DUBLIN CITY UNIVERSITY

Ollscoil Chathair Bhaile Atha Cliath

DEVELOPMENT OF HORSERADISH PEROXIDASE AND TYROSINASE-BASED ORGANIC-PHASE BIOSENSORS

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 Ph.D., 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 own work.

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Olubunmi Adeyoju

Date: 26/9/95

DEDICATED TO MY PARENTS AND 'BANJI

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LIST OF SYMBOLS

Symbol	*Description*	*Dimensions*
A	electrode area	cm^2
C	bulk concentration of substrate	M
D_M	diffusion coefficient	cm^2/s
E	enzyme	-
EI_n	enzyme-inhibitor complex	-
ES	enzyme-subsrate complex	-
ESI_n	enzyme-substrate-inhibitor complex	-
E_i	initial potential	V
E_λ	switch/final potential	V
E_p	peak potential	V
$E_{p\,a}$	anodic peak potential	V
$E_{p\,c}$	cathodic peak potential	V
$E_{p/2}$	potential at half the peak height	V
$E_{1/2}$	half-wave potential	V
f	frictional coefficient of the reactant	$J\ s/cm^2$
F	Faraday constant	C/mol
ΔG	Gibbs free energy	J/mol
I_{max}	limiting current	A
I_n	inhibitor	-
$I_{p\,a}$	anodic peak current	A
$I_{p\,c}$	cathodic peak current	A
I_{ss}	steady-state current	A
k	Boltzmann constant	J/K
k	heterogenous reaction rate constant	cm/s
k_{cat}	enzyme turnover number	cm/s
k'	capacity factor	-
K_i	inhibition constant	M
K_i'	apparent inhibition constant	M
K_m	Michaelis-Menten constant	M
K_m'	apparent Michaelis-Menten constant	M
l	thickness of enzyme layer	cm
M'	solvent hydrophobic properties	-

M''	solvent electronic properties	-
M'''	solvent steric properties	-
n	number of electrons transferred per molecule	-
R	gas constant	J/mol K
R_s	frictional force of resistance	J S
S	substrate	-
S'	substrate hydrophobic properties	-
S''	substrate electronic properties	-
S'''	substrate steric properties	-
S_{ox}	oxidised form of substrate	-
S_{Red}	reduced form of substrate	-
t	time	s
t_R	retention time of the analyte	s or min
t_M	retention time of the mobile phase	s or min
t_R'	adjusted retention time of the analyte	s or min
T	absolute temperature	K
v	velocity of the reactant	
v_o	initial enzyme reaction velocity	cm^2/s
V_{max}	limiting initial velocity	cm^2/s
V_{max}'	apparent limiting initial velocity	cm^2/s
x	Hill coefficient	
Y	fractional inhibition	
α	partition coefficient for the substrate	
β	partition coefficient for the mediator	
ε	dielectric constant	-
ν	scan rate	mV/s
η	absolute viscosity	
ω	angular velocity	/s
λ_{max}	wavelength of maximum absorption	
φ	kinematic viscosity	cm^2/s

LIST OF ABBREVIATIONS

Arg	arginine
BAS	Bioanalytical Systems
BSA	bovine serum albumin
BTP	butanone peroxide
CV	cyclic voltammetry
DEC	dielectric constant
DEDTC	diethyldithiocarbamate
DMFc	1,1-dimethylferrocene
DNA	deoxyribonucleic acid
EBDC	ethylenebisdithiocarbamate
ELISA	enzyme-linked immunosorbent assays
ETU	ethylenethiourea
FDA	Food and Drugs Administration
FIA	flow injection analysis
FMCA	ferrocene monocarboxylic acid
GCE	glassy carbon electrode
His	histidine
HLA	hydroxylamine
HPLC	high performance liquid chromatography
HRP	horseradish peroxidase
IMER	immobilised enzyme reactor
LC	liquid chromatography
L-DOPA	L-3,4-dihydroxyphenylalanine
Leu	leucine
LWB	Lineweaver Burk plot/equation
MCE	mercaptoethanol
MeSNC	methyl isothiocyanate
MetFc	ferrocenemethanol
o-PEDA	o-phenylenediamine
OPEE	organic-phase enzyme electrode
OTTLE	optically transparent thin layer electrochemical cell
PC	personal computer
PEG	poly(ethylene glycol)
Phe	phenylalanine
PTFE	poly(tetrafluoro-ethylene)

PVC	poly(vinyl chloride)
PVP	poly(vinyl pyridine)
RPM	rotations per minute
SCE	saturated calomel electrode
SDS	sodium dodecyl sulphate
TEATS	tetraethylammomum-p-toluenesulphonate
THU	thiourea
THF	tetrahydrofuran
Tyr	tyrosine
UV	ultraviolet

ABSTRACT

Horseradish peroxidase-and tyrosinase-modified electrodes were constructed by entrapping the enzyme within an Eastman AQ 55D polymer matrix The biosensors were used to detect their respective substrates (i e organic peroxides and phenols) and inhibitors in polar organic solvents Horseradish peroxidase (HRP) inhibitors investigated included thiourea, ethylenethiourea, mercaptoethanol, hydroxylamine and methyl isothiocyanate Similarly, diethyldithiocarbamate was detected as a tyrosinase inhibitor Electrochemical methods such as cyclic voltammetry and steady-state amperometry, as well as spectroelectrochemistry, were used to characterise the analytical performance of these biosensors in organic solvents, namely acetomtrile, methanol, acetone, tetrahydrofuran, 2-propanol and 2-butanol Operational parameters, such as the use of a mediator, the effects of the chemical nature of the utilised mediator and its concentration, water content of the solvent and working potential were investigated and used to optimise the catalytic performance of the biosensors The kinetic parameters for the sensors in the different organic media, such as the current as the substrate concentration approaches infinity (I_{max}), the apparent Michaelis-Menten constant (K_m'), the apparent inhibition constant (K_i') and Hill coefficients were determined The differences in the values of these parameters were linked to the physico-chemical properties of the organic media, i e solvent properties such as polarity, hydrophobicity, kinematic viscosity, dielectric constant, and solvent-enzyme interactions Finally, a glassy carbon electrode was modified with tyrosinase and a conducting poly(1-vinylimidazole)-based osmium polymer The ability of this biosensor to function as a detector for high performance liquid chromatographic (HPLC) analysis of phenolic compounds such as p-aminophenol, phenol, p-cresol, catechol and p-chlorophenol was characterised The phenol sensor exhibited a 100 to 200-fold improvement in sensitivity and detection limit when compared to an ultraviolet-visible spectrophotometric detector The effects of operational parameters, such as the concentrations of the modifying compounds i e the osmium polymer and the crosslinking compound, poly(ethylene glycol), the mobile phase composition and flow rate, the working potential and the biosensor's short-term stability were investigated and optimised The biosensor was used to measure phenol in an antiseptic cream, p-acetamidophenol (paracetamol) in a cold/flu relieving salt, and phenolic compounds in cigarette filter tips

CHAPTER ONE: ORGANIC-PHASE ENZYME ELECTRODES

1.1. INTRODUCTION

Recent advancements in technological innovations, along with their subsequent industrial and medical applications, have led to an increase in the need for the detection and quantification of chemical substances In addition to the traditional requirements of an analytical instrument/device such as sensitivity, selectivity and accuracy, modern analytical devices are required to be cheap, compact, simple to use, rapid, rugged, and in some instances disposable These needs have led to the development of biosensors Biosensors have helped scientists to achieve and improve the concept of bringing measurements out of the analytical laboratory and into everyday life This has resulted m the commercial availability of biosensors, which can be used directly by clinical patients to monitor their body glucose and cholesterol levels Biosensors have also opened up new potentials for continous on-line analysis This is useful for the monitoring and control of processes in industry, medical mtensive care units, and regulatory bodies such as the Food & Drugs Administration (FDA) and Environmental Protection Agencies (EPA) The analyte(s) in these areas of application are in some instances either highly hydrophobic or unstable m the presence of water Hence, the need for organic-phase biosensors

A biosensor is an analytical device that comprises a biochemical substance which is integrated within, or in intimate contact with, a suitable physical transducing system An organic-phase biosensor refers to the analytical device as described above but which functions predominantly in organic solvents The biochemical substance used in a

biosensor is capable of interacting directly with the analyte(s) of interest in complex sample matrices, thus eliminating the need for extensive sample pretreatment or clean up steps Therefore, it regulates the sensitivity and selectivity of the device [1-4] The biochemical substance is either based on a catalytic system, e g enzymes, cells and tissues, organisms, or on an affinity system, e g immune proteins, nucleic acids and cell receptors Enzymes, to date, are the most widely utilised and studied in the development of organic-phase biosensors The interaction between an enzyme and the analyte usually results in a change in one or more physico-chemical parameters associated with the interaction, e g ions, electrons, gases, heat, mass etc The physical transducer converts these changes (and hence the extent of the enzymatic reaction or catalysis) into an electronic signal that can be suitably processed, monitored and quantified The signal output is then directly dependent on the concentration of the analyte(s) of interest The various transducers that have been used in the development of organic-phase biosensors include electrochemical, thermal and spectroscopic-based devices Electrochemical detection methods hold a leading position among the methods presently available This is because they have been proven to be highly selective, cheap and easily miniaturized and automated [4-7]

1.2. ENZYMES

Enzymes are proteins which act as catalysts in biological systems A few enzymes, such as chymotrypsin and ribonuclease, are catalytically active without any need for cofactors However, most enzymes require the

presence of additional non-protein component(s), i e cofactors, to be active The cofactor may be small organic molecules referred to as a coenzyme, or a metal ion For instance, carboxylpeptidase A requires a zinc(II) ion, while lactate dehydrogenase requires nicotinamide adenine dinucleotide (NAD^+), as cofactors for catalytic activity There are a few enzymes known to require the presence of both a metal ion and a coenzyme for activity Coenzymes such as nicotinamide adenine dinucleotide phosphate ($NADP^+$) and NAD^+ bind weakly to the enzymes Consequently, after participating in the catalytic redox reactions, their altered forms are released from the enzyme However, there are coenzymes which bind firmly to the enzymes These type of coenzymes are referred to as prosthetic groups, e g flavin adenine dinucleotide (FAD) Prosthetic groups cannot be separated from the enzyme without denaturing the protein The flavin nucleotides are not released at the end of the catalytic redox reactions, instead they pass on the hydrogen atoms to a specific external acceptor to complete the sequence and then revert to their original form [8-10]

Enzymes are individually unique in terms of the type(s) of reaction(s) they catalyse, hence they have been generally classified by the Enzyme Commision (EC) into six main groups These are oxidoreductases, transferases, hydrolases, lyases, isomerases and ligases Examples of some enzymes in each class and the type of reaction they catalyse are illustrated in Table 1 1 As a result of enzymes having a high substrate/product specificity and catalytic efficiency, they can carry out a series of specific interrelated reaction chains and produce only the desired products This inherent ability, along with the ability of enzyme-

catalysed reactions to occur at near neutral pH and at around room/body temperature, make enzymes easy to adapt into analytical procedures Enzyme-based biosensors may be designed to determine the concentrations of substrates, coenzymes, activators and inhibitors The performance of such biosensors is governed by the properties of the sensing enzyme and the kinetics of the enzyme-analyte interaction [11]

Table 1 1 Classification and functions of enzymes

CLASS	REACTION TYPE	EXAMPLES
OXIDOREDUCTASES	catalyse oxidation-reduction reactions, i e reactions involving the transfer of hydrogen atoms, oxygen atoms or electrons from one substrate to another	oxidases, dehydrogenases, peroxidases, oxygenases
TRANSFERASES	catalyse reactions involving transfer of a group, e g a methyl group from one compound to the other compound	phospho-transferases, alkyl-transferases acyl-transferases, glycosyl-transferases, sulpho-transferases
HYDROLASES	catalyse reactions involving the addition or removal of water	esterases, thiolesterases, glycosidases, peptidases
LYASES	catalyse non-hydrolytic removal of groups from substrates, often leaving double bonds or addition of substance across double bonds	decarboxylases, aldehyde-lyases, oxo-acid-lyases, hydro-lyases
ISOMERASES	catalyse isomerization reactions and inversion at an assymetric carbon atom	racemases, epimerases, intramolecular oxidoreductases, transferases and lyases
LIGASES	catalyse synthesis of new bonds coupled to breakdown of ATP or nucleoside triphosphate	aminoacyl-tRNA synthetases

1.2.1. STEADY-STATE ENZYME KINETICS

All energetically favourable biochemical reactions (i e those occuring with a decrease in the free energy, ΔG) need to overcome a potential energy barrier, known as the activation energy, before the reaction can take place Enzymes act as catalysts by allowing the formation of different, more stable, transition states, and thus reduce the activation energy In effect, the position of chemical equilibrium remains unchanged, but it is reached much faster than in the corresponding uncatalysed reaction Enzymes react with substrates to form enzyme-substrate complexes These are quite distinct from the transition states which also occur as part of the process of enzyme catalysis A single-substrate enzyme-catalysed reaction can therefore be described by the equation

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_2} E + P$$

At time t, it is assumed that the rate of formation of the enzyme-substrate complex ES is $k_1[E][S]$, and the rate of formation of the enzyme, E, and the substrate, S, from ES is $k_{-1}[ES]$, where [E], [S] and [ES] are the concentrations of the enzyme, substate and the enzyme-substrate complex at time t, respectively Michaelis and Menten, in 1913, hypothesised that an equilibrium was obtained and maintained between E, S and ES This was modified by Briggs and Haldane in 1925, who assumed that [ES] is broken down as fast as it was being formed and therefore maintains a steady-state equilibrium Therefore, a hyperbolic

relationship exists between the initial velocity, v_o, and initial substrate concentration [S], such that at a constant total enzyme concentration, [E]

$$v_0 = (V_{max}[S])/[S] + K_m \quad (1\text{-}1)$$

Equation 1 1 is referred to as the Michaelis-Menten equation K_m is the Michaelis-Menten constant and V_{max} is the limiting initial velocity at a particular total enzyme concentration This equation holds for single enzyme-catalysed reactions where

- [S] >> [E], therefore [ES] ~ constant
- there is a high affinity between E and S
- [E] remains constant
- for enzyme molecules with several binding sites for the substrate, there is no interaction between the sites
- concentration of cosubstrate(s), where present, remains constant
- ES $\rightarrow$ E + P is the rate limiting step
- there are no mass transfer limitations
- enzyme inhibitors are absent
- investigations are performed during the initial period of the reaction, i e when [P] ~ 0

K_m is independent of enzyme concentration, while V_{max} increases as the total concentration of the enzyme present increases, i e $V_{max} = k_{cat}[E]$ It has been shown [12-14] that equation 1 1 can be rewritten such that

$$v_0 = (k_{cat}/K_m)\,[E][S] \qquad (1\text{-}2)$$

where k_{cat} is the turnover number, and [E] is the concentration of the free enzyme The turnover number describes the maximum number of substrate molecules which can be converted to products per molecule of enzyme per unit time These parameters, because they are unique to the system being studied, are usually used to characterise and identify a particular enzyme K_m values describe the affinity of the enzyme for the substrate, i e a low K_m value indicates a high affinity of enzyme for substrate, while a high K_m value indicates a low affinity For most systems, K_m values lie in the range 10^{-2} - 10^{-6} mol/cm^3 Similarly, for most enzymes, k_{cat} lies in the range 1-10^4 per second [12] The term k_{cat}/K_m is known as the catalytic efficiency A high value indicates that the limiting factor for the overall reaction is the frequency of collisions between enzyme and substrate molecules A comparism of k_{cat}/K_m values for different substrates can be used as a measure of the specificity of the enzyme Hence, the value of the kinetic parameters V_{max}, K_m and k_{cat} often have to be determined The most up-to-date method for their determination involves non-linear curve fitting of v_o and [s] values to the Michaelis-Menten equation using a computer programme However, in the absence of such programmes, approximate values of these parameters can be obtained by manipulating the Michaelis-Menten equation

(without changing the underlying assumptions) to forms that correspond to the equations of a straight line graph y = mx + c The commonly used manipulations include the Lineweaver-Burk equation

$$1/v_0 = (K_m/V_{max})\ 1/[S] + 1/V_{max} \quad \textbf{(1-3)}$$

the Eadie-Hofstee equation

$$v_0 = -K_m(v_0/[S]) + V_{max} \quad \textbf{(1-4)}$$

and the Hanes equation

$$[S]/v_0 = (1/V_{max})[S] + (K_m/V_{max}) \quad \textbf{(1-5)}$$

The values of V_{max} and K_m vary with the nature of enzymes and substrates The other factors that affect the values of these kinetic parameters for a particular enzymatic reaction include

- enzyme immobilisation techniques
- presence of activators and inhibitors
- the nature of the reaction medium, the effects could be in terms of its chemical properties, e g aqueous/organic nature, and/or physical properties such as pH, temperature, viscosity and polarity/hydrophobicity

This thesis is primarily concerned with the study of enzyme-catalysed reactions in organic media, and as such, the former two factors and further topics will be discussed in the context of organic-phase enzymatic reactions

1.2.1.1. *ENZYME CATALYSIS IN ORGANIC MEDIA*

The study of enzymes, which involves enzyme extraction, purification, characterisation, reactions and industrial applications, has mostly been carried out in aqueous media However, the ability of enzymes to catalyse reactions in organic solvents, first reported by Dastoli and Price in 1967 [15], along with their vast potential in organic synthesis and bioanalytical uses, continues to generate enormous interests and research activities The advantages of enzyme catalysis in organic solvents include catalysis of previously inaccessible substrates (e g organic compounds with a low solubility in water), increased thermal stability for the enzyme, decreased chances of microbial contamination, ability of enzymes to catalyse reactions that were impossible in aqueous media because of unfavourable thermodynamic or kinetic equilibrium, prevention of undesirable side reactions e g hydrolysis and nucleophilic addition of hydroxide ion, and finally the insolubility of enzymes in organic solvent which permits their easy recovery and subsequent recycling [16] Organic-phase enzyme catalysis has been carried out in several forms of non-aqueous media These include the use of enzymes in aqueous solutions containing a water-miscible organic cosolvent [17], microaqueous-organic biphasic mixtures [18,19], organic biphasic

mixtures [19], reversed micelles [19, 20] and finally anhydrous organic solvents [21, 22] But more importantly, the influence of the solvent pH, water content and physico-chemical properties on the specificity and catalytic activity of the enzyme are crucial for optimising the biocatalytic reaction

The specificity of an enzyme is dependent on the ionisable side chains of amino acid residues being in a particular form, making enzymatic reactions pH dependent Organic solvents do not in themselves have a defined pH, but according to Klibanov [23], during orgamc-phase enzymatic reactions, the enzyme acts based on the pH of the last aqueous medium to which it has been exposed In other words, the pH acquired by an enzyme before its introduction into an organic medium remains unaltered Therefore, to present the enzyme to the organic solvent in a catalytically competent state, it is recommended that the enzyme be dissolved in an aqueous phase of optimal pH for activity [24] It is widely accepted that while the catalytic efficiency of enzymatic reactions in organic media is comparable to and in some cases higher than, that displayed in aqueous media, some amount of water is still required for enzymatic activity in these solvents [24-26] A thin layer of water around the enzyme is essential to preserve the three-dimensional structure of the protein in a catalytically active form The catalytic activity of an enzyme increases as the water content of the organic solvent increases However, the minimum amount of water required for enzymatic activity in an organic solvent varies with enzymes as well as solvents For instance, the activity of alcohol dehydrogenase in the presence of 0 5 % water in isopropyl ether is approximately 3 orders of magnitude higher than in

methyl acetate or acetomtrile at the same water concentration [26] Also, while the activity of tyrosinase in chloroform requires about 3 5 x 10^7 molecules of water per enzyme molecule, chymotrypsin needs only 50 molecules of water per enzyme molecule for activity in octane [27] Indeed, immobilised glucose oxidase (GOx) has been shown to remain active in 100 % polar organic solvents such as acetomtrile and 2-butanol [28] This has been attributed to the presence of a carbohydrate shell around the active site of GOx, which tends to shield it from the effects of the organic solvents Similar shielding effects have been observed in the organic-phase catalytic behaviour of subtilisin [29] The differing activities of enzymes in these solvents have been attributed to the varying ability of organic solvents to strip essential water from the active site of the enzyme, thereby deactivating the biocatalyst The ability of organic solvents to strip essential water is believed to be a function of their hydrophobicity/polarity [30, 31] The less hydrophobic (i e more polar) the solvent, the higher its affinity for water, and hence it is more likely to strip the essential water from the enzyme molecules [32] However, most enzymes remain active in both hydrophobic and hydrophilic solvents for as long as the essential amount of water required for catalytic activity is maintained in the reaction medium This is achieved for hydrophilic solvents by the addition of some water, usually between 2 - 40 % v/v of the reaction medium This tends to satisfy the solvent's "thirst" [31] and makes it less likely to strip essential water layer in the enzyme microenvironment It is known that apart from solvent hydrophobicity, other physical properties of the solvent, such as kinematic viscosity and dielectric constant (ε), also influence the activity

of the enzymes in organic media [28, 33] The kinetic viscosity influences the diffusion/partition coefficients of the reacting species (i e the enzyme, substrate and/or inhibitors) and reaction products in the reaction media This ultimately affects the kinetic and thermodynamic parameters of the catalytic process Also, ε is a measure of relative permittivity, and governs the weakening of electrostatic forces around the enzyme's charged and polar active site Other factors, such as substrate and enzyme hydrophobicity, also significantly affect biocatalytic activity in organic media Ryu and Dordick [34] have shown that in the horseradish peroxidase (HRP) catalysis of phenols, a linear free energy relationship exists between the catalytic efficiency and both substrate and solvent hydrophobicity The catalytic efficiency decreased as the hydrophobicity of the phenols increased This effect became more pronounced as the solvent hydrophobicity increased Similarly, an increase in HRP hydrophobicity has been shown to increase catalytic activity in water-immiscible hydrophobic solvents [35] This is believed to be as a result partitioning of substrate between the bulk of the reaction medium and the enzyme active site The substrate partitioning to the enzyme's hydrophilic active site would tend to decrease as substrate and solvent hydrophobicity mcreases It would therefore take a greater amount of the substrate to saturate the enzyme, resulting in an increase in K_m

Several attempts have been made to correlate these solvent properties (i e polarity, viscosity and ε) to the activity of enzymes in organic solvents Attempts have been made to use solvent polarity, as expressed by the Hildebrand solubility parameter, to correlate solvent

hydrophobicity and enzyme catalysis [36] Similarly, Campanella *et al* [37] have consistently correlated ε of organic solvents to the sensitivity and bioactivity of tyrosinase-based sensors in such solvents However, to date, the most widely used and accepted guide in predicting biocatalytic activity in a given organic solvent is the use of the logarithm of partition coefficient (log P) of the test organic medium in a standard octanol-water biphasic system [38] It is based on solvent hydrophobicity such that solvents with a log P < 2 are expected to be unfavourable for enzymatic reactions because they would generally be hydrophilic and therefore tend to distort the water-enzyme interactions Solvents with a log P between 2 and 4 are weak water distorters, hence affecting enzymatic activity in an unpredictable manner, and solvents with a log P > 4 would favour enzymatic reactions, because they are hydrophobic and therefore tend not to distort the water-enzyme interactions While the enantioselectivity, as exhibited by some proteolytic enzymes, has been found to correlate to the log P of the reaction medium [39], the model fails to account for the surprisingly high activity of certain enzymes in some organic solvents e g , subtilisin in DMF (log P = -1 0), porcine pancreatic lipase in pyridine (log P = 0 71) Indeed, Narayan and Klibanov [40] recently explained that the correlation between the enzyme catalytic activity and log P values do fail for organic solvents within a narrow range of log P values In another study, Ryu and Dordick [41] used an equation which took into account the physico-chemical properties of both substrates and solvents

$$\log (V_{max}/K_m) = M'S' + M''S'' + M'''S''' + C_{eff} \qquad \textbf{(1-6)}$$

where S', S'' and S''' describe the hydrophobic, electronic and steric properties (i e Hansch, Hammett and Taft constants), respectively of the substrate Similarly, M', M'' and M''' describe the hydrophobic, electronic and steric properties of the solvent C_{eff} is the catalytic efficiency of the enzyme on the "standard-state" substrate in which S', S'' and S''' are zero It was shown that the effect of electrostatic forces, as predicted by the ε values, is prevalent on the enzyme's exterior and is a primary effect, while, hydrophobicity, as expressed by log P, as well as the solvent's surface tension effects are secondary Therefore, organic solvents primarily affect electrostatic interactions, and only after some denaturation has occurred, will hydrophobic interactions, such as the water stripping phenomenon, be effected However, hydrophobicity governs the substrate partitioning into the active site of the enzyme in organic solvents and plays a major role in enzyme-substrate interactions involving hydrophobic substrates in organic solvents In the same study [41], an equation that would predict the catalytic efficiency of peroxidase catalysis in acetomtrile-water and methanol-water reaction media was therefore hypothesised

$$V_{max}/K_m = 0\ 48 \times 10^{[0\ 089\varepsilon - (1\ 75 + 0\ 012\varepsilon)S'' - 0\ 21(\log P + 4\ 10S')]} \qquad \textbf{(1-7)}$$

This equation gave rise to a correlation coefficient of 0 92 with the experimental catalytic efficiencies of peroxidase catalysis of phenols in the solvents

In view of these discussions, and considering the other ways by which an organic reaction medium affects biocatalysis, it can be concluded that

any parameter that would rightly correlate with biocatalytic activity in organic media (provided there is no chemical interaction between the solvent and the reacting species including the immobilisation material) must embrace the solvent, enzyme, substrate and immobilization material polarity, the kinematic viscosity, surface tension and dielectric constant of the solvent, as well as the size of the reacting species

1.2.1.2. *ENZYME IMMOBILISATION*

Enzyme immobilisation is simply the confinement of an enzyme molecule within an insoluble medium which is distinct from the bulk reaction medium An exchange of charged/neutral species is allowed between the enzyme molecule and the substrate, activator or inhibitor molecules which are dispersed and being monitored in the bulk reaction medium [42] In enzyme-based analytical devices using electrochemical detection methods, the enzyme is usually confined on the surface of the electrodes which include glassy carbon, platinum and carbon paste electrodes Immobilisation often causes a dramatic change in the measured kinetic parameters of the enzyme-catalysed reaction Therefore, the kinetic parameters K_m and V_{max} for immobilised enzymes are referred to as apparent or effective K_m (denoted K_m') and apparent V_{max} (denoted V_{max}') The apparent K_m of an immobilized enzyme is usually significantly higher than those for the same enzyme in free solution [12] The effects of immobilisation on the kinetic behaviour of an enzyme has been attributed to one or a combination of several factors [12, 43] These include

Partitioning effects these result from the fact that the equilibrium concentrations of the substrate within the immobilised enzyme layer may be different from that in the bulk reaction medium This effect is usually dependent on the chemical nature of the immobilisation material, as well as hydrophobic interactions between the immobilisation material and low molecular weight substances in the reaction medium

Conformational and Steric effects the conformation of an enzyme may be altered during immobilisation or it may be embedded within the immobilisation medium such that the enzyme either gets denatured or its active sites are less accessible to the substrate and/or other reacting species

Diffusional/Mass transfer effects m fast enzymatic reactions where the particle size or membrane thickness are relatively large, there are diffusional resistances to the movement of the substrate and other reacting species to and from the site of enzymatic reaction Hence, the immobilised enzyme, even in steady-state, would be exposed to local concentrations of substrate, product or inhibitor different from those m the bulk reaction medium

Microenvironmental effects when the enzyme is immobilised, the enzyme-substrate interactions are now taking place in a different microenvironment This results in a change in the catalytic pathway of the reaction and ultimately a change in the intrinsic kinetic parameters For instance, the K_m is affected by the electrostatic field of immobilisation material, such that when the immobilisation material is of

opposite charge to that of the substrate, K_m significantly decreases and vice-versa when the immobilization material has the same charge as the substrate Similarly, it has been shown [44] that the pH optimum of many enzymes shift to a more alkaline value if the immobilisation material is anionic, and to a more acid value if it is cationic, due to changes in the degree of ionisation of amino acid residues of the active site

In addition to these effects, enzyme immobilisation in organic-phase reactions protects the biocatalyst from aggregation, which is one of the mechanisms of enzyme inactivation by organic solvents and solvent-induced conformational change, by increasing the rigidity of the enzyme molecule and thereby hindering unfolding [45-48] For instance, while free chymotrypsin precipitates in aqueous-organic biphasic media (with concentrations of dioxane greater than 30 %), reactions of the same enzyme when immobilised can be successfully carried out in 95 % (v/v) dioxane-water medium [45] Mionetto *et al* [49] reported a comparison of the catalytic activity of free and immobilised acethylcholme esterase in a variety of organic solvents The free enzyme displayed little or no activity in hydrophilic solvents such as acetonitrile, butanol and methanol However, the immobilised enzyme under the same conditions retained some of its activity in these solvents The retained activity was as high as 50 % of its original activity in acetonitrile The degree and nature of the effects of immobilisation depends not only on the enzyme reaction but also on the immobilisation technique used Enzyme immobilisation can be achieved by several methods but those that have been used for organic-phase enzyme catalysis are mainly based on

physical adsorption, covalent attachment, cross-linking and gel/polymer entrapment

1.2.1.2.1. *Physical adsorption*

This method was the earliest form of immobilisation technique, and was introduced in 1916 [12] It involves mixing the enzyme solution with an inert carrier such as activated charcoal, or physically adsorbing the enzyme solution directly on to the electrode surface without any change in enzymic activity Immobilisation is achieved by the formation of weak van der Waals and hydrogen bonds between the enzyme and the electrode surface This method is effective, simple and rapid, and high enzyme loadings are achievable However, because of the weak nature of the bonds, the enzyme is easily leached from the electrode surface and is susceptible to fouling by the solvent and other substances which either easily adsorb or interact with the electrode/carrier as the immobilised enzyme is being used It also tends to be ineffective when the water content of the solvent is high An example of the application of this form of immobilisation in organic-phase enzyme catalysis, is the immobilisation of alcohol dehydrogenases (i e from horseliver, yeast and *thermoanaerobium brockii*) on porous glass beads for catalysis of cinnamyl alcohol in 99 9 % (v/v) n-hexane [33] Also, this method has been well utilised by Turner and coworkers for the organic-phase enzyme catalysis and detection of peroxides, phenols and cholesterol [50, 51]

1.2.1.2.2. *Covalent attachment*

Immobilisation with this method is achieved by the formation of permanent linkages between the enzyme and the immobilisation material This is usually done by forming covalent bonds between the functional groups of the enzyme such as free α- or ε- amino groups, hydroxyl, imidazole or free carboxyl groups, and those of the immobilisation material such as diazonium and carboxyl groups It can also be achieved by chelation, such that strong metal bridges are formed between hydroxyl oxygen atoms of the immobilisation material and amino nitrogen atoms on the enzyme [52] The enzyme active site is usually protected with a substrate or substrate-analogue during immobilisation This is to ensure that it remains free from covalent bonds during immobilisation Covalent attachment, compared to physical adsorption, gives rise to a more stable immobilised enzyme layer However, the inevitable loss of enzymatic activity during immobilisation resulting in low enzyme loadings remains a major drawback of this method This technique has for instance been used for chymotrypsin catalysis in dioxane [45]

1.2.1.2.3. *Cross-linking*

This method involves using a bifunctional agent such as glutaraldehyde to form Schiff's base linkages with free ammo groups on the enzyme molecule [12]

$$2(E\text{-}NH_2) + CHO\text{–}(CH_2)_3\text{–}CHO \rightarrow E\text{-}N\text{–}CH\text{–}(CH_2)_3\text{–}CH\text{–}N\text{-}E$$

Since each enzyme molecule has several free amino groups, a cross-linked network is formed This method particularly favours high enzyme loading as well as stability The major drawbacks of the method include the unselective nature of the bifunctional agents, as well as intra-molecular bonding, which makes the enzyme active site inaccessible to the substrate resulting in a lower catalytic efficiency To overcome this limitation, non-enzymatic, low molecular weight proteins like bovine serum albumin (BSA) are usually used along with the cross-linking agents BSA allows more inter-molecular bonding and less crowding of the enzyme It is possible to combine cross-linking with other techniques such as physical adsorption and gel/polymer entrapment For instance, glucose oxidase was immobilised by cross-linking with glutaraldehyde with [53] and without [28] BSA for catalysis of glucose oxidation in 90 % (v/v) acetonitrile- and 2-butanol-water reaction media

1.2.1.2.4. *Gel/Polymer entrapment*

This is the most popular method of enzyme immobilization both in aqueous and organic-phase enzyme catalytic reactions [1, 54] The advantages of this method include

- the stability of polymers and their ability to function at neutral pH, mild temperatures and other conditions favourable to enzyme catalysis

- the ease of incorporation of enzymes, as well as application

of the polymer onto the electrode surface, the thickness/concentration of the polymer and the concentration of the enzyme and/or other modifying substances can be easily adjusted to suit each particular system

- the chemical and physical properties of the polymers can be easily modified to suit the needs of the enzyme, reaction or analytical procedures/devices, for instance, its ion exchange and size exclusion properties could be modified to ensure permselectivity or to eliminate electrode fouling, the polymers can be functionalized to improve their hydrophobicity, stability, permeability, compatibility with enzyme, electrochemical properties etc

- availability of a wide variety of polymers, and hence the possibility of combining polymer films to improve stability and efficiency of enzyme catalysis

Synthetic polymeric films, in terms of their mode of preparation, are either premade /preformed films or electropolymerised films The premade films may again be non-functionalised, i e contain only an organic moiety such as poly(vinyl pyridine) (PVP) or functionalised i e contain redox or ion exchange active sites e g poly[vinylpyridine $Os(2,2'\text{-bipyridyl})_2Cl]Cl$, ferrocene-modified polysiloxane, poly(ester sulphomc acid) ionomer [55-59] Polymer films are usually formed by casting, spin-coating or dip-coating the electrode surface with a solution

of the polymer, with or without the enzyme and other modifying agents Polymers can be broadly divided into 4 major types based on their "mode of action"/ physico-chemical properties [60] Each type may again be premade or electropolymerised depending on how it is prepared These are

- **Conducting polymers** these are polymers that have conjugated π-electron systems and hence possess reversible redox properties e g poly(pyrrole) [61]

- **Non-conducting polymers** these polymers do not have redox capabilities, they are usually used to protect immobilized enzyme from electroactive interferents and fouling species-examples include insulating poly (phenol) films [62] and poly(vinyl chloride) (PVC) films [63]

- **Redox polymers** these are polymers that can transfer or accept electrons from their surrounding environments, they are also called electron exchange polymers and redoxites-examples include ferrocene-modified poly(ethylene oxide) [64]

- **Ion-exchange polymers** these are permselective polymers with the ability to incorporate or reject ions based on whether they carry positive or negative charges, the two most widely used ion-exchange polymers are Nafion [65,66]

and Eastman AQ polymers [58], however, of these two, Eastman AQ polymers exhibit exceptional stability in organic solvents, hence it is the most widely used immobilization technique for organic-phase enzyme catalysis [67]

1.2.1.2.5. *Eastman AQ ion-exchange polymers*

These are poly(ester sulphonic acid) anionomers produced by Eastman Kodak There are 3 different types of Eastman AQ polymers Eastman AQ 29D, AQ 38D and AQ 55D Their molecular weights, as determined from percent sulphonation, are 2500, 2500 and 1500, respectively [68] The complete structure of the three different AQ polymers is not known, but their proposed backbones are as shown in Figure 1 1 [68] However, of the three, AQ 55D is the most studied and applied in organic-phase enzyme catalysis This is because the AQ 55D polymer is stable in organic solvents, i e it does not swell, and unlike Nafion it is easily dispersed in water and insoluble in most of the solvent Its other properties include its ability to preconcentrate catalysts into the film, lower the overpotential of substances which otherwise are difficult to reduce or oxidise, and selectively exclude anionic and large substances [58, 68] In aqueous media, the AQ 55D membrane (because of its hydrophobic alkyl chains) selectively binds to hydrophobic cations However, in organic media, the membrane tends to be more selective for smaller less hydrophobic cations [68] A modified form of the Gibbs-Donnan equation [69] accounts for the effects of the

hydrophobic/hydrophilic nature of organic solvents on the extent of the ion-exchange reaction between the AQ 55D membrane and the substrate/inhibitors Hence, for a simple ion-exchange reaction

$$X^+ + {}^*Y^+ \longrightarrow {}^*X^+ + Y^+$$

the extent of the reaction, as described by the modified Gibbs-Donnan equation is

$$RT \ln K_{x,y} = -RT \ln\{ (\delta_x^* \delta_y)/(\delta_x \delta_y^*)\} - P\Delta V - \Delta G_H \qquad \textbf{(1-8)}$$

where X^+ and Y^+ are the reacting species with different hydrophobic properties, $K_{x,y}$ is the ion selectivity coefficient, δ_x and δ_y are the activity

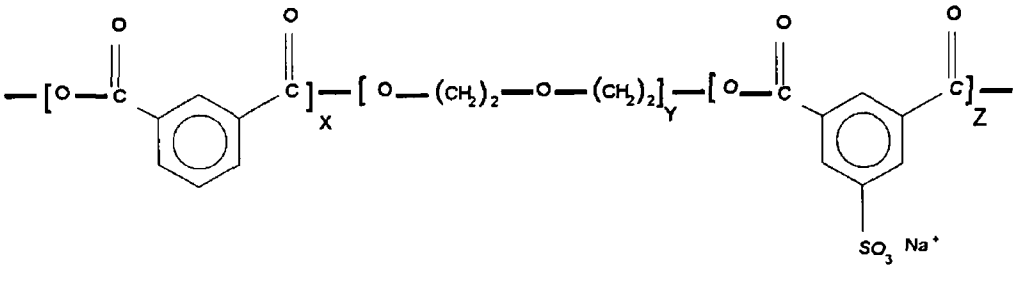


Figure 1 1 The hypothesised backbone structure of Eastman AQ polymers

coefficients of the respective ions, and the asterik refers either to the ions or their activity coefficient within the ion-exchange polymeric film P is the membrane swelling pressure and ΔV is the partial molar volume difference of the membrane ion-counter ion salts in the membrane phase ΔG_H describes the contribution of the hydrophobic nature of an ion to the free energy change associated with the incorporation of this ion into the polymeric film The contribution of this term will also be directly dependent on the hydrophobic/hydrophilic nature of the solvent and any significant solvation effects Hence, the selectivity of Eastman AQ 55D polymeric films (which ultimately affects the catalytic performance of the immobilised enzyme) is also dependent on the nature of the organic reaction medium Eastman AQ 55D polymeric films have been well utilised in entrapping tyrosinase and horseradish peroxidase for catalysis in organic media [70-73] This thesis reports the development of biosensors for the organic-phase detection of peroxide, phenols, thiourea, ethylenethiourea and diethyldithiocarbamates by entrapping horseradish peroxidase and tyrosinase within AQ 55D polymeric films

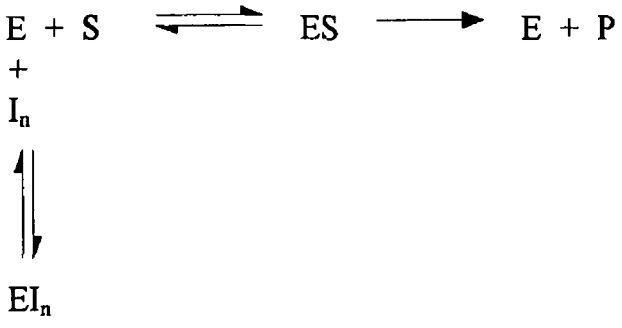
1.2.1.3. *ENZYME INHIBITORS*

Enzyme inhibitors are compounds which decrease the rate of an enzyme-catalysed reaction, by interacting with the enzyme, cofactor or substrate An enzyme-bound inhibitor affects the ability of the enzyme to bind the substrate and/or the catalytic ability of the enzyme hence it affects K_m', k_{cat} and ultimately the catalytic efficiency of the enzymatic reaction There are two types of inhibitors reversible and irreversible Irreversible

inhibitors cannot be physically separated from the enzyme and their degree of inhibition tends to increase with time In contrast, reversible inhibitors can be physically separated from the enzyme, e g by dialysis, to restore full enzymatic activity They induce a definite degree of inhibition such that a steady-state equilibrium is usually obtained Hence, they obey Michaelis-Menten kinetics On the basis of the inhibition mechanism and its effects on the kinetic parameters (i e K_m' and V_{max}), reversible inhibition for a single-intermediate enzymatic reaction can be described as competitive, uncompetitive, non-competitive, mixed effects or allosteric inhibition [74-76]

1.2.1.3.1. *Competitive inhibition*

Competitive inhibitors are usually structurally similar to the substrates whose reactions they inhibit The enzyme-bound inhibitor can therefore act so as to inactivate the enzyme or be held in an unfavourable position for a catalytic reaction to occur with other potential substrates The general effect of a competitive inhibitor depends on both the inhibitor and substrate concentrations and their relative affinities for the enzyme At a particular enzyme and inhibitor concentration, if the substrate concentration is low relative to that of the inhibitor, then the inhibitor will compete favourably with the substrate for the enzyme's binding site and the degree of inhibition is high This effect is reversed when the substrate concentration is high For a single-substrate, single-binding site enzymatic reaction, in the presence of a competitive inhibitor, I_n, the reaction scheme can be described [64] as



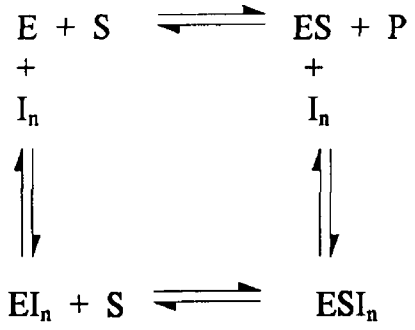
and the initial reaction rate is given by a modified form of the Michaelis-Menten equation

$$V_o = V_{max}[S]/\{[S] + K_m(1 + [I_n]/K_i)\} \quad (1\text{-}9)$$

where $[I_n]$ is the bulk concentration of the inhibitor and K_i is the inhibition constant The lower the value of K_i, the greater the degree of inhibition at any given [S] and $[I_n]$ Other terms remain as they were in the Michaelis-Menten equation Hence, in competitive inhibition, V_{max} is unaffected while K_m is increased by a factor of $(1 + [I_n]/K_i)$ K_i in this instance is not equivalent to the concentration of inhibitor that yields 50 % inhibition

1.2.1.3.2. *Non-competitive inhibition*

In this type of inhibition, the inhibitor acts by binding to the active site or by inducing a conformational change which affects the active site, but either way it does not affect substrate binding Therefore, for a single-substrate reaction in the presence of a non-competitive inhibitor, the reaction scheme is as shown



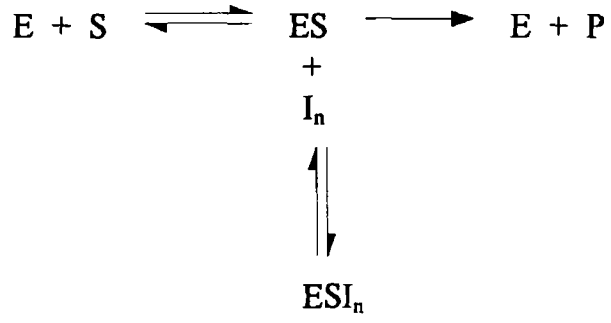
and the initial reaction rate is given as

$$V_o = \{V_{max}/(1 + [I_n]/K_i)\}\{[S]/([S] + K_m \quad (1\text{-}10)$$

Therefore, while K_m remains unchanged, V_{max} decreases in the presence of a non-competitive inhibitor The degree of inhibition depends on the concentration of the inhibitor and its affinity for the enzyme or the enzyme-substrate complex When the bulk concentration of the inhibitor is equal to K_i, a 50 % inhibition at all substrate concentrations is observed

1.2.1.3.3. *Uncompetitive inhibition*

In this type of inhibition the inhibitor does not affect the enzyme-substrate binding It binds to the enzyme-substrate complex but not to the free enzyme, i e



The initial reaction rate is therefore given by

$$V_o = \{[V_{max}/(1 + [I_n]/K_i)]\ [S]\}/\{[S] + [K_m/(1 + [I_n]/K_i)\} \qquad (1\text{-}11)$$

Hence, both V_{max} and K_m are altered Uncompetitive inhibition is rarely observed with single-substrate systems However, multi-substrate enzymatic reactions usually show the same characteristics in the presence of inhibitors

1.2.1.3.4. *Mixed effects inhibition*

This is used to describe inhibition systems that obey Michaelis-Menten kinetics but do not show characteristics that are similar to competitive, non-competitive or uncompetitive inhibitions

1.2.1.3.5. *Allosteric inhibition*

The inhibitor in this case acts by binding to the enzyme at a site distinct from the substrate-binding site It influences conformational changes which in turn alter the binding characteristics of the enzyme for the substrate and/or the subsequent reaction properties The Michaelis-Menten plot becomes less hyperbolic and more sigmoidal i e the rate of reaction is slow at low substrate concentration This type of inhibition plays an important role in metabolic regulation in living cells It is also possible for a competitive, non-competitive or uncompetitive inhibition to be allosteric [77]

The apparent K_m and I_{max} values, and hence the inhibition mechanism for enzyme-inhibitor reactions, is usually determined from the Lineweaver-Burk plots for the uninhibited and inhibited enzymatic reactions Figure 1 2 shows typical Lmeweaver-Burk plots for competitive, non-competitive, uncompetitive and mixed effect inhibitions

1.2.1.3.6. *Applications of Enzyme Inhibition*

Enzyme inhibition phenomena have been widely applied in medicine, industry and other applications In has been used for instance in the clinical treatment and control of diseases Gout, which occurs as a result of increased production of uric acid, is treated with allopurinol, a competitive inhibitor of xanthine oxidase (the enzyme which converts xanthine to uric acid) Similarly, angiotensin converting enzyme (ACE)

and monoamino oxidase inhibiting drugs are also being used in the treatment of hypertension and depression, respectively In the fruit industry, small amounts of sulphur dioxide and sodium bisulphite are used to prevent the browning of fruits by inhibiting polyphenol oxidase Research scientists use inhibitors (reversible and irreversible) in the investigation of the kinetic mechanism of the enzyme-substrate reaction(s) as well as the study and modification of the chemical attributes of enzyme active sites [78, 79] Inhibitors have been used in organic-phase enzymology to improve enzyme-substrate specificity and stability An inhibitor-induced activation was used to "lock" chymotrypsin and subtilisin in a favourable conformation for catalysis before introducing the enzymes into anhydrous organic solvent reaction medium [30, 80] The catalytic activity of subtilisin lyophilised from aqueous solutions containing the competitive inhibitors such as N-ethyl-L-tyrosine amide was found to be 55 times greater than that of the enzyme lyophilised without the inhibitor in an anhydrous octane reaction medium More significantly, the inhibition effects of toxicants and drugs on enzymes is being exploited to determine the extent of their toxicity and impact in biological systems and the environment This principle is also being used to develop analytical devices for the detection of such compounds [72,81]

