Use of Stable Isotopes to Assess Phytoremediation of Soils Contaminated with Cadmium and Zinc

by

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Declaration

This thesis is a record, composed by myself, of work carried out at The Robert Gordon University, Aberdeen and The Macaulay Land Use Research Institute, Aberdeen. It has not been accepted in any previous application for a higher award. All quotations have been distinguished by quotation marks, and all resources of information specifically acknowledged by reference to the authors.

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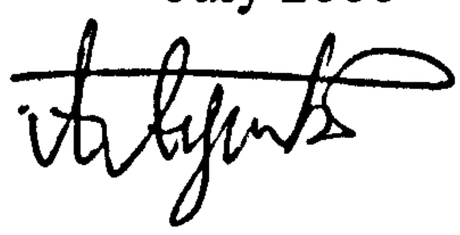

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Abstract

Land contaminated with heavy metals such as Cd and Zn can be remediated using a number of different approaches. Many of these strategies are very expensive (e.g. removal and disposal of the soil, covering the soil with uncontaminated soil, extraction with acids or chelates) whilst others provide only short term solutions (e.g. reduction of metal bioavailability by liming or adding organic matter). An alternative approach is the use of hyperaccumulator plants to remove the heavy metals. Phytoremediation, as this process is known, is an attractive method for remediation of contaminated land since it is relatively inexpensive and has the potential through the appropriate selection of plant species to be effective. However, there are many factors that need to be considered for phytoremediation, perhaps the most important is the bioavailability of metals from different component or functional pools within the soil. The bioavailability of heavy metals has been assessed through a variety of approaches, with extraction using chemicals being the most common. Another approach is the isotopic exchange method which has been regularly used for estimating the bioavailable P in soil. This technique relies on the assumption that isotopically exchangeable P is available to a growing plant and yields the so called E-value for a soil. In past studies, particularly in highly P fixing soils, the E-value has been shown to overestimate the soil available P. To overcome this a second procedure based on the measurement of isotope uptake by a plant growing on a spiked soil has been proposed and yields the so called L-value. As well as P, this method has been applied to Ni and only very recently to Cd and Zn using radioisotopes. In the present work, stable isotopes are used for the first time to measure the Cd and Zn available for plant uptake using isotopic exchange principles based on the E and L-values using a contaminated (Great Billings) and a non-contaminated (Countesswells) soil. These values were compared to Cd and Zn bioavailable pool determined using more traditional chemical extraction methods (5 extractants). Of the chemical extractants 0.1M $NaNO_3$, 0.01M $CaCl_2$, 0.5M NaOH, 0.43M CH_3COOH and 0.05M EDTA (pH=7.0), the highest amounts of Cd and Zn were extracted by CH_3COOH and EDTA. In addition, these two extractants extracted the Cd and Zn amounts close to the L-values determined using the isotopic exchange method.

An isotope dilution thermal ionisation mass spectrometric (ID-TIMS) method for an

accurate and precise determination of Cd and Zn in soils and plants via isotope ratio measurements was developed. This was then validated by quantifying Cd and Zn in soils, plant and animal Certified Reference Materials digested by three different methods. In addition, there was no significant difference between open tube *aqua regia*, closed microwave bombs and HF digestion procedures which was related to the accurate definitive method of ID. Also, an approach for the sequential isotopic analysis of Cd and Zn on the same filament within the TIMS was developed, allowing more rapid analysis and reducing running time on the instrument. Cd and Zn dual analysis on the same filament proved to be successful and produced results comparable to single analysis.

Isotopic ratios of Cd and Zn were used to determine both E and L-values in two soils. Although the E-value determined (in soil solution system) was higher than the L-value (using plants) for the 2 soils tested, the 2 values were generally within agreement. L-values were determined using 3 different plant species, the hyperaccumulator alpine penny cress (*T. caerulescens*), dandelion (*T. officinale*) and spring barley (*H. vulgare*). The available pool of Cd and Zn for plant uptake was determined to be the same for the 3 different plant species examined although plant species had markedly different abilities to uptake metals.

In addition to using the plant species to estimate the bioavailable metals, the potential of these plant species for phytoremediation of Cd and Zn was also evaluated. Among the plants tested, only *T.caerulescens* proved to be a Cd hyperaccumulator. However, *T. officinale* removed an equivalent amount of Cd and more of Zn than the other two plant species; although its Cd and Zn content was not sufficiently high to be considered as a hyperaccumulator. Based on this, dandelion has a good potential for phytoremediation, which lead to an attempt to study Cd and Zn uptake in the rhizosphere of dandelion roots. However, the experiment which used soil between glass plates did not show a clear depletion in Cd and Zn concentrations towards the root surface or if metal concentration was related to the amount of root present in any soil section.

Abbreviations

AA: atomic absorption.

CRM: certified reference material.

DM: dry matter.

EDTA: ethylenediaminetetraacetic acid.

FAAS: flame atomic absorption spectrometry.

GFAAS: graphite furnace atomic absorption spectrometry.

ICP-MS: inductively coupled plasma mass spectrometry.

ID: isotope dilution.

LOD: limit of detection.

LOQ: limit of quantification.

RSD: relative standard deviation (100×SD/mean).

SD: standard deviation.

SE%: standard error % (=100×SD/$\sqrt{n}$, n: number of samples).

TIMS: thermal ionisation mass spectrometry.

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1. INTRODUCTION

1.1 HEAVY METAL POLLUTION:

Heavy metals are natural constituents of the earth crust, the concentration of which depends on the geological history, but individual concentrations rarely exceed 1000 mg/kg. Selected concentrations of several elements in major rock types are shown in Table 1.1.1. Of the rocks on the earth's surface, 75% are sedimentary rocks. However, both sedimentary and igneous rock types form ore minerals containing high levels of one or more heavy metals and represent important commercial sources of these elements.

Table 1.1.1: Mean heavy metal content (mg/kg) of major rock types (Alloway, 1995a).

Element	Earth's crust	Igneous rocks			Sedimentary rocks		
		Ultramafic	Mafic	Granitic	Limestone	Sandstone	Shales
Ag	0.07	0.06	0.1	0.04	0.12	0.25	0.07
Cd	0.1	0.12	0.13	0.09	0.028	0.05	0.22
Cr	100	2890	200	4	11	35	90
Ni	80	2000	150	0.5	7	9	68
Pb	14	14	3	24	5.7	10	23
Zn	75	58	100	52	20	30	120

Natural processes such as bedrock weathering and volcanic eruptions circulate heavy metals in the atmosphere and the biosphere. In addition, humans have used metals for thousands of years dating back to our prehistoric ancestors who learned how to recover metals such as gold, silver and tin. The industrial revolution created a high demand for coal and metals at the turn of the nineteenth century, increasing heavy metal production and inevitably the introduction of these metals into the environment. Although heavy metals existed in soils and rocks, human activities greatly enhanced their transfer from tightly bound elements in the geosphere to active elements within the biosphere. In the twentieth century, demand for metals has steadily increased and as a consequence so has the pollution

associated with mining and purification activities in some parts of the world. The disposal of heavy metal contaminated waste also represents a serious problem.

While some heavy metals are required in many biochemical processes such as Zn, other metals like Cd are pollutants, potentially toxic and have no known function in any biological organism (Wagner, 1993). When natural or anthropogenic activities release heavy metals into the biosphere, they may enter the food chain through leaching into groundwater or by direct uptake by plants. Because plants obtain nutrients from soil, man can be exposed to heavy metals either through stock grazing on contaminated plants or directly through edible crops and vegetables. An incident in Japan in 1913 illustrated this, where farmers grew rice irrigated with wastewater from a Zn mine. This resulted in a disease known as "itai-itai " and was found to be caused by Cd poisoning (Cunningham *et al*, 1998a). In the Netherlands, Cd contamination from industrial emissions was found to cause increased body burdens and altered kidney function (Kreis *et al*, 1992). In these examples the incidents were localised close to industrial sites (such as a Zn smelter area), however pollutants are not restricted to local areas and can be distributed over a wide area by a variety of mechanisms including air and water. It was found that, for example, the Arctic environment and its inhabitants contains surprisingly high levels of heavy metals, and other pollutants, as these contaminants are swept northward by winds from industrial regions (Tenenbaum, 1998). In northern Europe, the highest concentrations were found in the south western part of Scandinavia, Cd and Pb in particular, which was assumed to have resulted from metal deposition transport from the densely populated areas of central Europe (Nordic Council of Ministers, 1990).

As a consequence of metal pollution and inappropriate waste disposal practices, many waste sites exist and many represent a serious health hazard. Around 25,000 contaminated sites in the United States have been identified, between 50,000 and 100,000 in Britain and 5,000 to 6,000 sites in the former western part of Germany (Alloway, 1996). This growing problem has lead to legislation towards providing an acceptable waste disposal policy. The lack of affordable effective environmental remediation has created a need for the development of novel approaches (Raskin, 1996). Among them is the innovative approach of phytoremediation (Section 1.3), which offers a relatively inexpensive solution with

minimum environmental disturbance. The major risks from heavy metal pollution results from the fact that:

- metals are persistent pollutants that cannot be biodegraded,
- they cannot be eliminated by incineration, and
- they are progressively accumulating in humans as they are the top of the food chain.

Excessive metal intake in humans results from crops and vegetables being grown on contaminated land. However, other direct sources involve the direct ingestion of contaminated soil (by gardeners, children and animals) or even through the inhalation of dust from contaminated sites (Alloway, 1995a).

Another risk of heavy metal pollution comes from alterations to the diversity of ecosystems and ecotoxicological implications to the environment. Changes in species diversity, vegetation cover and biomass can occur. For example, the soil microbial biomass can be affected; concentrations of Zn higher than 300 μg/g in soils treated with sewage sludge can cause a marked decrease in the activity of the nitrogen-fixing bacteria such as *Rhizobium leguminosum bv trifolii* (Alloway, 1995b). Although several trace metals are required for algal growth and photosynthesis, marked increases in concentration have been shown to affect metabolic functions (Rand and Petrocelli, 1985). On heavily polluted sites, few species may exist (exemplified by *Thlaspi cearulescens*, a hyperaccumulator that tolerates high levels of Zn and other metals, and was found to be the one species able to grow on Zn smelting site (Salt *et al*, 1998)). Also, as part of the ecosystem, earthworms have been found to contain higher levels of Cd, Zn and other heavy metals in sludge treated soils leading to further concentration in tissues of birds moving metals up the food chain (Brady, 1990). However, polluted land may in some cases be unable to support any plant life, which may in turn lead to desertification, restriction of land use and soil erosion.

The work reported in this thesis focuses particularly on Cd and Zn, these metals were named as "Priority Pollutants" by the US Environmental Protection Agency (USEPA) (Lue-

Hing *et al*, 1992). In the UK, the Ministry of Agriculture, Fisheries and Food classified Cd as one of the priority inorganic pollutants (Ministry of Agriculture, Fisheries and Food, 1995). Zn is relatively non-toxic when compared to Cd, however high concentrations can cause ill health effects.

1.1.1 General sources of Zn and Cd pollution:

Pollution generated by industrial activities has accelerated as a result of the expanding uses of Cd and Zn in all areas of industry. Wherever Zn ores are mined or smelted, there is a possibility of Cd pollution (Alloway, 1995b); as both come from the same geological source and, in general, have similar chemistry. The primary sources of Cd and Zn pollution are:

1. The burning of fossil fuels.
2. Metalliferous mining and smelting industries.
3. Manufacturing industries such as: electronics industries, glass and ceramics making, textile plants, pigments and paints industry, tanneries, plating and stainless steel manufacture.
4. Production of fertilisers and pesticides.
5. Waste disposal: Municipal wastes, radioactive waste, sewage sludge and waste processing.

1.1.2 Properties of Zn and Cd:

Zn was known in India and China before 1500 AC; its name came from the German "zink" while Cd comes from the Latin word cadmia, which means calomine and was discovered in 1817 by Stomeger in Germany. Both Zn and Cd belong to group IIB of the periodic table. While Zn is an essential element for human beings, animals and higher

plants, Cd has no biological function and is highly toxic to plants and animals (Wagner, 1993). Table 1.1.2 summarises the important properties and uses of Zn and Cd.

Table 1.1.2: Properties and uses of Zn and Cd.

PROPERTY	Zn	Cd
CHEMICAL		
Atomic number	30	48
Relative atomic mass, g/mol	65.5	112.41
Melting point, °K [a]	692.73	594.1
Boiling point, °K [a]	1180	1038
No. of stable isotopes	5	8
1st Ionisation energy, kJ/mol [c]	906.4	867.7
PHYSICAL		
Colour	Bluish white -silvery	Bluish-white
Characteristics	Brittle	Soft
BIOLOGICAL		
Essentiality [b]	Essential for humans, animals, higher plants	Not essential, toxic
Level in humans (muscles), µg/g [b]	240	0.14-3.2
Daily dietary intake, mg [b]	5-40	-
Toxic intake, mg [b]	150-600	3-330
Lethal intake, g [b]	6	1.5-9
GEOLOGICAL		
Abundance in the earth's crust, µg/g [b]	75	0.11
Major source [c]	ZnS (Sphalerite)	ZnS (Sphalerite)
World production, tonnes/year [b]	4.9×10^6	1.4×10^4
USES [c, d]	Batteries, galvanising, brass alloys, paints, pharmaceuticals.	Plating, alloys, pigments, Ni-Cd batteries, control rods in nuclear reactors.

Compiled from: [a] Emsley, 1991; [b] Anglene and Bini, 1992; [c] Alloway, 1995b; [d] World Resources Institute, 1992.

1.1.3 Origin of Zn and Cd in soils:

Parent rock materials such as ZnS minerals (sphalerite) and $ZnCO_3$ (smithsonites) represent the major source of both Zn and Cd in soils. The average Zn: Cd ratio for most rocks is around 500:1, but ranges from 27:1 to 7000:1 (Alloway, 1995b). Natural processes such as weathering, wind/water erosion and volcanic activity release heavy metals into the environment. However, their contribution is negligible to the quantity introduced by the anthropogenic activities. For example, Cd release from anthropogenic sources is about 10-fold greater than that predicted from natural sources on a global scale (Wagner, 1993). Nriagu and Pacyna (1988) reviewed the worldwide trace metals release into air, soil and water from different industrial sources. They concluded that human activities now have a major impact on the global and regional cycles of trace metals. The relative contribution of Cd from different anthropogenic sources in western countries was estimated to be: atmospheric deposition 39-41%, phosphatic fertilisers 54-58% and sewage sludge 2-5% (Alloway, 1995b).

A. Atmospheric fall-out:

This arises as a result of, for example, emissions from metal recovery plants and from vehicles. Zn concentrations in the atmosphere measured in different locations showed a wide variation in concentration. For example, Zn concentrations ranged from (ng/m^3): 0.002-0.05 in the South Pole, 15 in Shetland Islands and 14-6800 in Japan (Kabata-Pendias, and Pendias, 1992). These figures indicate the relationship between the element fall-out within an area and the industrial activities in the immediate locality. Compared to Zn, the problem of atmospheric fall out is even worse with Cd which is more volatile. The estimated worldwide atmospheric deposition of Cd onto soils is 5700 tonnes/year over land and 2400 tonnes/year over the ocean (Nriagu and Pacyna, 1988). Winds also play an important role in transferring pollutants.

B. Agrochemicals:

Agrochemicals are the chemicals used in agriculture to fight diseases, suppress weed growth and increase productivity. The contribution from fertilisers and pesticides depends on the origin of the phosphates (rock phosphates) used for the manufacture of fertilisers and pesticides. Zn concentration in phosphate fertilisers ranges from 50-1450mg/kg, in limestone from 10-450mg/kg while in manure values range from 15-250mg/kg. Some pesticides contain up to 25% Zn (Kiekens, 1995) which can have significant effects on Zn concentration in soils. For Cd, fertilisers have been estimated to contribute 4.3g/ha/y in the UK (Hutton and Symon 1986) and a total annual world-wide input of 30-250 tonnes/year (Nriagu and Pacyna, 1988). This is highly significant given the high toxicity of Cd at relatively low concentrations (Table 1.1.2).

C. Sewage sludge:

Sewage sludge is produced from liquid and water carried waste following a range of sewage treatment processes applied to both domestic and industrial sewage. The process ranges from simple treatments like the separation of solid particles to complicated procedures involving longer chemical, physical and biological treatments. The main objectives of sludge treatment are to make it easier and cheaper to dispose of with minimal effect on the environment. Conventional sewage treatment, for example, removes 40-75% Zn from the influent (Kiekens, 1995). Household sewage is not a major source of heavy metal contamination when compared to the industrial effluents; for example, Cd and Zn concentrations in sewage sludge from a small village has been estimated to contain 7 and 560mg/kg, respectively, compared to 444 and 6890mg/kg, respectively, from larger industrial cities (Brady, 1990).

In northern Europe, sewage sludge has often been disposed of to the sea. This was prohibited by the new European Community legislation on 1998 (Ministry of Agriculture, Fisheries and Food, 1991). The UK produces a total of 40 million tonnes of sludge every year (Lester, 1996a), and disposed over 50% of it into the sea (Towers, 1994). Alternative

methods for sewage sludge disposal considered include landfill and incineration. Sludge can be applied to land in liquid form either by spraying or injecting into the soil to give uniform distribution and to eliminate odour problems. Dry sludge can also be applied to land, where it is ploughed in to speed up incorporation of sludge into land and reduces odour. Most of the sludge used in agriculture receives some form of pre-treatment, however, it may contain appreciable amounts of heavy metals such as Zn and Cd. Sewage sludge that has been applied to agricultural land has been shown to be of benefit to plants and soils by increasing crop production (Hernandez *et al*, 1991). It slows down the decline in organic matter content and introduces appreciable amounts of nitrogen and phosphorus into the soil. However, the benefit may be reduced if the nutrients are present in forms that are unavailable to the plants or if the heavy metals accumulated in the plant tissues (Omran and Waly, 1988, Sanders *et al*, 1986). In fact, Cd content is the principal factor limiting use of sludge on agricultural land (Wagner, 1993). A limit to the amount of metal in agricultural soil has been derived from consideration of the normal soil metal content and the level at which negative health effects are likely to occur. Table 1.1.3 shows the limits placed on Cd and Zn addition to arable soils from sewage sludge. The cumulative limit is based on a period of 30 years. The guidelines also specify that the maximum quantity, which can be applied in any one year, is six times the annual average.

Table 1.1.3: Limits on metal additions to arable soils through sewage sludge disposal (Lester, 1996b).

Metal	Cumulative limit (kg/ha)	Annual limit (kg/ha)	Soil concentration limit (mg/kg)
Cd	5	0.17	3.5
Zn	560	18.6	280

Table 1.1.3 illustrates the need to monitor the amount of sewage sludge added and the concentration of heavy metals it contains. Sewage Cd and Zn content varies depending on where the sewage sludge originated; for example Cd and Zn concentrations were reported up to 444mg/kg (Brady, 1990) and 49000mg/kg (Berrow, and Webber, 1972) for sewage

from industrial wastes. The impact of contamination of agricultural soil by potentially toxic levels of Zn and Cd depends not only on the type and amount of sludge applied, but also on soil properties. These properties include the pH (Narwal *et al*, 1983), cation exchange capacity, chemical transformation of metals and availability to plants (White and Chaney, 1980). These factors will be considered in more detail later in this chapter.

Once the heavy metals associated with sewage sludge are incorporated into the soil, extractability appears to decrease with time indicating a possible change in the chemical form in the soil (Chang *et al*, 1984). This may be related to the fact that significant amounts of heavy metals are associated with humic and fulvic acid fractions of soil organic matter (Holtzclaw *et al*, 1978). As a result, the uptake of Zn and Cd by plants from sewage treated soils are consistently lower than from soil treated with comparable amounts of metal elements in the form of inorganic salts which are more readily available to plants (Chang *et al*, 1984, Dowdy and Larson, 1975). This suggests that metals exist in more unavailable forms in sewage sludge (mainly as organic complexes). This is however subject to the origin of the sewage sludge.

1.2 REMEDIATION OF CONTAMINATED SOIL:

Different factors must be considered when building strategies to remediate contaminated soils. These include: soil properties (the pH being critical), type of contaminant (organic/inorganic), contaminant levels, its physical state and the way the site was polluted. Ultimately, the cost of any remediation strategy is also of paramount importance.

1.2.1 Methods:

A. Excavation:

This *ex-situ* method involves the physical removal of the contaminated soil into a landfill or a permitted hazardous waste site. This method transfers the contaminants from one place to another and is usually laborious and very expensive.

B. Incineration:

Contaminated soil is transferred to a treatment plant, where the excavated soil is treated in two stages. The first is aimed at evaporating the contaminants from the soil particles at temperatures between 200°C and 700°C, organic pollutants are then completely oxidised at temperatures between 900°C and 1100°C. The clean soil is then cooled with water. This method may be appropriate for organically contaminated soils, but is not effective for soils contaminated with heavy metals.

C. Extraction:

i. Ex-situ treatment:

Contaminated soil is transferred to the treatment plant, a liquid extracting agent, typically an acid, is then mixed into the soil. The extracting agent and the soil particles are then separated. Chemicals such as detergents and complexing agents may be added to the extracting agent to enhance its extracting ability. This method is laborious and needs large quantities of extracting solvent. In addition the nutrient content of the extracted soil may be substantially diminished depending on the strength of the extracting agent used.

ii. In-situ treatment:

Before this treatment is started, the site is physically isolated so that pollutants cannot leach into the ground water. The treatment process consists of infiltration of a suitable aqueous extraction media (such as acids with/without chelates) into the contaminated soil, which dissolves the pollutant as it percolates through the soil. The percolate, is then pumped into a water treatment system to remove pollutants. The procedure is repeated until the required clean up is achieved. The method is suitable for high permeability sandy soil which allows water or aqueous reagents to sweep through (Rulkens *et al*, 1995), and can take up to a year to completely remove contaminants from a soil.

Another extraction approach, "soil vapour extraction", makes use of the volatility of a contaminant. The gas phase between soil particles in a contaminated site is in phase equilibrium with the pollutants adsorbed into the soil particles. The injection of air into the soil creates an airflow pattern through the contaminated soil, the air is then pumped out to a suitable filtering system. The method is only applicable for organic volatile compounds and possibly volatile compounds of mercury and arsenic (Rulkens *et al*, 1995).

D. Electroreclamation:

This method involves the set-up of vertically or horizontally placed electrodes in the soil, each provided with a housing system (a well) containing a solution to enhance the deposition of the contaminants onto the electrodes through facilitating their movement. The method is basically a form of electroplating. The electrodes are electrically connected, where a suitable electric current is passed through to form an electrochemical cell. Moisture, groundwater and the housing solutions help transport ions and electrically charged small particles towards the electrodes, which are then removed and their metal content is removed by washing. Treatment times vary from weeks to several months and are suitable for removing a range of heavy metals (Rulkens *et al*, 1995). The same principle is applied in an approach named as "electrowinning" where an electric current is passed through electrodes immersed in the waste stream of a plant. Metals deposited can then be recovered from the electrodes (Krishnan *et al*, 1993).

E. Fixation:

Immobilisation of pollutants can be achieved by chemical processing of soils. The pollutant is held within the soil surface profile and is not allowed to leach deeper into the ground (Salt *et al*, 1995). Soil amendments such as phosphate, lime and organic matter are sometimes used to immobilise toxic metals such as Cd and Zn (Schnoor, 1997). Like most heavy metals, Cd and Zn can be immobilised if the soil pH is kept near neutral or above, as they are strongly adsorbed to the soil particles at these pH levels (Brady, 1990). This approach can be used sometimes as a transition measure to stop pollutants from moving until a more permanent treatment method can be adopted.

F. Bioremediation:

Bioremediation is the use of living organisms to treat contaminants. It is favoured by both the public and private sectors as an alternative method for waste treatment due to low

cost and minimal environmental impact. Biodegradation of contaminants and phytoremediation are two basic aspects of bioremediation. In the early stages of this approach, the focus was primarily on the use of microorganisms for the conversion of organic contaminants into simple non-polluting breakdown products such as water and carbon dioxide. Although land contaminated with organic pollutants such as pesticides, are additionally polluted with metals, only recently, has attention turned to the treatment of these contaminants using microorganisms (Roane *et al*, 1998). This may be related to the fact that microorganisms can destroy organic contaminant by oxidising them to carbon dioxide, whereas heavy metals cannot be chemically degraded. Microbial bioremediation of heavy metals is mainly concerned with immobilisation by precipitation / reduction (Summers, 1992), or by converting heavy metals to volatile forms (Lovley and Coates, 1997). Although microbial activity can be harmed by high concentrations of heavy metals impacting growth, morphology and biochemical activities as a result of specific interactions with cellular components (Roane *et al*, 1998), this may be overcome by genetic manipulation technique. However, the release of genetically altered microbial organisms into the environment is a subject currently of intense public concern. Another aspect of bioremediation which has attracted a great deal of attention has been the use of selected plant species to absorb metals from soil. This is know as phytoremediation and is discussed further in the next section.

1.3 PHYTOREMEDIATION:

Phytoremediation is a combination of the Greek word phyton, "plant", and the Latin word remediare, "to remedy" (Cunningham *et al*, 1998c). Raskin *et al* (1994) defines phytoremediation, as the use of plants for environmental remediation and involves removing organic compounds and metals from soils and water. This technology is based on plants that have the ability to tolerate high levels of heavy metals (Salt *et al*, 1995). The aim of current phytoremediation efforts is to develop innovative, economical and environmentally compatible approaches to remove heavy metals and radionuclides from the environment (Ensley, 2000).

Phytoremediation involves a number of biological mechanisms including direct uptake, release of exudates into the rhizosphere (to enhance bacterial and fungal processes), and metabolitic processes within the root and shoot cells. Selected plants are grown on the contaminated site, where they draw up pollutants and concentrate them within various tissues. The plants are then harvested and may be further treated by burning them in a controlled system. The residue of the plants would then be very rich in metals and could be recycled or placed in landfills. Designing a phytoremediation system varies according to a wide range of factors including the contaminant conditions at the site, the level of clean up required and the plant species to be used. All these factors need to be considered for effective phytoremediation.

1.3.1 Hyperaccumulators:

In the early 1970s, T. Jaffre discovered a tree in New Caledonia (South Pacific), *Sebertia acuminata*, that exudes a sap containing 26% nickel on dry weight basis. This discovery lead to the growing of specific plants with a high metal content, a technique known as "phytomining" (Robinson, 1999). Although all plants uptake essential metals from soils and waters (such as Zn, Mg, etc.), only certain plants can accumulate heavy metals, which have no known biological functions, and may be toxic (Salt *et al*, 1995).

When plants assimilate phytotoxic amounts of metals, growth becomes inhibited and biomass production decreases (Marr *et al*, 1999). Some species can tolerate high levels of heavy metals and accumulate them to unusually high concentrations, these are known as hyperaccumulators. This extraordinary behaviour allows certain plants to survive on smelt or mining lands that contain very high levels of particular metal(s) (Brown *et al*, 1994). This adaptation has resulted in populations of certain plant species with high resistance to the effects of toxic metals (Bezel *et al*, 1998). Although this behaviour is not fully understood, metallothioneins and phytochelatins are probably involved in the uptake process in the plant (Salt *et al*, 1995, Tomsett and Thurman, 1988 and Jackson *et al*, 1990). These compounds are the subject of a great deal of research so that their role and function in resistance to metal toxicity and hyperaccumulation can be understood (Chaney *et al*, 1997). For a plant to resist the toxic effects of heavy metals it must either limit cellular uptake, or develop a heavy metal resistant metabolism (Salt *et al*, 1995). While the first aspect is very limited, metabolic processes to detoxify heavy metals once they enter the cells involve: chelation, compartmentalisation or precipitation (Salt *et al*, 1995). Trace elements can be stored as inactive chemical compounds in cell walls of plant tissues without actually causing physical changes to the plant (Marr *et al*, 1999). The root system plays an important role in the mechanism in which plants can accumulate and resist heavy metals. As heavy metals penetrate thorough the root system, many are fixed by the formation of poorly soluble organic compounds, which leads to a local increase in the heavy metals concentration but inhibits transport to other parts of the plant (Bezel *et al*, 1998).

Recent studies focusing on the biological mechanisms of transport within the hyperaccumulating plants (particularly *T. caerulescens*) have shown that histidine plays the major role in transportation of Zn within root cells, while organic acids complex with Zn during long-distance xylem transport and storage in shoots (Brown *et al*, 1995; Salt *et al*, 1999). The same was found for *B.juncea*, another hyperaccumulating species (Salt *et al*, 1995).

The term hyperaccumulator has been defined in different ways by different authors. A Zn and Cd hyperaccumulator will contain more than 10000mg/kg (1.0%) Zn and

>100mg/kg (0.01%) Cd in the dry matter of above ground tissues (Raskin *et al*, 1994, Baker and Brooks, 1989). The definition of hyperaccumulators varies according to the particular metal, as this could be affected by the biochemical functions of the metal in the plant. Zn for example, is essential to plants and therefore, for a plant to be a hyperaccumulator, it has to have >1% dry weight compared to Cd, a non-essential metal, where >0.01% dry weight indicates hyperaccumulator activity. Also, the relative abundance of metal in the soil plays a role in defining hyperaccumulators, for example, Zn is much more abundant than Cd in soil. For a hyperaccumulator to have a good potential for phytoremediation, it must have a rapid uptake rate for elements in soil solution and be capable of translocating metals from roots to shoots or to a harvestable root component (i.e. root crop).

Ongoing research in the phytoremediation field revolves around determining which plant works most efficiently at remediating a given pollutant. Plant species differ in the way they metabolise, volatise and/or accumulate pollutants. Baker and Brooks (1989) have listed about 400 hyperaccumulating plant species of different metals. More recently, Reeves and Baker (2000) reviewed hyperaccumulators in recent studies. Selected hyperaccumulator plants of Cd and Zn are shown in Table 1.3.1.

The capability of a plant for phytoremediation is normally expressed as amount of metal per unit mass of dried plant material. Another way is to use the mean accumulation factor defined as: metal concentration in the shoots / initial metal concentration in soil (Alloway *et al*, 1988; Knight *et al*, 1997). This factor describes the plant phytoremediation potential in a more accurate way, as plants grown on highly contaminated soil will have higher metal content compared to plants grown on slightly contaminated or uncontaminated soil. Expressing the plant ability to hyperaccumulate metals in this way makes it easier and more accurate when comparing different plants grown on different soils as the comparison is based on the same criteria.

Table 1.3.1: Cd and Zn content of selected hyperaccumulator plants (mg/kg).

Plant species	Cd	Zn	Reference
Chicory			
(Cichorium intybus L)	10-300 (Shoots)	nd[a]	Simon *et al*, 1996
	10-890 (Roots)	nd	Simon *et al*, 1996
Dandelion		nd	
(Taraxacum officinale)	20-410 (Shoots)		Simon *et al*, 1996
	20-1360 (Roots)	nd	Simon *et al*, 1996
Alpine penny cress			
(Thlaspi caerulescens)	1020	up to 18,455	Brown *et al*, 1994
(*Thlaspi calaminare*)	nd	39,600	Baker and Brooks, 1989
Indian mustard			
(Brassica juncea)	200-1400	nd	Zaurov *et al*, 1999

[a] nd = not determined.

1.3.2 Phytoremediation mechanisms:

As well as extraction processes plants can be used in a variety of ways to deal with heavy metal pollution. The following section discusses the range of phytoremediation mechanisms.

A. Phytoextraction:

Phytoextraction is the uptake and storage of pollutants in the plant stems, leaves or a harvestable root component. Plants are grown on contaminated sites where they uptake heavy metals. The plants parts are then collected and disposed of appropriately. Phytoextraction may produce an economically viable technique for metal recycling particularly when dealing with high value metals such as thallium, gold and nickel (Brooks *et al*, 1999), a method known as phytomining. Growing hyperaccumulating plans followed

by incineration and disposal of the ash can still be a cheaper option than other traditional remediation methods (Black, 1995). However, such an approach is not without problems, currently even the best hyperaccumulators (*Thlaspi caerulescens* capable of attaining 1022mg/kg Cd and 18455mg/kg Zn (Brown *et al*, 1996)) would take 13 to 14 years of continuous cultivation to clean sewage sludge contaminated sites to normal metal levels (Salt *et al*, 1995). In addition, despite the ability of *T. caerulescens* to accumulate high levels of metals, in the present experiments it proved difficult to grow and produces a low biomass yield.

Recently, metal chelators have been added to the heavy metal contaminated soil prior to planting. The role of chelators (such as EDTA) is to increase the metal concentration in the soil solution and aid the translocation of metals from roots to the leaves. This chelate-assisted phytoextraction increases metal uptake. Results with *Brassica juncea* (Indian mustard) showed that seedlings grown for 4 weeks in soil had a Cd content of 875µg/g dry weight after the addition of chelating agent compared to 164µg/g in the absence of a chelator (Salt *et al*, 1995). This approach has been implemented commercially (Salt *et al*, 1998). Some plant species (*Poaceae*) secrete phytosiderophores which are natural chelating agents and are able to solubilise and accumulate Fe in root cells. The potential exists therefore to develop plants which secrete metal-selective ligands into the rhizosphere and specifically solubilise elements of interest for phytoremediation (Chaney *et al*, 1997).

B. Phytostabilisation:

Phytostabilisation evolved from the fact that contaminated sites often lack established vegetation cover. This may cause soil erosion, spreading the pollutants even further into the environment. Phytostabilisation uses plants to limit the mobility and bioavailability of metals and prevents them from leaching into the ground water. This is not a comprehensive treatment of a contaminated site, but can be helpful in holding pollutants until a more suitable remediation process can be implemented. Phytostabilisation reduces the bioavailability of metals by converting the metal from a soluble oxidation state to an insoluble one (Salt *et al*, 1995). Three week old *B.juncea* were able to reduce the Pb level in

the leachate from 740μg/ml in the absence of a plant to 22μg/ml in the presence of a plant (Salt *et al*, 1995). This mechanism is similar in principle to chemical fixation where soil amendments are added to immobilise toxic metals (as discussed in Section 1.2.1E), but has the advantage of cost and not introducing chemicals to the soil.

C. Rhizofiltration:

Rhizofiltration is the use of plant roots to absorb, concentrate and precipitate metal contaminants from surface or groundwater. The ideal plant for rhizofiltration should have a large and rapidly growing root system that can absorb or adsorb the contaminants strongly (Schnoor, 1997). The process can involve either the absorption of a metal by complexation with root binding materials or adsorption on the root surface. In rhizofiltration, the ability of certain plant cellular components within the roots, including proteins, to bind metals is very important (Raskin, 1996). Sunflower roots have been shown to dramatically reduce the levels of Cr (1300 to 0), Cd (250 to 200) and Ni (1200 to 250) μg/l in aqueous solution within 24h (Salt *et al*, 1995). Provided plants that can produce significant amount of roots or surface area can be found, rhizofiltration can provide an efficient, practical and inexpensive solution for large water volumes as a form of water treatment technology (Dushenkov and Kapulnik, 2000).

D. Phytovolatilisation:

Phytovolatilisation is the uptake and vaporisation of pollutants by a plant. This mechanism takes a solid or liquid contaminant and transforms it to an airborne vapour. Phytovolatilisation is suitable for contaminants that can form volatile compounds with the plant constituents; and is a limitation to the application of this technique.

The vapour can either be the pure pollutant, as in the case of Hg^0 (Boyajian and Carriera, 1997), or metabolised by the plant before it is vaporised such as Se as dimethylselenide (Zayed and Terry, 1994; Wantanabe, 1997). However, the environmental

implications of metal volatilisation have to be considered before this approach becomes accepted by the regulators and the public (Salt *et al*, 1998).

1.3.3 Advantages and disadvantages of phytoremediation:

The potential benefits of phytoremediation are numerous, however the technique is not without problems. Although the technique is still relatively new, several field trials have confirmed the applicability and feasibility of using plants for environmental clean up. Salt *et al* (1998) listed examples of field trials on metals including Cd, Zn, Pb, Ni, Se, Cr and U. The advantages and disadvantages of phytoremediation are discussed next.

<u>Advantages:</u>

1. Relatively inexpensive:

   Phytoremediation is potentially cheaper than conventional treatment approaches such as incineration and soil washing. It has been estimated that using phytoextraction to clean up one acre of sandy loam soil to a depth of 50 cm will cost $60,000-100,000 compared to $400,000 for excavation and storage using traditional soil removal methods (Salt *et al*, 1995). In another study, phytoremediation can cost $10-100 per cubic yard whereas metal washing can cost $30-300 per cubic yard (Wantanabe, 1997). In fact, phytoremediation could result in the production of a 'crop' of a metal rich plant. This is known as "Phytomining" and the residue could be recycled and used (Brooks *et al*, 1998 and Baker *et al*,1988).

2. Volume of remaining ash:

   Compared to removing the soil into a landfill, for example, removing heavy metal contaminated soil from two and a half acres to a depth of about 18 inches creates about 5,000 tons of soil that must be disposed of in a hazardous landfill. In contrast, plants that take up the metal and are burned leave a residue of between 25 and 30 tons of ash to be disposed of with a huge reduction of mass and volume (Black, 1999).

3. Has been shown to work with a broad range of metals and organic pollutants.

4. Elimination of secondary air or water-born wastes.
5. Planting vegetation on a site reduces erosion by wind and water and leaves usable topsoil intact.
6. Public acceptance and minimal environmental disturbance.

Disadvantages:

1. Time-consuming: may take several growing seasons to clean up a site.
2. Hyperaccumulators normally have low biomass limiting their effectiveness in uptaking metals. Studies in biotechnology may help overcome this disadvantage as discussed next.
3. Plants suitable for phytoremediation often have relatively short roots, which limits the phytoremediation potential to the surface.
4. Phytovolatilisation of compounds can transform a soil or groundwater pollution problem to an air pollution problem.
5. Risk of transfer into the food chain:

   Vegetation use to remediate a site may be consumed by wildlife that feed on these plants, therefore, humans, animals and birds should be prevented from consuming phytoremediating plants.

1.3.4 Biotechnology to improve phytoremediation:

Traditional plant breeding can only use the available genetic diversity within a species. By identifying the gene(s) responsible for metal tolerance and accumulation it may be possible to transfer these genes and "engineer" extremely efficiency hyperaccumulating plant species (Raskin, 1996). This can be achieved through the introduction of genes responsible for metal tolerance and accumulation from hyperaccumulators into high biomass plant species such as trees. Some success have already been achieved in this area, for example genes encoding the Cd-binding protein, metallothionein, have been expressed in plants as a means of increasing Cd resistance (Salt *et al*, 1995). Increasing the

concentrations of the metal binding protein or peptides in plant cells would increase metal binding capacity and accumulation (Chaney *et al*, 1997). Genetically engineered plants of *Arabidopsis thaliana* L., *Nicotiana tabacum* L. (tobacco) and *Liriodendron tulipifera* L. (yellow polpar) have been shown to reduce mercurial compounds from spiked growth media (Rugh et al, 2000). Such progress would increase the commercial ability of phytoremediation technology. Phytostabilization is another area where genetically modified plants with enhanced metal binding capacity could be used to prevent heavy metals from entering the food chain (Chaney *et al*, 1997). Biotechnology approaches to the subject of phytoremediation have been discussed at length elsewhere (Ortiz *et al*, 1995; Rauser, 1995; Salt *et al*, 1995; Cunningham and Ow, 1996; Raskin 1996; Chaney *et al*, 1997 and Raskin and Ensley, 2000).

1.4 BIOAVAILABILITY:

Bioavailability can be defined as the quantity of a soil nutrient that is accessible to the plant roots over some useful period such as the growing season (Welch *et al*, 1991). Another definition leading to the same conclusion defines availability as that part of, for example, water or mineral nutrient in the soil or fertiliser, which can be drawn upon by a plant (Walker, 1995).

1.4.1 Zn and Cd uptake by plants:

Soils vary in both content and ability to supply metals and nutrients (N, P, K, Mn, Fe, and Zn) to the plants according to soil characteristics and plant root ability to mobilise them. The total metal content in a soil is a contribution of both the parent rock material and anthropogenic sources. As a result of these parameters, metals exist in soil in different forms whether associated with the soil adsorption sites or as organic/inorganic complexes. Some, all, or none of these pools may be available to the plant (Kennedy *et al*, 1997).

Zn is an essential trace element for higher plants and is predominantly absorbed as a free divalent cation Zn^{+2} in solution and forms either a functional, structural or regulatory cofactor for a large number of enzymes (Tagwira *et al*, 1992). Crops, such as maize, flax, hops, cotton, legumes, grapes, citrus and fruit trees are sensitive to Zn levels. Zn deficiency symptoms are inter-veinal chlorosis, stunted growth, malformation of stems and leaves and violet red points on the leaves. Although Cd is not essential to plants, it is readily taken up and unlike some heavy metals does not show phytotoxic symptoms at low concentrations (Chaney and Giordano, 1977). This can be particularly important in food crops since significant Cd levels may be present, yet no visible symptoms exist. The Zn and Cd content of plants varies considerably as a function of different soil and climatic factors and also of plant genotypes (Kiekens, 1995). Factors that govern plant uptake of Cd and Zn are given in Table 1.4.1 and soil factors are discussed in the following section.

Table 1.4.1: Factors that govern Cd and Zn plant uptake (adapted from McLaughlin *et al*, 1999).

Soil and crop factors	
SOIL	
pH	
Metal partitioning (K_d) (distribution of metal between soil particles and soil solution)	
Total metal concentration	
Metal sorption capacity of soil for:	a. organic matter
	b. cation exchange capacity
	c. clay, Fe, Mn oxides
Complexation reactions	
Nutrients: Zn, Cu, Mn, Ca, N, P, K	
Temperature, redox potential, moisture content, soil texture, aeration	
Application of fertilisers containing Cd and Zn	
CROP	
Species and cultivar	
Plant tissue (root, leaf, grain, etc.) and age	

The mechanism with which plants absorb metals through roots can be by both passive and active (metabolic) processes (Alloway, 1995b). Passive (non-metabolic) involves the diffusion of soil solution ions into the root endodermis and transportation to different plant parts. Metabolic processes include metal chelation with phytosiderophores which can be secreted into the rhizosphere by roots, or with proteins, such as metalloenzymes and various metal storage and carrier proteins (Raskin, 1996). Also plant roots can solubilise heavy metals by acidifying the soil environment with protons extruded from the roots (Salt *et al*, 1995; Krishnamurti *et al*, 1997). Metals have different mechanisms, which could be either passive such as Pb or metabolic such as Zn or a combination of both (Kabata Pendias and Pendias, 1992). Once metals are in the roots they are either stored or exported to the shoots through the xylem which has a high cation exchange capacity and hence tend to retard the transportation of metals (Salt *et al*, 1995).

1.4.2 Factors affecting metal bioavailability:

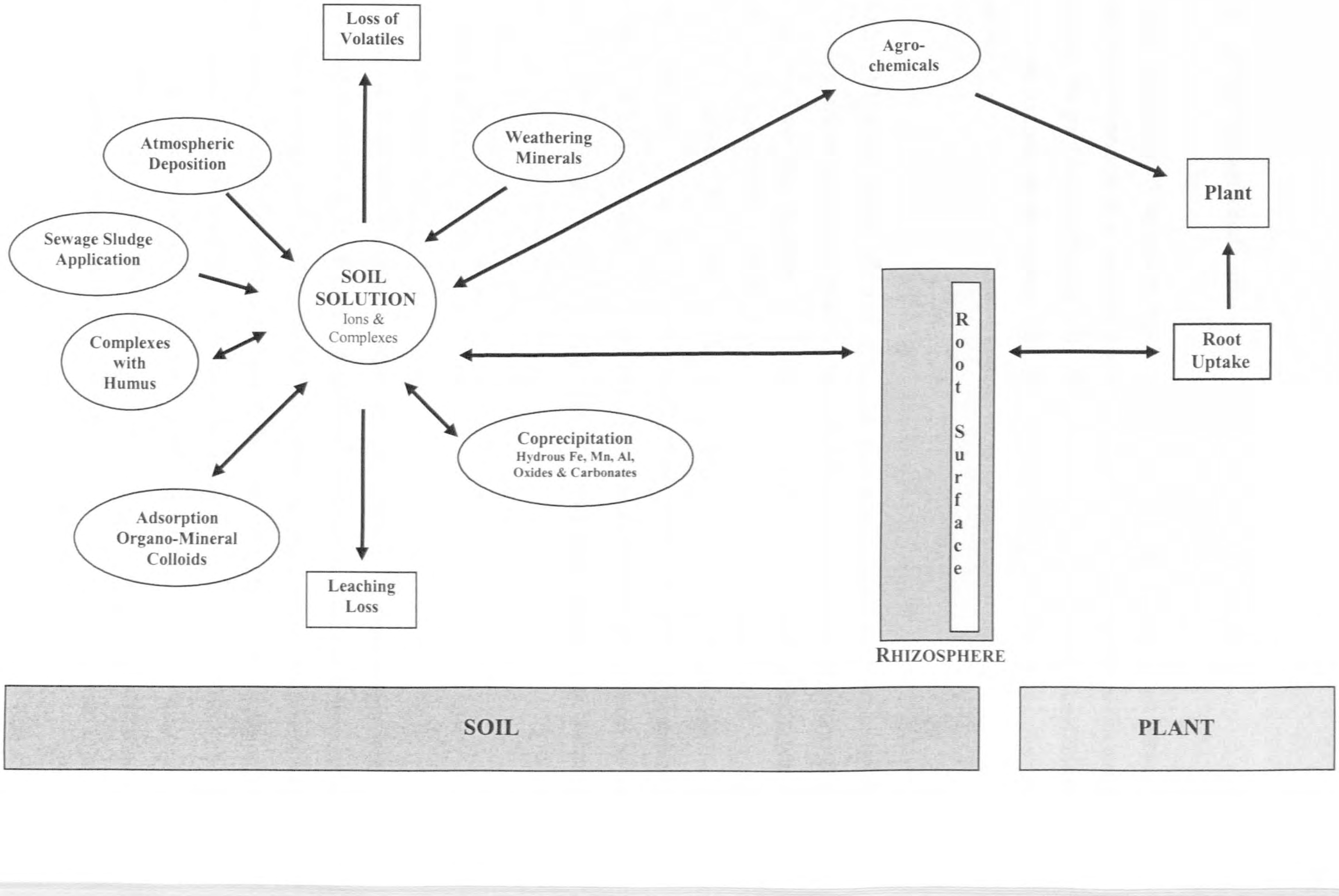
Bioavailability is closely related to solubility, which is in turn related to the soil solution pH and other chemical interactions within the soil system. The main factors affecting bioavailability are discussed separately, however, these factors are highly interactive and cannot be separated in a natural plant/soil system. The major interrelationship of heavy metals between plant and soil are shown in Fig. 1.4.1.

A. Soil pH:

pH is acknowledged to be the principal factor governing concentrations of soluble and plant available metals (Brallier *et al*, 1996). It affects metal solubility, cation exchange capacity and metal speciation, which in turn affect plant available metal.

For metals, solubility normally increases at low pH, resulting in increased bioavailability. Sanders *et al* (1986) showed that the equilibrium between solution and exchangeable Zn was strongly pH dependent. At high pH, Zn, for example is precipitated as $Zn(OH)_2$, $ZnCO_3$ or calcium zincate (Saeed and Fox, 1977) and is unavailable for plant uptake. The bioavailability of metal is dependent on the chemical form, which in turn is affected by the pH. Zn hydroxy and carbonate species occur at pH 6-8.5 but do not occur at pH 3.5-6.0 (Ritchie *et al*, 1995). As for Cd, lowering the soil pH increases the solubility, as less metal is adsorbed on soil particles. This is reflected in plant uptake where Cd concentrations in above ground biomass at soil pH 5.5 were three times more than at pH 7.5 using *B. juncea* (Indian mustard) plant (Zaurov *et al*, 1999). Other metals present in the soil solution system can affect the equilibrium of the adsorption of Zn and Cd between the aqueous and solid phases, i.e. the key factor in availability. This equilibrium is, in turn, highly affected by the pH.

Figure 1.4.1: The soil-plant interactions of heavy metals (adapted from Alloway, 1990 and Rieuwerts *et al*, 1998).



B. Soil-soil solution metal partitioning:

Metal bioavailability in soils is largely dependent on the partition of metals between the solid and solution phases as mentioned above. This partition has been assessed in terms of measuring the partition coefficients and adsorption isotherms. The partition coefficient, K_d can be used to compare the sorptive capacities of different materials and is described by the equation:

$$K_d = \frac{[M]_s}{[M]_l}$$

where $[M]_s$ is the amount of metal sorbed per unit weight of soil (or other material) and $[M]_l$ is the amount of metal in solution per unit volume of liquid, at equilibrium. This partitioning has been also discussed in terms of cation exchange capacity (CEC), where electroneutrality equilibrium is maintained between the soil surface negative charges and the soil solution cations. This charge equilibrium is pH dependent. Also, Evans (1989) and Alloway (1990) state that the adsorbing solid will usually prefer one cation over another; the preference being proportional to the valency (charge) and inversely proportional to the hydrated ionic radius (larger in cations with smaller ionic radii due to increased polarisation power).

C. Precipitation:

As well as adsorption, metals can leave the soil solution *via* precipitation with a chemical agent (generally an anion such as phosphate, carbonate or sulphate) to form solid metal-containing phases. Metals may also be precipitated as metal hydroxides (Basta and Tabatabai, 1992). Precipitated metals are unavailable for plant uptake. Precipitation is unlikely to occur in acidic conditions or without the presence of large quantities of both anions and cations (Rieuwerts *et al*, 1998). For example, precipitation of Cd is unlikely to occur in neutral or acid soils unless there are very high concentrations of carbonates, phosphates or sulphates present Boekhold *et al* (1993). This again emphasises the role of

pH on availability. For Zn, Brümmer *et al* (1983) reported that very high concentrations of Zn could be adsorbed before precipitation occurs.

D. Complexation:

Complexation can have two effects on the bioavailability. Dissolved inorganic and organic metal complexes introduce more metal in a form that could be uptaken by plants, and increase bioavailability. Whilst insoluble metal complexes are unavailable for plant uptake. Chelating agents that forms soluble metal-chelate complexes prevent precipitation thereby increasing bioavailability for plant uptake. It was shown that the shoots of *Brassica juncea* seedlings uptake of Cd was increased after the addition of chelating agent compared to that in the absence of a chelator (Salt *et al*, 1995). Chloride and dissolved organic carbon (DOC) in municipal waste leachates are efficient ions for solubilising heavy metals (Bourg, 1995). However, organic matter applied to soil in sewage sludge can increase the adsorption capacity of soil and through the formation of stable organo-metallic complexes actually decrease availability (Elliott and Singer, 1988).

E. Redox potential:

The reduction-oxidation reaction, which involves the flow of electrons from a reducing agent to an oxidising agent, known as redox potential E_h, affects the availability of heavy metals to plants. High redox potentials are typically recorded in dry, well-aerated soils, whilst soils prone to waterlogging and rich in organic matter tend to have low E_h values (Evans, 1989). In a review, Bjerre and Schierup (1985) found contradicting reports regarding the effect of redox conditions on metal solubility: some studies reported that Zn availability increases in a poorly drained, waterlogged soils, whilst others arrived at opposite conclusions. However this could be due to the varying conditions and soil properties in the studies. The decrease in redox potential resulted in small increases in acetic acid extractable Pb, Cd and Zn (Bjerre and Schierup, 1985).

