METAL CHELATION IN SEPARATION SCIENCE

by

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Declaration

"I hereby certify that this material, which I now submit for assesment 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: M. Deacon *ID No.* ***89700848***

Marian Deacon

Date: 19/9/94

To my Parents, Abraham and Sarah

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CONTENTS

Abstract

The science of transition metal chelation finds applicability in many analytical areas where the determination of agents which will chelate transition metal ions, or the determination of transition metal ions themselves, is desired. This thesis details the use of transition metal chelation in solving analytical problems encountered in the adhesives, fertiliser and biomedical laboratory.

In the adhesives industry, transition metal ions in anaerobic adhesives can initiate the polymerisation process, resulting in premature setting of products in their packaging. Addition of a chelating agent such as ethylenediaminetetraacetic acid, however, renders the metal ion inactive with respect to its catalytic properties. A novel method was developed for the simultaneous determination of the seven metal cations Cu(II), Pb(II), Ni(II), Zn(II), Co(II), Cd(II), and Mn(II), with limits of detection as low as 30 ppb for certain metal ions. The method has been shown to give no response in the presence of excess EDTA. This was achieved by the development of a solid-phase extraction procedure, and separation on a dynamically coated C_{18} reversed-phase high-performance liquid chromatography column.

In the fertiliser industry, chelates are added to commercial fertilisers for the supply of micronutrients to plants. The determination of the iron chelates of ethylenediamine-tetraacetic acid (EDTA), diethylenetriaminepentaacetic acid (DTPA), hydroxy-2-ethylenediaminetriacetic acid (HEEDTA), ethylenediaminedi(o-hydroxy-phenylacetic) acid (EDDHA), and ethylenediaminedi(o-hydroxy-p-methylphenyl) acetic acid (EDDHMA), and the Cu, Zn and Mn chelates of EDTA was investigated. The ion-pairing reagent tetrabutylammonium hydroxide, gave a separation of all iron chelates on a Chromspher C_{18} column using a solvent switching system. Cu-, Zn-, and MnEDTA were separated using the ion-pairing reagent tetradecyltrimethylammonium bromide. For the iron chelates both limit of determination and linear range studies, showed that the method is capable of analysing the concentration range found in commercial fertilisers. The capabilities of gel permeation chromatography for the separation and purification of the above chelates, was also investigated. Of the two gels Bio-Gel P2 and Fractogel HW-40 (S), Fractogel HW40 (S) gave the best separation of FeEDDHA, FeEDDHMA, FeDTPA and FeHEEDTA. CuEDTA, ZnEDTA and MnEDTA were found to co-elute with FeDTPA.

Capillary electrophoresis (CE) has become very important in the analysis of peptides, as it is a highly powerful mechanism of separation, and can analyse nanoliter sample quantities. However, detection methods employing UV, spectrofluorimetry, radio-labelling and mass spectrometry have limited sensitivity. A copper-coated capillary was developed for the determination of peptides by CE with electrochemical detection. A simple washing procedure produces a copper-coated column which is stable for 12 hours. Under alkaline conditions, peptides complex with Cu(II) from the walls of the column to form Cu(II)-peptide complexes which are subsequently oxidised at a carbon fibre electrode to form copper(III)-peptides. The system was shown to be applicable for the analysis of small peptides (five amino acids), and protected peptides, for which typical detection limits were in the $1x10^{-7}M$ range.

CHAPTER 1
METAL CHELATION IN SEPARATION SCIENCE

1.1 METAL CHELATION IN SEPARATION SCIENCE

The importance of metal chelation is widely apparent throughout the literature of analytical chemistry. By far the largest interest at present is in the area of the analysis of metal ions. Metal chelation is used to aid analysis at all stages of the analysis process, i.e. in metal ion extraction, preconcentration, separation and detection. Obviously the opposite case is also true, and thus when the analytical problem is the determination of chelating agents, chelation with metal ions can also be used. Formation of a chelate between the chelating agent with a metal ion aids both separation and detection. Alternatively, investigators may be interested in the quantity of a metal in a sample which is present as the free ion, or that which is chelated. Thus there are many areas in analytical chemistry where metal chelation is applicable.

This thesis deals with three applications of metal chelation chemistry. The first application deals with the analysis of the free transition metal ion content of anaerobic adhesives. The anaerobic adhesive contains a chelating agent such as EDTA, which is used as a masking agent, since the presence of free transition metal ions can cause premature curing of the adhesive. Thus, the task here was to determine only the free metal ion content. This was done by development of a solid-phase extraction technique which could distinguish between the free and chelated metal, with minimal disruption of the equilibrium between free and chelated metal in the sample. The extracted metal ions were then separated by reversed phase ion-pair chromatography using the complexing agent tartaric acid to aid separation. Detection was then achieved by post-column derivatisation with the complexing agent 4-(2-pyridylazo)resorcinol (PAR). Thus the chemistry of metal chelation was used throughout the determination process.

The second application involves the determination of the metal chelate composition of fertilisers. Here, attention to the metal chelate equilibrium, and the change of this equilibrium under imposed chromatographic conditions, was of paramount importance for accurate quantification. Reversed phase ion-pair chromatography was again used for the determination.

The third application involved the determination of peptides by capillary electrophoresis. Here the peptides were complexed in situ with copper(II) ions to enable electrochemical detection. The copper(II) ions were immobilised on the silica capillary column due to their attraction for the silanol group.

1.2 METAL CHELATION CHEMISTRY

A chelate has many properties. These include its solubility, stability, specificity, kinetics of reaction, and its photometric, electrochemical or fluorescent properties which enable detection. These factors will obviously vary from chelate to chelate. However, for a given chelate, they will also change depending on the environment (solvent, pH, ionic strength of the medium) of the chelate. Thus, chelation chemistry is far from simple, and the analytical chemist working with a metal chelate must firstly know the chemical and physical properties of a metal chelate, and secondly the effect which the manipulations he/she will carry out during the analysis will have on the chelate. This chapter gives a brief introduction to metal chelate chemistry. It explains how metal chelation chemistry can be used in various analytical applications. Since the largest area of analytical application is the analysis of samples containing trace metal ions, an explanation is given of the importance of the properties of the chelating agent used to aid extraction, separation and detection. Some samples may contain both free metal ion and metal chelate, and the information required is the quantity of each (i.e. speciation). This is a particularly difficult analytical problem, as analysis must be carried out without disruption of the balance between free and chelated metal, and thus where disruption occurs this must be accounted for. To understand metal chelate chemistry, one must first examine the main properties of the chelating agent, the metal ion and finally the formation of the metal chelate.

1.2.1 Ligands and Chelating Agents

A ligand donates electrons to an electron-deficient metal and thus forms a complex. Chelating agents are compounds that contain at least two atoms which are electron donating. In a chelating agent, the donor atoms are connected intramolecularly by chains of other atoms, and a chelate ring is formed for each donor atom, after the first which coordinates with the metal ion. Thus the word "chelate" (meaning crabs claw) is given to this structure. A stable chelate usually contains a five- or six- membered ring. The electron pair bonds formed between the electron-accepting metal and the electron-donating chelating agent may be "essentially ionic" or "essentially covalent", depending on the metals and the donor atoms involved. Among the various organic groups which may unite with metals by the replacement of hydrogen are the carboxyl, sulphonic hydroxyl, oxime, primary amino and secondary amino groups. Among the groups which combine by coordination are primary amino, secondary amino, tertiary amino, cyclic tertiary amino, oxime, alcoholic hydroxyl, carbonyl and thioether groups. Practically all the chelate rings known involve combinations

of the functional groups given. A common case is where the chelating agent has at least one acidic donor group (-OH) as well as one or more basic donor atoms, such as a nitrogen atom. During chelation, the acidic group loses a proton and becomes anionic donor, thus resulting in charge neutralisation. The basic nitrogen donates a pair of electrons to the metal ion. An example of such a chelating agent is 8-hydroxyquinoline (oxine).

1.2.2 Metal Ions in Aqueous Solution

Metal ions in aqueous solution are usually highly solvated, i.e. in aqueous solution a metal ion is more or less strongly bound to a number of water molecules so oriented that the negative (oxygen) end of water dipole is directed toward the positive metal ion. Due to the positive charge of metal ions, electrons in the surrounding solvated water molecules are displaced toward the metal, and loss of hydrogen ions from the bound water molecules takes place more readily than from free solvent molecules. Stepwise dissociation of hydrogen ions from the coordinated water molecules results in effect in the successive replacement of water molecules by hydroxide groups. Each hydroxyl group reduces the charge of the metal ion by one. When the positive charge of the metal ions has been neutralised by the hydroxyl groups, the ionic forces keeping the metal ions apart are essentially removed. The simple molecules thus formed usually polymerise into an infinite three dimensional lattice of metal ions, hydroxyl ions, and water molecules and precipitate from solution as the hydrous metal oxide or hydroxide as shown below.

$$M(H_2O)_x^{2+} \underset{H^+}{\overset{OH^-}{\rightleftharpoons}} M(OH)(H_2O)_{x-1}^{+} \underset{H^+}{\overset{OH^-}{\rightleftharpoons}} M(OH)_2(H_2O)_{x-2}\downarrow$$

A shift in the equilibria from left to right is favoured by an increase of the pH of the medium. The hydroxyl ion may also be considered a ligand or complexing agent, and the hydroxo forms of the metal ions may be considered a simple type of complex. When the hydroxyl ion concentration is high, many insoluble metal hydroxides react further to produce negative ions which are again soluble.

The equilibrium between an aquo metal ion and a chelating agent may be considered to involve the displacement of a weakly coordinating group, a water molecule, by a strongly coordinating group i.e. the chelating agent. Reaction of the metal ion with hydroxyl ions is prevented or decreased. For example, upon reaction of EDTA (ethylenediaminetetra acetic

acid) with the divalent transition metals, the resulting chelate compound is stable even in the highest pH solutions. However, the tendency of the iron(III) ion to hydrolyse is so great that ferric hydroxide is formed in solutions with a hydroxyl concentration greater than 10^{-6}M. For this reason, iron(III) hydroxide precipitates from iron(III)-EDTA solutions above pH 8.

1.2.3 The Formation of a Metal Chelate

The most important fact to consider when dealing with metal chelates is that the formation of metal chelates is an equilibrium reaction. This equilibrium is affected by such factors as pH, solvent and ionic strength. These factors either strengthen or weaken the chelate. In homogeneous solution, the equilibrium constant for the formation of the chelate complex from the solvated metal ion and the ligand in its fully dissociated form is called the formation or stability constant. Thus for FeEDTA

$$Fe^{3+} + Y^{4} \rightleftharpoons FeY^{-} \qquad K = [FeY^{4-}]/ [Fe^{3+}] [Y^{4-}]$$

The $\log_{10}$ stability constant for this reaction is 25.1 [1]. This is a relatively high value, which means there will be a large ratio of chelated to unchelated form of the metal when equivalent amounts of metal and ligand are present.

However, the stability constant is a very simplistic method of describing metal chelate reactions, and may often be misleading. This is because it is not possible in the majority of cases to prepare a solution in which all the metal is in the form of aquo ions and all the EDTA in the form of Y^{4-} ions. Side reactions caused by factors such as pH, buffers, masking agents and disturbing metal ions, can all affect the metal chelate equilibrium. Of these, pH has been studied the most extensively. In the pH range, chelating agents such as EDTA will exist in both protonated and unprotonated forms which are in equilibrium with each other. Metal ions compete with hydrogen ions for the available donor atoms. The hydroxyl ions in the water will act as competing ligands for the metal ions and will also form hydroxy compounds with the ligands. These simultaneous equilibria must be considered when calculating the stability constant which will obviously be affected by changes in pH. To describe this relationship, conditional stability constants are used. The calculation of the conditional stability constant requires a detailed knowledge of the system. For example to calculate the equilibrium constant of iron (III)-EDTA with regard to pH, one has to know the side reactions of both the metal ion and the chelate with both hydrogen and hydroxyl

ions. For example iron(III) forms with EDTA (H_4Y^-) not only the complex FeY^-, but also the complex FeHY, and two complexes $[Fe(OH)Y]^{2-}$ and $[Fe(OH)_2Y]^{3-}$, the stability constants all of which are known [2]. Figure 1.1 shows the stability constants of various metal EDTA complexes as functions of pH. This figure shows how maximum $\log_{10}$ values of many conditional constants such as 25.1 for iron(III)-FeEDTA differ greatly from the values of the concentration constants.

Because various species in solution are in a formation-dissociation equilibrium, displacement reactions of one metal or ligand by another are possible. Thus reactions such as

$$ML + M' \rightleftharpoons M'L + M$$

or

$$ML + L' \rightleftharpoons ML' + L$$

are possible. For equivalent amounts of M and M', the ratio of the formation constants for the two complexes must be 10^4 for 99% complete displacement of one metal in a complex by another.

Thus, when applying metal chelation chemistry to solve an analytical problem, these factors must be taken into consideration. It must be remembered that a metal chelate is not a static system, but will change in the degree to which it is associated/dissociated with changes in solvent conditions, pH, ionic strength and the presence of competing ions.

1.2.4 Kinetics of the Reaction

For most metal chelate applications in analytical chemistry it is important that the equilibrium condition is achieved quickly. Complexation of many metal ions is almost instantaneous, particularly for the divalent ones . However, reaction rates of many ions with higher valences may be slow. For example, reaction rates with Cr(III), and Co(III), may be very slow. Systems that equilibrate rapidly are termed "labile", and those that are slow to equilibrate are termed "inert". Inertness may give the appearance of stability, but a complex that is apparently stable because of kinetic inertness may be unstable in the thermodynamic equilibrium sense.

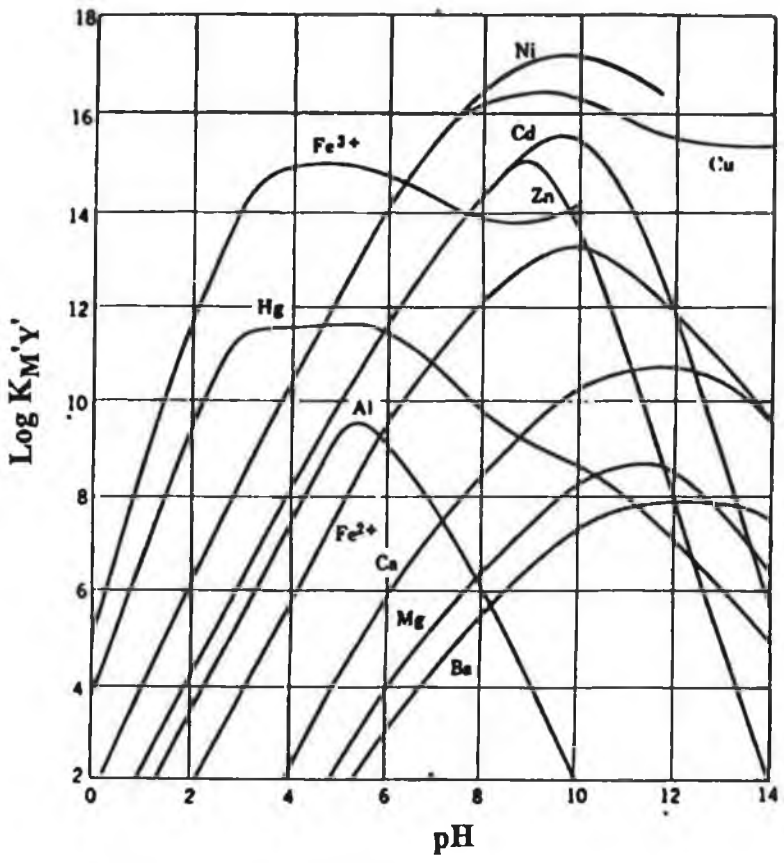

Figure 1.1. Stability constants of various metal EDTA complexes as functions of pH. From [1].

1.3 APPLICATION OF METAL CHELATION IN THE ANALYSIS OF TRACE METAL IONS

1.3.1 Chelation in Trace Metal Ion Extraction

The two most common extraction techniques are liquid-liquid extraction and solid-phase extraction. The formation of a chelate, is one of the many methods used to achieve extraction of trace metals. Extensive use is made of reagents such as dithizone, dithiocarbamates, 8-hydroxyquinoline etc. In liquid-liquid extraction studies, for quantitative assessment of extraction, use is made of the distribution coefficient i.e. the ratio of total analytical concentration in the organic phase to the total analytical concentration in the aqueous phase. Chelates are characterised by high distribution coefficients for extraction. The extraction theory of chelates is quite well developed. The extraction equation for a chelate is given by

$$M^{n+} + nHA_{(o)} \rightleftharpoons MA_{n(o)} + nH^{+}$$

where A stands for the anion of the reagent which is a weak acid (pK_a = 3-13), and n represents the metal ion charge, (o) denotes the organic phase. The extraction constant is equal to

$$K_{ex} = \frac{[MA_n]_o\,[H^+]^n}{[M^{n+}]\;\;[HA]^n}$$

Under conditions when all forms of a metal in the aqueous phase can be disregarded, excepting the M^{n+} ion, the ratio $MA_{n(o)}/M^{n+}$ represents the distribution coefficient.

$$D = \frac{K_{ex}\,[HA]^n_o}{[HA]^n_o}$$

$$\log D = \log K_{ex} + n\log [HA]_o + npH$$

This is the main equation which describes the extraction of chelates. The extraction constant depends on the stability constant of the chelate being extracted β_n, of the complex being extracted, reagent dissociation constant K_{HA}, distribution constant of the chelate $K_{D,MA}$ and the chelating agent distribution constant $K_{D,HA}$. This dependence can be expresssed by [3]

$$K_{ex} = \frac{\beta_n K_{D,MA} K^n_{HA}}{K^n_{D,HA}}$$

Thus, the better the extraction, the higher the stability of the complex and the greater its distribution constant. The greater the acidity of the reagent aand the less it goes into the organic phase, the greater will be the extraction. As the concentration of the reagent increases so does the degree of extraction. The control of pH is very important. Extraction efficiency will be found to increase with increasing pH until a plateau is reached, and then decrease. This is due to the fact that as the pH increases new metal species begin to appear in the aqueous phase, for example MA_{n-1}, and finally MA_n (the plateau region). At still higher pH values metal hydrolysis is seen; sometimes the formation of MA^-_{n+1} metal ion complexes takes place and extraction decreases.

Thus for optimum trace metal extraction, the reaction conditions must be optimised to favour maximum chelation. The chelating agent must also be carefully selected to ensure the necessary degree of selectivity towards the metal ions to be extracted in the presence of interfering ions. Similarly, the competition of other chelating agents within the sample matrix must be considered. Thus the chelating agent used for extraction must be sufficiently strong to displace any other ligands or chelating agents in the sample matrix. Such sources of interferences can only be accounted for by a knowledge of the sample matrix and recovery studies. Thus the sample should be spiked with the metal ion to be determined and the percentage recovered used to aid quantification. Such studies should be carried out over the concentration range to be determined. Where a mixture of metal ions is to be extracted by using a single chelating agent then the effect of different ratios of the individual metal ions in a sample containing a mixture of the metal ions should be determined. This is due to the fact that each of the metals ions will have a different stability constant with the chelating agent. Thus, there will be a preferential chelation of certain metals ions over others. When the ratios of metal ions changes, the recoveries of metal ions may also change.

Solid phase extraction is becoming very popular, and for many applications is replacing liquid-liquid extraction. This is due to the fact that the quantities of glassware and solvents used is less, it is more easily performed, and automation is simpler. Several sorbent methods have been developed for the preconcentration of transition metals. Much research has focused on the use of chelate sorbents, where a chelating agent is chemically bound to a support material. Alternatively, chelating agents may be adsorbed to a general sorbent such as C_{18}, silica, alumina, activated carbon or cellulose, and the metal ion chelated as the sample passes through. Addition of the chelating agent to the sample, followed by adsorption of the chelate on a general sorbent, is also used. Cation exchange may obviously also be used; however, anion exchange of the metal in its chelate form can also be performed. As with liquid liquid extraction the same principles apply for optimisation where chelation is used. Sample conditions are thus adjusted to ensure optimum chelation, and the sample is passed through a pre-conditioned solid phase extraction cartridge. The metal is retained as the chelate on the cartridge. The cartridge is then washed with appropriate solvents to remove matrix contaminants which may also have been retained. Finally the cartridge is washed with a solvent to remove the metal ion. Thus extraction and preconcentration are achieved. Unfortunately many of the extraction materials which have been developed, are not commercially available in a pre-packed cartridge

Where quantitation of the metal ion is of interest, then extraction is optimised to achieve maximum recovery of the metal ion. However, where the analysis involves distinguishing between free and chelated metal in the sample, the extraction of metal ion must be carried out without disruption of the metal chelate equilibrium. This is a much more difficult situation, as a chelating agent or complex must be used which does not abstract metal ion from the chelate present in the sample, but which extracts free metal ion under the conditions present within the sample. This area is discussed in more detail in chapter 2.

1.3.2 Chelation in Trace Metal Separation

Various methods have been developed in which metal chelation is used to aid the separation of metal ions. The two principal approaches involve pre- or on-column chelation with a strong chelating agent, followed by separation on a reversed-phase HPLC column. The other most popular approach involves the use of an ion-exchange column, with the addition of weak complexing agents to the eluent to aid separation. Both applications require a knowledge of the properties of chelating agents, and thus the appropriate chelating agents

to use. For the analysis of metal ions by reversed-phase chromatography, after complexation with a strong chelating agent, the following conditions and prerequisites can be deduced from the important properties of the most successful ligands used [4]:

- formation of stable neutral metal chelates
- control of complex formation by pH-variation or by use of masking reagents
- formation of well defined chelates with clear stoichiometry
- formation of conjugated, five membered chelate rings between metal and ligand, because these possess the highest stability. Four and six membered chelate rings reduce stability or range of application (an exception being the S,S-four-ring of the dithiocarbamates or six-rings with β-diketone structure)
- chelating agents with N-, O-, S- or Se-atoms as coordination sites possess good complex chemical properties. In addition these ligands are for analytical application sufficiently stable under normal circumstance
- to receive compact, well resolved elution profiles, the metal chelates have to be reasonably soluble in the mobile phase
- A ligand must not be too voluminous, so that the element specific properties of the central atom are maintained and levelling of the chromatographic behaviour of the complexes will be avoided
- the chelating conjugated-π-system should cover the complete ligand for sensitive photometric detection (high extinction coefficients)

Numerous chelating agents have been used. The most popular chelating agent developed are the dithiocarbamates. Other popular reagents include dithizone, oxine, beta-diketones, beta-ketoamines, hydrazones, diphosphinates, crown ethers, pyridine, 4-(2-pyridylazo)-resorcinol (PAR), 1-(2-pyridylazo)-2-naphthol (PAN),porphyrins, and EDTA. Many excellent reviews have been produced on this area [4-7]

Metal complexing agents, such as tartaric acid, citric acid and oxalic acid, have also been used to aid the separation of metal ions by ion-exchange chromatography. The stability constants for the formation of metal ion complexes govern the effectiveness of the separation of metal ions by ion-exchange chromatography. Complexing agents are chosen which have substantially different stability constants for the metal ions of interest. The kinetics of complexation reactions are also critical as separations occur typically within fifteen minutes. Riviello [8] studied the kinetic effects of the complexation reactions of Mn(II), Fe(II), Co(II), Ni(II), Cu(II) Cd(II), and Zn(II) with oxalic acid on the separation of

these metal ions by ion-exchange chromatography. In a well packed cation exchange column the major contributor to band spreading, and thus column inefficiency was found to be mass transfer in the mobile phase. Thus the rate of diffusion of the sample ion between the resin and eluent are important when considering complex equilibria and ion-exchange reaction. With an eluent containing a complexing agent various equilibria are established as shown in Figure 1.2. In order to maintain reasonable column efficiency, the rates of complexation and diffusion must be similar. While oxalate allows for the effective separation of many transition metals by cation exchange, a loss in column efficiency is observed for all metals except Cu(II). Similar diffusion and complexation rates minimise the finite time for the equilibrium distribution of the metal ion between the complexing eluent and the stationary phase. Minimising the finite time for equilibrium distribution reduces the contribution of complex equilibria to band spreading.

When using this system for determination the presence of chelating agents in the sample must be considered. Chelating agents within the sample may compete with the complexing agents in the eluent and thus removal before analysis may be necessary.

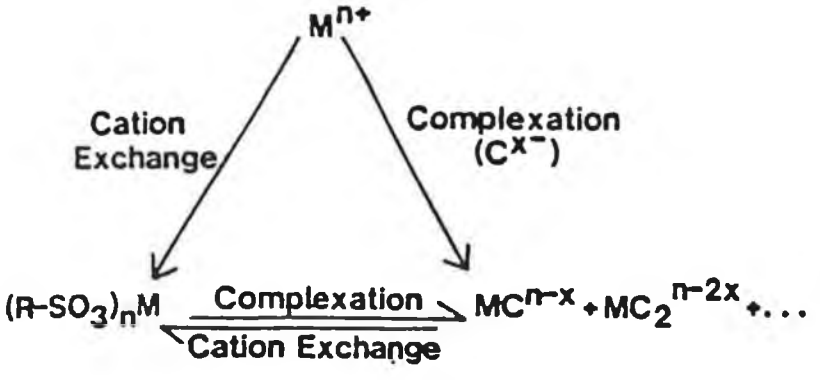

Figure 1.2 Equilibria in the separation of transition metals by cation exchange chromatography [8]

1.3.3 Chelation in Trace Metal Ion Detection

1.3.3.1 Photometric Detection

For metal chelates, two types of light absorption usually occur in the visible and ultraviolet regions - absorption arising from electronic transitions in a conjugated system in which the metal may or may not take part, and absorption arising from electronic transitions in the metal ion itself. The transition in conjugated systems is usually very intense and is frequently observed both in the ligand and in the metal chelate. When the electrons are tightly bound, these transitions occur in the ultraviolet region, since the frequency and energy of the light absorbed are relatively large. A number of factors are involved in decreasing the energy of electron transition sufficiently to give absorption in the visible range. One is the loosening of the binding of electrons so that they are more easily displaced. This accounts for the intense colour of the chelate compounds of sulphur donor atoms, in which the valence electrons are loosely held. Also, a long conjugated system involving an electron shift between two or more polar resonance forms would give rise to absorption in the visible region. It is important that the electron shifts which result in absorption of light involve a change in the dipole moment in the molecule, since such a shift is required to interact with the electromagnetic field in light radiation. This requirement is usually met when it is possible to write one or more structural formulas involving a shift of positive or negative charges. The absorption bands characteristic of a metal ion alone are usually relatively weak, and they result from electron shifts in unfilled *d*-orbitals of the transition metals. An example of such a metal is copper. These transitions are frequently greatly intensified by coordination of the metal ion.

A photometric reagent commonly used in chromatographic applications for post-column detection of transition metals is 4-(2-pyridylazo-)-resorcinol. This reagent has become very popular due to its solubility in water and its ability to complex with a range of transition metals. There are four chromophoric species of the chelate depending upon the pH, and thus the formation and detection of transition metals with this reagent is very pH-dependent [9]. Although the reaction kinetics are fast, the peak height will increase with increased reaction coil length [10]. Reaction coil temperature may also affect the result achieved with a spectrophotometric reagent. Usually PAR is used as a post-column reagent when separation is achieved by addition of complexing agents to the eluent. Thus the equilibrium constant for the replacement of the on-column anionic complex by the PAR complex should be large and the PAR complex should be kinetically stable.

The scope of metal ions determined by ion chromatography using post-column derivatisation with PAR has been limited to Mn(II), Fe(II), Co(II), Ni(II), Zn(II), Cd(II), Pb(II) and the lanthanide metals [11,12,13]. However, it is well known that PAR is a sensitive colorimetric indicator for many other transition metals. Investigation of the post-column chemistry of PAR has shown that this limitation has been due primarily to the kinetics of complexation of PAR with metal ions. It has been found that the use of the organic chelators in the eluent significantly reduces the kinetics of complexation of PAR with many metals ions, such that detection cannot be achieved [14]. It has been observed, however, that certain ligands can be added to the post-column solution which enhance the kinetics of complexation formation in the presence of chelators in the eluent. It has been proposed that these ligands solvate the metal-eluent complex in such a way as to enhance the kinetics of ligand exchange between the metal and PAR. Ligands which have been found to have such a property include carbonate, ammonia, phosphate and alkanolamines. Thus chromium, mercury, vanadium and gallium can be determined using PAR containing 1 M phosphate buffer at pH 8.8, using the complexing agent pyridine-2,6-dicarboxylic acid. Other systems have also been used such as Eriochrome Black T [15] and mixtures of Eriochrome Black T with MnEDTA [16]. These systems are dealt with in more detail in chapter 2.

1.3.3.2 Electrochemical Detection

The combination of a metal ion with an electron donor always increases the electron density around the metal, and complex formation will tend to increase the tendency toward oxidation to a higher valence state. Thus the oxidation potential of the metal complex, or of the metal ion in equilibrium with complexing agent, is usually increased by complex formation.

A second factor which stabilises the higher-valence state of a metal is the formation of a negative complex or chelate compound. If the metal complex formed is negative, an increase in the oxidation state of the complexed metal ion results in a stabilising reduction in the charge of the complex. Therefore the increase in oxidation potential which usually accompanies complex formation will be greater when the complex formed is negatively charged. A third factor, namely the tendency to form a stable electron configuration in the metal orbitals may result in either an increase or a decrease in the oxidation potential. The symmetrical inert gas configuration with completed $3d$, $4s$ and $4p$ shells is much more stable than arrangements having one electron more or less. For example, the covalent iron and cobalt complexes with an octahedral arrangement of donor groups have stable inert gas

electronic orbitals when iron is in the divalent state and cobalt is trivalent. Thus the tendency to complete the available orbitals will favour the higher valence state for cobalt, but will stabilise the lower valence state for iron. Detection by electrochemical reaction of metal chelates is not as popular as UV spectrophotometric detection. A well known application involves the electrochemical detection of Ni(II), Cu(II), Co(II), Cr(VI) and Cr(III) as dithiocarbamate chelates which was popularised by Bond and Wallace [17,18]. In this thesis complexation of peptides with copper is used to enable electrochemical determination.

1.4 Application of Metal Chelation Chemistry in the Analysis of Chelating Agents and Metal Chelate Content.

Chelating agents are important in many areas such as tinned food, health care preparations (creams, shampoos etc.) and adhesives. The chelating agents are added to stabilise the product by complexing the metal ions present and thus preventing deleterious degradation of the product. In these situations the total quantity of chelating agent within the sample needs to be assessed. Conversion of the chelating agent to the metal chelate is usually used to aid separation and detection. Here the important factor to consider is that all the chelating agent is present in one metal form. Thus the interfering metal ions must be removed, or alternatively analytical conditions must ensure that the metal chelate being used for detection has the strongest formation constant. Thus for example complexation with iron is typically used for the determination of EDTA as within a certain pH range this has a higher formation constant with EDTA than typical interferences such as copper, zinc or manganese. Analysis is thus achieved by addition of excess iron prior to analysis [19]. This area is dealt with in more detail in chapter 3.

Many of the above applications also require a knowledge of the specific metal chelate content and the content of free metal ion. This information can be used to assess the necessity of the addition of more chelating agent to ensure stabilisation, and also to investigate the effectiveness of the chelating agent used. In such situations, analysis must not alter the metal chelate equilibrium present within the sample. Where the analysis, such as separation using liquid chromatography, will disrupt the metal chelate equilibrium, this must be accounted for by the use of standards [20,21]. Association and dissociation reactions of metal chelates which occur due to manipulations and imposed analytical conditions should be considered. [20,21] This type of analysis is discussed in more detail in Chapter 3.

1.5 Concluding Remarks

Thus the complexity of applications involving metal chelate chemistry may be visualised. Due to the variety of chelating agents available there is a wide range of applications of chelation in analytical chemistry. This brief introduction has shown how when using an analytical procedure in which metal chelation is involved, an understanding of metal chelation chemistry is essential to achieve a meaningful result.

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CHAPTER 2

THE ANALYSIS OF TRANSITION METAL IONS IN THE PRESENCE OF THEIR EDTA CHELATES IN ANAEROBIC ADHESIVES

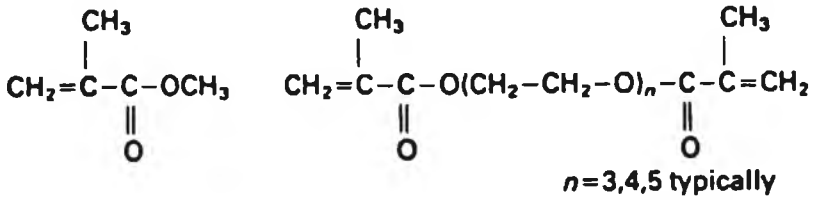
2.1 APPLICATIONS OF ANAEROBIC ADHESIVES

Anaerobic adhesives are used in the manufacture of many mechanical products, such as cars, aeroplanes and typewriters, to name but a few. Their use increases the speed of production and increases reliability of the finished product. There are four main areas of application. These are locking threaded assemblies, sealing threaded porous and flanged assemblies, strengthening cylindrical assemblies, and structural bonding. Threaded assemblies may loosen or unwind due to vibrations or overload; placing an adhesive between the metal parts locks them together and increases the resistance to such behaviour. Impregnation sealants, when painted over a surface under vacuum, migrate into pores, cracks, and surface imperfections in castings, or welds and improve mechanical properties such as machineability, which result from the smoothing of surfaces due to the filling of voids and crevices. Designers of cylindrical assemblies, such as bearings or gears, have traditionally relied upon interference fits to provide structural integrity. This requires careful machining and a high grade surface finish. However, adhesives can be used to fill the voids between the metal parts, thus increasing metal to metal surface contact, and decreasing the necessity of a high grade surface finish. Structural bonding involves the assembly of flat structurally bonded parts, and may involve dissimilar substrates such as glass to metal or wood to metal. Thus it can be realised how anaerobic sealants simplify assembly procedures and increase assembly rates by lowering scrap and rework which normally occur with mechanical methods.

Many other adhesive systems are two component systems, the components of the adhesive which initiates the curing of the adhesive being added just before use. Anaerobic sealants, however, are one component systems, as the cure process is inhibited by the presence of oxygen. Thus, the adhesive will remain stable in liquid form until confined between for example a screw and a thread where it is starved of oxygen. At this point the cure process is no longer inhibited, and thus the adhesive solidifies. Thus anaerobics fit easily into high volume production operations, because the single component material eliminates mixing and pot life problems occurring with two component adhesive systems. This results in cost savings for users.

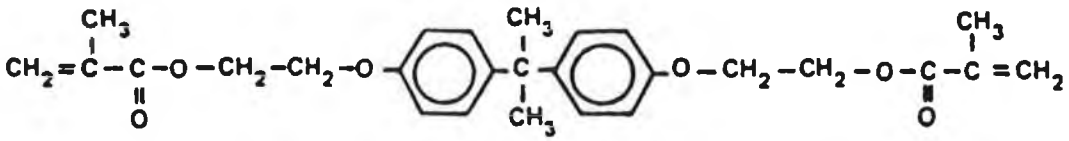
2.2 TYPICAL COMPOSITION AND CHEMISTRY OF AN ANAEROBIC ADHESIVE

The purpose of this section is to give the analytical chemist an appreciation of the matrix of anaerobic adhesives and thus the considerations when extracting metal ions in the presence of their chelates from this matrix. Anaerobic adhesives are predominantly organic liquids, as the main ingredient in an anaerobic adhesive is a liquid monomer. The remainder of the components in the adhesive are present to ensure that in the absence of oxygen the liquid monomer will polymerise to form a tough heat and solvent resistant solid polymer - the adhesive. Methacrylate esters, and less commonly acrylate esters, are the monomers of choice. Some examples of commonly used methacrylate esters are reproduced in figure 2.1

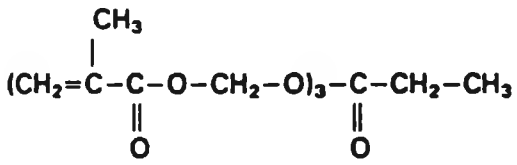


Methyl methacrylate (I)

Polyethylene glycol dimethylacrylate (II)



Ethoxylated bisphenol A dimethyacrylate (III)



Trimethylolpropane trimethacrylate (IV)

Figure 2.1 Methacrylate esters commonly used in the synthesis of anaerobic adhesives from [1]

The remaining components of an adhesive are free radical initiators, accelerators, free radical inhibitors, metal chelating agents, plasticisers, thickeners, inert fillers, pigments and dyestuffs. These are formulated in rigidly controlled concentrations to give the correct balance between speed of cure and stability.

Initiators, as the name suggests, initiate polymerisation. A wide variety of free radical initiators have been described, of which hydroperoxides are the most important. Cumene hydroperoxide, as discovered by Krieble in the 1950's, is commonly used . The equations given below represents a simplified schematic of the reaction when a mixture of the monomer and hydroperoxide are placed between active metal surfaces and air is excluded.

$$R\text{-}O\text{-}O\text{-}H + H^{+} + M^{n+} \rightarrow RO^{\bullet} + M^{(n+1)+} + H_2O$$

$$R\text{-}O\text{-}O\text{-}H + M^{(n+1)+} \rightarrow M^{n+} + R\text{-}O\text{-}O^{\bullet} + H^{+}$$

$$2ROO^{\bullet} \rightarrow 2RO^{\bullet} + O_2$$

$$RO^{\bullet} + C=C \rightarrow RO\text{-}CH_2\text{-}CH_2^{\bullet} \quad \text{etc.}$$

Thus the active metal surface participates in the cure process. Active metals are those having two adjacent oxidation states such as the transition metals Fe(II)/Fe(III), Cu(I)/Cu(II), and Co(II)/Co(III) . When the hydroperoxide comes in contact with the active metal, free radicals are generated via a reduction-oxidation reaction involving metal ions. The metal has a catalytic effect on the hydroperoxide initiator, one transition metal ion being able to produce many free radicals. In the absence of oxygen the free radicals generated initiate polymerisation of the monomer by direct addition to one of its terminal double bonds. If oxygen is present, however, the methacrylate free radicals formed will react more rapidly with oxygen than with the terminal double bonds, and they thus form peroxy free radicals.

These peroxy free radicals are capable of reacting with further terminal double bonds. However the rate of this reaction is very slow,compared with that of the formation of the peroxy radicals and so a bottleneck is created.

The formulation of this basic monomer-hydroperoxide system was followed rapidly by work that identified the trialkylamines as accelerators of the polymerisation process. The exact mechanism of this acceleration has never been completely defined. It was also found that the presence of oxygen alone was frequently inadequate to assure non-gelation of the product during storage. The problem was solved by the addition of free-radical stabilisers, for example quinones, to the anaerobic adhesive formulations. Hydroquinone is a commonly used stabiliser.

The anaerobic formulations prepared containing the above ingredients were low viscosity liquids which could cure to only a single strength. To obtain a range of viscosities and ultimate cure strengths, the use of a range of polymeric thickeners including polystyrene, and polymethacrylates were used. Plasticisation of the adhesives, with for example the polyethyleneglycol octanoates, gave the capability of producing formulations with a range of different cure strengths.

On inactive surfaces, such as zinc and cadmium plate and passivated stainless steel, where the concentration of transition metal ions is extremely low, the adhesives often remain essentially uncured for long periods. The solution to the problem of slow cures on inactive surfaces was discovered in the mid-1960's when it was found that a dialkylarylamine in combination with saccharin afforded systems with the desired curing characteristics and long-term shelf stability.

As transition metals play an active part in the cure process, it is necessary to eliminate transition metal contamination. Every effort is made to exclude the metals from the sealant during the manufacturing process through rigorous control of both raw materials and plant and processing conditions. However, there will always be some contamination and thus chelating agents such as EDTA are added to complex the transition metals.

2.3 THE REQUIREMENTS OF AN ANALYTICAL METHOD FOR THE ANALYSIS OF TRANSITION METAL IONS IN ANAEROBIC ADHESIVES

As explained in section 2.2, transition metals form an intrinsic part of the cure process and thus the presence of transition metal ions in the adhesive may cause premature polymerisation before use. To tackle transition metal ion contamination a chelating agent such as EDTA is added to inactivate the metal ions.

Thus to support the production process, analytical capability should provide the following information on the transition metal content of adhesives:

- the method of analysis developed should determine copper, iron, lead, nickel, cobalt, zinc, cadmium and manganese at sub-trace levels
- the analytical method used should determine free metal ions only, i.e. it should be capable of distinguishing between free and chelated metal. To give a true picture of the quantity of free metal ion, the method must not disturb the existing metal-chelate equilibrium in the adhesive as manufactured
- the valence state of the metal should be determined and the method of analysis should not alter the valence of the metal
- the method should provide simultaneous measurement of the above metal ions, and be easy to perform.

Atomic absorption spectroscopy (AAS) was the original approach used for the determination of metal content of sealant at Loctite. However, the method only determines total metal content. Whether the metal is chelated is not known, and the valency of the metal cannot be determined. Also each element must be determined individually, making the method time consuming and laborious.

Thus investigation of chromatographic methods was initiated. The following sections provide a background to the choice of chromatographic methods which have been developed for the analysis of metal ions.

2.4 AN INTRODUCTION TO CHROMATOGRAPHIC ANALYSIS OF TRANSITION METALS

As transition metal ions have very similar properties, chromatographic methods using metal complexation or chelation have been developed. Metal chelation may be used pre-columnb or "in-situ" [2,3] to aid separation [4-6] and "post-column" to aid determination [7-9]. The application of gas chromatography following the conversion of metal ions to volatile chelates, has also been used but has not gained the same popularity. The last three years, however, has produced a small number of references to the use of capillary electrophoresis involving metal chelation [10-16].

Analysis of trace metals by chromatographic means has become popular as it has many advantages when compared to spectroscopic methods such as atomic absorption spectroscopy (AAS), inductively coupled plasma (ICP) or direct current plasma (DCP). Separations developed to date typically allow the simultaneous determination of eight to ten metal ions. This is both labour and time saving, especially when the typical metal composition of a sample is not known. Spectroscopic techniques such as AAS only give the total amount of metal present; however, with chromatographic analysis, speciation is possible. Thus the oxidation state of a metal may be determined, which is very significant in many industrial, environmental and biological applications. Also, by careful optimisation of chromatographic conditions, a trace amount of one metal may be determined in the presence of a large quantity of another. The presence of matrix interferences can also be more easily detected and removed than with spectroscopic techniques. Chromatographic techniques are also very sensitive and allow determinations at the ppb level. Also chromatographic methods have low operational costs. However, if one is only interested in high throughput for the analysis of metals in ideal matrices, such chromatographic techniques fall short of the speed offered by AAS.

Chromatographic trace metal analysis has thus found application in many fields of study. For instance toxicology where a change in the oxidation state of the metal can change the toxicological properties of the metal ion. In the cycling of metals in the environment and the transformations which they undergo as they pass through the environment or mammalian systems, the ability to determine metal speciation becomes very important. Another area of application is the analysis of industrial effluents where it is necessary to monitor for a range of heavy metals prior to discharge. Also in the area of pharmaceuticals for the analysis of inorganic drugs which have active metal ingredients, or organic drugs used in chelation

therapy which have functional groups with high affinities for metal binding. Many products contain chelating agents which act as masking agents preventing the deleterious effects of free metal ion contaminants. Chromatographic analysis can thus be used to distinguish the chelating agent content, the free metal ion content and the quantity of chelate.

2.4.1 Gas Chromatography

To enable determination by gas chromatography, the analyte must be volatile and stable at the temperature of the separation. It must also be neutral to guard against unwanted column interactions. Most metallic compounds or moieties, as a direct result of their ionic nature, cannot be directly volatilised at the temperatures used in commercially available gas chromatographs. Uden and Henderson [17] have listed eight constraints which prevented the development of GC in this application area. These include the limitation of chelating agents to neutral, highly stable species, unhelpful column interactions, and the need to synthesise most derivatising reagents. The range of organic ligands that have been found to be suitable for GC analysis has been relatively limited, but within these classes a considerable amount of development and application has been done. Beta-diketonates, beta-thioketonates, beta-ketoaminates, dialkyldithiocarbamates, dialkyldithiophosphinates and metalloporphyrins have all been used. These have been well reviewed by Schwedt [18], Uden [19], and Nickless[20]. However, the high hopes placed on GC in the 1960's as a method of elemental analysis have remained largely unfilled, as multielement analysis of practical utility have been described in just a few isolated cases.

2.4.2 Liquid Chromatography

2.4.2.1 Analysis of Metal Chelates

The development of liquid chromatography (LC) for the determination of metal ions began in earnest in the early 1970's and has grown steadily since that time. The original approach to analysis involved pre-derivatisation of the metal ion with a chelating agent, followed by chromatographic separation, usually by reversed-phase LC. Certain conditions and prerequisites can be deduced from the important properties of the most successful ligands used [21]: These conditions have been outlined in section 1.3.2.

Numerous chelating agents have been used in this regard, the most popular class of chelating agent developed being the dithiocarbamates. Others popular reagents include dithizone, oxine, beta-diketones, beta-ketoamines, hydrazones, diphosphinates, crown ethers, pyridine, 4-(2-pyridylazo)-resorcinol (PAR), 1-(2-pyridylazo)-2-naphthol (PAN),porphyrins, and EDTA. Many excellent reviews have been produced in this area [18,20,21,22]

Generally, the method consists of a pretreatment step involving addition of the chelating agent to the sample in a solution similar to the mobile phase, or in some other aqueous solution, followed by solvent or solid-phase extraction. The sample containing the chelate dissolved in the mobile phase or in the same solvent used for extraction is then injected onto the column . The derivatised sample is usually separated using a reversed phase column with an organic modifier such as methanol or acetonitrile. A second approach involves "in-situ" formation of the complex [4,5]. Here the complexing agent is added directly to the mobile phase. The free metal ion may then be injected and complexation thus takes place "on-column". For this approach to be successful, the kinetics of the reaction must be fast enough for formation during the separation time. As shown by Bond and Wallace [5] for a mixture containing Cu(II), Ni(II), Co(II), Cr(III) and Cr(VI) to be determined by complexation with sodium dithiocarbamate, "in-situ" complexation was only suitable for the determination of Cu(II) and Ni(II). In the case of Cr(III) and Cr(VI), complexation was found to take up to three hours. While on-column complexation simplifies the analysis procedure, there are some disadvantages. Sensitivities for on-column techniques may not be as good as for pre-column techniques, [5]. Several authors have reported peak splitting and spurious peaks. Hutchins et al. suggested that the peak shapes were poor because of exchange reactions of the chelates with the nickel in the stainless steel columns [23]. They tried to suppress this effect by the addition of EDTA to the mobile phase and the use of plastic-walled columns. However the EDTA totally displaced cadmium and lead ions form their chelates and they were unretained. Smith et al. [24] tested this hypothesis by leaving samples containing lead ions in the injector loop for varying times before injection. They also used different flow rates to change the residence time of the chelate in the column. In each instance the results were effectively the same with no evidence of an interaction changing the peak shape or reproducibility. It was shown that the problem was due to the low solubility of the heavy metal chelates in the typical methanol-water mobile phase mixtures. The problem was overcome for the determination of lead and mercury by addition of chloroform (10%) to the mobile phase. In the case of precolumn derivatisation involving a solvent extraction, this complicates the automation of the chromatographic system. In addition, the extraction efficiency of the pretreatment step must be considered, especially when dealing with samples

containing competing ligands or very high or low pH values. Many matrix species absorb in the UV region and pose interference problems when present in the sample. However despite these disadvantages, examination of the literature over the last five years since the review of Marina et al. [25], shows that determination by chelation still remains a popular technique for the analysis of trace metals. New chelating agents have been investigated and improvements have been made especially in the area of separation methods. Separation methods, such as ion-pair reversed-phase HPLC [21,26] are becoming more popular, as have methods where the column is precoated with the chelating agent [27,28]. Improved separations have resulted from the use of supercritical fluid chromatography [29], micellar electrokinetic chromatography [10] and capillary electrophoresis [11-15].

Hoshino et al. [30] investigated the use of six 2,2'-dihydroxyazobenzene derivatives for the determination of Al(III), Co(III), Cu(II), Fe(III), Mo(VI), V(V), Zn(II) by ion-pair reversed-phase chromatography (IP-RPHPLC). However the separation achieved was not very efficient.

Siren and Riekkola [26] investigated the use of ion-pair chromatography for the separation of Cu(II), Pd(II), Fe(III) and Co(III) as ion-associates of their 1-nitroso-2-naphthol-6-sulphonate (126NNS) and 2-nitroso-1-naphthol-6-sulphonate (216NNS) anionic complexes with organic ammonium and inorganic cations. Very efficient separations were achieved using the 126NNS reagent ion-paired with cetyltrimethylammonium bromide in a gradient elution from 70-90% methanol. The compounds were identified spectro-photometrically with a diode-array detector, with detection limits in the ng/ml level.

