SEDE AMMINISTRATIVA: UNIVERSITÀ DEGLI STUDI DI PADOVA

DIPARTIMENTO DI AGRONOMIA ANIMALI ALIMENTI RISORSE NATURALI E AMBIENTE

CORSO DI DOTTORATO IN ANIMAL AND FOOD SCIENCE

CICLO XXXII

Method development for thiols analysis: identification and quantitation in plant products through mass spectrometry techniques

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“To my grandmother Agnese”

ACKNOWLEDGEMENTS

I would like to express my thanks to my supervisor Prof. Antonio Masi for his support during this three years path and for encouraging my research. I would like to thanks Prof. Stefano Dall'Acqua for following me during my approach in the amazing mass spectrometry world and for his useful suggestions.

I would like to thank my colleagues Nisha Sharma and Anna Rita Trentin and the Prof. Rossella Ghisi for their support and encouragements during the bad moments. In particular thank you Nisha, we shared lots of moments, a long-distance flight, lot of food and the same path. Thank you my friend.

I would like to thank my colleagues and friends from Padova University, Sarah Currò and Eros Borsato, for their constant support and friendship.

I gratefully acknowledge to all the people I met in Australia, expecially Prof. David Jeffery who followed me during this experience. Also a special thanks to Dimitra Capone for her help in software utilization and useful suggestions, to Liang Chen who followed me during my lab activity, to all the people in Wine Science group.

I also acknowledge the founding sourced that made my PhD possible. I was founded by University of Padova grant during these three years. I also want thank Aldo Gini Foundation grant during my six months period abroad.

Finally, a special thanks to my family. Thanks to my mother, my father, my sister and my grandparents for supporting me during this experience. Thank you with all my heart!

CONTENTS

LIST OF ABBREVIATIONS

4-TP = 4-thiopyridine

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

APS = 5'-phosphosulfate

ATP = adenosine monophosphate

ATPS = adenosine-5'-triphospahte sulfurylase

CMPI = 2-chloro-1-methylpyridinium

CMQT = 2-chloro-1-methylquinolinium

Cyis = Cystine

Cys = Cysteine

CysGly = Cysteinylglycine

CysT = Cyateamine

DTDP = 4,4'-dithiodipyridine

DTNB = 5,5'-dithio-bis-(2-nirobenzoic acid)

DTT = Dithiothreitol

EC = Electrochemical

ECD = Electrochemical detection

FL = Fluorescence

γ-GC = γ-glutamylcysteine

GGCT = γ-glutamyl ciclo-transferase

GGT = γ-glutamyltransferase /transpeptidase

GR = Glutathione Reductase

GSH = Reduced Glutathione

GSSG = Ozidized glutathione

Hcys = Homocysteine

HPLC = High Performance Liquid Chromatography

HPLC-FL= High Performace Liquid Chromatography- Fluorimetric detector

HPLC-MS = High Performace Liquid Chromatography- Mass Spectrometer detector

HPLC-MS/MS = High Performace Liquid Chromatography- Tandem Mass Spectrometer detector

HRMS = High Resolution Mass Spectrometry

LMW = Low Molecular Weight

mBBr = monobromobimane

ME = mercaptoethanol

Met = Methionine

MS = Mass Spectrometry

NADPH = nicotinamide adenine dinucleotide phosphate

PAPS = 3'-phosphoadenosine 5'-phosphosulfate

PCs = phytochelatins

QqQ = triple quadrupole

QToF= quadrupole – time of flight

RDS = relative standard deviation

ROS = reactive oxygen species

RT = retention time

SAM = S-adenosylmethionine

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

SDS = sodium dodecyl sulfate

SiR = sulfite reductase

SPE = solid phase extraction

SRM = Selected Reaction Monitoring

TCEP = tris(2-carboxyethyl)phosphine

ABSTRACT

Thiols are a class of organic compounds, which play a crucial role in maintenance of the redox homeostasis in plants. Furthermore, they are involved in oxidative stress response and in regulation of cellular metabolism. These compounds are characterized by a nucleophilic -SH group and they represent the principal source of reduced sulfur in plants, in the forms of protein thiols and low molecular weight (LMW) thiols. The latter are involved in deactivation of xenobiotics, in enzymatic reactions and in the modification of redox state of sensitive molecules in plants.

Several analytical methods were developed for the quantification of thiols and disulfides in different matrices. The most studied thiols in plants are glutathione and cysteine, whereas the knowledge related to other LMW thiols are limited. They are biological thiols, such as homocysteine, cysteamine and cysteinylglycine, or volatile thiols, studied in food and beverages (e.g. wine) for their flavouring properties.

Recent studies show that a huge amount of LMW thiols exists, but several of them still need to be identified and, due to their low concentration in plants, their identification represent a major challenge.

In order to deepen the study of this class of compounds, in this work two new analytical methods, for the identification and quantification of thiols, were carried out through the utilization of mass spectrometry techinques. After the derivatisation with 4,4'-dithiodipyridine (DTDP) of plant extracts, samples were analysed by high performance liquid chromatography (HPLC) coupled with triple quadrupole (QqQ) mass spectrometer.

The preventive analysis of authentic thiol standards led to the definition of instrumental parameters and to 4-TP-thiol-derivatives fragmentation pattern. Compounds as glutathione, cysteine and cysteinylglycine were used as references in order to define a fragmentation rule for the development of both quantification and identification methods.

The quantification methods was developed through the analysis in SRM mode of authentic thiol standards. The fragmentation pattern for each compounds were defined after the direct infusion of standards in QqQ. This method allows to determine the concentration level of glutathione, glutathione disulfide, cysteine, cystine, cysteamine, cysteinylglycine and homocysteine in plant extracts. The method was validated for cauliflower leaves, rocket, garlic, onion and walnut, showing high sensitivity and selectivity.

The identification method was developed through the distinctive fragmentation spectra produced by 4-TP-thiols-derivatives, characterised by the formation of m/z 144, m/z 112, m/z 111 or the neutral loss of 111 from the 4-TP part of the molecules. These signals were used as markers to confirm the presence of thiol-containing compounds in plant extracts. By the analysis of cauliflower leaves, rocket, garlic and onion extracts in precursor ion scan and neutral loss scan, a list of m/z values was obtained for each matrix. Unknown thiols were identified with the exact molecular mass and molecular formula determined using high-resolution mass spectrometer (HRMS) quadrupole-time of flight (QTOF). Identified compounds were confirmed by the analysis of authentic standards in SRM scan. Furthermore, any matrix was spiked with the authentic standard and then analysed after derivatisation.

Identification method was applied to different *Brassicaceae* species from the Botanical Garden of the University of Padova, showing distinct thiol compositions, with several species-specific compounds.

CHAPTER I

GENERAL INTRODUCTION

Metabolism of low-molecular-weight thiols in plants and analytical techniques for their detection

Introduction

Thiols are a class of organic sulphur compounds, characterized by a sulfhydryl residue (-SH) which makes the thiol moiety one of the strongest nucleophilic molecule in living cells. They are implicated in the maintenance of cellular redox homeostasis and in plant responses to stress factors. Moreover, they are essential in the regulation of cellular metabolism.

Biological thiols (generally called biothiols) can be classified as low molecular weight free thiols or large molecular weight protein thiols. The latter are characterized by the occurrence of several disulphide linkages (-S-S-) between two sulfhydryl residues, which affect the protein structure and properties. The sulfur containing amino acids cysteine (Cys) and methionine (Met) are essential components of protein, with important regulatory and structural functions in biological systems. Nevertheless, it was proven that about 2% of organic sulfur reduced molecules in plants do not occur in protein form, but in low molecular weight (LMW) thiols (Rennenberg, Brunold, Dekok, & Stulen, 1990). The most studied LMW thiols in plants are glutathione (GSH) and its related compounds, such as Cys, cysteinylglycine (CysGly), γ-glutamylcysteine (γ-GC) and phytochelatins (PCs), but the existence of hundreds of unidentified LMW thiols was proven in recent studies (Inoue et al., 2013; Kanawati, Kubo, Schmitt-kopplin, & Grill, 2014). Their features are still unknown, but recent advances in analytical techniques can provide a new way for identifying these compounds and shed light on their role(s) in plant physiology and sulfur metabolism.

This thesis aims to describe the known LMW thiols in plants and to suggest new analytical approaches for identifying new thiol compounds through the combination of different Mass Spectrometry techniques. Moreover, a new method in MS for quantification of

several known thiols has been developed, in order to detect seven characteristic sulfur metabolites (GSH, oxidize glutathione (GSSG), Cys, cystine (Cyis), cysteamine (Cyst), homocysteine (Hcys) and CysGly) in the same analysis. The innovative contribute of these two method allows to investigate sulphur metabolism in plants, both in quantification of known compounds, and in order to discover new molecules.

- **Sulfate Assimilation and Plant Sulfur Metabolism**

Sulfur assimilation is an essential and critical part of plant primary metabolism, which aims to convert oxidised form of sulfur from the soil into the principal two sulfur-containing amino acids: cysteine and methionine (Leustek, Martin, Bick, & Davies, 2000; Takahashi, Kopriva, Giordano, Saito, & Hell, 2011).

The assimilation of inorganic sulfur begins from the soil, where specific membrane-bound sulfate transporters carry them to the leaves. The sulfates, after their inclusion into the cell, can become part of the vacuolar sap or they can be transported to the plastids where the reduction steps happen. Here, the adenosine-5'-triphospahte sulfurylase (ATPS) catalyzes the transfer of the sulfate moiety to the adenosine monophosphate molecule (ATP) in order to form 5'-phosphosulfate (APS). Afterwards, APS can be reduced to sulfide (S^{2-}) after a middle reduction step of its sulfite form through sulfite reductase (SiR). Otherwise, sulphate compounds can be reduced to sulphide form through the action of APS kinase catalyst, which uses the APS as a substrate for the production of 3'-phosphoadenosine 5'-phosphosulfate (PAPS) an highly activated sulphate form that acts in order to modify sugars, proteins and secondary metabolites (Leustek, 2002). The reaction of PAPS with sulfotransferases leads to the synthesis of glucosinolates (Klein & Papenbrock, 2004).

After its formation, sulphide is involved in the reaction for Cys synthesis, which occurs in plastids, mitochondria and in the cytoplasm (Figure 1).

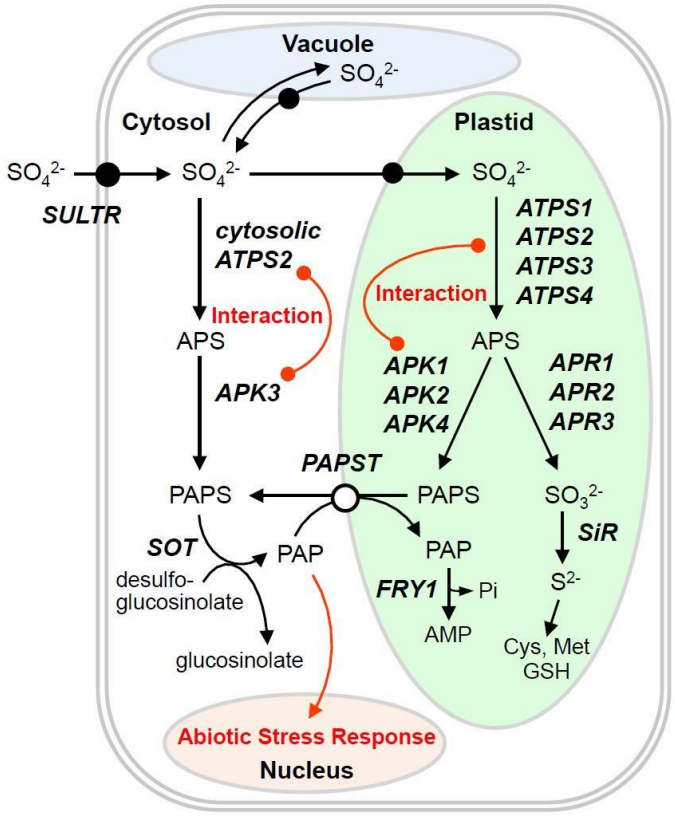

Figure 1: Sulfur metabolism in plants.

Cys is the main product of sulfur assimilation in plants and represents an essential amino acid for protein synthesis. Because of its rapid incorporation in protein or its conversion in other compounds, the concentration of Cys in plants is quite low (Pilon-smits & Pilon, 2006). Cysteine is one of the major components of glutathione (GSH), a tripeptide with a primary role in sulfur metabolism thanks to its function of sulfur transporter between organs, regulating the plant sulfur homeostasis. It can be easily converted into Cys or in other LMW thiols and it is used to regulate protein function, detoxify xenobiotic and to protect cells against oxidative stress.

Moreover, Cys is the precursor of methionine (Met), a sulfur-containing amino acid that, despite Cys, does not contain the thiol moiety. Met, as Cys, is an important molecule for the protein structure and it acts as a precursor for the synthesis of S-adenosylmethionine (SAM), a non-thiol compound involved in regulation of plant growth and stress response. Morover, SAM is also a methyl donor for several molecules, including polysaccharides, nucleic acids and proteins (Brzezinski, Bujacz, & Jaskolski, 2008).

Met and GSH represent the major source of sulfate compounds in plants. Furthermore, they can be easily converted into Cys by specific enzymes, in order to return part of plant sulfur metabolism.

- **Thiol proprieties and disulfidide bond**

As already mentioned, thiols are sulfur compounds characterized by a sulfhydryl (-SH) functional group. The high polarization of the sulfur atom makes thiols more acidic (pK_a = 9-12) then their corresponding alcohols (pK_a = 15). Therefore, the addition of a base during an organic synthesis generates the S- form, which is much more nucleophilic than the SH one.

The oxidation of thiol moiety can occur easily, leading to the formation of a disulfide bond. The generation of disulfide linkage between two cysteine residues of the same chain (intra-molecular linkage) stabilizes the protein tertiary structure; on the contrary, the creation of the bond between cysteine residues belonging to different chains (inter-molecular linkage) supports the proteins quaternary structure.

The reversible interchange reaction between disulfides and sulfhydryl groups is pH dependent: at physiological pH, Cys residues are protonate, but the pH increment (due to

the presence of basic or polar amino acids nearby) involves the disulfides loss, with the reformation of thiolate anion (Colville & Kranner, 2010). The nature of this linkage shows a temperature dependence as well: the higher temperature increase the interchanges with a consequent denaturation of proteins (Morel, Bonicel, Micard, & Guilbert, 2000).

- **Sulfur Compounds and antioxidant activity**

The detoxification of reactive oxygen species (ROS) is led by the ascorbate, the primary ROS scavenger in plants. However, GSH is also a another major and widely known redox component that performs several functions in the organism, including regulation of the cell cycle and the storage and transportation of reduced sulfur (Noctor et al., 2012).

Through the GSH peroxidase catalyst, GSH reacts directly with ROS, transferring the electrons from reduced nicotinamide adenine dinucleotide phosphate (NADPH) to dehydroascorbate. GSH peroxidase can also regenerate oxidized glutathione (GSSG) from GSH. On the contrary, Glutathione Reductase (GR) utilizes NADPH to convert GSSG to GSH. As evidenced by several studies, the GSH concentration in plants tissue increases in response to stress conditions, due to an increased synthesis of GSH in these situations (Ballmoos, Ru, Szalai, & Brunold, 2001). The importance of the measurement of GSH/GSSG redox couple is essential for the assessment of cellular redox potential and of the antioxidant activity in a specific cellular compartment.

- **Metal coordination**

Thiols are well known to coordinate metals such as Cu, Ag, Zn, Pb and Hg. The affinity with these metals has been used to develop several procedures for thiol selective

extractions from complex matrices. Nevertheless, as thiolate anion, cysteine is not able to interact with group 1 and 2 metal ions (Proteins, Jacob, Giles, Giles, & Sies, 2003). Cysteine enriched proteins involved in metal transport are called metallothioneins. They also play an important role in detoxification and storage of excessive amounts of essential metals (Hassinen, Tervahauta, Schat, & Ka, 2011).

LMW thiols in plants

Most of LMW thiols in plants derive from Cys or GSH and are known for their property to react with reactive electrophilic species (such as reactive oxygen or nitrogen species) or xenobiotic compounds. Several of them are identified and they will be described in this paragraph.

- **Cysteine**

Cysteine is the principal product of the sulfur assimilation in plants. It is synthesized in a two steps pathway, occurring in mitochondria (first step) and in cytosol and chloroplasts (second step). The first step includes the formation of O-acetylserine through the acetylation of serine from acetyl-CoA supported by acetyltransferase catalysis. Then reduced sulphur is added to O-acetylserine-(thiol)-lyase, which eliminates the acetate moiety with the consequent formation of cysteine (Hell & Wirtz, 2011). As already mentioned, the concentration of Cys in tissues is usually low because of its rapid conversion into other compounds or incorporation in proteins. Indeed, Cys is the primary source of sulfur for the production of Met, vitamins (thiamine and biotin), lipoic acid,

coenzyme A and GSH. Moreover, it covers an important structural role in thiol-containing proteins (Pilon-smits & Pilon, 2006).

Cysteine can dimerize into cystine through the formation of a reversible disulfide linkage.

In plants, it was demonstrated that the cleavage of cysteine's β-carbon-sulfide link, catalysed by cystine lyase, results in the formation of thiocysteine, pyruvate and ammonia. Thiocysteine can be further metabolized into cysteine, hydrogen sulphide, thiocyanate or elemental sulfur (Lyase et al., 2003).

As last, cysteine may act as a precursor for several secondary metabolites, such as the volatile thiols, that play a primary role in food flavouring.

- **Homocysteine**

Homocysteine (Hcys) is the intermediate compounds produced during the synthesis of Met from Cys. The three steps that form methionine include the reaction of Cys with O-phosphohomoserine (OPHS) to form thioether cystathionine. From here, the production of Hcys is fast with the formation of pyruvate and ammonia as by-products (Mudd, 2014; Pilon-smits & Pilon, 2006). Hcys is then transported to plastids for Met formation. Met can be involved in protein formation or it can be converted to SAM for methylation of several molecules. SAM, as a consequence, can produce S-adenosylhomocysteine, that can be easily converted in adenosine and Hcys through enzymatic hydrolysis (Hesse & Hoefgen, 2003). Met acts as a substrate for the synthesis of biologically active compounds, such as S-methylmethionine, S-adenosylmethionine and ethylene. S-methylmethionine, similar to GSH, is responsible of long-distance transport of reduced sulfur, playing a central role in plants sulfur homeostasis (Brzezinski et al., 2008).

The studies regarding the analysis of Hcys have received considerable attention after the determination of this compound in high level in human plasma, in correlation with the occurrence of cardiovascular diseases (Guan, Hoffman, Dwi, & Matthees, 2003).

- **Glutathione cycle**

Glutathione is a tripeptide (γ-glutamyl-cysteine-glycine) which plays a pivotal role in sulfur metabolism in plants. In plant tissues, it is found in a concentration range between 3 and 10 mM in the principal cellular compartments, where it regulates the cellular homeostasis (Grill, Tausz, Centre, Street, & Faculty, 2004; Noctor, Queval, Mhamdi, Chaouch, & Foyer, 2011).

The sulfhydryl group supplied by the cysteine residue exists in its reduced form under physiological conditions but, in conditions of oxidative stress, the dimer GSSG can be created through the formation of the disulfide bond between cysteine residues of two GSH molecules. The biosynthesis of GSH in plants is regulated by GSH1 and GSH2, two ATP-dependent enzymes that add progressively cysteine (Cys) and glycine (Gly) residues to glutamyl (Glu) part in two steps reaction. The γ-Glu-Cys synthetase (GSH1) acts in plastids for γ-glutamylcysteine formation. Subsequently, the addition of glycine can occur in both chloroplasts or cytosol after the transfer of γ-Glu-Cys (Hothorn, Wachter, & Stuwe, 2006).

The properties related to GSH are several and various: it can participate in plant growth, in detoxification of xenobiotics and heavy metals; moreover, its important role in reduced sulphur storage and long-distance transportation makes GSH a fundamental molecule in plant sulphur metabolism (Noctor et al., 2011).

The degradation of GSH in cells is operated by GGT (γ-glutamyltransferase /transpeptidase) and GGCT (γ-glutamyl ciclo-transferase). These two enzymes are located in different compartments of the cell. The GGT catalyses the separation of γ-glutamyl bond, generating the thiol cysteinylglycine (CysGly). As a consequence, the determination of CysGly concentration can indicate the state of degradation of GSH in cells (Masi, Ghisi, & Ferretti, 2002).

- **Cysteamine**

Cysteamine represents the simplest aminothiol compounds in plants. It is produced in plants from two different biological pathways: the degradation of Coenzyme A and the decarboxylation of Cys. The degradation of Coenzyme A leads to the production of pantetheine as intermediate, that is converted in cysteamine. Cysteamine is subsequently oxidized in hypotaurine, with the final production of taurine (Besouw, Masereeuw, Heuvel, & Levtchenko, 2013).

