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INYUVESI YAKWAZULU-NATALI

Identification and quantification of chelidonic acid and other ligands with potential for Ni uptake in the hyperaccumulator, *Berkheya coddii*

by

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As the candidate's supervisor I have/have not approved this thesis/dissertation for submission.

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Abstract

B. coddii is a known nickel hyperaccumulator, a plant that is able to take up high levels of Ni (< 37 000 mg kg $^{-1}$) without exhibiting toxic effects. It grows in serpentine soil and is endemic to the Barberton Region, Mpumulanga Province, South Africa.

One of the key factors governing hyperaccumulators is the mechanism of uptake. This study investigates the uptake mechanism of *B. coddii*. Plants and soil from this region were collected for analysis and uptake studies (Ni spiking).

A characterisation of amino and organic acids, both commonly associated with uptake in hyperaccumulators, was carried out using a range of chromatographic techniques namely, reverse phase-high performance liquid chromatography (RP-HPLC) and liquid chromatography-mass spectrometry (LC-MS). Amino acid analysis results showed a high proline concentration (12 400 mg kg^{-1}) in younger leaves of *B. coddii*, and organic acid results showed high levels of ascorbic acid (2100 mg kg^{-1}) present in the stem, indicating a stress response to high levels of Ni. Analysis carried out on *B. coddii*, using methods of size exclusion chromatography (SEC), high resolution-mass spectrometry (HR-MS) and HPLC indicated that the ligand responsible for Ni uptake is the organic acid, chelidonic acid.

This study has shown that a leaf-water extract contains chelidonic acid in a 3:1 molar ratio to Ni. Nickel uptake studies were performed to further investigate the relationship between Ni and chelidonic acid. Upon treatment of *B. coddii* with soluble Ni the concentrations of Ni and chelidonic acid increased. This resulted in an increase of the molar ratio between chelidonic acid and Ni to 6.76:1.

Preface

The experimental work described in this dissertation was carried out in the School of Chemistry & Physics, University of KwaZulu-Natal, Durban, from March 2013 to June 2014, under the supervision of Dr. L Pillay and Prof. A Kindness.

These studies represent original work by the author and have not otherwise been submitted in any form for any degree or diploma to any tertiary institution. Where use has been made of the work of others it is duly acknowledged in the text.

HR-MS work was carried out by H.Fletcher at the Central Analytical Facility (Stellenbosch).

Amino acid analyses were carried out by M. Patience the Central Analytical Facility (Stellenbosch).

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DECLARATION 1 - PLAGIARISM

I, Chrisanne Naicker, declare that:

1. The research reported in this thesis, except where otherwise indicated, is my original research.

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Signed: _____

Chrisanne Naicker

For you, my dearest uncle Eddie, my source of happiness for memorable 16 years

Death be not proud, though some have called thee
Mighty and dreadfull, for, thou art not soe,
For, those, whom thou think'st, thou dost overthrow,
Die not, poore death, nor yet canst thou kill mee.
From rest and sleepe, which but thy pictures bee,
Much pleasure, then from thee, much more must flow,
And soonest our best men with thee doe goe,
Rest of their bones, and soules deliverie.
Thou art slave to Fate, Chance, kings, and desperate men,
And dost with poyson, warre, and sicknesse dwell,
And poppie, or charmes can make us sleepe as well,
And better then thy stroake; why swell'st thou then?
One short sleepe past, wee wake eternally,
And death shall be no more; death, thou shalt die.

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Abbreviations

Amino Acids:

His - Histidine

Pro - Proline

Arg - Arganine

Glu – Glutamic acid

Phe - Phenylanaline

Tyr - Tyrosine

Asp – Aspartic acid

Met -Methionine

Lys - Lysine

ILe - Isoleucine

Thr - Threonine

Cys - Cysteine

Ser - Serine

Gly – Glycine

Miscellaneous:

APCI - Atmospheric pressure chemical ionization

ESI-MS - Electrospray ionization mass spectrometry

HPLC – High performance liquid chromatography

HR-MS - High resolution mass spectrometry

ICP-OES - Inductively couple plasma optical emission spectrometry

IR - Infrared

LC-MS - Liquid chromatography mass spectrometry

MP – Melting point

MS - Mass spectrometry

NAAT - Nicotianamine aminotransferase

NAS - Nicotianamine synthase

NMR - Nuclear magnetic resonance

PC – Phytochelatins

PIXE – Particle induced X-ray emission

Q-TOF - Quadrupole Time of flight

RP - Reverse phase

RT - Retention time

SAM - S-adenosyl-methionine

SEC - Size exclusion chromatography

SEM – Scanning electron microscopy

TOF - Time of flight

UV- Ultra violet

XAS – X-Ray absorption spectroscopy

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Chapter 1
Introduction

Plants have a complex system for growth and development. A major part of this system is the ability to regulate appropriate concentration levels of essential metals *e.g.* Ni (< 500 mg kg^{-1}), Fe, Mn, Cu and Zn (10-1000 mg kg^{-1}), as well as non-essential metals and metalloids *e.g.* Hg, Cd, Pb and As (< 100 mg kg^{-1}) (Anderson *et al.* 2001). Essential metals play an important role in enzymatic processes critical to plant sustainability. Although these metals are essential for plant life, high concentrations may have adverse effects such as inhibiting enzyme function and causing oxidative damage leading to plant death (Wei *et al.* 2009).

1.1 Plant Responses to metal uptake and transport

Plants growing in soil with high levels of metals can respond in one of three ways: exclusion, unrestricted uptake or hyperaccumulation (Baker 1981).

Plants that exclude high levels of essential metals, do not transport them to plant tissues above ground. This prevents them from experiencing any toxic effects *e.g.* the Pb excluder *Matricaria chamomilla* (Zarinkamar *et al.* 2013). Plants that have an unrestricted uptake take up metals present irrespective of their nutrient value and will eventually experience toxic effects and die. Hyperaccumulating plants, can take up large levels of metal (greater than essential metal concentrations) without experiencing toxic effects, because it stores the metal in a non-labile form (Watanabe and Osaki 2002). This allows the plant to survive in soil that has a high metal concentration. The process of hyperaccumulation is deemed an extreme physiological response in heavy metal tolerance (Baker 1981).

Hyperaccumulators are known to grow in four main types of soils *viz* serpentine soils (originating from Fe and Mg rich ultramafic rocks and containing high concentrations of Ni, Cr and Co); seleniferous soil (originating from Se rich rocks); calamine soils (high in concentrations of Zn, Cd and Pb); as well as Co and Cu containing soils (originating from argillite and dolomite rocks) (Reeves and Baker 2000).

Hyperaccumulators have been identified throughout the world with various metals sequestered as well as levels accumulated. The most common element involved in hyperaccumulation is Ni (Table 1), with 360 known Ni hyperaccumulating plants (Reeves 2006).

Table 1: Metals associated with hyperaccumulating plants (Baker *et al.* 1999)

Metal	**Metal concentration/ dry weight mg kg^{-1}**	
	Regular Plants	**Hyperaccumulators**
Ni	1-10	>1000
Co	0.03-2	>1000
Cu	5-25	>1000
Pb	0.1-5	>1000
Zn	20-400	>10 000
Mn	20-400	>10 000
As	0.1-1.2	>1000

1.2 Uptake Mechanisms in hyperaccumulators

In order for the metal to be transported and ultimately stored within the plant, ligands are required to bind the metal (Callahan *et al.* 2006). The plant root interacts with the soil in the rhizosphere. The availability of metals are dependent on the presence of microbes, a low pH (metals are less soluble at pH >5), the transudation of ligand as well as a change in redox potential (Whiting *et al.* 2001). Two pathways of metal uptake from the soil have been identified (Romheld and Marschner 1986). The first pathway solubilises the metal by reducing it while simultaneously decreasing the pH by releasing H^+. For every drop in the pH by 1 unit, the solubility of the metal increases by 1000 (Rengel 1999). This then causes the metal to be directly absorbed into the roots. The second pathway involves the production of specific ligands; commonly phytosiderophores that originate from the mugineic acid family (Reichman and Parker 2005). Ligands are exudated from the roots and bind to the metal in the rhizosphere and the co-ordinated complex is then absorbed by the roots (Reichman *et al.* 2005).

1.3 Metal storage in plants

The exact physiological process involved in hyperaccumulation is unknown. Plants store excess metal ions in non-labile complexes in order to prevent toxic effects (Watanabe *et al.* 2002). Storage areas include the cell wall, vacuole and cytosol (Robinson *et al.* 2003). In order to be successfully stored within plant tissues, the metal must initially be in a mobile form ensuring uptake from the soil, then compartmentalised and sequestered through the roots, transferred to the xylem to be transported, distributed then stored in leaf cells (Clemens *et al.* 2002). Each stage of transport affects hyperaccumulation. To combat the high metal concentration some hyperaccumulators have been reported to produce amino acids that combat the damaging effects of excess metal concentrations (Sharma and Dietz 2006).

1.4 Uptake and Transport

Most metals are considered to be bound to ligands such as low molecular mass compounds or proteins (Salt *et al.* 1999). Selective ligands are involved in the storage mechanism. The mechanism of storage occurs such that high concentrations of metals are stored within the plant(Küpper *et al.* 2004). Plants contain a number of ligands required for physiological processes such as organic acids, amino acids, proteins and peptides. Several organic acids and amino acids (Fig 1) have been reported to bind to the accumulated metal in hyperaccumulating plants (Schaumlöffel *et al.* 2003).

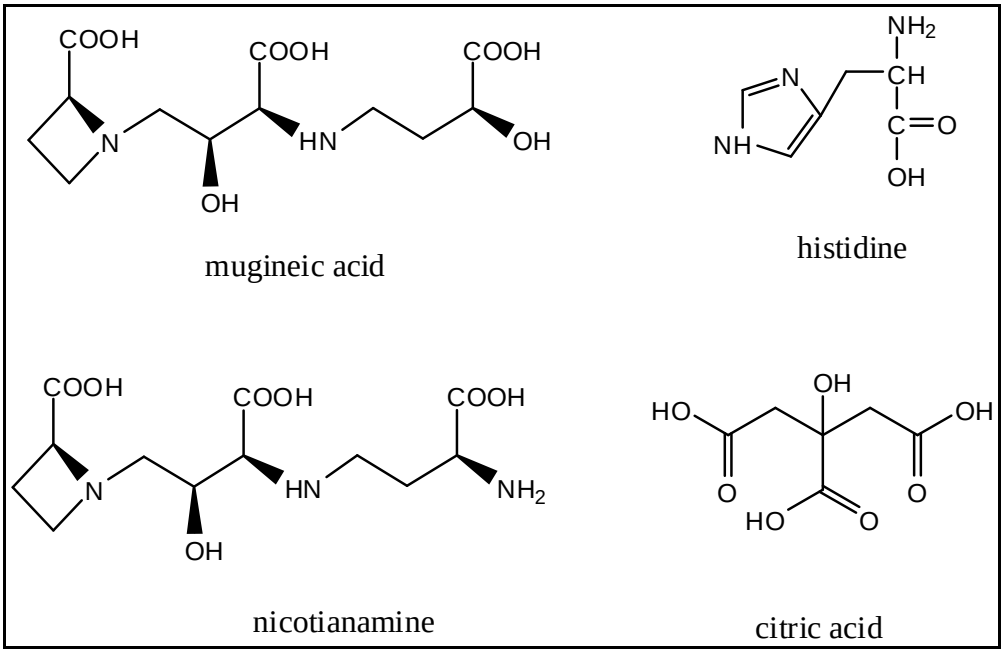


Figure 1: Common metal binding ligands found in hyperaccumulators

1.4.1 Amino Acids

Amino acids play important roles in various plant processes and are essential to plant life. The role of amino acids are particularly important in hyperaccumulators. When exposed to metals, plants synthesise peptides such as glutathione and phytochelatins (PC), amines (nicotianamine,

and mugineic acids) and amino acids (proline and histidine). These peptides and amino acids accumulate (concentrations in the millimolar range) to assist with detoxifying high metal levels within the plant (Sharma *et al.* 2006).

1.4.1.1 Histidine

Histidine is an important free amino acid involved in the hyperaccumulation process (Ute *et al.* 1996, Kameda *et al.* 2002, Kerkeb and Krämer 2003). It is a ligand with a number of different co-ordination states which make it very versatile. It commonly occurs in plants in a tridentate state; $M(His)_2$ (Fraser *et al.* 1965). Some hyperaccumulating plants *e.g. Thlaspi caerulescens* have been reported to contain $Ni(His)_2$ complexes (Salt *et al.* 1999). *A. lesbiacum* (Ute *et al.* 1996), *Pisum sativum* (Kameda *et al.* 2002) and *Brasicca juncea* (Kerkeb *et al.* 2003) have a directly proportional relationship between histidine and Ni concentrations. This implies that the concentration of histidine increased when plants were exposed to increasing Ni concentration.

1.4.1.2 Proline

Proline is a proteinogenic (precursor to proteins) amino acid. The role of proline in plants is to act as an osmolyte, radical scavenger, electron sink, stabiliser of macromolecules and a component of the plants cell wall (Sharma *et al.* 2006). It has also been linked to heavy metal tolerance in plants (Verbruggen and Hermans 2008). When a heavy metal accumulates in a plant, proline concentrations increase from 10 to 50 mM. It is therefore proposed to be a response to the increase in heavy metal concentration(Sharma *et al.* 2006). Proline is synthesised in plants from three reactions involving glutamate (α-Ketoglutarate cycle) (Fig 2). Studies inducing proline accumulation in response to metal uptake are generally comparable, in magnitude.

When a plant takes up high levels of heavy metals its process of osmoregulation is affected. This results in a water deficit within plants which can lead to toxicity and death. Proline serves to reinstate the osmoregulation balance whilst the plant taking up excess heavy metals (Sharma *et al.* 2006). Proline content in stems is higher than in roots with the highest concentration in the leaves (Rai 2002). An increase of up to >20-fold in the free proline content in the leaves of metal in the accumulator *Silene vulgaris* was observed (Schat *et al.* 1997). The authors showed that Cu was most effective in inducing proline production followed by Cd and Zn (Schat *et al.* 1997). The magnitude of the metal-induced increase in proline content in non-hyperaccumulator species was lower than that in *Silene vulgaris*, although they were exposed to higher metal concentrations (Delauney and Verma 1993).

1.4.1.3 Nicotianamine

Nicotianamine (NA) is formed by the condensation of three S-adenosyl-methionine molecules (SAM), catalysed by the enzyme nicotianamine synthase(Callahan 2007). Nicotianamine aminotransferase (NAAT) catalyses the conversion of NA to mugineic acid (Fig 2) derivatives and is present only in graminaceous (Strategy II) plants. The products of the reaction are converted to a range of potential ligands including alternating amino and carboxylato groups. NA was originally found and isolated from the leaves of the tobacco plant *Nicotiana tabacum L*. in 1970, and has now been reported in all naturally occurring plants (Kristensen *et al* 1970). It was recently reported to play a role in Ni storage in the hyperaccumulator *B. coddii* (Callahan 2007).

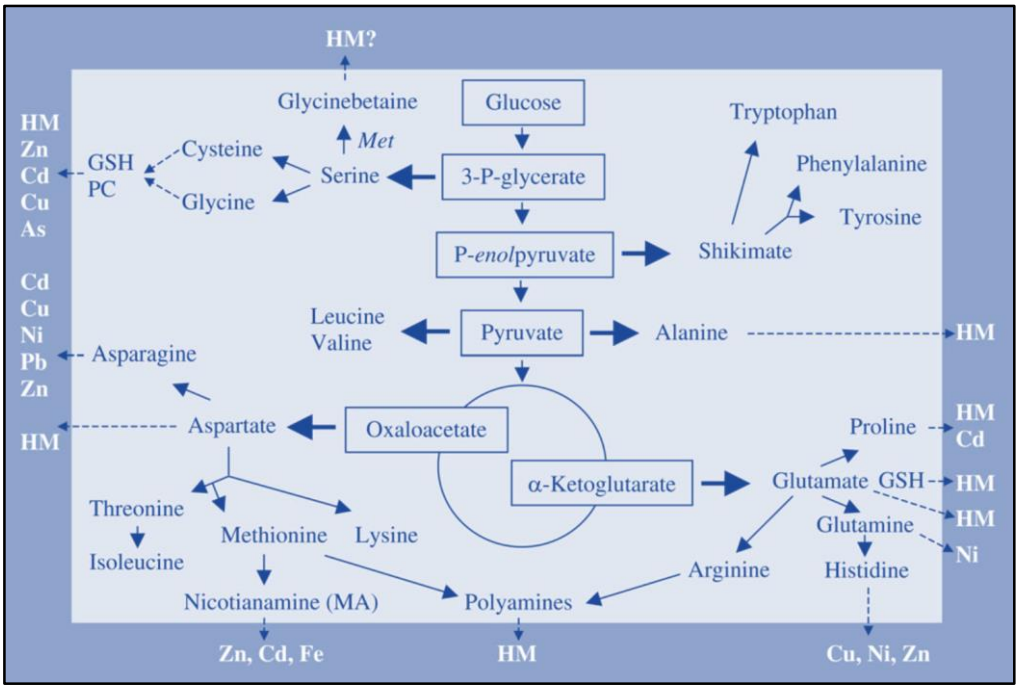


Figure 2: Amino acids produced in plants from various metabolic cycles and their relevant heavy metals (Sharma *et al.* 2006)

1.4.2 Organic Acids

Organic acids (including citric, ascorbic, oxalic, tartaric, malic, malonic and aconitic acids) are typically carboxylic acids found in the cell tissues of plants, (Terry and Bañuelos 1999). These acids have been reported to play roles in the process of metal accumulation (Jones 1998, Krämer *et al.* 2000, Ma 2000, Chen *et al.* 2003). The Zn hyperaccumulator, *A.halleri* was found to have increasing concentrations of common organic acids (citric, malic acid) when exposed to increasing Zn concentrations (Zhao *et al.* 2000). Studies on hyperaccumulators using X-Ray Spectroscopy showed the presence of O- or N- based ligands implying the presence of organic acids.

