Chapter 4
Metallomics Study in Plants Exposed to Arsenic, Mercury, Selenium and Sulphur

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Abstract This chapter is focussing on the interaction of arsenic, mercury and selenium with plans. Aspects of biotransformations are discussed, before the analytical methodologies are listed and critically appraised in the second part. A holistic view is given, starting from the soil environment and continuing to the plant roots and the translocations into the upper part of the plants. Under different soil conditions, different kinds of elemental species are identified, which have an impact on how the elemental species are taken up by the plant. The uptake mechanisms of these elemental species are explained and compared before the biotransformation reactions of all elemental species in the plant root; their transport into the vacuoles and translocation to the leaves and grains are discussed. Here in particular the interaction with sulphur-rich phytochelatins is described for all three elemental species. Since the sulphur chemistry is so important for the uptake, bioaccumulation and translocation of the metals and metalloids, a subchapter about sulphur chemistry in plants has been added. All aspects of biotransformation dealt with in this chapter is finally rounded up by a thorough description of the analytical methodology given with a focus on the use of HPLC-ICPMS/ESI-MS for both quantitative and molecular analysis.

Keywords Arsenic · Phytochelatins · Mercury · Selenium · Sulphur · ICP-MS · ESI-MS · Hyphenated techniques · HPLC-ICPMS/ESI-MS

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Abbreviations

As(III)	Arsenite
As(V)	Arsenate
DMA(V)	Dimethylarsinic acid
ESI-MS	Electrospray ionisation mass spectrometry
GSH	Glutathione
HPLC	High performance liquid chromatography
ICPMS	Inductively coupled plasma mass spectrometry
MeHg	Methylmercury
MMA(III)	Monomethylarsonious acid
MMA(V)	Monomethylarsonic acid
PC	Phytochelatins
Se(IV)	Selenite
Se(VI)	Selenate
Se^o	Elemental selenium
TETRA	Tetramethylarsonium
TMA	Trimethylarsine
TMAO	Trimethylarsine oxide

4.1 Introduction

Metallomics, the molecular forms of metals and metalloids in plants, can be viewed from different angles with different degrees of complexity. In this chapter, we have focussed on the exposure to mainly toxic elements such as arsenic and mercury, whilst selenium and sulphur were covered as well. Selenium is mainly considered toxic, but there is evidence that selenium might be essential to plants in a similar way as in animals and humans. There are a lot of similarities on how plants react to direct exposure to these elements so that common schemes can be developed.

In this book chapter, we do not cover other toxic elements such as cadmium or essential elements such as zinc or copper. Furthermore, we exclude the formation of essential metalloproteins, since other analytical methods and platforms are necessary for the analysis of those elements, which goes beyond the merit of this chapter.

The focus of our chapter is on arsenic, mercury, selenium and sulphur; thus we cover the most studied elements with regard to speciation analysis in plants. We will describe the speciation in which these elements occur in soil and soil porewater and the form in which they are taken up by the roots. The process of accumulation in vacuoles including long-range transport (roots/shot translocation) will be covered by identifying their molecular forms including their biotransformations. Here we have added this subchapter on sulphur since this element seems important for biotransformation of thiophilic elements arsenic, mercury and selenium.

4.2 Biotransformation of Arsenic

Arsenic is a metalloid with ubiquitous occurrence in our environment – of either natural or anthropogenic origin. Thomas and Troncy (2009), referring to the ambivalent character associated with arsenic, called it "a beneficial therapeutic poison" in their review about arsenical-based medicine, recent and past. Its "fame" as poison, however, is undisputable. Marie Lafarge (Griffin 2015) and Madelaine Smith (MacGowan 2007; Scotsman 2005) were probably not the only women who rid themselves of their husband or lover using arsenic. With such a reputation, it is not surprising that arsenic even made it on stage. In "Arsenic and Old Lace" (Kesselring 1939), two elderly ladies take it in their skilled hands to remedy old bachelors from their suffering, with home-made organic elderberry wine doing the trick. Not all arsenic-related incidents were as intentional, to just name the Bradford sweets poisoning in 1858 (Jones 2000) as one. In recent days, the widespread arsenic poisoning in Southeast Asia has been of great concern, with arsenic-contaminated drinking water being one of the culprits (McCarty et al. 2011). The considerable daily consumption of rice poses another significant route of arsenic uptake (Meharg and Rahman 2003; Ohno et al. 2007), which on a second thought raises the question of how plants defend themselves against arsenic exposure.

4.2.1 Arsenic Species in Bulk Soil and Rhizosphere

The arsenic uptake by terrestrial plants mainly takes place via the roots. The speciation and bioavailability of arsenic in any given soil depend among other things on the soil type, pH, redox potential and the content of metal oxides/hydroxides (e.g. Fe, Al). The most prominent arsenic species found in the soil environment are the two inorganic forms As(III) and As(V). Under aerobic conditions, arsenate is the major arsenic species, whereas under anaerobic conditions (e.g. flooded rice paddy fields), arsenite prevails (Zhao et al. 2008; Masscheleyn et al. 1991). Both forms are readily adsorbed by metal oxides such as Fe oxides/hydroxides, an often-found soil constituent, as was demonstrated by a study undertaken by Pierce and Moore (Pierce and Moore 1982). In general, arsenate showed a stronger sorption behaviour than arsenite. At a pH of 4, the adsorption of arsenate to Fe oxides/hydroxides was the highest and decreased with increasing pH. The adsorption of arsenite to Fe oxides/hydroxides was most efficient at a pH of 7. Having said that, Pierce and Moore also revealed that the sorption behaviour of the two inorganic arsenic species is more complex than that. The findings of the study showed that the adsorption behaviour of both species is influenced by their concentration: the inorganic arsenic – adsorbent ratio as well as the presence of ions competing for the sorption sites on Fe oxides/hydroxides, such as sulphate.

Adsorption of inorganic arsenic to metal oxides/hydroxides puts plants out of harm's way – a false sense of safety. Adsorbed arsenate no longer being bioavailable can be mobilised again by reduction to arsenite due to either reducing condi-

tions or microbial activities. Reductive dissolution of Fe oxides/hydroxides is another process that increases the bioavailability of inorganic arsenic in soil (Masscheleyn et al. 1991).

Apart from inorganic arsenic, also organic species are found in soils. Here, monomethylarsonic acid (MMA(V)) and dimethylarsinic acid (DMA(V)) are to be named as the two prominent methylated arsenic species found in arsenic-contaminated soils. If not a result of arsenical treatment of the soil (e.g. biocides), MMA(V) and DMA(V) are formed from arsenite by microorganisms (Abedin et al. 2002). These two organo-arsenic species, as the inorganic ones, can be further metabolised to volatile (methylated) arsines, removing arsenic from the soil (Cheng and Focht 1979). Mestrot et al. (2013) have reviewed biovolatilisation of arsenic from soils and sediments, concluding the amount of volatile arsenic species being very low in comparison to the total arsenic content of the bulk soil.

Rhizosphere is the soil in the immediate proximity of plant roots and should be regarded as an independent soil entity. The rhizosphere serves among other things as nutrient pool for plants. Root exudates, such as organic acids, are means used by plants to increase the bioavailability of nutrients by changing pH and redox potential of the rhizosphere. These two soil parameters affect the adsorption and desorption characteristics and thereby the bioavailability, of not only nutrients but also contaminants such as arsenic. In their pursuit to optimise their nutrient uptake, plants often team up with fungi and microorganisms, increasing the complexity of arsenic speciation and bioavailability even further. Mechanistic studies regarding a. arsenic speciation in soil and/or plants and b. arsenic uptake and translocation by plants require a well-designed experimental set-up considering above-mentioned information, to ensure that no false conclusions are drawn. A study ran by Lomax and co-workers (Lomax et al. 2012) demonstrated that when looking at methylated arsenic species in plants, axenic exposure media should be chosen to rule out any microorganism- or fungi-derived contamination of the growth media with methylated arsenic species, such as MMA(V) or DMA(V). This work and the findings by Zhu and co-workers (Jia et al. 2012) indicate that plants are not able of arsenic methylation, thereby refuting previously made assumptions (Raab et al. 2005).

Methodical investigations regarding the effect of selected root exudates and microbial siderophores on the dissolution of arsenic from As-goethite were conducted by Liu et al. (2017). Dissolution testing in the presence of selected root exudates and/or siderophores demonstrated their beneficial effect on the solubilisation of inorganic arsenic. Analogous experiments with the arsenic hyperaccumulator *P. vittata* indicated continuous dissolution of As from goethite due to its depletion from the solution by *P. vittata.* The investigated ligands also affected the As uptake by *P. vittata* as well as its translocation in the plant with differing extent.

Gonzaga and co-workers (Gonzaga et al. 2006) studied the influence of the As hyperaccumulator *P. vittata* and the non-accumulator *N. exaltata* on bulk soil and rhizosphere parameters as well as on arsenic soil distribution. After 8 weeks of growth on As-contaminated soil in a greenhouse, the water-soluble arsenic con-

centration, pH and dissolved organic carbon (a measure for root exudates) were determined in bulk soil and rhizosphere and compared to soil with no plants growing. For the bulk soil, no changes were observed. In the rhizosphere, however, plant species-dependent changes were monitored. Noticeable was also that both plant species depleted the most abundant arsenic pool, the amorphous Al and Fe hydrous-oxide bound, rather than the most available one, an observation that could be explained by the release of root exudates.

