in plant species, but can be extended to any biological sample and food products.

More work needs to be done regarding the hypothetical molecules identified and also about the peaks which still remain unknown, as for example the peak with m/z 363.

References

- Feng, S., Zheng, X., Wang, D., Gong, Y., Wang, Q., Deng, H. (2014). Systematic analysis of reactivities and fragmentation of glutathione and its isomer GluCysGly. *The Journal of Physical Chemistry A*, **118**, 8222–8228.
- Gallaher, C.M., Gallaher, D.D., Peterson, S. (2012). Development and validation of a spectrophotometric method for quantification of total glucosinolates in cruciferous vegetables. *Journal of Agricultural and Food Chemistry*, **60**, 1358–1362.
- Jezek, J., Haggett, B.G.D., Atkinson, A., Rawson, D.M. (1999). Determination of glucosinolates using their alkaline degradation and reaction with ferricyanide. *Journal of Agricultural and Food Chemistry*, **47**, 4669–4674.
- Murphy, C.M., Fenselau, C., Gutierrez, P.L. (1992). Fragmentation Characteristic of glutathione conjugates activated by high-energy collisions. *Journal of the American Society for Mass Spectrometry*, **3**, 815–822.
- Pivato, M., Fabrega-Prats, M., Masi, A. (2014). Low-molecular-weight thiols in plants: Functional and analytical implications. *Archives of Biochemistry and Biophysics*, **560**, 83–99.
- Rellán-Álvarez, R., Hernández, L.E., Abadía, J., Álvarez-Fernández, A. (2006). Direct and simultaneous determination of reduced and oxidized glutathione and homogluthathione by liquid chromatography–electrospray/mass spectrometry in plant tissue extracts. *Analytical Biochemistry*, **356** (2), 254–264.
- Thakur, S.S., Balaram, P. (2008). Fragmentation of peptide disulfides under conditions of negative ion mass spectrometry: studies of oxidized glutathione and contryphan. *Journal of the American Society for Mass Spectrometry*, **19** (3), 358–366.
- Xie, C., Zhong, D. & Chen, X. (2013). A fragmentation-based method for the differentiation of glutathione conjugates by high-resolution mass spectrometry with electrospray ionization. *Analytica Chimica Acta*, **788**, 89–98.

List of Tables

Table 1. Parameters and conditions used in the MS.

Table 2. Method used in HPLC to have the good separation of the standards.

Table 3. Masses in the negative ion mode from the standard-SBD, and their respective retention times in HPLC-MS, after using the diluted buffer.

List of Figures

Figure 1. Workflow of the methods used in the study.

Figure 2. Chromatogram obtained in HPLC-FL, where it is possible to see a good separation of all the standards tried in this study.

Figure 3. GSH in negative ion mode (m/z 504). (A) HPLC chromatogram, which shows the peak of the molecule obtained. (B) Mass spectra.

Figure 4. GSH in positive ion mode (m/z 506). (A) HPLC chromatogram, which shows the peak of the molecule obtained. (B) Mass spectra.

Figure 5. Cys in negative ion mode (m/z 318). (A) HPLC chromatogram, which shows the peak of the molecule obtained. (B) Mass spectra.

Figure 6. Hcys in negative ion mode (m/z 332). (A) HPLC chromatogram, which shows the peak of the molecule obtained. (B) Mass spectra.

Figure 7. Cysteamine in negative ion mode (m/z 274). (A) HPLC chromatogram, which shows the peak of the molecule obtained. (B) Mass spectra.

Figure 8. Cys-Gly in negative ion mode (m/z 375). (A) HPLC chromatogram, which shows the peak of the molecule obtained. (B) Mass spectra.

Figure 9. γ -Glu-Cys in negative ion mode (m/z 447). (A) HPLC chromatogram, which shows the peak of the molecule obtained. (B) Mass spectra.

Figure 10. NAC in negative ion mode (m/z 360). (A) HPLC chromatogram, which shows the peak of the molecule obtained. (B) Mass spectra.

Figure 11. Fragmentation (MS^2 in green) from the GSH standard and the fragments obtained.

Figure 12. Fragmentation from the fragment m/z 231 corresponding to the SBD-S. MS^2 in green, MS^3 in purple and MS^4 in blue.

Figure 13. Fragmentation (MS^2 in green) from the Cys-Gly standard and the fragments obtained.

Figure 14. Fragmentation (MS^2 in green) from the Hcys standard and the fragments obtained.

Figure 15. Fragmentation (MS^2 in green) from the γ -Glu-Cys standard and the fragments obtained.

Figure 16. Fragmentation (MS^2 in green) from the cysteamine standard and the fragments obtained.

Figure 17. Fragmentation (MS^2 in green) from the Cys standard and the fragments obtained.

Figure 18. Fragmentation (MS^2 in green) from the NAC standard and the fragments obtained.

Figure 19. Fragments obtained from the SBD-S (m/z 231).

Figure 20. Mass spectra from the molecule m/z 393 in the cauliflower (*Brassica oleracea*).

Figure 21. Mass spectra from the molecule m/z 393 in the wild rocket (*Diplotaxis tenuifolia*).

Figure 22. Mass spectra from the molecule m/z 393 in the cultivated rocket (*Eruca sativa*).

Figure 23. Ion 393 from *Eruca sativa*. (A) HPLC obtained showing the peak corresponding to the ion 393 at 6 min (B) ion $[M-H]^-$ 393 and its dimer $[2M - H]^-$ 787.

Figure 24. Spontaneous degradation of the glucosinolates in alkaline conditions produces the thioglucose, which is then attached to the fluorophore through the free $-SH$ group.

Figure 25. Mass spectra from the ion with m/z 762 from *Solanum tuberosum*. MS^2 in green and MS^3 in purple.

Figure 26. Detail from (A) the ion present in the MS^2 corresponding to the GSH-SBD (m/z 504) (B) the ion present in MS^3 corresponding to the SBD-S (m/z 231).

Figure 27. Hypothetical structure from the molecule with m/z 762.

Figure 28. Fragments obtained in the MS^2 spectra from the molecule with m/z 762.

Figure 29. The pattern of fragmentation from the molecule with m/z 744 in green chilly (*Capsicum* L.). (A) The ion path for the ions, MS^2 from 744 in green and MS^3 from 504 in blue. (B) Detail from the MS^2 (C) Details from the MS^3 .

Figure 30. Fragments obtained in the MS^2 spectra from the molecule with m/z 744.

Figure 31. Hypothetical structure from the molecule with m/z 744.

Figure 32. Other possible hypothetical structures from the molecule with m/z 744.

Figure 33. Mass spectra from the molecule m/z 363 in cultivated arugula (*Eruca sativa*).

CHAPTER III

APPLICATIONS TO WINEMAKING PROCESSES

“Method optimization and quantification of low-molecular-weight thiols in grapes by HPLC-FL detection”

Marta Fabrega-Prats, Antonio Masi, Heike Semmler, Ernst Rühl, Claus-Dieter Patz, Doris Rauhut*

Under submission in *Australian Journal of Grape and Wine Research*

Method optimization and quantification of low-molecular-weight thiols in grapes by HPLC-FL detection

Marta Fabrega-Prats¹, Antonio Masi¹, Heike Semmler², Ernst Rühl³, Claus-Dieter Patz⁴, Doris Rauhut^{2*}

¹ DAFNAE University of Padua, Viale dell'Università 16, 30520 Legnaro (PD), Italy

² Hochschule Geisenheim University, Department of Microbiology and Biochemistry, Von Lade Straße 1, 65366 Geisenheim, Germany

³ Hochschule Geisenheim University, Department of Grapevine Breeding, Von Lade Straße 1, 65366 Geisenheim, Germany

⁴ Hochschule Geisenheim University, Department of Wine Analysis and Beverage Research, Von Lade Straße 1, 65366 Geisenheim, Germany

Keywords: antioxidant, grapes, glutathione, HPLC, sample preparation, thiols

Abstract

Low-molecular-weight (LMW) thiols are highly reactive compounds involved in the maintenance of the cellular redox balance. These compounds and specifically glutathione (GSH) have an antioxidant function which is not limited to grapes but extends in must and wine. They may influence the susceptibility to oxidative browning by exerting a protective function during the fermentation and aging processes. GSH and other metabolically related LMW thiols like cysteine (Cys), γ -glutamylcysteine (γ -Glu-Cys) and cysteinylglycine (Cys-Gly) have an antioxidant role, by protecting white wines from oxidative aging and detrimental organoleptic modifications (Vallverdú-Queralt *et al.*, 2015).