Studies reported that metal coordination is different for leaves of varying ages (Schaumlöffel *et al.* 2003). The hyperaccumulator *T. caerulescens* showed different metal co-

ordination environments in tissues of different ages (Küpper *et al.* 2004). In older tissues, metals bonded to O were in high concentrations suggesting that rather than producing a specific ligand the plant instead sequestered, detoxified and stored the metals into vacuoles (Küpper *et al.* 2004). In younger tissues, sulphur ligands – phytochelatins (cysteine) and histidine were produced to bind to the metal. This occurs because younger tissues require stronger ligands for detoxification (Küpper *et al.* 2004). X-ray absorption spectroscopy (XAS) analysis on *S.acuminata* showed that most of the Ni in the plant is not bound to one main ligand but bound to the citrate, malate and histidine ligands (Schaumlöffel *et al.* 2003). The role of organic acids with hyperaccumulators vary according to species (Callahan 2007). They have been suggested to play a role in sequestration of metals but not as the main binding ligands and transporters of metal complexes (Callahan 2007).

Other findings show that organic acids are responsible for detoxifiying hyperaccumulators in two ways: organic acids (citric and malic acid) are exuded in the rhizosphere allowing high levels of metals to be transported to the storage sites within the leaves were a different ligand bonded to the metal (Chen *et al.* 2003). The other path would be the organic acids binding to the metal from the initial stages of uptake and storing the metal within the leaves in a non-labile form (Ma 2000).

1.4.2.1 Citric, Ascorbic, Tartaric & Chelidonic Acid

Citric acid is one of the most common organic acids found to play a role in hyperaccumulation (Schaumlöffel *et al.* 2003). Citric acid along with tartaric acid play important roles in the citric acid plant cycle which is important for producing energy that plants require to survive (Chen *et al.* 2003). These organic acids are involved in the detoxification processes of various metals (Lee *et al.* 1977, Ma 2000, Chen *et al.* 2003).

In addition, they appear to act as a metal transporter to storage sites within the plant by binding to the metal and rendering it non-labile (Chen *et al.* 2003). Upon addition of citric acid to a Pb accumulating plant, increasing Pb concentrations were found in the leaves along with increased organic acid concentrations (citric, ascorbic and tartaric acid) found in the roots.

Ascorbic acid, (vitamin C) is essential for plant growth and is used to deal with stresses that plants experience. It occurs in areas of the plant that is used to transport metals to prevent metal toxicity *e.g.* stems in hyperaccumulators (Jones 1998).

Chelidonic acid is a naturally occurring heterocyclic organic acid found in plants. It has been detected in over 688 species of plants (Roberts and Wink 1998). It mainly occurs in the vacuole and is thought to be related to the mechanism of moving alkaloids from the cytosol into the vacuoles. Studies have indicated that the acid may act as a growth inhibitor to plants of other species in close proximity to plants that secrete the acid (Roberts *et al.* 1998). The full metabolic pathway of the acid is unclear. Studies have shown that the acid originates from a complex carbohydrate (Shen *et al.* 2001). *B. coddii* is the first known hyperaccumulator that chelidonic acid has been associated with (this study).

1.5 Ni Hyperaccumulators

Alyssum bertolonii (Fig 3) was the first metal rich plant discovered (Minguzzi and Vergano 1948). With the leaf material containing 1 % m/m of NiO compared to 0.42 % m/m NiO in the soil (Minguzzi *et al.* 1948).

Figure 3: ***Alyssum bertolinii***
(Adapted from: http://www.associazionebotanicabresciana.it/photos/p6.html)
(Date accessed: 29/04/2014)

The highest concentration of Ni was found in the hyperaccumulating plant *Sebertia acuminata* (~25% dry mass) (Sagner *et al.* 1998). Ni concentration was visibly evident with Ni "leaking" from the sap in the tree (Fig 4).

Figure 4: ***Serbertia acuminata*** **with Ni enriched sap**
(Adapted from: http://www.cnrs.fr/cw/dossiers/dosbiodiv/index.html)
(Date accessed: 29/04/2014)

The accumulation in *S. acuminata* led to the term hyperaccumulator and its concentration based definition "A concentration of greater than 1,000 mg Ni kg^{-1} of dry leaf tissue" (Brooks *et al.* 1977). This threshold was set at 100-1,000 times higher than the normal Ni concentration in plants grown on non-metalliferous soils and 100 times greater than the expected levels from non-accumulating plants on serpentine soils (Callahan 2007). Other Ni hyperaccumulators include *Hybanthus floribundus* (Severne and Brooks 1972), *Psychotria douarrei*, *Hybanthus caledonicus* (Kelly *et al.* 1975), *H. austrocaledonicus* (Jaffré and Schmid 1974) and *S. acuminata* (Jaffré *et al.* 1976).

1.6 *Berkheya coddii*

B. coddii is a nickel hyperaccumulating plant endemic to serpentine soil and is reported to accumulate over 37 000 mg kg^{-1} (Pillay 2005). It is found primarily in the Barberton Region in Mpumulanga Province, South Africa. The plant was originally studied to assess its role in phytomining and phytoremediation (Robinson *et al.* 1997, Brooks *et al.* 1998, Brooks and Robinson 1998, Salt *et al.* 1998, Brooks *et al.* 2001).

Figure 5: *B. coddii* in bloom

1.6.1 Previous Work

A number of studies have been carried out on *B. coddii* for a range of applications.

1.6.1.1 Phytomining

Phytoextraction is a process whereby plants extract contaminants released into soil and water either through natural or anthropogenic sources (Keeling *et al.* 2003). The use of hyperaccumulators as phytoextractors was first discussed by Chaney (1983) and then later on by (McGrath *et al.* 1993). When metal is extracted from soil and used for commercial use the process is then known as phytomining (Harris *et al.* 2008). Plants used in phytomining are grown in contaminated soil and then ashed to extract the metal (Nemutandani *et al.* 2006). *B. coddii* has been reported to have a biomass production of 22 t ha per annum (Robinson *et al.* 1997). Due to the high uptake of nickel, *B. coddii* has shown potential for being a very important tool in phytomining (Keeling *et al.* 2003).

Several studies have been performed to test uptake capabilities of *B. coddii*.

Reports indicate that nickel uptake is dependent on the bioavailability of nickel in the soil (Pillay 2005). The addition of the chelating agent (ethylenediaminetetraacetic acid) EDTA as well as citric acid to the soil decreased nickel uptake because of both competed with the plants nickel binding ligands (Robinson *et al.* 1997). Another cause for decreasing uptake is the addition of calcium and magnesium carbonates, calcium directly competes with the nickel pathway for uptake (Robinson *et al.* 1999). Sulphur can be added to the soil to assist in uptake when the soil has a soluble metal fraction less than the needed uptake levels. The addition of elemental sulphur to the soil is economically feasible and makes a difference to the amount of nickel (< 1200 mg kg^{-1}) taken up by the plant (Robinson *et al.* 1999).

1.6.1.2 Distribution of Ni in plants

Dimethylglyoxime (DMG) staining has been used to identify Ni deposits within the plant (L'Huillier *et al.* 1996). The Ni-DMG bond is a very strong bond and is capable of dissociating most Ni complexes (Krämer *et al.* 1996). The Ni-DMG complex forms red insoluble crystals and can be seen wherever Ni is present in a plant. Precipitating Ni out with DMG however proved inadequate in terms of quantifying Ni in the plant and can thus be only used for the sole purpose of qualitative identification (Gramlich *et al.* 2011). Micro-particle induced X-ray emission (Micro-PIXE) analysis of the plant showed that most Ni is stored in tissues that have no physiological role within plant processes. Some Ni was present in areas of the plant that are involved in transport and detoxification (Mesjasz-Przybylowicz and Przybylowicz 2011). Previous work indicated that Ni had a high presence in the xylem (the main route of transportation) and is mainly stored in the outer cuticles within the leaf (Fig 6) (Angle *et al.* 2003). In leaves of a younger age, nuclear microprobe studies indicated Ni to be concentrated in the mesophyll, margins and midrib epidermis (Mesjasz-Przybylowicz *et al.* 2001). Laser ablation coupled with DMG staining and inductively coupled plasma-mass spectrometry (ICP-MS) on the roots gave an insight into the plants uptake mechanism. Ni was concentrated in the stele (central part of stem) as opposed to the cortex of the roots, which indicated the presence of a selective or active uptake mechanism (Moradi *et al.* 2010). Contradicting results were found when the technique of magnetic resonance imaging (MRI) was used on the roots. Results indicated a passive uptake mechanism and a specific pattern of Ni concentrating at the plane of the roots which showed the ability of hyperaccumulation (Moradi *et al.* 2010). Thus far imagining has only been moderately useful.

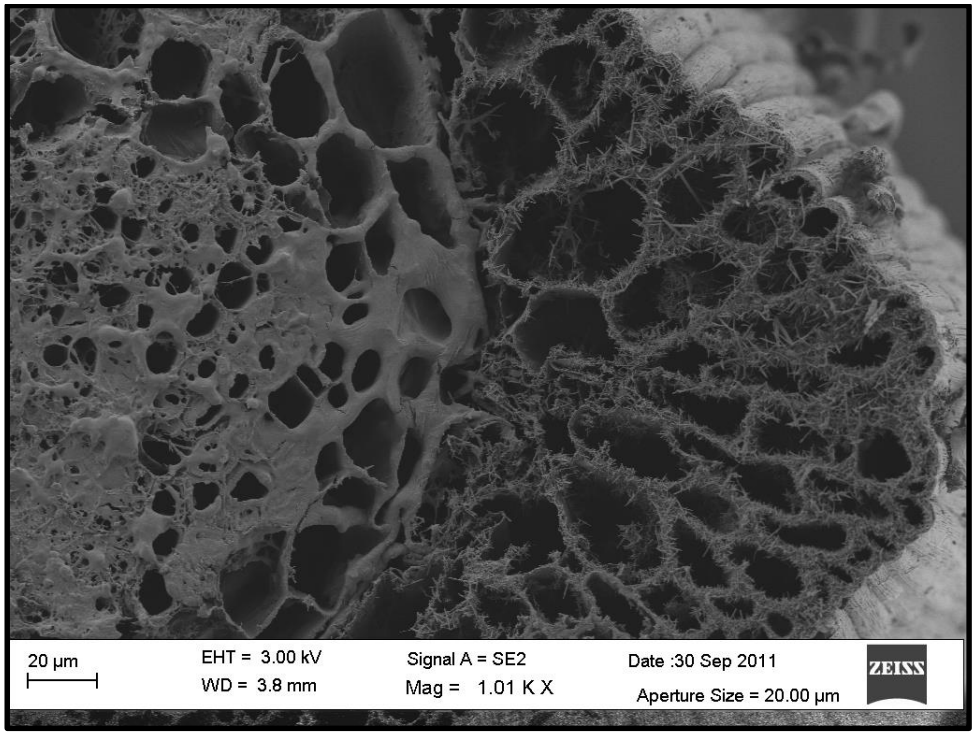


Figure 6: SEM of precipitated Ni crystals in the upper epidermis of *B. coddii* *(Naicker and Pillay 2011)*

B. coddii was reported to have effective root to shoot translocation of Ni (Angle *et al.* 2003). The higher content of Ni in the soil, the lower uptake of macro and micronutrients in the plant (Robinson *et al.* 2003).

1.6.1.3 Soil- Metal Uptake Relationship

Pillay (2005) carried out an extensive total and bioavailable metal analysis on serpentine soil from the Barberton region. Bioavailability studies were carried out to assess the actual fraction of the total metal concentration that was available to the plant for uptake. Three different techniques were carried out to ensure reliability of metal bioavailability namely the Bureau of Community Reference (BCR) sequential extraction technique, ammonium acetate and EDTA extractions. Ammonium acetate extractions concluded that Mn and Ni are most available for uptake and EDTA showed an availability trend of Mn, Fe and Ni are most available. The BCR extraction was carried out to assess the potential availability of metals held within the soil.

Results concluded that Ni and Co are associated with Mn and Fe oxides, Fe and Cr occur primarily as refractory minerals and Cu as a sulphide or complexed to organic matter.

Based on soil findings, uptake studies were carried out to assess *B. coddii's* uptake capacity. A set of plants were spiked with different concentrations (100 mM, 500 mM and 1000 mM) of Ni solution over a period. The total Ni content in each plant was ascertained along with Ni content in the soil. Findings suggested that rate of uptake of Ni by *B. coddii* is dependent on the availability of Ni in the soil. The maximum level of Ni stored within in the plant based on uptake studies was 37 000 mg kg^{-1}.

1.6.1.4 Identification of Ni-Ligand complex

Slatter (1998) reported the ligand responsible for nickel storage (malic acid) however, upon analysis using reversed phase HPLC, malic acid was quantified and yielded a ratio of 1:0.013 Ni to malic acid indicating malic acid cannot be responsible for binding to Ni (Pillay 2005). *B. coddii* was later investigated as a model plant to look at the coordination of Ni to nicotianamine which was identified as the ligand responsible for binding to Ni (Callahan 2007). The association constant of Ni-NA was reported by Callahan (2007) to be much higher than common organic acids and complexing agents; this added to the idea of NA binding to Ni. However no supporting data of the quantification of Ni and NA was reported, neither were molar ratios between the two calculated. Pillay (2005) reported an unidentifiable major peak (RP-HPLC) in from a plant-water extract of *B. coddii* with a unique UV spectrum (Fig 7).

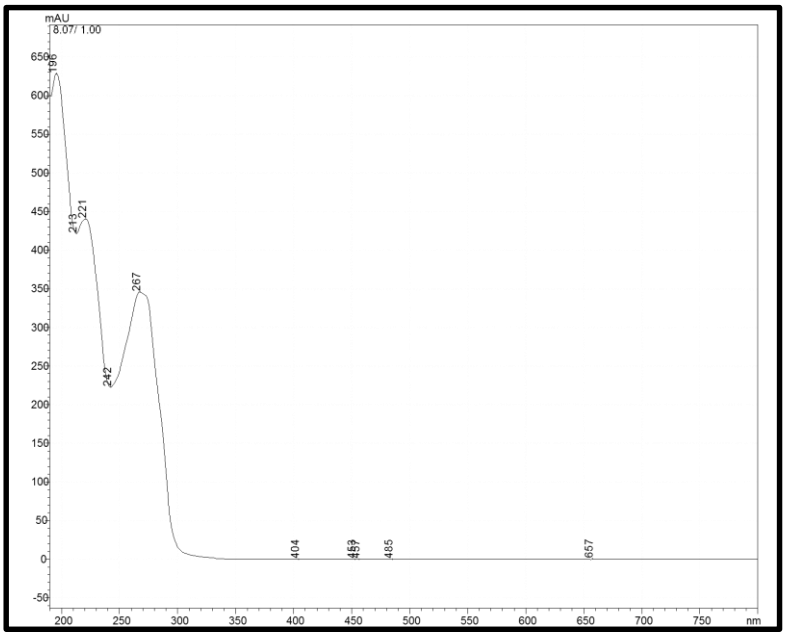


Figure 7: Unique UV-Spectrum of unidentifiable peak in *B. coddii (Pillay 2005)*

1.7 Aims and objectives

Understanding how nickel is sequestered and stored within *B. coddii* can be very useful for the plants role in phytoremediation. By finding the ligand that renders the metal non-toxic and quantifying it can lead to other studies regarding uptake and maximum nickel storage capacity within the plant.

The objectives of the project:

i. To identify the ligand responsible for complexing to nickel in *B. coddii*.
ii. To quantify the ligand in *B. coddii*.
iii. To investigate the relationship between amino acids and nickel in *B. coddii*.
iv. To investigate the relationship between organic acids and transport of nickel in *B. coddii*.
v. To investigate the response of the ligand in *B. coddii* to an increase in nickel exposure.

1.8 Dissertation Overview

Chapter 1: an introduction covering a background on metals that are taken up and transported within plants; the uptake mechanism of hyperaccumulators, physiological processes involved in metal storage in plants, uptake and transport of metals by various ligands in the plants, Ni hyperaccumulators such as *B. coddii*.

Chapter 2: describes the experimental work carried out including the materials and instrumentation and describes various analytical techniques used during the course of the study. Experimental and sampling site, extraction procedures, sample preparation, analysis of organic and amino acids, and metals.

Chapter 3: discusses the results of identification and quantification of amino and organic acids with various techniques including size exclusion chromatography, mass spectrometry and HPLC.

Chapter 4: discusses the uptake results of Ni in *B. coddii* as well as the correlation between the levels of Ni and those of chelidonic acid.

Chapter 5: presents general conclusions and suggestions for future work.

References: Listed after Chapter 5

Appendices: 1-5 are placed after the bibliography

Chapter 2

Experimental

2.1 Introduction

This chapter outlines experimental and instrument details used in the project.

2.2 Materials and Instrumentation

2.2.1 Consumable Materials

During the course of the project, various chemicals and consumable materials were used. Analytical grade reagents were used unless chromatography grade reagents were required.

2.2.2 Instrumentation

The different instrumentation used to carry out analyses during the project are listed and the theory of each technique is explained in this section.

