Some Analytical Studies

on

Sensors and Speciation

by

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of

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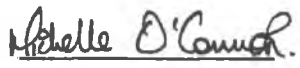
Declaration

I hereby declare that the contents of this thesis, except where otherwise stated, are based entirely on my own work, which was carried out in the School of Chemical Sciences, Dublin City University, Dublin and in New Mexico State University, Las Cruces, New Mexico, USA.



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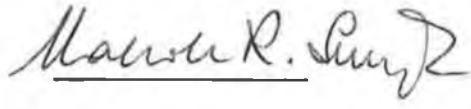

Malcolm R. Smyth

(Supervisor)

To my mother, father and brother

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Abstract

Some Analytical Studies on Sensors and Speciation

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This thesis is divided into two parts. Part A describes the development of some biosensors based on the immobilisation of enzymes, tissue, microbes and plant materials in carbon paste. The performance characteristics of these biocatalytic devices were investigated in both batch and flowing systems for the detection of neurotransmitters, mono-phenols and glucose using amperometry. A new immobilisation procedure involving the use of spectroscopic graphite and silicone grease is also described. Anodic Stripping Voltammetry was employed to illustrate the detection of Pb(II) and Cu(II) at lichen-modified carbon paste electrodes.

Continuing the theme of metal analysis in Part B, comparison of chromatographic and spectrometric methods is described for the detection of Fe(III) and Al(III) in soils and clays. Information regarding speciation of these metals within the matrices of soils and clays was obtained. Subsequently, a chromatographic method for the separation of Fe(II) and Fe(III) was investigated involving the use of an ion-pairing reagent.

PART A

BIOSENSORS

CHAPTER 1

THEORY, INSTRUMENTATION AND APPLICATIONS OF BIOSENSORS

1.1. Introduction

The use of immobilised enzymes and intact cells is a vast and rapidly growing area in the field of biosensors. Such systems have found many analytical applications, particularly in the field of clinical analysis. It is an area which has seen a major growth over the past decade manifested in the number of publications which have appeared on the subject to date. As such, it is beyond the scope of this thesis to delve too much into a detailed discussion on biosensors. This chapter is intended as an introduction to the area of electrochemical biosensors in connection with the four chapters which follow, and will include discussions on : the use of enzymes, tissues and microbes as sensors, methods of detection (including amperometry and anodic stripping voltammetry) and also a short discussion on enzyme kinetics.

1.2. Electrochemical Biosensors

The definition of a "biosensor" is somewhat ambiguous, essentially consisting of "a biocatalyst which is held in close contact with a transducer" (e.g. carbon paste electrode). A more formal description would be "a device which makes use of some biochemical recognition property as a basis for selective detection". Traditionally, electrode based

biosensors have made use of immobilised enzymes, but new developments have led to the use of intact microorganisms [1-5], biological tissues [6-13], antigens or antibodies [14-18] and other molecular recognition elements. The first enzyme electrode was introduced in 1962, and since then a wide variety of electrodes have been developed for the analysis of various compounds (Table 1.1).

Biosensors have several advantages which make them particularly suited for analytical applications. These include simple and low cost instrumentation, fast response times, minimum sample pretreatment, high sample throughput, and most importantly selectivity of response.

There are, however, many problems associated with electrochemical biosensors which do not allow their use in routine methods of analysis, in particular the working lifetime of the electrode, typically determined by the stability of the biological component of the biosensor. However, despite the fact that real-world applications of most of these biosensors have been delayed by such disadvantages as fragility, reproducibility and stability, several examples of commercially available biosensors are shown in Table 1.2.

Enzyme immobilisation was originally carried out for economic reasons. Isolated or purified enzymes are often expensive, so that analytical procedures which do not reuse enzymes can be prohibitive in cost. The

use of an enzyme electrode not only concentrates the biocatlaytic activity in the area of the sensing device, but also provides the possibility of making reagentless probes.

Table 1.1. A selection of enzyme electrodes

ANALYTE	IMMOBILISED CATALYST	REFERENCE
Ascorbate	L-Ascorbate oxidase	[20]
Bilirubin	GOD + PO : membrane	[21]
Cholesterol	Cholesterol oxidase Cholesterol esterase	[22]
Creatinine	Creatinase	[23]
Ethanol	ADH, HRP	[24]
Glucose	GOD	[25]
Lactate	Lactate dehydrogenase	[26]
Urea	Urease	[27]
Uric acid	Uricase	[28]

Abbreviations:

GOD: glucose oxidase, PO: peroxidase, ADH: alcohol dehydrogenase, HRP: horseradish peroxidase.

Table 1.2. A selection of commercially available biosensors

MANUFACTURERS	ANALYTE
Analytical Instruments Co. Toyo Jozo, Japan	Glucose, alcohol glycerol, lactate
Daiichi, Kagaku, Japan	Glucose, alcohol, acetic acid
Gambro,Sweden	Glucose
Genetics International	Glucose
Midwest Research Institute	Pesticides
Oriental Electric Co., Japan	Fish freshness
University of Lund, Sweden	Sugars, alcohol, penicillin, urea, oxalate, lactate
Yellow Springs Instruments Co., USA	Glucose, lactate, alcohol, sucrose

1.3. Tissue-Based Biocatalytic Electrodes

During the 1960's and early 1970's, several biosensors were developed using the basic principle of coupling isolated or purified enzymes to electrochemical transducers [29]. In the mid 1970's,

microbiologists showed that whole viable cells could be immobilised [30].

The use of whole cells as opposed to purified enzymes has important advantages such as stabilising the biocatalytic activity [31, 32], increasing the amount of biocatalytic activity [33], simplifying the electrode fabrication [34] and eliminating the need for additional expensive cofactors [35]. Because of these advantages, the use of subcellular fractions, bacterial cells and tissue sections as biocatalysts (see Table 1.3) has greatly increased the range of compounds for which biocatalytic electrodes membrane can be developed.

Table 1.3 Tissue-based Biosensors

SUBSTRATE	TISSUE	SENSOR	REFERENCE
Glutamine	porcine kidney	NH_3	[32]
Adenosine	mouse small intestine	NH_3	[37]
Glutamate	yellow squash	CO_2	[38]
Guanine	rabbit liver	NH_3	[39]
Pyruvate	corn kernels	CO_2	[40]
Cysteine	cucumber leaves	NH_3	[41]
Dopamine	banana pulp	O_2	[42]
Tyrosine	sugar beet	O_2	[41]
Phosphate/ fluoride	potato / glucose oxidase	O_2	[43, 44]

A particular enzyme may be more stable in its

natural environment, and indeed some enzymes lose their activity if isolated or purified. Required cofactors or multi-step reactants may already be present in the cell and do not need to be immobilised separately. The cost of whole cells may be considerably less than that of the purified enzyme. On the other hand, whole cells may not possess the selectivity of the purified enzyme.

The use of animal tissue slices as biocatalysts retains all the positive features shown by microorganisms and has the added advantage of structural integrity and mechanical ruggedness. Moreover, biochemical knowledge of mammalian metabolic properties of such tissues can often be sufficiently predicted in advance to permit the proper tissue to be selected for specific applications.

Plant tissues were generally thought to have slow metabolic rates and less bioactivity than animal tissues, hence would be less effective as biocatalysts. In fact, the opposite was shown to be the case where tissues taken from the growing parts of plants or from fruit and vegetables that store nutrients were shown to have a high level of bioactivity [12, 30].

In some cases the enzyme activity is found to be concentrated in certain areas of the plant [13]. Wang et al. [36] recently developed a scanning electrochemical microscopic technique for monitoring biological processes. This technique was used to

ascertain the distribution of biocatalytic activity. The use of natural materials as biocatalytic layers in biosensors was received into the scientific world with much scepticism. However, over the last decade, numerous results have allayed such doubts and shown that natural materials in certain cases can be more advantageous than the use of isolated enzymes.

A comparison of sensors utilising enzymes, mitochondria and bacterial and tissue slices carried out by Rechnitz et al. [34] showed these materials to be favourable over their purified enzymatic counterparts. Very often, the immobilisation procedure is simplified when using a natural material as opposed to the isolated enzyme.

A simple physical confinement of the biocatalyst between two membranes or a single membrane may be all that is required compared to cross-linking / covalent attachment of the pure enzyme to a support. This in turn means that the cost of making such a sensor is considerably reduced by virtue of the fact that it is not necessary to use expensive purified enzyme.

One problem of using this type of biocatalytic sensor in the presence of other enzymes in the tissue. Thus selectivity is not as good as for the pure enzyme. This is where a combination of liquid chromatography and electrochemical detection using these biosensors may be used to advantage since interfering analytes may be separated prior to detection.

1.4. Carbon Paste Electrodes

There are many different types of graphite electrodes used at present in voltammetric and amperometric detector cells as working electrode materials. They may be classified as follows :

* paraffin or wax impregnated graphite;
* epoxy resin impregnated graphite;
* graphite embedded into silicone rubber;
* graphite paste;
* glassy carbon;
* pyrolytic graphite;
* carbon fibre;
* reticulated vitreous carbon; and
* carbon foil.

Carbon paste was the electrode material of choice in the work reported in this thesis, except in Chapter 3, where the use of spectroscopic graphite is discussed. Carbon paste is a mixture of graphite powder with an inert binding material such as paraffin oil, ceresin wax, silicone grease or mineral oil (e.g. Nujol). These electrode materials show high reproducibility, are easy to prepare and maintain, and have good anodic potential ranges with small background (residual) currents. One disadvantage of these materials is that they can disintegrate in non-aqueous solvents. A carbon paste working electrode

needs to be repacked from time to time, and generally a batch of paste is prepared to minimise differences from one surface to another. The lifetime of the electrode is dependent to a large degree on the mobile phase and the applied potentials. For example, whenever a large potential (> +2.0 V) is applied to the electrode, the surface is rapidly destroyed.

Fouling of the electrode surface may also occur where components of the mobile phase or products of the electrode reactions adsorb onto the surface. Packing of the paste is important to ensure as smooth a surface as possible, in order to obtain reproducible results. Smoothing is usually carried out on a deck of computer cards or weighing paper.

1.4.1. Construction / Preparation of Enzyme Electrodes

The construction of each individual electrode for the systems investigated in this thesis is described under Experimental sections; however, some important points to note when preparing an enzyme electrode are given below.

A base sensor electrode is chosen which is suitable for the enzyme reaction being studied, e.g. whether detection is amperometric or potentiometric. Generally either reactant or product need to be electroactive; if not, the use of a mediator should be considered. Detection may be monitored by either consumption or

production of a particular species involved in the reaction at the electrode surface. A suitable enzyme is selected taking into consideration its specificity, selectivity, adaptability to immobilisation, availability, and of course cost (purified enzyme versus tissue-containing enzyme). Some enzymes may be used in the impure state e.g. jack bean urease or glucose oxidase for the food industry (General Mills), or in its natural state e.g. polyphenol oxidase in banana or tyrosinase in mushroom. In certain cases, it may be possible to purify the enzyme. During the working lifetime of the enzyme electrode, it should be stored in a buffer solution (type, pH and concentration appropriate for the enzyme being used) in a refrigerator.

1.4.2. The Use of Mediators

The use of oxido-reductase enzymes such as polyphenol oxidase, tyrosinase and alcohol dehydrogenase is particularly suited to mediated amperometric biosensors, since they readily donate electrons to electrochemically active artificial electron acceptors. A mediator must show a number of characteristics to be suitable for such systems. It should :

(i) readily participate in redox reactions with both the biocomponent and electrode, effecting

rapid electron transfer;

(ii) be stable under the experimental conditions;

(iii) undergo no side reactions;

(iv) have a suitable redox potential such that it does not interfere with other electrochemically active species;

(v) be unaffected by a wide range of pH;

(vi) not affect the bioactivity of the immobilised enzyme; and

(vii) preferably be recyclable.

Examples of mediators include :

* ferrocene and its derivatives [45, 46];
* ferricyanide [47];
* N,N,N',N'-tetramethyl-4-phenylenediamine [48];
* benzoquinone [49];
* tetrathiafulvalene [50];
* nicotinamide adenonine dinucleotide [47];
* tetracyanoquinodimethane [51].

1.5. Enzyme Kinetics

1.5.1. Introduction to Enzyme Kinetics

This section gives a short discussion on enzyme kinetics and different parameters that affect the activity of the immobilised enzyme. The primary function of enzymes is the catalysis of chemical reactions. Being susceptible to conditions such as pH

and / or temperature, they have a tendancy to undergo denaturation and inactivation. Hence parameters that require optimisation when using enzyme-based sensors include pH, compatability with mobile phase / carrier solution, and percentage enzyme loading in the carbon paste.

1.5.2. Theory

Enzyme kinetics are based on a theory put forward by Michaelis and Menten [52] in 1953, which suggests that the enzyme (E) first forms a complex with the substrate (S). This complex (ES) subsequently breaks down giving the free enzyme and products (P) according to reaction 2.

$$\text{Reaction 1 : } E + S \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} ES$$

$$\text{Reaction 2 : } ES \overset{k_{+2}}{\rightleftharpoons} E + P$$

where k_{+1}, k_{-1} and k_{+2} represent the rates of reaction for the individual reactions. This theory was based on two important assumptions :

(i) The effect of reaction 2 on reaction 1 is assumed to be negligible i.e. the reaction between the enzyme and its substrate remains in

equilibrium and is not affected by the rate of breakdown of the enzyme-substrate complex to give the enzyme and products;

(ii) the concentration of free substrate remains essentially constant during the initial period of the reaction and the concentration of free substrate (S) can be taken as being equal to the total substrate concentration. This holds true when the total substrate concentration is much greater than the total enzyme concentration.

At a fixed enzyme concentration, the velocity of the enzyme catalysed reaction (V) is given by :

$$V = V_m S/(K_m + S) \quad \ldots\ldots\ldots (1)$$

where K_m is the Michaelis-Menten constant (which is the concentration of substrate at which half the maximal reaction rate is observed) and V_m is the maximum velocity obtained when the enzyme is saturated with substrate.

The response at an enzyme-modified electrode is dependent on a number of parameters, e.g. pH, temperature, inhibitors and the concentration of the substrate. It will also depend on the thickness of the immobilised membrane (if one is used) in which the reaction takes place. If a membrane is involved in the immobilisation of the enzyme or tissue, the time

required to reach a steady state response is strongly dependent on the membrane thickness. This is due to the effect this has on the rate of diffusion of the substrate through the membrane to the electrode surface. Work has been done to investigate the effects of membrane thickness on the electrode response [53].

When an enzyme is immobilised, one generally observes an increase in K_m. This increase is usually related to the charge on the substrate and / or carrier, diffusion effects, and in some cases, tertiary changes in enzyme configuration. However, in some cases no change in K_m is observed. The effects of immobilisation are discussed in Chapter 3.

1.5.2.1. Effect of Substrate Concentration

Increasing the substrate concentration in an enzyme catalysed reaction increases the rate of reaction as seen in Figure 1.1. It is not uncommon to find that, while the Michaelis-Menten equation is obeyed at lower substrate concentrations, the velocity falls off again at high concentrations. Possible causes for this effect are (i) the formation of ineffective enzyme-substrate complexes and (ii) since all enzymes act in aqueous media, a very high substrate concentration will imply a reduction in the concentration of water, which may lower the velocity, especially if one if the reactants is water. All response curves level off at high substrate

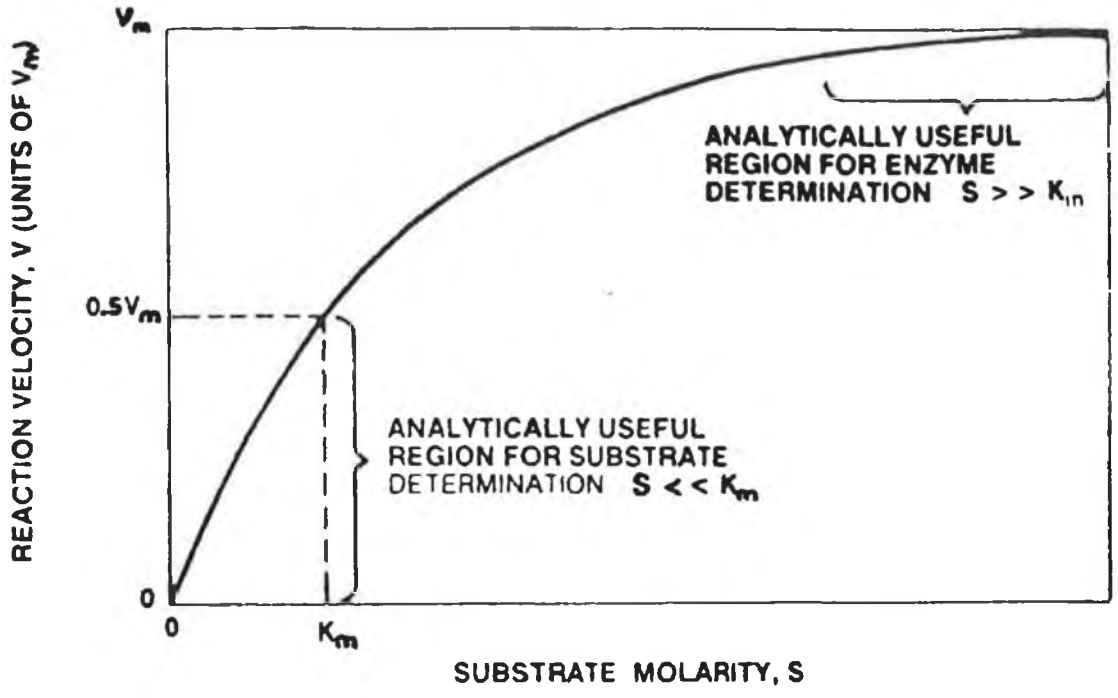

Figure 1.1. Dependence of the velocity of an enzyme-catalysed reaction upon substrate concentration (at a constant level of enzyme activity).

concentrations as predicted by the Michaelis-Menten equation, which in effect states that the reaction rate becomes independent of substrate at high substrate concentrations.

1.5.2.2. Effect of pH

In general, enzymes are only active over a limited range of pH and in most cases a definite optimum pH is observed. Like all pH effects, the effect on enzymes is due to changes in the state of ionisation of the components of the system as the pH changes, whether this be on the free enzyme or substrate or the enzyme-substrate complex.

The immobilised enzyme will have a different pH range from the range of the soluble enzyme because of its changed environment. Generally, a compromise is necessary between the pH at which the sensor reaction is optimum and that at which the enzyme reaction proceeds with maximum efficiency. It is important not to force the enzyme to conform with the requirements of the sensor otherwise the enzyme will lose activity if not within its optimum pH range [54, 55].

1.5.2.3. Effect of Temperature

The effect of increasing temperature is to increase the reaction rate which results in a faster response. In some cases no great improvement in response is

observed for increasing temperature [56, 57], so measurements are usually made at room temperature or $25^{o}C$.

1.6. Flow Injection Analysis

Flow injection analysis (FIA) is based on injection of a sample into a moving non-segmented carrier stream of a reagent or a mobile phase containing a reagent. The injected sample zone disperses on its way to the detector. A simple FIA system (see Figure 1.2) consists of a pump (P) or gravity feed reservoir that propels the carrier stream (R) via an injection port (S) through which a well defined volume of sample solution (usually by means of a sample loop) is injected. The sample zone is then dispersed (and reacts with components of the carrier stream where relevant) in a coil and is detected downstream at a flow through detector.

FIA is typified by sharp peaks, the heights of which are related to the analyte concentration. The dispersion coefficient, D, is the ratio of the concentration of sample solution before (C^{o}) and after (C^{max}) the dispersion process has taken place [58] (see Figure 1.3). Response times for these systems are usually less than 30 s (depending on the biocatalyst) which makes them ideal for high sample throughput.

Because of this rapid response characteristic, its

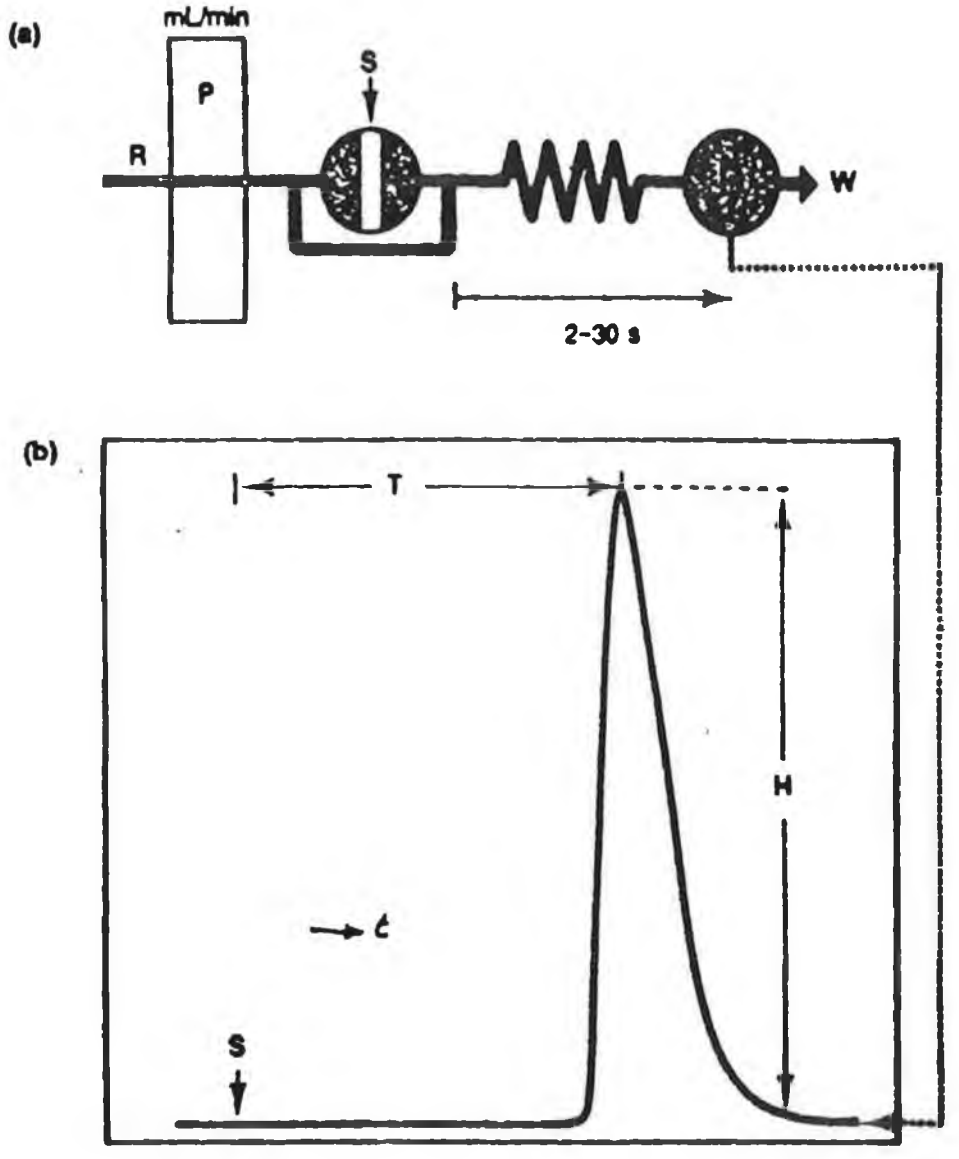

Figure 1.2. (a). The components of the simplest FIA system :R, the carrier stream of reagent; P, pump; S, injection port; D, detector; and W, waste. (b). Typical recording showing one sampling cycle, commencing at the point of injection (S) and ending when the sample zone has passed into the waste (W). The readout, peak height (H), is available within 30 s (T) after sample has been injected.

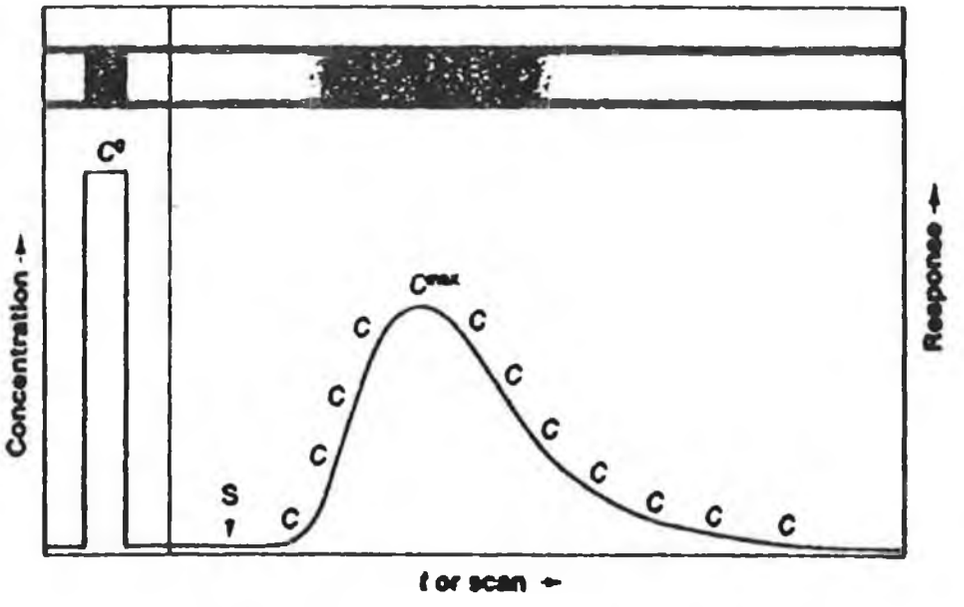

Figure 1.3. FIA gradient techniques. While the zone prior to injection (left) is homogeneous and will therefore yield a square readout, the dispersed zone (right) is composed of a continuum of changing concentrations.

versatility and ability to handle microlitre volumes, FIA has proven itself an ideal technique for applications involving biosensors.

Along with the selectivity of biocatalysts for specific substrates, this system is particularly suited to analytical problems such as those found in clinical, pharmaceutical, agricultural and environmental situations. It offers the selectivity of enzymatic reactions with the economy and stability gained by immobilising the often costly catalysts and, applied in the FIA mode, they ensure strict repeatability. Also, when a high concentration of enzyme is immobilised within a small volume, the ensuing high activity facilitates extensive and rapid conversion of substrate at minimum dilution of sample.

Aside from detection, FIA is dependent on three factors - sample injection, sample zone dispersion and reproducible timing of the movement of the sample zone from the injection port into the detector. Because of the very short sampling cycle, there is only one sample zone in the FIA flow channel at any one time. The shape of the dispersion zone depends on the geometry of the flow channel and the flow velocity and reflects a continuum of concentrations.

The reproducibility of peaks is excellent since the zone disperses in an identical manner on each injection. The theoretical description of the time-dependent concentration profile of analytes reagents and products under these flow condition is

still under debate. One approach is that of Van der Slice [59], in which the flow is assumed to be completely laminar and the dispersion of a sample zone is based on numerical solutions of the diffusion - convection equations in the regions in which FIA systems are usually operated.

The two key parameters predicted by Van der Slice are, the time from injection to the initial appearance of the sample zone at the detector (t_a) and the baseline to baseline time (Δt_b) for each sample zone at the detector (see Figure 1.4), where:

$$t_a = 109\ a^2\ D^{0.025}\ (L/q)^{1.025} \quad .....(2)$$

$$\Delta t_b = ([35.4\ a^2]/D^{0.036}).([L/q]^{0.64}) \quad ...(3)$$

D = diffusion constant of the analyte (cm^3)

a= internal radius of tubing (cm)

q = flow rate (cm^3/min)

L = length of reaction tubing (cm).

Not only does the sample zone change in profile (with changing parameters) as it moves down the tubing, but it also changes in terms of the concentration profile. FIA is considered to be a kinetic measurement system in which the concentration within the sample zone is a function of time.

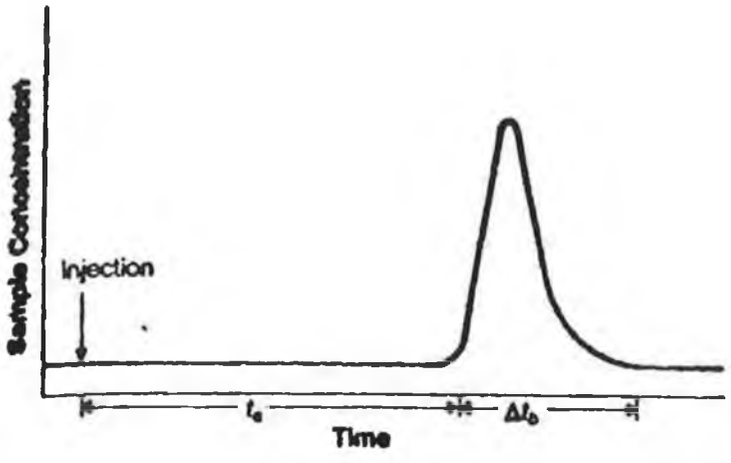

Figure 1.4. Showing the two parameters predicted by Van der Slice, Δt_b and t_a.

1.7. Chromatography

1.7.1. Introduction to Liquid Chromatography

Liquid chromatography (LC) refers to any chromatographic procedure in which the moving phase is a liquid. This method dates back to the 1930's and has been used for an enormous variety of applications. Over the last 40 years, the practice of chromatography has witnessed a continuing growth in a number of areas, including the number of chromatographers, publications, the variety and complexity of samples being separated, and separation speed.

Figure 1.5 shows the hypothetical separation of a three-component sample in an LC column. The rate of migration of each component is dependent on the equilibrium distribution of that component between the stationary phase (which may be a solid or a liquid) and the flowing stream or mobile phase. This rate of migration is affected by a number of experimental parameters including the mobile phase composition and the nature of the stationary phase. Each component also disperses as it moves through the column. This is known as band broadening. The type of chromatography used in this thesis is based on liquid-solid or adsorption chromatography which involves high surface area particles with retention of sample molecules occurring by attraction to the surface of the particle.

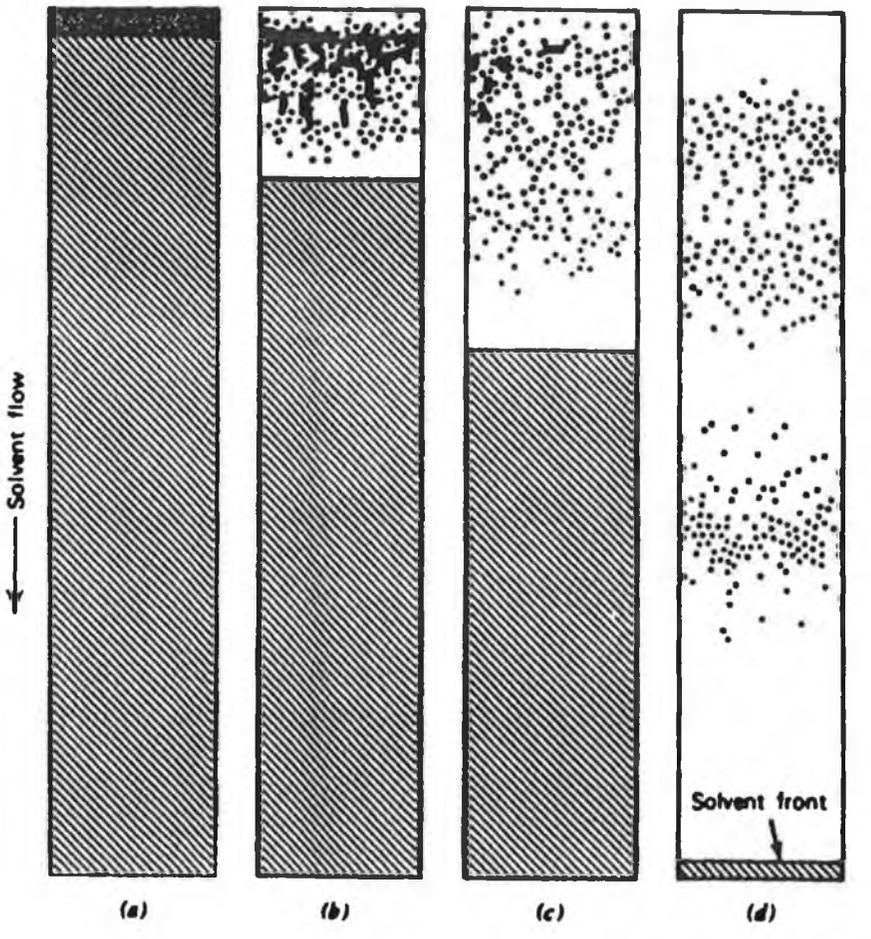

Figure 1.5. Hypothetical separation of a three component mixture in an LC column.

1.7.2. Theory

1.7.2.1. Retention Characteristics

The relative polarities of solute and solid stationary phase determine the rate of movement of that solute through a column. Thus the mobile phase and the solute molecules are in competition for the active sites of the adsorbent. The more strongly the mobile phase interacts with the adsorbent (such as silica $(SiO_2)_x$ and alumina $(Al_2O_3)_x$), the quicker the solute will elute from the column. Thus more polar solvents have higher elution strength, such as in reversed-phase chromatography which is the technique used throughout this thesis.

The solvent of the mobile phase is termed the eluent. The time required for the solute to pass through the column is called the retention time, t_r (Figure 1.6). The retention volume, V_r, is the volume required to elute a solute from the column. It may be obtained directly from t_r and the mobile phase flow rate, F.

$$V_r = t_r F \quad \ldots\ldots (4)$$

The retention time and retention volume of the unretained components are given by t_m and V_m respectively. Thus the adjusted retention volume V_r' and adjusted retention time t_r' are given

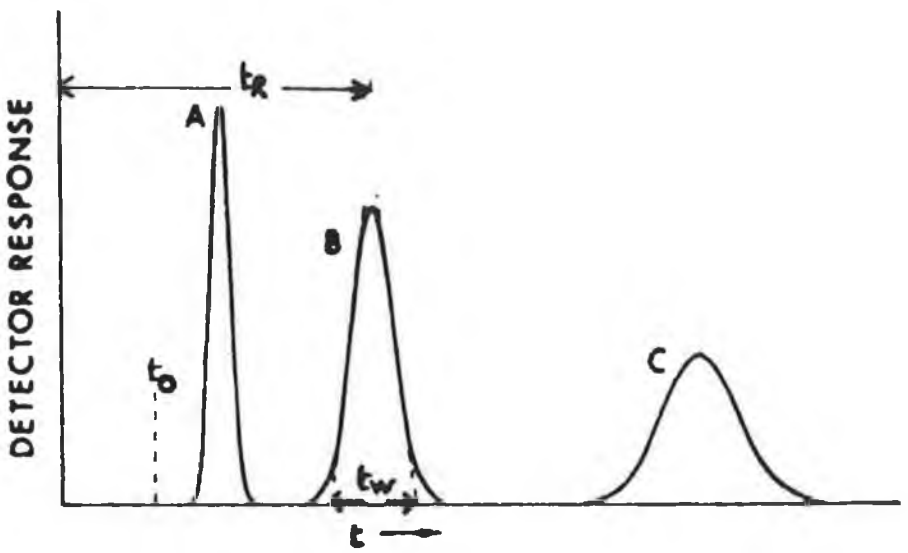

Figure 1.6. Characteristic features of a typical chromatogram. The time t_0 marks the elution of any unretained sample bands.

by :

$$V_r' = V_r - V_m \text{ ; and } .....(5)$$

$$t_r' = t_r - t_m .....(6)$$

The width of the chromatographic peak at the base is given by t_w and that at half height as $t_{w_{1/2}}$.

1.7.2.2. Partition Coefficient

When a solute enters a chromatographic system, it immediately distributes between the stationary and mobile phase. If the mobile phase is stopped sometime during elution, then the sample will be in equilibrium between the stationary and the stopped mobile phase. In this state, the concentration in each phase is given by the partition coefficient K,

$$K = C_s / C_m .....(7)$$

where C_s is the concentration of solute in the stationary phase and C_m is the concentration of solute in the mobile phase. K determines the average velocity of each solute zone as the mobile phase moves down the column. At the appearance of a peak maximum at the column exit, one half of the solute has eluted in the retention volume V_r and half remains in the volume of the mobile phase. Thus

$$V_r C_m = V_m C_m + V_s C_s \quad \text{.....(8)}$$

By rearrangement and insertion of **K** :

$$V_r = V_m + KV_s \text{ (or } V_r - V_m = KV_s) \quad \text{.....(9)}$$

In adsorption chromatography, **K** is termed the adsorption coefficient and V_s the surface area of the packing material.

1.7.2.3. Capacity Factor

The capacity factor k' is the most important quantity in column chromatography. k' is a measure of the time spent in the stationary phase relative to the time spent in the mobile phase. It is also defined as the ratio of the total amount of solute in the stationary phase to the total amount of solute in the mobile phase at equilibrium.

$$k' = C_s V_s / C_m V_m = K(V_s / V_m) \quad \text{.....(10)}$$

$$k' = t_r - t_m / t_m = V_r - V_m / V_m \quad \text{.....(11)}$$

1.7.2.4. Column Selectivity and Relative Retention or Separation Factor

Column selectivity α is measured by the relative separation of peaks and is given by

$$\alpha = k_2'/k_1' = k_2/k_1$$

and also by

$$t_{r_2}-t_m/t_{r_1}-t_m = t_{r_2}'/t_{r_1}'$$

$$= V_{r_2}'/V_{r_1}' \quad .....(12)$$

Column selectivity depends on two conditions : the nature of the two phases and the column temperature.

1.7.2.5. Column Efficiency (Plate Theory)

The plate theory of chromatography envisages a chromatographic column as being composed of a series of discrete but contiguous narrow horizontal layers called theoretical plates. At each plate, equilibration of the solute between the stationary and mobile phases is assumed to take place. N, the number of theoretical plates, is a measure of the efficiency of the column, as is the height equivalent to a theoretical plate HETP (or H) where

$$N = (t_r/\delta_t)^2 = 16(t_r/w_b)^2 \quad .....(13)$$

$$\delta_t = W_b/4 \quad .....(14)$$

where δ_t^2 = the chromatographic band variance in time unit.

$$H = L/N \quad \ldots\ldots\ldots\ldots\ldots\ldots \quad (15)$$

where L is the length of the column.

1.7.2.6. The Rate Theory of Chromatography

The rate theory describes the effects of variables which affect the width of the elution band (zone broadening) as well as the elution time. The migration rate is given by :

$$v = L/t_r \quad \ldots\ldots(16)$$

The retardation factor or retention ratio R is given by :

$$R = (L/t_r)/(L/t_m) = t_m/t_r = V_m/V_r \quad \ldots(17)$$

R is a measure of the fraction of time that an average solute resides in the mobile phase. Therefore, (1-R) is the fraction of time in which the solute is held in the stationary phase. Assuming dynamic equilibrium exists, the ratio of the fractions of time spent in each phase is equal to the ratio of quantity of solute in each phase.

$$R/(1-R) = C_m V_m / C_s V_s \quad \ldots\ldots(18)$$

Since $K = C_s/C_m$,

$$R = V/(V_m + KV_s) \text{ .....(19)}$$

$$\text{and } (1-R) = KV_s/(V_m + KV_s) \text{ .....(20)}$$

1.7.2.7. Band Broadening

The ideal chromatographic process is one in which the components of a mixture form narrow bands which are completely separated from one another. The ultimate width of a peak is determined by the total amount of diffusion occurring during movement of the solute through the system and on the rate of mass transfer between the two phases. Attempts have been made to define efficiency in terms of diffusion and mass transfer. The plate height, H, is affected by the following contributions.

$$H = A + B/U + CU$$

A is the multiple path term, which accounts for different portions of the mobile phase, and consequently the solute, travelling different total distances because of the various routes taken around the particles of stationary phase. U is the mobile phase flow rate. In a poorly packed column, some molecules will travel more rapidly by going along the more open or low resistance path near the column wall. This is called channelling. Others may diffuse into stagnant areas and lag behind; called Eddy diffusion.

B/U is the molecular diffusion term or longitudinal or axial diffusion of solute molecules within the mobile phase caused by local concentration gradients.

CU is the mass transfer term which is the dominant cause of band broadening at high flow rates. When the molecule is in the stationary phase it is retained and falls behind the band centre as the band centre continues to migrate down the column. When the molecule is in the mobile phase, its velocity is faster than the band centre, since the flow velocity is always greater than the band velocity. The random transfer back and forth between the mobile and stationary phases causes dispersion in the chromatographic peak as some molecules will by chance migrate ahead of the average and others will migrate more slowly than the average.

1.7.2.8. Resolution

In well behaved chromatographic system (Figure 1.7), the resolution of two adjacent peaks is defined as :

$$R_s = t_{r_2} - t_{r_1} / 0.5(w_1 + w_2) \quad ....(21)$$

The resolution, if inadequate, can be improved in two independant ways :

(i) increasing separation (thermodynamic effect);

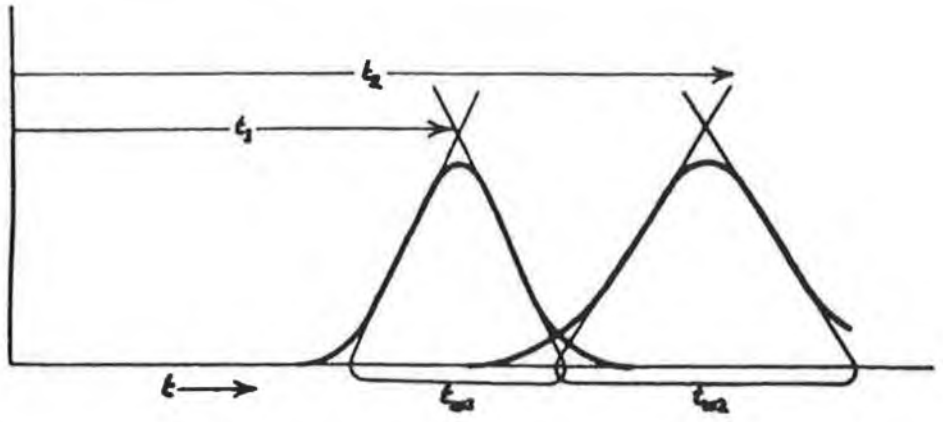

Figure 1.7. Resolution in LC.

(ii) decreasing peak width (kinetic effect).

The use of chromatography as a separation technique has several advantages :

(i) The availability of a variety of sensitive and selective detectors for both FIA and HPLC;
(ii) advanced column technology;
(iii) relative ease of method development; and
(iv) low running cost.

1.8. Amperometry

1.8.1. Introduction to Amperometry

There are two types of electrochemical sensors: potentiometric and amperometric. Ion-selective electrodes are an example of potentiometric sensors where the potential developed (relative to a reference electrode) at zero current, at a selective membrane or electrode surface is related to the analyte concentration.

Amperometric sensors consist of an electrode held at a constant potential (relative to a reference electrode) sufficient for the oxidation or reduction of an analyte of interest. The current produced as a result of this electrochemical reaction is thus proportional to the analyte concentration. The research reported in this thesis has been concerned with the area of amperometric sensors involving the incorporation of enzymes, tissues and microbes in carbon paste as the selective element of the biosensor.

Stripping Voltammetry was also used for the detection of metals at the lichen-modified carbon paste electrode. This technique is therefore also discussed.

1.8.2. Liquid Chromatography / Electrochemical Detection (LCEC)

Amperometric detection was carried out under both static (batch) and flow systems. In the following section, the theory, requirements, limitations and characteristics of electrochemical (EC) detection are described along with a discussion on carbon paste electrodes.

The suitability of EC detection to a given problem ultimately depends on the voltammetric characteristics of the molecule(s) of interest in a suitable mobile phase and at a suitable electrode surface.

EC detection may only be used for compounds which are electroactive. Not all compounds are electroactive, so this appears to impose a limitation on the technique. However, there are ways of overcoming such problems, in particular, by the use of an enzyme or biocatalyst. While the substrate of an enzymatic reaction may be electroinactive, the product of that reaction may well be electroactive. The use of mediators also provides an alternative route to an electroactive species. These features are illustrated in the following sections.

EC detection is not suitable for normal phase LC separations since non-polar organic solvents are not well suited to many electrochemical reactions. However, it is compatible with reversed phase and ion-exchange separations.

LCEC may be considered in terms of electrolysis occurring at a fixed point along a flowing stream. In this case the stream is the mobile phase eluting from the analytical column, a sequence of solute zones separated with varying degrees of resolution.

These zones pass into for example, a very low volume thin-layer cell, where the flow is constrained to a thin film passing over a planar electrode held at a fixed potential (see Figure 1.8). If the potential is greater (more positive for oxidations, more negative for reductions) than that required for the electrolysis of the analyte, a measurable current passes from the electrode to analyte (or vice versa). Ideally, the resulting current is directly proportional to the concentration of solute passing through the channel.

The fundamental law governing all amperometric determinations is Faradays Law :

$$Q = n F N \quad \ldots\ldots\ldots (22)$$

where Q is the number of coulombs used in converting N moles of material, n is the number of moles of electrons lost or gained in the transfer process per mole of material and F is Faradays constant (96500 coulombs / mole of electrons).