Uehara et al. [2] investigated the use of salicylaldehydebenzoylhydrazone (SAB) as a pre-derivatising reagent for the determination of aluminium by ion-pair IP-RPHPLC. This reagent is highly fluorescent, and detection limits of 0.3ng/ml were obtained. The separation was achieved on a C_{18} column with an eluent consisting of tetrabutylammonium bromide, EDTA and sodium acetate in aqueous acetonitrile solution. A chromatogram obtained for a solution of eleven metal ions showed the method to be highly selective for aluminium. Although iron, vanadium and cobalt were also detectable, it was thought that the other SAB chelates decomposed on the column owing to their labile nature. Increased sensitivity and selectivity were achieved when fluorescence detection was used as opposed to absorbence measurement. It is very surprising that more fluorescent and electrochemically active derivatising reagents have not been developed.

Faltynski and Jezorek [27] investigated the separation of Mn(II), Cd(II), Zn(II), Co(II), Ni(II), and Pb(II) on several silica-bound azo-coupled chelating stationary phases. The stationary phases used were 2-methyl-8-quinolinol silica gel, N-(1-naphthyl)ethylenediamine silica gel, o-acetoacetylphenol (diketone) silica gel, thenoyltrifluoroacetone silica gel and diphenylthiocarbazone (dithizone) silica gel. Contrary to the claims of the author, however, none of these stationary phases gave efficient separations of the above metal ions.

The chromatographic properties of silica-immobilised 2-pyridinecarboxyaldehyde phenyl hydrazone as stationary phase for the separation of Mn(II), Fe(II), Cd(II), Zn(II), Co(II), Pb(II) and Cu(II) were investigated by Simonzadeh and Schilt [28]. Separations achieved, however, were inefficient in mobile phases containing chloride, perchlorate or oxalate anions.

Laintz et al. [29] proposed to have found the solution to many of the problems associated with liquid chromatography of diethyldithiocarbamates (DDC) by using bis(trifluoroethyl)dithiocarbamate (FDDC) in combination with supercritical fluid chromatography (SFC). The authors demonstrated that FDDC forms stable complexes with As(III), Bi(III), Co(III), Fe(III), Hg(II), Ni(II), Sb(III) and Zn(II) and achieved a very efficient separation of these complexes by capillary supercritical fluid chromatography using CO_2 as mobile phase. The fluorinated ligand is superior to its hydrogenated form DDC, with respect to thermal stability and solubility in supercritical CO_2. While FDDC was used in the 1980's for determination of metal ions by GC, SFC has the advantage of much lower injection temperature (room temperature) and oven temperature (100 °C), which reduces the risk of sample decomposition. It also overcomes problems associated with the HPLC separation of DDC complexes such as solubility and instability in the mobile phase. Metal-DDC complexes are sparsely soluble in water, and decompose depending on the pH of the solution. These problems are not encountered in SFC using CO_2 as a mobile phase. An efficient separation of all the above metal ions was obtained in less than twenty minutes.

During the last five years a number of references have been published, dealing with the analysis of trace metals by capillary electrophoresis. Saitoh et al. [10] developed the first application of micellar electrokinetic capillary chromatography for the separation of metal chelates. The resolution of PAR chelates was found to be excellent on a 60 cm x 0.05 mm i.d. silica capillary filled with a 0.02M sodium dodecylsulphate micellar eluent at an applied voltage of 16.5kV (driving current 12mA). The theoretical plate number for the chelates

reaches to more than 1 x 10^5 per 60cm. The elution behaviour was discussed in terms of electrokinetic and micellar partition characteristics of the chelates. A very efficient separation of Co(II), Ni(II), Cr(II) and Fe(II) was obtained. a-Hydroxyisobutyric acid has also been used for the separation of Cd(II), Pb(II), Zn(II), and Cu(II) [19,20]. Swaile and Sepaniak [12] used CZE and on-column chelation with 8-hydroxyquinoline-5-sulphonic acid for the determination of Ca(II), Mg(II), and Zn(II). The complexes were selectively and sensitively detected using laser-based fluorometry. Limits of detection in the ppb range were achieved. Iki et al. [14] used PAR with UV detection for the determination of Ni(II) and Co(III). Copper was found to co-elute with the reagent peak. Zelensky [15] et al. employed on-column complexation with xylenol orange for the photometric detection of Zn(II), Mn(II) and Cd(II), with separation by capillary isotachophoresis. Regan et al. [16] used chelation with PAR for the determination of Co(II), Fe(II), Cu(II) and Zn(II) by CE with UV detection. Peak stacking was used to decrease the limit of detection. Detection limits of 1 x 10^{-8} M were obtained for Co(II), Fe(II) and Zn(II) and 4 x 10^{-7} M for Cu(II).

2.4.2.2 Ion Chromatography

To determine ions directly a detectable property had to be used. Conductivity of the ion was the obvious solution, however, the problem was distinguishing the conductivity of the analyte from the mobile phase after elution of the metal from an ion-exchanger. Small et al. [31] developed a chromatographic procedure that used a novel two-column ion-exchange system wherein the first column serves to separate the ions of the sample, while the second serves to suppress the conductance of the eluent while actually enhancing the conductance of the sample ions. A cation suppressor, however, contains a high capacity strong base anion exchange resin which exchanges the eluent anion (typically Cl^-) with a hydroxide ion. Thus this system was unsuitable for the analysis of transition metals, and in many cases due to practical reasons such as maintenance of the suppressor, undesirable. Also, one single manufacturer held the patent for the suppressor device design. For these reasons, alternative solutions to the analysis were quickly sought. Alternative approaches have involved low conductivity eluents or the use of different detectors. Investigation of low conductivity eluents proved that the sensitivity is not good, as the background is still relatively high. A major development occurred when post-column derivatisation was introduced based on post-column derivatisation of the metals with PAR reagent before photometric detection, this method extended the scope of ion-chromatography to include transition metal ions [32].

As the selectivity coefficients for separation of transition metal complexes having the same charge are essentially the same on a cation exchanger, an anionic complexing ligand was added to the mobile phase to aid separation. The criteria for choosing a complexing ligand are as follows;

- metal ion and ligand must form neutral or anionic complexes
- large differences in the formation constants of the complexes of the various metals lead to increased selectivity
- the transition metal-ligand complex should be thermodynamically stable but kinetically labile (i.e. the formation of the complex must be thermodynamically possible but reversible under the chromatographic conditions used)
- if the detection of the eluting species is achieved by postcolumn derivatisation with an appropriate indicator, the equilibrium constant for the replacement of the on-column anionic complex by the indicator complex should be large and the indicator complex kinetically stable.

Typical complexing agents are the weak organic acids which preferentially form anionic complexes with the transition metals i.e. citric, tartaric and oxalic acid. Separation may be achieved by using a mixture of two different complex forming acids. Separation relies on the significant differences of the complexes formed (see section 1.3.2). Thus metal bands migrate through the column at different rates determined by their affinity to complex with the ligand added to the eluent vs their electrostatic affinity for the stationary ion-exchange sites. By the use of different complexing agents, this method offered great flexibility with regard to selectivity. The optimum separation of a particular metal ion is achieved by adjusting the pH of the eluent. As weak organic acids are employed, a reduction in the pH of the eluent reduces the effective concentration of the organic acid and leads to longer retention times. An increase in the concentration of the complexing ligand leads to a loss in resolution as the concentration of the counterion (Na^+ ,H^+) will also increase. This would lead to a faster displacement of the metal ions from the exchange sites on the resin which would result in a loss of separation capability.

Elchuk and Cassidy [7] have obtained excellent chromatographic separation of the lanthanides with a-hydroxyisobutyric acid using postcolumn derivatisation and spectrophotometric detection. Stevenich and Fritz [8] achieved a separation of Zn(II), Co(II), Mn(II), Cd(II), Ca(II), Pb(II), and Sr(II) using a low-capacity ion-exchange column with an eluent of ethylenediamine and tartrate at pH 4.0. Separation here is achieved by

what is called a "push-pull" mechanism where the "pushing effect" is provided by the ethylenediamine cation and the "pulling effect" is provided by the weakly complexing organic acid anion. O'Dea et al. [9] used this method also to achieve a separation of Fe(III), Fe(II), Ni(II), Zn(II), Co(II), and Mn(II) after extraction from an adhesives sample.

The scope of metals determined by ion chromatography using post-column derivatisation with PAR has been limited to Mn(II), Fe(II), Co(II), Ni(II), Cu(II), Zn(II), Cd(II), Pb(II) and the lanthanide metal ions. However it is well known that PAR is a sensitive colorimetric indicator for many other transition metals. Investigation of the post-column chemistry of PAR with metals has shown that this limitation has been due primarily to the kinetics of complexation of PAR with metals rather than the universality of PAR itself. It has been found that the use of the organic chelators in the eluent significantly reduces the kinetics of complexation of PAR with many metals such that detection cannot be achieved. [33]. It has been observed, however, that certain ligands can be added to the post-column solution which enhance the kinetics of complex formation in the presence of chelators in the eluent. It is proposed that these ligands solvate the metal-eluent complex in such a way as to enhance the kinetics of ligand exchange between the metal and PAR. Ligands which have been found to have such a property include carbonate, ammonia, phosphate and alkanolamines. Thus chromium, mercury, vanadium and gallium can be determined using PAR containing 1M phosphate at pH 8.8 using the complexing agent pyridine-2,6-dicarboxylic acid (PDCA)

PAR is not the only reagent which has been used for post column derivatisation. Post column ion-displacement (PCID) may also be used. The requirements of the post-column reagent mixture are a metal chelate with a lower formation constant than the corresponding chelates of the analyte metals, together with an indicator that will form a radiation-absorbing complex with the displaced cation. Bowles and Bader [34] used a reagent mixture of Eriochrome Black T and magnesium-EDTA complex. Eluted metal cations displace the magnesium which then forms a complex with the Eriochrome Black T. Ca(II), Co(II), Cu(II), Fe(II), Mg(II), Ni(II), Sr(II), and Zn(II) could be detected (separation of all these components was not achieved).The absorbance of this complex was measured at 520nm. Detection limits for several cations are in the mg/ml range. Jones et al. [35] used Eriochrome black-T on its own with detection at 610nm for the determination of Mg(II), Mn(II), Co(II), Zn(II), Cu(II). These were separated very efficiently on a Partisil 10 SCX cation exchange column with a mobile phase containing lactic acid at pH 3.6. Detection of Fe(III), Cu(II), Zn(II), Ni(II), Co(II), Cd(II), Fe(II), Mn(II) and Mg(II) was also achieved after separation using tartaric acid on the same stationary phase at pH 4.3. Wang et al. [36]

used 3-(2-arsenophentlazo]-4,5-dihydroxy-2,7-naphthalene-disulphonic acid trisodium salt (Arsenazo) for the determination of the 14 rare-earth metals at the mg/ml level. The cations were separated on a Nucleosil SA column employing gradient elution. Transition and heavy metals, Fe(II), Cu(II), Zn(II), Ni(II), Co(II), Pb(II), and Mn(II) were eluted with a solution of sodium lactate followed by post-column reaction with PAR-Zn-EDTA.

A further development in separation technology was achieved when Cassidy and Elchuk [37] discovered that a reversed phase column coated with a hydrophobic molecule containing an ionic functional group could be used for the separation of metal ions. Several sulphate (C_{12}, C_{20}) and sulphonate (C_6 , C_8) cation systems were investigated. These dynamically coat the column giving it cation exchange character. A separation of Cu(II), Pb(II), Zn(II), Ni(II), Co(II), and Mn(II) was achieved using a C_{18} column and a mobile phase containing octanesulphonate and tartaric acid at pH 3.4. Detection was by post-column reaction with PAR. Schmidt and Scott used hexanesulphonate with tartaric acid to separate Cu(II), Pb(II), Zn(II), Ni(II), Co(II), Cd(II), Fe(II) and Mn(II). However the separation between Zn and Ni, and Cd and Fe was not very efficient. Krol [38] modified the conditions of Cassidy and Elchuk slightly to achieve an efficient separation of Fe(III), Cu(II), Pb(II), Zn(II), Ni(II), Co(II), Cd(II), Fe(II) and Mn(II). Cassidy and coworkers [39] used 1-octanesulfonate for the determination of Pr, Ce and La using a mobile phase containing a-hydroxyisobutyric acid at pH 3.8.

Using a dynamically coated column has been shown to give more efficient separations [38] than using low capacity ion-exchangers [9]. This is due to the fact that when using a dynamically coated column the capacity of the column can be easily varied by changing the concentration of the coating reagent. Another advantage of a dynamically coated column is that the column is less susceptible to degradation. With an ion-exchange column degradation can occur by irreversible binding of contaminants to the ion-exchange sites. With a dynamically coated column, regeneration of the ion-exchange sites when contaminated is easily performed by washing off the coating reagent by addition of organic solvent to the mobile phase. The column can then be freshly coated. Thus this, and the fact that ion-exchange resins are more expensive than reversed-phase columns, makes the dynamically coated column a more attractive option.

2.5 Previous Methods of Analysis Investigated at Loctite

The following chromatographic techniques had been investigated at Loctite for the analysis of transition metals

- determination by liquid-liquid extraction followed by pre-column complexation with 8-hydroxyquinoline and determination by reversed phase chromatography [40]
- cation exchange separation using a low capacity polymeric cation exchange column with a "push pull" mechanism. [9]

The first approach involved solubilising the adhesive in dichloromethane and extracting with 0.1M hydrochloric acid. The extract was passed through a C_{18} Sep-Pak and the eluate mixed with 8-hydroxyquinoline to form the metal chelates. To further clean-up the sample, the complexes were extracted into dichloromethane and this organic phase was then passed through a silica Sep-Pak cartridge. The cartridge was dried and the complexes removed using a methanol wash. Separation was carried out on a reversed-phase column with an acetonitrile/acetate mobile phase, pH 6.0, containing 8-hydroxyquinoline. Cu(II) and Fe(III) were determined with limits of detection of 0.1 and 0.5 ppm respectively. Cr(II) and Zn(II) were also separated however no detection limit were given. Cobalt and nickel could not be determined. Also Fe(II) ions were not detected (Figure 2.2).

The second method involved the use of an Ion-210 low capacity polymeric cation exchange column [9]. This gave rise to a very inefficient separation of the transition metals Cu(II), Fe(II), Ni(II), Zn(II), Co(II), and Mn(II). The mobile phase consisted of 3.9 mM ethylenediamine with 17 mM citric acid, pH 3.0. The use of other hydroxy acids did not improve the separation. Detection was achieved by post-column reaction with 4-(2-pyridylazo) resorcinol (PAR). The extraction procedure developed involved extraction of the adhesive solubilised in chloroform with 0.1 M HCl. The acid extract was passed through a C_{18} Sep-Pak prior to injection. However the column performance was found to quickly and irreversibly degenerate due to contamination by organics (Figure 2.3).

It was decided from an examination of the literature to investigate the analysis of the adhesives using a dynamically coated column. Cassidy and Elchuk [39] achieved a separation of Cu(II), Pb(II), Ni(II), Zn(II), Co(II), Fe(II), Cd(II), and Mn(II) using a dynamically coated reversed-phase column. In essence, a cation exchange capacity is formed

by coating the column with sodium octanesulfonic acid. The eluent was made 2 mM in sodium octanesulfonic acid, 50 mM in tartaric acid, and 2% acetonitrile, and had a pH of 3.4. Detection was achieved using post-column addition of PAR reagent. Thus it was decided to investigate the use of this method for the determination of metal ions in adhesives. Apart from achieving a separation of the metal ions of interest, the method also had two intrinsic advantages. Firstly, unlike the Ion 210 cation exchange column, the capacity of the column could be easily varied by changing the concentration of the sodium octanesulphonic acid. Secondly, the column could be easily regenerated when contaminated by the carry over of organics. Thus this was the method of choice for the present investigation.

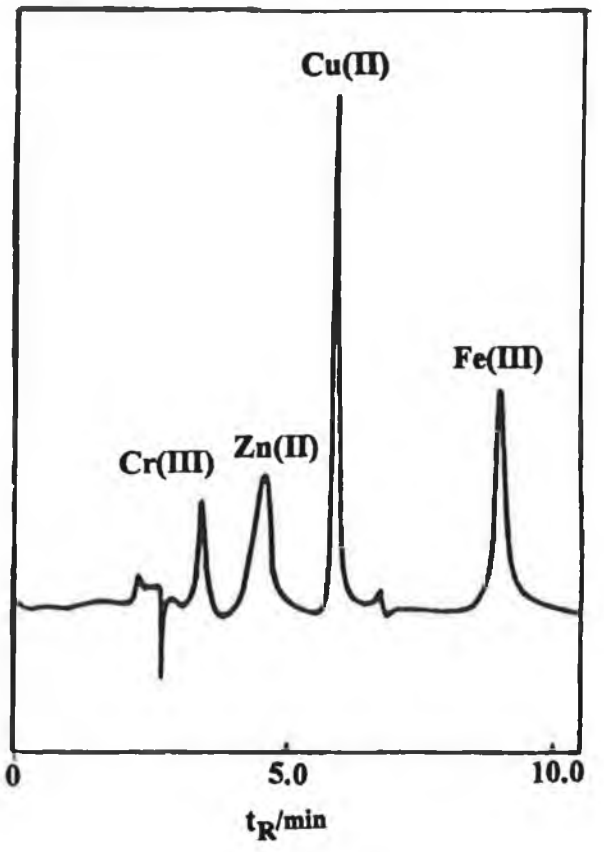


Figure 2.2. HPLC separation of Cu(II), Fe(III), Zn(II) and Cr(III) as their 8-hydroxyquinoline complexes. Eluent 50:50 mixture of acetonitrile (1 x 10^{-2} in 8-hydroxyquinoline) and 0.02M acetate acetate buffer (pH 6.0 made 0.2M in KNO_3) Column: Supelchem 25 cm x 4.6 mm i.d. 5 μm particle size. Detection 400nm [40].

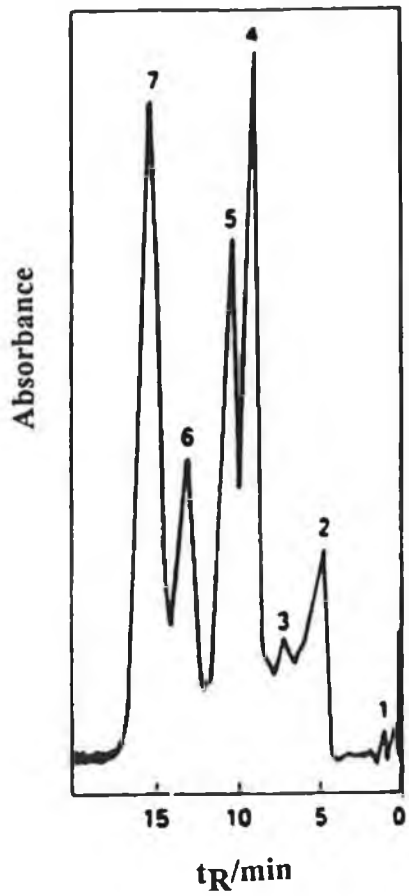


Figure 2.3 Separation of 1) Fe(III), 2) Fe(II), 3) Ni(II), 4) Zn(II), 5) Co(II), 6) Cu(II) and 7) Mn(II) on Ion 210 cation exchange column. Eluent of 3.9mmol/L EDA and 17mmol /L citric acid, pH 3.0. Detection with 3 x 10^{-4} M PAR, pH 10.14. [9].

2.6 DEVELOPMENT OF A SOLID PHASE EXTRACTION AND PRECONCENTRATION TECHNIQUE FOR THE DETERMINATION OF FREE METAL IONS IN THE PRESENCE OF THEIR CHELATES IN ANAEROBIC ADHESIVES

2.6.1 Expectations of the Developed Method

For the determination of the free metal ions in an adhesive by the method developed by Cassidy and Elchuk, it was obvious that an organic adhesive sample cannot be injected into an aqueous separation system. Thus an extraction of the metal ions into an aqueous system is necessary. The extraction method developed had to meet the following demands:

- the extraction method developed should not disturb the metal-chelate equilibrium in the sample; thus extraction must be carried out from an unmodified matrix
- only free metal ions should be extracted. If metal chelates were co-extracted, subsequent chromatography of the chelates could alter the metal-chelate equilibrium and thus give a false high or low result. Metal chelates should thus be considered as interferences
- concentrate the metal ions for sensitive detection
- prevent contamination of the analytical column
- materials for use in the method should be commercially available
- the method should be fast and easy to perform

2.7 LITERATURE SURVEY OF EXTRACTION AND PRECONCENTRATION TECHNIQUES FOR TRANSITION METAL IONS

Solid-phase extraction was introduced in the late 1970's, and has become very popular for the extraction and preconcentration of transition metals. The method involves passing the sample through a small quantity of a solid phase which enables isolation of the metal ion. This may be achieved by one of two mechanisms. The solid phase may retain the matrix components and allow only the metal ion to pass through. Alternatively the solid phase has a greater attraction for the analyte than the solvent in which the analyte is dissolved. The goal is to retain an isolate on a sorbent strongly enough that the isolate does not move through the sorbent bed. Thus, as the sample solution passes through the sorbent bed, the analyte concentrates on the surface while the other sample components pass through the bed. A washing procedure of different solvents is used to selectively elute interferences from the sorbent. The analyte may then be removed in a small volume of an appropriate solvent. Thus extraction, purification and preconcentration are simultaneously achieved. The method has many advantages over liquid-liquid extraction, which is tedious, time consuming, involves large solvent and sample volumes, and is thus costly [40]. Liquid-liquid extraction is also difficult to perform with samples which contain surfactants because of the formation of emulsions.

Several sorbent methods have been developed for the preconcentration of transition metals. Much research has focused on the use of chelate sorbents where a chelating agent is chemically bound to a support material. Alternatively, chelating agents may be adsorbed to a general sorbent such as C_{18}, silica, alumina, activated carbon or cellulose, and the metal ion chelated as the sample passes through. Addition of the chelating agent to the sample, followed by adsorption of the chelate on a general sorbent, is also used. Cation exchange may obviously also be used; however, anion exchange of the metal in its chelate form can also be performed.

2.7.1 Synthetic Ion Exchangers

Synthetic ion exchangers are usually made of copolymers of styrene and divinylbenzene, containing acidic (SO_3H^+, COOH) or basic ($N(CH_3)_3{}^+OH^-$) side groups. Cassidy and Elchuk [41] used a home-packed 13 mm Aminex A-5 cation resin to achieve preconcentration of Co(II), Ni(II), Zn(II), Pb(II) and Mn(II). Quantitative recoveries of metal ions at the ng/ml and pg/ml levels from aqueous samples were obtained. Samples were eluted from the cartridge with the mobile phase 0.06 M citrate, pH 4.8, and detected by post-column derivatisation with PAR reagent. The concentration cartridge was placed in the sample loop of the HPLC system. In a separate investigation, a commercially available 4 mm x 30 mm guard column from Brownlee filled with acid ion exchanger was used [42]. 0.35 to 0.5 M tartrate was used as the eluent. Detection limits of 1 to 15 pg/ml were obtained for Co(II), Ni(II), Zn(II), Pb(II), Cu(II) and Mn(II). Contamination from reagents and the chromatographic system was found to be a problem at the pg level. Also, memory effects were found due to adsorption onto to wetted components of the sampling system with subsequent desorption into a more dilute sample. Specialised washing procedures were thus introduced, and steel tubing was replaced where possible by glass-lined steel to reduce these interferences.

2.7.2 Complex forming sorbents

The use of a complexing or chelating agent to remove a metal ion from the sample matrix allows very selective extraction of the metal ion. With many complexing agents, distinction between different metal ions is possible. This chemistry has been adopted for use with solid-phase extraction. There are three different types of sorbents which have been developed: (1) sorbents with complex-forming groups inoculated to the polymeric or inorganic matrix [43-55]; (2) polymer heterochain sorbents, where the complex forming centers enter into the composition of the polymer chains [56]; and (3) sorbents modified or impregnated with complex-forming reagents [57-63].

2.5.2.1 Complex-forming sorbents with inoculated groups

This involves the introduction of chelate groups into a finished (ready-made) polymer matrix. The matrices most often used are cross-linked styrene-divinylbenzene copolymers;

however, cellulose, polyvinylalcohol and other polymeric materials may also be used. Reactive groups (chloromethyl, chlorohydrin, epoxy, amino, etc.) are usually introduced into the matrix which are then reacted with monomeric reagents containing chelate-forming groups. The extent to which the active groups in the polymer are replaced by chelate groups is often small; nevertheless, these sorbents exhibit a fairly high capacity.

Much work has been published on resins containing iminodiacetate groups. Florence and Batley [43] used Chelex-100, a commercially available resin, for the determination of Zn(II), Cd(II), Pb(II), and Cu(II) in seawater using direct anodic stripping voltammetry for detection. The seawater at natural pH (pH 8.1) was passed through a 6 cm x 1.2 cm diameter column of Chelex-100 at a rate of 3 ml/min, and the metal ions eluted with 2 M HNO_3. Retention of metals found naturally in seawater was found to be less than that of spiked samples. It was concluded that this was due to metals being bound by organics in the seawater. Detection was achieved at the 0.2 to 0.8 mg/ml level.

Hirata et al. [44] used the iminodiacetate chelate resin Muromac A-1 for the preconcentration of Cd(II), Zn(II), Cu(II), Mn(II), Pb(II), Fe(III) and Cr(III). The column was a homepacked 7 mm x 4 mm (i.d.) column, placed on-line with an atomic absorption spectrometer. Retention of the metal ions was found to be pH dependent. All divalent metals examined were recovered quantitatively in the pH range 3-5, and the trivalent metals were recovered at a maximum pH of 1. Elution was achieved using 2 M nitric acid. Enrichment factors were in the range 90-180 fold for the seven elements with detection limits in the range 0.14-2.1 mg/L.

Kumara et al. [45] also used Muromac for the preconcentration of Cu(II) and Pb(II) in an on-line column preconcentration system for detection by ICP-AES. The sample solution was introduced at pH 2 and eluted with nitric acid. The recovery of metals was found to be dependent on the concentration of nitric acid. Mass detection limits were 3 ng for copper and 190 ng for lead. In the case of copper, negative interferences were encountered, severely from iron(II) and moderately from chromium (III).

Chan et al. [46] synthesised a cellulose-based iminodiacetic acid sorbent derived from sawdust. Cellulose was used as it is hydrophilic, unlike polymer-based resins, and thus is more suited for applications involving aqueous samples. The sorption behaviour of the metal ions Pb(II), Cu(II), Ni(II), and Cr(III) were investigated in aqueous solution in the pH range 2-8. The working pH for Cu(II), Pb(II), Cr(III) and Ni(II) was chosen at 4.0, 4.0, 5.0, and

7.0 respectively. Nitric acid was found to be the most effective acid for elution. 85% recovery was achieved for Cu(II), Pb(II) and Ni(II) by washing the sorbent with 3 mls of concentrated nitric acid. The same treatment gave only a 30% recovery for Cr(III). For 200 mg/L metal ion solutions, a preconcentration factor of over 30 can be achieved for lead and nickel.

Sorbents with several other complex-forming groups have been synthesised and used. Chwastowska and Rozowski [47] synthesised a styrene-divinylbenzene copolymer inoculated with dithizone. The preconcentration of Cu(II), Zn(II), Pb(II), Co(II) and Ni(II) in water-alcohol media was investigated. Copper and lead were retained quantitatively over the pH range studied i.e. 0.8-5.4. Quantitative sorption of zinc occured above pH 4.6, and for cobalt and nickel above 5.4. The use of a water-alcohol medium improved the optimum column flow rate and sample volume. Whereas in an aqueous medium only sorption of copper and lead was independent of flow rate (up to 10 ml/min) and sample volume (up to 500 ml), in a water-alcohol medium only the sorption of nickel depended on flow rate. This indicates more advantages conditions for complex formation.

Mentasi et al. [48] developed a sorbent disk by chemically binding diethylenetriamine (DIEN) to cellulose. The DIEN disks were obtained by functionalising Whatman 41 cellulose filters. These were then used in a Pyrex glass millipore filtering device for the preconcentration of copper. This system was compared to immobilising 4-dodecyl-diethylenetriamine on C_{18} functionalised silica. Both adsorption and chemical bonding achieved satisfactory concentration factors. However, the elution of the metal with hydrochloric acid (20 ml) caused protonation of the immobilised ligand which was then in part eluted with the metal. Thus the column must be reloaded for each run. Binding capacity was found to increase up to pH 5. 10 mg of Cu(II) could be concentrated by a factor of 200. Diethylenetriamine was found to have a very low affinity for alkaline earth metals, unlike Chelex 100.

Cellulose powder inoculated with 2,2'-diaminodiethylamine (DEN) functional groups was used for the preconcentration of transition metals from water [49]. The sorbent was added to the 250 ml samples and stirred overnight. Co(II), Fe(III), and Cr(III) were collected quantitatively above pH 6, Cd(II) and Zn(II) above pH 7, and Mn(II) above pH 9 Elution was carried out using 3 to 4 ml of 1 M HNO_3. Only Cd(II) and Zn(II) were eluted quantitatively.

2.7.2.2 Chelate sorbents formed from inorganic matrices

Complex forming groups can be inoculated not only to organic, but also to mineral matrices, such as silica gel and glass beads. These sorbents have several advantages over organic polymers. They reach equilibrium rapidly in carriers with wide pores, they have a higher sorption capacity, they do not swell in water and other solvents, and they have high thermal stability. Silica is modified chemically by organic compounds at the surface hydroxyl groups. In many cases, chemical modification is achieved by reacting the silica with 3-chloropropyltrimethoxysilane. The resulting silica is then functionalised with the complexing agent.

Iamamoto and Gushikem [50] used silica gel modified with pyridinium ion for the preconcentration of cobalt, zinc, cadmium and mercury from ethanol. All metals, except mercury could be eluted quantitatively from the column with pure water. The metals were adsorbed as their anionic chloride complexes (M^+Cl^-). The adsorption is strongly dependent on the dielectric constant of the solvent and the stability constant of the metal chloride complex. In aqueous solution, anionic metal ion complexes occur with the addition of a large amount of the electrolyte Cl^-. In ethanol, the metal ions are associated to a much greater extent than in aqueous solution, because of the lower dielectric constant, and therefore these ions are adsorbed from the solvent without the addition of any electrolyte. The recovery of the metal ion by elution from the column with pure water is higher than 97% in every instance, except for Hg(II), where it was necessary to use 0.1M HNO_3 in order to obtain a recovery of 99%.

Moreira and Gushikem [51] investigated the preconcentration of copper, nickel, iron, zinc and cadmium from ethanol using silica gel modified with 3(1-imidazolyl)propyl groups. Elution was carried out using 0.1 M hydrochloric acid in ethanol/water with a mole fraction of water of 0.85. Quantification was carried out using AAS with detection limits in the low mg/L range.

Luhrmann and Kettrup [52] described a simple method for the preparation of silica immobilised 8-hydroxyquinoline by reaction of the surface groups with aminopropyltrimethoxysilane. The resulting material was investigated for its affinity to copper (II), nickel (II), cobalt (II), iron (III), manganese (II), chromium (III), zinc (II),

cadmium(II), lead(II) and mercury(II). The metal uptake capacities were found in the range from 0.2 to 0.7 mmol/g,

Fang et al, [53] investigated preconcentration of Cu, Zn, Pb and Cd on three different chelate exchangers - Chelex 100, an 8-quinolinol chelating exchanger (the 8-quinolinol was azo-immobilised to controlled pore glass) and the weakly acidic 122 resin, which had a phenol-formaldehyde base with salicylate functional groups. These chelate exchangers were used in a flow injection mode and detection was achieved on-line with an AAS. The columns were home-packed. The system was used for the analysis of sea water. Chelating resins are especially suited to this application as they retain heavy metal ions in the presence of alkali and alkaline earth metal ions. The samples were loaded in an ammonium acetate buffer and eluted with 2 M nitric acid. The 8-quinolinol material was found to have the highest concentration factor. This is probably due to the faster exchange rate of the surface bound chelating functional groups. The analytes were eluted more readily from the surface, thus being present in a smaller volume of eluate. However, due to the smaller exchange capacity and comparatively high stability of magnesium 8-quinolinolate, the recoveries of the elements from sea water was unacceptable. The recoveries of the elements decreased roughly in decending order of their stability constants. Good recoveries were obtained for all metals when Chelex 100 was used, due to the lower stability of the iminodiacetate complex and the higher exchange capacity of the column. The acidic 122 resin behaved similarly to Chelex 100, except for cadmium, for which no satisfactory recovery could be obtained. The degree of preconcentration ranged from 50 to 105 fold for different elements with detection limits for Cu, Zn, Pb and Cd of 0.07, 0.03, 0.5 and 0.05 mg/ml respectively. A subsequent paper [54] used the 8-quinolinol for the determination of cobalt. A 48-fold enrichment was achieved with a detection limit of 20 mg/ml.

The metal complexation capacities of biochelating silicas utilising hydroxamate complexation were investigated by Glennon and Srijaranai [55] Hydroxamic acid functional groups have been immobilised by the esterification of modified hydroxylated silochrome silica and reaction with hydroxylamine. Unsubstituted (HA-Si) and N-methyl-substituted monohydroxamate silicas (NMHA-Si) were compared with the biochelator, desferrioxamine, immobilised onto silica (DFA-Si). A comparison of the metal complexation capacities of Fe(III) and Cu(II) found the complexing order to be HA-Si > NMHA-Si (DFA-Si). Studies on the extraction of Fe(III), Cu(II), and Zn(II) from seawater showed recoveries of 98%, 97%, and 77% respectively. Elution of the metals from the cartridge was obtained using 0.08M EDTA for Fe(III) and acidified water (pH 2) for Cu(II) and Zn(II).

2.7.2.3 Polymeric heterochain sorbents

Complex forming sorbents can be obtained not only by inoculating complex-forming groups to any inert matrix. Active groups may constitute a component of the matrix proper; often the complex forming centres enter into the composition of polymer chains as heteroatoms. Tsisin et al. [56] developed an aminocarboxylic heterochain polymer called POLAC-1. These sorbents gave greater sorption capacity. When compared to Dowex A-1 (aminocarboxylic acid groups inoculated) the metal distribution on POLAC-1 was 10^4 higher and the pH region optimum for metal extraction was wider. Zn(II), Cd(II), Co(II), and Cu(II) were all extracted at pH 5 from a 0.025M solution of potassium phthalate and subsequently eluted with 5 ml of 1.0 M HNO_3. Thus concentration coefficients of up to 500 were achieved. The greater efficiency of the resin was explained by higher compactness and mobility of the functional groups interacting with the metal ion.

2.7.2.4 Sorbents Modified with Complex-forming Reagents

It is not necessary to chemically bind the complex-forming groups to the matrix by stable covalent bonds. Such reagents can be firmly fixed on the surface of an ion exchanger as a second layer i.e. as a layer of counterions. Alternatively they may simply be dissolved in an organic solvent or water and fixed as a thin layer on the surface of a porous carrier.

Singh et al. [57] investigated the chelation properties of aluminium oxide fixed with diethylenetriaminepentaacetic (DTPA), for the preconcentration of transition metals. The DTPA was immobilised by shaking the aluminium oxide with 0.2% (W/V) DTPA for 12h. The behaviour of sixteen metal ions were investigated. Pt(IV), Cr(III), Ag(I), Au(III), Os(VIII) and Ru(VIII) are strongly absorbed by the exchanger. Mn(II), Fe(III), Co(II), Ni(II), Cu(II), Zn(II) and Cd(II) only partially adsorbed while Cr(VI), V(V), Fe(II), Hg(II) and Mo(VI) were scarcely absorbed at all. The reason for selective adsorption was attributed to the large difference in stability constants. The system was thus used to selectively preconcentrate Pt(IV) and Cr(III) in the presence of Mn(II), Fe(III), Fe(II), Co(II), Ni(II), Cu(II), Zn(II) and Cr(VI). All foreign metals were eluted with 40 ml of sodium citrate and ammonia. The Pt(IV)-DTPA chelate was eluted with 40 ml of 5% KI in 0.1M HCl and Cr(III)-DTPA in 40 ml of 0.5 M perchloric acid.

Giraudi et al. [58] described a preconcentration technique for the transition metal ions Cu(II), Ni(II), Co(II), Cd(II), Zn(II), Mn(II), Fe(II) and Hg(II). The trace metal ions were adsorbed in the form of 1-(2-pyridylazo)-2-naphthol) (PAN) complexes on C_{18} bonded silica gel. The sample was treated with an excess of PAN at pH 9.0 adsorbed on the column and eluted with 0.5 ml of 8 M $HClO_4$. A 200 fold concentration was easily achieved with 100% recovery within experimental error. Alkali metal ions were found not to interfere.

Abollino et al. [59] compared the adsorption of transition metals complexes of 8-hydroxyquinoline on polystyrene-divinybenzene resin (Amberlite XAD-2) and an anion exchange resin (Bio-Rad AG MP-1). It was found that the contamination levels of metal ions in these resins was lower than those with silica-based supports. The anion exchanger was found to be the better sorbent for the metal ions Cd(II), Cu(II), Mn(II), Ni(II), Pb(II) and Zn(II), on the basis of selectivity and much lower sensitivity to interferences. A metal ligand ratio of 1:20 was used for isolation with the sample adjusted to pH 9. Elution was achieved by washing the sorbent with 5.0 ml of 2.0M HNO_3. Enrichment factors of up to 100 were achieved enabling detection with ICP in the mg/L range. The experimental recoveries for Cu(II), Cd(II) and Mn(II) were 115%, 92.4 and 94.9% respectively.

Sturgeon et al. [60] achieved the preconcentration of transition metals from sea-water by complexation with 8-hydroxyquinoline and adsorption on C_{18} bonded silica gel. Adsorption was achieved at pH 8.9 and elution of the complex was achieved with 5 ml of methanol. Cd, Zn, Cu, Ni, Co, Mn and Fe were quantitatively recovered from seawater. Co gave a 67% recovery while the recovery of lead was erratic. Enrichment factors of 50-100 were readily obtained

Terada and Nakamura [61] used 1-nitroso-2-naphthol supported on silica gel for the preconcentration of Co(II) from natural water samples. Co(II) was quantitatively retained on the column of chelating silica at pH > 3.0, and eluted with glacial acetic acid or a mixture of acetone and hydrochloric acid (9:1 v/v). A concentration factor of 100 was achieved by passing 2.5 litres of water through the column. The effect of EDTA, tartrate, citrate, cyanide and ammonium ions was examined. These species had no effect on the retention of cobalt.

Vanderborght and Grieken [62] used complexation with 8-hydroxyquinoline with subsequent adsorption on activated carbon for the determination of Mn, Fe, Co, Ni, Cu, Zn, and Cd in natural waters. Enrichment factor of 10,000 and recoveries of 85 to 100% were achieved. The pH of the sample was adjusted to a value of 8. An optimal quantity of oxine

was added to provide a 5 mg/L free oxine concentration. The oxine dissolved by heating the sample to a temperature of 60C and the metal oxinate precipitate filtered off after cooling. An optimal quantity of activated carbon was added and allowed to equilibrate for 1 hr. The method was shown to be free from interferences from alkali and alkaline earth ions.

Fang et al. [63] used a silica C_{18} microcolumn to collect diethylammonium diethyldithiocarbamate complexes of heavy metals in aqueous samples. The system was used in a flow injection configuration for the determination of cadmium, lead and copper by atomic absorption spectroscopy. Ethanol and methanol were used for elution. Enrichment factors of 19-25 were achieved at sampling frequencies of 120/hr. Detection limits for Cd, Pb, and Cu were 0.3, 3, and 0.2 mg/L respectively.

2.8 DEVELOPMENT OF A SOLID PHASE EXTRACTION AND PRECONCENTRATION TECHNIQUE FOR THE DETERMINATION OF TRANSITION METAL IONS BY ION-PAIR REVERSED-PHASE CHROMATOGRAPHY

2.8.1 General Considerations

Thus an examination of the literature shows that solid-phase extraction for the preconcentration of transition metals has been applied mostly to the analysis of aqueous samples. No methods have been developed for the extraction of metal ions from an organic matrix such as an adhesive. Most applications developed to date are concerned with the isolation of metal ions from natural or sea waters with on-line solid-phase extraction followed by analysis by atomic absorption spectroscopy.

The main focus of attention, therefore, has been the selective extraction of the transition metal ions in the presence of interfering ions, such as alkali and alkaline earth metals. Thus the majority of methods developed involve the use of a complexing agent which has a high formation constant and is selective to transition metal ions. These are either a part of the polymer matrix, chemically bound to a polymer or inorganic matrix such as silica, or adsorbed to a polymer or a sorbent such as C_{18} , activated carbon or cellulose. The pH of the sample is adjusted for optimum complexation and the sample passed through the sorbent. Due to the high formation constants, a strong acid is usually necessary to elute the metal ion.

There are many factors which make this general approach unsuitable for the present investigation. Firstly, one is dealing with a highly organic matrix and thus the use of a sorbent which has a non-polar base such as polystyrene divenylbenzene or C_{18}, will retain the matrix. This therefore eliminates many of the developed applications where the metal complexing agent or the ion-exchanger is bound to a polymer base, or fixed to a C_{18} matrix. Furthermore, the use of a sorbent with a non-polar base would result in the retention of the metal chelates of EDTA together with the metal ions.

The use of a chelate for metal ion abstraction may upset the metal chelate equilibrium within the adhesive sample by competing with the EDTA for complexation of the metal ions. This behaviour is very probable for chelating agents such as 8-hydroxyquinoline, PAN, and dithiocarbamates. Terada and Nakamura [61] found that EDTA did not interfere with the

analysis of cobalt when 1-nitroso-2-naphthol was used for extraction. This means that 1-nitroso-2-naphthol was able to compete with EDTA for the copper. An examination of the conditional constants under the conditions of analysis would be the only means of predicting exact behaviour. For applications where the complexing agent is merely fixed to the support, then leaching of the complexing agent can occur which would interfere with the subsequent chromatography and detection with PAR reagent. In most aqueous applications involving complexing agents there is a narrow optimum pH range at which optimal complexation occurs, thus the sample pH must be adjusted accordingly. In this application, complexation within the organic matrix cannot be optimised in such a manner, as extraction must be carried out with minimal disturbance of the sample, such that the metal chelate equilibrium within the sample is not changed. Furthermore for the elution of metal ions from these chelate sorbents, a strong acid must be used. This introduces a further sample preparation step for preparation for chromatography in the chosen system. Thus, for these reasons, the use of a complexing agent is not suitable for analysis of the free metal ion content of adhesives.

Furthermore, it was necessary that the sorbent used be commercially available. This rules out many of the developed methods which require synthesis of the sorbent. Thus use of a commercially available product ensures a more consistent sorbent. The use of disposable cartridges ensures a fresh solid phase for each sample which avoids build up of irreversibly adsorbed material or the release of late eluting compounds.

It is obvious that a cation exchange resin can be used for extraction. However, when choosing a cation exchanger, the matrix of the sorbent must be considered as the sample matrix will interact with this. Thus many of the commercially available cation exchange solid phase extraction cartridges, which consist of a polymer matrix are unsuitable. Ion-exchanger which are synthesised by attachment of the cation exchange group through a carbon chain to a silica matrix are also unsuitable as these exhibit secondary non-polar interactions. The only commercially available sorbent which exhibits cation exchange without also exhibiting non-polar interactions is silica itself.

Schindler [64] studied the adsorption of Fe(III), Cu(II), Cd(II) and Pb(II) on silica. Figure 2.4 shows the adsorption of these ions as a function of pH Thus it was decided to investigate silica for the extraction of the free metal ions. Waters Alumina A (a low capacity cation exchanger) and Accell Plus CM (a weak cation exchanger) were also investigated.

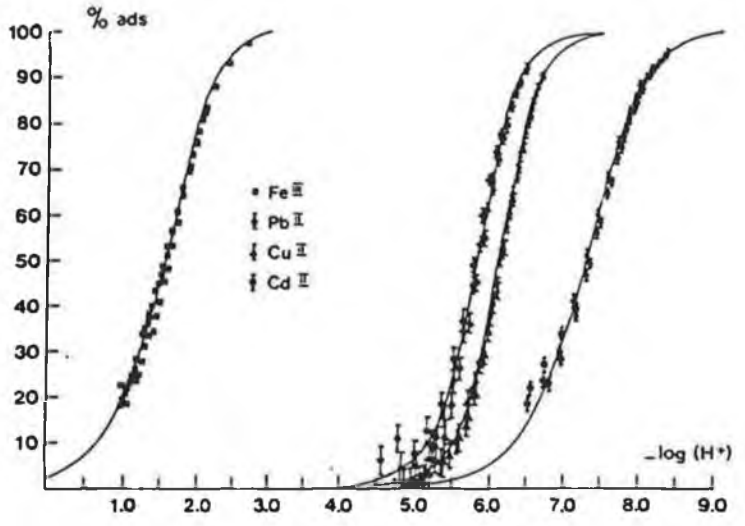


Figure 2.4. Adsorption of Fe(III), Cu(II), Cd(II) and Pb(II) as a function of log [H^+] on silica. From [64]

2.8.2 EXPERIMENTAL

2.8.2.1 Reagents

All aqueous solutions were prepared with water obtained by passing distilled water through an ELGA-STAT (Elga, High Wycombe, UK) water purification apparatus. 4-(2-pyridylazo)resorcinol (PAR) was obtained from Aldrich. Sodium octyl sulphonate was obtained from Rohm Chemicals. Tartaric and citric acids and ultrapure NaOH pellets were obtained from Riedel-de Haen. Metal standards were prepared from atomic absorption standard metal ion solutions from May and Baker, with the exception of Fe(II) which was made from ammonium iron(II) sulphate hexahydrate. Isooctane and diethyl ether were obtained from Riedel-de-Haen, whereas chloroform was obtained from Aldrich.

2.8.2.2 Apparatus

The high-performance liquid chromatography (HPLC) system consisted of a Waters 501 HPLC pump, a Waters RCSS Guard Pak Module, and a Waters mBondapak C_{18} cartridge (4.6 mm x 10 cm) containing within a Waters Z module. The eluted metal ions were detected after post-column derivatisation with PAR reagent. The post-column reactant was delivered using a Waters reagent delivery module to a T-piece situated between the end of the column and the reaction coil. The T-piece was connected such that the column effluent and the PAR reagent entered at 180° to each other. The reaction coil consisted of HPLC tubing. The reagent delivery module was pressurised with nitrogen to produce a flow rate of PAR reagent exiting from the module of 0.5 ml/min. The complexed metal ions were detected using a Shimadzu 8PD-6AV ultraviolet detector at 520nm. The detector output was monitored with a Drew Scientific data capture unit and the chromatograms were processed with a Roseate Chromatography data management package.

All sample preparation cartridges were obtained from Waters. Plastipak syringes from Becton Dickinson were used for the introduction of samples onto the sample preparation cartridges.

2.8.2.3 Preparation of Reagents

Mobile Phase: 2 mM sodium octyl sulphonate (NaOS), 20 mM citric acid and 30 mM tartaric acid, 2% Acetonitrile, pH 3.4, as described by Krol [38]. 700 mls of Milli-Q water

was added to a one litre volumetric flask. The NaOS, tartaric acid and citric acid were added and disolved. 20 mlsof acetonitrile was added and the solution diluted to the mark with Milli-Q water. The eluent pH was adjusted to pH 3.3 with NaOH pellets. 1M NaOH was used for the final adjustment to pH 3.40 +/-0.01. This was filtered using a 0.45 µm Millipore HA filter.

PAR Reagent: 0.2 mM PAR, 1 M acetic acid, and 3 M ammonium hydroxide. In a well ventilated fume hood 100 mls of Milli-Q water was added to a beaker.103 mls of concentrated ammonium hydroxide was added followed by 0.026 g PAR. The PAR was disolved by stirring. 29 mls of concentrated acetic acid was added slowly. The solution was then transfered to a 500 ml volumetric flask and diluted to the mark with Milli-Q water. This was filtered using a 0.45µm Millipore HA filter.

2.8.2.4 Sample Preparation Procedure

The silica Sep-Pak cartridge was firstly wetted with 2 ml of ethanol, then conditioned by washing with 5 ml of de-ionised water. Adhesive samples solubilised in 30 mls of an appropriate solvent (typically iso-octane or chloroform) were then applied to the top of the cartridge. The cartridge was then washed with a series of solvents depending upon the interferences present in the adhesive sample being analysed. The metal ions were eluted with 3 ml of the mobile phase used for separation (2 mM sodium octyl sulphonate (NaOS), 20 mM citric acid and 30 mM tartaric acid, 2% Acetonitrile, pH 3.4). The first 1 ml (representing the dead volume of the cartridge) was discarded and the remaining 3 ml used for injection into the HPLC system. The spiking of metal ions into adhesive formulations was achieved using standards prepared in ethanol-water (90 + 10).

