Metal and metalloid speciation in plants: Overview, instrumentation, approaches and commonly assessed elements

Kroukamp EM[1,2], Wondimu T[2], Forbes PBC*[1]

[1]Department of Chemistry, Faculty of Natural and Agricultural Sciences, University of Pretoria, Lynnwood Road, Pretoria, 0002, South Africa
[2]Department of Chemistry, Faculty of Science, University of Johannesburg, Kingsway Road, Johannesburg, 2006, South Africa

Abstract

The ability of plants to hyper-accumulate metals and metalloids from the surrounding environment may pose a significant health risk to both humans and animals since plants form a substantial component of diet. This attribute, however, has also been identified as a useful tool in bioremediation and biomonitoring studies; where assimilated metal(loid)s in plants often correlate to environmental exposure. Since the bioavailability and toxicity of these elements depends upon their chemical form, speciation studies are essential in determining mobility and metabolic pathways. This can be done in a number of ways where sampling, pre-treatment and storage are all important factors affecting speciation. Appropriate analytical techniques for speciation studies can either be direct methods such as XAS, or indirect methods which require species separation prior to analysis. Separation techniques can be in the form of sequential extractions or column separation and analyte detection often utilises instrumentation such as ESI-MS, ICP-MS and ICP-OES.

Key words:
Speciation
Metals
Metalloids
Plants
Biomonitoring
Bioremediation
Extraction

List of Abbreviations:

µXRF	Micro-X-ray Fluorescence
AFS	Atomic Fluorescence Spectroscopy
API	Atmospheric Pressure Ionization
CEC	Capillary Electrochromatography
CID	Collision Induced Dissociation
CRI	Collision Reaction Interface
CRM	Certified Reference Material
CZE	Capillary Zone Electrophoresis
CZE-ICP-MS	Capillary Zone Electrophoresis Inductively Coupled Plasma Mass Spectrometry
ESI-MS	Electrospray Ionization Mass Spectrometry
EXAFS	Extended X-ray Absorption Fine Structure
FAAS	Flame Atomic Absorption Spectroscopy
FTIR	Fourier Transform Infrared Spectroscopy
GC-MS	Gas Chromatography Mass Spectrometry
GC-MS-HGAAS	Gas Chromatography Mass Spectrometry Hydride Generation Atomic Absorption Spectroscopy
GFAAS	Graphite Furnace Atomic Absorption Spectrometry
HEN	High Efficiency Nebulizer
HFBA	Heptafluorobutanoic acid
HG-GC-QFAAS	Hydride Generation Gas Chromatography Quartz tube Flame Atomic Absorption Spectrometry
HPLC	High Pressure Liquid Chromatography
HPLC-HG-AFS	High Pressure Liquid Chromatography Mass Spectrometry Atomic Fluorescence Spectroscopy
HPLC-ICP-MS	High Pressure Liquid Chromatography Inductively Coupled Plasma Mass Spectrometry
HPLC-UV-HG-AFS	High Pressure Liquid Chromatography Ultra Violet Hydride Generation Atomic Fluorescence Spectroscopy
IC	Ion Chromatography
ICP-MS	Inductively Coupled Plasma Mass Spectrometry
ICP-OES	Inductively Coupled Plasma Optical Emission Spectrometry
IEC	Ion Exchange Chromatography
IEF	Isoelectric focussing
IPC	Ion Pairing Chromatography
IP-RPLC-ICP-MS	Ion Pairing Reversed Phase Liquid Chromatography Inductively Coupled Plasma Mass Spectrometry
IS	Ion Spray
ITP	Isotachophoresis
LC-VG-ICP-MS	Liquid Chromatography Vapour Generation Inductively Coupled Plasma Mass Spectrometry
MAE	Microwave Assisted Extraction
MECC	Micellar Electrokinetic Capillary Chromatography
NMR	Nuclear Magnetic Resonance
PCA	Principal Component Analysis
PM	Particulate Matter
QTOF-MS	Quadrupole Time of Flight Mass Spectrometry

RPLC	Reversed Phase Liquid Chromatography
RTILS	Room Temperature Ionic Liquids
SEC	Size Exclusion Chromatography
SE-HPLC-ESI-MS-MS	Size Exclusion High Pressure Liquid Chromatography Electrospray Ionization Tandem Mass Spectrometry
SeMC	Methyl-L-selenocysteine
SEM-EDX	Scanning Electron Microscope Energy Dispersive X-ray Microanalysis
SeMet	Selenomethionine
SWV	Square Wave Voltammetry
TA	Target Analysis
TDS	Total Dissolved Solids
TFA	Trifluoroacetic acid
TOF-SIMS	Time of Flight Secondary Ion Mass Spectrometry
UAE	Ultrasonic Assisted Solvent Extraction
XANES	X-ray Absorption Near Edge Structure
XAS	X-ray Absorption Spectrometry

Highlights:

- Metal(loid) speciation in plants is used in bio-accumulation, -remediation and -fortification studies
- Sample preparation for metal(loid) speciation studies is critical
- Metal(loid) species can either be separated by sequential extractions or column separations
- Detection methods for speciation include XAS, HPLC-ICP-MS, CE-ICP-MS, HPLC-ICP-OES and AAS
- Commonly speciated elements in plants are As, Se, Sb, Cd, Pb, Mn and Ni

1 Introduction

The importance of speciation studies is evident from the increasing number of publications focussing on this type of analysis. This is because total elemental concentrations do not provide sufficient information about the bioavailability and fate of metals and metalloids in the environment [1], whereas speciation studies are able to [1; 2; 3; 4; 5; 6; 7]. Such analyses can be described as the identification and measurement of specific forms of an element according to its molecular or complex structure, electronic or oxidation state or its isotopic composition [8]. The interest in metal and metalloid species in plants (which are generally collectively referred to as “metals” in this paper) is well established, as plants are natural, dietary sources for major, minor and trace essential minerals [9]. Since metals are non-biodegradable [10; 11], metal loading in plants is therefore an important consideration when assessing potential health impacts [12; 13], where metals can be assimilated either as elements or as metabolites [14].

The uptake of metal(loid)s by plants is either a passive or active process. Vascular plants take up metals from their roots, transpiration through stomata on the leaf surface, and deposition on the surface of leaves and bark. Non-vascular plants, however, often lack cuticle and stomata, [15] and factors such as surface water, rain and passive diffusion from substrates facilitate the movement of metals into these plants [16]. The uptake, translocation, transformation and accumulation of metallic species in crop plants is extremely important from an animal and human health perspective [17; 18]. Since a number of plants and their produce are distributed globally, their composition needs to be determined to meet import and export criteria [19]. The testing procedures employed can prove to be challenging, as the toxicity or beneficial nature of metals is not necessarily determined solely by its organic or inorganic form [6; 20] or the oxidative species present [6; 21]. Therefore toxicity is often linked to the solubility of the chemical form and the ligand species. Knowledge of potential sources of pollutants within a particular area is advantageous as it can provide information about the compounds present and their physical and chemical properties.

Metal(loid)s in the environment usually occur as a mixture of inorganic and organic compounds with varying degrees of toxicity. Natural sources of these elements in plants include that arising from soil microbial activity or the weathering of geological formations, as was investigated by Eiche *et al.* [18], who evaluated the speciation of Se in edible crops growing in seleniferous soils in India (Table 1). Such studies of metal speciation and metal homeostasis in plant crops can also be used in the biological engineering, otherwise known as biofortification, of food crops to increase the uptake of beneficial metals by plants into key plant components [22]. Metals can also occur in the environment as a result of anthropogenic activities, and biomonitoring studies are often undertaken to assess the risks associated with such exposures.

Biomonitoring studies make use of direct monitoring of biological organisms, which are able to assimilate contaminants over a long period of time and remain in equilibrium with the system [23; 24; 25; 26]. These types of studies are therefore not as subjective as those involving ecosystems which are only sampled at a specific point in time [27; 28]. By using plants as biomonitors, information on metabolic pathways, detoxification mechanisms and source apportionment can be obtained. A study by Bergqvist *et al.* [29] showed that higher concentrations of inorganic As in edible crops correlated with the concentrations of As in the soils from an abandoned glassworks in which they were grown. Thus a clear link between cause and effect could be observed. These concentrations where higher than those in plants grown in less contaminated soils; namely greenhouse and agricultural alum shales (Table 1). Plants are not only used to assess soil contamination in biomonitoring studies, but can also be used in the biomontoring of atmospheric pollution. Botanical matrices such as moss, pine needles, tree bark, leaves, grass, ferns and lichens have been employed in this regard [30; 31]. Metal speciation studies in such cases can be either intra-, inter- or extra-cellular. An example of the latter is a study by Schreck *et al.* [32], who determined the speciation of Pb in atmospheric fallout dust on the surface of edible plants.

Table 1: Summary of published studies dealing with the separation of species of metals in plants

Metals speciated	Plant material	Plant species	Location	Extraction method	Sample mass (g)	Column	Mobile Phase	Analytical technique	Reference
As	Lichens and green plants	*Alectoria ochroleuca, Usnea articulate, Achillea millefolium, Alnus incana, Asplenium viride, Dryopteris dilate, Equisetum pratense, Fragaria vesca, Rubus idaeus, Vaccinium myrtilis, Vaccinium vitis idaea, Picea abies, Larix decidua, Deschampsia cespitosa.*	Austria	MeOH: H_2O (9:1)	0.5, 0.8,1	Anionic species: Hamilton PRP-X-100 (250 x 4.1 mm; 10 µm Cationic species: Zorbax 300-SCX cation-exchange column (150 x 4.6 mm)	Anionic: pyridine (20 mM, pH 2.6, CHOOH) Cationic: $NH_4H_2PO_4$ (20 mM, pH 5.6, 25 % NH_4	HPLC-ICPMS	[65]
As	Brake Fern	*Pteris vittata*	Greenhouse study using soils from Florida, USA	MeOH:H_2O (1:1)	0.010	Anionic species: Hamilton PRP-X-100 (250 x 4.6 mm, 10 µm particle size)	K_2HPO_4 & KH_2PO_4 (0.015 M, pH 5.9)	HPLC-HG-AFS and HPLC-ICP-MS	[37]
As	Lichens	*Parmelia sulcate*	Portugal	Sequential	0.2-0.5	Separation carried out on pre-column. Anionic species: Hamilton PRP-X-100 (250 x 4.1 mm) Cationic species: Altech Adsorbosphere SCX 5U (250 x 4.6 mm)	Anionic: KH_2PO_4 (15 mmol/L, pH 6.1, NH_4OH) Cationic: pyridine (2.5 mmol/L, pH 2.65, HCL)	HPLC-UV-HG-AFS	[77]

