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Trace Metal Speciation In Environmental Systems

By

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Declaration

I hereby certify that this material, which I now submit for assessment on the programme of study leading to the award of Doctor of Philosophy, is entirely my own work and has not been taken from the work of others save and to the extent that such work has been cited and acknowledged within the text of my work.

Signed: Nicking Rogers

Date: 16/12/96

For my parents

Generations have trod, have trod, have trod;
And all is smeared with trade; bleared, smeared with toil;
And wears man's smudge and shares man's smell: the soil
Is bare now, nor can foot feel, being shod.

Gerard Manley Hopkins

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ABSTRACT

Trace Metal Speciation in Environmental Systems

Aisling Rogers

The adverse environmental impact of trace metals is an issue of current concern. The increasing recognition that it is the chemical form of a metal which determines its toxicity and bioavailability presents a new challenge for analytical chemists. Techniques are required which are sufficiently sensitive and selective to enable the quantitation of metal species at lower levels than ever before. The importance of trace metal speciation in environmental samples is reviewed in Chapter 1. The effects of inorganic complexation, organic complexation and oxidation state on metal uptake and toxicity to the biota are discussed. Special reference is made to two currently topical metals, cadmium and selenium. Cadmium is recognised as a particularly toxic metal with no beneficial effects on living organisms while selenium is a cumulative toxin which is essential at low levels. In both cases, however, toxicity is clearly dependent on the metal species present.

The complexation of cadmium by Suwannee River fulvic acid is reported in Chapter 2. Metal complexation by aquatic humic materials has important consequences for metal transport and mobility in the environment. Due to the polyelectrolyte nature and heterogeneity of these naturally occuring organic ligands, characterisation of their metal binding ability is complex. The application of ^{113}Cd NMR for this purpose is presented. The complexation of cadmium by the International Humics Substances Society fulvic acid standard is examined.

The concept of selenium speciation is explored in Chapter 3. The analytical techniques currently used for selenium determination are reviewed and two novel techniques are presented. An extraction technique, termed matrix solid phase dispersion, is applied to selenium determination in milk and fish samples. A procedure is also described to enable the simultaneous determination of the two inorganic selenium species, selenite and selenate in aqueous samples.

The uptake of copper by the filamentous fungus *Rhizopus arrhizus* is discussed in Chapter 4. Microbial biomass has the ability to sequester metal ions from aqueous solution by a variety of mechnisms. Much of the research to date relates to batch systems. In this chapter the use of lyophilised fungal cells for the removal of metal ions from aqueous solution under continous flow conditions is investigated.

Chapter 1

Trace metals in the environment

1.1 Introduction

Trace metals are widely dispersed throughout the environment. Increased global industrialisation has resulted in a large scale elevation of natural levels. Anthropogenic activity, such as fossil fuel combustion, mining, electroplating processes, iron and steel production and the use of pesticides in agriculture have all contributed to increased levels of metals in environmental systems. This has given much cause for concern since metals can be neither degraded nor metabolised. In contrast to other environmentally important chemicals they are an example of ultimate persistence. They are readily accumulated by plants and organisms and are extremely toxic in excess. Metals undergo biogeochemical cycling in the environment during which they are transformed between various chemical species. They may exist in the free, uncomplexed form or as both inorganic and organic complexes. It is becoming increasingly apparent that it is the physico-chemical form of a metal which determines its fate, mobility and toxicity.

Natural waters are largely responsible for the transfer of metals in the environment. Rivers, lakes, ground water and the sea all contribute significantly to the cycling of metals. Atmospheric cycling is also of great importance. Industrial sources emit a large amount of metals directly into the atmosphere. This emission occurs primarily in the form of fine aerosol particles. Thus, metals can be transported large distances and are ultimately deposited on both land and sea. According to Nurnberg, precipitation in the form of rain and/or snow provides the most important deposition mode for Cd, Pb, Cu, Zn and Ni [1]. This is significant since metals in dissolved form are more readily taken up by vegetation.

All metals are toxic to living organisms if present in excess. Metals such as Cd, Pb, and Hg, however, are toxic even at low levels [1]. Some metals are essential and are actively taken up by the organism. Of these, Ca, Fe, and Zn are needed in the largest amounts. Se and Cu, although also essential, are required in much smaller quantities [2]. Metal absorption may change with the organism's need for a specific element at a given time. Under deficit conditions a given metal will be absorbed more readily. This increased absorption may also extend to other metals, occupying the same relative position in the periodic table. Chemical similarities in a series of metals, for example between Zn and Cd or between Ca and Pb, frequently results in the uptake of toxic metals to target organs. The metals which have most severely affected human health in the last 25 years include Pb, Hg, Cd, Se and Sn. Of these, Cd, Se and Sn are "new" problems [2]. Previously, their presence in the environment had not been considered to be a hazard.

1.2 Speciation

According to Florence [3], the speciation of an element can be defined as the determination of the concentrations of the individual physico-chemical forms of an element in a sample which, together, constitute its total concentration. Metal speciation, particularly in water and biological systems, has become particularly important due to the realisation that the environmental behaviour of a metal (i.e. toxicity, bioavailability, bioaccumulation and geochemical transport) is often critically dependent on its physico-chemical form and transport mechanisms between the available forms.

The Minamata accident in Japan, induced by the bioaccumulation of mercury as methylmercury, reinforced the importance of speciation. Effluent containing

methylmercury was released into Minamata Bay by a factory making acetaldehye. Bioaccumulation by fish and shellfish led to methylmercury entering the foodchain. Ultimately, at least 798 people died or suffered permanent neurological damage from post-natal methylmercury exposure. This incident triggered huge international interest in speciation and in the importance of determining individual metal species rather than total metal concentration. Inorganic complexation may also alter metal toxicity. Lead halide aerosols, emitted by automobiles, are extremely toxic. Exposure is via the lungs from which they are readily absorbed into the bloodstream [3]. The oxidation state of an element can have a profound effect on bioavailability and toxicity. Chromium (III) is known to be an essential element while chromium (VI) is highly toxic. Arsenic (III) is also much more toxic than arsenic(V) [3]. In order to understand the impact of metals on living systems, therefore, it is necessary to know both the chemical speciation of the metal in solution and the toxicity of the various chemical species [4].

1.2.1 Inorganic complexation

Inorganic complexation has a significant influence on the bioavailability and toxicity of trace metals. Copper toxicity to the freshwater crustacean, *Daphnia magna*, has been shown to be directly related to the activities of free and hydrated copper ions in aqueous solution [5]. Complexation with the inorganic ligands, pyrophosphate and bicarbonate significantly reduced toxicity. The total copper concentration was maintained at 5 μM. On addition of bicarbonate in the range 0-10 mM at pH 7.4, decreased mortality rates were observed with a corresponding increase in median survival time from approximately 50 mins to 544 mins. Pyrophosphate was added in the range 2.5-500 μM. A decrease in the rate of death with a corresponding increase in median survival time was observed up to 50 μM pyrophosphate. At this point

> 99% of the copper was calculated to be complexed and the toxicity was negligible. Upon addition of 500 μm pyrophosphate the calculated ionic Cu^{2+} activity was less than 0.001 μm and no deaths were observed during the course of the three day experiment. Copper toxicity to *Daphnia magna* would, therefore, appear to be directly related to ionic copper activities. The bioaccumulation of mercury by two types of bait fish has been shown to decrease upon formation of mercury phosphate complexes. The fish examined were the fathead minnow, *Pimephales promelas* and the emerald shiner, *Notropis atherinoides* [6]. Increasing phosphate concentrations by a factor of 100 decreased uptake of mercury to one-third of its original value. Uptake was also found to fall off rapidly between pH 4 and 6 in distilled water. This was attributed to the formation of $Hg(OH)_2$ and the subsequent decrease in free mercury concentration.

Cadmium toxicity to the grass shrimp, *Palaemonetes pugio*, has been reported to decrease with increased salinity and upon addition of the chelator NTA (nitrilotriacetic acid) [4]. Experiments were carried out in diluted UV -treated seawater over a four-day period. Free cadmium concentration was measured using a cadmium ion-selective electrode. Toxicity was found to directly proportional to the concentration of $CdCl_2$ (0-50 μM) in seawater media. However increased salinities in the range 4-29% were found to result in a substantial decrease in *Palaemonetes pugio* mortality. At a $CdCl_2$ concentration of 2×10^{-6} M and a salinity of 5%, a mortality of 50% was observed. At an increased salinity of 29%, a much greater $CdCl_2$ concentration (1×10^{-5} M) was required to produce the same effect (50% mortality). This $CdCl_2$ concentration increased to 3×10^{-5} M on the addition of 1×10^{-4} M NTA under the same conditions (5% salinity, 50% mortality). This is 15 times the amount of $CdCl_2$ required to produce the same mortality in the absence of the chelator. It was concluded that cadmium toxicity to *Palaemonetes pugio* was

dependent on the free cadmium ion concentration and that mortality decreased at high salinities and in the presence of the NTA. The presence of inorganic chelators can therefore significantly affect metal uptake and accumulation in microorganisms leading to altered toxicities and death rates.

1.2.2 Organic complexation

Naturally occurring organic ligands, such as humic and fulvic acids, readily form complexes with metals and are known to exert a significant influence on trace metal speciation in the environment. Humic matter is a complex mixture formed by random condensation of the degradation products of plants and animals. Aquatic humic substances result mainly from the decomposition of plankton while terrestrial humic substances arise largely from the decomposition of lignite [7]. They are, therefore, very heterogeneous in nature. Their strong ability to complex metal ions is largely due to the presence of carboxylic and phenolic groups, additionally, sulphur and nitrogen atoms may also be present in different functional groups [8]. Aquatic humic substances play an important role in water quality since they are the major constituents of dissolved organic carbon. A study carried out by Mantoura et al. reported that more than 90% of copper and mercury are complexed by humic material in freshwater [9]. This is very significant since many studies have shown that the complexation of metals by humic substances largely determines their bioavailability, toxicity and mobility.

Copper toxicity to the Atlantic salmon, *Salmo salar*, has been reported to decrease with increasing concentration of humic acid in the test water [10]. Acute copper toxicity was examined following the addition of commercially obtained humic acid (Aldrich). Toxicity was measured in terms of the incipient lethal concentation of

copper which was defined as the level beyond which 50% of the population could not live for an indefinite time. In the absence of any humic acid the incipient lethal concentration of copper was 25 μg/l. This concentration increased to 90 and 165 μg/l on the addition of 5 and 10 mg/l of humic acid, respectively. The reduction in copper toxicity was attributed to the decrease in cupric ion availability. Brown et al. also reported that the addition of 4.5 mg/l of humic acid (HA) to test water increased the median survival time of rainbow trout (*Salmo gairdneri*) from 470 to 1050 minutes when exposed to 2000 μg Cu/l [11]. The humic acid used in these studies had been obtained commercially.

Similarly cadmium toxicity to *Salmo salar* is reduced in the presence of aquatic humic material. Natural humus, equivalent in concentration to approximately 78 mg/l, significantly reduced the toxicity of cadmium (1 mg/l as $Cd(NO_3)_2.6H_2O$) to *Salmo salar* over a 90 hour exposure period [12]. In a later study, Sedlacek, Kallqvist and Gjessing studied cadmium uptake by the green alga *Selenastrum capricornutum* [13]. They found that the logarithm of the amount of cadmium accumulated in the algae was proportional to the logarithm of the algal biomass. However, aquatic humic acid was found to significantly reduce the rate of accumulation and toxic effects of cadmium by changing the chemical speciation of the metal in solution. In the absence of any humic material, more than 80% of the ^{109}Cd labelled solution of cadmium chloride was taken up by the algae over a period of seven days. The concentration of the aquatic humic material was expressed in terms of its total organic carbon content (mg C/l). The percentage uptake of cadmium upon the addition of 1.6 mg C/l was in the range 40-55%. Higher levels of humic acid (8.1 mg C/l) decreased uptake further to less than 20%. Toxicity was evaluated by determination of the concentration of cadmium that reduced the algal growth rate to 50% of the control (i.e. EC_{50}). For a growth medium without humus

an EC_{50} value of 6 mg/l Cd was obtained. This concentration increased to 150 mg/l in growth medium to which 8.1 mg C/l of humus material had been added.

The uptake of cadmium by barley plants is also species dependent. The effect of complex formation with terrestrial humic acid and with chloride ions was studied [14]. The barley plants were transferred periodically between a nutrient solution and a test solution containing humic acid (HA) in the range 0-1710 μgcm^{-3} and cadmium in the range 0-5 μgcm^{-3}, in either a $NaNO_3$ or NaCl medium (0.006 M). The nutrient solution did not contain either cadmium or humic acid. Growth was observed over a period of nineteen days. An ion-selective electrode could not be used to determine the level of free Cd^{2+} in the test solutions due to interference by HA. Instead dialysis equilibrium was used. Solutions containing humic acid in the same concentration range as the test solutions were sealed in air-free dialysis tubing. Each tube was added to corresponding solutions of $Cd(NO_3)_2$ and shaken for 24 hours. AAS was used to determine the equilibrium concentration of Cd outside the dialysis bag and, by difference ,the concentration of Cd inside the bag was calculated. At the end of the 19-day growth period the plants were harvested, digested using an acid mixture and analysed by AAS. The Cd concentration was related to the calculated Cd speciation in the test solutions. Humic acid complexation (0-1710 μg/ml) was found to reduce Cd uptake across the range of cadmium concentrations (0-5 μg/ml) by 25-66%. Speciation, therefore, has a significant impact on metal uptake. This study indicates that free ionic Cd^{2+} is the form in which cadmium is absorbed by barley plants.

The presence of humic acid has also been found to decrease cadmium absorption by snap beans [15]. Solution culture experiments were carried out in order to examine the effect of naturally occurring components of soil solutions such as calcium ions,

hydrogen ions and organic acids on cadmium uptake. No significant reduction in uptake was observed upon the addition of calcium or altering the pH in the range 4 to 7, indicating that neither Ca^{2+} or H^{+} ions compete for uptake with Cd^{2+}. Addition of humic acid, however, decreased the cadmium activity in solution and the subsequent absorption of cadmium by corn roots, indicating that cadmium ion activity in solution directly affects uptake.

The toxicity of both Cu and Cd to the daphnids *Daphnia magna* and *Daphnia pulex* was investigated upon addition of humic acid to test water [16]. The humic acid used was obtained commercially (Aldrich) and concentrations were expressed as ash-free dry weight. Both acute and chronic toxicity were investigated. Acute toxicity was studied over a period of three days and was evaluated in terms of the concentration of metal in the test water required to cause death in 50% of the population (LC_{50}). For Cu the mean LC_{50} increased from 28.3 μg/l in water to which no humic acid (HA) had been added to 53.2 μg/l in water containing 1.5 mg/l HA. Conversely the addition of HA at the same concentration (1.5 mg/l) increased the acute toxicity of Cd with a corresponding decrease in the mean LC_{50} from 87.8 μg/l to 7.1 μg/l. In terms of chronic toxicity, which was evaluated over a 42 day period, the addition of HA in the range 0-1.5 mg/l increased the survival time of the daphnids exposed to 30 μg Cu /l. However mortality was significantly more rapid in water containing Cd at a concentration of 20 μg/l upon the addition of 0.75 and 1.5 mg/l of HA. Although the addition of HA affected the relative toxicities of both Cu and Cd, it had no significant effect on the bioaccumulation of either metal. This indicates that uptake and final body loading are not related to toxicity in this case. Cu and Cd have significantly different binding affinities for humic acid. Cd-HA complexes are relatively less stable than Cu-HA complexes. Following uptake it is possible that Cd

could be displaced from the HA complex resulting in an increase in free Cd^{2+} ion concentration and subsequent toxicity to the daphnids.

Humic material is widespread in both aquatic and terrestrial environments. This naturally occurring organic material has a significant impact on metal uptake and toxicity to plants and animals. Its composition is dependent on source, however, resulting in widely varying metal binding abilities. A comparison between studies carried out using humic acid from different locations is therefore difficult. In general however it would appear that complexation with humic acid decreases the bioaccumulation and toxity of metals in a wide variety of plants and animals. This has been attributed to the fact that complexation results in a decrease in the concentration of free metal ions, indicating that it is this species which is most toxic to living organisms.

1.2.3 Oxidation State

Metal bioavailability and toxicity can vary significantly with oxidation state. In biological systems chromium can exist as either Cr(III) or Cr(VI). The health effects of these two forms of chromium are so fundamentally different that they must be considered separately. Trivalent chromium is more prevalent than the hexavalent form as Cr(VI) is easily reduced on entering the body. The occurrence and importance of chromium in the functioning of biological systems has been studied in detail by Gutherie [17]. Cr(III) is essential for efficient glucose, lipid and protein metabolism [18]. However Cr(VI) is toxic and affects the lungs, liver and kidneys. Anionic CrO_4^{2-} diffuses through negatively charged cell membranes where it oxidises and binds other important biological molecules with toxic results. Inhalation of chromium may result in bronchial carcinomas [19]. Occupational dermatitis in

bricklayers has also been attributed to Cr(VI) in wet cement [20]. The mechanisms of chromium metabolism, genotoxicity and carcinogenicity have been extensively described by De Flora and Wetterhahn [21]. In occupational health the OEL (occupational exposure limits) for water soluble and certain water insoluble compounds in indoor air is limited to 0.5 mg/m^3 for Cr(III) and 0.05 mg/m^3 for Cr(VI) reflecting the different toxicity of both species. Hexavalent chromium is such a potent carcinogenic agent for the respiratory tract that continuous monitoring is imposed [22].

Arsenic occurs in various organic and inorganic species with several oxidation states (-3, 0, +3 and +5). The most common forms are arsenite and arsenate ions (As(III) and As(V)), monomethylarsonic and dimethylarsinic acids (MMA and DMA), arsine, di- and trimethylarsine as well as other organoarsenical compounds such as arsenobetaine, arsenocholine, arsenolipids and arsenosugars. The toxicity of arsenic depends on its chemical form. Inorganic species of arsenic are more toxic than organic compounds [23]. The relative toxicities of arsenite (As(III)) and arsenate (As(V)) in rats was studied [24]. The dose which was fatal to 50% of the population (LD_{50}) was determined in each case. An LD_{50} of 10 mg/kg was determined for arsenite. Arsenate was much less toxic with an LD_{50} of 100 mg/kg being obtained. Arsenobetaine, the arsenic compound commonly found in marine animals, had no toxic effect when administered orally to mice at a dose of 400 mg/kg body weight [25]. A study carried out on rat embryos also found that they were unaffected by arsenobetaine [26].

Selenium toxicity is also species dependent. The predominant forms of selenium in nature are selenite and selenate which contain selenium in tetravalent and hexavalent forms, respectively. A study carried out by Niimi and LaHam [27] found that

selenite compounds were more toxic to the zebrafish (*Brachydanio rerio*) than selenates. Toxicity was measured over 96 hours and the concentrations lethal to 50% of the fish population (LC_{50}) were determined. For sodium and potassium selenite the LC_{50} was in the range 15-23 μg/ml. However the corresponding selenates were much less toxic with LC_{50}'s of 81-82 μg/ml.

The role of metals in living systems is not only dependent on exposure and uptake but on the chemical form of the metal in question. For elements which exist in many different oxidation states the determination of total concentration is therefore no longer sufficient. There is a need to develop analytical techniques which differentiate in terms of oxidation state as well as metal identity.

1.3 Cadmium

1.3.1 Cadmium in the environment

Cadmium is the sixty-seventh most abundant element in the earth's crust [28]. Natural cadmium levels in soil are in the range 0.01-0.5 mg/kg [29]. However in polluted areas concentrations of 0.2 - $\geq$ 50 mg/kg have been reported [29, 30]. Contamination has been attributed to the high levels of cadmium in fuels, fertilisers and mine tailings [31]. In industry cadmium is widely used in the production of alloys, paints and plastics [32]. Due to its relatively high vapour pressure cadmium is readily vaporised during smelting operations or coal combustion. In industrial areas airborne cadmium has been reported at levels of 20-300 ng/m^3 [33]. This compares with 0.1-50 ng/m^3 in urban areas and 0.003-4 ng/m^3 in rural areas.

Cabera et al. have reported that industrial pollution from the spoil heaps of 19th century mining activities has resulted in cadmium levels of up to 540 mg/kg being detected in topsoils in N.E. Clwyd [14]. Agricultural activity has also resulted in elevated levels of cadmium in soil, through the addition of superphosphate and sewage sludge. A study carried out by De Haan and Swerman indicates that superphosphate contains cadmium in the range 50-170 mg/kg [34]. Sewage sludges in England and Wales have been reported to have cadmium levels of up to 1500 mg/kg [35].

The concentration of cadmium in river water, is generally in the range < 0.05-0.2 μg/l [36]. In estuarine water concentrations of < 0.04-2 μg/l have been reported [37, 38]. In seawater cadmium levels at the surface (≤ 0.001-0.05 μg/l) are very much lower than at depths below 1000m (< 0.15 μg/l) [39, 40]. Cadmium concentrations exceeding 10 μg/l are not recommended in water for human consumption [32].

1.3.2 Cadmium in biological systems

Cadmium is not an essential element for plants, animals or man [28]. The main sources of cadmium for humans are food, water and tobacco smoke. The latter is very significant since fractional absorption of inhaled cadmium is much higher than that of the ingested metal. Cadmium in food does not readily cross the intestinal barrier. Only 1-5% of ingested cadmium is absorbed [31]. The biological half-life of cadmium in the body is of the order of 20 years. Therefore cadmium acts as a cumulative poison.

The distribution of cadmium in the body depends on the form in which it enters the blood. Inorganic cadmium accumulates in the liver, however cadmium which is

administered as a thiol complex is more readily taken up by the kidneys. Following prolonged exposure, the major portion of total body cadmium is accumulated in the kidney cortex. Ultimately this results in the production of renal lesions following which the accumulated cadmium may be released from the kidney and gradually excreted in urine [31]. Cadmium is readily complexed by a low molecular weight, cysteine-rich, protein called metallothionein. This protein sequesters, through thiolate bonds, a variety of metallic cations such as cadmium, zinc, copper and mercury. Much research has been carried out using ^{113}Cd NMR in an effort to characterise these metalloprotein complexes [41, 42]. The precise function of metallothionein in cadmium metabolism, however, is not yet conclusive.

1.3.3 Toxicity

In the body, cadmium reacts readily with proteins and other biological molecules. Its chemical similarity to zinc enables displacement of zinc from certain enzymes, thus rendering them inactive [43]. Cadmium exposure may result in health effects at multiple sites including the lungs, prostate, testes, heart, liver and kidneys [31]. After long term chronic exposure the kidneys accumulate a major portion of total body cadmium. The renal cortex is the major target organ. Cadmium accumulation results in the inhibition of various proximal tubular transport mechanisms. In particular, cadmium interferes with the tubular reabsorption of low molecular weight proteins [31]. In Japan, high levels of cadmium in irrigation water has resulted in "itai-itai" disease [44]. The symtoms are osteomalacia and renal tubular malfunction and result from eating cadmium enriched rice.

As discussed in the previous section, metallothionein has a high affinity for cadmium. This has important consequences for the treatment of cadmium toxicity

since it reduces the efficacy of coventional chelating drugs like EDTA in removing cadmium from the body [31]. Cadmium-metallothionein (Cd-MT) has also been shown to be more nephrotoxic than cadmium chloride ($CdCl_2$) [45]. It would appear that the high affinity of metallothionein for cadmium inhibits complexation with non-filterable plasma protein and as a result, plasma cadmium remains filterable and is reabsorbed in the renal tubule resulting in an increase in renal toxicity. The concentrations of complexed and free cadmium may, therefore influence toxicity in living systems.

1.3.4. Speciation.

The oxidation state of cadmium is +2 in all compounds [28]. The most common species are bivalent cadmium ions (e.g $CdCl_2$), chlorocadmium complexes, cadmium bound to proteins, cadmium bound to colloidal substances and acid cadmium complexes. While cadmium salts with strong acids are readily soluble in water the sulphide, carbonate, fluoride and hydroxide salts are much less soluble. The solubility of the hydroxide salt is improved, however, through complex formation in ammonium hydroxide.

Experimental determination of cadmium species in natural waters is very difficult due to the exceptionally low total concentrations of cadmium in unpolluted sea and fresh waters (< 1 μg/l) [18]. In seawater, however, it is generally considered that cadmium exists largely complexed with chloride ions. In freshwater, the predominant species are free, hydrated Cd^{2+} ions and carbonate complexes. Humic compounds and suspended muds also have the ability to adsorb ionic cadmium strongly. A study carried out by Gardiner demonstrated that, in fresh waters,

adsorption onto suspended muds by humic substances is a major factor in controlling cadmium concentrations in fresh waters [46].

Electrochemical techniques have been employed to investigate cadmium speciation in seawater. Batley and Gardner carried out a study on lakewater using anodic stripping voltammetry [47]. It was reported that 70-90% of cadmium in the water analysed was in the form of free, hydrated ions or labile complexes. A study of the Yarra River [48] determined cadmium levels in the range 2-12 μg/l. They concluded that up to 65% of the metal was associated with organic matter. Complexation by organic matter is also very significant in soil plant systems and is a major factor affecting the bioavailability and toxicity of heavy metals. Humic substances with molecular weights ranging from several hundreds to millions constitute the bulk of natural organic matter and can appreciably affect the fate of heavy metals in the environment [49]. Relan et al. investigated the complexation of cadmium by humic material derived from farmyard manure, a common source of organic matter added to soil in India [50]. The strength of binding was found to be quite strong. This was determined by the calculation of the stability constant (log K) which had a value of 7.18. The order of stability of the metals tested was Cu > Zn > Fe > Pb = Cd > Mn. Although a substantial fraction of cadmium found in soil is known to be complexed by humic material, very little is known about the nature of the chemical interactions involved. This is due to the complex composition of humic substances.

Cadmium pollution in the environment is of concern due to the toxic nature of this trace metal. It is not an essential element in mammalian nutrition and accumulates in the body predominantly in the kidneys. Cadmium has a biological half-life of the order of 20 years and acts as a cumulative poison. Long-term cadmium exposure can cause irreversible renal effects. Atmospheric emissions of cadmium are largely due

to coal combustion and zinc smelting and subsequent deposition of the metal results in the enrichment of soils in the surrounding areas. Cadmium levels in soil are also increased through the addition of superphosphate and sewage sludge. Runoff from contaminated soil results in the pollution of natural waters together with pollution by effluent released from industries producing, for example, alloys, paints and plastics. In the natural environment cadmium is widely complexed by humic material, resulting in reduced toxicity to the biota. Much of the literature to date has studied metal complexation by humic acids in terms of copper, due to the high affinity of humic material for this metal. There is a need for further study of the mechanism of cadmium complexation to enable greater understanding of the pathway and mobility of this toxic metal in the environment.

1.4 Selenium

The element selenium was discovered in 1818 by Berzelius. It was named after the moon from the Greek "selene", since it resembled, chemically, tellurium, which had already been named after the earth, from the Latin "tellus" [51]. Study of the chemistry of selenium revealed the remarkable susceptibility of its electrons to excitation by light, resulting in the generation of an electric current. This has led to the widespread use of selenium in the electronics industry. It is used in the production of solar batteries, photoelectric cells and photocopiers. It is also widely used in the glass industry, either as a decolouring agent or, in excess, as a colourant. Combined with cobalt, selenium removes the greenish tint in glass, while in excess it is used to create the deep red colour used in traffic lights and car tail lights. In agriculture, selenium compounds are used as insecticides, fungicides and as soil additives [52]. The classification of selenium has changed over the past two decades. Previously, it was considered to be a primarily toxic element with possible benefits

at low levels. Today, however, it is recognised as a beneficial element with serious toxic effects in oversupply [2].

1.4.1 Selenium in the environment

Selenium ranks seventieth in adundance among the elements and constitutes approximately 1 x 10^{-5}% of the earth's crust [53]. It is generally found in metal-sulphide deposits of the metals Cu, Zn, Ag, Hg and Pb. Natural processes, such as the weathering of minerals, contribute to selenium levels in the environment. The largest man-made source of selenium is coal combustion, accounting for about half of the total anthropogenic emissions [54]. Its widespread use in industry also contributes to elevated selenium levels in the environment.

In soil selenium is generally present in the range 0-80 μg/g [55]. Selenium levels in soil give a good indication of animal and human exposure since they determine the level of plant uptake. Soils that supply sufficient selenium to produce plants containing toxic amounts of selenium in their tissues are commonly referred to as "toxic seleniferous soils". These soils are generally alkaline in nature and occur in regions of low rainfall [56]. Selenium generally occurs as selenate. Since this is the most water-soluble form of selenium, it is readily translocated into vegetation and leached into groundwater. In the Central Valley of California, selenium levels up to 3800 μg/l in groundwater have been reported [56]. Seleniferous soils are found extensively in South Dakota, Wyoming, Montana, Nebraska and Arizona in the U.S.A. and in areas of Alberta, Saskatchewan and Manitoba in Canada [57]. Non-toxic seleniferous soils are soils which contain large quantities of selenium but low levels of water-soluble selenate. These soils do not produce toxic vegetation. In general, they are characterised by a zone of accumulated iron and aluminium

compounds. They tend to have an acid pH, in the range 4.5 to 6.5 which, in the presence of ferric hydroxide, renders the selenium unavailable to plants. Some soils contain selenium only at sub-ppm levels. In these areas, disorders in livestock have been attributed to selenium deficiency [58].

Selenium levels in natural waters have been extensively reviewed by Robberecht and Van Grieken [59]. In river water selenium concentration is generally in the range 0.016-20 ng/l. Lake water contains selenium at levels < 0.1-1.85 ng/l while the concentration in ocean waters is in the range 0.025-0.2 ng/l. Reddy et al. recently reported selenium levels of 3-330 μg/l in shallow post-mining groundwater from coal mines in seleniferous areas of the Powder River Basin, Wyoming [60]. This has given cause for concern since an estimated 70% of Wyoming's population use groundwater as their source of drinking water. Federal regulations state that selenium in drinking water should not exceed 0.01 mg/l [60].

1.4.2 Selenium in biological systems

Selenium is an essential trace element in the human diet [61]. The US National Research Council (1980) has estimated that a safe and adequate daily intake of selenium for adults is between 50-250 μg [62]. Overt clinical deficiency is associated with blood levels of <10-15 mg/ml whereas toxicity occurs at levels > 300 ng/ml [63]. Selenium is widely available in food supplements, either organically bound in the form of seleno-methionine as in Brewer's yeast, or it may be present in an inorganic form as selenite or selenate [64]. Because selenium is derived from a wide variety of foodstuffs including meat, poultry, grains, seafood, vegetables, fruits and milk, very few cases of human selenosis or deficiency have been reported.

1.4.2.1 Distribution in the body

In the body, the highest levels of selenium are found in the kidney. Selenium is also found in the glandular tissues, particularly the pancreas, the pituitary and the liver. Low levels of selenium are found in the muscles, bones and blood with even less accumulating in the adipose tissue. Cardiac muscle contains higher levels of selenium than skeletal muscle. Studies indicate that the duodenum is the main site of absorption [65]. Absorbed selenium is transported with plasma proteins from which it enters all the tissues including the bones, the hair and the erythrocytes [66]. It is generally accepted that selenium replaces sulphur in sulphur-containing compounds in the tissues and occurs predominantly as selenocysteine and selenomethionine in both protein-bound and non-protein bound forms.

The main biochemical role of selenium in the body is as an inherent component of the enzyme glutathione peroxidase (GSPHx). GSPHx contains 4 gram-atoms Se/mol, present as selenocysteine [63]. This enzyme works in conjunction with other antioxidants and free radical scavengers in the cell and, in the presence of reduced glutathione, is responsible for the reduction of hydrogen peroxide and organic peroxides to water and corresponding alcohols, respectively. GSPHx activity in the cell occurs in the mitochondrial matrix and the cytosol.

1.4.2.2 Deficiency

Although selenium deficiency is rare, in severe cases it has been associated with a degenerative disease of heart muscle (cardiomyopathy), which may be fatal [67]. Results of research in China have suggested a relationship between low selenium status and the prevalence of Keshan disease, an endemic cardiomyopathy that

primarily affects children [68]. Symptoms include acute or chronic cardiac insufficiency, heart enlargement, gallop rhythm, arrythima and ECG changes. The disease exhibits a regional distribution and occurs in a belt-like zone reaching from northeastern China to the southwestern part of the country. The most severely affected areas were found to be those where the incidence of selenium deficiency diseases in farm animal was also high [68]. Dietary supplementation with as little as 500 μg of selenium per week, given orally as sodium selenite reduced significantly the incidence of cardiomyopathies in Keshan [69]. Selenium deficiency is also suspected to be one of the main causes of Kashin-Beck disease, an endemic osteoathropathy that occurs in eastern Siberia and in certain parts of China and again mainly affects children [70]. The residents in these areas have low selenium status characterised by low blood and hair selenium levels, low blood glutathione peroxidase activity and low urinary excretion. Although supplementation has been found to be successful in the prevention of deficiency states, it is essential that the levels of selenium in the blood of patients is closely monitored, to ensure that toxicity states are not produced [67].

Selenium has also been implicated in the prevention of human cancers [63]. Although the actual mechanism is not known, tumour prevention is not thought to be related to selenium antioxidant activity. Instead it appears that selenium interacts directly with metabolites of tumour-inducing chemicals. In a study by Willett et al., blood samples were collected from more than 10,000 individuals in the U.S. and stored at low temperature [71]. Over the period of study, 111 new cases of cancer were identified and the stored prediagnostic blood samples were analysed for selenium. The mean serum selenium level was found to be significantly lower in cancer cases than in the controls (129 ± 2 μg/l compared with 136 ± 2 μg/l). It was concluded from this study that low selenium intake increases the risk of cancer. A

further study by Salonen et al. collected blood samples from over 8000 individuals in Finland [72]. Six years later, 128 cases of cancer among these individuals, had been reported. The mean concentration of selenium for these cases was 50 μg/l compared with 54.3 μg/l for the controls. An association has also been suggested between low serum selenium levels and the risk of death from acute coronary heart disease, as well as the risk of fatal and non-fatal myocardial infarction [73]. In this study, case-control pairs were taken from a population of 11,000 people. The individuals who had a non-fatal myocardial infarction or died or coronary heart disease or other cardiovascular conditions over the seven year study period were found to have a mean serum selenium concentration of 51.8 μg/l. This compares with 55.3 μg/l among the controls.

Although these studies give some indication of the effects of low serum selenium levels in humans, the results presented are not conclusive. In each case a very limited population was studied and analysis of blood serum was not carried out when the health effects in question were most prevalent. It is possible that low serum selenium levels may only be a marker for other dietary factors not yet examined.

1.4.3 Toxicity

Selenium is known to be a cumulative toxic substance in living systems. Overexposure in humans results in loss of hair and nails, skin lesions, abnormalities of the nervous system, disturbances of the digestive tract and possible tooth decay [74]. Major symptoms such as necrotic degeneration of the liver, fibrosis of the kidney and mycocardial congestion have also been reported [75]. The extent of toxicity is, however, species dependent [49].