The aim of this study was to develop an easy and fast method to quantify the amount of these compounds in different grapevine varieties and clones used for the production of white and red wines. LMW thiols were extracted from grapes and analyzed by HPLC-Fluorescence separation of the SBD-derivatives (derivatized with the fluorescent dye SBD-F; ammonium 7-fluoro 2,1,3-benzooxadiazole-4-sulfonate). The fermentable sugars and organic acids were also extracted and measured by HPLC separation.

The results show that the developed method has a very good reproduction for sampling and measuring LMW from the grape berries and that it can be applied both for

must and wine. Moreover, it not only allows to quantify the amount of GSH, which is the most important LMW thiol implicated in the antioxidant activity, but also to determine quantification of Cys, Cys-Gly, γ -Glu-Cys. In conclusion, this study on grape berries of different varieties and clones demonstrates that GSH is the major LMW thiol. Its content varies between varieties. Other LMW thiols could play an essential role too, due to their antioxidant properties. GSH and related LMW thiols are an important factor which can be considered in the evaluation of the grapes used for wine making.

Introduction

LMW thiols are highly reactive compounds involved in the maintenance of the redox balance in cells and in stress response. GSH is the mostly known LMW thiol and its physiological function in living cells has received a lot of attention during recent years. But also its related compounds such as Cys, γ -Glu-Cys and Cys-Gly have a relevant function both in animals and in plants (Pivato *et al.*, 2014).

In plants, GSH is the main non-protein intracellular LMW thiol (0.2-10 mM) which can be oxidized to form a dimer, the glutathione disulfide (GSSG), with a disulfide bond between the two identical GSH molecules. In oxidative stress conditions, GSSG can be accumulated in the cell. GSH is implicated in the sulfur metabolism of the cell, but also in the control of intracellular redox and in detoxification (Kritzinger *et al.*, 2013; Pivato *et al.*, 2014).

GSH has an antioxidant function, which is not limited to grapes; it is also important in must and wine. It can influence the susceptibility to oxidative browning by exerting a protective function during the fermentation and aging processes. GSH and other metabolically related LMW thiols protect white wines from oxidative aging and detrimental organoleptic modifications (Vallverdú-Queralt *et al.*, 2015).

Cheyrier and colleagues (1989) quantified for the first time GSH in the grape berries. It is well known that it is being accumulated during ripening and it is related to nitrogen (Adams and Liyanage, 1993). Its content can exceed 100 mg/kg in mature grapes and in grape juice GSH ranges from 10 to 100 mg/L (Cheyrier *et al.*, 1989; Lavigne *et al.*, 2007). Its concentration very much depends on grape variety, environmental conditions and viticultural practices (Cheyrier *et al.*, 1989). After fermentation, GSH concentration in wine is lower and ranges from 1 to 20 mg/L (Cassol *et al.*, 1995; Du Toit

et al., 2007). GSH content can be also affected by *Saccharomyces cerevisiae* activity during alcoholic fermentation and during aging on the lees (Lavigne *et al.*, 2007).

During recent years there has been a high interest in GSH in wine production because it protects wine from oxidative browning and flavor loss, particularly in the variety Sauvignon blanc (Pons *et al.*, 2015). GSH has a positive effect in wine aging by preserving wine color and aromas protecting volatile thiols. It protects grape must from oxidation, because it traps caftaric acid and quinones by oxidizing them. This reaction is called grape reaction product (GRP) and it is already described (Singleton *et al.*, 1984; Fracassetti *et al.*, 2011). It is very important to take GRP into account when grape berries are mashed, as this reaction is really fast. It has been shown that more than 90% of the total original GSH in grape juice is lost within 5 min without protection against oxidation (Okuda *et al.*, 1999).

GSH can be naturally present in higher concentrations in certain grape varieties. GSH is recommended as an additive to must and wine to limit the extent of oxidation (Vaimakis and Roussis, 1996; Hosry *et al.*, 2009). Moreover, it also preserves the varietal aroma compounds and reduces the occurrence of ageing off-flavors (Lavigne *et al.*, 2008; Rodríguez-Bencomo *et al.*, 2015). Furthermore the addition of GSH in sparkling wine seems to retain higher levels of SO₂ (Webber *et al.*, 2014). According to the OIV General Assembly in 2015 in Mainz the addition of GSH in musts and wines (Resolutions OIV-OENO 445-2015 and 446-2015), but not exceeding 20 mg/L was recommended (OIV General Assembly, 2015).

There are several methods already reported in literature to quantify GSH but less are described to quantify the related LMW thiols together with GSH in the same analysis. There is a lack of studies done on grape berries, as a result of the difficulty to work with this material due to the fast conversion of the GSH during browning. To avoid this, for the first time, Cheynier *et al.* (1989) used perchloric acid. In 2015, Roland and Schneider described a method using benzene sulfinic acid and sodium metabisulfite and in the same year Pons *et al.*, (2015) published another method with the application of EDTA together with HCl as pretreatments before analysis. Since the determination is challenging but at the same time really important, the aim of this study was to find a fast and easy method, which permits the measurement of more LMW thiols, not only GSH, in grape berries. Different grape varieties and clones were used as sample material to establish and

optimize the analytical method to investigate and quantify natural occurrence of LMW thiols, in grapevine varieties.

Material and methods

Sampling of the grapes

Several bunches of grapes from 11 white and 4 red varieties and clones were sampled at the day of harvesting from the experimental vineyard at Geisenheim, Germany, without damaging any berry (Table 1). Then the pedicel of each berry was cut accurately without damaging the berry skin. Exactly 50 berries were counted in a food safe plastic box and weighted. After that, two other boxes were filled with collected berries of the same weight. Argon was used to purge the boxes to remove the air completely and then frozen immediately with liquid nitrogen. Boxes with samples were kept at -80°C until their analysis to prevent any type of reaction.

Table 1. List of all grape varieties used in this study, the abbreviations used and their pedigree.

Grape variety	Abbreviation	Pedigree
White		
White Riesling	WR	
Pinot blanc (Weißer Burgunder)	PB	
Pinot gris (Ruländer)	PG	
Auxerrois	Au	Heunisch Weiss x Pinot
Hibernal	Hi	(Seibel 7053 x Riesling) F2
Saphira	Sa	Arnsburger x Seyve Villard 1-72
Gm 9620-14	G1	Saphira x Bronner
Gm 8107-3	G2	Ehrenbreitsteiner x Fr 52-64
Gm 9116-1	G3	Prinzipal x Gm 8107-3
Gm 9224-2	G4	Gm 7116-29 x Bronner
Gm 9620-5	G5	Saphira x Bronner
Red		
Pinot noir (Spätburgunder)		
Mixed berry size clone	PN-m	
Loose cluster clone	PN-l	
Compact cluster clone	PN-c	
Dakapo	Da	Deckrot x Portugieser

Sample preparation

From each deep frozen box, still frozen berries were taken out and placed inside a plastic bag to unfreeze during 25 min. After thawing, berries were mashed for 1 min in a plastic bag from the outside by hand and must extracted for subsequent analyses.