As	Lichens and tree bark	*Parmelia caperata, Platanus hybrida*	Portugal	MeOH:H_2O (1:1)	0.2-0.5	Anionic species: Hamilton PRP-X-100 (240 x 4.1 mm) Cationic species: Altech Adsorbosphere SCX 5U (250 x 4.6 mm)	Anionic: KH_2PO_4, (20 mmol/L, pH 6.0) Cationic: pyridine (2.5 mmol/L, pH 2.65)	HPLC-HGAFS	[21]
As	Flowering plant	*Calluna vulgaris,*	Spain	MAE and DB[1] Orthophosphoric acid (0.3 M)	0.2	Anionic species: Hamilton PRP-X-100 (250 x 4.1 mm; 10 µm)	TRIS(hydroxymethyl amionoethane) pH 7, adjusted with glacial acetic acid.	HPLC-ICP-MS	[48]
As	Rice, wheat	*Rice Flour CRM, NIST SRM 1568a Rice flour, commercial rice, Wheat*	USA, Czech Rebublic (commercial rice), wheat (Italy)	MAE and Water bath extraction	0.25	Anionic species: Hamilton PRP-X-100	Malonic acid (2, 5 or 10 mM at pH 5.6 adjusted with aqueous ammonia)		[62]
As	Signalgrass	*Brachiaria brizantha*	Brazil	HNO_3 (2 % v/v)	0.2	Anionic species: IonoSpher A (250 x 4.6 mm)	Phosphate buffer 12.5 mmol/L, pH 5.4	HPLC-ICP-MS	[49]
As	Carrot, lettuce, spinach	*Daucus carota L, Lactuca sativa L., Spinacia oleracea*	Sweden	MeOH:H_2O (1:1)	0.1- 5	Anionic species: Hamilton PRP-X-100 (250 x 4.6 mm)	$H(NH_4)_2PO_4$ (pH 5.8)	XANES and Sequential extraction with HPLC-AAS	[29]
As	Rice	SRM 1568b and CRM 7503-a and rice from local market	USA	Enzyme-assisted water phase microwave extraction (0.5 % amylase solution)	0.2	7100 CE system. 60 cm coated fused silica capillary with i.d. 100 µm	Na_2CO_3 (8 mM, pH 11) buffer	CE-ICP-MS	[84]
As	Rice	*Yerua, Camba, ZHE733, Puita and El Paso 144*	Argentina	Heat assisted extraction (sand bath)	~1.0	Heated PRP-X-100 column	NaH_2PO_4/Na_2HPO_4 buffer	HPLC-HG-AFS	[113]
Cd	Flowering plants, grasses,	*Silene cucubalis, Agrostis tenuis, Rauwolfia*	France	Sequential and online	0.010	SEC column: Superdex Peptide HR 10/30 (300 x 10	TRIS-HCL buffer (30 mM, pH 8.5) ES-MS-MS: Orifice	SE HPLC-ICP-MS, SE HPLC-ES-	[9]

[1] DB: Digestion block

	maize	*serpentina, Zea mays L.*					mm). Superdex 75 HR 10/30 and Superdex 200. Guard column: TSK PWXL (40 x 3 mm)	potential 40 V, Ionspray voltage 4100 V.	MS-MS	
Cd	Edible plant	*Arabidopsis thaliana*	Poland	Sequential extraction		0.040	Superdex 75 HR 10/30 (300 x 10 mm)	30 mM Tris buffer with 10 mM of NaCl 7.4	SEC-RPLC-ESI-MS	[89]
Cd	Flowering plant	*Arabidopsis halleri*	Japan	Extraction					113Cd NMR	[115]
Mn	Hazelnuts		Turkey	Sequential online	and	0.1	Heated column (26 °C) 200 column oven and a vacuum degasser. Brownlee DB Aq. C-18 (150 x 4.6 mm i.d. and 5 µm film thickness). Guard column (40 mm) with the same stationary phase material.	MeOH/H_2O (gradient mobile phase 100:0 (A%/B%, v/v) for 0-1 min, 95:5 (A%/B%, v/v for 1 min, 97:3 (A%/B%, v/v) for 2 min and 98:3 (A%/B%, v/v) for 2 min)	ICP-MS and HPLC-ICP-MS	[17]
Ni	Green plant	*Thlaspi arvense L.. Thlaspi goesingense*	Germany:						XAS	[41]
Ni	Tree	*Sebertia acuminata*	New Caledonia	Sequential on-line	and	0.127	SEC column: Superdex Peptide HR 10/30 (300 x 10 mm). Preparative SEC on Superdex 30 preparative grade column (600 x 26 mm, Mx< 10000Da) lypholized at – 50 °C	SEC: $NH_4C_2H_3O_2$ (5 mmol/L, pH 6.8) and degassed ultrasonically.	SE HPLC-ES MS-MS, CZE-ICP-MS	[90]
Ni	Green plant	*T. caerulescens*	France:	Sequential on-line	and	1	SEC column: Superdex Peptide HR 10/30 (300x 10 mm).	SEC: $NH_4C_2H_3O_2$ (5 mmol/L, pH 7) and degassed ultrasonically.	SE HPLC-ICP-MS,	[4]
Ni	Flowering plant	*Alyssum serpyllifolium ssp.*	Portugal						SWV	[120]

		Lusitanicum							
Sb	Flowering plant, moss, vascular plants, lichens, fungi	*Mimulus sp., Funaria hygrometrica, Drepanocladus sp. Typha latifolia, Bidens cernua,Cladonia sp, Lycoperdon s. Coprinus comatus.*	Canada	MeOH: H_2O (1:1)	0.5 ± 0.005, 1-2 for moss	GC-MS: PTETM (-5.30 m x 0.32 mm, 0.25 µm Supelco)	Buffer: $NaBH_4$ (2% w/v) and $NaBH_4$ (6%)	HG-GC-AAS, Headspace HG-GC-MS	[92]
Se	Garlic and mustard	*Allium sativum and Brassica juncea*	Spain	UAE	0.2	Ion pairing reverse phase column C8 (250 x 2.0 mm, 5 µm) and SEC Shodex Asahipak GS-220 HQ (300 x 7.6 mm, >3000 Da)	IPRLC: 0.2 % (v/v) heptafluorobutyric acid, 10 % (v/v) MeOH (pH 2.5) SEC: 10 mM ammonium acetate buffer (pH 6.5)	HPLC-ICP-MS	[91]
Se	Yeast and Clover	*Not mentioned*	China	Water extraction and enzymatic extraction	Not mentioned	C18 column (shim-pack CLC, 5_m, 4.6 × 150 mm, Japan)	0.4% (v/v) [BMIM]Cl, 0.4% (v/v) [BMMIM]BF_4, and 99.2% high purity deionized water (pH = 6) was used for isocratic elution at a flow rate of 1.0mLmin−1	RTILs improved RPLC-ICP-MS	[86]
Se	Kale	*Brassica oleracea var. alboglabra L.*	Thailand	0.1 M HCl in 10 % v/v MeOH	0.1	Reverse phase column Inertsil® C18 (250 x 4.6 mm, 5 µm)	8 mM BSA, 4 mM TFA pH 4.5	HPLC-ICPMS	[95]
Se	Wheat and mustard	*Triticum aestivum and Brassica juncea*	India					SE HPLC-ES MS-MS, CZE-ICP-MS	[18]
U	Sunflower and oilseed rape	*Helianthus annuus and Brassica napus.*	France					HG-GC-AAS and HG-GC-MS	[59]

Biomonitoring can also be employed in aquatic ecosystems such as estuarine, salt water and freshwater systems through the use of aquatic plants such as the water hyacinth, *Eichhornia crassipes* [33]. Here, *in situ* water hyacinth was used to evaluate and remediate mercury contaminated waters from a chloralkali plant. Since certain species of plant have been shown to accumulate higher concentrations of metals than others, plants have also played a large role in bioremediation efforts [34]. Bioremediation-phytoremediation projects exploit the ability of plants to assimilate metals in concentrations higher than their metabolic requirements. Hyper-accumulating, metal tolerant plants are commonly used in the phyto-extraction of heavy metals from metal-loaded soils, such as those found in mining [35] and sawdust heaps [36] Certain plant species are known to possess higher bioconcentration potentials than others, such as was found in the fern, *Pteris vittata*; where these plants are commonly used in the removal of As from contaminated soils and groundwater [37]. Livestock and agricultural farmers often populate catchment areas with riparian vegetation such as bulrushes and reeds to form man-made wetlands; reducing water flow, downstream silt build-up and the eutrophication of rivers [38; 39; 40].

To determine a suitable plant species to be used for the mentioned applications, familiarity with the elements of interest, as well as an understanding of their speciation will greatly assist with the selective extraction of elements [41]. Moreover, hyper-accumulation of metals in different parts of the plant may vary quite considerably, and knowledge of these properties can be beneficial. There are concerns, however, that the process of assimilation of metals by plants may result in the biotransformation of certain metallic and metalloid species [9; 42], as was found in the case of As in the lichen species *Parmelia caperata* (Table 1) [21]. This biotransformation is a potential issue for other elements as well [21] and as a result, non-living biomass has also been assessed for its suitability in bioaccumulation and bioremediation studies.

Inactivated mosses and lichens have been employed in bioremediation studies as biosorptive agents; absorbing metal ions from industrial waste waters to reduce toxicity [43; 44]. A study assessing copper (as Cu II) in lichen biomass, *Cladonia rangiformis hoffm*., was used to illustrate this, as copper is a common by-product of industry, processing and mining wastes [44]. The effect of pH was found to be a defining parameter in the absorption of Cu [44; 45] and is also likely to have an influence on the chemical species present. The absorption of Cu at low pHs was found to be inhibited, assumedly due to a competition between H_3O^+ and Cu (II) at the lichen surface, whilst at pH > 5, bioabsorption was also found to be inhibited [44]. Similar effects were observed in studies by Uluozlu *et al.* [45] who monitored the bioabsorption of Pb (II) and Cr (III) into the lichen *P. tiliaceae* using Fourier Transform Infrared Spectroscopy (FTIR). Since a number of different factors can disturb the equilibrium in the chemical species, appropriate means of sampling and sample pre-treatment are important in any chemical speciation analysis.

2 Sampling and sample pre-treatment

A number of sample pre-treatment techniques have been established for the speciation of simple matrices; however, complex matrices still present a significant challenge. This is partly due to shifts in chemical equilibria which may occur during this step, affecting the quality of the results, the extent of which are often difficult to quantify [1; 46; 47]. Consequently, sampling and sample stabilization in complex matrices are considered to be the most important steps in speciation analyses [1; 47]. To prevent alterations, the pre-treatment procedures are often not as aggressive as one would use for total element determinations [48; 49].

In biological materials, contamination during sampling and from sampling apparatus may result in altered species distributions [1; 47; 50]. The following recommendations in terms of sample collection have been made:

- Samples should be collected using acid-washed plastic [51], nylon, ceramic or teflon tweezers [58].
- Sampling time should be kept to a minimum [1].
- Powder free latex, nitrile or vinyl gloves should be worn.
- Samples such as roots and stems, which are in close contact with the ground, should be gently washed of adhering materials so as to limit contamination [30].
- Samples such as leaves, bark, epiphytic mosses and lichens, should be collected 1-2 m above the ground to limit contamination by “back-splash”, and are generally not washed [30; 31].
- Samples of similar sizes should be chosen to reduce variations which can be introduced from longer growth periods [15].
- Storage should take place in acid washed polyethylene or polypropylene bottles or paper bags [1; 52].
- Samples should be stabilized as soon as possible which can involve steps such as drying, placement in an inert environment or freezing.

Sample pre-treatment should involve as few steps as possible; thereby limiting the chances of sample loss and species alteration [53]. There are conflicting approaches to the washing of samples, where some authors prefer to remove extraneous materials in this manner [30], while others are concerned that washing steps may result in the dissolution of ionic compounds, altering their concentrations [52]. Inevitably, it comes down to the desired information. If the assessment of the adhering particles, isotopic ratios, or the speciation of metals within the sample are of interest, then it has been recommended that the samples are not washed [54]. If the bioconcentrated fraction is of interest then it is recommended that the samples are washed [55]. To assess the distributions of metallic species within the bioconcentrated fraction, conditions which maintain the original species, but avoid contamination

by soil, bark or other particles need to be employed. For this reason other cleaning procedures, such as nitrogen air jets and ultrasonic baths or probes may be recommended.