Best selectivity is attained where the electrode reactions are carried out at the fastest rate in order that the current produced is limited not by reaction

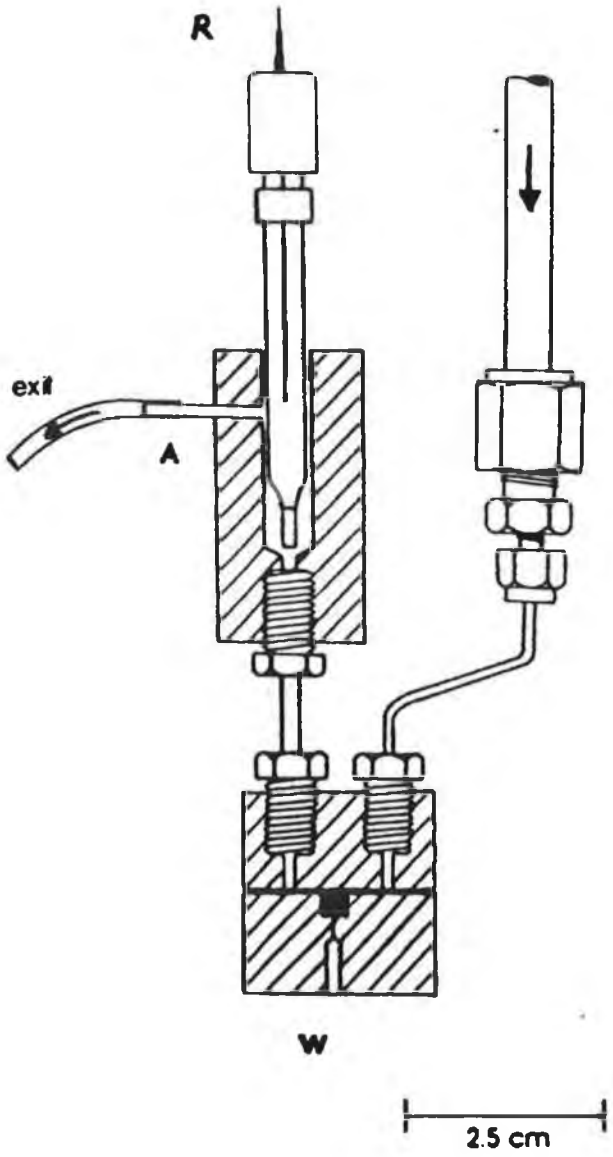

Figure 1.8. Cross-section of a transducer showing the working (W), reference (R) and auxiliary (A) electrodes.

at the surface, but by the mass transport of the molecules to the surface. The choice of electrode material is important primarily from the point of view of ruggedness and long term stability.

In LCEC determinations, points to consider when choosing electrode material include the potential range, compatability with the mobile phase and long term stability. Carbon paste, glassy carbon, mercury, amalgamated gold and platinum have been popular choices for electrode material. In this case, carbon paste was used which is a mixture of spectroscopic graphite powder (carbon) and a mineral oil such as Nujol.

For most published applications of carbon paste electrodes, the useful lifetime of the electrode surface can extend to many months. If extremes of potential are used (>1.0 V vs Ag/AgCl) and / or if the mobile phase contains high concentrations of organic solvent, the electrode surface may need to be renewed more frequently. Each electrode material has distinct advantages.

Glassy carbon provides a useful alternative to carbon paste in situations where the solvent strength of the mobile phase is unsuitable for carbon paste. Glassy carbon is a hard, amorphous carbon material capable of being polished to a mirror-like finish. It offers good solvent resistance, particularly to mobile phases containing a high percentage of organic solvents.

One of the main advantages of using EC detection in conjunction with a flow system in comparison to voltammetry in quiescent solutions is the continuous "cleansing" of the electrode surface by the mobile phase. In voltammetry for instance, sample molecules, lipids or proteins may cause "poisoning" of the electrode surface after a number of sample determinations.

In some cases, the electrode reaction itself leads to polymer formation which "passivates" the surface causing unreliable results.

The electrode system usually consists of a working electrode set at a fixed potential and varying the potential of the neighbouring solution with respect to a reference electrode. The important point to note is that it is the potential difference between an electrode and the solution which is of importance in electrochemistry. Normally, this potential difference extends across an interphase region, the electrical double layer.

Following application of a fixed potential to the electrode, there is usually a considerable charging current region. This is the current required for the electrode solution interface to achieve the applied potential difference.

1.8.2.1. Theory

A hydrodynamic voltammogram (Figure 1.9), obtained

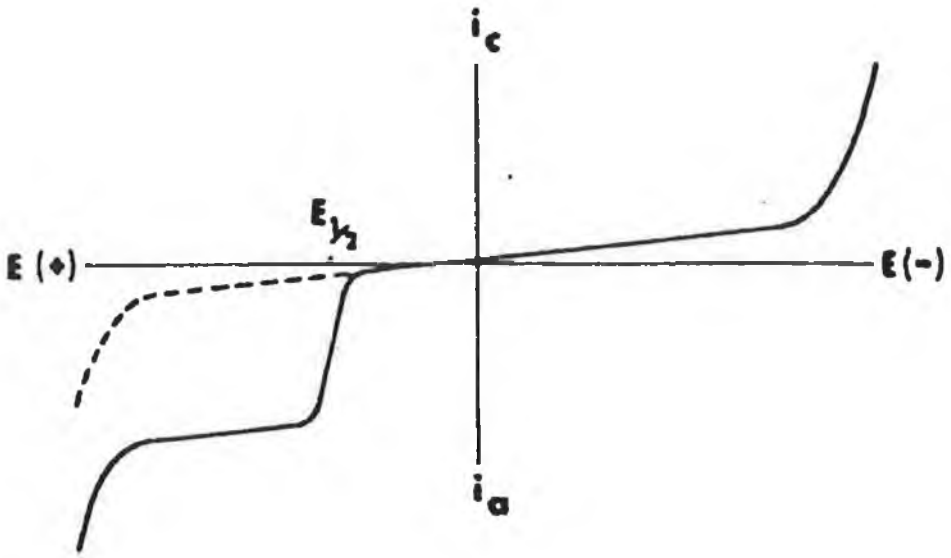

Figure 1.9. Example of an hydrodynamic voltammogram, characterised by the half-wave potential, $E_{1/2}$ of solute of interest.

by measuring the current at a series of fixed potentials has three distinct regions:-

(i) A zero currrent region where the potential is not sufficient to promote oxidation;

(ii) an intermediate region where the peak height increases with increasing potential - here the potential is controlling the kinetics of the heterogeneous electron transfer from solute / analyte to the electrode surface;

(iii) a plateau region in which, the peak height is independent of the potential and where diffusion to the electrode surface is the rate determining factor, i.e. the current is proportional to the rate of transport of molecules per unit surface area and per unit time.

Figure 1.10 shows the hydrodynamic voltammograms for some hypothetical compounds which can be oxidised (A, B, C and D). The magnitude of the current is directly proportional to the bulk concentration at every point along the voltammetric curve. Although it is often desirable to operate an electrochemical detector on the limiting current plateau (region 3, Figure 1.10), (E_2 for compound A), it may be more beneficial to use a potential taken from the rising part of the curve (E_1 for compound A).

Generally, the lower the potential, the greater the

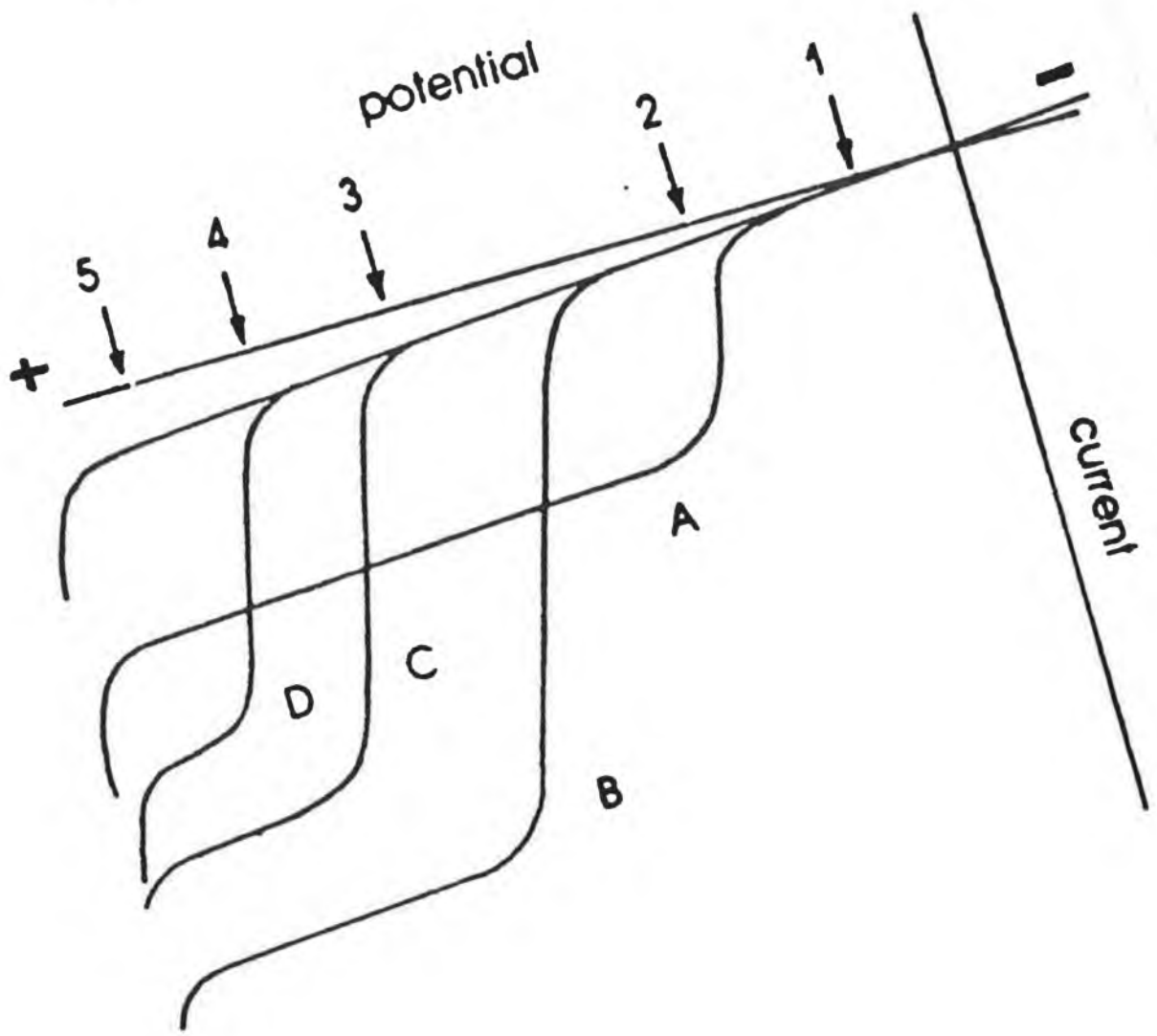

Figure 1.10. Hypothetical hydrodynamic voltammogram for oxidisable solutes A, B, C and D.

selectivity. If, for example, A and B are not well resolved by chromatography, it may be difficult to detect A at E_2 in the presence of a thousandfold excess of B. Lowering the potential to E_1 will decrease the sensitivity to A severalfold, but the sensitivity to B may be decreased by many orders of magnitude. At E_3, A and B will be detected with comparable sensitivity and at E_4 it would also be possible to detect C. In that case, selectivity for C will not be good and a greater reliance will have to be placed on the chromatography.

Sensitivity to C will be further decreased due to a larger background current resulting from more easily oxidisable substances. At E_5 and beyond, it is likely that the EC detector will not be useful for trace analysis as the background oxidation (D) becomes excessive.

There are special experimental problems associated with LCEC detection of trace amounts of reducible substances , because reduction of dissolved oxygen, trace metal ions and hydrogen ions can interfere. The use of the reductive mode is one of the highlights of this section, illustrating the effective "removal" of interferences.

1.8.2.2. Mobile Phase Limitations

Amperometric detection relies on the transfer of electrons between a solute and the electrode surface,

hence the choice of solvent / mobile phase needs to be such that allows the electrode reaction to proceed. Some important considerations are listed below:

(i) the electrolyte must be present usually at 0.01 M to 0.1 M concentrations, to convey charge through the electrochemical cell;

(ii) the solvent must have a sufficiently high dielectric constant to freely permit ionisation of the electrolyte;

(iii) the mobile phase must be electrochemically inert;

(iv) the electrolyte should be compatible with the electrode material being used.

Even with these limitations, LCEC still has a broad range of use, since all ion-exchange and most reversed-phase separations employ these types of mobile phases.

1.8.2.3. Performance Characteristics

Sensitivity is defined as the slope of the response versus the concentration curve. The detection limit refers to the lowest amount of analyte required to give a signal X times (usually X=3) greater than the standard deviation of the noise (see Figure 1.11).

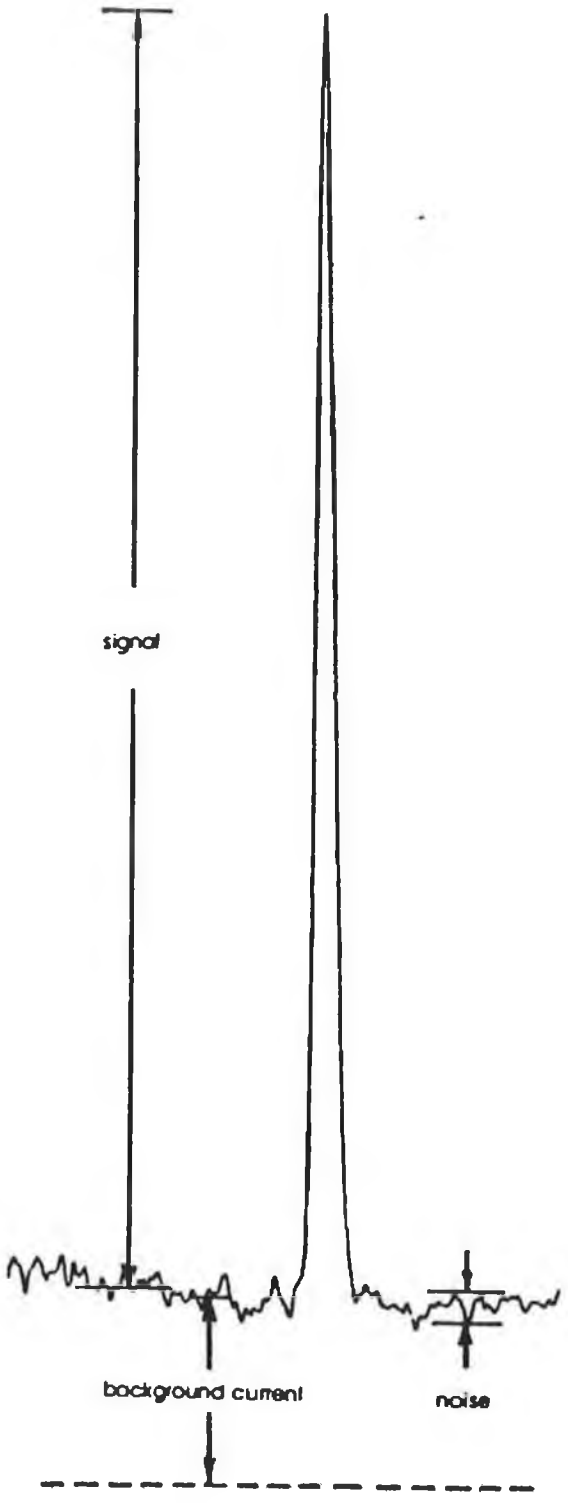

Figure 1.11. Performance characteristics for detector evaluation.

1.8.2.4. Current Response

There are two main contributions to the current response of a thin-layer electrochemical cell:-

(i) the Faradaic response - due to the redox processes either from analyte or solvent impurities; and

(ii) the charging current, which is the current required to charge the double layer capacitance at the electrolyte / electrode interface.

Normally the charging current is not an issue since the detection is operated at fixed potential. Once the potential is supplied to the cell and the background decays to an acceptably flat level, only Faradaic contributions remain.

Background current usually arises from the oxidation or reduction of electroactive impurities which are present in the mobile phase. Some impurities include, as mentioned previously, metal ions, dissolved oxygen or contributions from the oxidation or reduction of mobile phase constituents.

Noise is a summation of contributions from pump pulsation, flow cell hydrodynamics, surface reactions, static electricity, power line noise and electronic amplification. In spite of the many advantages that LCEC has using a single working electrode, in certain cases greater selectivity and lower detection limits

can be attained using dual electrodes. The use of a dual electrode may also provide additional qualitative information previously unavailable. Dual electrodes may be used either in the parallel adjacent, parallel opposed or series mode (see Figure 1.12). In the parallel-adjacent dual electrode mode, two electrodes are placed adjacent to each other on one side of the flowstream across the channel from a corresponding auxiliary electrode. In this way, a chromatogram may be recorded at two different potentials.

Applications for this include the simultaneous determination of substances with significantly different oxidation (or reduction) potentials, and the simultaneous measurement of peak current ratios in order to confirm the identity of eluted compounds.

In the series dual electrode mode, the two electrodes are placed in a series configuration so that the downstream electrode can be used to study the product(s) resulting at the first electrode. This approach was investigated using tissue and plain carbon paste at the first and downstream electrode respectively in chapter 4.

Not only is it a means of detecting compounds which have electrochemically reactive products, but may also be used to see the effects an immobilisation procedure has on a "biosensor" such as banana or mushroom tissue.

In the parallel opposed dual electrode mode the two working electrodes are placed opposite each other such

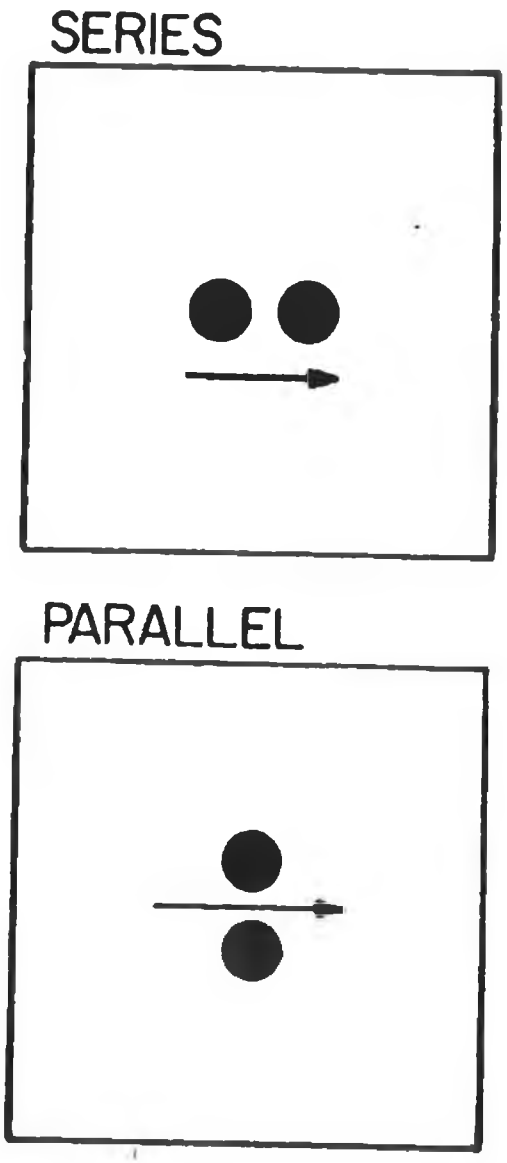

Figure 1.12. Cell configuration for parallel-adjacent and series dual electrode detection.

that "redox" cycling can be used to enhance the response for components in which both sides of the redox couple are stable (e.g. hydroquinone / quinone). The effect of this approach will obviously depend on the number of cycles which can be achieved during the "residence time" in the active region of the detector cell.

1.8.3. Oxidative Mode LCEC

The oxidation of a compound involves the electron transfer from a compound to the electrode surface with an increase in the oxidation state. The major advantages of oxidative electrochemistry are that oxygen is not electrochemically active and solid electrodes can be used. Working electrodes are usually based on carbon, such as graphite or an anisotropic solid in carbon paste and glassy carbon respectively.

In general, for electrochemical oxidation to take place, one looks for the same features and reactive centres as one would for a homogeneous oxidation reaction e.g. delocalised electrons and stability of product (see Figure 1.13 and 1.14 for examples of electroactive compounds).

1.8.4. Reductive Mode LCEC

The reduction of a compound involves the gain of electrons by a compound with a concommittant decrease

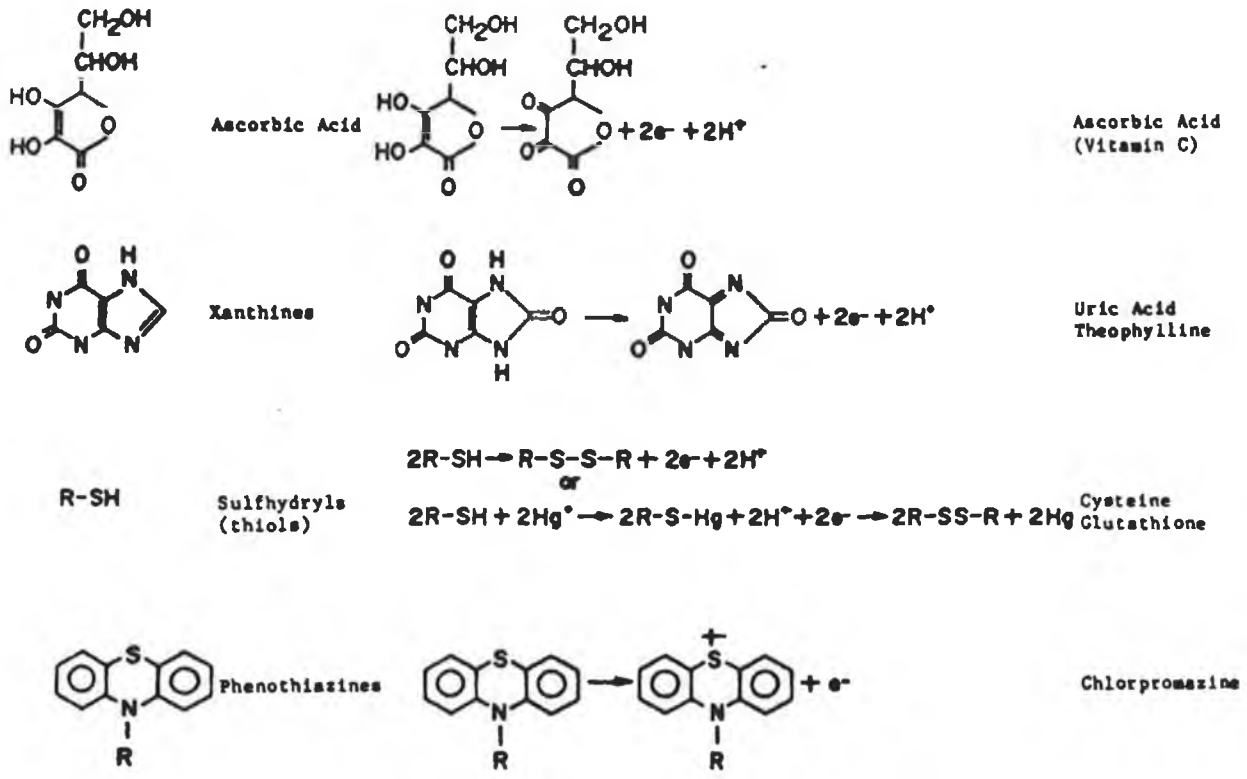

Figure 1.13. Examples of some electroactive compounds.

in oxidation state. Generally, the more delocalised the electrons become, the more easily reducible the compound. In addition, electron withdrawing groups on an aromatic ring enhance the reduction reaction. Oxygen is not really a problem in reductive mode LCEC, since the mobile phase is always degassed prior to use, usually by both vacuum filtration and sonication for at least 20 min. The potentials at which the electrodes will be sensitive to oxygen will however depend on the applied potential.

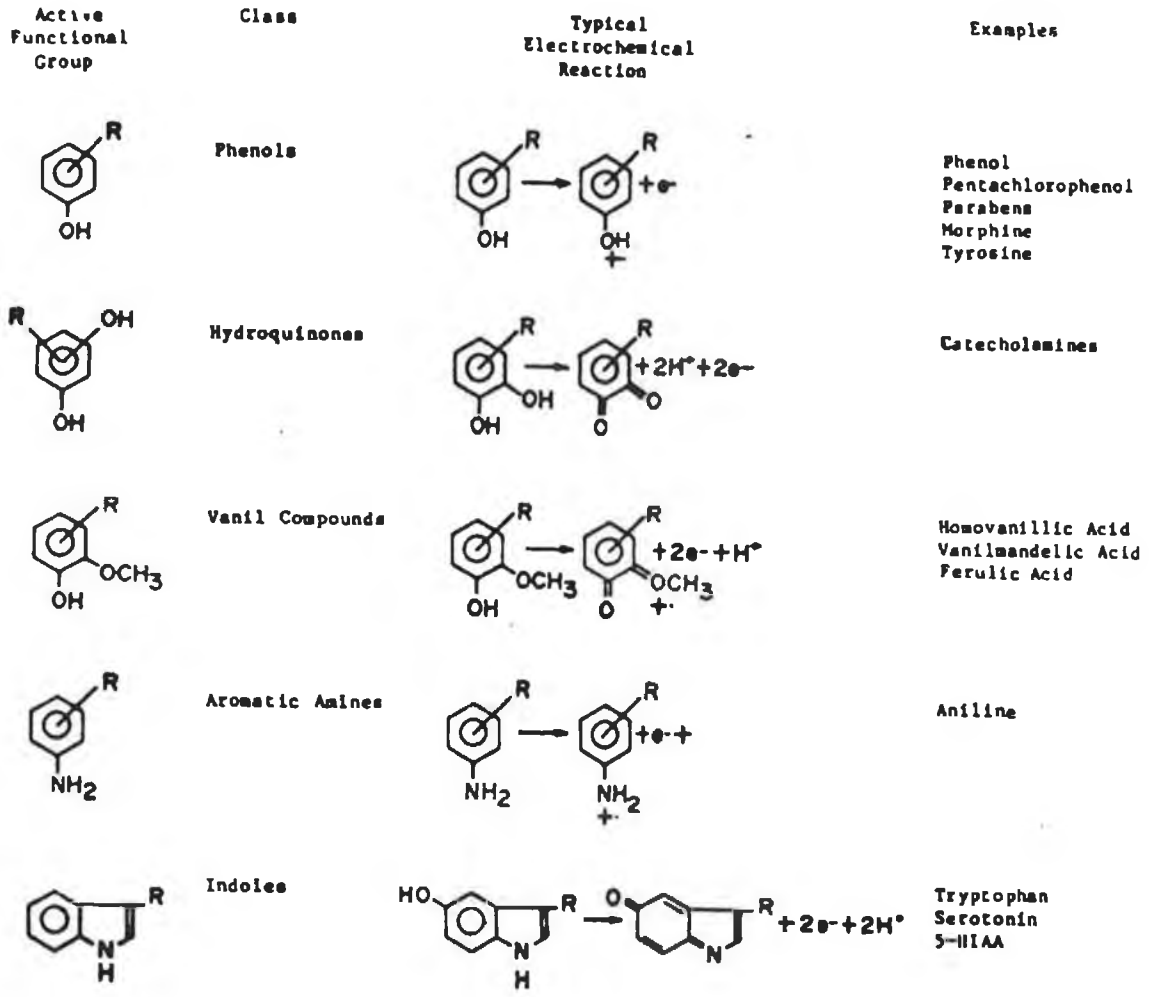

Figure 1.14. Examples of some electroactive compounds.

1.9. Voltammetry

1.9.1. Introduction to Voltammetry

Voltammetry is the measurement of current at a working electrode over a range of applied potential. A plot of current versus applied potential results in a voltammogram and is used to obtain both qualitative (peak potential) and quantitative (peak current) information about the analyte species. The technique involves the use of three electrodes, the working electrode which may be solid, stationary or dropping mercury, a reference electrode such as Ag/AgCl or calomel, and an auxiliary electrode which could be platinum or carbon.

Polarography is the field of voltammetry concerned with electrode processes at mercury and was devised by J. Heyrovsky in 1922 [60]. Since then, numerous methods have evolved and play key roles in analytical situations.

Pulse techniques [61] in particular exhibit excellent sensitivities and find wide application, particularily in the area of environmental analysis.

1.9.2. Anodic Stripping Voltammetry (ASV)

Anodic stripping voltammetry has been applied to a variety of analytical situations; in particular, the analysis of trace heavy metals in the environment

[62, 63]. What follows is a discussion on the principles of ASV by way of an introduction to Chapter 5, with particular reference to the application of this technique for the determination of Pb(II) and Cu(II) at lichen-modified carbon paste electrodes.

ASV is a two step process, in which the metal ion of interest is first preconcentrated at the electrode surface in what is referred to here as the accumulation step. The potential is then scanned anodically, during which time the deposited metals are stripped out of the electrode in an order according to each individual metals standard electrode potential.

The resulting voltammogram shows the current response obtained versus time (Figure 1.15). The peak current, i_p, is proportional to the metal concentration, from which quantitative measurements may be made, while the peak potential, E_p, yields qualitative information.

The potential E of any electrode is given by the generalised form of the Nernst equation :

$$E = E_0 - [(0.0591/n)\log_{10}([red]/[ox]) \quad .....(23)$$

where E_0 = the standard electrode potential, n = the number of electrons transferred in the electrode reaction and [red]/[ox], the ratio of the concentrations of the reduced and oxidised species.

The total current, i_t, produced during the stripping step, is a combination of contributions from

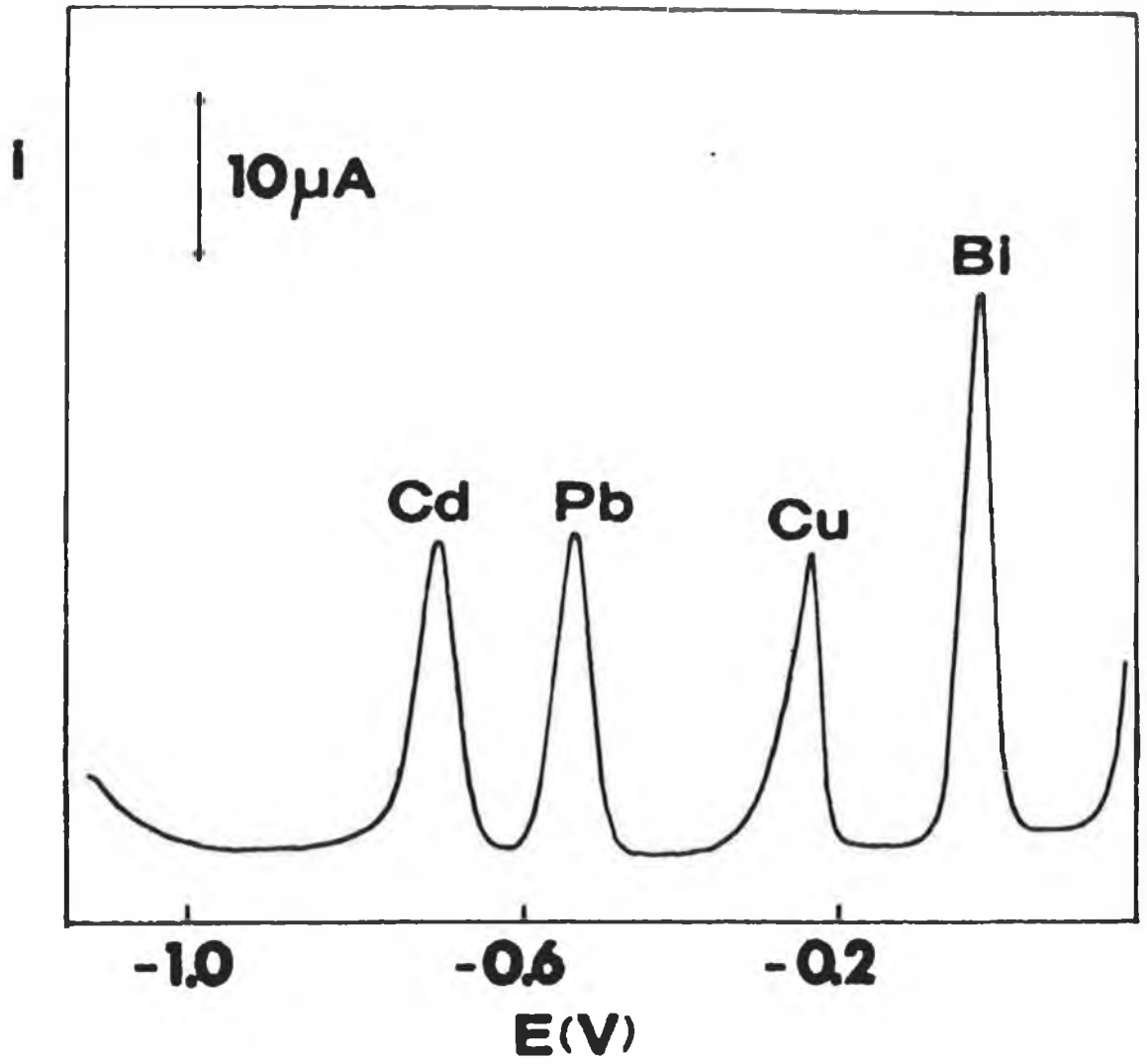

Figure 1.15. A typical stripping voltammogram.

the stripping peak current, i_p, and the background current, i_{bkg}.

$$i_t = i_p + i_{bkg} \quad \text{.....(24)}$$

The background current itself is given by :

$$i_{bkg} = i_c + i_f \quad \text{.....(25)}$$

where i_c is the charging current and i_f the faradaic current. The limit of detection is determined by the ratio of i_f / i_c. The charging current is that current which must flow to charge or discharge the electrical double layer formed by the orientation of equal and opposite charges (at a given potential) at the electrode / solution interface.

1.9.2.1. Differential Pulse Anodic Stripping Voltammetry (DPASV)

In differential pulse anodic stripping voltammetry, the charging current is effectively diminished and the current response is mainly faradaic. Pulses of equal amplitude are combined with a potential scan as shown in Figure 1.16. Variables include pulse height (amplitude), pulse duration (order of ms) and pulse rate (0.5-5 s). Scan rates are usually slow (1-5 mV/s), to ensure that the potential does not change significantly for the duration of the pulse. Currents

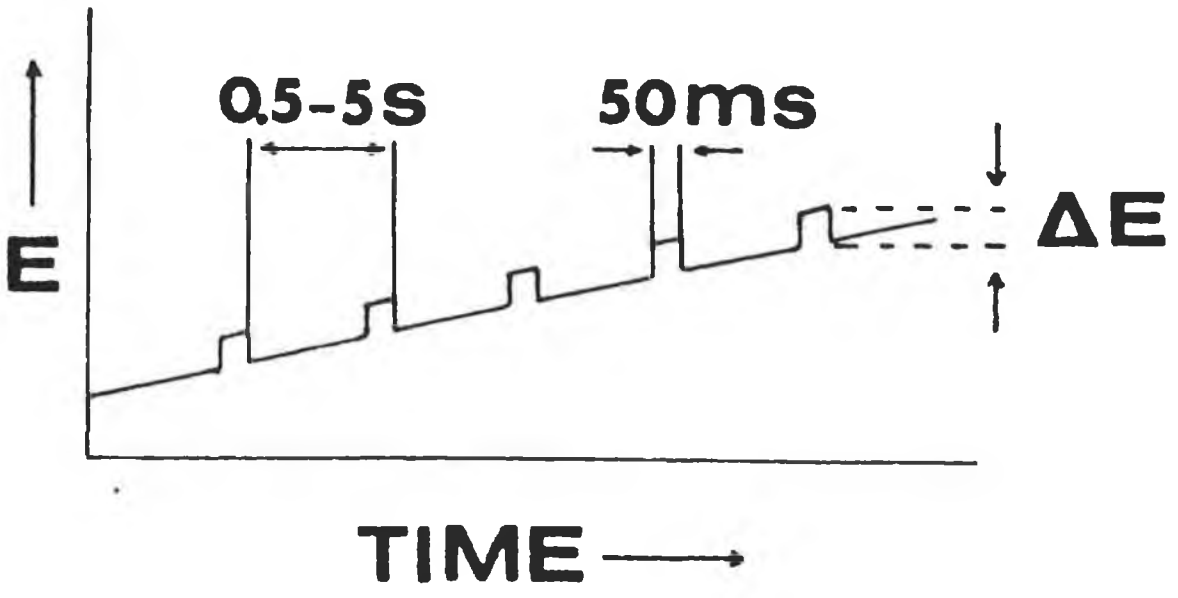

Figure 1.16. Potential-time sequence for differential pulse stripping analysis.

are sampled twice; just before pulse application and just before the pulse ends. The response (current difference) is a peak corresponding to concentration of the metal ion.

A significant advantage of this technique is the ratio i_f / i_c. When a potential pulse is applied, the total current increases (as does i_f and i_c). However, during the pulse, i_c decays much more rapidly than i_f (see Figure 1.17), with the result that when the current is sampled at the end of the pulse, the contribution from i_c is essentially the same as before the pulse. Hence the current response is mainly faradaic in nature, with the result that the limit of detection is greatly lowered.

The faradaic current is a direct measure of the rate of the redox reaction taking place at the electrode and of the concentration of electroactive species. This faradaic current depends on two factors :

(i) mass transport, which is the rate at which the species move to the electrode surface and

(ii) charge transfer, or the rate at which electrons transfer from the electrode to solution species and vice versa.

The electrochemical cell for ASV consists of three electrodes i.e. a working electrode, which in the case of the work reported here is carbon paste-based, a reference electrode (Ag/AgCl), which ideally is of known and constant potential with negligible variation

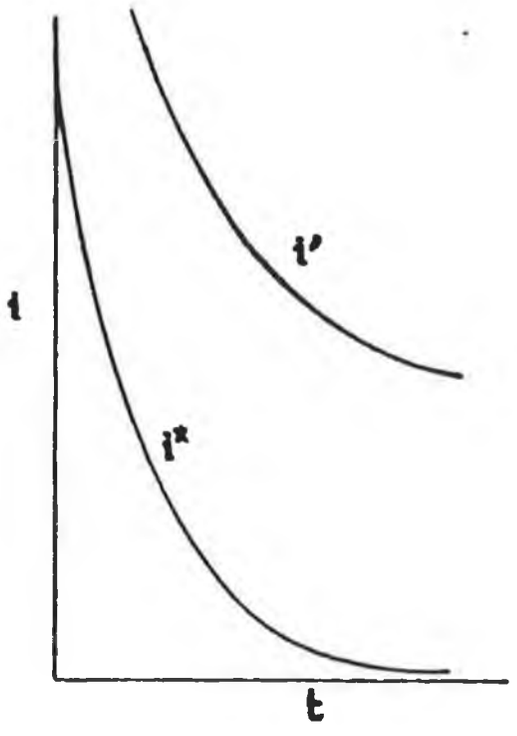

Figure 1.17. Decrease in the faradaic current (i') and the capacitive current (i^*) with elapsed time (t) from the application of a potential pulse.

in the liquid-junction potential from one test or calibration solution to another, and an auxiliary electrode which is made of an inert conducting material.

1.9.2.2. Procedure for Differential Pulse Anodic Stripping Voltammetry

The stripping step is carried out in the electroyte which may be an inorganic salt (such as potassium chloride of potassium nitrate), mineral acid, base or buffer, usually about 0.1 M in concentration to provide a conducting medium. The removal of oxygen is generally necessary and facilitated by purging the electrolyte with an electroinactive gas (e.g. nitrogen or argon). The deposition (accumulation) step can either be carried out under electrolytic conditions, or at open circuit. In this case it was carried out at open circuit and based on the ability of the lichen to complex metal ions. In the deposition step at a mercury electrode for example, the metal of interest is reduced at a controlled potential (negative) for a pre-selected length of time. The reaction may be defined as follows :

$$M^{n+} + ne^- \rightleftharpoons M(Hg)$$

The formation of an amalgam, as seen in the above equation, is dependent upon the solubility of the

metal of interest in the mercury. Under normal conditions of deposition (e.g. at a mercury electrode), a potential more negative than the stripping potential of the metal is applied to the working electrode to cause formation of the amalgam. The deposition time is also a critical parameter. Too long may result in saturation of the electrode surface, whereas too short a time may not be sufficient to obtain a suitable response, both of which are likely to produce irreproducible results.

Deposition is usually carried out under conditions involving convective transport (e.g. rotating disk electrode, magnetic follower) which must be reproducible. The deposition current is influenced by two conditions : diffusion and convection. Diffusion, by virtue of the fact that at the electrode solution interface, a diffusion layer is formed, across which there is a concentration gradient. Because of this gradient, ions move by diffusion towards the electrode surface.

The next stage in the process can involve medium exchange. In this case, deposition is carried out in one solution, while the stripping step is carried out in a different medium. This procedure may be used to minimise interferences. In this work, it was carried out by simply removing the electrode from the accumulation cell, rinsing with distilled water and immediately placing in the electrolyte (measurement) cell.

Following medium exchange, the working electrode undergoes a rest period prior to the stripping step, which involves an anodic potential scan to strip off the metals. The resulting peak current and peak potential may be used to obtain quantitative and qualitative information respectively.

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CHAPTER 2

THE DETERMINATION OF GLUCOSE AT A BIENZYME-MODIFIED CARBON PASTE ELECTRODE

2.1. Introduction to Glucose Sensors

Biosensors have attracted considerable attention over the past decade, particularily in the area of clinical chemistry, and none more so, than in the area of the development of glucose sensors.

The monitoring of glucose levels is important for both diagnostic and therapeutic purposes. Exact and rapid determination of glucose is essential not only in analytical clinical laboratories, but also for on-line supervising of diabetic patients. Blood glucose levels are usually in the range of 3.5-5.0 mM/l. Over the last few years, there has been an intensive drive to develop implantable glucose sensors with a built-in insulin supply.

The first reported glucose sensor was that developed by Updike and Hicks in 1967 [1]. This and other glucose sensors are generally based on immobilised glucose oxidase (GOx) and the following reaction :

$$\text{D-glucose} + O_2 + H_2O \xrightarrow{\text{GOx}} \text{Gluconic acid} + H_2O_2.$$

where either consumption of oxygen or production of hydrogen peroxide may be monitored amperometrically. Detection of glucose has also been carried out using potentiometric sensors and mediated amperometric sensors, some of which will be discussed.

Glucose oxidase is a flavoprotein with a reaction

pH optimum of 5.6. The natural electron acceptor is oxygen, which is reduced to hydrogen peroxide. However, other artificial electron acceptors may be used. The enzyme requires neither cofactors nor activators and is readily immobilised, making it suitable for use as a biosensor. Table (2.1) lists a selection of some glucose sensors [2-9].

Table 2.1. Some reported glucose sensors

METHOD	DETECTION	REFERENCE
GOD / membrane	O_2 / H_2O_2	[2]
GOD / membrane	O_2	[3]
GOD / membrane	H_2O_2, in vivo	[4]
GOD / membrane	mediated (ferrocenium) amperometric probe	[5]
Concanavalin A + fluorescein-labelled dextran	optical fibre, fluorimetry	[6]
GOD + PO	F^-	[7]
GOD, Pt, cellulose diacetate	H_2O_2	[8]
GOD	mediated (benzoquinone)	[9]

Abbreviations : GOD, glucose oxidase, PO, peroxidase.

Nagy et al. [10] developed a self-contained electrode for the determination of glucose, based on an iodide membrane sensor. The highly sensitive iodide sensor monitors the decrease in iodide activity, however, it does suffer interference from metabolites such as ascorbic acid and uric acid. Nilsson et al. [11] described the immobilisation of glucose oxidase in a polyacrylamide gel mounted on a pH glass electrode.

Detection of glucose was investigated in an FIA system by Massoom and Townshend [12], whereby glucose oxidase was cross-linked to controlled porosity glass via glutaraldehyde, with detection based on the monitoring of the production of hydrogen peroxide. Another flow cell based on a glucose oxidase-modified carbon fibre ultramicroelectrode was described by Wang et al. [13]. This electrode exhibited a response time of 11 s, a detection limit of 1.3×10^{-5} M and activity for 12 days.

Another amperometric glucose sensor was developed by Matuszewski and Trojanowicz [14], where glucose oxidase was incorporated into carbon paste and detection based on the amperometric monitoring of hydrogen peroxide. Monitoring hydrogen peroxide was also used by Gunasingham et al. [15], enabling the detection of glucose in whole blood, based on the immobilised reactor of Massoom and Townshend [12], but including a pre-electrolysis cell of carbon fibres to remove interferants.

