ELEMENTAL DISTRIBUTION IN SELECTED EDIBLE *TRIFOLIUM* SPECIES (CLOVER) AND THE IMPACT OF SOIL QUALITY ON THE CHEMICAL CHARACTERISTICS OF *TRIFOLIUM DUBIUM*

by

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As the candidate's supervisor, I have approved this thesis for submission

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PREFACE

The research contained in this thesis was completed by the candidate while based in the Discipline of Chemistry, School of Chemistry and Physics of the College of Agriculture, Engineering and Science, University of KwaZulu-Natal, Westville, South Africa.

The contents of this work have not been submitted in any form to another university and, except where the work of others is acknowledged in the text, the results reported are due to investigations by the candidate.

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DECLARATION 1: PLAGIARISM

I, Thaveshan Gounden, declare that:

(i) the research reported in this dissertation, except where otherwise indicated or acknowledged, is my original work;

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DECLARATION 2: PUBLICATIONS

Publication 1

Title: Elemental analysis and nutritional value of edible *Trifolium* (clover) species

Authors: Thaveshan Gounden, Dr. Roshila Moodley and Prof. Sreekantha Babu Jonnalagadda

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Publication 2

Title: The distribution and toxicological assessment of heavy metals in *Trifolium dubium* (little hop clover) and the impact of soil quality

Authors: Thaveshan Gounden, Dr. Roshila Moodley and Prof. Sreekantha Babu Jonnalagadda

Journal: Manuscript prepared for Analytical Letters

In preparation of the above manuscripts, I performed all the experiments and interpreted the data. The co-authors assisted in editing and verifying the scientific content in the manuscript.

Signed: Thaveshan Gounden

Date: December 2017

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ABSTRACT

Trifolium (clover) species are edible herbs that are rich in proteins, phytocompounds and essential macro and micro-nutrients. These herbaceous plants can therefore contribute significantly to the nutritional needs of people and can play a significant role in traditional medicine. With malnutrition becoming a growing problem amongst poverty-stricken communities in African countries where food is unaffordable, the use of *Trifolium*, a common perennial herb, can provide much needed nutrition and can ameliorate food insecurity. The bioactive phytocompounds in *Trifolium* species promote its use in traditional medicine as this is an affordable and, debatably, better form of treatment. South Africa has a rich diversity of *Trifolium* species, however there is little to no information regarding the nutritional value of these herbs and the uptake of toxic heavy metals which can cause adverse health effects if ingested.

The main aim of this study was to evaluate the elemental distribution and nutritional value of five edible *Trifolium* species, namely *Trifolium africanum*, *Trifolium burchellianum*, *Trifolium repens*, *Trifolium dubium* and *Trifolium pratense* in KwaZulu-Natal, South Africa. The results obtained were used to assess the potential of these herbs to alleviate malnutrition in KwaZulu-Natal. The chemical composition of the five edible herbs was determined by analysing for proteins, lipids, dry ash and carbohydrates. *T. dubium* proved to accumulate the highest concentrations of toxic heavy metals (Cd and Pb), therefore the elemental distribution of essential and toxic metals in *T. dubium* were assessed for impact of soil quality on uptake by the plant by obtaining samples from ten different geographic locations in KwaZulu-Natal.

Trifolium species investigated in this study were rich in essential nutrients with concentrations of elements varying significantly ($p<0.05$) and were found in decreasing order of Ca > Mg > Fe > Mn > Zn > Se > Cu > Cr > Pb > Ni > Co > Cd > As. The species had adequate levels of lipids (4.2 to 8.6%), proteins (35.1 to 45.4%) and carbohydrates (26.7 to 47.0%). The results obtained suggested that *T. pratense* was the most suitable species for human consumption due to it having undetectable levels of toxic metals (As, Cd and Pb) and being rich in macro and micro-nutrients, especially Fe (7.84 mg/10 g, dry mass) and Se (0.36 mg/10 g, dry mass). The elemental uptake in *T. dubium* varied significantly with geographic location ($p<0.05$) and were found to be in descending order of Ca > Mg > Fe > Zn > Mn > Se > Ni > Cr > Cu > Co > Pb > Cd > As, where the availability and accumulation of heavy metals from soil were controlled by the plant to meet physiological needs. Soil quality parameters such as pH ranged from 6.15 to 6.68, soil organic matter ranged from 0.9 to 2.6% and cation exchange capacity ranged from 4.25 to 9.05 meq/100g. In general, this study indicated that *Trifolium* species investigated contain adequate concentrations of essential elements and low to undetectable levels of toxic elements which make it a safe and valuable source of nutrition. Therefore, these plant species can contribute positively to the diet of poverty-stricken communities and can aid in improving food insecurity in KwaZulu-Natal.

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ABBREVIATIONS

ANOVA	Analysis of variance
BAF	Bioaccumulation factor
CCD	Charged coupled device
CEC	Cation exchange capacity
CRM	Certified reference material
DL	Detection limit
DRI	Dietary reference intake
EDTA	Ethylenediaminetetraacetic acid
ICP-AES	Inductively coupled plasma-atomic emission spectrometry
ICP-OES	Inductively coupled plasma-optical emission spectrometry
ISO	International Organisation for Standardisation
PCA	Principal component analysis
PFA	Perfluoroalkoxy
RDA	Recommended dietary allowance
SOM	Soil organic matter
UL	Upper intake level
WHO	World Health Organisation

CHAPTER 1

1.1 Introduction

The world's growing population, specifically in developing countries, has increased the need for essential nutrients to sustain a healthy lifestyle (Bongaarts, 2007). These nutrients can be found in forage plants and edible herbs which are a rich source of phytonutrients. Phytonutrients that are found in edible herbs and fruits are known to be a better source of nutrients than those found in dietary supplements. Consumption of these natural resources contributes towards maintaining a healthy diet and preventing diseases. Forage plants may also improve food security especially in rural societies which are dependent on natural resources to meet basic dietary needs. (Kader, 2001).

Trifolium species, forage herbs commonly known as clover species, is a genus of the Leguminosae family. These species are abundant with approximately 255 different species occurring naturally in temperate and subtropical regions of the northern and southern hemispheres with the Mediterranean region having the greatest diversity (Ellison et al., 2006). *Trifolium species,* namely *Trifolium repens* (white clover) and *Trifolium pratense* (red clover) have been used in folk/traditional medicine for many centuries due to the presence of flavonoids and isoflavanoids in the plants (Kolodziejczyk-Czepas, 2012). *T. repens* contains a high mineral and protein content which is predominantly due to the plant's root nodules having nitrogen fixing bacteria. This not only contributes to the plant's nitrogen-fixation capabilities, but also inhibits the use of fertilizers and other concentrate feeds (Shrestha, 2013). In terms of the herb's medicinal applications, it can be used in the treatment of pneumonia, fever, sore throat, meningitis and menstrual pain (Bahmani et al., 2015, Cakilcioglu et al., 2011, Sabudak and Guler, 2009). Engelmann et al. (2009) reported that *T.*

pratense is used as a plant-based alternative in herbal and dietary supplements due to the species containing isoflavanoids that are useful in treating estrogen-related diseases. Medicinal applications of *T. pratense* include the use as an antidiabetic herb (Bahmani et al., 2014), the treatment of cardiovascular disease (Sõukand and Kalle, 2013) and bronchitis (Bahmani et al., 2014). Other medicinal applications of *Trifolium* species are the use of *T. burchellianum* as an anticancer and blood purifying remedy (Magama et al., 2013) and that of *T. angustifolium* in treating stomach aches and diarrhoea (Barros et al., 2010). Additionally, *Trifolium* species are also used as an ingredient in soups, salads, desserts and drinks (Kelley et al., 2002).

Regular assessment of medicinal herbs and fruits for heavy metal contamination is important as they are natural food sources. The elemental content of edible plants needs to be evaluated in order to establish if plants are safe for human consumption and to assess their contribution to the diet (Roberts, 1981). Several inorganic elements responsible for plant growth and development are macronutrients (N, P, K, S, Mg and Ca) and micronutrients (As, Cr, Co, Cu, Fe, Mn, Mo, Ni, Se and Zn). The plant accumulates a higher concentration of macronutrients compared to micronutrients which are usually found at trace levels. However, exposure to concentrations higher than the recommended threshold values set by health organisations for essential elements in humans can have detrimental effects and can affect an entire population if consumed for either medicinal or dietary purposes (Rengel et al., 1999). Inorganic elements that are nonessential (As, Cd and Pb) can also be found in trace amounts (Jadia and Fulekar, 2009). Daily anthropogenic activities that contribute towards heavy metals in soil include poor waste disposal by industrial or urban areas, the use of pesticides and motor vehicle emissions (Cui et al., 2004, Pakade et al., 2013). The accumulation and exclusion of these heavy metals from the soil is controlled by the plant to meet physiological requirement levels and also depends on soil concentrations. The chemical properties of soil which include the moisture content, soil organic matter (SOM), pH and cation exchange capacity (CEC) affect the mobility of essential/nonessential nutrients which influence uptake by plants (Greger, 1999, Imtiaz et al., 2003).

1.2 Problem Statement

Most inhabitants of poverty-stricken communities in South Africa are both economically and physically unable to purchase commercially available food that is essential for a healthy and balanced diet (Moodley et al., 2007). South Africa is one of the many developing African countries that require alternative food sources to combat malnutrition and to overcome food insecurity (Goggin et al., 2009). It has been reported that about 14 million people (of which 1.5 million are children) do not meet basic nutritional and dietary needs (Gqaleni et al., 2007). Therefore, the need to promote the consumption and medicinal use of locally grown herbs that are easily accessible for local inhabitants is critical.

Plants have the ability to accumulate high concentrations of heavy metals from contaminated soil and the primary exposure pathway to these metals is generally through ingestion. At elevated levels, these heavy metals (whether toxic or essential) can have adverse health effects however; many factors govern whether adverse health effects may occur. If a plant has a tendency to accumulate toxic metals and if ingested regularly, be it for medicinal or nutritional value, a person’s susceptibility to exposure and adverse health effects increases.

There are standards and upper limitations set for metals in plants by the World Health Organisation (WHO) to prevent exposure to high concentrations of metals (WHO, 2005). Despite these regulations, there is little to no information on the elemental content, classification (accumulator, excluder or indicator) or nutritional value of *Trifolium* species in South Africa. Evaluating the chemical characteristics of these plants can contribute to ameliorating food insecurity in South Africa and may provide greater knowledge in food science and agriculture.

1.3 Aims

The aim of the study was to compare the elemental distribution of selected elements (As, Ca, Cd, Co, Cr, Cu, Fe, Mg, Mn, Ni, Pb, Se and Zn) in five different *Trifolium* species (*Trifolium africanum* (African clover), *Trifolium burchellianum* (burchell's clover), *Trifolium repens* (white clover), *Trifolium dubium* (hop clover) and *Trifolium pratense* (red clover)) found in KwaZulu-Natal, South Africa. *T. dubium*, which accumulated higher levels of toxic heavy metals (Cd and Pb), was chosen to determine the impact of soil quality on elemental uptake as a function of geographical location.

1.4 Objectives

- To validate the method used for the analysis of metals in *Trifolium* species and corresponding soil samples using certified reference materials.
- To determine the concentration of selected elements (macro, micro and toxic) in the five *Trifolium* species and to evaluate for metal toxicity.
- To determine the proximate chemical composition (moisture, lipid, protein and dry ash) of the five *Trifolium* species.
- To determine the nutritional value of the five species analysed by comparing elemental results to recommended dietary allowances.
- To determine the concentration of elements (macro, micro and toxic) in *T. dubium* leaves and corresponding growth soil (total and exchangeable) from the ten sampling sites in KwaZulu-Natal.

- To determine the pH, soil organic matter and cation exchange capacity of the soil samples from the ten sampling sites.
- To perform statistical analyses to assess the impact of soil quality on elemental uptake by *T. dubium*.

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CHAPTER 2

LITERATURE REVIEW

2.1 Medicinal plants

Traditional medicine uses plant-based drugs that are developed using traditional knowledge, the benefits of which are recognised by the World Health Organisation (WHO), hence the current push for incorporating it into healthcare systems, specifically in developing countries (Akerele, 1987). The main reason for this is that in most developing countries, local communities do not have proper healthcare systems or facilities due to lack of accessibility and affordability (Graham, 1991). The move towards traditional medicine to serve healthcare needs has resulted in an increase in research on folk or traditional medicine and the medicinal benefits of plants which are commonly used to treat various conditions (Gyasi et al., 2011).

In South Africa, it has been reported that about 80% of the country's inhabitants use traditional medicine for their healthcare needs (Goggin et al., 2009, Gqaleni et al., 2007). It is evident that most South Africans, especially black South Africans, prefer to use folk medicine over conventional medicine to treat a wide range of diseases and conditions. An example is the study conducted at a local clinic in KwaZulu-Natal, where 84% of the patients chose to be treated using traditional medicine. The most popular form of treatment needed was for HIV/AIDS patients who also preferred the use of traditional medicine to boost their immune system. The study concluded with only 18% of patients stating that they may consider reducing their use of plant-based drugs in the future (Mander et al., 2007).

Plants are the main source of medicine for traditional healers and various parts of the plant are utilised in the South African medicinal plant trade (Table 2.1) (Mander, 1998). Traditional healers obtain their medicinal plants from nature. They either use various parts of the plant directly or they produce medicinal remedies or concoctions. It is reported that approximately 1000 medicinal plants found in the wild are being sold in local markets in Southern Africa (Chen et al., 2004).

Table 2.1: The percentage of each part of the plant utilised in the medicinal plants trade (Mander, 1998)

Plant parts used	**Percentage in trade (%)**
Bark	27
Roots	27
Bulbs	14
Whole plant	13
Leaves and stems	10
Tubers	6
Mixture of parts	3

Medicinal plants used to prepare traditional medicine can also have negative effects that are not considered. Plants have the ability to accumulate toxic trace elements (As, Pb and Cd) due to growing and developing in heavy metal contaminated soil. The ingestion of these toxic elements (even in trace amounts) can cause severe illnesses to people who consume or utilise medicinal plants. Heavy metal poisoning can be carcinogenic, cause damage to the brain, kidneys and liver (Khan et al., 2008). A study on herbal drugs reported that all medicinal plants collected for preparation of traditional medicine should be tested for heavy metals since contamination of medicines is a common problem (Sahoo et al., 2010). Abou-Arab and Abou Donia (2000) determined the heavy metal contamination in medicinal plants in Egypt and found that the levels of toxic metals, particularly Pb and Cd, exceeded maximum levels. The study concluded that the uptake of toxic metals from soil was highly dependent on the plant species. An efficient method for reducing the metal content was by boiling the plant in water instead of submerging the plant in hot water. Chizzola et al. (2003) monitored the heavy metal concentration in medicinal plants growing in Austria and found them to contain Cd and Pb. It was reported that Cd contamination only occurred in some plant species, showing that the soil did not play a role in Cd uptake by the plants. However, proper soil management was recommended for the growth of these medicinal plants since toxic metals were taken up which causes severe health risks if consumed in large amounts. Arpadjan et al. (2008) determined the concentration of As, Cd and Pb in seven different species of edible Bulgarian herbs. The three toxic metals were found to be present in all species analysed, with Pb having the highest concentration.

2.2 Leguminosae family

There is a wide spread of the Leguminosae plant family across the world where it has approximately 750 genera with an estimated 16,000 to 19,000 species (Allen and Allen, 1981). Lewis (2005) reported that the Leguminosae plant family is the third largest, containing 727 genera and approximately 19,325 species. The plant family has been subdivided into three distinctive groups by taxonomists, namely Mimosaceae, Caesalpiniaceae and Fabaceae (Hutchinson, 1964). The general characteristics of plants that belong to the Leguminosae family include trees, shrubs, woody vines and perennial or annual herbs. The genus *Trifolium* (clover) belongs to the subfamily Fabaceae and has been reported to be one of the largest genera in the Leguminosae family, with approximately 255 species (Gillett and Taylor, 2001).

2.3 *Trifolium* species

The genus name, *Trifolium*, was derived from the three leaflets of the plant (trifoliolate). *Trifolium* species, commonly known as clover, are annual or perennial herbs that can reach up to 50 cm in height. The flowers grow between 0.3-2.5 cm and are organised in capitate to spicate heads. The fruits are usually present with 1-2 seeds, but can have up to 9 seeds. The plants contain pods that are regularly dehiscent; however they can be irregularly dehiscent if there is a lack of sutures (Zohary and Heller, 1984).

A wide range of different *Trifolium* species have a native spread in temperate and subtropical (Northern and Southern hemispheres) habitats, whilst these species are not usually present around Southeast Asia. There are three main habitats that have the largest diversity of *Trifolium* species namely, western North America, east Africa and the Mediterranean region (Polhill et al., 1994). These abundant species tend to grow and spread around mountains, alpine peaks, meadows, prairies, woodlands and semi-deserts. A small group of selected *Trifolium* species can grow in shady areas, hence these species prefer to be exposed to high solar radiation like most herbaceous plants (Ellison et al., 2006). Many *Trifolium* species grow as forage plants or green manure crops. Gillett and Taylor (2001) reported that 16 species are being cultivated, with more species becoming naturalised across the world. Animals also tend to graze around areas rich in diversity of *Trifolium* species (Taylor, 1985).

2.4 Uses of *Trifolium* species

These herbs are well known for their biological activity and phytochemicals, making these species an important medicinal plant that has been utilised in folk medicine (Kolodziejczyk-Czepas, 2012). These species are also consumed as a herb making them an important source of nutrients (Tundis et al., 2015). A frequent use of *Trifolium* in the environment would be its role as a bioindicator, where the plants are able to determine the bioavailability of toxic heavy metals in contaminated soil. These studies using bioindicators not only provide information on toxicity and heavy metal exposure to inhabitants that consume the plant, but also for identifying pollutants that cause DNA damage in living plant species (Karlsson et al., 2003). Some of the uses in agriculture include nitrogen fixation and the growth of *Trifolium* in and around pastures for animal grazing (Brock et al., 1988).

2.4.1 Medicinal uses

The use of clover in traditional medicine is predominantly due to its anti-inflammatory, antioxidant, cytotoxic and estrogenic activity, and its use in minimising the side effects of chemotherapy and preventing the development of cardiovascular disease (Sabudak and Guler, 2009). Table 2.2 is a summary of some of the basic medicinal uses of different *Trifolium* species (Kolodziejczyk-Czepas, 2016).

Table 2.2: Ethnopharmacological recommendations for use of different Trifolium species in humans

***Trifolium* species**	**Parts of the plant used**	**Traditional uses**	**References**
T. alexandrinum (Berseem clover)	Whole plant, seeds and flowers	Antidiabetic remedy, treatment of asthma, cough, ulcers and wound healing	Khan et al. (2014a)
T. angustifolium (Narrow clover)	Aerial parts	Stomach aches and diarrhoea	(Barros et al., 2010)
T. burchellianum (Burchell's clover)	Aerial parts	Anticancer and blood purifying remedy	Magama et al. (2013)
T. dubium (Hop clover)	Leaves	Swellings and acne	Moravec et al. (2014)
T. fragiferum (Strawberry clover)	Seeds and aerial parts	Wound healing and burns	Tugba (2016)
T. minus (Suckling clover)	Whole plant	Blood clot disorders	Ummara et al. (2013)
T. pannonicum (Hungarian clover)	Aerial parts	Wound healing	Menković et al. (2011)
T. philistaeum	Flowers	Treatment of ovarian and breast cancer	Jaradat et al. (2016)
T. pratense (Red clover)	Whole plant and flowers	Disorders in the reproductive and nervous system, sore throat, expectorant, antiseptic, analgesic, pneumonia, meningitis, lung disease, heart disease and polycystic ovarian syndrome	Cakilcioglu et al. (2011), Goswami et al. (2017), Sõukand and Kalle (2013)

***Trifolium* species**	**Parts of the plant used**	**Traditional uses**	**References**
T. purpureum (Purple clover)	Leaves	Antidiabetic	Bahmani et al. (2014)
T. repens (White clover)	Whole plant, flowers and seeds	Treatment of sore throat, fever, coughs, colds, leucorrhoea, eye disease, deworming remedy, analgesic, stomach disorders, menstrual pain and improvement of memory	Amjad et al. (2015), Bahmani et al. (2015), Papp et al. (2013)
T. resupinatum (Persian clover)	Whole plant and flowers	Skin sores, constipation, digestive disorders and curing liver ailments	Gulshan et al. (2012)
T. rueppellianum	Whole plant	Meningitis	Giday et al. (2009)

2.4.2 Food

Trifolium species are a popular source of food for inhabitants who consume leafy vegetables. The plant species is an important nutraceutical that contains minerals and nutrients that can contribute positively to the diet (Kelley et al., 2002). These herbaceous species contain important phytonutrients, namely flavonoids and phenols that can be ingested through consumption, resulting in a more effective and affordable route to nutrients in comparison to commercial dietary supplements (Kader, 2001). The plant polyphenols also contribute to food characteristics, namely the bitterness, astringency and colour (Pandey and Rizvi, 2009).