Selenium toxicity in livestock has been recognised for many years. As far back as 1856, Dr. Madison observed a number of toxic signs, including hair loss in cavalry horses at Fort Randall in the old Nebraska Territory [57]. It was not until 1934 that "Alkali disease", which caused widespread lameness and death of livestock in the Dakotas and Wyoming, was assigned to high levels of selenium in plants grown in the area [75]. Mainly horses, cattle, sheep and chickens were affected. The manifestations of this disease were extreme tenderness, inflammation of the feet, softening and loss of hoofs and horns and loss of hair (mane, tail). Diminution in vision has also been reported for cattle and sheep feeding on seleniferous plants [75]. This disease has been termed "blind staggers" since further intoxication results in a weakening of the limbs and eventually respiratory failure, resulting in death.

Leaching of selenium from irrigated fields has resulted in serious environmental consequences at the Kesterson National Wildlife Refuge, California [76]. Selenium levels of up to 1400 μg/l have been reported and have been blamed for unusually high rates of mutations, deformation and even death of wild waterfowl. In 1984, the ingestion of super-potent selenium tablets resulting in 12 cases of human selenium toxicity was reported [77]. On analysis, each tablet was found to contain 27-31 mg of selenium, approximately 182 times the amount stated on the label. Symptoms included nausea and vomiting, nail changes, hair loss, fatigue and irritability.

1.4.4 Speciation

Selenium has four formal oxidation states, elemental selenium (0), selenide (II), selenite (IV) and selenate (VI). Selenium also exists in organometallic compounds such as dimethylselenide, dimethyldiselenide, dimethylselenone, selenomethionine,

selenocysteine and trimethylselenonium [55]. Methylated selenium compounds are generally produced as a result of microbial activity. They are extremely volatile and readily released into the atmosphere [55]. Selenium rapidly changes oxidation state with respect to pH [54]. In highly basic solutions the predominant species is selenate, in mildly basic or acid solutions it occurs mainly as selenite and in highly acidic solutions, elemental selenium or selenide are preferred. In addition to thermodynamic equilibria, other factors controlling redox state are biochemical reactions, chemical treatment, surface interaction and adsorption [78].

The two most common forms of selenium in natural waters are Se(IV) and Se(VI) [76]. Se(IV) exists as the weak selenious acid H_2SeO_3 and as a number of inorganic selenites. Selenites are generally less soluble than their corresponding selenates. Dissolved selenites in aqueous waters (pH range 3.5-9.0) exist predominantly as the hydrogen selenite ion. Under acidic conditions selenites are rapidly reduced to elemental selenium by mild reductants such as ascorbic acid or sulphur dioxide. Formation of selenate is favoured in alkaline and oxidising conditions. Selenates are very soluble in water and do not tend to form stable complexes [78]. The speciation of selenium in natural waters is varied. In oxygenated waters Se(IV) is thermodynamically favoured and it is known that the element is dissolved from rocks in this form [79]. A study carried out on the Irish Sea and the English Channel measured 0.34 μgl^{-1} and 0.50 μgl^{-1} of selenium, respectively. It was concluded that this was composed almost entirely of Se (IV), since the iron(II) hydroxide coprecipitation technique used removed less than 2% of an added Se(VI) spike. Similarly, Se(IV) has been reported to be the predominant species in Japanese coastal waters. Reduction of selenium in the range 0.04-0.08 μgl^{-1} resulted in no detectable increases in the concentration of Se(IV), indicating the absence of Se(VI). The level of Se(IV) was determined by extraction as piazselenol derivatives,

followed by reaction with 4-nitro-o-phenyldiamine, with analysis by gas chromatography [80]. In seawater samples from the North Pacific Ocean, the level of total selenium in surface waters was in the range 0.06-0.12 μgl^{-1} which increased to 0.20 μgl^{-1} in deeper layers. While Se(IV) was uniformly distributed with depth , the Se(VI) content in deep waters was approximately three times the level at the surface. This distribution of selenium has been attributed to biological processes and the fact that oxidation phenomena are restricted to the surface layers [78]. Significant differences have been observed between the speciation of selenium in river water compared to seawater. While Se(IV) predominates in seawater, it has been reported that less than 8% of total dissolved selenium in riverwater can be attributed to Se(IV) [18]. Previously a study by Measures and Burton had concluded that only about 10% of total dissolved selenium in a river water sample was due to Se(IV) [80]. The remainder was attributed to Se(VI), as the selenate ion. However the presence of organically associated species or selenium adsorbed on colloidal matter could also contribute significantly. A study carried out on two rivers in east central Japan found that Se(IV) comprised only 2-16% of the total selenium content [81]. Se(VI) has been reported to be the predominant form of total selenium in drinking water [82]. This is possibly due to the chlorination carried out on potable water. In a study carried out on waste water, 30-100% of total selenium was found to be Se(IV) [83].

The predominant inorganic selenium species in soil are selenate and selenite. Selenium exists predominantly as selenate (SeO_4^{2-}) in alkaline soils of regions with low rainfall [56]. Since selenate is more water-soluble than selenite, it is more readily available for plant uptake and potentially more toxic to the biota. Selenite (SeO_3^{2-}) is generally found in soils of low pH. It has a lower bioavailability than selenate since it readily absorbs onto ferrous oxides, like geothite, and hydrous

oxides of aluminium in acid environments [54]. Dimethylselenide and dimethyldiselenide are the predominant forms of selenium in air. These volatile compounds account for 40% of all selenium emitted into the atmosphere [53].

The natural environment is currently being exposed to increased levels of metals as a result of increased industrialisation and intensified agricultural processes. Since metals cannot be degraded they pose a substantial health risk. Some metals are more toxic even at low levels (Hg, Cd, Pb) while others are cumulative toxins (Se). However it is now widely accepted that it is the chemical form of a metal which largely dictates its bioaccumulation and toxicity. Metals can exist in a number of oxidation states each of which may have very different effects on living systems. For example the role of chromium, arsenic and selenium in mammalian systems is defined by the valence state of the metal. European directives are now acknowledging this distinction [22] and the onus is currently on analytical techniques to respond with increased selectivity and sensitivity. Inorganic complexation of metals has a significant effect on metal accumulation and toxicity. This has particular relevance in aquatic environments where varied salinities can affect metal uptake by microorganisms. Metal accumulation at this stage has implications for entry into the food chain at higher levels. Finally metal complexation by humic substances is of great significance due to the widespread occurrence of both aquatic and terrestrial humic material in nature together with the substantial metal-binding ability of this natural material.

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Chapter 2

Cadmium complexation by aquatic fulvic acid

2.1 Introduction

Humic substances play an important role in water quality since they are the major constituents of dissolved organic carbon. These soluble organic acids leach into ground and surface waters from soil and plant organic matter. The scientific definition of humic substances is an operational one. They are coloured, polyelectrolyte, organic acids isolated from water by adsorption onto XAD or weak base ion-exchange resins, or by a comparable procedure [1,2]. Humic and fulvic acids are distinguished by their solubility at low pH. Following concentration and purification on an ion-exchange column, the pH of a concentrated solution of humic and fulvic acids is reduced to pH 1 with concentrated HCl, resulting in the precipitation of the humic acid fraction [2]. Humic acid is, in fact, defined as the fraction of humic substances that is not soluble in water under acidic conditions (pH < 2) but is soluble at higher pH values. Fulvic acids, on the other hand, are defined as the fraction of humic substances that are soluble in water under all pH conditions. Humic materials are distinguished therefore based on the pH dependence of their water solubility. These substances are inherently heterogeneous in nature and their molecular character varies greatly with source.

Humic materials play an important role in the transport and bioavailability of anthropogenic organic contaminants. They interact with organic pollutants such as pesticides and herbicides, increasing their solubility [3-6]. Humic substances are also known to complex trace metals. A detailed understanding of the cation binding properties of humic substances has particular importance for developing long-term solutions to the problems of waste storage and remediation as well as the more fundamental characterisation of metal ion transport and bioavailability [7]. Humic

substances also play a major role in trace metal toxicity to plants and living organisms [8].

2.1.1 Metal complexation by aquatic humic substances

The metal ion complexation properties of humic and fulvic acids are complex, both because of the heterogeneity of the samples and the polyelectrolyte nature of these mixtures. The most prevalent metal coordination sites in aquatic humic and fulvic acids are carboxylic groups and phenolic groups [9-11]. Since humic samples have a high degree of variability in their composition, amino, sulphydryl and quinone groups can also be present, however, since these groups are much less prevalent in humic materials their contribution to ion-binding is generally not very substantial.

Metal ion complexation has been studied primarily by electrochemical methods, in particular ion-selective electrodes. A study by Bresnahan et al. investigated copper complexation by soil and aquatic fulvic acid samples [12]. Conditional stability constants were calculated based on data obtained from ISE measurements. Scatchard plots indicated the presence of two classes of binding sites with stability constants of 1×10^6 and 8×10^3 M^{-1}. In the pH range 4-6, the total number of binding sites per molecule increased from 0.6 to 2.6 and from 0.8 to 4.2, for aqueous and soil fulvic acids, respectively. This increase in binding sites was most pronounced between pH 5 and 6. Bresnahan et al. speculate that the increase in binding sites may be due to a structural change in the fulvic acid macromolecules whereby more functional groups are exposed and available for metal binding. Similarly cadmium complexation by fulvic acid has also been found to be pH dependent [13]. From pH 4-8, the overall conditional stability constant increased from 1.4×10^3 to 12×10^3 M^{-1} for aquatic fulvic acid and from 1.7×10^3 to 43×10^3 $M^{-1}$for soil fulvic acid. Complexation was

also found to be dependent on fulvic acid concentration. Increasing the concentration from 20 to 70 mg/l reduced the conditional stability constant by 50%, at constant pH. This was attributed to the polyelectrolyte nature of fulvic acids. At high concentrations, it would appear that conformational changes occur, thereby reducing the number of sites available for binding. Ion-selective electrodes have also been used to investigate the effect of counter-ion concentration on copper complexation by fulvic acid from the Suwannee River, Georgia [14]. Competitive binding experiments were carried out by potentiometric titration of copper with fulvic acid, using calcium and potassium nitrate solutions as background electrolytes. The concentration of fulvic acid was measured in terms of carbon composition and was maintained as 25 mg C/l throughout the experiment. Copper was titrated at pH 6 in the range 1 $x10^{-6}$ to 4 x 10^{-4} M, as $Cu(NO_3)_2$. The use of KNO_3 (1 x 10^{-2}-1 x 10^{-3} M) as a background electrolyte had no effect on copper complexation. $Ca(NO_3)_2$ reduced copper complexation however at concentrations of 1 x 10^{-2} M and 1 x 10^{-3} M. At lower concentrations (1 x 10^{-4}-1 x 10^{-6} M), $Ca(NO_3)_2$ had no effect. The authors concluded that calcium may compete with copper for some of the binding sites, but not all. This study illustrates the heterogeneity of the possible binding sites in fulvic acid since calcium and copper do not appear to compete for all of the same coordination sites.

Fluorescence spectroscopy has been used to characterise the interactions of fulvic acid with metal ions by measuring the quenching of the fulvic acid fluorescence as a function of the complexation of a paramagnetic ion, for example Cu^{2+} [15,16]. A broad emission peak was observed for fulvic acid at 445-450 nm upon excitation at 350 nm [15]. Emission intensity was at a maximum at pH 5 and dropped off significantly at lower pH levels. Complexation with Cu^{2+}, Pb^{2+}, Co^{2+}, Ni^{2+} and Mn^{2+} was investigated. Of these only Cu^{2+} was found to dramatically quench the

fluorescence. At a threefold molar excess of Cu^{2+} (pH 5) the fluorescence of 5 x 10^{-5} M fulvic acid decreased by 60%. Further studies utilised a fluorescence titration technique to enable the determination of a conditional stability constant (K) for Cu^{2+} with soil fulvic acid [16]. At pH 5 the calculated stability constant was 0.48 x 10^{-5} M^{-1}. Titrations of the model compound L-tyrosine were carried out to validate the procedure. The conditional stability constant obtained was within 1.5% of the theoretical value expected for L-tyrosine. Overall the major advantage of fluorescence is that free ligand concentration, rather than free metal ion concentration, is measured. Complexation of metals by humic substances often results in the formation of large aggregates or precipitates [16]. Many analytical methods, particularly electrochemical methods, which measure free metal ion activity will subsequently detect a decrease in the free metal ion levels which is incorrectly interpreted as further complexation. Once adsorption or precipitation occurs, therefore, the model of a solution equilibrium between free and bound metal ion fails. The losses of metal ion associated with precipitation can often be avoided by making measurements in slightly acidic solution (i.e. pH 5-6) [16].

2.1.2 Characterisation of aquatic humic substances

Much work has been carried out in an effort to examine the chemical nature of the functional groups responsible for the acidity and metal binding properties of aquatic humic substances. Humic materials are inherently heterogeneous in nature. They are composed of a highly complex mixture of non- identical acidic functional groups. In addition the chemical composition of humic materials varies significantly with source. Therefore, it is not generally possible to compare results obtained by different authors. Variations in isolation and titration procedures also prevents direct comparison. In general humic substances are characterised by their molecular weight

distribution, elemental analysis, aromatic to aliphatic ratio, total acidity and functional group distribution [3].

2.1.2.1 Molecular weight distribution

The molecular weight distributions of humic substances are usually determined by separation techniques such as gel permeation chromatography, ultrafiltration and ultracentrifugation [17]. Flow field-flow fractionation has also been used to determine the size distribution of humic and fulvic acid samples [18]. More recently, small angle x-ray scattering, vapour-pressure osmometry and fast-atom bombardment mass spectrometry have been used to characterise the Suwannee River fulvic acid standard [19]. An average molecular weight of 800 daltons has been determined for this standard. In the case of aquatic humic materials, there is a general size differential with number average molecular weights of 800-1000 daltons for the fulvic acid fraction and 2000-3000 daltons for the humic acid fraction [20].

2.1.2.2 Total acidity

The general consensus among authors is that the acidic nature of humic substances is primarily due to carboxyl and phenolic groups [21-23]. Sulphur and nitrogen acids do not contribute significantly to acidity. It has been estimated that aquatic humic and fulvic acid samples contain less than 1% sulphur and 2% nitrogen [9]. Due to the prevalence of oxygen (approximately 44%), it is the oxygen-containing ligands that are considered to be the predominate acid groups. Humic materials have an irregular polymeric structure with no readily identifiable monomer units [24]. The various functional groups exist in very different structural environments and are rarely chemically identical. Recent studies carried out on carboxyl group structures

in Suwannee River fulvic acid estimate that the strong acid characteristics ($pK_a \leq$ 3.0) can be separated into 57% aliphatic carboxyl groups and 43% keto acid and aromatic carboxyl-group structures [25].

2.1.2.3 Infrared spectroscopy

IR spectroscopy is widely used in the characterisation of humic substances. This technique provides information regarding the nature, reactivity and structural arrangement of oxygen-containing functional groups. It is also used to indicate the presence or absence of inorganic impurities (metal ions, clay) in isolated humic fractions. A classification system has been devised by Stevenson [26] which sub-divides humic substances into three groups, humic acids, fulvic acids and humic materials containing proteins and carbohydrates. IR spectra of fulvic acids are characterised by a very strong absorption band at 1720 cm^{-1}. Absorption in the 1600 cm^{-1} region is weak and centred near 1640 cm^{-1}. A major problem in the use of IR spectroscopy for the analysis of humic substances is interference caused by absorbed moisture, which produces bands in the 3300 to 3000 cm^{-1} and 1720 to 1500 cm^{-1} region.

2.1.2.4 ^{1}H and ^{13}C NMR

NMR has been used extensively to determine the functional group distribution and ratio of aliphatic to aromatic residues for a variety of humic and fulvic acids including the Suwannee River fulvic acid standard [27,28]. ^{1}H NMR is more sensitive than ^{13}C NMR because of the greater inherent sensitivity of the ^{1}H nucleus and because of its greater natural abundance. The major proton resonances which have been assigned in solutions of humic materials include aliphatic protons attached

to benzene rings (CHγ 0.90 ppm, CHβ 1.3 ppm and CHα 2.6 ppm), methoxyl protons (3.7 ppm), lactone protons (4.0-5.5 ppm), aromatic protons (6.0-7.5 ppm), hydroxyl protons (10 ppm) and carboxylic acid protons (13 ppm) [27].

The advantage of ^{13}C NMR compared with ^{1}H NMR is that the much wider chemical shift range of carbon provides better resolution of the resonances of individual functional groups [28]. However ^{13}C NMR has a lower sensitivity and natural abundance relative to protons. In addition the acquisition of quantitative ^{13}C NMR spectra is complicated by variable spin-lattice relaxation times (T_1) and variable nuclear Overhauser enhancement (NOE). The spin-lattice relaxation time, T_1, is a time constant which is inversely proportional to the rate at which energy is transferred from the spins to the lattice, allowing the magnetization to return to its equilibrium value after a radiofrequency pulse. If the delay between pulses is too short , carbon nuclei with shorter T_1's will be exaggerated in intensity relative to carbon nuclei with longer T_1's. A pulse delay on the order of 3 to 5 times the longest T_1 present in the sample is generally required for quantitative spectra. The T_1 values of Suwannee River fulvic acid were investigated by Thorn [28]. The longest T_1 was reported to be 1.8 seconds and was determined for carboxyl and ketone groups. A pulse delay of 8 seconds in conjunction with a 45° pulse allowed for complete relaxation of nuclei between pulses. Differential nuclear Overhauser enhancement (NOE) effects were eliminated by employing inverse-gated decoupling. The decoupler was turned on during the acquisition but off during the pulse delay. The NOE effect occurs because proton decoupling causes changes in the population distribution of the ^{13}C spins, resulting in an enhancement of the ^{13}C signals.

Concentrations of various hydroxyl groups present in humic substances cannot be directly determined from natural abundance ^{13}C-NMR spectra of underivatised

humic substances because of the problem of overlapping resonances. Carboxylic acid carbons overlap with ester, amide, lactone and some phenolic carbons (160-180 ppm). Phenolic carbons overlap with other non-protonated aromatic carbons (135-165 ppm) and carbohydrate and alcohol carbons may overlap with ether carbons (60-77 ppm) [28]. Methylation of hyroxyl functional groups has been carried out to enable the observation of the corresponding methyl esters and methyl ethers [29]. Humic and fulvic acids were methylated with ^{13}C-enriched reagents and the intensities of the peaks in the region 50-62 ppm were measured. NOE for all OCH_3 groups in this region was found to be uniform so that integration of the peak areas should give a good representation of the relative abundance of functional groups, provided the spin-lattice relaxation time has been accurately determined. ^{13}C-enriched diazomethane was used to enable the analysis of hydroxyl functional groups in the Suwannee River fulvic acid standard [28]. The methyl esters of carboxylic acids and phenolic hydroxyls were observed at 52 ppm and 56 ppm, respectively.

Overall the measurement of functional group concentrations in humic substances by both ^{13}C and ^{1}H NMR spectroscopy is at best semiquantitative. The effect of variations in NOE in liquid samples must be evaluated by gated decoupling experiments on every sample to be measured. In addition the relaxation times of all functional groups must be measured accurately for each sample and the delay times chosen to allow for adequate relaxation of the group with the longest spin-lattice relaxation time. The main advantage of NMR for the study of of humic substances is the fact that it is a non-destructive technique and generally enables functional group analysis without separation or chemical alteration of the sample.

2.1.3 ^{113}Cd NMR

^{113}Cd NMR was used in these studies to characterise Cd(II)-ion complexation by aquatic fulvic acid. ^{113} Cd is an excellent metal probe due to its ability to form complexes with many different conformations and ligand numbers [30]. Cd^{2+} has a filled d-shell and is not subject to ligand field effects. Its nuclear properties are similar to ^{13}C. ^{113}Cd is diamagnetic and has a nuclear spin I = 1/2. Its inherent sensitivity is 1.09×10^{-2} with respect to ^{1}H for an equal number of nuclei at constant field. Naturally occurring cadmium contains *ca.* 12 % of the ^{113}Cd isotope, 7.6 times the natural abundance of ^{13}C. This abundance can be increased eight-fold by the use of isotopically-enriched ^{113}Cd (96 atom %).

^{113}Cd NMR was first described in the early seventies [31-33]. Preliminary studies were carried out by Maciel and Borzo [31] using pulsed fourier-transform NMR (FT-NMR). A number of inorganic and organic ^{113}Cd compounds were examined. A large chemical shift range of over 538 ppm was reported. Individual ^{113}Cd resonances were found to be concentration dependent. $Cd(ClO_4)_2$, $Cd(NO_3)_2$ and $Cd(Cl)_2$ were measured at concentration levels of 0.1 and 4.2 M. Concentration had the least effect on the chemical shift of $Cd(ClO_4)_2$ with a difference of only 8.11 ppm being observed between the two levels. For $Cd(NO_3)_2$ and $Cd(Cl)_2$ however, altering the concentration produced significant chemical shift differences of 45.69 ppm and 67.72 ppm, respectively. Kostelnick and Bothner-By also studied the effect of concentration on the chemical shift of ^{113}Cd in aqueous solutions of its salts [32]. On increasing concentration, it was found that the $Cd(NO_3)_2$, $Cd(ClO_4)_2$ and $CdSO_4$ complexes gave signals with shifts at higher fields whereas the halide complexes (i.e. CdI_2, $CdBr_2$ and $CdCl_2$) gave downfield shifts. The slopes for $Cd(ClO_4)$ and

$CdCl_2$ were small. These complexes are generally used as chemical shift references in NMR studies. The effect of organic and amino-acid ligands on the chemical shift of 2 M $Cd(SO_4)$ was also investigated. An important general trend was discovered. Ligands which bind through oxygen cause increased shielding of the Cd nucleus, while ligands that bind through nitrogen produce a marked deshielding of the Cd nucleus. Finally ligands which bind via sulphur produce very large deshielding [30].

2.1.3.1 Chemical Shift

^{113}Cd chemical shifts cover a wide range from 750 ppm to -200 ppm [30]. This broad chemical shift dispersion reflects the tremendous sensitivity of the shielding of the ^{113}Cd nucleus to its local environment. Subtle changes in coordination can produce substantial shifts in ^{113}Cd resonances. The extreme sensitivity of ^{113}Cd chemical shifts to substituent effects was recognised even in early studies [33]. The replacement of methyl groups by ethyl groups in dimethyl cadmium produced an upfield chemical shift of almost 100 ppm. Replacement of the ethyl groups by n-propyl moieties produced a further shift upfield of 40 ppm. In all it was estimated that the ^{113}Cd chemical shifts are at least 15 times more sensitive to substituent effects than ^{13}C shifts.

Based on these initial qualitative observations, it was hoped that once the chemical shifts of a sufficient number of Cd^{2+} complexes were measured there would emerge a reliable structure-versus-shift correlation that would allow the interpretation of ^{113}Cd chemical shifts for complexes in terms of the number, identity and geometric arrangement of the ligands at the binding site. The main obstacle hindering this development is the effect of chemical dynamics on ^{113}Cd chemical shifts. Facile chemical exchange is frequently encountered with model cadmium complexes in

aqueous solutions. Therefore the chemical shift observed for a cadmium compound with a known crystal structure is rarely indicative of that complex alone but instead is a weighted average of the chemical shifts of multiple species in rapid equilibrium [32,34]. Known equilibrium constants must, therefore, be used to calculate the chemical shifts for the individual complexes. The uncertainty in chemical shifts obtained in this way is dependent on the extent to which the equilibrium constants actually describe the system under investigation. Additional solution complexes may also be present which are unaccounted for by the known equilibrium constants [35].

Much effort has been made to circumvent the adverse effects of chemical exchange, including the application of solid-state NMR methods, the use of super-cooled solutions and the synthesis of less-labile ligands [30]. The use of super-cooled solutions (-80°C) enabled the observation of four distinct signals in a solution containing cadmium iodide. Distinct signals were observed which correspond to CdI_3 (122 ppm), CdI_4 (101 ppm), CdI_2 (43 ppm), CdI (20 ppm) and free Cd (or $Cd(NO_3)_n$, -86 ppm) [36]. Solid-state ^{113}Cd NMR spectra using the cross-polarisation magic angle spinning technique (CP-MAS) has been used to study a $[Cd_{10}(SCH_2CH_2OH)_{16}](ClO_4)_4$ complex [37]. The solid state spectrum showed three resonances for the three different Cd^{2+} sites in this complex whereas the solution spectrum showed only two, as a result of fast exchange between two of the three sites under the latter conditions.

The broad chemical shift range for ^{113}Cd means that excellent spectral resolution can be obtained even when $^{113}Cd^{2+}$ is bound to multiple coordination sites of very similar structure. Thus all the binding sites in a system can be observed simultaneously. In addition, the nature of the coordinating ligands and their coordination number are strongly reflected in the chemical shift [30]. It is generally

agreed in the literature that the ^{113}Cd chemical shift induced by various ligands increases in the order O > N > S. Therefore, the resonance signal obtained in solutions of cadmium containing coordination compounds is reflective of the directly bonded heteroatom of the ligand. Thus the specific binding sites involved in the complexation can be identified.

2.1.3.2 Chemical Exchange

Chemical exchange can have a significant effect on ^{113}Cd NMR spectra due to the wide range of chemical shifts observed and because of the molecular degrees of freedom of the central $^{113}Cd^{2+}$ atom or of its surrounding ligands. Both the chemical shifts and the line widths of ^{113}Cd resonances can be affected. The extent of the effect depends on the rate of the exchange processes relative to the chemical shift difference between the exchanging species. Intermediate-exchange conditions can result in broadening of the resonances to such an extent that observation of the resonance may be prevented. Under conditions of fast exchange, however, a single resonance is observed with a chemical shift which is a statistical average of the chemical shift values for the free and complexed species [38]. It is possible to add one complexing agent in excess until the chemical shift value remains essentially constant at which point it is assumed that the final chemical shift value is due entirely to the complex. The binding site constant can then be evaluated from the chemical shift data.

2.1.3.3 Biological Applications

^{113}Cd NMR has been used extensively to study metalloprotein complexes in which the native diamagnetic metal has been replaced by an isotopically-enriched spin -1/2

^{113}Cd nucleus. Cd^{2+} is particularly suited to these studies due to the fact that its ligand preferences and coordination geometry is quite similar to that of Zn^{2+} and Ca^{2+}. Cd^{2+} and Ca^{2+} also have similar ionic radii of 0.97 and 0.99 angstroms, respectively. In all known Zn^{2+} metalloenzymes, Cd^{2+} can be substituted for the native Zn^{2+} , with only altered catalytic efficiency and specificity [35]. In addition to providing information on the number and type of coordinating groups, ^{113}Cd NMR has afforded valuable information on protein conformational changes, accessibility of solvent and anions to the metal and the nature of protein interactions with substrates and inhibitors [30]. Some interesting characteristics have been reported including both high-affinity and low-affinity sites, high, medium and slow rates of exchange and separate chemical shifts for different binding sites (under conditions of slow exchange). Substrate and inhibitor binding have also been observed by chemical shift changes [30].

The largest ^{113}Cd chemical shift reported to date is for Cd^{2+} bound to the non-catalytic site of horse liver alcohol dehydrogenase [39]. A resonance was observed at 751 ppm, where Cd^{2+} is coordinated to four cysteine residues. The most deshielded resonance has been observed in horseradish peroxidase [40]. A chemical shift of -193 ppm was obtained where Cd^{2+} is coordinated only to oxygen ligands at a high affinity Ca^{2+} site.

The metal ion complexation chemistry of fulvic acids is complex due to its structural heterogeneity and polyelectrolyte nature. ^{113}Cd has been widely used as a metal probe for many metalloproteins due to its excellent NMR spectroscopic properties. These studies provide excellent models for the investigation of metal ion binding in fulvic acid because of its aggregate size and complex structure. In this work ^{113}Cd NMR was used to examine cadmium ion binding to aquatic fulvic acid by

monitoring the ^{113}Cd chemical shift as a function of the ligand-metal ion ratio [41]. Since the metal ion binding characteristics of humic materials vary significantly with source a previously characterised fulvic acid standard was used [43]. Use of a standard material whose composition has been extensively studied enabled meaningful comparison with the work of other authors as well as the accumulation of data on one particular type of humic material.

2.2 Experimental

2.2.1 Chemicals and solutions

The fulvic acid used in this research was obtained from the International Humic Substances Society (I.H.S.S.). It is a standard fulvic acid sample which was isolated from the Suwannee River, Georgia. The isolation and characterisation of this material is described in detail elsewhere [42]. ^{113}CdO (93.4% enriched) was purchased from Cambridge Isotopes. All solutions for NMR analysis were prepared in D_2O (99.96 atom *% d* , Sigma). Concentrated solutions of DCl and NaOD, purchased from Isotec, were diluted in D_2O and used to adjust the solution pH of the NMR samples. For the pH titrations, potassium hydrogen phthalate (Fisher, Certified A.C.S.) was used to standardise the sodium hydroxide (Fisher, Certified A.C.S.) titrant solution.

2.2.2 Procedure

2.2.2.1 pH titrations

The acidic nature of the fulvic acid sample was determined by pH titration. Since the acidity of fulvic acid is mostly due to the presence of carboxylic acid and phenolic functional groups, titration with NaOH allows the determination of the total number of these groups per milligram of the sample. All solutions were prepared from boiled and cooled, distilled, deionised water to avoid any contribution to acidity from dissolved carbon dioxide. All titrations were carried out in triplicate under a controlled N_2 atmosphere in a polyethylene glove bag and the sample solutions were bubbled with N_2 gas before analysis. Ionic strength effects were not controlled in these experiments and activity effects were not taken into consideration.

A combination pH electrode (Fisher 13-620-288) and Fisher Acumet 10 pH meter were used for these studies. The electrode was calibrated daily using aqueous pH 1.00, 4.00, 7.00 and 10.00 buffers (Fisher). NaOH (0.0982 N) was delivered to the sample solution in 10, 20 and 50 μl aliquots. The autopipettes used for titrant delivery were calibrated and, based on an average of 12 replicate measurements, were found to deliver 9.8, 24.4 and 48.8 μl of solution, respectively. Titrant volumes recorded were adjusted accordingly. The titrant was standardised in duplicate against the primary standard, potassium hydrogen phthalate (KHP) immediately prior to each use using standard quantitative analytical techniques. The fulvic acid solutions were prepared by dissolving 10 mg in 2 ml of CO_2-free water. A blank was also prepared containing 2 ml of CO_2-free water. Prior to the titration, the pH of the sample was adjusted to 2 using 1 μl of 1 M HCl. No attempt was made to control or correct for ionic strength effects.

2.2.2.2 NMR studies

All ^{113}Cd solutions were diluted from a 0.0875 M stock solution prepared by dissolving ^{113}CdO in a small volume of DCl (35%) and diluting the solution to 1000 µl using D_2O. Samples containing 4.38 mM $^{113}Cd^{2+}$ and fulvic acid in the range 2.25 to 50 mg/ml were prepared from cadmium and fulvic acid stock solutions which were made up to 500 µl with D_2O. All pH measurements in D_2O solution were corrected for the deuterium isotope effect using the relationship pD = pH meter reading + 0.4 [43]. For simplicity, the corrected pD values for D_2O solutions are referred to as pH throughout. The pH of each D_2O solution was adjusted to 6.4 (i.e. a pH meter reading of 6.0) using concentrated NaOD. pH measurement of the samples prepared for NMR analysis were made using a 3 mm glass pH microelectrode (Ingold) and a Fisher Scientific Acumet 10 pH meter at ambient temperature. The electrode was calibrated daily using aqueous buffers as described above.

The NMR studies were carried out using a Bruker AM-500 MHz spectrometer, equipped with a broad band multinuclear probe and operated at a spectral frequency of 110.93 Hz. Chemical shifts were reported relative to 1 M $Cd(ClO_4)_2$ used as an external reference. The spin-lattice relaxation time, T_1, of the $Cd(ClO_4)_2$ reference solution was 65 seconds, measured using the standard inversion recovery method. The relaxation times of the fulvic acid complexed Cd are much less than this value (approximately 1 second) but were not precisely determined due to the low concentrations and low signal-to-noise of the spectra obtained for these solutions. Typically 8,000-20,000 scans were recorded using a 3 µsec (40°) pulse and a 0.85 second repetition time. The data files were transferred to a Silicon Graphics Indigo workstation and processed using Felix 2.30 (Biosym). The cadmium free induction decays were apodized by multiplication with a decaying exponential equivalent to 50

Hz line-broadening unless otherwise stated. Baseline distortions were corrected by fitting selected baseline points to a fifth order polynomial.

2.3 Results

2.3.1 Determination of the number of titratable functional groups

As fulvic acids are by nature heterogeneous samples, apparent equilibrium constants calculated using ligand concentrations in units such as molarity are not really meaningful. An alternative approach is to determine the total number of ionizable functional groups per mass unit of the ligand [44]. Potentiometric pH titrations of the fulvic acid standard were carried out in triplicate using NaOH as titrant. Preliminary standardisation of the NaOH with 0.1 M KHP indicated that the molarity of the NaOH was 0.0982M.

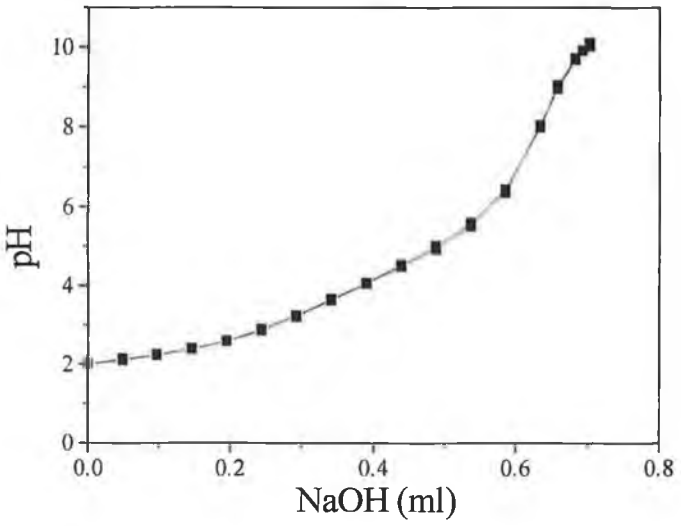

Figure 2.1 Potentiometric pH titration of Suwannee River fulvic acid standard. Conditions: 5 mg/ml fulvic acid (pH 2) titrated with 0.0982 M NaOH in controlled N_2 atmosphere.

Table 2.1 Potentimetric pH titration of fulvic acid standard

Vol. NaOH (ml)	pH	pH	pH
0.0000	2.00	2.00	2.01
0.0488	2.14	2.09	2.11
0.0976	2.24	2.22	2.25
0.1464	2.40	2.38	2.41
0.1952	2.60	2.58	2.59
0.2440	2.85	2.88	2.91
0.2928	3.20	3.24	3.25
0.3416	3.62	3.64	3.67
0.3904	4.05	4.08	4.08
0.4392	4.48	4.51	4.54
0.4880	4.92	4.97	5.01
0.5368	5.50	5.58	5.60
0.5856	6.35	6.42	6.44
0.6344	7.98	8.05	8.03
0.6588	8.95	9.02	9.05
0.6832	9.68	9.70	9.72
0.6930	9.90	9.90	9.91
0.7028	10.01	10.11	10.09

Fulvic Acid = 5 mg/ml

NaOH = 0.0982 M.