F. Soil physical properties:

Soil structure could affect the migration of metals from soil to plant. In compacted soil, root growth will be restricted and penetration through the surface soil horizon may be greatly reduced. This will decrease the amount of soil exposed to root activity and exudates which can mobilise metals. The influence of soil texture on metal solubility (and thus availability) is best expressed in terms of the division of the soil into clay, silt and sand fractions. These fractions are defined by particle size fractions of <2μm, 2-50μm and >50μ m, respectively (Alloway, 1995). The affinity of metals to adsorption is demonstrated by the ranking: clay > silt > sand (Rieuwerts *et al*, 1998). According to Eriksson (1989) metals may be bound to the surface hydroxyl groups along the edges of clay particles as well as being directly bound to the clay surface. This is exemplified by fine textured soils, which are rich in clay and contain more Zn than coarse textured soils derived from sandy materials (Tagwira *et al*, 1992).

Other factors that could affect metal bioavailability include soil temperature. Temperature has been found to be positively correlated with plant uptake of Pb and Cd (Hooda and Alloway, 1993). Biologically, microorganism activity in the rhizosphere can improve heavy metal uptake by plants (Lodenius and Autio, 1989; Deighton and Goodman, 1995 and Ernst, 1996).

1.4.3 Speciation of Zn and Cd:

Chemical speciation is important to understand the potential health hazards and bioavailability of metals based on the specific chemical form rather than the total element. As discussed in previous sections, changes in the environmental conditions such as the pH, can alter the chemical form of a metal and strongly affect its behaviour. As an example, arsenic is extremely toxic in its inorganic forms, but relatively less harmful as aresenobetaine (a common form in fish) (Ure and Davidson, 1995). Organo-tin compounds, such as the antifouling agent tributyltin, are generally more toxic than inorganic species

(Ure and Davidson, 1995). Although such an example for either Cd or Zn could not be found, the same basic principles apply. However, it should be noted that speciation is often associated with the chemical oxidation state and since Zn and Cd only exist as 2+ ions, this restricts the scope for chemical speciation.

A. Definition and introduction:

Speciation has been defined from different perspectives. The following is a summary.

1. The species are defined by function regardless of the different chemical forms (Ure, 1991).
2. Operationally defined according to the physical or chemical fractionation process applied to obtain a certain fraction. This applies particularly to sequential extraction, where it is possible to define metals as "water/acid soluble", "reducible", "oxidisable" etc.
3. According to chemical compounds or oxidation states:
   This is the hardest to achieve since the analytical methodology requires a great selectivity and sensitivity (Ure and Davidson, 1995), as many metal species could be below the limit of detection of the instrument used.

According to Bernhard *et al* (1986), the term speciation encompasses three aspects:

A. The processes of transformation from one species to another (species distribution).
B. The analytical methods used.
C. The actual distribution of different compounds in a given matrix.

Aspects B and C are compatible with Ure definitions above, but the first aspect is largely confined to Biological Science (Kersten and Forstner, 1995). When speciation is concerned, it should be precisely defined to clear any misinterpretation. The definition

sought depends on the objectives of the work, the level of details and precision required and the field of application.

B. Zn and Cd chemical forms in soil solution:

The key factors in the chemical forms of Zn and Cd in soil have been discussed earlier under factors affecting bioavailability (Section 1.4.3). The complexity of soil structure, wide range of soil properties, different sources of heavy metals, climatic conditions and soil:soil solution:root interactions all contribute to the different metal chemical forms or species in the soil. Kabata-Pendias (1995) described the relative mobility-bioavailability of the different metal species to plants as in Table 1.4.2.

Table 1.4.2: Relative bioavailability and mobility of trace metals species in soils (Kabata-Pendias, 1995).

Metal species and association	Bioavailability-mobility
Simple or complex cations in solution phase	Easy
Exchangeable cations in organic and inorganic complexes	Medium
Metal compounds precipitated on soil particles	After dissolution
Metals bound or fixed inside organic substances	After decomposition
Metals bound or fixed inside mineral particles	After weathering and/or decomposition

Zn exists in the soil solution mainly as the free cation Zn^{2+}. The major forms are (in descending order of solubility) (Kiekens, 1995):

$Zn(OH)_2 > Zn\,CO_3 > ZnO > Zn_2SiO_4 > ZnFe_2O_4$

Zn also forms complexes with chloride, phosphate, nitrate and sulphate. Zn has been described as having the greatest variety of biochemical functions (catalytic, regulatory and structural) of any trace element (Bryce-Smith, 1989).

After weathering, Cd exist in the soil solution mostly as a divalent cation Cd^{2+} which is readily taken up by plants (Cabrera *et al*, 1988), or as a complex with inorganic ligands, the most important in soil solution being complexes of Cl^-, SO_4^{2-} or HCO_3^- (McLaughlin *et al*, 1996). As Cd is a soft Lewis acid it reacts and complexes readily with soft Lewis bases, such as chloride and hydroxyl group (Puls and Bohn, 1988). In contrast to other trace elements like Cu or Zn, Cd organic ligands seem not to have great significance in the overall speciation of Cd in soil solutions (Holm *et al*, 1995). For most agricultural soils, Cd solubility is controlled by sorption/desorption reactions with the soil solid phase (Brummer *et al*, 1988).

The analysis of the different metal species has been linked to sequential extraction, where selective extractants are used to quantify the element content associated or bound to a particular phase, which comes under the "operationally" defined speciation. Different analytical techniques have been used for the quantification of metal species like HPLC, GFAAS, ICP-AES and others depending on the extraction scheme and the limit of detection of the instrument used.

Based on the fact that the concentration and speciation of the metal depends partly on concentration of ligands in the soil solution and the stability constants of the ligand-metal complex (Alloway, 1995b), computer simulation models have been developed to predict metal species existing in the soil environment. Although, problems arise when these models are applied to a complex media such as the natural soil, they remain the best available methods for predicting the observed processes in soil (Lumsdon and Evans, 1995).

1.4.4 Methods to estimate metal bioavailability to plants:

As heavy metals are introduced to the soil through atmospheric deposition, sewage sludge application or other sources, complex chemical and biochemical reactions take place where heavy metals bind to the soil solid phase or complex with other chemicals in the soil solution as described above. The distribution of metals between soil solution and soil solid phase gradually reaches equilibrium. This process may take years (Kennedy *et al*, 1997). There is no generally accepted method of estimating the bioavailability of heavy metals (Houba *et al*, 1996). The methods developed reflect in part the level of knowledge regarding processes occurring in the soil and processes of plant uptake, but more especially the capabilities of analytical methods (Houba *et al*, 1996).

A. Soil total metal content:

Measuring the metal total concentrations is not a reliable indicator of the bioavailable and mobile metal fraction in soils (Rieuwerts *et al*, 1998; Marr *et al*, 1999). The method becomes even less effective when additional metals are added to the soil from different sources (e.g. sewage sludge (McBride, 1995) which contain metals in unavailable complexed forms). However, the total metal content is a measure of the metal build up, and provides an estimate of the extent or degree of saturation of total cation exchange capacity of soil colloids (Gupta and Aten, 1993). As the percentage of metal saturation increases, fewer metals are bound to the soil solids; hence the proportion of metal in the soil solution increases, which is reflected in turn in bioavailability.

B. Direct plant uptake:

A simple approach to measure the availability of a metal in a soil to a particular plant species is to grow the plant and see how much of the metal is accumulated. This direct metal uptake by plant is not an accurate measure of the metal availability to plants for many reasons:

1. The uptake depends on how long the plant is grown for and the crop yield.
2. Unavailable metal at some point can become bioavailable due to continuous changes of soil conditions.
3. Metals in soil can be in excess of that which the plant needs or can uptake.
4. Plant species differ widely in their ability to mobilise metals in the rhizosphere depending on growth conditions as well as the plant itself.

Nevertheless, this method is used to build relationships between different plant species uptake and different soils in an attempt to build mathematical models to evaluate element availability (Gupta and Aten, 1993).

C. Ion exchange resins:

Exchange resins were first introduced in 1955 as a method to simulate P uptake by plant roots (Amer *et al*, 1955). A soil:solution ratio of 1:80 is used with typically 1g of resin and mixed thoroughly. The resin is then washed with a suitable reagent, where adsorbed metals are displaced, and the metal content of the washing solution is measured. The method uses anion, cation, and even mixed anionic-cationic exchange resins. The amount of metal extracted depends on the desorbing anion, soil pH and the nature of cation on the soil exchange sites (Sen Tran *et al*, 1992). Despite their accuracy in determining the plant-available P, resin extraction methods have not been widely adopted by soil-test laboratories (Kamprath and Watson, 1980). Mehlich-3 is an example of a resin which may be used for multiple element extraction procedure to predict the availability of essential plant nutrients

from soils (Sen Tran *et al*, 1992). The technique was used to provide information about sorption sites and release rates for radio-caesium (Livens *et al*, 1996). This method has been used in conjunction with the isotopic exchange methods to compare the measure of predicted available P in a soil (Sen Tran *et al*, 1992). There is also little evidence in the literature for the use of this technique to assess heavy metal bioavailability.

D. Extraction:

One of the most widely used methods to predict bioavailability is to chemically extract the metal from the soil. A wide range of chemical extractants has been used to achieve this. These include: CH_3COOH, NH_4OAc, $CaCl_2$, $MgCl_2$, NaOH, NH_4NO_3, EDTA, DTPA, HCl, KNO_3, $NaNO_3$ and many others (Kennedy *et al*, 1997). Despite the enormous number of extractants used, the extraction technique is widely used and accepted as a method to estimate bioavailability. If practical, analysis of the soil solution is the ideal way of measuring heavy metal bioavailability (Kennedy *et al*, 1997). Unfortunately, this small volume of soil solution may have metal concentrations below the detection limit, and has lead to the development of laboratory based extraction methods as a compromise (Kennedy *et al*, 1997). However, the availability determined by extractants may not be universally applied to all plant species or all elements (Ure, 1996). The plant available metal will be any or a combination of the following metal fractions: water soluble, exchangeable soil-soil solution, organically and inorganically bound to soil, oxide bound, carbonate bound and bound to iron and manganese oxides. Extractants have the ability to extract one or more of these fractions (Kennedy *et al*, 1997) which explains why there are a large number of extractants used in the literature. The search for a suitable extractant becomes even more difficult when a variety of metal inorganic and organic complexes are added into the soil plant system from sewage or other sources.

In a review, Kennedy *et al* (1997) summarised most of the extractants used to predict heavy metal bioavailability. The single extractants cover a wide range of strength, ranging from weak extractants such as water, to moderate salts like $CaCl_2$, to the stronger acetic

acid and ammonium compounds. These different solvents extracted different amounts of metals from the same soil, making it a challenge to find a "suitable" extractant that would be the "universal" extractant of choice for bioavailability studies. In their study, Gupta and Aten (1993) attempted to establish a model to correlate between plant uptake and the data from different extractants in order to get a better understanding for factors affecting bioavailability. Single extraction and the extractants used in the present work are discussed in further detail in Section 3.1.

Sequential extraction is another approach to categorising metal forms in a soil. Extractants of increasing strength in succession are divided into defined fractions, commonly: soluble, exchangeable, weakly adsorbed ions, organically bound, oxide, carbonate, and finally the residual which is determined by the most aggressive extractant like HF or HNO_3, H_2O_2 or $HClO_4$ or a combination of these. Here, metal extracted by the weakest extractant will be available to a plant whilst at the other end of the scale, metal associated with the residual components are unlikely to be available. Because of the number of separate steps and analytical procedures necessary to estimate the different fractions, sequential extraction procedure is not commonly used for assessment of plant-available micronutrients (White and Zasoski, 1999). Instead, it has been used more to identify the solid-phase chemical forms (speciation) of heavy metals in soils (Haq and Miller, 1972; McLaren and Crawford, 1973 and 1974; Sims and Patrick, 1978 and Cottenie *et al*, 1980).

E. Isotopic exchange methods:

The isotopic exchange approach represents a non-extraction procedure to evaluate the amount of nutrients and heavy metals potentially available for plant uptake. The technique was first used in 1950 (Wiklander, 1950) to predict P bioavailability. More recently, Fardeau and co-workers have used this approach to quantify the available soil P in soils using radioisotopes (Fardeau *et al*, 1991; Frossard *et al*, 1994; Morel *et al*, 1995; Fardeau, 1996; Fardeau *et al*, 1996 and Sinaj *et al*, 1997). The method is based on relating the isotopically exchangeable P in soil-solution "the E-value" with the available soil P for plant uptake "the L-value" determined in pot experiments and correlating the 2 values using

different types of soils and a variety of plant species. It is assumed with this technique that the soil isotopically exchangeable P is equivalent to the available soil P (Larsen, 1952). This assumption was verified by different workers (Frossard *et al*, 1994, Fardeau, 1996, Fardeau *et al*, 1991, Morel *et al*, 1995, Fardeau *et al*, 1996 and Sinaj *et al*, 1997). The E-value is determined in a relatively uncomplicated soil/water system, and allows prediction of the available metal for a wide range of soil and environmental conditions (Echevarria, 1997). To determine the E-value, a radioisotope (with known specific activity) is added to the soil-solution system in a steady state (which is achieved by shaking the soil in water for 18h). As time proceeds, the soil solution isotopic composition changes and the specific activity in the soil-solution is reduced as radioactive isotopes are exchanged into the soil. From the specific activity at a given time, the concentration of the isotopically exchangeable metal can be calculated. This increases with time as more exchange occurs, until equilibrium state of isotopic exchange is reached, which represents the highest concentration (E-value). In this method, it is important to know when the isotopic equilibrium in the soil solution is achieved which could be hours, weeks or infinity depending on soil properties (Sinaj *et al*, 1997).

Determination of the "L-Value" has been suggested as an alternative because the E-value have been shown to overestimate the soil available P, especially in high P fixing soils (Fried, 1964). The L-value can be directly assessed by growing a plant on isotopically spiked soil (labelled with radioactive ^{32}P), where the specific activity of the plant, is used to determine the available element "L-value". When a radioactive isotope is added to a soil, exchange starts immediately with the organic, ionic, or inorganic forms of the element and ultimately equilibrium is reached. Two "pools" develop in which the element exists: an isotopically exchangeable (available to the plant) and non-exchangeable pool (not available to the plant) (Fardeau *et al*, 1996). After complete isotopic exchange between the added isotope and the element in the soil, plants are then grown on this spiked soil. The plants uptake elements from the soil and a portion of the added isotope. The isotopic composition in the plants is then used to calculate the available metal to the plant "L-value". The transfer factor (plant activity concentration (Bq/kg) / total soil activity concentration) have been

used to quantify the availability of radionuclides for plant uptake. P availability experiments are normally undertaken using a P carrier, however adding a carrier should be limited or avoided as it may modify the system diffusion processes (Fardeau *et al*, 1996). Recently, the E- and L-value method has been used to characterise bioavailable Ni, using a radioactive isotope. The results showed that the pool of isotopically exchangeable soil Ni is the pool of available Ni, and it was concluded that the method could also be applied to other metals (Echevarria *et al*, 1997). Very recently, the technique has been used for the determination of the labile Cd and Zn in soil, but only in a soil solution system (E-value) (Young *et al*, 2000). The study did not involve any plant experiments. All applications of the technique to plant studies have in the past been restricted to using radioisotopes.

The principles of E & L-values in Fardeau's work formed the basis of the methodology in the work reported here which aimed at bioavailability assessment of Cd and Zn using the stable isotopes ^{111}Cd and ^{67}Zn. These isotopes were added to both soil solution system, and plant soil system in exactly the same procedure as in radioisotopes. In the present work, the determination of the E or L-value is based on isotope dilution (ID) principles using stable isotopes. In this procedure the isotopic ratio (instead of measuring radioactivity) is measured and from this the E-value (in soil:water system) and the L-value (in soil:plant system) can be determined. E and L-value determination for Cd and Zn are discussed in further details in Sections 3.3.3 and 3.3.4.

In the present experiments, stable isotopes instead of radioisotopes were used for many reasons:

- Availability of isotope ratio measurement facilities at the Macaulay Institute using the thermal ionisation mass spectrometer (TIMS) with high analytical precision and accuracy.
- The shorter experimental procedure for the determination of the E-value (*See* Section 3.3.3). Using stable isotope dilution principles, soil solution metal content and the E-value are determined in 1 step using the stable isotope procedure, while each is determined separately using the usual radioisotope procedure.

- Safety precautions. No special safety precautions or special growth cabinets are required for handling stable isotopes compared to procedures involving radioactive isotopes.
- Short half lives of many metals, restricting their use for the determination of the E and L-values.

1.5 QUANTITATIVE ANALYSIS OF Cd AND Zn

Analysis of trace metals did not start until the 1940s when a number of studies concerning trace metal deposition in relation to health and disease were made by employing spectrographic methods of analysis (Berman, 1980). Since then, developments in analytical instrumentation and techniques have resulted in a greater understanding of the essentiality and function of heavy metals in living organisms. Also, inorganic pollution arising from industrial activities has led to more demand for the quantification of pollutant metals such as Cd and Zn in the biosphere. Precise, accurate, and reliable analytical data are required in all aspects of research. Inaccurate analytical results can lead to misinterpretations of the ecological and economic impacts of heavy metals. Techniques of quantitative analysis can be broadly categorised into (Ure, 1995):

(i) Single element methods such as atomic absorption spectrometry (AAS), and

(ii) Simultaneous multielement methods such as inductively coupled plasma atomic emission spectrometry (ICP-AES) or inductively coupled plasma mass spectrometry (ICP-MS).

Other examples of analytical methods include calorimetric, electrochemical and mass spectrometric methods. The choice of method for a particular analysis should take into account factors such as how the sample is introduced to the instrument, the level of precision and accuracy required, method availability and cost. After choosing an appropriate method of analysis, the sample is prepared accordingly.

1.5.1 Sample preparation:

The vast majority of analysis procedures require varying degrees of sample preparation, to ensure the sample is in an appropriate form to introduce to the instrument. However, sample preparation requirements and the amount of work involved is different from one technique to another. Sample preparation is time consuming, it can be expensive, and there is always the possibility of introducing contamination at different stages of the

procedure. Errors during sample preparation have been reviewed by Tschopel (1992), and include:

I. Inadequate sampling, sample handling and storage.

II. Contamination of sample by tools, apparatus, reagents and airborne dust during the analytical procedure.

III. Adsorption and desorption effects at the surface of the vessels and phase boundaries (e.g. filters or precipitates).

IV. Losses of elements such as Cd if they are volatile.

V. Undesired or incomplete chemical reactions (e.g. change of valency, ion exchange).

VI. Matrix effects on the analyte signal.

VII. Incorrect calibration and standard materials.

As samples are susceptible to contamination during preparation, care must be taken to avoid contamination, especially at the trace or ultra trace analysis level. This involves the use of high purity reagents and glassware and appropriate procedures for cleaning tools and glassware. The following discussion of methodology relates to the analysis of solid materials, in particular in soils, which represent complex and from an analytical point of view, difficult material to work with.

A. Decomposition methods:

The aim of the decomposition procedure is to transfer the sample that contains the elements of interest into the appropriate form for the chosen method of analysis. Decomposing the sample is achieved first, by heating at a high temperature (450°C) to decompose organic materials and convert metals to the same chemical form. This is followed by a suitable digestion method to release analytes from the matrix components. Different digestion procedures are used depending on the sample type and analysis required. Table 1.5.1 summarises the decomposition methods usually used in inorganic analysis.

Table 1.5.1: Decomposition methods used in inorganic analysis (adapted from Tschopel, 1992 and Ure, 1995).

Decomposition technique	Procedure/method	Reagents
Fusion decomposition	Acidic	$NaHSO_4$
	Alkaline	$NaCO_3$
	Oxidising	$NaCO_3 + NaNO_3$
	Sulfonating	"Freiberger" decomposition, $KCO_3 + S$
Wet chemical decomposition	Open systems with acids	
	Non oxidising	HCl, *aqua regia* ($HCl:H_2O:HNO_3$)
	Oxidising	$HNO_3/KMnO_4$, H_2O_2, $HClO_4$
	Closed system (Pressure bomb)	HF and/or HNO_3
Miscellaneous		
Pyrolysis/pyrohydrolysis	(Heat/ heat + H_2O)	
Decomposition with halogens	(e.g. F, Cl_2, $HCCl_3$)	
Decomposition by reduction	(e.g. H_2, C, NH_3, metals)	
Electrolytic oxidation		

The use of sealed containers is important to prevent the loss of the analyte during sample decomposition, especially for volatile elements (Fassett and Paulsen, 1989) such as Cd. The techniques employed in this study were: open tube *aqua regia*, microwave HNO_3 and hydrofluoric acid (HF) digestion. These are discussed below in greater details.

Open tube aqua regia digestion:

Aqua regia ($HCl:H_2O:HNO_3$) is an effective oxidising agent due to the production of nitrosyl chloride (NOCl) which upon heating dissociates into the toxic and highly corrosive chlorine gas. In addition, *aqua regia* oxidises many materials more efficiently than either hydrochloric or nitric acid alone (Kingston and Jassie, 1988). Samples are digested with different acid combinations of *aqua regia* solutions (3:3:2 (used in the present work) at

about 140°C on a heating block and a reflux system. Safety precautions involve trapping acidic vapours in NaOH solution, and the whole digestion procedure is carried out in fume cupboard. According to Ure (1995), *aqua regia* extracts between 70 and 90% of the total contents of seven trace elements including Cd (the study did not include Zn). The main advantages of *aqua regia* digestion are the large throughput of samples, ease of automation, and the simplicity and safety of the technique compared to HF digestion (McGrath and Cunliffe, 1985).

Microwave HNO_3 digestion:

Decomposition procedures that make use of microwave ovens have to some extent replaced conventional oven and hot plate methods. This technique has been recommended by the NIST for contaminated environmental materials since it is less subject to contamination in the laboratory (Ure, 1995). Closed-system microwave digestion (and closed HF digestion bombs) benefit from the fact that acids such as HNO_3 or HCl digest materials more quickly at elevated temperatures and pressures (Jackwerth and Gomiscek, 1984). Microwaves are electromagnetic radiation (with typical energy output of 600-700W), the energy is being used to supply heat to the sample mixture causing sample decomposition (Neas and Collins, 1988). However, appropriate safety precautions should be taken with the microwave digestion procedure. Available commercial systems are equipped with specially designed Teflon disc to release excess pressure and to prevent explosions. In addition, the microwave oven is equipped with a special filtration (ventilation) system preventing hazardous vapours polluting the working environment. Samples are transferred into microwave bombs using a suitable amount of conc. HNO_3 (or mixed acids) and heated in the microwave oven. The method is fast, uses less sample and less consumables than open tube *aqua regia* digestion.

Hydrofluoric acid (HF) digestion:

Since some elements in soils are associated with silicates, breakdown of these silicate minerals is important for determining the total metal content. Total dissolution requires a powerful reagent such as hydrofluoric acid (HF) that is capable of attacking these recalcitrant materials. Because it attacks silicates, HF must be stored in vessels made of Pt, PTFE, propylene or polyethylene and not in glass vessels. If the samples required for analysis contain organic matter and sulphides, it is necessary to use an oxidising agent such HNO_3 or $HClO_4$ in combination with HF. In these cases, samples are heated in a mixture of concentrated HF and HNO_3 in tightly sealed PTFE bombs at approximately 180°C for 6h followed by evaporating reagents by further heating for approximately 6h. Although HF is a strong oxidising agent, some minerals such as chromite ($FeCr_2O_4$) and those containing elements such as Al, Cr and Mn, resist acid attack and special dissolution procedures must be followed (Yamasaki, 1996).

As a result of the health risks involved in HF handling, special safety precautions must be taken which include the use of specially designed fume cupboards, the use of protective gloves and clothing, appropriate disposal of materials containing HF acid and adequate training of staff. In addition, the recommended sample size must not be exceeded because of the danger of explosion.

B. Chromatographic separation:

Although the purification of elements of interest is not essential for many methods of analysis (e.g. GFAAS, AAS, ICP-AES), these elements must be separated from the majority of other inorganic components present in the digest for thermal ionisation mass spectrometry (TIMS) employed in the present work. In particular high concentrations of alkali and alkaline earth metals as well as other trace metals may potentially interfere with the ionisation process within the mass spectrometer preventing or obstructing the analysis of the element under investigation (Bacon, 1996). Separation is achieved using one or more of the following procedures: ion exchange chromatography, extraction, electrodeposition,

precipitation and high performance liquid chromatography (Bacon, 1996). Ion exchange resins are most frequently used for this purpose. Ion exchange is a stoichiometric reaction between a solid and a solution whereby an ion is taken up by the solid and another goes into solution (Lederer, 1994).

Ion exchange resins are available in many ionic forms, and may be converted from one to another. Cation exchange resins are negatively charged attracting positively charged ions, whereas anion exchange resins attract negatively charged species. The ionic form of the resin refers to the ion presently adsorbed by the resins functional group. A sample ion will displace the existing ion if it has more selectivity (affinity) for the functional group on the resin. This sample ion can then be displaced (and eluted using different reagents) by a third ion (from the eluting solvent) with a higher selectivity for the resin. This selectivity is dependent on the equilibrium constant (K_{eq}) of each individual element. For example the cation exchange reaction with a sulfonic acid group is:

$$SO_3^- H^+_{(s)} + M^{x+}_{(aq)} \Leftrightarrow SO_3^- M^{x+}_{(s)} + H^+_{(aq)} \quad \text{.....1.5.1}$$

where M^{x+} is a cation of charge x. The equilibrium constant (K_{eq}) for this reaction is:

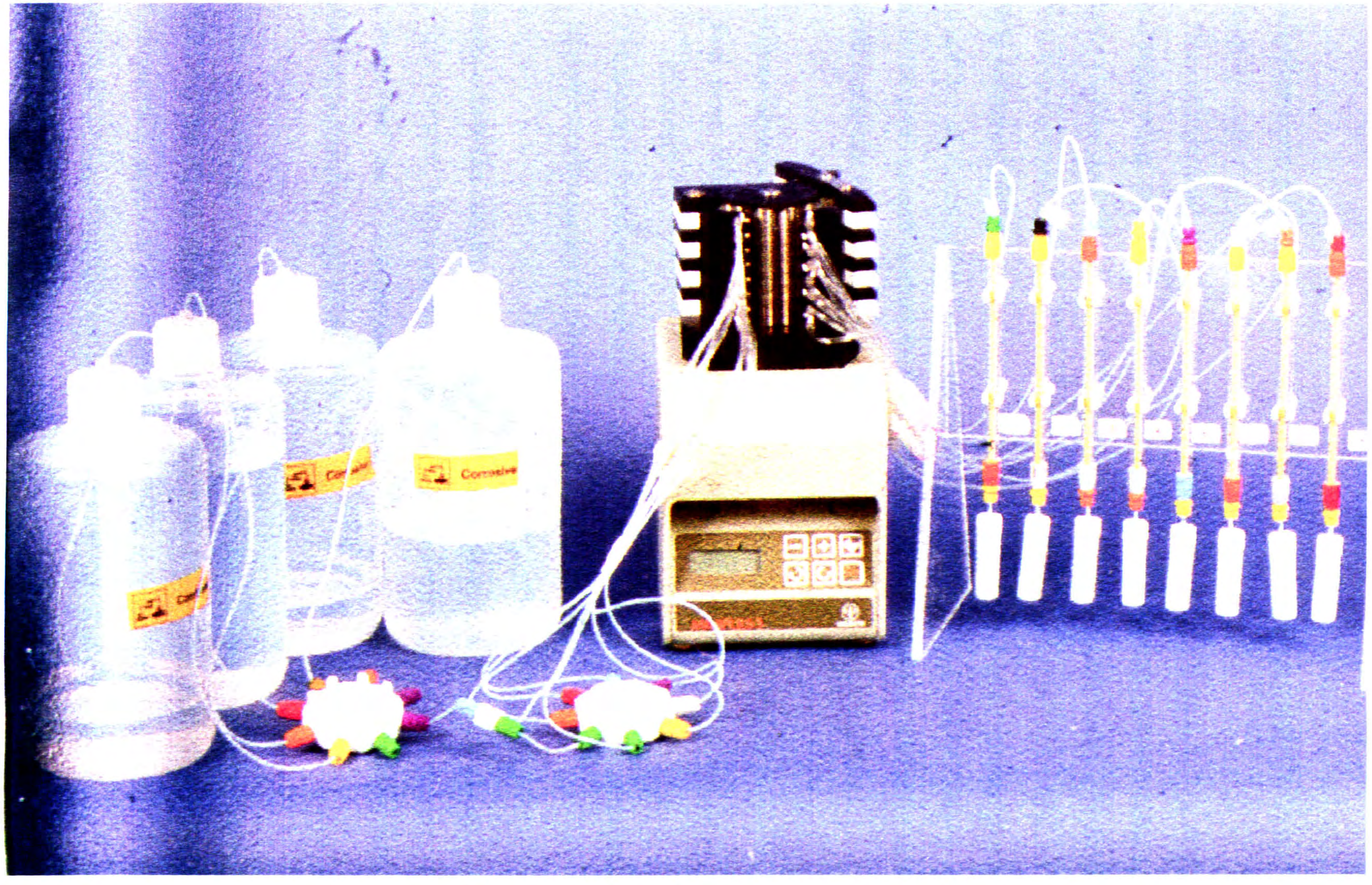
$$K_{eq} = \frac{[SO_3^- M^{x+}]_s [H^+]_{aq}}{[SO_3^- H^+]_s [M^{x+}]_{aq}} \quad \text{.....1.5.2}$$

Different cations have different values of K_{eq} and are therefore retained differently on the exchange resin. K_{eq} is affected by the pH, and this is used to elute different elements with different strengths of acid solutions in column ion exchange chromatography. The more commonly used resins are made of a crosslinked polymer support, such as styrene divinylbenzene (used throughout the experiments), with different functional groups (SO_3^- H^+, $RCH_2N^+(CH_3)_2$) and ionic forms (H^+, Na^+, Cl^-). These are used for the separation of metals as well as separation or concentration of peptides and amino acids. Conventional resins could be used for many elements, however some resins have been manufactured to be element specific (Adriaens *et al*, 1992), where the functional group has a very high

selectivity for a specific element. Other adsorbents include silica, alumina (Al_2O_3), zirconium oxide, titanium hydroxide or titanium dioxide (Lederer, 1994).

In the current study an anion exchange resin was used. After the introduction of the sample onto the ion exchange column, the procedure involves sequential elution with reagents such as HCl, HBr and HNO_3 solutions to separate the analyte(s) of interest. The ion exchange chromatography system used in sample purification in the present work is pictured in Photograph 1.5.1. For analysis with TIMS, the solution fraction containing the analyte of interest is dried, and the analyte recovered is then redissolved in a suitable reagent to be loaded onto filaments. This system is discussed in further details in Section 3.2.2C).

Photograph 1.5.1: Ion exchange chromatography used in sample purification.



1.5.2 Chemical methods of trace element analysis:

A. Flame and graphite furnace atomic absorption spectrometry (FAAS, GFAAS):

Dramatic progress in both instrumentation and applications have occurred since the early 1960s when the first atomic absorption instrumentation became commercially available (Milner and Whiteside, 1989). Atomic absorption spectrometry (AAS) uses the fact that free atoms of an element absorb light at a wavelength characteristic of that element which is determined by its outer electronic structure. The extent of absorption is a measure of the number of atoms in the light path and hence concentration. AAS, in many research areas, is the predominant method for Cd analysis. The reasons been reliability and speed, also with a graphite furnace versatility and detection power (Stoeppler, 1992b).

The most important drawbacks in flame AAS are (Ure, 1995):

- The poor efficiency of sample introduction by pneumatic nebulisation (<10% efficient).
- The chemical interference effects intrinsic to the low energy of flame atomisers.
- Incomplete atomisation of the analyte with low-temperature flames.

Interference from the constituents of biological materials enhanced the Cd absorption signal many fold (Berman, 1980) which lead to inaccurate results. Sample requirements in the 2-10ml range are usual, but samples of about 50-100µl can be analysed with pulse nebulisation techniques (Ure, 1995).

The limitations of atomisation efficiency of the sample and the large dilution introduced by the expanding flame gases in flame AAS have been overcome by the use of an electrothermal atomiser that takes the form of a cylinder of graphite for which only microlitres (5-100µl) of sample are required. Because the whole sample is atomised, and the atomic vapour produced is partly confined within the graphite tube, the sensitivity of

GFAAS is 100-1000 fold greater than that of flame AAS (Ure, 1995). Also, introduction of Zeeman background correction has reduced or eliminated most of the interferences previously associated with GFAAS (Boss and Fredeen, 1989). However, the determination of Cd in the graphite furnace is often difficult due to matrix interferences during atomisation which required the addition of chemicals (such as ammonium nitrate or nitric acid), to reduce its volatility (Stoeppler, 1992b). Despite the high sensitivity and small sample requirements, GFAAS application in studies of soils and heavy metals pollution has been slow to develop because of severe and complex interferences (Urc, 1995).

With coverage of about 70 elements and quite good precision, flame AAS is one of the most reliable methods. However, the graphite furnace is slower than flame AAS or ICP-AES in terms of the speed of analysis, and it can only analyse one element at a time. AAS is gradually being replaced or complemented with other techniques, especially inductively coupled plasma atomic emission spectroscopy (ICP-AES) systems (Stoeppler, 1992a) or inductively coupled plasma mass spectrometry (ICP-MS); as these techniques have the advantage of being multielement techniques (Beaty, 1988).

B. Inductively coupled plasma atomic emission spectrometry (ICP-AES):

The plasma source was introduced as a means of element excitation and ionisation in the early 1960s with the first commercial appearance in 1974. Since then, ICP-AES has become a major analytical technique for both sequential and simultaneous multielement analysis of up to 70 elements (Stoeppler, 1992a). The plasma source was introduced because it has a high degree of stability, an ability to overcome depressive interference effects caused by the formation of stable compounds and can excite several elements that are not excited by conventional chemical flame techniques (Greenfield *et al*, 1964). When compared to AAS, the fact that radiation intensity is directly proportional to the atom concentration is an advantage (Stoeppler, 1992a). However, when atoms are ionised by plasma, numerous atomic transitions produce spectra which are, particularly with heavier elements, very complex (Stoeppler, 1992a).

Interferences in ICP-AES occur and are divided into: translational (or additive) caused by spectral overlaps and by stray light in the spectrometer and rotational (or multiplicative) interference caused by general matrix effects (HMSO, 1996). Translational interferences are proportional to the concentration of the interferent and independent of the concentration of the analyte. The interferent produces a signal that is additive to the analyte signal and causes a simple translation of the calibration curve. Rotational interferences occur when the effect of a given concentration of the interferent is to multiply the analyte signal by a constant factor. This factor is also independent of the concentration of the analyte. In general, matrix interference problems, which can affect precision, accuracy and detection limit, are still encountered in ICP-AES. However, a variety of techniques are available to minimise the effects and include optimisation of nebulisation, preconcentration, coupling with chromatographic systems, flow injection and others (Stoeppler, 1992a). Multielement determination in samples with low elemental levels, such as in seawater or uncontaminated soil, may require preconcentration procedure prior to ICP-AES analysis (Stoeppler, 1992b). ICP-AES remains a powerful technique capable of rapid, highly accurate and precise trace element analysis.

ICP-MS uses a mass spectrometer rather than an optical spectrometer as the detector. The main advantages over other analytical techniques include increased sensitivity, high selectivity, rapid multielement analysis and the ability to measure specific isotopes (Yamasaki, 1996). However, matrix effects are the main disadvantage of ICP-MS instruments. This is discussed in further details in Section 1.5.4.

1.5.3 Isotope Dilution (ID) Technique:

Geological and nuclear investigations in the 1950s led to the development of the ID technique with first applications to the determinations of organic compounds being carried out in the 1970s. Isotope dilution mass spectrometry (IDMS) is today used for the determination of great number of elements and organic substances (Heumann, 1986b). It can be employed using both stable and radioactive isotopes, however the present discussion

is focused on the use of stable isotopes. As discussed earlier, different analytical methods have been applied to determine metal concentration, however, reproducible, sensitive and precise data are not necessarily accurate. Therefore, a method that would give the "true" value is always required so that the variation of results from different analytical methods or different laboratories, especially when lower concentrations are to be determined, could be avoided. One way to validate a method is to use certified reference materials (CRMs). Alternatively, a definitive method such as IDMS (Moody and Epstein, 1991) which produces highly accurate quantitative data can be employed (Heumann, 1986a). Although the definition of a definitive method varies, it should have the following characteristics (Moody and Epstein, 1991):

- A well described theoretical foundation.
- Be evaluated on an experimental basis.
- Its sources of errors are easily identified.
- It incorporates a high level of accuracy, precision and reliability.

The favourable analytical conditions of IDMS allowed the method to be accepted internationally as a method of highest quality and is used for the certification of the CRMs themselves (Heumann, 1986a).

IDMS determination of metals have been carried out using various MS techniques, including thermal ionisation (TIMS), spark source (SSMS), laser resonance ionisation (RIMS), secondary ionisation (IS) and inductively coupled plasma (ICP-MS) (Beary *et al*, 1994). Although ID using ICP-MS is used method in industrial analysis, spectral interferences can produce erroneous results (Horn and Heumann, 1994). On the other hand, ID carried out using TIMS does not suffer from interferences (as metals are purified prior to analysis and also because of differences in the ionisation potential), which makes it a suitable method where high precision and accuracy are required. ID has been applied for the determination of Cd and Zn in samples using both ICP-MS and TIMS on trace or ultratrace levels. Samples analysed included CRMs (Sands and Rosman, 1997 and Okamoto, 1991), food (Gotz and Heumann, 1987), soils (Gotz and Heumann, 1986), pigments (Beer and Heumann, 1994), seawater (Stukas and Wong, 1983), biological samples (Hwang and

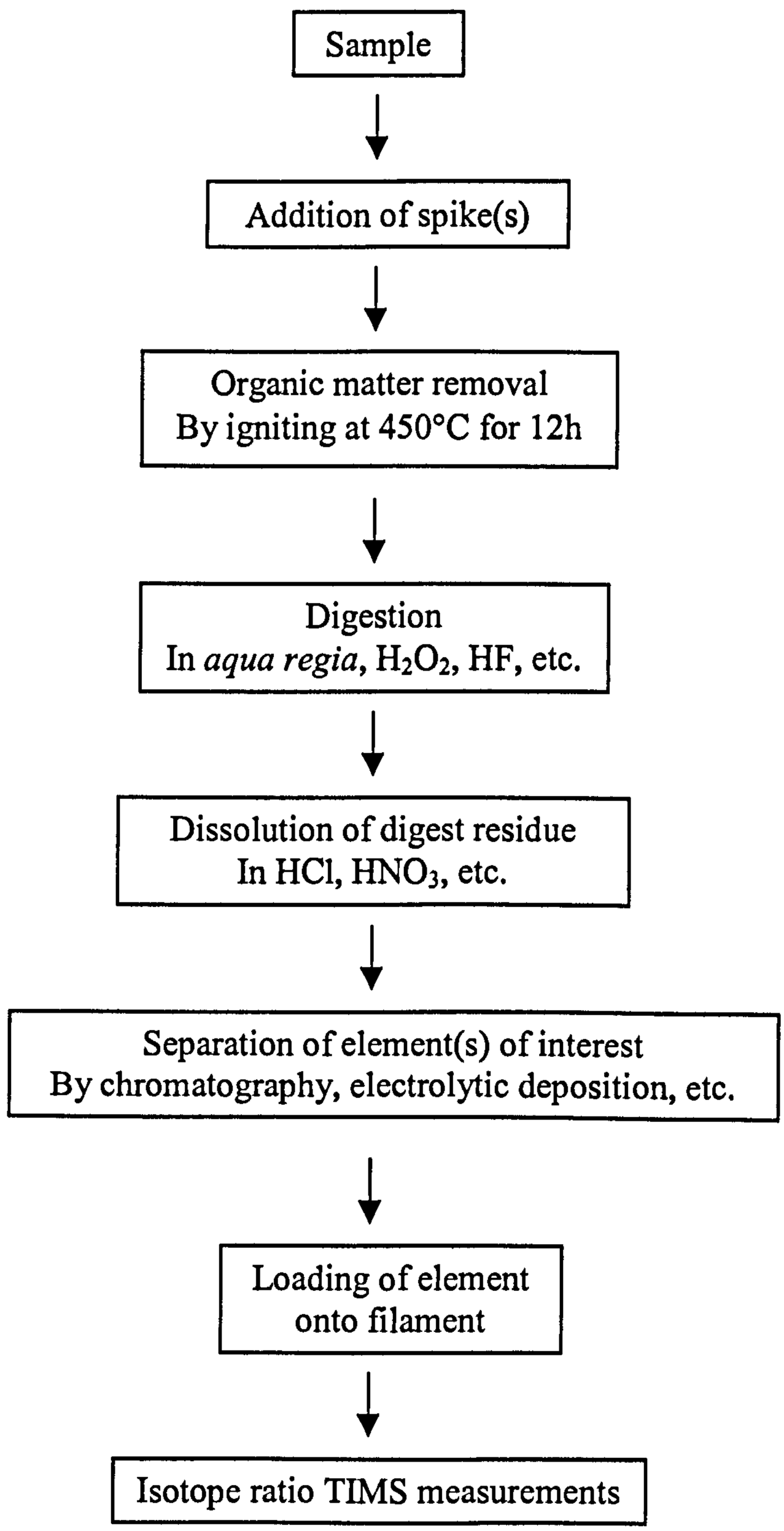
Jiang, 1996), and have been used for metabolism studies in man and animals (Turnlund, 1983).

The power of the IDMS technique comes from the fact that it relies on the measurement of a change in isotope ratio. One isotope acts as an internal standard so that systematic errors such as losses during separations are essentially eliminated, hence quantitative recovery is not required. This is particularly useful if an analyte is very difficult to extract quantitatively or when sample preparation involves many stages. With optimisation of spike addition, separation method and loading technique, low limits of quantification (LOQ) can be obtained. However, due to the expensive instrumentation and the time consuming sample preparation, IDMS is not a routine method of analysis (Stoeppler, 1992a).

A. Sample treatment and principles of ID :

The general aspects of sample preparation discussed earlier (Section 1.5.1) apply also in the preparation of samples for ID. ID involves adding a known amount of a fully characterised enriched isotope (spike) to the sample. This is followed by good mixing (so isotopic exchange takes place), digestion of the sample and finally measurement of the isotopic ratio by a mass spectrometric technique from which the metal content can be calculated. Fig. 1.5.1 describes the general analytical procedure for the determination of isotope ratios using ID-TIMS (which has been followed throughout the experiments). In this procedure, the analyte(s) separation from the matrix is essential and ion exchange chromatography is widely used for this purpose (Lederer, 1994).

Fig. 1.5.1: Analytical procedure used to measure the isotope ratio on TIMS.



ID can be applied to any element that has more than one stable isotope (about 60 elements) or long-lived radioactive ones (Fassett and Paulsen, 1989). The term ID comes from the fact that the isotopic ratio of the original sample is altered by the addition of an artificially enriched isotope (spike). Although quantitative recovery of the analytes of interest is not required in ID, complete isotopic exchange between the analyte in the sample and the added spike must occur. This requires both the analyte in the sample and the spike to be in the same chemical form. This is generally achieved by decomposing the mixture and driving the element to the same oxidation state (Fassett and Paulsen, 1989). The principles of ID technique are described below, however, more detailed discussion can be found elsewhere (Tolgyessy *et al* 1972; Habfast; 1982; Heumann, 1992; and Barker, 1999).

In ID, an element that has two isotopes of masses m_1 and m_2, where m_1 is more abundant in the sample, an exact known amount of artificially enriched isotope m_2 (called spike) is added to the sample. The sample and the spike are isotopically equilibrated. If N is the number of atoms, h is the isotope abundance, the subscripts X and SP symbolises the sample and the spike, respectively, the superscript *1* and *2* symbolise isotopes *1* and *2*. Then the isotopic ratio R of m_2/m_1 in the sample would be:

$$R = \frac{N_X h_X^2 + N_{SP} h_{SP}^2}{N_X h_X^1 + N_{SP} h_{SP}^1} \quad \text{.....1.5.3}$$

Arranging the equation above gives

$$N_X = N_{SP}\left(\frac{h_{SP}^2 - R h_{SP}^1}{R h_X^1 - h_X^2}\right) \quad \text{.....1.5.4}$$

At the same time

$$N_X = \frac{W_X C_X N_A}{M} \quad \text{.....1.5.5}$$

Where W_X sample weight (in g),C_X the concentration of the sample, N_A Avogadro constant and M the atomic mass of the natural element.

As the number of atoms of the added spike (N_{SP}) is known from the spike concentration (calculated using the spike atomic weight), h for isotopes *1* and *2* is known from characterising the spike and a natural element, equations 1.5.4 and 1.5.5 could be transformed to calculate the concentration of the element in the sample C_x (μg/g) as follows (Heumann, 1992):

$$C_X = 1.66 \times 10^{-18} \frac{M}{W_X} N_{SP} \left(\frac{h^2_{SP} - Rh^1_{SP}}{Rh^1_X - h^2_X} \right) \quad \ldots\ldots 1.5.6$$

B. Optimisation of spike addition:

For an isotope to be selected, the following points are to be considered:

1. It should have low abundance in the natural sample.
2. No interference with other isobaric nuclides in the sample (Heumann, 1986a).
3. Availability and cost.

In IDMS, errors are mainly caused by measuring isotopic ratios, and for this reason a certain number of ratio measurements (usually about 100) should be made to achieve good precision and accuracy. Other sources in the uncertainty of an IDMS measurement (equation 1.5.6) come from errors in the amount of spike added and the isotopes abundance measured (Heumann, 1992). To minimise errors in the amount of spike added, additions are made by weighing aliquots from the stock solution. Moreover the spike concentration is verified by reversed IDMS using standard solutions of natural isotopic abundance (*See* Section 3.2.2A) Normally, the statistical error of the isotope ratio (R) measurement has the greatest influence on the analytical results than all other sources of error (Heumann, 1992).

An error multiplication factor f(R) (the propagation of the uncertainty in the concentration from error uncertainty in the R measurements) was derived by Heumann (1992) and is dependent on the atomic ratio N_S/N_{SP} in the analysis and on the isotope enrichment of the spike. The error multiplication factor is decreased with higher isotope enrichment in the spike and the closer the isotopic ratio (after spike addition) to 1. Also the more enriched the tracer spike, the larger the differences in the ratio and the greater is the accuracy of the analysis (Tolgyessy *et al*, 1972) which is reflected on the error multiplication factor. Heumann (1986b) demonstrated that the error multiplication factor decreased with higher isotope enrichment of the spike with an optimum mixing ratio of 1:1 using thallium as an example. The ratio is not necessarily 1:1 depending on both the spike and the natural element isotopic abundances. Also "overspiking" (where an excess amount of spike has been added) may be used where the spike acts as a carrier during the isolation process. This may be advantageous especially when very small amounts of the analyte have to be determined in the sample (Heumann, 1986b).

Different equations are used to calculate the optimum theoretical isotope ratio (R_{opt}) derived from the spike (*SP*) and the natural element (*S*) isotope abundances. According to Heumann (1992), R_{opt} is calculated using the following equation, where 1 is the lighter isotope:

$$R_{opt} = \sqrt{\left(\frac{h^2}{h^1}\right)_S \times \left(\frac{h^2}{h^1}\right)_{SP}} \quad \text{.....1.5.7}$$

Using the characterised atomic abundances for the spike and a natural element, R_{opt} values for ^{111}Cd/ ^{114}Cd and ^{66}Zn/ ^{67}Zn of 8.6:1 and 0.6:1 are obtained, respectively. However, a ratio close to 1:1 was targeted with both elements throughout the experiments reported in this thesis to retain reasonable counts for both isotope signals in the detector.

C. Advantages and sources of error of ID technique:

The benefits of using the ID technique have been discussed above, and can be summarised as follows:

1. No need for quantitative recovery of the sample.
2. The reduction of systematic errors. Errors in the sample preparation procedure or losses of the analyte are essentially eliminated.
3. No need to establish a calibration curve for analyte concentration determinations.

Like any other analytical technique, ID can suffer from errors associated with sample preparation (Section 1.5.1), although they are minimal. Nevertheless, the following possible sources of error should be addressed when applying the technique. These include:

1. Non-equilibration of the ID process. The isotope added should be isotopically exchanged with the sample different species and is usually achieved by decomposing the sample into one element form. Incomplete decomposition could result in significant errors (Fassett and Paulsen, 1989).
2. Isotopic fractionation in the ion source. This is most significant in elements of low relative atomic mass. Using a double or triple filament instead of a single one can reduce this effect (Crews *et al*, 1994). Different approaches have been suggested to account for fractionation in ID. These include internal normalisation (Moore *et al*, 1974; DeBievre, 1990); iterative method (Henrion, 1994; Pickup and McPherson, 1976); and double spiking (Woodhead *et al*, 1995).
3. Isotopic abundance variation in nature. This can usually be neglected with a few exceptions (e.g. Lead) (Heumann, 1988).
4. Blank Corrections. A blank is usually determined by the same analytical process that is used in the analysis of the sample, but without any of the sample component present.
5. Interferences. In TIMS isobaric interferences are negligible if appropriate separation of the analyte is carried out.

1.5.4 Inductively coupled plasma mass spectrometry (ICP-MS):

The main advantage of ICP-MS lies in its multielement analysis ability which makes it one of the dominant techniques for IDMS (Heumann, 1992). Other advantages of ICP-MS include: the plasma vacuum interface, which allows sample introduction to MS to be performed outside the vacuum (Fassett and Paulsen, 1989), the fact that there is no need for the isolation of individual metals prior to analysis and also it is a rapid technique allowing the use of multiple spikes/ ID determinations if required at once. Despite these advantages, the technique still suffers from matrix effects which can have a significant impact on precision. For example, in a study comparing both ICP-MS and TIMS using ID, $^{52}Cr^{+}$ was interfered with by $^{36}Ar^{16}O^{+}$ and $^{40}Ar^{12}O^{+}$, while $^{53}Cr^{+}$ was interfered with by $^{40}Ar^{13}O^{+}$, leading to incorrect results from ICP-MS whereas there was no interferences using the TIMS (Horn and Heumann, 1994). In ICP-MS, $^{64}Zn^{+}$ can potentially suffer from interferences from $^{64}Ni^{+}$ or $^{31}P^{16}O_2{}^{1}H^{+}$, $^{32}S^{16}O^{16}O^{+}$, $^{32}S^{32}S^{+}$, also, $^{66}Zn^{+}$ from $^{34}S^{16}O^{16}O^{+}$, $^{32}S^{34}S^{+}$, $^{35}S^{16}O^{16}O^{+}$ (Barker, 1999). Interference effects can be reduced or overcome using a variety of techniques including using a cool plasma, high resolution ICP-MS and sample pre-treatment techniques. In addition, the precision of TIMS for the isotope ratio measurements is usually better by one order of magnitude or more than by ICP-MS (Heumann, 1992). Nevertheless, although drifts in measured isotope ratio of a few percent are sometimes observed, precision of isotope ratio measurement of 0.25% can be achieved in ICP-MS (Fassett and Paulsen, 1989).