Bakoglu et al. (2009) investigated the fatty acids, protein and nutrient content in *T. aureum*, *T. repens* and other plant species belonging to the Leguminosae family. The results showed both *Trifolium* species to be the richest in proteins and nutrients, namely Cu, Mn, Ni, Mg, Zn, Cr and Fe. These results correlated with previous studies on the Leguminosae family, indicating that these plants are rich in proteins and nutrients (Chang and Satterlee, 1979, Tewatia and Virk, 1996). Engelmann et al. (2009) reported on the use of plant extracts from *T. pratense* as a food additive in herbal and dietary supplements. The plant extracts contain isoflavones that have potent estrogenic effects that can help lower the risk of breast and ovarian cancer, and treat other menopausal disorders. These phytoestrogens are natural remedies which can be an alternative to woman who want to avoid hormonal therapy containing estrogen. Promensil (Figure 2.1) is a dietary supplement of plant phytoestrogens extracted from *T. pratense*. Examples of food that contain or use *Trifolium* species in the ingredients are salads, soups, entrees and desserts (Belsinger and Coca, 1991).

Figure 2.1: Dietary supplement containing extracts from *T. pratense*
(https://www.luckyvitamin.com/p-4979-dropped-promensil-menopause-40-mg-60-tablets)

2.4.3 Bioindicators

Plants are utilised as bioindicators to assess heavy metal toxicity in soil ecosystems. This technique evaluates the effect of anthropogenic activities in the environment by measuring the elemental uptake by hyperaccumulating plants. Plants that accumulate toxic heavy metals from soil assist in the prevention of metal toxicity in humans and indicate the potential or existence of environmental pollution (Knight et al., 1997). *Trifolium* species are hyperaccumulating plants that have been used in biomonitoring investigations as they are sensitive to pollutants in the environment (Piraino et al., 2006).

T. repens was utilised as a bioindicator in a study where the toxic and genotoxic effects of Cd and As were investigated (Ghiani et al., 2014). Plant species were planted in soils that were spiked with known concentrations of Cd and As to determine the uptake of these toxic heavy metals by the plant. There was a synergistic or additive relationship between the metals, where an increase in As uptake decreased the concentration of Cd in the plant, whilst an increase in Cd showed an increase in As uptake. In summary, all plant species indicated the simultaneous presence of the toxic heavy metals in soil.

Contaminated soil in the vicinity of a Pb smelter was investigated using *T. repens* and *Lolium perenne* (Poaceae plant family) to assess the uptake of Cd, Pb and Zn to determine the metal uptake by the plant and its phytotoxicity (Bidar et al., 2007). The concentrations of the metals accumulated by the plants were in the order of Cd > Zn > Pb. Between the two plant species studied, *T. repens* accumulated the lowest concentration of these heavy metals. Malizia et al. (2012) assessed the usefulness of *T. pratense* in monitoring the heavy metal content in soil and to evaluate pollution in the environment. Inductively Coupled Plasma Atomic Emission Spectroscopy (ICP-AES) was used to determine the elemental uptake of heavy metals (Cu, Zn, Mn, Pb, Cr and Pd) from soil by the plant. The results obtained in the study showed the metal uptake by the plant species and provided knowledge on the extent of soil pollution. It was concluded that herbaceous plants are useful analytical tools that can be utilised in assessing selected heavy metals in soil.

2.4.4 Agriculture

Trifolium species were used in a study conducted in Fukushima, Japan, where the plants were being evaluated for their phytoremediation ability. *T. hybridum*, *T. incarnatum* and *T. repens* were grown in a cultivated farmland that had been contaminated with radiocesium. These plants together with 10 other species from different families decreased the concentration of radiocesium in the soil, however, the complete removal of radiocesium was unsuccessful (Kobayashi et al., 2014). Pasture legumes have been recognised for ameliorating the nitrogen content of soils without the extensive use of nitrogen fertilizer. The importance of preventing the use of nitrogen fertilizer in agriculture increases sustainability and minimizes environmental problems relating to the use of nitrogen fertilizers. *T. repens* enhances the nitrogen-fixation process in soil and in grass pastures (Ledgard and Steele, 1992), and it can also improve pastures for grazing animals (Cave et al., 2015, Hammond et al., 2013, Lane et al., 1997).

2.5 *Trifolium* species in this study

The five *Trifolium* species selected for this study are *Trifolium africanum* (African clover), *Trifolium burchellianum* (Burchell’s clover), *Trifolium repens* (white clover), *Trifolium dubium* (hop clover) and *Trifolium pratense* (red clover).

2.5.1 *Trifolium africanum*

The perennial herb, commonly known as the African clover, is an indigenous plant species that is native to Africa. The common plant name 'African clover' originated from the fact that these species are indigenous to Southern Africa, where it is mainly found in KwaZulu-Natal, Lesotho, Orange Free State and Cape Province. The herb is grown around grassy areas, hills and along roadsides where it is readily grazed by livestock in the wild and on farms. The flowers of *T. africanum* are between light purple and pink in colour which can be used to distinguish them from other species. The flowers can be found in small groups usually in a spherical shape where they tend to start growing on an elongated stem in the middle of spring up until summer (Jones et al., 1974).

Figure 2.2: *Trifolium africanum*
(http://www.ngkenya.com/flora/trifolium-01.html)

2.5.2 *Trifolium burchellianum*

The common name of *T. burchellianum* is Burchell's clover which originated from the plant's scientific name. The other common name given to these species by local people in Southern Africa is 'Cape clover' since the species grows in the Cape Province. This species is native to Africa, where it is located in South Africa, Lesotho, Angola, Kenya, Tanzania and Uganda (Wilson and Bowman, 1993). The herb grows in loamy sands with an acidic pH (approximately 6) in grasslands, forests, grazed fields (common plant for animal grazing) and along roadsides (Ryding, 1991). The distinguishing feature of this *Trifolium* species is the dark purple colour of the flower petals growing from the plant's elongated stem.

Figure 2.3: *Trifolium burchellianum*
(http://redlist.sanbi.org/species.php?species=363-10)

2.5.3 *Trifolium repens*

One of the most commonly known *Trifolium* species is white clover which is distributed across the temperate regions. *T. repens* was given its common name ‘white clover’ due to the white flower heads that sprout from the stem that make these species distinguishable. The perennial herbaceous plant, which originated in Europe, can be found growing along roadsides, pastures, grassy fields, lawns and parks (Carlsen and Fomsgaard, 2008). The plants contribution to nature is the nitrogen-fixation ability where it converts atmospheric nitrogen to the form needed by plants (Almeida et al., 2000). The plant is used for many traditional medicine applications (Table 2.2) and possesses a high protein and carbohydrate content which make it a nutritious and valuable forage herb (Cook, 1966).

Figure 2.4: *Trifolium repens*
(https://keyserver.lucidcentral.org/weeds/data/media/Html/trifolium_repens.htm)

2.5.4 *Trifolium dubium*

These annual herbaceous legumes are known as Little Hop clovers. *T. dubium* can be found in dry habitats containing shallow, coarse-textured soil. The name 'Hop clover' was originated from the plants dried flower heads to dried hops. The 'Little' in the common name of hop clover differentiates the plant from other *Trifolium* species containing yellow flower heads. *T. dubium* and *T. procumbens* both contain yellow flowers, but *T. dubium* has the smallest flower heads and the least amount of flowers in the head. The leaflets are different to most clovers where the centre leaflet is on a stalk, with the leaves (1-2 cm long) being serrated around the edges. The herb prefers warm temperate grasslands where it grows in pastures during spring and summer and are distinguished by their yellowish flowers (Kemp et al., 1999). The forage plant has similar characteristics to other *Trifolium* species; the leaves are used in traditional medicine, the plant has nitrogen fixation capabilities and livestock graze on them (Brock, 1973, Moravec et al., 2014, Uysal et al., 2010).

Figure 2.5: *Trifolium dubium*
(https://keyserver.lucidcentral.org/weeds/data/media/Html/trifolium_dubium.htm)

2.5.5 *Trifolium pratense*

Red clover is the common name given to *T. pratense* since the plant is distinguished by the red colour of its flowers. Red clover is a forage herb that is distributed and cultivated globally. Grazing by livestock and being a potent nitrogen-fixer are the main contributions to ecosystems, whilst the plants medicinal properties are exploited in traditional medicine. The perennial herb can be found growing in pastures and grasslands where they are enjoyed by bees that use them to produce honey. The flowers of red clovers tend to grow 10-15 mm long (Taylor and Quesenberry, 1996). Extracted constituents from *Trifolium pratense* are commercialised and sold in American and European markets as dietary supplements (Polasek et al., 2007). It is one of the few *Trifolium* species from which phytochemicals have been extracted and commercialised into tablets that are taken by woman for hormonal therapy (Coon et al., 2007). The flowers and other parts of the plant have also been used in the production of tea which is sold in Europe (Figure 2.7).

Figure 2.6: *Trifolium pratense*
(http://drmgoeswild.com/the-top-30-vascular-plant-species-in-britain-fabaceae/trifolium/)

Figure 2.7: Red clover tea sold in Europe
(https://www.swansonvitamins.com/alvita-tea-red-clover-tea-24-bag-s)

2.6 Soil

Soil is the material found on the surface of the earth that is differentiated by the structure, thickness, texture and colour. The components that primarily make up soil are inorganic and organic constituents generated from weathered parent material, gases, moisture and essential nutrients that are required by plants (Bronick and Lal, 2005). The different layers of soil are divided into four regions (Figure 2.8). The organic materials (O) layer (approximately 0.5 to 3 inches thick) present at the top of the soil profile consists of leaves, twigs and needles. Organic soils consist of about 20% organic matter. The topsoil (A) layer represents the region of accumulation of biological activity, where it is defined as the mineral horizon. This layer is darker and coarser than other underlying layers. Consisting of approximately 1-5% organic matter, this layer is significantly thinner or may not be present due to soil erosion.

The subsoil (B) layer is also known as the region of accumulation, consisting of clay (red or brown in colour), iron and any other constituents that are penetrated by water. Organic matter is found in trace amounts. The weathered or decomposed rock (C) layer is the region that contains the most minerals. There is little to no biological activity in this layer which mainly consists of weathered rock, sediments, and saprolite. The solid rock (R) layer consists of granite, limestone, basalt and sandstone. This region is attached to the earth (Rumpel and Kögel-Knabner, 2011). Humus is one of the major organic components in soil which is produced from decaying organic matter that has further broken down into organic compounds. These contribute to the dark brown/black colour of soils and also improve the capacity to retain water within the soil (Zsolnay, 1996).

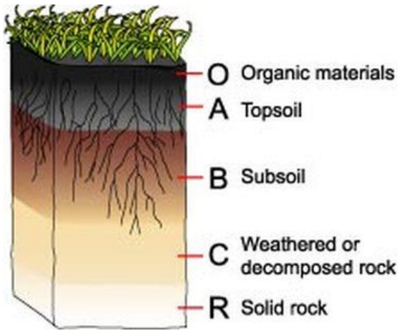

Figure 2.8: Soil profile showing the different soil horizons (https://valeriaapes.weebly.com/apes-in-a-box-soil-profile.html)

Soil is the source of many nutrients that are absorbed by the plant through the root system which is in direct contact with soil (Figure 2.9), which is not only responsible for the transportation of nutrients to the plant, but also offers protection and assists in the growth and development of plant life. Elements can be accumulated by the plant in three ways, namely mass flow, diffusion and root interception. During mass flow, the mobility of ions (Ca^{2+}, Mg^{2+}, NO_3^- and Cl^-) occurs from the soil to the root surface through water where nutrients are absorbed by the plant. Diffusion occurs when nutrients in the soil are transported from a region of high concentration (soil) to a region of low concentration (plant roots). Root interception is the process that occurs when the root system of a plant has grown into different regions of the soil which causes the direct uptake of nutrients (Weil et al., 2016).

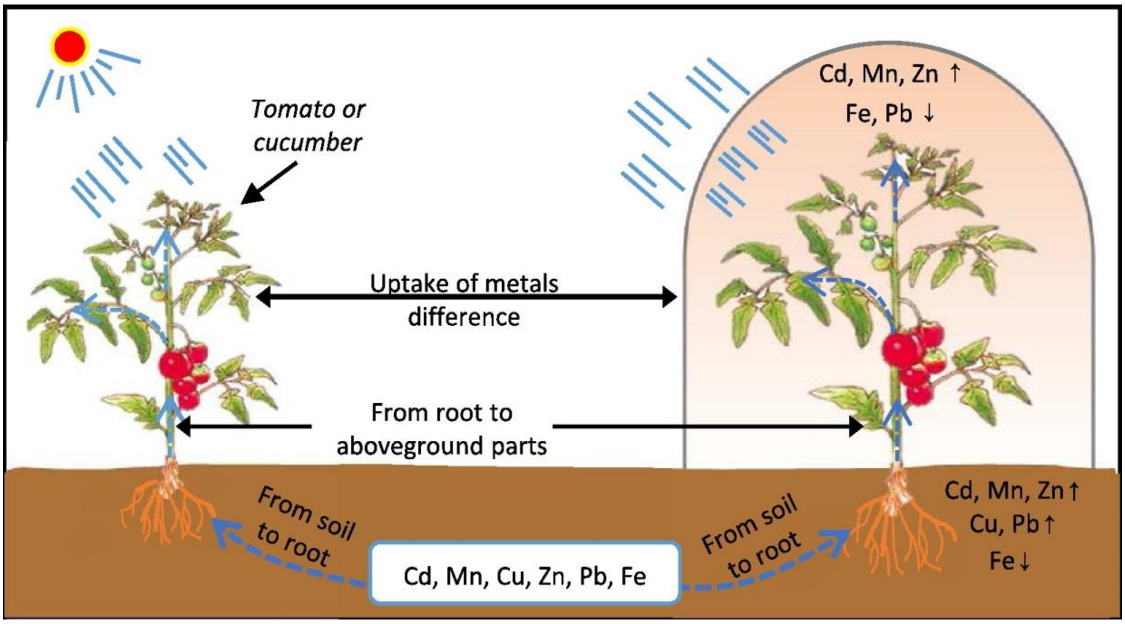

Figure 2.9: The uptake of elements from the soil through the roots of the plant (https://ars.els-cdn.com/content/image/1-s2.0-S0375674216304010-fx1.jpg)

2.7 Soil analysis

Assessment of soil can be performed using either chemical or physical techniques. Evaluating the moisture, pore spacing and density of soil are common physical techniques utilized in assessing soil. The general chemical techniques can be performed by determining the elemental composition and distribution in soil, organic matter content, salinity, pH, cation exchange capacity (CEC) and nutrient content (N, P, K) (Fageria et al., 2010).

2.8 Soil quality

The quality of soil is important for many functions that are performed, namely forming an excellent medium for the growth of plants, allowing the regular flow of water in the ecosystem, storing and recycling nutrients within the soil and maintaining a balanced elemental content that will not negatively impact animal and human health. High organic matter content in soil is preferable since it allows for water and the roots of the plant to penetrate the soil more efficiently. Soil organic matter (SOM), soil pH and CEC are techniques used to assess the quality of soil (Lewandowski et al., 1999).

2.8.1 Soil organic matter (SOM)

Soil organic matter (SOM) has a major influence on the functions of soil, which include the ability to hold water and the ability for water to infiltrate the soil (Rees et al., 2000). Decaying animals and plant material enhance the SOM content which also improves the ability to maintain plant nutrients from leaching out (Poole, 2001). These nutrients are maintained and made available for plant uptake due to the breakdown of organic matter into compounds in the soil, allowing the roots of plants to absorb available nutrients. The process occurs all over again when decaying plant material or animal waste is introduced into soil which is then consumed by microorganisms in the soil, breaking down organic matter (Beldin and Perakis, 2009). High clay content tends to increase organic matter in soil due to the fact that clay binds cations (Ca^{2+} and Mg^{2+}) more effectively than sandy soils (Kabata-Pendias and Pendias, 2001). Figure 2.10 indicates how sources of SOM (decaying organisms) are decomposed by microorganisms into inorganic forms or humus. The ions that form either leach out, undergo chelation to form organometallic complexes or are released to the surface of the soil, which then becomes available for uptake by the plant.

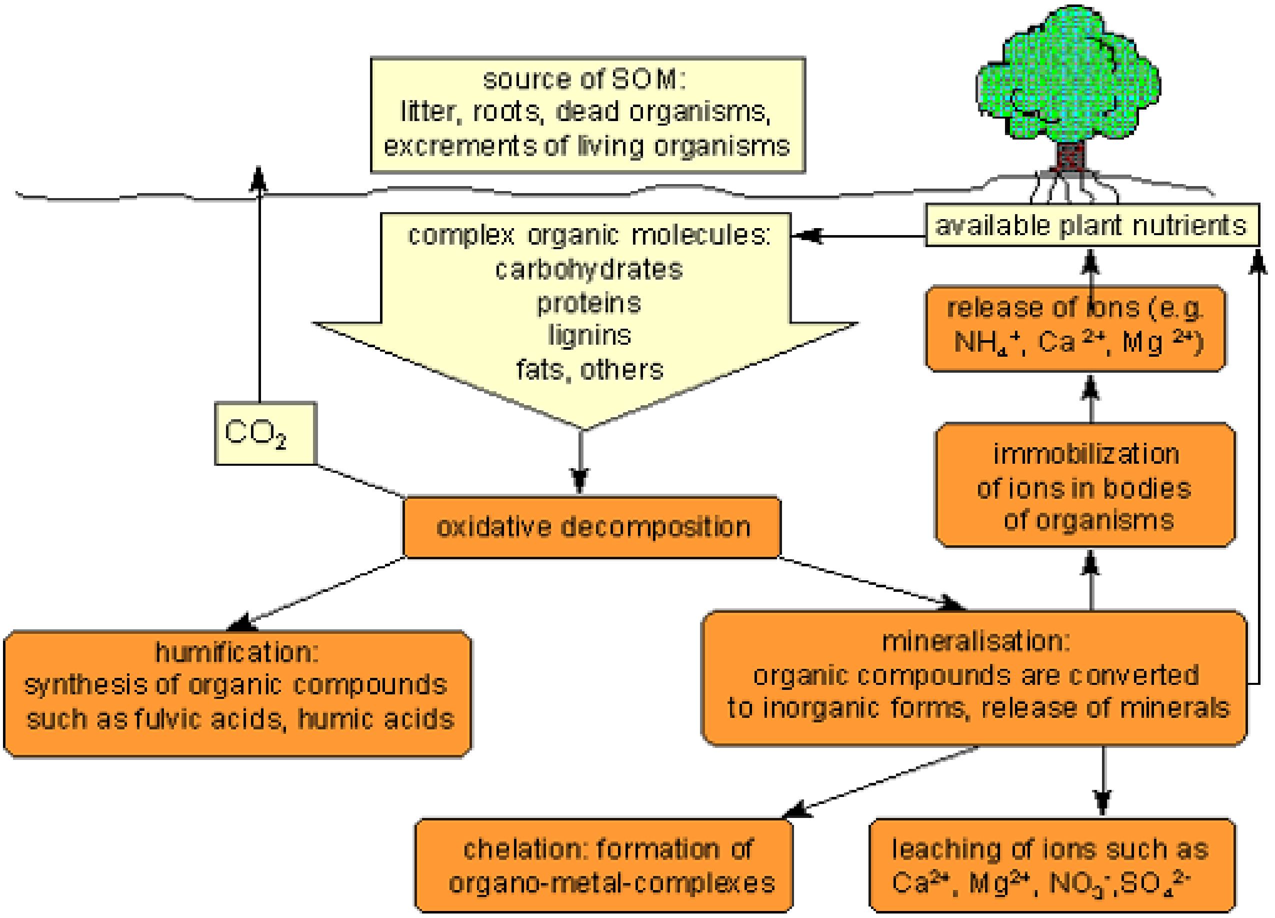

Figure 2.10: The formation of organic matter in soil (https://theorangegardener.org/topics/soil/organic-matter/)

2.8.2 Soil pH

Soil pH indicates if the soil is acidic (0 – 6.0), alkali (8 – 14) or neutral (about 7). The most optimum pH for vegetation and fruits to grow in soil is between 5.5 – 8.0 (Figure 2.11) (McKenzie, 2003). The formation of acidic and alkali ions in soil affect pH of soil. Soils with a pH below 6 have an excess of H^+ ions which causes acidic cations (Al^{3+} and Fe^{2+}/Fe^{3+}) to occupy negative exchange sites. An increase in soil pH due to the addition of basic cations (Ca^{2+}, Mg^{2+}, K^+ and Na^+) in soil causes the Al^{3+} and Fe^{2+}/Fe^{3+} to combine with OH^- and form insoluble Al and Fe oxides (Equation 1).

Equation 1: $Al^{3+} + 3H_2O \rightarrow Al(OH)_3 + 3H^+$

This creates free space in negative charged sites in soil, allowing for nutrient exchange. The formation of basic cations in soil is mostly due to the presence of carbonates (Equation 2) and bicarbonates (Equation 3) which naturally occur in soils (McCauley et al., 2009).

Equation 2: $2H^+ + Na_2CO_3 \rightarrow CO_2 + H_2O + 2Na^+$

Equation 3: $NaHCO_3 + H^+ \rightarrow Na^+ + CO_2 + H_2O$

Soil pH is an important tool to evaluate soil quality since pH levels in soil have a direct influence on nutrient availability due to the H^+ ions occupying the negative charges on the surface of soil. Nutrients that stick strongly to the surface of soil when the pH of soil is high (basic, low H^+ concentration) are Cu, Fe, Mn and Zn. This is mainly due to the charge to size ratio of the molecules being high, where each molecule is small but contains a charge of two to three. This results in the nutrients being unavailable for uptake by the plant. A decrease in soil pH (acidic, high H^+ concentration) will result in these nutrients being made available for uptake by the plant due to the fact that the nutrients do not stick as tightly as compared to when the soil pH is high. Sulfur and the basic cations Ca^{2+}, Mg^{2+}, K^+ and Na^+ are large in size, therefore these nutrients do not bind strongly to soil particles. A high pH will result in the nutrients being readily available for uptake since they easily come off the soil particles. Nutrients exposed to low pH soil are displaced by H^+ and are lost through nutrient uptake by the plant or leaching (Lake, 2000, McCauley et al., 2009).