Due to the fact that humic substances are composed of a mixture of polyprotic acids, no discrete end points are observed on titration with alkali (Figure 2.1). Instead, the individual pK_a values for each class of functional group extend over a relatively wide and apparently continuous range. In fulvic acids the predominate acid groups are carboxyl and phenolic functional groups. A pH of 8 is generally chosen as the equivalence point for the carboxyl groups [45]. This is based on the assumption that the pK_a values of carboxyl groups are centred around 5.0 and that the pK_a values for phenolic functional groups are centred around 10.0. If phenols have an average pK_a of 10.0 then, assuming monoprotic behaviour, they would be 50% titrated at pH 10.0. Accordingly, the carboxyl content was determined from the alkali consumed to pH 8 while the phenol content was calculated as twice the alkali assumption between pH 8.0 and 10.0.

As shown in Figure 2.1 the volume of NaOH titrated at pH 8 and 10 was 0.639 and 0.700 ml, respectively. This indicates that the fulvic acid standard contains 6.3 meq/g carboxyl groups and 1.2 meq/g phenolic groups. These results compare favourably with previously reported values of 6.1 meq/g carboxyl groups and 1.2 meq/g phenolic groups for the Suwannee River fulvic acid standard [45]. At pH 6.4, the pH at which the metal ion complexation studies were carried out, the fraction of carboxylic acid groups (α_{coo^-}) which are deprotonated is 0.89. Thus it would appear that pH 6.4 is a suitable pH at which to study cadmium complexation.

2.3.2 Metal ion-fulvic acid binding constants

The ability of ^{113}Cd NMR to give widely separate chemical shifts for various types of functional groups will enable the simultaneous determination of metal ion complex formation constants for different binding sites. In typical fast exchange

NMR studies of equilibrium constants, the observed chemical shift, δ_{obs}, represents an average of the free chemical shift, δ_{free}, and the bound (metal ion complex) chemical shift, δ_{bd} [38].

For an n:1 complex, the observed chemical shift, δ_{obs}, is a weighted average of δ_{free} and δ_{bd} as follows:

$$\delta_{obs} = \{[Cd_{bound}]\delta_{bd} / [Cd_{total}]\} + \{[Cd_{free}]\delta_{free} / [Cd_{total}]\} \quad \text{(Eq. 1)}$$

If the total Cd concentration is held constant and the fulvic acid concentration is varied then

$$K = [Cd_{bound}] / [Cd_{free}] \{n[\text{Fulvic Acid}] - [Cd_{bound}]\} \quad \text{(Eq. 2)}$$

where [Fulvic Acid] refers to the total fulvic acid concentration and n is the number of cadmium binding sites per fulvic acid molecule. Eq. 2 can be rearranged into a Scatchard equation ,

$$[Cd_{bound}] / [Cd_{free}][\text{Fulvic Acid}] = nK - K[Cd_{bound}] / [\text{Fulvic Acid}] \quad \text{(Eq. 3)}$$

so that a plot of $[Cd_{bound}] / [Cd_{free}][\text{Fulvic Acid}]$ vs $[Cd_{bound}] / [\text{Fulvic Acid}]$ will yield K and n from the slope (-K) and intercept (nK), respectively.

In this study, the total cadmium concentration, Cd_{total}, was maintained at 4.38 mM while the fulvic acid concentration was varied from 2.25 to 50 mg/ml at pH 6.4. The preliminary spectra obtained and the conditions of measurement are illustrated in the Appendix. The chemical shift was measured relative to $Cd(ClO_4)_2$ at 25°C in all

cases. As the concentration of fulvic acid was increased a gradual change in chemical shift was observed (Figure 2.2).

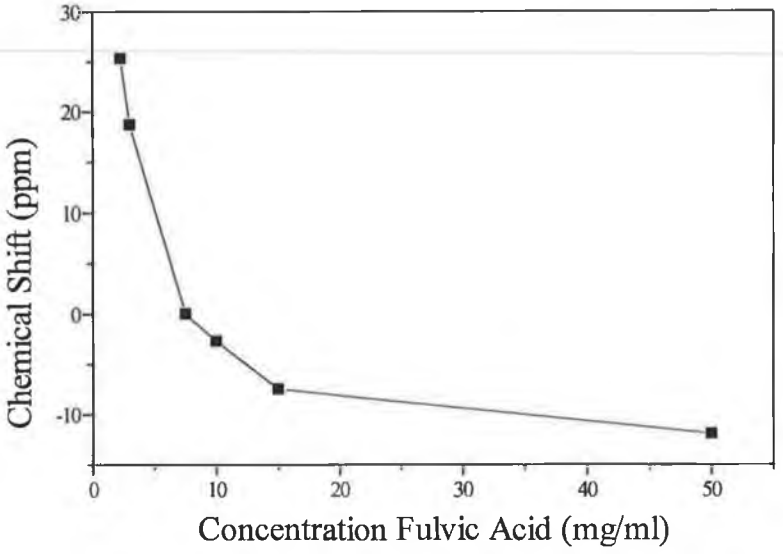

Figure 2.2 Plot of ^{113}Cd NMR chemical shifts at increasing concentrations of fulvic acid. Conditions: Cd 4.38 mM; Fulvic acid 2.25 mg/ml-50 mg/ml; pH 6.4; Reference solution, $Cd(ClO_4)_2$, 1 M; Temperature 25°C; Spectral frequency 110.93 Hz; Pulse 3 μsec (40°C); 0.85 sec repetition time.

An upfield change in chemical shift was observed at increasing concentrations of fulvic acid. At a fulvic acid concentration of 2.25 mg/ml a chemical shift of 25.36 ppm was obtained. As Cd became increasingly bound, as the concentration of fulvic acid was increased to 50 mg/ml, a chemical shift of -11.9 ppm was observed. Upfield chemical shifts are generally associated with Cd binding to carboxyl groups.

By definition from Eq. 1, as the concentration of the fulvic acid approaches infinity (while the Cd concentration is constant), the observed chemical shift is due only to that of the pure complex [38]. As a result, a plot of observed chemical shift versus reciprocal fulvic acid concentration yields the chemical shift of the pure complex at a value of 1/[Fulvic Acid] = 0, the y intercept. A plot of measured chemical shift versus reciprocal fulvic acid concentration gives an intercept of -13.6 ppm for the chemical shift of the "pure" complex (Figure 2.3). In a secondary plot, the observed chemical shift was fitted to a polynomial expansion of reciprocal fulvic acid concentration, yielding a value of 48.3 ppm for the chemical shift of free ^{113}Cd.

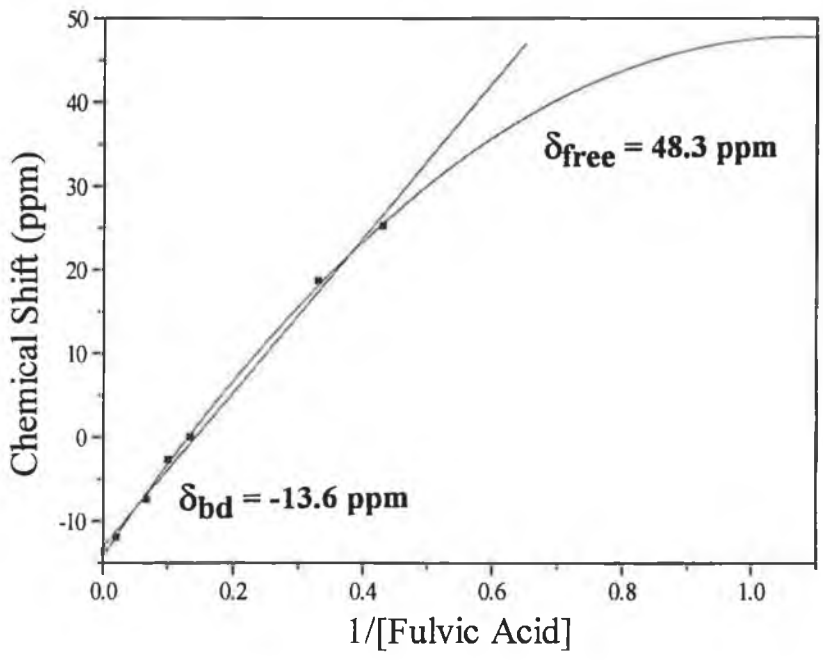

Figure 2.3 Plot to determine the chemical shifts of free ^{113}Cd (δ_{free}) and ^{113}Cd-Fulvic Acid complex (δ_{bd}). Conditions: Cd 4.38 mM; Fulvic acid 2.25 mg/ml-50 mg/ml; pH 6.4; Reference solution, $Cd(ClO_4)_2$, 1 M; Temperature 25°C; Spectral frequency 110.93 Hz; Pulse 3 μsec (40°C); 0.85 sec repetition time.

Substitution of the values for δ_{free} and δ_{bd} into Eq. 4 allowed the concentration of free and bound cadmium to be calculated at each fulvic acid concentration (Table 2.2).

$$[Cd_{bound}] = [Cd_{total}](\delta_{free} - \delta_{obs})/(\delta_{free} - \delta_{bd}) \qquad \text{(Eq. 4)}$$

Table2.2

[Fulvic Acid] (mg/ml)	δ_{obs} (ppm)	[Cd total] (mM)	[Cd bound] (mM)	[Cd free] (mM)
2.25	25.36	4.38	1.62	2.76
3.0	18.72	4.38	2.09	2.29
7.5	0.06	4.38	3.41	0.97
10	-2.66	4.38	3.61	0.77
15	-7.43	4.38	3.94	0.44
50	-11.90	4.38	4.26	0.12

where

δ_{free} = 48.3 ppm

δ_{bd} = -13.6 ppm

To enable the calculation of the conditional stability constant (K) and the number of binding sites per fulvic acid molecule (n) a Scatchard plot of the data was carried out. According to Eq. 3, this plot will yield K and n from the slope (-K) and the intercept (nK), respectively. The data for this plot was calculated as shown in Table 2.3.

Scatchard plot: $[Cd_{bound}] / [Cd_{free}][\text{Fulvic Acid}]$ Vs $[Cd_{bound}] / [\text{Fulvic Acid}]$

Table 2.3

[Fulvic acid] (mg/ml)	$[Cd_{bound}]$ (mM)	$[Cd_{free}]$ (mM)	$[Cd_{free}]$. [Fulvic acid]	$[Cd_{bound}]/$ [Fulvic acid] $[Cd_{free}]$	$[Cd_{bound}]/$ [Fulvic acid]
2.25	1.65	2.73	6.21	0.2609	0.7200
3.00	2.08	2.30	6.87	0.3042	0.6967
7.50	3.41	0.97	8.63	0.4708	0.4551
10.0	3.61	0.77	9.10	0.4658	0.3606
15.0	3.94	0.44	7.05	0.6021	0.2662
50.0	4.25	0.13	3.50	0.7100	0.0852

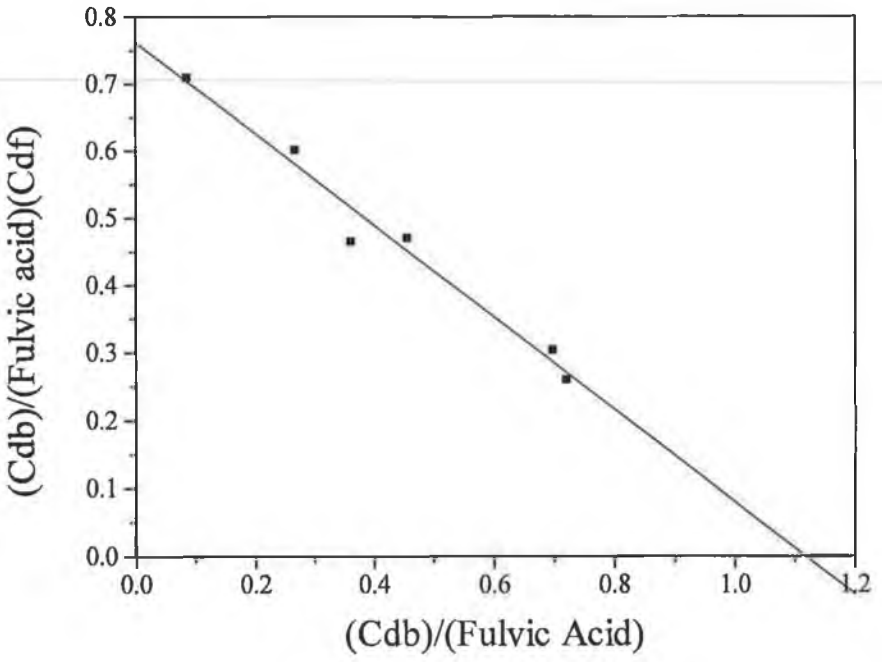

Figure 2.4 Scatchard Plot for the calculation of the conditional stability constant (K) and the number of binding sites per fulvic acid molecule (n). Slope = -6.7×10^2 M^{-1}. Intercept = 0.737.

The conditional stability constant (K) for the Cd-Fulvic acid complex under these experimental conditions was 6.7×10^2 M^{-1}. The number of Cd binding sites per Fulvic acid molecule was approximately 1.1 indicating that a 1:1 complex was formed.

2.4 Discussion

2.4.1 Carboxylic acid binding sites

The upfield chemical shift of the pure complex (-13.6 ppm) indicates that Cd is binding to carboxyl groups [30, 35, 46]. This is in good agreement with the results obtained for the potentiometric titrations which showed that carboxyl groups were the most prevalent acidic groups in the fulvic acid standard (6.3 meq/g). Leenheer et al. have also reported that the strong-acid characteristics ($pK_a \leq 3.0$) of Suwannee River fulvic acid can be separated into 57% aliphatic carboxyl groups and 43% keto acid and aromatic carboxyl-group structures [25]. Characterisation of the Suwannee River fulvic acid standard has shown that an important fraction of the carboxyl groups are cyclic aliphatic α-ester and α-ether structures [47]. Carboxylate groups have also been reported to be the primary binding sites for Ca^{2+} and Cd^{2+} in peat humic acid [8] and in aqueous fulvic acid at pH 6.0 [48].

2.4.2 Concentration of binding sites

The potentiometric pH titrations indicate that there are approximately 6.3 meq of carboxyl groups per gram of fulvic acid. Using a number average molecular weight of approximately 800g/mole for the Suwannee River standard material there are on average 5 carboxylic acid ligands per fulvic acid molecule [19]. At pH 6.4, approximately 89% of these carboxylate groups are deprotonated suggesting that the effective number of carboxylate binding sites per fulvic acid molecule is 4. Under the conditions of our experiments a 1:1 cadmium-fulvic acid complex was formed (n=1.1). This can be attributed to the large variation in component molecular weights for fulvic acid.

2.4.3 Metal ion stability constants and multiple binding sites

Studies of metal ion complexation by humic acids in estuaries has only recently been a subject of systematic investigations, and these are primarily concerned with the binding of Cu^{2+}. In an early study [12] the coordination of copper(II) ions in soil and water fulvic acids was investigated and two classes of binding sites were identified with stability constants of 1×10^6 and 8×10^3 M^{-1} over the pH range of 4 to 6. Luminescence spectroscopy was used to investigate metal binding sites on fulvic acid obtained from air-dried Okchun Metamorphic belt (Korea) topsoil samples. Both 1:1 and 1:2 carboxylate moieties were identified using deconvoluted ^7F_o - ^5D_o excitation spectra of Eu(III). The weaker binding species was quite abundant and showed a rapid increase from pH 2.9 to 6.3 [49].

The apparent Cd-fulvic acid complex formation constant of 6.7×10^2 M^{-1} obtained at pH 6.4 is reasonable in view of weaker stability constants obtained for Cd^{2+} versus Cu^{2+} reported previously [48]. We do not observe a second Cd^{2+} binding site as determined by NMR in this sample, however this is possibly due to the limited Cd^{2+} concentration range used in this study [48,49]

2.4.4 Fast Exchange

The change in chemical shift with changing concentration is clear evidence for fast exchange. This contrasts sharply with ^{113}Cd studies of binding to proteins [50,51] and in soil humic acids [52] where there is little, if any, evidence for such exchange. The fact that such exchange occurs in fulvic acids suggests that a competitive binding study with other metals will yield additional equilibrium constants.

2.5 Conclusion

This study represents a new approach for the characterisation of the metal ion complexation chemistry of humic substances isolated from water. Although ^{1}H and ^{13}C NMR have been used extensively for the characterisation of humic and fulvic acids, NMR spectroscopic methods have not been widely used in the examination of metal ion complexation by naturally occurring organic matter. A recent paper describes the use of ^{113}Cd NMR to investigate the binding of cadmium to two soil humic acid samples [52]. In these experiments, varying amounts of soil humic acid were added to solutions containing equal amounts of Cd^{2+}. The soil humic acid samples were much less soluble than the aquatic fulvic acid standard used in this study. The amount of cadmium complexed by the soil humic acids was determined by measuring the decrease in the amount of free ^{113}Cd in solution using NMR spectroscopy. Only the free $^{113}Cd^{2+}$ was detected in this study and no evidence for chemical exchange between free and bound cadmium was detected since the bound cadmium was removed from solution by precipitation as the humic acid complex.

In this work ^{113}Cd NMR was used to examine metal ion binding by monitoring ^{113}Cd chemical shifts as a function of the ligand - metal ion ratio. The specific groups involved in the binding were identified as carboxyl groups. Complexation was found to occur under conditions of fast exchange. Under these conditions the observed chemical shift represents a statistical average of the free and complexed cadmium species. The chemical shift of the free species can be measured in the absence of the ligand. By adding the ligand in excess the chemical shift of the pure complex can be obtained. A conditional binding constant of 6.7×10^{2} M^{-1} was determined by monitoring the change in the chemical shift as $^{113}Cd^{2+}$ became increasingly bound.

This investigation represents a fundamental departure from standard methods of analysis of humic materials. NMR measurements of metal ion complexation by fulvic acids enables a more detailed characterisation of the number and type of binding sites to be obtained. Competitive binding experiments may also be carried out in which the $^{113}Cd^{2+}$ ion could be replaced by other metal ions. This would allow the determination of the relative affinity of different metal ions for the same site. Increased characterisation of the metal binding ability of aquatic fulvic acids is essential in order to obtain a greater understanding of the fundamental solution chemistry of these environmentally important compounds.

2.6 References

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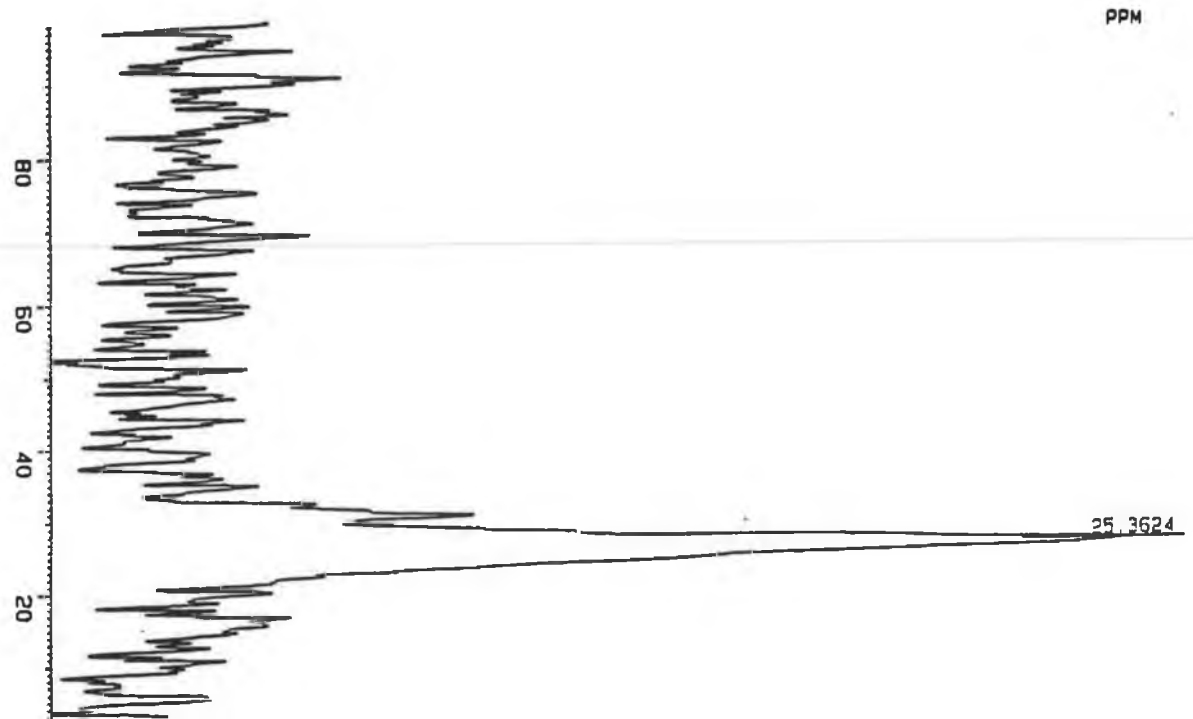
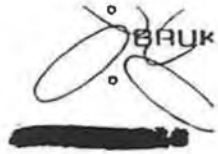
Appendix

Appendix A.

^{113}Cd NMR spectrum of 4.38 mM Cd : 2.25 mg/ml Fulvic Acid





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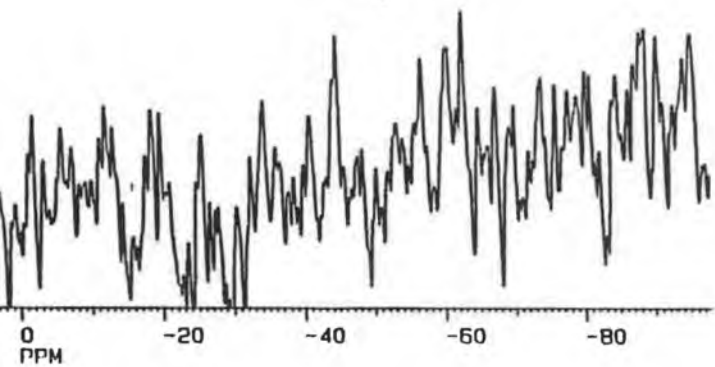
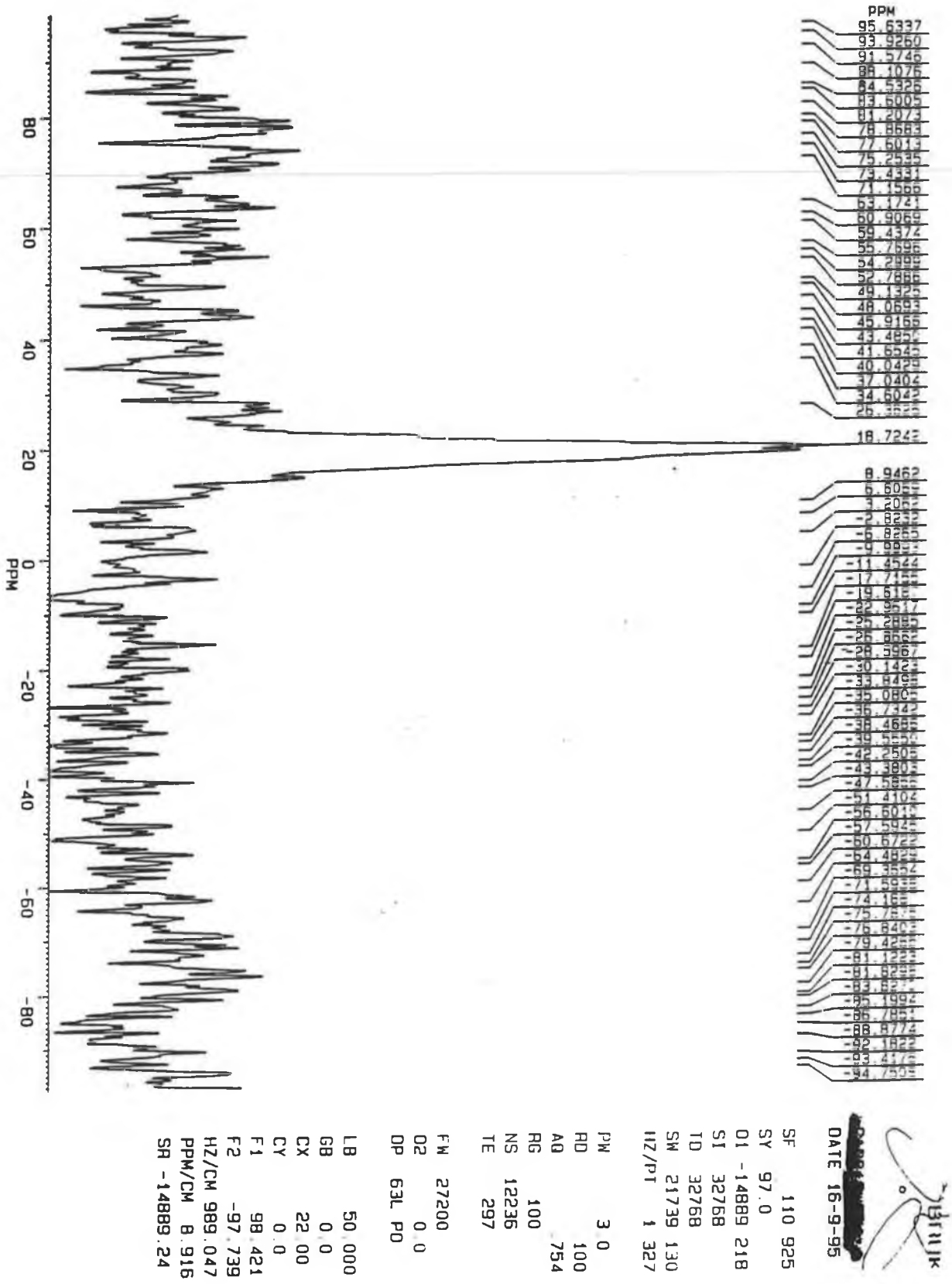


Appendix B.

^{113}Cd NMR spectrum of 4.38 mM Cd : 3.0 mg/ml Fulvic Acid



Appendix C.

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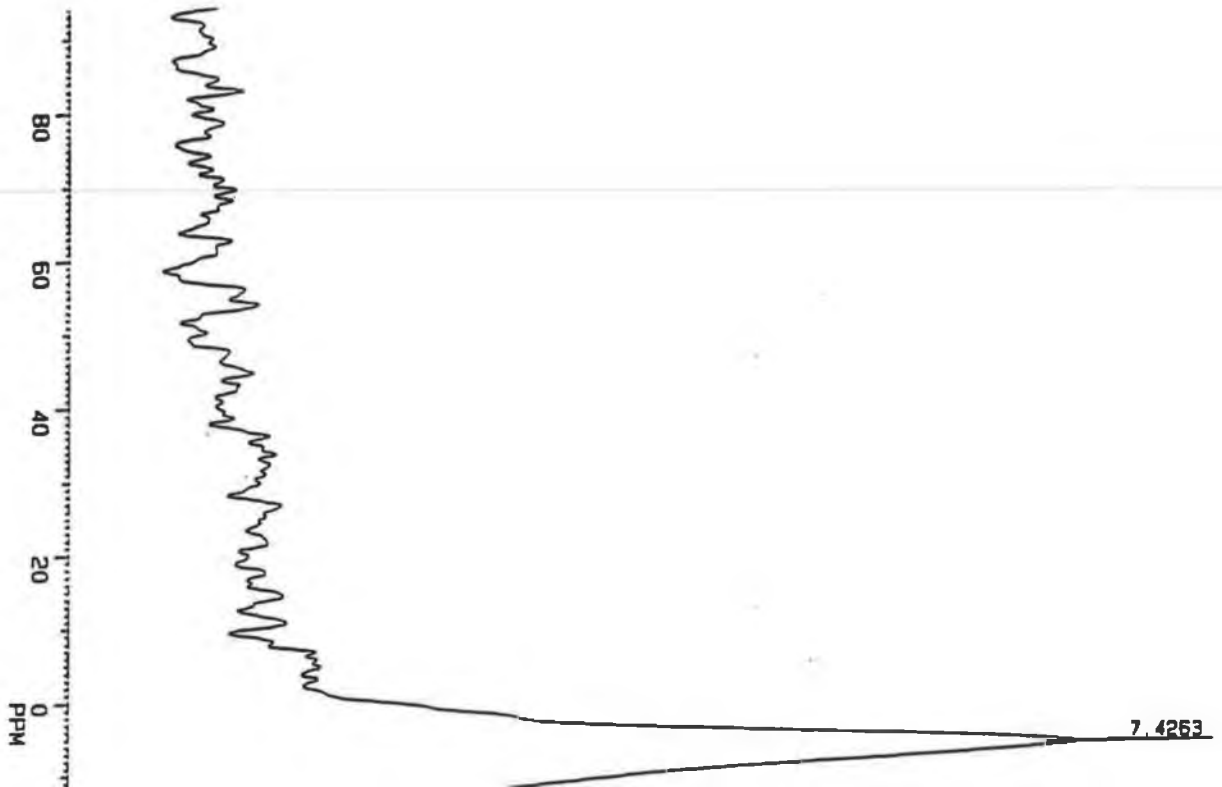

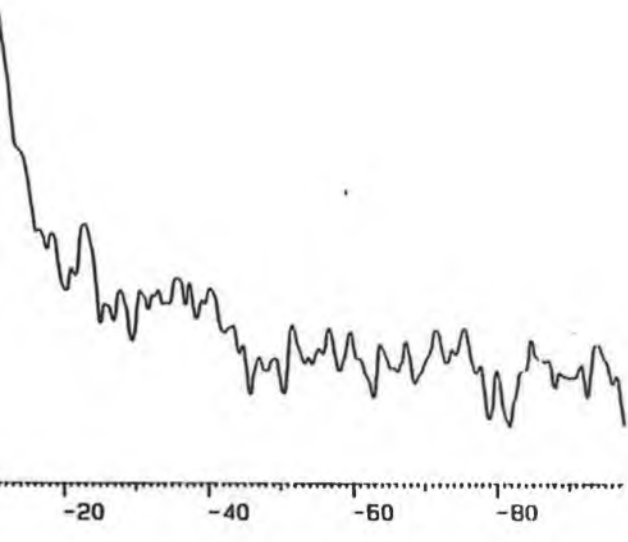

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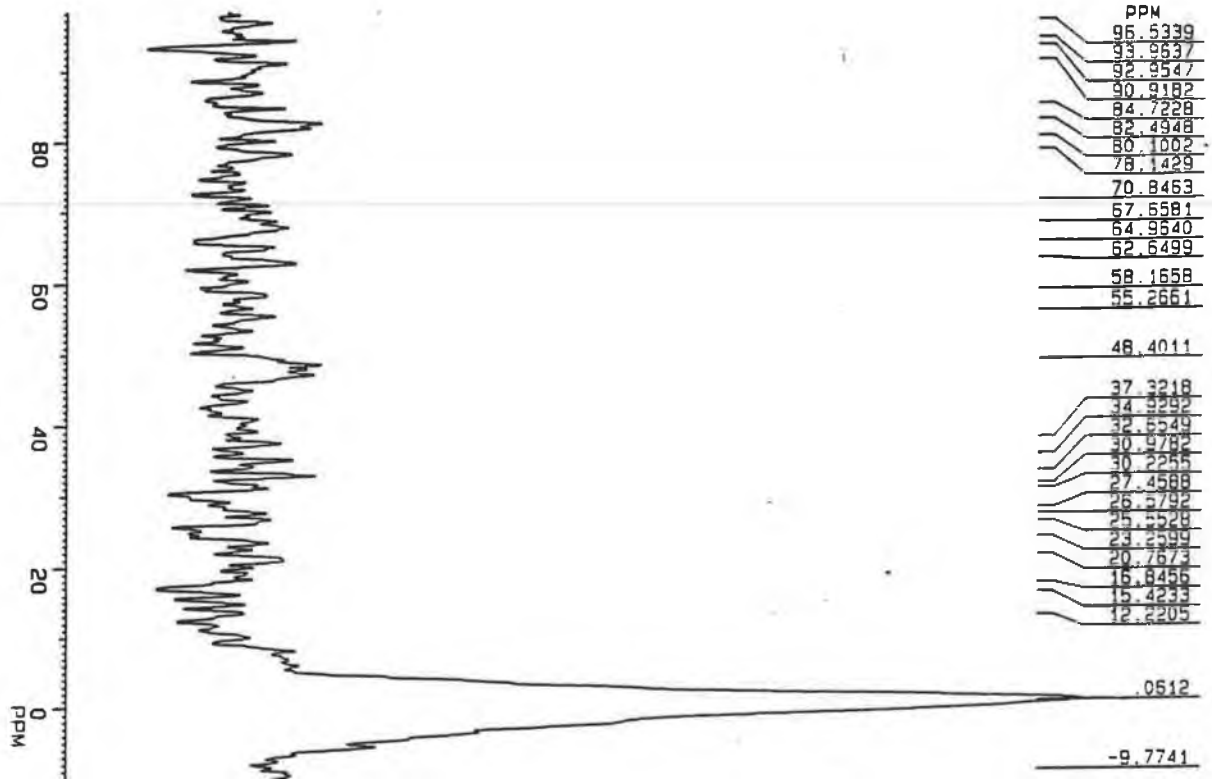
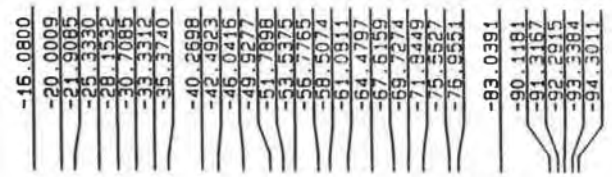
Appendix D.