An LCEC method for the separation of glucose, and such interferants as uric acid and ascorbic acid, was reported by Huang and Kissinger [16] based on the detection of H_2O_2 at a Pt electrode. Gorton et al. [17] described a carbon paste electrode modified with glucose oxidase and a ferrocene-containing siloxane polymer. This sensor was further coated by a poly(ester-sulphonic acid) cation exchanger to remove interferences from ascorbic and uric acids. The mediator tetrathiafulvalene was co-immobilised with glucose oxidase for the detection of glucose in a flowing system by Gunasingham et al. [18]. A 30 s response time was obtained. In a mixed-ferrocene-glucose oxidase carbon paste electrode developed by Wang et al. [19], interfernce from ascorbic acid was removed by incorporating stearic acid into the carbon paste.

Yao et al. [20] described a bienzyme-modified enzyme reactor involving both glucose oxidase and peroxidase and using the mediator hexacyanoferrate(III) with amperometric detection at -50 mV. Scheller et al. [21] reported that the use of two enzymes may enhance sensitivities as a result of substrate amplification. In one example mentioned, the combination of glucose oxidase and glucose dehydrogenase inreased the sensitivity of a glucose sensor by a factor of 10 [22]. Rennenberg et al. [23] reported the use of an enzyme sequence electrode for the detection of bilirubin based on the

coimmobilisation of glucose oxidase and peroxidase in a gelatine membrane over a Pt electrode with detection at +0.60 V.

As can be seen from all the cases mentioned above, detection of glucose is normally by catalytic conversion involving the enzyme glucose oxidase. However, Bindra and Wilson [24] developed a non-enzymatic sensor based on a Nafion coated gold electrode and pulsed amperometric detection. A collagen membrane prevented interference from proteins normally found in whole blood samples.

2.2. Current Developments in Glucose Sensors

In the future, it is likely that "closed-loop" systems will appear on the market, where the blood glucose levels may be monitored with a biosensor and feedback control of insulin delivery rates from a "pump" is achieved, so as to maintain blood levels within the normal range.

Implantable sensors have been developed (see Table 2.2), but such devices must possess a number of characteristics to be of value for the purpose intended.

(i) A suitable site for implantation so as not to risk infection;

(ii) size of probe, e.g. needle-type-sensor;

(iii) biocompatability;

(vi) linear within the range 2.0-30.0 mM/l;

(v) level of resolution of 1 mM;

(vi) specific for glucose, not affected by metabolites;

(vii) drift less than 10 % per day;

(viii) response time of less than 2 min;

(ix) calibration easily facilitated;

(x) amenable to mass production; and

(xi) cheap.

Table 2.2. Characteristics of implantable sensors

	REFERENCE			
	Bessman [31]	Fischer [32]	Kondo [33]	Shichiri [28]
Detector	Galvanic cell	Pt anode/ H_2O_2	O_2 (Clark)	Pt anode/ H_2O_2
Enzymatic membrane	Nylon	Sepharose	Nylon	Cellulose acetate
Immob^{n}	Glut.	CNBr	Glut.	Glut.
Non-enzy^{c} membrane (1)	PP	CA	PP	PU
Non-enzy^{c} membrane (2)		PT	PT	PVA
Sensor Geometry	Plane geometry	Plane geometry	Vessel-shaped	Needle-shaped

Abbreviations : Glut, glutaraldehyde; PP, polypropylene; PU, polyurethane; PT, perforated Teflon; PVA, polyvinyl alcohol.

Several sensors have been developed for glucose monitoring situations. Soeldner et al. [25] were one of the first to investigate a glucose sensor in vitro and in vivo. Disc-shaped sensors were placed in the subcutaneous tissue of monkeys and rabbits. A glucose oxidase enzyme electrode developed by Bessman et al. [26] based on the monitoring of oxygen consumption, showed some problems associated with oxygen tension, which is one of the main issues to be overcome in the development of these devices. One possibility, is the direct oxidation of glucose, such as was carried out by Lerner [27] at a platinum electrode and by Bindra and Wilson [24] at a Nafion-coated gold electrode.

Another problem to be surmounted is the correlation between the glucose levels at the site of probe insertion and that in the bloodstream. Shichiri et al. [28] reported that there was a significant relationship between subcutaneous glucose and blood glucose levels in dogs, although, after acute intravenous glucose dosage, the subcutaneous increases were delayed by 5-15 min and were about 65 % lower than the blood levels. In similar work carried out at a later stage by the same workers [29], they found that, using the needle-type sensor, the glucose levels were approximately 25 % lower in subcutaneous tissue compared to blood, but normal glucose levels were resumed almost immediately.

Pickup and Claremont [30] have developed a sensor based on the co-immobilisation of glucose oxidase and

ferrocene on graphite foil, covered by a polyurethane membrane. This sensor proved to be free from any constraints imposed by oxygen levels and, changes in blood glucose levels on administration of glucose or insulin were almost concurrent.

Several problems remain to tackled, however, the future does look optimistic for the application of these in vivo sensors and their routine use, not just by professional people, but also by patients.

2.3. Method

In this section, the use of co-immobilised glucose oxidase (GOx) and horseradish peroxidase (PO) for the detection of glucose, based on the amperometric reduction of ferricyanide (-0.20 V) (see reaction sequence below) is investigated.

$$\text{D-Glucose} + H_2O + O_2 \xrightarrow{\text{GOx}} \text{Gluconic acid} + H_2O_2$$

$$H_2O_2 + Fe(CN)_6^{4-} \xrightarrow{\text{PO}} H_2O + Fe(CN)_6^{3-}$$

$$Fe(CN)_6^{3-} + e^- \longrightarrow Fe(CN)_6^{4-} \text{ (-0.20 V)}$$

The use of a mediator and a second enzyme has an important advantage in that the detection potential is reductive. In the oxidative mode, interferants such as

other metabolites (e.g. uric acid) normally present in the serum are detected in the presence of glucose. This electrode system has several advantages including excellent response times, ease of preparation, stability and low cost.

The effects of mediator concentration, electrode composition, pH of electrolyte, linear range and % GOx are investigated. Experiments were carried out under both static and flowing conditions. The method, with slight modification, was also used for the detection of bilirubin which is important for diagnostic purposes and therapeutic monitoring. The reaction sequence for the detection of bilirubin is shown :

$$\text{D-glucose} + H_2O + O_2 \xrightarrow{\text{GOx}} \text{Gluconic acid} + H_2O_2$$

$$H_2O_2 + \text{bilirubin} \xrightarrow{\text{PO}} \text{product} + H_2O \ (+0.80V)$$

2.4. Experimental

2.4.1. Apparatus

Batch experiments were performed in a Bioanalytical Systems (BAS) Model VC-2 electrochemical cell. The bienzyme-working electrode, reference electrode (Ag/AgCl) (Model RE-1, BAS) and platinum wire auxillary electrode joined the cell through holes in

its cover. A magnetic stirrer and a stirring bar (7 mm long) provided the convective transport. The flow injection system consisted of the carrier reservoir, a Rheodyne Model 1010 injector (20 µl loop), 1 mm i.d. Teflon connecting tubing and the thin-layer electrochemical detector. The Ag/AgCl reference and stainless steel auxillary electrodes were located in a downstream compartment (Model RC-2A, BAS).

Flow of the carrier stream was maintained by gravity. The current response was measured with an IBM Instruments Inc. EC/225 Voltammetric analyser and a Houston Omniscribe strip chart recorder.

The enzyme electrode was prepared in the following manner. The bienzyme-containing paste was made by thoroughly mixing the enzymes, glucose oxidase (GOx) and horseradish peroxidase (PO) with graphite powder (Fisher, grade *38) and mineral oil (Aldrich) (the ratio being 10:10:40:40 (w/w) respectively). A portion of this paste was packed into the cavity of a 3 mm i.d. glass tube and smoothed on a deck of weighing paper - likewise for the thin-layer electrochemical flow cell.

Contact was provided by a copper wire embedded in plain carbon paste which was also packed in the glass tubing. The current response for the FIA experiments was measured with an EG&G PAR Model 364 Polarographic analyser and a Houston Omniscribe strip chart recorder.

2.4.2. Reagents and Procedure

All solutions were prepared with doubly distilled water. Glucose oxidase (Sigma EC 1.1.3.4. No. G-6125), peroxidase (Sigma EC 1.11.1.7. No. P-8125), potassium ferrocyanide trihydrate (Baker), glucose (No. G-5250, Sigma, B-D(+) glucose), uric acid (Sigma, No. U-2625), bilirubin (Sigma, No. B-4126) and hydrogen peroxide (Aldrich, 30 wt % solution in water.) were used without further purification. All measurements were performed in a 0.05 M phosphate buffer (pH 7.4) solution containing 0.01 M ferrocyanide, except the study on interferences where only phosphate buffer was used. Amperometric detection was performed by applying a potential of -0.20 V for all measurements except the determination of bilirubin and interferants where a potential of +0.90 V was applied.

2.5. Results and discussion

2.5.1. Effect of pH

Amperometric measurements were carried out by spiking glucose (final concentration of 5 mM) into phosphate buffer of different pH and measuring the current response produced. Figure 2.1.a shows a graph of pH of the electrolyte vs. current response in the pH region 2-12. A maximun response at pH 7.4 is obtained which corresponds to the optimum pH for PO

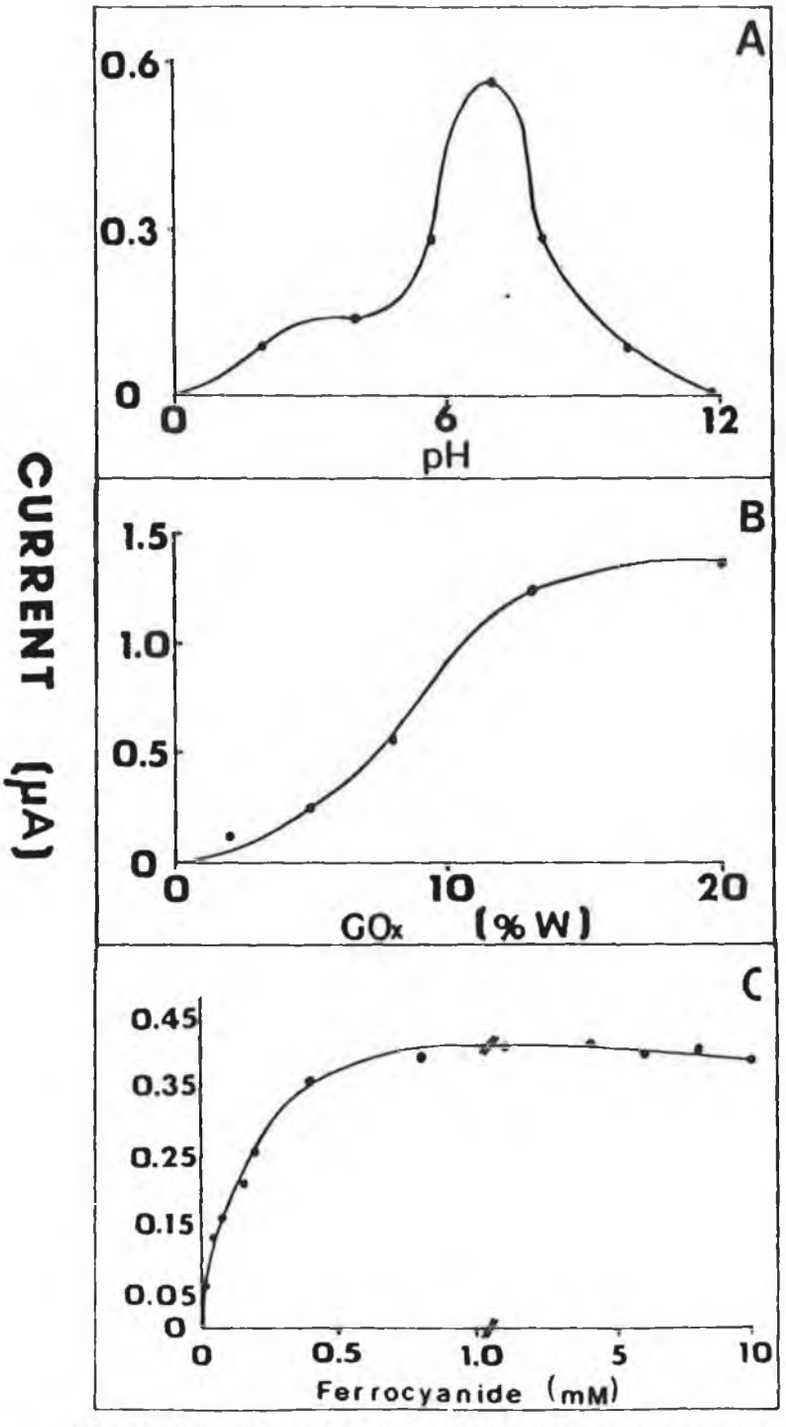

Figure 2.1. : Plots for batch experiments showing the effects of varying pH of the electrolyte, % GOx (w/w) and concentration of mediator. Final concentration of glucose, 5 mM; detection, -0.20 V; electrolyte, 0.05 M phosphate buffer (pH 7.4, b and c only); enzyme loadings, 10 % GOx (w/w) (a and c only), 10 % PO (w/w); ferrocyanide concentration, 0.01 M (a and b only).

activity. A pH of 7.4 was chosen for further studies.

2.5.2. Effect of Glucose Oxidase Loading

The PO loading (10 % w/w) was kept constant and the GOx varied from 0 - 20 % (w/w). The current responses obtained for a final glucose concentration of 5 mM in a phosphate buffer (pH 7.4) under batch conditions are shown in Figure 2.1.b. The current response increases with increasing enzyme loading and begins to level off around 12 % (w/w). A GOx loading of 10 % (w/w) was used thereafter.

2.5.3. Effect of Mediator Concentration

The effects of varying the concentration of the mediator ferrocyanide was investigated by spiking glucose into a phosphate buffer electrolyte (pH 7.4) to obtain a final concentration of 5 mM. As may be seen from Figure 2.1.c, increasing the mediator concentration resulted in an increase in current response up to a ferrocyanide concentration of 0.4 mM. A further increase in response was not observed and a mediator concentration of 10 mM was used for further studies.

2.5.4. Calibration

Calibration experiments were carried out for both

batch (static) (Figure 2.2) and flow systems (Figure 2.3). Conditions as shown in Figure 2.3 for flow injection analysis. Batch experimental conditions were as follows : 10 % (w/w) GOx, 10 % (w/w) PO, detection potential : -0.20 V, electrolyte : phosphate buffer (pH 7.4) containing 10 mM ferrocyanide.

For batch experiments, the GOx/PO-modified electrode showed linearity over the concentration range ($2x10^{-4}$ - $2x10^{-3}$ M), (slope 1.2 μA/mM, correlation coefficient 0.999) investigated.

Linearity was also observed up to $5x10^{-3}$ M glucose, over the concentration range ($1x10^{-3}$ - $10x10^{-3}$ M), (slope 0.2 μA/mM, correlation coefficient 0.991). For calibrations carried out under flow conditions, linearity was observed over the entire concentration range ($5x10^{-3}$ - $25x10^{-3}$ M) investigated. (Slope 0.7 μA/mM, correlation coefficient 0.9952.).

The GOx/PO-modified carbon paste electrode exhibited very fast response times as seen by the shape of the FIA peaks (see Figure 2.3), producing 95 % of the current response in under 30 s with a possible sample throughput of 60 samples per hour. This indicates a rapid reaction mechanism which includes transport of the substrate to the reactive surface, conversion to a product and transport of the final product (shown earlier in the reaction sequence to be the reduced ferricyanide) away from the electrode. An important factor in this mechanism is

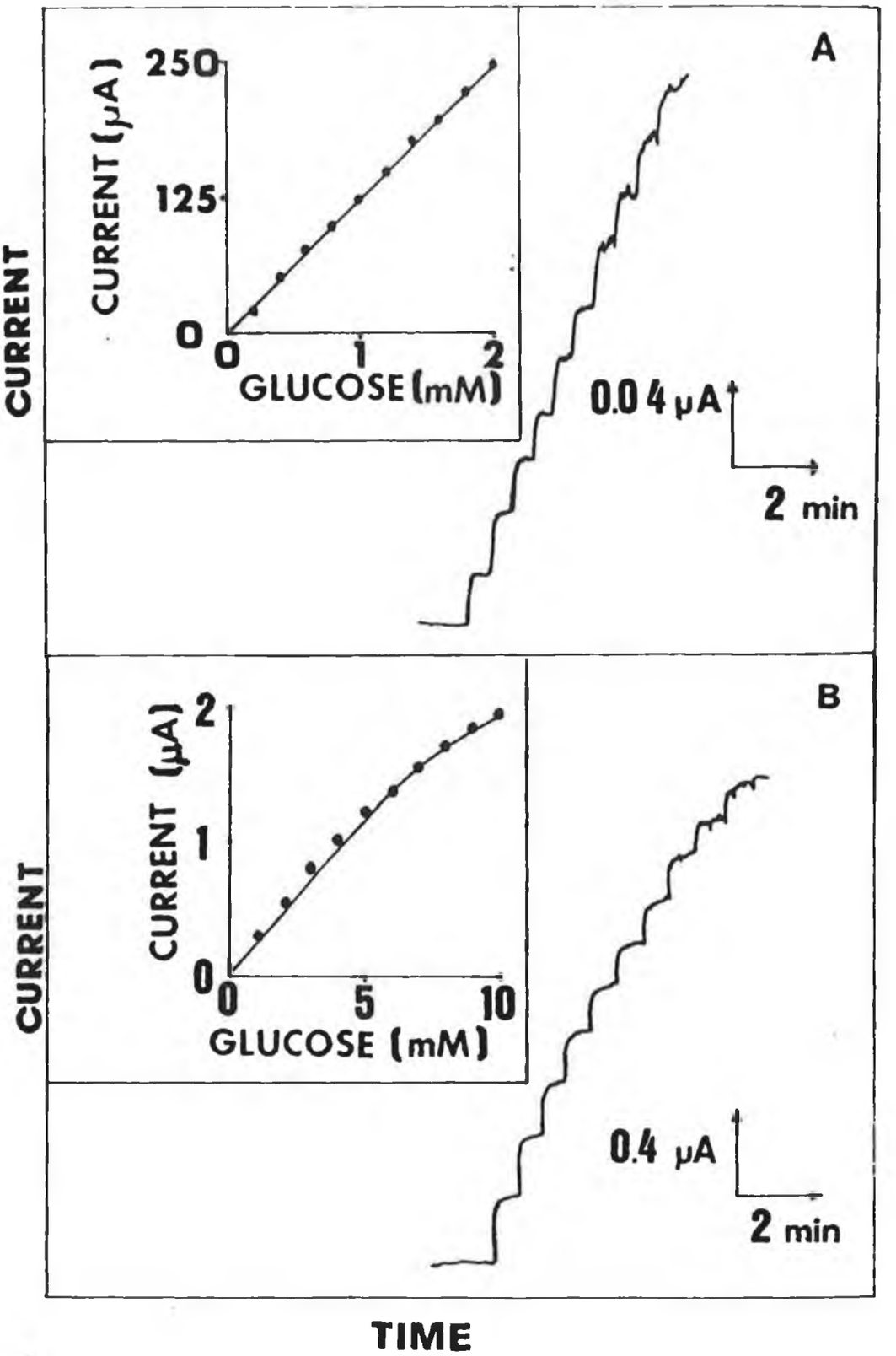

Figure 2.2. : A. Calibration curve for glucose concentration in the range 0-2 mM for batch conditions. 0.01 M ferrocyanide in phosphate buffer (pH 7.4), 10 % GOx, 10 % PO (w/w), -0.20 V. B. Calibration curve for glucose concentrations in the range 0-10 mM. Conditions as in A.

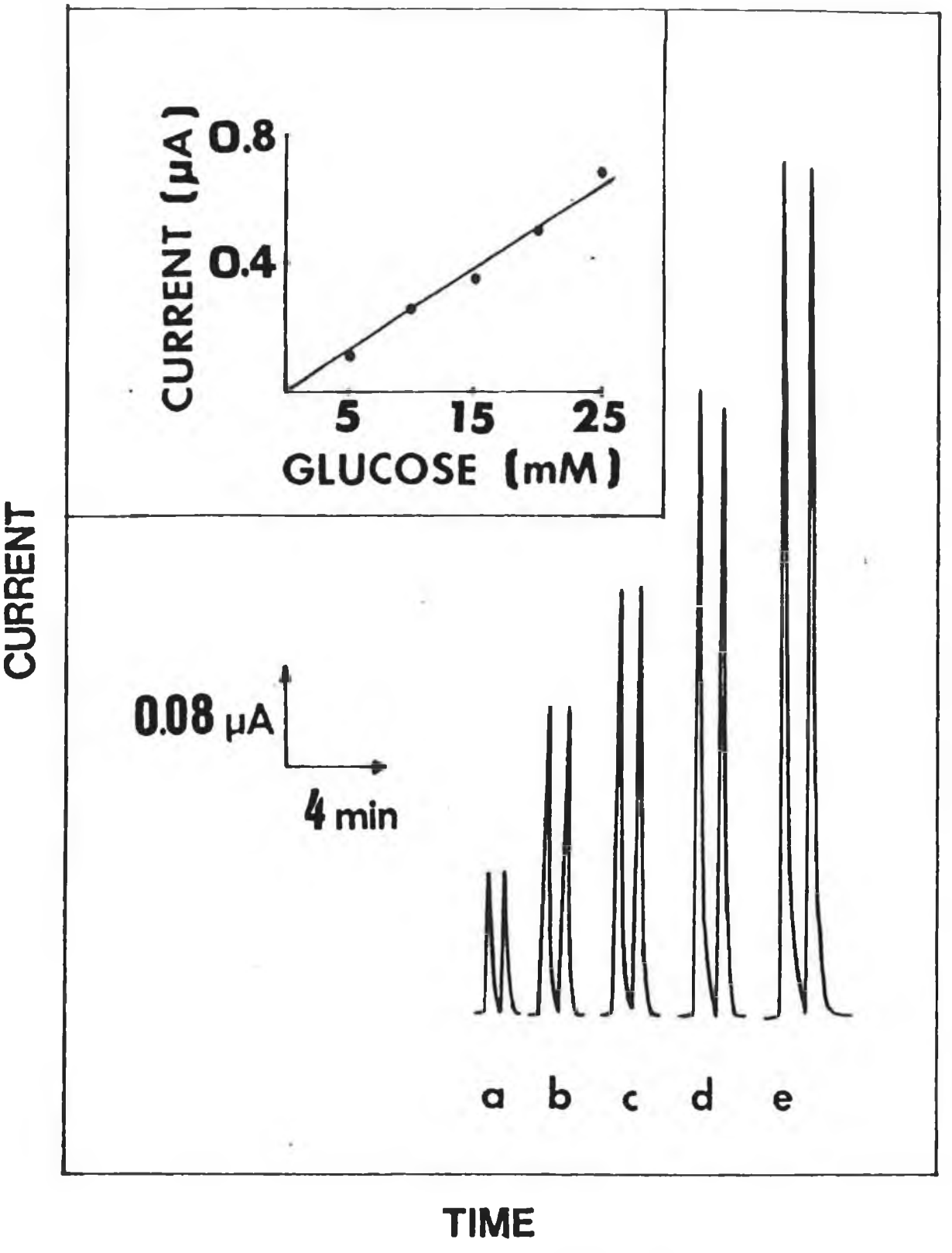

Figure 2.3. : Flow injection peaks obtained for increasing concentrations of glucose at the GOx/PO (10 % (w/w) each)-modified carbon paste electrode. Electrolyte, 0.05 M phosphate buffer (pH 7.4) with 0.01 M ferrocyanide; detection potential, -0.20 V; correlation coefficient, 0.9952; limit of detection, 60 μM glucose.

the recycling of the mediator as seen in the reactions involved. A detection limit of $6x10^{-5}$ M glucose was calculated based on a signal-to-noise ratio (S/N) of 3.

2.5.5. Interference Studies

At a plain carbon paste electrode both uric acid and glucose are detected (+0.90 V) under the conditions shown in Figure 2.4. However, at the GOx/PO-modified electrode, glucose may be detected in the presence of the normally interfering uric acid, also shown in Figure 2.4. There are two reasons for this, one being the bioselective nature of glucose oxidase (uric acid is not a substrate of this enzyme) and secondly, the fact that the analysis is carried out in the reductive mode. (Uric acid is not detected at -0.20 V.).

2.5.6. Stability Test

A stability test was carried out over a period of three weeks on the bi-enzyme-modified electrode, without any surface renewal. Between measurements, the working electrode was stored in phosphate buffer (pH 7.4) at $4^{o}C$. For each measurement, glucose was spiked into the electrolyte to obtain a final concentration of 4 mM. Over the first three days of the test, the electrode exhibited an increase in

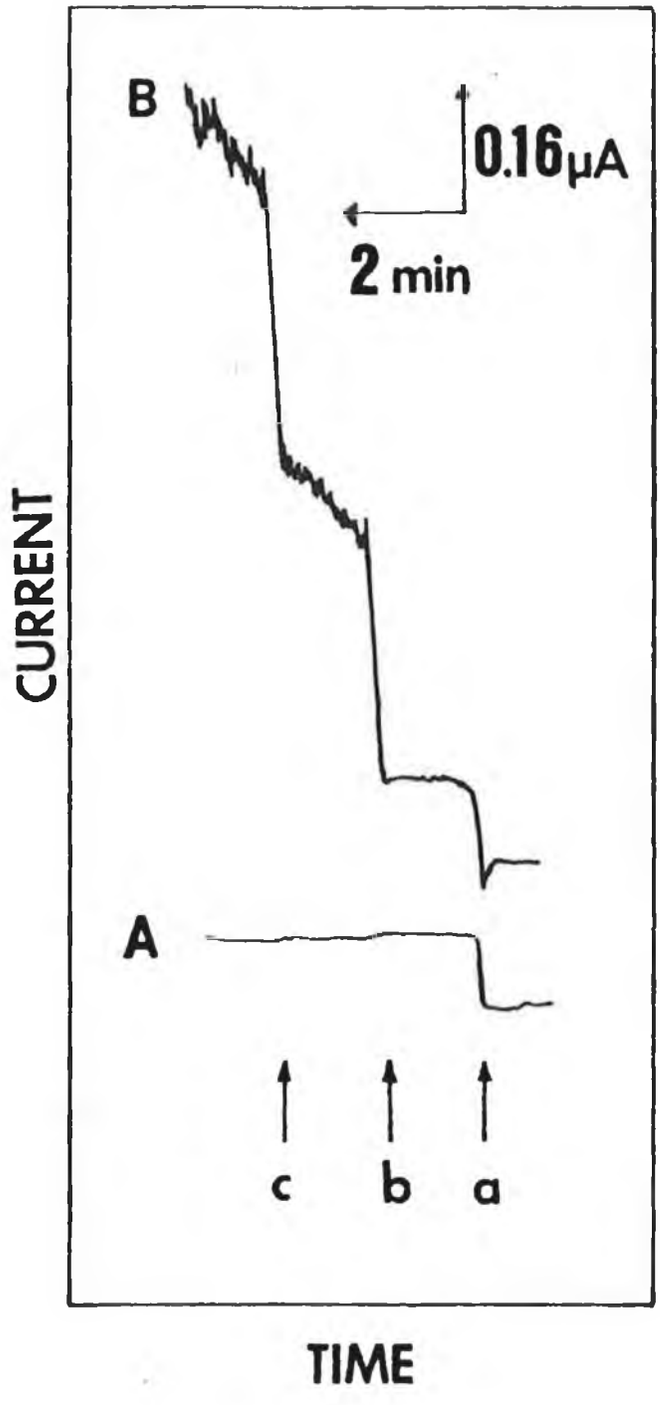

Figure 2.4. : Current responses for batch experiments carried out to investigate interference by Uric acid. A : 10% GOx/10% PO (w/w); 0.01 M ferrocyanide in phosphate buffer (pH 7.4), -0.20 V. B : no mediator, +0.90 V; 10% GOx. a. 3 mM glucose. b. 5 μM uric acid. c. 5 μM uric acid.

current response which could possibly be attributed to "settling" of the enzyme within the matrix of the carbon paste. After four days, the electrode exhibited excellent stability showing no significant change in response.

2.5.7. Detection of Bilirubin

Bilirubin is a toxic substance and is normally detected by spectrophotometry. Detection of bilirubin is important for diagnostic purposes and therapeutic monitoring and was possible to detect using the GOx/PO-modified carbon paste electrode. Conditions and results are shown in Figure 2.5. Note that no mediator is necessary, since detection of bilirubin is based on the consumption (decrease in current response) of H_2O_2, (in the presence of PO) which has been produced as a result of the conversion of glucose in the presence of GOx and O_2 to gluconic acid. Under batch conditions, glucose is spiked in and the current response allowed to equilibrate (due to the oxidation of H_2O_2 at +0.90 V). Bilirubin is then added and the decrease in current response, as shown in Figure 2.5, used for quantitative analysis.

2.6. Conclusions

The excellent response characteristics and stability of this bienzyme electrode indicates the

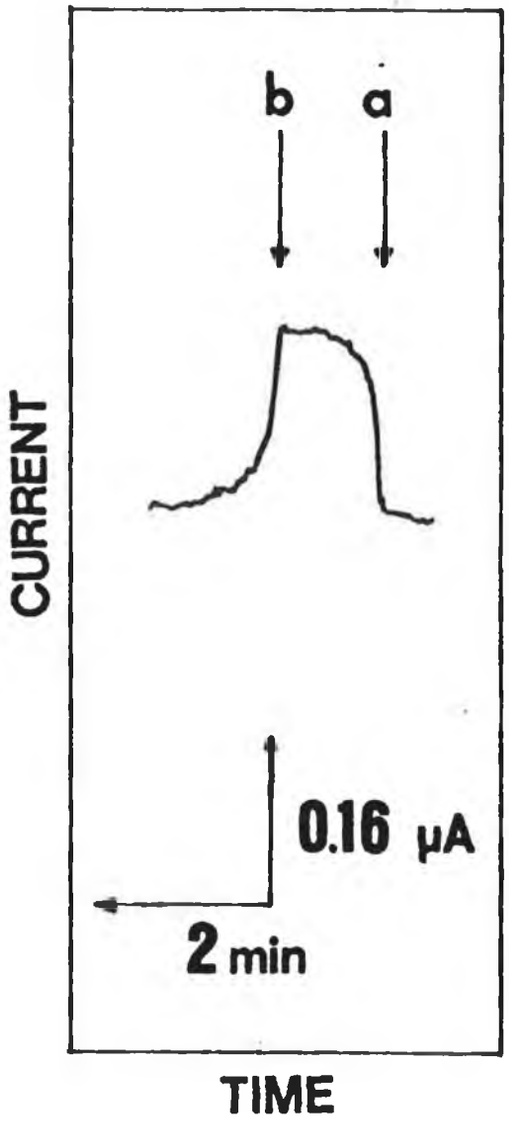

Figure 2.5. : Trace showing the detection of bilirubin at 10% GOx/10% PO (w/w) modified carbon paste electrode. No mediator; +0.90 V; 0.05 M phosphate buffer (pH 7.4). a. 2 mM glucose. b. 5 µM bilirubin.

feasability of using enzyme coupled reactions for the detection of glucose. Interferences due to metabolites (such as uric acid) are eliminated because amperometric detection is carried out in the reductive mode (-0.20 V).

Fast response times lend the possibility of high sample throughput and the electrode exhibited excellent linearity within a clinically applicable range.

The use of ferrocyanide as a mediator has the advantage in that it is recycled in the series of reactions that are involved in the detection of glucose. The use of modified carbon paste, facilitates easy and simple electrode surface renewal which does not involve any complex immobilisation procedure such as cross-linking of the enzyme to a support or electrode surface.

In effect, a very useful sensor has been developed involving the coimmobilisation of GOx and PO. It may also be feasible to include the mediator in the carbon paste which would eliminate the need for addition of the mediator to the electrolyte or mobile phase.

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CHAPTER 3

SILICONE-GREASE BASED IMMOBILISATION METHOD FOR THE PREPARATION OF ENZYME ELECTRODES

3.1. Introduction to Immobilisation Methods

Enzyme-based amperometric sensors offer many possibilities in areas such as clinical diagnosis or biotechnology. Such probes consist of a thin layer of the enzyme immobilised on the surface of the sensing electrode [1-3]. The performance of enzyme electrodes is strongly dependent on the immobilisation procedure used.

Over the years, numerous methods for enzyme immobilisation have been developed [4]. Enzymes may be immobilised in a variety of ways with little or no immediate loss of their biocatalytic activity. Most enzymes in their natural environment are immobilised to some support, be it a membrane or some cell organelle. An enzyme is affected by changes in its environment and as such, its behaviour may change. Thus the selection of an immobilisation method must at least not affect the biocatalytic activity of the enzyme and if possible, even mimic its natural environment.

One of the main advantages of immobilising an enzyme is cost. Purified enzymes are expensive; so the ability to add, remove and reuse enzymes at will is particularily attractive. Enzyme immobilisation started over 70 years ago with work done by Nelson and Griffin [5]. Through a process of adsorption, invertase was immobilised on charcoal with no loss of bioactivity. Later on in the 1950's, Grubhofer and

Schlecht [6, 7] reported on the immobilisation of several enzymes on polyaminopolystyrene and a chlorinated resin by covalent attachment.

3.2. Classification of Immobilisation Methods

There are several methods of enzyme immobilisation classified under two main divisions: that of chemical immobilisation and physical entrapment. Among the techniques available are : absorption, adsorption, cross-linking, ion-exchange, entrapment, microencapsulation, copolymerisation, covalent attachment, electrochemical deposition and immunological attachment. However, to simplify matters, immobilisation technology is based on four main techniques :

(i). Adsorption;

(ii). Cross-linking;

(iii). Entrapment; and

(iv). Covalent attachment.

3.2.1. Adsorption

The adsorption of enzymes onto surfaces (see Figure 3.1) is dependent on many variables, including pH, type of solvent, ionic strength, temperature and enzyme concentration. The stability of the adsorbed enzyme and its activity are dependent on assay and storage conditions, substrate concentration and ionic

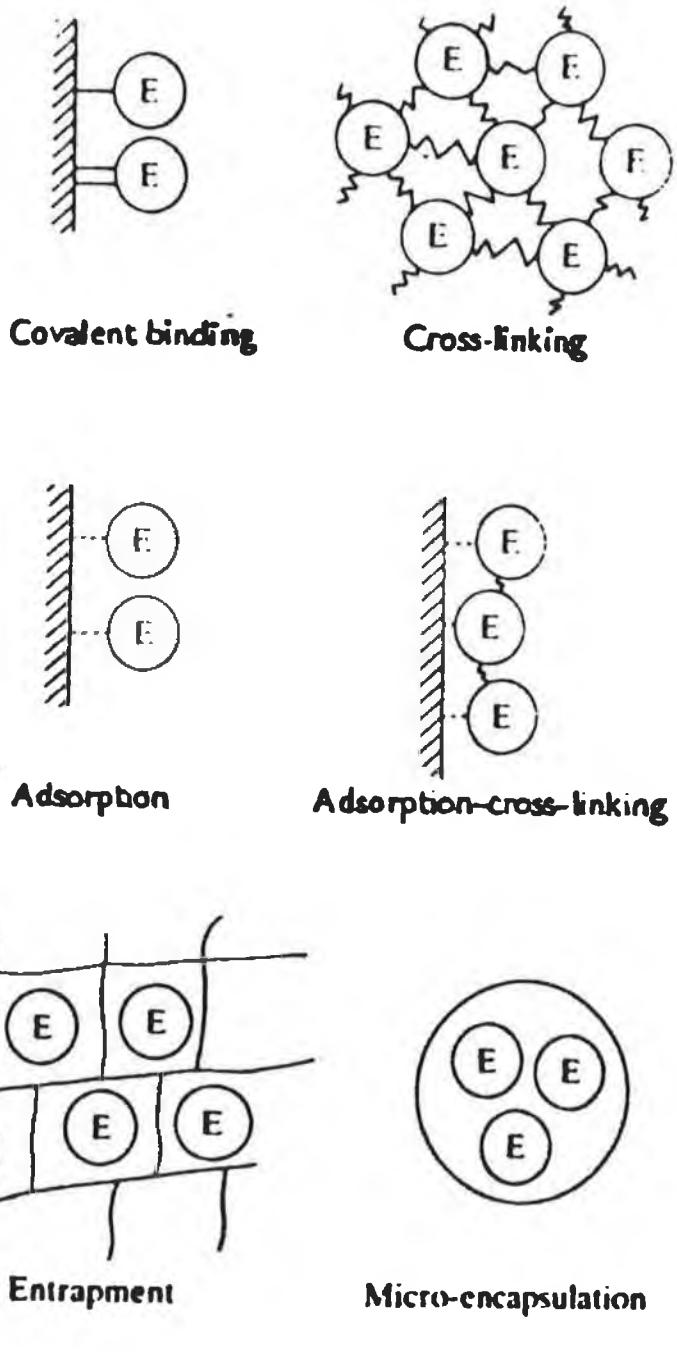


Figure 3.1. Methods of enzyme immobilisation.

strength of storage solution.

Commonly used adsorbents include alumina, carbon, celluloses, clays, hydroxyapatite and glasses such as controlled pore glass. Messing et al. [8, 9] have shown that enzymes adsorbed to glass surfaces have extremely long half-lives and can be successfully used repeatedly without loss of activity.

The great advantage of adsorption is that usually, no reagents are required and it also tends to have less of an effect on the nature of the enzyme compared to chemical methods. Binding forces are due to hydrogen bonds, multiple salt linkages and Van der Waal's forces. Provided that the experimental conditions are strictly controlled, ionic interaction may be exploited in the immobilisation step [10]. Reports are also available on the use of hydrophobic interactions as a means of immobilising an enzyme [11]. In these methods, irrelevant proteins may be adsorbed along with the enzyme. Furthermore, displacement of the enzyme may take place during the subsequent operation due to competition for binding sites on the matrix.

3.2.2. Cross-Linking

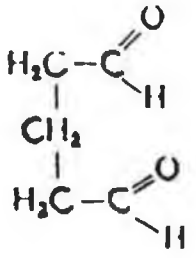
Enzymes have also been immobilised using a combination of adsorption and "cross-linking" with for example glutaraldehyde (a bifunctional reagent) [12]. Enzymes linked via glutaraldehyde to glass beads

have been reported [13-16]. The enzyme is coupled to the primary amino groups of commercially available alkylamino glass by two different methods.

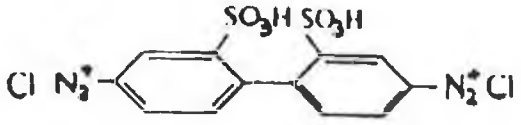
(i) the enzyme and glass beads are allowed to react with glutaraldehyde which forms Schiff base linkages both with the primary amino groups of the enzyme protein and with those of the glass beads.

(ii) the carbohydrate moiety, if present in the enzyme molecule to be immobilised, is first oxidised with periodate to form dialdehyde groups which are then coupled with the primary amino groups of the glass beads through Schiff base formation.

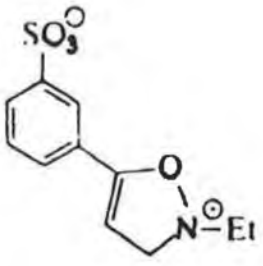
Enzymes can be immobilised using only cross-linking (see Figure 3.1). This is achieved using with low molecular weight multi-functional reagents (Figure 3.2), producing covalent bonds with intermolecular cross-links between the reagent and the enzyme. The activity of the cross-linked enzyme is dependent on a number of parameters, including enzyme concentration, pH and ionic strength. Reagents used for this method include : diisocyanates, diisothiocyanates, disulphonic acids and carbodiimides, as well as glutaraldehyde.



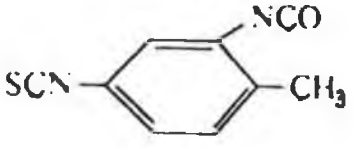
Glutaraldehyde



Bisdiazobenzidine-2.2'-disulphonic acid

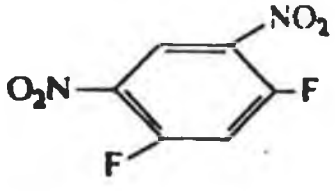


N-ethyl-5-phenylisoxazolium 3'-sulphonate (woodward reagent K)



Toluene-2-isocyanate 4-isothiocyanate

Hexamethylene diisocyanate



1.5-Difluoro-2.4-dinitrobenzene

Figure 3.2. Some common bifunctional reagents used for cross-linking.

3.2.3. Entrapment

Enzymes may also be immobilised by entrapment within a cross-linked water molecule polymer [17-19]. The method of preparation usually involves the cross-linking of the polymer in the presence of the enzyme, thus physically entrapping the enzyme (Figure 3.1).

If a polymeric film is prepared in a solution containing a biocatalyst, the biocatalyst becomes trapped within the 3-dimensional lattice of the gel matrix. The polymer lattice structure is such that the large enzyme molecules cannot diffuse out, but small substrate molecules can diffuse through the polymer.

Entrapment may also be carried out by immobilising the enzymes onto a solid support and covering this layer with a polymeric film such as cellulose acetate [20]. Other examples include starch gels and nylon. However, this method does have some disadvantages :

(i) the presence of a large diffusion barrier between the enzyme and substrate; and
(ii) some loss of enzyme from the active surface.

The latter may be overcome by cross-linking with glutaraldehyde. One important advantage of using this method is that the film may be used to prevent electroactive interferents from reaching the sensing surface. It may also protect the electrode surface

from passivation, caused by adsorption of protein molecules. Various polymers have been used to both immobilise and protect the enzymatic layer, where selectivity of response may be achieved by excluding interfering analytes and allowing detection of analytes of interest on the basis of analyte size or charge or both [21].

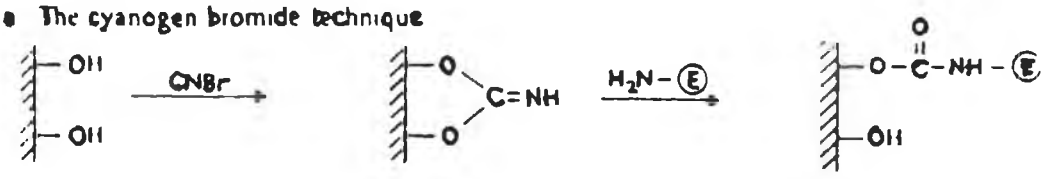
3.2.4. Covalent Attachment

Probably one of the most commonly used methods of immobilisation is via covalent attachment (Figure 3.1). Theoretically, covalent coupling offers the most stable, most versatile method of immobilising enzymes. Covalent bonding between the enzyme and support matrix is accomplished through functional groups in the enzyme which are not essential for its biocatalytic activity. Examples of some of the more common methods of such attachment are shown in Figure 3.3.

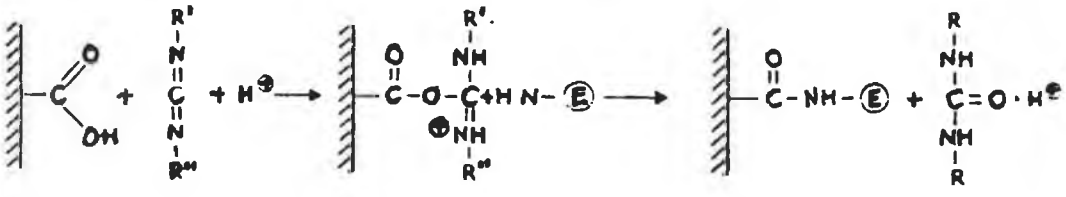
3.2.4.1. Carboxymethylcellulose azide

The carboxymethylcellulose is converted by the reactions shown in Figure 3.3.c. The method is relatively simple. The final coupling step is carried out at a slightly alkaline pH. The final product is an amide formed primarily to the -amino group of lysine.

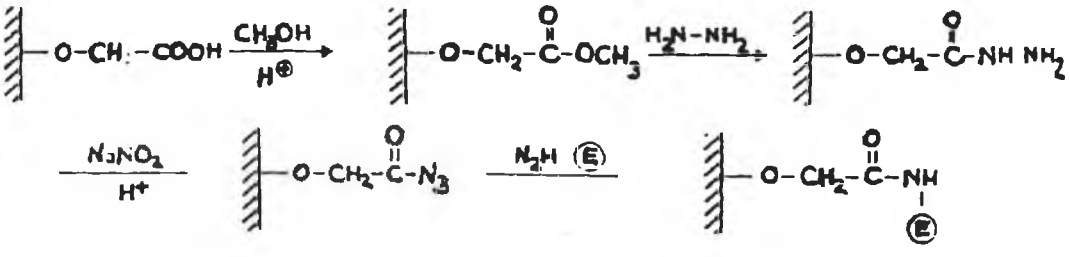
a The cyanogen bromide technique



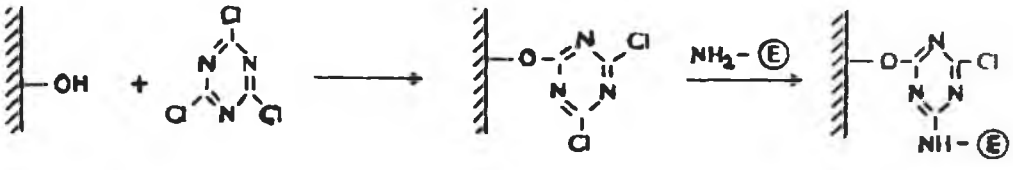
b The Carbodi-imide method



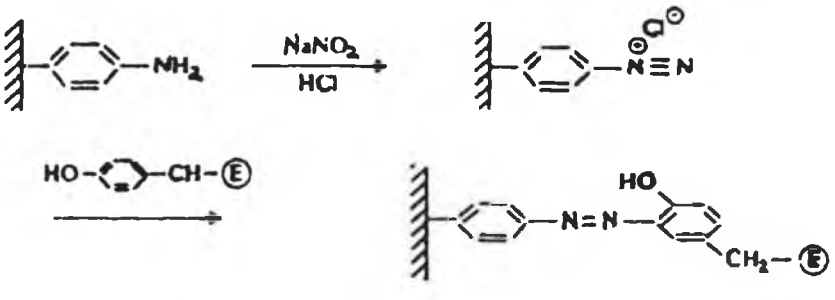
c. Via acyl groups by treatment of hydrazides with nitrous acid



d. Coupling using cyanuric chloride



e. Coupling through diazonium groups from aromatic amino groups



f. Coupling via thiol groups

Figure 3.3. Some common reactions used for covalent binding.