For LMW thiol analysis of the grapes, samples were treated with sodium metabisulfite (20 mg/mL); EDTA 1 mM and HCl 0.1 M immediately after mashing them. A volume of 250 μ L of each of them was added together with the same amount of sample to avoid their oxidation. Then, they were centrifuged for 5 min at 13.000 rpm and the supernatant collected. For the analyses of organic acids and sugars, the sample after mashing was used, without any pretreatment.

Analysis of low-molecular-weight thiols

The centrifuged samples were processed with an adaptation of the method developed by Masi *et al.*, 2002. LMW thiols and all compounds were reduced in alkaline conditions using tributylphosphine (TBP) and then derivatized using the fluorescent dye SBD-F (ammonium 7-fluoro 2,1,3-benzoxadiazole-4-sulfonate).

From each box with frozen berries, two analytical samples were taken to be derivatized and analyzed. In this way for each sample three biological replicates were used.

A HPLC analysis was performed using the Agilent 1200 Series (Agilent Technologies Inc., Santa Clara, CA) with a Luna Phenomenex column, RP C18 x 3 μ m, 150 x 3.0 mm and a pre-column C18 4 x 3.0 mm (Phenomenex, Aschaffenburg, Germany) at a temperature of 35°C. The mobile phase was NH₄-formiate 75 mM at pH 2.9: methanol (97:3) as eluent A and methanol 90% as eluent B. The runs had a duration of 80 min with a flow rate of 0.3 ml/min using the gradient reported in Table 2.

A volume of 20 μ L of each sample was injected in the HPLC system. Qualitative and quantitative analyses were done with the application of external standard mixture of the investigated compounds: GSH, Cys, Hcys, Cys-Gly, NAC, Cysteamine and γ -Glu-Cys. The calibration was done using lineal regression of all standards. The conditions for the fluorescent detector were λ exc. 386 nm and λ em. 516 nm. The chromatograms were

then analyzed using the software ChemStation for LC systems (Agilent Technologies Inc., Santa Clara, CA).

Table 2. Method used in HPLC for the thiol analysis. Eluent A was NH₄-formiate 75 mM at pH 2.9: methanol (97:3); eluent B was methanol (90%).

Time (min)	Eluent A (%)	Eluent B (%)
0	100	0
17	100	0
36	88	12
50	85	15
65	90	10
70	100	0
80	100	0

Analysis of organic acids and sugars

The analyses were carried out using the method described previously by Schneider *et al.*, 1987 and modified by Sponholz *et al.* (unpublished). The analyses were performed using a HPLC Agilent 1100 Series and with the column Allure Organic Acids (Rested GmbH, Bad Homburg, Germany) 5 µm, 250 x 4.6 mm at a temperature of 46°C and using the same pre-column similar to thiol analysis. The method used isocratic conditions and the mobile phase was composed of water with 0.5% ethanol and 0.0139% H₂SO₄ concentrated. The flow rate was 0.6 ml/min and the duration time was 30 min for each sample. The injection volume was 5 µL. Two detectors were used: Variable Wavelength Detector (MWD) measured with UV at wavelength 210 nm and Refractive Index Detector (RID). Then, the chromatograms were analyzed using the same software as for the thiol analysis, ChemStation (Agilent Technologies Inc., Santa Clara, CA). This method enables the quantification of fructose and glucose, ethanol and the organic acids shikimic acid, tartaric acid, malic acid, lactic acid, citric acid and acetic acid.

Statistical analysis

For the LMW thiols an analysis of variance (ANOVA) was done for all the variables: Cys, Cys-Gly, γ-Glu-Cys and GSH. Normality for the entire trait was checked through visual inspection of histogram density plots and by the Shapiro Wilk test and a

threshold of 0.05 was used. GSH in the white varieties was not normal and so a Log_{10} transformation was used. In case of the red grapes, there was a bimodal distribution (three Pinot noir clones vs. Dakapo) and an ANOVA could be done for all variables. A Tukey test was applied to check for significant differences between samples. The analyses were performed with R statistical software (R Core Team, 2015).

After that, a principal component analysis (PCA) with all data was calculated. In this case, the sum of glucose and fructose were put together as “Total Sugar” because they were not giving different information for the PCA. This analysis was performed with R-based chemometric software, RGui (Chemometrics of the Italian Chemical Society).

Results and Discussion

The experiments started after adapting the HPLC method from Masi *et al.*, 2002 in the laboratory. The method was optimized adjusting the HPLC conditions. The first step was changing the flow, then analysis time, column temperature and isocratic (with elution buffer) vs. different gradients using elution buffer and the methanol. Once the best method for the HPLC analysis was found, the next step was to find the best method for sample preparation for thiol extraction from the grapes. The method had to permit thiol extraction and quantification by avoiding the reaction of GSH with quinones and polyphenol oxidase reaction (PPO), which is causing browning. As mentioned previously, there were already studies trying to avoid this problem. The first group who tried to avoid these reactions, quantified GSH in grapes using perchloric acid at 6%, which has the advantage to keep sample intact (Cheynier *et al.*, 1989). But recent studies by several groups showed that there are better reagents; for example benzene sulfinic acid (1mg/mL) together with sodium metabisulfite (4.5mg/mL) (Roland and Schneider, 2015); Pons *et al.*, (2015) used a solution of HCl /EDTA (0.1M / 1 mM). After comparing several methods with the same sample, it was decided to use a procedure, which is best for quantifying LMW thiols and especially GSH (Figure 1). Once different combinations of these compounds were tried, the final result was to use sodium metabisulfite (20 mg/mL), EDTA 1 mM and HCl 0.1 M in the same proportion together with the sample (1:1:1:1).

After trying this combination with different berry colors; red, white and different varieties; in the first instance it was shown that red varieties have less GSH than white varieties as it has been already reported by Cheynier *et al.*, (1989). Afterwards, all

calibration curves using all the standards already mentioned were conducted following the described sample preparation and with a linear regression (Figure 2).

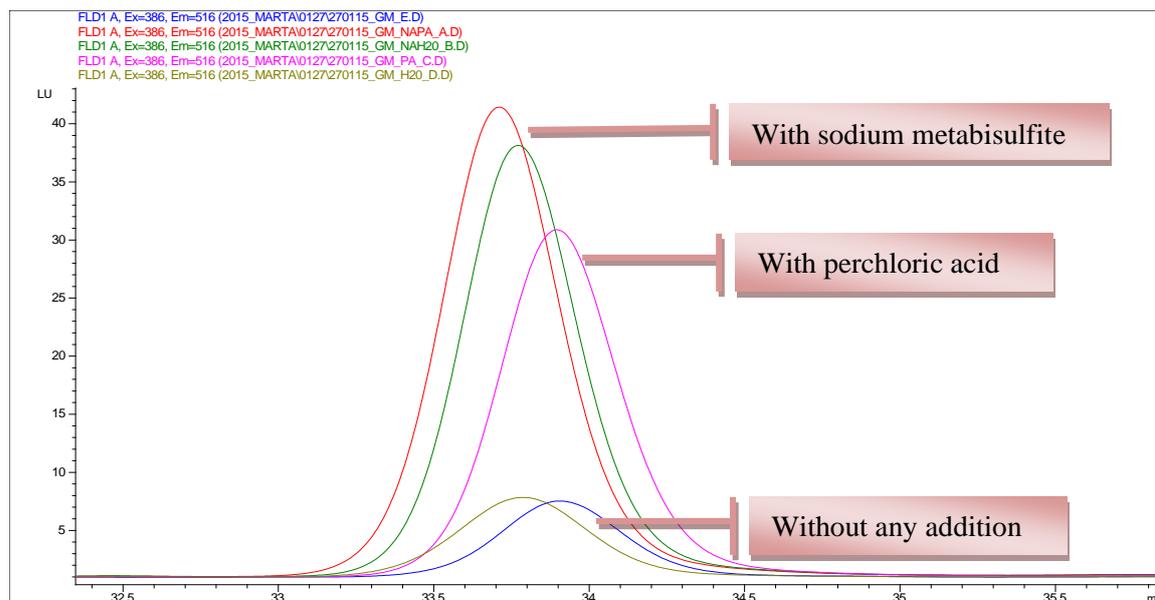


Figure 1. Detail of a GSH peak showing an example of different extraction conditions compared in this study. In red and olive green the analysis of GSH without any pretreatment of the sample can be seen. In pink with a pretreatment of perchloric acid and in red and dark green with sodium metabisulfite in different concentrations. The samples pretreated were diluted 1:4.