^{113}Cd NMR spectrum of 4.38 mM Cd : 10 mg/ml Fulvic Acid





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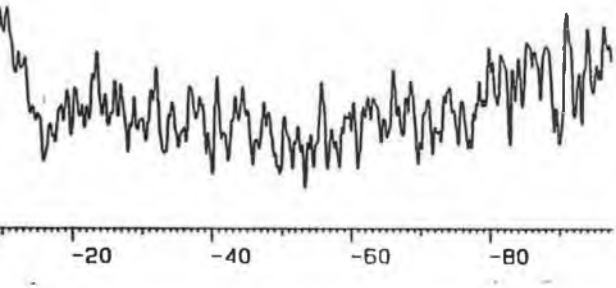
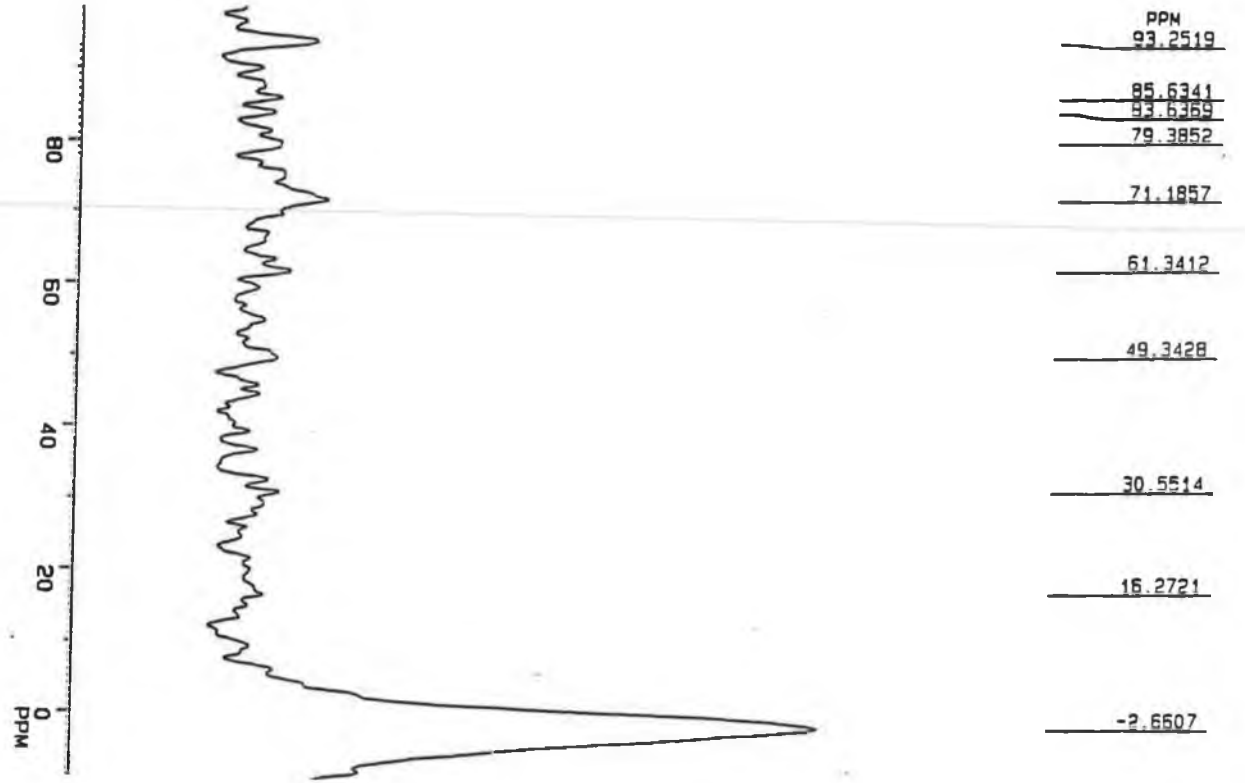


Appendix E.

^{113}Cd NMR spectrum of 4.38 mM Cd : 15 mg/ml Fulvic Acid



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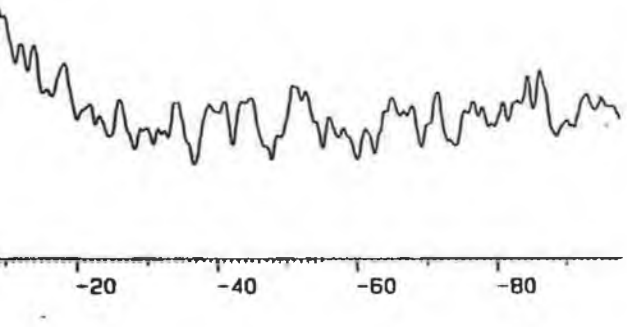

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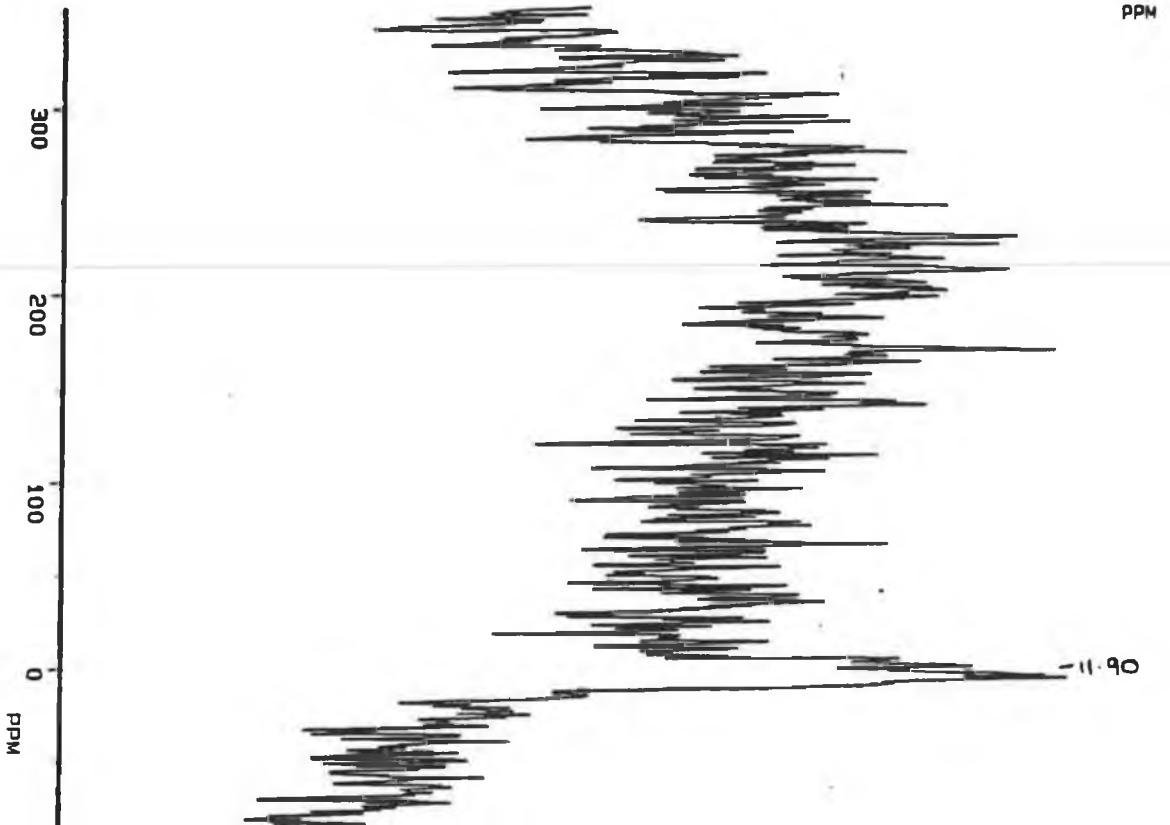
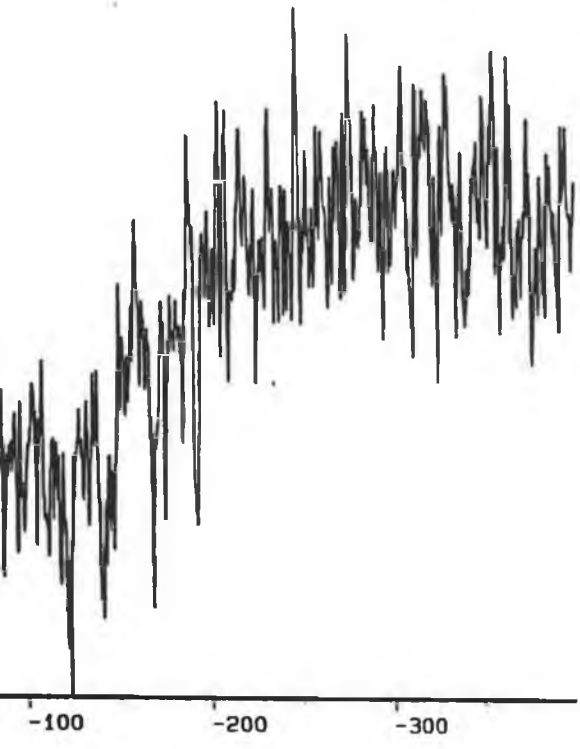
Appendix F.

^{113}Cd NMR spectrum of 4.38 mM Cd : 50 mg/ml Fulvic Acid





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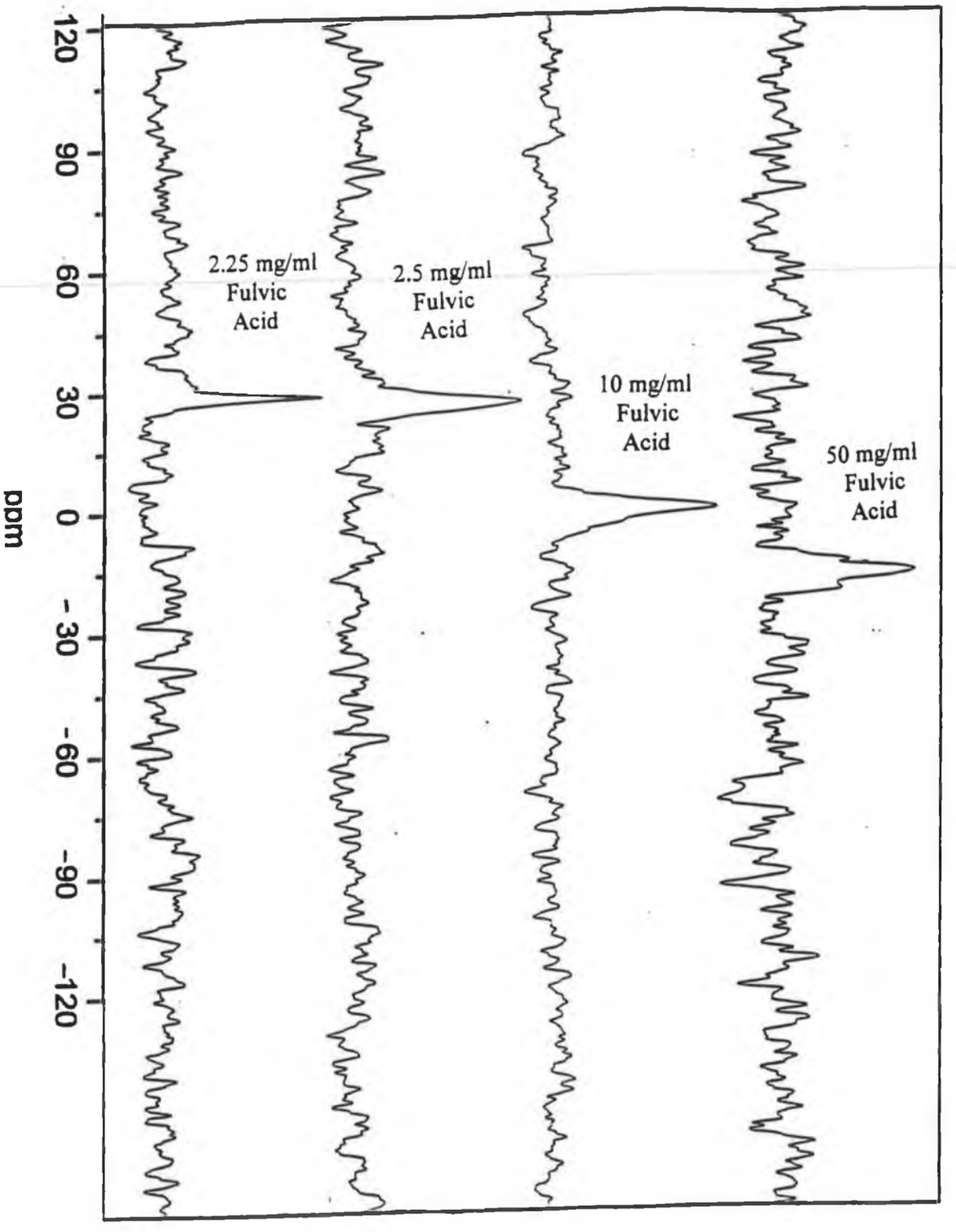
Appendix G.

^{113}Cd NMR chemical shift measurements of Cd-Fulvic Acid complexes



4.38 mM Cd

Chapter 3

Selenium speciation

3.1 Introduction

The speciation of selenium with respect to oxidation state is important in assessing its bioavailability and toxicity. In the environment, inorganic selenium can exist in four different valence states, Se(IV), Se(VI), Se(II) and Se(0). In order to understand the behaviour of selenium in the environment, speciation information is essential. Selenium can be a cumulative toxic substance when found in high concentrations in food and water. At low levels, however, it is recognised as an essential trace element in animal nutrition. For an accurate assessment of the effects of selenium, both on the body and in nature, the speciation and concentration of each species present must be known.

In this chapter matrix solid phase dispersion (MSPD) is investigated as a suitable extraction technique to enable selenium determination in food samples. The procedure described is simple, rapid, inexpensive and is particularly suitable as an initial screening process to confirm the presence of selenium in environmental samples.

An on-line speciation procedure is also described which enables selenium determination in aqueous samples. The inorganic fraction of dissolved selenium in natural waters consists predominantly as selenate (SeO_4^{2-}), designated here as Se(VI), and as selenite (SeO_3^{2-}), Se(IV). Separation of these species is achieved by ion-exchange chromatography with detection by hydride generation atomic absorption spectrometry. Since only tetravalent selenium produces volatile hydrides, the hexavalent form must be reduced prior to detection. A completely on-line procedure is described.

3.1.1 Matrix Solid Phase Dispersion

Matrix solid phase dispersion (MSPD) is an extraction technique that has been largely applied to the isolation of drug residues, environmental contaminants and naturally occurring component molecules from biological materials [1-4, 6, 8]. The extraction is accomplished by blending the sample with a derivatised silica support. The mechanical forces, generated from the grinding of samples with irregular shaped particles (silica or polymer-based solid supports), combined with the lipid solubilizing capacity of a support-bound polymer allows the isolation of dispersed components from the sample matrix [1]. Extraction is attained in a stepwise fashion and is based on the solubility characteristics of the different molecules in the matrix.

This approach has several advantages over classical methods of extraction. The sample is distributed over a large surface area and is therefore much more exposed to the extraction process. Blending of the sample with the packing material is easy to perform and does not require expensive equipment or special devices. MSPD is a chemically and physically mild process. The sample is not exposed to excessive heating, grinding or strong chemical or detergent based reagents as in other methods of extraction [1]. Such physical and chemical harshness not only complicates the isolation procedure but can lead to the destruction of the analyte and/or the generation of sample artifacts. The use of very small volumes of extracting solvents generates less waste and reduces costs. Classical extraction techniques are relatively more time consuming and labour intensive than MSPD. MSPD is a quick, simple and inexpensive method and is particularly suited for initial screening applications.

MSPD techniques have been applied to the extraction of a wide variety of antibiotics from animal tissues. Five sulfonamides have been determined in salmon muscle

tissue [2]. Sulfadiazine (SDZ), sulfamerazine (SMRZ), sulfamethazine (SMTZ), sulfadimethoxine (SDMX) and sulfapyridine (SP) were the compounds studied. These antibiotics are widely used on fish farms to control the spread of fish diseases and so a rapid, efficient method of analysis in fish tissue is essential. C_{18} was used as the packing material and was blended with the spiked muscle tissue until a homogeneous mixture was obtained. Dichloromethane was used for elution. The extracted sulfonamides were analysed by HPLC with photo-diode array detection. Detection limits were 48, 66, 100, 150 and 228 ppb for SP, SMRZ, SDZ, SDMX and SMTZ, respectively. Average recoveries were from 66 to 82%. This technique can also be applied to salmon liver tissue. Extraction from kidney tissue was not successful, however, due to excessive interferences.

The use of matrix modifiers has been reported for the MSPD extraction of oxytetracycline from milk [3]. EDTA and oxalic acid were added to the C_{18} packing material prior to blending. EDTA was used to chelate any inorganic ions and to remove tetracycline from the matrix constituents. Oxalic acid was also used to complex inorganic ions and to lower the pH of the milk, thereby enhancing the extractability of tetracycline into the eluting solvent. The advantage of this method is that unlike classical methods a heating step is not required to convert tetracyclines to their anhydro form [4].

The drug nicarbazin (DNC) has been extracted from chicken liver and muscle tissue [5]. Again C_{18} was the packing material used. Hexane was used to wash any matrix interferences from the sample/sorbent column. Acetonitrile was used for elution. Recoveries of DNC were 95.8 and 83.7% from liver and muscle tissues, respectively. The conventional ethyl acetate technique involves time-consuming homogenization and multiple solvent partition cleanup steps [6]. Comparison

between the two methods gave comparable results when four chicken liver and three muscle tissues were examined.

The isolation of the antiparasitic drug, Ivermectin, from bovine liver using MSPD has also been reported [7]. The overall recovery of the spiked samples was 74.6% and the limit of detection was 1 ppb. Again comparison of this procedure with a classical extraction method [8] yielded comparable results. Levels of 13.0 and 12.8 ppb Ivermactin were determined using MSPD and the traditional extraction method, respectively. This method involved multiple homogenization and centrifugation steps, followed by evaporation of large volumes of solvent and further cleanup by hexane-acetonitrile and (acetonitrile + water)-hexane solvent partition steps.

MSPD has also been applied to the extraction of environmental contaminants in food samples. Lott and Barker [9] have reported the isolation of a wide variety of chlorinated pesticides from oysters (*Crassostrea virginica*) at levels of 31.3-500 ng/g. The pesticides examined included α-BHC, β-BHC, lindane, hepatochlor epoxide, p,p'-DDE, dieldrin, endrin, 4,4'-DDD, endrin aldehyde, p,p'-DDT, endosulfan sulfate and methoxychlor. The fortified oyster samples were blended with 2 g C_{18} and packed into a syringe barrel column that contained 2 g activated Florisil (Mg_2SiO_3). Elution was carried out by gravity flow with 8 ml of acetonitrile-methanol (90:10). Average percentage recoveries were in the range 66-84%. The eluate was analysed by GC/ECD.

The large number of polymer phases bound to solid supports currently available, together with the high degree of flexibility of the elution procedure, ensures that MSPD can be applied to a wide variety of analytical problems. It is a rapid, simple

and inexpensive method and is a very suitable initial screening technique for many naturally occurring and incurred components in complex matrices.

3.1.2 Analytical speciation techniques

Chromatographic separation techniques are widely used for speciation purposes. Liquid chromatography (LC) is generally applied to metal speciation while gas chromatography (GC) is used for the ultra-trace determination of organometallic compounds. The coupling of chromatography to an element-specific detector, such as atomic spectrometry, results in a highly sensitive method for elemental speciation.

3.1.2.1 Gas chromatography

Study of the environmental aspects of organometallic compounds has intensified in recent years. The use of GC for this purpose was recently reviewed by Y.K. Chau [10]. The various columns used, derivatisation techniques required and atomic spectrometric techniques employed were discussed.

Both packed and capillary columns can be used for organometal speciation purposes. The most suitable packed columns have been found to be non-polar or low polarity inert stationary phases such as dimethylsilicone gum (OV-1, OV-101 etc.) coated on an inert support such as Chromosorb W [11]. Improved efficiency and resolution has been found with capillary or megabore open-tubular columns with thin-film coatings such as polymethylsiloxane (DB-1) [12]. To avoid possible catalytic decomposition of organometals on heated metal surfaces, the use of glass columns is preferable. The main advantage of packed columns is that they can be conveniently cleaned by replacing part of the packing materials if fat, oil or organic debris is deposited.

Capillary or megabore columns, on the other hand, have better resolution, with sharper peaks, leading to improved sensitivity.

While many volatile organometallic compounds can be separated directly by GC, other organometallic species, particularly the ionic organometals (e.g. di- and trialkyl metals) will require derivatisation for conversion into volatile forms amenable to GC separation. Hydride formation and alkylation are commonly used [13]. Hydride formation applies only to organometals which readily form covalent hydrides (As, Sb, Bi, Sn, Pb, Se, Te and Ge). Alkylation is the addition of an alkyl group to the mono-, di- and trialkyl metals to form tetraalkyl-substituted metals, which are more volatile than their parent compounds. New techniques for derivatisation have been reported including on-column hydride formation and on-column ethylation [14,15].

GC is an efficient separation method for the speciation of naturally volatile organometals or for those species that can be readily converted into volatile derivatives. It is not suited to the determination of inorganic species, however. Off-line derivatisation would have to be initially carried out, followed by extraction into an organic solvent, before analysis could take place [16]. The advantage of GC is that it can be easily interfaced to any mode of atomic spectrometric detector because the GC effluent containing the analytes is in a gaseous state. The effluent can be directly transported to either AAS or AES in flame or furnace modes without serious compatibility problems [17].

3.1.2.2. Liquid chromatography

The advantages of LC over GC for metal speciation purposes are discussed in a recent review by Chau [10]. With LC little sample preparation is required since there

is no need to isolate the analytes from the sample matrix. High boiling compounds can be separated without the need for derivatisation. LC has more variable operational parameters. Both the stationary and mobile phases can be varied simultaneously to achieve better separation. LC can also be operated at ambient temperatures and is, therefore, more suitable for environmental studies. The main limitation is that LC is relatively more difficult to interface with atomic spectrometric detectors. Since a continuous flow of liquid eluate is delivered at a certain flow-rate, the atomic spectrometric detector must be compatible with the liquid flow.

Continuously operating atomic spectrometric detectors such as AAS or AES in the flame mode are more adaptable to receiving liquid flows [18]. Using a HPLC pump the eluant flow-rate can be matched with the operating requirements of the detector. There are inherent difficulties in interfacing HPLC to furnace AAS, however, due to the discontinuous nature of sample introduction. Discrete sampling procedures are usually employed [19-22]. These designs are indirect interfaces and generate pulse signals, the sum of which is used to quantify a chromatographic peak. In the case of a very narrow component peak, the intermittent sampling may miss part or all of a component. The disadvantage of these systems, therefore, is that they do not provide continuous, real-time analysis.

3.1.2.3 Atomic Spectrometry

Atomic spectrometry is the most commonly used mode of detection in speciation studies. This is because it is an element specific technique and is thus highly selective. The many forms of atomic spectrometry include flame AAS (FAAS), electrothermal AAS (ETAAS), graphite furnace AAS (GFAAS), inductively coupled

plasma (ICP) with atomic emission (ICP/AES) or mass spectrometric (ICP/MS) detection.

3.1.2.3.1 Atomic Absorption Spectrometry

Atomic absorption spectrometry (AAS) can be subdivided based on the atomisation cell used. Externally heated quartz tubes and graphite furnaces are most commonly used. Quartz tubes can be externally heated either by a flame (FAAS) or electrothermally (ETAAS). Electrical heating is carried out either by means of a resistance wire wound around the tube or by a tailored furnace. This has the advantage that the temperature can be easily controlled to provide the optimum temperature for the analysis and the tube is heated uniformly. Flame heating, on the other hand, usually means that the top of the tube is cooler than the directly heated underside.

Graphite furnaces can also be used for atomisation purposes. These consist of a hollow graphite cylinder placed horizontally with the light path passing through longitudinally. The interior of the cylinder is coated with pyrolytic graphite. Electrodes at the end of the cylinder electrically heat the cylinder walls. Samples are inserted through an orifice in the top centre of the cylinder. The main advantages of this type of atomisation are high sensitivity and the ability to analyse small sample volumes. Matrix effects can be more severe, however, and precision tends to be lower. As discussed previously, GF-AAS is usually more difficult to interface to a chromatographic technique, since it requires discrete sample volumes for analysis. Externally heated quartz tubes can be coupled to a continuous flow system which makes interfacing easier for speciation studies.

3.1.2.3.2 Inductively Coupled Plasma

Inductively coupled plasma (ICP) is a relatively recent development with commercial systems only becoming available in 1974. In contrast with AAS, ICP is capable of simultaneous multi-element determination. For metal analysis, ICP is usually coupled either to a mass spectrometer (ICP/MS) or an atomic emission spectrometer (ICP/AES). Inductively coupled plasma provides a uniquely effective excitation source for emission spectrometry. This is because plasma jets generate higher energy of excitation, resulting in better sensitivity than that previously obtained using flames or arcs. While sensitivities are comparable, one aspect of atomic emission surpasses atomic absorption , and that is its multi-element analysis capability. ICP/MS is also a powerful technique for trace element determination due to the two-fold identification built into the system. Despite their potential as powerful metal speciation techniques, the high cost of the instrumentation renders ICP systems unsuitable for routine analyses.

3.1.3 Selenium determination in environmental samples

3.1.3.1 Hydride generation

Hydride generation, coupled to atomic spectrometry, is commonly used for selenium determination. This technique is based on the formation of volatile hydrides following reaction with sodium borohydride in the presence of acid. It is widely used for the determination of many elements including selenium, antimony, arsenic, bismuth, germanium, lead, tellurium and tin. Hydride generation is highly selective since only certain elements have the ability to form volatile hydrides. Therefore, it acts not only as a very useful sample introduction method, but also separates the

analyte of interest from other potential interfering components in the sample, resulting in preconcentration.

Hydride generation was first described by Holak in 1969 for the determination of arsenic [23]. A batch generator was used to convert arsenic to arsine. This was adsorbed in a liquid nitrogen trap, from which it was swept into the flame by nitrogen gas and detected by AAS. Introduction of the analyte into the flame in the gaseous state was much more efficient than conventional nebulisation techniques. The sensitivity was also greatly improved due to the fact that the arsine entered the flame as a narrow plug of sample. Since then the technique of hydride generation has become widespread. Batch generators have largely been replaced by flow injection systems. In flow injection hydride generation AAS the acid and reducing solution flow continuously at a constant rate to the gas/liquid separator and a small volume of sample is injected into the acid stream. A transient signal is produced. The advantages of this system are low sample and reagent consumption, high sample throughput and increased sensitivity, since an excess of sample is not required to produce a transient signal. A flow injection system is also more suitable for automation.

3.1.3.1.1 Hydride generation AAS

A recent paper by Chan and Sadana describes the use of an automated flow injection hydride generation AAS system for the determination of selenium in samples such as soil, vegetation, waters, sediments and industrial wastes [24]. An electrically heated atomiser was used. A quartz tube, 10cm x 1cm i.d., with an inlet tube fused into the centre, was enclosed in a 22-guage Chromel wire and insulated with Thermofab (an asbestos substitute) string. The temperature of the tube was controlled by a variable

transformer. The optimum temperature was found to be 850°C. A large sample loop was incorporated into the system in order to maximise the sensitivity, although the volume of this loop is not stated in the paper. The limit of detection, defined as three times the standard deviation for a sample containing a low concentration of analyte, was calculated as 0.3 ng ml^{-1} for Se in solution and 75 ng g^{-1} in solid sample. This is based on total selenium, however, and not on the concentration of the individual species.

A similar technique was used by Yamamoto et al. involving flow-injection analysis, this time combined with a gas segmentation method [25]. An electrically heated quartz cell unit was used for atomisation. Total selenium was determined in several standard reference materials obtained from the National Bureau of Standards. The matrices analysed included low-alloy steel, coal fly ash, wheat flour rice flour and orchard leaves. The analysis of the materials was in good agreement with certified values. A detection limit of 0.3 ng was obtained for selenium. The relative standard deviation, estimated from ten replicate measurements of synthetic samples, was 0.8%. The use of gas segmentation enabled an increase in the sampling rate and allowed the analysis of 120 samples per hour. However only total selenium content was determined.

A slightly modified FIA-HG-AAS system has been developed by Narasaki and Ikeda [26]. They have described it as a "flow-injection batch system". The hydride gas is stored in a gas-liquid separator up to an appropriate pressure and then swept automatically into an atomic absorption furnace. The aim is to improve the sensitivity of the technique, however, the limit of detection obtained was still only 0.4 ppb for total Se with a relative standard deviation of 5% for duplicate measurements of one sample solution. No species information was obtained.

The analysis of inorganic selenium species, dissolved dimethylselenonium (DMSe) and methylated organoselenium compounds has been achieved using a continuous flow method [27]. The technique involved hydride generation, cryogenic condensation, separation based on boiling points and quantitation by AAS. The advantage of this technique is that a single apparatus can be used for the determination of both inorganic and organic selenium compounds. However the experimental set-up involved is quite complex. The procedure is as follows. DMSe was purged out of a solution and trapped on a U-tube immersed in liquid nitrogen. After controlled heating of the sample trap, DMSe was quantified in a quartz flame-in-tube atomiser aligned in the optical path of an AAS. Selenite (Se(IV)) and oxidised methylated Se compounds were reduced by $NaBH_4$ in 4M HCl to H_2Se and DMSe, respectively. Entrained by a helium carrier gas, the H_2Se and DMSe were trapped in a liquid nitrogen cooled U-tube. Their separation was accomplished by controlled heating of the sample trap. Since only Se(IV) is capable of hydride formation, the other selenium species present must be estimated by difference. The detection limit was found to be 5 ng of Se. A relative standard deviation of 4.8% was obtained for six absorbance measurements at the 50 ng level. Triplicate analyses (as total Se) of two EPA water pollution quality control reference samples for Se yielded values of 12 +/- 2.1 and 48.6 +/- 4.2 μgl^{-1} compared with true values of 10.9 and 50.2 μgl^{-1}, respectively. Although useful as a preconcentration technique, the limitations of cryogenic condensation are prolonged analysis time and poor recoveries of some elements after the trapping step.

3.1.3.1.2 Hydride generation ICP

ICP techniques have also been applied to selenium determination in environmental samples. Studies carried out using both ICP-MS and ICP-AES have shown that

sample introduction via hydride generation produces a substantial gain in sensitivity [28]. A detection limit of 0.02 $ngml^{-1}$ as total selenium has been reported by HG-ICP-MS, compared with 6.0 $ngml^{-1}$ via conventional nebulisation. ICP-AES was less sensitive, having detection limits of 0.8$ngml^{-1}$ and 70 $ngml^{-1}$ with hydride generation and conventional nebulisation, respectively. Both of these techniques were more sensitive than HG-AAS which had a detection limit of 1 $ngml^{-1}$ for selenium.

HG-ICP-AES has been used for the simultaneous determination of arsenic, antimony, bismuth, selenium and tellurium in aqueous solutions [29]. Hydrides were generated by continuous mixing of sample and reagent solutions and the analytical signals, when stabilised ,were integrated over a fixed period. Detection limits of 1 $ngml^{-1}$ or below were reported for each of the elements studied The limitations of this technique are that a continuous flow system was not used, a high plasma power was required (2.7 kW) and a separate process was required for the determination of selenium in sediments.

Goulden et al. [30], on the other hand, described the simultaneous determination of As and Se in water, fish and sediments using conventional continuous flow equipment and a plasma power of only 1.4 kW. Using a preconcentration step, detection limits of 0.03 μgl^{-1} for Se and 0.02 μgl^{-1} for As were obtained in water samples. Determination of As and Se in reference soil and sediment samples gave results that were in close agreement with certified values (i.e. 0.1-0.4 μgg^{-1} for Se and 1.9-7.1 μgg^{-1} for As). Interference studies have also been carried out for HG-ICP-AES [31]. Again the elements determined were As, Sb, Bi, Se and Te. Two types of possible interferences were investigated. Mutual interferences between the analytes was found to be negligible. The effect of other cations at specific concentrations (Cu, Fe and Pb) was found to lower the recoveries of some of the

analytes, especially Se and Te. This was overcome by prior separation of the analytes by co-precipitation on lanthanum hydroxide.

Schramel modified conventional HG-ICP/AES instrumentation by eliminating the gas-liquid separator from the system [32]. The advantage of this modification is that normal solution nebulisation can be easily converted to hydride generation without interrupting the plasma. As, Se, Sb, Bi and Sn were determined simultaneously in environmental samples. A detection limit of 0.5 $ngml^{-1}$ was obtained for selenium, using a plasma power of only 1.5 kW, compared with the detection limit of 0.8 $ngml^{-1}$ obtained by Thompson et al. [29], using a much higher power.

ICP/MS is a powerful technique for trace element determination due to the two-fold identification built into the system. Its main limitation is the high cost of the instrumentation which often makes it unsuitable for routine analyses. In addition to this, selenium is one of the more difficult elements to determine by ICP/MS because of isobaric interferences on several of its most abundant isotopes by polyatomic ions. Selenium also has a very high ionisation potential (9.75 eV). The use of the major isotopes ^{80}Se, ^{78}Se and ^{76}Se (isotopic abundances 49.82, 23.52 and 9.02%,respectively) is limited by the presence of argon dimers, $^{40}Ar_2$, $^{40}Ar^{38}Ar$ and $^{40}Ar^{36}Ar$. The resolution required to separate these ions from the corresponding selenium isotopes (i.e. 9690, 9970 and 7080, respectively) is unavailable on quadrupole ICP/MS instruments. The minor isotopes ^{74}Se, ^{77}Se and ^{82}Se (isotopic abundances 0.87, 7.58 and 9.19%, respectively) are normally used. In addition, the degree of ionisation of selenium in an argon plasma is calculated to be only 33% [33]. The use of mixed-gas plasmas [34,35] and the direct removal of polyatomic ions from the matrix [36] have both been suggested as ways to reduce polyatomic interferences.

Laborda et al.[34] reported a significant reduction in interference by adding nitrogen (8%) to the aerosol carrier flow. The addition of nitrogen is thought to reduce the temperature of the gas in the axial channel because some of the energy is used to dissociate the diatomic injector gas. This temperature drop could be responsible for both the reduction of the ionised polyatomic species which require more energy to ionise than atomic species as well as for the reported reductions in analyte sensitivities when N_2 is introduced in the aerosol carrier gas. Multi-element determinations in five reference materials were carried out and detection limits for elements along the mass range (from Li to Cl) were found to be , on average, 2-3 times higher with the mixed-gas plasma.

Platzner et al.[35] assessed the addition of trifluoromethane (CHF_3) to the aerosol carrier gas. The analyte response was significantly increased for all of the analytes examined, (i.e. As, Se, Cu and Zn), with a coincident decrease in the blank signal. This reduction of interference was attributed to competitive reactions between the matrix species and the CHF_3 or species derived from it in the plasma. An improved detection limit of 0.032 $ngml^{-1}$ for Se at m/z 78 was obtained. Previously the limit of detection for the determination of selenium by ICP/MS was 0.88 $ngml^{-1}$.

Matrix elimination has also been suggested for the removal of interferences due to polyatomic ions [36]. An activated alumina (acidic form) packed microcolumn was used to separate and preconcentrate the analytes of interest from interfering species present in the matrix, which are flushed to waste by the use of an appropriate buffer and switching valves in a typical flow-injection manifold. Analysis of certified reference materials for As, Cr, Se and V yielded results in good agreement with the certified values. Close to 100% recovery was obtained for all analytes. The limit of

detection for selenium, using a 200 µl loop was 65 $ngml^{-1}$. Using a preconcentration system, this was reduced to 1.0 $ngml^{-1}$ at m/z 78.

ICP/MS, therefore, is a highly sensitive multi-element detection method. However, the determination of selenium in particular is subject to many interferences. Currently research is being carried out in an effort to reduce the effects of these interferences. Despite its potential as an extremely sensitive metal speciation technique, ICP/MS remains a sophisticated and expensive system which only a few privileged laboratories could acquire.

3.1.3.1.3 Inteferences

A number of interferences have been identified which can affect selenium determination by hydride generation. Transition metals and other hydride-forming elements, such as Co, Cu, Sn, Sb and Ni, can cause some signal depression. Of these, Cu is the only one which produces a significant influence on the selenium signal [26]. This signal loss has been attributed to the formation of stable complexes in the gas-liquid reaction [37]. Agterdenbos et al. [38] have suggested that transition elements also interfere by decomposing the $NaBH_4$ before the reaction is complete. The addition of iodide to the sample was proposed since it catalyses SeH_2 formation even in the presence of interfering metal ions.