2.8.3 RESULTS AND DISCUSSION

2.8.3.1 Optimisation of Chromatographic Separation and Detection System

The choice of chromatographic conditions was made based on the work of Cassidy and Elchuk [37] and Krol [38]. Further optimisation was then carried out with respect to the size of the sample loop, the length of the reaction coil and the temperature of the reaction coil.

The large capacity of the column, and the large volume of eluents (3 ml), emanating from the solid-phase extraction cartridges used for sample preparation, required the use of a large sample loop. Sample loop sizes investigated were 200 ml, 500 ml, 1 ml and 2 ml. It was found that the peak height for each metal increased with increasing sample loop size up to 1 ml (Fig. 2.5). Further increase in the loop size to 2 ml resulted in the capacity of the column being exceeded resulting in a lack of resolution between various peaks, thus the results for the 2ml sample loop are not quoted. A 1 ml sample loop size was therefore used in all further investigations.

The length of the reaction coil from the mixing T-piece to the detector determines the reaction time of the PAR reagent with the metal ion. The length of the reaction coil was varied from 25 to 135cm. For all metal ions investigated, the peak height increased with increasing length of the reaction coil at room temperature (Fig 2.6). Due to frictional forces it was noted that a further increase in the mixing coil length was affecting the flow rate of the PAR reagent. Thus to maintain the PAR flow rate at 0.5ml/min the nitrogen pressure in the reagent delivery module would need to be increased which was not possible with the system used. Thus a 135cm reaction coil length was used.

The temperature of the reaction coil was varied from room temperature to 60°C (Fig 2.7). For most metal ions the peak height remained relatively constant; however a decrease in response was found for Zn(II) between 10 and 40°C. As the peak heights of most metal ions remain relatively constant, it was decided to maintain the reaction coil at room temperature.

The chromatographic separation of a 1ppm mixture of eight divalent metal ions achieved using a 1ml injection loop, and a 135cm reaction coil at room temperature is shown in Figure 2.8.

2.8.3.2 Optimisation of Sample Preparation

As outlined in section 2.6, silica was chosen as a solid phase extractant as it exhibits cation exchange interactions. Accel plus, and Florasil were also investigated. These sorbents are produced by Waters. Detailed information is not given on their composition: Accel plus is defined as a weak cation exchanger, and Florasil as a co-precipitate of magnesia and silica gel.

The extraction cartridges were investigated as to their ability to remove the metals under investigation from aqueous solutions using the procedure outlined in section 2.8.2.3 and spiking at the 100 mg/L level. The silica Sep-Pak proved the most promising in terms of recoveries and interferences. Accell Plus contained high concentrations of interfering metal ions, while very poor recoveries were obtained with Florasil.

The adsorption of transition metal ions on silica from aqueous solution was as expected [64]. The mobile phase for the chromatographic separation was ideal to use for elution of the adsorbed metal ions, as it had a pH 3.4, which as shown by Schindler (figure 2.4) will cause desorption of Pb(II), Cu(II) and Cd(II) but not Fe(III). The presence of tartaric acid in the mobile phase most probably assists elution. Recoveries of metals when adsorbed on silica were Cu(II) - 87%, Pb(II) - 73%, Ni(II) - 76%, Zn(II) - 110%, Co(II) -102%, Fe(II) 79%, and Mn(II) - 61%. A blank run showed the silica Sep-Pak to be contaminated by Zn(II). Thus it was necessary to run a blank on each new batch of Sep-Paks used. Cd(II) and Fe(III) were not investigated, however Figure 2.4 would suggest that Fe(III) would not be recovered under the conditions used.

The extraction of the metal ions from a typical plasticiser i.e. tetraethylene glycol di-(2-ethylhexanoate) was then investigated. The wetting-conditioning procedure was the same as that described above. A 30 ml sample of plasticiser solubilised in an equal volume of iso-octane was passed through the Sep-Pak with the aid of a 10 ml syringe at a rate of 2-3 ml/min. The Sep-Pak was then washed with 10 ml of iso-octane to remove any remaining plasticiser. The metal ions were eluted from the Sep-Pak with 3 ml of mobile phase, the first 1 ml representing the residual iso-octane being discarded and the remaining 3 ml collected. This fraction was passed through a prewetted C_{18} Sep-Pak in order to remove any remaining organic components. Iso-octane was chosen as a solubiliser as it was the most non-polar solvent in which the plasticiser was soluble. This ensured that any residual carryover in the

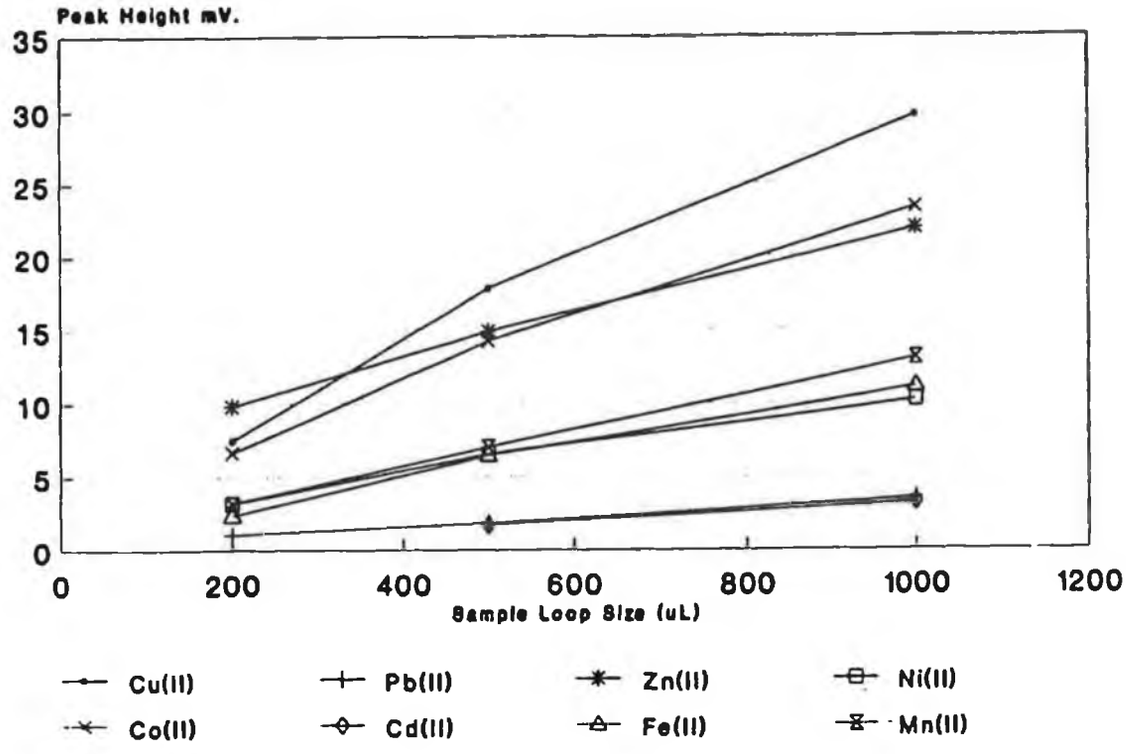


Figure 2.5. Effect of sample loop size on peak height obtained following separation of eight divalent metal cations, Cu(II), Co(II), Zn(II)., Mn(II), Fe(II), Ni(II), Pb(II), and Cd(II).

Conditions: Column μBondapak C_{18}, Eluent 2 mM sodium octanesulphonate, 30 mM tartaric acid, 20 mM citric acid, pH 3.4, flow rate 1.0 ml/min. Detection: post-column derivatisation with PAR reagent (0.2 mM PAR, 1 M acetic acid and 3 M ammonium hydroxide), at 520 nm, flow rate 0.5ml/min.

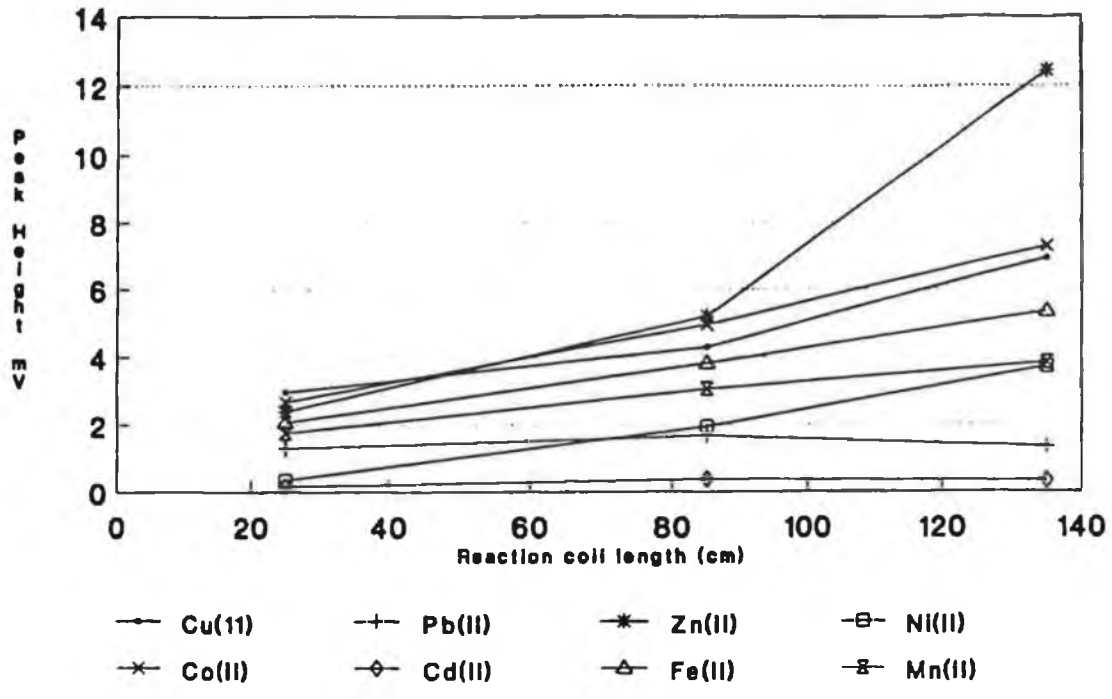


Figure 2.6. Effect of length of reaction coil on peak height obtained following separation of eight divalent metal cations. Zn(II), Co(II), Cu(II), Fe(II), Mn(II), Ni(II), Pb(II) and Cd(II).

Conditions: Column μBondapak C_{18}, Eluent 2 mM sodium octanesulphonate, 30 mM tartaric acid, 20 mM citric acid, pH 3.4, flow rate 1.0 ml/min. Injection loop 1.0 ml/min. Detection: post-column derivatisation with PAR reagent (0.2 mM PAR, 1 M acetic acid and 3 M ammonium hydroxide), at 520 nm, flow rate 0.5ml/min.

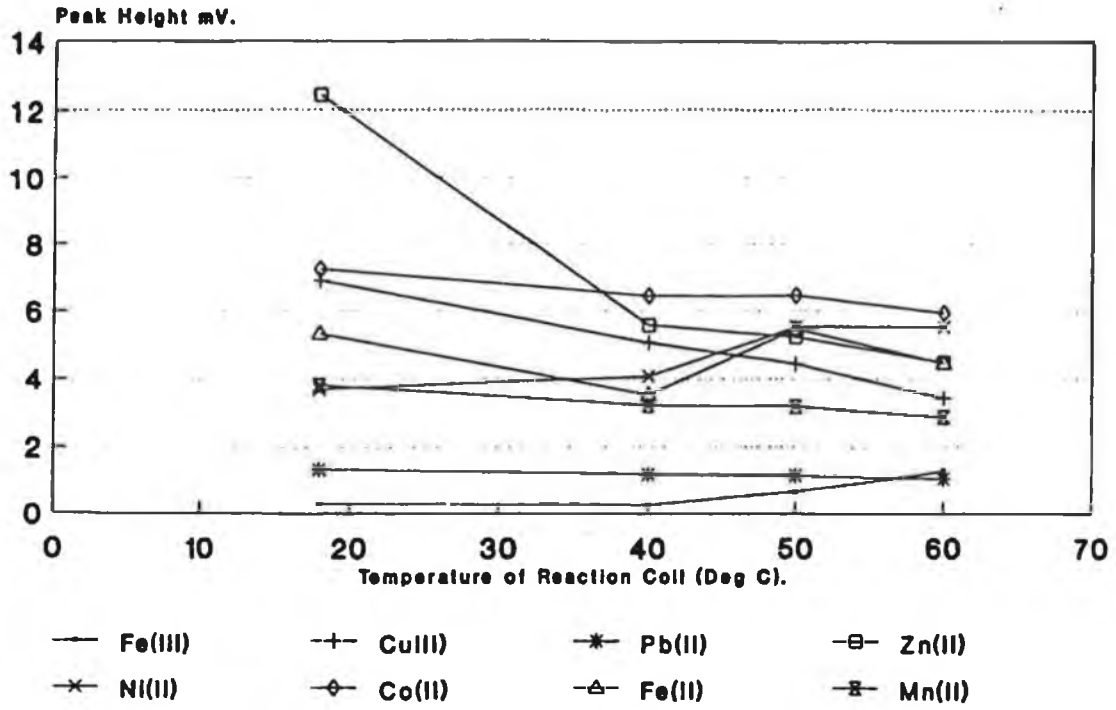


Figure 2.7. Effect of temperature of reaction coil on peak height obtained following separation of eight divalent metal cations. Zn(II), Co(II), Cu(II), Ni(II), Mn(II), Fe(II), Pb(II), Fe(III).

Column μBondapak C_{18}, Eluent 2 mM sodium octanesulphonate, 30 mM tartaric acid, 20 mM citric acid, pH 3.4, flow rate 1.0 ml/min. Injection loop 1.0 ml/min. Detection: post-column derivatisation with PAR reagent (0.2 mM PAR, 1 M acetic acid and 3 M ammonium hydroxide), at 520 nm, flow rate 0.5ml/min.

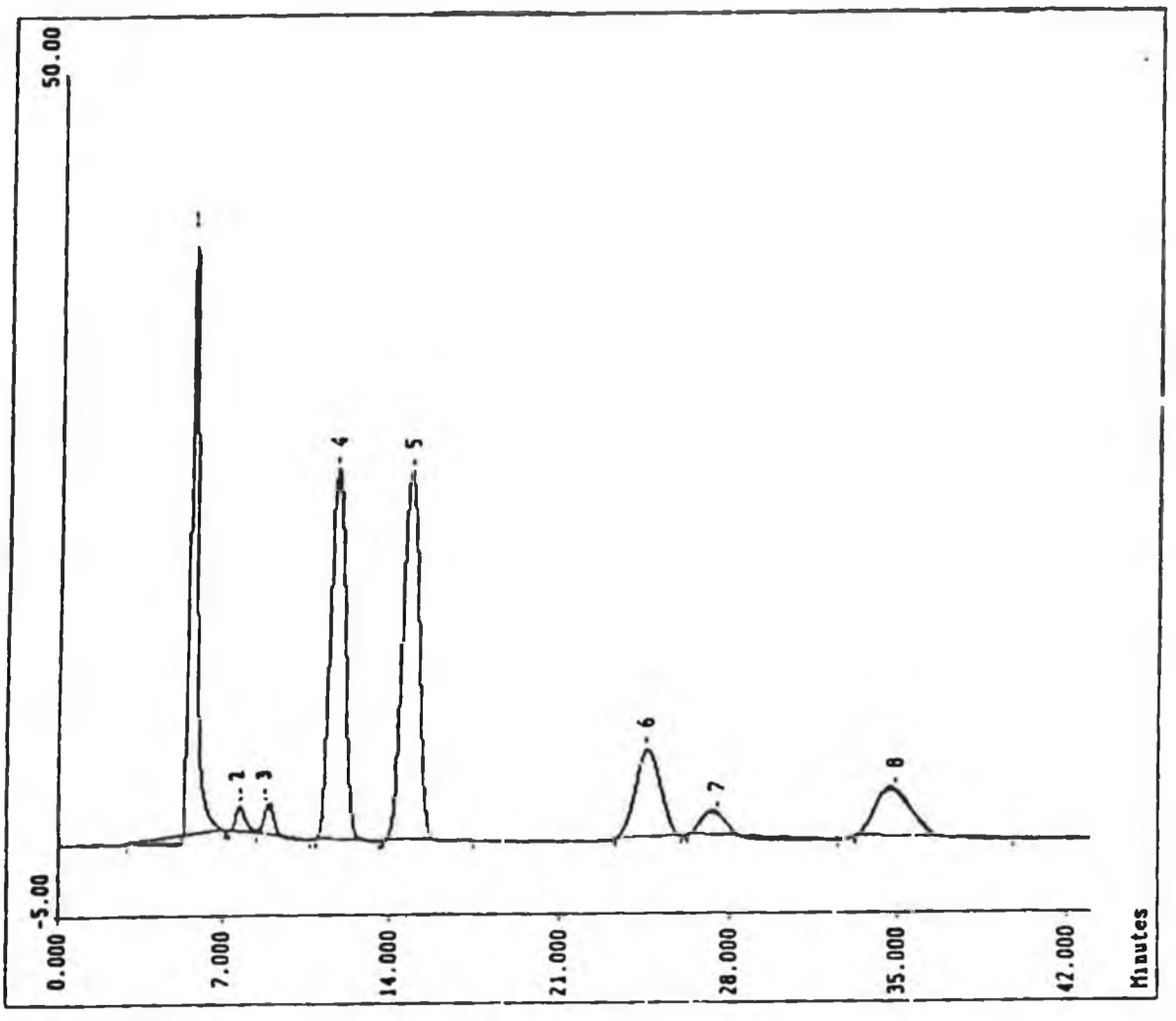


Figure 2.8. Separation of 1 mg/L aqueous mixture of 1. Cu(II), 2. Pb(II), 3. Ni(II), 4. Zn(II), 5. Co(II), 6. Fe(II), 7. Cd(II), and Mn(II).

Column μBondapak C_{18}, Eluent 2 mM sodium octanesulphonate, 30 mM tartaric acid, 20 mM citric acid, pH 3.4, flow rate 1.0 ml/min. Injection loop 1.0 ml/min. Detection: post-column derivatisation with PAR reagent (0.2 mM PAR, 1 M acetic acid and 3 M ammonium hydroxide), at 520 nm, flow rate 0.5ml/min.

elution of the metal ions with the mobile phase would be easily removed using the C_{18} Sep-Pak. A typical chromatogram, obtained following this procedure, is shown in Fig. 2.9, where it can be seen that there was a good recovery for most of the metal ions except Fe(II). As a recovery of 79% for Fe(II) was achieved from aqueous solution it was assumed that the problem involved the plasticiser matrix. A liquid-liquid extraction investigation involving the extraction of 50 ml of plasticiser solubilised in 50 ml of iso-octane with 5 ml of the mobile phase showed that while the other metals were extracted, Fe(II) was not extracted. It is believed that the Fe(II) is somehow held within the sample matrix or converted to Fe(III). The present chromatographic technique does not indicate the presence of Fe(III).

Procedures were developed for raw materials, intermediate and finished products. For formulations which were not soluble in iso-octane, more polar solvents, such as chloroform and dichloromethane, were used. It was found, however, that when such solvents were employed, splitting of the Cu(II) peak occurred. This was thought to be due to the carryover of these solvents because of their miscibility with water and decreased retention on the C_{18} Sep-Pak. This problem was counteracted by employing a 10 ml wash with iso-octane following the 10 ml wash of the Sep-Pak with the solubilising solvent (i.e. chloroform-dichloromethane). As chloroform is miscible with iso-octane, the chloroform was removed, and the traces of the less polar iso-octane were trapped by passage through the C_{18} Sep-Pak. A typical chromatogram obtained for six of the metal ions following solid-phase extraction from a formulation containing triethylene glycol dimethacrylate cumene hydroperoxide, saccharin, dodecyl methacrylate and a primary alcohol ethoxylate is shown in Fig 2.10. It is possible that the cumene hydroperoxide and saccharin may interfere with the adsorption of the metal ions as they will also be adsorbed by polar interactions to the silica. In hindsight, a wash with a more polar solvent such as acetone, may have helped to remove these interferences.

In samples containing aromatic amines such as N,N-dimethyl-o-toluidine and N,N-diethyl-p-toluidine, peak splitting again occurred. For the removal of these amines, solvents such as ethanol, acetone and diethyl ether were investigated. Diethyl ether proved to be the most suitable. Again the order in which the washing of the Sep-Pak was carried out proved to be critical to prevent the carryover of washing solvents. Therefore, samples containing amines were solubilised in chloroform and passed through the Sep-Pak, which was then washed with 10 ml aliquots of the chloroform, followed by diethyl ether and iso-octane.
The determination of Cu(II), Ni(II), Pb(II), Zn(II), Co(II), Cd(II) and Mn(II) was then investigated in a typical anaerobic sealant. Linear calibration graphs were constructed for all

the metal ions at concentration levels of between 60 and 500 mg/L. Limits of detection from 40 to 70 mg/L and recoveries of 61-110% were obtained for these metal ions using a sample size of 30 ml. Table 2.1.

It was found that the chromatographic separation was very susceptible to interference from organics. However, with the present sample preparation step using the C_{18} Sep-Pak and an in-system guard column, no problems were encountered with regard to contamination by organics from samples. The greatest source of contamination by organics was found to be the water used in the mobile phase. Even continuous use of the system for the analysis of aqueous samples was not possible if the water used in the preparation of the mobile phase was not of excellent quality. Contamination by organics was evident by a reduction in the peak height of the copper (II) peak which gradually disappeared with increasing contamination by organics. Washing of the column with a 50:50 mixture of acetonitrile:water and recoating the column resulted in a return to the original response. However with special attention to the quality of the water supply used in the mobile phase this problem should not occur.

In order to investigate the ability of the method to differentiate between free metal cations and their ethylenediaminetetracetic acid (EDTA) complexes, a sample of tetraethylene glycol-di-(2-ethylhexanoate) was spiked at the 100 μg/L level with the metal ions under investigation and an excess of EDTA (20ppm) was added. These samples were then extracted using the solid-phase extraction procedure described above. The chromatogram obtained (figure 2.11) showed no response for any of the metal ions investigated, indicating that metals complexed by EDTA are not detected by this procedure. Further studies would involve investigating the response as the EDTA concentration is lowered.

Table 2.1. Recoveries and limits of detection for metal ions spiked at 100 μg/L level in an adhesive sample containing triethylene glycol dimethacrylate, cumene hydroperoxide, saccharin, dodecyl methacrylate and a primary alcohol ethoxylate.

Metal Ion	Recovery (%)	Limit of detection/mg/L
Cu(II)	80	60
Pb(II)	93	70
Ni(II)	89	70
Zn(II)	110	40
Co(II)	75	40
Fe(II)	ND	--
Cd(II)	97	60
Mn(II)	61	60

ND = not determined

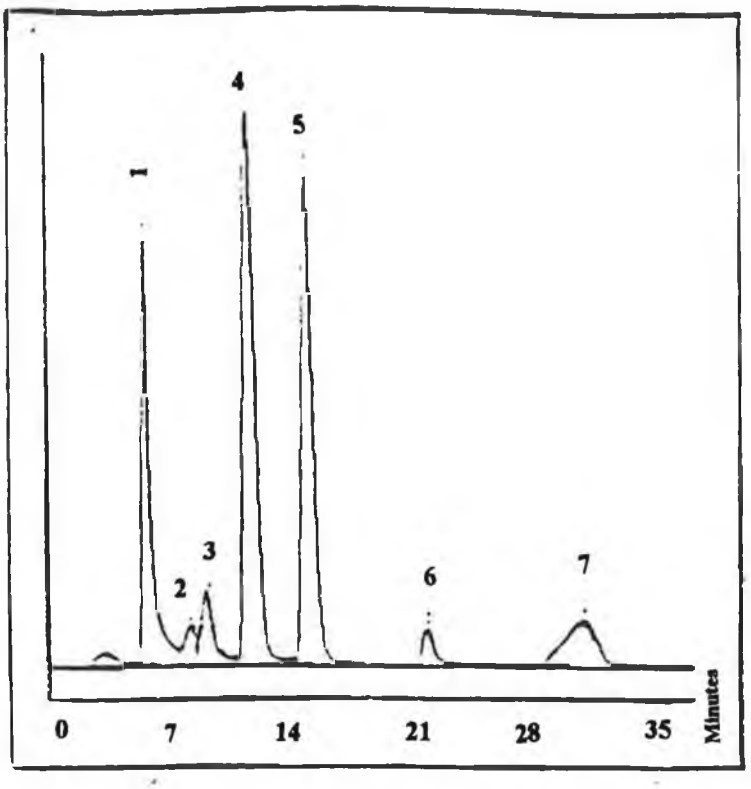


Figure 2.9. Chromatogram obtained following solid-phase extraction of metal ions present at the 100 mg/L level in a solution of tetraethylene glycol di-(2-ethylhexanoate). 1. Cu(II), 2. Pb(II), 3. Ni(II), 4. Zn(II), 5. Co(II), 6. Cd(II) and 7. Mn(II).

Column μBondapak C_{18}, Eluent 2 mM sodium octanesulphonate, 30 mM tartaric acid, 20 mM citric acid, pH 3.4, flow rate 1.0 ml/min. Injection loop 1.0 ml/min. Detection: post-column derivatisation with PAR reagent (0.2 mM PAR, 1 M acetic acid and 3 M ammonium hydroxide), at 520 nm, flow rate 0.5ml/min.

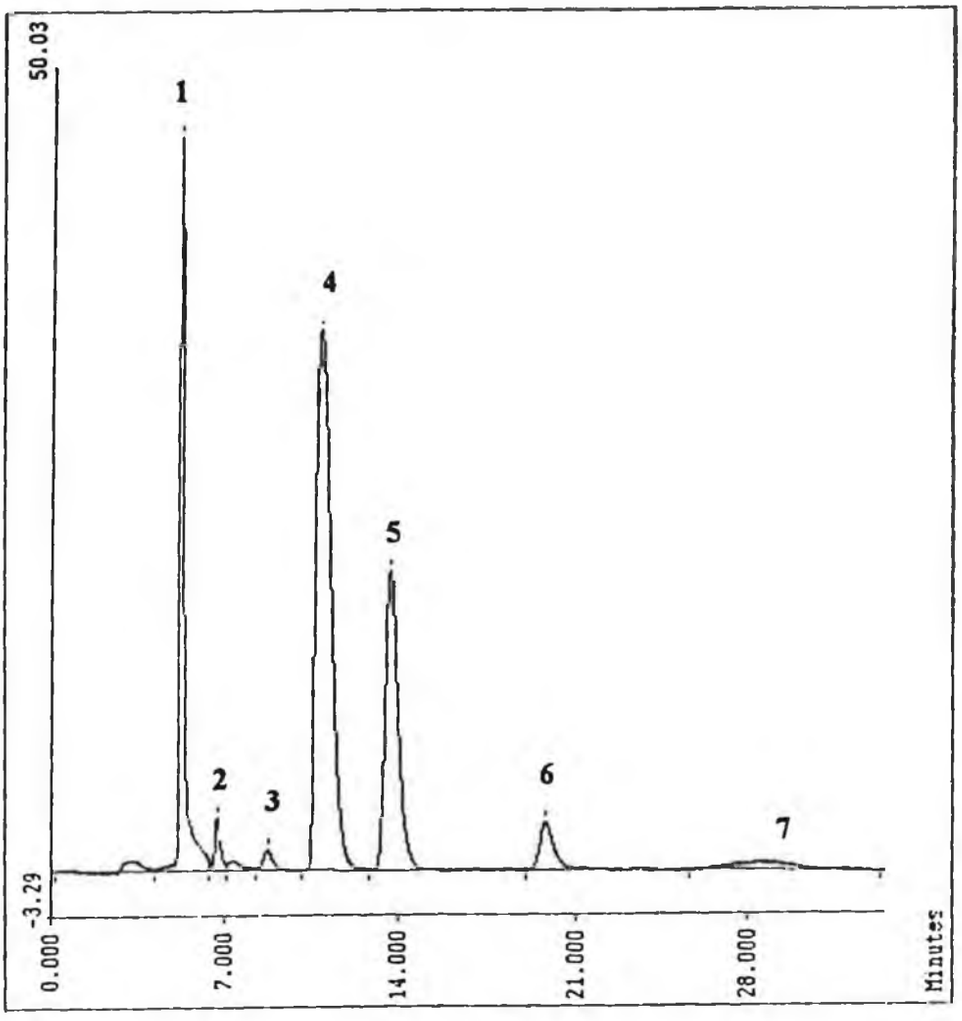


Figure 2.10. Chromatogram obtained following solid-phase extraction of metal ions present at the 100 μg/L level in an anaerobic sealant formulation containing triethylene glycol dimethacrylate, cumene hydroperoxide, saccharin, dodecyl methacrylate and a primary alcohol ethoxylate. 1. Cu(II), 2. Pb(II), 3. Ni(II), 4. Zn(II), 5. Co(II), 7. Cd(II) and 8. Mn(II).

Column μBondapak C_{18}, Eluent 2 mM sodium octanesulphonate, 30 mM tartaric acid, 20 mM citric acid, pH 3.4, flow rate 1.0 ml/min. Injection loop 1.0 ml/min. Detection: post-column derivatisation with PAR reagent (0.2 mM PAR, 1 M acetic acid and 3 M ammonium hydroxide), at 520 nm, flow rate 0.5ml/min.

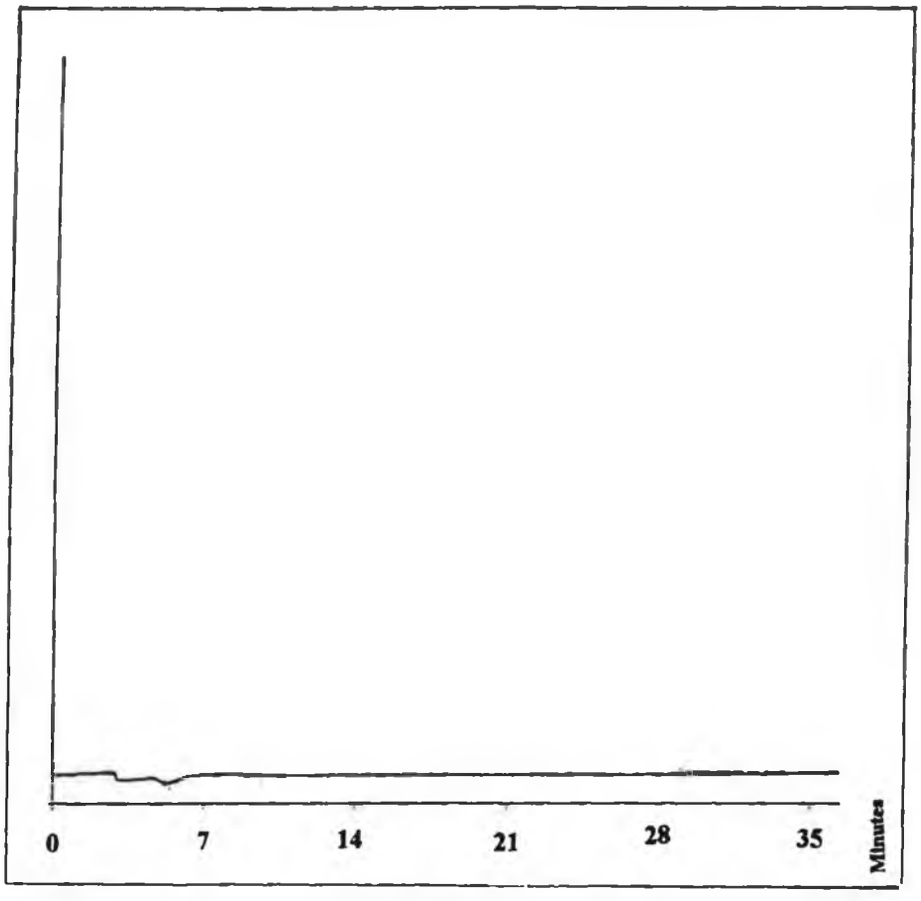


Figure 2.11. Chromatogram obtained following solid-phase extraction of a sample of anaerobic sealant formulation containing 100 mg/L of 1. Cu(II), 2. Pb(II), 4. Zn(II), 5. Co(II), 7. Cd(II), and 8. Mn(II) in the presence of 20ppm EDTA.

Column μBondapak C_{18}, Eluent 2 mM sodium octanesulphonate, 30 mM tartaric acid, 20 mM citric acid, pH 3.4, flow rate 1.0 ml/min. Injection loop 1.0 ml/min. Detection: post-column derivatisation with PAR reagent (0.2 mM PAR, 1 M acetic acid and 3 M ammonium hydroxide), at 520 nm, flow rate 0.5ml/min.

2.9 CONCLUSIONS

Simplicity is one of the major advantages of the developed method. The method involves relatively little sample preparation for the amount of information achieved. The method has much fewer preparation steps than the method developed by Mooney et al. [40]. Furthermore the method may in future be easily automated by using a specialised commercially available vacuum manifold. Also a lot of information is gained in a single analysis as Cu(II), Pb(II), Co(II), Ni(II), Zn(II) Cd(II) and Mn(II) can be determined simultaneously. In the presence of a large excess of EDTA no signal was obtained showing that the method does not respond to metal contamination present in the form of a complex.

Further investigation may reveal the reason for the loss of Fe(II). All investigations were carried out by spiking the sample at the 100 mg/L level. Thus the effect of one metal on the recovery of another was not seen. For example, if copper has a greater attraction for the silica surface, then a higher concentration of copper may result in a lesser recovery of another metal, e.g. zinc. This effect could be investigated by spiking samples with different ratios of metal ions.

A more detailed study of washing solvents may improve the chromatograms obtained. For samples containing polar compounds such as cumene hydroperoxide and saccharin there is always the possibility of retention, as silica is a polar sorbent. The chromatograms may benefit from the introduction of a more polar wash solvent such as isopropanol, such that the wash sequence would involve for example 10 ml chloroform, 10 ml isopropanol, 10 ml iso-octane, and 3 ml mobile phase. Special attention should be paid to the quality of the water supply used with regard to contamination by organics. A deterioration in the peak height of Cu(II) is an indicator of unsatisfactory organic contamination in the water supply. The silica Sep-Pak itself was found to be contaminated by zinc. This was accounted for by performing a blank measurement on each new batch of Sep-Paks.

The method has been shown to give no response in the presence of excess of EDTA. Further investigation may indicate that the method can thus distinguish between free and chelated metal. This would involve investigations at various levels of EDTA and metal ion. However there are still many questions unanswered as the area of metal chelation in non-aqueous systems is relatively unknown. A true determination of the free metal content will only be achieved if the metal-chelate equilibrium is not disturbed. In this investigation, the effect of the solubiliser is not known. It is known that the stability of a metal chelate is affected by the

solvent system. This is due to the fact that metal ions are electron pair acceptors, and if the solvent has donor properties, then the chelating agent must displace the donor solvent to achieve complexation. The greater the donor properties of the solvent then the less stable the complex. Also it must be remembered that unless the metal is in the cationic form, it may not be determined. Thus metal which is not complexed, but not present as the free metal ion, may be undetected.

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CHAPTER 3

THE ANALYSIS OF THE MICRONUTRIENT CONTENT OF FERTILISERS

3.1 MICRONUTRIENTS AND THE GROWTH OF PLANTS

The elements iron, copper, zinc, manganese, molybdenum and boron, although needed in extremely small amounts, are essential for the growth of plants [1]. Deficiencies in these elements can cause reduced crop yields and in severe cases plants will not grow. Many soils contain thousands of times more micronutrients than most crops require. The problem of micronutrient deficiency is obviously not one of total supply. For the micronutrients in the soil to be available to plants they must be solubilised in soil solution, and thus they may be taken up by the plant by the mass-flow of water to the roots. However, to understand why solubilisation may not be achieved, the nature of soil must be examined. The area of soil research is in its infancy, and is a very complicated subject.

The soil is not a static system. It is a constantly active dynamic system, involving biological, physical and chemical changes. The manner in which inorganic solid phases interact with other components in soils is depicted diagrammatically in Figure 3.1. The soil solution is the focal point in this diagram. As plants remove a nutrient from the soil solution (reaction 1), the concentration of that nutrient drops. If that nutrient is held on the ion-exchange sites of soils, some of the adsorbed nutrient is released (reaction 4) to re-establish its level in solution. Depletion of a nutrient from solution also causes crystalline minerals and other precipitates to dissolve (reaction 6) in order to replenish the soil solution and resaturate the depleted soil exchange sites (reaction 3). Concurrently, micro-organisms may remove nutrients from the soil in the course of their metabolic activities (reaction 7), or during the decomposition of organic materials, nutrients may be released to the soil solution (reaction 8). Equilibrium in its true sense is probably never achieved in soils. If it were, soils would be a poor medium for plant growth, because many of the essential plant nutrients would be too insoluble. Fortunately the environment in soils is constantly changing due to fluctuating temperatures, growing roots, respiring micro-organisms, changing O_2 and CO_2 levels, alternating moisture levels and other important changes. In soils where there are deficiencies the solubility of the micronutrient in the soil solution, and thus the bioavailability of the micronutrient, is affected by many factors. Table 3.1 pinpoints the major factors involved. It can be seen that the issue of micronutrient deficiency is far from simple.

For the analytical scientist trying to examine the possibility that poor crop growth is due to a micronutrient deficiency is a difficult task. Soil tests for micronutrients have shown that various methods of extraction have been used. These include extraction with water, EDTA, $NH_4C_2H_3O_2$, ,HCl, etc. However, each method of extraction produces a different result,

and to date no single method has been shown to be correct when predicting micronutrient deficiency. All that can be achieved is a correlation between plant response and the method of extraction for a particular metal [2]. These field calibration studies have been achieved in localised areas, but often results have not been applicable elsewhere. Thus, even when using the most logical extractant, the level of nutrient in the soil has not provided all the information necessary for proper interpretation. This has pointed out the necessity of considering other soil properties and environmental factors when attempting to interpret micronutrient soil tests.

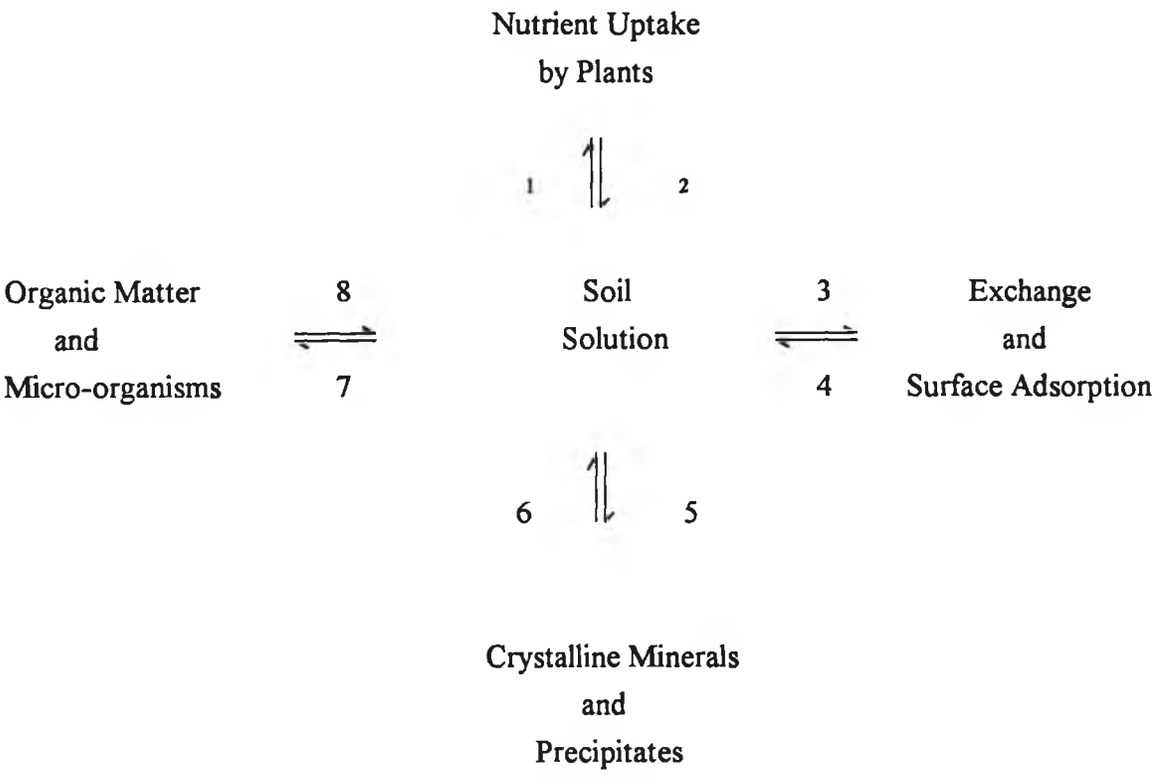

Figure 3.1. Nutrient distribution within the soil. From [2]

Mn	Fe	B	Cu	Zn	Mo	Cause of deficiency
	•		•			high nitrogen
	•		•	•		high phosphorus
	•					low potassium
	•	•				high calcium
				•		high magnesium
	•		•		•	high manganese
•			•			high iron
•	•				•	high copper
			•			low zinc
•	•		•			high zinc
•					•	low pH
•	•	•	•	•		high pH
					•	high sulphur
•						high sodium
	•					high bicarbonates
	•					iron:copper:manganese imbalance
	•	•	•			low organic matter
	•		•	•		high organic matter
•						poor drainage
•		•				drought
•	•					cold, wet soils
	•					poorly aerated soils
				•		exposed subsoil's
•						heavy manuring
•	•	•	•			heavy rainfall
•		•	•	•		light and sandy soil

Table 3.1 Factors which affect micronutrient availability. From Stoller Chemical Company, Houston, Texas, Catalogue

3.1.2 The use of metal chelates as micronutrient fertilisers.

The amount of soluble metal present in soils or nutrient solutions can be increased by the addition of water soluble metal chelates. Chelating agents can increase the solubility of added or indigenous metal cations. The organic chelate molecule surrounds the positively charged metal and protects the new chelated form of cation from precipitation or adsorption within the soil. In the search to find suitably stable chelates, the chelating agents which have been studied most fall into two categories: the aminocarboxylates and the hydroxycarboxylates. The European Union has recommended the use of the chelating agents EDTA, DTPA, HEEDTA, EDDHA, EDDHMA and EDDCHA (see Table 3.2). Not all the chelates work equally well under the same conditions, and therefore a range of chelating agents are needed. The critical factors for the use of a given chelate are its stability and the cost of preparation of the chelate. Usually the more stable chelates, such as FeEDDHA, are the most expensive, and therefore it is important that a sufficiently stable chelate of minimum cost is used. Thus a good understanding of metal chelate equilibria is essential to the effective use of chelating agents.

There are three principle uses of micronutrient fertilisers - as soil fertilisers, as foliar sprays and in hydroponics. Hydroponics is the growth of plants in water culture, i.e. in the absence of soil. Unlike the growth of plants in soil this represents a very controlled environment where the constituents and the parameters controlling the growth of the plant are known. Foliar application is used as it eliminates complicated soil reactions, irrigation is not required to move the compounds into the root zone, the response is rapid and there is less wastage. However the disadvantages of foliar application are the greater risk of toxicity, incomplete coverage and subsequent uneven response and the need for repeated applications.

Numerous studies have been made to test the effectiveness of various chelates in supplying micronutrients to plants. Empirical methods; i.e., the chelates are tested in different soils and nutrient solutions to see what would happen, may be used [2], or alternatively theoretical models [3,4]. Theoretical studies produced stability diagrams of the chelates in soil solution based on a knowledge of the formation constants of the chelated species and the activity of the competing metal ions expected in soils. According to Norvell [4], in soils a cation must satisfy two requirements in order to compete successfully for an appreciable fraction of a chelating agent. Firstly, an adequate quantity of the cation must be present in solution or in equilibrium with the soil solution to permit formation of a significant concentration of metal chelate. Secondly the chelates of this cation must possess sufficient stability to exist in

equilibrium with the concentrations of the cation and free ligand in soil solution . Thus according to Norvell [4], in soils the pH of the solution, and the concentrations of Ca^{2+}, Mg^{2+}, Al^{3+}, and Fe^{3+} are the main factors affecting the stability of an added metal chelate. However, this does not include the reactions of the natural complexes formed by soluble organic matter in the soil. However, studies such as those by Norvell [4], have been shown to be realistically accurate by practical laboratory studies. Figures 3.2 to 3.4 show how the stability's of different chelating agents vary in the soil.

Thus the complexity of the use of metal chelates can be appreciated. The choice and use of the correct chelate is critical from the point of view of its effectiveness and from an economic point of view.

CHELATING AGENT	ABBREVIATION
Ethylenediamine tetraacetic acid	EDTA
Diethylene triamine pentaacetic acid	DTPA
Hydroxy-2-ethylenediamine triacetic acid	HEEDTA
Ethylenediamine di(o-hydroxyphenyl) acetic acid.	EDDHA
Ethylenediamine-di(o-hydroxy-p-methylphenyl) acetic acid.	EDDHMA
Ethylene diamine di (5-carboxy-2-hydroxyphenyl) acetic acid	EDDCHA

Table 3.2. Chelating agents recommended by directive 76/116/EC of the European Union.

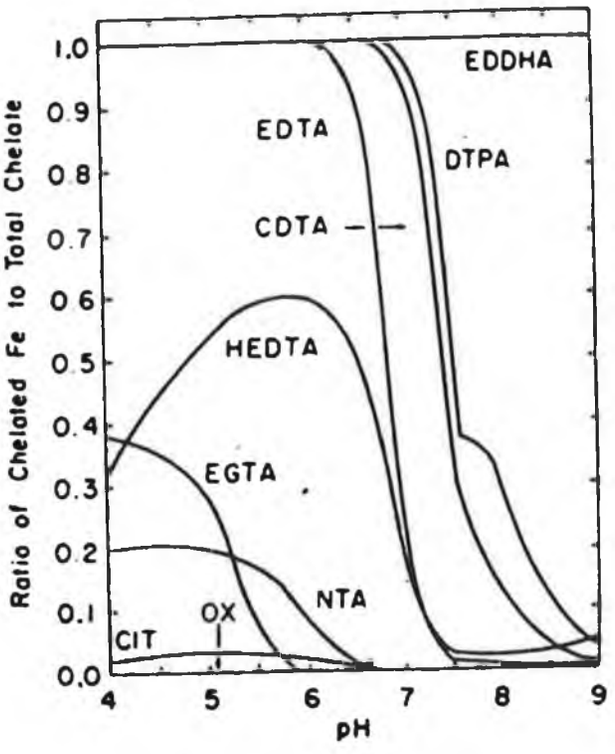

Figure 3.2. Comparison of Fe-chelate stability's in soil solution. Equilibrium between H^{+}(varied between pH 4 to pH 9), Ca^{2+}(3 x 10^{-3}M), Mg^{2+}(1 x 10^{-3} M), Al^{3+}, Fe^{3+} (concentrations were assumed to be controlled by the solubility of hydrous oxides with solubility products of $10^{-33.4}$ and $10^{-39.4}$ respectively), and 10^{-4}M concentrations of each chelating agent is assumed [4].