1.5.5 Thermal ionisation mass spectrometry (TIMS):

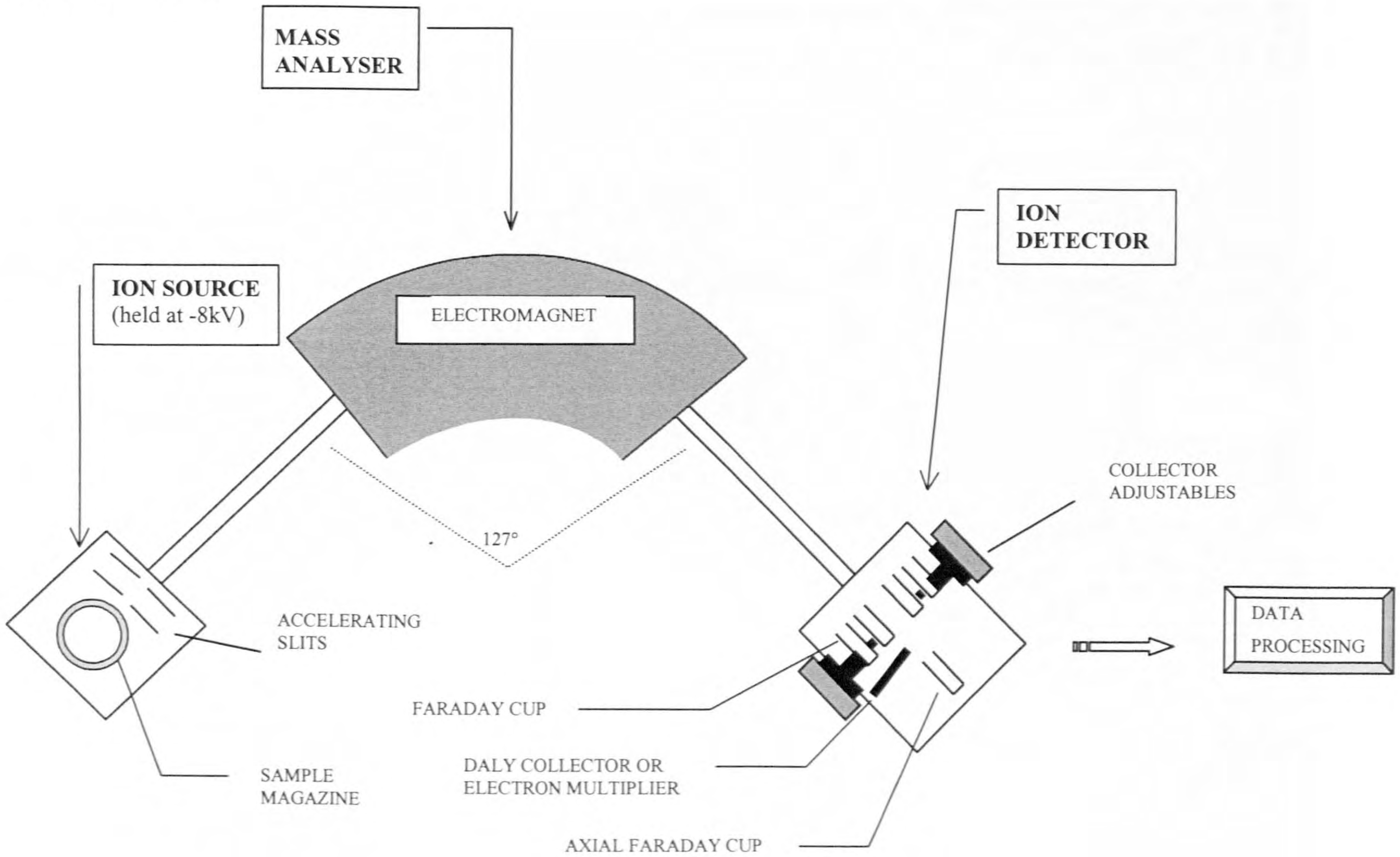
TIMS was among the first mass spectrometric techniques developed, often referred to as surface ionisation, and was used by Dempster since the beginning of the 20th century (Bacon, 1996). Since then, TIMS has been the principal technique for isotope ratio measurement and is generally accepted to be the mass spectrometric technique that yields the highest precision (0.001-0.5% relative standard deviation, Heumann, 1986b; Hachey *et al*, 1987).

Compared to other techniques (Crews *et al*, 1994), TIMS does not suffer from:

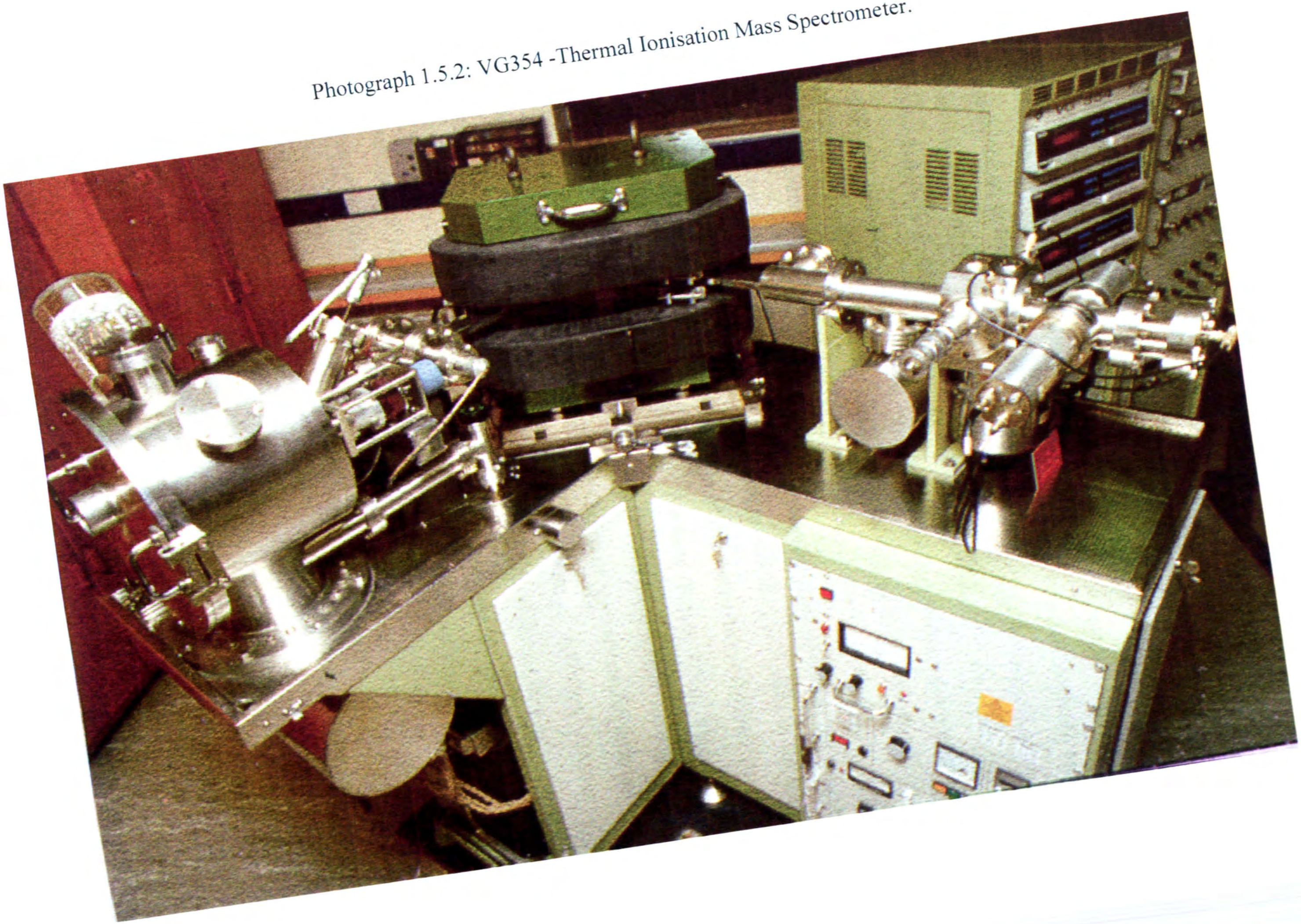
- Isobaric interference (provided proper separation of the analyte is carried out) as in ICP-MS.
- Hydride interference and low sensitivity as in fast atom bombardment MS (FABMS).
- The need to prepare volatile derivatives of the element as in gas chromatography MS (GCMS).

However, the high accuracy and precision in TIMS comes at a cost in terms of its high purchase price and in its relatively slow analysis time, therefore, applications must be justified in terms of the uniqueness or the value of information obtained (Bacon, 1996). For example, it is necessary to isolate the analytes of interest from the matrix and also from other metallic elements making the TIMS relatively time consuming (Horn and Heumann, 1994, Beary *et al*, 1994). Finally, the number of samples that can be analysed by the TIMS (about 8 samples per day) is much lower than other techniques (20-100 samples per day for ICP-MS) (Crews *et al*, 1994). The mass spectrometer in general consists of the three major parts: the ionisation source, the mass analyser, where the different masses are separated according to their mass to charge ratio (m/*z*), and the detector. The data from detectors is fed into a computer where a final calculation of the isotopic ratios is performed. Two different types of mass analysers are employed in commercial TIMS instrumentation: magnetic sector (most widely used) and quadrupole. The latter is cheaper, smaller and easier to operate, but provides lower precision compared to the magnetic sector instruments (Crews *et al*, 1994). In addition, only one isotope signal measurement can be made at a time unlike multi-detector instruments with magnetic sector analysers. The present discussion focuses on magnetic sector TIMS, since this is the type of MS employed throughout the experiments. Fig. 1.5.2 is a schematic diagram of a magnetic sector TIMS. The VG354-TIMS at MLURI used for isotope ratio measurements in this thesis is pictured in Photograph 1.5.2. All TIMS instruments use a computer to control the major instrument functions: turret sample selection, heating up filaments, setting the magnetic field, peak searching and focusing, data collection and processing (Fassett and Paulsen, 1989).

Fig. 1.5.2: Schematic diagram of a magnetic sector TIMS components: ion source, mass analyser and ion detectors. All are kept under low pressure (10^{-6}-10^{-7}mBar).



Photograph 1.5.2: VG354 -Thermal Ionisation Mass Spectrometer.

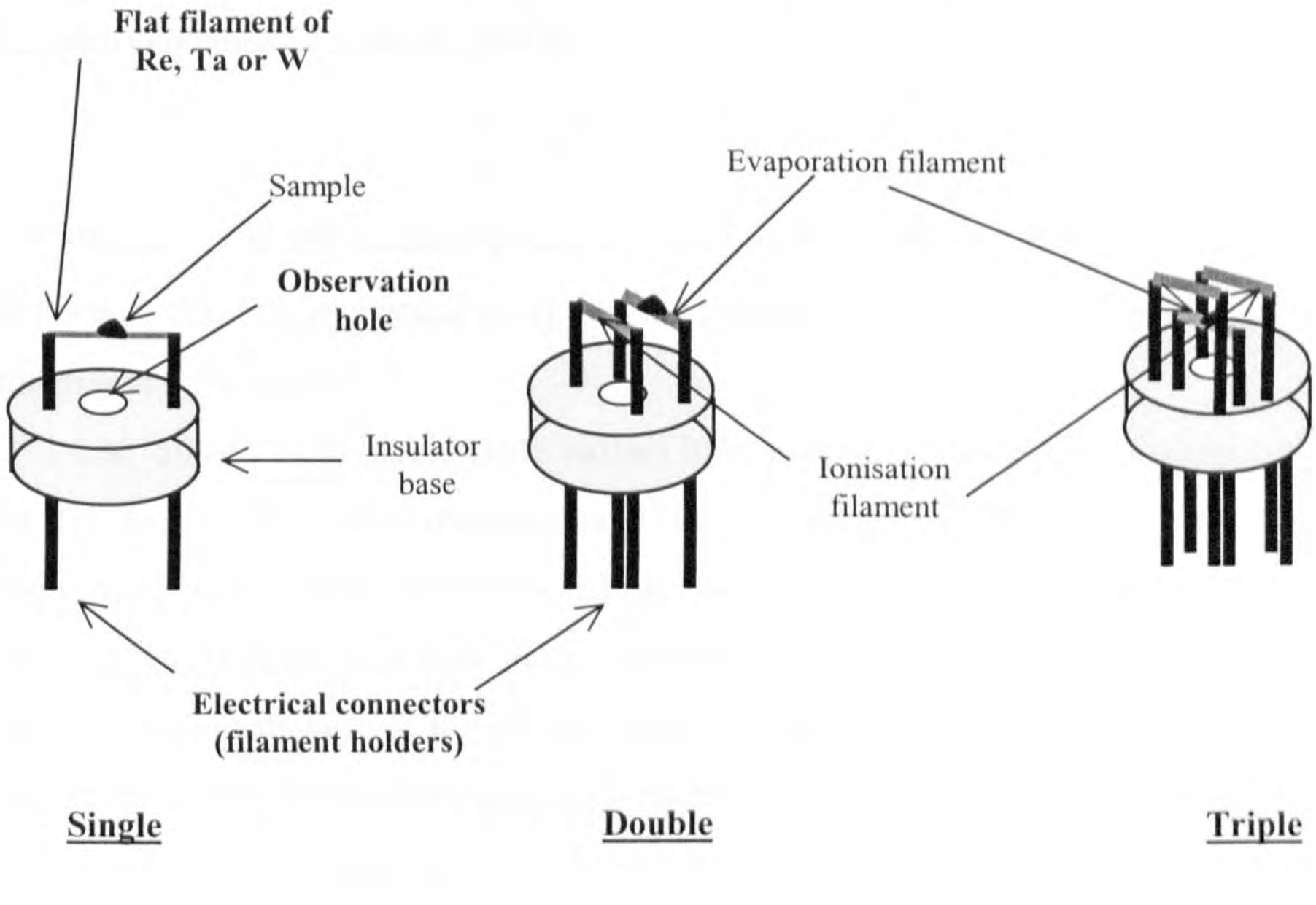


A. Loading of filaments and the ion source:

Three types of filaments (Fig. 1.5.3) are used in TIMS, the choice being dependent on the analyte and analysis protocol. In a single filament, the sample bead has a single filament used for both evaporating and ionising the sample, and was the technique used in our experiments. Double filaments have two filaments (wires), one is used for evaporating the sample, while the other for ionising. In the triple filament, the two side filaments are used for evaporating the sample while the centre filament is used for ionisation (Crews *et al*, 1994). Use of triple or double filaments separates the evaporation and ionisation processes and is especially important when dealing with elements that evaporate at low temperature such as Ca or Se, while on the other hand, sufficient ion yield is reached only at high filament temperatures (Crews *et al*, 1994).

Various methods are used to enhance ionisation and stabilise the ion beam particularly for metals with first ionisation energies greater than 7eV, such as Zn, 9.39; Cd, 8.99; Au, 9.22; Pt, 9.0eV. These techniques include: the use of dual and triple filaments and the addition of chemicals or enhancers to the filament (Bacon, 1996; Heumann, 1992). Heumann (1988) has reviewed chemicals used to enhance ionisation. These include silica gel, H_3PO_4, HBO_3, $AlCl_3$, and $Al(NO_3)_3$. The choice of method results from experiments aimed at optimising the loading procedure to obtain a sufficient number of ratio measurements accompanied by acceptable precision. The chemical form of the analyte, any additional reagents added, the nature of the filament metal and temperature used for evaporation all rather unpredictably influence the ionic species formed, the ionisation efficiency and the ion beam stability (Thirlwall, 1997).

Fig.1.5.3: Single, double and triple filament assemblies in glass beads.



Also, as TIMS analysis is time consuming, techniques that can speed up the analysis are valuable. In the present work, the possibility of analysing more than one element on the same filament was investigated as Cd and Zn were deposited onto the same filament. By analysing two elements together the instrument utilises half the normal number of filaments, and there is no need to reload the sample turret between samples, thereby avoiding the need to re-establish high vacuum conditions. This approach makes use of the fact that Cd and Zn have different first ionisation potentials.

Ion sources in TIMS instruments, are equipped with multi-sample turrets that accommodate a carousel containing pre-loaded filaments and allow automated processing of 13-20 samples per batch (Crews *et al*, 1994). The mass spectrometer used in this study has a 16 place carousel in the source chamber which is evacuated to $<3\times10^{-7}$mBar. The analyte deposited onto a filament is heated slowly under vacuum conditions by passing an electrical current through the filament. Analyte ions, emitted from filament surface, are

accelerated into the mass analyser by a high voltage (8kV) applied between the ion source and the accelerating slit. The fraction of ionised vaporised atoms is given by the Saha-Langmuir equation (Thirlwall, 1997):

$$\frac{n^+}{n^\circ} = e^{(W-E)/kT} \quad \text{.....1.5.8}$$

Where n° is the neutral atoms, n^+ the positive ions, W the work function of the filament, E the first ionisation energy of the material evaporated, T the temperature and k Boltzmann's constant.

Like all MS techniques, TIMS suffers from isotopic fractionation. Lighter isotopes are slightly more efficiently volatilised and ionised compared to heavier ones, so at the beginning of the analysis, the measured ratio of the heavier compared to the lighter isotope will be lower than the true ratio in the sample. By the end of the analysis, the ratio will be higher than the true ratio in the sample prior to analysis as a result of the enrichment in the heavier isotope as the analysis proceeds. Although the degree of fractionation is dependent on the element, it is typically 0.1-1% per atomic mass difference (Bacon, 1996), which means elements with low atomic mass will suffer from fractionation more than elements with higher atomic mass. However, fractionation usually does not influence the result substantially (Heumann, 1988). Fractionation can be taken into account either by calibration through using isotopic standards or by using internal normalisation (Fassett and Paulsen, 1989). The latter is only appropriate when at least 3 isotopes for a given element are available (Faure, 1986).

Filament temperatures range from 700-2000 °C depending on element ionisation energy, therefore filaments are made of elements with a high work function, and low volatility. Typical filament metals are rhenium, tantalum and tungsten (Thirlwall, 1997). As only singly charged ions are formed, because of the high ionisation energy of the element relative to the filament temperature, simple spectra are produced compared to other techniques such as ICP-MS (Crews et al, 1994). Ionisation methods result in forming both positive and negative ions as electron transfer can occur between the analyte and the filament in either direction depending on filament loading conditions and instrument set-up (Bacon, 1996).

Positive ionisation is used for metals with low first ionisation energy, <7eV, whereas negative ionisation is applied for non-metals and a number of transition metals (Heumann, 1992).

C. Mass analysers:

Magnetic sector analysers can be either single or double focusing. A single focusing analyser could have a circular path of 180, 90 or 60°, while the double focusing is only at 90° due to design structure (Thirlwall, 1997). The double focusing analyser gives higher resolution between the different ion masses as ions are first passed through a radial electrostatic field that focuses particles of the same kinetic energy into the slit leading to the magnetic sector. It works as an energy filter and decreases the broadening of the ion beam reaching the collector. This makes it possible to resolve ions differing by small fraction of a mass unit (Skoog, 1985). In the TIMS used throughout the experiments, an extended geometry mass analyser was used with a circular path of 127° instead of 90°. This design allows better resolution between the different masses. The principles of mass resolution in a magnetic field are described below (adapted from Skoog, 1985 and Faure, 1986).

When an ion of mass m and charge ze (where z is the number of electrons lost in the ionisation and e the electron charge) is affected by a potential difference of V volts, it gains energy E equal to

$$E = zeV = \frac{1}{2}mv^2 \quad \text{.....1.5.9}$$

where v is velocity. Re-writing the equation as

$$v = \sqrt{\frac{2zeV}{m}} \quad \text{.....1.5.10}$$

shows that ions of different masses have different velocities, but the same kinetic energy because they were accelerated by the same potential difference V.

When ions enter a magnetic field, the magnetic force F_M deflects them into circular path

$$F_M = Hzev \quad \text{.....1.5.11}$$

where H is the magnetic field strength and e is the charge on the ion. At the same time, this is balanced by the centrifugal force F_c expressed as

$$F_C = \frac{mv^2}{r} \quad \text{.....1.5.12}$$

where m is the ion mass and r is the radius of curvature. A particle must fulfil the condition that the two forces must be equal in order to traverse the circular path, this means:

$$Hzev = \frac{mv^2}{r} \quad \text{.....1.5.13}$$

and

$$v = \frac{Hzer}{m} \quad \text{.....1.5.14}$$

substituting equation 1.5.14 in equation 1.5.9 gives

$$\frac{m}{z} = \frac{H^2 r^2 e}{2V} \quad \text{.....1.5.15}$$

Different masses could be detected by varying one of the three (H, V or r).

The magnetic sector mass analyser consists of a flight tube that is curved into an arc of a circle between the poles of an electromagnet and is pumped to attain a high vacuum

(10^{-6}-10^{-7} Pa). The applied magnetic field deflects the ion beam along this flight tube at appropriate accelerating potential and magnetic field and resolving the ion beam according to the mass/charge (*m/z*) ratio. Either the magnetic field is set to a value such that the most abundant isotope is detected and the collimator focus voltage adjusted or the opposite where the magnetic field is scanned. Once a peak is located, it must be centred, that is the exact magnetic flux density determined for the ion beam to pass symmetrically into the collector (Thirlwall, 1997).

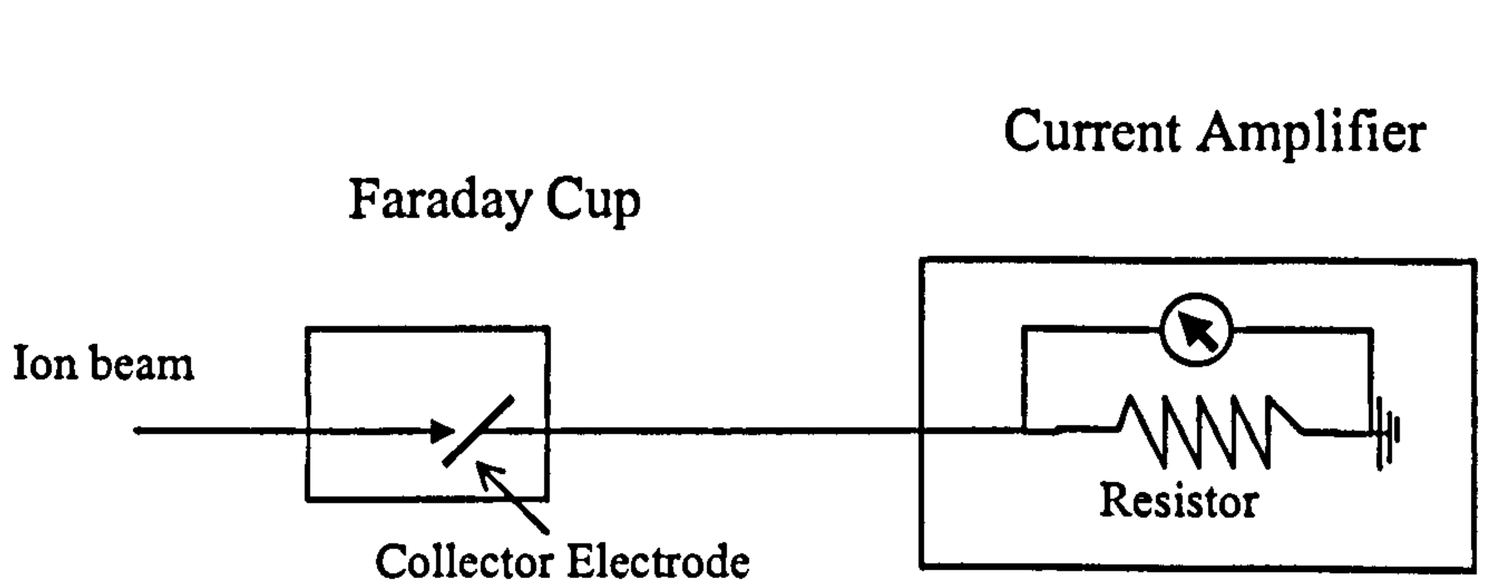
D. Ion detectors:

The three types of ion detection systems applied in TIMS are designed so that different ionic masses and signal strengths can be detected. These are:

- Faraday cups:

A Faraday cup Fig. 1.5.4 consists of a carbon-coated metal box with an inclined metal electrode that collects incoming ions (Thirlwall, 1997). Positive ions strike the electrode, which accumulates positive charge neutralised by an electric current flowing from earth through a large resistor ($10^{11}\Omega$), where according to Ohm's law, the potential difference across the resistor is proportional to the ion current. Single or multiple Faraday cups allow high precision measurements and are used when sufficient ion current is available (Crews *et al*, 1994). However, the electronic noise in the resistor restricts the Faraday cup to measurement of ion beam currents greater than 10^{-15}A (Thirlwall, 1997).

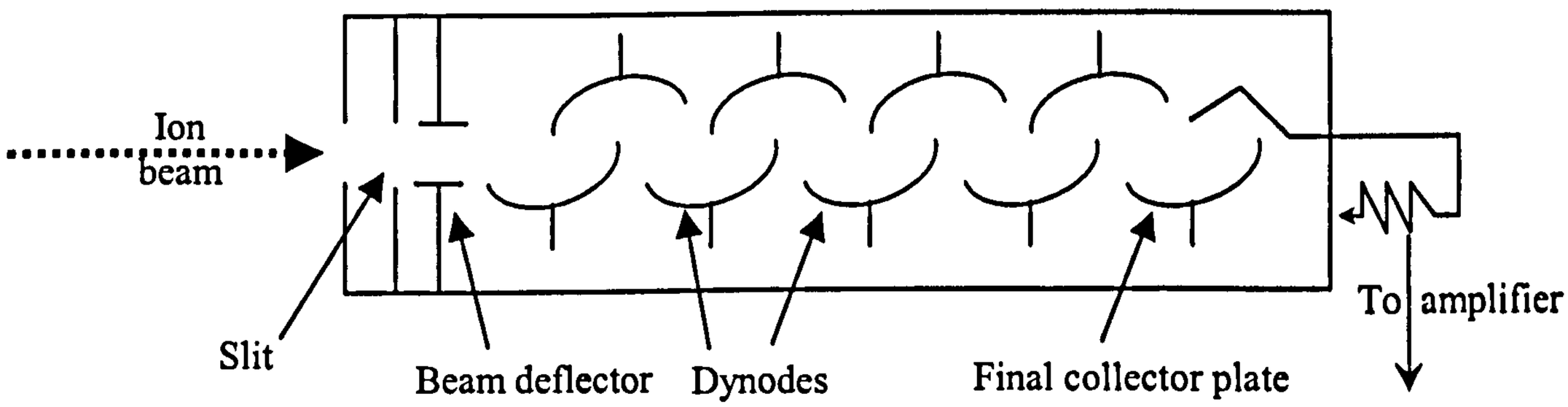
Fig. 1.5.4: Faraday cup collector schematic diagram.



- Electron multiplier:

This detector is used for measuring low intensity ion currents from small samples or low abundance isotopes (Crews *et al*, 1994). In the electron multiplier (Fig. 1.5.5) positive ions are accelerated to the first plate of multiplier, which are made of copper/beryllium alloy, which generates 2 electrons for each positive ion, which in turn are accelerated toward another plate and so on (Barker, 1999). After 20 stages, current amplification of about 10^7 is realised (Skoog, 1985).

Fig. 1.5.5: Schematic diagram of the discrete dynode electron multiplier (Barker, 1999).



- Daly multiplier detectors:

Like the electron multiplier, the Daly detector is used for measuring low intensity ion currents or low abundance isotopes (Crews *et al*, 1994). In the Daly multiplier system, an aluminised cathode at a negative potential is used to generate secondary electrons, which are deflected towards a scintillator emitting photons which are in turn counted by a photomultiplier (Thirlwall, 1997). The Daly system provides better peak flat, greater detector efficiency and stable gain than electron multipliers (Thirlwall, 1997).

Modern instruments could have one or more types of collectors, including up to 9 faraday collectors (Newman, 1996). Multicollector instruments allow simultaneous measurement of intensities of more than one ion beam over an array of collectors. In the instrument (a VG 354 magnetic sector thermal ionisation mass spectrometer, Micromass UK Ltd.) used throughout the experiments, 5 Faraday cup collectors system was used.

Multicollector systems offer three main advantages (Thirlwall, 1997):

1. The analysis time is reduced as more available ions are collected.
2. Short term instabilities in ionisation in the ion source could be overcome.
3. The ability to measure very large isotope ratios (up to 10^8) by Faraday and electron multiplier combinations.

Once the corresponding isotope peak is found, the ion beam intensity is increased by raising the filament current to obtain acceptable precision, provided that the sample loaded on the filament is not exhausted too rapidly as the more isotopic ratio measurements made, the more precise the measurement (Thirlwall, 1997). A major requirement of the collector analyser configuration for isotope ratio analysis by TIMS is "flat-topped peaks", which means that an entire ion beam of a specific isotope should pass the collector over a significant range in magnetic flux density (Thirlwall, 1997). The signal from the detectors is integrated over a few seconds interval and the current generated by the ions arriving at each detector is recorded. These data are fed into a computer and isotope ratios are then accumulated, usually in blocks of 10-25. Statistical analysis of the ratios is then made by the instrument software before averaged ratio data are printed out with associated precision estimates.

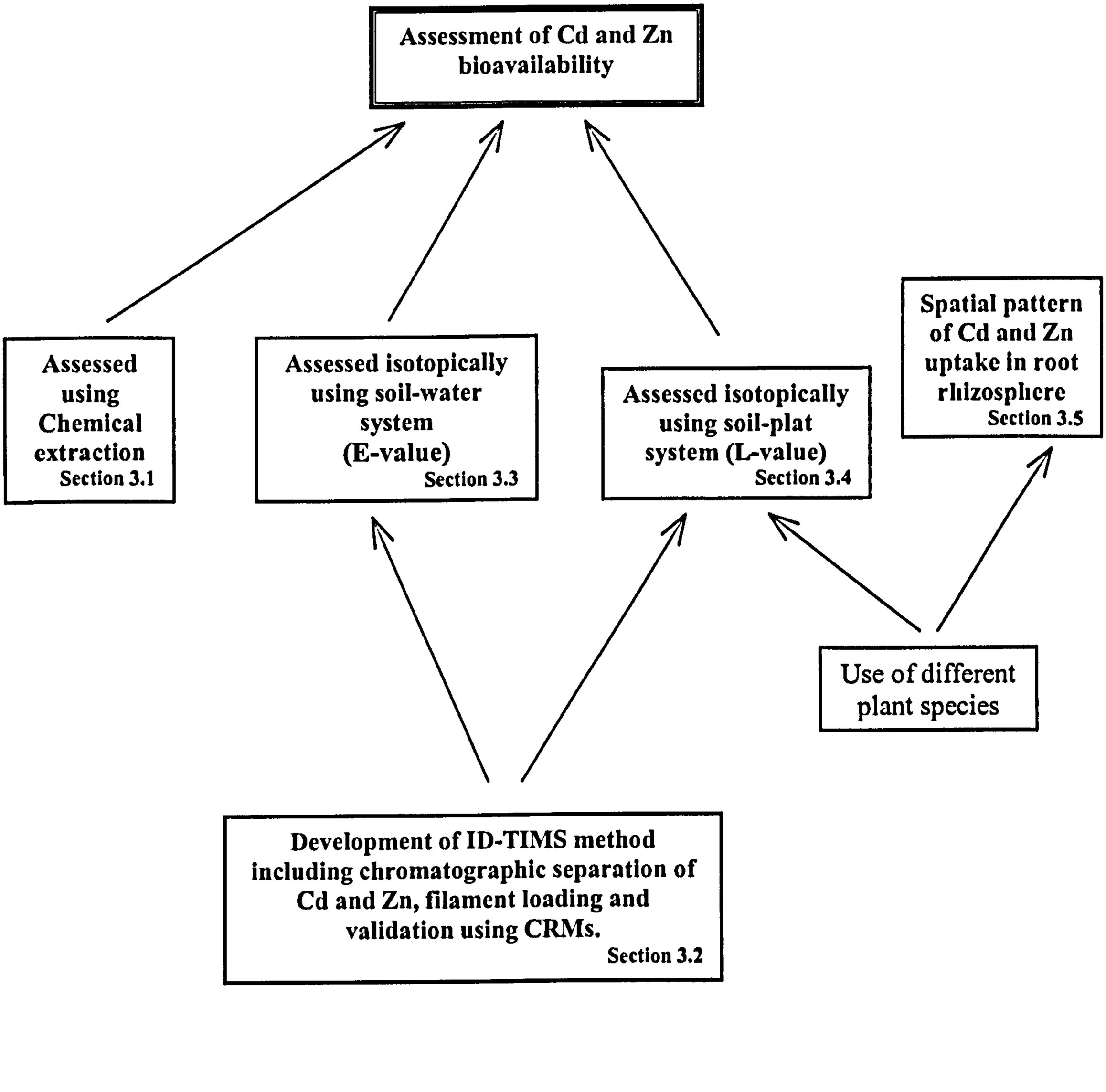
2. AIMS AND OBJECTIVES

The work presented here is to evaluate the measurement of Cd and Zn bioavailability using a novel stable isotope dilution technique. To achieve this, specific isotope analysis methodology using thermal ionisation mass spectrometry will be developed and validated. Cd and Zn bioavailability in two soils will then be assessed using routine chemical extractant methods and compared to that determined isotopically. Isotopic evaluations of bioavailability will then be made and be based on both a simple soil waster system and a soil plant system. The uptake of heavy metals by different plant species will be assessed and compared. Also, the spatial pattern of heavy metal uptake within the rhizosphere will be evaluated using a polluted soil. The overall plan to the thesis is given in Fig. 2.1.1 together with an indication of the sections which deal with each heading.

The main objectives of this project can be summarised as:

1. Develop methodology for the accurate and precise determination of isotope ratios of Cd and Zn using thermal ionisation mass spectrometry.
2. Validate this methodology by quantification of Cd and Zn in certified reference materials and assess the efficiency of various digestion procedures using the principles of isotope dilution.
3. Estimate the bioavailability of Cd and Zn in two soils using chemical extraction techniques.
4. Estimate the bioavailability of Cd and Zn in the same two soils using isotopic techniques in both a simple soil water system, and in a soil plant system. Compare results with objective 3.
5. Compare and contrast the uptake of Cd and Zn by different plant species; a Cd and Zn hyperaccumulator, a pollution indicator species and a crop plant.
6. Evaluate the spatial uptake of Cd and Zn within the root rhizosphere using a polluted soil.

Fig. 2.1: Overview of the scheme of the expreiments in this project.



3. EXPERIMENTS

3.1 SOIL EXTRACTION

3.1.1 Introduction:

Extraction remains the most commonly used method to estimate nutrient and metal availability to plants. In the present experiment, 5 extractants were used to compare their ability to extract Cd and Zn in two different soils. Apart from NaOH, these extractants have been extensively used in the literature for this purpose and represent a range from weak acids to neutral salts to strong bases. Both $CaCl_2$ and $NaNO_3$ have the advantage that the soil solution pH, which is of special importance, is unchanged by these extractants, and would represent a more accurate measure of the activity of the metal in the soil solution. In contaminated soils, these unbufferred salts results were more strongly correlated with appearance of toxicity symptoms compared to results achieved with dilute acid solutions or solutions containing complexing agents (Houba *et al*, 1996). Extraction methods are based on the assumption that ions associated with soil components will be displaced from the adsorption sites by the presence of an excess of competing ions in the extract (Kennedy *et al*, 1997). The extracting efficiency depends on many factors such as soil preparation, the extractant strength and volume, soil particle size and extracting time. In most procedures, the sampled soil is dried overnight at 105°C to remove moisture. Nevertheless, Ure (1996) explains the benefits of the use of field-moist soil samples in analysis as oxidation/reduction processes and the loss of volatile components during the drying process would be minimised. However, this is impractical from analytical point of view, as it is difficult to homogenise moist samples for representative sub-sampling. A 2mm sieve particle size, which we followed, is the norm for agricultural laboratories (Ure, 1996). A finer powder would allow more of the soil metal to exist in the soil water more easily. The time the extractant is kept in contact with soil particles is also important for adsorption-desorption processes to reach equilibrium.

0.1M sodium nitrate ($NaNO_3$):

Gupta and Aten (1993) recommended $NaNO_3$ for bioavailability studies of soils contaminated with heavy metals because it showed a higher correlation with plant metal concentration when compared to other extractants. Other reasons are:

1. 0.1M $NaNO_3$ being a neutral salt will not affect the soil pH (when compared to acids or bases), which plays an important role for the equilibrium between soil solution and soil solid phases.
2. The concentration of metals in the $NaNO_3$ solution extractant are found to be uniform (as a percentage of total) especially in anthropogenically contaminated soils having different soil properties (with regard to clay, organic matter and oxides content and the soils environment).
3. The 0.1M concentration of $NaNO_3$ in the extractant solution is comparable with that in soil solution under field conditions (which ranges between 0.05-2M).
4. Suitability for many metal detection techniques (such as GFAAS).
5. Very often low metal concentration predicts successfully the ecotoxic concentrations.

0.01M calcium chloride ($CaCl_2$):

Houba *et al* (1996) suggested that 0.01M $CaCl_2$ could be chosen as a universal extractant for many reasons:

1. The soil is extracted with a solution that has more or less the same concentration (0.01M) as the average salt concentration in a soil solution.
2. The divalent cation causes good coagulation in the suspension compared to salts of monovalent cations (Na^+, NH_4^+).
3. Since Ca^{++} is the primary cation on the adsorption complex of soils, the $CaCl_2$ solution is better able to extract other adsorbed cations than would solutions with other cations.
4. In addition to nutrients and heavy metals, soluble organic C, N, P and S can be determined, as they can be important for interpreting the influence of extracted metals and for evaluation of microbial transformations.

5. Since different nutrients and metals are measured, interpretation can also consider relationships between them.

$CaCl_2$ was used in 3 different molar strengths: 0.1, 0.05 and 0.01M and were proposed either as a simulation of soil solution or as an index of availability of Cd and Zn to plants (Young *et al*, 2000). However, all have extracted higher concentrations of Cd and Zn than expected. This may be due to the formation of chloro-complexes (Gupta and Aten, 1993) as chloride anions have a very high complexing ability (1meq/l Cl^- forms 5mg $CdCl^+$/l) (Verloo and Eeckhout, 1990).

0.5M sodium hydroxide (NaOH):

Extraction with NaOH is usually part of sequential extraction procedure of soil heavy metals and can extract the metal organically bound fraction (Chang *et al*, 1984; Kennedy *et al*, 1997). NaOH was chosen as a base solution to be compared with the neutral salts and the acetic acid.

0.43M acetic acid (CH_3COOH):

Acetic acid dissolves the exchangeable species but releases, in addition, the more tightly bound exchangeable forms (Ure, 1996). The pH of the soil solution has an important effect on the amount of metal extracted with acetic acid. This was the motive for using unbufferred solutions of inorganic salts (such as $CaCl_2$ and $NaNO_3$) to measure the extractable content as the pH of the soil suspension would be determined by mainly the soil and not by the extractant (Houba *et al*, 1996). The pH of soil solution being affected by acetic acid is the main concern where acetic acid may overestimate bioavailability.

0.05M ethylenediaminetetraacetic acid (EDTA) (pH = 7.0):

EDTA has been a basis for considering both metal toxicity and deficiency (Rowell, 1997). EDTA forms strong complexes with metal ions in solution causing metal desorption from soil surface particles. Ure *et al* (1993) indicated that EDTA can extract Cd (and possibly Zn) from oxide sites, organically bound and also carbonate bound Cd. This lead to the observation that EDTA may overestimate Cd plant available fraction and may be more indicative of the total Cd pool in the soil (Mann and Ritchie, 1993).

3.1.2 Materials and methods:

A. Great Billings and Countesswells soils:

The 2 soils chosen for the extraction procedure were also used in all the experiments in this thesis. The Countesswells soil is a non-contaminated soil, while the Great Billings soil is a sewage sludge amended soil with high levels of Cd and Zn.

Great Billings soil was collected by the soil science group at the Macaulay Land Use Research Institute (MLURI) from Great Billings sewage farm (Northampton) in 1991. 300kg of field moist soil was collected by multiple sampling to a depth of 10cm and bulked into polyethylene bags for transport to MLURI. The whole soil was air dried then sieved through a 2mm round hole sieve, mixed thoroughly and stored in tightly sealed polyethylene bags. This soil preparation forms part of the Community Bureau of Reference (BCR), Commission of the European Communities programme to make available a certified reference soil for a number of heavy metals determinations. The soil was named CRM 483 by the BCR (Quevauviller *et al*, 1997a) and a number of extraction results based on 0.05M EDTA and 0.43M acetic acid were then published (Quevauviller *et al*, 1997b). Indicative values were also given for extractions carried out using $CaCl_2$ and $NaNO_3$. Both certified and indicative values are summarised in Table 3.1.1a.

Countesswells soil was chosen as a non-contaminated soil. As part of this project, approximately 6kg were sampled from a field, under grass, adjacent to MLURI to a depth of 15cm and bulked into polyethylene bags. Plant debris was removed and the soil thoroughly mixed by hand and dried at 60°C overnight. After sieving through a 2mm sieve, the soil was air dried for seven days at 30°C with occasional mixing by hand. The soil Cd and Zn extractable content has not been certified, however the general properties of the soil have been discussed elsewhere (MacLeod *et al,* 1998). Both Great Billings and Countesswells soils were dried overnight at 105°C prior to all experiments including extraction.

B. Reagents:

Apart from $NaNO_3$, which was obtained from Fisons, Loughborough, UK, all other reagents used in this study were obtained from BDH, Poole, UK.

C. Extraction procedures:

For extraction, 10 replicates of each soil along with 4 blanks were carried out for each type of extractant. Raw materials of $NaNO_3$, $CaCl_2$, NaOH and EDTA used were dried overnight in a dry seal desiccator, with silica gel beads under vacuum, prior to preparing solutions.

0.1M sodium nitrate:

The procedure followed here is described by Gupta and Aten (1993) who recommended 0.1M $NaNO_3$ for bioavailability studies of soils contaminated with heavy metals. A 40g soil sample was transferred to a 250ml polyethylene extraction bottle, where 100ml of 0.1M $NaNO_3$ was added and the mixture was shaken on an end-over-end shaker (40rpm) for 2h at room temperature. The suspended soil samples were centrifuged at

3000×g for 10min and the supernatant filtered (using Whatman No 542, 12.5cm filter paper, previously rinsed with $NaNO_3$), into polyethylene bottles. Samples were then acidified with 2ml of 69%(w/v) HNO_3 to prevent microbial growth and stored at 5°C prior to analysis. Concentration calculations were based on a final volume of 102ml.

0.01M calcium chloride:

The procedure followed here follows that described by Novozamsky *et al* (1993). A 10g soil sample was transferred into a 250ml polyethylene extraction bottle, to which 100ml of 0.01M $CaCl_2$ were added and the mixture was shaken on an end-over-end shaker for 2h at room temperature. The extracts were left on the bench to settle then decanted into centrifuge tubes and centrifuged at 3000×g for 10min. The supernatant was then decanted into polyethylene bottles and stored at 5°C prior to analysis. Samples were clear and did not require filtration.

0.5M sodium hydroxide:

The procedure used here follows that described by Chang *et al* (1984). A 2g soil sample was transferred to a 250ml polyethylene extraction bottle, then 25ml of 0.5M NaOH were added and the suspension was shaken on an end-over-end shaker for 16h at room temperature. The suspension was centrifuged at 3000×g for 10min and 20ml of the supernatant were carefully transferred into a 25ml volumetric flask. The volume was made up to 25ml using 0.5M NaOH, and transferred to polyethylene bottles. Samples were stored at 5°C prior to analysis. The final dilution step was included so that we have sufficient amount of solution for analysis. All dilution factors were considered when calculating the final concentration.

0.43M acetic acid:

The procedure used here follows that described by Quevauviller *et al* (1997b). A 5g soil sample was transferred to a 250ml polyethylene extraction bottle to which 200ml of 0.43M acetic acid were added. The mixture was shaken in an end-over-end shaker for 16h at room temperature. The suspension was then immediately filtered through a filter paper (Whatman No 542, 12.5cm), previously rinsed with 0.05M EDTA followed by Millipore (Milli-Q) water, into 250ml volumetric flasks overnight. The volume was then made up to the mark with 0.43M acetic acid. The filtrates were then transferred to polyethylene bottles, and stored at 5°C prior to analysis.

0.05M ethylenediaminetetraacetic acid (EDTA) (pH = 7.0):

The procedure used here follows that described by Quevauviller *et al* (1997b). A 5g soil sample was transferred to a 250ml polyethylene extraction bottle to which 50ml of 0.05M EDTA (pH adjusted to 7.0) solution were added. The suspension was then shaken on an end-over-end shaker for 1h at room temperature, then immediately filtered through filter paper (Whatman No 542, 12.5cm), previously rinsed with 0.05M EDTA followed by Millipore water into 50ml volumetric flasks overnight. The volume was made up to 50ml using EDTA, then transferred to polyethylene bottles and stored at 5°C prior to analysis.

D. Analysis:

Cd in Countesswells soil extracted using $NaNO_3$, acetic acid and EDTA were analysed using GFAAS (GTA-96, Varian UK, at a wavelength of 228.802nm) because concentrations were below the limit of quantification (LOQ) of ICP-AES (around 0.003mg/l). However, Countesswells soil extracts using NaOH were lower than the LOQ of GFAAS (0.00037mg/l) and were not determined. The rest of the extractants were analysed

for Cd and Zn using ICP-AES (IRIS, Thermo Jarrel Ash UK, at a wavelength of 228.802nm for Cd and 213.856nm for Zn), with LOQ around 0.003mg/l for both elements.

3.1.3 Results:

The extractable Cd and Zn content for Great Billings and Countesswells soils with the corresponding standard error (SE = SD/√n) are shown in Table 3.1.1a and b, respectively.

Table 3.1.1a: Extractable Cd and Zn content (µg/g) of Great Billings soil, where values are the mean ± SE and n =10 for each determination.

	Determined		Certified		Indicative	
Extractant	**Cd**	**Zn**	**Cd**	**Zn**	**Cd**	**Zn**
0.1M $NaNO_3$	0.147±0.002	4.10±0.02	[a]	[a]	0.08±0.03	2.7±0.8
0.01M $CaCl_2$	0.427±0.003	8.16±0.1	[a]	[a]	0.45±0.05	8.3±0.7
0.5M NaOH	12.8 ±0.22	273 ± 2.6	[a]	[a]	[a]	[a]
0.43M CH_3COOH	19.5±0.15	608±4.9	18.3±0.3	620±12	[a]	[a]
0.05M EDTA	23.0±0.12	581±2.5	24.3±0.7	612±10	[a]	[a]

[a] No data available.

Table 3.1.1b: Extractable Cd and Zn content (µg/g) of Countesswells soil, where values are the mean ± SE and n =10 for each determination.

	Determined	
Extractant	**Cd**	**Zn**
0.1M $NaNO_3$	0.0061±0.0002	0.051±0.003
0.01M $CaCl_2$	0.016±0.001	0.21±0.010
0.5M NaOH	[a]	2.15±0.032
0.43M CH_3COOH	0.148± 0.002	4.28±0.051
0.05M EDTA	0.097± 0.004	2.05±0.022

[a] Below LOQ of GFAAS of 3.7µg/l.

Despite the fact that these extractants (with the exception of NaOH), are frequently used to determine bioavailability, the results show the big difference between the amount of Cd and Zn extracted by each extractant. For both soils, acetic acid and EDTA extracted the most while $NaNO_3$ extracted the least. When compared with the certified values for Great Billings soil, Cd extracted with acetic acid (19.5±0.15µg/g) and Zn extracted with EDTA (581±2.5µg/g) were significantly different from the certified values (at $P<0.05$, using CoStat 3.03, 1986, CoHort Software, Berkley, USA). Table 3.1.2 lists the t-test values for Cd and Zn. However, Cd extracted by EDTA (23.0 ±0.12µg/g) and Zn extracted by acetic acid (608±4.9µg/g) were not significantly different from the certified values. Although comparison with the indicative values can not be made, good agreement for extraction by $CaCl_2$ was obtained.

Table 3.1.2: T- test ($P<0.05$) for Cd and Zn between the determined and the certified values.

Metal	$t_{Critical}$	$t_{Calculated}$	
		0.43M CH_3COOH	0.05M EDTA
Cd	2.26	6.76	1.58
Zn		1.93	7.70

Because of the similarity of the amounts of Cd or Zn extracted with acetic acid and that extracted with EDTA in Great Billings soil, a t-test was carried out to see if there was a significant different between the 2 extractants. Both extractants were significantly different for both elements ($t_{Calculated}$: 22.9 for Cd, 4.88 for Zn, $t_{Critical}$: 2.26, at $P<0.05$). For Countesswells soil, no t-tests were carried out as there are no certified values for extraction, and the Cd and Zn extracted with the different extractants were different.

The relative standard deviations RSD% (RSD%=100SD/mean) for replicates of each extractant (n=10) are shown in Table 3.1.3. There was no evidence of gross contamination as blanks had both elements below the limit of quantification of the instruments used.

Table 3.1.3: RSD% values for the different extractants.

	RSD%			
	Great Billings		Countesswells	
Extractant	**Cd**	**Zn**	**Cd**	**Zn**
0.1M $NaNO_3$	3.2	1.6	8.9	19.3
0.01M $CaCl_2$	3.3	3.4	14.9	15.8
0.5M NaOH	5.5	3.1	[a]	4.8
0.43M CH_3COOH	2.4	2.6	3.6	3.6
0.05M EDTA	1.6	1.4	14.6	3.2

[a] Not determined.

The concentration of Cd and Zn in the various extractants appeared to be correlated in that the higher Cd content, the higher Zn content and *vice versa*. This might have been caused by the similarity in Cd and Zn chemistry and the co-occurrence of the both elements in the ores and rocks. To test this correlation, a graph of Cd versus Zn concentrations was plotted for each extractant (n=10) and a straight line fitted to the data from which the correlation coefficient (R^2) was calculated (Table 3.1.4). The correlation coefficient for EDTA was markedly higher than that of the other extractants (0.92 compared to that of 0.23 in $NaNO_3$ for Great Billings soil).

Table 3.1.4: Correlation coefficients (R^2) between Cd and Zn.