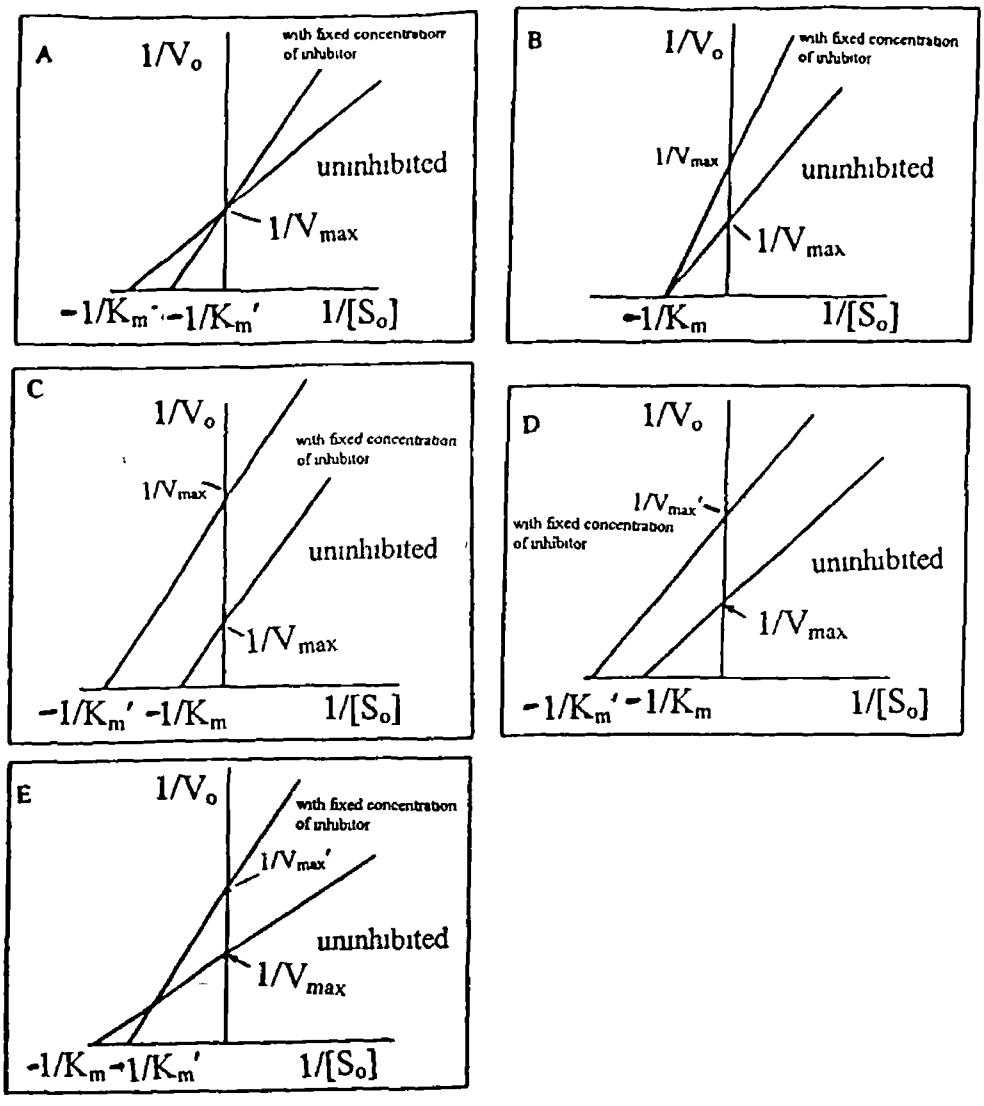


Figure 1 2 Lineweaver-Burk plots showing the characteristics of competitive (A), non-competitive (B), uncompetitive (C), mixed non-competitive-uncompetitive (D), and mixed competitive-non-competitive (E) inhibitions Reproduced from [8]

1.3. ELECTROCHEMICAL TRANSDUCTION METHODS

The two major types of electrochemical detection methods that have been exploited in the development of organic-phase biosensors are based on potentiometric and voltammetric principles Potentiometric devices relate the potential difference existing between the working electrode and the reference electrode to the quantity of the electroactive species/analyte The measured potential is proportional to the logarithm of the analyte activity present in the sample [82, 83] Miyabayashi *et al* [84] have for example reported a potentiometric chymotrypsin electrode for monitoring enzyme esterification catalysis in diisopropyl ether and toluene containing 0-2 % (v/v) water However, voltammetric transduction methods are increasingly becoming more popular and have been most widely used in the development and study of organic-phase biosensors This is because voltammetry as a transducing system for enzyme-catalysed reactions has proven itself to be more sensitive, rapid (there is no need to wait for thermodynamic equilibrium) and accurate than potentiometry [85, 86] Voltammetric responses are directly proportional to the analyte concentration, hence control and data analyses of the sensors are easily automated Also voltammetric methods permit the determination of both kinetic and thermodynamic parameters of the electrode process In contrast, potentiometric methods furnish only thermodynamic data

Voltammetry, or polarography (when a mercury electrode is used), is used to describe measurements of the current-time or current-concentration response of an electrode at a fixed potential as well as the

current-potential relationship in an electrochemical cell The use of voltammetry as an electroanalytical method m organic solvents continues to generate research interests and activities in the fields of electrochemistry and organic-phase biosensors The factors that have fuelled these increased interests include [51, 87]

- the ability of the solvents to dissove hydrophobic analytes, which are unstable and/or undergo degradation via hydrolysis or redox processes in water

- ability of the solvents to solubilise organic, and to a lesser extent inorganic, compounds

- the ability to scan over a wide potential range with these solvents because of their resistance to oxidation and reduction

- the possibty of using inert supporting electrolytes and microelectrodes to overcome solution resistance associated with organic solvents

Voltammetric measurements are usually carried out in a glass electrochemical cell with a three-electrode configuration, linked to a potentiostat and a X-Y (for current-potential measurements) or X-t (for current-time/concentration measurements) recorder A typical conventional electrochemical cell (shown in Figure 1 3) with a volume

capacity range of 1 - 50 ml contains the reaction medium, i e the deaerated solvent and supporting electrolyte It is fitted with a Teflon cover, which has O-ring adapters to hold three electrodes The three electrodes are the working/indicator, reference and counter/auxilliary electrodes The working electrode could be a solid electrode (diameter > 2 mm) or a microelectrode (diameter < 2 mm) There are a wide variety of electrode materials that could be used as working electrodes These are broadly divided into four major groups i e metals, carbonaceous materials, semiconductors and organic conducting salts [88-90] Examples of materials under each group is shown in Table 1 2 The choice of an electrode material in organic-phase bioelectroanalysis depends significantly on the nature and useful potential range of the electrode, solvent, supporting electrolyte and the reacting species i e enzyme, substrate, inhibitor etc The most common materials for organic-phase bioelectroanalysis are platinum, glassy carbon and pyrolytic graphite The electrodes made from these materials are usually non-porous, gas impermeable and inert towards chemical/solvent attack at the desired operatmg potential The disadvantage of these electrodes is their susceptibility to protein fouling and deactivation This could be solved by polishing the exposed electrode surface with alumina or diamond, somcation, chemical oxidation with nitric and/or chromic acid, electrochemical oxidation, thermal oxidation, laser activation and radio frequency-oxygen plasma oxidation [86]

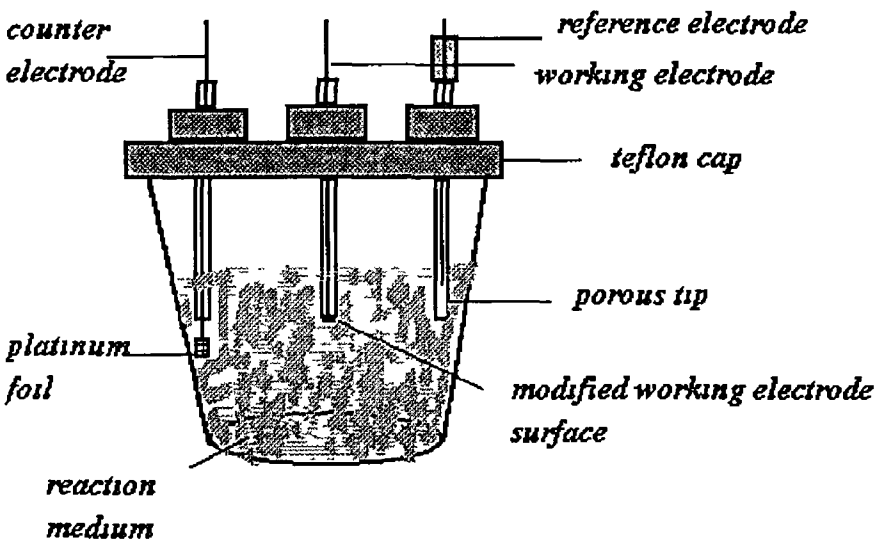


Figure 1 3 A typical electrochemical cell

Table 1 2 Classification of the different types of electrochemical electrode materials

MATERIALS	EXAMPLES
Metals	platinum, gold, nickel, silver
Carbonaceous materials	glassy carbon, pyrolytic graphite (basal or edge plane), carbon paste, carbon fibres
Semiconductors	metal oxides such as ruthenium oxide, tin oxide
Conducting organic salts	N-methyl phenazinium / 7,7,8,8-tetracyano-p-qumodimethane i e $(NMP)^+$ TCNQ

The reference electrode is usually placed as close as possible to the working electrode to ensure minimal resistance between them The electrode is either a saturated calomel electrode (SCE, $Hg/Hg_2Cl_2/KCl$), a mercury-pool electrode, or a silver/silver(I) chloride electrode (Ag/AgCl/KCl), which has a relatively lower sensitivity to current loadings The potential of the reference electrode is usually known and must remain constant during electroanalysis The counter electrode is the current-carrying electrode It is usually a simple noble metal electrode e g platinum foil or gauze, with a larger surface area than the working electrode It is possible for the counter electrode to be placed in a glass vessel and separated from the reaction medium by a porous frit to prevent contamination by reaction products generated at the surface of the electrode The potentiostat holds the potential of the working electrode against the constant potential of the reference electrode It also measures the current at the working electrode

Voltammetry represents a wide range of electrochemical techniques as shown in Table 1 3 [91] However, the most widely utilised techniques in organic-phase bioelectroanalysis are cyclic voltammetry (CV), fixed-potential amperometric and spectroelectrochemical methods

Table 1 3 Types of voltammetric methods Reproduced from [91]

METHOD	EXAMPLES
POTENTIAL STEP METHODS	normal pulse voltammetry, square-wave voltammetry, differential pulse voltammetry, chronocoulometry
LINEAR POTENTIAL SWEEP STRIPPING CHRONOAMPEROMETRY	anodic and cathodic stripping voltammetry
PHASE SENSITIVE AC VOLTAMMETRY	fundamental AC voltammetry
LINEAR POTENTIAL SWEEP (DC) VOLTAMMETRY	cyclic voltammetry, classical DC polarography, current sampled (Tast) voltammetry
CONTROLLED POTENTIAL METHODS	amperometric titrations, fixed-potential amperometric detection

1.3.1. CYCLIC VOLTAMMETRY

Cyclic voltammetry is a cyclic potential scan technique It involves changing the potential of the working electrode linearly at a scan rate, v, from a starting potential, i e the initial potential E_i, to a predetermined final potential i e the switch potential E_λ, and then returning to the starting potential The scan rate, v, varies from 1 mV/s to as high as 10^6 V/s A simultaneous measurement and recording of the working electrode current is made along with the potential sweep The resulting current-voltage diagram is called a cyclic voltammogram (CV) A typical CV is shown in Figure 1 4

The measured current is diffusion and mass transfer controlled For a simple reversible reaction

$$S_{ox} + ne^- \rightleftharpoons S_{Red}$$

at the beginning of the CV experiment, the starting potential is chosen such that there is no electrolysis of the electroactive species in the cell Then the potential is scanned initially in the negative (or forward) direction At a sufficiently negative potential, there is a reduction of the electroactive species, S_{OX}, at the electrode surface Hence, a diffusion controlled reduction current (denoted I_{pc}) increases rapidly until the surface concentration of S_{OX} approaches zero The current is diffusion-controlled because at fast scan rates ($v > 20$ mV/s), the rate of diffusion

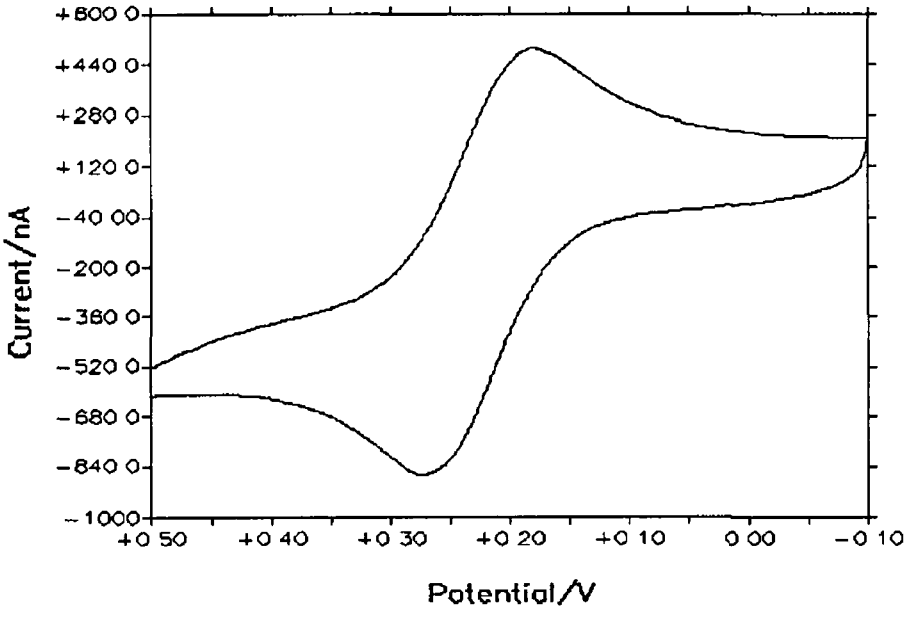


Figure 1 4 A typical cyclic voltammogram of a peroxidase/Eastman AQ polymer-modified electrode in 98 % v/v acetonitrile, containing 4 mM 1,1-dimethylferrocene and 0 1 M TEATS The concentration of horseradish peroxidase on the electrode surface was 34 U/cm^2 E_i was -100 mV and E_λ was 500 mV *vs* SCE, while the voltage scan rate was 20 mV/s The cathodic peak potential, E_{pc} and the anodic peak potential, $E_{p,a}$,were +275 and +180 mV, respectively The cathodic peak current, I_{pc} and the anodic peak current, I_{pa} were -770 and + 680 nA, respectively

of S_{OX} is not high enough to replace its depleting concentration around the electrode surface as quickly as it is being reduced Therefore, the cathodic current decays giving rise to a peak-shaped voltammogram At the switch potential, scanning is performed in the positive (reverse) direction When the electrode potential is sufficiently positive to bring about the oxidation of accumulated S_{Red}, an anodic current (denoted $I_{p\,a}$) flows and counteracts the cathodic current The diffusion-controlled anodic current increases until the surface concentration of S_{Red} approaches zero and then decays as the solution surrounding the electrode is depleted of the S_{Red} formed during the cathodic scan However, if the potential scan is carried out at relatively slow scan rates (i e $v < 10$ mV/s), when the surface concentration of the redox species equals zero, the curent reaches a plateau resulting m a sigmoidal-shaped cyclic voltammogram as shown m Figure 1 5 The cathodic/anodic peak currents obtained are essentially steady-state and could be used for measuring the concentration of the electroactive substance/analyte [92]

The steady-state current (in amperes) is then described by the equation [92]

$$I_{ss} = nFA[S_{ox}]^{*}[E](kD_{ox})^{1/2} \qquad \textbf{(1-12)}$$

where F is the Faraday constant (96 485 C/mol), A is the electrode area in cm^2, $[S_{OX}]^{*}$ is the bulk concentration of the oxidant in mol/cm^3, k is the reaction rate constant m mol/s, D_{OX} is the diffusion coefficient of S_{OX} in cm^2/s and [E] is the enzyme concentration in mol/cm^3

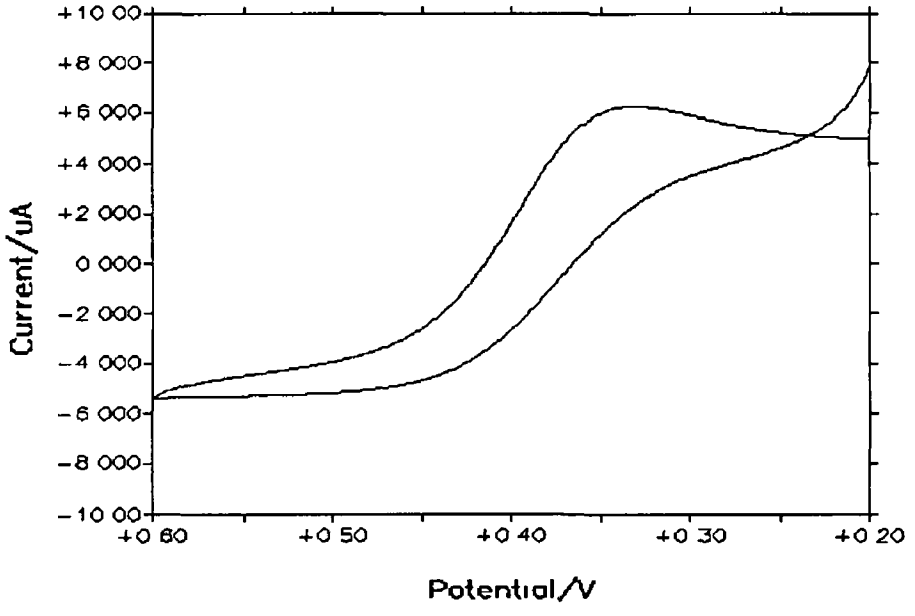


Figure 1 5 A sigmoidal cyclic voltammogram for an enzyme modified glassy carbon electrode, when 100 μM of butanone peroxide was added to the reaction cell The electrode was modified with 68 U/cm^2 of horseradish peroxidase The initial potential was +200 mV, and the switch potential was +600 mV The scan rate was 5 mV/s The reaction medium was 98 % v/v acetonitrile containing 0 1 M TEATS and 0 5 mM ferrocenemethanol

The important parameters of a cyclic voltammogram are the cathodic and anodic peak potentials, $E_{p\,c}$ and $E_{p\,a}$, the cathodic and anodic peak currents, $I_{p,c}$ and $I_{p,a}$, the potential at half the cathodic or anodic peak height, $E_{p/2\,c}$ or $E_{p/2\,a}$, and the half-wave potential, $E_{1/2}$ These parameters are particularly useful in determining if an electrochemical reaction is reversible, irreversible or quasi-reversible The diagnostic features of the cyclic voltammogram for reversible irreversible and quasi-reversible chemical reactions are shown in Table 1 4 [85, 93]

For a reversible reaction, the peak current (in amperes) for S_{OX} is given by

$$I_p = (nF)^{3/2} (\pi \nu D_{OX}/RT)^{1/2} \chi(\sigma t)\, A[S_{OX}] \qquad \textbf{(1-13)}$$

where $\chi(\sigma t)$ is 0 4463, and R is the gas constant For a simple electron transfer reaction at 298 K, the peak current is given by a simplified expression of eqn 1-13 above, i e the Randles-Sevcik equation

$$I_p = -(2\ 69 \times 10^5) n^{3/2} A\, D_{OX}^{1/2}\, \nu^{1/2} [S_{OX}] \qquad \textbf{(1-14)}$$

where ν is the scan rate in V/s These equations and characteristics all hold when the experiments are carried out with a planar working electrode and require slight modifications when there is adsorption or when using microelectrodes

Table 1 4 The diagnostic features for reversible, irreversible and quasi- reversible reactions as obtained from cyclic voltammetry

FEATURE	REVERSIBLE	IRREVERSIBLE	QUASI-REVERSIBLE
plot of I_p versus $v^{1/2}$	linear	linear	non-linear
plot of E_p versus $v^{1/2}$	E_p is independent of $v^{1/2}$	E_p varies with v	E_p varies with v
/E_p - $E_{p/2}$/ at 25°C	56 6/n mV	47 7/(αn_a) mV	26$\Delta(\Lambda,\alpha)$/n mV
$E_{p\,a}$ - $E_{p\,c}$	57 0/n mV (provided E_λ << $E_{p,c}$	$E_{p\,a}$ or $E_{p\,c}$	≠57 0/n mV
$I_{p\,a}/I_{p\,c}$ =	1	$I_{p\,a}$ or $I_{p\,c}$	≠ 1

where n is the number of electrons transferred per molecule of the electroactive substance, n_a is the number of electrons transferred in the rate determining step, α is the cathodic/anodic electrochemical charge transfer coefficient and $\Delta(\Lambda, \alpha) = (E_{p/2} - E_p)nF/RT$

Cyclic voltammetry is particularly useful in the initial characterisation and qualitative study of the catalytic/redox properties of biosensors It is used to determine the formal potentials of biosensors Also, it is used in the evaluation of electron transfer kinetics and detection of chemical reactions that occur prior to or follow electron transfer [94] It has been used for instance to study the electrochemistry and determine heterogenous electron transfer rate constants of the redox reactions of microperoxidase [95-97] The redox and kinetic properties of mediators and their suitability for a particular biosensor can also be usually studied with this technique [98, 99]

1.3.2. AMPEROMETRY

Amperometry, also called voltamperometry, is a fixed potential voltammetric technique It involves the measurement of current produced as a result of electron transfer reactions at the working electrode The measured current, usually the steady-state current (I_{ss}), is directly proportional to the bulk concentration of the electroactive substance/analyte in the cell The current is affected by (i) the rate of mass transfer of the analyte(s) from the bulk of the reaction medium to the electrode surface, (ii) the rate of electron transfer at the electrode surface and (iii) the rate of the surface and/or chemical reactions that occur prior to or follow the electron transfer process, e g adsorption, protonation and catalytic decomposition However, a steady-state current is obtained when the rates of all the reaction steps contributing to its magnitude are equal Hence, for a reversible homogenous electrode reaction which exhibits fast heterogenous electron transfer kinetics, I_{ss} is

governed only by the rate at which the electroactive substance is brought to the electrode surface by mass transfer There are three types of mass transfer methods

- **Migration** -movement of charged ions as a result of electrical potential gradient
- **Convection** - movement of the electroactive substance brought about by solution stirring or hydrodynamic transport
- **Diffusion** - movement of the electroactive substance as a result of a concentration gradient

Organic-phase amperometric experiments are usually performed in the presence of an excess inert supporting electrolyte relative to the amount of the analyte Usually, the concentration of the supporting electrolyte is at least 100 times more than that of the analyte This eliminates the effect of migration of charged ions In a similar way, by either stirring the reaction medium at a constant rate, making use of rotating disk electrodes or using controlled flow arrangements as in high pressure liquid chromatography (HPLC) and flow injection analysis (FIA), the effect of convective transport is kept constant Hence, the current is limited by the rate of diffusion of the analyte Therefore, for a reversible reaction

$$S_{OX} + ne^- \rightleftharpoons S_{Red}$$

the cathodic current is proportional to the area of the electrode A, the number of electrons transferred n and the difference between the bulk concentration $[S_{OX}]^*$ and the surface concentration $[S_{OX}]_{(0\ t)}$

$$I = nFAm_{OX}([S_{OX}]^* - [S_{OX}]_{(0,t)}) \quad \textbf{(1-15)}$$

where m_{OX} is the mass transport coefficient of the analyte, S in the oxidised form [100-103] The potential of the working electrode is fixed at a predetermined value, such that every molecule of S_{OX} that reaches the electrode surface is immediately reduced to S_{Red} Therefore, the mass transport steady-state current or limiting current (I_L) is reached I_L is a linear function of the bulk concentration of S_{OX} provided m_{OX} remains constant, i e ,

$$I_L = nFAm_{OX}[S_{OX}]^* \quad \textbf{(1-16)}$$

In stirred amperometric experiments, a stagnant layer (diffusion layer) is formed close to the electrode surface If the conditions of linear diffusion apply over this layer then $m_{OX} = D_{OX}/\delta_{OX}$, where D_{OX} is the diffusion coefficient of S_{OX} and δ_{OX} is the thickness of the S_{OX} layer formed However, for rotating disk electrode experiments $m_{OX} = 0\ 617 D_{OX}^{2/3}\omega^{1/2}\varphi^{-1/6}$ Therefore, the limiting current for a rotating disc electrode is given by the equation

$$I_L = 0\ 62nFAD_{OX}^{2/3}\ \omega^{1/2}\ \varphi^{1/6}[S_{OX}]^* \quad \textbf{(1-17)}$$

where ω is the rotation speed in revolutions per second and φ is the kinematic viscosity in cm^2/s In steady-state amperometric experiments the selection of the operating potential allows only limited selectivity This is usually further improved in biosensors by the catalytic properties of the enzyme or other biological substances and the controlled mass transport through functionalized polymeric films [98] A typical amperometric current-concentration profile is shown in Figure 1 6 The values of the measured current when plotted against the bulk concentration of the analyte gives a calibration plot for the analyte Most biocatalytic systems would give a Michaelis-Menten type, or hyperbolic calibration plot The steady-state current is directly proportional to the initial rate of reaction, v_o, such that

$$I_{ss} = I_{max} [S_{OX}]/K_m' + [S_{OX}] \qquad \textbf{(1-18)}$$

For an immobilised enzyme electrode, I_{max} is directly proportional to V_{max}, i e

$$I_{max} = (nFA/k_{cat} [E]) = (nFA/V_{max}) \qquad \textbf{(1-19)}$$

where *l* is the thickness of the enzyme layer and [E] is the total enzyme concentration [104]

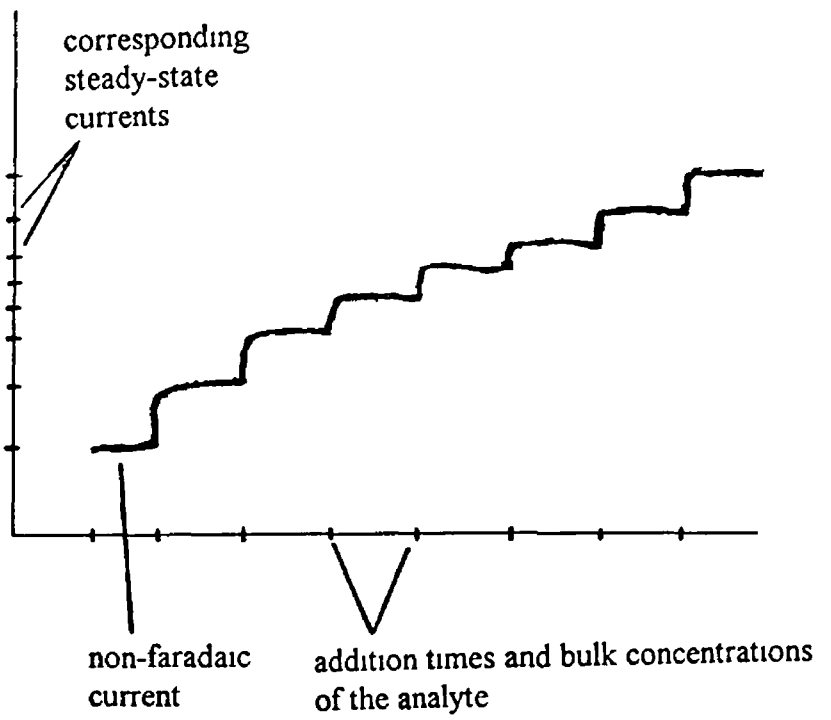


Figure 1 6 A typical current-time plot as obtained in a fixed-potential amperometric experiment Aliquots of known analyte concentrations are added at the equal time intervals Hence, a calibration plot of steady-state current versus bulk concentrations of the analyte can be obtained

1.3.3. SPECTROELECTROCHEMISTRY

This is a technique which combines optical and electrochemical methods, the most commonly used optical method being ultraviolet-visible (UV-VIS) absorption spectroscopy It involves applying a fixed potential to the reaction medium and simultaneously following changes in absorbance as a result of species being depleted or produced in the redox process Experiments are done with a "funnel-shaped" optically transparent thin layer electrochemical (OTTLE) cell The working electrode is either a transparent platinum or gold minigrid, which is placed between two quartz microscopic slides The reference and counter electrodes are placed in contact with the reaction medium in the upper half of the cell The working volume of the cells range from 30 μl to 7 ml Spectroelectrochemistry is useful for studying the absorption characteristics of the electroactive species The absorption measurements could be used to determine the concentration ratio of oxidized/reduced species at each applied potential Therefore, the formal potential and the number of electrons transferred during the redox process can be determined [94, 105] Deng and Dong [106] have for instance used it to determine the redox potentials and heterogenous electron transfer constant of HRP at a poly (o-phenylenediamine) modified platinum electrode

1.4. ORGANIC-PHASE ENZYME ELECTRODES

Organic-phase enzyme electrodes, (OPEEs), are biological devices, in which the enzyme effects the generation of electrochemically active species by interacting with the analyte(s) The electrochemically active intermediates or products are then monitored amperometrically at the electrode surface The organic-phase may be a gel or organic moiety, incorporated into the microenvironment of the immobilized enzyme This has been used in development of a tyrosmase-based OPEE, in which the enzyme was mixed with silicone grease prior to immobilization on graphite disc electrodes [107] Phenols (the analytes) partitioned into the organic enzyme microenvironment from an aqueous reaction medium The sensitivity of this type of OPEE is largely determined by the hydrophobic interactions between the gel and the analyte(s) This is because the hydrophobic interactions determine the extent of analyte partitioning into the enzyme layer However, the most common type of OPEEs are those in which the organic-phase is in the form of an organic solvent reaction medium to which the enzyme is exposed An example of this is the first OPEE reported [50, 108] This was a tyrosinase-based biosensor for phenol detection in chloroform In organic solvents, tyrosinase catalyses the oxidation of phenols to the corresponding quinones, which are then electrochemically detected at the electrode surface This form of phenol detection in aqueous medium is impossible because o-quinones undergo polymerisation to form enzyme deactivating polyaromatic pigments [109] It is important that the essential amount of water in the immobilised enzyme layer (which depends on the

hydrophobicity of the organic solvent) is maintained for high sensing efficiency

There are three different classes of OPEEs based on the nature of the electron transfer process employed This is similar to the first, second and third generation types of aqueous-phase enzyme electrodes [90]

- OPEEs based on the measurement of the increase or decrease in the concentrations of oxygen or hydrogen peroxide, e g the Clark-type oxygen sensors

- OPEEs in which the enzyme undergoes an initial redox reaction with the analyte and then another redox reaction with a mediator, the mediator is in turn oxidised/reduced by the electrode to generate a current that is directly proportional to the concentration of the analyte

- OPEEs in which the enzyme undergoes a redox reaction with the analyte and (in the absence of a mediator) then gets oxidised/reduced to its original form by the modified or unmodified electrode

Campanella *et al* have used an amperometric, tyrosinase-modified, gas diffusion oxygen electrode for phenol detection in several organic solvents [37, 110] This OPEE was based on measurements of the decrease in current as oxygen is used up in the enzymatic reaction

$$\text{phenol} + O_2 \xrightarrow{\text{tyrosinase}} \text{hydroquinone} + H_2O$$

Another OPEE of this type was used in the determination of cholesterol in butter and margarine samples [111] A significant feature of these OPEEs is their ability to function (in the absence of added water [110] and supporting electrolyte) in hydrophobic solvents like n-pentane, n-hexane, n-heptane, toluene and chloroform The response time of the biosensors in these solvents varied between 1 5 and 3 minutes These sensors are, however, limited to aerobic systems in which the presence of oxygen is not detrimental to the reaction or stability of the reactants/products Also, their responses are sensitive to changes in the partial pressure of dissolved oxygen in the cell The second type of OPEEs, i e those that make use of mediators, are more common Mediators are low-molecular weight redox compounds, which shuttle electrons from the enzyme's active site to the surface of the electrode Hence, an electrical connection between the enzyme's active site and the electrode surface is established A potential mediating compound should be chemically stable in its reduction and oxidation states within the pH range of 5-8 and at the operating potential and temperature, be a specific substrate for the sensing enzyme, not react with the solvents or supporting electrolyte, and exhibit fast reaction kinetics with both the

enzyme and the electrode [112] The mediator may be immobilised along with the enzyme at the surface of the electrode to produce reagentless OPEEs Schubert *et al* [113] for instance used a water soluble mediator, pottasium hexacyanoferrate (II) co-adsorbed with HRP on a graphite electrode to detect hydrogen peroxide in non-polar organic solvents such as chloroform, chlorobenzene and 1-octanol The biosensor exploited the oxidation of hexacyanoferrate(II) ion by hydrogen peroxide in the presence of HRP The hexacyanoferrate(III) ion was rereduced at graphite electrodes at -20 mV The response time of the biosensor was 2 minutes Hexacyanoferrate(II) ion [114] has also been used co-immobilised with HRP within an Eastman AQ membrane The biosensor detected peroxides in polar organic solvent(s) e g 90 % v/v acetonitrile in water A response time of 5-20 seconds was achieved indicating fast reaction kinetics Mediators may also be added to the reaction medium, such that electron transfer between the enzyme active site and the electrode surface is effected by a freely diffusmg mediator o-Phenylenediamine (o-PEDA) has been well utilised in this manner for the detection of peroxides and HRP-inhibitors such as thiourea, ethylenethiourea, mercaptoethanol and methyl isothiocyanate [73, 81,115-117] The response times in polar solvents e g 95-98 % acetonitrile, methanol, 2-butanol, tetrahydofuran (THF), and acetone was about 5-15 seconds in all cases Hydrophobic compounds, such as ferrocenes, have also been used as soluble freely diffusing mediators in OPEEs Ferrocene monocarboxylic acid (FMCA) was used in the determination/detection of glucose in polar solvents such as acetonitrile and 2-butanol [53, 118] Also, ferrocene was used in the determination of peroxides using a HRP-based biosensor in acetonitrile [71] The third

type of electrodes are those that make use of electrode materials or immobilization media on which the oxidised/reduced enzyme can be directly re-reduced/oxidised Iwuoha and Smyth [119, 120] have developed GOx-based OPEEs in which a direct unmediated electrical communication between the enzyme and the electrode is achieved by "molecular wiring" This is formed by some kind of electrostatic interaction between polycationic Os-polymer and polyanionic enzyme molecule A large biomolecular complex was formed by reacting ("wiring"), and in the process entrapping, GOx with the redox/conducting osmium polymer, $[Os(bpy)_2(PVP)_{20}Cl]Cl$, on a glassy carbon electrode The active site of GOx was, therefore, connected electrically to the electrode surface via the formed conducting biocomplex The biosensor was used to determine glucose in acetonitrile containing 0-35 % water (v/v) Also, a HRP-based biosensor for the detection of BTP and hydroxylamine, based on direct electron transfer between HRP and the platinum electrode has been reported [121] The biosensor operated in polar solvents such as 98 % (v/v) acetonitrile and methanol

The type of electron transfer exploited in the development of enzyme-based biosensors depends on the physico-chemical properties of the organic media desired, the nature and stability of the sensing enzyme and the desired sensor response time and sensitivity A variety of amperometric organic-phase enzyme electrodes are shown in Table 1 5

Table 1 5 Examples of developed organic-phase enzyme electrodes indicating the mode of immobilization and mediation exploited

ENZYME	ANALYTE	SOLVENT	METHOD OF IMMOBILISATION	MEDIATOR	REFERENCE
Tyrosinase	phenol	n hexane	entrapment with a dialysis membrane	oxygen	37
Acetyl choline esterase	paraoxon, aldicarb	variety of hydrophobic solvents	entrapment in photocrosslinkable polymer PVA SbQ	-	49
Tyrosinase	phenols	buffer saturated chloroform	adsorption on graphite foil	oxygen	50
GOx	glucose	2 butanol	crosslinking with BSA and glutaraldehyde	FMCA	53
HRP	BTP, MCE THU and ETU	acetonitrile	entrapment in Eastman AQ 55D polymer	o-PEDA	73
Tyrosinase	phenols	pentane, hexane toluene, chloroform, acetonitrile and water	entrapment in Kappa carregenan gel	oxygen	110
Cholesterol oxidase	cholesterol	chloroform hexane (1 1)	adsorption on alumina	oxygen	111
HRP	peroxide	dioxane, chloroform and octanol	coadsorption with mediator on graphite foil	$[Fe(CN)_6]^{4-}$	113
HRP	peroxides	acetonitrile	entrapment in graphite-epoxy resin	o-PEDA	117
GOx	glucose	acetomtrile	entrapment m a conducting osmium polymer	osmium redox centres	119
HRP	BTP HLA	acetonitrile and methanol	entrapment in Eastman AQ 55D polymer	-	121
Laccase	catechol and hydroquino ne	butanol ethanol and propanol	entrapment in *Eastman* AQ 55D polymer		122
Alcohol dehydrogen ase	secondary alcohols	acetonitrile	entrapment with mediator in Eastman AQ 55D polymer	$NADP^+$ cofactor	123
Tyrosinase	phenol	chloroform	entrapment in cellulose acetate	oxygen	124
HRP	BTP	acetonitrile	entrapment in graphite teflon electrode	ferrocene	125

HRP	peroxides	chloroform and acetonitrile	adsorption on carbon fibre electrode	ferrocene	126
Tyrosinase	phenols	chloroform and acetonitrile	adsorption on carbon fibre	-	126

1.4.1. APPLICATIONS OF OPEEs

OPEEs are indeed capable of being used in any application, provided the analyte of interest is a substrate or inhibitor for an organic-phase stable enzyme The critical and deciding factor being the solubility of the sample matrix or analyte in organic solvents An OPEE can be used as a batch/disposable sensing device or as a detection system for continuous on-line flow system as in sequential injection analysis (SIA), FIA and HPLC systems There is a wide application/potential for OPEEs in environmental monitoring Toxic agricultural chemicals, e g pesticides and their degradation products, as well as industrial wastes in food, water and soil, can be easily determined with these sensors The toxicants are detected based on their inhibitory effects on the enzyme activity/catalysis, hence there is a potential of organic-phase biosensors being used to monitor air quality and professional hazards in workplaces When used in this way, OPEEs could provide a cheaper and faster pollution indication than living organisms such as fish and guinea pigs or vegetation OPEEs have being reported for the determination of diethyldithiocarbamate, dichlorophenoxyacetic acid, organophosphorus and organocarbamate pesticides, pesticide degradation products such as methyl isothiocyanate and ethylenethiourea, and industrial wastes like

phenols, hydroxylamine, mercaptoethanol and benzoic acid [49, 73, 81, 110], phenols being detected as substrates of tyrosinase-based biosensors The major drawback in the application of OPEEs in the determination of environmental analytes is that, since most of the poisonous analytes are mimicking their toxic mechanism in living systems, their inhibitory effects on the enzyme catalysis are not usually fully reversible Hence, OPEEs can only be used as batch sensors for most applications of this nature This neccesitates some form of sample pretreatment for complex sample matrices containing either more than one analyte or forms of interferences

The most common clinical application of an OPEE to date has been in the determination of cholesterol [111] However, its potential in monitoring other clinically relevant hydrophobic substances, such as the lipophilic vitamins A, D, E and K, bilrubin and fatty acids, cannot be overemphasised Similar applications for veterinary uses can also be envisaged

There is a great application potential for these sensors in process monitoring and quality control laboratories of pharmaceutical, food and petrochemical industries In terms of pharmaceutical applications, tyrosinase and peroxidase-based OPEEs have being developed as detection systems for FIA of phenols and peroxides in a range of anti-infective pharmaceutical formulations and cosmetic products [71] Other potentials for OPEEs m the pharmaceutical industry includes monitoring of the production of steroids and steroid-based drugs Its possible application to other cosmetic ingredients, such as allantom, glycerol and

triglycerides is also envisaged In addition, Wang *et al* [127] have reported on the FIA determination of phenols in olive oils The biosensor allowed an injection rate of 60 samples per hour and exhibited a detection limit of 4 x 10^{-7} mol/cm^3 In another study [128] they reported the FIA determination of the peroxide value of vegetable oils in chloroform The sensor permitted an injection rate of 120 samples per hour and exhibited a detection limit of 2 5 ppm OPEEs have the potential of being used to determine optically active products in food products m addition to monitoring the shelf life of certain foods Their ability to detect glucose and alcohols in organic solvents would enable their use in process momtoring and quality control of hydrophobic fermentations in the beverage/wine industry The use of OPEEs to determine low water concentrations has also been demonstrated [129], thus providing a more simple and rapid alternative method to the Karl Fisher method Finally, an alcohol dehydrogenase-based OPEE was used to detect secondary alcohols such as 2-butanol, 2-propanol, 2-pentanol, etc , in untreated petrol samples [123] Similar detection systems would enable an expansion of the application of OPEEs to the petrochemical industry The potentials and capabilities of OPEEs m defense and military applications have also being projected [51]

1.5. OBJECTIVES OF THE THESIS

The main objectives of this thesis are

- to determine polar organic solvents, in which horseradish peroxidase and tyrosinase-modified electrodes would maintain catalytic activity

- to evaluate biosensor sensitivities as well as kinetic parameters such as K_m', K_i', I_{max} etc , in detecting analytes (i e enzyme substrates and inhibitors) in these solvents

- to relate the evaluated biosensor sensitivities and kinetic parameters to the physical properties of the solvents

- to use the developed biosensor as a detection unit for reversed-phase chromatographic analysis of analytes

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CHAPTER TWO: BIOCHEMICAL PROPERTIES OF THE SENSING ENZYMES

2.1. HORSERADISH PEROXIDASE AND TYROSINASE

The sensing enzymes used in this thesis are horseradish peroxidase and mushroom tyrosinase In this section, the structure, biochemistry and reaction kinetics of these enzymes, as well as the chemistry, structure and analytical significance of their respective substrates and inhibitors, (i e the analytes) will be discussed

2.1.1. OCCURRENCE OF THE ENZYMES

2.1.1.1. *HORSERADISH PEROXIDASE*

Enzymes referred to as peroxidases are oxidoreductases and belong to the EC class 1 11 The major characteristic of peroxidases is their ability to catalyse reactions of a wide variety of electron donating compounds in the presence of peroxide These enzymes possess a redox active site, which can undergo alternate oxidation by peroxide and reduction by the electron donating compound Peroxidases are widely distributed in the plant and animal kingdoms There are peroxidases isolated from animal sources such as thyroid, bacterial and glutathione peroxidases, and plant sources such as turnip, peanut, alfalfa, tobacco, horseradish and fungal-lignin peroxidases [1, 2] The major difference between the several types of peroxidases lies in their specificity (and subsequently their physiological roles) for peroxide and electron donor For instance, glutathione peroxidase, the enzyme that controls the auto-oxidation of unsaturated lipids, exhibits a high specificity for lipid hydroperoxides as oxidants and glutathione as an electron donor Most of the peroxidases

isolated from plants sources play an important physiological role in lignin biosynthesis, with the exception of fungal peroxidases which are functional in lignin biodegradation of the plants Among the plant peroxidases, horseradish peroxidase (HRP) is the most extensively studied The enzyme was discovered by Bach in 1903, and is readily obtained in horseradish (*Armoracia rusticana*) roots The inherent properties of HRP, such as its stability over a pH range of 5-10, and good thermostability, have allowed its wide use in dry-reagent chemistry "dipsticks" and biosensors for different areas of analytical biochemistry, e g enzyme immunoassays

2.1.1.2. *MUSHROOM TYROSINASE*

Tyrosinases are also oxidoreductases and belong to the EC class 1 14 A major feature of these metalloproteins is their widespread presence in plants and living organisms Tyrosinases catalyse either the oxidation of monophenols or ortho-diphenols to their respective ortho-quinones The qumones formed then undergo further enzymatic and non-enzymatic reactions that lead to the formation of polymeric pigmented materials These reactions are responsible for the distinctive pigmentation of the skin, eyes and hair in animals [3] In a similar way, these reactions control the browning and ripening processes of fruits and vegetables Tyrosinases are therefore of great economic and practical importance in the food and agricultural industry Amongst the tyrosinases isolated from plant/fruit sources such as banana, beet, potato, mushroom, carrot, broad bean and spinach, the mushroom tyrosinase is the most utilised in the development and study of tyrosinase-based biosensors Mushroom

(*Agaricus bisporus*) tyrosinase is relatively stable and commercially available in a preparation of high specific activity

2.1.2. BIOCHEMISTRY AND STRUCTURE

2.1.2.1. *HORSERADISH PEROXIDASE*

HRP is a glycoprotein containing iron (III) protoporphyrin IX (shown in Figure 2 1) as the prosthetic group [4] The unpurified enzyme has about 40 different isoenzymes But the three most important are acidic isoenzyme A (HRP-A), neutral or slightly basic isoenzyme C (HRP-C), and a strongly basic HRP, i e cyanoperoxidase HRP-C has an isoelectric point of 8 5 and is responsible for about half the catalytic activity of the unpurified enzyme [5] Most commercial preparations of the enzyme are HRP-C, therefore, all further discussions of the enzyme refer to the isoenzyme C, unless otherwise specified

A molecule of HRP consists of 308 amino acid residues, the iron(III) protoporphyrin, 18 % carbohydrate content, 4 intramolecular sulphide bonds, 2 calcium(II) ions and some bound water The sequence of the 308 amino acid residues of HRP has been determined by Welinder [6, 7] This sequence has been confirmed in another study by Fujiyama *et al* [8], in which 3 different *c*DNA clones of HRP were isolated and characterised One of the isolated *c*DNA clones showed a similar amino acid sequence

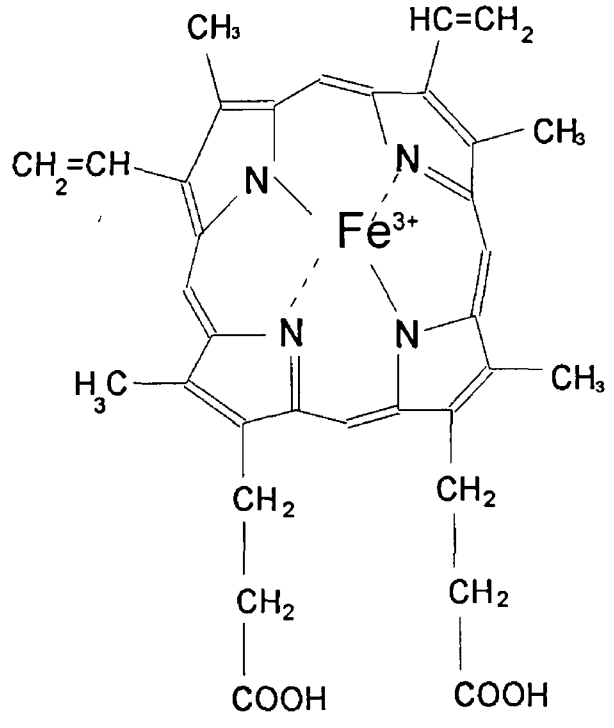


Figure 2 1 The chemical structure of iron(III) protoporphyrin IX

Also, on the basis of this sequence, a HRP-C encoded gene has been synthesized and expressed in *Escherichia coli* The unglycosylated, insoluble and inactive recombinant enzyme was solubilised and folded to give the active enzyme An overall yield of 2-3 % was reported [9] The diverse specificity of peroxidases and all heme-containing proteins has been linked to the effects of the surrounding amino acid residues on the reactivity of the prosthetic heme group Amino acid residues that are

crucial to the catalytic reactivity of HRP have been identified as the axial His 170, Leu 237 and Tyr 185 on the proximal side and Arg 38, His 42 and Phe 41 on the distal side [10] The prosthetic heme active site is non-covalently attached through the iron(III) fifth coordination position to the imidazole side of His 170 The heme-active site has been shown [11] to be buried within the HRP molecule, such that only the heme-edge is accessible to substrates, thus making the catalytic activity of the enzyme susceptible to the effects of the hydrophobic and ionic properties of surrounding protein Indeed, it has been shown [10, 12] that the binding of aromatic donor molecules, e g resorcinol, 2-methoxy-4-methyl phenol, and competitive inhibitors, e g benzhydroxamic acid, occur in the vicinity of the heme peripheral 8-methyl group The extent and nature of the bindmg being affected by hydrophobic interactions with Tyr 185 and hydrogen bonding with adjacent amino acid residues such as Arg 183 The presence of 2 calcium(II) ions per molecule of HRP has never been linked to reaction mechanisms [13] It is, however, believed to be responsible for the structural stability of the enzyme In a study by Shiro *et al* [14], the removal of the calcium ions was shown to lead to a decrease in the specific activity and thermal stability of the enzyme The carbohydrate content of HRP consists of 8 neutral oligosaccharide side chains attached to Asparagine residues 13, 57, 158, 186, 198, 214, 255 and 268 Kurosaka *et al* [15] reported the structure of the major glyco-components of HRP The proposed structure is shown in Figure 2 2 It has been shown that the absence of the carbohydrate side chains does not affect the catalytic ability of HRP [9] Yeast cytochrome *c* peroxidase is the only peroxidase for which X-ray crystallography has been used to obtain high resolution structural information [16] Amino acid sequence

homology between plant peroxidases and this yeast peroxidase has been used to model the structure of HRP-C [10, 13] Figure 2 3 shows the predicted active site structure of HRP-C The enzyme has a total molecular weight of about 42,100