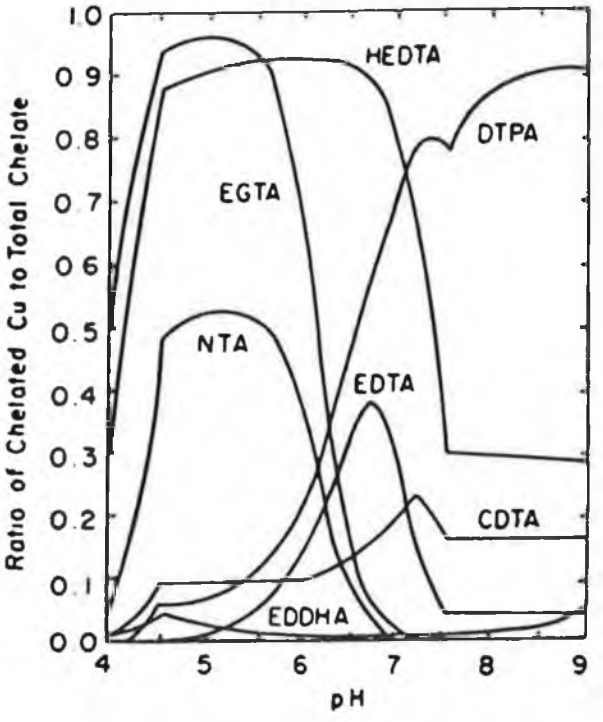

Figure 3.3. Comparison of Cu-chelate stability's in soil solutions. This figure is based on the Cu^{2+} solubility relationship: $[Cu^{2+}] = 10^{3}(H^{+})^{2}$ with a maximum concentration of 10^{-6}M. Equilibrium between H^{+}(varied between pH 4 to pH 9), Ca^{2+}(3 x 10^{-3}M), Mg^{2+}(1 x 10^{-3} M), Al^{3+}, Fe^{3+} (concentrations were assumed to be controlled by the solubility of hydrous oxides with solubility products of $10^{-33.4}$ and $10^{-39.4}$ respectively), Cu^{2+} and 10^{-4}M concentrations of each chelating agent is assumed.[4]

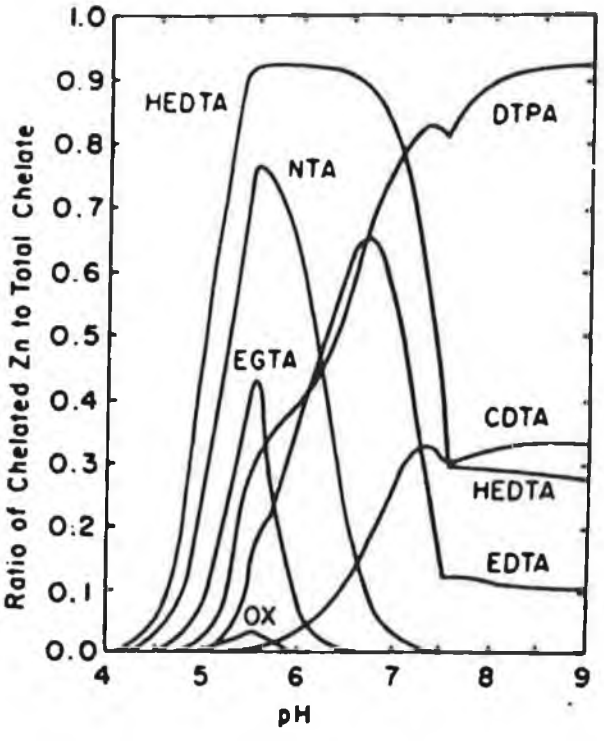

Figure 3.4. Comparison of Zn-chelate stability's in soil solutions. This figure is based on the Zn^{2+} solubility relationship: $[Zn^{2+}] = 10^{6}(H^{+})^{2}$ with a maximum Zn^{2+} concentration of 10^{-5}M. Equilibrium between H^{+}(varied between pH 4 to pH 9), Ca^{2+}(3 x 10^{-3}M), Mg^{2+}(1 x 10^{-3} M), Al^{3+}, Fe $^{3+}$ (concentrations were assumed to be controlled by the solubility of hydrous oxides with solubility products of $10^{-33.4}$ and $10^{-39.4}$ respectively), Zn^{2+} and 10^{-4}M concentrations of each chelating agent is assumed.[4]

3.2 DEVELOPMENT OF AN ANALYTICAL METHOD

3.2.1 Requirements of the Directive

This investigation was undertaken to ensure that the analytical method used for the determination of fertilisers satisfied the requirements of the EU directive 76/116/EC. This directive dictates that there are two categories of fertiliser which may be marketed: fertilisers containing only one of the trace elements listed, and secondly fertilisers containing at least two different trace elements. To evaluate the trace element content of the fertiliser, the directive recommends that the total content in respect of each nutrient, the water soluble content, the chelated form in which the trace element is present, and the quantity of the trace element which is chelated must be determined. This investigation is concerned with the latter two requirements.

Theoretically the analytical method developed must be able to distinguish between any combination of the micronutrients copper, iron, zinc, manganese, cobalt and molybdenum with the chelating agents EDTA, DTPA, HEEDTA, EDDHA, EDDHMA and EDDCHA. In practice, however, an examination of fertilisers available on the market from 1989 showed that fertilisers containing iron in the form of EDTA, DTPA, HEEDTA, EDDHMA and EDDHA may be found, while copper, zinc, and manganese were added as EDTA chelates. No samples were found to contain boron or molybdenum. Thus the present investigation set about quantifying the chelates FeEDTA, CuEDTA, ZnEDTA, MnEDTA, FeDTPA, FeHEEDTA, FeEDDHA and FeEDDHMA.

3.2.2 Discussion of Methods of Analysis Existing within the EU

Methods of analysis for chelates in fertilisers have been submitted to the EU by the Netherlands, France, Spain and Belgium.

The choice of method in The Netherlands is based on a gel permeation chromatographic (GPC) method developed by Boxma [5] and has been used to determine the iron chelates of EDTA, DTPA, HEEDTA, EDDHA and EDDHMA and the zinc, manganese and copper chelates of EDTA. The total concentration of metal ion is firstly determined by ICP. The sample is then passed through a Sephadex G10 column 42 x 2.5 cm and the sample eluted with 0.035M calcium chloride, pH 7.0. The fraction containing the complex is detected

spectroscopically (UV) as it elutes from the column and is collected and analysed by ICP for metal ion content. However, FeEDTA coelutes with FeDTPA, and FeEDDHA coelutes with FeEDDHMA. The Dutch method [5] also involves an increased in the flow rate compared with the Boxma method from 2 ml/min to 4 ml/min but the analysis time is still very long at eighty minutes. Therefore the shortcomings of the method are that it can only identify what metal ions are present, but it cannot identify what chelating agents are present. Furthermore the fate of the free metal ions have not been determined. It is probable that they are retained by the gel, if this retention was reversible then misleading results would be obtained.

The choice of method in France determines the chelated fraction of the elements copper, iron, manganese and zinc, and identifies the chelating agent present. The sample is analysed by AAS to determine the total metal content of the sample. The chelating agents EDTA, HEEDTA, DTPA, EDDHA, EDDHMA, and EDDCHA are identified using a thin layer chromatographic (TLC) technique. To the extract solution calcium hydroxide is added to precipitate any metal ion present, in either the free or chelated forms. Separation of the chelating agents is achieved by chromatography on a thin layer of DEAE (DEAE-cellulose in formic form) with formic acid as the eluent. The developing solution contains sarcosine cresol red and copper sulphate. The aminocarboxylic acid chelating agents will form a more stable complex with Cu(II) than with sarcosine creol red, and will thus appear as yellow spots on a purple background. A second aliquot of the sample is then adjusted to pH 7.0. This is then agitated with the sodium form of Amberlite IR-120-P, a strong cationic sulphonic type exchange resin, to remove metal cations. The trace element content of the resulting solution is determined by AAS, thus giving the chelated content of the sample. This is a very long and labour intensive method. The cation exchanger takes three hours to prepare and the sample must be shaken for four hours with the cation exchanger. It must also be remembered that the cation exchanger only retains cations. This means that the negatively charged chelates of any chelating agent which is stable at pH 7 are not retained and are thus included in the value obtained. Also, if a trace element is present in neutral form, then this will not be retained, thus giving falsely high results.

The choice of method in Spain determines the quantity of free and chelated iron present in a sample. The total iron is determined gravimetrically in the form of ferric oxide with prior removal of organic material by treatment with an oxidising acid mixture. The total iron content may also be determined by AAS. Again the organic material is removed with an oxidising acid mixture before analysis. The chelated iron is determined by taking an aqueous sample of the ferrous chelate. The non-chelated iron is precipitated as iron (III) hydroxide

using sodium hydroxide at pH 8.5 and the solution precipitate removed by filtration. An aliquot of the resulting solution is then treated with an oxidising acid mixture to destroy the organic material. The iron may then be determined by AAS, or gravimetrically as iron (III) oxide. Thus the method does not identify or quantify the chelating agents present.

The method of choice in Belgium determines the quantity of chelating agent both qualitatively and quantitatively. A TLC method using the same principle as the French method is used to identify the chelating agents present. A titration procedure is used to determine the quantity of the of the chelating agents NTA (nitrilotriacetic acid), EDTA, HEEDTA and DTPA. The pH of a sample solution is adjusted to pH 2.0 and a known excess of bismuth is added. The bismuth will bind with all the chelating agents present displacing copper, mangenese, zinc, cobalt, etc. from their respective complexes as it has a higher conditional stability constant with the chelating agent at this low pH. The excess of bismuth is then back titrated with a solution of polycarboxylic acid of known concentration using a metallochromic indicator. This therefore determines the total amount of chelating agent present. It can only be used in cases where the sample contains only one chelating agent, the only disadvantage that it cannot be used where there is a mixture of chelating agents, as the bismuth may preferentially bind with one chelating agent. It also does not give an indication of the composition of the fertiliser. Also it is probable that the bismuth will chelate with more than just the recommended chelates, the presence of which may or may not be discovered by the TLC method.

3.3 CHROMATOGRAPHIC TECHNIQUES FOR THE DETERMINATION OF METAL CHELATES

Ion-exchange, ion-pair, reversed-phase, gel permeation, thin layer and gas chromatography have all been used for the determination of aminocarboxylic acid chelates. The majority of applications involve the use of these methods for the quantitative determination of chelating agents by the addition of metal ions (usually iron or copper) to aid detection, or alternatively for the determination of metal ions by the addition of a chelating agent such as EDTA to aid separation.

3.3.1 Thin layer chromatography and paper chromatography

Thin layer and paper chromatography are normally used as spot tests to confirm identification of a compound. Hill-Cottingham [6], using paper chromatography with a 4:1:5 butanol-acetic acid-water eluent separated NaFeEDDHA into two isomer bands, coloured red ($R_f = 0.54$) and violet ($R_f = 0.66$), which were identified as the meso- and dl-racemic isomers of NaFeEDDHA, although assignment of isomer to band was unsuccessful. Rajabalee et al. [7] used TLC on silica gel in a benzene-ethanol-water mixture for the separation of various metal chelates of EDTA and NTA. The chelates were detected by spraying with PAN (1-(2-pyridylazo)-2-naphthol) reagent, with detection limits of 0.5 μg for the chelates of NTA and 2 μg for the chelates of EDTA. Camacho et al. [8] used TLC combined with ion-exchange chromatography for the detection of Ca(II)-, Cu(II)-, Fe(III)-, Mg(II)-, Mn(II)- and Zn(II)-EDTA in human faeces. TLC separations were achieved on silica gel and cellulose precoated plates using a mixture of water-ethyl methyl ketone-butanol-acetone or water-ethylene glycol monoethyl ether -butanol-acetone and a developing spray containing PAN, murexide or benzidine. In either solvent system a detection limit of 0.5 mg was achieved. However as TLC is a laborious technique, and quantitation is difficult then this method was not considered suitable for the determination of metal chelates in fertilisers.

3.3.2 Gas chromatography

Gas chromatographic (GC) procedures have been used for the determination of aminocarboxylic acid complexing agents, mainly by formation of the respective alkyl esters [9,10,11]. Gardiner [9] determined EDTA in sewage by formation of the ethyl ester, while

Sniegoski separated EDTA, HEIDA (N-(2-hydroxyethyl)iminodiacetic acid and IDA (iminodiacetic acid) in the form of their n-butyl esters [11]. However, Cassidy et al. [10] found that in the presence of Fe(II) or Fe(III) (10-300 μg/ml), irreproducibility and low results were found when determining EDTA at the 10-100 μg/ml level, as in the presence of air and light iron caused decomposition of the EDTA by a cyclic photoreduction/air oxidation of the iron (III) complex. Standards containing copper (II) did not, however, exhibit similar losses. Aue et al [12] found that even in the presence of a ten-fold excess of iron, zinc and copper, nitrilotriacetic acid could be determined at the 1 ppm level when the sample was previously acidified to remove the metal ions before esterification Therefore, GC has only been used as a method of determining the chelating agent.

3.3.3 Gel permeation chromatography

Yoza et al. [13] investigated the behaviour of magnesium ions, EDTA ions and their complex on a Sephadex G-15 column with a 0.1M sodium chloride solution as eluent. Each of the species was injected and the elution behaviour observed. It was found that the three species eluted at different retention times; the EDTA ion eluting first after 46.0 minutes, followed by MgEDTA at 50.0 minutes and the Mg ion at 64.3 minutes. Yoza et al. [14] also chromatographed magnesium (II), strontium (II) and barium (II) ions (i.e. no complexes involved) on a Sephadex G-15 column with a 0.1 M sodium chloride solution as eluent. The shapes of elution curves and K_d values were found to be dependent on sample concentrations. This was explained in terms of the interaction of the solutes with the gel matrix by adsorption and the variation in effective sizes of the solutes with the variation in ionic strength of the surrounding medium. In a separate investigation, Yoza [15] demonstrated the use of gel permeation chromatography for the determination of the stability constant of a complex such as MgEDTA. A gel chromatographic column of Sephadex G-10 was pre-equilibrated with a buffered eluent containing a known concentration of magnesium ion [C_{Mg}]. EDTA was dissolved in a solution of magnesium ion of the same concentration as that in the eluent. Thus the concentration of free magnesium ion in the sample is reduced to a level lower than [C_{Mg}] by an amount corresponding to the MgL complex formed. The eluent of the column was monitored by AAS. As MgL emerges from the column a positive peak is seen. Behind the MgL peak the magnesium level continues to be constant and then decreases at the elution position of free magnesium ion, to below the baseline level to form a negative peak that corresponds to the amount of magnesium consumed to form MgL. Thus as [C_{Mg}] is known and [MgL] may be

measured, [L] may be calculated from ($[L]_t$ - [MgL]) where $[L]_t$ is the total concentration of ligand. Thus the stability constant may be calculated.

Boxma [5] developed a gel chromatographic method for the separation of free and complexed metal which is still used as the standard method by state laboratories in The Netherlands for the determination of chelates in fertilisers. The chelates FeEDTA, FeDTPA, FeEDDHA and FeEDDHMA were separated from free iron (II)/(III) on a Sephadex G10 column. The chelates were eluted with a 0.15 M sodium chloride solution adjusted to the pH of the chelate solution. FeEDTA coeluted with FeDTPA, and FeEDDHA coeluted with FeEDDHMA, making it impossible to determine a mixture of the chelates. Also FeEDDHA and FeEDDHMA had a very long retention time of four hours. It was found that interferences from iron chelates of moderate stability could be eliminated by eluting with 0.035 M calcium chloride at pH 7.0.

The chromatographic behaviour of the EDTA complexes of Co(II), Ni(II), Cu(II), Cr(III), Fe(III), Co(III), and Bi(III) on Sephadex G-10, -15, and -25 in NaCl-containing solvents has been studied by Deguchi [16]. The $M(II)EDTA^{2-}$ complexes were easily separated from the $M(III)EDTA^{-}$ complexes. Apart from these two types of complexes, fractionation is more difficult. In some cases, e.g. in the chromatography of $BiEDTA^{-}$ and $CrEDTA^{-}$, the use of a long column results in a good separation. Increase of the NaCl concentration increases the elution volumes, which Deguchi explained as a result of a reduction of the effective size of the complexes.

3.3.4 Ion exchange chromatography

In an aqueous environment, within a given pH range, aminocarboxylic acid chelates appear as an anion. This makes it possible to use an anion exchange column for separation.

The development of the analysis of chelates using anion exchange chromatography started with low pressure columns. Vanderdeelen [17] showed that iron(II), copper(II), zinc(II), manganese(II) and cobalt(II) could be separated in the form of EDTA chelates using a Dowex 2-X8, (200-400 mesh) anion exchange resin. The effect of the eluents potassium chloride, sodium acetate, ammonium acetate and hydrochloric acid were investigated, the best resolution being obtained using 0.5 M ammonium acetate. Using this eluent, the chelates were completely resolved and investigations were carried out in the mM range.

However, the column was manually packed and a long retention time of five hours was obtained. The development of high pressure systems, however, overcame the problem of long retention times.

Longbottom [18] investigated the use of anion exchange chromatography as a way of separating NTA from the chelates ethylenediamine diacetic acid (EDDA), HEEDTA, and EDTA in sewage samples and solutions of detergent formulations. As it was the chelating agents which were of interest, all chelates were converted to the iron(III) form by reaction with iron(III) nitrate. A 1m x 0.25 in stainless steel column packed by DuPONT with SAX, a strong anion exchange resin coated with Zipax, was used. The mobile phase was 0.02 M $Na_2B_4O_7.10H_2O$ at pH 9.0. The chelation with Fe(III) prior to injection was used to overcome metal interferences, as NTA preferentially chelates with iron(III) ions when available. After injection, the iron precipitated due to the high pH, releasing the chelating agent which is separated from the iron(III) ion for UV detection at 254 nm. A good separation of EDDA, HEEDTA, EDTA and NTA was obtained in four minutes. The limit of detection of the method was 1.0 mg/l.

Jones and Manahan [19] have demonstrated that EDTA, NTA (nitrilotriacetic acid), EGTA [ethylene-bis(oxyethylenenitrilo)tetraacetic acid] and CDTA [(1,2 cyclohexylene-dinitrilo)tetraacetic acid] could be separated as their copper(II) complexes by HPLC using a 5-cm weak anion-exchange Aminex A14-type resin column and an aqueous 0.05M ammonium sulphate, pH 5.5, mobile phase. The eluted copper (II) chelates were detected by AAS. The chelates were observed to elute from the column as a function of charge and size in the order: Cu_2(EGTA), $Cu(NTA)^-$, $Cu(EDTA)^{2-}$ and $Cu(CDTA)^{2-}$. This analysis was developed to determine chelating agents as pollutant components of natural and waste waters. The chelated copper(II) ion added to the mixture served as an indicator metal to allow detection by AAS. AAS was used as it is a very selective detector as only the metal-containing compound is seen. Therefore this method does not have the background absorption problems associated with UV detection. The detection limit for EDTA was 0.196 mg/l. However, as the parameter being measured is the concentration of metal per unit volume of liquid introduced into the flame at a specific instant, band spreading in the chromatographic system must be maintained at a minimum which dictates the use of a small column (5 x 0.21 cm) in this case. However, the instrumentation is not difficult to set up as the column eluent simply feeds directly to the AAS nebuliser.

Van Loon et al. [20] described a method of separation and detection of Cu(II), Ni(II) and Zn(II) in the form of their EDTA chelates using a Partisil-10 SCX cation exchange HPLC system interfaced with atomic fluorescence spectroscopy (AFS). In this approach, the HPLC instrument was directly interfaced to the nebuliser capillary of the flame burner of the AFS. The AFS contained three different channels for monitoring emissions from three different elements simultaneously. The mobile phase consisted of pure water for the first minute followed by a 5 minute convex gradient up to 100% 1M ammonium nitrate. Introduction of instruments which combine AFS with an inductively coupled plasma atomic source means that this approach could be extended to monitor for more than just three elements simultaneously in a single HPLC run. The total run time was ten minutes. Detection limits were not discussed.

Harmsen et al. [21] determined EDTA in the form of its iron chelate using an anion exchanger, i.e. Partisil 10 SAX (Whatman) (25 x 4.6 mm) using the eluent 0.5M NaCl and 3 ml glacial acetic acid in 1L of water, pH 3.1. The EDTA was converted to its iron(III) chelate by reaction with iron (III) chloride. The analysis time was seven minutes and detection limit was 0.2 mg/l at 258 nm. It was found that the addition of the metal ions Cu(II) and Zn(II) did not cause any interferences, while Co(II) caused a slight interference.

Barak et al. [22] separated the two isomers of FeEDDHA on a 30 x 4.6 mm Wescan Ion-Guard anion exchange cartridge with an eluent containing 5 mM H_2SO_4 and 0.01 M $Fe_2(SO_4)_3$ the isomers eluting at four and six minutes respectively at a flow rate of 3 cm/min. FeEDDHA was well resolved from the chelates NaFeCDTA, NaFeEDTA and NaHFeDTPA. However, the elution behaviour of the latter three chelates were not shown. Uncomplexed EDDHA could be distinguished from FeEDDHA, as EDDHA being protonated and uncharged passes down the column with the solvent. The detection limit was 0.0012 mmol/l FeEDDHA. Standards of the two isomers were prepared for this investigation by separating commercial grade NaFeEDDHA into the meso- and dl-racemic isomers using paper chromatography [6]. Using a procedure for the production of a standard containing only the dl-racemic form developed by Bailey et al [23], the red band was assigned as the dl-racemic isomer, while the violet band was the meso isomer. The two bands were dried and re-dissolved in water. Thus positive identification of the separated sample could be achieved.

Matsushita [24] determined a range of transition metal cations by complexation with EDTA. The eluent consisted of 1 mM EDTA at pH 6.0, and separation was achieved on a TSKgel IC-Anion-SW column. UV spectroscopic detection at 210 nm was found to be more sensitive than conductivity detection, with detection limits in the 5.0-60 ng range for conductivity detection and detection limits for UV in the 20 to 250 ng range.

Yamaguchi et al. [25] used a CuO-loaded cation exchanger (2.5 x 1 cm) for the determination of EDTA. The cation exchanger, prepared by treating the Cu(II)-form ion-exchange resin Dowex 50W-X4 with a hot alkaline solution converted EDTA to the equivalent amount of Cu(II)-EDTA chelate. The copper in the eluent was determined spectrophotometrically as its diethyldithiocarbamate complex, and thus EDTA at the 10^{-5}M level was indirectly determined. This CuO-loaded cation exchanger was found to be stable and conversion to take place in the pH range 5.5-9.6. The H-form of the resin (Dowex 50W-X8) was used to decompose metal-EDTA complexes for the determination of EDTA. At pH 4.0, the complexes of Co(II), Cd(II), Zn(II), Pb(II), Cu(II) and Hg(II) were decomposed namely the metal ion was fixed on the resin and the liberated EDTA was found in the eluent. However the recovery of EDTA from Fe(III) complexes was poor.

Yoza et al. [26] used an anion-exchange column (50 cm x 2.6 mm i.d.. TSK-GEL, IEX220SAA) for the determination of the relative complexing abilities of individual ligands in multicomponent samples, although quantitative results were not given. Detection was achieved following a post-column reaction of the ligand with the coloured methylthymol blue complex of copper (II) at pH 5.5. Thus the conditional complexing abilities of the ligands at pH 5.5 was determined. A gradient technique was employed with eluents 0.05M $(CH_3)_4NCl$ and 0.20M $(CH_3)_4NCl$ at pH 5.5. A separation of EDDA, EDTA-OH, EDTA, DTPA-OH and DTPA was achieved.

3.3.5 Separation on reversed-phase silica columns

The reversed-phase systems using chemically bonded silica gel as the stationary phase have been widely used, especially in conjunction with an ion-pairing reagent (this will be discussed in more detail in the next section)

Ohzeki et al. [27] achieved a very efficient separation of the iron (III) complexes of EDTA, methyl-EDTA, DTPA and CyDTA (1,2-cyclohexylenedinitrilo)-tetraacetic acid on a silica

column (Zorbax SIL) using $1x10^{-2}$M phosphate buffer solution, pH 3 as the mobile phase. The retention of the chelates was enhanced with increasing concentration of the buffer solution and with increasing amount of sodium sulphate added to the eluent. Ohzeki et al. [27] concluded that the retention of the chelates depended largely on the hydrophobic moiety of the chelate, as the complex forming properties are essentially invariant. Detection was carried out at 260 nm achieving mM detection limits.

Dai et al. [28] determined NTA and EDTA on a reversed-phase column with an aqueous trichloroacetic acid mobile phase at pH 2.0. Detection was carried out using an amperometric detector employing a carbon-paste electrode. The aminopolycarboxylic acids are directly oxidised at the detector electrode without involving an intermediate species such as a metal ion. Formation of a free radical leading to decarboxylation was suggested to be the primary electron transfer process. The detector response was found to be strongly dependent on pH decreasing with increasing pH in the pH range 1.8-3.0. The low pH was used to maximise hydrophobicity of the analytes and to destabilise interactions between the ligands and cations present in the sample. Where such interferences occur, DTPA can be used to suppress it. DTPA was found to successfully suppress interference from Cu(II), but only indicate interference from Fe(III). Amperometric detection was seen as being more selective than UV methods, which rely on complexation with a metal ion for detection. This is due to the fact that many metal ions can compete for the chelating agents, and miscellaneous metal ions present in samples can thus cause interference. Another reported analytical problem with UV spectrometric detection is the limited linear range of the calibration curves. The minimum detectable amounts were 0.1 ppm NTA and 0.15 ppm EDTA.

3.3.6 Ion Pair Chromatography

Ion-pair chromatography (IPC) is now replacing many of the applications which were formerly reserved for ion-exchange chromatography, because much more efficient and reproducibile separations are achieved with the former. Both the normal and reversed-phase modes have been used, although the latter is the most popular. To date, reversed-phase high performance ion-pair chromatography (RP-HPIPC) has been used in the analysis of metal chelates, mostly in cases where the chelating agents present are the species of interest. Here

the chelating agents present are converted to chelates by addition of a metal salt, usually copper or iron.

The most common ion-pairing reagent is the tetrabutylammonium ion (TBA^+) used in combination with a C18 column. The earliest relevant reference found was by Perfetti et al. [29] and referred to the determination of EDTA in the form of its copper complex in crab meat and mayonnaise. The EDTA was extracted from the food sample with water and converted to the copper chelate by addition of a five-fold excess of $CuSO_4$. The chelate was then separated using a C18 column and the ion-pairing reagent tetrabutylammonium hydroxide (TBAOH) at a pH of 4.7. UV spectrometric detection was achieved at 254 nm, with detection limits of 50-100 ng EDTA.

Vanezky et al. [30] developed a method for the determination of EDTA in boiler water in the form of the iron(III) chelate. The chelating agent was converted to the iron(III) form by reaction with $FeCl_3$. A C18 column was used along with the ion-pairing reagent tetrabutylammonium bromide (TBABr) at a concentration of 0.0162M in 0.05M sodium acetate at a pH of 4.5. They decided to convert EDTA to the iron(III) form rather than the copper(II), form, as it was found that CuEDTA was not stable in the presence of iron(III). This is due to the fact that an exchange equilibrium occurs between the iron(III) and the CuEDTA resulting in the formation of FeEDTA, as FeEDTA has a higher formation constant than CuEDTA at a pH of 4.5. It was found that when the FeEDTA was used for the analysis, copper(II), nickel(II), calcium(II) and magnesium(II) did not interfere. Detection was carried out at 254 nm with a detection limit of 35 ng EDTA.

Van Waren et al. [31], using very similar conditions to Vanezky et al. [30], also found interference from iron when EDTA was determined in mayonnaise.

Inman et al. [32] again determined EDTA in the form of the iron(III) complex using TBAOH on a reverse-phase column. However, the method of detection, using absorbance ratioing, was a new concept. The ratiogram, a point by point ratio of chromatograms detected at two different wavelengths, provides additional information to supplement the retention time data and was used to help differentiate the analyte peak from vancomycin-related compounds.

Yamaguchi et al. [33] achieved a separation of EDTA and NTA in the form of their iron(III)- complexes using a C18 column with 0.175M TBABr, 0.02M phosphate buffer, pH

2.5. A pH of 2.5 was used as it was found that the peak area of Fe(III)-NTA broadened with increasing pH and was no longer detectable above pH 3.5. The complexing agents were converted to their iron(III) complexes by heating a sample solution in the presence of excess iron(III). The excess iron was then removed by a cation exchange resin column and the eluent directly subjected to analysis. The retention time of both compounds increased with increased concentration of TBA^+, and decreased with increasing concentration of acetonitrile. The method was found to be essentially free from interference from the metal ions Mg(II), Ca(II), Co(II), Cd(II), Zn(II), Ni(II) and Cu(II). Among the anions studied, a high concentration of nitrate anion gave a peak which had almost the same retention time as iron(III)-NTA. Detection was carried out at 255 nm with detection limits of 2 mM and 6 mM for EDTA and NTA respectively.

Parkes et al. [34] achieved a very efficient separation of HEEDTA, NTA and EDTA in the form of their copper(II)complexes again using the mobile phase 10% methanol/90% water/0.01M TBA^+ ion with a C18 column. However a higher pH of 7.5 was used. At this pH it was found that recovery of above 80% was obtained even in the presence of iron(III). Detection was carried out at 254 nm with a detection limit of 0.1 mg/g NTA.

Chinnick [35] achieved a separation of the copper complexes of DHEG, NTA, EDTA, DTPA and HEEDTA using a C18 column with the ion-pairing reagent benzyltrimethylammonium chloride (BTAC) (0.05M), at a pH 3.5-4.0. The eluent contained copper(II) chloride (0.01 M), making it possible to form the copper complexes *in situ*. Detection at 760 nm was used to eliminate interferences from ultraviolet absorbing compounds. Detection limits were in the low mM range. The efficiency of the separation between DTPA and HEDTA however requires improvement.

Yamazaki et al. [36] achieved resolution of the two enantiomers of the two complexes $[Co(EDTA)]^-$ and $Cis[Co(IDA)_2]^-$ where IDA = iminodiacetate, using quinine as the ion-pairing reagent. Quinine is a chiral counter ion which produces diastereoisomer ion-pairs with two kinds enatiomeric eluate ions. The degree of formation of these diastereoisomeric ion-pairs are generally different, thus the enantiomeric separation through the ion-exchange column is achieved.

Buchberger et al. [37] used EDTA to extract Cu(II), Fe(III), Pb(II), Cd(II), Co(II), Ni(II), and Zn(II) from soil sediments with subsequent determination by ion-pair chromatography using a mobile phase consisting of 1% hexadecyltrimethylammonium bromide in 1.2 mM

phosphate buffer pH 7.2:acetonitrile:methanol (12:5:3). Detection of Cu(II), Fe(III) and Pb(II) was obtained by direct detection at 250 nm and for Cd(II), Co(II), Ni(II), Pb(II) and Zn(II) also at 250 nm after post-column reaction with copper(II) sulphate at low pH. Direct detection limits were 1.5 to 4.0 ng or 30 to 50 ng for post-column reaction detection.

3.4 DEVELOPMENT OF A METHOD OF ANALYSIS FOR THE DETERMINATION OF METAL CHELATES IN FERTILISERS.

3.4.1 Metal Chelate Chemistry and the Development of an Analytical Procedure

Section 1.2.3 gives an introduction to metal chelate chemistry. From this discussion it was concluded that metal chelation is an equilibrium reaction and the position of t equilibrium is influenced by pH, ionic strength, solvent and the presence of other chelating agents and metal ions.

These factors must be considered when developing a chromatographic technique. Thus, for example, the quantity of metal that will be chelated during a chromatographic separation carried out at pH 8 will differ from that which will be chelated during a chromatographic separation carried out at pH 6. Also, inherent reactions within a method such as adsorption of metal ions to the gel material in GPC must be considered [38]. Thus it is my opinion that it may be incorrect, as has previously been the case (section 3.2.2), to collect the chelate fraction eluting from a separation column, analyse for metal content and claim this is the chelate composition of the fertiliser. This quantity will actually represent the chelate composition only under the conditions of analysis. This behaviour may be accounted for by the use of standards and peak height or area measurements. A standard of FeEDTA of 95% purity, for example, may dissociate on equilibration in the analytical system, such that 80% of the metal remains chelated. The detector response achieved, however, may be taken as that corresponding to a sample of 95% purity as manufactured. Thus samples of fertilisers in the solid form may be analysed.

However, where a fertiliser contains a mixture of metal ions and chelating agents, the competing reactions of the metal ions and ligands must be considered. Again the mixture of metal ions and chelating agents will achieve an equilibrium dependent upon the conditions employed in the analytical method. However, the analytical conditions not only have an influence on the extent of association/dissociation of the chelate but also on the extent of replacement reactions of metal chelates. Thus, for example, if a mixture of FeEDTA and ZnEDTA are to be analysed, a determination carried out at pH 6 may differ from that carried out at pH 8 as the conditional constant of ZnEDTA increases from pH 6 to pH 8 ($10^{11.8}$ to $10^{14.1}$), while the stability of FeEDTA decreases ($10^{14.7}$ to $10^{13.7}$) (see Figure 1.1). An investigation of this behaviour would be very involved, especially as there are so many

possible combinations of chelates in fertilisers. Therefore for the purpose of this investigation it has been presumed that the analysis of a mixture of metal ions and chelating agents is impossible using a chromatographic method of fixed pH, as the analysis will reflect the equilibrium achieved by the metals under the conditions of analysis and not the composition as manufactured.

Once the solid fertiliser is analysed the significance of this analytical result must also be questioned, as the composition of the fertiliser given is the composition as manufactured. However, as discussed in section 3.1, and as seen to some extent in figures 3.2 to 3.4, the quantity of chelate available to the plant, i.e. the bioavailabiliity of the chelate, will vary with the soil conditions. Also, if the fertiliser is to be used for hydroponics, then the pH of the hydroponic solution may vary from pH 4.0 to pH 10.0. In other words the analytical result does not reflect the value of a fertiliser in the field. To replicate conditions of use in analysis would be impossible. It is not the job of the analytical scientist to solve these anomalies as this is the area of expertise of a soil scientist. However, it is the duty of an analyst to know the significance of the result obtained, and to point out such anomalies. It would be incorrect to supply a result without pointing out the applicability of the result. The analysis of micronutrient availability will therefore require a holistic approach including all of these factors. A meaningful analysis would perhaps involve the determination of the total quantity of chelating agent and the total quantity of micronutrients. These results could then be used, together with a model of the behaviour of metal chelates in soils, such as those developed by Norvell and Halvorson et al. [3,4], to predict the effectiveness of the fertiliser under various conditions of usage.

3.4.2 The Aim of this Analytical Investigation.

Thus the aim of this analytical investigation was to develop a separation of the chelates Fe-EDTA, Fe-DTPA, Fe-HEEDTA, Fe-EDDHA, Fe-EDDHMA, Cu-EDTA, Zn-EDTA and Mn-EDTA. This method could then be used with the aid of standards to qualitatively and quantitatively determine the quantity of any of these metal chelates in a fertiliser which contains one chelating agent in combination with one trace element.

In the case of the chelating agent EDDHA, which occurs as a mixture of two diastereoisomers, manufacturers wish to separate the isomers as one isomer is a more

effective fertiliser than the other. Therefore a separation of the two diastereoisomers is needed.

A possible future application of this work would be to convert the chelates present in a fertiliser or soil sample to the iron(III) form and use the separation achieved for the determination of the total amount of chelating agent present.

3.5 PROPOSAL FOR THE DEVELOPMENT OF NEW METHODS OF ANALYSIS FOR THE DETERMINATION OF METAL CHELATES IN FERTILISERS

When deciding on a separation method for metal chelates, one can choose from GC, ion-exchange or ion-pair chromatography. To date most analytical methods involving the separation of metal chelates have been concerned with either the determination of the metal or the chelating agent. Gas chromatography has been used for the determination of chelating agents; however, metal ions cause interference. These can be removed by previously acidifying to remove the metal ions before esterification. However, where iron is present, this does not apply, as for example FeEDTA forms a complex at low pH. Thus GC is not suitable for this application.

In HPLC determinations of chelating agents, complexation with a metal ion has been used to aid detection, whereas for the determination of metal cations, complexation with a chelating agent has been used to aid separation. Thus mixtures of transition metals have been separated as their EDTA chelates. Chelating agents have been determined mostly by the formation of their respective iron(III) or copper(II) chelates. Research has shown [19,28] that chelation with iron at low pH suffers from less interferences than complexation with copper(II).

Ion-exchange chromatography has largely been replaced by ion-pair chromatography, as it is recognised as providing a more efficient separation due to the flexibility of the method. Therefore it was decided to investigate the use of ion-pair chromatography for the determination of chelates in fertilisers. However the ion-exchange method developed by Barak [22] for the separation of the isomers of EDDHA may be easily adapted for the determination of these chelates in the present possible mixture of chelates.

Whereas ion-exchange and ion-pair chromatography both provide a method based on the interaction of the chelate anion with a counter ion, gel permeation chromatography provides a totally different principle for separation, i.e. that of separation on the basis of size. The method is still generally associated with biological applications and the determination of polymer size, and no further investigations have been carried out on the merits of this technique as a method for the determination of metal chelates since the work of Boxma [5] and Deguchi [16]. Since then, however, many new gels have come on the market which are more rigid, making the separation faster and which have smaller pore sizes thus making the

method more applicable to smaller molecules such as chelates. Therefore the development of a gel permeation technique was also investigated.

In this investigation detection limits are not critical as chelates are present in fertilisers at the % level and there is unlimited quantity of sample.

3.6 DEVELOPMENT OF A GEL PERMEATION CHROMATOGRAPHIC SEPARATION METHOD FOR THE IDENTIFICATION OF METAL CHELATES IN COMMERCIAL FERTILISERS.

3.6.1 Introduction

If the Boxma separation is studied in detail, it can be seen that it does not exhibited gel permeation (GPC) behaviour, i.e. separation on the basis of size. It can be seen that contrary to GPC, where the larger components of a sample have a shorter elution time compared to the smaller components, in the Boxma separation the smaller chelates FeEDTA and FeDTPA elute before the larger chelates FeEDDHA and FeEDDHMA. Thus FeEDTA and FeDTPA are being excluded from the gel. Hydroponic, ionic and structural interactions all play a part in the behaviour observed by Boxma [5] and also Deguchi [16]. This is thought to be due to the presence of a low number of carboxylic acid groups in the gel matrix. These will repel anions from the gel phase when they are applied to the gel in low quantities and eluted with ion-free water [39]. Spitzy et al. [40] found that this effect can be removed for inorganic anions by the addition of other electrolytes to the eluent. This is possibly why Boxma and Deguchi used calcium chloride and sodium chloride. However, this behaviour or the investigation of it, was not mentioned in either paper [5,16]. Therefore this behaviour was investigated in a gel other than Sephadex to see if this phenomenon can be used to help achieve a separation.

The long retention times of FeEDDHA and FeEDDHMA was considered to be due to the interaction of the aromatic groups with the Sephadex matrix. Similar long retention times for aromatic compounds has been reported by Gellotte [39] and Determann and Walter [41] in the gel permeation chromatography of compounds with aromatic groups. Williams [42] suggests that this is due to the involvement of the π electron system of the solute with the ether oxygen's in the glyceryl bridge of the Sephadex. This effect increases as expected as the pore size of the gel decreases, and therefore the amount of polymer in the gel increases. However, for the smallest pore sizes of Sephadex G10 and Sephadex G15, the retention of aromatics increases dramatically and cannot be explained only by interaction of the π electron system. Determann and Walters [41] have suggested that the added increase in retention was due to structural considerations. However, they were not able to observe this very large increase for the Bio-gels.

Therefore two types of gel were investigated. Bio-gel P2 which is the gel with the smallest pore size of the Bio-gel range (200-2000u), and Fractogel HW40, one of the new ranges from Merck of small pore sized very rigid gels which can operate at a flow rate relatively high for GPC i.e. 1 ml/min.

3.6.2 Experimental

Chemicals and Reagents

Deionised water was obtained by passing distilled water through a Waters Milli-Q water purification system. Sodium hydroxide (40%) solution was obtained from BDH. Potassium dihydrogen phosphate was obtained from Merck. Sodium chloride was analytical grade. FeEDTA and ZnEDTA were obtained from Koch-Light, FeDTPA was obtained from Aldrich, CuEDTA was obtained from Sigma, FeHEEDTA, FeEDDHA and FeEDDHMA were obtained from private sources (Instituut voor Bodemvruchtbaarheid, Oosterweg 92, Postbus 30003, 9750 RA, Haren, (Gr), The Netherlands). All standards and samples for analysis were made up in the mobile phase and filtered with a 0.45 μm Millipore HA filter prior to analysis.

Apparatus

A Waters M45 pump, a Waters solvent select valve, and a Gilson Model 231 injector with a 20 μl injection loop were used. The detector used was a Merck Hitachi L-4000 UV detector at 210 nm. The system was interfaced to a Nelson Analytical 900 series interface. Fractogel TSK HW-40(S) was obtained from Merck and Bio-Gel P2 from Bio-Rad. Data were processed using a Nelson Analytical 3000 Series Chromatography data system. A Corning 10 x 450 mm adjustable column casing was used. Both gels were packed in this column give a column of 1 x 35 cm.

Methods

Pretreatment of sample. The sample weight approximately equivalent to 3 mg of the metal present in the chelate was taken. The weight of the sample in grms was estimated using the calculation $1/(U/(3x10^{-1})$ where U was an estimate of the percentage of chelated metal in the sample. The sample was dissolved in approximately 20 ml deionised water and placed in an ultrasonic bath for 30 minutes. The solution was made up volumetrically to 25 ml and filtered through a 0.45 μm filter.

Analysis of sample. 20 µl of the pretreated sample was injected onto a Fractogel column (1x35 cm) in an aqueous mobile phase that was 0.05M in NaCl and 0.005M in KH_2PO_4, pH 8.0 at a flow rate of 0.5 ml/min. The chromatogram was recorded by measuring the absorbance at 210 nm. With the aid of the chromatogram the chelate fraction was collected, 1 ml of 5M HCl added, and the sample made up to 10 ml with the mobile phase. Analysis of the fraction by AAS/ICP was used to identify the metal present. This information, in combination with the retention time of the fraction may be used to identify the chelate present. Quantification can be achieved by comparison of peak height/area with that of standards.

3.6.3 Separation on Bio-Gel P2

Figure 3.5 shows the influence of the concentration of NaCl on the retention of the chelates on Bio-gel P2 with an aqueous mobile phase at pH 7.0 (adjusted with NaOH) at a flow rate of 0.25 ml/min. The plot of Ve/Vt (elution volume/total column volume) gave a measure of retention independent of column dimensions or flow rate. It can be seen that as NaCl concentration was increased, the retention of the chelates increased steadily. This indicates that ionic interactions between the chelates and negatively charged groups in the gel was being overcome and therefore the anions were not being repelled from the column to the same extent. As with the Boxma method [5], the iron chelates of EDDHA and EDDHMA eluted with longer retention times than the chelates of EDTA, DTPA and HEEDTA. However, the retention times of FeEDDHA and FeEDDHMA were significantly less than those obtained with the Boxma method employing Sephadex G10. The increase in retention time of both EDDHA and EDDHMA chelates with increased sodium chloride concentration is much more dramatic than that for the EDTA, DTPA and HEEDTA chelates. This is possibly due to the fact that as ionic interactions are overcome, hydrophobic interactions increase and EDDHA and EDDHMA will have a much greater hydrophobic interaction due to their respective aromatic groups.

There was very little change in resolution once the NaCl concentration was increased above 0.05M, and therefore it can be concluded that this is the optimum NaCl concentration for separation, as there is no further decrease in ionic repulsion with increased NaCl concentration. FeEDTA and FeDTPA are well resolved using this system. FeDTPA and FeHEEDTA have a resolution factor (R_S) of 1.38. FeHEEDTA and FeEDTA are poorly resolved, having a R_S value of 0.52. FeEDDHA and FeEDDHMA have a R_S value of 0.8.

3.6.4 Separation on Fractogel HW-40(S)

The chelates behaved similarly on Fractogel, the retention time increasing as the sodium chloride concentration was increased (Figure 3.6). The separation increases up to a salt concentration of 0.05M. A further increase in salt concentration does not bring any improvement in resolution but only causes an increase in retention time; therefore the optimum salt concentration for separation was found to be 0.05M. Under these conditions, FeDTPA and FeEDTA are resolved having a R_S value of 2.0. FeEDDHA and FeEDDHMA are also resolved having a R_S value of 2.1. However, as with Bio-gel, FeEDTA and FeHEEDTA are not resolved, having a R_S value of 0.3. Comparison of the retention of the chelates on Fractogel and Bio-Gel is shown in Table 3.3. The chelates FeEDDHA and FeEDDHMA have a long retention time on Fractogel However this interaction is helping to achieve greater resolution than on the Bio-Gel. Due to the better resolution of FeEDDHA and FeEDDHMA with Fractogel HW-40(S), and the greater rigidity of the gel which enabled higher flow rates to be employed than with the Bio-Gel, thus decreasing analysis time, the Fractogel was chosen instead of the Bio-Gel for further study.

CHELATE	BIO-GEL Ve/Vt	FRACTOGEL Ve/Vt
Fe(III)EDTA	0.67	0.46
FeDTPA	0.52	0.40
FeHEEDTA	0.62	0.48
FeEDDHA	1.1	1.77
FeEDDHMA	0.98	2.93

Table 3.3 Comparison of Ve/Vt retention values of iron(III) chelates on Bio-Gel P2 and Fractogel HW-40S columns..
Bio-Gel P2 conditions: aqueous eluent 0.05M in NaCl, pH 7.0. Column: 1 x 35 cm. Flow rate 0.25 ml/min.
Fractogel TSK HW-40 (S) conditions: eluent 0.05M in NaCl, pH 7.0. Column 1 x 35 cm. Flow rate 0.5 ml/min.

As FeEDDHA and FeEDDHMA were well resolved, the improvement of the separation of FeEDTA, FeDTPA and FeHEEDTA was then investigated. As buffer was added to the mobile phase it was found that the order of elution of the chelates changed. It was found that as the buffer concentration was decreased, the separation improved for FeDTPA and FeHEEDTA (Figure 3.7). The best separation was achieved at a buffer concentration of 0.02M; however FeHEEDTA and FeEDTA are still poorly resolved under these conditions.

Using this buffer concentration the pH was varied from pH 6.5 to pH 8.0 to try to improve the separation (Figure 3.8). It can be seen from this figure that the chelates changed elution order over this pH range. The best separation was found at pH 6.5. However, it was found that if pH 8.0 was used together with a low buffer concentration (figure 3.7), thus encouraging the elution order FeDTPA, FeEDTA, FeHEEDTA, a better separation was achieved (Figure 3.9). Figure 3.10 shows the separation of FeEDDHA and FeEDDHMA. CuEDTA, ZnEDTA and MnEDTA, were found to coelute with FeDTPA under these conditions (Fig. 3.11).

The relative standard deviation of the retention time based on ten injections of the same standard was less than 1% for the iron(III) chelates of EDTA, DTPA and HEEDTA. The analysis of fertiliser samples showed that matrix interferences were not a problem for the separation as the standard deviation did not increase. However, to avoid matrix interferences, a minimum run time of fifty minutes is recommended. Figures 3.12 to 3.14 show examples of three samples analysed. Quantitation using this method should be carried out by a comparison of peak height/area to standards as previously discussed, and not by collection and analysis of the metal content of chelate fractions.

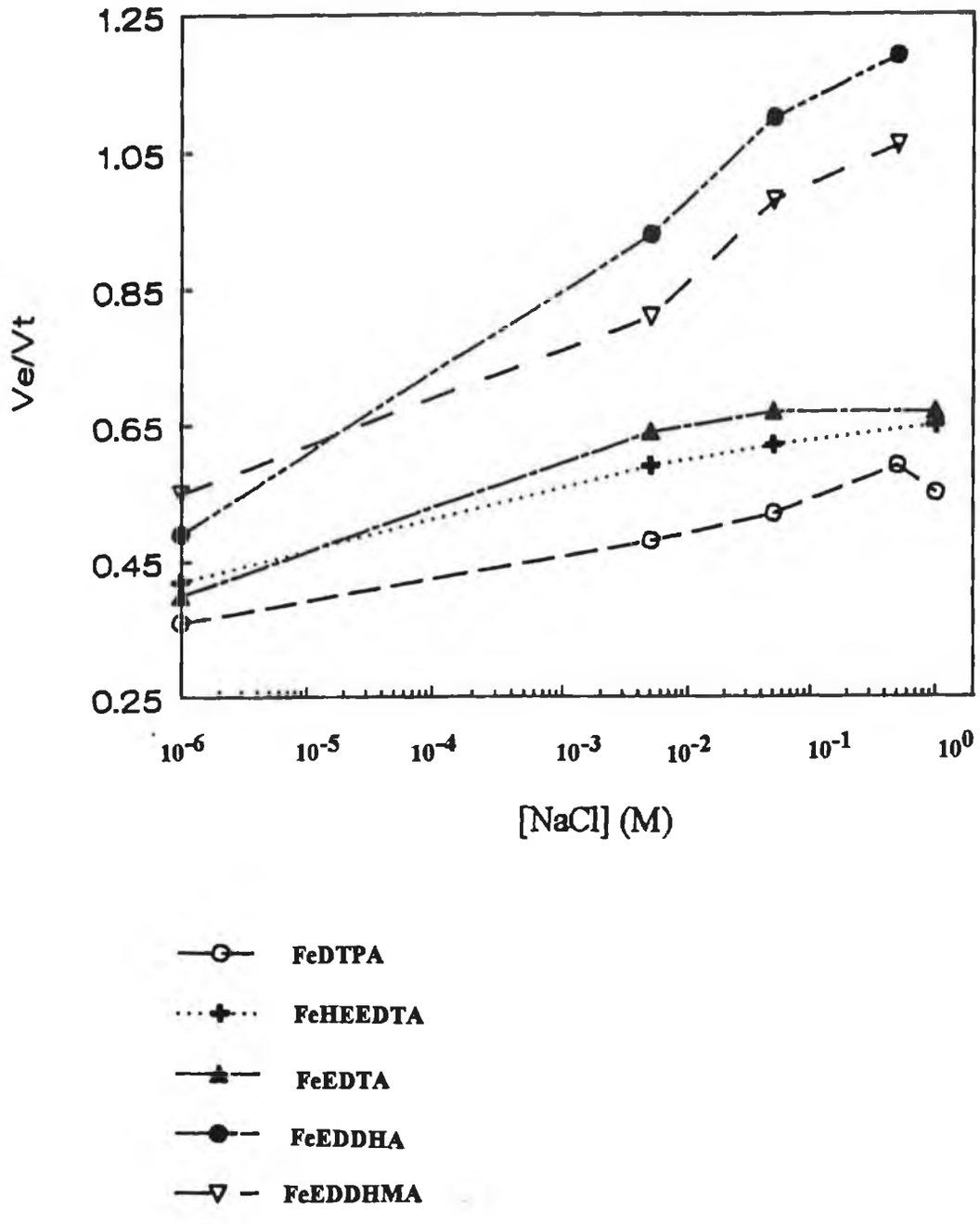

Figure 3.5 The influence of the concentration of sodium chloride on the retention of iron(III) chelates on Bio-gel P2. Column: 1 x 35 cm.. Mobile Phase: sodium chloride in aqueous solution pH 7.0. Flow rate 0.25 ml/min. Detection 210 nm.