Nitrite has also been found to interfere significantly with selenium determination [39]. This interference has been attributed to the reaction of nitrite and the generated hydride. Nitrate also interferes indirectly since it is reduced to nitrite during the reduction of Se(VI) to Se(IV). Sulphanilamide can be used to eliminate these interferences.

Organic substances present in the sample matrix suppress the selenium signal due to the formation of an adduct involving the organic component and SeH_2. Alternatively a strong interaction between Se and the organic substituents may actually inhibit SeH_2 formation [37].

3.1.3.2 Hyphenated techniques

3.1.3.2.1 Gas chromatography / Atomic detection

As discussed previously, GC is widely used for speciation studies. The speciation of dimethylselenide (DMSe) and dimethyldiselenide (DMDSe) has been described at nanogram levels [40]. These volatile selenium compounds are produced from inorganic selenide salts by fungi, plants and animals and are widely present in the atmosphere. The GC served to separate the organo-selenium compounds with a silica-furnace AAS system used for detection. A sample trap was used to adsorb the volatile analytes. This consisted of a glass U-tube (6mm x 26cm) packed with 3% OV-1 on Chromosorb W at -80°C. Desorption was achieved by heating the trap to 100°C and sweeping the adsorbents into the separation column. A precombustion tube was used to "burn-off" any organic solvents which would interfere with selenium determination. The sample trap was found to give good recoveries of 95-98%. The accuracy of the method was determined by duplicate analyses of a synthetic air sample (250ml) containing 10 ng and 16 ng (as Se), respectively of dimethylselenide and dimethyldiselenide. The results were 10 +/- 0.5 and 16 +/- 0.7 ng, respectively, for these two compounds. The precision was also determined by duplicate analyses of a synthetic air sample spiked with 10 ng and 16 ng, as Se, of DMSe and DMDSe, respectively. The relative standard deviation was 8% and 7%,

respectively. It is proposed that 0.1 ng of Se could be detected with certainty by this method.

Total selenium and dissolved selenite species have been determined in sea water samples by GC using an electron capture detector [41]. The level of selenate present in the samples was calculated by difference. The experimental procedure involved some initial sample preparation. The sample solution was reacted with 4-nitro-o-phenylene-diamine to form 5-nitropiazoselenol which was extracted into toluene. Photo-oxidation of the samples under controlled pH conditions was used to release any organically-bound selenium. A detection limit of 10×10^{-12} M of selenium (100 ml sample) was obtained and precision was estimated to be +/- 2.4% at a concentration of 394×10^{-12} M. The limitations of this technique are the time-consuming sample preparation procedure and the indirect selenate determination. ECD detection is not an element-specific detection technique and lacks selectivity.

3.1.3.2.2 Liquid chromatography / Atomic detection

LC is widely used for metal speciation studies. Selenium speciation has been carried out using paired-ion reversed phase HPLC with DCP detection [42]. A C_{18} column was used (Bondapak C_{18}, 10μm, 15cm x 3.9mm i.d.). The mobile phase was 2.5 mM TBAHS (tetrabutylammonium hydrogen sulphate) with 0.01 M of dipotassium hydrogen phosphate and potassium dihydrogen phosphate at pH 6.55. The HPLC pump was interfaced directly to the DCP system via a short, flexible Tefzel connector. This allowed continuous eluant introduction into the DCP spray chamber. Animal feed samples were analysed for their selenite and selenate content. Detection limits of 0.5 ppm was reported for each species. The incorporation of a post-column hydride formation step would improve the sensitivity of this technique.

Ion-exchange chromatography (IEC) is widely used for metal speciation. Laborda et al. used a nucleosil 100-SB anionic exchange column (250mm x 4.1mm, 10μm particle size) for the separation of trimethylselenonium ($TMSe^{+}$), selenite (Se(IV)) and selenate (Se(VI)) [43]. Detection was by ET-AAS, using a sampling procedure based on fraction collection and hot injection into a graphite furnace. $TMSe^{+}$ was eluted using ammonium citrate (0.01 M, 2 ml/min) at pH 3. The pH of the eluant was then altered to pH 7 using a linear gradient of 1 min. Fractions of 1ml were collected, 10μl of which was injected into the furnace. A matrix modifier composed of Ni and $Mg(NO_3)_2$, (10μl), was also used to eliminate interference from the eluant. For a 100μl HPLC injection, containing 200 ng of each selenium species, the detection limits obtained were 85.3 μgl^{-1}, 96.3 μgl^{-1}, and 79.4 μgl^{-1} for $TMSe^{+}$, Se(IV) and Se(VI), respectively. Separation of the three compounds was also carried out in water and urine matrices, containing 16 ng Se each. A human urine sample, with a total selenium content of 58.5 μgml^{-1}, was also analysed. To enable determination at these levels the experimental conditions were altered . Fractions of 0.5 ml were collected as opposed to the 1 ml fractions collected previously. The volume of the aliquot injected into the furnace was increased to 50 μl. For the water sample, detection limits of 16.7 μgl^{-1}, 12.7 μgl^{-1} and 7.6 μgl^{-1} were obtained for $TMSe^{+}$, Se(IV), and Se(VI) respectively. Se(IV) was not eluted from the urine matrix but $TMSe^{+}$ and Se(VI) were detected at levels of 10 and 7.3 μgl^{-1}. Only $TMSe^{+}$ was detected in the human urine sample, however, the levels are not reported. The low recoveries obtained in this study appear to be due to the discontinuous nature of the monitoring process. This study emphasises the drawbacks of coupling HPLC with ETAAS. The use of an on-line system, rather than one based on fraction collection, would be likely to provide even lower detection limits.

The use HPLC coupled to an on-line hydride generation ICP-AES system has been compared with off-line detection by ETAAS [44]. Inorganic selenium (selenate and selenite) and a number of selenoaminoacids (selenocystine, selenomethionine and selenoethionine) were determined. A vesicle-mediated HPLC technique was used. The cationic surfactant didodecyldimethylammonium bromide (DDAB) was used both to modify the C_{18} stationary phase and as a mobile phase component. A combination of hydrophobic and electrostatic interactions were responsible for separation of the species. Gradient elution was carried out using 0.01 M sodium acetate at pH 5 (10^{-5} M DDAB) and 0.2 M sodium acetate at pH 7.5 (10^{-5} M DDAB). The selenocompounds were detected on-line using a continuous hydride generation ICP-AES system with focused microwave reduction. A limit of detection of 1 µg/ml Se was reported. The corresponding detection limit by the HPLC-ETAAS method was 5 µg/ml Se. In this case fractions of 250 µl were collected manually from the chromatographic column every 15 seconds. The on-line system was not successful when applied to the analysis of selenium in urine samples, however, due to the high ionic strength of the matrix. The inorganic selenium species could not be sufficiently resolved from the selenoaminocids.

Frankenberger et al. described the use of single column ion chromatography (SCIC) with conductometric detection for the determination of selenite and selenate [45-47]. Initial studies investigated the determination of selenite (SeO_3^{2-}) in aqueous soil extracts [45]. Due to its availability for plant uptake, mobility in soil and toxicity to the biota, routine monitoring of selenium in soil is essential. Selenite was determined in the presence of phosphate, chloride, nitrate and nitrite anions. Separation was carried out on a low capacity anion-exchange column (Vydac 302IC, 4.6 x 250mm), preceded by an ion guard column (Wescan 269-003) which served as a concentrator column. Conductometric detection was used for quantification. The optimum mobile

phase was found to be 1.5 mM phthalic acid, adjusted to pH 2.7 using formic acid. Chloride ions were found to interfere and were removed by reaction with a silver saturated cation-exchange resin. The detection limit for SeO_3^{2-} was 3 μgl^{-1}. The relative standard deviation, using a 500 µl loop was 2% with standards (3 mgl^{-1}) and 6.7% in soil extracts (0.5 mgl^{-1}). Further work [46] involved the determination of selenate (SeO_4^{2-}). This was achieved in the presence of high background levels of other ions including chloride, nitrite, nitrate and sulphate. Separation and quantification were carried out as before, this time using a mobile phase of 4 mM phthalic acid, adjusted to pH 4.6 with sodium borate. Sulphate was found to be an interferent. However a quantitative analysis of selenate was possible with background levels of up to 40 μgl^{-1} of SO_4^{2-}. The detection limit was 18 μgl^{-1} for SeO_4^{2-} with a relative standard deviation of 1.46%.

More recently, the simultaneous determination of selenite and selenate by this method has been reported [47]. A low capacity, resin-based anion-exchange column was used (Wescan 260-029, 250mm x 4.1mm). Conductometric detection was employed. The eluant used was PHBA (4mM) at pH 8. Previous investigations reported interferences on selenite and selenate quantification by chloride and sulphate anions, respectively. In this study, Se(IV) and Se(VI) were clearly resolved in the presence of interfering anions. It was found that 55 μgml^{-1} of Cl^-, 32 μgml^{-1} of NO^{3-} and 68 μgml^{-1} of SO_4^{2-} did not cause any interference in the determination of the two selenium species. The detection limits for a 2ml sample injection were 60 μgl^{-1} and 110 μgl^{-1} for Se(VI) and Se(IV), respectively. The relative standard deviation, using a 500 µl loop, was 0.90 to 1.86% for Se(VI) and Se(IV). This method was applied to the analysis of seleniferous soil samples. The analytical recovery for Se(IV) was examined with known amounts of spiked analyte and was found to range from 91 to 97%.

The separation of selenate and selenite by HPLC has been coupled to hydride-generation atomic fluorescence detection [48]. An anion-exchange column was used (BAX-10, 50 mm x 4.6 mm i.d.). Gradient elution was employed with 25 mM K_2SO_4 at pH 5 (2 ml/min), switching to 100 mM K_2SO_4 (pH 5). Reduction of Se(VI) to Se(IV) was carried out by mixing the eluate with 7 M HCl (8 ml/min) and pumping through a heating coil inside a microwave unit. The heated solution then passed into a reaction coil where it was mixed with 1% $NaBH_4$ in 0.1 M NaOH (4 ml/min). A gas-liquid separator was used to enable detection of SeH_2 by AFS (atomic fluorescence spectrometry). Detection limits of 0.2 and 0.3 $ngml^{-1}$ were obtained for Se(IV) and Se(VI), respectively, with relative standard deviations of 1.5 and 2%, based on five replicate injections. This technique represents an improvement on a previously reported procedure where total Se and Se(IV) were determined by on-line microwave reduction followed by hydride generation-quartz furnace atomic absorption spectrometry [49]. In this case Se(VI) was determined by difference. Limits of detection were not reported but the procedure was validated by the analysis of a certified reference material, NIST 1643c Trace elements in Water. The selenium concentration obtained (12.3 μgl^{-1}) was in good agreement with the certified value of 12.7 ± 0.7 μgl^{-1}.

The separation of Se(IV) and Se(VI) prior to detection has led to a significant improvement in speciation techniques. Both HPLC and GC have been applied for this purpose. HPLC is more suited to direct interfacing with most modes of detection due to its flowing streams and anion-exchange columns in particular have been shown to be very suitable for selenium speciation.

3.1.3.3 Electrochemical techniques

The most common electrochemical methods of selenium speciation in natural waters are differential pulse polarography (DPP) and differential pulse cathodic stripping voltammetry (DPCSV). Both are based on the electrochemical determination of Se(IV) which can be reduced at a mercury electrode in two discrete steps

$$Se^{4+} + Hg + 4e^- \quad ===> \quad HgSe$$

This reaction is irreversible. However the selenide layer (HgSe) can be accumulated at the electrode surface and stripped cathodically as follows

$$HgSe + 2H^+ + 2e^- \quad <===> \quad Hg + H_2Se$$

DPP has been reported for the determination of Se(IV) in HCl and $HClO_4$ media [50]. A detection limit of 10 μgl^{-1} was obtained. Deposition for 2-12 minutes prior to cathodic stripping lowered the detection limits to 0.1-2 μgl^{-1}. The addition of EDTA eliminated interference from Pb(II). Se(IV) has also been determined by DPP as 4-chloro-o-phenylenediamine piazselenol [51]. Pretreatment of the sample was carried out on a Chelex-100 resin column (Bio Rad) to remove inteferences from Cr, Cu, Mo, Ni, Zn, Te and V. A detection limit of 0.4 μgl^{-1} was obtained. This method was applied to fresh, estuarine and sea waters.

Chelex-100 resin (Bio Rad), in the iron (III) form, has also been used to preconcentrate selenium from river water [52]. Microwave reduction was carried out to convert all the selenium in the sample to Se(IV) and Se(VI) was determined by difference. Detection was by DPCSV. The reduction procedure was carried out by

the addition of 7.4 ml of concentrated HCl to 10 ml of sample to give a resulting HCl concentration of 4 M. The limit of quantitation for the DPCSV procedure was 0.1 μgl^{-1}. A relative standard deviation of 3% at 0.4 μgl^{-1} was reported. The preconcentration procedure was found to be quantitative down to ngl^{-1} levels. Recoveries of 99% and 98% were obtained at 20 μgl^{-1} and 500 μgl^{-1}, respectively. This procedure was applied to river water samples taken from the Tevere River. Total dissolved selenium was determined as 117 ngl^{-1} and 25 ngl^{-1} in the samples analysed. In both cases practically all the soluble selenium was found to be Se(IV) (115 ngl^{-1} and 22 ngl^{-1}, respectively).

Cathodic stripping voltammetry has been used for the analysis of Se(IV) and total dissolved selenium in sea water samples [53]. A deposition time of 15 minutes was reported and $Cu(I)_2Se$ was preconcentrated on a hanging mercury drop electrode. The presence of copper resulted in a very linear response and a detection limit of 0.01mM Se. UV irradation of the samples was carried out at pH 8 prior to analysis to convert all selenium species to Se(IV). This also eliminated interferences from natural organic surfactants and electroactive organic compounds.

Holak et al. recently described the determination of selenium in food supplements, also by cathodic stripping voltammetry (CSV) [54]. The samples were in 1 M HCl and copper was added to the solution. It was found that the deposition of Se(IV) as HgSeCu increased the sensitivity of the technique. After a deposition time of 1 minute, 0.2 $ngml^{-1}$ of selenium could be detected. The method was applied to the analysis of food supplements. Digestion of the samples was carried out using

HNO_3 - $HClO_4$ and all species were converted to Se(IV) by heating with HCl. For the selenium tablets a limit of detection of 1 $\mu g g^{-1}$ selenium, as selenomethionine, was obtained. A deposition time of 300 seconds was used.

A focused microwave system was used for the reduction of selenate to selenite in spiked milk and sausage samples as well as a certified reference material (lyophilised pig kidney) [55]. A flow injection manifold was used and detection was by DPCSV. Digestion of the samples was also carried out with the microwave system using HNO_3 and H_2O_2. The on-line reduction was carried out using a teflon coil (1 metre) placed in the microwave chamber. A stream of sample in 6 M HCl was passed through the coil in which reduction of Se(VI) to Se(IV) took place. The stream was then diluted five-fold prior to loading the loop of the injection valve and determination of total selenium. The linear range for Se(IV) was between 0.5 and 30 $\mu g l^{-1}$ ($r = 0.999$). The method is less sensitive for Se(VI) with a linear range of 3 - 120 $\mu g l^{-1}$ ($r = 0.994$) being reported. The limiting factor for this process is the five-fold dilution of the sample required to reduce the acid matrix form 6 M to 1.2 M prior to detection. In addition no separation of the species was carried out prior to detection and Se(VI) was determined as the difference between Se(IV) and total selenium.

3.1.3.4 Capillary Electrophoresis

Capillary electrophoresis (CE) has been applied to the separation of selenium compounds [56, 57]. Although CE is a fast, efficient technique it is not as sensitive as other methods such as HPLC or GC. This is largely due to the smaller sample

volumes used (nanolitres compared with microlitres). The determination of selenium in tap water has been reported using CE with preconcentration using the field-amplified injection technique [56]. The samples were initially stacked at a polarity of 10 kV. After preconcentration, separation was carried out at 20 kV on a fused silica column (50cm x 50μm i.d., 30cm length) using sodium tetraborate (25 mM)/sodium dihydrogen phosphate (75 mM) as the electrophoretic buffer solution. Detection was by UV at 195 nm. A limit of detection of 25 ppb was reported for selenate and selenite in tap water samples. It was proposed that this method would be suited to the analysis of fresh water, rain water and groundwater samples due to the fact that these samples have relatively weak matrices and low conductivities.

A detection limit of 10 μg/l for selenate and selenite has been reported using CE with electromigrative injection and UV detection at 254 nm [57]. The method was less sensitive for selenocysteine and selenomethionine with a reported detection limit of 300 μg/l. Separation was carried out on a fused silica capillary (60 cm x 75μm i.d., 52 cm to detector). The running buffer was sodium chromate (5 mM)/ trimethyltetradecylammonium hydroxide (0.5 mM) at pH 10.5 and the applied potential was - 20 kV. This procedure was found to be subject to interferences from nitrate ions.

The advantages of CE are high efficiency and fast analysis times. However this separation technique is less sensitive than either HPLC or GC and off-line preconcentration of the analyte is often required prior to analysis.

3.1.3.5 Neutron activation analysis

Neutron activation analysis (NAA) is not very sensitive for selenium speciation in environmental samples. The selenium species must be separated prior to detection. Preconcentration techniques are generally used to improve the sensitivity of the technique [37]. The radioisotopes most commonly used for activation analysis are ^{77m}Se and ^{75}Se. ^{77m}Se provides the highest sensitivity but has a very short half-life of 17.5 s. It is generally used in instrumental NAA. ^{75}Se is more widely used as its very long half life (120.4 days) enables chemical separation. However, long activation times are required. Massee et al. [58] determined Se(IV) and Se(VI) by NAA. Reduction of Se(VI) to Se(IV) was carried out by refluxing with concentrated HCl. Se(IV) was then reduced to elemental selenium with ascorbic acid. Finally, activated charcoal was used to adsorb the elemental selenium which was then detected by NAA. A detection limit of 10 μgl^{-1} was obtained and both sea and fresh water were analysed.

3.1.3.6 Fluorimetric methods

For spectrofluorimetric detection, Se(IV) is complexed with 2,3 diaminonaphthalene (DAN) to form 4,5 benzopiazselenol. This complex is extracted into cyclohexane and the fluorescence is measured at an excitation wavelength of 380 nm and an emission wavelength of 520 nm [37]. Only Se(IV) can be determined by this method, so speciation is limited by the ability to transform each species to Se(IV).

Fluorimetric detection has been used by Takayanagi et al. for selenium speciation in marine and estuarine environments [59-61]. The technique described involves a

number of steps. Initially, the samples are filtered to remove colloidal material so that only dissolved selenium is detected. In order to determine all the selenium species present the samples were oxidised by UV irradiation from a 1200 watt mercury lamp for 5 hours in the presence of hydrogen peroxide. More than 92% of dissolved organic carbon is destroyed by this procedure and organic selenium is transformed into inorganic species. The inorganic species are then converted to Se(IV) prior to detection by reduction using HCl. UV irradiation need not be carried out if only inorganic selenium species are required. Since the levels of selenate and selenite in natural waters are too low to be measured directly by fluorimetry, preconcentration must be carried out. Se(IV) was preconcentrated by complexation with ammonium 1-pyrrolidine dithiocarbamate (APDC), extracted into chloroform, followed by back-extraction into nitric acid [59]. Fluorimetric detection was then carried out. Total inorganic selenium, on the other hand, was precipitated from solution using tellurium in the presence of hydrazine sulphate. Following dissolution using nitric and perchloric acid, the species were reduced to Se(IV) and determined by fluorimetry. The difference between the value obtained for total inorganic selenium and Se(IV) is taken as the level of Se(VI). Following preconcentration the limit of detection, taken as twice the signal to noise ratio was 20 x 10^{-12} M. Precision, measured as the relative standard deviation, was about 2% at concentrations above 274 x 10^{-12} M.

3.1.4 Selenium speciation

Selenium is an essential nutrient at low concentrations and a toxic species at higher concentrations for both animals and humans [62]. Its intake is consistent with health in only a very narrow concentration range and outside this range, deficiency or

toxicity occurs [52]. Since it is the chemical form of an element which determines its role in living organisms speciation is of great importance. The difficulties associated with selenium speciation are the low levels in environmental samples, relative stabilities and interconversion between species [37]. A wide range of analytical techniques have been applied to selenium determination including differential pulse polarography (DPP), differential pulse cathodic stripping voltammetry (DPCSV), capillary electrophoresis (CE), fluorimetry and neutron activation analysis (NAA). Techniques involving hydride-generation, however, have received the most attention in the literature. The generation of volatile selenium hydrides enhances sensitivity and selectivity as selenium is removed from interfering matrix components. This procedure has been coupled to a wide range of analytical techniques including flame atomic absorption spectrometry (FAAS), electrothermal atomic absorption spectrometry (ETAAS), graphite furnace atomic absorption spectrometry (GFAAS), ICP-AES, ICP-MS and AFS. The limiting factor in all cases is the fact that only tetravalent selenium produces volatile hydrides. Reduction of the sample is usually carried out prior to detection to convert all Se(VI) to Se(IV). The level of Se(VI) is determined as the difference between reduced samples (total selenium) and those on which no pre-reduction was carried out (Se(IV)). The inherent error in this method of determination has led to the development of chromatographic techniques to enable separation of the Se(IV) and Se(VI) species prior to detection. Coupling of chromatographic techniques such as HPLC and GC to element specific detectors such as FAAS, GFAAS and ICP-AES has led to a significant improvement in sensitivity as well as more reliable speciation information. In this work an on-line system has been developed to enable the determination of Se(IV) and Se(VI) in aqueous samples. The quantitative reduction of Se(VI) to Se(IV) was investigated following their separation by HPLC and prior to determination by FAAS. The

compatibility of these analytical techniques with liquid flow enabled an on-line flow injection system to be developed.

3.2 Experimental

3.2.1 Chemicals and solutions

3.2.1.1 MSPD

The anion-exchange (SAX), cation-exchange (SCX), C_{18} and cyanopropyl (CN) preparative packing materials (30-70 μm) used were obtained from Alltech. The reverse-phase packing was prewashed using methanol (HPLC grade, Fissons) and distilled deionised water. The ion-exchange packing materials were washed with distilled deionised water. The sample/packing homogenate was packed into the barrel of a 6 ml syringe. The syringes used were Varian Bond Elut SPE cartridges from which the original packing material had been removed. Analar grade hexane (BDH) was used to wash the columns and elution was carried out using HCl (BDH). The extraction procedure was optimised using milk and cod fillets obtained from the local supermarket. These were stored in the freezer until needed and a portion of sample defrosted prior to use.

Se (IV) and Se (VI) solutions were prepared from sodium selenite and sodium selenate, respectively (Aldrich). The purity of these solutions was confirmed by comparison with standards prepared using sodium salts obtained from the BCR program on selenium speciation. For hydride generation, sodium tetrahydroborate powder (98%, Aldrich) and sodium hydroxide pellets (Lennox) were used. The

selenium solutions were acidified using HCl obtained from BDH. Concentrated HCl was also used for the reduction of Se(VI) to Se(IV). In an effort to eliminate interferences from free chlorine, which can cause reoxidation of Se(VI), the HCl solution was bubbled with helium at 100 ml/min for 3 hours before use.

3.2.1.2 Selenium speciation

Separation of the selenium species was carried out using an anion-exchange column, PRP-X100 (250mm x 4mm, 10μm) purchased from Hamilton. This is a poly(stryrene divinylbenzene) trimethylammonium anion-exchanger. The mobile phase used was p-Hydroxybenzoic acid, PHBA, (99%, BDH). Concentrated solutions of NH_4OH or HCl were used to adjust the pH of the mobile phase.

3.2.2 Procedure

3.2.2.1 Hydride Generation AAS

Flow injection hydride generation-atomic absorption spectroscopy (HG-AAS) was employed for the detection of selenium. A schematic diagram of the apparatus is shown in Figure 3.1. A Varian AA-275 atomic absorption spectrometer was used for these studies. The burner was suitably modified to allow a silica atomisation cell to be supported in an air-acetylene flame approximately 5 mm above the slot of a 5 cm single-slot burner. This atomisation cell consisted of a "T"-shaped silica tube (150 x 2 mm i.d.). The signal from the spectrometer was displayed on a Philips chart recorder (PM 8251). A selenium hollow cathode lamp (S. & J. Juniper & Co.) was operated at a lamp current of 6 mA, a wavelength of 196 nm and a band pass of 1 nm. Air and acetylene flow rates of 6 ml/min and 2ml/min, respectively, were used.

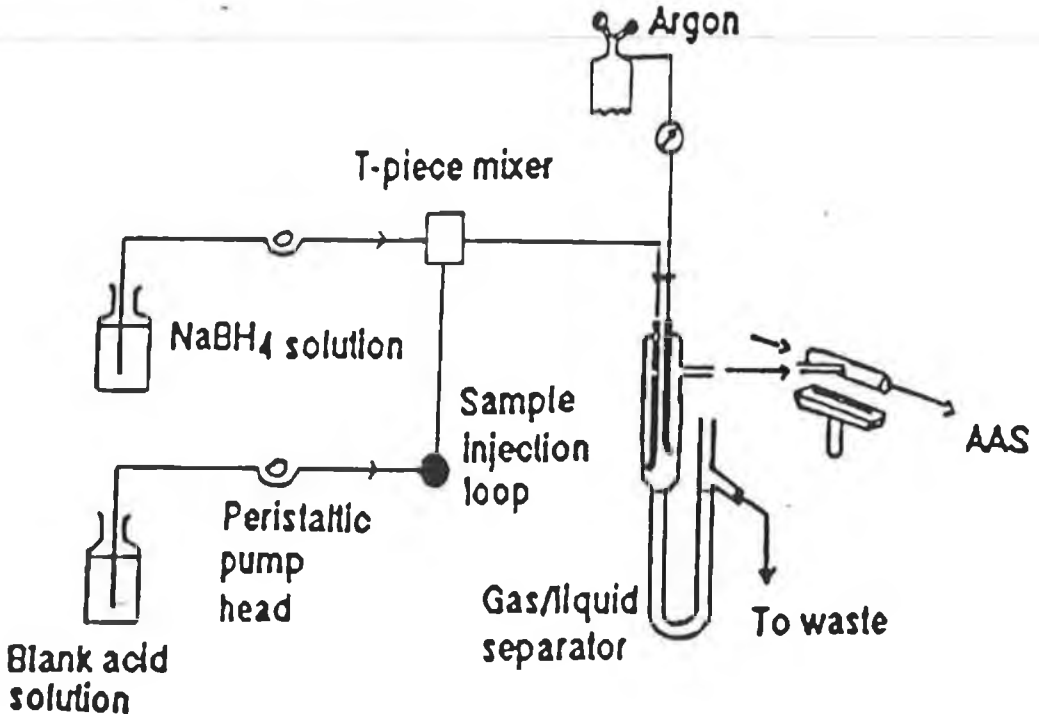


Figure 3.1 Schematic diagram of flow-injection hydride-generation AAS system

The flow injection system consisted of a two-channel peristaltic pump (Miniplus 3), a four-way rotary valve (Tecator 5001) with an external loop for sample injection, a Kel-F mixing T (Plasma-Therm) and a gas-liquid separator (Plasma-Therm). A sample loop consisting of Teflon tubing of 1 mm i.d. and a volume of 360 µl was used for analysis. Teflon tubing was used throughout the flow injection system. The sodium tetrahydroborate and hydrochloric acid solutions were pumped at 1.8 and 6.2 ml/min, respectively. The carrier gas used was argon. A flow rate of 600 ml/min gave the optimum sensitivity while allowing the signal to return to baseline within a 20 second integration time. 2% sodium tetrahydroborate solution, prepared in 1% (w/v) NaOH, was used for these studies. This solution was prepared daily and filtered before use through a 0.45 µm filter. 3 M HCl was used in the carrier stream.

3.2.2.2 Reduction of Se(VI) to Se(IV)

Only tetravalent selenium is capable of producing volatile selenium hydrides. Therefore Se(VI) must be reduced to Se(IV) prior to detection by hydride-generation AAS. A number of off-line reduction procedures were investigated. Initially, direct heating of the sample mixed with conc. HCl was investigated as described by Cutter [62]. A hot-plate was used and the ratio of sample to acid was 1:1. Thus the final acid concentration used for reduction was 6 M. The samples were reacted for 10 mins at 95°C. Se(VI) standards were reduced in the range 50-200 ppb.

According to Vijan [63], it is the concentration of HCl that has the greatest effect on the reduction procedure. He has suggested the use of momentary high concentrations of acid followed by dilution with water. A modified version of this method was investigated. Se(VI) was reduced in the range 50-250 ppb. 12.5-62.5 µl of a 100 ppm Se(VI) solution was made up to 250 µl with distilled water in a beaker. 2 ml of

concentrated HCl was added to obtain a momentary acid concentration of 10.7 M. This mixture was allowed to stand for 3 minutes and made up to 25 ml with distilled water.

3.2.2.3 MSPD

3.2.2.3.1 Extraction procedure

The sample was placed in a mortar and spiked directly with the selenium solution. The mixture was allowed to equilibrate for one minute before 0.4 g of packing material was added. This was then blended for about 30 seconds until a homogeneous mixture was obtained. The resultant sorbent/sample mix was quantitatively transferred into a 6 ml syringe barrel plugged with a 0.45 μm filter disc and glass fibre prefilter. The column head was covered with a filter paper disc. The column was tapped on the bench a number of times to allow the material to settle. Selenium species were eluted using 1M HCl. The flow through the column was controlled by means of a 5 ml plastic syringe attached to the end of the column by rubber tubing. The eluate was made up to 10 ml with 1 M HCl and analysed by HG-AAS. Blank controls were prepared using Milli-Q water in place of the spiking solution.

3.2.2.3.2 Selenite extraction from milk samples

Optimal extraction conditions were established for selenium in milk samples. Initially only Se(IV) was extracted since a pre-reduction step would have to be incorporated in order to enable Se(VI) determination by HG-AAS. A spiking level of 25 μl of a 200 ppm Na_2SeO_3 solution was used. 0.4 g of SAX packing material was

used for the optimisation studies. The concentration and volume of HCl used for elution was optimised. The volume of sample used was also investigated since this will affect the homogeneity of the mixture. A number of packing materials were evaluated for their capacity to extract selenium from milk. Composite solid phases were also tested.

In order to validate the extraction procedure, intra- and inter-assay variabilities were determined. The intra-assay variability was determined from the analysis of replicates over one day while the inter-assay variability was determined over a three day period. All extractions were carried out using 100 µl of milk and a spiking range of 5-25 µl of a 200 ppm Se(IV) solution. Since the final volume of solution is 10 ml this corresponds to 100-500 ppb Se (IV). Standards were also run in this range.

In order to determine the intra-assay variability, analysis was carried out in triplicate at each concentration level. The % recovery was calculated in each case by comparison with the absorbance obtained for the corresponding standard. The mean % recovery and standard deviation were then calculated at each concentration level. By dividing each standard deviation by its corresponding mean and expressing the result as a percentage, the % coefficient of variation (CV) was calculated. The intra-assay variability is defined as the mean % CV $\pm$ the standard deviation.

The inter-assay variability was determined over a three day period. The % recovery was averaged on a daily basis. The mean and standard deviation of these averages over the three-day period was evaluated. As before, the % CV was calculated. This was defined as the inter-assay variability.

3.2.2.3.3 Selenite extraction from fish samples

Conditions were optimised for the extraction of selenium from fish samples. Fresh cod fillets were used. In order to evaluate the presence of any interfering components in the fish matrix, 0.01g of sample was spiked with 25 µl of blank solution (distilled water)and 0.4 g of SAX was added to the mixture. The packing material was then washed with distilled water (2 ml) followed by hexane(8 ml) and with hexane alone (8 ml) prior to elution. In both cases, the columns were dried in an oven for one hour at 60°C followed by drying under a stream of nitrogen. The absorbances obtained with and without washing steps were measured. Byrne et al. [64] reported low recoveries of arsenic when increased amounts of fish were used. This was attributed to binding of trivalent arsenic to the sulphydryl groups in the fish tissue. Thus the arsenic was becoming bound to the cellular proteins in the tissue matrix and these complexes were being strongly retained by the stationary phase. The amount of fish tissue used for the extraction of selenite was varied in the range 0.025-0.4g. The equilibration time was increased to five minutes to allow thorough mixing of the sample and the spiking solution. A number of different fish samples were evaluated.

3.2.2.3.4 Selenate extraction from milk samples

The extraction of Se(VI) as selenate from milk samples was investigated. A reduction procedure was carried out prior to detection by HG-AAS, since only Se(IV) is capable of forming volatile hydrides. The reduction procedure used was a modified version of the procedure optimised in section 3.2.2.2. The anion-exchange sorbent, SAX, (0.4 g) was used for the MSPD extraction and 100 µl of milk sample was used in all cases. Following extraction the eluate was not made up to 10 ml as

previously described, since the post-column reduction procedure involves a substantial dilution of the original analyte. Initially only selenate was determined.

50 µl of a 200ppm selenate solution was spiked into the milk sample (100 µl). Following elution in 8 ml of 1 M HCl, a 250 µl aliquot of the eluate was mixed with 2 ml of concentrated HCl and made up to 25 ml with distilled water. The expected concentration, following reduction, would therefore be 12.5 ppb. A combined solution containing both selenite and selenate was also analysed. In this case, 25 µl each of 200 ppm Se(IV) and Se(VI) were spiked into the milk sample and the eluate reduced as before. Again the expected final concentration would be 12.5 ppb Se(IV).

The reduction procedure was modified in order to increase the final concentrations of both species. A 500 µl aliquot of the eluate was reacted with 2 ml of concentrated HCl and the solution was made up to a final volume of 12 ml using distilled water. Thus the expected concentration of each species would be 52.1 ppb. The precision of the technique was evaluated based on five replicate analyses of 50 µl of 200 ppm Se(VI).

3.2.2.4 Selenium speciation

3.2.2.4.1 Ion-exchange chromatography

Chromatographic conditions for selenate (SeO_4^{2-}) and selenite (SeO_3^{2-})were optimised by fraction collection followed by analysis using hydride generation AAS. The mobile phase was prepared in the range 3-12 mM PHBA at pH 8.5. NH_4OH and HCl were used to alter the pH of the solutions. A selenium standard was prepared containing 10 ppm SeO_3^{2-} and SeO_4^{2-}. Using a 20 µl injection loop, this standard was analysed in duplicate at each mobile phase concentration. The flow-rate through

the column was set at 1.5 ml/min and fractions were collected for 2 minutes, so that the volume of each fraction was 3 ml. In order to determine the approximate retention times for SeO_3^{2-} and SeO_4^{2-}, each fraction was analysed before and after reduction. The reduction procedure used was as described in the previous section. 1.5 ml of conc. HCl was added to 3mls of sample and the solution boiled at 100°C for 4 minutes. The retention time of an unretained component (t_o) was obtained by injecting water onto the column and measuring the absorbance at a wavelength of 308 nm. PHBA exhibits weak absorbance at this wavelength and, by injecting water, the mobile phase will be diluted and give a negative peak at t_o. Anionic capacity factors (k') were calculated for SeO_3^{2-} and SeO_4^{2-}, to determine which mobile phase concentration gave adequate separation with a fast analysis time.

