

**CRITICAL STUDIES IN CARBON ELECTRODE
MATERIALS WITH APPLICATIONS IN THE
ELECTROANALYSIS OF THE MYCOTOXIN
CITRININ**

MICHAEL JOHN NILAND

**CRITICAL STUDIES IN CARBON ELECTRODE MATERIALS WITH
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MYCOTOXIN CITRININ**

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MICHAEL JOHN NILAND

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Abstract

Guided by increasing legislation, the analysis of food borne toxins, including mycotoxins, seeks to address market related demands for the development of analytical systems to monitor this threat to food security and human health. This Thesis is directed at the assessment of the application of electrochemistry for direct electroanalysis and characterisation of the mycotoxin citrinin (CIT) in aqueous media as well as fundamental investigations of the surface of polished and oxidised glassy carbon electrodes (GCE).

This study provides the first known account of CIT detection through electrochemical methods. Although electrochemically active, CIT current responses (I_p) were highly irreproducible at polished GCE with a coefficient of variation (C.V.) of 20.16 %. As stability of I_p across multiple electrode preparations is a key requirement in electroanalysis, investigations were directed at attaining stability in CIT I_p . Achieving stability in CIT I_p was investigated via two approaches, including: accounting for I_p variability between electrode preparations as a result of variable GCE surface conditions as a post-data-acquisition analysis and secondly, removing I_p variability through modification of GCE.

Accounting for variability in I_p was investigated through the application of double layer capacitance as an indicator of the activity of an electrode, and in so doing serving as a relative mediator of I_p responses between electrodes. Application of this procedure dropped CIT C.V. to a third of starting value across polished GCE (C.V. = 7.18 %), chemically oxidised GCE (Pi-GCE, C.V. = 8.47 %) and functionalised multi-walled carbon nanotube modified GCE (fMWCNT, C.V. = 25.79 %) and was effective with analysis of structurally distinct molecules, 2,4-dimethylaniline (2,4-DMA) and 1,2,4-trihydroxybenzene (Triol). Furthermore, it afforded the ability to determine discreet solution overlapping data sets of I_p . Stabilising I_p through GCE surface modification was achieved by anodic electro-oxidation of GCE and allowed for direct electroanalysis of CIT and subsequent characterisation and analysis of CIT in complex media as it reduced C.V. of CIT I_p to 0.73 %.

Fundamental investigations of the electrode surface condition are described such that the source of variability could be identified and the interactions of CIT with the electrode understood. Two surface oxidation techniques were applied in modification of GCE; anodic electro-oxidation (EOx GCE) and chemical oxidation using piranha solution (Pi-GCE), analysis of which has previously not been reported. Fundamental analyses to determine surface morphology and chemistry of Pi-GCE, EOx-GCE and polished GCE were conducted using high resolution scanning electron microscopy (HRSEM), scanning electrochemical microscopy (SECM), energy dispersive X-ray spectroscopy (EDX), X-ray photoelectron

spectroscopy (XPS), fourier transform infrared spectroscopy (FTIR) and via electroanalytical methods. These studies showed that both oxidation procedures introduced a variety of oxide species at GCE surface, and further that the extent of those species was similar with total % O being 27.67 % and 33.47 % at Pi-GCE and EOx-GCE respectively. Although chemically similar, each surface was morphologically distinct. Electrochemical analyses at the surfaces revealed Pi-GCE to behave more similarly to polished GCE than EOx-GCE. As CIT responses were found to be stable at EOx-GCE (C.V. = 0.73 %) as opposed to Pi-GCE (C.V. = 22.87 %), stability of CIT I_p was likely to be as a result of a physical interaction with electrode morphology rather than interaction on a chemical basis. Morphological analyses revealed polished GCE and Pi-GCE to be highly morphologically irregular at the micro-scale. Although comparatively smooth, the surface morphology of EOx-GCE does not account for the stability of I_p . This study thus proposed a theory to describe the mechanism by which the limited conductivity and porosity of EOx-GCE allow for it to provide a relatively stable surface area within the oxide layer, adjacent to the electrode surface, and thus provided a stable platform for electroanalysis.

Voltammetric characterization of CIT at EOx-GCE revealed that anodic oxidation in aqueous media involved an uneven number of electrons to protons via an ECE mechanism. This was illustrated to be $n_t = 2e^-$ accompanied by the transfer of $1H^+$ per molecule oxidised. A proposed reaction scheme for the initial stages of CIT oxidation was suggested to involve both hydroxyl and carboxyl moieties of the CIT molecule. CIT oxidation was shown to arise as a result of a relatively complex mass transport regime which included both adsorptive and diffusive derived I_{p1} . The LOD in buffered aqueous media was found to be 16 nM, a highly competitive result in relation to chromatographic techniques.