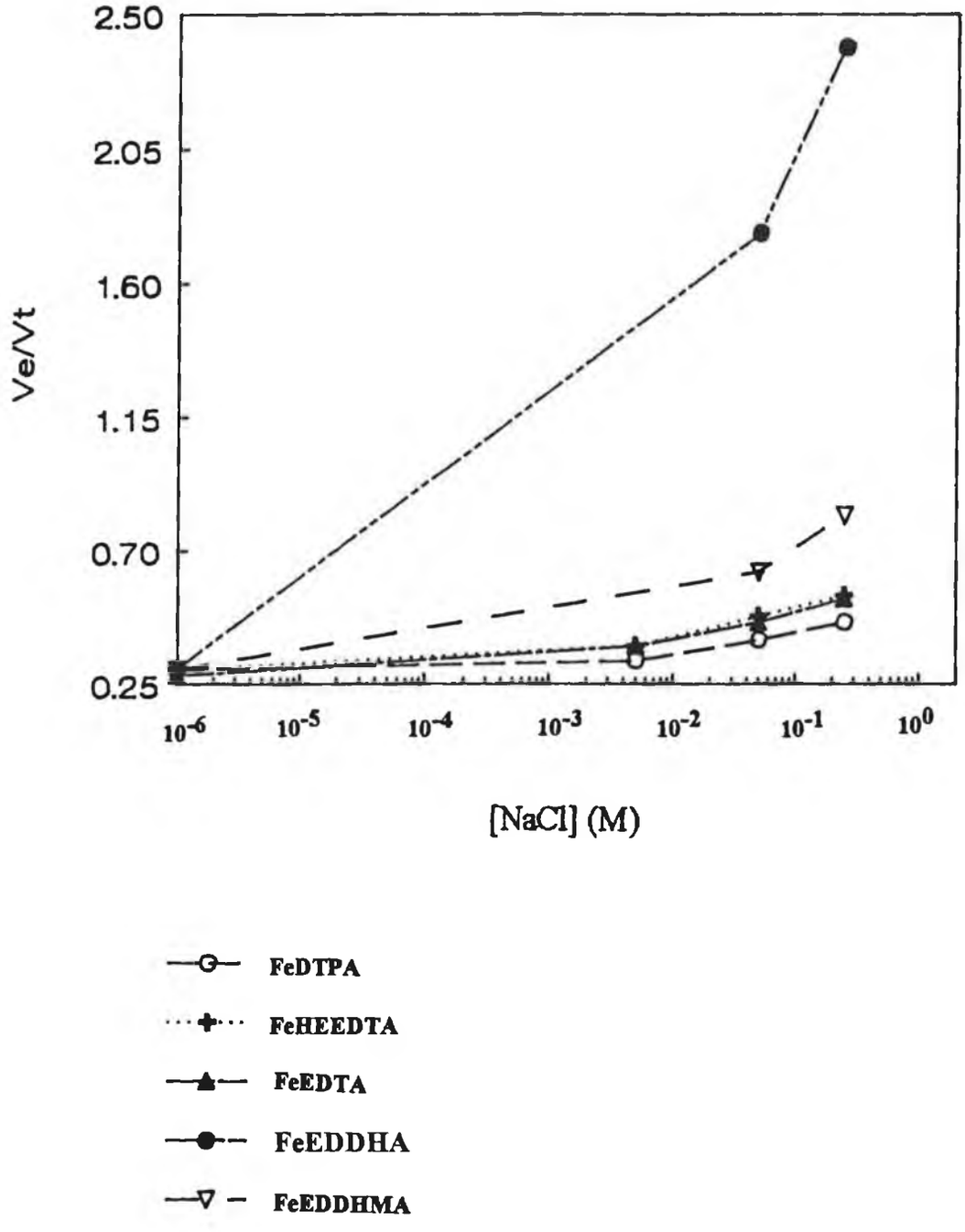

Figure 3.6. The influence of sodium chloride concentration on the retention of chelates on Fractogel TSK HW-40 (S). Column: 1 x 35 cm. Flow rate 0.5 ml/min. Mobile Phase: sodium chloride solution at pH 7.0. Detection 210 nm

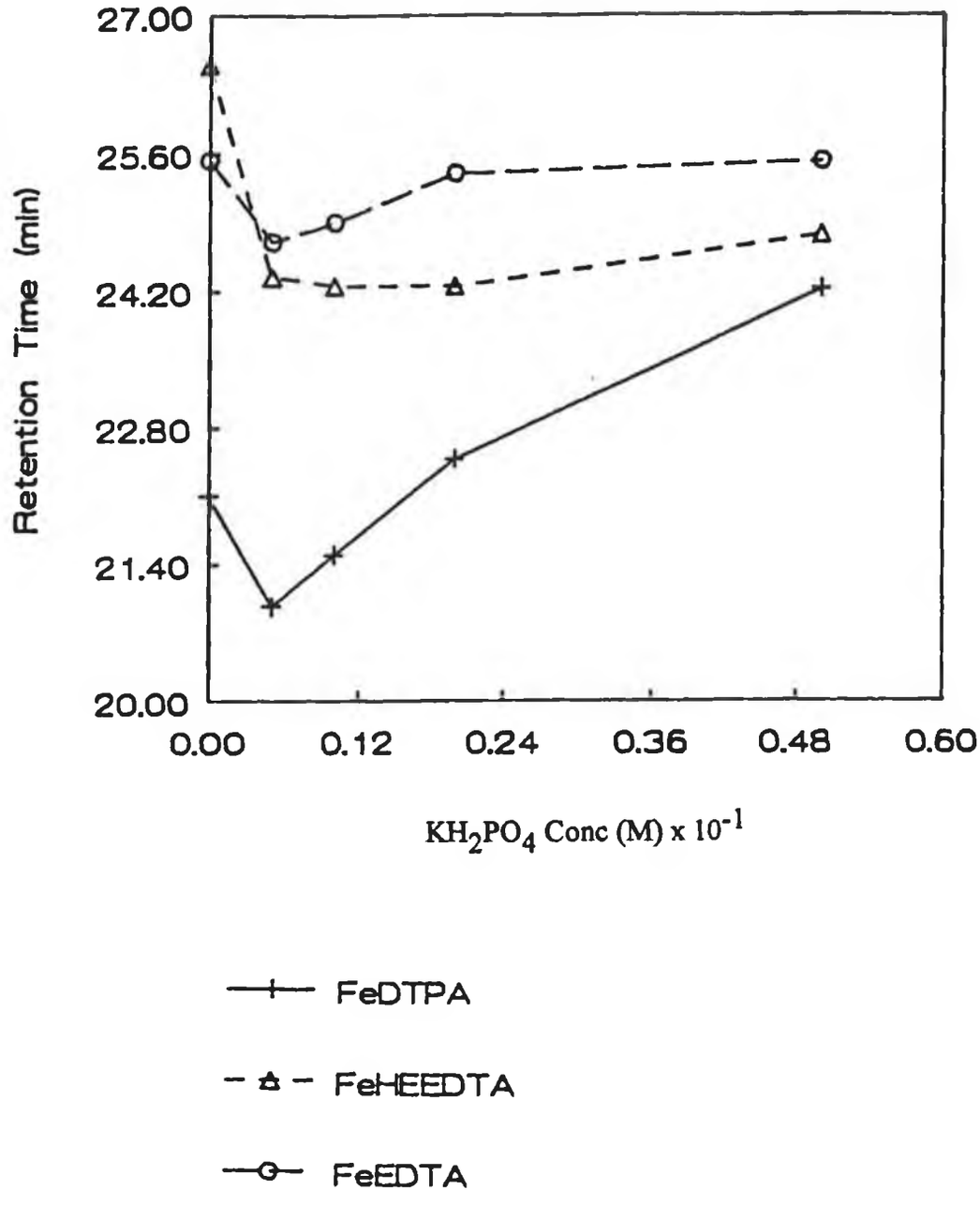

Figure 3.7. The influence of the phosphate buffer concentration on the retention of iron(III) chelates on Fractogel TSK HW-40 (S). Column: 1 x 35 cm. Mobile phase 0.05 M NaCl, pH 7.0, containing variable KH_2PO_4 concentrations. Flow rate 0.5 ml/min. Detection 210nm.

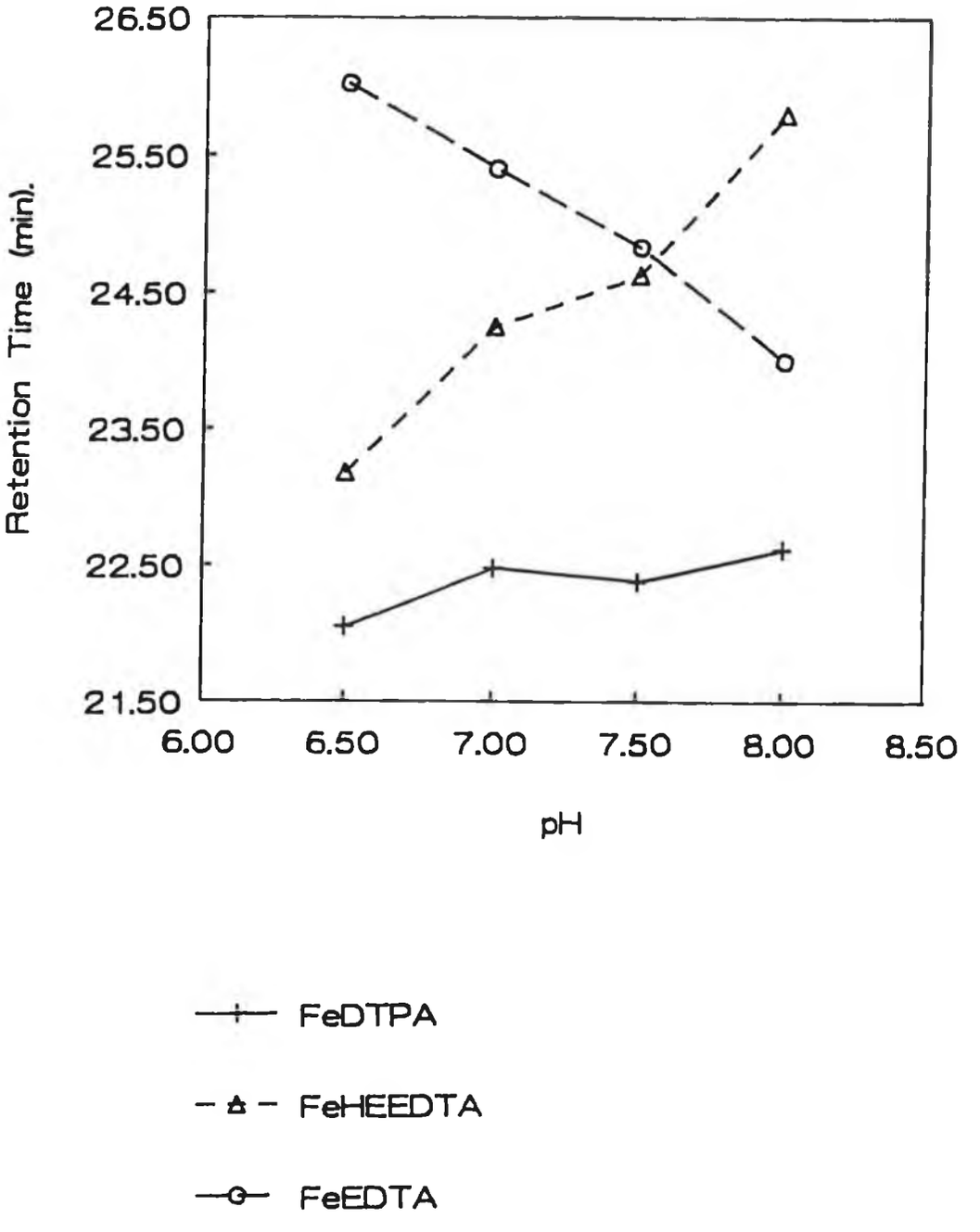

Figure 3.8. The influence of pH on the retention of the iron(III) chelates on Fractogel TSK HW-40(S). Column: 1 x 35 cm. Flow rate 0.5 ml/min. Mobile phase: 0.05M NaCl, 0.02M KH_2PO_4, pH variable. Detection 210nm.

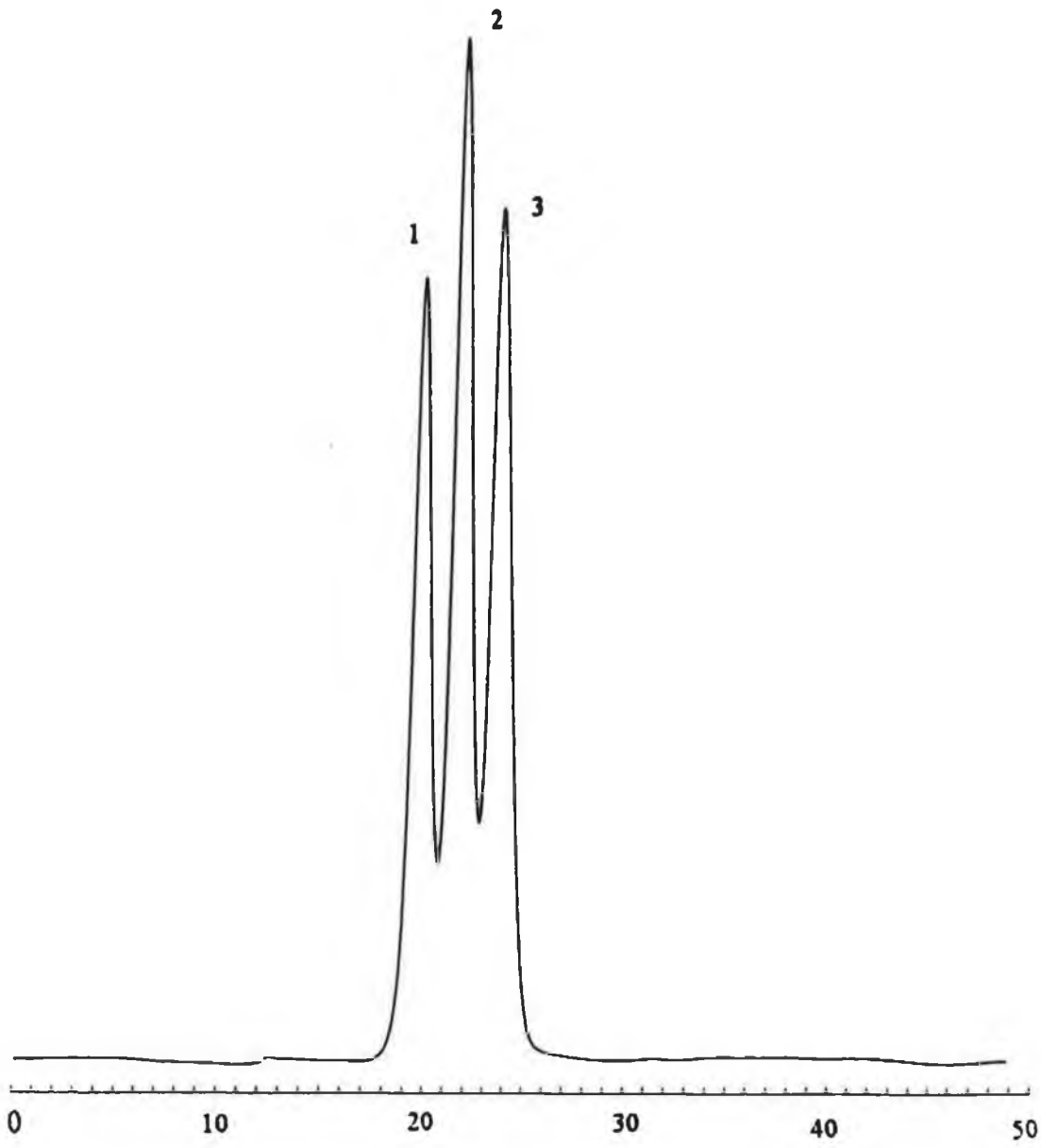

Figure 3.9. Separation of 1) 19 µg FeDTPA, 2) 26 µg FeEDTA, and 3) 28 µg FeHEEDTA. Column 1 x 35 cm Fractogel TSK HW-40(S). Mobile Phase: 0.05M NaCl, 0.005M KH_2PO_4, pH 8.0. Injection volume 20 µl. Flow rate: 0.5 ml/min. Detection at 210 nm.

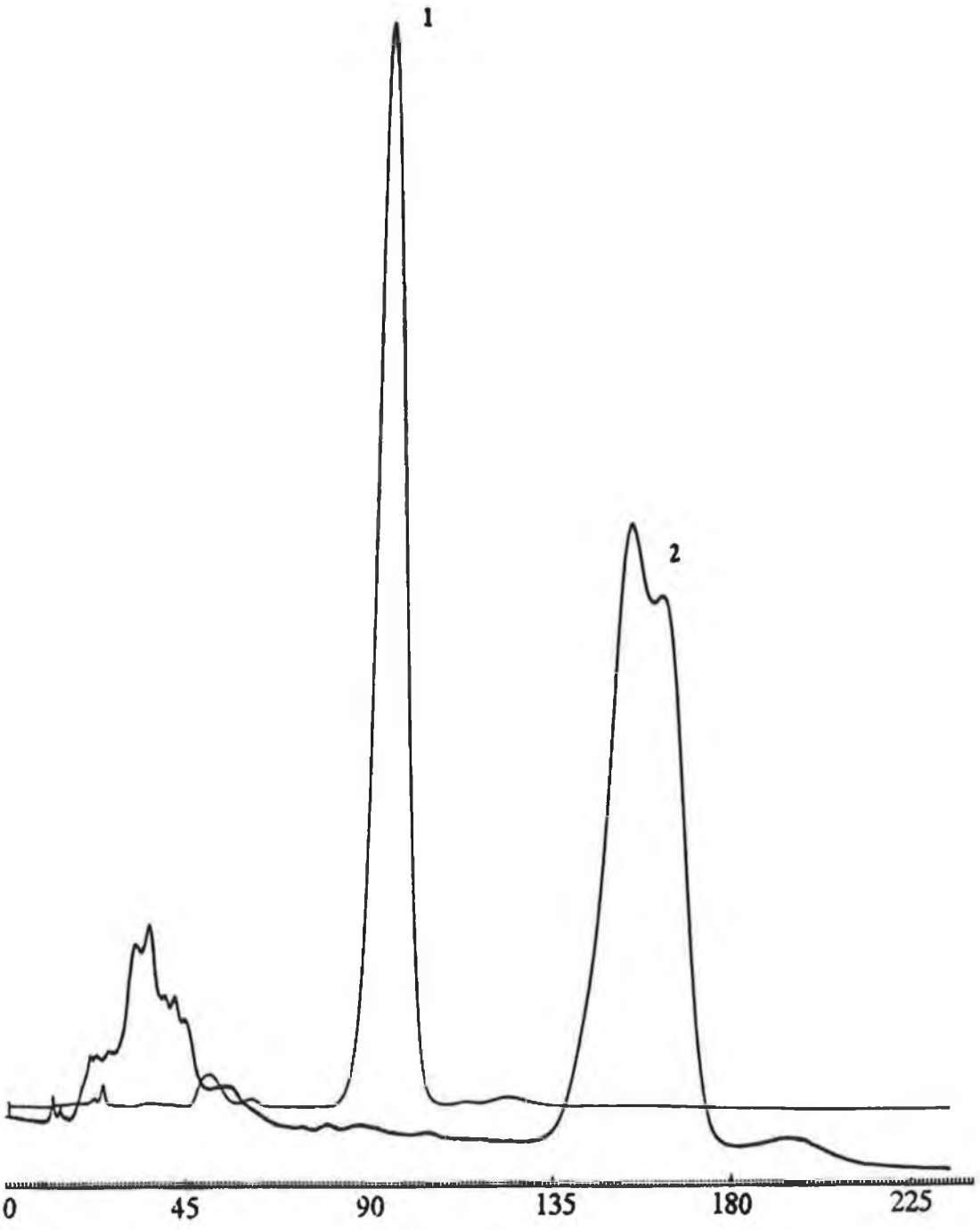

Figure 3.10. Separation of 1) 390 µg FeEDDHA, 2) 44 µg FeEDDHMA. Column 1 x 35 cm Fractogel TSK HW-40(S). Mobile Phase: 0.05M NaCl, 0.005M KH_2PO_4, pH 8.0. Injection volume 20 µl. Flow rate: 0.5 ml/min. Detection at 210 nm.

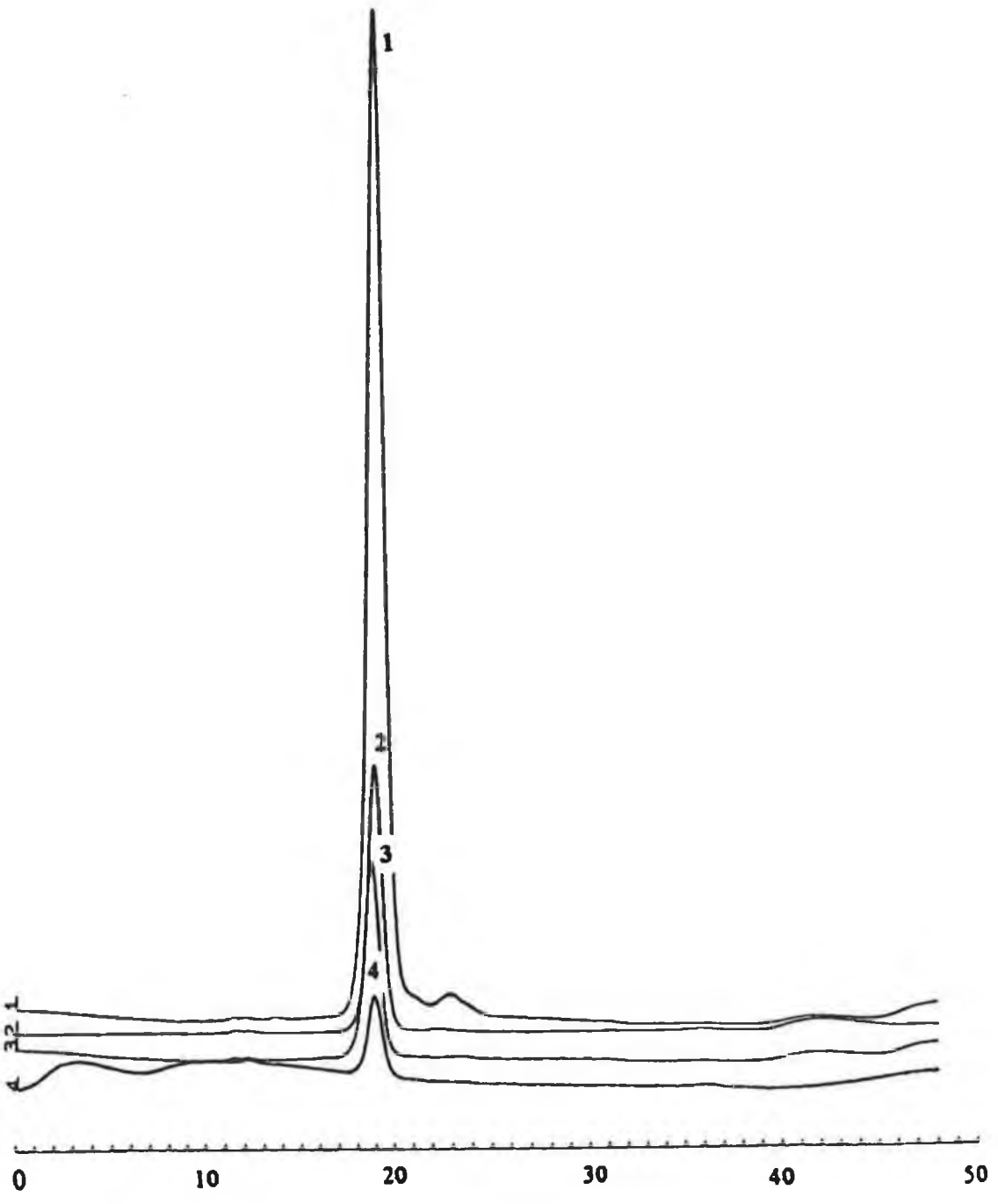

Figure 3.11 Separation of 1) 19 µg FeDTPA, 2) 13 µg CuEDTA, 3) 15 µg MnEDTA, 4) 13 µg ZnEDTA. Column: 1 x 35 cm Fractogel HW40S. Mobile phase: 0.05 M NaCl, 0.005 M KH_2PO_4, pH 8. Injection volume: 20 µl. Flow rate 0.5 ml/min. Detection 210 nm.

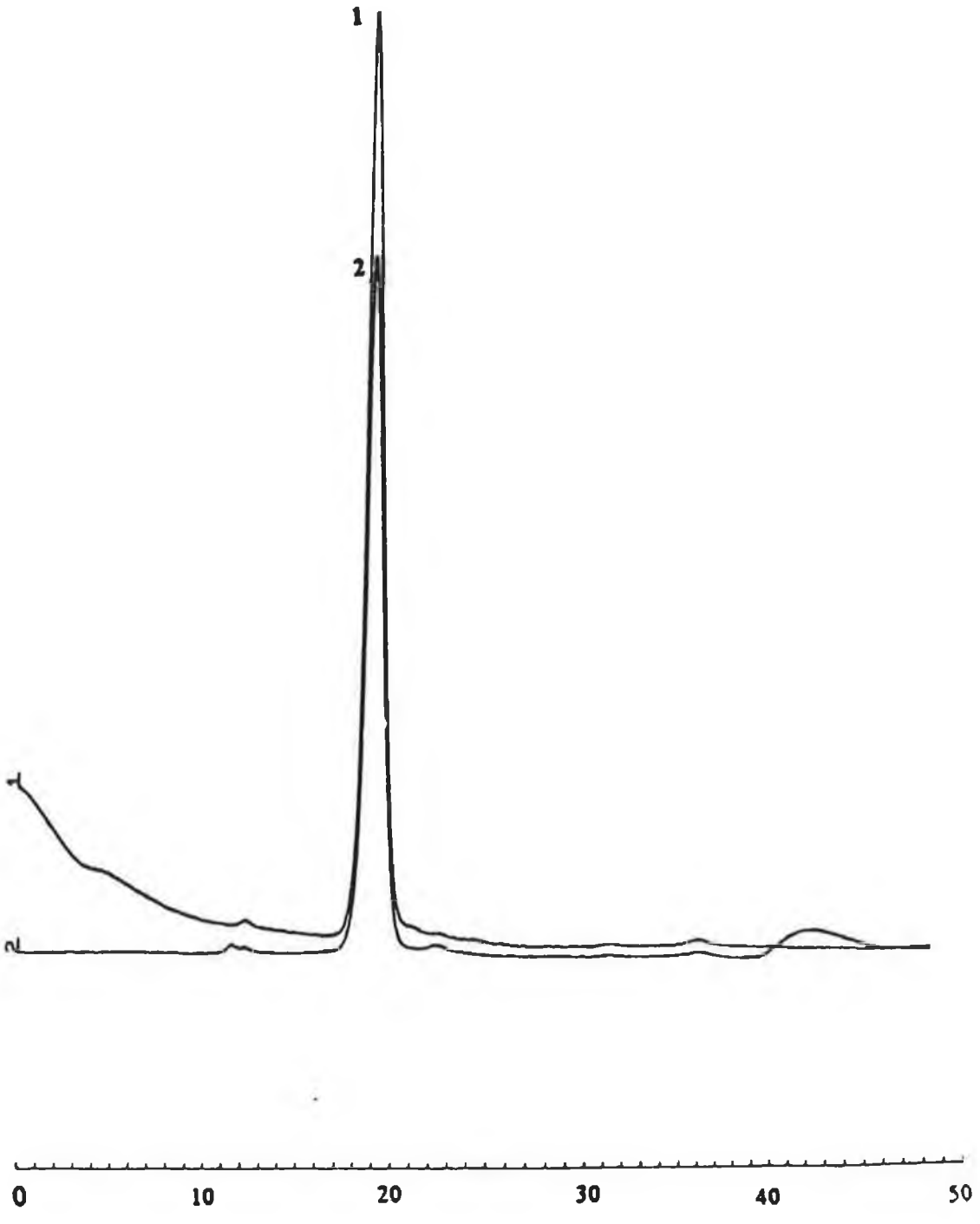

Figure 3.12. Overlay of 1) fertiliser sample containing CuEDTA with 2) CuEDTA standard. Column: Fractogel HW40S 1 x 35 cm. Eluent: 0.05 M NaCl, 0.005 M, pH 8. Flow rate 0.5 ml/min. Injection volume 20 μL. Detection 210 nm.

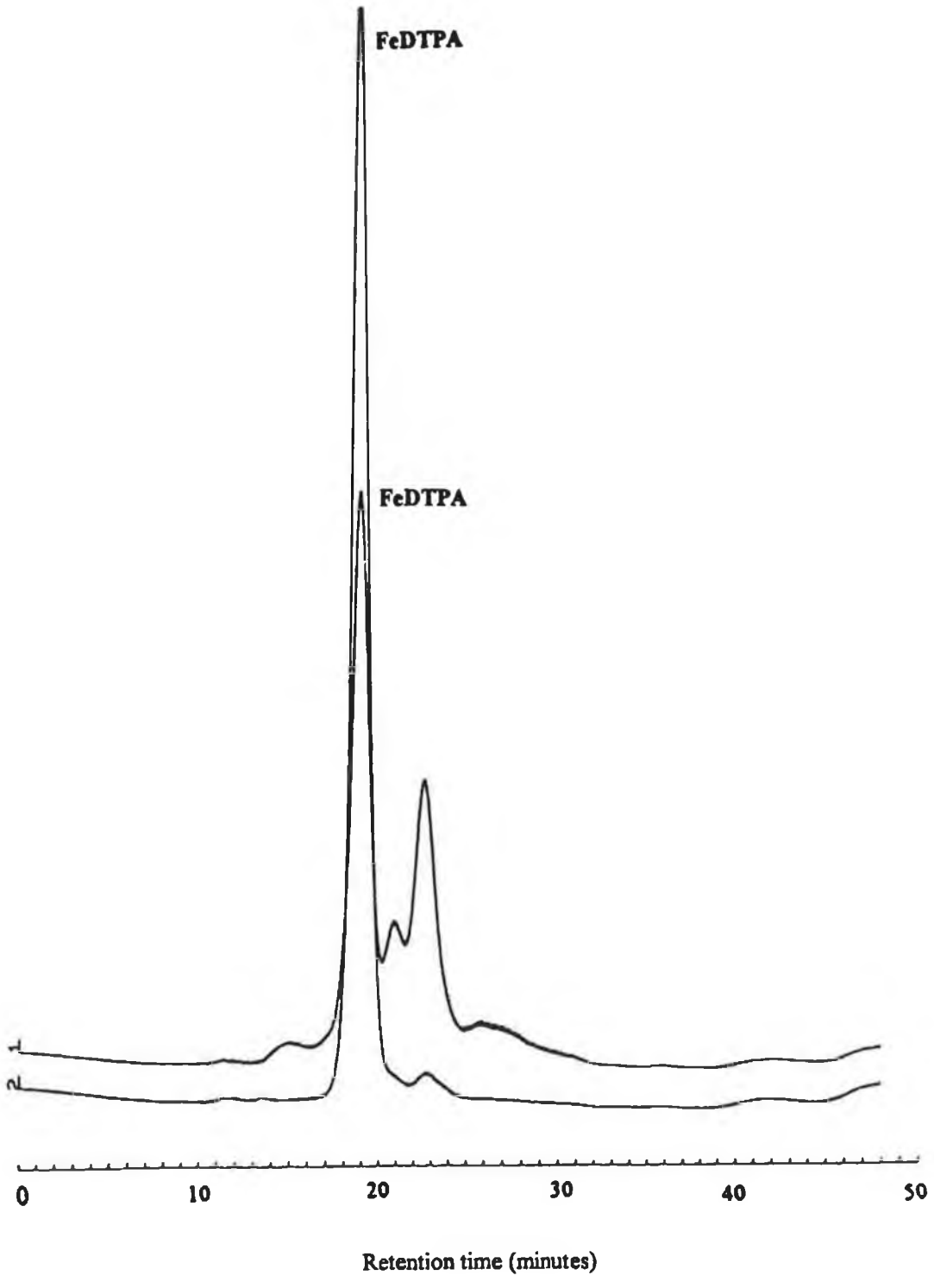

Figure 3.13. Overlay of 1) sample containing FeDTPA with 2) FeDTPA standard. Column: Fractogel HW40S 1 x 35 cm. Eluent: 0.05 M NaCl, 0.005 M KH_2PO_4, pH 8. Injection volume: 20 µl. Detection: 210 nm.

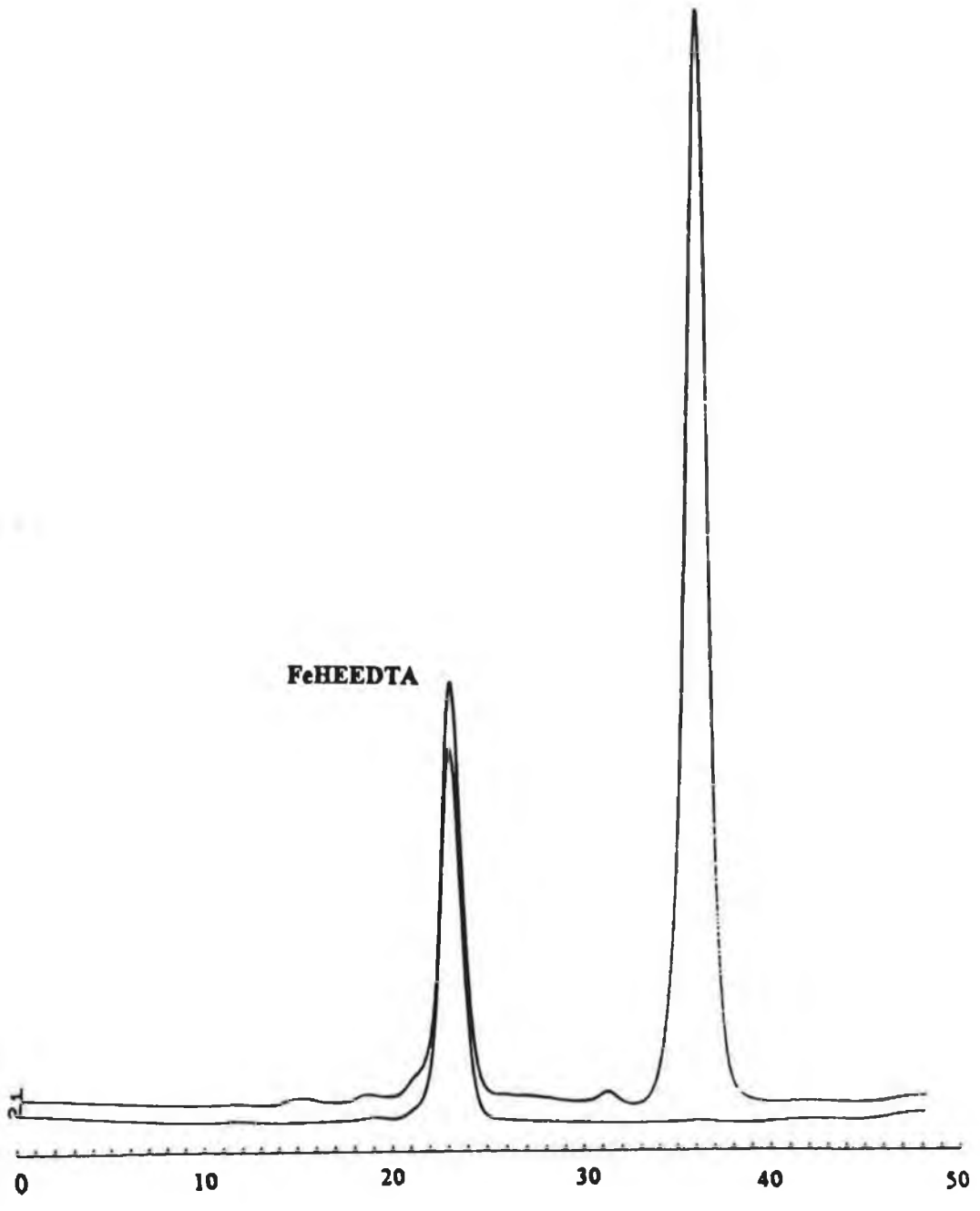

Figure 3.14. Overlay of 1) sample containing FeHEEDTA with 2) FeHEEDTA standard. Column: Fractogel HW40S 1 x 35 cm. Eluent: 0.05 M NaCl, 0.005 M KH_2PO_4, pH 8. Injection volume: 20 µl Flowrate: 0.5ml/min. Detection 210nm.

3.6.5 Conclusion

The method developed shows a large improvement on the Boxma method. All chelates in a mixture containing FeDTPA, FeHEEDTA, FeEDDHA and FeEDDHMA can be separated. As outlined, the method is intended for the analysis of fertilisers containing a single micronutrient in combination with a single chelating agent. Therefore the retention time of the chelate, in combination with identification of the micronutrient present by AAS/ICP, may be used to identify any of the chelates FeEDTA, FeDTPA, FeHEEDTA, FeEDDHA, FeEDDHMA, CuEDTA, ZnEDTA and MnEDTA. Quantitation can be achieved by peak height/area analysis. The analysis time of three hours for a sample containing FeEDDHMA is however a disadvantage of the method.

An investigation of the elution behaviour of the free metal ions Fe(III), Cu(II), Zn(II) and Mn(II) should be carried out on the optimised system to ensure that all free metal ion has eluted before analysis of another sample.

The detection of Mn(II)EDTA and Zn(II)EDTA could be improved by postcolumn reaction with copper [37].

3.7 DEVELOPMENT OF AN ION PAIR CHROMATOGRAPHIC SEPARATION FOR THE SIMULTANEOUS DETERMINATION OF METAL CHELATES IN FERTILISERS.

3.7.1 Introduction

Ion-pair chromatography has been used to date for the separation of aminocarboxylic acid chelating agents in the form of the copper or iron chelates. No separation of the mixture of chelating agents in this investigation has been found in literature.

Buchberger [37] used ion-pair chromatography for the determination of a mixture of transition metal ions by conversion to their EDTA chelates. In studies on the determination of the chelating agent, the tetrabutylammonium ion (TBA^+) has been the most popular ion-pairing reagent used. Parkes et al. [34] achieved a separation of EDTA, HEEDTA and NTA in the copper form using TBA^+. EDTA, DTPA, HEEDTA, DHEG and NTA have also been separated as their copper chelates using benzyltrimethylammonium chloride (BTAC) [33]. The determination of the total chelating agent by conversion to the copper form cannot be used in this particular application as, unlike the applications mentioned [34,35], a fertiliser sample may already contain a mixture of metals. On examination of the conditional formation constants available for the chelates of iron and copper, it is evident that a method converting the chelates to the copper form will suffer from interference from iron. Therefore a separation of the chelating agents in the iron form would be required.

When developing a method where the Fe(III)- chelates of EDTA, DTPA, HEEDTA, EDDHA and EDDHMA and the Cu(II)-, Zn(II)- and Mn(II)- chelates of EDTA must be separated, the acidic pH's used in former investigations are not suitable. A pH above pH 6 is more suitable for detection as the conditional stability constants are higher and thus detection will be more sensitive. In previous investigations acidic pH's were used when complexing with iron to avoid interferences. Similarly, a pH below 9 should be used to ensure detection of some of the iron chelates which will hydrolyse above this pH, and also to prevent decomposition of the column. [18].

In this investigation the ion-pairing reagents TBA^+, BTAC and tetradecyltrimethylammonium bromide (TDTMABr) were therefore investigated for the separation of the Fe(III)- chelates of EDTA, DTPA, HEEDTA, EDDHA and EDDHMA and the Cu(II)-, Zn(II)- and Mn(II)- chelates of EDTA. A possible future application of this

work would be to convert the chelates present in a fertiliser or soil sample to the iron(III) form and use the separation achieved for the determination of the total amount of chelating agent present.

3.7.2 Experimental

Reagents

Deionised water was obtained by passing distilled water through a Waters Milli-Q water purification system. Sodium hydroxide (40%) solution was obtained from BDH. Potassium dihydrogen phosphate (analar) was obtained from Merck. Sodium chloride was analytical grade. Benzyltrimethylammonium chloride, potassium dihydrogen phosphate and acetonitrile (pro analyst) were obtained from Merck. Tetrabutylammonium hydroxide (40% solution in water; 1.5M) was obtained from Fluka. FeEDTA was obtained from Koch-Light, and FeDTPA was obtained from Aldrich. CuEDTA was obtained from Sigma. ZnEDTA was obtained from Koch-light. FeHEEDTA, FeEDDHA and FeEDDHMA standards could not be found commercially or within the network of experts working on this analysis. However, they are being manufactured industrially, but not to high purification levels. Samples of these chelating agents were obtained from Instituut voor Bodemvruchtbaarheid, Oosterweg 92, Postbus 30003, 9750 RA, Haren, (Gr), The Netherlands. All standards and samples for analysis were made up in the mobile phase and filtered through a 0.45 μm Millipore HA filter prior to analysis.

Preparation of Mobile Phases for Ion-Pair Chromatography

Mobile phase A: 32.5 ml of 1.5 M solution of tetrabutylammonium hydroxide was added to 200 ml of water. The pH was adjusted to 7.5 with phosphoric acid and made up to just below the mark in a 1 L in a volumetric flask. A final pH adjustment to pH 7.5 was made and the solution made up to the mark. This was filtered with a 0.45 μm filter.
Mobile phase B: 32.5 ml of 1.5 M tetrabutylammonium hydroxide solution was added to 200 ml of water. This was made up to approximately 650 ml, the pH adjusted to 7.5 with phosphoric acid and the solution filtered with a 0.45 μm filter. 300 ml of acetonitrile was added and made up with filtered water to 1 L in a volumetric flask. All mobile phases were degassed in an ultrasonic bath.

Ion-Pair Chromatography System

A Waters M45 pump, a Waters solvent select valve, and a Gilson model 231 injector with a 20 μl injection loop were used, in conjunction with a Merck Hitachi L-4000 UV detector. A Chromspher C18 column was used. The system was interfaced to a Nelson Analytical 900

Series interface. Data were processed using a Nelson Analytical 3000 Series Chromatography data system.

3.7.3 Investigation of the Ion-Pairing Reagent Benzyltrimethylammonium Chloride

A mobile phase of 0.05 M BTAC, 0.05M KH_2PO_4, pH 7.5 gave good resolution of the Fe(III) chelates of EDTA, DTPA and HEEDTA. A pH of 7 was also investigated however resolution between FeDTPA and FeHEEDTA increased at pH 7.5. A higher pH was not investigated as hydrolysis of Fe(III)-EDTA occurs in solutions where the pH exceeds pH 7.5 [43].

The influence of phosphate buffer concentration was then investigated (Figure 3.15). It was found that as the concentration of the buffer was increased from 0.02M to 0.3M, the retention time of the chelates decreased. Bidlingmeyer and Warren [44] also found this behaviour for the retention of alkylsulphonates with the ion-interaction reagent cetylpyridinium chloride above 10^{-3}M KH_2PO_4. With addition of phosphate there is an increase in the amount of reagent adsorbed as the charge repulsion between the reagent ions on the stationary phase is reduced. However, the increase in ionic strength of the eluent also results in a decrease in electrostatic attraction between the analyte and the ion-interaction reagent such that retention of the sample by adsorptive forces increases. The overall result in this investigation, as was that of Bidlingmeyer and Warren [44] is a decrease in retention of the analyte. In the present investigation this decrease in electrostatic attraction and increase in adsorptive forces was accompanied by a decrease in peak width and increased resolution up to a buffer concentration of 0.1M. Beyond this concentration the decrease in peak width was not enough to compensate for the decrease in retention time, so that the resolution started to again decrease. Therefore at this concentration the combination of adsorptive and electrostatic forces is optimum.

To improve resolution further, especially between FeHEEDTA and FeEDTA, the concentration of BTAC was increased, as shown in Figure 3.16. It was expected that as the concentration of BTAC was increased, the sample would have more interaction with the ion-pairing reagent, and therefore have a longer retention time. It was found that there was very little change in the retention time, however peak width decreased and thus resolution increased.. This would suggest that the increase in BTAC concentration causes faster mass transfer by increasing competition for the reversed phase.

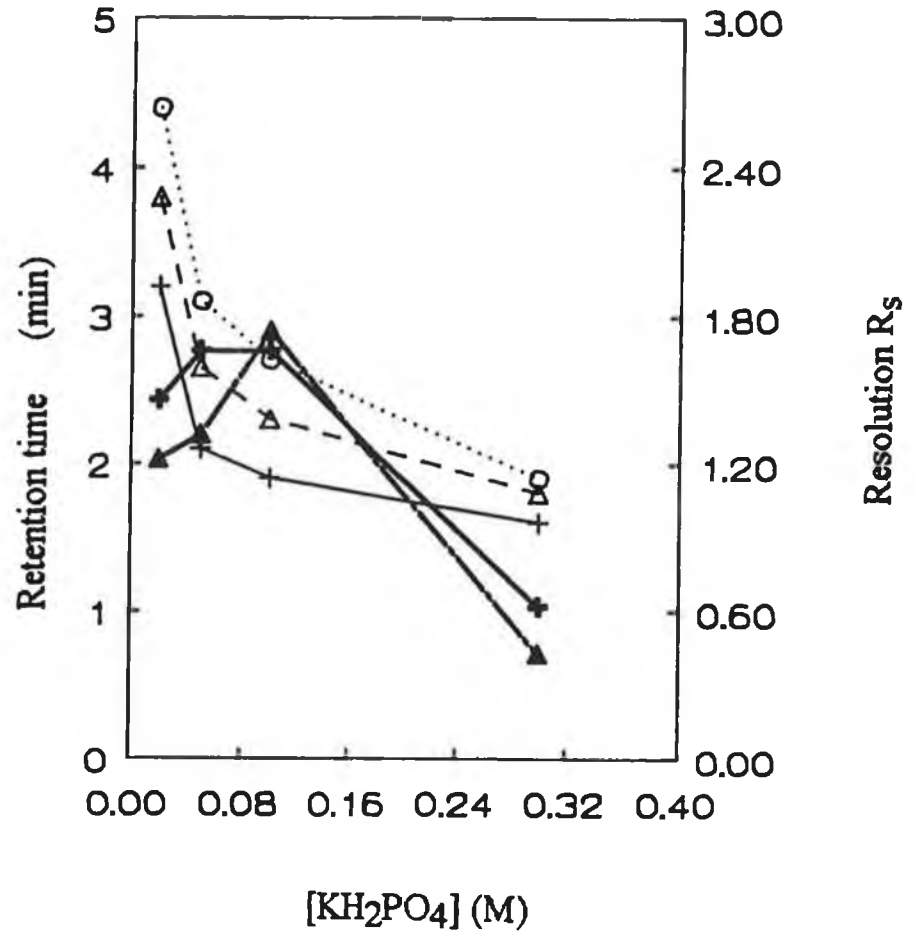

Figure 3.15. The influence of buffer concentration on the retention time (t_R) and resolution (R_S) of iron(III)- chelates. Column: Chromspher C18. Eluent: 0.05M BTAC, pH 7.5, KH_2PO_4 variable. Flow rate: 1 ml/min. Injection loop: 20 µl. Detection 300 nm.