2.1.2.2. *MUSHROOM TYROSINASE*

Mushroom tyrosinase is known to be localised in several tissues of the mushroom fruiting body The different tissues have been reported to contain varying amounts of active and inactive (latent) forms of the enzyme, and different forms of tyrosinase isoenzymes which in turn exhibit varying specificities for L-3, 4-dihydroxy phenylalanine (L-DOPA), tyrosine and catechol [17, 18] The inactive enzyme forms can be reactivated by treatment with proteases or detergents such as sodium dodecyl sulphate (SDS) [19, 20] Reactivation with proteases is believed to occur via proteolysis, and with SDS, a minor conformational change in the enzyme has been suggested However, the mechanisms of activation still remain to be elucidated In a recent study, Rodriguez and Flurkey [21] reported that tissues in the stalk of the mushroom plant contained faster migrating isoenzyme forms than other morphological regions This isoenzyme, however, exhibited the least specificity for L-DOPA

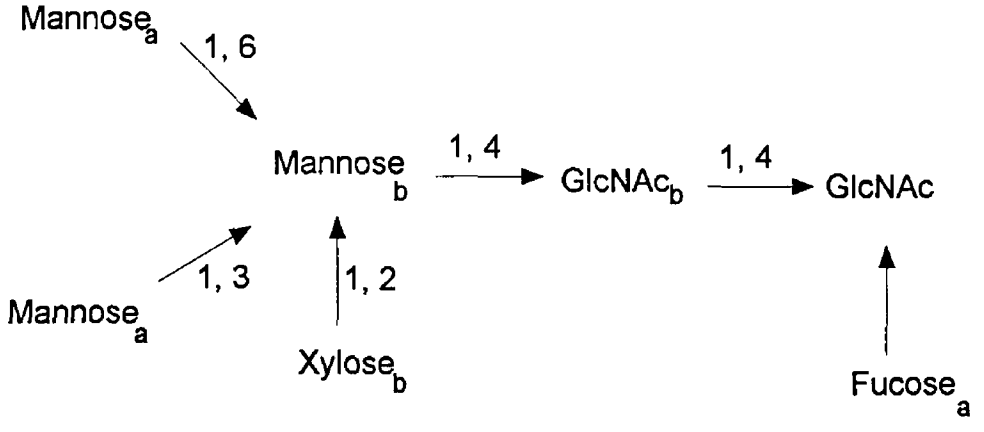


Figure 2 2 Proposed structure for the major oligosaccharide from horseradish peroxidase The numbers indicate the coding for the respective monosaccharides and GlcNAc = N-acetylglucosamine

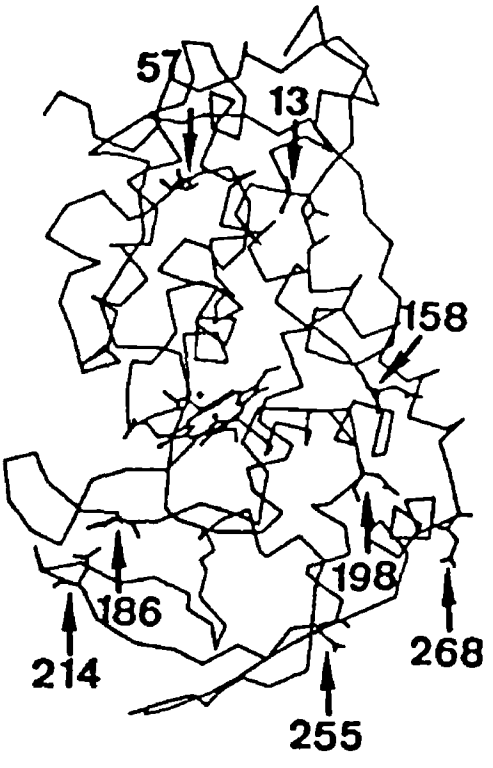


Figure 2 3 The predicted backbone structure of horseradish peroxidase Glycosylation sites are indicated by the numbers Reproduced from [5]

It has been reported that these variations, i e different isoenzyme forms, specificities and ratio of active to inactive enzyme forms m mushrooms, changes markedly during the development of the plant from small pins to the mature stage [22] They may also be considerably affected by growth conditions As a result of these multiplicity, there has been a rather poor progress in unravelling the complete primary structure of mushroom tyrosinase and other higher plant tyrosinases However, Strothkamp *et al* [23] investigated the quaternary structure of the enzyme using SDS-acrylamide gel electrophoresis Mushroom tyrosinase was found to contain two different types of polypeptide chains, designated "heavy" (H) and "light" (L), with molecular weights of 43,000 and 13,400, respectively It was concluded that the predominant form of the enzyme, in aqueous solutions has a molecular weight of about 120,000 and a quaternary structure, L_2H_2 In contrast, significant progress have been made with fungal tyrosinases A full description of the primary structure of tyrosinase from *Neurospora crassa* [24-26] and *Streptomyces glaucescens* [27] has been reported However, unlike m mushroom tyrosinase, the *Neurospora* tyrosinase has been shown to contain two similar polypeptide chains [28, 29] Hence it remains to be established if the mechanism described for *Neurospora,* its copper binding and several other features also apply to mushroom tyrosinase Tyrosinase enzyme from mushrooms have an isoelectric point of 4 7

2.1.3. KINETICS OF CATALYTIC REACTIONS

2.1.3.1. *HORSERADISH PEROXIDASE*

The site of catalytic activity in HRP is the sixth coordination position of the iron(III) ion within the protoporphyrin group This site can be occupied by water, peroxides, fluorides and cyanides A normal HRP catalysis of peroxide involves the formation of different oxidation states of the enzyme There is an initial formation of HRP **compound I** (HRP-I), an mtermediate-spin iron(IV) compound, which has two oxidizing equivalents above iron(III) ion in the native enzyme HRP-I further undergoes a single electron reduction step to give a low-spin, iron(IV) compound referred to as HRP **compound II** (HRP-II) Finally, HRP-II undergoes another single electron reduction step to give HRP in its resting state [30-32] Hence, the enzymatic reaction takes place via a modified peroxidase shuttle mechanism as shown m the equations below

$$HRP + ROOH \xrightarrow{k_1} HRP\text{-}I + ROH$$

$$HRP\text{-}I + AH_2 \xrightarrow{k_2} HRP\text{-}II + {}^{\bullet}AH$$

$$HRP\text{-}II + AH_2 \xrightarrow{k_3} HRP + {}^{\bullet}AH + H_2O$$

$${}^{\bullet}AH + {}^{\bullet}AH \xrightarrow{k_4} AH_2 + A$$

where AH_2 is a hydrogen donor or electron donor compound Compounds that have been used with HRP as hydrogen or electron donors include o-dianisidine, guiacol, p-fluorophenol, iodide, luminol, dihydrofluorescein, 2,2'-azmo-di-[3-ethyl-benzothiazme-(6)-sulphonic acid] (ABTS), p-chloronaphthol, ferrocyanide, o-phenylenediamine and ferrocenes [5] The latter three are commonly used as mediators in HRP based amperometric biosensors The presence of HRP **compound III** (HRP-III), an oxy-iron(II) compound, also referred to as oxyperoxidase has also been reported [33, 34] It is formed from excess peroxide and HRP-II HRP-III is catalytically inactive and decomposes slowly to give HRP in its resting state as shown below

$$\text{HRP-II} + \text{ROOH} \longrightarrow \text{HRP-III} + \text{ROH}$$

$$\text{HRP-III} \longrightarrow \text{HRP} + O_2^-$$

However, the rate of reaction in the latter step is very slow Hence, any accumulation of HRP in the HRP-III state would result in a loss in catalytic efficiency The direct regeneration of HRP from HRP-I by some substrates, e g iodide, is also known Peroxides also undergo similar reactions with HRP on carbon paste, platinum, platinized carbon paste and graphite electrodes [4, 35, 36] These forms of enzymatic reaction involve a single two-electron oxidation and reduction steps, i e

$$\text{HRP} + \text{ROOH} + 2H^+ \longrightarrow \text{HRP-I} + \text{ROH} + H_2O$$

$$\text{HRP-I} + 2e^- \longrightarrow \text{HRP}$$

2.1.3.2. *MUSHROOM TYROSINASE*

A pair of copper(II) ions are known to be the functional units in the active site of tyrosinase [37] A normal tyrosinase catalysis of phenol involves the shuttling of these copper ions between the +2 and +1 oxidation states [38] There is an initial formation of deoxytyrosinase and catechol Deoxytyrosinase has one oxidation equivalent below copper(II) ion in the native enzyme (met-tyrosinase) and further binds molecular oxygen to give oxytyrosinase Finally, oxytyrosinase undergoes another electron transfer reaction with the pre-formed catechol to give the enzyme in its resting state and water The reaction scheme proceeds via a modified shuttle mechanism as follows

phenol + met-tyrosmase ⟶ catechol + deoxytyrosinase

O_2 + deoxytyrosinase ⟶ oxytyrosinase

catechol + oxytyrosmase ⟶ 1, 2-benzoqumone + met-tyrosinase + H_2O

The use of one electron mediating compounds, such as ferrocene monocarboxylic acid, ferrocyanide and osmium bis (2, 2'-bipyridine), in amperometric tyrosinase-based biosensors have been reported [39, 40] However, most tyrosmase-based biosensors depend on the re-reduction of the formed o-quinone at the electrode surface The amount of o-quinone reduced is directly proportional to the concentration of phenol

2.1.4. ANALYTICAL APPLICATIONS

2.1.4.1. *HORSERADISH PEROXIDASE*

HRP has been well utilised as a sensing or "reporting" enzyme One of its earliest applications is its use in bienzyme colorimetric assays in a "dip-stick" format The technique involves the initial reaction of an oxidase with the analyte(s) to generate hydrogen peroxide after a fixed period of incubation The generated peroxide then reacts with HRP to oxidise a leuco dye, e g ABTS or p-aminoantipyrine, to its coloured form The colour intensity is then directly proportional to the concentration of the analyte [33] HRP has also found use as an alternative to radioactive labels in DNA probes and enzyme-linked immunosorbent assays (ELISA) The ELISA technique uses an enzyme-labelled antibody with the antigen attached to a solid phase, the binding of enzyme-labelled antibody to immobilized antigen is competitively inhibited by an added standard or test antigen The product concentrations measured at the end are inversely proportional to the concentration of the standard or test antigen in the incubation solution [41, 42] The enzyme has also been extensively used in amperometric biosensors For instance, the use of HRP in aqueous media as a monoenzyme biosensor for peroxides and its inhibitors [43, 44], in bienzyme biosensors for glucose, D-amino acids etc [45], and in trienzyme biosensors for cholesterol in serum [46], have been reported In a similar way, HRP has been well utilised in monoenzyme OPEEs for the detection of hydrogen peroxide, organic peroxides and HRP

inhibitors [47-50] This has been attributed to its ability to function and relative stability in organic solvents

2.1.4 2. *MUSHROOM TYROSINASE*

The major analytical application of tyrosinase is its use as a sensing enzyme for its natural substrates i e L-tyrosine, L-DOPA, catechol, phenol and their structural analogues The detection of L-tyrosme and some catechol-containing compounds is of great importance in clinical chemistry For instance, Toyota *et al* [51] reported the use of tyrosine detection by a tyrosinase-based biosensor to determine the total protein content in serum Similarly, its use for the selective elimination of acetaminophen interference during the amperometric biosensmg of glucose in biological fluids has been reported [52] More recent applications involves its use as a sensing enzyme for the detection of phenols and catechols as well as its inhibitors in organic solvents [49]

2.1.5. PEROXIDES

Peroxides are the primary substrates for horseradish peroxidase They are compounds with the -O-O- functional group and are described by the general formula R_1-O-O-R_2 The peroxide function may be part of a ring or polymeric system, and in some cases, there is more than one peroxide group present These compounds can be broadly divided into inorganic peroxides (when R_1 and R_2 are hydrogen or inorganic groups) and

organic peroxides (when either or both R_1 and R_2 are organic groups) R_1 and R_2 may be the same or different chemical groups to give symmetrical and unsymmmetrical peroxides, respectively

2.1.5.1. *INORGANIC PEROXIDES*

There are different types of inorganic peroxides or peroxy-compounds These include (i) peroxides formed from dioxygenyl ion, O_2^+, examples include $O_2^+AsF_6^-$, $O_2^+BF_4^-$ and $O_2^+PF6^-$ (these compounds are stable under an inert atmosphere at temperatures up to 100°C and are known to be powerful fluorinating agents), (ii) ionic superoxides containing the O_2^- ion, (iii) ionic peroxides containing the O_2^{2-} ion and ionic hydroperoxides containing the O_2H^- ion, and (iv) non-stoichiometric peroxides, formed by treatment of solutions of salts of thorium or of some actinide elements with hydrogen peroxide The type of peroxy-compounds formed by different elements is shown in Table 2 1 Inorganic peroxides are generally used as oxidising agents However, hydrogen peroxide is the most popular and commonly used inorganic peroxide [53] Hydrogen peroxide (H_2O_2) may be described as a saturated molecule, with single bonds between the hydrogen and the oxygen atoms, and between the two oxygen atoms Its structure is represented by H-O-O-H In its pure form, H_2O_2 is obtained as a syrupy and almost colourless liquid It is soluble in water and several organic solvents, such as alcohols, esters, ethers and amines and exhibits an extensive redox chemistry In aqueous solutions, as well as in its solid, liquid and gaseous states, H_2O_2 would readily decompose into water

Table 2 1 The types of peroxy-compounds formed by different elements Reproduced from [53, 54]

Type of peroxy-compound	Elements
ionic superoxides	alkali metals except lithium, alkali-earth metals, Zr, Hf, Fe, Co, Ag etc
ionic peroxides and hydroperoxides	alkali and alkali-earth metals, Zn-Hg
solid non-stoichiometric peroxides	rare earth metals, Th, Pa
"ether" and "alcohol" type hydroperoxides	Be, B, Al, Ga, C-Pb, F
peroxyacids, peroxy salts	C, P, S, V, Nb, Ta, Cr, Mo, W, Mn, U
peroxy complexes (neutral or cationic)	Ti, Zr, Hf, V, Nb, Ta, Cr, Mo, W, Re, Ru, Co, Rh, Ir, Ni, Pd, Pt, Cu, U

and oxygen The decomposition reaction is catalysed by metals, metal oxides or metal ions

$$2H_2O_2\ (l) \rightleftharpoons 2H_2O\ (l) + O_2\ (g) \qquad \Delta G^0_{298} = -27\ 92\ \text{kcal/mole}$$

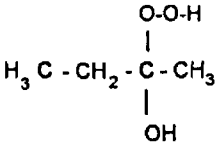
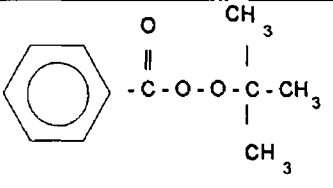
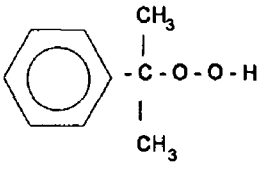
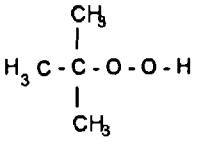
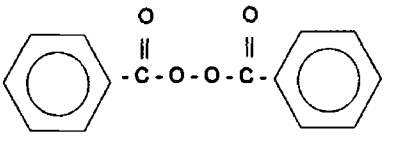
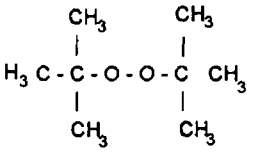
The inherent properties of H_2O_2, such as its bleaching and oxidising/catalytic abilities, have lead to its widespread use as an industrial reagent/material H_2O_2 has been widely used as a commercial bleaching agent Indeed, it has been described as the traditional bleaching agent in European detergents [55] Also, as a result of environmental restrictions, H_2O_2 is gradually being used to replace chlorine as a bleaching agent in pulp and paper, cellulose, domestic bleach and sterilants, textile and wine cork manufacturing industries as well as in water treatment plants [56-58] Recently, its use as a bleaching/whitening agent in Mentadent and Colgate toothpastes and mouthwashes was launched [59] Other products, in which H_2O_2 is used as a bleaching agent, include leather, furs, human and animal hair, tripe and sausage skin H_2O_2 is also used in small quantities as a mild disinfectant and antiseptic in pharmaceutical and cosmetic formulations It is used as a catalyst in polymerization reactions, in addition to being a starting material in the production of epoxides, organic peroxides, peroxyacids and sodium perborate Finally, H_2O_2 is used as a source of oxygen in rocket fuels and submarines

2.1.5.2. *ORGANIC PEROXIDES*

There are two major types of organic peroxides the hydroperoxides, with a general structure R_1-O-O-H and the dialkyl peroxides with a general structure R_1-O-O-R_2 For both types of organic peroxides, R_1 and R_2 could be primary, secondary or tertiary alkyl, cycloalkyl and aryl groups Organic peroxides are usually liquids, and where they are solids they characteristically have low melting points They are soluble in water and most organic solvents, particularly alcohols They are, however, unstable and prone to explosions, the most stable being the di-tertiary dialkyl peroxides

Organic peroxides decompose readily to give free radicals, and have been well studied and used as initiators of free radical reactions in organic chemistry Hence, they are used commercially to initiate polymerisation of vinyl monomers to produce polystyrene, poly(vinyl) chloride, poly(propylene) and poly(ethylene) [60] They are also utilised as polymerisation initiators in the adhesive and sealants industry [61] The commonly used organic peroxides for polymerisation initiation and polymer curing include butanone peroxide, tert-butyl peroxybenzoate, cumene hydroperoxide, tert-butyl hydroperoxide, benzoyl peroxide and di-tert-butyl peroxide (see Table 2 2 for their structures)

Table 2 2 Chemical structures of some organic peroxides

ORGANIC PEROXIDE	STRUCTURE
2-butanone peroxide	
tert-butyl peroxybenzoate	
cumene hydroperoxide	
tert-butyl hydroperoxide	
benzoyl peroxide	
di-tert-butyl peroxide	

The concentration of these compounds in adhesive formulations ranges from 0 5 to 4 % w/w [62] Organic peroxides are also used as bleaching agents for textiles and paper pulp, in the production of oxygen for rocket fuels and as antiseptics m pharmaceutical formulations It has been estimated that the world-wide industrial consumption of organic peroxides is worth about $75 million every year [63] However, despite their technological and industrial usefulness, organic peroxides are known to be intermediates in the air oxidation of some synthetic and natural organic compounds They have been linked to the degradation of certain vitamin products, rancidity of fats, and gum formation in lubricating oils [62, 64] Also, because of the growing replacement of chlorine by ozone as a disinfectant in water treatment plants, there is a growing environmental interest in the quantitative analysis of organic peroxides in drinking water [65] Organic peroxides and hydroperoxides are formed when ozone reacts with some natural organic substances m water such as alcohols, ethers, carbonyl and organometallic compounds In a similar way, organic peroxides and peracids are likely to be biochemically active and can cause ecosystem damage because of their phytotoxicity Peroxyacetyl nitrate (PAN), a known potent lachrymator and phytotoxin, has been observed to be an important pollutant in urban atmospheres [66] This widespread use/presence of organic peroxides in industrial materials and in the environment has led to the need for rapid and reliable methods of analysis

Organic peroxides have been determined using a variety of approaches These include (i) physical and instrumental methods, such as

chromatographic (i e gas, adsorption, liquid-liquid partition, paper and thin layer) and polarographic methods, (ii) chemical reduction methods, which involves the titrimetric reduction of the peroxide using iodide, arsenate, iron(II) or stannous ions, organic phosphines, catalytic hydrogenation and lithium aluminium hydride, and (iii) colorimetric and photometric methods, which are usually used to determine trace levels of organic peroxides, typically in autoxidized natural products, rancid fats and vinyl polymerisation systems, and involves the use of aromatic diamines, leuco methylene blue, iodine and iron(II) thiocyanate [67, 68] However, these analytical techniques are generally time consuming and are not particularly suitable for routine or on-line analysis HRP-based OPEEs offer an alternative and relatively faster and more sensitive method for the quantitative analysis of organic peroxides In a previous study by Wang *et al* [69], the sensitivity of a HRP-based biosensor towards some organic peroxides in aqueous media, was in the order 2-butanone peroxide > tert-butyl peroxy benzoate > cumene hydroperoxide > tert-butyl hydroperoxide

2.1.6. PHENOLS

Monophenols and ortho-diphenols are the primary substrates for tyrosinase The term "phenol" is used to refer specifically to the monohydroxy derivative of benzene However, it is also used as a general term for all derivatives of benzene and its structural analogues containing nuclear hydroxy groups These compounds are termed mono-, di-, tri-hydric phenols etc , depending on the number of hydroxy groups

present Phenols are generally soluble in aqueous alkali (except bicarbonate solutions) and most organic solvents Phenol and its lower analogues are weak acids Its acidic property is enhanced by the presence of electron-withdrawing groups such as fluoro-, chloro-, nitro- and carbonyl groups in the ortho and/or para positions In contrast, electron-donating groups such as ammo- and methoxy-groups suppress phenol acidity Dihydric phenols are generally slightly more acidic than phenol [70] The pK_a values of some phenolic compounds are shown in Table 2 3

Table 2 3 The pK_a values of some phenolic compounds

Name	pK_a
phenol	9 94
p-fluorophenol	9 95
p-chlorophenol	9 38
p-bromophenol	9 36
pentachlorophenol	5 26
o-nitrophenol	7 23
2,4-dinitrophenol	4 01
p-methoxyphenol	10 21
p-aminophenol	10 3
catechol (1,2-dihydroxy benzene)	9 25

Phenolic compounds are present in nature as polymers, i e anthocyanins, flavolans and tannins They are responsible for the colours and organoleptic properties of many flowers and fruits Phenol (C_6H_5OH) itself is found in pine needles, mammalian urine, and the essential oil of tobacco leaves Phenols are important starting materials in the manufacture of fine chemicals, drugs, dyestuffs, textiles, plastics, detergents, disinfectants, herbicides, insecticides, fungicides, bactericides, and antioxidants They have, however, also been identified as major industrial wastes from the above industries, coal conversion plants, petroleum refineries and ore mines [40, 70, 71] Phenols are toxic to fish at levels above 2 mg/L and can be tasted in fish flesh at concentrations much lower than the toxic level The ingestion of one gram of phenol can be fatal in humans [72, 73] Indeed, a recent European Union directive (76/464/CEE) indicates that the maximum admissible individual concentration for organic contaminants (including phenols) m drinking water is 0 1 μg/l [74] Hence, the need for routine quantitative analysis of these compounds for effective raw material, on-line process and product quality controls in the industry, as well as in environmental quality monitoring and evaluation Most of the analytical methods that have been used for the determination of phenols such as gas chromatography, spectrometry and spectrofluorimetry [75-77] are expensive and unsuitable for *in situ* and on-line analysis

2.1.7. INHIBITORS OF HORSERADISH PEROXIDASE AND TYROSINASE

HRP is inhibited by a number of anionic, cationic and uncharged species These include mercury(II), cadmium(II), cobalt(II), copper(II), iron(II), iron(III), maganese(II), nickel(II) and lead(II) ions, p-amino benzoic acid, cyanide, azide, cyclopropanone hydrate, L-cysteme, dichromate, hydroxylamine, sulphides, sulphites and thiourea [78-81] In a similar way, compounds that inhibit the activity of the tyrosinase enzyme include azide, cyanide, benzoic acid, 1-methyl-2-mercaptoimidazole, benzhydroxamic acid, L-cysteme, diethyldithiocarbamate, 2-mercaptoethanol, cobalt(II) ion, hydroxylamine, L-mimosine and phenylthiourea [39, 82-85] The decrease in the activity of both enzymes m the presence of these compounds has enabled the use of HRP- and tyrosmase-based OPEEs as sensors for these inhibitors HRP inhibitors that have been detected in this thesis are thiourea, ethylenethiourea, mercaptoethanol, hydroxylamine and methyl isothiocyanate, while diethyldithiocarbamate was detected as an inhibitor of tyrosinase The primary analytical significance of these compounds is in environmental pollution and quality evaluation

Thiourea (THU) has been shown to be carcinogenic to laboratory animals, although its carcinogenicity to humans has yet to be evaluated [86] THU is the parent compound of a number of pesticides such as thiophanates, phenylthiourea and α-naphthylthiourea In addition, THU, mercaptoethanol (MCE) and hydroxylamine (HLA) are by-products of

industrial activities such as organics synthesis, industrial boiler cleaning and metal mining [87-89] Further, hydroxylamine and its derivatives are well known mutagens and moderately toxic substances They induce highly specific mutations with the nucleic acid cytosine, and are known to cause both reversible and irreversible physiological changes associated with methemoglobinemia [89] Ethylenethiourea (ETU) is also a derivative of thiourea ETU is the primary metabolic and degradation product of ethylenebisdithiocarbamate pesticides (EBDCs) [90] EBDCs and diethyldithiocarbamates (DEDTCs) are the most widely used group of fungicides and have been classified as probable human carcinogens (group B_2) by the U S Environmental Protection Agency [91] This is mainly because of the inevitable presence of ETU in their formulations ETU is a known potent cause of cancers, birth defects and thyroid disorders Also, some EBDCs such as Maneb and Zineb are linked to increases in various reproductive effects such as sterility and stillbirths More significantly, EBDCs and ETU cannot presently be detected in foods using routine Foods and Drugs Administration (FDA) testing methods [91] Methyl isothiocyanate (MeSNC) is a soil fumigant, usually applied before the crops are planted It controls soil insects, nematodes, fungi and weed seeds It is also the active component and degradation product of the pesticidal formulations of 3, 5-dimethyl-1, 3, 5-thiadiazinane-2-thione (Dazomet) and sodium methyl cabadithioate (metam-Na) [92]

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CHAPTER THREE: EXPERIMENTAL

3.1. CHEMICALS / REAGENTS

Horseradish peroxidase type II, EC 1 11 1 7 (100 U/mg or 200 U/mg,), mushroom tyrosinase EC 1 14 18 1 (2400 U/mg), p-acetamidophenol and poly(ethylene glycol) diglycidyl ether were obtamed from Sigma Chemical co , St Louis Mo, USA Poly(ester sulphomc acid) Eastman Kodak AQ 55D polymer was obtained as a 28 % aqueous solution from Eastman Kodak Chemicals, Rochester NY, USA Glutaraldehyde (25 % solution in water), tetraethylammonium-p-toluenesulphonate (TEATS), o-phenylenediamme hydrochloride (o-PEDA), ferrocenemonocarboxylic acid (FMCA), 1,1-dimethylferrocene (DMFc), ferrocenemethanol (MetFc), 2-butanone peroxide, phenol, catechol, p-cresol, m-cresol, p-chlorophenol, ethylenethiourea, methyl isothiocyanate, hydroxylamine sulphate, 2-mercaptoethanol, diethyldithiocarbamic acid-sodium salt and 2-propanol were supplied by Aldrich Chemicals, Gillingham, UK AnalaR grade anhydrous disodium hydrogen orthophosphate and sodium dihydrogen orthophosphate as well as p-aminophenol were purchased from Merck, Poole, UK Thiourea was purchased from M&B Chemicals, Dagenham, UK Acetonitrile, methanol, chloroform and tetrahydrofuran (THF) were obtamed as HPLC grade from Lab-Scan, Stillorgan, Dublin Also, super purity grade acetone and 2-butanol were obtamed from Romil Chemicals, Loughborough, UK and Riedel-de-Haen, Hannover, Germany, respectively Poly(1-vinyl imidazole)-based osmium polymer was obtained from Dr D Leech, Universite du Montreal, Montreal, Canada Antiseptic cream, Secaderm salve (Fisons, Loughborough, UK) and the cold/flu relieving salt, Beechams hot lemon cold remedy (Beechams, Dublin, Ireland) were obtained from a local pharmacy

Eastman AQ 55D polymer and glutaraldehyde were diluted with water to 2 8 % and 5 % before being used Also, anhydrous disodium hydrogen orthophosphate and TEATS were dried in an oven at 110°C for three hours and in a desiccator for at least two weeks, respectively before being used Other chemicals were used without any form of pretreatments, and where required, de-ionised water was used

3.2. EXPERIMENTAL PROCEDURES

The experimental work reported in this thesis has been divided into 4 sections on the basis of the analytical/electrochemical technique utilised These are cyclic voltammetric, steady-state amperometric, spectroelectrochemical and HPLC experiments The procedures for carrying out these experiments, and all further discussions, will therefore be discussed in respect of these analytical techniques

3.2.1. APPARATUS

3.2.1.1. *CYCLIC VOLTAMMETRY*

Cyclic voltammetric (CV) measurements were performed using a BioAnalytical Systems (BAS, Lafayette IN) CV-50 W Voltammetric Analyzer interfaced to a Taxan PC 788 computer Experiments were

carried out in a 10 ml or 20 ml thermostatted conventional electrochemical cell obtained from BAS or EG&G Princeton Applied Research Company (Princeton, NJ) The cells used had a three-electrode configuration and had a poly-(tetrafluroethylene) cover In all experiments, the working electrode (i e prior to modification) was a BAS glassy carbon (GCE) or platinum disc electrode (3 mm diameter) The reference and auxiliary electrodes were KCl-type saturated calomel electrode (SCE) and platinum wire mesh, respectively

3.2.1.2. *STEADY-STATE AMPEROMETRY*

Steady-state amperometric experiments were carried out using an EG&G electrochemical detector (Model 400) connected to a WPA (Model CQ 95) or Phillips PM 8261 X-t recorder All experiments (except those involving rotating disc working electrodes) were performed using the same electrochemical cell set-up as described for cyclic voltammetric experiments A magnetic stirrer and stirring bar (1 mm long) provided the convective transport Rotating disc electrodes experiments were carried out using rotating platinum (8 mm or 4 mm diameter), Model 6 1204 010 or glassy carbon (4 mm diameter), Model 6 1204 000, disc electrodes from Metrohm AG, Herisau, Switzerland

3.2.1.3. *SPECTROELECTROCHEMISTRY*

Spectroelectrochemical experiments were performed with a Shimadzu UV-VIS-NIR recording spectrophotometer (Model UV 3100) linked to an EG&G scanning potentiostat (Model 362) and an Elonex PC 433

computer Experiments were carried out in a 7 ml quartz optically transparent thin-layer electrochemical (OTTLE) cell A platinum wire mesh was used as working electrode, while a platinum wire and silver/silver(I) chloride electrodes were used as auxiliary and reference electrodes, respectively

3.2.1.4. *HPLC ANALYSIS*

HPLC experiments were performed with a Beckman System Gold liquid chromatograph (Beckman, San Ramon, CA) It consists of a solvent module (Model 118) used for delivery of the mobile phase and samples, and a diode-array UV detector (Model 168), both interfaced to an Elonex PC 466 computer A sample volume of 20 µl was injected into the mobile phase by a motorised injection valve The analytical column was a Beckman Ultrashere 243533 stainless-steel column, (0 46 cm x 4 5 cm), packed with reversed-phase octadecylsilyl material (5 µm particle size) The waste line from the UV detector was linked to a thin-layer liquid chromatography electrochemical cell, Model K0234 (EG&G) The working electrode was an enzyme modified-dual glassy carbon electrode (EG&G, Model MP-1304), placed parallel to each other The reference and auxiliary electrodes were as previously specified for the spectroelectrochemical experiments Amperometric responses from the modified-working electrode was monitored via a linked electrochemical detector (EG&G, Model 400), and the signal was recorded on a Phillips PM 8261 X-t recorder All the connections between the different parts of

the system were made of stainless steel tubings (0 5 mm I D) and Altrex screw couplings

3.2.2.2. *PREPARATION OF THE BIOSENSORS*

The surface of either a glassy carbon or platmum electrode was prepared for enzyme immobilisation by polishing with aqueous slurries of 0 1 and 0 05 μm particle size alumina (Metrohm AG) The electrode was pressed face-down onto an alumina impregnated microcloth pad, and moved m a figure-8 pattern (to ensure an even grinding action) for about 2-3 minutes It was subsequently rinsed m distilled water and wiped using Kleenex medical wipes

Horseradish peroxidase was immobilised by (i) adsorption onto prepared GCE and (ii) entrapment within an Eastman AQ 55D polymer matrix on prepared glassy carbon and platinum electrodes Also, tyrosinase was immobilised on a prepared GCE by (i) entrapment within an Eastman AQ 55D polymer matrix (ii) crosslinking with glutaraldehyde and (iii) crosslinking with a poly(1-vinylimidazole)-based osmium polymer Adsorption of HRP on a GCE was achieved by placing 5 μl of a prepared HRP solution (10 mg/ml, made up in 0 05 M phosphate buffer, pH 7 05), on the electrode surface The enzyme coating was dried in a silica-packed desiccator for 2 hours The final HRP loading on the electrode was 68 U/cm^2, where 1 U = 16 67 nkat

To prepare the enzyme-Eastman polymer-modified electrodes, HRP (5 mg/ml) and tyrosinase (5 5 mg/ml) solutions were made up in 0 05 M phosphate buffer, pH 7 05 An enzyme-polymer solution was prepared by mixing equal volumes of the prepared enzyme solution and 2 8 % Eastman AQ polymer A 5-20 μl aliquot of the mixed enzyme-polymer solution was placed on the surface of the polished platmum or glassy carbon electrode The enzyme coating was then dried by using a heat gun (Steinel, Model HL 1800E) placed approximately 30 cm away for 30-40 minutes or in a desiccator for about 2 hours The tyrosinase-modified electrode, immobilised by crosslinking with glutaraldehyde, was prepared m a similar manner, with 5 % glutaraldehyde replacing the 2 8 % Eastman polymer These HRP and tyrosmase modified-electrodes had a final enzyme loading of 20 or 34 U/cm^2 and 170 U/cm^2, respectively

Osmium polymer/tyrosinase-modified electrodes were prepared by placing 5 μl of the poly(1-vinylimidazole)-based osmium polymer (1 mg/ml), poly(ethylene glycol) (2 5 mg/ml) and tyrosinase (5 5 mg/ml) onto the surface of prepared GCE The osmium polymer and poly(ethylene glycol) solutions were prepared with water, while the enzyme solution was prepared with phosphate buffer as previously specified The modified GCE was allowed to air dry for 3 hours The final tyrosinase loading on the electrode was 120 U/cm^2

3.2.3. ELECTROCHEMICAL MEASUREMENTS

3.2.3.1. *CYCLIC VOLTAMMETRY*

Cyclic voltammetry (CV) was used to study the electrochemical behaviour of the solvents and analytes The initial and switch potentials were -300 and +300 mV, respectively Experiments were performed with an Eastman AQ polymer-modified platinum electrode in 98 % v/v acetomtrile, acetone, methanol, 2-butanol and THF, in the absence and presence of 1 mM BTP Thiourea (THU), ethylenethiourea (ETU), hydroxylamme (HLA) and methyl isothiocyanate (MeSNC) were also studied in 98 % v/v acetonitrile In a similar way, the electrochemical features of phenol and DEDTC, were studied with an Eastman AQ polymer-modified GCE in 80 % v/v acetomtrile, acetone, THF and 2-propanol The specified solvents were made up to 100 % with water and also contamed 0 1 M TEATS as an electrolyte A potential scan rate of 10 mV/s *versus* SCE was used for the experiments

CV was also used to characterise the electrochemical behaviour of the mediating compound, 1,1-dimethylferrocene (DMFc) in acetomtrile, methanol and acetone containing 1 5 % v/v water and 0 1 M TEATS Experiments were performed with bare GCEs and Eastman AQ polymer/HRP-modified GCEs, m the absence and presence of 1 mM BTP The electrodes were scanned between initial and switch potentials of 0 and 500 mV, respectively The concentration of DMFc was 4 mM in all the experiments, unless otherwise specified The scan rates were varied from 10 to 100 mV/s, and from the linear plots of the

corresponding I_p against $v^{1/2}$, the diffusion coefficients of DMFc were evaluated Electrochemical data obtained at a scan rate of 10 mV/s were used to estimate parameters, such as the ratio of the anodic and cathodic peak currents, $I_{p,a}/I_{p,c}$ and the difference in redox potentials, ΔE_p

Finally, the catalytic behaviour of HRP and tyrosinase-modified electrodes was studied using CV For both biosensors, the ratio of the catalytic current, I_k, observed in the presence of their respective analytes i e BTP and phenol, to that observed in their absence, I_d, was used to characterise the catalytic performance of the biosensors in different organic media I_k/I_d values for the Eastman AQ polymer/HRP-modified GCE were obtained in 98 5 % v/v acetonitrile, acetone and methanol The initial and switch potentials were 0 and 500 mV, respectively, while the potential scan rate was 10 mV/s *vs* SCE In a similar way, I_k/I_d values, for the Eastman AQ polymer/tyrosinase-modified GCE were obtained in 80 % v/v, oxygen-saturated acetone, acetonitrile, THF, 2-propanol and 2-butanol As a result of the poor stability of Eastman AQ 55D polymer in aqueous media, I_k/I_d in 0 05 M phosphate buffer, pH 7 05 was evaluated with a glutaraldehyde/tyrosinase-modified GCE The tyrosinase-modified electrodes were cycled between initial and switch potentials of +300 mV and -300 mV, respectively, and at a low scan rate of 5 mV/s The specified organic solvents were made up to 100 % by addition of water and contained 0 1 M TEATS For the HRP-based biosensors, the reaction medium also contained 4 mM DMFc as a soluble electron transfer mediator At the same experimental conditions described above, the change in the observed catalytic current, ΔI_k, for the

BTP and phenol sensors in the presence of their respective enzyme inhibitors were evaluated CV studies of the inhibition of thiourea (THU) and ethylenethiourea (ETU) on a DMFc-mediated peroxidase-based biosensor were performed in the presence of 1 mM BTP, unless otherwise stated Similarly, the inhibition of diethyldithiocarbamate (DEDTC) and its mercury, nickel chromium, arsenic and lead complexes on a tyrosinase-based biosensor were performed in the presence of 100 μM catechol

Also, CV was used to compare the electron mediating abilities of DMFc, ferrocenemonocarboxylic acid (FMCA) and ferrocenemethanol (MetFc) for a peroxidase-modified GCE For these experiments, HRP was immobilised by adsorption onto the electrode, with a final enzyme concentration of 68 U/cm^2 I_k/I_d for the detection of BTP, and ΔI_k for the detection of THU and ETU were evaluated m acetomtrile containing 2 % water, 0 1 M TEATS and 0 5 mM of the respective mediators The initial and switch potentials for the DMFc-based sensor were 0 and 500 mV, respectively The same parameters for the MetFc and FMCA-based sensors were +200 mV and +600 mV, respectively A potential scan rate of 5 mV/s was used in the experiments

All the CV experiments were done in a 10 ml electrochemical cell The cell and its contents (except for experiments with the tyrosinase-modified electrodes) were thoroughly degassed by passing nitrogen gas through it, for 15-20 minutes before the experiments The measurements of the peak currents and potentials presented were obtamed with the computer using a BAS CV-50 W program

3.2.3.2. *STEADY-STATE AMPEROMETRY*

Kinetic and analytical properties of both the HRP and tyrosinase-based biosensors were studied using this technique In all experiments, the background current was allowed to decay to a steady-state before starting the experiments The measured background current is subsequently deducted from the measured biosensor responses Therefore, the biosensor responses (i e the measured currents) presented are a result of the faradaic processes at the enzyme modified-electrodes

The Eastman AQ polymer/HRP-modified electrodes were polarised at -250 mV *versus* SCE (unless otherwise stated) Reagentless, i e "mediatorless", o-PEDA- and DMFc-mediated peroxidase-modified electrodes were studied Experiments were performed to determine the optimal working conditions for these biosensors For instance, the working electrode potential was optimised, by measuring the responses of the sensors to BTP as their potential was varied from -200 mV to -400 mV The effect of water content of the reaction solvent on the sensor response was tested by carrying out experiments in which buffer concentrations were varied from 0 % to 3 % Finally, the concentration of the respective mediators was varied, m order to determine the optimal concentration for BTP detection The concentration of o-PEDA was varied between 0 and 2 mM, while DMFc concentration was varied between 0 and 5 5 mM In order to obtain calibration plots for BTP, the responses of the DMFc-mediated biosensor to aliquots of 0 1 mM up to a final concentration of 1 mM BTP was measured in acetonitrile, acetone and methanol In a similar way, the responses of the o-PEDA-mediated

biosensor to aliquots of 1 mM BTP up to a final concentration of 10 mM was measured in acetomtrile, acetone, methanol, THF and 2-butanol Responses of the reagentless biosensor to aliquots of 2 mM BTP up to a final concentration of 20 mM was measured m acetonitrile and methanol The organic media in all the experiments contained 1 5 % - 2 % water, 0 1 M TEATS and where a mediator is required, 4 mM DMFc or 0 5 mM o-PEDA was used

The effects of the presence of 0 2 mM THU, 0 2 mM ETU and 0 4 mM mercaptoethanol (MCE) on the response of the o-PEDA-mediated peroxidase-based sensor to BTP was studied These experiments were performed in 98 % v/v acetomtrile containing 0 1 M TEATS and 0 5 mM o-PEDA The enzyme loading was 20 U/cm^2 Kinetic parameters such as the apparent Michaelis-Menten constant, K_m', and the limiting current, I_{max}, were used to determine the inhibition mechanisms of THU, ETU and MCE on the HRP-modified electrodes Also, the decrease in the response of the Eastman AQ polymer/HRP-modified electrodes towards BTP upon successive additions of 0 1-0 2 mM THU, ETU, MCE and methyl isothiocyanate (MeSNC) was measured m several organic media The measured current decreases were used to obtain calibration plots for these compounds m solvents such as acetonitrile, acetone, methanol and 2-butanol These experiments were done m the presence of 1 mM BTP unless otherwise stated

The steady-state catalytic responses of an Eastman AQ polymer/tyrosinase-modified electrode were measured on addition of aliquots of 10 μM phenol up to a final concentration of 100 μM These

measurements were performed in acetonitrile, acetone, and THF containing 20 % v/v water and 0.1 M TEATS. For these experiments, a rotating disc GCE was modified with an Eastman polymer entrapped tyrosinase. The concentration of tyrosinase on the electrode was 170 U/cm^2. The modified electrode was polarised at -150 mV *versus* SCE, and the experiments were performed at a rotation speed of 1500 rpm.

The effects of the presence of 20, 30, 40 and 50 μM DEDTC on the response of the biosensor to phenol (10-100 μM) in 80 % v/v 2-propanol was also evaluated. The changes in the kinetic parameters I_{max} and K_m' were used to determine the inhibition mechanism of DEDTC on the tyrosinase-modified electrode. The experimental conditions are as previously described except that the working electrode was polarised at -200 mV *vs* SCE. Experiments on the detection of DEDTC were performed by measuring the decrease in the responses of the tyrosinase-modified electrode towards 100 μM phenol as standard additions of DEDTC were added to the reaction cell. Aliquots of 5 μM DEDTC up to a final concentration of 70 μM were added. The reaction cell contained 80 % v/v 2-propanol and 0.1 M TEATS. Also, the effects of varying phenol concentrations from 70 μM to 200 μM on the catalytic efficiency of the sensor for DEDTC detection were studied. Changes in the response of the biosensor to DEDTC when 100 μM phenol was replaced with other tyrosinase substrates such as 100 μM catechol, m-cresol and p-cresol was also assessed.