Other possible attachment sites include cysteine, serine and tyrosine.

3.2.4.2. Azo compounds

Arylamines can be diazotised and coupled via azo linkage to proteins [19]. The technique has been successfully used for covalent attachment of protein to polyaminostyrene, p-aminobenzoylcellulose and arylamine glass [23]. The coupling to protein occurs at a slightly alkaline pH. Coupling occurs through an available tyrosine residue, although lysine, arginine, cysteine and serine have also been implicated in the reaction.

3.2.4.3. Isocyanates and Isothiocyanates

Arylamines and alkylamines can be converted to isocyanates and isothiocyanates. These activated derivatives will then react with amine on the protein to form substituted ureas.

3.2.4.4. Carbodiimides

This occurs through amide linkage as seen in Figure 3.3.b.

3.2.4.5. Cyanogen Bromide

The activation of cross-linked dextrans, including agarose and even cellulose, is a simple and attractive method of covalently coupling proteins to water insoluble carriers [24]. The reaction sequence is shown in Figure 3.3.a.

3.2.4.6. Glutaraldehyde

Glutaraldehyde (Figure 3.2) was mentioned previously as a cross-linking agent for attachment of an enzyme to a support such as glass beads. It is one of the simplest and most gentle of coupling methods [25-27].

3.2.5. Some Methods of Immobilisation related to Enzyme Electrodes

Blaedel et al. [28] immobilised alcohol dehydrogenase on cyanogen activated sepharose gel which was packed into a reticulated vitreous carbon (RVC) disk. A 15 s response time was reported. A recent development by Wang et al. [29] involved the use of a modified graphite epoxy electrode which proved to be robust, fast in response (18 s) and capable of acting in organic media.

Savangikar et al. [30] reported on the immobilisation of pepsin in paraffin wax, which when

molten was applied as a thin film to a solid support. Gunasingham et al. [31] described a glucose sensor based on the immobilisation of glucose oxidase by simultaneous electrodeposition of the enzyme and platinum onto a glassy carbon electrode, combining the electrocatalytic activity of platinum and the stability of glassy carbon.

3.2.6. Effect of Immobilisation on the Enzyme

The physical or chemical confinement of an enzyme within a matrix has a number of effects on the characteristics of the enzyme catalysis. Such changes may arise from interactions between the enzyme and the support which affect the catalytic reaction, for example, by distortion of the active site. Another possible cause of such changes in the enzyme could be a difference between the bulk solution and the area immediately surrounding the enzyme.

3.2.6.1. Effect of Diffusion on Kinetics

The rate of conversion of substrate to product is affected by both mass transfer of the substrate in the bulk solution (external) and within the support matrix (internal). External mass transport is partly attributed to diffusion. If the fluid to be analysed is near the surface of the support because of bulk flowing or stirring, convective transport will also

contribute to the delivery of the substrate to the enzyme.

Slow mass transfer invariably increases the apparent Michaelis constant, K_m^{app}. Mass transfer within the support matrix is under the influence of parameters such as substrate size and concentration and enzyme loading. Increasing the enzyme loading usually results in an increase in the analytical response, but may result in a decrease in the efficiency of enzyme use.

3.2.6.2. Effect on Stability

In certain cases, a particular immobilisation method may lead to enhanced stability of the enzyme. Stability may be defined in terms of :

(i) storage - which is the ability of the enzyme to retain its biocatalytic activity under set storage conditions.

(ii) thermal stability - which is a measure of the ability of the immobilised enzyme to withstand elevations in temperature often in excess of those that would denature the enzyme proteins.

(iii) operational stability - which is a measure of how the enzyme sensor functions under experimental conditions i.e. analysis.

Although the inactivation process (loss of activity

of the enzyme) may be different for each individual enzyme, the generally accepted reason involves the unfolding of the three-dimensional structure of the protein which ultimately disfigures the active site of the enzyme. However, loss of activity may not simply be due to such an effect as the immobilisation procedure used, but may be a function of other factors such as the presence of certain solvents.

It is interesting to note that the use of either miscible [32] or immiscible [33] solvents can increase the observed rate of reaction because of increased stabilisation of either substrate, product or enzyme.

3.3. Background to Proposed New Immobilisation Procedure

The method proposed in this chapter differs from traditional enzyme electrode schemes, in that it is based on mixing the enzyme with silicone grease and using the enzyme-containing grease to "fill" the micropores of a graphite surface. This method results in a self-supported (membrane-free) enzymatic layer, which is in close contact with the conducting graphite sites. The ability of enzymes (e.g. xanthine oxidase, tyrosinase) to catalyse reactions in a non-polar organic environment has long been recognised [34, 35]. The immobilisation of pepsin has also been described [30]. The new method is used for the construction of a tyrosinase electrode. Tyrosinase (mushroom

polyphenol-oxidase, E.C. 1.14.18.1) is an extremely interesting enzyme because many of its phenolic substrates are of analytical significance.

The biocatalytic activity of tyrosinase in organic phases has recently been exploited for synthetic organic work [35] and improved quantification of p-cresol [36]. Some other work in this thesis desribes a mushroom-modified carbon-paste electrode for the detection of phenolic compounds based on the presence of this enzyme in the natural material [37].

The new immobilisation procedure combines the extreme simplicity of preparation with the high speed of response. Its characteristics are investigated in the following sections.

3.4. Experimental

3.4.1. Apparatus

Batch experiments were performed in a Bioanalytical Systems (BAS) Model VC-2 electrochemical cell. The enzyme-based working electrode, reference electrode (Ag/AgCl, Model RE-1, BAS) and platinum wire auxiliary electrode joined the cell through holes in its Teflon cover. A magnetic stirrer and stirring bar (7 mm long) provided the convective transport.

The flow injection system consisted of a carrier reservoir, a Rheodyne Model 7010 injector (20 µl loop), 1 mm i.d. Teflon connecting tubing and the thin

layer electrochemical detector. The Ag/AgCl reference and stainless steel auxiliary electrodes were located in a downstream compartment (Model RC-2A, BAS). Flow of the carrier solution was maintained by gravity.

The current response was measured with an EG & G PAR Model 174 Voltammetric Analyser connected to a Houston Omniscribe strip chart recorder.

3.4.2. Preparation of the Enzyme Electrode

The enzyme electrode was prepared in the following manner (see Figure 3.4 for configuration of both batch and flow electrodes). The enzyme containing grease was made by thoroughly mixing the enzyme tyrosinase and Dow Corning high vacuum silicone grease (usually 10 % enzyme by weight). A portion of this paste was packed into the micropores of the 3 mm diameter graphite (RW001, Ringsdorf-Werke) disk electrode.

The surface micropores were generated by polishing on a silicon carbide paper No. 320 and were filled by dipping in the enzyme-grease paste followed by smoothing on a deck of weighing paper. A copper wire provided electrical contact to the graphite disk.

A glass tube and electrode cavity of a thin layer detector housed the working electrode in batch and flow experiments respectively. Removal of the grease layer from the surface was easily carried out by sonication for 5 min in doubly-distilled water.

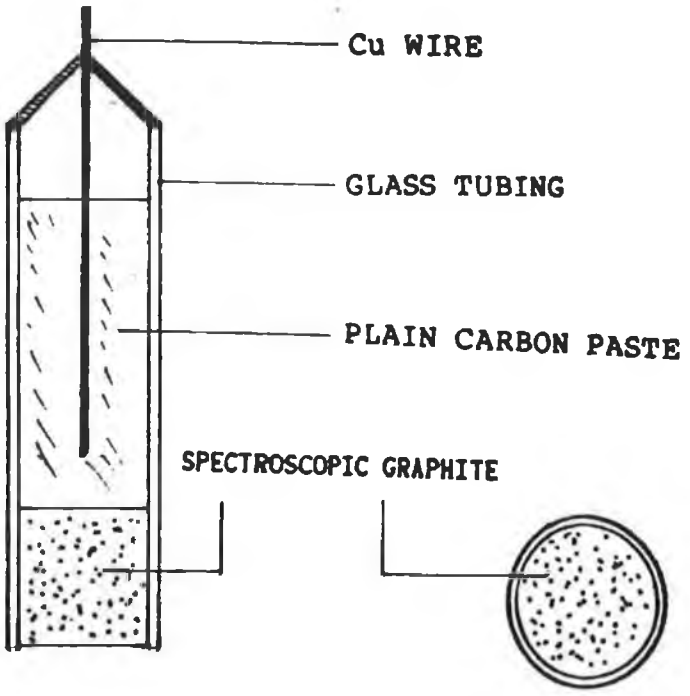


Figure 3.4. Structure of spectroscopic graphite enzyme-grease electrode.

3.4.3. Reagents and Procedure

All solutions were prepared with doubly distilled water. Tyrosinase (220 U/mg), dopamine, norepinephrine (Sigma), phenol (Fisher), p-cresol (Aldrich) were used without further purification. All measurements were performed in a 0.05 M phosphate buffer (pH 7.4) solution. Amperometric detection was performed at room temperature by applying a potential of -0.20 V and allowing the background current to decay.

3.5. Results and Discussion

3.5.1. Batch studies

Tyrosinase is sensitive to a broad range of phenolic compounds. Such compounds can be detected by reduction (at low potentials) of the enzymatically produced quinone species (see Figure 4.2 in Chapter 4.).

Figure 3.5 shows the response of the tyrosinase grease-based electrode to successive additions of dopamine, each addition effecting a 4×10^{-5} M increase in concentration. The electrode responds rapidly to the dynamic changes in the dopamine concentration, producing 95 % of the steady state current response in 5 s.

The high surface bioactivity is indicated from the large response for micromolar changes in the

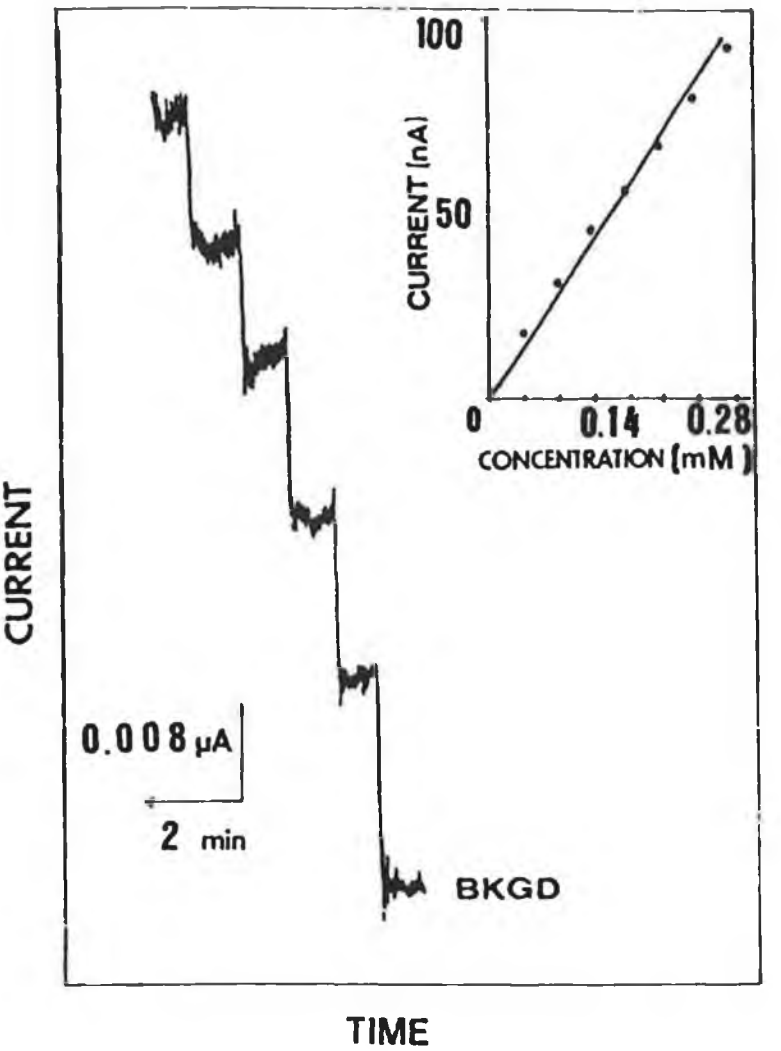


Figure 3.5. Typical strip-chart recording showing the dynamic response of the enzyme-grease electrode to successive increments of dopamine concentration in 4×10^{-5} M steps. Applied potential, -0.20 V; solution stirring, 400 rev. min^{-1}; electrolyte, phosphate buffer (pH 7.4). Also shown is the resulting calibration graphs over the range 4×10^{-5} - 28×10^{-5} M.

concentration. Based on a signal-to-noise ratio of 3, these data correspond to a detection limit of 6 x 10^{-6} M (10 µg in the 10 ml solution used). The five additions shown came from a series of seven concentration increments, the results of which are shown as an insert in Figure 3.5.

The response is linear with respect to dopamine concentration over the entire (4 x 10^{-5} - 2.8 x 10^{-4} M) range examined (slope, 317 nA/mM; intercept, 0.3 nA; correlation coefficient, 0.998).

Similar response characteristics were observed in analagous measurements of the monophenols, p-cresol and phenol (sensitivities of 373 and 913 nA/mM, respectively). The effect of the enzyme loading on the amperometric response was evaluated.

Calibration plots for dopamine constructed at the 2, 5 and 10 % (w/w) enzyme containing grease electrodes yielded slopes of 39, 47 and 317 nA/mM, respectively. Hence, the response increases with the increase in the bioactivity (but not with the expected direct proportionality). A 10 % (w/w) tyrosinase loading was used in all subsequent work.

3.5.2. Flow Injection Studies

The fast response of the tyrosinase / grease-based electrode makes it suitable for use in dynamic flow systems. Figure 3.6 depicts flow injection response peaks at the tyrosinase based thin-layer detector for

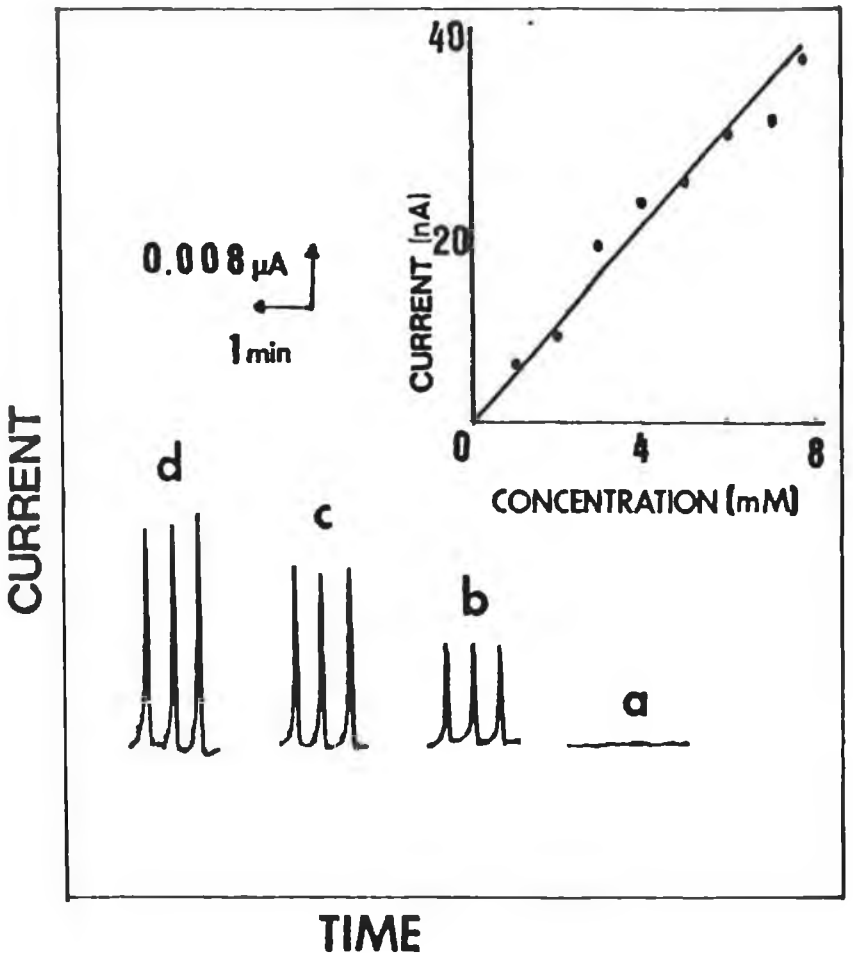


Figure 3.6. A. Blank solution response and flow-injection peaks for solutions containing increasing norepinephrine concentrations of B, $2x10^{-3}$; C, $4x10^{-3}$; and D, $6x10^{-3}$ M. Flow rate, 1.0 ml/min; other conditions as in Figure 3.5. Also shown is the resulting calibration graph over the range $1x10^{-3}$ - $8x10^{-3}$ M.

norepinephrine solutions of increasing concentration (2-6 mM). Short response times and a rapid return to the baseline were observed, indicating a facile transport of the substrate and removal of the product.

The peak half width was 4.8 s, allowing an injection rate of 120 samples per hour. The three measurements shown in Figure 3.6 are part of a series of eight concentration increments over the 1×10^{-3} to 8×10^{-3} M range. The resulting linear calibration plot is shown in Figure 3.6 (slope, intercept and correlation coefficient of 5 nA/mM, 0.15 nA and 0.990 respectively).

A series of 20 repeat injections of a 2.5×10^{-3} M norepinephrine solution yielded a mean peak current of 10.1 nA, a range of 9.4-10.4 nA and a relative standard deviation of 2.4 %. Because of the negative potential used for monitoring the quinone product, such measurements of cathecolamines are not affected by otherwise interfering species (e.g. ascorbic acid).

The tyrosinase / grease-based electrode had an analytically useful response for up to 12 days (with intermittent usage and storage at $4^{o}C$ in phosphate buffer (pH 7.4) between measurements). A slowly decreasing sensitivity was observed during this period. It is not clear, at the present time, whether micro-environmental effects (of the grease) contributed to the loss of bioactivity.

Earlier studies of tyrosinase in nonaqueous media (e.g. chloroform) indicate a highly stable bioactivity

[35, 36]. Unlike hydrophilic solvents, which strip the essential water molecules from the surface of the enzyme (thereby inactivating it), tyrosinase retains its catalytic activity in hydrophobic environments, possessing no affinity for water [35].

The electrode design permits easy and fast replacement of the grease based enzyme layer. Alternatively, because of its extremely low cost, it could be used as a disposable device. At this point, the response mechanism of the enzyme grease electrode has not been determined. It is reasonable to assume that it includes transport of the substrate and product into, within and away from the grease layer. The extremely fast response indicates that diffusional resistance (both external and internal) have negligible impact on the actual reaction rate.

Alternatively, it is possible that the fast response is associated only with a thin film of the enzyme which is retained on the grease layer (and not with that suspended in the bulk of the layer). Obviously, changes in kinetic parameters and substrate specificity are expected upon exposure to the hydrophobic environment. These, and other environmental effects of the grease layer on the bioactivity, remain to be explored.

3.6. Conclusions

A new approach for constructing amperometric

biosensors based on the incorporation of an enzyme into silicone grease and using the grease to fill the micropores on a graphite surface, has been described. The enzyme-grease electrode concept, illustrated with the enzyme tyrosinase, offers a very simple, rapid and inexpensive approach for the fabrication of enzyme electrodes.

The tyrosinase electrode responds very rapidly to changes in the concentration of phenolic compounds. Response times ($t_{95\ \%}$) as low as 5 s have been determined.

In flow injection analysis, 120 samples per hour can be processed with a relative standard deviation of 2.4 %. The electrode remains active for about 12 days. The detection limit for dopamine is 6×10^{-6} M. This method of biosensor construction should be applicable to other enzyme / substrate systems.

In summary, the observations and discussion presented above point to the potential of the enzyme / grease electrode concept. This approach could be advantageously employed for biosensing applications where very fast response times and operating simplicity are desired. In view of earlier observations of the biocatalytic activity of various enzymes in non-polar media, this immobilisation procedure could be adapted to other enzyme / substrate systems. Other porous surfaces (of different materials or porosities) may provide a useful support for the enzyme containing grease.

3.7. References

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CHAPTER 4

TISSUE- AND MICROBE-BASED ELECTROCHEMICAL DETECTORS FOR LIQUID CHROMATOGRAPHY

4.1. Introduction

Liquid chromatography with electrochemical detection (LCEC) has become a well established technique for trace organic analysis [1, 2]. To date, most reports on LCEC have dealt with ordinary solid- (carbon or metal) and mercury-based electrodes. Although EC detection has the advantage of good sensitivity for amenable molecules, it sometimes suffers from problems of selectivity.

An interest in using modified electrodes for detection in both FIA and HPLC is therefore increasing because they provide the opportunity to tailor the electrode surface to meet more selective detection needs.

Most work in this area to date has focussed on electrocatalytic surfaces that enhance the detection of compounds with kinetically-hindered redox reactions [3, 4] and permselective coatings that offer improved selectivity and stability based on size or charge exclusion [5, 6]. Halbert and Baldwin used carbon paste electrodes containing cobalt phthalocyanine (CoPC) to detect cysteine, homocysteine, N-acetylcysteine and glutathione by catalysing the electrooxidation of these sulphydryl-containing compounds [3]. When used as the sensing electrodes in amperometric detection following liquid chromatography, the CoPC electrodes permitted detection of the compounds at the same potential.

Cox et al. [4] used modified electrodes for the determination of thiocyanate in flow systems, based on an iodine-modified platinum electrode coated with cellulose acetate and a glassy carbon electrode modified by anodisation in $RuCl_3/K_4Ru(CN)_6$.

Wang and Hutchins [5] described the use of a cellulose acetate-coated thin-layer glassy carbon detector in terms of its permeability and analytical use. The pore size of the cellulose acetate film could be controlled by varying base hydrolysis times of the film. As a result of excluding macromolecules from the surface, the stability and selectivity were greatly improved, electrode fouling was minimised and greater selectivity towards smaller analytes demonstrated in both flow injection and LC systems.

Wang et al. [6] also developed a Nafion-coated working electrode, which acted by excluding anionic and neutral interferences. The advantages of using a permselective polymeric film coated electrode for amperometric monitoring in flowing streams were demonstrated using its selective response to cationic neurotransmitters such as dopamine, epinephrine and norepinephrine. A bilayer electrode was also investigated combining the protective property of cellulose acetate with the selectivity of Nafion.

Biological tailoring of detector surfaces has been rarely used for LCEC, with the exception of immobilised enzyme flow cells e.g. those based on L- and D-amino acid oxidases [7]. These were used by Yao

and Wasa [7] as amperometric detectors for HPLC following splitting of the flow after elution from the column, and detecting D-isomers in one line and L-isomers in the other.

In this chapter, the selectivity improvements associated with the use of tissue- and microbe-based detectors for LCEC will be described. Recently there has been a growing interest in using cellular materials in conjunction with electrochemical sensors. Tissue- and microbe-based biocatalytic devices possess several advantages over their enzymatic counterparts, including improved stability, higher biocatalytic activity and reduced cost [8-10]. Enzyme sequence reactors have also been used where more than one enzyme is immobilised. Long response times and recovery times - associated with long diffusional pathways - precluded the use of early bioelectrodes for monitoring flowing streams such as chromatographic eluents. Recent efforts aimed at eliminating diffusional barriers have facilitated the development of fast-responding cellular-based devices.

In particular, mixed biocomponent / carbon paste surfaces [11] and bioreactor / electrode collector designs [12] have been shown suitable for measuring dynamic changes in concentration that characterise flow injection systems.

The former design by Wang and Lin [11] involved the use of a mixed-plant tissue-carbon paste electrode which gave a response time ($t_{95\ \%}$) as low as 12 s,

making rapid detection in flowing streams feasible. The elimination of a diffusion barrier allows rapid response to changes in the substrate concentation.

In a paper by the same authors [12], a dual electrode, consisting of a tissue "reactor" which generated electroactive species that were subsequently carried in the flow stream and detected amperometrically at a plain carbon paste "collector" electrode was described. Again, due to the absence of a diffusional barrier, fast response times were observed. The possibility of using these bioelectrodes in conjunction with commercially available, low dead volume thin-layer amperometric devices holds a great potential for LCEC. These features and the presence of multiple metabolic pathways or "class" selective enzymes in tissues and microorganisms are advantageous for LCEC operation. Indeed LCEC may be one approach to address the specificity limitation of some cellular materials.

A new selectivity dimension based on bioactivity is thus added to LCEC detectors, as only a few components in a complex sample are likely to be substrates of the enzyme(s) present. For LCEC one should consider the compatability of the cellular materials with the chromatographic mobile phase and post-column addition of co-factors and redox mediators whenever required.

4.2. Experimental

4.2.1. Apparatus

The liquid chromatographic system (Bioanalytical Systems (BAS) LC-303) consisted of a dual piston pump (PM-30A), an ODS guard cartridge, a Rheodyne Model 7125 injector (20 µl loop), a Biophase C_{18} column (25 cm x 4.6 mm) and a BAS Model LC-3 amperometric controller. The mixed biocomponent / carbon paste thin-layer detector was prepared as follows.

A 5 mm thick section of the banana pulp was taken from near the centre of the fruit. 0.1 g of the pulp was crushed and mixed with 0.9 g of mineral oil (Aldrich) using a spatula. 1.1 g graphite powder (Acheson graphite, Fisher) was added to the banana-oil slurry and thoroughly mixed for 15 min. The mixed tissue-carbon paste was stored at $0^{o}C$.

A portion of this modified paste was packed into the end of a 4 mm i.d. glass tube (for batch experiments) or into the electrode cavity of the thin layer detector (for flow measurements),- Figure 4.1. A copper wire provided the electrical contact for the voltammetric measurements. New carbon paste surfaces were smoothed on a deck of computer cards or weighing paper. The series bioreactor / carbon paste collector flow cell was prepared as follows: two circular cavities, each 2.5 mm in diameter and 2.0 mm deep, were positioned in series in the thin-layer channel.

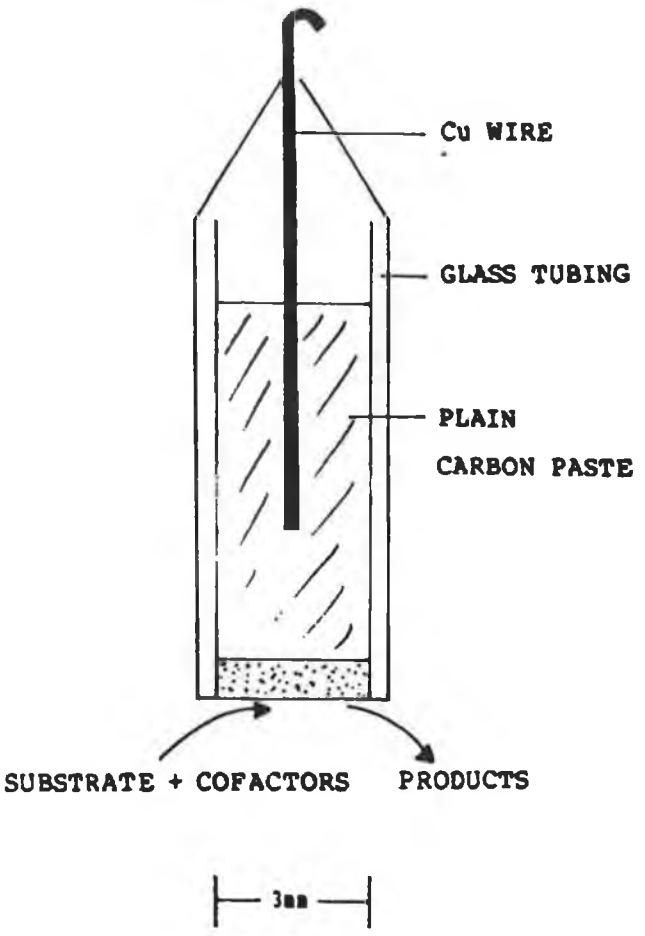

Figure 4.1. Configuration of carbon-paste-based batch and flow electrodes.

These were filled with the disk-shaped tissue slice (upstream) and carbon paste (downstream) respectively. The tissue slice was obtained as before.

A distance of 0.6 mm separated the tissue reactor and electrode collector. The Ag/AgCl (3 M NaCl) reference electrode was positioned downstream in the conventional manner.

Pastes containing 5 % banana, 2 % yeast and 5 % mushroom by weight were employed. Mushroom paste was prepared by crushing a 1/4 section of a fresh mushroom, removing excess water and mixing as for the banana with mineral oil and graphite powder. Yeast (Red Star, Quick rise) was prepared in a similar manner, by crushing dried yeast to a fine powder and thoroughly mixing with mineral oil and graphite powder.

A post-column addition of a solution containing 2 mM nicotinamide adenine dinucleotide (NAD^+) and 2 mM ferricyanide was employed in connection with the yeast detector. A 2 m coil of 0.5 mm i.d. Teflon tubing was placed between the column and the detector when oxidase-containing tissues were used as a surface modifier, since oxygen is removed from the mobile phase for the chromatographic separation.

4.2.2. Reagents

All solutions were prepared using distilled deionised water. Standards of dopamine,

norepinephrine, dopac, epinephrine, uric acid, gallic acid (Sigma), ascorbic acid, n-propanol (Baker), phenol, p-cresol, chlorophenol (Aldrich) and ethanol (US Industrial Chemicals Co.) were prepared daily.

The supporting electrolyte was 0.05 M phosphate buffer prepared using dipotassium hydrogen ortho-phosphate (Mallinkrodt) and the pH adjusted by addition of o-phosphoric acid or sodium hydroxide (banana, pH 4.5; mushroom, pH 5.0; yeast, pH 7.4).

All mobile phases (banana, 100 % phosphate buffer; mushroom, 70:30, phosphate buffer : acetonitrile (HPLC grade, Aldrich); yeast, 100 % phosphate buffer and post-column addition of 50:50, NAD^+ : $Fe(CN)_6^{3-}$.) were deaerated under vacuum for 7-10 min. The bananas and mushrooms used throughout the study were purchased at a local store and kept under refrigeration (5^oC) until required. All compounds investigated and the post column additives were dissolved in their respective mobile phases. Urine samples were obtained from healthy volunteers, filtered by passing through a 10-15 μm glass filter and diluted with mobile phase.

4.3. Results and Discussion

4.3.1. Banana Electrode

Phenol oxidases are copper proteins which occur widely in nature and catalyse the oxidation of certain

phenolic compounds to quinones which are then autooxidised to dark brown pigments, generally known as melanins.

The phenol oxidase known to be present in banana pulp is by substrate specificity a polyphenol oxidase. Another type of phenol oxidase is tyrosinase, typically found in mushrooms, which catalyses the oxidation of both mono- and di-phenols [14].

Yasunobu [14] has reviewed the substrate specificity of a number of phenol oxidases from various sources, both plant and animal. He concluded that these enzymes catalyse the oxidation of a wide variety of substrates, but that each individual enzyme tends to catalyse the oxidation of one particular phenol (or a particular type of phenolic compound) more readily than others.

Dopamine has been shown to be the most readily oxidised substrate of banana polyphenol oxidase. Figure 4.2 shows the general enzymatic reaction involved.

Amperometric measurements of biogenic amines (dopamine, epinephrine, norepinephrine, dopac, uric acid and ascorbic acid), primary alcohols (ethanol, n-propanol) and monophenols (gallic acid, phenol, p-cresol and chlorophenol) at banana-, yeast- and mushroom-modified carbon paste bioelectrodes respectively, were employed to demonstrate the utility of cellular-based detectors for LCEC.

Figure 4.3 illustrates the LCEC system used,

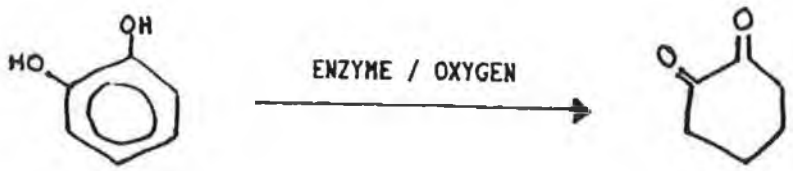

Figure 4.2. Enzymatic conversion reaction to a quinone species.

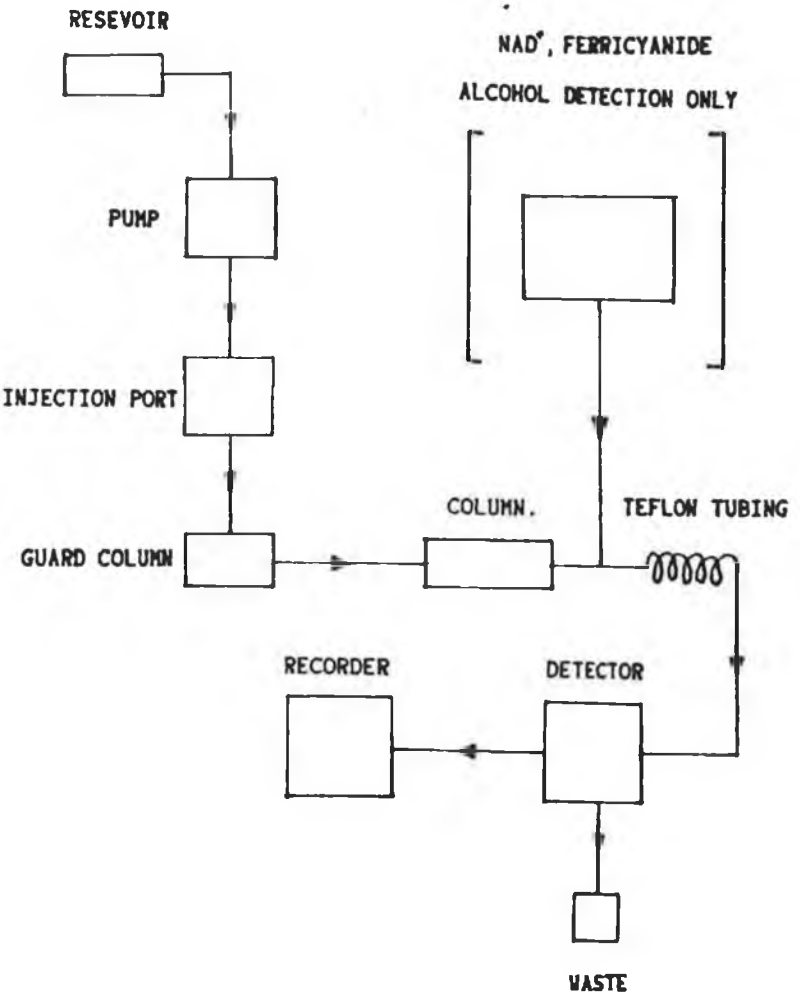

Figure 4.3. LC system used with tissue- and microbe-based detection.

including the reservoir for post-column addition of NAD^+ for detection of ethanol at the yeast-modified carbon paste thin layer detector. The 2 m coil of Teflon tubing not only allows for mixing of post-column additions with the mobile phase, but also for permeation of the oxygen necessary for the bioactivity of the oxidase enzymes.

Figure 4.4 shows the chromatogram obtained for a diluted (1:12) urine sample, spiked with dopamine, norepinephrine and epinephrine, as obtained at the mixed-banana carbon paste thin layer detector. Only three peaks, corresponding to the reductive monitoring of the quinone products of the biocatalytic reaction are observed despite the complex physiological matrix.

In contrast, five peaks, including sizeable and overlapping ascorbic acid and uric acid ones (t_r of 4 and 10 min respectively) were observed in conventional measurements of the oxidative current at the unmodified carbon paste detector (operating at a potential of +0.90 V) as seen in Figure 4.5. The chromatogram is thus greatly simplified by incorporating the plant tissue into the surface, as only substrates of the enzyme present, i.e. polyphenol oxidase, present are detected at the low negative reduction potential.

A similar simplification was observed for the same mixture, using the series banana reactor / carbon paste collector thin-layer cell. This cell consists of two cavities. Plain banana tissue was placed in the

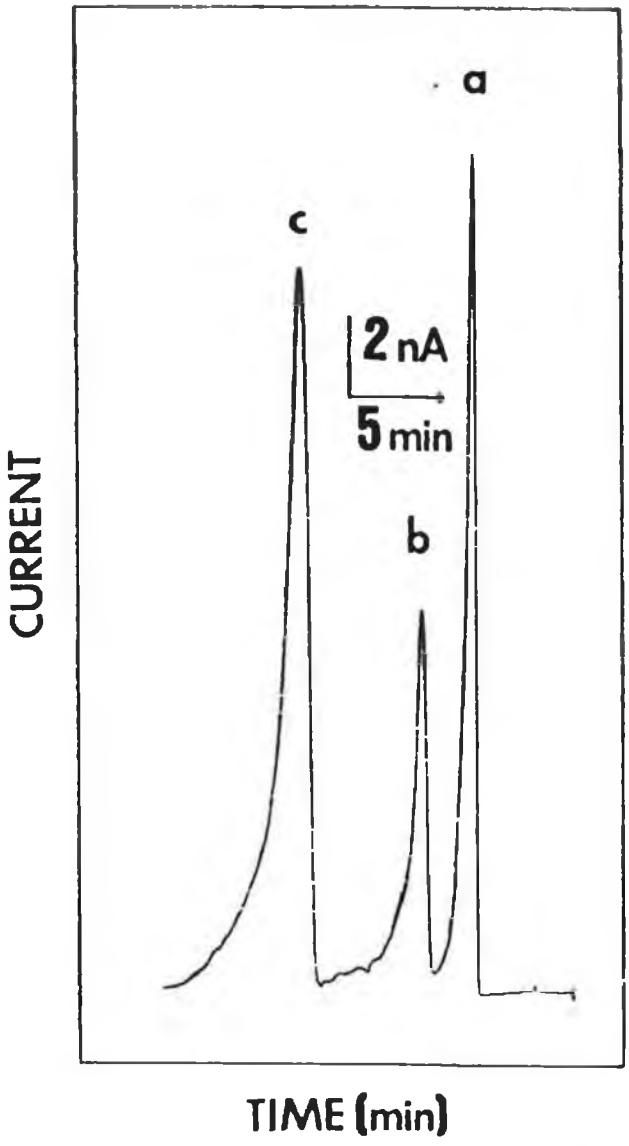

Figure 4.4. Chromatogram of a diluted (1:12) urine sample, spiked with norepinephrine, epinephrine and dopamine (5 µg injected; peaks a, b and c respectively) obtained at the mixed banana-carbon paste thin-layer detector. Mobile phase, 0.05 M phosphate buffer (pH 4.5); flow rate, 1.0 ml/min; applied potential, -0.20 V.

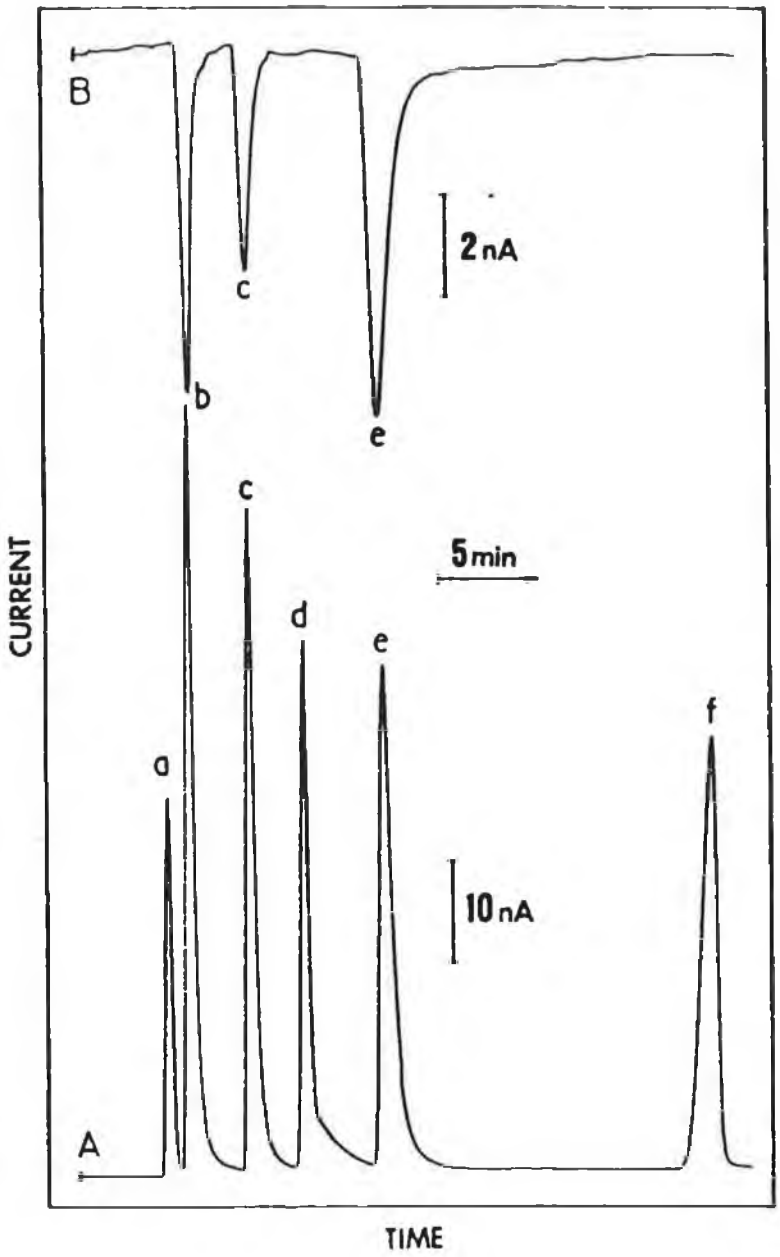

Figure 4.5. Chromatograms obtained at the unmodified (A) and banana-modified (B) carbon-paste detectors. Peak identities, (a) ascorbic acid; (b) norepinephrine; (c) epinephrine; (d) uric acid; (e) dopamine and (f) dopac. Mobile phase, 0.05 M phosphate buffer (pH 4.5); flow rate 1.0 ml/min; applied potential (A), +0.90 V and (B) -0.20 V.

first cavity and plain carbon paste in the second. As the flowing stream / mobile phase containing the analytes passes over the first cavity, they are catalytically oxidised to their respective quinones which are then reduced electrochemically at the plain carbon paste collector cavity (-0.20 V). It was interesting to note that there was no significant difference between these results and when the banana was immobilised in carbon paste. This would seem to indicate that the immobilisation procedure used, does not affect the bioactivity of the enzymes contained in the plant tissue.

All subsequent work was performed with mixed biocomponent / carbon paste electrodes. The new dimension of information based on bioactivity is particularily attractive in connection with dual electrode operation [15, 16]. Simultaneous operation of two electrodes (e.g. one bare and the second biologically modified) greatly improves the ability to obtain qualitative information about eluting chromatographic peaks.

For example, an estimate of peak purity can be obtained through generation of peak ratios (modified / unmodified). These provide a unique characterisation of the individual components based on their bioactivity through their K_m values. The merits of this concept were illustrated for a mixture of six solutes, using the banana-modified and unmodified electrodes (held at -0.20 V and +0.70 V respectively).

Current ratios of 0.06, 0.035, 0.025, 0.0, 0.0 and 0.0 were estimated for dopamine, norepinephrine, epinephrine, dopac, ascorbic acid and uric acid (all at 2 mM) respectively. This trend in current ratios is according to what is expected based on the substrate specificity of the banana polyphenol oxidase. Note again, that the bioelectrode does not respond to ascorbic acid, uric acid and dopac.

4.3.2. Mushroom Electrode

The mushroom / carbon paste thin layer cell offers similar selectivity improvements towards the detection of monophenolic compounds (see Figure 4.6). Many such compounds are substrates of the enzyme mushroom tyrosinase [17] and can thus be detected by reduction (at -0.20 V) of the enzymatically produced quinone species.