3.2.2.4.2 HPLC-Hydride generation AAS

The HPLC apparatus was coupled directly to the HG-AAS system. Initially, this system was optimised only for the determination of SeO_3^{2-}, as Se(IV), since the determination of SeO_4^{2-}, as Se(VI), would require the incorporation of an on-line pre-reduction step. The eluate from the HPLC column was pumped at 1.5 ml/min into the 3 M HCl line using a "T"-piece, thereby removing the original sample loop from the HG-AAS system. A 100 µl injection loop was fitted on the HPLC in an effort to compensate for any loss in sensitivity due to the increased path length of the sample. Standards containing Se(IV) in the range 0.2-1 ppm were determined.

3.2.2.4.3 Incorporation of on-line reduction procedure

The apparatus was set up as shown in Figure 3.2.

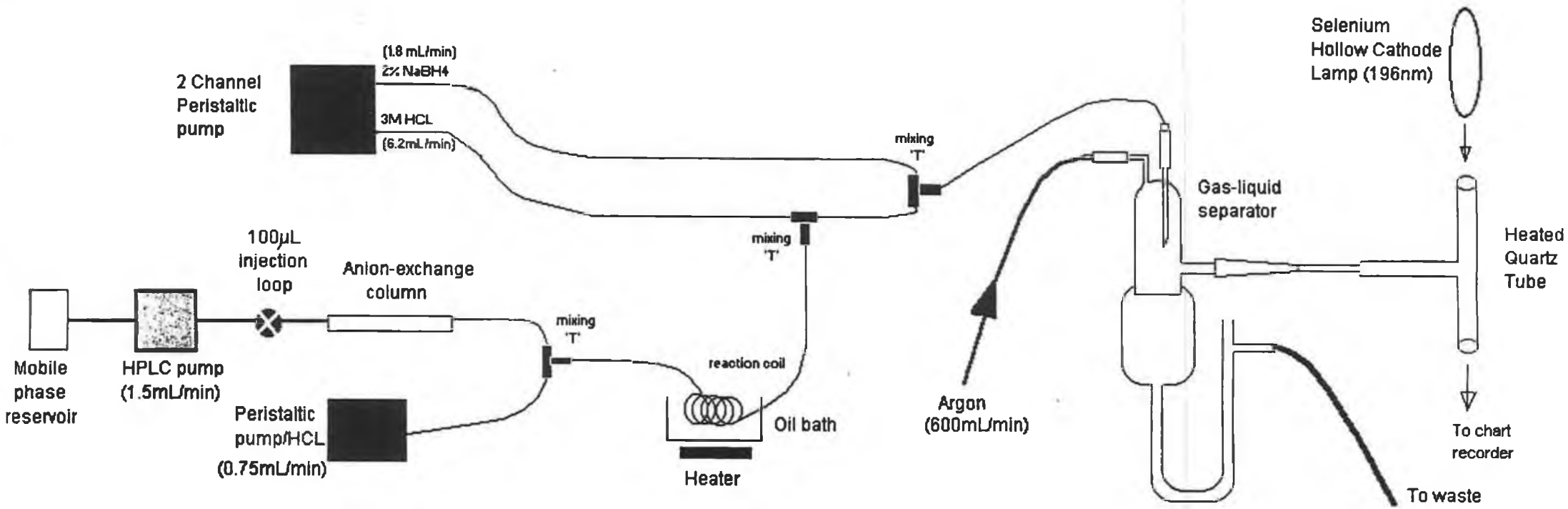


Figure 3.2 Schematic diagram of HPLC-hydride generation apparatus for simultaneous determination of Se(IV) and Se(VI) with on-line reduction procedure

An on-line reduction procedure was incorporated into the system. This involved reacting the column effluent with concentrated HCl and boiling the mixture at 100°C for 10 minutes in a reaction coil made of teflon tubing. The procedure was as follows. Following elution of SeO_4^{2-} (i.e. at approximately 5 minutes) concentrated HCl was pumped into the lines using a peristaltic pump. The acid solution, flowing at 0.75 ml/min was mixed with the column effluent at 1.5 ml/min, by means of a "T"-piece. The mixture was then directed into a reaction coil over a period of 2 minutes. This coil was 241 cm long with an internal diameter of 0.154 cm. Its capacity was therefore 4.5 ml [i.e. 2 min x (1.5 + 0.75) ml]. Once the coil was full the flow was stopped and the tubing was heated in an oil bath. Temperatures in the range 80-100°C were investigated. The length of time of stopped flow was also varied from 4 to 12 minutes in order to maximise the reducton of Se(VI). Following reaction the solution was pumped into the 3 M HCl line from which it was reacted with $NaBH_4$. The volatile SeH_2 produced was directed using a stream of argon into the quartz atomisation cell where it was detected by AAS. Standard solutions containing 1-5 ppm each of Se(IV) and Se(VI) were run under these conditions.

3.3 Results and discussion

3.3.1 Reduction of Se(VI) to Se(IV)

A number of off-line reduction methods were investigated. The first procedure involved reacting Se(VI) with concentrated HCl in an open vessel on a hot plate. Acid was added to the sample in a ratio of 1:1 and the resulting mixture was boiled for 10 minutes at 95°C. The results obtained for the Se(VI) solutions were compared with the corresponding absorbances for Se(IV) standard solutions in the same concentration range (50-200 ppb) (Figure 3.3).

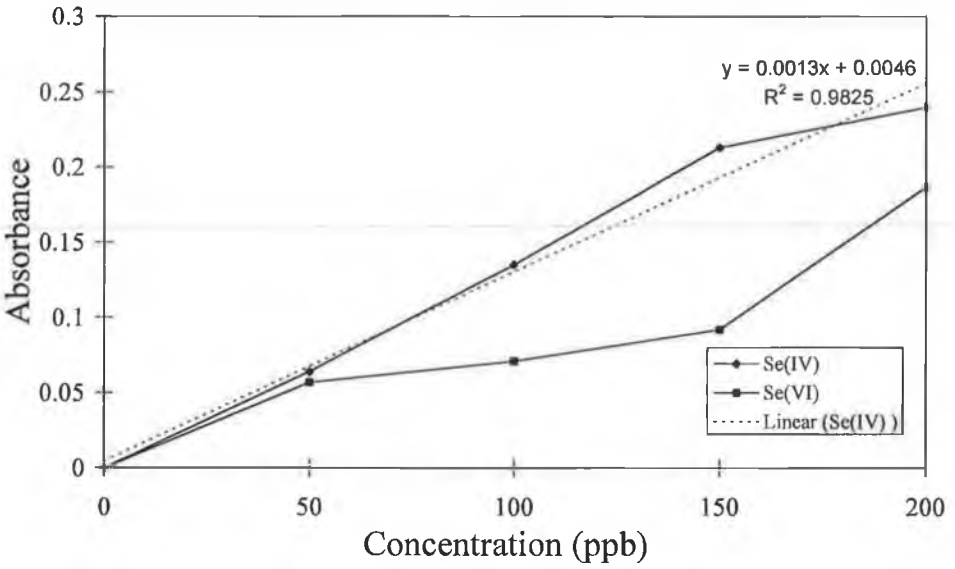


Figure 3.3 Plot of Absorbance Vs Concentration for (a) Se(IV) (b) Reduced Se(VI). Reduction conditions: 6 M HCl, 10 min, 95°C.

Although some conversion appears to be occurring, the results are not reproducible and a linear response was not obtained. It would appear that boiling the samples in open vessels was resulting in the loss of volatile selenium hydrides.

Since highly concentrated HCl is required for complete conversion of Se(VI) to Se(IV), the addition of relatively large volumes of acid to small volumes of sample, followed by a significant dilution step, was investigated. Reductions were carried out on Se(VI) solutions in the range 50-250 ppb and the results were compared with the responses obtained for Se(IV) solutions in the same range (Figure 3.4).

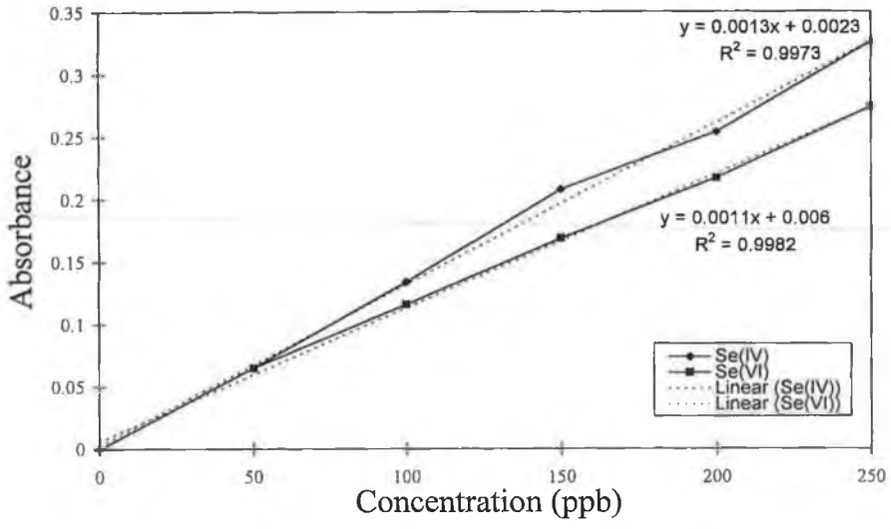


Figure 3.4 Plot of Absorbance Vs Concentration for (a) Se(IV) (b) Reduced Se(VI). Reduction conditions: 12.5-62.5 µl of 100 ppm Se(VI) in 250 µl water reacted with 2 ml HCl (10.7 M), 3 min; final volume of solution, 25 ml; 50-250 ppb Se(VI).

A linear response was obtained (R^2 = 0.9982) with % conversion in the range 82-97%, based on a comparison between the reduced solutions and Se(IV) standards in the same concentration range (Table 3.1). The precision of the reduction procedure was very good with a coefficient of variation (CV) of 2.5 % over the range 50-250 ppb Se(VI), based on triplicate analyses at each concentration.

Table 3.1 Reduction of Se(VI) to Se(IV) in 10.7 M HCl (50-250 ppb)

Conc. Se(VI) (ppb)	Conc. Se(VI) (post-reduction) (ppb) x	Mean Conc. (ppb) x'	s (ppb)	CV %	Conversion %
50	51.3 45.9 48.2	48.5	2.70	5.6	97
100	89.0 85.9 86.7	87.2	1.61	1.8	87
150	126.7 129.8 128.2	128.2	1.55	1.2	85
200	163.6 165.9 165.2	164.9	1.18	0.7	82
250	210.5 207.5 208.2	208.7	1.57	0.8	84

where

Standard deviation (s) = $[\Sigma (x-x')^2 / n-1]^{1/2}$

Coefficient of variation (% CV) = 100 (s / x')

Reduction was also carried out on Se(VI) standards at a lower concentration range (10-50 ppb). A linear response was obtained (R^2 = 0.9943) with conversion in the range 81-99%. Based on triplicate analyses at each concentration level the overall % CV was 8.8% (Table 3.2, Figure 3.5).

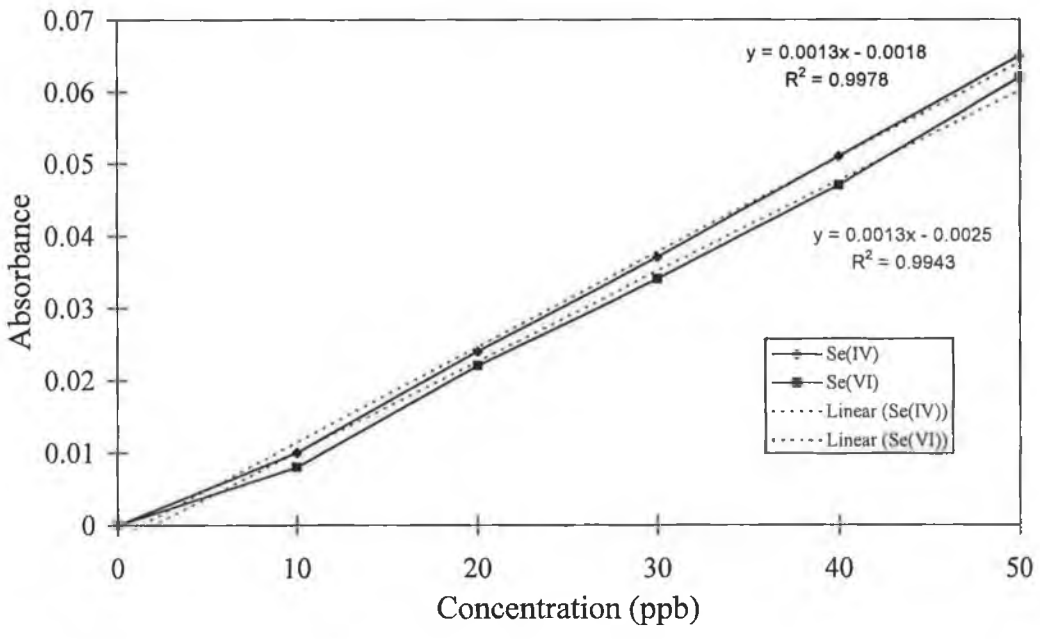


Figure 3.5 Plot of Absorbance Vs Concentration for (a) Se(IV) (b) Reduced Se(VI). Reduction conditions: 2.5-12.5 µl of 100 ppm Se(VI) in 250 µl water reacted with 2 ml HCl (10.7 M), 3 min; final volume of solution, 25 ml; 10-50 ppb Se(VI).

3.3.2 MSPD

3.3.2.1 Selenite extraction from milk samples

3.3.2.1.1 Optimisation of the procedure

The extraction of 25 µl of 200 ppm Se(IV) from milk was investigated using an anion-exchange sorbent (SAX). The concentration and volume of the eluant (HCl) were varied in the range 1-5 M and 1-10 ml, respectively. The % recovery dropped sharply when 5 M HCl was used (Figure 3.6). Since the lower concentrations of acids were comparable it was decided to use 1 M HCl for the remainder of this work. No significant increase in recovery was observed above an eluant volume of 8 ml (Figure 3.7). The volume of milk sample used was varied in the range 50-300 µl since this will affect the homogeneity of the mixture. The highest recovery was obtained when 100 µl of sample was used (Figure 3.8).

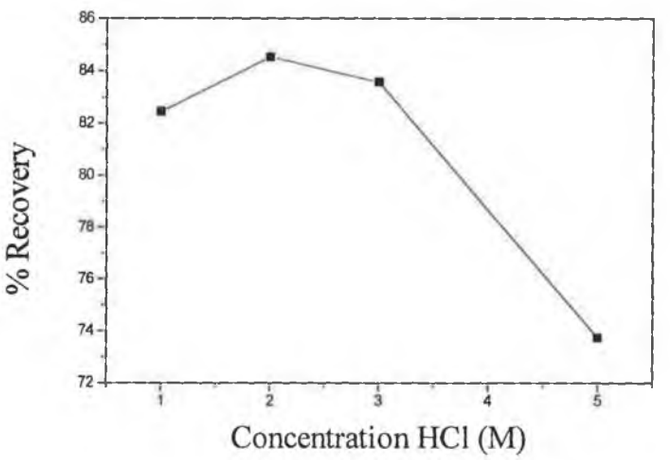
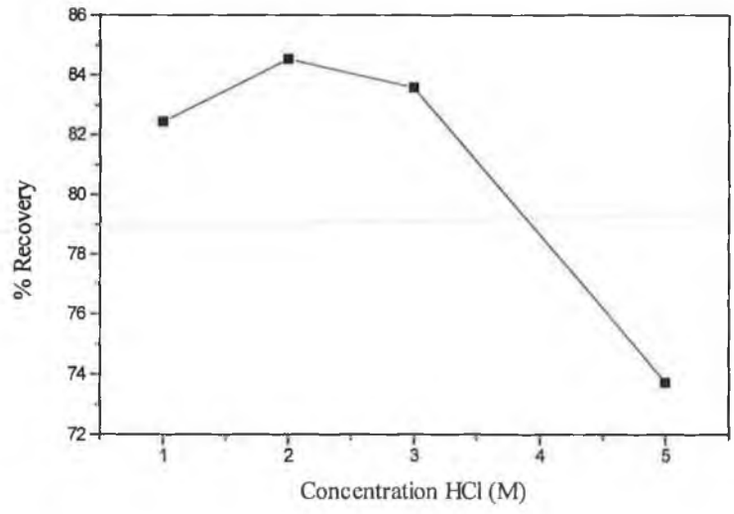


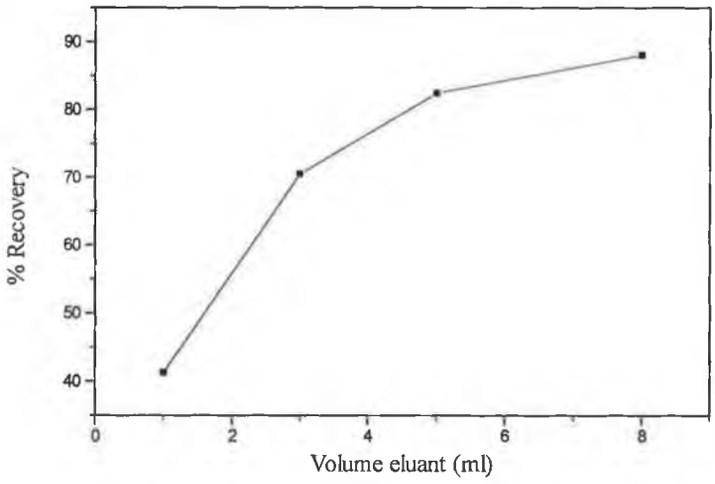
Figure 3.6 Extraction of Se(IV) from milk samples. Conditions: 200 ppm Se(IV), 25 µl; Packing material 0.4 g SAX (anion-exchange); Eluant HCl, 1-5 M, 10 ml.

Figure 3.6 Extraction of selenite from milk samples using HCl (1-5 M)



Extraction of Se(IV); 25 µl of 200 ppm solution. Packing material; 0.4 g SAX (anion - exchange). Eluant; HCl, 1-5 M, 10 ml.

Figure 3.7 Extraction of selenite from milk samples using HCl (1-10 ml)



Extraction of Se(IV); 25 µl of 200 ppm solution. Packing material; 0.4 g SAX (anion - exchange). Eluant; HCl, 1M, 1-10ml.

A number of different packing materials were also examined. The weight of packing used was 0.4 g and 25 µl of 200 ppm Se(IV) was extracted from a 100 µl milk sample. The expected concentration of Se(IV) in the final solution would be 625 ppb. The highest % recovery of Se(VI) (93.3%) was obtained using an anion-exchange (SAX) packing material (Table 3.3).

Table 3.3. Se(IV)extraction from milk samples.

Packing material	**Conc. Se(IV) (following extraction) (ppb)**	**% Recovery**
SAX (anion-exchange)	583	93.3
CN	515	82.4
C_{18}	573	91.7
SCX (cation-exchange)	280	44.8
SCX / C_{18}	399	63.8
SCX / CN	396	63.3
SAX / C_{18}	567	90.7
SAX / CN	559	89.4

Expected concentration Se(IV) = 625 ppb.

3.3.2.1.2 Validation

Inter- and intra-assay variabilities were determined on the optimised extraction procedure. The intra-assay variability was calculated based on the analysis of replicates over one day. The mean % recovery and its corresponding standard deviation were calculated based on the analysis of Se(IV) standards in the same concentration range.

The standard deviation, s, was calculated as follows

$$s = [\Sigma (x - x')^2 / n - 1]^{1/2}$$

where

x = % recovery

x' = mean % recovery

n = number of replicates

By dividing each standard deviation by its corresponding mean the % coefficient of variation (CV) can be calculated

$$\% CV = 100 (s / x')$$

The intra-assay variability, defined as the mean % CV was calculated as 3.7 % over the concentration range 10-500 ppb (Table 4.4).

Table 3.4. Intra-assay variability

Conc. (ppb)	Conc. (post-extraction) (ppb)	x	x'	x-x'	$(x-x')^2$	s	s/x'	% CV
100	96.5	96.5	99.2	2.7	7.29	2.70	0.0272	2.74
	99.2	99.2		0.0	0.00			
	93.3	101.9		2.7	7.29			
200	190.2	95.1	93.3	1.8	3.24	1.80	0.0193	1.93
	183.0	91.5		1.8	3.24			
	186.6	93.3		0.0	0.00			
300	300.1	100	98	2.0	4.00	3.09	0.0315	3.15
	292.0	97.3		0.7	0.49			
	290.2	96.7		1.3	1.69			
400	348.8	87.2	90.6	3.4	11.56	2.98	0.0329	3.29
	368.8	92.2		1.6	2.56			
	370.4	92.5		1.9	3.61			
500	452.4	90.5	87.8	2.7	7.29	6.49	0.0739	7.38
	462.3	92.5		4.7	22.09			
	401.9	80.4		7.4	54.76			

where

x = % recovery

x' = mean % recovery

s = standard deviation

% CV = coefficient of variation

The inter-assay variability was determined over three days. The mean % recovery was calculated per day and the overall average determined over three days.

Table 3.5. Inter-assay variability

Day	Conc (ppb)	Conc (post-extraction) (ppb)	% recovery	x	(x')	$(x-x')^2$	s	% CV
1	100	110.9	110.9	100.16	97.97	4.80	2.10	2.14
	200	191.2	95.6					
	300	298.3	99.4					
	400	390.2	97.6					
	500	486.6	97.3					
2	100	115.4	115.4	97.78		0.04		
	200	183.0	91.6					
	300	310.9	103.6					
	400	375.8	94.0					
	500	421.7	84.3					
3	100	92.0	92.0	95.58		3.96		
	200	188.4	94.2					
	300	313.6	104.5					
	400	374.9	93.7					
	500	477.6	95.5					

where

x = % recovery per day

x' =mean % recovery over three days

s = standard deviation

% CV = coefficient of variation

The inter-assay variability, defined as the % CV, was calculated as 2.14 %. Since both the inter and intra-assay variabilities and their standard deviations are well below 10% this extraction procedure can be deemed valid.

3.3.2.2 Selenite extraction from fish samples

3.3.2.2.1 Evaluation of interfering components in fish matrix

The packing material was washed prior to elution in an effort to eliminate any possible interferences from the sample matrix. A blank solution (distilled water, 25 µl) was spiked onto 0.01 g of fish to which 0.4 g of SAX packing material was added. The results indicate that the matrix components will not produce a false positive response when the washing step is omitted from the procedure.

Table 3.6

Wash solution	Absorbance of blank
Hexane (8 ml)	0.002
Water (2 ml) + Hexane (8 ml)	0.003
No washing step	0.001

3.3.2.2.2 Retention of selenium by fish matrix

Studies carried out on arsenic extraction from fish samples have shown that some of the analyte was irreversibly bound by the matrix resulting in low recoveries [57]. The quantity of fish used for selenium extraction was varied in the range 0.025-0.4 g

in order to estimate the effect of the matrix on the % recovery. Since the composition of fish differs with the type of fish analysed, a number of different varieties were examined. In all cases the sample was spiked with 50 μl of a 200 ppm Se(IV) solution. Following extraction the eluate was made up to 10 ml with 1 M HCl, giving a final expected Se(IV) concentration of 1000 ppb. In all cases the % recovery decreased with increased weight of fish. For fresh cod as the weight of sample was increased from 0.025 to 0.1 g, the % recovery decreased from 74.65% to 0 %. Retention of selenite was comparable with all the fish examined.

Table 3.7

Type of fish	Weight (g)	Conc Se(IV) (ppb)	% Recovery
Fresh cod	0.025	746.5	74.65
	0.04	42.2	4.48
	0.1	0.0	0.00
Dried cod	0.025	748.7	74.87
	0.04	89.7	8.97
Dried tuna	0.025	820.6	82.06
	0.04	300.9	30.09
	0.4	281.9	28.19
Dried dogfish	0.025	875.4	87.54
Dried monkfish	0.025	757.1	75.71

Expected final concentration = 1000 ppb Se(IV)

3.3.2.3 Selenate extraction from milk samples

The extraction of 50 µl of 200 ppm Se(VI) from a 100µl milk sample was investigated. Following reduction with 10.7 M HCl the expected concentration would be 12.5 ppb, as Se(IV). The extraction procedure was carried out in triplicate and recoveries of 83.2-88 % were obtained (Table 3.8).

Table 3.8

Concentration Se(IV) (ppb)	% recovery
11.0	88.0
10.4	83.2
10.6	84.8

Expected concentration = 12.5 ppb Se(IV)

A combined selenium solution containing 25 µl each of 200 ppm Se(IV) and Se(VI) was also extracted and reduced under the same conditions. The expected final concentration would be 12.5 ppb Se(IV), as before. Recoveries, based on three replicate injections, were in the range 59.2-64.8% (Table 3.9).

Table 3.9

Concentration Se(IV) (ppb)	% Recovery
8.1	64.8
7.4	60.0
7.5	59.2

Expected concentration = 12.5 ppb Se(IV)

The precision of the combined extraction/reduction procedure was evaluated based on five replicate analyses of a 50 µl solution of 200 ppm Se(VI). The reduction conditions were modified to increase the concentration of Se(IV) in the final solutions. The volume of eluate reacted with HCl was doubled to 500 µl and the volume of the final solution was halved (12 ml). Thus the final expected concentration would be 52.1 ppb Se(IV). Recoveries were obtained in the range 67-79% (Table 3.10). A standard deviation of 2.03 ppb was calculated based on the analysis of five replicates with an average concentration of 38 ppb Se(IV) (Relative standard deviation = 5.3%).

Table 3.10 Evaluation of the precision of the technique

Run	Conc. (ppb)	x	x'	$(x - x')^2$	s	% CV	Recovery %
1	31.00 45.97 39.67	38.9	38.0	0.81	1.82	4.8	74.6
2	31.80 37.31 35.73	35.0		9.0			67.1
3	37.31 38.09 45.97	40.5		6.25			77.7
4	34.94 39.67 37.31	37.3		0.49			71.6
5	35.73 38.88 39.67	38.1		0.01			73.1

where

$s = [\Sigma (x-x')^2 / n-1]^{1/2}$

% CV = 100 (s / x')

Expected concentration = 52.1 ppb Se(IV)

3.3.3 Speciation

3.3.3.1 Ion-exchange chromatography

The chromatographic conditions for selenate (SeO_4^{2-}) and selenite (SeO_3^{2-}) determination were optimised by varying the concentration of the mobile phase (p-Hydroxybenzoic acid) in the range 3-12 mM. The anionic capacity factor, k', was calculated for SeO_3^{2-} and SeO_4^{2-} at each mobile phase concentration. k' is defined as the ratio of the time spent by the solute in the stationary phase relative to the time it spends in the mobile phase. It is calculated as follows

$$k' = t_r - t_o / t_o \qquad \text{Eq. 1}$$

where

k' = anionic capacity factor

t_r = retention time (minutes)

t_o = retention time of unretained component (minutes)

Water was used to determine the value of t_o. A negative absorbance was produced at 1.433 minutes. A comparison of k' between two species is a good indication of their separation. As the concentration of the mobile phase increased, the value for k' decreased (Figure 3.9). The retention times (t_r), shown in Table 3.11, are only approximate since the fractions collected had a volume of 3 ml, corresponding to 2 minute intervals. The optimum mobile phase would appear to be 12 mM PHBA (pH 8.5).

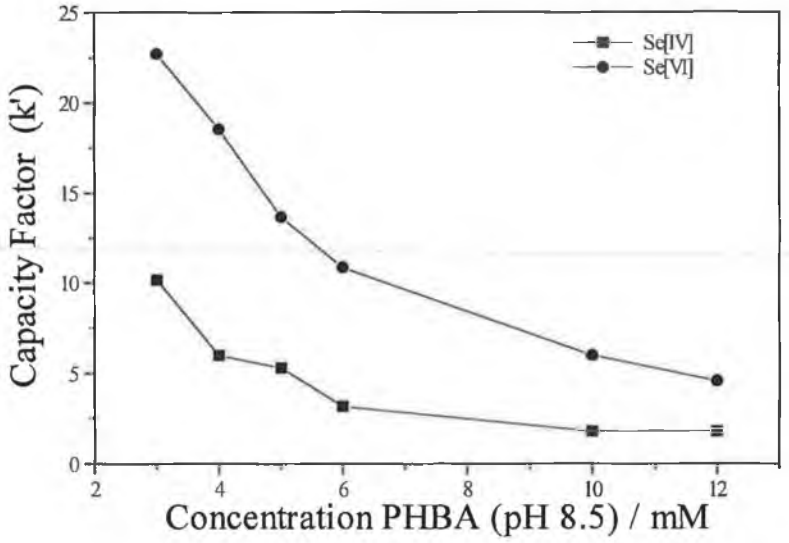


Figure 3.9 Optimisation of separation of selenite and selenate. Conditions: 10 ppm SeO_3^{2-} and SeO_4^{2-}; Mobile phase 3-12 mM PHBA, pH 8.5; Fraction collection 3 ml; Flow-rate 1.5 ml/min; Reduction in 6 M HCl, 100°C, 4 min; Detection by HG-AAS.

Table 3.11

Concentration PHBA / mM	t_r (min)		k'	
	SeO_3^{2-}	SeO_4^{2-}	SeO_3^{2-}	SeO_4^{2-}
3	16	34	10.165	22.726
4	10	28	5.978	18.539
5	9	21	5.281	13.654
6	6	17	3.187	10.863
10	4	10	1.791	5.978
12	4	8	1.791	4.538

3.3.3.2 Coupling of HPLC to HG-AAS

The HPLC pump was coupled to the HG-AAS system. Standards containing Se(IV) in the range 0.2-1 ppm were analysed (Figure 3.10).

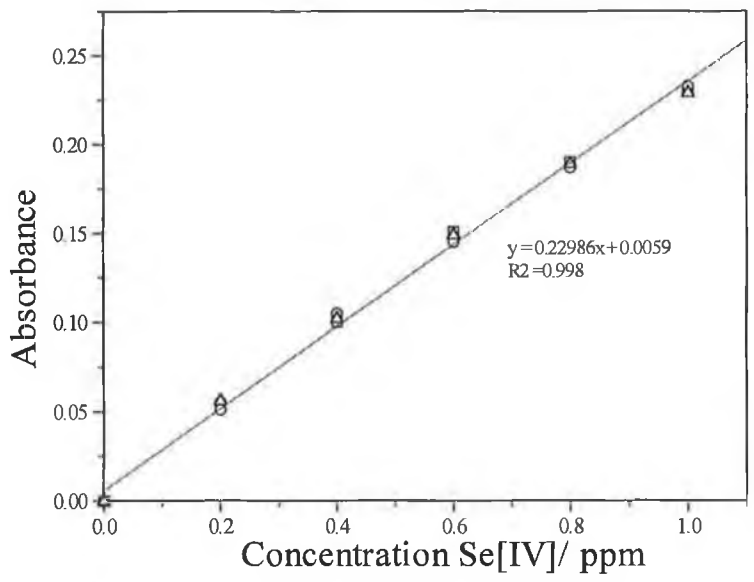


Figure 3.10 Se(IV) analysis by HPLC-HG-AAS. Conditions: 0.2-1 ppm Se(IV); Mobile phase 12 mM PHBA, pH 8.5; Flow rate 1.5 ml/min; 2% (v/v) $NaBH_4$ in 1% (w/v) NaOH, 3 M HCl.

A linear response was obtained ($R^2 = 0.998$). The precision of the technique was determined, based on three replicate analyses at each concentration. The % CV over the concentration range 0.2-1 ppm Se(IV) was 2.2%. The limit of detection under these conditions was 0.033 ppm Se(IV), calculated as three times the standard deviation of the lowest standard.

Table 3.12 Evaluation of precision for determination of Se(IV) by HPLC-hydride generation AAS

Conc. Se(IV) (ppm)	s (ppm)	% CV
0.2	1.1×10^{-2}	5.3
0.4	1.1×10^{-2}	2.6
0.6	7.7×10^{-3}	1.2
0.8	6.7×10^{-3}	1.0
1.0	8.9×10^{-3}	0.7

where

Standard deviation (s) = $[\Sigma (x-x')^2 / n-1]^{1/2}$

Coeffcient of variation (% CV) = 100 (s / x')

3.3.3.3 Incorporation of on-line reduction procedure

In order to provide a completely on-line system for selenium speciation, it was necessary to incorporate an on-line reduction procedure so that Se(VI) is reduced to Se(IV) after leaving the anion-exchange column and before entering the hydride generation system. The reduction procedure involved reaction with concentrated HCl (6 M) and heating under closed conditions. The dependence of this procedure on temperature was investigated as shown in Figure 3.11. At temperatures above 100°C the response was found to level off. Studies also showed that a reaction time of 10 minutes was required to ensure optimum reduction of Se(VI) (Figure 3.12).

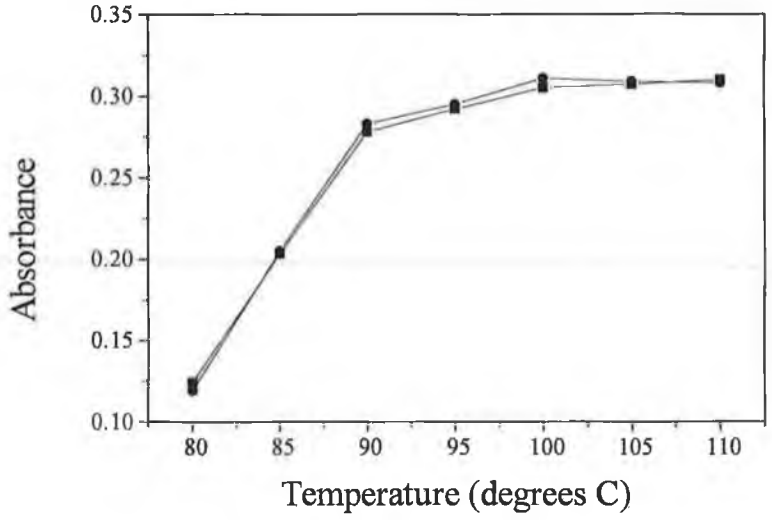


Figure 3.11 Effect of temperature on reduction of Se(VI) to Se(IV). Conditions: 5 ppm Se(VI), 6 M HCl, 8 min.

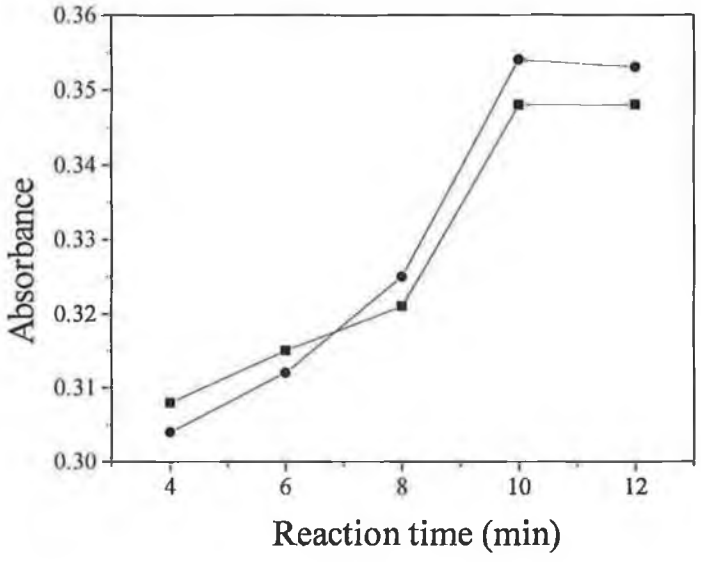


Figure 3.12 Effect of reaction time on reduction of Se(VI) to Se(IV). Conditions: 5 ppm Se(VI), 6 M HCl, 100°C.