Further application of EOx-GCE in complex media illustrated that CIT associates non-specifically with the components of food samples, primarily proteins. As a result of this, extraction of CIT from such media is mandatory. Liquid-liquid extraction illustrated a recovery in CIT I_{p1} and in so doing provided a means of accurately and sensitively detecting CIT from food samples with an LOD of 20 nM. These responses were corroborated by HPLC analyses on the same extractions and illustrate the applicability of electroanalysis as an analytical technique.

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List of Abbreviations and Symbols

In alphabetical order:

2,4-DMA	2,4-Dimethylaniline	BR	Britton-Robinson buffer
°C	Degrees Celsius	BSA	Bovine Serum Albumin
α	Electron transfer coefficient	BSE	Back scattered electrons
α	CPE exponent	C	Coulomb
Γ	Amount of analyte adsorbed at electrode (mol.cm ⁻²)	C	Carbon
ε	Dielectric constant	C°	Concentration of analyte (mol.cm ⁻³)
ε_0	Permittivity of free space	C_{dl}	Double layer capacitance (F)
μ	Mean	CE	Counter electrode
μ	Micro (unit)	CIT	Citrinin
ν	Linear potential scan rate (V.s ⁻¹)	cm	Centimetre
ρ	Roughness factor of an electrode	CO	Carbonyl
σ	Standard deviation (from mean)	COOH	Carboxyl
σ	CPE coefficient (Y ₀ ; s ^{α} . Ω^{-1})	CPE	Carbon paste electrode
τ	Transition time in chronoamperometry (s)	CPE	Constant phase element
Ω	Ohm	CV	Cyclic voltammetry
ω	Frequency range (s ⁻¹)	C.V.	Coefficient of variation
A	Amperes	D	Diffusion coefficient (cm.s ⁻²)
A	Surface area (cm ²)	d	Diameter (cm)
a	Radius of the tip of a probe UME in SECM	d	Substrate-UME tip separation in SECM
AE	Auxiliary electrode	dH ₂ O	Distilled water
AFB _{1/2}	Aflatoxin B ₁ or aflatoxin B ₂	DMF	N, N-dimethylformamide
AFM ₁	Aflatoxin M ₁	DSMZ	Deutsche Sammlung von Mikroorganismen und Zellkulturen
AFM	Atomic force microscopy	E	Potential of an electrode versus a reference (V)
Ag/AgCl	Silver/Silver chloride reference electrode	E_b	Binding energy of an electron in matter
Ag-QRE	Silver wire quasi-reference electrode	E_f	Final potential (V)
atm	Atmospheres (pressure)	E_i	Initial potential (V)
AuE	Gold working electrode	E_{kin}	Kinetic energy of an ejected electron
BAS	Bioanalytical systems	E_p	Faradaic peak potential (V)
BET	Burnauer, Emmett and Teller method of measuring surface area	$E_{p1/2/3 \dots n}$	Potential at which a faradaic peak with numerical notation (1, 2, 3 ... n) arises (V)

E_{pa}	Anodic peak potential (V)	I	Current (A)
E_{pc}	Cathodic peak potential (V)	IHP	Inner Helmholtz plane
E_{pzc}	Potential at zero charge (V)	I_{max}	Maximum current response (A)
E_{step}	Magnitude of a potential step (V)	$I_{p1/2/3 \dots n}$	Current response of a faradaic peak with numerical notation (1, 2, 3 ... n) (A)
ΔE	Difference in potential ($E_1 - E_2$, V)	I_{pa}	Anodic peak current (A)
$\Delta E_{p,1/2}$	Breadth of a peak at the half peak height	I_{pc}	Cathodic peak current (A)
EC	Heterogeneous electron transfer followed by homogenous chemical reaction	I_T	Ultramicroelectrode tip current (A)
ECE	Heterogeneous electron transfer, homogenous chemical reaction then heterogeneous electron transfer	$I_{T,\infty}$	Ultramicroelectrode tip steady-state current (A)
EDX	Energy dispersive x-ray microanalysis	J	Flux
EIS	Electrochemical impedance spectroscopy	K	Kelvin
EOx-GCE	Electrochemically oxidised GCE	KCl	Potassium chloride
ETD	Everhart-Thornley detector	KOH	Potassium hydroxide
F	Farads	LD ₅₀	Dose that kills 50 % of animals or cells
FT-IR	Fourier transform infrared spectroscopy	LiClO ₄	Lithium perchlorate (electrolyte in organic solvent)
fMWCNT	Functionalised multi-walled carbon nanotube	LLE	Liquid-liquid extraction
FRA	Frequency response analyser	ln	Natural logarithm
g	Grams	LOD	Limit of detection
GC	Gas chromatography	LOQ	Limit of quantification
GCE	Glassy carbon electrode	Log	Logarithm
GPES	General Purpose Electrochemical System (potentiostat control software)	LSV	Linear sweep voltammetry
H ₂ SO ₄	Sulphuric Acid	M	Molarity
HNO ₃ ⁻	Nitric Acid	m	Mass (g)
HOPG	Highly ordered pyrolytic graphite	MB	Maize broth
HPLC	High performance liquid chromatography	MeOH	Methanol
HRSEM	High resolution scanning electron microscopy	MS	Mass spectroscopy
HV	High voltage	mV	Millivolts
h ν	Quantum energy	MWCNT	Multi-walled carbon nanotube
Hz	Hertz	N	Number of samples assessed
		n	Total number of electrons transferred
		n_a	Number of electrons transferred in the rate limiting step
		NaOAc	Sodium acetate buffer
		NaOH	Sodium hydroxide