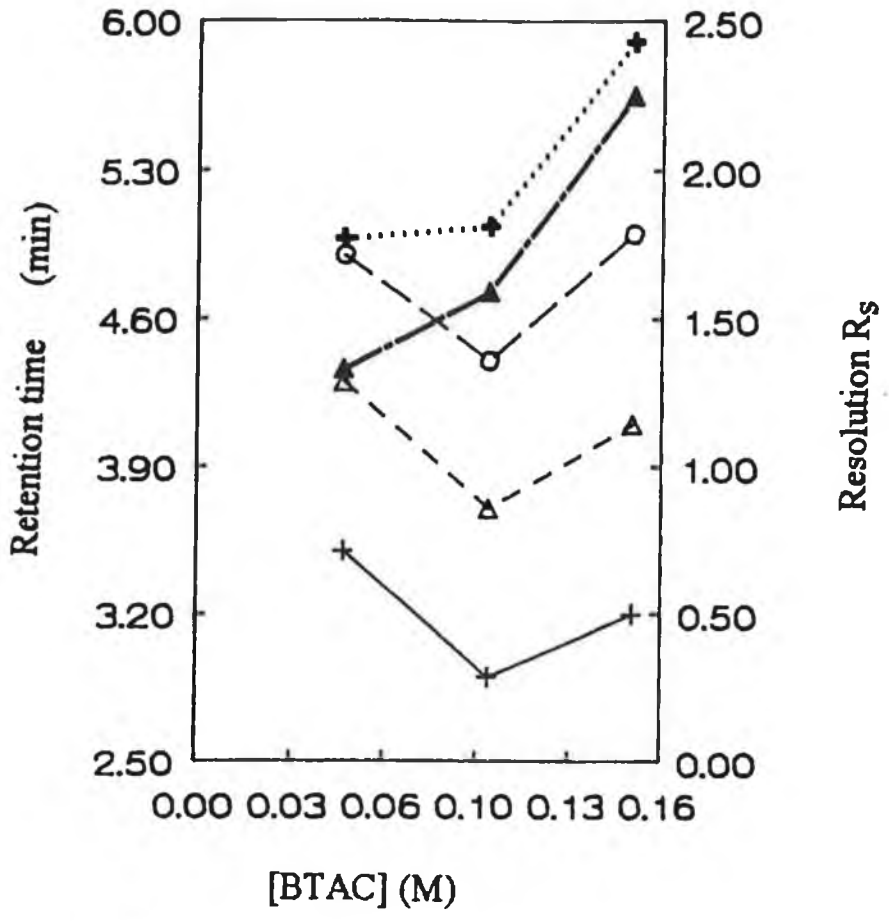

Figure 3.16. The influence of the concentration of BTAC on the retention time (t_R) and resolution of chelates. Column: Chromspher C18. Eluent: 0.1 M KH_2PO_4, pH 7.5, BTAC variable. Flow rate: 0.5 ml/min. Injection loop 20 µl. Detection 300 nm

3.7.4 Development of a solvent switching system

The retention times of Fe(III)EDDHA and Fe(III)EDDHMA were extremely long using an eluent of 0.15M BTAC, 0.1M KH_2PO_4, pH 7.5, (2.5 hours for EDDHA). Addition of an organic modifier to decrease the retention time of Fe(III)-EDDHA and Fe(III)-EDDHMA was achieved using a solvent switching system. A switching valve was placed between the solvent reservoir and the pump, which was activated by a pulsed signal (given in this case by the Gilson auto-injector). Using this system the column was firstly equilibrated with mobile phase A (0.15M BTAC, 0.1M KH_2PO_4, pH 7.5) and the Fe(III)-EDTA, Fe(III)-DTPA and Fe(III)-HEEDTA chelates were allowed to elute. The solvent was then switched to mobile phase B (0.15M BTAC, 0.1M KH_2PO_4, pH 7.5 with variable acetonitrile concentration). Investigation of the addition of 10%,20%, 25% and 30% acetonitrile showed 25% acetonitrile to be the optimum. Elution with 25% acetonitrile for 15 minutes was necessary to elute FeEDDHA and FeEDDHMA. A further 25 minutes was needed to re-equilibrate the system with mobile phase A. The switching sequence was thus as follows: - 5 minutes mobile phase A, 15 minutes mobile phase B followed by 25 minutes mobile phase A. Obviously when transferring this method to another chromatographic system the exact timing of the switching sequence will depend on the dead volume of the tubing connecting the switching valve and the pump. Figure 3.17 shows the separation of the iron chelates achieved. Two peaks were obtained for both FeEDDHA and FeEDDHMA, it is thought that these represent the separation of the respective diastereoisomers. This is important as for FeEDDHA it has been shown that only one of the isomers is effective as a fertiliser. The optimum wavelength for determination of the iron chelates was found to be 283 nm. Figure 3.18 shows how CuEDTA is well separated from FeEDTA, FeDTPA and FeHEEDTA chelates. However, it was found that the Zn(II)-EDTA and Mn(II)-EDTA chelates only exhibit an absorption below 220 nm. However, as BTAC also has a high background absorbance at 220 nm, determination of Zn(II)-EDTA and Mn(II)-EDTA was not possible using this system.

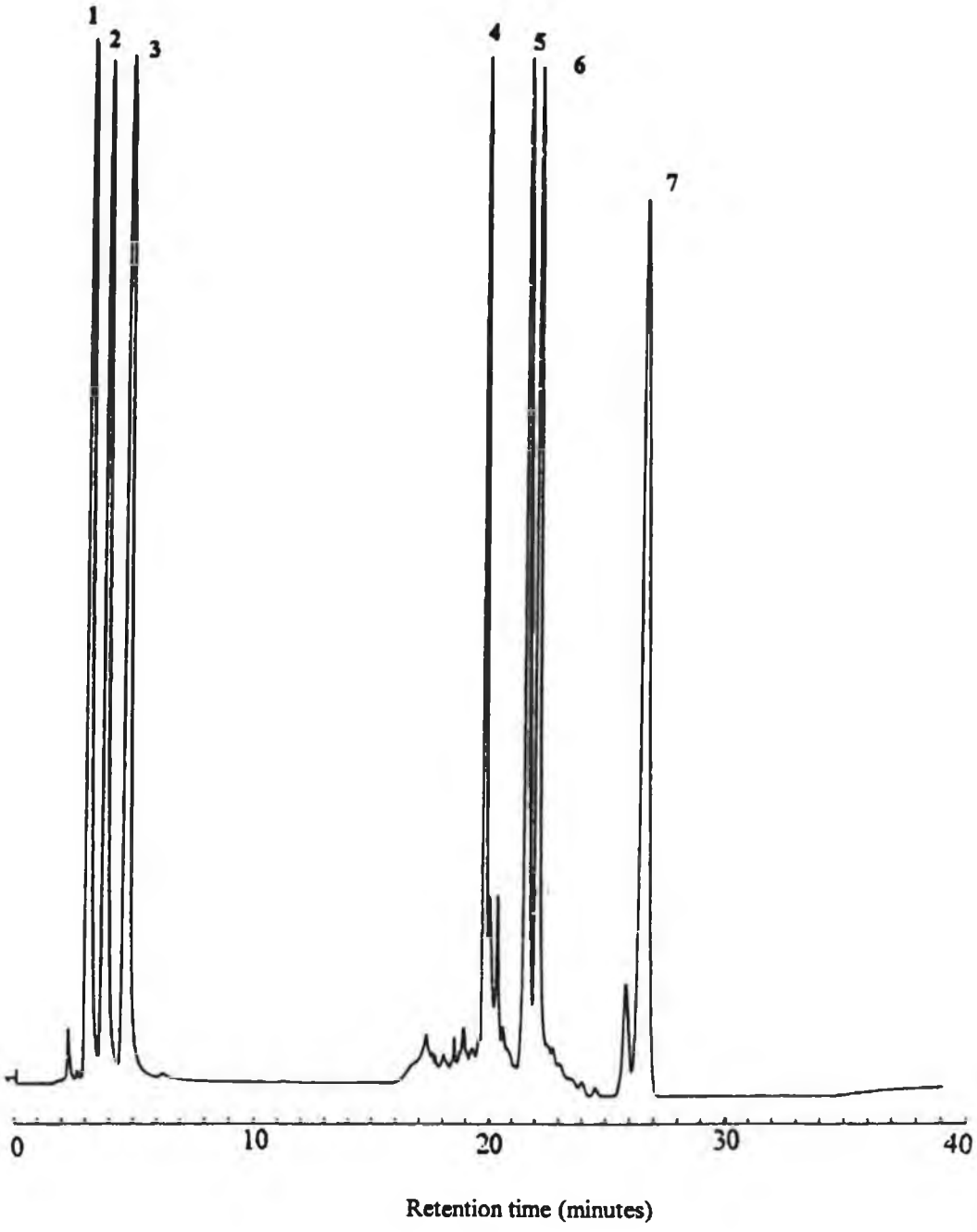

Figure 3.18. Separation of 1) 24 µg FeDTPA, 2) 45 µg FeHEEDTA, 3) 18 µg FeEDTA, 4),5) 80 µg FeEDDHMA, 6), 7) 74.5 µg FeEDDHA. Eluent: Mobile phase A: 0.15 M BTAC, 0.1 M KH_2PO_4, pH 7.5, mobile phase B: 0.15 M BTAC, 0.1 M KH_2PO_4, pH 7.5. 25% acetonitrile. Switching sequence: 5 minutes mobile phase A, 15 minutes mobile phase B, 25 minutes mobile phase A. Flow rate: 0.5 ml/min. Injection loop 20 µl. Detection 283 nm

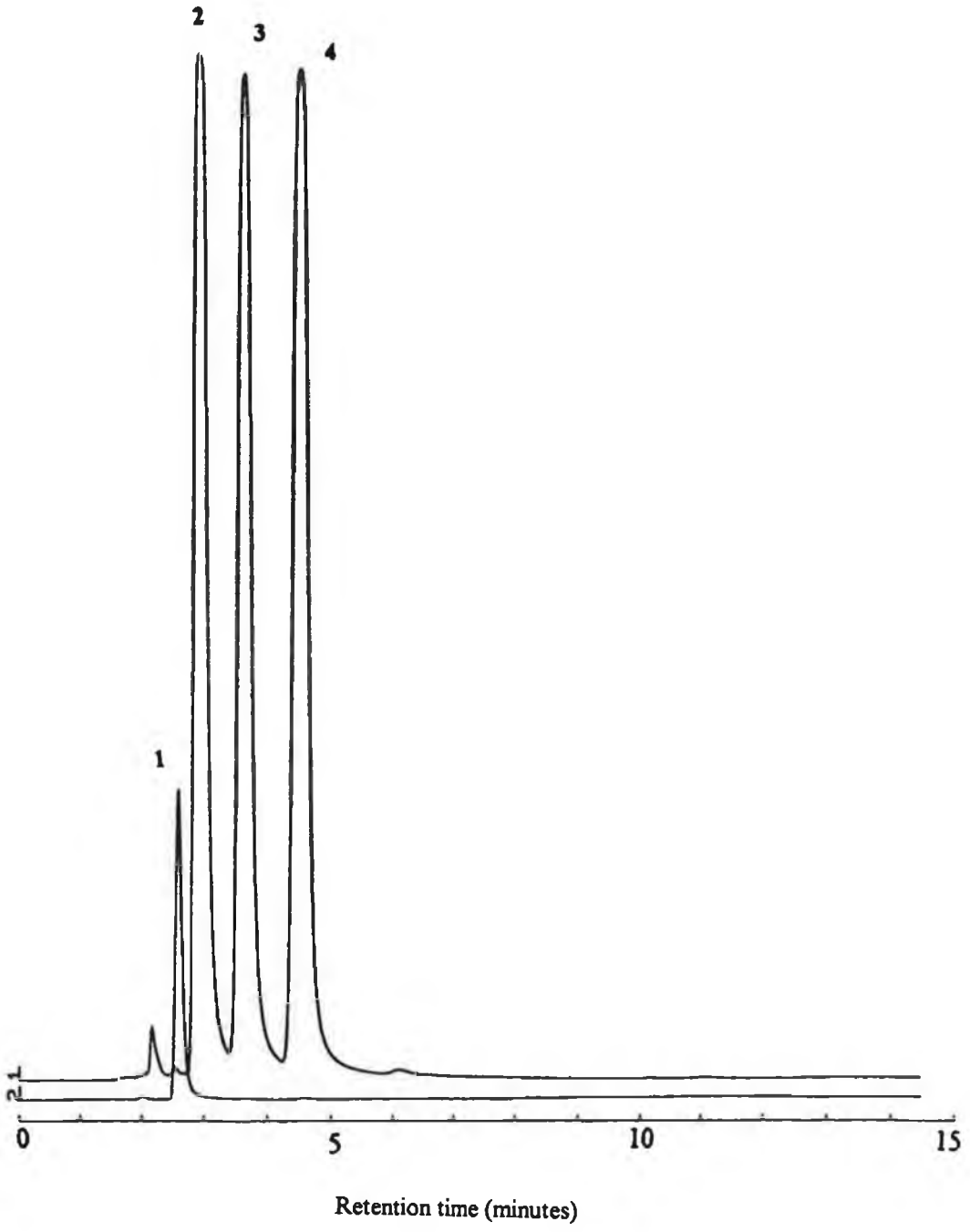

Figure 3.19. 1) 14.7 µg CuEDTA, 2) 23.6 µg FeDTPA, 3) 45 µg FeHEEDTA, 4) 18.1 µg FeEDTA. Eluent: mobile phase A: 0.15 M BTAC, 0.1 M KH_2PO_4, pH 7.5. Mobile phase B: 0.15 M BTAC, 0.1 MKH_2PO_4, , pH 7.5. Switching sequence: 5 mins A, 15 mins B, 25 mins A. Flow rate 0.5 ml/min. Injection loop 20 µl. Detection: 283 nm.

3.7.5 Investigation of the ion-pairing reagent tetrabutylammonium hydroxide

The ion-pairing reagent TBAOH was then investigated. Good resolution of the chelates was obtained. As with BTAC, the Fe(III) chelates of EDTA, DTPA and HEEDTA eluted very quickly, while the EDDHA and EDDHMA chelates had a longer retention time and were well separated. At an equivalent concentration of TBAOH the retention time of all the chelates was longer with respect to BTAC.

The dependence of the separation on the TBAOH concentration is shown in Figure 3.20 Unlike the behaviour of BTAC, where the retention remained almost constant, it was found that as the concentration of TBAOH was decreased, the retention time and resolution of the chelates increased. This is contrary to what would normally be expected and cannot be explained by such theories as ion-pair, ion-exchange or the ion-interaction mechanism proposed by Bidlingmeyer et al. [45], which would all predict that the retention time should increase as ion-pair reagent concentration increases. It has however, been shown by Hung and Taylor [46], that over wide ranges in mobile phase ion-pairing concentration, the dependence of capacity factors on ion-pairing concentration is complex. It may reach a plateau or even pass through a maximum. It was predicted that the mechanism occurring was a combination of ion-exchange and desolvation. As the concentration of ion-pairing reagent increases, the C_{18} surface area available for desolvation subsequent to ion-exchange will decrease. Also, the capacity factor will decrease as the counter ion concentration increases as a result of added pairing salt. This mechanism was modelled for aqueous systems and over concentration ranges similar to that used in this study. Bidlingmeyer et al. [45] carried out studies over a smaller concentration range of 0-20 mM and in the presence of an organic modifier thus this explains the differences in observations. The optimum separation achieved in this study, was at 0.05M TBAOH. Below this concentration the retention time continued to increase but resolution decreased. This may be explained by increased hydrophobic interaction of the chelates with the stationary phase due to increased area available for desolvation and less competition from the ion-pair reagent. However, there are not enough ion-exchange sites to maintain selectivity.

Due to the longer retention time of the chelates, the chelates of EDDHA and EDDHMA again needed addition of acetonitrile. Acetonitrile was added to a second mobile phase and a solvent switching system used. The amount of acetonitrile added was 30%. The final conditions were thus : mobile phase A: 0.05M TBAOH, pH 7.5. mobile phase B: 0.05M

TBAOH, pH 7.5, 30% acetonitrile, using a switching sequence of 5 minutes mobile phase A, 20 minutes mobile phase B, 25 minutes mobile phase A. The separation achieved is shown in Figure 3.22.

Investigation of the separation of Mn(II)EDTA, Cu(II)EDTA and Zn(II)EDTA with TBAOH found that these species coeluted (Figure 3.23)

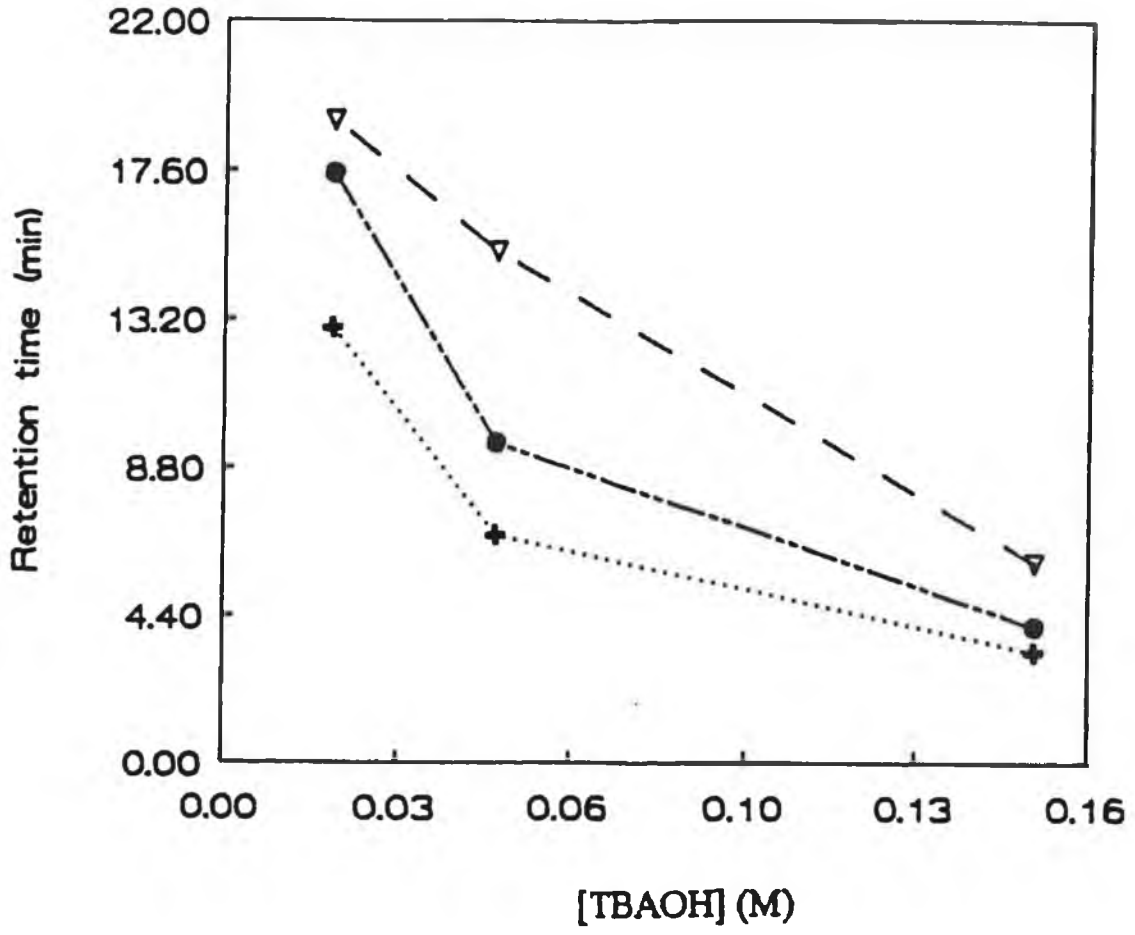

Figure 3.20. The influence of TBAOH concentration on retention time of iron(III)- chelates. Column: Chromspher C18. Eluent: mobile phase A: TBAOH variable, pH 7.5, mobile phase B: TBAOH variable, pH 7.5, 25% acetonitrile. Flow rate 0.5 ml/min. Injection loop 20 µl. Detection 300 nm.

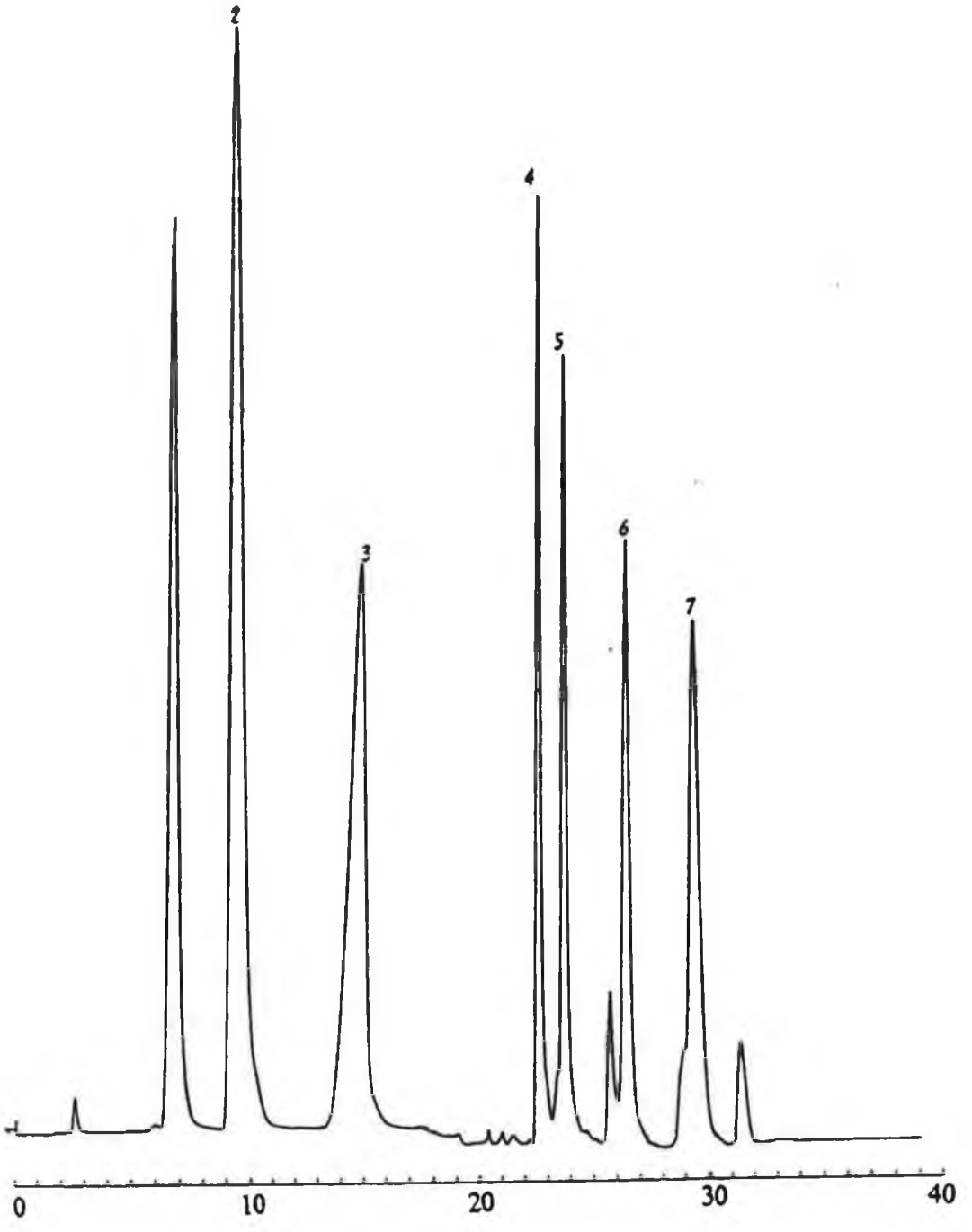

Figure 3.21. Separation of 1) 67 µg FeHEEDTA, 2) 61 µg FeDTPA, 3) 47 µg FeEDTA, 4),5) 94 µg FeEDDHA, 6), 7) 120 µg FeEDDHMA. Column: Chromspher C18. Eluent: mobile phase A: 0.05 M TBAOH, pH 7.5, mobile phase B: 0.05 M TBAOH, pH 7.5, 30% acetonitrile. Switching sequence: 5 minutes A, 20 minutes B, 25 minutes A. Flow rate: 0.5 ml/min. Injection loop: 20 µl. Detection: 300 nm.

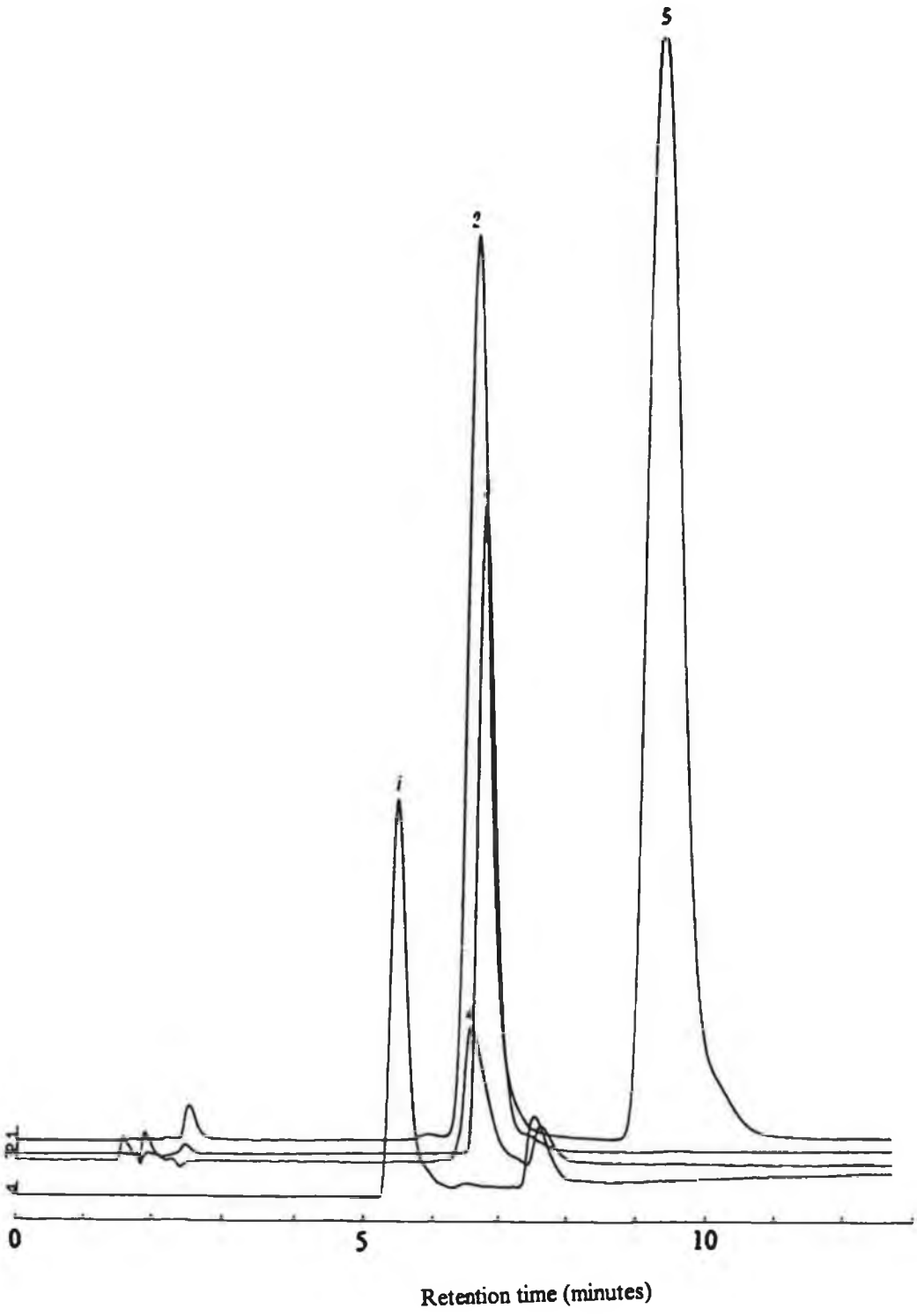

Figure 3.22. 1) 15.4 µg MnEDTA, 2) 67 µg FeDTPA, 3) 14 µg CuEDTA, 4) 13 µg ZnEDTA 5) 61 µg FeHEEDTA. Column: Chromspher C18. Eluent: mobile phase A: 0.05 M TBAOH, pH 7.5, mobile phase B: 0.05 M TBAOH, pH 7.5, 30% acetonitrile. Switching sequence: 5 minutes A, 20 minutes B, 25 minutes A. Flow rate: 0.5 ml/min. Injection loop: 20 µl. Detection: 225 nm.

3.7.6 Investigation of the ion pairing reagent tetradecyltrimethylammonium bromide

To separate Mn(II)-EDTA, Cu(II)-EDTA and Zn(II)-EDTA, an alternative ion-pairing reagent was needed. It has been shown that the larger the ion-interaction reagent the greater the amount adsorbed on the stationary phase for a given concentration [45]. Therefore, at equivalent concentrations, TDTMABr will provide more ion-exchange sites than TBAOH. From the behaviour of TBAOH, it was predicted that as the concentration decreased the increased area available for desolvation improved the separation. However, below 0.05M, there was a lack of ion-exchange sites. Therefore, an ion-pairing reagent which allows sufficient interaction with the reversed phase and gave sufficient ion-exchange sites was needed. Thus TDTMABr was chosen, as at low concentration it would coat the column more efficiently. Figure 3.23 shows how the retention of Mn(II)-EDTA, Zn(II)-EDTA, Cu(II)-EDTA and Fe(III)-HEEDTA increases as the concentration of TDTMABr decreases. Figure 3.24 shows the separation achieved. It can be seen that the detection of ZnEDTA is very insensitive; this could possibly be improved by the development of a post-column reaction with copper [37].

3.7.7 Sample Analysis

The ion-pair chromatographic method described using TBAOH as ion-pairing reagent gave rise to a linear calibration for the determination of the iron(III)- chelates in the range 1.79-179 mmoles Fe in the form of chelate with correlation coefficients of FeEDTA - 0.999, FeDTPA - 0.997, FeHEEDTA - 0.999, FeEDDHA - 0.998 and FeEDDHMA - 0.999. 1.79 mmoles Fe in the form of chelate was the limit of detection for FeEDDHA and FeEDDHMA while for FeEDTA, FeDTPA and FeHEEDTA it was below this. No interferences were encountered in the analysis of real samples and sample retention times agreed very closely with those of standards. Figures 3.25 to 3.27 show examples of three samples analysed. No quantitative results have been quoted, as official standards were not available to carry out such studies.

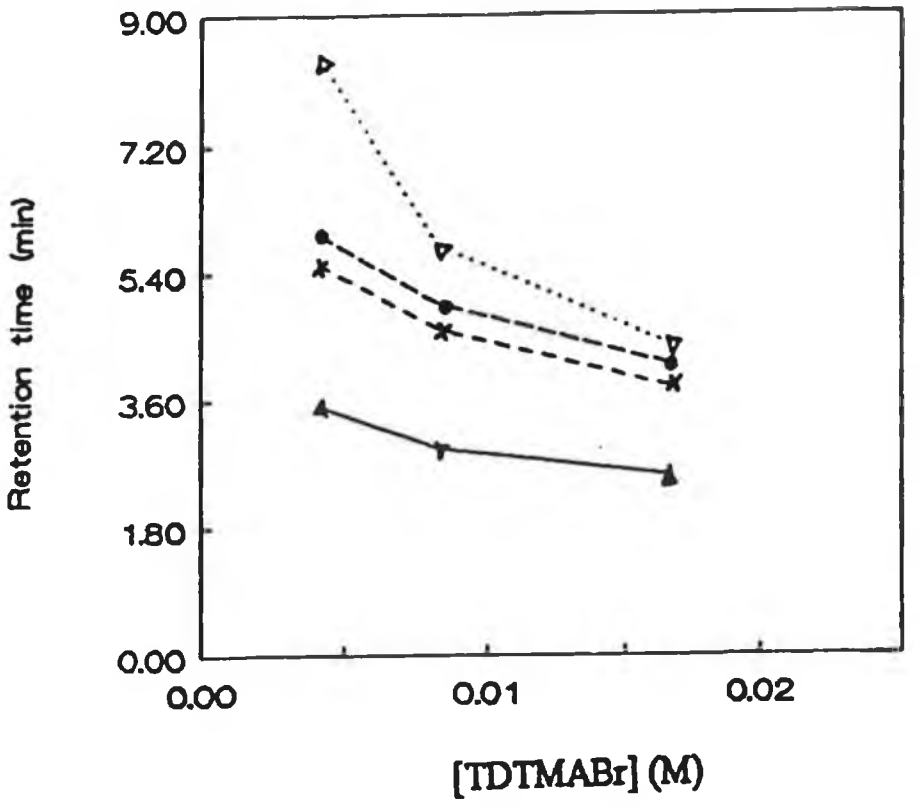

Figure 3.23. Influence of TDTMABr concentration on the retention time of various chelates. Column: Chromspher C18, Eluent: [TDTMABr] variable, 0.1M KH_2PO_4, pH 7.5. Flow rate 0.5 ml/min. Injection loop: 20 µl. Detection 220 nm.

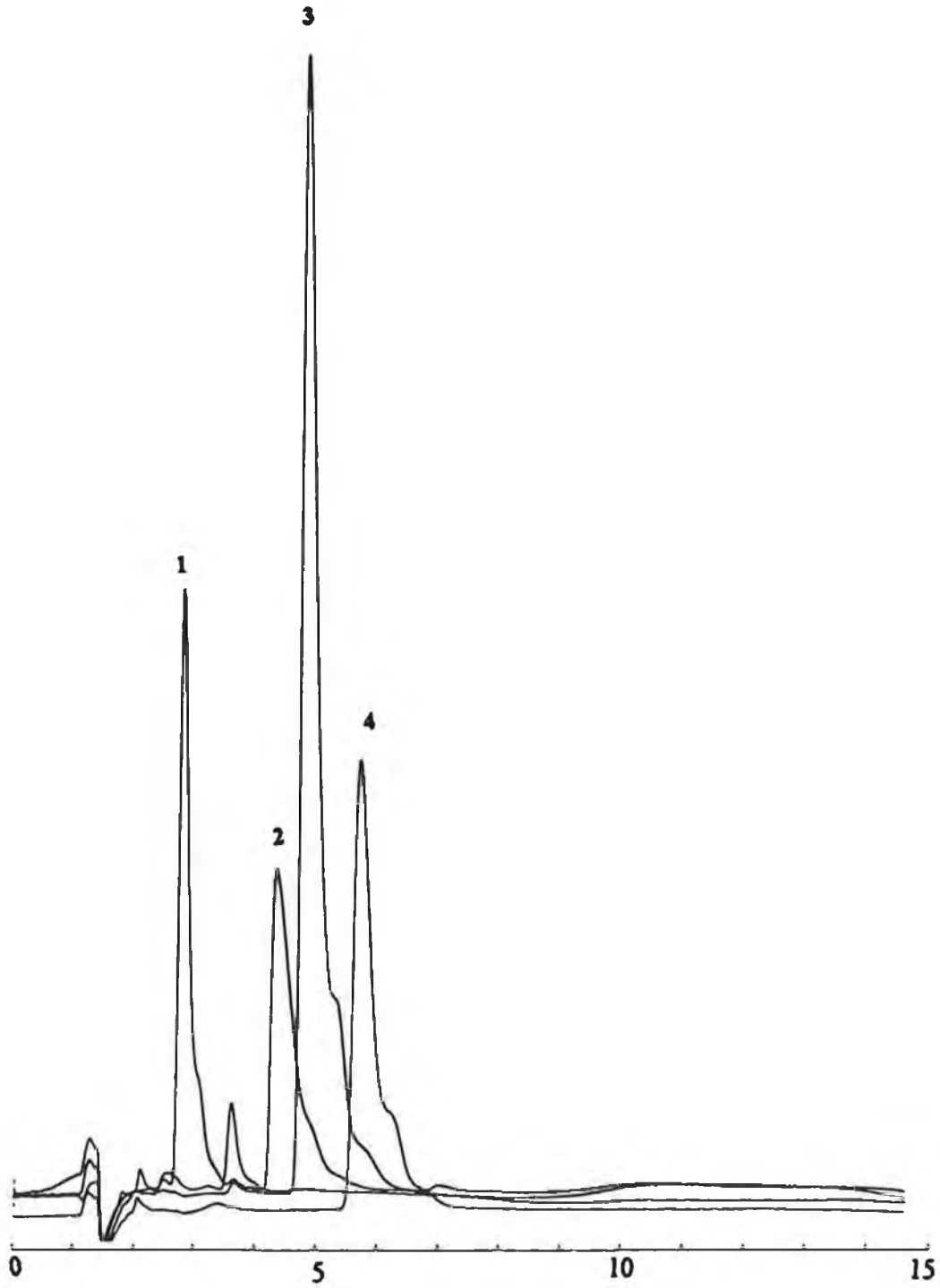

Figure 3.24. Separation of 1) 46 µg MnEDTA, 2) 330 µg ZnEDTA, 3) 41 µg CuEDTA, 4) 67 µg FeHEEDTA. Column: Chromspher C18. Eluent: 0.01 M TDTMABr, 0.1 M KH_2PO_4, pH 7.5. Flow rate 0.5 ml/min. Injection loop: 20 µl. Detection 220 nm.

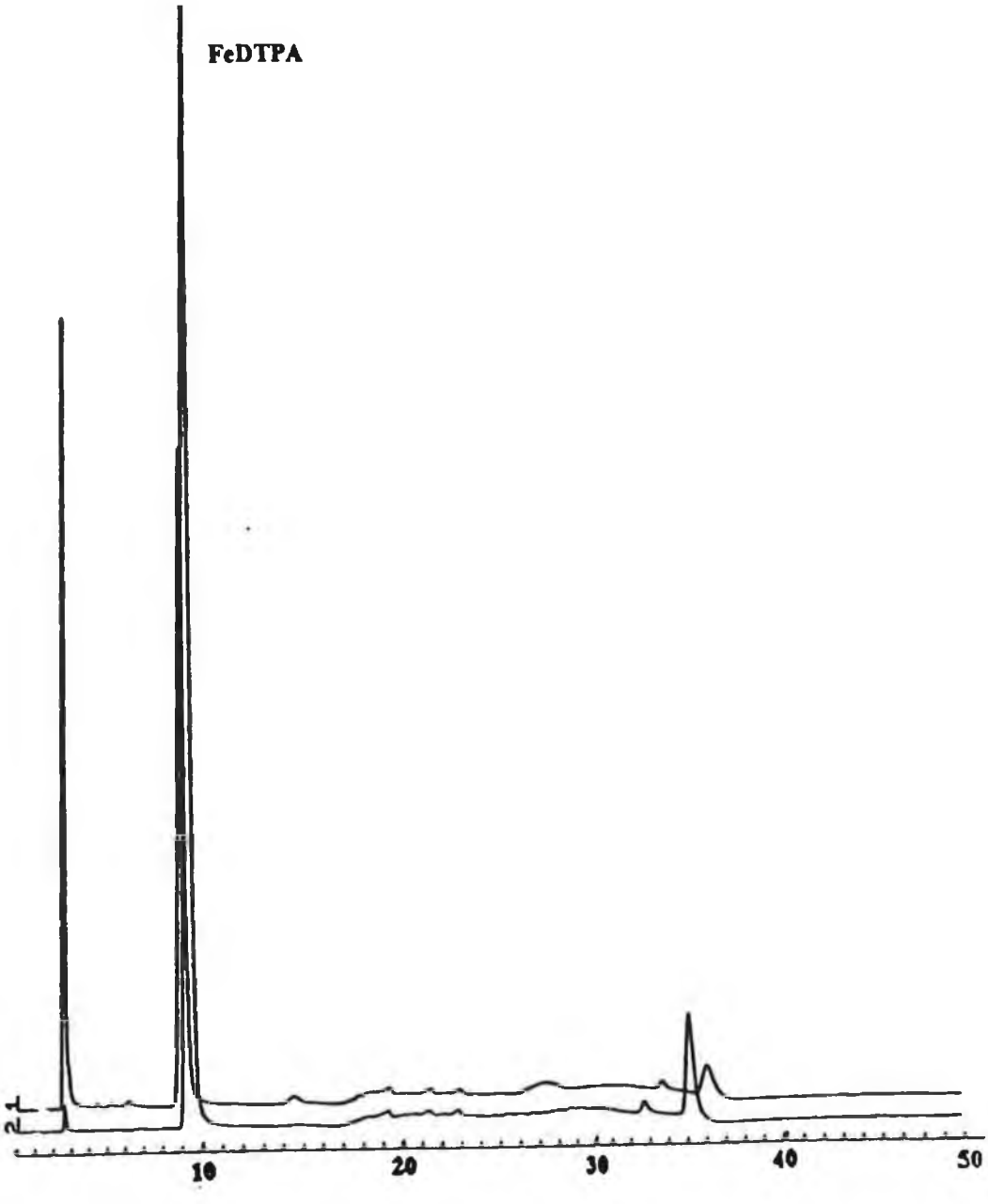

Figure 3.25. Overlay of 1) fertiliser sample containing 3.2% iron in the form of FeDTPA with 2) FeDTPA standard. Column: Chromspher C18. Eluent: mobile phase A: 0.05 M TBAOH, pH 7.5, mobile phase B: 0.05 M TBAOH, pH 7.5, 30% acetonitrile. Switching sequence: 5 minutes A, 20 minutes B, 25 minutes A. Flow rate: 0.5 ml/min. Injection loop: 20 µl. Detection: 300 nm.

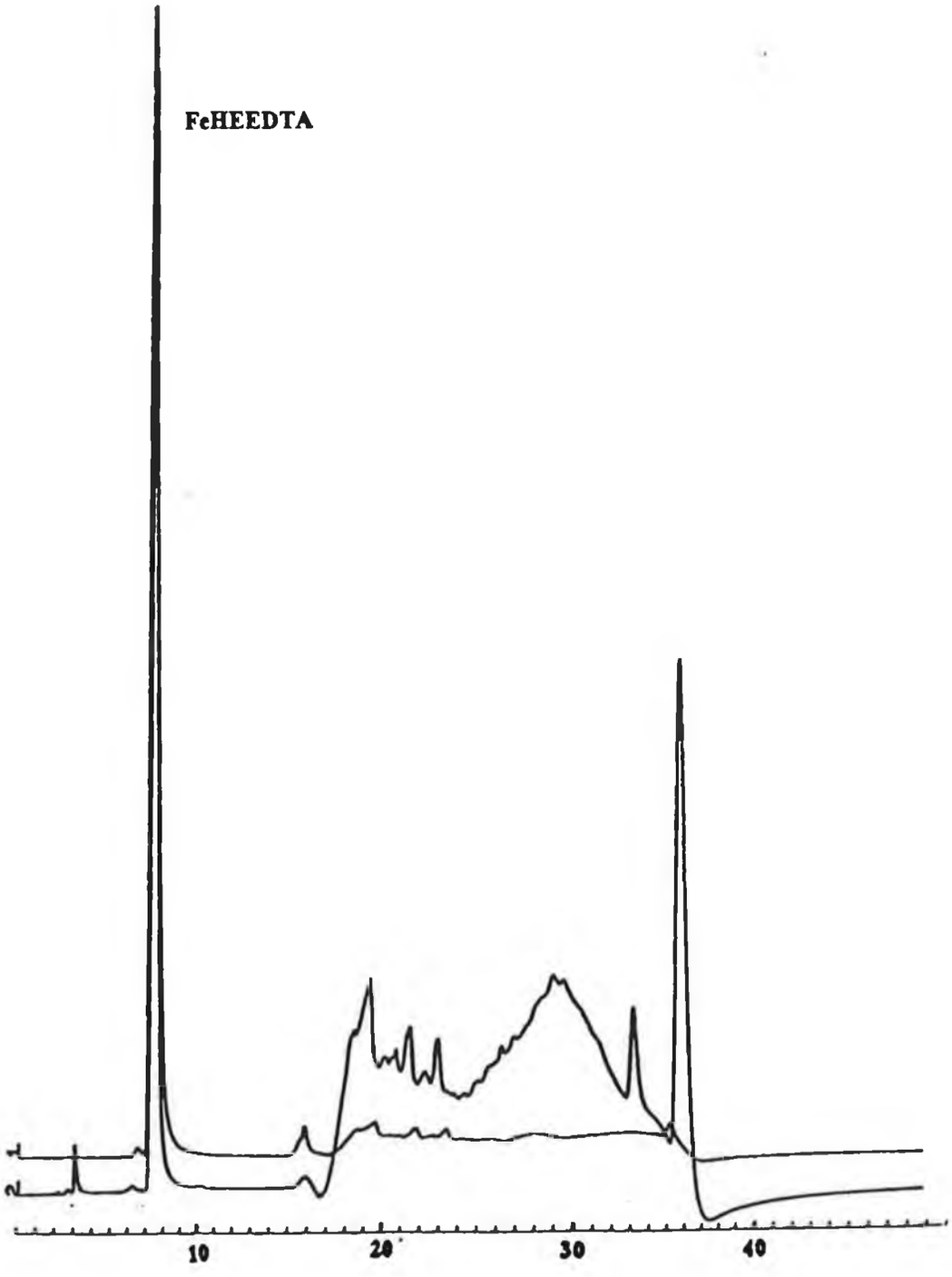

Figure 3.26 Overlay of 1) FeHEEDTA "standard" with 2) fertiliser sample containing approximately 5.4% iron in the form of FeHEEDTA. Column: Chromspher C18. Eluent: mobile phase A: 0.05 M TBAOH, pH 7.5, mobile phase B: 0.05 M TBAOH, pH 7.5, 30% acetonitrile. Switching sequence: 5 minutes A, 20 minutes B, 25 minutes A. Flow rate: 0.5 ml/min. Injection loop: 20 µl. Detection: 300 nm.

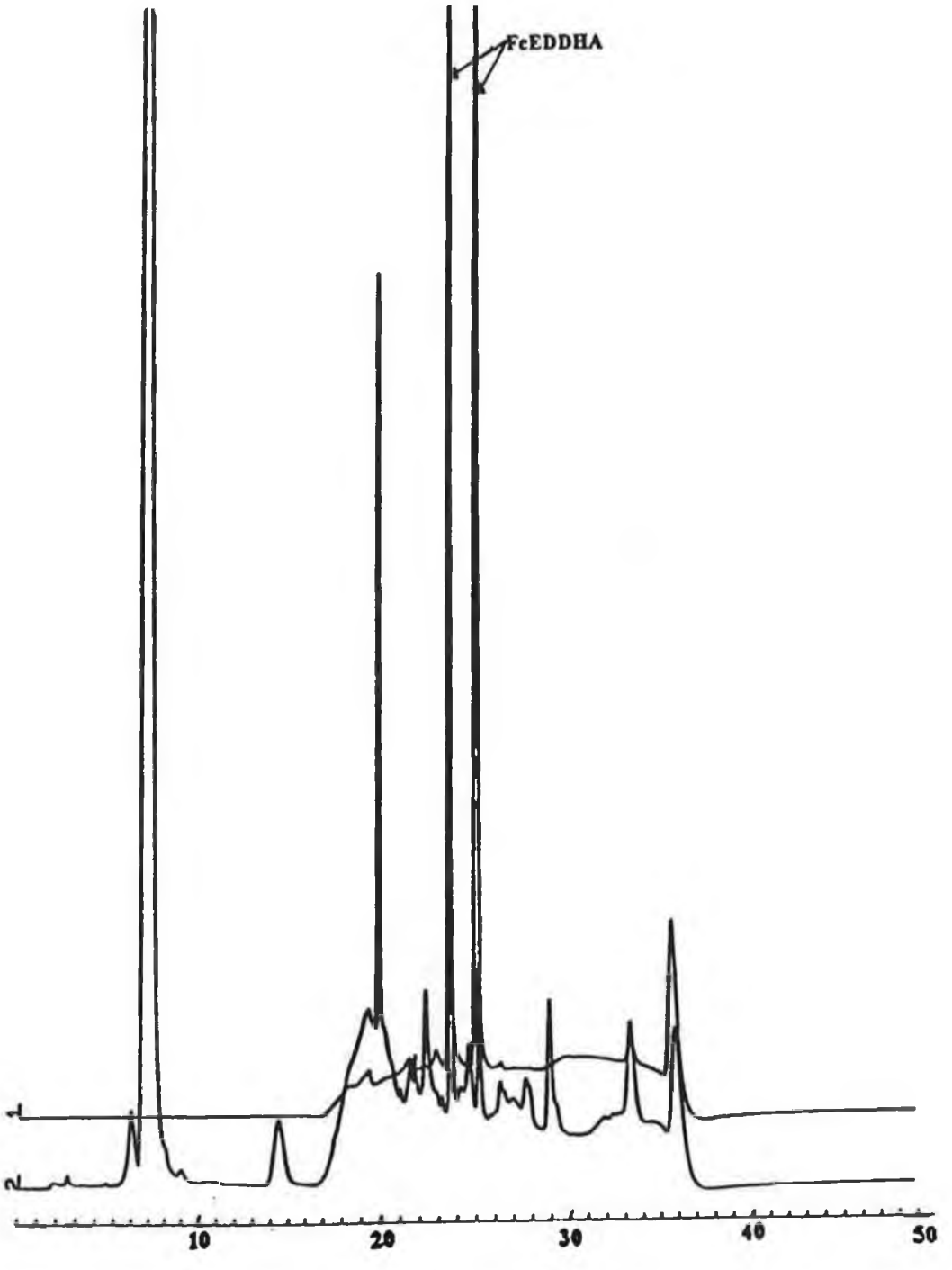

Figure 3.27 Overlay of 1) FeEDDHA "standard" with 2) fertiliser sample containing approximately 0.2% iron in the form of FeEDDHA. Column: Chromspher C18. Eluent: mobile phase A: 0.05 M TBAOH, pH 7.5, mobile phase B: 0.05 M TBAOH, pH 7.5, 30% acetonitrile. Switching sequence: 5 minutes A, 20 minutes B, 25 minutes A. Flow rate: 0.5 ml/min. Injection loop: 20 µl. Detection: 300 nm.