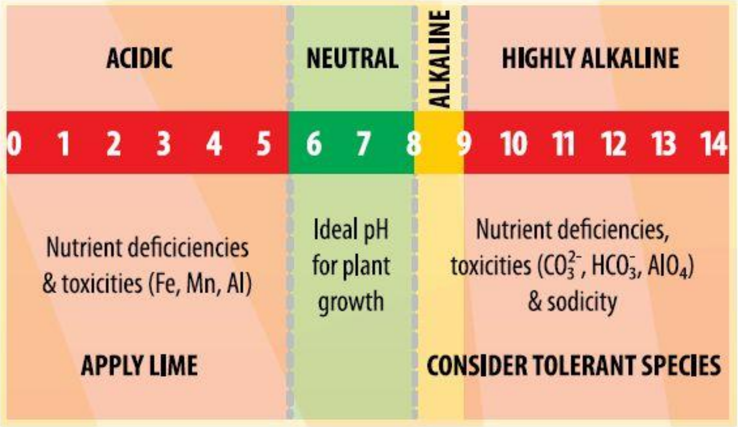

Figure 2.11: Soil pH scale and plant growth (http://soilquality.org.au/factsheets/soil-ph-south-austral)

2.8.3 Cation exchange capacity (CEC)

The cation exchange capacity (CEC) of soil is determined by evaluating the amount of negatively charged sites on the surface of soil that can hold cations by utilising the forces of attraction. These positively charged ions (Ca^{2+}, Mg^{2+}, K^{+}, Na^{+} and Al^{3+}) are exchangeable within the soil which increases the ability of the plant to accumulate positively charged cations from the soil (Ross and Ketterings, 1995). The negatively charged sites on the surface of soil are mainly due to the clay and organic matter content (Shaw and Andrews, 2001). The relationship between soil pH and CEC is controlled by the amount of negative charges in clay and organic matter in the soil, where an increase in soil pH increases CEC. Clay contains high CEC levels based on the fact that organic matter has a high number of negative charges, whilst sand has very low CEC levels due to it not having an electrical charge (Figure 2.12) (Thomas et al., 2007).

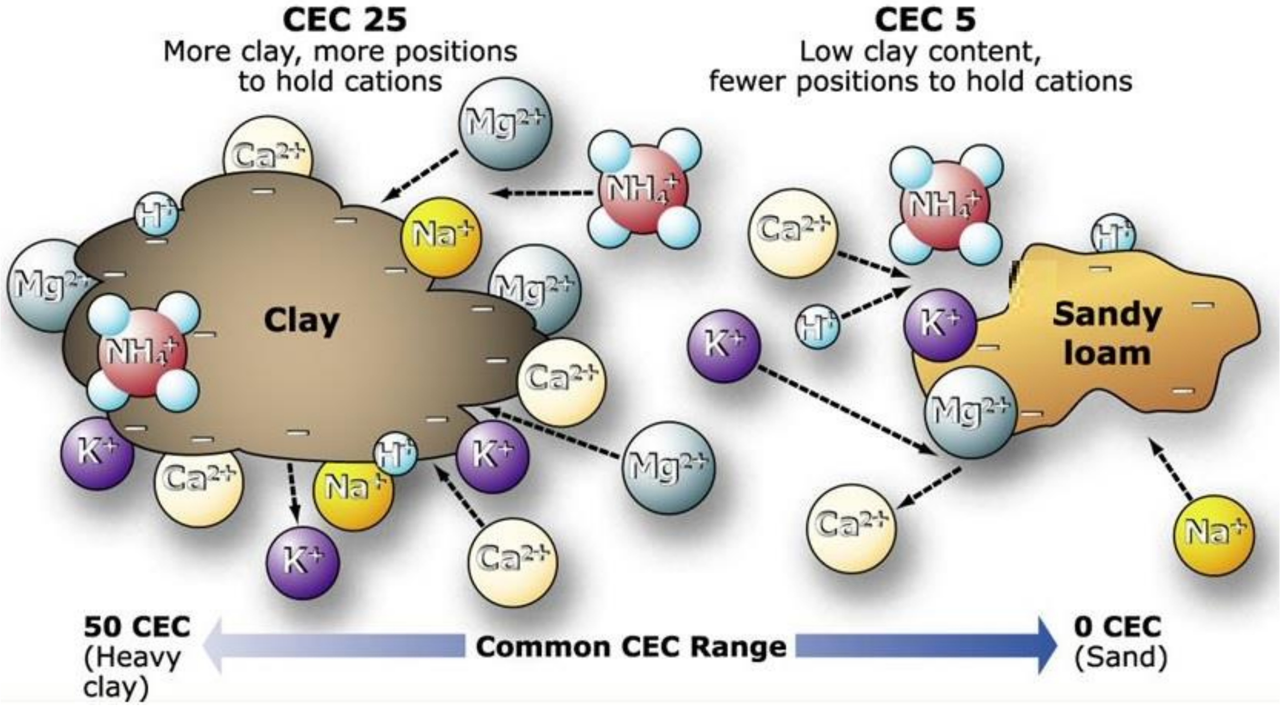

Figure 2.12: The different CEC levels between clay and sand (http://slideplayer.com/slide/6996635/)

2.9 Total and exchangeable metals in soil

Elements with densities larger than five and atomic weights of 23 and higher are classified as heavy metals (Passow et al., 1961). There are approximately 38 heavy metals of which As, Cd, Co, Cr, Cu, Fe, Mn, Ni, Pb, Se and Zn are a few. These metals enter ecosystems via natural or anthropogenic activities. Metals can accumulate in various compartments of soil and mobilization of these metals is possible due to changes in the environment such as weather conditions (Fedotov and Miró, 2007). Concentrations of trace elements in soil are dependent on the climate, the form and intensity of weathering and other aspects that prevailed during soil formation. The key source of trace elements is parent rock material which is susceptible to weathering and tends to form fine textured soils. On the other hand, rocks that are resilient to weathering have low micronutrient content and tend to form rough textured soils (Sillanpää, 1982). A small portion of the total concentration of heavy metals in the soil that is accessible for incorporation into biota is known as the bioavailable concentration (Leventhal and John, 1995). Biochemical and chemical processes direct, for the most part, the mobility and availability of heavy metals. These processes are subjective to the pH, SOM, CEC and other biological activities (Violante et al., 2010).

Table 2.3: Chemical forms of metals in soil (Gunn et al., 1988, Salomons, 1995)

	Fraction	**Mobility**
TOTAL	In pore water (dissolved)	High
	Weakly adsorbed (exchangeable)	High
	Associated with carbonates	High
	Associated with Fe, Mn oxides	Moderate
	Complexed by organics	Moderate
	Associated with sulfide	Low
	In the mineral lattice	Low

The chemical forms of metals in soil in their various solid phases are shown in Table 2.3 (Gunn et al., 1988, Salomons, 1995). Metals are related to numerous sites in soil; variations in the cation composition could potentially result in ion exchange which releases weakly adsorbed cations into the soil. The mobility and availability could increase due to changes in redox activities and environmental conditions resulting in the release of metal ions. Metal incorporated in the mineral lattice are typically unavailable to biota (Leventhal and John, 1995).

2.10 Soil extraction techniques

In determining which ions in the soil are exchangeable, the soil needs to be submerged in an extractant, typically an ionic solution. The ions in the extractant solution will displace the weakly held ions on the surface of the soil or adsorbed ions. At this point, both the exchangeable ions and ions in the extractant will be contained in the solution. The extractant chosen is dependent on the targeted ions in the soil (Gleyzes et al., 2002).

The 1M ammonium acetate (NH_4OAc) extractant solution is preferred as it is highly concentrated and the acetate ion which has a metal complexing power inhibits re-adsorption and precipitation of free metal ions (Ure, 1996). Acetic acid typically dissolves exchangeable species and has the ability to release more firmly bound exchangeable forms (Rapin and Forstner, 1983). A strong chelating agent that forms durable complexes with various metals is ethylenediaminetetraacetic acid (EDTA), which is known to be one of the most successfully used phytoremediation chelating agents (Rashid, 1974). This is mainly due to the molecule possessing a strong ability to chelate to a variety of metals as well as the ability to increase plant uptake and the bioavailability of metals in soil. The reagent binds to metal ions and is able to form complexes whereby the ions are held securely (Liphadzi and Kirkham, 2006).

For the formation of these complexes, the molecules in the extractant solution (made up of ammonium acetate, EDTA and acetic acid) form the ligand which will thereby bind to the metals from the soil.

A typical reaction for complex formation is:

$M + nL \leftrightarrows MLn$

With an equilibrium expression of:

$$K = \frac{[MLn]}{[M] \times [L]} n$$

Where K = equilibrium constant and logK = formation (stability) constant.

The higher formation constant results in the metal ion being held more tightly within the newly formed complex. Hence, among other sequestering reagents, such as nitrilotriacetic acid and gluconic acid, EDTA contains one of the highest stability constants and therefore highest sequestering effect (Kocialkowski et al., 1999). Combining EDTA and acetic acid has been shown to attack the carbonate phase and extract metals in non-silicate bound plates. Therefore, the available metal forms are better represented by combining all three extractants in solution (Rauret et al., 2000).

2.11 Heavy metal contamination in soil

Heavy metals have the tendency to be adsorbed onto different particles, usually forming hydroxo complexes or existing as free ions in soil. The different species of metals present in the environment are influenced by the redox reactions that occur. These chemical reactions taking place within the environment causes a change in the oxidation state of the metal present. The metal species that are present as free ions lead to contamination since these metals are regarded as toxic, therefore it is important to assess the heavy metal uptake by plants as well as the toxicity in soil (Valentão et al., 2002).

Heavy metals present in the environment can exist in different species of that specific element, where the species formed is influenced by factors that include ionic strength, soil pH, temperature, redox chemical reactions occurring within soil and chemical composition (Blais et al., 2008). Table 2.4 below gives examples of heavy metals and their species that can possess toxic effects in the environment.

Table 2.4: Heavy metals that occur in the environment and their relative species (Blais et al., 2008)

Metal	Species
As	As^{3+}, As^{5+}
Cd	Cd^{2+}
Cr	Cr^{6+}
Cu	Cu^{2+}, Cu^{+}
Ni	Ni^{2+}
Pb	Pb^{2+}
Se	Se^{2-}, Se^{4+}, Se^{6+}
Zn	Zn^{2+}

The mining and chemical industry in South Africa produces waste that may contain high concentrations of toxic heavy metals and other pollutants. The irregular disposal of waste from these industries contaminate the environment due to the leaching of these toxic heavy metals into soil and water systems (Naicker et al., 2003, Roychoudhury and Starke, 2006). Living organisms that consume or utilise plants can experience severe health risks due to exposure to these toxic heavy metals if they have been accumulated by plants. Due to this reason, it is essential that plants be assessed for heavy metal contaminants before they are used for medicinal purposes or consumed as part of the diet (Hussain et al., 2011).

2.12 Relationship between plants and soil

Nutrients that are widely distributed in soil can be subdivided into two groups, namely major elements and trace elements. These nutrients, which are vital for plant growth and development, are obtained from decaying plant material and the weathering of parent rock. The roots of the plant are responsible for the absorption of nutrients from the soil by plant, therefore accumulation of major and trace elements by the plant are dependent on the mobility of elements from the soil to the root system. Nutrients absorbed by the root system are then transported to different parts of the plant by plant cells (Vitousek et al., 2009).

2.13 Elements in plants

The metabolic functions that occur within plants require elements for these functions that are known as essential elements. Deficiencies in essential elements can cause growth defects, however elevated concentrations can also lead to toxic effects. Plants have a high acceptance for specific elements which allows for the selective uptake (in the roots) of certain ions and the exclusion of others (Figure 2.13) (Mendez and Maier, 2008). Many developments have been made in order to understand the molecular mechanisms which are used by plants for the uptake and accumulation of micronutrients, for example, zinc, iron, manganese and copper. There are a few of these mechanisms that also assist in not only the uptake of essential elements but also in the accumulation of non-essential (or toxic) elements, in the likes of elements such as As, Pb and Cd (Liñero et al., 2017).

In human beings, the intake of elements such as Cd, As and Pb has been related to cases with many disorders which typically include failure of organs such as the kidneys, diseases like diabetes and cancer, and also mental health problems (Järup, 2003). Research conducted in the past few years have shown that transporters within the plant are able to discriminate between essential metals and toxic elements like the ones mentioned, but this is not in all plants. Furthermore, a process called sequestration, especially of non-essential elements, in the roots of plants has been referred to in many different plant species as one of the main processes which is responsible for the limiting of the translocation (movement and placement) of non-essential elements to edible tissues which exist above ground level in the plant, including its seeds. By increasing the concentration of the micronutrients which are bioavailable (commonly known as biofortification) in grains while depressing the accumulation of toxic elements will most likely require the functioning of a few different transporters in the plant. The selectivity for root uptake in most plants is fairly good but there is room for improvement for selectivity towards essential elements. This is the reason why non-essential elements which are not needed and are toxic are still taken up by plants.

There exists a few toxic elements which have similar properties to that of essential elements and therefore uptake specificity becomes a real challenge. For instance, As can be found in soils in different oxidation states (commonly as arsenate, As^{5+} or arsenite, As^{3+}) and arsenate is extremely similar to phosphate (an essential element) with regard to the thermochemical radii, charged oxygen atoms and their p*K*a values. This is just an example, among others, illustrating why plants can often allow for the uptake of non-essential elements. Hence, sequestration of non-essential elements in many different types of plant roots is described as a key process for the limitation of translocation of toxic elements being taken up into the aboveground edible plant tissues and the seeds (Khan et al., 2014b).

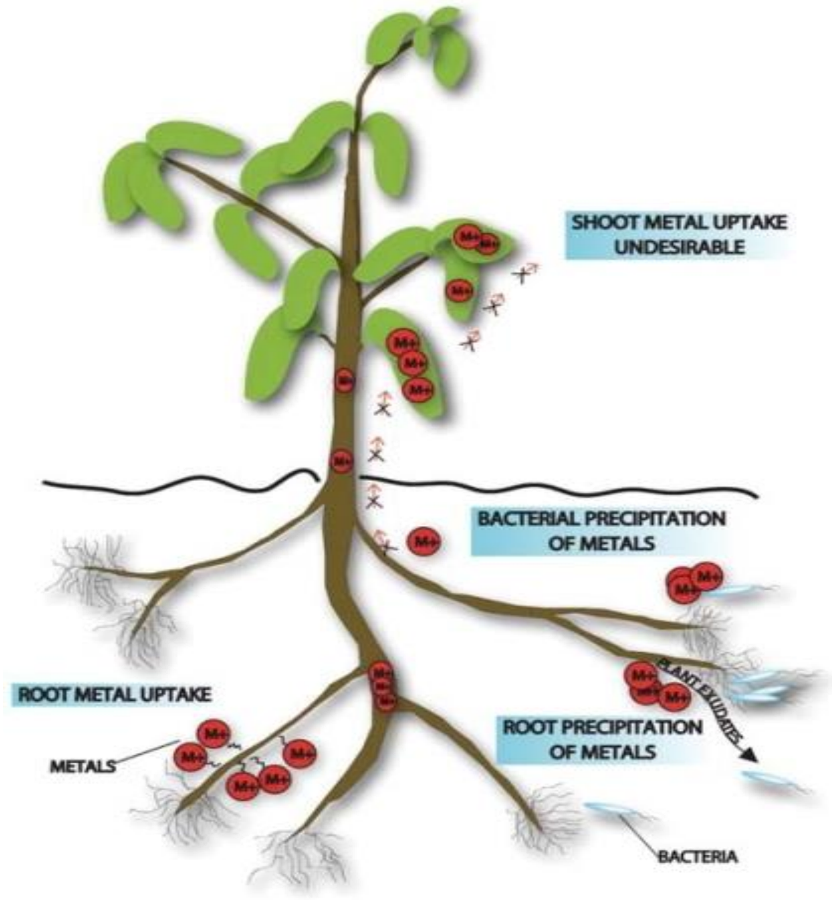

Figure 2.13: The sequestration and the root uptake of metals (https://www.semanticscholar.org/paper/Phytostabilization-of-Mine-Tailings-in-Arid-and-Se-Mendez-Maier/)

2.14 Accumulators and excluders

Plants that accumulate heavy metals are called accumulators, with the term hyperaccumulators being given to the extreme accumulators. These types of plants thrive in seriously contaminated soils and in proximity to ore deposits. On the other hand, there are plants that have established prevention or barring mechanisms making them unresponsive to wide concentration ranges of heavy metals – these are called excluders (Bradl, 2005).

Massoura et al. (2005) assessed the transfer of Ni from soil to *Trifolium pratense, Triticum aestivum* and *Alyssum murale*. The objective of the study was to determine which, from the three plant species, would accumulate or exclude Ni. These plants were grown for three months on four soils that were spiked with a range of total and bioavailable Ni. The results obtained indicated that the accumulation of Ni was highly dependent on the available Ni in the soil since all plant species accumulated Ni. Due to the three plant species proving to be Ni hyperaccumulating plants, their application in the phytoextraction of Ni in soil can be exploited. This lowers the toxicological risk of excess Ni in soil systems since the concentration of Ni is reduced by the hyperaccumulating plants.

2.15 Bioaccumulation factor

Bioaccumulation is crucial as it allows for the uptake and storage of specific nutrients for growth and development, namely vitamins, trace elements, fats and amino acids, by the organisms (McDowell, 2012). The net result of a chemicals uptake, storage and removal is also known as bioaccumulation. Bioaccumulation depends on solubility, agility and contact of metals with the various explicit sites in the organism's body. Trace elements like Pb, Cu and Cd causes difficulty with bioaccumulation as they are able to accumulate within organisms at high concentrations resulting in toxicity (Berthet et al., 1992).

The ratio of a metal's concentration in a plant to its concentration in soil is known as the bioaccumulation factor (BAF) (Timperley et al., 1970).

$$BAF = \frac{[Metal]_{plant}}{[Metal]_{soil}}$$

2.16 Essential elements in humans

Seeds and plants are the leading source of nutrients that are essential for humans and for livestock as well. More than 21 elements, including C, N, O, P, K, S, Ca, Mg, Fe, Cu, Co, Mn, Mo, B, Na, Cr, F, I, Ni, Se and Zn have been established as essential elements in both animal and human life (Abdulla et al., 1996) and are commonly known as nutrients. The essential elements are employed in the metabolism or structure of organisms and fit within either of the following two categories, namely macro-elements and micro-elements. Macro-elements are defined as the elements that are required in large quantities within the body, whereas microelements are required in smaller amounts.

Some elements like Ca and Mg are required in the human body in greater quantities than others (Table 2.5). Regular growth and development of the human skeleton requires Ca, whereas Mg is vital in developing and preserving the bones and crucial for a variety of enzymatic reactions (Gibson, 2005).

Table 2.5: Recommended Dietary Allowances (RDAs) of individuals[a,b]

Life stage	**Ca (mg/d)**	**Cr (μg/d)**	**Cu (μg/d)**	**Fe (mg/d)**	**Mg (mg/d)**	**Mn (mg/d)**	**Se (μg/d)**	**Zn (mg/d)**
Males								
14-18 y	1300	35	890	11	410	2.2	55	11
19-50 y	1000	35	900	8	400	2.3	55	11
>51 y	1200	30	900	8	420	2.3	55	11
Females								
14-18 y	1300	24	890	15	360	1.6	55	9
19-50 y	1000	25	900	18	320	1.8	55	8
>51 y	1200	20	900	8	320	1.8	55	8

[a] Sources from: Food and nutrition board, Institute of Medicine, National Academies (2011)

[b] RDA- Average daily intake level sufficient to meet the requirement of 97-98% of healthy individuals in a group

Iron is contained in haemoglobin within erythrocytes and deposited in macrophages too. It is utilised by cells in the body and is crucial in specific enzymatic processes. The trivalent form of Cr (Cr^{3+}) is vital in carbohydrate, lipid and nucleic acid metabolism and functions in the regulation of insulin in patients with diabetes (Strain and Cashman, 2009).

Selenium acts as an antioxidant and has significant interactions with other antioxidant micronutrients. Selenocysteine comprises at least 30 selenoproteins. Zinc plays a vital role in basic cellular processes and the synthesis of lean tissue in the human body. In the human body, Zn functions in three key groups – catalytic, structural and regulatory (Strain and Cashman, 2009).