NB	Nutrient broth	SEM	Scanning electron microscopy
NOVA	Potentiostat control software	S_p	Peak sensitivity ($A.M^{-1}$)
O	Oxidant	SCE	Saturated calomel electrode (reference electrode)
O	Oxygen	SPE	Solid phase extraction
OCH A	Ochratoxin A	STM	Scanning tunnelling microscopy
OH	Hydroxyl	STP	Standard temperature pressure
OHP	Outer Helmholtz plane	T	Temperature (K)
PAT	Patulin	t	Time (seconds)
PDA	Potato dextrose agar	TE	Transmitted electrons
Pi-GCE	GCE chemically oxidised with piranha solution	TEAP	Triethylammonium phosphate (electrolyte in organic solvent)
Pt	Platinum auxiliary electrode	TLC	Thin layer chromatography
Q	Charge passed in electrolysis (C)	TLD	Through lens detector
Q_{dl}	Pseudocapacitance of the double layer derived from CPE (F)	Triol	1,2,4-trihydroxybenzene also 1,2,4-benzenetriol
r	Radius	UME	Ultramicroelectrode
R	Molar gas constant ($J.mol^{-1}.K^{-1}$)	UV-Vis	Ultra violet-visible
R	Reactant	V	Volt
RB	Rice broth	v/v	Volume per volume
RE	Reference electrode	WE	Working electrode
R_p	Polarisation resistance (Ω)	XPS	X-ray photoelectron spectroscopy
R_s	Solution resistance (Ω)	Z	Impedance vector (Ω)
r_s	Spearman's correlation coefficient	Z_{Im}	Imaginary part of impedance (Ω)
R^2	Correlation coefficient	Z_{Re}	Real part of impedance (Ω)
s	Second(s)	Z_{CPE}	Impedance of a constant phase element
SE	Secondary electrons	ZEA	Zearalenone
SECM	Scanning electrochemical microscopy	Z_w	Warburg impedance

List of Equations

Physical constants:

e	Elementary charge	$1.60218 \times 10^{-19} \text{ C}$
F	Faraday constant	$9.64853 \times 10^4 \text{ C}$
N_A	Avogadro's number	$6.02214 \times 10^{23} \text{ mol}^{-1}$
R	Molar gas constant	$8.31447 \text{ J.mol}^{-1}.\text{K}^{-1}$

Equations as they appear in Thesis:

Eq. 1.1	$m\text{C} + n\text{H}_2\text{O} - a_1e^- \rightarrow (m-x)\text{C} - \text{OH}_{\text{surf}} + \text{CO}_2$
Eq. 1.2	$(m-x)\text{C} - \text{OH}_{\text{surf}} - n\text{H}_2\text{O} - b_1e^- \rightarrow q\text{CO}_2 + r\text{H}^+$
Eq. 1.3	$\text{C} + \text{O}_2 \rightarrow \text{CO}_2$
Eq. 1.4	$\text{C} + \text{OH}^- - e^- \rightarrow \text{C} - \text{H}_{\text{ads}}$
Eq. 1.5	$\text{C} - \text{OH}_{\text{ads}} + \text{OH}^- - e^- \rightarrow \text{C} + \text{H}_2\text{O} + 1/2\text{O}_2$
Eq. 1.6	$\text{C} + \text{O}_2 \rightarrow \text{CO}_2$
Eq. 2.1	$\text{C.V.} = \frac{\sigma}{\mu} \times 100$
Eq. 2.2	$I_{pa} = (2.69 \times 10^5)n_t^{3/2}AD^{1/2}C^0v^{1/2}$
Eq. 2.3	$R(\%) = 100 - \text{C.V.}$
Eq. 2.4	$S_p = \frac{I_p}{C^0}$
Eq. 2.5	$LOD = \frac{3\delta}{m}$
Eq. 2.6	$\Gamma = \frac{Q}{nFA}$
Eq. 2.7	$\text{Degree of Fouling}(\%) = \frac{P_{a \text{ final}}}{P_{a \text{ Initial}}} \times 100$
Eq. 4.1	$f(x) = H\left(\frac{1}{\sigma\sqrt{2\pi}}\right)e^{-\frac{(x-\mu)^2}{2\sigma^2}}$
Eq. 4.2	$I_{T,\infty} = 4nFD_0C_0a$
Eq. 5.1	$Q = C_{dl}A(E - E_{pzc})$
Eq. 5.2	$i = \left(\frac{E}{R_s}\right)e^{-\frac{t}{R_sC_d}}$
Eq. 5.3	$\tau = R_sC_d$