Despite the known metabolic pathway for the production of taurine in animals, the role of cysteamine in plants is not completely understood. It has been proved that the presence of cysteamine/cystamine buffer can deactivate RuBisCO, an enzyme involved in the Calvin Cycle (Moreno, Garcìa-Murria, & Marìno-Navarro, 2008).

Nevertheless, the cysteamine has been detected at high concentration level in apple skins and the acquisition of new knowledge about the process of cysteamine biosynthesis and its metabolism in plants may help to define its role in sulfur metabolism (Pivato, Fabrega-Prats, & Masi, 2014).

❖ **Phytochelatins**

In response to high concentrations of toxic metals in the cytoplasm, GSH through the enzyme PC synthase (γ-glutamylcysteine dipeptidyltranspeptidase) produces Phytochelatins (PCs), small peptides characterized by the repetition of the dipeptide Glu-Cys and having $(\gamma\text{-Glu-Cys})_n$-Gly as a general structure. The number of didpeptide ripetitions is indicated by n, which can range from 2 to 11 but it is usually not more than 5 (Potesil et al., 2005). PCs exist in higher plants, some fungi, algae and they play a pivotal role in sequestering heavy metals (such as Cd, Cu, Hg, As and Pb) by forming metal-PC complexes through the activity of cysteine's thiol moiety (Frank, 2005; Petraglia et al., 2014). Moreover, they have an important part in transportation of heavy metals into vacuole and in maintenance of cellular ionic homeostasis (Pal & Rai, 2010) .

Thiols analytical approaches and detection techniques

The quantitation and identification of LMW thiols in plants are essential for the assessment of the biological functions and the redox state of free thiols. Several analytical methods have been developed using different detection techniques. Most of them follow five principal steps: (i) extraction; (ii) reduction; (iii) derivatisation; (iv) separation; (v) detection. The above mentioned steps are described below:

(i) Extraction

Free thiols in plants are ectracted through acidic solutions of chlorhydric, perchloric, sulfosalicylic or metaphosphoric acid. The acidic environment is necessary to avoid the formation of disulphide bonds and to keep the sulfhydryl group protonated in its reduced

form. At the same time, acidic conditions lead to proteins precipitation. Thiolathion studies can be conducted after the resuspension of the pellet with a detergent (such as SDS or Tween) or other agents (e.g. guanidine).

(ii) Reduction

The reduction step is necessary to ensure the availability of thiol compounds in their reduced form, avoiding the formation of disulphide linkages. The disulphide reduction requires to be specific, quantitative and rapid. The most used reducing agents for this purpose are usually thiol-containing reductants (such as dithiothreitol, DTT, or 2-mercaptoethanol, ME) or various substituted phosphines such as tris(2-carboxyethyl)phosphine, TCEP. Thiol-based reductants are needed in large excess and demand an additional removal step to avoid undesirable reactions with the derivatising agent. Moreover, they usually require pH $\geq$ 7 for obtaining efficient reactions, with the risk of occurrence of undesirable side-reactions (Getz, Xiao, Chakrabarty, Cooke, & Selvin, 1999).

On the contrary, TCEP is widely used as substitute for DTT because of its low pKa value, which promotes the reduction of disulphide bonds at low pH, depending on the phosphine's substituent (the increasing of chain substituents decreases the pKa value). Phosphines are irreversible reductants of disulfides linkages; they perform a nucleophilic attack on one of the two sulphur atoms. The resulting phosphonium ion sulphur adduct is then hydrolysed to the corresponding phosphine oxide (Cline et al., 2004).

In addition to these two classes of reductants, several other methods are known even if not so widely used. Sodium borohydride for example is a good reductant and its excess can be easily removed; but the reaction takes place at high pH and this environment can

lead to undesirable side-reactions (Hansen, Østergaard, Nørgaard, & Winther, 2007). Another way can be the metallic zinc: its excess can be easily removed by centrifugation and the reaction occurs at low pH, which suggests its attitude for mass spectrometer post analysis (Erlandsson & Ha, 2005).

(iii) Derivatisation

The derivatisation of thiol compounds consists of the chemical labelling of the sulfhydryl group with a specific reagent selected for reacting with thiol moiety. This step demonstrates to be a useful tool for thiols detection, since the label acts as a marker for these specific compounds. Most of these labels prompt an irreversible thiol-disulfide exchange reaction, with a subsequent increment of the molecular mass of the thiol compound. A wide range of derivatising agents are used for thiol labelling, depending on the detection approach adopted. For examples, for MS techniques labels are not strictly required, but they can be useful in the way to distinguish oxidized thiols from reduced forms.

An example of a suitable MS label is the 4,4'-dithiodipyridine (DTDP), an aromatic disulphide compounds that reacts with the free reduced sulphur of the sulfhydryl group (Grassetti, 1967). This derivatising agent requires a neutral pH to react with the thiol group and it represents a valid option for thiols analysis through MS techniques. Nevertheless, DTDP is also used in thiols analysis with UV detection, along with 5,5'-dithio-bis-(2-nirobenzoic acid) (DTNB), known as Ellman's reagent, 2-chloro-1-methylpyridinium (CMPI) and 2-chloro-1-methylquinolinium (CMQT) (Probes, 2012).

The analysis through fluorimetric detector differs from the UV detection for the high sensitivity and the thiol-selectivity of the derivatising agents. The most common used

fluorescent thiol-specific label is the monobromobimane (mBBr), which is used for thiol quantitation and for the analysis of thiol-containing proteins (Fahey & Newton, 2019; Hansen & Winther, 2009). Other two important labels are ammonium 7-fluoro 2,1,3-benzooxadiazole-4-sulfonate (SBD-F) and 4-aminosulfonyl-7-fluoro-2,1,3-benzoxadiazole (ABD-F). The benzofurazanes have the great advantage, compared to mBBr, of emitting light only after their linkage to thiols. On the contrary, mBBr has a weak fluorescence of its own and this aspect can create inconveniences in chromatograms interpretation (Boersma et al., 2010; Toyo, 2009).

(iv) Separation

Derivatised thiols are separated through two different approaches: electrophoresis or chromatographic techniques (Boersma et al., 2010; Eaton, 2006). The latter are the most used, usually in high performance liquid chromatography (HPLC), which is often coupled with a preventive thiol-selective affinity chromatography (e.g. solid phase extraction, SPE) in order to concentrate the sulphur compounds and to purify them (Huang et al., 2010) . The gas chromatography technique is included in the chromatographic approaches and it is used especially for the determination of volatile compounds; for this reason, it is usually implicated in flavouring analysis of food and beverages (e.g. wine) (Rafii, Elango, Courtney-martin, & House, 2007).

(v) Detection

The absence of specific physicochemical proprieties, such as its own fluorescence or a high UV-Vis absorption, makes the detection of thiols in a selective and sensitive way a

major challenge. Several detectors can be used, depending on the nature of the derivatising agent used: UV, electrochemical (ECD), fluorimetric (FL), mass spectrometer (MS) detector connected to chromatographic separative techniques. The most widely used method for thiol determination is the analysis through fluorimetric detection, because of its high sensitivity and selectivity. The electrochemical detection is particularly suitable for thiols analysis, since it does not require the derivatization step because of the easily oxidation of thiol groups. Despite it, the removal of this step leads to the instability of thiol compounds, with the consequent insecurity on results accuracy (Diopan et al., 2010) .

Recently, accurate identification methods have been developed with the use of mass spectrometry techniques, which can detect biological thiols without a preventive derivatisation step (Rellán-álvarez, Hernández, Abadía, & Álvarez-fernández, 2006). Despite it, the labelling is still suggested for clearly quantify oxidize thiols and reduced thiols. The most used derivatising agents used in MS detection are the aromatic disulfides reactives, that were employed in various studies for the quantitation of thiols in biological samples (Capone, Ristic, Pardon, & Jeffery, 2015; Guan et al., 2003; Zahradnı, 2008). The thiols detection methods through MS are gradually increasing and further developments with this technique can lead to robust methods for quantitation and identification of new compounds.

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CHAPTER II

QUANTIFICATION OF THIOLS AND DISULFIDES

"Simultaneous quantification of reduced and oxidized thiols in plant extracts by HPLC-MS/MS analysis"

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Simultaneous quantification of reduced and oxidized thiols in plant extracts by HPLC-MS/MS analysis

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Abstract

In this study a new HPLC-MS/MS method was developed for the quantification of seven compounds belonging to sulfur metabolome in plants. The simultaneous detection of Cysteamine, Homocyseine, Cysteinylglycine, Glutathione, Cysteine and the disulfides form of the latter two, Glutathione disulphide and Cystine, is provided after the derivatisation of thiol compounds with 4,4'-dithiodipyridine to avoid the oxidation of thiols to disulphide forms. The method was applied to plant samples such as cauliflower leaves, rocket, garlic, onion and walnut but it will be an useful tool for the investigation of these biological thiols and disulfides in several types of matrices.

Introduction

Biological thiols play an important role in cellular redox homeostasis in plants and in other biological systems. In particular, the alteration of the concentration of two of the most abundant low-molecular-weight (LMW) thiols, glutathione (GSH) and cysteine

(Cys), is associated to the appearance of disease in human beings or to stress conditions in plant systems.

Glutathione is a tripeptide composed of glutamic acid, cysteine and glycine residues and it is implicated in cellular defence against free radical hydroperoxides and xenobiotics. The ratio between GSH and its oxidized form, GSSG, is a crucial indicator of oxidative stress in biological systems and its alteration can be due to the effects of different stresses (Foyer et al., 1995; Xiang, Werner, Christensen, & Oliver, 2001). In general, the measurement of GSSG concentration in tissues was led through the reduction of GSH with a specific reducing agent. The difference between the total GSH (after the reduction step) and the initial GSH values was attributed to GSSG concentration in the sample.

Cysteine is one of the main products of the sulfur assimilation in plant system. It represents the metabolic precursor of several molecules, among which GSH, cysneinylglycine (CysGly) and homocysteine (Hcys) and it can dimerize into its disulfide form, cystine (Cyis), whose metabolic function in plant is still not totally understood. The role of cystine is not the only one to be partially defined: cysteamine (CysT) is an aminothiol produced through an alternative pathway; its role in plant metabolism is not fully understood, but evidences suggest it has regulatory and physiological properties.

Recent methods used the high performance liquid chromatography (HPLC) for biothiols separation, coupled with a variety of detection techniques, such as UV-Vis detection (Raggi, Mandrioli, Casamenti, Musiani, & Marini, 1998), fluorimetry (FL) (Pastore et al., 1998; Salazar et al., 1999), electrochemical detection (EC) (Kleinman & Richie, 1995; Xing, Zhong, Han, & Yang, 2014) and mass spectrometry (MS) (Guan, Hoffman, Dwi,

& Matthees, 2003; Lee, Yim, Lim, & Kim, 2016; Rellán-álvarez, Hernández, Abadía, & Álvarez-fernández, 2006).

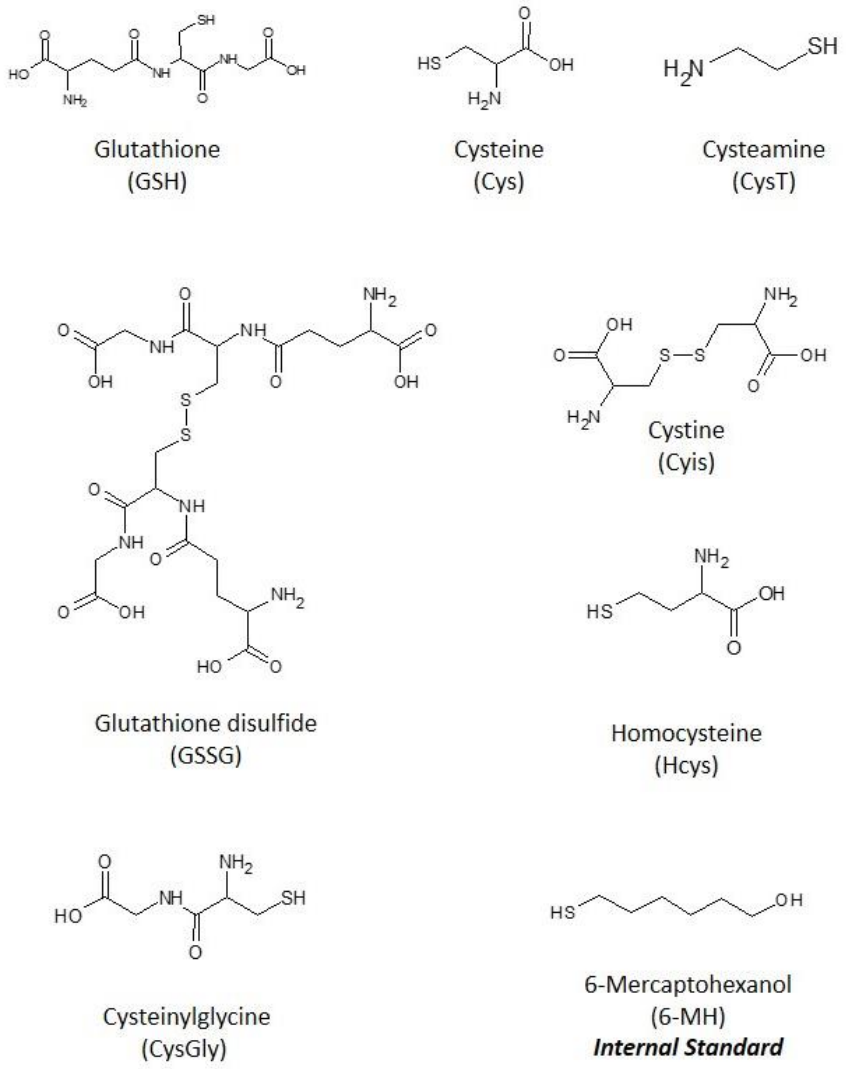

Figure 1: Chemical structures of thiols and disulfides described in the text.

In particular, in HPLC-MS methods the auto-oxidation of thiols is usually prevented by the derivatisation of the thiol moiety with a specific label, such as iodoacetic acid (Loughlin, Skiles, Alberts, & Schaefer, 2001), Ellman's reagent (Guan et al., 2003) or N-ethylmaleimide (Robotham & Kelly, 2019; Sutton et al., 2018). The recent increment in the use of mass spectrometers as detectors significantly increases the selectivity, avoiding

the reduction step for the detection of disulfides. These techniques improve the sensitivity and can quantify these compounds at low concentrations.

In this study, we describe a HPLC-MS/MS method for the simultaneously quantification of GSH, GSSG, Cys, Cyis, CysT, Homocys, CysGly in plant samples (cauliflower leaves, rocket, garlic, onion, walnut) after their derivatisation with 4,4'-dithiodipyridine (DTDP). The internal standard 6-mercaptohexanol (6-MH) was used in addition to the samples (Figure 1). The concentrations of disulfide species are directly detected without the intermediate reduction step. step. The method can be applied even to food samples after acidic thiol extraction or directly to beverages, such as wine.

Materials and methods

Chemicals and Materials

Chemicals and reagents used in the analysis were purchased from commercial suppliers: 4,4'-Dithiodipyridine (DTDP), Formic Acid (≥ 98%), Hydrochloric acid (HCl, ≥37%), were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Thiols standards included L-Gutathione reduced (≥ 98.0%), L-Cysteine (97%), Cys-Gly (≥ 85%), Cysteamine (~ 95%), Homocysteine (≥ 98%), L-Glutathione Oxidized (≥98%), L-Cystine (≥ 98%) and 6-Mercaptohexanol (97%) were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Ammonium Acetate and EDTA were purchased from USB Corporation (Cleveland, OH, USA) and from AppliChem (Darmstadt, Germany) respectively.

Plant samples were collected from supermarkets in Padova between April and May 2019.

Samples Extraction and Derivatisation

- Derivatising Agent

A 10 mM solution was prepared by adding 220 mg of DTDP in a mixture of 20 mL water and 100 µL of concentrate HCl (37% w/w). After the sonication and the dissolution, Milli-Q water was added for achieve the final volume of 100 mL. The DTDP solution was divided in several aliquots, stored at -20° C until required.

- Extractive Solution HCl-EDTA

An extractive solution of HCl 0.1 M - EDTA 1 mM was prepared adding 1.657 mL of concentrate HCl (37% w/w) and 75 mg of EDTA in 100 mL water. After sonication, Milli-Q water wad added to give a final volume of 200 mL. The extractive solution was stored at room temperature.

- Thiols Extraction from Plant Samples

Every sample was weighted for a total amount of ~ 700 mg and placed on the mortar, previously cooled with liquid nitrogen. Then liquid nitrogen was added to the sample and homogenisation was carried out with pestle. The extractive solution was added: the ratio between sample weight (mg) : extractive solution (µL) was respectively 1:4. After the addition, the mixture was further homogenised until it became liquid and transferred in a tube for the centrifugation step. The supernatant, containing extracted free thiols, was separated from the pellet and stored at -20° C until the derivatisation.

- Derivatisation procedure

In a tube 300 µL of thiol extract (or thiol standard) and 640 µL of ammonium acetate (CH_3COONH_4, 0.16 M, pH=6.4) were mixed, and after stirring 50 µL of freshly prepared 10 mM DTDP were added. The sample was stirred and left at room temperature for 60 min before 10 µL of concentrate HCl was added. Prior to the HPLC-MS/MS analysis, the derivatised sample was filtered with a 0.2 µm nylon filter.

Thiol Standards preparation for LC-MS Parameters optimization.

Mass spectrometer parameters were optimized with MS/MS infusion experiments of 10 ppm solutions of derivatised thiol standards, using an infusion pump operating at 10 µL/min.

Instrumentation and Chromatography

- HPLC Conditions:

The Quantitative Analyses were performed using a 150 mm x 4.6 mm i.d., 5 um, 100 Å Kinetex C18 column operated at 25 °C and protected by a 4 mm x 3.0 mm i.d. guard cartridge of the same material, with a Thermo Fisher Scientific LC connected to an QqQ MS system. The electrospray ionization was set in positive ion mode. The analysis were led with a flow rate of 0.200 mL/min. The solvents were: solvent A (0.5% aqueous formic acid) and solvent B (0.5% formic acid in acetonitrile)

The gradient for solvent B was as follows: 0 min, 2%; 5 min, 2%; 20 min, 80%; 25 min, 80%; 26 min, 2%; followed by 10 min of column equilibration with 2% B. For the analysis, the injection volume of 10 µL was set.

❖ Mass Spectrometer Conditions

The QqQ (TSQ Quantiva) used N_2 was used for sheath gas, 35 arbitrary units; auxiliary gas, 20 arbitrary units and sweep gas, 2 arbitrary units; the ion spray voltage, source fragmentation, ion transfer tube temperature and vaporizer temperature were set at 4500 V, 0 V, 300 °C, 275 °C, respectively. Argon was used as collision gas with CID set at 2.5 mTorr. For Selected Reaction Monitoring (SRM) mode, Q1 and Q3 had 0.7 resolution.

MS/MS Infusions of standard solutions were used for obtaining the mass transitions to insert in the SRM method (Table 1).

Compound	Retention Time (min)	Precursor (m/z)	Product (m/z)	Collision Energy (V)
Cysteamine	8.8	187	111 112 144	35
Cysteine	8.2	231	112 144	35
Cystine	6.8	241	122 152	15
Homocysteine	14.5	245	112 134	35
Cysteinylglycine	9.8	288	112 144	20
Glutathione	17.3	417	112 306	35
Glutathione disulfide	15.3	613	177 232	35
6-Mercaptohexanol	21	244	111 144	35

Table 1: Mass Transitions and Retention Times for HPLC-MS/MS analysis with SRM scan mode.

Results and Discussion

Several trials were led for the optimization of the derivatisation procedure. After the selection of CH_3COONH_4 for pH increment, the addition of concentrated HCl was inserted as last step reaction. Indeed, the use of the acid for stopping the reaction is necessary for ensuring the measurement accuracy . The analysis of a mixture of derivatised standards (CysT, Cys and GSH) was also planned to optimize the procedure. Standards were analysed through HPLC-MS/MS, with and without the final addition of concentrate HCl. The two samples were analysed several time, in a time range of 15 hours. Results show the stabilization of the signal after the introduction of concentrate HCl; on the contrary, without the acid addition the signal was not stable during the time for all the analysed standards (Figure 1).

Method Validation

The mass transitions for the analytical method were determined through infusion MS/MS experiments with purchased standard (Table 1). As internal standard, 6-mercaptohexanol (6-MH) was used, since no evidence from literature shows its natural existence in plants.