The extent of the biocatalytic reaction depends on the specific phenolic substance (dependent on the nature of the substituents and their position [17]) which is the key for the selectivity improvement. The enzyme polyphenol oxidase catalyses the conversion of phenols to o-quinones via a hydroxylation and subsequent dehydrogenation reaction. Peak current ratios (mushroom-modified / unmodified) of 0.19, 0.08, 0.07 and 0.0 were estimated for p-cresol, p-chlorophenol, phenol and gallic acid respectively. Note that gallic acid, which yields the largest

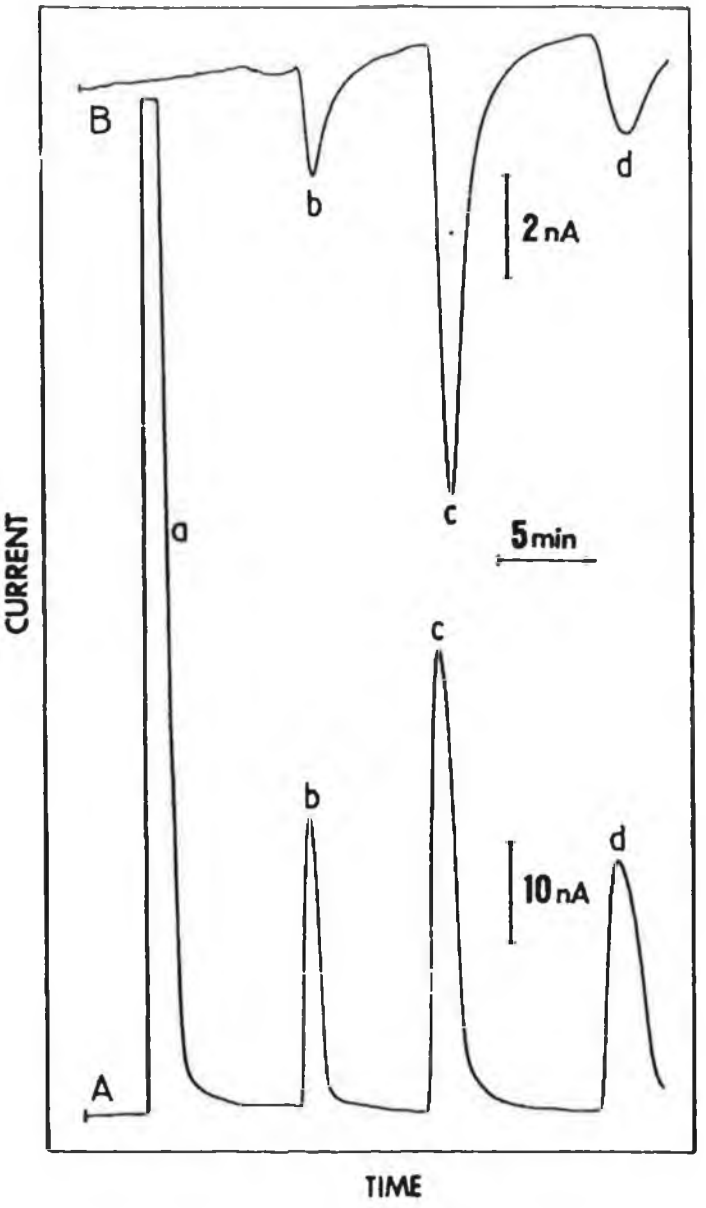

Figure 4.6. Chromatograms obtained at the (A) unmodified and (B) mushroom-modified carbon-paste detectors. Peaks: (a) gallic acid; (b) phenol; (c) p-cresol; (d) p-chlorophenol (all present at the 7 μg level). Mobile phase, 0.05 M phosphate buffer (pH 5.0)-acetonitrile (70:30); flow rate, 1.0 ml/min; applied potential, (A) +0.90 V and (B) -0.20 V.

response at the unmodified electrode, is not detected at the bioelectrode. Vanillic acid exhibited a similar behaviour.

The highest peak ratio, obtained for p-cresol is not surprising considering the known regioselectivity of the enzyme [17]; o- and m-cresols in contrast are not reactive, since the substituents must be in the para position.

Despite these substrate specificity trends, a LCEC operation is essential where "class" selective enzymes are concerned; an analogous flow-injection detection of the mixture illustrated in Figure 4.6 yielded the expected additive response. Unlike the mushroom tyrosinase, monophenols are not oxidised by the banana polyphenol oxidase [18].

Phenol oxidase is the generic term used to include all enzymes which catalyse the oxidation of phenols; tyrosinase catalyses the oxidation of both mono- and di-phenols; polyphenol oxidase catalyses the oxidation of ortho-dihydric phenols only. It may thus be possible to couple several tissue-containing bioelectrodes, e.g. mushroom- and banana-based surfaces, into an array detector for obtaining additional supplementary information.

The data shown in Figure 4.6 indicates also the feasability of using "organic-rich" mobile phases (e.g. 30 % acetonitrile) in conjunction with tissue bioelectrodes for LCEC. The ability of enzymes to catalyse reactions in organic solvents has been

recognised for more than two decades.

Dastoli and Price [19] found that milk xanthine oxidase could catalyse the oxidation of crotonaldehyde when the enzyme was present as a suspension in various organic solvents. The presence of organic solutions may actually be advantageous for the operation of the enzyme mushroom tyrosinase [17, 20].

Hall et al. [20] found that operating an enzyme electrode in an organic solvent enables a simpler and faster enzyme immobilisation procedure to be used. Because the enzyme is insoluble in the organic phase, it remains in a thin aqueous film retained on a hydrophilic support.

4.3.3. Yeast Electrode

Measurements of ethanol (and other primary alcohols) in complex samples can be obtained in a similar manner at the yeast-based detector for LCEC. Such measurements are not feasible at conventional amperometric detectors. Utilising the alcohol dehydrogenase (ADH) activity in Bakers yeast, it has recently been demonstrated that yeast-modified carbon paste electrodes permit convenient detection of primary alcohols in simple samples.

The incorporation of dry yeast into a carbon paste electrode results in an effective sensor for ADH substrates such as primary alcohols; secondary and tertiary alcohols are not oxidised. However, since

this procedure is based on the anodic detection of the reduced cofactor (NADH) or the redox mediator (ferricyanide) assays of complex samples require chromatographic separation of interfering oxidisable constituents.

Figure 4.7.A shows the chromatograms obtained at the yeast-modified carbon-paste thin layer detector for a diluted urine sample, (a) before and (b) after spiking with ethanol. A defined ethanol peak was observed at t_r=5.5min and simultaneous measurements of ascorbic and uric acids were also feasible (t_r of 3.0 and 4.0 min respectively). An ethanol response was not observed in analagous measurements at the conventional carbon paste detector as shown in Figure 4.7.B.

The post-column reagent addition does not affect the response times at the modified electrode. Alternatively, it may be possible to immobilise (and regenerate) the co-factor and redox mediator in the detector compartment and thus circumvent the need for post-column addition.

Because of their mechanism of action i.e. catalytically-controlled enzymatic reactions, it is essential to examine the concentration dependance of whole-cell based detectors for LCEC.

Calibration experiments were performed to evaluate the response at the banana, mushroom and yeast flow cells. The mushroom detector yielded a linear response for p-cresol over the entire concentration range

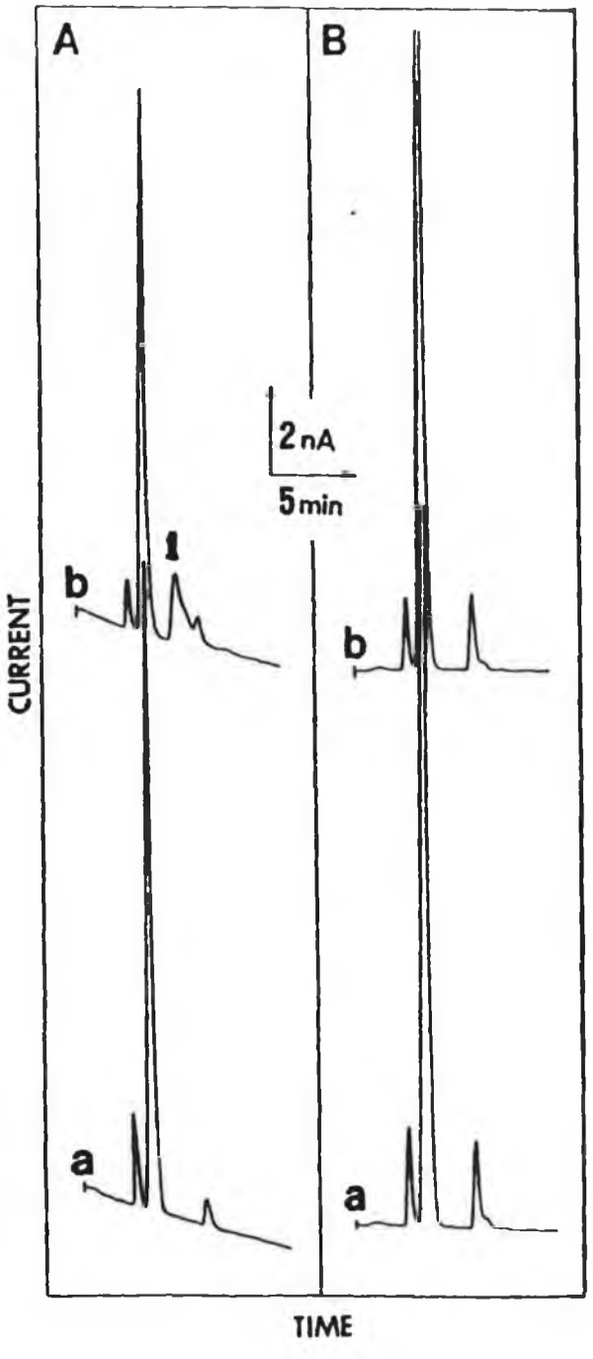

Figure 4.7. Chromatograms of a diluted (1:12) urine sample before, (a) and after (b), after spiking with ethanol (9 µg injected, peak 1), obtained at the yeast-modified (A) and unmodified (B) carbon- paste detector. Mobile phase, 0.05 M phosphate buffer (pH 7.4); flow rate, 1 ml/min; applied potential, +0.70 V.

($5x10^{-4}$-$5x10^{-3}$ M) examined (slope, 1.6 nA/mM; correlation coefficient, 0.997). For dopamine and ethanol (at the banana and yeast flow cells respectively), linearity prevailed up to $2x10^{-3}$ M and $2.5x10^{-2}$ M respectively. Reciprocal ("Lineweaver-Burk" type) plots may be used to extend the linear range. Detection limits of $7x10^{-5}$ M dopamine, $6x10^{-4}$ M ethanol and $1.5x10^{-4}$ M p-cresol were estimated from the above calibration experiments. These values correspond to 0.24, 0.50 and 0.33 µg respectively in the 20 µl injected volume. Such values are above the nanogram (and often subnanogram) detection limits that characterise conventional amperometric detectors.

4.4. Conclusions

A novel electrochemical detection approach for liquid chromatography has been described which involves the use of rapid responding tissue and microbe-based carbon paste bioelectrodes. This approach adds a new dimension of selectivity to liquid chromatography / electrochemistry based on bioactivity or substrate specificity. Complex chromatograms (e.g. of urine samples) are greatly simplified as only substrates of the enzyme (present in the cellular material) are detected.

Selectivity can be greatly improved by obtaining two chromatograms, one profiling only the biologically

active solutes. Because of their inherent biocatalytic activity and stability, cellular materials are more suitable for liquid chromatography detection than isolated enzymes. This concept was tested on several classes of analytes using banana, mushroom and yeast-modified electrodes. Results are also given for a series configuration with a tissue-generator / electrode collector. These developments should lead to increased use of natural materials for monitoring chromatographic eluents.

The main power of the concept reported in this chapter, lies in utilising the substrate specficity of enzymes. This can help to provide additional information (via peak ratios and measurements) on new analytes. Sensitivity enhancement may be achieved via enzymatic recycling of the analyte in two-enzyme systems [21, 15]. The amplification makes use of some of the excess enzyme which is otherwise unused in diffusion-controlled enzyme electrodes.

While the work presented here is within the framework of few cellular materials, the concept could be extended to the detection of numerous solutes via a judicious choice of the biological modifier. Other biosensing schemes e.g. chemoreceptive processes, may also be suitable for monitoring chromatographic eluents.

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CHAPTER 5

THE DETERMINATION OF Pb(II) AND Cu(II) AT LICHEN-MODIFIED CARBON PASTE ELECTRODES

5.1. Introduction

Lichens are plants formed by the symbiotic association of an alga and a fungus. Their general structure is that of an upper cortex, a protective central fungal medulla (of loosely packed hyphae) and a lower cortex (see Figure 5.1). The algae may form a distinct layer beneath the upper cortex or can be dispersed throughout. Most lichens have an extracellular matrix which is a gelatinous secretion containing polysaccharides such as lichenan and isolichenan together with glucans and galactomannose and lichen acids.

For years, lichens have been known to accumulate metal ions and have been used extensively as biomonitors of environmental pollution [1, 2]. The diversity of lichen species close to a suspected pollution source has been used to assess the levels of gaseous air pollutants with fewer lichen species being found closer to the emission source [3, 4]. They are considered useful biomonitors of sulphur dioxide [5, 6], acid rain [7, 8, 9], radionuclides [10, 11, 12], chlorinated hydrocarbons [13, 14, 15] and ozone [16, 17, 18]. Both lichens and mosses have been used as monitors of uranium contamination by Boileau et al. [21] around two centres of uranium mining in Canada. Samples were analysed for metals such as Ti, Fe, Ni, Pb and U by X-ray fluorescence spectroscopy.

A review by Richardson [22] on the pollution

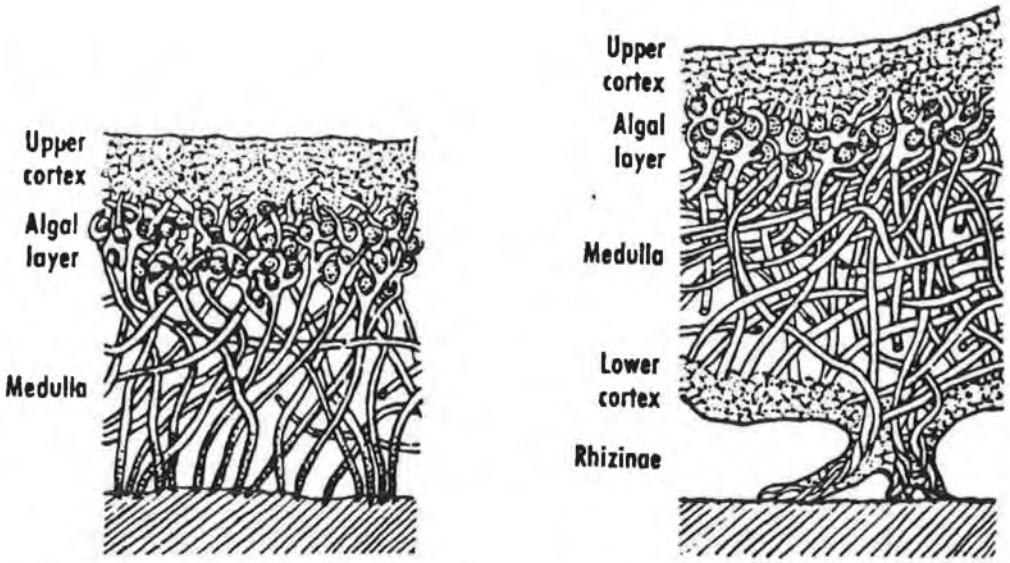

Figure 5.1. Cross-section of lichen thallus.

sensitivity of lichens discusses the effects of sulphur dioxide and acid rain on the lichens, along with the mechanism of elemental accumulation by these plants. More recently, Beck and Ramelow [23] used lichens enclosed in porous PVC tubes as monitors of dissolved metals in natural waters. The PVC tubes were suspended at different sampling points along a river. After two weeks, the lichens were analysed by atomic absorption spectroscopy for a wide variety of metals ions including Pb(II) and Cu(II).

Wang et al. [24] carried out electrocatalytic experiments at algae-modified electrodes to investigate the incorporation of anionic and cationic metal complexes by algae. Gardea-Torresday et al. [25, 26] reported voltammetric measurements at an algae-modified electrode capable of preconcentrating Cu(II) and Au(I).

These studies suggested that the development of lichen-modified electrodes might open up a new area of sensor development based on the non-electrochemical bioaccumulation of metal ions.

In this chapter, lichen-modified carbon paste electrodes are described for the detection of lead(II) and copper(II) using differential pulse anodic stripping voltammetry (DPASV). These electrochemical biosensors incorporate the biological selectivity of lichen species such as Cladonia portentosa, Lobaria pulmonaria, and Roccella. The voltammetric response was evaluated with respect to the sensitivity of

voltammetric detection, pH of accumulation solution, pH of electrolyte solution, metal ion concentration, percentage lichen loading in the carbon paste, interferences and surface renewal.

5.2. Experimental

5.2.1. Preparation of Lichen-Modified Electrodes

Working electrodes were prepared by packing 3 mm i.d. glass tubing with plain carbon paste (40:60, mineral oil : graphite powder) with a Cu wire providing electrical contact. The modified paste (containing lichen) was packed as a thin layer at the sensing end of the electrode and smoothed on a deck of non-oil absorbing material, such as weighing paper. Modified pastes were prepared from the three lichens investigated, i.e. Cladonia portentosa, Lobaria pulmonaria and Roccella, as follows.

Collected lichen samples were cleaned of any non-lichen material and crushed to a fine powder with a mortar and pestle. Appropriate quantities of lichen were mixed with mineral oil, then graphite powder (e.g. 0.2 g lichen : 0.4 g mineral oil : 0.4 g graphite powder) and thoroughly mixed for 15 min to ensure equal distribution of the lichen within the carbon paste. Modified pastes were generally made in 1 g batches and stored at room temperature.

5.2.2. Chemicals

Metal ion solutions were prepared from Spectrosol AA standard solutions and stored in polyethylene bottles. Buffers were prepared from potassium dihydrogen phosphate, di-potassium hydrogen phosphate and sodium acetate (pH adjusted with o-phosphoric and acetic acids respectively). The reference (Ag/AgCl), auxiliary (Pt) and working electrodes were placed in the electrochemical cell through holes in a plastic cover with a fourth inlet for nitrogen, required for purging of the electrolyte. Accumulation was carried out in a separate cell with a magnetic stirrer and stirring bar (7 mm long) providing convective transport.

5.2.3. Method

The working electrode was immersed in the accumulation cell containing a stirred metal ion solution for a preselected length of time (see accumulation time study), after which it was removed, rinsed with deionised water and placed in the measurement cell containing the supporting electrolyte.

Accumulation was carried out under open circuit conditions (no applied potential) for a preselected length of time (accumulation time). The accumulation cell contained the stirred metal ion solution of

interest, while the measurement cell contained the electrolyte.

Differential pulse voltammograms were recorded with an EG&G Model 264A Polarographic Analyser / Stripping Voltammeter and a Houston Instrument Omnigraphic 2000 XY recorder. Deionised distilled water was used throughout to prepare all solutions.

Scans were run after an equilibration time of 15 s from a starting potential of -0.8O V (Pb(II)) or -0.3O V (Cu(II)). The electrode surface was easily renewed by removal of the old surface using a spatula and repacking as before.

5.3. Results and Discussion

5.3.1. Effect of Accumulation Time

The effect of varying the accumulation time for the uptake of Pb(II) at the Roccella-modified (20% w/w) working electrode is shown in Figure 5.2. From this it can be seen that there is an initial rapid increase in the response followed by a plateau and a further increase.

The initial uptake could represent either specific or fungal binding and the latter rise in the curve represent the slower non-specific or perhaps algal binding of the metal ion.

Algae have been shown to follow this pattern of initial rapid uptake and subsequent slow uptake [27].

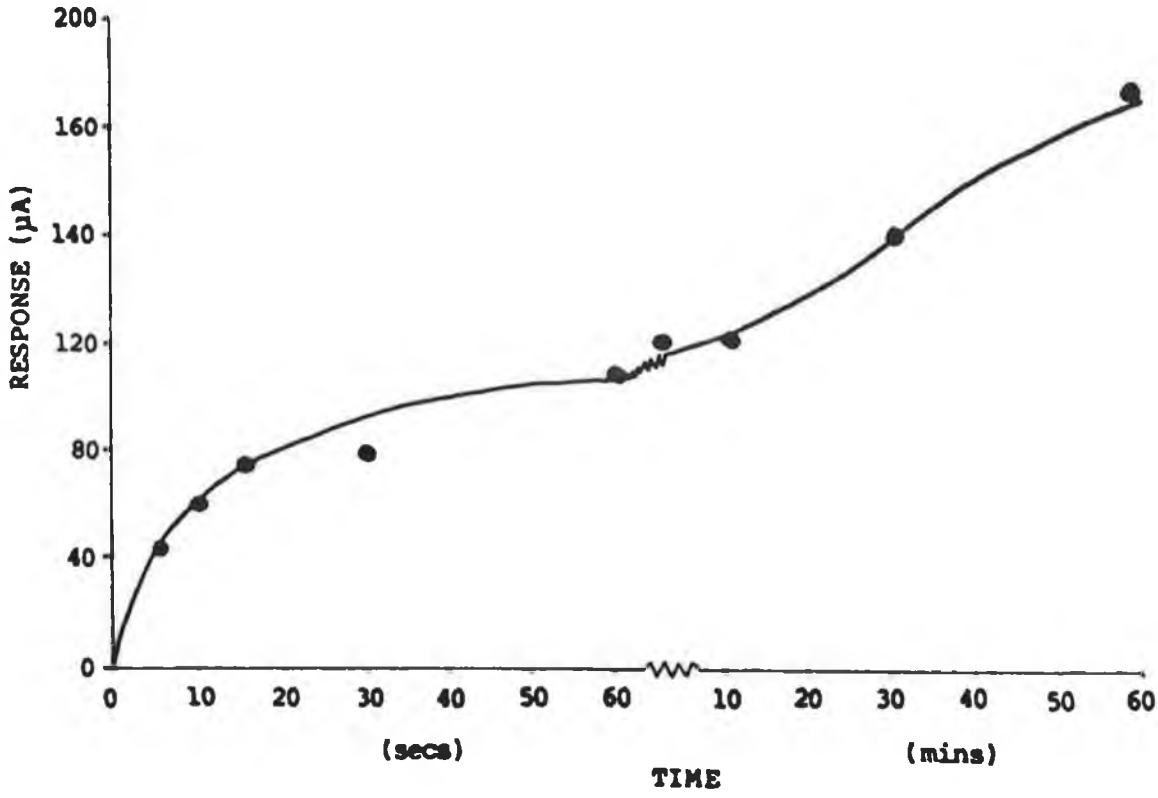

Figure 5.2. Graph showing the effect of varying the accumulation time for a 1 mM Pb solution (pH_{acc} 6.3, pH_{elec} 4.0 in phosphate buffer, 0.02 M) at a Roccella-modificd (20% w/w) carbon paste electrode. Pulse amplitude, 100 mV; scan rate, 5 mV/s; scan, -0.80 V ---- -0.20 V.

A similar curve was observed for the uptake of Cu(II) by the Lobaria-modified (20 % w/w) carbon paste electrode. A two stage uptake mechanism has also been reported for the moss Sphagnum [28]. In the case of Pb(II) accumulation at the Roccella-modified electrode, uptake of Pb(II) reached 80 % of its equilibrium value at 2 min after which slower uptake proceeded.

5.3.2. Effect of Accumulation and Measurement pH

Figure 5.3 (a) and (b) shows the effect of varying the electrolyte pH for the determination of Pb(II) and Cu(II) at each of the three lichen-modified electrodes. The variation in response is most likely due to the wide range of ligands and binding sites present in each lichen.

Functional groups are most likely oxygen and nitrogen donor atoms such as listed below (pK_a values are shown in brackets) : $-PO_3H$ (2-3), $-COO^-$ (4-6), aromatic amines (5-6), phenolic and enolic groups (6-9), $-PO_3^{2-}$ (7-8) and aliphatic amines (7-9) [3]. For the measurement of Cu(II) uptake, the best response was obtained at pH 4.8 (Figure 5.3.b).

The optimum pH for Cu(II) accumulation at the Lobaria-modified electrode (as seen in Figure 5.4.b) was at pH 6.0. If we consider these two points (pH_{acc} 4.8 and pH_{elec} 6.0), it would appear that

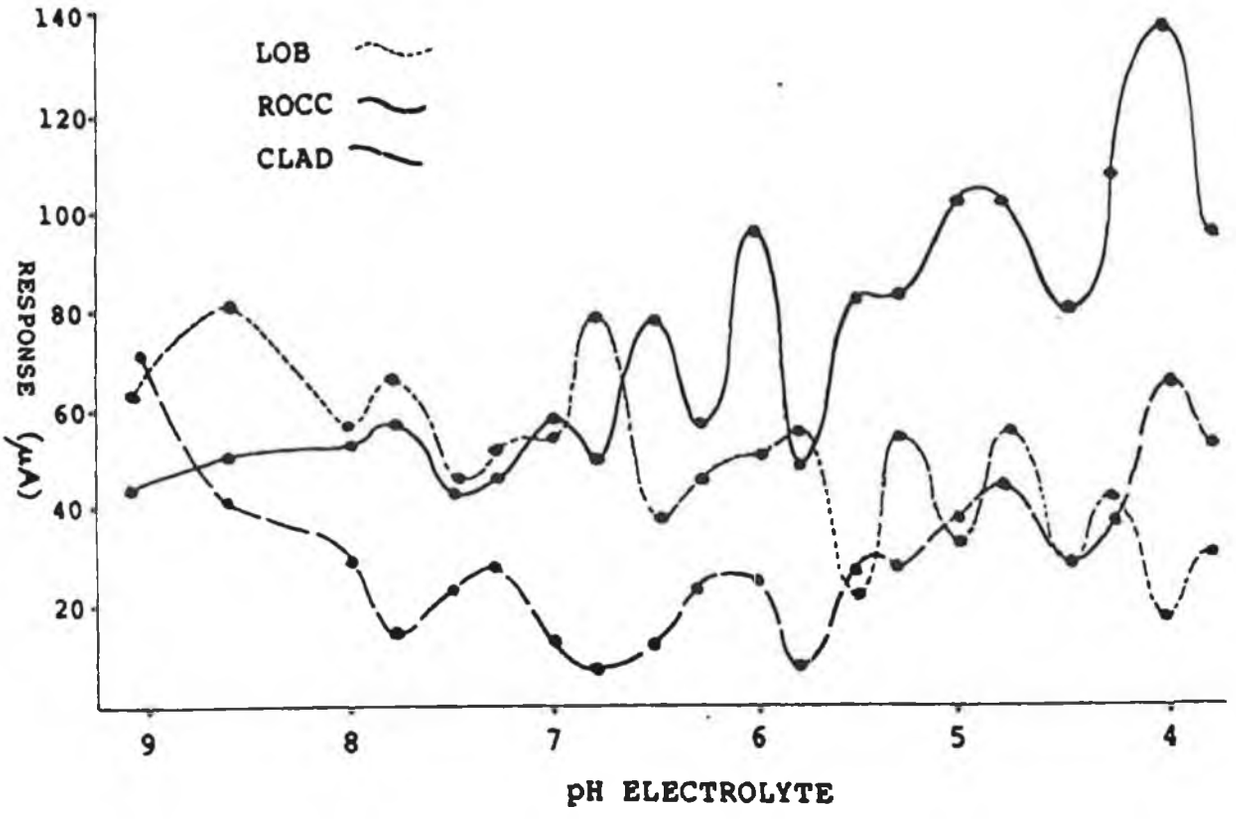

Figure 5.3. (a) Graph showing the effect of varying the pH of electrolyte for the detection of Pb(II) at each lichen-modified carbon paste electrode. Detection conditions: accumulation solution, 1 mM Pb(II)(aqueous); electrolyte, 0.02 M phosphate buffer; scan, -0.80 V to -0.20 V; scan rate, 5 mV/s; pulse amplitude, 100 mV; equilibration time, 15 s; degassing time (N_2), 10 min; accumulation time, 2min.

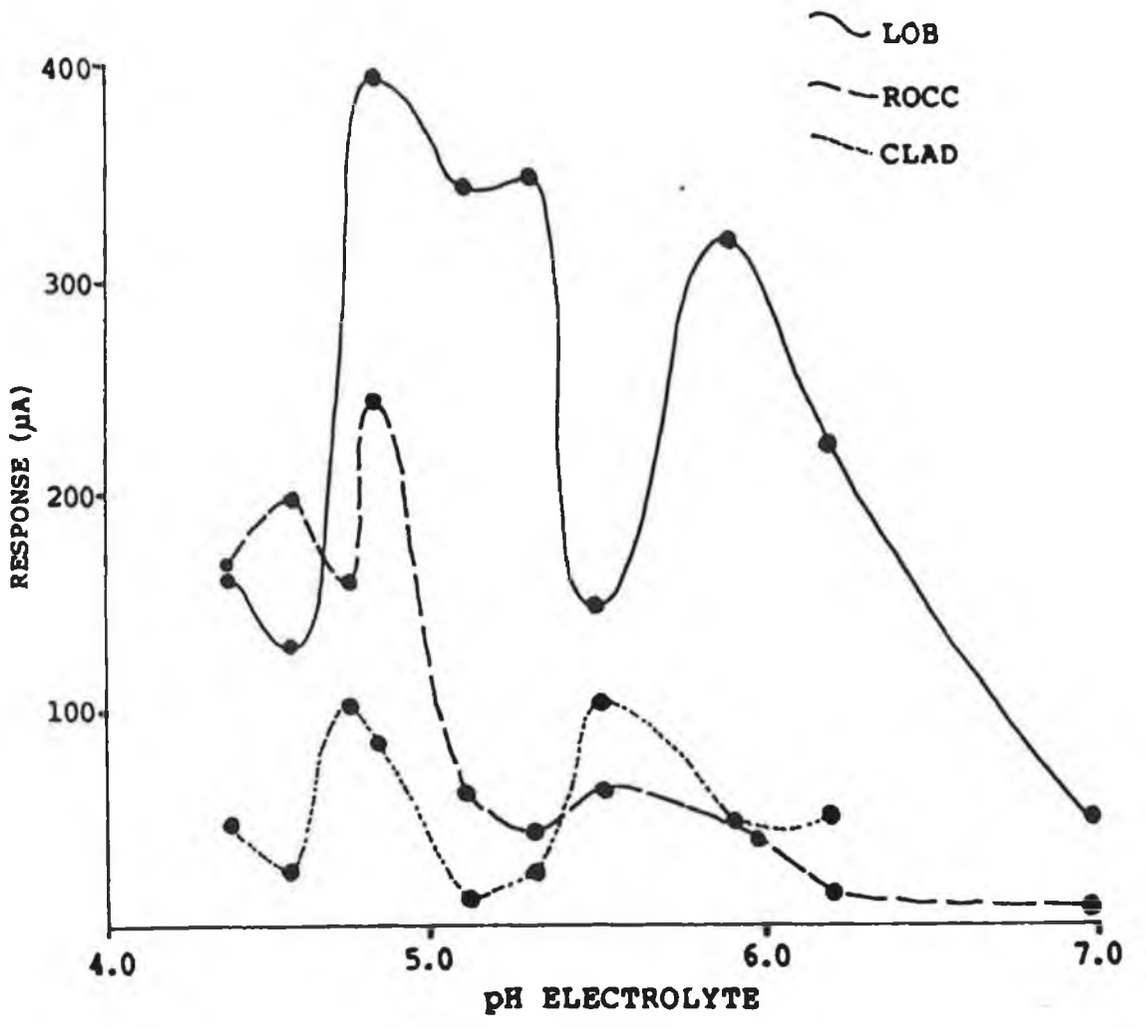

Figure 5.3. (b) Graph showing the effect of varying the pH of electrolyte for the detection of Cu(II) at each lichen-modified carbon paste electrode. Detection conditions: accumulation solution, 1 mM Cu(II)(aqueous); electrolyte, 0.02 M acetate buffer; scan, -0.30 V to +0.40 V, scan rate, 5 mV/s; pulse amplitude, 100 mV; equilibration time, 15 s; degassing time, 10 min; accumulation time, 5 min.

the binding sites are most likely carboxylate in nature (pK_a in the range 4-6).

This is in agreement with the conclusions of Puckett et al. [31]. Looking at the responses obtained for Pb(II), it can be seen that the best accumulation occurs in the region 5-7, with a maximum at pH 6.3 for the Roccella-modified electrode (see Figure 5.4.a).

Phenolic and enolic groups are likely candidates for binding considering this accumulation pH of 6.3. Where there are concurrent peaks (e.g. at pH 7.0 Figure 5.4.a), this may represent similar binding sites in different lichens. Optimum pH for measurement of Pb(II) occurs at pH 4.0, as shown in Figure 5.3.a.

Looking at some of the constituents of these lichens one finds in Lobaria : gyrophoric acid, norstictic acid and stictic acid: and in Roccella : isolichenan, lichenan, roccellic acid and lecanoric acid. Considering that carboxylate and hydroxycarboxylate groups are most likely responsible for metal ion binding, it is interesting to note that gyrophoric, roccellic and lecanoric acids in particular are carboxylate by nature.

Finally, comparison of all three lichens for Pb(II) and Cu(II) uptake shows that Pb(II) is preferentially bound by Roccella at pH_{elec} 4.0 and pH_{acc} 6.3 (Figure 5.5), whereas Lobaria has the greatest affinity for the uptake of Cu(II) at pH_{elec} 4.8 and pH_{acc} 6.0 (Figure 5.6).

The nature of the binding is not clear, but it is

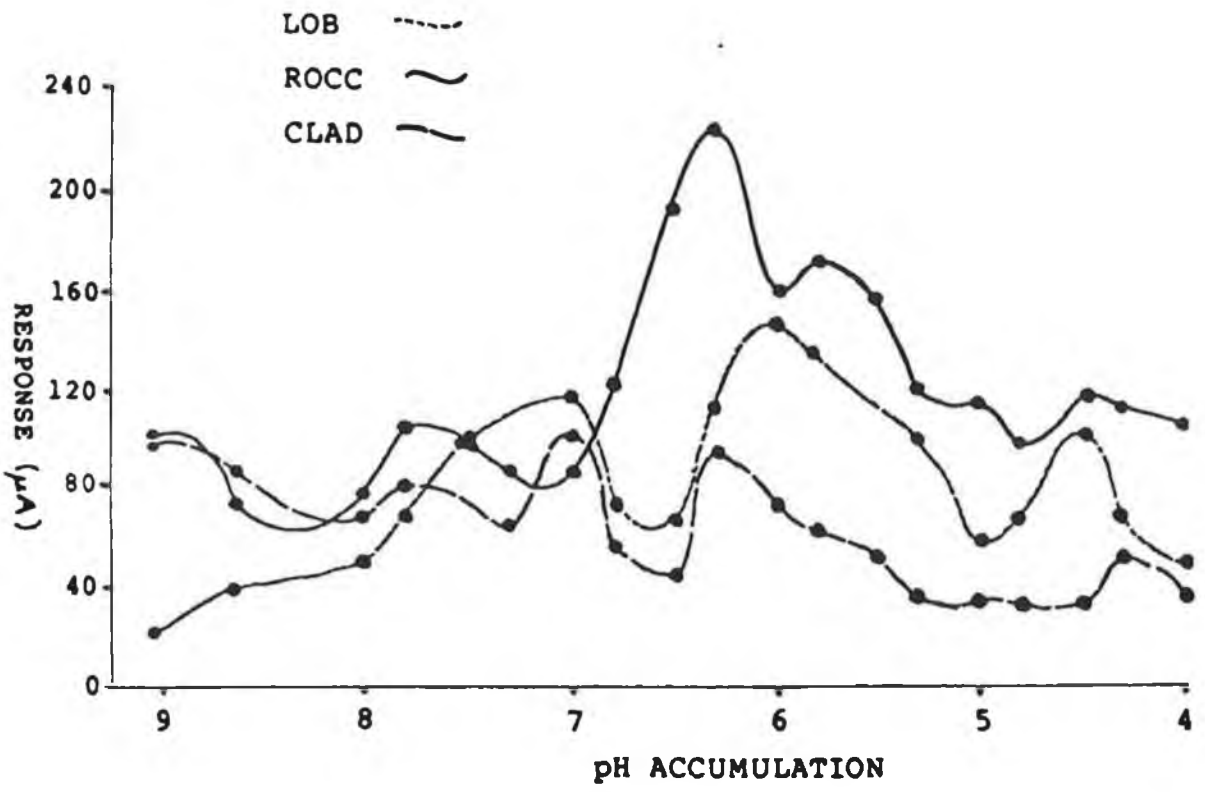

Figure 5.4. (a) Graph showing the effect of varying the pH of accumulation for the detection of Pb(II) at each lichen-modified (20% w/w) carbon paste electrode. Detection conditions: as in Figure 5.3. (a) except; accumulation solution, 1 mM Pb(II) (phosphate buffer, 0.02 M); electrolyte, phosphate buffer (0.02 M), Cladonia Portentosa, pH 4.8; Lobaria Pulmonaria, pH 6.8; Roccella, pH 4.0.

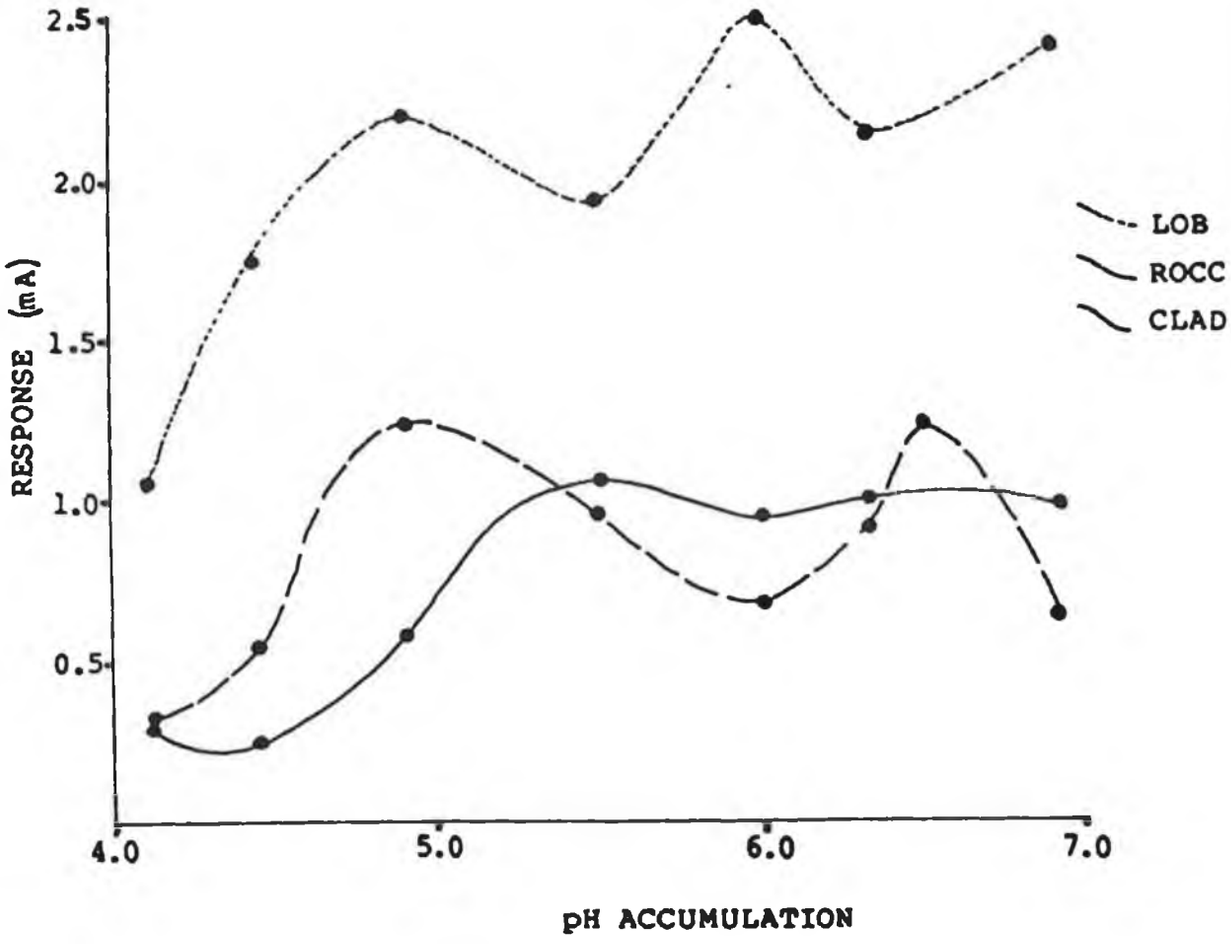

Figure 5.4. (b) Graph showing the effect of varying the pH of accumulation for the detection of Cu(II) at each lichen-modified (20% w/w) carbon paste electrode. Detection conditions: as in Figure 5.3. (b) except; accumulation solution, 1 mM Cu(II) (acetate buffer, 0.02 M); electrolyte, acetate buffer (0.02 M), Cladonia Portentosa. pH 5.5; Lobaria Pulmonaria. pH 4.8; Roccella. pH 4.8.

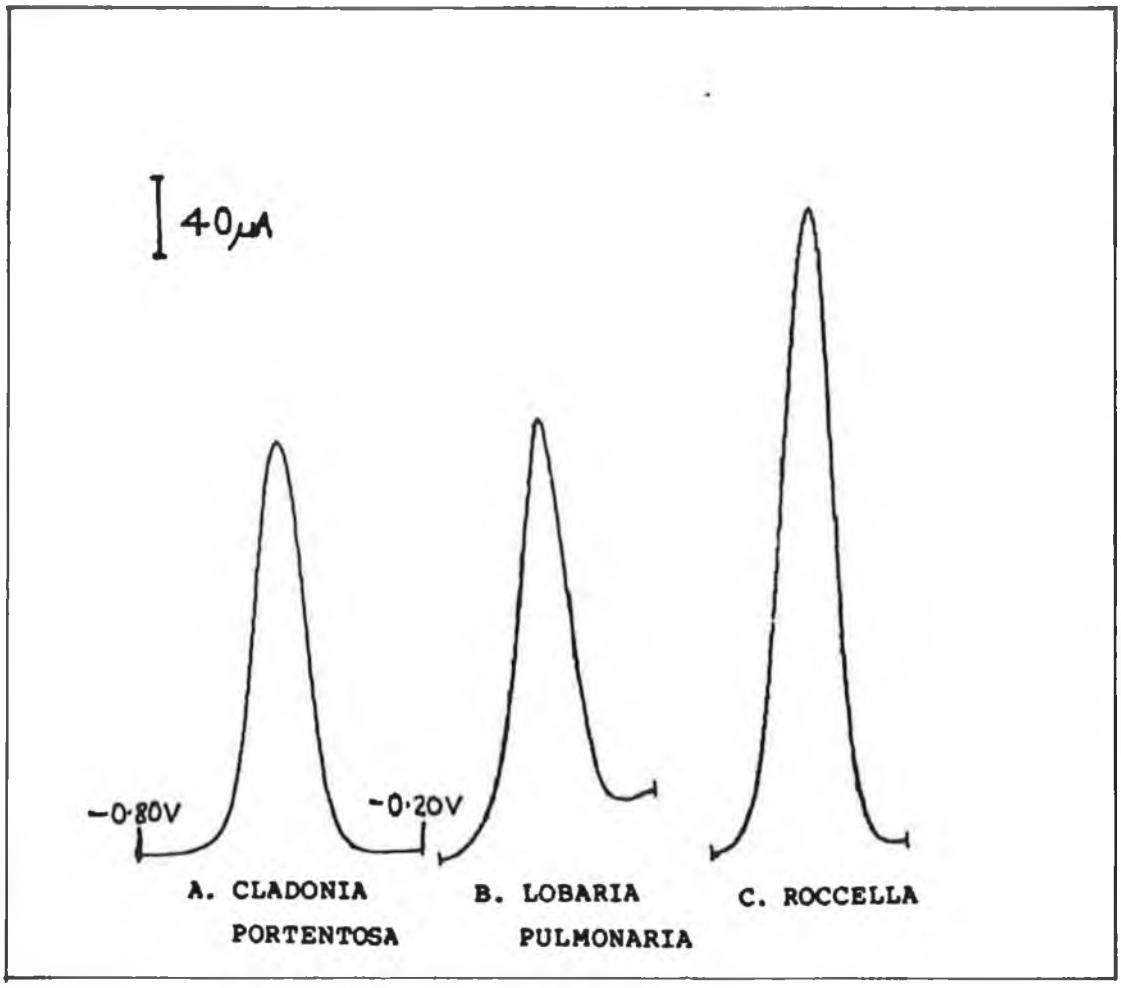

Figure 5.5. Comparison of the three lichens (20% w/w) (Cladonia Portentosa, pH_{elec} 4.8, pH_{acc} 7.0; Lobaria Pulmonaria, pH_{elec} 6.8, pH_{acc} 6.0; Roccella, pH_{elec} 4.0, pH_{acc} 6.3) for the uptake of Pb(II).