Eq. 5.4	$i_{step}(t) = \left(\frac{\Delta E_{step}}{R_s} \right) e^{-\frac{t}{R_s C_{dl}}}$
Eq. 5.5	$\ln[i_{step}(t)] = \ln\left(\frac{\Delta E_{step}}{R_s} \right) - \frac{t}{R_s C_{dl}}$
Eq. 5.6	$\ln[i_{step}(t)] = \left(\frac{-1}{\tau} \right) t + \ln\left(\frac{\Delta E_{step}}{R_s} \right)$
Eq. 5.7	$R_s = \frac{\Delta E_{step}}{e^{C_{int.}}}$
Eq. 5.8	$C_{dl} = \tau \frac{e^{C_{int.}}}{\Delta E_{step}}$
Eq. 5.9	$i = \nu C_{dl} \left[1 - e^{\frac{-t}{R_s C_{dl}}} \right]$
Eq. 5.10	$i = \nu C_{dl}$
Eq. 5.11	$C.V. = \mu(\Theta_1, \Theta_2, \Theta_3 \dots \Theta_n)$
Eq. 5.12	$\Theta = \left(\frac{I_{p,n} - I_{p \text{ mod },n}}{I_{p,n}} \right) \times 100 \%$
Eq. 5.13	$Z_{CPE}(\omega) = \left(\frac{1}{\sigma} \right) (i\omega)^{-\alpha}$
Eq. 5.14	$I_p = \frac{\alpha F^2 A \nu \Gamma_0}{2.718 RT}$
Eq. 6.1	$A = L \times B$
Eq. 6.2	$A = \pi r^2$
Eq. 6.3	$\Gamma = \Gamma_{max} \frac{Kc}{1 + Kc}$
Eq. 6.4	$I_{p1} = (2.99 \times 10^5) n_t (\alpha \cdot n_a)^{1/2} A D^{1/2} C^o \nu^{1/2}$
Eq. 6.5	$\Delta E_{p, \frac{1}{2}} = 3.53 \frac{RT}{n_t F} = \frac{90.6}{n_t}$
Eq. 6.6	$E_p = E_{\frac{1}{2}} - \frac{RT}{n_t F} 0.780 + \frac{RT}{2n_t F} \ln \lambda$
Eq. A5.1	$h\nu = E_b + E_{kin}$

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CHAPTER 1

General introduction

1.1 Contamination of agricultural food products

The loss of agricultural produce due to contamination is one of the greatest liabilities to the agricultural sector. This is true through all stages of production and processing, from crops in the field to processed and packaged product in retail outlets. The scope of contamination of foodstuffs can be envisaged by considering the ability of contaminants to disperse in nature, affect almost any conceivable environment, be taken up by crops, be found as residues in livestock and the often recalcitrant nature of many toxins, pesticides or herbicides. The reality remains that contamination of foodstuffs to some degree is generally considered unavoidable (Torres *et al.* 1996; Arrebola *et al.* 2003; Korta *et al.* 2003; Pugliese *et al.* 2004; Xu *et al.* 2006; Murphy *et al.* 2006; Prieto-Simón *et al.* 2006; Krska & Molineli, 2007; Krska *et al.* 2008; Wagacha & Muthomi, 2008; Kumar *et al.* 2008). Due to the huge number of possible sources of toxins and the vast diversity of severity of their impacts, simple, inexpensive and routine analysis of commonly-encountered food-borne toxins is of crucial importance as a measure to reduce their consumption by humans and livestock alike.

Contaminants can be classed broadly into contamination by whole pathogenic organisms, or by compounds which may be toxic or otherwise spoil the quality of foods. Toxic compounds can be further subdivided into those arising from biological sources (bacteria and fungi that grow on or in the food, plant toxins and higher vertebrates such as some types of fish) and toxins arising from anthropogenic sources (chemical contaminants from other human practices e.g. poisons applied to crops or livestock to limit weeds and pests). A vast majority of biological contaminants are secreted by micro-organisms, particularly fungi, associated with foods which release toxins in order to gain a competitive advantage or as a defence against other organisms. Toxins from anthropogenic sources are generally a result of poor industrial and agricultural practices, resulting in the contamination of water courses which may later be used in agricultural practices. Due to the variety of toxins which are present in foods, this is an area of analytical research which has vast scope for further studies to be conducted.