Nitrogen air jets are especially useful in speciation studies as they are thought to provide an inert and clean environment, limiting contamination and changes in the species pattern distributions. Some samples, such as mosses, are especially porous and as a result are difficult to clean effectively [56], and so a number of techniques have been employed to remedy this. Spagnuolo *et al.* [56] found that nitrogen air jets were too aggressive for the cleaning of mosses at higher pressures (4-8 bars) and were unable to remove adhering particles at lower pressures [56] and results were found to be irreproducible and unpredictable. In comparison, the use of ultrasonic probes for cleaning procedures was found to produce reproducible data and ensured the integrity of the cell membrane [56]. The procedural requirements for immersing the sample in a liquid phase however, was found to encourage the leaching of metals from the sample, compromising the quality of the results [56]. Ultrasonic baths have also been used as a gentler alternative to ultrasonic probes, and have been used to clean foliose lichens such as *Parmelia sulcata* [57]. Consequently they are often the preferred cleaning procedure for speciation analysis [58].

The choice of preparation techniques also depends upon the type of analytical instrumentation which will be used for a particular chemical species. Laurette *et al.* [59], for example, recommended that root samples be washed with distilled water and soaked in 10 mM Na_2CO_3 to remove adsorbed uranium from the root surfaces of oilseed rape and sunflower plants prior to analysis with X-ray absorption spectrometry (XAS) with X-ray Absorption Near Edge Structure (XANES) and Extended X-ray Absorption Fine Structure (EXAFS) (Table 1).

A degree of heterogeneity in chemical species may be expected between samples within a population [7; 43]. To account for this, it is recommended that a large, representative set of samples be collected, stabilized, pulverized and homogenised [60]. However these steps require the manipulation of the sample, which can lead to contamination [51]. Techniques commonly used in retaining species integrity and preventing analyte losses include enzymatic hydrolysis, volatilization with and without pre-concentration, as well as different leaching and extraction methods [1; 47; 61; 62]. Some of the challenges surrounding homogenization can be addressed by drying or freezing samples prior to this step; increasing the ease by which homogenization can take place, and ending microbial activity which may otherwise disrupt the chemical equilibrium between species. Commonly employed techniques are air drying [15; 58], oven-drying [63], freezing to between -20°C to -80 ˚C [64], freeze-drying [65] and cryogenic freezing with liquid nitrogen [59; 66].

It has been recommended that biological tissue samples be freeze dried and stored under cold conditions until processing [1; 53]. The freeze drying process removes water molecules by freezing the water within the sample. By keeping the pressure below the triple point and gradually increasing the temperature, the ice crystals are sublimated [67], which limits water-based reactions [53]. Freeze drying, however, may remove certain volatile species from the sample [68], and in some cases has been found to alter the major chemical speciation patterns [53]. This observation was confirmed by Eiche *et al.* [18], who noted that the drying step (air drying and subsequent freeze drying) resulted in poor recoveries of some species of Se in wheat and Indian mustard plants (Table 1). For this reason, techniques involving the formation of water on the surface of the sample are often avoided and some researchers have chosen to rather freeze their biological materials in liquid nitrogen [41; 59], and store under cold conditions until processing. Presumably this is based on the assumption that the species distributions will experience little alteration during cryofixation and thus the original species integrity can be ensured. Sample stabilization steps for transition elements require further consideration as they are especially susceptible to oxidation, which can compromise the species integrity. Therefore, studies which focus on evaluating the species distribution patterns for these elements should be conducted in an inert environment [67].

Quality control procedures in the analysis of chemical species should ensure that the data captured is an accurate and true reflection of the species concentrations. Mass balances should be determined throughout the procedure and species spikes are avoided as spikes may affect the species equilibrium [1; 53]. If the study is to involve the validation of a new technique, cross correlation of results with an existing technique is highly recommended [46] and certified reference materials should be analysed where possible.

There are a variety of analytical techniques which may be employed in the determination of appropriately collected and pre-treated samples, and these are discussed in Section 3.

3 Direct analytical techniques

3.1. XAS

Synchrotron X-ray Absorption Spectroscopy is a powerful, element-specific speciation tool [7], and is commonly used in the assessment of metal-uptake, toxicity and tolerance in plants, particularly in hyper-accumulation studies [7; 69]. Measurements using XAS provide a plethora of information about the target analyte in the sample, such as ligand identification in terms of oxidation state and symmetry,

as well as information about neighbouring atoms [69]. XAS follows the Beer-Lambert Law, where signals from XAS are deconvoluted using chemometrics [7].

XAS is able to provide quantitative metal or metalloid speciation data for environmental samples, irrespective of their physical state and provided that the original species integrity is maintained throughout the entire analytical procedure [2; 7]. XANES or EXAFS is typically used after principal component analysis (PCA) and target analysis (TA) to determine the analyte species identity. XANES is the analysis which occurs within the energy region of 50 eV, below the elements absorption edge to 200 eV above this [70].

Although this technique has shown to be a valuable tool in speciation analysis, poor detection limits, maintenance of the species integrity and limitations on the number and variety of species within a sample which are quantifiable make calibration a challenge and reduce its applicability. The quantification of transition elements has been especially problematic, although new instrumentation may address this [7]. Where these elements are of interest, sample and species integrity is maintained by cryofixation of specimens and sample runs taking place at low temperatures [7]. The limited availability of certified reference materials (CRMs) for the analytes and species of interest for this technique presents an additional challenge [7]. Consequently, laboratories need to carefully synthesize in-house standards with low errors and appropriate matrix matching.

4 Separation techniques

Two separation techniques which are commonly employed in speciation analysis are extractions and chemical separation using chromatography [71]. Sequential extractions characterize molecular groups according to similar chemical traits; where the fractions are linked to bioavailability [1; 3; 71; 72]. These techniques, however, are not fully selective, since two or more species are often extracted together, and thus do not allow for the identification of any single chemical species [1]. For this reason, extractions are not regarded as true chemical speciation analyses [1] whereas separations using chromatography are [71; 73]. Moreover, sequential extraction procedures are not as suitable for routine analysis as chromatographic techniques because they are often involved and time consuming processes [74].

The measurement of analytes in extracts from sequential extraction procedures have mostly used Inductively Coupled Plasma Optical Emission Spectrometry (ICP-OES) [75], Graphite Furnace Atomic Absorption Spectrometry (GFAAS) [71] and Flame Atomic Absorption Spectroscopy (FAAS) [76] for quantification, whilst a number of hyphenated techniques such as High Pressure Liquid Chromatography

- Inductively Coupled Plasma - Mass Spectrometry (HPLC-ICP-MS) [21; 73] and Hydride Generation - Gas Chromatography – Quartz Tube Flame Atomic Absorption Spectrometry (HG-GC-QFAAS) [73] have been widely used for the detection and characterization of individual chemical species in extracts. The extraction and separation techniques are described further in Sections 4.1 and 4.2, whilst the detectors employed are discussed in Section 4.3.

4.1. Extraction techniques

Extractions can be either single step or sequential; where the latter treats the sample with leaching agents of increasing strength of interaction [1; 71; 72]. The sequential extraction method optimized by Tessier *et al.* [72] is most commonly used, however some issues were the lack of selectivity with respect to individual species and the pH dependency of the method [1; 76]. Several adaptations have thus been suggested for different matrices including plants such as lichens [77]. Improvements have included the use of less aggressive reactants and extractants not being replaced in each step but rather added to each step, with small aliquots set aside for analysis [77].

Ultrasonic-Assisted Solvent Extraction (UAE) has gained popularity in the last few years as an attractive alternative to Microwave Assisted Extraction (MAE) techniques as it does not require high temperatures, pressures, or harmful chemicals [56; 78; 79]. A solid sample is placed into a solution and is exposed to ultrasonic energy, which results in the formation of imploding bubbles, leading to high localized temperatures and pressures [79]. In this way compounds or elements can be extracted from the sample matrix [56]. Ultrasonic methods also increase the rate of reaction in sequential extractions [78] and assist with the homogenous suspension and distribution of slurries [80]. The application of UAE in the extraction of weakly bound organic compounds from their respective matrices is also possible [78], provided that the temperature does not exceed the tolerances of the analyte [80]. UAE has been used in the extraction of selenium species from *Allium sativum* and *Brassica juncea* prior to chromatographic separation using HPLC-ICP-MS [82] (Table 1).

Factors such as particle size, extractant concentration, sonication time, sample mass to extractant ratio [57; 81; 82], and the use and choice of surfactants [82] all impact on the extraction efficiency. Extractions using ultrasonic probes are more efficient, reproducible and repeatable than the use of ultrasonic baths [57] which do not have a uniform distribution of ultrasound energy and also lose power over time [83]. An additional benefit of using this technique is that it involves fewer analytical steps than many other extraction techniques, making it less susceptible to contamination [57].

4.2. Hyphenated techniques

Reactions of metals and metalloids are seldom related to their elemental properties, but rather to their oxidation state or their role as a component of larger macromolecules [1]. Therefore coupling of different separation techniques with either element or molecule-selective analytical techniques, or both, form the basis for most speciation analyses [1; 46]. Methods of separation commonly include Liquid Chromatography (LC), Capillary Electrophoresis (CE) and Gas Chromatography (GC), where detection methods can either be elemental or molecular [1]. Coupling methods require ease of interfacing between the separation and detection system, and adequately low detection limits [84]. Some of these techniques which are commonly employed are briefly discussed in this section.

4.2.1. Liquid Chromatography (LC)

Liquid chromatography is an extremely useful technique because both the mobile and stationary phases used in the separation can be altered depending on the analyte; improving species stability and analyte separation [1]. It can be used to separate thermally labile and non-volatile compounds and is easily interfaced with ICP-MS making it suitable for speciation studies [85]. Unlike GC, derivatization to produce volatile compounds is not required. It is also more reproducible than CE [86]. Species alterations may still occur, however. Contamination of stationary phases or denaturing of biomolecules brings with it the risk that the species of interest may not be quantitatively recovered [1; 7; 46]. It is therefore necessary to ensure that the metal-ligand bond is stronger than any bonds which can form between these components and the stationary phase [46]. LC columns commonly used to separate chemical species are based upon factors such as retention time, size and mobility [1; 5; 46]. Different columns, their benefits and short fallings will be discussed in the following sections.

4.2.1.1. Size Exclusion Chromatography (SEC)

Size Exclusion Chromatography (SEC) is a technique which often precedes detection, differentiating between free metal ions and elements bound within a macromolecular structure [46]. In this way, separation is dependent upon the molecular size of the analyte [87; 88]. Its ease of coupling to on-line detection systems makes this type of separation extremely efficient [46]. Speciation is gentle, and does not result in high element species losses, or alterations to the species type on the column [1]. The mobile phase is determined by assessing sample-solvent solubility and its chemical compatibility with the stationary phase [87]. As shown in Table 1, mobile phases commonly employed in SEC techniques are Tris-HCL buffer [9], Tris buffer with NaCl [89], ammonium acetate [4; 90; 91] and sodium borohydride [92].

