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Electroanalysis of Some Compounds of Biological and Industrial Importance

by

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DECLARATION

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

Signed *February 1997*

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For my parents
Victor and Anne

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Abstract

This thesis is a study of the application of modern electroanalytical techniques to the determination of analytes in complex media. The recent large increase in publications applying electroanalytical techniques to environmental, pharmaceutical and clinical applications is discussed in Chapter 1, including a discussion of electrode materials and their applications, the use of modified electrodes to improve selectivity and reduce electrode passivation, in addition to an overview of the use of electrochemical detection in flowing solution analysis.

Chapter 2 describes the use of polarography at a dropping mercury electrode to investigate the cure chemistry of anaerobic adhesives. The influence of transition metal ions, in the presence of accelerators, on the catalytic decomposition of the free radical initiators *t*-Butyl perbenzoate, *t*-Butyl hydroperoxide and benzoyl peroxide is discussed.

The determination of clenbuterol in bovine urine is discussed in Chapter 3. The electrochemical characteristics of the β-agonist were investigated at a bare carbon paste electrode. The application of a thin Nafion film to the electrode surface permitted accumulation of clenbuterol onto the electrode surface while at the same time selectively excluding anionic interferences present in the urine sample. Differential pulse voltammetry was used to detect low levels of clenbuterol in urine.

Amperometric and voltammetric detection coupled to capillary electrophoresis, applied to the determination of anionic phenolic acids in beer and wine, is outlined in Chapter 4. The passivation of the carbon fibre working electrode by the large concentration of neutral molecules present in the sample was overcome by injecting a large volume of diluted sample into the capillary, reversing the polarity to remove cations and neutral molecules from the capillary, and separating the remaining anions under normal conditions. Hydrodynamic voltammetry obtained amperometrically by the step-wise increase in potential were compared to voltammetric detection in the production of *i-E* curves for analyte identification.

Conclusions drawn from the work carried out in the thesis and suggestions for future research are outlined in Chapter 5.

CHAPTER ONE

SOME ANALYTICAL APPLICATIONS OF ELECTROCHEMICAL DETECTION

1.1 INTRODUCTION

In the past, electroanalytical techniques have not been as popular as chromatographic or spectroscopic techniques. The recent large increase in publications applying electroanalytical techniques to environmental, pharmaceutical and clinical applications indicates, however, that electroanalytical techniques are now becoming more widely accepted. This large range of applications, along with modern, automated instrumentation with computerised data analysis capability, has had a large impact in the introduction of electrochemistry into the analytical laboratory. Electroanalytical techniques can now offer both quantitative and qualitative information, very low detection limits relative to spectroscopic and chromatographic detection techniques (0.1 pM levels), a large linear dynamic range (10^{-3} - 10^{-8} M for voltammetric techniques), good selectivity and easy sample preparation. These attributes will be discussed in more detail further on in the text.

Electrochemical detection can be applied to any compound which is oxidised or reduced at an electrode surface. The choice of an electroanalytical technique for the detection of an analyte depends very much on the sample matrix in which it is present, the sensitivity required, and, to a lesser extent, the analysis time. It is the purpose of this chapter to give a brief overview of the analytical applications of electroanalytical techniques and to discuss some of the advances in electroanalytical techniques in recent times.

1.2 ELECTRODE MATERIALS FOR ELECTROANALYSIS

The development of electroanalytical techniques began in the 1920's, with the development of the dropping mercury electrode (DME). Applications of electroanalytical techniques then expanded in the 1950's with the development of various solid electrodes. The recent use of microelectrodes has significantly contributed to the coupling of electrochemistry with microseparation techniques, in addition to providing selective and sensitive applications. The type of working electrode applied to a specific analysis depends on the identity of sample to be analysed, with the main requirements being sensitivity and selectivity at the applied potential, in addition to having a stable and reproducible working surface.

1.2.1 Dropping mercury electrode

Mercury as a working electrode material was first introduced by Kucera in 1903.[1] This then lead to the development of the DME in 1922 by Heyrovsky.[2] In modern instruments, a static dropping mercury electrode (SDME) is usually employed. This is based on the same principle as the DME, the underlying difference being that the mercury drop is first allowed to expand to a certain area and is then held stationary up to a pre-selected drop-time, at which point the drop is knocked off from the capillary and a new one formed. If the current is then measured using any one of the available polarographic techniques (DC polarography, normal pulse or differential pulse polarography), the high non-faradaic current associated with a continually changing electrode surface is largely eliminated.

Solenoid valves are used to control the mercury drop-size and drop-time. Once activated, the solenoid valves lift a plunger allowing mercury to flow very rapidly (due to large bore sizes usually used) and the drop to grow. Once the required drop-size is obtained, the solenoid valves are deactivated resulting in a static drop. At the end of the chosen drop-time, the drop is dislodged, as for the DME.

The application of mercury as a working electrode has developed mainly due to its wide reduction potential range, attributed to the very high overvoltage evolution of hydrogen in comparison to other solid noble metal electrodes.[3] According to Kissinger *et al.*,[4] the overvoltage for the reduction of the aqueous hydrogen ion on mercury relative to platinum can be as much as -1.0 V, thereby extending the applicable potential range for alkali metal cation reduction, which can be as low as -2.0 V vs SCE in alkaline electrolytes. This negative potential may be extended even lower by the use of quaternary ammonium salts as the background electrolyte, with potentials as low as -3.0 V being attainable in non-aqueous media.

The DME cannot usually be applied as a working electrode in the study of oxidation processes, due to the oxidation of mercury at very low positive potentials. In electrolytes void of anions capable of forming insoluble salts with mercury cations (e.g. perchlorates), oxidation occurs at 0.4 V. However, this can be as low as -0.3 V in the presence of salts such as potassium chloride. This limitation of the DME is the main reason why solid electrodes prepared with noble metals such as gold, platinum, or carbon are used for oxidation reactions.

The constantly renewable surface (the frequency of which is governed by the drop life-time) obtained with the DME results in highly reproducible *i-E* curves, independent of the effects of time or electrode history. Unlike the DME, non-

renewable solid electrode surfaces often show *i-E* curves distorted over time from surface effects. The current due to an analyte at the DME is given by the Ilkovic equation, which describes the limiting current at an expanding spherical electrode:

$$i_d = 0.627nFC\,D^{1/2}\,m^{2/3}\,t^{1/6} \qquad (1\text{-}1)$$

where i is the current (A), n is the number of electrons consumed in the oxidation/reduction per ion/molecule, F is Faraday's constant, C is the analyte concentration in milli moles (mM), D is the diffusion coefficient in cm^2sec^{-1}, m is the rate of mercury flow in mg sec^{-1} and t is the mercury drop time in s. As the limiting current is proportional to the analyte concentration, the DME can be applied to quantitative analysis. Unlike stationary planar electrodes, the current at a DME is an increasing function with time, with the maximum value reached just before the end of the drop life.

Despite the complicated instrumentation required for application of the DME compared to solid electrodes, the increased reduction potential range has ensured its application to a wide range of analytical applications, some of which will be discussed further on in the chapter.

A variation of the DME is the hanging mercury dropping electrode (HMDE), in which the mercury drop develops from the end of the capillary, up to a particular surface area, with the radius usually not exceeding 1 mm. Once the predetermined size is reached, growth stops and the mercury drop is suspended up to experimental completion, at which point the drop is mechanically knocked off and

a new one grown. A SMDE set to the HMDE mode produces a very reproducible surface.

The primary application of the HMDE is in trace analysis, where analytes are present in very dilute solutions. By initially preconcentrating the analyte ions at the electrode surface, the concentration can be determined by consequently "stripping" the analyte from the electrode surface, with the electrochemical signal obtained being proportional to the concentration of analyte at the electrode surface which in turn is proportional to its concentration in solution. This is known as stripping voltammetry. Cationic heavy metal ions are preconcentrated, in a stirred solution, by reduction onto the mercury surface to form an amalgam with mercury. A positive potential scan is applied to strip the dissolved metals by re-oxidation to their ionic form. This is known as anodic stripping voltammetry (ASV).

Anionic species may also be preconcentrated at a mercury electrode surface by oxidation to form insoluble salts with mercury. Cathodic stripping (CSV) is then applied and the peak current measured. In addition to ASV and CSV, differential pulse techniques may be applied to give the techniques known as DPASV and DPCSV. As an alternative to the HMDE, mercury film electrodes (MFE) may also be used. Electrode preparation is carried out by mercury electrodeposition onto a noble metal electrode.[5] The application of mercury as an electrode for stripping analysis is limited to potentials below the oxidation potential of mercury, and this has led to the development of techniques involving the use of solid electrodes, such as carbon, for the preconcentration of analytes onto the electrode surface and will be discussed in a later section of the chapter.

1.2.2 Carbon electrodes

The popularity of solid electrodes (over the dropping mercury electrode) has developed since 1950 mainly due to the number of reactions which can be studied using such electrodes which are not available at mercury surfaces. Nowadays there is a large variety of electrochemical techniques which employ stationary electrodes, and much research has been carried out into the interpretation of reactions occurring at such surfaces. Although a wide variety of substances have been used as solid electrodes, noble metals such as platinum and gold, and carbon electrodes remain the most popular.

The properties of the various carbon electrodes make it the material chosen for many applications, as it possesses the necessary criteria for satisfactory electrode production i.e. easy preparation, easily reproduced surface, wide useful potential range, and finally, a low electrical resistance. Carbon was first used as a working electrode in 1952 by Lord and Rogers[6] who used a spectroscopic graphite electrode to obtain voltammograms for organic compounds. A reproducible surface was obtained by breaking off the tip of the electrode (then seen as an advantage over the platinum electrode). Carbon, in the form of graphite, is versatile and inexpensive,[7] and has been used in a variety of ways in the development of electroanalytical techniques. Hart and Wring have described the fabrication of several different types of carbon electrode (among them a disposable glassy carbon electrode) which have been applied to the detection of biological molecules.[8,9,10,11,12,13] Although a wide variety of carbon electrodes are available, the most commonly used in electrochemical analysis include those prepared from

pyrolytic graphite, glassy carbon and carbon paste. A discussion on the preparation and application of carbon paste electrodes (CPE) is given below.

1.2.2.1 Carbon paste electrode

The carbon paste electrode was first reported in 1958 by Adams.[14] In general, the electrode consists of a plastic cylinder with a well of about 0.5 mm in diameter and 3 mm deep. Electrical contact is made through a platinum, copper, steel or graphite contact. To pack the electrode, a small, but excess amount of carbon paste is pressed into the well; the electrode is then inverted and the paste is pressed onto a flat, clean surface. The electrode is pressed with a circular motion on this surface in order to remove any excess paste and to attain a flat, shiny surface with no holes in the paste. It is important not to apply too much pressure as this separates the carbon and oil, resulting in high resistance contact between the paste and metal. Any excess paste remaining on the electrodes body can be wiped away carefully with a tissue.

Electrodes are renewed by removing a small layer from the surface and re-packing as before. If a completely new electrode is required, the well may be cleaned out in an ultrasonic bath, dried and re-packed as before. Although reproducible packing remains an art, it is possible to obtain reproducibility of approximately ±2 % with a 30 sec preparation time, although reproducibilities also depends on the compound under study, as well as the medium employed.

Carbon paste is prepared by mixing graphite powder with a suitable mulling liquid. Buchalik and Darlewski[15] carried out a voltammetric study on carbon paste

electrodes incorporating mulling agents such as parafin oil, silicone oil, bromonaphthalene and mineral oil (Nujol). Their results showed that the preparation made with Nujol performed the best. Rice *et al.*[16] carried out similar experiments to compare electron-transfer rates of each of the mulling agents. They discovered that straight-chain hydrocarbons gave rise to faster rates than Nujol, with the rate decreasing with increasing chain length. Kinsley and Curran[17] reported, using AC voltammetry, that Nujol gave rise to the lowest background currents. It has been reported that, for practical applications, a mulling agent should have low volatility, very low solubility in the medium to be employed, and no electroactive impurities.

Carbon paste can be prepared by thoroughly mixing approximately 2 ml of Nujol and 5 g of graphite powder in a mortar and pestle until the mixture is uniformly wetted (approximately 30 min). When prepared, the paste should have a "peanut butter" appearance. High purity graphite should be used when using pulse techniques to detect very low concentrations, as reagent grade pastes may result in large background peaks. Gritty graphite types should not be used as they result in an irregular electrode surface, resulting in lower peak currents.

The range over which a CPE can be used is quite large, and depends slightly on the mulling liquid (although there is little variation with mulling agent) as well as the background electrolyte. Reversible potential limits for aqueous solutions can be defined in terms of two possible background processes - reduction of the hydrogen ion and the oxidation of water. In general, the anodic limit for CPEs in 0.1 M acid is approximately +1.3 V, with cathodic limits being about -1.1 V in 1 M acid. Due to extremely low residual currents, the complete anodic range is usually available

for study. A small residual wave is almost always seen, however, on cathodic background scans, even after deaeration (therefore complicating cathodic potential limits), which appears to be due to oxygen reduction and caused by a small amount of dissolved oxygen adsorbed onto the surface resulting in a significant residual current. Deaeration allows the limit to be expanded to about -1.5 V. However, the fact that reduction waves of electroactive species can be seen without great difficulty means that the cut-off limits are usually specified at the point where sudden current increases, indicating real background reduction.

CPEs can be used in aqueous solutions containing up to 25 % alcohol or some other solvent. When used in non-aqueous media, the carbon paste tends to disintegrate either due to the wetting of the graphite by the solvent or the dissolution of the mulling agent. A solution to this problem is the addition of surfactant, e.g. sodium lauyrl sulphate, which causes equal wetting of the graphite by both the mulling agent and the solvent.

Electron-transfer rates for redox processes at carbon electrodes are slower than those at metal surfaces. Rice *et al.*[16] showed that pretreatment of the electrode at positive potentials greater than +1.2 V results in increased electron-transfer rates. Ravichardran and Baldwin[18] enhanced the voltammetric response for hydroquinone, ascorbic acid, hydrazine and NADH using preanodisation in buffer at +1.75 V for 5 min followed by precathodisation at -1.2 V for 10 s. Results proved that the enhancement of the signal was for a shorter period of time than for glassy carbon electrodes.

In summary, CPEs have essentially zero resistance over the entire anodic potential range. Drawbacks such as oxide dissolution or hydrogen desorption are rare. CPEs

are believed to be one of the most simple and practical of solid electrodes, yet are still capable of precision in limiting-current measurements rarely equalled by noble metals. Therefore, for routine applications, the CPE is far superior to platinum or gold.

1.2.3 Carbon fibre electrodes

The carbon fibre electrode (CFE)was introduced in 1979 by Ponchon *et al.*[19] in their determination of catecholamines *in vivo*. Since then, many applications of microelectrodes to *in vivo* analysis have been realised. Most carbon fibres used are home-made and many variations have been reported;[3,4,19,20] most involve the threading of the carbon fibre through a glass capillary of very narrow bore size (carried out under a microscope), until approximately 1 mm of fibre extends out the other side. The fibre is then held in place using epoxy glue between the fibre and capillary tip. Electrical contact is usually made with a copper wire connected with silver epoxy to the fibre. The required length of fibre protruding from the tip can then be cut to the required length. Carbon fibre disc electrodes are prepared by cutting the fibre to the glass tip. Usually, the electrode is passive and requires pretreatment before use. The construction of the carbon fibre electrode used throughout these experiments is shown in Figure 1-1.

Careful electrode preparation is necessary, as bad construction reflects in the electrode response. Incomplete sealing of joints may result in solution seeping into the glass capillary causing an increase in resistance and large signal fluctuations.[21]

The potential range of the carbon fibre electrode is similar to a carbon paste electrode of conventional size i.e. approx. -0.8 V to 1.2 V vs SCE.[18] Carbon fibre electrodes show improved S/N ratios relative to conventional electrodes, which is attributed to very low charging currents and increased mass transfer rates (which increase as electrode size decreases). Small electrode surfaces also reduce IR losses which allow electrodes to be used in solutions of higher resistivity, further expanding the range of applications.

The current-voltage curves obtained with carbon fibre electrodes are sigmoidal rather than peak shaped, as exhibited with a macro electrode. This is due to diffusion effects. In the former case, at slow scan rates the rate of electrolysis equals the rate of diffusion to the surface, so that steady-state is reached in shorter

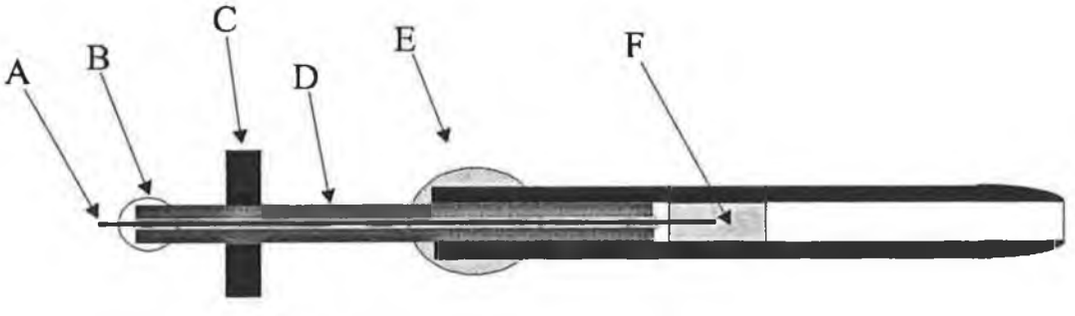


Figure 1-1 *Carbon fibre electrode:* (A) *exposed carbon fibre;* (B) *silicone seal;* (C) *rubber septum;* (D) *fused capillary 50 μm i.d.;* (E) *epoxy glue;* (F) *silver epoxy glue for electrical contact.*

times.[22] On the reverse scan, the reduction current follows the oxidation curve, as the oxidised compound has diffused from the surface. As the fibre has a cylindrical surface, cylindrical rather than planar diffusion occurs.[19] This sigmoidal shape allows for more accurate concentration measurements, and the peak current as defined by Aoki *et al.*[23] can be evaluated from:

$$i_p = r^{-1}nFCD = 0.466/nFr^2\upsilon(RTD)^{-1/2} + 0.335nFr^2\upsilon(RTD)^{-1/2} \qquad (1\text{-}2)$$

where r is the electrode radius, υ is the scan rate, T is temperature in Kelvin and the remaining terms as in Eqn. 1-1. The background charging current for carbon fibre electrodes, as with other carbon electrodes, is small on the second scan due to conditioning of the surface.[19] If an increase in this charging current is evident, it may be due to fractures in the electrode caused during electrode preparation or electrochemical pretreatment.

1.3 MODIFIED ELECTRODES

When an electroanalytical technique requires the detection of analyte concentrations in the low nanogram range, bare solid electrodes usually do not permit detection at the required level of sensitivity, especially when the analyte of interest exists in complex matrices such as environmental samples or biological fluids. By chemically modifying the electrode surface, greater sensitivity and selectivity may be achieved. Electrode modification entails either the irreversible

chemisorption of the modifier onto the electrode material, or the covalent binding of the modifying molecule to functional groups present on the electrode surface.

The first chemically modified electrode was introduced in 1973 by Lane *et al.*[24] who studied the chemisorption of electroactive allyl compounds on platinum electrodes. Moses and co-workers[25] followed suit two years later by chemically modifying tin oxide electrodes by (chemically) bonding amine, pyridine and ethylenediamine onto a Pt surface. Both the above applications involved transforming heterogeneous, unpredictive surfaces into chemically predictive ones such as those required for electrochemical studies. Electrode modification may be classified into four categories, according to their specific functions: electrocatalysis, biosensors/selective recognition (by antibodies, enzymes, nucleic acids or protein receptors), permselectivity and selective preconcentration. The general area of biosensors will not be dealt with in this chapter.

1.3.1 Electrocatalysis

Electrocatalysts may be integrated into an electrode process by chemisorption onto an electrode surface or the addition to electrode constituents, in order to accelerate otherwise sluggish electron transfer rates which brought about the effects of overpotential, which is usually seen at bare electrode surfaces. Electrocatalysts are small, electroactive compounds (usually redox mediators) that shuttle electrons between the electrode surface and the electroactive analyte. Although electrocatalysts do increase sensitivity, they do not often result in improved selectivity, which is vital to real sample analysis. Conducting polymers may be used

to increase electron transfer rates as well as eliminate interferences, therefore increasing selectivity. The use of clay-modified electrodes with incorporated electrocatalysts has received much attention in recent years, and was first reported in 1983 by Ghosh and Bard[26] who proved that $Ru(bpy)_3^{2+}$ incorporated into a platinum/clay mixture and coated onto a SnO_2 electrode was indeed electroactive. Clay as a modifier boasts high chemical stability as well as possessing specific structural characteristics which allow immobilisation of catalysts. Ghosh and Bard claimed that the rate-limiting process for charge transfer through the clay was either by an electron hopping mechanism or by the diffusion of the redox species through the clay to the electrode surface. It has also been shown that clay can actually influence catalyst specificity.

1.3.2 Permselective membranes

The electroanalytical detection of analytes in complex biological/environmental samples is often hindered by electrode surface fouling due mainly to binding of matrix macromolecules to the electrode surface, in addition to oxidation/reduction of electrochemical interferences present in the sample. By depositing a permselective membrane on the electrode surface, many of these difficulties are alleviated. Such membranes are polymeric in nature and many show selectivity on both size and charge exclusion basis. Ion-exchange membranes, however, selectively exclude specific compounds solely on the basis of charge.

1.3.2.1 Size-exclusion membranes

Cellulose acetate (CA) membranes screen electrochemical interferences, not only by anion-repulsion, but also by exclusion of neutral molecules according to their size. Reddy *et al.*[27] compared the application of poly(vinylchloride) (PVC) and cellulose acetate as electrode modifiers for the analysis of oxalate in urine. Oxalate oxidase, incorporated into the electrode surface, catalyses the reaction of oxalate with molecular oxygen to give hydrogen peroxide which is amperometrically determined at 550 mV. The effectiveness of plasticised (pPVC), unplasticised PVC (uPVC) and cellulose acetate in the elimination of common electrochemical interferences present in urine, namely the low molecular weight organic compounds ascorbate, homovanillic acid (HVA) and direct oxidation of oxalate itself, was investigated. Although pPVC successfully excluded the anionic interferents, ascorbate and oxalate, the neutral HVA did partition into the membrane. The unplasticised membrane, devoid of lipid, although excluding urine molecules and HVA, was ineffective in excluding the anionic compounds. CA, on the other hand, allowed an increased response for hydrogen peroxide with reduced interferences from all three organic urine compounds.

The suppression of protein interference in the determination of cadmium and lead by anodic stripping voltammetry was investigated by Hoyer and Jensen[28] using a mercury deposited CA membrane-modified glassy carbon electrode. Traditional electrode coating with a bulk polymer solution results in a thick film which often retards mass transport of the analyte through the film. CA, however, allows rapid diffusion of small molecular weight compounds. Their investigation proved, however, that casting the polymer *in situ* by a phase-inversion method in which a

swelling agent is added to the membrane solution prior to casting, results in minimal analyte peak depression owing to protein interferences. Variation of the casting procedure allows manipulation of CA porosity, and therefore mass transport of analytes through the film to the electrode surface. The LOD using this technique was found to be 9 nM for cadmium and 5 nM for lead.

A base-hydrolysis casting technique has been developed by Wang and Hutchins[29] to vary the porosity, and therefore permeability characteristics, of the CA membrane. Base-hydrolysis with 0.07 M potassium hydroxide solution for a pre-determined length of time breaks the polymer backbone into small fragmented chains. The porosity of the membrane, and hence its molecular weight cut-off, is controlled by variation of base-hydrolysis time. This technique permitted the detection of smaller biological molecules, such as uric acid, in the presence of larger ones, such as serum albumin, without signal suppression due to electrode fouling.

The importance of permselective membranes in modifying electrodes for *in vivo* analysis has been shown by Abdel-Hamid[30] in the development of a glucose needle-type biosensor in the monitoring of hemorrhagic shock. A matrix of 1,3-phenylenediamine containing entrapped glucose oxidase was electropolymerised onto the surface of a platinum wire at + 0.65 V for 15 minutes. Following the addition of another enzyme-free polymer layer, films of polyurethane, polyvinylchloride or CA were coated onto the sensor surface by dipping the electrode into the polymer solution and allowing it to air dry. CA showed a longer electrode response time with a shorter linear range in comparison to the PU and PVC coatings, when tested in glucose solutions of 2.2 mM to 11.1 mM. This is

probably due to the thick CA film retarding mass transport of glucose through the film.

A screen-printed (enzyme free), CA-coated amperometric sensor, reported by Gilmartin and Hart[31] has been developed for the detection of paracetamol in urine to diagnose drug overdose and renal failure. A 1.8% CA membrane was sufficient to eliminate interferences from thiols such as cysteine, gluthathione, salicylic acid, uric acid, ascorbic and gentisic acid.

The above examples all indicate the effectiveness of CA as a permselective electrode modifier by size exclusion. It has been shown that by variation of the polymer casting procedure, manipulation of membrane porosity is possible, thereby permitting selective molecular cut-off values allowing for the analysis of a wide variety of biological compounds in complex biological matrices.

1.3.2.2 Ion-exchange membranes

Selective exclusion is also attainable by modifying electrode surfaces with ion-exchange polymers. These polyelectrolytic membranes exclude molecules mainly on the basis of charge. Anionic exchangers exclude positively charged molecules in solution through charge repulsion and allow negative ions to permeate the film, without hindrance of analyte diffusion through the membrane onto the electrode surface. To date, the application of anion-exchangers as permselective membranes in chemical analysis has been limited; three specific polymers and examples of their applications are given below.

Oyama *et al.*[32] in 1986 reported the application of a polycationic perfluoropolymer coated onto a platinum electrode. The anionic exchange properties of the polymer arise due to a quaternary ammonium moiety in the polymer side chain, with the chloride counterion exchanging with the solution anions. The exchange properties of the film were studied by carrying out cyclic voltammetric experiments using 0.2 M $Fe(CN)_6^{3-}$, and it was concluded that a steady state current reached after 1 hour preconcentration under open circuit conditions involved only 10 % of the quaternary ammonium sites. The modified electrode only partially excluded a cationic redox couple, $Ru(NH_3)_6^{3+/2+}$, and a neutral couple, hydroquinone/quinone, from the electrode surface.

A Tosflex® (TOSOH Co., Ltd., Tokyo, Japan) film was reported by Schiavon[33] as being more effective than Nafion® in the development of an anodic stripping voltammetric technique to detect copper and lead in high resistive deionised water; the membrane coating acting as a solid polymer electrode. The 0.01 M perchloric acid electrolyte was situated at the inner surface of the ion-exchanger surrounding the reference and counter electrodes, with the glassy carbon working electrode placed at the outer membrane surface. Mercury was electrodeposited at the modifier/working electrode interface. Initial pre-electrolysis of the cations at - 0.9 V at the Nafion modified electrode showed a considerable memory effect due not only to cations remaining in the polymer following the redissolution step of anodic stripping voltammetry, but also due to cations migrating into the internal electrolyte as a result of longer pre-electrolysis times; thereby contaminating the internal filling solution. Tosflex, on the other hand, did not retain the Pb^{2+} and Cu^{2+}

ions following stripping redissolution, therefore permitting the detection of metal ions in high resistive samples.

A nitrate concentration level of 0.5 μg/ml in drinking water has been detected by incorporating Amberlite LA2, an anion exchange polymer, into the carbon paste mixture during preparation.[34] The direct differential pulse voltammetric detection of nitrate was based on its catalytic reduction of cationic thallium, in the form of tetrachlorothallate(III) (adsorbed into the Amberlite under open circuit conditions), to Tl(0) and reoxidation to Tl(I) by nitrate in the sample. The increase in re-oxidation current was proportional to the nitrate concentration, with a limit of detection of 0.5 mg/L nitrate for 0.5 mg/L thallium concentration. Although nitrate is not toxic, its presence in drinking water polluted with fertilisers may be as high as 200 mg/L. However, reduction of nitrate forms nitrite which can lead to the production of toxic N-nitroso compounds as well as other ailments in the body.

Cation exchange polymers make up the majority of ion-exchange polymer modified electrodes. Among the most commonly reported, Eastman AQ polymer (Eastman Chemical Products, N.Y., U.S.A.), has found widespread use in the development of organic-phase biosensors. Wang *et al.*[35] have discussed how the poly(ester-sulfonic acid) has the ability to entrap enzymes with a high loading value as well as being stable in a number of organic solvents such as acetonitrile. The ion-exchange properties of the ionomer cause it to discriminate against anionic interferences.

The preliminary studies by Wang *et al.*[36] were then applied in the development of a biosensor to evaluate the peroxide concentration in vegetable oil. Lipid hydroperoxides are the main products of lipid oxidation, the presence of which can be used to identify product rancidity. An Eastman AQ polymer solution was coated

onto a glassy carbon electrode and used in an FIA system with a chloroform carrier stream at 0.7 ml/min, saturated with a ferrocene mediator, 0.1 M phosphate buffer and 0.1 M TBAB. The vegetable oil sample was injected without prior sample clean-up and was detected at - 0.1 V.

Electroanalysis in clinical chemistry has benefited immensely from the application of permselective membranes for electrode modification. Enhancement of selectivity in this area is hindered at bare electrode surfaces as a result of fouling by adsorption of large organic molecules, as well as interference from electrochemically active molecules (whose oxidative response curves overlap those of the analyte) present in abundance in complex biological samples. Permselective membranes are a means of controlling these interferences, as well as enhancing sensitivity through incorporating electrocatalysts within the membrane to speed up electron-transfer reactions, or through preconcentration of the analyte within the membrane. A vital prequisition of the membrane for *in vivo* analysis is its biocompatability with body fluids, along with its ability to prevent the degradation of biosensing enzymes and electrodes.

An example of the practical importance of such electrodes is their application to glucose sensing in the diagnosis of diabetes and the prevention of hypoglycaemia. A wide variety of sensors are now available, including disposable needle-type sensors for self-monitoring by patients and *in vivo* probes for continuous blood-glucose monitoring.

The most widely reported modifier for such electrodes is Nafion, a cross-linked perfluorosulphonate cation-exchange resin, a product of E.I. du Pont de Nemours and Co. (USA). Nafion as a modifier has been widely used for analytical purposes

and shows considerable potential for use in analytical techniques due to its chemical and electrochemical inertness, its insolubility in water, its hydrophilicity, and chemical and thermal stability, which all lead to it possessing almost ideal properties as a chemical modifier.[37] Its chemical and thermal stability arise due to the fluorocarbon backbone. Nafion, due to its high selectivity for H_2O_2, has been used in conjunction with various other electrode modifiers of varying characteristics to prepare multi-layer probes for the enhancement of sensitivity and selectivity of glucose sensors. In addition to ascorbic and uric acid, acetaminophen is oxidised between 700 and 800 mV, which interferes dramatically with the oxidation response of H_2O_2. Zhang *et al.*[38] have reported an implantable tri-layer glucose sensor consisting of a cellulose acetate/Nafion composite inner layer containing immobilised glucose oxidase and an outer biocompatable layer. The CA/Nafion composite allows selective passage of H_2O_2 to the electrode surface, while discriminating against acetaminophen. The sensor has been successfully applied to the *in vivo* monitoring of glucose in rats.

A needle-type glucose biosensor, based on the same tri-layer design, had been developed in 1993 by Moussy and co-workers.[39] The sensor, implanted sub-cutaneously in female dogs, consists of a coiled platinum wire working electrode coated with poly(o-phenylenediamine) film into which glucose oxidase immobilised in bovine serum albumin and glutaraldehyde was deposited. A coiled Ag/AgCl wire served as the reference electrode and the entire electrode body was dipped in liquid Nafion. The sensor proved to be stable *in vivo* for up to 14 days after which failure to respond to glucose resulted from degradation of the AgCl layer of the reference electrode.

Wang and Wu[40] deposited rhodium microparticles onto a glassy carbon electrode modified with a Nafion/glucose oxidase mix. Rhodium particles show selective catalytic behaviour towards the production of H_2O_2 in the presence of glucose oxidase. A calibration curve carried out *in vitro* in 0.05 M phosphate buffer, pH 7.4, with an applied potential of + 0.3 V, was linear from $1x10^{-3}$ M to $3x10^{-3}$ M glucose, while efficiently excluding the interferents uric acid, ascorbic acid and acetominophen.

1.3.3 Preconcentration

Trace analysis involves the detection of an analyte in very low quantities, often at the picomolar level. Although modifying electrodes may increase selectivity for a specific species in a sample matrix, through biorecognition or selective exclusion from permeating a membrane, electrochemical techniques often do not allow direct detection at such low levels. By preconcentrating the analyte onto the modified surface by chemical or physiochemical methods, detection levels can be lowered to the ultratrace level. Preconcentration of a very dilute solution of a particular organic species at solid electrodes is usually based on the principle of non-electrolytic deposition (at a constant potential) of analytes at a mercury surface, previously discussed in Section 1.2.1, prior to their being stripped from the surface by oxidation/reduction. This preconcentration step results in a much higher concentration of analyte at the electrode surface than in solution, therefore the sensitivity of the technique is increased dramatically. For instance, CSV at a hanging mercury drop electrode (HMDE) for the detection of the anticancer drug,

daunorubicin, in urine was reported by Wang *et al.*[41] A five minute preconcentration time at -0.30 V in acetate buffer, pH 4.4, achieved a limit of detection of $1x10^{-9}$ M.

Mercury as an electrode material has been discussed previously in Section 1.2.1. Despite its widespread application in electroanalytical reductions, its limitations in the positive potential range has lead to the application of modified solid electrodes such as the noble metals gold or platinum, or carbon. Preconcentration at solid electrodes is achieved through complexation,[42,43] partitioning, bioaccumulation by organisms e.g. mosses,[44] adsorption into an inorganic layer such as zeolites[45] and sepiolites,[46] as well as through ion-exchange.

The preconcentration of ionic species in dilute solutions onto an ion-exchange membrane has allowed detection of analytes at the ultratrace level. The anion-exchanger Tosflex, coated onto a glassy carbon disc electrode has been applied by Ugo *et al.*[47] to the determination of trace mercury, in the organic form $HgCl_4^{2-}$, by its electrodeposition at -0.20 V for 10 minutes at 2000 rpm. Differential pulse stripping voltammetry from -0.10 V to 0.30 V vs Ag/AgCl in sea water samples gave a mercury level of $1.6x10^{-10}$ M in the North Adriatic Sea.

The cation, hexacyanoferrate(II), is used as a fining agent in wines; however overdosage can lead to the production of cyano compounds/potassium cyanide, necessitating a sensitive analytical method for its routine detection. By incorporating liquid Amberlite LA2 into the carbon paste mix for electrode preparation, ion-aggregates between the analyte and ion-exchanger allowed analyte preconcentration under open circuit conditions. Detection of hexacyanoferrate(II)

by differential pulse voltammetry with $2.5x10^{-2}$ M HCl as a supporting electrolyte gave a limit of detection of 25 μg/L of $[Fe(CN)_6]^{4-}$ in Austrian wine.[48]

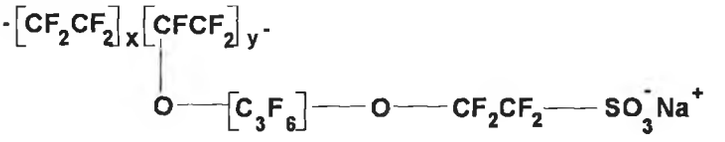
Boyd *et al.*[49] studied the accumulation behaviour of the β-agonists salbutamol, feneterol and metaprotenerol at a Nafion-modified carbon paste electrode. The higher affinity of salbutamol for accumulation was attributed to the different substitution of the hydroxy groups on the aromatic ring. Their results were applied to the cathodic stripping analysis of $9x10^{-9}$ M feneterol in spiked urine and serum samples, with feneterol oxidation at approx. 0.80 V in Britton-Robinson buffer, pH 2.0.[50]

1.3.3.1 Ion-exchange mechanism in membranes

Preconcentration of cations and anions by ion-exchange membranes such as Nafion and Tosflex, occurs due to electrostatic interactions between the analyte ion and a counter ion of the membrane. The rate of oxidation/reduction of such ions at an electrode surface is governed by both its rate of diffusion through the solution and diffusion through the film. The latter is dependent on the permeability of the analyte through the membrane; a function of its partitioning at the solution/film interface which is measured by the distribution constant, K_d, and its diffusion coefficient through the film, D_f. The selectivity of a modifying film for an analyte depends on the identity and concentration of the competing buffer counterion in solution, with K^+, Na^+ and Li^+ showing decreasing competition effects, with increasing buffer ion concentrations permitting less analyte permeability.[51]

Nafion (I), consists of a hydrophobic fluorocarbon backbone and a hydrophilic side chain containing the negative sulphonato moiety involved in cation-exchange. The

high selectivity of Nafion for hydrophobic cations results from their hydrophobic interaction with the fluoro backbone, along with electrostatic interaction between the negative sulphonato groups and the cations.



I

This cation-exchange resin allows accumulation and transportation of cations yet restricts the interaction of other anions and neutral species. According to Leddy *et al.*,[52] ionic transport through exchange membranes is governed by the microstructure of the Nafion and the charge of the ionomer. They have described how Nafion consists of a hydrophilic sulphonic acid phase and a hydrophobic fluorocarbon phase. From the structure of the polymer it can be seen that every SO_3^- group is a fixed anionic site, which is connected by a "pendant side chain" to the fluorocarbon backbone. The microstructure of the polymer chain is formed by a balance between the astringent fluorocarbon forces and the repulsive interactions between the sulphonic acid groups. The latter interactions are shielded somewhat by water molecules which are absorbed into the polymer. The fluorocarbon sites and the hydrated sites are somewhat segregated, and in studies of mass and charge transport which involved coating an electrode surface with Nafion solution, these two phases were intermixed without any order. Leddy *et al.*[52] applied some order to the microstructure by supporting the Nafion on Nuclepore polycarbonate

membranes. Results proved that increasing the ordering of the microstructure resulted in increased transport rates of ions through the membrane.

In multilayer films, charge transfer can occur via electron transfer and diffusion.

1.3.3.1.1 Electron-transfer

Extensive research has been carried out into the study of electron transfer mechanisms through films containing uniformly distributed sites that may be oxidised or reduced. Kaufmann and Endler[53] originally proposed that in polymeric films with redox sites anchored to the polymeric chains, the charge is carried by electrons that "hop" between adjacent reduced and oxidised sites within the polymer. They discovered that "electrons and appropriate counterions for charge neutrality can move through the polymer matrix in response to changes in applied potential". More recent studies which have been carried out[54,55,56] have indicated that this mechanism may also be involved in charge transfer when ionic species are electrostatically bound within polyelectrolyte films (Nafion is an anionic sulphonate group-containing polyelectrolyte).

The rate of electron transport in the Nafion film is known as the ***electron diffusion coefficient***.[57] It describes the kinetic behaviour of a modified electrode system and is an important feature when carrying out electrolysis at an electrode surface.

1.3.3.1.2 Diffusion

Another charge conducting mechanism in polymer films such as Nafion is the molecular diffusion of the electrostatically bound ions through the film. Martin *et al.*[56] have described how ion-containing polymers tend (on a microscopic level) to separate into two phases: a bulk polymer phase and a low density ionic "cluster" phase. It is this low density phase which contains the electroactive species, and since ion-pair interactions are not significant in polymers such as Nafion, the electroactive species is able to diffuse through the polymer. Therefore, diffusion rather than electron hopping could be responsible for charge transport.

Yeager and Steck[58] carried out studies into the influence of polymer structure on cation and water diffusion in Nafion. They concluded that cations and water may exist in two different regions in the polymer: the first region exists as ionic clusters, whereas the second is an interface between the ionic clusters and the fluorocarbon backbone. The proportion of cations in each region depends on its size and charge density, with ions of low charge density and large size preferring the interfacial region and vice versa.

Martin and Dollard[59] have likened the diffusion of ions through Nafion films to the movement of analyte molecules through a reversed-phase liquid chromatography column. In both cases, hydrophobic interactions with a non-polar stationary phase impede the progress of a an analyte species through (in the case of film diffusion) or with (in the case of RPLC), a polar phase. Also, common to both, the speed at which the analyte reaches the substrate (film diffusion) or the detector (RPLC) depends on the strength of hydrophobic interactions.

Usually, according to White *et al.*,[55] both electron transfer *and* diffusion contribute to the conduction process in Nafion films, with the relative contributions depending on the nature of the incorporated ion. The concentration of ions preconcentrated in the membrane is limited, however, due to crosslinking occuring and very high loading concentrations. The crosslinking properties of Nafion and Tosflex films have been reported by Oyama[32] and Dunsch,[60] respectively. Anion-induced crosslinking of Tosflex occurred when large concentrations of $Fe(CN)_6^{3-/4-}$ were preconcentrated onto the membrane, which resulted in a decrease in ion mobility through the film, depicted by a decrease in current flow at the electrode surface. This irreversible cross-linking was accounted by Dunsch to be due to structural changes in the film brought about by side-chain position rearrangement by the multiply charged anions. Crosslinking by methylviologen cations in the Nafion membrane, however, was found by Oyama *et al.*[32] to be reversible and regeneration of the polymer to the original Nafion-K^+ state was readily brought about by soaking the electrode in saturated KCl solution.

1.4 ELECTROCHEMICAL DETECTION IN FLOWING SOLUTIONS

Since the potential of electrochemistry as a detection technique in flowing solution analysis was envisaged by Kissinger in the 1960's,[61] the original application to clinical neurochemistry has been expanded to all aspects of analytical chemistry. Electrochemical detection is often applied to the detection of an analyte in flowing solutions as an alternative to the "universal" UV/vis spectroscopic and refractive

index (RI) detectors. Many criteria must be met by a detection system if it is to be applied to routine analysis of complex media. A detection method for segmented flow analysis (SFA) and flow injection analysis (FIA) must be chosen so that selectivity for a particular analyte is attained without sample component separation. The refractive index method of detection is based on measuring bulk properties of the sample, and therefore lacks selectivity for an analyte. Although UV/vis spectroscopic detection measures a specific property of the analyte, i.e. absorbance at a particular wavelength, the attainable sensitivity is limited to the ng range and many compound types, such as hydroperoxides and mercaptans,[62] either do not contain chromophores, or absorb at very low wavelengths.

As samples became more complex and the need for trace analysis arose, the importance of high performance liquid chromatography (HPLC) as a technique to separate sample compounds prior to detection became evident. Isocratic separation conditions are often not sufficient to separate compounds of structural similarity, and so complex separation methods of gradient elution and column switching are often applied. These complex systems place many demands on the detection system employed, which must have a fast response rate so as not to interfere with the component separation achieved by the column, as well as a size compatible with the HPLC system. This last point, in particular, has become of increasing importance with the development of microbore columns and capillary electrophoresis (CE).

The selectivity of electrochemical detection arises from the fact that only compounds oxidisable/reducible at an applied potential are detected. The application of a high oxidation/reduction potential results in less interfering

electrochemically active compounds than compounds absorbing at a particular wavelength, therefore requiring less stringent sample clean-up procedures which are often time-consuming and costly.

Electrochemical detectors for flowing solutions can be classified as one of two categories. The first measures the charge transfer between a conducting liquid and an immiscible solid conductive phase (the working electrode), which includes the popular techniques of coulometry, potentiometry and voltammetry. Amperometry monitors the current when electrons pass to or from an electrode and usually involves only a small fraction of analyte molecules in solution. Coulometry, on the other hand, involves the oxidation/reduction of 100 % of the analyte in solution, with the electric charge passed being proportional to the concentration of analyte in solution. Potentiometric measurements, involving the measurement of potential difference representing concentrations of both oxidisable/reducible species in solution, are rarely applied to flowing solution analysis due to their slow response times (which decrease at lower concentrations) affecting sample throughput.

The monitoring of electrical properties of liquids, such as impedance, constitutes the second category of electrochemical detectors. Conductometric detection is often coupled to ion chromatography to measure charged species. The non-selectivity of this technique restricts its application to continuous monitoring.

The improved selectivity and sensitivity afforded by electrochemical detection is restricted by two major limitations. Firstly, the carrier solution in the flowing analysis system must have good electrical conductivity, thus eliminating detection in non-polar media. An aqueous media or a polar solvent must be used in which a base electrolyte can dissolve. An approach to overcoming this problem is the use of

microelectrodes.[63] The decrease of ohmic drop at these electrodes reduces the need for conductive solvent systems, thereby increasing the analyte application range. Secondly, the maintenance of a constant electrode activity can be hindered by neutral molecules in samples, or by ion-exchange occurrences, which all alter the electrochemical properties of the surface. This problem is of somewhat less significance in HPLC analysis as the electrode surface is continually being rinsed with carrier solution. This problem has recently been diminished with the use of modified electrodes, which have been discussed in Section 1.3.

1.4.1 Electrochemical Detection in Microseparations

As the need to detect analytes in the pico- and femto- molar range increases, especially in clinical applications, so too does the incentive for the development of separation techniques which can analyse such concentrations in nanolitre volumes.

The conventional detection systems developed for flowing systems necessitate relatively large flow-through volumes and therefore may not be directly applied to microvolume analysis. The miniaturisation of HPLC is achieved through the use of open-tubular capillary liquid chromatography. A window for UV-Vis and LIF detection is made by removing the polyimide protective coating from the capillary end, with the small capillary internal diameter resulting in a small pathlength, limiting the detectable concentrations for UV-Vis detection to the 10^{-6} M range.[64] The two detection methods which can be miniaturised for coupling to LC and CE microbore columns are fluorescence and electrochemistry. Because not all compounds fluoresce or possess an electrophore, in an attempt to make these

techniques universally applicable to all types of compounds, the development of analyte derivatisation and indirect detection methods became a necessity. Derivatisation of compounds, carried out off-line before injection onto the column or by on-line by pre- or post-column derivatisation,[65] can be complicated by reaction side products and small volumes thereby producing inaccurate results. Indirect detection methods may be universally applied and involve the measurement of the displacement of a mobile phase background signal by the solution analyte. The technique requires the addition of an electroactive ligand (e.g. dithiocarbamate) to the mobile phase to produce a steady signal.[66] The ligand complexes metal ions in solution to form an electroinactive product; seen as a decrease in background current. Olefirowicz and Ewing[67] applied this method to the simultaneous detection of electroactive catechols and electroinactive peptides; the former directly and the latter indirectly by complexation with the electrophore dihydrobenzylamine.