Table 2.6: Tolerable Upper Intake levels (ULs)[a,b]

Life stages (M/F)	**Ca (mg/d)**	**Cr (µg/d)**	**Cu (µg/d)**	**Fe (mg/d)**	**Mg (mg/d)[c]**	**Mn (mg/d)**	**Ni (mg/d)**	**Se (µg/d)**	**Zn (mg/d)**
14-18 y	3000	ND	8000	45	350	9	1	400	34
19-50 y	2500	ND	10000	45	350	11	1	400	40
>51 y	500	ND	10000	45	350	11	1	400	40

[a] Sourced from: Food and nutrition board, Institute of Medicine, National Academies, 2011

[b] UL- Highest level of daily nutrient intake that is likely to cause no adverse health effects

[c] Represents intake from a pharmacological agent only

ND- Not determinable

2.17 Instrumentation

In order for a sample's analyte concentrations to be determined, the sample needs to be digested and metals extracted into solution. The choice of the different techniques and reagents for use in the dissolution and decomposition of the sample is important in determining the analyte content in the sample. Concentrations of analytes in the sample can be found by utilising specialised qualitative and quantitative techniques. In this study, decomposition was carried out by microwave digestion while inductively coupled plasma – optical emission spectrometry (ICP-OES) was the chosen technique for the determination of the elemental concentrations in the plant and soil samples due to its high efficiency, selectivity, rapid analysis times and convenience.

2.17.1 Microwave digestion

Sample preparation has been revolutionised ever since the microwave digestion technique first came to light during the mid-1970s. Utilisation of microwave decomposition has allowed the sample preparation step to be done in minutes instead of hours. The technique makes use of either sealed or open vessels, although sealed vessels have since gained more popularity due to minimal outside contamination and little loss of volatile materials. These sealed vessels also increase the efficiency of digestion, allowing for higher elevated temperatures and pressures to be reached (Lamble and Hill, 1998, Levine et al., 1999, Matusiewicz, 2003).

The decomposition of a sample is usually carried out by use of a hotplate, which could take hours of an analyst's time, thus one of the chief benefits of using microwave digestion is that it is time efficient. In this method, decomposition of the sample occurs due to the microwave energy being directly transported to all of the solution's molecules simultaneously, in the absence of heat transfer onto the vessel itself (Figure 2.14), while with the utilisation of conventional heating methods (hotplate) conduction exists, thereby allowing for the transfer of heat energy to the vessel. More time is needed for the heating of these vessels themselves compared to the time needed for the heating of the solution in the vessel, this is due to the vessel being a poor heat conductor. Conventional heating techniques (hotplates) also result in the solution being heated unevenly (Skoog et al., 2013).

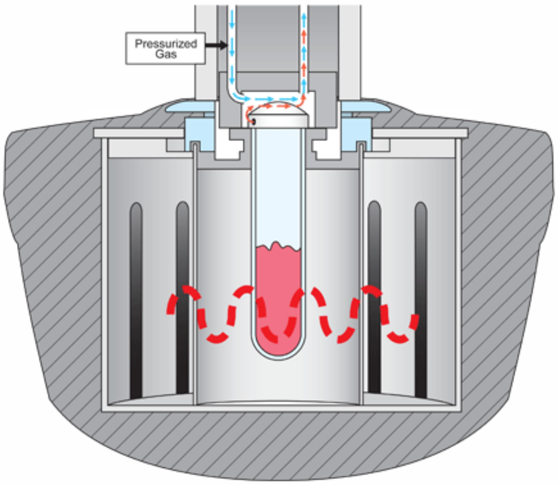

Figure 2.14: The pathway of microwave energy (http://cem.com/en/discover-sp-features/)

These vessels, which are used in microwave digestion, usually consist of a two-piece design, whereby the caps and liners are made up of Teflon or perfluoroalkoxy (PFA) of superior grade purity and the outer jackets are composed of polyetheramide or selected resilient microwave transparent composite material. Two of the main advantages of these materials are that they are able to withstand high pressures and temperatures and that they are resistant to chemical attack from any of the acids which are commonly used for digestion, for example, nitric acid. Teflon has a melting point of around 300 °C and is usually not attacked by many of the more commonly used digestion acids, this is why Teflon is the material of choice. If phosphoric or sulfuric acid is utilised, vessels composed of quartz or borosilicate glass are used due to the acids exhibiting high boiling points, which are higher than the melting point of Teflon (Matusiewicz, 2003).

2.17.2 Inductively coupled plasma-optical emission spectrometry (ICP-OES)

The ICP-OES instrument is often used to determine the concentration of a range of elements in a sample. The instrument requires the sample to be introduced in the liquid form, therefore the sample (in the case of a solid) has to be digested in an appropriate acid in order to be brought into an aqueous form (Rouessac and Rouessac, 2013, Skoog et al., 2013).

Mahlangeni et al. (2017) used nitric acid (HNO_3) to digest edible mountain nettles by microwave digestion and analysed the samples for 13 elements using ICP-OES. The concentrations of elements found in the herbal plants were in decreasing order of Ca > Mg > Fe > Mn > Zn > Cr > Cu > Ni > Pb > Co > As > Cd > Se. The elemental analysis of the herbal plants *Trifolium repens* and *Lolium perenne* were performed using ICP-OES by Bidar et al. (2007). These plants were growing in a heavy metal contaminated field, where the concentration levels of Cd, Pb and Zn were investigated and found to be in decreasing order of Cd > Zn > Pb.

A nebulising gas flow introduces the sample for analysis into the capillary tube. The sample solution is transformed into an aerosol by the high speed gas which is at the tip of the capillary tube. Isolation of the aerosol droplets then occurs based on their size whereby the finer droplets are transferred to the plasma while the larger droplets go to the drain. Streams of Ar gas move through three concentric quartz tubes which sit in the plasma unit. An induction coil, powered by a radio frequency generator, surrounds the tube. This generator is able to produce up to 2 kW of energy (Figure 2.15). Atoms and ions exist in the plasma vapour and are often highly excited to a state of radiated light (photon) emission. Analysis of the sample is made through spectral observations which commonly occur at 15 to 20 mm above the induction coil. This is where elevated temperatures such as 5 000 to 6 000 K are attained (Skoog et al., 2013).

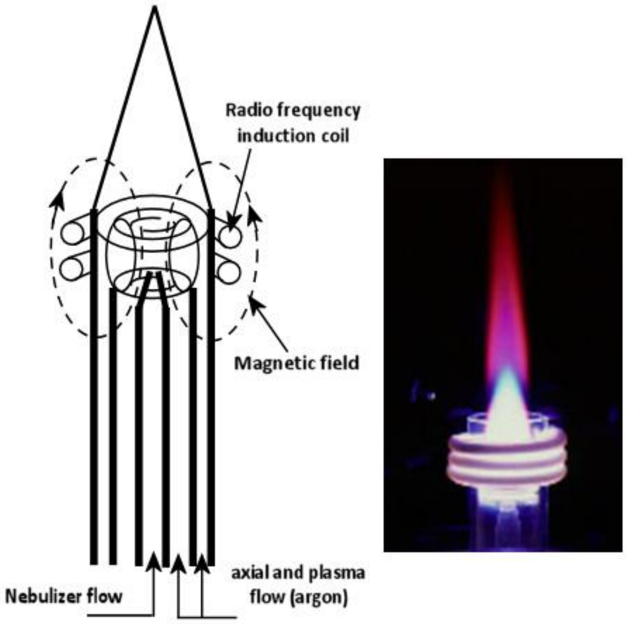

Figure 2.15: Inductively coupled plasma source with a flame-like tail topping a brilliant white opaque core (http://www.chemiasoft.com/chemd/node/52)

The wavelength isolation device then differentiates between the atomic or ionic emission from the plasma and, from this, separation of the different wavelengths of the constituents occurs. This wavelength separation can occur in either a monochromator, a polychromator or in a spectrograph. A simultaneous spectrometer is capable of scanning a wide selection of wavelengths and it can do this through the use of a polychromator or spectrograph. These simultaneous spectrometers contain dispersive devices and these devices can either be a combination of gratings and a prism or, just the gratings alone (Figure 2.16). In this way, multi-elements in the sample can be found instantaneously (Skoog et al., 2013).

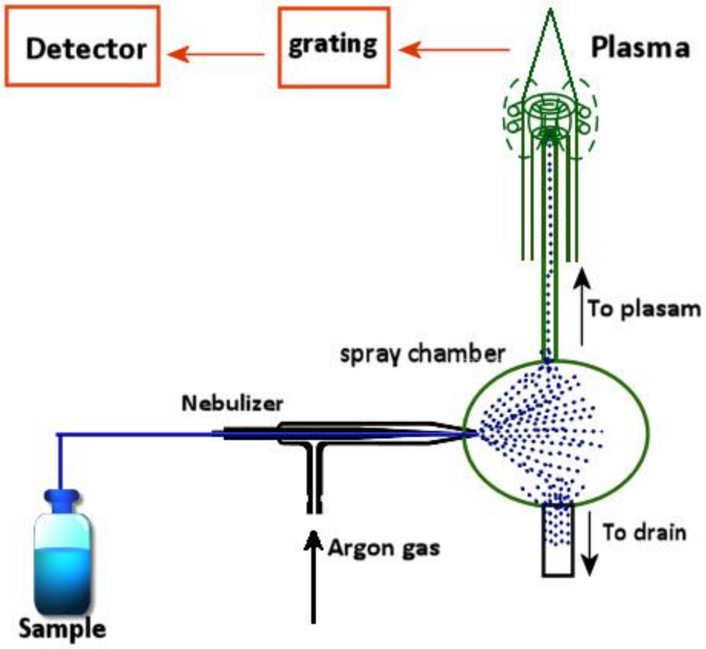

Figure 2.16: Basic depiction of the path that a sample solution would take through the ICP-OES instrument
(http://www.chemiasoft.com/chemd/node/52)

One of the most preferred array detectors is the charged coupled device (CCD) and it is commonly used for most simultaneous and for selected sequential spectrometers. The CCD uses a certain amount of electrical charge which would denote an analogue quantity, for instance light energy, sampled at different times. The memory utility comes from shifting these charges simultaneously, down a row of cells, also in discrete time (Rouessac and Rouessac, 2013).

2.17.2.1 Limit of detection

A detection limit (DL) can be defined as the minimum concentration which can be stated with a certain level of confidence. Detection limits for the ICP-OES are typically in the sub ppb-ppm range.

Table 2.7: Typical detection limits reported in ICP-OES

Element	Wavelength (nm)	IDL[a] (µg/L)
As	193.691	5.7
Cd	226.499	0.1
Ca	315.880	0.5
Co	228.612	0.4
Cr	205.557	0.3
Cu	324.747	0.3
Fe	259.933	0.3
Mg	279.071	0.1
Mn	257.604	0.04
Ni	231.602	0.5
Pb	220.350	1.5
Se	196.022	8.8
Zn	213.855	0.2

[a] IDL = Instrument Detection Limit

2.17.2.2 ICP-OES interferences

Background emissions cause spectral interferences. These are caused by the occurrence of an element other than the analyte, stray light emerging within the band-pass of the wavelength selection device and unresolved overlap of molecular band spectra. It is often a requirement to subtract background emission for most of the analytical emission lines. Alternative lines can be chosen in order to avoid spectra overlap (Skoog et al., 2013).

Upon a shift in the ionization process, physical interferences occur whereby substances within the sample are able to change the viscosity of the solutions. Two of the parameters which could then be altered are the nebulisation process efficiency and the flow rate. Organic solvents, which are constituents that are combustible, can influence the temperature of the atomiser thereby indirectly affecting the efficiency of the atomization process. In other cases, the flow rates of the solutions could differ due to dissimilar matrices, where the sample could be containing acid yet the standard solutions do not (Rouessac and Rouessac, 2013, Skoog et al., 2013).

In the event of a single element bringing in to the plasma additional electrons, chemical interferences ensue. This occurs due to the less concentrated alkali metal having its neutral atom population raised, thereby bringing about an enhancement in the emission intensity, in relation to the standard. In the uncommon case where this chemical interference takes place, it may be necessary to reduce the inner Ar gas flow and/or to raise the radio frequency power in order to eliminate the chemical interference (Rouessac and Rouessac, 2013, Skoog et al., 2013).

2.18 Quality assurance

The analysis of a standard whose analyte concentration is reliably known still remains one of the best ways in which an analytical method can be validated. The overall composition and concentration of analytes of the standard should, ideally, be as close as possible to that of the sample (Skoog et al., 2013). Certified reference materials (CRMs) are thus a requirement. A number of laboratories are responsible for the authentication of the analyte concentrations in CRMs.

A CRM is as reference material according to the International Organisation for Standardisation (ISO). This reference material is accompanied by a certificate and has one or more of its property values officially stated by a method which would establish its traceability to a precise realisation of the unit in which the property values are expressed, and for which each certified value is supplemented by uncertainty at a stated level of confidence.

2.19 Walkley-Black Method

The analysis of SOM is typically carried out using this method. The solvent used is typically potassium dichromate in acid medium. The dichromate ion reacts with the carbon in soil in an oxidation step as shown in reaction (1).

(1) $2Cr_2O_7^{2-} + 3C + 16H^+ \rightarrow 4Cr^{3+} + 3CO_2 + 8H_2O$

Ideally, the organic soil matter would be reacting with a known quantity of the reagent due to the surplus of chromate ions. Thereafter, the estimation of the amount of organic matter in that specific soil is done by performing a back-titration between the ions that are left behind from the previous step and a known concentration of ferrous solution (Schulte, 1995). The excess dichromate ion reacts with the ferrous ion as shown in the reaction (2).

(2) $6Fe^{2+} + Cr_2O_7^{2-} + 14H^+ \rightarrow 2Cr^{3+} + 6Fe^{3+} + 7H_2O$

The following equation is used for the conversion of organic carbon to organic matter to be made:

$$\text{Soil organic matter} = \%\ \text{Total carbon} \times 1.72$$

2.20 Chapman method principles

Soil CEC determinations can be made in a variety of different ways. There exists a method by Chapman (1965) which employs ammonium acetate at neutral pH (pH of 7.0). Over the years, a well-built database has been composed for soil CEC for this method, especially since it has been extensively used in the United States (Ross and Ketterings, 1995). The method consists of three simple steps. In the first step, ammonium acetate solution is used for soil saturation, thereafter allowing for the replacement of the ammonium cations by the metal cations which are adsorbed on the surface of the soil. The second step allows for the elimination of the surplus of ammonium acetate solution (which is the saturating solution) by the addition of ethanol. In the third step, potassium cations (from a KCl solution) substitute the adsorbed ammonium cations and a Kjeldahl distillation would then determine the quantity of ammonia that is released. In analytical chemistry, a Kjeldahl digestion is whereby the distillation process converts organic nitrogen into ammonia (Chapman, 1965, Ross and Ketterings, 1995).

Gaseous ammonia is formed from the conversion of ammonium ions:

$NH_4OH + CH_3OOH \rightarrow (CH_3COO)^- + NH_4^+ + H_2O$,

$NH_4OH\ (aq) \rightarrow NH_3\ (g) + H_2O$

Distillation occurs in the next step together with the reaction of a known quantity of excess hydrochloric acid with ammonia:

$NH_3 + HCl \rightarrow NH_4Cl + HCl_{(left)}$

A back-titration then occurs with a standard sodium hydroxide solution and the left-over acid:

$HCl_{(left)} + NaOH \rightarrow NaCl + H_2O$

2.21 References

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CHAPTER 3

ELEMENTAL ANALYSIS AND NUTRITIONAL VALUE OF EDIBLE *TRIFOLIUM* (CLOVER) SPECIES

Abstract

Trifolium species, commonly known as clover species, have a cosmopolitan distribution and, as such, are used in many different traditional systems of medicine and are also consumed by different communities all over the world. In this study, the elemental distribution and nutritional value of five edible *Trifolium* species, namely *Trifolium africanum*, *Trifolium burchellianum*, *Trifolium repens*, *Trifolium dubium* and *Trifolium pratense* were determined to evaluate the potential of these plant species to alleviate malnutrition in South Africa, thereby contributing towards the global battle against food insecurity. *Trifolium* species were found to be a rich alternate source of essential nutrients with concentrations of elements being in decreasing order of Ca > Mg > Fe > Mn > Zn > Se > Cu > Cr > Pb > Ni > Co > Cd > As and with adequate levels of lipids (4.2 to 8.6%), proteins (35.1 to 45.4%) and carbohydrates (26.7 to 47.0%). *Trifolium* species were found to be rich in Se (contributing >516% towards its RDA) with *T. dubium* having a concentration of 0.53 mg/10 g, dry mass, which is higher than Brazil nuts. *T. pratense* was found to be the most suitable species for human consumption due to it having low levels of toxic metals (As, Cd and Pb) whilst being rich in macro and micro-elements, especially Fe (7.84 mg/10 g, dry mass) and Se (0.36 mg/10 g, dry mass).

Keywords: Traditional medicine, nutrition, food insecurity, elemental distribution

3.1 Introduction

Malnutrition is a common health problem amongst rural communities in African countries and this condition has the potential to result in noncommunicable diseases such as diabetes, cancer, heart and chronic respiratory diseases (Mayosi et al., 2009, Norman et al., 2007). Rural households are substantially dependent on food plants to meet their dietary requirements, provide a diverse diet and maintain a healthy lifestyle. Food plants are a rich source of phytocompounds, micronutrients and essential elements and uptake of these plants assist in the prevention of diseases through ingestion of the phytocompounds and also help to meet nutrient demands (Moodley et al., 2007, WHO, 2005). Scientific investigations on the effects of plants on human health showed a decrease in nutrition-related diseases on frequent consumption which correlated with lower risk of diseases (Bosman et al., 2011).

Human activities (anthropogenic sources), which include industrial, agricultural, automobile emissions, improper waste disposal, and mining, have resulted in environmental pollution including soil contamination (Bidar et al., 2009). Heavy metal contamination of soil is a concern due to toxic, non-biodegradable, ubiquitous metals, and their negative impact on the environment (Bidar et al., 2007). The prevalence and accumulation of metals in the edible parts of plants is predominantly due to them not being easily metabolized after uptake. Furthermore, toxic metals can be carcinogenic and mutagenic if they occur at elevated levels in the body (Mahlangeni et al., 2012). Uptake of heavy metals from soil by accumulator or hyperaccumulator plants that are consumed by humans is a major route of exposure to these heavy metals. Therefore, uptake of high concentrations of heavy metals by plants from soil will pose a risk to human health (Moodley et al., 2012).

The genus *Trifolium* (Fabaceae), commonly known as clover, is a genera of the Leguminosae family (Zohary and Heller, 1984). *Trifolium* species occur in temperate and subtropical regions of both hemispheres with the Mediterranean region having the greatest diversity (Kolodziejczyk-Czepas, 2012). It is an important forage plant and is used as a medicinal herb (Tundis et al., 2015). *Trifolium* species are rich in bioactive compounds that impart medicinal value and this together with potential nutritional value can potentiate its nutraceutical properties (Kolodziejczyk-Czepas, 2016).

Studies on *Trifolium* species from other regions of the world have been widely conducted, however, there is a lack of information on the species found in South Africa. Previously, we reported on the nutraceutical value of edible nettles in an effort to ameliorate food insecurity (Mahlangeni et al., 2016, Mahlangeni et al., 2017). In this study, we investigated the elemental distribution in five edible clover species (*Trifolium africanum*, *Trifolium burchellianum*, *Trifolium repens*, *Trifolium dubium* and *Trifolium pratense*) found in KwaZulu-Natal by determining the concentrations of 13 analytes (As, Ca, Cd, Co, Cr, Cu, Fe, Mg, Mn, Ni, Pb, Se and Zn) in these species. The contribution of these plants to the nutritional needs of humans was evaluated by comparing to Recommended Dietary Allowances (RDAs).

3.2 Materials and methods

3.2.1 Sampling

Five different plant species, namely *Trifolium africanum*, *Trifolium burchellianum*, *Trifolium repens*, *Trifolium dubium* and *Trifolium pratense* were gathered and sampled accordingly from four sampling sites in KwaZulu-Natal, South Africa. All samples were collected on warm, sunny days during the season of spring. *T. repens* and *T. pratense* were collected from Japanese Gardens, Durban North (29.795º S, 31.039º E); *T. dubium* from Westville (29.817º S, 30.945º E); *T. burchellianum* from Port Shepstone (30.727º S, 30.432º E) and *T. africanum* from the University of KwaZulu-Natal, Howard College, Glenwood (29.870º S, 30.977º E). Plant samples were stored and refrigerated in sealed polyethylene bags at 4 ºC for less than a week before digestion.

3.2.2 Proximate chemical composition of leaves of *Trifolium* species

The lipid content was determined by soxhlet extraction (Marinho-Soriano et al., 2006). The protein content was obtained by converting nitrogen to protein (6.25 × % N) where nitrogen was determined using an elemental analyser (Thermo Scientific Flash 2000). The ash content was determined by incinerating a known mass of sample in a muffle furnace at 600 ºC for 6 h and the carbohydrate content was determined by difference (subtracting the percentage lipid, protein, and ash from 100 g of sample, dry mass).

3.2.3 Reagents and standards

The solvents used in the experiments were of analytical reagent grade and were purchased from Sigma Aldrich (St. Louis, USA) and Merck (Kenilworth, USA). All glassware and plastic bottles used were soaked in 3M HNO_3, rinsed with double distilled water, and air dried until use. Calibration standards were prepared from 1000 mg L^{-1} stock standard solutions (Fluka Analytical, Sigma, Switzerland) using double distilled water.

3.2.4 Sample preparation

Plant material was thoroughly washed with double distilled water before drying in an oven at 50 ºC, overnight. Some unwashed leaves were dried in the oven at 50 ºC to constant mass to determine the moisture content. Dried samples were packaged into polyethylene bags after being crushed using a mortar and pestle to achieve homogeneity, and placed in the refrigerator until digestion the next day.