Concerns surrounding SEC, however, are that electrostatic effects may be observed in a non-neutral column, giving an uncontrolled recovery of metallo-complexes [1; 9]. Furthermore, artefacts may be formed due to interactions between the packing material of the column and the metal or ligand [9]. The chemical conditions of the mobile phase also affect the composition of the complex and may further impact the quality of the results [9]. Other issues with SEC are the lack of standards for analysis, causing the resulting information to be based upon the volume eluted, and is therefore regarded as being strictly qualitative [9; 88].

4.2.1.2. Ion Exchange Chromatography (IEC)

In Ion Exchange Chromatography (IEC), cations or anions are exchanged between the ionic sites on the stationary phase and the mobile phase [1; 5]. This technique is widely used, and can be applied to both ionic and non-ionic compounds, although ligand exchange reactions are needed to resolve non-polar compounds [87]. IEC generally has high separation efficiency, where the retention of the ionic species is determined by pH, ionic strength of the mobile phase and the nature of the ion exchanger [1; 87; 93]. Properties such as cross-linkage, particle size, and functional groups in the stationary phase resins determine the distribution coefficient, and thus the efficiency of the chromatographic separation [94]. Salt-buffered solutions containing methanol or acetonitrile are commonly used as mobile phases [87]. Separations carried out using higher cross-linked resins possess greater mechanical strength, and experience less swelling in solution than other resin grades, although it should be noted that the ion exchange is not as efficient in these resins [94].

Disadvantages of using this technique are the possible retention of some organic ions due to hydrophobic interactions between the sample and the stationary phase [1; 94] and poorer selectivity in comparison to Ion Pairing Chromatography (IPC) [93], which is discussed in Section 4.2.1.3.

4.2.1.3. Reversed Phase Liquid Chromatography (RPLC)

Reversed Phase Liquid Chromatography (RPLC) can be used to separate both ionic and non-ionic compounds [1; 85]. Separations are quick, efficient and highly reproducible [1]. In this technique the mobile phase commonly consists of aqueous mixtures of methanol, acetonitrile, tetrahydrofuran or heptafluorobutyric acid (HFBA) [1; 87; 91]. The stationary phase is generally a carbon chain bound to the stationary phase support material and is less polar than the mobile phase solvents [5]. The ion pairing reagent in the mobile phase promotes ionic interaction, which is achieved through both the ionic and organic regions of the analyte [5]. Ionizable analytes interact with the ionic end, and the organic end of the mobile phase interacts with the stationary phase [5]. This allows the analysis to resemble an ion exchange column with the added benefit of being cheaper, having higher resolving power and being

more robust than IC columns [5; 85]. Columns typically used in this technique are C_8 [91] and C_{18} [86; 95] (Table 1).

An adaptation of this technique, Ion Pairing Reversed Phase Liquid Chromatography ICP-MS (IP-RPLC-ICP-MS), can be used for the concurrent separation of anionic, cationic and neutral molecules provided that an appropriate ion-pair reagent is added to the mobile phase [5; 86]. Trifluoroacetic acid (TFA) is a useful ion pairing reagent for IP-RPLC; achieving good separations, with the drawback of having long retention times and consequently, poor peak resolution [86]. HFBA, in comparison, is a stronger ion-pairing reagent with better chromatographic resolution than TFA [86].

Poor tolerances to high concentration matrices, and less effective separation than that achieved for IC [5] are disadvantages of this technique. Organic solvents and acids may alter the chemical species of the analyte [1] and the variation of buffer solutions, pH control and the addition of salt to the mobile phase is often required [1; 5; 87]. Consequently, this technique is limited to strongly bound metals, where covalent element-ligand bonds are preferred [1]. In terms of detection, coupling this technique with ICP-MS may cause carbon loading on the cones and cooling of the plasma, resulting in ionization effects [1; 5]. This poses a challenge in analysis both in terms of increased interferences and degraded detection limits [1; 5] and will be discussed further in Section 4.2.1.4

4.2.1.4. Addressing analytical challenges in LC

There are a number of ways to improve the detection limits and reduce artefact formation in liquid chromatographic techniques. These include, but are not limited to, the preparation of the samples in the LC buffer which allows the eluent and organic modifier to be in a diluted state [1; 96]. Peltier-cooled spray chambers are also recommended in ICP-MS systems when dealing with organic matrices, as well as the entraining of small quantities of oxygen into the plasma to burn off excess carbon from the cones [1; 5]. The introduction of oxygen into the plasma is not expected to alter the chemical species since the introduction of the chemical species is dependent upon the elution time of the column. However, additional oxygen in the plasma may result in additional polyatomic formation and can also alter the ionization conditions of the plasma which may impact quantification. Lastly, for volatile elements such as Hg, better LODs can be achieved by using alternative techniques such as Liquid Chromatography Vapour Generation ICP-MS (LC-VG-ICP-MS), allowing for direct plasma introduction [96].

4.2.2. Capillary Electrophoresis (CE)

CE is a powerful separation technique with a high resolving power and is commonly employed in the speciation of macromolecules, although the separation of ions possessing different charges is also possible [84; 96]. In this technique, different modes of separation such as Capillary Zone Electrophoresis (CZE), Micellar Electrokinetic Capillary Chromatography (MECC), Isoelectric Focussing (IEF), Isotachophoresis (ITP) and Capillary Electrochromatography (CEC) are possible on a single instrument [1; 97]. CE techniques only slightly disturb the equilibrium between different elemental species, and the absence of a stationary phase eliminates possible interactions between the stationary phase and the analyte [1]. The coupling of CE to MS is determined by the degree to which the analyte is electrophoretically mobile and the differences in the structure of macromolecules [97]. Dead-volumes on ICP-MS are low; with flow rates of less than 0.5 µL/min [96]. However, techniques such as Electrospray Ionisation (ESI), Ion Spray (IS) and Atmospheric Pressure Ionization (API) are often employed to change the phase to a gas prior to mass spectrometric detection [97]. Mobile phases can be in the form of sodium carbonate and columns such as 60 cm coated fused silica capillary have been reported [84].

Limitations of this technique relate to small sample volume allowances, and ensuring that the sample is adequately homogenous [96]. Detection limits and peak resolution are inferior to those in LC, and alterations to the chemical species may occur due to the application of high voltages [1; 84]. The choice of nebulizer is important as those used in coupling CE to ICP-MS often introduce backpressure which may compromise electrophoretic separation by producing laminar flow in the capillary [96]. Since the analyte mobility is dependent upon pH and the strength of the electric field, migration time shifts may also occur with CE analysis, although this is commonly dealt with by introducing internal standards and using the method of standard additions for additional quality control [1].

4.2.3. Gas Chromatography (GC)

Hyphenation of GC to ICP-MS has increased sensitivity and allowed for higher matrix tolerances than that found in GC-MS [98]. Although GC is used to separate volatile species, the species of interest in these applications are commonly not volatile [1; 98]. To achieve a gaseous sample, complex sample preparation is required, where this may put the sample at risk of contamination and species loss or alteration [1]. The separation of the analyte species often takes place at elevated temperatures which, when coupling to detectors such as ICP-MS, requires that the transfer line be heated [1]. As an alternative, some studies have coupled GC to other detectors such as AAS or AFS, and this has been found to exhibit promising results [98].

4.3. Detection Techniques

4.3.1. Electrospray Ionization Mass Spectrometry (ESI-MS)

Electrospray Ionization is a sensitive, soft ionization technique for ionisable analytes, which is easily coupled to HPLC [1; 99]. Extremely advantageous is the ability of this technique to allow for the conversion of a liquid sample into its gaseous form, thereby ensuring that little of the original species information is lost [100]. It is especially useful for organic solvents which often pose problems in ICP-MS, and is used in the identification of unfragmented molecules, where further fragmentation can be induced and controlled through collisions (Collision Induced Dissociation; CID) [1]. Lead, for instance, can be directly speciated without any previous analyte separation [101]. ESI-MS is often used as a complementary detection method for species evaluation by HPLC-ICP-MS [101; 102; 103; 104]. Information obtained by ESI-MS is highly dependent upon the species of the analyte and therefore requires a substantial amount of off-line investigations for quality assurance [97].

In ESI there are a number of important factors affecting ionization and the formation of an appropriate spray. These include the solvent type, flow, composition and type of the capillary, applied potential, distance to counter-electrode, surface tension, analyte and electrolytes in the sample, as well as electrochemical processes at the probe tip [99]. The positioning of the CE capillary also needs to be optimized, where studies by Schramel *et al.* [97] have shown that the ideal position for the analysis of Se, Cu and Sb species was 0.5-0.7 mm outside the ESI stainless steel tip.

An undesirable effect in this type of analysis is the formation of ion-solvent clusters, where a single species may be split into multiple signals due to the solvent forming a gas and expanding in the vacuum [100]. This, along with electrolytic processes occurring at the tip of the ESI needle, may result in the transformation of chemical species [1]. In comparison to ICP-MS, ESI-MS also has poorer detection limits [103]. Ionization in the presence of salts is inefficient and further degrades these limits [46; 103]. For this reason it is often recommended that either SEC or RPLC is run with salt-free buffers, and that the analyte is pre-concentrated through freeze-drying prior to analysis [46].

4.3.2. Atomic Absorption Spectrometry (AAS)

Although Flame Atomic Absorption Spectroscopy (FAAS) offers a cheap alternative to a number of other techniques, detection limits are generally insufficient for the determination of environmentally relevant concentrations of elements [1; 29; 85]. The addition of matrix modifiers in the analysis of some elements may compromise species integrity and the high flow requirements of such systems also pose a

significant problem when coupled with other techniques such as HPLC [1]. For this reason, hydride Generation Gas Chromatography Atomic Absorption (HG-GC-AAS) techniques are often preferred, and are considered to be a valuable method for detecting hydride forming elements such as As [105] and Hg [106].

Graphite Furnace Atomic Absorption Spectrometry (GFAAS), on the other hand, requires only a few microliters of sample, offers low detection limits, is capable of analysing slurries and solids and requires little sample pre-treatment [1; 82; 85]. Caution in the use of this technique should be exercised to ensure that the samples are representative of the sample population and are homogenous [82]. It is important to note that GFAAS cannot be coupled to HPLC because the data points are generated too infrequently for peak identification [1; 85]. For these reasons, the use of AAS systems is uncommon in species characterization studies.

4.3.3. Inductively Coupled Plasma (ICP)

ICP technologies have the benefit of being capable of high sample throughput, multi-element detection, possess a wide linear dynamic range, exhibit long term stability and have greater sensitivity in comparison to many other techniques [107; 108]. In comparison to flame techniques, argon plasma techniques (such as ICP) are relatively inert with plasma temperatures reaching 10 000 K; breaking any molecular bonds; including those of refractory compounds which is typically not possible with techniques such as FAAS [1; 107].

4.3.3.1. Inductively Coupled Plasma Optical Emission Spectrometry (ICP-OES)

ICP-OES is one of the most common techniques used in the analysis of macroelements in plant biomonitoring studies. It is a multi-element technique where most modern instruments allow for simultaneous measurements of emission spectra, and background corrections can be easily applied through the software [1; 107]. It is a relatively robust technique and is utilised for a wide range of analytes from numerous applications [109] and is sometimes a preferred technique to ICP-MS in situations where monoisotopic elements are of interest. Most modern ICP-OES systems allow for the optimization of parameters such as plasma, auxiliary and nebulizer gas flows; sampling height; peristaltic pump flows; and RF power, all of which can significantly improve the detection limits of specific elements [107; 109].