Having discussed the increased bioavailability of inorganic arsenic by means of root exudates, there has got to be an example demonstrating the opposite. Liu et al. (2004) suggest that the release of O_2 by the roots of rice plant can further the formation of an iron plaque in the presence of Fe(II) due to oxidation processes. The formation of an iron plaque on the root surface could facilitate the trapping of arsenate, thereby reducing its bioavailability and uptake by rice plants.

More detailed information on the subject of "arsenic species in bulk soil and rhizosphere" can be found in review articles, for example, by Zhao et al. (2008) and Fitz and Wenzel (2002).

4.2.2 Arsenic Speciation in Terrestrial Plants

It might not come as a surprise that different plant species have developed different mechanism to cope with soil-derived arsenic exposure – ranging from low arsenic uptake to hyperaccumulation (Zhao et al. 2010a). Inorganic arsenic, when taken up as As(V), is readily reduced, enzymatically and non-enzymatically, to As(III) inside the plant. Both forms are toxic, by different modes of action though, as explained in more detail in a review paper by Finnegan and Chen (2012). As(III) as the major arsenic species is generally found in plant roots (Xu et al. 2017 b) after exposure to inorganic arsenic is dealt with in various ways by plants. One option is its efflux from the roots by, for example, aquaporin channels that also facilitate arsenite uptake. Zhao et al. (2010b) investigated the efflux capacity of rice aquaporin Lsi1, concluding bidirectional permeability of this passive, but major, arsenite transport route. Their findings also indicated that arsenite efflux will depend on the arsenite concentration in the external medium – the higher the concentration gradient the higher the efflux rate. Also, the existence of further efflux mechanisms was brought forward by the researchers. The review by Chen et al. gives a more comprehensive account on arsenite efflux mechanisms in plants, also covering the findings from transgenic plants (Chen et al. 2017). The work by Liu et al. (2010) addressed the impact of phytochelatins on arsenite efflux and root-to-shoot translocation in *Arabidopsis* using wild-type as well as GSH-deficient *cad2-1* and PC-deficient *cad3-1* mutants. The experiments meddling with the GSH and PC synthesis showed that the arsenite efflux is more pronounced in *Arabidopsis* plant with insufficient GSH/PC supply, suggesting the trapping of arsenite by complexation with GSH and PCs.

Glutathione (GSH) and phytochelatins (PCs) are often discussed cysteine-rich peptides, contributing to arsenic detoxification in plants, non-tolerant, tolerant and hyperaccumulating ones. Their contribution to arsenic detoxification, however, probably differs depending on the plant species, as the varying contribution of As(III)-PC complexes to the total arsenic content suggests (Raab et al. 2004, 2005). The complexation of arsenite by GSH and/or PCs and their subsequent sequestering from the cytosol into vacuoles, whether in roots or shoots, are another arsenic detoxification pathway found in plants. Song and co-workers identified the two vascular transporters AtABCC1 and AtABCC2 in *Arabidopsis thaliana*, supporting the hypothesis of As(III)-PC vacuole sequestering (Song et al. 2010). The complexation of As(III) by PCs does not only impact on the arsenite efflux, but it also reduces its mobility in terms of root-to-shoot translocation- an aspect of particular interest for crop plants such as rice, where the above-ground tissues (e.g. rice grains) are of vital importance for the nutrition of millions of people. Batista et al. concluded from their study on six rice cultivars that the complexation of arsenite by phytochelatins suppresses the arsenite root-to-shoot translocation (Batista et al. 2014).

The number of possible phytochelatins is vast. The core of each phytochelatin is the repetitive y-glutamylcysteine unit whereas the terminal amino acid can vary, depending on the plant and metal(loid) species. Apart from the most frequently discussed PCs ($[y\text{-Glu-Cys}]_n$-Gly), iso-PCs ($[y\text{-Glu-Cys}]_n$-Glu; ($[y\text{-Glu-Cys}]_n$-Gln), desglycine-PCs ($[y\text{-Glu-Cys}]_n$) and hydroxymethyl-PCs ($[y\text{-Glu-Cys}]_n$-Ser) have been associated with arsenic exposure in higher plants (Batista et al. 2014; Mishra et al. 2017). Arsenite requires the formation of three As-S bonds to either PCs or glutathione to ensure that its effectiveness to interact with enzymes and proteins is diminished. With plants having GSH, PCs, iso-PCs, desglycine-PCs and hydroxymethyl-PCs at their "hand", the number of conceivable As(III)-"PC" complexes is considerable. Raab et al. (2005) detected not only As(III)-PC complexes in root and above-ground tissue of *Helianthus annuus* but also a mixed complex with arsenite being chelated by GSH and PC_2. They also detected for the first time the coordination of MMA(III) with phytochelatins – namely, the MMA(III)-PC_2 complex. Mishra et al. (2017) studied arsenic speciation in *Oryza sativa L.* after exposure to As(V), DMA(V) and MMA(V). Their findings confirmed the capability of (some) plants to reduce MMA(V) to MMA(III) and subsequent complexation by thiols (including, e.g. GSH, PCs, hydroxymethyl-PCs) of the latter. DMA(V) taken up by the rice plants was mainly detected as such in the plant tissue extracts, with one unidentified compound being the exception. Could it have been dimethylthioarsinoyl glutathione (DMA(V)S-GS), which was detected in *Brassica oleracea* by Raab et al. (2007a).

Despite the arsenic species found in roots and above-ground tissues being quite diverse, the two shuttles, xylem and phloem exudates, are only entered by As(V), As(III), MMA (V) and DMA(V) (Ye et al. 2010). Even though free oxidised glutathione and phytochelatins were found, they did not detect any arsenic-thiol com-

plexes in phloem and xylem exudates of castor beans. The authors discuss that the relative fractions of As(III) and As(V) found in xylem sap are most likely determined by several factors such as:

- As(V)-to-As(III) reduction capacity in roots
- As(III) vs As(V) xylem-loading transporters
- Availability of As(III) in roots, determined by thiol complexation in roots
- Competing ions

MMA(V) and DMA(V) are two arsenic species produced by soil microorganisms. They are taken up more slowly than inorganic arsenic, the efficiency with which they are transported via xylem and phloem, however, is higher (Raab et al. 2007b).

Arsenic hyperaccumulating plants translocate most of the arsenic entering the roots into above-ground tissues. Thiol complexation of arsenic can be considered as secondary in hyperaccumulators, as the low fraction (1%) of As(III)-PCs detected in *Pteris cretica* suggests (Raab et al. 2004). Lombi et al. (2002) looked into the arsenic distribution in the fronds of another arsenic hyperaccumulator, *Pteris vittata*. The majority (75%) of inorganic arsenic found in the fronds was As(III), with the remaining being As(V). A large fraction (96%) of total arsenic in the fronds was located in the pinnae. There, most of the inorganic arsenic was detected in upper and lower epidermal cells. X-ray microanalysis results suggest that the arsenic in these cells is compartmentalised in the vacuole. It has been suggested that the ACR3 found in duplicate in the vacuolar membrane of *Pteris vittata* is the key to arsenite translocation from cytosol into the vacuole in hyperaccumulating plants (Indriolo et al. 2010).

In summary, inorganic arsenic is as ubiquitous in plants as it is in our environment. Thiol complexation occurs in roots and above-ground tissues, but not in xylem and phloem. The two methylated arsenic species MMA(V) and DMA(V) are less abundant in plants but were detected in all tissues, including xylem and phloem. MMA(V) and DMA(V) are not produced by plants. They are taken up from the soil environment. MMA(V) can be reduced to MMA(III) which in turn is available for thiol complexation. After thiol activation DMA(V) can also bind to thiols. Diversity, abundance and distribution of the various arsenic species are determined among other things by plant species, soil parameters, other nutrients, available arsenic species, and its concentration, in soil environment as well as exposure time.

Arsenic species found in plants also comprise less spoken of species such as trimethylarsine oxide (TMAO), tetramethylarsonium ion (TETRA), arsenobetaine and arsenocholine (Geiszinger et al. 2002). Investigation on arsenic volatilisation from rice plants revealed that when TMAO was taken up by rice plants, it was reduced to the volatile species trimethylarsine (TMA) (Jia et al. 2012). The researchers also discovered that neither inorganic arsenic nor MMA or DMA was further methylated to TMA. Due to their absence in the scientific limelight, up to now little is known about their compartmentalisation and fate, once they are taken up by terrestrial plants. These compounds are, however, not the result of plants metabolising other arsenicals but originate from the soil surrounding the root system, where they are most likely produced and released by microorganisms.

4.3 Biotransformations of Mercury

Mercury (Hg) occurs in a variety of stable chemical species, with the main species being elemental mercury Hg^o, inorganic salts (e.g $HgCl_2$) and organic Hg compounds in which Hg can be bound to one or two carbon chains. Organic mercury is mainly present as methylmercury ($MeHg^+$, here defined as HgMe) and is well known to bioaccumulate through the aquatic food chain. In the environment, MeHg can be bound to a variety of anions, e.g. with chloride anions, whilst Hg^{2+} can bind with a variety of anions, often as well with Cl anions to form a stable $HgCl_4^{2-}$ complex in water. Mercury is also thiophile and forms strong complexes and/or chemical bonds with sulphur or selenium in the environment and in biota. The bioaccumulation of MeHg in biota is driven by binding through sulphur-containing proteins, as the cysteine moiety of amino acids was shown to bind with MeHg, e.g. in protein-rich rice seeds (Clarkson and Magos 2006).