2.2.2.1 Inductively Coupled Plasma – Optical Emission Spectroscopy (ICP-OES)

Instrument: Perkin Elmer, Optical Emission Spectrophotometer (OES), Optima 5300DV

Table 2: Operating parameters of the ICP-OES

Spectral Profiling	No
Resolution	Normal
Read Delay Time	60 s
Source Equilibration Delay	15 s
Plasma Aerosol Type	Wet
Nebulizer Start-up Conditions	Instant
Element	Ni (231.06 nm)
Plasma	15 L/min
Auxiliary Gas	0.2 L/min
Nebuliser	0.80 L/min
Power	1500 W
Viewing Distance	15.0
Plasma View	Axial
Sample Flow Rate	1.50 mL/min
Wash Rate	1.50 mL/min
Wash Time	30 s

2.2.2.2 High Performance Liquid Chromatography

Instrument: Shimadzu – Prominence HPLC

Table 3: Operating parameters of the HPLC

Solvent Delivery Unit	LC-30AD
	Flow Rate: 0.0001 – 5.000 mL/min
Degasser	DGU-20A3 – 3 lines
Liquid Chromatograph	CBM-20A
Autosampler	SIL-20A
	Variable Injection Volume: 0.1 – 100 µL
Diode Array Detector	SPD-M20A
	Wavelength Range: 190 – 800 nm
	Lamp: D2, W
Cell	Path length: 10 mm
	Volume: 13 ìL
	Pressure 1740 psi
Flow Rate	1.0 mL/min
Injection volume	10 µL
Mobile Phase	KH_2PO_4 adjusted to pH 2.7 with ortho-H_3PO_4
Column	Reverse Phase C_{18}, Phenomenex, Luna, 5 µm, 25
	X 0.46 mm
Detector Wavelength	220 nm
Run Time	20 minutes

2.2.2.3 Mass Spectrometry

Table 4. LC-Peak Identification & MS-MS (ES-TOF-MS)

Instrument	**Waters Aquity UPLC connected to a Waters Micromass LCT Premier**
Mobile phase	70% H2O / 30% MeOH
Flow Rate	0.25 mL/min.
Injection Volume	4 µL
Column Temp	35 °C
Column	Kinetex C_{18} 2.5 µm
Mass Range	100 – 600 amu
MS mode	Electrospray W+ and W-

Table 5: Ligand Identification (HR-MS)

Instrument	**Waters Synapt G2 connected to a Waters UPLC**
Mobile Phase	70% water / 30% methanol
Flow Rate	0.25 mL/min
Column	Waters BEH C_{18}, 2.1x100 mm column
Source	Electrospray negative, Capillary voltage: 3 kV, Cone Voltage: 15 V
Lock mass	Leucine enkaphelin

Table 6: Amino acid analysis (APCI-TOF-MS)

Instrument	**Waters API Quattro Micro**
Mobile Phase	Water and Acetonitrile (A1, A2, B1, B2)
Solvents A1 and A2	Eluent A2 (100ml Eluent A concentrate and 900ml Water)
Solvents B1 and B2	Eluent B (Supplied by AccQ Tag Kit)
Column	AccQ Tag C_{18}, 1.7µm, 2.1x100mm
Injection Volume	1 µL
Source	Electrospray positive, Capillary voltage: 3.5kV, Cone voltage: 15 V, Temp: 120°C
Desolvation Temp	350°C, Desolvation gas: 350 L/h, Cone gas: 50 L/h

2.2.2.4 Nuclear Magnetic Resonance (NMR)

Table 7: characterisation of chelidonic acid - NMR:

Instrument	**Bruker 400Hz**

2.3 Sampling Site and Sample Collection

Sampling was carried out in Barberton, Mpumalanga Province, South Africa. *B. coddii* plants were collected from the sites in January (summer). Samples of serpentine soil were also collected, along with the plants. The plants were grown at the University of KwaZulu-Natal (Westville Campus), School of Chemistry & Physics. The plants were situated in an area that received maximum sunlight.

2.3.1 Transplanting of *B. coddii*

Plants were transplanted into large pots along with the serpentine soil collected. The base of the pots were lined with stones before addition of soil, to ensure drainage occurs.

2.3.2 Watering

B. coddii plants were watered once a day with tap water, and the leaves were sprayed using a plant mister. The plants grew well in a warm environment.

2.3.3 Ni Spiking in *B. coddii* plants

B. coddii plants of the same age, size and height were individually potted for spiking analysis. A 100 mM $NiCl_2$ solution was made up using tap water and $NiCl_2$. Every second day 20 mL of the solution was added to the plant for a total of 4 weeks. A control plant was also present with no addition of Ni solution. Every seven days a leaf from each plant on the same part of the plant was collected. A total of 280 mL (28 mmol) of the solution was added to the plant.

2.3.4 Plant sample preparation

The procedure was adapted from Pramanik *et al.* (2004). Leaves from *B. coddii* were randomly selected, washed thoroughly with tap water to remove any soil and dirt residue. Washed leaves were air dried and amino and organic acids were extracted in an agate mortar and pestle using Millipore water.

2.4 Identification of ligand in *B. coddii*

The target compounds (amino and organic acids) were extracted from the leaves following the stated procedure (section 2.3.4).

2.4.1 Preparation of column for Size Exclusion Chromatography

A mass of 10 g of Sephadex G-10 (Sigma) was used as the stationary phase. An aqueous, 4 M solution of a Tris(hydroxymethyl)methylamine (Sigma) solution was made using Millipore water in a 100 mL volumetric flask. The pH was adjusted using a concentrated HCl (Sigma) to pH 4. A volume of 10 mL of the solution was added to the Sephadex beads and mixed to form a slurry. The slurry was left to swell for > 3 hours followed by the addition of another 10 mL of the solution into the swollen gel.

After the addition of the slurry into the column, the column was flushed with Millipore water to ensure the removal of the Tris buffer.

2.4.2 Sample elution through a Size Exclusion Column

The sample was loaded onto the column using a Pasteur pipette. The sample (yellow in colour) moved through the column with the mobile phase (Millipore water).

Fractions each containing a volume of 5 mL were collected until the colour of the eluent was clear. A total of nine fractions were collected.

2.4.3 Ni analysis of collected fractions

From the collected fractions a volume of 2 mL was removed and made up to the mark in a 10 mL volumetric flask using Millipore water. The samples were analysed using ICP-OES.

2.4.4 HPLC Analysis of collection fractions

A solution of 0.05 M KH_2PO_4 mobile phase was made up using Millipore water, the pH adjusted to 2.7 with concentrated H_3PO_4 (Sigma).The mobile phased was filtered through a 0.45 µM filter. Samples were run at a wavelength of 220 nm and chromatograms obtained.

2.5 Organic Acid Analysis in *B. coddii*

2.5.1 Standard preparation for qualitative analysis of organic acids in HPLC

To identify the different organic acids present in the plant extract, 200 ppm single standards were made from a number of different organic acids (Appendix 1). Salts of each acid were weighed out and dissolved in Millipore water.

2.5.2 Standard preparation for quantitative analysis of organic acids in HPLC

Ranges of standards for quantitative analysis were based on peak area of the related peak in the sample chromatogram. A range of standards (10 – 500 ppm) were prepared for analysis of each peak (Exact range was dependant on the individual peak area). A total of five standards were used for calibration curves used for the quantification of the acid.

2.6 Amino Acid Analysis in *B. coddii*

Samples were extracted with 1mL 50% acetonitrile, 0.1% formic acid solution, for 1 hour in a sonicating bath. After which the samples were centrifuged and diluted (10x). A volume of 10µL of the samples were added to the Waters AccQ Tag Kit constituents and placed in a heating block at a temperature of 55°C, for ten minutes. Heated samples were then analysed using LC-MS.

2.7 Synthesis of Chelidonic acid

Synthesis was adapted from (Riegel and Zwilgmeyer 1943).

2.7.1 Sodium ethoxide synthesis

Pure sodium was weighed out (4.6 g) cut into small pieces and slowly dissolved in 60 mL of dry ethanol. The addition of sodium was carried out in a gently heated 250 mL round-bottomed flask fitted with a reflux condenser and sealed with drying tube ($CaCl_2$).

Sodium ethoxide solution (30 mL) was stored in a 250 ml round-bottomed, flask constantly stirring under a reflux condenser. The remaining solution was kept warm using a heating mantle.

2.7.2 Acid synthesis

The first half of the solution was cooled until the appearance of a solid. To this solidifying solution, 5.8 g of dry acetone mixed with 15 g of ethyl oxalate was added whilst constantly stirring resulting in the formation of a clear brown solution. (The reaction is exothermic so heat was evolved). As soon as the mixture began to become turbid, 16 g of ethyl oxalate was added to the other solution of sodium ethoxide and poured into the flask with the turbid

solution. After the solution was stirred for 30 minutes the mixture solidified. The round-bottomed flask was then fitted with a distillation apparatus and heated to 110°C until 15 mL of ethanol was distilled off. The apparatus was fitted with a drying tube ($CaCl_2$). The mixture was then cooled to 20°C.

The mixture was transferred to a 400 mL beaker to which 30 mL of concentrated HCl was added along with 80 g of cracked ice. Any lumps formed were crushed until a creamy yellow suspension was obtained. The suspension was filtered using a Buchner funnel and flask under vacuum. The filtered product was mixed with 10 mL of ice water and filtered once more. The filtered product was mixed with 30 mL of concentrated HCl (Sigma) in a 250 mL round-bottomed flask and heated on a steam bath for 20 hours under reflux. The mixture was then cooled to 20°C. The solid product observed was beige in colour. The product was filtered using a Buchner funnel and flask, washed twice with 50 mL of ice water, dried first at 100°C for two hours then at 160°C to remove the water of crystallisation. The synthesis of the acid was confirmed by NMR and HPLC. No other products or impurities were revealed. Melting point = (253-257)°C, yield = 77%.

2.8 Ni uptake studies in *B. coddii*

2.8.1 Sample preparation

All samples collected were washed thoroughly with tap water and left to air dry. Samples were then crushed with 5 mL of Millipore water using a mortar and pestle to form a pulp. The pulp was then filtered and samples made up to the mark using Millipore water.

2.8.2 Standard preparation of Ni for quantitative analysis of spiked leaves

A range of Ni standards were made up (1-100 ppm) from a 1000 ppm stock solution and used to quantify Ni using ICP-OES. A total of five standards were used for the calibration curve.

2.9 Sample Analysis Instrumentation

2.9.1 ICP-OES – metal analysis

ICP-OES has several advantages over other common metal analysis instrumentation. ICP-OES is a multi-element analysis method compared to other techniques such as atomic absorption spectroscopy (Skoog, *et al*. 2004). For most metals the ICP has a low limit of detection which makes it more sensitive than other common methods (Skoog, *et al*. 2004). This allows for trace analysis studies to be carried out.

2.9.2 Emission Spectroscopy

The ICP-OES is an emission spectroscopy technique which results in the formation of emission spectra. The plasma supplies energy, which excites the analyte atoms. Upon application of the energy, the analyte atoms move from ground state to a higher excited state back to ground state (energy is given of as photons of visible light or ultraviolet radiation). The exact movement of the electrons depends on the metal and the amount of orbitals it contains. The analyte atoms then relax back to ground state and the (Murray *et al.* 2000).The ICP-OES comprises of three main parts i.e. the sample introduction system, the torch and the spectrometer (Fig 8).

The sample is introduced into the ICP-OES by a peristaltic pump (Skoog, *et al*. 2004). The sample is pumped into the nebuliser where it is vaporized. The vaporized sample collects in the spray chamber along with argon gas. Heavy vapour particles settle as waste and the smaller, lighter particles move to the torch with the argon gas (Sneddon and Vincent 2008). The plasma can reach temperatures as high as 8000 K. The high temperature ensures complete vaporization and desolvation. The heat energy causes the excitation of electrons, which when relaxing gives off the energy at wavelengths specific to the metal (Thomas 2013).

The light emitted from the plasma is focused through the entrance slit into the spectrometer which is a polychromator (used for simultaneous metal analysis). The diffraction grating in the spectrometer refracts the light into component colours (Holmes 2001).The photomultiplier tube, which is the detector, scans all the wavelengths simultaneously, which is then interpreted by the computer and displayed as various metal concentrations (Skoog, *et al*. 2004).

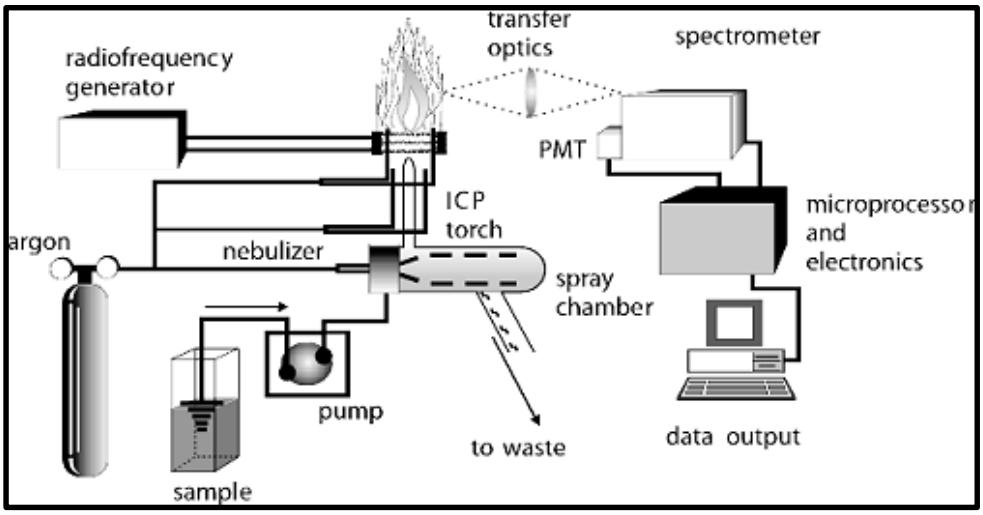


Figure 8: Schematic of an ICP-OES
(Adapted from http://www.balticuniv.uu.se/environmentalscience/ch12/chapter12_g.htm***)***
(Date accessed: 1/05/2014)

2.9.3 Advantages and Disadvantages of ICP-OES

Atomization of the analyte occurs in an inert environment as compared to flames which are unstable, the cross section of the plasma is also uniform and the plasma has a thin optical path length which keeps self-absorption to a minimum (Skoog, *et al*. 2004). However ICP-OES are susceptible to interferences which can affect results. There are two types of interferences that have an effect on results *i.e.* spectral and transport interferences (Sneddon *et al.* 2008).Spectral interferences occur when two emission lines overlap or when the background of an emission line is affected. It can decrease or increase the signal which in turn decreases or increases the actual concentration (Pillay, 2006). The best way to minimise for the interference is to use another emission line. Transport interferences are related to the viscosity of the samples and standards. If the viscosity is different, the rate at which they are pumped to the nebuliser will also be different (Pillay, 2006). This will result in the less viscous solution having a higher concentration.

2.9.4 Standard Solutions

For every analysis carried out, an instrument calibration curve was carried out. A calibration is used to measure the response of the instrument's signal against the standard solutions, which contains known concentrations (Skoog, *et al*. 2004). The calibration curve is a plot of concentration against signal response.

2.10 Column Chromatography

Size exclusion Chromatography (SEC) was used to isolate the Ni-ligand complex from the leaves of *B. coddii*. The column of choice was a Sephadex G-10 column.

Size exclusion Chromatography (SEC) also known as gel-filtration chromatography is a type of partition chromatography. The technique is used to separate molecules of different molecular sizes which is generally based on molecular weight (Vasudevan *et al.* 2002, Rojas *et al.* 2004). Molecules from the analyte of concern are found between a mobile phase and a stationary phase (consisting of a matrix (porous)). A column is made up of a stationary phase, which is in a bead form *e.g.* Sephadex (Rojas *et al.* 2004).

Once the analyte enters the column, molecules that are larger in size than the pores of the stationary phase it moves at a fast rate through the column due to the lack of interaction. The smaller molecules that remain will interact with the matrix pores and be retained (Mori and Barth 1999, Vasudevan *et al.* 2002, Rojas *et al.* 2004).

2.11 High Performance Liquid Chromatography (HPLC)

2.11.1 Principles

Analysis of plant extracts was carried out using HPLC for identification of the Ni-ligand complexes and organic acids present. HPLC is a separation technique, which separates on the basis of polarity of analytes with respect to the stationary phase (Skoog, *et al*. 2004).

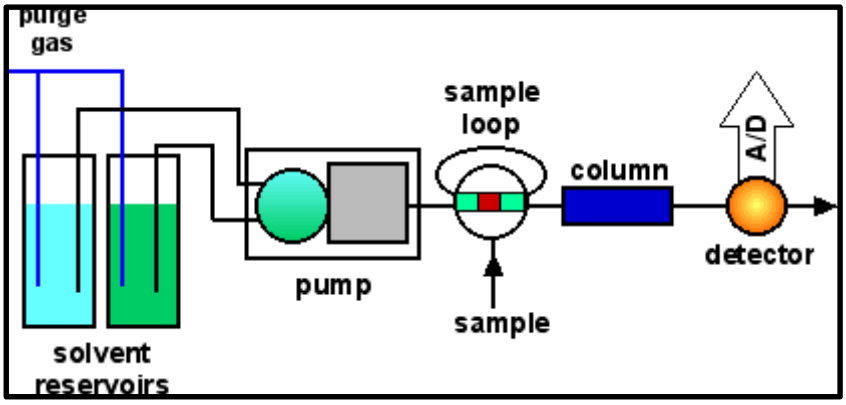


Figure 9: Schematic of a HPLC setup
(Adapted from: http://www.chem.utoronto.ca/coursenotes/analsci/chrom/hplc.html)
(Date accessed: 1/05/2014)

The system (Fig 9) consists of a mobile phase (a solvent), a pump, a sample injection port, a column, a detector, waste line and a data output device (usually a computer) (Snyder *et al.* 2012). The injected sample is pumped through the column with the mobile phase. Separation is based on analyte interaction with the column(Kazakevich and Lobrutto 2007). The most common type of detector used is a UV detector. The detector measures the UV light of the compound eluting from the column. RP-HPLC was chosen based on the analyte. Organic acid analysis required a non-polar column and polar mobile phase because of the nature of an acid.