The steady-state currents measured from these biosensors were analyzed by non-linear curve-fitting to a Michaelis-Menten equation using a

Sigma plot computer programme A fresh enzyme surface was used for each experiment Also, the cell and its contents (except for experiments with the tyrosinase-based electrode) were thoroughly degassed by passing nitrogen gas through it for 15-20 minutes before each experiment

3.2.3.3. *SPECTROELECTROCHEMISTRY*

The working electrode was polarised at -250 mV versus SCE for all experiments unless otherwise stated HRP (1 mg/ml) was used in solution and a fresh cell solution was used for each spectral run The change in absorbance was measured from an initial wavelength of 300 nm to a final wavelength of 650 nm However, spectral changes in the Soret region, i e λ_{max} around 400 nm, was used to determine the effects of organic solvents on the physico-chemical stability of HRP These experiments were performed in 100 % phosphate buffer and in increasing concentrations (50 % - 90 % v/v) of acetomtrile, acetone and methanol The solvents were made up to 100 % with 0 025 M phosphate buffer, pH 7 05 Changes in the spectra of HRP (1 mg/ml), when 1 mM BTP and then 1 mM ETU and THU were added to the cell, were monitored The observed changes were used to explain HRP-BTP and HRP-inhibitor interactions The experiments were done in the phosphate buffer as specified above

3.2.3.4. *HPLC ANALYSIS*

Experiments were performed to optimise the chromatographic separation of a 50 μM standard solution of catechol, phenol, p-cresol and p-chlorophenol The retention times, t_R, of these analytes with different mobile phase compositions as well as the retention time of the pure organic component of the mobile phase, t_M, were measured The retention time of the organic component was measured as the first deviation in the baseline after their respective injections The ratio, $(t_R-t_M)/t_M$, i e the capacity factor (k') values, were used to evaluate the performance of the mobile phases The mobile phases that were assessed were acetonitrile water (50 50), methanol water in the ratios of 60 40, 50 50, 40 60 and 30 70 Further, the effects of having 10 mM and 25 mM of the electrolyte TEATS in the mobile phase on the retention properties of the phenolic compounds was studied The mobile phase composition for this study was methanol water (50 50) Also, changes in the separation/retention of these compounds, when the 50 % water concentration of the mobile phase was replaced with 0 025 M phosphate buffer, at pH values of 5, 6, 7 and 8, respectively was studied In these experiments (described above) the flow rate of the eluents was 1 ml/min and detection was with a UV detector at a fixed wavelength of 280 nm

The optimal wavelength for UV spectral detection of catechol, phenol, p-cresol and p-chlorophenol was also determined The UV spectrometric detector response to a 50 μM concentrations of the four analytes was

measured as the detector wavelength was varied from 210 nm to 300 nm The mobile phase was methanol water (50 50) at a flow rate of 1 ml/min

The ability of an osmium polymer/tyrosinase-modified GCE to function as an electrochemical detector for the phenols after their LC separation was investigated The modification parameters of the biosensor such as the concentration of the osmium polymer and the crosslinking agent, poly(ethylene glycol) (PEG) were optimised This was done by measuring the simultaneous response of the biosensor to 0 1 μM -0 6 μM concentrations of p-aminophenol, phenol, p-cresol and p-chlorophenol The biosensor sensitivities, which were obtained from the slopes of the straight-line calibration plots for the analytes, was then measured as the concentrations of the osmium polymer and PEG were varied Experiments were performed at osmium polymer concentrations of 0 04, 0 07 and 0 11 mg/cm^2, and PEG concentrations of 0 04, 0 11 and 0 18 mg/cm^2 The mobile phase was a binary mixture of methanol and 0 025 M phosphate buffer, pH 6 5 (50 50) containing 0 025 M TEATS The mobile phase flow rate was 0 80 ml/min, while enzyme concentration on the electrode was 120 U/cm^2 Experiments were performed at -200 mV *versus* SCE

Experiments were performed to optimise the operating conditions of the biosensor such as the mobile phase flow rate and the working potential The response of the biosensor to 0 2 μM phenol, p-aminophenol, p-cresol, and p-chlorophenol was measured, as the flow rate of the mobile phase was varied from 0 4 ml/min to 1 6 ml/min and as the working potential was varied from +50 mV to -300 mV Finally, the short term

operational stability of the biosensor was measured by injecting 0 6 μM concentrations of catechol, p-cresol and p-chlorophenol, consecutively over a ten-hour period The GCE was modified with 0 11 mg/cm^2 of osmium polymer, 0 18 mg/cm^2 of PEG and 120 U/cm^2 of tyrosinase The mobile phase was methanol phosphate buffer, (50 50) The concentration of the phosphate buffer and its pH were as previously specified

A solution of the antiseptic ointment 500 mg/ml was made up in ethanol and further diluted 1 3000 with the mobile phase before it was injected In a similar way the cold relieving salt (2 mg/ml) was dissolved in the mobile phase and then diluted 1 200 before it was injected The mobile phase for the determination of phenol in the antiseptic cream was methanol phosphate buffer, 50 50, while for the determination of p-acetamidophenol in the cold/flu salt, the ratio was 30 70 Cigarette filter tips were collected from the university bar as they were being discarded Each cigarette filter stud was then soaked in 5 ml methanol for 24 hours and then analysed The extract was further diluted 1 50 before it was injected A standard mixture containing phenol (0 1 μM-0 6 μM), m-cresol (0 1 μM-0 6 μM), catechol (5 nM-30 nM) and p-cresol (5 nM-30 nM) were also analysed The concentration of these compounds in the cigarette filter tips was calculated by extrapolation from the obtained straight-line calibration plots of the analytes The mobile phase was a mixture of methanol and 0 025 M phosphate buffer, pH 6 5, (30 70) The GCE was modified with 0 11 mg/cm^2 of osmium polymer, 0 18 mg/cm^2 of PEG and 120 U/cm^2 of tyrosinase The mobile phase flow rate was 0 8 ml/min and the working potential was -200 mV *vs* SCE

In all these experiments, the mobile phase was always degassed by sonicating for 3-5 minutes before it was used Also, all specified reagents and solutions were prepared daily

CHAPTER FOUR: CYCLIC VOLTAMMETRY

4.1. ELECTROCHEMICAL BEHAVIOUR OF THE ANALYTES

The faradaic current measured during the amperometric detection of analytes utilising biosensors can be generated by

(i) biocatalysis of the analyte,
(ii) a direct reduction/oxidation of the analyte at the underlying electrode surface,
(iii) a combination of (i) and (ii)

The operating conditions of amperometric biosensors are, however, usually designed such that the direct reduction/oxidation of the analyte(s) on the electrode surface is either non-existent or minimized considerably Therefore, cyclic voltammetry (CV) has been used to investigate the electrochemical behaviour of the analytes detected in this thesis Figure 4 1 shows the cyclic voltammograms of an AQ polymer-modified platinum electrode in 98 % v/v acetonitrile, methanol, acetone, 2-butanol and THF The CVs did not show any distinctive electrochemistry in the absence and presence of 1 mM BTP, thus indicating that the electrochemical behaviour of Eastman AQ 55D polymer, the organic solvents, as well as BTP, does not interfere with biosensor responses when operating within the potential range of +300 to -300 mV *vs* SCE Similar scans obtained in the absence and presence of the inhibitors, THU, ETU, HLA and MeSNC, in acetonitile are shown in Figure 4 2 While little or no difference was observed in the presence of ETU and MeSNC, an anodic current started forming at +150 mV and -50 mV for

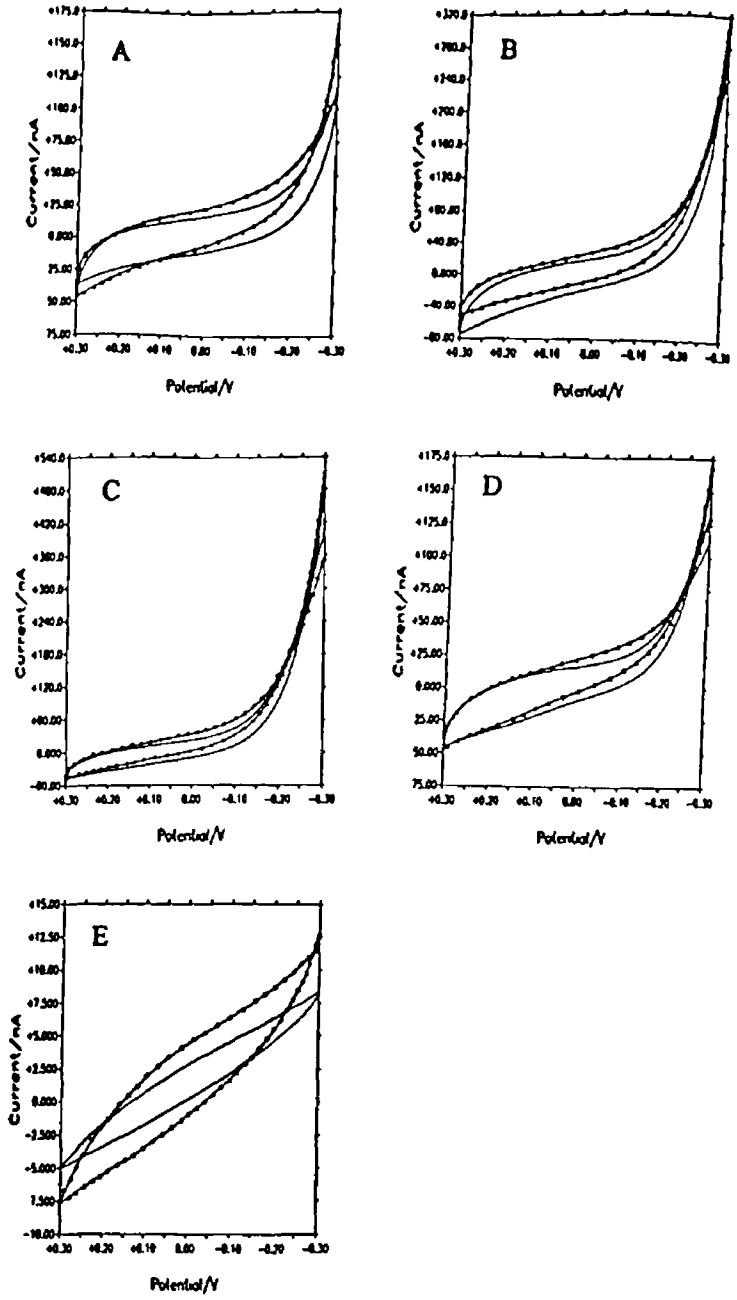

Figure 4 1 Cyclic voltammograms of an Eastman AQ polymer-modified platinum electrode in 98 % v/v acetonitrile (A), acetone (B), methanol (C), THF (D) and 2-butanol (E), in the absence (——) and prescence of (+++++) of 1 mM butanone peroxide E_i and E_λ are -300 mV and +300 mV, respectively, while the scan rate was 5 mV/s The 10 ml reaction cell also contained 0 1 M TEATS, and was maintained at at 20°C

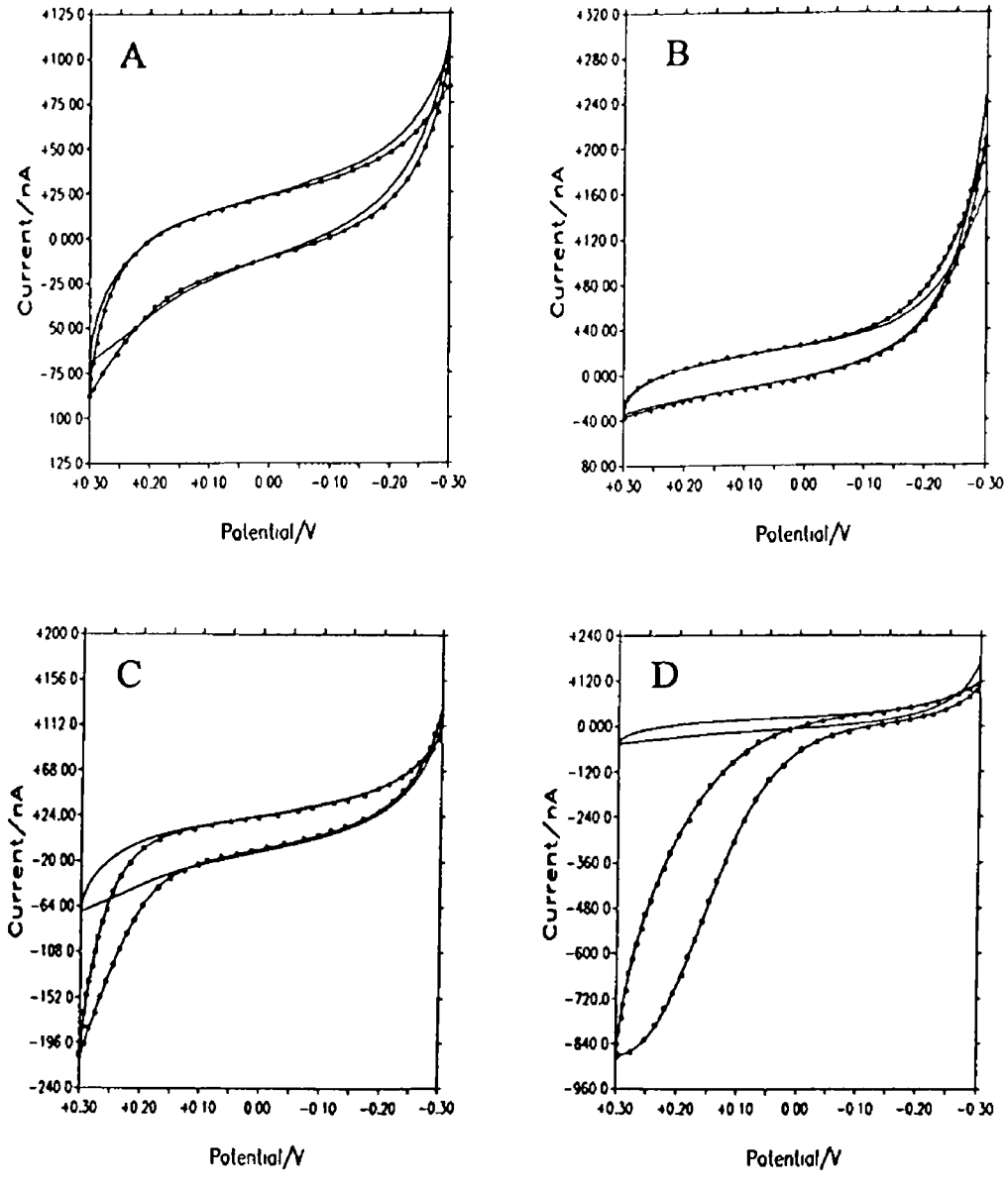

Figure 4 2 Cyclic voltammograms of an Eastman AQ polymer-modified platinum electrode in the absence (——) and presence (•••••) of 1 mM ethylenethiourea (A), methyl isothiocyanate (B), thiourea (C) and hydroxylamine (D) Other experimental conditions are as specified in Figure 4 1

THU and HLA, respectively This indicates that a direct oxidation of these compounds at the platinum electrode occurs at about this potential Therefore, potentials more positive than the stated values would be unsuitable for their biocatalytic detection Phenol and DEDTC were then studied with an AQ polymer-modified glassy carbon electrode in 80 % v/v acetonitrile, acetone, THF and 2-propanol As was observed with BTP, there was no appreciable difference in the CVs obtained in the absence and the presence of 100 μM phenol (not shown) However, the voltammograms in the presence of 100 μM DEDTC (Figure 4 3) showed an anodic current with E_p at +212 mV, +160 mV, +122 mV and +155 mV in acetonitrile, acetone, THF and 2-propanol, respectively Thus biocatalytic detection of DEDTC in these solvents is best done at lower negative potential values The immobilised Eastman AQ polymer in these solvents had noticeably "hardened" up at the end of each experiment This effect was more pronounced in 2-butanol, and in general it was more noticeable with the BTP experiments The hardening of the polymer layer is most likely a result of the "water-withdrawing" effects of the solvents

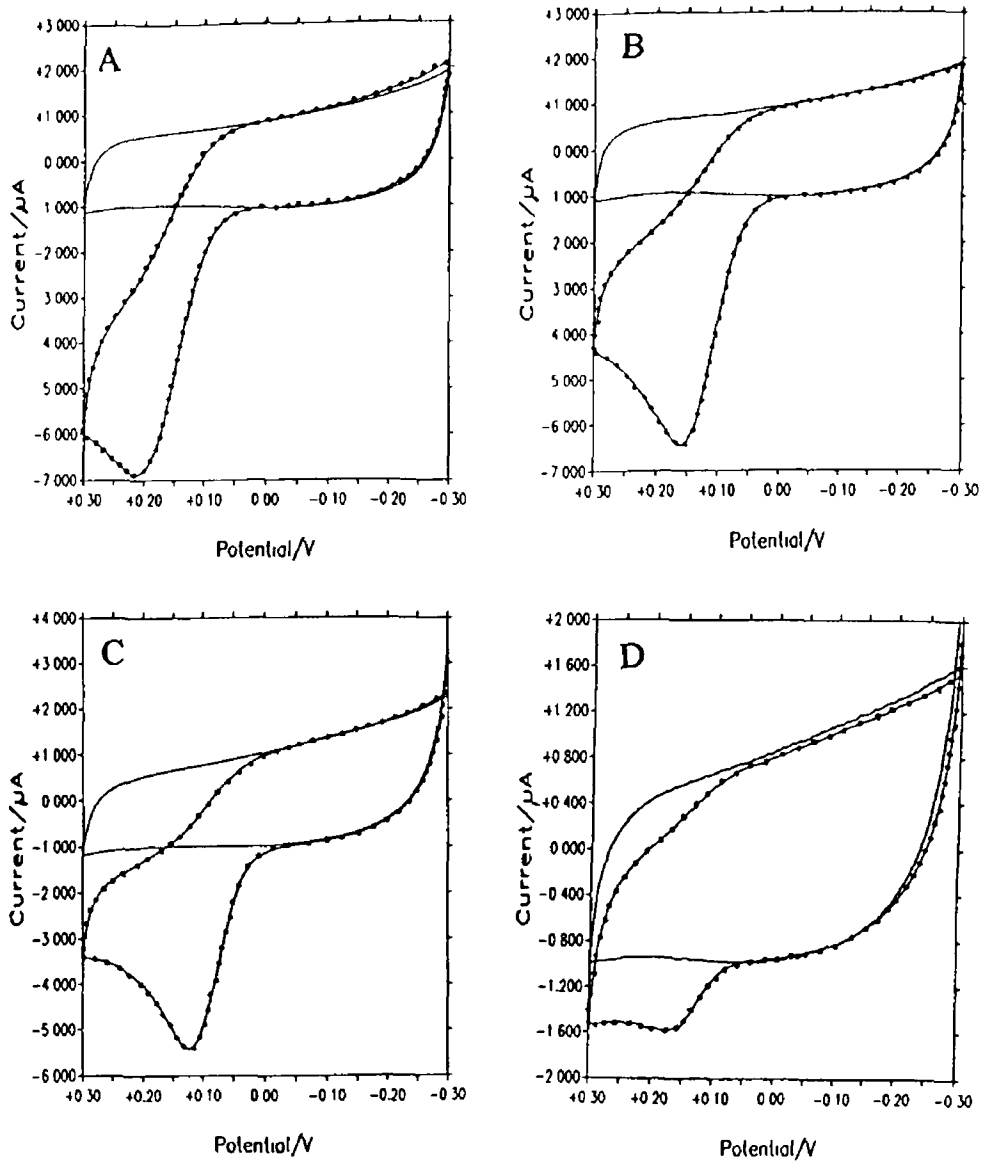

Figure 4 3 Cyclic voltammograms of an AQ polymer-modified glassy carbon electrode in the absence (——) and presence (••••••) of 100 μM DEDTC in 80 % v/v acetonitrile (A), acetone (B), THF (C) and 2-propanol (D) Other experimental conditions are as in Figure 4 1

4.2. ELECTROCHEMICAL BEHAVIOUR OF 1,1-DIMETHYLFERROCENE

Ferrocene (bis (η^5-cyclopentadienyl) iron) and its structural analogues act as electron donors in the reduction of HRP compounds I and II Hence they have been used as mediators in peroxide sensors using peroxidase as the sensing enzyme [1-3] 1,1-dimethylferrocene (DMFc), was introduced as a mediator in amperometric biosensors by Cass *et al* in 1984 [4] In aqueous media, DMFc is reversibly oxidised in a single electron transfer reaction to yield dimethylferricinium ion It is stable in the reduced form and is not light-, oxygen-, or pH-sensitive Figures 4 4-4 6 are the respective cyclic voltammograms of DMFc at the bare glassy carbon electrode (A), HRP-modified electrode in the absence (B) and presence (C) of BTP in mainly acetonitrile, methanol and acetone media The values of the parameters, ΔE_p, $I_{p,a}/I_{p,c}$ and diffusion coefficients as evaluated from the cyclic voltammograms are summarised in Table 4 1 These values indicate a quasi-reversible electrochemistry of DMFc on both plain and HRP-modified GCE in the three solvents Hence, there is no significant difference in the rate of charge transfer and movement of the electroactive species [5] The peak separation, ΔE_p, increases with increasing potential sweep rate, while the oxidation peak potential shifts towards more positive values ΔE_p and $I_{p,a}/I_{p,c}$ were in all cases lowest in acetonitrile ΔE_p values of the CV of DMFc at the bare GCE were larger than those at the HRP-modified electrodes This indicates that the DMFc, which diffused from the bulk solution into the polymer film of the biosensor, tends to behave like a surface-bound species [6] On the

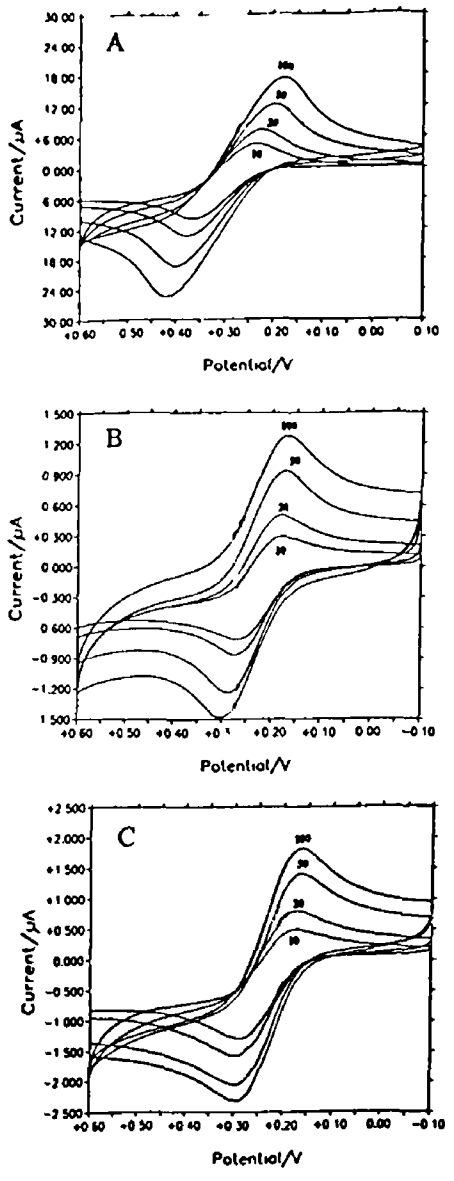

Figure 4 4 Cyclic voltammograms of 4 mM 1,1-dimethylferrocene in acetonitrile, at bare glassy carbon electrode (A), horseradish peroxidase/Eastman AQ polymer-modified electrode in the absence of butanone peroxide (B), and the presence of 0 5 mM butanone peroxide (C) The numbers on the CVs are the scan rates in mV/s The concentration of horseradish peroxidase on the electrode was 34 U/cm^2 The 10 ml reaction cell also contained 0 1 M TEATS and 1 5 % v/v water The experiments were performed at 20°C

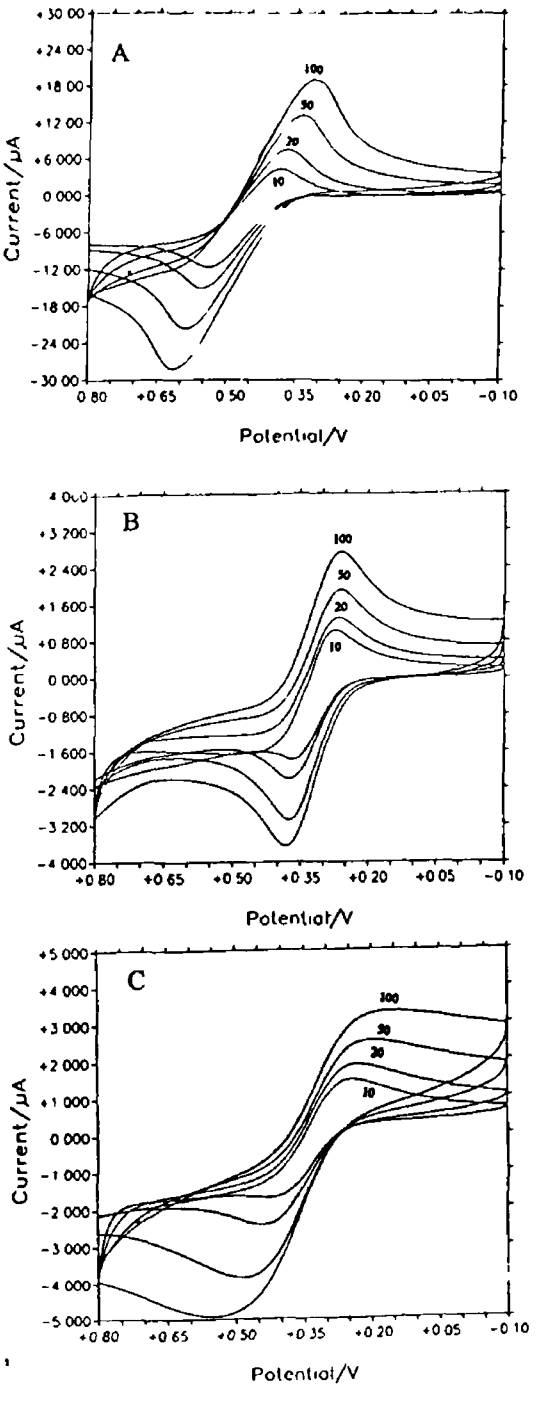

Figure 4 5 Cyclic voltammograms of 4 mM 1,1-dimethylferrocene in acetone, at bare glassy carbon electrode (A), horseradish peroxidase/Eastman AQ polymer-modified electrode in the absence of butanone peroxide (B) and in the presence of 0 5 mM butanone peroxide (C) Other experimental conditions are as described in Figure 4 4

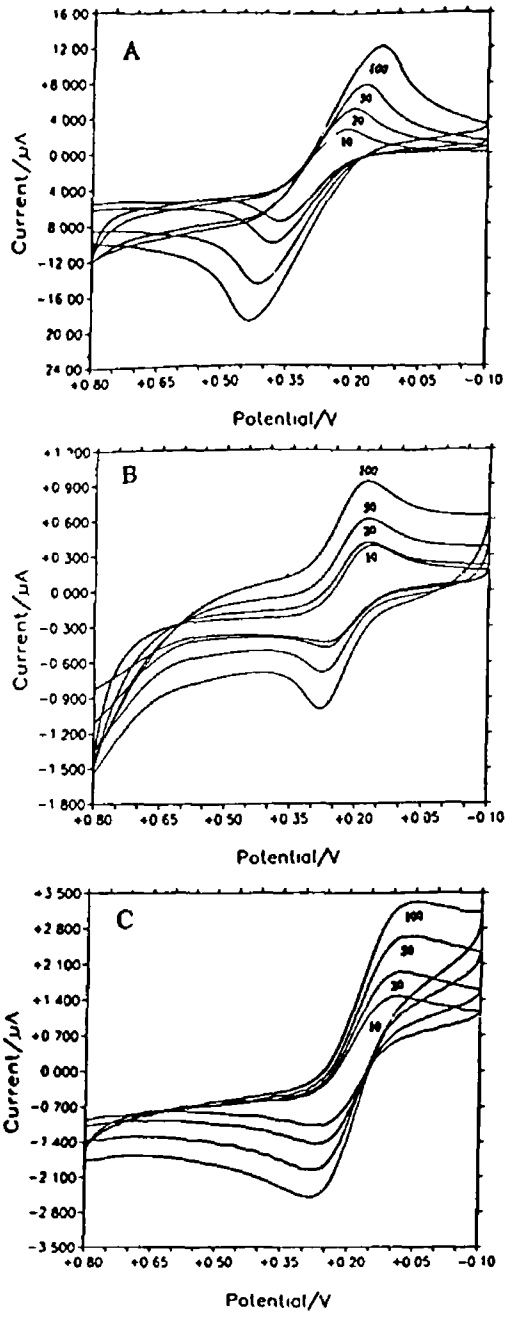

Figure 4 6 Cyclic voltammograms of 4 mM 1,1-dimethylferrocene in methanol, at bare glassy carbon electrode (A), horseradish peroxidase/Eastman AQ polymer-modified electrode in the absence of butanone peroxide (B) and in the presence of 0 5 mM butanone peroxide (C) Other experimental conditions are as described in Figure 4 4

Table 4 1 Cyclic voltammetric parameters for DMFc on bare and HRP-modified GCE in acetomtrile, acetone and methanol (experimental conditions are as in Figure 4 4)

SOLVENT	BARE GCE			HRP MODIFIED GCE			HRP MODIFIED GCE (+ BTP)	
	ΔE_p (V)[a]	$I_{p,a}/I_{p,c}$	D_M (cm^2/s)	ΔE_p (V)	$I_{p,a}/I_{p,c}$	D_M (cm^2/s)	ΔE_p (V)	$I_{p,a}/I_{p,c}$
Acetonitrile	0 15	0 80	9×10^{-7}	0 092	0 87	$3\,4 \times 10^{-10}$	0 10	0 78
Methanol	0 18	0 97	4×10^{-7}	0 095	1 28	$1\,1 \times 10^{-10}$	0 15	1 31
Acetone	0 18	0 82	8×10^{-7}	0 100	1 20	$1\,2 \times 10^{-10}$	0 19	1 14

a ΔE_p and $I_{p\,a}/I_{p\,c}$ values were evaluated from data obtained at a scan rate of 10 mV/s

other hand, the CV of DMFc on the bare GCE is as expected for a diffusion controlled electrochemical reaction In the presence of BTP, the ΔE_p's for HRP-modified GCEs approach those calculated for the bare GCE This behaviour is attributed to the influence of the diffusion characteristics of BTP in the various organic solvents on the electrochemistry of the system

Greet *et al* [7] reported that for a quasi-reversible electrochemical reaction, the plot of I_p vs $v^{1/2}$ is characterized by a transition from a reversible (at low v) to an irreversible (at high v) reaction profile, passing through a quasi-reversible region (at intermediate v) Hence, I_p shows a linear relationship with $v^{1/2}$ at low and high potential sweep

rates, the two linear zones being separated by an inflection region at intermediate scan rates Accordingly, the theory of reversible kinetics was applied to DMFc electrochemistry at relatively low scan rates of 10-100 mV/s The diffusion coefficient, D_M, of DMFc was evaluated from linear plots of I_p against $v^{1/2}$ by applying the Randles-Sevcik equation [7,8]

$$I_p = 2\ 69 \times 10^5 n^{3/2} D_M^{1/2} C_M v^{1/2} \quad (4\ 1)$$

where n is the number of electrons transferred during the redox reaction, D_M is the diffusion coefficient of the elecroactive substance (cm^2/s), C_M is the concentration of the electroactive substance (mol/l), and v is the potential scan rate (mV/s) At the bare GCE, the D_M values of DMFc in acetonitrile, methanol and acetone were $9x10^{-7}$, $4x10^{-7}$ and $8x10^{-7}$ cm^2/s, respectively The corresponding values for the HRP-modified electrode were $3\ 4x10^{-10}$, $1\ 1x10^{-11}$ and $1\ 2x10^{-10}$ cm^2/s, respectively The difference between the two sets of D_M values (i e the difference between D_M obtained at the bare and HRP-modified GCE in each solvent) describes the solvent-dependent diffusion ability of DMFc within the enzyme layer The D_M results imply that the rate of electron transfer was highest in acetonitrile

4.3. CATALYTIC BEHAVIOUR OF THE BIOSENSORS

4.3.1 HORSERADISH PROXIDASE-MODIFIED ELECTRODE

Figure 4 7 shows the CVs of DMFc at the peroxidase-modified electrode in the absence (I) and presence (II) of BTP The polarographic wave in the absence of BTP depends on the diffusion of DMFc to the electrode (I_p increases with $v^{1/2}$) Hence the peak cathodic current is a diffusion controlled current, I_d In the presence of BTP, the current is controlled by the enzyme kinetics at the steady state Hence the peak cathodic current in this case is kinetically controlled current, or catalytic current The catalytic current, I_k,was measured at the optimal cathodic peak potential, $E_{p,c}$, the value of which depends on the solvent medium $E_{p,c}$ values in acetonitrile, methanol and acetone were 0 15 V, 0 13 V and 0 32 V, respectively The I_k for the peroxide sensor in acetone (6 8 μA) was greater by a factor of 2 5 than in acetomtrile (2 69 μA) and methanol (2 60 μA), thus implying that the sensor showed a better response in acetone compared to methanol or acetomtrile It is known that for a biosensor the ratio of the catalytic current to the diffusion controlled current, I_k/I_d, may be taken as a measure of catalytic efficiency [9-11] The I_k/I_d evaluated in this study were 3 57, 2 58 and 2 93 for acetonitrile, methanol and acetone, respectively There is a good correlation between the I_k/I_d values and the diffusion coefficient values obtamed for DMFc at the bare and HRP-modified GCE as shown in Table 4 1, in that the I_k/I_d of the biosensor increases as the D_M of DMFc mcreases

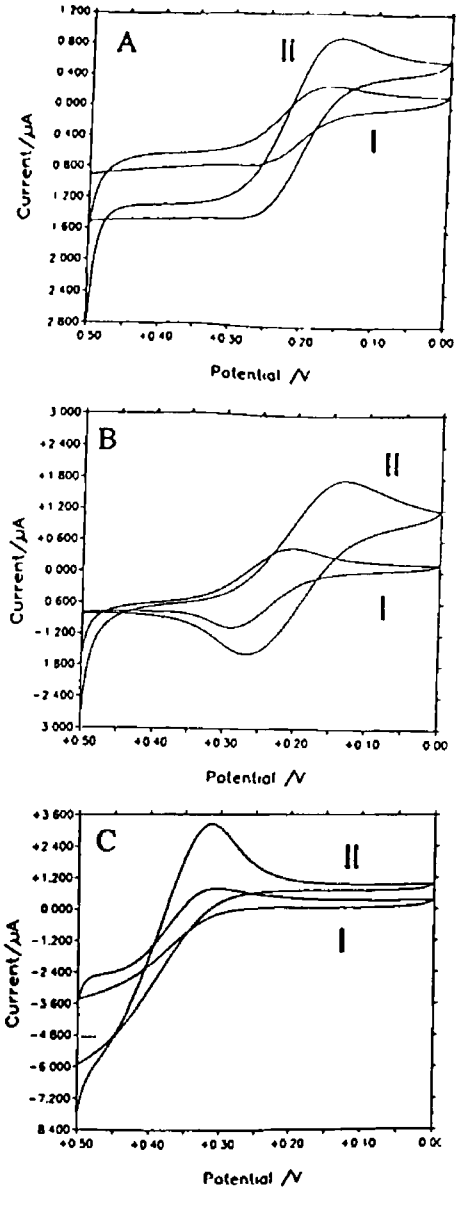

Figure 4 7 Cyclic voltammograms of 4 mM 1,1-dimethylferrocene on a horseradish peroxidase/Eastman AQ polymer-modified electrode in acetonitrile (A), methanol (B) and acetone (C) in the absence (I) and in the presence (II) of 1 mM butanone peroxide The concentration of horseradish peroxidase on the electrode was 34 U/cm^2 E_i and E_λ were 0 mV and 500 mV, respectively, while the scan rate was 5 mV/s The 10 ml reaction cell also contained 1 5 % v/v water and 0 1 M TEATS

In a separate study, HRP was immobilized by adsorption on GCE and the values of I_k/I_d of the biosensor when ferrocenemethanol (MetFc), ferrocenemonocarboxylic acid (FMCA) and DMFc were employed as electron transfer mediators was evaluated The values shown in Table 4 2 indicate that the catalytic efficiency of the biosensor with MetFc (3 3) is 14 7 % and 50 % greater than in FMCA (2 8) and DMFc (1 7), respectively, thus implying that HRP undergoes a considerably more efficient electron transfer with MetFc in a mainly acetonitrile medium than with FMCA and DMFC

Table 4 2 A comparison of the catalytic performance of the biosensor in detecting butanone peroxide, thiourea and ethylenethiourea when 0 5 mM MetFc, FMCA and DMFc are utilised as soluble electron transfer mediators Experimental conditions are 98 % v/v acetomtrile, 0 1 M TEATS, horseradish peroxidase, 68 U/cm^2 immobilised by adsorption on GCE, E_i for 1,1-dimethylferrocene experiments was 0 mV while E_λ was 500 mV These parameters were +200 mV and +600 mV, respectively for MetFc and FMCA experiments A voltage scan rate of 5 mV/s was used in all experiments

Mediator	**Butanone peroxide, I_k/I_d**	**Ethylenethiourea, ΔI_k (%)**	**Thiourea, ΔI_k (%)**
Ferrocenemethanol	3 3	+ 30	- 15
Ferrocenemonocarboxylic acid	2 8	+ 45	- 12
1,1-dimethylferrocene	1 7	+ 51	- 20

4.3.1.1. *THIOUREA AND ETHYLENETHIOUREA INHIBITION STUDIES*

The inhibitory effects of THU on the HRP-generated catalytic current are shown in Figure 4 8 In the presence of 1 mM THU, the I_k of the biosensor in acetonitrile, methanol and acetone decreased by factors of 2 02, 1 21 and 4 80, respectively Conversely, Figure 4 9 shows that in the presence of 1 mM ETU, I_k increased by factors of 1 99, 1 38, and 1 14, respectively, thus implying that when DMFc is employed as an electron mediator, ETU acts as a co-substrate for HRP rather than as an inhibitor in the these solvents Similar observations were made for ETU (acting as a co-substrate) and THU (being an inhibitor) when MetFc and FMCA were utilised as mediators as shown in Table 4 2 The greatest percentage change in I_k for both THU and ETU was observed with DMFc

4.3.2. TYROSINASE-MODIFIED ELECTRODE

The electrochemical behaviour of the tyrosinase-based phenol sensor was studied in oxygen-saturated 0 05 M phosphate buffer, pH 7 05, as well as in acetone, acetonitrile, THF, 2-propanol and 2-butanol containing 20 % v/v water In the phosphate buffer experiments, the enzyme was immobilised onto the GCE with glutaraldehyde because of the instability of AQ polymer in aqueous media The biosensor did not show any distinct electrochemistry in these solvents in the absence of

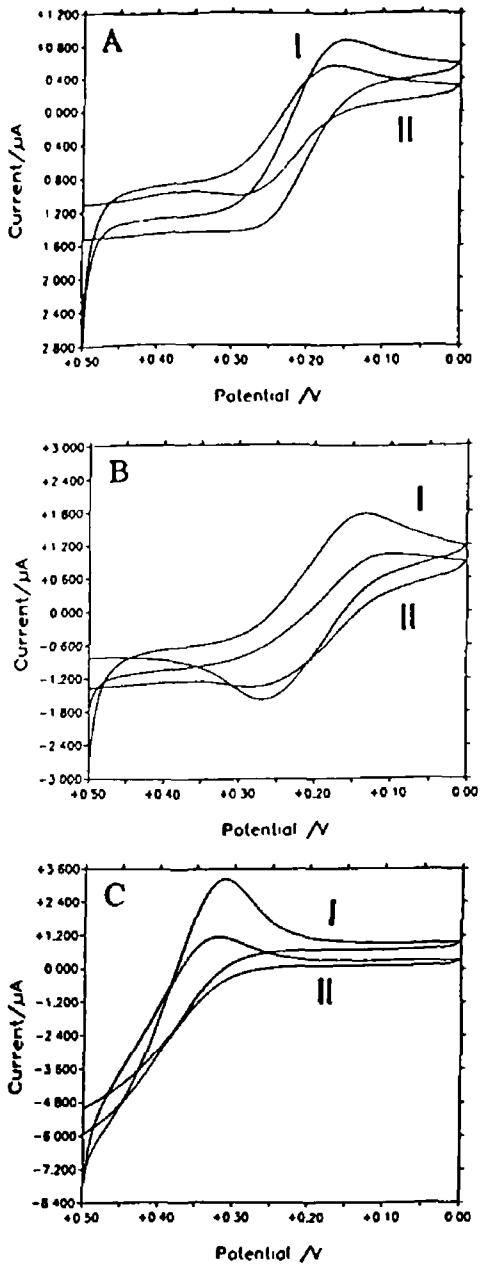

Figure 4 8 Cyclic voltammograms at a horseradish peroxidase/Eastman AQ polymer-modified eletrode in acetomtrile (A), methanol (B) and acetone (C) in the presence of 1 mM butanone peroxide (I) and on addition of 1 mM thiourea (II) Other experimental conditions are as specified in Figure 4 7

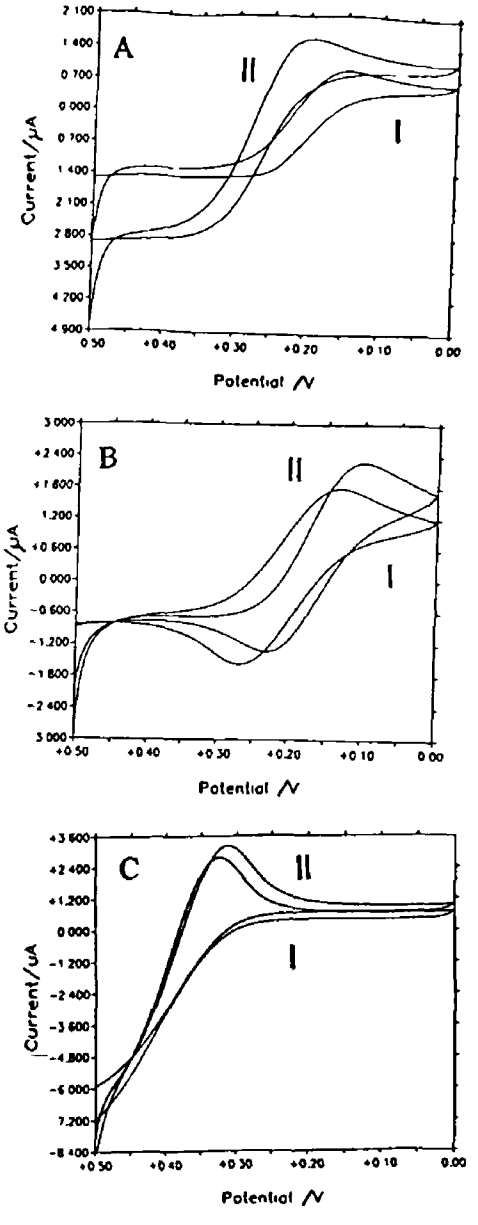

Figure 4 9 Cyclic voltammograms at a horseradish peroxidase/Eastman AQ polymer-modified electrode in acetonitrile (A), methanol (B) and acetone (C) in the presence of 1 mM butanone peroxide (I) and on addition of 1 mM ethylenethiourea (II) Other conditions are as specified in Figure 4 7

phenol However, in the presence of 100 μM phenol, I_k starts forming at +100 mV *vs* SCE, as shown in Figure 4 10 The observed current is attributed to the reduction of benzoquinone formed by the enzymatic action of tyrosinase on phenol I_k observed for the reduction of phenol in phosphate buffer and THF did not show a maximum value within the potential range studied However, it reached a maximum value at -200, -150, -120 and -45 mV in acetonitrile, acetone, 2-propanol and 2-butanol, respectively Plots of the logarithm of the I_k density against potential (i e Tafel plot) in these solvents were linear, indicating completely irreversible kinetics This implies that the anodic process contributed less than 1 % of I_k [12] Therefore the observed I_k resulted from the reduction of catalytically formed benzoqumone at the underlying GCE Cooper and Hall [13] have previously reported that the reduction of benzoquinone starts at about +100 mV The values I_k and I_d were obtained by measuring the respective currents at -150 mV *vs* SCE (except in 2-butanol, in which they were measured at -45 mV) Table 4 3 shows the values of I_k/I_d in the utilised reaction media Hence, the catalytic efficiency of the biosensor in detecting phenol was highest in acetone, and least in THF Also, the efficiency of the biosensor in detecting phenol in the organic solvents increased by factors of 4 1 (acetone), 3 0 (acetonitrile), 2 5 (2-propanol) and 1 1 (2-butanol) over that of the aqueous medium

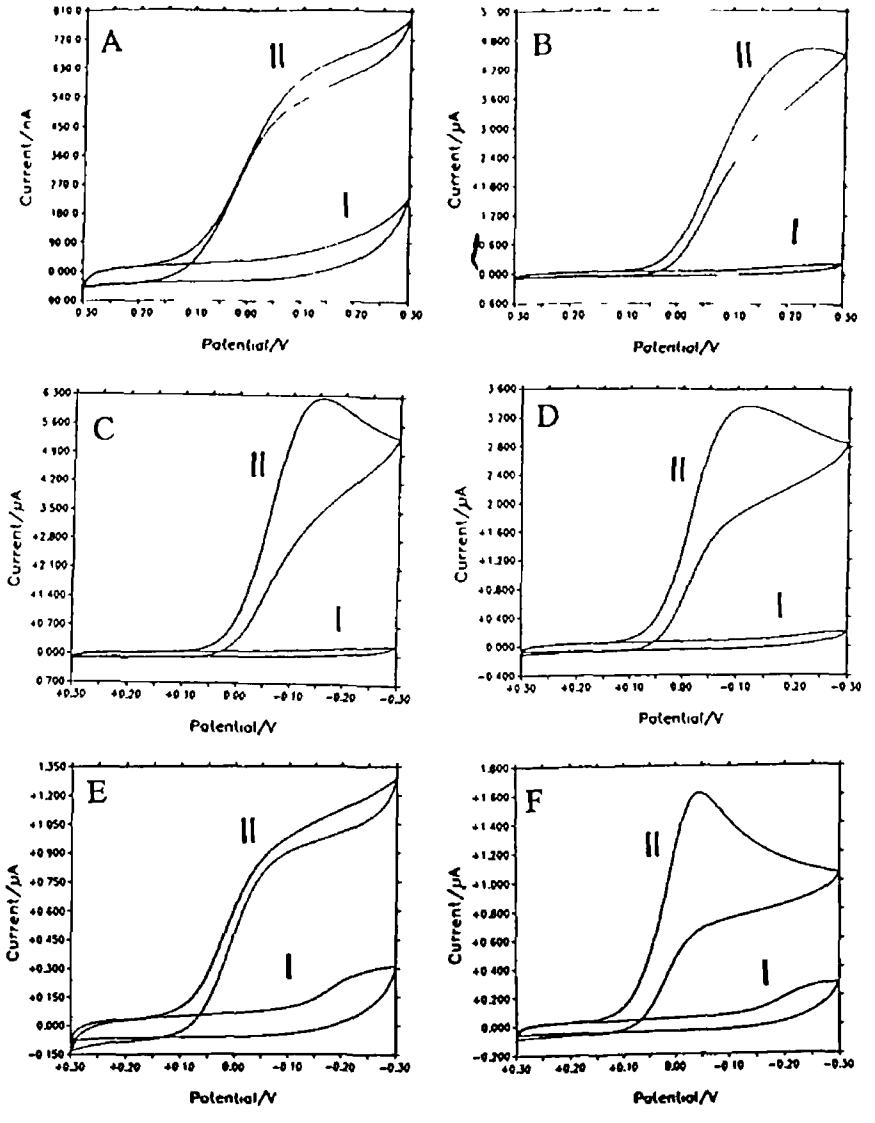

Figure 4 10 Cyclic voltammograms of tyrosinase/Eastman AQ polymer-modified electrode in 0 05 M phosphate buffer, pH 7 05 (A), acetone (B), acetonitrile (C), 2-propanol (D), THF (E) and 2-butanol (F), containing 20 % v/v water, in the absence (I), and presence (II) of 100 μM phenol The concentration of tyrosinase on the electrode was 170 U/cm^2 E_i and E_λ were -300 mV and +300 mV, respectively, while the potential scan rate was 5 mV/s The 10 ml reaction cell also contained 0 1 M TEATS and was maintained at 20°C for all experiments

Table 4 3 A comparison of the catalytic performance of the phenol sensor in several reaction media Experimental conditions are as in Figure 4 10

SOLVENT	I_k/I_d
acetone	34 4
acetomtrile	26 0
2-propanol	22 0
2-butanol	9 1
phosphate buffer	8 8
THF	7 7

4.3.2.1. *DIETHYLDITHIOCARBAMATE INHIBITION*

Figure 4 11 shows the effects of DEDTC inhibition on the enzyme-generated I_k in acetonitrile, acetone, THF, 2-propanol and 2-butanol The respective percentage decrease in I_k measured at -100 mV (except in acetone which was measured at -60 mV) are 57, 41, 42, 27 and 19 % These values predict that at the stated potential(s), DEDTC inhibition will be highest in acetomtrile and lowest in 2-butanol In addition to its pesticidal applications, DEDTC is also used as a chelating agent in the determination of metal ions by UV spectrophotometry [14-16] However, a major drawback of this analytical method is that DEDTC-metal complexes are water insoluble Hence, a solvent extraction step is usually required Figure 4 12 shows the inhibitory effect of DEDTC-