Elemental mercury is highly volatile; Hg^0 volatilises in ambient temperatures; however $HgCl_2$ is similarly volatile and can be found in the air. Coal-fired power plants release several tonnes of Hg per year, and Hg does not remain in the ash but forms volatile Hg^0, $HgCl_2$ and other reactive Hg species (Pavageau et al. 2002).

Mercury species in general undergo a most complicated cycle in the environment: volatile Hg, e.g. from coal-fired power plants, is oxidised by the sun or other radicals and is removed from the atmosphere with rain as wet deposit but can also be deposited dry; as Hg and its species are volatile or can be volatilised, the Hg species can deposit into the seas or lands; inorganic Hg is prone to methylation in water and sediment, forming MeHg which bioaccumulates in the food chain. Crucially, airborne Hg is very mobile and undergoes a depositing-volatilising cycle, in which Hg is "distilled" to the poles, evidenced by Hg analysis of polar ice sheets and long-lived animals, e.g. polar bears or whales (Swain 2007).

In general, any Hg compound is toxic, with MeHg the most critical Hg compound as it is the most toxic, and known to accumulate. MeHg can also pass through the blood-brain barrier and can damage brain functions in the foetus. This has been evidenced tragically when the Minamata bay was contaminated with MeHg, taken up into the staple food, fish, and caused death or severe brain damage for hundreds of people. However, biota have developed detoxification strategies, e.g. the formation of HgSe nanoparticles or binding in the form of phytochelatin or other sulphur or selenium compounds. For example, HgSe nanoparticles were evidenced in the liver and brain of a pod of long-finned pilot whales, which suggests that MeHg is transported to the brain where it is detoxified through the formation of biochemical inert HgSe (Gajdosechova et al. 2016).

4.3.1 Hg in Soil and Biotransformation

Hg in soil mainly occurs in inorganic form of Hg^{2+} and can bind to anions, form stable complexes or form salts. In anaerobic conditions, Hg^{2+} can be methylated, and it is thought that the methylation process is microorganism induced, probably in

an attempt to detoxify the inorganic mercury which is more toxic for the plant than MeHg (Xu et al. 2016). Rice cultivation practices are important. In wet conditions, MeHg is formed and can be taken up throughout, whilst it was found that alternative dry-wet conditions can mitigate MeHg formation however spikes upon re-establishing wet conditions (Rothenberg et al. 2014). Recently, it was suggested that S in soils may control Hg speciation in rice (Xu et al. 2017a, b).

4.3.2 Hg Species Accumulation in Rice

Rice is a staple food for most Asian countries, which makes it the most important food commodity worldwide. This underpins the importance of mercury analysis in rice and the research on uptake and accumulation processes of inorganic and methylmercury.

In a pot experiment using flooded conditions, rice plants were challenged with either inorganic Hg^{2+} or $MeHg^{+}$, which evidenced a stark difference in terms of uptake into different plant parts of the rice plant and showed a selective accumulation for MeHg into the grain, whilst Hg^{2+} was rather accumulated in roots. It was shown that rice plants challenged with Hg^{2+} was efficiently bound with a variety of phytochelatins, using two cysteine functional groups to bind Hg in the oxidised form, Hg^{2+}, which is deposited in the roots. In contrast, $MeHg^{+}$ does not form any complexes with phytochelatins but is transported and deposited mainly in the rice grains (Krupp et al. 2009; Krupp et al. 2008). This has important implications for food security in rice.

In this exposure experiment, it was evidenced that Hg^{2+} is bound to phytochelatins in the root but that MeHg was transported into the grains, probably as self-defence. This clearly demonstrates that the knowledge of molecular binding and speciation is of utmost importance to understand detoxification pathways.

MeHg in rice grain is accumulated to the highest concentrations and is uniformly distributed; some Hg2+ is also accumulated in bran and husk, similar to the As accumulation in bran and husk, but very different from grain where As is mainly found in husk and bran as discussed in Chap. 2 in this book. Whilst it is easy to remove As by husking and bran removal, MeHg cannot be removed as it is bound to proteins in the grain.

The way how the uptake and accumulation of Hg in roots and MeHg in rice grains works is shown in Fig. 4.1.

The picture is further complicated by the fact that Hg is taken up from the atmosphere: in a stable isotope experiment, it was evidenced that airborne Hg is taken up through aerial parts of the rice plant, showing that Hg^{2+} in rice grains is almost entirely from atmospheric Hg sources and is taken up through the leaves (Strickman and Mitchell 2017). This adds to the Hg burden in the rice plant, especially where coal-fired plants are exhaling mainly Hg^{0}, e.g. in the Wuhan District in China. Recently, Nature reported the uptake of atmospheric elemental Hg into vegetation in the tundra is approximately 70%, explaining high burdens of Hg in soil and water run-off in this part of the world (Obrist et al. 2017).

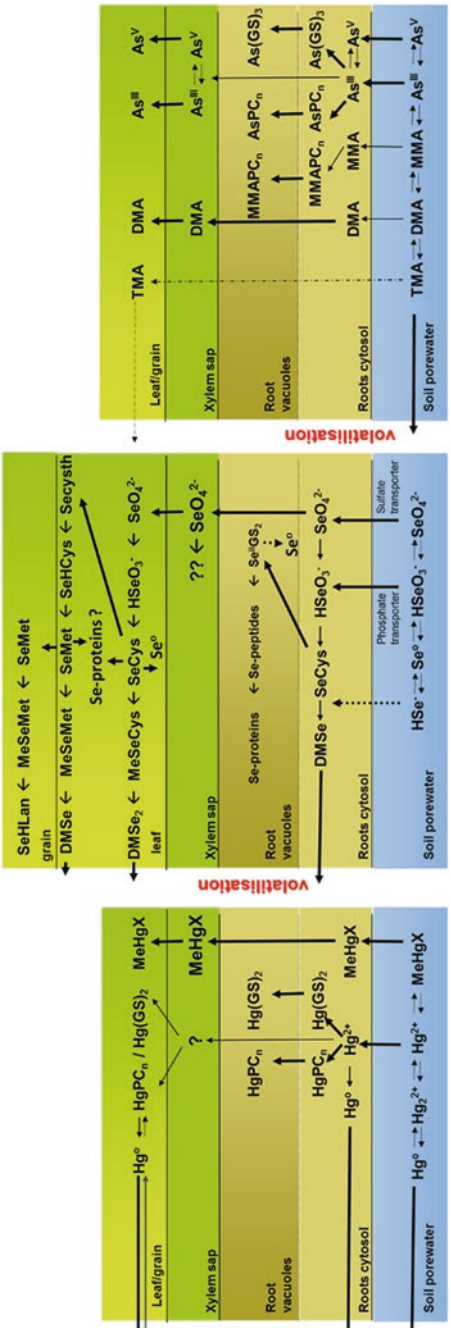


Fig. 4.1 Uptake, accumulation in roots, translocation into shoots and seeds for mercury, selenium and arsenic

4.3.3 Hg -Accumulating Plants in Terrestrial Plants and Phytoremediation

A variety of terrestrial plants are able to grow on highly Hg-contaminated soil; e.g. *Arabidopsis*, *Brassica*, *Datura*, *Marrubium* and *Sesbania*. For example, *Sesbania drummondii* was shown to accumulate up to 1000 mg/kg of Hg in their shoots but 40 times more in its roots. The search for hyperaccumulator plants for Hg is driven by the interest to reclaim contaminated lands. *S. drummondii* is a potential Hg bioaccumulator; however there are no confirmed hyperaccumulator plants for Hg (Venkatachalam et al. 2009).

There are attempts to detoxify Hg in plants using engineered transgenic plants which are bacterial genes *merA* and *merB* which are known to volatilize Hg^0, with the argument that Hg0 is less toxic than inorganic Hg or MeHg (Meagher 2000). Whilst this strategy may help to decontaminate a certain area, the volatile Hg^0 will be far more mobile and only dilutes the contamination, whilst Hg contamination in the atmosphere rises.

4.4 Selenium Biotransformations

Selenium is an important micronutrient and an essential element for animals including humans. It has been established almost 60 years ago (Schwarz and Foltz 1957) that the selenium is an integral part of glutathione peroxidase, an enzyme which is important to deter oxidative stress. However, so far selenium has not been established to be essential for plants. Although there are plants which hyperaccumulate selenium, as far as the authors know, there is neither a specific transporter known for the uptake of selenium and their species into the plant, nor a selenium-specific transporter known for the translocation of selenium inside the plant. Selenium is a very redox-active element, and it exist in many different oxidation stages from –II (as Se^{2-}), 0 (as Se^o), +IV ($HSeO_3^-$) and + VI (SeO_4^{2-}). In addition +II exists when selenium binds to sulphur ($SeSO_3^{2-}$). But it forms also a rather stable Se-C bond; hence we can expect a plethora of organoselenium species in environmental and biological samples (Wallschlaeger and Feldmann 2010).