2.12 Mass spectrometry

Mass spectrometry is used to identify molar masses of compounds. This technique is based on the formation of ions from the analyte, the analysis of ions formed in comparison to the mass to charge ratio (m/z) and the detection of ions produced (Traldi *et al.* 2006). There are three basic components of a mass spectrometer namely the ion source, mass analyser and detector.

The type of data obtained from an MS is dependent on the way ions are produced therefore the choice of ion source is very important. The choice of ion source needs to be dependent on the volatility, molecular weight and thermal ability of the analyte as well as the nature of the matrix (Dams *et al.* 2003). There are two main categories of ionisation sources namely those that require the analyte to be in a gaseous form and those that can handle a low volatile and high molecular weight analyte (Souverain *et al.* 2004).

Three different mass spectrometers were employed in various analyses conducted during this project *i.e.* TOF-ES-MS, APCI-TOF-MS and HR-MS.

2.12.1 Time of Flight-Electrospray-Mass Spectrometry

For this ionisation technique *i.e.* Electrospray Ionisation (ESI). The mass analyser used was a Time of Flight (TOF) analyser. TOF measures the time taken for an ion to travel a specific distance.

2.12.1.2 Ionisation source: Electrospray Ionisation (ESI)

The principle of ESI results when an analyte is injected through a capillary line in the presence of a magnetic field. ESI comes about in three distinct steps (Fig 10).

Step 1: charged drops are formed in the near the end of the metal capillary.

Step 2: solvent evaporation results in a rapid decrease of the size of the charged drop due to columbic repulsion.

Step 3: ions originated in a gaseous phase from the small charged drops (Ardrey 2003).

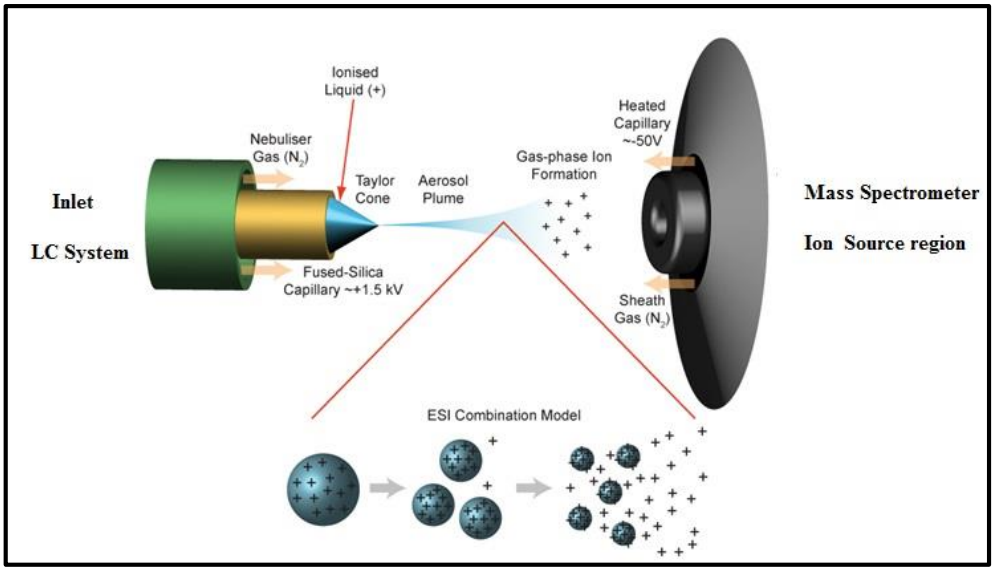


Figure 10: Schematic of an electrospray ionisation source

(Adapted:http://www.lamondlab.com/MSResource/LCMS/MassSpectrometry/electrosprayIonisation.php)

(Date accessed: 3/05/2014)

The capillary tube which generally has an external diameter of about 10^{-4} m, has a potential of approximately 1.5 kV. The counter electrode (a heated capillary) is placed at a distance of about 1-3 cm away from the capillary tube, and found at the entrance to the mass analyser.

A high electric field is present at the capillary due to the capillary thickness. Within the region a Taylor cone forms (Ardrey 2003). This occurs because of an interaction between the analyte solution and the electric field which causes the charged particles within the solution to move in the direction of the electric field (Fig 10). A high enough electric field will cause the analyte to disperse as a spray from the apex of the cone. The droplets formed will carry either a negative or positive charge, the charge is determined by whether the needle is placed at a positive or negative voltage (Ardrey 2003). The solvent evaporation decreases the size of the drop as well as increases the force of the electrical field perpendicular to the surface of the drop.

2.12.1.3 Mass Analyser: Time of Flight

Time of Flight was used in plant analysis for multiple analyses (section 2 2.2.3). It consists of a linear configuration. Between the ionisation source and detector is a region that is under vacuum without any electric field (Fig 11).

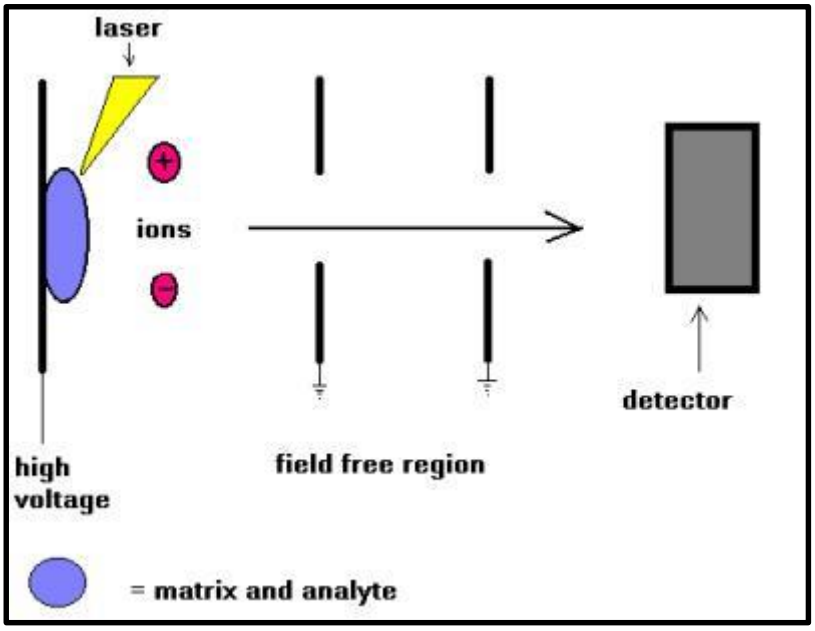


Figure 11: Drift region in a Time of Flight setup where gas ions gain an acceleration

(Adapted from: http://www.astbury.leeds.ac.uk/facil/MStut/mstutorial.htm)

(Date accessed: 3/05/2014)

Ions formed in the ionisation source have a m/z accelerated by the action of a field and produce a speed (Ardrey 2003, Traldi *et al.* 2006). The potential energy of the ions are equal to the kinetic energy. Ions with different m/z values will have different speeds. This means that the time taken for ions to reach the detector is based on their m/z values and thus their time of flight (Ardrey 2003, Traldi *et al*. 2006).

2.12.2 Ionisation Source: Atmospheric Pressure Chemical Ionisation (APCI)

APCI was developed based on findings in a chemical ionisation (CI) source. The amount formed (yield) in a reaction in the gaseous phase not only depends on the partial pressures of reactants involved but also on the surrounding total pressure of the reaction environment. Changing the pressure from 0.1-1 Torr in a CI ion source to the atmospheric pressure increased the amount of ions produced as well as sensitivity (Ardrey 2003, Vékey *et al.* 2011).

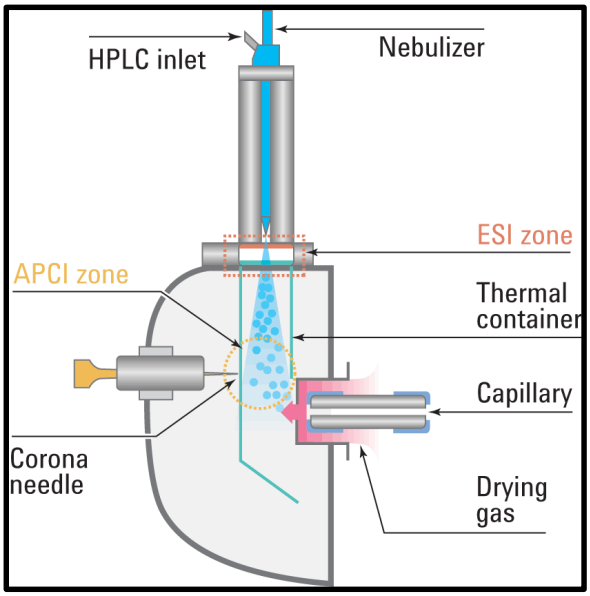


Figure 12: Schematic of an APCI ionisation source

(Adapted: http://www.chem.agilent.com/enUS/Newsletters_old/pharmaanalysis/issue8/Pages/g31035.aspx) (Date accessed: 3/05/2014)

The ionisation instrument used in APCI is known as a corona discharge. The method is useful for analytes dissolved in suitable solvents. Analyte solution is injected into a heated capillary tube (350-400°C) which vaporises the solution, (Fig 12). The vaporised solution moves out from the capillary and enters the atmospheric pressure region where corona discharge occurs (Ardrey 2003, Vékey *et al.* 2011).

The vaporised solution is carried with the assistance of a flow of N_2 gas. Solvent molecules come into contact with an electron beam emitted from the corona discharge and form ions which then react with other solvent molecules (protonated or deprotonated depending on analysis mode).

One of the drawbacks found with APCI was the existence of some of the analyte clustered with some solvent (Ardrey 2003, Vékey *et al.* 2011) . Declustering of the mixture (sample and solvent) can be brought about by colliding the clusters with N_2 gas or thermal treatments. APCI is a preferred method when derivatised samples are analysed, thus proving useful for amino acid analysis.

2.12.3 High Resolution Mass Spectrometry (HR-MS)

HR-MS was used to identify the ligand chelidonic acid in *B. coddii*.

HR-MS is a system that uses mass analysers based on several principles of separation *i.e.* multiple fragmentation. The system is sometimes known as Quadrupole-Time Of Flight (Q-TOF).

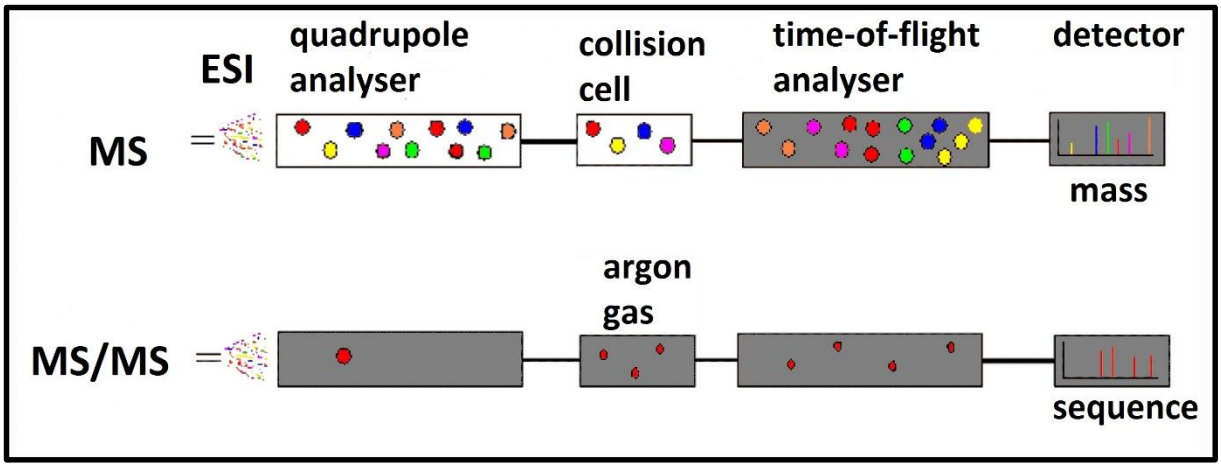


Figure 13: Schematic of the ion path in a Q-TOF instrument

(adapted from: http://www.chem.pitt.edu/facilities/mass-spectrometry/introduction) Date accessed: 3/05/2014)

The ion under analysis is generated in the source then chosen by the first quadrupole mass filter (MS1) (Fig 13). Collisions with the ion occur in a collision cell, a quadrupole that only operates in RF mode (Ardrey 2003). The products from MS2 are analysed using the TOF analyser. The accurate masses obtained by the product ions from the collisions are due to the high resolution of TOF (Aiken *et al.* 2007). These masses allow the elemental composition of the analyte to be discovered which results in very specific MS/MS output (Aiken *et al.* 2007).

Chapter 3: RESULTS & DISCUSSION Identification & Quantification of Ligands

Metal analysis focused only on nickel, since it is the only metal accumulated by the plant. Amino and organic acid analysis studies quantified in various parts of the plant determine the ligand responsible for Ni accumulation, and the effect of the age of the plant (leaves) was studied. A variety of MS techniques were performed, along with RP-HPLC for various objectives in the study.

Upon identification of the ligand responsible for Ni uptake, synthesis and characterisation (chelidonic acid) were performed, followed by quantification of chelidonic acid in *B. coddii*. A plant-water extract was used for all plant analyses in this study.

3.1 Amino Acid Analysis in *B. coddii*

Concentrations of the 17 free amino acids found in plants were quantified in *B. coddii* in various parts of the plant as well as different ages of leaves. The role amino acids play in *B. coddii* are not well known (apart from essential plant functions) and an insight to various concentrations can be of use for possible phytoremediation work. It is important to investigate the role of amino acids in hyperaccumulators in order to ascertain more information about plant responses to metal uptake. Leaves, roots and stems were randomly picked to ensure a representative sample. Plant-water extracts on sections of *B. coddii* were analysed for amino acids. Samples

were first derivatised to create fluorescent derivatives (AccQ•Fluor™) then quantified using LC-MS.

Table 8. Free amino acids and Ni concentrations present in *B. coddii* (mg kg^{-1})

Amino Acid	**roots**	**stems**	**young leaf**	**middle leaf**	**older leaf**	**seeds**
Ni	**8.59**	**25.59**	**94.58**	**101.4**	**137.2**	**30.74**
His ł	28.36	49.64	258.1	87.78	152.7	562.1
Pro ł	23.63	289.5	12 447	3 150	89.05	491.8
Arg ł	75.62	13.24	401.5	219.5	197.2	585.5
Glu ł	104.0	385.5	669.2	324.1	1 469	2763
Phe ◊	23.63	26.47	133.8	50.64	50.88	491.8
Tyr ◊	23.63	47.98	86.04	27.01	50.88	421.6
Asp ϖ	85.07	216.7	372.8	195.8	286.2	1405
Met ϖ	4.73	13.24	38.24	13.51	12.72	93.68
Lys ϖ	28.36	71.14	296.4	64.15	95.41	398.1
ILe ϖ	18.91	84.38	95.60	30.39	127.2	491.8
Thr ϖ	23.63	71.14	133.8	87.78	101.7	445.0
Cys Ł	0.000	6.62	19.12	3.38	6.36	70.26
Ser Ł	42.51	115.8	506.7	178.9	184.4	796.3
Gly Ł	33.08	74.45	153.0	37.14	190.8	538.6

ł = α-ketoglutarate, ◊ = *P-enol*pyruvate, ϖ = oxaloacetate, Ł = 3-P-glycerate (plant metabolic cycles)

The distribution of amino acids showed no apparent trend in *B. coddii* (Table 2). However high concentrations of proline were apparent in certain plant sections. Proline was predominant in the leaves collected from young plants compared to the leaves from older plants. This amino acid was also the predominant acid in the middle leaf. Proline accumulates in plants that

undergo stress (Verbruggen *et al.* 2008).These stresses can be brought about by drought, high levels of salt, excessive light, oxidative and biotic stresses and most importantly heavy metal stresses (Hare and Cress 1997). The function of proline in the younger plants can be to protect enzyme activities and protein integrity (Kishor *et al.* 2005). Younger plants experience more stress to metal accumulation than older leaves because of the sudden response to accumulation of metals and having lower concentrations of proteins required for enzymatic activities (Deinlein *et al.* 2012). The distribution of proline suggests as *B. coddii* grows, physiological processes within the leaves adapt to Ni accumulation more easily than younger leaves. In addition, a larger concentration of the Ni binding ligand could be present in older leaves thus relieving plant stresses and requiring lower concentrations of proline within the leaves (Sharma *et al.* 2006).

Histidine was reported to be involved in the binding of Ni several hyperaccumulators with concentrations reaching over 1200 mg kg^{-1} (Ute, Janet *et al*. 1996, Kameda, Ito *et al*. 2002, Kerkeb and Krämer 2003). Concentrations of histidine in *B. coddii* correlate with regular levels in plants (< 400 mg kg^{-1}) (Richau *et al.* 2009). These levels of histidine indicate that the function of the acid in *B. coddii* is to aid in plant growth and development (Muralla *et al.* 2007, Bikard *et al.* 2009).

Amino acid analysis did not yield any substantial concentrations of nicotianamine, the ligand reported to be responsible for Ni coordination (Callahan 2007). Nicotianamine is an amino acid part of the oxaloacetate cycle in plants and originates from its precursor methionine. Concentration of methionine in various sections of *B. coddii* is < 100 mg kg^{-1}. If nicotianamine was being produced for uptake of Ni, it is assumed that the concentrations of methionine will be higher in the plant (mol ratio methionine:Ni $1.54X10^{-6}$:1). The concentration of cysteine

compared to other amino acids in its cycle is low. Cysteine is generally associated with a high sulphur content and sulphur ligands, which have been reported not to be present in Ni hyperaccumulators (Callahan *et al.* 2006). Other amino acids that belong to the same cycles within plants were similar in concentration (Appendix 1).

Concentrations of the acids in the plant correlate with findings in other Ni hyperaccumulators where no role is played by amino acids (Callahan 2007). Ni concentrations of the plant-water extracts were quantified using ICP-OES to investigate the relationship between amino acids and Ni in *B. coddii*.