The development of on-column electrochemical detection is based on the same principle for both liquid chromatography and capillary electrophoresis i.e. the positioning of a working electrode (usually a carbon fibre) into the end of a separation capillary. For LC analysis, a thin gasket was often used in conventional thin-layer cells to reduce the cell volume from 1 μl to between 0.15-0.25 μl to accommodate the small elution volume. This has been improved recently by Bioanalytical Systems (BAS) in their production of the Unijet detection cell[68] for microbore HPLC, shown in Figure1-2. The cell is column end-fitting and operates with mobile phase flow rates as low as 200 μl/min. A variety of disk electrode materials including glassy carbon, platinum and gold can be used. This

miniturised flow-cell allows detection of neurotransmittors to concentrations as low as 10^{-11} M. The application of electrochemical detection to capillary electrophoresis, however, is more complicated due to the high levels of noise arising from the high voltage applied accross the capillary. Efficient shunting of this noise has been successfully achieved by decoupling the separation current through a number of different means, the majority of which involve the application of a conducting polymer layer at the capillary exit. Another method of decreasing current is by decreasing the capillary internal diameter.

An overview of the application of microbore HPLC with electrochemical detection to the analysis of biogenic amines in dialysates, recently published by Cheng and Kuo,[69] reports on the detection of the neurotransmitters adrenaline,

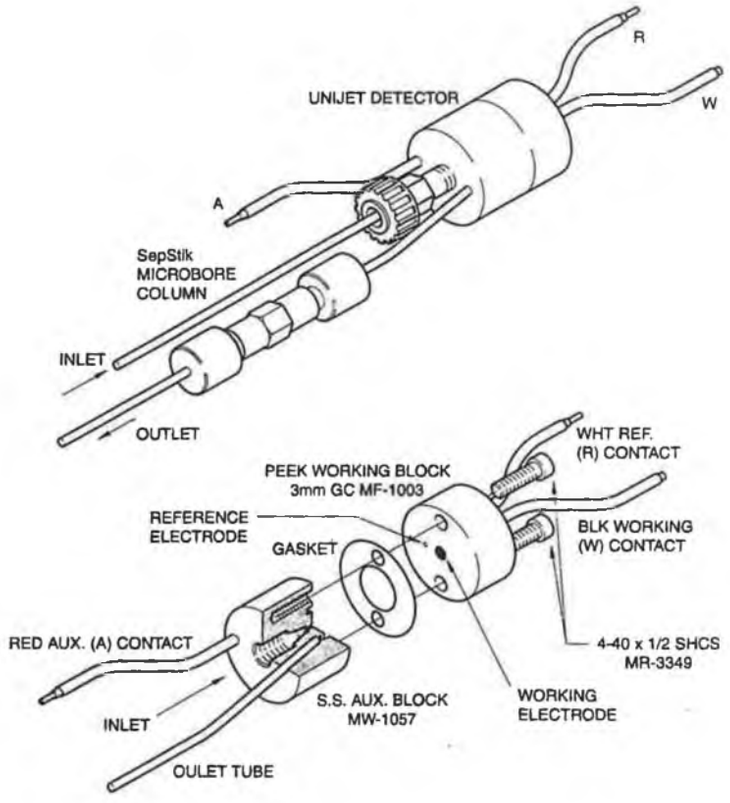


Figure 1-2 *The Unijet detection cell by Bioanalytical Systems (BAS) for microbore HPLC amperometric detection.*[68]

noradrenaline, dopamine and serotonin, in addition to precursors and metabolites in blood, tissue and cerebrospinal fluid samples. Detection of these compounds by conventional HPLC-ED, existing at trace levels in humans, is hindered by the large quantities of proteins present in samples. Large sample volumes are therefore required in order for sample clean-up and analyte preconcentration. *In vivo* microdialysis sampling produces clean, small- volume samples, which are then separated on a microbore column to attain the low detection limits required.

1.4.2 Voltammetric Detectors for Flowing Solution Analysis

Detector criteria for an analytical system is ruled by the chemical nature and concentration of the analyte, separation column efficiency in addition to the working electrode material and geometry. The elution volume of the separation method limits the detector cell flow-through volume, which must be low enough so as not to effect peak resolution or sensitivity. A necessary increase in cell volume dilutes the eluted analyte peak thereby decreasing sensitivity; with too small a cell volume resulting in band broadening.[70]

The application of a dropping mercury electrode to flowing solution analysis, despite its many attractive electrochemical characteristics, has been hindered due to the instability of the drop in flowing solutions, complicated cell design and geometry, problems with dissolved oxygen and metal ion impurities, in addition to mercury toxicity. A wall-jet design where the column eluent is directed either vertically or horizontally onto the dropping mercury electrode eliminates the necessity to remove dissolved oxygen from the background electrolyte. Although a

hanging mercury drop electrode or a surface coated gold electrode have simpler cell design and are less susceptible to mechanical failure than the dropping mercury electrode, the elimination of background current is unattainable.

Thin layer and wall-jet cells are the most common detector design applied to electrochemical detection with solid electrodes for flowing solutions and may be applied to both amperometric and coulometric analysis. Both these designs meet the requirements for liquid chromatography detectors, i.e. robust design, good signal-to-noise ratios, high mass transfer rates and high sensitivity. The small cell-volume geometry allows for the use of a variety of electrode materials and permits surface cleaning by the carrier stream flowing over the electrode surface. The positioning of the reference and working electrode in close proximity is vital to ensure consistent polarisation along the complete length of the working electrode, with the counter electrode positioned opposite the working electrode, thus eliminating iR drop. Often, the electrochemical requirements are neglected due to the priority of cell volume and hydrodynamics in minimising peak dispersion,[71] with the most common design used in amperometric mode seeing the auxiliary electrode placed opposite the working electrode, with the reference electrode positioned downstream. Detector cell design for flowing solutions has been discussed in detail by Kissinger and Heinemann.[72]

1.4.3 Working electrodes and their applications

The type of working electrode chosen, as well as the electrochemical technique applied are dependent on the type of sample to be analysed, the overall

requirements being chemical and physical inertness to mobile phase constituents at the applied potential. According to Warner in a review of electrochemical detectors for liquid chromatography,[73] three conditions must be met by the chosen working electrode. Primarily, it must be applicable to analysis in the required potential range; secondly, it should be compatible with the chromatographic mobile phase, and finally, a stable and reproducible working surface should be attainable. As a result, glassy carbon electrodes find most applications in routine analysis of easily oxidisable/reducible compounds.

Mercury electrodes find most applications for compounds whose reduction at solid electrodes is hindered by the low hydrogen overvoltage at such surfaces. As the dropping mercury electrode is not easily applied to small volume cells and flowing solutions, mercury films amalgamated onto gold electrodes are often applied. Bratin *et al.*[74] have reported the application of a thin-layer amperometric detection system with a gold amalgamated electrode for the reductive quantitation of the insecticides, parathion and methylparathion, in water and the thyphoid antibiotic, chlorampenicol in plasma. Mercury was amalgamated onto the polished gold surface either by physical dropping or electrolytically at - 0.7 V vs Ag/AgCl for 10-15 min. Large problems were encountered with surface cleanliness and reproducibility, with the admission of oxygen to the system resulting in a large interfering current at - 0.3 V.

The area of forensic analysis has benefited greatly from the selectivity and sensitivity that electrochemical detection at a DME offers to liquid chromatography. The identification of explosive and firearm propellant constituents present in skinswabs, clothing as well as other personal possessions, to

incriminate a suspect is routinely carried out by reductive electrochemistry. The main constituents of explosives, namely nitramines, aromatic nitro-compounds and alkyl nitrates; as well as those of firearm propellants, nitroglycerin and nitrocellulose, are readily reduced at a mercury electrode.[75] Stabilisers, such as diphenylamine, are often detected amperometrically upstream of the mercury electrode to facilitate a two-component identification.

Metal electrodes such as gold, platinum and nickel, may be applied to the analysis of compounds not detectable at carbon electrodes, such as carbohydrates, alcohols, sulphur compounds, amino acids and glycols.[64] It is thought that the electrochemical inactivity of these aliphatic amines and alcohols at carbon electrodes is due to the absence of π-bonding to facilitate the stabilisation of free radical oxidation products, thereby increasing the activation barrier for such processes.[76] Noble metal electrodes, possessing unsaturated surface d-orbitals, successfully interact with and therefore stabilise oxidation intermediates, thus permitting the oxidation of aliphatic compounds at lower potentials. Widespread routine application of these electrodes is hindered by their unpredictable surfaces, which are passivated rapidly during flowing analysis by sample constituent adsorption, a direct consequence of the high catalytic activity of clean electrode surfaces.

Johnson and LaCourse[77] have reported a means of electrode surface cleaning by the application of a multi-step potential-time waveform, known as pulsed amperometric detection (PAD), a typically applied 3-step waveform is shown in Figure 1-3(i). The technique initially involves analyte detection at E_{det} over a time t_{det}, followed by the anodic desorbtion of absorbed organic foulants by the

application of large positive potential pulses, E_{ox}, over a time t_{ox}. The monolayers of surface oxides thus formed passivate the electrode, which is cathodically reactivated to its original reduced form by the application of negative pulses, E_{red}. PAD is restricted to use in flowing solutions where sample components have previously been separated, due to similar voltammetric responses by all such surface controlled oxidations. An alternative form of PAD is outlined in Figure 1-3(ii) which involves potential scanning between E_{det} and E'_{det}, with the former potential chosen such that oxide is formed on the electrode surface during the potential scan, which is consequently stripped from the surface on the reverse scan. An alkaline pH is required for carbohydrate oxidation at an Au electrode, whereas

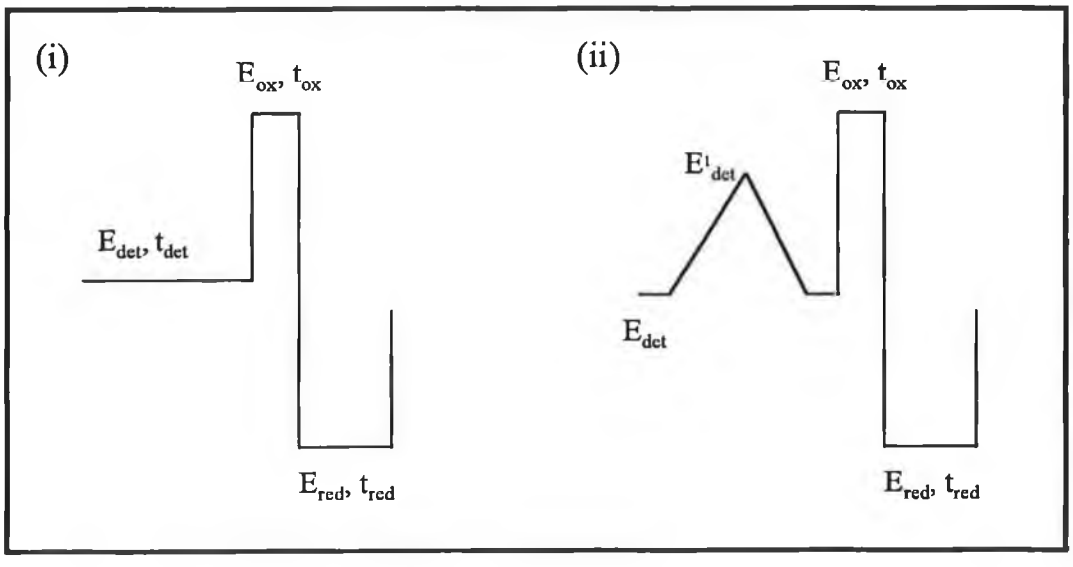


Figure 1-3 *Typical waveforms applied in Pulsed Electrochemical Detection (PED) involving the application of* (i) *a constant detection potential and* (ii) *cyclic potential scanning, where E is the applied potential for analyte detection (det), surface oxidation (ox) and reduction (red) for a time, t.*[77]

they are readily oxidised at a Pt electrode in acidic media. This is due to the different electronic configuration of the two metals which affects their affinity for free radical adsorption. As the technique is based on the initial adsorption of analyte onto the electrode surface, a prerequisite is a lone pair of electrons, which eliminates the detection of compounds such as sulphonates and quaternary aliphatic amines.

The qualitative and quantitative analysis of controlled substances, in both biological fluids and as bulk preparations, has been reviewed by Selavka and Krull.[78] Most forensic laboratories rely on low-cost, fast, highly accurate and universally sensitive techniques to obtain high certainty results which will stand up in court. Drugs of abuse such as morphine, heroin, cannabis, benzodiazapines and tricyclic antidepressants, are all electrochemically active and readily detected at a working electrode. Other substances, however, such as cocaine, barbiturates, and amphetamine stimulants which are not easily oxidised or reduced are transformed into electroactive compounds using the post-column photolytic derivatisation technique. Dual electrodes have also been applied to increase selectivity for drugs.

Many excellent reviews of the applications of electrochemical detection to flowing solution analysis have been published, including one by Radzik and Lunte,[79] highlighting the widespread applicability of amperometric and coulometric detection to drug reaction schemes, pharmaceutical and biomedical analysis, as well as the study of xenobiotic metabolism. The direct and indirect determination of amino acids, peptides and to a certain extent, proteins, have been reported by Dou *et al.*[80] which emphasises the diverse range of analytes which may be detected by electrochemical means.

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CHAPTER TWO

DECOMPOSITION OF PEROXIDES BY TRANSITION METAL IONS IN ANAEROBIC ADHESIVE CURE CHEMISTRY

2.1 INTRODUCTION

Anaerobic adhesives find most applications in the mechanical engineering industry[1] where they are applied to sealing, threadlocking, and retaining applications. Anaerobic adhesives are referred to as single component systems, in so far as curing is initiated by surface chemistry. Formulations generally consist of a monomer, initiator, accelerator, active metal surface and inhibitor/stabiliser, in addition to such performance modifiers as plastisers, fillers and thickeners. These formulations rely on a stringent balance of reactivity and stability for optimum curing.

The monomer chosen for an adhesive formulation determines the overall properties of the cured polymer, with methacrylate esters being the most common used by Loctite (Ireland) Ltd. A polymer consisting of 2-ethylhexyl methacrylate chains is tactile, whereas monomers may be chosen, such as methacrylate-capped urethanes, to promote crosslinking which results in a rigid solid.

Readily reducible organic hydroperoxides, including cumene hydroperoxide, *t*-butyl hydroperoxide, *t*-butyl perbenzoate and benzoyl peroxide, are the most commonly applied initiators, reacting through a one electron transfer with lower state metals to initiate polymerisation:

$$ROOH + M^{n+} \rightarrow RO^{\cdot} + OH^{-} + M^{(n+1)+}$$

Once a free radical has been formed through redox decomposition, it either reacts with molecular oxygen, thereby discontinuing further reaction, or attacks the monomer to initiate polymerisation. A combination of saccharin (benzoic sulfimide) and a reducing aromatic amine such as tetrahydroquinoline (THQ) and *N,N*-dimethyl-*p*-toluidine

(DMPT) are often incorporated into the cure system to accelerate the redox initiation process. In conjunction with hydroperoxides, reduction of the higher oxidation state transition metal ions to a lower state occurs by the amine; the electron transfer being possibly aided by a saccharin/amine charge transfer.[2]

Premature curing can occur as a result of low levels of transition metal ions present in formulations. For this reason, chelating agents, such as EDTA, are often added as stabilisers in adhesive formulations to sequester such contaminants. Other free radical inhibitors added may include substituted phenols such as *p*-methoxyphenol, hydroquinone or pyrogallol. Oxygen also plays as important role in storage. Viscosity and strength of the polymer is fashioned by varying the type and concentration of fillers and thickeners.

Anaerobic adhesive formulations remain stable in liquid form, for extended periods of time in the presence of oxygen (therefore they are stored in polyethylene containers through which oxygen can permeate); however, oxygen elimination results in rapid polymerisation by redox decomposition of the initiator by the active substrate metal surface to form strong, solvent resistant solids. Dissolved molecular oxygen prevents polymerisation through converting the active radical ion to an unreactive peroxy radical, $ROO^{\cdot}$. Once the adhesive formulation is confined between two mating surfaces, the majority of molecular oxygen is eliminated and the remainder is rapidly depleted through reaction with free radical initiators and rapid adhesive curing occurs. The role of oxygen in curing inhibition is shown in Figure 2-1.

A simplified outline of the curing system of the methacrylate ester based adhesives is shown in Figure 2-2. The reaction scheme highlights the importance of transition metal ions in the catalytic decomposition of the peroxide, generating free radicals. Free radical ions thus formed attack a monomer to begin polymerisation. The rate of curing is very

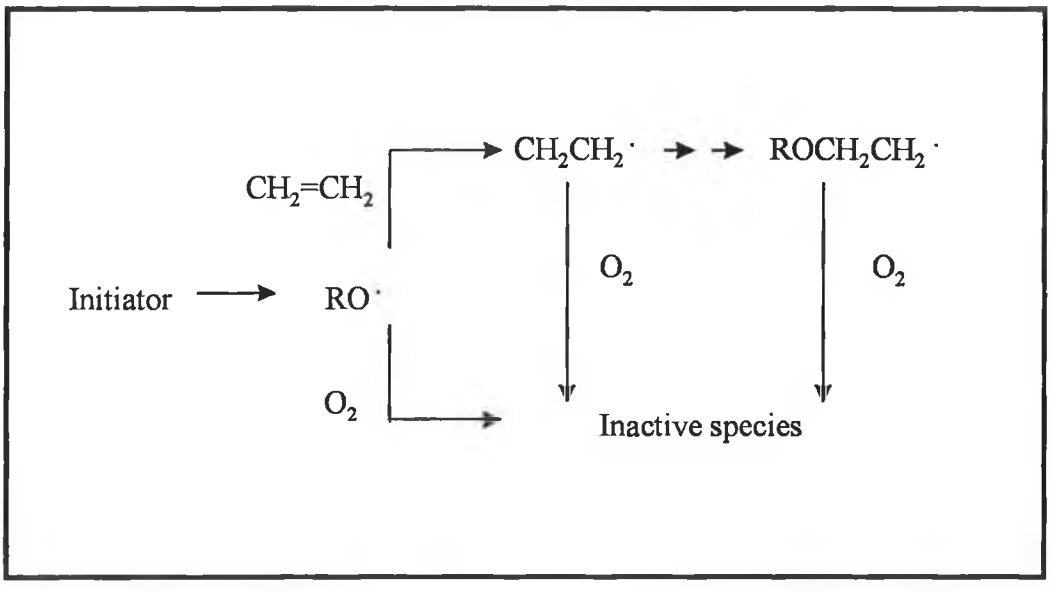

Figure 2-1 *Role of O_2 in anaerobic adhesive curing inhibition.*

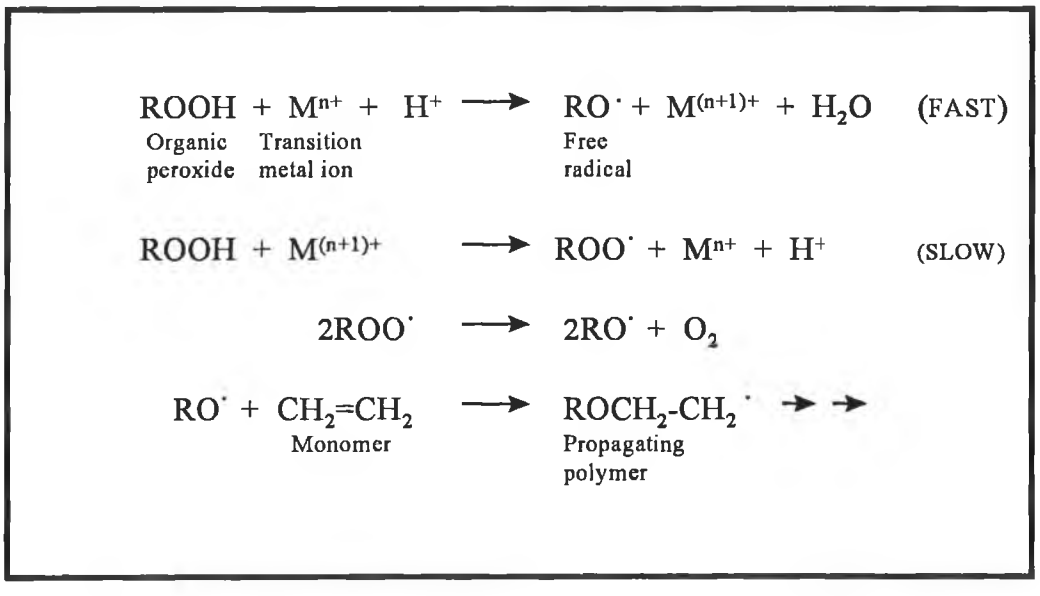

Figure 2-2 *Anaerobic adhesive curing scheme.*

much dependent on the nature of the metal substrate, with copper and iron ions aiding rapid curing, and zinc and cadmium ions resulting in slower polymerisation rates. Due to the stringent balance of formulation constituent ratios required for prolonged storage and efficient curing, effective quality control procedures are necessary during production to ensure the concentration of contaminating metal ions and oxygen are below the required level, as well as the concentration of sequestering agents and stabilisers. Levels may be between 0.01 - 10 % of the formulation composition.[1]

Hai-lin *et al.*[3] have developed a differential pulse voltammetric technique for the monitoring of Cu^{2+} and Fe^{3+} in adhesive formulations by coating a glassy carbon electrode with the formulation. In a supporting electrolyte of 0.1 M sodium dodecyl sulphate, Cu^{2+} was detected at a level of 0.1 mg/L and Fe^{3+} at 2.0 mg/L. Previously, identical ions were detected by reversed-phase HPLC with spectral detection at 400 nm to a level of 100-250 ppb and 250-600 ppb for Cu^{2+} and Fe^{3+}, respectively.[4] The procedure involved reacting the ions with 8-hydroxyquinoline to form oxine complexes, which were separated on a C_{18} column in a acetonitrile/ 0.02 M acetate buffer, pH 6.0, containing $1x10^{-2}$ M oxine and 0.2 M KNO_3. Although Cu and Fe ions could be detected, similar complexes of Zn^{2+}, Ni^{2+}, Cr^{3+} and Co^{3+} were not resolvable.

Similar problems with resolution were encountered by O'Dea *et al.*[5] in the development of an ion-exchange chromatographic technique for transition metal ions. A mobile phase consisting of ethylenediammonium (EDA) cation and citric acid ensured "pushing" and "pulling" of ions along the column. Detection was at 520 nm following post-column derivatisation by 2-(2-pyridylazo)resorcinol (PAR). Of the eight ions examined, Cu^{2+}, Zn^{2+}, Co^{2+}, Fe^{2+} and Mn^{2+} were separated. Resolution of Cu^{2+}, Pb^{2+}, Ni^{2+}, Zn^{2+}, Co^{2+}, Fe^{2+}, Cd^{2+} and Mn^{2+} was obtained by solid-phase extraction on a C_{18} Sep-Pak cartridge

and injection onto a dynamically coated C_{18} column by Deacon *et al.*[6] As with the ion-exchange method, a PAR post-column derivatisation detection system was applied.

Gas chromatography with thermal conductivity detection has been applied to the monitoring of dissolved oxygen in anaerobic formulations.[7] A sealant sample, dissolved in acetonitrile, was injected onto a 10 % OV-17 Chromosorb W stationary phase with a helium gas carrier phase. A LOD of 0.3 ppm was attainable.

To date, much of the research on organic peroxides in cure formulations has been carried out on cumene hydroperoxide, as this is the most common peroxide used as a polymerisation initiator in cure systems. Little is known about the activity of other peroxides and their possible use in the development of new anaerobic adhesives with faster sealing ability and applicability on a wider variety of surfaces. It is the aim of this chapter to discuss the rate of decomposition of the organic peroxides t-butyl perbenzoate, t-butyl hydroperoxide and benzoyl peroxide by the transition metal ions Fe(II), Fe(III), Cu(I), Cu(II) and Co(II) over time and to identify the ions which cause rapid peroxide decomposition. To the reaction mixtures of metals which are slow in decomposing each peroxide, the accelerators 1-acetyl-2-phenylhydrazine (APH), N,N-dimethyl-p-toluidine (DMPT) and 1,2,3,4-tetrahydroquinoline (THQ) were added in a 1:1 and a 2:1 molar ratio to metal ion, in order to monitor the increase in decomposition. Finally, saccharin and maleic acid were also incorporated into the mixtures (also in a 1:1 and 2:1 ratio to metal) to observe their effect. The structures of the organic peroxides, accelerators and acids used are shown in Figure 2-3.

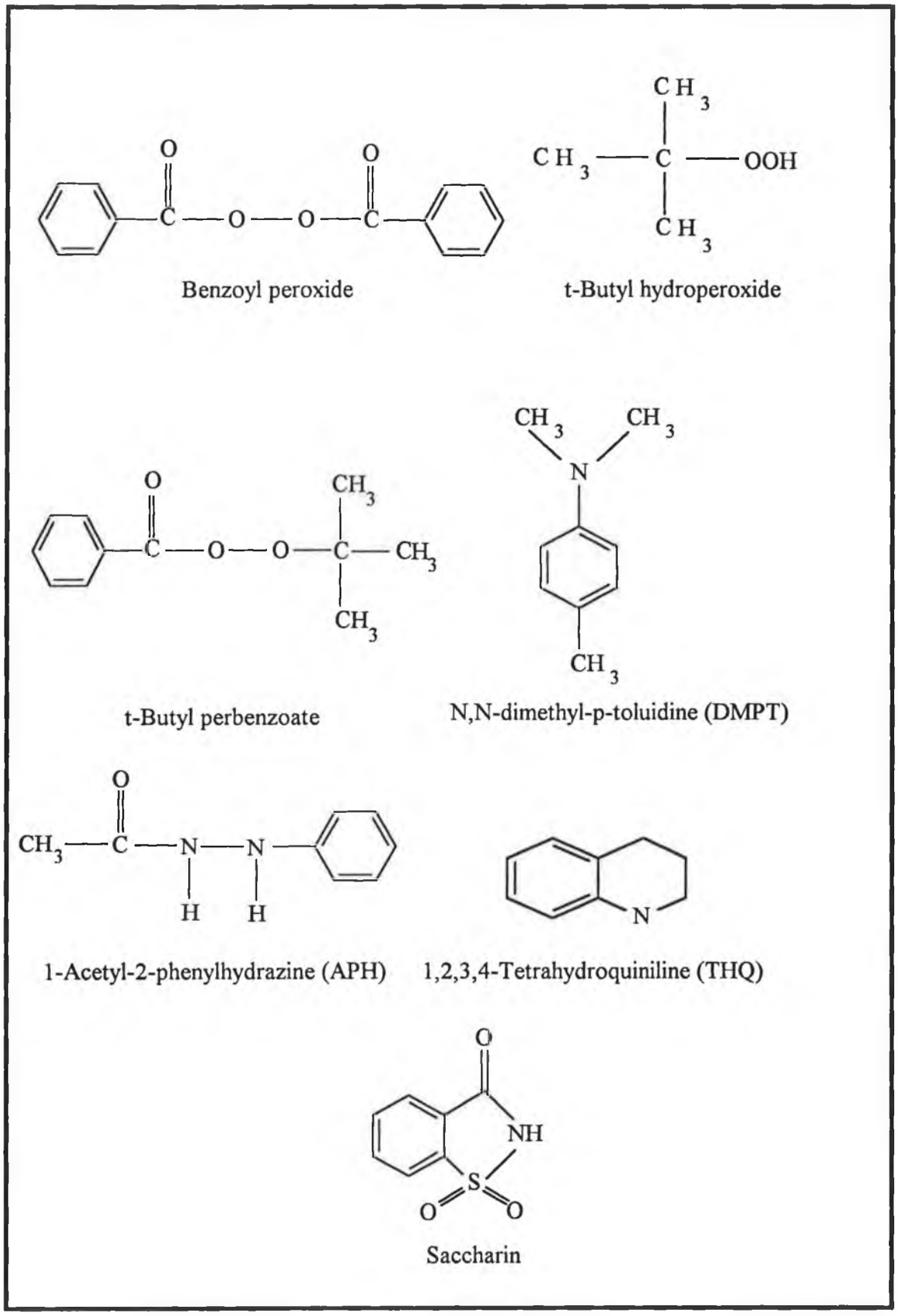

Figure 2-3 *Structures of some initiators and accelerators used in anaerobic adhesives*

2.2 EXPERIMENTAL

2.2.1 Apparatus

The decomposition of t-butyl perbenzoate by metal ions was studied using an EG & G PARC (Princeton Applied Research, Princeton, NJ) Model 264A Polarographic Analyser/Stripping Voltammeter, linked to an EG & G PARC, Model 303 Static Mercury Drop Electrode. An Ag/AgCl reference electrode was filled with non-aqueous filling solution of 0.1 M $AgNO_3$ in acetonitrile. The counter electrode used was a platinum wire. Differential pulse polarograms were recorded using a Houston X-Y chart recorder with a medium drop size set to a drop time of 1 s, a pulse amplitude of 50 mV, with a quiet time of 15 s before scanning.

A BAS (Bioresearch Analytical, West Layfette) Electrochemical Analyser controlled the EG & G PARC SDME for the analysis of t-butyl hydroperoxide and benzoyl peroxide. The system was run using software installed on an Elonex 486 PC, with a medium drop size set to a pulse period of 200 ms, pulse width of 50 ms, pulse amplitude of 50 mV, sample width of 17 ms and a quiet time of 2 s.

2.2.2 Reagents

Triply distilled mercury was purchased from Aldrich Chemicals Ltd. Analar grade APH was obtained from Eastman Kodak. t-butyl perbenzoate, t-butyl hydroperoxide, benzoyl peroxide, saccharin, maleic acid, DMPT, THQ and metal salts, all of Analar grade, were supplied by Loctite (Ireland) Ltd.

In the decomposition of t-butyl perbenzoate and t-butyl hydroperoxide, solutions of Fe(III) and Co(II) were prepared by dissolving the nitrate salts in methanol. Cu(II)

acetate was dissolved in a 9:1 methanol:de-ionised water mixture, which was heated slightly. Cu(I) chloride dissolved in a 3:7 ammonia: methanol solution. A 1 M ammonium Fe(II) sulphate solution was prepared in deionised water, which was diluted 1 in 10 with methanol to allow for dissolution of the peroxides.

Due to the non-polar properties of benzoyl peroxide, which is insoluble in methanol, a 3:2(v/v) solution of chloroform: methanol was used in place of 100 % methanol to dissolve both the peroxide and metal salts.

2.2.3 Procedures

Decomposition experiments were carried out at room temperature in an electrolyte consisting of 0.3 M lithium chloride dissolved in a 1:1 methanol: toluene mixture. All solutions were purged with oxygen-free nitrogen for 2 min prior to scanning, with the solutions blanketed with the gas throughout the scan to avoid oxygen absorption.

Peroxides were dissolved in their appropriate solution ion in a 100 cm^3 acid-washed beaker, from which a 100 µl aliquot was removed and added to 10 cm^3 of the electrolyte to give a final cell concentration of 1 x 10^{-3} M peroxide. After purging, differential pulse polarograms were obtained by scanning in the appropriate potential range: t-butyl perbenzoate from -0.90 V to -1.50 V, t-butyl hydroperoxide from -0.8 V to -2.00 V and benzoyl peroxide from +0.25 V to -0.70 V, all at a scan rate of 20 mV/s. The peak height of the broad organic peroxide peak was used to evaluate the percentage of peroxide decomposition at pre-determined time intervals on addition of the following cure components:

i) organic peroxide + metal salt

ii) organic peroxide + metal salt + acid (1:1 and 2:1 molar ratio to metal)

iii) organic peroxide + metal salt + accelerator (1:1 and 2:1 molar ratio to metal)

iv) organic peroxide + metal salt + accelerator + acid (1:1 and 2:1 molar ratio to metal)

The reducing amines investigated included APH, DMPT and THQ and the acids were saccharin and maleic acid.

2.3 RESULTS AND DISCUSSION

The reduction of organic peroxides at mercury electrodes in non-aqueous media has been widely investigated,[8,9] with differential pulse polarography (DPP) of organic peroxides producing broad but well-defined symmetrical peaks,[10] without the need for sample clean-up. For this reason, DPP was used for investigating the reaction of transition metal ions with organic peroxides, by monitoring the change in peroxide concentration over a period of time.

As the decomposition of organic peroxides occurs mainly due to interaction with transition metal ions in lower oxidation states,[11] the main function of accelerators is to reduce a higher oxidation state metal ion in order to lower its activation energy and increase its activity. The initial accelerators used in the 1960's were based on trialkylamines, such as tributylamine (TBA) and DMPT, which are not very active reducing agents, but had to be used due to restrictions in product stabilisation. However, with more advanced stabilisation methods such as the use of EDTA to sequester

contaminating metal ions, more active reducing agents can be added, such as THQ and APH.

The incorporation of acids, such as saccharin and maleic acid, into cure formulations is necessary as they play a key role in the release of transition metal ions from substrate surfaces, with the type of acid responsible for the concentration and oxidation state of the transition metal ion released. It has been found that these acids may play a part in the acid-catalysed decomposition of such peroxides. It has also been proposed that the presence of both tertiary amine and saccharin results in the formation of a charge transfer complex which promotes reduction of the higher state transition metal ion. Each of these processes produce free radicals to initiate polymerisation.

2.3.1 Decomposition of t-butyl perbenzoate

The reduction of t-butyl perbenzoate at a mercury electrode occurs at a $E_{1/2}$ of -0.75 V, and its decomposition by iron, copper and cobalt metal ions is shown in Figure 2-4. Addition of both Cu(I) and Fe(II) resulted in very rapid decomposition of the peroxide, the former causing immediate decomposition upon addition and the latter resulting in 65 % decomposition after 2 min. The reaction of Fe(III) was slower with the reaction proceeding no further than 49 % after 60 min. However, the addition of any of the three accelerators APH, DMPT or THQ in a 1:1 molar ratio to metal dramatically increased the reaction rate to result in a 100 % decomposition of t-butyl perbenzoate in 5 min.

Co(II) proved to be ineffective in reacting with the peroxide, resulting in less than 20 % decomposition after 120 min, while Cu(II) proved to be inactive. It was decided to investigate the acceleratory effects of the reducing amines and the acids on the peroxide decomposition by these transition metals.

2.3.1.1 Decomposition by Cu(II)

The addition of either saccharin or maleic acid to the Cu(II) reaction had little acceleratory effect on the decomposition of t-butyl perbenzoate (Figure 2-5), despite reports that saccharin, a weak acid, acid-catalyses the decomposition of cumene hydroperoxide by protonation of the hydroperoxide, thereby lowering the activation energy for cleavage of the peroxide bond.[12] Saccharin in a 1:1 molar ratio did not push the decomposition to above 18 %, whereas a 2:1 molar ratio had no effect on inactivity. It was decided to investigate the effects of a stronger acid, maleic acid, which in a 1:1 ratio was less effective than saccharin 1:1, and a 2:1 ratio caused only 7.1 % decomposition after 140 min. Raftery[12] has correlated reducing agent oxidation potential and reduction activity, over a wide range pH scale. It was discovered that the most active reducing agent, APH, had the lowest oxidation potential over the pH range, with the potential increasing as the pH decreases. Reducing agents in decreasing order of activity were APH, THQ and DMPT. The incorporation of organic acids would lower the medium pH, thereby increasing the oxidation potential, which may be representative of the amine's ability to reduce metal ions, although this is still speculative.

The incorporation of amine accelerators into the reaction improved decomposition rates dramatically (Figure 2-6). APH in both a 1:1 and 2:1 ratio caused total substrate decomposition in 2 min. THQ rapidly decomposed t-butyl perbenzoate, with 97 % decomposition in 60 min when present in a 1:1 ratio with Cu(II). This was increased to 96 % in 20 min when the ratio of amine to metal ion was doubled. Saccharin and maleic acid were added to the 1:1 THQ reaction to investigate their effect. The addition of 1:1 saccharin:Cu(II) pushed the time of total decomposition to 2 min, whereas THQ/maleic acid showed a less dramatic increase in reaction rate. Total decomposition of the organic

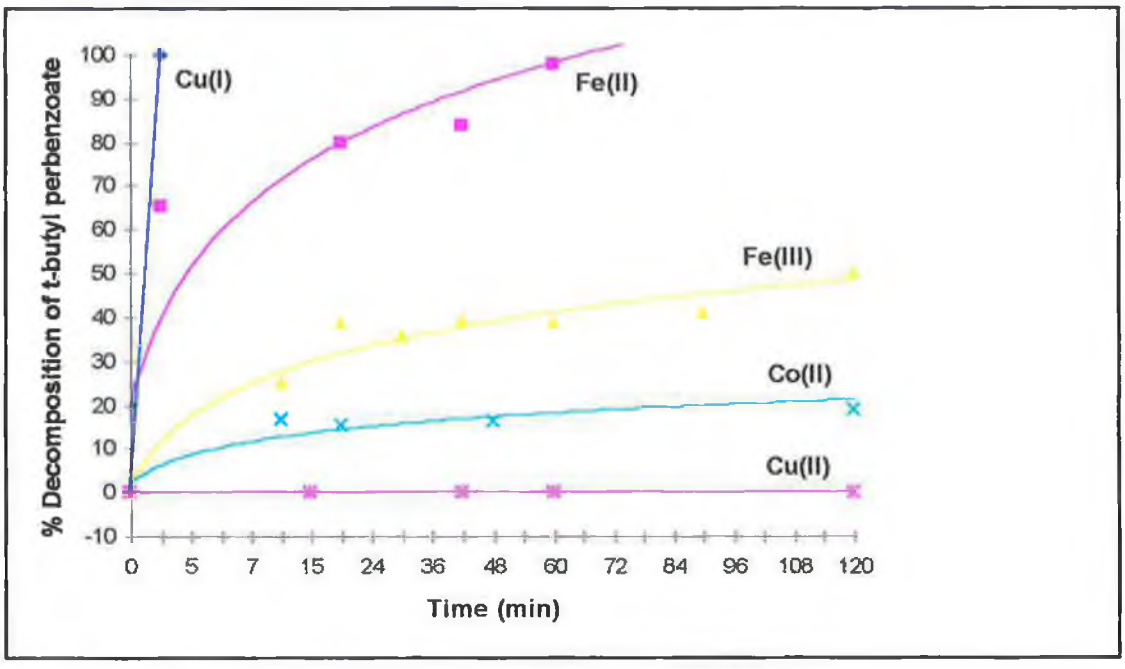

Figure 2-4 *Decomposition profiles of t-butyl perbenzoate following incorporation of transition metal ions into the reaction mixture.*

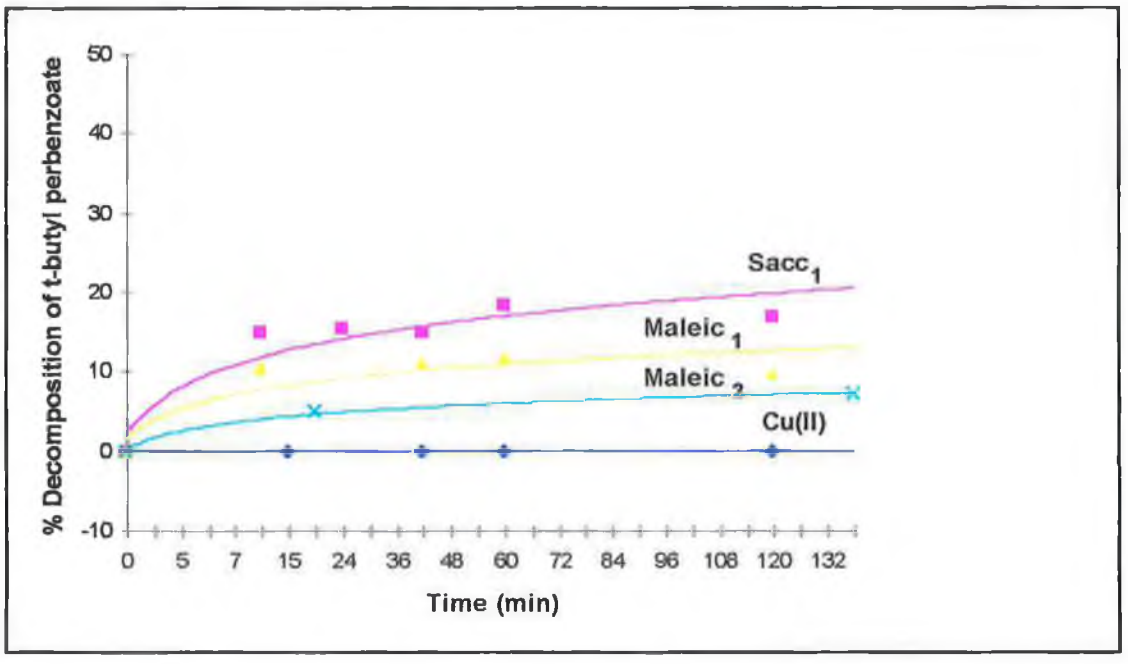

Figure 2-5 *Effect of the addition of weak acids saccharin and maleic acid to the peroxide-Cu(II) mixture. Subscript numbers denote molar ratio to metal.*

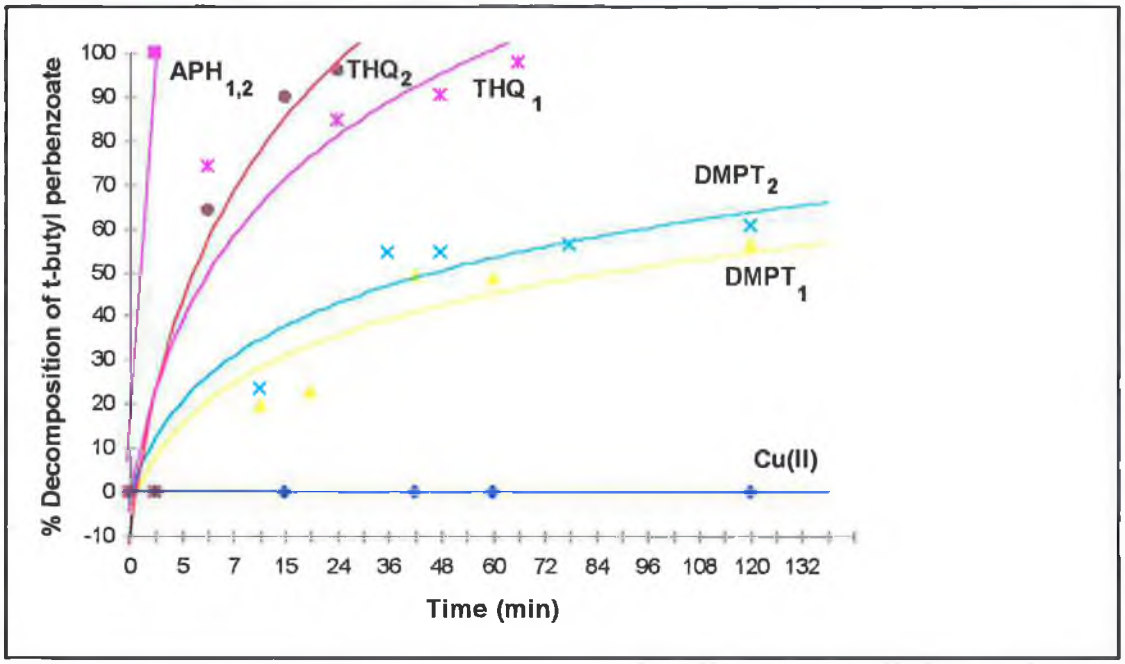

Figure 2-6 *Effect of APH, DMPT and THQ accelerators on decomposition of t-Butyl perbenzoate by Cu(II). Subscript numbers denote molar ratio to metal.*

peroxide took place in 12 min when maleic acid was present in a 2:1 ratio with the metal ion, with the 1:1 ratio requiring twice the time to achieve the same effect.

The reaction of DMPT was much slower than the other amines. Little difference was evident in the decomposition effects of a 1:1 or 2:1 molar ratio to Cu(II). Maximum decomposition was achieved with a 2:1 ratio at 60 % after 120 min. The inclusion of maleic acid, examined at both ratios, retarded the reaction. A combination of DMPT and saccharin successfully increased decomposition to 100 % after 10 min and 15 min for 2:1 and 1:1 saccharin ratios, respectively. These results point to a unique role of saccharin in the reduction of the Cu(II) to Cu(I) by DMPT, as the addition of maleic acid actually retarded metal reduction, indicating that this trend is not a result of its acidic nature. A similar observation was made by Raftery[12] in the reduction of Cu(II) by DMPT in polarographic cumene hydroperoxide studies. Although DMPT showed no reduction capabilities, when it was reacted with saccharin to form a new product, aminal, very efficient Cu(II) reduction occurred. The reducing capabilities of aminal were first reported by Wellman and Brockmann[13] when they discovered a new compound on reaction of saccharin with DMPT. This compound formed metal chelates and caused rapid decomposition of cumene hydroperoxide by Cu(II).

These results proved that Cu(II) alone plays an inactive role in t-butyl perbenzoate decomposition, but the addition of APH at an equi-molar concentration to metal was ample to cause complete decomposition. The effectiveness of THQ as an accelerator depends on the presence of saccharin. DMPT, much less effective than APH or THQ in decomposition, also relies on the presence of saccharin to increase the rate of reaction.