3.2.5 Digestion and elemental analysis

Acid digestions were performed using the CEM Microwave Accelerated Reaction System (MARS 6, CEM Corporation, Matthews, NC, USA) with MARSXpress™ vessels and infrared temperature sensors. Approximately 0.25 g (dry mass) of plant material and certified reference material (CRM) were weighed and placed into microwave vessels, to which 10 mL HNO_3 was pipetted. Each sample was pre-digested for 30 min before digestion (Kisten et al., 2015, Magura et al., 2016). The power output was set to 100 % at 1600 W and the temperature was set to reach 180 ºC in 20 min. The holding time was set for 15 min and cooling time was set for 15 min. Plant material was digested in triplicate, filtered through 0.45 µm, 25 mm nylon syringe filters into 25 mL volumetric flasks, made to the mark using double distilled water, transferred into 15 mL polypropylene vials with screw caps then refrigerated at 4 ºC prior to elemental analysis which was done within a week of digestion.

Analysis of elements (As, Ca, Cd, Co, Cr, Cu, Fe, Mg, Mn, Ni, Pb, Se, and Zn) was carried out using an Inductively Coupled Plasma-Optical Emission Spectrometry (Perkin Elmer® Optima™ 5300 with Dual View, Billerica, Massachusetts, USA). Three of the most sensitive analytical wavelengths were initially chosen and the one that produced optimum CRM results was finally selected for the analysis of samples.

3.2.6 Quality assurance

Quality assurance was performed using the CRM, *white clover* BCR-402 (Community Bureau of Reference of the Commission of the European Communities, Brussels, Belgium).

The experimental data (n=3, p=0.05) obtained for the reference material expressed in mg kg^{-1} was 0.182 ± 0.004 for Co, 4.97 ± 0.221 for Cr, 241.06 ± 20.96 for Fe, 8.21 ± 0.08 for Ni, 6.64 ± 0.21 for Se and 24.80 ± 0.97 for Zn compared to their corresponding certified values 0.178 ± 0.008 for Co, 5.19 for Cr, 244 for Fe, 8.25 for Ni, 6.70 ± 0.25 for Se and 25.2 for Zn. The experimental values were within the acceptable range of that stipulated for the CRM at the 95% confidence interval.

3.2.7 Statistical analysis

A comparative study and grouping of results were performed by one way ANOVA and Tukey’s posthoc test to determine and evaluate significant differences between means. Statistical analysis was done using the Statistical Package for the Social Sciences (PASW Statistics 24, IBM Corporation, Cornell, New York).

3.3 Results and discussion

3.3.1 Chemical composition

The results obtained for the analysis of the five *Trifolium* species is presented in Table 1. The moisture content was 10.5% for *T. burchellianum*, 10.7% for *T. africanum*, 12.5% for *T. repens*, 13.7% for *T. dubium* and 13.8% for *T. pratense*. The results show *Trifolium* species to be rich in proteins and carbohydrates with *T. dubium* being richer in proteins than carbohydrates, *T. pratense* having the lowest fat content and *T. burchellianum* having the lowest ash content indicating lower metal content.

Table 3.1: Proximate chemical composition (g per 100 g, dry mass) of the five *Trifolium* species analysed (Mean ± SD, n=3)

Species	Lipid (%)	Protein (%)	Ash (%)	Carbohydrate* (%)
T. africanum	6.3 ± 0.6	39.2 ± 0.2	14.0 ± 0.4	40.5 ± 1.1
T. burchellianum	5.1 ± 0.3	40.1 ± 0.5	7.9 ± 0.2	47.0 ± 0.3
T. repens	7.2 ± 0.4	40.6 ± 1.5	14.5 ± 0.2	37.9 ± 1.2
T. dubium	8.6 ± 0.2	45.4 ± 1.2	19.3 ± 0.5	26.7 ± 0.6
T. pratense	4.2 ± 0.3	35.1 ± 1.6	23.6 ± 0.4	37.1 ± 1.63

*Carbohydrate determined by difference from the dry mass (100 g).

3.3.2 Elemental distribution

Calcium is an essential macro-element that improves and maintains skeletal health because it contributes to bone strength (Heaney, 2002). Of the nutrients studied, Ca concentrations were found to be the highest in all species, ranging from 6008 mg kg^{-1} in *T. repens* to 15477 mg kg^{-1} in *T. pratense*, which was significantly higher than the other *Trifolium* species ($p<0.05$) (Fig. 3.1). Magnesium is vital for the metabolic functioning of cells in the body (Burch and Giles, 1977). *T. burchellianum* was found to be richer in Mg (5079 mg kg^{-1}) and Mn (252 mg kg^{-1}) relative to the other species analysed (Fig. 3.1 and 3.2). Iron is used to produce red blood cells in the body and transport oxygen to cells (Adamson, 1994) while Cr is responsible for the transportation of glucose in the blood to cells and prevents Ca deficiency in the body (Mertz, 1969). Concentrations of Ca, Cr and Fe were significantly higher ($p<0.05$) in *T. pratense* relative to the other species with low levels of Mn and Ni (Fig. 3.4). Zinc is used for wound healing and cell growth and division (Burch et al., 1975), whilst Cu is used to produce enzymes and repair connective tissues in the body (Uauy et al., 1998). Both these elements showed an antagonistic effect in two *Trifolium* species; in *T. africanum* when Cu was high, Zn was low and in *T. repens*, when Zn was high, Cu was low. Cobalt which is a fundamental component of vitamin B12 that assists in the production of red blood cells that help prevent anaemia (Mertz, 1981), was lowest in *T. pratense* and highest in *T. repens*. Selenium is an essential micronutrient that is used in the production of antioxidant enzymes that help prevent cancer and heart disease (Arvilommi et al., 1983). *T. dubium* was found to be a rich source of Se (52.5 mg kg^{-1}) which was significantly higher ($p<0.05$) than the other *Trifolium* species studied.

The concentrations of Cd and Pb in *T. africanum*, *T. burchellianum*, *T. repens* and *T. dubium* species are illustrated in Figure 3.3. If present, As, Cd and Pb were below the instrument detection limit for all samples of *T. pratense*. Cadmium concentration was highest in *T. burchellianum* (0.5 mg kg^{-1}) and Pb was highest in *T. dubium* (3.0 mg kg^{-1}) which exceeded threshold values set by the Department of Health, South Africa, for leafy vegetables which is 0.2 mg kg^{-1} for Cd and 0.3 mg kg^{-1} for Pb (Motsoaledi, 2016). The concentration of elements analysed in the five clover species were generally found to be in decreasing order of Ca > Mg > Fe > Mn > Zn > Se > Cu > Cr > Pb > Ni > Co > Cd > As.

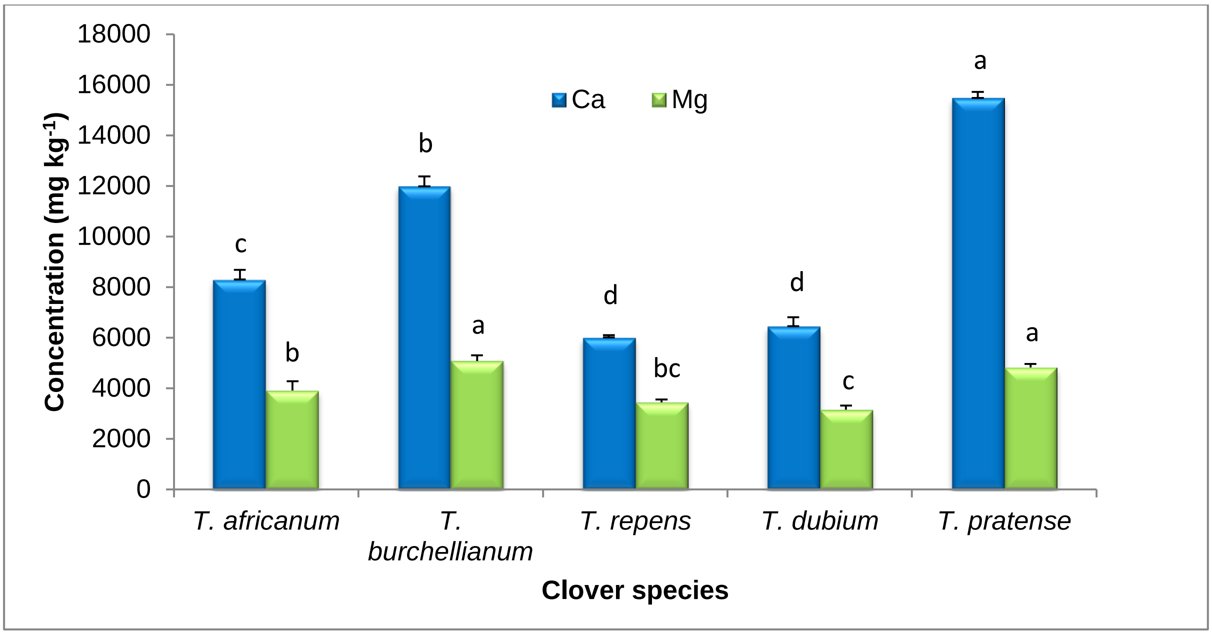

Figure 3.1: Elemental concentration of macro-elements (Mean ± SD, n = 3, in mg kg^{-1}) in the five different *Trifolium* species. Different letters for each element show significant differences amongst means of the *Trifolium* species (Tukey's posthoc comparisons, $p<0.05$)

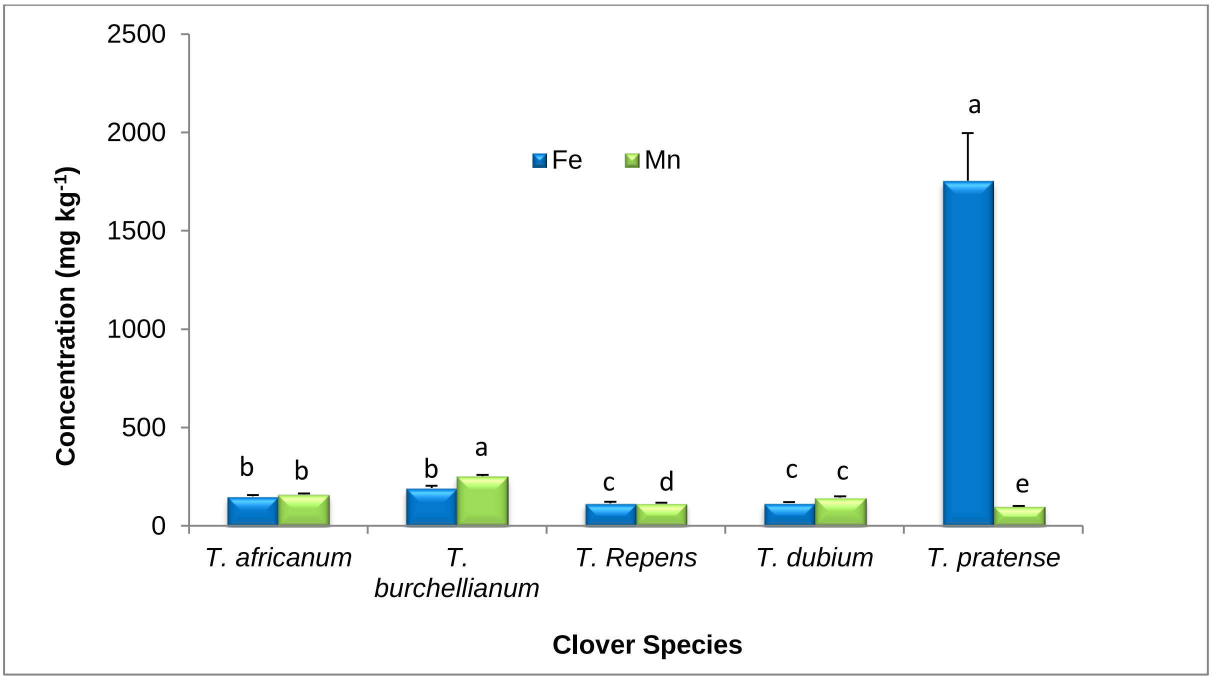

Figure 3.2: Elemental concentration of Fe and Mn (Mean ± SD, n = 3, in mg kg^{-1}) in the five different *Trifolium* species. Different letters for each element show significant differences amongst means of the *Trifolium* species (Tukey's posthoc comparisons, $p<0.05$)

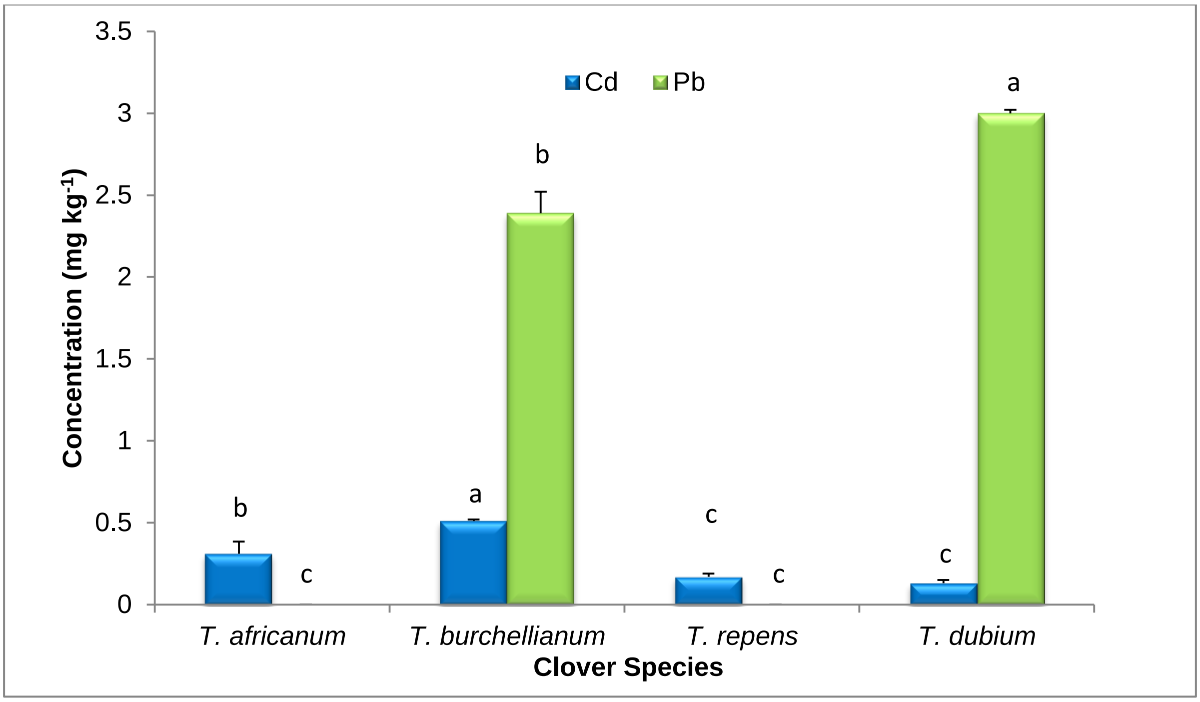

Figure 3.3: Elemental concentration of Cd and Pb (Mean ± SD, n = 3, in mg kg^{-1}) in the four different *Trifolium* species. Different letters for each element show significant differences amongst means of the *Trifolium* species (Tukey's posthoc comparisons, $p<0.05$)

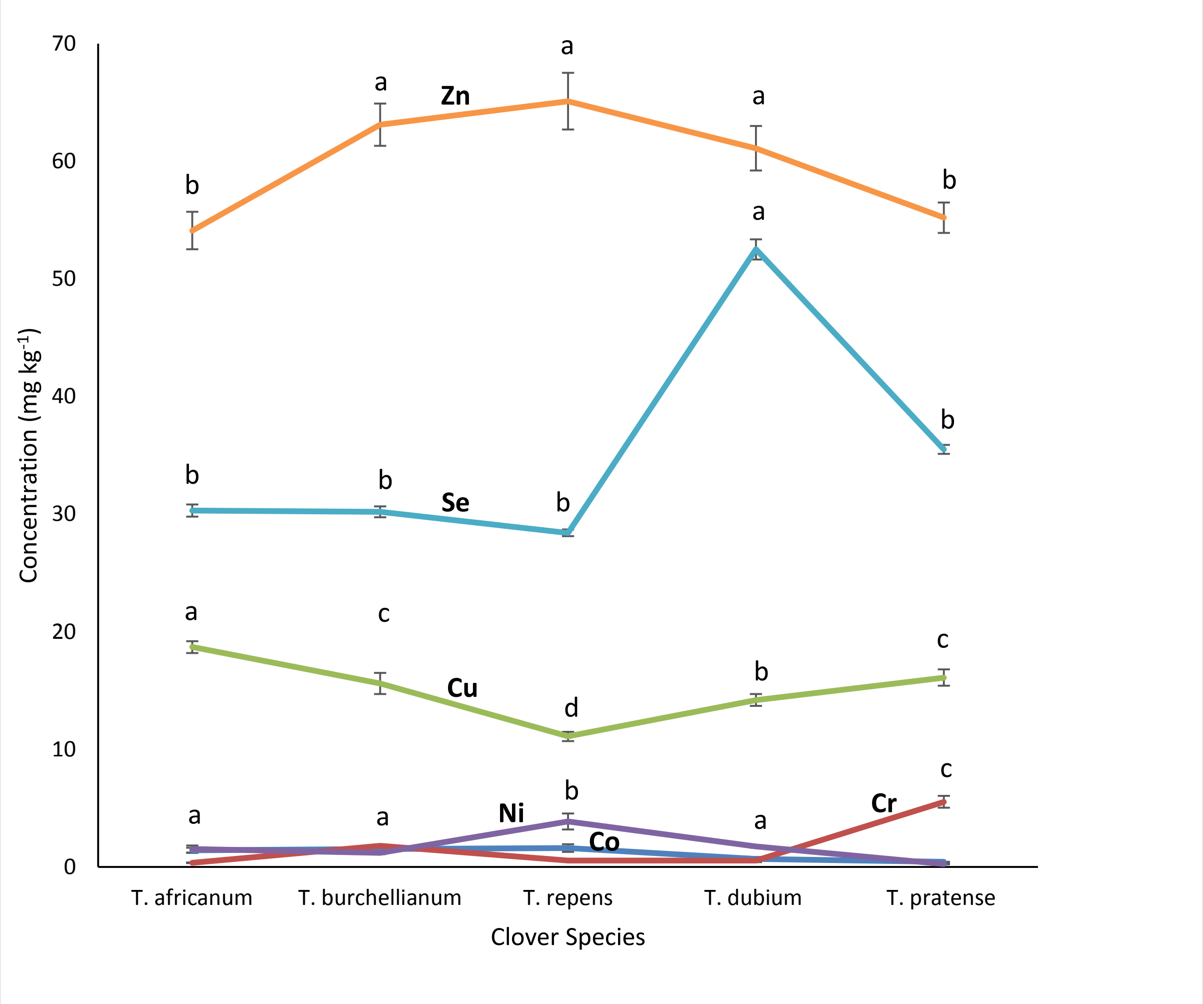

Figure 3.4: Elemental concentration of the micro-elements (Co, Cr, Cu, Ni, Se and Zn; Mean ± SD, n = 3, in mg kg^{-1}) in the five different *Trifolium* species. Different letters for each element show significant differences amongst means of the *Trifolium* species (Tukey's posthoc comparisons, $p<0.05$)

3.3.3 Contribution of elements in *Trifolium* species to the diet

The Dietary Reference Intakes (DRIs) and Upper Intake Levels (ULs) for selected elements are presented in Table 2 and the estimated contribution of 10 g (estimated serving size) of *Trifolium* species, dry mass, to Recommended Dietary Allowances (RDAs) was evaluated.

Iron deficiency can result in anaemia and increase an individual's susceptibility to infection. However, an excess of Fe may be carcinogenic and/or increase an individual's risk of heart disease (Calabrese et al., 1985). Consumption of 10 g of *T. pratense* may contribute 98% (7.84 mg/10 g) towards the RDA for Fe without exceeding the UL. Iron deficiency is a growing health concern as commercial foods rich in Fe such as spinach, peas and beans are unaffordable or are not readily available in rural communities (Hurrell, 1997). *T. pratense* can be an alternative source of Fe due to the plant being environmentally abundant and rich in this nutrient (Thomas and Bowman, 1998).

Chromium is an important micro-element required for protein and carbohydrate metabolism in humans. The concentrations of Cr exceed the RDA for the element in all *Trifolium* species. However, high intake of Cr is not a health issue, therefore a UL for Cr has not been established due to it not being linked with any adverse health and toxic effects even at high concentrations (Levine et al., 1968). Manganese is an essential nutrient necessary for brain and nerve functioning of the body (Takeda, 2003). Approximately 10 g of *T. burchellianum* species may contribute approximately 110 % (2.52 mg/10 g, dry mass) towards the RDA for Mn without exceeding the UL for the element, therefore making it safe for human consumption.

Selenium’s antioxidant properties make it an important nutrient, however, if in excess, Se can cause gastrointestinal disturbances, tooth decay and depression (Tank and Storvick, 1960). Brazil nuts (*Bertholletia excelsa*) are known to be rich in Se and consumption of Brazil nuts is known to protect the body against free radical damage and tumour development (Chang et al., 1995). The other health benefits of Se in the body include the reduction of asthma symptoms, improved fertility and regulation of thyroid function (Rayman, 2000). The concentration of Se in Brazil nuts have previously been reported to be as high as 0.36 mg/10 g, dry mass (Moodley et al., 2007). About 10 g of *Trifolium* species may contribute >516% towards the RDA for Se with *T. dubium* having a concentration of 0.53 mg/10 g, dry mass, which is higher than Brazil nuts and *T. pratense* having a concentration of 0.36 mg/10 g, dry mass, similar to Brazil nuts.