Selenium standards containing Na_2SeO_3 and Na_2SeO_4 in the range 1-5 ppm were analysed under the above conditions. The peaks obtained were well separated with retention times of 6.5 min and 9.25 min for SeO_3^{2-} and SeO_4^{2-}, respectively. The run time per sample was 20 minutes, including 10 minutes of stopped flow to enable reduction (Figures 3.13, 3.14).

The resolution between the peaks was calculated using the following equation

$$R_s = 2[t_{r1} - t_{r2}] / [w_1 + w_2] \qquad \text{Eq. 2}$$

where

Rs = resolution between peaks

t_{r1} = retention time of peak 1 t_{r2} = retention time of peak 2

w_1 = width at base of peak 1 w_2 = width at base of peak 1

The resolution between peaks was determined as 1.1.

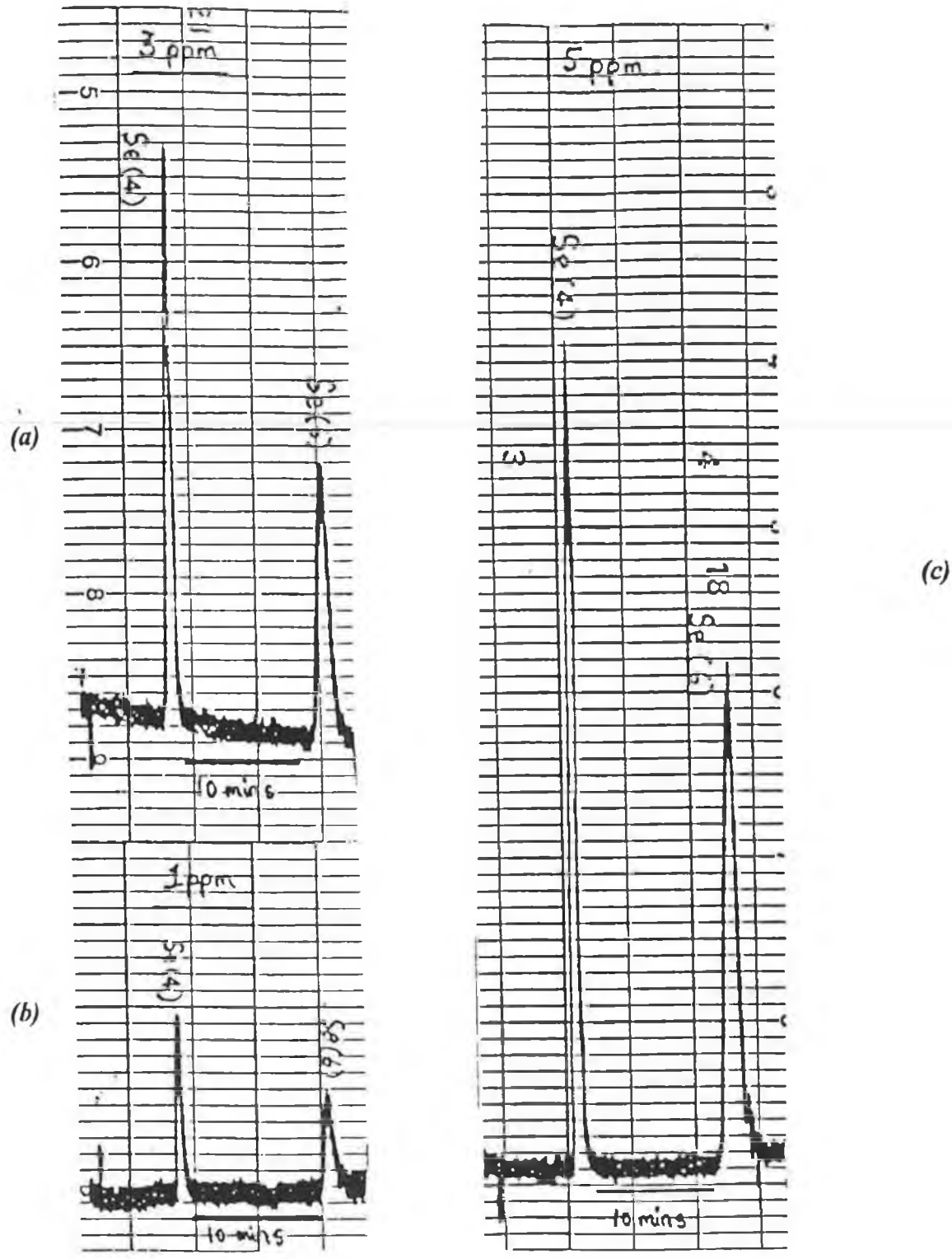


Figure 3.13 Chromatogram illustrating the separation of Se(IV) and Se(VI) by HPLC-HG-AAS (a) 3 ppm (b) 1 ppm (c) 5 ppm. Conditions: 100 µl injection volume; Mobile phase 12 mM PHBA, pH 8.5; Anion-exchange column (Hamilton PRPX-100); HPLC flow-rate 1.5 ml/min; Reduction using conc. HCl added at 5-7 min, flow-rate 0.75 ml/min, temp 100°C, stopped flow 10 min; Hydride generation using 3 M HCl and 2% $NaBH_4$, argon flow-rate 600 ml/min.

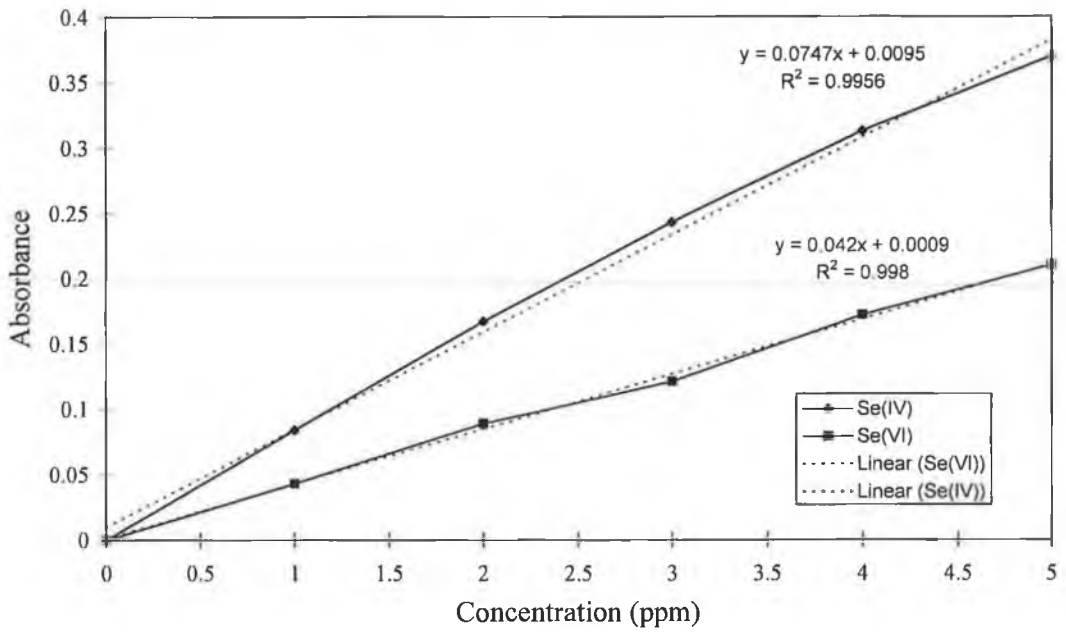


Figure 3.14 Simultaneous analysis of Se(IV) and Se(VI) using HPLC-HG-AAS system with on-line reduction. Conditions: Anion-exchange column, 12.5 mM PHBA, pH 8.5; Reduction in 6 M HCl, 100°C, 10 min; Hydride generation using 2 %(v/v) NaBH4 in 1% (w/v) NaOH and 3 M HCl; Detection by FAAS.

The linearity at this concentration range was good for both Se(IV) and Se(VI) with R^2 values of 0.9956 and 0.998, respectively. The precision of the technique was evaluated, based on triplicate injections at each concentration level (Tables 3.13, 3.14). The overall % CV for Se(IV) and Se(VI) over the concentration range 1-5 ppm was 2.1% and 1.8%, respectively. The % conversion of Se(VI) to Se(IV) was in the range 75-85% when the dilution of Se(VI) by concentrated HCl was taken into account (Table 3.15).

Table 3.13 Evaluation of precision for Se(VI) determination by HPLC-HG-AAS

Conc. ppm	x	x'	$(x-x')^2$	s	% CV
1	0.043 0.045 0.041	0.043	0 4×10^{-6} 4×10^{-6}	2×10^{-3}	3.8
2	0.087 0.089 0.091	0.089	4×10^{-6} 0 4×10^{-6}	2×10^{-3}	1.83
3	0.119 0.120 0.123	0.121	4×10^{-6} 1×10^{-6} 4×10^{-6}	2.25×10^{-3}	1.43
4	0.170 0.172 0.174	0.172	4×10^{-6} 0 4×10^{-6}	2×10^{-3}	0.95
5	0.208 0.209 0.212	0.210	4×10^{-6} 1×10^{-6} 4×10^{-6}	2.25×10^{-3}	0.82

where

x = absorbance

x' = mean absorbance

Standard deviation (s) = $[\Sigma (x-x')^2 / n-1]^{1/2}$

Coefficient of variation (% CV) = 100 (s / x')

Table 3.13 Evaluation of precision for Se(IV) determination by HPLC-HG-AAS

Conc. ppm	x	x'	$(x-x')^2$	s	% CV
1	0.084	0.084	0	2.55×10^{-3}	3.04
	0.081		9×10^{-6}		
	0.086		4×10^{-6}		
2	0.175	0.167	6.4×10^{-5}	5.67×10^{-3}	3.40
	0.160		4.9×10^{-5}		
	0.165		4×10^{-6}		
3	0.250	0.243	4.9×10^{-5}	6.25×10^{-3}	2.57
	0.241		4×10^{-6}		
	0.237		3.6×10^{-5}		
4	0.319	0.313	3.6×10^{-5}	3.1×10^{-3}	0.99
	0.309		1.6×10^{-5}		
	0.312		1×10^{-6}		
5	0.366	0.370	1.6×10^{-5}	2.05×10^{-3}	0.55
	0.375		2.5×10^{-5}		
	0.370		0		

where

x = absorbance

x' = mean absorbance

Standard deviation (s) = $[\Sigma (x-x')^2 / n-1]^{1/2}$

Coefficient of variation (% CV) = 100 (s / x')

The % conversion of Se(VI) to Se(IV) was in the range 75-85% as shown in Table 3.15. An average value of 80% was obtained.

Table 3.15 Reduction of Se(VI) to Se(IV)

Conc ppm	Absorbance Se(IV)	Absorbance Se(VI)	x dilution factor (1.5)*	% conversion
1	0.084	0.043	0.065	77
2	0.167	0.089	0.134	80
3	0.243	0.121	0.182	75
4	0.313	0.172	0.258	82
5	0.370	0.209	0.314	85

* 1.5 ml Se(VI) was reacted with 3 ml conc. HCl.

3.4 Conclusion

This work illustrates the applicability of MSPD extraction procedures for the isolation of selenium from food samples. An analytical technique has also been developed for the simultaneous determination of Se(IV) and Se(VI) in aqueous samples.

MSPD is a simple, rapid technique which enables selenium extraction from complex matrices. No expensive instrumentation is required, compared with many extraction procedures (e.g. microwave digestion). The speed of analyis renders MSPD a suitable technique for the initial screening of samples. In addition this work illustrates the good recoveries that can be obtained. For the extraction of 200 ppb of

Se(IV) from milk samples, over a period of three days, recoveries of 91.6-95.6% were obtained. Precision both between runs and within runs was also good with variabilities of 2.1% and 3.7% being obtained, respectively. While the recovery of selenium from milk samples was high, in the case of the fish samples, irreversible binding was observed at higher weights (> 0.025g). This was attributed to selenium binding by matrix components, for example, cellular proteins in the tissue matrix. Information regarding selenium species can be obtained by combining the MSPD extraction technique with a reduction procedure prior to detection. Under the conditions described recoveries of 67-79% were obtained for the extraction of Se(VI) from milk samples. Reproducibility was good with a relative standard deviation of 5.3%, based on the analysis of five replicates with an average concentration of 38 ppb Se(IV), following reduction. Overall this technique represents a rapid, reproducible extraction procedure for selenium, from which some speciation information can be obtained.

An analytical technique is described which enables the simultaneous determination of Se(IV) and Se(VI). Most analytical methods, including hydride generation AAS, are only capable of detecting Se(IV). Se(VI) must be obtained as the difference between total selenium and Se(IV). In general the error for the species determined by difference is always higher than for the species determined directly. In this work a chromatographic technique was developed which gave good resolution between the species allowing selective post-column treatment prior to detection. The only limiting factor is the necessity of long path lengths for the incorporation of a reduction procedure and hydride generation reaction prior to detection. The resulting decrease in sensitivity is illustrated by a comparison of the response obtained for 1 ppm Se(IV) before and after the addition of the reaction coil (on average, 0.231 and 0.084, respectively). The sensitivity for Se(VI) is decreased further due to dilution

with HCl during reduction. However the precision of the technique is very good with relative standard deviations of 3.80 and 2.47%, for Se(VI) and Se(IV) respectively, based on three replicate injections at a concentration of 1 ppm. The % conversion of Se(VI) to Se(IV) is also adequate (75-85%) considering the simplicity of the system and the lack of expensive instrumentation required. The advantages of this technique are the simultaneous determination of Se(IV) and Se(VI) without the need for a difference measurement, the simplicity of the system and the reproducibility of the analyses. The apparatus could also be easily modified, by the addition of a preconcentration column, prior to the analytical column, which would increase the sensitivity and render the system more applicable to real samples.

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Chapter 4

Copper complexation by *Rhizopus arrhizus*

4.1 Introduction

Microbial biomass has the ability to absorb a wide variety of metal ions from aqueous solution. This process has been termed biosorption [1]. Virtually all microorganisms have a significant biosorptive ability. Bacteria, fungi, algae and yeasts all exhibit high metal uptake capacities. Living and dead cells, as well as excreted or derived products, are capable of metal ion removal from solution. A wide range of metals may be accumulated. Some are essential for microbial metabolism at low concentrations (e.g. Cu, Zn, Mn) while others have no known biological function (e.g. Au, Ag, Cd, Pb, Hg, Al) [2].

The ability of microrganisms to sequester these metal ions has been applied to the removal of metals from waste or process solutions. Subsequent treatment of the loaded biomass using, for example, dilute acids, carbonate/bicarbonate solutions or chelating agents, enables the recovery of valuable elements or further containment of highly toxic species. Thus metals can be recycled and effluent successfully detoxified [1].

Due to industrial and agricultural activities, metal pollution in the environment has intensified in recent years [3]. As a result legal restraints on these long-term environmental hazards have become increasingly strict. Since microbial biomass is largely produced as waste product from industrial processes (e.g. fermentation) it provides an attractive, cost-effective alternative to existing forms of emission control.

To date, both living and non-living microbial systems have been utilised for metal ion removal from solution. One typical application of living systems is the use of

microorganisms in sewage treatment plants. The intentional cultivation of microorganisms and higher plants in meandering streams, impoundments and wetlands has also been reported [2]. Due to their susceptibility to metal poisoning, however, living-cell systems are generally used to decontaminate waste waters where metal concentrations are below toxic levels. The use of dead cells has a much broader scope since they are not affected by metal toxicity or adverse operating conditions. In addition factors such as nutrient supply and culture maintenance need not be taken into account.

4.1.1 Mechanisms of metal ion removal by microorganisms

In the environment, metal interactions with microorganisms take place by a variety of mechanisms. Of these, chemical transformations, extracellular precipitation, extracellular complexation and binding to the cell surface will be considered. The application of each of these mechanisms for metal ion removal from solution will also be discussed.

4.1.1.1 Metal transformations

Metals are chemically transformed in the environment through the action of microorganisms. These transformations include oxidation, reduction, methylation and dealkylation reactions. Microbial oxidation has been applied to the removal of arsenic from sewage [4]. Arsenite-oxidase producing bacteria were used. These bacteria catalyse the conversion of As(III) to As(V), thus facilitating the precipitation of arsenic, as As(V), with Fe(III). The bacteria *Enterobacter cloacae* have been used to detoxify chromate-containing effluents [5]. These bacteria are resistant to high levels of chromate (10mM) which is removed from contaminated

effluent by precipitation as Cr(III). The methylation of some metals by microorganisms results in their volatilisation and subsequent removal from solution. The conversion of selenium into forms such as dimethylselenide, dimethyldiselenide and trimethylselenonium occurs in this way. Similarly Hg(II) can be transformed into methylmercury compounds such as dimethylmercury [2]. Volatilisation is an important mechanism for the transformation of metals in the environment, particularly in soils and sediments. However since methylated metals are difficult to capture, due to their volatility, this mechanism of metal removal has not been widely developed in aqueous systems. Finally, microbial dealkylation processes result in the detoxification of organometallic compounds such as organomercurial complexes. The production of organomercurial lyase by bacteria catalyses the conversion of these compounds to Hg(II) which is subsequently reduced to elemental Hg by mercuric reductase [6, 7].

4.1.1.2 Extracellular precipitation

Microorganisms have the ability to immobilise metals through the excretion of metabolic products. Sulphate-reducing bacteria, such as *Desulphovibrio* and/or *Desulphotomaculum* sp. precipitate metals in this way. These bacteria are found in anaerobic environments such as bogs, anoxic soils and sediments. They oxidise organic matter and reduce sulphates to sulphites. As a result H_2S is produced which reacts with soluble heavy metals to form insoluble metal sulphides. The formation of mineral deposits such as covellite (CuS) and sphalerite (ZnS) has been attributed to the action of these bacteria [2]. A study by Jackson [8] found that sulphate-reducing bacteria were responsible for the removal of metals from a contaminated lake in Canada. Mine and smelter wastes containing Zn, Cd, Cu, Hg and Fe had been discharged into the surface waters. The presence of sewage in the lake promoted the

growth of algae which accumulated the heavy metals. When the algae died they were oxidised by sulphate reducing bacteria. The H_2S produced reacted with the metals forming metal sulphides which were precipitated into the sediment. Jackson proposed that the construction of impoundments with the addition of organic nutrients to simulate algal growth would be a feasible, low-cost mechanism for the removal of metals from contaminated water.

A purpose-designed, 9 m^3 stainless steel, sludge-blanket reactor, using sulphate-reducing bacteria has been developed by Shell Research Ltd. (Sittingbourne, Kent, U.K.) and Budelco BV (Budel-Dorplein, The Netherlands) [9]. A selected, but undefined consortium of sulphate-reducing bacteria are used with ethanol as the growth substrate. This reactor was able to tolerate a wide range of inflow pH and operating temperature and yielded outflow concentrations below the parts per billion range. This process has since been expanded to a commercial pilot-plant scale using a 1800 m^3 reactor built by Parques BV (Balk, The Netherlands). This plant is capable of treating effluent at the rate of 7000 m^3 per day [3]. In another pilot-plant study, carried out by the U.S. Bureau of Mines, a 4500 m^3 reactor was built using spent mushroom-compost as a substrate and support for sulphate-reducing bacteria. This reactor removed 95% of the metals and 20% of the sulphate from 19.3 m^3 of coal-mine drainage waters [10].

Similarly *Citrobacter sp.* precipitate metals as metal phosphates. These bacteria have a surface-located acid-type phosphatase enzyme which catalyses the release of HPO_4^{2-} from a supplied substrate (e.g. glycerol 2-phosphate). As a result, divalent cations are precipitated as $MHPO_4$ (where M represents the divalent metal cation) and removed from solution. This process could be applied to metal removal from effluents in which phosphate-containing organic substrates are present [11,12].

4.1.1.3 Extracellular complexation

Microbial complexation may also occur extracellularly through the secretion of metal-specific ligands. Extracellular polysaccharides and siderophores complex metals in this way.

4.1.1.3.1 Siderophores

Siderophores are relatively low molecular weight, iron-specific coordination compounds, excreted under low iron stress by aerobic and facultative anaerobic microorganisms for the purpose of securing iron from the environment [2]. These chelating agents have a strong affinity for iron, as Fe(III), which is solubilised and converted into a form suitable for uptake.

Siderophores complex metals via hydroxamate, catecholate and amino-acid functional groups. Chelating agents have been synthesised based on siderophore structures for the purpose of metal complexation. Such a model was the subject of a patent application in 1985 [13]. A catechol derivative was modified by substituting electrophilic ions on the benzene ring including Cl^-, Br^-, and NO_2^- groups. This synthetic chelating agent was immobilised using silica gel. In addition to Fe(III), Th(IV) and UO_2 were also removed from solution. The removal of Fe(III) to a concentration of less than 0.1 mM was reported. Since microbial growth is substantially restricted at this level it was propsed that this synthetic siderophore could be used to prevent spoilage in a wide range of liquid media including fruit and vegetable juices, wine, beer, drinking water, ophthalmic solutions and antibiotic preparations. Currently used products such as Chelex 100 (BioRad), have a low

selectivity for iron and important cations, such as Mn^{2+} and Mg^{2+}, which are desirable components of the medium, may be removed. Commercial ion-exchange products may also liberate sodium or potassium ions into the liquid medium being treated.

A low molecular mass model of a siderophore has been used for the separation of transition metals by HPLC [14]. The model used was aspartic acid β-hydroxamate. This reagent is a model of the naturally occurring siderophore Pseudobactin which contains aspartic acid and hydroxamate functional groups. The separation of Al, Co, Cu, Fe and Mo was achieved using ion-pairing reversed-phase liquid chromatography on a polymeric column. The method was applied to the analysis of Cu in laboratory tap water following preconcentration using solid-phase extraction. The results obtained compared favourably with analyses carried out by flame atomic absorption spectroscopy.

4.1.1.3.2 Metal binding polymers

Bacterial extracellular polymers play a significant role in the adsorption of metal ions from solution. This mechanism of metal removal has been applied in activated sludge systems for biological wastewater treatment. Extracellular polymers are generally polysaccharide in nature. They remove metals either by adsorption or by physical entrapment of precipitated or insoluble metals.

The bacterium *Zoogloea ramigera* is widely used for flocculation purposes in activated sludge treatment systems. This bacterium produces an extracellular polysaccharide composed of galactose, glucose and pyruvate. It acts as an efficient adsorbing agent for heavy metal ions. A continuous process for metal accumulation

using pregrown *Zoogloea ramigera* removed approximately 3 mM per gram of dry weight copper at a biomass concentration of <1 g dry weight per litre [15].

4.1.1.4 Binding to the cell surface

Complexation of metals to microbial cell surfaces has been widely studied since this mechanism of metal removal is exhibited by both living and dead cells. As discussed previously, the use of non-living microbial cells is preferable in situations where metals are present at toxic levels. The use of living cells is limited by adverse operating conditions such as high temperatures and extreme pH.

Microbial cell walls are composed of a wide variety of functional groups including phosphate, carboxylate, and hydroxyl groups. As a result a multiplicity of non-equivalent binding sites are available for metal complexation. Cell surface binding of metals by bacteria, algae and fungi has been extensively studied.

4.1.1.4.1 Bacterial Cell Walls

Isolated cell walls of *Bacillus subtilis* have been investigated for their ability to accumulate metal ions. In a study carried out by Beveridge and Murray the uptake of 18 metals was examined [16]. Of these it was found that substantial amounts of Mg, Fe, Cu, Na and K were accumulated. Lesser amounts of Mn, Zn, Ca, Au and Ni were retained with only small amounts of Hg, Sr, Pb and Ag being adsorbed onto the cell wall. Some active sites also acted as nucleation centres for Au accumulation with the formation of microscopic Au crystals. Carboxyl groups have been shown to be the major sites of metal uptake in the *Bacillus subtilis* cell wall [17]. Chemical derivatisation of these groups resulted in a significant decrease in metal uptake.

Conversely, the chemical modification of amine groups had no effect on metal sorption. Cell wall teichoic acids (anionic polymers of α-D-glycopyranosyl glycerol phosphate) complex metals through phosphodiester bonds. Mild alkali treatment removed approximately 94% of these groups resulting in a 44, 52 and 60% decrease in Na, Fe and K uptake, respectively.

Due to the structural differences between gram-negative and gram-positive bacteria the mechanism of uptake by these microbes is significantly different. Gram-negative envelopes consist of two membrane bilayers (the outer and plasma membranes), which are chemically distinct from one another, with a thin peptidoglycan layer sandwiched between them in the periplasmic space. The cell envelopes of *Escherichia coli* K-12 have been studied for metal uptake [18]. The accumulation of 32 metals was examined. Large amounts of Hf and Os (> 0.9 μmol/mg, dry weight) were accumulated. Intermediate amounts (0.1-0.4 μmol/mg, dry weight) of Pb, Zn, Zr, Fe, Mn, Mo, Mg, Co, and Ce were taken up, with only small amounts (< 0.1 μmol/mg, dry weight) of Na, K, Rb, Ca, Sr, Cu, Sc, La, Pr, Sm, U, Ru, Ni, Hg, Pt, Pd, Au and In being detected. Energy dispersive X-ray analysis suggested that most metal deposition occurred at the polar head group regions of the constituent membranes or along the peptidoglycan layer.

4.1.1.4.2 Algal Cell Walls

The significant biosorptive ability of algae is well known. A marine alga (seaweed) has been reported to accumulate cobalt to approximately 17% of its dry weight [19]. Good recoveries were obtained when the algal material was reused five times, comparing favourably with a conventional ion-exchange resin. A non-living brown seaweed *Sargassum natans* has been shown to have an extraordinary capacity for Au

uptake [20]. Values of 420 mg Au/g dry biomass have been reported, outperforming the ion-exchange resin IRA-400 and equalling the performance of activated carbon.

As with most biosorbent materials the exact molecular mechanism for metal-algae interactions remain elusive. The reversibility of the reaction suggests electrostatic bonding [20]. Complexation has been attributed to carboxyl, amide, hydroxyl, phosphate, amino, imidazole, thiol and thioether moieties present in the cell wall in the form of proteins, carbohydrates and lipids [2].

The contribution of carboxyl groups to the binding process has been investigated [21]. Methanol esterification was carried out on the carboxyl groups of five different non-living algae, *Chlorella pyrenoidsa, Spirulina platensis, Eisenia bicyclis, Laminaria japonica and Cyanidium caldarium*. The uptake of three metal cations , Cu, Al and Au, was examined. The extent of esterification was monitored by analysing the amount of methanol released in the sample hydolysates by gas chromatography. It was found that the uptake of Cu and Al decreased dramatically following chemical modification of the carboxyl groups. In contrast Au binding capacities slightly increased suggesting that ionised carboxyl groups on algal biomass play a minor role in Au binding. This is in agreement with previous studies which proposed that nitrogen and sulphur are the ligating atoms for Au on algal cell walls [22]. The increase in binding is probably due to the incease in the overall anionic charge on the cell surface upon esterification. In any case, the results clearly indicate that carboxyl groups are mainly responsible for Cu and Al binding to algal biomass.

Freeze-dried *Chlorella* species exhibit some selectivity in metal accumulation from multicomponent aqueous solutions [23]. At pH 5.0, in a solution containing 1 mmol of each respective ion, uptake was as follows

$$UO_2 > Cu > Zn > Ba = Mn > Cd = Sr$$

It was found that solution pH strongly influences metal binding by *Chlorella* species. Metal cations were found to bind strongly at pH $\geq$ 5.0 but were readily eluted at pH $\leq$ 2.0. Conversely anions such as $PtCl_4^{2-}$, CrO_4^{2-} and SeO_4^{2-} were complexed at pH $\leq$ 2.0 and removed at pH 5.0. A third group of metals were independent of pH (i.e. Ag, Hg and $AuCl_4^-$). Alkaline earth metal cations, such as Mg and Ca, were found to have little binding affinity with algal cell walls.

4.1.1.4.3 Fungal Cell Walls

The main site of metal accumulation in fungi appears to be the cell wall [9]. The use of fungal biomass for microbial biosorption has received considerable attention since it is produced as a waste material from several industrial fermentation processes [24]. Biomass derived from commercial fermentations involving the use of organisms such as *Aspergillus niger*, *Aspergillus oryzae*, *Penecillium chrysogenum* and *Saccharomyces cerevisiae* are capable of binding up to 200mg uranium/g dry weight of biomass from solutions at concentrations as low as 0.4 mM [1]. Tzesos and Keller investigated the use of an undefined mixed culture, obtained as waste from a fermentation process, for the uptake of radium from aqueous solutions [25]. Uptake was measured in terms of radioactivity (i.e. Curie, denoted as Ci). Maximum uptake was observed between pH 7 and 10. Radium biosorptive uptake capacities of 4.5×10^4 nCi/g were reported at pH 7 at an equilibrium radium concentration of

1000 pCi/l. *Penecillium chrysogenum* biomass adsorbed 5 x 10^4 nCi/g radium under the same conditions.

Pre-treatment of biomass before use has a significant impact on subsequent metal uptake. Galun et al. investigated uranium uptake by *Penecillium digitatum* following a number of different pre-treatment procedures [26]. The fungus was placed in boiling water and treated separately with methanol, dimethyl sulphoxide and potassium hydroxide. All three treatments resulted in increased uranium uptake. It was proposed that these agents may expose binding sites either by configurational change or by the removal in solution of masking groups. The use of formaldehyde as a denaturing agent resulted in cross-linking and masking of the functional groups. Sodium azide, an inhibitor of electron transport did not affect uptake significantly indicating that it is not a metabolic process.

Rhizopus arrhizus biomass has been shown to be very efficient for radionuclide uptake. The removal and recovery of caesium, strontium and uranium from aqueous solution by naturally pelleted mycelial biomass has been studied [27]. Uptake of uranium was in excess of 90%. The presence of caesium and strontium in mixed metal solutions did not interfere indicting that *Rhizopus arrhizus* exhibits some selectivity towards uranium. Volesky reported a uranium and thorium uptake capacity in excess of 200 mg/g of *Rhizopus arrhizus* [28]. This uptake is approximately four times higher than the maximum loadings exhibited by the ion-exchange resin IRA-400 which is widely used in the uranium ore extraction process.

The adsorption of 17 different metal species from aqueous solution by *Rhizopus arrhizus* has been investigated [29]. All of the cations were adsorbed (La, Mn, Cu, Zn, Cd, Ba, Hg, Pb, Ag , Cr, Ba, V, and $UO_{2)}$ with the exception of four metal

cations (Na, K, Rb, Cs). The highest uptake was observed for UO_2 (0.82 mM/g). The studies were carried out at a pH as close as possible pH 4 to avoid precipitation of metal oxides. Buffers were not used however since previous studies had shown their ability to complex many of the metal ions and thus interfere with uptake. The fact that alkali metal ions were not adsorbed by the biomass is significant since these metals will not interfere with the uptake of desired metal ions from industrial effluents.

A recent study has indicated that carboxyl groups are the primary metal binding groups in the fungal cell wall [30]. Chemical modification of these groups was carried out with a HCl/methanol solution. The extent of esterification was determined by measuring the methanol released after hydrolysis using gas chromatography. The uptake of zinc under these conditions was investigated at pH 5.5. It was concluded that carboxyl groups are responsible for at least 30% of *Rhizopus arrhizus* and *Mucor miehei* zinc binding properties. The contribution is even higher for *Penecillium chrysogenum* and *Trichoderma reesi*, with 55% and 70% of these groups participating in zinc binding, respectively. The role of several other functional groups has also been investigated by Tobin et al. [31]. The uptake of four cations was studied, La, Zn, Pb and UO_2. Esterification of phosphate groups, using triethylphosphite-nitromethane, resulted in a significant decrease in the uptake of each of the cations. For UO_2, Zn, Pb and La, uptake was reduced by 19, 26, 13 and 38%, respectively. The inhibition of carboxyl groups using formaldehyde also diminished uptake, although to a lesser extent. The adsorption of Zn and La was reduced by 24 and 33%, respectively. A smaller reduction of 3.6% was reported for UO_2 while Pb uptake was unaffected by this treatment. It would appear that both phosphate and carboxyl groups contribute to some degree to metal binding in *Rhizopus arrhizus*. The authors proposed that association with hydroxyl and other

groups may reinforce binding and electrostatic attraction may further augment cation uptake, however, these are only secondary mechanisms. Scatchard plots indicate that complexation occurs via a multiplicity of non-equivalent sites with differing affinities for metal ions. The mode of binding appears to be a reversible association between the metals and the functional groups within the biomass. It was concluded that binding sites consist of a number of different functional groups each participating to various degrees in metal complexation.

Metal interactions with microorganisms therefore take place through a wide variety of mechanisms. The precipitation of metals by chemical transformation or the secretion of enzymes and extracellular ligands has been largely applied to metal removal in sewage systems. Metal binding to the surface of microbial cells is currently of great interest, however, as this form of metal uptake occurs even in non-viable cells. Biosorption can therefore be applied under much more flexible conditions since non-living cells are not affected by adverse conditions such as extremes of pH and temperature. Bacteria, algae and fungi all exhibit significant metal uptake at the cell surface. Of these, fungal cells have been most extensively studied since fungal biomass is readily available as a waste product from industrial processes, such as fermentation. Metal biosorption by these cells provides an efficient, inexpensive alternative to existing methods of metal removal from waste streams.

4.1.2 Immobilisation of microorganisms in solid supports

For industrial applications microbial biomass is usually immobilised in an inert solid support. Freely suspended microbial biomass has many limitations including small

particle size, low mechanical strength and low density. Separation of biomass from effluent is difficult and continuously stirred reactors are generally used. Immobilisation technology has enabled the use of biomass in packed-bed and fluidised-bed reactors [2]. Binding to an inert support serves to improve the physical characteristics of biomass and modifies the particle size so that it is similar to other commercial adsorbents (0.5 - 1.5 μm). Supports such as agar, cellulose, silica, alginate, polyacrylamide. toluene diisocyanate, collagen, liquid membranes, metal hydroxide precipitates and glutaraldehyde have been employed for this purpose. Both living and non-living microbial cells can be immobilised.

4.1.2.1 Immobilised living systems

Immobilisation of living cells is carried out by culturing the microbial cells in the presence of an inert support material resulting in the formation of a biofilm. A wide variety of solid supports can be used. Anthracite coal was used to immobilise a mixed culture of denitrifying bacteria [32]. Uranium was accumulated from solution at a capacity of 140 mg/g dry cells. Biosorption was rapid with a solution containing 25 mg U/l being reduced to 0.5 mg U/l over a period of eight minutes. Polyvinylchloride granules were used to immobilise viable *Psedomonas fluorescens* cells [33]. Cell loadings of 0.1-0.4 g dry weight per gram of plastic was reported for the heavy metals investigated. The concentration of $Pb(NO_3)_2$ was reduced from 1.0 mg to 0.05-0.1 mg/l while $ZnSO_4$ concentration was reduced from 10 to 5 mg/l. However copper was highly toxic to the bacterial population. Living *Citrobacter* species have been immobilised on rigid supports such as reticulated foams, stainless steel wire and wood shavings. The biosorption of uranium was examined and it was found that immobilisation on reticulated foam gave the best results. 90% U removal was attained and U precipitation was reported as 3.9 g U/g dry weight of reticulated

foam [34]. Pb removal from solution was investigated using *Citrobacter* species immobilised on glass helices and entrapped on polyacrylamide gel [35]. Metal accumulation of 3.9 g Pb/g dry weight and 3.8 g U/g dry weight was reported.