The concentration of Ni in leaves of *B. coddii* increases as the leaf grows and ages (Table 2). The larger the surface area of the leaf, the higher the concentration of accumulated Ni. Ni concentrations in the plant follows the same pattern as previous findings (Pillay 2005). Seeds of *B. coddii* contain higher levels of Ni than the stems and roots, because it requires Ni for its physiological processes during germination. Roots and stems have lower concentrations of Ni because they have no apparent role in the storage process. For proline to be involved in Ni uptake and storage it needs to bind in a minimum molar ratio of 1:1 to enable complex formation. Molar ratios between Ni and proline did not yield any ratios of significance, particularly for the young leaf (0.006535 Pro: 1 Ni) and middle leaf (0.0001468 Pro: 1 Ni). The results of this study indicate that proline does not appear to be the primary ligand in involved in Ni uptake in *B. coddii*. It may be only involved in physiological processes as a result of stressful environment of *B. coddii*. Molar ratios between amino acids and Ni were calculated, with none being of any significance to indicate a role in Ni binding.

3.2 Organic Acid Analysis in *B. coddii*

The organic acid content in *B. coddii* was analysed in an attempt to identify the ligand responsible for Ni uptake and to ascertain the distribution within the plant. A total of 25 organic acids which play a role in plant processes were analysed, with those detected shown below (Table 3). The organic acids typically associated with hyperaccumulators (ascorbic, citric and tartaric acid) were quantified in *B. coddii*.

Table 9. Detected organic acids analysed in *B. coddii*

Organic Acid	**Retention Time / min**	**Organic Acid**	**Retention Time / min**
ascorbic acid	5.29	citric acid	20.12
cinnamic acid	7.00	oxalic acid	6.39
dinitrobenzoic acid	7.12	tartaric acid	7.32
furoic acid	2.92	succinic acid	21.92, 26.86*
gallic acid	2.17	fumaric acid	8.46
hydroxynicotinic acid	3.12	maleic acid	9.71
nicotinic acid	3.31	malic acid	4.70
mandelic acid	2.89	malonic acid	4.85
phthalic acid	2.79	tannic acid	5.04
Plant -water extract	**5.29, 7.70, 7.32, 20.12**		

**Two peaks were detected for succinic acid one of which was an impurity*

All peaks were clearly resolved. The retention times of the detectable organic acids did not correspond with the retention time of the unknown peak (7.70 min) from the plant-water extract (Table 3). This indicated that none were associated with Ni uptake.

The plant-water extract yielded a chromatogram with 3 additional peaks of substantial peak area apart from the unknown peak. The peaks were identified as tartaric, citric and ascorbic acid.

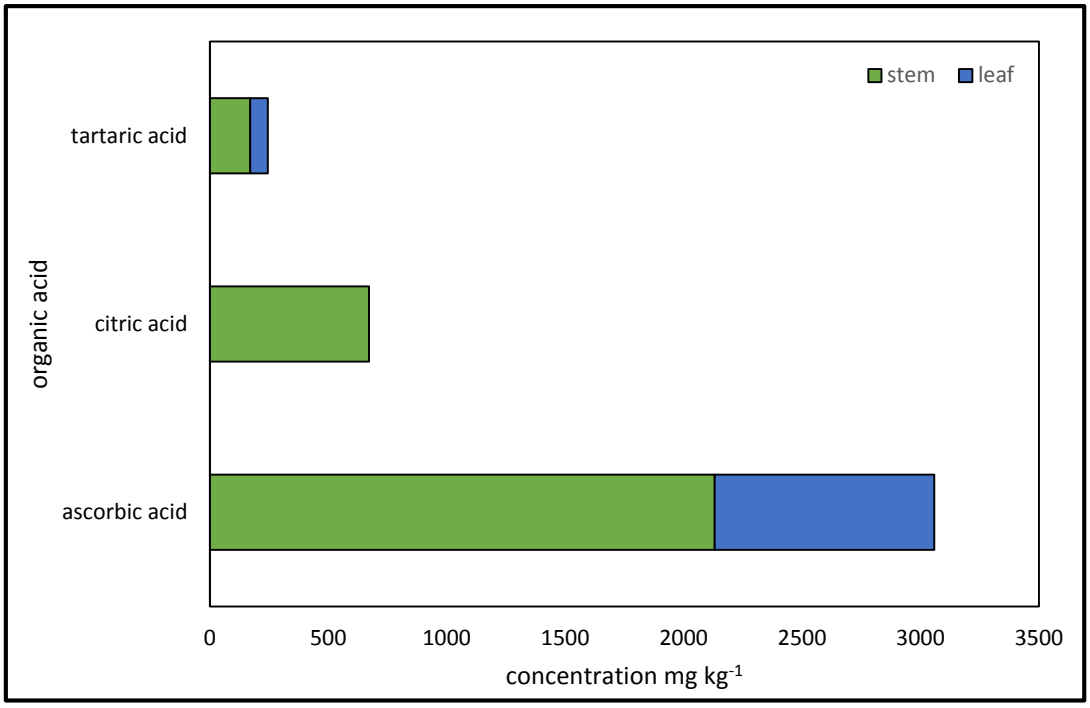


Figure 14: Major Organic Acids present in *B. coddii* (mg kg^{-1})

Compared to the stems and leaves, organic acid content in the roots of *B. coddii* were negligible indicating that organic acids are not involved in the translocation of Ni from the roots.

Malic acid, thought to originally be responsible for Ni uptake (Slatter 1998), was not present in the stems and was of negligible concentrations in the leaves.

Organic acid content was higher in the stem compared to the leaves particularly ascorbic acid (Fig 14). Compared to other findings (Ma 2000), ascorbic acid is unusually high in the stems of *B. coddii*. This indicates that it may play a role in sequestering Ni from the stem to the leaves before it is rendered into a non-toxic form.

Citric acid has been identified as a metal chelator in some hyperaccumulators (Schaumlöffel *et al.* 2003). In *B. coddii* its concentration is too low to be responsible for the sequestering in *B. coddii*.

Tartaric acid, present in both stem and leaf but in lower concentrations compared to citric and ascorbic acid also plays a role in the Krebs cycle in plants (Ma 2000). If it did play a role in Ni sequestration it would be a minor role due to the low concentration (Fig 14) of the acid present in *B. coddii*.

3.3 Identification of unknown ligand

3.3.1 Extraction and isolation of Ni-unknown ligand

Amino and organic acids were extracted from leaves of *B. coddii* using size exclusion chromatography (Section 2.5) yielding 9 fractions. Extracts were analysed for Ni content by ICP-OES then analysed by RP-HPLC to identify any relationship between Ni and organic acids eluting from SEC.

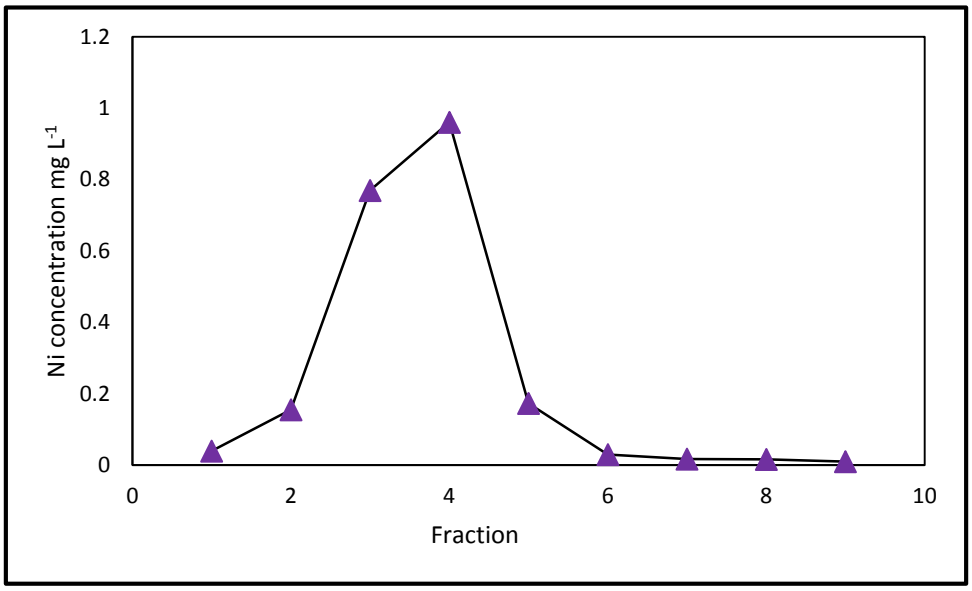
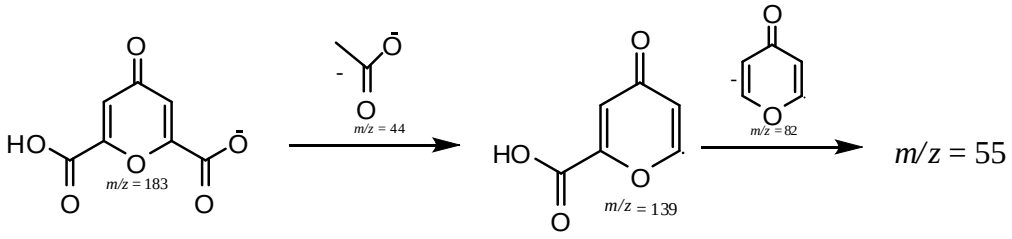


Figure 15: Extracts from SEC with corresponding Ni concentrations (mg L^{-1})

Fraction 4 had the highest nickel concentration (0.961 mg L^{-1}) from all extracts collected (Fig 15). Each fraction was subsequently analysed by RP-HPLC using UV detection. Findings obtained for fraction 3 and 4 (highest Ni levels) showed a peak indicated that a ligand had eluted with Ni. The UV-spectrum of this unidentified ligand (now reported in this study as chelidonic acid) matched the spectrum of the unidentified ligand reported by Pillay (2005). Fraction 4 had a higher peak area (866614 pA*s) of the unknown peak compared to fraction 3 (124411pA*s). These findings indicated a relationship between nickel and the unknown peak in *B. coddii*.

3.3.2 MS Analysis of the SEC extract

The plant-water extract (fraction 4) from the size exclusion column was injected into HR-MS which yielded an elemental analysis based on fragmentation patterns. Results from the injection indicated the unknown peak has a molar mass of 184.10 g mol^{-1} and a molecular formula of $C_7H_4O_6$. MS-MS was performed after HR-MS to help elucidate the unknown compound based on smaller fragmentation patterns. No metal isotopes were viewed in either spectra indicating the complex dissociates upon interaction with the LC column. Fragmentation patterns from the HR-MS and MS-MS spectra (Appendix 2) indicated the following fragmentation patterns:



Scheme 1: Fragmentation pattern of unknown

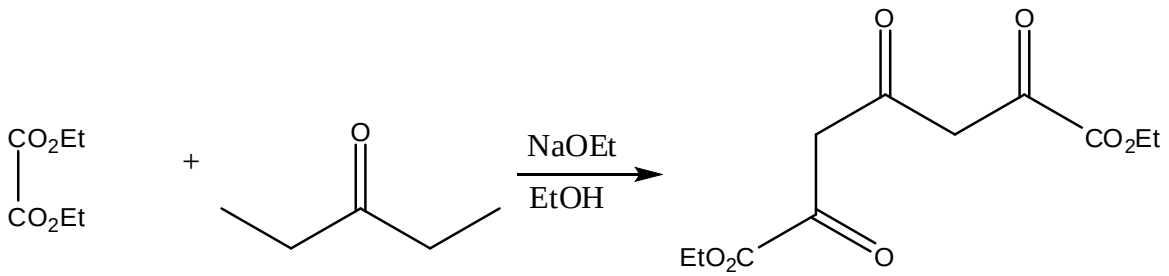
Based on fragmentation patterns along with mass obtained from HR-MS the compound was identified to be an organic acid chelidonic acid.

3.3.3 Synthesis and characterisation of chelidonic acid

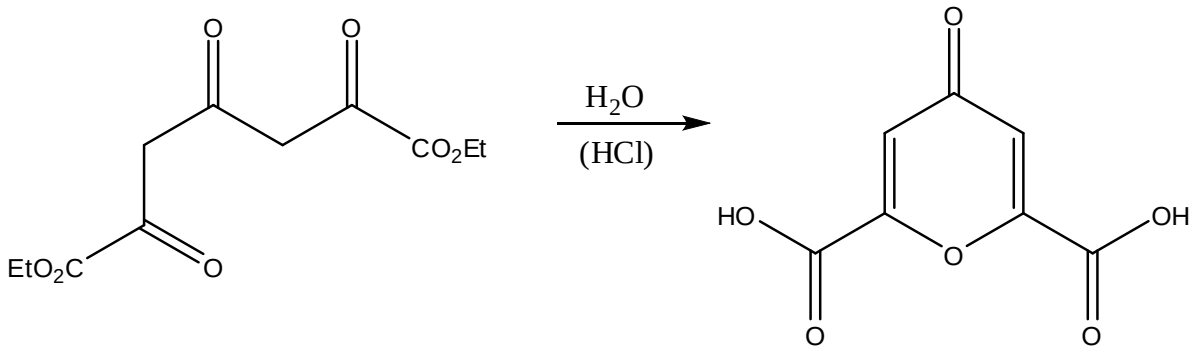
Synthesis of chelidonic acid was performed for qualitative (to match the suggested acid to the unknown peak using RP-HPLC) and quantitative analyses. Chelidonic acid is a naturally occurring heterocyclic organic acid found in plants. It can also be synthesised in a two-step reaction using acetone and ethyl oxalate.

Steps for synthesis

Step 1:



Step 2:



Scheme 2: Reaction steps for synthesis of chelidonic acid

Chelidonic acid binds to Ni in a 2:1 ratio by simply mixing the acid and Ni in a polar solvent (Belian *et al.* 2014). Previous work reported characteristics of the ligand as water soluble and polar (Pillay 2005). Studies show the acid has λ maxima of: 206, 225 and 266 cm^{-1}. The lambda max for the unknown peak in the HPLC reported in current findings is 205, 224 and 264 cm^{-1}.

Characterisation

H^1, C^{13} NMR and IR was performed to characterise the formation of chelidonic acid. Shifts indicating bond formations indicate the formation of the acid (Table 4, Table 5 and Table 6). Chelidonic acid is a symmetrical compound therefore only one peak was seen for each shift in NMR. IR data shows the stretching frequencies corresponding to relevant bonds in chelidonic acid.

Table 10. H^1 NMR Shifts

Shift / ppm	Bond
7.1482	C-H (symmetrical) on ring
2.2794	OH (symmetrical)

Table 11. C^{13} NMR Shifts

Shift / ppm	Bond
117.2090	C=C
158.1628	C-O
184.5361	C=O
215.4492	COOH

Table 12. IR Shifts for chelidonic acid (cm^{-1})

Shift/ cm^{-1}	**Bond**
3566	C-O-H
3073	C=C-H
1640	C=C
1217	C-O

3.3.4 HPLC Identification of chelidonic acid

RP-HPLC was carried out on the plant-water extract. Acidic pH values (pH <3) are favoured during analysis of organic acids to ensure acids (acids are protonated) interact with the stationary phase of the column, which has an effect on separation and resolution.

A 100 ppm standard of chelidonic acid was injected in a RP-HPLC. The resulting chromatogram and UV spectrum matched the unknown chromatogram and UV spectrum.

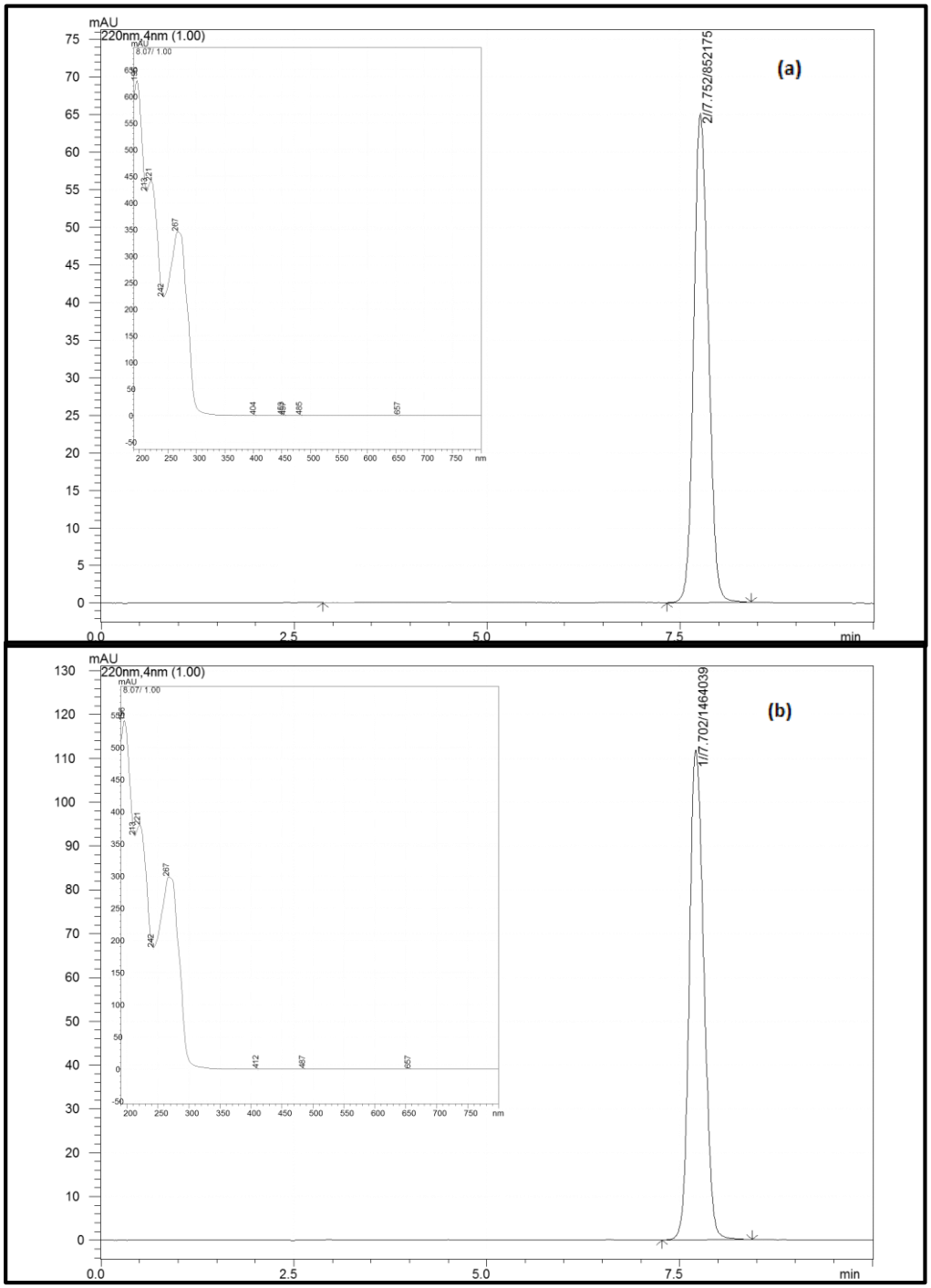


Figure 16: HPLC chromatograms and UV-Spectra of unknown plant extract (a) and chelidonic acid standard (b)

Based on retention time and matching UV spectra (Fig 16), it can be concluded that the unknown peak in the plant-water extract is chelidonic acid.