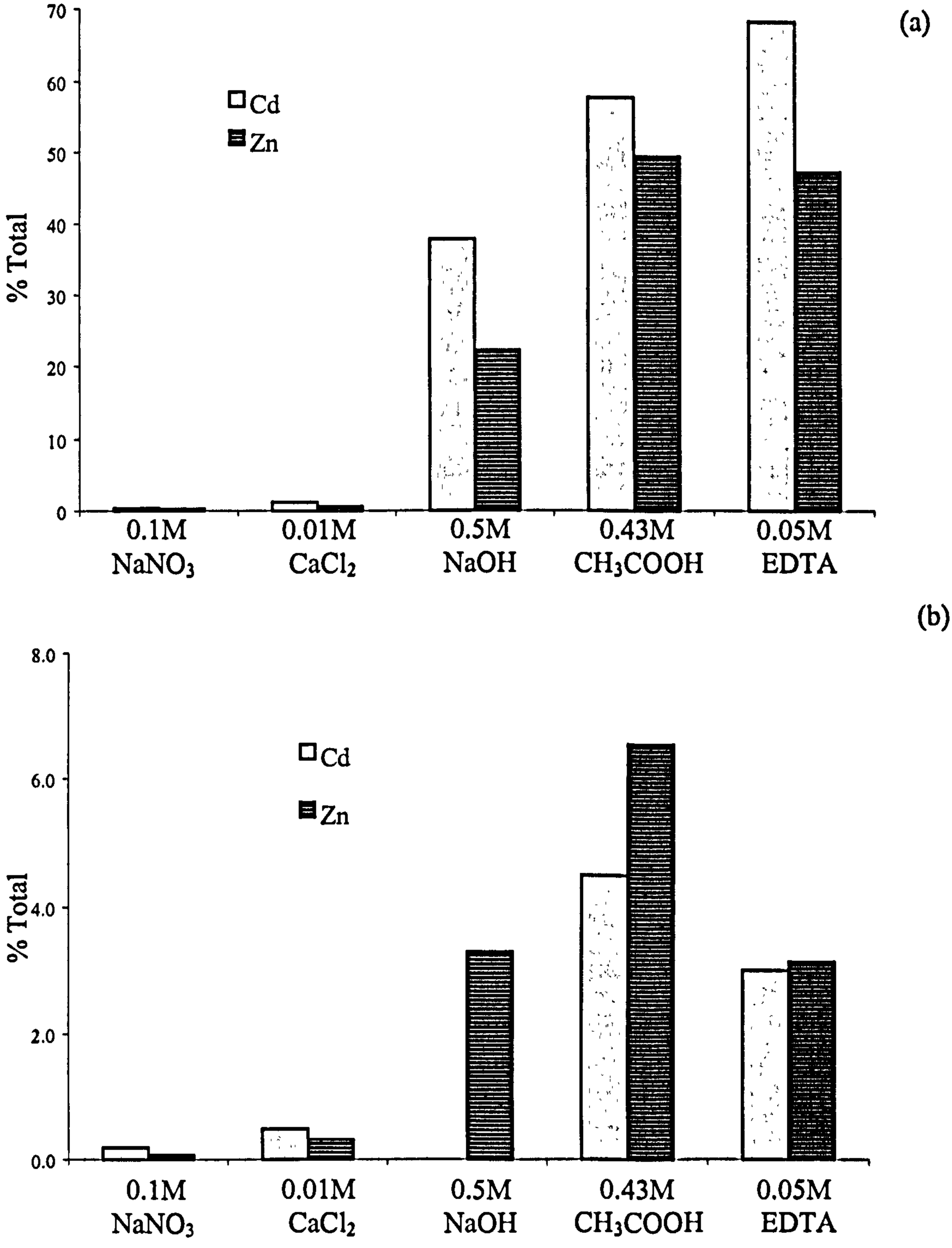
	Great Billings	Countesswells
Extractant	**R^2**	**R^2**
0.1M $NaNO_3$	0.23	0.13
0.01M $CaCl_2$	0.62	0.19
0.5M NaOH	0.30	[a]
0.43M CH_3COOH	0.83	0.48
0.05M EDTA	0.92	0.90

[a] Not determined.

Another way to show the different efficiency of the extractants used, a graph of the extractable Cd and Zn was plotted as a percentage of the total Cd or Zn soil content (total

metal was determined by ID *aqua regia* digestion, Section 3.2.4E). This is shown in Fig. 3.1.1 (a) for Great Billings soil and (b) for Countesswells soil.

Fig. 3.1.1: Percentage of Cd and Zn extractable content compared to totals of Great Billings (a) and Countesswells (b) soils.



3.1.4 Discussion:

The agreement between the certified and the experimentally determined acetic acid and EDTA values (Table 3.1.1a) was generally good (less than 6% difference from the certified value for either Cd or Zn) and is an indication of the robustness of these techniques. However, Cd extracted with acetic acid and Zn extracted with EDTA were significantly different from the certified values (at $P<0.05$, Table 3.1.2). T-test showed also that the amount of Cd extracted with acetic acid or with EDTA was significantly different (at $P<0.05$), despite the closeness of the 2 values. The same also applies to Zn. This is related to the different complexing ability of the extractant with the metal in a given soil.

In the extraction with $CaCl_2$, the indicative values for Cd and Zn (0.45 and 8.3µg/g) compare well with the experimental values (0.43µg/g Cd and 8.2µg/g Zn). In the extraction with $NaNO_3$, the experimental values for Cd and Zn (0.147 and 4.1µg/g) were higher than the indicative values (0.08µg/g Cd and 2.7µg/g Zn). This may be referred to the different end-over-end shaker speed and the different centrifuges (in terms of g) used in the 2 procedures.

Extractable Cd ranged from 0.44 to 68% and from 0.19 to 4.5% of total Great Billings and Countesswells soils, respectively. Extractable Zn ranged from 0.33 to 49.4% and 0.08 to 6.6% of total Great Billings and Countesswells soils, respectively. The wider range for both elements in Great Billings compared to Countesswells may have risen from the fact that Cd and Zn in Great Billings were introduced to the soil by sewage sludge addition. Sewage sludge application introduces metal in organic and inorganic complexes which affects the amount of metal extracted depending on their solubility in the extractant present and how strongly are they bound to the soil particles. The amount of Cd or Zn extracted by each extractant was different with EDTA and acetic acid extracting the most. The percentage of the extracted metal compared to the total soil metal was consequently different (*See* Fig. 3.1.1). EDTA extracted as much as 70% of total Great Billings Cd compared to only 3% of that in Countesswells soil. The amount extracted with EDTA for Great Billings soil agrees with a similar study where Cd ranged from 16 to 63% of total Cd

using 10 different soils from predominantly pasture sites (Gray *et al*, 1999). However, the mount of Cd extracted with EDTA in the Countesswells soil is still very low compared to the Gray *et al* (1999) results. In addition, each extractant had extracted a different percentage of the total for the same element among the 2 soils. EDTA, for example, extracted 47.2% of total Zn in Great Billings compared to only 6.1% in Countesswells soil. This may be attributed to the difference in Cd and Zn species present as well as the physical and chemical properties of each soil.

The 2 salts ($CaCl_2$ and $NaNO_3$) extracted much lower amounts of Cd and Zn than the other 3 extractants with $CaCl_2$ extracting higher amounts than $NaNO_3$. This may be related to the very high ability of chloride anion in the formation of Cd or Zn complexes (Ure, 1996; Verloo and Eeckhout, 1990). Cd extracted with calcium chloride was about 1.3% and 0.5% of total Cd in Great Billings and Countesswells soils, respectively. In a similar study, Cd extracted with 0.01M $CaCl_2$ was in the range 0.9 to 8.4% of total soil Cd in 10 different soils (Gray *et al*, 1999). Cd extracted with $NaNO_3$ was 0.4% and 0.2% of total Cd in Great Billings and Countesswells soils, respectively, which is lower than that of 4% found by Gupta *et al* (1996). The determined values of Zn were 1.3% and 0.1% of total Zn, compared to that of 0.25% found by Gupta *et al* (1996).

The big difference in the amount of extractable Cd or Zn for the above tested extracting agents is related to the individual extractant ability to complex and hence mobilise Cd or Zn. Both EDTA and acetic acid are capable of complexing with the metal exchangeable, organically bound and the carbonate bound phases in the soil (Ure, 1996) thus extracting a wide range of metal species. In addition, 0.43M acetic acid is suggested to extract the more tightly bound inorganic soil heavy metals (Kennedy *et al*, 1997). Although NaOH and $NaNO_3$ are suggested to complex with the same organically bound metals (Kennedy *et al*, 1997), the 2 extractants showed a big difference in their Cd and Zn extraction efficiency.

From the previous data, the tested extractants could be classified into 3 bands according to their ability to extract Cd and Zn. $NaNO_3$ and $CaCl_2$ salts could be classified as weak extractants, NaOH as a moderate extractant while both acetic acid and EDTA can be considered strong extractants as they extracted the highest levels of Cd and Zn. Both

EDTA and acetic acid had nearly identical Cd and Zn extractable content. However from analytical point of view, EDTA produced more precise and reproducible results (in terms of RSD% values) in soils with high levels of Cd and Zn, while acetic acid was better in non-contaminated soils. The extraction results are discussed in relation to the other approaches in this thesis for bioavailability determination of Cd and Zn in the general discussion in Section 4.1.

From the results above, the extractants were either too weak (such as 0.01M $CaCl_2$) or too strong (such as 0.05M EDTA), resulting in huge differences in estimating the metal bioavailable pool (Young *et al*, 2000). This in fact puts a question mark on the reliability of these extractants in bioavailability studies. Gupta *et al* (1996) categorised the metal fractions within the soil dynamic system into:

- the mobile fraction (soluble, very active and bioavailable),
- the mobilisable fraction (potentially available and partly active) and
- the pseudo total content (inactive and inert).

The neutral unbuffered salts of $NaNO_3$ and $CaCl_2$ were classified to be able to characterise the mobile fraction (active and readily available to plant) of soil metal. On the other hand, the strong EDTA and acetic acid chelating agents were described to be suitable to predict the mobilisable metal fraction in soil (Gupta *et al*, 1996). The pseudo total fraction or the residue, other than the mobile and mobilisable fractions, is extracted using more vigorous extractants such as concentrated nitric acid. Although this characterisation is similar to that in sequential extraction, it is aimed at predicting the metal available to plant using single extractants.

The above categorising of the soil fractions can also be useful in the risk assessment of polluted soils to human health. The metal fraction extracted by neutral salts for example represents an immediate health risk, while the fraction extracted with strong chelating agents predicts the potential health risk in the future. This would help to roughly distinguish between harmful, potentially harmful and harmless heavy metal concentrations in the soil (Gupta *et al*, 1996).

Within samples extracted with each extractant, the correlation between Cd and Zn concentrations was observed where higher amounts of Cd were associated with higher amounts of Zn and *vice versa*. In addition, the higher the extractable amounts of Cd and of Zn, the more correlation between them. Among all the extractants tested, EDTA represented the highest correlation between Cd and Zn extractable content, with R^2 values of 0.92 and 0.90 for Great Billings and Countesswells soils, respectively. Although the strong chelating agents of EDTA and acetic acid extracted similar amounts of the 2 elements, the correlation between Cd and Zn in acetic acid was poorer than that in EDTA (*See* Table 3.1.4). However, the reason for such behaviour is not clear.

Precision values, expressed as RSD%, were presented in (Table 3.1.4) above and represent the reproducibility of extracting Cd and Zn using each extractant (Ure, 1996). In general, extraction procedures produced more precise results for both Cd and Zn in Great Billings compared to Countesswells soil. This may be because of the higher Cd and Zn concentrations in Great Billings compared to Countesswells soils. Within Countesswells soil, the lowest precision was observed in the extraction with the relatively weak extractants $NaNO_3$ and $CaCl_2$. The losses in Cd and Zn during sample preparation such as decanting the supernatant and/or filtering after centrifuging may have caused this. Although Cd extracted with acetic acid, EDTA and sodium nitrate in Countesswells soil was determined on GFAAS, the instrument does not seem to have improved the RSD% compared to that using the ICP-AES (See Table 3.1.4 above). This in fact decreases the significance of the analytical instrumentation used and its impact on the RSD% values, hence precision and reproducibility. In the contaminated Great Billings soil, the RSD% values were comparable despite large differences in extractable metal from each extractant (where all measurements were made on the ICP-AES). For example, Cd extracted by EDTA was 156 times that extracted by $NaNO_3$ although their RSD% values are not very different (1.6% compared to 3.2%).

3.1.5 Main findings:

1. The poor extraction of Cd or Zn in Countesswells soil compared to the contaminated Great Billings soil indicating a difference in behaviour of the 2 soils.
2. In general, precision, expressed as the RSD%, was better where Zn and Cd were extracted at higher levels.
3. In Great Billings soil, the experimental values compared well with certified values.
3. The extractable content is arranged in the following ascending order:

- Great Billings:

  Cd: $NaNO_3$ <$CaCl_2$ <NaOH< Acetic Acid <EDTA

  Zn: $NaNO_3$ <$CaCl_2$ <NaOH< EDTA < Acetic Acid

- Countesswells:

  Cd: $NaNO_3$ <$CaCl_2$ < EDTA < Acetic Acid

  Zn: $NaNO_3$ <$CaCl_2$ <NaOH< EDTA < Acetic Acid

4. In general, the higher the extractable amounts of Cd and Zn, the greater the corrclation between the concentration of these elements.

3.2 QUANTIFICATION OF Cd AND Zn BY ID-TIMS

This chapter has been split into two sections; the first covers the characterisation of the isotope spikes (^{111}Cd and ^{67}Zn) used throughout this project in terms of the isotopic and elemental composition. The second section goes on to use these isotope spikes to quantify Cd and Zn in certified reference materials (CRMs). The CRMs were digested using three different methods; open tube *aqua regia*, sealed bomb microwave and HF techniques. Finally, the elements of interest were separated from the resulting digests and analysed on the thermal ionisation mass spectrometer (TIMS). This analysis was undertaken by loading a single filament either with Cd or Zn alone, or by dual loading a single filament with both elements and analysing them sequentially.

3.2.1 Materials and reagents:

The CRMs chosen in this study were of contrasting materials and had differing Cd and Zn content (Table 3.2.1).

Table 3.2.1: CRMs codes, material, Cd and Zn content (μg/g, mean ± 95% confidence interval).

Code	Material	Cd	Zn
GBW07401 [a]	Soil	4.3±0.2	680±11
GBW07405 [a]	Soil	0.45±0.04	494±11
CRM 60 [b]	Aquatic Plant	2.20±0.10	313±8
SRM 1566a [c]	Oyster tissue	4.15±0.38	830±57

Obtained from: [a] Institute of Geophysical and Geochemical Exploration, Langfang, China; [b] Laboratory of the Government Chemist, Middlesex, UK; [c] National Institute of Standards and Technology, Gaithersburg, MD, USA.

Enriched isotopes spikes of ^{111}Cd and ^{67}Zn were supplied by CK Gas Products (Wokingham, Berkshire, UK). Cd and Zn atomic absorption (AA) standards and all

analytical reagents used in this study were obtained from BDH chemicals (Poole, Dorset, UK). High purity Millipore (Milli-Q) water was used throughout with a metered resistivity ≥18MΩ.cm and was obtained from an Elgastat UHP system (Elga Ltd, Bucks, UK). Anion exchange resin: AG 1-X8 strong anion exchange resin- chloride form, 200-400 mesh (Biorad Laboratories, Hercules, CA, USA) was used for the purification of Cd and Zn. Silica gel suspension was prepared by shaking 1g of finely ground silica gel in 15ml of 0.66M H_3PO_4 overnight. The suspension decanted after a settling period of 28min was then used.

3.2.2 Methods:

Throughout the present work, samples were prepared on clean benches in an environment suitable for trace analysis. Glassware and other equipment used were cleaned by soaking in "Decon" detergent overnight, rinsed with deionised water, soaked in conc. HNO_3 for 3h, and finally rinsed thoroughly with high purity Millipore water and air dried. Protective gloves and clothing were also used. In addition, the number of steps required for sample preparation was also minimised to reduce systematic errors and contamination possibilities.

A. Preparation and characterisation of Cd and Zn spikes:

Stock solutions of the artificially enriched stable isotope spikes were prepared as follows: 10.233mg of ^{111}Cd (95 Atom %) and 18.828mg of ^{67}Zn (90 Atom %) were weighed into separate 50ml volumetric flasks. 1.5ml of 69% HNO_3 were then used to dissolve each spike, and the volume was made up to the mark using Millipore water to give a 0.5M HNO_3 solution. Working standard solutions of the spikes were prepared by weighing an accurate amount of each spike into a 50ml volumetric flask and the volume was made to give a 0.1M HNO_3 solution. Both stock and working standard solutions were stored in well-sealed polyethylene bottles, in a cold room. The exact isotopic composition of

each spike, and as a check a natural standard, was determined using the TIMS. Five replicates of each spike and a natural atomic absorption standard were analysed for their isotopic composition on the TIMS (following loading procedures described later in Section D below), from which the average atomic masses were calculated.

The exact concentration of both working solution spikes (Cd and Zn) was determined by Reverse-IDMS and as a check using ICP-AES. Reverse isotope dilution mass spectrometry (R-IDMS) uses a natural standard, typically an atomic absorption standard, as the 'spike' and the actual enriched spike is treated as a sample. Using this approach, and the following known parameters: the elemental composition of the natural 'spike', its isotopic composition which in most cases can be taken as the IUPAC values and the isotopic composition of the enriched spike, the elemental composition of the latter can be determined. A check was conducted using ICP-AES of the made up enriched spike solutions. For all future calculations, the concentrations determined by R-IDMS were used.

Having characterised the made up spike solutions (in terms of both isotopic and elemental composition) and AA standard solutions, a further check was made to ensure the isotopic characterisation was correct. A series of check solutions containing a mixture of spike and AA standard were prepared for each element. The atom% of Cd (the number of atoms of ^{111}Cd divided by the number of atoms of both ^{111}Cd and ^{114}Cd) was in the range 40-93%, while from 9-83% for Zn (atoms of $^{66}Zn/(^{67}Zn$ and $^{66}Zn)$) over the prepared solutions. As the amounts of both spike and AA standard solutions mixed are precisely known, the theoretical expected isotope ratio of selected isotopes ($^{111}Cd/^{114}Cd$ and $^{66}Zn/^{67}Zn$) were calculated. At the same time, the isotopic ratios of these solutions were measured on the TIMS. The two values were compared to check that both the isotopic and elemental characterisation of the spike and the AA standard solutions was correct. This is an essential pre-requisite of any IDMS work.

B. Digestion methods:

Each CRM was dried in the oven at 105°C overnight to remove moisture. Replicate aliquots of 0.5-2g of each CRM were dried at 105°C for 8h in silica crucibles, to which an

exact amount of the fully characterised ^{111}Cd and ^{67}Zn spike solutions were added. The amount of spike solution added (2-5g) was adjusted to provide a ratio of 1:1 for $^{111}Cd/^{114}Cd$ and $^{66}Zn/^{67}Zn$. The amount of spike added was based on certified Cd and Zn content of the material. Each sample was then mixed with a plastic spatula. The spiked CRMs were then dried again and organic matter removed by igniting in a muffle furnace for 12h at 450°C. The CRMs were then digested using three different digestion procedures described below.

Open tube (aqua regia) digestion:

The samples were transferred into the digestion tubes, to which 20ml of *aqua regia* (3:3:2, $HCl:H_2O:HNO_3$) were added, the samples were then heated at 140°C for 10h on a heating block (Gerhardt digestion system, Gerhardt UK Ltd., UK). 10ml of 50% HNO_3 were then used to dissolve the residue by heating at 80°C for 40min. Samples were left to cool to room temperature then filtered overnight (Whatman No 542, 12.5cm). The filtrate was then dried under heat lamps in preparation for separation of Cd and Zn by ion chromatography.

Microwave HNO_3 digestion:

Samples were transferred into polytetraflouroethene (PTFE) microwave bombs, to which 12.5ml of 69% (w/v) HNO_3 were added. The contents were then heated for 1min at 70% power, then 9min at 100% power (600W) using a microwave oven equipped with a fume extraction system (Floyd Inc., UK). After cooling overnight, the contents were filtered (Whatman No 542, 12.5cm) into glass containers and dried under heat lamps ready for chromatographic separation.

Closed bomb (HF) digestion:

The samples were transferred into PTFE bombs, to which 10ml of 48% HF, and 2ml of 69% HNO_3 were added. The bombs were then tightly sealed and placed on a hot plate at approximately 180°C for 6h. After cooling overnight, the bombs were carefully opened and the contents evaporated using a hot plate. 3ml of 69% HNO_3 followed by 5ml of Millipore water were then used to transfer and filter the residue into glass containers. Again the sample solution was dried under heat lamps to be loaded onto the ion exchange column. Because of the hazards involved in handling HF, extreme care was taken during the procedure.

C. Chromatographic separation:

This perhaps is the most challenging aspect of sample preparation for TIMS analysis; the isolation of a relatively pure element from a complex matrix can be extremely difficult. An anion exchange chromatography procedure was developed to isolate Cd and Zn from digested materials. This procedure used a combination of different strength HCl acid solutions (Kraus and Moore, 1953) and a HBr/HNO_3 mixture (Strelow, 1978).

To condition the ion exchange columns (10mm internal diameter ×100mm length, Omnifit, Cambridge, UK) prior to separation procedures, 6M HCl, H_2O, 6M HCl each for 10min was pumped through the columns using a peristaltic pump set at a flow rate of 0.5 ml/min. Digested samples, dissolved in 1-2 ml of 6M HCl, were loaded onto the exchange columns. The separation of Cd and Zn from other elements present in samples was achieved after several trials using different reagents and different strength. To asses the efficiency of Cd and Zn separation following a specific elution protocol, fractions were collected every 2 min and analysed on the ICP-AES for Cd, Zn and other elements present. The best separation procedure developed involved sequential elution of HCl, HBr and HBr/HNO_3 through the ion exchange column following the elution protocol described in Table 3.2.2. Zn was collected from 90-104min from the start of the elution protocol using 0.01M HCl, while Cd was eluted

using HBr/HNO_3 mixture from 125min till the end. Cd and Zn fractions collected were dried under heat lamps to be loaded onto filaments in preparation to measure isotope ratios by TIMS.

Table 3.2.2: Elution protocol for the separation of Cd and Zn.

Reagent	Time (min)
6M HCl	20
4M HCl	14
2.5M HCl	20
0.5M HCl	30
0.01M HCl	20
1M HBr	10
0.01 M HBr / 4M HNO_3	30

D. Filament single and dual loading procedures:

The filament loading procedure plays an important role in obtaining a successful analysis (i.e. sufficient number of ratio measurements and acceptable precision). Initially, a number of trials using atomic absorption standards were conducted before moving on to the CRMs. Filament loading started with an investigation of a single loading procedure for Cd and Zn, then proceeded to the dual loading technique to allow the sequential analysis of both elements on the same filament.

Single loading procedure:

The minimum amounts of Cd or Zn required to give 50 and 100 ratio measurements (considered as sufficient number of ratio measurements) for Cd and Zn, respectively using AA standards and real (soil) samples was initially investigated. Rhenium filaments were

loaded with decreasing amounts of Cd and Zn and analysed on the TIMS. The best loading procedure developed for both elements was as follows:

- 1µl of silica gel suspension in 0.66M H_3PO_4 was loaded onto a single rhenium filament using a microsyringe, a drop (0.3µl) at a time and dried into a spot using a current of 1.1A.
- A quantity equivalent to 1µg of Zn in 0.1M HNO_3 was added in the same manner.
- The filament was then gradually heated to a current of 2.4A and held at this current momentarily, to fuse the sample to the filament.

Care was taken to ensure loading the sample onto the filament occurred in as small a spot as possible as broad spots seemed not to work as efficiently as small spots when analysed on the TIMS.

- Cd was dissolved in 10-200µl of the silica gel suspension and a quantity equivalent of 0.064-0.10µg Cd was directly loaded onto a single rhenium filament and dried into a spot using 1.1A.
- The sample was fused to the rhenium wire by heating gradually to a current of 2.4A.

Dual loading procedure:

In dual loading, Cd and Zn were deposited onto the same single rhenium filament following the procedure above but proceeded by loading 1µl of the silica gel solution. To ensure that Cd or Zn analysis was unaffected by the presence of each other, a range of Cd : Zn concentrations were made up using AA standards. Seven solutions containing 0% Cd (100% Zn) ranging to 100% Cd (0% Zn) (single element) were prepared. For each solution, both elements were loaded onto the same rhenium filament and their ratios measured on the TIMS. Cd, having the lower ionisation potential (8.99eV), was analysed first followed by Zn (9.39eV). The impact of any differences on an ID experiment were assessed using plant and soil samples which had been spiked with both Cd and Zn for quantitative analysis.

E. Isotopic analysis:

All analyses were carried out using a VG 354 magnetic sector thermal ionisation mass spectrometer (Micromass UK Ltd.) operated using a single collector, peak jumping routine. The instrument major components were described in Section 1.5.5 earlier. The TIMS is equipped with a 16 place carousel in the source chamber, and the instrument is kept evacuated to $< 3\times10^{-7}$mBar during analysis.

Filaments loaded with Cd or Zn were inserted in the sample turret and the turret inserted into the source chamber which is then evacuated to $< 3\times10^{-7}$mBar. After an initial preheat period (1min at 1.25A) the analysis starts by scanning the calibrated magnetic field over a range of values close to that of the desired mass. Once a signal is detected by the axial Faraday collector, the ion beam is then focused automatically and the intensity of the ion beam increased by increasing the filament current to match a set target beam strength. The target beam strength was selected so that the sample is not exhausted too rapidly and significant isotope ratios are collected. The maximum ion current which is allowed to flow through each filament to achieve this target beam was 4.5A for both Cd and Zn. Isotope ratios were measured in a single collector mode, where isotopic masses are focused sequentially into the axial collector at different currents generating "flat-topped peaks". The different mass peaks are cyclically measured and each mass ion beam intensity is counted for an integration time of 10 seconds. Between each measurement a delay time was inserted (2 second) to allow the voltage to stabilise and the previous ion signal to decay in the collector. The isotope ratios were collected in blocks of 10 ratio measurements at 1.0×10^{-11}A for Zn while in blocks of 5 ratio measurements at 5.0×10^{-12}A for Cd. Finally, a total of 100 ratio measurements of the following isotopic ratios were measured for Zn (*m/z*): 64/66, 64/67, 64/68, 64/70, 66/67. As for Cd, 50 ratios of (*m/z*) 111/110, 111/114, 110/116 were collected.

F. Determination of CRMs Cd and Zn content by ID-TIMS:

After the CRMs have been spiked, digested, and Zn and Cd separated and loaded onto filaments, the *m/z* 66/67 for Zn and *m/z* 111/114 for Cd were measured on the TIMS to determine Cd and Zn content in the CRMs using the following ID equation (equation 1.5.6 described in details in Section 1.5.3A):

$$C_X = 1.66\times10^{-18}\frac{M}{W_X}N_{SP}\left(\frac{h^2_{SP}-Rh^1_{SP}}{Rh^1_X-h^2_X}\right).$$

To ensure that the isotope ratio measured is the true ratio and that it has not been altered or affected by the use of different chemical reagents (through digestion or separation processes), a blank correction was carried out. Cd and Zn spikes (n=3) were put through the same sample preparation procedure and their isotope ratio measured. The measured isotope ratios were compared with Cd and Zn spikes (that have not been put through sample preparation steps) to determine any contamination from reagents and apparatus used.

G. Determination of Cd and Zn in Great Billings and Countesswells soils by ID-TIMS:

The method validated using CRMs was used to determine total Cd and Zn content in the 2 soils used throughout the present experiments. Cd and Zn spikes were added to 0.5-2g of soil, 5 replicates of each soil, and mixed well. Samples were then digested following the microwave digestion procedure described above. Cd and Zn were then separated by ion chromatography and their isotope ratios measured on the TIMS, from which Cd and Zn content was calculated using the above equation.

3.2.3 Results:

A. Spikes isotopic and elemental composition:

Isotopic analysis of the spikes produced the results shown in Table 3.2.3 and 3.2.4.

Table 3.2.3: Isotopic characterisation of Cd spike and a natural atomic absorption (AA) standard compared to the IUPAC values. The elemental atomic masses (g/mol) are calculated.

Isotope	Exact Mass (g)	Isotopic Composition (Atom %)		
		Spike	AA	IUPAC[a]
106	105.906	0.00	1.26	1.25
108	107.904	0.00	0.89	0.89
110	109.903	0.60	12.54	12.50
111	**110.904**	**96.53**	**12.83**	**12.80**
112	111.903	1.78	24.18	24.14
113	112.904	0.42	12.20	12.22
114	113.903	0.58	28.68	28.73
116	115.048	0.09	7.42	7.50
Elemental Atomic Mass		110.945	112.342	112.382

[a] IUPAC, 1991.

Table 3.2.4: Isotopic characterisation of Zn spike and a natural atomic absorption (AA) standard compared to the IUPAC values. Spike Isotope Characterisation. The elemental atomic masses (g/mol) are calculated.

Isotope	Exact Mass (g)	Isotopic Composition (Atom %)		
		Spike	AA	IUPAC[a]
64	63.929	1.88	49.55	48.63
66	65.926	4.43	27.70	27.92
67	**66.927**	**89.44**	**3.99**	**4.11**
68	67.925	4.22	18.17	18.84
70	69.925	0.04	0.59	0.61
Elemental Atomic Mass		66.870	65.363	65.47

[a] IUPAC, 1991.

Fig. 3.2.1 (a) and (b) show the percentage difference between TIMS characterised isotope abundance (using AA standard) and that described by IUPAC for both elements. The representative isotopic composition described by the IUPAC is based on the chemicals and materials most commonly encountered in the laboratory (IUPAC, 1991). The percentage difference (% Difference) is calculated using the equation below. Differences ranged from –1.0% for ^{116}Cd to 0.6% for ^{108}Cd, while for Zn the differences were –3.5% for ^{68}Zn and 1.9% for ^{64}Zn.

$$\%\ Difference = \frac{charcterised\ value - IUPAC\ value}{IUPAC\ value} \times 100\% .$$

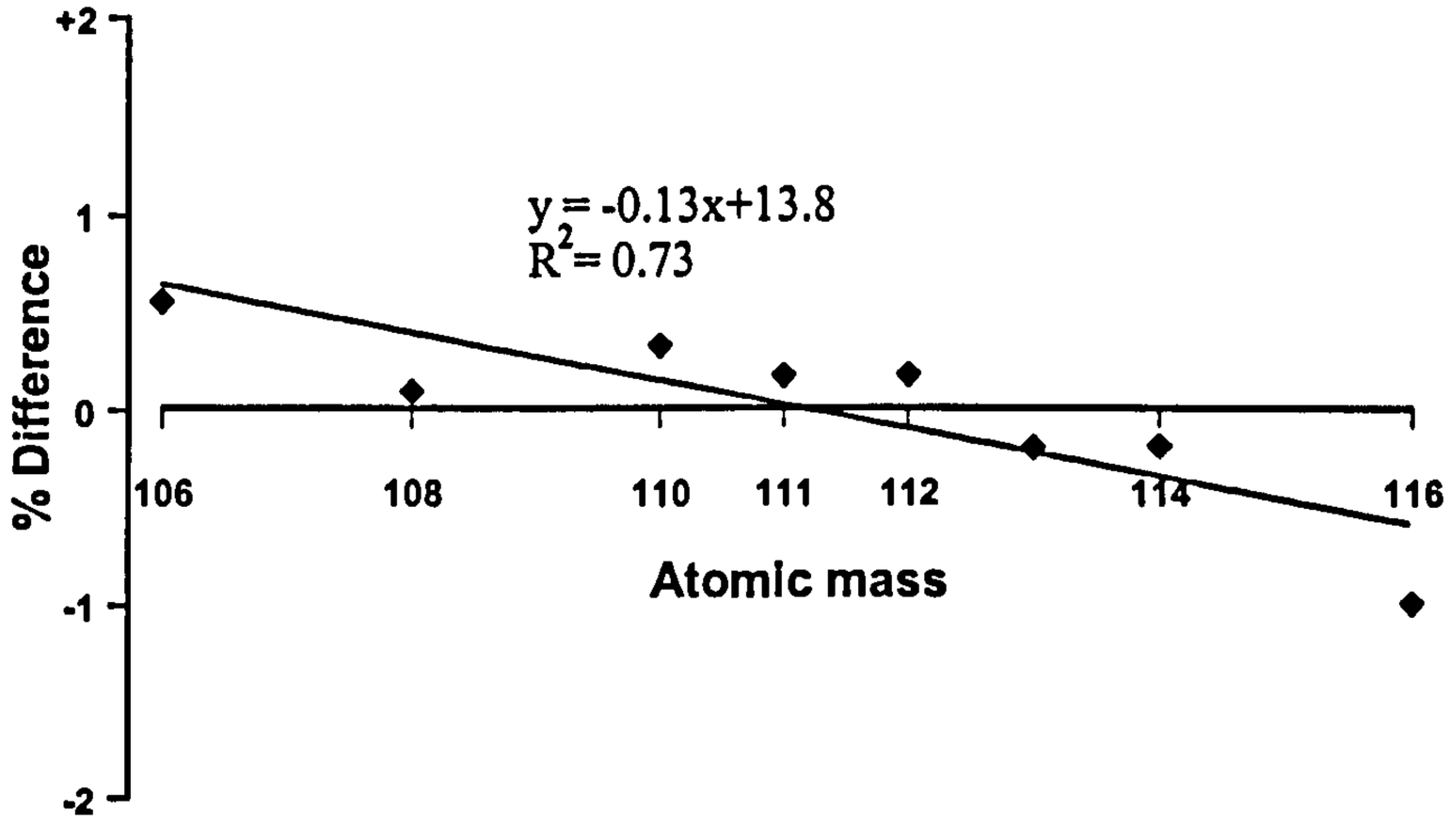
The elemental composition of the spike solutions was determined using reverse isotope dilution mass spectrometry (R-IDMS). These concentrations were used for all future calculations. For comparative purposes, the spike solutions concentration was also determined using ICP-AES. The concentrations determined by the R-IDMS and ICP-AES are shown in Table 3.2.5.

Table 3.2.5: ^{111}Cd and ^{67}Zn Spikes elemental content (µg/g) determined by ICP-AES and R-IDMS. Values in parenthesis are SD (n=3).

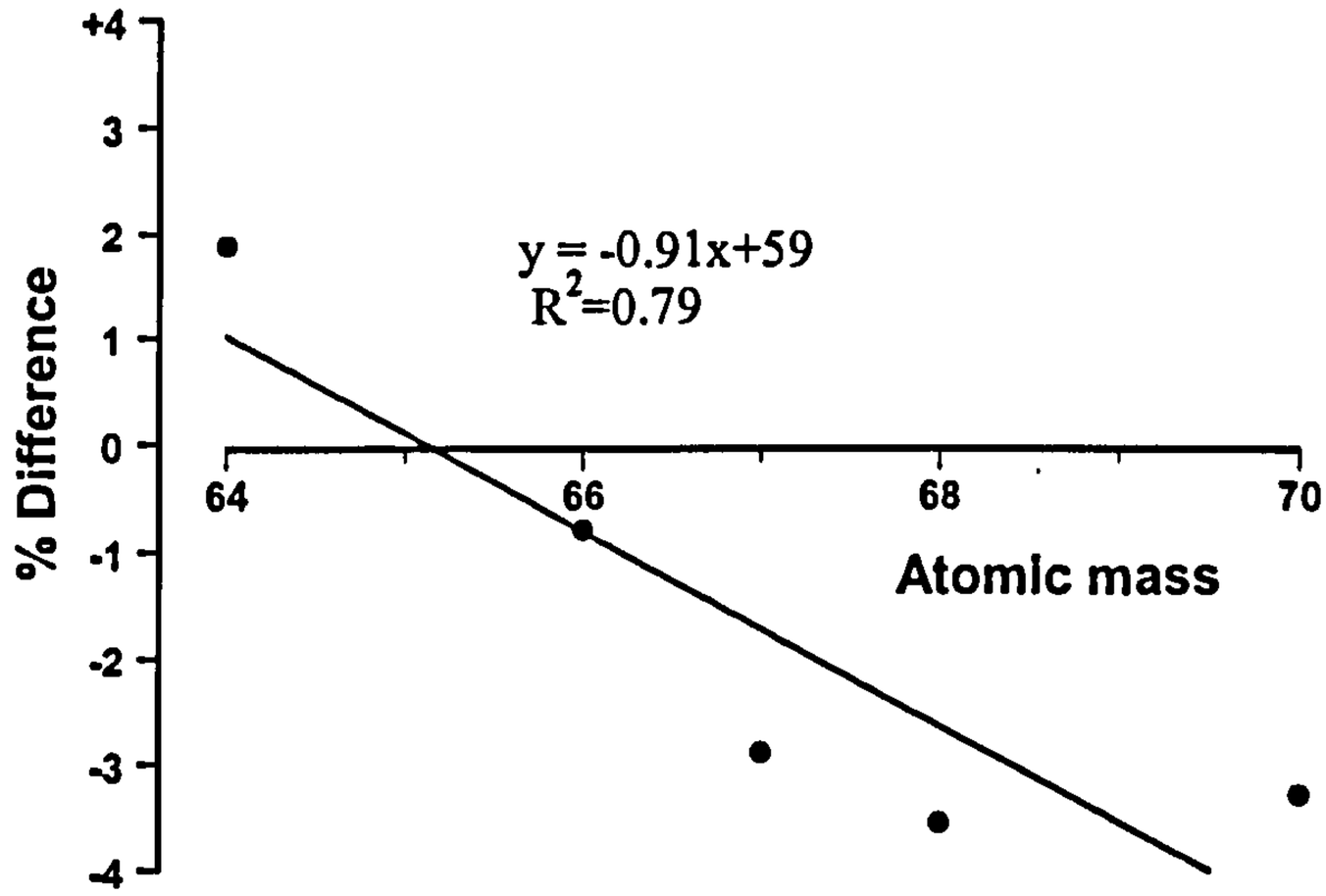
Element	ICP-AES	R-IDMS
Cd	0.157 (0.004)	0.158 (0.001)
Zn	15.76 (0.05)	15.93 (0.008)

Fig. 3.2.1: Isotopes percentage difference when compared to IUPAC values for Cd (a) and Zn (b). The line drawn through data is a linear best fit model, the equation and R^2 of this model are provided for comparative purposes.

(a)



(b)



To ensure the isotopic and elemental composition of spike and AA standard solutions was correct, the isotopic ratio of mixed solutions was measured on the TIMS (R_{Measd}) and compared to that calculated theoretically (R_{Calcd}) as shown in Fig. 3.2.2 (a) and (b) for Cd and Zn respectively. The isotope ratio is plotted against the Atom% (the number of atoms of one isotope divided by the number of atoms of the 2 isotopes). Cd showed a steady increase in the difference between the theoretical $^{111}Cd/^{114}Cd$ ratio and the experimentally determined using TIMS analysis with the increase in ^{111}Cd atom % (a difference up to 3.9% at 92 atom %). In addition the theoretical ratios were always higher than the measured ones. On the other hand, Zn showed excellent agreement between the theoretical and the measured isotopic ratios (<0.5% difference).

B. Separation of Cd and Zn:

The separation of Cd and Zn from other heavy metals present in the sample and from each other achieved by the sequential elution of acids through the ion exchange column. The results obtained from the separation of the contaminated Great Billings soil are shown schematically in Fig. 3.2.3. The figure presented here represents the final successful separation procedure, however it must be noted that the amount of work involved was tremendous. The isolation of Cd and Zn involved a series of trials with a large number of elution sequences with different solutions, exchange resins and solutions flow rate. Each procedure was evaluated according to the improvements achieved in the separation of Cd and Zn, and results were examined carefully in the planning for the next trial. The time devoted to the ion exchange chromatography procedure was about 8 months and represented a very challenging task. This work generated a huge amount of data in terms of analyses and separation chromatograms and is not presented here because of shortage of space.

Fig.3.2.2a: ^{111}Cd / ^{114}Cd measured (R_{Measd}) and calculated (R_{Calcd}) ratio against isotopic compositon.

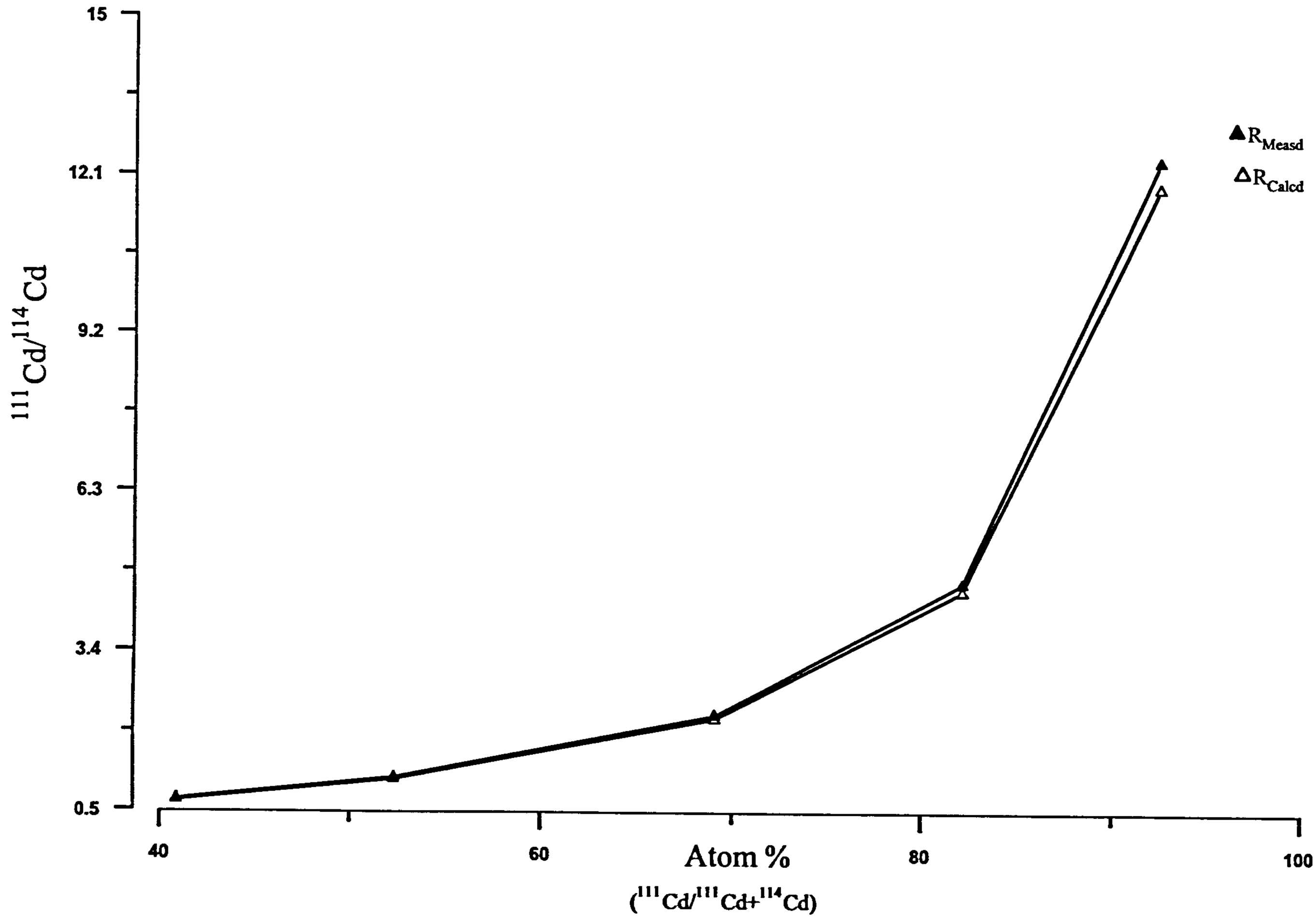


Fig.3.2.2b: $^{66}Zn/^{67}Zn$ measured (R_{Measd}) and calculated (R_{Calcd}) ratio against isotopic compositon.

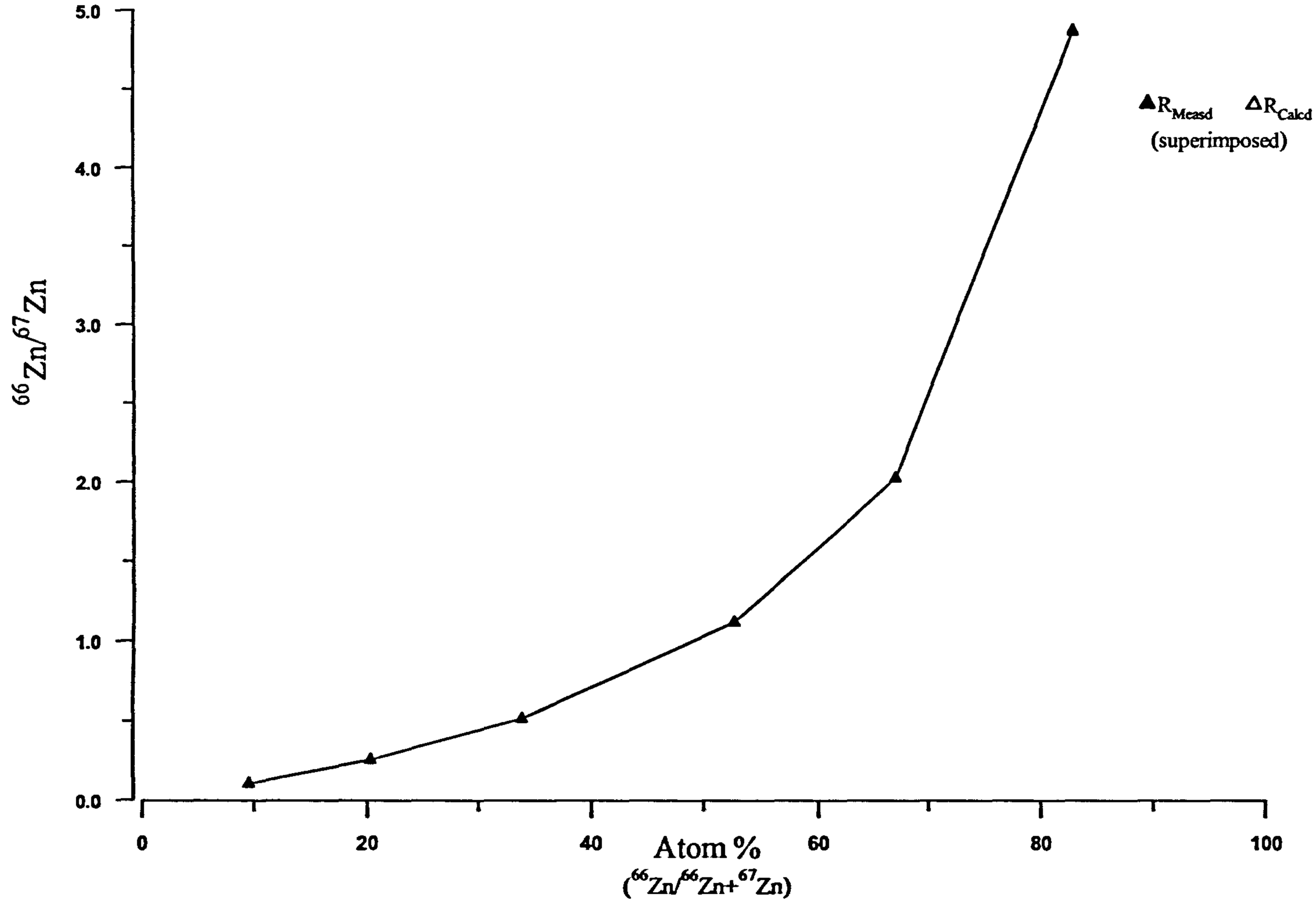
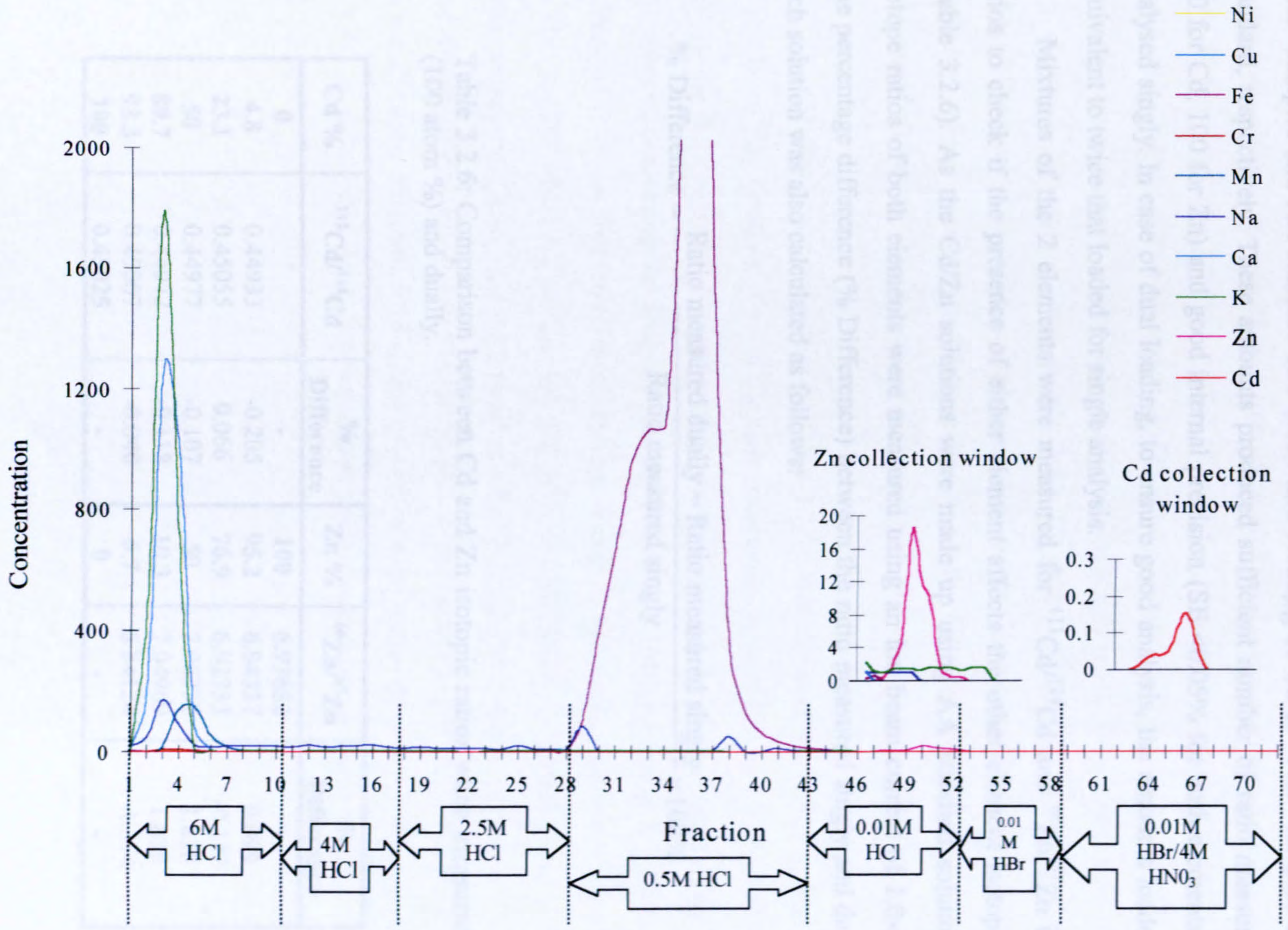


Fig. 3.2.3: Cd and Zn chromatographic separation (Concentration in μg/ml; each fraction was collected over 2min).



C. Single and dual loading:

The lowest amount of Cd or Zn needed for a good analysis was 1μg of Zn in both "real" samples and AA standard and 0.064 and 0.025μg of Cd in "real" samples and AA standard, respectively. These amounts produced sufficient number of ratio measurements (50 for Cd, 100 for Zn) and good internal precision (SE <0.05% for both elements) when analysed singly. In case of dual loading, to ensure good analysis, the amounts loaded were equivalent to twice that loaded for single analysis.