4.4.1 Selenium Speciation in Soil and Soil Porewater

As can be seen from the Pourbaix diagram (Fig. 4.2), selenium can be thermodynamically stable as selenide (HSe^-) in water-logged soils which display strongly reducing conditions such as paddy soils. Under slightly reducing conditions, also elemental selenium is the most stable species, whilst selenite and selenate are the most common selenium species in aerobic soils. In acid soils selenite as $HSeO_3^-$ is the most prevalent form, whilst in alkaline soils selenate is much more stable.

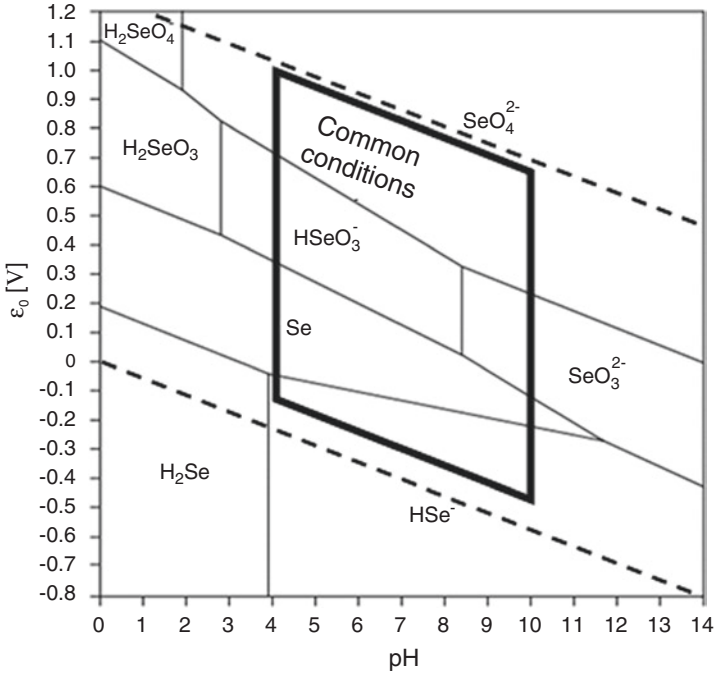


Fig. 4.2 Pourbaix diagram of selenium, which illustrates the thermodynamic stable species in soil porewaters in dependence of the redox potential and the pH

4.4.2 Selenium in Terrestrial Plants

Since selenium has not shown so far that it is an essential micronutrients for plants, no specific transporter of selenite or selenate has been identified. However, selenite is known to be taken up by phosphate transporters. In rice it has been shown using a rice mutant overexpressing OsPT2 to have enhanced selenite uptake (Zhang et al. 2014), and overexpression in OsPT8 in tobacco enhances also the selenite uptake as recently been reported (Song et al. 2017). Even the marine coccolithophore *E. huxleyi* uses a phosphate transporter, and selenite uptake is not influenced by selenate, sulphate or sulphite (Araie et al. 2011). Selenate on the other hand is taken up by the efficient sulphate transporter Sultr1;2 as established for *Arabidopsis thaliana* (Shibagaki et al. 2002).

Once in the roots, the different selenium species have also different long-range transport in the plants. Selenite is not well transported in the xylem to the shoots whilst selenate is efficiently transported (Li et al. 2008). However, inside the roots already biotransformation of the selenium species may take place. Selenate is reduced to selenite and further to elemental selenium in the root cells.

Elemental selenium can make up almost 20% in plant cells (e.g. *Thunbergia alata)* (Aborode et al. 2015). This reduction is not very efficient; hence there is the tendency that selenate is transported into the shoots whilst selenite is biotransformed into organoselenium species. This transformation encompasses a further reduction to selenide, which however is too reactive and consequently forms other organoselenium compounds and has therefore not been identified in plants.

Several organoselenium compounds can be identified in the roots, for example, selenium diglutathione ($Se(GS)_2$) and selenium phytochelatin2 ($SePC_2$), but additional selenium could replace sulphur in cysteine and form Se-containing glutathione (Aborode et al. 2016; Bluemlein et al. 2009b). This compound has however never been isolated on its own but in its oxidised form, when bound via the Se-S bond to another peptide chain. This finding illustrates that Se potentially be incorporated as selenocysteine (SeCys) or as selenomethionine (SeMet) in plant proteins. This however has not been intensively researched in contrast to selenium speciation in yeast (Bierla et al. 2012). What we know is that selenate (SeO_4^{2-}) gets reduced inside the roots to selenite ($HSeO_3^-$); this can be transferred via $Se(GS)_2$ and very reactive selenide (HSe^-) to form SeCys. SeCys can also be methylated to MeSeCys and to volatile dimethyldiselenide ($DMSe_2$) (Pilon-Smits et al. 1999). SeCys could however transfer to selenohomocysteine (SeHCys) which could convert to SeMet and eventually also to the volatile dimethylselenide (DMSe). This volatilisation is considered as a detoxification. Whilst selenium excluder usually generates only DMSe, hyperaccumulator tend to generate $DMSe_2$, which is more efficient to excrete selenium.

One aspect of selenium biotransformation and translocation is to introduce bioavailable forms of selenium into the edible parts of the plants. For example, a recent study showed the influence of nitrogen and sulphur fertilisers on the speciation of selenium compounds in wheat grains (Duncan et al. 2017). In the grains, up to 70 mg/kg selenium could be accumulated, and besides the expected SeMet and Se-methylselenocysteine (MeSeCys), selenohomolanthionine (SeHLan) was characterised to occur in the grain. Usually, in plants with lower selenium concentration, the main selenium species in the grain or seed is SeMet.

The transformation of SeCys into selenoproteins in plants is still controversially discussed, although it has been shown that in the algae *Chlamydomonas reinhardtii* glutathione peroxidase has been characterised (Fu et al. 2002). One obstacle is the reactivity of the selenium in the selenoproteins, and their small concentrations make it difficult to establish novel proteins. Using multidimensional HPLC coupled to ESI-qTOF and ICPMS established a selenium-containing peptide in which SeMet replaces methionine in soybean (Chan and Caruso 2012). There have been reports that glutathione peroxidase exists in *Aloe vera* (Sabeh et al. 1993), but later reports have concluded that this GPx protein does not contain SeCys. Hence, so far no specific selenoprotein in which selenium is incorporated specifically as SeCys has been established for terrestrial plants, and that is why the essentiality of selenium in plants is still controversially discussed.

4.5 Sulphur Biotransformation

Sulphur is important for plants as an essential, often growth-limiting, macronutrient and has indirect effects on the use efficiency of other plant nutrients. Plants are, contrary to animals and humans, capable of incorporating sulphur in organic compounds like the amino acids cysteine and methionine, as well as in essential vitamins and cofactors (Lewandowska and Sirko 2008; Blum et al. 2013).

In *Brassicaceae* (e.g. cabbage, broccoli, mustard) and *Allium* (e.g. onion, garlic, leek) species, sulphur compounds are additionally relevant as secondary metabolites. Glucosinolates and alliins are both enzymatically hydrolysed after tissue rupture to form a wide array of mostly volatile products. These are not only responsible for giving these vegetables their flavour but are also commonly associated with health benefits like reducing the risk of cancer (Hirai and Saito 2008; Kopriva et al. 2015).

The occurrence of sulphur in the environment ranges from the oxidation states −II in sulphides to +VI in sulphates. The biogeochemical cycle of sulphur as described by Takahashi et al. (2011) shows the free mobility between lithosphere, hydrosphere and atmosphere. Inputs of sulphur through atmospheric deposition that are less than the requirements of the crops lead to a sulphur deficiency in soils. The main causes of this deficit are the reduction of anthropogenic SO_2 emissions, the use of highly concentrated fertilisers with low sulphur content and the increased sulphur removal due to high-yielding crops (Scherer 2009; Solomon et al. 2009; Blum et al. 2013).

4.5.1 Sulphur Species in Soil

Of the total sulphur content in soil that ranges between 0.01% and 0.1%, only less than 5% is usually present as inorganic S, whereas organic sulphur compounds make up more than 95% (Balík et al. 2009; Solomon et al. 2009).

Sulphate constitutes the most important S source for the direct uptake by plants. Therefore, the process of mineralisation is pivotal to make organic S plant-available. Mineralisation can take place biologically as the breakdown of compounds with C-bonded S by microorganisms or biochemically as the hydrolysis of sulphate esters by sulphatase enzymes in the soil (Assefa et al. 2014).

The organic S compounds can be divided in the already mentioned two main groups of ester sulphates (C-O-S) and C-bonded S (C-S) (Scherer 2009). The traditionally used method for the soil sulphur speciation analysis is a wet-chemical sequential extraction. Prietzel et al. (2003) listed the commonly used extractions to determine sub-fractions of inorganic as well as organic S. Since not all important fractions are covered by these, indirect quantification by calculation from other fractions is necessary. Furthermore, the fractions are operationally defined and do not quantify distinct S species (Prietzel et al. 2009).