Since the early 1990s, interest in the area of food toxins, particularly those of microbial origin, has grown rapidly with a number of review papers emerging toward the later 2000's directed at investigations into the impacts of food borne contaminants (Smith & Henderson, 1991; Stollof & van Egmond, 1991; Wood, 1992; Park, 1993; D'Mello & MacDonald, 1997; D'Mello *et al.* 1999; Bankole & Adebajo, 2003; Leung *et al.* 2006; Xu *et al.* 2006; Murphy *et al.* 2006; Prieto-Simón *et al.* 2006; Richard, 2007; Krska, & Molineli, 2007; Krska *et al.* 2008; Wagacha & Muthomi, 2008; Kumar *et al.* 2008, Mayer *et al.* 2009; Sherif *et al.* 2009; Turner *et al.* 2009). These reviews show that contamination of foods remains a global issue with severe and far reaching impacts. Accordingly, means of accurately assessing the levels of such toxins in foods is of crucial importance to public health and food safety and forms the broader field within which this Thesis is situated.

1.1.1 Toxins from microbial sources

a) Fungi

Fungi are a broad and diverse group of eukaryotic organisms with over 70 000 described species in the kingdom (Alexopoulos *et al.* 1996; Madigan, *et al.* 2000). Although specific environmental conditions such as favourable nutrient sources, temperature, moisture, aeration, pH and light conditions are typically required for a given fungus to proliferate (Robinson, 1967), due to the vast variety of fungi, there are species capable of colonising almost any conceivable combination of environmental conditions, resulting in them generally being considered ubiquitous organisms (Madigan, *et al.* 2000).

All fungi share a common mode of nutrition in that they are heterotrophic, synthesising sufficient organic material for growth from available organic matter (Hawksworth, 1991). Fungi are primarily saprophytic and have fundamental roles in the cycling and exchange of nutrients within terrestrial and aquatic ecosystems. They are typically terrestrial and spread across or through a substrate as the local environment becomes nutrient-depleted (Madigan, *et al.* 2000).

Fungi commonly exploit living plants and harvested products, resulting in spoilage of foodstuffs as they grow. Direct contamination takes the form of parasitic infection (mycosis) of living organisms (including livestock and people) (Madigan, *et al.* 2000). Secondary impacts to fungal infections of agricultural products stem from exposure to toxic fungal metabolites (mycotoxins) through dietary, respiratory, dermal and other means, collectively termed mycotoxicosis (Bennett & Kilch, 2003). Mycotoxins are a diverse group of biologically active, low molecular weight, secondary metabolites produced by a number of fungal species (primarily *Fusarium*, *Penicillium* and *Aspergillus* species). Although fungal growth is a prerequisite for the production of mycotoxins, not every strain of a

species has the capacity to produce mycotoxins, and those which do produce mycotoxins often produce multiple forms of mycotoxins at a time (Hof & Kupfahl, 2009). Mycotoxicosis is characterized as non-contagious, non-transferable, non-infectious, and non-traceable to microorganisms other than fungi (Hussein & Brasel, 2001). The symptoms are highly variable and dependent on the type of mycotoxin, or combination thereof, duration of exposure, physical condition of the exposed individual and other poorly understood synergistic effects. In general, symptoms are analogous to disease caused by exposure to pesticides or heavy metal residues (Bennett & Kilch, 2003).

b) Mycotoxins

Mycotoxins comprise of a group of several hundred toxic and carcinogenic compounds of a variety of chemical structures and biosynthetic origins and result in a number of chemical and physicochemical effects in biological systems, and were only grouped together as they cause disease and death in human beings and other vertebrates (Huwig *et al.* 2001; Bennett & Klich 2003; Xu *et al.* 2006; Krska & Molineli, 2007; Prieto-Simón *et al.* 2006; Krska *et al.* 2008; Wagacha & Muthomi, 2008; Kumar *et al.* 2008). They are generated both pathogenically on crops and saprophytically on stored foods (Gardiner *et al.* 2005; Krska & Molineli, 2007; Krska *et al.* 2008; Wagacha & Muthomi, 2008; Kumar *et al.* 2008). Mycotoxins are considered distinct from other low molecular weight fungal metabolites, such as alcohols that are toxic to vertebrates in high concentrations as well as fungal metabolites which are primarily toxic to invertebrates, plants and other microorganisms (Bennett, 1987).