2.3.1.2 Decomposition by Co(II)

The decomposition of t-butyl perbenzoate by Co(II) ions, can be seen from results to be highly dependant on the presence of accelerators and acids in the reaction mixture in order for any decomposition to occur. A maximum of 30 % peroxide decomposition was attained by the addition of APH and saccharin, each at a concentration ratio of 2:1 to metal. APH alone achieved a 21 % and 24 % peak reduction in 120 min when added in a 1:1 and 2:1 ratio, respectively. With APH present in a 2:1 molar ratio to Co(II), the addition of saccharin in a 1:1 ratio retarded decomposition to 20 %, and although maleic acid added in a 1:1 ratio gave a similar result to the APH alone, doubling this ratio caused total retardation of decomposition.

Surprisingly, THQ, considered to be a stronger reducing agent than DMPT, did not have the same acceleratory effect as DMPT. With only 4 % substrate decomposition after 105 min in a 1:1 ratio, THQ in a double concentration to metal caused no decomposition. Maleic acid, in conjunction with THQ, at either concentration did not affect Co(II) inactivity; neither did saccharin in a 2:1 ratio. Present in an equi-concentration with Co(II), THQ/saccharin resulted in an overall decomposition of 14 % after 90 min.

DMPT added at the same concentration as the metal reached 16.8 % decomposition of the peroxide after 60 min, while doubling this concentration retarded the reaction completely. Further studies on the former showed DMPT/saccharin and DMPT/maleic acid, both in 1:1 ratio to metal, to have similar acceleratory effects, achieving a maximum of 18 % decomposition after 90 min. Saccharin in a 2:1 ratio with the metal, in the presence of DMPT, accelerated the reaction to only 5.1 % decomposition after 90 min, while maleic acid in the same concentration caused no decomposition. It can be concluded from these results that Co(II) is not very effective in the decomposition of t-butyl perbenzoate, even in the presence of accelerators and acids. This has lead to the

use of primers on such inactive surfaces. Primers usually consist of solvent-soluble metal salts, such as copper naphthenate, which are brushed onto the bonding surface before application of the adhesive formulation.[14]

2.3.2 Decomposition of t-butyl hydroperoxide

Reduction of t-butyl hydroperoxide at the dropping mercury electrode occurs at a $E_{1/2}$ of -1.25 V. A decomposition profile of cumene hydroperoxide, of similar structure to t-butyl hydroperoxide, by transition metal ions has previously been carried out Raftery,[12] who found that Fe(II) and Fe(III) ions rapidly catalysed the decomposition reaction, with Cu(I) and Cu(II) ions relying more on the presence of accelerators to increase the reaction rate. Similar results were obtained for iron and copper ions in the decomposition study of t-butyl hydroperoxide, shown in Figure 2-7. Fe(II) decomposed the hydroperoxide by 80 % after 15 min. Although the presence of Fe(III) caused 64 % decomposition in 10 min, no further decomposition occurred up to 120 min. The incorporation of either APH, DMPT or THQ successfully pushed the reaction to completion within 2 min (Figure 2-8).

The decomposition of the hydroperoxide by copper ions was not as spontaneous, and although Cu(I) resulted in 93 % decomposition in 120 min, Cu(II) showed virtually no reactivity, with the t-butyl hydroperoxide peak reduced by only 7.5 % in the same time.

2.3.2.1 Decomposition by Cu(I)

Preliminary studies carried out with Cu(I) involved the addition of saccharin and maleic acid, in a 1:1 and 2:1 molar ratio to metal, without the presence of reducing agents. The presence of saccharin at a 1:1 ratio showed a decrease in decomposition efficiency,

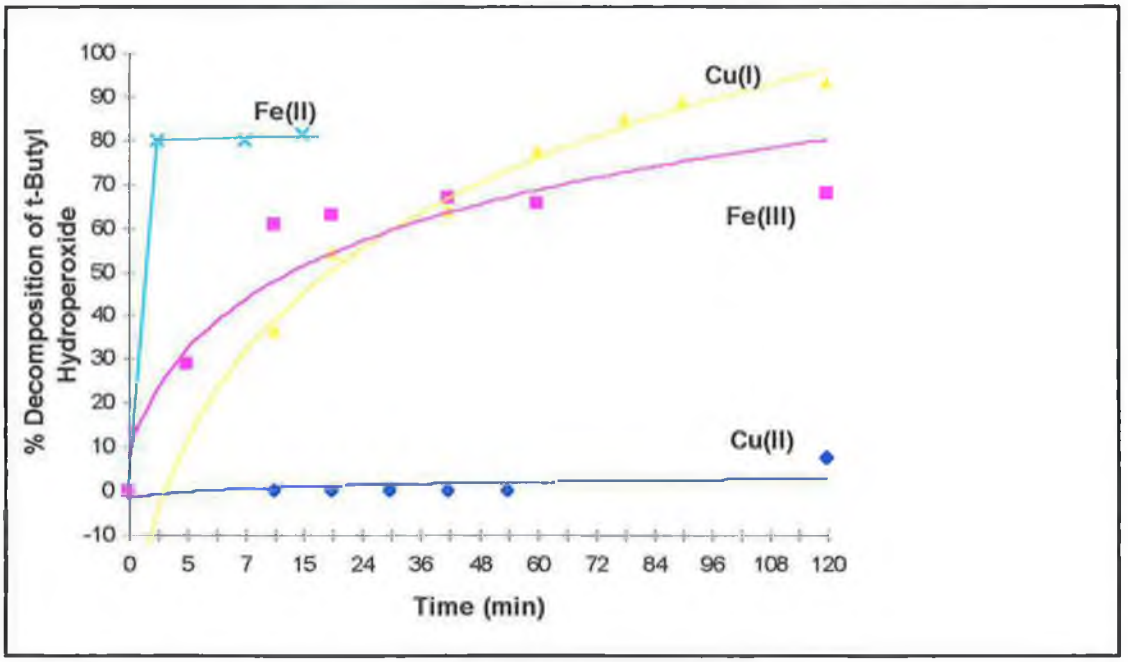

Figure 2-7 *Decomposition of t-butyl hydroperoxide by transition metal ions.*

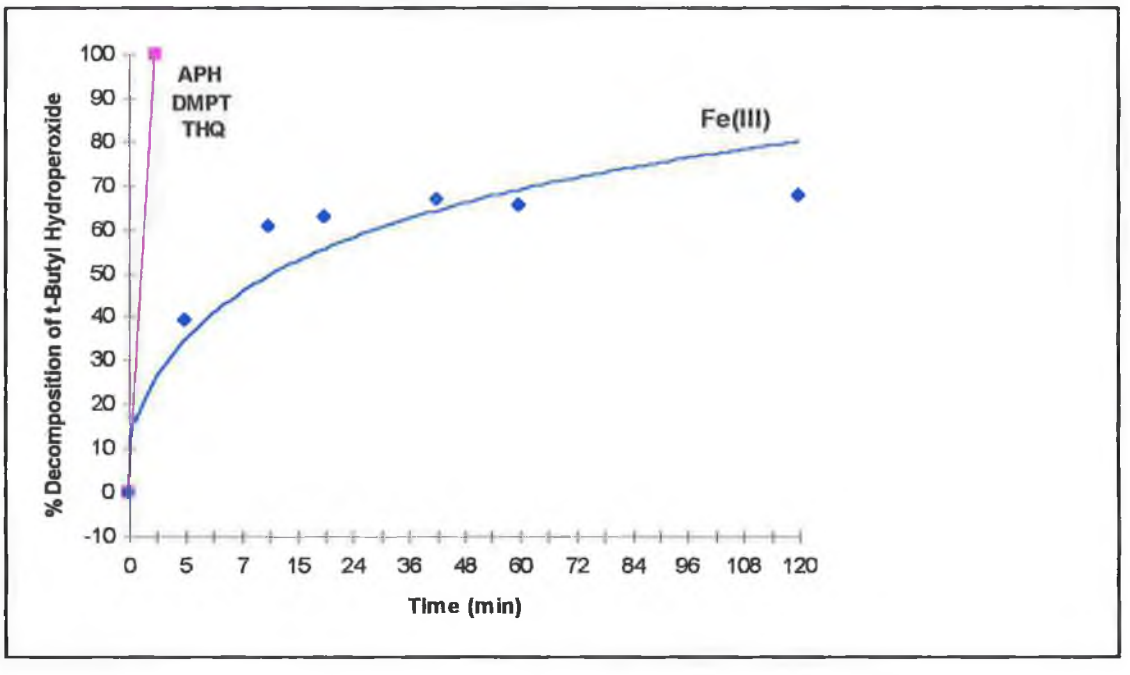

Figure 2-8 *Effect of the addition of accelerators on the decomposition of t-butyl hydroperoxide by Fe(III).*

allowing only 40 % in 120 min. As with the decomposition study of t-butyl perbenzoate by Cu(II), the addition of saccharin in a 2:1 ratio reduced Cu(I) activity further to only 28 % after 120 min. Maleic acid, being a stronger acid than saccharin, had a more detrimental effect on decomposition, resulting in only 13 % after 60 min. Cu(I) in the presence of the stronger reducing agents APH and THQ rapidly decomposed t-butyl hydroperoxide. APH in a 1:1 ratio was ample to cause immediate decomposition, whereas THQ in a 1:1 ratio reduced the hydroxide concentration to 97 % in 17 min. THQ in a 2:1 ratio achieved the same percentage in 5 min less. To investigate if the addition of saccharin or maleic acids to a 1:1 THQ-Cu(I) mixture would increase reactivity, these acids were added, as usual, in a 1:1 and 2:1 ratio to metal. It was observed that the incorporation of the acids inhibited the rate of decomposition in accordance with the strength and concentration added. Saccharin in an equi-molar concentration with metal ion achieved 96 % decomposition in 75 min, whereas double this concentration limited the decomposition to 67 % in the same time. The stronger acid, maleic acid, when present with Cu(I) alone, slowed down the reaction to 43 % decomposition in 75 min when added in a 2:1 ratio.

Although the accelerator DMPT increased the rate of t-butyl hydroperoxide decomposition when added to the Cu(I)-hydroperoxide mixture, its effect was less than APH or THQ owing to its weaker reducing ability. Whether present in a 1:1 or 2:1 ratio, DMPT increased the decomposition rate similarly (Figure 2-9). The incorporation of either saccharin or maleic acid into the 2:1 DMPT reaction mixture had a similar deceleratory effect as when added to THQ. Results showed that the addition of saccharin in a 1:1 or 2:1, and maleic acid in a 1:1 or 2:1 ratio, reduced the decomposition of hydroperoxide to 49 %, 47 %, 28 % and 16 % after 60 min, respectively.

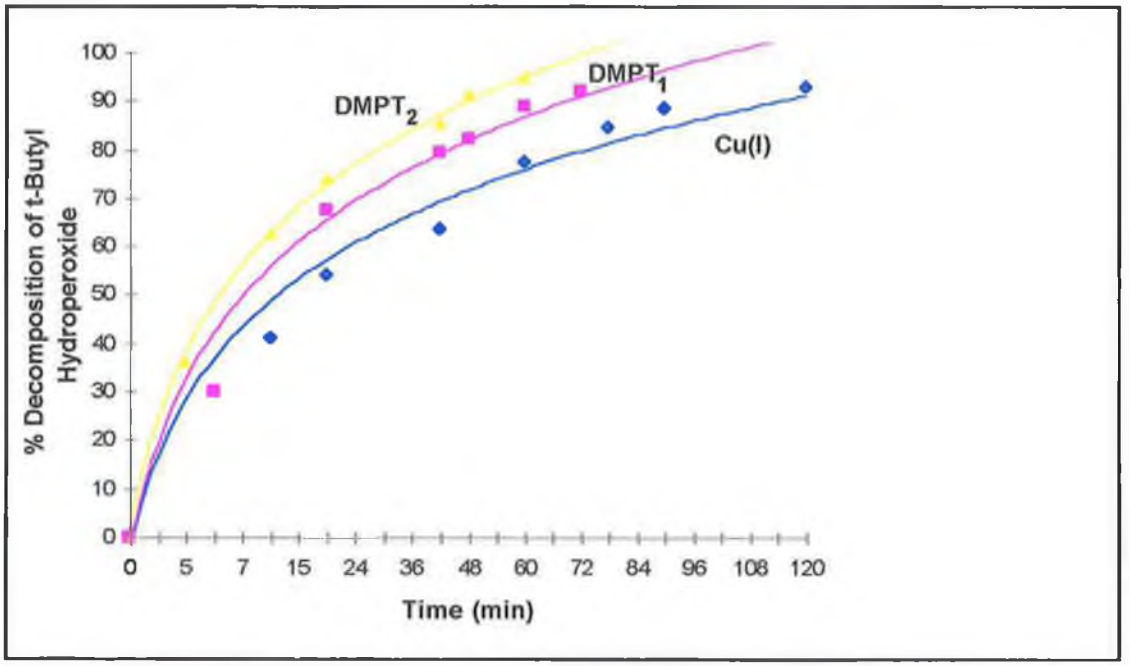

Figure 2-9 *The effect of DMPT on the Cu(I) catalysed decomposition of t-butyl hydroperoxide. Subscripts indicate molar ratio to metal.*

From these studies it can be seen that although total t-butyl hydroperoxide decomposition can be achieved by Cu(I) ions, the rate of reaction is too slow to be efficient in an anaerobic cure formulation. The addition of APH results in 100 % decomposition within 2 min. Although THQ, at twice the concentration of metal, achieves 97 % decomposition in 12 min, this rate cannot be improved by the addition of saccharin or maleic acid, both of which slow down the decomposition reaction. DMPT is not a very efficient accelerator of t-butyl hydroperoxide decomposition by Cu(I) ions, with maximum decomposition obtained in 60 min in a 2:1 ratio to Cu(I). Once more, the presence of acids in the reaction mixture decreases the reaction rate.

2.3.2.2 Decomposition by Cu(II)

The inability of Cu(II) to decompose t-butyl hydroperoxide is probably due to its presence in a higher oxidation state, thereby relying on the presence of reducing agents to provide activity. Results obtained support this theory, with the presence of APH in a 1:1 or 2:1 ratio showing similar decomposition trends, as seen in Figure 2-10, both obtaining a reduction in peak height of above 85 % in 2 min, thereby omitting the need for co-accelerators. THQ did not accelerate the reaction between Cu(II) and the hydroperoxide to the same extent as it did Cu(I), nevertheless, 70 % decomposition was achieved in 5 min. As THQ in a 1:1 ratio was less effective, the addition of maleic acid and saccharin to the reaction was studied, with spontaneous decomposition occuring upon addition.

DMPT showed the predictably less acceleratory effects, with a 1:1 DMPT ratio increasing activity to only 31 % in 100 min. Doubling the DMPT concentration increased the decomposition to 52 % after 120 min, with the addition of maleic acid to the DMPT-

Cu(II) mixture causing a decrease in activity to 30 % for a 1:1 ratio and 16 % with a 2:1 ratio after 120 min. Surprisingly, the incorporation of saccharin at both ratios dramatically increased the decomposition, to 96 % in 15 min for a 1:1 ratio and 100 % in 8 min for a 2:1 ratio to metal, shown in Figure 2-11.

It can be summarised, therefore, that Cu(II) is not capable of catalysing t-butyl hydroperoxide decomposition unless complexed to accelerators. The incorporation of APH alone to the Cu(II)-t-butyl peroxide mixture is ample to cause complete decomposition, with the addition of THQ, in the presence of either saccharin or maleic acid, to the mixture achieving complete decomposition. The addition of DMPT in the presence of saccharin (both at a 2:1 ratio with metal ion) also reduces Cu(II) to Cu(I) to cause rapid decomposition of the peroxide.

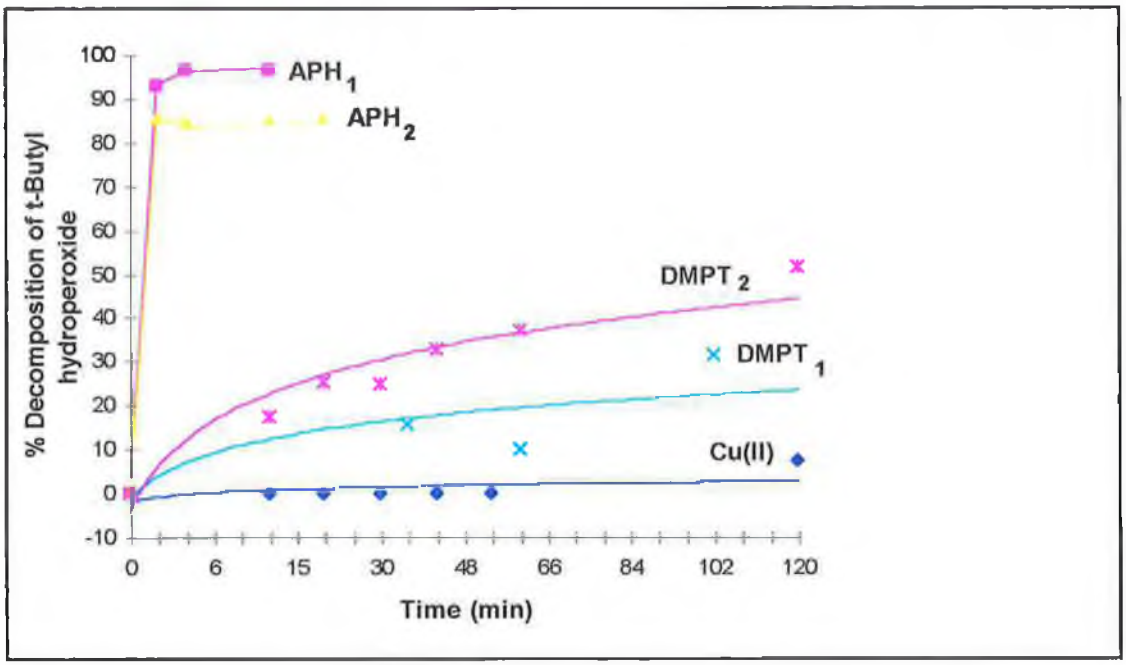

Figure 2-10 *Acceleration of t-butyl hydroperoxide decomposition by APH and DMPT in 1:1 and 2:1 molar ratio to Cu(II). Subscript numbers denote ratio to metal.*

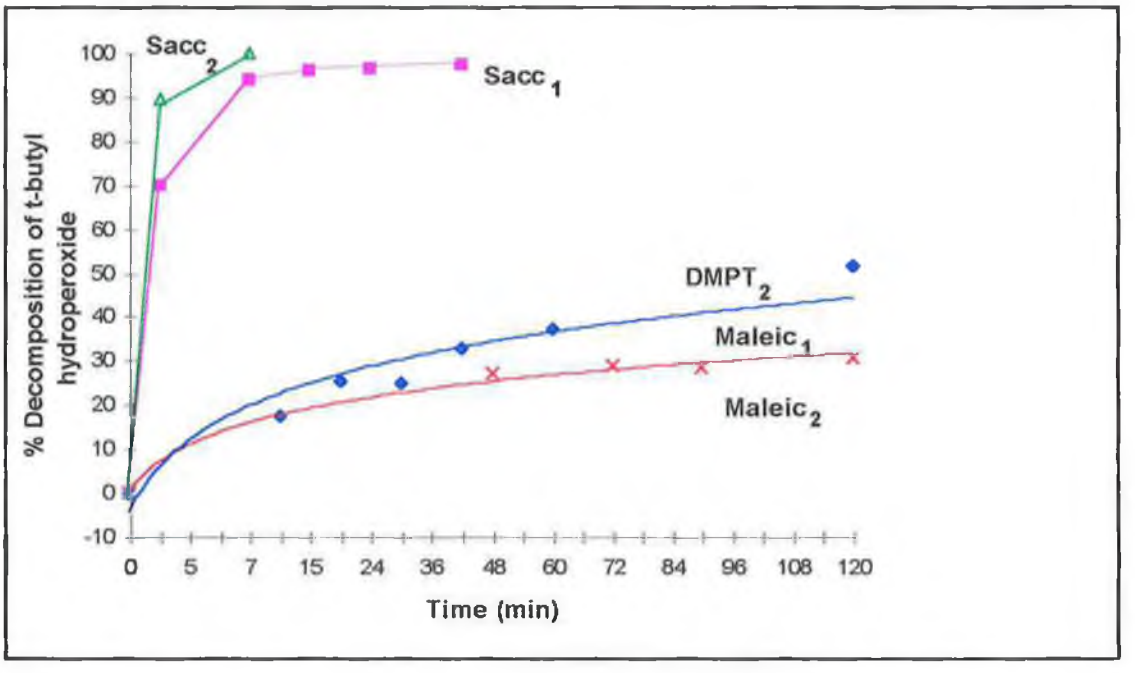

Figure 2-11 *Incorporation of Saccharin and Maleic acid to 2:1 DMPT-Cu(II) mixture in the decomposition of t-butyl hydroperoxide.*

2.3.3 Decomposition of benzoyl peroxide

The reduction of benzoyl peroxide occurs in non-aqueous solutions at an $E_{1/2}$ of -0.07 V. Figure 2-12 shows decomposition by Fe(III), Cu(I), Cu(II) and Co(II) transition metal ions. Due to solubility incompatibilities, Fe(II) decomposition studies could not be carried out. Copper ions had similar decomposition profiles to the studies carried out on t-butyl perbenzoate, i.e. Cu(I) ions readily decomposing the peroxide and Cu(II) ions showing no activity. The inactivity of Cu(II) was overcome by the addition of accelerators, with all three resulting in immediate decomposition. The cleavage of the peroxide bond by Fe(III) ions was much slower than the other two organic peroxides, resulting in less than 10 % decomposition after 120 min. The observed Co(II) decomposition profile showed predictable inactivity, with the 20 % reduction in peak height seen after a reaction time of 10 min not exceeded after 120 min. It was decided to observe further the decomposition profiles of Fe(III) and Co(II) ions following the addition of APH, THQ and DMPT, with and without the presence of weak acids.

2.3.3.1 Decomposition by Fe(III)

The effect of accelerators on the decomposition of benzoyl peroxide by Fe(III) is plotted in Figure 2-13. The addition of APH caused rapid peroxide decomposition with a 2:1 ratio showing a slightly increased rate of reaction, while the incorporation of saccharin or maleic acid did not alter the decomposition rate. THQ at a 1:1 ratio caused ready decomposition of the peroxide. An interesting response curve was observed when DMPT was added to the Fe(III) ions. Although the benzoyl peroxide was decomposed to nearly 70 % on addition of a 2:1 ratio of accelerator, a 1:1 ratio hindered any peroxide

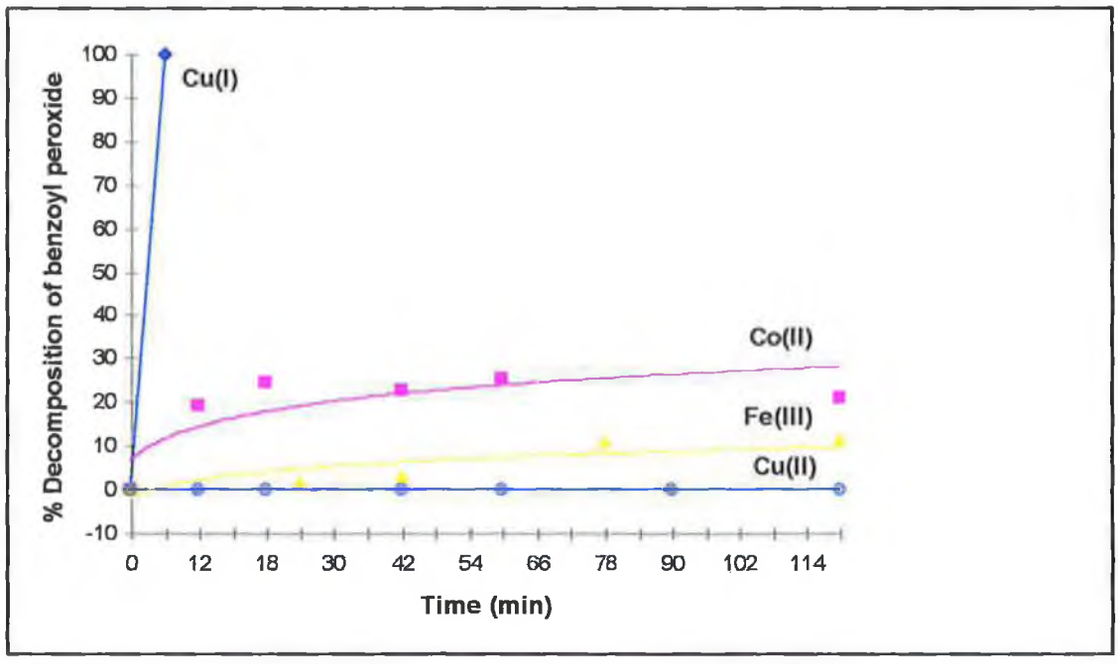

Figure 2-12 *Decomposition of benzoyl peroxide by metal ions.*

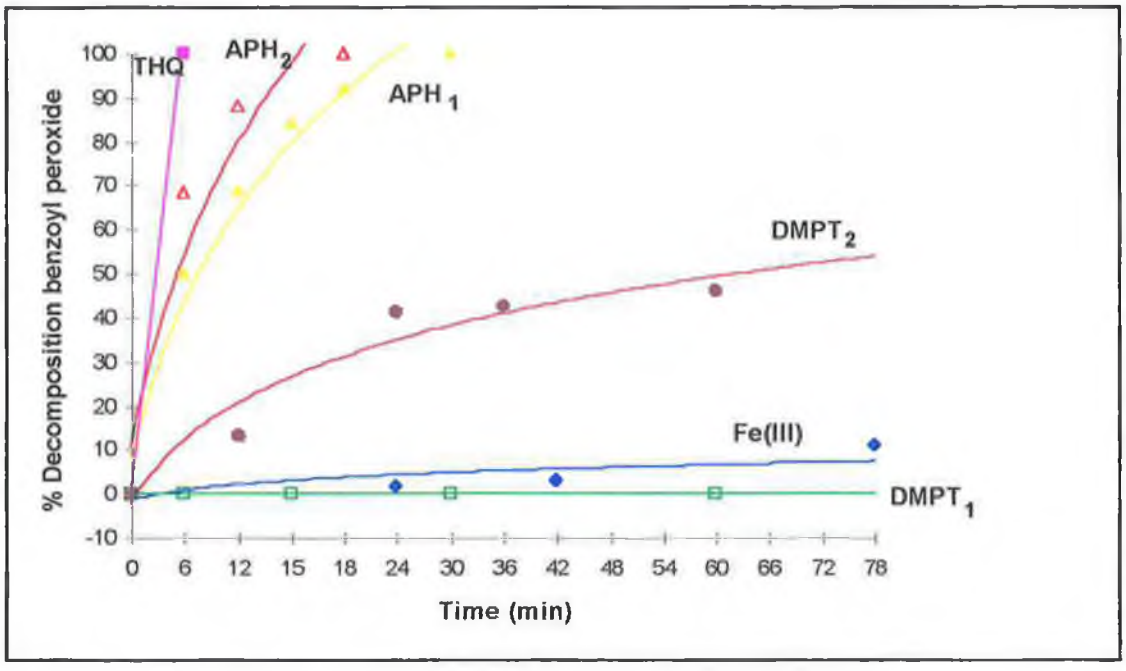

Figure 2-13 *Acceleration of benzoyl peroxide decomposition by Fe(III) in the presence of APH, THQ and DMPT. Subscripts indicate molar ratios to metal.*

decomposition. These experimental results lead to the suggestion that a 2:1 complex is formed between the DMPT and Fe(III), which is necessary for decomposition to occur. Beaunez *et al.*[11] reported in a study of polymer initiation by cumene hydroperoxide and copper saccharinate that the Cu(I) ions were complexed 2:1 with DMPT and these complexed ions were very strong reductants of the hydroperoxide in comparison to uncomplexed Cu(I) ions. Similar results were obtained by Raftery[12] in his study of the decomposition of cumene hydroperoxide by Cu(I), which indicated that Cu(I) is capable of decomposing cumene hydroperoxide only when present in a 2:1 complex with APH and DMPT. The apparent decomposition inhibition effect of saccharin and maleic acid was evident also in this study, with the degree of decomposition inhibition increasing with acid strength.

2.3.3.2 Decomposition by Co(II)

The limited decomposition of benzoyl peroxide by Co(II) ions could not be overcome by the addition of APH, with or without the presence of saccharin or maleic acid. APH in a 1:1 molar ratio completely inhibited the decomposition reaction, again pointing to a 2:1 complex formation between APH and Co(II). Further studies were thereby carried out on APH present in double concentration to metal ion. Although a 1:1 saccharin ratio and 2:1 saccharin and maleic acid ratios showed similar decomposition profiles, a 2:1 ratio of saccharin added to the APH-Co(II) mixture, inhibited the reaction completely.

Both THQ and DMPT very effectively decomposed the peroxide, the former resulting in 100 % decomposition in 2 min. DMPT in both ratios showed similar trends, with both reaction rates slightly slower than THQ. The incorporation of saccharin to the reaction mixture in a 1:1 ratio slowed the reaction to 80 % and in a 2:1 ratio, inhibited the

reaction completely. Maleic acid showed a great inhibitory effect with both ratios reducing decomposition to lower than 20 %.

2.4 CONCLUSIONS

The results of this investigation highlight the intricacy of the chemistry involved in the curing of anaerobic adhesives, and it is obvious that generalisation of reaction mechanisms is not possible. The main influence of transition metal ions is from the lower oxidation state, which reacts with organic peroxides to initiate polymerisation by a free radical mechanism. Certain acids, such as saccharin and maleic acids, liberate these transition metal ions from the substrate surface; however, the quantity of metal and its oxidation state depends on the acid used.[15] Accelerators reduce metals from their inactive higher oxidation state to the active lower one.

In this study of the initiators, t-butyl perbenzoate, t-butyl hydroperoxide and benzoyl peroxide, it was found that decomposition is greatly catalysed by Fe(II) ions, whereas Fe(III) ions are less reactive, with the presence of accelerators resulting in rapid decomposition. In the case of t-butyl perbenzoate and benzoyl peroxide, Cu(I) causes immediate decomposition, with the rate being slower for t-butyl hydroperoxide. In all three cases, Cu(II) was inactive and relied heavily on complexation with APH, THQ or DMPT to become active. The generation of free radicals by Co(II) was very slow and the incorporation of accelerators did not affect the rate of reaction to any great extent.

Maleic acid reduced the rate of peroxide decomposition when added to most accelerator-metal complexes, with the exception of the decomposition of t-butyl perbenzoate by Cu(II) with THQ in a 1:1 ratio to metal, where activation occurred due to peroxide

protonation. Saccharin also had an overall curtailing effect on decomposition rates; however, it did act to accelerate the decomposition of t-butyl perbenzoate by THQ-Cu(II) and DMPT-Cu(II) complexes, in addition to the decomposition of t-butyl hydroperoxide by DMPT-Cu(II) and benzoyl peroxide by APH-Fe(III).

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CHAPTER THREE

VOLTAMMETRIC ANALYSIS OF CLENBUTEROL IN BOVINE URINE

3.1 INTRODUCTION

Clenbuterol, $C_{12}H_{18}Cl_2N_2O$ (Figure 3-1), is a β-agonist drug used in the treatment of asthma in man, and has been under considerable investigation over the past few years. Clenbuterol, and other β-agonists promote muscle growth and reduce body fat in cattle, thereby reducing the cost of animal production. However, clenbuterol residues which accumulate in animal tissues may have either an antiasthmatic or tocolytic effect in humans, as discussed by Meyer *et al.*[1] Hooijerink *et al.*[2] stated that "the increase in carcass weight of calves, orally treated with clenbuterol as a growth promoter, is about 10% without any change in food intake". Hooijerink has also pointed out some of the restrictions on the therapeutic use of clenbuterol in animals. In order to prevent exploitation of the growth promotion effects, efficient screening procedures and quantification techniques, which are sensitive enough to detect the very low concentrations of the drug in biological samples, are crucial.

To date, many methods for the detection of clenbuterol in routine analysis have been reported, including HPLC coupled with UV spectrometric,[2,3,4] mass spectrometric,[5,6,7] and electrochemical[8,9,10,11] detection, thin-layer chromatography with UV and mass spectroscopic[12]detection and gas chromatography.[13,14,15] None of these techniques, however, offer the very low limits of detection required for the ultratrace (sub ng/g) analysis of clenbuterol in biological matrices, following its use as a growth promoter.

These very low limits of detection for clenbuterol can only really be achieved at present using radioimmunoassay (RIA) or enzyme immunoassay (EIA). These

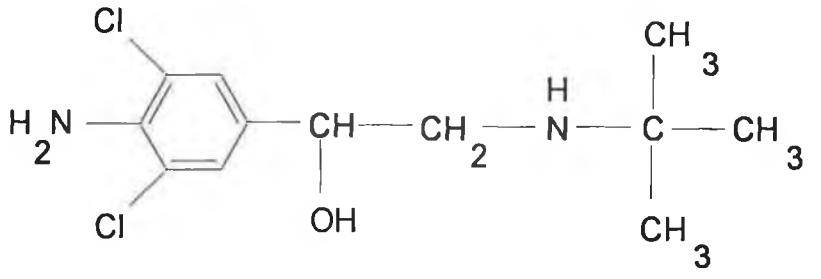


Figure 3-1 *Chemical structure of clenbuterol*

immunoassays, which have been used alone[16,17,18,19] or as detection techniques coupled to HPLC,[1,20] can detect clenbuterol in the sub ng/g range. However, such immunoassays require much reagent and sample preparation, and therefore have long analysis times.

In this chapter, the electrochemical behaviour of clenbuterol has been studied in detail, and the development of a sensitive electrochemical technique for the analysis of clenbuterol in biological samples has been examined. Bacon and Adams[21] have previously discussed the oxidation of aromatic amines at a carbon paste electrode and Qureshi and Eriksson[11] has briefly reviewed the electrochemical behaviour of clenbuterol at bare carbon paste electrodes. A comparison was also made in this chapter between the electrochemical behaviour of clenbuterol at bare carbon paste and a Nafion-modified carbon paste electrode. Nafion has been often used in the preparation of modified electrodes owing to its many advantageous chemical and physical properties, such as electrochemical inertness, insolubility in water,

hydrophobicity, and chemical and thermal inertness, which all lead to it possessing almost ideal properties as a chemical modifier.[22] This perfluorosulphonated cation-exchange resin can be used to selectively preconcentrate positively charged molecules through electrostatic interaction due to the hydrophilic negatively charged sulphonato groups in the polymer structure, whereas its ionic selectivity for hydrophobic organic cations is achieved through interactions with the hydrophobic fluorocarbons of the film. A very thin film of Nafion is ample to offer minimal obstruction to the diffusion of the analyte to the electrode surface, while at the same time preventing adsorption/desorption processes of organic species in biological fluids.[23] The use of Nafion-modified CPEs for the detection of other β-agonists has been examined by Boyd *et al.*[24] As with the β-agonists salbutamol, fenoterol and metaproterenol, clenbuterol displayed an increased response in signal with the Nafion-modified electrode. Various investigations showed that clenbuterol accumulates linearly at the Nafion-modified electrodes, therefore promising the detection of low concentrations of clenbuterol.

Cyclic voltammetry (CV) is often the first experiment performed in the development of an electroanalytical technique to investigate electrochemical processes occuring at the electrode surface. It is based on the continuous variation of the potential that is applied across the electrode-solution interface and a measurement of the resulting current. In CV, the current is measured during a potential scan (applied linearly from an initial value E_i to a final value E_f) at a constant scan-rate (υ). Kissinger and Heineman[25] has described how a potential-time excitation signal for CV causes a sweeping of the working electrode potential

back and forth between two predetermined values (E_i and E_f), known as the *switching potentials*, which can be ended after the first scan or continue for any number of scans. Each cycle consists of:

(i) Positive scan from $+E_i$ to $+E_f$.

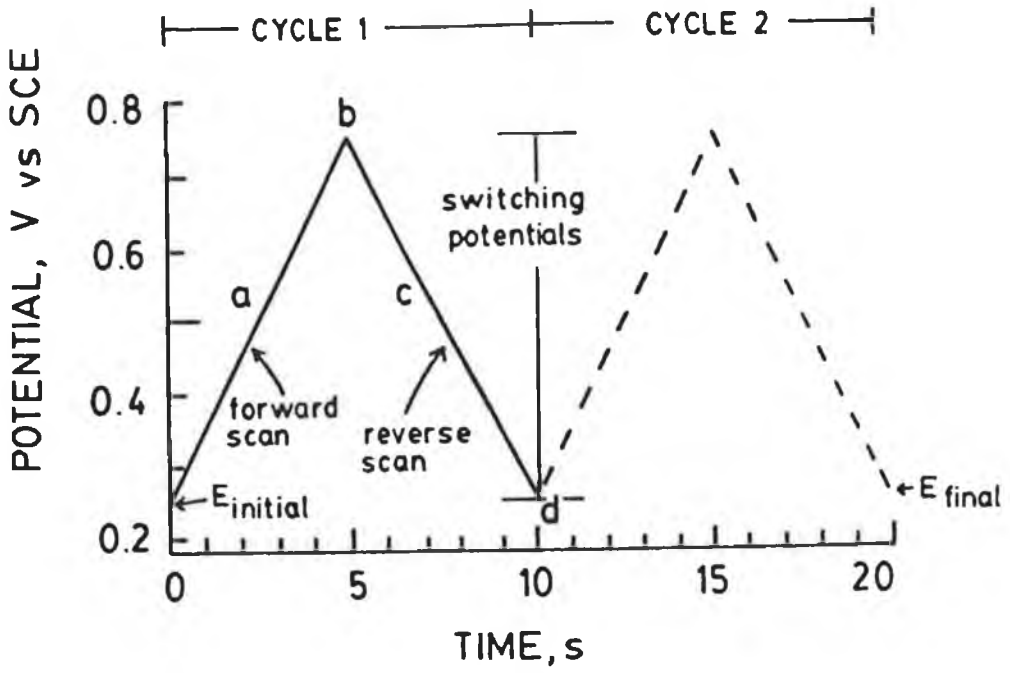
(ii) Scan direction reversed at switching potential $+E_f$.

(iii) Negative scan from $+E_f$ to $+E_i$.

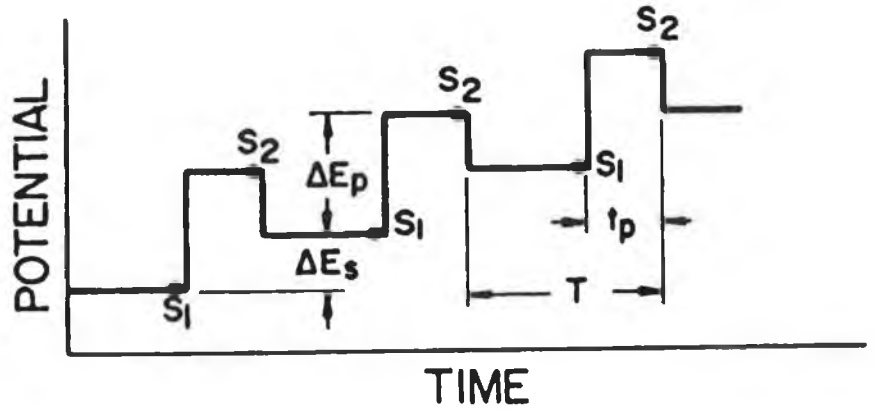
(iv) End of first cycle.

A typical potential-time excitation signal for cyclic voltammetry is shown in Figure 3-2(i). A particular scan profile is chosen so that no electrolysis occurs at E_i, and during the scan the applied potential becomes sufficiently positive to cause oxidation of the species at the electrode surface. Oxidation gives rise to an anodic current which increases until all the species at the electrode surface has been oxidised. The decay of the current is a result of the lack of unoxidised analyte surrounding the electrode surface. In the case of redox couples, oxidation continues (even after switching to a negative scan) until the applied potential is sufficiently negative to cause reduction of the oxidised species, resulting in a cathodic current.

The above example points out that CV can quickly generate a new species on the initial positive scan, with the reverse (and subsequent) scan(s) portraying the type of species formed. In the case of a redox couple, oxidation occurs on the initial



(i)



(ii)

Figure 3-2 *Potential-time excitation signals for* (i) *Cyclic Voltammetry and* (ii) *Differential Pulse Voltammetry.*[25]

positive scan, and reduction of the oxidised form on the reverse negative scan. Adams[26] states that "in the absence of *coupled chemical reactions*, continuing cycles merely gradually alter the concentration profiles near the electrode surface". He continues to point out that there is a steady decrease in peak height until a steady state is reached after about 5-10 cycles. For reversible reactions, there is only a few millivolts in the difference between initial and steady state conditions. Quasi-reversible couples show a greater separation in peak potentials (E_p), with irreversible reactions displaying complete separation in both processes (anodic and cathodic). Whether a process is reversible, quasi-reversible or irreversible depends on its rate of charge transfer, which can be altered by background electrolyte and electrode material.

CV is a very effective method of examining both electrochemical *and* chemical processes occuring at an electrode surface. With a complex electrode reaction, there is a significant difference between the first and subsequent scans. Such is the case for an irreversible oxidation of an organic molecule at an electrode surface, caused by a *chemical follow-up reaction*.[27] This mechanism involves the addition/removal of an electron from the molecule to create a new redox state which may be chemically reactive with another component of the solution. Follow-up reactions have been divided into four main classes: EC (*following chemical reaction*) mechanisms, ECC (*half-generation*) mechanisms, ECE mechanisms and, finally, CE (or *preceding chemical reaction*) mechanisms. CV can be used to determine the type of follow-up reaction occuring, therefore resulting in information on *both* chemical and electrochemical processes.

Qualitative information is obtained from cyclic voltammograms by measuring peak currents (i_p) and the potentials at which these peaks appear (E_p). CV is restricted to being basically a qualitative technique due to the difficulty in obtaining accurate peak current measurements; a result of the changing baseline as the scan number increases. Kissinger and Heineman[25] has stated that "CV is the most versatile electroanalytical technique thus far developed" due to its good sensitivity, reproducibility, and the little time required to scan in a potential range for any oxidisable or reducible species in solution.

Quantitative measurements (such as concentration) are usually attained using more sensitive techniques, such as differential pulse voltammetry (DPV). This technique involves application of a dc voltage ramp of 2-10 mV/sec to the working electrode (Figure 3-2(ii)), with a small fixed potential pulse (in the range 10-100 mV) applied, just before which a current sample is recorded (s_1). A second sample is taken at the end of the pulse (s_2), and the difference between the two currents is calculated. The pulse width (t_p) has traditionally been around 50 msec. With increasing pulse size comes increasing sensitivity but increased charging currents and broader peaks (a result of slower electron-transfer kinetics), resulting in a decrease in resolution.[25] For most analysis, a pulse size of 50-100 mV is optimum; above this there is no large increase in response. Unlike normal pulse voltammetry, the result is a peak, not a wave (as the measured signal is the difference in current measured before and after application of the pulse), and the pulse steps are clearly visible. Detection limits with this technique are as low as 1×10^{-8} M for routine purposes and lower under ideal circumstances.

One of the main advantages of differential pulse voltammetry is the increase in faradaic-to-charging current ratio, therefore allowing detection of lower concentrations. One of the main disadvantages of DPV, however, is slow electron-transfer kinetics, which lowers the response (peak current vs sample concentration) and therefore increases the detection limit, as well as increasing the peak width. Also, slow electron-transfer kinetics tends to make an electrochemical process dependant on other species which may be present in solution when analytes are detected in environmental samples, where variation of the matrix can alter the DPV analyte response.

3.2 EXPERIMENTAL

3.2.1 Reagents and materials

1 M, 0.1 M and 0.01 M perchloric acid (70%; Riedel-de Haën, Analar grade) solutions were prepared using deionised water obtained by passing distilled water through a Milli-Q water purification system (Millipore).

Glacial acetic acid and boric acid were obtained from Panreac, Barcelona. Purchases from Sigma included chloroacetic acid and nitric acid (60%). Clenbuterol was a gift from The National Food Centre, Dublin.

Britton-Robinson buffer was prepared using 11.5 ml acetic acid (99%), 13.5 ml phosphoric acid (85%) and 12.44 g boric acid per litre. 2 M sodium hydroxide was used to adjust the pH of the solutions from pH 2-12. All solutions were prepared

using de-ionised water and stored at 4°C. The Nafion stock solution (5%) was purchased from Aldrich. Solutions were prepared in a 1:1 water-isopropanol (Riedel-de Haën, spectranalar grade) dilution of the stock solution. Spectroscopic grade graphite powder (Ultra carbon, Dicoex, Bilbao) and Nujol (Riedel-de Haën, spectranalar grade) were of analytical grade and carbon paste was prepared by mixing 5 g of carbon with 1.8 ml of Nujol.

3.2.2 Apparatus

Cyclic voltammetry (CV) was carried out using a Metrohm VA Scanner E612 and VA detector E611, coupled to a Graphtec WX4421 recorder.

Differential pulse voltammetric analysis of the clenbuterol oxidation peak appearing at approximately 1.10 V was carried out using a Metrohm Polarecord differential stripping analyser.

Analysis of the oxidation peak of the quasi-reversible couple at 0.42 V by DPV was conducted with a BAS CV-50 voltammetric analyser linked to a Taxan 788 PC.

All experiments were carried out in a glass cell designed to suit a three electrode potentiostatic unit. In all cases, a bare/Nafion-modified electrode served as the working electrode, and the counter electrode was a platinum electrode. An Ag/AgCl reference electrode was used with the Metrohm system and a saturated KCl reference electrode was used with the BAS system.

Spectroelectrochemistry experiments were performed using an EG & G Princeton Applied Research Model 362 scanning potentiostat attached to a Shimadzu

UVVIS-NIR spectrometer. The system was controlled by a UV-3101PC software package for Windows. The working electrode was a platinum gauze electrode, the reference a silver wire and the counter electrode a platinum wire, each of which was connected to the potentiostat. The electrodes were placed in a 2 ml cell, especially designed to fit the system, which was placed in the path of the optical beam.