Mixtures of the 2 elements were measured for $^{111}Cd/^{114}Cd$ and $^{66}Zn/^{67}Zn$ isotopic ratios to check if the presence of either element affects the other element isotopic ratio (Table 3.2.6). As the Cd/Zn solutions were made up using AA standard solutions, the isotope ratios of both elements were measured using an ion beam current of 1.0×10^{-11}A. The percentage difference (% Difference) between the ratio measured singly and dually for each solution was also calculated as follows:

$$\%\ \text{Difference} = \frac{\text{Ratio measured dually} - \text{Ratio measured singly}}{\text{Ratio measured singly}} \times 100\%$$

Table 3.2.6: Comparison between Cd and Zn isotopic ratios when measured singly (100 atom %) and dually.

Cd %	$^{111}Cd/^{114}Cd$	% Difference	Zn %	$^{66}Zn/^{67}Zn$	% Difference
0	-	-	**100**	**6.93652**	-
4.8	0.44933	-0.205	**95.2**	6.94337	0.099
23.1	0.45055	0.066	**76.9**	6.92733	-0.132
50	0.44977	-0.107	**50**	7.06332	1.828
89.7	0.44972	-0.118	**10.3**	7.04969	1.632
93.3	0.45007	-0.040	**6.7**	6.84124	-1.374
100	**0.45025**	-	**0**	-	-

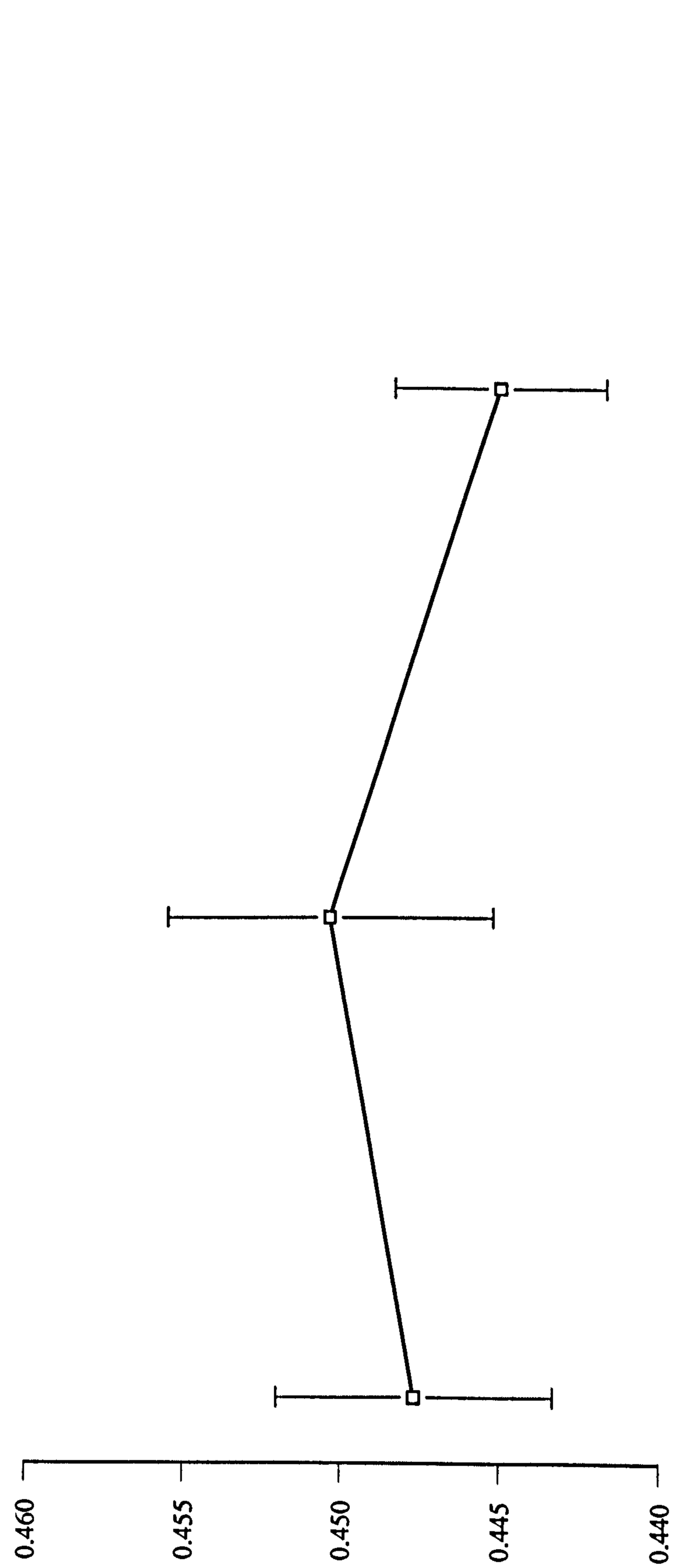
Although the change in the isotopic ratio was small, the effect it has on calculating the concentration using ID was investigated using both soil and plant samples (Table 3.2.7). For each ratio measurement the standard error (%) is also listed to allow the precision of both analyses to be compared. The isotope ratios measured singly and then dually for each element were tested for significant difference using t-test (at $P<0.05$, using CoStat 3.03, 1986, CoHort Software, Berkley, USA). The ratios measured singly and dually were not significantly different. The same was also found for corresponding concentrations. Overall, the %difference between concentrations determined singly or dually was less than 0.9% for both elements. Exceptions were sample 2 for Cd (2.4% difference) and sample 8 for Zn (2.0% difference). The difference in these 2 particular samples may be attributed to the number of ratio measurements, where it was 30 for the Cd sample (compared to 50 for the other samples), and 60 for Zn (usually 100 ratio determinations).

The Cd data presented earlier in Table 3.2.6 were obtained using a beam ion current of at 1.0×10^{-11}A, however, due to the low concentration of Cd in soil and plant samples used in the dual analysis, a lower target current was used for this analysis. This has the advantage of decreasing the analysis time so that data is obtained before all the sample is evaporated from the filament. To ensure this change in the analysis protocol had no effect on the results obtained, a Cd sample was loaded onto a filament and 100 ratio measurements of $^{111}Cd/^{114}Cd$ were made at 5.0×10^{-13}, 5.0×10^{-12} and at 1.0×10^{-11}A. The data were then averaged (Fig. 3.2.4) and the isotope ratio measured over the above currents were not significantly different. All Cd isotopic ratios were collected over an aiming current of 5.0×10^{-12}A throughout the following experiments (including Cd isotopic ratios in Table 3.2.7 below).

Table 3.2.7: Cd and Zn concentration determined using isotopic ratio measured singly or dually in soil (1-6) and plant (7-8) samples. The standard error (SE%) of each ratio measurement is also included.

Sample	Singly (s) or Dually (d)	Cd			Zn		
		$^{111}Cd/^{114}Cd$	SE %	Concentration (μg/g)	$^{66}Zn/^{67}Zn$	SE %	Concentration (μg/g)
1	s	0.909210	0.005	4.20	3.794638	0.046	674
	d	0.912824	0.011	4.17	3.797954	0.044	675
2	s	0.873707	0.023	4.63	3.810657	0.041	675
	d	0.883326	0.008	4.52	3.797487	0.048	670
3	s	0.695895	0.023	4.16	2.157344	0.021	1228
	d	0.697041	0.035	4.14	2.159262	0.020	1230
4	s	0.690896	0.007	3.47	2.558959	0.017	1277
	d	0.692133	0.031	3.46	2.563136	0.012	1280
5	s	0.687270	0.005	3.36	2.639795	0.011	1272
	d	0.688051	0.018	3.35	2.651414	0.016	1281
6	s	0.788697	0.004	3.28	2.236543	0.034	1217
	d	0.788687	0.023	3.28	2.249241	0.012	1227
7	s	1.034974	0.016	2.24	0.99779	0.005	320
	d	1.041001	0.023	2.22	1.010938	0.044	325
8	s	0.695121	0.009	4.36	5.713496	0.022	847
	d	0.696598	0.014	4.33	5.693211	0.078	830

Fig. 3.2.4: $^{111}Cd/^{114}Cd$ isotope ratio at different aiming currents (error bars are standard deviation).



D. Validation of the methodology and comparison of digestion procedures:

Cd and Zn concentrations in the 4 CRMs were calculated using the ID equation (described in details in Section 1.5.3). Results of the three different digestion methods are presented in details in Tables 3.2.8a and b to show the precision of each ratio measurement. The mean value of each digestion method for each CRM are shown schematically in Fig. 3.2.5a, b, c and d associated with the standard deviation.

Table 3.2.8a: Certified reference materials (CRMs) sample weight, spike added, the digestion method used, number of ratio measurements and current (A), standard error (SE %) and the calculated concentration for Cd.

CRM	Sample weight (g)	^{111}Cd spike added (g)	Digestion method [a]	111/114 Ratio	No. ratios @current	SE %	Concentration (µg/g)
GBW07401	0.5316	1.9305	AR	0.909210	140@1E-11	0.005	**4.205**
	0.5429	1.9851	AR	0.914039	120@1E-11	0.021	**4.190**
	0.5326	1.9961	AR	0.931004	50@1E-12	0.006	**4.144**
	0.5213	2.0204	M	0.932294	120@1E-11	0.011	**4.274**
	0.4671	2.0025	M	0.991630	120@1E-11	0.010	**4.211**
	0.5159	2.0170	M	0.941355	120@1E-11	0.006	**4.232**
	0.5293	2.0250	M	0.906284	140@1E-11	0.009	**4.458**
	0.4438	2.1487	M	1.026792	100@1E-11	0.033	**4.467**
	0.5870	2.1239	HF	0.880372	300@1E-11	0.003	**4.468**
	0.5436	2.005	HF	0.873707	70@1E-11	0.023	**4.626**
	0.5327	2.0023	HF	0.861212	50@1E-12	0.006	**4.857**
	0.6122	2.1208	HF	0.866042	120@1E-11	0.009	**4.425**
GBW07405	0.5755	0.2185	AR	0.889885	50@1E-11	0.035	**0.459**
	0.5376	0.2197	AR	0.952930	50@1E-12	0.003	**0.432**
	0.5412	0.2153	AR	0.918580	40@1E-11	0.009	**0.451**
	0.4943	0.2672	AR	1.084220	180@1E-11	0.016	**0.454**
	0.6162	0.2275	M	0.932949	50@1E-12	0.014	**0.407**
	0.5109	0.2259	M	0.975895	50@1E-12	0.015	**0.455**
	0.4423	0.2340	M	1.069510	60@1E-11	0.027	**0.447**
	0.5415	0.2253	HF	0.978257	50@1E-12	0.004	**0.419**
	0.5368	0.2198	HF	0.952403	50@1E-12	0.012	**0.433**
	0.5459	0.2232	HF	0.889341	50@1E-12	0.029	**0.495**

Table 3.2.8a (*Continued*)

CRM60	0.5438	1.0343	AR	0.918092	120@1E-11	0.006	**2.161**
	0.4924	1.0867	AR	0.962872	110@1E-11	0.021	**2.289**
	0.5042	1.1463	AR	1.015738	120@1E-11	0.008	**2.138**
	0.4362	1.0892	AR	1.058610	220@1E-11	0.006	**2.184**
	0.4679	1.1547	M	1.034974	120@1E-11	0.016	**2.245**
	0.5328	1.1957	M	0.990888	120@1E-11	0.005	**2.207**
	0.5549	1.1815	M	0.997945	120@1E-11	0.008	**2.067**
	0.5896	1.3568	HF	1.015679	120@1E-11	0.019	**2.165**
	0.4150	1.1057	HF	1.061427	50@1E-12	0.012	**2.319**
	0.5948	1.3864	HF	1.000912	50@1E-12	0.016	**2.251**
SRM1566a	0.5494	1.1099	AR	0.695121	50@1E-12	0.009	**4.358**
	0.4780	1.0933	AR	0.727500	50@1E-12	0.017	**4.365**
	0.5062	1.0806	AR	0.717519	50@1E-12	0.011	**4.224**
	0.4741	1.0156	M	0.722680	50@1E-12	0.013	**4.160**
	0.4777	1.0615	M	0.725846	50@1E-12	0.067	**4.266**
	0.4593	1.0448	M	0.731590	50@1E-12	0.005	**4.279**
	0.4739	1.1273	HF	0.734952	50@1E-12	0.006	**4.423**
	0.5081	1.1045	HF	0.725617	50@1E-12	0.006	**4.177**
	0.4948	1.1056	HF	0.722935	50@1E-12	0.013	**4.335**

[a] AR: *aqua regia*, M: Microwave, HF: hydrofluoric acid.

Table 3.2.8b: Certified reference materials (CRMs) sample weight, spike added, the digestion method used, number of ratio measurements and current (A), standard error (SE %) and the calculated concentration for Zn.

CRM	Sample weight (g)	^{67}Zn spike added (g)	Digestion method [a]	66/67 Ratio	No. ratios @current	SE %	Concentration (µg/g)
GBW07401	0.5316	0.8627	AR	3.794638	100@1E-11	0.046	**674.059**
	0.5429	0.8451	AR	3.863061	100@1E-11	0.031	**673.015**
	0.5326	0.8681	AR	3.781202	100@1E-11	0.036	**671.707**
	0.5213	0.8493	M	3.782786	100@1E-11	0.039	**672.027**
	0.5757	0.8905	M	3.866933	100@1E-11	0.027	**669.511**
	0.4671	0.8772	M	3.532189	100@1E-11	0.023	**670.288**
	0.5159	0.8657	M	3.722738	100@1E-11	0.043	**668.331**
	0.5042	0.8725	M	3.683469	100@1E-11	0.040	**673.622**
	0.5436	0.8756	HF	3.810657	100@1E-11	0.041	**675.336**
	0.5327	0.8816	HF	3.783195	100@1E-11	0.039	**682.819**
	0.6122	0.8701	HF	4.058741	100@1E-11	0.036	**689.885**
GBW07405	0.5376	4.3010	AR	1.097122	100@1E-11	0.008	**500.420**
	0.6191	4.3188	AR	1.224101	100@1E-11	0.012	**500.097**
	0.4882	4.3975	AR	0.969762	63@ 1E-11	0.008	**484.367**
	0.4969	4.3985	M	0.970028	100@1E-11	0.021	**476.154**
	0.6162	4.3194	M	1.205429	100@1E-11	0.006	**492.922**
	0.5109	4.3210	M	1.038686	100@1E-11	0.006	**494.566**
	0.5583	4.2105	HF	1.147287	70@1E-11	0.064	**498.596**
	0.5415	4.3320	HF	1.101391	100@1E-11	0.009	**503.466**
	0.5368	4.3201	HF	1.097254	100@1E-11	0.006	**502.803**
	0.5459	4.3156	HF	1.114816	100@1E-11	0.004	**504.363**
CRM60	0.5438	2.7401	AR	1.107192	100@1E-11	0.005	**318.754**
	0.4924	2.7198	AR	1.030575	100@1E-11	0.006	**319.906**
	0.5042	2.6988	AR	1.035265	100@1E-11	0.006	**311.736**
	0.3766	2.8039	AR	0.807407	100@1E-11	0.017	**320.997**
	0.7286	2.8124	M	1.332627	100@1E-11	0.039	**320.316**
	0.4679	2.6921	M	0.997790	100@1E-11	0.005	**321.358**
	0.5328	2.6998	M	1.109448	100@1E-11	0.012	**319.647**
	0.5549	2.696	M	1.142795	100@1E-11	0.004	**308.137**
	0.5896	2.6901	HF	1.207616	100@1E-11	0.006	**321.569**
	0.4933	2.6464	HF	1.063197	100@1E-11	0.028	**322.818**
	0.4150	2.7088	HF	0.895502	100@1E-11	0.006	**318.703**
	0.5948	2.7045	HF	1.234106	100@1E-11	0.006	**329.316**

Table 3.2.8b (*Continued*)

SRM1566a	0.5494	0.2889	AR	5.713496	100@1E-11	0.022	**846.954**
	0.4780	0.2618	AR	5.655825	100@1E-11	0.030	**833.967**
	0.5062	0.2715	AR	5.656457	50@1E-11	0.027	**817.179**
	0.4741	0.2787	M	5.575821	70@1E-11	0.042	**830.600**
	0.4777	0.2792	M	5.583974	100@1E-11	0.032	**832.009**
	0.4593	0.2801	M	5.584960	100@1E-11	0.030	**868.916**
	0.4739	0.2869	HF	5.559869	100@1E-11	0.043	**843.074**
	0.5081	0.2926	HF	5.622801	100@1E-11	0.027	**849.850**
	0.4948	0.2808	HF	5.683154	100@1E-11	0.019	**887.209**

[a] AR: *aqua regia*, M: Microwave, HF: hydrofluoric acid.

Results obtained by the 3 digestion methods were tested to see if there was a significant difference between the amount of Cd or Zn extracted using each procedure and the certified values. Among all results, only Cd in GBW07401 digested using HF proved to be significantly different at the $P<0.05$ level from the certified value. All the other analyses generated results which were not significantly different from the certified values. In addition to comparing the experimental results with the certified values, the results of Cd or Zn from each digestion procedure were compared. In all CRMs, there was no significant difference between the 3 digestion methods for both elements apart from 2 exceptions: HF dissolved more Cd in GBW07401 and more Zn in SRM 1566a than the other 2 methods using *aqua regia* or microwave digestion.

Fig. 3.2.5a: GBW07401 Cd and Zn content determined by ID-TIMS.

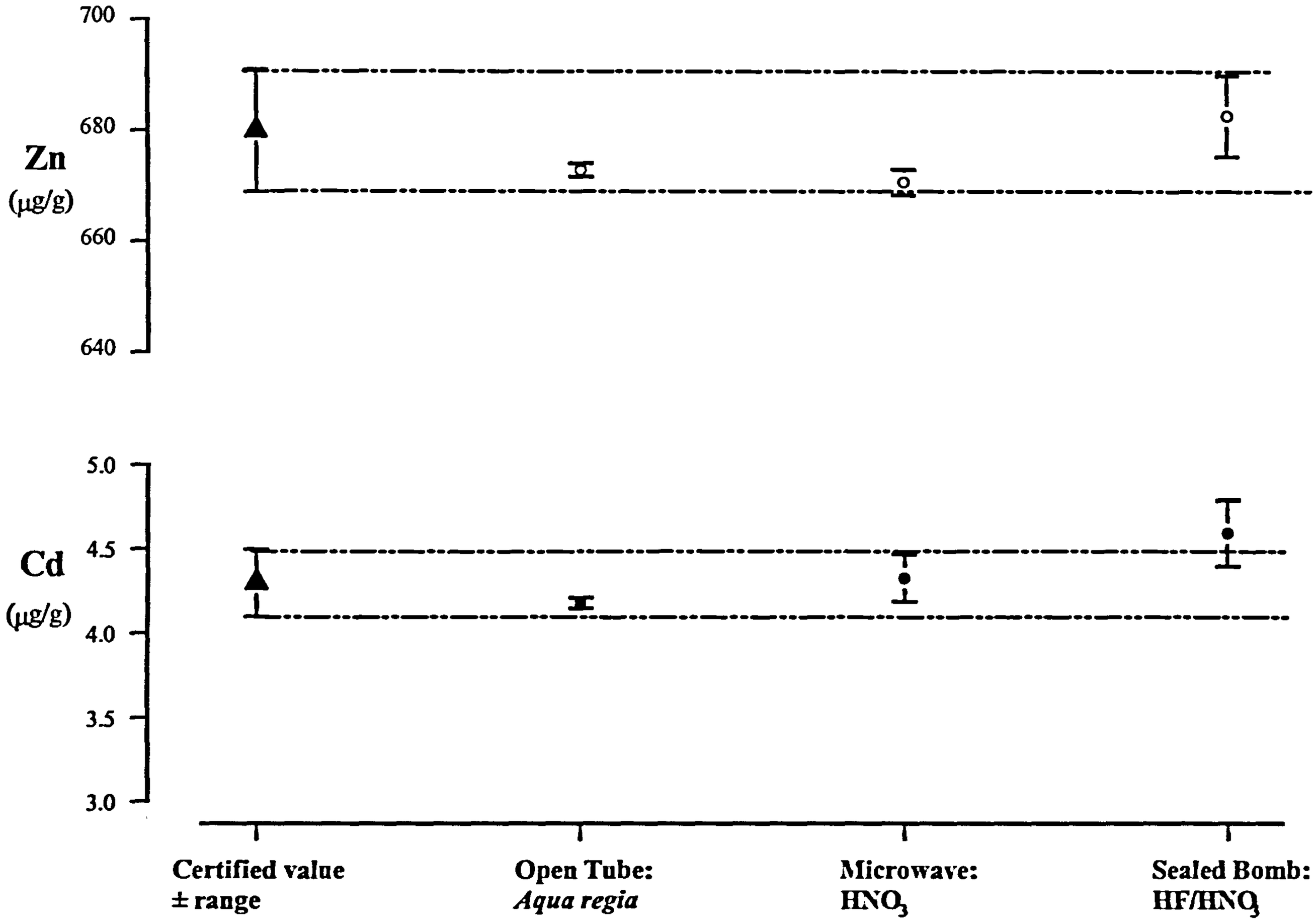


Fig. 3.2.5b: GBW07405 Cd and Zn content determined by ID-TIMS.

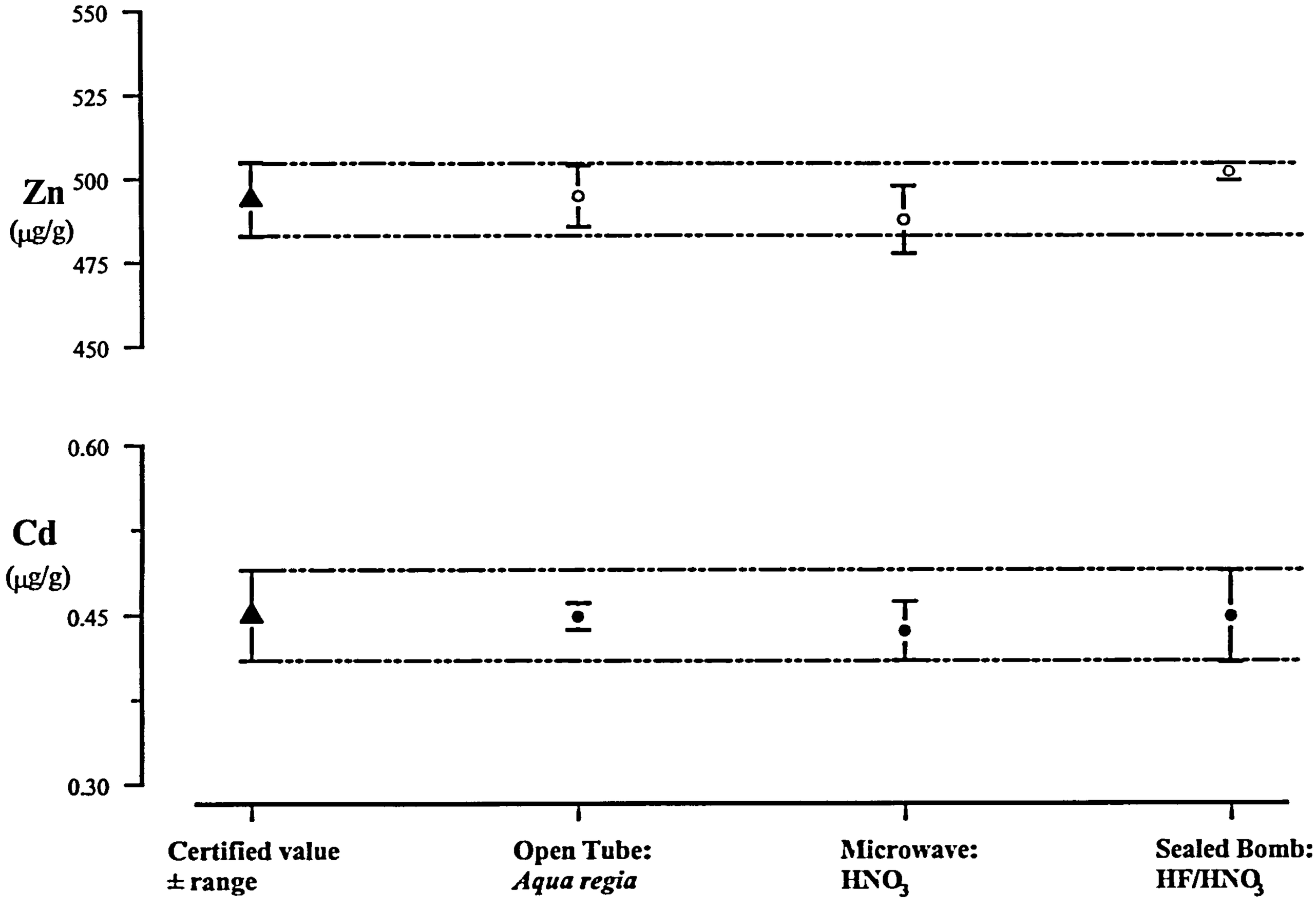


Fig. 3.2.5c: CRM60 Cd and Zn content determined by ID-TIMS.

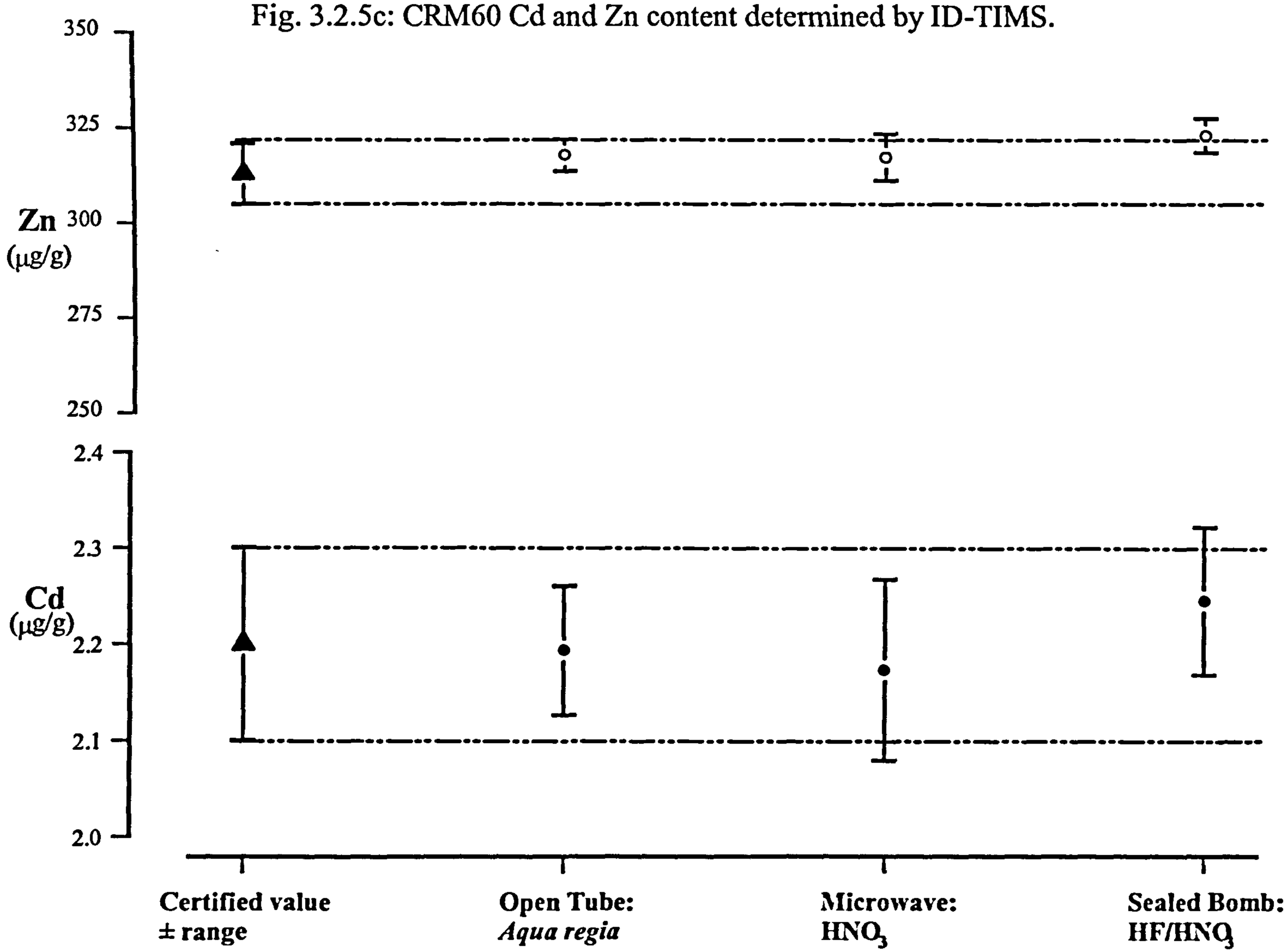
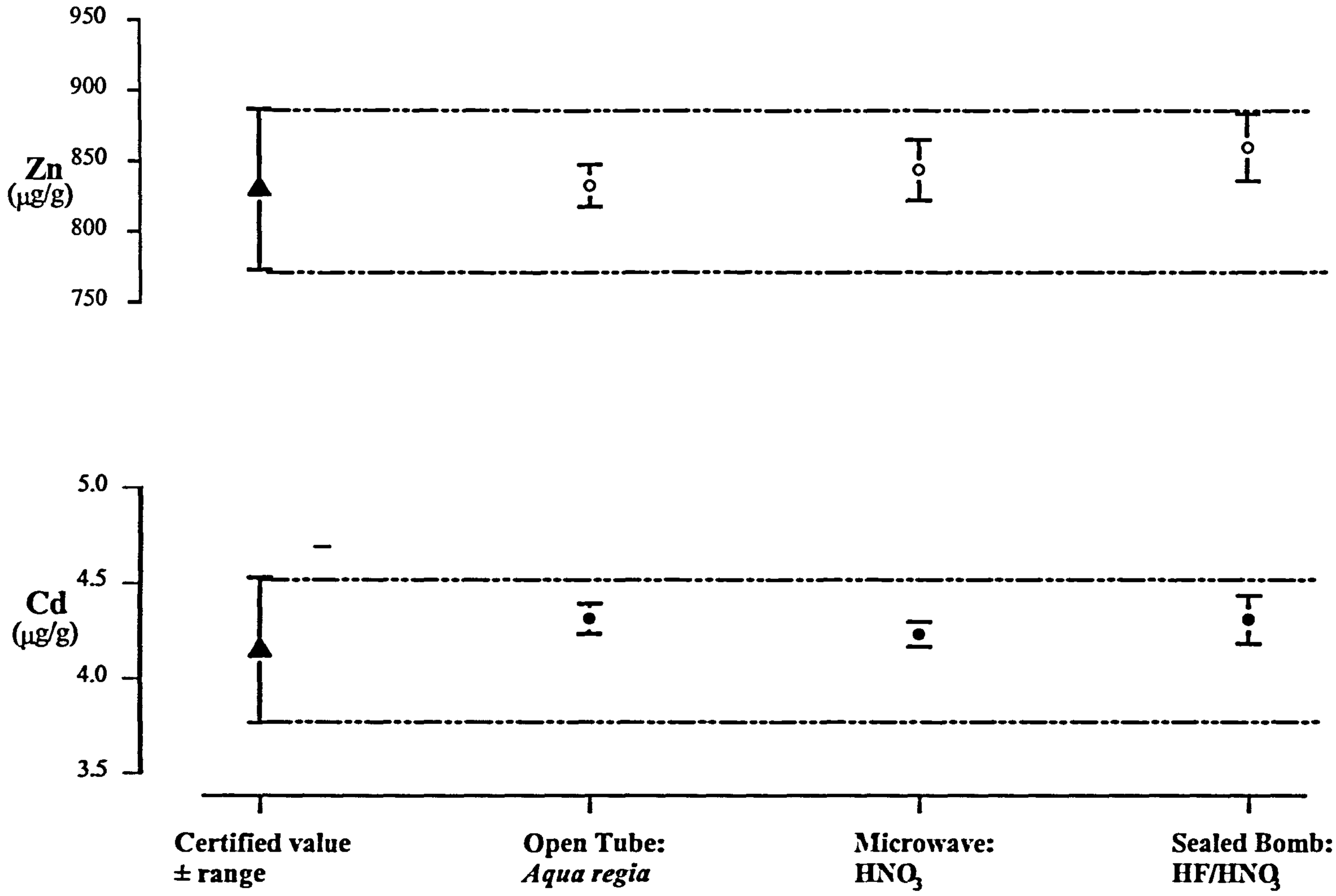


Fig. 3.2.5d: SRM1566 Cd and Zn content determined by ID-TIMS.



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For blank correction, Table 3.2.9 shows Cd and Zn isotope ratio of spike samples that have been put through the sample preparation procedure and that before. The 2 ratios showed no significant difference (at $P<0.05$) indicating that the sample preparation procedure followed did not affect the isotope ratios through contamination from reagents, columns or vessels used. If the isotope ratio in the blank samples was affected, the change in the isotopic ratio would have been used to correct for such variation in the calculation of the results, as no significant change was observed no such correction was made.

Table 3.2.9: Cd and Zn spike isotope ratio before and after undergoing sample preparation procedure. Mean ± SD, n = [a] 10, [b] 8, [c] 3.

	$^{111}Cd/^{114}Cd$	$^{66}Zn/^{67}Zn$
Before	166.76±2.8[a]	0.049504 ±0.00056[a]
After	169.95±2.9[b]	0.049018±0.00098[c]

E. Determination of Cd and Zn in Great Billings and Countesswells soils by ID-TIMS:

Cd and Zn total content in the 2 soils used throughout the experiments was determined following the isotope dilution technique is presented in Table 3.2.10.

Table 3.2.10: Cd and Zn content in Great Billings and Countesswells soils determined by ID-TIMS. Mean ± SD (n=5).

Soil	Cd	Zn
Great Billings	33.8±1.4	1231±54
Countesswells	3.29±0.5	65.3±5.8

3.2.4 Discussion:

Both the spike solutions and unlabelled AA standard solutions were successfully characterised for their isotopic composition. The spikes purchased had high percentages of the required isotopes (95 atom% for ^{111}Cd and 89 atom % for ^{67}Zn) necessary for the ID work proposed. The isotopic characterisation of a natural Cd and Zn was necessary to determine the spike solutions concentrations (using R-IDMS). For both Cd and Zn, the isotopic abundance in the AA standards determined was lower than the IUPAC values toward higher isotopic masses, whilst the isotopic abundances at the lower end of the mass spectrum were overestimated (Fig 3.2.1). In addition, the extent of the difference was higher in Zn at both ends of the mass spectrum compared to Cd. A possible cause of this may have been fractionation during analysis of the sample. The effect may have been more noticeable for Zn since it has a lower atomic mass than Cd hence this effect is magnified for lighter elements as the relative difference in mass between isotopes is highest (Fassett and Paulsen, 1989). The difference between the true isotopic abundance "IUPAC values" and that characterised by the TIMS is known as instrumental fractionation and results from the ionisation process. More ions of the lighter isotopes are produced at the beginning of the analysis, while as the analysis proceeds, the number of ions of the heavier isotopes increases. For this reason, a large number of isotope ratio measurements are required over the analysis period so that the mean value would be a representative ratio and would minimise fractionation effects. This fractionation can be corrected through the use of isotopic standard reference material, where isotopic ratios are corrected by a suitable factor or by using internal normalisation, where another isotope ratio is used to correct for the ratio used in ID calculations (Fassett and Paulsen, 1989).

Comparing the isotopic ratio measured with that expected theoretically (Fig 3.2.2) provided further proof that the isotopic and elemental characterisation of the spike standards was correct as illustrated by the excellent agreement between the measured and theoretical values. This forms the essential basis for any ID work. In addition, the elemental composition of the spikes determined by R-IDMS agreed very closely with the ICP-AES values thus providing an independent check and confidence in the isotopically determined results.

The isotopic ratios measured either singly or dually were of good precision (SE<0.050%). In addition, the concentrations determined using both filament loading techniques (Table 3.2.6) agreed well with each other. There was no significant difference between the isotope ratios when measured singly or dually or between the concentrations determined using either measurement. This also shows no evidence of partial evaporation or fractionation of Zn during Cd analysis. The presence of Cd or Zn did not affect the isotope ratio as Cd and Zn have different atomic masses (mass difference of 47 atomic mass units) and different first ionisation energy. The dual analysis method was successful in saving sample running times (up to 24h) and the amount of consumables used (filaments and reagents). There have been few studies on the analysis of more than one element on the same filament. Ramakumar *et al* (1994) reported the simultaneous isotopic analysis of uranium and plutonium. However, a correction in the isotopic ratio was made to compensate for the some isobaric interference between the 2 elements. Also, Vanhaecke *et al* (1998) reported an oligo-element method for the determination of Cr, Pb and Cd in photographic AgCl emulsions using ID-TIMS. However, there is no literature available on the dual analysis of Cd and Zn sequentially on the same filament.

Due to difficulties in Cd analysis at the full current (1.0×10^{-11}A), measuring $^{111}Cd/^{114}Cd$ isotope ratio at lower aiming currents (Fig. 3.2.4) was investigated. Isotopic ratios collected at 3 ion beam intensities were not significantly different with only 0.57% difference between that measured at 1.0×10^{-11}A and that measured at 1.0×10^{-12}A. Cd has been analysed over a range of beam strengths including 10^{-14}- 10^{-12}A (Rosman and Kempt, 1991) and 10^{-13}- 10^{-11}A (Wiadmann *et al*, 1994). In addition the precision obtained (in terms of SE%) was similar. In proving that Cd analysis at low current matches that at high current, it is particularly important to make sure that Cd analysed does not suffer from isotope fractionation. The way the isotope ratio measures against the current (Fig. 3.2.4) implies that fractionation (for ^{111}Cd and ^{114}Cd) is minimal, as the isotope ratio does not change under increasing current values.

Chromatographic isolation of Cd and Zn from the digested samples illustrated that the elution of strong acids (such as 6M HCl) at the beginning of the separation procedure was effective in washing through the sample alkali and alkaline earth elements while retaining

transition metals. The developed procedure separated Cd and Zn efficiently from each other and from other inorganic components present. The purity of the separated analyte is essential to minimise the isobaric interferences and to maximise ionisation efficiency of the analyte (Bacon, 1996; Thirlwall, 1997). The similar chemistry of Cd and Zn made it very difficult to separate them, especially because Cd is generally present in very low concentrations accompanied with high levels of Zn. However, this problem could be exploited in dual loading if Cd and Zn could be eluted together so that both are loaded onto filaments. This requires more investigation as it could save preparation and expensive instrument time.

IDMS of Cd and Zn quantification in all 4 CRMs yielded accurate results within the uncertainty range for the certified value proving that the technique was successful. However, the relative standard deviation (RSD%) in Zn case was generally lower than that for Cd, which may be due to the much higher concentration of Zn in samples compared to that of Cd. This difference in terms of RSD% occurred despite the fact that the amount of spike added for both Cd and Zn were adjusted to roughly give close isotope ratios. At the same time, the difference in RSD% was also observed within Cd samples themselves, where higher Cd content resulted in lower standard deviation. This was observed despite the fact that filament loading procedure (including the amount of Cd loaded) was the same. Further investigation is needed to explain such variation.

To date, no independent data are available on analysing the present CRMs using ID-TIMS for Cd and Zn content. The method has been used for other CRMs such as marine sediments (SD-M-2/TM, BCSS-1) and dogfish muscle (DORM-1). Results agreed with the certified values for Cd and Zn (in addition to other metals) in the marine sediments but were slightly higher for Zn in the dogfish muscle (Rosman and Kempt, 1991). The RSD% in the latter study was up to 2% for Zn while up to 5% for Cd. These values are close to our determined RSD%, in addition they agree with the fact that the present observed RSD% values are better for Zn than Cd, however the other authors did not comment on this. This was also the case in the determination of Cd and Zn in specimens from the limnic environment using ID-TIMS (Waidmann *et al*, 1994). The data obtained with the ID technique is within 1-3% of the certified value generally associated with this technique using TIMS (Heumann, 1986b; Heumann, 1992). Analysis of a sewage sludge CRM (BCR

144) for Pb yielded accurate results within the certified values with RSD% of 0.2-2.8% (Heumann, 1986a).

Despite the different procedures and reagents in the 3 digestion methods investigated, there was no significant difference between them in the determined Cd and Zn content. This is contradictory to Ure's (1996) findings where microwave oven digestion was not entirely equivalent to the aqua regia as it dissolved somewhat less of some heavy metals in a reference material (between 70 and 90% of the total contents). This may indicate that, for the CRMs used, the proportion of Cd and Zn associated with silica (that are only dissolved using HF) is low. However, a closer look at the results shows that the CRMs analysed following HF digestion, the most vigorous method, have produced marginally higher results than the other methods. In addition, the combination of HF and HNO_3 (an oxidising agent) was added to the samples so that organic matter and sulphides metals would be released (Ure, 1995) may have also contributed to this. Microwave digestion, even though it produced lower results than the other techniques in most cases, would be the technique of choice as it has the added benefit of shorter digestion times and uses less consumables. Also, the data produced were accurate and not significantly different from the certified values or the *aqua regia*/HF methods.

Like any analytical method, precision is a key factor in the quality of data obtained. The precision of the ID-TIMS method is dependent on the confidence in the isotope ratio measurement. This uncertainty arises from the difference between the isotope ratio measured over the analysis period (the standard deviation (*SD*)). As the reported isotope ratio is the mean of a large number (n) of individual ratio determinations, the standard error (*SE* %) of the mean is used to express the analytical error or internal precision. It is evident that the precision of the isotope ratio improves as the number of ratios measured increases, hence the reason why high numbers of ratio measurements are normally required. This is clear in the analysis report as the precision increases with more isotope ratio determinations. In the present work a minimum of 100 ratio determinations for Zn and 50 ratios for Cd were used (provided a SE <0.050% is obtained). This criterion was used for all isotope ratio determination throughout the present work.

Finally, the main advantage of the ID technique lies in that there is no need for the quantitative separation of the analyte compared to other analytical techniques. This is particularly important in techniques that require several stages in sample preparation, where loss of the analyte is likely to occur. TIMS, which requires an extensive procedure for sample preparation, benefits the most from such a technique.

3.2.5 Main findings:

1. It has been shown that the isotope dilution- thermal ionisation mass spectrometry (ID-TIMS) can give precise and accurate data in the quantification of Cd and Zn in soil and plant materials.
2. Open tube *aqua regia*, microwave HNO_3 and closed bomb HF digestion procedures showed no significant difference in the recovery of Cd and Zn using ID-TIMS.
3. Cd and Zn content in the 2 soils have been determined.

3.3 DETERMINATION OF THE ISOTOPICALLY EXCHANGEABLE Cd AND Zn "THE E-VALUE"

3.3.1 Introduction:

As discussed in Section 1.4.4E earlier, by allowing an isotope label to exchange with metal contained within a soil and then assessing the extent to which isotopic dilution occurs, an estimate of the isotopically exchangeable metal pool can be made. This estimate can then be used to predict the bioavailability of the metal. The experimental procedure carried out here is based on similar procedures used for the determination of available P in soils using ^{32}P radioisotope (Morel *et al*, 1995; Fardeau, 1996; Fardeau *et al*, 1996; Frossard *et al*, 1994). In Fardeau's work, ^{32}P is added to a soil solution mixture, the added radioactivity distribution between soil and soil solution PO_4^{-3} is then used to calculate the isotopically exchangeable P. This is known as the E-value. In the present experiments, stable isotopes are used and have the advantage of calculating the E-value in 1 step compared to 2 steps as with radioisotopes, where there is a need to determine the total amount of P in soil solution. There is no literature available on the use of stable isotopes to measure the E-value, which makes the present work unique.

Radioisotopes procedure for determination of isotopically exchangeable P:

The radioisotope method (Morel *et al*, 1995; Fardeau *et al*, 1996) involves 2 steps. The isotopically exchangeable P (mg/kg soil) is determined by shaking 1 g of soil with 100ml of water for 18h to reach a steady state, then a solution of P labelled with ^{32}P of known activity is added to the soil suspension, and mixed thoroughly. At selected times, a portion of the soil suspension is removed with a syringe and the solution separated immediately from the solid phase by Millipore filtration (0.2μm). The amount of radioactivity introduced (*R*) is known, and the amount of radioactivity remaining in the

solution at time t (r_t) is measured. The isotopically exchangeable P at a given time *t* (E_t) is calculated using the principle of isotopic dilution, assuming that:

at time *t*, the ratio of the radioactivity in solution to the amount of isotopically exchangeable P in time *t* is identical to that in soil solution. This is expressed in the following equation:

$$\frac{R}{E_t} = \frac{r_t}{100\,C_P} \qquad \text{.....3.3.1}$$

where the factor 100 accounts for the soil:solution ratio of 1g of soil in 100ml of water and C_P is the concentration of water soluble P (mg/l). Therefore, $100C_P$ is equivalcnt to the water soluble P content of the soil expressed in mg/kg. Water soluble P (C_P) is determined by shaking 1g of soil with 10ml of water (or similar water:soil ratio) for 18h (to reach a steady state), and determining P concentration.

Rearranging equation 3.3.1 gives the isotopically exchangeable P:

$$E_t = \frac{100\,C_p}{(r_t/R)} \qquad \text{.....3.3.2}$$

The method could be performed with or without adding a carrier (Morel *et al*, 1995).

The use of stable isotopes of metals such as ^{111}Cd and ^{67}Zn, as discussed here, has the advantage over the radioisotope technique in that there is no need to determine the metal content of a soil:solution suspension. The concentration in a stable isotope study can be based on isotope dilution principles as discussed earlier in Section 1.5.3. In this chapter the E-value is determined in 2 soils (Great Billings and Countesswells) which have been used throughout this thesis. To investigate the dynamics of the isotopic equilibration between spikes added and the soil solution Cd and Zn, the isotopic ratio was measured over a period of just under 1200h.

3.3.2 Materials and methods:

A. Soils, isotopes and reagents:

Preparation of the Great Billings and Countesswells soils for this experiment is identical to that described in Section 3.1.2A. The Cd and Zn isotope spikes used are described in Section 3.2.4A. Reagents used are described in Section 3.1.2B.

Change in the isotopic ratio:

Calculation of the amount of spikes to be added to each soil was based on the amount of spike which would cause a significant measurable change in the isotope ratio. The targeted isotopic ratio change was based on the standard deviation (SD) derived from 20 analyses of $^{111}Cd/^{114}Cd$ and $^{66}Zn/^{67}Zn$ of different samples (independent replicates). An isotopic ratio change equivalent to 25×SD for Cd and 100×SD for Zn was targeted. In this way the change (about 15% of baseline ratio) would be easily detected with relatively small spike addition and allow the full dynamics of the isotope equilibration to be described. The natural baseline and targeted isotope ratios are described in Table 3.3.1. The amount of Cd and Zn spikes to be added to achieve the targeted isotopic ratio change was based on the acetic acid Cd and Zn extractable content.

Table 3.3.1: Baseline and targeted isotopic ratios of Cd and Zn.

	Baseline Ratio		**Targeted Ratio**	
Soil	**$^{111}Cd/^{114}Cd$**	**$^{66}Zn/^{67}Zn$**	**$^{111}Cd/^{114}Cd$**	**$^{66}Zn/^{67}Zn$**
Great Billings	0.447261	6.942371	0.518880	6.1219367
Countesswells			0.513875	6.1904019

B. Experimental procedure:

Approximately 1.25g of Great Billings soil and 5g of Countesswells soil (due to the low Cd and Zn content) samples were weighed into separate 500ml polyethylene bottles. 200ml of Millipore water was added to each sample and the suspension was shaken end-over-end (40 rpm) for 18h to reach a steady state. The suspensions were then spiked with a known amount of ^{111}Cd and ^{67}Zn as described in Table 3.3.2.

Table 3.3.2: Weight of spike isotopes solution (g) added to each soil. Values in parentheses are the spike solutions concentration (μg/g).

Soil	Spike added	
	^{111} Cd	^{67} Zn
Great Billings	4.0 (0.158)	0.3 (15.929)
Countesswells	1.0 (0.013)	1.0 (0.049)

The suspensions (n=13) were then shaken to allow isotopic exchange and each sampled at specified time intervals ranging from 1h to 7 weeks. The solution from each suspension was filtered immediately, first through Whatman No.542 filter paper using a large Buchner funnel (200mm diameter) and secondly through a Millipore filtration system (0.2μm pore size and 47mm diameter). Filtration of the soil suspension was done as quickly as possible, to stop any further isotopic exchange occurring during filtration, particularly in the first 3 samples (1, 2.5 and 5.5h). The filtrate volume was reduced on a hot plate and then evaporated to dryness under heat lamps. The residue was taken up in 6M HCl, and the Cd and Zn separated using the anion exchange procedure described in Section 3.2.4B. Isotopic ratio measurements were carried out using a VG 354 magnetic sector thermal ionisation mass spectrometer operated using a single collector and peak jumping routine. The instrument has been described in Section 3.2.2E. From the measured isotopic ratios, the weight of each soil sample and the amount of Cd/Zn spikes added, the principles of isotope dilution (equation 1.5.6 Section 1.5.3A) were then used to calculate the soil isotopically exchangeable Cd or Zn (the E-value).

3.3.3 Results:

The measured Cd and Zn isotopic ratios are presented in Tables 3.3.3a and b with the calculated E-values for Great Billings and Countesswells soils, respectively (The within measurement standard error (SE) % of each ratio is also included). Cd in Countesswells soil could not be measured successfully on the TIMS due to the very low amount of Cd in the soil water suspension.

Table 3.3.3a: Great Billings soil suspension Cd and Zn isotopic ratio with the corresponding standard error expressed as (SE % = (SD/√n)×100) and the calculated E-value against time (at least 50 and 100 ratios for Cd and Zn, respectively).

	Cd			Zn		
Time (h)	$^{111}Cd/^{114}Cd$	SE (%)	E-value (μg/g)	$^{66}Zn/^{67}Zn$	SE (%)	E-value (μg/g)
1	0.547263	0.041	16.9	[a]		
2.5	0.534622	0.046	19.2	5.911920	0.039	471
5.5	0.516318	0.017	22.8	5.936510	0.025	569
21	0.51843	0.019	24.2	6.153628	0.051	658
45	0.51747	0.012	24.0	6.131457	0.028	625
69	0.506928	0.025	27.2	6.251171	0.048	721
165	0.506966	0.008	28.1	6.247172	0.028	740
357	0.501943	0.010	31.1	6.265456	0.067	775
501	0.511623	0.017	26.1	6.295898	0.042	803
693	0.505411	0.010	28.9	6.299957	0.036	809
837	0.505379	0.011	29.1	6.342486	0.056	880
1005	0.502222	0.010	30.8	6.314957	0.023	837
1197	0.503481	0.010	30.0	6.348452	0.033	887

[a] TIMS analysis was not successful below LOD of 1μg.

Table 3.3.3b: Countesswells soil suspension Zn isotopic ratio with the corresponding standard error expressed as (SE % = (SD/√n)×100) and the calculated E-value against time (at least 100 ratios).