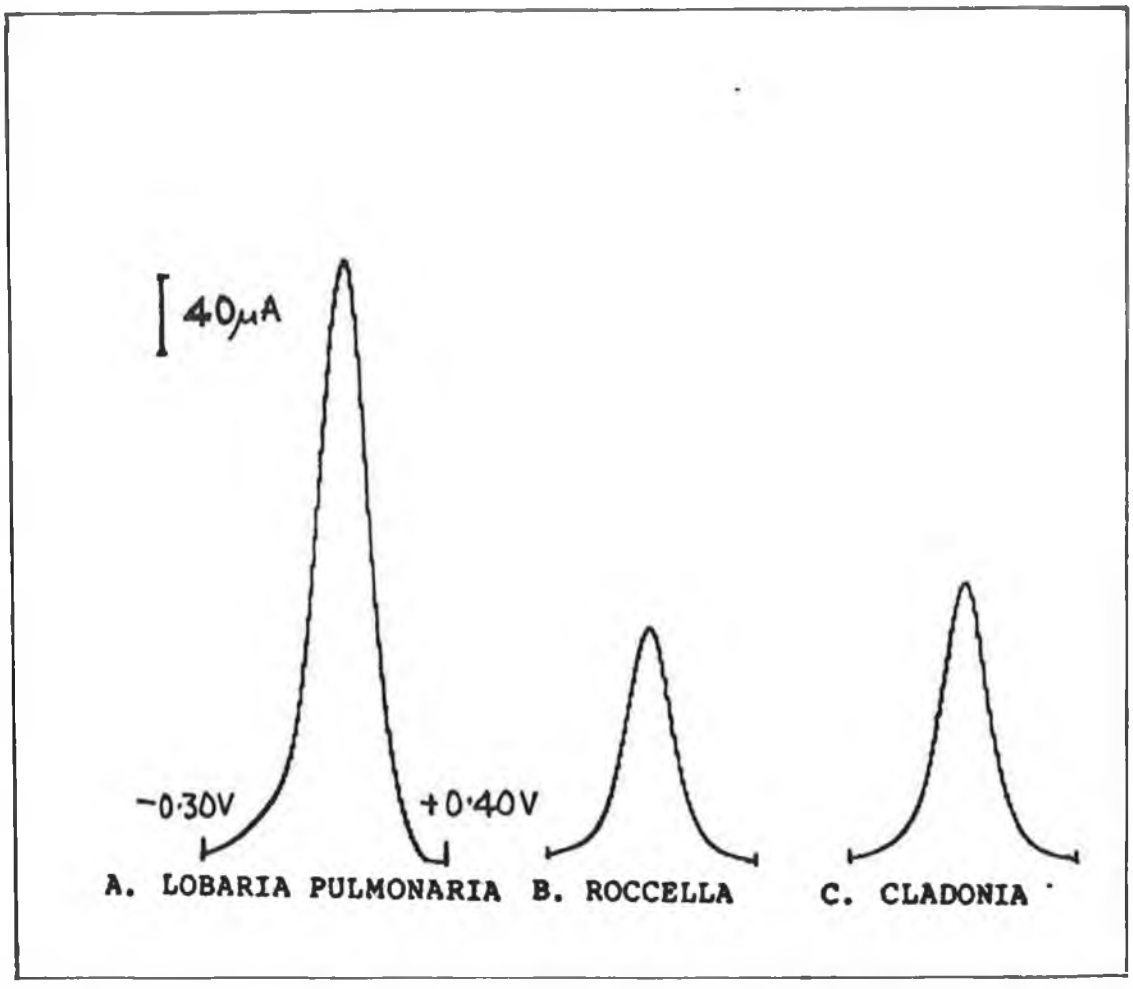

Figure 5.6. Comparison of the three lichens (20% w/w) (Cladonia Portentosa, pH_{elec} 5.5, pH_{acc} 6.5; Lobaria Pulmonaria, pH_{elec} 4.8, pH_{acc} 6.0; Roccella, pH_{elec} 4.7, pH_{acc} 5.5) for the uptake of Cu(II).

thought to occur via a process of ion-exchange [29, 31]. Richardson et al. removed the extracellular matrix [10] and found that for a particular lichen, the binding capacity increased which seemed to indicate that the main metal binding sites were on the cell wall rather than in the extracellular matrix.

The most likely binding sites are compounds containing carboxylate and hydroxycarboxylate groups which form part of the protein component of the fungal cell wall [30]. Richardson et al. have described the uptake of lead and uranium by lichens [10, 19, 20]. Attempts were made to identify which fractions of lichen cell walls were responsible for accumulation of the metals ions. The main metal-binding sites proved to be associated with the protein component of the fungal cell wall, especially since proteins are sources of anionic functional groups capable of binding metal ions, e.g. carboxylic acid residues of amino acids which make up the cell wall components.

It is only possible at this stage to propose possible ligands based on the comparison of accumulation and electrolyte pH measurements with pK_a values. If the process is one of ion-exchange, the uptake of the metal ion may be represented as

$$M^{n+} + H_2R \rightleftharpoons 2H^+ + M(R)$$

where R represents the functional group responsible for binding.

On placing the working electrode in the accumulation solution, a slight increase in pH was noted, but generally by no more than 0.3 of a pH unit.

The relative capacities of competing metal ions is determined by the free energies of the HR complex (or pK_a values) and the stability of the metal-ligand complex. For both Pb(II) and Cu(II) uptake (Figure 5.4 (a) and (b)), there appears to be a region (5-7) within which greatest accumulation occurs. This concurs with the natural pH of most lichens, so it may be possible to accumulate over a broad pH range.

The difference between buffered metal solutions and aqueous (non-buffered) solutions showed an increase of 47 % in the response for the former. However, it may be possible to simply use this working electrode as a "dip type" device (e.g. for natural waters where pH is in the range 3.6-7.0) which would obviate the need for sample pretreatment or even sample collection. For example, at relatively low pH values, carboxylate groups are protonated and capable of binding metal ions.

It may be possible to characterise lichens based on their accumulation profiles (Figure 5.4 (a) and (b)) or bioaffinities for certain metal ions.

5.3.3. Effect of Lichen Loading

As seen in Figure 5.7 a linear response was obtained for the accumulation of Pb(II) under the

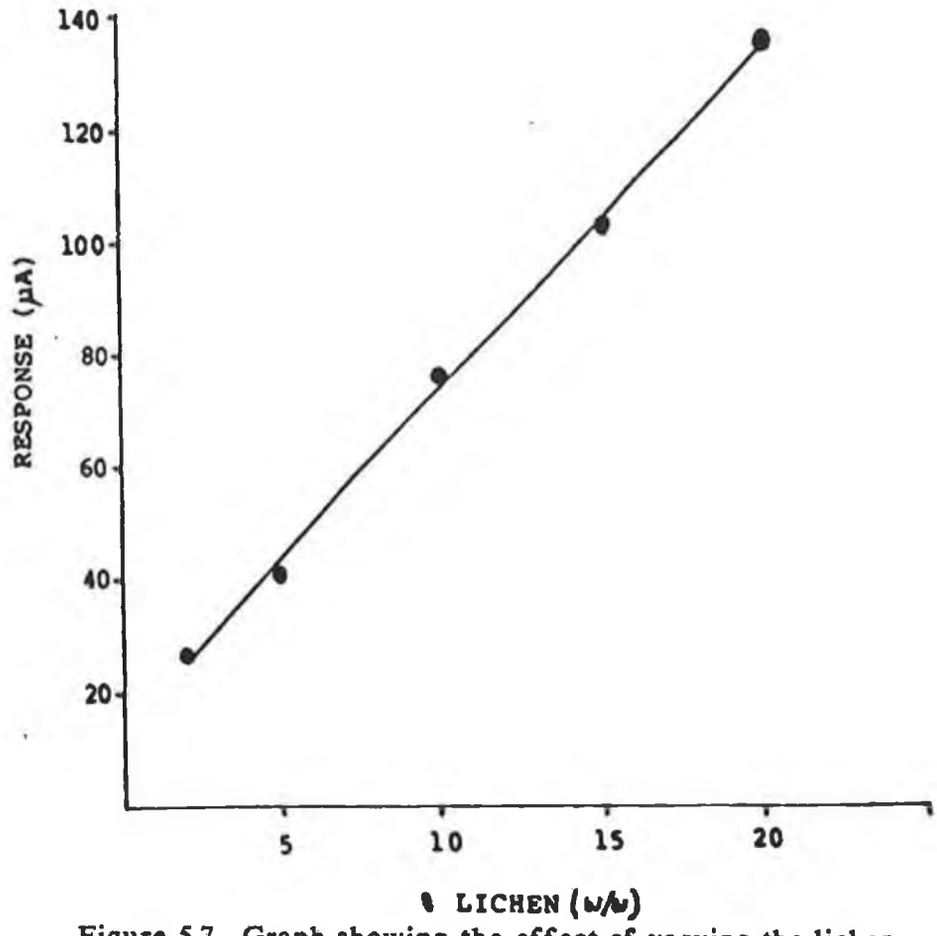

Figure 5.7. Graph showing the effect of varying the lichen loading (% w/w) in the carbon paste for a 1 mM Pb(II) solution (phosphate buffer 0.02 M, pH 6.3) at a Roccella-modified carbon paste electrode. Detection conditions: electrolyte, phosphate buffer 0.02 M, pH 4.0; pulse amplitude, 100 mV; scan rate, 5 mV/s; accumulation time, 2min; scan, -0.80 V to -0.20 V.

conditions stated, with increasing lichen loading (% w/w) in the carbon paste. As would be expected, the more lichen available for binding, the greater the binding capacity and hence the amount of metal accumulated.

5.3.4. Surface Renewal

The possibility of renewing the electrode surface was investigated by washing in HCl. For Pb(II) accumulation at the Roccella-modified electrode, a 5 min wash in 0.25 M HCl resulted in almost total removal of complexed Pb. A series of 10 accumulations on the same surface yielded a % CV of 14 %. For Cu(II), a 5 min wash in 0.25 M HCl resulted in almost total removal of bound Cu, and a further scan was found to strip off any remaining Cu. Increasing the HCl concentration to 0.5 M resulted in total removal of surface bound metal. This experiment confirms that the nature of the binding is via a process of ion-exchange, where under very acidic conditions, the bound metal is replaced once again by a proton.

5.3.5. Effect of Interferences

The effects of interfering metal ions on both the Pb and Cu responses (using Roccella- and Lobaria-modified electrodes respectively, under optimum conditions of accumulation and measurement)

were investigated by measuring the % change in the normal response for a 1 mM Pb(II) or Cu(II) solution on addition of 0.5 mM metal ion. The results are shown in Table 5.1.

Table 5.1. The effect of interfering metal ions on the Pb(II) and Cu(II) responses

INTERFERING METAL ION	% CHANGE IN RESPONSE Pb(II)	Cu(II)
Cd(II)	0.0	+13.0
Al(III)	+4.6	-28.0
Fe(II)	0.0	-5.2
Hg(II)	+25.0	-3.9
Zn(II)	0.0	-27.0
Cu(II)	0.0	0.0
Pb(II)	0.0	-75.4

It is interesting to note that the Pb(II) response is greatly enhanced in the presence of Hg(II), possibly due to the formation of a Hg layer on the surface of the electrode.

This effectively preconcentrates the Pb as an amalgam which is subsequently stripped of on the anodic scan. No such effect was observed for Cu in the presence of Hg.

Most metals ions interfered with the determination of Cu(II), in particular Pb(II) (Figure 5.8), which produced a 75 % decrease in the Cu peak. This is not

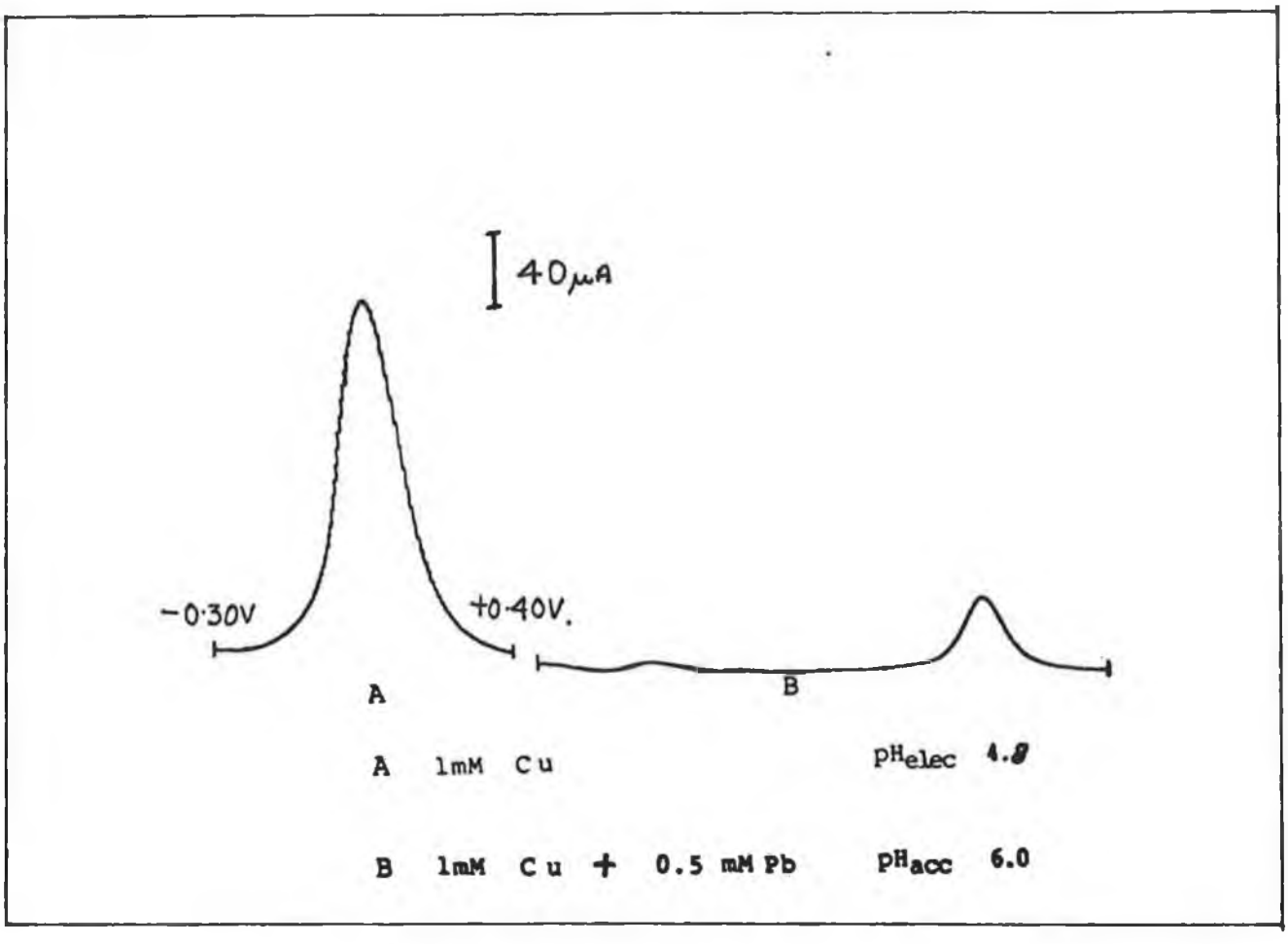

Figure 5.8. Voltammogram showing the effect of 0.5 mM Pb(II) on the copper response at a Lobaria Pulmonaria-modified (20% w/w) carbon paste electrode. pH_{elec} 4.8, pH_{acc} 6.0. Other conditions as in Figure 5.3.b.

surprising if the selectivity sequence for the binding of Pb(II) and Cu(II) to carboxylate groups is investigated [31]. Here we see that Pb(II) is preferentially bound over Cu(II) and may in fact displace any Cu(II) that is already bound.

In fact, the effects of different interfering metal ions could possibly be used to classify the nature of the functional groups involved in the binding based on a selectivity sequence. Using the Hg layer may improve the sensitivity of the method.

An interesting point to note, is the effect of Cu on the Pb response. While Cu does not affect the height of the Pb peak, there is a broadening of the peak (Figure 5.9). This most likely represents competition between the ions for binding sites resulting in slower reaction kinetics. While investigating the interfering metals, Hg was nicely resolved from Cu and gave good peak shape at the Lobaria-modified electrode.

5.3.6. Calibration

Calibrations were carried out for both Pb(II) and Cu(II) for metal ion concentrations in the range 0-1 mM. As seen in Figure 5.10, the response for Pb(II) was linear over the range investigated (slope 113.7 μA/mM, correlation coefficient 0.9932). At lower concentrations (0-100 μM Pb), linearity was less evident with a correlation coefficient of 0.974. A

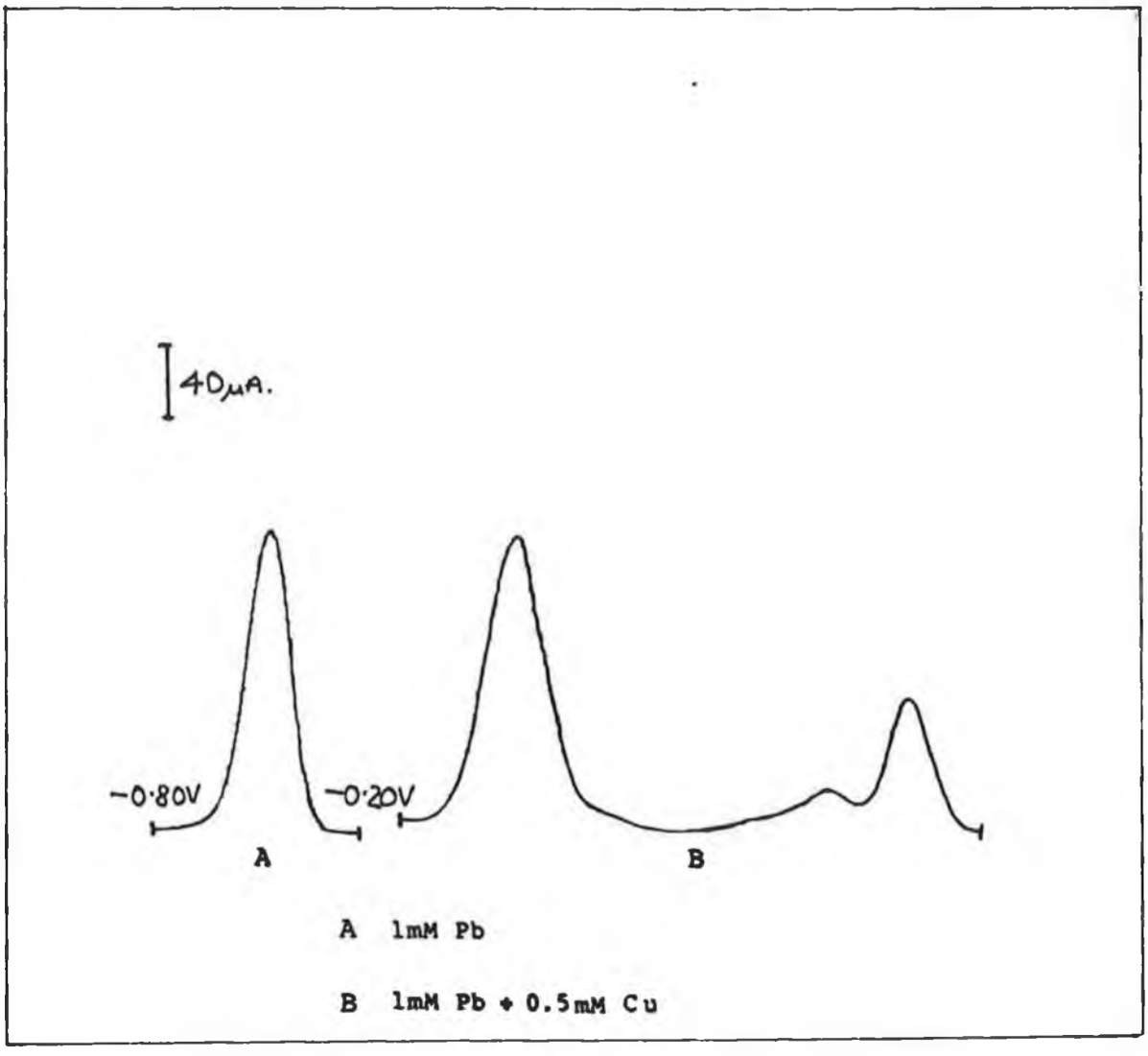

Figure 5.9. Voltammogram showing the effect of 0.5 mM Cu(II) on the lead response at a Roccella-modified (20% w/w) carbon paste electrode. pH_{elec} 4.0, pH_{acc} 6.3.

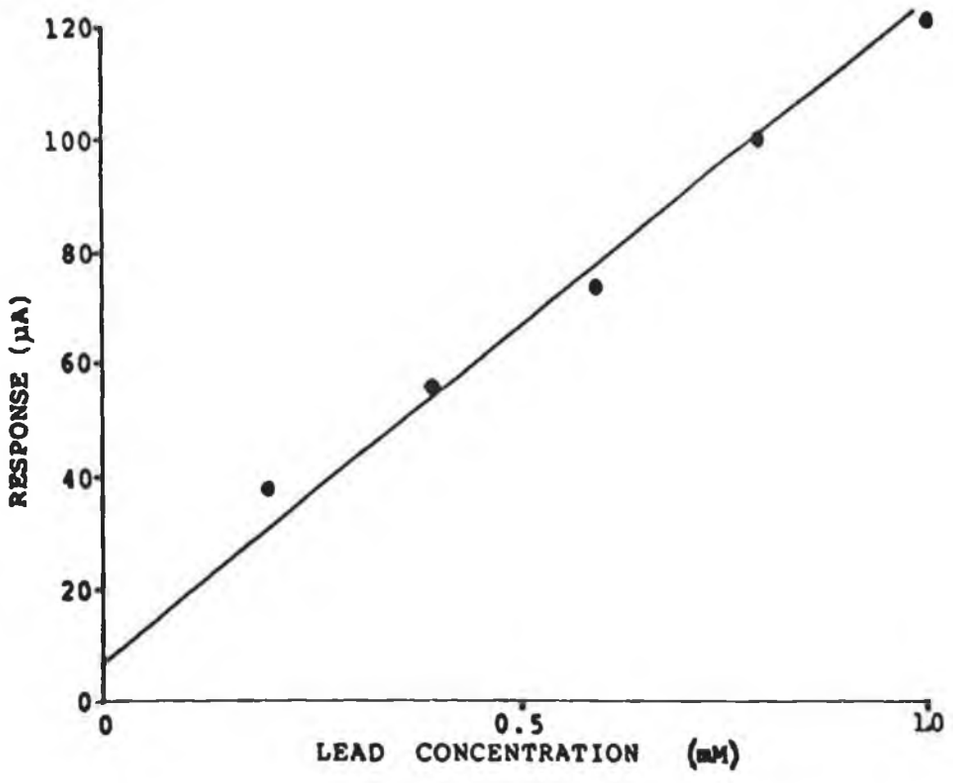

Figure 5.10. Calibration curve for Pb(II) concentrations in the range 0-1 mM at the Roccella-modified (20% w/w) carbon paste electrode. Detection conditions as in Figure 5.7.

detection limit of 20 μM Pb(II) was calculated based on a S/N ratio of 3. For detection of Cu(II), there was a slight response at plain carbon paste. Taking this into account, calibrations were linear over the concentration range investigated (0-1 mM) with a slope of 1399.8 μA/mM and correlation coefficient of 0.9357.

5.4. Conclusions

A lot of work still remains to be done on this system, in particular on improving the sensitivity of the method. However, it lends possibility to use as a rapid method for "on site" analysis as a "dip type" device, since the surface is easily renewable and highly stable. A change of surface may not be necessary since an acid wash removes bound metal.

Greater selectivity may be attained by perhaps combining a variety of lichens in carbon paste. For the present study, three lichens containing different algae and distribution were chosen. Cladonia portentosa contains trebouxia, the commonest lichen photobiont and is a temperated / based forest species. Lobaria pulmonaria contains myrmecia and has a Western Atlantic distribution while Roccella contains trentepohlia and the lichen has a typically Mediterranean distribution.

By using one or more lichens to formulate the lichen paste, it may be possible to detect a range of metal ions which are well resolved on the one

voltammogram. The fact that accumulation is carried out under open circuit conditions and solely dependant on the bioaffinity of the lichen for the metal also simplifies sample analysis and pretreatment.

Extractions of various fractions of the lichens need to be carried out and compared to the response at the total lichen. It may be possible to carry out speciation studies, since accumulation of the metal ions is very much a pH-dependent process using careful selection of pH conditions and other parameters.

This work on metal analysis using biosensors, leads into the next section which investigates the use of chromatography and spectrophotometry for the analysis of metals in soil and clay and also, the development of a method for the separation of Fe(II) and Fe(III).

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PART B

SPECIATION

CHAPTER 6

THE ANALYSIS OF TRACE METAL IONS IN SOILS AND CLAYS

6.1. Introduction

The analysis of trace metals in soil may be used either to diagnose a problem that has already occurred or evaluate a soil for a particular purpose. In several instances, analysis of soil has been used to monitor pollution. There are a variety of methods available for the analysis of trace metals in soil, the most commonly used being atomic absorption spectrometry (AAS) and graphite furnace AAS (GFAAS). Polarographic and voltammetric techniques have also been applied successfully, particularily in the chemical speciation of metals in soil.

Chromatographic techniques used include high performance liquid chromatography (HPLC), flow injection analysis (FIA) and gas chromatography (GC). These techniques generally involve the prior complexation of the metal ion of interest with a ligand species. Those ligands commonly used include dithiocarbamates, dithizonates and oxines.

Neutron activation analysis, X-ray fluorescence and inductively coupled plasma (ICP) spectrometry are other techniques used for the analysis of trace metals. In most references, it appeared that the aim was to improve existing methods in terms of sensitivity, limit of detection and capability of being used routinely. One of the most important aspects of soil analysis, particularly for trace

constituents, is the sampling and storage of samples. Sampling is therefore discussed in this chapter and some procedures are outlined for different soil types to account for factors such as the variability of soil. A section is also included on extraction and digestion techniques and a discussion on the four main extraction techniques used in soil analysis.

The critical factor in soil and plant analysis is to try and equate metal ion availability to the plant with extractability in chemical terms. There is no one extractant that can totally solve this problem. Most methods aim at extracting slightly soluble fractions and avoiding decomposition of the primary minerals. The dilute acid methods tend to give coloured solutions if the organic matter content of the soil is more than 3-5 %. For this reason, atomic spectroscopic methods are preferred to colorimetric methods.

It is often favoured, therefore, to use a combination of methods. For some elements, e.g. cobalt, the total content present may be important. In such cases, ashing followed by strong acid extraction can be used. There are many elements which occur in plants and soils, but it is by no means certain if they all have the same function. Sometimes, the chemical composition of plants can be used to locate minerals, e.g. Pb or Li. This is the basis of geoprospecting.

Metals function as very specific catalysts in enzyme systems in plants e.g. copper in catalase, iron

in porphyrin. A deficiency of copper in soil can lead to certain ailments in cattle, e.g. friesian cattle turning brown where the normal coat colour is black and white, reduced milk yields or poor carcasses. Sometimes plants can be poisoned by an excess of a particular element. Analytical methods for trace metals in soils properly applied can be extremely useful in ensuring better crops and animals.

There are many different reasons for requiring analysis of different fractions for metals in soil. In recent years, the large increase in intensive farming around the world has resulted in the depletion of many essential elements required for the growth of plants. To counteract this, minerals have been added to the soil, but if the correct balance is to be maintained, the exact percentage of the elements of interest in the soil must be known. However, this is a very difficult task, as the total metal in the soil does not represent that percentage which is available to plants.

6.2. Soil Structure

Since the increase in agricultural productivity in the 1940's, it has been seen how man's intervention can vastly improve soil fertility on all of the types of soil with which he works. The actual productivity does, however, vary widely, which shows that there are aspects of productivity outside man's direct control.

One intrinsic factor in yield variation is soil type, which is related to the soils composition and its position in the landscape.

Soil is a mixture of particles ranging in size from macroscopic (gravel and sand, 2000-200 μm) to microscopic particles (silt and clay, 100-<2 μm), with significant variation in surface area among these particles.

The amount of clay present is a prime consideration in the composition of the soil and has a highly important effect on its properties. Soils with more than 30-35 % by mass weight of clay tend to take on the properties of the clay itself, i.e. waterlog more easily during periods of excess rainfall, stay wet longer, require greater draft in cultivation and form large aggregates. Soils containing 15-25 % clay are the most productive in that they provide an adequate surface for interaction with water and nutrients.

Soil forming processes usually cause an accumulation of hydrogen ions in the soil. Biological activity releases organic acids and HCO_3^- into the soil solution, and rainwater also contains acids and CO_2. When there is leaching in the soil, bases are carried down with the leachate and there is a net accumulation of hydrogen ions. Thus in soils not containing free calcium carbonate, colloids in the upper soil layers tend to retain aluminium compounds complexed with organic matter and sorbed on clay surfaces.

For example, if soil acidity is allowed to increase and fall below pH 5.0, the point at which hydrogen ions begin to depolymerise the hydroxyaluminium complexes, free Al(III) is released into the soil solution. Even in small concentrations, Al(III) is toxic to many agricultural crops, and generally speaking, concentrations in the range 2-5 µg/ml inhibit the growth of sensitive crops like barley.

In combination with organic matter, clay contributes coherence and structural stability which enables the soil to resist the mechanically destructive effects of rain and wind. Because clay has a large specific surface that is predominantly negatively charged, they retain cationic nutrients like K(I) and NH_4^+ and also absorb toxic substances.

In general, soil clays are mixtures of several components, and each of these components may have a relatively low order of regularity in its structure.

The solid phases that exhibit surface reactivity in soils are to be found primarily in the clay fraction. Such phases found in soil clay mainly consist of polymers i.e. compounds and mixtures of compounds formed from the binding together of repeating molecular units. If the repeating structure in the solid phase persists throughout a molecular region whose diameter is at least 3 nm, the solid phase is said to be "crystalline". If structural regularity does not exist over molecular distances of this

dimension, the solid phase is termed "amorphous" [1]. The most important molecular structural units in the inorganic polymers found in clays are silica which exists in the tetrahedron form, i.e. SiO_4^{4-}, and the octahedral complexes of the type MX_6^{m-6b}, comprising a metal cation M^{m+}, and six anions, X^{b-}. Both of these compounds can polymerise to form polymeric structures as illustrated in Figure 6.1.

Crystalline structures that contain only silica tetrahedral sheets do not exist in soils, but carbonate, oxide, oxyhydroxide and hydroxide structures that have metal cations in an octahedral coordination are widespread [2].

Aluminium, iron and manganese form the most important oxide, oxyhydroxide and hydroxide minerals in soil clays. Among the iron compounds listed, goethite (Figure 6.3) is the one most often found in soils. Goethite is the most thermodynamically stable of the iron oxides.

Gibbsite is the most important of the aluminium minerals (see Figure 6.3 and Table 6.1) [2]. The polymeric sheet structures combine to form the phyllosilicate class of minerals. These can be classified into three layer types distinguished by the number of tetrahedral and octahedral sheets combined. The 1:1 layer type consists of one tetahedral and one octahedral sheet (Figure 6.2). In soil clays it is represented by the kaolinite group, with the unit cell chemical formula, $[Si_4](Al_4)O_{10}(OH)_8$, where

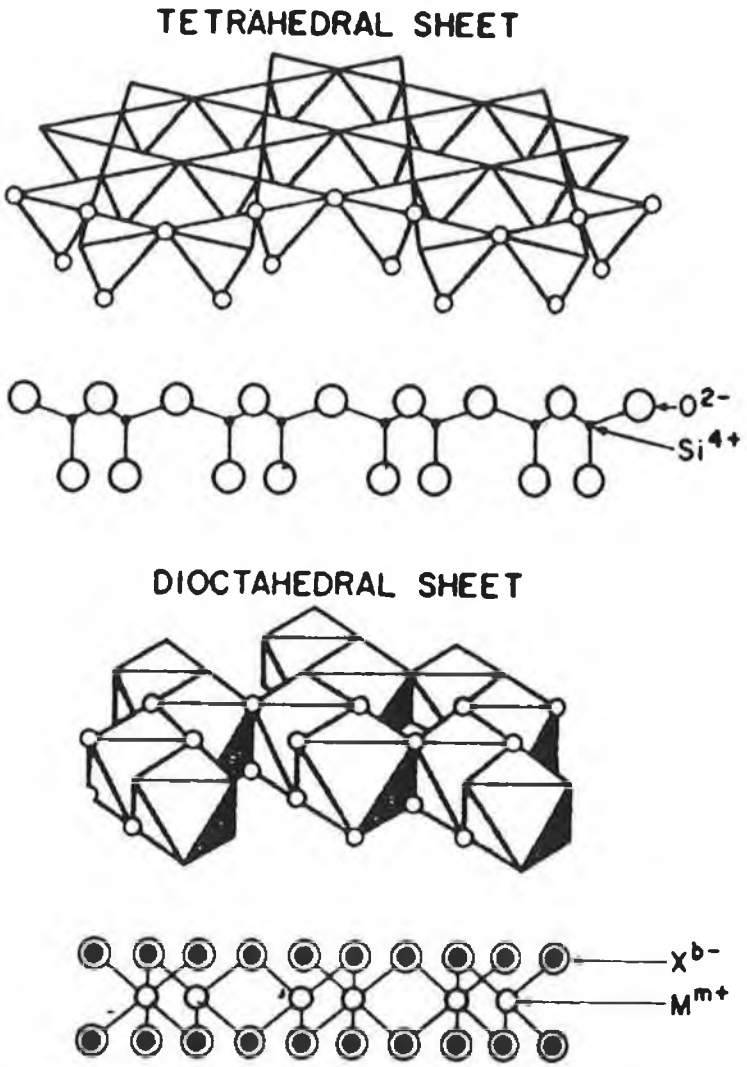

Figure 6.1. Sheet polymeric structure of SiO_4^{4-} and $MX_6^{(m-6b)}$.

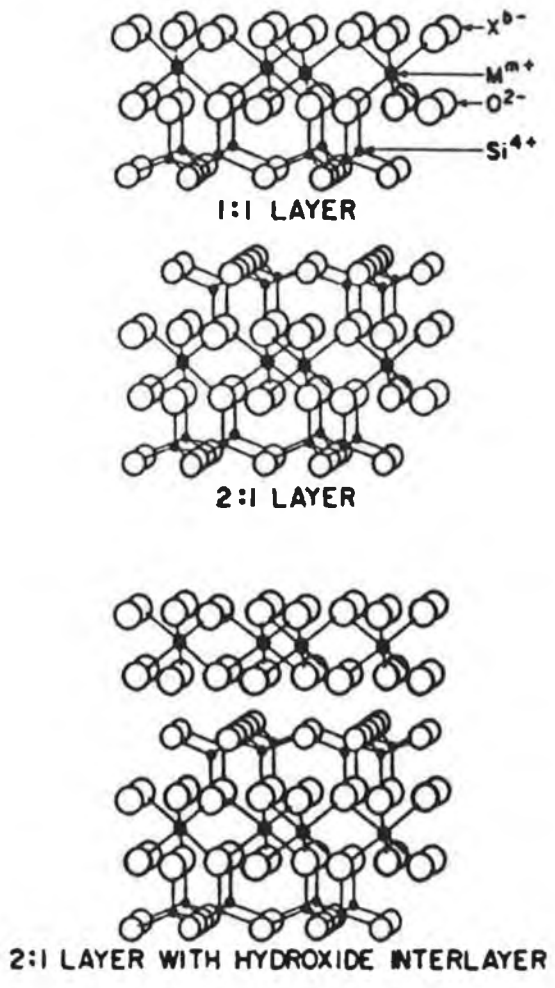

Figure 6.2. The three layer types for phyllosilicate structures in soil clays.

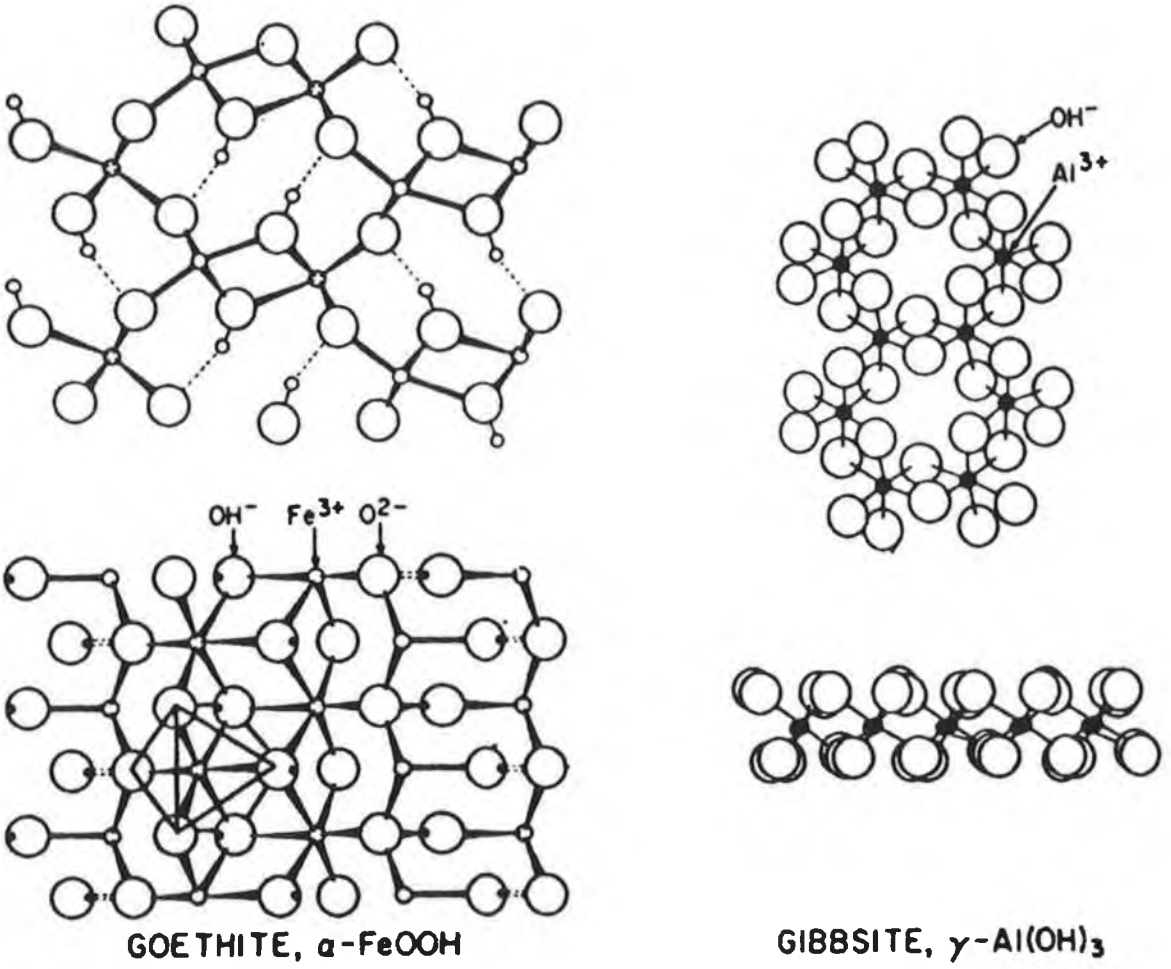

Figure 6.3. The molecular structures of goethite and gibbsite. Hydrogen bonds in goethite are indicated by dashed lines.

the cation enclosed in brackets is in tetrahedral coordination and that enclosed in parentheses is in octahedral coordination. The 2:1 layer type has two tetrahedral sheets sandwiching an octahedral sheet (Figure 6.2). The three clay groups with this structure are mica (illitic), vermiculite and smectite (montmorillonite), each with the general unit cell formula:

$$M_x[Si_aAl_{8-a}](Al_bFe(III)_cFe(II)_{c'}Mg_{4-b-c-c'})O_2O(OH)_4$$

The 2:1 layer type with hydroxide interlayer is represented by dioctahedral chlorite in clays (Figure 6.2). The unit cell formula of this mineral can be expressed as:

$$[Si_aAl_{8-a}](Al_bFe(III)_cMg_{c'})O_{20}(OH)_{16}$$

Table 6.1. Metal oxides, oxyhydroxides and hydroxides commonly found in clay

NAME	CHEMICAL FORMULA
Anatase	TiO_2
Birnessite	$Na_{0.7}Ca_{0.3}Mn_7O_{14}.2.8H_2O$
Boehmite	γ-AlOOH
Ferrihydrite	$Fe_2O_3.2FeOOH.2.6H_2O$
Gibbsite	$\gamma Al(OH)_3$
Goethite	$\alpha FeOOH$
Hematite	αFe_2O_3
Ilmenite	$FeTiO_3$
Lepidocrocite	FeOOH
Lithiophorite	$(Al,Li)MnO_2(OH)_2$
Maghemite	Fe_2O_3
Magnetite	$FeFe_2O_4$

6.3. Chemical Equilibria in Soils

Soils differ in their total chemical composition. These differences are important in equilibrium considerations because they help to determine which elements control the solubility of other elements. In soil, two very important parameters influence the availability of an element to the plants. These are :

(i) the intensity factor, which is the concentration of an element in the soil solution;

(ii) the capacity factor, which is the ability of solid phases in soils to replenish that element as it is depleted from solution.

As plants remove ions from solution, the concentration of those ions in the immediate vicinity of roots is reduced and diffusion gradients are established.

6.4. Aluminium in soil

Aluminium constitutes approximately 7.1 % by weight of the earth's crust. During weathering, aluminium is released from primary minerals and is precipitated as secondary minerals, largely as aluminosilicates, into which many other ions can be incorporated. Weathering results in the loss of silica from aluminosilicates,

leaving aluminium to precipitate as oxides and hydroxides.

The amorphous aluminium oxides γ-Al_2O_3 (crystalline) and α-Al_2O_3 (corundum) are high temperature minerals that normally do not form in soils. The most soluble form of aluminium hydroxide in soils is $Al(OH)_3$ (amorphous). Other minerals include, α-$Al(OH)_3$ (bayerite), γ-AlOOH (boehmite), $Al(OH)_3$ (norstrandite), γ-$Al(OH)_3$ (gibbsite) and α-AlOOH (diaspore) (Table 6.2).

Since silicon is removed from soils more rapidly than aluminium, intense weathering causes the eventual disappearance of aluminosilicates. The iron and aluminium that are released generally precipitate as oxides and hydroxides. Gibbsite occurs as an important mineral in many highly weathered soils (Figure 6.3).

In aqueous solution, Al(III) does not remain as a free ion (see Table 6.3), but is surrounded by six molecules of water forming $Al(H_2O)_6^{3+}$ which may form several different hydrolysis products. Fluoride complexes of aluminium can be extremely important in soils. Ions other than OH^- and F^- also complex Al(III) such as SO_4^{2-} and NO_3^-.

6.5. Iron in soil

The average content of iron in soils is estimated at 3.8 %. In primary minerals it occurs mainly as ferromagnesium minerals (see Table 6.3). Iron,

	Other Equilibria	
26	$CaSO_4 \cdot 2H_2O(gypsum) \rightleftharpoons Ca^{2+} + SO_4^{2-} + 2H_2O$	- 4.64
27	$CaF_2(fluorite) \rightleftharpoons Ca^{2+} + 2F^-$	- 10.41
28	$Al^{3+} + 3e^- \rightleftharpoons Al(c)$	- 86.02

Table 6.2 Equilibrium reactions of aluminium minerals and complexes at 25°C.