Thiol standards were divided into two standard mixture (Mix A and Mix B) because of the incompatibility of glutathione and cysteinylglycine quantitation in the same analysis: indeed, the spontaneous degradation of glutathione produces cysteinylglycine and the quantitation of all the compounds in the same mixture may not be accurate for cysteinylglycine. For this reason, plant samples were analysed using two different mixtures of standards. In particular, Mix A was composed of Cyis, Cys, GSSG, GSH

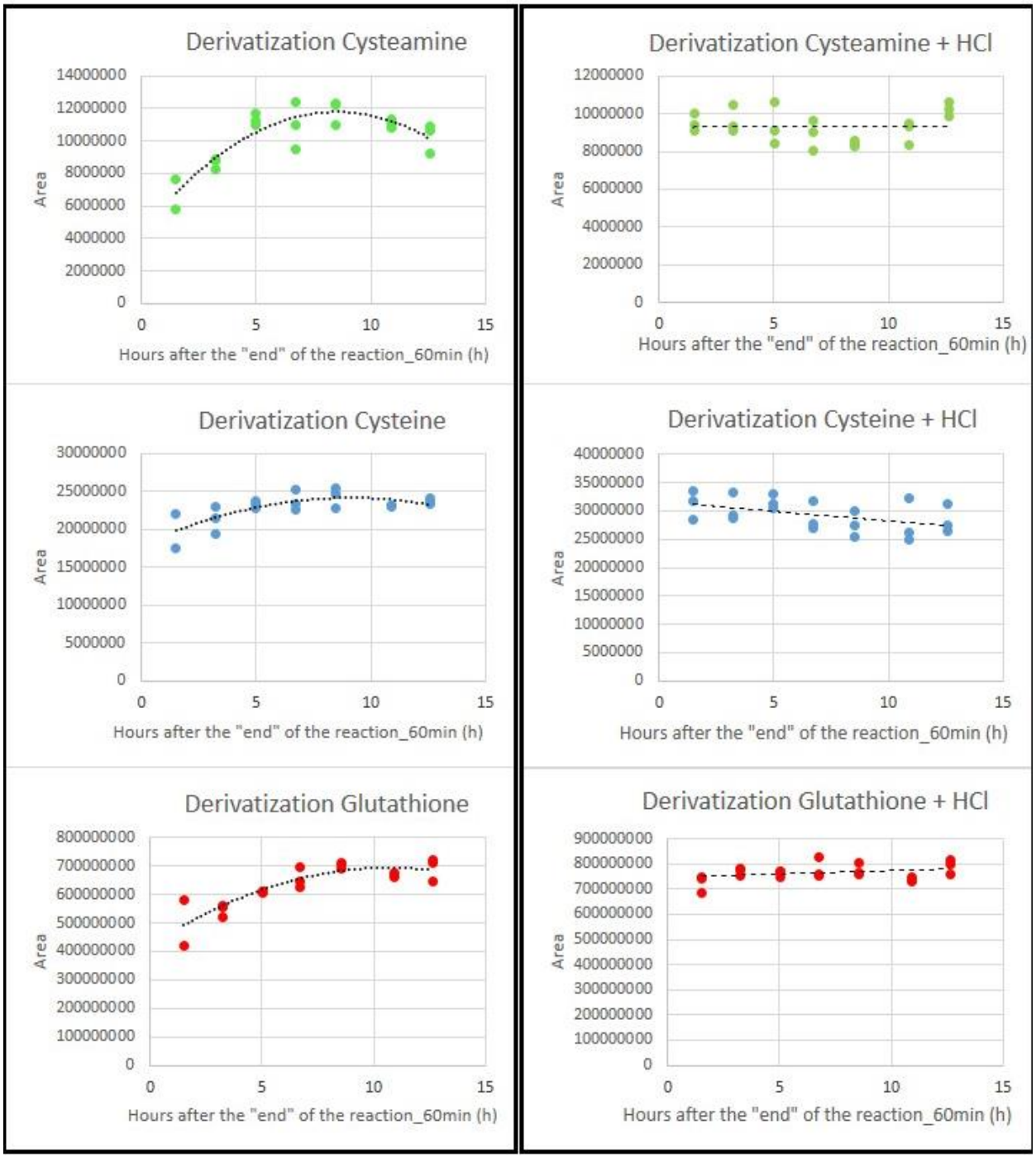

Figure 2: Graphics representing the trend line without HCl final addition (left) and with HCl final addition (right).

and Mix B of CysT, CysGly, Hcys. Chromatograms of the two standard mixtures are shown in Figure 3.

As shown in Figure 3, CysGly can be detected both in mixture A and B, but only the signal from Mix B was considered for the method validation.

For method validation, linearity, recovery, limit of detection (LOD) and limit of quantification (LOQ) were determined.

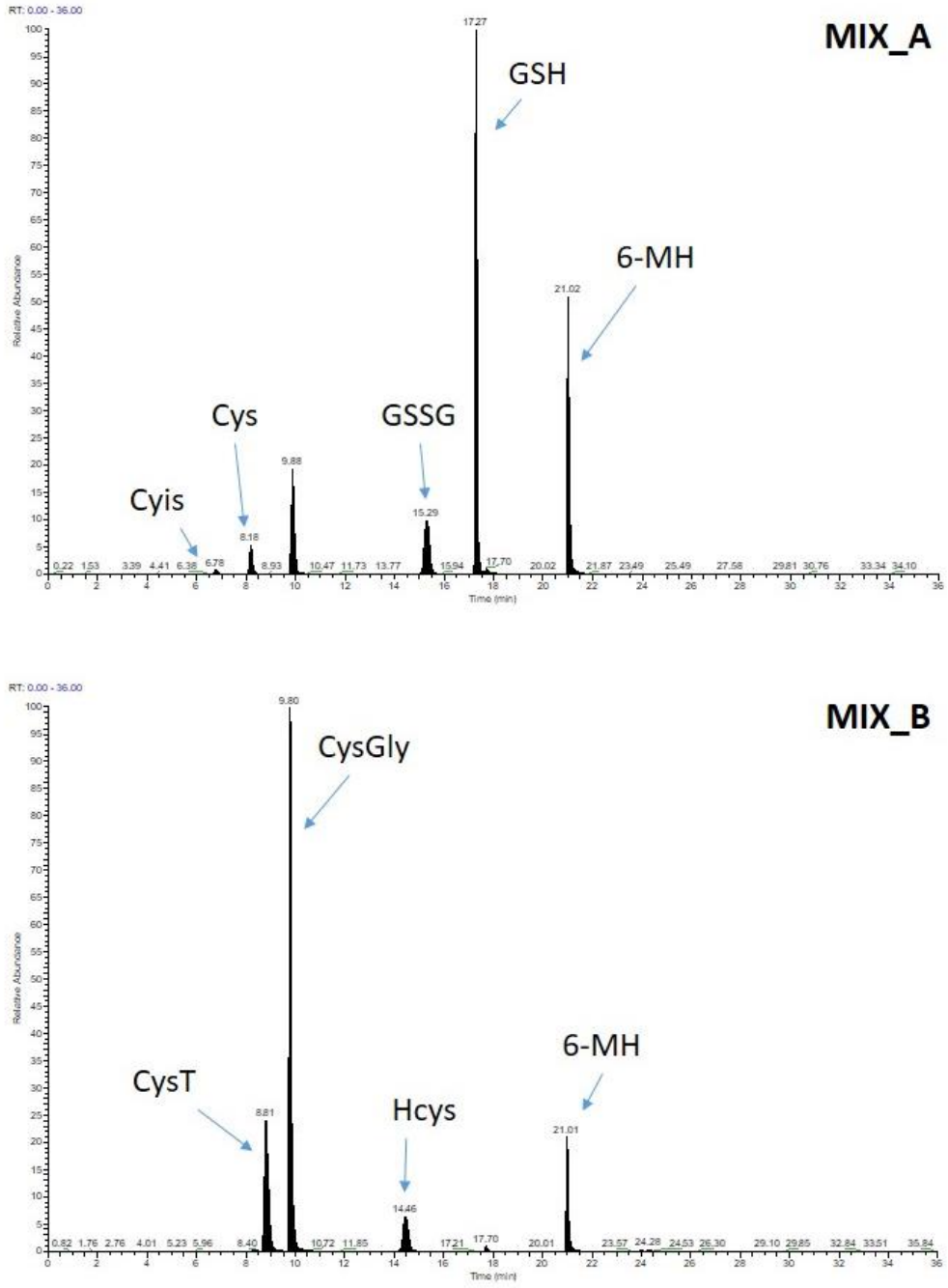

Figure 3: Chromatograms of Mix A (containing 6-MH, Cyis, Cys, GSSG and GSH) and Mix B (6-MH, CysT, CysGly and Hcys).

❖ Linearity

Thiol standard mixtures were prepared at ten concentrations: Cyis (0, 0.5, 1.25, 2.5, 5, 6.25, 10, 12.5, 18.75, 25 µM), Cys (0, 0.5, 1.25, 2.5, 5, 6.25, 10, 12.5, 18.75, 25 µM), GSSG (0, 4, 10, 25, 40, 50, 80, 100, 150, 200 µM), GSH (0, 10, 25, 50, 100, 125, 200, 250, 375, 500 µM), CysT (0, 1, 3, 5, 10, 12.5, 20, 25, 50, 75 µM), CysGly (0, 1, 3, 5, 10, 12.5, 20, 25, 50, 75 µM), Hcys (0, 1, 3, 5, 10, 12.5, 20, 25, 50, 75 µM) and were added to extractive solution (HCl/EDTA, blank) and to the extracts of cauliflower leaves, rocket, garlic, onion and wolnut before the derivatisation.

❖ Limits of detection (LODs) and quantification (LOQs)

LODs, defined as the concentration of analyte which produce a S/N ratio of 3, were calculated from the visual evaluation method. LODs and LOQs values for each standard are shown in Table 2.

Analyte	LOD (µM)	LOQ (µM)
Cyis	0.165	0.5
Cys	0.165	0.5
GSH	3.3	10
GSSG	1.32	4
CysT	0.33	1
CysGly	0.33	1
Hcys	0.33	1

Table 2: LODs and LOQs in each matrix.

❖ Precision and Recovery

The determination of precision and recovery was led on seven replicates samples spiked in each matrix with the two mixtures at low and high levels: 1.25 µM and 12.5 µM for Cyis and Cys, 10 µM and 100 µM for GSSG, 25 µM and 250 µM for GSH, 3 µM and 25 µM for CysT, CysGly and Hcys. Precision was expressed through relative standard deviation (RSD). Results of linearity and recovery are shown in Table 3, Table 4, Table 5, Table 6.

	Cyis					Cys				
		% Recovery (RSD)					% Recovery (RSD)			
	Curve R^2	1,25 uM	SD	12,5 uM	SD	Curve R^2	1,25 uM	SD	12,5 uM	SD
Blank	0,9787	165,56	(10,84)	110,37	(11,43)	0,9926	125,94	(4,41)	109,29	(19,40)
Cauliflower	0,9895	82,23	(25,85)	78,81	(4,02)	0,9915	74,59	(31,87)	103,71	(7,96)
Rocket	0,9858	79,43	(28,29)	90,74	(12,94)	0,9954	103,44	(6,57)	81,22	(12,94)
Garlic	0,9884	108,08	(20,71)	94,73	(11,38)	0,9776	137,85	(43,47)	126,10	(42,72)
Onion	0,9966	101,52	(48,86)	81,42	(33,38)	0,9871	97,36	(54,19)	91,40	(19,44)
Walnut	0,9853	170,87	(27,47)	114,06	(8,33)	0,9914	133,31	(44,35)	109,59	(5,92)

Table 3: Analytical method validation parameters for Cyis and Cys in different vegetal matrices.

	GSSG					GSH				
		% Recovery (RSD)					% Recovery (RSD)			
	Curve R^2	10 uM	SD	100 uM	SD	Curve R^2	25 uM	SD	250 uM	SD
Blank	0,9961	111,49	(5,89)	89,69	(7,09)	0,9987	80,10	(5,80)	100,11	(2,22)
Cauliflower	0,9831	24,25	(113,92)	75,68	(13,22)	0,9903	29,77	(32,80)	95,07	(6,61)
Rocket	0,9893	99,81	(15,30)	79,39	(8,09)	0,9903	74,97	(12,10)	67,54	(6,44)
Garlic	0,9952	120,97	(9,47)	89,98	(35,88)	0,9924	60,46	(15,33)	116,41	(23,46)
Onion	0,9923	95,45	(17,57)	79,44	(38,17)	0,9796	88,61	(18,50)	62,53	(22,05)
Walnut	0,9878	120,34	(22,83)	93,33	(37,24)	0,9906	74,04	(20,59)	113,03	(8,22)

Table 4: Analytical method validation parameters for GSSG and GSH in different vegetal matrices.

	CysT					CysGly				
		% Recovery (RSD)					% Recovery (RSD)			
	Curve R^2	3 uM	SD	25 uM	SD	Curve R^2	3 uM	SD	25 uM	SD
Blank	0,975	140,92	(66,28)	121,89	(34,27)	0,9877	119,99	(56,85)	110,23	(28,84)
Cauliflower	0,9918	59,56	(17,95)	102,20	(14,56)	0,9914	66,76	(14,36)	105,23	(9,94)
Rocket	0,9929	136,40	(5,40)	94,23	(8,25)	0,9964	125,37	(5,36)	91,13	(5,78)
Garlic	0,9467	176,60	(14,47)	130,35	(32,99)	0,985	132,65	(7,02)	111,88	(24,30)
Onion	0,9989	86,73	(14,81)	108,82	(16,07)	0,9773	55,83	(18,65)	105,30	(13,98)
Walnut	0,9956	93,67	(7,92)	110,47	(15,53)	0,9955	123,76	(4,10)	109,91	(14,06)

Table 5: Analytical method validation parameters for CysT and CysGly in different vegetal matrices.

	Hcys				
		% Recovery (RSD)			
	Curve R^2	3 uM	SD	25 uM	SD
Blank	0,995	135,03	(50,14)	111,02	(40,86)
Cauliflower	0,9926	126,55	(7,17)	99,42	(7,72)
Rocket	0,9903	139,38	(6,19)	95,06	(6,11)
Garlic	0,9884	131,65	(12,86)	103,72	(34,79)
Onion	0,9955	108,87	(8,15)	102,05	(14,50)
Walnut	0,9989	103,24	(2,20)	107,15	(13,33)

Table 6: Analytical method validation parameters for Hcys in different vegetal matrices.

Quantitative analysis in plant samples

The importance of GSH/GSSG ratio determination led to the definition of a new method for determining the level of these two compounds and of Cys, Cyis, CysT, Hcys and CysGly. In this work, we developed and validated a new HPLC-MS/MS method for the quantification of these compounds in plant tissue extracts. The separation of biothiols through reverse-phase HPLC, with electrospray ionization setting in positive ion mode, is followed by the analysis in SRM mode with triple quadrupole mass spectrometer.

To our knowledge, this is the first method developed in HPLC-MS/MS which aims to quantify Cyis, CysGly, CysT, Hcys, GSH (after their derivatisation with DTDP) and to

detect simultaneously the disulfides GSSG and Cyis in plant extracts. Disulfides are measured directly, without any reduction step. The method has been validated through the determination of LODs, LOQs, analyte recoveries, linearity and calibration curves.

LODs for analyte concentrations were calculated between 0.165 – 3.3 μM (Table 2), which confirms the good sensitivity of the method. The LODs for GSH and GSSG are respectively 3.3 and 1.32 μM, very similar to the LODs found in literature.

The recoveries for analytes spiked in plant extracts are different from each matrix, with and from ideally recovery values. Generally, we observed more stability at high concentration, with the exception of GSSG. This may be due to the increment of ionization efficiency of GSSG at high concentrations (authors' personal observation). The low recovery rates at low concentration spikes can be justified by the large difference between the signal from the Internal Standard and the signal from some analytes; in these cases, the ratio calculation may lead to some errors. The Relative standard deviation (RSD) values are usually higher than 8%. The linearity for each matrix was confirmed by the function based on ten points, with coefficients of determination (R^2) included in the range 0.9467 – 0.9989.

The validated method was then applied to five plant samples: cauliflower leaves, rocket, garlic, onion and walnut. For quantitation, three sample replicates were analysed in the same batch with the calibration curves. Six-points calibration curves are obtained in duplicate by analysing solutions of standard in the ranges of 0.5-12.5 μM (Cys and Cyis), 4-100 μM (GSSG), 10-250 μM (GSH), 1-25 μM (CysT, CysGly and Hcys). Results show coefficients of determination (R^2) of 0.9816 (Cyis), 0.9401 (Cys), 0.977 (GSH), 0.9661 (GSSG), 0.9419 (CysT), 0.9623 (CysGly) and 0.964 (Hcys). The concentrations of analytes in samples were calculated using these calibration curves (Table 7). The results

show the presence of Cyis only in cauliflower leaves, with a concentration of 2.60 μmol kg^{-1} FW. The shortage of studies regarding this compounds in plants doesnot give the possibility of compare this concentration level. CysT and Hcy were not detected in these samples. Literature reports that Hcys can be found in different legumes (Matamoros, Moran, Iturbe-Ormaetxe, Rubio, & Becana, 1999), whereas CysT was found in apple skin (Pivato, Fabrega-Prats, & Masi, 2014).

The concentration of CysGly is in the range (~ 5 μmol kg^{-1} FW for each saple) expected by the comparison with the quantitation of this compound in *Arabidopsis* leaves (Tolin et al., 2013) and the same is for Cys levels, with a content range in plant samples close to the expected values (Leustek, Martin, Bick, & Davies, 2000; Yarmolinsky, Brychkova, Fluhr, & Sagi, 2013). However, the concentration of GSSG varies between the samples, showing the highest level in cauliflower leaves. Concentration values are very close to sugar beet roots (Zaharieva & Abadía, 2003) for rocket, garlic and onion, but never high as found in *Vigna radiata* leaves (Shanker, Djanaguiraman, Sudhagar, Chandrashekar, & Pathmanabhan, 2004). The concentrations of GSH among these samples are very similar (20-30 μmol kg^{-1} FW) but much lower than the level found in other species (Fricker, May, Meyer, Sheard, & White, 2000; Hartmann, Fricker, Rennenberg, & Meyer, 2003; Yarmolinsky et al., 2013), except for *Medicago sativa* mature leaves (Pasternak, Asard, Potters, & Jansen, 2014).

Plant tissue	**Cyis** µmol kg^{-1}	**Cys** µmol kg^{-1}	**CysT** µmol kg^{-1}	**CysGly** µmol kg^{-1}	**Hcys** µmol kg^{-1}	**GSSG** µmol kg^{-1}	**GSH** µmol kg^{-1}
Cauliflower leaves	2.60 ± 2.25	7.57 ± 2.55	/	5.12 ± 0.12	/	85.57 ± 62.93	24.12 ± 2.75
Rocket	/	3.50 ± 0.18	/	5.07 ± 0.06	/	16.39 ± 0.91	21.18 ± 0.83
Garlic	/	17.50 ± 5.47	/	5.28 ± 0.5	/	21.56 ± 5.35	29.26 ± 10.84
Onion	/	8.50 ± 0.59	/	5.54 ± 0.12	/	16.73 ± 1.44	25.74 ± 0.52
Walnut	/	4.41 ± 0.16	/	5.15 ± 0.15	/	58.35 ± 6.94	21.45 ± 1.02

Table 7: Contents of Cyis, Cys, CysT, CysGly, Hcys, GSSG, GSH found in plant tissues using HPLC-MS/MS method.

Conclusions

In this study, a novel HPLC-MS/MS method was developed for the simultaneous detection and quantification of Cyis, Cys, GSH, GSSG, CysT, CysGly and Hcys in plant tissue extracts. The assay requires the derivatisation with DTDP and offers a good selectivity and sensitivity. Further adjustments of method can be considered, such as the introduction of a second internal standard with lower concentration for the analysis of analytes at very low levels. This method will be an useful tool in the investigation of the quantities of these sulfur compounds, with possible applications in other types of biological matrices.

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CHAPTHER III

IDENTIFICATION OF UNKOWN THIOL COMPOUNDS

"A novel HPLC-MS/MS approach for the identification of biological thiols in vegetables"

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Under Submission in *Food Chemistry*

A novel HPLC-MS/MS approach for the identification of biological thiols in vegetables

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Abstract

Thiols in nature are important molecules with diverse functions, ranging from acting as crucial antioxidants that prevents chronic diseases in humans to contributing aroma and flavours to foods and beverages. Biological thiols such as glutathione (GSH), cysteine, and related compounds are of interest due to the participation of GSH in the maintenance of cellular redox homeostasis, acting as detoxification agents against reactive oxygen species. This antioxidant defence mechanism also occurs in plants, and GSH and other thiols are important dietary phytochemicals, which can readily be obtained from consuming various fruits and vegetables. However, knowledge of thiol metabolism in plants is limited to known compounds, and a large number of unidentified thiol-containing metabolites (including potential flavour compounds) can exist in vegetables, as evidenced by previous studies. Using precursor ion scan mode, a high-performance liquid chromatography-tandem mass spectrometry (HPLC-MS/MS) approach has been proposed for the screening of thiols in vegetal samples, including cauliflower leaves, rocket, garlic and onion, after extraction and thiol-specific derivatisation with 4-4'-dithiodipyridine. Compound identity was proposed using HPLC with high resolution MS

and verified by analysis of authentic standards, after their derivatisation. Results showed a different thiol composition between vegetal samples, which was indicative that thiol profiles might be species-specific. Furthermore, 1-β-thio-D-glucose was identified for the first time in Brassica. Given the interest in the contribution of thiols to the nutritional value and flavouring of foods, this approach could lead to prospecting studies that identify new thiols so their role in the nutritional value of vegetables can be defined.