3.2.3 Preparation of working electrodes

Carbon paste was prepared by thoroughly mixing (approx. 30 min) 5 g of graphite powder with 1.8 ml of Nujol in a mortar and pestle.

The carbon paste electrode (CPE) was thoroughly cleaned before packing with new paste by sonicating in dichloromethane to remove all the paste from the previous experiment. After being dried with tissue paper, the electrode was then packed tightly with carbon paste and the surface was smoothed on a cardboard sheet until there were no cracks apparent and the surface had a shiny appearance.

The modified electrode was prepared by pipetting 10 μl of the appropriate percentage Nafion solution onto the electrode surface and allowing to dry for 15 min under a domestic hairdryer. Nagy *et al.*[28] has demonstrated that this heat-pretreatment step improved both the electrochemical performance and the stability of the Nafion-modified electrode, with increased background currents where this step was omitted.

3.2.4 Methods

3.2.4.1 Electrochemical behaviour of clenbuterol at bare- and Nafion-modified CPEs

3.2.4.1.1 Cyclic voltammetry at bare carbon paste electrodes

To study the electrochemical processes of clenbuterol at various pH values, $1x10^{-5}$ M solutions of clenbuterol were prepared in Britton-Robinson (BR) buffers of pH 2-12. The CV behaviour of clenbuterol at bare carbon paste electrodes was then studied using a scan rate of 50 mV/sec in the potential range -0.20 V $\rightarrow$ +1.35 V. After each new electrode had been prepared, it was placed in the background solution and the solution stirred for 5 min at -0.20 V. A background scan was always taken in order to ensure that there was no contamination from the previous experiment. After injection of the clenbuterol, the solution was stirred for 5 s to ensure thorough mixing of the drug within the solution.

3.2.4.1.2 Spectroelectrochemistry of clenbuterol at a platinum electrode

Spectroelectrochemistry experiments were carried out by scanning a blank background solution, at each oxidation potential, in the UV range 220 nm - 350 nm and subtracting the spectra from the sample spectra. Scans were made at a slow, medium or fast scan-rate, as chosen with the software. UV spectra, consisting of four consecutive scans, were obtained for a clenbuterol cell concentration of 5.6×10^{-3} M made up 0.1 M perchloric acid at each of 0.00 V, 0.30 V, 0.60 V, and 0.80 V. At an applied potential of + 1.20 V, 12 scans were run to follow product formation. Spectra were also obtained at 0.60 V and 0.00 V

after clenbuterol oxidation. The electrodes were rinsed with deionised water following each experiment.

3.2.4.1.3 Cyclic voltammetry at Nafion modified carbon paste electrodes

Using $1x10^{-5}$ M clenbuterol, a pH study (same conditions as above) was carried out using Britton-Robinson buffer solutions of pH 2-12. After having established that linear accumulation occurs, accumulation studies were carried out at various background compositions and pH values. Once a pH for analysis had been chosen, studies were carried out to establish the relevant Nafion concentration, accumulation potential, scan rate and stirring speed to give rise to the highest peak current for this pH value.

3.2.4.2 Differential pulse voltammetric detection of clenbuterol in biological samples

All experiments were carried out at room temperature (approximately 25°C). It was unnecessary to remove dissolved oxygen from the solutions before experimental analysis. After preparation, the electrode was conditioned by stirring at 0.00 V in the background electrolyte for 5 min before a background scan was recorded. On injection of clenbuterol, the solution was stirred at 0.00 V (established as the optimum deposition potential) for the appropriate preconcentration time. After a 5 s rest period, an anodic scan was made from 0.00 V to +1.3 V and the peak current was measured from the background baseline.

For the analysis of the oxidation peak at +0.42 V, clenbuterol was initially preconcentrated onto the Nafion membrane at 0.00 V, followed by conversion to the clenbuterol oxidation product by stirring under electrolysis at +1.05 V for the appropriate conversion time. The oxidation of the quasi-reversible couple could then be seen by scanning from 0.10 V to +0.60 V, with the peak current being evaluated as before.

3.2.4.2.1 Electrode Renewal

The rate-controlling process within the Nafion membrane was proven in this research (by medium-exchange experiments) to be the diffusion of the clenbuterol through the modified layer to the surface of the electrode. Once the oxidation potential of clenbuterol (+1.10 V) has been passed, the follow-up chemical reaction product is formed, and subsequent scans from 0.00 V to +1.20 V revealed no oxidation peak without further preconcentration of clenbuterol onto the Nafion membrane. At the low concentrations of clenbuterol present in biological samples, reproducibility tests indicated that the adsorbed products formed after clenbuterol oxidation did not affect the preconcentration of clenbuterol onto the Nafion membrane, indicating that adsorbed products are not a problem in this analysis.

However, the adsorption of the quasi-reversible couple proved to be a problem when analysing the oxidation peak at +0.42 V. It was found that stirring the electrode in NaOH under electrolysis at -0.60 V for 120 s was sufficient to remove the adsorbed material. 0.10 M NaOH was established as the optimum cleaning solution, with 1 M NaOH destroying the electrode. Lower cleaning potentials also

proved hazardous to the electrode. After cleaning, the electrode was placed in a clean background electrolyte and stirred for 10 s before scanning from +0.10 V to +0.60 V. If adsorbed products remained on the surface, the cleaning process was repeated again. With increasing clenbuterol concentrations, more adsorbed products are produced on the electrode surface which may require longer cleaning times. This renewal procedure was found to be reproducible when tested with 1 x 10^{-7} M clenbuterol, with a standard deviation of 2.1 % when n = 7.

3.2.4.2.2 Urine Sample Clean-up

Urine clean-up was carried out using liquid/liquid extraction followed by a selective mixed-mode solid-phase extraction procedure, as developed by Collins *et al.*[29] To 5 ml of urine was added 4 ml of acetate buffer, pH 4.80, plus 0.66 ml of *suc d'Helix pomatia* enzyme (this enzyme deconjugation step was taken as a precaution, although it has been reported that clenbuterol does not form conjugates;[29] this step would also be required for a multi-residue analysis of β-agonists in urine). This was incubated overnight at 37 °C. After having saturated the solution with 3.47 g of NaCl, the pH was adjusted to pH 10.5 with 1 M NaOH. The liquid/liquid extraction step involved the addition of 10 ml of ethyl acetate-propan-2-ol (3:2), with the test tubes being subsequently shaken for 10 min, followed by centrifugation at 2000 rpm for 15 min. The organic phase was withdrawn and evaporated at 40 °C under nitrogen and the ethyl acetate-propan-2-ol extraction step repeated. Finally, 6 ml of phosphate buffer, pH 6.0, was added and the solution was vortexed for 1 min.

The mixed-mode solid-phase XtractT extraction column was conditioned by the addition of 5 ml methanol, 5 ml water and 2 ml phosphate buffer (pH 6.0, 0.1 M). The urine sample was then applied at a rate of 2 ml/min and the eluate dried under vacuum for 10 min. After having been washed with 5 ml water and 5 ml 100 % methanol, the column was eluted with 5 ml of 3 % ammonia-methanol solution. The 100 % methanol removes organic compounds present in the urine. If this wash step was carried out on a reversed-phase solid-phase extraction column, the analytes would be removed with the interferences. However, the cation-exchange properties of the column ensure that the β-agonists remain on the column until they are eluted with the ammonia-methanol solution. The solvent was evaporated under nitrogen as before and reconstituted into 300 µl methanol-water (1:1). This clenbuterol extraction procedure displays recoveries of greater that 77 %.[29]

3.3 RESULTS AND DISCUSSION

3.3.1 Electrochemical behaviour of clenbuterol

3.3.1.1 Electrochemical behaviour of clenbuterol at bare carbon paste electrodes

The electrochemical behaviour of clenbuterol at bare CPEs was found to be similar to that reported by Qureshi and Eriksson,[11] who carried out CV in both phosphate buffer, pH 3.97 and 4.00, and acetate buffer, pH 3.98. Their studies showed that clenbuterol is irreversibly oxidised at high potentials, this process being followed by a chemical follow-up reaction, which they state was either an EC

or an ECE process. Figure 3-3 shows the electrochemical behaviour of clenbuterol at bare carbon paste electrodes. These results show that clenbuterol undergoes an ECE process at carbon paste electrodes. This mechanism involves the

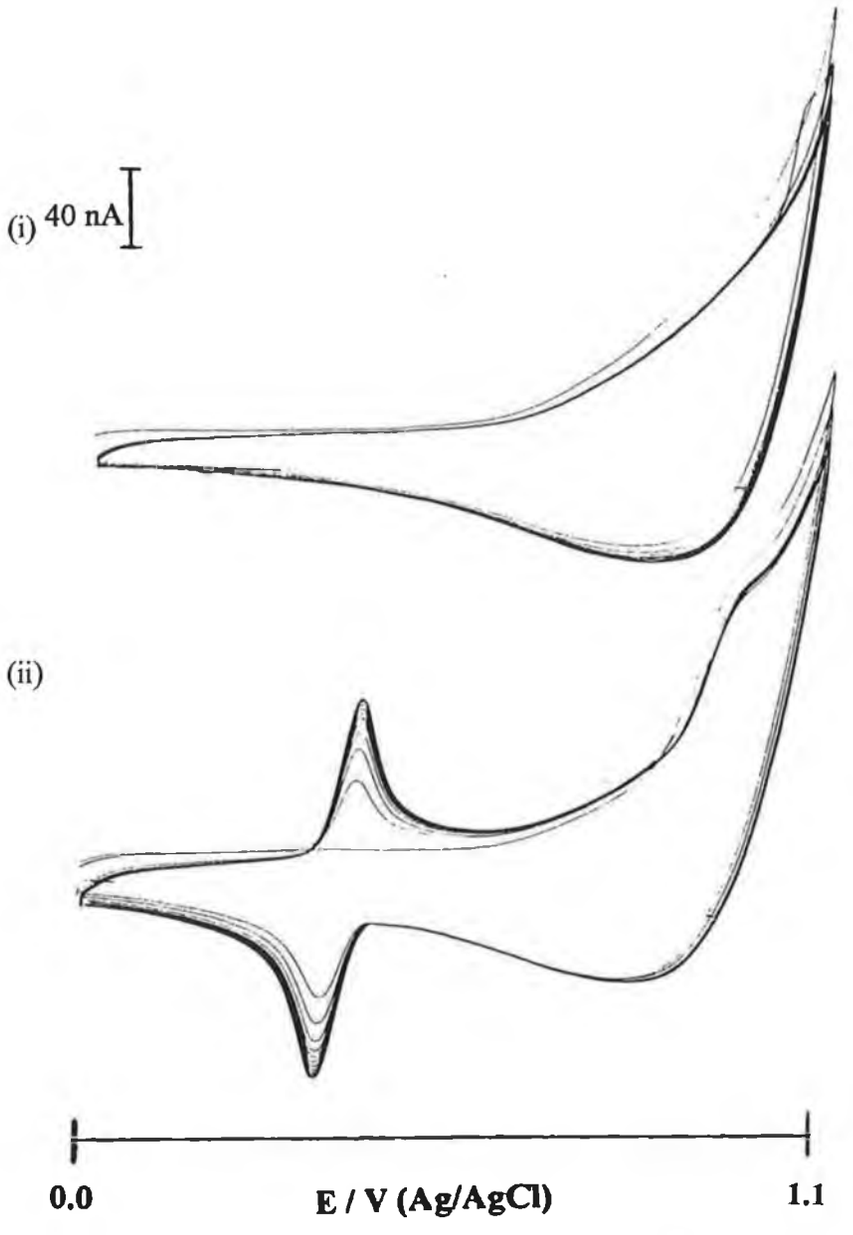


Figure 3-3 *Electrochemical behaviour of 1 x 10^{-5} M clenbuterol in 0.1 M perchloric acid at a bare carbon paste electrodes, showing* (i) *voltammogram of background electrolyte and* (ii) *oxidation of clenbuterol at +1.00 V with oxidation product being reduced on the reverse scan at +0.45 V. Scan range: 0 V → +1.1 V, scan rate: 50 mV/s.*

electrochemical generation of a product which then reacts with another constituent of the solution to form a product which is more easily oxidised/reduced than the original "starting" compound and so is immediately electrolysed.[25] Figure 3-3(i) shows the voltammogram of the background electrolyte (0.1 M perchloric acid). In Figure 3-3(ii) the oxidation of clenbuterol is evident at 1.00 V. An obvious feature of this scan is the non-appearance of a corresponding reduction wave on the reverse scan, which would arise if the compound was reversibly oxidised at the electrode surface. Another feature is the absence of an oxidation peak at 0.475 V on the initial scan, which is evident in subsequent ones. From this it can be deduced that clenbuterol is irreversibly oxidised, with the irreversibility being due to a chemical follow-up reaction of the oxidation product. This means that the oxidation product (formed at approx. 1.00 V) undergoes a chemical reaction to a second product which exhibits a quasi-reversible couple at considerably lower positive potentials. This was proven by scanning (with a new electrode) to a potential lower than the oxidation potential of clenbuterol. The absence of reduction waves on the reverse scan, and subsequent reoxidation waves on the forward scan, indicates that the quasi-reversible couple is due to a product formed at higher potentials. Figure 3-4 shows the electrochemical behaviour of clenbuterol at bare carbon paste electrodes as the pH is changed from acidic to basic pH values. Scan 3-4(i) was carried out in an acidic medium, with the accumulation of the quasi-reversible couple onto the surface clearly visible. Figure 3-4(ii) represents the electrochemical behaviour in BR buffer, pH 6.0. From this one can see that at almost neutral pH, very low traces of quasi- reversible couple are apparent, as well as a shift in oxidation potential to less positive potentials.

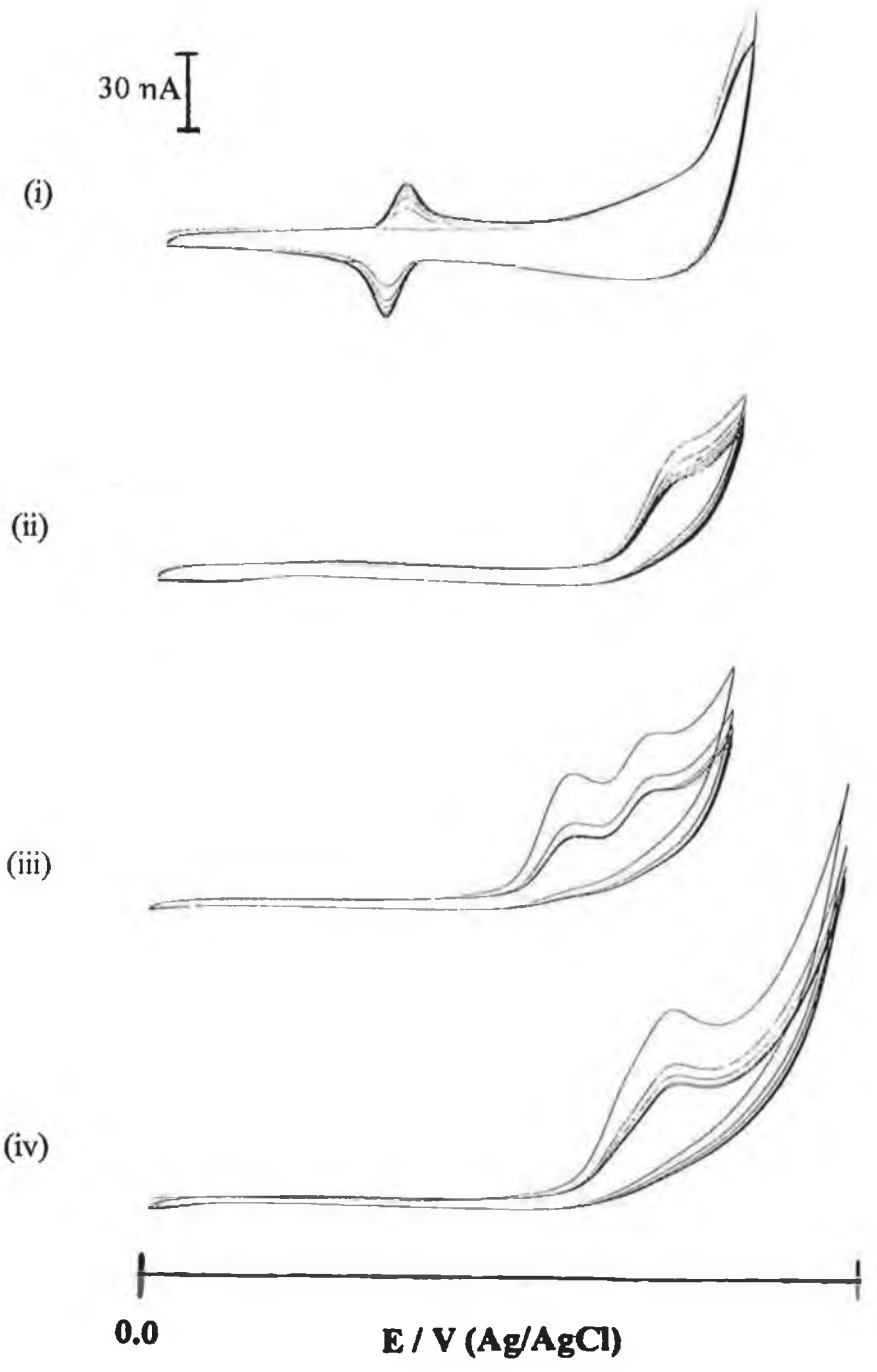


Figure 3-4 *Effect of variation of pH on electrochemical behaviour of 1 x 10^{-5} M clenbuterol:* (i) *1 M perchloric acid,* (ii) *BR buffer, pH 6.0,* (iii) *BR buffer pH 10.0 and* (iv) *BR buffer pH 12.0. Conditions as for Figure 3-3.*

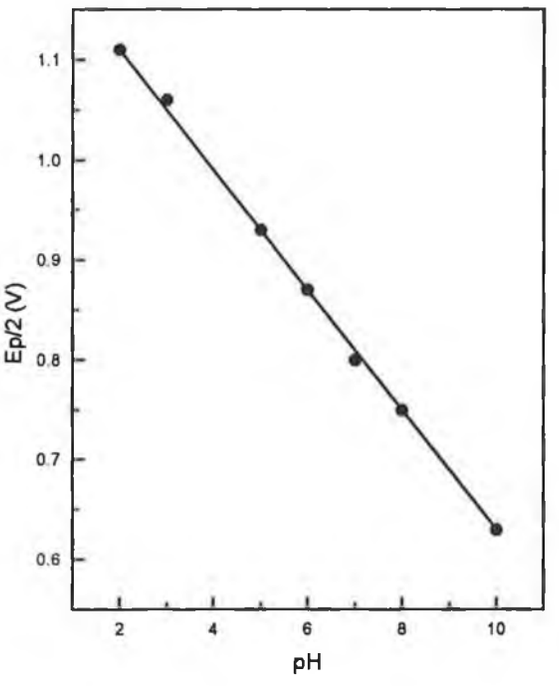


Figure 3-5 *The relationship between half-peak potential and pH for 1 x 10^{-5} M clenbuterol at a bare carbon paste electrode (scan rate = 50 mV/s).*

In addition to the main oxidation peak, a second clenbuterol oxidation peak was observed between pH 7 and 10 (Figure 3-4(iii)), which is probably related to the oxidation of the second amino group on the molecule. There were no traces of chemical follow-up reaction products at this pH. The single peak appearing at pH 12.0, shown in Figure 3-4(iv), indicates that this is the only process which exists at this pH.

Figure 3-5 represents the relationship of half-peak potential and pH for clenbuterol at a bare CPE. Linearity of $E_{p/2}$ vs pH for clenbuterol was observed in the pH

range 2-12 with a negative slope of 60.9 mV per pH unit, corresponding to a Nernstian behaviour involving a process with an identical number of protons and electrons. This trend was also observed by Boyd *et al.*[24] in the electrochemical study of fenoterol. An experiment carried out under the same conditions as the above study, but using 0.1M perchloric acid as the background electrolyte, displayed a shift in oxidation potential. This shift is likely to be due to the influence of the buffer solution on clenbuterol, suggesting that there is an interaction between the molecule and one of the constituents in the Britton-Robinson buffer.

Further studies of the quasi-reversible couple over the pH range 2-12 show that the peaks are more defined and show a current increase as the pH moves towards more acidic values. The amount of chemical product formed is therefore very pH dependant. This is further evidence of the theory that the quasi-reversible couple may be due to "head-to-tail radical coupling" of para-substituted anilines, with the resulting aminodiphenylamines being oxidised at less positive potentials, as described by Bacon *et al.*[21] They saw that the amount of "head-to-tail" coupling increases with acidity, and decreases rapidly as the pH moved towards neutral values. In the case of clenbuterol, only a trace of product is seen above pH 3.

3.3.1.2 Spectroelectrochemistry of clenbuterol

To prove that the quasi-reversible couple was indeed formed by a chemical follow-up reaction occuring after clenbuterol oxidation, spectroelectrochemical experiments were carried out on 1.5 x 10^{-5} M clenbuterol with a platinum gauze electrode. Cyclic voltammetry proved clenbuterol to be oxidised at + 1.20 V at a

platinum working electrode in 0.1 M perchloric acid. Spectroelectrochemistry involves the coupling of a spectroscopic technique to an electrochemical experiment to monitor the absorption of a compound in a set wavelength range in the ultra-violet (UV), infrared (IR) or near-infrared (NIR) region of the spectrum, when a fixed potential is applied. This technique can be used to monitor the electrogeneration of a product and many mechanistic characteristics of electrode reactions have been studied,[30] along with analytical applications.[31]

Multiple scans were initially made in the range 220 nm to 350 nm at 0.00 V to reveal the wavelength of maximum absorption for clenbuterol to be 243 nm with absorption to a lesser extent at 296 nm in the acidic background. As the applied potential was increased stepwise from 0.00 V to 1.20 V in 0.20 V increments, no change in the absorption spectra was evident. However, over the 20 consecutive scans carried out at + 1.20 V, shown in Figure 3-6(i), it is evident that as clenbuterol is being oxidised (as seen in a decrease in absorption of clenbuterol at 243 nm), a product which absorbs at 277 nm is being formed. This product turns the solution yellow, in contrast to the colourless solution before product production.

Figure 3-7 compares the spectra of clenbuterol oxidation at 0.60 V (i) before and (ii) following oxidation at + 1.20 V. The product absorbing at 277 nm is only present in the spectrum obtained after oxidation at + 1.20 V, indicating that it is, as predicted, a result of a chemical follow-up reaction to clenbuterol oxidation. This product was removed from the electrode surface when the applied potential was maintained at 0.00 V in blank 0.1 M perchloric acid, which was used in the

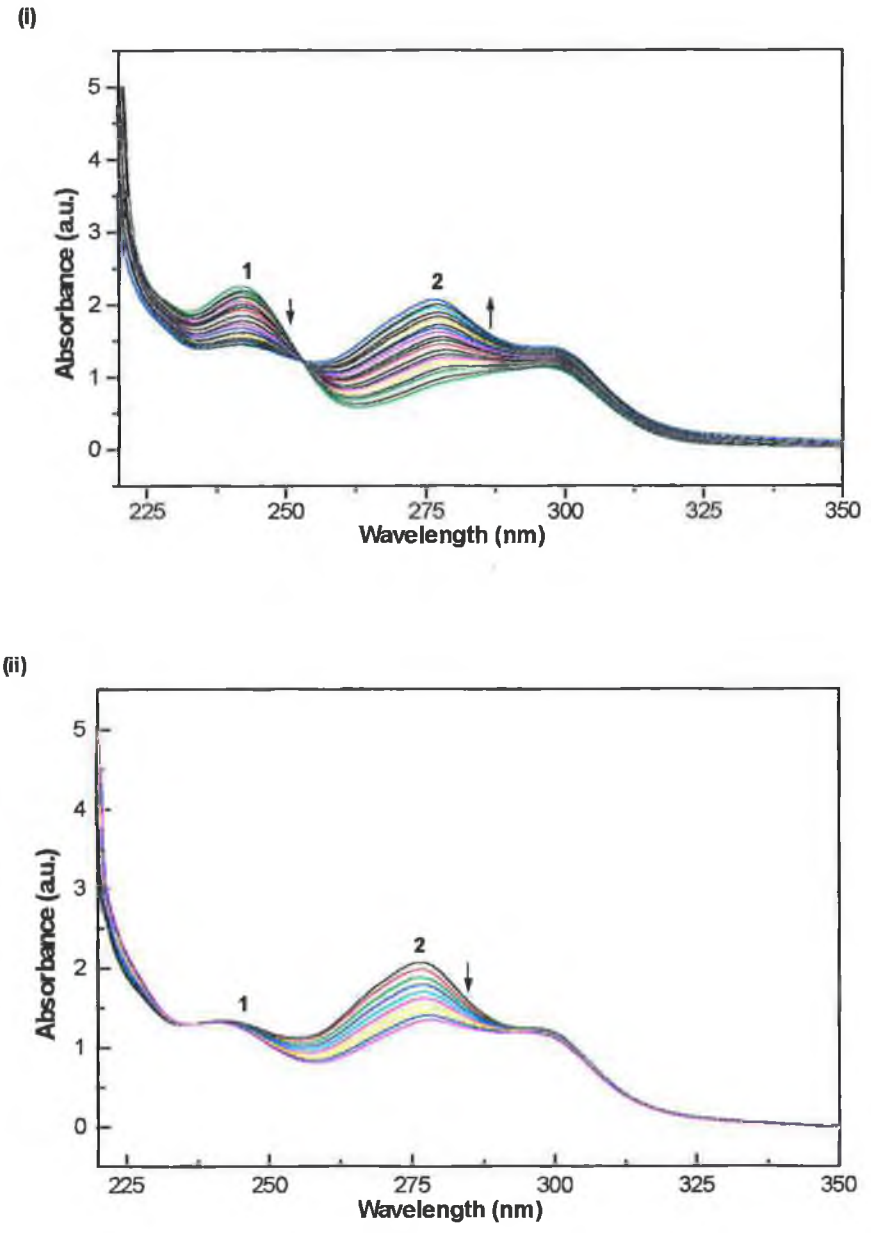


Figure 3-6 *UV spectra of 1.5 X 10^{-5} M clenbuterol obtained using spectroelectrochemistry showing maximum clenbuterol absorption at 243 nm at* (**1**) *and with the follow-up reaction product absorbing at 277 nm* (**2**). *Application of a fixed potential of + 1.20 V, as seen in* (i)*shows the reduction in clenbuterol absorption as product is formed. Holding the potential at 0.00 V in blank 0.1 M perchloric acid removes this product from the electrode surface* (ii).

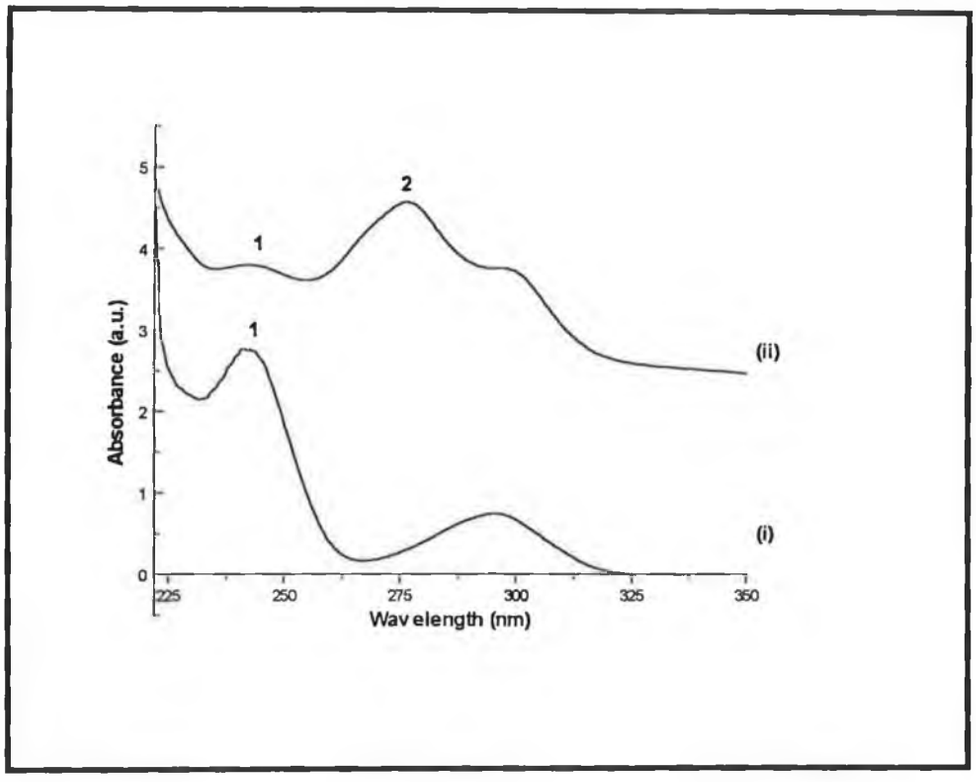


Figure 3-7 *Comparison of clenbuterol absorption spectra at 0.60 V* (i) *before and* (ii) *after oxidation at + 1.20 V showing* (1) *clenbuterol absorption maximum and* (2) *product absorption. Conditions as for Figure 3-6.*

development of an electrode renewal technique in the study of the accumulation of the product onto the electrode surface.

After the research reported in this chapter had been completed, McGrath *et al.*[32] attempted to further characterise the clenbuterol oxidation process at a porous carbon electrode using liquid chromatography with coulometric detection. A mass spectrum obtained for clenbuterol (oxidised at 880 mV, pH 5.0) displayed a fragmentation pattern indicative of a compound containing 4 chlorine atoms,

resulting from a dimer formed via an azo bond from the head-to-tail coupling of 2 clenbuterol radical cations formed after clenbuterol oxidation.

McGrath[32] obtained UV spectra for clenbuterol, similar to those shown in Figure 3-6, without electrolysis, at 880 mV and 1000 mV. The dimer absorbed at 295 nm and 285 nm for oxidation at 880 mV and 1000 mV, respectively indicating that 2 different electrode processes occur. The "shoulder" appearing at 265 nm in Figure 3-7(ii) can possibly be attributed to the further oxidation of the azo bond at higher potentials,[32] which could be confirmed by carrying out the spectroelectrochemical experiments at higher potentials for longer periods of time.

3.3.1.3 Electrochemical behaviour of clenbuterol at Nafion-modified electrodes

3.3.1.3.1 pH study

Using $1x10^{-5}$ M clenbuterol, a pH study was carried out using Britton-Robinson buffer solutions of pH 2-12 using a carbon paste electrode modified with a thin layer of 0.05 % Nafion. A background scan was carried out on the pre-treated electrode, followed by injection of 20 µl of 1 x 10^{-2} M clenbuterol into 20 ml of background electrolyte (i.e. 1 : 1000 dilution). After 5 sec stirring, the initial scan was recorded from 0.00 V to +1.30 V. Electrolysis was terminated once this scan was complete and the solution stirred, followed by multiscanning in the same range. Large increases in peak current were achieved for the Nafion-modified electrodes. Figure 3-8 shows the electrochemical behaviour of clenbuterol at Nafion-modified carbon paste electrodes. At all pH values, each initial scan

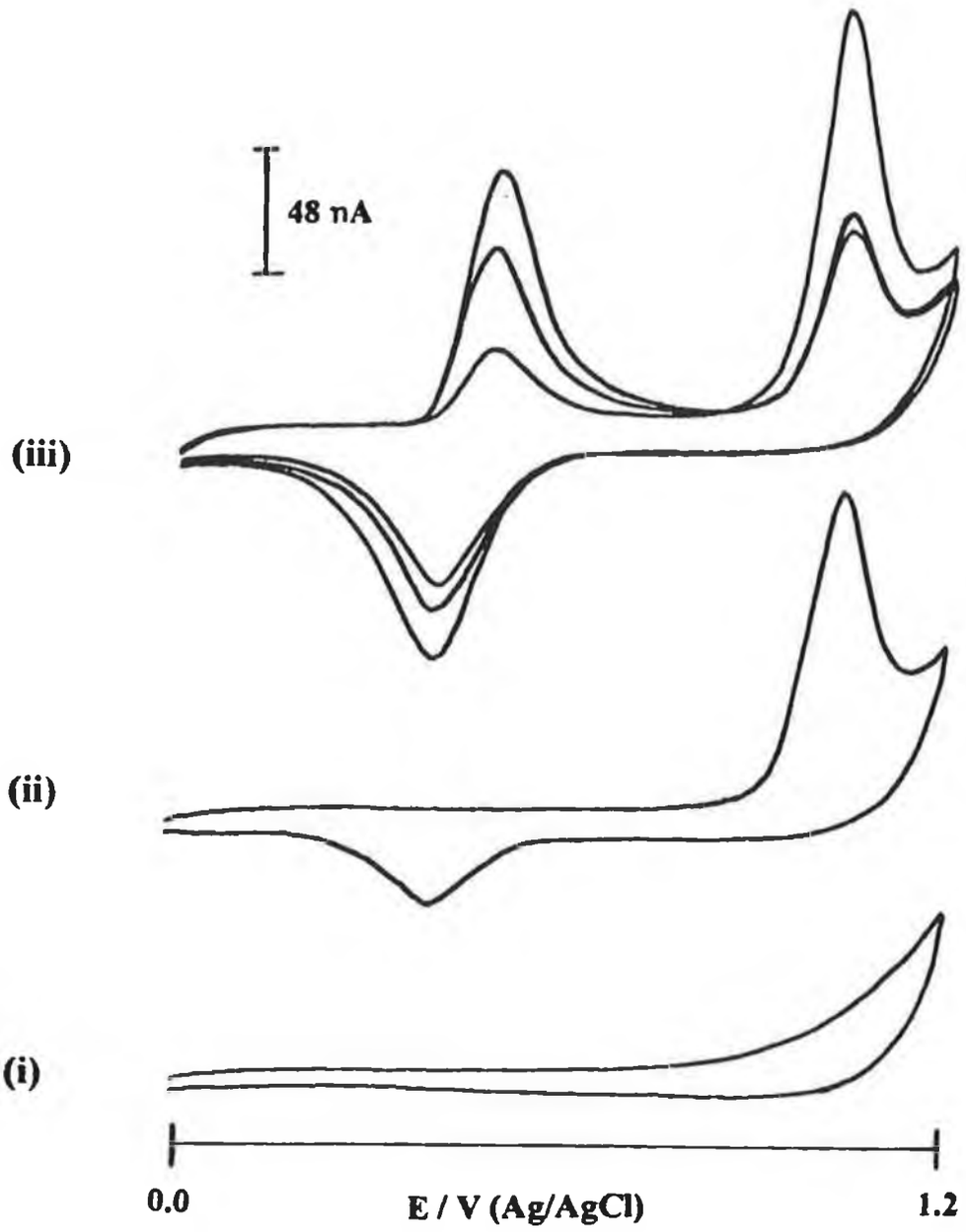


Figure 3-8 *Electrochemical behaviour of 1 x 10^{-5} M clenbuterol in 0.1 M perchloric acid at a 0.05 % Nafion-modified electrode:* (i) *background scan;* (ii) *initial scan on injection following a 5 s stirring; and* (iii) *multiscan showing clenbuterol irreversible oxidation and adsorption of product onto electrode surface as no of scans increases.*

revealed no quasi-reversible couple at low potentials, but a reduction peak was present on the reverse scan after the clenbuterol had been oxidised at +1.10 V, indicating that the reduction peak is due to a product of clenbuterol oxidation. On re-application of electrolysis, a further 5 s stirring was carried out to re-establish the diffusion layer and multiple scanning in the range 0.00V → +1.20V was carried out to study the accumulation behaviour on the Nafion film. From pH 2-6, the quasi-reversible couple displayed an increasing signal as the scan number increased. The signals decreased in intensity and clarity as the pH moved towards alkaline media, and from pH 7-12 no quasi-reversible couple was seen. This coincides with the pH at which the second process appears at bare carbon paste. The second process is not distinguishable at Nafion-modified carbon paste electrodes, but its presence was observed by a broadening of the main clenbuterol oxidation peak. This peak decreases in intensity after the second scan (regardless of pH) due to the fact that all the clenbuterol in the Nafion layer has been converted into the product.

Figure 3-9 shows that the half-peak potential of the clenbuterol oxidation peak remains constant between pH 1-5, but the oxidation potential was shifted to more negative values after pH 5 with a slope of -46 mV/pH. Across the pH scale, there was a considerable increase in peak current using a Nafion-modified electrode over an unmodified carbon paste electrode, which was even more significant as the acidity of the background electrolyte increased.

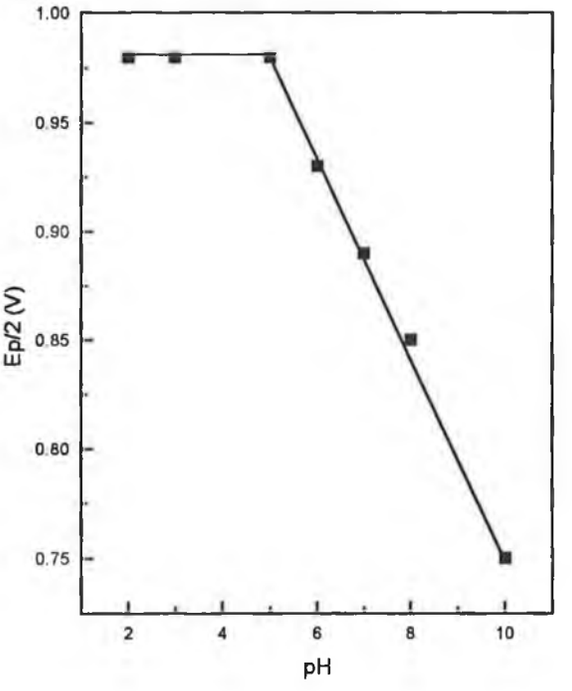


Figure 3-9 *The relationship between half-peak potential and pH for Nafion-modified carbon paste electrodes.*

3.3.1.3.2 Effect of pH and background electrolyte on accumulation

After having established that linear accumulation occurs, the effect of pH and background electrolyte on the accumulation behaviour of clenbuterol at Nafion-modified CPEs was investigated using a clenbuterol cell concentration of 1 x 10^{-6}M and accumulating at 0.00 V for regular time intervals up to 10 min. Accumulation onto the surface was carried out, under stirring conditions, by holding a potential for a specific accumulation time after which the stirring was stopped and the accumulated clenbuterol was stripped from the surface by scanning from 0.00 V to +1.30 V.

Experiments were carried out in 1 M, 0.1 M and 0.01 M perchloric acid, monochloroacetic acid/0.1 M sodium hydroxide buffer, pH 1.8 and 3.8, and acetic acid/0.1 M sodium hydroxide buffer, pH 3.5 and 5.6. As with all cyclic voltammetric experiments that were carried out, electrode preconditioning was carried out by stirring in the background solution at -0.20 V for 5 min. Bare carbon paste showed no accumulation of the oxidised product of clenbuterol. On application of a thin Nafion membrane, a large increase in peak current was observed, and it was demonstrated that accumulation of the analyte occurred onto the membrane. Accumulation curves obtained for the aforenamed electrolytes are shown in Figure 3-10.

By comparing accumulation trends of clenbuterol at Nafion-modified electrodes in the above background solutions, two important features were observed as the pH moved towards acidic values:

(i) the peak current (i_p) increased;

(ii) a linear range was achieved for longer accumulation times.

Therefore, the results of the above study indicate that acidic background electrolytes provide optimum ion-exchange conditions for the analysis of samples. From this result, it was decided to choose between 1 M, 0.1 M and 0.01 M perchloric acid as the electrolyte for use in future studies.

On choosing a background electrolyte for electrochemical analysis, the chosen solution must possess a number of important qualities. These include a long linear range, a steep slope indicating sensitivity, an analyte peak well separated from the

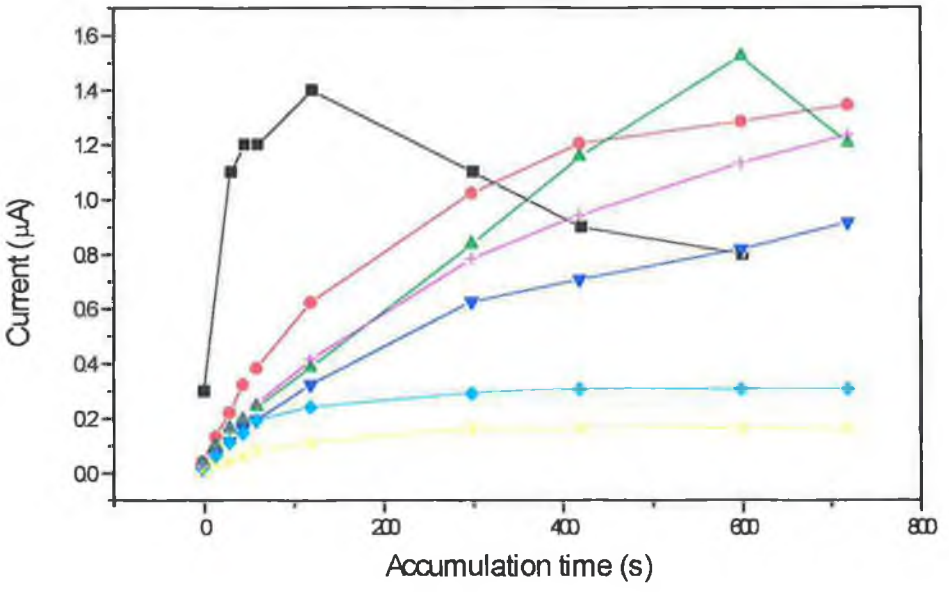


Figure 3-10 *Accumulation studies of 1 x 10^{-6} M clenbuterol at a 0.05 % Nafion-modified electrode in 1 M perchloric acid* (■); *0.1 M perchloric acid* (●); *0.01 M perchloric acid* (Δ); *0.1 M chloroacetic acid, pH 1.9,* (∇); *0.1 M chloroacetic acid, pH 3.8,* (◆); *0.1 M acetic acid, pH 3.5,* (+) *and 0.1 M acetic acid, pH 5.6* (×).

background signal, a small peak half-width and a maximum enhancement factor (i.e. $(i_p)_{max} / (i_p)_o$). The results shown in Table 3-1 were obtained. 0.1 M perchloric acid was chosen as the optimum background electrolyte for further analysis based on its long linear range, high sensitivity, peak potential and position, small peak half-width and large enhancement factor in comparison to other electrolytes. Under

	1 M perchloric acid	**0.1 M perchloric acid**	**0.01 M perchloric acid**
Linear range	0.0 - 1.0 min	0.0 - 0.75 min	0.0 - 0.50 min
Slope of linear section	9.0×10^{-7} A/min	5.3×10^{-7} A/min	3.2×10^{-7} A/min
Peak potential	1.1 V (not separated from background)	1.05 V (separated from background)	1.02 V (separated from background)
Peak half-width (after 1 min accumulation)	1.4 cm	1.0 cm	1.3 cm
$(i_p)_{max} / (i_p)_o$	4.0	8.0	6.3

Table 3-1 *Effect of variation of background electrolyte acidity on peak characteristics.*

	0.1 M perchloric acid	**0.1 M nitric acid**
Linear range	0.0-0.75 min	0.0-0.75 min
Peak potential	1.05 V	1.05 V
Peak half-width	1.0 cm	1.0 cm
Slope of linear range	0.36 μA/min	0.35 μA/min

Table 3-2 *Variation of acidic background electrolyte*

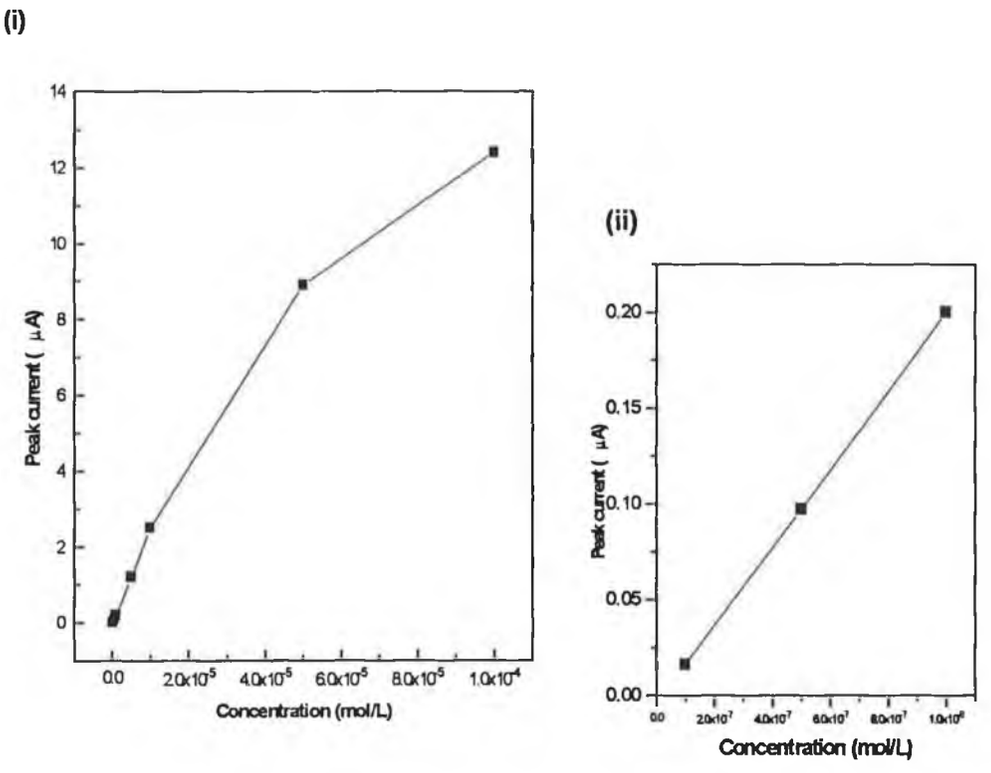


Figure 3-11 (i) *Calibration curve, carried out using CV, in the range 1 x 10^{-7} M to 1 x 10^{-4} M clenbuterol in 0.1 M perchloric acid with an accumulation time of 30 s;* (ii) *Calibration curve from 1 x 10^{-7} M to 1 x 10^{-6} M clenbuterol.*

identical experimental conditions, 0.1 M nitric acid was investigated as an electrolyte to establish whether the composition of the electrolyte had an affect on the clenbuterol response. The results obtained did not show any significant difference, as shown in Table 3-2.