	Zn		
Time (h)	**$^{66}Zn/^{67}Zn$**	**SE (%)**	**E-value (μg/g)**
1	6.749161	0.073	7.5
2.5	6.757498	0.050	7.7
5.5	6.728115	0.073	6.6
21	6.767260	0.074	8.3
45	6.737821	0.066	7.0
69	6.787645	0.033	9.3
165	6.763685	0.036	8.1
357	6.763685	0.039	8.0
501	6.752360	0.051	7.5
693	6.779111	0.014	8.9
837	6.806223	0.076	10.7
1005	6.763030	0.052	8.0
1197	6.769837	0.018	8.4

For Great Billings soil, the isotopic ratios were of good precision with an average SE of 0.018 and 0.040% for Cd and Zn respectively, and 0.050% for Zn in Countesswells soil. The change in E-value with time and the corresponding isotopic ratio are presented schematically in Fig. 3.3.1a, b and c. For Great Billings soil, although $^{111}Cd/^{114}$ Cd was measured on the TIMS, it is plotted as $^{114}Cd/^{111}Cd$ (Fig 3.3.1a), to allow a clearer visual comparison of the data. A logarithmic regression equation ($y = a\ Ln(x) + b$) was fitted to the data and the correlation coefficients (R^2) were calculated. The tangent line drawn is based on the Mean Value Theorem calculations as described late in this section.

Fig. 3.3.1a: Great Billings soil $^{114}Cd/^{111}Cd$ isotopic ratio with the corresponding E-values (μg/g) against time (h). The logarithmic equation and the correlation coefficient (R^2) are also included.

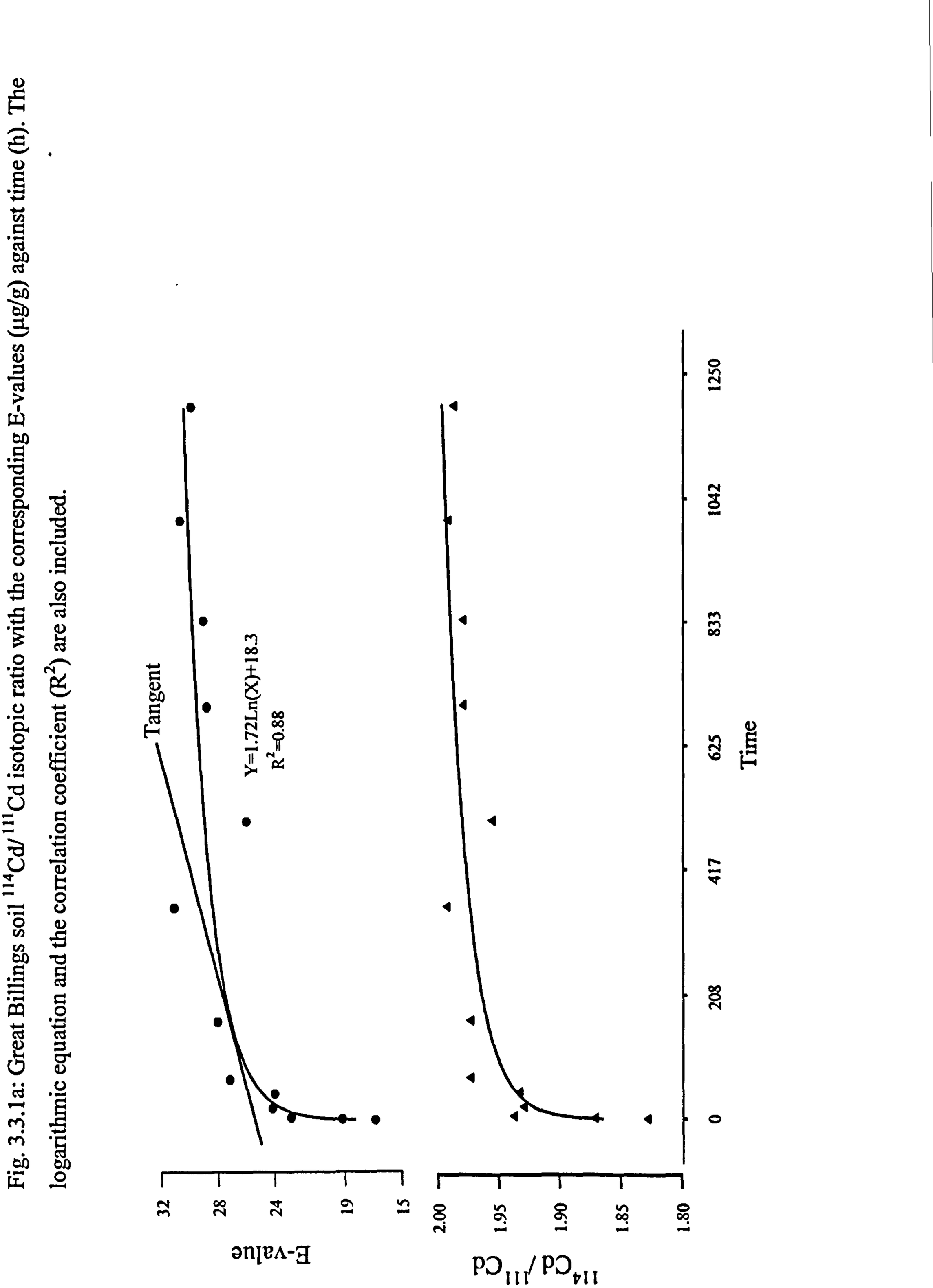


Fig. 3.3.1b: Great Billings soil $^{66}Zn/^{67}Zn$ isotopic ratio with the corresponding E-values (μg/g) against time (h). The logarithmic equation and the correlation coefficient (R^2) are also included.

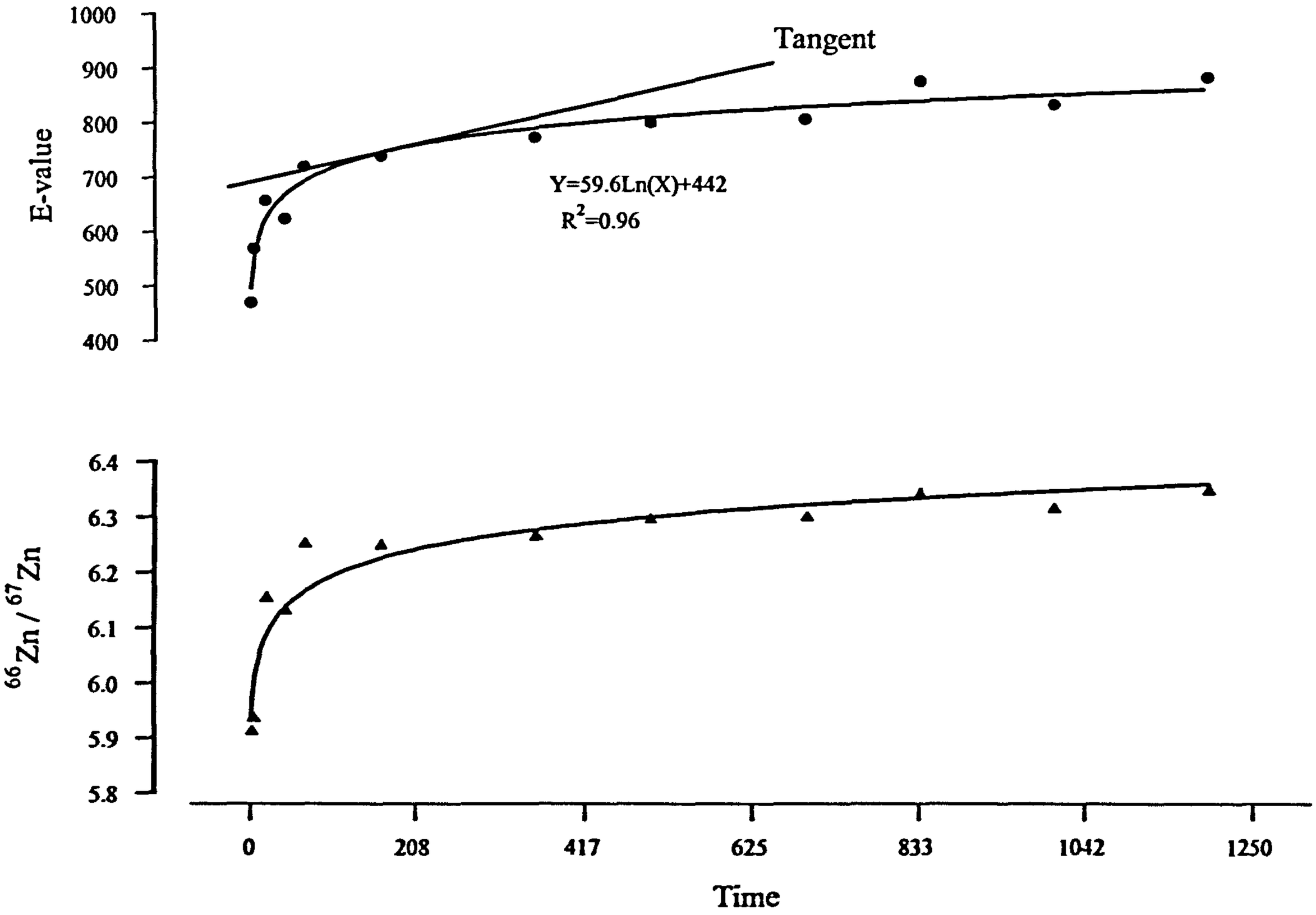
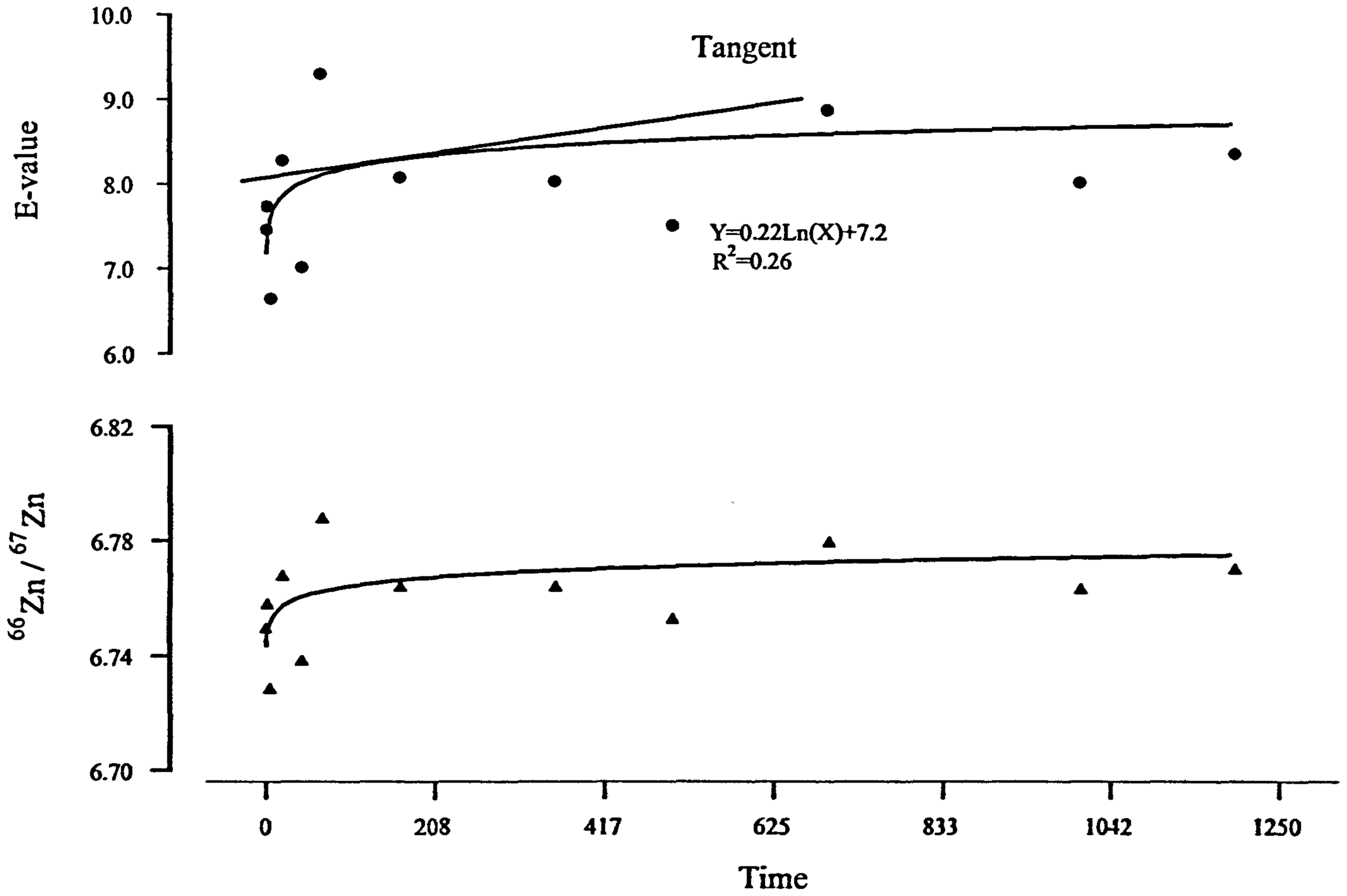


Fig. 3.3.1c: Countesswells soil $^{66}Zn/^{67}Zn$ isotopic ratio with the corresponding E-values (μg/g) against time (h). The logarithmic equation and the correlation coefficient (R^2) are also included.



The data shows the rapid rate of change in the E-value at the beginning of the experiment compared to that at the end of the experiment as time proceeds. E-value change rate (slope), for example, in the up to 21h is 0.37mg/kg per hour compared to 4.9×10^{-3}mg/kg per hour afterwards for Cd in Great Billings soil. However, the data above does not show clearly when the isotopic equilibrium has been reached, therefore the mean of the E-values was determined using 2 methods. In the first, the Mean Value Theorem (Swokowski *et al*, 1994) was used to draw a tangent line that is parallel to a straight line between the first and the last experimental E-values (secant). The point at which the tangent line touches the fitted line is the average slope. The E-value was estimated at this point. In the second approach, according to Young *et al* (2000), the E-value for Cd and Zn (using radioisotopes) reaches equilibrium after 48h of spike addition. A mean E-value was determined from 69h to the end of the experiment (1197h). Mean values using both methods are presented in Table 3.3.4. and are also expressed as percentages of the total Cd and Zn content. Soils total Cd and Zn content has been determined earlier by acid digestion using ID-TIMS (Section 3.2.4G).

Table 3.3.4: Mean E-values (μg/g) of Cd and Zn in Great Billings soil and Cd in Countesswells soil and their percentage of total Cd and Zn determined by 2 methods.

	E-value							
SOIL	**METHOD A [a]**				**METHOD B [b]**			
	Cd	**% Total**	**Zn**	**% Total**	**Cd**	**% Total**	**Zn**	**% Total**
Great Billings [c]	**27**	**80**	**730**	**60**	**29**	**86**	**806**	**65**
Countesswells [d]	**-**	**-**	**8.3**	**13**	**-**	**-**	**8.6**	**13**

[a] Using Mean Value Theorem; [b] Using values obtained after 69h; [c] Total of 33.8 and 1231μg/g of Cd and Zn, respectively; [d] Total of 65.3μg/g of Zn.

The E-values obtained by the 2 methods in Table 3.3.4 above were not significantly different. In addition, the extrapolated isotopic exchange times for the mean E-values obtained by the Mean Value Theorem (using the tangent) were obtained at relatively the

same corresponding times; Cd and Zn at 160 and 176h respectively in Great Billings soil, while Zn in Countesswells soil at 160h.

The theoretical equations derived for each metal (*See* Fig. 3.3.1a, b and c) were used to estimate E-values for times longer than the present experiment. Table 3.3.5 lists selected projected times with the corresponding E-values for Cd and Zn in Great Billings soil and Zn in Countesswells soil. These theoretically estimated E-values are also expressed as a percentage of the total soil Cd or Zn content (Table 3.3.5).

Table 3.3.5: Estimated E-values (µg/g) using the theoretical equation at selected times and their percentages compared to the total Cd and Zn.

	Great Billings				Countesswells	
Time	**Cd**	**% Total**	**Zn**	**% Total**	**Zn**	**% Total**
1 min	5.9	17.5	8.6	0.7	5.6	8.6
5 min	8.6	25.4	104.5	8.5	6.0	9.6
1 h	12.9	38.2	252.7	20.5	6.5	10.0
1 day	23.8	70.4	631.7	51.3	7.9	12.1
1 week	27.2	80.5	747.7	60.7	8.3	12.7
1 month	29.6	87.6	834.5	67.8	8.6	13.2
3 months	31.5	93.3	900.0	73.1	8.8	13.6
6 months	32.7	96.7	941.3	76.5	9.0	13.8
1 year	33.9	100.3	983.5	80.0	9.2	14.0

3.3.4 Discussion:

A soil solution suspension at a steady state, considering Zn as an example, will have a natural isotopic ratio of ^{66}Zn / ^{67}Zn of 6.942371. Immediately after the addition of a spike (^{67}Zn) it disperses in the solution phase causing the ^{66}Zn / ^{67}Zn ratio to drop sharply. As the isotopic exchange between the solution and the soil particles occurs, more spike (^{67}Zn) is

exchanged into the soil, causing the solution ^{66}Zn / ^{67}Zn ratio to increase. This increment in the soil solution isotopic ratio continues to increase until equilibrium is reached. The objective of monitoring this change with time was to determine the "critical" time at which this equilibration is reached. Filtration of the soil solution at the proposed times was done quickly to stop any further isotopic exchange occurring during filtration, particularly in the first 3 samples (1, 2.5 and 5.5h). This was done using a large Buchner funnel, followed by submicron vacuum filtration for further separation. The targeted isotope ratio of Cd and Zn (Table 3.3.1) was close to that measured (Table 3.3.3a) for Great Billings soil. However this was not the case for Zn in Countesswells soil (Table 3.3.3b) which may be related to the lower bioavailable Zn (isotopically exchanged) compared to that extracted by acetic acid on which the spike addition was based.

The time following the spike addition at which E-value is determined is different in published literature, here we estimated equilibration had been reached after 69h. In experiments investigating E-value determination for P, the E-value was determined after 1min (Morel *et al*, 1995), 4h and after 3 weeks of spike addition (Frossard *et al*, 1994). For Ni, it was estimated after an equilibration time of 17h (Echevarria *et al*, 1997). In recent publications, Cd E-value was measured after 7 days of Cd radioisotope spike addition (Smolders *et al*, 1999) while Young *et al* (2000) determined the E-value for Cd and Zn using radioisotopes after 48h of spike additions, assuming that the added isotopes had fully equilibrated with the soil Cd and Zn. In the experiments investigating P isotopic exchange, the frequent determination of the isotopically exchangeable P occurring in 1min (expressed as E_1) may be related to the different behaviour of P in soil solution compared to heavy metals. In addition the rate of isotopic exchange in the first minute may be so rapid that equilibration is essentially complete after this period and little exchange occurs afterwards. The wide range of suggested equilibration times for P and heavy metals lead to the present experiments of the isotopic exchange monitoring with time. However, the present data could not predict a precise isotopic equilibration time.

Initially, the isotopic ratio in general (hence the E-value) increased sharply (a rate of 0.37mg/kg E-value per hour in the first 21h for Great Billings Cd) with time, rising to almost a plateau toward the end of the experiment time of just over 7 weeks (a rate of

4.9×10^{-3}mg/kg E-value per hour in the period after 21h, for Great Billings Cd). Although it is not possible to specify a critical time for when the isotopic exchange is complete, the time corresponding to the point where the tangent touches the fitted curve (Fig. 3.3.1a, b and c) may be used. These values were 160 and 179h for Cd and Zn in Great Billings soil respectively and 160h for Zn in Countesswells soil. The fact that these values are similar for Cd and Zn in the same Great Billings soil and with that of Cd in Countesswells may suggest that a time of about 160-180h could be sufficient for the determination of E-values for these 2 elements. This agrees with times found recently by Smolders *et al* (1999) for Cd (7 days) while different from that found by Young *et al* (2000) for Cd and Zn (48h) using radioisotopes. The comparison would have been clearer if the E-value of Cd in Countesswells had been determined. The reason why this was not possible was the particularly low levels of Cd in the soil suspension which proved to be too low to be measurable on the TIMS.

The use of a "stronger" reagent, such as acetic acid or calcium chloride that would extract more Cd and metals in general into the solution and may have been an option to attempt to obtain a Cd result for the Countesswells soil. However, the use of a solvent other than water might affect the soil solution equilibrium or the isotopic exchange equilibrium, hence affecting the results. In published work, the E-value has been always determined only in a soil:water suspension for P (Fardeau, 1996, Fardeau *et al*, 1996; Morel *et al*, 1995; Frossard *et al*, 1994) and Ni (Echevarria *et al*, 1997). Very recently, the E-value has been determined for Cd and Zn in $Ca(NO_3)_2$ and $CaCl_2$ solutions (Young *et al*, 2000). Suspending the soil in an electrolyte rather than water has the advantage of increasing the metal concentration in the solution, making it easier to measure metals in the solution. In addition, the use of an electrolyte makes the isotopic distribution between the solid and solution phases faster (Young, 2000). In addition, Smolders (2000) compared Cd E-values measured in 2 different strengths of electrolytes (0.001M and 0.01M $CaCl_2$) and found no difference between them. However, the difference in the E-value, if any, when measured in water and in other electrolytes still needs to be investigated.

The mean E-value determined either using the Mean Value Theorem or values determined after 69h of isotopic exchange were similar. In fact the E-value determined for

Zn in Countesswells soil using the 2 methods is exactly the same. However, the mean E-value determined after 69h of isotopic exchange (Method B, Table 3.3.4) will be used for comparison purposes with the other present experiments. The E-values of Cd and Zn in Great Billings soil were 86% and 65% of total soil metal. Zn in Countesswells had an E-value of 13% of total Zn and very different from Zn in Great Billings soil. The fact that the total Cd or Zn in the 2 soils is greater than the isotopically exchangeable pool signifies the presence of fixed or unavailable forms of Cd and Zn that were not isotopically exchangeable. Smolders *et al* (1999) found Cd E-value in the range 62-90% of total soil Cd in 8 different types of soils. Young *et al* (2000) found E-values in the range of 6-81% of total for Cd and 6-48% of total for Zn, using sewage sludge amended soils. These values compare well with the values obtained in the present experiments. E-values for Cd in both the present and published experiments were estimated to be higher than values estimated for Zn. This may suggest that Cd binds less strongly to the soil particles making it more mobile compared to Zn.

The E-value determined using the logarithmic equation (Table 3.3.5) continuously increased with time. The percentages of values calculated theoretically compared to totals were different for Zn or Cd between the 2 soils. In Great Billings soil, the Cd E-value after a year (33.9mg/kg) was estimated to be slightly more than the soil total Cd (33.8mg/kg), and taking into consideration that these estimations are based on the mathematical model and are not measured, the value is not that unreasonable. The fact that a large proportion of the Cd in the soil was added by sewage sludge application may make a significant amount of Cd readily available for plant uptake. As Cd levels in "normal soils" such as Countesswells are about 3.3mg/kg, this indicates that approximately 31mg/kg of the Great Billings Cd was not an original constituent of the soil and could easily be available. In addition, the plant would exude acids and enzymes which would make the original soil Cd (not added by sewage sludge application) available over time. Also, as EDTA extracted about 70 % of total Cd (Section 3.1.3), it might not be surprising that the other 30% would become available over a year by the chemical and physical changes in the soil environment. However, this was not the case for Zn in both soils, in fact Zn behaved differently in the

two soils exchanging about 76 and 14% of its total content in Great Billings and Countesswells soils, respectively, in 1 year.

Finally, E-value in the past has been determined using radioisotopes, and has been described to provide the best indicator of elements chemical reactivity (bioavailability) (Young *et al*, 2000). In addition E-value determined for Cd and Zn were found to be robust to changes in the soil:solution equilibrium caused by electrolyte strength and soil:solution ratio (Young *et al*, 2000). In this study we have shown that stable isotopes can be used equally well if not better for E-value determinations. As mentioned, stable isotopes have the advantage over radioisotopes in that ID principles can be simultaneously adopted to quantify elements as well as assess equilibration.

3.3.5 Main findings:

1. E-value can be determined following the isotopic exchange principles using stable isotopes.
2. The rate of isotopic exchange between added Cd and Zn spikes and that of a soil solution is very fast at the addition of the spikes. This rate decreases with time until equilibrium is reached.
3. The isotopic equilibration between added spikes of Cd and Zn and these of a soil solution suspension is estimated to occur after approximately 180h of spikes addition.
4. Estimated Cd E-value expressed as a percentage of total was higher than that estimated for Zn.
5. For Great Billings contaminated soil, Cd E-value estimated theoretically after a year, came to a value close to the soil total Cd.
6. The rate of which isotopic exchange occurs for Cd in Great Billings soil or Zn in the Great Billings and Countesswells soils was different.

3.4 DIRECT DETERMINATION OF AVAILABLE Cd AND Zn "THE L-VALUE"

3.4.1 Introduction:

A direct way to measure the bioavailability of heavy metals in soils is to grow plants on the soil and determine the metal uptake. Limitations (discussed earlier in Section 1.4.4A) including the extent to which plant species differ in their ability to accumulate metals may be overcome by using isotope spikes. This approach provides a term known as "L-value" and was proposed because E-value overestimated the bioavailable P in a simple soil:water mixture (Section 1.4.4E). Determining the bioavailable metal in a soil:plant system (L-value) has the advantage of measuring that fraction of metal in the natural field conditions and not in a simple hydroponic system (E-value) using water or nutrient solutions (Brooks *et al*, 1999). Similar to E-value determination, the L-value experimental procedure, which has been used extensively with P is based on a radioisotopic method (Frossard *et al*, 1994; Fardeau *et al*, 1996). Basically, a radioisotope is used to label the soil. Plants are then grown in the soil and the aerial parts are analysed for their radioactivity. The transfer factor of the specific activity in the plants compared to that of the soil is then used to calculate the L-value (Frossard *et al*, 1994; Echevarria *et al*, 1997).

In addition to the determination of L-values, this experiment also aims at assessing the potential for phytoremediation (Section 1.3) of Cd and Zn in the 3 plant species used. Plants that can uptake heavy metals and accumulate them to high levels are useful in remediating contaminated soils. *Thlaspi caerulescens* (alpine penny cress) has been used for this purpose (Salt *et al*, 1998; Chaney *et al*, 1997; Raskin *et al*, 1994; Baker and Brooks, 1989). *Taraxacum officinale* (dandelion) has a wide distribution (Dalby, 1999) and an ability to tolerate a broad range of climatic and soil conditions (Simon *et al*, 1996). It is often found growing on industrial waste sites and can clearly tolerate potentially phytotoxic conditions. It's potential for phytoremediation has not been investigated, however it has been used as a

phytoindicator[a] of pollution (Kabata-Pendias and Dudka, 1991; Benzel *et al*, 1998; Normandin *et al*, 1999; Murray *et al*, 2000). In this respect, dandelion plant tissue content of metals is directly proportional to the level of pollution, and thus it can serve as indicator of metal contamination in soils (Adriano, 2000). Dandelion's worldwide distribution and its ability to tolerate high levels of contamination make it well suited for phytoremediation. *H. vulgare* (spring barley) tolerated high levels of heavy metals including Cd and Zn, and has been suggested to have a phytoremediation potential equal to the hyperaccumulator *B.juncea* (Indian mustard) in a study involved the amendment of soil by EDTA (Ebbs and Kochian, 1998).

In the following experiments L-value estimations were made using 2 soils and the 3 plant species described above. The distribution of Cd and Zn uptake into the plant parts was also assessed and discussed in terms of the phytoremediation potential.

3.4.2 Materials and methods:

A. Seeds:

T. caerulescens (alpine penny cress) was chosen because it is known as a hyperaccumulator of Cd and Zn. Seeds of a germination rate of 75% were purchased (Guy, D., Mas des Avinieres, France). *T. officinale* (dandelion) was chosen because it grows easily and is commonly found in meadows and waste land. Seeds were purchased from John Chambers Wild Flower Seeds (Northamptonshire, UK). *Hordeum vulgare* (spring barley) was chosen for comparison and seeds were obtained from Dr L. Morrice (Scottish Agricultural College, Aberdeen).

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[a] Phytoindicators or indicator species or biomonitors are plant whose presence or absence in an area indicates certain environmental conditions, such as soil type or high levels of pollution (Dresner and Avison, 1998).

B. Experimental procedure:

The spikes used here are identical to those described in Section 3.4.1 and were added to cause the same change in the isotopic ratio. The amount of spikes added to each soil is tabulated in Table 3.4.1.

Table 3.4.1: Cd and Zn spike isotopes added to each soil. Values in paranthesis are the percentage of spike added to the total metal present (%).

SOIL	Soil Total Weight (kg)	Amount of Spike Added (μg)	
		Cd	Zn
Great Billings	2.183	858 (1.16)	8966 (0.33)
Countesswells	2.250	6.68 (0.10)	28.7 (0.02)

Table 3.4.1 also shows the relative amount of Cd and Zn spike added compared to the total which was added in very small amount so that the total metal content is not highly affected by the spike addition. Also, Cd and Zn spikes were added in a volume of about 500ml of very dilute HNO_3 (<0.002M) so that the spikes were equally distributed in each soil and to make sure that the soil pH was not affected. The spiked soil was mixed then air dried at 30°C for about a week and mixed thoroughly by hand twice a day. The soil was then left at room temperature for 2 days prior to potting. This relatively long mixing procedure was used so that a homogenous distribution of the spikes was achieved. Each spiked soil was then transferred into plastic pots (about 120g for each pot), 6 replicates each for *T. caerulescens* and *T. officinale* and 5 for *H. vulgare* were potted.

Seeds were germinated in acid washed sand (using 0.1M HCl) soaked in water for 2 weeks for *T. caerulescens* and 1 week for both *T. officinale* and *H. vulgare* at room temperature. The seedlings were then carefully transplanted into pots. The plants were grown in the controlled environment cabinet and watered with deionised water daily. The growth cabinets (Conviron Growth Chamber, Model S10H, Controlled Environments Ltd, Canada) were operated using the following parameters: 12:12h day/night cycle, air temperature 18°C, relative humidity 75%, light intensity of 160μmol/m^2.s

photosynthetically active radiation (PAR). Any water that drained from pots was collected in dishes and poured back into the pot regularly.

After 3 months, plant shoots were harvested and the majority of roots were removed from each pot by hand and rinsed with Millipore water. Both roots and shoots were dried at 70°C for 72h, weighed and milled. About 0.5g of dry plant material was ashed at 450°C for 12h, then transferred quantitatively into microwave bombs. Microwave digestion was carried out following the procedure described in Section 3.2.2A. The digest was filtered into 25ml volumetric flasks and made up to volume using Millipore water. 5ml of this digest were transferred into 25ml volumetric flasks, made up to volume with Millipore water and used to measure plant Cd and Zn concentration. This was measured using inductively coupled plasma atomic emission spectrometer (ICP-AES, IRIS, Thermo Jarrel Ash). Solutions containing low concentrations of Cd were determined using graphite furnace atomic absorption spectrometer (GTA-96, Varian) using ammonium phosphate as a chemical modifier (Stoeppler, 1992b). The remaining filtrate (approximately 20ml) was evaporated then dissolved in about 4ml of 6M HCl, and Cd and Zn separated following the chromatographic procedure developed as in Section 3.2.2B. Cd and Zn isotopic ratios were measured using TIMS (Micromass UK Ltd) described in Section 3.2.2E, from which the pool of the isotopically available metal was calculated.

3.4.3 Results:

In this experiment, plants were grown in small pots (containing about 120g of soil) so that plants roots were able to access metals within the whole pot, and eventually all the soil was exposed to roots. This was particularly obvious in *H. vulgare* and *T. officinale* plants where a net of roots within and around the soil formed, but not the same for *T. caerulescens*, which has small fine roots and the upper part of the pots were absent from

any roots. *T. officinale* was grown in especially designed conical pots (*See* Photograph 3.4.1B) that have the same shape as the taproot, to allow the root to develop naturally. Plants grown in the 2 soils appeared healthy and showed no visible symptoms of toxicity under the conditions used in the experiment.

A. Plants Cd and Zn content:

The plant species replicates recorded weight and their Cd and Zn content in both soils are tabulated in Tables 3.4.2a and b. The 3 plant species are also pictured in Photographs 3.4.1. The individual replicates are included in order to show the difference in both yield and metal concentration. *T. caerulescens* roots were not easy to separate, as they were very fine, easily damaged (brittle), and did not yield sufficient amount (maximum of 150mg) for analysis.

Table 3.4.2a: Plants weight (g) and Cd and Zn content (μg/g) in shoots and roots of plants grown in Great Billings soil. The mean and RSD% are also included.

Plant Species		Shoots			Roots		
	Replicate	Weight	Cd	Zn	Weight	Cd	Zn
T. caerulescens	1	0.18	237	3425	[a]		
	2	0.15	178	2070			
	3	0.66	433	6297			
	4	0.39	492	6265			
	5	0.45	418	4324			
	6	0.22	581	5326			
	Mean	*0.34*	*390*	*4618*			
	RSD%	*59*	*40*	*36*			
T. officinale	1	1.22	69.3	992	1.53	56.2	1210
	2	1.11	43.6	711	1.46	39.7	880
	3	1.00	69.2	787	1.66	27.9	538
	4	1.41	39.4	753	2.17	44.4	1182
	5	0.77	68.4	882	0.60	70.2	1543
	6	1.51	68.0	1103	2.23	70.2	1405
	Mean	*1.17*	*59.6*	*871*	*1.61*	*51.4*	*1126*
	RSD%	*23*	*24*	*18*	*37*	*33*	*32*
H. vulgare	1	1.22	10.0	817	0.21	145	4472
	2	2.02	66.6	636	0.59	131	3321
	3	1.97	9.1	695	0.43	95	3366
	4	1.91	7.2	642	0.56	116	2958
	5	1.43	10.7	722	0.34	212	3910
	Mean	*1.71*	*20.7*	*702*	*0.43*	*140*	*3605*
	RSD%	*21*	*124*	*11*	*37*	*32*	*16*

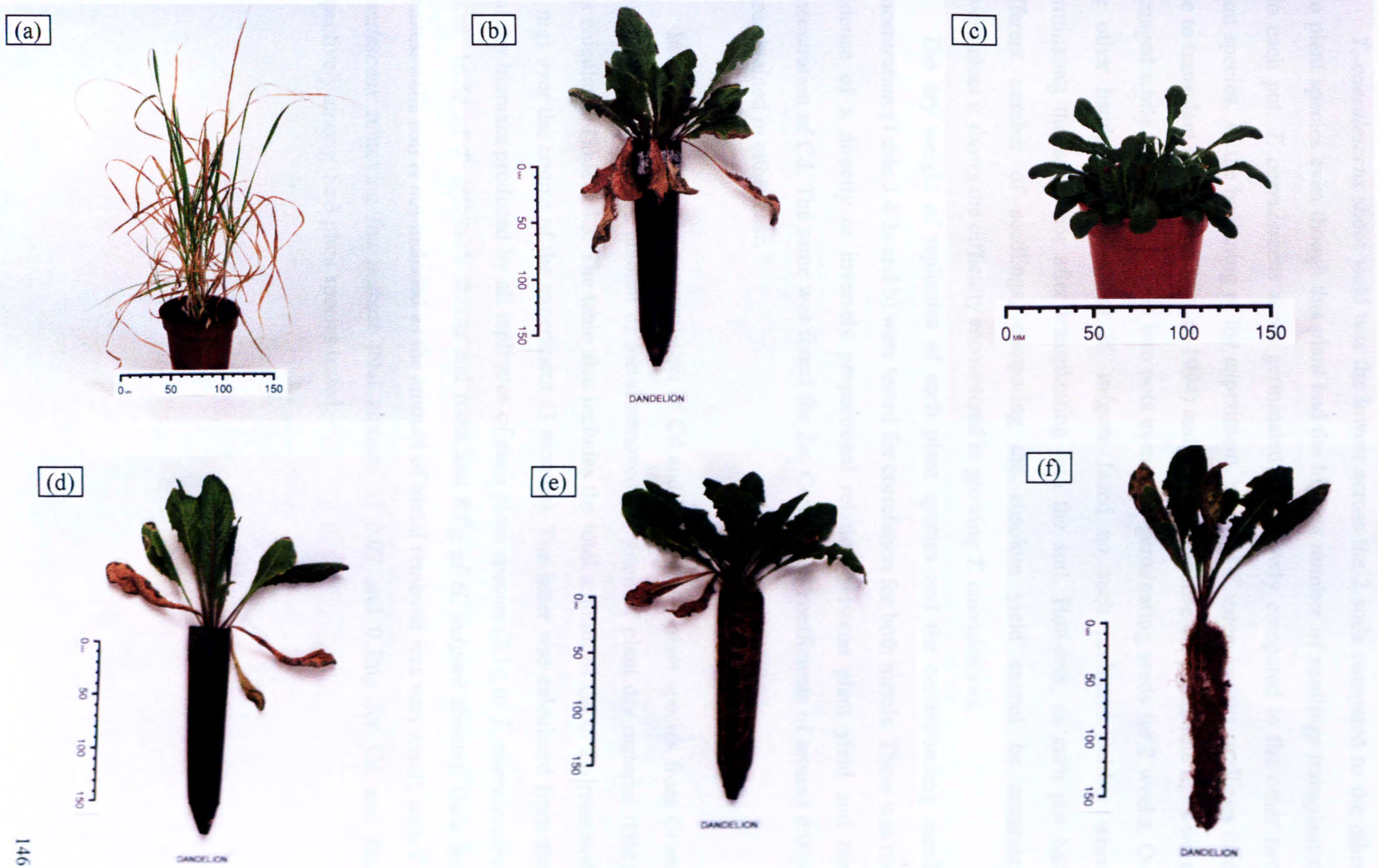
[a] Root weight was very small.

Table 3.4.2b: Plants weight (g) and Cd and Zn content (μg/g) in shoots and roots of plants grown in Countesswells soil. The mean and RSD% are also included.

Plant Species		Shoots			Roots		
	Replicate	Weight	Cd	Zn	Weight	Cd	Zn
T. caerulescens	1	0.41	29.7	253	[a]		
	2	0.32	24.0	234			
	3	0.62	20.9	331			
	4	0.32	36.9	272			
	5	0.42	25.9	321			
	6	0.47	34.6	302			
	Mean	*0.43*	*28.7*	*286*			
	RSD%	*26*	*22*	*0.1*			
T. officinale	1	0.84	0.47	20.9	2.45	0.36	17.3
	2	0.97	0.54	17.7	2.26	0.30	16.4
	3	0.80	0.54	17.4	2.07	0.36	16.7
	4	0.76	0.65	21.0	1.98	0.28	18.1
	5	0.81	0.57	22.2	2.18	0.29	17.4
	6	0.79	0.56	18.4	1.31	0.36	22.6
	Mean	*0.83*	*0.56*	*19.6*	*2.04*	*0.32*	*18.1*
	RSD%	*8*	*11*	*10*	*19*	*12*	*13*
H. vulgare	1	1.89	0.13	21.4	0.74	0.85	50.4
	2	1.58	0.10	25.4	0.78	0.74	44.7
	3	1.89	0.16	22.1	1.00	0.32	31.0
	4	1.86	0.14	21.2	0.70	0.41	29.3
	5	1.95	0.13	22.3	0.97	0.09	12.6
	Mean	*1.83*	*0.13*	*22.5*	*0.84*	*0.48*	*33.6*
	RSD%	*8*	*15*	*8*	*17*	*65*	*44*

[a] Root weight was very small.

Photograph 3.5.1: (a) *H. vulgare* (barley); (b) *T. officinale* (dandelion) and (c) *T. caerulescens* (alpine penny cress).
(d-f) show *T. officinale* root system.



T. caerulescens shoot yield was the lowest across the 2 soils compared to the other two plant species even though this plant had the highest number of seedlings transplanted into each pot. *T. caerulescens* seeds germinated very slowly compared to the other two plant species. At the beginning of the experiment, many of *T. caerulescens* seedlings died due to transplant shock (Brown *et al*, 1994) and possibly because the plants fine roots were damaged while transplanting them into pots even after germinating seeds for 2 weeks. On the other hand, *T. officinale* or *H. vulgare* faced no such problems neither when germinating the seeds nor after transplanting into the soil. However, as each pot had different number of seedlings, comparing the absolute yield cannot be accurate, nevertheless it shows the difficulty encountered in growing *T. caerulescens*.

The dry weight of replicates of each plant species and the corresponding metal concentration (Table 3.4.2a and b) were tested for correlation for both metals. There was no evidence of a directly or inversely proportional relation between plant yield and the concentration of Cd. The same was found for Zn. Correlation coefficients of around 0.003 were obtained in either case.

In order to assess the accumulation of Cd and Zn in the 3 plant species from Great Billings soil, the average amount of metal removed per gram of plant dry material (DM) was calculated Table 3.4.3. The table also includes the total amount of Cd or Zn removed (in mg) over the course of the experiment (3 months). The latter was calculated from the total dry biomass produced by all replicates of each plant species (2.1g of *T. caerulescens* shoots, 16.6g of *T. officinale* shoots and roots and 8.6g of *H. vulgare* shoots). Data for Countesswells soil is not included as the amount of metal removed was very small, with *T. caerulescens* removing the highest total amount of 0.07 and 0.7mg for Cd and Zn, respectively among the 3 plant species tested.

Table 3.4.3: The plant species ability to remove Cd and Zn from the contaminated Great Billings soil.

Plant Species	**Metal removed per gram of DM** (mg/g)		**Total Metal Removed** (mg)	
	Cd	**Zn**	**Cd**	**Zn**
T. caerulescens	0.41	5.2	0.86	10.7
T. officinale	0.05	1.0	0.90	16.8
H. vulgare	0.02	0.7	0.20	5.9

An alternative way to assess the plant species ability to accumulate metals is to use the metal accumulation factor (A_f) (Baker *et al*, 1994):

$$A_f = \frac{metal\ concentration\ in\ the\ shoots}{initial\ total\ metal\ concentration\ in\ soil}$$

The mean A_f values of the 3 plant species in the 2 soils are presented in Table 3.4.4. The initial total metal concentration of each soil was determined by ID TIMS (Section 3.2.4E). In the table below, the hyperaccumulator species *T. caerulescens* has much higher A_f in both soils than the other 2 plant species for both soils.

Table 3.4.4: The mean accumulation factor A_f of *T. caerulescens*, *T. officinale* and *H. vulgare* plants in Great Billings and Countesswells soils. Values in parentheses are the standard deviations (SD).

Species	A_f			
	Great Billings		Countesswells	
	Cd	**Zn**	**Cd**	**Zn**
T. caerulescens	11.5 (4.5)	3.8 (1.4)	8.7 (1.9)	4.4 (0.6)
T. officinale	1.8 (0.4)	0.7 (0.1)	0.17 (0.02)	0.30 (0.03)
H. vulgare	0.6 (0.8)	0.6 (0.1)	0.04 (0.01)	0.34 (0.03)

In addition to the plants ability to accumulate Cd and Zn, the partitioning of Cd, for example, between shoots and roots is important. This shoots/roots distribution indicates the efficiency of translocation of metals which is very important particularly because phytoremediation is mainly concerned with the above the ground parts of the plant. Plant species shoots/roots ratios of both Cd and Zn in the 2 soils is presented in Table 3.4.5. The relatively large SD shows the difference between replicates of the same plant species in the distribution of metal in their shoots and roots.

Table 3.4.5: Mean values of Cd and Zn shoots/roots ratio for *T. officinale* and *H. vulgare* grown in Great Billings and Countesswells soils. Values in parentheses are the SD.

Species	Shoots/Roots			
	Great Billings		Countesswells	
	Cd	Zn	Cd	Zn
T. officinale	1.3 (0.6)	0.8 (0.3)	1.7 (0.4)	1.1 (0.2)
H. vulgare	0.16 (0.16)	0.20 (0.02)	0.5 (0.6)	0.8 (0.5)

Due to the similarities in the chemistry of Cd and Zn, the correlation between Cd and Zn uptake within the roots and that within the shoots was examined. The correlation coefficients between Cd and Zn concentration in the shoots and roots were calculated (Table 3.4.6). In this table, the higher R^2 values observed in the roots of *T. officinale* and *H. vulgare* plants show that Cd and Zn concentrations are more correlated in the roots than they are in the shoots.

Table 3.4.6: Correlation coefficient (R^2) between Cd and Zn in shoots and roots of the plant species grown in Great Billings and Countesswells soils.

Plant Species	Great Billings		Countesswells	
	Shoots	Roots	Shoots	Roots
T. caerulescens	0.86	[a]	0.05	[a]
T. officinale	0.56	0.90	0.05	0.08
H. vulgare	0.22	0.28	0.57	0.96

[a] Roots were not analysed.

B. L-values:

Cd and Zn isotopic ratios in the plants shoots grown in both soils were used to calculate the L-values (Tables 3.4.7a and b) according to the equation 1.5.6 (described earlier in Section 1.5.4A). In the tables below, the individual replicates isotopic ratios and the corresponding L-values are presented to show their similar L-values despite their different Cd and Zn content (Tables 3.4.2a and b above). This highlights the fact that direct metal uptake or plants Cd and Zn content can not be taken as a method to determine the bioavailability. In addition, the metal content of the plants has no effect on the L-value, which is a great advantage of this methodology.

Table 3.4.7a: Cd and Zn isotopic ratio of plants shoots grown in Great Billings soil with the corresponding standard error (SE %) and the calculated L-value expressed as μg/g of dry soil.

Plant Species		Cd			Zn		
Replicate		$^{111/114}$Cd	SE (%)	L-value	$^{66/67}$Zn	SE (%)	L-value
T. caerulescens	1	0.499831	0.037	25.0	6.192616	0.031	739
	2	0.490363	0.018	30.4[a]	6.204149	0.032	752
	3	0.505265	0.024	22.7	6.152576	0.036	697
	4	0.500444	0.017	24.7	6.182503	0.039	728
	5	0.504248	0.051	23.1	6.163997	0.044	708
	6	0.500877	0.014	24.5	6.184152	0.063	730
	Mean			*24.0*			*726*
	RSD%			*4.3*			*2.8*
T. officinale	1	0.506421	0.011	22.3	6.181868	0.029	727
	2	0.503150	0.017	23.6	6.225869	0.025	777
	3	0.508077	0.018	21.7	6.133477	0.024	678
	4	0.503387	0.013	23.5	6.224783	0.033	776
	5	0.503493	0.021	23.4	6.217918	0.035	767
	6	0.508153	0.017	21.6	6.158944	0.031	703
	Mean			*22.7*			*738*
	RSD%			*4.1*			*5.7*
H. vulgare	1	0.508043	0.016	21.7	6.139404	0.027	684
	2	0.502978	0.023	23.6	6.191698	0.070	738
	3	0.506495	0.016	22.2	6.141883	0.015	686
	4	0.502873	0.016	23.7	6.203113	0.050	751
	5	0.508262	0.047	21.6	6.145274	0.044	690
	Mean			*22.6*			*710*
	RSD%			*4.5*			*4.5*

[a] Data point rejected as an outlier using the following Dixon's Q test (Miller and Miller, 1988):

$$Q = \frac{|\text{Suspect value} - \text{Nearest value}|}{\text{Largest value} - \text{Smallest value}}$$

Table 3.4.7b: Cd and Zn isotopic ratio of plants shoots grown in Countesswells soil with the corresponding standard error (SE %) and the calculated L-value expressed as µg/g of dry soil.

Plant Species		Cd			Zn		
	Replicate	$^{111/114}$ Cd	SE (%)	L-value	$^{66/67}$ Zn	SE (%)	L-value
T. caerulescens	1	0.468190	0.029	0.46	6.648008	0.025	6.3
	2	0.465528	0.042	0.53	6.679901	0.019	7.1
	3	0.47673	0.030	0.33	6.694184	0.132	7.5
	4	0.466076	0.023	0.52	6.697025	0.030	7.6
	5	0.471184	0.018	0.41	[a]		
	6	0.464360	0.017	0.56	6.705599	0.041	7.9
	Mean			*0.47*			*7.3*
	RSD%			*18.3*			*8.5*
T. officinale	1	0.503461	0.020	0.18	6.715007	0.009	8.3
	2	0.503758	0.012	0.18	6.700512	0.015	7.7
	3	0.503428	0.021	0.18	[a]		
	4	0.511753	0.017	0.16	6.669184	0.040	6.8
	5	0.494632	0.030	0.21	[a]		
	6	0.467616	0.021	0.48[b]	6.653331	0.057	6.4
	Mean			*0.18*			*7.3*
	RSD%			*11.0*			*11.8*
H. vulgare	1	[a]			6.707612	0.015	8
	2	0.470024	0.016	0.43[b]	6.704824	0.128	7.9
	3	0.496357	0.063	0.20	6.686447	0.077	7.3
	4	0.492612	0.031	0.22	6.718347	0.075	8.4
	5	0.494414	0.008	0.21	6.685154	0.156	7.3
	Mean			*0.21*			*7.8*
	RSD%			*4.0*			*6.1*

[a] TIMS analysis was not successful, [b] Data point rejected as an outlier using Dixon's Q test (Miller and Miller, 1988).

The measured isotopic ratios in the shoots (*See* tables 3.4.7a and b) had good precision. An average standard error (SE) of 0.049% and 0.037% for Cd and Zn,

respectively, for plants grown in Great Billings soil was obtained. For plants grown in Countesswells soil, the average SE was 0.035% for Cd and 0.059% for Zn.

T-test (at $P<0.05$, using CoStat 3.03, 1986, CoHort Software, Berkley, USA) was used to see if the L-value determined by the different plant species was different. For Great Billings soil, there was no significant difference between the L-values measured using the 3 plant species for both Cd and Zn. The same was found for Zn in Countesswells soil. As for Cd in Countesswells soil, there was no significant difference between L-values measured using *T. officinale* (0.18μg/g) and *H. vulgare* (0.21μg/g), however, there was a significant difference (at $P<0.05$) between L-value measured using the hyperaccumulator *T. caerulescens* (0.47μg/g) and the other plant species.

The L-values of all replicates determined using the 3 plant species (apart from the significantly different Cd value determined using the hyperaccumulator *T. caerulescens* in Countesswells soil and rejected values) were averaged and are presented in Table 3.4.8. The L-values are also presented as a percentage of the total Cd and Zn soil content. This value is different in the 2 soils for either Cd or Zn as was the case in the extraction and E-value determination experiments earlier.

Table 3.4.8: L-values of Cd and Zn in Great Billings and Countesswells soil. Mean ± SD. Values in paranthesis are the mean expressed as a percentage (%) of total soil metal.

	L-value (μg/g)	
Soil	**Cd**	**Zn**
Great Billings	23.1±1.1 (68%)	725±33 (59%)
Countesswells	0.19±0.02 (6%)	7.46±0.63 (11%)

Although the above L-values were based on the shoot material, a few samples of *T. officinale* and *H. vulgare* roots grown in Great Billings soil were analysed for their isotopic composition and their L-values calculated. *T. officinale* mean value of 24.0µg/g and 811µg/g for Cd and Zn (n=5) and for *H. vulgare* values of 23.9µg/g (n=3) and 725µg/g (n=2) for Cd and Zn, respectively were calculated. However, any slight contamination of roots with soil particles (especially the Great Billings contaminated soil) would greatly affect the isotopic ratio, the isotopic composition of the roots Cd and Zn was not measured or used in calculating the L-values.