Keywords: 4.4'-dithiodipyridine, thiol derivatisation, vegetable, identification

Introduction

It is well known that a significant correlation exists between human nutritional habits and the development of chronic diseases. Damage to cell membranes by the action of free radicals is thought to be the cause of many diseases, and it has been demonstrated that the introduction of a ample fruits and vegetables in the diet can decrease the risk of the occurrence of such disorders (e.g., diabetes, cancer and Alzheimer's disease) (Steinmetz & Potter, 1991; Willett, 2002). Fruits and vegetables contain phytochemical substances that enhance nutritional value by preventing radical formation as a result of their antioxidant effect.

Thiols constitute an important class of phytochemicals, and are organic compounds characterized by a sulfhydryl functional group with strong nucleophilic proprieties. They protect cells from oxidative damage and have a pivotal role in the maintenance of cellular redox homeostasis (Jobe & Kopriva, 2018; Sen & Packer, 2000; Włodek, 2002). The presence of these so-called biological thiols (biothiols) in fruits, vegetables and spices has been addressed through the quantification of some of the most widely studied biothiols,

especially glutathione (GSH) and cysteine (Cys) (Demirkol & Cagri-Mehmetoglu, 2008; Manda, Adams, & Ercal, 2010; Qiang, Demirkol, Ercal, & Adams, 2005).

GSH is a tripeptide (L-glutamyl- L-cysteinyl-glycine) and represents the main low molecular weight non-protein thiol in all living cells. It is involved in protection against oxidative stress and in the detoxification of heavy metals and xenobiotics in plants (Meister, 1994; Sen & Packer, 2000). Cys, a thiol-containing amino acid, is one of the main products of the sulfur assimilation pathway in plants and serve as sulfur donor in several reactions (Pilon-smits & Pilon, 2006). Additionally, a large number of thiols from secondary metabolism are is especially important to the flavor impact in various foods and beverages (Lampe, 2003; Vermeulen et al., 2007).

Several specialized studies have been reported on the different classes of thiols, assessing their efficacy in preventing human disease or their contribution to food flavor properties. However, few biothiols have been described in the literature, and an array of thiol molecules likely exist that are still unknown (Chen, Capone, & Jeffery, 2019; Inoue et al., 2013; Kanawati, Kubo, Schmitt-kopplin, & Grill, 2014). Chromatographic and spectrometric evidence has revealed that thiol profiles change between different plants, indicating how these molecules are specifically related to the nature of the samples (Pivato, Fabrega-Prats, & Masi, 2014).

Their low concentrations in plants and vegetables combined with their reactivity mean that the identification of these compounds represents a major challenge that, if solved, could open new prospective studies that aim to understand how thiols could be involved in plant metabolism.

Numerous analytical methods for thiol analysis have been developed using liquid chromatography (LC) with electrochemical (EC), fluorescence (FL), or mass spectrometry (MS) detections along with different derivatizing agents (Winther &

Thorpe, 2014). In the order to discover new thiols, different approaches can be taken, such as using sulfur-specific isolation techniques or selective detectors (Chen et al., 2019). In particular, precursor ion scan methodology using a triple quadrupole (QqQ) MS appeared to be a promising strategy, with the detection of unique ions such as those arising after thiol-specific derivatisation with a reagent like 4,4'-dithiodipyridine (DTDP) (Capone, Ristic, Pardon, & Jeffery, 2015).

This work aimed to test the hypothesis that a novel analytical HPLC-MS/MS strategy could be utilised for identifying unknown thiols in plants and food samples, after their derivatization with DTDP. Initial identification and then confirmation of analytes were undertaken with different mass spectrometry techniques – precursor ion scan followed by molecular formula determination and analysis of authentic standards (Figure 1). The effective pH range of DTDP means the approach can be used for matrices that range from acidic to neutral, making this a broadly applicable technique for thiol discovery.

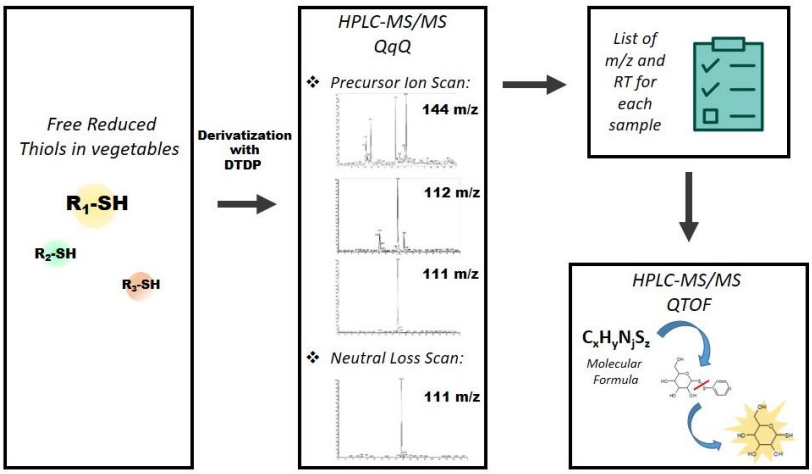


Figure 1: Proposed approach to the identification of unknown thiols. After the derivatization with DTDP, thiols were analysed in high performance liquid chromatography in combination with a triple quadrupole tandem mass spectrometer (HPLC-MS/MS QqQ) in precursor ion scan mode. The mass/charge ratio (m/z) listed as results, were further analysed through high resolution quadrupole-time of flight (QToF) mass spectrometer for molecular formula determination.

Materials and methods

Chemicals and materials

4,4'-Dithiodipyridine (DTDP), formic acid (≥ 98%), and concentrated hydrochloric acid (HCl, ≥37% w/w) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Thiol standards included 1-thio-β-D-glucose sodium salt, allyl mercaptan (≥ 90%), 1-Propanethiol (99%), L-Gutathione reduced (≥ 98.0%), L-Cysteine (97%) and Cys-Gly (≥ 85%), purchased from Sigma-Aldrich (St. Louis, MO, USA), and γ-Glutamylcysteine, which was purchased from Kohjin Co. (Shimbashi, Minatoku, Tokyo). Ammonium Acetate was purchased from USB Corporation (Cleveland, OH, USA) and EDTA disodium salt hydrate from AppliChem (Darmstadt, Germany). Water was obtained from a Milli-Q water purification system. Solutions and standards were prepared volumetrically. All vegetal samples were purchased fresh from major chain supermarkets in Padova, Italy.

Plant samples were collected in the same way described in Chapter 2 and the same derivatization procedure was adopted for all the samples.

Only cauliflower leaves, rocket, garlic and onion were considered in this study, since the walnut didn't show an interesting thiol profile from a preliminary screening.

Preparation of thiol standard derivatives for LC-MS parameter optimization.

Thiol standard solutions were prepared by dissolving the respective thiol in HCl 0.1 M. Mass spectrometer parameters were optimized with infusion MS/MS experiments of 10 mg/L solutions of thiol standards derivatized according to the procedure in Section 2.2.4, using an infusion pump operating at 10 μL/min.

Instrumentation and Chromatography

- HPLC Conditions

Qualitative analyses were performed with a Thermo Fisher Scientific HPLC system connected to a TSQ Quantiva QqQ MS system with electrospray ionization in positive ion mode. Separations were conducted using a 150 mm × 4.6 mm i.d., 5 μm, 100 Å Kinetex C18 column operated at 25 °C and protected by a 4 mm x 3.0 mm i.d. guard cartridge of the same material The solvents were 0.5% aqueous formic acid (solvent A) and 0.5% formic acid in acetonitrile (solvent B), with a flow rate of 0.200 mL/min. The linear gradient for solvent B was as follows: 0 min, 2%; 5 min, 2%; 20 min, 80%; 25 min, 80%; 26 min, 2%; followed by 10 min of column equilibration with 2% B. An injection volume of 10 μL was used. These conditions were based on previous analysis of thiol-4-TP derivatives (Capone et al., 2015).

An Agilent 1290 Infinity UHPLC system connected to a Waters Xevo G2-S Q-TOF MS system was used for accurate mass determinations. The same conditions were applied as specified above, but with a decreased column equilibration time (for a total run time of 31 min).

- Mass Spectrometer Conditions

The TSQ Quantiva MS had the following conditions: N_2 was used for sheath gas, 35 arbitrary units; auxiliary gas, 20 arbitrary units and sweep gas, 2 arbitrary units; the ion spray voltage, source fragmentation, ion transfer tube temperature, and vaporiser temperature were set at 4500 V, 0 V, 300 °C, and 275 °C, respectively. Argon was used

as collision gas with CID set at 2.5 mTorr. For precursor ion scan and neutral loss scan mode, Q1 and Q3 had 0.7 resolution and a collision energy ramp was set from 10 eV to 45 eV.

The Waters Q-TOF had the following conditions: sampling cone voltage, source offset, desolvation temperature and source temperature set at 40 V, 80 V, 450 °C and 300 °C, respectively. The capillary voltage was set to 4500 V and the nebuliser gas used was N_2. A collision energy ramp from 20 eV to 40 eV was applied. Data were collected in full-scan and MS^e mode simultaneously.

Results and Discussion

As showed in several studies, plants belonging to Brassicaceae family show various thiol composition (Aghajanzadeh, Hawkesford, Kok, Bloem, & Kühn-institute, 2014; Bednarek, 2012; Diopan et al., 2010). Rocket, leaves of cauliflower (both belonging to Brassicaceae family), garlic and onion (both well known for thiols contribute to their flavoring proprieties), were selected as samples for this study in order to explore their sulfur metabolome.

First, representative thiols such as L-cysteine (Cys), L-glutathione (GSH) and cysteinyl-glycine (Cys-Gly) were studied to determine the optimal experimental conditions and to verfiy the efficacy of the new derivatization protocol. Using the data from the HPLC-MS/MS (QqQ) analysis, the loss of specific fragments generated by the cleavage of the 4-thiopyridine (4-TP) part of the molecules could be used as a "fingerprint" for the identification of thiol compounds in vegetal samples, with further analysis using high resolution mass spectrometry (HRMS).

The derivatization of thiols with DTDP followed by HPLC-MS/MS analysis was previously used for several methods developed to quantify flavor compounds in wine (Capone et al., 2015; Chen, Capone, & Jeffery, 2018). Nevertheless, DTDP derivatization of biothiols from vegetal samples and the subsequent analysis by mass spectrometry was without precedent. First, the investigation focused on the definition of a new protocol of derivatization with DTDP, in order to increase the pH of the extracted samples (pH~1) to within the pH values often used with this reagent (pH 4.6 and 7.0) (Egwim & Gruber, 2001; Grassetti, 1967), although moderately acidic conditions (i.e., pH 3.5) can also be effective (Capone et al., 2015). A solution of ammonium acetate in water was selected for buffering purposes and was included in the derivatization protocol. Because the reaction of DTDP with thiols occurs over a wide pH range (Egwim & Gruber, 2001), only a brief evaluation of pH value was conducted and the derivatisation reaction was terminated by adding concentrated. After the optimization of the derivatization step, the samples were directly analysed without any purification or concentration process.

Thiol standard solutions of GSH, Cys and Cys-Gly were analysed by HPLC-MS/MS after their derivatization with a QqQ in product ion scan mode, to identify the principal transitions produced by the thiol-4-TP species, generating a "fingerprint" of DTDP-derivatised compounds. MS/MS provided fragmentation data that was consistent with the previous work (Capone et al., 2015), with an intense fragment at mass/charge ratio (*m*/*z*) 144 and a second lower intensity fragment at m/z 111 (or m/z 112) , each arising from the derivatized portion of the molecule. In contrast, GSH was the only thiol the yielded m/z 306 as the most intense fragment rather than m/z 144, which arose from the neutral loss of *m*/*z* 111, consistent with the 4-TP part of the molecule. This distinctive fragmentation pathway could be related to gas-phase conformation of the GSH-4-TP molecule, which may influence the way it undergoes collision induced dissociation such that is does not

as easily generate the fragment with m/z 144. A similar argument has been proposed previously to account for the different fragmentation patterns for glutathionylated phenolic compound isomers in wine, based on the relative accessibility of collision gas to the thioether bond of GSH (Cejudo-Bastante, Pérez-Coello, & Hermosín-Gutiérrez, 2010).

The results from the analysis of biothiol standards led to the setting of a new precursor ion scan – neutral loss scan methods that can be applied to vegetal matrices in order to investigate their thiol profiles.

Hence, the values of m/z 144, m/z 112 and m/z 111 were set as "precursor" for precursor ion scan mode and *m/z* 111 was set as "neutral loss" in neutral loss scan mode. Characteristic chromatograms from the analysis of cauliflower leaves, rocket, garlic and onion were generated for each scan mode (Figure 2). The assessment of the chromatographic profiles led to the collection of a list of peaks, characterized by a specific retention time (RT) and one or more m/z values related to the scan mode (Table 1). Based on the initial analysis of thiol standards, different compounds could be readily recognized in vegetal sample profiles: GSH was detected in precursor ion scan mode of m/z 112 for all the samples, revealing a precursor ion at m/z ~ 417 and RT ~17.2 min; Cys precursor ion was detected with m/z ~ 231 and a RT ~ 8.5 min using precursor ion scan mode of m/z 144 (for all the samples) and m/z 112 (only for cauliflower leaves).

The most intense peak in the chromatograms appearing at ~ 17.48 min (Figure 2) with precursor ion m/z ~ 222 was due to the presence of excess DTDP from the derivatisation stage. As previously reported (Capone et al., 2015), this was an important observation because excess reagent is required to ensure the complete derivatization of all thiols among vegetal samples (to prevent scrambling of the thiol-4-TP disulfides by additional thiol-disulfide exchange reactions forming other mixed disulfides no longer

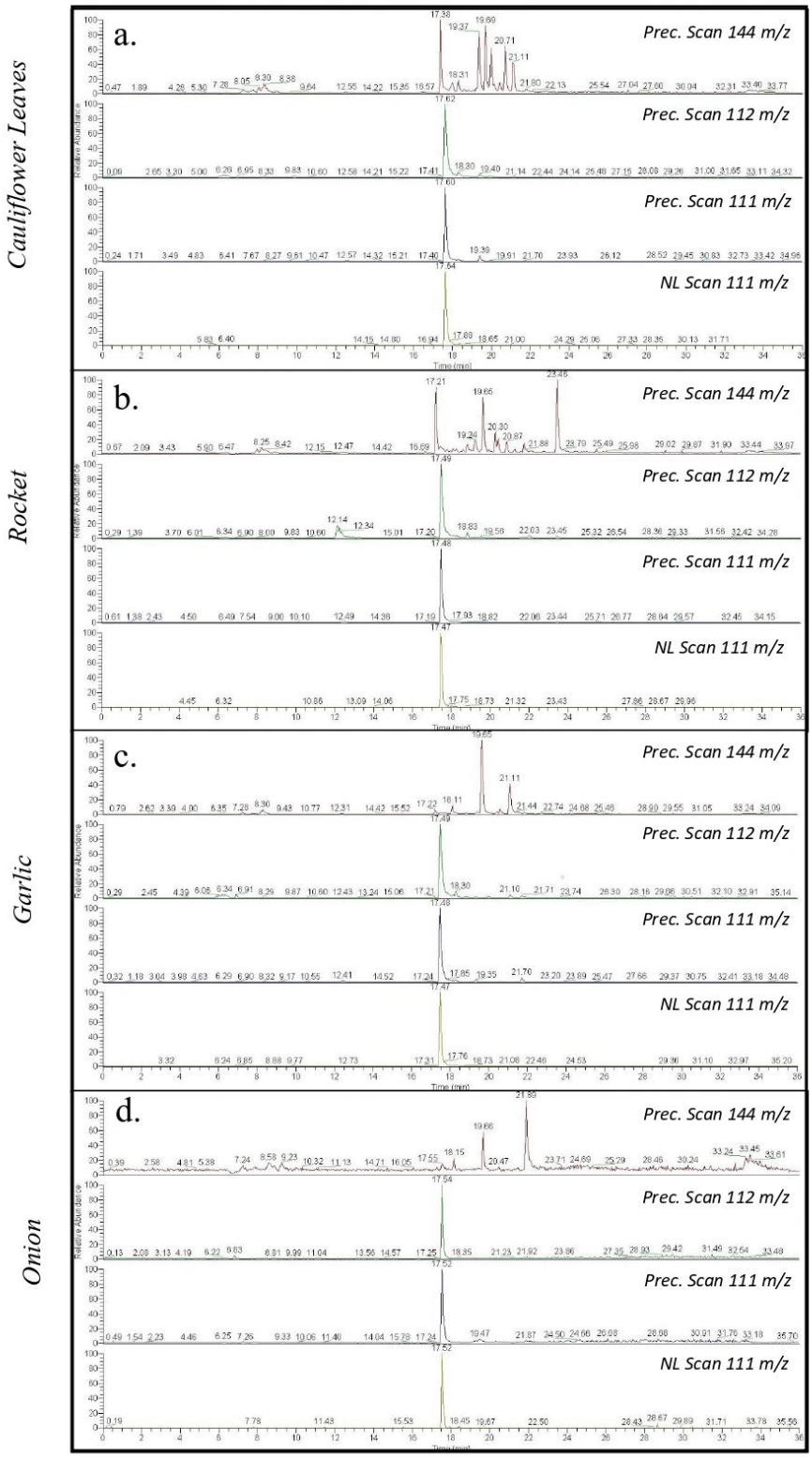


Figure 2: Screening of thiols through HPLC-MS/MS QqQ. Thiol profile from a) Cauliflower leaves; b) Rocket; c) Garlic; d) Onion samples. The samples were analysed through Precursor Ion scan (m/z 144; m/z 112; m/z 111) and Neutral Loss scan of 111.

Cauliflower Leaves		Rocket		Garlic		Onion	
Precursor m/z 144		**Precursor m/z 144**		**Precursor m/z 144**		**Precursor m/z 144**	
m/z	RT (min)	m/z	RT (min)	m/z	RT (min)	m/z	RT (min)
189.64	7.28	265.78 ; 246.56 ; 185.38 ; 162.88	8.01	189.68	7.28	414.42 ; 276.17	7.24
246.65	7.69	231.65	8.25	231.23	8.30	423.22 ; 226.29 ; 203.14 ; 180.71	8.62
185.63 ; 349.03 ; 543.51	8.05	203.74 ; 262.73	8.42	438.25 ; 308.30	12.31	231.85 ; 203.50	8.82
231.48	8.30	322.49 ; 278.12 ; 180.45	8.94	360.55 ; 231.71 ; 181.03	17.22	322.43 ; 278.31 ; 203.58	9.23
306.64	17.38	288.46	9.65	454.39 ; 185.44	17.54	419.00 ; 226.50	17.26
392.67	17.99	438.24 ; 393.94 ; 308.04	12.47	469.09 ; 352.27 ; 266.61 ; 190.38 ; 254.16	17.75	394.73 ; 226.51 ; 427.45	18.15
371.43 ; 308.53 ; 291.46	18.27	306.60	17.21	394.68	18.11	188.76 ; 205.63 ; 226.41	19.66
277.79 ; 217.56	19.37	438.38	17.46	450.60 ; 358.21 ; 159.22	19.37	186.49	21.89
188.7 ; 205.59	19.65	225.50 ; 257.31	18.84	246.50 ; 205.71 ; 188.75	19.65		
318.79	19.98	243.49 ; 277.56	19.24	231.69	20.59		
217.6	20.42	246.76 ; 205.67 ; 188.69	19.65	184.47 ; 226.44	21.11		
418.54; 203.5 ; 185.46 ; 162.63	20.71	244.52	20.26	274.25 ; 184.47	21.40		
246.48 ; 287.42	21.11	225.55	20.83	454.27 ; 249.12 ; 226.31 ; 186.30	21.84		
601.47 ; 449.52 ; 226.37 ; 192.64	21.80	436.90	21.27	313.40 ; 272.44	22.74		

226.46 ; 185.64	25.50	453.24 ; 226.55	21.72				
226.39	27.04	257.66	23.46				
Precursor m/z 112		**Precursor m/z 112**		**Precursor m/z 112**		**Precursor m/z 112**	
m/z	RT (min)	m/z	RT (min)	m/z	RT (min)	m/z	RT (min)
203.67	3.06	324.60 ; 244.57	9.83	171.38	6.34	175.64	6.83
175.58	6.95	417.20	17.27	175.60	6.91	417.20	17.25
306.38 ; 231.31 ; 346.20	8.33	222.63	17.49	244.51	9.87	222.4	17.54
487.47 ; 244.65	9.83	437.63 ; 517.31	18.83	192.31	15.02		
192.63	15.22	276.67	22.03	360.50 ; 231.52 ; 180.98	17.21		
417.15	17.20	257.45	23.45	417.23	17.25		
222.58	17.62			222.66	17.49		
				450.35	19.32		
				184.53 ; 153.48	21.75		
Precursor m/z 111		**Precursor m/z 111**		**Precursor m/z 111**		**Precursor m/z 111**	
m/z	RT (min)	m/z	RT (min)	m/z	RT (min)	m/z	RT (min)
221.65	17.6	221.62	17.48	224.34 ; 221.21 ; 170.52	6.41	221.62	17.57
253.38	18.33	253.7	17.93	253.55 ; 221.59	18.29	152.52	19.47
158.41	19.39	257.61	23.48	221.45 ; 158.55	19.35		
				184.55	21.70		
				216.57 ; 257.44	23.20		
Neutral Loss 111		**Neutral Loss 111**		**Neutral Loss 111**		**Neutral Loss 111**	
m/z	RT (min)	m/z	RT (min)	m/z	RT (min)	m/z	RT (min)
251.83	6.40	257.29	23.43	221.87	17.47	221.97 ; 331.19	21.52
665.78	16.94			184.05	21.08	223.11	28.67

Table 1: List of Retention time (RT) and mass/charge ratio (m/z) of the compounds detected through HPLC-MS/MS QqQ. Data were collected from chromatograms reported in Fig. 2.

containing the 4-TP moiety. As shown in Figure 2, the derivatising agent did not produce the m/z 144 fragment.