3.3.1.3.3 Optimisation of conditions

A calibration curve which was carried out over wide concentration in the range 1 x 10^{-7} M to 1 x 10^{-4} M can be seen in Figure 3-11. This plot displayed greatest

linearity at low concentrations, with a linear regression value of 0.9999 (n=3) from 1 x 10^{-7} M to 1 x 10^{-6} M. The concentration chosen for the evaluation of the most favourable experimental conditions was 5 x 10^{-7} M, as this was the centre point of the curve. This concentration was used in the optimisation of percentage Nafion, scan-rate, stirring speed and starting potential.

3.3.1.3.3.1 Percentage Nafion

Table 3 shows the results of a study of the effect of increasing percentage Nafion concentration, which showed that 0.05 % resulted in a maximum peak current at a peak potential well separated from the non-faradaic current of the background solution. It was noted that as the percentage Nafion increased, so did the peak potential. This trend was also observed by Hoyer[23] in relation to the thickness of

% Nafion	Peak current (nA)	Peak potential (V)
0.01	75	+1.020
0.02	97	+1.030
0.05	216	+1.045
0.10	140	+1.050
0.20	108	+1.065
0.50	100	+1.080
1.00	100	+1.090

Table 3-3. *Variation of percentage Nafion on carbon paste electrodes and the effect on the accumulation behaviour of 5 x 10^{-7} M clenbuterol for 30 s at 0.00 V in 0.1 M perchloric acid.*

the Nafion coating, and is probably due to the Nafion membrane obstructing the mass transport of the analyte molecules. It was observed that the charging current also increased as the percentage Nafion increased, and above a 0.5 % Nafion membrane, this current restricted measurement of the analyte signal.

3.3.1.3.3.2 Accumulation potential

Of the seven accumulation potentials examined, 0.00 V proved to be the most efficient at accumulating the molecule over a 30 s period of time. From Figure 3-12 it can be seen that accumulation under electrolysis at -0.20 V accumulates

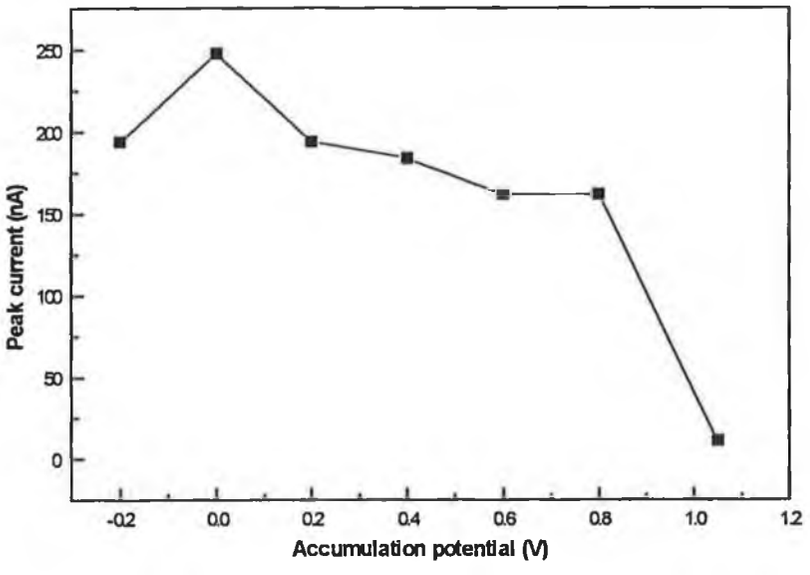


Figure 3-12 *Effect of accumulating clenbuterol onto the modified electrode surface at alternative potentials. Experimental conditions: 0.1 M perchloric acid as background electrolyte, a clenbuterol concentration of 5 x 10^{-7} M and a 0.05 % Nafion-modified carbon paste electrode.*

considerably less efficiently than at 0.00 V. This therefore eliminates the theory that clenbuterol may be accumulated onto the electrode surface during the potential scan from 0.00 V $\rightarrow$ +1.20 V. Very little accumulation occurs close to the primary oxidation potential of the clenbuterol molecule.

3.3.1.3.3.3 Scan-rate and stirring speed

A study of the effect of varying the scan rate on peak current allowed the determination of the rate-controlling process within the Nafion layer. A plot of peak current (i_p) versus the square-root of the scan rate ($\nu^{1/2}$) was linear (Figure 3-13) indicating the rate controlling process to be the diffusion of the clenbuterol through the Nafion layer. As shown in Figure 3-14, there was a non-linear relationship between current (i_p) and scan rate (ν), concluding that adsorption is not the rate-controlling process. Confirmation of the process classification was obtained when the dependence of the peak current (i_p) on the stirring speed (rpm) was also found to be linear, as shown in Figure 3-15 a stirring speed of 800 rpm was chosen for all future experiments.

3.3.1.3.4 Medium exchange

Medium exchange experiments which were carried out were similar to those described by Boyd *et al.*[24] Clenbuterol was accumulated onto the newly modified electrode surface from a concentration of 1 x 10^{-7} M for 30 s at 0.00 V and the

peak current recorded. Following transferral of the electrode into a fresh background solution, electrolysis was applied for a further 30 s before scanning in the usual range. The resulting peak current was measured.

After having incorporated medium exchange (with 30 s accumulation), a peak was observed which was 28 % of the original peak. This indicates that diffusion does indeed occur through the Nafion membrane, and from the above results it can be

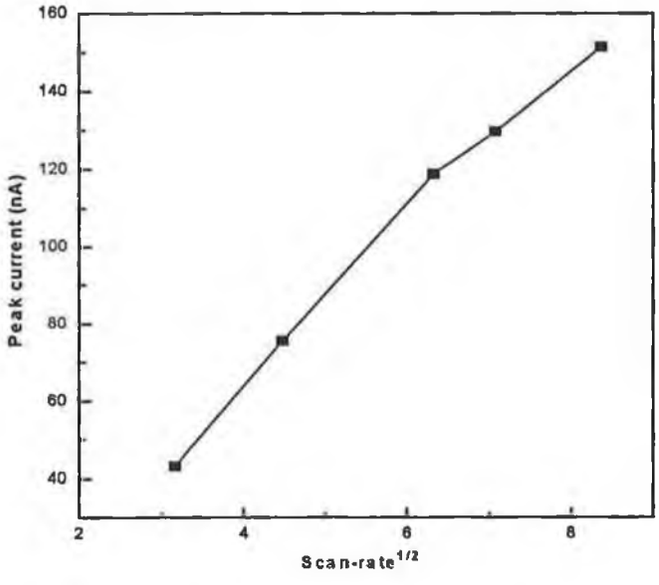


Figure 3-13 *Linear relationship between scan-rate$^{1/2}$ and peak current indicating diffusion through Nafion membrane.*

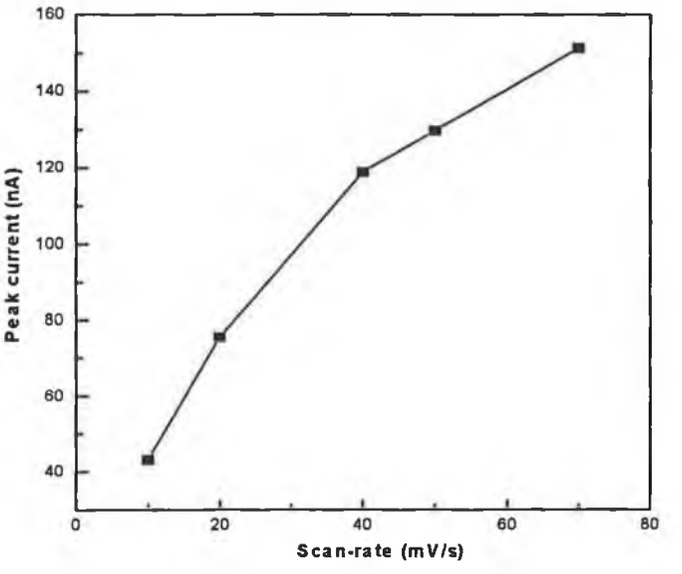


Figure 3-14 *Non-linear relationship between scan-rate and peak current.*

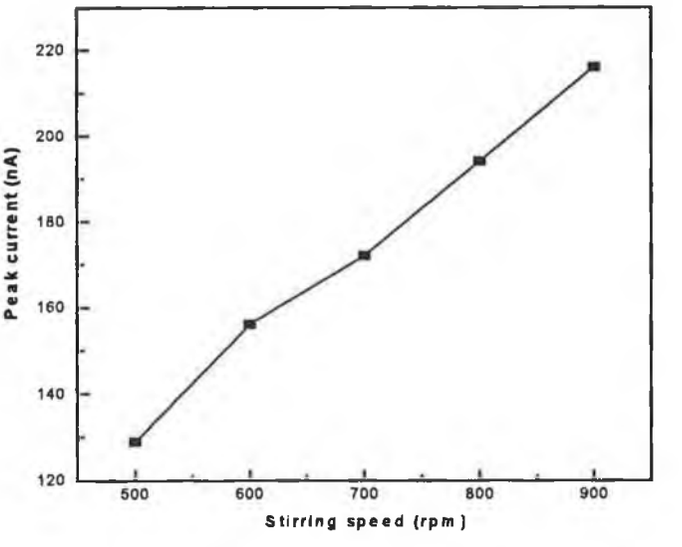


Figure 3-15 *The linear relationship between the stirring speed of the solution and the peak current. Experimental conditions: 5 x 10^{-7} M clenbuterol accumulated for 30 s onto a 0.05 % Nafion-modified carbon paste electrode, carried out in 0.1 M perchloric acid.*

concluded that the rate-controlling process is the diffusion of the clenbuterol through the Nafion layer to the surface of the electrode.

3.3.1.3.5 Accumulation studies at different concentrations

Accumulation curves were obtained for 1 x 10^{-7} M, 2 x 10^{-7} M, 5 x 10^{-7} M and 1 x 10^{-6} M clenbuterol in 0.1 M perchloric acid, using a 0.05 % Nafion-modified electrode, a scan range from 0.00 V to +1.30 V and a scan-rate of 50 mV/sec.

Figure 3-16 shows accumulation studies which were carried out for four different concentrations of clenbuterol on an electrode coated with 10 µl of 0.05 % Nafion in 0.1 M perchloric acid. The highest concentration, 1x10^{-6} M, displayed a linear accumulation response up to 30 s, whereas a concentration of 5x10^{-7} M clenbuterol was linear up to 45 s.

For both concentrations, saturation occurred after 180 s. As the concentration was lowered, the linear ranges lengthened (as is usually the case) with 2x10^{-7} M and 1x10^{-7} M clenbuterol concentrations achieving linear responses up to 60 s and 120 s respectively, with saturation apparent after 300 s. Results showed a linear increase in the slope of the current versus time curves with increasing concentration, indicating that accumulation is linear (for a chosen, fixed accumulation time) over a given concentration range. This allows the use of accumulation on the Nafion membrane to develop a quantitative method to determine levels of clenbuterol in biological samples.

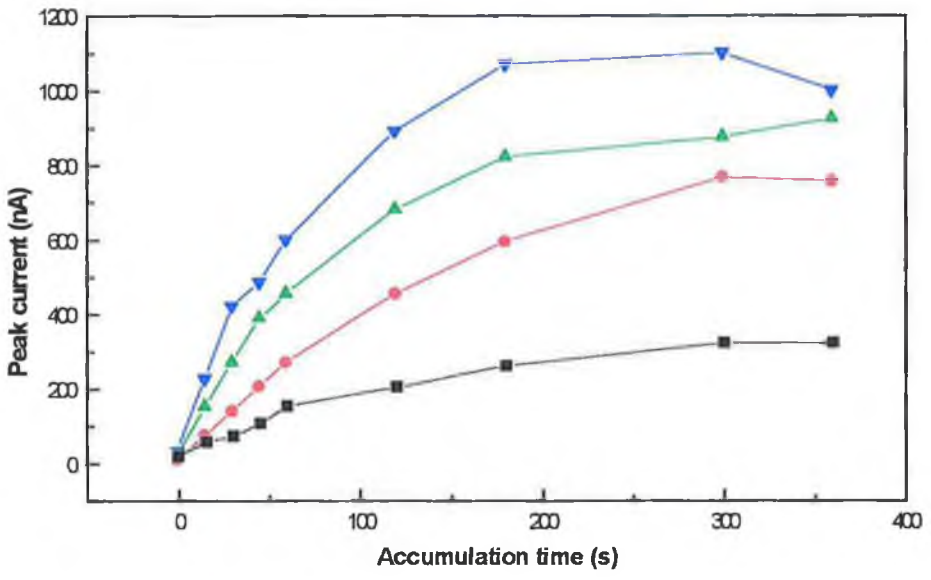


Figure 3-16 *Accumulation curves of clenbuterol concentrations of 1 x 10^{-7}M* (■), *2 x 10^{-7} M* (●), *5 x 10^{-7} M* (Δ) *and 1 x 10^{-6} M* (∇). *Experiments carried out in 0.1 M perchloric acid at a Nafion-modified carbon paste electrode.*

A calibration curve carried out in 0.1 M perchloric acid in the range $4x10^{-8}$ M - $3x10^{-7}$ M proved to be linear from $4x10^{-8}$ M - $2.4x10^{-7}$ M clenbuterol, following the equation:

$$i_p/nA = 1.25x10^{+7}\ C/mol.dm^{-3} + 0.039 \qquad (n = 10, r = 0.9991)$$

From this graph, a limit of detection of 5.8 x 10^{-9} M was calculated based on the clenbuterol concentration giving a signal equal to the blank signal plus three standard deviations of the blank. The limit of quantitation is regarded as the

"lower limit for precise quantitative measurements, as opposed to qualitative detection".[33] This was calculated based on the analyte concentration giving a signal equal to the blank signal plus ten standard deviations of the blank and was evaluated to be 2.7×10^{-8} M.

3.3.1.3.6 Reproducibility tests

1×10^{-7} M clenbuterol was accumulated onto a clean electrode surface at 0.00 V for 30 s and stripped anodically following a 5 s rest period. The analysis was carried out a further nine times and the peak current measured each time.

Reproducibility tests showed the peak to have a standard deviation of 2.4 %, when n = 10.

3.3.2 Differential pulse voltammetric determination of clenbuterol in bovine urine

Differential pulse experiments were carried out in 0.1 M perchloric acid with a carbon paste electrode modified with 10 µl of a 0.05 % Nafion solution. Preconcentration was at 0.0 V with a stir speed of 800 rpm. These conditions were found to be optimum for cyclic voltammetry experiments.

Figure 3-17 shows the differential pulse voltammetric behaviour of clenbuterol in 0.1 M perchloric acid at a 0.05 % Nafion-modified carbon paste electrode. Figure 3-17(i) was a result of stirring the electrode in the background solution for 5 min at 0.00 V. On injection of 6×10^{-6} M clenbuterol, the drug was accumulated onto the surface at 0.00 V for 2 min, followed by a scan from +0.20 V to +1.20 V (ii). The

potential was then held at 1.02 V for 1 min (under stirring) and the reaction products were then stripped anodically from the electrode surface by scanning as above (iii).

Variation of the percentage Nafion concentration showed 0.05 % as the optimum, due not only to the fact that this concentration resulted in the highest peak current, but also as increased Nafion concentrations resulted in increased charging currents in addition to higher oxidation potentials. Accumulation studies carried out on the main clenbuterol oxidation peak evaluated the limit of quantification of using this method to be 1 x 10^{-8} M clenbuterol. The use of this peak for the determination of

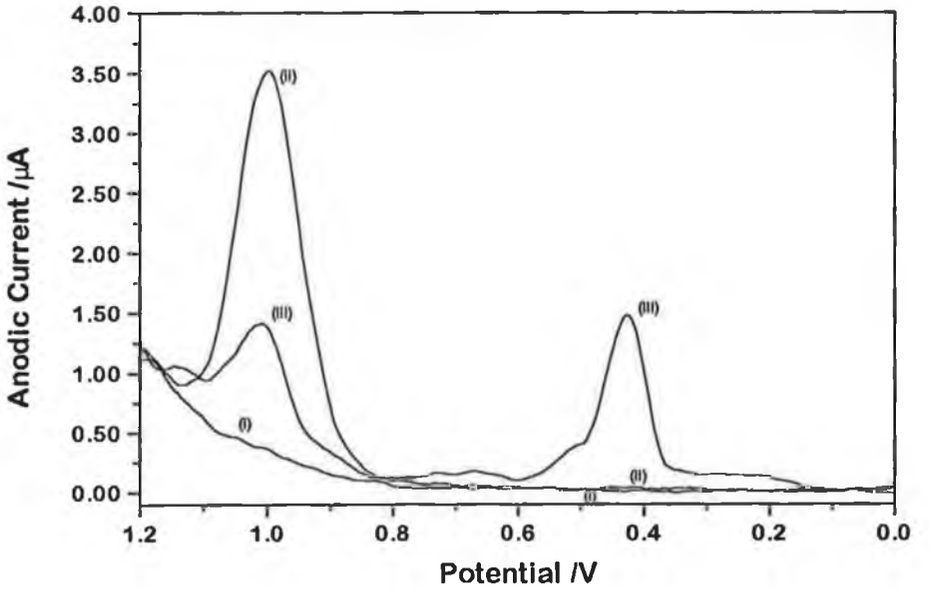


Figure 3-17 *Differential pulse voltammetry of clenbuterol at Nafion-modified CPEs (0.05 %) in 0.1 M perchloric acid:* (i) *stirred background solution at 0.00 V for 5 min and scanned from 0.00 V to 1.20 V;* (ii) *accumulation of clenbuterol onto the surface at 0.0 V for 2 min, with stirring and* (iii) *electrolysis at 1.05 V for 1 min, with stirring.*

clenbuterol in biological samples is not recommended, however, due to its presence on a large sloping background, and also due to the fact that many interfering compounds present in urine matrices undergo oxidation at these high positive potentials of around 1.00 V. For these reasons, it was decided to investigate the use of the product peak for analysis.

3.3.2.1 Differential Pulse Voltammetry of the quasi-reversible couple at +0.42 V

It has already been discussed how clenbuterol is irreversibly oxidised at high positive potentials, the oxidation process being followed by a chemical reaction which results in a chemical product oxidisable at much lower positive potentials. Some pH studies carried out on Nafion-modified electrodes using CV have indicated that the quantity of product formed is very pH dependent, with optimum conditions arising in acidic media (c.f. Section 3.3.1.1). It was decided to investigate the optimum conditions for product generation and the subsequent accumulation of this product onto the electrode surface. The low positive potentials at which the quasi-reversible is oxidised is a very attractive feature for analytical applications, as it is far removed from the slope of the non-faradaic background current, therefore omitting problems of this nature. The oxidation peak appearing at 0.40 V using DPV is a well-defined adsorption peak which is present even at very low concentrations of clenbuterol.

The initial stage of the investigation involved ascertaining the conditions for maximum product generation. Knowing that 0.1 M perchloric acid resulted in the

clenbuterol oxidation peak of greatest intensity, experiments were carried out in this electrolyte using a 0.05 % Nafion-modified electrode (prepared in the usual manner) and a clenbuterol concentration of 1.2 x 10^{-5} M. Using a fresh electrode each time and treating the electrode under electrolysis at 0.00 V for 300 s, the tests carried out involved: (i) a 5 s stirring period on injection of the drug (i.e. no accumulation onto the surface) followed by scanning from 0.00 V to +1.20 V, and rescanning to determine the quantity of the product on the electrode surface; (ii) as in (i) above, again without accumulation of clenbuterol onto the surface, but scanning from 0.00 V to +1.20 V followed by holding the potential at 1.00 V (without stirring) to further generation of the product and finally rescanning from 0.00 V to +1.20 V to determine peak height; and (iii) a repeat of (ii) but stirring when electrolysing at 1.00 V. Results showed that the quantity of the quasi-reversible couple formed was enhanced by holding the electrode at the potential at which the clenbuterol is oxidised while stirring the background solution. Therefore, by initially accumulating clenbuterol onto the electrode surface, and subsequently converting the oxidised clenbuterol into the reaction product by holding at a predetermined high positive potential and detecting it at approx. 0.40 V, it can be seen that there is potential to detect much lower concentrations of clenbuterol in aqueous samples than using the main oxidation peak.

Optimisation of the pH was then carried out using 1 M, 0.1 M and 0.01 M perchloric acid solutions: only acidic pHs were examined due to the fact that the quasi-reversible couple is seen only below pH 3. Each test was carried out by injecting a 1.2 x 10^{-5} M clenbuterol solution (giving an overall cell concentration of 1.2 x 10^{-5} M clenbuterol) into the appropriate background solution (containing a

freshly prepared electrode), accumulating at 0.00 V for 120 s and at the oxidation peak potential for 60 s (with stirring), followed by scanning from 0.00 V to + 1.20 V. A background electrolyte of 0.1 M perchloric acid resulted in the maximum peak current, indicating that product production is dependent on optimum conditions for clenbuterol oxidation.

Once these conditions had been chosen, the optimum potential for product formation was established by studying peak current after electrolysis at 0.95, 1.00, 1.05 and 1.10 V. Of these, 1.05 V proved to be the most favourable and therefore was used in all experiments which followed.

Pulse amplitude variation indicated 70 mV to result in the most intense peaks and 20 mV/sec was chosen as the scan rate due to shorter scan times.

3.3.2.1.1 Accumulation studies

Figure 3-18 shows the accumulation of the oxidised product onto the electrode surface after the accumulation of 5×10^{-8} M clenbuterol onto the Nafion film at 0.00 V for 60 s, followed by electrolysis at 1.05 V for 30 s, up to 300 s. The relationship between accumulation time and peak current is shown in Figure 3-18(ii). Accumulation studies were carried out on four clenbuterol concentrations, as seen in Figure 3-19, with 0.1 M perchloric acid as the supporting electrolyte, and the remaining experimental conditions were those that were found to be optimum as have been previously discussed. Preconcentration times of 60 s at 0.00 V and 30-300 s at 1.05 V were applied throughout each experiment; however, adsorption of the product onto the electrode surface meant that the accumulation

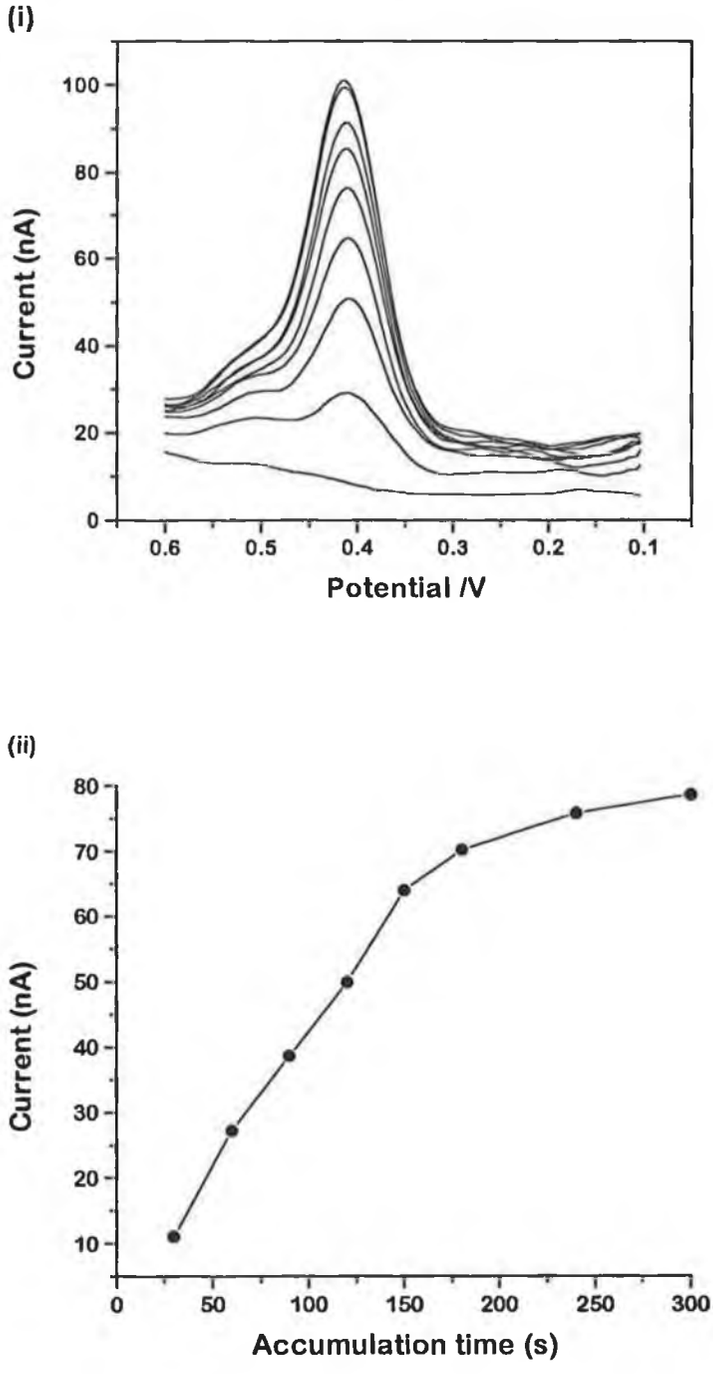


Figure 3-18 (i) *Accumulation of oxidised product of 5 X 10^{-8} M clenbuterol onto a 0.05 % Nafion-modified carbon paste electrode in 0.1 M perchloric acid. Accumulation at 0.00 V for 60 s, followed by electrolysis at 1.05 V for 60 s before scanning from 0.10 V to 0.60 V. Scan-rate was 20 mV/s; pulse amplitude, 70 mV;* (ii) *the relationship between accumulation time at 1.05 V and peak current.*

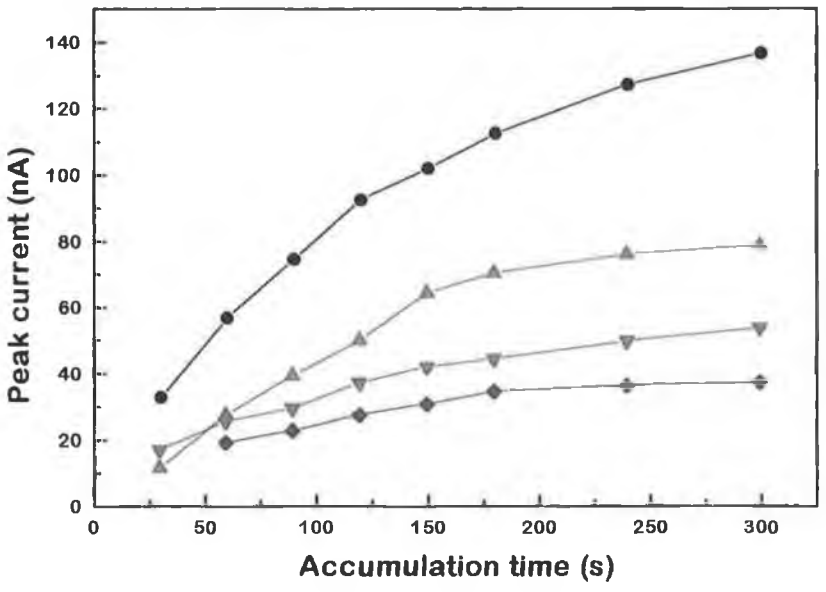


Figure 3-19 *Accumulation curves of four clenbuterol concentrations describing the accumulation of the quasi-reversible couple oxidation peak onto the membrane surface: 5 X 10^{-8} M (◆); 1 X 10^{-7} M (∇); 5 x 10^{-7} M (Δ) and 1 x 10^{-6} M (●). Scan-rate, 20 mV/s; pulse amplitude, 70 mV.*

time at any given point in the experiment was the sum of all the accumulation times applied before that particular time. As with the accumulation studies carried out on the clenbuterol oxidation peak at 1.10 V, linearity increased with decreasing concentration. The peak current of 1 x 10^{-6} M clenbuterol solution increased linearly with time for up to 90 s and there was still an increase in current after 300 s. Below this concentration, however, after 150 s the peak current seemed to level off, indicating that no additional product was adsorbed onto the surface after this time. A concentration of 5 x 10^{-7} M displayed linear accumulation up to 120 s; lowering the concentration to 1 x 10^{-7} M increased this linearity up to 150 s and the lowest concentration, 5 x 10^{-8} M, accumulated linearly up to 180 s.

The relationship between the slopes of these accumulation curves (which increased proportionally with concentration) and the concentration of the drug was linear (n = 4, r = 0.9989), as is to be expected.

A calibration curve was carried out in 0.1 M perchloric acid with accumulation times of 6 min at 0.00 V and 6 min at 1.05 V was linear in the range 1 x 10^{-9} M to 7 x 10^{-9} M with electrode renewal between each new concentration, according to the equation:

$$i_p/nA = 5.26 \times 10^{+10}\ C/mol.dm^{-3} + 32.8 \qquad (n = 6, r = 0.9987)$$

From this graph, a LOD of 2.33 x 10^{-10} M clenbuterol was calculated as being the clenbuterol concentration giving a signal equal to the blank signal plus three times the standard deviation of the blank.[33] It can be seen that much lower levels of clenbuterol can be detected using this method than by studying the primary oxidation peak.

3.3.2.2 Determination of clenbuterol in bovine urine

The effect of the urine matrix on the electrode response was investigated by injecting amounts of 50, 100, 200, 500 and 1000 µl of extracted urine into the electrochemical cell containing that amount of 0.1 M perchloric acid to maintain the cell volume constant at 4 ml. A carbon paste electrode modified with 0.05 % Nafion solution was used as the working electrode. Initially, the solution was stirred for 5 s after urine injection and a direct scan was carried out in the range

0.00 V → +1.20 V. Subsequently, electrolysis was carried out at 0 V (the accumulation potential of clenbuterol) for 5 min and the scan repeated in the same potential range. Finally, the potential was held at 1.05 V for 5 min. Figure 3-20 shows the response of 1000 µl of urine when injected into the 0.1 M perchloric acid background. Results showed that the quantity of interferences on the

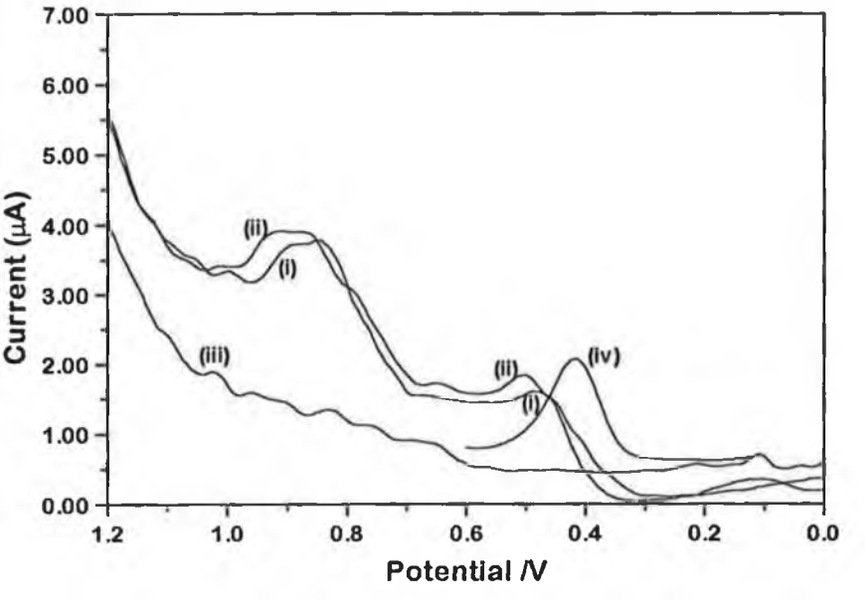


Figure 3-20 *The electrode (modified with a 0.05 % Nafion layer) response to 1000 µl (5 ml equivalent) of extracted urine injected into 3000 µl of 0.1 M perchloric acid* (i) *direct scan from 0.00 V to 1.02 V after stirring for 5 s on injection;* (ii) *electrolysis at 0.00 V for 5 min;* (iii) *electrolysis at 1.05 V for 5 min and* (iv) *the location of the product peak after injection of 1 X 10^{-6} M clenbuterol followed by electrolysis at 0.00 V for 5 min and 1.05 V for 5 min.*

electrode surface increased as the concentration of urine in the cell increased. The interferences appeared on the direct scan, and were still to be seen after electrolysis at 0.00 V. However, electrolysis for 5 min at 1.05 V removed the interferences from the electrode surface. A large oxidation peak can be seen at +0.90 V (Figure 3-20), which would interfere with the quantification procedure, if it were the main clenbuterol oxidation peak that was being used for analysis. As can be seen from the diagram, no oxidation of compounds present in urine occurs at +0.42 V where the analytical peak appears. To choose a Nafion percentage for further analysis, investigations were carried out on the effect of Nafion percentage on the urine interferences. Nafion concentrations of 0.01, 0.02, 0.05, 0.10, 0.20, 0.50 and 1.00 % were investigated by applying 10 µl of the appropriate solution onto the electrode surface and evaporating in the usual manner. Identical scans were carried out as in the previously mentioned experiment after 1 ml of urine had been injected into the cell. As was to be expected, the amount of interferences on the electrode surface increased as the Nafion concentration increased. It was decided to maintain a 0.05 % Nafion membrane on the surface as this resulted in minimal interferences.

A calibration curve was constructed in the range 2 x 10^{-9} M to 2 x 10^{-8} M clenbuterol by injecting 100 µl of urine containing a known quantity of clenbuterol and accumulating for 660 s at 0.00 V and 660 s at 1.05 V. A scan was carried out in the range 0.10 V $\rightarrow$ 0.70 V and the peak current was measured in the usual manner. The response was linear in the range 4 x 10^{-9} M to 1.5 x 10^{-8} M ($r = 0.997$, $n = 6$). This corresponds to a limit of detection of 1.02 x 10^{-9} M, calculated as before.

3.4 CONCLUSIONS

In this chapter, an analytical method using differential pulse voltammetry has been described for the determination of clenbuterol in bovine urine extract. Previous reports on the electrochemical behaviour of clenbuterol at Nafion-modified carbon paste electrodes have described how, in acidic media, clenbuterol is irreversibly oxidised at high positive potentials. The irreversibility of the oxidation is caused by a chemical follow-up reaction (an ECE mechanism). The oxidation product undergoes a chemical reaction to a second product which is more readily oxidised/reduced than the original compound.

Preliminary studies of the use of the main oxidation peak for clenbuterol determination limited quantification to a concentration of 1×10^{-8} M and had the additional drawback of having to measure a peak which appeared on a sloping background current. The accumulation of the product formed after clenbuterol oxidation onto the Nafion layer involved preconcentrating the clenbuterol onto the surface, followed by the conversion of the oxidised form of clenbuterol into product by applying a high positive potential for a specific time. The product was oxidised by scanning from 0.00 V to 0.60 V, with the oxidation peak appearing at 0.40 V. In this case, a limit of quantitation of 1×10^{-9} M clenbuterol was achieved, which corresponded to a LOD of 2.33×10^{-10} M. This process presents many advantages from an analytical point of view as it involves oxidation at a very low positive potential well separated from background interferences.

This method was then applied to the analysis of clenbuterol in the presence of a urine matrix, the latter having been cleaned up successfully using a two-step

extraction procedure involving a liquid/liquid extraction step followed by a mixed-mode solid-phase extraction step. It has previously been shown that this method results in a clenbuterol recovery of greater than 77 % from bovine urine samples.[29] The two-step extraction procedure ensures very efficient urine clean up with minimal matrix compounds remaining in the extract to interfere with subsequent analysis.

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CHAPTER FOUR

ANALYSIS OF PHENOLIC ACIDS IN BEER BY CAPILLARY ELECTROPHORESIS

4.1 INTRODUCTION

Capillary electrophoresis (CE) is an attractive separation technique due to its extremely low mass detection limits, high separation efficiency without the need for gradient elution and small sample volume requirement (typically in the nl range), which makes it very attractive to bioanalysis where sample volumes may be limited. A further advantage of CE is that no organic solvents are required, in contrast to high performance liquid chromatography (HPLC), which widens the application range as well as reducing operational costs.

CE separates compounds on the basis of their mass-to-charge ratio in a buffer-filled, narrow bore (20-200 μm i.d.) capillary, across which an electric field has been applied.[1] The electrophoretic separation of compounds depends on the mobility of each ion, μ_i, which is determined by its charge and Stokes radii, with positive ions migrating towards the cathode, situated at the detection end of the capillary, under the influence of the electric field. Due to differences in mobility, ions of opposite charge are completely resolved. Ions of equal charge polarities are still separated according to the number of charges and Stokes radii.

The electrophoretic velocity of an ion, ν_i, is the product of its mobility, μ_i, and the applied field, E. However, the electroosmotic flow (EOF), caused by buffer interaction with the charge fused silica capillary walls, results in the electromigration of buffer ions towards the cathode. For this reason neutral molecules, although not separated, will migrate the length of the capillary due to EOF, which is stronger than the electrophoretic force. Anionic compounds will elute the capillary later than neutral molecules, as they are attracted to the anode,

situated at the injection end of the capillary, but again move the length of the capillary due to the effect of EOF. The observed analyte velocity is therefore given by:

$$v_i = [\mu_{eo} + \mu_{ep}(i)]E \qquad (4\text{-}1)$$

where μ_{eo} is the electroosmotic mobility, $\mu_{ep}(i)$ is the electrophoretic mobility of the ion and E is the voltage applied across the capillary.

Besides the charge and Stokes radii of a compound, other factors which effect separation include temperature, the applied electric field, capillary length and buffer viscosity. In contrast to the parabolic flow caused by a pressure-driven HPLC system, the flow velocity in the electrically driven CE is uniform across the capillary, therefore reducing band broadening and resulting in very high separation efficiencies, often in the 500,000 - 1,000,000 theoretical plates range. One of the major limitations to the applications of CE is joule heating which limits the voltage applied across the capillary to 30 kV. If not dissipated, this heat results in a temperature gradient across the capillary, causing band broadening. Methods of curtailing heat generation include the use of longer capillaries and lower separation voltages. Smaller i.d. capillaries increase the effectiveness of heat dissipation.

Although capillary zone electrophoresis (CZE), as described above, is the simplest method involving an open-tube capillary filled with buffer, a large variety of variations to the technique have developed over recent years. One of the greatest reasons for reduction in operation costs in comparison to HPLC is that adaptations to the capillary for the detection of specific analytes can be carried out by the incorporation of additives to the electrophoretic buffer, unlike HPLC which

requires the use of selective separation columns. Due to the fact that neutral molecules cannot be separated by this technique, the addition of a charged surfactant, such as the negatively charged sodium dodecyl sulphate (SDS), to the electrophoresis buffer, comprises the technique Micellar Electrokinetic Capillary Chromatography (MECC). Neutral molecules form adducts with surfactant micelles, formed when the critical micelle concentration is exceeded.

Chiral separations are also readily applicable to CE, again involving the addition of a chirally selective agent such as cyclodextrins to the electrophoretic buffer. Other forms of CE include capillary isotacophoresis (CITP), capillary isoelectric focusing (CIEF) and capillary gel electrophoresis (CGE).

In terms of detection strategies, the use of electrochemical methods in conjunction with CE has greatly increased in recent times. Previously, ultraviolet (UV) spectral detection was most frequently used; however, due to the short path lengths required for CE, the technique is generally limited to high detection concentrations. The use of fluorescence detection, first investigated by Brownlee and Sunzeri,[2,3] resulted in similar sensitivities to UV detection, but limits of detection were subsequently greatly improved with the application of laser induced fluorescence (LIF), with mass levels of a few hundred molecules being detected.[4] This technique is not widely applicable, however, due to the fact that derivatisation of the analyte is often necessary in order to ensure fluorescence at the laser wavelength.

Amperometric detection using a single working electrode is by far the most widely used electrochemical detection method for capillary electrophoresis.[5,6,7,8,9] The use of a dual electrode system, however, can improve both the detection limits and selectivity, as well as giving qualitative information.[10] Voltammetric detection

offers much more qualitative data than the single potential detection mode in amperometry, and therefore can be very advantageous in capillary electrophoresis where migration times vary, especially when very complex sample matrices are involved. However, the development of voltammetric detection methods has been hindered due to the large charging currents involved with potential scanning in thin-layer cells resulting in high detection limits. Lunte *et al.*[11] have reported a voltammetric-amperometric dual-electrode detection system for flow injection analysis and liquid chromatography, and this system has since been modified by Park for application as a detection technique for CE.[12]

The majority of procedures reported for phenolic acid identification in complex matrices (such as alcoholic beverages and soil samples) involves gas chromatography (GC) or HPLC analysis. The analysis of phenolic acids by GC requires derivatisation due to their non-volatile nature.[13] HPLC separation is usually coupled with UV detection, and analysis of such mixtures involves elaborate extraction procedures,[14,15] as well as complicated gradient elution[16,17,18] and lengthy analysis times. Profiling of phenolic acids by HPLC with UV detection necessitates detection at 2 wavelengths, due to variance in absorption maxima.[16,19]

Electrochemical detection with HPLC is well developed for phenolic acid analysis due to the low detection levels attainable.[20,21,22] The complex matrices associated with beer and wine samples make it difficult, however, to reliably identify analyte peaks due to varying retention times, and often additional component characteristics must be determined.[13] Amperometric detection permits the verification of component identity by the comparison of sample and standard characteristic *i-E* curves obtained by hydrodynamic voltammetry experiments. This

procedure can be time-consuming, so the use of scanning, rather than fixed potential detection can be particularly advantageous in peak identification.

The identification of a selection of phenolic acids (Figure 4-1) in beer using CE with electrochemical detection is described in this chapter. CE is an attractive alternative to HPLC for the analysis of phenolic acids in beer and wine samples as the acids are negatively charged at neutral pH values and therefore elute separately to the high concentration of compounds, such as flavanoids, which add colour and flavour and are neutral around pH 7. This reduces the need for elaborate clean-up procedures as well as reducing analysis times.

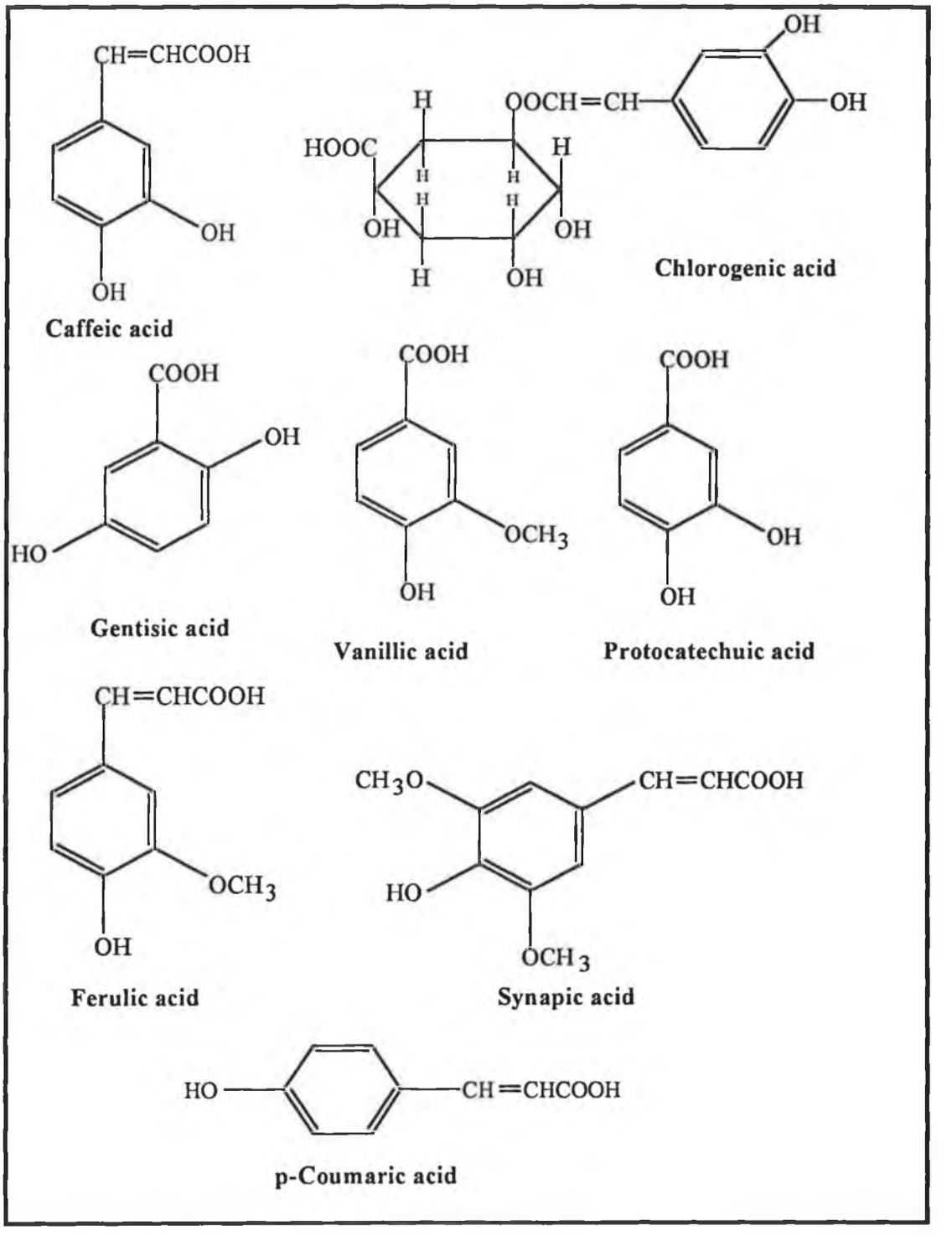


Figure 4-1 *Chemical structures of phenolic acids.*

4.2 EXPERIMENTAL

4.2.1 Chemicals

Gentisic, protocatechuic, vanillic, p-coumaric, caffeic, ferulic, synapic and chlorogenic acids were all purchased from Sigma (St. Louis, MO). Dopamine and catechol were also received from Sigma. Phosphoric and nitric acids were both obtained from Fisher Scientific (Fair Lawn, NJ). Nafion perfluorinated ion exchange powder (5 % stock) was obtained from Aldrich (Milwaukee, WI). All other chemicals were of reagent grade or better and were used as received.