3.4.4 Discussion:

A. Phytoremediation potential:

Although replicates of each plant species were grown exactly under the same controlled experimental conditions, Cd and Zn contents had a wide range (e.g. 2070-6300mg/kg Zn in *T. caerulescens* grown in Great Billings soil, Table 3.5.1a). This is demonstrated by the relatively high standard deviation for both elements content in shoots and roots (Table 3.4.2a and b). Roots Cd and Zn content in particular could have been affected by how efficient they were separated from soil particles, especially in Great Billings as small residues of soil could affect appreciably Cd and Zn content. However, this effect (in terms of RSD) was no bigger than that in shoots indicating good cleaning of roots from soil. The variation in metal uptake of individual replicates is not clear, however it may have been caused by the different number of seedlings in each pot, the individual plant root activity or other biological factors.

T. caerulescens above the ground tissues contained notably higher Cd and Zn content than both *T. officinale* or *H. vulgare* (Table 3.5.1a). Under the present experimental and growth conditions *T. caerulescens* was demonstrated to be a hyperaccumulator of Cd (accumulating >100µg Cd/g DM), but not a Zn hyperaccumulator (containing <10000µg

Zn/g DM). These definitions of hyperaccumulators are based on those given by Baker and Brooks (1989) (*See* Section 1.3.1). Although recent studies for *T. caerulescens* showed that the species is a hyperaccumulator of Zn and Cd (Brown *et al*, 1994; Brown *et al*, 1995; McGrath *et al*, 1997; Knight *et al*, 1997; Shen *et al*, 1997; Brooks *et al*, 1999) using contaminated soils and sometimes nutrient solutions, the results obtained here are not as high as those published. However, factors affecting metal availability (Section 1.4.2), the different soil properties, growth conditions and soil total metal may have affected the plants metal uptake. In particular this latter parameter may be particularly important since in a similar study, shoot Zn and Cd concentrations of up to 18455 and 1020μg/g, respectively, were reported by Brown *et al* (1994) for *T. caerulescens* grown on a Zn smelter-contaminated soil. This soil had a total Zn and Cd content of 48000 and 1020μg/g, respectively, which is much higher than the soil used here, where the total Zn and Cd content were 1231 and 33.8μg/g, respectively.

However, although *T. caerulescens* has accumulated high levels of Cd and Zn from Great Billings soil, low Cd and Zn content was found in *T. caerulescens* plants grown in the non contaminated, Countesswells soil. This indicates that this species is not able to accumulate Cd or Zn from soils with low total Cd or Zn content. This was also found by other workers (Hajar, 1987; McGrath *et al*, 1997; Brown *et al*, 1994; Baker *et al*, 1994).

T. officinale and *H. vulgare* are both considered non-hyperaccumulators of either Cd or Zn using the same definition. However, *T. officinale* has accumulated about 70% of the threshold accumulation value used to define a Cd hyperaccumulator. This indicates that the plant has at least the potential for phytoremediation, which may be increased by altering the experiment conditions (such as soil pH or indeed the soil itself). In addition, the larger biomass production of *T. officinale* compared to *T. caerulescens* could result in actually removing more metal in net terms. The ability of *T. officinale* for phytoremediation would be markedly increased if a means of harvesting the taproots from the ground were available. The total metal removed (translocated) is used to evaluate the potential effectiveness of each plant for phytoremediation (Brown *et al*, 1995). In this respect our study shows that *T. officinale* and *T. caerulescens* have the same phytoremediation potential for Cd (both removing in net terms about 0.9mg), while *T. officinale* is a better phytoremediator for Zn

(accumulating 16.8mg compared to 10.7mg) Table 3.4.3. In terms of metal removed per gram of dry matter, the experiments conducted have shown that *T. caerulescens* had the highest phytoremediation potential. However, when remediating a contaminated site, the major concern is the amount of metal removed and not its concentration in plant material. A careful balance between the 2 factors must be made.

In literature, *T. officinale* has not been tested as a hyperaccumulator, but has been demonstrated to be a potential indicator plant for heavy metal contaminated sites (Simon *et al*, 1996). It accumulated up to 410 and 1360μg/g of Cd in shoots and in its roots/rhizomes, respectively (Simon *et al*, 1996). *H. vulgare* did not accumulate high amounts of Cd and Zn and, within the experimental conditions, it is not a hyperaccumulator in spite of the relatively high Cd and Zn content in the roots. However, *H. vulgare* grown on Zn contaminated soil accumulated >2mg of Zn per plant, 2-4 times more Zn than what was observed in the hyperaccumulator *B.juncea* (Indian mustard) indicating the plant's good potential for phytoremediation (Ebbs and Kochian, 1998). The latter study, did not include Zn content of shoots (μg/g) and was mainly concerned with the absolute amount of Zn removed.

The phytoremediation ability of *T. caerulescens* of Cd or Zn can also be seen from the accumulation factor (Table 3.4.4). *T. caerulescens* had by far the highest A_f for both Cd and Zn in both soils. This indicates that this species has the greatest ability to access, mobilise and translocate Cd and Zn to the shoots than the other species considered here. *T. caerulescens* had particularly high A_f for Zn (4.4) in Countesswells soil compared to that of the other species. This may have occurred because *T. caerulescens*, having survived on Zn smelter soils, may have lead Zn to play a more major role in this species (plant metabolism and function) than others. As a result the plant may have been forced to mobilise and translocate Zn more than it usually does particularly with the low Zn content of Countesswells soil. This was not proved, however in an experiment by Pollard (2000) *T. caerulescens* plants grew better and with larger yield when grown in solutions of high Zn concentration than in low Zn concentration solutions. The different A_f of the plant species in the 2 soils may be attributed to the different soil properties, particularly the pH. Although the determined A_f values for Cd were higher than these for Zn, Knight *et al* (1997) found

the mean A_f for Zn as much as 60 times that for Cd. This is again can be related to the different soil properties and plant species studied.

The metal shoots/roots distribution from the phytoremediation point of view indicates the efficiency of translocation of metals from roots to shoots and is used to evaluate the potential effectiveness of each plant for phytoremediation (Brown *et al*, 1995). In addition, comparisons of internal distribution of Cd and Zn may provide an understanding of different internal tolerance mechanisms (Baker and Walker, 1990). Even though it was not possible to analyse *T. caerulescens* roots to compare root and shoot Cd and Zn composition. Zn concentration in shoots has been reported to be 2-10 times that in roots (Knight *et al*, 1997; Brown *et al*, 1995; Shen *et al*, 1997), while 1-6 times for Cd (McGrath *et al*, 1997; Knight *et al*, 1997). In the present experiments, *T. officinale* had higher shoots/roots ratios of both Cd and Zn in both soils compared to *H. vulgare* (Table 3.4.5). However a ratio of about 1 in *T. officinale* indicates that the metal is distributed almost evenly between shoots and roots making roots as important to phytoremediation as shoots. Although *H. vulgare* did not accumulate appreciable amounts of Cd and Zn, the root content was as much as 30 and 7 times that in the shoots, respectively. However, as *H. vulgare* roots are not easy to harvest, their potential for phytoremediation cannot be compared to that of *T. caerulescens* or *T. officinale*. Nevertheless, this weak translocation of metal from roots to shoots indicates that *H. vulgare* grown on a soil contaminated with Cd and Zn has the ability to confine these metals in the roots and not to transport them to the above the ground parts of the plant (particularly the edible grains). *H. vulgare* could potentially be grown on "contaminated" soil without the risk of plant containing high levels of Cd or Zn.

In Table 3.4.6 above, correlation coefficients (R^2) between Cd and Zn in the shoots and in the roots were presented, where higher correlation found in the roots indicating high Cd and Zn association in the plant roots. Within the shoots, Cd and Zn correlation in *T. caerulescens* grown in the Great Billings contaminated soil was the highest which is probably related to the plants' ability to translocate and accumulate high levels of both elements. This could also be related to suggestions that *T. caerulescens* has a non-specific uptake mechanism which scavenges several metals (Knight *et al*, 1997). The high Cd and Zn correlation in the roots compared to the shoots might be explained as follows. Due to the

similar chemistry of both elements, the flux of Cd and Zn from soil solution into the plant root through exudates is very much similar. However, once both elements are within the plant root, the root cells may have the ability to selectively transport each element to a different extent to the shoots. The variation in the correlation coefficient shows that Cd and Zn correlation is not simply influenced by the plant species on its own or by the soil properties. This is clear from the fact that no plant species had the same correlation coefficient in the 2 soils and also any soil had failed to have the same correlation coefficient regardless of the plant species. Both the plant and soil factors need to be investigated for a further more precise conclusion.

B. L-values:

The RSD% (100 SD/mean) values (Table 3.4.7a and b) represent the difference in the L-values obtained from individual replicates (pots) of each plant, which is frequently used in the comparison of results precision (Miller and Miller, 1988). Replicates of plants grown in Great Billings soil had less variation in the calculated L-value compared to that in Countesswells soil for both elements (highest RSD% value: 5.7 in Great Billings soil compared to 18.3 in Countesswells soil). The small variation of L-value within plant replicates (pots) could be related to the isotopic equilibration between the spikes added and the original soil metals. The greater isotopic equilibration (the lowest RSD%) in the sewage sludge amended Great Billings soil might have been enhanced by the fact that the soil Cd and Zn were introduced to the soil which might have made the isotopic exchange easier.

The L-values determined using the 3 plant species showed no significant difference (analysis of variance, P=0.05) for both metals in the Great Billings soil. However, for plants grown in Countesswells soil, a significant difference (P=0.05) was only found for Cd between *T. caerulescens* and each of *T. officinale* and *H. vulgare*. The fact that similar L-values were obtained with 3 different plant species (Table 3.4.7a and b) despite the species

Cd ad Zn uptake and tolerance (Tables 3.4.2a and b) clearly illustrate that the metal pool available for any plant species uptake is the same. The difference lies in the extent to which each plant species is able to mobilise and accumulate the elements from that bioavailable pool. However, the fact that the hyperaccumulator *T. caerulescens* had different Cd L-value from *T. officinale* and *H. vulgare* in the Countesswells soil is surprising and needs further investigation. This might indicate that *T. caerulescens* plants have the ability to access a pool of soil Cd that was unavailable for plant uptake by other species. However, this is not confined to hyperaccumulating plants, Hamon *et al* (1997) found that the bioavailable Cd for canola plants was nearly double that available for other plants such as capeweed, lettuce and wheat using radioisotopes.

The percentage of total Cd and Zn in bioavailable form was calculated to be 68% and 59% in Great Billings soil and 6% and 11% in Countesswells soil, respectively. This indicates that a significant amount of these metals exist in forms available for plant uptake in the Great Billings soil. In contrast, in Countesswells soil only a small proportion is in fact available. This can again be related to difference in the original sources of Cd and Zn in the 2 soils. Although stable isotopes have not been used to estimate bioavailable Cd or Zn, recent studies used radioisotopes for this purpose. Hamon *et al* (1997) used 5 plant species on a fine sandy loam soil and estimated that 36% and 12% of total soil Cd and Zn was available for plant uptake. In another study using 1 plant species (wheat) and 8 different soils, the L-value of Cd varied from 55-108% of total soil Cd (Smolders *et al*, 1999). In a similar experiment to estimate the bioavailable Ni using radioisotopes, the L-value ranged from 15% (in a silty loam soil) to 40% (in a clay soil) of soil total metal using red clover (Echevarria *et al*, 1997). These results, despite the wide range, show that our Cd and Zn L-values are comparable since the different soil properties can produce different results.

Comparing the values obtained here with the other part of the isotopic exchange method (the E-value, Section 3.3) shows that L-values obtained are lower than E-values. This however, has been generally the case for work investigating P availability (Sen Tran *et al*, 1988; Fardeau *et al*, 1996; Fardeau, 1996). In contrast, a recent study found that Cd behaves differently where L-values always exceeded the E-values between 1.05 and 1.4-

fold. (Smolders *et al*, 1999). The L-values of Cd and Zn in relation to the E-values and soil extraction have been discussed in further details later (Section 4.1).

In this experiment we have applied the isotopic kinetics in a soil plant system to determined the pool of bioavailable Cd and Zn in 2 soils using 3 plant species. This is the first time stable isotopes are used for this purpose rather than radioisotopes. Stable isotopes offer the added advantage of not requiring the special care when handling radioisotopes or the need for special growth cabinets. In addition, the present experiment reports for the first time, the use of a hyperaccumulating plant species in the evaluation of bioavailable metal in comparison to non-hyperaccumulating plant species. The agreement of the bioavailable pool between the 3 different plant species is very significant.

3.4.5 Main findings:

1. *T. caerulescens* shows much greater accumulation of both Cd and Zn than *T. officinale* and *H. vulgare*.
2. A higher yielding crop with *T. caerulescens* Cd and Zn content, could be used to remediate Zn and Cd contaminated sites *in-situ* more effectively and within a shorter time frame.
3. *T. officinale* removed approximately the same amount of Cd when compared to *T. caerulescens*, while it removed more Zn.
4. *T. caerulescens*, *T. officinale* and *H. vulgare* grown on Great Billings soil have the same available Cd and Zn (L-value).
5. *T. caerulescens*, *T. officinale* and *H. vulgare* have the same available Zn (L-value). However, *T. caerulescens* available Cd was different and higher from the other similar two plant species.

3.5 LOCALISATION OF Cd AND Zn AROUND *T. OFFICINALE* (DANDELION) ROOTS

Phytoremediation of contaminated soil is dependent on plant roots accessing the pollutant heavy metal. The root activity in the rhizosphere will affect the efficiency by which metal uptake occurs. Among the 3 plant species studied in the determination of L-values (Section 3.4), *T. officinale* was chosen to investigate root development and the effect it has on Cd and Zn concentration. Due to root activity, Cd and Zn concentration was expected to deplete in the rhizosphere area, where the lower concentrations were expected to be closer to the root surface. In addition Cd and Zn concentration in relation with the amount of root present (weight) in each soil section was also to be investigated to see if the root weight is related to activity.

T. officinale was chosen for this experiment because it has a tendency to develop an extensive taproot system. As mentioned earlier, this species is found growing on contaminated soil and has the ability to withstand potentially phytotoxic conditions. In addition, the relatively rapid growth ability of *T. officinale* would make monitoring the effect of the root activity on Cd and Zn depletion easier within short period of an experiment.

The Great Billings sewage sludge amended soil was used to study the amount of Cd and Zn taken up by the plant and its potential for phytoremediation. For a small plant, dandelion taproot system can penetrate into the soil making it a good candidate for phytoremediation of contaminated soils. The thickness of the soil in between the glass sheets was designed to be small to allow a high surface of soil to be exposed to the roots so that the system encloses the entire rhizosphere. Having measured the extractable Cd and Zn before growing dandelion (Section 3.1.3), each section of the soil around the root was extracted using acetic acid to detect any decrease in the extractable Cd and Zn content.

3.5.1 Materials and methods:

Polystyrene strips, 8mm thick, were placed on the 2 sides and the bottom edge of a glass plate (200 × 200 × 2mm). Dried and sieved Great Billings soil used in this experiment has been described earlier in Section 3.1.2A. A layer of the soil, about 4mm thick, was spread on the glass plate. Seedlings of *T. officinale* which had been germinated for 3-4 weeks with a root of 4-10mm in length, were then placed in the centre of the glass plate. The roots were then covered with another 4mm of soil and sandwiched with the upper glass plate, which was covered with cling film to prevent soil from sticking to the glass. The two plates were then sealed with masking tape on the 2 sides and the bottom leaving the top open to enable watering of the plant. A few holes were made at the bottom to allow excess water to drain. Three glass plates were constructed each containing a total amount of soil between 225-230g of dry soil. Plates 1 and 3 contained 1 plant, plate 2 contained 2 plants.

The plates were covered with a black bag to protect the roots from light, positioned vertically and kept in the growth cabinet (Section 3.4.2) and watered daily for 4 weeks. Water was added in small quantities so leaching (drainage) was minimised. Photographs 3.5.1a, b and c were taken 2 and 4 weeks after the start of the experiment to monitor the root development.

After 4 weeks, the upper glass plate was carefully removed to minimise disturbing the soil or breaking the roots. Each plate was then divided into 12 soil sections (3 vertical × 4 horizontal, *See* Photographs 3.51a,b,c). The roots were carefully removed from each section and dried at 30°C for 72h, and dry weight recorded. The soil in each section was dried at 105°C overnight, left to cool to room temperature (in a sealed container to prevent soil absorbing moisture) and dry weight recorded. Three subsamples of each section were then extracted with 0.43M acetic acid following the procedure described earlier (Section 3.1.2C). Cd and Zn concentrations were analysed using ICP-AES (IRIS, Thermo Jarrel Ash, UK).

3.5.2 Results:

The acetic acid extractable Cd and Zn content of Great Billings soil after growing *T. officinale* is tabulated in Table 3.5.1. The table also includes the corresponding root and soil weights in each section. The glass plates are shown in Photograph 3.5.1a, b and c for plates 1, 2 and 3, respectively, this also includes a key to the different sections on each plate.

Table 3.5.1: The 3 glass plates experiment results with the weight (g) of both root and soil in each section, and the corresponding extractable Cd and Zn content[a] (μg/g). Also *See* Photograph 3.5.1a, b and c.

Plate Section	Plate 1				Plate 2				Plate 3			
	Weight		Concentration		Weight		Concentration		Weight		Concentration	
	Root	Soil	Cd	Zn	Root	Soil	Cd	Zn	Root	Soil	Cd	Zn
A	0.02	17.1	18.8	575	0.03	17.0	19.7	607	0.01	17.0	18.3	548
B	0.25	21.9	19.7	612	0.45	18.0	19.8	611	0.11	18.5	19.1	576
C	0.01	18.2	18.3	565	0.03	15.7	18.9	551	0.02	17.7	18.8	562
D	0.03	17.4	19.1	611	0.04	19.6	19.6	599	0.02	18.4	18.5	548
E	0.08	22.3	18.0	574	0.17	20.3	19.2	579	0.03	20.4	19.6	593
F	0.02	19.0	19.0	582	0.03	15.7	19.8	611	0.02	18.9	19.9	604
G	0.01	18.0	17.2	537	0.04	19.8	18.9	573	0.02	19.4	18.3	557
H	0.06	21.0	17.7	561	0.07	20.8	19.4	602	0.02	21.8	19.7	601
I	0.04	17.2	19.3	599	0.03	15.5	19.3	600	0.02	18.3	20.8	613
J	0.02	18.7	19.5	614	0.08	23.5	19.3	582	0.01	19.8	19.4	584
K	0.06	22.3	19.0	582	0.08	23.0	19.0	557	0.03	23.5	20.0	614
L	0.02	17.5	19.7	610	0.04	16.4	19.0	568	0.02	18.0	20.2	615

[a] Cd and Zn measured had maximum standard deviations of 0.6 and 6μg/g, respectively (n=3).

The above data for each plate showed a different pattern of Cd and Zn concentration and of root development. For example, although the root existed mainly in the central sections (B, E, H and K), Cd or Zn was not the lowest. The relationship between Cd or Zn concentration and the root weight was tested and the correlation coefficients between Cd or Zn concentration in each section and the amount of root present were calculated for each plate (Table 3.5.2).

Table 3.5.2: Correlation coefficients (R^2) between root weight and Cd and Zn concentration in the three plates.

Plate Number	Correlation coefficients (R^2)			
	Roots : Concentration		Root/soil : Concentration	
	Cd	Zn	Cd	Zn
1	0.087	0.091	0.10	0.011
2	0.12	0.08	0.14	0.09
3	0.005	0.009	0.002	0.004

As the R^2 values between the weight of root present in each section and metal concentration did not indicate a correlation, the root/soil ratio in each section was tested for correlation with Cd or Zn concentration. The root/soil weight ratio was chosen because of differences in soil weight in each section as a result of packing. However, the poor correlation was not enhanced by this approach.

Despite the poor correlation between Cd or Zn concentration with either the root weight or with root/soil ratio, it was noticed that wherever there was a decrease in Zn concentration, the same was observed in Cd. A regression between Zn and Cd concentration in each section of each plate was established and correlation coefficients (R^2) of 0.87, 0.83 and 0.93 were calculated for plates 1, 2 and 3, respectively. The high correlation coefficients suggest a similar behaviour in the uptake pattern between Cd and Zn.

In addition to investigating the Cd and Zn concentration around the *T. officinale* roots, the plant phytoremediation ability, despite the short term of this experiment, was investigated. The total amount of Cd and Zn removed after 4 weeks of growing dandelion in between glass plates is shown in Table 3.5.3 below along with the Cd and Zn content in the soil before and after plant growth. The amount of Cd or Zn removed was calculated by subtracting the total Cd or Zn in the plate after growing the plant from that present before.

Table 3.5.3: The average[a] 0.43M Acetic acid extractable Cd and Zn content in soil before[b] and after growing *T. officinale* and amount of Cd and Zn removed in each plate (mean ± SD). Total soil weight of each plate is also given.

		Cd			Zn		
Plate	Soil weight (g)	Before (µg/g)	After (µg/g)	Removed (µg)	Before (µg/g)	After (µg/g)	Removed (µg)
1	231	19.5 ±0.15	18.8± 0.8	161	608±5	585± 25	5304
2	225		19.3± 0.3	45		587± 21	4731
3	232		19.4± 0.8	23		585± 25	5329

[a] The average extractable Cd or Zn was calculated by multiplying each section Cd or Zn concentration by the amount of soil present, the sum of these values for all sections was then divided by the total soil in each plate.
[b] *See* Table 3.1.1a.

For Zn, the 3 plates had significantly reduced ($P<0.05$) extractable Zn content after growing *T. officinale*. there was a significant reduction in the ($P<0.05$) in the extractable Cd content of the soil in plate 1 after growing *T. officinale*. However, no significant differences were observed in plates 2 and 3.

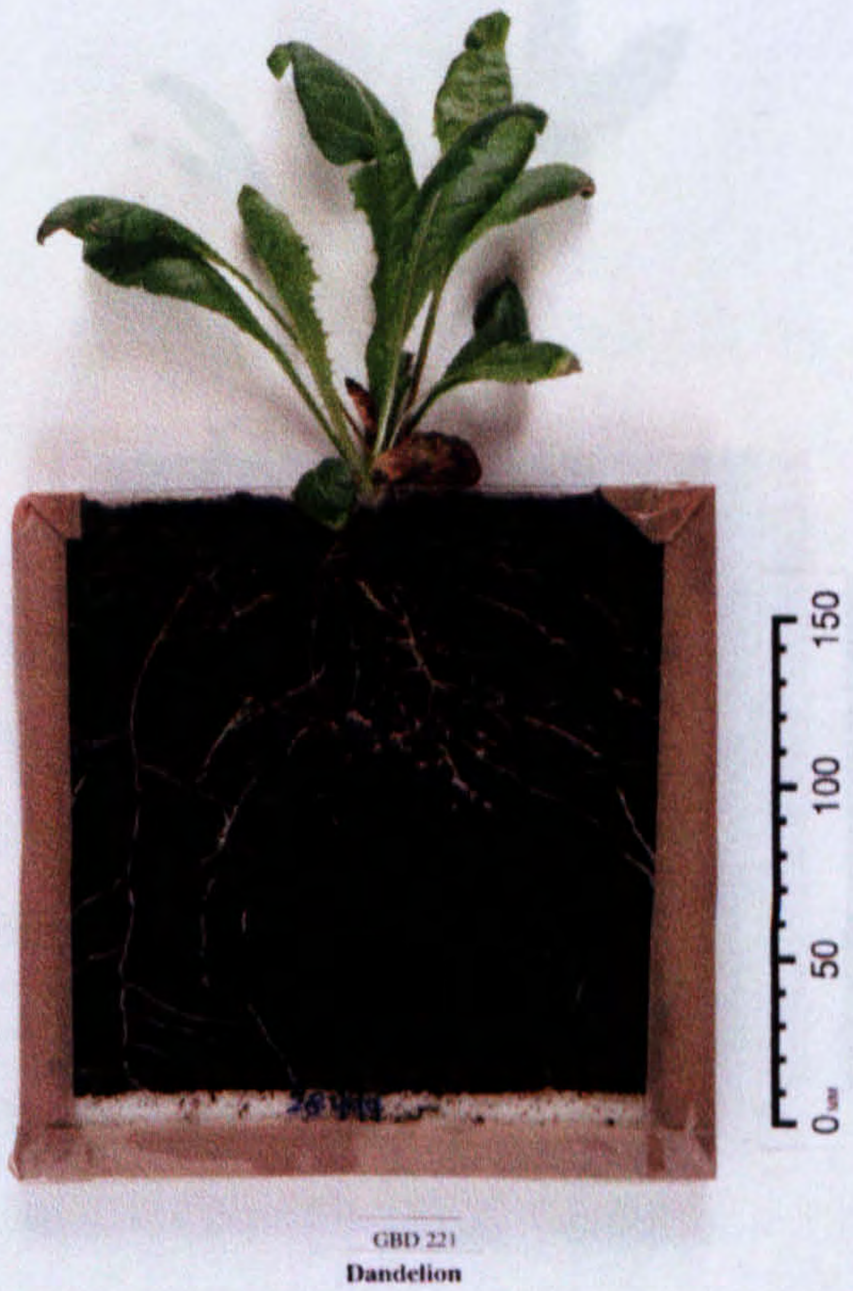
Photograph 3.5.1a: Glass plate 1 root growth.

GBD 331
Dandelion

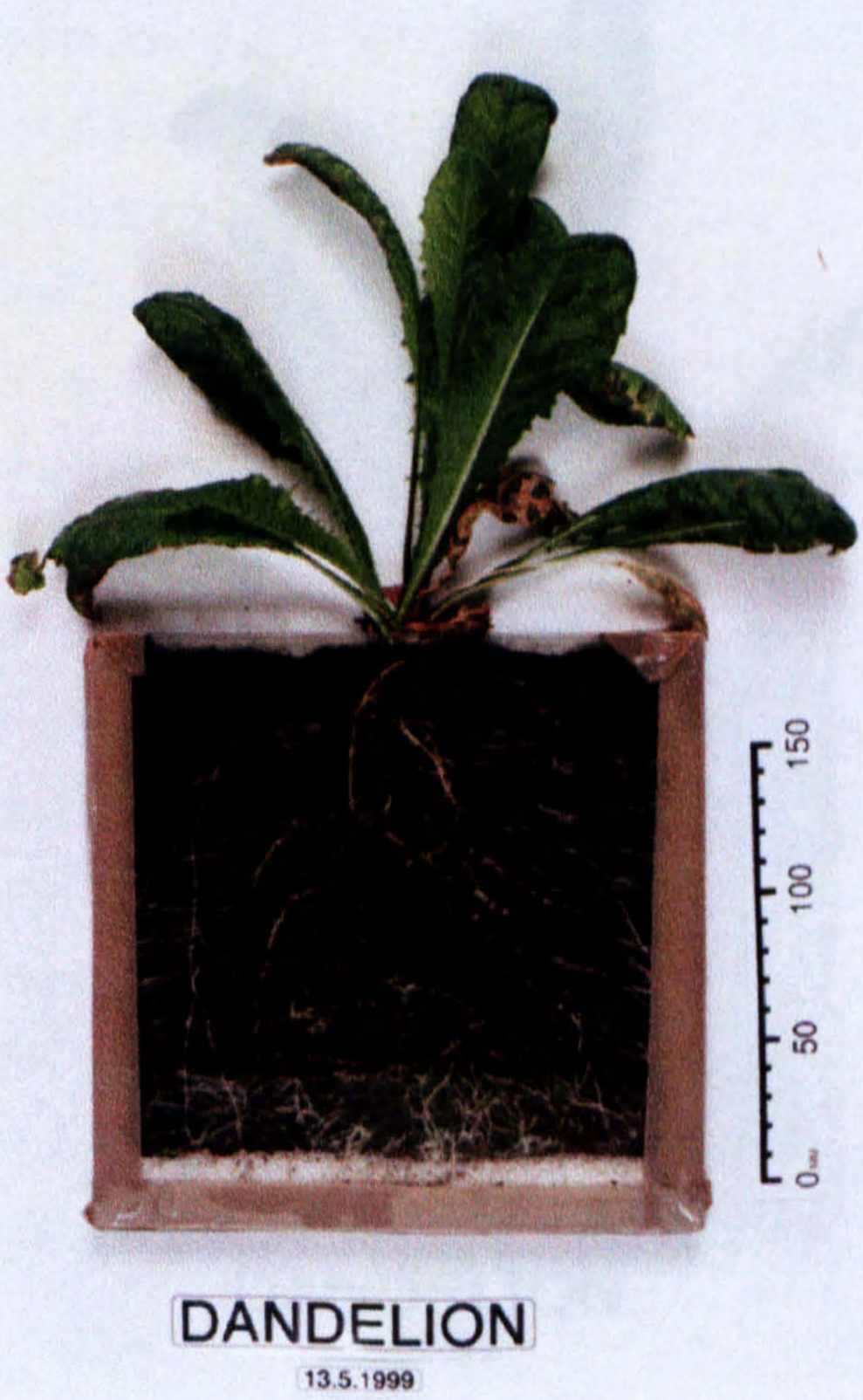
A	**B**	**C**
D	**E**	**F**
G	**H**	**I**
J	**K**	**L**

DANDELION
13.5.1999

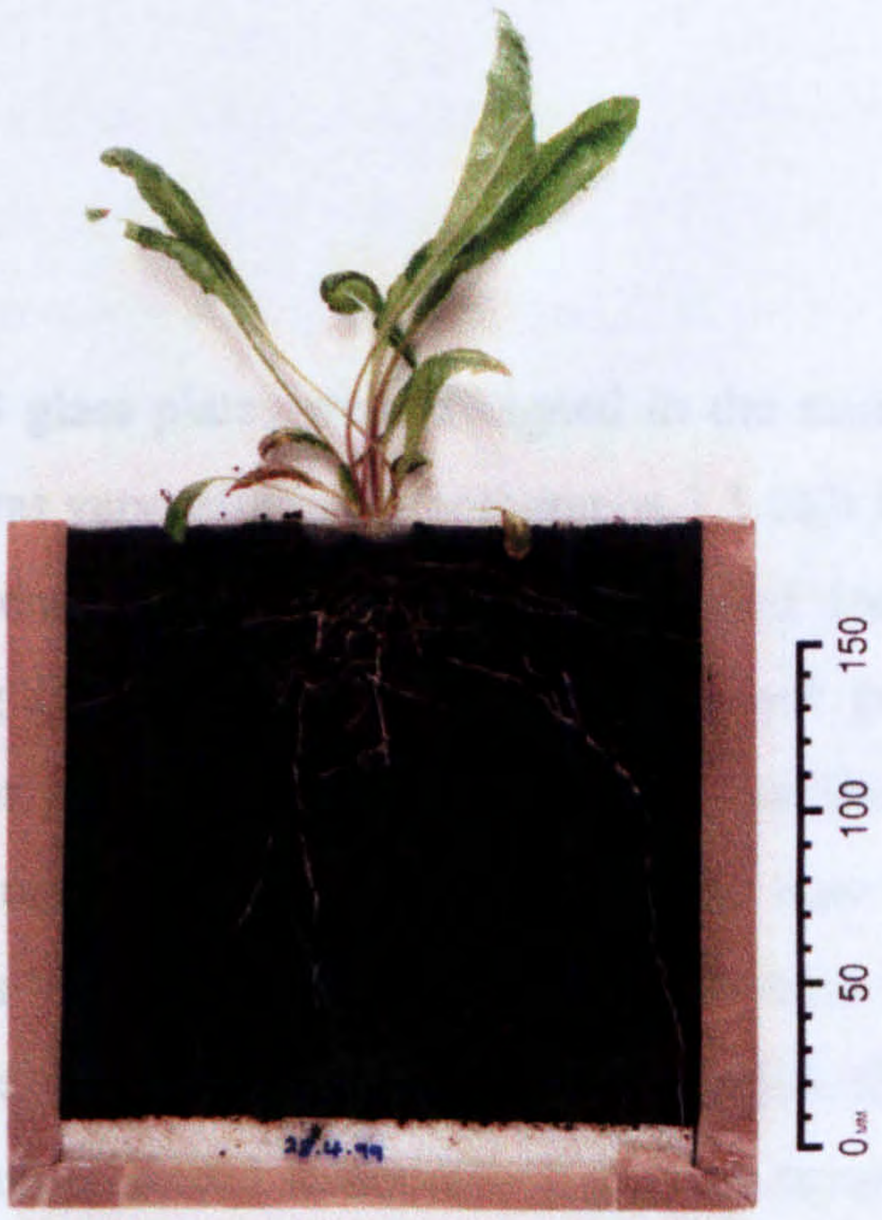
Photograph 3.5.1b: Glass plate 2 root growth.



A	B	C
D	E	F
G	H	I
J	K	L



Photograph 3.5.1c: Glass plate 3 root growth.



A	B	C
D	E	F
G	H	I
J	K	L

3.5.3 Discussion:

Although the 3 glass plates were designed in the same way, unfortunately the root growth in each one was very different (Photographs 3.5.1a,b and c; Table 3.5.1) and did not produce similar concentration pattern results as expected. Due to this difference, a general conclusion regarding Cd or Zn concentration with root growth was not possible. The difference in the root growth and the concentration distribution may be related to many factors such as the number of seedlings in each plate, how compact the soil was in each section and variations in drainage. Also the individual plants may have varied in relation to heavy metal tolerance. In addition, the fact that the plates did not have only one seedling and each seedling plant produced sometimes 3 major roots and not only one, made the soil root interaction system more complex than originally anticipated.

Among the different sections, uptake of Cd and Zn from the more compacted sections was lower than the other less compacted sections. In two cases (Plates 1 and 3), Cd and Zn concentrations were found to be relatively lower in the top 3 sections, A, B and C, which may be related to a leaching effect, watering the plant may have resulted in water washing metals toward the bottom of the plate. The taproot weight did not have a direct effect on decreasing Cd and Zn within a soil section. When Cd or Zn concentration was tested for correlation with the root weight or with the root/soil ratio, poor correlation was found indicating that the root weight is not simply a measure of their activity. The section where the majority of taproot existed (B) did not have lower levels of Cd or Zn, compared to other sections. This is perhaps not surprising since the taproot is likely to have a storage role rather than been involved in metal uptake. In the central sections (E, H and K), a gradual decrease in Cd and Zn concentration was observed in plate 1 indicating the small roots high activity toward the bottom, however this was not the case in plates 2 and 3. This behaviour however is not clear.

Despite the poor correlation between Cd or Zn with the root present in soil, correlation coefficients values between the concentration of Cd and that of Zn (R^2 of 0.87, 0.83 and 0.93) indicate a close relationship between Cd and Zn uptake by roots. This indeed

agrees with the correlation coefficient determined earlier in the extraction of Cd and Zn using acetic acid (0.83 in Table 3.1.4) and with that in the roots of *T. officinale* (0.90 in Table 3.4.6). This agreement shows that Cd and Zn are correlated to a similar extent in soil and in roots and provides further evidence that the correlation obtained is correct and is not as a result of the analytical method used in the analysis. The similarities in the chemistry of the 2 elements could be responsible to this behaviour, although they differ significantly in their role within the plant as Zn is essential while Cd has no known function and indeed is phytotoxic (Wagner, 1993). Similar correlation was established earlier in the extracting the soil with acetic acid and EDTA (Section 3.1.3).

The average Cd and Zn concentration in each plate was lower than that before growing the plants (Table 3.5.2) indicating the plant uptake of Cd and Zn. However, the data were significantly different only for Cd (in plate 1) and for Zn (in the 3 plates) between before and after growing the plant. Also, each plate had different total amounts of Cd and Zn removed and do not seem to be related to the total root weight. The amount of Cd and particularly of Zn removed over a period of 4 weeks (about 5 mg of Zn), shows *T. officinale* high ability to remove Cd and Zn from contaminated soil, and a high potential for phytoremediation and reinforces data presented earlier (Section 3.4.3A).

An important issue which may have given a better understanding of the uptake of Cd and Zn in the rhizosphere is the investigation of root exudates and other metabolites into each section of the soil and the quantitative and qualitative effect this might have on the mobilisation of Cd and Zn. This approach have been investigated for hyperaccumulating plants such *T. caerulescens* (Brown *et al*, 1995; Raskin, 1996; Shen *et al*, 1997; McGrath *et al*, 1997; Knight *et al*, 1997; Salt *et al*, 1999). However, there is a lack in the literature of studies investigating *T. officinale* metal mobilisation and uptake in the rhizosphere.

In this study we detected no evidence of any pattern to the metal uptake, it appeared relatively uniform across the entire root rhizosphere. No zone of high activity or metal depletion were identified. However, although the experiment failed to show any pattern of Cd or Zn concentration distribution in relation to the amount of root present, many interesting points have been raised as a result. Growing 1 plant in each plate instead of 2 or 3 would make it easier to demonstrate the root distribution of Cd and Zn. Also, as there was

many roots of different sizes in any soil section, using means (such as rhizosphere bag, McGrath *et al*, 1997) to restrict a certain root size in a particular soil section. This is based on the assumption that roots of the same size (diameter) have the same function on metals present in the soil. A less complex system would be studying the uptake of Cd and Zn by *T. officinale* in solution culture instead of the present more complex soil system.

3.5.4 Main findings:

1. There was a decrease in the acetic acid extractable Cd and Zn content after growing the plant compared to that before.
2. There is a correlation between Cd and Zn depletion pattern.

4. DISCUSSION AND FUTURE WORK

4. GENERAL DISCUSSION

Thermal ionisation mass spectrometry (TIMS) is generally accepted as the mass spectrometric method that yields isotope ratio measurements with the highest precision for isotope ratio measurement (Heumann, 1986b). Combined with the definitive method of isotope dilution, TIMS provides a powerful analytical technique for metal quantification. An ID-TIMS method was developed here and shown to produce highly accurate and precise results, which were validated through the analysis of CRMs. The method developed using the principles of ID was used to measure the available Cd and Zn for plant uptake from a contaminated and a non-contaminated soil. This was determined in a soil:solution system (known as E-value) and in a plant:soil system (known as L-value). In the following discussion, results from the different experiments are summarised and compared. As a hyperaccumulator, *T. caerulescens* was used to estimate the bioavailability of Cd and Zn to be compared with the non-hyperaccumulating species of *H. vulgare* and *T. officinale*. *H. vulgare* is a crop plant and was included for comparative purposes. *T. officinale* was chosen as it is found growing on contaminated land. In addition, the ability of the 3 plant species to accumulate Cd and Zn and the potential for phytoremediation is discussed.

4.1 Bioavailability of Cd and Zn assessment:

The characterisation of heavy metal fractions in soil is very important to predict the amount of bioavailable metal for plant uptake. Extraction with various chemicals is most commonly used for this purpose. Alternatively, determining the bioavailable metal pool can be achieved using isotopic dilution method (Young *et al*, 2000). In the present work, 5 chemical extractants were used to estimate the bioavailability of Cd and Zn in 2 soils and compared with the bioavailable Cd and Zn estimated by the isotopic exchange methods (E

and L-value). The data obtained is summarised in Table 4.2.1a and b for Great Billings and Countesswells soils, respectively.

Table 4.1.1a: Extractable Cd and Zn, E-value and L-value content (μg/g of dry soil) for Great Billings soil. Values in parenthesis are percentages of soil total[a] metal.

	Extraction					E-value	L-value		
	CH_3COOH	EDTA	NaOH	$CaCl_2$	$NaNO_3$		*T. caerulescens*	*T. officinale*	*H. vulgare*
Cd	19.5 (58)	23.0 (68)	12.8 (38)	0.15 (0.4)	0.43 (1.3)	29 (86)	24.0 (71)	22.7 (67)	22.6 (67)
Zn	608 (50)	581 (47)	273 (22)	4.1 (0.3)	8.2 (1)	806 (65)	726 (59)	738 (60)	710 (58)

[a] Total of 33.8 and 1231μg/g of Cd and Zn, respectively.

Table 4.1.1b: Extractable Cd and Zn, E-value and L-value content (μg/g of dry soil) for Countesswells soil. Values in parenthesis are percentages of soil total[a] metal.

	Extraction					E-value	L-value		
	CH_3COOH	EDTA	NaOH	$CaCl_2$	$NaNO_3$		*T. caerulescens*	*T. officinale*	*H. vulgare*
Cd	0.15 (5)	0.10 (3)	nd	0.02 (0.6)	0.01 (0.3)	nd	0.47 (14)	0.18 (6)	0.21 (6)
Zn	4.3 (7)	2.1 (3)	2.2 (3)	0.21 (0.3)	0.05 (0.1)	8.6 (13)	7.28 (11)	7.3 (11)	7.78 (12)

[a] Total of 3.3 and 65.3μg/g of Cd and Zn, respectively.
nd= not determined, below LOD of 0.064μg.

The amount of Cd or Zn extracted by the 5 extractants was very different. CH_3COOH and EDTA for example, extract the exchangeable, organically bound and the tightly bound inorganic heavy metals, while $CaCl_2$ extracts only the exchangeable pool, and $NaNO_3$ is capable of extracting only the organically bound metals (Ure, 1996; Kennedy *et al*, 1997). This explains the higher amounts of Cd and Zn extracted with CH_3COOH and EDTA compared to other extractants. In Great Billings soil, for example, Cd extracted with the

relatively strong extractant 0.05M EDTA (23.0μg/g) was 153 times greater than Cd extracted with the weak extractant $CaCl_2$ (0.15μg/g). Similar differences in the extractable Zn in Great Billings soil, or Zn and Cd in Countesswells soil were also found. In addition, Cd or Zn (expressed as percentage of total soil metal) extracted with the same extractant was different in the 2 soils. For example, CH_3COOH extracted 50% of total Zn in Great Billings soil while only 7% of total Zn in Countesswells soil. The percentage of total extracted in Great Billings soil was always higher than that extracted in Countesswells soil for all the extractants. This may be related to the fact that most of Great Billings soil Cd and Zn were introduced to the soil through sewage sludge application, which makes them more mobile and more easily extracted. The different extractants tested can be categorised into: extractants able to predict the mobile fraction of Cd and Zn in the soil ($CaCl_2$, $NaNO_3$), while others estimate the mobilisable fraction (NaOH, EDTA, CH_3COOH).

In contrast to the widespread use of extractants in the determination of Cd and Zn for bioavailability studies, there have been relatively few studies of utilising radiolabile Cd and Zn (Young *et al*, 2000). In addition, there have been no studies on the use of stable isotopes for bioavailability determinations, the fact that makes this approach unique.

In the determination of the E-value, the isotopic equilibration for Cd and Zn was estimated to occur after 160-180h of spike addition. The E-value for Zn, expressed as a percentage of total, was different in Great Billings (65%) from that in Countesswells (13%) soil. L-values determined for Zn in Great Billings soil were not significantly different in the 3 plant species studied. Similar results were found for Cd in Great Billings soil and for Zn in Countesswells soil. However, the L-value for Cd determined with the hyperaccumulator *T. caerulescens* (0.47μg/g,) was significantly different from that determined using the other 2 plant species (approximately 2.5 times). This different result needs further investigation to determine if the hyperaccumulation ability of *T. caerulescens* could have caused this or if this resulted from the soil properties. In Great Billings soil, the L-value percentage of totals was 70% for Cd and 60% for Zn. In Countesswells soil, Cd was 6% (apart from *T. caerulescens* value) while Zn was 11% of total soil metal. In a similar study, Hamon *et al* (1997) found approximately 36% and 12% of total Cd and Zn, respectively, available for plant uptake using radiolabeling. Although Cd and Zn values were different in the present

and Hamon study, different soil properties may have caused this. None of the plant species studied in our experiment was investigated by Hamon *et al* (1997). However, the similar L-values obtained despite the difference in the amount of Cd and Zn taken up by the plants was also found by Hamon *et al* (1997). This suggests that the soil has a specific metal fraction available for plant uptake, however plant species are capable of uptaking only certain amounts of that metal fraction (pool).

The extraction, E-value and the averaged L-value (from the 3 plant species) results were tested for significant difference using T-test (Table 4.1.2). Because the L-value was measured in a natural soil plant system, other values were tested against it. The E-values (determined in a soil:solution system) were significantly higher (at $P<0.05$) than L-values (determined in a soil:plant system) for both elements. This agrees with published work investigating P (Fardeau *et al*, 1996) and also Ni (Echevarria *et al*, 1997). The E-value for Cd was about 1.3 times its L-value for Great Billings soil, Zn on the other hand had E-values of 1.1 and 1.2 times its L-value for Great Billings and Countesswells soils, respectively. However, in a recent publication Cd was found to behave differently. Smolders *et al* (1999) found that the L-values always exceeded the E-values between 1.05 and 1.4-fold. Nevertheless, the fact that the 3 different plant species have similar L-values generally close to the E-values gives credit for the ability to use stable isotopes in the determination of bioavailable Cd and Zn.

The relatively weak extractants 0.1M $NaNO_3$, 0.01M $CaCl_2$ and the "mild" 0.5M NaOH underestimated the bioavailable Cd or Zn in the determination of either by E or L-values and, as a result, are not included for comparison. On the other hand, the stronger extractants CH_3COOH and EDTA extracted Cd and Zn in amounts comparable to the E and L-values. However, although EDTA extractable Cd (23.0µg/g) matched very closely the L-value in the 3 plant species (24.0, 22.7 and 22.6µg/g in *T. caerulescens*, *T. officinale* and *H. vulgare*, respectively), it was significantly different (at $P<0.05$) from the mean L-value (as the number of samples and SD have an important impact on T-test). However, although Table 4.1.2 shows that L-values were significantly different from the extraction and the E-values, the observed t-values show that Cd extracted with EDTA is the closest to the L-value determined in Great Billings soil while Zn extracted with CH_3COOH is the closest to

the L-value in Countesswells soil. Although T-test is applied here, care should be taken in interpreting the statistical output as different variables are tested.

Table 4.1.2: T-test between E-value, L-value and extraction results for Cd and Zn in (a) Great Billings and (b) Countesswells soils (at $P<0.05$). t-observed (t-critical).

(a)

	Cd			Zn		
	E-value	Extraction		E-value	Extraction	
		CH_3COOH	EDTA		CH_3COOH	EDTA
L-value	8.6 (2.3)	11.3 (2.2)	2.6 (2.1)	3.5 (2.3)	12.6 (2.2)	17.3 (2.1)

(b)

	Cd			Zn		
	E-value	Extraction		E-value	Extraction	
		CH_3COOH	EDTA		CH_3COOH	EDTA
L-value	nd	6.0 (2.4)	11.3 (2.3)	2.9 (2.3)	18.3 (2.2)	32.1 (2.2)

nd= not determined.

In the present work, it is shown that among the extractants tested, CH_3COOH and EDTA are the most appropriate extractants to estimate the availability of Cd and Zn for plant uptake in the light of the L-values obtained. It is also clear for a particular soil, one of these 2 extractants would be more suitable in predicting the bioavailable metal pool depending on soil properties. However, the T-test shows that the fast and relatively easily determined E-value was as good for Cd and even better for Zn than chemical extraction with CH_3COOH or EDTA. The fact that the 3 plant species estimated the bioavailable Cd and Zn to be the same is the most significant conclusion of this work.

4.2 Plant species potential for phytoremediation:

Phytoremediation is an emerging technology built on environmentally sound basis. As promising field experiments have been implemented, research in this area is expected to expand dramatically in the future (Salt *et al*, 1998). In the present experiments, 3 different plant species were tested for their ability to accumulate and distribute Cd and Zn between shoots and roots (Table 4.2.1). The data in this table is confined to the Great Billings soil because plant species grown on the non-contaminated Countesswells soil did not accumulate high levels of Cd and Zn.

Table 4.2.1: Cd and Zn maximum content (μg/g) and distribution between shoots and roots for the plant species studied (in Great Billings soil).

Plant species	Cd		Zn	
	Max. Content	Shoots : Roots Distribution	Max. Content	Shoots : Roots Distribution
T. caerulescens (alpine penny cress)	581	nd	6297	nd
T. officinale (dandelion)	70	1.3	1543	0.8
H. vulgare (spring barley)	67	0.2	817	0.2

nd= not determined, root content not analysed.

The present experiment demonstrates the ability of *T. caerulescens* to accumulate Cd and Zn, however, according to hyperaccumulators definition (Section 1.3.1), this plant species is a hyperaccumulator of Cd, but not of Zn. *T. officinale* (dandelion), according to definition is not a hyperaccumulator of either Cd or Zn. However, it accumulated up to 70mg/kg of Cd (a hyperaccumulator must accumulate 100mg/kg) which can not be ignored. In addition, dandelion has produced 8 times biomass compared to that produced by *T.*

caerulescens and this increases its potential for phytoremediation. In fact, within the present experimental conditions, the total Cd removed by *T. officinale* (dandelion) (0.90mg) was slightly higher than that removed by the hyperaccumulator *T. caerulescens* (0.86mg). For Zn, dandelion removed a total of 17mg compared to 11mg removed by *T. caerulescens*. In addition, the ability of dandelion to grow in various climatic and soil condition and its worldwide spread makes it suitable for phytoremediation in comparison to *T. caerulescens*, which was difficult to grow as was encountered in the present experiments.

When phytoremediation is concerned with the above the ground parts of the plant, the distribution of metal between shoots and roots of the plant is particularly important. This demonstrates the plants ability to extract metals and transfer them into the shoots so they can be easily harvested. However, this becomes less important in plants that accumulate metals in roots or equally between roots and shoots (such as *T. officinale*) where mechanical means can be available to harvest the roots. Both *H. vulgare* have low concentrations of Cd or Zn in the shoots compared to that in the roots while *T. officinale* have a ratio close of 1:1, indicating a poor ability to transfer metals from roots to shoots. As it was not possible to measure Cd or Zn concentration in the roots of *T. caerulescens*, this typical hyperaccumulator has high efficiency in accumulating metals in the shoots (Salt *et al*, 1998; Chaney *et al*, 1997).

4.3 General conclusions:

1. ID-TIMS produced accurate and precise results for the determination of Cd and Zn concentrations in soil and plant materials.
2. Stable isotopes can be used in bioavailability studies of Cd and Zn (and possibly other metals) and offers advantages over radioisotopes in terms of safety and excluded use. Stable isotopes also allow direct quantification of metals using ID principles.
3. The E-values determined were slightly higher than the L-values for both Cd and Zn in the two soils.

4. Despite the big difference between Cd and Zn content in the plant species studied, L-values were similar implying that plant species have the same metal bioavailable pool.
5. Among the extractants tested, EDTA and CH_3COOH had the nearest extractable Cd and Zn content to the determined E and L-values.
6. *T. caerulescens* (alpine penny cress) is a hyperaccumulator of Cd.
7. *T. officinale* (dandelion) has a good potential for phytoremediation. It removed the same amount of Cd and more Zn compared to the hyperaccumulator *T. caerulescens*.

4.4 Future work:

This work has laid the basis for bioavailability determination of Cd and Zn using stable isotopes which, compared to radioisotopes, neither require special safety procedures upon handling nor special growth facilities for experiments involving plants. Investigating the E-value experimental conditions (such as soil:solution ratio, solvent used and equilibration time) could build a solid basis for the determination of bioavailable metal. This easy, cheap and quick E-value determination could replace the long extraction procedures or even pot experiments. Stable isotopes could be used to study different plant species uptake and L-values over a range of soil types. This can be used to determine if the bioavailable metal is different or the same (as found in the present experiments) for a wider variety of plant species. Studying the changes in isotopic ratio in the plant with time may provide vital information about the availability of metals to plants and could show if the plant is continuously involved in transferring "unavailable" metal pool into "available" forms through root exudates. In addition, stable isotopes could be used as tracers to determine exactly in which part of the plant the metal is accumulated.