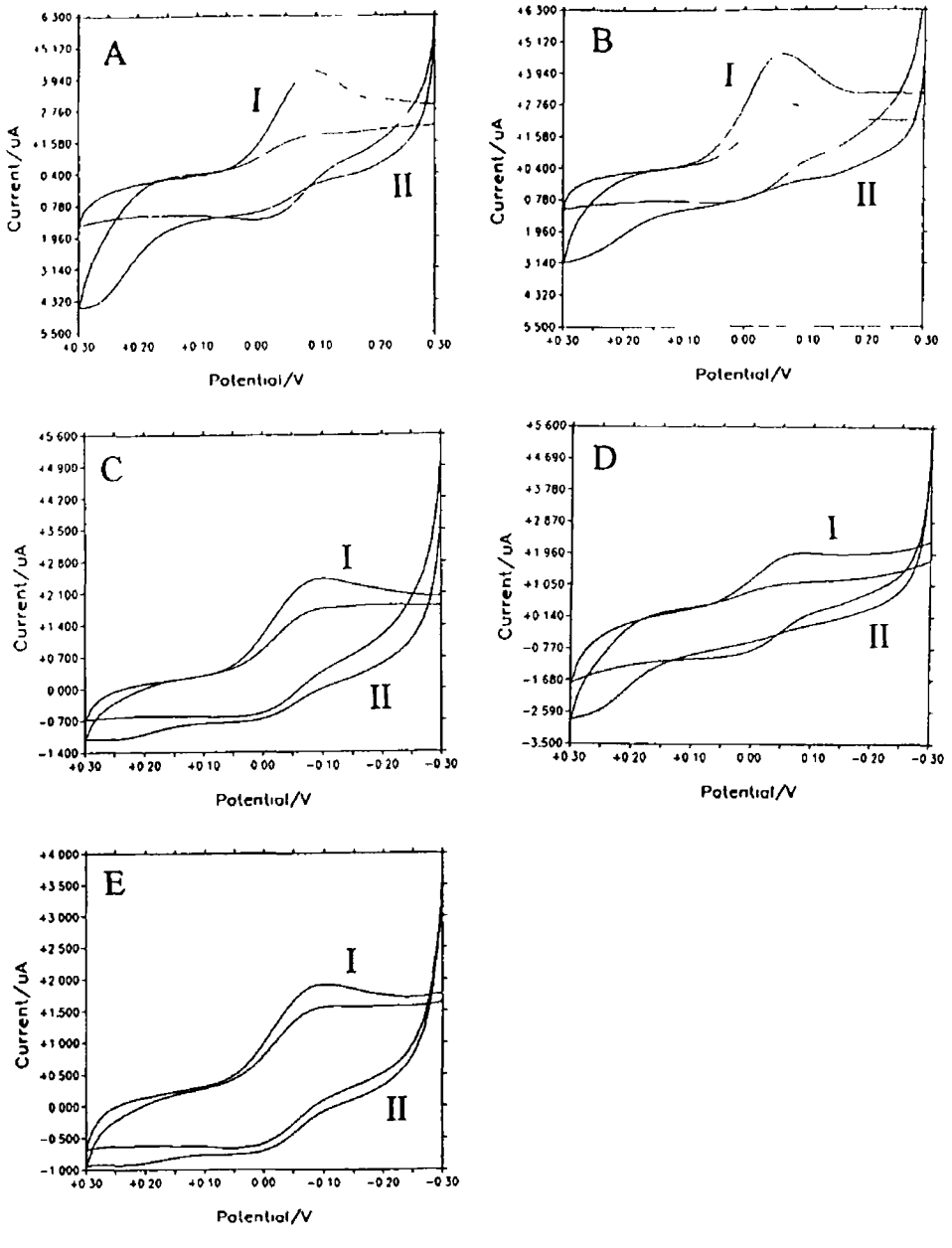

Figure 4 11 Cyclic voltammograms of tyrosinase/Eastman AQ polymer-modified electrode in acetonitrile (A), acetone (B), 2-propanol (C), THF (D) and 2-butanol (E) in the presence of 50 μM catechol (I) and on addition of 10 μM diethyldithiocarbamate (II) Other experimental conditions are as specified in Figure 4 10

lead(II) complex on I_k Similar scans (not shown) were obtained with DEDTC-nickel(II), DEDTC-mercury(II), DEDTC-arsenic(II) and DEDTC-cobalt(II) complexes This preliminary study indicates the possibility of having an organic phase tyrosinase-based biosensor as a detection system for HPLC analysis of these metals

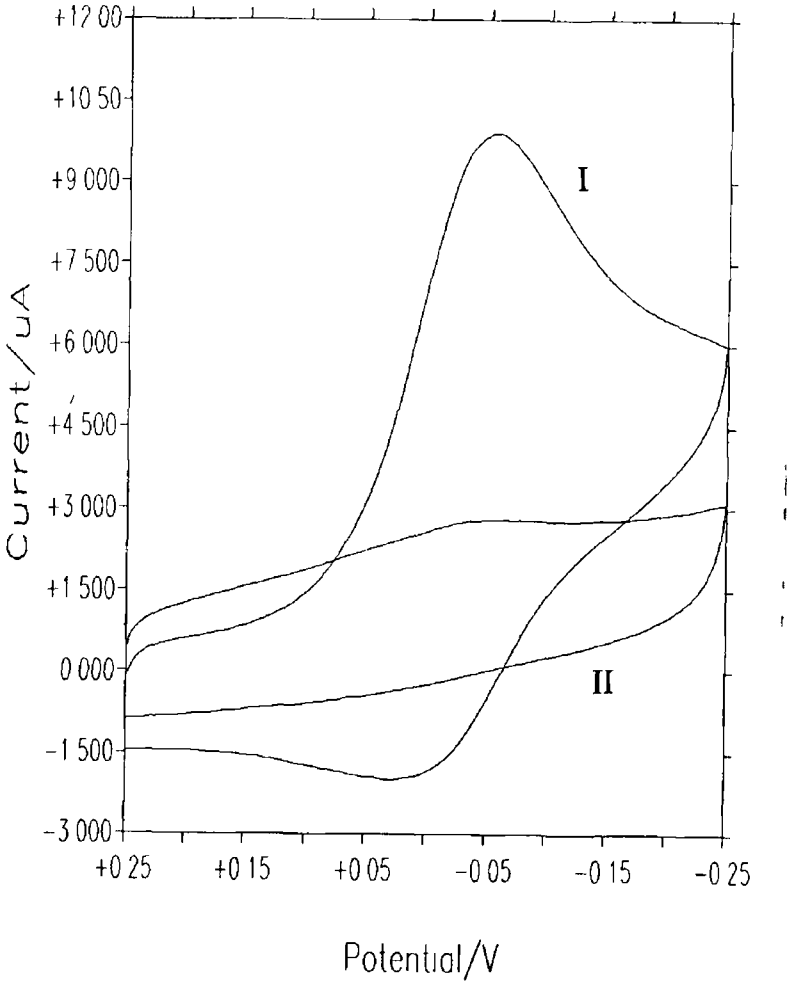

Figure 4 12 Cyclic voltammograms of tyrosinase/Eastman AQ polymer-modified electrode in acetonitrile in the presence of 100 mM catechol (I) and on addition of 50 ppm diethyldithiocarbamate-lead complex (II) Other experimentals conditions are as in Figure 4 10

4.4. CONCLUSIONS

Cyclic voltammetry results, as obtained with the bare glassy carbon electrodes and HRP-modified electrodes, shows that the electrochemistry of the mediator (DMFc) varies with the organic solvents used Significant differences in electrochemical parameters such as the redox peak potentials and electron transfer rates were observed There were also changes in the reversibility of the DMFc reduction/oxidation processes, which was more noticeable in acetone at a scan rate of 5 mV/s (Figure 4 7C) Similar changes in reversibility were observed in acetonitrile and methanol at lower scan rates i e < 5 mV/s This behaviour suggests that the a considerable amount of the oxidised form of DMFc is used up or depleted as it is being formed This may be as a result of adsorption onto GCE or side-reactions between these species and the organic solvents, BTP or the electrolyte However, at higher scan rates, the voltage scan is rapid compared to the rate of the "side-reaction", therefore the CVs are identical to those for uncomplicated charge transfer [17] The catalytic performance of the HRP-modified electrodes, in detecting peroxide and the inhibiting compounds was dependent on the electron transfer mediator employed However, while thiourea displayed inhibitory abilities, ethylenethiourea acted as cosubstrate for the ferrocene-based peroxide biosensors This suggests the possibility of different reaction mechanisms for HRP inhibition by ETU and THU Also, it shows that the mediator can play a significant role in the inhibitory process, since the inhibition of HRP by these compounds had previously been reported [18] Nonetheless, the chemical structure of ferrocenes, allows for the synthesis of a wide variety of

analogues that can be designed to suit a particular organic medium as well as the enzymatic applications The catalytic performance of both the peroxide and phenol biosensors varied with the organic solvents utilised This shows that the physico-chemical properties of these solvents play an important role in the extent of biocatalysis While the catalytic performance of the HRP-modified electrode in detecting BTP and THU in the organic solvents followed the same trend, there was no correlation between the biosensor detection of phenol and DEDTC, thus suggesting that the rate of diffusion of the analytes to the electrode surface is not the sole determinant of the biosensor performance In conclusion, these studies indicate the need for a careful choice of solvent medium, electron-mediating compound and simple immobilisation technique to develop sufficiently selective and sensitive enzyme-based biosensors for analytes acting as enzyme substrate or inhibitor

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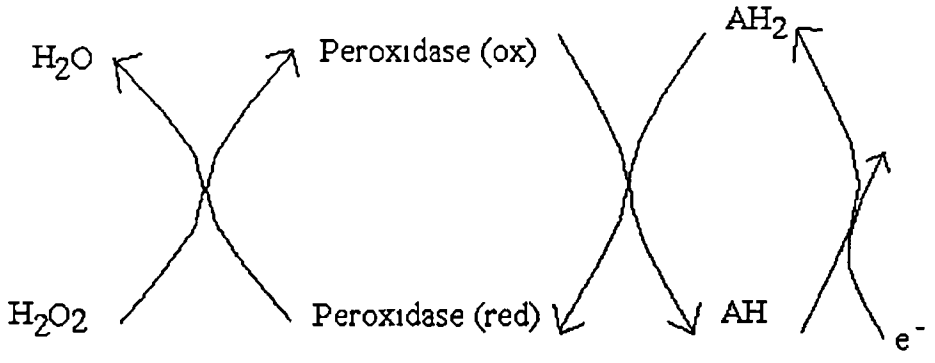
CHAPTER FIVE: FIXED-POTENTIAL TECHNIQUES

5.1. STEADY-STATE AMPEROMETRY

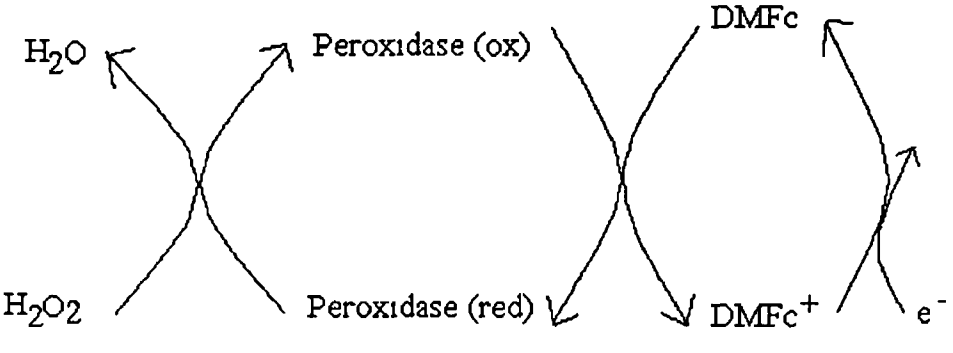
5.1.1. PEROXIDE BIOSENSOR

The mediating compound in a biosensor is usually co-immobilised with the enzyme on the electrode surface or added as a soluble component of the reaction media. A major problem with the immobilisation of mediators is their tendency to leach from the enzyme layer. The resulting uncertainties in the concentration of the mediator makes it difficult to investigate the biosensor response as a function of the concentration of the mediator. Also, it makes theoretical analysis and discussions of the biosensor's kinetic features difficult [1]. Hence, the preference for soluble mediator-based biosensors.

Three major types of OPEEs (on the basis of the type of mediation) have been developed for the detection of butanone peroxide. In the first two, o-phenylenediamine and 1,1-dimethylferrocene were each employed as soluble electron transfer mediators. The third OPEE was a "reagentless" HRP-modified platinum electrode, which functioned by direct electron transfer between the enzyme redox centre and the platinum surface. o-Phenylenediamine is a hydrophilic compound, and is generally classified as a "hydrogen donor" type of mediator. The reaction mechanism for an o-PEDA-based biosensor can be summarised as



where AH_2 represents o-phenylenediamine The peroxidase reduces hydrogen peroxide to water, then o-phenylenediamine, while reducing the oxidised peroxidase, is itself oxidised by loss of a hydrogen atom to form a free radical The free radical finally undergoes oxidation at the electrode surface to its original form, ready for another catalytic cycle In contrast, DMFc is hydrophobic and is classified as an "electron donor" type of mediator The reaction mechanism of a DMFc-based biosensor is described by the scheme



5.1.1.1. *o-PHENYLENEDIAMINE-BASED PEROXIDE BIOSENSOR*

o-PEDA has recently become one of the mediators of choice for amperometric peroxidase sensors Its mediating abilities, when co-immobilised with the enzyme as well as in its soluble forms, has been well documented for organic-phase detection of peroxides [2-5] In all experiments, o-PEDA remained stable in both its oxidised and reduced states under normal operating conditions In addition, it exhibited fast reaction kinetics with peroxidase and the electrode, such that 95 % of the steady-state signals were achieved within 8 ± 2 seconds at all times A typical peroxide response trace, i e current vs time, is shown in Figure 5 1 for an Eastman AQ polymer/HRP-modified platinum electrode with o-PEDA as a mediator From response traces such as this, the steady-state current response at various BTP concentrations have been determined The measured steady-state currents of this biosensor (and in the rest of the study) were analysed by non-linear least square curve fitting to the Michaelis-Menten equation with a Sigma plot computer programme

5.1.1.1.1. *Effect of o-PEDA concentration on biosensor performance*

Figure 5 2 shows the calibration curves for BTP at different concentrations of the mediator A linear response to BTP concentration up to 1 mM was observed throughout The catalytic I_{max}/K_m' and the K_m'

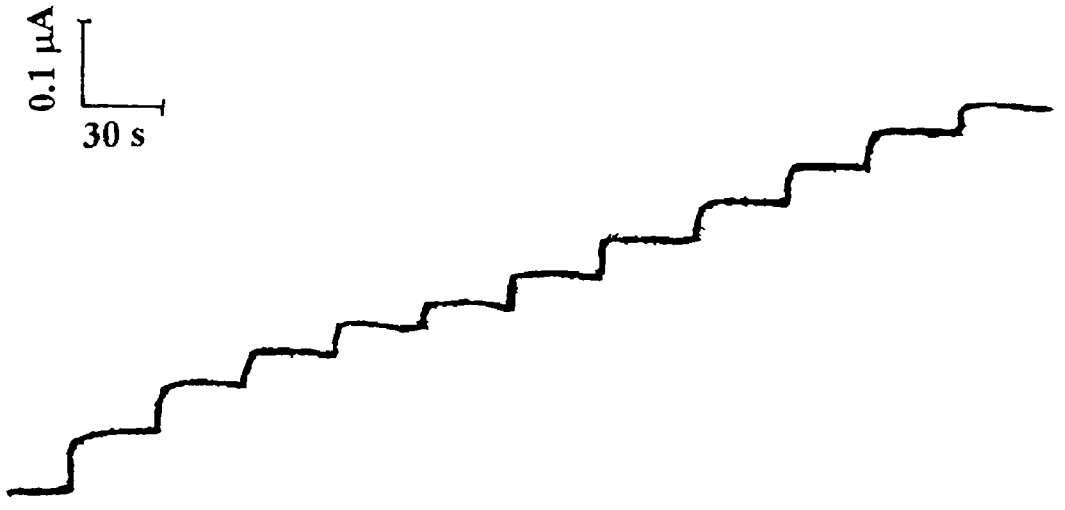


Figure 5 1 Current-time responses of the o-phenylenediamine-based peroxide sensor in 98 % v/v acetonitrile containing 0 1 M TEATS and 0 5 mM o-PEDA to successive additions of 0 1 mM butanone peroxide aliquots to the 20 ml reaction cell The sensor was a Eastman AQ polymer/HRP-modified electrode The concentration of HRP on the electrode was 20 U/cm^2 and the working potential was -250 mV *versus* SCE

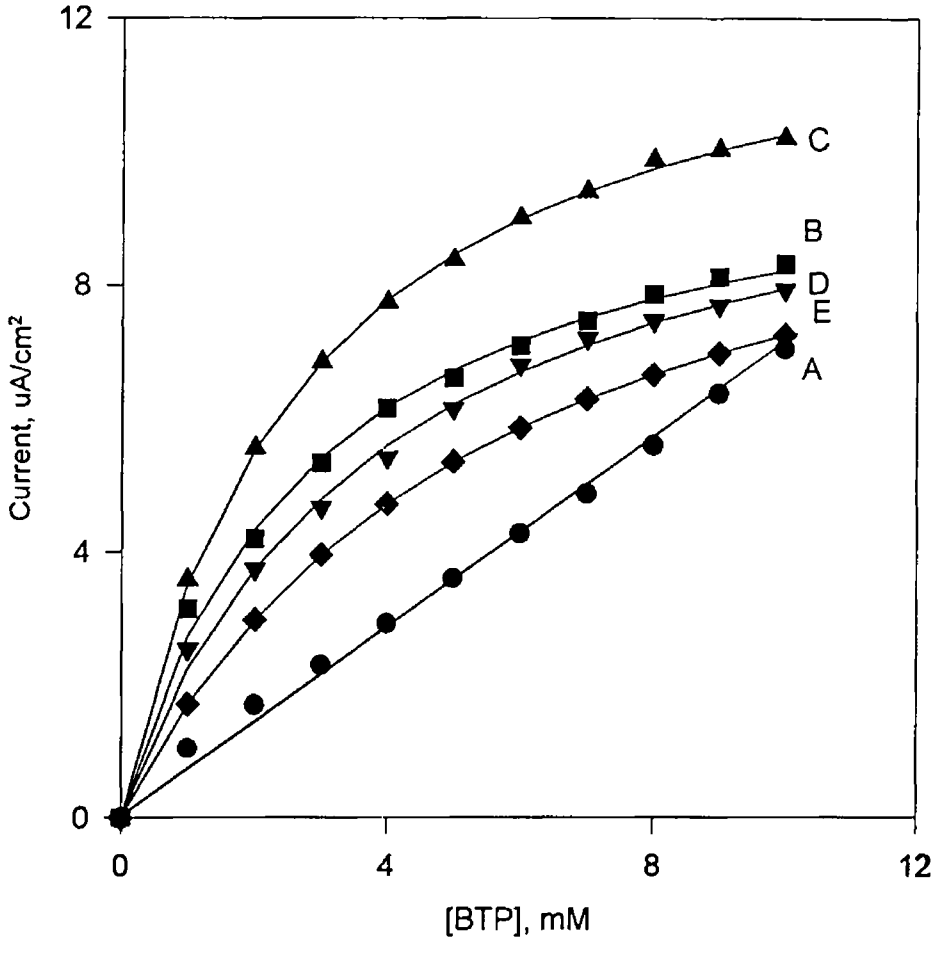


Figure 5 2 Calibration curves for butanone peroxide at 0 (A), 0 25 (B), 0 50 (C), 0 75 (D), and 1 (E) mM o-phenylenediamine concentrations, respectively The responses were obtained with an Eastman AQ polymer/HRP-modified platinum electrode in acetonitrile-phosphate buffer (98 2 % v/v) containing 0 1 M TEATS Other experimental conditions are as described in Figure 5 1 K_m' was 2 9, 2 7, 3 9 and 5 5 mM respectively, and catalytic efficiency was 3 7, 4 8, 2 8 and 2 0 μA/mM cm^2, respectively

values obtained were used a measure of the sensor performance At o-phenylenediamine concentrations of 0 25, 0 50, 0 75 and 1 0 mM the corresponding K_m' values obtained were 2 9,2 7, 3 9 and 5 5 mM/cm^2 and I_{max}/K_m' values were 3 7, 4 8, 2 8 and 2 0 μA/mM cm^2, respectively Analyses of these values and the calibration curves (Figure 5 2) show that the optimal sensor performance was obtained when o-phenylenediamine concentration is 0 50 mM Above 1 mM mediator concentration, there was no significant difference in the magnitude of biosensor response to additions of 1 mM BTP as shown in Figure 5 3 However, further experiments were carried out with the optimal mediator concentration, i e 0 50 mM o-PEDA

5.1.1.1.2. *Effect of water concentration on biosensor performance*

Figure 5 4 shows increased biosensor responses to BTP as the water concentration of the reaction medium was increased from 0 to 3 % v/v As discussed in section **1.2.1.1.**, peroxidase, and indeed most enzymes, are known to require added water for activity in non-aqueous solvents Hence, enzymatic activity in non-aqueous media increases as the amount of added water increases However, the minimal amount of water required for activity varies from one enzyme to the other The minimal water concentration required for this peroxide biosensor was 0 % v/v as shown in Figure 5 4 This is because the HRP-based sensor responds to BTP in the absence of added water However, the catalytic performance (evaluated from the I_{max}/K_m' values) of the biosensor on changing from 100 % acetonitrile to acetonitrile containing 3 % water increased by a factor of 13

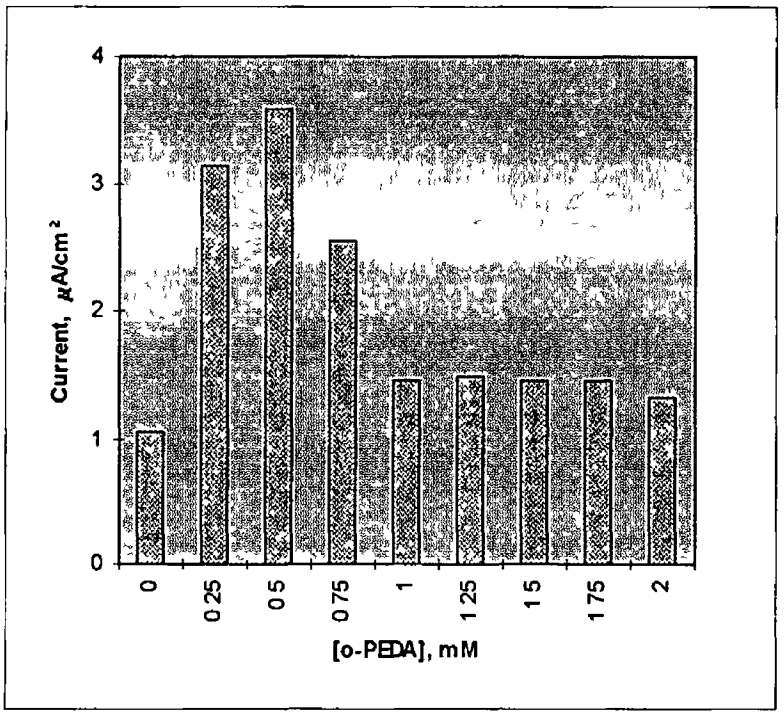


Figure 5 3 Responses of the Eastman AQ polymer/HRP-modified platinum electrode to additions of 1 mM butanone peroxide at different o-phenylenediamine concentrations Experimental conditions are as in Figure 5 2

5.1.1.1.3. *Effect of electrode potential on biosensor performance*

Figure 5 5 shows the calibration curves obtained for BTP concentrations at different electrode potential values At less negative potentials (i e as the potential approaches zero), decreases in the sensor responses were observed Conversely, as the potential was decreased to -400 mV, increases in the biosensor responses were observed This trend is in

agreement with the redox potential values suggested for HRP and its intermediate compounds in other studies For instance, Durliat *et al* [6], using thin layer spectroelectrochemistry, predicted that the redox potential range for HRP-I, HRP-II and HRP-III at a pH of 7 2, is in the

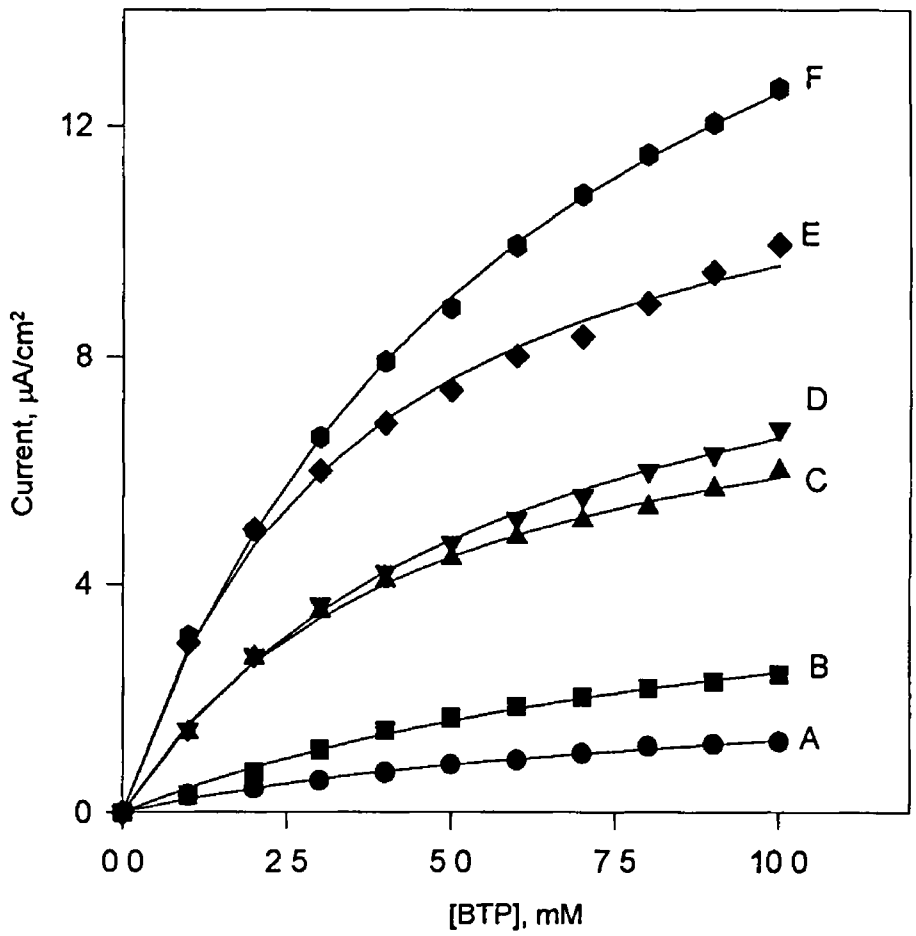


Figure 5 4 Calibration curves for butanone peroxide at 0 % (A), 1 % (B), 1 5 % (C), 2 % (D), 2 5 % (E) and 3 % (F) water concentrations, respectively, o-PEDA concentration was 0 5 mM Other conditions are as described in Figure 5 2 The respective K_m' values were 9 9, 11 3, 4 5, 5 9, 3 5, and 6 3 mM, while I_{max} values were 2 5, 5 2, 8 5, 10 5, 12 9, and 20 4 µA/cm^2, respectively

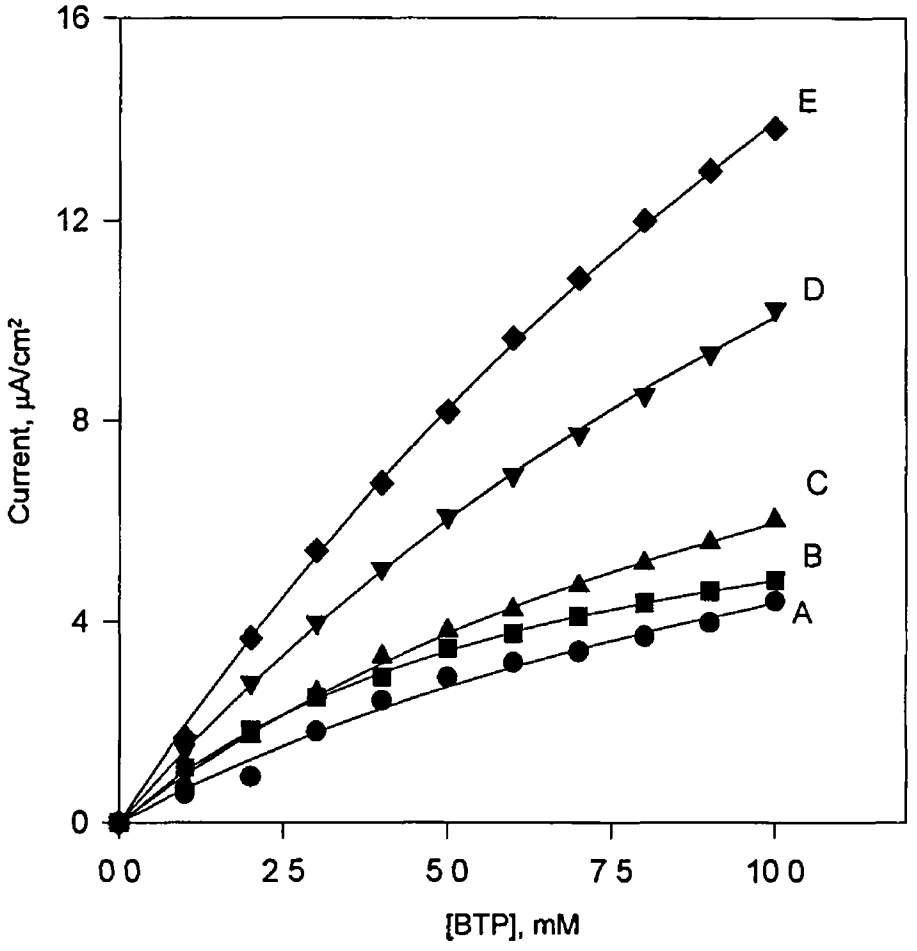


Figure 5 5 Calibration curves for butanone peroxide at -200 mV (A), -250 mV (B), -300 mV (C), -350 mV (D), and -400 mV (E) Other conditions as in Figures 5 2 and 5 4

region of 150 mV to -500 mV In a similar way, Deng and Dong [7] using the same technique, showed that at pH 7 0, the reduction of HRP begins at -400 mV reaching an equilibrium at -700 mV However, in order to avoid interference as a result of oxygen reduction at higher negative potentials, the working electrode was polarised at -250 mV *versus* SCE in all subsequent experiments

5.1.1.1.4. *Effects of nature of organic solvents on biosensor performance*

The amperometric responses of the AQ polymer/HRP-modified platinum electrode towards BTP in methanol, acetomtrile, acetone, 2-butanol and THF are shown in Figure 5 6 The biosensor sensitivities (slopes of the plots) were evaluated as 2 6, 3 8, 6 7, 0 1 and 4 6 $\mu A/ cm^2$ mM in the solvents as listed above, respectively (see Table 5 1) The highest sensitivity was therefore found in acetone and the least in 2-butanol These results were obtained in reaction media containing 98 % v/v of the respective organic solvent Ryu and Dordick [8] reported that in water/polar solvent mixtures, a solvent content greater than 90% v/v alters the transition-state structure and active-site microenviroment of HRP during phenolic oxidations This decreases enzymatic activity, which in turn lowers the sensor sensitivity This form of solvent effect on the active site structure is expected to increase as the polarity of the solvent increases In terms of polarity, the solvents in this study were rated as methanol > 2-butanol > acetonitrile > acetone > THF, such that the greatest solvent effect and hence least biosensor sensitivity, would be expected in methanol Conversely, the least solvent effect and

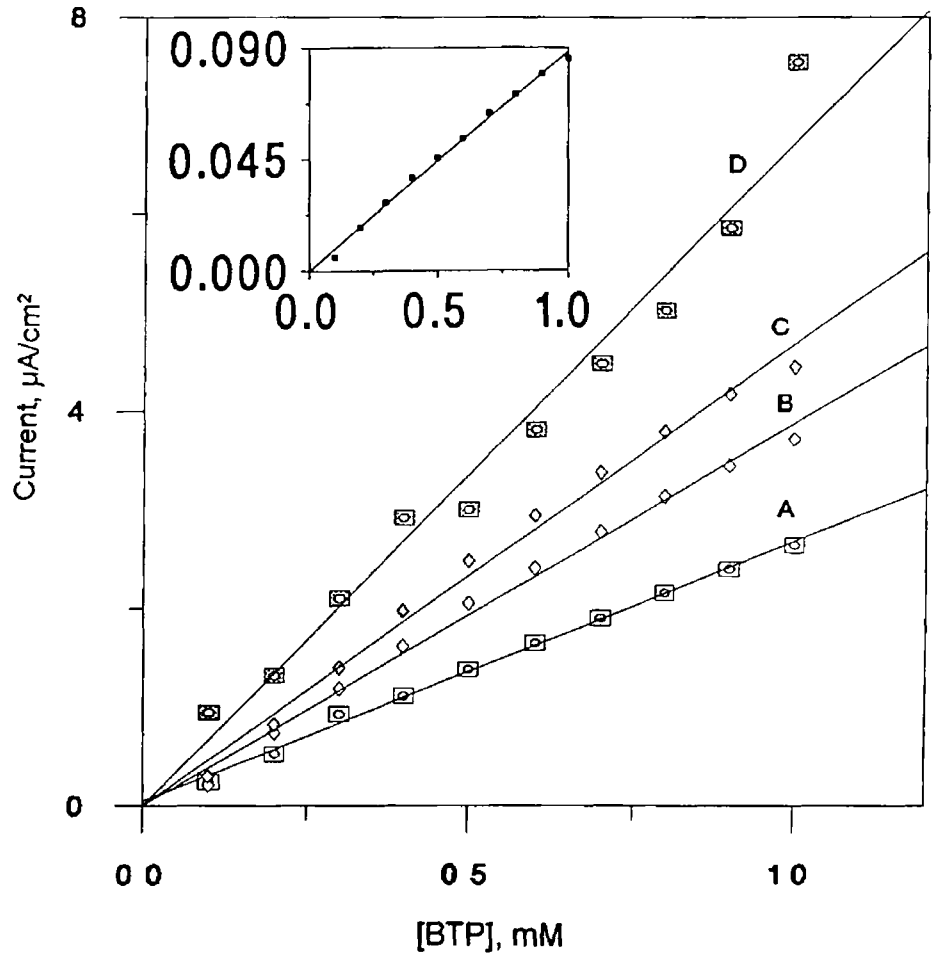


Figure 5 6 Calibration curves for successive 0 1 mM BTP additions in methanol (A), acetonitrile (B), THF (C), acetone (D) and 2-butanol (*inset*) The Eastman AQ polymer/HRP-modified platinum electrode was polarised at -250 mV vs SCE The solvents contained 1 5 % v/v water, 0 1 M TEATS and 0 5 mM o-PEDA

subsequently the highest sensitivity is expected in THF This does not correlate with the observed sensitivities As discussed in section **1.2.1.1.**, the sensitivity of an organic-phase biosensor is influenced by the polarity of the organic medium as well as its other physical properties such as kinematic viscosity and dielectric constant The kinematic viscosity influences the diffusion and partition coefficients of the reacting species (i e the substrate and mediator) across and within the immobilised enzyme layer The dielectric constant is a measure of relative permitivity and governs the ability of solvents to weaken electrostatic forces around the substrate and the enzyme's charged and polar active site In a recent study, Iwuoha *et al* [9] showed that there was a correlation between the diffusion coefficient of solvated glucose molecules and the dielectric constant of some polar organic solvents A low kinematic viscosity and high dielectric constant would be expected to favour high diffusion/partition coefficients which results in better sensor sensitivity Acetone has a lower kinematic viscosity and higher dielectric constant than THF, while methanol has a much lower kinematic viscosity than 2-butanol, hence the trend observed with the sensitivities Similarly, the frictional force of resistance (R_s) exerted on the reactant molecule by the solvent medium could be used to explain the sensitivity trend The frictional force is the product of the frictional coefficient of the reactant (f) and the velocity of the reactant (v) in the solvent medium i e

$$R_s = fv \qquad (5.1)$$

where f = kT/D The quantity kT is a measure of the kinetic energy of the reactant, k being the Boltzmann constant and T the temperature in

degrees Kelvin D is the diffusion coefficient of the reactant in the solvent However, from Stokes law, f is directly proportional to the absolute viscosity, η, and v is dependent on the dielectric constant, ε [10] The function $1/\varepsilon\eta$ has therefore been used as a measure of the frictional force of resistance exerted on the reactants by the solvents The higher the $1/\varepsilon\eta$ value the lower the frictional effect of the solvents on the substrate This would bring about higher diffusibility of the substrate, thereby resulting in greater sensor sensitivity The results show a positive correlation between $1/\varepsilon\eta$ of the solvents and the sensor performances in methanol, acetone, acetomtrile and 2-butanol (see Table 5 1) $1/\varepsilon\eta$ also followed the same trend as the log P (an indication of solvent hydrophobicity) values [11] for all the solvents except 2-butanol The correlation between the physical properties of solvents (including kinematic viscosity and dielectric constant [12]) and the evaluated kinetic parameters are presented in Table 5 1

Table 5 1 The values of the physical properties of the organic solvents, and the kinetic parameters for BTP detection with a HRP-modified platinum electrode in the organic solvents

Parameters	*Methanol*	*Acetone*	*Acetonitrile*	*2-butanol*	*THF*
[a]Dielectric constant,ε (25°C)	32 6	20 7	37 5	15 8	7 6
[b]Kinematic viscosity x10^2, cm^2/s	0 75	0 38	0 46	5 22	0 51
Sensitivity, $\mu A/cm^2$, mM	2 6	6 7	3 8	0 1	4 6
K_m', mM	28 7	10 2	7 1	7 3	5 9
I_{max}, $\mu A/cm^2$	75 1	71 9	31 1	0 7	31 7
$(1/\varepsilon\eta)$ x10^2, $(mPa\ s)^{-1}$	5 5	15 2	7 2	1 5	23 9
[c]log P	-0 77	-0 24	-0 34	0 88	0 46

a The dielectric constant of solvents at 25°C are those reported in reference [12], *b* kinematic viscosity was calculated from absolute viscosity and density of pure solvents at 25°C as given in reference [12], *c* log P values as reported by Laane *et al* in reference [11]

5.1.1.2. *DMFc-BASED PEROXIDE SENSOR*

In the previous chapter DMFc was shown to be capable of acting as a mediator in HRP-based organic-phase biosensors Steady-state amperometry has been used to determine optimal DMFc concentrations and subsequently calibration curves for BTP detection in acetomtrile, methanol and acetone The time required to reach 95 % of the steady-state signals was in the range of 12 ± 2 seconds in all experiments This is very similar to the observed response time with the o-PEDA-based sensor, indicating that DMFc also exhibits fast reaction kinetics with HRP and the electrode Figure 5 7 shows the current-time recordings obtained with the biosensor upon successive additions of 0 1 mM of BTP in acetonitrile

5.1.1.2.1. *Optimisation of DMFc concentration*

Figure 5 8 shows the effect of DMFc concentration on the sensor response to 1 mM BTP in acetomtrile The apparent Michaelis-Menten constant for DMFc, $K_m'_{DMFC}$, was 2 1 mM There was no significant increase in the biosensor response at DMFc concentrations ≥ 3 75 mM This implies that at these concentrations, the sensor's response to BTP is operating under enzyme kinetic-limited conditions Therefore, the steady-state current described by the equation [1]

$$I_{ss} = I_{max}/\{(K_{m\ BTP}/\alpha[BTP]) + (K_{m\ DMFC}/\beta[DMFc]) + 1\} \qquad \textbf{(5.2)}$$

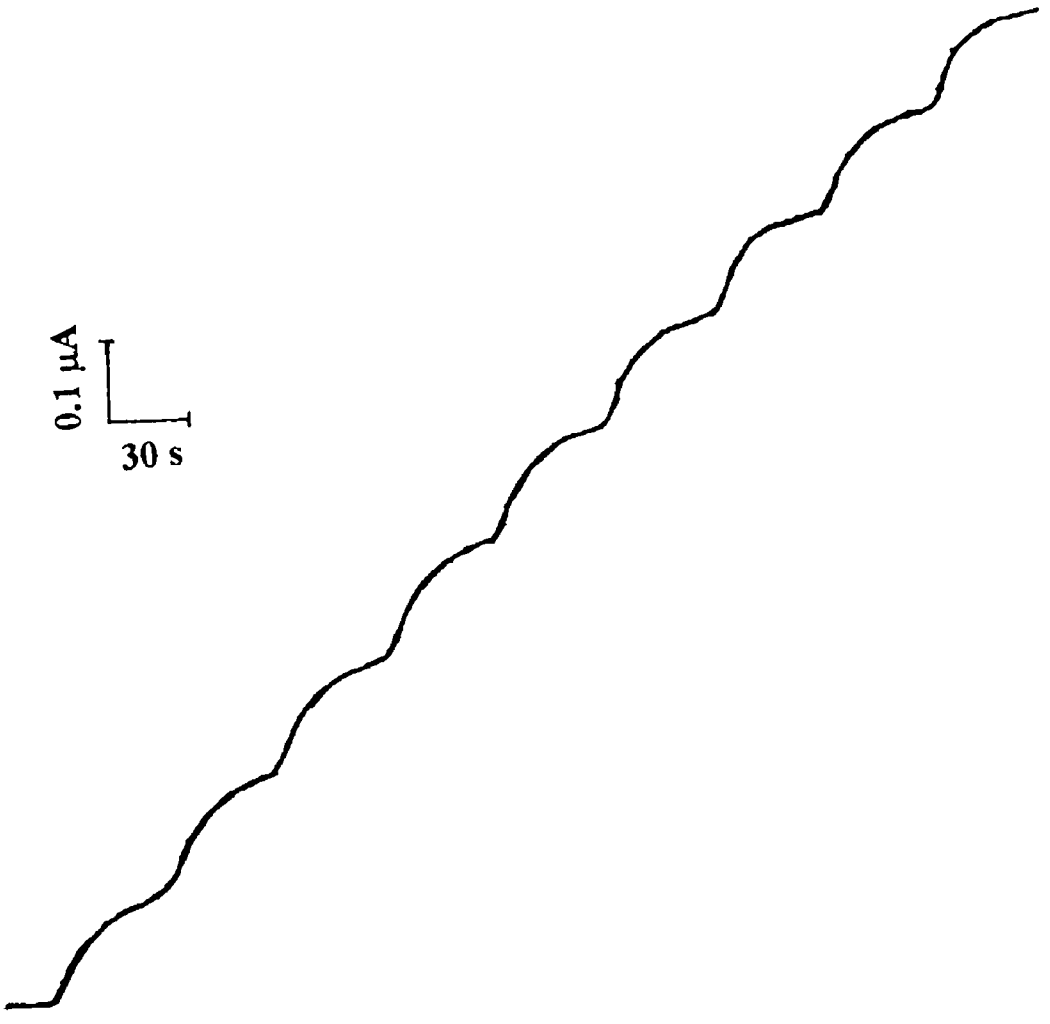


Figure 5 7 Current-time resonses for the peroxide sensor in 98 5 % acetonitrile containing 4 mM 1,1-dimethylferrocene and 0 1 M TEATS to succesive additions of 0 1 mM butanone peroxide aliquots to the 20 ml reaction cell The sensor was a Eastman AQ polymer/HRP-modified electrode The concentration of HRP on the electrode was 20 U/cm^2 and the working potential was +110 mV *versus* SCE

can be rearranged to

$$1/I_{ss} = (K_m'_{BTP}/I_{max}[BTP]) + (1/I_{max}) \quad (5\,3)$$

Equation 5 3 is a linear form of the Michaelis-Menten equation, and α and β are the partition coefficients for butanone peroxide and the mediator, respectively The apparent K_m for BTP is defined as K_m/α In order to induce this conditions, DMFc concentration of 4 mM was used in all experiments

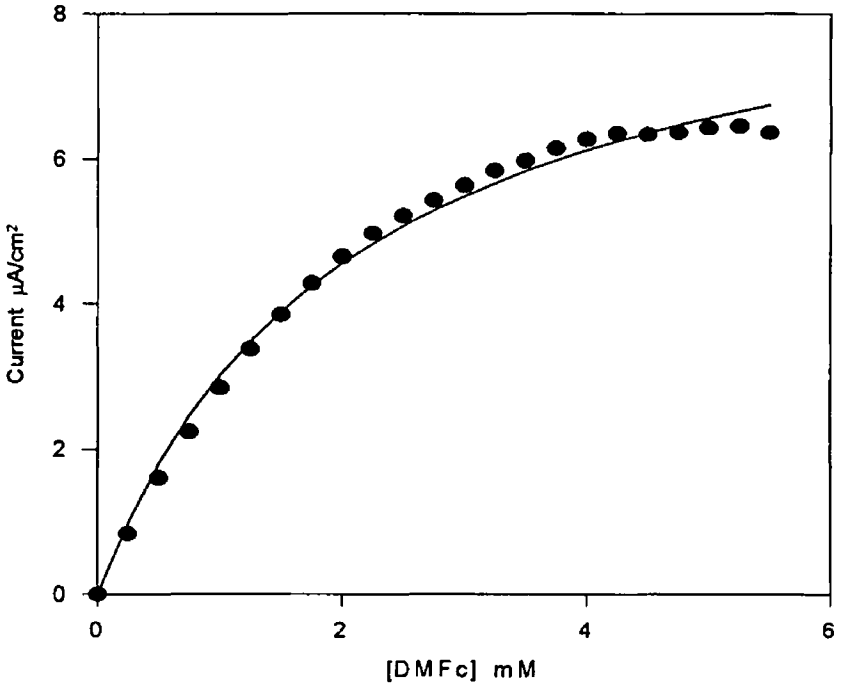


Figure 5 8 A plot of the response of an Eastman AQ polymer/peroxidase-modified electrode to 1 mM BTP with increasing 1,1-dimethylferrocene concentrations Experiment was performed in 98 5 % v/v acetomtrile containing 0 1 M TEATS Other experimental conditions are as described in Figure 5 7

5.1.1.2.2. *Effect of the working potential on the biosensor response*

Figure 5 9 shows the responses of the biosensor to 1 mM BTP at several applied potentials At potentials between 60 mV and 150 mV the was no significant differences in the biosensor responses However, as the potential increased beyond 180 mV, a dramatic reduction in the response of the biosensor was observed This profile reflects the reductive detection of the oxidised form of the mediating compound i e dimethylferricinium ion A potential of 110 mV gave the most favourable signal to noise characteristics and background current, and was therefore used in all subsequent experiments

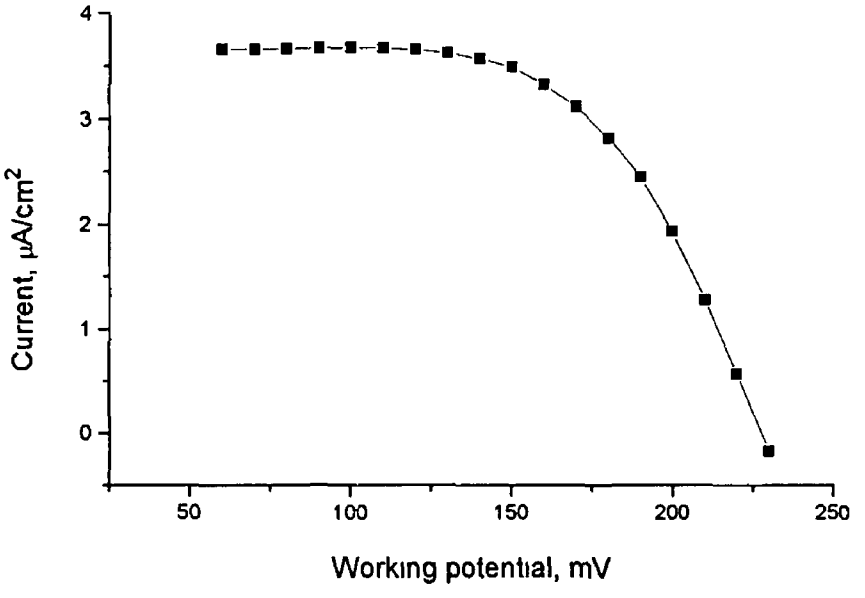


Figure 5 9 Plot of sensor response to 1 mM butanone peroxide as the polarisation potential increases from 60 mV to 230 mV Other experimental conditions are as in Figure 5 7