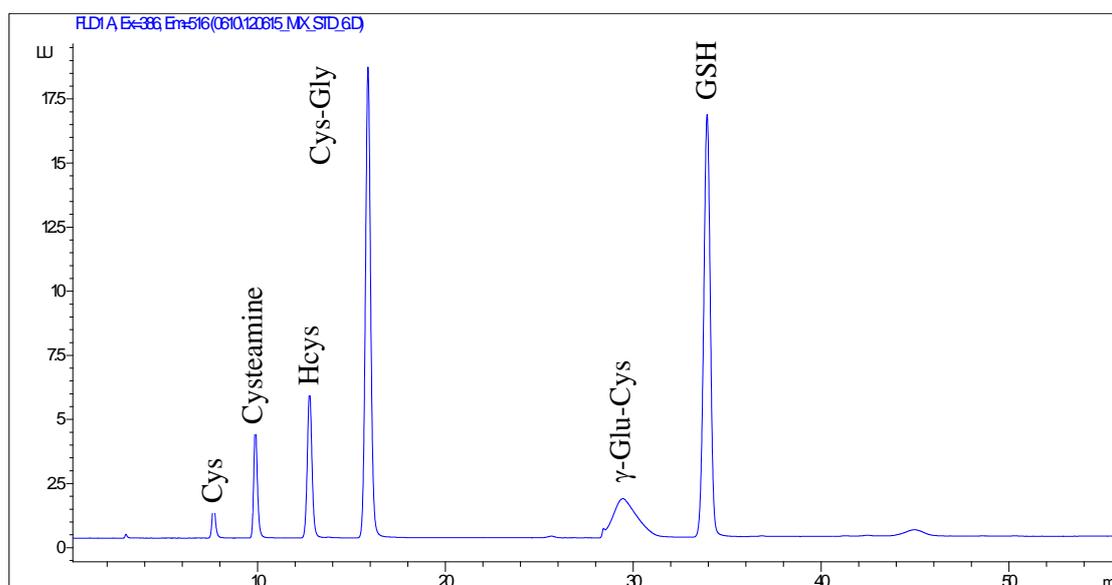


Figure 2. Chromatogram with all the LMW thiol standards used in the HPLC-FL method.

Table 3. Means and standard deviation of LMW thiols of grapes from different varieties (consisting in three biological replicates).

VARIETY	Cys [$\mu\text{mol/L}$]	Cys-Gly [$\mu\text{mol/L}$]	γ -Glu-Cys [$\mu\text{mol/L}$]	GSH [$\mu\text{mol/L}$]
White				
WR	1.37 \pm 0.14	0.23 \pm 0.02	0.33 \pm 0.06	45.0 \pm 6.6
PB	1.68 \pm 0.20	0.20 \pm 0.03	0.26 \pm 0.06	38.2 \pm 10.6
PG	1.83 \pm 0.46	0.12 \pm 0.01	0.17 \pm 0.05	23.0 \pm 6.5
Au	1.14 \pm 0.16	0.15 \pm 0.01	0.26 \pm 0.04	27.7 \pm 1.9
Hi	2.62 \pm 0.25	0.32 \pm 0.03	0.64 \pm 0.19	84.9 \pm 16.6
Sa	2.82 \pm 0.10	0.37 \pm 0.03	1.92 \pm 0.38	129.8 \pm 16.3
G1	1.97 \pm 0.28	0.21 \pm 0.01	0.60 \pm 0.11	91.5 \pm 4.5
G2	2.56 \pm 0.31	0.24 \pm 0.02	0.22 \pm 0.09	29.5 \pm 3.1
G3	1.43 \pm 0.22	0.16 \pm 0.03	0.14 \pm 0.01	11.2 \pm 0.7
G4	2.83 \pm 0.16	0.45 \pm 0.04	0.91 \pm 0.16	122.1 \pm 9.4
G5	3.34 \pm 0.39	0.44 \pm 0.04	1.27 \pm 0.20	150.8 \pm 23.6
Red				
PN-m	0.93 \pm 0.04	0.09 \pm 0.02	0.18 \pm 0.02	15.4 \pm 4.0
PN-l	1.40 \pm 0.17	0.14 \pm 0.01	0.17 \pm 0.05	15.1 \pm 5.3
PN-c	1.29 \pm 0.11	0.14 \pm 0.01	0.19 \pm 0.04	11.0 \pm 0.8
Da	2.31 \pm 0.19	0.24 \pm 0.02	1.19 \pm 0.10	35.6 \pm 6.3

Table 4. Means and standard deviation of sugars and organic acids of grapes from different varieties (consisting in three biological replicates).

VARIETY	SUGARS			ORGANIC ACIDS		
	Glucose [g/L]	Fructose [g/L]	Tartaric acid [g/L]	Malic acid [g/L]	Shikimic acid [mg/L]	Citric acid [g/L]
White						
WR	106 \pm 5	119 \pm 5	6.7 \pm 0.5	5.4 \pm 0.1	81.0 \pm 4.0	0.27 \pm 0.02
PB	101 \pm 6	117 \pm 5	4.8 \pm 0.7	6.0 \pm 0.2	17.0 \pm 0.9	0.29 \pm 0.03
PG	100 \pm 4	116 \pm 5	4.6 \pm 0.4	6.0 \pm 0.3	17.2 \pm 1.1	0.27 \pm 0.02
Au	101 \pm 1	113 \pm 1	5.3 \pm 0.2	3.1 \pm 0.2	29.7 \pm 4.2	0.20 \pm 0.01
Hi	92 \pm 1	99 \pm 1	4.3 \pm 0.1	6.6 \pm 0.2	33.0 \pm 1.3	0.28 \pm 0.03
Sa	99 \pm 5	114 \pm 6	4.4 \pm 0.3	10.1 \pm 0.4	19.1 \pm 0.9	0.26 \pm 0.10
G1	107 \pm 4	122 \pm 5	4.5 \pm 0.2	5.0 \pm 0.1	24.2 \pm 0.7	0.25 \pm 0.02
G2	93 \pm 2	107 \pm 1	5.2 \pm 0.7	3.3 \pm 0.2	13.8 \pm 0.8	0.15 \pm 0.03
G3	95 \pm 2	112 \pm 1	5.3 \pm 0.1	5.3 \pm 0.3	8.8 \pm 0.3	0.20 \pm 0.05
G4	120 \pm 9	128 \pm 5	4.3 \pm 0.5	3.2 \pm 0.3	27.1 \pm 1.8	0.27 \pm 0.03
G5	112 \pm 3	127 \pm 3	4.6 \pm 0.6	8.5 \pm 0.4	14.6 \pm 0.8	0.35 \pm 0.04
Red						
PN-m	103 \pm 1	110 \pm 1	5.3 \pm 0.4	5.6 \pm 0.2	9.7 \pm 1.2	0.34 \pm 0.01
PN-l	104 \pm 9	105 \pm 9	4.5 \pm 0.4	5.1 \pm 0.3	11.4 \pm 0.8	0.31 \pm 0.03
PN-c	99 \pm 2	94 \pm 3	5.2 \pm 0.2	6.0 \pm 0.2	10.3 \pm 0.4	0.34 \pm 0.02
Da	90 \pm 4	96 \pm 3	4.6 \pm 0.2	6.9 \pm 0.5	68.5 \pm 3.0	0.41 \pm 0.03