A balanced diet is critical for healthy living and this is accomplished by consuming adequate amounts of essential nutrients. Unfortunately, rural communities in South Africa require alternative sources of nutrients due to financial constraints and inaccessibility. This study shows *Trifolium* species to be rich in essential nutrients thereby providing an alternate source for satisfying the nutritional needs of people. Furthermore, these plant species do not have a tendency to accumulate high concentrations of toxic metals making them safe for human consumption.

Table 3.2: Estimated contribution of essential elements analysed in 10 g of *Trifolium* species to the diet of most individuals

Element	**DRI[a] (mg/day)**		**Estimated contribution to RDA[b] (10 g / day), %**				
	DRI	**UL[c]**	**TA**	**TB**	**TR**	**TD**	**TP**
Ca	1000	2500	6.4	9.2	4.6	4.9	11.9
Cr	0.024	ND[d]	10.3	51.4	15.4	15.7	158
Cu	0.9	8	20.8	17.3	12.3	15.8	17.9
Fe	8	45	8.3	10.7	6.4	6.4	98
Mg	310	350	12.2	15.9	10.8	9.8	15.1
Mn	1.6	9	70	110	50	62	43.1
Se	0.055	0.4	551	549	516	955	646
Zn	8	34	4.9	5.7	5.9	5.6	5

[a] Institute of Medicine of the National Academies: Dietary Reference Intakes (DRIs) (2001).
[b] RDA = Recommended Dietary Allowance
[c] UL = Tolerable Upper Intake Level
[d] ND = Not determined
TA – *Trifolium africanum*, TB – *Trifolium burchellianum*, TR – *Trifolium repens*, TD – *Trifolium dubium*, TP – *Trifolium pratense*

3.4 Conclusion

The elemental distribution and proximate chemical composition of edible *Trifolium* species revealed the plants to be rich in essential nutrients, proteins and carbohydrates. All species contained high concentrations of Se but *T. dubium* was the only species to exceed the tolerable upper intake level. These species can therefore contribute positively to the diet of people, especially in the rural and poverty-stricken communities where there is a lack of minerals and health foods. Cadmium and Pb were not detectable in *T. pratense*, which also had the lowest Co concentration compared to other species. In summary, the concentrations of elements in the five *Trifolium* species studied were found to be in decreasing order of Ca > Mg > Fe > Mn > Zn > Se > Cu > Cr > Pb > Ni > Co > Cd > As. This study shows *Trifolium* species to be a rich, alternate source of essential nutrients with *T. pratense* being rich in Fe and Se with undetectable amounts of the toxic metals (As, Cd and Pb) studied.

3.5 Acknowledgements

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CHAPTER 4

THE DISTRIBUTION AND TOXICOLOGICAL ASSESSMENT OF HEAVY METALS IN *TRIFOLIUM DUBIUM* (LITTLE HOP CLOVER) AND THE IMPACT OF SOIL QUALITY

Abstract

Trifolium dubium is an annual herbaceous legume that is utilised in traditional medicine to treat wounds and as an anti-inflammatory, and it is also consumed as a vegetable. In this study, *T. dubium* was assessed to determine the elemental uptake by the plant and the chemical characteristics of soil. Across ten geographic locations in KwaZulu-Natal, South Africa, the concentration of elements in *T. dubium* were found to be (descending order) Ca > Mg > Fe > Zn > Mn > Se > Ni > Cr > Cu > Co > Pb > Cd > As. The sites in this study had an influence on elemental uptake by the plant, however the availability and accumulation of elements from soil were controlled by the plant in order to meet physiological requirements. Soil chemical characteristics were evaluated and were found to have a pH range of 6.15 to 6.68, soil organic matter content of 0.9 to 2.6% and cation exchange capacity ranged from 4.25 to 9.05 meq/100g. This study has indicated that *T. dubium* contains adequate concentrations of essential elements and undetectable levels of toxic elements, making the herb safe for consumption and allowing its usage in traditional medicine without causing any adverse health effects. The plant can contribute to poverty-stricken communities by ameliorating food insecurity.

Keywords: Traditional medicine, elemental uptake, availability, accumulation, physiological

4.1 Introduction

Edible herbs play a significant role in the diet of individuals due to their nutritional value. Developing countries that are continuously increasing in population require more natural vegetation to ensure that the dietary needs of the population are met (Kruger et al., 2003, Vorster, 2002). This demand has led to increased consumption of forage plants for their nutrients. The nutrition of edible herbs is dependent on several factors that include the capability of the soil to provide these nutrients, the rate at which nutrients are absorbed by the plant, the movement of nutrients within the plant and the spread of nutrients to different functional sites (Imtiaz et al., 2003). Soil quality can have an influence on the mobility of nutrients in the soil because the elemental uptake by the plant is dependent on various interactions between the different nutrients in the soil which are bound to soil particles. This variation of nutrients in the soil is controlled by organic matter content, pH and cation exchange capacity of the soil matrix (Greger, 1999).

The use of pesticides, poor waste disposal, automobile emissions and urban and industrial effluent are anthropogenic activities that contribute to the elevated levels of toxic heavy metals in soil (Cui et al., 2004, Pakade et al., 2013). Plants that are widely used for consumption and medicinal purposes have the ability to bioaccumulate heavy metals from the soil which can directly influence the health of an individual (Hongyi et al., 2009). There have been many toxicological studies on the impact of heavy metal contamination in soils due to the fact that these elements are non-biodegradable and they tend to accumulate in the body and cause severe diseases (Mathee et al., 2002, Türkdoğan et al., 2003).

Trifolium dubium, commonly known as hop clover or suckling clover, is a yellow flowering herb of the Leguminosae family (Fabaceae) (Caradus, 1995). This particular species is an abundant forage plant that is native to Europe, but can be found in temperate and subtropical regions of the northern and southern hemispheres with the Mediterranean region having the greatest diversity. *Trifolium* species are known for their nutritive value and are used in traditional medicinal applications due to the herb having potent antioxidant and anti-inflammatory properties. *T. dubium* is a well-known haemostatic agent that is applied to open wounds to help stop the bleeding (Kolodziejczyk-Czepas, 2012, Mustafa et al., 2012, Sabudak and Guler, 2009).

There is a general lack of information on the distribution of elements in *Trifolium* species and the impact of soil quality parameters on elemental uptake, therefore an investigation on elemental uptake by the plant and assessment of the soil matrix will valuably contribute to agriculture and food science. Previously, we reported on the elemental distribution in indigenous fruits and medicinal plants of South Africa and the impact of soil quality on elemental uptake (Mahlangeni et al., 2016a, Moodley et al., 2013, Reddy et al., 2014). In this study, we determine the elemental accumulation and potential metal toxicity in *Trifolium dubium* as a function of geographical location by collecting plant and soil samples across the east coast of KwaZulu-Natal and analysing them for 13 analytes (As, Ca, Cd, Co, Cr, Cu, Fe, Mg, Mn, Ni, Pb, Se and Zn) to evaluate the impact of soil quality parameters on elemental uptake.

4.2 Materials and Methods

4.2.1 Sample collection and preparation

T. dubium and soil samples from directly below the root of the plant were collected from 10 different sites in KwaZulu-Natal (Figure 4.1); A – Ballito, B – Tongaat, C – Verulam, D – Umhlanga, E – Umgeni, F – Westville, G – Glenwood, H – Queensburgh, I – Isipingo and J – Port Shepstone. The collection of samples was performed in May with the average temperature being approximately 24 ºC with sunny skies. Plant and soil samples were stored in labelled polyethylene bags and transported to the laboratory where the leaves were washed with double distilled water and oven dried at 40 ºC to completely remove moisture. The dried leaves were crushed using a food processor (Russel Hobbs range) and stored in polyethylene bags and refrigerated at 4 ºC for two days before digestion. Soil samples (loamy in texture) were collected with a plastic spade at a depth of 10-15 cm, passed through a 2 mm mesh sieve to remove gravel and dried in oven at 40 ºC. The sieved samples were crushed using a mortar and pestle to reduce particle size before being stored in labelled polyethylene bags and refrigerated at 4 ºC for two days before digestion.

Figure 4.1: Sampling sites in KwaZulu-Natal, South Africa

4.2.2 Reagents and Standards

All solvents (analytical-reagent grade) used in this study were purchased from Sigma Aldrich (St. Louis, USA) and Merck (Kenilworth, USA). Laboratory glassware and plastic bottles were cleaned with 3M HNO_3, rinsed with double distilled water and air dried until use. The preparation of calibration standards was done using 1000 mg L^{-1} stock standard solutions (Fluka Analytical, Sigma, Switzerland) and double distilled water.

4.2.3 Extraction of exchangeable metals

The preparation of the solution used for extracting exchangeable metals from soil samples was performed by dissolving 38.543 g of ammonium acetate salt ($NH_4CO_2CH_3$), 37.224 g ethylenediaminetetraacetic (EDTA) and 25 mL acetic acid (CH_3COOH, 96 %) in double distilled water in a 1 L volumetric flask. The extraction was done by adding 30 mL of the prepared extracting solution to 3.0 g of dry soil sample in labelled 50 mL polyethylene bottles and shaken for 2 h using an orbital shaker. The extraction was performed in triplicate for each site. Each extracted solution was filtered through 0.45 µm Millipore™ filter membranes into labelled polyethylene bottles and stored until analysis.

4.2.4 Soil pH, soil organic matter (SOM) and cation exchange capacity (CEC)

The pH of the soil was determined using a calibrated glass electrode pH meter. A 0.01 M calcium chloride ($CaCl_2$) solution was prepared and 5 mL was added to 5.0 g of soil to form a slurry which was stirred and allowed to stand for 30 min before the pH was recorded. Soil organic matter (SOM) was determined using the wet chemistry extraction procedure (Walkley and Black, 1934). Cation exchange capacity (CEC) of the soil was performed using ammonium acetate at pH 7. (Chapman, 1965).

4.2.5 Analysis of essential and toxic elements

Plant and soil samples were acid digested using the CEM Microwave Accelerated Reaction System (MARS 6, CEM Corporation, Matthews, NC, USA) with MARSXpress™ vessels with IR temperature sensors. Approximately 0.25 g (dry mass) plant material, certified reference material (CRM) and soil sample were weighed and placed into vessels, to which 10 mL of 70 % HNO_3 was pipetted. Each mixture was pre-digested for 30 min before microwave digestion. The power output was set to 100% at 1600 W and the temperature was set to reach 180 ºC for the plant material and 200 ºC for the soil samples in 20 min. All samples were digested in triplicate, with the holding time set for 15 min and cooling time set for 15 min. The digested samples were filtered through 0.45 µm, 25 mm nylon syringe filters into 25 mL volumetric flasks, diluted to the mark using double distilled water and transferred into labelled polyethylene vials which were stored in the refrigerator at 4 ºC prior to elemental analysis. The concentration of the analytes (As, Ca, Cd, Cr, Cu, Fe, Mg, Mn, Ni, Pb, Se, Co, and Zn) were determined by inductively coupled plasma – optical emission spectrometry (ICP-OES) (Perkin Elmer® Optima™ 5300 with Dual View, Billerica, Massachusetts, USA) for all digested samples.

4.2.6 Quality assurance

The analytical method was validated by determining the elemental content of the CRMs which was chosen due to similarities to the sample matrix and comparing the results obtained for each wavelength to certified values. For plant analysis, the *white clover* BCR-402 (Community Bureau of Reference of the Commission of the European Communities, Brussels, Belgium) was used. For soil analysis, the CRM (D081-540) used was from Era quality control. The data obtained for the reference materials is illustrated in Table 4.1. The results were within the acceptable range prescribed for the CRMs, which indicated the accuracy of the method at the 95% confidence interval.

Table 4.1: Concentrations obtained for the plant (BCR-402) and soil (D081-540) certified reference materials to perform method validation (Mean ± SD, n=3)

Element	**Concentration in BCR – 402 (mg kg^{-1})**		**Concentration in D801 – 540 (mg kg^{-1})**		
	Measured	Certified	Measured	Certified	Acceptable limit
As	-	-	75.5 ± 5.9	88.4	71.5 – 105
Ca	-	-	6776 ± 368	7530	6210 – 8850
Cd	-	-	138 ± 7.0	143	116 – 169
Co	0.182 ± 0.004	0.178 ± 0.008	189 ± 4.8	199	166 – 233
Cr	4.97 ± 0.221	5.19	76.6 ± 2.9	86.8	69.3 – 104
Cu	-	-	264 ± 3.3	268	219 – 317
Fe	241 ± 21	244	14129 ± 408	12800	5380 – 20100
Mg	-	-	2679 ± 25.2	2850	2210 – 3490
Mn	-	-	362 ± 8.0	425	347 – 502
Ni	8.21 ± 0.08	8.25	221 ± 10.5	236	194 – 279
Pb	-	-	96.9 ± 5.6	97.9	80 – 116
Se	6.64 ± 0.21	6.70 ± 0.25	120 ± 3.3	127	98.4 – 156
Zn	24.8 ± 0.97	25.2	133 ± 3.9	130	106 – 155

4.2.7 Bioaccumulation factor

Bioaccumulation is the uptake and storage of specific nutrients from the soil by the plant, namely vitamins, trace elements, fats and amino acids (McDowell, 2012). The ratio of the metal’s concentration in the plant to the metals concentration in soil (total) is known as the bioaccumulation factor (BAF) (Timperley et al., 1970).

$$BAF = \frac{[Metal]_{plant}}{[Metal]_{soil}}$$

4.2.8 Statistical analysis

Concentrations of heavy metals in *T. dubium* leaves and soil (total and exchangeable) were grouped by one way ANOVA and Tukey's posthoc test to determine significant differences between means. The relationship between the concentrations (leaves and soil) and soil quality parameters (pH, SOM and CEC) was established by applying Pearson's correlation analysis to the results obtained. Principal component analysis (PCA) was utilised to find patterns in data of high dimension. Statistical analyses were performed using the Statistical Package for the Social Sciences, (PASW version 24, IBM Corporation, Cornell, NY, USA).

4.3 Results and Discussion

4.3.1 Elemental analysis

The concentrations of the eight different analytes in the plant leaves and corresponding soil samples from the ten sampling sites are reported in Table 4.2. Calcium and Mg (Figure 4.2) in leaves of *T. dubium* were the nutrients with highest concentration, which is as expected as these are major nutrients in plants (Marschner, 2011). Plant Ca ranged from 2180 to 13520 mg kg^{-1}, which exceeds the average Ca concentration of 5000 mg kg^{-1} found in plants (Akenga et al., 2014). Site B showed exclusion of Ca, as it was the only sampling site that contained higher total soil than plant Ca (BAF = 0.5) with highest exchangeable percentage (66.7%). Total soil and plant Mg ranged from 641 to 6765 mg kg^{-1} and 1134 to 2252 mg kg^{-1}, respectively, with exchangeable percentages being less than 5.9%. *T. dubium* accumulated Mg which was indicated by high BAFs at most sites. The BAFs for Ca and Mg indicated that the plant tends to accumulate these macro-elements in order to meet physiological requirement levels, which has been reported in previous studies (Mahlangeni et al., 2016b). Site E, G and H were the only sites exceeding the maximum permissible concentration levels set for Mg in South African soils (1300 – 1500 mg kg^{-1}) (Guo et al., 2016).

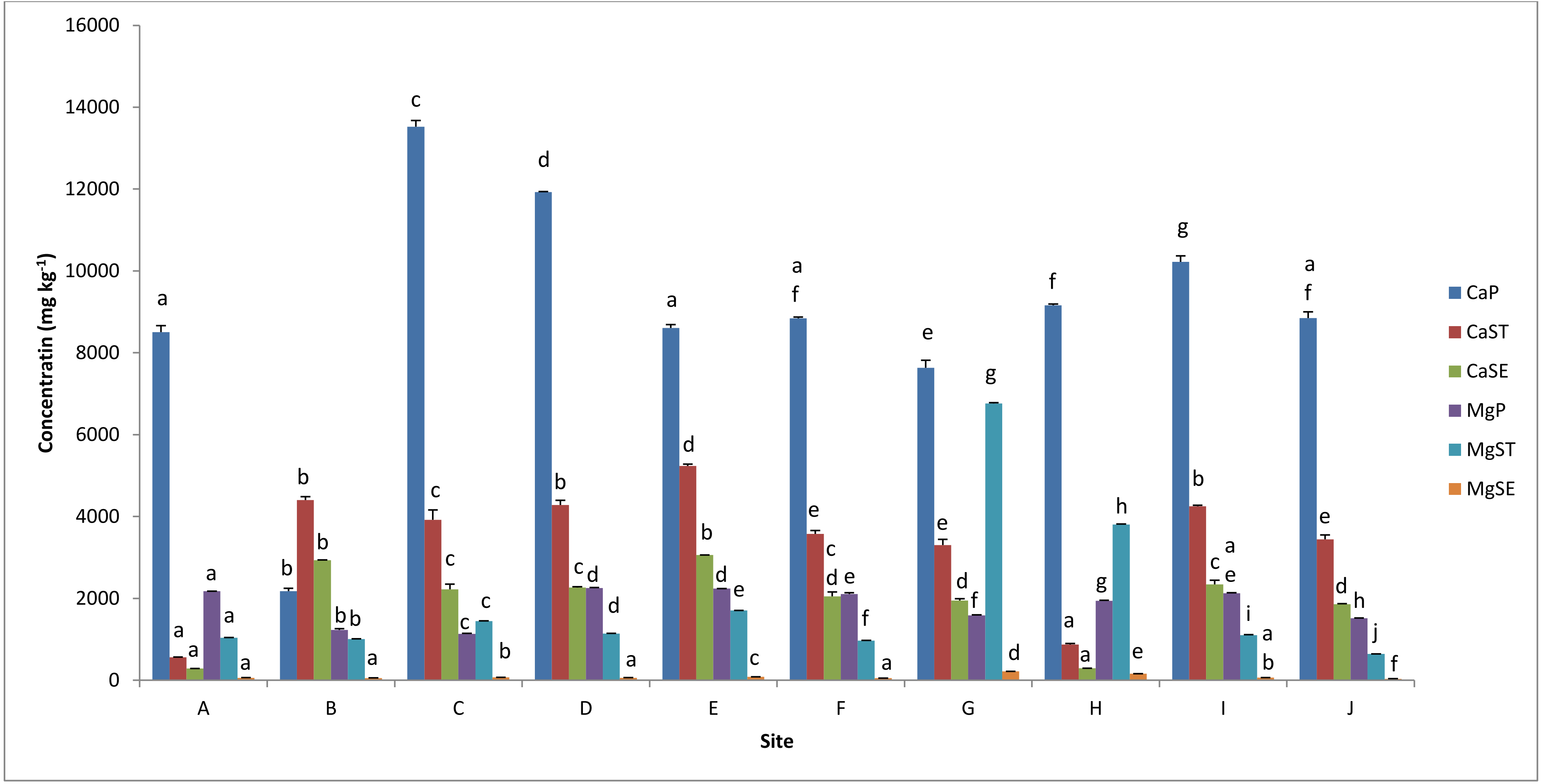

Figure 4.2: Elemental concentrations of macro-elements in *T. dubium* (Mean ± SD, n = 3, in mg kg^{-1}) from ten sites in KwaZulu-Natal, South Africa. Different letters show significant differences amongst means (Tukey's posthoc comparisons, $p<0.05$)

P – Plant, ST – Soil Total, SE – Soil Exchangeable

Trace elements such as Cu, Mn, Se, Fe, Ni and Zn are essential for plant growth and are required by humans. However, high concentrations of these metals that exceed the maximum levels set by health organisations can have negative effects if ingested (Rengel et al., 1999). Total soil Cu ranged from 4.1 to 21.6 mg kg^{-1} with 8.2 to 45.5% being in exchangeable form. The concentration levels of Cu and Cr accumulated by the plant are shown in Figure 4.3. The concentrations of Cu (0.85 – 6.1 mg kg^{-1}) and Cr (0.47 – 6.47 mg kg^{-1}) were similar (Figure 4.3) indicating that the plants requirement levels for these two nutrients are similar, however 19.4 to 81.2% of Cr was in available form in comparison to Cu (8.2 – 45.4%). For Cr, all sites were below the maximum permissible concentration (80 mg kg^{-1}) for soils in South Africa, however site H was the only site that did not exceed the maximum threshold for Cu in South African soils which is set at 6.6 mg kg^{-1} (Van Der Waals and Snyman, 2004).

Concentrations of Mn at all sites ranged from 15.3 to 45.4 mg kg^{-1}. An average BAF of 0.3 indicated that the plant has the tendency to exclude this element in order to meet physiological requirements. Selenium and Zn concentrations in the plant revealed that *T. dubium* tends to accumulate both these elements. Exchangeable Se and Zn ranged from 4.5 to 8.1 mg kg^{-1} and 6.1 to 43 mg kg^{-1}, respectively. The concentration of Se and Zn in the plant ranged from 9.4 to 26.1 mg kg^{-1} and 5.9 to 54.1 mg kg^{-1}, respectively. The average BAFs for Se (3.0) and Zn (1.1) indicated that these essential elements are accumulated by the plant. Five sites (D, E, F, G and J) exceeded the maximum permissible concentration set for Zn in South African soils (Van Der Waals and Snyman, 2004).