5.1.1.2.3. *Effects of organic solvents on the biosensor response*

Figure 5 10 shows the dependence of the steady-state amperometric response of the biosensor to BTP on the operating solvent medium As expected for biocatalytic reactions, the initial steady-state currents increased linearly with BTP concentration, and then levels off at higher BTP concentrations The linear limits for BTP detection in acetone, acetonitrile and methanol was 0 2 mM, 0 5 mM and 0 4 mM, respectively The values of I_{max}, K_m' and the sensitivities (I_{max}/K_m') of the DMFc-based biosensor in acetonitrile, methanol and acetone are given in Table 5 2 The highest sensitivity was observed in the least polar solvent used in the study, acetone However, in contrast to the trend observed with the o-PEDA-based biosensor, the lowest sensitivity was in acetonitrile This indicates that other effects, which are dependent on DMFc characteristics such as steric bulk, electronic effects and hydrophobicity are present These factors complicate the enzyme-mediator free energy dependency and its interpretation

Table 5 2 Sensitivity and kinetic parameters for butanone peroxide detection with a Eastman AQ polymer/HRP-modified electrode in 98 5 % v/v acetone, acetonitrile and methanol containing 0 1 M TEATS and 4 mM DMFc Other experimental conditions are as in Figure 5 10

Solvents	K_m', mM	I_{max}, μA/cm^2	I_{max}/K_m', μA/mM cm^2
Acetone	0 5	21 3	42 6
Acetonitrile	5 5	56 5	10 3
Methanol	2 9	44 5	15 3

The sensitivity of the DMFc based peroxide sensor, when compared to the o-PEDA-based sensor had increased by factors of 6 3, 2 7 and 5 8 in acetone, acetomtrile and methanol, respectively In section **4.2**, the electrochemical behaviour of DMFc in the presence of BTP was shown (CV studies) to be similar to that of surface-bound species This implies favourable Eastman AQ-polymer/DMFc interactions thus leading to the preconcentration of DMFc into the immobilised enzyme layer Also, because of the hydrophobic nature of both DMFc and the Eastman AQ polymer, BTP, which is also hydrophobic, partitions easily into the enzyme layer The result is a lower K_m' and higher sensitivity values as observed with these sensor relative to those of the o-PEDA-based sensor

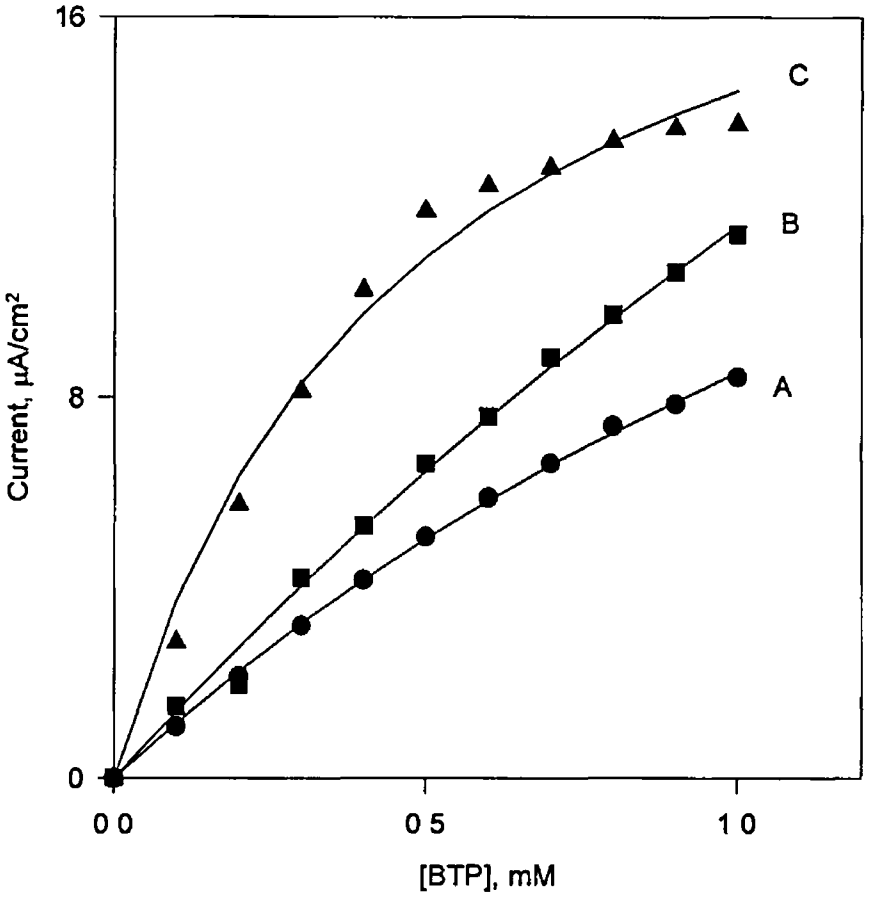


Figure 5 10 Calibration curves for butanone peroxide in 98 5 % v/v acetonitrile (A), methanol (B) and acetone (C) The respective sensitivities are 6 1, 22 7 and 7 8 µA/mM cm^2 Other experimental conditions are as in Figure 5 7

5.1.1.3. *"REAGENTLESS" PEROXIDE BIOSENSOR*

Direct electron transfer between HRP and a bare electrode surface (thus obviating the need for mediators) was first reported for a carbon paste electrode by Yaropolov *et al* [13] Similar results have since been reported for HRP immobilised by adsorption on spectographic graphite and pyrolytic graphite electrodes, electropolymerisation on platinum electrodes, entrapment on graphite epoxy resin electrode, and by adsorption on platinised carbon paste electrodes [14-17] Figure 5 11 shows the current vs time plots obtained with a reagentless Eastman AQ-polymer/HRP-modified platinum electrode for successive additions of 2 mM BTP The response time was < 20 seconds in all experiments, again indicating fast reaction kinetics The interaction between the enzyme active site and the platinum electrode is believed to be possible, because the active site in HRP (unlike in most redox enzymes) is not in its centre, but is positioned at some distance close to the outer surface of the HRP molecule This distance is low enough to allow a reaction between the active site and the electrode surface [18] The exact reaction mechanism between platinum and the heme active site is still not fully understood However, other metals such as ruthenium, palladium and tin oxide are also known to exhibit similar effects on peroxidases The calibration curves of the biosensor responses to BTP in acetonitrile and methanol is shown in Figure 5 12 A linear response was obtained up to 10 mM in acetonitrile and 8 mM in methanol This is an improvement over the previously reported linearity up to 0 5 mM peroxide concentrations obtained with HRP-modified platinised carbon paste electrodes [14] The increase in both response and linearity may be attributed to rapid

electron transfer rate between the HRP active site and platinum surface

K_m' values were 19 9 and 9 9 mM

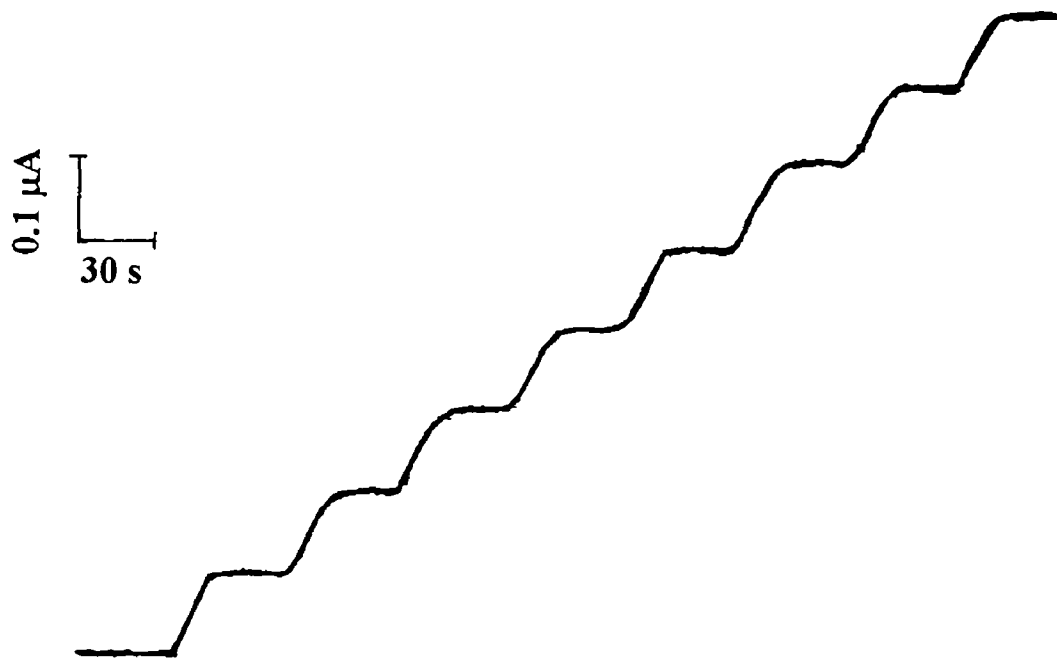


Figure 5 11 The current-time responses of the peroxide sensor in 98 % v/v acetonitrile containing 0 1 M TEATS, to successive additions of 2 mM butanone peroxide aliquots to the reaction cell The biosensor was a reagentless Eastman AQ polymer/HRP-modified platmum electrode HRP loading was 20 U/cm^2 and the working electrode was polarised at -250 mV vs SCE

Also, I_{max} values were 18 0 and 44 7 $\mu A/cm^2$ in acetomtrile and methanol, respectively Therefore, the catalytic performance (I_{max}/K_m') of the biosensor was 2 2 in acetonitrile and 1 8 in methanol These values, when compared to those of the o-PEDA and DMFc-based sensors, imply that there is no significant difference m the catalytic performance of this biosensor in the two solvents This suggests that the differences m the catalytic performance as observed with the o-PEDA and DMFc-based biosensors to a significant extent is a result of the diffusion characteristics of the respective soluble mediators *vis-à-vis* that of BTP in the organic solvents

Figure 5 13 shows the effect of the working potential on the reagentless biosensor It indicates that the sensor response to 20 mM BTP increased by a factor of 8 when the potential is decreased from 0 mV to -300 mV This behaviour is similar to the effect of electrode potential on the response of the o-PEDA-based peroxide sensor However, to avoid interference from oxygen reduction at more negative potentials the workmg electrode was polarised at -0 25 V for all experiments

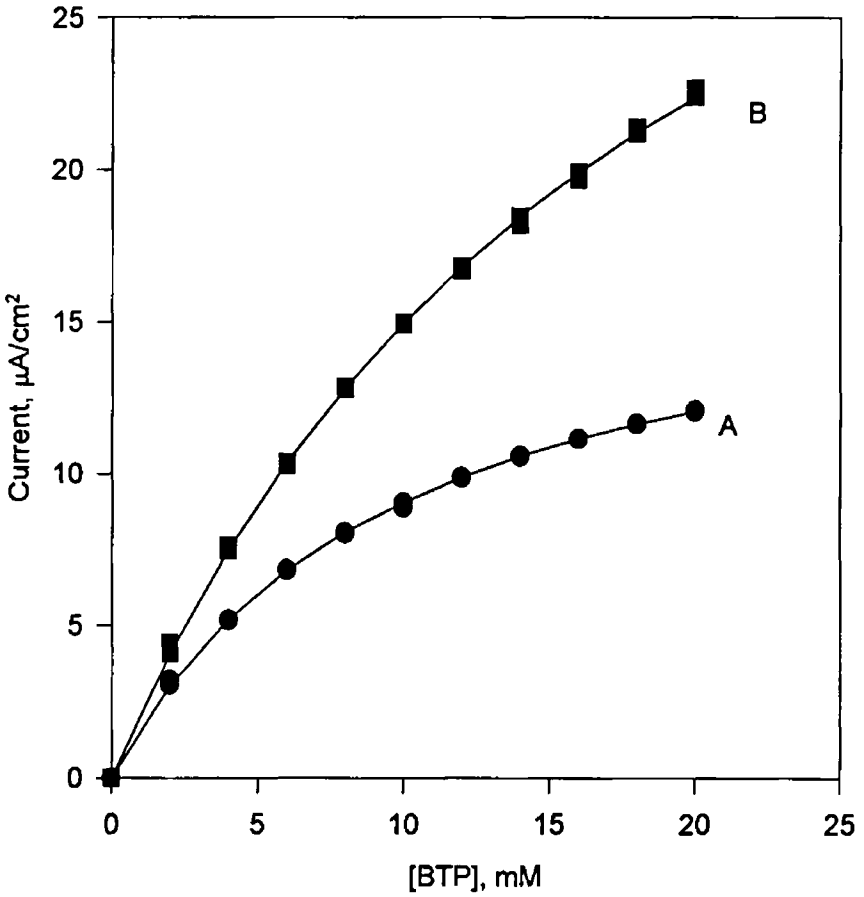


Figure 5 12 Calibration curves for butanone peroxide in methanol (A) and acetonitrile (B) The biosensor was a reagentless AQ polymer/HRP-modified platinum electrode in a solution containing 0 1 M TEATS and 2 % v/v water Other experimental conditions are as in Figure 5 11 The catalytic performance is 1 8 and 2 2 in methanol and acetonitrile, respectively

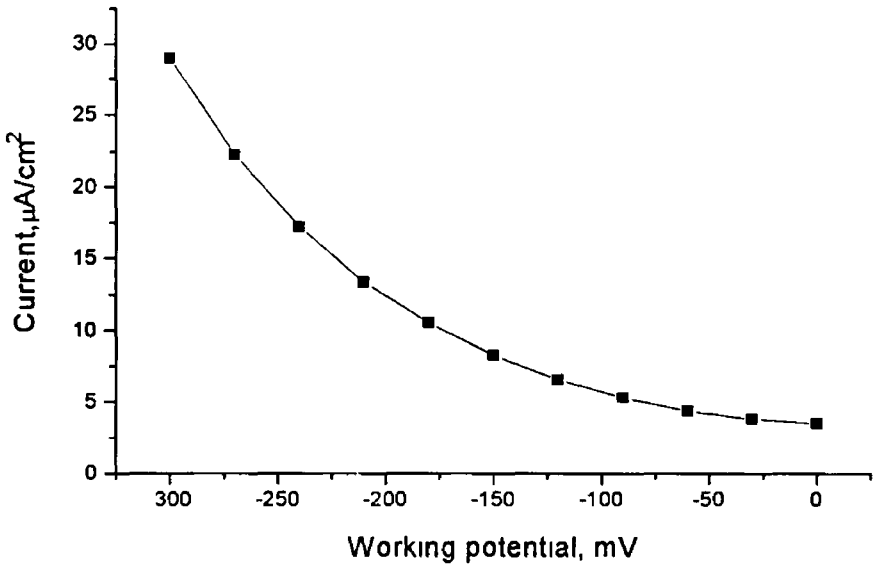


Figure 5 13 Profile of the response of the reagentless AQ polymer/HRP-modified platinum electrode to 20 mM butanone peroxide as the working potential is decreased from 0 to -300 mV in acetonitrile Other operating conditions are as Figure 5 11

5.1.2. PHENOL BIOSENSOR

The steady-state current responses of the biosensor in the presence of phenol was monitored at -150 mV in air-saturated solutions of 80 % v/v acetomtrile, acetone and tetrahydrofuran Figure 5 14 shows the time responses of the sensor additions of 10 μM phenol The sensor exhibited fast responses to phenol, such that 95 % of the steady-state signals were attained in 25 ± 5 seconds in all solvents However, the size of the signal is large for less hydrophobic acetone (log P = -0 23) and acetonitrile (log

P = -0 33) On the other hand, the sensor gave very small signals in tetrahydrofuran, which is a solvent that has a positive log P value

The calibration curves of the phenol sensor are shown in Figure 5 15 The sensor exhibits a very large linear range in THF in spite of the low current output in this medium compared to acetomtrile and acetone The linear ranges are >110 μM, 55 μM and <40 μM in THF, acetone and acetonitrile, respectively The highest biosensor response to phenol was in acetone followed by acetonitrile and then THF This trend correlates with that predicted by the CV results The calibration currents were measured with a Eastman AQ polymer/tyrosinase-modified platinum disk electrode rotating at 1500 rpm A rotating disk profile was applied because, for a diffusion controlled current, the current should increase as the rotation speed of the electrode increases provided that the electroactive species in this case o-benzoquinone, is not lost to the bulk of the solution At the rotation speed of this experiment, the loss of benzoqumone is insignificant This is because the catalytic current increases as the electrode rotation speed is stepped up from 0 to 3000 rpm Electrochemical Michaelis -Menten kinetics has been employed in the analysis of the steady-state currents obtained with the sensor in the different reaction media I_{max} and K_m', for the systems are shown in Table 5 3 These results show that the phenol sensor is about five times more sensitive in acetonitrile and acetone than in THF The sensitivities of the tyrosinase electrode in acetomtrile and acetone do show a positive correlation with both their log P and $1/\varepsilon\eta$ values

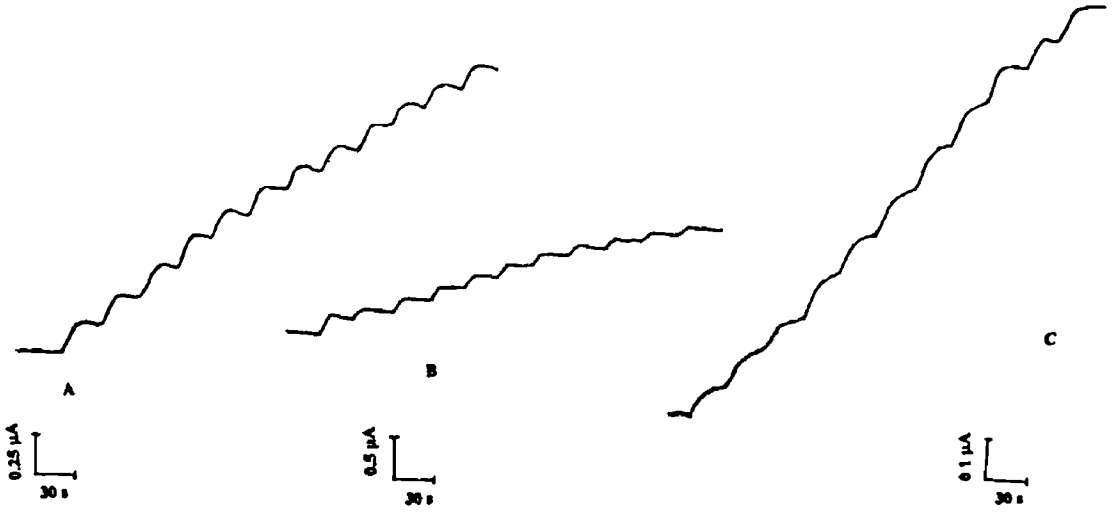


Figure 5 14 Current-time responses of the amperometric phenol sensor in 80 % v/v (A), acetone, (B) acetonitrile, (C) tetrahydrofuran containing 0 1 M TEATS to additions of 10 µM phenol aliquots to the 10 ml reaction cell The sensor was a tyrosmase-Eastman AQ polymer modified platinum disk electrode The concentration of tyrosinase on the electrode was 170 U/cm^2 The rotation speed of the electrode was 1500 rpm and the working potential was -150 mV *versus* SCE

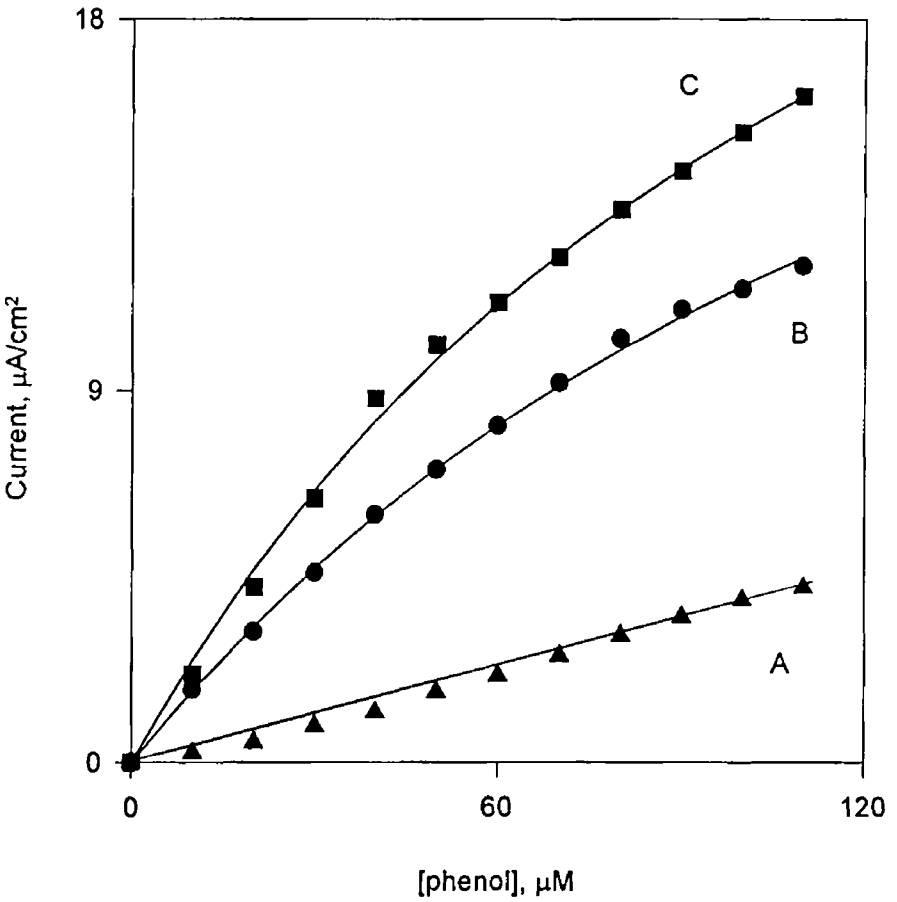


Figure 5 15 Calibration curves of the tyrosinase-modified platinum electrode in (A) tetrahydrofuran, (B) acetonitrile, (C) acetone Experimentals conditions are as described in Figure 5 14

However, as observed with the peroxide sensor, the sensitivity of this sensor (when comparing the sensitivities in either acetonitrile or acetone to that of THF) decreases with increases in both log P and $1/\varepsilon\eta$ values of the solvent This suggests that other solvent properties play a significant role in organic-phase biosensing The diffusion coefficient of phenol, D_{phenol} values, in the respective solvents, which was measured by chronocoulometric potential step method [19] are also shown in Table 5 3 As expected, the sensitivity of the phenol sensor increases as D_{phenol} in the various solvents increases However, the differences in the D_{phenol} values are too small to account alone for the the drop is sensitivity by a factor of five on changing from acetomtrile or acetone to THF D_{phenol} theoretically depends primarily on the $1/\varepsilon\eta$ values as previously discussed in section **5.1.1.1.4** On the other hand, the sensitivity of the biosensor depends not only on the flux of phenol to the enzyme electrode surface, but also on the Gibbs energy of activation of the enzyme-substrate reaction [20] Thus the sensitivity of the phenol biosensor will be determined by the stability of the transition state of the electroenzymatic reaction *vis-a -vis* the ground-state stability of the phenol molecule in the various solvent media

Table 5 3 Kinetic parameters of the amperometric organic-phase phenol sensor based on the immobilisation of Eastman AQ polymer-entrapped tyrosinase on a platinum disk electrode The experimental conditions are as in Figure 5 14

Parameters	*Acetonitrile*	*Acetone*	*Tetrahydrofuran*
K_m', μM	159 1	285 1	-
I_{max}, μA cm^2	29 5	63 9	-
[a]Sensitivity, A cm^2/μM	185 2	224 1	41 6
[b]log P	-0 34	-0 24	0 46
1/εη	7 2	15 2	23 9
[c]D_{phenol} x10^6, cm^2/s	8 8	9 9	6 3

[a]I_{max} /K_m' is the sensitivity of the tyrosinase sensor in acetonitrile and acetone The sensitivity in tetrahydrofuran is the slope of the linear calibration curve of the sensor

[b]log P values are as reported in [11]

[c]D_{phenol} values were calculated from the slopes of the Anson plots of chronocoulograms obtained with an unmodified 25 μm diameter platinum microdisk electrode in the respective solvents [19]

5.1.3. INHIBITION BIOSENSORS

5.1.3.1. *EFFECT OF INHIBITORS ON PEROXIDE AND PHENOL BIOSENSORS*

Figure 5 16 shows how the presence of thiourea, ethylenethiourea and mercaptoethanol affects the response of the biosensor to BTP The largest decrease in the biosensor response was observed in the presence of 0 2 mM thiourea, while the lowest was in the presence of 0 4 mM mercaptoethanol The values of I_{max}, K_m' and the biosensor sensitivities (I_{max}/K_m') are shown in Table 5 4

Table 5 4 Values of kinetic parameters for the peroxide biosensor, in the absence and presence of thiourea, ethylenethiourea and mercaptoethanol Experimental conditions are as described in Figure 5 16

	I_{max}, $\mu A/cm^2$	K_m', mM	I_{max}/K_m', $\mu A/mM\ cm^2$
absence of any inhibitor	13 5	3 0	4 5
presence of 0 2 mM thiourea	4 9	1 9	2 6
presence of 0 2 mM ethylenethiourea	6 6	2 2	3 0
presence of 0 4 mM mercaptoethanol	7 7	2 1	3 7

These values indicate that the sensitivity of the peroxide sensor decreased by 42 %, 33 % and 18 % in the presence of thiourea, ethylenethiourea and mercaptoethanol, respectively The decrease in I_{max} implies that the total enzyme concentration is effectively reduced by the presence of the inhibitors, while the decrease in K_m' shows that the binding of the inhibitor affects that of BTP These changes in the values of both K_m' and I_{max} implies that the inhibition mechanism is a mixed effects form of inhibition Figure 5 17 gives the Lineweaver-Burk plots of the data presented in Figure 5 16 The features of this plots shows that the mechanism of inhibition is the same for all three inhibitors Also, the negative values of the intersection points of the plots in the presence of the inhibitors with that in the absence of inhibition is characteristic of non-competitive-uncompetitive mixed-effects inhibition mechanism [21]

There are two processes by which an inhibitor may bind to an enzyme

(I) $E + I_n \rightleftharpoons EI_n$ (inhibitor constant K_i)

(II) $ES + I_n \rightleftharpoons ESI_n$ (inhibitor constant K_I)

hence,

$$K_i = ([E][I_n])/[EI_n] \quad \text{and} \quad K_I = ([ES][I_n])/[ESI_n] \qquad \textbf{(5.4)}$$

A non-competitive-uncompetitive reaction mechanism indicates that $K_i > K_I$ in the inhibition of the peroxide sensor by THU, ETU and MCE

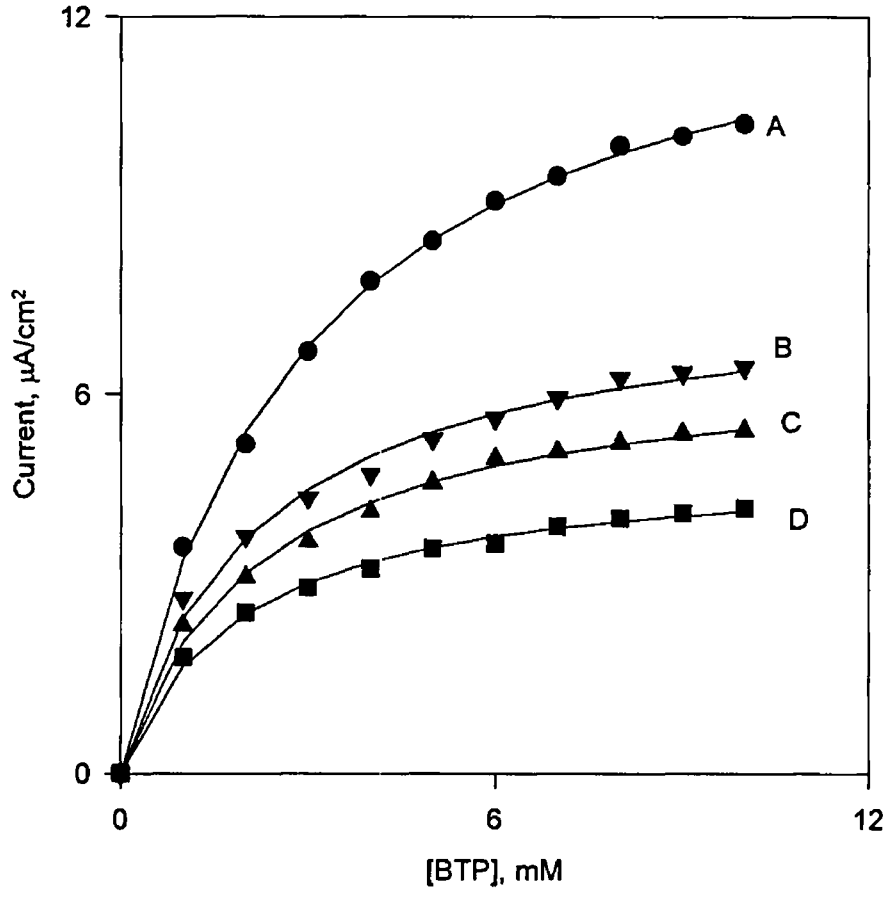


Figure 5 16 Calibration curves for butanone peroxide (A) without any inhibitor, (B) with 0 4 mM mercaptoethanol, (C) with 0 2 mM ethylenethiourea, and (D) with 0 2 mM thiourea The experiments were performed with an Eastman AQ polymer/HRP-modified platinum electrode in 98 % acetonitrile containing 0 1 M TEATS and 0 5 mM o-PEDA The enzyme loading was 20 U/cm^2 and the working potential was -250 mV

The inhibitory effects of varying concentrations of diethyldithiocarbamate (20-50 μM) on the calibration curve of the phenol sensor showed the same characteristics as those in Figure 5 16 (plots not shown) Table 5 5 shows the sensitivity, K_m' and I_{max} values obtained from the plots The high values of I_{max} at 20 and 30 μM DEDTC suggests that there is an increase in the catalytic turnover rate of the enzyme, k_{cat} This means that the rate at which phenol reacts with the enzyme in the presence of these concentrations of the inhibitor is higher than in the absence of DEDTC Hence an increase in the corresponding K_m' values The sudden decrease in the I_{max} values at 40 and 50 μM DEDTC is possibly as a result of substrate inhibition Substrate inhibition occurs when a molecule of substrate binds to one site of the enzyme and then another molecule of substrate (the inhibitor) binds to a separate site on the enzyme to form a dead-end complex The presence of substrate inhibition is supported by the assymetrical "V" shape of the Lineweaver-Burk plots (a feature of substrate inhibited enzymes) of the responses of the phenol sensor when this concentrations of DEDTC are added (plots not shown) The I_{max}/K_m' values show that the sensitivity of the phenol sensor decreased by 57 % and 86 %, in the presence of 20 μM and 50 μM DEDTC, respectively

The Lineweaver-Burk plots of the responses of the sensor to phenol in the absence and presence of 20 and 30 μM DEDTC are shown in Figure 5 18 In contrast to the peroxide sensor, intersection points of the plots in the presence of DEDTC with that obtained in its absence is positive This is the predicted feature for a competitive-non-competitive mixed

effects inhibition mechanism This implies that for DEDTC inhibition of the phenol sensor, $K_I > K_i$

Table 5 5 The values of kinetic parameters for the phenol sensor, in the absence and presence of 20-50 μM diethyldithiocarbamate The experiments were performed with an Eastman AQ polymer/tyrosinase-modified platinum electrode in 80 % 2-propanol containing 0 1 M TEATS The enzyme loading was 170 U/cm^2 and the working potential was -200 mV

[DEDTC], μM	K_m', μM	I_{max}, μA/cm^2	I_{max}/K_m', μA/μM cm^2
0	49	32	0 7
20	174	59	0 3
30	441	88	0 2
40	68	14	0 2
50	175	18	0 1

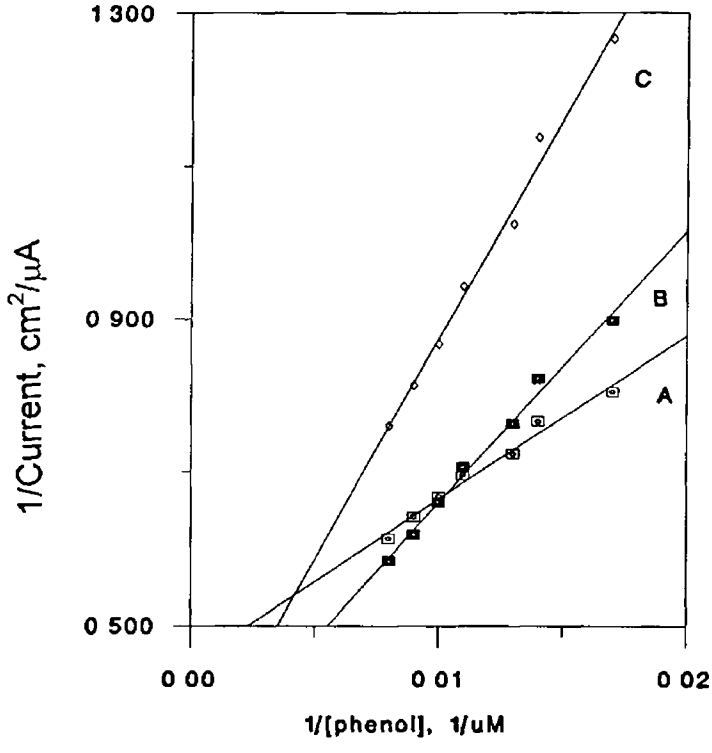


Figure 5 18 Lineweaver-Burk plots of calibration curves for phenol with an Eastman AQ polymer/tyrosinase-modified electrode in the absence of DEDTC (A), m the presence of 20 μM DEDTC (B), and in the presence of 30 μM DEDTC (C) Other experimental conditions are as described in Table 5 5

5.1.3.2. *BIOSENSOR DETECTION OF THE INHIBITORS*

A biosensor, based on the inhibitory effects of the analyte is expected to operate under enzyme-limited conditions The concentrations of o-phenylenediamine (0 5 mM), 1,1-dimethylferrocene (4 mM) and butanone peroxide (1 0 mM) for the peroxide sensor as well as 100 μM phenol, for the phenol sensor used in this study have been found to induce this condition as shown in our previous discussions Under these enzyme-limited conditions, the concentration of both HRP and tyrosinase does not affect the degree of inhibition exhibited by their respective inhibitors These are thiourea, ethylenethiourea mercaptoethanol, hydroxylamine and methyl isothiocyanate (for the peroxidase-based sensor) and diethyldithiocarbamate for the tyrosinase-based sensor Standardizing the enzyme-generated catalytic current and its inhibition by these compounds implies that the relative magnitude of inhibition is governed primarily by the affinity of the inhibitor for the enzymes Therefore, variations during the large-scale production of the biosensor would not be important In steady-state binding kinetics, the fractional inhibition, Y, is the ratio of the concentration of the enzyme-inhibitor complex, $E\text{-}I_n$, to the total concentration of the enzyme, $[E]_t$

$$Y = n[E\text{-}I_n]/([E\text{-}I_n] + [E]) = n[I_n]/([I_n] + K_i') \qquad \textbf{(5.5)}$$

where n is the number of binding/active sites m the biosensor, [E] is the concentration of free HRP, and $[I_n]$ is the concentration of the inhibitor K_i', the apparent inhibition constant, is directly

proportional to the dissociation constant of the E-I_n complex It is the free concentration of the inhibitor required for 50 % inhibition and is a measure of the sensor's efficiency in detecting the inhibitor The lower the apparent inhibition constant, the higher the sensor efficiency in detecting the respective inhibitor Also, n is directly proportional to I_{max}, i e the current when the active sites are fully saturated with peroxide (in the absence of any inhibition) Since $[I_n]$ is directly proportional to the change in current (ΔI) before and after its addition and Y = current due to inhibition binding/total current when the sites are fully saturated with substrate then

$$Y = I_{max}[I_n]/([I_n] + K_i') = \Delta I/I_{ss} \qquad \textbf{(5.6)}$$

where I_{ss} is the observed catalytic steady-state current in the absence of any inhibition It is also possible for inhibition to be expressed as percentage inhibition, $\%I_n$, which is Y × 100 Equation 5 6 implies that plots of the fractional inhibition or percentage inhibition against the bulk concentration of several inhibitors would be hyperbolic The hyperbolic nature of such plots indicates that there is one active site per enzyme molecule and that the immobilised enzyme layer is very thin, such that all the binding/active sites are equivalent in the interaction between the enzyme and the respective inhibitor The apparent inhibition constant, K_i', was evaluated by direct curve fitting of the biosensor responses to equation 5 6 using computer programs or from linear forms of the equation such as the Scatchard equation

$$Y/[I_n] = -Y/K_i' + I_{max}/K_i' \quad (5.7)$$

and the Hughes-Klotz equation

$$1/Y = 1/I_{max} + K_i'/I_{max}[I_n] \quad (5.8)$$

5.1.3.2.1. *Detection of horseradish peroxidase inhibitors*

A typical current versus time response as obtained during the detection of thiourea and ethylenethiourea is shown in Figure 5 19 Steady-state responses were achieved in < 30 seconds for ETU, THU and other inhibitors at all times Figures 5 20-5 22 shows the plots of the fractional inhibition of the peroxidase-modified electrode (with o-phenylenediamine as mediator) against the bulk concentrations of ETU, THU and MeSNC in organic solvents The apparent inhibition constant, K_i', values are shown in Table 5 6 K_i' for ETU were 0 13, 0 09, 0 45 mM and for THU were 0 12, 0 05 and 0 21 mM in methanol, acetone and 2-butanol, respectively In a similar way, K_i' for MeSNC were 0 27, 0 18 and 0 37 mM in acetonitrile, methanol and 2-butanol, respectively The sensor's efficiency in detecting both ETU and THU was highest in acetone and least in 2-butanol, the same trend observed in the catalytic reduction of BTP The K_i' values in all the solvents except acetonitrile, were generally lowest for THU This suggests that in instances where their inhibition of HRP mimics their toxicity mechanism, THU is more toxic than either ETU or MeSNC The higher catalytic efficiency of the

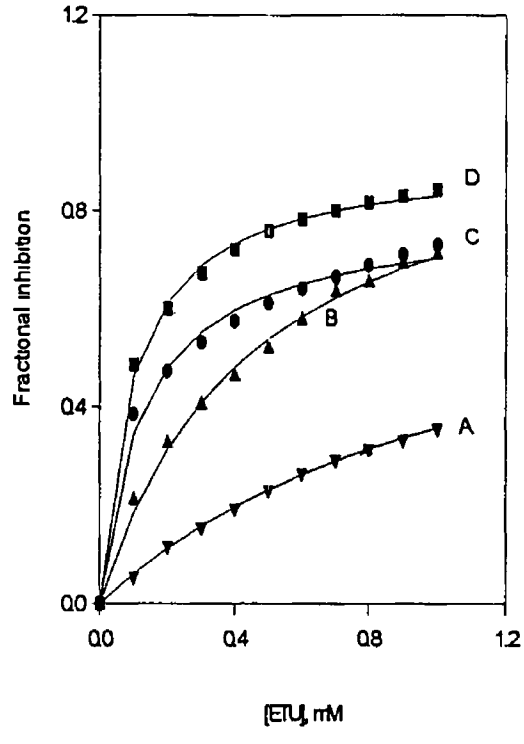


Figure 5 20 The calibration curves for successive 0 1 mM additions of ethylenethiourea in acetonitrile (A), 2-butanol (B), methanol (C), and acetone (D) The solvents contained 1 5 % v/v water, 1 mM butanone peroxide, 0 1 M TEATS and 0 5 mM o-phenylenediamine The Eastman AQ polymer/HRP-modified electrode was polarised at -250 mV vs SCE The enzyme loading was 20 U/cm^2

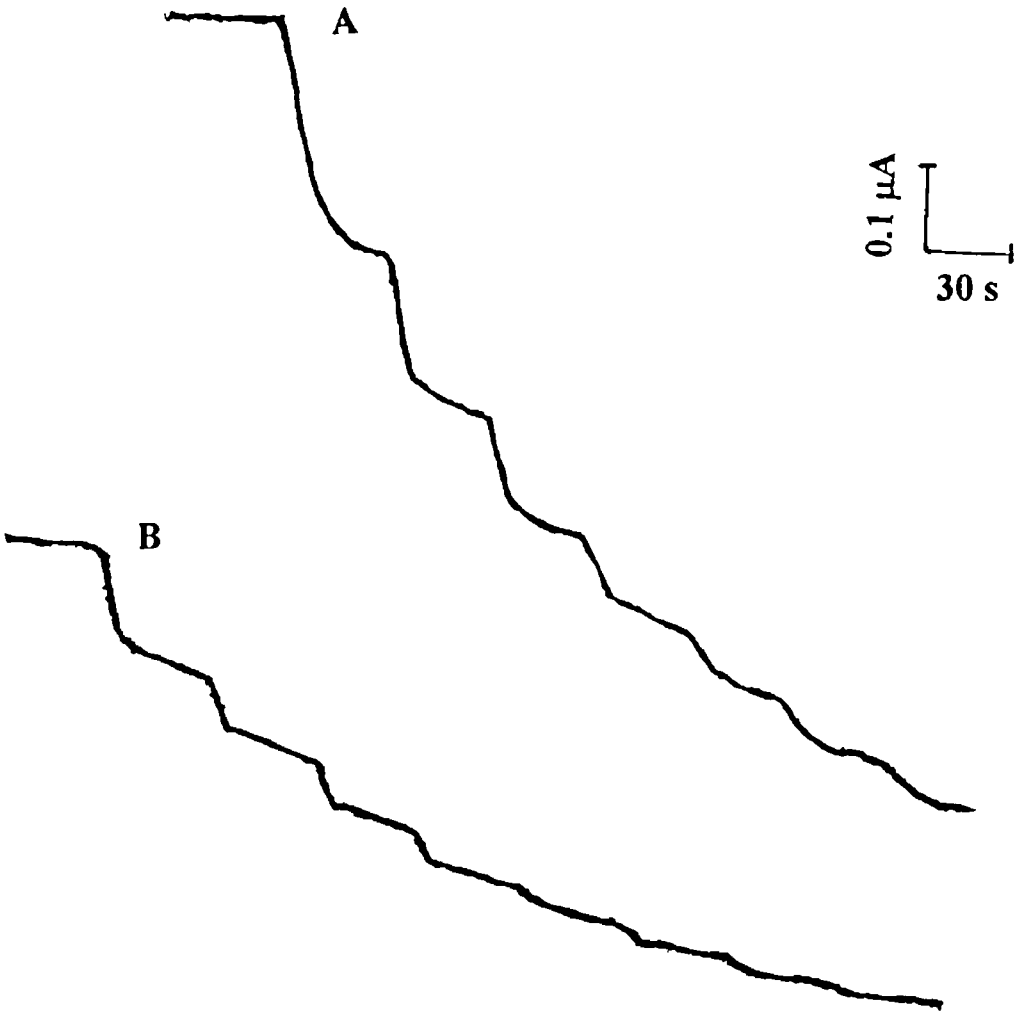


Figure 5 19 Current-time recordings obtained with the o-phenylenediamine-based peroxide sensor upon successive additions of 0 1 mM thiourea (A) and ethylenethiourea (B) Experiments were performed in 98 % v/v acetonitrile containing 1 mM butanone peroxide, 0 1 M TEATS and 0 5 mM o-phenylenediamine The concentration of HRP on the electrode was 20 U/cm^2 and the working potential was -250 mV vs SCE

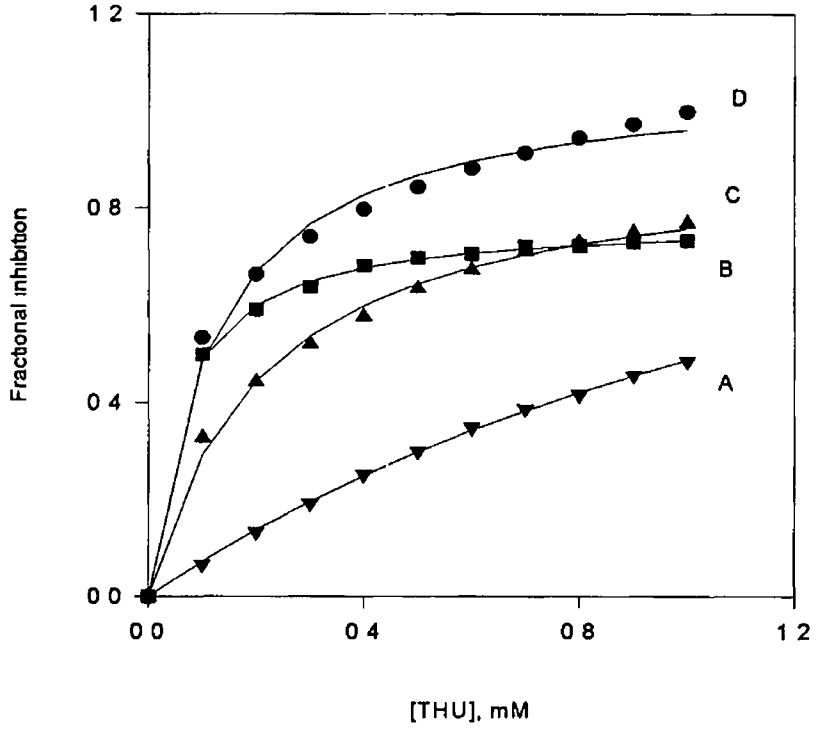


Figure 5 21 Calibration curves for successive 0 1 mM additions of thiourea in acetomtrile (A), acetone (B), 2-butanol (C), and methanol (D) Other experimental conditions are as m Figure 5 20