Table 4.1 Sulphur speciation in soils with different oxygen availability (Cambisol > Stagnosol > Histosol): (a) reduced inorganic S and reduced organic S, (b) intermediate S and oxidised S (Prietzel et al. 2009)

(a)	Inorganic sulphide S^{2-}	Inorganic sulphide S_2^{2-}	Organic disulphide S	Organic monosulphide/ thiol S
Oxidation state	−2	−1	0.2	0.5
	mg S kg^{-1}			
Cambisol	0 ± 0	695 ± 49	842 ± 60	0 ± 0
Stagnosol	434 ± 15	954 ± 34	607 ± 21	0 ± 0
Histosol	0 ± 0	2347 ± 83	1369 ± 48	0 ± 0

(b)	Sulphoxide	Sulphite	Sulphone	Sulphonate	Ester sulphate	Sulphate
Oxidation state	2	3.7	4	5	6	6
	mg S kg^{-1}					
Cambisol	476 ± 34	439 ± 31	146 ± 10	329 ± 23	73 ± 5	659 ± 47
Stagnosol	520 ± 18	390 ± 14	260 ± 9	564 ± 20	87 ± 6	564 ± 20
Histosol	652 ± 23	652 ± 16	0 ± 0	587 ± 21	196 ± 14	717 ± 29

X-ray absorption near-edge spectroscopy (XANES) can be used for the direct speciation of soil S (Jokic et al. 2003; Prietzel et al. 2003) or the analysis of extracts of the humic substances in soil (Solomon et al. 2003). The energy of the maximal absorption of irradiated X-rays gives information on the oxidation state of S atoms. A quantitative analysis of samples of different soil types in Fichtelgebirge, Germany, shows the variability of S compounds in soil with varying oxygen availability. The results for the upper sections of the organic layer of the soil are depicted in Table 4.1. With decreasing levels of oxygen availability and higher influence of the groundwater in the sequence Cambisol-Stagnosol-Histosol, the contribution of reduced S species to the total S content increases (Prietzel et al. 2009).

4.5.2 Sulphur Metabolism and Speciation in Plants

Uptake of S takes place mainly in the form of sulphate through the roots of the plants. It is distributed throughout plant tissues by dedicated sulphate transporters. Excess sulphate is stored in vacuoles, but the majority is reduced in the shoot tissues (Maathuis 2009; Kopriva et al. 2015).

Reduction of sulphate takes place in a multistep pathway to form sulphide, which is used to form cysteine out of *O*-acetylserine. Cysteine is the starting material for the biosynthesis of all other reduced sulphur-containing metabolites like the amino acid methionine or the redox buffer glutathione. An alternative way of sulphur assimilation is sulphation, the addition of sulphate to a hydroxyl group. Sulphation plays a minor role in higher plants compared to reduction but is important in the biosynthesis of a range of metabolites including the glucosinolates in *Brassicaceae* (Leustek and Saito 1999; Kopriva et al. 2015).

Glucosinolates are secondary sulphur metabolites of importance to humans because of their presence in *Brassicaceae* crops like cabbage, broccoli and mustard. Their molecular structure consists of a thioglucoside group bound to an *N*-hydroximinosulphate ester plus a variable R group derived from different amino acids. Based on the amino acid, the compounds are classified as aliphatic, aromatic or indole glucosinolates. Tissue damage leads to the hydrolysis of glucosinolates by myrosinase to unstable intermediates that rearrange to isothiocyanates. The isothiocyanates are primarily responsible for flavour and taste of these plants (Prestera et al. 1996; Halkier and Gershenzon 2006).

Aires et al. (2006) analysed leaves and roots of broccoli sprouts (*Brassica oleracea*) and the effect of nitrogen and sulphur fertilisation on glucosinolates. The fertilisation showed a detrimental effect on the glucosinolate levels, and broccoli sprouts react sensitively to higher salt concentrations due to the fertilisation. The analysis of leaves and roots showed that aliphatic glucosinolates predominate in aerial parts of the plants, whereas aromatic and indole glucosinolates prevail in the roots. The total glucosinolate concentrations found in leaves and roots ranged from 21.8 to 56.1 μmol/g DW. The seven identified glucosinolates with their proportion of the total glucosinolate content in the broccoli sprouts are shown in Table 4.2.

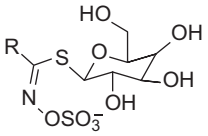
Allium vegetables like garlic and onion are universally consumed because of their flavour but also as traditional medicine. Both flavour and beneficial health effects are attributed to *S*-alk(en)yl-$_L$-cysteine sulphoxides, another group of secondary sulphur metabolites. Their flavour and lachrymatory characteristics are connected with the transformation to thiosulphinates (e.g. allicin) and other volatile organosulphur compounds (e.g. diallylpolysulphides) by the enzyme alliinase when the plant tissue is damaged (Jansen et al. 1989; Ichikawa et al. 2006; Yamazaki et al. 2010).

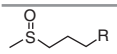
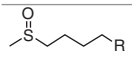
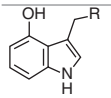
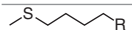
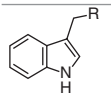
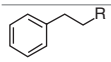
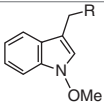
The most common *S*-alk(en)yl-$_L$-cysteine sulphoxides in *Allium* plants are alliin, isoalliin and methiin. The three best known γ-glutamyl peptides occurring in *Alliums* γ-glutamyl-*S*-allyl-$_L$-cysteine (GSAC), γ-glutamyl-*S*-1-propenyl-$_L$-cysteine (GSPC) and γ-glutamyl-*S*-methyl-$_L$-cysteine (GSMC) are thought to be precursors for the corresponding sulphoxides and/or sulphur storage peptides (for structures, see Fig. 4.3) (Ichikawa et al. 2006; Yamazaki et al. 2010; Raab et al. 2017).

The common analysis methods involved the use of species-specific standards for quantification and identification (Ichikawa et al. 2006; Yamazaki et al. 2010). A recently developed method by Raab et al. (2017) uses a parallel ICP-MS/MS and ESI-MS system to gain elemental and molecular information simultaneously. Some of the newly identified sulphur compounds found in *Allium sativum* (garlic) are depicted in Fig. 4.3 together with the structures of the most commonly found sulphur compounds in *Allium* plants. The experiments with a changing sulphur fertilisation under hydroponic growth conditions show that the sulphur content in the bulbs was hardly affected, whereas the sulphur content in the roots was positively influenced (Raab et al. 2017).

The heavy metals arsenic, selenium and mercury are influenced by sulphur regarding the uptake and accumulation in plants. Thiols like glutathione play an important role in the detoxification process, and an increase in S supply improved the As tolerance in *Hydrilla verticillata* by enhancing the thiol metabolism (Srivastava and D'Souza 2009; Srivastava and D'Souza 2010). Reversely arsenic

Table 4.2 Basic structure of glucosinolates and different R groups occurring in aerial part and leaves of broccoli sprouts including the proportion of the total glucosinolate content (Aires et al. 2006)



Structure of R	Trivial name	Aerial part/%	Roots/%
	Glucoiberin	20.4	2.7
	Glucoraphanin	48.2	6.8
	4-Hydroxy-glucobrassicin	4.8	4.9
	Glucoerucin	11.2	25.5
	Glucobrassicin	5.7	5.5
	Gluconasturtiin	8.0	38.5
	Neoglucobrassicin	1.7	16.1

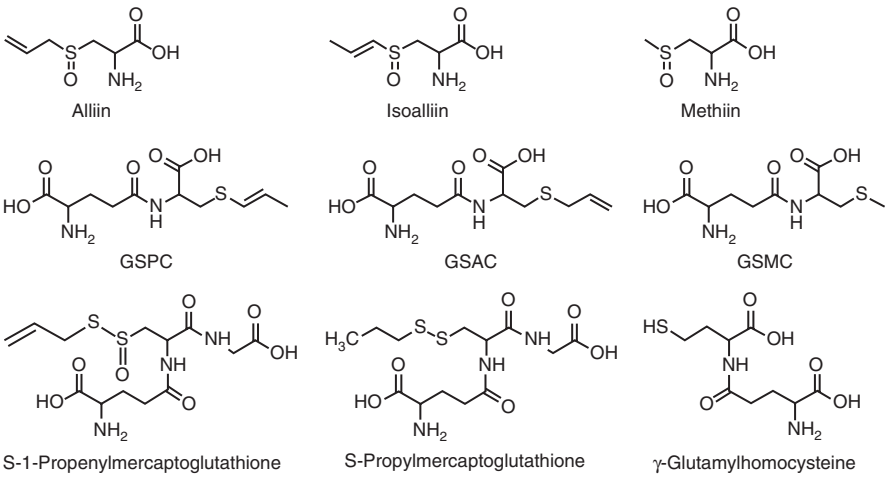


Fig. 4.3 Molecular structures of alliin, isoalliin and methiin, their precursor γ-glutamyl peptides and proposed structures for three newly identified sulphur compounds in garlic (Raab et al. 2017)

also affects the uptake of S. Watanabe et al. (2014) found an enhanced uptake of sulphur in the As hyperaccumulator *Pteris vittata* with As in the growth medium. Selenium reacts in a different way on higher sulphur fertilisation levels. Selenium concentrations in rapeseed (Liu et al. 2017a) as well as wheat (Liu et al. 2014; Yang et al. 2017) decreased significantly through the application of S fertilisers. Liu et al. (2014) explain these findings with changes in the soil resulting in the binding of Se to Fe-Mn oxide and organic matter. The effects of sulphur treatment on the accumulation of mercury in rice show a similar outcome. Both total mercury and methylmercury concentrations in the plants decrease due to the transformation of mercury to the form of RS-Hg-SR. Additionally mercury is inactivated in the soil to HgS and therefore reducing the health risk from these crops (Li et al. 2017).