3.3.5 Quantification of Chelidonic Acid in *B. coddii*

Quantification of chelidonic acid was carried out by RP-HPLC with external linear calibration.

Plants were collected from the serpentine outcrop for analysis. Plant-water extracts of *B. coddii* were used to quantify the acid. This was preferred over a complete acid digestion to ensure the Ni-ligand complex remained intact which would give a better indication of the mol ratio of Ni to chelidonic acid upon Ni quantification. Concentrations of chelidonic acid in the root, stems and leaves of *B. coddii* were quantified.

Table 13. Ni and Chelidonic Acid Concentrations in unspiked *B. coddii* (mg kg^{-1})

Plant Section	Ni	Chelidonic acid
Root	8.590	179.1
Stem	25.59	168.5
Leaf	2944	11889

Concentrations of chelidonic acid in leaves of *B. coddii* are almost 10 fold higher compared to other plant sections (Table 7) indicating the storage of the acid within the leaves. Reported concentrations of chelidonic acid in leaves, stems and root of non-hyperaccumulators are < 200mg kg^{-1} (Leopold *et al.* 1952). Ni levels in leaves are over 100 fold higher in leaves compared to the stems and significantly higher than roots. These high levels are indicative of Ni storage within leaves. Uptake studies with Ni in *B. coddii* would give an indication to type of relationship between high levels of chelidonic acid in leaves and Ni.

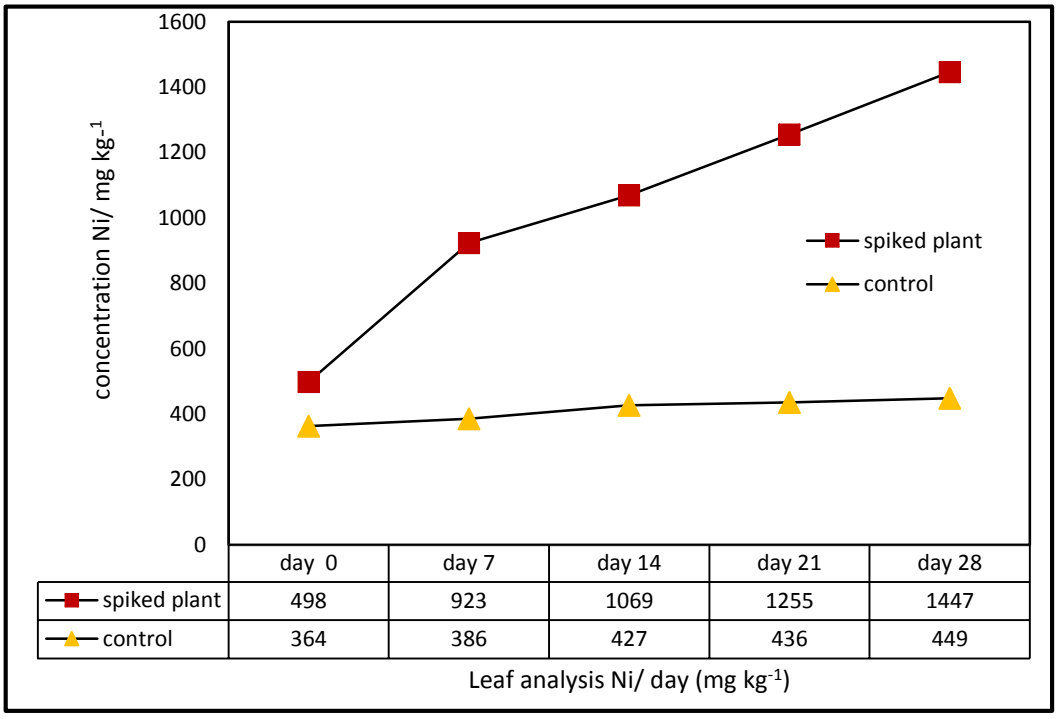
Chapter 4: RESULTS & DISCUSSION

Uptake Analysis

Spiking studies were carried out to examine the relationship between Ni and chelidonic acid. Spiked plants were treated with 100 mM Ni solution for a period of 28 days prior to analysis *i.e.* 20 mL of Ni solution was added to the plants every second day (section 3.3.3). A plant-water extract was used for all plant analyses in this study.

4.1.1 Ni concentrations in *B. coddii*

Over a period of one month *B. coddii* was spiked with a known concentration of a 100 mM Ni solution ($NiCl_2.6H_2O$) to investigate the relationship between Ni and chelidonic acid.



	day 0	day 7	day 14	day 21	day 28
spiked plant	498	923	1069	1255	1447
control	364	386	427	436	449

Figure 17: Concentration of Ni in *B. coddii* leaves over a one month treatment of 100mM soluble Ni (mg kg^{-1})

Discrepancies between the concentrations of Ni on day 0 could be due to small variations in plant size, sample preparation (extraction) and/or physiological uptake within the plants. Upon the addition of Ni (Fig 17), *B. coddii* increases its uptake of Ni. Previous studies indicate *B. coddii* can accumulate up to 10 fold more Ni when the soil is exposed to higher levels of bioavailable Ni (Pillay 2005). This shows that the plant exhibits an increased uptake response to elevated Ni concentrations. As the plant takes up and stores more Ni, it needs to also produce a chelator/ligand to bind to Ni in order to render it non-toxic to the plant. Without a ligand to bind to Ni, high levels soluble Ni would be present within the leaves of *B. coddii* ultimately resulting in Ni toxicity and plant death.

4.1.2. Relationship between Ni increase and amino acids in *B. coddii*

Amino acid analysis was performed on spiked Ni leaves of *B. coddii*. A total of 17 amino acid concentrations were quantified to investigate the relationship between an increase in Ni and amino acids.

Table 14. Amino acid concentrations of *B. coddii* at day 0 and day 28 (mg kg^{-1})

Amino Acid	Day 0	Day 28
His	830	890
Pro	31840	29160
Arg	920	1140
Glu	630	450
Phe	180	160
Tyr	110	110
Leu	180	190
Ala	630	920
Val	1000	1030
Asp	1020	980
Met	290	270
Lys	30	90
ILe	140	110
Thr	570	470
Cys	40	20
Ser	960	970
Gly	170	160

Amino acid levels between day 0 and day 28 do not differ significantly (Table 8). Over a period of 28 days, some levels decrease and others increase by small margins. Whether this is a response to increasing Ni is unclear. The concentration of proline, shows a slight decrease over time, this is in line with earlier suggestions that its production is a response to plant age and

stress. To ascertain a clearer understanding of amino acid response to Ni uptake, spiking experiments need to be conducted for a longer period of time (future work).

4.1.2 Relationship between elevated Ni levels and Chelidonic acid in *B. coddii*

In conjunction with quantification of Ni in *B. coddii*, chelidonic acid was also quantified (RP-HPLC) during the spiking experiment.

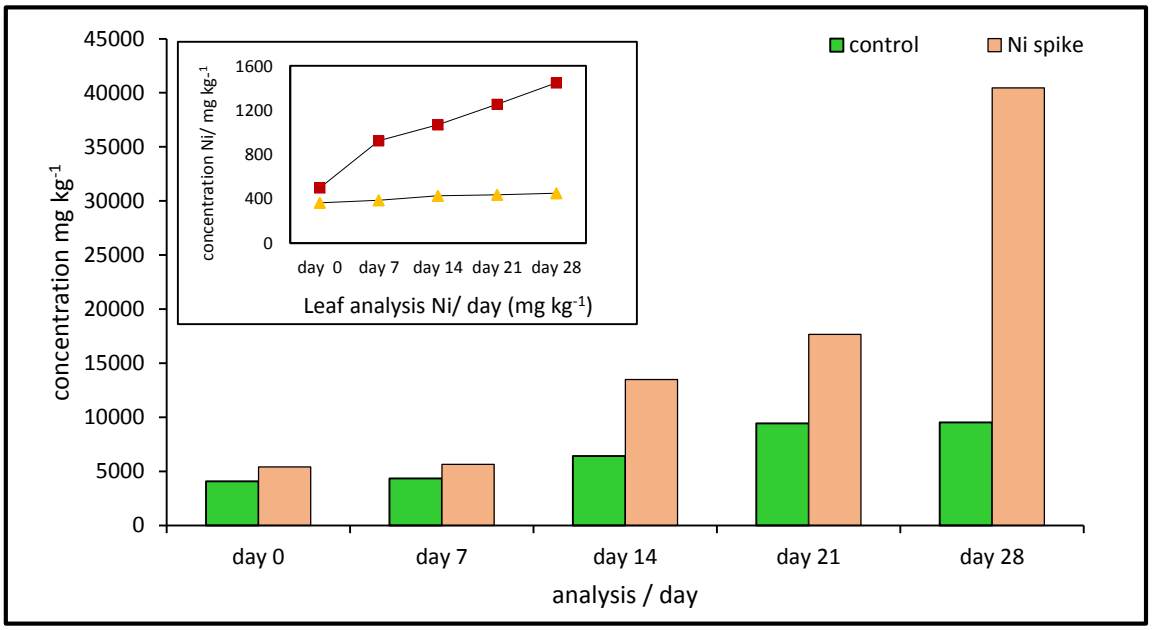


Figure 18: Concentration of chelidonic acid in *B. coddii* during spiking experiment (mg kg^{-1})

Spiking studies with metals have been carried out on other hyperaccumulators (Peng *et al.* 2008). When a high level of metal has been exposed to the plant, the plant responds by increasing its concentration of some other organic or amino acid. These studies have been carried out to identify the metal-ligand complex as well as assess the plants physiological response to accumulation (Bhatia *et al.* 2005, Peng *et al.* 2008).

B. coddii exhibits a response in the production of chelidonic acid when exposed to increasing Ni levels (Fig 18), Ni concentrations from Fig 17 are included to help compare the change in concentrations. Molar ratios give an indication to the ability of a ligand to complex to a metal. Chelidonic acid complexes with Ni in a 2:1 ratio. As the amount of Ni added increases, the production of chelidonic acid increases over 3 fold compared to the control. This shows that chelidonic acid plays a role in Ni uptake in *B. coddii* and is a response to stress. Molar ratios between Ni and chelidonic acid were determined during spiking studies.

Table 15. Molar ratio of chelidonic acid: Ni in a control and spiking study

Day of Analysis	**Molar Ratio Chelidonic Acid : Ni**	
	Control	**Spike**
0	3.46:1	3.59:1
7	3.81:1	3.87:1
14	4.0:1	4.80:1
21	4.49:1	4.90:1
28	4.93:1	6.76:1

Slatter (1998) states that if a ligand is responsible for complexation it will be found in excessive levels in *B. coddii*. The control analysis (Table 9) showed a constant increase in the molar ratio chelidonic acid to Ni.

The ratio of chelidonic acid reached levels of 6.76:1 to Ni (Table 9) indicating that *B. coddii's* response to a sudden increase in Ni was to produce excess concentrations of chelidonic acid to prepare for an influx of Ni uptake. Based on quantification and spiking studies it can be

concluded that chelidonic acid is in fact the ligand responsible for complexing to Ni in *B. coddii*.

Results in this chapter show a contrast between the control (*B. coddii* plants no addition of 100mM Ni solution) and the spiked plant (100mM Ni addition). As the control plant ages a constant increase of Ni results in an increase of chelidonic acid, with mol ratios being relatively constant, however when exposed to increasing Ni levels, *B. coddii* produces higher levels of chelidonic acid (28 days) which may be due to a stress response by the plant. Further uptake studies with varying Ni concentrations need to be carried out to ascertain the maximum amount of Ni and chelidonic acid *B. coddii* can produce. These results give insight into the plants uptake capabilities.

Chapter 5
Conclusions

This study found several new insights into plant accumulation by *B. coddii*. The primary objective was achieved and the ligand responsible for uptake identified as chelidonic acid. The acid has not been associated with uptake in other hyperaccumulators. In addition, this study has successfully shown that ligands (malic acid and nicotianamine) previously reported to be involved in Ni uptake are not present in sufficient amounts to adequately complex Ni. Typically molar ratios for Ni and ligands should be a minimum of 1:1. Nicotianamine was present in the plant at levels too low to successfully quantify.

Chelidonic acid was successfully synthesised and used to qualitatively and quantitatively measure the concentration in *B. coddii*. Chelidonic acid was quantified and exists in a 3:1 mol ratio compared to Ni. Uptake studies investigating the response of chelidonic acid to an increase in Ni indicated *B. coddii* produces more chelidonic acid as soluble Ni concentrations increase. The molar ratio between chelidonic acid and Ni increase from 3:1 (normal) to 6.76:1 after 28 days of spiking *B. coddii* with 100mM of Ni solution.

In addition, other important amino and organic acids present in the plant were also identified and quantified. This analysis indicated that no amino acids played a significant role in Ni accumulation. Only proline appears to have a significant role to play in the hyperaccumulation process and is present at elevated concentrations in the leaves (12000 mg kg^{-1} in young leaves). Molar ratios calculated for proline indicate that proline can play no role in uptake and storage

of Ni. Numerous studies have reported proline production in hyperaccumulators as a stress response and this seems apparent for *B. coddii*.

Organic acid analysis showed high levels of ascorbic and citric acid in the stems of *B. coddii*. The molar ratios of these two acids indicate that there is no relationship between either acid with Ni uptake. The high ascorbic acid levels may be due to a stress response and an aid to plant growth and development, with citric acid present due to the vital role it plays in the Krebs cycle.

Amino acids and organic acids are the most predominant ligands associated with hyperaccumulation. Results from this study show that chelidonic acid is clearly associated with the uptake mechanism in *B. coddii*.

5.1 Further Work

The identification of chelidonic acid as the ligand responsible for uptake in *B.coddii* leads to several other avenues of research.

- Using *B. coddii* as a plant for phytoremediation

  The maximum concentration of Ni that *B. coddii* can tolerate should be investigated to estimate the plants' capacity as a phytoremediator. This can be carried out by uptake studies similar to the one carried out in this research but for a longer period of time and with a wider range of solutions.

- Enhancing the uptake of Ni by the plant

Studies into increasing the uptake capacity and rate may improve the phytoremediation capability of the plant. Investigation into manipulating the soluble Ni levels and/or the chelidonic acid levels may provide improved uptake.

References

Aiken, A. C., DeCarlo, P. F. and Jimenez, J. L. (2007). "Elemental analysis of organic species with electron ionization high-resolution mass spectrometry." Analytical Chemistry **79**(21): 8350-8358.

Anderson, C., Deram, A., Petit, D., Brooks, R., Stewart, R. and Simcock, R. (2001). "Induced hyperaccumulation: metal movement and problems." Trace Elements in Soils: Bioavailability, Flux and Transfer **4**: 63-76.

Angle, J. S., Baker, A. J. M., Whiting, S. N. and Chaney, R. L. (2003). "Soil moisture effects on uptake of metals by *Thlaspi, Alyssum, and Berkheya*." Plant and Soil **256**(2): 325-332.

Ardrey, B. (2003). Liquid Chromatography-Mass Spectrometry: An Introduction. West Sussex, England, Wiley.

Baker, A. J. M. (1981). "Accumulators and excluders - strategies in the response of plants to heavy metals." Journal of Plant Nutrition(3): 643-654.

Baker, A. J. M., McGrath, S. P., Reeves, R. D. and Smith, J. A. C. (1999). Metal hyperaccumulator plants: a review of the ecology and physiology of a biological resource for phytoremediation of metal-polluted soils. Boca Raton, Lewis Publishers.

Belian, M. F., Silva, W. E., de Sá, G. F., Alves, S. and de Farias, R. F. (2014). Synthesis and characterization of Cr(III), Mn(II), Fe(III), Co(II), Ni(II), Cu(II) and Zn(II) complexes with 2,6-pyridinedicarboxilic acid, chelidamic acid and chelidonic acid. Synthesis and Reactivity in Inorganic, Metal-Organic, and Nano-Metal Chemistry, Taylor & Francis. **44:** 1461-1463.

Bhatia, N. P., Walsh, K. B. and Baker, A. J. (2005). "Detection and quantification of ligands involved in nickel detoxification in a herbaceous Ni hyperaccumulator S*tackhousia tryonii Bailey*." Journal of Experimental Botany **56**(415): 1343-1349.