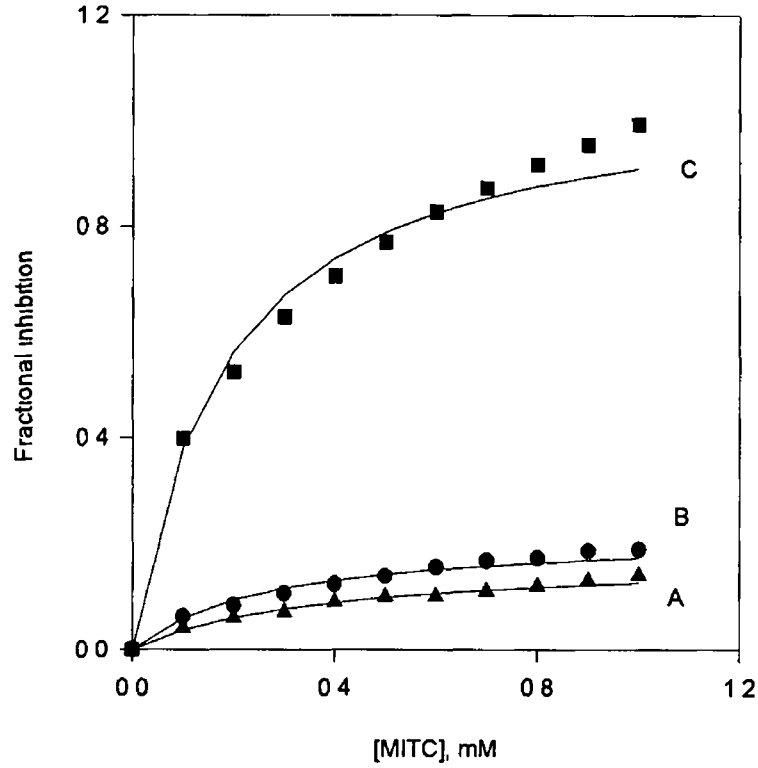


Figure 5 22 Calibration curves for successive additions of 0 1 mM methyl isothiocyanate in methanol (A), acetonitrile (B), and 2-butanol (C) The solvents contain 1 5 % v/v water, 1 mM butanone peroxide, 0 5 mM o-phenylenediamine and 0 1 M TEATS The Eastman AQ polymer/HRP-modified electrode, had an enzyme loading of 20 U/cm^2 and was polarised at -250 mV

biosensor in detecting THU may be attributed to favorable hydrophobicity and electronic characteristics Ryu and Dordick [8] have shown in a peroxidase-catalysed oxidation of phenols that a direct relationship exists between the catalytic efficiency and the substrate hydrophobic and electronic characteristics The catalytic efficiency decreases as phenol hydrophobicity increases These differences may also be as a result of the influence of the Eastman AQ 55D polymer on the biosensor performance The polymer selectively excludes anionic species from the immobilised enzyme layer and exhibits a strong affinity for hydrophobic substances ETU and THU exist as resonance forms and in their most stable forms they exist as anions [23] However, the polymeric film is permeable to ETU, THU, MeSNC and similar inhibitors probably because of their low molecular weight and hydrophobic nature The permeability of these inhibitors, which in turn influences the biosensor performance, differs as the steric hindrances and the stability of their resonance forms change in different organic media

Hydroxylamine was also detected using a mediator-free peroxidase-modified platinum electrode Figure 5 24 shows the response-time recording obtained for the biosensor upon successive additions of 0 1 mM hydroxylamine in acetomtrile and methanol A steady-state response was achieved in 30 ± 5 seconds The biosensor response was standardised by expressing it as percentage inhibition The calibration curve obtamed from these data is shown in Figure 5 24 (inset) The response was linear up to 0 08 mM and 0 06 mM m acetomtrile and methanol, respectively Values of K_i' for hydroxylamine were 0 1 mM in both solvents

Table 5 6 Values of the apparent inhibition constant, K_i' for thiourea, ethylenethiourea and methyl isothiocyanate in methanol, acetone, 2-butanol and acetonitrile Experimental conditions are as described for Figures 5 20-5 22

Solvent	K_i', mM		
	THU	**ETU**	**MeSNC**
methanol	0 12	0 13	0 18
acetone	0 05	0 09	-
2-butanol	0 21	0 45	0 37
acetonitrile	1 75	1 19	0 27

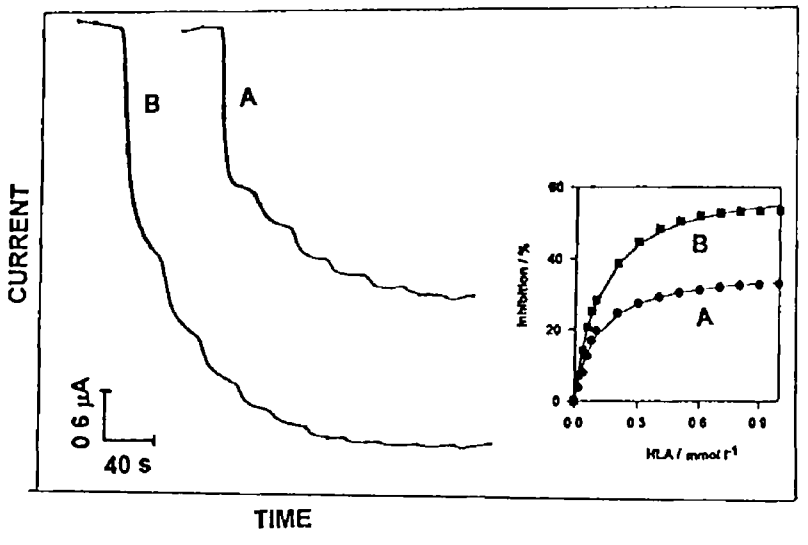


Figure 5 24 Response-time recordings obtained with the Eastman AQ polymer/HRP-modified electrode upon successive additions of 0 1 mM of hydroxylamine, in acetomtrile (A) and methanol (B) Inset is the resulting calibration curves for hydroxylamine m acetonitrile (A) and methanol (B) The respective reaction medium contamed 0 1 M TEATS, 20 mM butanone peroxide and 2 % v/v water HRP loading was 20 U/cm^2 and the operating potential was -250 mV *vs* SCE

The deviation of the response of the o-PEDA-based biosensor to ETU, THU and MeSNC from Michaelis-Menten kinetics in organic media was determined from their respective Hill coefficient, x

$$Y/(1-Y) = (\Delta I/I_{50})^x \qquad (5.9)$$

In the steady-state ligand binding kinetics, the Hill coefficient for an ideal, hyperbolic, Michaelis-Menten theory obeying enzyme catalysis is 1 0 [24, 25] Figures 5 25-5 27 shows the Hill plots for the peroxidase-modified electrode in the detection of ETU, THU and MeSNC in methanol, acetone, 2-butanol and acetonitrile The Hill coefficients of the biosensor (the slopes of the plots) for these compounds are listed in Table 5 7 The least deviations for the three compounds was observed in acetonitrile These values imply that the deviation of the kinetic behaviour of the sensors in acetone, methanol and 2-butanol from Michaelis-Menten kmetics is remarkably high Amidst other factors, these deviations may be as a result of chemical interactions between the enzyme and the organic solvents The presence of organic solvents is known to induce a disruption of the non-covalent forces (i e hydrogen bonding, hydrophobic, van der Waals and ionic interactions) which maintain the native, secondary and tertiary structures of enzymes

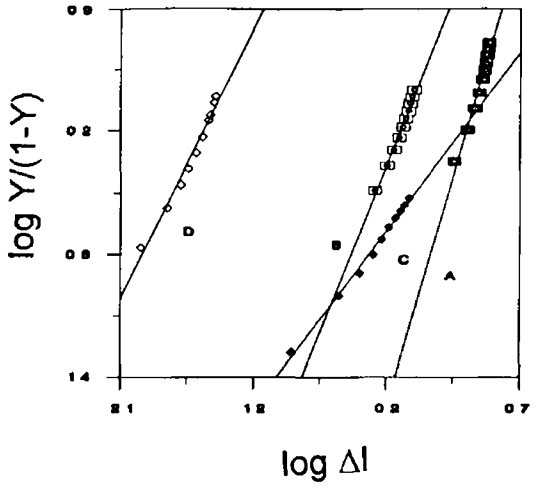


Figure 5 25 Hill plots for the determination of ethylenethiourea with the Eastman polymer/HRP-modified electrode in acetone (A), methanol (B), acetonitrile (C) and 2-butanol (D) containing 0 5 mM o-phenylenediamine Experimental conditions are as in Figure 5 20

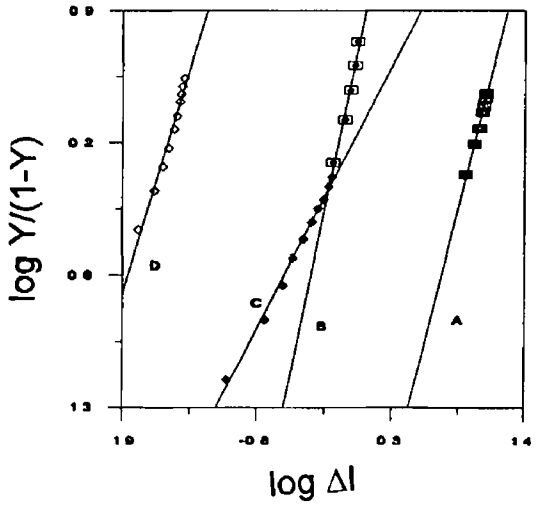


Figure 5 26 Hill plots for the determination of thiourea with the Eastman AQ polymer/HRP-modified electrode in acetone (A), methanol (B), acetonitrile (C) and 2-butanol (D) containing 0 5 mM o-phenylenediamine Experimental conditions are as in Figure 5 21

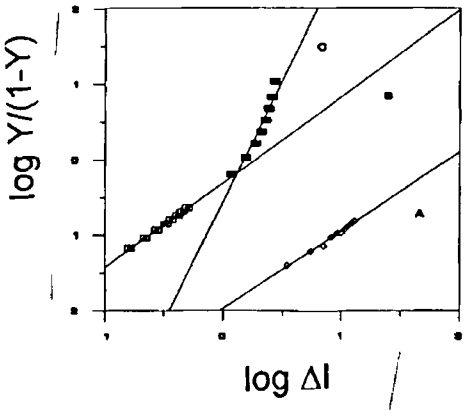


Figure 5 27 Hill plots for the determination of of methyl isothiocyanate with the Eastman AQ polymer/HRP-modified electrode in 2-butanol (A), acetonitrile (B) and methanol (C) containing 0 5 mM o-phenylenediamme Experimental conditions are as in Figure 5 22

Table 5 7 Values of the Hill coefficients for thiourea, ethylenethiourea and methyl isothiocyanate in acetomtrile, acetone, methanol and 2-butanol Experimental conditions are as in Figures 5 25-5 27

Solvent	**Hill coefficient (x)**		
	MeSNC	**THU**	**ETU**
methanol	3 19	3 2	2 2
acetone	-	2 7	3 1
2-butanol	1 04	2 2	1 8
acetonitrile	1 13	1 3	1 2

5.1.3.2.2. *Detection of diethyldithiocarbamate*

Figure 5 28 shows the current-time recording as obtained during successive additions of 5 μM aliquots of DEDTC with the Eastman AQ polymer/HRP-modified electrode Steady-state signals were obtained in < 30 seconds in all experiments It is essential that the inhibitor-detecting biosensor operates at conditions at which only the enzyme kinetics is limiting Therefore, the effect of varying saturation concentrations of phenol on the performance of the biosensor in detecting DEDTC was studied Table 5 8 shows the values of I_{max}/K_i' obtained at phenol concentrations between 80-150 μM These values indicate that the optimal catalytic performance of the biosensor was obtained in the presence of 100 μM phenol Hence, all subsequent experiments were performed with 100 μM phenol

Table 5 8 Values of K_i' for DEDTC detection at varying concentrations of phenol

[PHENOL], μM	I_{max}/K_i', μA/μM
80	0 2
100	0 3
120	0 1
150	0 1

Figure 5 29 shows the calibration curves for DEDTC detection when catechol, phenol, p-cresol and m-cresol are used as enzyme substrates Linear responses for the biosensor extended up to DEDTC concentrations of 15, 10, 10 and 25 μM in the presence of each of the substrates as listed above, respectively The I_{max}/K_i' values (shown in Table 5 9) indicates that the highest catalytic performance of the biosensor was obtained when m-cresol was used while the lowest performance was in the presence of p-cresol The catalytic efficiency of peroxidase-catalysed oxidation of phenols, has been shown to decrease as the substrate hydrophobicity increases [8] In terms of hydrophobicity, catechol > phenol > p-cresol > m-cresol The trend of the sensitivity values for catechol, phenol and p-cresol is in agreement with this prediction However, the significantly higher sensitivity m the presence of m-cresol may be attributed to favourable steric and electronic characteristics, for the formation of the tyrosinase-DEDTC complex

Table 5 9 Values of the apparent inhibition constant and Hill coefficient for the detection of DEDTC in the presence of different types of phenols Experimental conditions are as in Figure 5 28

SUBSTRATE	I_{max}/K_i', μA/μM
CATECHOL	1 5
PHENOL	1 2
p-CRESOL	1 1
m-CRESOL	1 9

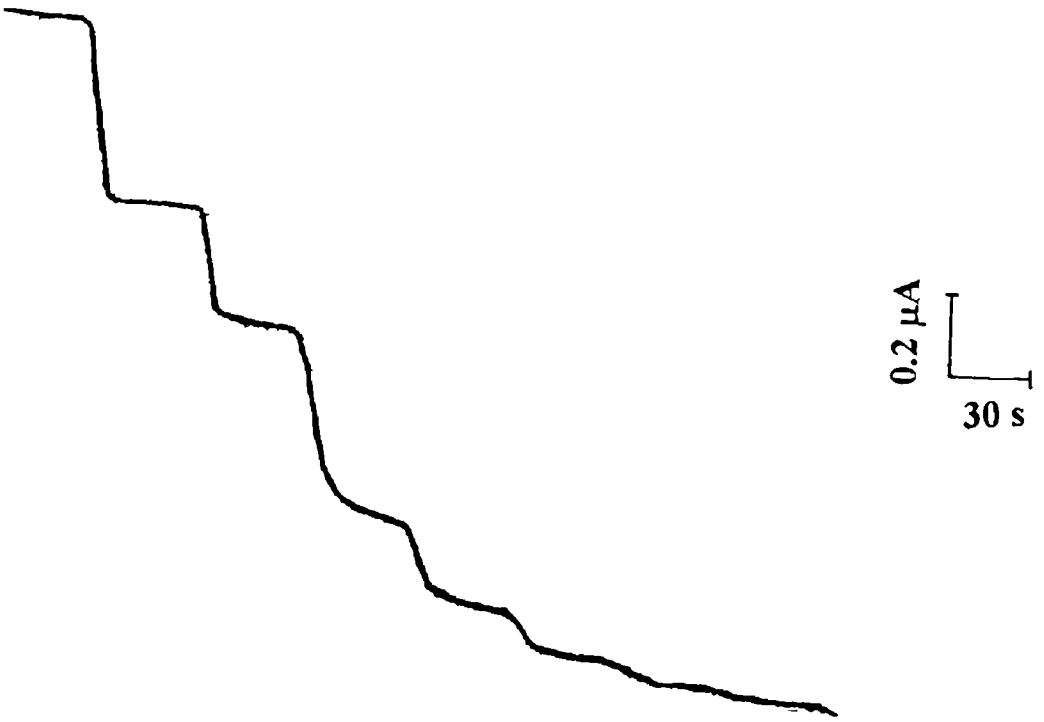


Figure 5 28 Current-time recording as obtained during successive additions of 5 µM aliquots of DEDTC with the Eastman AQ polymer/HRP-modified electrode The experiment was performed in 2-propanol containing 20 % v/v water, 0 1 M TEATS and 100 µM phenol The working potential was -200 mV vs SCE

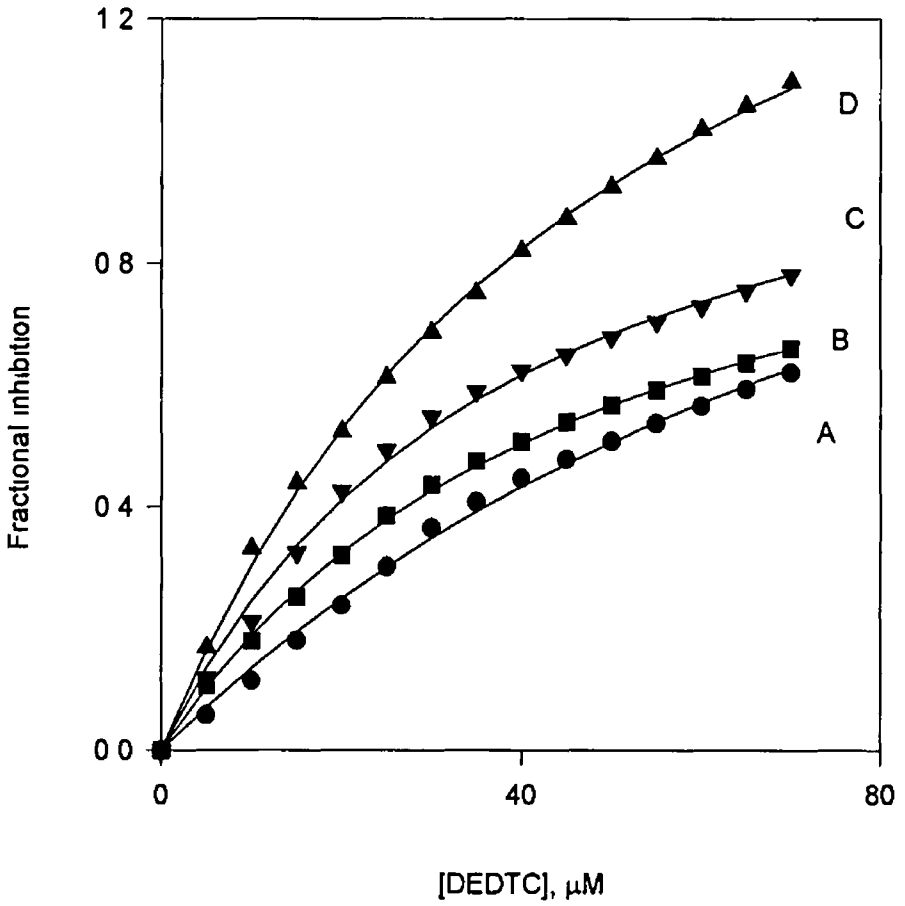


Figure 5 29 Calibration curves for DEDTC in the presence of 100 μM m-cresol (A), p-cresol (B), phenol (C) and catechol (D) Experimental conditions are as described in Figure 5 28

5.2. SPECTROELECTROCHEMISTRY

5.2.1. EFFECTS OF ORGANIC SOLVENTS ON HRP ACTIVITY

The spectral absorbance of native HRP at 403 nm is known to be a measure of its activity [26, 27] Similarly, the physico-chemical changes in the enzyme active site in the presence of organic solvents can be followed by momtoring shifts in the wavelength and changes in the intensity of the absorbance associated with the heme content of the enzyme The changes m absorbance at 403 nm (i e the Soret region) were studied m 100 % aqueous buffer, 50 %, 60 %, 75 % and 90 % acetomtrile, methanol and acetone at a fixed potential of -250 mV The organic solvents were made up to 100 % with 0 025 M phosphate buffer, pH 7 05 Figure 5 30 shows the spectra of HRP m 100 % aqueous phosphate buffer, 60 % methanol and acetonitrile, and 90 % acetonitrile The absorbance intensity m 60 % methanol and acetonitrile decreased by ca 90 % and 76 %, respectively There was no absorbance at this wavelength in 50-90 % acetone, 90 % methanol (spectra not shown) and 90 % acetonitrile, thus indicating a total loss of the enzyme's activity at these concentrations of organic solvents In 50 % methanol and 60 % acetomtrile, there was no shift m λ_{max}, however m 60 % methanol there was a shift in λ_{max} to 420 nm There was also some peak broadening in methanol with little or no such effect m acetomtrile These changes may be due to a solvent-induced exposure of the enzyme active site to the reaction medium This exposure may lead to the dissociation of the active site heme, dimerization of the dissociated free heme and/or other

physico-chemical interactions Previous studies [8], based on electron paramagnetic resonance (EPR) spectral changes, have shown that methanol changes the spin state of the native HRP to a low-spin iron(III) and acts as a ligand to the haem iron in HRP In the same study, acetonitrile was believed to form a cyano-complex with the enzyme

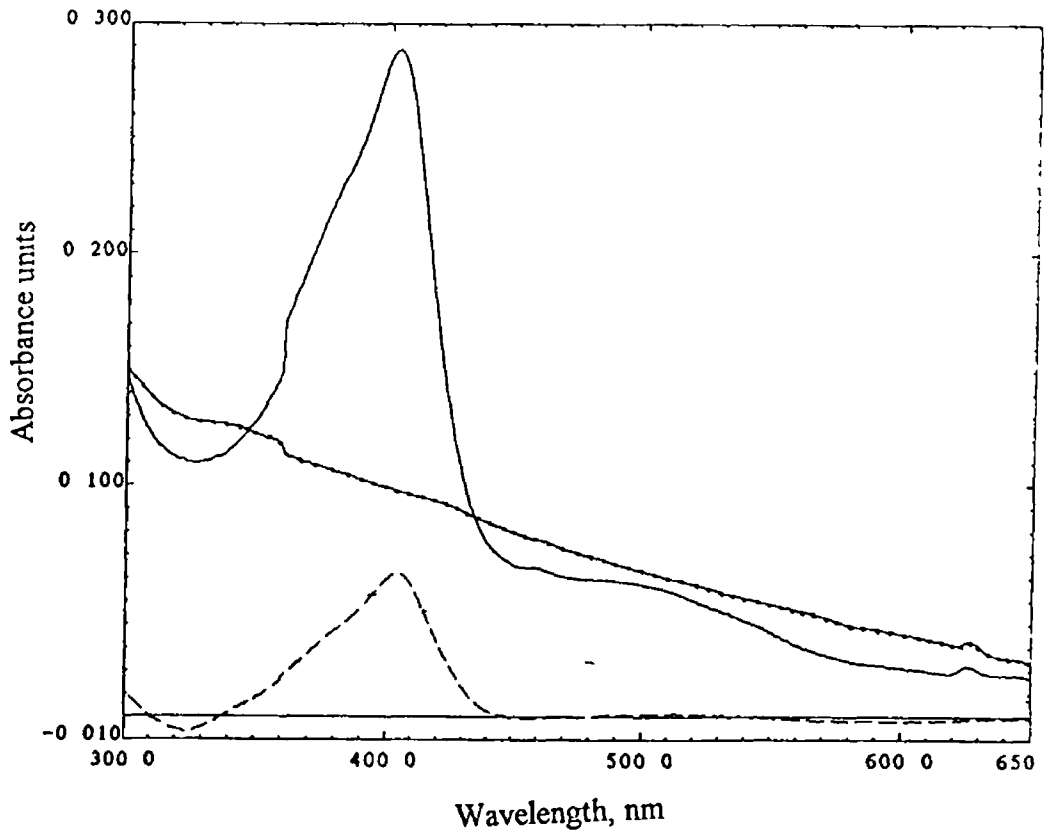


Figure 5 30 The absorption spectra of horseradish peroxidase (1 mg/ml) in 100 % phosphate buffer 0 05 M, pH 7 05 (——), 60 % acetonitrile (-), 60 % methanol (---) and 90 % acetonitrile (.....) The applied potential was -250 mV

5.2.2. EFFECTS OF INHIBITORS ON HRP ACTIVITY

The spectral changes following polarisation of the electrodes at -250 mV was also monitored in aqueous media after consecutive additions of the mediator, o-phenylenediamine (o-PEDA), the substrate, BTP, and the inhibitors, THU and ETU as presented in Figure 5 31 In the presence of o-PEDA (spectrum not shown), there was a reduction in the absorbance intensity at 403 nm However, on addition of BTP, there was in addition to the reduction in absorbance intensity, a broadening of the spectrum from ca 370 nm to 550 nm On addition of ETU and THU, the absorbance intensity around this region again increased with no change to the peak broadening effects There is a wide speculation [28, 29] that sulphides and compounds containing the S^{2-} group act as HRP inhibitors by serving as a reductant for the peroxidase compounds I and II The increase in absorbance intensity in the Soret region (i e λ_{max} around 400 nm) when ETU and THU were added as observed in this study implies the reduction of the oxidised forms of the HRP (i e compounds I and II) in the presence of these inhibitors Table 5 10 shows the values of λ_{max} and the absorbance values obtained for the various systems

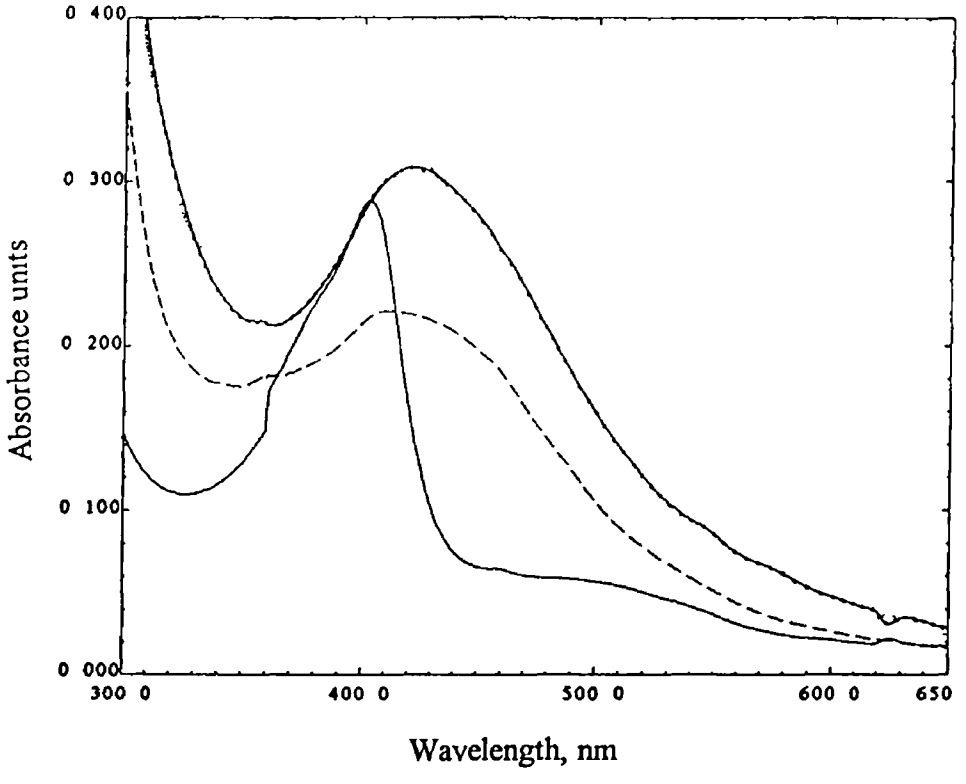


Figure 5 31 The absorption spectra of HRP (1 mg/ml) in 100 % phosphate buffer (—), HRP after addition of 0 5 mM o-PEDA and 1 mM BTP (----), HRP containing 1 mM BTP and 1 mM ETU (++++), HRP containing 1 mM BTP and 1 mM THU (· ·) The applied potential was -250 mV

Table 5 10 The values of λ_{max} and absorbance obtained for HRP-mediator, HRP-substrate and HRP-inhibitor interactions

System	λ_{max}, nm	Absorbance	Remarks
HRP	403	0 29	sharp peak
HRP+o-PEDA	403	0 21	sharp peak
HRP+o-PEDA+BTP	410	0 22	broad peak
HRP+o-PEDA+BTP+ETU	420	0 31	broad peak
HRP+o-PEDA+BTP+THU	410	0 32	broad peak

5.3. CONCLUSIONS

Organic-phase peroxide and phenol sensors have been presented A simple immobilisation technique, which involves entrapment of the respective sensing enzyme i e HRP and tyrosinase, within a cation-exchange polymer (Eastman AQ polymer) was employed The peroxide sensors were an o-PEDA-based, DMFc-based, and reagentless sensor, while the phenol sensor was reagentless The values of the kinetic

parameters, K_m', I_{max} and I_{max}/K_m' were evaluated in polar, water-miscible organic solvents namely, acetonitrile, methanol, acetone, THF and 2-butanol In particular, it has been demonstrated that the catalytic performance of these sensors can be greatly affected by the nature of the solvent and its water content

It is generally accepted that (i) water is required for enzymatic catalysis (ii) hydrophilic solvents strip the enzyme's active site of water, thereby inactivating the biocatalyst (iii) the amount of water required for an enzyme to function in non-aqueous media needs to be determined [32-34] The o-PEDA-based peroxide sensor presented in this study, was active in 100 % acetonitrile Although its sensitivity improved by a factor of 13 in the presence of 3 % water In contrast, spectroelectrochemical studies show that HRP dissolved in 90 % acetonitrile did not show any significant catalytic activity In line with previous studies [35, 36], these results indicate that enzyme fixation helps to retain the required active site water, for enzymes to function in polar organic solvents Therefore, a careful choice of immobilisation material and method is crucial in the design and development of organic-phase enzyme electrodes

The trend of the sensitivity values obtained for the o-PEDA-based peroxide sensor in acetonitrile, acetone and methanol was different from that of the DMFc-based sensor While a similarity was observed in the trend of the sensitivity values obtained for the o-PEDA-based peroxide sensor and the phenol sensor The diffusion coefficients of phenol in acetone, acetonitrile and THF were correlated to the biosensor sensitivities However, the differences in the diffusion coefficient values

in these solvents were considered too small to alone account for the sensitivity variations as observed in these solvents Therefore, the sensitivities of these sensors have been correlated to some physical properties of these solvents namely polarity, hydrophobicity, dielectric constant and kinematic viscosity In a recent study [37], Klibanov and co-workers accepted that solvent hydrophobicity (and therefore log P values) does not explain adequately the effects of the nature of organic media on enzymatic catalysis Indeed this study advocated for parameters that would take into account the solvent-enzyme interactions and the solvent dielectric constant This thesis introduces a new parameter, $1/\varepsilon\eta$, which takes into account solvent effects on the enzyme's non-covalent interactions and the diffusion of the analytes $1/\varepsilon\eta$ was better than log P in predicting the sensitivities of these biosensors, in 2-butanol relative to the other solvents However, both $1/\varepsilon\eta$ and log P failed to account for the low sensitivity of both sensors in THF relative to acetone This deviation is attributed to specific interactions that occur between the studied enzymes and THF

The sensitivities of the DMFc-based sensor were generally higher than those of the o-PEDA-based and reagentless sensor The low K_m' values of the DMFc-based sensors indicate a relatively more favourable HRP-BTP reaction kinetics compared to that obtained in the other two sensors This feature has been attributed to hydrophobic interactions/partitioning between the mediator, the immobilisation material and the substrate, BTP

Inhibition biosensors for the detection of thiourea, ethylenethiourea, mercaptoethanol, methyl isothiocyanate, hydroxylamine and diethyldithiocarbamate, were also presented The peroxidase-based biosensor was used to detect the first five analytes as shown above In a similar way, the tyrosinase-based biosensor was used to detect diethyldithiocarbamate The values of K_m' and I_{max} for the peroxide and phenol biosensors, that were obtained in the absence of inhibitors were compared to those obtained in their presence The differences in these values were used to explain enzyme-substrate interactions of the biosensors Also, these values indicated that the inhibitory actions of these compounds is a mixed-effects type of inhibition mechanism This is in agreement with other studies of this type [5, 38] The mixed-effects inhibition mechanism implies that enzyme inhibition as exhibited in this study by thiourea, ethylenethiourea, mercaptoethanol, methyl isothiocyanate, hydroxylamine and diethyldithiocarbamate, are not completely reversible inhibitions

The inhibition biosensors were studied in acetonitrile, acetone, methanol and 2-butanol The kinetic parameters, K_i', I_{max}/K_i' and the Hill coefficients were evaluated Also, the trend of the sensitivity values of the biosensors and their respective deviations from Michaelis-Menten kinetics was different for the analytes The spectroelectrochemical study gives some indication to the possibility of the HRP-inhibitor interactions being a redox type of reaction

These studies have in general revealed the complexity of organic-phase biosensor behaviour, with multiple factors competing for the influence of

the catalytic performance Variations in the kinetic parameters of the presented biosensors with, the nature of the organic media, nature and type of mediation employed and the nature of the analytes, shows the importance of the enzyme-analyte, enzyme-mediator and the enzyme-solvent binding on the performance of the biosensor This is further complicated by other factors such as the steric bulk, hydrophobicity, charge in the organic medium, the mediators redox potential as well as the overall influence of the immobilisation material and/or procedures However, there is a broad indication that a careful manipulation and engineering of the wide variety of organic solvents [39], enzymes[40], mediators and immobilisation materials can be used to design biosensors to meet specific industrial and analytical requirements

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CHAPTER SIX: HIGH PERFORMANCE LIQUID CHROMATOGRAPHIC ANALYSIS (HPLC) OF PHENOLIC COMPOUNDS

6.1. INTRODUCTION

Chromatography was first described in 1903 by Tswett [1], and has since developed as a very important analytical technique It presently exists in several forms such as column liquid, ion-exchange, size exclusion, affinity and gas chromatography as well as electrophoresis [2] Chromatographic separations generally make use of two immiscible phases, one phase is held stationary while, the second phase moves under gravity, pressure or by capillary action past the stationary phase and is referred to as the mobile phase A multi-component mixture, when introduced into this system is carried with the mobile phase However, the components will be partially retained dependmg on their interaction with the stationary phase, such that small differences in their distribution coefficients will cause them to be separated The extent of interaction between the analyte and the stationary phase is characteristic of the individual compound Therefore, retention properties are used to identify the components of a mixture by comparison with pure standards An instrumental method of detection is usually linked to chromatography to increase reproducibility and sensitivity Hence, present day chromatographic techniques serve as separation as well as analytical methods

The stationary phase can be either a solid or a liquid placed in a column or over an inert support The mobile phase can be a gas, a liquid or a supercritical fluid Hence, analytical chromatographic methods are broadly divided into two main groups dependmg on whether the mobile phase is a gas (gas chromatography) or a liquid (liquid chromatography)

Liquid chromatography (LC) is the earliest form of chromatography The distribution and retention of an analyte when using this technique may result from partition, ion-exchange, size exclusion, or adsorption onto a solid stationary phase Liquid chromatography is usually subdivided into liquid-solid and liquid-liquid chromatography In normal phase LC, the stationary phase is relatively polar and the mobile phase relatively non-polar In contrast, reversed-phase LC uses a non-polar stationary phase and a polar mobile phase

High performance liquid chromatography (HPLC) simply describes an experimental technique in which the efficiency of classical liquid chromatography is improved It involves the use of stationary phase materials with small particle sizes, usually between 3-10 μm These small-sized particles, when packed into a column impart a considerable resistance to solvent flow, such that a high pressure is required to pump the mobile phase through the column HPLC can often easily achieve separations and analyses that would be difficult or impossible using other forms of chromatography It has been described [3] as the most powerful of all chromatographic techniques

6.1.1. HPLC INSTRUMENTATION

The instrument used for HPLC experiments is called a high performance liquid chromatograph It comprises several individual components which are often obtained separately and then linked together using connecting tubes and valves (see Figure 6 1) The major components are the mobile phase, a high presure pump, an injector, a column pre-packed with the

stationary phase, a detector, and a recorder/integrator to display the detector signals

6.1.1.1. *MOBILE PHASE*

The mobile phase (also called eluent) is usually kept in 1L glass bottles i e the reservoir The caps of these bottles have holes drilled into them through which a poly(tetrafluoro-ethylene) (PTFE) tube is fitted to carry the mobile phase to the pump The mobile phase is a significant parameter in the design of HPLC experiments This is because its polarity and properties can be easily manipulated to improve the efficiency of separations Water, buffers and/or organic solvents, in varying proportions, are normally used as the eluent to achieve the required separation The choice of organic solvents for mobile phase depends on the type of separation desired For instance, non-polar organic solvents are usually the choice for normal phase HPLC, while relatively polar organic solvents are used for reversed-phase separations

6.1.1.2. *HIGH PRESSURE PUMP*

Analytical HPLC separations at flow rates between 0 1-10 ml/min require pressures of up to 600 psi In addition, the pump flow rate must be highly reproducible to give reliable retention times These requirements are satisfied by pumps of different designs such as pressure amplication, syringe, diaphragm and reciprocating pumps However, the latter two designs are the most commonly used types

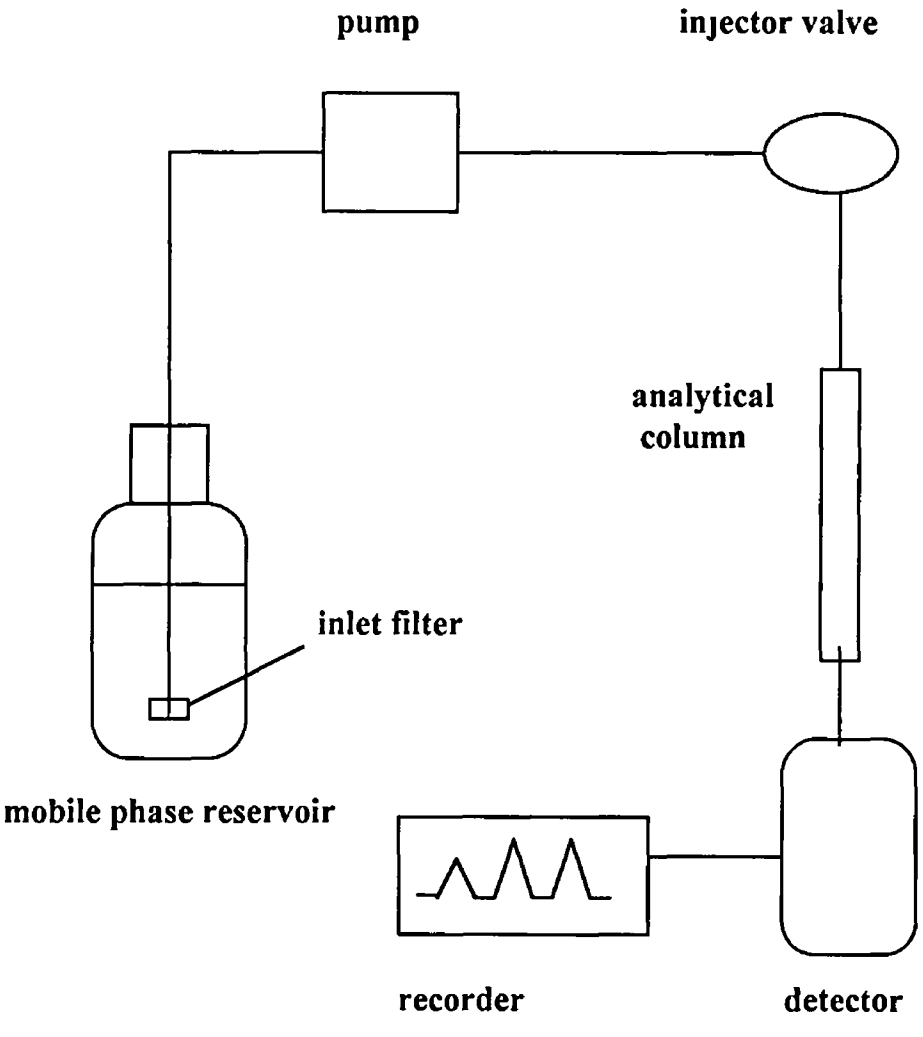

Figure 6 1 Schematic diagram of a high performance liquid chromatograph

6.1.1.3. *INJECTION UNIT*

This is simply the unit for introducing samples onto the column The most poplular type is the six port valve injector system It is easy to use and has a high level of precision The injector loop volume HPLC systems varies from 10 to 50 µl

6.1.1.4. *COLUMN*

Dependmg on the mode of application, there are three types of HPLC columns These are the pre-column, post-column and the analytical column The pre- and post- columns are employed either to remove impurities/interferences in the sample or used in derivative preparation Hence, they improve the separation and/or detection efficiency in an analysis The analytical column is where the desired separation takes place The pre-column is normally placed between the injector and the analytical column, while the post-column is placed between the analytical column and the detector HPLC columns must be able to withstand the applied pressures in addition to being chemically resistant to the mobile phase solvents Therefore, they are usually made of stainless steel, glass or plastic A typical analytical column is 10-25 cm long and 0 30-0 50 cm in internal diameter

The type of stationary phase material packed into the column also depends on the type of separation desired i e normal or reversed-phase separation Normal phase separations are carried out with either silica gel or neutral alumina as the stationary phase (silica gel being the most

common) In contrast, reverse d-phase separations are usually carried out with hydrocarbonaceous alkyl-bonded silica particles The most popular of these materials is the octadecylsilyl (ODS or C_{18})-substituted silica In addition to hydrocarbons, other chemical groups such as cyano, nitro, diol and amino groups can also be bonded to silica Stationary phases of this type can often be used in either normal or reversed-phase modes However, the pH of the mobile phase used with stationary phases containing a silica gel matrix is usually limited to a pH range of 2 5 to 8 This is because of the instability of silica at extremely low and high pH levels This has led to the development of polymer stationary phases such as crosslinked polystyrene-divinylbenzene and polyacrylates These can be used over a wide mobile phase pH range of 1 to 13

6.1.1.5. *DETECTORS*

These are devices that are used to monitor changes in the concentration of the mobile phase emerging from the column i e the column efluent The changes in the concentration are converted into observable electrical signals The detector is the most essential component during analytical HPLC experiments It must be unaffected by small changes in the operating conditions such as flow rate or column temperature, it must be compatible with a wide range of mobile phases, cheap, reliable and easy to use The performance of a detector is usually assesed using the following parameters

sensitivity the ratio of the detector signal to the analyte concentration, it is desirable that

the detector gives a high signal for a small amount of analyte,

linear range — the concentration range of the analyte in which the detector response is directly proportional to the amount of the analyte, the wider the linear range the better the detector performance,

dead volume — the dead volume of the detector includes its cell volume and the length/bore of associated tubings, a high dead volume in the detector gives rise to extra column dispersion and therefore must be kept to a minimum

A large number of devices have been used as detectors in HPLC However, the most commonly used types are ultraviolet-visible spectroscopy, fluorescence spectroscopy, refractive index, conductivity, and amperometry [2-4] The performance characteristics of these detectors are shown in Table 6 1 Other forms of detection systems include infrared and mass spectrometry, vapour phase nephelometry, flame ionisation and more recently enzyme-based biosensors The main interest in this thesis was the use of the latter type of detection system Therefore, the use of enzyme-based biosensors as HPLC detectors are now discussed in greater detail

6.1.1.5.1. *Enzyme-based biosensors as HPLC detectors*

Enzyme-based biosensors have been employed as detectors for HPLC and indeed other analytical flow methods such as flow injection analysis (FIA) in either of two ways (i) as immobilised enzyme reactors and (ii) as enzyme-based amperometric electrodes

Immobilised enzyme reactors (IMERs) are prepared by immobilising the sensing enzymes on inert supports such as liquid chromatography silica or polymer matrices The enzyme-embedded support is then packed into a stainless steel or glass column, which is operated either as a pre-column or post-column during HPLC experiments IMERs are described as catalytic reaction or chemical derivitisation detection systems This is because the detection principle is based on a reaction between the immobilised enzyme and the analyte(s) to give products that are more easily detected Hence, IMERs are essentially employed to aid the sensitivity and selectivity of the conventional detection systems, i e UV-VIS spectroscopy, amperometry, fluorescence, refractive index and conductivity [5] However, Heller and coworkers [6] in a recent study described the use of oxidase-based IMERs with peroxidase-modified-electrodes as detectors Immobilised glucose oxidase, lactate oxidase and acetylcholine esterase in the reactors reacted with their respective substrates, i e the analytes, to give hydrogen peroxide The produced hydrogen peroxide was then detected with an osmium polymer "wired" peroxidase electrode The analytes were glucose, lactate and acetylcholine/choline, respectively The sensitivity and detection limit of the reported enzyme electrode in this study, increased by magnitudes of

Table 6 1 Performance characteristics of detectors used in HPLC analysis [4]

Characteristics	UV-visible absorption	Fluorescence	Refractive index	Conductivity	Amperometry
Response	selective	selective	[a]universal	selective	selective
Sensitivity, g/cm³	10^{-8}	10^{-12}	10^{-6}	10^{-7}	10^{-10}
Linear range, g/cm³	10^{4}-10^{5}	10^{3}-10^{4}	10^{3}-10^{4}	10^{3}-10^{4}	10^{4}-10^{5}
Typical flow cell volume	1-8	8-25	5-15	1-5	0 5-5
[b]*Noise level*	10^{-4} a u	10^{-7} a u	10^{-7} r i u	10^{2} μS/cm	0 1 nA
[c]*Usage, %*	78	31	37	21	15

a there must be a difference between the refractive index of the analyte and that of the mobile phase

b full meaning of the units are a u = absorbance, r i u = refractive index units, nA = nanoamperes

c usage means the proportion of HPLC operators who use each kind of detector The total is greater than 100 % because many operators use more than one type of detector Data obtained from reference [3]

2-10, when compared to a conventional platinum amperometric electrode Other examples of the applications of IMERs are shown in Table 6 2

Table 6 2 Examples of components analysed by the use of IMERs pre- or post- liquid chromatographic separation

Analyte	*Enzyme*	*Detection method*	*Reference*
Nicotinamide coenzymes	glucose-6-phosphate dehydrogenase	Amperometry	[7]
Oligosaccharides	amylglucosidase	Electrochemical	[8]
Glycerol	glycerol dehydrogenase	Fluorescence	[9]
Acetylcholine Choline	acetylcholine esterase	Amperometry	[10]
Zinc	carboxypeptidase A	UV-VIS	[11]

The use of enzyme-based amperometric electrodes as detection units in HPLC has a wide application potential, and is generating more interest Enzyme-based electrodes offer a sensitivity that is comparable to those of IMERs, but at cheaper costs (since there is no need for post-columns and an extra detector) These electrodes operate in special electrochemical cells called thin-layer or flow cells A typical thin-layer cell consists of two basic units, the working electrode unit and a connection block unit The working electrode may be glassy carbon,

carbon paste or metal (i e platinum, gold) electrodes The unit usually consists of two separate electrodes embedded in solvent resistant plastic materials The two electrodes may be made from the same or different materials The electrode surface(s) is subsequently modified with the sensing enzyme(s) and the immobilising agents The connection block is usually made of stainless steel It contains the reference and counter electrodes, and in addition the column effluent flows through it The working electrode unit is mounted and adjusted, so that its surface is level with that of the connection block The column effluent passes through a pipe into the connection block, in a direction that is perpendicular to the centre of the electrode surface It then passes the electrodes and flows toward another cylmdenical layer, where the outlet is connected A typical thin-layer amperometric cell is shown m Figure 6 2

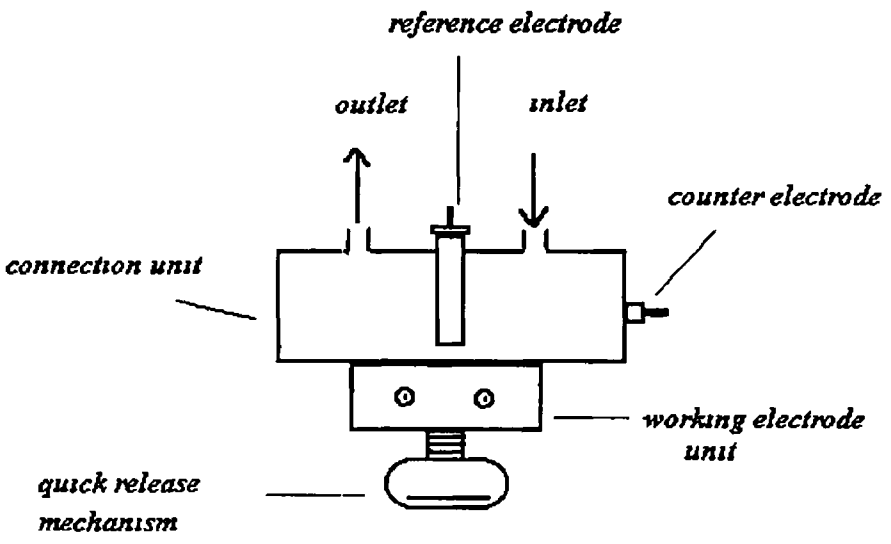

Figure 6 2 A thin-layer/flow amperometric cell

Yao and Wasa [12] reported the application of L- and D- amino acid oxidase-modified glassy carbon electrode to the HPLC analysis of L- and D- isomers of several amino acids The amino acids detected included methionine, tyrosine, leucine, phenyalanine, valme, proline, histidine and arginine The detection limit of the first four acids was 2 pM The detection did not require any form of complicated sample pretreatment In a similar way, Nordlmg *et al* [13] reported on a dual-electrode detection system for the simultaneous determination of glucose and soluble cellodextrines after LC separation The cellodextrins detected were cellobiose, cellotriose, cellotetraose and cellopentaose The sensing enzymes, glucose oxidase and cellobiose oxidase, were immobilised by "wiring" with an osmium polymer onto glassy carbon electrodes