4.6 Overview of the Analytical Approaches

4.6.1 Introduction

From an analytical perspective, metal(loid)-thiol complexes are species of sharp contrast depending on the metal(loid) being bound. With varying levels of stability in vivo and throughout the analytical procedure, the identification and quantification of such complexes can provide the analyst with a number of issues. This is exemplified by metal(loid)-phytochelatin complexes, formed by plants in the response to cellular influx of metal(loid) species.

The "traditional" complexes of phytochelatins, with metals such as cadmium and lead, are stable at high pH (>7), with little to no stability at low pH (<7) (Rauser 1990; Steffens 1990). Alkaline plant extracts can be stored at room temperature over a period of days with insignificant levels of PC complex degradation (Chassaigne et al. 2001; Johannig and Strasdeit 1998). The inherent stability of such complexes allows for 2D chromatography: size-exclusion chromatography (SEC) as the initial clean up and fraction collection step, followed by reverse phase LC-ESI-MS and LC-ICP-MS analysis as the identification and quantification techniques, respectively (de Knecht et al. 1994; Barałkiewicz et al. 2009).

In contrast, this is not the case for phytochelatin complexes of arsenic, mercury or selenium. Extracts containing such complexes show no stability at high pH (>7) and limited stability at low pH (<7) and must be stored chilled (<5 °C) prior to analysis to preserve the speciation profile (Meharg and Hartley-Whitaker 2002; Raab et al. 2004). However, the combination of low pH and temperature only extends sample stability to 24 h, after which time PC complex speciation is all but lost and only free unbound PC species are observed (Sneller et al. 1999). Sample storage at ambient temperatures leads only to faster sample speciation degradation. Attempts to extend the period of sample stability, such as freeze-drying of samples or extracts or their immediate storage at −78 °C, yield no appreciable extension in their shelf lives (Bluemlein et al. 2009a).

The instability of arsenic and selenium phytochelatin complexes is linked to the stability of their various oxidation states. In the natural environment, which would be primarily oxidising and where pH would be close to 7, the predominant forms of arsenic and selenium are the arsenate ($[AsO_4]^{3-}$, As(V)) and selenite ($[SeO_3]^{2-}$, Se(IV)) anions, respectively (Spuches et al. 2005; Zhu et al. 2008). However, the species bound by phytochelatins are arsenite ($As(OH)_3$, As(III)) and selenium (Se(II)), respectively, both showing better stability under reducing conditions (low pH and anoxic environments) (Aborode et al. 2016). The instability of PC-As and PC-Se complexes is linked to the driving force towards oxidised species: for arsenic, transformation of As(III) → As(V), and for Se, transformation of Se(II) → Se(IV). This can be mitigated against, but not removed completely, through the use of acidic diluents and low temperatures during extraction.

The peculiarity of the Hg-PC complex instability versus the inherent stability of Zn-PC or Cd-PC complexes could be due to the difference in their redox behaviours. Whereas Zn and Cd will displace H^+ from acidic solution, forming the Zn^{2+} and Cd^{2+} ions, respectively, Hg does not unless in the presence of oxidising acids. Furthermore, whilst Zn and Cd are amphoteric and can dissolve at low and high pH, mercury is not soluble at high pH, producing insoluble mercury oxides.

Whilst low pH conditions stabilise PC complexes with arsenic, selenium and mercury, there is competition for the binding sulphur atoms between hydrogen ions and the bound metal(loid). This destabilises the PC complex, leading to its eventual disintegration, and explains their transient stability at low pH.

Arsenic-PC complexes were the first identified to show inherent instability during isolation and extraction (Raab et al. 2004). In contrast to metal(loid)s such as lead and cadmium, which exist as the cations Cd^{2+} and Pb^{2+}, respectively, arsenic exists in the environment predominantly as the oxoanion arsenate, As(V), and smaller quantities of the neutral species arsenite, As(III) (Quaghebeur and Rengel 2005). Complexation requires the in vivo reduction of As(V) to As(III), which is then bound by thiols in the ratio of 1:3 with regard to As/S, with the loss of water as by-products (Scott et al. 1993). However, the driving force in most non-reducing environments is for the oxidation of As(III) to As(V), explaining As-PC complex instability at high pH and in oxygen-rich environments (Mandal and Suzuki 2002).

For arsenic and mercury, the case becomes complicated further, in that there is the potential for methylated analogues to exist within the environment. Monomethylarsenate ($CH_3AsO(OH)_2$, MMA(V)) and dimethylarsenate ($(CH_3)_2AsO(OH)$, DMA(V)) can exist in the environment as a result of natural methylation of arsenate in soils or from anthropogenic sources (e.g. historical herbicide use) (Le et al. 2000; Mandal and Suzuki 2002). Monomethylmercury (CH_3Hg^+) is almost exclusively produced via natural methylation of mercury in the environment but can be produced via anthropogenic actions such as burning of mercury wastes (Krupp et al. 2008). PC complexes can form with methylated arsenic and mercury species; however their stability is drastically reduced versus their inorganic analogues, as the number of M-S bonds decreases (Scott et al.

1993; Delnomdedieu et al. 1993). For arsenic, complex stability can be summarised as follows:

$$\mathrm{As(III)-PC} > \mathrm{MMA(III)-PC} > \mathrm{DMA(III)-PC}$$

4.6.2 General Overview

Rapid sample preparation and analytical protocols are required to deal with the transient stability of PC complexes with arsenic, selenium or mercury. Low pH media are required to stabilise any complexes that may occur but not so low that they are detrimental to either the speciation profile or the analytical instrumentation used.

Current research suggests that the timeframe in which samples must be analysed is 24 h, and this makes the choice of analytical techniques crucial (Raab et al. 2004). The chromatographic method must resolve a number of nonbinding PC species and PC-metal(loid) complexes within a short period of time. Additionally, the detection step(s) must identify and quantify species in the extract simultaneously.

4.6.3 Sample Preparation

Given that the sample preparation step can impact heavily upon the subsequent chromatography, there must be compatibility between both the extraction and analytical steps, whilst maintaining conditions suitable for the isolation and stabilisation of PC-metal(loid) species.

Sample extraction is typically performed with chilled 1% formic acid solution (Bluemlein et al. 2008a; Wood et al. 2011). Plant material is frozen with liquid nitrogen and ground to a fine powder with a mortar and pestle. Extraction time is typically between 40 and 60 min, with intermittent shaking of the sample by hand. The extraction solution is around pH 2, which is tolerable by most LC columns, LC systems and analytical instrumentation. Furthermore, the sample injection volumes used are typically between 10 and 50 μL and are rapidly diluted by the eluent during chromatography, which is of a slightly higher pH. Finally, the use of a low pH extraction solution helps to minimise the uptake of large peptides and proteins into solution, which would typically be achieved via a clean-up step such as SEC, which could precipitate within the analytical system during measurement.

Samples can be filtered prior to analysis; however this may raise the concern of loss of species via adsorption to the filter discs used. Centrifugation is more commonly used, with samples spun at typically 3000–4000 rpm for 5 min. There is

expected to be minimal change to the speciation, given the brief period of time that samples will be at ambient. However, this has not been proven.

4.6.4 Chromatographic Method

The instability of PC-As, PC-Se and PC-Hg complexes precludes the use of any additional chromatography techniques, such as SEC, prior to analysis. The chromatographic method must resolve a variety of free PC species and PC-M complexes in a single run, under conditions that not only preserve the speciation profile of the extract but are compatible with the subsequent analytical instrumentation.

For the chromatographic method, reverse phase analysis using a C_{18} column is typically employed (Raab et al. 2005). A gradient elution system, utilising 0.1% formic acid and methanol as eluents, can resolve a number of free PC species and PC-metal(loid) complexes in around 30–60 min based on a 1 mL/min flow rate, depending on the metal(loid) under investigation. The pH of the eluent system is typically around 4, which is tolerable by the C_{18} column, the LC system and the subsequent detection systems. The eluent system also helps mitigate the higher pH of the extraction solution (50 μL of pH 2 solution) via its rapid dilution once in the LC system.

UV data is generally not gathered nor required during analysis of plant extracts, as neither the poisoning metal(loid), free PC species, nor PC-M complexes are significantly UV active. Detection is garnered on both an elemental and molecular basis.