Table 1 lists a large number of ions observed by using precursor ion and neutral loss scan methods, meaning they could be new unidentified thiols as per this proposed thiol discovery approach. However, the mass resolution of QqQ MS is relatively low, and the protonated derivative ions listed may have more than one molecular formula. To identify the unknown compounds, samples were further analyzed with high-resolution mass spectrometry (HRMS), which allows the m/z values to be accurately determined and molecular formulas to be calculated. Thus, using HPLC connected to a quadrupole-time of flight (QToF) mass spectrometer, a full-scan/MS^e method was set with the same chromatographic and mass spectrometer parameters used previously in QqQ analysis. By calculating the mass errors (measured in parts per million (ppm)) between the accurate m/z value measured by HRMS analysis and the theoretical value of the candidate compound, it was possible to assign the molecular formula. A mass error of less than 5 ppm confirmed the plausibility of the determined molecular formula.

After this evaluation, the molecular formulas of four different thiols compounds were defined (Table 2). In cauliflower leaves and rocket, the peak at ~ 17.4 min with precursor ion of m/z 306 found based on m/z 144 was associated with 1-β-thio-D-glucose-4-TP molecule. In garlic, the peak at 17.22 min and precursor ion of m/z ~ 360 detected with m/z 144 and m/z 112 was related to γ-glutamyl-cysteine-4-TP, whereas the peak at ~ 21 min with precursor ion of m/z ~ 184 and detection with *m/z* 144 was related to allyl mercaptan-4-TP. In onion, the peak at 21.89 min with precursor ion of m/z ~ 186 based on m/z 144 was associated with 1-propanethiol-DTDP (Table 2). After the exclusion of the m/z values related to “non-thiol” compounds, and due to the low concentration of

thiol molecules in plant tissues, only these four thiols were identified through HRMS analysis in the studied samples. Nonetheless, the concept of thiol discovery using thiol specific derivatisation, precursor ion scan analysis and then HRMS measurements was shown to be viable.

As reported in the literature, allyl mercaptan has been widely studied in garlic for its anticancer effect and as flavor character impact compound (Nian, Delage, Pinto, & Dashwood, 2008; Tamaki & Sonoki, 1999), whereas γ-glutamyl-cysteine causes a decrease of blood pressure (Ashraf, Khan, Ashraf, & Qureshi, 2013). Similarly, 1-propanethiol is known as volatile compound in onion for its high odorant activity and for being the main source of characteristic onion flavors (Boelens, de Valois, Wobben, & van der Gen, 1971; Cannon et al., 2015; Farkas, Hradský, & Kovác, 1992; Løkke, Edelenbos, Larsen, & Feilberg, 2012; Wermes et al., 2017). Importantly, no reports were found regarding the presence of free 1-β-Thio- D -glucose in Brassica species, but it may be produced by the spontaneous degradation of glucosinolates, which are sulfur-containing metabolites that play a role in plant defense (Hopkins, van Dam, & van Loon, 2009; Ishida, Hara, Fukino, Kakizaki, & Morimitsu, 2014).

In order to confirm the compounds assigned by HRMS analysis, commercial standards were purchased and subsequently analysed. In particular, a Selected reaction monitoring (SRM) method was developed in QqQ and used for the analysis of both the mixture of the four standards after derivatization and the four treated (i.e., extracted and derivatised) vegetal samples. The mass transitions for each derivatized standard were first determined after a brief evaluation of the mixture in product ion scan mode (as was mentioned earlier for GSH, Cys, and Cys-Gly).

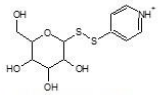
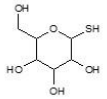
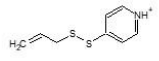
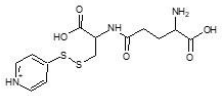
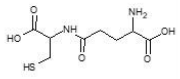
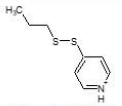
	Identified compound+DTDP	Identified Compound	Parts per milion (ppm)	Sample
Thiol-DTDP Molecular Formula $C_{11}H_{16}NO_5S_2$ Thiol Molecular Formula $C_6H_{12}O_5S$	 Exact Mass: 306,046993 HRSM Mass: 306,0470	 Name: 1-β-Thio-D-glucose	0,02	- Cauliflower Leaves - Rocket
Thiol-DTDP Molecular Formula $C_8H_{10}NS_2$ Thiol Molecular Formula C_3H_6S	 Exact Mass: 184,025468 HRSM Mass: 184,0255	 Name: Allyl Mercaptan	0,17	- Garlic
Thiol-DTDP Molecular Formula $C_{13}H_{18}N_3O_5S_2$ Thiol Molecular Formula $C_8H_{14}N_2O_5S$	 Exact Mass: 360,068791 HRSM Mass: 360,0688	 Name: Γ-Glutamyl-Cysteine	0,02	- Garlic
Thiol-DTDP Molecular Formula $C_8H_{12}NS_2$ Thiol Molecular Formula C_3H_8S	 Exact Mass: 186,041118 HRSM Mass: 186,0411	 Name: 1-Propanethiol	-0,09	- Onion

Table 2: Identified Compounds from High Resolution Mass Spectrometry (HRMS) analysis.

The selected fragments for each sample were: m/z 306 → m/z 144 and m/z 306 → m/z 111 for 1-β-Thio- D -glucose- DTDP; m/z 360 → m/z 144 and m/z 112 for γ- glutamyl-cysteine-DTDP; m/z 184 → m/z 143 and m/z 111 for allyl mercaptan-DTDP; m/z 186 → m/z 144, m/z 112 for 1-propanethiol-DTDP. Generally, a hypothetical structure can be confirmed by matching the RT and the abundance ratio of the SRM transition ions for the analyte to those of the corresponding authentic standard compound. SRM mode was thus used to obtain this specific information, and both RT and abundance ratio criteria were satisfied (data not shown). As further confirmation of the veracity of the identifications, extracted vegetal samples were spiked with the standard compounds, then derivatised and analysed, to compare with unspiked samples. For all the samples, the intensity of the original peaks from the natural analytes increased significantly and no additional peaks were observed in the chromatogram of the spiked samples (Fig. 3).

The findings of the present study highlight how a combination of different mass spectrometry techniques and a novel derivatisation and HPLC-MS/MS approach can lead to an exploration of the thiol profile of vegetal samples, with the possibility of identifying new thiols. Studies can then subsequently occur to understand the contribution of such thiols to the nutritional value of vegetables.

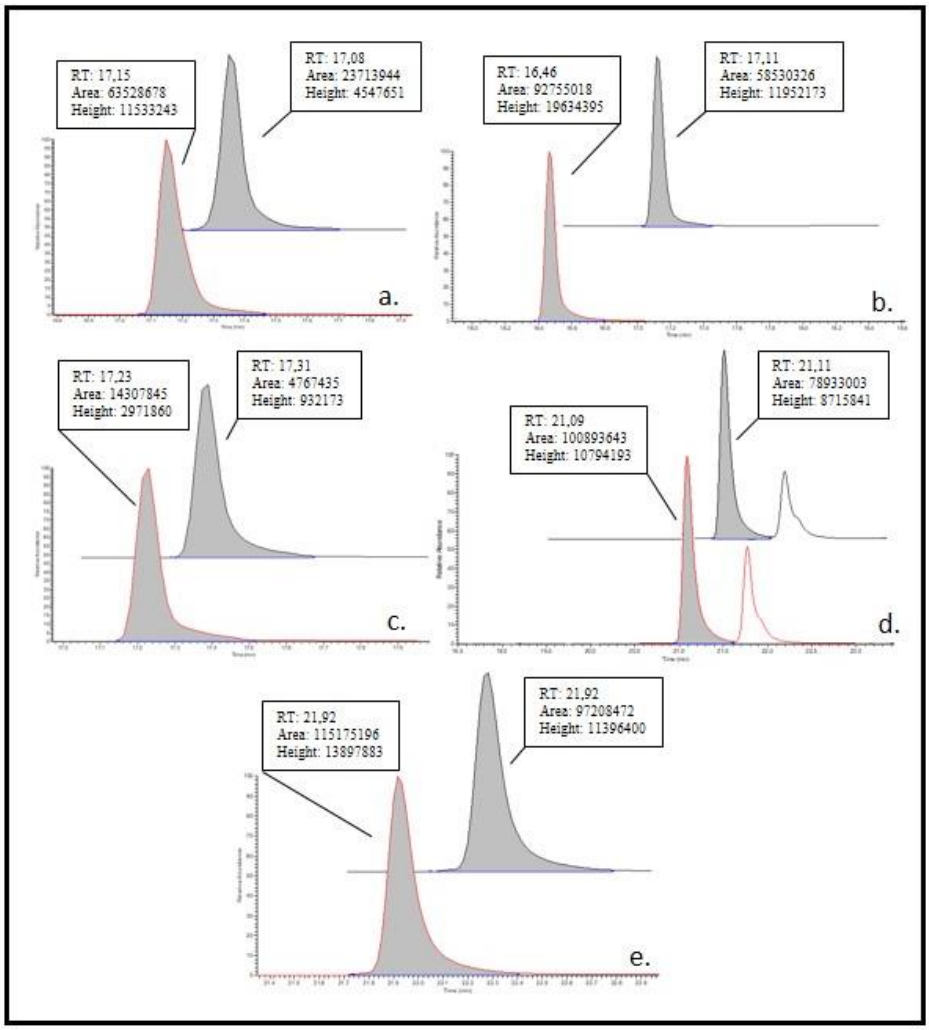


Figure 3: SRM chromatograms of natural and spiked samples. Retention time (RT), Area and Height of Natural sample (in the foreground) and spiked sample (in the background) were compared for every standard. a) Confirmation of 1-Thio-β-D-glucose in Cauliflower leaves; b) Confirmation of 1-Thio-β-D-glucose in Rocket; c) Confirmation of γ-Glutamyl-Cysteine in Garlic; d) Confirmation of Allyl Mercaptan in Garlic; e) Confirmation of 1-Propanethiol in Onion.

Conclusions

From this study, an original HPLC-MS/MS method for discovering thiol-containing compounds has been proposed. The free (reduced) thiol profiles can be ascertained after the simple extraction of vegetal samples and thiol-specific derivatization with DTDP. The thiol-4-TP disulfides were targeted due to the characteristic fragments produced from derivatized part of the molecule. The results show the identification of four thiols and their confirmation through the combination of different MS techniques, and analysis of and spiking with authentic standards. However, this was a proof of concept study, and the identification of all the prospective thiol compounds would perhaps require an additional concentration step and then matching with authentic standards after following the proposed approach, to confirm their identities. This methodology is a promising tool for future studies that aim to discover new sulfur metabolites, just as 1-β-thio-D-glucose was identified in Brassica for the first time in this study.

APPENDIX: APPLICATION OF IDENTIFICATION METHOD

HPLC-MS/MS identification of unknown thiols in Brassicaceae species from the Botanical Garden of the University of Padova

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Introduction

The HPLC-MS/MS method developed for identifying new thiols in cauliflower leaves, rocket, garlic and onion (described at the beginning of this Chapter) was subsequently applied on 30 plant samples from Botanical Garden of Padova, belonging to *Brassicaceae* family.

As mentioned at the beginning of this chapter, glucosinolates are a class of secondary metabolites with a primary role in plant defense in Brassicaceae species. All glucosinolates share the same basic structure, consisting in three principal parts: a β-thioglucose moiety, a sulfonate oxime moiety and a side chain that can vary depending on the nature of the compound (Hopkins et al., 2009).

The thiol profiles of several plants belonging to this family wad studied in order to discover new coumpounds, that could derive from glucosinolates degradation or from unknown sulphur pathways in plants.

The plants selected with the aim of screening their thiols profile were:

1) *Aurinia leucadea;*
2) *Alliaria Petiolata;*
3) *Arabis Alpina;*
4) *Arabis Hirsuta;*
5) *Arabis Turrita;*
6) *Armoracia Rusticana;*
7) *Aubrieta Deltoidea;*
8) *Barbarea Vulgaris;*
9) *Berteroa Incana;*
10) *Cardamine bulbifera;*
11) *Crambe Cordofolia Steven;*
12) *Crambe Hispanica;*
13) *Crambe Maritima;*
14) *Diplotaxis tenufolia;*
15) *Eruca Sativa;*
16) *Erucastrum Palustre;*
17) *Erysimum Cheiri;*
18) *Fibigia Comune;*
19) *Hesperis Matronalis;*
20) *Iberis Semperflorens;*
21) *Iberis Umbellata.*
22) *Iberis Sempervirens;*
23) *Isatis Tinctoria;*
24) *Lepidium Draba;*
25) *Lunaria Annua;*
26) *Matthiola tricuspidata;*
27) *Orychophragmus Violaceus;*
28) *Peltaria alliacea;*
29) *Rorippa austriaca;*
30) *Sinapis Alba;*

The samples were collected at the Botanical Garden of Padova and, after extraction, each was divided into two aliquots. The first was derivatised with ammonium 7-fluoro-2,1,3-benzoxadiazole-4-sulfonate (SBD-F), a fluorescent label thiol-specific. The derivatisation procedure with SBD-F includes a reduction step, in order to obtain reduced thiols from disulphide compounds. The analysis through liquid chromatography coupled with fluorimetric detector (HPLC-FL) can show the total amount of thiols in a given sample after reduction.

The second aliquot was derivatized with DTDP and analysed through HPLC-MS/MS tecniques. The initial screening through the fluorimetric analysis indicates the most characteristic thiol profiles, in order to lead the MS analysis only with the most promising samples.

Materials and methods

Samples Treatment

- Collection of the samples

The collection of the samples took place at the Botanical Garden of Padova. Leaves of each plant were cut and strictly frozen in liquid nitrogen and then stored at -20°C until extraction.

- Thiols extraction

Thiols were extracted following the extraction procedure described in Chapter 2.
The extraction solution was divided into two aliquots.

- Derivatisation with SBD-F

A volume of 50 μL of extraction solution was added to 117 μL of 1M borate buffer (pH=10.5). Sample reduction was achieved adding 33 μL of 1% tributylphosphine. Then 33 μL of derivatising agent (SBD-F, 0.3%) were included in the solutions. After 1 hour of incubation at 60°C, the reaction was stopped with 17 μL of 4 M HCl.

- Derivatisation with DTDP

The derivatisation procedure with DTDP it is described in Chapter 2.

Instrumental Conditions

- HPLC-FL conditions

The HPLC analysis (Agilent 1260) was led with a mobile phase of 75 mM NH_4-formiate (pH = 2.9) methanol (97:3) in isocratic condition for 15 minutes, using a 150 mm x 4.6 mm i.d., 5 um, 100 Å Kinetex C18 column operated at room temperature and protected by a 4 mm x 3.0 mm i.d. guard cartridge of the same material.

Fluorimetric detector (Agilent 1260 infinity ii) was setted with excitation wavelength = 386 nm and emission wavelength = 516 nm.

- HPLC-MS/MS techniques

The HPLC-MS/MS parameters are described at the beginning of this Chapter.

Results and Discussion

Plant samples from the Botanical Garden were analysed through HPLC-FL. The chromatograms acquired are shown in Figure 1; Figure 2; Figure 3.

From the preliminary analysis of SBD-F derivatised samples, it was possible to screen the thiol compounds in the 30 plant samples.

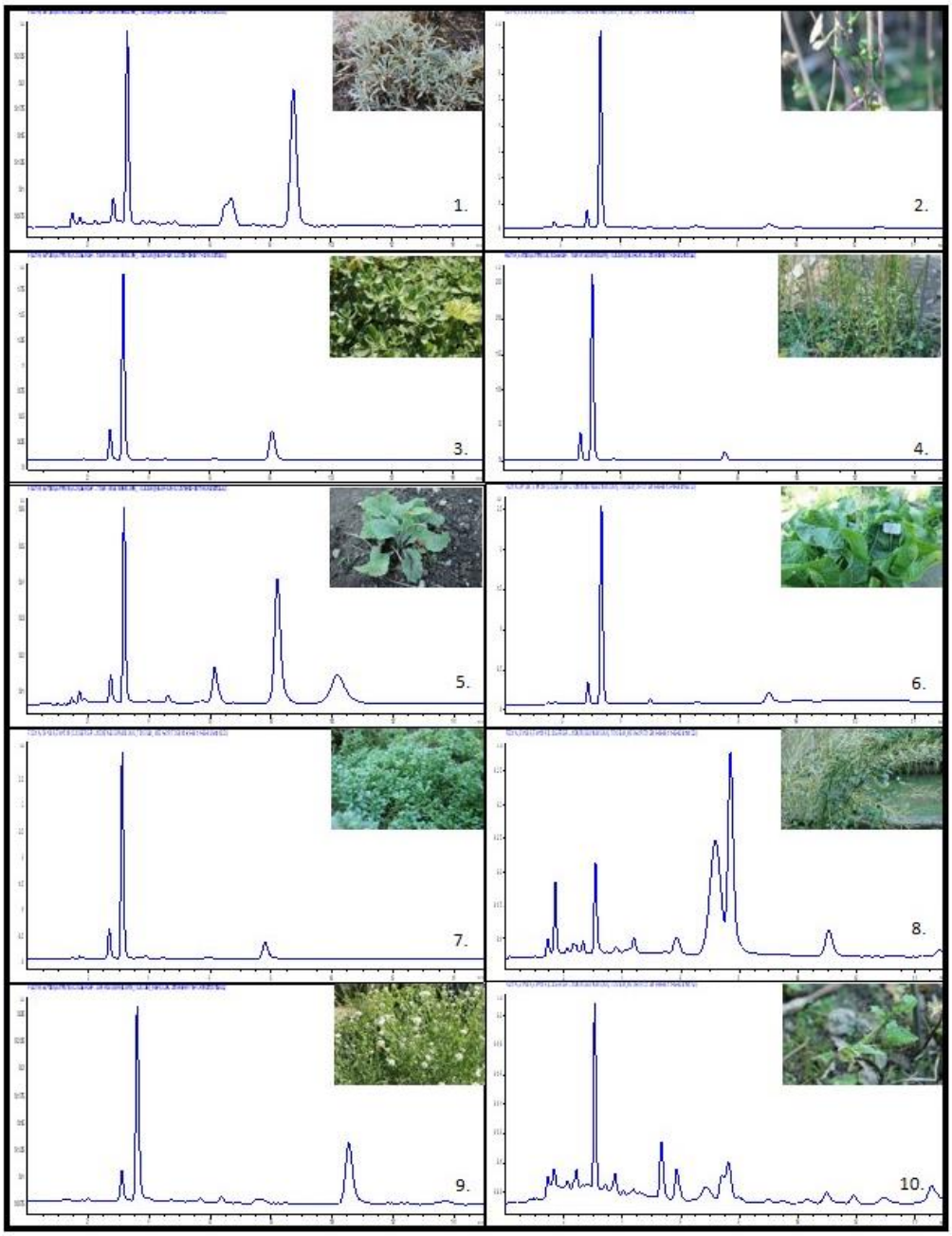


Figure 1: Chromatograms of 1) *Aurinia leucadea;* 2) *Alliaria Petiolata;* 3) *Arabis Alpina;* 4) *Arabis Hirsuta;* 5) *Arabis Turrita;* 6) *Armoracia Rusticana;* 7) *Aubrieta Deltoidea;* 8) *Barbarea Vulgaris;* 9) *Berteroa Incana;* 10) *Cardamine bulbifera* acquired through HPLC-FL analysis.