The mycotoxins of greatest concern in agriculture are listed in Table 1.1 with relation to the regions affected, foods contaminated and medical implications.

c) Prevalence of mycotoxin contamination and exposure

The Food and Agricultural Organization (FAO) of the United Nations has estimated that up to 25% of the world's food crops are significantly contaminated with mycotoxins (WHO, 1999). Table 1.1 illustrates both the wide range of foods affected, though their presence is more pronounced in grains such as wheat, maize, barley and rice (Kumar *et al.* 2008). The wide variety of countries affected illustrates the global nature of their impacts. Although a worldwide phenomenon, exposure to mycotoxins is especially high in developing countries in Western Europe, Africa, South America and Asia. This is due to a number of factors including tropical and subtropical climates (high humidity and ambient temperatures promotes fungal growth), poor farming practices, limited pest control, poor food storage practices, a higher dependence on staple foods combined with less varied diets and a lack of effective infrastructural monitoring (Doko *et al.* 1996; Hell, *et al.* 2000; Shephard, 2008; Sherif *et al.* 2009).

Table 1.1: Prevalence, source and toxicological impacts of selected major food borne mycotoxins.

Mycotoxin	Fungal Species	Contaminated Foodstuffs	Countries Affected	Recorded Toxicological Effects
Alflatoxins (B ₁ , B ₂ , G ₁ , G ₂ , M ₁ , M ₂)	<i>Aspergillus flavus</i> , <i>Aspergillus parasiticus</i> , <i>Aspergillus nomius</i>	Cassava, cottonseed cake, figs, cereals: maize, wheat, rice, sorghum and cereal products, nuts and nut products (brazils, peanuts, peanut butter, pistachios), soybean, spices, pasta, quince, tobacco, oilseeds, watermelon seeds	Argentina, Benin, Brazil, Botswana, China, Egypt, Gambia, Guinea, India, Italy, Kenya, Korea, Malaysia, Nigeria, Portugal, Qatar, South Africa, Senegal, Sierra Leone, Turkey, USA, Uganda, Vietnam	Humans: Carcinogenicity (hepatic and renal), Cirrhosis, hepatitis, jaundice, kwashiorkor, immunosuppression, Reye's syndrome Animals: Carcinogenicity (hepatic and renal), death, teratogenesis, mutagenesis, reduced rate of reproduction, weight loss, immunosuppression
Ochratoxin A	<i>Penicillium verrucosum</i> <i>Penicillium cyclopium</i> <i>Penicillium viridicatum</i> <i>Aspergillus ochraceus</i> <i>Aspergillus alliaceus</i> <i>Aspergillus auricomus</i> <i>Aspergillus carbonarius</i> <i>Aspergillus glaucus</i> <i>Aspergillus niger</i>	Cereal crops and products: (Maize, rice, rye, wheat, buckwheat, barley, millet, oats, sorghum, beer), cocoa, coffee, Fruits and fruit products (grapes, ginseng, raisins, wine, currants,), nuts, spices, pork, cheese	Bulgaria, Canada, Denmark, USA, Germany, Sweden, Morocco, Norway, Romania, Sierra, South Africa, Leone, Vietnam	Humans: Balkan endemic nephropathy Animals: Carcinogenicity, genotoxicity, mutagenicity, nephrotoxicity, teratogenesis reduced rate of reproduction, immunosuppression
Citrinin	<i>Penicillium verrucosum</i> <i>Penicillium citrinum</i> <i>Aspergillus citrinum</i> <i>Aspergillus niveus</i> <i>Aspergillus carneus</i> <i>Aspergillus terreus</i> <i>Monascus purpureus</i> , <i>Monascus kaoliang</i> , <i>Monascus sanguineus</i>	Cereal crops and products: (Maize, wheat, rice, oats, peanuts, rye, barley), fruits, coconut, spices, aged ham, sausages, cheese, sake, dry copra, medicinal plants (indigenous medicines)	Argentina, Brazil, China, Canada, Egypt, Denmark, France, Germany, Ghana, Ireland, Italy, India, Japan, Norway, Portugal, South Africa, Sweden, Taiwan, USA, Vietnam	Humans: Balkan endemic nephropathy, fatal kidney disease, Shoshin-Kakke beriberi, yellow rice toxicosis Animals: Abortion, apoptosis, bronchial constriction, carcinogenicity (Renal and hepatic), enzyme inhibition, fetotoxicity, nephropathy, necrosis, renal damage, teratogenesis, vasodilatation
Patulin	<i>Penicillium griseofulvum</i> <i>Penicillium expansum</i> <i>Aspergillus sp.</i> <i>Byssoschlamys sp.</i>	Cereal grains and products, apples, apple juice, pears, grapes, cherries, bilberries, pies, jam, silage	Japan, South Korea, India, Iran, Italy, Belgium, Spain, Nigeria, Saudi Arabia, South Africa	Humans: Gastrointestinal hyperaemia, haemorrhages, nausea and vomiting, protein synthesis inhibition, ulceration Animals: Carcinogenicity, death, haemorrhages, hepatic, intestinal, renal and tubular congestion, epithelial degeneration, neurotoxicity, immunotoxicity, pulmonary oedema
Zearalenone	<i>Fusarium graminearum</i> , <i>Fusarium culmorum</i> <i>Fusarium crookwellense</i> <i>Fusarium equiseti</i> <i>Fusarium sporotrichioides</i>	Cereal crops and products: (Maize, wheat, flour, ginseng, bread, noodles, rice, barley, oats, sorghum, maize beer), walnuts, milk, meat	Canada, Mexico, Morocco, New Zealand, Puerto Rico, South Africa	Humans: Oxidative stress, cell death, DNA fragmentation Animals: Lymphocytic infiltration in the liver, kidney, and uterus, chromatin abnormalities, oocyte maturation inhibition, hepatocellular swelling,