4.6.5 Analytical Detection

PC-metal(loid) complexes with arsenic, mercury or selenium in plant extracts are typically identified via ESI-MS and quantified on the metal(loid) via ICP-MS. Unlike PC-Cd complexes, which can be analysed sequentially by HPLC-ICP-MS and HPLC-ESI-MS (Barałkiewicz et al. 2009), the inherent instability of As, Se and Hg complexes means that both elemental and molecular data must to be collected simultaneously. The chromatography established by Raab et al., as described in the previous section, permits such analyses (Raab et al. 2004). Using LC-ESI-MS-ICP-MS (hyphenated mass spectrometry), both molecular and elemental information can be collected in a single sample run. The eluents used are tolerable by both systems, and the addition of formic acid helps species identification through enhanced protonation. However, the ICP-MS has to be configured to handle organic eluents. To achieve simultaneous identification and quantification, the

eluent flow is split post-column, diverting portions of the eluent to both MS systems. Given the greater sensitivity of the ICP-MS versus the ESI-MS, eluent flow is typically spilt 2:8, respectively, between the systems.

Inevitably, there are drawbacks. The first is sensitivity. Given that the sample flow is split between two systems, there is a detriment to the signal intensities recorded by both the ESI-MS and ICP-MS, which disproportionately affects the ICP-MS more. Secondly, the use of methanol as an eluent leads to the formation of soot particles in the ICP-MS plasma, which could block the sampler and skimmer cones. This can be negated through the addition of oxygen into the plasma during analysis but does require that the ICP-MS hardware is capable of such a function. Additionally, as the sampler and skimmer cones are traditionally made of nickel which can be corroded when oxygen is added into the plasma, there is a requirement to change them to more expensive and delicate platinum cones for speciation analysis.

4.7 Analytical Strategy in Action

To put the analytical strategy into context, the precise requirements for arsenic, selenium and mercury-PC complex analysis are discussed.

4.7.1 ESI-MS Analysis: General Considerations

The predominant role of ESI-MS is to provide species identification for the array of glutathione, unbound PC and metal(loid)-bound PC species present in the plant extracts under investigation. Independent of the metal(loid)s to which the plant are being exposed, the ESI-MS should be optimised to detect unbound glutathione and PC species. Analysis is conducted in positive mode with species identification predominantly made on the protonated molecular ion $[M + H]^+$, due to the combination of formic acid in the eluent system with the multiple amide group nitrogen atoms present within GSH and PC structures (e.g. Raab et al. 2007c; Batista et al. 2014). Parameters applicable to the aforementioned species would apply equally to PC-metal(loid) complexes.

No direct quantification of species can be realistically made. Since ESI-MS is a relatively gentle ionisation technique and there is a substantial level of unrelated species present in the formic acid extracts, matrix suppression of the target PC and GSH species is expected. As no standards of PCs are readily available, it is not possible to counteract this by means of internal standard addition. Whilst bound PC and GSH can be quantified by ICP-MS on the central metal(loid), unbound species are largely unquantifiable due to the lack of a suitable heteroatom for analysis.

4.7.2 ICP-MS Analysis: General Considerations

The role of ICP-MS is to provide quantification for the various PC-metal(loid) complexes found in the plant extracts. The high temperature plasma efficiently desolvates, atomises and ionises the sample stream passed to it by the LC system (Gray 1989; Hill et al. 2005). Quantification is thus conducted on an elemental basis and can be applied across a range of PC-metal(loid) species provided a suitable elemental standard is used. The sensitivity of the ICP-MS can reveal PC-metal(loid) species that may not be evident initially from the ESI-MS data and is an important confirmatory tool in the analysis of such species.

There are, however, a number of factors which must be assessed and addressed during ICP-MS analysis: the isotope upon which to perform elemental identification and quantification, the possibility of ion suppression during analysis and the effect of methanol on plasma behaviour during analysis.

Phytochelatins and glutathione contain predominantly carbon, hydrogen, oxygen and hydrogen, none of which are readily quantified due to their relative ubiquity. The exception is sulphur; however, quantification is not a simple task. The main isotope is ^{32}S with a natural abundance of 95%, which is indistinguishable from $^{16}O_2$ when a single quadrupole instrument is used (Bluemlein et al. 2008b). Quantification is thus only routinely possible on PC-metal(loid) species with standard ICP-MS hardware, with identification and quantification being performed on the bound metal(loid).

As with ESI-MS analysis, there is the potential for ion suppression due to the sheer number of components that can be extracted during sample preparation. However, ICP-MS has two distinct advantages over ESI-MS that help to circumvent this problem:

1. The argon plasma is a considerably more powerful ionisation source, with a much higher degree of ionisation efficiency over ESI-MS.
2. Analysis is conducted on an elemental basis and is species-independent.

With ICP-MS, there should be less ion suppression effects due to the ferocity of the argon plasma, which operates with temperatures of up to 10,000 K. Where suppression becomes an issue, the application of an appropriate internal standard can correct for this. Typically, the internal standard is an element that is unlikely to be found in the sample matrix but has similar ionisation properties inside the plasma to that of the metal(loid) under investigation and is added post-column, as a separate continual stream throughout the duration of analysis. For arsenic, selenium and mercury, a common internal standard is rhodium with measurement on the only stable isotope, ^{103}Rh (Krupp et al. 2008; Bluemlein et al. 2009b).

Chromatographic separation of the PC complexes of arsenic, selenium and mercury utilises a gradient elution system composed of 0.1% formic acid and methanol as eluents, the latter of which has significant impacts on the plasma: eluent droplet size, degree of nebulisation and addition of fuel. Methanol levels of up to 10% in the eluent generally enhanced the signal area for arsenic when running equivalent

standards. The effect was not linear, and gains in signal area intensity were lost with increasing methanol levels up to 20% (Bluemlein et al. 2008b). With mercury-PC complex analysis, increasing methanol levels up to 50% actually resulted in significantly diminished signal areas for standards compared to those at 0% methanol (Krupp et al. 2008). Initially, the addition of methanol to the plasma acts as a fuel, increasing the temperature of the plasma and enhancing the atomisation and ionisation processes (Forsgard et al. 2006). At higher levels, methanol removes energy from the plasma through combustion, diminishing the ionisation capability of the plasma. As described previously, increasing methanol levels requires ICP-MS hardware capable of allowing the addition of oxygen flows into the plasma to remove carbon and the use of expensive and delicate platinum sampler and skimmer cones for analysis.

4.7.3 ICP-MS Analysis: Arsenic-Phytochelatin Complexes

Arsenic is a monoisotopic element, being quantified on ^{75}As, m/z 75. There is the potential for interference of the m/z 75 signal by the argon chloride complex, $[^{40}Ar^{35}Cl]^+$, in systems where a substantial amount of chloride could be present (May and Wiedmeyer 1998). Interference of m/z 75 can readily be determined during analysis by comparison of the background isotope ratios for selenium between ^{77}Se, ^{78}Se and ^{82}Se. As chlorine has a significant component of ^{37}Cl, formation of $[^{40}Ar^{37}Cl]^+$ with mass m/z 77 would be expected and would impact on the observed signal for ^{77}Se. Whilst not routinely observed during analysis of terrestrial plants, where such argon chloride complex formation is observed, this can be dealt with through the use of a hydrogen collision cell.

The chromatographic method developed by Raab et al. (2004) utilises a gradient composed of 0.1% formic acid and methanol running from 0 to 20% methanol over the first 20 min and is able to resolve a number of free PC species, PC-As complexes and glutathione. A variety of plant species, including *Helianthus annuus* (sunflower), *Thunbergia alata* (black-eyed Susan) and *Arabidopsis thaliana* (thale cress), have shown a wide array of complexes, such as GS-As-PC_2, As-$(PC_2)_2$, As-PC_3 and As-PC_4, as determined by ESI-MS data (Raab et al. 2005; Bluemlein et al. 2008a; Liu et al. 2010).

The arsenic triglutathione complex As-SG_3 was hypothesised to exist in vivo but had never been observed equivocally. Liu et al (2010) demonstrated that the complex was present in extracts from the roots of the *cad2-1* mutant of *Arabidopsis thaliana*, a species deficient in PC synthase such that there was little to no PC production by the plant when exposed to arsenic (Liu et al. 2010). This result highlighted that only when there were no PCs available to bind arsenic would the As-SG_3 complex form, presumably as a result of its instability relative to other As-PC complexes.

Research to date has identified As-PC complexes where only 1 atom of arsenic is bound per complex, i.e. poly-arsenic complexes have not been identified. This simplifies quantification of the overall As-PC, given the 1:1 ratio between arsenic and complex. Where unknown As-containing species are identified, quantification by ICP-MS can still be achieved, and it is assumed that the complex contains only 1 central arsenic atom which may help with species identification.

4.7.4 ICP-MS Analysis: Selenium-Phytochelatin Complexes

The major isotope of selenium is ^{80}Se with a normal abundance of 49.6%. However, this is heavily interfered by $[^{40}Ar_2]^+$ during ICP-MS analysis (May and Wiedmeyer 1998). Analysis is typically conducted on ^{77}Se or ^{78}Se, with abundances of 7.6% and 23.7%, respectively. The latter can be interfered by $[^{40}Ar^{38}Ar]^+$, even though the abundances of both argon isotopes are 99.6 and 0.6%, respectively. This is due simply to the sheer volume of argon used to maintain the plasma, making ^{77}Se the preferred isotope for quantification by ICP-MS.