In terms of environmental analytes, the detection limits of this technique are poor when compared with ICP-MS, although markedly superior to AAS. Interferences arising from many wavelengths of light being emitted simultaneously may occur [109]. Rare earth elements, for example have numerous emission wavelengths and are often poorly quantified at sub-ppm levels unless complex background corrections are applied [109]. The nebulizer and spray chamber needs to be carefully selected for each specific matrix, and argon humidifiers may be used in samples where salt concentrations are high. It is important to ensure that standards and samples are closely matrix-matched in both ICP-OES and ICP-MS analyses, where any deviations may result in signal drift [110]. Poor transport efficiency and tolerance of organic solvents can be dealt with through the use of ultrasonic or glass frit nebulizers [107] and cooled spray-chambers. Solvent loads on the plasma from organic matrices can be further reduced through aerosol thermostating, introduction of a condenser, use of a micro-HPLC column and increased RF power [107].

4.3.3.2. Inductively Coupled Plasma Mass Spectrometry (ICP-MS)

ICP-MS is often the favoured analytical technique for speciation studies due to its wide availability, low detection limits, wide dynamic range, versatility and ability to determine isotope ratios, which allows it to be used in source apportionment studies [64]. It is also easily interfaced with LC columns [84]. It is a highly sensitive, multi-element analytical technique, where analyte concentrations can be determined either sequentially (quadrupole/hexapole MS) or simultaneously (double-focussing or magnetic sector MS) [1; 5; 85].

As a stand-alone technique, ICP-MS can only provide information about the total metal content and not the oxidation state of the metal or its interactions in biological systems including the metal binding sites in biomolecules or its alkalyted form [5; 103]. Coupling to HPLC, however, allows for the identification of individual metallic species, which is achieved through the determination of the retention time of species [1]. Further advantages are the allowance of ICP-MS to remove difficult matrices on-line [85]. HPLC-ICP-MS requires samples to be in the liquid form and thus an extraction step is required for non-aqueous samples [7], and so the species of interest should be sufficiently stable so as to avoid species alterations [7; 46], as is the case in all speciation analyses. On-line detection systems reduce the chance of species alterations resulting from interaction time of the matrix with the analyte, and contamination in terms of storage and losses [1]. Off-line systems (fraction collection and subsequent delivery to detection), however, allow quality control checks to be maintained throughout the entire separation procedure [1]. To benefit from both of these aspects it is recommended that methods are developed off-line for overall quality control, and then adjusted for the on-line mode [1].

Disadvantages of ICP-MS are that it is not as robust as ICP-OES and has poor tolerance for total dissolved solids (TDS); typically in the range of 0.1-0.2 % [110]. High concentrations of acids in samples may also

have a negative effect on the lifetime of the sampling cones [1]. When using HF in sample digestion, for example, it is recommended that platinum cones be used [1]. Chloride ions which are present in HCl matrices are not only extremely corrosive but also pose a problem in the analysis of low concentrations of ^{75}As which has a polyatomic overlap of $^{40}Ar^{35}Cl$. Unfortunately, As is monoisotopic and so the use of an alternative isotope is not an option. Correction equations, collision or reaction gases may therefore be applied to reduce or remove this interference [84]. Some instruments have a Collision-Reaction Interface (CRI) which uses modified sampler and skimmer cones to eliminate these interferences prior to mass separation [49] whereas others use a collision or reaction cell prior to the quadrupole/hexapole.

Salt and carbon loading in the plasma from organic extraction media, as one often finds in HPLC techniques, may result in changes in the plasma temperature and consequently affect ionization. Resulting deposits on the cones from these matrices distort the ion beam and degrade the mass resolution of the instrument [102]. Several techniques are employed to reduce this, such as sample dilution, the use of ion-exchange columns, and the introduction of oxygen into the plasma to burn off excess carbon [1]. Physical changes, such as viscosity and surface tension may also affect sample transfer and analysis [1; 5].

In the development of an HPLC-ICP-MS method there are a number of parameters which require optimization. The column flow of the HPLC needs to be matched to the sample uptake rate of the ICP-MS, therefore requiring careful selection of the nebulizer and optimization of instrumental parameters [5; 102]. Nebulizers in this application are usually concentric, and in the form of high efficiency nebulizers (HEN) as these types have good reproducibility due to improved aspiration in comparison to the crossflow types [1; 5; 102]. Other types of couplings to ICP-MS are also possible such as CE-ICP-MS. In such cases, the electrical circuit at the end of the capillary and flow rates between the column and the ICP-MS need to be optimized.

5 Commonly assessed metals and metalloids

5.1 Arsenic

Arsenic is a carcinogenic metalloid [50] which occurs in the environment as a result of human activities and naturally through the weathering of arsenic based geological structures [47]. Prolonged exposure to low concentrations of bioavailable arsenic species has been linked to a number of toxic effects, the extent of which is dependent upon the speciation [47; 50].

In terrestrial environments, inorganic forms of As are dominant [50], toxic [21; 50; 73], and competitively inhibit phosphate metabolism [112]. As (V) is most commonly found in soils under oxidising conditions [50], making it the predominant form to be taken up by plants, however it may be reduced to lesser toxic arsenite via plant metabolic processes [48]. As (III), on the other hand is found under reducing conditions [50], has a high affinity for the thiol groups in proteins and consequently inactivates a number of enzymes [112].

Instrumental techniques for the separation of arsenic species have been primarily in the form of HPLC- or Gas Chromatography Hydride Generation Atomic Absorption Spectroscopy (GC-MS-HGAAS), HPLC or GC -ICP-MS, HPLC or GC -ICP-OES [112] or High Performance Liquid Chromatography Hydride Generation Atomic Fluorescence Spectroscopy (HPLC-HG-AFS) [113]. HPLC is the most common of these techniques in the separation of As species, where Ion Chromatography (IC) is mostly used for plant extracts [50]. By coupling these techniques with ICP-MS, detection is completed on-line and is highly sensitive [47; 50]. Other separation techniques used in the determination of non-volatile species are ion pairing liquid chromatography (IPLC) and SEC [47; 111]. Due to the variety of As compounds available in nature, adequate separation is sometimes difficult to achieve, where co-eluted peaks may require further separation and identification [50; 112].

An example of the use of HPLC-ICP-MS, shown in Table 1 is in the determination of arsenic species in lichens and plants near a decomissioned arsenic smelter site in Austria [65], whilst sequential extractions were used to separate As species in lichens to assess As air pollution in another study [77] (Table 1). Extractions were performed on powdered lichens using water, $CaCl_2$ and H_3PO_4 respectivelyIt was concluded that biotransformation of As may have occured, where the lichens were thought to methylate the As species as a protective measure.

XAS has also gained popularity in the analysis of As species as it allows for the direct analysis of As species in plant material with little sample preparation [50]. It is also able to provide an indication of the locality of As species within the complex plant matrix [50]. Detection limits with this method, however, are rather poor and are limited to the dominant As species [50]. Furthermore, this technique is not as widely available as ICP-MS and standards are difficult to come by. As shown in Table 1, Bergqvist *et al.* [29] investigated the presence of As in carrots, lettuce and spinach using XANES and HPLC-AAS. Organic forms of As were not found in any of the plant species and only inorganic forms were present. The concentrations of As were found to be higher in impacted sites and was linked to higher extractable As in the soils. However, the results from HPLC-AAS and XANES spectrometry did not correlate. Analysis via HPLC-AAS indicated that As (V) was the major form of As in the samples, whereas XANES readings found As (V) and As (III) to be more or less equally represented in the sample, which may be indicative of poor detection limits of HPLC-AAS for this species. Alternatively the extraction procedure used for HPLC-AAS itself may have altered the oxidative species of As in the sample.

Although to a much lesser extent, Capillary Electrophoresis (CE) [84] and on-line speciation using HPLC-HG-AFS [113] have also been explored as alternative methods for As speciation.

5.2. Selenium

Selenium is an essential micronutrient which is toxic at elevated concentrations [18; 47] and its uptake from soil is dependent on a number of parameters, including speciation [18]. As shown in Table 1, a number of different methods have been used in the determination of selenium species. A common method of detection is XAS, where short scan times reduce the likelihood of species alteration from redox reactions, and also prevents the volatilization of Se [18]. IPRP-HPLC-ICPMS is also commonly used [95]. In a study by Maneetong *et al.* [95], the most abundant form of Se in hydrophonically grown kale seedlings was selenomethionine (SeMet) which correlated with the findings by Eiche *et al.* [18]. In contrast to the latter study, however, Maneetong *et al.* [95] did not find any major inorganic species of Se. Other identified species were methyl L-selenocysteine (SeMC) and two unknown species. It was also found that these species of Se decreased after 15 days, which was attributed to biotransformation of the selenium species. The study by Eiche et al. [18] further proved that Se in these soils is highly bioavailable and that hyper-accumulation of the metal will occur. It was hypothesized that Se is taken up in the form of selenite and is stored and biotransformed in the root into the organic forms of Se. The two most mobile forms of Se; namely selenite and SeMet; where found to be in highest concentrations in the leaves, thus confirming their mobility. In comparison to concentrations found in the roots and leaves, Se in the stems were low; implying that this part of the plant is merely a passage between the roots and leaves.

Room temperature ionic liquids (RTILs) have been found to be an attractive additive in HPLC columns in the analysis of selenium species, as found in studies such those conducted by Chen *et al.* [86] which analysed Se species in yeast and clover, where good resolution of Se species could be achieved in 8 min. The RTILs did not alter the pH of the mobile phase and therefore ensured species integrity. No memory effects were observed and an injection volume of only 10 µL was required [88].

5.3. Antimony

The speciation of antimony in plants has also received some attention. Industrial sources of Sb are from mining and smelting where Sb is applied in the production of flame retardants, plastics, textiles, semiconductors and parasite drug treatments [92]. As shown in Table 1, a study by Koch *et al.* [92] found Sb (V) to be the dominant form of Sb in biota using HG-GC-AAS and headspace HG-GC-MS for

quantification. This finding was unexpected as this is the most stable oxidation state of Sb under normal environmental conditions, which should limit its mobility in biological systems therefore there may be some level of biotransformation of the oxidative species by the biomonitor organisms [92]. Antimony (III) and dimethylantimony were also found in moss from this study [92], however, some of the recoveries of Sb using this method were very poor; and varied from 0.7 % in *Funaria hygrometrica* to 95 % in *Typha latifolia*. This could point to some issues with sampling or extraction; for example, it was thought that the extraction method using methanol and water (1:1) may be inadequate to extract the antimony species. Furthermore, the use of sonication for 20 minutes could result in heat build-up and cause species alterations or losses. Although not considered in this study, the addition of the matrix modifier $NaBH_4$ in the confirmation of methylantimony species using HG-GC-AAS may also have introduced undesired effects on the antimony species present.