Other mycotoxins of importance in agricultural products: Alternaria mycotoxins, (altenuene, alternariol, alternariol monomethyl ether, altertoxin I, tenuazonic acid), Cyclopiazonic acid, Citreoviridin, Ergot alkaloids (agroclavine, ergocornine, ergocristine, ergocryptine, ergometrine, ergotamine), Lolitrem, Moniliformin, Sterigmatocystin (precursor of AFB₁), Fumonisin (B₁, B₂), Trichothecenes (Type A: diacetoxyscirpenol, HT-2 toxin, monoacetoxyscirpenol, neosolaniol, T-2 toxin, Type B: 3- and 15-acetoxynivalenol, vomitoxin, fusarenol X).

Table was compiled from the following references: Saito *et al.* 1971; Scott *et al.* 1972; Kroch *et al.* 1973; Nelson *et al.* 1985; Aleo *et al.* 1991; Sweeney & Dobson, 1998, Abdel-Sater & Saber 1999; Minervini *et al.* 2001; Heber *et al.* 2001; Odhav & Naicker, 2002; Bennett & Klich, 2004; Williams *et al.* 2004; Xu *et al.* 2006; Prieto-Simon *et al.* 2006; Wagacha *et al.* 2008; Trucksess & Scott, 2008; Chen *et al.* 2008; Shephard, *et al.* 2008; Abid-Essefi *et al.* 2009; Flajs & Peraica, 2009; Dong *et al.* 2010.

The prevalence of mycotoxin exposure in Africa, and the sub-Saharan sub-region in particular has been noted through several studies (Doko *et al.* 1996; Sibanda *et al.* 1997; Bankole & Adebajo, 2003; Strosnider *et al.* 2006; Wagacha & Muthomi, 2008). Commonly reported in these studies were the high levels of a variety of mycotoxins in staple foods particularly aflatoxins, zearalenone, patulin, ochratoxin, citrinin and fumonisins, the absence of regulatory bodies and, in many cases, the absence of any mitigation procedures to limit mycotoxin contamination. The extent of the problem is well illustrated by reports which have stated that 78 % of raw peanut samples in Botswana (Mphande *et al.* 2004), 65 % of pre-harvest maize in Nigeria (Bankole & Mabekoje, 2004) and 85 % of peanut oil in Senegal (Muleta & Ashenafi, 2001) containing Aflatoxin B1. Furthermore, Bankole & Adebajo (2003) reported that 98 % of blood tests carried out on persons in Gambia, Guinea, Conakry, Nigeria and Senegal tested positive to aflatoxin markers.

A two year survey program was conducted to evaluate various animal feed producers situated throughout Europe, Asia, Oceania and the Mediterranean region found that more than half of the grains and cereals sourced from Europe contained mycotoxin levels above the limit of quantification of methods applied, while one third of samples originating from the Asian-Pacific region tested positive for mycotoxin contamination (Binder *et al.* 2007). Animal products such as milk, meat and eggs can also be contaminated by mycotoxins when the animals are fed contaminated feeds (Shreeve *et al.* 1979).

d) Mycotoxin toxicity

The medical symptoms of mycotoxin exposure are varied depending on the type of mycotoxin, the organism being affected, dose received and co-occurrence of mycotoxins. They are however documented as being cyto-, nephro- and neurotoxic, carcinogenic, mutagenic, and immunosuppressive and have estrogenic properties, the syndrome of which is collectively termed mycotoxicosis (D'Mello & MacDonald, 1997; Huwig *et al.* 2001; Bennett & Klich, 2003; Williams *et al.* 2004; Strosnider *et al.* 2006; Xu *et al.* 2006; Krska & Molineli, 2007; Prieto-Simón *et al.* 2006; Krska *et al.* 2008; Wagacha & Muthomi, 2008; Kumar *et al.* 2008). When foods containing certain mycotoxins (such as citrinin) are cooked, the chemical structure may often be altered to a secondary structure which may be equally, and in some cases more, toxic (Xu *et al.* 2006).