Reaction No.	Equilibrium Reaction	log K^*
	Oxides and Hydroxides	
1	$0.5\gamma\text{-}Al_2O_3(c) + 3H^+ \rightleftharpoons Al^{3+} + 1.5H_2O$	11.49
2	$0.5\alpha\text{-}Al_2O_3(\text{corundum}) + 3H^+ \rightleftharpoons Al^{3+} + 1.5H_2O$	9.73
3	$Al(OH)_3(\text{amorp}) + 3H^+ \rightleftharpoons Al^{3+} + 3H_2O$	9.66
4	$\alpha\text{-}Al(OH)_3(\text{bayerite}) + 3H^+ \rightleftharpoons Al^{3+} + 3H_2O$	8.51
5	$\gamma\text{-}AlOOH(\text{boehmite}) + 3H^+ \rightleftharpoons Al^{3+} + 2H_2O$	8.13
6	$Al(OH)_3(\text{nordstrandite}) + 3H^+ \rightleftharpoons Al^{3+} + 3H_2O$	8.11
7	$\gamma\text{-}Al(OH)_3(\text{gibbsite}) + 3H^+ \rightleftharpoons Al^{3+} + 3H_2O$	8.04
8	$\alpha\text{-}AlOOH(\text{diaspore}) + 3H^+ \rightleftharpoons Al^{3+} + 2H_2O$	7.92
	Sulfates	
9	$Al_2(SO_4)_3(c) \rightleftharpoons 2Al^{3+} + 3SO_4^{2-}$	20.84
10	$Al_2(SO_4)_3 \cdot 6H_2O(c) \rightleftharpoons 2Al^{3+} + 3SO_4^{2-} + 6H_2O$	3.45
11	$KAl_3(SO_4)_2(OH)_6(\text{alunite}) + 6H^+ \rightleftharpoons K^+ + 3Al^{3+} + 2SO_4^{2-} + 6H_2O$	3.04
	Hydrolysis	
12	$Al^{3+} + H_2O \rightleftharpoons AlOH^{2+} + H^+$	−5.02
13	$Al^{3+} + 2H_2O \rightleftharpoons Al(OH)_2^+ + 2H^+$	−9.30
14	$Al^{3+} + 3H_2O \rightleftharpoons Al(OH)_3^0 + 3H^+$	−14.99
15	$Al^{3+} + 4H_2O \rightleftharpoons Al(OH)_4^- + 4H^+$	−23.33
16	$Al^{3+} + 5H_2O \rightleftharpoons Al(OH)_5^{2-} + 5H^+$	−34.24
17	$2Al^{3+} + 2H_2O \rightleftharpoons Al_2(OH)_2^{4+} + 2H^+$	−7.69
	Complexes	
18	$Al^{3+} + F^- \rightleftharpoons AlF^{2+}$	6.98
19	$Al^{3+} + 2F^- \rightleftharpoons AlF_2^+$	12.60
20	$Al^{3+} + 3F^- \rightleftharpoons AlF_3^0$	16.65
21	$Al^{3+} + 4F^- \rightleftharpoons AlF_4^-$	19.03
22	$Al^{3+} + 3NO_3^- \rightleftharpoons Al(NO_3)_3^0$	0.12
23	$Al^{3+} + SO_4^{2-} \rightleftharpoons AlSO_4^+$	3.20
24	$Al^{3+} + 2SO_4^{2-} \rightleftharpoons Al(SO_4)_2^-$	1.90
25	$2Al^{3+} + 3SO_4^{2-} \rightleftharpoons Al_2(SO_4)_3^0$	−1.88

Equilibrium Reaction	log K°
Fe(III) Oxides and Hydroxides	
$Fe(OH)_3(amorp) + 3H^+ \rightleftharpoons Fe^{3+} + 3H_2O$	3.54
$Fe(OH)_3(soil) + 3H^+ \rightleftharpoons Fe^{3+} + 3H_2O$	2.70
$\frac{1}{2}\gamma\text{-}Fe_2O_3(maghemite) + 3H^+ \rightleftharpoons Fe^{3+} + \frac{3}{2}H_2O$	1.59
$\gamma\text{-}FeOOH(lepidocrocite) + 3H^+ \rightleftharpoons Fe^{3+} + 2H_2O$	1.39
$\frac{1}{2}\alpha\text{-}Fe_2O_3(hematite) + 3H^+ \rightleftharpoons Fe^{3+} + \frac{3}{2}H_2O$	0.09
$\alpha\text{-}FeOOH(goethite) + 3H^+ \rightleftharpoons Fe^{3+} + 2H_2O$	−0.02
Other Fe(III) Minerals	
$FeCl_3(molysite) \rightleftharpoons Fe^{3+} + 3Cl^-$	13.25
$Fe_2(SO_4)_3(c) \rightleftharpoons 2Fe^{3+} + 3SO_4^{2-}$	2.89
$KFe_3(SO_4)_2(OH)_6(jarosite) + 6H^+ \rightleftharpoons K^+ + 3Fe^{3+} + 2SO_4^{2-} + 6H_2O$	−12.51
Fe(III) Hydrolysis	
$Fe^{3+} + H_2O \rightleftharpoons FeOH^{2+} + H^+$	−2.19
$Fe^{3+} + 2H_2O \rightleftharpoons Fe(OH)_2^+ + 2H^+$	−5.69
$Fe^{3+} + 3H_2O \rightleftharpoons Fe(OH)_3^\circ + 3H^+$	−13.09
$Fe^{3+} + 4H_2O \rightleftharpoons Fe(OH)_4^- + 4H^+$	−21.59
$2Fe^{3+} + 2H_2O \rightleftharpoons Fe_2(OH)_2^{4+} + 2H^+$	−2.90
$Fe(c) \rightleftharpoons Fe^{2+} + 2e^-$	15.98
$Fe^{3+} + e^- \rightleftharpoons Fe^{2+}$	13.04
$Fe_3O_4(magnetite) + 8H^+ + 2e^- \rightleftharpoons 3Fe^{2+} + 4H_2O$	35.69
$Fe_3O_4(magnetite) + 8H^+ \rightleftharpoons 3Fe^{3+} + e^- + 4H_2O$	−3.42
$Fe_3(OH)_8(ferrosic\ oxide) + 8H^+ + 2e^- \rightleftharpoons 3Fe^{2+} + 8H_2O$	43.75
$Fe_{0.95}O(wustite) + 0.10e^- + 2H^+ \rightleftharpoons 0.95Fe^{2+} + H_2O$	12.42
Fe(II) Hydrolysis	
$Fe^{2+} + H_2O \rightleftharpoons FeOH^+ + H^+$	−6.74
$Fe^{2+} + 2H_2O \rightleftharpoons Fe(OH)_2^\circ + 2H^+$	−16.04
$Fe^{2+} + 3H_2O \rightleftharpoons Fe(OH)_3^- + 3H^+$	−31.99
$Fe^{2+} + 4H_2O \rightleftharpoons Fe(OH)_4^{2-} + 4H^+$	−46.38
$3Fe^{2+} + 4H_2O \rightleftharpoons Fe_3(OH)_4^{2+} + 4H^+$	−45.39

Table 6.3. Equilibrium reactions of iron minerals and complexes at 25°C.

released as a result of weathering, precipitates as Fe(III) oxides and hydroxides. Solubility of iron in soils is governed mainly by Fe(III) oxides, while hydrolysis and complexation are important modifying factors.

The percent composition of various iron oxides in a soil may have little bearing on the Fe(III) activity maintained by that soil. Soils generally contain some of several different iron oxides. The solubility of Fe(III) is usually controlled by the most soluble oxide present. For this reason, soil iron ($Fe(OH)_3$) generally controls the activity of Fe(III) in most soils. Only in well drained, highly weathered soils are haematite and goethite expected to lower the solubility of Fe(III) toward their equilibrium levels.

The solubilities of haematite and goethite are nearly identical, but goethite is generally considered the ultimate weathering product of iron in soils.

The solubilities of $FeCl_3$ (molysite) and $Fe_3(SO_4)_2$ (c) are much too high to allow their formation in soils. The mineral $KFe_3(SO_4)_2(OH)_6$ (jarosite) is often found in acid sulphate soils. Normally, jarosite can only form in soils below pH 4.0, but depending on SO_4^{2-} and K(I) activities, it may form at slightly higher pH's.

In aqueous solution, Fe(III), like Al(III), is surrounded by a hydration sheet of six molecules of water giving rise to $Fe(H_2O)_6^{3+}$ (Table 6.3.). Increasing the pH removes H^+ from the complexed

water and gives rise to various hydrolysis products. In the pH range of soils (4.0 - 8.0), the hydrolysis species are more abundant than free Fe(III), and are important because they increase the total iron in solution.

In the case of insoluble nutrients, transport from soil to plant roots is generally the rate limiting step in nutrient uptake. Since diffusional and mass-flow transport processes depend upon total solubility, the hydrolysis species are very important in plant nutrition.

Iron has a minimum solubility in the pH range of 7.4 - 8.5 which is the pH range of soils in which iron deficiencies are most common.

Fe(III) also combines with various anions, e.g. Cl^-, Br^- and F^-, to form complexes and ion pairs (see Table 6.3). The Fe(III) complexes of nitrate, sulphate and phosphate are in small quantities in comparison to the hydrolysis species and do not contribute a great deal to total iron.

6.6. Soil Analysis

6.6.1. Soil Sampling

For any analysis, the sample taken must be representative of the system being analysed. Thus, because of the variabilities that occur within the soil system, a sampling scheme must be established in which the sample reflects the system from which it is taken. Depending on the soil type, different sampling procedures are employed.

(i) arable soils and short term leys (an area of land temporarily under grass) should be sampled at a depth of about 15 cm using a screw auger (25 mm diameter). At least 25 samples should be taken and mixed thoroughly;

(ii) permanent grass or long leys need to be sampled to a depth of approximately 7.5 cm and this to include the top-layer;

(iii) direct drilled crops require special attention. It is generally wise to test separately the top 2-3 cm of soil when taking samples of either grassland or the stubble.

Once the samples reach the laboratory, they undergo a number of important sample preparation steps before the analytical determination, including coding, identification and recording. Samples must be properly

stored in order to prevent contamination or decomposition. Normally, the soil samples are air dried and crushed prior to sieving. Three types of sieving may be used:

(i) mechanical sieving where the soils are passed through rubber rollers to separate aggregates prior to sieving through a 2 mm mesh;
(ii) manual sieving where the soil is rolled manually prior to sieving; or
(iii) sieving through a hand sieve which is more generally used for small samples.

Once the samples are sieved, they are then ready for analysis.

6.6.2. Extraction Techniques

In order to be able to distinguish between the different forms of metals in soils and clays, selective extraction procedures have been developed, some of which give an indication of the concentration of metal "available" to crops. These extraction techniques act only as guidelines, because, owing to the complexity of soil, it is impossible for any single technique to selectively extract one fraction in the presence of the other. Dissolution techniques may be divided into two main groups:

(i) extraction techniques; and

(ii) digestion techniques.

Extraction techniques do not break down the structures of the soil and tend to represent "available" metals, whereas digestion techniques which are destructive and represent total metal fractions. The four main extraction techniques are :

(i) oxalate extraction;

(ii) dithionite-citrate-bicarbonate extraction;

(iii) pyrophosphate extraction;

(iv) ethylenediamine-tetraacetic acid (EDTA) extraction.

An extraction technique is considered selective if its extraction profile reaches a plateau after some time.

6.6.2.1. Oxalate Extraction

Oxalate has been found to complex metals resulting in clear extracts [3]. Minor changes in pH appear to have little effect on the "oxalate-extractable" metal if kept around pH 3.0. Oxalate-extractable iron has been found to be very light sensitive [4] and as such extracts should be stored in the dark before analysis. With oxalate, complete extraction may take weeks to achieve. There is an initial high extraction rate which indicates preferential extraction of a certain metal fraction.

Oxalate extracts were found to contain mainly ferrihydrite with minor amounts of lepidocrocite and goethite. Oxalate was also found to extract some of the more well crystallised iron oxides. Being a strong ligand, oxalate has also been found to dissolve metals in organic complexes.

This technique could be a useful aid for separating compounds prior to identification by X-ray diffraction. As with the other extraction techniques, this technique may help to quantify that fraction of metals available to the plants.

6.6.2.2. Pyrophosphate Extraction

According to Jeanroy and Gillet [5], this extraction is thought to involve the adsorption of pyrophosphate onto soil particles resulting in

increased negative charges which increases the solubility of metal ions associated with these particles in water. Another possibility is that iron contained in insoluble organic complexes is released by pyrophosphate, leaving negatively charged complexes which may be water soluble.

The main problem associated with this technique, however, lies in the fact that the extracts contain suspended materials and this leads to poor reproducibility [6]. Much work has been done [5, 7, 8] to improve reproducibility, especially by trying to clarify the extracts prior to the measurement of the metal concentration. Pyrophosphate does not dissolve iron by forming iron-pyrophosphate complexes, or if so, only to a limited extent. In addition, such complexes have been shown to be highly unstable [9].

6.6.2.3. Ethylenediamine Tetraacetic Acid (EDTA) Extraction

The use of EDTA as an extraction technique is too slow for routine analysis. It may however be used as a reference technique. The identity of the fraction extracted is still rather obscure. According to Borggaard [10, 11, 12] it allows selective extraction of amorphous iron oxides in soils and clays.

Like oxalate, EDTA forms strong complexes with iron and aluminium as well as other di- and tri-valent metals [13]. Experiments on synthetic and natural iron

oxides and silicates have shown that EDTA only dissolved amorphous iron oxides leaving the well crystallised iron oxides and silicates unchanged [10]. The major part of EDTA-extractable metal seems to consist of ill-defined amorphous or poorly ordered crystalline oxides [14, 15].

6.6.2.4. Dithionite-Citrate-Bicarbonate (DCB) Extraction

This extraction technique is based on the fact that iron(II) compounds are more soluble than iron(III) compounds. This method was proposed by Mehra and Jackson [16] in 1960 for estimating "free" iron oxides in soil and clays.

Iron compounds meant to be dissolved by this technique include mainly oxides of different crystallinity though they may also include a small fraction of water-soluble, exchangeable and organic-bound metals. However, the ability of this technique to dissolve oxides, is affected by the size of the oxide crystals and several investigators [17, 18, 19] have shown that large crystals, especially of magnetite, goethite and haemitite, are only partially dissolved by DCB, even though they have been crushed prior to extraction.

6.6.2.5. Other Extraction Methods

Mehra et al. [20] used potassium hexacyanoruthenium(II) as an analytical reagent for spectrophotometric determination of iron at 550 nm.

Uhlemann et al. [21] used N-thio-benzoyl-N-phenylhydroxylamine for the extraction of metals such as Mn, Fe, Co, Ni, Cu, Zn, Cd or Pb. An aqueous solution of the corresponding nitrate salts of these metals (40 μM) containing potassium sodium tartrate as a complex forming agent, $NaNO_3$ to maintain constant ionic strength and HCl or NaOH to establish the optimum pH, was extracted for 30 min with an equal volume of $CHCl_3$ with N-thio-benzoyl-N-phenylhydroxylamine. Metal ions were extracted into the aqueous phase and determined by AAS.

Piccolo et al. [22] compared four digestion procedures on soil extracts for spectrophotometric determination of Fe and Al. These involved digestion with (i) HNO_3-H_2O_2 ; (ii) HNO_3-HCl ; (iii) HNO_3-$HClO_4$; (iv) H_2O_2.

The metal ions were then complexed using pyrophosphate, oxalate and dithionite. Fe(II) and Al(III) were determined by 1,10-phenanthroline and aluminon methods respectively. The results were compared with those obtained directly on soil extracts using AAS. No significant differences were obtained using oxalate and dithionite. Digestion with H_2O_2

was found to be the most suitable digestion procedure since it was found to be both rapid and accurate. No correlation was evident between spectrophotometric and AAS methods for the pyrophosphate extracts.

In recent work by Berrow et al. [23], 2-oxagluconic acid (2-OG), ammonium acetate, acetic acid, EDTA and diethylenetriaminepentaacetic acid (DTPA) were investigated as extractants for various trace elements. At the same pH, Co, Ni, Zn, Fe and Ti were better extracted into 2-OG than into ammonium acetate. Bruce et al. [24] used KCl to extract Al, Ca and Mg from soil. Salinas et al. [25] determined Fe by the extraction of the ion-pair formed between the benzohydroxamic acid-Fe(III) complex and methyltrioctylammonium ions (Adogen 464) in isobutylmethylketone.

Hydroxylamine and ammonium oxalate solutions were studied for use as extractants of Fe and Al from soils by Ross et al. [26]. The acid hydroxylamine and acid ammonium oxalate were extracted for four hours with a soil : solution ratio of 1:250 as an alternative to the extraction with oxalate for four hours with a soil : solution ratio of 1:40. The advantage of the former method was that, although both methods gave comparable results, the hydroxylamine solutions were more easily analysed by AAS and did not require heating overnight.

The determination of Al(III) has been achieved using 8-hydroxyquinoline (8-HQ) as a complex forming

agent, followed by extraction of the complex with an organic solvent and measurement of either the absorbance or fluorescence of the complex. Chloroform was used to extract the complex in separatory funnels. The spectrophotometric methods are similar in sensitivity to the more commonly used aluminium methods [27], but the fluorimetric methods were up to 100 times more sensitive [28]. Both methods have been applied to the analysis of Al in soil extracts [27, 29, 30, 31]. In 1978 Bloom et al. [32] used butyl acetate (instead of chloroform) as the solvent for the extraction of the complex in the determination of Al(III) in soil extracts.

In this 8-hydroxyquinoline method, a 15 min reaction time was used to allow reduction and complexation of interfering Fe(III), before the samples were analysed spectrophotometrically. This interference is due to the reaction of metals, such as iron, with 8-hydroxyquinoline, resulting in falsely high absorbance values.

May et al. [33] modified the 8-hydroxyquinoline method to measure low concentrations of monomeric aluminium in neutral waters. In 1983, James et al. [34] a reported a further modification of the 8-hydroxyquinoline method to determine labile and total aluminium in soil extracts. A second wavelength was used to account for Fe interference.

6.7. Methods of Soil Analysis

6.7.1. Atomic Absorption Spectrophotometry (AAS)

From literature it would appear that atomic absorption methods are the most commonly used for the determination of metals. Since the introduction of graphite furnace atomic absorption spectrophotometry (GFAAS), it has become very widely used, as has inductively coupled plasma (ICP) spectroscopy.

Since AAS determines total metal concentration, speciation within the soils is generally carried out using selective extractions. Many of the extraction techniques already mentioned have been used.

Das et al. [38] reported the AAS determination of trace metals in raw sewage. The method involved pre-treatment of the sample with HNO_3 and HCl. The metal ions were then converted into tartrate complexes which were adsorbed on columns of Dowex 2x-8 resin for subsequent elution and determination by AAS.

Schmidt et al. [39] also used nitric acid as an extractant in the determination of cadmium, copper, iron, lead and zinc. Cd, Co, Ni and Pb were extracted using EDTA by Iu et al. [40]. The detection limits for the determination of these metals was further improved using GFAAS following the EDTA extraction. Petrov et al. [41] used acetate to extract Cd, Co, Cu, Mn, Ni, Pb and Zn from soil followed by detection using AAS. A limit of detection of 1-2 ppm was obtained using

preconcentration techniques, but low recoveries and irreproducible results were obtained when measuring manganese and zinc after preconcentration.

Detection limits of 0.5-1.0 ppm were attained by Portnaya et al. [42] where trace metals were determined by AAS following co-precipitation with chrompyrazole II. Magistrelli et al. [43] compared AA and ICP as methods for the determination of trace metals. AAS was recommended for the determination of less than 10 elements per sample, otherwise ICP was more rapid. For most elements, detection limits were of the same order for both. However, for very low levels, GFAAS is most useful. The ICP technique does not require pre-treatment of the sample, but the instrumentation is more expensive than AAS.

Wolf et al. [44] determined trace metals in soil as indicators of environmental pollution. Moss and soil samples were dissolved by pressure ashing with HNO_3 at 14^oC, digestion with aqua regia or total ashing with HF. The metals were then determined by AAS. Aluminium, calcium and magnesium were determined in KCl extracts by Bruce et al. [45]. A method of direct solid analysis was described by Stupar et al. [46] involving aspiration of soil suspensions for the determination of Cu, Fe, Mn and Mg. Particle size, flame temperature and position of the flame were found to be critical in determining the fractions of particular elements atomised. The method was found to be suitable for the determination of Li, Ca, Sr, Ba,

Al, Cr and Ti.

Kenneth et al. [47] developed a method for the determination of lead in soil by GFAAS with direct introduction of slurries. The sensitivity of this technique was 0.6 μg/g soil in a typical slurry.

Cadmium was also analysed by this method [48], where analyte recoveries were shown to be quantitative provided that the soil was finely ground prior to analysis. The importance of soil particle size was studied and it was shown that insufficient grinding led to poor recoveries, in particular from larger particle sizes.

A further improvement in the sensitivity and accuracy of this technique could be achieved for the analysis of cadmium, lead, mercury, antimony and arsenic, by diluting the solid soil sample with graphite powder [49].

6.7.2. Chromatography

The first reported separation of metal co-ordinated complexes by HPLC was in 1972 by Huber et al. [50]. Six metals, including Al(III), were separated in 25 minutes using acetylacetonates as ligands with UV detection at 310 nm. Since then, many other ligands such as ketoamines, dithiocarbamates, oxinates, ethylenediamines, 1,10-phenanthrolines, and porphyrins have been used for the separation of metals as metal complexes using HPLC.

6.7.2.1. High Performance Liquid Chromatography (HPLC)

6.7.2.2. Dithiocarbamates

Liske et al. [51] determined Zn(II), Cu(II), Mn(II), Ni(II), Pb(II), Cr(II), Co(II), Cd(II) and Fe(III) as diethyldithiocarbamate complexes using reversed-phase chromatography. Both Tande et al. [52] and Schwedt et al. [53] have speciated chromium using diethyldithiocarbamate and ammonium pyrrolidine dithiocarbamate respectively with UV detection. Bond and Wallace [54] used electrochemical detection for the determination of Cu(II) as a dithiocarbamate complex using both external and in situ formation of the complex. The technique was extended [55] by the same workers to determine Cu(II), Ni(II) and Co(II) as well as Cr(III) and Cr(VI).

Bond and Wallace have reported the determination of a number of metals using dithiocarbamates as ligands, including an automated system [56] for the determination of Ni(II) and Cu(II) in a variety of water sources. They have also reported on the separation of Cu(II) and Hg(II) [57] using morpholinecarbodithiocarbamate and pyrrolidinecarbodithiocarbamate.

The determination of trace levels of Cd(II), Pb(II) and Hg(II) as diethyldithiocarbamate complexes using reversed-phase HPLC was reported by Drasch [58]. Detection limits of < 1 ng/ml were achieved using "in

situ" complexation. Inatimi [59] carried out multi-element analysis in some environmental samples using normal phase HPLC and a mobile phase of benzene. Dithiocarbamate complexes of Hg(II), Cu(II), Pb(II), Ni(II) and Mn(II) were analysed following extraction at pH 8.5.

6.7.2.3. 8-Hydroxyquinolates

Berthod et al. [60] were the first to report on the separation of metal-8-hydroxyquinolates by HPLC in 1979. This method has the advantages of forming neutral chelates and having native fluorescence. Al(III) and Co(III) were separated by Hambali and Haddad [61] using a silica column and UV detection at 254 nm.

Bond and Nagaoasa [62] separated Fe(II), Cu(II) and Al(III) using 8-hydroxyquinoline (oxine) and in situ complexation. Hoffman and Schwedt [63] compared pre-column and on-column derivatisation for the separation of oxinate complexes of Cr(VI), Co(II), Mn(II), Zn(II), Cu(II), Al(III) and Mn(III). Using on-column derivatisation, the separation of all the above metal complexes could be achieved on a reversed-phase column with a mobile phase containing methanol, borate buffer (pH 9.0) and oxine. Pre-column derivatisation suffered from problems associated with changes in the oxidation states of some metals during the derivatisation step.

Baiocchi et al. [64] and Mooney et al. [65] investigated the separation of metals on a C_{18} column, using pre-column chelation with oxine and a mobile phase containing an excess of oxine.

6.7.2.4. 1,10-phenanthroline

Paired-ion HPLC using 1,10-phenanthroline has been used by O'Laughlin and Hanson [66] in the determination of Fe(II), Ni(II) and Ru(II). Rigas and Pietrzyk [67] reported on the use of Fe(II)-1,10-phenanthroline salts as mobile phase additives for the HPLC separation of inorganic analyte anions on a reversed-phase column. Indirect detection was used to detect analyte anions by monitoring the eluent at 510 nm where the Fe(II)-1,10-phenanthroline complex absorbs.

1,10-phenanthroline has been added to a mobile phase to enhance the stability of metal xanthate complexes [68]. The separation of Fe(II), Hg(II), Ni(II) and Cu(II) was achieved on a reversed-phase column with a mobile phase containing acetonitrile, tetraethyl ammonium perchlorate, xanthate and 1,10-phenanthroline. Detection limits of 0.5 ppm were attained for each metal using UV detection at 290 nm.

Trace amounts of iron were pre-concentrated from an aqueous phase using 1,10-phenanthroline and tetraphenylborate supported on naphthalene by Naghiro

et al. [69]. The adsorbed Fe(II) was dissolved withacetonitrile from the column together with the adsorbent and the absorbance read at 508 nm.

6.7.2.5. Other Ligands used for HPLC

The acetylacetone complexes of Mn(II), Be(II), Co(III), Rh(III), Ru(III), Pd(II) and Pt(II) benzoylacetene complex of Cr(III) were studied by Gurira et al. [70]. Acetonitrile / water and methanol / water mobile phases were investigated and detection limits in the ng range were achieved with UV detection at 280 nm.

Heinzmann and Ballschmiter [71] used 1,2-diketo-bisthiobenzocarbazones and 1,2-diketo-bisthiosemicarbazones for the determination of Hg(II), Ni(II), Cu(II), Zn(II) and Pb(II). However, due to problems with solubility of the complexes in organic solvents, these ligands are not extensively used.

Pb(II), Cd(II) and Zn(II) were chelated with EDTA and studied by Beckett et al. [72]. Saitoh et al. [73] used tetraphenylporphyrin for the separation of a number of metals including Fe(II). The separation was achieved in 8 minutes using a Lichrosorb RP-18 column. Detection was achieved using UV detection at 420 nm.

6.7.3. Other Chromatographic Techniques

Den Bleyker et al. [74] investigated the TLC of metal ions on bonded stationary phases. The separation of Fe(III), Cu(II), Zn(II) and Ni(II) was studied. Best separation was on a phase prepared by the reaction of (3-((2-aminoethyl)amino)propyl) trimethoxysilane with silica gel . Chromatograms were developed with 10 % solution of trifluoroacetyl acetone in acetone. The spots were located by spraying with ethanolic 1 % diphenylcarbazide and for Fe with aqueous 1 % $K_4Fe(CN)_6$.

Dunemann et al. [75] studied element species analysis on soil solutions by gel chromatography and chemical reaction detectors. Gel chromtography was used to fractionate organic matter in soil equilibrium solutions by molecular weight. The fractions so obtained were analysed for metals in a continuous flow system of pumps, reagent additions and a variable wavelength detector.

Table 6.4. Reagents and detection wavelengths used for the determination of Zn, Al and Fe

METAL	REAGENT	WAVELENGTH
Zn	Zincon solution pH 10	
	HCN + chloral hydrate	600 nm
Al	Chrome azurol S	546 nm
Fe	Ferrozine	562 nm

6.8. References

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CHAPTER 7

COMPARISON OF HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC AND ATOMIC SPECTROMETRIC METHODS FOR THE DETERMINATION OF Fe(III) AND Al(III) IN SOIL AND CLAY SAMPLES

7.1. Introduction

The identification and quantitation of bioavailable metals in soil and clay samples proves to be of continuing interest [1, 2]. Consequently, the development of methodology for the "speciation" and quantitation of metal ions held in amorpous or semi-crystalline forms outside the formal, structural packets in soil and clay samples (i.e. "extra framework" forms) is of considerable importance for bioavailability studies.

To this end, a variety of different extraction procedures have been developed, principally to investigate the nature of iron oxides in soil and clay matrices. Mehra and Jackson [3] introduced the dithionite-citrate-bicarbonate (DCB) method for the extraction of iron oxides of different crystallinites, encompassing water-soluble, exchangeable and organic-bound iron species.

McKeague and Day [4], however pioneered the use of oxalate to extract amorphous, non-crystalline or poorly ordered iron oxides. In addition, various strong acid extractions have been reported which are supposed to approximate the "total" iron content [5, 6], whereas the use of pyrophosphate is supposed to extract only organic-bound Fe(III) complexes [7]. Bloom et al. [8] developed a spectrophotometric method for the determination of "available" aluminium, which involved the use of 8-hydroxyquinoline (oxine) as

extractant, at a wavelength of 395 nm. However, a 15 min extraction period was necessary to surmount the interference form Fe(III), and probably resulted in a change in the speciation of Al(III) during the extraction process.

In an attempt to overcome this, James et al. [9] used a 15 s extraction period combined with an estimate of the Fe(III) interfernce from the absorbance at 600 nm. This type of background correction is, however, laborious and prone to large error.

The extraction method employed in the analysis of metal ions in soils and clays is therefore of obvious importance in the development of any "speciation" scheme. The detection of metal ions following these extraction procedures may be carried out using a variety of techniques ; the most commonly employed methods being based on colorimetry or atomic absorption spectrometry (AAS). In recent years, however, much interest has been shown in the application of high-performance liquid chromatography (HPLC) for trace metal analysis. A variety of ligands have been investigated in this regard, including the dithiocarbamates [10, 11], dithizone [12], 1,10-phenanthrolines [13] and oxine [14].

The use of oxine as a ligand in an HPLC method for the determination of Cu(II) and Fe(III) in anaerobic adhesive formulations has recently been reported [15]. In this section, the use of this particular ligand for

the determination of Fe(III) and Al(III) in soil and clay samples is investigated. A major potential advantage of using this ligand for this application, is that it may also be used as the extractant of the metal ions from the original soil or clay sample.

7.2. Experimental

7.2.1. Materials

All chemicals used were of analytical grade. Soil samples were taken from a fixed locality on the DCU campus, sieved to obtain a particle size of 2 mm or less, and dried in an oven at 110°C. The clay sample used was an untreated Wyoming montmorillonite described by Breen et al. [16]. All aqueous solutions were prepared in distillled water, further purified by passage through a Milli-Q water purification system.

All organic solvents used were of HPLC-grade. Sample preparation cartridges (Sep-Pak) were obtained from Waters. The C_{18} column used in this study was obtained from Supelchem, and was a 25 cm x 4.6 mm steel column containing LC-18-DB (5 μm particle size) packing material. A guard column of C_{18} packing material was used to protect the analytical column.

7.2.2. Apparatus

The HPLC system used in this study consisted of an Applied Chromatography Systems (ACS) Model 352 ternary gradient pump connected to a Rheodyne 7125 injection valve and a Shimadzu Model SPD-6A variable-wavelength spectrophotometric detector. AAS was carried out using an Instrumentation Laboratory (IL) Model 357 AA/AE spectrophotometer.

7.2.3. Methods

7.2.3.1. Digestion / Extraction Procedures

(i) Hydrofluoric acid digestion

Dried soil or clay (0.1 g) was shaken with 5.0 ml 40 % hydrofluoric acid in a PTFE vessel for 24 h, and the extract diluted to 1 part in 300 parts water, prior to analysis.

(ii) Hydrochloric acid digestion

Dried soil or clay (0.1 g) was shaken with 100 ml 36 % hydrochloric acid for 24 h. Analysis was carried out on 1:100 and 1:25 dilutions in water for soil and clay samples, respectively.

(iii) Dithionite-citrate-bicarbonate extraction

This was carried out according to the method of Mehra and Jackson [3].

(iv) Oxalate extraction.

This was carried out according to the method of McKeague and Day [4].

(v) Oxine extraction for Fe(III)

Dried soil or clay (0.5 g) was extracted for 4 h with 40.0 ml of (0.5 %) oxine dissolved in 0.02 M acetate buffer, pH 4.0. A 2.0 ml aliquot of each extract was then passed through a silica Sep-Pak. The metal ion-oxine complex was then eluted with 4 ml methanol before analysis.

(vi) Oxine extraction for Al(III).

This was carried out according to the method of James et al. [9], with the following modifications : (i) the oxine concentration was reduced to 0.5 %, (ii) the reaction was stopped by centrifugation instead of extraction with butyl acetate, which interfered with subsequent HPLC analysis.

7.2.4. High Performance Liquid Chromatographic (HPLC) Analysis

The conditions used for the HPLC analysis of Fe(III) and Al(III) were the same as those reported in [15]. The mobile phase contained acetonitrile (containing 1×10^{-2} M oxine)-0.02 M acetate buffer pH 6.0 (containing 0.2 M potassium nitrate) (50:50).

Standard solutions of metal ions or extracts from soil and clay samples were injected directly onto the column through the injection port without any external formation of the complex.

7.2.5. Atomic Absorption Spectrophotometric (AAS) Analysis

The conditions used for AAS measurements for Fe(III) were : light source, hollow cathode; lamp current, 8 mA; wavelength, 248.3 nm; slit width, 80 μm; burner head, single slot; band pass, 0.3 nm; flame description, air-acetylene, oxidising, fuel lean, blue. For Al(III) they were : light source, hollow cathode; lamp current, 8 mA; wavelength, 309.3 nm; slit width, 320 μm; burner head, nitrous oxide; band pass, 1.0 nm; flame description, nitrous oxide-acetylene, reducing, fuel rich, red.

7.2.6. X-Ray Diffraction (XRD) Analysis

Oriented samples for X-ray diffraction (XRD) analysis were prepared by evapourating an aqueous slurry of soil or clay onto a microscope slide (15 x 10 mm). The slide was then placed in the goniometer of a Philips Model PW1050 diffractometer operating at 40 kV and 20 mA using CuK_{α} radiation (λ= 1.5418). The XRD profiles were recorded at 2^{o} (2θ) min^{-1} from 0-60^{o} (2θ). In the case of the hydrofluoric acid

digestion, the extract contained almost no solid matter, and a small portion of the extract was poured onto a glass slide, where some crystals formed.

7.3. Results and Discussion

7.3.1. X-Ray Diffraction Studies

The XRD profile of the untreated soil sample is shown in Figure 7.1, and illustrates that the major, indexable, crystalline component of this soil is α-quartz (Q).

In contrast, the XRD profile of the untreated clay (C) sample (Figure 7.1.b) indicates the presence of several impurities including mica (M), kaolin (K), quartz (Q) and feldspar (F), but exhibits no peaks commensurate with crystalline Fe- or Al-containing species such as goethite, lepidocrocite or gibbsite, respectively.

The effect of increasing severity of three of the extraction/digestion procedures reported in this section on the XRD traces obtained for the clay sample is illustrated in Figure 7.1.c-e. The DCB extract was not washed prior to collecting the XRD data, and this accounts for the three characteristic sodium chloride (N) peaks shown in Figure 7.1.c. The XRD trace obtained for the hydrochloric acid digest is shown in Figure 7.1.d, and shows that treatment with this 36 % hydrochloric acid causes no noticeable degradation of

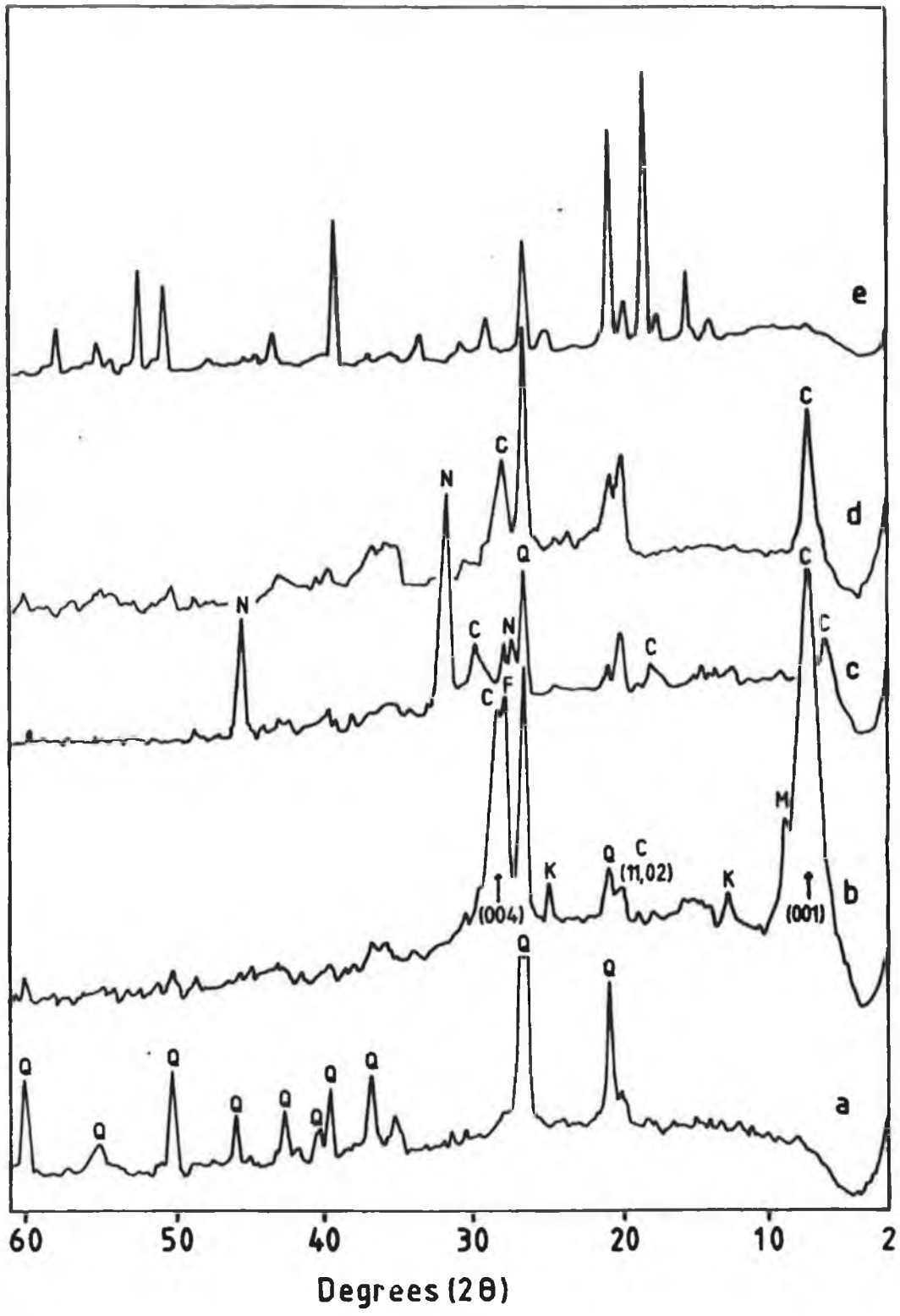

Figure 7.1. XRD profiles for (a) untreated soil, (b) untreated clay, (c) DCB-extracted clay, (d) hydrochloric acid digested clay, and (e) hydrofluoric acid digested clay.

the structural clay lattice, and reflects the greater resistance of aluminous silicates to acid attack compared to the magnesium rich analogues.

The reduced intensity of the Q and F peaks in the DCB extract most probably reflects the physical loss of sample rather than the preferential extraction, whilst the Feldspar peak for the hydrochloric acid digested clay (Figure 7.1.d) lies below the 005 reflection marked C at around 28^{o} (2θ).

The XRD trace obtained for the hydrofluoric acid digest of the clay (Figure 7.1.e) illustrates emphatically that treatment with hydrofluoric acid has a devastating effect on the clay, leaving few identifiable reflections. In a similar manner, only the hydrofluoric acid digestion procedure had any marked effect on the diffraction profile of the soil sample.

7.3.2. Digestion / Extraction Methods

The results obtained using both HPLC and AAS for the percentages of Fe(III) and Al(III) in the soil and clay samples following the various digestion / extraction procedures detailed under Experimental are given in Table 7.1. A typical trace obtained for the separation of Fe(III) and Al(III) using HPLC is shown in Figure 7.2. A detection wavelength of 400 nm was employed, which lies between the λ_{max} values for the oxine complexes of Fe(III) and Al(III) at 450 nm

n.d. = not detected.

Extractant	*Sample*	*HPLC*		*AAS*	
		Fe(III) *(%)*	*Al(III)* *(%)*	*Fe(III)* *(%)*	*Al(III)* *(%)*
HF	Soil	2.38 ± 0.18	0.09 ± 0.002	2.36 ± 0.12	2.64 ± 0.09
	Clay	1.6 ± 0.05	0.25 ± 0.005	1.64 ± 0.12	7.51 ± 0.44
HCl	Soil	1.69 ± 0.04	0.20 ± 0.01	1.71 ± 0.02	0.42 ± 0.01
	Clay	0.75 ± 0.01	0.14 ± 0.01	0.75 ± 0.01	0.15 ± 0.01
DCB	Soil	1.39 ± 0.06	1.33 ± 0.04	1.46 ± 0.03	1.70 ± 0.04
	Clay	0.18 ± 0.01	0.004 ± $1 \cdot 10^{-4}$	0.16 ± 0.01	0.003 ± $1 \cdot 10^{-4}$
Oxalate	Soil	0.08 ± 0.01	0.04 ± 0.005	0.08 ± 0.005	0.07 ± 0.005
	Clay	0.04 ± 0.005	0.04 ± 0.001	0.04 ± 0.001	0.08 ± 0.001
Oxine	Soil	0.05 ± 0.005	n.d.	0.05 ± 0.005	n.d.
	Clay	0.02 ± 0.001	n.d.	0.02 ± 0.001	n.d.

Table 7.1. Concentrations of Fe(III) and Al(III) obtained using HPLC and AAS following digestion / extraction procedures.

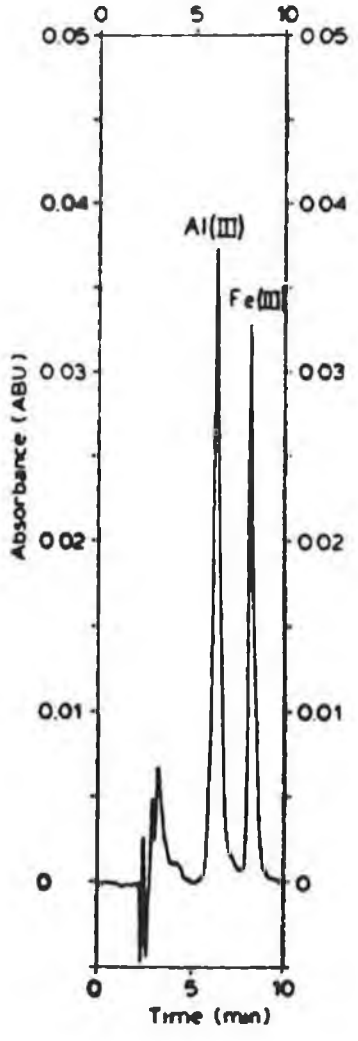

Figure 7.2. HPLC separation of a 10 ppm mixture of Fe(III) and Al(III) with spectrophotometric detection.

Conditions : flow rate 1.0 ml/min, detection wavelength 400 nm.

and 375 nm, respectively. Limits of detection of the order of 1-2 ppm for Fe(III) and Al(III) were typically achieved using both methods.

7.3.2.1. Hydrofluoric Acid Digestion

The values obtained for the percentage Fe(III) in the soil and the clay represent "total" metal content, because the hydrofluoric acid completely digests the samples as shown from the XRD trace in Figure 7.1.e. The results obtained using both HPLC and AAS are in good agreement, and show that the Fe(III) content of the soil sample exceeds that of the clay.

The values obtained for the percentage Al(III) in the soil and clay using HPLC were consistently lower than those obtained using AAS. This is probably due to the fact that Al(III) forms a much stronger complex with fluroride ions than with oxine, and hence prevents any in-situ formation of the Al(III)-oxine complex on the column.

With the high temperatures used in the rich nitrous oxide-acetylene flame, the Al(III)-fluoride complexes will be atomised, hence giving rise to a more accurate value of the Al(III) content in the soil or clay. Consequently, greater cognisance should be afforded the AAS results, particularily since the value of 7.51 % Al(III) in the clay sample is close to that reported previously.

7.3.2.2. Hydrochloric Acid Digestion

The values obtained for the percentage Fe(III) in the soil and clay were shown to be in good agreement using both instrumental methods of analysis. In the case of Al(III), the results for the clay were in good agreement, but the percentage Al(III) in the soil was found to nearly double using AAS when compared to HPLC. The reason for this discrepancy is not clear, but may be due to the nature of the "extra-framework" Al(III) in the respective matrices.

From the results of the XRD studies, it is clear that 36 % hydrochloric acid digestion is not as effective at breaking down the structural units of the soil and clay matrices, and thus this digestion procedure can only yield results that approximate to "total" metal ion content.

7.3.2.3. Dithionite-Citrate-Bicarbonate Extraction

This extraction technique, which is based on the strong reducing properties of dithionite, was developed to extract oxides of different crystallinity, including water-soluble, exchangeable and organic-bound metal [3]. The optimum pH for this extraction is 7-8, and this is maintained by the buffering capacity of the bicarbonate anion. Citrate is employed to complex the reduced metal.

The values obtained for the percentages of Fe(III)

and Al(III) (Table 7.1) show that whilst the HPLC and AAS results for percentage Fe(III) are in good agreement, the HPLC results for percentage Al(III) in the soil sample are somewhat lower than those obtained by AAS.

This probably reflects competition between citrate and oxine for Al(III), which is a problem in the HPLC assay, but not in AAS, where the citrate complex would be broken down. Consequently, since the HPLC method relies on complexation of free metal ion with oxine, the difference between the two results may relate to the amount of Al(III)-citrate complex present following extraction. One further interpretation which cannot be ruled out, is that the DCB method extracts organic-bound Al(III) which would be determined by AAS but not by HPLC.

The higher values for the percentage Al(III) in the soil compared to those obtained following hydrochloric acid digestion may be explained by the possible presence of these phases, because as Schulze and Schwertmann [17] have found in both naturally occurring and synthetic goethites, the substitution of Fe(III) by Al(III) substantially reduces the intensity of peaks in the diffraction profile.

7.3.2.4. Oxalate Extraction

The oxalate extraction was introduced by McKeague and Day [4] to extract the "active" fraction of metal

ions from soils. This includes amorphous, non-crystalline or poorly ordered metal oxides and also includes organic-bound metal.