5.4. Cadmium

Cadmium commonly enters the environment from the burning of fossil fuels and municipal wastes [114] and is carcinogenic, teratogenic and mutagenic in high concentrations [89]. Its speciation in plants has been investigated both in terms of analytical method development [9] and translocation mechanisms in the plant itself [115]. Due to the biological and biochemical transformations of Cd in plant roots, the bioavailability and toxicity trends in roots are different to those in soil [116]. A study by Ueno *et al.* [115] identified Cd species using ^{113}Cd Nuclear Magnetic Resonance (NMR). In this study, the inorganic form of free Cd ions was found to be dominant, where 85.7 % was present as the free metal, 7.7 % was complexed with sulfate, 3.2 % was present as a Cd-citrate complex and 0.1 % was present as a Cd-Histidine (Cd-His) complex. This implied that the mobility of Cd is not dependent on the ability of the ion to complex with organic ligands, and rather suggests that the transfer of Cd to the xylem is an energy dependant process. It was also surmised that Cd uptake is competitively inhibited by Zn, which is likely due to Cd and Zn following similar metabolic pathways. There is also a close relationship between Fe and Cd homeostasis in plants, where Fe deficiency caused a significant increase in the amount of Cd present in the xylem sap. In this study, the main organic anion present in the xylem sap was citrate, followed by His. Cysteine and gluthathione metal complexes were below detection limits. A study by Połeć-Pawlak *et al.* [89] found that 89 % of cadmium accumulated in the roots and inhibited growth of root hairs. Furthermore, this study found that the majority of the accumulated cadmium was found within the water-soluble peptide fraction [89].

5.5. Lead

Lead enters the environment primarily through the production of lead batteries, paints and gasoline [117]. The toxicity of Pb is well documented and can affect every organ; although the toxic effects are primarily neurological [117]. A study by Schreck *et al.* [32] investigated smelter activities in battery

recycling operations as potential sources of Pb air pollution. Lead containing particulate matter (PM) within and on the surface of leaves were determined using Micro-X-ray Fluorescence (μXRF), Scanning Electron Microscopy coupled with Energy Dispersive X-ray Microanalysis (SEM-EDX) and Time-of-Flight Secondary Ion Mass Spectrometry (TOF-SIMS). The species identified within this study were $PbSO^{2+}$, PbO^{+} and $PbCO^{+}$ and Pb^{+} [32].

5.6. Manganese

Manganese is ubiquitous in the environment and is commonly used in the production of steel and aluminium alloys, and has also been introduced into gasoline as a lead replacement [118]. Fractionation of Mn species is used in bioavailability and mobility studies in food crops, such as hazelnuts, as shown in Table 1 [17], where Mn species were determined using HPLC-ICP-MS. Mn was extracted using fractionation methods such as water, diethyl ether, n-hexane and methanol. In this study, most of the Mn present was found to be associated with proteins (54-87 %) with a small component associated with oils.

5.7. Nickel

Nickel is commonly assessed in metal speciation studies. It is released into the environment through activities such as battery production, electroplating, development of alloys and colouring of ceramics [119]. A study by Alves *et al.* [120] determined the speciation of Ni in xylem sap using Square Wave Voltammetry (SWV), where citric, oxalic, malic and malonic acids were found to have the greatest effect on species distributions. Nickel has also been assessed in hyper-accumulating plant species, *T. goesingense* and *T. arvense,* using XANES [41]. It was found that 87 % of the Ni which was not bound to the cell wall was chelated by citrate, and thus confirmed that Ni-organic complexes play an important role in Ni tolerance mechanisms. This study also found that 25 % of intracellular Ni was bound to histidine, and this bond was thought to assist the movement of Ni from the cytoplasm into the vacuole. At low pHs it was found that Ni-His complexes were destabilized and resulted in Ni ions favouring complex formation with organic acids such as citrate. It was noted that the accumulation of Ni by a non-accumulator takes place in the cytoplasm, and often causes the death of the plant [41].

Nickel speciation has also been assessed in a metal hyper-accumulating tree species, *Sebertia acuminate,* which is endemic to New Caledonia [90]. This study by Schaumöffel *et al.* [90] used complementary techniques such as SE-HPLC-ESI-MS-MS and CZE-ICP-MS to accurately quantify various Ni species at both the molecular and elemental level. Stable nickel compounds such as nicotianamine and nickel citrate complexes were identified along with 5 other stable complexes, implying that this

technique cannot be used for the identification of non-stable complexes. It was also found that a combination of these methods was required for adequate separation and identification of nickel species. This study recommended that future studies involve a Quadrupole Time of Flight Mass Spectrometer (QTOF-MS) or ESI-MS for more sensitive and accurate measurements of less concentrated Ni complexes. Similar experiments were conducted by Vacchina *et al.* [4], regarding a different plant species, *T. caerulescens* [4] using SE-HPLC-ICP-MS and SE-HPLC-ESI-MS. It was found that metal complexes could be identified through the complementary use of HPLC-ICP-MS, CZE-ICPMS and ESI-MS-MS. Results from CZE-ICP-MS showed that the nickel species present was Ni (II) and a second unidentified nickel peak was thought to be a stable nickel complex. This complex was further characterized using ESI-MS-MS and was found to be a previously unreported nickel compound which is produced by this plant assumedly in response to nickel stress.

6 Conclusions and future outlook

It is clear that the application of plant speciation studies is extremely important in a number of fields, however the accurate quantification of metallic species is a complex and challenging task. Quality control is critical in speciation analyses, which highlights the use of certified reference materials (CRMs), whenever possible, to improve reliability of analytical results. In the absence of a suitable CRM, physically different methods should be employed for comparison.

Future directions in metal speciation studies are likely to focus on the development of improved sample preparation and analytical techniques so as to ensure that the original species profile is not altered. Literature reports on speciation of metals in plant materials have focused on a limited number of elements, primarily As, Cd, Mn, Ni, Pb, Se and Sb. In view of the importance of speciation studies highlighted here, future studies are likely to focus on additional elements which are of toxiciological and environmental relevance. Moreover, speciation studies are also likely to become widely applied in the evaluation of food commodities with plant origins; the biofortification of desired chemical species in food crops; and is also likely to find a place in the development of pharmaceuticals since bioavailability and metabolic pathways are dependent upon chemical speciation.

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References:

1. B. Michalke, Element speciation definitions, analytical methodology, and some examples, Ecotoxicol. Environ. Safe. 56 (2003) 122-139.

2. T. Ressler, J. Wong, J. Roos, I.L. Smith, Quantitative speciation of Mn-bearing particulates emitted from autos burning (Methylcyclopentadienyl)manganese tricarbonyl-added gasolines using XANES Spectroscopy, Environ. Sci. Technol. 34 (2000) 950-958.

3. A.J. Fernández Espinosa, M.T. Rodriguez, F.J.B. de la Rosa, J.C. Jiménez Sánchez, A chemical speciation of trace metals for fine urban particles, Atmos. Environ. 36 (2002) 773-780.

4. V. Vacchina, S. Mari, P. Czemic, L. Marqués, K. Pianelli, D. Schaumlöffel, M. Lebrun, R. Łobinski, Speciation of nickel in a hyperaccumulating plant by high-performance liquid chromatography-inductively coupled plasma mass spectrometry and electrospray MS/MS assisted by cloning using yeast complementation, Anal. Chem. 75 (2003) 2740-2745.

5. R. Thomas, Practical guide to ICP-MS: A tutorial for beginners, second ed., CRC Press, London, 2008.

6. M. Stanislawska, B. Janasik, W. Wasowicz, Application of high performance liquid chromatography with inductively coupled plasma mass spectrometry (HPLC-ICP-MS) for determination of chromium compounds in air at the workplace, Talanta 117 (2013) 14-19.

7. M. Gräfe, E. Donner, R.N. Collins, E. Lombi, Speciation of metal(loid)s in environmental samples by x-ray absorption spectroscopy: A critical review, Anal. Chim. Acta 822 (2014) 1-22.

8. EVISA, Speciation as a discipline in Analytical Chemistry – Definitions. EVISA. [Online] [Cited: 26 08 2015.] http://www.speciation.net/Public/Document/2003/09/11/495.html.

9. V. Vacchina, K. Połeć, J. Szpunar, Speciation of cadmium in plant tissues by size-exclusion chromatography with ICP-MS detection, J. Anal. Atom. Spectrom. 14 (1999) 1557-1566.

10. C.H. Walker, S.P. Hopkin, R.M. Sibly, D.B. Peakall, Principles of Ecotoxicology, third ed., Taylor & Francis Group, USA, 2006.

11. M. Marchese, A.M. Gagneten, M.J. Parma, P.J. Pavé, Accumulation and elimination of chromium by freshwater species exposed to spiked sediments, Arch. Environ. Contam. Toxicol. 55 (2008) 603-609.

12. P.R. Palaniappan, S. Karthikeyan, Bioaccumulation and depuration of chromium in the selected organs and whole body tissues of freshwater fish, *Cirrhinus mrigala*, individually and in binary solutions with nickel, J. Environ. Sci. 21 (2009) 229-236.

13. A. Valvanidis, T. Vlachogianni, Metal pollution in ecosystems. Ecotoxicology studies and risk assessment in the marine environment, Sci. Advance. Environ. Toxicol. Pharmacol. 21 (2010) 241-245.

14. G. Rejomon, M. Nair, T. Joseph, Trace metal dynamics in fishes from the southwest coast of India, Environ. Monit. Assess. 2010 (2010) 243-255.

15. S. Loppi, S.A. Pirintsos, Epiphytic lichens as sentinels for heavy metal pollution at forest ecosystems (central Italy), Environ. Pollut. 121 (2003) 327-332.

16. E. Nieboer, P. Lavoie, R.L.P. Sasseville, K.J. Puckett, D.H.S. Richardson, Cation-exchange equilibrium and mass balance in the lichen *Umbilicaria muhlenbergii*, Can. J. Botany 54 (1976) 720-723.

17. U.S. Erdemir, S. Gucer, Fractionation analysis of manganese in turkish hazelnuts (*Corylus avellana L.*) by inductively coupled plasma-mass spectrometry, J. Agric. Food Chem. 62(2014) 10792-10799.

18. E. Eiche, F. Bardelli, A.K. Nothstein, L. Charlet, J. Göttlicher, R. Steininger, K.S. Dhillon, U.S. Sadana, Selenium distribution and speciation in plant parts of wheat (*Triticum aestivum*) and Indian mustard (*Brassica juncea*) from a seleniferous area of Punjab, India, Sci. Total Environ. 505 (2015) 952-961.

19. CFIA, Canadian Food Inspection Agency, Government of Canada, [Online] 2015. [Cited: 24 08 2015.] http://www.inspection.gc.ca/food/fresh-fruits-and-vegetables/food-safety/sampling-fresh-fruit-and-vegetables/eng/1353610539095/1353610619804.

20. C. Rodriguez Álvarez, M. Jiménez Moreno, F.J. Guzmán Bernado, R.C. Rodriguez Martin-Doimeadios, J.J. Berzas Nevado, Mercury methylation, uptake and bioaccumulation by the earthworm *Lumbricus terrestris* (Oligochaeta), Appl. Soil Ecol. 84 (2014) 45-53.

21. A. Machado, Z. Šlejkovec, J.T. van Elteren, M.C. Freitas, M.S. Baptista, Arsenic speciation in transplanted lichens and tree bark in the framework of a biomonitoring scenario, J. Atmos. Chem. 53 (2006) 237-249.

22. T. Punshon, M.L. Guerinot, A. Lanzirotti, Using synchrotron X-ray fluorescence microprobes in the study of metal homeostasis in plants, Ann. Bot. –London, 103 (2009) 665-672.

23. L. Connell, P. Lam, B. Richardson, R. Wu, Introduction to Ecotoxicology, first ed., Blackwell Science Ltd., Oxford, 1999.

24. T.V. Nedelkoska, P.M. Doran, Characteristics of heavy metal uptake by plant species with potential for phytoremediation and phytomining, Miner. Eng. 13 (2000) 549-561.