All solutions were prepared using deionised water obtained by passing distilled water through a NANOpure water purification system (Sybron-Barnsted, Boston, MA). 100 μM stock solutions of phenolic acids were prepared in 0.1 M perchloric acid and refrigerated until use. Stock solutions were diluted as required with buffer and filtered through a 0.45 μm pore size Acrodisc syringe filter (Fisher, Fair Lawn, NJ) before injection. CE run buffers were prepared by titrating the free acid to the desired pH with sodium hydroxide pellets. For capillary activation, an EDTA solution was prepared by titrating 0.5 M disodium EDTA with 0.5 M sodium hydroxide to pH 13.0. Beer samples were bought commercially.

4.2.2 CE-EC apparatus

A schematic diagram of the CE-EC system is shown in Figure 4-2. The system was driven by a high voltage dc (0-30 kV) dual polarity power supply (Spellman High Voltage Electronics Corporation, Plainview, NY). The anodic high voltage

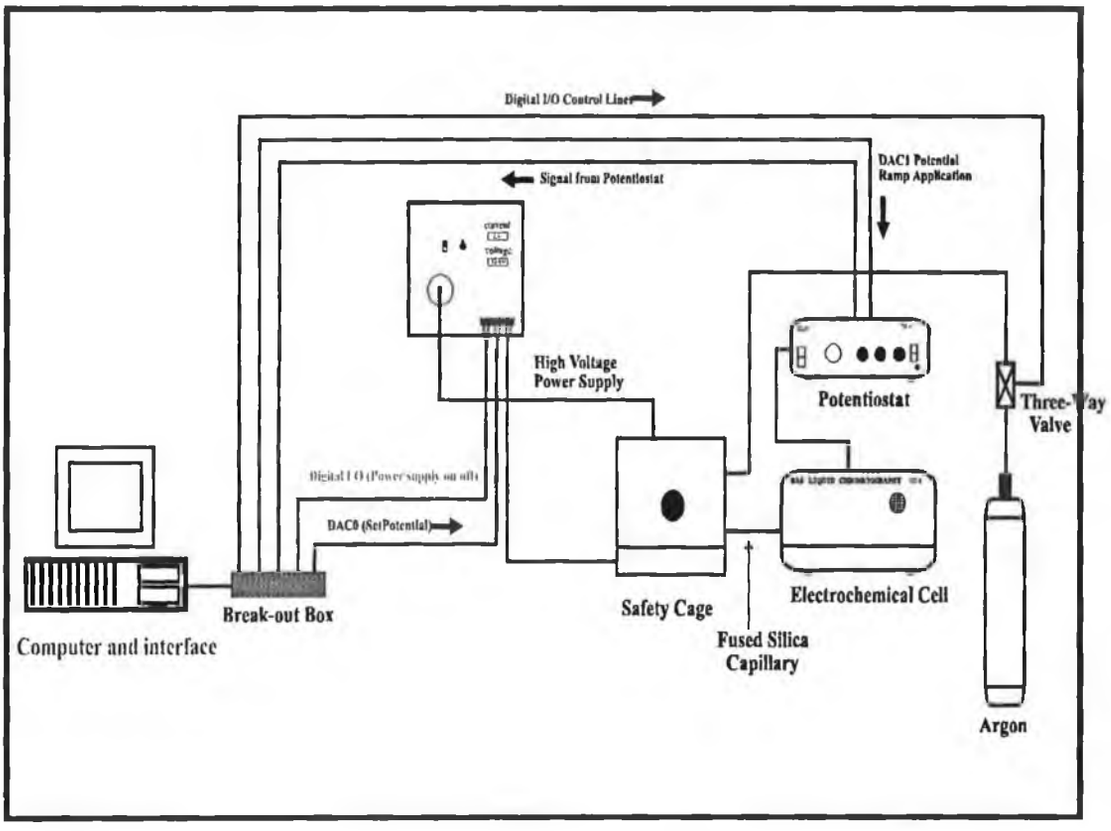


Figure 4-2 *Schematic diagram of CE-EC system.*

end was isolated in a plexiglass box fitted with an interlock for operator safety. The outlet of the capillary, containing a Nafion decoupler, was introduced into the electrochemical cell via a septum. The cell was filled with electrolyte as stated in the text. The fused silica capillary of 65 cm length and 50 μm i.d. was preconditioned before use by flushing it with 0.1 M sodium hydroxide for 5 min and then run buffer for 2 hours to ensure equilibration. Sample was introduced into the capillary via pressure or electrokinetic injection as described in the text. The working electrode was inserted into the CE electrochemical cell via a hole pre-drilled in the wall of the cell directly opposite the capillary end-column decoupler and was held in place with a septum. The electrode was inserted to the correct

depth (0.15 mm from capillary end) inside the decoupler using an X-Y-Z micromanipulator (Newport, Fountain Alley, CA). This was carried out under a microscope.

4.2.2.1 Electrochemical detection

Voltammetric studies were carried out in a separate conventional three electrode cell with an Ag/AgCl (with 3 N NaCl as the internal solution) reference electrode (RE-4 Bioanalytical Systems, West Lafayette, IA), a platinum wire counter electrode and a free standing carbon fibre working electrode. To eliminate environmental noise, the cell was placed in a BAS CC-4 Faraday cage. The system was controlled by a BAS preamplifier system (Bioanalytical Systems, West Layfayette, IA) which was connected to an IBM compatible computer. Data acquisition and control of the system was controlled by software programmed locally with Turbo C2.0. This set-up was also used to condition electrodes following preparation before insertion into the electrical decoupler.

4.2.2.2 Preparation of Carbon Fibre Electrodes

Preparation of the working electrodes used in all experiments involved initially threading a 33 μm o.d. carbon fibre (Avco Speciality Products, Lowell, MA) through a 5 cm length of 50 μm i.d. fused-silica capillary, leaving 50 mm of fibre exposed at each end of the capillary. Silicone glue was applied at each end of the capillary to join it with the fibre.

A hole (5 mm) was drilled approximately halfway down the length of a 30 cm standard syringe needle to expose the inner channel. Loose metal was prized out of the opening with another needle to allow free movement of the capillary within the channel after it had been inserted through the blunt end of the needle. After having inserted the capillary along this channel until the fibre could be seen through the hole, silver epoxy glue was coated onto the fibre in order to establish electrical contact and also to hold it in position. Superglue was applied between the needle and the capillary and left to dry. Silicone glue was applied once more to the capillary/carbon fibre joint and the electrode was hung upside down overnight to dry in a teardrop shape to ensure proper sealing.

4.2.3 Methods

4.2.3.1 Voltammetric studies

Prior to use in voltammetric experiments, each new electrode was initially sonicated for 2 min in 33 % (v/v) "Micro" cleaning solution (International Products, Trenton, NJ) followed by electrode activation involving the application of a stream of pulses ± 2.0 V in amplitude at a speed of 1 kHz for 30 s. Once activated, the electrode was transferred to the detection cell containing the capillary.

For pH studies, 100 μM of standard solution was placed in the detection cell and voltammograms were scanned in the range 100 mV → 1200 mV at 10 mV/s with a sensitivity of 10 nA/V and a gain of 1. Between each voltammetric scan, the

electrode was activated using a potential pulse of ± 2 V at 10 kHz for 30 s. These activation conditions were evaluated to be adequate to maintain a sufficiently activated electrode for routine analysis.

For hydrodynamic voltammetric experiments, detection was carried out using the CE-EC detection cell, with 100 μM standards being injected at 55 psi for 2 s. 50 mM phosphate buffer, pH 7.2, was used as the run buffer as well as the detection electrolyte. Applied working potentials are specified for each experiment in the text.

4.2.3.2 Method of Large Injection Volume

A 10 μM standard solution mixture containing dopamine, catechol, caffeic acid and protocatechuic acid was prepared and diluted accordingly with deionised water. Samples were prepared as described below. 25 mM phosphate buffer, pH 7.2, was used as the run buffer. Each time a new buffer was prepared, the steady state current was evaluated by carrying out electrophoresis on the run buffer at a separation voltage of 25 kV and monitoring the steady current. Two buffer bottles were used during the experiment - one containing the run buffer; the other containing buffer to collect waste. Fresh buffer was placed in both vials after each run.

Experiments were carried out as follows:- the electrochemical cell was first filled with phosphate buffer and the electrode was electrochemically pretreated by applying a switching potential of ±2 V at a frequency of 5 kHz for 30 s. The sample was then injected under pressure at 20 psi for the appropriate time. The

sample bottle was replaced with the waste buffer bottle and 25 kV was applied under reversed polarity conditions. The current was monitored until it reached 99 % of its steady state value, at which point electrophoresis was stopped. The electrochemical cell was then refilled with 1 M nitric acid and the polarity switched to normal mode. Electrophoresis was performed at 25 kV and eluting compounds detected with the electrode set at the appropriate working potential. A stationary potential of 0.45 V was applied when the sample was injected in order to avoid fouling of the electrode by the oxidation of sample matrix components. The capillary was flushed for 60 s at 20 psi with 0.5 M EDTA, pH 13.0, followed by 120 s with run buffer, between each run.

4.2.3.3 Beer sample clean-up

Sep-Pak® C_{18} cartridges were conditioned by passing through 20 ml of deionised water, followed by 3 ml of methanol and a further 20 ml of water. Stout samples were diluted appropriately with deionised water and neutralised by the dropwise addition of 0.1 M NaOH to pH 6.5. They were then passed through the conditioned cartridge 5 times.

4.3 RESULTS AND DISCUSSION

4.3.1 Electrode Characterisation

4.3.1.1 Electrode preparation

Prior to the use of an electrode as a detection device for analytical purposes, it is first necessary to evaluate certain characteristics of the electrode. A freshly made carbon fibre electrode must be effectively activated to remove adsorbents present on the electrode surface as a result of electrode preparation, which serve to reduce the voltammetric signal dramatically due to a decrease in amount of solution species reaching the surface. Experiments were carried out to determine an effective cleaning method for this purpose. It is thought that electrode activation functions not only by removing contaminants from the electrode surface, but also to increase the number of oxygen surface functionalities as well as altering the structure of the carbon surface.[23] Activation studies were carried out using 100 μM protocatechuic acid in 100 mM phosphate buffer, pH 2.1. Scans were performed in the range 100→1200 mV at 20 mV/s. The effect of activation was studied by monitoring the response following activation using a square-waveform with various potential pulses from ± 1.5 V to ± 2.0 V and varying activation time from 10 to 60 s at a frequency of 10 kHz. Results indicated that although electrochemical activation did initially result in increased electron-transfer rates (indicated by lower $E_{3/4}$-$E_{1/4}$ values), further activation resulted in a deterioration in response, i.e. the activation process was not reproducible. The higher the

activation potential applied the faster the electron transfer rates became, and the steeper the response slope of the *i-E* curve.

A combination of both physical and electrochemical pretreatments resulted, however, in adequate electrode surface activation. As it was not practical to physically activate the electrode between each electrophoretic run, once the electrode was inserted in the Nafion decoupler, only the electrochemical pretreatment was applied. Figure 4-4 shows the *i-E* curve of 100 μM protocatechuic acid carried out in identical conditions to above using a freshly made electrode without a history of pretreatment.

This was used as an indication of the effectiveness of activation, as the response depends on the condition of the electrode surface. As a result of sonication in 33 % (v/v) "Micro" cleaning solution for 2 min, followed by sonication in deionised water for the same time period, a large increase in response was seen with protocatechuic acid being oxidised at a $E_{1/2}$ value of 0.50 V, corresponding to the removal of physically bound contaminants from the electrode surface. Continual electrochemical activation at ± 2 V for 30 s at a frequency of 10 kHz further increases the response until a steady response curve is obtained after 5 consecutive repetitions of this procedure.

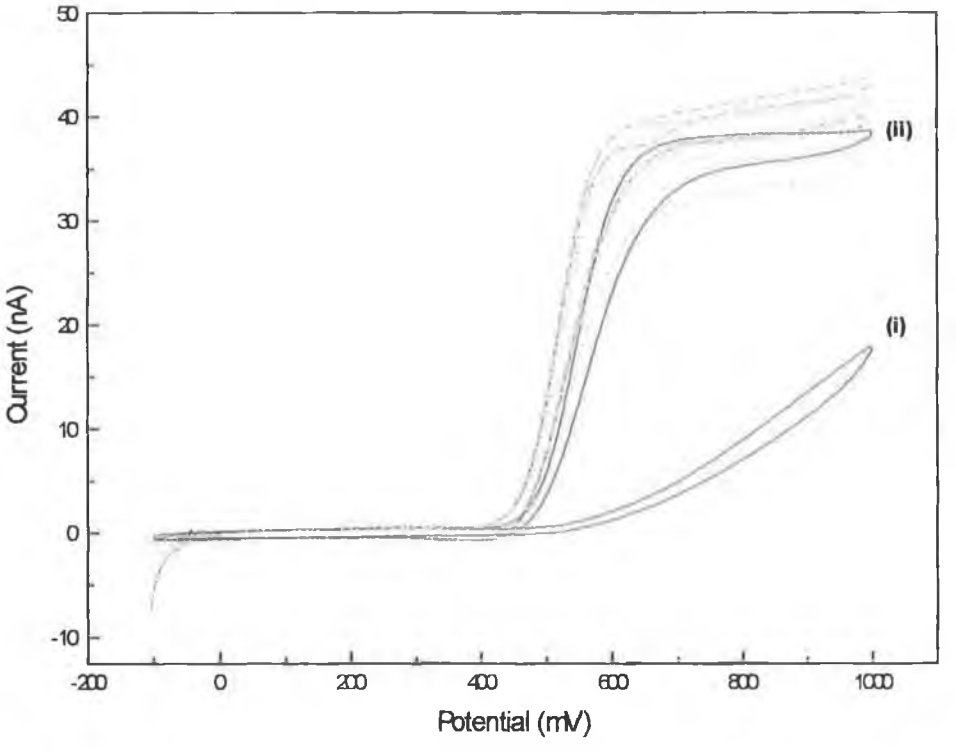


Figure 4-4 *Voltammetric response of 100 μM protocatechuic acid in 100 mM phosphate buffer, pH 2.5, from -100 to 1000 mV at 20 mV/s. The carbon fibre electrode showed no response to the analyte before activation* (i). *Physical methods of activation, e.g. sonication, are effective as initial activation methods to remove physically bound contaminants from surface, followed by electrochemical activation at ± 2 V, 10 kHz for 30s. Multiscans obtained with this activation are shown in* (ii).

4.3.1.2 Electrode activation

During analysis, the electrode surface is modified to a certain extent as a result of adsorption of solution species which occurs during potential scans. These species may effectively block some active sites and may be seen in a decrease in oxidation current of the analyte. It is therefore necessary to clean the electrode surface of contaminants between each analysis. The chosen activation method must allow for maximum signal enhancement, indicating a well activated surface, without overactivation of the electrode. Overactivation is thought to cause fracturing of the carbon fibre resulting in a large increase in surface area of the electrode. O'Shea *et al.*[23] reported such an increase in their study of the pretreatment of carbon fibre microelectrodes for the determination of folic acid. A large increase in charging current was observed, along with an increase in faradaic current. Swain and Kuwana[24,25] have investigated how severe, anodic pretreatment can cause fracturing along the principle axis of the fibres, thereby increasing the surface area of the electrode. Furbee *et al.*[26] immobilised glucose oxidase in the fractures of an overactivated electrode and applied it to the detection of glucose. In the latter case, experiments were carried out in stationary solutions, therefore allowing time for solution species to diffuse from the outer electrode surface into the fractures. The increase in charging current attributed to the extra surface area was compensated by an increase in faradaic current, however, species in solution do not have ample time to interact with the new surfaces within the fibres, the net result being an increase in charging current with no corresponding increase in faradaic current.

A similar increase in charging current resulted when low activation frequencies were used repeatedly for activation of the carbon fibre electrode in this application.

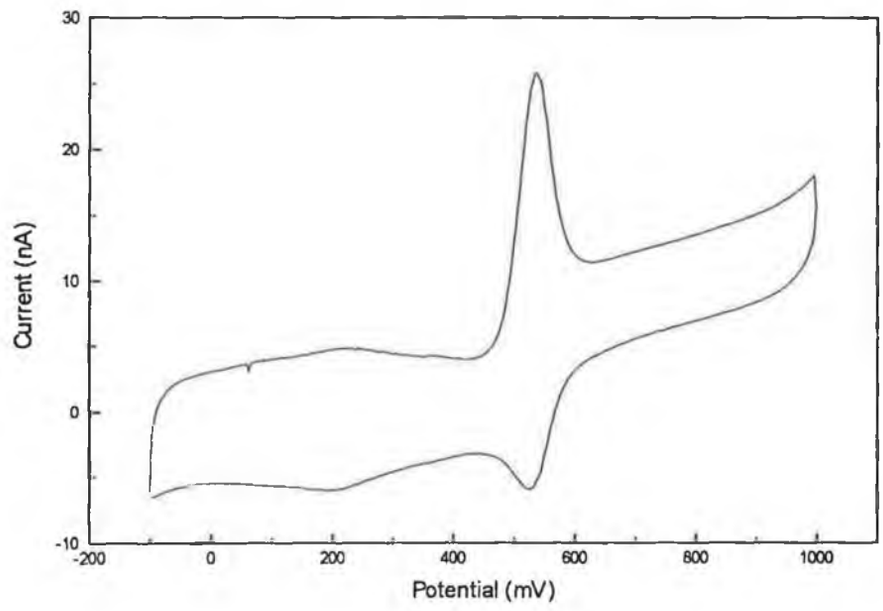


Figure 4-5 *Voltammogram of an over-activated electrode following successive activation at a low frequency (1 kHz) at ± 2 V for 30 s. Sample: 100 μM protocatechuic acid in 100 mM phosphate buffer, pH 2.1.*

It was observed that repeated activation using lower frequencies such as 1 kHz caused fracturing of the electrode, so care had to be taken to avoid overactivation. The voltammogram of an overactivated electrode can be seen in Figure 4-5. It is evident from this that the response curve is similar to the peak-shape of a macro electrode rather than the sigmoidal wave-form of a microelectrode, in addition to the presence of a high charging current. As electrode overactivation is irreversible, once the response curve shown in Figure 4-5 was obtained, a new electrode was activated using the procedure outlined in section 4.3.1.1. An important feature of

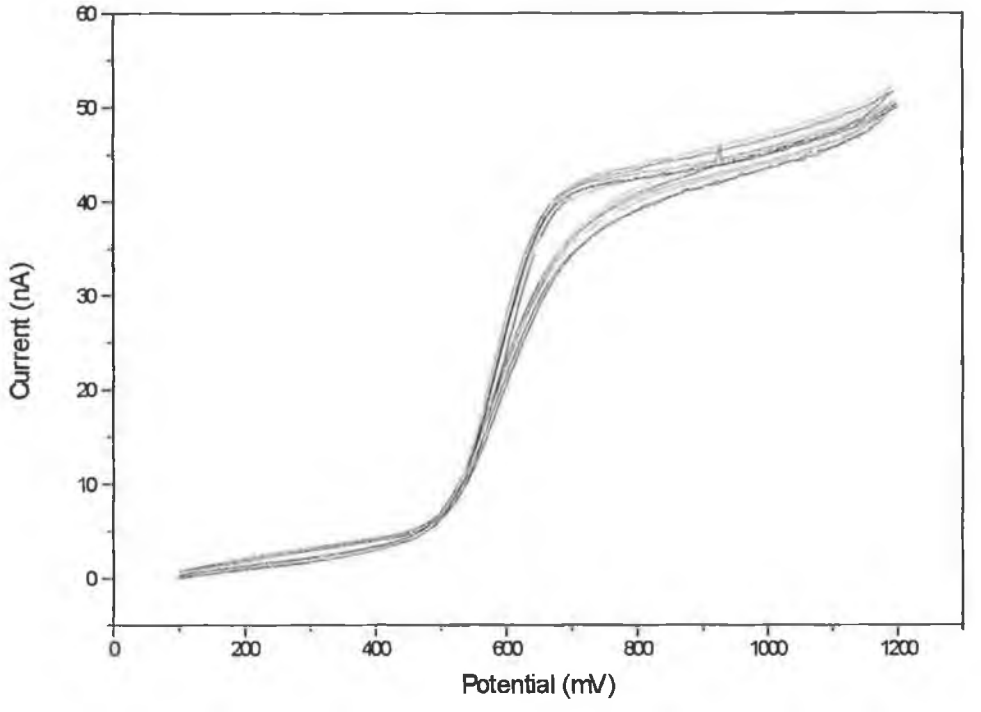


Figure 4-6 *Reproducibility of electrode activation: ± 2 V applied at 10 kHz for 30 s between voltammetric scans in the range 100 mV → 1200 mV. Electrode treatment history - sonication in "Micro" cleaning solution for 2 min, followed by activation at ± 2 V at 1 kHz for 30s. Analyte was 100 μM protocatechuic acid in 100 mM phosphate buffer, pH 2.1.*

an activation method used in routine analysis for quantitative and qualitative purposes is reproducibility. Higher frequency activation for longer times proved to be much more effective in maintaining a reproducibly active surface. As shown in Figure 4-6, with 100 μM protocatechuic acid as the analyte and conditions identical to above, seven consecutive activations at a potential pulse of ± 2 V for 10 s at 10 kHz showed little variation of response curve.

4.3.1.3 Voltammetry of phenolic acids

Stability studies were carried out on phenolic acid standards by obtaining cyclic voltammograms of a test 100 μM protocatechuic acid standard and comparing it to that of a 100 μM protocatechuic acid solution containing 1 mM EDTA as a preservative and a freshly prepared 100 μM acid solution. Cyclic voltammograms were scanned at half-hour intervals up to a period of 9 hours and left overnight before being scanned the next morning. As the voltammetric response remained stable overnight, it was concluded that fresh standard solutions, prepared daily, was ample to ensure stable solutions were used.

Prior to attempting the electrophoretic separation of phenolic acids, it was necessary to investigate their voltammetric characteristics by carrying out a pH study in the range pH 2.1 - pH 7.2 and comparing the results to the response of positive and neutral ions. 100 μM standard solutions of dopamine, catechol and protocatechuic acid were prepared in 100 mM sodium phosphate buffer, pH 2.1, 100 mM sodium acetate buffer, pH 4.75, 100 mM sodium acetate buffer, pH 5.5, 50 mM sodium phosphate buffer, pH 6.5 and 50 mM sodium phosphate buffer, pH 7.2. The potential range varied with pH, but the potential window was maintained at 1.10 V and scans were made at a rate of 10 mV/s. Figure 4-7 shows the *i-E* curves for the three compounds across the pH range given above. It is evident that while dopamine which is positive in the pH range studied ($pK_a \approx 9$-10) showed an increase in oxidation current as the pH moved towards neutral values (Figure 4-7(i)), catechol, which is neutral from pH 2.1-7.2, shows a relatively constant current across the pH scale (Figure 4-7(ii)). In the case of protocatechuic acid,

however, with a pK_a value in the range 4-5, it is evident that there is a dramatic increase in oxidation current in acidic media (Figure 4-7(iii)), showing that the electrochemical oxidation of phenolic acids is very dependent on the pH of the supporting electrolyte. This is most likely due to the negative charge on the phenolic acid above pH values of 4-5 affecting its interaction with the electrode surface.

It is also evident from a comparison of the response slope of the three compounds that there appears to be a significant retardation of electron transfer kinetics in more basic media for protocatechuic acid, as shown in Table 4-1, which is not seen for both dopamine and catechol. In each case, the linear relationship between pH and $E_{p1/2}$ was negative and almost 60 mV/pH unit (-62.51, -53.31 and -59.60 mV/pH for dopamine, catechol and protocatechuic acid respectively), corresponding to a Nernstian behaviour involving a process with an identical number of protons and electrons.

To investigate if this pH dependence is a characteristic specific to the carbon paste electrode, identical studies were carried out using a gold microelectrode. Results indicated that the response of phenolic acids using this gold electrode do not follow the same trend. This phenomenon shows that a specific characteristic of the carbon fibre electrode is responsible for the observed retardation in electron transfer kinetics. One possibility to explain this is charge-charge repulsion between the negatively charged phenolic acids and the electrode surface. Carboxyl moieties on the carbon fibre surface vary according to pH, and therefore at certain pH values these surface groups are charged, bringing about repulsion. Phenolic acids

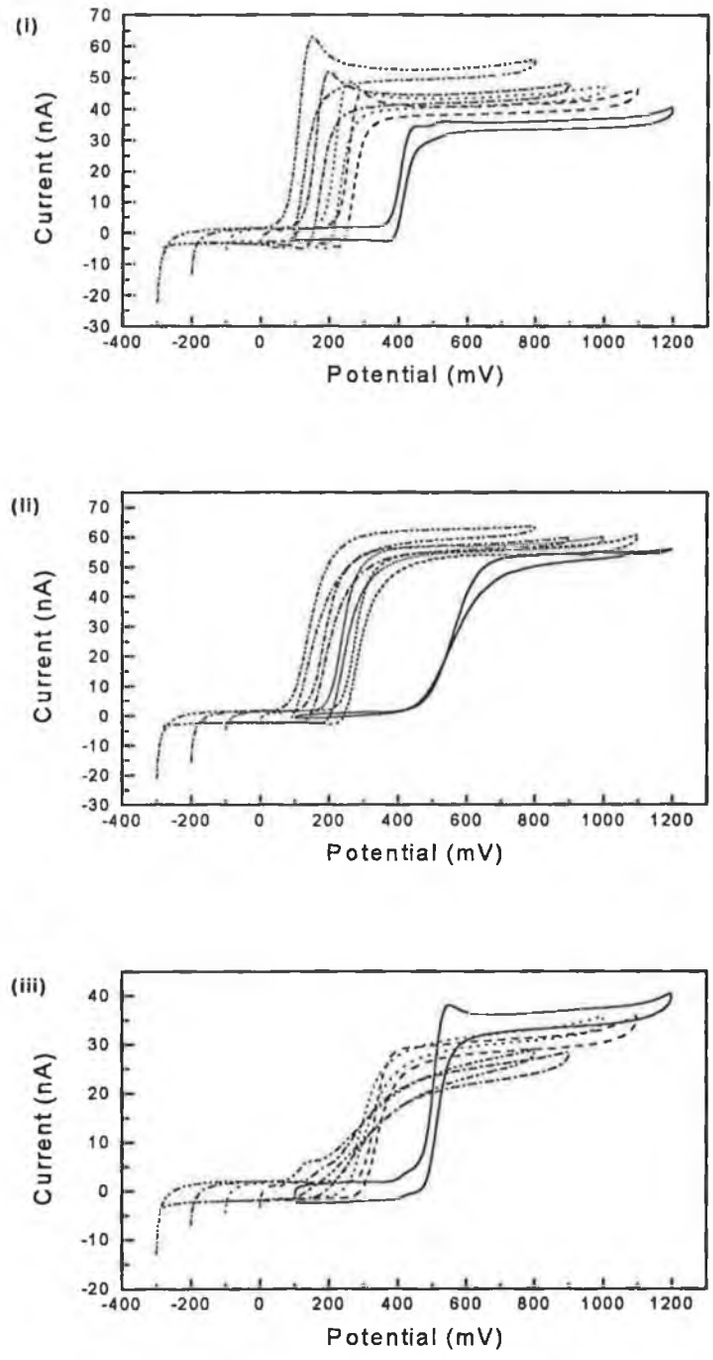


Figure 4-7 *Cyclic voltammograms of 100 μM standard solutions of* (i) *dopamine,* (ii) *catechol and* (iii) *protocatechuic acid in* (——) *100 mM phosphate buffer pH 2.5;* (-------) *100 mM sodium acetate buffer pH 4.75;* (……) *100 mM sodium acetate buffer pH 5.5;* (— - —) *50 mM phosphate buffer pH 6.5 and* (— - - —) *50 mM phosphate buffer pH 7.2.*

pH	$E_{3/4} - E_{1/4}$ mV	$E_{1/2}$ mV	i_p nA
2.1	32.12	494.19	34.58
4.75	41.23	333.69	29.82
5.5	64.79	299.54	30.08
6.5	134.92	275.57	23.50
7.2	237.19	275.16	24.51

Table 4-1 *Effects of pH on standard electrochemical characteristics of protocatechuic acid.*

are negatively charged above their pK_a value. There are no such moieties on the gold electrode surface to cause such a pH-dependence. Both the phenolic acids and the carbon fibre electrode are uncharged in acidic media, so electrode interaction is not hindered. These results indicate that although an acidic detection electrolyte for the CE-EC system improves sensitivity for the negatively charged phenolic acids, it does not eliminate the interference of the oxidation of high concentrations of neutral compounds present in beer samples, as electrode interaction of these compounds is independent of electrolyte pH. As the oxidation of these compounds in high concentrations can foul the electrode, it is necessary to remove them by some other method.

4.3.2 CE-amperometric detection of phenolic acids

As concluded by the cyclic voltammetry experiments, the electrochemical response for phenolic acids is greatly reduced in solutions of pH 7.0 relative to catechol, even though both contain the same electroactive moiety (dihydroxyphenol groups). This difference in response, which is most likely to be a result of charge-charge repulsion of the deprotonated, negatively charged phenolic acids (a result of their pK_a value around 4-5) and the negatively charged electrode surface carboxyl group in neutral media.

Below the pK_a of both these groups, i.e. in acidic media, this repulsion no longer exists and accessibility of the acids to the surface is greatly increased. However, this acidic pH is not practical for normal CE separations, which restricts run buffers to above pH 5.7. To overcome this drawback, 25 mM sodium phosphate buffer, pH 7.2, was used as the run buffer with 1 M nitric acid as the cell electrolyte. The use of a Nafion end-column decoupler aided in post-column pH modification by titrating the run buffer with protons rapidly diffusing from the acid in the cell across the Nafion membrane.

Once a voltage is applied across a capillary in normal CE mode, i.e. the anode at the injection end and the cathode at the detection end of the capillary, ions separate and elute according to their charge/size ratio, although structural shape and degree of hydration also plays an important role. As phenolic acids are negatively charged at the run buffer pH, they are attracted to the anode end. However, hydrogen bonding interaction is responsible for the phenolic acids being swept forward towards the cathodic end with the EOF. The structural differences between the two smallest phenolic acids, protocatechuic and gentisic acid, are responsible for

the former being eluted first. Although vanillic acid has a larger molecular weight than p-coumaric acid, it is thought that the linear chain structure of the p-coumaric acid increases its degree of solvation, and therefore it migrates more rapidly than vanillic acid. The migration order of the chosen phenolic acids in a 25 mM sodium phosphate, pH 7.2, run buffer at a separation voltage of 30 kV is chlorogenic acid (354 $gmol^{-1}$); synapic acid (224 $gmol^{-1}$); ferulic acid (194 $gmol^{-1}$); caffeic acid (180 $gmol^{-1}$); p-coumaric acid (164 $gmol^{-1}$); vanillic acid (168 $gmol^{-1}$); protocatechuic acid (154 $gmol^{-1}$) and gentisic acid (154 $gmol^{-1}$).

Figure 4-8 represents electropherograms of seven phenolic acids separated electrophoretically in 25 mM phosphate buffer, pH 7.2, and detected in 0.1 M nitric acid at five oxidation potentials. If the electropherogram recorded at an oxidation potential of 1050 mV is studied, it can be seen that this potential is high enough to correspond to the limiting current plateau of the *i-E* curves of the dihydroxyphenolic acids, i.e. chlorogenic, caffeic, protocatechuic and gentisic acids, as the response for these peaks increase and becomes constant at lower potentials. However, it is evident from the diagram and throughout the research that the oxidation current for all the acids is not the same. The reduced current obtained for chlorogenic acid oxidation relative to other phenolic acids is more than likely due to the molecule's larger size and geometric shape, affecting both its diffusion towards and interaction with the electrode surface. It is also obvious from the electropherogram that vanillic acid, containing a monohydroxyphenol group, is not yet being oxidised at its $E_{1/2}$ - therefore highlighting the need for detection at high oxidation potentials to ensure oxidation of both mono- and di-hydroxyphenol compounds.

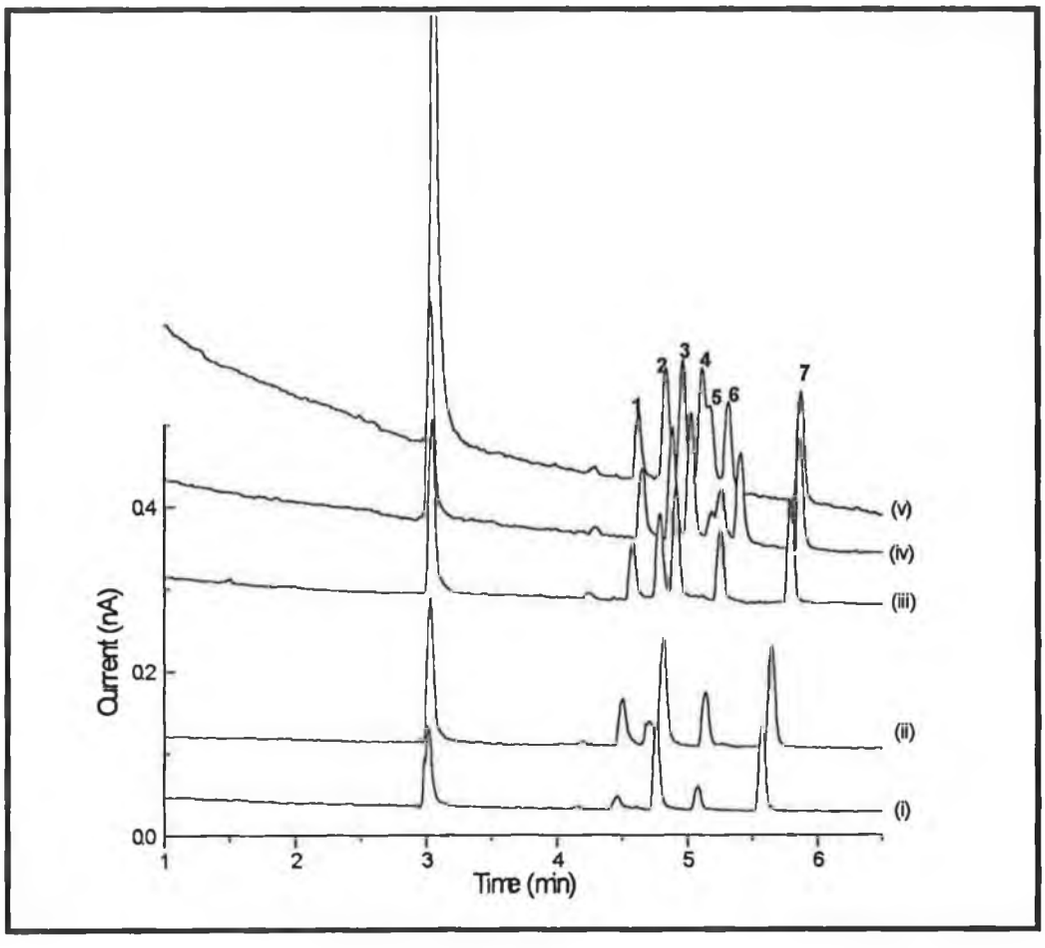


Figure 4-8 *CE separation of phenolic acids 1-chlorogenic; 2-ferulic; 3-caffeic; 4-p-coumaric; 5-vanillic; 6-protocatechuic and 7-gentisic acids at* (i) *650 mV;* (ii) *750 mV;* (iii) *850 mV;* (iv) *950 and* (v) *1050 mV. Electrokinetic injection at 30 kV for 3 s with 25 mM phosphate run buffer, pH 7.2, and 1 M nitric acid in detection cell.*

4.3.2.1 Variation of detection electrolyte composition

Figure 4-9 compares the effect of the composition and pH of the detection cell solution on the electropherograms of the phenolic acid mixture. It has been previously shown with voltammetric experiments in stationary solutions how a reduction in current resulted with neutral pH solutions in the electrochemical cell,

more than likely a result of charge-charge repulsion between the negatively charged phenolic acids and carboxyl groups on the electrode surface. When these pH studies are applied to flowing solutions, such as CE, the effect is more pronounced as the analytes are flowing over the electrode surface, resulting in less ions interacting, and therefore being oxidised, at the electrode surface at any given time. This results in less oxidation current. It is also apparent from Figure 4-9(i) that both background current and noise are much higher in phosphate buffer, pH 7.2, than in acidic media. This is more than likely due to less efficient shunting of the noise due to CE current by the decoupler in basic media.

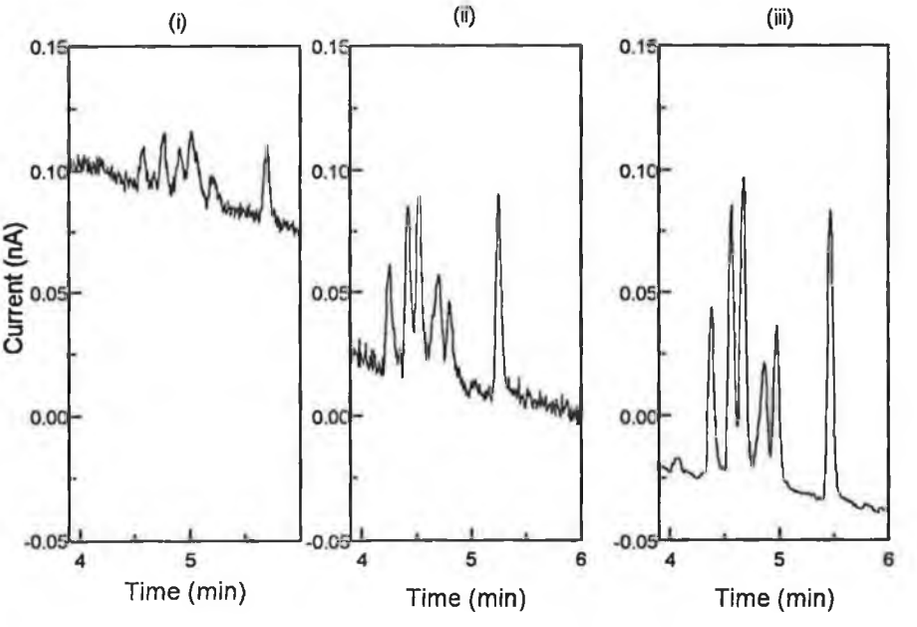


Figure 4-9 *Effect of composition of detection electrolyte on the electropherogram of phenolic acids:* (i) *50 mM phosphate buffer, pH 7.2,;* (ii) *0.1 M nitric acid and* (iii) *1 M nitric acid. 1 μM phenolic acid mixture injected at 30 kV for 3 s with 25 mM phosphate, pH 7.2, as run buffer. Detection was at 950 mV.*

4.3.3 Real sample analysis

Figure 4-10 shows the electropherogram of a beer sample, without any prior clean-up, injected electrokinetically at 30 kV for 3 s and separated at 30 kV with 25 mM phosphate buffer, pH 7.2, as the run buffer. Detection was at 850 mV in 0.1 M nitric acid. The migration rate of ions in normal mode CE is cations, neutrals with anions eluting last presents a problem for the detection of negatively charged phenolic acids.

It is apparent from this figure that the negatively charged phenolic acids, eluting between 6-10 min are eluted on a steeply sloping background as a result of the large concentration of neutral compounds present in alcoholic samples. This steep slope hinders the accurate quantification of the phenolic acids, in addition to the fouling of the working electrode to a certain degree caused by the irreversible oxidation and/or adsorption of sample matrices. This results in irrecoverable changes in background signal, therefore affecting electrode response. This electrode fouling can only be reversed by low frequency activation of the carbon fibre electrode, such as 1 kHz. Characterisation of the electrode has shown that regular low frequency activation results in irreversible fracturing of the electrode surface, thus hindering it useless for further studies.

For this reason, sample clean-up is a necessary part of the analysis of phenolic acids in beverages. Conventional sample clean-up techniques involving solid-phase extraction are expensive and tedious. The removal of positive and neutral peaks from the capillary can be achieved by the use of a large injection volume method. This method was first introduced in 1992 by Chein *et al.*[27] as a stacking technique; the principle of which is shown in Figure 4-11.

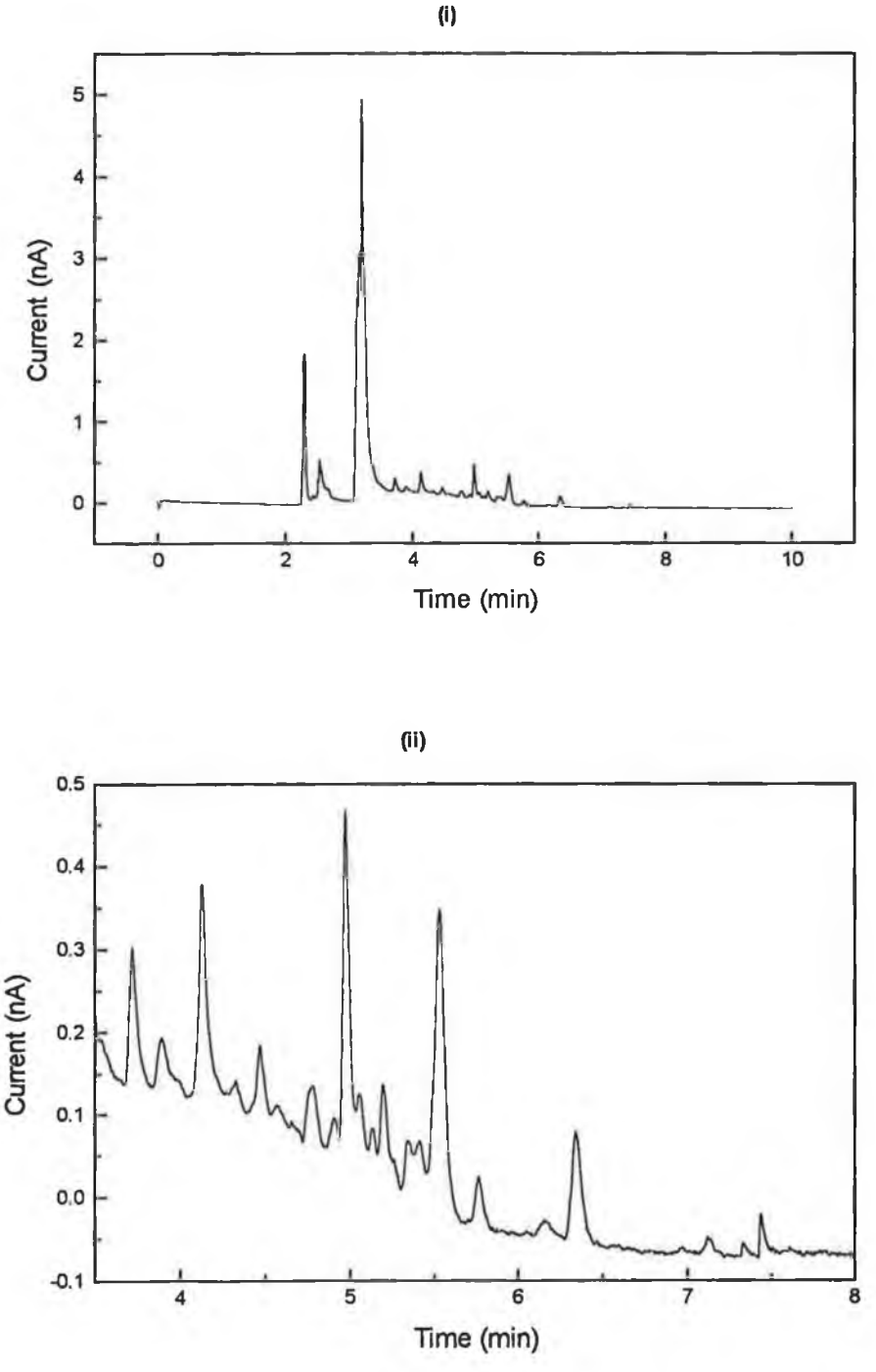


Figure 4-10 (i) *CE separation of a stout sample injected without clean-up for 3 sec at 30 kV;* (ii) *close-up of anions eluting on a steep slope. Experimental conditions: 25 mM phosphate run buffer, pH 7.2, detection in 1 M nitric acid at 750 mV.*

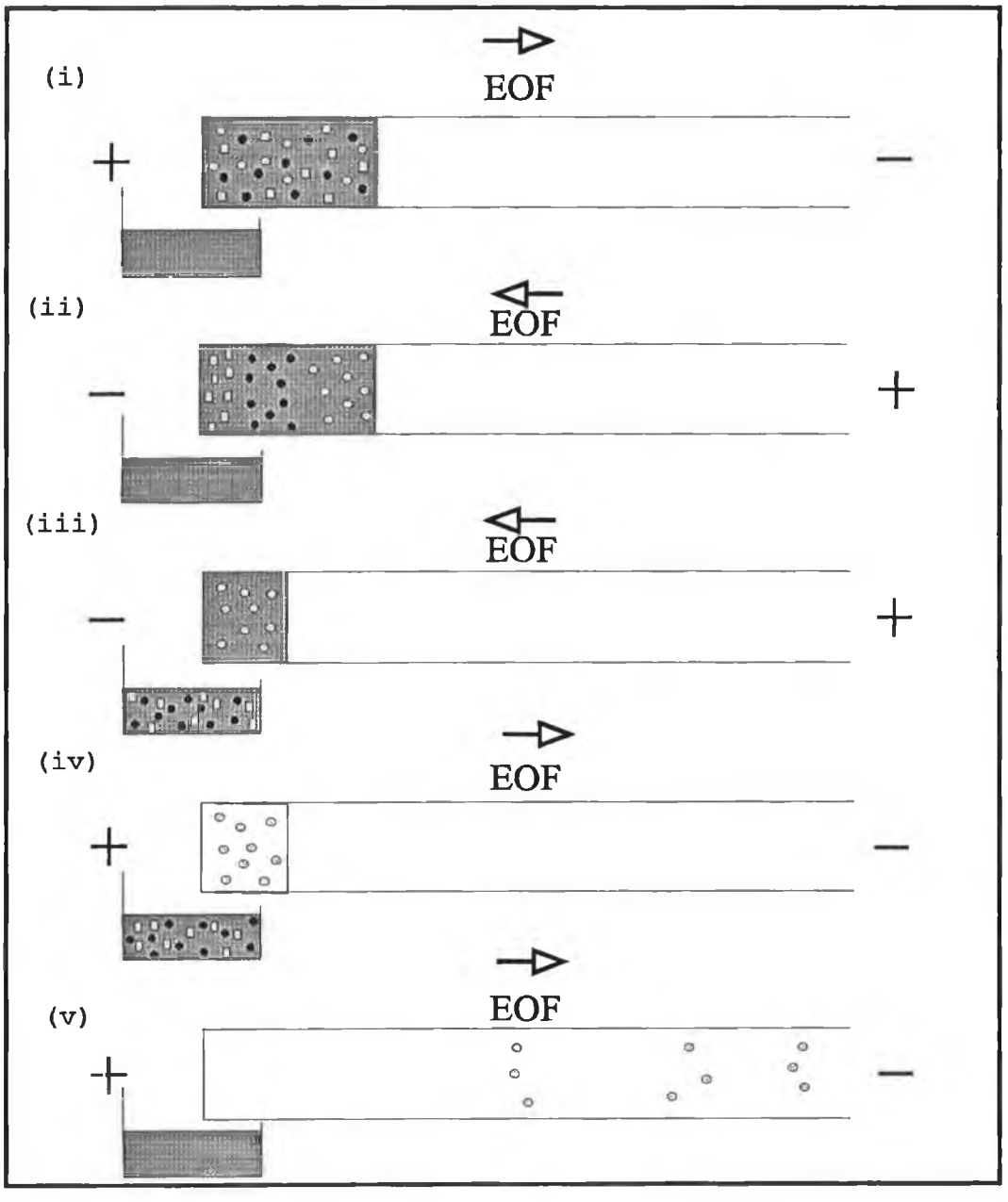


Figure 4-11 *Principle of cation* (□) *and neutral* (●) *ion removal with large injection volume of sample:* (i) *diluted sample injected so as to nearly fill the capillary;* (ii) *polarity switched causing reversed EOF;* (iii) *reversed EOF draws sample buffer carrying cations and neutrals from capillary;* (iv) *with only anions remaining in capillary, polarity is again reversed and* (v) *normal electrophoresis in phosphate buffer, pH 7.2, separates anions.*

The technique involves diluting the sample up to a 100-fold with a very low conducting medium, such as water. Due to the high resistivity of the water, and therefore its high electric field strength, the electrophoretic velocity of the ions within the sample zone is much faster than the velocities within the electrophoretic buffer zone. As a result, the ions migrate very rapidly until they reach the water/buffer interface, at which point their electrophoretic velocity is greatly reduced. This results in a "stacking" of ions at the interface. Figure 4-12 shows the effectiveness of large volume injection in both the removal of cation and neutral compounds from solution as well as the stacking of anions. Figure 4-12(i) shows "normal" electrophoresis; "normal" implying the injection end of the capillary being the anode and the detection end the cathode.