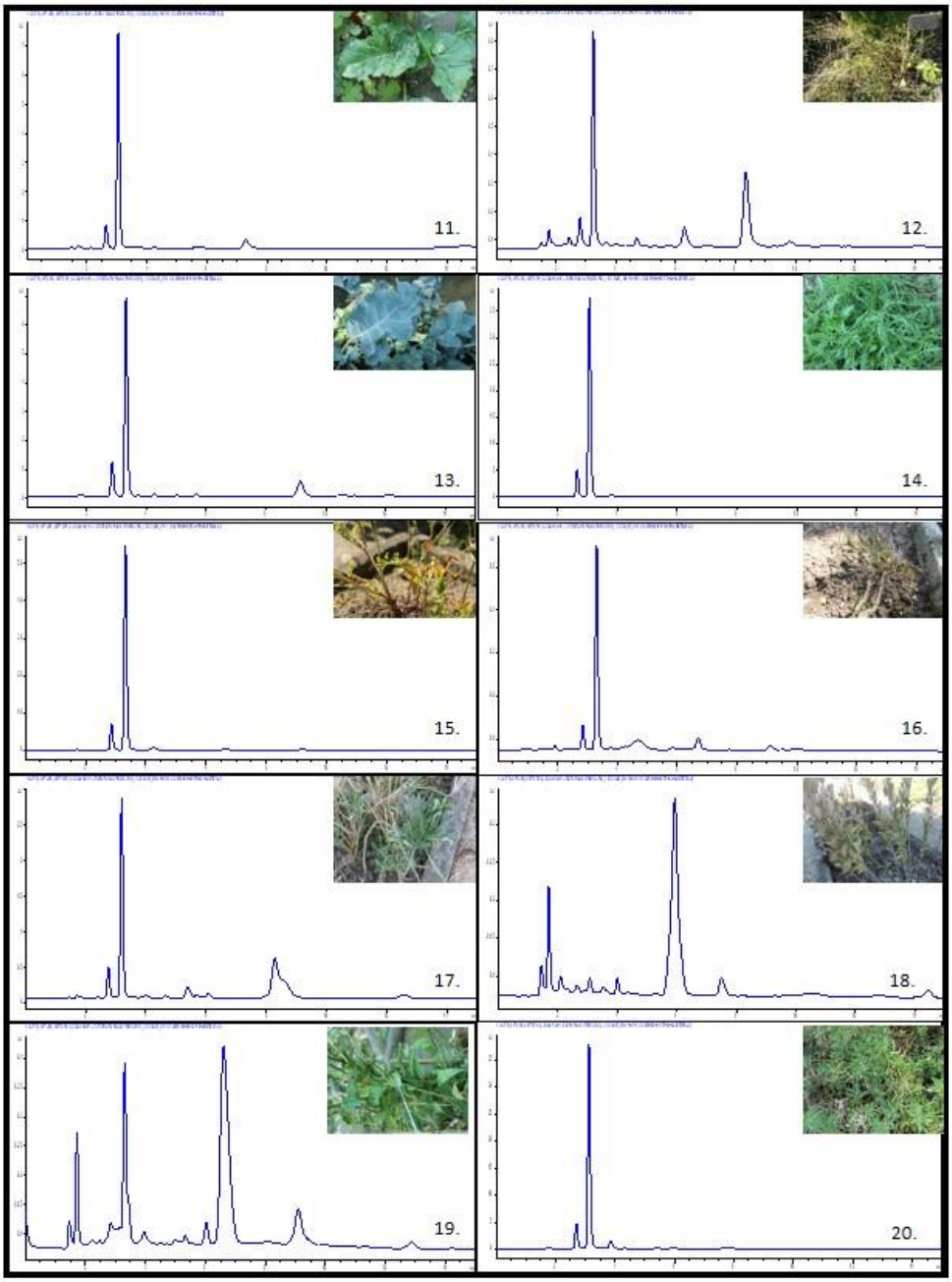


Figure 2: Chromatograms of 11) *Crambe Cordofolia Steven;* 12) *Crambe Hispanica;* 13) *Crambe Maritima;* 14) *Diplotaxis tenufolia;* 15) *Eruca Sativa;* 16) *Erucastrum Palustre;* 17) *Erysimum Cheiri;* 18) *Fibigia Comune;* 19) *Hesperis Matronalis;* 20) *Iberis Semperflorens* acquired through HPLC-FL analysis.

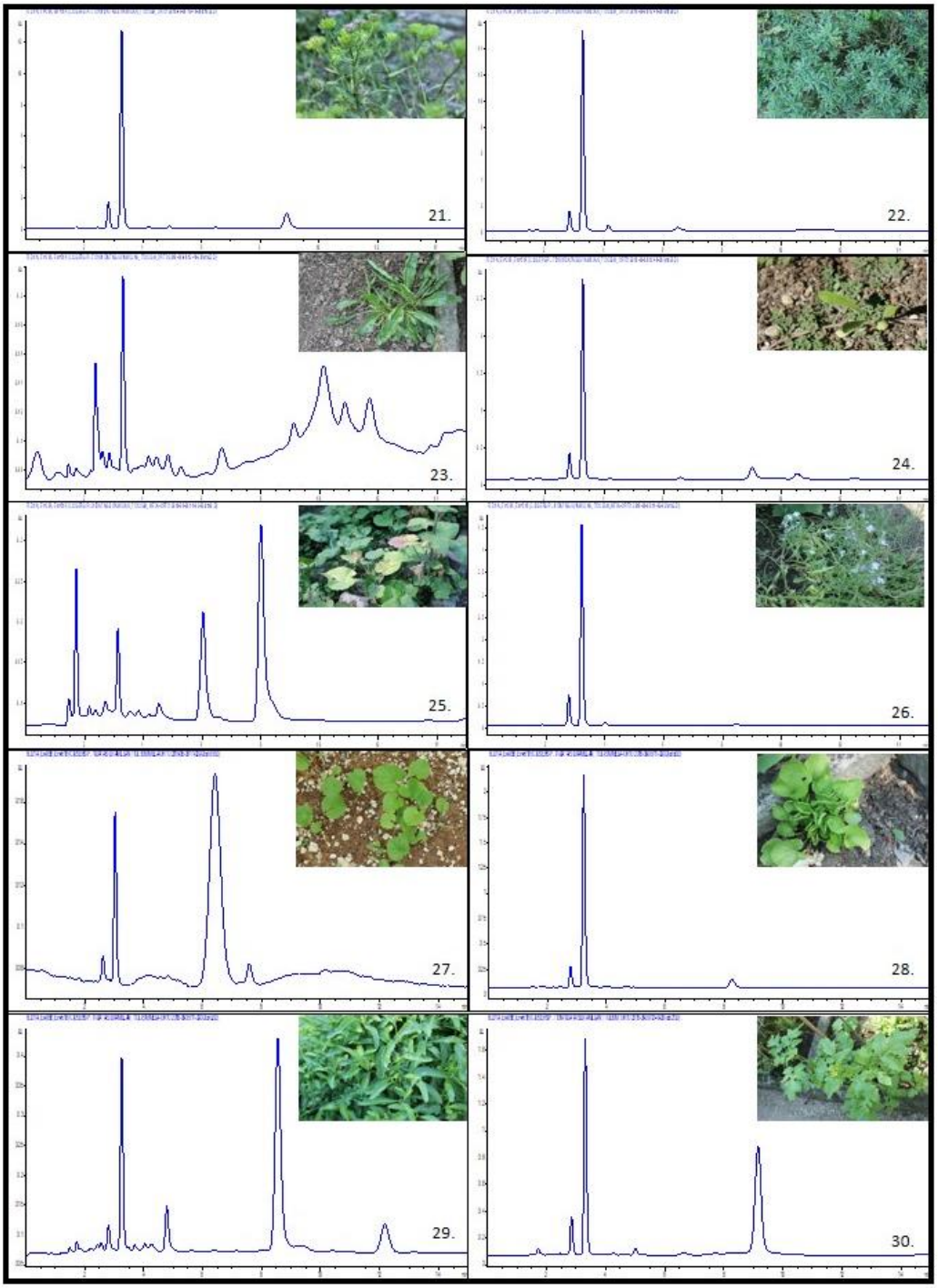


Figure 3: Chromatograms of 21) *Iberis Umbellata;*.22) *Iberis Sempervirens;* 23) *Isatis Tinctoria;* 24) *Lepidium Draba;* 25) *Lunaria Annua;* 26) *Matthiola tricuspidata;* 27) *Orychophragmus Violaceus;* 28) *Peltaria alliacea;* 29) *Rorippa austriaca;* 30) *Sinapis Alba* acquired through HPLC-FL analysis.

Among them, it was exstimated that *Aurinia leucadea, Arabis Turrita, Barbarea Vulgaris, Cardamine bulbifera, Crambe Hispanica, Erysimum Cheiri, Fibigia Comune, Hesperis Matronalis, Isatis Tinctoria, Orychophragmus Violaceus, Rorippa austriaca* were the species with the most interesting thiol profile. Indeed, the chromatograms showed several peaks related to the presence of unknown thiols.

In order to identify these compounds, the selected species were further analysed through MS techniques. The second aliquot of each sample was derivatised with DTDP using the procedure described at the beginning of Chapter 2. The procedure with DTDP derivatising agent can consider only the free reduced thiols naturally existent in the plants, since no reduction step is included in the derivatisation procedure.

After derivatisation, the samples were analysed in HPLC-MS/MS method using the identification method developed with cauliflower leaves, rocket, garlic and onion samples (described at the beginning of this Chapter).

The chromatograms were acquired with triple quadrupole MS in precursor ion scan of m/z 144, precursor ion scan of m/z 112, precursor ion scan of m/z 111 and neutral loss scan of 111 for each sample. The results are shown in Figure 4; Figure 5; Figure 6; Figure 7; Figure 8; Figure 9; Figure 10; Figure 11; Figure 12; Figure 13; Figure 14; Figure 15.

In most of the 4-TP-thiol derivatised samples , the chromatograms acquired in precursor ion scan of m/z 144 and the precursor ion scan of m/z 112 indicate the most diversified composition of thiols.

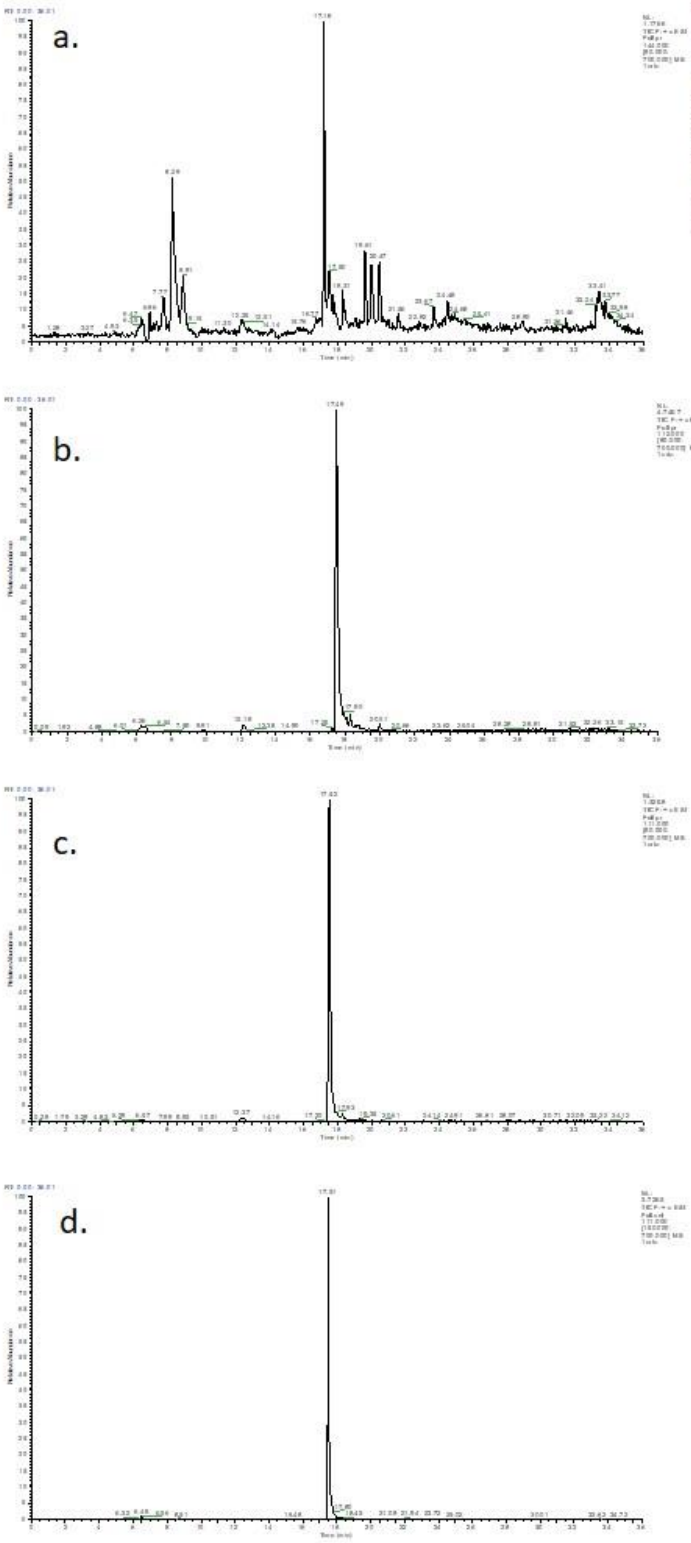


Figure 4: Chromatograms of *Aurinia leucadea* in
a) Precursor Ion scan of m/z 144;
b) Precursor Ion scan of m/z 112;
c) Precursor Ion scan of m/z 111;
d) Neutral Loss scan of 111.

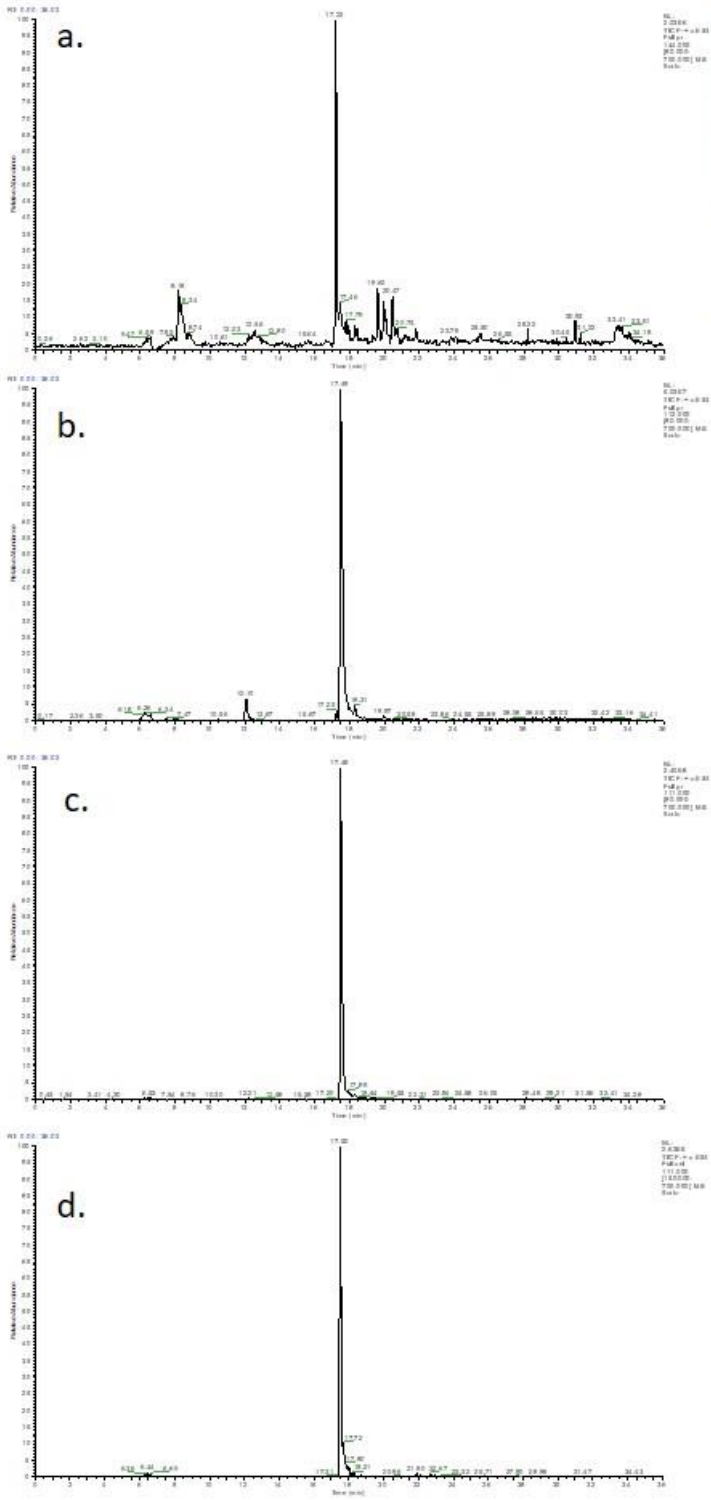


Figure 5: Chromatograms of *Arabis Turrita* in
a) Precursor Ion scan of m/z 144;
b) Precursor Ion scan of m/z 112;
c) Precursor Ion scan of m/z 111;
d) Neutral Loss scan of 111.

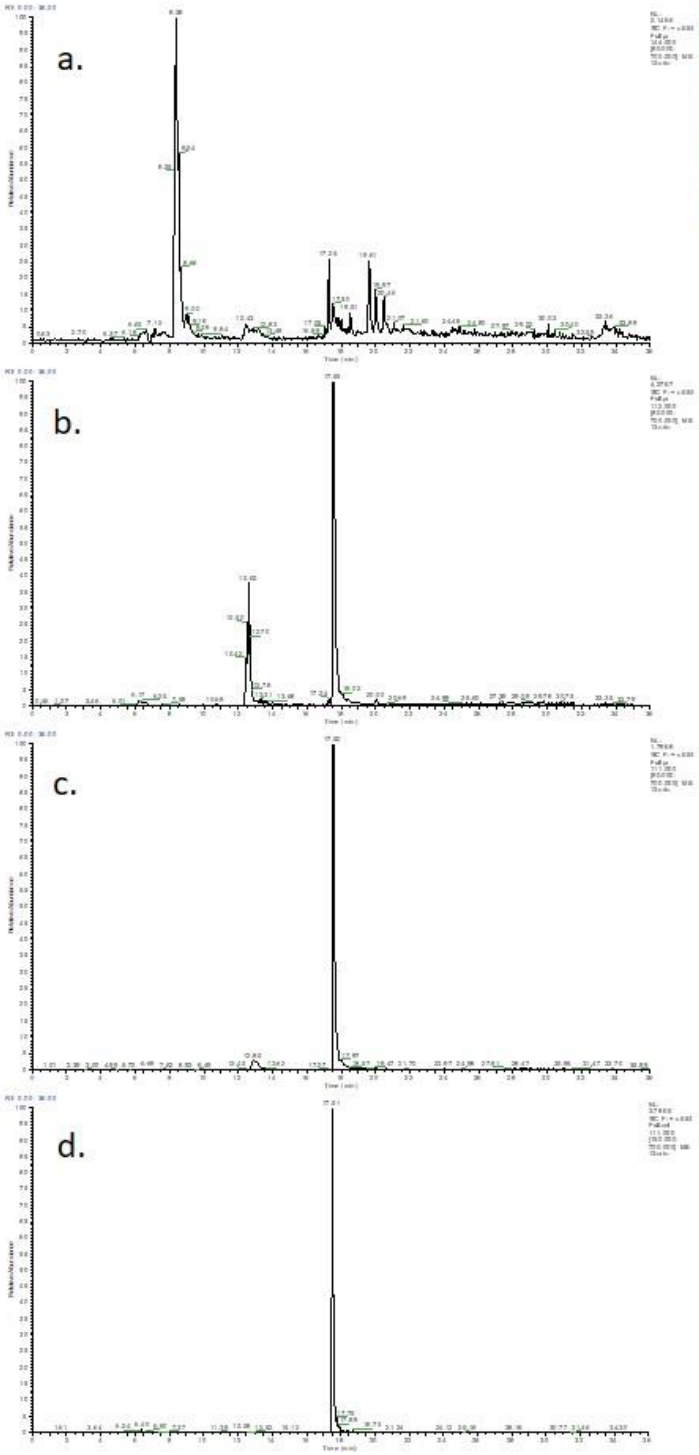


Figure 6: Chromatograms of *Barbarea Vulgaris* in
a) Precursor Ion scan of m/z 144;
b) Precursor Ion scan of m/z 112;
c) Precursor Ion scan of m/z 111;
d) Neutral Loss scan of 111.