Bikard, D., Patel, D., Le Metté, C., Giorgi, V., Camilleri, C., Bennett, M. J. and Loudet, O. (2009). "Divergent evolution of duplicate genes leads to genetic incompatibilities within *A. thaliana*." Science **323**(5914): 623-626.

Brooks, R. R., Chambers, M. F., Nicks, L. J. and Robinson, B. H. (1998). "Phytomining." Trends in plant science **3**(9): 359-362.

Brooks, R. R., Lee, J., Reeves, R. D. and Jaffré, T. (1977). "Detection of nickeliferous rocks by analysis of herbarium specimens of indicator plants." Journal of Geochemical Exploration **7**: 49-57.

Brooks, R. R. and Robinson, B. H. (1998). Plants that hyperaccumulate heavy metals: Their role in phytoremediation, microbiology, archaeology, mineral exploration and phytomining. New York, CABI Publishing.

Brooks, R. R., Robinson, B. H., Howes, A. W. and Chiarucci, A. (2001). "An evaluation of *Berkheya coddii Roessler* and *Alyssum bertolonii Desv.* for phytoremediation and phytomining of nickel." South African Journal of Science **97**(11 & 12): p.558-560.

Callahan, D., Baker, A. M., Kolev, S. and Wedd, A. (2006). "Metal ion ligands in hyperaccumulating plants." Journal of Biological Inorganic Chemistry **11**(1): 2-12.

Callahan, D. L. (2007). The coordination of nickel in hyperaccumulating plants. PhD, University of Melbourne.

Chaney, R. L. (1983). Land Treatment of Hazardous Wastes. Park Ridge, New Jersey, Noyes Data Corp.

Chen, Y., Lin, Q., Luo, Y., He, Y., Zhen, S., Yu, Y., Tian, G. and Wong, M. (2003). "The role of citric acid on the phytoremediation of heavy metal contaminated soil." Chemosphere **50**(6): 807-811.

Clemens, S., Palmgren, M. G. and Krämer, U. (2002). "A long way ahead: understanding and engineering plant metal accumulation." Trends in plant science **7**(7): 309-315.

Dams, R., Huestis, M. A., Lambert, W. E. and Murphy, C. M. (2003). "Matrix effect in bio-analysis of illicit drugs with LC-MS/MS: influence of ionization type, sample preparation, and biofluid." Journal of the American Society for Mass Spectrometry **14**(11): 1290-1294.

Deinlein, U., Weber, M., Schmidt, H., Rensch, S., Trampczynska, A., Hansen, T. H., Husted, S., Schjoerring, J. K., Talke, I. N., Krämer, U. and Clemens, S. (2012). "Elevated Nicotianamine Levels in *Arabidopsis halleri* Roots Play a Key Role in Zinc Hyperaccumulation." The Plant Cell Online **24**(2): 708-723.

Delauney, A. J. and Verma, D. P. S. (1993). "Proline biosynthesis and osmoregulation in plants." The plant journal **4**(2): 215-223.

Fraser, K., Long, H., Candlin, R. and Harding, M. M. (1965). "The structures of bishistidino-nickel (II),-cobalt (II), and-cadmium (II)." Chemical Communications (London)(15): 344-345.

Gramlich, A., Moradi, A. B., Robinson, B. H., Kaestner, A. and Schulin, R. (2011). "Dimethylglyoxime (DMG) staining for semi-quantitative mapping of Ni in plant tissue." Environmental and Experimental Botany **71**(2): 232-240.

Hare, P. D. and Cress, W. A. (1997). "Metabolic implications of stress-induced proline accumulation in plants." Plant Growth Regulation **21**(2): 79-102.

Harris, A. T., Naidoo, K., Nokes, J., Walker, T. and Orton, F. (2008). "Indicative assessment of the feasibility of Ni and Au phytomining in Australia." Journal of Cleaner Production **17**(2): 194-200.

Holmes, L. (2001). "Determination of thorium by ICP-MS and ICP-OES." Radiation protection dosimetry **97**(2): 117-122.

Jaffré, T., Brooks, R. R., Lee, J. and Reeves, R. D. (1976). "*Sebertia acuminata*: a hyperaccumulator of nickel from New Caledonia." Science **193**(4253): 579-580.

Jaffré, T. and Schmid, M. (1974). "Nickel accumulation by a *Rubiaceae* of New Caledonia." Psychotria douarrei, Comptes Rendus de l'Académie des Sciences **278**: 1727-1730.

Jones, D. (1998). "Organic acids in the rhizosphere – a critical review." Plant and Soil **205**(1): 25-44.

Kameda, Y., Ito, E., Takeshita, A., Nakazawa, R. and Takenaga, H. (2002). "Detoxicational Effect of the Exogenous Histidine on the Growth of Pea (*Pisum sativum L.*) Treated with Nickel." Journal of agricultural science-Tokyo University of agriculture **47**(1): 45-48.

Kazakevich, Y. V. and Lobrutto, R. (2007). HPLC for pharmaceutical scientists, John Wiley & Sons.

Keeling, S. M., Stewart, R. B., Anderson, C. W. N. and Robinson, B. H. (2003). "Nickel and cobalt phytoextraction by the hyperaccumulator *Berkheya coddii*: implications for polymetallic phytomining and phytoremediation." International Journal of Phytoremediation **5**(3): 235-244.

Kelly, P. C., Brooks, R. R., Dilli, S. and Jaffré, T. (1975). "Preliminary observations on the ecology and plant chemistry of some nickel-accumulating plants from New Caledonia." Proceedings of the Royal Society B: Biological Sciences **189**(1084): 69-80.

Kerkeb, L. and Krämer, U. (2003). "The Role of Free Histidine in Xylem Loading of Nickel in *Alyssum lesbiacum* and *Brassica juncea*." Plant Physiology **131**(2): 716-724.

Kishor, P. K., Sangam, S., Amrutha, R., Laxmi, P. S., Naidu, K., Rao, K., Rao, S., Reddy, K., Theriappan, P. and Sreenivasulu, N. (2005). "Regulation of proline biosynthesis, degradation,

uptake and transport in higher plants: its implications in plant growth and abiotic stress tolerance." Current Science **88**(3): 424-438.

Krämer, U., Cotter-Howells, J. D., Charnock, J. M., Baker, A. J. and Smith, J. A. C. (1996). "Free histidine as a metal chelator in plants that accumulate nickel." Nature **379**(6566): 635-638.

Krämer, U., Pickering, I. J., Prince, R. C., Raskin, I. and Salt, D. E. (2000). "Subcellular localization and speciation of nickel in hyperaccumulator and non-accumulator *Thlaspi* species." Plant Physiology **122**(4): 1343-1354.

Küpper, H., Mijovilovich, A., Meyer-Klaucke, W. and Kroneck, P. M. (2004). "Tissue-and age-dependent differences in the complexation of cadmium and zinc in the cadmium/zinc hyperaccumulator *Thlaspi caerulescens* (Ganges ecotype) revealed by X-ray absorption spectroscopy." Plant Physiology **134**(2): 748-757.

L'Huillier, L., d'Auzac, J., Durand, M. and Michaud-Ferrière, N. (1996). "Nickel effects on two maize (*Zea mays*) cultivars: growth, structure, Ni concentration, and localization." Canadian journal of botany **74**(10): 1547-1554.

Lee, J., Reeves, R. D., Brooks, R. R. and Jaffré, T. (1977). "Isolation and identification of a citrato-complex of nickel from nickel-accumulating plants." Phytochemistry **16**(10): 1503-1505.

Leopold, A. C., Scott, F. I., Klein, W. H. and Ramstad, K. (1952). "Chelidonic Acid and its Effects on Plant Growth." Physiologia Plantarum **5**(1): 85-90.

Ma, J. F. (2000). "Role of Organic Acids in Detoxification of Aluminum in Higher Plants." Plant and Cell Physiology **41**(4): 383-390.

McGrath, S. P., Sidoli, C. M. D., Baker, A. J. M. and Reeves, R. D. (1993). Integrated Soil and Sediment Research; A Basis for Proper Protection. Amsterdam, The Netherlands, Kluwer Academic Publishers.

Mesjasz-Przybylowicz, J. and Przybylowicz, W. J. (2011). "PIXE and metal hyperaccumulation: from soil to plants and insects." X-Ray Spectrometry **40**(3): 181-185.

Mesjasz-Przybylowicz, J., Przybylowicz, W. J. and Pineda, C. A. (2001). "Nuclear microprobe studies of elemental distribution in apical leaves of the Ni hyperaccumulator *Berkheya coddii*." South African Journal of Science **97**(11/12, Pt. 2): 591-593.

Minguzzi, C. and Vergano, O. (1948). "Nickel content in the ash of *Alyssum bertolonii*." Atti della Società Toscana de Scienze Naturali di Pisa **55**: 49-74.

Moradi, A. B., Oswald, S. E., Nordmeyer-Massner, J. A., Pruessmann, K. P., Robinson, B. H. and Schulin, R. (2010). "Analysis of nickel concentration profiles around the roots of the hyperaccumulator plant *Berkheya coddii* using MRI and numerical simulations." Plant and Soil **328**(1-2): 291-302.

Moradi, A. B., Swoboda, S., Robinson, B., Prohaska, T., Kaestner, A., Oswald, S. E., Wenzel, W. W. and Schulin, R. (2010). "Mapping of nickel in root cross-sections of the hyperaccumulator plant *Berkheya coddii* using laser ablation ICP-MS." Environmental and Experimental Botany **69**(1): 24-31.

Mori, S. and Barth, H. G. (1999). Size exclusion chromatography. Berlin, Springer.

Muralla, R., Sweeney, C., Stepansky, A., Leustek, T. and Meinke, D. (2007). "Genetic dissection of histidine biosynthesis in *Arabidopsis*." Plant Physiology **144**(2): 890-903.

Murray, R., Miller, D. J. and Kryc, K. (2000). "Analysis of major and trace elements in rocks, sediments, and interstitial waters by inductively coupled plasma–atomic emission spectrometry (ICP-AES)." ODP Technical Notes **29**: 1-27.

Naicker, C. and Pillay, L. (2011). Ni in *Berkheya coddii*. Honours, Westville, University of KwaZulu-Natal**.**

Nemutandani, T., Dutertre, D., Chimuka, L., Cukrowska, E. and Tutu, H. (2006). "The potential of Berkheya coddii for phytoextraction of nickel, platinum, and palladium contaminated sites." Toxicololgy and Environmental Chemistry **88**(2): 175-185.

Peng, K., Luo, C., You, W., Lian, C., Li, X. and Shen, Z. (2008). "Manganese uptake and interactions with cadmium in the hyperaccumulator—*Phytolacca Americana L*." Journal of Hazardous Materials **154**(1–3): 674-681.

Pillay, L. (2005). Uptake and distribution in the Ni hyperaccumulator *Berkheya coddii*. Masters, University of Natal (Howard College).

Pramanik, B. K., Matsui, T., Suzuki, H., Kusugi, Y. and Tomohiro, M. (2004). "Seasonal fluctuations of some sucrose metabolizing enzymes and sugar andorganic acid contents in broccoli." Asian Journal of Plant Sciences **3**(5): 549-555.

Rai, V. K. (2002). "Role of Amino Acids in Plant Responses to Stresses." Biologia Plantarum **45**(4): 481-487.

Reeves, R. D. (2006). Hyperaccumulation of trace elements by plants. Dordrecht, Springer.

Reeves, R. D. and Baker, A. J. M. (2000). Metal Accumulating Plants. Phytoremediation of Toxic Metals - Using Plants to Clean up the Environment. New York, Wiley**:** 193-229.

Reichman, S. M. and Parker, D. R. (2005). Metal complexation by phytosiderophores in the rhizosphere. Toronto, Elsevier.

Rengel, Z. (1999). Heavy Metals as Essential Nutrients. Heavy Metal Stress in Plants, Springer Berlin Heidelberg**:** 231-251.

Richau, K. H., Kozhevnikova, A. D., Seregin, I. V., Vooijs, R., Koevoets, P. L., Smith, J. A. C., Ivanov, V. B. and Schat, H. (2009). "Chelation by histidine inhibits the vacuolar sequestration of nickel in roots of the hyperaccumulator *Thlaspi caerulescens*." New Phytologist **183**(1): 106-116.

Riegel, E. R. and Zwilgmeyer, F. (1943). "Chelidonic acid." Organic Syntheses: 40.

Roberts, M. F. and Wink, M. (1998). Alkaloids: biochemistry, ecology, and medicinal applications, Springer.

Robinson, B. H., Brooks, R. R. and Clothier, B. E. (1999). "Soil Amendments Affecting Nickel and Cobalt Uptake by *Berkheya coddii*: Potential Use for Phytomining and Phytoremediation." Annals of Botany **84**(6): 689-694.

Robinson, B. H., Brooks, R. R., Howes, A. W., Kirkman, J. H. and Gregg, P. E. H. (1997). "The potential of the high-biomass nickel hyperaccumulator *Berkheya coddii* for phytoremediation and phytomining." Journal of Geochemical Exploration **60**(2): 115-126.

Robinson, B. H., Lombi, E., Zhao, F. J. and McGrath, S. P. (2003). "Uptake and distribution of nickel and other metals in the hyperaccumulator *Berkheya coddii*." New Phytologist **158**(2): 279-285.

Rojas, E. E. G., dos Reis Coimbra, J. S., Minim, L. A., Zuniga, A. D. G., Saraiva, S. H. and Minim, V. P. R. (2004). "Size-exclusion chromatography applied to the purification of whey proteins from the polymeric and saline phases of aqueous two-phase systems." Process biochemistry **39**(11): 1751-1759.

Romheld, V. and Marschner, H. (1986). "Evidence for a specific uptake system for iron phytosiderophores in roots of grasses." Plant Physiology **80**(1): 175-180.

Sagner, S., Kneer, R., Wanner, G., Cosson, J.-P., Deus-Neumann, B. and Zenk, M. H. (1998). "Hyperaccumulation, complexation and distribution of nickel in *Sebertia acuminata*." Phytochemistry **47**(3): 339-347.

Salt, D. E., Prince, R. C., Baker, A. J. M., Raskin, I. and Pickering, I. J. (1999). "Zinc Ligands in the Metal Hyperaccumulator *Thlaspi caerulescens* As Determined Using X-ray Absorption Spectroscopy." Environmental Science & Technology **33**(5): 713-717.

Salt, D. E., Smith, R. D. and Raskin, I. (1998). "Phytoremediation." Annual Review of Plant Physiology and Plant Molecular Biology **49**(1): 643-668.

Schat, H., Sharma, S. S. and Vooijs, R. (1997). "Heavy metal-induced accumulation of free proline in a metal-tolerant and a nontolerant ecotype of *Silene vulgaris*." Physiologia Plantarum **101**(3): 477-482.

Schaumlöffel, D., Ouerdane, L., Bouyssiere, B. and Łobiński, R. (2003). "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." Journal of Analytical Atomic Spectrometry **18**(2): 120-127.

Severne, B. C. and Brooks, R. R. (1972). "A nickel-accumulating plant from Western Australia." Planta **103**(1): 91-94.

Sharma, S. S. and Dietz, K.-J. (2006). "The significance of amino acids and amino acid-derived molecules in plant responses and adaptation to heavy metal stress." Journal of Experimental Botany **57**(4): 711-726.

Shen, Z.-W., Fisinger, U., Poulev, A., Eisenreich, W., Werner, I., Pleiner, E., Bacher, A. and Zenk, M. (2001). "Tracer studies with 13C-labeled carbohydrates in cultured plant cells. Retrobiosynthetic analysis of chelidonic acid biosynthesis." Phytochemistry **57**(1): 33-42.

Slatter, K. (1998). Nickel Accumulation and Tolerance in *Berkheya coddii* and its Application in Phytoremediation. Masters, University of Natal.

Sneddon, J. and Vincent, M. D. (2008). "ICP-OES and ICP-MS for the determination of metals: Application to oysters." Analytical Letters **41**(8): 1291-1303.

Snyder, L. R., Kirkland, J. J. and Glajch, J. L. (2012). Practical HPLC method development, John Wiley & Sons.

Souverain, S., Rudaz, S. and Veuthey, J.-L. (2004). "Matrix effect in LC-ESI-MS and LC-APCI-MS with off-line and on-line extraction procedures." Journal of Chromatography A **1058**(1): 61-66.

Terry, N. and Bañuelos, G. S. (1999). Phytoremediation of contaminated soil and water. Boca Raton, FL, Lewis Publishers.

Thomas, R. (2013). Practical guide to ICP-MS: a tutorial for beginners, CRC press.

Traldi, P., Magno, F., Lavagnini, I. and Seraglia, R. (2006). Quantitative Applications of Mass Spectrometry, Wiley.

Ute, K., Janet, D. C.-H., John, M. C., Alan, J. M. B. and Smith, J. A. C. (1996). "Free histidine as a metal chelator in plants that accumulate nickel." Nature **379**(6566): 635-638.

Vasudevan, P. T., Palekar, A. A. and Yan, S. (2002). "Optimization of lipase purification by rotating annular size-exclusion chromatography." Biocatalysis and Biotransformation **20**(3): 189-199.

Vékey, K., Telekes, A. and Vertes, A. (2011). Medical applications of mass spectrometry. Amsterdam, The Netherlands, Elsevier.

Verbruggen, N. and Hermans, C. (2008). "Proline accumulation in plants: a review." Amino Acids **35**(4): 753-759.

Watanabe, T. and Osaki, M. (2002). "Mechanisms of adaptation to high aluminum condition in native plant species growing in acid soils: a review." Communications in Soil Science and Plant Analysis **33**(7-8): 1247-1260.

Wei, W., Wang, Y., Wei, Z.-G., Zhao, H.-Y., Li, H.-X. and Hu, F. (2009). "Roles of Organic Acids and Nitrate in the Long-Distance Transport of Cobalt in Xylem Saps of *Alyssum murale* and *Trifolium subterraneum*." Biological Trace Element Research **131**(2): 165-176.