3.7.8 Conclusion

From the above two investigations it may be concluded that the chelates FeEDTA, FeDTPA, FeHEEDTA, FeEDDHA, FeEDDHMA, CuEDTA, ZnEDTA and MnEDTA, may be determined in a fertiliser containing one of the above chelates. Of the two separations developed with BTAC and TBAOH for the iron chelates the separation with BTAC is the better of the two as it will not suffer interference from ZnEDTA and MnEDTA as seen with the TBAOH method (figures 3.20 and 3.23), also the method can be used for the determination of CuEDTA. An investigation of the exact elution of ZnEDTA and MnEDTA could be carried out by using a post-column reaction of these chelates with copper. This could reveal a separation of these chelates using the BTAC method.

For FeEDDHA and FeEDDHMA, it is thought that a separation of the diastereoisomers has been achieved, which is important as research shows only one of these isomers is effective as a fertiliser. Identification of the isomers could be achieved by separation of the FeEDDHA standard by TLC into its isomers [6]. This was not studied due to lack of time.

This method will be useful for quality control in the manufacture of fertilisers. Further studies should aim to develop a method for the determination of chelating agent. This would involve the development of a sample preparation procedure for conversion of all chelating agent to the iron form at low pH, with removal of interfering ions [47] and subsequent determination by the method developed.

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CHAPTER 4
DETERMINATION OF PEPTIDES BY CAPILLARY ELECTROPHORESIS/ELECTROCHEMISTRY USING ON-COLUMN COMPLEXATION

4.1 INTRODUCTION TO PEPTIDE RESEARCH

Peptide research today is big business. This is due to the discovery of the wide role of peptides in practically all life processes. Peptides act as poisons, antibiotics, food additives, neurotransmitters, hormones and controllers of various activities in the body. It is their potential pharmacological properties which have attracted most attention. Their powerful medicinal properties continue to stimulate considerable interest in the pharmaceutical industry to come up with various effective analogues of the natural peptides. The peptide Angiotensin II, for example, was found to increase blood pressure. Glutathione is the most widespread and probably best studied among the naturally occurring peptides. This tripeptide, γ–L-glutamyl-L-cysteinyl-glycine, has a multitude of biochemical functions within the cell. It is the most abundant intercellular thiol in almost all aerobic biological species. Another well known peptide, penicillin is continually investigated to find alternatives for resistant bacteria. The neurotransmitter-like enkephalins, discovered in 1975 to control the sensation of pain and to have potent opiate activity, have become the subject of intensive research. Innumerable enkephalin analogues were synthesised in the hope of finding a harmless substitute for morphine. Indeed, the research into neurotransmitters is one of the largest areas in peptide research. Exciting developments in this area include the direct monitoring of neurotransmitter concentrations in an alert freely moving animal.

Thus, in the search to understand how the body functions, peptide research has become very important. Pharmaceutical industries use knowledge gained through research of peptides to develop drugs to mimic the activity of naturally occurring peptides or perhaps to block their action. The development of a new drug usually involves the isolation of the newly discovered peptide, the determination of the amino acids present, the determination of the sequence of these amino acids, and finally the synthesis and determination of the purity of the drug. Peptide research is also important in the study of proteins. Numerous research problems of modern biochemistry involve the characterisation of proteins through a technique called peptide mapping. This involves site-specific fragmentation of a protein followed by a high-resolution separation and quantitative measurement of the individual cleaved peptides. The determination of post translational modifications and identification and localisation of genetic variants are among the most important areas benefiting directly from acquisition of accurate peptide maps. Thus the demand for the development of highly sensitive analytical techniques to determine peptides is ever increasing

4.2 CHROMATOGRAPHIC AND ELECTROPHORETIC ANALYSIS OF PEPTIDES.

There are many criteria which must be considered when selecting or developing an analytical method for the analysis of peptides, or indeed any analyte. The method must be adequately sensitive, selective but yet general, and place minimal restrictions on solvent usage, pH range, ionic strength, and viscosity. In peptide analysis it is of particular advantage if the method applies to a broad range of peptide size, and is non-destructive. Finally the method should be stable, easy to perform, run with minimal operator attendance, be commercially available and inexpensive. Unfortunately in the real world this combination is rarely if ever found ! However many techniques will have various combinations of these criteria. This section discusses the developments in the determination of peptides. As the research involved the development of a method of detection for peptides, particular attention is given to the discussion of the various detection methods developed.

In the 1930's electrophoretic techniques were introduced for the determination of peptides. Modern electrophoresis is a collection of manual, intensive methodologies that cannot be run unattended and that cannot be readily automated. Casting of gels, application of samples, running of gels, and staining of gels are time consuming tasks prone to irreproducibility and poor quantitative accuracy. The 1950's saw the introduction of chromatographic methods. During the last two decades the analytical methodologies in this area have considerably strengthened through the rapid advances of modern liquid chromatography. The 1970's saw the introduction of capillary electrophoresis (CE) which has brought enormous advances to the field of peptide analysis. This method is a very powerful separation technique, and in general method development is much faster. Due to the different separation mechanisms involved, CE and HPLC are complementary rather than competitive, thus broadening the spectrum of molecules which may be separated by combining the two methods. The detection limits quoted for many CE applications with electrochemical and fluorescence detection are now reaching attomole levels. As injection volumes are in the nanoliter to picoliter range concentration molar detection limits should be calculated when comparing detection limits to HPLC. However, the low injection volumes are where the strength of the method lies, as it opens up the field of analysis to areas such as single cell analysis. The major challenges for the determination of peptides by CE lie in the area of detection systems. Demands for high sensitivity detection in CE are due to the nanolitre sample volumes used. Dealing with these low sample volumes in the presence of an applied electric field

necessitates the development of new detectors. However, many of the principles for detection with HPLC applications can still be used.

4.3 Detection Methods used in the Determination of Peptides

4.3.1 Introduction

Peptides have been determined by chromatography with direct detection, indirect detection, radiolabelling, derivatisation, and by chemically modified electrodes. Direct detection has been carried out using the innate properties of UV absorption, fluorescence and electrochemical (EC) detection. However, in many cases, direct detection has proved to be insufficiently sensitive. Indirect detection using a background of an absorbing species, a fluorescent species or an electrochemically active species has many limitations. Radio-labelling, while in many cases still the most sensitive approach to the detection of peptides, is not used routinely. This is due to the inherent expense of the technique, the difficulty of obtaining commercially available antibodies, and the disposal problems. Thus much research at present has concentrated in two areas. Firstly, the development of new derivatising reagents which form peptide derivatives with electrochemical or fluorescent properties [20-34]. Secondly the determination of peptides with novel electrode materials and chemically modified electrodes [43,44].

4.3.2 Direct Detection of Peptides

4.3.2.1 UV detection

Direct UV detection carried out at 215 nm (π to π^* transition of the amide carbonyl) may be used for the determination of peptides. The method however is very unselective. For peptides containing the amino acids phenylalanine, tyrosine and tryphtophan, greater selectivity may be achieved. These amino acids contain an aromatic group and thus may be detected at 280 nm. The UV spectrum of tyrosine is strongly dependent on pH. The absorption of tryphtophan is nearly independent of pH, it shows a maximum at 280 nm with and a second maximum at 288 nm. However, despite the lack of sensitivity, UV detection is still widely used as a post-column detection method with HPLC, as it is simple, non-destructive and detects both primary and secondary amino acids.

4.3.2.2 UV detection with CE

Thus, with the development of CE, UV detection was adapted for detection purposes. The capillary itself is used as the flow cell. This involves stripping off a small section (1-2 cm) of the exterior coating on the capillary with acid or by burning, and inserting this zone into a UV detector which has been adapted for the capillary. Ludi et al. [1] adapted a typical UV detector used with HPLC for CE by connecting it to the on-column flow cell with optical fibres. With CE, detection wavelengths as low as 200 nm may be used. This is due to the short pathlength and absence of refractive index changes due to solvent density pulses that result from HPLC pumping. However, as UV detection is dependent on the path length of the flow cell, which in typical capillaries is 25-100 μm, the method has limited sensitivity with typical detection limits of the order of 5-20 ng/μl. It is used mostly for the observation of the behaviour of various peptides under different conditions and thus to draw models of peptide behaviour in general. Attempts to improve sensitivity include sample stacking [2]. This involves raising the pH of the sample solution to a value well above the isoelectric point of the peptides by the addition of an appropriate amount of base. Application of a potential gradient over the capillary initiates the movement of the peptides towards the anode. The direction of electrophoretic mobility is reversed as soon as the peptides migrate into the acidic electrophoresis buffer leading to the concentration of the applied peptides at the sample solution electrophoresis buffer interface. With this scheme, detection limits of 1 ng/ml can be obtained.

4.3.2.3 Fluorescence detection.

Fluorescence generally gives rise to lower limits of detection (LOD) than UV detection. This arises from the fact that the signal of interest is observed on a very small background. High excitation intensity may be employed without a significant increase in background signal and noise. Certain unmodified peptides can be detected fluorometrically . Only marginal gains (relative to UV detection) are realised using this approach because intrinsic fluorescence of peptides is generally weak and varies with structure. Therefore, fluorescence detection is usually indirect or employed with pre- or post-column derivatisation.

4.3.2.4 Fluorescence Detection with CE

With CE laser induced fluorescence may be used where excitation maxima are situated close to wavelengths of readily available lasers, e.g. that of a helium/cadmium laser or an argon laser. Helium /cadmium lasers are most popular as they are relatively inexpensive and emit in the UV region (325 nm). Increased sensitivity is attributed to the ability to tightly focus the intense laser beam directly onto the CE separation capillary. The method is more suitable for adaptation to CE than UV as the intensity of the excitation radiation can be increased to account for losses in pathlength. Hogan and Yeung [3] determined sub-femtomolar quantities of tryptic digest mixtures using indirect fluorescence detection. Indirect fluorescence is based upon the charge-induced physical displacement of a coeluting, fluorescent buffer ion by the non fluorescent, non absorbing analyte ion. Hogan and Yeung used the fluorescent salicylate ion. Mass detection limits of 165-550 attomoles for peptides were achieved. Most fluorescent applications with CE involve the use of a derivatising reagent.

4.3.2.5 Electrochemical Detection

Direct electrochemical detection is made possible by oxidation of phenolic, indole or thiol moieties, or the reduction of disulphides within a peptide. The amount of current produced is related to the quantity of analyte. Tyrosine and tryptophan contain electro-oxidisable phenolic and indole moieties respectively. Cysteine contains the electro-oxidisable thiol moiety while cystine contains the electroreducible disulphide bridge. EC detection is used with HPLC as it is both more sensitive and selective than UV detection, and more generally applicable than fluorescence detection. Sensitivity in many cases in the picomolar or nanomolar range which is equivalent to that achieved with radio immunoassay, while the technique is also less expensive. The greater selectivity arises from the fact that only compounds with an oxidation potential at or below the set potential will be detected. Greater selectivity has been achieved by the use of two electrodes in series. The first is held at a potential slightly below the second and thus only analytes with oxidation potentials between the two potentials or at the second potential will be detected [4].

Many direct EC determinations have been carried out for peptides containing tyrosine and tryptophan [5-8]. Bennett et al. [5] investigated the electrochemistry of twenty synthetic neuropeptides. The study showed that a large number of peptides known to occur within

brain neurons are oxidised at micro-carbon paste working electrodes between +0.6 V and +0.9 V. Peptides containing only tyrosine, for example neurotensin, exhibited single oxidation peaks comparable with tyrosine peaks (+0.66 V at pH 7.4; +0.84V at pH 4.6). Those containing tryptophan such as cholecystokinin tetrapeptide, caerulein and samatossin, were comparable with tryptophan peaks (+0.74V at pH 7.4; +0.88V at pH 4.6) and those containing both tyrosine and tryptophan, such as cholecystokinin octopeptide, gonadotrophin releasing hormone, α-melanocyte stimulating hormone, and synacthen, produced oxidation profiles with two comparable peaks at pH 7.4, but one broad peak at pH 4.6. However, individual peptides showed characteristic oxidation profiles with regard to peak shape and position, indicating the contribution of other amino acids to their electroactivity. This is particularly true for cyclic peptides containing cysteine, consistent with the observation that cysteine produced a small broad oxidation profile. Detection limits of between 1×10^{-6} to 1×10^{-4} M were obtained. Sauter and Frick [6] also determined cholecystokinin tetrapeptide and cholecystokinin octapeptide in rat cortex, hyppocampus, striatum and brain stem at the 0.1 pmol level, and thus showed direct detection by EC to be comparable to RIA. Spatola and Benovitz [7] determined the tyrosine-containing peptides leucine enkephalin, and leuteinising hormone-releasing hormone, after separation by reversed phase chromatography at a glassy carbon electrode, using an operating potential of +1.06 V. Detection limits in the pmol range were an improvement of at least 20-fold or greater over UV detection limits.. One must remember, however, that this type of detection is limited to the detection of peptides containing the electroactive amino acids. This may however be an advantage as in the case of peptide mapping of a protein where the peptide map is made much simpler.

Measures to improve selectivity include using dual coulometric-amperometric cells and peak current ratio measurements. Schieffer [4] devised a system where the upstream electrode was held at a lower potential than the analytical cell to completely oxidise and make undetectable other species oxidisable at potentials lower than that of the analyte. This provided subnanogram detection limits for L-dopa. The use of peak current ratios may also improve selectivity. Fleming and Reynolds [8] showed that endorphins methionine-enkephalin-lysine and methionine-enkephalin-arginine, which are pentapeptides differing by only a methyl group, exhibit characteristic oxidative behaviour. Therefore peak current ratios are characteristic for each compound.

4.3.2.6 Disadvantages of Electrochemical Detection.

Detection limits with EC detection are reduced when using reversed phase chromatography with compounds having widely different hydrophobic properties. Such analytes require the use of gradient elution, as isocratic conditions can result in abnormally long chromatographic runs. Gradient elution may only be successfully achieved with EC by maintaining comparable ionic strengths in both organic and buffer mobile phases. Drumheller et al. [9] determined neurotensin, a thirteen amino acid peptide containing two tyrosine residues down to 250 pg level using isocratic elution and down to 25 ng using gradient elution. Bombesin, which contains tryptophan, was however detected at the 25 ng using EC detection, compared with 5 μg for UV detection.

Another drawback with EC detection is that large peptides are often found to be chemically inactive. This is because the diffusion of large biomolecules to the electrode, where electron transfer takes place is slow and the peptide is perceived as too large to orient its EC active site with the electrode surface. Measures to counteract this include post-column photolysis and the use of additives. Dou et al performed post-column photolysis on-line and post-column with a 1.4 ml knitted open tubular reactor [10,11]. Both a Zn lamp (having a main irradiation line at 210 nm and a mercury lamp (having a maximum irradiation line at 254 nm) were used. The overall sensitivity of the method was comparable to fluorescence detection and better than UV detection at 210 nm. The degradation, conformational change or modification of side chains of a peptide or protein by photolytic reaction produce electroactive photoproducts having their own electrochemical properties which can be used for detection. The processes occurring have not been determined, but they are thought to include cleavage of disulphide bridges in the protein structure to produce thiols, exposure of electrochemically active amino acids such as tyrosine and tryptophan to the electrode surface, which would result from unfolding of the protein upon irradiation. It is also possible that photohydroxylation of non-EC active amino acids into EC active derivatives, and photocleavage of the peptide backbone to generate smaller, now EC active peptides, takes place. It has been shown that amino acids with inherent electrochemical activity, such as tyrosine and tryphtophan, show increased activity after irradiation.

Large negatively charged peptides are especially difficult to detect at graphite electrodes as the charge on the electrode itself is negative. Armstrong et al. [12] showed, however, that it is possible to detect the negatively charged proteins rubredoxin, ferrodoxin and flavodoxin

by addition of multivalent cations to the eluent. The approach of the proteins to the electrode surface is assisted by the high positive charge density in or near the outer Helmholtz plane that may be generated by multivalent cations. Multivalent ions, such as Mg^{2+} and $Cr(NH_3)_6^{3+}$, which are redox inactive over the potential range of interest, were used. Maximum peak currents for these proteins were obtained at Mg^{2+} concentrations ranging between 40 and 80 mM. and between 5 and 10 mM for the trivalent cation. Measurements were carried out by square wave voltammetry at pyrolytic graphite electrodes.

4.3.2.7 Electrochemical Detection with CE

Adaptation of electrochemical detection for use with CE is complicated by the scale of the system and the presence of the large applied potential across the capillary with its associated current. This current through the capillary is typically several orders of magnitude greater than the faradaic current to be measured at the electrode. Wallingford and Ewing [13] have developed a system utilising a porous glass coupler to isolate the electrode. The porous glass coupler, located in the cathodic reservoir divides the capillary into two segments permitting the electrophoresis potential to be independently applied across only the first capillary segment. This segment is then used as the separation capillary. The joint in the capillary is placed at ground potential, so that the second section of the capillary is effectively isolated from the high potential field of the separation capillary. Electroosmotic flow generated in the first segment of the capillary acts as a pump to transport analytes and buffer through the second segment, which is approximately 2 cm in length, to the detector. Typically cylindrical shaped carbon fibre electrodes are used with amperometric detection. These are inserted into the end of the capillary by the use of a micromanipulator and a microscope. The coulometric efficiency of this type of detection scheme increases as the capillary size decreases. This is due to a decrease in the annular region around the electrode. The difficulties associated with this construction, according to O'Shea et al. [14], are that fabrication of the porous glass coupler is difficult and intricate, the joint is fragile and the porous glass capillary is not readily available. An alternative system was developed by O'Shea et al. consisting of a Nafion joint. This consisted of a piece of Nafion tubing (a conducting polymer) placed over a fracture in the capillary. The system operates in a similar manner to that of Wallingford and Ewing's construction. Wang and Chen [15] proposed an alternative method of coating the fracture using cellulose acetate porous polymer. The film was applied as cellulose acetate solution in acetone to a thickness of 80 μm. The durability of the Nafion joint, i.e. two

months without deterioration, was quoted as being longer than that of the cellulose acetate joint - which was quoted as being greater than one week.

A method of coulometric detection was proposed by Huang et al. [16]. Conductivity measurements are made on-column by placing the electrodes on opposite sides of the capillary wall. The on-line conductivity cell was constructed by fixing platinum wires through diametrically opposite holes in 50 or 75 μm i.d. capillary tubing. These holes were made using a computer controlled CO_2 laser. Under a microscope, two 25 μm Pt wires were placed opposite each other. The deviation of the distance along the capillary between the electrodes is less than 10 μm. This means that the potential difference between the two electrodes can be minimised to less than 0.3 V in a 300 V cm^{-1} electric field. This feature eliminates most of the electrochemical reactions occurring at the electrodes. Another advantage of this structural form is that excellent resolution is made possible by the very small cross-sectional area of the electrodes. This system can be used to detect and quantify any species causing a conductivity change with respect to the background. The authors used this system for the determination of Rb^+, K^+, Na^+, and Li^+. The system has not been used for peptide determination.

Despite the difficulties in the adaptation of EC detection to CE, the technique has advantages over EC detection with HPLC. The slower flow rate of CE means that reaction kinetics do not need to be as fast with CE as with HPLC. Also with CE, gradient elution is not used. As CE with electrochemical detection is so new, very little work has been done on the direct electrochemical determination of peptides. Wallingford and Ewing [17] determined the biogenic amines serotonin and norepinephrine using a capillary column of 12.7 μm with a carbon fibre electrode. The narrow column diameter has the advantages of efficient heat dissipation, and lower mass detection limits due to enhanced coulometric efficiency. Detection limits of 8.5×10^{-9}M (or 6 attomole) for serotonin and 11 attomole or (1.6×10^{-8}M) for norepinephrine were obtained. The system was used for acquiring and separating cytoplasmic samples removed from the giant dopamine neuron of the pond snail. This was achieved by attaching a 7.5 μm microinjector tip inside the cell membrane. A further investigation [18], where the column diameter was reduced to 9 μm, resulted in a detection limit for serotonin of 0.7 attomole and 3.0 attomole for norepinephrine. However, while the mass detection limits were lower for this system relative to the 12.7 μm capillary, the concentration detection limits were not as low.

Wallingford and Ewing [17] have also investigated the effect of micellar solutions on the electrochemistry of neutral and cationic catechols. It was found that the micellar buffer caused band broadening of the solute bands. This was thought to be due to frictional forces, with the capillary wall creating a back pressure. At high surfactant concentration there was a shift in $E_{1/2}$ values such that lower electrode potentials may be employed for the oxidation of neutral electroactive species. This translates to lower background currents with electrochemical detection. However at low surfactant concentrations, an increase in the limiting current was observed for non-ionic catechols, while a decrease was observed for the cationic catechols. These differences in voltammetric limiting current as a function of SDS concentration warrants careful characterisation of EC detection in MECC (Micellar Electrokinetic Capillary Chromatography). Detection limits of 0.2-0.4 fmol were achieved for dopamine, norepinephrine and epinephrine. In a further paper, Wallingford and Ewing [18] suggested that this effect may be caused by adsorption of the micellar agent to the carbon electrode surface. Surface modification of the electrode with Nafion however was suggested to rectify the problem, since it would repel the negatively charged anion..

Indirect amperometric detection has been introduced for CE by Olefirowicz and Ewing [19]. To carry out indirect amperometric detection, a cationic electrophore, i.e. 3,4-dihydroxybenzylamine (DHBA), was added to the electrophoretic buffer. A constant background current is produced by continuous oxidation of DHBA at the carbon fibre working electrode, which is held at +0.7 V vs SSCE. Zones of non-electroactive cations displace the cationic DHBA during migration to preserve electroneutrality in the zone. Thus, as these cationic zones pass through the detector region, a decrease in the current for DHBA oxidation is observed as a negative peak. Indirect amperometric detection of several cationic amino acids and dipeptides have been accomplished using this system with 26 μand 9 μm i.d. capillaries. Detection limits for the dipeptides Lys-Phe and His-Phe were 5.2 and 5.5 fmol respectively. Indirect amperometric detection is limited to analytes with the same charge as the detectable ionic component. Therefore only cations or only anions can be indirectly detected in the same separation.

4.3.3 Detection Following Derivatisation.

To improve limits of detection for in particular, the use of derivatisation reactions has been investigated. This usually involves reaction of the analyte with a derivatising reagent to produce a compound which is fluorescent or electrochemically active. However, the search for the "perfect" derivatising reagent still continues. Factors which must be considered when developing or choosing a derivatising reagent include the selectivity of the derivatising reagent for different functional groups, the stability of the derivatives formed, and whether the derivatising reagent is itself fluorescent or electrochemically active. The conditions necessary for derivatisation, particularly in relation to pH and solubility, may place restrictions on the chromatographic or electrophoresis conditions which may be used for separation. The speed and simplicity of the derivatisation reaction and the detection limits which may be achieved must also be considered. The purity of the derivatising reagent and the formation of fluorescent side products may pose problems. This section discusses some of the derivatising reagents which have been developed with different combinations of the above properties. Most of these reagents rely on amine-specific chemistry for derivative formation, and thus suffer from interference from amino acids and other amines which are present in biological matrices. These reagents also cannot be used for the detection of secondary amines, which include peptides containing pyroglutamate and those that are formylated.

4.3.3.2 The Development of Derivatising Reagents

Spectrofluorometric determination of peptides with ninhydrin was introduced in the 1960's. It reacts upon heating with primary amines to produce unstable derivatives. The reaction takes place between pH 8 and 9 and is thus carried out post-column, necessitating the use of two pumps. Ammonia is an interferent. Today, however, ninhydrin has been replaced by fluorescamine, which resulted from studies of the structure of the fluorescent derivatives formed with ninhydrin. The properties of fluorescamine derivatives are similar to those of the ninhydrin derivatives, however, the derivatives are relatively stable (hours) and reaction rates are fast without the necessity of heating. The derivatives absorb light at 390 nm and emit fluorescence at 475 nm. Udenfriend et al. [20] used fluorescamine for the determination of such peptides as oxytocin, vasopressin, bradykinin, angiotensin and insulin by HPLC. Detection limits in the picomolar range were achieved. Ammonia was found not to interfere. Interference from amino acids could be minimised by operation at pH 7. Jorgenson and

Lukacs [21] used fluorescamine for the first demonstration of fluorescence detection with CZE, and demonstrated the separation of several dipeptides at the 50 μg/ml level. The detector was home-made, consisting of a high-pressure mercury arc lamp as the source of UV light, glass filters for isolation of excitation and emission wavelengths, and a photomultiplier tube detector.

Other derivatising reagents produced for fluorescence detection include 9-fluorenyl methylchloroformate (FMOC), phenyl isothiocyanate (PITC) and 1-dimethyl aminonaphthalene-sulfonyl chloride (DANSYL). All form stable derivatives; however, FMOC and DANSYL react with water to produce large hydrolysis peaks. Both FMOC and PITC are fluorescent, and thus excess reagent must be removed prior to analysis. Volgt et al. [22] achieved a detection limit of 500 fmol for angiotensin II when derivatised with FMOC at pH 9.5, and determined on a column of miniaturised diameter (125x2 mm). FMOC reacts with both primary and secondary amines, and depending on reaction conditions, hydroxy functions are also reactive. Therefore its selectivity is very poor.

For ultrasensitive applications, there is considerable advantage if the reagent is not fluorescent or electrochemically active, while the products of the reaction are. This advantage is met through the reactions of primary amines with dialdehydes or arylaldehydes that result in the formation of highly fluorescent and electrochemically active isoindoles. When a primary amine is reacted with o-phthalaldehyde (OPA) in the presence of mercaptoethanol, the product is a 1-(alkylthio)-2-alkylisoindole, which has both fluorescent and electrochemical properties, while the reagent itself does not interfere. OPA is presently used extensively for the derivatisation of peptides. The derivatisation reaction is carried out in aqueous solution buffered with borate at pH 10. However the derivatives are not stable, and therefore derivatisation must be carried out post-column or pre-column with careful timing. Orwar et al. [23] used OPA for the determination of γ-glutamyl peptides, including glutathione, achieving detection limits of 200 fmol for fluorescence detection and 425 fmol for electrochemical detection (low nM).Stability of the derivatives exposed to daylight at room temperature revealed slow degradation ($t_{1/2}$ > 6h). This is not the case in general, however, with the signal for many amino acids, whose signals decrease by 50-75% in forty minutes. Allison et al. [24] showed that the electrochemical properties of the derivative were far less susceptible than its fluorescence to changes in the structure of the derivative. This group proposed that an increase in the steric bulk of the thiol used would increase the stability. Therefore OPA was reacted with the analyte in the presence of a tert-butylthiol (2-

methyl-2-propanethiol). This resulted in an isoindole that was rapidly and irreversibly oxidised, with half-lives of several hours and detection limits of 500 fmol for amino acids.

Naphthalene-2,3-dicarboxyaldehyde/cyanide ion (NDA/CN) was developed by Carson et al. [25] as an improvement of the OPA reaction, with regard to the stability of the derivatives. The reagent reacts with primary amines to produce N-substituted 1-cyanobenz[f]isoindole derivatives (CBI). As with OPA, the reagent itself is not fluorescent or electroactive. An increase in the $t_{1/2}$ of glycine from 1.5 hr with OPA to 10 hr with NDA/CN was realised. The derivatives produced have substantially higher quantum efficiencies than OPA derivatives. Montigny et al. [26] determined the CBI derivatives of 18 amino acids with detection limits of less than 200 fmol injected on column.

Zhang et al. [27] developed a derivatisation method specific for N-terminal tyrosine-containing peptides. The method was used for the determination of opoid peptides, as each opoid peptide has a tyrosyl residue at the N-terminus of its amino acid sequence. The method involves post-column derivatisation with hydroxylamine, cobalt(II) ion and borate at 100°C. Opoid peptides, such as leucine enkephalin and methionine enkephalin, were determined with detection limits of 0.6-1.8 pmol.

4.3.3.3 Derivatisation with CE

When using derivatisation to improve detection with CE, many of the reagents used with HPLC are unsuitable. Firstly, with CE, postcolumn derivatisation cannot be performed as easily, due to the dimensions involved. Development of a postcolumn reactor which would not cause significant broadening due to connections is very difficult. Secondly the reagent must not hinder the separation. Thus attention must be given to the size to charge ratio of the derivatising reagent. In CZE the matching of the sample and the running buffer are critical. Therefore, this rules out a lot of present derivatisation techniques used with HPLC, which require the use of an organic solvent for pre-derivatisation. The micromanipulation involved in pre-derivatisation is extremely difficult when one is dealing with nanolitre sample quantities, and thus pre-derivatisation methods are prone to irreproducibility. For fluorescence detection, the excitation wavelengths of derivatising reagents should correspond with the output of available lasers.

Liu et al. [28] developed the reagent 3-(4-carboxybenzoyl)-2-quinlinecarboxaldehyde (CBQCA) especially for fluorometric determination of peptides by CE. The excitation spectrum of the derivatives formed with this reagent are coincident with the 442 nm output of the helium/cadmium laser. The derivatives exhibit optimum migration behaviour in CE due to the low hydrophobicity of the reagent. The stability of the derivative exceeds 24 hr. The optimum pH for reaction of small peptides is pH 8.5-9.5; for peptides of greater than ten amino acids there is no distinct optimum pH. The peptide glycine-glycine-tyrosine-arginine was determined in borate buffer at pH 9.5 with a detection limit of 13.8 attomole's.

Cobb and Novotny [29] developed selective reagents for the determination of arginine- and tyrosine- containing peptides. One utilised a selective reaction of benzoin with the guanidine moiety to derivatise arginine residues occurring in a peptide. Tyrosine residues are detected by formylation, followed by reaction with 4-methyl-1,2-phenylenediamine. A helium-cadmium laser used for detection is particularly suited to the determination of the arginine-containing peptides which have an excitation maximum close to 327 nm. A detection limit of 270 attomole's for arginine- containing peptides, and 390 attomole's for tyrosine- containing peptides was obtained. This type of derivatisation is very selective and allows abbreviated peptide maps to be obtained.

Postcolumn derivatisation has generally been avoided with CE, as the use of connecting devices (tubing, fittings etc.) would cause bandbroadening. Pentoney et al. [30] developed a low volume, on-column connector, constructed by drilling a hole (approximately 60 μm in diameter) through a 75 μm i.d. capillary with a laser. A stainless steel wire was inserted through the hole to act as a guide for two additional 75 μm i.d. fused capillaries that butt against the main capillary. The three capillaries are then glued together, and the wire removed. Hydrostatic pressure was used to introduce the reagent stream into the separation channel by raising the reagent reservoirs above the buffer reservoirs. Zone broadening by the connector was determined to be approximately 10%. The system was used for the determination of amino acids by derivatisation with OPA followed by fluorescent detection. A detection limit of 9×10^{-7} M (14 fmol injected) was obtained for L-histidine. For analytes that form stable adducts there was a factor of 3 decrease in sensitivity for on-column derivatisation versus pre-column derivatisation. However, greater sensitivity was achieved for analytes that react to produce unstable adducts.

4.3.3.4 Post-column derivatisation with copper

Post-column derivatisation with copper, followed by UV or electrochemical detection, has also been used [31-36]. The copper is used in the form of a reagent, the most popular of which is the Biuret reagent. Here the copper exists as copper sulphate, in the presence of sodium hydroxide to provide the high pH for reaction, and sodium tartrate for stabilisation of the copper. Peptides react with the copper to produce copper(II)-peptides which may be detected at 555 nm or by oxidation to the Cu(III) complexes at modest potentials [31]. Reaction must take place post-column due to the high pH necessary for complexation. Warner and Weber [31] used post-column complexation with the Biuret reaction at pH 9-10, for the electrochemical determination of peptides. Cu(II) forms complexes with amines and thus the method may suffer from interference. However, the electrochemistry was found to be reversible, and thus to improve selectivity, dual electrode amperometric detection was used. In this scheme an upstream anode oxidises the Cu(II)-peptides to Cu(III)-peptides, the Cu(III)-peptides are then transported downstream across the surface of a cathode and reduced back to copper(II)-peptides. Current at the cathode is measured. Of the amino acids tested using this method only histidine was found to interfere at the cathode because its electrochemical behaviour is reversible. Although tyrosine and tryptophan interfere at the anode, their interference at the cathode is minimal because their oxidation products undergo rapid chemical reactions to form species that are difficult to reduce. The anode was held at a potential of 0.80 V, and the cathode at 0 or 0.10 V and the method could detect tripeptides and longer. The detection limit for the tripeptide alanine-alanine-alanine was 12 nM or 0.25 pmol injected. By increasing the pH of the post-column Biuret reagent to pH 12, Tsai and Weber [32] showed that detection of dipeptides was possible with sensitivities an order of magnitude lower than that of tripeptides. With amine-specific reagents, peptides that do not contain lysine and that have a pyroglutamate residue on the amine terminus, or the amine terminus of which has been acetylated or formylated, will not react. Tsai and Weber found that by raising the pH to 12.1, it was possible to detect N-acetyl dipeptide amides by complexation with Cu(II). However it was not possible to determine N-carbobenzoxy dipeptides or N-carbobenzoxy dipeptide amides. Dipeptide amides were found to give the Biuret reaction at pH 9.2 and be oxidisable at an E^0 of 0.67 V. Dipeptides were found to be oxidisable at the higher potential of 0.90 V at pH 10. However, detection sensitivity was an order of magnitude lower.

The relatively slow reaction kinetics of formation of the electroactive Cu(II)-peptide complexes for larger peptides (six amino acids and greater) however requires a relatively high temperature and long reaction times. Bradykinin, for example, requires at least 2 min for reaction at 20.8 °C. Tsai and Weber [33] discovered that the pre-column incubation of bradykinin, Tyr-bradykinin and insulin A chain with Biuret reagent for 20 minutes at 60 °C leads to the formation of biuret complexes which can then be subjected to chromatography in acidic eluents. However, with acidic eluents, the post-column addition of basic copper tartrate was still necessary to achieve a signal. In flow injection experiments it was found that the sensitivities of bradykinin and tyr-bradykinin are greater than for trialanine, implying that more than one copper binds to each molecule of these peptides. A ZrO_2-polybutadiene (PBD) column, which is stable to base was also investigated. The mobile phase was 1.0 mM Cu^{2+}, 3.0 mM KNa tartrate, 50 mM Na_2HPO_4, and NaOH was added for adjusting the pH to 10.5. This was found to be successful; however,the PBD column caused fouling of the electrode.

For peptides containing tyrosine, unusual behaviour was observed by Tsai and Weber [34] when using dual electrode detection. At the anode, for a peptide containing tyrosine, both the tyrosine and the Cu(II) moieties will be oxidised, and thus tyrosine increases the anodic sensitivity. However, the cathodic sensitivity is decreased. It was discovered that the tyrosine moiety undergoes an initial oxidation at the anode, followed by a chemical reaction which yields a reductant capable of reducing the Cu^{3+} to Cu^{2+}.

Athanasiou-Malaki and Koupparis [35] used on-column derivatisation with copper, for the determination of amino acids. Instead of the direct reaction of amino acids with Cu(II) ions in solution, the amino acids were reacted with excess of copper(II) phosphate suspension, and the copper ions produced on acidifying the filtrate were then measured This allows the determination of a number of α-amino acids in the concentration range 5×10^{-4} to 1×10^{-2} M. As it does not depend on the α-amino acid forming a soluble copper complex, this method is more widely applicable.

4.3.4 Novel Electrode Materials

4.3.4.1 Electrochemical Detection with a Copper Electrode

When a copper electrode is polarised at a slightly positive potential in a buffer solution, different layers of copper oxide (or hydroxide) are formed. First a layer of copper(I) oxide is deposited on the copper surface; its thickness does not depend on the potential. The layer is extremely insoluble and is reduced only at fairly negative potentials. Because such potentials are not normally applied, the copper electrode can be regarded as covered with an inert layer of copper(I) oxide. At more positive potentials, copper (II) ions are formed, and these can diffuse through the copper(I) oxide layer. The solubility of the copper (II) ions in weakly acidic or alkaline buffers is limited. Therefore, if the electrode potential is increased, the copper(II) concentration at the electrode surface will increase only up to a certain value $[Cu^{2+}]_{max}$ at a potential E_{max}. Above this potential, a layer of copper(II) oxide or hydroxide will be deposited on top of the copper(I) oxide layer. A further increase in potential will only lead to a thicker oxide layer. In the presence of substances that form stable complexes with cupric ions, the solubility of the oxide film is enhanced, and thus the anodic current increases. A similar effect also occurs with other metals, e.g. mercury, cobalt and nickel, but these materials are less suitable. The oxidation of mercury produces free mercuric ions that are rapidly transported from the electrode surface and the oxidation of cobalt and nickel is slow and complicated. The oxidation of copper is relatively simple and rapid and cupric ions form stable complexes with a number of ligands. Stulik et al. [36] used a copper electrode for the determination of amino acids. The copper electrode was activated before measurement by polarisation for 5 minutes at -0.30 V, followed by polarisation for fifteen minutes at +0.15 V in the flowing mobile phase. Detection sensitivity increased with decreasing buffer concentration, with increasing pH above pH 6, and decreasing flow rate. Detection limits in the low nanogram range were achieved. The authors found that this method of detection would be most useful when used with anion exchangers and ammoniacal mobile phases, as unlike the use of chemically bonded phases, there was no discrepancy between the pH required for separation and detection. Kok et al. [37] found that the buffer used had a large effect on the electrode behaviour. The limiting current for carbonate buffers was considerably higher than phosphate buffers. This is because copper(II) ions form much stronger complexes with carbonate than with phosphate. For borate buffer, the limiting current was found to be lower than in phosphate buffer; however, the electrode kinetics were observed to be slower. The authors concluded that the different electrode behaviour is due to the oxide layers on the electrode surface; copper ions formed by the

electrode reaction must diffuse through the oxide (or hydroxide) layers and the permeability of these layers will therefore determine the rate of electrode kinetics. Thus participation of phosphate in film formation, but not borate explains the higher permeability of the oxide layer. Stulik et al. [38] also investigated the determination of peptides, and found that detection of dipeptides is most sensitive. The sensitivity decreases with increasing length of peptide and it was impossible to detect peptides longer than tetrapeptides. Detection limits for the dipeptides studied were in the range 5-25 ng while detection limits for tri- and tetrapeptides were in the range 50-250 ng. The peptides containing -S- or two carboxyl groups were detected with highest sensitivity. Dipeptides with protective terminal groups, e.g. Gly-Gly-Z, cannot be detected. Sensitivity could not be explained only on the basis of differing stability constants of the complexes formed, but primarily on the basis of differing complexation kinetics.

Engstrom-Silverman and Ewing [39] used a copper wire electrode for the determination of amino acids and dipeptides with CE. As the sensitivity of the copper electrode has been found to increase with a decrease in flow rate, this makes CE particularly suitable for detection with a copper electrode. However since the copper electrode detection principle relies on an adsorption-like process for solutes, copper(II) complexation peaks are often broader than typically observed in CE, and significant tailing is often observed particularly for the separation of cations. The separation and detection of four charged dipeptides, cationic Lys-Gly and Arg-Leu, and anionic Asp-Leu and Asp-Gly, gave theoretical plates values of 8,300, 10,300, 35,000 and 25,000 respectively. The low theoretical plate counts for the cationic peptides compared to the anionic peptides is probably a result of slower complexation kinetics for the peptides as a result of charge. Lys-Gly has the lowest number of theoretical plates of all the peptides, which is probably due to the cationic charge present on the peptide. A higher number of theoretical plates was obtained for the anionically charged peptides, which supports the observation that the copper electrode has faster complexation kinetics and a greater sensitivity towards anionic species. Detection limits ranged from 0.99 to 3.4 fmol. These detection limits correspond favourably with those observed for cationically charged dipeptides in a 9 μm capillary with indirect amperometric detection [40].

4.3.4.2 Detection at Mercury Amalgam Electrodes

The thiol moiety can be electrochemically determined using a mercury electrode. This is believed to happen by the oxidation of mercury in the presence of the thiol. The thiol groups

of cysteinyl residues are among the most reactive side chains of proteins, and undergo alterations that profoundly affect the structure and reactivity of proteins. Thiols form disulphide cross-linkages, and determining that disulphide cross-linkages have been formed is a common task in biotechnology. Although carbon electrodes can be used for the direct oxidation of thiols the potential needed is much higher than the potential needed at a mercury electrode. Allison and Shoup [41] used two mercury/gold (gold amalgam) electrodes in series arrangement with the reduction of the disulphide to thiol at the upstream electrode (-1.0 V), followed by thiol detection downstream (+0.15 V). The authors applied this to the separation and determination of glutathione in whole blood. with detection limits of 3.5 pmol for the reduced form of glutathione (GSH) and 5.7 pmol for the oxidised form of glutathione (GSSG), well below the normal μM quantities. Whether a peptide has a thiol or disulphide can only be determined by separate analysis, in which the detector is operated in the one and subsequently in the two-electrode modes. Sun et al. [42] developed an EC detector cell that includes three electrodes and thus can be used to distinguish between thiol- and disulphide-containing peptides in a single chromatographic analysis. Electrodes were mercury-silver amalgam. Although the sensitivity of the EC detector depends on the size and composition of the peptides 10-20 pmol of thiols and 20-30 pmol of disulphides could be detected. In this investigation a decapeptide was detected, showing that peptide size is not as critical to detection as with copper electrodes. No indication of the size limitation for detection of a peptide was given.

Wang et al. [43] compared UV detection with EC detection at both a copper and a hanging mercury drop electrode for detection of peptides separated by ion-exchange chromatography. UV detection was found to be approximately ten times more sensitive (1 ng) on column than electrochemical detection; the sensitivities of amperometric detection with a copper electrode and tensammetric detection being comparable. It was found that a hanging mercury drop could not be used as it rapidly becomes passivated and the measuring sensitivity and reproducibility are poor. However, a dropping mercury electrode operated between -0.36 to -0.40 V was satisfactory. The tensammetric response increases with increasing surface area of the mercury drop, with increasing drop time and with increasing amplitude of the applied alternating current (AC) voltage. The maximum response was obtained at a d.c. potential of -0.38 V, a drop time of 3 s and an AC voltage amplitude of 30 mV. A response was obtained above pH 3; however, the optimum pH for detection is pH 5-6 where the solutes are mostly uncharged which supports their adsorption at the electrode. The advantage of tensammetric detection over detection at a copper electrode is that the response does not depend on the length of the peptide chain for a series of glycine peptides.

4.3.4.3 Detection at Chemically Modified Electrodes

Chemically modified electrodes involve the use of surface bound redox mediators. These mediators catalyse the oxidation or reduction of specific solute species at substantially reduced potentials. Thus slow electron-transfer kinetics are improved and analytes which require an overpotential that is beyond that of solvent electrolysis can be detected.

Halbert and Baldwin [44] determined glutathione and cysteine in whole blood and plasma using an electrode modified with cobalt phtalocyanine (CoPC). The electrode was constructed by mixing CoPC into the graphite powder/nujol oil matrix used to fabricate conventional carbon paste electrodes. The blood samples were treated with a mixture containing EDTA and o-phosphoric acid, passed through a nylon filter and onto the column. However, the electrode response was found to decrease to between 70-80% of the initial current when sequential injections of 20 or more samples were made. This was thought to be due to leaching of the CoPC from the electrode during analysis. However, the electrode surface may be easily renewed in less than ten minutes. O'Shea and Lunte [45] adapted this idea for the EC detection of cysteine and glutathione by capillary electrophoresis. The electrodes were synthesised by the immobilisation of CoPC into a carbon paste matrix.

4.4 DETERMINATION OF PEPTIDES BY CAPILLARY ELECTROPHORESIS/ELECTROCHEMISTRY USING ON-COLUMN Cu(II) COMPLEXATION

4.4.1 General Considerations.

When developing a method of determination of peptides by CE, there are many factors which must be considered. As with all methods, sensitivity, selectivity, reproducibility and ease of performance are of paramount importance. Naturally, previously developed HPLC methods should be examined; however, direct transfer of methods between HPLC and CE is often not possible. This is due to the fact that the separation achieved by CE is dependent upon size charge differences, whereas with reversed-phase HPLC, analytes are separated on the basis of differences in hydrophobicity and polarity. Also CE places restrictions on method development which are not encountered with HPLC, in particular with regard to choice of pH, solvent, and ionic strength.

In capillary electrophoresis, a high voltage is applied across a buffer-filled fused silica capillary. Thus ionisable silanol groups are in contact with the buffer contained within the capillary. The negatively charged wall attracts positively charged ions from the buffer, creating an electrical double layer. When a voltage is applied across the capillary, cations in the diffuse portion of the double layer migrate in the direction of the cathode, carrying buffer with them. The result is a net flow of buffer solution in the direction of the negative electrode. This is called the electroosmotic flow (EOF) and makes possible the simultaneous analysis of cations, anions and neutral species in a single analysis.

Thus a CE separation is highly dependent on pH [46]. Silanols become progressively more protonated as the buffer pH is made more acidic, thereby reducing the charge on the wall and concomitantly the EOF. The flow rapidly decreases as the pH is reduced to pH 4.5, and then continues to decrease more slowly down to pH 1.5. The ideal condition for separation would be a slow EOF with a high charge to size ratio of the analyte; however, a slow EOF will mean longer analysis times. The pH must also be carefully chosen to avoid adsorption interactions of the analyte with the wall of the capillary, a major source of band broadening in CE [47]. This results in operating pH's for peptides and proteins usually being at low or high pH. At low pH much of the negative charge has been titrated off the silica walls of the capillary and there is very little coulombic interaction between the positively charged analytes and the negatively charged wall. Alternatively, at high pH, the solute acquires a

negative charge, and is thus repelled by the negatively charged wall. Intermediate pH values may be used depending on the isoelectric pH of the analytes. Much research is being focused on the development of wall coatings to overcome this problem.

Unlike HPLC, where a wide variety of solvents and solvent mixes may be used, with CE an aqueous buffer system must be used. The ionic strength of the buffer must be maintained low as Joule heating occurs and increases with increasing ionic strength, as a consequence of the resistance of the buffer to the flow of current. Ineffective dissipation of this heat through the walls of the capillary results in temperature gradients across the capillary which results in band broadening.

Thus it becomes obvious that CE, although a very powerful method of separation, has many restrictions when developing a method . Transfer of methods developed for HPLC analysis of peptides in many cases is not possible. However, HPLC studies have shown that the innate UV, fluorescent and electrochemical properties of peptides provide sensitive detection for a very limited range of peptides. Thus derivatising reagents which react with the analyte to produce an electrochemically active of fluorescent derivative have been developed. Another approach has involved the use of electrode materials, such as copper and mercury, and limited studies on chemically modified electrodes.

Many of the derivatising reagents developed to date are unsuitable for use with CE due to their hydrophobic nature. This necessitates derivatisation to be carried out in the presence of organic media which is unsuitable for use with CE. Derivatising reagents which use an organic solvent for prederivatisation are also unsuitable as the matching of the sample and running buffer are critical in CE. Also the addition of a large hydrophobic derivatising reagent to a charged peptide will not improve the charge to size ratio which is critical to attaining a separation. The interactions of the derivatising reagent with the walls of the silica capillary may also render it unsuitable.

In addition to the chemistry of derivatisation, the practical performance of the derivatisation must also be considered. When dealing with nanolitre sample quantities, manipulation of the sample to perform prederivatisation becomes difficult and is prone to irreproducibility. Post - column derivatisation has been performed [30], but has been shown to be only useful for unstable derivatives, as sensitivity for stable derivatives was shown to be one third that of pre-column derivatisation, due to the band broadening introduced.

4.4.2 Reasons for Developing a Method Involving On-Column Derivatisation with Copper

On-column derivatisation is the obvious solution to problems posed by pre- and post-column derivatisation with CE. The derivatising reagent should maintain the optimum size to charge ratio of the analyte. Thus it was decided to investigate on-column derivatisation of the analyte with copper. The HPLC method developed by Weber et al. [31] has been shown to have many inherent advantages. Copper will form a stable derivative, and the copper itself is not electroactive at the detection potentials used. Many derivatising reagents are amine specific reagents. Thus peptides that do not contain an amine functionality, i.e. due to acetylation or formylation, will not be detected. However, Tsai and Weber have shown [32] that N-acetylated dipeptides may be detected by complexation with copper. The method is general for peptides, i.e. it is not dependent on the presence of a particular amino acid for detection but will react with all peptides. However, while being general for peptides the method is selective over non-electroactive amino acids in the single electrode mode, and is selective over all amino acids with the exception of cysteine in the dual electrode mode. Also, nanomolar detection limits can be achieved.