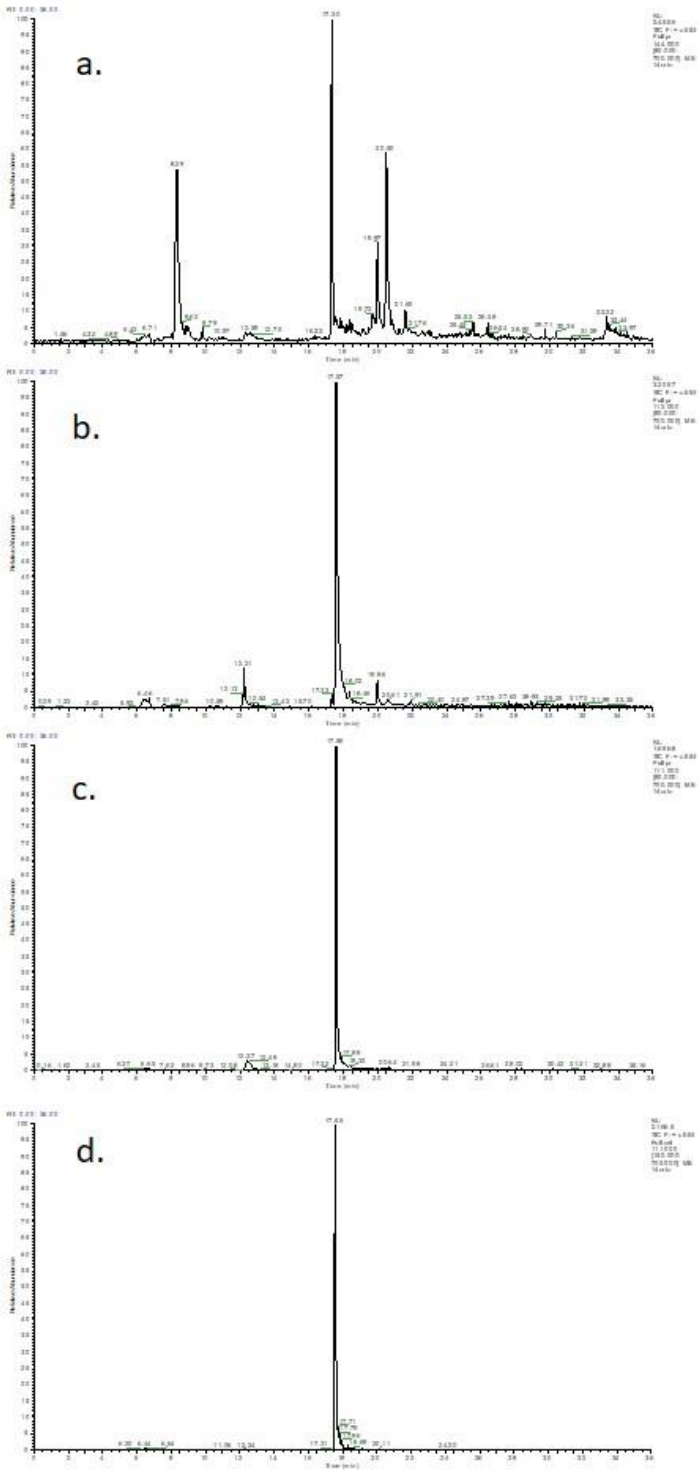
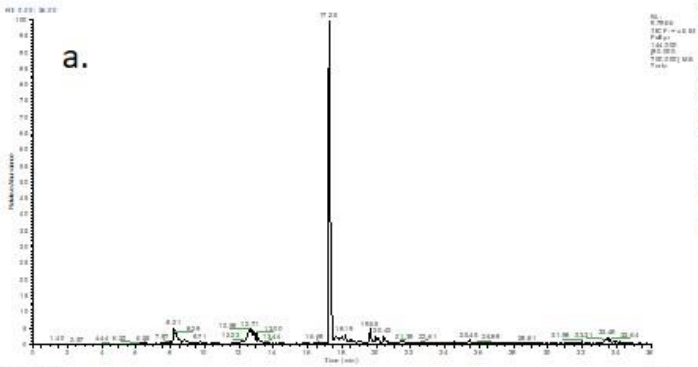
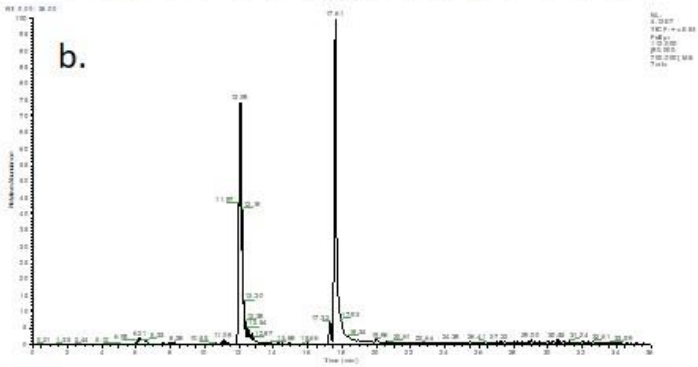
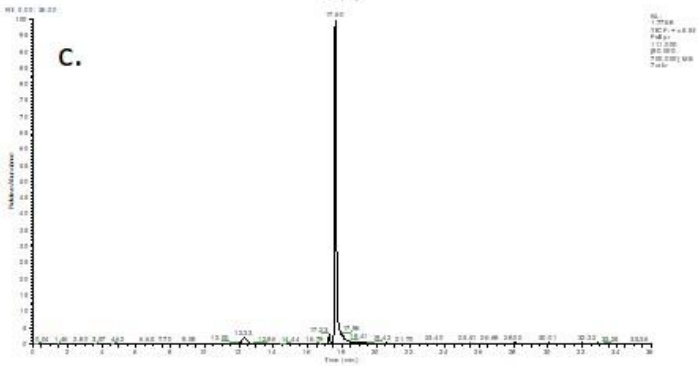


Figure 7: Chromatograms of *Cardamine bulbifera* in
a) Precursor Ion scan of m/z 144;
b) Precursor Ion scan of m/z 112;
c) Precursor Ion scan of m/z 111;
d) Neutral Loss scan of 111.







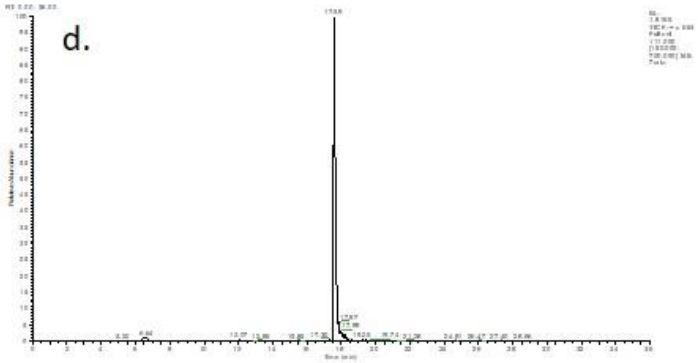


Figure 8: Chromatograms of *Crambe Hispanica* in
a) Precursor Ion scan of m/z 144;
b) Precursor Ion scan of m/z 112;
c) Precursor Ion scan of m/z 111;
d) Neutral Loss scan of 111.

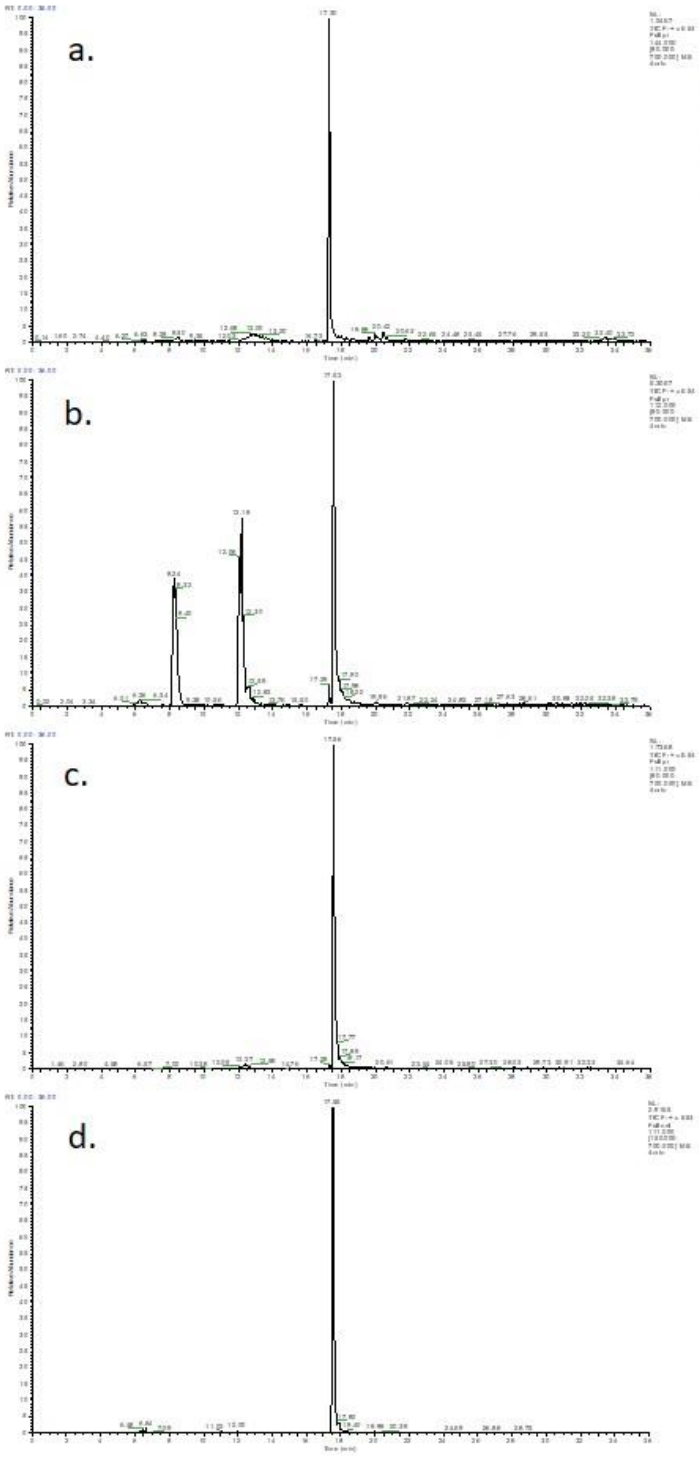


Figure 9: Chromatograms of *Erysimum Cheiri* in
a) Precursor Ion scan of m/z 144;
b) Precursor Ion scan of m/z 112;
c) Precursor Ion scan of m/z 111;
d) Neutral Loss scan of 111.

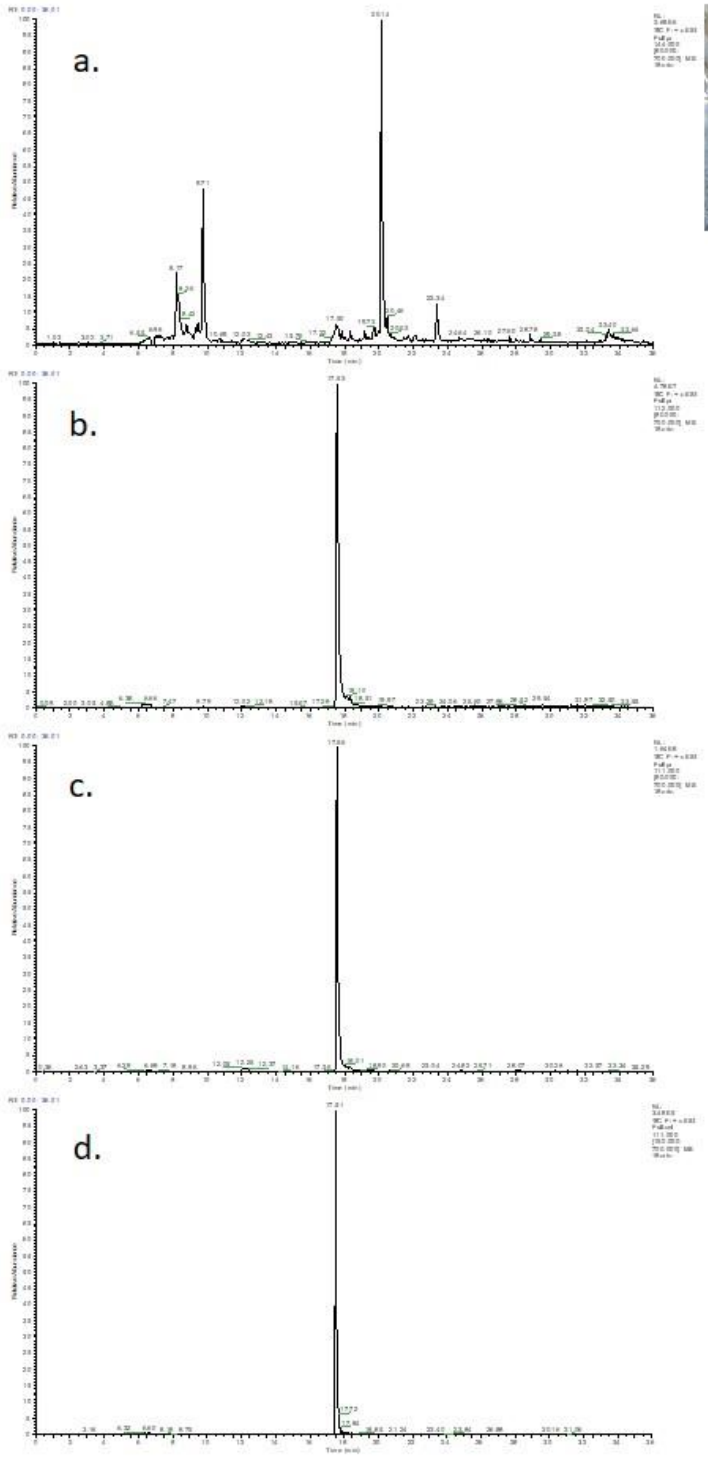


Figure 10: Chromatograms of *Fibigia Comune* in
a) Precursor Ion scan of m/z 144;
b) Precursor Ion scan of m/z 112;
c) Precursor Ion scan of m/z 111;
d) Neutral Loss scan of 111.

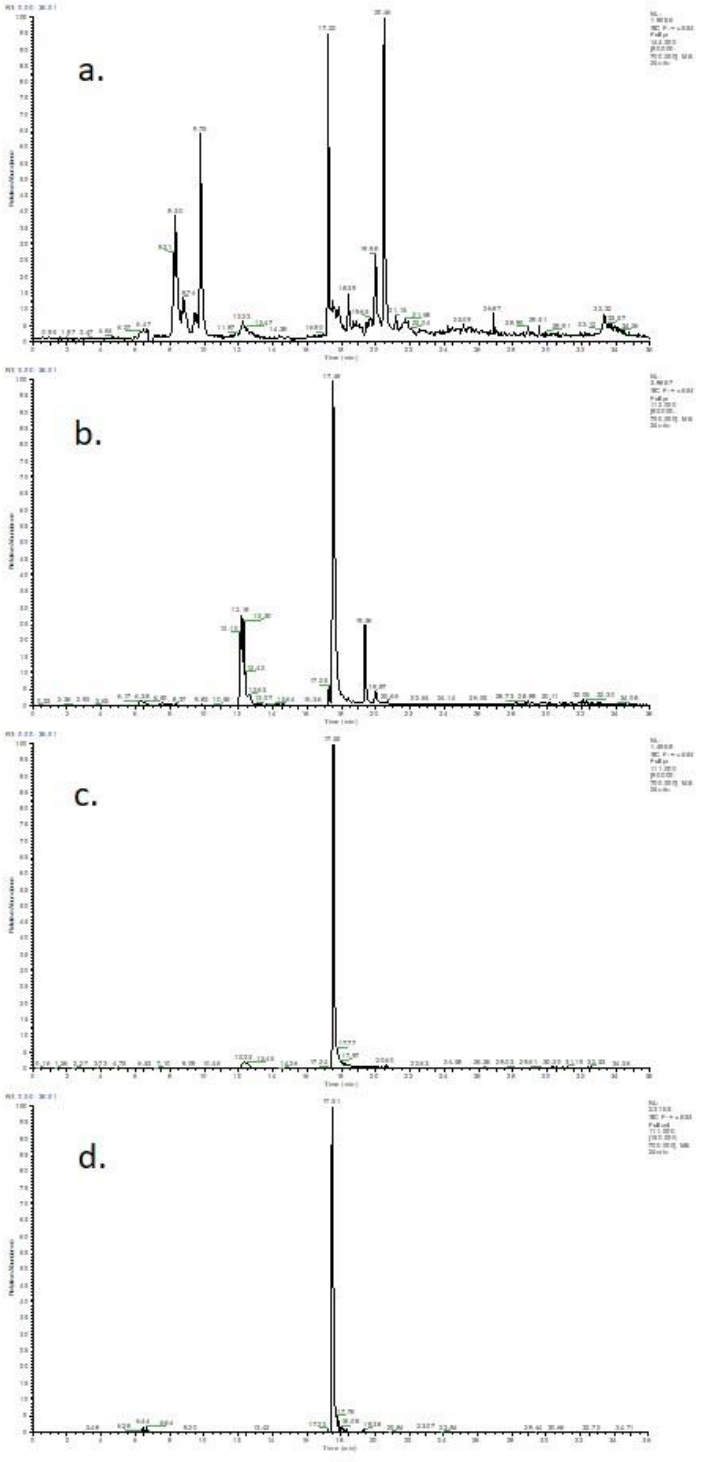


Figure 11: Chromatograms of *Hesperis Matronalis* in
a) Precursor Ion scan of m/z 144;
b) Precursor Ion scan of m/z 112;
c) Precursor Ion scan of m/z 111;
d) Neutral Loss scan of 111.

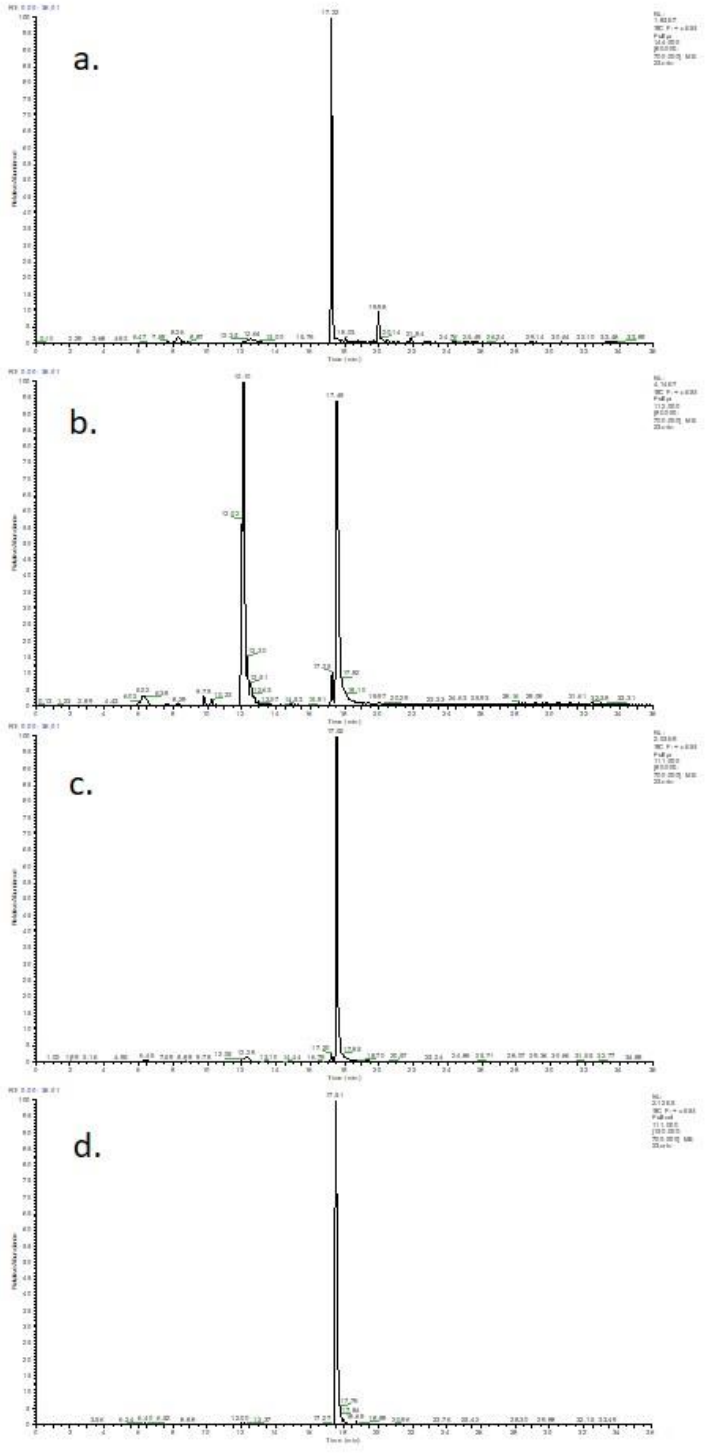


Figure 12: Chromatograms of *Isatis Tinctoria* in
a) Precursor Ion scan of m/z 144;
b) Precursor Ion scan of m/z 112;
c) Precursor Ion scan of m/z 111;
d) Neutral Loss scan of 111.