Marko-Varga and coworkers [14] used carbon paste electrodes modified with alcohol oxidase and horseradish peroxidase as HPLC detectors for methanol and ethanol The enzymes were covalently immobilised onto carbon paste, which was then packed into a plastic tube and inserted mto the thin-layer cell The developed detector was used to monitor ethanol produced from paper pulp industrial waste water after fermentation with *Saccharomyces cerevisiae* The results obtamed were comparable with those obtained with a refractive mdex detector In another study [15], the same group described the use of phenol oxidase-based biosensors as detection units in LC for the determination of phenolic compounds The best sensitivity and stability was obtained when the enzyme was immobilised by covalent coupling and cross-linking with glutaraldehyde on solid graphite electrodes Catechol, phenol and p-cresol were detected

with this biosensor after their reversed-phase separation on a C_{18} bonded silica analytical column The phenol oxidase-modified graphite electrodes exhibited better selectivity compared to the UV detector in detecting the analytes as listed above

The ability of immobilised enzymes to retain catalytic activity in polar organic solvents (as discussed in chapters 4 and 5) has generated renewed interests in the application of enzyme-based amperometric electrodes as detectors in reversed-phase HPLC This is because the mobile phase in reverse-phase HPLC is usually a polar organic solvent such as acetonitrile and methanol in combination with water, or in some cases buffers This section of the thesis reports on the development of a reversed-phase HPLC separation of phenolic compounds using binary mixtures of methanol and water, and their subsequent detection with a tyrosinase-osmium polymer-modified glassy carbon electrode The selectivity and sensitivity of the enzyme-based biosensor detection system was compared with a UV detector for the determination of phenolic compounds in cigarette smoke

6.2. HPLC ANALYSIS OF PHENOLIC COMPOUNDS

6.2.1. OPTIMISATION OF CHROMATOGRAPHIC SEPARATION

The effects of operating conditions such as the column packing, temperature, or more importantly the mobile phase composition, on the

retention property of an analyte is usually measured in terms of the capacity factor, k', value k' is defined as the ratio of the adjusted retention time of the analyte (t_R') to that of the mobile phase (t_M) [4]

$$k' = (t_R - t_M)/t_M = t_R'/t_M \qquad (6.1)$$

where t_R is the time after injection, for the peak maximum of a analyte, to be separated i e the unadjusted retention time The capacity factor is independent of the mobile phase flow rate or the physical dimensions of the column Its values are usually between 1 and 10 to achieve adequate resolution and reasonable analysis time

Table 6 3 shows the capacity factor values for catechol, phenol, p-cresol and p-chlorophenol with different mobile phase compositions These values indicate that the most favourable k' values were obtained with binary mixtures of 30-50 % methanol and 70-30 % water The mobile phase containing 60 % methanol gave high retention times, but there was a poor resolution between the analyte peaks However, resolution increased as the amount of water in the mobile phase increased The substitution of 50 % methanol with 50 % acetonitrile resulted in shorter retention times with very little resolution between the analyte peaks This indicates that acetonitrile, compared to methanol, competes effectively with the analytes for occupation of the octadecyl groups on the stationary phase [16], and therefore reduces the amount of interaction occurring between the analytes and the stationary phase

Table 6 3 Evaluated capacity factor values for catachol, phenol, P-cresol and P-chlorophenol with different mobile phase compositions

SOLVENT	[a]CAPACITY FACTOR VALUES			
	catechol	phenol	P-cresol	P-chlorophenol
acetonitrile water (50 50)	0 4	0 5	0 6	0 7
methanol water (60 50)	0 7	0 9	1 1	1 3
methanol water (50 50)	0 4	0 6	1 0	1 4
methanol water (40 60)	0 4	0 7	1 5	2 3
methanol water (30 70)	0 5	1 2	3 0	4 7
methanol water (50 50), + 10 mM TEATS	0 4	0 6	0 9	1 3
methanol water (50 50), + 25 mM TEATS	0 4	0 6	0 9	1 3
[b]methanol buffer pH 5 (50 50)	0 8	1 0	1 5	2 1
methanol buffer pH 6 (50 50)	0 8	1 0	1 5	2 0
methanol buffer pH 7 (50 50)	0 4 -	0 6	0 9	1 3
methanol buffer pH 8 (50 50)	0 5	0 8	1 1	1 5

a the retention times of the respective organic components was measured as the retention time of the first deviation of the baseline after its injection

b the buffer used m all experiments was 0 025 M phosphate buffer and the pH was adjusted with diluted phosphoric acid and sodium hydroxide

Organic phase amperometric tyrosinase-based sensors have been shown to operate within a pH range of 5-8 [15] and in the presence of some amount of conducting salts, usually tetraalkylammonium salts [17] Similarly, the chromatographic separation of phenols is dependent on the mobile phase pH and the presence of tetraalkylammomum salts [18] Therefore the effects of the mobile phase pH and the presence of 10 and 25 mM tetraethylammomum-p-toluenesulphonate (TEATS) on the retention of the analytes was evaluated (see Table 6 3) The k' values indicate that the addition of 10 and 25 mM TEATS to the mobile phase did not result in any significant change m the retention and resolution of these components Also, there was little variation in the retention of the analytes as the pH was varied The four compounds are all weakly acidic with pK_a values in the range of 9-10 Therefore, at the stated pH values, they are unionized and are expected to undergo hydrophobic interactions with the C_{18} groups on the stationary phase The slightly higher retention times as observed with the mobile phases that had a pH of 5 and 6 may be as a result of decreased electrostatic repulsion between the analytes and the sulphonate groups on the column Methanol/0 025 M phosphate buffer, pH 6 0 mixtures, (50 50 and 30 70) was used as mobile phase in the rest of the experiments

6.2.2. OPTIMISATION OF UV DETECTION WAVELENGTH

The wavelength used for UV detection usually requires a careful selection This is because the wavelength primarily determines both the sensitivity and selectivity of the detector response to a particular analyte Most of the previous studies have reported UV detection of phenols at

either 254 nm [19,20] or 280 nm [21,22] Figure 6 3 shows the response of the UV detector to a mixture of 0 5 mM catechol, phenol, p-cresol and p-chlorophenol at wavelengths between 210 and 300 nm The absorption spectra showed local maxima at 220 nm and at 270-290 nm, with a sharp decrease between 230 and 260 nm The response at 220 nm was higher by a factor of 7-9 than that observed at 280 nm for all the analytes However, in order to avoid eluent absorption (which occurs for most solvents at wavelengths < 230 nm), 280 nm was chosen as the optimum wavelength for UV detection of the phenolic compounds

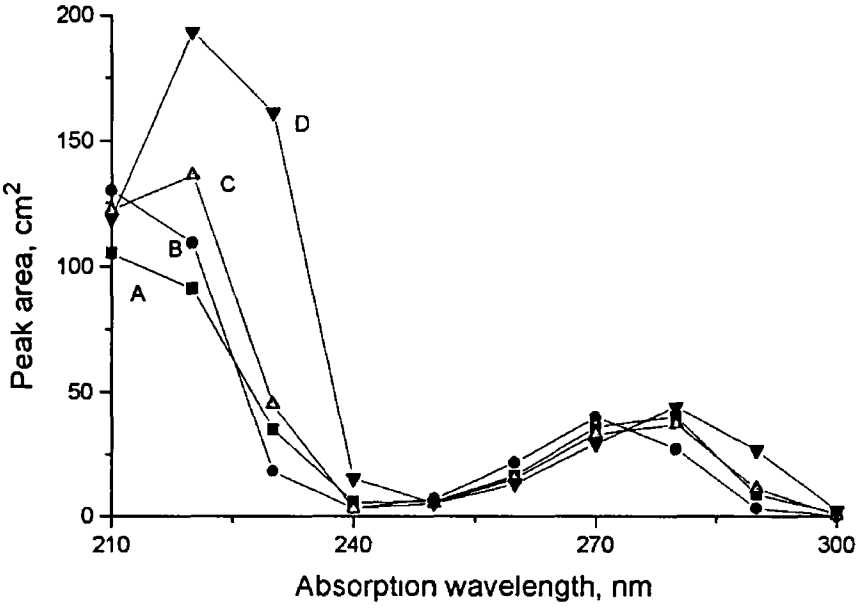

Figure 6 3 Response of a UV detector to 0 5 mM of catechol (A), phenol (B), p-cresol (C) and p-chlorophenol (D) at wavelengths of 210 nm to 300 nm

6.2.3. TYROSINASE-BASED SENSOR FOR THE DETECTION OF PHENOLS

The detection mechanism for the tyrosinase-based phenol sensor varies from one study to the other Nonetheless, they can be generally grouped into three main classes (i) those that detect the consumption of oxygen during the catalytic oxidation of phenolic compounds [23, 24], (ii) those based on detecting o-quinoid products of the enzyme reaction at a cathodically potentiostated carbon electrode (as described in section **5.3.2**), and (iii) those that use redox mediators [25-27] or redox conducting polymers [28] to reduce the o-qumones In this study, tyrosinase was immobilised onto a glassy carbon electrode, with a conducting poly(1-vinylimidazole)-based osmium redox polymer, $[Os(bpy)_2(PVI)_{10}Cl]^{+/2+}$ The polymer is basically a complex of osmium 2,2-bipyridyl chloride, $[Os(bpy)_2Cl]^{+/0}$ and poly(1-vinylimidazole) It is known to combine its mediating functions with being an immobilisation matrix for the enzyme [29] The immobilization process, involves the use of a cross-linking agent, poly(ethylene glycol) (PEG), to link the amino groups of the enzyme with the free imidazole nitrogen of the polymer, resulting in a "reagentless", portable and disposable phenol sensor Poly(vinyl imidazole)-based osmium polymers, compared to the more popular poly(vinyl pyridine)-based ones are water soluble, and therefore do not require modifications to bind easily with water soluble cross-linkers such as PEG [30, 31] In addition the redox potentials of poly(vinyl imidazole)-based osmium polymers are lower by about 80-100 mV Figure 6 3 shows the chemical structure of $[Os(bpy)_2(PVI)_{10}Cl]^{+/2+}$ used in this study Robinson *et al* [32] have

described an enzyme inhibition electrode based on the co-immobilization of this polymer with tyrosinase The electrode was used to detect azide, a potent respiratory poision at concentrations as low as 5 μM

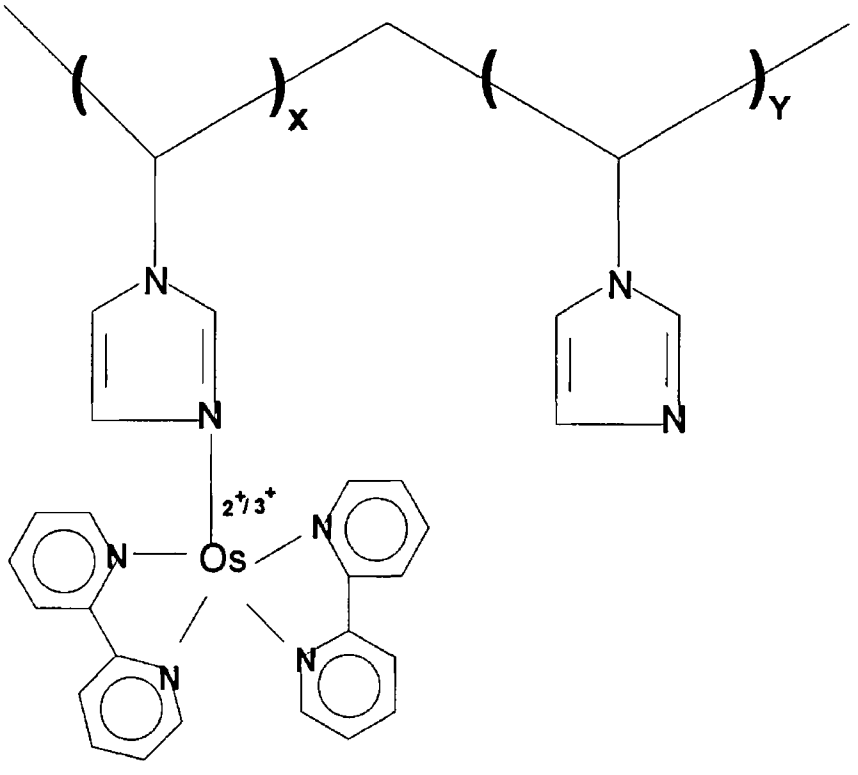

Figure 6 3 Chemical structure of the poly(1-vinylimidazole) and osmium bipyridyl chloride polymeric complex, where x = 1 and y = 10

6.2.3.1. *OPTIMISATION OF ELECTRODE MODIFICATION PARAMETERS*

The most significant variable parameters in the electrode modification process are the concentrations of the osmium polymer and the cross-linking agent, poly(ethylene glycol) glycidyl ether Figure 6 4 shows the effect of changes in the osmium polymer concentrations on the sensitivity of the detector response to p-aminophenol, phenol, p-cresol and p-chlorophenol The sensitivity values are the slopes of the straight-line calibration curves obtained for the analytes The sensitivity of the sensor to p-aminophenol decreased by 22 5 % when the osmium polymer concentration was increased from 0 04 to 0 11 mg/cm^2 This may be due to electrostatic attraction/repulsion of this analyte or its quinoid intermediate with the chain constituents of the redox polymer or with the enzyme-polymer complex In contrast, and as expected, the sensitivity of the sensor for the other three analytes increased as the concentration ratio of the osmium polymer to the enzyme increased Phenol was not detected with an osmium concentration of 0 04 mg/cm^2, while the largest increase of 200 % was observed with p-cresol

The sensitivity of the sensor for all the analytes increased as the concentration of PEG was increased from 0 04 to 0 18 mg/cm^2 (see Figure 6 5) It is believed that at the lower PEG concentration, a poor network structure was formed because the cross-linker was not sufficient to form strong intermolecular bridges between the enzyme molecules [33] However, as the PEG concentration increased, the network structure and its charge-mediating abilities improved, thus leading to

increases in the sensor sensitivity This increase, however, differed significantly between the analytes Phenol p-cresol, p-chlorophenol and p-aminophenol increased by factors of 8, 10, 2, and 1 4, respectively

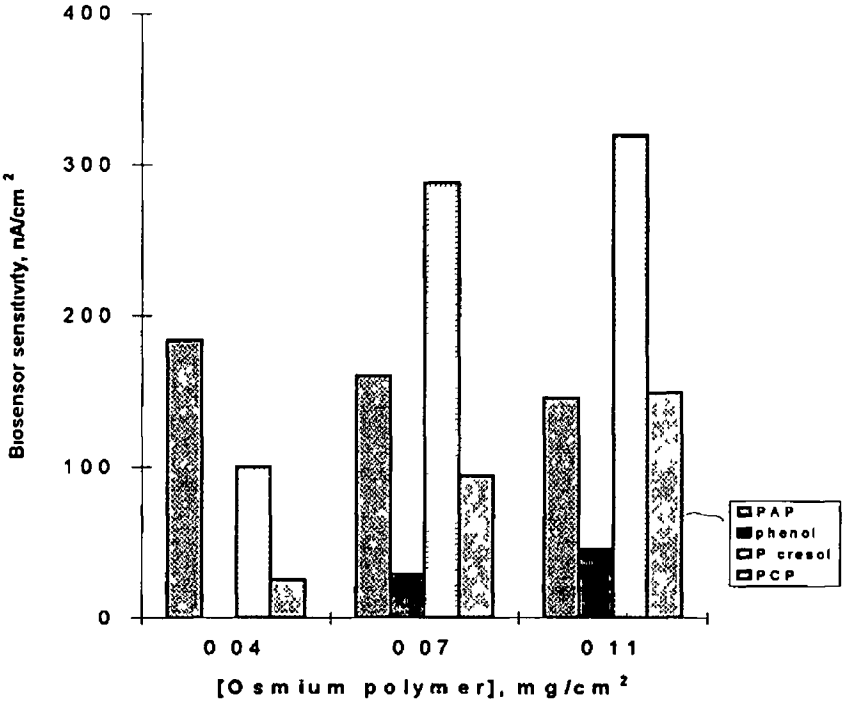

Figure 6 4 The biosensor sensitivities for the detection of p-aminophenol (PAP), phenol, p-cresol and p-chlorophenol (PCP), at different concentrations of the osmium polymer The mobile phase was a binary mixture of methanol and 0 025 M phosphate buffer pH 6 5 (50 50) containing 0 025 M TEATS The mobile phase flow rate was 0 80 ml/min The analytes were separated on 5 μm C18 column The biosensor was a glassy carbon electrode modified with tyrosinase (120 U/cm^2), PEG (0 18 mg/cm^2), and the osmium polymer Experiments were performed at -200 mV *versus* SCE

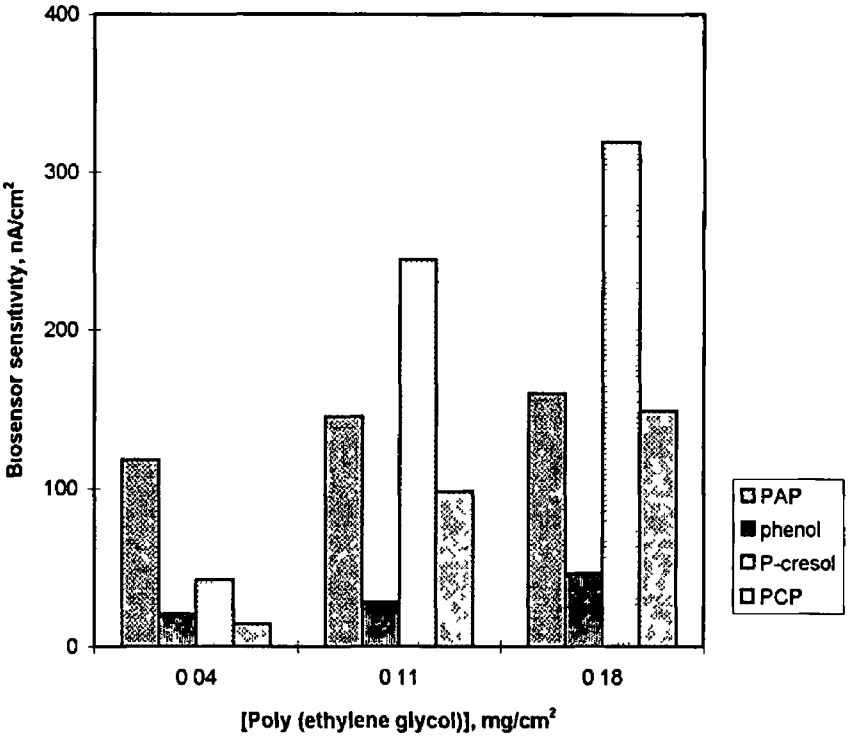

Figure 6 5 The biosensor sensitivities for the detection of p-aminophenol (PAP), phenol, p-cresol and p-chlorophenol (PCP) at different concentrations of poly(ethylene glycol) The osmium-polymer concentration was 0 11 mg/cm^2 Other experimental conditions are as described in Figure 6 4

6.2.3.2. *CHARACTERISTICS OF THE PHENOL SENSOR*

A typical chromatogram as obtained with this sensor, for a 20 µl sample containing 1 µM phenol and p-aminophenol and 0 1 µM p-cresol and p-chlorophenol is shown in Figure 6 6 Similar measurements performed with an osmium polymer/poly(ethylene glycol)-modified electrode (i e in the absence of tyrosinase) did not give any response

The phenol sensor exhibits favourable dynamic properties, and as indicated in Figure 6 6, the analysis time is less than 5 minutes, thus allowing for 15-20 sample injections per hour The response time of the biosensor was estimated by comparing the retention times of the analytes obtained with a UV detector and those obtained with the modified electrodes (Table 6 5) The difference in the retention times was less than 3 seconds for all the analytes If the time the sample plug spends in the connection tubings (linking the UV detector to the modified electrode) is considered, then these values imply that the response time of the phenol sensor was far less than 3 seconds This is a 10-fold improvement over the response time obtained with Eastman polymer/tyrosinase-modified electrodes It can therefore be concluded that the properties of the osmium polymer immobilization matrix results in significantly faster enzymatic redox reactions and electron transfer kinetics

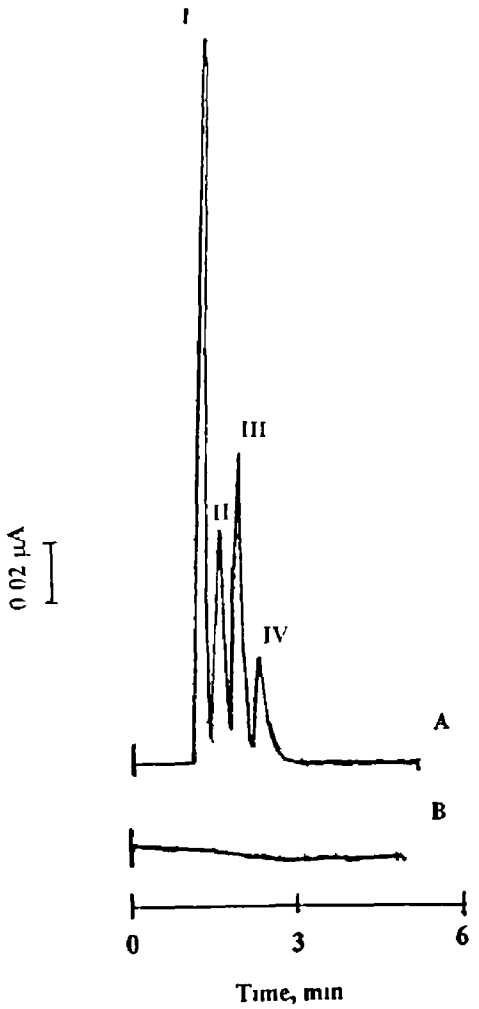

Figure 6 6 A is the response of the biosensor to 1 0 µM p-aminophenol (I), 1 0 µM phenol (II), 0 1 µM p-cresol (III) and 0 1 µM p-chlorophenol (IV) B is the response obtained with the "enzyme-free" osmium polymer/ poly(ethylene glycol)-modified electrode The injected sample volume was 20 µl The electrodes was modified with 0 11 mg/cm^2 osmium polymer, 0 18 mg/cm^2 poly(ethylene glycol), and for the phenol sensing electrode, 120 U/cm^2 of tyrosinase Other experimental conditions are as specified in Figure 6 4

Table 6 5 A comparison of the retention time values of the phenols obtained with a UV detector and the tyrosinase-modified electrode The UV detection wavelength was 280 nm and the mobile phase flow rate was 1 ml/min Other experimental conditions are as described in Figures 6 4 and 6 6

ANALYTE	RETENTION TIMES (t_R), seconds		
	[a]UV detector	*Tyrosinase-modified electrode*	Δt_R
p-aminophenol	72 72	74 4	1 68
Phenol	93 84	96 6	2 76
p-cresol	115 08	117 6	2 52
p-chlorophenol	141 12	144	2 88

a the UV detector retention times were obtained from the computer, based on Beckmann's system gold chromatographic program

6.2.3.2.1. *Effect of mobile phase flow rate on the sensor's response*

The response of the biosensor is also affected by experimental variables, such as the mobile phase flow rate as well as the operating potential In a flow system with a fixed geometry and constant sample volume, the flow rate determines the level of convection and the time of contact between the modified electrodes and the analyte(s) These two factors ultimately determine the sensor response/sensitivity Elmgren *et al* [34] reported that the dependence of biosensor responses on the flow rate depends on the concentration of the analyte as well as the enzyme kinetics Figure 6 7 shows the dependence of the biosensor response to p-aminophenol,

phenol, p-cresol and p-chlorophenol The lower flow rates were found to enhance the sensitivity of the modified elctrode for these analytes except p-aminophenol, which showed an optimum at about 1 ml/min Also, there was significant peak width broadening at the low flow rates, hence a flow rate of 0 8 ml/min was used in all subsequent experiments

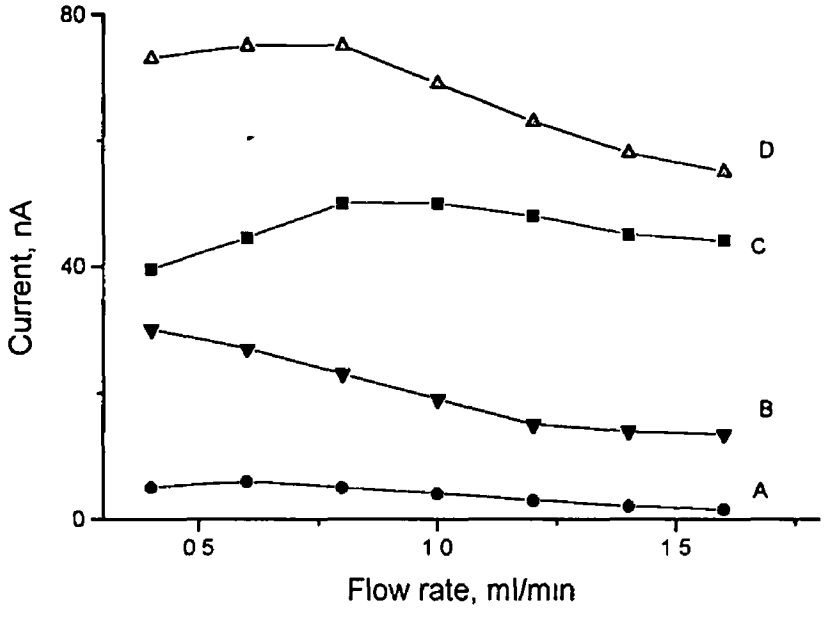

Figure 6 7 The effect of variations in mobile phase flow rate on the response of the tyrosinase/osmium polymer-modified electrode to 0 2 μM phenol (A), p-chlorophenol (B), p-aminophenol (C) and p-cresol (D) Other experimental conditions are as specified in Figures 6 4 and 6 6

6.2 3.2.2. *Effect of the working potential on the sensor's response*

Figure 6 8 shows the effects of the operating potential on the biosensor responses The expected sigmoidal shape was observed with p-cresol, which indicates negligible ohmic distortions The reponses to p-aminophenol increased as the potential shifted to the higher negative values On the contrary, phenol and p-chlorophenol showed an optimum sensor response at -150 mV and -200 mV, respectively Therefore, the working electrode was polarised at -200 mV *versus* SCE for all experiments

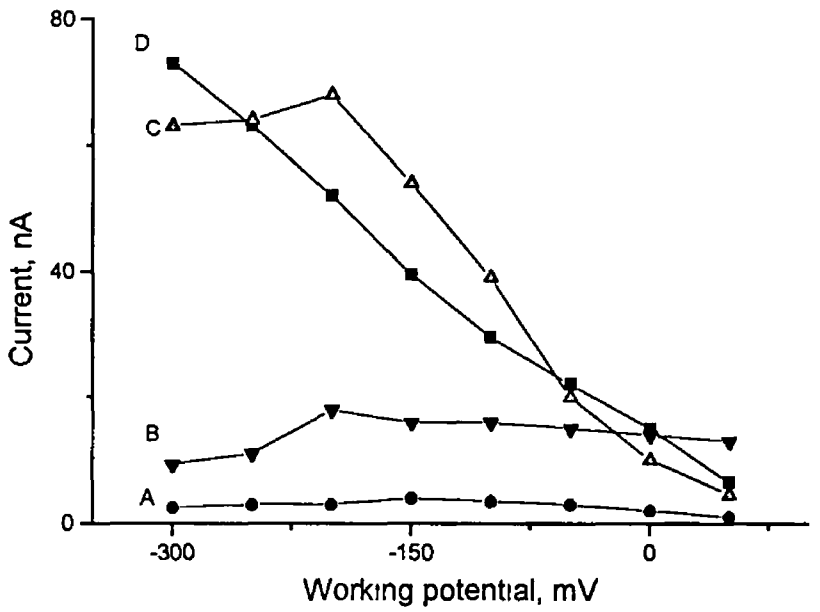

Figure 6 8 The response of the modified electrode to 0 2 μM phenol (A), p-chlorophenol (B), p-cresol (C) and p-aminophenol (D), as the working electrode potential was shifted to the higher negative potentials Other experimental conditions are as described in Figures 6 4 and 6 6

6 2.3.2.3. *Calibration characteristics of the biosensor*

Table 6 6 shows the calibration characteristics of the tyrosinase/osmium polymer-modified glassy carbon electrodes for the named analytes Sensitivity of the sensor for these compounds was measured from the slope of their respective straight-line calibration curves These values indicate that the sensor was most sensitive to p-cresol and least sensitive to phenol The observed sensitivity trend i e p-cresol > p-aminophenol > p-chlorophenol > phenol is similar to that reported for the detection of these compounds (except p-aminophenol) with a tyrosinase-graphite-epoxy biocomposite electrode [35] The selectivity pattern was believed to have been induced by the hydrophobic nature of the immobilization matrix

Table 6 6 Comparison of calibration features for phenolic compounds as obtained with a osmium polymer/tyrosinase-modified electrode Experimental conditions are as described in Figures 6 4 and 6 6

Features	p-aminophenol	Phenol	p-cresol	p-chlorophenol
Sensitivity, nA/ μM	160	46	319	149
Correlation coefficient	0 9974	0 9940	0 9934	0 9968
Detection limit, nM	42	58	4	13
Coefficient of variation (%) for n = 8	1 1	4 5	1 3	11

Favourable signal to noise characteristics allowed detection limits in the range of 4 nM (obtained with p-cresol) to 58 nM (obtained with phenol), the detection limits being the analyte concentrations that would generate a current three times the noise of the background current The precision of the detection was evaluated on the basis of variations in 8 consecutive measurements The coefficient of variation values indicate that the sensor's precision is higher in detecting p-aminophenol and p-cresol While it was considerably lower in detecting phenol and in particular p-chlorophenol, i e the two compounds to which the sensor is least sensitive The lower sensor precision in detecting these analytes may be due to unfavourable electronic and/or steric effects within the enzyme layer

The accuracy of the responses of the phenol detector was validated by its use in the analysis of phenolic compounds in pharmaceutical samples The samples were an antiseptic cream, with a known phenol concentration of 2 4 % w/w, and a cold/flu relieving salt, that contained 10 % w/w p-acetamidophenol (paracetamol) The percent relative error was determined as $\{(x_d-x_t)/x_t\} \times 100$, where x_t is the true/known concentration of the analyte and x_d is the experimentally determined concentration of the analyte The percent relative error of the biosensor in determining phenol and paracetamol in the stated samples were -10 % and +14 %, respectively

6.2.3.2.4. *Operational stability of the biosensor*

The operational stability of the sensor was investigated by performing consecutive assays of a standard sample containing 0 6 μM, of catechol, p-cresol and p-chlorophenol The sample was injected 120 times over a ten-hour period i e each analysis taking 5 minutes Initially, the response of the sensor to catechol increased (relative to the initial response) as the number of injections increased And then it appeared to stabilise after 60 injections (Figure 6 9) On the contrary, the responses to p-cresol and p-chlorophenol continued to decrease gradually At the end of the ten-hour period, the biosensor retained only 78 % and 45 % of its initial response to p-cresol and p-chlorophenol, respectively The structure of the microenvironment of polymers and/or enzymes tends to change towards more favourable and reactive conformations within the first 24 hours of biosensor preparations [36] These changes are believed to be responsible for the increase/decrease in the sensor response as observed in this study Tyrosinase has two different active centres (i e catecholase and cresolase activities), both acting independently in the presence of their analytes The observed increases in the response to catechol, and the concurrent decrease for p-cresol and p-chlorophenol implies these changes m the conformation of the polymer and/or enzyme, are favourable for the catacholase activity and unfavourable for the cresolase activity of the enzyme

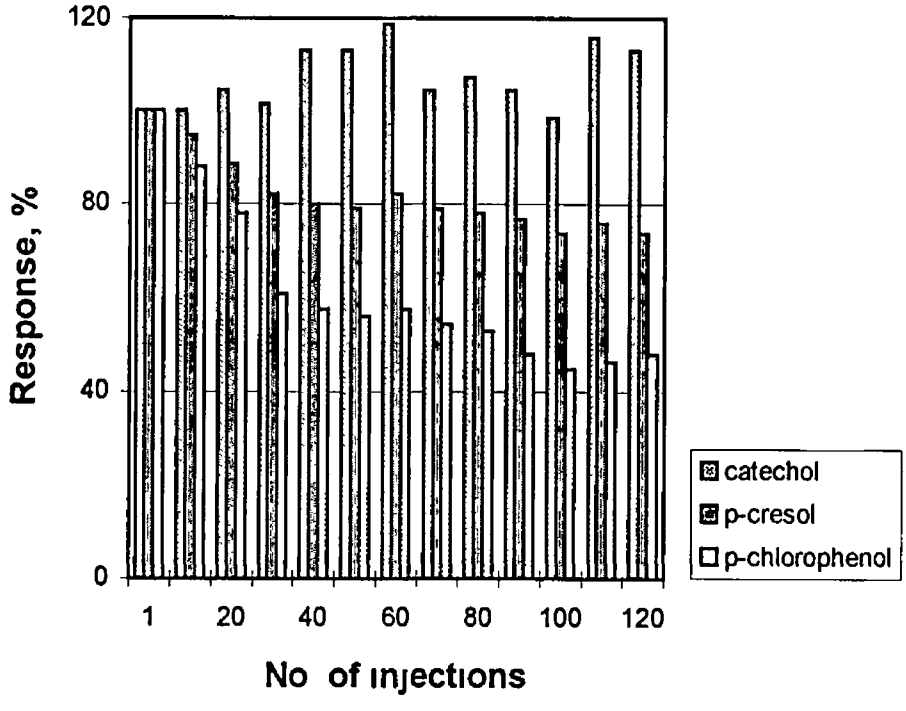

Figure 6 9 Operational stability of the phenol sensor as determined by consecutive assays of 0 6 μM catechol, p-cresol and p-chlorophenol Experimental conditions are as described in Figures 6 4 and 6 6

6.2.3.3. DETERMINATION OF THE PHENOLIC COMPOUNDS IN CIGARETTE FILTER TIPS

The possible application of the phenol biosensor to complex sample matrix was demonstrated by its use in the liquid chromatographic analyses of cigarette filter tips Phenolic compounds are known to contribute to the aroma, flavour and biological activity of tobacco smoke [37] In order to protect the smoker from inhaling large doses of these compounds, cellulose acetate-based filters are placed at one end of commercial cigarettes These filters act by selectively removing phenols and other carcinogenic compounds These filters are also used as sample collectors during evaluations of the residual phenolics in the mainstream tobacco smoke [38,39]

The mobile phase in this study was 30 70, methanol 0 025 M phosphate buffer, pH 6 5 to ensure satisfactory resolution between the analytes Table 6 7 (A) shows the calibration features for the analytes The mobile phase change resulted in better sensitivity and detection limits for phenol and p-cresol, while there was a considerable loss in the precision However, the general features follow a similar trend to that described initially for the biosensor in Table 6 6 The UV detector was found to be less sensitive than the phenol biosensor The analytes could not be detected within the biosensor's linear range on the UV detector The calibration features of 10-80 μM of these compounds as obtained with the UV detector is shown in Table 6 7 (B)

Table 6 7 (A) Calibration features of the sensor in the analysis of phenolic compounds in cigarette filter tips The mobile phase was 30 70 binary mixture of methanol 0 025 M phosphate buffer pH 6 5 Other experimental conditions are described in Figures 6 4 and 6 6

FEATURE	CATECHOL	PHENOL	p-CRESOL	m-CRESOL
Slope, nA/nM	2 99	0 23	3 63	0 05
Intercept	1 21	2 64	2 14	-1 82
Correlation coefficient	0 9983	0 9992	0 9992	0 9904
Limit of detection, nM	1 6	14 6	1 1	156 4
Coefficient of variation, %	2 8	10 2	10 2	5 7

Table 6 7 (B) Calibration features for the analytes as obtained with a UV detector The detection wavelength was 280 nm Other experimental conditions are as specified in Table 6 7(A) and Figure 6 4

FEATURE	CATECHOL	PHENOL	p-CRESOL
Slope, absorption units/μM	0 11	0 06	0 19
Correlation coefficient	0 9989	0 9979	0 9961
Limit of detection, μM	6	8	8

The filter of four different brands of commercial cigarettes were analysed For each brand, one spent filter stub was soaked m 50 ml of methanol and left overnight at 4°C Figure 6 10 shows the typical chromatogram as obtained with the biosensor for a 20 µl injection of the cigarette filter extract By comparison with chromatograms obtained with the standards, peaks IV, V VI and VII have been identified as those of catechol, phenol, p-cresol and m-cresol, respectively From available literature, [39-43], the presence of approximately 20 phenolic compounds have been identified However, tyrosinase is specific for (and would therefore respond to) only 8 of these compounds These are 3-ethylphenol, 4-ethylphenol, 4-methoxyphenol, 3-methyoxyphenol and the four analytes detected in this study Peaks I-III and VIII as observed in Figure 6 10 are possibly those of the above-named compounds

Table 6 8 shows the evaluated concentrations of catechol, phenol, p-cresol and m-cresol in the 4 different brands of cigarettes analysed

BRAND	CONCENTRATION, µM/cigarette			
	Catechol	Phenol	p-Cresol	m-Cresol
Benson & Hedges	1 6	32 9	0 6	1 5
Marlboro	1 3	27 7	0 6	9
Silk cut	0 75	12 46	0 2	1 2
Marlboro lights	1 3	28 5	0 65	1 5

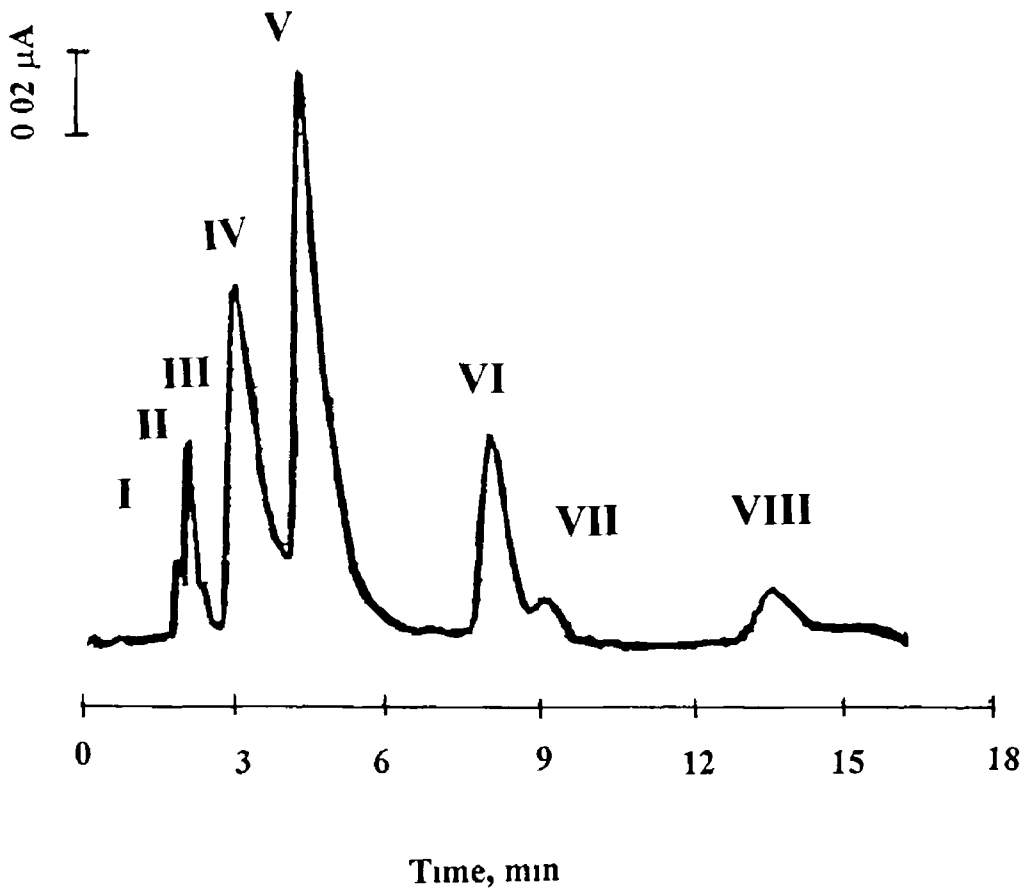

Figure 6 10 The response of the biosensor to a 20 µl injection of a cigarette filter extract (Marlboro Lights brand) I, II, III and VIII are unidentified phenolic compounds, while IV, V, VI and VII are catechol, phenol, p-cresol and m-cresol, respectively All experimental conditions are as specified in Table 6 7(A)

6.3. CONCLUSIONS

An enzyme-based unit for the HPLC analysis of phenolic compounds has been described This biosensor is capable of detectmg a wide variety of phenolic compounds However, those that have been identified in this study (by running pure standards), are catechol, phenol, p-aminophenol, p-chlorophenol, p-cresol p-acetamidophenol and in-cresol In comparison to a UV detector, overwhelmingly mcrease in sensitivity and detection limits was obtained with the tyrosinase-based biosensor It also displayed good reproducibility However, the differential loss m the sensitivity of this sensor within the first 12 hours of usage as well as its comparatively high percent relative error are problems that still need to be addressed This might be done by mproving the cross-linking of the enzyme and the uniformity of the network The developed biosensor was able to detect catechol, phenol, p-cresol, m-cresol and 4 other phenolic compounds in cigarette filter tips This shows that the sensor is capable of being used as a detection unit in the analysis of complex sample matrices without any form of sample pre-treatment or purification It is envisaged that this biosensor would be useful in areas where high sensitivities and detection limits for complex phenolic-compound matrices is required i e forensic, environmental and clinical applications

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CHAPTER SEVEN: CONCLUSIONS AND FUTURE TRENDS

7.1. CONCLUSIONS AND FUTURE TRENDS

The greatest advantage in the use of organic-phase enzyme electrodes as analytical tools, is the possibility of directly analysing samples that are insoluble or only partially soluble in water, but soluble m organic solvents However, to date, the only commercial organic-phase biosensor reported is an alcohol oxidase-based sensor, for the determination of alcohols m petrol and other petroleum products [1, 2] This sensor was developed by Phillips Petroleum and was constructed by immobilising the enzyme onto the surface of an oxygen electrode with a semi-permeable membrane The slow commercialisation and development of industrial orgamc-phase biosensors is because these sensors are a relatively recent feature in analytical chemistry Nonetheless, it is established that the greatest drawback for organic-phase biosensors, is the inadequate knowledge of the physico-chemical behaviour of enzymes in non-aqueous media [1,3] This thesis has provided insights into (i) the sensor performance-determining processes such as analyte diffusion as well as enzyme kinetics in organic solvents and (ii) the application of OPEEs as detectors for HPLC analysis It is expected that future research work would target addressing crucial questions that have emanated in the course of this work

For instance, it is important to determine if the catalytic mechamsms of enzymes like horseradish peroxidase and tyrosinase is comparatively the same in the various organic media, as well as m water Similar studies using Hammett analysis and kinetic isotope effect examination, have been reported for subtilisin carlsberg (serine protease from *Bacillus*

licheniformis) [4,5] In addition , solid-state NMR and electron spin resonance techniques could be used to ascertain that the active site structures of these enzymes in the organic solvents and water are really indistinguishable [6] These facts when combined with results reported in chapters 4 and 5 of this thesis, is sure to provide possible routes to improving (i) analyte specificity, as well as the detection range and limits, (ii) the stability, and (iii) the response times of organic-phase biosensors

In this study, the biosensor responses have been analysed using simple Michaelis-Menten kinetics model Future interests in the kinetic studies and analysis of organic-phase biosensors may be directed at considering coupled mass transport and enzyme kinetics as well as the effects of the finite enzyme layer thickness Similar analysis and studies have been developed by Bartlett and coworkers for aqueous-phase enzyme electrodes [7, 8]

The on-going trend of developmg immobilisation materials and methods that would allow both aqueous and non-aqueous applications of developed enzyme electrode is desirable [9, 10] In a similar way, Deng and Dong [11] have also reported an immobilisation technique that allowed tyrosinase to maintain its biocatalytic activity for up to three months in pure chloroform and chlorobenzene These findings would allow direct theoretical comparisons of the performance of biosensors in pure organic, biphasic organic-water and aqueous media And more importantly, it would allow for the easy adaptation of developed sensors as detectors in reversed-phase as well as normal phase HPLC analysis

In a similar way, the possible application of biosensors as detection units in HPLC analysis might provide a solution to the problems of interferents and multi-analyte sample matrices It is expected that studies on the application of biosensors as HPLC detection units would be extended to other analytes, such as organic peroxides and hydroperoxides, alcohols etc In addition, the ability of enzymes to function in supercritical, reverse micelle and detergentless microemulsion fluids [12,13], coupled to recent analytical developments and interests in reverse micelles and supercritical fluid chromatography would undoubtedly lead to more research work in these area

Finally it would be important to extend the enzymes (and ultimately the analytes) being used in the development and study of organic-phase enzyme electrodes Future interests may be directed at the use of new enzymes such as lipases and other lipid-catalysing enzymes found in humans Studies of this nature would definitely be of analytical and clinical significance

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APPENDIX

List of Publications

1 "Determination of Kinetic Parameters for the Inhibitory Effects of Organic Sulphides on an Amperometric Peroxide Biosensor in Non-aqueous Media," Adeyoju O , Iwuoha E I and Smyth M R , *Talanta* **41** (1994) 1603-1608

2 "Amperometric Determination of Butanone Peroxide and Hydroxylamine via Direct Electron Transfer at a Horseradish peroxidase-Modified Platinum Electrode," Adeyoju O , Iwuoha E I and Smyth M R , *Analytical Proceedings Including Analytical Communications* **31** (1994) 177-179

3 "Kinetic Study of the Inhibitory Effects of Methyl Isothiocyanate on a Peroxidase-Modified Platinum Electrode in Non-aqueous Media," Adeyoju O , Iwuoha E I and Smyth M R , *Analytical Letters* **27**(11) (1994) 2071-2081

4 "Reactivities of Amperometric Organic-phase Peroxidase-Modified Electrodes in the Presence and Absence of Thiourea and Ethylenethiourea as Inhibitors," Adeyoju O , Iwuoha E I and Smyth M R , *Analytica Chimica Acta* **305** (1995) 57-64

5 "Amperometric Glucose Biosensors Based on an Osmium (2+/3+) Redox polymer-Mediated Electron Transfer at Carbon Paste Electrodes," Pravda M , Adeyoju O , Iwuoha E I , Vos J G , Smyth M R and Vytras K , *Electroanalysis* **7** (1995) 619-625

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Ethylenethiourea," Adeyoju O , Iwuoha E I and Smyth M R , *Electroanalysis* in press

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