A standard solution containing 10 μM each of dopamine, catechol, caffeic acid and protocatechuic acid prepared in run buffer was injected electrokinetically at 30 kV for 3 s and separated at 30 kV with detection at 750 mV. The positive dopamine and neutral catechol are evident at 2.5 and 3.0 min respectively. In Figure 4-12(ii), a 1/10 dilution of the standard solution with deionised water was pressure injected for 30 s at 20 psi. Electrophoretic conditions were identical to 4-12(i). Once the voltage is applied across the capillary, stacking occurs as described above. However, this long plug of non-conductive buffer which essentially fills the capillary causes most of the electric field strength to drop as a result of high resistivity. This causes the electric field strength in the buffer solution to approach zero, the result of which is a drop in the electrophoretic mobility of ions once they leave the sample plug. The ions therefore stack and move only under the influence of the EOF. This effect can be seen in Figure 4-12(ii) with a broadening of the

analyte peaks. Shorter migration times evident for this diluted sample are also due to the low electric field around the sample as a result of the capillary being 10 % filled with water. This results in a large decrease in the electromigration of ions, causing them to move predominantly under the influence of the EOF. As the zeta potential (ξ) is higher in the water medium due to less buffer ions, the EOF is very fast in the water zone. The net EOF, being the vector sum of the EOF in the buffer and in water, results in the ions eluting faster. Another contributing factor may be the sample ions injected further into the capillary resulting in less capillary length

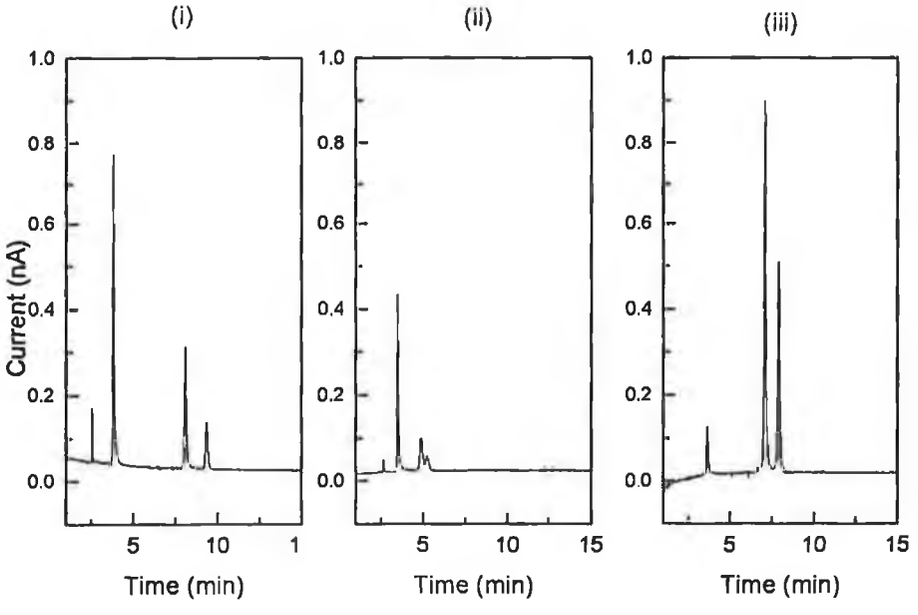


Figure 4-12 *Elimination of cations and neutrals in addition to sample stacking shown with 10 uM standard of dopamine, catechol, caffeic and protocatechuic acid:* (i) *normal electrophoresis;* (ii) *sample diluted 1/50 with water followed by electrophoresis;* (iii) *large injection volume with reversed polarity.*

for separation. This is reinforced by broader peaks resulting from insufficient separation time.

To overcome this effect, Chein *et al.*[27] have described how removing the sample buffer removes the problem of reduction in resolution by distributing the field strength and electrophoretic velocities. By applying a reversed polarity, i.e. the injection end of the capillary being the cathode and the detection end the anode, the sample buffer ions are essentially "pulled out" of the capillary while the stacking is taking place. If the reversed polarity is applied for a sufficiently long time, the positive and neutral ions may be removed from the capillary. This is controlled by monitoring the current. Upon initialisation of reversed polarity conditions, the current initially drops towards zero due to the low conductivity of the sample buffer. As the buffer is removed from the capillary, the current rises towards a steady value. At a certain point, the neutrals and cations have been removed from the capillary and only the anions remain. If the polarity is then switched to normal and electrophoresis is carried out as before, the anions are effectively separated in the absence of cations and neutral compounds. This, along with the stacking effect, is shown in Figure 4-12(iii).Variation of experimental parameters such as separation potential, reversed polarity application time, sample dilution, injection time and detection potential was carried out prior to hydrodynamic voltammetry experiments for peak identification.

Each experiment was carried out with a 10 μM standard solution of dopamine, catechol, caffeic acid and protocatechuic acid prepared by diluting a 100 μ M stock solution freshly each day. Working standards were prepared by diluting stock to the appropriate concentration with water.

4.3.3.1 Separation potential

Variation of both the separation potential and the reversed polarity stacking potential was carried out by preparing a 1/50 dilution of the 10 μM standard mixture and pressure injecting for 30 s at 20 psi. Initially, the stacking potential was maintained at 30 kV while the separation voltages 30 kV, 25 kV and 20 kV were examined. Results indicated that the faster EOF achieved with higher separation potential gave rise to faster elution times, and sharper peaks, but poor resolution between the two phenolic acids. As the potential was decreased, a longer elution time and broader peaks were evident but baseline resolution was attained. For this reason, the intermediate voltage 25 kV was applied for stacking potential variation. Of the electrophoresis potentials investigated (30 kV, 25 kV, 20 kV and 15 kV), 25 kV resulted in maximum peak current and separation.

4.3.3.2 Stacking time

One of the most critical parameters to control is the reversed polarity application time as this affects the degree of sample stacking. Having near-filled the capillary with sample diluted in a low conductivity buffer, initial application of potential (reversed conditions) resulted in very little current flow. As the sample buffer is removed from the capillary and replaced by run buffer (drawn from the detection cell) the conductivity in the capillary slowly rises, and therefore by monitoring the current flow one can control when only the negative phenolic acids remain in the capillary.[27] To optimise this condition for this analysis, the steady state current of the run buffer was determined by applying 25 kV across the capillary and

monitoring the current flow in μA. The current values at 25 %, 50 %, 75 %, 90 %, 99 % and 100 % were calculated and experiments were carried out by allowing the current to reach the respective values before applying normal electrophoresis. Responses are shown in Figure 4-13. Dilution of a 10 μM standard solution containing dopamine, catechol, caffeic acid and protocatechuic acid by 50 and injection for 30 s at 20 psi before the polarity was reversed at 25 kV for the appropriate time and then separated under normal conditions at 25 kV, resulted in the peak height for both caffeic and protocatechuic acids remaining constant until the current was allowed to reach 95 % of the steady state value, from which point a large increase in current was evident. This increase was maintained until the potential was held for 30 s after steady state had been reached, at which point the anions were removed from the capillary in addition to the cations and the neutral ions. At the 25 % value, peaks were broad with little resolution due to inadequate stacking. Ions migrated faster when shorter application times were used. This is again due to the overall EOF in the capillary being faster due to the volume of water remaining in the capillary. It is also evident from this figure that the amount of positive ions removed from the capillary increases as the reversed polarity application time is increased.

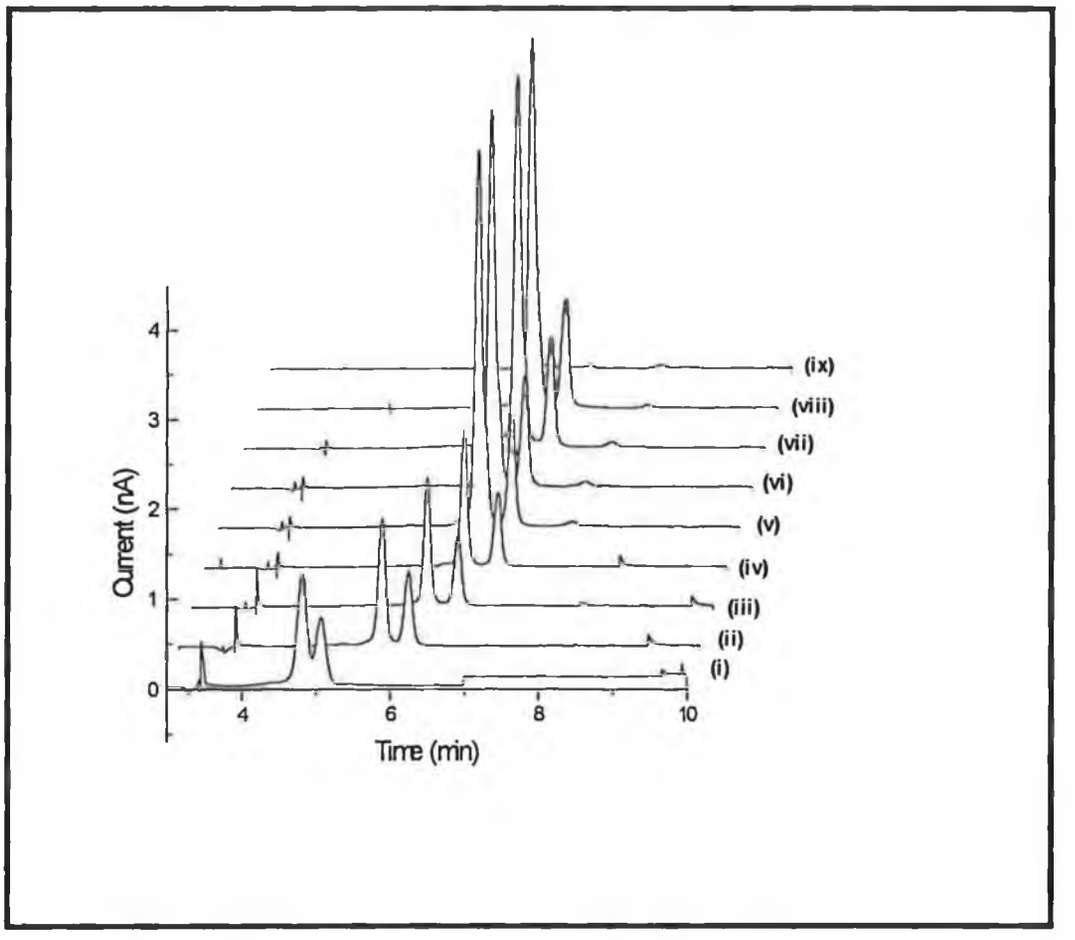


Figure 4-13 *Variation of stacking time under reversed polarity conditions with a 10μM standard solution of dopamine, catechol, caffeic and protocatechuic acid diluted 1/50 with water and injected at 20 psi for 30 s. Reversed polarity was applied at 25 kV to* (i) *25 %;* (ii) *50 %;* (iii) *75 %;* (iv) *90 %;* (v) *95 %;* (vi) *99 %;* (vii) *100 %;* (viii) *100 % + 10 s and* (ix) *100 % + 30 s.*

4.3.3.3 Dilution factor and injection time

By varying the injection time in 5 s intervals from 5 s to 60 s, the current for both phenolics increased steadily up to 30 s above which point no further increase was seen indicating that at this point the capillary was full. A more detailed study was

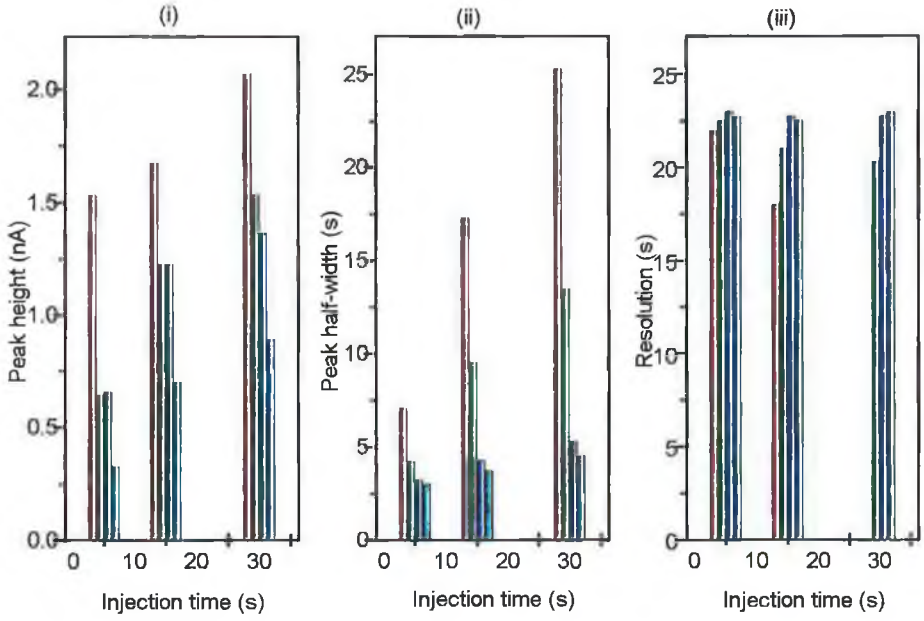


Figure 4-14 *Optimisation of stacking conditions; effects of dilution factors 1/10 (red), 1/25 (green), 1/50 (navy), 1/100 (blue) and injection time (5 sec, 15 sec and 30 sec) on* (i) *peak height;* (ii) *peak half-width and peak resolution* (iii). *Experimental conditions: 25 mM phosphate buffer, pH 7.2, run buffer, 25 kV separation voltage, injection at 20 psi, detection at 750 mV in 1 M nitric acid.*

then carried out by preparing dilutions of 1/10, 1/25, 1/50 and 1/100 and injecting each for 5 s, 15 s and 30 s. The results are shown graphically in Figure 4-14. Although there was not a large variation between the dilutions with respect to peak resolution, the 1/10 dilution showed much broader peaks as the injection time increased resulting in less resolution. A 1/10 dilution injected for 30 s gave no resolution between peaks. The peak half-width decreased dramatically in more dilute solutions giving much sharper peaks indicating more efficient stacking. However, as the dilution factor increased, the peak current decreased therefore affecting sensitivity. With smaller dilutions, shorter injection times were required to

attain sensitivity and resolution. For further standard experiments, a 1/50 dilution was injected for 30 s. Dilutions less than 1/10 did not permit stacking and it was impossible to remove cations and neutrals during polarity reversal.

4.3.3.4 Effect of buffer ionic strength

The effect of buffer ionic strength on stacking and resolution was investigated by varying the concentration of buffer from very low ionic strength (5 mM) to the highest applicable to electrochemical detection (i.e. without the electrophoretic current reaching very high values which would damage the electrode), which was 30 mM phosphate buffer, pH 7.2. The capillary was equilibrated with each new buffer by flushing for 3 min after EDTA washing. The experiment involved injecting a 1/50 dilution of the 10 μM standard solution of dopamine, catechol, caffeic and protocatechuic acid for 30 s at 20 psi. 25 kV was applied across the capillary for both reversal of polarity and electrophoresis. Detection was at 750 mV. Electropherograms are shown in Figure 4-15. The diagram shows that there is a dependency between both the degree of stacking and peak resolution on ionic strength, with both improving as the latter increases. This effect is related to the relative resistivities between the sample and run buffer. The lower degree of stacking obtained with lower ionic strength buffers is more than likely a result of the smaller difference in ionic strengths between the two buffers. In the low-conductivity sample buffer, i.e. water, the electric field strength is high due to high resistivity and therefore the ions migrate rapidly. In the case of a high conductivity run buffer, the ions migrate at a slower rate causing a slower migration rate once

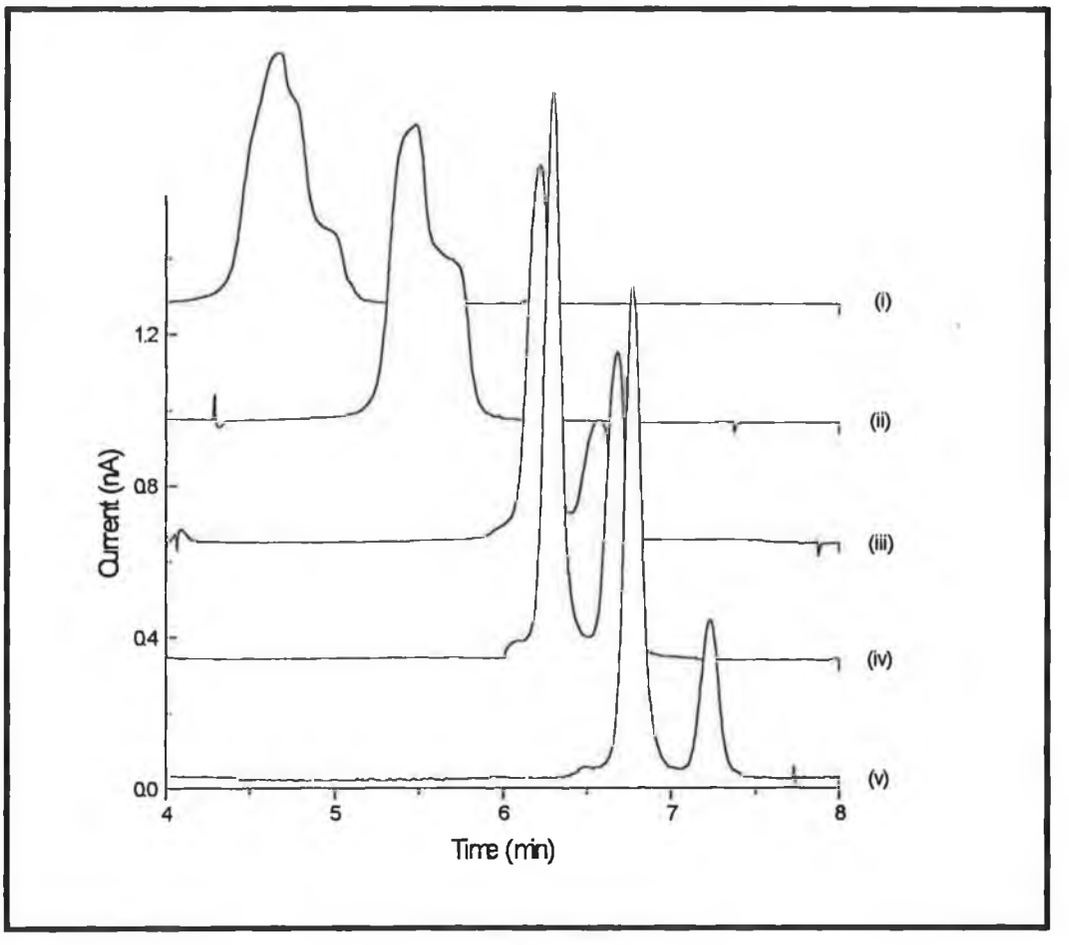


Figure 4-15 *Effect of buffer ionic strength on degree of stacking and peak resolution. Standard solution of 10 μM dopamine, catechol, caffeic and protocatechuic acid diluted 1/10 with water and separated by the Large Injection Volume Method with run buffers of* (i) *5 mM;* (ii) *10 mM;* (iii) *20 mM;* (iv) *25 mM and* (v) *30 mM sodium phosphate buffer, pH 7.2.*

the sample-run buffer boundary is met. This difference in migration speed affects whether ions are stacked into a narrow zone to be separated during conventional electrophoresis. However, when the difference in resistivities is less there is a lower degree of stacking. This can be clearly seen in Figure 4-15(i) and 4-15(ii).

One aspect which effects resolution is the amount of high-resistivity sample buffer remaining in the capillary after polarity reversal. Chein *et al.*[27] discussed how a

long plug of water (in this case 30 s at 20 psi which almost fills the capillary) injected into the capillary causes a redistribution of the electric field strength, resulting in the field strength in the run buffer region to approach zero. Therefore, the electrophoretic mobility of the analyte ions, once conventional electrophoresis is applied, is zero resulting in the ions stacking together and moving along the capillary solely under the influence of the EOF. This effect may be a reason for the loss of resolution evident for low conductivity buffers in the experiment,

implying that sample buffer may remain in the capillary. As stated previously, the removal of sample buffer from the capillary is monitored by the increase in current flow as a result of sample buffer being replaced by higher conductivity run buffer. If the difference in conductivities between buffers is low, then it is difficult to ascertain when all the sample buffer has been removed. This theory is re-enforced by the fact that the unresolved peak in Figure 4-15(i) is, in fact, three peaks implying that catechol was not drawn out of the capillary. Therefore, longer reversed polarity times must be applied when low ionic strength buffers are applied, but this may not ensure good peak separation. Very good peak resolution was obtained for higher ionic strength buffers which means that more sample may be injected into the capillary, an important feature when low levels of analyte are contained in samples. The slower migration times at higher concentration buffers is due to the inverse relationship between the EOF and buffer concentration, resulting in slower EOF at higher concentrations.

4.3.4 Oxidation at higher potentials

Although the di-hydroxphenolic acids (caffeic, protocatechuic and gentisic acid) are easily oxidised at lower potentials, mono-hydroxyphenolic acids (vanillic, ferulic, synapic and p-coumaric acid) require higher potentials, up to 1000mV. This means that if mono-hydroxphenolic acids are to be identified by CE-EC, an efficient removal of neutral ions from the sample must be obtained as these ions

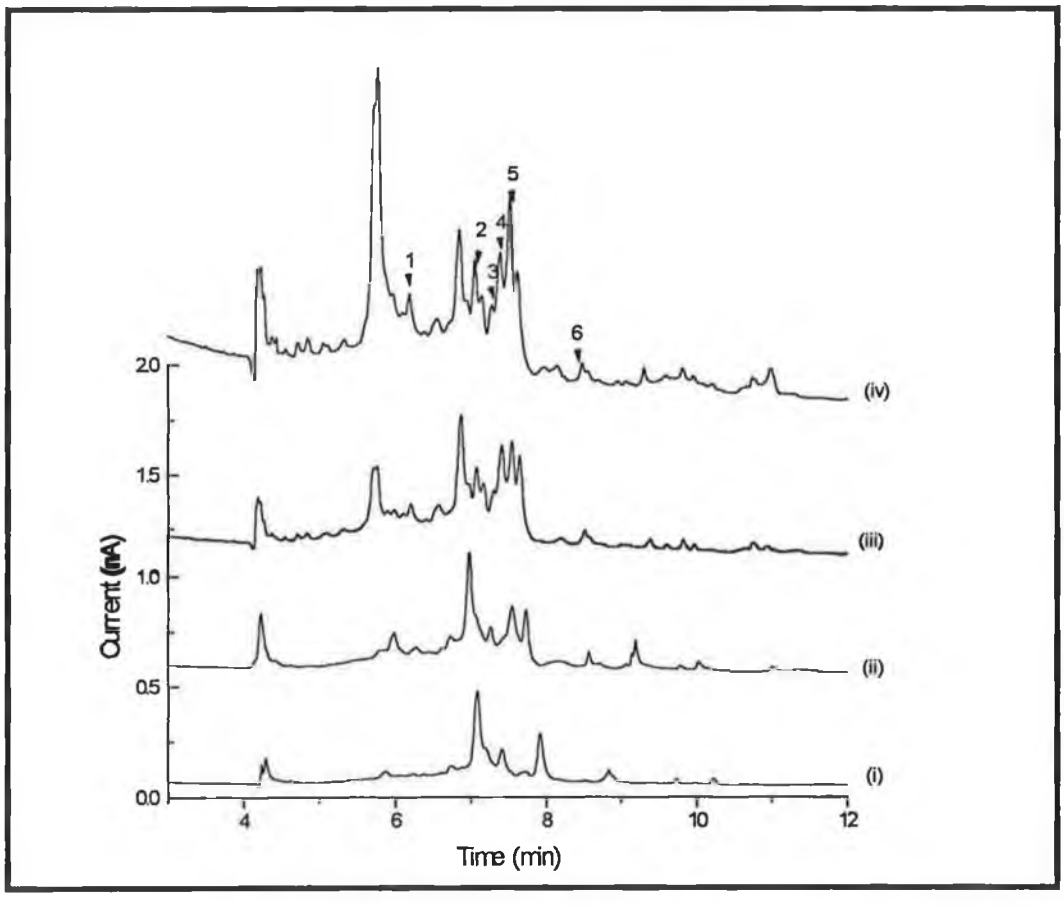


Figure 4-16 *CE of stout sample, diluted 1/10 with water, adjusted to pH 6.5 with 0.1 M NaOH and passed through a C18 cartridge before injection onto the capillary at 20 psi for 3 s. Reversed polarity applied followed by normal mode electrophoresis at 25 kV. Detection at* (i) *850 mV;* (ii) *950 mV;* (iii) *1050 mV and* (iv) *1150 mV in 1 M nitric acid. Peak identification by spiking sample with 1 μM standard solutions. Peak identity - 1, synapic; 2, caffeic; 3, p-coumaric; 4, vanillic; 5, protocatechuic; 6, gentisic acid.*

greatly affect electrode performance. The reason that the stout sample had to be passed through the C_{18} cartridge was to remove a "lump" of hydrophobic anions which greatly interfered with peak identification as the oxidation potential was increased. This problem did not arise with any other beverage sample investigated. Figure 4-16 above shows a stout sample, diluted 1/10 with water and injected for 3 s at 20 psi. It is evident from the diagram that the number of compounds being oxidised is greatly increased at the higher potentials, as to be expected.

4.3.5 Hydrodynamic amperometry

Hydrodynamic voltammetry was carried out on both standards and samples by varying the oxidation potential stepwise by 25 mV from 425 mV to 1050 mV and measuring the current of each peak. A 10 μM standard solution consisting of chlorogenic, ferulic, vanillic, caffeic, protocatechuic and gentisic acids and a stout sample were diluted 1/10 with deionised water before injection onto the capillary for 10 s at 2 psi with detection at the appropriate potential. *i-E* curves were plotted from the measured current against the applied potential. Figure 4-17(i) and 4-17(ii) shows *i-E* curves obtained for the standard mixture (see diagram for peak identification). An electropherogram of the stout sample with detection at 1050 mV, along with numbers assigned to peaks is shown in Figure 4-18(i). It is evident that the large injection volume technique is successful in eliminating the majority of neutral components from the capillary, which is highlighted by the small peak for a very high oxidation potential. It can be seen from the diagram that the hydrophilic anionic "lump" is present even though it has been shown in the previous section

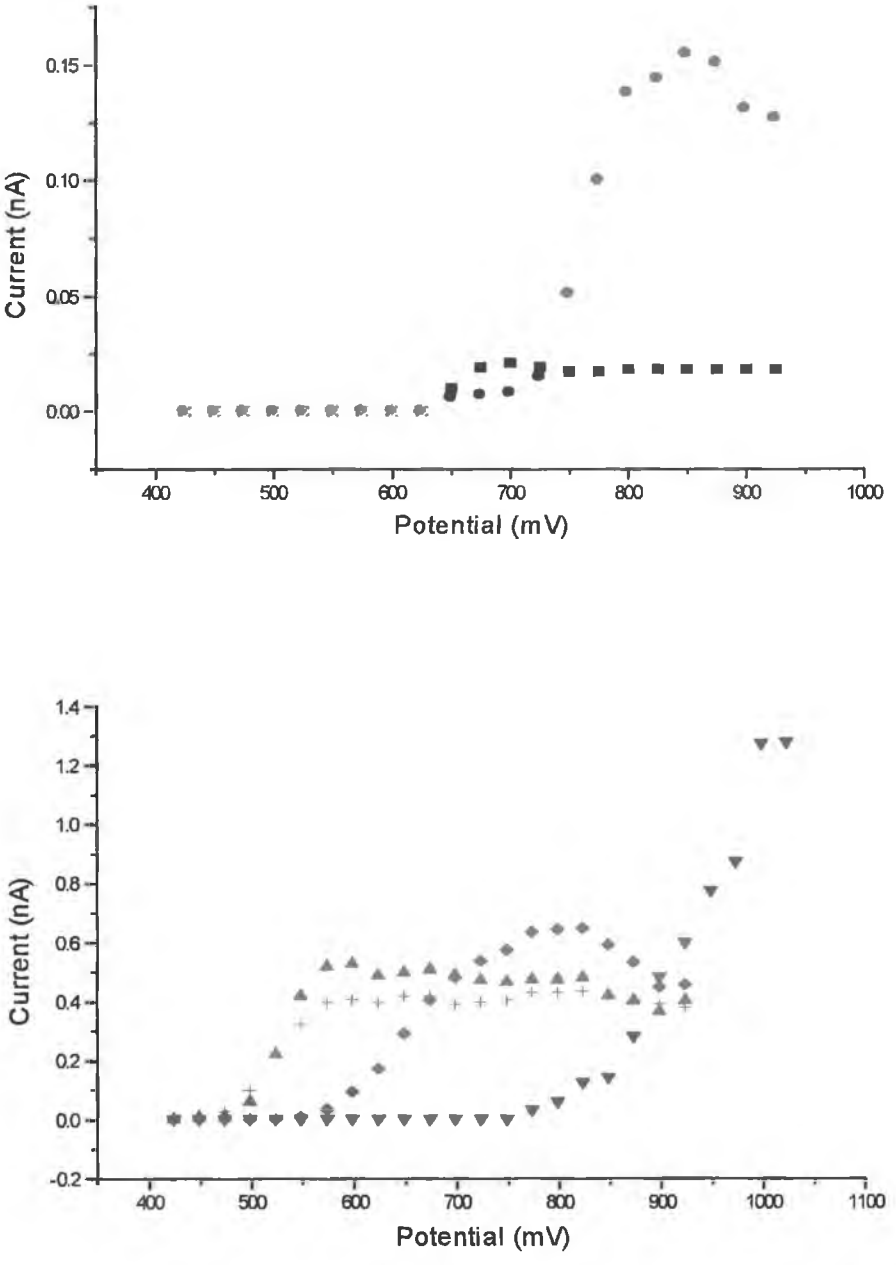


Figure 4-17 *i-E curves obtained by hydrodynamic amperometry of phenolic acid standards:* (i) *chlorogenic acid* (■) *and ferulic acid* (●) *and* (ii) *caffeic acid* (Δ); *vanillic acid* (∇); *protocatechuic acid* (◆) *and gentisic acid* (+). *Standards diluted 1/10 with water, injected for 10 s at 2 psi. Polarity reversal at 25 kV with separation in 25 mM phosphate buffer, pH 7.2, at 25 kV. 1 M nitric acid in detection cell.*

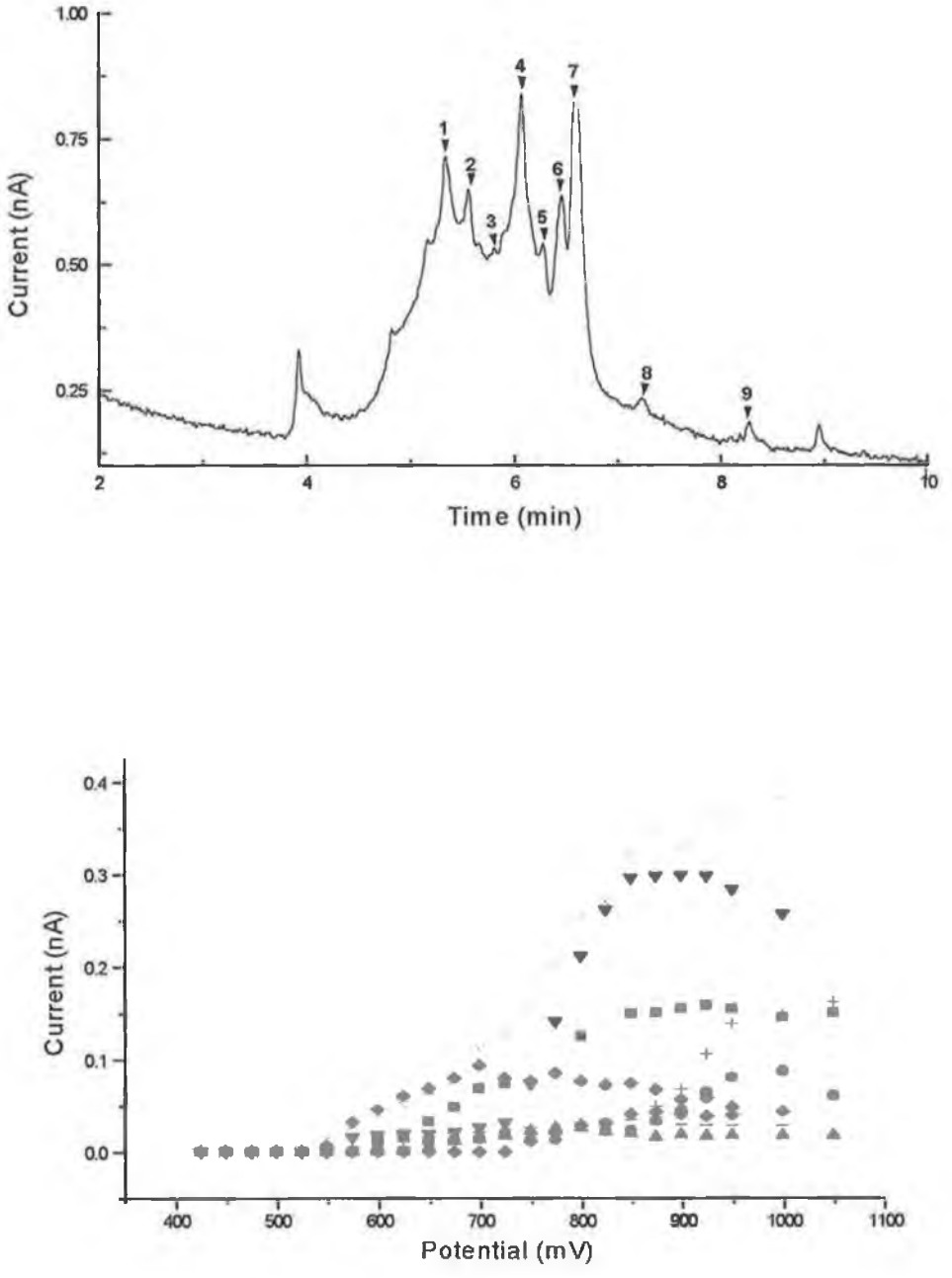


Figure 4-18 *Hydrodynamic amperometry of stout sample showing* (i) *electropherogram at 1050 mV and* (ii) *i-E curves evaluated for the selected peaks. Injection and experimental conditions as for Figure 3-17.*

that passing the stout sample through a C_{18} cartridge preceded by adjusting the pH of the solution to pH 6.5 is effective in decreasing it. As no optimisation of this clean-up was carried out, it was decided to eliminate this step for the hydrodynamic amperometric experiments to avoid analytes remaining on the cartridge. Figure 4-18(ii) shows the *i-E* curves plotted for the peaks numbered in 4-18(i).

Comparison of curve shape and $E_{1/2}$ values of both standards and sample leads to the identification of three of the chosen phenolic acids in the stout sample. Peak no.3 of the sample represents chlorogenic acid, with the very low current resulting either from a much lower concentration compared to other sample components, or from its hindered interaction with the electrode surface, as discussed previously in Section 4.3.2. Peak no. 4 was identified as ferulic acid with an $E_{1/2}$ of 750 mV (evaluated from the peak maximum of the *i-E* plot 1st derivative). Sample peak no. 6 was assigned as vanillic acid, this peak clearly being due to a mono-hydroxyphenolic acid due to the very high $E_{1/2}$ value. Although protocatechuic acid had a very similar curve to that obtained for sample peak no. 5, it is expected to migrate faster that vanillic acid due to its structure, therefore eliminating the peak correlation. There may, of course, be other phenolic acids present in the unidentified sample peaks, which could be confirmed by obtaining hydrodynamic voltammograms of various other acids.

A comparison of sample spiking and hydrodynamic experiments for peak identification accenuates the inaccuracy associated with the former. Peak no. 7 in Figure 4-18(i) was identified by spiking to be protocatechuic acid; however, the non-matching of *i-E* curves proves this to be inaccurate. It was decided therefore,

to investigate whether voltammetric information, in addition to migration times, could confirm many sceptical peak identities.

4.3.6 CE-Voltammetry of phenolic acids

It has already been discussed how electrochemical detection for CE using amperometry presents improved sensitivity over UV techniques and due to the simple instrumentation required compared to other electrochemical detection systems, it is by far the most popular. However, in samples of complex matrices such as those of beverage and food origin, analyte peaks are not fully resolved, migration times vary and peak identification with 100 % confidence is not feasible without further peak characterisation. Evaluation of *i-E* curves gives electrochemical information such as the $E_{1/2}$ value and curve shape unique to each sample component which increases the probability of confident peak identification. To obtain voltammetric information in the amperometric mode (where a single fixed potential is applied between the working and reference electrode) it is necessary to obtain electropherograms at different potentials by stepwise variation of the potential between each electrophoretic run. This procedure, as carried out for phenolic acid standards in Section 4.3.5, is insufficient when electropherograms at only a few potentials are obtained, and are extremely lengthy and laborious when a wide range of potentials are applied in order to obtain more detailed curves. In addition, this work requires a very stable working electrode with very reproducible surface activation methods to avoid response fluctuations over the prolonged use.

With the application of voltammetric detection, however, where the potential domain is added to the time domain of the CE or HPLC system, a potential range is scanned for each point in the time domain resulting in characteristic *i-E* curves for both background and sample components, which allows for highly confident peak identification. Many researchers have studied the application of voltammetry to detection in flowing solutions,[28,29,30,31] with the development of CE-voltammetry raising increasing interest in recent years.

It has already been shown how neutral compounds can be removed from a complex beverage sample by injecting a large volume of sample, diluted in low conductivity buffer, into the capillary and reversing the polarity to draw out the sample buffer containing these ions, before separating the remaining anions in normal CE mode. However, we can see from the diagrams that a wide range of anionic compounds exist in these samples, and to selectively extract phenolic acids using solid phase extraction would involve intricate and time-consuming techniques, especially when analyte concentrations are low. It was the aim of this research to investigate the use of voltammetric detection to distinguish between two co-eluting peaks under differing concentration levels and to investigate the effect of relative concentrations on the *i-E* curves extracted for each compound.

4.3.6.1 Coupling voltammetry to CE

One of the most difficult problems to overcome when coupling voltammetric detection to CE is ohmic potential (iR) drop leading to distortion of *i-E* curves

which can mask important electrochemical characteristics, therefore reducing the selectivity of the technique.

iR drop is caused by a potential gradient occuring along the length of the electrode which results in lower peak currents and a shifting of the $E_{1/2}$ to higher values. White *et al.*[32] have given examples of voltammetric waves distorted by iR drop. This lack of uniformity of potential is most at risk in thin layer cells where the reference electrode is held upstream of the working electrode in the path of the working electrode current.[33] The actual potential difference between the working and reference electrode varies according to Ohm's law i.e. the product of *i* and *R* of the solution. Therefore, the potential at the downstream end of the electrode is nearest the one applied by the potentiostat but the potential at the upstream end can be below the $E_{1/2}$ of the analytes, resulting in complete distortion of the *i-E* curves. The problem increases as the concentration of the analytes increase due to the increase in faradaic current. iR drop can be minimised by the use of higher conductivity buffers but this is limited in CE applications due to Joule heating. Placing the working and auxiliary electrodes as close as possible together is one way of reducing the problem.

Another approach to reducing iR drop is to decrease the electrode surface area by miniaturizing the electrode size, therefore decreasing the current flow. Carbon fibre electrodes as working electrodes for rapid potential-scanning detection systems in flowing solutions are particularly advantageous due to the cylindrical diffusion towards the fibre producing a high rate of steady-state mass transport to the surface resulting in rapid signal stabilisation. These completely steady state voltammograms are of sigmoidal shape which simplifies the characterisation of

electrode processes such as $E_{1/2}$, i_{lim} and the differentiation between reversible and irreversible processes through wave shape and $E_{1/4}$ - $E_{3/4}$ values.[33]

However, both Ferris *et al.*[34] and White *et al.*[32] have reported distortion of voltammetric curves due to the ohmic drop when using microelectrodes for potential scanning. Ferris *et al.* used a 5 μm i.d. microelectrode to detect dopamine, adrenaline and catechol for detection in CE. She found the correlation coefficient representing linearity of concentration and current to be less than 1, thus proving that the currents obtained for higher concentrations were less than expected due to iR drop. White *et al.*[32] showed an increase in peak current with oxidation at lower $E_{1/2}$ for hydroquinone when higher conductivity buffers were used with a 9 μm o.d. carbon fibre electrode. This variation with buffer ionic strength proves that iR drop is still a problem, even with miniaturized working electrodes. Both these reports prove that, although microelectrodes can reduce the problem of iR drop, they do not in fact eliminate it.

4.3.6.2 On-column electrical decoupling

The problem of iR drop addressed above when coupling CE to electrochemical detection was overcome by Park *et al.*[35] in 1995 with the development of an on-column electrical decoupler made using a 1 mm long cast Nafion membrane located 2 mm from the capillary end separating the electrophoresis capillary with the capillary end housing the working electrode. This membrane was sufficient to completely isolate the electrochemical cell from the electrophoresis current with its large conduction channels. A cast Nafion diffusion barrier was used by Park *et al*[35]

in the same set-up, 1 mm in length and placed at the end of the capillary so as to completely enclose the detection electrode, to act as an enhancer of electrical conductivity. The CE cathode is placed in the cathodic buffer reservoir. The preparation of both decouplers has previously been described.[35]

4.3.6.3 CE-voltammetry experimental conditions

The experimental conditions for voltammetry were very similar to those of amperometry. A 50 μM i.d. capillary 65 cm in length was used in the separation of the phenolic acids for system optimisation to investigate various parameters such as effect of separation voltage, buffer ionic strength and scan number. For co-eluting peak distinction, the capillary was cut by 10 cm. A dual-electrode system with digital background subtraction was used to minimise charging current and instrument noise. Two carbon fibre electrodes with very similar background currents and waveforms were used- one placed in the path of the analytes in the end-column Nafion diffusion barrier as for amperometric detection and the other secured in the electrochemical cell.