Extraction of spiked soil with different extractants may provide information about which pool is extracted with a particular extractant. Comparing this pool with that of the

same soil after growing plants, may explain where the plant is uptaking its metal from (exchangeable, organically bound etc.) and which particular extractant is associated with that pool. In addition, the successful methodology used for the determination of bioavailable Cd and Zn could be expanded to investigate the availability of other heavy metals to plant species.

Another direction that resulted from the present work would be investigating root exudates and the mechanisms involved in the uptake of metals into the plant. This is under continuous investigation for the hyperaccumulating species *T. caerulescens* (McGrath *et al*, 1997; Knight *et al*, 1997; Salt *et al*, 1999) but not for promising *T. officinale* (dandelion). A closer inspection of the metabolism involved might lead to considerably increase dandelion's ability in phytoremediation.

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Appendix

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A1. Cd isotopic analysis using TIMS.

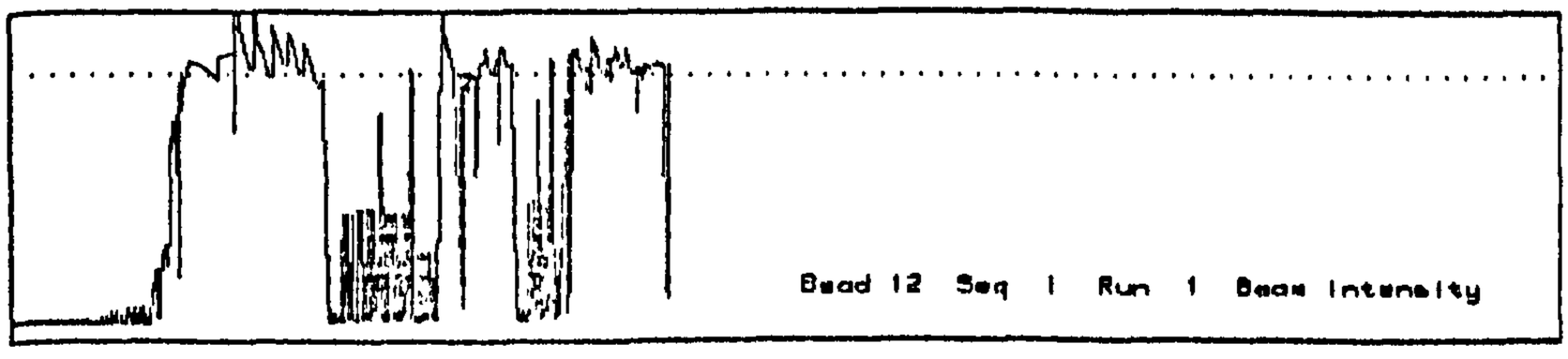
```
Bead No. 12      Cd1113   21 Sep 1997   Cd Single C:\HTB386\TIMS\DATA\AA1111.PRN
** MASS112.1 located at field 3.6705
** MASS114.1 located at field 3.7047
** MASS110.1 located at field 3.6359
** MASS111.1 located at field 3.6533
** MASS112.1 located at field 3.6705
** MASS113.1 located at field 3.6877
** MASS114.2 located at field 3.7047
** MASS116.2 located at field 3.7386

----> Bead 12  Seq  1  Run  1  Typ16   Aiming current 1.0E-12  on

Z bias    Z focus   Slit      D focus   D bias    Extract   Source
250       752       548       786       338       766       547

Filaments :  Center 2.075  Side 0.000 amps   Rhenium aiming  0.0E+00

Time =     43 mins       6 secs
```




```
Centering Peak
-

Baseline zero is     99.6

Mean intensity of 114 peak is  3.0E-13 amp

Ratio       Mean          St. Error        Mean          St. Error
            (before rejection)             (after  rejection)
106/108     1.331573       .578 %          1.331573       .578 %      on   5
110/111      .977272       .060 %           .977272       .060 %      on   5
112/114      .844401       .066 %           .844401       .066 %      on   5
113/114      .431052       .070 %           .431052       .070 %      on   5
116/114      .263066       .256 %           .263066       .256 %      on   5

                        106/108      110/111      112/114      113/114      116/11
Unbiassed ratio       1.331573       .977272      .844401      .431052      .26308
Bias value used       1.000000      1.000000     1.000000     1.000000     1.00000
  Biassed ratio       1.331573       .977272      .844401      .431052      .26308
** MASS110.1 located at field 3.6357
** MASS111.1 located at field 3.6531
** MASS112.1 located at field 3.6703
** MASS113.1 located at field 3.6874
** MASS114.1 located at field 3.7045
** MASS116.1 located at field 3.7383
```

----> Bead 12 Seq 2 Run 1 Typ16   Aiming current 3.0E-12 on

Z bias	Z focus	Slit	D focus	D bias	Extract	Source
234	744	464	786	346	776	547

Filaments : Center 2.143 Side 0.000 amps   Rhenium aiming 0.0E+00

Time =   54 mins   40 secs

Baseline zero is   102.4

Mean intensity of 114 peak is 8.3E-13 amp

Ratio	Mean (before rejection)	St. Error	Mean (after rejection)	St. Error		
106/108	1.457019	.673 %	1.457019	.673 %	on	5
110/111	.976582	.047 %	.976582	.047 %	on	5
112/114	.843015	.019 %	.843015	.019 %	on	5
113/114	.425153	.129 %	.425153	.129 %	on	5
116/114	.256145	.259 %	.256145	.259 %	on	5

	106/108	110/111	112/114	113/114	116/114
Unbiassed ratio	1.457019	.976582	.843015	.425153	.256145
Bias value used	1.000000	1.000000	1.000000	1.000000	1.000000
Biassed ratio	1.457019	.976582	.843015	.425153	.256145

** MASS110.1 located at field 3.6357
** MASS111.1 located at field 3.6531
** MASS112.1 located at field 3.6703
** MASS113.1 located at field 3.6874
** MASS114.1 located at field 3.7044
** MASS116.1 located at field 3.7383

----> Bead 12 Seq 3 Run 1 Typ16   Aiming current 1.0E-11 on

Z bias	Z focus	Slit	D focus	D bias	Extract	Source
248	718	448	784	344	796	547

Filaments : Center 2.257 Side 0.000 amps   Rhenium aiming 0.0E+00

Time =   65 mins   22 secs

Baseline zero is   93.8

Mean intensity of 114 peak is 2.6E-12 amp

Ratio	Mean (before rejection)	St. Error	Mean (after rejection)	St. Error		
106/108	1.397262	.195 %	1.397262	.195 %	on	10
110/111	.978933	.060 %	.979358	.046 %	on	9
112/114	.841660	.100 %	.841660	.100 %	on	10
113/114	.427029	.179 %	.426438	.128 %	on	9
116/114	.258918	.146 %	.259191	.113 %	on	9

	106/108	110/111	112/114	113/114	116/114
Unbiassed ratio	1.397262	.979358	.841660	.426438	.259191
Bias value used	1.000000	1.000000	1.000000	1.000000	1.000000
Biassed ratio	1.397262	.979358	.841660	.426438	.259191

** MASS110.1 located at field 3.6357
** MASS111.1 located at field 3.6531
** MASS112.1 located at field 3.6703
** MASS113.1 located at field 3.6874

** MASS114.1 located at field 3.7044
** MASS116.1 located at field 3.7383
** MASS106.1 located at field 3.5659
** MASS108.1 located at field 3.6010
** MASS110.1 located at field 3.6357
** MASS111.1 located at field 3.6532
** MASS112.1 located at field 3.6704
** MASS113.1 located at field 3.6875
** MASS114.1 located at field 3.7045
** MASS116.1 located at field 3.7384

----> Bead 12 Seq 4 Run 1 Typ16 Aiming current 3.0E-11 on

Z bias	Z focus	Slit	D focus	D bias	Extract	Source
242	684	454	790	324	826	547

Filaments : Center 2.414 Side 0.000 amps Rhenium aiming 0.0E+00

Time = 84 mins 4 secs

Baseline zero is 64.3

Mean intensity of 114 peak is 7.7E-12 amp

Ratio	Mean (before rejection)	St. Error	Mean (after rejection)	St. Error		
106/108	1.399503	.168 %	1.399503	.168 %	on	10
110/111	.974999	.130 %	.974999	.130 %	on	10
112/114	.844953	.192 %	.844953	.192 %	on	10
113/114	.428664	.265 %	.427147	.114 %	on	8
116/114	.259189	.249 %	.258725	.193 %	on	9

	106/108	110/111	112/114	113/114	116/114
Unbiassed ratio	1.399503	.974999	.844953	.427147	.258725
Bias value used	1.000000	1.000000	1.000000	1.000000	1.000000
Biassed ratio	1.399503	.974999	.844953	.427147	.258725

** MASS106.1 located at field 3.5658
** MASS108.1 located at field 3.6009
** MASS110.1 located at field 3.6358
** MASS111.1 located at field 3.6531
** MASS112.1 located at field 3.6704
** MASS113.1 located at field 3.6875
** MASS114.1 located at field 3.7045
** MASS116.1 located at field 3.7383

----> Bead 12 Seq 5 Run 1 Typ16 Aiming current 3.0E-11 on

Z bias	Z focus	Slit	D focus	D bias	Extract	Source
284	692	478	790	332	826	547

Filaments : Center 2.422 Side 0.000 amps Rhenium aiming 0.0E+00

Time = 99 mins 31 secs

Baseline zero is 99.9

Mean intensity of 114 peak is 7.6E-12 amp

Ratio	Mean (before rejection)	St. Error	Mean (after rejection)	St. Error		
106/108	1.414551	.065 %	1.414551	.065 %	on	10
110/111	.975591	.103 %	.975591	.103 %	on	10

112/114	.842644	.043 %	.842644	.043 %	on 10
113/114	.425642	.110 %	.425642	.110 %	on 10
116/114	.257975	.111 %	.257975	.111 %	on 10

	106/108	110/111	112/114	113/114	116/114
Unbiassed ratio	1.414551	.975591	.842644	.425642	.257975
Bias value used	1.000000	1.000000	1.000000	1.000000	1.000000
Biassed ratio	1.414551	.975591	.842644	.425642	.257975

** MASS106.1 located at field 3.5658
** MASS108.1 located at field 3.6009
** MASS110.1 located at field 3.6358
** MASS111.1 located at field 3.6531
** MASS112.1 located at field 3.6703
** MASS113.1 located at field 3.6874
** MASS114.1 located at field 3.7045
** MASS116.1 located at field 3.7383

----> Bead 12 Seq 5 Run 2 Typ16 Aiming current 3.0E-11 on

Z bias	Z focus	Slit	D focus	D bias	Extract	Source
286	698	478	790	332	832	547

Filaments : Center 2.450 Side 0.000 amps Rhenium aiming 0.0E+00

Time = 115 mins 23 secs

Baseline zero is 123.4

Mean intensity of 114 peak is 7.4E-12 amp

Ratio	Mean (before rejection)	St. Error	Mean (after rejection)	St. Error	
106/108	1.412801	.064 %	1.412801	.064 %	on 10
110/111	.977666	.036 %	.977666	.036 %	on 10
112/114	.842293	.032 %	.842072	.020 %	on 9
113/114	.425652	.043 %	.425652	.043 %	on 10
116/114	.258262	.057 %	.258262	.057 %	on 10

	106/108	110/111	112/114	113/114	116/114
Unbiassed ratio	1.412801	.977666	.842072	.425652	.258262
Bias value used	1.000000	1.000000	1.000000	1.000000	1.000000
Biassed ratio	1.412801	.977666	.842072	.425652	.258262

Ratio	Grand Mean (before rejection)	St.Error	Grand Mean (after rejection)	St.Error	
106/108	1.413676	.047 %	1.413328	.042 %	on 19 of 20
110/111	.976628	.058 %	.977258	.031 %	on 16 of 20
112/114	.842468	.026 %	.842468	.026 %	on 20 of 20
113/114	.425647	.058 %	.425678	.035 %	on 16 of 20
116/114	.258118	.062 %	.258312	.038 %	on 18 of 20

	106/108	110/111	112/114	113/114	116/114
Unbiassed ratio	1.413328	.977258	.842468	.425678	.258312
Bias value used	1.000000	1.000000	1.000000	1.000000	1.000000
Biassed ratio	1.413328	.977258	.842468	.425678	.258312

** MASS106.1 located at field 3.5658
** MASS108.1 located at field 3.6009
** MASS110.1 located at field 3.6358
** MASS111.1 located at field 3.6531
** MASS112.1 located at field 3.6703
** MASS113.1 located at field 3.6874
** MASS114.1 located at field 3.7045
** MASS116.1 located at field 3.7383

----> Bead 12 Seq 5 Run 3 Typ16   Aiming current 3.0E-11 on

Z bias	Z focus	Slit	D focus	D bias	Extract	Source
280	692	470	788	330	838	547

Filaments : Center 2.492 Side 0.000 amps Rhenium aiming 0.0E+00

Time = 131 mins 28 secs

Baseline zero is 51.3

Mean intensity of 114 peak is 7.3E-12 amp

Ratio	Mean (before rejection)	St. Error	Mean (after rejection)	St. Error	
106/108	1.398701	.055 %	1.398701	.055 %	on 10
110/111	.977158	.033 %	.977158	.033 %	on 10
112/114	.842792	.015 %	.842769	.008 %	on 8
113/114	.426601	.046 %	.426601	.046 %	on 10
116/114	.259314	.039 %	.259314	.039 %	on 10

	106/108	110/111	112/114	113/114	116/114
Unbiassed ratio	1.398701	.977158	.842769	.426601	.259314
Bias value used	1.000000	1.000000	1.000000	1.000000	1.000000
Biassed ratio	1.398701	.977158	.842769	.426601	.259314

Ratio	Grand Mean (before rejection)	St.Error	Grand Mean (after rejection)	St.Error			
106/108	1.408684	.100 %	1.408684	.100 %	on	30	of 30
110/111	.976805	.040 %	.977115	.021 %	on	25	of 30
112/114	.842576	.018 %	.842503	.014 %	on	25	of 30
113/114	.425965	.045 %	.426145	.037 %	on	28	of 30
116/114	.258517	.059 %	.258670	.045 %	on	28	of 30

	106/108	110/111	112/114	113/114	116/114
Unbiassed ratio	1.408684	.977115	.842503	.426145	.258670
Bias value used	1.000000	1.000000	1.000000	1.000000	1.000000
Biassed ratio	1.408684	.977115	.842503	.426145	.258670

** MASS106.1 located at field 3.5658
** MASS108.1 located at field 3.6009
** MASS110.1 located at field 3.6358
** MASS111.1 located at field 3.6531
** MASS112.1 located at field 3.6703
** MASS113.1 located at field 3.6874
** MASS114.1 located at field 3.7044
** MASS116.1 located at field 3.7383

----> Bead 12 Seq 5 Run 4 Typ16   Aiming current 3.0E-11 on

Z bias	Z focus	Slit	D focus	D bias	Extract	Source
288	682	472	788	330	838	547

Filaments : Center 2.544 Side 0.000 amps Rhenium aiming 0.0E+00

Time = 147 mins 47 secs

Baseline zero is 94.0

Mean intensity of 114 peak is 6.8E-12 amp

Ratio	Mean (before rejection)	St. Error	Mean (after rejection)	St. Error

106/108	1.411104	.091 %	1.411104	.091 %	on	10
110/111	.977849	.020 %	.977702	.015 %	on	9
112/114	.842255	.012 %	.842329	.009 %	on	9
113/114	.425690	.024 %	.425690	.024 %	on	10
116/114	.258429	.045 %	.258429	.045 %	on	10

	106/108	110/111	112/114	113/114	116/114
Unbiassed ratio	1.411104	.977702	.842329	.425690	.258429
Bias value used	1.000000	1.000000	1.000000	1.000000	1.000000
Biassed ratio	1.411104	.977702	.842329	.425690	.258429

Ratio	Grand Mean (before rejection)	St.Error	Grand Mean (after rejection)	St.Error				
106/108	1.409289	.078 %	1.409289	.078 %	on	40	of	40
110/111	.977066	.031 %	.977448	.015 %	on	33	of	40
112/114	.842496	.014 %	.842401	.009 %	on	32	of	40
113/114	.425896	.035 %	.425805	.022 %	on	33	of	40
116/114	.258495	.045 %	.258607	.036 %	on	38	of	40

	106/108	110/111	112/114	113/114	116/114
Unbiassed ratio	1.409289	.977448	.842401	.425805	.258607
Bias value used	1.000000	1.000000	1.000000	1.000000	1.000000
Biassed ratio	1.409289	.977448	.842401	.425805	.258607

** MASS106.1 located at field 3.5659
** MASS108.1 located at field 3.6009
** MASS110.1 located at field 3.6358
** MASS111.1 located at field 3.6531
** MASS112.1 located at field 3.6703
** MASS113.1 located at field 3.6874
** MASS114.1 located at field 3.7045
** MASS116.1 located at field 3.7383

----> Bead 12 Seq 5 Run 5 Typ16 Aiming current 3.0E-11 on

Z bias	Z focus	Slit	D focus	D bias	Extract	Source
288	668	474	788	342	838	547

Filaments : Center 2.633 Side 0.000 amps Rhenium aiming 0.0E+00

Time = 164 mins 25 secs

Baseline zero is 123.5

Mean intensity of 114 peak is 6.3E-12 amp

Ratio	Mean (before rejection)	St. Error	Mean (after rejection)	St. Error		
106/108	1.415247	.085 %	1.415247	.085 %	on	10
110/111	.977950	.020 %	.977950	.020 %	on	10
112/114	.842399	.011 %	.842399	.011 %	on	10
113/114	.425372	.046 %	.425372	.046 %	on	10
116/114	.258231	.043 %	.258231	.043 %	on	10

	106/108	110/111	112/114	113/114	116/11
Unbiassed ratio	1.415247	.977950	.842399	.425372	.25823
Bias value used	1.000000	1.000000	1.000000	1.000000	1.00000
Biassed ratio	1.415247	.977950	.842399	.425372	.25823

Ratio	Grand Mean (before rejection)	St.Error	Grand Mean (after rejection)	St.Error				
106/108	1.410481	.069 %	1.413426	.041 %	on	40	of	50
110/111	.977243	.026 %	.977565	.013 %	on	43	of	50
112/114	.842477	.012 %	.842400	.007 %	on	42	of	50
113/114	.425792	.030 %	.425675	.020 %	on	42	of	50
116/114	.258442	.038 %	.258428	.026 %	on	44	of	50

A2. Zn isotopic analysis using TIMS.

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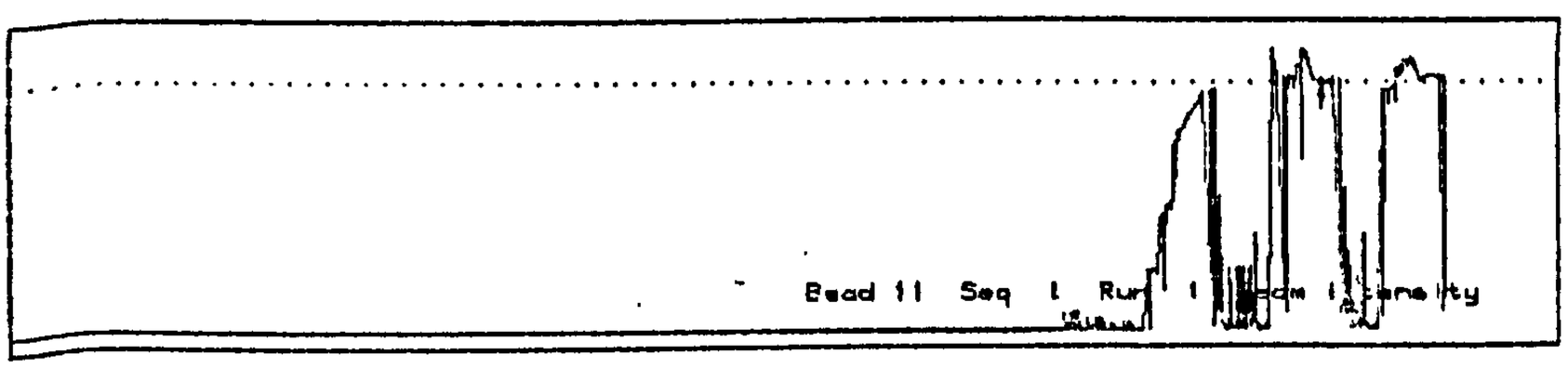
Bead No. 11      72  24 Aug 1999  Zn Single-AHMED      272A
** MASS 64.1 located at field 2.7330
** MASS 66.1 located at field 2.7768
** MASS 68.1 located at field 2.8201
** MASS 64.1 located at field 2.7330
** MASS 66.1 located at field 2.7768
** MASS 67.1 located at field 2.7986
** MASS 68.1 located at field 2.8201

----> Bead 11  Seq  1  Run  1  Typ 7   Aiming current 2.0E-12  on

Z bias   Z focus  Slit     D focus  D bias   Extract  Source
380      856      618      774      412      750      606

Filaments :  Center 2.739  Side 0.000 amps   Rhenium aiming  0.0E+00

Time =     28 mins      11 secs
```




```
Centering Peak
-

Baseline zero is     56.9

Mean intensity of  64 peak is  7.6E-13 amp

Ratio        Mean          St. Error        Mean          St. Error
             (before rejection)              (after  rejection)
64/66      1.785058     .089 %          1.785058     .089 %    on  10
64/67      4.165157     .315 %          4.165157     .315 %    on  10
64/68      2.719976     .236 %          2.719976     .236 %    on  10
64/70    140.179603   10.432 %        129.190125    8.350 %    on   9
66/67      2.333096     .245 %          2.333096     .245 %    on  10

                  64/66        64/67        64/68        64/70        66/67
Unbiassed ratio   1.785058     4.165157     2.719976     129.190125   2.333096
Bias value used   1.000000     1.000000     1.000000     1.000000     1.000000
  Biassed ratio   1.785058     4.165157     2.719976     129.190125   2.333096
** MASS 64.1 located at field 2.7330
** MASS 66.1 located at field 2.7768
** MASS 67.1 located at field 2.7986
** MASS 68.1 located at field 2.8202
```

----> Bead 11 Seq 2 Run 1 Typ 7   Aiming current 5.0E-12 on

Z bias	Z focus	Slit	D focus	D bias	Extract	Source
358	844	584	774	444	734	602

Filaments : Center 2.842 Side 0.000 amps Rhenium aiming 0.0E+00

Time = 43 mins 58 secs

Baseline zero is 6.0

Mean intensity of 64 peak is 2.1E-12 amp

Ratio	Mean (before rejection)	St. Error	Mean (after rejection)	St. Error		
64/66	1.776553	.072 %	1.776553	.072 %	on	10
64/67	4.101229	.140 %	4.101229	.140 %	on	10
64/68	2.702160	.101 %	2.702160	.101 %	on	10
64/70	70.782030	.789 %	70.049816	.392 %	on	8
66/67	2.308485	.157 %	2.308485	.157 %	on	10

	64/66	64/67	64/68	64/70	66/67
Unbiassed ratio	1.776553	4.101229	2.702160	70.049816	2.308485
Bias value used	1.000000	1.000000	1.000000	1.000000	1.000000
Biassed ratio	1.776553	4.101229	2.702160	70.049816	2.308485

** MASS 64.1 located at field 2.7330
** MASS 66.1 located at field 2.7768
** MASS 67.1 located at field 2.7986
** MASS 68.2 located at field 2.8202

----> Bead 11 Seq 2 Run 2 Typ 7   Aiming current 5.0E-12 on

Z bias	Z focus	Slit	D focus	D bias	Extract	Source
380	838	566	772	474	698	594

Filaments : Center 2.864 Side 0.000 amps Rhenium aiming 0.0E+00

Time = 59 mins 10 secs

Baseline zero is 33.5

Mean intensity of 64 peak is 1.7E-12 amp

Ratio	Mean (before rejection)	St. Error	Mean (after rejection)	St. Error		
64/66	1.783916	.060 %	1.783916	.060 %	on	10
64/67	4.141233	.266 %	4.141233	.266 %	on	10
64/68	2.714133	.140 %	2.714133	.140 %	on	10
64/70	96.931756	3.405 %	96.931756	3.405 %	on	10
66/67	2.321517	.244 %	2.316953	.162 %	on	9

	64/66	64/67	64/68	64/70	66/67
Unbiassed ratio	1.783916	4.141233	2.714133	96.931756	2.316953
Bias value used	1.000000	1.000000	1.000000	1.000000	1.000000
Biassed ratio	1.783916	4.141233	2.714133	96.931756	2.316953

** MASS 64.1 located at field 2.7330
** MASS 66.1 located at field 2.7768

** MASS 64.1 located at field 2.7330
** MASS 66.1 located at field 2.7769
** MASS 67.1 located at field 2.7987
** MASS 68.2 located at field 2.8202

----> Bead 11 Seq 3 Run 1 Typ 7   Aiming current 1.0E-11 on

Z bias	Z focus	Slit	D focus	D bias	Extract	Source
402	818	530	772	522	634	590

Filaments : Center 2.931 Side 0.000 amps Rhenium aiming 0.0E+00

Time = 74 mins 46 secs

Baseline zero is 60.3

Mean intensity of 64 peak is 3.5E-12 amp

Ratio	Mean (before rejection)	St. Error	Mean (after rejection)	St. Error		
64/66	1.781754	.069 %	1.781754	.069 %	on	10
64/67	4.131940	.180 %	4.131940	.180 %	on	10
64/68	2.714711	.082 %	2.716481	.056 %	on	9
64/70	90.159185	1.110 %	90.159185	1.110 %	on	10
66/67	2.318636	.140 %	2.318636	.140 %	on	10

	64/66	64/67	64/68	64/70	66/67
Unbiassed ratio	1.781754	4.131940	2.716481	90.159185	2.318636
Bias value used	1.000000	1.000000	1.000000	1.000000	1.000000
Biassed ratio	1.781754	4.131940	2.716481	90.159185	2.318636

** MASS 64.1 located at field 2.7331
** MASS 66.1 located at field 2.7769
** MASS 67.1 located at field 2.7987
** MASS 68.2 located at field 2.8202

----> Bead 11 Seq 3 Run 2 Typ 7   Aiming current 1.0E-11 on

Z bias	Z focus	Slit	D focus	D bias	Extract	Source
396	826	502	772	558	596	592

Filaments : Center 2.943 Side 0.000 amps Rhenium aiming 0.0E+00

Time = 89 mins 53 secs

Baseline zero is -2.4

Mean intensity of 64 peak is 3.8E-12 amp

Ratio	Mean (before rejection)	St. Error	Mean (after rejection)	St. Error		
64/66	1.771887	.045 %	1.771887	.045 %	on	10
64/67	4.088864	.078 %	4.088864	.078 %	on	10
64/68	2.689062	.035 %	2.689062	.035 %	on	10
64/70	77.200317	1.660 %	77.200317	1.660 %	on	10
66/67	2.307496	.077 %	2.307496	.077 %	on	10

	64/66	64/67	64/68	64/70	66/67
Unbiassed ratio	1.771887	4.088864	2.689062	77.200317	2.307496
Bias value used	1.000000	1.000000	1.000000	1.000000	1.000000
Biassed ratio	1.771887	4.088864	2.689062	77.200317	2.307496

Ratio	Grand Mean	St.Error	Grand Mean	St.Error

	(before rejection)		(after rejection)				
64/66	1.776820	.075 %	1.776820	.075 %	on	20	of 20
64/67	4.110402	.154 %	4.110402	.154 %	on	20	of 20
64/68	2.701886	.117 %	2.701886	.117 %	on	20	of 20
64/70	83.679751	2.012 %	83.679751	2.012 %	on	20	of 20
66/67	2.313066	.096 %	2.313066	.096 %	on	20	of 20

	64/66	64/67	64/68	64/70	66/67
Unbiassed ratio	1.776820	4.110402	2.701886	83.679751	2.313066
Bias value used	1.000000	1.000000	1.000000	1.000000	1.000000
Biassed ratio	1.776820	4.110402	2.701886	83.679751	2.313066

** MASS 64 1 located at field 2.7331
** MASS 66.1 located at field 2.7769
** MASS 67.1 located at field 2.7987
** MASS 68.2 located at field 2.8202

----> Bead 11 Seq 3 Run 3 Typ 7 Aiming current 1.0E-11 on

Z bias	Z focus	Slit	D focus	D bias	Extract	Source
426	834	492	772	574	574	606

Filaments : Center 2.955 Side 0.000 amps Rhenium aiming 0.0E+00

Time = 105 mins 2 secs

Baseline zero is 44.9

Mean intensity of 64 peak is 3.9E-12 amp

Ratio	Mean (before rejection)	St. Error	Mean (after rejection)	St. Error		
64/66	1.774207	.056 %	1.774207	.056 %	on	10
64/67	4.108434	.078 %	4.108434	.078 %	on	10
64/68	2.695250	.055 %	2.695250	.055 %	on	10
64/70	90.144246	1.219 %	90.144246	1.219 %	on	10
66/67	2.316052	.115 %	2.316052	.115 %	on	10

	64/66	64/67	64/68	64/70	66/67
Unbiassed ratio	1.774207	4.108434	2.695250	90.144246	2.316052
Bias value used	1.000000	1.000000	1.000000	1.000000	1.000000
Biassed ratio	1.774207	4.108434	2.695250	90.144246	2.316052

Ratio	Grand Mean (before rejection)	St.Error	Grand Mean (after rejection)	St.Error			
64/66	1.775949	.054 %	1.775578	.052 %	on	29	of 30
64/67	4.109746	.105 %	4.102631	.075 %	on	26	of 30
64/68	2.699674	.082 %	2.699674	.082 %	on	30	of 30
64/70	85.834582	1.512 %	85.834582	1.512 %	on	30	of 30
66/67	2.314061	.074 %	2.314061	.074 %	on	30	of 30

	64/66	64/67	64/68	64/70	66/67
Unbiassed ratio	1.775578	4.102631	2.699674	85.834582	2.314061
Bias value used	1.000000	1.000000	1.000000	1.000000	1.000000
Biassed ratio	1.775578	4.102631	2.699674	85.834582	2.314061

** MASS 64.1 located at field 2.7331
** MASS 66.1 located at field 2.7769
** MASS 67.1 located at field 2.7987
** MASS 68.2 located at field 2.8202

----> Bead 11 Seq 3 Run 4 Typ 7 Aiming current 1.0E-11 on

Z bias	Z focus	Slit	D focus	D bias	Extract	Source
402	834	494	772	578	574	612

Filaments : Center 2.967 Side 0.000 amps Rhenium aiming 0.0E+00

Time = 120 mins 9 secs

Baseline zero is 31.7

Mean intensity of 64 peak is 4.4E-12 amp

Ratio	Mean (before rejection)	St. Error	Mean (after rejection)	St. Error		
64/66	1.771295	.023 %	1.771295	.023 %	on	10
64/67	4.089568	.021 %	4.088901	.015 %	on	9
64/68	2.686128	.040 %	2.686128	.040 %	on	10
64/70	80.968559	.169 %	80.968559	.169 %	on	10
66/67	2.308868	.031 %	2.308868	.031 %	on	10

	64/66	64/67	64/68	64/70	66/67
Unbiassed ratio	1.771295	4.088901	2.686128	80.968559	2.308868
Bias value used	1.000000	1.000000	1.000000	1.000000	1.000000
Biassed ratio	1.771295	4.088901	2.686128	80.968559	2.308868

Ratio	Grand Mean (before rejection)	St.Error	Grand Mean (after rejection)	St.Error			
64/66	1.774786	.045 %	1.772859	.028 %	on	33 of	40
64/67	4.104701	.086 %	4.096397	.051 %	on	33 of	40
64/68	2.696287	.071 %	2.691632	.046 %	on	33 of	40
64/70	84.618077	1.213 %	84.618077	1.213 %	on	40 of	40
66/67	2.312763	.058 %	2.309595	.039 %	on	33 of	40

	64/66	64/67	64/68	64/70	66/67
Unbiassed ratio	1.772859	4.096397	2.691632	84.618077	2.309595
Bias value used	1.000000	1.000000	1.000000	1.000000	1.000000
Biassed ratio	1.772859	4.096397	2.691632	84.618077	2.309595

** MASS 64.1 located at field 2.7331
** MASS 66.1 located at field 2.7769
** MASS 67.1 located at field 2.7987
** MASS 68.2 located at field 2.8202

----> Bead 11 Seq 3 Run 5 Typ 7 Aiming current 1.0E-11 on

Z bias	Z focus	Slit	D focus	D bias	Extract	Source
400	826	492	772	582	582	622

Filaments : Center 2.967 Side 0.000 amps Rhenium aiming 0.0E+00

Time = 135 mins 20 secs

Baseline zero is 29.2

Mean intensity of 64 peak is 4.1E-12 amp

Ratio	Mean (before rejection)	St. Error	Mean (after rejection)	St. Error		
64/66	1.771776	.005 %	1.771764	.003 %	on	8
64/67	4.101704	.034 %	4.102702	.027 %	on	9
64/68	2.689056	.016 %	2.689056	.016 %	on	10
64/70	87.767326	.902 %	87.767326	.902 %	on	10
66/67	2.315014	.035 %	2.315014	.035 %	on	10

	64/66	64/67	64/68	64/70	66/67
Unbiassed ratio	1.771764	4.102702	2.689056	87.767326	2.315014
Bias value used	1.000000	1.000000	1.000000	1.000000	1.000000

Biassed ratio	1.771764	4.102702	2.689056	87.767326	2.315014

Ratio	Grand Mean (before rejection)	St.Error	Grand Mean (after rejection)	St.Error				
64/66	1.774184	.037 %	1.772472	.021 %	on	42	of	50
64/67	4.104102	.069 %	4.096959	.036 %	on	40	of	50
64/68	2.694841	.059 %	2.689748	.025 %	on	40	of	50
64/70	85.247926	1.000 %	85.247926	1.000 %	on	50	of	50
66/67	2.313213	.047 %	2.311127	.033 %	on	42	of	50

	64/66	64/67	64/68	64/70	66/67
Unbiassed ratio	1.772472	4.096959	2.689748	85.247926	2.311127
Bias value used	1.000000	1.000000	1.000000	1.000000	1.000000
Biassed ratio	1.772472	4.096959	2.689748	85.247926	2.311127

** MASS 64.1 located at field 2.7330
** MASS 66.1 located at field 2.7768
** MASS 67.1 located at field 2.7986
** MASS 68.2 located at field 2.8202
** MASS 70.2 located at field 2.8627

----> Bead 11 Seq 3 Run 6 Typ 7 Aiming current 1.0E-11 on

Z bias	Z focus	Slit	D focus	D bias	Extract	Source
360	812	490	772	580	596	626

Filaments : Center 2.971 Side 0.000 amps Rhenium aiming 0.0E+00

Time = 150 mins 11 secs

Baseline zero is 56.1

Mean intensity of 64 peak is 4.1E-12 amp

Ratio	Mean (before rejection)	St. Error	Mean (after rejection)	St. Error		
64/66	1.771150	.014 %	1.771150	.014 %	on	10
64/67	4.097301	.026 %	4.097301	.026 %	on	10
64/68	2.685982	.018 %	2.685982	.018 %	on	10
64/70	85.481517	.523 %	85.481517	.523 %	on	10
66/67	2.313353	.028 %	2.313353	.028 %	on	10

	64/66	64/67	64/68	64/70	66/67
Unbiassed ratio	1.771150	4.097301	2.685982	85.481517	2.313353
Bias value used	1.000000	1.000000	1.000000	1.000000	1.000000
Biassed ratio	1.771150	4.097301	2.685982	85.481517	2.313353

Ratio	Grand Mean (before rejection)	St.Error	Grand Mean (after rejection)	St.Error				
64/66	1.773678	.032 %	1.772008	.016 %	on	50	of	60
64/67	4.102968	.058 %	4.097027	.029 %	on	50	of	60
64/68	2.693365	.052 %	2.688365	.019 %	on	47	of	60
64/70	85.286858	.836 %	86.295018	.633 %	on	51	of	60
66/67	2.313237	.039 %	2.311765	.025 %	on	49	of	60

	64/66	64/67	64/68	64/70	66/67
Unbiassed ratio	1.772008	4.097027	2.688365	86.295018	2.311765
Bias value used	1.000000	1.000000	1.000000	1.000000	1.000000
Biassed ratio	1.772008	4.097027	2.688365	86.295018	2.311765

** MASS 64.1 located at field 2.7330
** MASS 66.1 located at field 2.7768
** MASS 67.1 located at field 2.7986
** MASS 68.1 located at field 2.8201
** MASS 70.2 located at field 2.8627

----> Bead 11 Seq 3 Run 7 Typ 7   Aiming current 1.0E-11 on

Z bias	Z focus	Slit	D focus	D bias	Extract	Source
358	810	482	772	576	606	628

Filaments : Center 2.979 Side 0.000 amps   Rhenium aiming 0.0E+00

Time =   165 mins   7 secs

Baseline zero is   18.3

Mean intensity of 64 peak is 4.4E-12 amp

Ratio	Mean (before rejection)	St. Error	Mean (after rejection)	St. Error		
64/66	1.772056	.050 %	1.772056	.050 %	on	10
64/67	4.097156	.110 %	4.101042	.063 %	on	9
64/68	2.688143	.088 %	2.690046	.058 %	on	9
64/70	84.068754	1.378 %	84.068754	1.378 %	on	10
66/67	2.312109	.079 %	2.312543	.040 %	on	8

	64/66	64/67	64/68	64/70	66/67
Unbiassed ratio	1.772056	4.101042	2.690046	84.068754	2.312543
Bias value used	1.000000	1.000000	1.000000	1.000000	1.000000
Biassed ratio	1.772056	4.101042	2.690046	84.068754	2.312543

Ratio	Grand Mean (before rejection)	St.Error	Grand Mean (after rejection)	St.Error			
64/66	1.773446	.029 %	1.772098	.014 %	on	57	of 70
64/67	4.102138	.052 %	4.097640	.026 %	on	59	of 70
64/68	2.692619	.047 %	2.689155	.021 %	on	59	of 70
64/70	85.112843	.743 %	85.664898	.563 %	on	59	of 70
66/67	2.313076	.035 %	2.312193	.021 %	on	55	of 70

	64/66	64/67	64/68	64/70	66/67
Unbiassed ratio	1.772098	4.097640	2.689155	85.664898	2.312193
Bias value used	1.000000	1.000000	1.000000	1.000000	1.000000
Biassed ratio	1.772098	4.097640	2.689155	85.664898	2.312193

** MASS 64.1 located at field 2.7330
** MASS 66.1 located at field 2.7768
** MASS 67.1 located at field 2.7986
** MASS 68.1 located at field 2.8201
** MASS 70.2 located at field 2.8626

----> Bead 11 Seq 3 Run 8 Typ 7   Aiming current 1.0E-11 on

Z bias	Z focus	Slit	D focus	D bias	Extract	Source
352	810	484	772	578	608	632

Filaments : Center 2.983 Side 0.000 amps   Rhenium aiming 0.0E+00

Time =   179 mins   51 secs

Baseline zero is   56.9

Mean intensity of 64 peak is 4.1E-12 amp

Ratio	Mean (before rejection)	St. Error	Mean (after rejection)	St. Error		
64/66	1.770126	.021 %	1.770126	.021 %	on	10
64/67	4.104190	.046 %	4.104190	.046 %	on	10
64/68	2.687117	.045 %	2.686140	.029 %	on	9

64/70	86.729935	.677 %	86.729935	.677 %	on	10
66/67	2.318543	.044 %	2.318543	.044 %	on	10

	64/66	64/67	64/68	64/70	66/67
Unbiassed ratio	1.770126	4.104190	2.686140	86.729935	2.318543
Bias value used	1.000000	1.000000	1.000000	1.000000	1.000000
Biassed ratio	1.770126	4.104190	2.686140	86.729935	2.318543

Ratio	Grand Mean (before rejection)	St.Error	Grand Mean (after rejection)	St.Error			
64/66	1.773031	.026 %	1.771667	.013 %	on	67	of 80
64/67	4.102395	.046 %	4.098572	.024 %	on	67	of 80
64/68	2.691931	.042 %	2.688405	.017 %	on	66	of 80
64/70	85.314980	.657 %	85.819251	.492 %	on	69	of 80
66/67	2.313759	.033 %	2.313101	.021 %	on	65	of 80

	64/66	64/67	64/68	64/70	66/67
Unbiassed ratio	1.771667	4.098572	2.688405	85.819251	2.313101
Bias value used	1.000000	1.000000	1.000000	1.000000	1.000000
Biassed ratio	1.771667	4.098572	2.688405	85.819251	2.313101

** MASS 64.1 located at field 2.7330
** MASS 66.1 located at field 2.7768
** MASS 67.1 located at field 2.7986
** MASS 68.1 located at field 2.8201

----> Bead 11 Seq 3 Run 9 Typ 7 Aiming current 1.0E-11 on

Z bias	Z focus	Slit	D focus	D bias	Extract	Source
346	810	484	772	576	610	632

Filaments : Center 2.991 Side 0.000 amps Rhenium aiming 0.0E+00

Time = 194 mins 42 secs

Baseline zero is 13.8

Mean intensity of 64 peak is 4.3E-12 amp

Ratio	Mean (before rejection)	St. Error	Mean (after rejection)	St. Error		
64/66	1.769139	.019 %	1.769139	.019 %	on	10
64/67	4.086391	.078 %	4.086391	.078 %	on	10
64/68	2.680479	.046 %	2.680479	.046 %	on	10
64/70	81.475750	1.669 %	81.475750	1.669 %	on	10
66/67	2.309822	.062 %	2.309822	.062 %	on	10

	64/66	64/67	64/68	64/70	66/67
Unbiassed ratio	1.769139	4.086391	2.680479	81.475750	2.309822
Bias value used	1.000000	1.000000	1.000000	1.000000	1.000000
Biassed ratio	1.769139	4.086391	2.680479	81.475750	2.309822

Ratio	Grand Mean (before rejection)	St.Error	Grand Mean (after rejection)	St.Error			
64/66	1.772599	.024 %	1.771085	.011 %	on	71	of 90
64/67	4.100616	.044 %	4.097898	.023 %	on	74	of 90
64/68	2.690658	.040 %	2.687965	.017 %	on	72	of 90
64/70	84.888399	.629 %	85.582688	.482 %	on	76	of 90
66/67	2.313322	.030 %	2.312548	.020 %	on	72	of 90

	64/66	64/67	64/68	64/70	66/67
Unbiassed ratio	1.771085	4.097898	2.687965	85.582688	2.312548
Bias value used	1.000000	1.000000	1.000000	1.000000	1.000000
Biassed ratio	1.771085	4.097898	2.687965	85.582688	2.312548

** MASS 64.1 located at field 2.7330

** MASS 66.1 located at field 2.7768
** MASS 67.1 located at field 2.7986
** MASS 68.1 located at field 2.8201
** MASS 70.2 located at field 2.8627

----> Bead 11 Seq 3 Run 10 Typ 7   Aiming current 1.0E-11 on

Z bias	Z focus	Slit	D focus	D bias	Extract	Source
346	804	482	772	576	624	630

Filaments : Center 2.995 Side 0.000 amps   Rhenium aiming 0.0E+00

Time =   209 mins   28 secs

Baseline zero is   55.9

Mean intensity of 64 peak is 4.2E-12 amp

Ratio	Mean (before rejection)	St. Error	Mean (after rejection)	St. Error		
64/66	1.769268	.020 %	1.769268	.020 %	on	10
64/67	4.092659	.033 %	4.092659	.033 %	on	10
64/68	2.681341	.027 %	2.681341	.027 %	on	10
64/70	87.325191	.785 %	87.325191	.785 %	on	10
66/67	2.313130	.025 %	2.313130	.025 %	on	10

	64/66	64/67	64/68	64/70	66/67
Unbiassed ratio	1.769268	4.092659	2.681341	87.325191	2.313130
Bias value used	1.000000	1.000000	1.000000	1.000000	1.000000
Biassed ratio	1.769268	4.092659	2.681341	87.325191	2.313130

Ratio	Grand Mean (before rejection)	St.Error	Grand Mean (after rejection)	St.Error		
64/66	1.772266	.023 %	1.770815	.011 %	on	82 of 100
64/67	4.099821	.040 %	4.097071	.021 %	on	83 of 100
64/68	2.689727	.038 %	2.686929	.018 %	on	84 of 100
64/70	85.132078	.576 %	85.878736	.419 %	on	83 of 100
66/67	2.313302	.027 %	2.312619	.018 %	on	82 of 100

	64/66	64/67	64/68	64/70	66/67
Unbiassed ratio	1.770815	4.097071	2.686929	85.878736	2.312619
Bias value used	1.000000	1.000000	1.000000	1.000000	1.000000
Biassed ratio	1.770815	4.097071	2.686929	85.878736	2.312619

اهداء

.... هذه الرسالة مهداة الى روح امي الغالية ، تغمدها الله بواسع رحمته..

أحمد أيوب

السابق- معطية بذلك تحليلا اسرع وتوفيرا في وقت الجهاز الثمين • لقد اعطى هذا التحليـــل نتائج بنفس دقة وصحة تحليل كل معدن على حده •

تم استعمال قياس النسبة النظيرية لحساب قيمتي E & L- Value في تربتيـــن• رغـــم ان E-Value المقاسة في معلق التربة في الماء كانت اعلى من L-Value المقاسة بعـــد نمـــو النبات في التربتين المختارتين، الا ان القيمتين كانتا متقاربتين• لقد تـــم قيـــاس L- Value باستعمال ثلاث نباتات : نبات ذو قابلية عالية لامتصاص المعادن (Alpine penny cress) ونبات الهندباء البرية (Dandelion) ونبات الشعير (Spring barley)• ان كمية الكادميوم والزنك المتوفر للنباتات الثلاث كانت متشابهة بالرغم من الاختلاف الكبير في تركيز المعدنيـن في انسجة النبات •

اضافة الى استعمال النباتات لقياس المعدن المتوفر فانه تم ايضا تقييم قدرة هذه النباتات علـــى الاستصلاح الخضراوي (Phytoremediation) من بين الثلاث نباتات التي تم اختيارها فـــان Alpine كانت النبتة الوحيدة ذات القدرة العالية لامتصاص الكادميوم• بالرغم من هـــذا فـــان Dandelion قد ازال كمية متساوية من الكادميوم واكثر من الزنك من النبتتين الاخريتيـــن• اعتمادا على هذا فان هذه النبتة لها قدرة عالية لان تكون فعالة وذات كفاءة فـــي اســـتصلاح الاراضي الملوثة بالكادميوم والزنك و ربما معادن اخرى• ادى هـــذا الـــى اجـــراء تجربـــة لمحاولة دراسة كيفية امتصاص المعدن من خلال جذور Dandelion • غـــير ان التجربـــة باستعمال تربة ملوثة محصورة بين لوحين من الزجاج لم تظهر أي نمـــط لنقصـــان تركـــيز الكادميوم والزنك باتجاه سطح الجذر ولم تظهر التجربة كذلك ان تركيز المعدنين كان مرتبطـــا بكمية الجذر الموجودة في مقطع معين من التربة •

مـــلخـــص

يمكن استصلاح الأراضي الملوثة بالمعادن الثقيلة مثل الكادميوم والزنك بطرق مختلفة، غـــير أن الكثير من هذه الطرق باهظة التكاليف (مثل ازالة التربة الملوثة وتغطيـــة الارض بطبقـــة جديدة من التربة الجيدة، او استخلاص المعادن الثقيلة بمواد كيماوية كالأحمـــاض)، أو تمثـــل حلولا قصيرة الامد (مثل اختزال المعدن من خلال تكليس التربة او اضافة مواد عضويـــة)، يمثل استعمال نباتات ذات قدرة عالية على امتصاص المعادن الثقيلة طريقة بديلة وجذابة فيمـــا يعرف بالاصلاح الخضراوي (Phytoremediation) اذ تمتاز هذه الطريقة بتكلفتها القليلـــة وكفاءتها العالية من خلال استعمال نباتات متخصصة، على الرغم من هذا فإن هناك عوامـــل عديدة يجب أخذها بعين الاعتبار، لعل من أهمها توافر المعدن –بأشكاله المختلفة في التربـــة– للامتصاص من قبل النبات ،

يتم تحديد قياس المعدن المتوفر للامتصاص من قبل النبات بطـــرق عـــدة اكثرهـــا انتشـــارا الاستخلاص بواسطة مواد كيماوية، يمثل التبادل النظيري (Isotopic Exchange) طريقـــة اخرى تستعمل باستمرار لقياس توافر الفوسفور في التربة، تفترض هذه الطريقة أن المعـــدن القابل للتبادل النظيري هو المعدن المتوافر للامتصاص من قبل النبات من خلال تجربـــة فـــي معلق التربة بالماء فيما يعرف بـــ E- Value ، على الرغم من هذا فان دراســـات عديـــدة ابدت ان E-Value تميل الى اعطاء قيمة اكبر من القيمة الحقيقية، من أجـــل تخطـــي هـــذه العقبة تم تجريب قياس المعدن القابل للتبادل النظيري من خلال انماء نباتات على تربـــة تـــم اضافة نظير المعدن لها، وعرفت هذه الطريقة بـــ L- Value ، بالإضافة للفوسفور فقـــد تم استعمال الطريقة للنيكل، وفي تجربة حديثة جدا لمعدني الكادميوم والزنك من خلال نظـــائر مشعة، في تجاربنا الحالية تم استعمال نظائر مستقرة (Stable Isotopes) لقيـــاس معدنـــي الكادميوم والزنك المتوافر لامتصاص النبات من تربة ملوثة (Great Billings) وتربة غـــير ملوثة (Countesswells) ، كذلك فإنه تم مقارنة هذه القيم بـــالطرق التقليديـــة مـــن خـــلال الاستخلاص بخمسة مواد كمياوية ، من بين $NaOH, CH_3COOH, NaNO_3, CaCl_2$ و EDTA فإنه تم استخلاص اكبر كمية مـــن الكـــادميوم والزنـــك بواســـطة CH_3COOH و EDTA ، بالإضافة لهذا فإن هاتين المادتين استخلصتا كمية كادميوم وزنك قريبة مـــن L- Value المقاسة بطريقة التبادل النظيري،

لقد طورنا طريقة صحيحة ودقيقة لقياس تركيز الكادميوم والزنك بطريقة التخفيـــف النظـــيري (Isotope Dilution) من خلال قياس النسبة النظيرية (Isotope Ratio) بواســـطة جـــهاز المطياف الكتلي بالتأين الحراري (TIMS) ، تم تقييم هذه الطريقة باستعمال عينـــات تربـــة ونبات وحيوان مقاسة (CRMs) وقد تم تحطيم وهضم هذه العينات بثلاث طرق حيث انتجـــت جميعها نتائج صحيحة ودقيقة، بالاضافة الى ذلك لم يظهر التحليل أي اختـــلاف فـــي نتـــائج الطرق الثلاث: الماء الملكـــي (*Aqua Regia*)، اســـتعمال حـــامض النيـــتريك فـــي فـــرن الميكروويف، واستعمال حامض الهيدروفلوريك، بالاضافة لما سبق فانه تم بنجـــاح اختيـــار تحليل معدني الكادميوم والزنك على نفس الشعيرة السلكية– مقارنة لكل منها على حـــده فـــي

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