The technique is based on complexation of metal ions by oxalate at pH 2-3. In this section, extractions were carried out at pH 2 and 3, but little difference was noticed in the percentage metal ion concentrations obtained. The results obtained for the percentage Fe(III) and Al(III) in soil and clay samples following this extraction procedure at pH 3 are given in Table 7.1. Once again there is good agreement between the HPLC and AAS values for the percentage Fe(III), but not for the percentage Al(III). However, it is unlikely that the source of this discrepancy is due to the competitive chelation observed in the DCB method because there is a digestion step using nitric acid-sulphuric acid prior to analysis, which should break down any Al(III)-oxalate complex and / or organic-bound Al(III).

Unfortunately, this acid digestion step lowers the pH of the extract prior to the analysis stage, and studies with comparable standard solutions indicate that these low pH values suppress absorbance readings in AAS and affect the complexation of Al(III) with oxine, resulting in lower values than anticipated for the HPLC method.

The reasons that the values obtained for the percentage Al(III) using HPLC are higher in the soil

and lower in the clay following the oxalate extraction compared with the dithionite-citrate-bicarbonate extraction may be due to the fact that oxalate is a better extractant of amorphous Al(III) species [4] which are suggested to be more prevalent in the soil than the clay from the XRD studies.

7.3.2.5. Oxine Extraction

Methods employing oxine as an extractant for "available" Al(III) in soils have been reported in the literature [8, 9], but this extractant has not been widely used for determinations of Fe(III) in soil and clay samples. Therefore, the use of oxine for Fe(III) determinations in these matrices, based on the method of James et al. [9] reported for Al(III) was investigated.

The extraction method involving oxine was optimised with respect to time of extraction, pH and isolation of the complex using Sep-Pak cartridges. The extraction time was varied between 2 and 72 h, but periods in excess of 4 h gave comparable results.

Previous studies [14, 15] have shown that the formation of the oxine-Fe(III) complex is optimal in the pH range 4-6, although analysis of the soil extracts indicated that slightly higher values were obtained at pH 4 than at pH 6.

Furthermore, the use of a silica Sep-Pak was found

to be an effective means of isolation of the complex formed, in addition to acting as a means of sample "clean-up", as observed previously [15]. It was found that 4 ml of methanol was required to quantitatively remove 20 µg of oxine-Fe(III) complex from the Sep-Pak.

The results in Table 7.1 show that there is good agreement between the HPLC and AAS results for the percentage Fe(III) in soil and clay samples using this extraction method. No detectable concentrations of Al(III) were found in any of the oxine extracts, by either HPLC or AAS, although spiking a soil sample with 5 ppm Al(III) resulted in HPLC and AAS values of 5.03 and 5.30 ppm Al(III), respectively. This suggests that oxine will only extract extremely labile Al(III) from these matrices.

7.4. Conclusions

A comparison of results in Table 7.1 show good agreement in the percentage Fe(III) values obtained from both soil and clay using both HPLC and AAS. The percentage Fe(III) extracted using the various methods decreased in the order HF > HCl > DCB > oxalate > oxine. This is to be expected considering the different mechanisms by which these extractions / digestions operate. It is interpreted from the results that the hydrofluoric acid extraction yields a value relating to the "total" Fe(III) content. Because of

the specififc nature of the DCB and oxalate extraction procedures for crystalline and non-crystalline oxides, respectively, the different values for the percentage Fe(III) arising from these procedures can be explained. The difference between the hydrofluoric acid results and those obtained using the DCB extraction can be attributed to the amount of Fe(III) which forms an integral part of the lattice structure of the clay, and perhaps also that of the soil.

The difference between the DCB and oxalate extractions for Fe(III) can be attributed to the amount of crystalline iron oxides present in these matrices, even though they were too small in particle size to be observed using XRD analysis. The difference between the oxalate results and those obtained using the oxine extraction is most probably due to "exchangeable" Fe(III) species.

The results obtained with the 36 % hydrochloric acid digestion suggest a small ingress of acid into the octahedral layer, thus leaching out a small amount of Fe(III) associated with the lattice structure. In the case of Al(III), the percentage values obtained in both soil and clay were found to be in good agreement for the hydrochloric acid digestion and the DCB extraction for the clay using both HPLC and AAS. The results obtained using the other extraction procedures, however, were found to be much lower using HPLC compared to AAS, especially for the soil. This is mainly due to competition between oxine and the

various extractants used for Al(III).

A comparison has been made of high-performance liquid chromatgraphic and atomic spectrometric methods for the determination of Fe(III) and Al(III) in soil and clay samples, following five different digestion / extraction procedures.

Good correlations were obtained in the case of Fe(III) determinations in both matrices, but correlation was only achieved for Al(III) determinations following an hydrochloric acid digestion and a dithionite-citrate-bicarbonate extraction of the clay sample.

Attempts have been made to explain the differences in results between the two methods in terms of the Al(III) species which are likely to be present following extraction. Confirmatory evidence to support some of the conclusions made with respect to the speciation of these metal ions in these matrices has been obtained using X-ray diffraction studies.

Although this investigation has necessarily limited itself to the analysis of a single soil and a single clay sample, it has, however, highlighted the possibility of employing HPLC (i) as a multi-element approach to the determination of metal ions in soils and clays, and (ii) to provide information on the speciation of metal ions, provided that experiments have been carried out taking into consideration the matrix involved as well as the sample preparation. If lower limits of detection were required than are

possible using the approaches described in this investigation, then these could be achieved for the HPLC method by employing the technique of "external formation" of the oxine-Fe(III) or oxine-Al(III) complex prior to injection onto the column, and for the AAS method by using a flameless approach to atomisation.

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CHAPTER 8

THE SPECIATION OF IRON

8.1. Introduction

Iron occurs in aqueous solution in the +2 and +3 oxidation states. Fe(III) shows a greater affinity for oxygen-containing ligands, unlike Fe(II) which shows a slight preference for nitrogen donor atoms. Both oxidation states form strong mainly octahedral complexes, the majority of which are chemically inert.

In the presence of oxygen or oxidising acids, some conversion of Fe(II) to Fe(III) occurs, but non-oxidising acids in the absence of oxygen give only Fe(II). Compounds of Fe(III) are generally more stable.

Speciation of an element in a sample may be defined as "the determination of the concentrations of the different physio-chemical forms of an element which together make up its total concentration in the sample". Speciates may include dissolved forms such as simple inorganic species, organic complexes and the element adsorbed on a variety of colloidal particles.

One of the main reasons for studying the speciation of metals in environmental samples is to understand either the biological or the geochemical cycling of the metals.

Determination of the total concentration of a metal does not provide sufficient information to predict toxicity, as each different physio-chemical form of an element may have a different toxicity.

Speciation may also provide information on the

adsorption of a metal on particulates or its overall transport in a water system. This could potentially be used to source effluent discharge.

Terms that are used in speciation studies include "labile" or "reactive" metal. This consists of the free metal ion and metal that can dissociate from complexes or colloidal particles. Labile metal is usually expressed as a percentage of total dissolved metal and the difference between total and labile metal is termed "inert" or "unreactive" metal. It has been found that labile metal correlates well with the toxic fraction of metal.

In this report, a literature survey of recent developments on the subject of speciation of iron is included, along with short discussions on the ligands used in this study, i.e. 1,10-phenanthroline and 5-sulphosalicylic acid. Some points are also discussed on the technique and theories of ion-pair chromatography and metal-ion buffers. The next chapter presents the development of a technique for the HPLC separation of iron(II) and iron(III).

8.2. Ligands for the Determination of Iron

Table 8.1 summarises some reactions that Fe(II) and Fe(III) undergo, as well as giving a short description of some of the sensitive and selective methods available for their determination.

Table 8.1. Some methods for the determination of iron(II) and iron(III)

REACTANT	Fe(II)	Fe(III)
potassium thiocyanate	no reaction (N.R.)	Blood red colour (due to the formation of Fe(III)-SCN complexes such as $FeSCN^{2+}$)
1,10-phen	intense red colour due to the formation of the tris $Fe(phen)_3^{2+}$ complex	very pale blue colour, does not with observation of the red $Fe(phen)_3^{2+}$ complex
oxidants such as $KMnO_4$ or $K_2Cr_2O_7$	oxidation of Fe(II) to Fe(III) in acidic solutions	N.R.
2,2-bipyridyl	deep red colour, similar complex to that formed with 1,10-phenanthroline	colourless complex
5-sulphosalicylic acid	N.R.	red / violet colour in acidic solution

REACTANT	Fe(II)	Fe(III)
bathophenanthroline	red / violet colour	colourless complex
reducing agents such as H_2S, SO_2 and $SnCl_2$	N.R.	reduces Fe(III) to Fe(II)
sodium salicylate	N.R.	intense violet colour in neutral solution which is discharged on acidification

8.2.1. Thiocyanate

In moderately acidic media, Fe(III) ions react with thiocyanate ions to give a red colour which for a long time was the basis for the determination of Fe(III); Fe(II) being determined after oxidation to Fe(III). Complexes formed by the reaction of Fe(III) and thiocyanate include $[Fe(SCN)]^{2+}$ and $[Fe(SCN)_2]^{+}$ up to $[Fe(SCN)_6]^{3-}$. Some complexes may be more abundant in solution than others depending on parameters such as pH and the concentration of the reagents.

In order to ensure stable complexes, excess SCN^- must be present. The molar absorptivity of the Fe(III)-thiocyanate complex solution in methyl iso-butyl ketone (MIBK) is 2.4×10^4 at λ_{max} = 495 nm. The λ_{max} varies between 460 and 530 nm depending on the medium. An aqueous medium gives a λ_{max} = 465nm.

Fluoride, phosphate, citrate and oxalate interfere with thiocyanate by forming stable complexes with Fe(III), as can high concentrations of acetate, chloride and sulphate albeit to a lesser degree.

8.2.2. Bathophenanthroline

4,7-diphenyl-1,10-phenanthroline (Figure 8.2) (bathophenanthroline) reacts with Fe(II) in a very similar manner to 1,10-phenanthroline (Figure 8.1). Both ligands are selective but the former gives rise to more sensitive methods of analysis. The λ_{max} of the Fe(II)-bathophenanthroline complex is 533 nm with an optimum pH for the colour reaction in the range 4-7.

Copper(I) also forms a very stable colourless complex with bathophenanthroline, which gives rise to interference. This may be prevented by either precipitation as cuprous thiocyanate or masking e.g. with thiourea. Bathophenanthroline may be used for the determination of Fe(II) in the presence of a large excess of Fe(III).

Figure 8.1. 1,10-phenanthroline and 2,2-bipyridyl.

Figure 8.2. Bathophenanthroline

(4,7-diphenyl-1,10-phenanthroline).

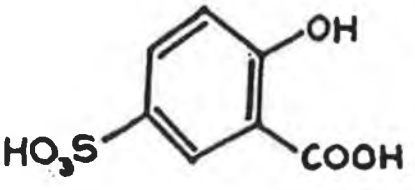

Figure 8.3. 5-sulphosalicylic acid.

8.2.3. 1,10-Phenanthroline and 2,2-Bipyridyl

Both 1,10-phenanthroline (phen) and 2,2-bipyridyl (bipy) contain nitrogen donor atoms which are selective towards Fe(II) (Figure 8.1). Both reagents react rapidly with Fe(II) ions over a wide pH range (2-9) to give coloured complexes. Three molecules of the ligand bind through their nitrogen donor atoms to one Fe(II) ion thus forming a stable octahedral complex. The λ_{max} values are given as 512 nm (Fe(II)-phen) and 522 nm (Fe(II)-bipy). One of the important features of these complexes is that once formed, they are stable and the complexed iron is resistant to oxidation.

Of all the metal ions that are complexed by 1,10-phenanthroline, those of iron, ruthenium and osmium give the most intensely coloured species and are among the most stable. Under strongly oxidising conditions, the tris-chelates of Fe(II) are converted into the corresponding Fe(III) chelates. For a given separation system, it may be important to know the formation or stability constants.

Such constants given in Table 8.2 are determined using the following equations.

$$M^{n+} + B \rightleftharpoons M(B)^{n+}$$

$$K_1 = [\, M(B)^{n+} \,] \,/\, [\, M^{n+} \,] \, [\, B \,]$$

$$M(B)^{n+} + B \rightleftharpoons M(B)_2^{n+}$$

$$K_2 = [\ M(B)_2^{n+}\ ] / [\ M(B)^{n+}\ ]\ [\ B\ ]$$

$$M(B)_2^{n+} + B \rightleftharpoons M(B)_3^{n+}$$

$$K_3 = [M(B)_3^{n+}]/[M(B)_2^{n+}][B]$$

$$\beta_2 = K_1K_2 = [M(B_2)^{n+}]/[M^{n+}][B]^2$$

$$\beta_3 = K_1K_2K_3 = [M(B_3)^{n+}]/[M^{n+}][B]^3$$

Table 8.2 Stability constants for Fe(II)-phen and Fe(III)-phen complexes

Metal ion	Ligand	$\log\beta_2$	$\log\beta_3$
Fe(II)	phen	5.85	21.3
Fe(III)	phen		14.1

The complex formed between Fe(II) and 1,10-phenanthroline has been shown to be of the inert type, i.e. once formed, and the rate of formation may be very rapid, these complexes tend to undergo ligand or metal ion-exchange reactions very slowly. Some rate constants for formation and dissociation of Fe(phen) chelates are shown in Table 8.3.

Table 8.3 Rates of formation and dissociation

Fe(II)-phen

Ion	Ligand	$logk_f$	$logk_d$	Solution	T^oC
Fe(II)	phen	17.3	-4.1		25
			-0.1	2M H_2SO_4	100.7
			-3.5	83% H_2SO_4	100.7
			-5.8	98% H_2SO_4	100.7

Table 8.4 Absorption maxima for Fe(II)-phen chelates

Chelate	λ max	ϵ extinction coefficient
$[Fe(phen)_3]^{2+}$	510nm	11100
$[Fe(phen)_2]^{3+}$	590nm	600

1,10-phenanthroline and related compounds have been used extensively in analytical chemistry as colorimetric reagents for a number of reasons. Most important is the fact that they may are formed quantitatively even in dilute solutions, provided a suitable pH and excess of reagent are used.

For example, the formation of $Fe(phen)_3^{2+}$ is found to go to 99.9 % completion at pH 5.0 independent of the Fe(II) concentration, so long as excess phenanthroline is at least 10^{-6} M. Another feature is that sensitivity may be improved by extraction of certain salts of the metal chelates into water immiscible solvents. Sensitivity and limit of detection may be dependent on the molar absorptivity

since formation of the chelate can be made quantitative by the use of a slight excess of reagent and proper pH control

8.2.4. Determination of Iron

Where excellent sensitivity and unusual conditions are not required, 1,10-phenanthroline may be used as a general reagent, as it is less expensive but just as suitable for the requirement. Generally, an acetate buffer (pH 3-6) is employed to enhance formation of the red Fe(II)-phen chelate.

In acidic solution, the competition between hydrogen and Fe(II) ions for 1,10-phenanthroline precludes formation of the tris-chelate. Several reducing agents are available to convert Fe(III) to Fe(II). Hydroxylamine hydrochloride, ascorbic acid and hydroquinone are but a few of those reported in several papers. The rate of formation of the coloured complex is dependent on which ligand the Fe(II) is already complexed to, but once formed, the colour is stable for several months.

Interferences such as colour formation by other ions with 1,10-phenanthroline, consumption of 1,10-phenanthroline by ions other than iron, and complexation of iron by other species can quite often be eliminated by for example, the use of masking agents, effective pH control or removal of interfering substances.

8.2.5. 5-Sulphosalicylic Acid

5-Sulphosalicylic acid, which forms a stable complex with Fe(III) ions in acid media, can be used successfully in the presence of Fe(II). Different complexes are formed depending on the pH of the medium.

At pH 2-3 a red-violet (1:1) complex is formed; pH 4-7, a brown-orange (1:2) comlpex; and at pH 8-10, a yellow (1:3) complex.

The violet complex, which is stable in acidic media is used to determine Fe(III) in the presence of Fe(II). 5-Sulphosalicylic acid forms water soluble complexes with most multivalent metal ions. Coloured complexes are obtained with metals having chromophoric properties.

8.3. Metal-Ion Buffers

In a solution containing a metal ion, M, and a chelating agent or ligand L, metal complexes are formed.

$$M + nL \rightleftharpoons ML_n$$

and the equilibrium can be expressed quantitatively by the appropriate stability constant

$$\beta_n = (ML_n) / (M)\,(L)^n$$

This can be written as:

$$pM = \log\beta_n + \log [(L)^n / (ML_n)]$$

where pM is the negative logarithm of the free metal ion activity. When the ligand is in excess and almost all the metal ion is bound in a complex, the free metal ion is "buffered" in a manner similar to hydrogen ions in pH buffers.

Very often the ligand L can undergo protonation which is the case with 1,10-phenanthroline. In acidic solutions, the principal species is the phenanthrolium ion, PhH^+, which is a weak base capable of acting as a mild reducing agent. This was one of the problems encountered during development of the method for the speciation of iron(II) and iron(III) since 1,10-phenanthroline (while protonated) could reduce non-complexed Fe(III) to Fe(II) giving rise to false results, hence the need for a buffer to control pH.

8.4. Ion-Pair Chromatography

When the sample is a strong acid or a strong base it is possible to alter the chromatographic retention by introducing long chain ionic alkyl compounds into the mobile phase. Addition of these reagents to the mobile phase will alter retention behaviour of ionic compounds but not of non-ionic compounds.

Reversed-phase ion-pair chromatography (RPIPC) was

the technique used for the speciation of iron(II) and iron(III) in chapter 9 and involves using a bonded phase packing as the stationary phase and an aqueous mobile phase containing the "ion-pairing" reagent.

For RPIPC, a non-polar surface (e.g. C_8 or C_{18}) is used as a stationary phase and an ionic alkyl compound is added to the aqueous mobile phase as a modifier. It is essential to operate the system after equilibration of the mobile phase and stationary phase has occurred in order to obtain reproducible analyses.

Most applications of RPIPC involve the addition of long chain alkyl sulphonate ions to the mobile phase to give enhanced separation of oppositely charged sample ions. The exact mechanism to describe the ion-pair phenomenon is still uncertain. There are however, three popular hypotheses.

8.4.1. The Ion-Pair Model

This proposes the formation of an ion-pair in the aqueous mobile phase prior to its adsorption onto the bonded hydrophobic stationary phase (see Figure 8.4). Retention is governed by the amount of non-polarity of the "ion-pair", which determines the affinity to the stationary phase. A longer alkyl chain on the pairing reagent simply makes a less polar ion-pair and the retention of the pair increases as a result of its greater affinity for the stationary phase.

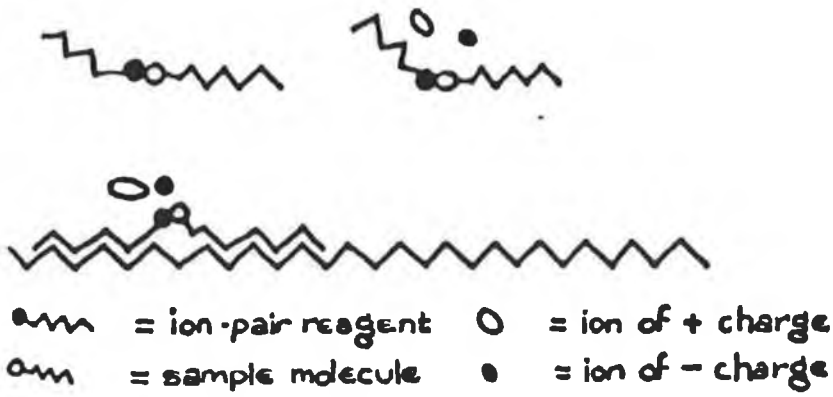

Figure 8.4. The ion-pair model.

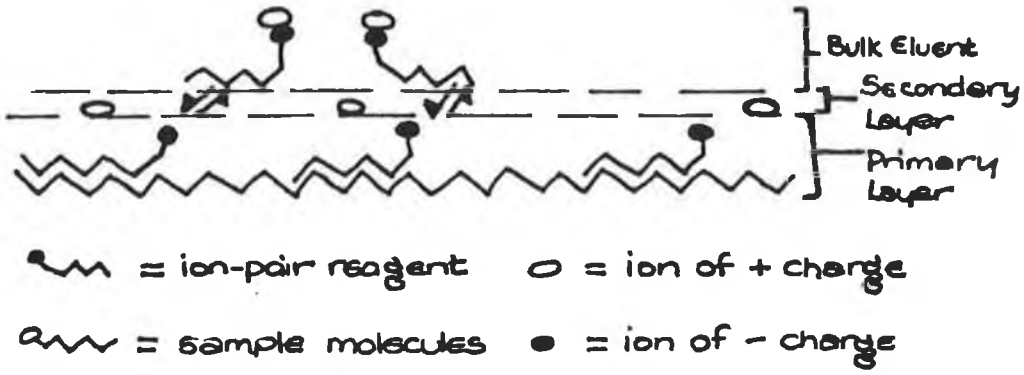

Figure 8.5. The ion-interaction model.

8.4.2. The Ion-Interaction Model

In the ion-interaction model (Figure 8.5), a layer of ion-pair reagent (lipophilic ions) is adsorbed onto the non-polar surface. Because these lipophilic ions have the same charge, they are well spaced from one another, and most of the surface area is still the original non-polar packing surface, with only a small amount of the surface being coated with the reagent. A primary ion layer and an oppositely charged counter-ion layer are formed on top of the non-polar surface. This is an electrical double layer model. Transfer of samples through this double layer is a function of electrostatic and Van der Waals forces.

Chromatographic retention results from this coulombic attraction and from an additional "sorption" of the lipophilic portion of the sample molecule onto the non-polar surface. The net result is that a pair of ions (not necessarily an ion-pair) has been adsorbed onto the stationary phase.

8.4.3. The Dynamic Ion-Exchange Model

In this model (Figure 8.6), it is the unpaired lipophilic alkyl ions that adsorb onto the non-polar surface and cause the column to behave as an ion exchanger. The longer the chain length of the ion-pairing reagent, the more surface coverage of ion-exchanger will occur, and the longer will be the retention of the ionic sample.

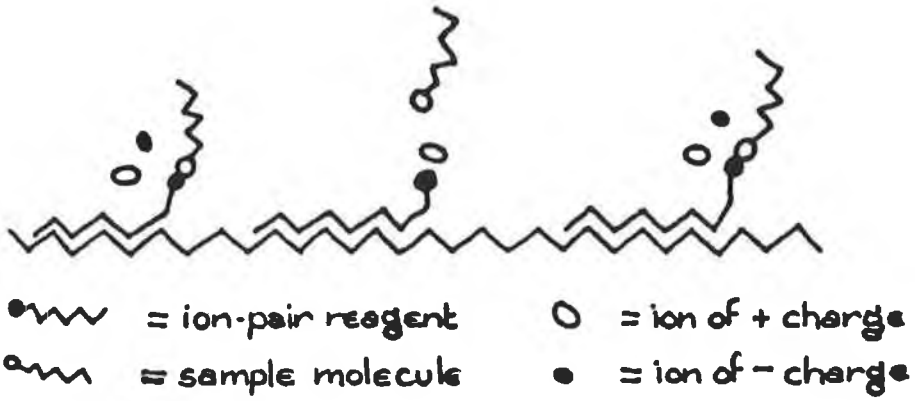

Figure 8.6. The dynamic ion-exchange model.

CHAPTER 9

REVERSED-PHASE LIQUID CHROMATOGRAPHIC SEPARATION OF Fe(II) AND Fe(III) AS THEIR RESPECTIVE 1,10-PHENANTHROLINE AND 5-SULPHOSALICYLATE COMPLEXES

9.1. Introduction

The speciation of Fe(II) and Fe(III) is of importance in industrial and environmental analysis. In the past, methods were developed for the determination of "total" iron. For instance, Mortatti et al. [1] developed a method which involved prior reduction of Fe(III) to Fe(II) with ascorbic acid, followed by complexation of the resulting total iron present in natural water and plant samples with 1,10-phenanthroline. The complex was subsequently detected spectrophotometrically at 512 nm.

More recently, flow injection analysis (FIA) with both spectrophotometric and atomic absorption spectrometric (AAS) detection has been introduced for the determination of Fe(II) and Fe(III). For example, in an FIA system described by Burgeura and Burgeura [2], Fe(II) was determined by measuring the absorbance of its 1,10-phenanthroline complex at 510 nm, whereas total iron was determined by measuring the atomic absorbance in the same flow line at 248.3 nm; Fe(III) could therefore be determined by difference.

Lynch et al. [3] described a similar method, but subsequently described a different approach in which parallel flow-injection manifolds were employed with simultaneous sample introduction using an autosampler coupled to two injection valves [4]. Detection of both species was carried out spectrophotometrically following complexation of Fe(II) with

1,10-phenanthroline and Fe(III) with thiocyanante. This system allows for direct quantitation of each species, resulting in better accuracy and precision.

Faizullah and Townshend [5] described FIA methods for the determination of Fe(III) and for the simultaneous determination of Fe(II) and total iron. Fe(III) was determined by passing the carrier stream containing the sample through a Jones reductor mini-column prior to complexation with 1,10-phenanthroline.

For the simultaneous determination of Fe(II) and total iron, the carrier stream was first split in two, with one portion passing through a Jones reductor and delay coil before recombining with the other stream. The combined stream, which then contained the unreduced portion preceding the remainder of the sample, was reacted with 1,10-phenanthroline, prior to spectrophotometric detection at 512 nm.

Fe(II) and Fe(III) have also been determined by ion chromatography with post-column reaction [6]. After separation on a cation-exchange column, the Fe(III) was reduced to Fe(II) by ascorbic acid prior to complexation with bathophenanthrolinesulphonic acid and subsequent spectrophotometric detection at 530 nm. Alternatively, the ions can be detected using 4-(2-pyridyl-azo)resorcinol (PAR) following ion-exchange separation [7].

In this section, a method is described for the simultaneous determination of Fe(II) and Fe(III) using

reversed-phase liquid chromatography (LC). This method obviates the need to reduce Fe(III) to Fe(II) or to use delay coils, and involves direct spectrophotometric detection without the requirement for a post-column addition.

9.2. Experimental

9.2.1. Materials

All chemicals were of analytical reagent-grade and solutions were prepared in water obtained by passing through a Milli-Q (Millipore) water purification system. A 0.08 % (w/v) solution of 1,10-phenanthroline was prepared by dissolving 0.400 g of 1,10-phenanthroline (Riedel de Haen) in 500 ml of 0.02 M sodium acetate buffer (pH 6.9)-acetonitrile (1+1).

A 0.1 % (w/v) solution of sulphosalicylic acid was prepared by dissolving 0.500 g of 5-sulphosalicylic acid (BDH) in 500 ml of 0.02 M sodium acetate buffer (pH 6.9)-acetonitrile (1+1). These solutions were then used to pre-complex the iron standards.

Standard solutions of Fe(II) and Fe(III) were prepared by dissolving 1.756 g of ammonium iron(II) sulphate hexahydrate (Riedel de Haen) or 1.804 g Ferric Nitrate in 20 ml of 3 M hydrochloric acid and diluting to 250 ml with water. The mobile phase consisted of acetonitrile [containing 0.1 % (w/v) 5-sulphosalicylic acid] - 0.02 M acetate buffer pH 6.9 [containing 0.1 % (w/v) tetramethylammonium chloride] (1+1). The mobile phase was deaerated by filtration through a 0.45 μm filter prior to use.

9.2.2. Apparatus

The LC system used in this study consisted of a Waters 501 HPLC pump connected to a Waters Associates model U6K injection port and a Shimadzu SPD-6A UV variable-wavelength spectrophotometric detector operated at 515 nm. A Phillips PM 8251 single pen recorder was used to record chromatograms.

The C_{18} column used in this study was obtained from Supelchem and was a 25 cm x 4.6 mm steel column containing LC-18-DB (5 µm particle size) packing material. A guard column of C_{18} packing material (10 um particle size) was used to protect the analytical column. The flow rate was maintained at 2.0 ml min^{-1}.

UV-visible spectra were obtained on a Shimadzu UV-240 spectrophotometer or a Hewlett-Packard diode array spectrophotometer.

9.3. Results and Discussion

9.3.1. Choice of Ligands and Buffer Constituents

Since 1,10-phenanthroline (phen) is well known to form a strong complex with Fe(II) [8], this compound was proposed as the ligand of choice for this ion. The selection of a ligand for the selective chelation of Fe(III) in the presence of Fe(II) therefore depended on the chosen ligand giving rise to a stable chelate

with a reasonable absorbance at a wavelength close to that of Fe(II)-phen.

Of the ligands investigated, i.e., ethyl xanthate, oxalic acid, pyrophosphate, quinolin-8-ol, thiocyanate and salicylic acid, oxalic acid and pyrophosphate were eliminated as no absorption spectra were obtained for their complexes in the region 190-800 nm.

Ethyl xanthate produced a complex which absorbed at 440 nm, a wavelength too far removed from that used to monitor the Fe(II)-phen complex, i.e., 510 nm. Quinolin-8-ol, although an excellent ligand for Fe(III) [9, 10], also gave a coloured complex with Fe(II) and hence could not be used. The only ligand which satisfied the criteria laid down was salicylic acid (SA), although it was subsequently found that this ligand suffers from limited water solubility, hence decreasing its usefulness in LC separations.

A variety of buffer systems based on acetate, citrate and phosphate between pH 3.5 and 6.0 were investigated with respect to the stability of the complexes and to prevent the reduction of Fe(III) to Fe(II).

No major change was observed in the molar absorptivity of the Fe(II)-phen complex on addition of the various buffers. No Fe(III)-SA complex was detected in the citrate buffer, but in all other buffer systems investigated complex formation was observed, with the highest apparent molar absorptivities being recorded in acetate buffer, and

this was therefore chosen as the most suitable for further study.

9.3.2. Choice of Ion-Pairing Reagent

Initial development of the LC method involved the production of a satisfactory peak only for the Fe(II)-phen complex. Ion-pair chromatography was necessary, since in the absence of an ion-pairing reagent the complex was completely retained on a C_{18} column. Various ion-pairing reagents were investigated including, dibutylamine (DBA), octanesulphonic acid (OSA) and tetramethylammonium chloride (TMAC), in a mobile phase of water-acetonitrile (1+1, v/v).

The use of DBA as an ion-pairing reagent gave rise to a decreased peak height for the Fe(II)-phen complex in comparison with TMAC (Figure 9.1). In addition, several peaks were obtained for the Fe(II)-phen complex when DBA was used, compared with the single peak obtained when TMAC was employed (Figure 9.1).

The use of OSA gave rise to even poorer chromatography than DBA in that the retention times were increased and the peaks broader. TMAC was therefore chosen as the ion-pairing reagent. The optimum concentration of TMAC in the mobile phase was found to be 0.16 % (w/v) ; similar retention behaviour was exhibited by the complex regardless of the

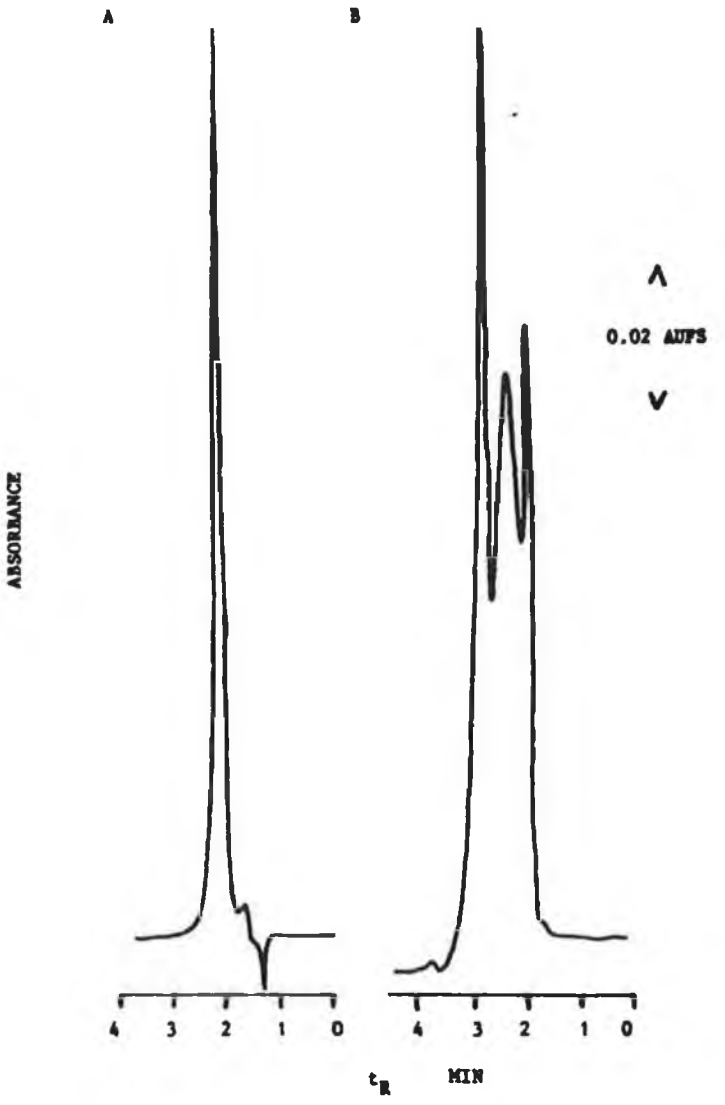

Figure 9.1. Influence of ion-pairing reagent on LC peak due to the Fe(II)-phen complex. (A) TMAC; (B) DBA. Mobile phase : water-acetonitrile (50:50) containing (A) 0.16% (w/v) TMAC and (B) 0.01% (v/v) DBA. AUFS is absorbance units full scale; t_r=retention time.

proportions (up to a maximum of 90 % of each) of water and acetonitrile in the mobile phase.

9.3.3. Separation of Fe(II) and Fe(III)

LC separation of Fe(II) and Fe(III) following direct injection of the ions into a mobile phase containing both phen and SA resulted in four peaks with retention times of 1.2 min (peak A), 1.4 min (peak B), 2.7 min (peak C) and 3.9 min (peak D) (Figure 9.2). When Fe(II) was injected alone, only one peak was obtained, i.e., peak C.

When Fe(III) was injected alone, all four peaks were obtained, indicating that some of the Fe(III) had been reduced to Fe(II), a process facilitated by the presence of 1,10-phenanthroline. It was therefore decided to attempt the separation using pre-complexed Fe(III), to minimise this effect.

Injection of the pre-complexed Fe(III)-SA complex on to the column with a mobile phase consisting of water-acetonitrile (1+1, v/v) which was 0.16 % (w/v) in TMAC and 0.1 % (w/v) in SA resulted in two peaks, i.e., peaks A and B. The height of peak B remained constant irrespective of the concentration of the Fe(III)-SA injected on to the column. When Fe(II)-phen samples were injected on to the column with the same mobile phase, a single peak, i.e., peak C, was obtained. When a mixture of Fe(II)-phen and Fe(III)-SA complexes were injected into a mobile phase which

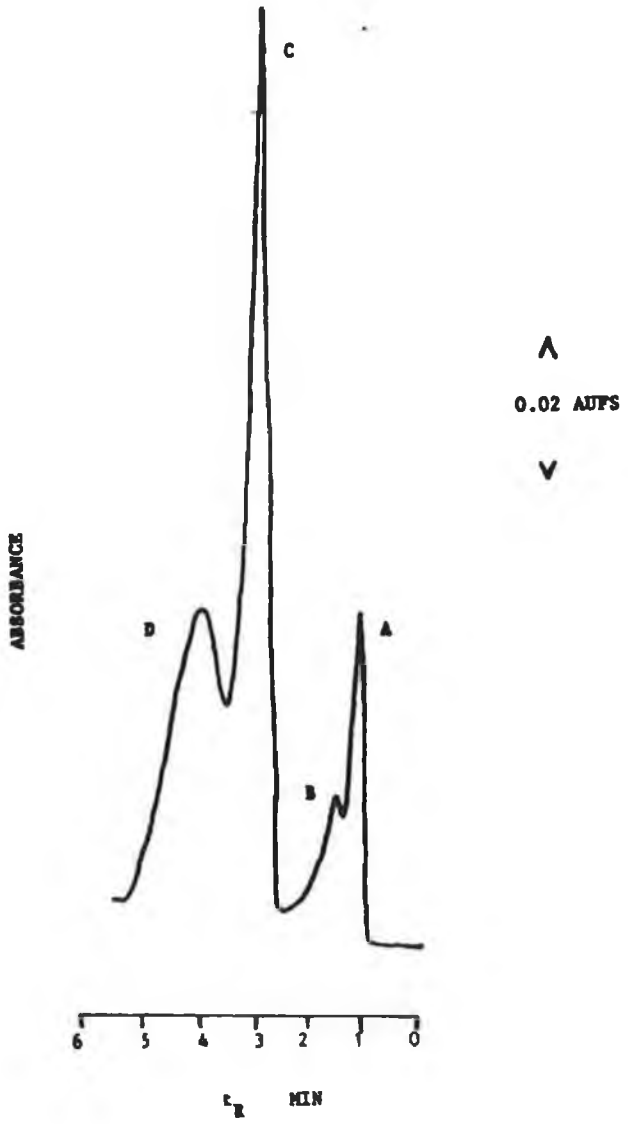

Figure 9.2. LC separation of Fe(II)-phen and Fe(III)-SA complexes. Mobile phase : water-acetonitile (50:50) containing 0.16% TMAC, 0.10% SA.

contained both phen and SA at the 0.1 % level, all four peaks were again obtained. This indicates that peak D results from the introduction of phen into the mobile phase, and could be due to a complex of Fe(III) with 1,10-phenanthroline. Calibration graphs for Fe(II) were linear (correlation coefficient typically 0.999, n=5) under these latter conditions.

It was next decided to replace SA by 5-sulphosalicylic acid (5-SSA), to see if the interfering peaks, i.e., peaks B and D, could be eliminated. 5-SSA has the advantage over SA, in that it is more soluble in aqueous solutions, and has been reported to give rise to a more stable complex with Fe(III) [11].

Using 5-SSA instead of SA in the mobile phase without 1,10-phenanthroline resulted in the elimination of peaks B and D, showing that 5-SSA does give rise to better stability for Fe(III) under these conditions. It was noted, however, that a small negative peak occurred immediately after the Fe(III)-5-SSA peak, probably caused by the consumption of 5-SSA as a result of complex formation.

Calibration graphs for Fe(III) under these conditions were linear with correlation coefficients of 0.9998 (n=5).

When 1,10-phenanthroline was also included in the mobile phase, a decrease in the height of the Fe(III)-5-SSA peak and a corresponding increase in the Fe(II)-phen peak was seen, again indicating reduction

of Fe(III) to Fe(II) in the presence of 1,10-phenanthroline.

Decreasing the concentraion of 1,10-phenanthroline in the mobile phase from 0.10 to 0.025 % (w/v) did not improve the situation. However, the use of acetate buffer in the pH range 6-7, in which region 1,10-phenanthroline is unprotonated, resulted in stabilisation of the Fe(III)-5-SSA peak height, but did not cause any further improvements.

It was decided, therefore, to use only one ligand (5-SSA) in the mobile phase, with the pH of the aqueous component adjusted to 6.9 by the use of an acetate buffer so that any excess of 1,10-phenanthroline remaining from the pre-complexation reaction of Fe(II) did not interfere, especially at low concentrations of Fe(III). The final separation is shown in Figure 9.3.

The choice of pH was made after finding conditions where the peak heights of the two complexes injected separately and in admixture were the same. The final system developed, incorporated 0.16 % (w/v) TMAC, 0.1 % (w/v) 5-SSA in an acetonitrile-0.02 M sodium acetate buffer (pH 6.9) (1+1).

It is important to add the 5-SSA first so as to prevent any reduction of Fe(III) to Fe(II). A typical chromatogram illustrating the separation of Fe(II)-phen and Fe(III)-5-SSA under these conditions is shown in Figure 9.3.

Calibration graphs were linear for Fe(II) and

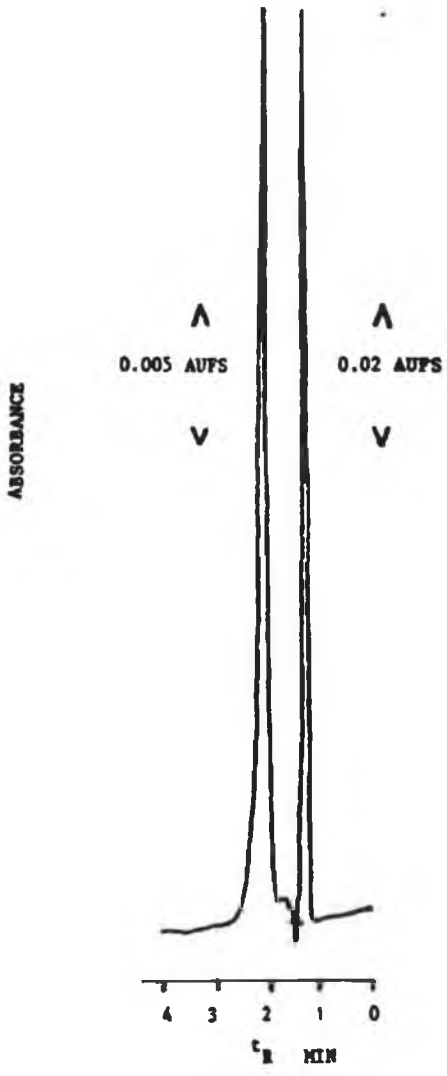

Figure 9.3. Optimised separation of Fe(II)-phen and Fe(III)-5-SSA complexes. Mobile phase : see Experimental.

Fe(III) in the range 1-12 μg/ml with correlation coefficients of 0.9995 and 0.9958, respectively (both n=5). Replicate (n=5) analyses of solutions containing 10 and 260 μg/ml of both Fe(II) and Fe(III) using the proposed method gave mean recoveries of 99 % and 97 % and relative standard deviations of 5 and 6 % respectively. Analysis of a digested soil extract gave Fe(II) and Fe(III) concentrations of 258 and 19 μg/ml respectively.

Table 9.1. Calibration Curves

	Fe(II)-Phen	Fe(III)-SSA
CONCENTRATION		PEAK HEIGHT
(ppm)	(cm)	
12	18.73	20.5
10	15.57	16.6
7	11.03	13.4
5	7.87	8.05
2	2.6	3.05
0	0.00	0.00

Fe(II)-phen

Slope=1.578

Intercept=-0.154

Correlation Coefficient=0.9995

Fe(III)-SSA

Slope=1.725

Intercept=-0.0749

Correlation Coefficient=0.9958

9.4. Conclusions

A method was developed for the separation of the complexes of Fe(II)-1,10-phenanthroline and Fe(III)-5-sulphosalicylate on the reversed-phase C_{18} column in the presence of an ion-pairing reagent, tetramethylammonium chloride.

In the final method, samples were injected onto the column in the pre-complexed form to ensure stability of both complexes and separated using a mobile phase consisting of acetonitrile [0.1 % (w/v) in 5-sulphosalicylic acid]- 0.02 M sodium acetate buffer (pH 6.9) [0.1 % (w/v) in tetramethylammonium chloride] (50 : 50). Spectrophotometric detection of the complexes was carried out at 515 nm. Calibration graphs were linear for both Fe(II) and Fe(III) in the concentration range 1-12 $\mu g\ mol^{-1}$.

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Appendix (i)

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1. *HPLC determination of trace metals in industrial and environmental samples.*
   M. Meaney, J. Mooney, M. Connor and M.R. Smyth.
   Analytical Proceedings, 1988, 25, 63.

2. *Comparison of HPLC and AAS methods for the determination of Fe(III) and Al(III) in soil and clay samples.*
   M. Meaney, M. Connor, C. Breen and M.R. Smyth.
   Journal of Chromatography, 449, 241, (1988).

3. *Reversed-phase separation of Fe(II) and Fe(III) as their respective 1,10-phenanthroline and 5-sulphosalicylate complexes*
   M. Connor, T. O'Shea and M.R. Smyth.
   Anal. Chim. Acta., 224, 65, (1989).

4. *Silicone-grease-based immobilisation method for the preparation of enzyme electrodes.*
   M.P. Connor, J. Sanchez, J. Wang, M.R. Smyth and S. Mannino.
   Analyst, 114, 1427, (1989).

5. *Tissue and microbe based electrochemical detectors for liquid chromatography.*
M.P. Connor, J. Wang, W. Kubiak and M.R. Smyth.
Anal. Chim. Acta., 229, 139, (1990).

6. *A bienzyme-modified carbon paste electrode for the detection of glucose and bilirubin.*
M.P. Connor, J. Wang, J. Sanchez and M.R. Smyth.
in press.

7. *The use of lichen-modified carbon paste electrodes for the detection of lead and copper by anodic stripping voltammetry*
M.P. Connor, E. Dempsey, M.R. Smyth and D.H.S. Richardson.
in press.