Whiting, S. N., de Souza, M. P. and Terry, N. (2001). "Rhizosphere Bacteria Mobilize Zn for Hyperaccumulation by *Thlaspi caerulescens*." Environmental Science & Technology **35**(15): 3144-3150.

Zarinkamar, F., Saderi, Z. and Soleimanpour, S. (2013). "Excluder Strategies in Response to Pb Toxicity in *Matricaria chamomilla*." Advances in Bioresearch **4**(3).

Zhao, F., Lombi, E. and Breedon, T. (2000). "Zinc hyperaccumulation and cellular distribution in *Arabidopsis halleri*." Plant, Cell & Environment **23**(5): 507-514.

Appendix 1: Amino acid analysis

Table A1. Complete list of amino acids identified (concentrations in mg mL^{-1})

	Young leaf	Seeds	Middle leaf	Roots	Older leaf	Stems
His	0.027	0.024	0.026	0.006	0.024	0.03
Ser	0.053	0.034	0.053	0.009	0.029	0.07
Arg	0.042	0.025	0.065	0.016	0.031	0.008
Gly	0.016	0.023	0.011	0.007	0.03	0.045
Asp	0.039	0.06	0.058	0.018	0.045	0.131
Glu	0.07	0.118	0.096	0.022	0.231	0.233
Thr	0.014	0.019	0.026	0.005	0.016	0.043
Ala	0.041	0.04	0.035	0.013	0.068	0.13
Pro	1.302	0.021	0.933	0.005	0.014	0.175
Cys	0.002	0.003	0.001	0.000	0.001	0.004
Lys	0.031	0.017	0.019	0.006	0.015	0.043
Tyr	0.009	0.018	0.008	0.005	0.008	0.029
Met	0.004	0.004	0.004	0.001	0.002	0.008
Val	0.026	0.03	0.026	0.007	0.034	0.076
ILe	0.01	0.021	0.009	0.004	0.02	0.051
Leu	0.026	0.024	0.019	0.01	0.027	0.059
Phe	0.014	0.021	0.015	0.005	0.008	0.016

Table A2. Ni concentrations for amino acid analysis at 231.604 nm in mg L^{-1}

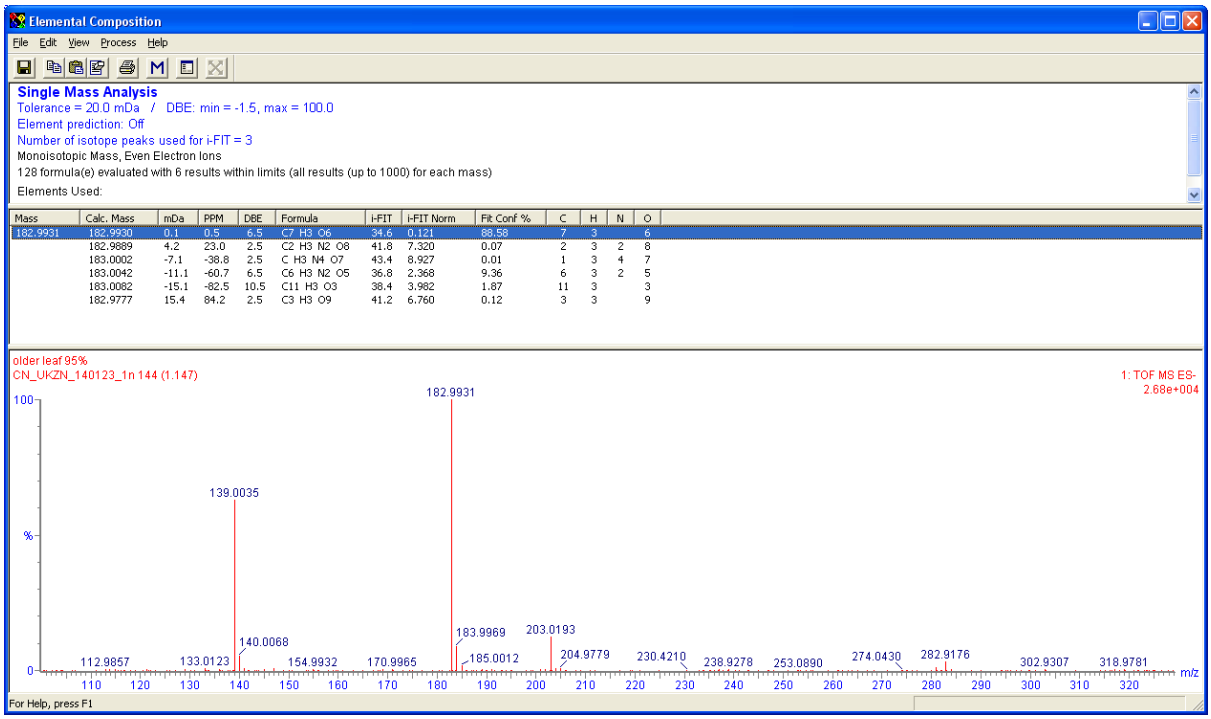
Plant section	**Ni**
Roots	1.819
Stems	15.47
Young leaf	28.81
Middle leaf	30.02
Old leaf	21.57
Seeds	1.313

Table A3. Organic acids used in the study

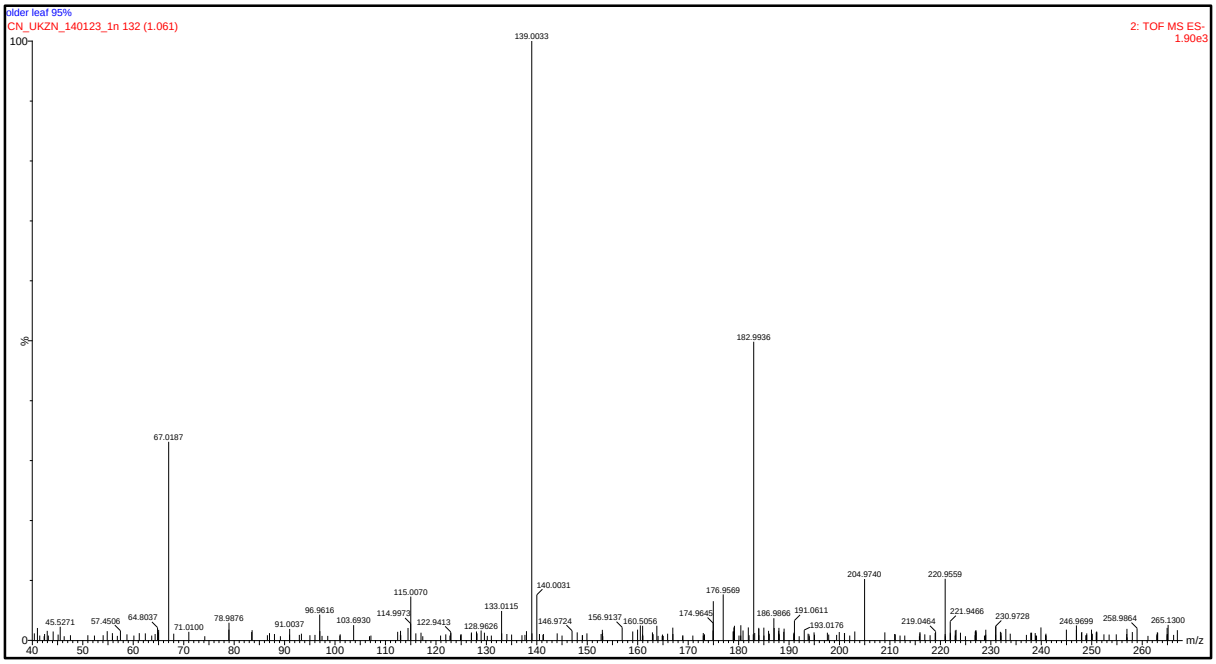
Organic Acid	**Supplier**
Ascorbic acid	Associated Chemical Enterprise
Cinnamic acid	Sigma
Dinitrobenzoic acid	BDH Chemicals Limited
Furoic acid	Sigma
Gallic acid	BDH Chemicals Limited
Hydroxynicotinic acid	Sigma
Nicotinic acid	Sigma
Mandelic acid	SaarChem
Phthalic acid	SaarChem
Tannic acid	BDH Chemicals Limited
Maleic acid	SaarChem
Malic acid	Fluka
Malonic acid	SaarChem
Citric acid	Continental Laboratory Suppliers
Oxalic acid	SaarChem
Tartaric acid	SMM Instruments
Succinic acid	SaarChem
Fumaric acid	Associated Chemical Enterprise

Appendix 2: MS Spectra

HR-MS of unknown peak in HPLC

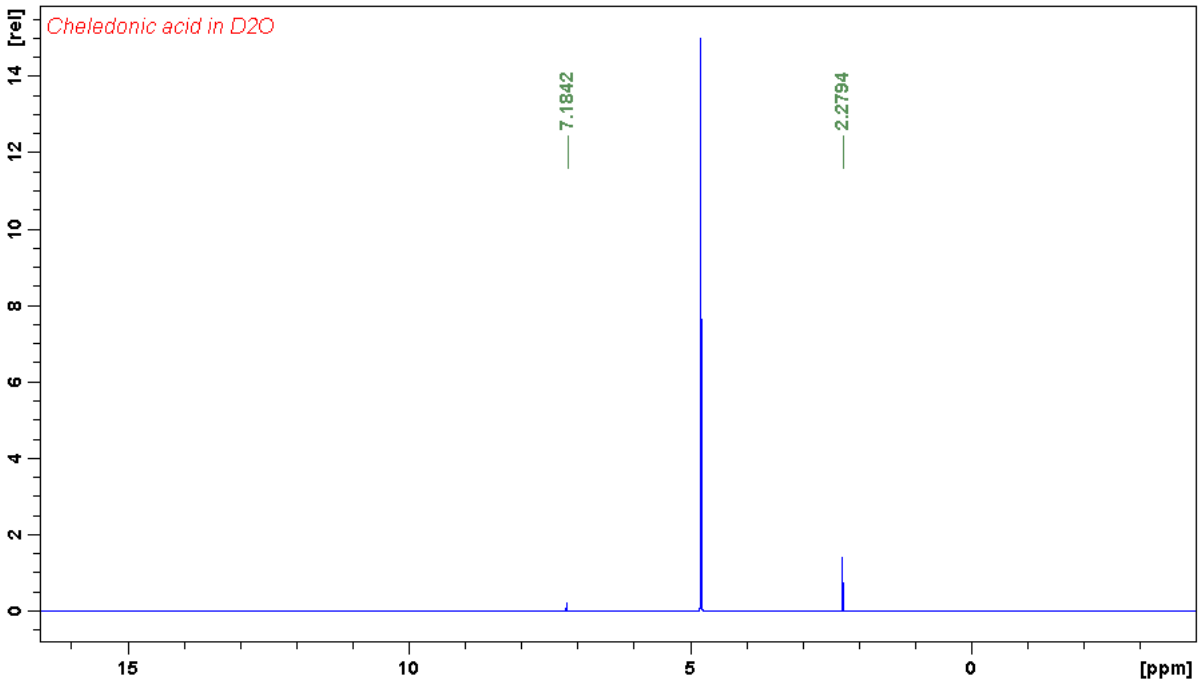


MS-MS of the unknown peak in HPLC

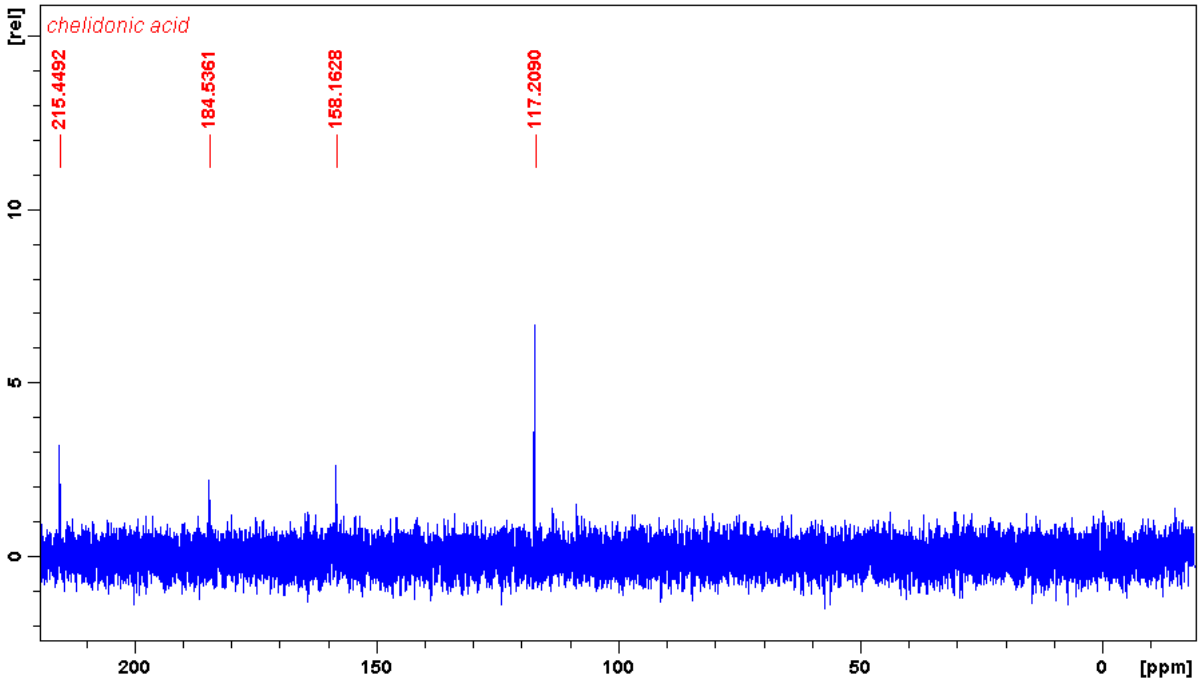


Appendix 3: NMR and IR Spectra

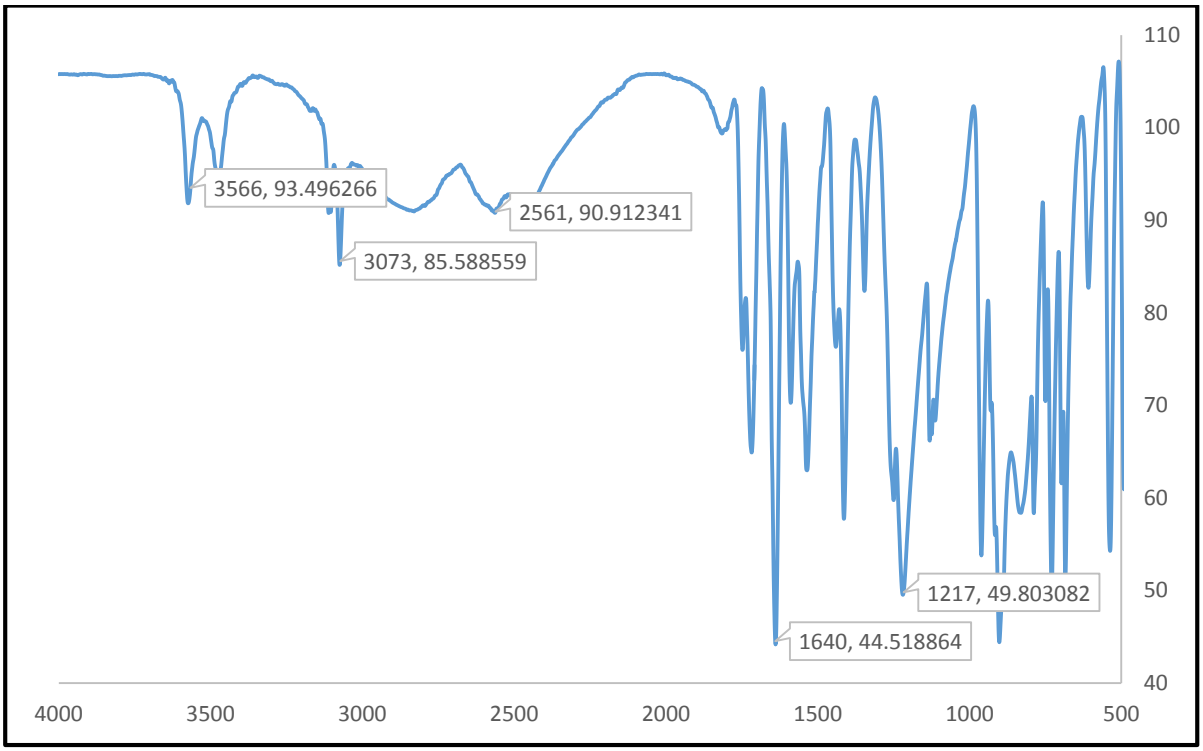
H^1 NMR spectrum of chelidonic acid:



C^{13} NMR of chelidonic acid



IR Spectrum of chelidonic acid



Appendix 4: Data for uptake studies

Table A3: Ni concentrations at 231.06 nm (in mg L^{-1})

Day	**Control**	**Spike**
Day 0	3.674	5.443
Day 7	4.146	10.734
Day 14	2.928	8.593
Day 21	2.04	9.245
Day 28	4.78	5.248

Table A4. Amino acid concentrations for spiking analysis (in g/100g)

Amino Acid	Day 0	Day 28
His	0.083	0.089
Ser	0.096	0.097
Arg	0.092	0.114
Gly	0.017	0.016
Asp	0.102	0.098
Glu	0.063	0.045
Thr	0.057	0.047
Ala	0.063	0.092
Pro	3.184	2.916
Cys	0.004	0.002
Lys	0.003	0.009
Tyr	0.011	0.011
Met	0.029	0.027
Val	0.1	0.103
ILe	0.014	0.011
Leu	0.018	0.019
Phe	0.018	0.016

Appendix 5: Permission for republication of figures

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