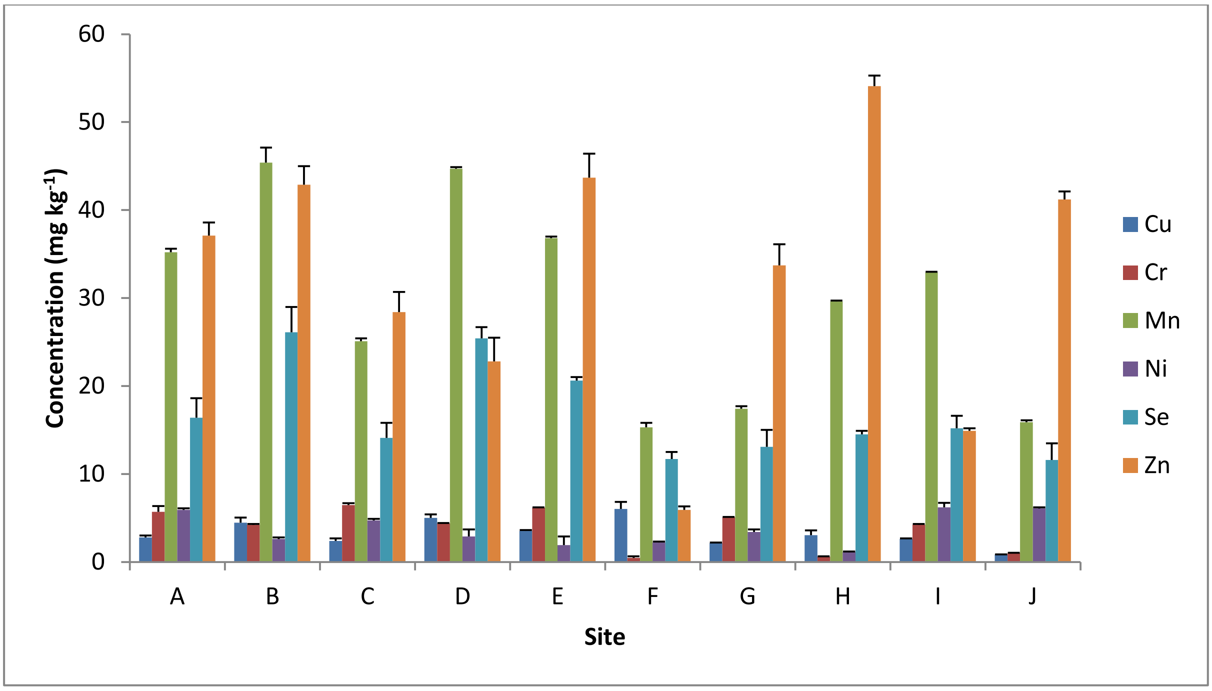

Figure 4.3: Elemental distribution of micro-elements in *T. dubium* (Mean ± SD, n = 3, in mg kg^{-1}) from ten sites in KwaZulu-Natal, South Africa

A – Ballito, B – Tongaat, C – Verulam, D – Umhlanga, E – Umgeni, F – Westville, G – Glenwood, H – Queensburgh, I – Isipingo and J – Port Shepstone

Iron is an essential element that can influence the growth and development of plants. Bioaccumulation of Fe is dependent on the root structure, salinity and soil pH. The availability of Fe for uptake is low due to the element tending to bind with mineral lattices in soil (Morrissey and Guerinot, 2009). This characteristic was observed in this study as exchangeable Fe was less than 6.7%. Iron in the plant ranged from 369 to 999 mg kg^{-1} with an average BAF of 0.1 indicating that the plant excludes Fe to meet low physiological needs. The increased levels of metals such as Cu and Mn in the plant and soil is just one of the contributing factors for low levels of Fe found in the plant (Fageria et al., 1990, Marschner, 2011). Elements such as these alter the accumulation of Fe by competing for the same membrane carrier during the process of uptake, as well as competing for the same soil absorption sites.

Concentrations of the toxic elements As and Cd were below the instrument detection limit for both plant and soil samples at all sites. This assessment indicated that all sites chosen along the east coast of KwaZulu-Natal were not contaminated with As and Cd (toxic heavy metals), allowing for the consumption and medicinal use of *T. dubium* from these areas. Concentration levels of Pb in soil (total) ranged from 0.44 to 3.15 mg kg^{-1}. These concentrations do not exceed the maximum tolerable level of 6.6 mg kg^{-1} set for South African agricultural soils (Mngadi et al., 2017). Site H (Queensburgh) and site J (Port Shepstone) were the only sites that accumulated Pb in the plant with concentrations of 7.57 mg kg^{-1} and 5.49 mg kg^{-1}, respectively which exceeded the maximum threshold concentrations for Pb in leafy vegetables (0.3 mg kg^{-1}) set by the Department of Health (Motsoaledi, 2016). This may have been caused by motor vehicle emissions along the roadside where these plant samples were collected. This suggests that *T. dubium* samples collected on high traffic sites should not be consumed due to Pb accumulation.

The results obtained indicate that *T. dubium* controls elemental uptake and translocation to the leaves of the plant with the elements being in the order of Ca > Mg > Fe > Zn > Mn > Se > Ni > Cr > Cu > Co > Pb > Cd > As. The BAFs for essential elements (Ca, Cu, Fe, Mn, Ni and Zn) showed that even for soil concentrations (total and available) which appeared to be below the requirements for the plant, the required physiological level was nonetheless reached due to the continuation of the accumulation process. On the contrary, if the plants' physiological requirements for soil concentrations were exceeded then the element was partially excluded by inhibiting uptake.

Table 4.2: Concentration of elements obtained at each site for *T. dubium* and surrounding soil (Total (T) and exchangeable (Ex) samples (Mean (SD), n=3) and bioaccumulation factor (BAF)

		Concentration (mg kg^{-1})				**BAF**
Site	**Element**	**Leaves**	**Soil (T)**	**Soil (Ex)**	**[Soil]$_{Ex}$/ [Soil]$_{T}$ %**	**[Leaves]/ [Soil]$_{T}$**
A	Co	1.61 (0.2)^{a}	0.99 (0.09)^{a}	ND	0	1.6
B		1.14 (0.37)^{b}	0.19 (0.11)^{b}	ND	0	6
C		1.69 (0.1)^{a}	1.39 (0.21)^{a}	ND	0	1.2
D		1.34 (0.04)^{a,b}	0.19 (0.04)^{b}	ND	0	7.1
E		0.17 (0.01)^{c,d}	0.58 (0.07)^{a,b}	ND	0	0.3
F		1.77 (0.04)^{a}	ND	ND	0	-
G		0.28 (0.01)^{d,e}	0.21 (0.13)^{b}	ND	0	1.3
H		0.64 (0.04)^{e}	1.18 (0.04)^{a}	ND	0	0.5
I		0.78 (0.08)^{b,e,f}	0.54 (0.08)^{b}	ND	0	1.4
J		1.88 (0.02)^{a}	0.28 (0.33)^{b}	ND	0	6.7
A	Cr	5.68 (0.66)^{a}	6.12 (0.07)^{a}	1.19 (0.18)^{a}	19.4	0.9
B		4.19 (0.11)^{b}	7.59 (0.02)^{b}	1.47 (0.39)^{a,b}	19.4	0.6
C		6.47 (0.20)^{c}	10.6 (0.05)^{c}	2.12 (0.14)^{b,c}	20	0.6
D		4.38 (0.06)^{d,b}	8.27 (0.11)^{d}	2.25 (0.43)^{c}	27.2	0.5
E		6.18 (0.04)^{a,c}	9.07 (0.07)^{e}	4.61 (0.28)^{d}	50.8	0.7
F		0.47 (0.16)^{e,f}	7.65 (0.08)^{b}	1.05 (0.03)^{a}	13.7	0.1
G		5.07 (0.05)^{a}	7.07 (0.06)^{f}	1.03 (0.04)^{a}	14.6	0.7
H		0.60 (0.04)^{f}	6.87 (0.09)^{f}	5.58 (0.11)^{e}	81.2	0.1
I		4.25 (0.08)^{b,g}	8.06 (0.08)^{g}	4.06 (0.09)^{d}	50.4	0.5
J		1.03 (0.01)^{f}	6.55 (0.06)^{h}	3.34 (0.29)^{f}	51	0.2

A	Cu	2.79 (0.22)^{a}	13.2 (0.15)^{a}	2.53 (0.65)^{a}	19.2	0.2
B		4.45 (0.61)^{b}	13.9 (0.11)^{a}	3.19 (0.31)^{a}	22.9	0.3
C		2.39 (0.28)^{a}	21.6 (2.6)^{b}	9.27 (0.22)^{b}	42.9	0.1
D		5.02 (0.39)^{b,c}	14.1 (1.1)^{a}	1.16 (0.07)^{c}	8.2	0.4
E		3.5 (0.13)^{a,b}	15.5 (0.1)^{a}	7.05 (0.08)^{d}	45.5	0.2
F		6.04 (0.77)^{c}	12.4 (0.03)^{a}	5.22 (0.48)^{e}	42.1	0.5
G		2.13 (0.08)^{a}	11.4 (0.53)^{a}	3.38 (0.19)^{a}	29.6	0.2
H		3.03 (0.55)^{a}	4.09 (0.13)^{c}	0.58 (0.17)^{c}	14.2	0.7
I		2.60 (0.08)^{a}	14.6 (0.05)^{a}	4.39 (0.31)^{e}	30.1	0.2
J		0.85 (0.02)^{d}	12.9 (0.22)^{a}	5.44 (0.39)^{e}	42.2	0.1
A	Fe	525 (5.8)^{a}	7063 (35)^{a}	450 (14)^{a}	6.4	0.1
B		999 (18)^{b}	7370 (45)^{b}	449 (2.7)^{a}	6.1	0.1
C		544 (1.9)^{a,c}	10129 (38)^{c}	648 (8.5)^{b}	6.4	0.1
D		763 (54)^{d}	7541 (36)^{d}	414 (12)^{a,c}	5.5	0.1
E		466 (1)^{e}	8313 (29)^{e}	547 (23)^{d}	6.6	0.1
F		369 (2.4)^{f}	8403 (49)^{e}	561 (25)^{d}	6.7	0
G		452 (4.1)^{e}	6765 (20)^{f}	394 (3.4)^{c}	5.8	0.1
H		585 (2.4)^{c}	3804 (12)^{g}	212 (7.3)^{e}	5.6	0.2
I		510 (0.64)^{a,e}	9404 (31)^{h}	575 (14)^{d}	6.1	0.1
J		645 (1.7)^{g}	6410 (24)^{i}	401 (19)^{c}	6.3	0.1
A	Mn	35.2 (0.4)^{a}	126 (1.2)^{a}	7.5 (0.3)^{a}	6	0.3
B		45.4 (1.7)^{b}	94.7 (1.1)^{b}	4.9 (0.6)^{b}	5.2	0.5
C		25.1 (0.3)^{c}	174 (1.4)^{c}	9.5 (1)^{c}	5.5	0.1
D		44.7 (0.2)^{b}	141 (4)^{d}	7.8 (0.04)^{d}	5.5	0.3
E		36.8 (0.2)^{a}	113 (0.9)^{e}	6.5 (0.7)^{a,e}	5.8	0.3

F		15.3 (0.5)^{d}	91.1 (0.23)^{b,f}	5.5 (0.3)^{b,e}	6	0.2
G		17.4 (0.3)^{e}	87.7 (0.49)^{f}	4.9 (0.5)^{b}	5.6	0.2
H		29.6 (0.1)^{f}	51.5 (0.48)^{g}	3.1 (0.7)^{f}	6	0.6
I		32.9 (0.1)^{g}	80.9 (0.7)^{h}	4.4 (0.2)^{b,f}	5.4	0.4
J		15.9 (0.2)^{d,e}	89.8 (0.3)^{f}	3.6 (0.2)^{b,f}	4	0.2
A	Ni	5.9 (0.2)^{a}	1.7 (0.2)^{a}	ND	0	3.5
B		2.6 (0.2)^{b}	3.6 (0.2)^{b}	ND	0	0.7
C		4.7 (0.2)^{a,c}	0.73 (0.2)^{c}	ND	0	6.4
D		2.9 (0.8)^{b}	0.29 (0.13)^{c}	ND	0	10
E		1.9 (1)^{b,d}	0.32 (0.01)^{c}	ND	0	5.9
F		2.2 (0.1)^{b,e}	1.2 (0.2)^{a}	ND	0	1.8
G		3.4 (0.3)^{b,c}	3.5 (0.2)^{b}	ND	0	1
H		1.1 (0.1)^{d,e}	4.3 (0.1)^{d}	ND	0	0.3
I		6.2 (0.5)^{a}	2.4 (0.2)^{e}	ND	0	2.6
J		6.1 (0.1)^{a}	3.7 (0.1)^{b}	ND	0	1.6
A	Se	16.4 (2.2)^{a}	8.6 (0.2)^{a}	4.8 (0.3)^{a}	55.8	1.9
B		26.1 (4.9)^{a,b}	12.2 (0.7)^{a,b}	5.3 (0.2)^{b}	43.4	2.1
C		14.1 (5.7)^{a}	15.4 (1.4)^{b,c}	8.1 (0.1)^{c}	52.6	0.9
D		25.4 (5.3)^{a,b}	11.6 (1.9)^{a,c,d}	4.5 (0.2)^{a}	38.8	2.2
E		20.6 (0.4)^{a,b}	14 (1.5)^{c,e}	7.4 (0.1)^{d}	52.9	1.5
F		11.7 (0.8)^{a,c}	1.5 (0.1)^{f}	ND	0	7.8
G		13.1 (1.9)^{a,c}	11.8 (1.8)^{a,c}	6.4 (0.01)^{e}	54.2	1.1
H		9.4 (0.4)^{a}	10.2 (1.3)^{a,e}	5.7 (0.1)^{b}	55.9	0.9
I		15.2 (4.4)^{a}	13.3 (2.7)^{b,d}	7.8 (0.01)^{c,d}	58.6	1.1
J		11.6 (1.9)^{a,c}	1.1 (0.03)^{f}	ND	0	10.5

A	Zn	37.1 (1.5)^{a}	20.4 (1.7)^{a}	11.2 (0.1)^{a}	54.9	1.8
B		42.9 (2.1)^{a}	17.4 (0.3)^{a}	9.2 (0.5)^{a,b}	52.9	2.5
C		28.4 (8.3)^{a,c}	21.6 (1.8)^{a}	12 (0.2)^{a}	55.6	1.3
D		22.8 (3.7)^{c,d}	88.4 (18)^{b}	43 (1.4)^{c}	48.6	0.3
E		43.7 (2.7)^{a}	61.5 (0.9)^{c}	25.9 (2.1)^{d}	42.1	0.7
F		5.9 (0.4)^{e}	67.6 (4.5)^{c}	32.9 (0.2)^{e}	48.7	0.1
G		33.7 (2.4)^{a,c}	56.3 (4.4)^{c}	23.4 (0.2)^{d}	41.6	0.6
H		54.1 (3.2)^{f}	29.2 (0.5)^{a}	11 (0.3)^{a}	37.7	1.9
I		14.9 (0.3)^{d,e}	11.7 (0.9)^{a}	6.1 (1.5)^{b}	52.1	1.3
J		41.2 (0.9)^{a}	70.9 (9.1)^{b,c}	37.1 (2.7)^{f}	52.3	0.6

Different letters show significant differences amongst means (Tukey's posthoc comparisons, $p<0.05$)
ND – Not detectable

4.3.2 Soil quality

The chemical properties of soil (pH, SOM and CEC) were assessed and the data obtained is shown in Table 4.3. The pH values reported confirm that the plant species *T. dubium* has the tendency to develop and grow in slightly acidic to neutral soil (pH range of 6.15 to 6.68). It has been reported that the optimum pH of soil for vegetation is between the pH range of 6.5 – 7.0 which is similar to the results in this study (McKenzie et al., 2003). The organic matter in soil has a major influence on the functions of soil, which include the ability to hold water and nutrients needed by plants. Studies relating to SOM have reported that there is a positive correlation between metal availability and SOM (Al-Yemeni and Hashem, 2006). The SOM content for the ten sampling sites ranged from 0.88 to 2.58%. The CEC (ranged from 4.25 to 9.04 meq/100g) is significant in determining the chemical properties of soil since it indicates an approximate quantity of cations, namely Ca^{2+} and Mg^{2+}.

Table 4.3: Comparison of pH ($CaCl_2$), SOM (%) and CEC (meq/100g) of the soil samples at ten chosen sites (Mean ± SD, n=3)

Sample site	pH ($CaCl_2$)	SOM (%)	CEC (meq/100g)
A	6.44 ± 0.03	1.41 ± 0.06	4.25 ± 0.05
B	6.25 ± 0.04	1.68 ± 0.01	6.83 ± 0.05
C	6.38 ± 0.01	2.10 ± 0.02	5.90 ± 0.03
D	6.15 ± 0.03	1.66 ± 0.04	7.08 ± 0.06
E	6.32 ± 0.01	1.75 ± 0.04	7.94 ± 0.03
F	6.22 ± 0.01	1.16 ± 0.03	6.11 ± 0.04
G	6.33 ± 0.02	1.10 ± 0.04	8.23 ± 0.07
H	6.39 ± 0.01	2.58 ± 0.02	5.67 ± 0.07
I	6.68 ± 0.08	2.37 ± 0.07	9.04 ± 0.03
J	6.47 ± 0.01	0.88 ± 0.02	6.91 ± 0.04

4.3.3 Statistical analysis

The correlations for the elemental concentrations in *T. dubium* leaves and soil are illustrated in Table 4.4. The correlation matrix was assessed by determining the correlation coefficient (r) which ranged between -1 and +1, where an r value greater than -0.7 shows a strong negative relationship and an r value greater than +0.7 shows a strong positive relationship. A synergistic relationship occurs when an increase in total soil concentration of one element reduces the soil retention capacity, indicating that the elements are competing for the same adsorption site (Moodley et al., 2012). A four way synergy was observed for metals Cu, Mg, Mn and Cr ($r \geq 0.7$). This was confirmed by the PCA analysis. There was a significant positive correlation between CEC and total soil Ca ($r = 0.7$) and between SOM and exchangeable Se ($r = 0.7$). Competition by elements for uptake by the plant via the same mechanism would be antagonistic. Generally, uptake of the element found in higher concentrations in the soil solution is favoured (Moodley et al., 2013). Total soil Mg (r = -0.8), Mn (r = -0.8), Cu (r = -0.7) and Fe (r = -0.7) negatively influenced the uptake of Ni.

The rotated component matrix of the elemental concentrations in soil of *T. dubium* is provided in Table 4.5 and the resultant loading scatter plots are given in Figure 4.4. For PCA, component loadings ≥ 0.6 were chosen. The rotated component matrix was performed on the soil using Varimax rotations. Three components were extracted with eigenvalues greater than one explaining 83.8% of the total variance. The first principal component comprising 43.6% of the variance had high loadings of Cr, Cu, Mg and Mn indicating that these elements were from a common source (soil rich in these elements). The second principal component comprising 22.1% of the variance had high loadings of Fe and Se whilst the third principal component comprising 18.2% of the variance had high loadings of Ca only. The elements clustered together in each principal component indicate that they are associated with a similar natural source.