Results indicate that the proposed sample preparation procedure yields good and reproducible results, as it is shown by the standard deviation from the several replicates and also between the thiol analysis and the organic acid analysis (Table 3 and 4). The analyses of sugars and organic acids was also conducted to confirm that the method is not only reliable for LMW thiol analysis, but it is also useful for analyzing other kind of compounds with other detection systems at the same time utilizing the same sample preparation. The amount of lactic acid and acetic acid were <0.1 , and are not shown in the Table 4. This result is good, as these two compounds are not present in grapes; they are only produced during fermentation procedure or berry rot.

In white varieties (Figure 3), Saphira, Gm 9620-5, Gm 9224-2 and also Hiberna were the ones with high amounts of LMW thiols.

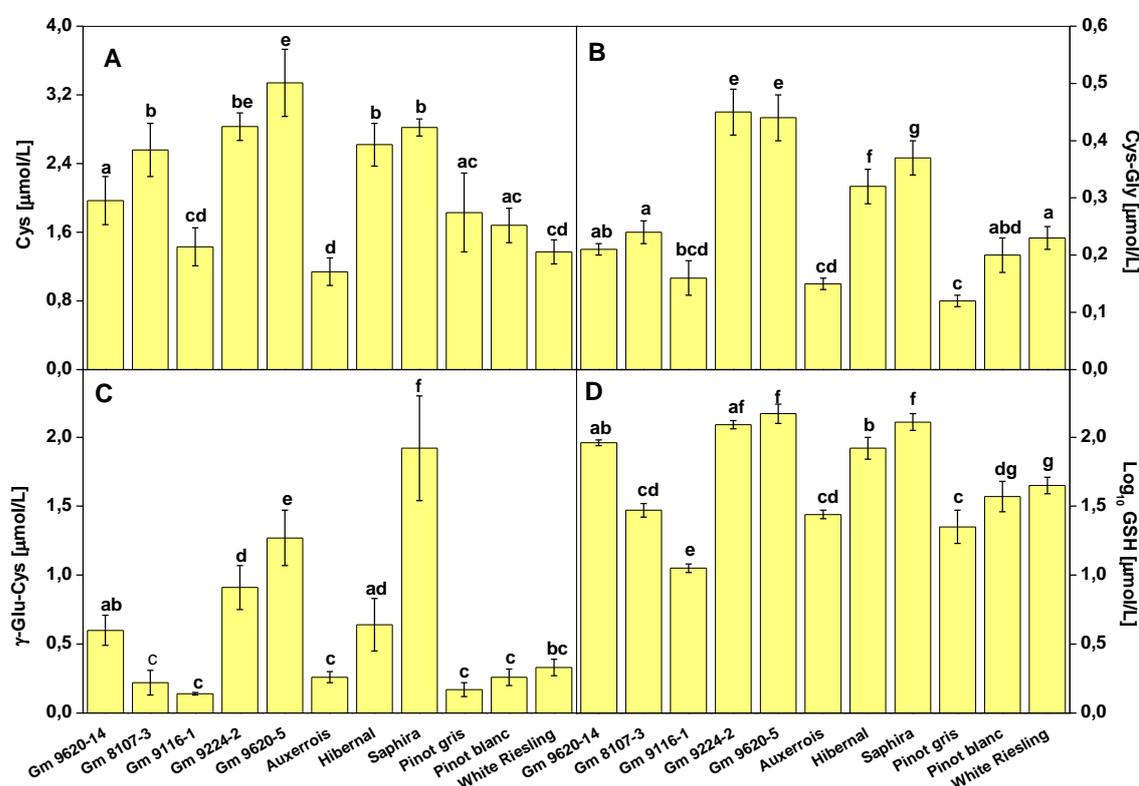


Figure 3. LMW content (A) Cys, (B) Cys-Gly, (C) γ -Glu-Cys and Log₁₀ GSH (D) in μ M in the white varieties analyzed. The bars show concentrations. Reported values are the mean \pm SD of three biological replicates. Different letters indicate significant differences between different varieties. In all the cases $P \leq 0.001$ ***

In red varieties (Figure 4), Dakapo is the variety with significantly higher amounts of the LMW thiols. The reason might be that this teinturier variety synthesizes anthocyanins in all plant parts and due to that; there is a high amount of GSH for transport to the vacuole (Marrs *et al.*, 1995). The other three genotypes are different clones of the same variety (Pinot noir), which in case of the GSH and γ -Glu-Cys have similar contents, but in case of Cys and Cys-Gly, the clone SB-m is significantly lower than the others.

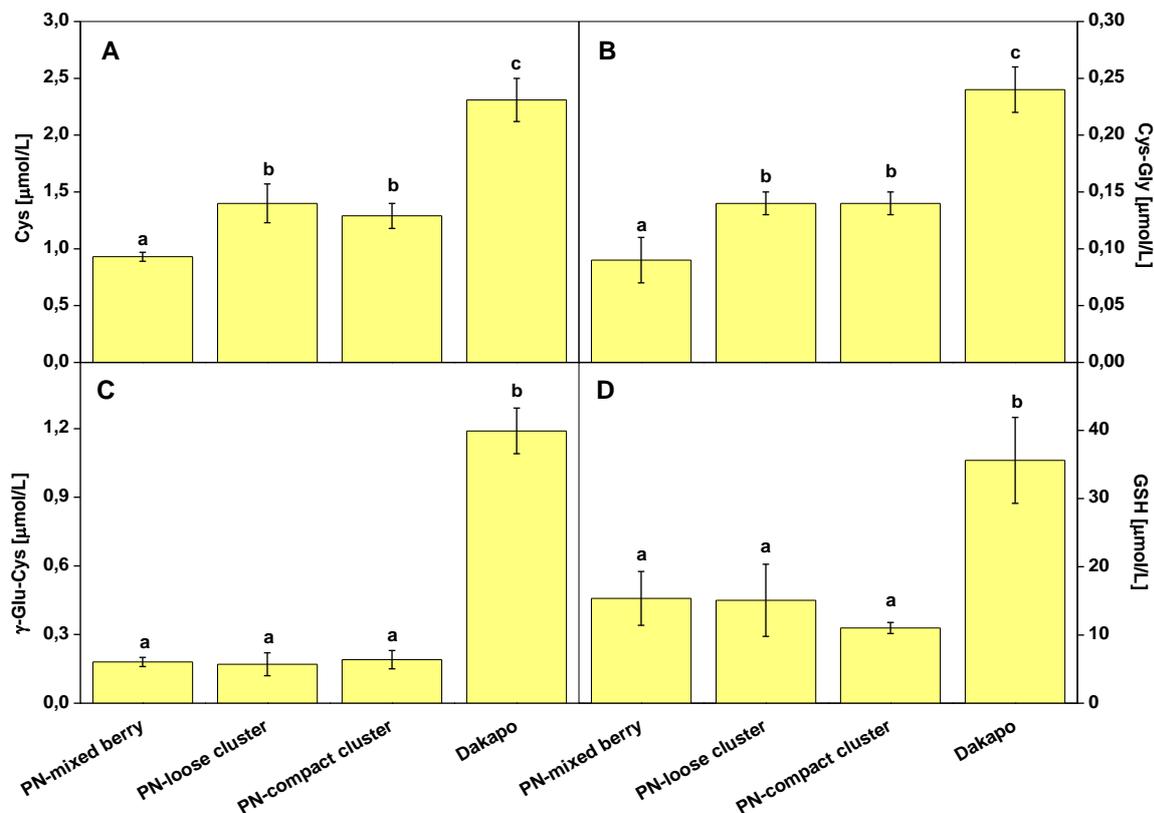


Figure 4. LMW content (A) Cys, (B) Cys-Gly, (C) γ -Glu-Cys and GSH (D) in μM in the red varieties analyzed. The bars show the total concentration. Reported values are the mean \pm SD of three biological replicates. Different letters indicate significant differences between different varieties. In all the cases $P \leq 0.001$ ***