Bluemlein et al. investigated the co-exposure of *Thunbergia alata* to selenite and arsenate, using the same methodology as described in the arsenic case study (Bluemlein et al. 2009b). The Se(II)-PC_2 complex and Se-cysteinylserine glutathione were found in root extracts of *T. alata*, the latter of which highlights a situation specific to selenium. As sulphur and selenium belong to the same group of the periodic table, there is a close synergy in their chemical behaviours, so much so that selenium can replace sulphur in a number of biothiols in vivo, rather than forming Se-SG or Se-PC complexes.

Selenium is not only bound by biothiols, it can replace sulphur in the same molecules. However, selenium does not replicate the same metal(loid)-binding function as sulphur. Work by Blümlein et al. observed no complexes or species containing Se and As simultaneously, which would suggest the formation of Se-As bonds in vivo as part of the plants overall detoxification strategy does not occur.

Further work by Aborode and co-workers investigated the exposure of *T. alata* to selenium as sodium selenite (Aborode et al. 2015). A number of selenium species were observed: selenopeptides, incorporating Se-S bonds; Se(II)-PC complexes, including selenocysteinyl-2,3-dihydroxypropionyl-glutathione, Se(II)-PC_2 and Se(II)-$(SG)_2$; and a significant proportion of elemental selenium (approx. 20%) which was not recovered by the 0.1% formic acid extraction procedure.

In common with arsenic, research to date has suggested the formation of Se-PC complexes where only 1 atom of Se is bound per complex, i.e. a 1:1 ratio between selenium and the complex. Quantification of known and unknown Se-containing species can thus be performed on a relatively simple basis.

4.7.5 ICP-MS Analysis: Mercury-Phytochelatin Complexes

Mercury belongs to the same group of the periodic table as cadmium and lead, PC complexes of which are stable under alkaline conditions. However, two separate studies demonstrated that Hg-biothiol complexes are stable under acidic conditions (Chen et al. 2009; Krupp et al. 2008). Consequently, whilst mercury does not exhibit the degree of oxoanion formation observed for arsenic and selenium, the biothiol complexes of $Hg^{2+}$and $MeHg^{+}$ can be stabilised and analysed in a similar fashion as is employed for As- and Se-biothiol complexes.

Mercury has seven stable isotopes, of which ^{200}Hg and ^{202}Hg are the most abundant at 23.13% and 29.8%, respectively (Chen et al. 2009). The higher atomic masses of mercury isotopes mean that there is minimal risk from interferences from other elements or polyatomic species and ICP-MS analysis can be conducted on either ^{200}Hg or ^{202}Hg without the need for a collision cell (May and Wiedmeyer 1998). Other common isotopes are ^{198}Hg (10.02%), ^{199}Hg (16.84%) and ^{201}Hg (13.22%). Quantitation is not routinely performed on these isotopes, but the spread of isotopes and their high relative abundances generates unique isotope patterns for mercury compounds, which can be useful when identifying mercury-containing compounds.

Whilst Hg-PC_2, Hg-PC_3 and Hg-PC_4 complexes had previously been demonstrated to exist by ESI-MS/MS (Chen et al. 2009), no work had been done to analyse samples by liquid chromatography and tandem mass spectrometry, allowing for the identification and quantification of a number of Hg-PC complexes. Krupp et al. first examined the synthetic Hg-biothiol compounds Hg-$(Cys)_2$, Hg-$(GS)_2$, MeHg-Cys and MeHg-GS by ESI-MS/MS (Krupp et al. 2008). Then, a chromatographic method was established to allow for their separation and detection. Similar to the method utilised by Raab et al., a gradient run-up to 50% methanol was developed and was able to resolve the aforementioned species for detection and quantitation (Krupp et al. 2009). This method was utilised to identify a number of Hg- and MeHg-PC in vivo complexes in *Oryza sativa* (rice) and *Marrubium vulgare* (horehound) by tandem HPLC-ESI-MS/ICPMS.

In common with arsenic and selenium, research to date has suggested the formation of Hg-PC complexes where only 1 atom of Hg is bound per complex, i.e. a 1:1 ratio between mercury and the biothiol. Quantification of known and unknown Hg-containing species can thus be performed on a relatively simple basis.

4.7.6 Quantification of Free PC Species

Bound PC and GSH species can be quantified by ICP-MS upon the central metal(loid). However, unbound thiol species are not readily amenable for quantification, given that hydrogen, carbon, nitrogen and oxygen are relatively ubiquitous in the eluent and sample stream. The only other suitable heteroatom upon which to quantify is sulphur; however, this has a number of issues (Bluemlein et al. 2008b).

Sulphur has poor ionisation characteristics, with a first ionisation potential higher than argon. The main isotope, ^{32}S, is heavily interfered by molecular oxygen, $^{16}O_2$. Finally, as PCs and similar biothiols contain no chromophore, quantification by LC-UV is not possible.

A significant number of attempts have been made to tag unbound thiol species to permit their quantification by LC-UV or LC-ICP-MS. Wood and Feldmann have reviewed and assessed a number of techniques available for biothiol quantification, each of which has their advantages and limitations (Wood and Feldmann 2012). Pre-chromatographic derivatisation of biothiols is one technique. One class of reagents for this purpose is bimanes, highly UV-active reagents which have been used in a wide array of analyses, permitting quantification of free thiol species down to picomolar levels in extracts (Delnomdedieu et al. 1993; Sneller et al. 1999; Steffens 1990). However, bimanes are expensive reagents. Additionally, the use of pre-chromatographic derivatisation may require conditions incompatible with PC-metal(loid) complex stability, which would drastically alter the speciation profile of the extract.

Post-chromatographic derivatisation is another route to quantify free biothiols. Reagents such as 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB) permit rapid derivatisation of biothiol species (~10 s) by rapid disulphide exchange between the reagent and the reduced biothiol (Bräutigam et al. 2010; Zhang et al. 2005). During this reaction, the mixed disulphide species 2-nitro-5-thiobenzoate – biothiol species – is formed, along with the anion 2-nitro-5-thiobenzoate. The latter has a yellow colour in solution and absorbs strongly at 412 nm, permitting reduced biothiol quantitation by HPLC-UV (Riddles et al. 1983). Whilst this method is rapid and sensitive, it can only be used to quantify reduced biothiol species and requires a continual stream of reagent mix for the entire analytical procedure.

Whilst the aforementioned techniques permit biothiol quantitation by HPLC-UV, the analysis is in indirect with respect to the biothiol being measured – quantitation is based upon the spectrophotometric properties of the reagent. Additionally, the conditions with which to achieve analysis may impact upon the speciation profile of the sample extract under investigation. But crucially, the reagents are specific to reduced biothiols. Any oxidised species, such as oxidised glutathione GSSG, are not bound by the reagents without the addition of a reducing agent, which again alters the speciation profile of the extract under investigation (Hansen et al. 2009)

Single quadrupole ICP-MS cannot resolve the main sulphur isotope ^{32}S from molecular oxygen $^{16}O_2$ during analysis, both of which are measured as m/z 32. Distinction between both species can be achieved through the use of high-resolution ICP-MS systems, which utilise a high strength electromagnetic field to resolve ^{32}S (m/z 31.9271) from $^{16}O_2$ (m/z 31.9898) (Bluemlein et al. 2008a). With HR-ICP-MS, it is possible to quantify sulphur in extracts down to concentrations of 5 µg/L.(Liu et al. 2010; Ye et al. 2010). As quantification is performed directly on sulphur, reduced and oxidised species can be quantified in the same run. Additionally, provided the eluent conditions are compatible with the ICP-MS, no modification of the sample extract is required.

There are, however, significant drawbacks with HR-ICP-MS (Wood and Feldmann 2012). The cost of an HR-ICP-MS is typically two to three times that of a standard quadrupole ICP-MS. Secondly, high resolution is achieved in part via an electromagnet which bends the ion beam. The speed at which the magnetic field can be altered, defined by the settling time required by the magnet when jumping between masses, thus selecting a specific m/z ratio, is slower than that of a standard quadrupole by a factor of 4–5. The mass range 2–260 amu can be scanned in 100 ms by a standard ICP-MS but takes around 300–600 ms with a HR-ICP-MS.

Whilst a number of methods exist to perform quantification of unbound thiol species, each has their own distinct advantages and limitations, making their analysis a far from routine task.

4.8 Concluding Remarks

Looking at the different elements, it becomes obvious that there are a lot of different compounds formed within plants. It is therefore necessary to have a nontargeted analysis for any metal(loid) biomolecule (Feldmann et al. 2017). The most versatile method is the use of ICPMS as an element-specific detector and electrospray MS as a molecular-specific detector in HPLC-ICPMS/ESIMS as explained above.

There has however been a complete lack of studies which focus on element/element interaction, with some exceptions, in which co-exposure of arsenic and selenium has been studied (Aborode et al. 2016, Bluemlein et al. 2009b). In terms of selenium-sulphur interaction, *Brassica* have been studied in detail (Tian et al. 2017).

Furthermore, it can be foreseen that genes will be identified for the biotransformations of arsenic seen in the different plants. The transport and accumulation of mercury and especially methylmercury will see more investigations in the future. Whilst the motivation to study mercury and arsenic in plants is the toxicity of the element species accumulating in the different parts of the plants, the focus for sulphur and selenium is more about essentiality. Especially it is expected that the determination of selenoproteins will be a focus of many studies in the near future in order to establish finally whether selenium is essential or beneficial for plants or not.

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