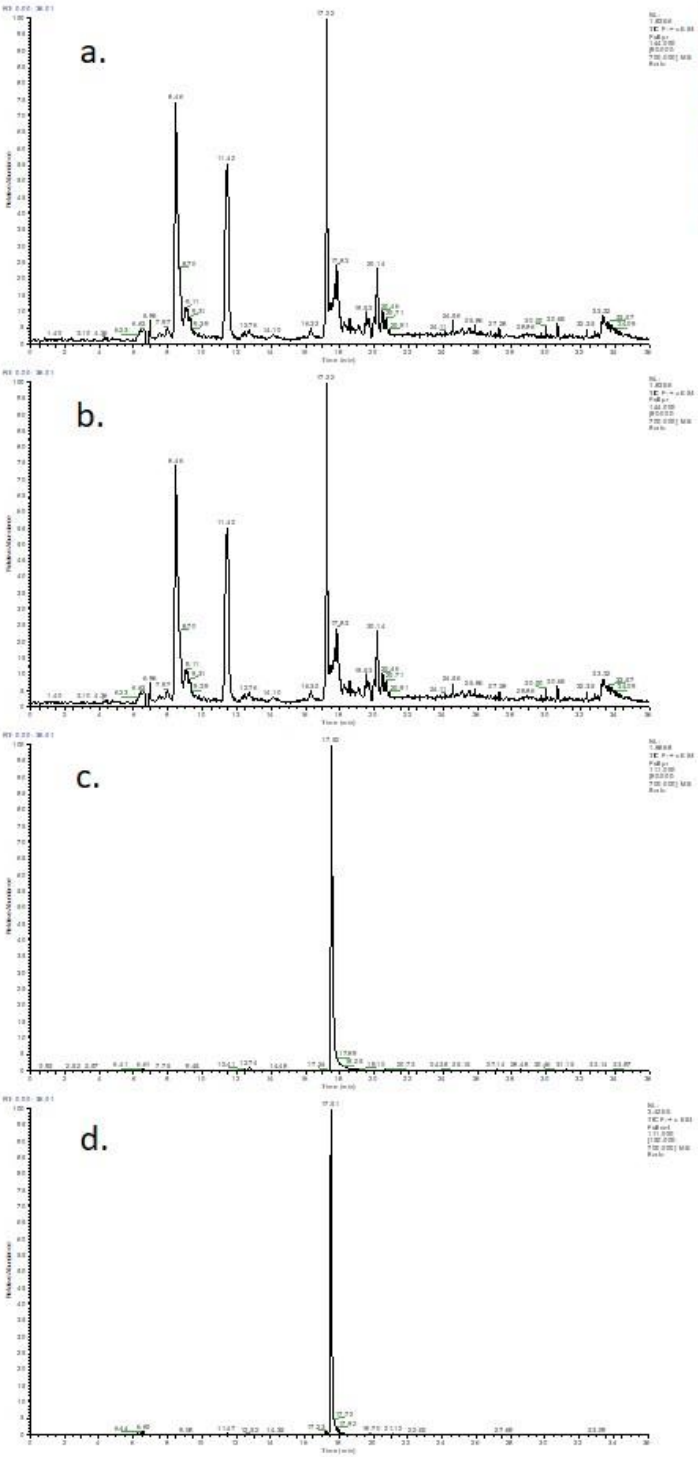


Figure 13: Chromatograms of *Lunaria Annua* in
a) Precursor Ion scan of m/z 144;
b) Precursor Ion scan of m/z 112;
c) Precursor Ion scan of m/z 111;
d) Neutral Loss scan of 111.

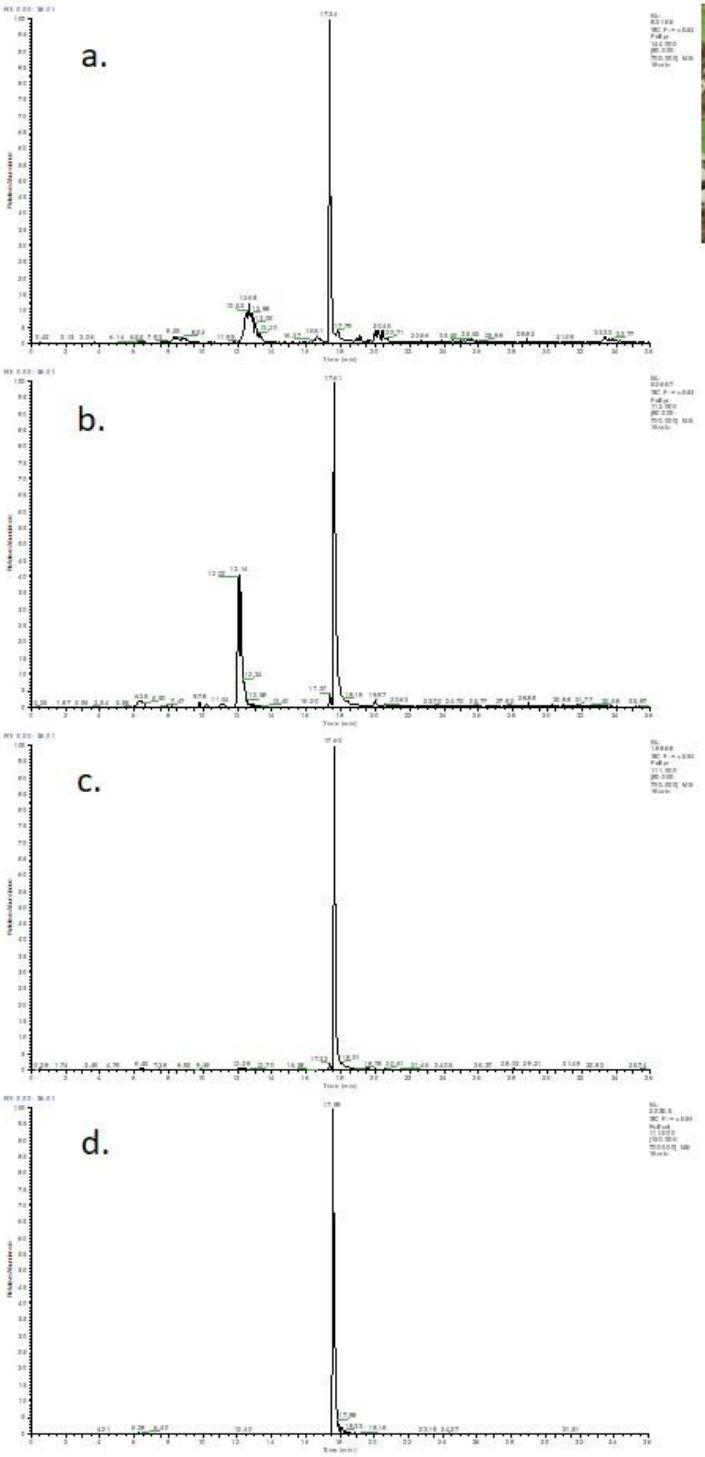


Figure 14: Chromatograms of *Orychophragmus Violaceus* in
a) Precursor Ion scan of m/z 144;
b) Precursor Ion scan of m/z 112;
c) Precursor Ion scan of m/z 111;
d) Neutral Loss scan of 111.

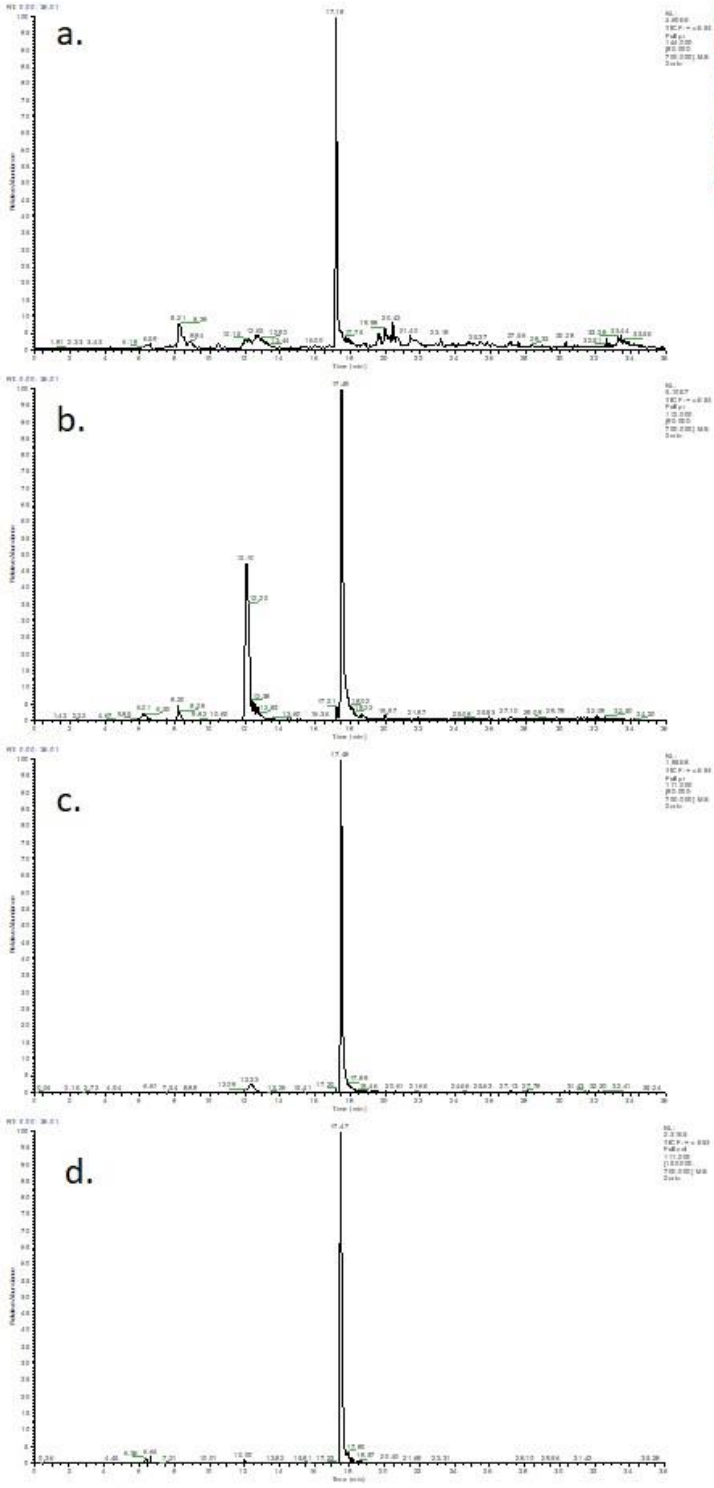


Figure 15: Chromatograms of *Rorippa austriaca* in
a) Precursor Ion scan of m/z 144;
b) Precursor Ion scan of m/z 112;
c) Precursor Ion scan of m/z 111;
d) Neutral Loss scan of 111.

From the chromatograms, it was possible to list a number of compounds described by retention time (RT) and mass/charge value (m/z) for all the type of scan (Table 1).

	Precursor 144 m/z		**Precursor 112 m/z**		**Precursor 111 m/z**		**Neutral Loss 111 m/z**	
Sample Name	**m/z**	**RT (min)**	**m/z**	**RT (min)**	**m/z**	**RT (min)**	**m/z**	**RT (min)**
Aurinia Leucadea	306,43	17,18	417,15	17,25	-	-	-	-
Erysimum Cheiri	306,63	17,30	417,49	17,29	-	-	-	-
Lunaria Annua	306,43	17,22	417,69	17,25	306,41	17,20	417,33	17,23
Barbarea Vulgaris	231,35	8,62	417,51	17,23	-	-	-	-
	308,68	13,16						
	306,48	17,25						
	306,61	17,30	417,45	17,33	306,54	17,32	417,44	17,00

Cardamine Bulbifera	286,42	19,97						
	224,33	20,46						
Fibigia Comune	231,64	8,30	-	-	-	-	-	-
Hesperis Matronalis	231,46	8,30	417,76	17,25	306,3	17,16	417,41	17,23
	308,71	12,19						
	306,61	17,22						
	452,28	17,83						
	290,60	17,83						
	286,44	19,94						
	266,61	20,46						

Table 1: List of Retention time (RT) and mass/charge ratio (m/z) of the compounds detected through HPLC-MS/MS QqQ. Data were collected from chromatograms reported in Figure 4; Figure 5; Figure 6; Figure 7; Figure 8; Figure 9; Figure 10; Figure 11; Figure 12; Figure 13; Figure 14; Figure 15.

As occurred with cauliflower leaver, garlic and onion, the mass obtained from the analysis through the triple quadrupole (QqQ) mass spectrometer did not achieve an high resolution for ready identifying the compounds. As a consequence, further analyses with high resolution mass spectrometry (HRMS) technique were led in order to obtain the exact mass of the unknown compounds and their molecular formula. The assessment of the mass error was considered in the way to evaluate the trueness of the matching between the expected result and the instrumental mass obtained by the instrument (mass error < 5 ppm) (Table 2).

The Analysis through quadrupole-time of flight (QToF) revealed the molecular formula and the possible structure of the compounds. As well as already studied biothiols (such as Cysteine and Glutathione), several of them could be related to the formation of thioglucose derivatives, possibly caused by the degradation of glucasinolates (such as 1-β-Thio-D-Glucose, 2- [4,5-hydroxy-2-(hydroxymethyl)- 6 -sulfanyloxan-3-yl] oxy-6-(hydroxymethyl)oxane-3,5-diol and Thiofucoside). The compound with m/z ~ 287 may be N-acetyl-homocysteine or N-acetyl cysteine methyl ester, derived from a rearrangement of N-acetylcysteine (Demirkol et al., 2004). The other identified coumpounds could be produced by rearrangements of biological molecules in plants, but their metabolic paths are still unknown.

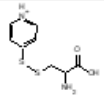
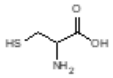
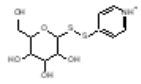
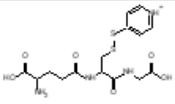
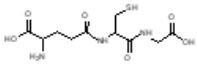
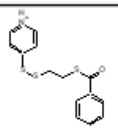
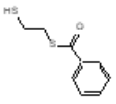
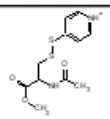
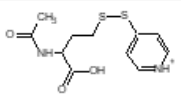
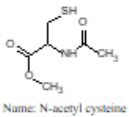
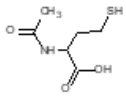
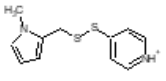
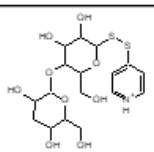
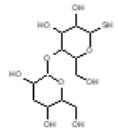
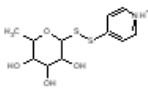
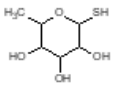
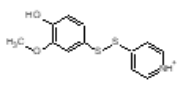
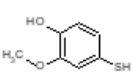
	Identified compound+DTDP	Identified Compound	Parts per milion (ppm)	Sample
Thiol-DTDP Molecular Formula $C_5H_{11}N_2O_2S_2$ Thiol Molecular Formula $C_3H_7NO_2S$	 Exact Mass: 231,026197 HRMS Mass: 231,0262	 Name: Cysteine	0,01	❖ *Barbarea Vulgaris* ❖ *Cardamine Bulbifera* ❖ *Hesperis Matronalis*
Thiol-DTDP Molecular Formula $C_{11}H_{18}NO_5S_2$ Thiol Molecular Formula $C_6H_{12}O_5S$	 Exact Mass: 306,046993 HRMS Mass: 306,0470	 Name: 1-β-Thio-D-glucose	0,02	❖ *Aurinia Leucadea* ❖ *Cardamine Bulbifera* ❖ *Erysimum Cheiri* ❖ *Hesperis Matronalis* ❖ *Lunaria Annua*
Thiol-DTDP Molecular Formula $C_{15}H_{23}N_4O_6S_2$ Thiol Molecular Formula $C_{10}H_{17}N_3O_6S$	 Exact Mass: 417,090254 HRMS Mass: 417,0903	 Name: Glutathione	0,11	❖ *Aurinia Leucadea* ❖ *Cardamine Bulbifera* ❖ *Erysimum Cheiri* ❖ *Hesperis Matronalis*
Thiol-DTDP Molecular Formula $C_{14}H_{12}NOS_3$ Thiol Molecular Formula $C_9H_{10}OS_2$	 Exact Mass: 308,023755 HRMS Mass: 308,0238	 Name: S-(2-Mercaptoethyl) thiobenzoate	0,14	❖ *Barbarea Vulgaris* ❖ *Hesperis Matronalis*
Thiol-DTDP Molecular Formula $C_{11}H_{15}N_2O_5S_2$ Thiol Molecular Formula $C_5H_{11}NO_3S$	 Exact Mass: 287,052412 HRMS Mass: 287,0524 or  Exact Mass: 287,052412 HRMS Mass: 287,0524	 Name: N-acetyl cysteine methyl ester  Name: N-acetyl-homocysteine	-0,04	❖ *Cardamine Bulbifera* ❖ *Hesperis Matronalis*
Thiol-DTDP Molecular Formula $C_{10}H_{11}N_2S_2$ Thiol Molecular Formula C_5H_7NS	 Exact Mass: 223,036366 HRMS Mass: 223,0364	 Name: 1-methyl-1H-2-pyrrolethiol	0,15	❖ *Cardamine Bulbifera*
Thiol-DTDP Molecular Formula $C_{17}H_{26}NO_9S_2$ Thiol Molecular Formula $C_{12}H_{22}O_9S$	 Exact Mass: 452,104903 HRMS Mass: 452,1049	 Name: 2-[4,5-hydroxy-2-(hydroxymethyl)-6-sulfanyloxan-3-yl]oxy-6-(hydroxymethyl)oxane-3,5-diol	-0,006	❖ *Hesperis Matronalis*
Thiol-DTDP Molecular Formula $C_{11}H_{17}NO_4S_2$ Thiol Molecular Formula $C_6H_{12}O_4S$	 Exact Mass: 290,052078 HRMS Mass: 290,0521	 Name: Thiofucoside	0,08	❖ *Hesperis Matronalis*
Thiol-DTDP Molecular Formula $C_{12}H_{12}NO_2S_2$ Thiol Molecular Formula $C_7H_8O_2S$	 Exact Mass: 266,030948 HRMS Mass: 266,0309	 Name: 4-Mercapto-2-methoxy-phenol	-0,18	❖ *Hesperis Matronalis*

Table 2: Identified Compounds from High Resolution Mass Spectrometry (HRMS) analysis.

Conclusions

This study represents a preliminary analysis and the standards of the identified compounds need to be purchased or synthesized in order to confirm their presence in the related samples. Glutathione and Cysteine were detected and confirmed through the comparison with the analysis of these standard evaluated during the method development step.

Most of the unknown thiols could derive from glucosinolate degradation or from internal rearrangement of biological thiols, but novel molecules from not yet described pathways could also be found. In facts, plant secondary metabolism is a huge source of molecular diversity which is still largely unexplored.

More studies must to be led in order to confirm these molecules. Nevertheless, it was proven how this method can be a useful tool for future study, which aim to explore the sulphur metabolism and to define new molecules in order to evaluate their role in plants as well as in several kinds of matrices (such as wine and food products).

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CONCLUSIONS

Two methods have been developed in this work for the analysis of thiol-containing compounds in plant species. Samples derivatization with DTDP label was followed by the analysis through HPLC-MS/MS, in order to quantify sulfur compounds and to identify new unknown species.

The quantification method focuses on the analysis of seven compound, important thiols and disulfides in plant sulfur metabolism. The analysis were led in SRM mmmode, based on the authentic standards fragmentations. The results show a high sensitivity and selectivity method, with LODs and LOQs very close to literature values. The inclusion of a second internal standard will be considered in order to minimize the errors during the recovery calculation.

The identification method was developed following MS/MS fragmentation of 4-TP-thiols-derivatives, in positive ion mode, and the formation of typical masses (m/z 144, m/z 112, m/z 111) used as markers of the presence of –SH group in thiol-containing molecules.

Following the fragmentation rules, several compounds were identified in plants samples and some of them were confirmed by authentic standards. Other putative compounds are in the process to be confirmed by the purchase or the synthesis of thiol standards.

These methods can be applied in the future for the quantification and identification of thiol compounds in plants and other matrices, such as food products and wine. Furthermore, the identification method can be an useful tool for the definition of new thiols in plants metabolism, in order to explore their role in plant physiology.