These results can also be confirmed from the PCA Plots (Scores and Loadings) (Figure 5 and 6), where these varieties with the higher amount of thiols are oriented in the positive direction of the first PC, while the others are on the negative side. The samples with higher sugar concentrations (e.g. Gm 9224-2) are found in the lower left quadrant. The variation of the replicates is acceptable. For the PCA, due to the low variation

between analytical replicates, the average between analytical samples was used for the three biological replicates (the three boxes).

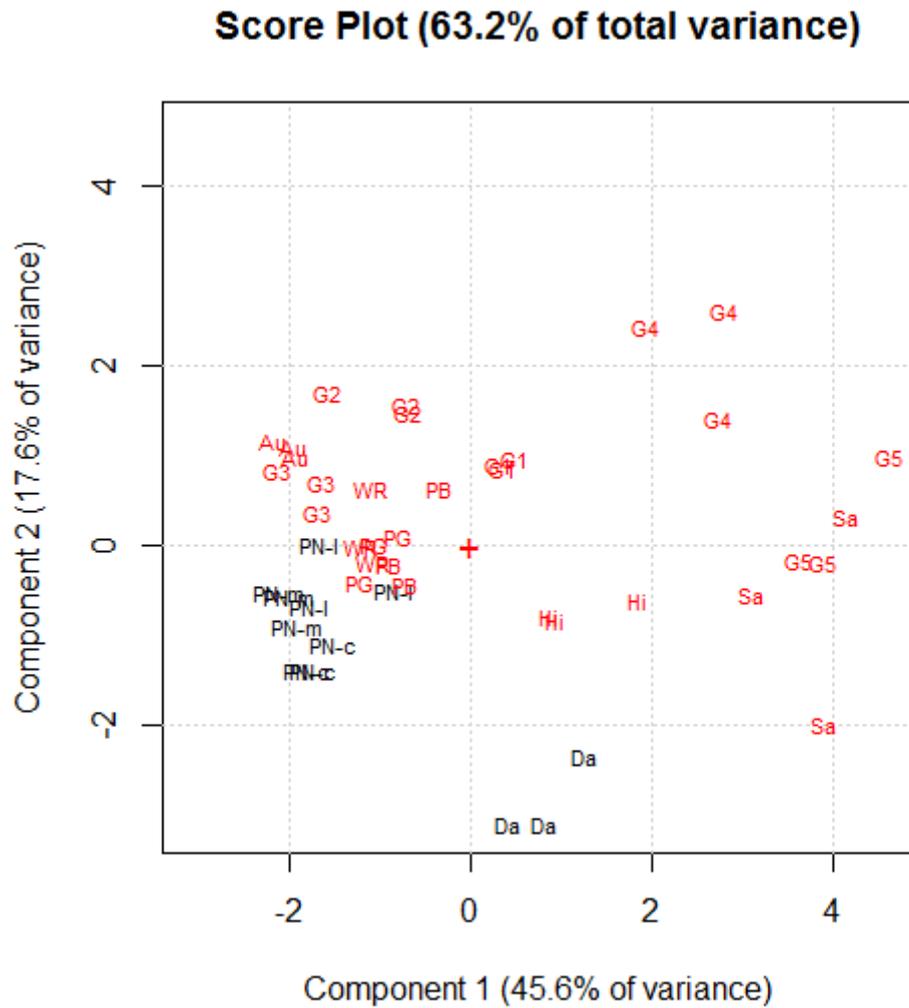


Figure 5. Score Plot from all the varieties analyzed in this study. Red varieties are indicated in black color and white varieties in red color. The PCA explains 63.2% of total variance.

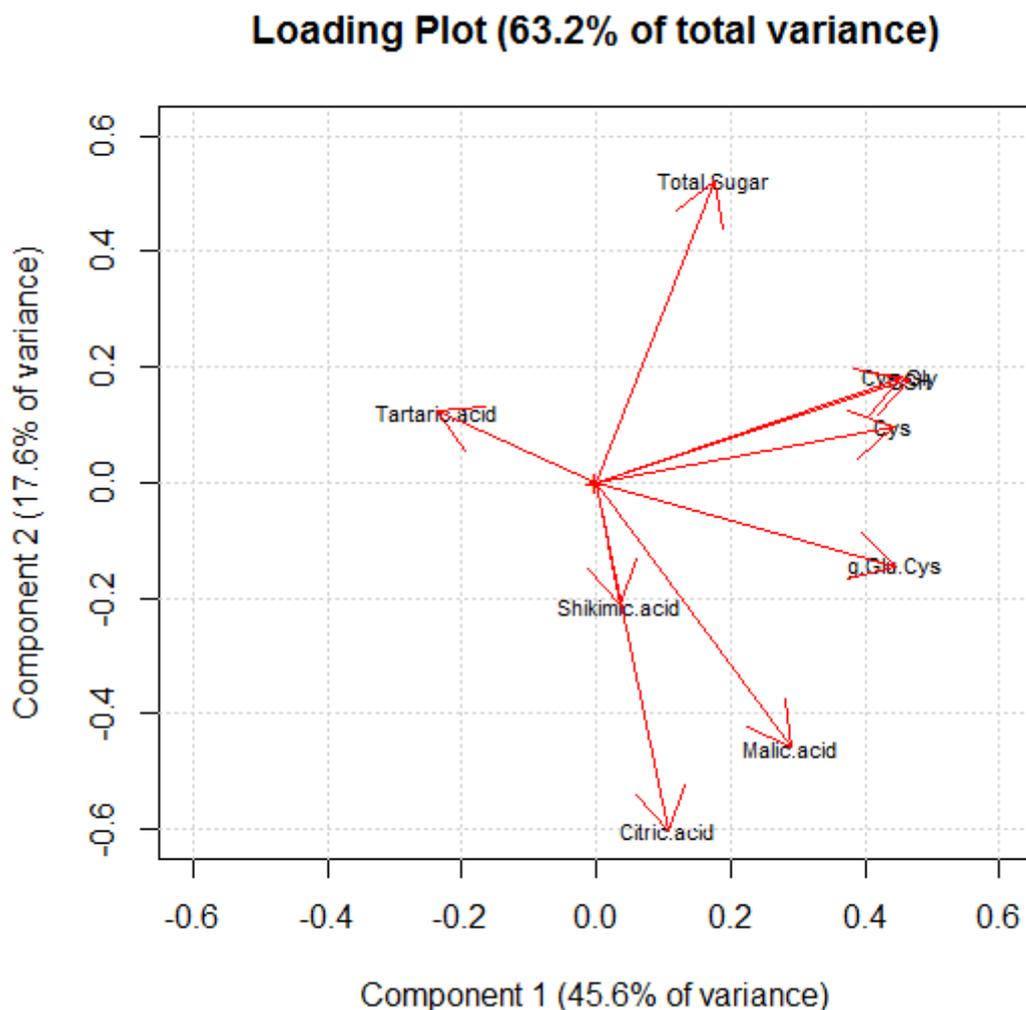


Figure 6. Loading Plot from all varieties analyzed in this study. The PCA (Component 1 and 2) explains 63.2% of total variance.