25. R. Figueira, P.C. Tavares, L. Palma, P. Beja, C. Sérgio, Application of indicator kriging to the complementary use of bioindicators at three trophic levels, Environ. Pollut. 157 (2009) 2689-2696.

26. K.I. Kularatne, C.R. de Freitas, Ephiphytic lichens as biomonitors of airborne heavy metal pollution, Environ. Exp. Bot. 88 (2013) 24-32.

27. R. van der Oost, J. Beyer, N.P. Vermeulen, Fish bioaccumulation and biomarkers in environmental risk assessment: a review, Environ. Toxicol. 13 (2003) 57-149.

28. B. Baraj, L.F. Niencheski, G. Fillmann, L. Hermanns, Biochemical normalization of trace metals in *Arctocephalus australis*, Braz. J. Oceanog. 57 (2009) 1-6.

29. C. Bergqvist, R. Herbert, I. Persson, M. Greger, Plants influence on arsenic availability and speciation in the rhizosphere, roots and shoots of three different vegetables, Environ. Pollut. 184 (2014) 540-546.

30. P.H. Kansanen, J. Venetvaara, Comparison of biological collectors of airborne heavy metals near ferrochrome and steel works, Water, Air, Soil Pollut. 60 (1991) 337-359.

31. F. de Nicola, V. Spagnuolo, D. Baldantoni, L. Sessa, A. Alfani, R. Bargagli, F. Monaci, S. Terracciano, S. Giordano, Improved biomonitoring of airborne contaminants by combined use of holm oak leaves and epiphytic moss, Chemosphere 92 (2013) 1224-1230.

32. E. Schreck, Y. Foucault, G. Sarret, S. Sobanska, L. Cécillon, M. Castrec-Rouelle, G. Uzu, C. Dumat, Metal and metalloid foliar uptake by various plant species exposed to atmospheric industrial fallout: Mechanisms involved for lead, Sci. Total Environ. 427-428 (2012) 253-262.

33. M. Lenka, K.K. Panda, B.B. Panda, Studies on the ability of water hyacinth (*Eichhornia crassipes*) to bioconcentrate and biomonitor aquatic mercury, Environ. Pollut. 66 (1990) 89-99.

34. J.Yoon, Z. Cao, Q. Zhou, L.Q. Ma, Accumulation of Pb, Cu, and Zn in native plants growing on a contaminated Florida site, Sci. Total. Environ. 368 (2006) 456-464.

35. W. Wisłocka, J. Krawczyk, A. Klink, L. Morrison, Bioaccumulation of heavy by selected plant species from uranium mining dumps in the Sudty Mts., Poland, Pol. J. Environ. Stud. 15 (2006) 811-818.

36. B. Robinson, S. Green, T. Mills, B. Clothier, M. van der Velde, R. Laplane, L. Fung, M. Deurer, S. Hurst, T. Thayalakumaran, C. van den Dijssel, Phytoremediation: using plants as biopumps to improve degraded environments, Aust. J. Soil Res. 41 (2003) 599-611.

37. W. Zhang, Y. Cai, C. Tu, L.Q. Ma, Arsenic speciation and distribution in an arsenic hyperaccumulating plant, Sci. Total Environ. 300 (2002) 167-177.

38. R.L. Knight, V.W.E. Payne Jr., R.C. Clarke Jr., J.H. Pries, Constructed wetlands for livestock wastewater management, Ecol. Eng. 15 (2000) 41-55.

39. E.J. Dunne, N. Culleton, G. O'Donovan, R. Harrington, A.E. Olsen, An integrated constructed wetland to treat contaminants and nutrients from dairy farmyard dirty water, Ecol. Eng. 24 (2005) 219-232.

40. N. Gottschall, C. Boutin, A. Crolla, C. Kinsley, P. Champagne, The role of plants in the removal of nutrients at a constructed wetland treating agricultural (dairy) wastewater, Ontario, Canada, Ecol. Eng. 29 (2007) 154-163.

41. U. Krämer, I.J. Pickering, R.C. Prince, I. Raskin, D.E. Salt, Subcellular localization and speciation of nickel in hyperaccumulator and non-accumulator *Thlaspi* species, Plant Physiol. 122 (2000) 1343-1353.

42. J.S. Weis, P. Weis, Metal uptake, transport and release by wetland plants: implications for phytoremediation and restoration, Environ. Int. 30 (2004) 685-700.

43. Y. Madrid, C. Cámara, Biological substrates for metal preconcentration and speciation, Trends Anal. Chem. 16 (1997) 36-44.

44. F. Ekmekyapar, A. Aslan, Y.K. Bayhan, A. Cakici, Biosorption of copper (II) by nonliving lichen biomass of *Cladonia rangiformis hoffm,* J. Hazard. Mater. B 137 (2006) 293-298.

45. O.D. Uluozlu, A. Sari, M. Tuzen, M. Soylak, Biosorption of Pb(II) and Cr(III) from aqueous solution by lichen (*Parmelina tiliaceae*) biomass, Bioresour. Technol. 99 (2008) 2972-2980.

46. R. Łobiński, J. Szpunar, Biochemical speciation analysis by hyphenated techniques, Anal. Chim. Acta. 400 (1999) 321-332.

47. K. Wrobel, K. Wrobel, J.A. Caruso, Pretreatment procedures for characterization of arsenic and selenium species in complex samples utilizing coupled techniques with mass spectrometric detection, Anal. Bioanal. Chem. 381 (2005) 317-331.

48. R. Larios, R. Fernández-Martínez, I. LeHecho, I. Rucandio, A methodological approach to evaluate arsenic speciation and bioaccumulation in different plant species from two highly polluted mining areas, Sci. Total Environ. 414 (2012) 600-607.

49. C.D. Amaral, J.A. Nóbrega, A.A. Noueira, Investigation of arsenic species stability by HPLC-ICP-MS in plants stored under different conditions for 12 months, Microchem. J. 117 (2014) 122-126.

50. M. Quaghebeur, Z. Rengel, Arsenic speciation governs arsenic uptake and transport in terrestrial plants, Microchim. Acta 151 (2005) 141-152.

51. F.J. Doucet, J. Carignan, Atmospheric Pb isotope composition and trace metal concentration as revealed by epiphytic lichens: an investigation related to two altitudinal sections in Eastern France, Atmos. Environ. 35 (2001) 3681-3690.

52. A. Bari, A. Rosso, M.R. Minciardi, F. Troiani, R. Piervittori, Analysis of heavy metals in atmospheric particulates in relation to their bioaccumulation in explanted *Pseudevernia furfuracea* thalli, Environ. Monit. Assess. 69 (2001) 205-220.

53. A. Ure, Chemical Speciation in the Environment, second ed., Blackwell Science, Oxford, 2002.

54. S.H. Boltersdorf, R. Pesch, W. Werner, Comparative uses of lichens, mosses and tree bark to evaluate nitrogen deposition in Germany, Environ. Pollut. 189 (2014) 43-53.

55. J. Bustamante, O. Liñero, I. Arrizabalaga, J.A. Carrero, G. Arana, A. de Diego, Sample pretreatment to differentiate between bioconcentration and atmospheric deposition of polycyclic aromatic hydrocarbons in mosses, Chemosphere 122 (2015) 295-300.

56. V. Spagnuolo, S. Terracciano, S. Giordano, Trace element content and molecular biodiversity in the epiphytic moss *Leptodon smithii*: Two independent tracers of human disturbance, Chemosphere 74 (2009) 1158-1164.

57. M.V. Balarama Krishna, J. Arunachalam, Ultrasound-assisted extraction procedures for the fast estimation of major, minor and trace elements in lichen and mussel samples by ICP-MS and ICP-AES, Anal. Chim. Acta 522 (2004) 179-187.

58. A. Huerga, I. Lavilla, C. Bendicho, Speciation of the immediately mobilisable As (III), As (V), MMA and DMA in river sediments by high performance liquid chromatography-hydride generation-atomic fluorescence spectrometry following ultrasonic extraction, Anal. Chim. Acta 534 (2005) 121-128.

59. J. Laurette, C. Larue, I. Llorens, D. Jaillard, P.H. Jouneau, J. Bourguignon, M. Carriére, Speciation of uranium in plants upon root accumulation and root-to-shoot translocation: A XAS and TEM study, Environ. Exp. Bot. 77 (2012) 87-95.

60. P. Pasławski, Z.M. Migaszewski, The quality of element determinations in plant materials by instrumental methods, Pol. J. Environ. Stud. 15 (2006) 154-164.

61. J.L. Gómez-Ariza, E. Morales, I. Giráldez, D. Sánchez-Rodas, A. Velasco, Sample treatment in chromatography-based speciation of organometallic pollutants, J. Chromatog. A 938 (2001) 211-224.

62. G. Raber, N. Stock, P. Hanel, M. Murko, J. Navratilova, K.A. Francesconi, An improved HPLC-ICPMS method for determining inorganic arsenic in food: Application to rice, wheat and tuna fish, Food Chem. 134 (2012) 524-532.

63. P. Adamo, S. Giordano, S. Vingiani, R. Castaldo Cobianchi, P. Violante, Trace element accumulation by moss and lichen exposed in bags in the city of Naples (Italy), Environ. Pollut. 122 (2003) 91-103.

64. M. Kunert, K. Friese, V. Weckert, B. Markert, Lead isotope systematics in *Polytrichum formosum*: An example from a biomonitoring field study with mosses, Environ. Sci. Technol. 33 (1999) 3502-3505.

65. D. Kuehnelt, J. Lintschinger, W. Goessler, Arsenic compounds in terrestrial organisms. IV. Green plants and lichens from an old arsenic smelter site in Austria, Appl. Organomet. Chem. 14 (2000) 411-420.

66. S. Loppi, L. Frati, L. Paoli, V. Bigagli, C. Rosetti, C. Bruscoli, A. Corsini, Biodiversity of epiphytic lichens and heavy metal contents of *Flavoparmelia caperata* thalli as indicators of temporal variations of air pollution in the town of Montecatini Terme (central Italy), Sci. Total Environ. 326 (2004) 113-122.

67. D.D. Ebbing, S.D. Gammon, General Chemistry, seventh ed., Houghton Mifflin Company, Boston, 2002.

68. T. Hjorth, Effects of freeze-drying on partitioning patterns of major elements and trace metals in lake sediments, Anal. Chim. Acta 526 (2004) 95-102.

69. T. Punshon, B.P. Jackson, A. Lanzirotti, W.A. Hopkins, P.M. Bertsch, J. Burger, Application of synchrotron x-ray beam microbeam spectroscopy to the determination of metal distribution and speciation in biological tissues, Spectrosc. Lett. 38 (2005) 343-363.

70. G. Sarret, E.A.H. Pilon Smits, H. Castillo Michel, M.P. Isaure, F.J. Zhao, R. Tappero, Use of synchrotron-based techniques to elucidate metal uptake and metabolism in plants, in: Advances in Agronomy. Elsevier Inc. 2013 , pp. 1-82.

71. A.J. Fernández, M. Ternero, F.J. Barragán, J.C. Jiménez, An approach to characterization of sources of urban airborne particles through heavy metal speciation, Chemosphere 2 (2000) 123-136.

72. A. Tessier, P.G. Campbell, M. Bisson, Sequential extraction procedure for the speciation of particulate trace metals, Anal. Chem. 51 (1979) 844-851.

73. T. Guerin, N. Molenat, A. Astruc, R. Pinel, Arsenic speciation in some environmental samples: a comparative study of HG-GC-QFAAS and HPLC-ICP-MS methods, Appl. Organomet. Chem. 14 (2000) 401-410.