30 kV was used for separation unless otherwise stated in the text. The cathodic reservoir was filled with 1 M HCL and the detection cell was filled with 100 mM phosphate buffer, pH 2.1.

To regenerate the capillary, flushing with 0.5 M EDTA, pH 13.0, was carried out for 30 s, followed immediately with flushing for 60 s with run buffer. Care was taken not to allow the basic solution to remain in the capillary for long periods of time to avoid salting out which may cause cracking at the capillary/Nafion joints.

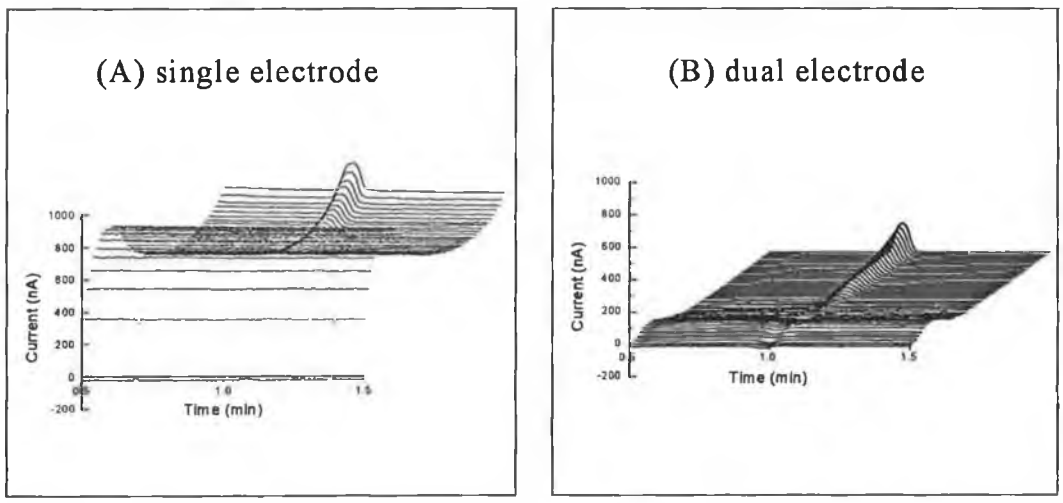
Electrode surface regeneration was carried out by applying electrochemical activation on both fibre electrodes simultaneously at ± 1750 mV at a frequency of 10 kHz for 60 s.

4.3.7 Optimisation of CE-voltammetry detection conditions

4.3.7.1 Electrochemical detection:dual versus single electrode

It has already been discussed how large charging currents associated with voltammetric detection limit the concentrations of analytes which can be detected. Although digital background subtraction can reduce the interference of charging current, it can be seen in Figure 4-19(A) that it does not effectively eliminate electronic or environmental noise. Dynamic charging current subtraction, carried out with a dual-electrode system controlled by a bipotentiostat has this ability, and therefore has the potential to obtain limits of detection in the high nanomolar range. With this subtraction system, any background signal recorded at the detection electrode (placed in the Nafion diffusion barrier) such as charging current or environmental and electronic noise, is also recorded at the second electrode and subtracted in real time so as not to hinder data acquisition. It can be seen in Figure 4-19(i) that even before digital background subtraction has been applied, there is a large improvement in background signal. Park *et al.*[12] saw an improvement of a factor of 20 for this subtraction method.

(i) Voltammograms of 10 μM protocatechuic acid
before digital background subtraction



(ii) Voltammograms of 10 μM protocatechuic acid
after digital background subtraction

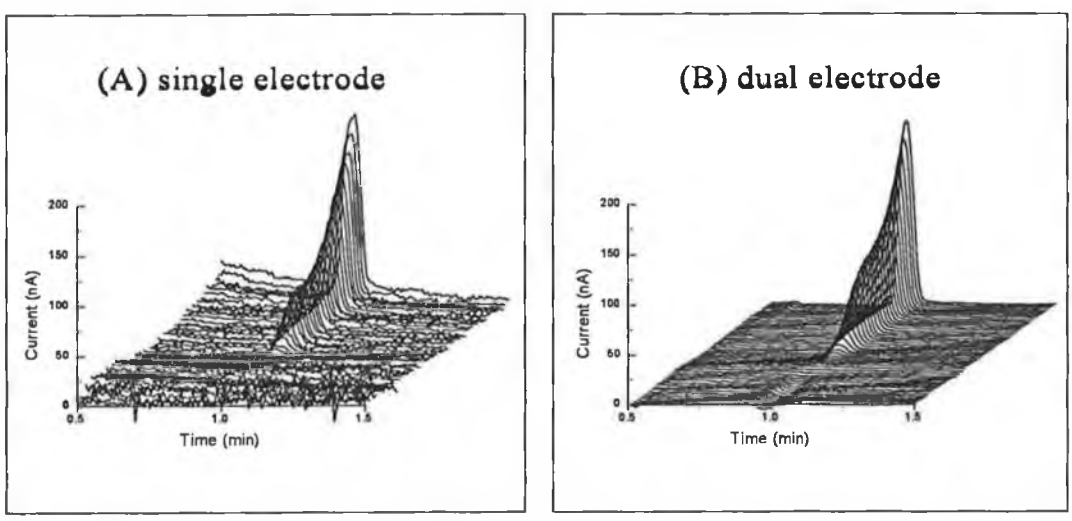


Figure 4-19 *Electropherograms of protocatechuic acid showing effect of background subtraction on signal using* (A) *a single- and* (B) *dual- working electrode. Potential scans were made in the range 100-1300 mV with detection in 100 mM phosphate buffer, pH 2.1. Injection was at 3 psi for 2 s, separation was at 30 kV in 25 mM phosphate buffer, pH 7.2.*

4.3.7.2 Buffer ionic strength

Once a freshly-made on-column electrical decoupler had been attached to the capillary, it was necessary to investigate its conductive properties for the elimination of iR drop. It has already been stated that increasing buffer concentration decreases iR drop but CE-voltammetry is limited to buffers of concentration below 500 mM. If the Nafion decoupler is effective in dispensing the electrophoresis current, then the analyte *i-E* curve shape should be independent of buffer ionic strength. A concentration of 100 μM protocatechuic acid was injected for 2 s at 2 psi and separated with 100 mM, 50 mM, 25 mM, 12.5 mM and 6.25 mM phosphate buffer, pH 7.2. Separation was carried out at 30 kV and detected in the range 0-1200 mV with a scan number of 5 and 100 mM phosphate buffer, pH 2.1 as the detection electrolyte. A dual electrode system with digital background subtraction was applied. Characteristic *i-E* curves were extracted from electropherograms of each buffer at the peak maximum using the Origin 3.5 plotting package. Results are shown graphically in Figure 4-20. It is evident that there is little variation in current magnitude and $E_{1/2}$ values across the range of ionic strengths. If iR drop was effecting this experiment, it would be expected that $E_{1/2}$ values would be increasing with decreasing buffer ionic strength, which is clearly not occuring in this case, and so it can be concluded that the Nafion on-column decoupler is efficient in dispensing the electrophoresis current once it leaves the capillary. The peak- rather than sigmoidal-shape of the wave indicates that the electrode is slightly overactivated.

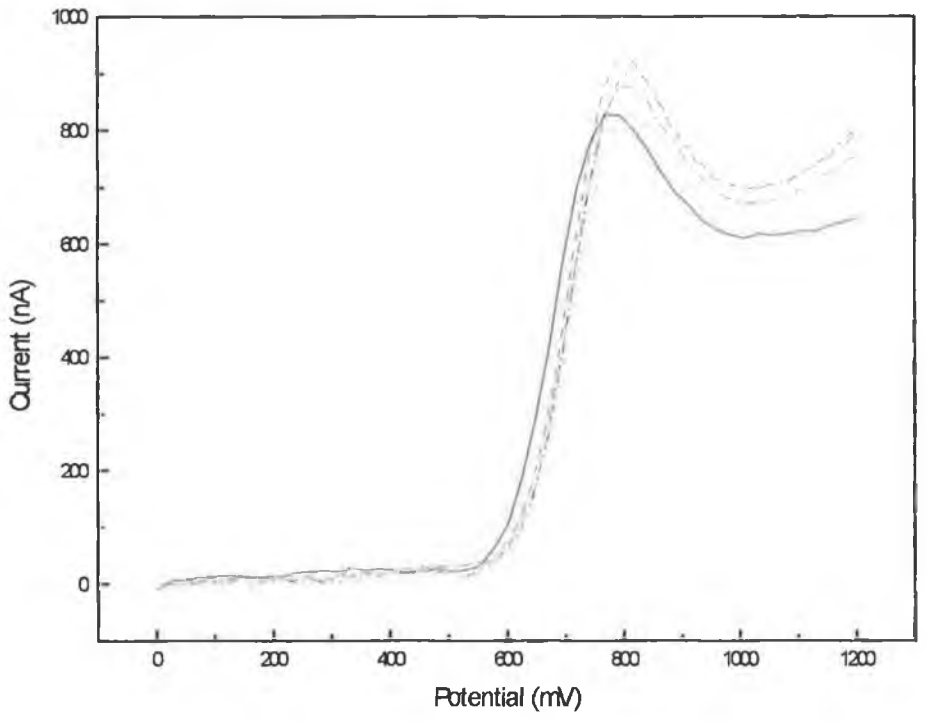


Figure 4-20 *Shunting of electrophoretic current by on-column Nafion decoupler seen by constant response slopes with varying run buffer ionic strength to 100 μM protocatechuic acid. Experimental conditions as for Figure 4-19.*

4.3.7.3 Effect of scan rate

Variation of scan rate and scan number in CE-voltammetric analysis can cause a large variation in both sensitivity and background current magnitude of analyte *i-E* curves. For each time point in the CE-domain, a potential scan was carried out in the chosen potential range with each scan consisting of 50 potential points. If multiple scans are taken at each time point, quantitation errors are reduced. However, if for example the scan range is 1000 mV and the scan time is 0.5 s

(restricted by hardware), then a faster scan rate is required for an increasing number of scans. Each scan requires 400 ms for single scan mode, 100 ms for 2 scan mode, 70 ms for 3 scan mode, 55 ms for 4 scan mode and 45 ms for 5 scan mode, with the time of one scan allocated at the beginning of each where the electrode is held at the initial potential to allow for nominal interference of large charging currents caused by the sudden potential drop from the highest potential of the previous scan. Time is also allocated at the end of each scan for data calculation and display. These scan times correspond to scan rates of 2.5 V/s, 10.0 V/s, 14.3 V/s, 18.2 V/s and 22.2 V/s for 1,2,3,4 and 5 scans per 0.5 s, respectively. A more detailed discussion of the hardware involved in the data acquisition has been carried out by Park[12] and is beyond the scope of this project.

Park[12] discovered that although background current magnitude increased linearly with scan rate, the sensitivity (measured by peak current) was linearly dependent on the log of scan rate from 2-20 V/s and independent above this rate. An exponential relationship exists between the ratio of peak current to background current and scan rate. This means that a slower scan rate achieves a higher signal to noise ratio, with higher scan rates resulting in higher faradaic signals but with much higher background currents. This drawback is insignificant, however, when dynamic charging current subtraction is applied as most of the background current is eliminated. To choose a scan rate number optimum to this analysis, the scan number was varied from 1 to 5.

Electropherograms were obtained for 100 μM standard solution of caffeic, protocatechuic and gentisic acids with injection for 2 s at 2 psi. Other experimental conditions remained constant as in Section 4. Figure 4-21 and 4-22 shows a

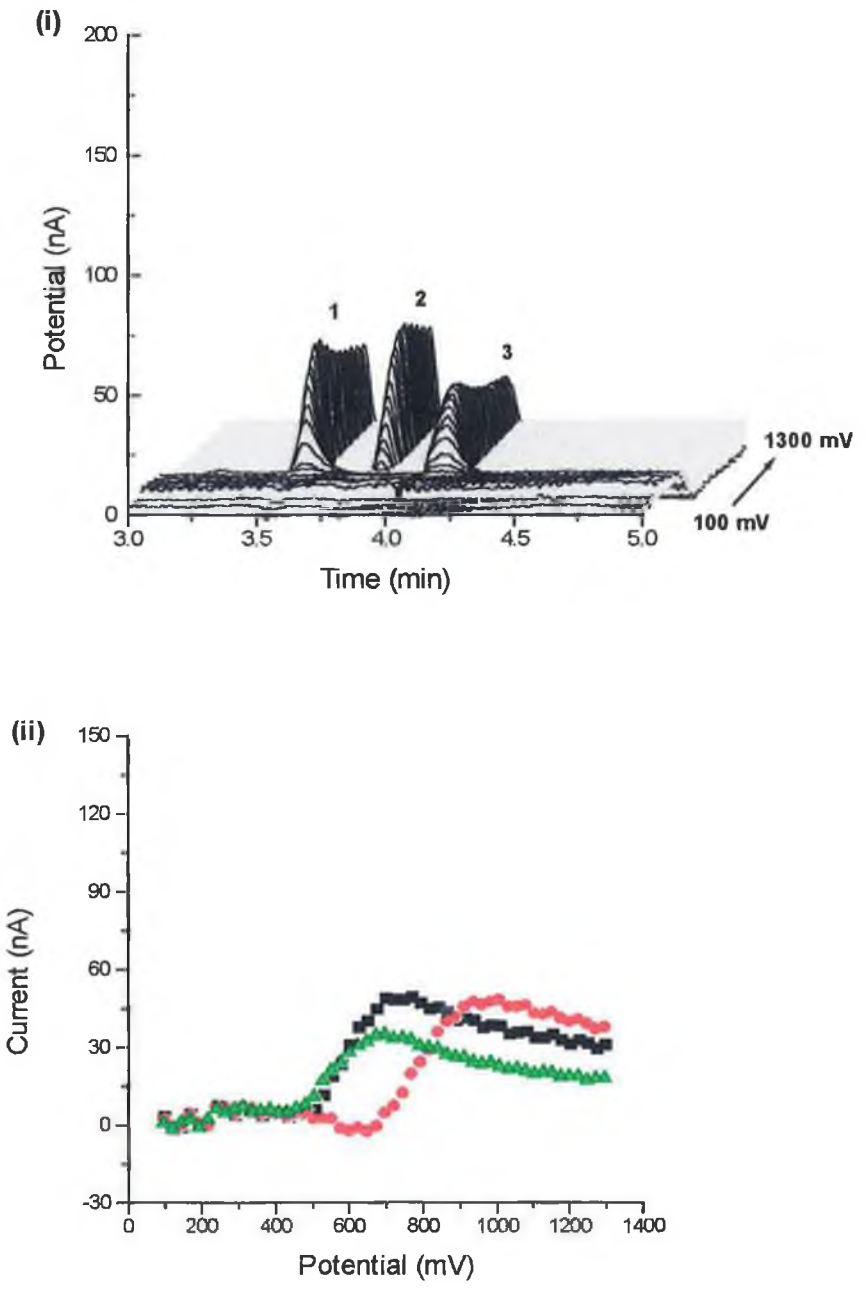


Figure 4-21 (i) *Electropherograms and* (ii) *extracted i-E curves of* 1, *caffeic acid* (■); 2, *protocatechuic acid* (●) *and* 3, *gentisic acid* (Δ) *in single scan mode. Separation conditions: 25 mM phosphate buffer, pH 7.2, 2 s injection at 2 psi, cathodic buffer reservoir filled with 1 N HCl and detection cell filled with 100 mM phosphate buffer, pH 2.1*

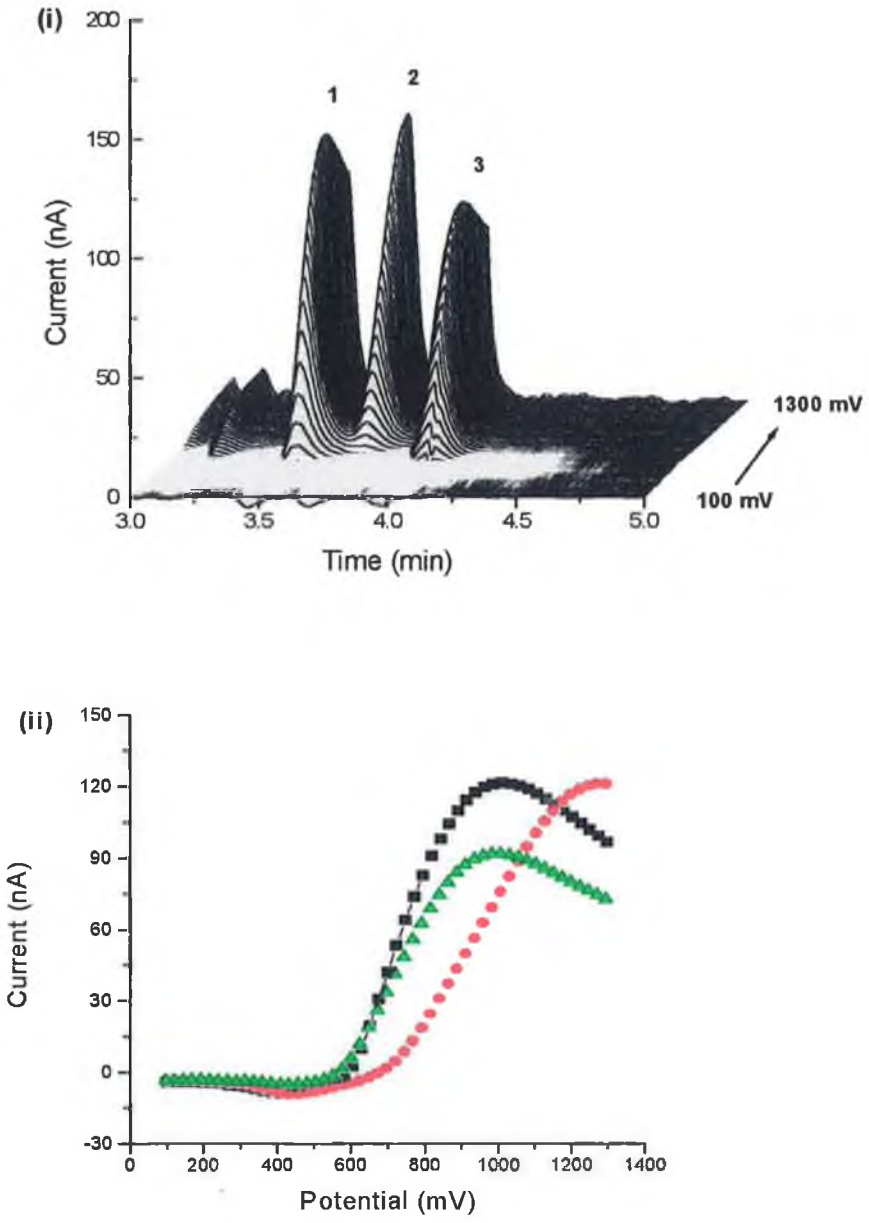


Figure 4-22 *Increased current without interference of an increase in background noise when the scan number is increased to 4 for* 1, *caffeic acid* (■); 2, *protocatechuic acid* (●) *and* 3, *gentisic acid* (Δ). *Conditions as for Figure 4-21.*

comparison between single scan and a 4 scan mode, showing (i) the electropherograms of each and (ii) the *i-E* curves of the 3 analytes extracted from the electropherograms. When choosing an optimum scan number (and therefore, scan rate), it is important to compare S/N ratio (more importantly, peak current), obtain the lowest noise following background subtraction, good peak separation in the CE domain and, most importantly, well defined and distinctive *i-E* curves. In the diagram, it is evident that a single scan results in low peak current and broad analyte peaks with indistinguishable *i-E* curves for caffeic and protocatechuic acid. A scan number of 4, however, shows large peak currents and very well defined *i-E* curves for all 3 analytes with very distinct $E_{1/2}$ values as well as very low background noise level permitting confident peak identification. Intermediate scan numbers (results not shown) resulted in intermediate peak current and although a scan number of 5 gave high peak currents, the background noise level increased dramatically. A scan number of 4 was used in all future experiments.

4.3.8 CE-voltammetric identification of phenolic acids

An on-column decoupler was used in the detection of a 100 μM mixture of 7 phenolic acids (Figure 4-23) to enable post-column pH modification for CE analysis with voltammetric detection. Separation conditions were chosen such that full peak resolution was not achieved so as to investigate the potential of voltammetry to distinguish between analytes in a mixture. 25 mM phosphate buffer, pH 7.2, was used as the run buffer with a separation voltage of 30 kV with

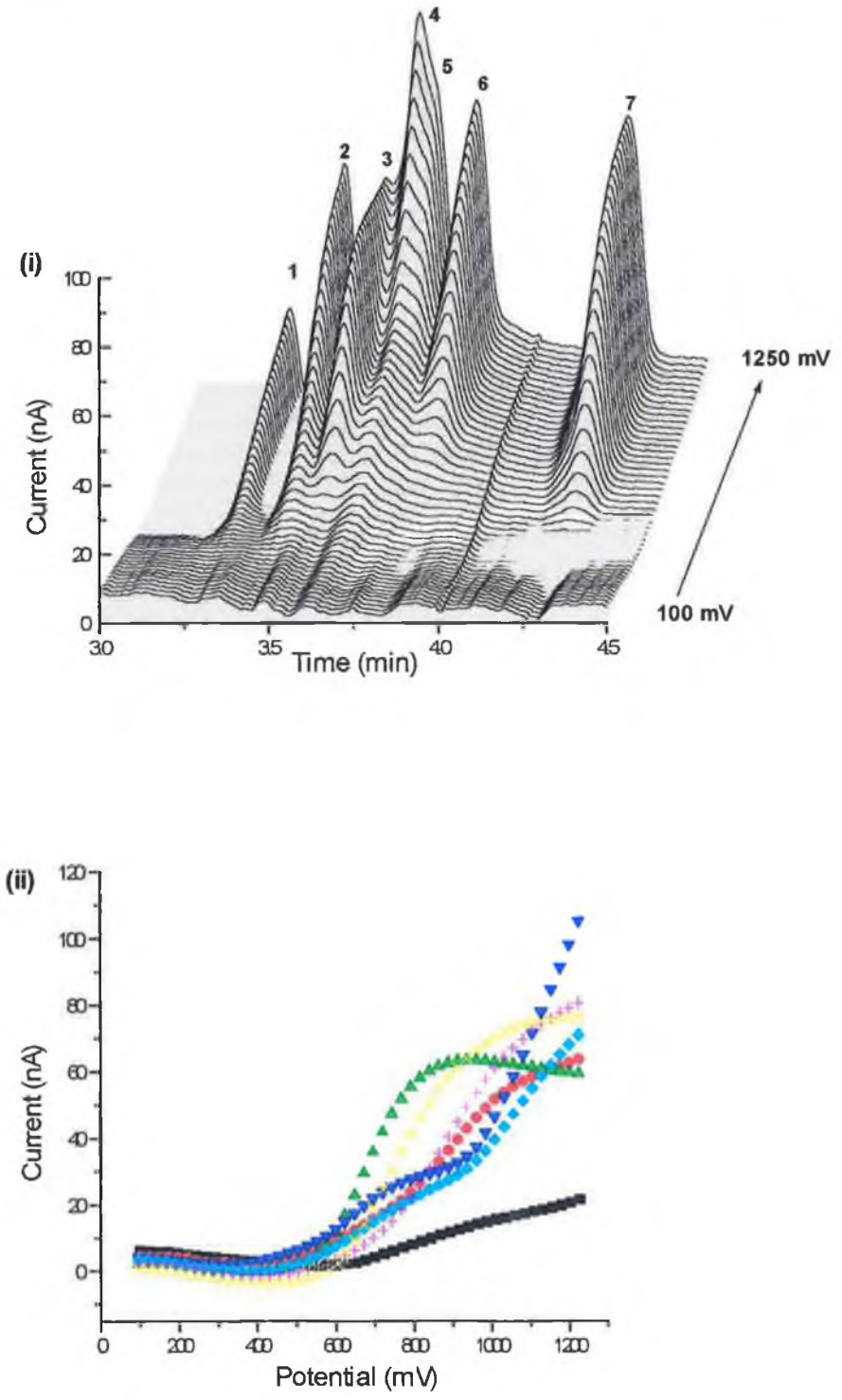


Figure 4-23 (i) *Electropherogram of 100 μM phenolic acid mixture and* (ii) *extracted i-E curves at peak maxima. Peak identities:* 1, *chlorogenic acid* (■); 2, *ferulic acid* (●); 3, *caffeic acid* (Δ); 4, *p-coumaric acid* (▽); 5, *vanillic acid* (◆); 6, *protocatechuic acid* (+) *and* 7, *gentisic acid* (×). *Conditions as before.*

pressure injection at 2 psi for 2 s. Because of the large concentration of analytes, broad peaks were obtained. The cathodic buffer reservoir was filled with 1 M HCl and the electrochemical cell was filled with 100 mM phosphate buffer, pH 2.1. The electropherogram of the mixture (following digital background subtraction) is shown in Figure 4-23(i). Data was transferred to the Origin plotting package, and the *i-E* curve for each peak was extracted from the data at the peak maximum. The resulting *i-E* curves are plotted in Figure 4-23(ii). It can be seen that each acid gives a very distinct *i-E* curve corresponding to its electrochemical properties. It is evident from the plot that the monohydroxy-phenolic acids, p-coumaric acid (▽) and vanillic acid (◆) have not yet reached their limiting current plateau at 1250 mV, and in subsequent experiments scans were made in the potential range 200-1400 mV. The characteristic low current response of chlorogenic acid (■) is evident and as stated previously is more than likely due to the molecule's large size hindering its interaction with the electrode surface.

Variation of run buffer concentration to 6.25 mM, 12.5 mM, 25 mM and 50 mM phosphate, pH 7.2, increased peak resolution, current and decreased migration time as was predicted. It was decided to investigate further the capacity of using this voltammetric scanning as a technique to distinguish between co-eluting peaks or peaks without baseline resolution. The application of this technique calls for analytes to possess differing and distinct electrochemical properties. If, for example, it is necessary to distinguish two mono-hydroxyphenolic acids in a mixture, both of which are oxidised at high positive potentials and have very similar *i-E* curves, it would be necessary to consider both migration time and migration order before being able to make a positive identification. If, however, the

co-eluting peaks have very distinct electrochemical characteristics (and therefore *i-E* curves), it is possible to make a more definite identification.

4.3.8.1 Co-eluting peaks

With a 55 cm long, 50 μM i.d. fused silica capillary, a run buffer of 10 mM phosphate pH 7.2 and 30 kV separation voltage, vanillic acid and protocatechuic acid co-elute. Injection was at 3 psi for 2 s. A 100 μM standard mixture of the acids resulted in two distinguishable peaks, without baseline resolution. Figure 4-24(i) shows the electropherograms of the acids, it being evident that a more sensitive response is obtained for protocatechuic acid. *i-E* curves, extracted at 9 different time points along the analyte peaks, investigate the influence of peak proximity on *i-E* curve shape. It is evident from Figure 4-24(i), where both acids are in equi-concentration, that the *i-E* curves extracted at the peak maxima for each compound (Δ, vanillic acid and ×, protocatechuic acid, respectively) are single component sigmoidal-shaped curves, without superimposition on each other. Both peaks are distinctly shaped, with protocatechuic acid being oxidised at lower potentials and reaching a limiting current at 1200 mV and vanillic acid being oxidised in the 1100 mV potential range without reaching its limiting value at 1300 mV.

However, the *i-E* curves extracted at intermittent points between the peak maxima show definite characteristics of both acid curves e.g. in the *i-E* curve (◆) extracted at point "e" of the electropherogram, the $E_{1/2}$ value is at higher potentials as expected for mono-hydroxyphenolic compounds with the peak shape being

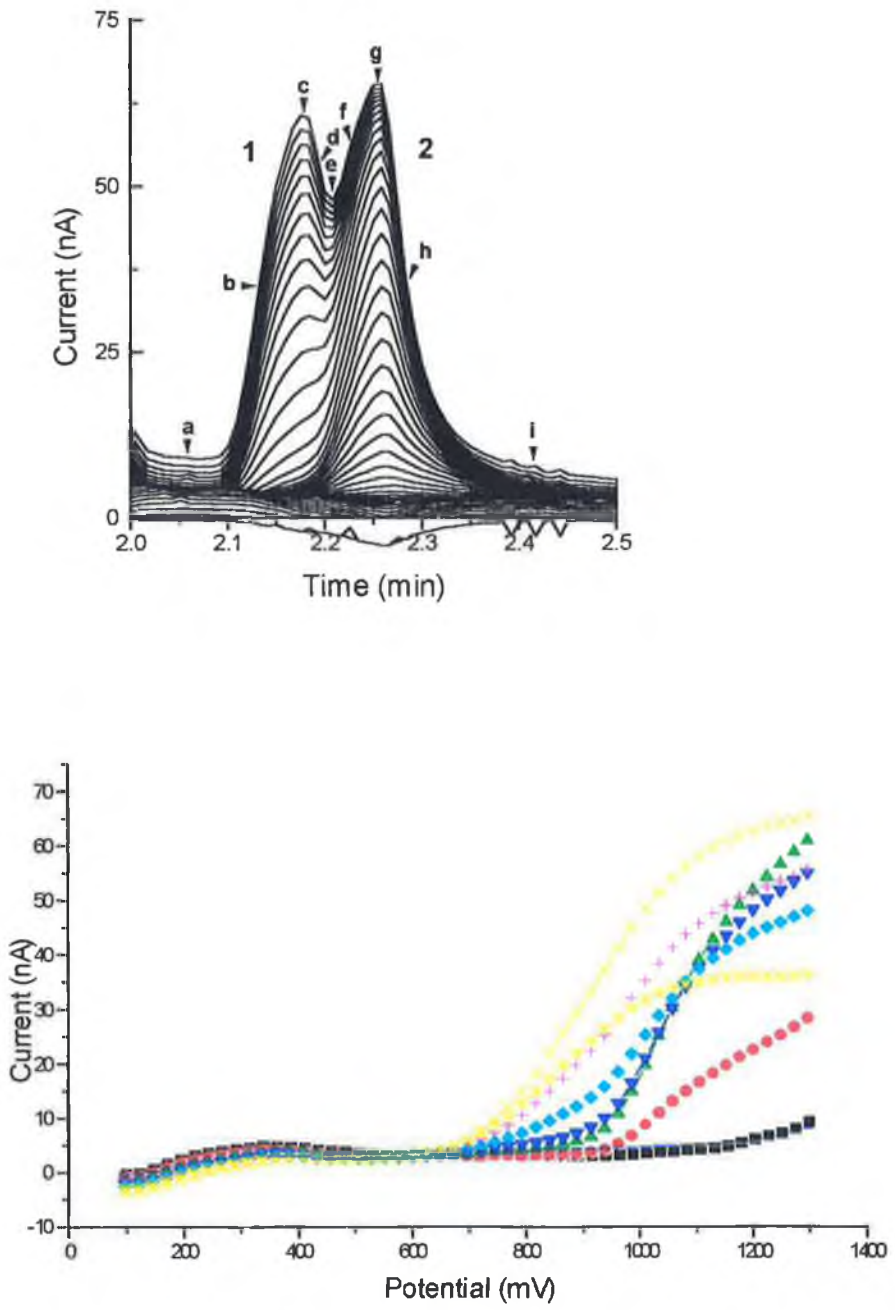


Figure 4-24 *Effect of peak proximity on shape of i-E curves when* 1, *100 μM vanillic acid and* 2, *100 μM protocatechuic acid co-elute. Curves* (ii) *were extracted from the electropherogram* (i) *at points a to i represented by* ■, ●, △, ▽, ◆, +, ×, ✱ and –, *respectively. Co-elution achieved using a 55 cm capillary with 10 mM phosphate run buffer, pH 7.2, and injection for 2 s at 2 psi.*

characteristic of protocatechuic acid. From the curves, it is evident that a marked curve distinction exists at points "b" and "h" on the electropherogram ((•) for vanillic acid and (*) for protocatechuic acid curves, respectively) although the oxidation current is lower. It is also evident from Figure 4-24(ii) that protocatechuic acid has much more dominant electrochemical characteristics in that its sigmoidal curve shape prevails in the curve extracted at the point of co-elution of the two acids. The symbols (▪) and (—) represent points on the electropherogram before and following peak elution, respectively, and therefore represent "blank" curves.

The above analysis was carried out on varying concentrations of protocatechuic acid while the vanillic acid concentration remained constant. The concentration of the former was varied to 50 μM, 25 μM and 10 μM and the *i-E* curves extracted at identical points along the electropherograms. Although a difference in concentration of up to 75 % resulted in distinctive *i-E* curves for both acids, therefore allowing confident distinction between the two, a concentration difference of 90 % (shown in Figure 4-25(ii)) results in too much interference from vanillic acid to allow confident peak distinction. Although the curve shape is characteristic of protocatechuic acid, the response shows an oxidation potential in a similar range to vanillic acid.

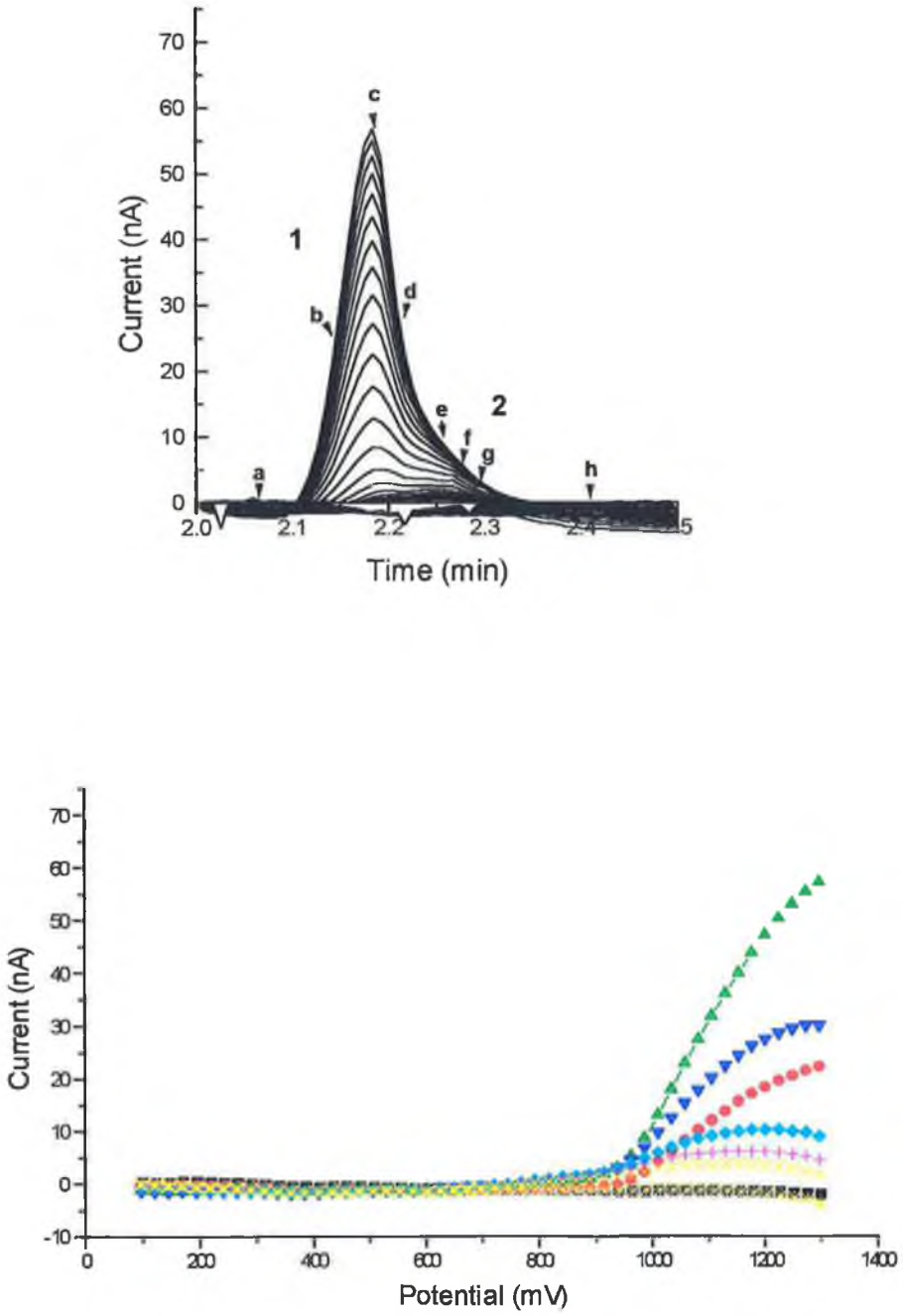


Figure 4-25 *Effect of reduction of protocatechuic acid concentration to 10 μM on i-E curves. Curve identification :* a = ■; b = ●; c = ▵; d = ▿; e = ◆; f = +; g = × and h = ⋆. *Experimental conditions as for Figure 4-24.*

4.4 CONCLUSIONS

A method has been described in this chapter which allows the identification of phenolic acids in beer using CE with amperometric detection, without the elaborate clean-up techniques required with other methods of analysis. The method involves the injection of a large volume of sample, as described by Chein *et al.*[27] The sample, diluted from 1/10 to 1/100 (depending on sample) with a low conductivity buffer such as water, results in stacking of the ions once a voltage is applied. Reversal of polarity removes positive and neutral ions, with the anions remaining in the capillary being separated once normal electrophoresis conditions are applied.

This technique may be applied to a wide variety of anionic analytes separated using CE, as an alternative to MECC. Many additives used to vary capillary wall charge have electroactive properties and therefore interfere with analysis. This method avoids such complications and by applying it to detection methods such as hydrodynamic voltammetry can be used to identify anions such as phenolic acids in complex matrices such as alcoholic and non-alcoholic beverages.

Investigations into the effectiveness of voltammetric detection using a dual-electrode system in distinguishing between the identities of 2 co-eluting peaks showed the potential of such a detection system for confident component identification in complex matrices, which eliminates the time-consuming procedure of obtaining hydrodynamic voltammograms by amperometric detection.

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CHAPTER FIVE

CONCLUSIONS

5.1 INTRODUCTION

This thesis represents an investigation into the role of electrochemical detection in modern analytical chemistry. Since the introduction of mercury as an electrode material by Kucera in 1903 and the development of the DME in 1921, electroanalytical chemistry has been applied to the detection of oxidisable/reducible compounds in all areas of analysis. The limitations of mercury in the anodic potential range led to the search of other electrode materials such as carbon, and the noble metals gold and platinum, as well as silver. Recent years has seen the development of carbon fibre electrodes for use in *in vivo* monitoring. Modern bioanalytical samples require the monitoring of increasingly low concentrations of analyte in increasingly complex sample matrices. This has brought about the use of methods to preconcentrate the analyte at electrode surfaces, which can be applied to both mercury and solid electrodes, the latter usually preconcentrating the analyte through ion-exchange mechanisms into a surface membrane. These membranes also reduce matrix interferences by excluding ions of opposite charge.

Spectroscopic detection systems for flowing solution analysis often do not reach the detection limits required to quantify an analyte in solution. Electrochemical detection can be effectively coupled to such instrumentation and with very low detection limits readily attainable. Modern electrochemical detection cells operate with very low cell volumes, and this, along with fibre electrodes, makes electrochemistry an ideal detection system for microseparations.

5.1.1 Decomposition of peroxides by transition metal ions in anaerobic adhesive cure chemistry

Anaerobic adhesive formulations are single component systems whose curing is initiated by surface chemistry but remains stable, in liquid form, in the presence of oxygen. Oxygen elimination results in rapid polymerisation by redox decomposition of the initiator by active substrate metal surface. Initiators used in formulations are usually readily reducible organic peroxides, which react through a one-electron transfer with lower state metals to initiate polymerisation. Reducing agents are often added to formulations to accelerate the initiation process by reducing metal ions to lower oxidation states. Saccharin, a weak acid, is often added to the mixture to release metal ions from substrate surfaces.

Differential pulse polarography was used to monitor the decomposition of t-butyl perbenzoate, t-butyl hydroperoxide and benzoyl peroxide by iron, copper and cobalt salts. Results showed that the type of metal ion, in addition to its oxidation state, had various effects on decomposition, with iron salts causing rapid peroxide decomposition. Cu(I) also results in rapid decomposition, however Cu(II) was inactive. Co(II) was also relatively inactive in peroxide decomposition.

The effect of the incorporation of the accelerators APH, THQ and DMPT to the peroxide-metal mixtures, in a 1:1 and 2:1 molar ratio to metal, showed APH to have the greatest acceleratory effect, followed by THQ and then DMPT.

The addition of saccharin to the reaction mixture reduced the rate of peroxide decomposition in most cases, however, it catalysed the decomposition of t-butyl perbenzoate by THQ-Cu(II) and DMPT-Cu(II) complexes, as well as the decomposition of t-butyl hydroperoxide by DMPT-Cu(II) and benzoyl peroxide by

APH-Fe(III). Maleic acid (a stronger acid) also curtailed decomposition, with the exception of when it was added to Cu(II)-THQ in the decomposition of t-butyl perbenzoate.

These results point to the intricacy of anaerobic adhesive cure chemistry with reaction mechanism generalisation not possible. Further investigations are necessary into the role of saccharin in the polymerisation process, as well as its interaction with transition metal ions. Further research into the effect of stronger reducing agents may also speed up adhesive curing. As the incorporation of acids into cure formulations is necessary for the liberation of transition metals from substrate surfaces, the fact that these acids for the most part actually reduce decomposition rates highlights the need for investigations into the effect of other acids.

5.1.2 Analysis of clenbuterol in bovine urine

A Nafion cation-exchange polymer was used to accumulate clenbuterol, the β-agonist drug used for the treatment of asthma in man and which also promotes the partitioning of cattle body fat into muscle. The electrochemical behaviour of clenbuterol, investigated by cyclic voltammetry at a bare carbon paste electrode, showed clenbuterol to be irreversibly oxidised at high positive potentials with the product of oxidation undergoing a chemical follow-up reaction. The product of this process was evident in acidic media as a quasi-reversible couple at much lower oxidation potentials.

The application of a thin Nafion layer on the electrode surface showed a large increase in clenbuterol response. Accumulation studies showed clenbuterol to accumulate linearly with time; however, a calibration curve carried out on the main oxidation peak did not allow the detection of amply low limits to detect levels in urine samples. Similar accumulation studies carried out on the product oxidation peak lowered the limit of detection to a level of 2.33×10^{-10} M. A cleaning step involving stirring of the electrode in a solution of 0.1 M NaOH at - 0.60 V for 120 s was applied after each experiment to remove adsorbed product from the electrode surface.

The method was applied to the detection of clenbuterol in a biological matrix. The bovine urine sample was initially cleaned-up by a two-step extraction procedure involving a mixed-mode solid-phase extraction procedure. The oxidation peak at + 0.40 V was free from interferences from the oxidation of urine constituents, due in part to exclusion by the protective Nafion barrier.

Further investigations could be made into the adaptation of the technique for the simultaneous detection of a number of β-agonists in a urine sample. In an attempt to lower the detection limits of the technique, the effect of incorporating the Nafion polymer into the carbon paste composite may be investigated further. Another interesting project would be the development of a clenbuterol immunosensor, incorporating antibodies raised against clenbuterol into the carbon paste.

5.1.3 Analysis of phenolic acids in beer by capillary electrophoresis

The majority of analytical procedures for phenolic acid profiling in beer involve HPLC analysis with UV detection. Due to the complexity of these samples, such techniques require lengthy extraction procedures and detection at more than one wavelength due to the varying absorbance maxima of the acids. The use of amperometric detection at high oxidation potentials permits the simultaneous detection of a large range of phenolic acids.

Capillary electrophoretic detection of a beer sample elutes components in the order of cations, neutral molecules and anions, when the cathode is situated at the detection end of the capillary. Amperometric detection with a carbon fibre electrode often leads to electrode fouling by the oxidation of large concentrations of neutral components present in the complex sample, especially when detection is at high oxidation potentials, such as needed for the oxidation of mono-hydroxy phenolic acids. This fouling necessitates harsh electrode cleaning procedures which often irreversibly damage the electrode. Elimination of these neutral components would increase the possibility of routine analysis of beer samples.

This elimination was achieved by dilution of the sample and standard solutions by 10 with water, followed by injection onto the capillary for long enough so as to nearly fill the capillary. By applying reversed polarity conditions i.e. the cathode at the injection end of the capillary, and monitoring the increase in current flow over time, the cations and neutral molecules are pulled from the capillary and the anions can be separated under normal polarity conditions.

Hydrodynamic voltammograms necessary for confident peak identification are time-consuming in amperometric mode, so it was decided to investigate the ability

of a voltammetric detection system to produce characteristic *i-E* curves from two co-eluting peaks, which the system was capable of carrying out up to a concentration difference of 90 % between the two peaks.

Further work can be carried out on the analysis of anionic components of beer samples by CE with voltammetric detection. At present, the life-time of the dual-electrode system is largely limited by the large concentration of neutral compounds present in the beer sample. The implication of a reversed polarity sample clean-up step would reduce such fouling effects, and eliminate the need for harsh electrochemical treatments. Optimisation of separation conditions and automation of the system could extend the application of electrochemical detection of anions by CE to many other complex samples, without the need for elaborate sample extraction procedures.

APPENDIX

Publications:

1. *Electrochemical Behaviour of Clenbuterol at Nafion-modified Carbon Paste Electrodes.*

   S. Moane, J.R. Barreira Rodriguez, A.J. Miranda Ordieres, P Tuñón Blanco and M.R. Smyth, *J. Pharm. Biomed. Anal.*, **14** (1995) 57.

2. *Differential Pulse Voltammetric Determination of Clenbuterol in Bovine Urine using a Nafion-modified Carbon Paste Electrode.*

   S. Moane, M.R. Smyth and M. O'Keeffe, *Analyst*, **121** (1996) 779.

3. *Identification of Phenolic Acids in Beer by Capillary Electrophoresis with Electrochemical Detection.*

   S. Moane, S. Park, M. R. Smyth and C.E. Lunte, in preparation.