The developed method for sampling and for measuring the LMW thiols from the grape berries gives good and reliable results. The same method can be also used to analyze LMW thiols in must and wine. The aim of this method was an optimization of sampling and sample preparation because sometimes only small amounts of grape berries are available. With this method it is possible to analyze only one box of 50 berries and get a reliable result. Sometimes in vineyard trials it is difficult to get larger amounts of berries, because there is a limitation in sampling due to several reasons: e.g. plot size or other studies. This method is fast and may be used to determine how these compounds are increasing during ripening or how a viticultural treatment in the field affects their amounts. It could even be used by winemakers to determine the optimum state of grape

harvesting. An additional advantage of the sampling method is that it fits to the analytical procedure and allows a minimum amount of samples for a good reproducibility (Table 5).

Table 5. Calibration and validation data of the HPLC-FL method used for the LMW thiol analysis.

Analyte	Calibration function	R ²	Calibration range [μmol/L]	LOD (noise *3) [μmol/L]	LOQ (noise *10) [μmol/L]	Reproducibility RSD (%)*
Cys	y=6.857x - 0.280	0.999	0.5 - 10	0.011	0.036	8.3
Cys-Gly	y=66.34x + 1.858	0.997	2 - 20	0.006	0.019	7.0
γ-Glu-Cys	y=13.86x+1.935	0.999	0.5 - 10	0.013	0.044	4.5
GSH	y=31.36x-73.96	0.999	1 - 500	0.015	0.050	2.6

* for n=2 and 5 different samples (red and white).

This method allows not only to quantify the amounts of GSH; but it is possible to determine in the same chromatogram the amount of other LMW thiols.

References

- Adams, D.O., Liyanage, C.(1993). Glutathione increases in grape berries at the onset of ripening. *American Journal of Enology and Viticulture*, **44**, 333-338.
- Cassol, T. and Adams, D.O. (1995). Detection of glutathione in white wines using an enzymatic analytical method. *American Journal of Enology and Viticulture*,**46**, 410.
- Cheyrier, V., Souquet, J.M., Moutounet, M. (1989). Glutathione content and glutathione to hydroxycinnamic acid ratio in *Vitis vinifera* grapes and musts. *American Journal of Enology and Viticulture*,**40**, 320–324.
- Du Toit, W.J., Lisjak, K., Stander, M., Prevoo, D. (2007). Using LC-MSMS to assess glutathione levels in South African white grape juices and wines made with different levels of oxygen. *Journal of Agricultural and Food Chemistry*,**55**, 2765–2769.
- Fracassetti, D., Lawrence, N., Tredoux, A.G.L., Tirelli, A., Nieuwoudt, H.H. Du Toit, W.J. (2011). Quantification of glutathione, catechin and caffeic acid in grape juice and wine by a novel ultraperformance liquid chromatography method. *Food Chemistry*, **128**, 1136–1142.
- Hosry, L.E., Auezova, L., Sakr, A., Hajj-Moussa, E. (2009). Browning susceptibility of white wine and antioxidant effect of glutathione. *International Journal of Food Science & Technology*, **44**, (12), 2459–2463.
- Kritzinger, E.C., Bauer, F.F., Du Toit, W.J. (2013). Role of glutathione in winemaking: a review. *Journal of Agricultural and Food Chemistry*, **61**, 269–277.
- Lavigne, V., Pons, A., Dubourdieu, D. (2007). Assay of glutathione in must and wines using capillary electrophoresis and laser-induced fluorescence detection. Changes in concentration in dry white wines during alcoholic fermentation and aging. *Journal of Chromatography A*, **1139**, 130–135.
- Lavigne, V., Pons, A, Darriet, P., Dubourdieu, D. (2008). Changes in the sotolon content of dry white wines during barrel and bottle aging. *Journal of Agricultural and Food Chemistry*, **56**, 2688-2693.
- Marrs, K.A., Alfenito, M.R., Lloyd, A.M., Walbot, V. (1995). A glutathione S-transferase involved in vacuolar transfer encoded by the maize gene *Bronze-2*. *Nature*, **375**, 397-400.
- Masi, A., Ghisi, R., Ferretti, M. (2002). Measuring low-molecular-weight thiols by detecting the fluorescence of their SBD-derivatives: application to studies of diurnal and UV-B induced changes in *Zea mays* L. *Journal of Plant Physiology*, **159**, 499-507.
- OIV-OENO 445-2015 Resolution. Treatment of must with glutathione.
- OIV-OENO 446-2015 Resolution. Treatment of wine with glutathione.

Okuda, T. and Yokotsuka, K. (1999). Levels of glutathione and activities of related enzymes during ripening of Koshu and Cabernet Sauvignon grapes and during winemaking. *American Journal of Enology and Viticulture*, **50**, 264-270.

Pivato, M., Fabrega-Prats, M., Masi, A. (2014). Low-molecular-weight thiols in plants: Functional and analytical implications. *Archives of Biochemistry and Biophysics*, **560**, 83-99.

Pons, A., Lavigne, V., Darriet, P., Dubourdieu, D. (2015). Glutathione preservation during winemaking with *Vitis vinifera* white varieties: example of Sauvignon blanc grapes. *American Journal of Enology and Viticulture*, **66**, 187-194.

R-based chemometric software developed by the group of Chemometrics of the Italian Chemical Society, freely downloadable from the site gruppochemiometria.it.

R Core Team (2015). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. URL <https://www.R-project.org/>.

Rodríguez-Bencomo, J.J., Andújar-Ortiz, I., Sánchez-Patán, F., Moreno-Arribas, M.V., Pozo-Bayón, M.A. (2015). Fate of the glutathione released from inactive dry yeast preparations during the alcoholic fermentation of white musts. *Australian Journal of Grape and Wine Research*, *Article in press*. doi: 10.1111/ajgw.12161.

Roland, A. and Schneider, R. (2015). Development and validation of a high-throughput analysis of glutathione in grapes, musts and wines by Stable Isotope Dilution Assay and LC-MS/MS. *Food Chemistry*, **177**, 152-157.

Schneider, A., Gerbi, V., Redoglia, M. (1987). A rapid HPLC method for separation and determination of major organic acids in grape musts and wines. *American Journal of Enology and Viticulture*, **38**, 151-155.

Singleton, V.L., Zaya, J., Trousdale, E., Salgues, M. (1984). Caftaric acid in grapes and conversion to a reaction product during processing. *Vitis*, **23**, 113-120.

Vaimakis, V., Roussis, I.G. (1996). Must oxygenation together with glutathione addition in the oxidation of white wine. *Food Chemistry*, **57** (3), 419-422.

Vallverdú-Queralt, A., Verbaere, A., Meudec, E., Cheynier, V., Sommerer, N. (2015). A straightforward method to quantify GSH, GSSG, GRP and hydroxycinnamic acids in wines by UPLC-MRM-MS. *Journal of Agricultural and Food Chemistry*, **63**(1), 142–149.

Webber V., Dutra S.V., Spinelli F.R., Marcon A.R., Carnieli G.J., Vanderlinde R. (2014) Effect of glutathione addition in sparkling wine. *Food Chemistry*, **159**, 391-398.

List of Tables

Table 1. List of all grape varieties used in this study, the abbreviations used and their pedigree.

Table 2. Method used in HPLC for the thiol analysis. Eluent A was NH₄-formiate 75 mM at pH 2.9: methanol (97:3); eluent B was methanol (90%).

Table 3. Means and standard deviation of LMW thiols of grapes from different varieties (consisting in three biological replicates).