Table 4.4: Correlation matrix for the elemental concentrations in *T. dubium* leaves (L) and soil (Total (T) and Exchangeable (E))

	CaE	CoT	CoL	CrT	CrL	CrE	CuT	CuL	CuE	FeT	FeL	FeE	MgT	MgL	MgE	MnT	MnL	MnE	NiT	NiL	SeT	SeL	SeE	ZnT	ZnL	ZnE	pH	SOM	CEC
CaT	1.0**	-0.5	-0.2	0.6*	0.3	0.0	0.6	0.2	0.5	0.4	0.2	0.6	0.7*	-0.1	-0.3	0.3	0.2	0.2	-0.4	-0.1	0.3	0.5	0.2	0.3	-0.3	0.3	-0.2	-0.1	0.7*
CaL	-0.2	0.5	0.2	0.5	0.1	0.2	0.3	-0.2	0.3	0.2	-0.5	0.3	0.2	0.2	-0.1	0.5	-0.2	0.5	-0.5	0.2	0.1	-0.3	0.2	0.2	-0.4	0.2	0.2	0.3	-0.1
CaE		-0.5	-0.2	0.6	0.3	-0.1	0.6	0.2	0.5	0.4	0.2	0.6	0.7*	-0.2	-0.3	0.2	0.2	0.2	-0.3	-0.1	0.3	0.6	0.2	0.2	-0.3	0.2	-0.2	-0.1	0.7*
CoT			0.0	0.3	0.3	0.4	0.1	-0.4	0.2	0.1	-0.2	0.0	-0.1	-0.1	0.1	0.3	0.1	0.3	-0.1	0.1	0.4	-0.3	0.5	-0.6	0.4	-0.6	0.4	0.6	-0.5
CoL				-0.1	-0.3	-0.4	0.3	0.1	0.2	-0.1	0.1	0.2	0.0	-0.2	-0.7*	0.4	-0.2	0.3	-0.2	0.4	-0.6	-0.1	-0.6	0.1	-0.3	0.2	-0.1	-0.3	-0.6
CrT					0.5	0.1	0.7*	0.2	0.7*	0.7*	-0.1	0.7*	0.7*	-0.2	-0.2	0.7*	0.2	0.6*	-0.6	-0.1	0.6	0.2	0.5	0.0	-0.3	0.0	-0.1	0.4	0.2
CrL						-0.2	0.7*	-0.2	0.3	0.6	0.0	0.5	0.7*	-0.1	0.0	0.7*	0.4	0.7*	-0.5	0.2	0.8**	0.5	0.7*	-0.3	0.0	-0.3	0.0	0.1	0.1
CrE							-0.3	-0.3	-0.1	-0.3	-0.1	-0.3	-0.2	0.3	0.1	-0.4	0.1	-0.4	0.2	-0.2	0.2	-0.2	0.3	-0.1	0.4	-0.1	0.4	0.6	0.2
CuT								0.0	0.8**	0.6	0.0	0.9**	0.9**	-0.3	-0.5	0.8**	0.1	0.8*	-0.7*	0.4	0.3	0.3	0.3	-0.1	-0.4	0.0	0.0	-0.1	0.1
CuL									-0.2	0.5	0.1	0.1	0.1	0.4	-0.2	0.1	0.3	0.2	-0.4	-0.6	-0.1	0.4	-0.2	0.2	-0.5	0.2	-0.7*	0.0	-0.1
CuE										0.3	-0.3	0.8**	0.7*	-0.4	-0.3	0.5	-0.4	0.4	-0.4	0.3	0.1	-0.1	0.1	-0.1	-0.2	0.0	0.2	-0.1	0.1
FeT											-0.2	0.7*	0.6	0.1	-0.1	0.5	0.3	0.6*	-0.7*	-0.1	0.6	0.3	0.6	-0.3	-0.6	-0.3	-0.1	0.3	0.2
FeL												-0.3	-0.1	-0.4	-0.3	0.1	0.7*	-0.1	0.3	-0.1	0.2	0.7*	0.0	-0.1	0.4	-0.1	-0.3	0.1	0.0
FeE													0.8**	-0.1	-0.5	0.6	0.0	0.6	-0.7*	0.3	0.2	0.2	0.2	-0.1	-0.6	-0.1	0.1	0.0	0.2
MgT														0.0	-0.4	0.7*	0.2	0.7*	-0.8**	0.2	0.4	0.5	0.3	0.1	-0.3	0.1	-0.1	-0.1	0.3
MgL															-0.1	-0.2	0.2	0.0	-0.4	-0.1	-0.1	0.1	-0.1	0.3	-0.3	0.3	0.0	0.1	0.1
MgE																-0.4	-0.3	-0.3	0.4	-0.4	0.3	-0.3	0.4	0.0	0.3	-0.2	0.0	0.1	0.2
MnT																	0.2	1.0**	-0.8**	0.3	0.4	0.4	0.2	0.1	-0.2	0.1	-0.3	-0.1	-0.2
MnL																		0.3	-0.2	-0.2	0.6	0.9**	0.4	-0.2	0.2	-0.2	-0.2	0.4	0.0
MnE																			-0.8**	0.1	0.4	0.4	0.3	0.0	-0.3	0.1	-0.3	0.0	-0.3
NiT																				0.0	-0.2	-0.4	-0.2	-0.3	0.5	-0.3	0.3	0.0	0.0
NiL																					-0.1	-0.2	0.0	-0.3	-0.2	-0.1	0.7*	-0.2	0.0
SeT																						0.4	1.0**	-0.4	0.2	-0.5	0.1	0.6*	0.3
SeL																							0.2	0.1	0.0	0.1	-0.5	0.0	0.2
SeE																								-0.5	0.2	-0.6	0.3	0.7*	0.3
ZnT																									-0.2	1.0**	-0.6	-0.6	0.2
ZnL																										-0.2	0.0	0.2	-0.2
ZnE																											-0.5	-0.6	0.1
pH																												0.3	0.2
SOM																													0.1

Table 4.5: Rotated component matrix for variables in soil samples (n = 30)

	PC1	**PC2**	**PC3**
Eigenvalues	4.355	2.209	1.818
Percentage of total variance	43.553	22.094	18.179
Percentage of cumulative variance	43.553	65.647	83.826
Ca	0.436	0.189	**0.846**
Co	0.181	0.426	-0.827
Cr	**0.733**	0.478	0.183
Cu	**0.855**	0.259	0.155
Fe	0.575	**0.599**	0.158
Mg	**0.841**	0.179	0.347
Mn	**0.930**	0.058	-0.179
Ni	-0.914	0.560	-0.77
Se	0.284	**0.834**	0.002
Zn	0.215	-0.777	0.422

Bold figures indicate values ≥ 0.6.

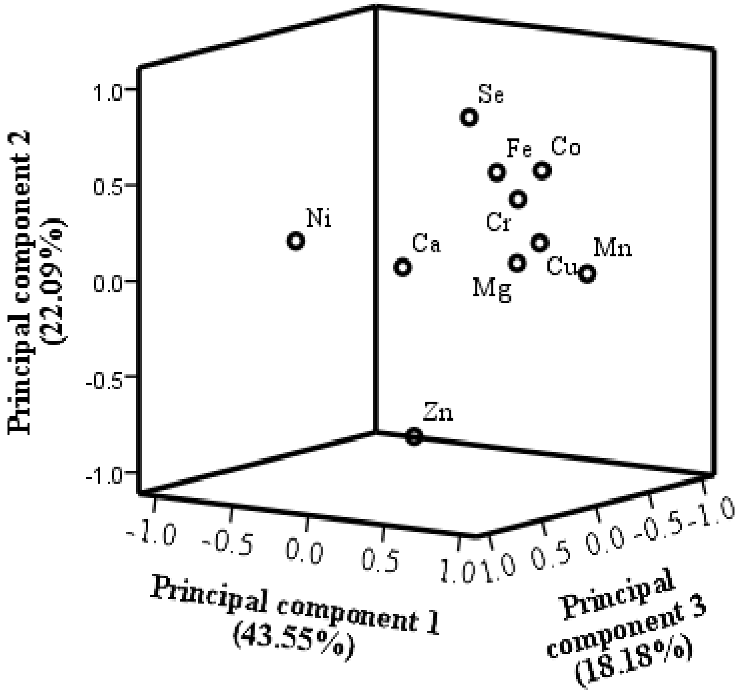

Figure 4.4: Principal component loading plot of metals in soil samples from ten different sites

4.4 Conclusion

The elemental uptake by *T. dubium* from ten sampling sites along the east coast of KwaZulu-Natal, South Africa was investigated. Heavy metal contamination was assessed at each site due to elevated levels of metals being a threat to human health. The concentration values obtained for the analytes in *T. dubium* were found to be (in descending order) Ca > Mg > Fe > Zn > Mn > Se > Ni > Cr > Cu > Co > Pb > Cd > As. The accumulation and exclusion of metals in this study indicated that the plant is in control of uptake of metals from the soil and this depends on the required levels needed for proper functioning of the plant. Elemental uptake of essential elements (Ca, Mg, Zn, Mn and Se) showed that *T. dubium* is a rich source of nutrients and, most importantly, the plant does not have the tendency to accumulate toxic heavy metals. This study shows that *T. dubium is* safe for human consumption, whether it’s used as a medicinal herb or as food, but picking these species from the roadside should be avoided due to Pb accumulation.

4.5 Acknowledgments

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CHAPTER 5

CONCLUSIONS AND RECOMMENDATIONS FOR FURTHER RESEARCH

5.1 Summary

This study aimed to investigate the elemental distribution of 13 analytes (As, Ca, Cd, Co, Cr, Cu, Fe, Mg, Mn, Ni, Pb, Se and Zn) in five edible *Trifolium* species, namely *T. africanum* (African clover), *T. burchellianum* (Burchell's clover), *T. repens* (white clover), *T. dubium* (little hop clover) and *T. pratense* (red clover) found in KwaZulu-Natal, South Africa. The chemical composition of the five species was determined by analysing for the moisture, lipid, protein, dry ash and carbohydrate content. The nutritional value of the selected *Trifolium* species was evaluated by obtaining concentrations of essential nutrients and comparing to RDAs. Toxic heavy metals accumulated in edible herbs can cause serious health issues if consumed. Arsenic, Cd and Pb are among the toxic heavy metals that are present in the environment. Therefore, the concentrations of these metals were determined and compared to maximum permissible limits in order to perform a toxicological assessment of heavy metals in *Trifolium* species. This study can provide nutritional information which is lacking on these herbaceous plants growing in KwaZulu-Natal. The impact of soil quality on elemental uptake as a function of geographical location was determined with focus on *T. dubium*. The parameters utilised in determining soil quality were pH, organic matter content and cation exchange capacity. The elemental content of these plants can be used to determine if they are safe for human consumption and if they can contribute positively to the diet and, if so, they can be promoted as an alternate source of nutrition in the areas in which they grow.

5.2 Findings from the study

The edible *Trifolium* species proved to be a rich source of lipids, proteins and carbohydrates. These herbaceous plants can be a potential source of nutrients if consumed as a leafy vegetable. The elemental distribution of five edible *Trifolium* species indicated that the plants are a rich source of essential nutrients. In summary, the concentrations of elements in the five *Trifolium* species studied were found to be in decreasing order of Ca > Mg > Fe > Mn > Zn > Se > Cu > Cr > Pb > Ni > Co > Cd > As. All species studied had a high Se content with *T. dubium* being the only species exceeding the tolerable upper intake level and containing the highest concentrations of the toxic elements Cd and Pb. Therefore, *T. dubium* was further investigated by evaluating the concentration of heavy metals from ten different geographic locations in KwaZulu-Natal, South Africa. The heavy metal concentrations in *T. dubium* varied with location and were found to be (descending order) Ca > Mg > Fe > Zn > Mn > Se > Ni > Cr > Cu > Co > Pb > Cd > As. Elemental uptake of essential elements (Ca, Mg, Zn, Mn and Se) showed that *T. dubium* is a rich source of essential nutrients but has a tendency to accumulate Pb.

5.3 Conclusion

This study is at the interface of food science, nutrition and health which is of increasing concern to the general population, government and the food industry. There is a worldwide increase in awareness of the impact of the diet to diseases and health so people are looking for alternate sources of nutritious foods. Also, for those communities that cannot afford vegetables that are commercially available, alternate natural sources of these foods should be explored and exploited. Therefore, this study provides significant scientific knowledge on edible *Trifolium* species that grow in KwaZulu-Natal, South Africa to food science, nutrition and health. *Trifolium* species can be a rich alternate natural source of nutrients that can contribute positively to the dietary needs of people thereby improving their nutritional status. However, due to these species having the tendency to accumulate toxic metals, it is important to exercise caution when utilising these plants if picked from contaminated sites or along the roadside due to metal toxicities.

5.4 Recommendations for further study

- Perform a phytochemical analysis on edible the *Trifolium* species investigated by isolating and identifying secondary metabolites in the plant.
- Determine the biological activity of isolated phytocompounds in *Trifolium* species.
- Investigate the heavy metal concentrations of other *Trifolium* species found in South Africa.

APPENDIX

Supporting information

The CRM certificate

EUROPEAN COMMISSION
JOINT RESEARCH CENTRE
Institute for Reference Materials and Measurements

Institute for Reference Materials and Measurements

CERTIFIED REFERENCE MATERIAL
BCR® – 402

CERTIFICATE OF ANALYSIS

225

WHITE CLOVER

	Mass fraction based on dry mass		Number of accepted sets of data p
	Certified value 1) [mg/kg]	Uncertainty 2) [mg/kg]	
As	0.093	0.010	15
Co	0.178	0.008	7
Mo	6.93	0.19	13
Se	6.70	0.25	15

1) Unweighted mean value of the means of p accepted sets of data, each set being obtained in a different laboratory and/or with a different method of determination. The certified value is traceable to the SI.

2) Half-width of the 95 % confidence interval of the mean defined in 1).

This certificate is valid for one year after purchase.

Sales date: 3 0. Juni 2014

The minimum amount of sample to be used is 100 mg.

NOTE

This material has been certified by BCR (Community Bureau of Reference, the former reference materials programme of the European Commission). The certificate has been revised under the responsibility of IRMM.

Brussels, November 1991
Revised: May 2007

Signed:

Prof. Dr. Hendrik Emons
Unit for Reference Materials
EC-JRC-IRMM
Retieseweg 111
2440 Geel, Belgium

Additional Material Information

	Mass fraction based on dry mass
	Value [1] [mg/kg]
Cr	5.19
Fe	244
Ni	8.25
Zn	25.2

1) The value is traceable to the SI.

DESCRIPTION OF THE SAMPLE

The material consists of a white clover powder in a glass bottle. The bottle contains about 25 g of powder and a small PTFE ball which has been added to facilitate the homogenisation prior to use.

ANALYTICAL METHOD USED FOR CERTIFICATION

- Direct current plasma atomic emission spectrometry
- Energy dispersive X-ray fluorescence
- Electrothermal atomic absorption spectrometry
- Electrothermal atomic absorption spectrometry with Zeeman background correction
- Hydride generation atomic absorption spectrometry
- Hydride generation inductively couples plasma emission spectrometry
- Inductively coupled plasma emission spectrometry
- Inductively coupled plasma mass spectrometry
- Instrumental neutron activation analysis
- Neutron activation analysis with radiochemical separation
- Visible light or U.V. spectrometry

PARTICIPANTS

- Agriculture and Food Development Authority, Wexford (IE)
- European Commission, Joint Research Centre, Institute for Reference Materials and Measurements, Geel (BE)
- Labor für Spurenanalytik, Bonn (DE)
- Forschungszentrum für Umwelt und Gesundheit, Neuherberg (DE)
- An Forais Taluntais, Wexford (IE)
- Aristotelian University, Lab. Anal. Chemistry, Thessaloniki (GR)
- Centre de Recherches Forestières, Nancy (FR)
- Ecole Européénne des Hautes Etudes des Industries Chimiques, Strasbourg (FR)
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- Landesanstalt für Ökologie, Recklinghausen (DE)
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- Università di Pavia, Chimica Generale, Pavia (IT)
- Universität Ulm, Sektion Analytik und Höchtsreinigung, Ulm (DE)

ERA
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Reference Materials

▪ Certificate of Analysis ▪

Product:	Metals in Soil
Catalog Number:	540
Lot No.	D081-540
Certificate Issue Date:	November 11, 2013
Expiration Date:	October 31, 2016
Revision Number:	Original

CERTIFICATION

Parameter	Total Concentration	Certified Value[1]	Uncertainty[2]	QC Performance Acceptance Limits[3]	PT Performance Acceptance Limits[4]
	mg/kg	mg/kg	%	mg/kg	mg/kg
Aluminum	68100	9390	7.22	5340 - 13400	4080 - 14700
Antimony	290	129	19.5	D.L. - 273	28.9 - 322
Arsenic	101	88.4	5.92	71.5 - 105	61.0 - 116
Barium	630	210	9.01	176 - 245	154 - 266
Beryllium	62.5	55.8	6.26	45.8 - 65.9	40.8 - 70.9
Boron	123	101	8.32	73.8 - 128	60.7 - 142
Cadmium	165	143	5.60	116 - 169	104 - 182
Calcium	26200	7530	7.27	6210 - 8850	5620 - 9440
Chromium	320	86.8	6.06	69.3 - 104	60.0 - 114
Cobalt	232	199	4.10	166 - 233	148 - 250
Copper	307	268	4.72	219 - 317	204 - 332
Iron	42900	12800	18.0	5380 - 20100	4040 - 21500
Lead	101	97.9	11.3	80.0 - 116	69.3 - 126
Magnesium	8960	2850	5.51	2210 - 3490	1860 - 3840
Manganese	1020	425	9.69	347 - 502	324 - 525
Mercury	9.20	9.03	21.9	6.45 - 11.6	4.63 - 13.4
Molybdenum	133	103	8.24	78.9 - 128	72.3 - 144
Nickel	296	236	4.17	194 - 279	175 - 302
Potassium	19400	2570	7.55	1860 - 3290	1570 - 3570
Selenium	145	127	4.47	98.4 - 156	84.6 - 170
Silver	73.7	66.2	18.9	49.6 - 82.8	44.4 - 88.0
Sodium	17900	1040	13.1	740 - 1340	628 - 1450
Strontium	300	167	12.0	133 - 202	118 - 216
Thallium	161	140	10.0	110 - 170	95.6 - 185

ISO/IEC GUIDE 34:2009
ACCREDITED

ISO/IEC 17025:2005
ACCREDITED

Parameter	Total Concentration	Certified Value[1]	Uncertainty[2]	QC Performance Acceptance Limits[3]	PT Performance Acceptance Limits[4]
	mg/kg	mg/kg	%	mg/kg	mg/kg
Tin	173	149	7.74	113 - 184	89.0 - 209
Titanium	3240	215	16.1	65.2 - 366	0.00 - 464
Vanadium	252	157	11.7	120 - 193	112 - 202
Zinc	141	130	11.5	106 - 155	87.0 - 173

ANALYTICAL VERIFICATION

Parameter	Certified Value[1]	Proficiency Testing Study			NIST Traceability	
		Mean	Recovery[5]	n	SRM Number	Recovery
	mg/kg	mg/kg	%			%
Aluminum	9390	9390	100	159	-	-
Antimony	129	129	100	179	-	-
Arsenic	88.4	88.4	100	201	-	-
Barium	210	210	100	191	-	-
Beryllium	55.8	55.8	100	181	-	-
Boron	101	101	100	125	-	-
Cadmium	143	143	100	200	-	-
Calcium	7530	7530	100	139	-	-
Chromium	86.8	86.8	100	197	-	-
Cobalt	199	199	100	176	-	-
Copper	268	268	100	192	-	-
Iron	12800	12800	100	159	-	-
Lead	97.9	97.9	100	203	-	-
Magnesium	2850	2850	100	142	-	-
Manganese	425	425	100	180	-	-
Mercury	9.03	9.03	100	138	-	-
Molybdenum	103	103	100	178	-	-
Nickel	236	236	100	195	-	-
Potassium	2570	2570	100	142	-	-
Selenium	127	127	100	199	-	-
Silver	66.2	66.2	100	186	-	-

Parameter	Certified Value[1]	Proficiency Testing Study			NIST Traceability	
		Mean	Recovery[5]	n	SRM Number	Recovery
	mg/kg	mg/kg	%			%
Sodium	1040	1040	100	135	-	-
Strontium	167	167	100	122	-	-
Thallium	140	140	100	178	-	-
Tin	149	149	100	135	-	-
Titanium	215	215	100	129	-	-
Vanadium	157	157	100	173	-	-
Zinc	130	130	100	192	-	-

1. The Certified Values are equal to the mean recoveries for the parameters as determined in an interlaboratory round robin study based on all applicable digestion techniques reported in the study. The Certified Values are based on an ""as received"" basis, assuming 100% solids content. The certified values are monitored and purchasers will be notified of any significant changes resulting in recertification or withdrawal of this certified reference material during the period of validity of this certificate.

2. The stated **Uncertainty** is the total propagated uncertainty at the 95% confidence interval. The uncertainty is based on the preparation and internal analytical verification of the product by ERA, multiplied by a coverage factor. The uncertainty applies to the product as supplied and does not take into account any required or optional dilution and/or preparations the laboratory may perform while using this product.

3. The **QC Performance Acceptance Limits (QC PALs™)** are based on actual historical data collected in ERA's Proficiency Testing program. The QC PALs™ reflect any inherent biases in the methods used to establish the limits and closely approximate a 95% confidence interval of the performance that experienced laboratories should achieve using accepted environmental methods. Use the QC PALs™ to realistically evaluate your performance against your peers.

4. The **PT Performance Acceptance Limits (PT PALs™)** are calculated using the regression equations and fixed acceptance criteria specified in the NELAC proficiency testing requirements. Use the PT PALs™ when analyzing this QC standard alongside USEPA and NELAC compliant PT standards. Please note that many PT study acceptance limits are concentration dependent (some non-linearly) and, therefore, the acceptance limits of this QC standard and any PT standard may differ relative to their difference in concentrations.

5. The **PT Data/Traceability** data include the mean value, percent recovery and number of data points reported by the laboratories in our Proficiency Testing study compared to the Certified Values. In addition, where NIST Standard Reference Materials (SRMs) are available, each analyte has been analytically traced to the NIST SRM listed. This product is traceable to the lot numbers of its starting materials. All gravimetric and volumetric measurements related to its manufacture are traceable to NIST through an unbroken chain of comparisons.
Traceability Recovery (%) = [(% recovery certified standard)/(% recovery NIST SRM)]*100
The traceability data shown were compiled by analyzing the ERA standards or their associated stock solutions against the applicable NIST SRMs.

6. * - These parameters are native elements and are present at high concentrations in the unspiked soil. As methods 3050 and 3051 do not normally dissolve elements bound in silicate structures, the recoveries of these elements will be low relative to the recoveries of the elements that are primarily spiked onto the soil.

7. The Total Concentrations are equal to the background concentrations in the blank soil matrix (measured using neutron activation, XRF, and total acid digestion techniques), plus the amount of each analyte spiked onto the soil. For Trace Metals, the values listed are only ""Theoretical Values"" based upon the methodologies listed.

8. For additional information on this product such as intended use, instructions for use, level of homogeneity, and safety information, please refer to the provided Instruction Sheet

If you have any questions or need technical assistance, please call ERA technical assistance at 1-800-372-0122 or send an email to info@eraqc.com.

Certifying Officer

Tom Widera

Thomas Widera

Quality Officer

Kristina Sanchez