74. M.B. Arain, T.G. Kazi, M.K. Jamali, N. Jalbani, H.I. Afridi, J.A. Baig, Speciation of heavy metals in sediment by conventional, ultrasound and microwave assisted single extraction methods: A comparison with modified sequential extraction procedure, J. Hazard. Mater. 154 (2008) 998-1006.

75. A.J. Fernández-Espinosa, M. Ternero-Rodriguez, Study of traffic pollution by metals in Seville (Spain) by physical and chemical speciation methods, Anal. Bioanal. Chem. 379 (2004) 684-699.

76. A.D. Banerjee, Heavy metals and solid phase speciation in street dusts of Delhi, India, Environ. Pollut. 123 (2003) 95-105.

77. M.M. Farinha, Z. Šlejkovec, J.T. van Elteren, H.Th. Wolterbeek, M.C. Freitas, Arsenic speciation in lichens and in coarse and fine airborne particulate matter by HPLC-UV-HG-AFS, J. Atmos. Chem. 49 (2004) 343-353.

78. C. Bendicho, I. Lavilla, Ultrasound-assisted metal extraction, Academic Press 3 (2000) 4421-4426.

79. T.G. Kazi, N. Jalbani, M.B. Arain, M.K. Jamali, H.I. Afridi, A.Q. Shah, Determination of toxic elements in different brands of cigarette by atomic absorption spectrometry using ultrasonic assisted acid digestion, Environ. Monit. Assess. 154 (2009) 155-167.

80. M. Blasco, C. Domeño, C. Nérin, Use of lichens as pollution biomonitors in remote areas: comparison of PAHs extracted from lichens and atmospheric particles sampled in and around the Somport tunnel (Pyrenees), Environ. Sci. Technol. 40 (2006) 6384-6391.

81. C. Domeño, M. Blasco, C. Sánchez, C. Nerín, A fast extraction technique for extracting polycyclic aromatic hydrocarbons (PAHs) from lichens samples used as biomonitors of air pollution: Dynamic sonication versus other methods, Anal. Chim. Acta 569 (2006) 103-112.

82. R.A. Sánchez-Moreno, M.J. Gismera, M.T. Sevilla, J.R. Procopio, Direct and rapid determination of ultratrace heavy metals in solid plant materials by ET-AAS ultrasonic-assisted slurry sampling, Phytochem. Anal. 21 (2010) 340-347.

83. J. Namiesnik, P. Szefer, Preparing samples for analysis-the key to analytical success, Ecol. Chem. Eng. 15 (2008) 431-474.

84. H. Qu, T.K. Mudalige, S.W. Linder, Arsenic speciation in rice by capillary electrophoresis/inductively coupled plasma mass spectrometry: Enzyme-assisted water-phase microwave digestion, J. Agric. Food Chem. 63 (2015) 3153-3160.

85. J.J. Thompson, R.S. Houk, Inductively coupled plasma mass spectrometric detection for multielement flow injection analysis and elemental speciation by Reversed-Phase Liquid Chromatography, Anal. Chem. 58 (1986) 2541-2548.

86. B. Chen, M. He, X. Mao, R. Cui, D. Pang, B. Hu, Ionic liquids improved reversed-phase HPLC on-line coupled with ICP-MS for selenium speciation, Talanta 83 (2011) 724-731.

87. N.P. Vela, L.K. Olson, J.A. Caruso, Elemental speciation with plasma mass spectrometry, Anal. Chem. 65 (1993) 585-597.

88. E.R. McKenzie, T.M. Young, Quality control considerations for size exclusion chromatography with online ICP-MS: a powerful tool for evaluating the size dependence of metal-organic matter complexation, Water Sci. Technol. 67 (2013) 1075-1082.

89. K. Połeć-Pawlak, R. Ruzik, K. Abramski, M. Ciurzyńska, H. Gawrońska, Cadmium speciation in *Arabidopsis thaliana* as a strategy to study metal accumulation system in plants, Anal. Chim. Acta 540 (2005) 61-70.

90. D. Schaumlöffel, L. Ouerdane, B. Bouyssiere, R. Łobiński, Speciation analysis of nickel in the latex of a hyperaccumulating tree *Sebertia acuminata* by HPLC and CZE with ICP-MS and electrospray MS-MS detection, J. Anal. Atom. Spectrom. 18 (2003) 120-127.

91. M. Montés-Bayón, M.J. Molet, E.B. González, A. Sanz-Medel, Evaluation of different sample extraction strategies for selenium determination in selenium-enriched plants (*Allium sativum* and *Brassica juncea*) and Se speciation by HPLC-ICP-MS, Talanta 68 (2006) 1287-1293.

92. I. Koch, L. Wang, J. Feldmann, P. Andrewes, K.J. Reimer, W.R. Cullen, Antimony species in environmental samples, Int. J. Environ. An. Ch. 77 (2000) 111-131.

93. D. Nette, A. Seubert, Determination of aminopolycarboxylic acids at ultra-trace levels by means of online coupling ion exchange chromatography and inductively coupled plasma-mass spectrometry with indirect detection via their Pd^{2+}-complexes, Anal. Chim. Acta 884 (2015) 124-132.

94. M. Schönbächler, M.A. Fehr, Basics of ion exchange chromatography for selected geological applications in: Treatise on Geochemistry, second ed., 2014, pp. 123-146.

95. S. Maneetong, S. Chookhampaeng, A. Chantiratikul, O. Chinrasri, W. Thosaikham, R. Sittipout, P. Chantiratikul, Hydrophonic cultivation of selenium-enriched kale (*Brassica oleracea var. alboglabra L*.) seedling and speciation of selenium with HPLC-ICP-MS, Microchem. J. 108 (2013) 87-91.

96. V.L. Dressler, F.G. Antes, C.M. Moreira, D. Pozebon, F.A. Duarte, As, Hg, I, Sb, Se and Sn speciation in body fluids and biological tissues using hyphenated-ICP-MS techniques: A review, Int. J. Mass Spectrom. 307 (2011) 149-162.

97. O. Schramel, B. Michalke, A. Kettrup, Analysis of metal species by using electrospray ionization mass spectrometry and capillary electrophoresis-electrospray ionization mass spectrometry, J. Chromatog. A 819 (1998) 231-242.

98. G.A. Zachariadis, *In situ* derivatization of metals and organometallic using borate reagents in gas chromatographic speciation studies, J. Chromatog. A 1296 (2013) 47-69.

99. E. de Hoffmann, V. Stroobant, Mass Spectrometry: Principles and Applications, third ed.,John Wiley & Sons Ltd, Sussex, 2007.

100. I.L. Stewart, Electrospray mass spectrometry: a tool for elemental speciation, Spectrochim. Acta B 54 (1999) 1649-1695.

101. A.L. Rosen, G.M. Hieftje, Inductively coupled plasma mass spectrometry and electrospray mass spectrometry for speciation analysis: applications and instrumentation, Spectrochim. Acta B 59 (2004) 135-146.

102. Q. Tu, T. Wang, C.J. Welch, P. Wang, X. Jia, C. Raab, X. Bu, D. Bykowski, B. Hohenstaufen, M.P. Doyle, Identification and characterization of isomeric intermediates in a catalyst formation reaction by means of speciation analysis using HPLC-ICPMS and HPLC-ESIMS, Anal. Chem. 78 (2006) 1282-1289.

103. Z. Chen, G. Owens, K. Kim, R. Naidu, Confirmation of lead aminocarboxylic complex formation using electrospray ionization mass spectrometry and speciation by anion exchange chromatography coupled with ICP-MS, Anal. Chim. Acta 599 (2007) 163-169.

104. A. Helfrich, J. Bettmer, Analysis of gold nanoparticles using ICP-MS-based hyphenated and complementary ESI-MS techniques, Int. J. Mass Spectrom. 307 (2011) 92-98.

105. A. Hernández-Zavala, T. Matoušek, Z. Drobná, D.D. Paul, F. Walton, B.M. Adair, D. Jiři, D.J. Thomas, M. Stýblo, Speciation analysis of arsenic in biological matrices by automated hydride-generation-cryotrapping-atomic absorption spectrometry with multiple microflame quartz tube atomizer (multiatomizer), J. Anal. Atom. Spectrom. 23 (2008) 342-351.

106. A.M. Carro, M.C. Mejuto, Application of chromatographic and electrophoretic methodology to the speciation of organomercury compounds in food analysis, J. Chromatog. A 882 (2000) 283-307.

107. X. Hou, B.T. Jones, Inductively Coupled Plasma/Optical Emission Spectrometry, In: R. A. Meyers, ed. Encyclopedia of Analytical Chemistry, John Wiley & Sons Ltd.,Chichester, 2000, pp. 9468-9485.

108. S. Marin, S. Lăcrimioara, R. Cecilia, Evaluation of performance parameters for trace element analysis in perennial plants using ICP-OES technique, J. Plant. Develop. 18 (2011) 87-93.

109. J. Nölte, ICP Emission Spectrometry: A Practical Guide, first ed., Wiley-VCH, Weinheim, 2003.

110. A.A. Ammann, Inductively coupled plasma mass spectrometry (ICP-MS): a versatile tool, J. Mass Spectrom. 42 (2007) 419-427.

111. T. Guerin, A. Astruc, M. Astruc, Speciation of arsenic and selenium compounds by HPLC hyphenated to specific detectors: a review of the main separation techniques, Talanta 50 (1999) 1-24.

112. V.M. Dembitsky, T. Rezanka, Natural occurrence of arseno compounds in plants, lichens, fungi, algal species, and microorganisms, Plant Sci. 165 (2003) 1177-1192.

113. S.S. Farías, A. Londonio, C. Quintero, R. Befani, M. Soro, P. Smichowski, On-line speciation and quantification of four arsenical species in rice samples collected in Argentina using a HPLC-HG-AFS coupling, Microchem. J. 120 (2015)34-39.

114. ATSDR, Agency for Toxic Substances and Disease Registery, Toxicological profile for cadmium, U.S. Department of Health and Human Services, Atlanta, 1997.

115. D. Ueno, T. Iwashita, F.J. Zhao, J.F. Ma, Characterization of Cd translocation and identification of the Cd form in xylem sap of the Cd- Hyperaccumulator *Arabidopsis halleri*, Plant Cell Physiol. 49 (2008) 540-548.

116. L. Hu, M.B. McBride, H. Cheng, J. Wu, J. Shi, J. Xu, L. Wu, Root-induced changes to cadmium speciation in the rhizosphere of two rice (*Oryza sativa L*.) genotypes, Environ. Res. 111 (2001) 356-361.

117. ATSDR, Agency for Toxic Substance and Disease Registry, Case studies in environmental medicine: Lead toxicity, U.S. Department of Health and Human Services, Atlanta, 2010.

118. B. Michalke, S. Halback, V. Nischwitz, Speciation and toxicological relevance of manganese in humans, J. Environ. Monitor. 9 (2007) 650-656.

119. ATSDR, Agency for Toxic Substances and Disease Registry**,** ToxGuide TM for Nickel, Department of Health and Human Services, Atlanta, 2005.

120. S. Alves, C. Nabais, M.D.L. Gonçalves, M.M. Correia dos Santos, Nickel speciation in the xylem sap of the hyperaccumulator *Alyssum serpyllifolium ssp. lusitanicum* growing on serpentine soils of northeast Portugal, J. Plant Physiol. 168 (2011) 1715-1722.