Table 4. Means and standard deviation of sugars and organic acids of grapes from different varieties (consisting in three biological replicates).

Table 5. Calibration and validation data of the HPLC-FL method used for the LMW thiol analysis.

List of Figures

Figure 1. Detail of a GSH peak showing an example of different extraction conditions compared in this study. In red and olive green the analysis of GSH without any pretreatment of the sample can be seen. In pink with a pretreatment of perchloric acid and in red and dark green with sodium metabisulfite in different concentrations. The samples pretreated were diluted 1:4.

Figure 2. Chromatogram with all the LMW thiol standards used in the HPLC-FL method.

Figure 3. LMW content (A) Cys, (B) Cys-Gly, (C) γ -Glu-Cys and Log_{10} GSH (D) in μM in the white varieties analyzed. The bars show concentrations. Reported values are the mean \pm SD of three biological replicates. Different letters indicate significant differences between different varieties. In all the cases $P \leq 0.001$ ***

Figure 4. LMW content (A) Cys, (B) Cys-Gly, (C) γ -Glu-Cys and GSH (D) in μM in the red varieties analyzed. The bars show the total concentration. Reported values are the mean \pm SD of three biological replicates. Different letters indicate significant differences between different varieties. In all the cases $P \leq 0.001$ ***

Figure 5. Score Plot from all the varieties analyzed in this study. Red varieties are indicated in black color and white varieties in red color. The PCA explains 63.2% of total variance.

Figure 6. Loading Plot from all varieties analyzed in this study. The PCA (Component 1 and 2) explains 63.2% of total variance.

CONCLUSIONS

In conclusion, the method developed in this work using the SBD-derivatives to investigate the unknown LMW thiols present in plant species, but also in animal and plant derived products, is good and it is working.

The method relies on the occurrence, following MS/MS fragmentation of SBD-derivatives in the negative mode, of a typical mass of 231 m/z corresponding to SBD-S and is therefore a genuine marker of the presence of an –SH group in any given thiol-bearing molecule.

Following this fragmentation rule, thioglucose in *Brassicaceae* plants was identified and confirmed by authentic standards. Other molecules whose putative structure has been proposed, are in the process of being confirmed.

Their occurrence in the plant kingdom and also their function in plant physiology and metabolism will be the next challenging work.

In the case of the thioglucose, identification of this molecule can be used for scientific research, for example for the simultaneous quantification of the total glucosinolate content and other LMW thiols, which may allow to investigate if any correlation with them exists.

The method for the quantification of LMW thiols using the SBD-F fluorescent dye can also be applied in grape analysis. Different grape varieties show different thiol, sugars and organic acids composition. This information adds more knowledge about LMW thiols and specially the GSH, which has a key role in the winemaking. This method can be applied in the future for the quantification of these compounds in musts, wines and yeasts. It can also be used to monitor a process from the microbiological point of view using different yeast strains in different fermentation and nutrients conditions.

LIST OF PUBLICATIONS

2013

- Anna Rita Trentin, Serena Tolin, Micaela Pivato, Sabrina Giaretta, **MARTA FABREGA-PRATS**, Mehdi Syed Muhammad Muntazir, Giorgio Arrigoni, Dinesh Prasad, Antonio Masi (2013). *Functions of the Gamma-Glutamyl Cycle in Plant Metabolism Explored by Proteomic Analysis of Arabidopsis Mutants*. VIII Italian Proteomic Association (ItPA) National Congress, Padua (Italy), 18-21th June 2013.
- Anna Rita Trentin, Micaela Pivato, Sabrina Giaretta, **MARTA FABREGA-PRATS**, Giorgio Arrigoni, Dinesh Prasad, Antonio Masi (2013). *GGT-assisted glutathione degradation is functional to apoplastic redox control and seed storage proteins accumulation*. 11th International POG conference Reactive Oxygen and nitrogen species in plants Warsaw (Poland), 17-19th July 2013.

2014

- Micaela Pivato, **MARTA FABREGA-PRATS**, Antonio Masi (2014). *Low-molecular-weight thiols in plants: Functional and analytical implications*. Archives of Biochemistry and Biophysics, vol. 560; pp. 83-99, ISSN: 0003-9861, doi: 10.1016/j.abb.2014.07.018.
- Anna Rita Trentin, Micaela Pivato, Leonard Barrabas Ebinezer, Sabrina Giaretta, Dinesh Prasad, **MARTA FABREGA-PRATS**, Antonio Masi (2014). *Role of apoplastic GGT1 in plant's response to UV-B radiation*. The final network conference of COST Action FA0906, UV4 Growth. Bled (Slovenia), 30th March-2nd April 2014.
- Anna Rita Trentin, Micaela Pivato, Sabrina Giaretta, **MARTA FABREGA-PRATS**, Leonard Barrabas Ebinezer, Mehdi Syed Muhammad Muntazir, Antonio Masi (2014). *Involvement of extracellular glutathione degradation in the response to UV-B radiation in A. thaliana wild-type and ggt1 mutant*. 9th International Workshop: Sulfur Metabolism in Plants. Freiburg im Breisgau (Germany), 14-17th April 2014.

- **MARTA FABREGA-PRATS**, Anna Rita Trentin, Antonio Masi, Stefano Dall'Acqua (2014). *Identification of low molecular weight thiols in plants by fluorescence derivatization and LC-MS/MS*. 20th International Mass Spectrometry Conference (IMSC). Geneva (Switzerland), 24-29th August 2014.

2015

- Anna Rita Trentin, Micaela Pivato, Mehdi Syed Muhammad Muntazir, Leonard Barrabas Ebinezer, Sabrina Giaretta, **MARTA FABREGA-PRATS**, Dinesh Prasad, Antonio Masi (2015) *Proteome readjustments in the apoplastic space of Arabidopsis thaliana ggt1 mutant leaves exposed to UV-B radiation*. *Frontiers in Plant Science*, vol. 6; article 128. doi: 10.3389/fpls.2015.00128.
- Anna Rita Trentin, Nisha Sharma, Sabrina Giaretta, **MARTA FABREGA-PRATS**, Giorgio Arrigoni, Antonio Masi (2015). *Gamma-glutamyl cycle and oxidative stress: investigations on apoplastic proteome composition in ggt1 mutants*. 12th International POG conference Reactive Oxygen and nitrogen species in plants. Verona (Italy), 24-26th June 2015.
- **MARTA FABREGA-PRATS**, Antonio Masi, Rossella Ghisi, Antonella Crapisi, Andrea Curioni, Simone Vincenzi (2015). *Low-molecular-weight thiol profiling in different Italian white grape varieties during maturation*. IX In Vino Analytica Scientia (IVAS) Congress. Trento (Italy), 14-17th July 2015.
- Sabrina Giaretta, Anna Rita Trentin, **MARTA FABREGA-PRATS**, Antonio Masi (2015). *Properties and functions of the γ -glutamyl cycle in plants: an overview*. "From Molecules to the Field" BOTANIKERTAGUNG 2015. Freising (Germany), 30th August-3rd September 2015.
- **MARTA FABREGA-PRATS**, Anna Rita Trentin, Antonio Masi, Stefano Dall'Acqua (2015). *Identification and quantification of low-molecular-weight thiols in plants HPLC-MS/MS and fluorescence derivatization*. EMBO Organized Congress "Thiol-based switches in life sciences". Sant Feliu de Guíxols (Spain), 12-17th September 2015.

- **MARTA FABREGA-PRATS**, Antonio Masi, Heike Semmler, Ernst Rühl, Claus-Dieter Patz, Doris Rauhut. *Method optimization and quantification of low-molecular-weight thiols in grapes by HPLC-FL detection*. Under submission in Australian Journal of Grape and Wine Research.