However the present investigation sought to increase the capabilities of the method. Apart from the obvious increase in separation efficiency achieved by using CE, there are other advantages. The chemistry of the method is ideally suited to CE, and the properties of CE may allow an improvement in the range of peptides which may be determined by this method when compared to HPLC.

The simplicity of the method can be improved by use of on-column derivatisation with CE. Derivatisation with copper requires an alkaline pH, and thus with HPLC the derivatisation must be performed post-column. However, the silica capillary will withstand high pH, and thus derivatisation may be performed on-column. This will not only be more convenient but will also allow greater reaction time which may broaden the range of peptides which may be detected.

Detection of the peptide copper complexes may be achieved by UV or electrochemically. Electrochemical detection was chosen as it gives greater selectivity, and unlike UV detection, is not pathlength dependent. The slow flow rates of CE are an advantage when using electrochemical detection. The detection of large biomolecules with HPLC was found to be difficult, because the diffusion of the large biomolecules to the electrode surface where

electron transfer takes place is slow. With the HPLC method Tsai and Weber found that peptides of six amino acids and larger could not be determined [32,33]. Bradykinin for example takes two minutes to react at 20.8 °C. However, with CE, the slow flow rate and the high degree of contact of the analyte with the electrode, leads to a longer reaction time with the electrode and high coulometric efficiency. This will give lower mass detection limits and may broaden the range of peptides which may be detected.

4.4.3 EXPERIMENTAL

4.4.3.1 Reagents

All solutions were prepared in deionised water and filtered with a 0.2 mm Acrodisc filter (Gelman). Biuret reagent (0.6M NaOH, 12 mM $CuSO_4$, 31.89 mM tartaric acid, 30.1 mM KI) was obtained from Sigma (St. Louis, MO, USA). Di-, tri-, and tetraglycine, Arg-Asp-Ser, Arg-Gly-Glu-Ser and Pro-Leu-Gly-amide were obtained from Sigma; and di-, tri-, and tetraalanine and Ala-Gly-Gly were obtained from Research Plus (Bayonne, NJ, USA). Ultrapure sodium hydroxide was obtained from Aldrich (Milwaukee, WI, USA). 3-[Cyclohexylamino]-1-propanesulfonic acid (CAPS), 2-amino-2-methyl-1-propanol (AMP) and glycine were obtained from Sigma, and used to prepare buffer solutions.

4.4.3.2 Capillary electrophoresis system

Electrophoresis in the capillary was driven by a high-voltage d.c. (0-30kV) power supply (Glassman High Voltage, Whitehouse Station, NJ, USA). The anodic high-voltage end of the capillary was isolated in a Plexiglas box fitted with an interlock for operator safety. A digital microampere current meter was positioned between the platinum wire ground cathode and ground. Experiments were performed at ambient air temperature (24°C). Sample introduction was performed using pressure injection. For UV work a CV^4 absorbance detector was employed.

Fused silica capillaries with an I.D. of 50 μm and an O.D. of 360 μm were obtained from Polymicro Technologies (Phoenix, AZ, USA). A capillary cutter (Supelco, Bellfonte, PA, USA) was used to score the polyimide coating approximately 1.5 cm from the end of the capillary column. A 1 cm length of Nafion tubing (I.D. 0.33 mm, O.D. 0.51 mm) (Perma Pure Products, Tom's River, NJ, USA) was then carefully threaded over the score mark. Both ends of the Nafion tubing were sealed to the capillary tubing using 815 epoxy resin (Mid-Con Plastics, Wichita, KS, USA) with 20% (v/v) triethylenetetramine. This was cured overnight Once cured, gentle pressure was applied to either end of the Nafion tubing, causing the capillary to fracture at the score. The Nafion tube holds the capillary joint securely in place and ensures correct alignment. For additional support the joint was epoxied to a small section of glass. The nafion joint was manipulated through two openings in opposite sides of a plastic beaker and subsequently sealed in place with epoxy. The joint was immersed in buffer solution and this assembly served as the cathodic buffer reservoir. The

detection capillary section was then inserted into the electrochemical detection cell.

The electrochemical detection cell was operated in a three electrode configuration, with platinum wire auxilary and an Ag/AgCl reference electrode from a HPLC electrochemical detector. Carbon fibre microelectrodes were constructed by adhering a 33 μm carbon fibre (Avco Speciality Products, Lowell, MA, USA) to copper wire with silver epoxy. This was then placed in a pulled capillary, pulled with a List-Medical Model 3A vertical pipette puller (Medical Systems, Greenvale, NY, USA), such that the fibre protruded from the capillary. Epoxy adhesive (Loctite) was then used to seal the fibre in the capillary. Once cured, the copper wire was secured at the opposite end of the capillary with epoxy. The fibre was then cut to the desired length using surgical scissors.

The microelectrode was then mounted on an X-Y-Z micromanipulator (Newport, Fountain Valley, CA, USA) and positioned in the electrochemical detection cell. With the aid of an optical microscope, the microelectrode was aligned and inserted into the capillary column. Electrochemical connections were made to a BAS LC-4C (Bioanalytical Systems, West Lafayette, IN, USA) amperometric detector. The low currents generated at the microelectrode required the electrochemical cell to be shielded in a Faraday cage to reduce noise contributions from external sources. The detection cell contained 0.1 M NaCl as the electrolyte.

Separations were performed on a fused-silica capillary, 1.2 m in length (i.d. 50 mm, o.d. 375 mm) (Polymicro Technologies, Phoenix AZ, USA). Unless otherwise indicated, the separation voltage was 25 kV and the detector potential was +0.90 V vs a Ag/AgCl reference electrode. All washings were accomplished using positive pressure.

4.4.3.3 Cyclic Voltammetry

A solution of $1x10^{-2}$ M Gly-Gly-Gly was prepared in 50 mM NaOH, 1 mM $CuSO_4$, 2.6 mM tartaric acid and 2.5 mM KI and allowed to react for 10 min. This solution was then diluted 1:10 in 10 mM NaOH, pH 9.5. Cyclic voltammetry experiments were conducted in a three-electrode configuration using a Model CySy-1 computerised electrochemical analyser (Cypress Systems, lawrence, KS, USA). The scan rate was 10 mV/s. A carbon fibre working electrode, a Ag/AgCl reference and a platinum auxiliary electrode were used in all studies.

4.4.3.4 Electrochemical pretreatment

Two different electrochemical pretreatments of the carbon fibre microelectrode were investigated:

(i) application of a 50-Hz square-wave waveform of 2V amplitude for 1 min. This pretreatment was performed while the microelectrode was inserted in the capillary column and operating buffer was flowing past its surface;

(ii) anodization of the microelectrode in an electrochemical cell at +900 mV in a solution of 1 M NaOH. This pretreatment was carried out for 15 min.

4.4.3.5 Pre-column derivatisation

Pre-column derivatisation was first evaluated for the detection of peptides. In this case, the sample was dissolved in 10 mM borate buffer, pH 10.3. To 900 ml of sample was added 100 ml of a solution consisting of 120 mM $CuSO_4$, 319 mM tartaric acid, 301 mM KI and 6 mM NaOH. The reaction was allowed to proceed for a minimum of 10 min and injected by pressure injection. The run buffer was 10 mM borate, pH 10.3.

4.4.3.6 On-column derivatisation

In this case, the Cu(II) was added to the buffer system so that the complexation would occur during the separation. The run buffer consisted of 10 mM borate, pH 9.5, 1 mM $CuSO_4$ and 3 mM tartaric acid. Samples were injected by pressure injection and the separation voltage was then applied.

4.4.3.7 Derivatisation using a Cu(II)-saturated capillary

The capillary was precoated with Cu(II) prior to the separation in this procedure. A solution of 1M NaOH was flushed through the capillary for 5 min, 0.1 M NaOH for 5 min and then a solution of 2.4 mM NaOH, 48 mM $CuSO_4$, 120 mM potassium tartrate and 120 mM KI was flushed through the capillary for ten min. Finally a 2.5 mM NaOH solution was pushed through for 15 min. Separations were performed in the 2.5 mM NaOH run buffer.

4.4.4 RESULTS AND DISCUSSION

4.4.4.1 Electrochemical Pretreatment

Electrochemical pretreatment of a carbon fibre electrode is well known to have a pronounced effect on the electron transfer properties of many solution species; in particular, enhancement of the electrochemical response [48-50]. In this study, two pretreatment regimes were investigated. The first was the application of a high frequency 2 V potential window to the electrode; this was considered a severe pretreatment. A second, milder pretreatment was also investigated, which involved pre-anodization of the microelectrode in 1M NaOH for 15 min at +900 mV. This second approach was utilised in subsequent studies, as it was found to provide activation of the microelectrode comparable to that of the harsher pretreatment, but was simpler to apply. The pretreatment was found to be necessary only for new or unused microelectrodes. Once pretreated, the microelectrodes did not require further pretreatment for reactivation, i.e., oxidation of the Cu(II)-peptide complexes did not foul the surface of the electrodes. It is most probable that the pretreatment removed an initial polymeric layer which inhibited electron transfer on the surface of the carbon fibre [50]. Figure 4.1 shows a the cyclic voltammetric behaviour of 1 mM GGG-Cu(II) at the carbon fibre microelectrode.

4.4.4.2 Pre-column complexation of peptides with copper

Initial studies focused on pre-column complexation of peptides with the biuret reagent. Borate buffer, a typical buffer used at high pH for CE studies, was chosen at a typical concentration of 10 mM. In CE, it is important that the ionic strength of the sample and run buffer are matched to avoid band broadening. Thus the biuret reagent a detection limit of $4x10^{-5}$ M was achieved (signal to noise ratio = 3) for the peptide Ala-Gly-Gly. It is obvious that the concentration of copper here is a limiting factor, as the concentration of copper is only one quarter the sample concentration. Typically there should be a ten fold excess of copper to peptide, however the ionic strength of the sample at this copper concentration would be prohibitive to analysis by CE.

was diluted to give a final sample concentration of 12 mM $CuSO_4$, 31.89 mM $C_4H_4KNaO_6.4H_2O$, 30.1 mM KI and 0.6 mM NaOH in 10 mM borate buffer, pH 10.3. Samples were derivatised with this diluted biuret, allowed to react for ten minutes, and then

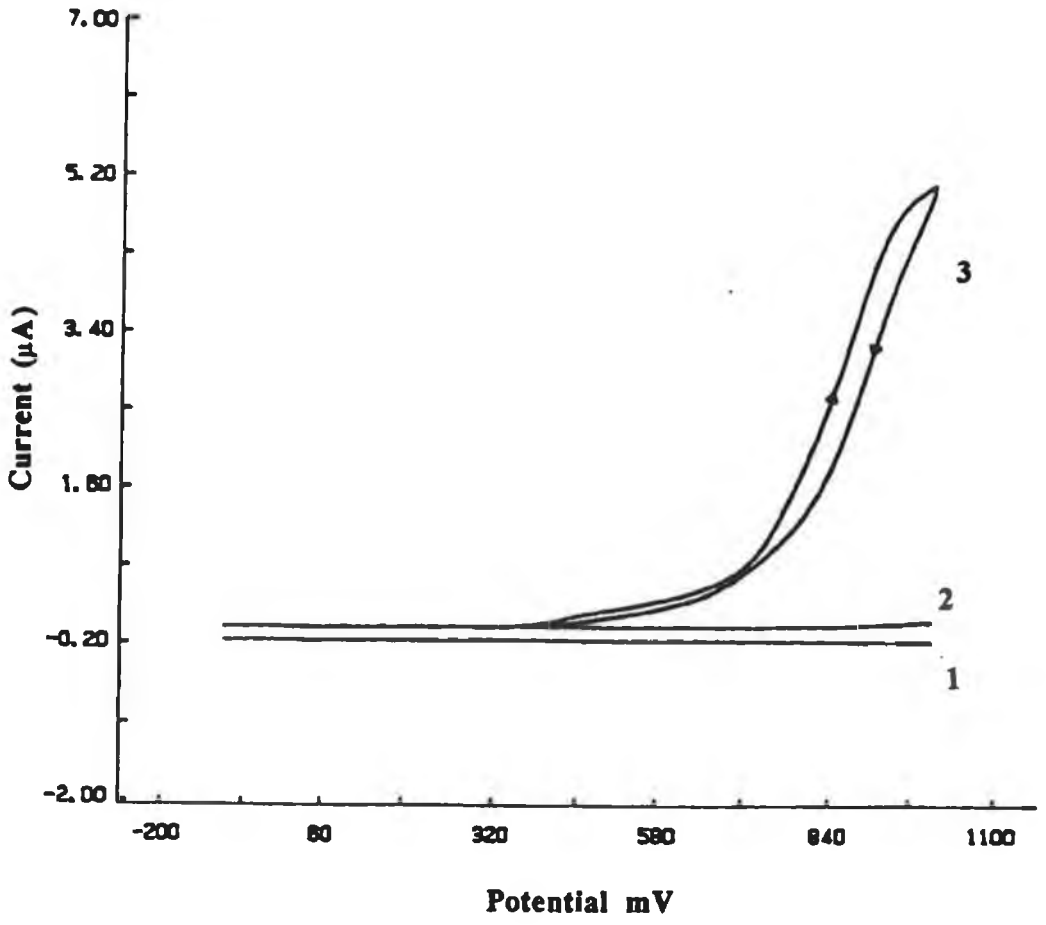


Figure 4.1. Cyclic voltammetric behaviour of 1 mM GGG-Cu(II) in 10 mM NaOH at a carbon fibre microelectrode. 1) Initial response to complex, 2) background electrolyte response after pretreatment of the electrode for 15 min in 1M NaOH with stirring 3) response to complex following electrochemical pretreatment. Scan rate 10 mV/s.

injected into the CE system where the run buffer was 10 mM borate pH 10.3. Using this method a detection limit of $4x10^{-5}$ M was achieved (signal to noise ratio = 3) for the peptide Ala-Gly-Gly. It is obvious that the concentration of copper here is a limiting factor, as the concentration of copper is only one quarter the sample concentration. Typically there should be a ten fold excess of copper to peptide, however the ionic strength of the sample at this copper concentration would be prohibitive to analysis by CE (however this was not investigated).

In an attempt to lower noise due to current produced in the capillary, zwitterionic buffers were investigated, including CAPS and AMP. However, it was found that when a buffer consisting of 10 mM CAPS (pH 10.3) was employed, the response for Cu(II)-Ala-Gly-Gly was reduced by 70%. AMP buffers produced high background after electrode pretreatment, which took >30 min to decay to baseline.

Thus prederivatisation had shown that detection of the peptide-copper complex was possible, however, the detection limit was limited by the ionic strength limitations of CE.

4.4.4.3 On-column complexation of peptides with copper

A buffer consisting of 10 mM borate, pH 9.5, 1 mM $CuSO_4$ and 3 mM potassium tartrate was investigated to find out if the peptides would form complexes at high pH in the absence of high concentrations of sodium hydroxide. However, this run buffer resulted in an extremely noisy baseline. The concentration of borate buffer was decreased to allow a higher concentration of biuret to be used than in pre-derivatisation studies. Detection limits of $1x10^{-4}$M for Ala-Gly-Gly (signal-to-noise ratio = 3) were obtained using a buffer consisting of 1 mM borate buffer, pH 10.0, 24 mM $CuSO_4$ 64 mM tartaric acid, 60 mM KI, and 1.2 mM in NaOH. However, with this buffer, there was a gradual increase in noise over time that was not eliminated by replacing the electrode with a new carbon fibre. Thus it was concluded that a component of the biuret reagent was building up at the Nafion joint, and hindering the flow of ions across the joint. It was noticed that the Nafion joint became blackened with use. Thus it was postulated that copper was depositing on the Nafion joint. This would occur as the Nafion contains sulphonate groups and perhaps the reaction was aided by the high negative potential at the joint.

4.4.4.4 Derivatisation using a Cu(II)-saturated capillary

The formation of a copper-coated capillary was then investigated. If a copper-coated capillary could be produced, then copper would be available for complexation with peptides without the need for it in the running buffer. This would also eliminate the need for prederivatisation. Coating of the column could be achieved by using a pressure injection system to push a solution containing copper through the column. While copper still comes in contact with the Nafion joint, in this case it is for a minimal period of time, without the presence of the applied voltage. Copper is thus available for complexation with the peptides, but does not accumulate at the Nafion joint.

The coating procedure described in the Experimental section produced a capillary which could run in 2.5 mM NaOH and was stable for >12h of continuous analysis. The column was coated on a daily basis and the electrode was left in the column during the coating procedure. Increasing the concentration of the Cu(II) solution used for washing did not result in an increase in response for the peptides tested. No response was obtained if the capillaries were not initially coated with Cu(II). Increasing the concentration of NaOH used to run the system to 10 mM decreased the migration time for the peptides, but also decreased the signal by 66%. However, NaOH was necessary to obtain the best signal. The use of other more conventional buffer systems such as borate, AMP, CAPS and glycine caused a decrease in response. Addition of 2.5% methanol or acetonitrile also caused a reduction in sensitivity.

Thus through this coating procedure, it is thought that copper becomes available for complexation and aids the electrochemical detection of the peptides. The interaction of copper with silica is well documented [51-53]. Silica consists of a network of joined SiO_4-tetrahedrons. At the surface of the silica capillary the oxygen atoms are only partially required to make up the Si-O-Si bridges. The rest of the oxygen atoms are free to bind ions from the solution it is in contact with. The Si^{4+} ion has a high electron affinity. This implies that the electron density around an oxygen atom bound to silicon is comparatively low. Thus the free electron pairs on the oxygen atoms are comparatively inactive and therefore the basicity relatively low. Hydrogen ions bound to a -Si-O^- group can therefore be replaced relatively easily by other positive ions. The basicity is in fact so low that even if one proton is bound with fair strength a second proton will not be taken up. Hence cation exchange is possible even at comparatively low pH, while anion exchange is not at all possible. In spite of this there are many reports of ions being strongly and even irreversibly held by forces in

addition to ionic attraction. This anomalous situation is not yet fully understood after half a century of research. Research has shown [52] that there are specifically bound ions which are held by other than ionic attraction forces. Covalent bonds may be formed between a metal cation and a silanol group. It seems to be a general rule that silica suspended in a solution of most polyvalent metal salts begins to adsorb metal ions when the pH is raised to within 1-2 pH units below the pH at which the polyvalent metal hydroxide is precipitated.

Schindler [51] studied the adsorption of copper at silica water interfaces using two silica samples - Silikagel H and Aerosil 200. It was discovered that above pH 4 copper is adsorbed on silica as $[(Si\text{-}O)Cu]^{+}$. As the pH increases, $[(Si\text{-}O)_2Cu]^{0}$ is formed, and above pH 7 all copper is adsorbed as this species. The authors showed that there was a correlation between the stability constants of the surface complexes and the corresponding values for simple hydroxo complexes. This correlation indicates that the ligand properties of the surface OH groups is not basically changed by the attached silica. surface. Moreover, it gives an explanation for the widespread observation of coincidence of adsorption and hydrolysis. The stability constant determined was as follows.

$$Cu^{2+} + 2SiOH \rightleftharpoons (Si\text{-}O)_2Cu + 2H+ \qquad \log \beta_2 = 11.19$$

When postulating a mechanism for coating of the column one has to consider that the column was flushed with diluted biuret reagent, which contains copper as the tartaric acid complex. Coordination with the tartrate anion can take place with the formation of two five membered rings, involving two weakly bound hydroxyl groups and one carboxyl group. The stability constant for this complex is $10^{4.5}$. However, at pH values exceeding 11, and in the presence of free caustic, tartaric acid becomes a very efficient sequestering agent At high pH it acts as a bidentate ligand with two tartaric acids molecules binding to one copper atom. The chelate structure is formed through the binding of strongly coordinating alkoxide groups, which gives the chelate a high degree of stability [54]. While a stability constant was not found for copper tartrate at high pH, it may be presumed that it is greater than $10^{4.5}$. While studies have shown that groups coordinated to a metal ion may be replaced by SiOH groups such studies all involved positively charged complexes [55,56]. However tartaric acid forms a negative complex with copper, there will be repulsion between it and the silica walls of the capillary. Thus this study shows that it is possible that the silanol group is able to abstract copper from a negatively charged tartaric complex. An interesting study which would help to elucidate the mechanism would involve the coating of the column with a copper solution that does not contain tartaric acid. Before tartaric acid was discovered to

stabilise the copper present in the Biuret reagent, high concentrations of NaOH (3%) were used to stabilise the copper. Thus coating of the column could be investigated using a copper solution 3% in NaOH.

To investigate the effect of coating the column with copper on the electroosmotic flow, a neutral solute - mesityl oxide was used. Mesityl oxide was injected and the time taken to reach the detector measured in a coated and uncoated column. Investigations showed that the coating procedure did not have any effect on the electroosmotic flow. This was contrary to expectations, as it was thought that the electroosmotic flow would be decreased due to a decrease in the negative charge of the capillary wall.

4.4.4.5 Separation of Peptides

A separation of di-, tri-, tetra- and pentaglycine is seen in Figure 4.2. A detection potential of +900 mV was chosen based on previous reports [31-34]. The best response was obtained for triglycine with a detection limit of $7x10^{-7}$ M. The response decreased with increasing size of the peptide. Detection limits for di-, tetra- and pentaglycine were 9.5×10^{-7} M, 1.6×10^{-6} M, and 5.5×10^{-6} M, respectively. As the stability constants for the complexation of copper with di-, tri-, and tetraglycine are log K =5.4, 5.5 and 5.4 respectively, the difference in sensitivity must be due to a difference in their electrochemical behaviour. For Gly-Gly-Gly, the response was linear from 1×10^{-4} to 5×10^{-6} M with a correlation coefficient of 0.998. The slope was 9 nA/mM. The responses for homogenous peptides of alanine were less than those for glycine. When a 50 mM solution of di-, tri-, and tetraalanine was injected, only the trialanine was detectable. This system can be employed for the detection of heterogeneous peptides as well. Figure 4.3 shows a separation of two peptides differing by a single amino acid, Arg-Gly-Asp-Ser (which supports fibroblast attachment and inhibits fibronectin binding to platelets; this is the target sequence for syphillis spirochete cytadherence), and Arg-Gly-Glu-Ser (an inhibitor of platelet aggregation).

Most methods of peptide analysis rely on the presence of a nucleophilic primary amine for derivative formation (8,57,58). The biuret reaction has an advantage over these methods, since it is selective for the peptide bond. Thus the determination of the amide protected Pro-Leu-Gly-amide was investigated. The detection limit of 2×10^{-6} M (signal-to-noise ratio = 3). The relative standard deviation for the reproducibility of detector response and migration time was 6.8% and 3.6% respectively (n=9). A comparison of electrochemical and UV

detection for the determination of Pro-Leu-Gly-amide is shown in Figure 4.4.

The interference from serum with electrochemical detection was, however, greater than expected. This is illustrated in Fig 4.1a and 4.1b where it can be seen that 2 x 10^{-6} M Pro-Leu-Gly-amide would be difficult to detect due to the background of the serum. The method developed by Weber et al [31] showed greater selectivity as two electrodes in series were used for detection. In their study the anode was held at 0.8 V and the cathode at 0.1 V. It was discovered that the reaction chemistry was reversible and thus a dual electrode system gave rise to greater sensitivity. This is not possible, however, with CE-EC at the present stage of development, as only one electrode may be used. However, in its present state, using only one electrode, from examination of Webers data [31], it can be seen that the selectivity for tripeptides using this method is on the order of 500 times better than for amino acids.

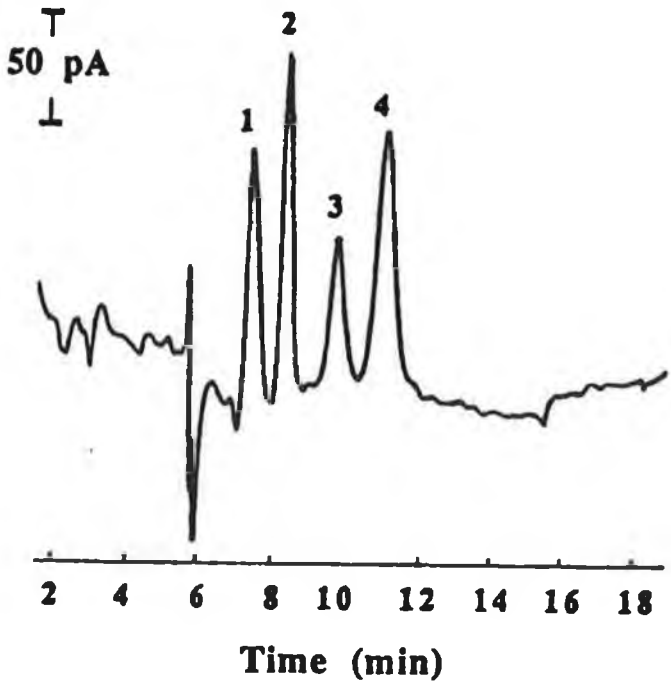


Figure 4.2. Separation of 9 x 10^{-6}M 1) di-, 2) tri-, 3) tetra- and 5 x 10^{-5}M 4) pentaglycine in 2.5 mM NaOH. Capillary 1.2 m, coated at 10 psi for 5 minutes in 1M NaOH, 5 minutes in 0.1M NaOH, 10 minutes in a solution 2.4 mM in NaOH, 48m M in $CuSO_4$, 120 mM in tartaric acid, 120 mM in KI, and finally 15 minutes in 2.5 mM NaOH. Applied potential 25 KV. Detection potential + 900 mV.

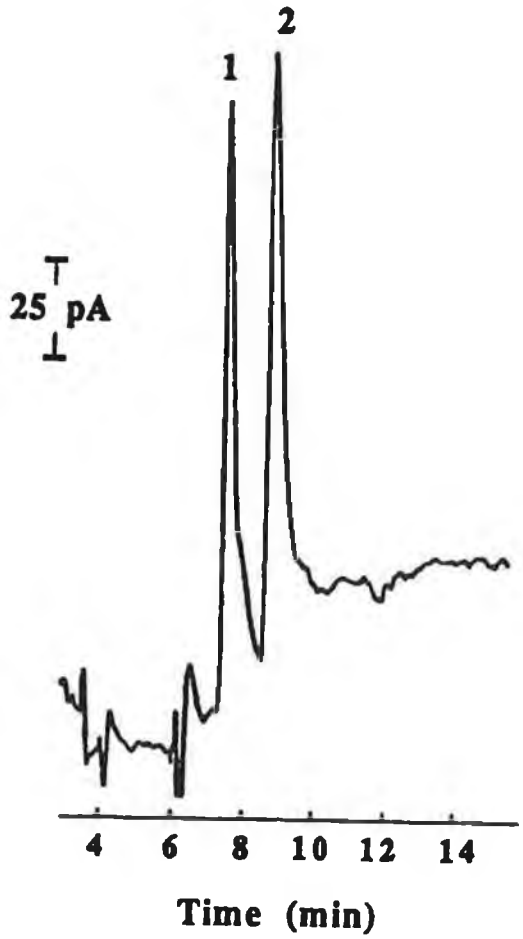


Figure 4.3. Separation of $1x10^{-5}$M 1) Arg-Gly-Asp-Ser and 2) Arg-Gly-Glu-Ser in 2.5 mM NaOH. Run conditions as in figure 2. Capillary 1.2 m, coated at 10 psi for 5 minutes in 1M NaOH, 5 minutes in 0.1M NaOH, 10 minutes in a solution 2.4 mM in NaOH, 48m M in $CuSO_4$, 120 mM in tartaric acid, 120 mM in KI, and finally 15 minutes in 2.5 mM NaOH. Applied potential 25 KV. Detection potential + 900 mV.

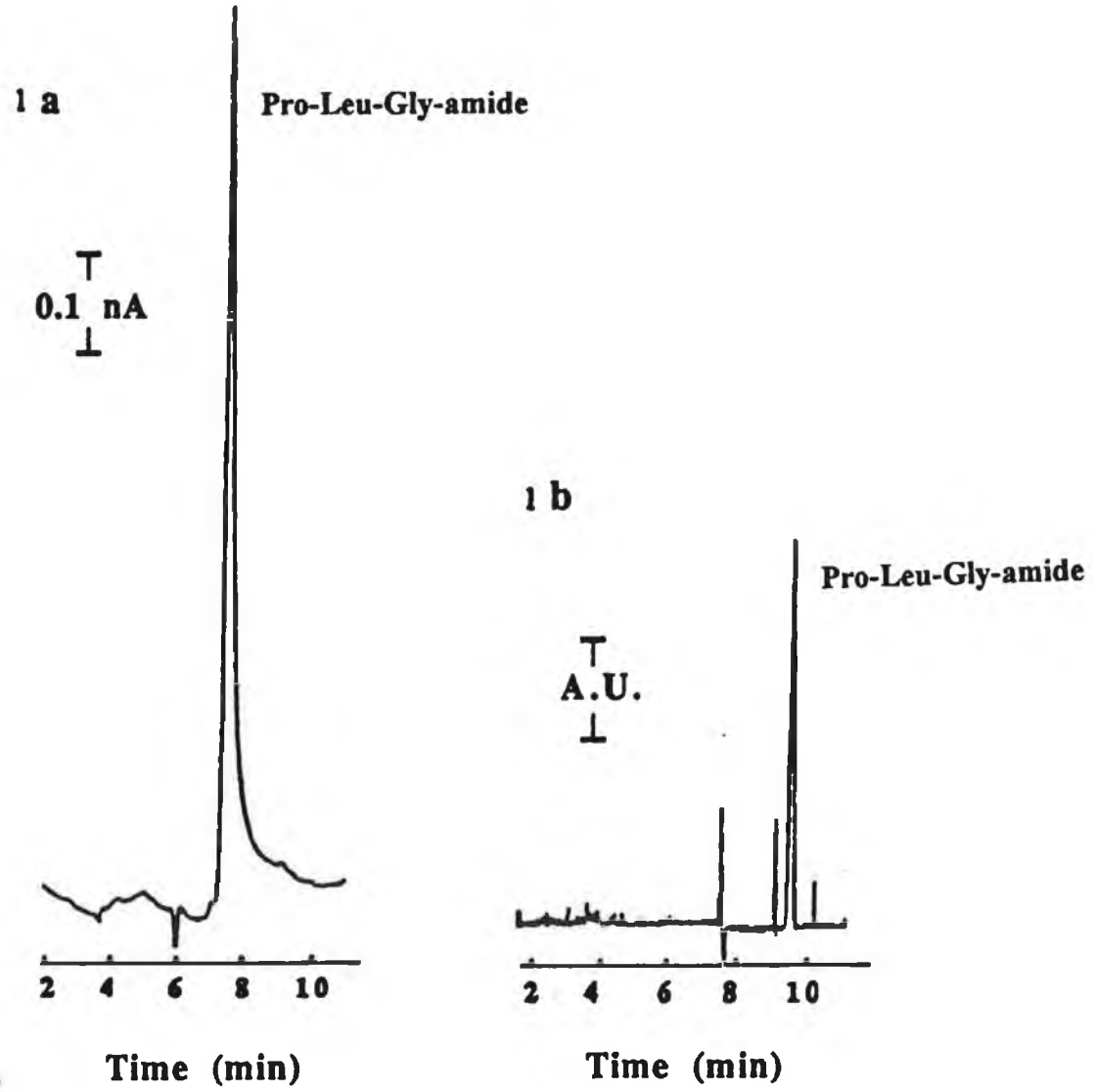
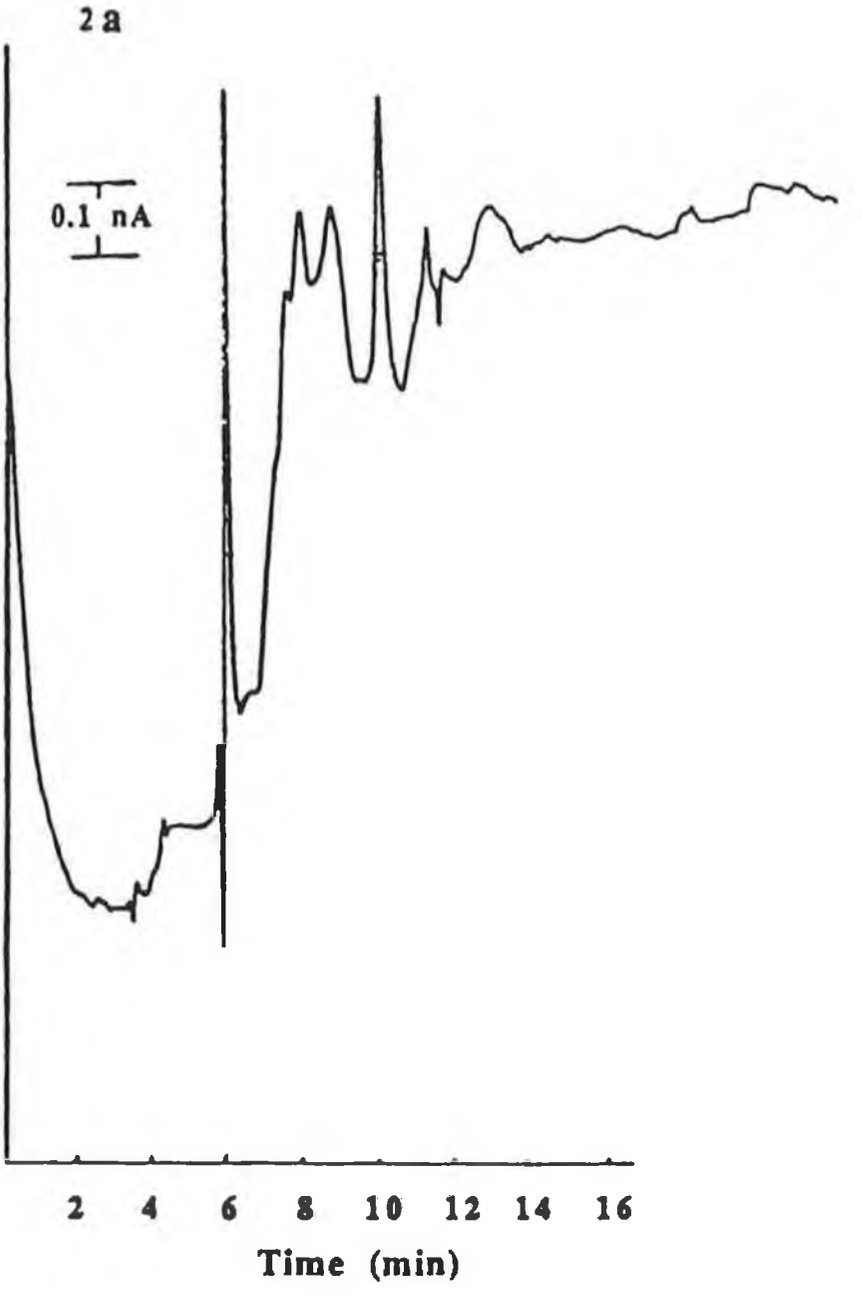


Figure 4.4. A comparison of UV and EC detection of $5x10^{-5}$M Pro-Leu-Gly-amide, 1a) detection EC, +900 mV. 1b) detection UV, 210 nm. 2) 1/100 dilution of serum containing $5x10^{-5}$M Pro-Leu-Gly-amide, 2a) detection EC, 900 mV. 2b) detection UV, 210 nm


2a
0.1 nA
2
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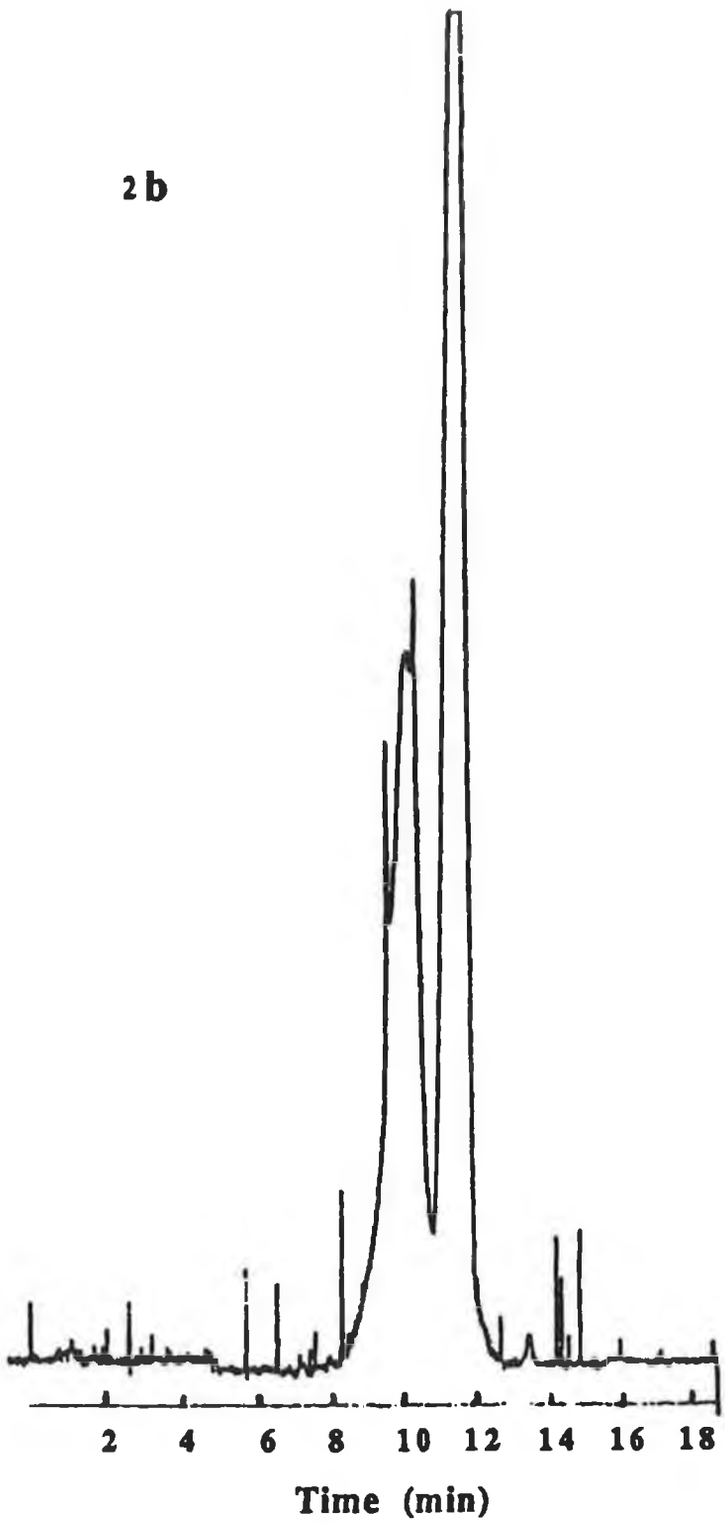
16
Time (min)


2b
2
4
6
8
10
12
14
16
18
Time (min)

4.4.5 Conclusion

In this Chapter it has been shown that by washing a silica capillary column with a copper solution, copper is made available for complexation with peptides. This provides a very simple method for the determination of peptides by CE where pre- and post-column derivatisation are not ideal options. The method shows a high separation efficiency, allowing a separation of homogeneous peptides differing by only one amino acid. Electrochemical detection was used, however it was shown to have no distinct advantage over UV detection with regard to sensitivity and selectivity. Investigations showed however, that the method can be used for the determination of N-terminally blocked peptides.

However, investigations using homogeneous peptides of alanine and glycine, show that sensitivity is best for tripeptides and decreases progressively for tetra- and penta- peptides. Thus contrary to expectations, detection was not improved by the longer reaction time and slower flow rate of the CE method. Detection limits of 7×10^{-7}M for triglycine are not as low as those achieved by HPLC of 1.2×10^{-8}M for trialanine. However the equipment used was home-made and detection limits may improve once commercial equipment becomes available. Similarly the selectivity of detection while better than with UV, can be improved by use of a dual electrode system which may be developed in future.

An investigation of the exact mechanism by which copper becomes available for complexation may bring about improvements to the method. At present the method is not flexible with regard to choice of running buffer which may limit separation and determination capabilities.

However the method under its present conditions, has been shown to be an extremely simple method for the determination of di-, tri-, tetra-, and penta-peptides and N-terminally blocked peptides.

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CHAPTER 5

THE FINAL ANALYSIS

5.1 THE FINAL ANALYSIS

5.1.1 Introduction

In this thesis three new applications of metal chelation in separation science have been developed.

The solid phase extraction method developed here for the analysis of adhesives is the first application which distinguishes between free and chelated metal. Unlike many application in literature where typical sample matrices are sea or fresh water, this application represents the analysis of free metal ion content in a complex organic matrix

In addition separation of FeEDTA, FeDTPA, FeHEEDTA, FeEDDHA and FeEDDHMA has never been achieved before. Also it is believed that the two peaks achieved for each of the chelates FeEDDHA and FeEDDHMA represent the diastereoisomers. Capabilities in analysis of fertilisers prior to the development of this technique was restricted to the identification of the chelating agent by TLC and determination of the total metal content. Methods existed for the determination of the quantity of metal chelated; however, the applicability of these to the present analysis has been challenge in section 3.2.2. This technique not only identifies the chelating agent present, but also can be used for quantification of the specific chelate content.

The coating of a capillary column with a metal ion to achieve on-column derivatisation is a new concept in capillary electrophoresis. The procedure developed for coating the capillary is extremely simple, as silica has an affinity for metal cations. The coated column was used for the amperometric detection of peptides. A separation of a series of peptide analogs, i.e. di-, tri-, tetra- and pentaglycine was achieved. This is a very difficult separation to achieve as each peptide contains the same amino acids and differs in size by just one amino acid. Thus the efficiency of the current technique is demonstrated.

5.2 Discussion of Scientific Approach and Further Studies

This thesis highlights the importance of the fact that metal chelation is an equilibrium reaction, and this should be considered when developing appropriate analytical techniques. While many applications exist for the determination of the quantity of free metal ion or the

quantity of chelating agent, this thesis has addressed the determination of the free metal ion, metal chelating agent and metal chelate content of the sample. The analyst must isolate the species of interest, i.e. the free metal ion, chelating agent, or chelate, without disruption of the metal-chelate equilibrium. Subsequent analytical manipulations will involve changes in pH, solvent and ionic strength. To obtain a meaningful result, the effect this will have on the isolated metal ion, chelating agent or chelate content must be accounted for by the use of standards. This is particularly important when analysing the specific chelate content. Attention to the potential for association and displacement reactions when a mixture of chelates are present is particularly important. Association and displacement reactions may make analysis of the specific chelate content of a sample containing a mixture of chelates impossible.

5.2.1 The Analysis of Transition Metal Ions in the Presence of their EDTA Chelates in Anaerobic Adhesives

The first application in this thesis involved the analysis of the free metal ion content of adhesives. A chelating agent such as EDTA is added to the adhesive to mask the presence of metal ion contamination. For the manufacturer it is important to know that all free metal ion has been chelated. The manufacturing procedures involve the addition of a large excess of EDTA to ensure that typical contamination levels are masked. The finished product is then analysed for total metal content to ensure that levels of contamination are within specifications. However, problems with this approach include the uncertainty that all free metal ion has been masked. For example, incomplete mixing of the product at manufacturing stage may lead to uneven levels of EDTA throughout the sample and subsequent high free metal ion content. Also, the formation constant of the chelate within this matrix is unknown and thus the efficiency of complexation is unknown. What is really needed therefore is a knowledge of the free metal ion content.

The method developed involved extracting the free metal ion by solid phase extraction, using its attraction for negatively charged silica, while the negatively charged chelate was not retained. This was confirmed by addition of excess EDTA to the sample, upon which no signal was obtained for metal ion. However, the question remains how can the analyst be sure that the metal-chelate equilibrium was not disturbed, and thus a true picture of the free metal ion content achieved. To the best of our knowledge this is the case. The only sample manipulation before abstraction involved the addition of a solubilising agent such as iso-octane or chloroform to the polymer matrix, which was necessary due to the high viscosity

of the finished product. However chelation has been shown to involve the displacement of the solvent molecule, where the solvent molecule has donor properties e.g. H_20 (section 1.2.2). The higher the donor strength of the solvent (i.e. the higher the stability of the solvate complex), the greater is the excess of ligand required for substitution of the solvent molecules situated in the first coordination sphere of the dissolved metal ion. It follows that with an increase in the donor strength of the solvent, the equilibrium stability's of the complexes in the solution decrease. The validity of the above correlation has been confirmed by many equilibrium studies, determinations of stability constants, and by data qualitatively reflecting the order of complex stability's, e.g., polarographic half-wave potentials, etc. [1,2]. Thus does the addition of chloroform or iso-octane to the adhesive change the equilibrium properties of the chelate?. This perhaps could be studied by polarographic measurements using a low viscosity methacrylate monomer, measuring the change in free metal ion in the presence of EDTA as chloroform or iso-octane is added.

Another future study would involve the analysis of the quantity of chelating agent. Thus a true picture of the behaviour of EDTA in adhesives could be achieved. Total chelating agent content could be achieved by conversion of the chelating agent content to one metal form. Conversion to the iron form should be investigated. Extraction could then be achieved with an anion exchanger followed by ion-pair chromatographic analysis.

5.2.2 The Analysis of the Micronutrient Content of Fertilisers

The second application of chelation chemistry involved the analysis of the micronutrient content of fertilisers. This work was initiated as a result of a new EC directive introduced to control the specifications of fertilisers. This directive dictates that there are two categories of fertiliser which may be marketed: fertilisers containing only one of the trace elements listed, and secondly fertilisers containing at least two different trace elements. To evaluate the trace element content of the fertiliser, the directive recommends that the total content in respect of each nutrient, the water soluble content, the chelated form in which the trace element is present, and the quantity of the trace element which is chelated must be determined. The investigation in this thesis was concerned with the latter two requirements.

An examination of the problem showed that it is not possible to determine the specific metal chelate content of a fertiliser containing a mixture of chelates using an analytical method such as chromatography (section 3.4.1). A method of separation for the desired metal

chelates was therefore developed. This method could be used only for chelates containing a single micronutrient in combination with a single chelating agent. A method of sample preparation must be developed involving the removal of the free metal ion before analysis. If this is not done then falsely high results may be obtained due to association during the chromatographic separation. The method of extraction developed for the analysis of the free metal ion content could be easily adapted for this application. Thus the liquid fertiliser should be prepared as for use e.g. in tap water, and then passed through a silica solid phase extraction cartridge.

However as discussed in section 3.4.1, it is my opinion that the analytical result obtained for the analysis of the specific chelate content of a fertiliser is only of interest from the point of view of manufacturing the product. It does not represent the value of the bioavailability of the micronutrient. Due to the competing reactions especially of H^+, Ca^{2+}, Mg^{2+} and Al^{3+}, the chelate content will change with changing soil conditions, especially pH. The laboratory result of the specific chelate content should thus be quoted as the maximum quantity available. A meaningful analysis with regard to micronutrient availability would perhaps involve the determination of the total quantity of chelating agent and the total quantity of micronutrients. These results could then be used, together with a model of the behaviour of metal chelates in soils, such as those developed by Norvell and Linsay [1,] to predict the effectiveness of the fertiliser under various conditions of usage.

Total chelating agent content could be determined by conversion of the chelating the chelating agent to the iron form at an optimum pH with removal of the remaining ions. The iron chelates can then be analysed by the developed method.

Confirmation of the separation of the isomers of EDDHA can be achieved by separation of an EDDHA standard by TLC using the method developed by Hill-Cottingham [2]. According to Barak the red band thus achieved is the dl-racemic isomer and the violet band is the meso-isomer. These bands can then be isolated and used as standards to identify the isomers.

5.2.3 Determination of Peptides by Capillary Electrophoresis/Electrochemistry using on-column complexation.

The third application involved the use of a copper-coated capillary for the determination of peptides. The use of copper complexation for the detection of peptides was not new, but has

been achieved by means of HPLC [3]. However, the HPLC method involves post-column derivatisation, due to the necessity for an alkaline pH. Thus the chemistry of this application was ideally suited for use with CZE, where the silica capillary used can withstand high pH. The derivatisation of the peptide with copper is ideally suited to separation by CZE. This is due to the fact that unlike other derivatisation techniques which involve the use of a large organic derivatisation reagent, the use of copper for derivatisation ensures that the size of the peptide is not excessively increased, thus minimising analysis times.

It is possible that the capillary may also be coated with other metal thus broadening the range of analytes which may be determined. The pH used in this application was very high. However this may not be necessary for other applications as adsorption of metal ion on silica takes place at lower pH's (Figure 2.8). Thus, for example, the determination of chelating agents by complexation with metal ions from the coated column could be achieved. For example determination of EDTA would be easily achieved by adsorption of Fe(III) to the column at pH 3. Direct injection of the sample would then ensure that at pH 3, Fe(III) would replace typical contaminants such as Cu(II) and Zn(II) and detection can be achieved photometrically.

The coating of the column also shows that analysis of free metal ions should not be attempted using a silica capillary due to adsorption of the metal ion on the capillary thus causing irreproducible results.

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