4.1.2.2 Immobilised non-living systems

The immobilisation of *Bacillus* species has been reported for the removal of metals from wastewaters [36, 37]. A non-living granular product has been developed which simultaneously removes several different toxic and heavy metals including Cd, Cr, Cu, Hg, Ni, Pb, U and Zn. Metal uptake is non-selective and nontoxic alkaline earth metals (Ca, Na, K, Mg) are not adsorbed. Loading capacities in excess of 10% of the dry weight of the biomass have been reported with a metal removal efficiency of >99%.

Fungal biomass has been immobilised by stiffening natural or dried cells with high molecular weight compounds such as aldehydes, glyoxal, glutaraldehyde or other polyfunctional aldehydes [2]. The stiffened or cross-linked products can be mechanically granulated. The dispersion of fungal biomass into a nonpolar medium such as xylene was the subject of a patent application [38]. The biomass dispersion was agglomerated using stiffening agents such as formaldehyde, formaldehyde-resorcinol solutions, formaldehyde-urea solutions and polyvinyl-acetate emulsions. Granules in the range 0.75 to 1.0 μm were obtained. Uranium biosorption of 90.5 mg/g was reported using this material. Reticulated foam was used to immobilise *Aspergillus oryzae* [39]. Biosorption of Cd was rapid with 90% of the metal being removed as $CdSO_4$ over a residence time of only 5 minutes. Uptake was pH dependent. In the range pH 5-8 there was an approximate increase of 2 mg of Cd per gram of mycelium (dry weight) for each unit increase in pH.

The immobilisation of *Rhizopus arrhizus* biomass has been investigated using alginate, polyacrylamide, epoxy resin and polyvinylformal materials [40]. The subsequent effects on cadmium uptake capacity were examined. Immobilisation using polyvinylformal gave the highest cell loadings with a maximum capacity of 80% (w/w) being achieved. Capacities of 66%, 50% and 10% (w/w) were obtained for epoxy resins, polyacrylmide and alginate respectively. Cadmium uptake by each of the biosorbents was measured relative to adsorption by the free biomass. Alginate-entrapped biomass exhibited the highest percentage removal of all the biosorbents tested. This is due to the fact that alginate itself is capable of removing cadmium from solution. Comparison of adsorption in terms of the total weight of biomass showed that little or no loss of uptake efficiency was caused by the immobilisation process. This has been attributed to the very high porosity of the alginate matrix and the open lattice structure which allows metals to penetrate to the immobilised cells. Similarly entrapment using polyacrylamide was not found to result in a loss in efficiency. Cell-free polyacrylamide beads did not adsorb cadmium. Although the epoxy resin biosorbents exhibited some degree of cadmium uptake the adsorption capacity values were significantly less than that of free biomass. This type of immobilisation resulted in almost total loss of biomass uptake efficiency. This was attributed to continuous coating of the resin. Polyvinylformal (PVF) provided the most successful sorbent material. Although the PVF matrix itself did not contribute to uptake it is apparent that immobilisation did not block or hinder binding to the biomass material. High cell loadings were achieved and metal uptake was 84% of that of free biomass.

Immobilisation of *Rhizopus arrhizus* using reticulated foam has also been reported [41]. Copper uptake from aqueous solution was investigated. Biosorption was found

to be pH dependent with maximum uptake in the pH range 6.7 - 7.0. Concentrated nitric acid was used to adjust the pH of the Cu solutions. It was observed that removal efficiency was higher at low influent metal concentrations indicating that a reasonably high ratio of fungal biomass to metal concentration is required. In this study saturation of the binding sites was observed at concentrations above 0.9 mg Cu/l. The presence of other metal ions including Mn, Zn, Cd, Mg and Ca inhibited Cu uptake indicating that *Rhizopus arrhizus* is not selective for Cu and all the metal ions are adsorbed by a similar mechanism.

Non-living algae have also been immobilised for use in commercial water purification systems. The immobilisation of *Chlorella* species has been reported using a polyacrylamide support [42]. The uptake of uranium from seawater was studied. A high uptake capacity was observed with 159 mg of uranium per g (dry weight) being adsorbed during 10 cycles. pH had no effect on uptake in the range 4 - 9. Desorption was achieved using 0.1 M Na_2CO_3. The dry weight of the cells decreased by 2% during 5 cycles compared with a decrease of 50% when free cells were used.

Immobilisation technology has broadened the scope of microbial biosorption in industrial applications. Immobilisation on a solid support has enabled biomass to be used in packed-bed and fluidised-bed reactors. A very diverse range of solid supports have been reported in the literature including synthetic polymers, reticulated foam, wood shavings and coal. Immobilisation is applicable to both living and non-living microbial systems.

4.1.3 Industrial applications

The use of biomass for toxic metal and radionuclide removal from waste streams has many advantages over existing technologies. Precipitation techniques using NaOH, lime, limestone or sulphides generate hazardous sludges which require disposal in specially classified waste landfill sites. With tightening of land disposal regulations the disposal of hazardous wastes is becoming increasingly expensive and unattractive. The use of microbial biomass provides a welcome alternative. Biomass is obtained as a waste product from many industrial processes (e.g. fermentation). It is inexpensive and widely available. From a performance point of view biomass based technologies compare favourably with existing procedures such as ion exchange, chemical precipitation and ultrafiltration [2]. Metal recovery following adsorption eliminates the production of solid hazardous waste and enables the containment of toxic species. Increased understanding of metal-microbe interactions, however, is required to enable fuller exploitation of these technologies in the future.

This work investigates the use of the filamentous fungus *Rhizopus arrhizus* for the uptake of metal ions from aqueous solution. The biosorptive ability of this fungal material has been widely studied under batch conditions. In this case, however, metal uptake is investigated under conditions of continuous flow. The retention and desorption characteristics of *Rhizopus arrhizus* are examined when used as a packing material in a precolumn attached to a HPLC system. Analysis of the metal solutions is carried out by flame atomic absorption spectrometry. The uptake and removal of Cu(II) ions are investigated and the adsorption of Fe(II) under identical conditions is also considered for comparison purposes.

4.2 Experimental

4.2.1 Chemicals and solutions

The biomass used in these studies (*Rhizopus arrhizus*, 25 μm and 200-400 μm) was kindly donated by the School of Biological Sciences, DCU. All Cu(II) solutions were prepared from $Cu(NO_3)_2$ obtained from BDH while the Fe(II) solutions were prepared from $FeCl_2.4H_2O$ (Aldrich). Water which had been filtered using a Millipore system (0.45 μm) was used in all cases.

Off-line studies were carried out using Varian Bond Elut cartridges (6 ml), the original packing material of which had been removed. Silanised glass wool was packed into the base of the empty syringes to prevent leakage of the biomass. The syringes were washed before use with dilute nitric acid (Aldrich) and water. For the Cu(II) studies the eluant used was sodium diethyldithiocarbamate (BDH, analar) prepared in methanol (HPLC grade, Fissons). For Fe(II) desorption 1, 10 phenanthroline monohydrate (Merck), prepared in methanol (HPLC grade, Fissons) was used. Fresh solutions were prepared daily. Nitric acid (Aldrich) was used to adjust the pH of the eluant and the metal solutions.

A stainless steel precolumn (10 mm x 1.5 mm i.d.) was attached to a Waters 501 HPLC pump for the on-line studies. To improve the porosity of the microbial packing material it was mixed with an inert packing support, molecular sieve 5A (0.5-5 μm). Distilled, deionised water and dilute nitric acid (Aldrich) were used to condition the column before use. Between runs the column was conditioned using methanol (HPLC grade, Fissons), HCl (Aldrich) and sodium acetate (BDH).

Analysis of the Cu(II) solutions was carried out using a Varian 252 atomic absorption spectrometer. The Fe(II)-1, 10 phenanthroline complexes were detected using a Shimadzu SPD-GAV UV-Vis spectrophotometer.

4.2.2 Procedure

4.2.2.1 Off-line studies

0.1 g of *Rhizopus arrhizus* biomass (25 μm) was packed manually into the base of a syringe which had been washed with equal volumes (5 ml) of dilute nitric acid (1 M) and water. 10 ml of water was loaded on to the column to ensure uniform settling of the packing material. A short length of rubber tubing connected to a second syringe was used to draw the solution through the column. Aqueous Cu(II) solutions (1 ppm, pH 3-5) were loaded onto the packing material in a volume of 2 ml. The effluent obtained was collected to enable the percentage uptake to be evaluated. Elution was carried out using 2 ml of sodium diethyldithiocarbamate (NaDDTC) in the range 0.001-0.1 M. The fractions collected were analysed by atomic absorption spectrometry.

4.2.2.2 On-line studies

A precolumn was packed manually with equal ratios of *R. arrhizus* (200-400 μm) and molecular sieve 5A to a total weight of 0.126g. The packing was settled using an engraver. The precolumn was sealed using metal frits and teflon o-rings and housed in a metal cartridge which was attached to a HPLC pump. Prior to loading the

packing material was conditioned using distilled and deionised water and dilute nitric acid (pH 4). Aqueous metal solutions (1 ppm, pH 4) were loaded in a volume of 2 ml and eluted in a volume of 2 ml (0.5 ml/min), unless otherwise stated. The effluent obtained on loading was collected and analysed by AAS to enable % retention to be calculated in each case. Desorption of Cu(II) was carried out using 0.1 M NaDDTC in the pH range 2-8. For Fe(II) complexation, the eluant used was 0.01 M 1, 10 phenanthroline in methanol at pH 8. Buffers were not used as previous studies have shown that they complex the metal ions to some degree and thus interfere with metal uptake [29]. Between runs the column was washed using methanol, 0.1 M HCl and sodium acetate at pH 4. This solution was prepared by mixing 25 mM sodium acetate with a 25 mM acetic acid until a pH of 4 was obtained.

4.3 Results and discussion

4.3.1 Off-line studies

4.3.1.1 Effect of pH on Cu(II) uptake from solution

Metal uptake by *Rhizopus arrhizus* biomass occurs at the cell wall. Fungal cell walls are composed of several potential ligands including phosphate, carboxylate and hydroxyl groups. A single uptake site could have several different functional groups all participating, to some degree, in metal complexation. Cu(II) uptake by *R. arrhizus* was investigated in the pH range 3-5. Basic pH's were not considered due to metal oxide precipitation at high pH values. Aqueous Cu(II) solutions (1 ppm, 2 ml) were loaded onto the fungal packing material and the % uptake was evaluated by analysis of the effluent following loading by AAS. Maximum uptake was observed

at pH 4. This is in agreement with previous reports which indicate that optimum metal uptake by *R.arrhizus* occurs at pH 4 [29, 43, 44]. In this study, 94% of a 1 ppm Cu(II) standard solution was adsorbed by *R. arrhizus* at this pH.

Table 4.1 Cu(II) uptake by *R. arrhizus* in the pH range 3-5

pH	Conc. Cu(II)* ppm	Mean Conc. ppm	% uptake
3	0.249 0.257 0.260	0.255	75
4	0.055 0.066 0.058	0.060	94
5	0.253 0.242 0.245	0.247	75

*Concentration of Cu(II) not retained by the packing material.

Concentration of loading solution = 1 ppm Cu(II)

At pH 4 most phosphate groups, present as mono- and diesters, would be negatively charged and available for binding with divalent metal ions. Many of the carboxylate groups would be neutral. Their acidic protons would be easily displaced, however, by metal ions. Chemical treatment of denatured *R. arrhizus* biomass has indicated that both of these functional groups contribute significantly to metal ion binding [31].

4.3.1.2 Desorption of Cu(II) from *Rhizopus arrhizus*

Sodium diethyldithiocarbamate (NaDDTC) was used to desorb Cu(II) from the microbial packing material. This complexing agent forms strong and neutral complexes with a large number of metal ions particularly Cu(II), Pb(II), Cd(II), Hg(II), Co(II) and Ni(II) [45]. Dithiocarbamate (DTC) ligands bind metals at two sulphur coordination sites with the formation of very stable chelate-4-rings [46]. The fast formation of stable complexes is the main reason for the widespread use of DTC ligands in chromatographic HPLC systems [47-51]. In this study NaDDTC was used as a chelating ligand due to its strong affinity for Cu(II) ions and its suitability for use for the subsequent separation of Cu(II) ions by HPLC. The effect of increasing ligand concentration on the % desorption of Cu(II) from *R.arrhizus* was studied.

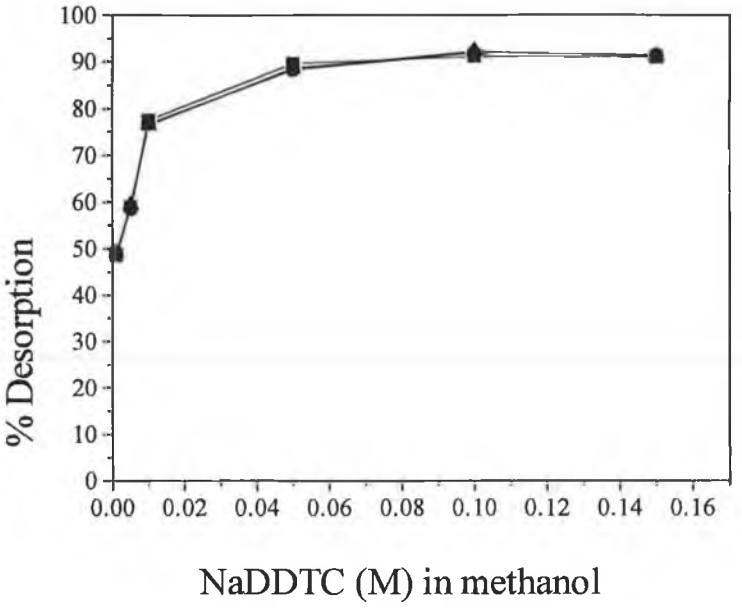

Figure 4.1 The % desorption of Cu(II) from *R.arrhizus* using NaDDTC in methanol. Conditions: 1 ppm Cu(II), pH 4; NADDTC in methanol, 0.001-0.15M; Flow-rate 0.5 ml/min; Adsorbent *R.arrhizus* (25 μm, 0.1 g)

Above a concentration of 0.1 M NaDDTC no further increase in desorption was observed. The ligand would appear to be in excess at this level and a further increase in concentration has no further effect on Cu(II) ion removal. The optimum desorption of Cu(II) from *Rhizopus arrhizus* was, on average, 92% under these conditions.

Table 4.2 Removal of Cu(II) from *R. arrhizus* using NaDDTC (0.001-0.15 M)

Conc. NaDDTC (M)	Conc. Cu(II) ppm	Mean Conc. ppm	Desorption %
0.001	0.485 0.494 0.492	0.490	49
0.005	0.590 0.585 0.594	0.591	59
0.01	0.776 0.768 0.765	0.770	77
0.05	0.897 0.883 0.887	0.889	89
0.10	0.912 0.920 0.923	0.918	92
0.15	0.910 0.915 0.911	0.912	91

Expected Cu(II) concentration = 1 ppm

4.3.2 On-line studies

4.3.2.1 Cu(II) uptake by *Rhizopus arrhizus*

The uptake and removal of Cu(II) ions by the fungal packing material was studied by attaching the precolumn to a HPLC pump, using flame atomic absorption spectrometry (AAS) for detection. Studies so far have indicated that maximum metal uptake occurs when the pH of the metal loading solution is 4. This has been attributed to the solution chemistry of the metal at a slightly acidic pH and the chemistry of the functional groups in the fungal cell wall. Preconditioning of the packing material with a dilute nitric acid solution (0.5 ml/min, 2 ml) at pH 4 was therefore investigated prior to loading of the metal ions. The % uptake of Cu(II) was determined by analysis of the effluent by AAS.

Table 4.3 Preconditioning of packing material

pH	Conc. * ppm	Mean Conc. ppm	Uptake %
7	0.18 0.20 0.19	0.19	81
4	0.04 0.07 0.04	0.05	95

*Concentration of Cu(II) not retained by the packing material.

Concentration of loading solution = 1 ppm

A significant increase in uptake was observed when the packing material was conditioned using dilute nitric acid (pH 4) compared with deionised water (pH 7). It would appear, therefore, that the functional groups in the fungal cell wall are most amenable to metal ion binding at this pH.

4.3.2.2 Effect of pH on desorption of Cu(II) from *R. arrhizus*

Metal complexation by dithiocarbamates is strongly pH dependent [52]. Cu(II) desorption from the fungal packing material was investigated using NaDDTC in the pH range 2-8. A 1 ppm Cu(II) solution (2 ml) was loaded onto the packing material at pH 4 and removed using 0.1 M NaDDTC (2 ml) at pH 2, 4 and 8. As before the HPLC flow-rate was 0.5 ml/min.

Table 4.4

pH	Conc. ppm	Mean Conc. ppm	Desorption %
2	0.245 0.265 0.270	0.260	26
4	0.400 0.385 0.415	0.400	40
8	0.930 0.970 0.950	0.950	95

Expected Cu(II) concentration = 1 ppm.

Optimum desorption occurred at pH 8 indicating that Cu(II) is readily complexed by NaDDTC at this pH. On average, 95% of Cu(II) is desorbed at this pH, indicating good recovery from the packing material. The low recoveries at more acidic pH's corresponds with previous reports that dithiocarbamate ligands are unstable in acidic solution [52].

4.3.2.3 Re-use of the fungal packing material

Re-use of the packing material was evaluated over six cycles. Methanol, HCl (0.1 M) and sodium acetate (pH 4) were used to regenerate the biomass between runs (10 ml). Aqueous Cu(II) solutions (1 ppm, pH 4) were loaded onto the precolumn in a volume of 2ml and eluted using NaDDTC (0.1 M, pH 8) also in 2 ml. The uptake of Cu(II) over six cycles was investigated (Figure 4.2).

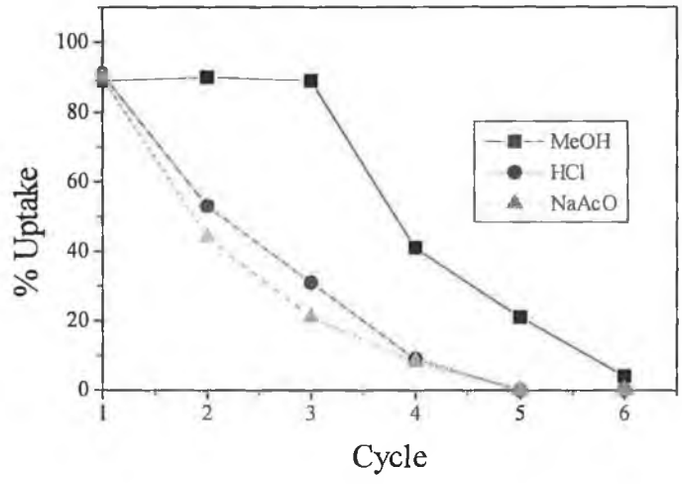

Figure 4.2 Plot of Cu(II) uptake over six cycles. Packing material was washed between runs using (a) Methanol (b) 1 M HCl (c) Sodium acetate, pH 4 (10 ml @ 1 ml/min). Conditions: 1 ppm Cu(II), pH 4; *Rhizopus arrhizus* (200-400 μm)/Molecular sieve 5A (0.5-5 μm), 0.126g.

Table 4.5.1 Regeneration of fungal packing material using methanol

Cycle	Conc. Cu(II)* ppm	Uptake %
1	0.112	89
2	0.101	90
3	0.106	89
4	0.591	41
5	0.792	21
6	0.962	4

*Concentration relates to Cu(II) not retained by the packing material

Concentration of loading solution = 1 ppm

Table 4.5.2 Regeneration of fungal packing material using HCl

Cycle	Conc. Cu(II)* ppm	Uptake %
1	0.091	91
2	0.472	53
3	0.691	31
4	0.912	9
5	-	-
6	-	-

*Concentration relates to Cu(II) not retained by the packing material

Concentration of loading solution = 1 ppm

Table 4.5.3 Regeneration of fungal packing material using sodium acetate

Cycle	Conc. Cu(II)* ppm	Uptake %
1	0.101	90
2	0.561	44
3	0.792	21
4	0.922	8
5	-	-
6	-	-

*Concentration relates to Cu(II) not retained by the packing material

Concentration of loading solution = 1 ppm

The use of HCl or sodium acetate was not successful for the regeneration of the fungal packing material. Uptake decreased to 53 and 44%, respectively, in subsequent runs. However, Cu(II) uptake remained constant over three cycles when methanol was used to wash the column between runs (Figure 4.2). This has been attributed to the fact that methanol would remove any excess DTC ligand remaining on the packing material. The build-up of DTC (pH 8) could result in Cu(II) ion precipitation on the packing material at basic pH. Thus the active sites would become blocked inhibiting further Cu(II) uptake. Alternatively, steric hindrance by DTC or binding of the negatively charged ligand to positively charged groups in the biomass would also inactivate the binding sites The lifetime of the packing material was short with negligible uptake in all cases after 6 cycles.

In an effort to extend the use of the precolumn, washing with increased volumes of methanol (5-15 ml) was investigated (Figure 4.3). Slower flow rates were also used in an effort to increase the contact time between the regenerant and the packing material (0.5-1.5 ml/min).

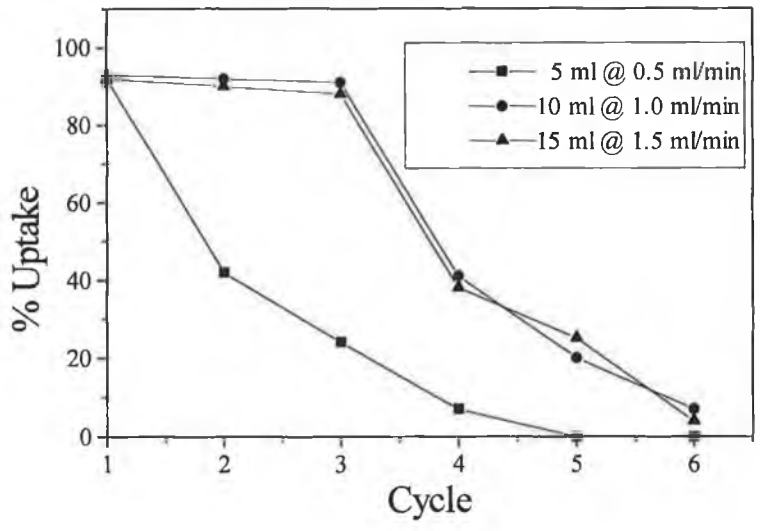

Figure 4.3 Plot of the uptake of Cu(II) by fungal packing material over six cycles. Packing material was washed between runs using methanol (a) 5 ml @ 0.5 ml/min (b) 10 ml @ 1 ml/min (c) 15 ml @ 1.5 ml/min. Conditions: 1 ppm Cu(II), pH 4; Packing material, *Rhizopus arrhizus* (200-400 μm)/Molecular sieve 5A(0.5-5 μm), 0.126g.

It was found that increasing the volume of methanol above 10 ml @ 1 ml/min did not improve uptake or extend the lifetime of the column. The uptake over three cycles was in the range 89-92%. This dropped sharply to 41% on the fourth cycle. The packing material was discarded in all cases after three runs.

Table 4.6 Regeneration of packing material using methanol

Cycle	Volume methanol (ml)	Conc. Cu(II)* ppm	Uptake %
1	5	0.079	92
	10	0.073	93
	15	0.084	92
2	5	0.585	42
	10	0.079	92
	15	0.177	90
3	5	0.762	24
	10	0.087	91
	15	0.209	89
4	5	0.930	7
	10	0.593	41
	15	0.564	38
5	5	-	-
	10	0.770	23
	15	0.801	25
6	5	-	-
	10	0.930	7
	15	0.959	4

*Concentration of Cu(II) not retained by the packing material

Concentration of loading solution = 1 ppm

4.3.2.4 Preconcentration

The potential use of *R. arrhizus* for preconcentration purposes was investigated. A 1 ppm Cu(II) solution (pH 4) was analysed in triplicate at loading volumes of 5-15 ml.

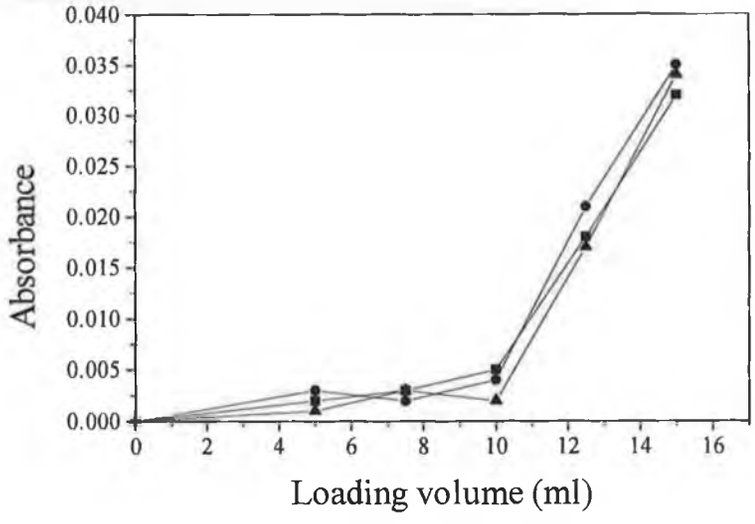

Figure 4.4 Plot of absorbance of Cu(II) in effluent Vs volume of loading solution. Conditions: 1 ppm Cu(II), pH 4, 2-15ml; *Rhizopus arrhizus* (200-400μm)/Molecular sieve 5A (0.5-5 μm).

Above a loading volume of 10 ml, the 1 ppm Cu(II) solution appeared to be washed off the column. The volume of NaDDTC required to desorb Cu(II) (10 ml of 1 ppm) was also investigated in an effort to determine the maximum preconcentration possible at this concentration level. The volumes used were in the range 0.5-3 ml (Table 4.7).

Table 4.7. Preconcentration of Cu(II) (1 ppm)

Volume NaDTC (ml)	Expected Conc.* Cu(II) /ppm	Conc. Cu(II) (ppm)	Recovery %
0.5	20	< 0.03	-
1	10	0.05	0.005
1.5	6.7	3.5	52
2	5	4.7	94
2.5	4	3.6	90
3.0	3.3	3.2	97

*This was calculated based on the fact that the 1 ppm Cu(II) solutions were loaded in a volume of 10 ml.

No significant desorption was observed at NaDDTC volumes below 1.5 ml. At this volume only 52% of the expected concentration of Cu(II) was removed from the packing material. However 94% of Cu(II) was desorbed in a 2 ml volume. The preconcentration factor obtained was 4.7, which compares very well with the expected value of 5. This would appear to be the maximum preconcentration possible for 1 ppm Cu(II) under these conditions. Good recoveries were also obtained at higher volumes of NaDDTC with 90 and 97% of Cu(II) being removed in volumes of 2.5 and 3 ml, respectively. In this case the 1 ppm Cu(II) standard solution was preconcentrated by factors of 3.6 and 3.2, respectively.

Preconcentration at lower levels was also investigated. Cu(II) standards (10-40 ppb, pH 4) were loaded onto the fungal packing material in a volume of 10 ml.

Desorption was carried out using 2 ml of 0.1 M NaDDTC (pH 8). In order to evaluate the precision of the technique the analysis was carried out in triplicate at each Cu(II) concentration level.

Table 4.8 Preconcentration of Cu(II) (10-40 ppb)

Conc. Cu(II) loaded (ppb)	Conc. Cu(II) desorbed (ppb)	Mean Conc. desorbed (ppb)	Expected Conc. Cu(II) (ppb)	Recovery %	s (ppb)	% CV
10	46 50 45	47	50	94	2.7	5.7
20	96 91 98	95	100	95	3.6	3.8
30	145 137 147	143	150	95	5.3	3.7
40	193 190 199	194	200	97	4.6	2.4

where

s (standard deviation) = $[\Sigma (x-x')^2 / n-1]^{1/2}$

% CV (Coefficient of variation) = 100 s /x'

x = Concentration Cu(II)

x' = Mean concentration Cu(II)

The desorption of Cu(II) under these conditions was very successful with recoveries of 94-97% being obtained. In all cases the Cu(II) solutions were loaded in a volume of 10 ml and desorbed in a volume of 2 ml. Preconcentration by a factor of five was therefore expected. The resulting increase in concentration was very close to expected values with factors of 4.7-4.85 being obtained. Validation of the procedure was carried out by evaluating the precision both within and between runs. The precision within each run was good with a % CV of 5.7, 3.8, 3.7 and 2.4% being obtained for 10, 20, 30 and 40 ppb Cu(II), respectively. Between runs an overall % CV of 3.9% was calculated. Since this is well below 10% it would appear that, under the conditions described, this represents a valid technique for Cu(II) preconcentration from aqueous samples.

4.3.3 Fe(II) uptake by *Rhizopus arrhizus*

For comparison purposes the uptake of Fe(II) by *R. arrhizus* was studied under identical conditions. The pH of the loading solution was maintained at 4 since this would appear to be the optimum pH for metal uptake by *R arrhizus*. A 1 ppm Fe(II) standard solution (pH 4) was loaded onto the packing material at a flow-rate of 0.5 ml/min (2 ml). The % uptake of metal by the fungal packing material was determined in triplicate using three separately prepared columns. AAS was used to determine the concentration of Fe(II) in the effluent in each case. The average uptake of Fe(II) by *R.arrhizus* (91%) was comparable with that obtained for Cu(II) (95%). It would appear therefore that both of these divalent metal ions are complexed at the same active sites on the biomass.

Table 4.9 Fe(II) uptake by *R.arrhizus*

Conc. Fe(II)* ppm	Mean Conc. ppm	Uptake %
0.065 0.105 0.073	0.081	92%
0.129 0.105 0.105	0.113	89%
0.065 0.105 0.097	0.089	91%

*Concentration of Fe(II) not retained by the packing material.

Concentration of loading solution = 1 ppm Fe(II)

4.3.3.1 Desorption of Fe(II)

In this case the complexing agent chosen to remove Fe(II) from the fungal packing material was 1, 10 phenanthroline. This heterocyclic base is known to form strong complexes with most transition metal ions over a broad pH range (3-9) [53]. Fe(II) reacts with 1, 10 phenanthroline to form an orange-red complex $[(C_{12}H_8N_2)_3Fe]^{2+}$. This complex is very stable and can be determined spectrophotometrically at 515 nm [54]. The characteristic stability of these complexes has led to their widespread use in analytical applications [55-57].

A 1 ppm Fe(II) standard solution (pH 4) was loaded onto the fungal packing material at 0.5 ml/min. As before the metal ions were loaded and desorbed in the same volume (2 ml) in order to determine whether quantitative removal of the metal from the packing material was possible. 0.01 M 1, 10 phenanthroline in methanol (pH 8) was used for desorption. The concentration of Fe(II) removed from the packing material was determined by measuring the absorbance of the Fe(II)-1,10 phenanthroline complex at 515 nm using UV-Vis spectrophotometry. This analysis was carried out in triplicate using three separately prepared columns. Good recoveries were obtained with an average desorption of 92%.

Table 4.10

Conc. Fe(II) ppm	Mean Conc. ppm	Desorption %
0.993 0.892 0.910	0.932	93
0.948 0.873 0.966	0.929	93
0.892 0.948 0.892	0.911	91

4.3.3.2 Preconcentration

The preconcentration of Fe(II) by *R. arrhizus* was investigated under the conditions previously optimised for Cu(II). Fe(II) standards (pH 4) were loaded in a volume of 10 ml (0.5 ml/min) and desorbed using 2 ml of 0.01 M 1, 10 phenanthroline in methanol (0.5 ml/min). The standards analysed were in the range 0.1-0.4 ppm since the limit of detection for Fe(II)-1, 10 phenanthroline by UV-Vis spectrophotometry is 0.1 ppm.

Fe(II) preconcentration in this concentration range was successful with recoveries of 84-94% being obtained (Table 4.11). On average the concentration of Fe(II) in the range 0.1-0.4 ppm was increased by a factor of 4.5. This compares very favourably with the expected preconcentration factor of five, based on the fact that the standards were loaded in a volume of 10 ml and eluted in a volume of 2 ml.

This procedure was validated based on the precision of the technique both between runs and within runs. Within each run % CV's of 2.8, 1.4, 0.8 and 3.0 were calculated at concentrations of 0.1, 0.2, 0.3 and 0.4 ppm, respectively (Table 4.11). The overall % CV was 2% indicating that the precision between runs is also very good.

Table 4.11 Preconcentration of Fe(II) (0.1-0.4 ppm)

Conc. Fe(II) loaded (ppm)	Conc. Fe(II) desorbed (ppm)	Mean Conc. desorbed (ppm)	Expected Conc. Fe(II) (ppm)	Recovery %	s (ppm)	% CV
0.1	0.41 0.45 0.39	0.42	0.50	84	0.011	2.8
0.2	0.86 0.90 0.84	0.87	1.0	87	0.011.	1.4
0.3	1.42 1.38 1.44	1.41	1.5	940	0.011.	0.8
0.4	1.79 1.85 1.90	1.85	2.0	93	0.055	3.0

where

s (standard deviation) = $[\Sigma (x-x')^2 / n-1]^{1/2}$

% CV (coefficient of variation) = 100 s /x'

x = Concentration Fe(II)

x' = Mean concentration Fe(II)

Similar uptake and removal was observed for the divalent cations Cu(II) and Fe(II) On average, the % uptake was 91% and 95% for Fe(II) and Cu(II) respectively. Recoveries were also comparable with an average % desorption of 92% and 95% being obtained for Fe(II) and Cu(II), respectively. Although this study dealt mainly with Cu(II) uptake by *R. arrhizus* it is clear that this fungal material could be applied to the accumulation of other metal ions from solution.

4.4 Conclusion.

The aim of this work was to investigate the applicability of *R. arrhizus* biomass as a precolum packing material for the preconcentration of Cu(II) ions from aqueous solution. The ability of this filamentous fungus to adsorb metal ions from aqueous solution is well known [29, 31]. These studies refer to batch systems, however. In this work a flowing system was used and the contact time betwen the biomass and the metal ions was minimal. Uptake of Cu(II) by *R. arrhizus* appears to be a rapid process with 95% of a 1 ppm standard solution being retained by the packing material over a period of 4 min (0.5 ml/min). Metal accumulation was found to be pH dependent with maximum uptake at pH 4 and desorption at pH 8. Binding was non-selective with similar uptake being observed for both Cu(II) and Fe(II) ions. These preliminary studies indicate the potential of *R.arrhizus* as an alternative LC packing material for the uptake of metal ions from aqueous solution. Since this fungal biomass is obtained as a waste material from many industrial processes it could provide an economically attractive alternative or adjunct to existing ion-exchange techniques.

4.5 References

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