Although rare, acute cases of mycotoxicosis are often lethal (Strosnider *et al.* 2006; Mayer *et al.* 2008). The main human and veterinary health burden of mycotoxins is related to chronic intoxication, characterized by low dose exposure over an extended period of time. As a result of a combination of factors, including the wide occurrence of mycotoxins and generally poor healthcare systems, the impact of mycotoxins is typically felt more strongly in developing nations across a number of sectors

including sociological, economic, agriculture and health. Secondary implications of mycotoxicosis include compounding the effect of, or aiding the infection of people with other diseases including HIV/AIDS, malaria, cholera, tuberculosis, haemorrhagic fevers and a range of malnutrition related illnesses to name but a few, all of which are prevalent across the African continent.

e) Mitigation of mycotoxin contamination

Aside from the direct medical implications of mycotoxin ingestion, there are profound economic consequences to mycotoxin contamination. These include the cost of human health care, veterinary health care, loss of production and abortions of livestock, food spoilage, loss of forage crops, rejection of feed and regulatory cost, as well as the requirement to fill the void left by contaminated food resulting in feed and food prices being forced up. Furthermore, according to a 2001 World Bank estimate, policy changes by the European Union will reduce imports of cereals, dried fruits, and nuts by 64% from nine African countries including Chad, Egypt, Gambia, Mali, Nigeria, Senegal, South Africa, Sudan and Zimbabwe due to mycotoxin presence, resulting in a projected cost of approximately 670 million USD in trade per year to these countries (Otsuki *et al.* 2001).

A number of strategies have been suggested to limit the exposure of people to mycotoxins which centre on a three-pronged approach including prevention of exposure, decontamination of foods and constant surveillance and monitoring of contaminated foods (Park, 1993; Huwig *et al.* 2001; Wagacha & Muthomi, 2008). Procedures for decontamination of foods are however typically either prohibitively expensive, unfeasible or not yet possible. As such, preventative measures are generally considered the only viable means of controlling mycotoxin exposure. Avenues for this include constant surveillance and monitoring of contaminated foods, good farming practices (early harvesting, insect and weed control, proper drying, processing, sorting and storage of foods), biological control (atoxicogenic fungi and insect biocontrol agents to outcompete or feed on mycotoxin producing fungi), chemical control (fungicide, herbicide and pesticide regimes), breeding for resistance, legislation and effective monitoring and awareness creation of toxins (Hussein & Brasel, 2001; Williams *et al.* 2004; Wagacha & Muthomi, 2008).

In order to limit the inevitable introduction of mycotoxin-contaminated commodities into the general food supply, regulatory agencies are empowered to survey the occurrence, and establish regulatory limits of mycotoxin contamination in food and animal feed. Guidelines for regulatory limits are determined by national and international institutions such as the European Commission (EC), the US Food and Drug Administration (FDA), the World Health Organisation (WHO) and the Food and Agriculture Organisation (FAO) of the United Nations. Such regulatory standards generally depend on several factors including the level of toxicity, degree of public exposure, distribution of

contamination and the availability of analytical methods used to detect and quantify each class of mycotoxin (Stoloff & van Egmond, 1991). In 2003, at least 100 countries worldwide adopted the advisory mycotoxin regulations set by the Joint FAO/WHO Expert Committee on Food Additives (JECFA) (van Egmond *et al.* 2007). Regulatory limits are often imposed for aflatoxins, ochratoxin A, fumonisins, ergot alkaloids, trichothecenes, zearalenone and patulin, however this panel requires broadening to other compounds such as citrinin. In Africa, 15 countries covering 59 percent of the continent's inhabitants had set regulatory limits for mycotoxins in 2003 (FAO, 2004). The establishment of mycotoxin regulations for the remaining African countries should be viewed in context of local food supply, food safety and available infrastructure which often makes implementation of such guidelines impractical. In addition, developing countries often adopt higher tolerance standards to accommodate for susceptibility to mycotoxin contamination, limited infrastructure locally available for monitoring, testing and certification required to comply with regulations and for economic development through trade (FAO, 2004; Strosnider *et al.* 2006).

f) Methods of mycotoxin detection

The inherent requirement for routine surveillance of foods for mycotoxin contamination emphasizes the need for rapid, accurate and sensitive analytical methods capable of identifying and quantifying the mycotoxins in various agricultural commodities. Due to the diverse chemical and physicochemical properties of mycotoxins, most analytical methods are comprised of separate extraction, cleanup, separation and detection procedures for each class of mycotoxin. These processes are typically chromatographic in nature, are characteristically expensive, time consuming and confined to laboratory conditions, requiring established laboratories, skilled persons to operate specialised equipment and analyse results. Laboratory-based methodologies, such as mass spectroscopy, gas chromatography and high pressure liquid chromatography each provide low limits of detection coupled with specific identification of mycotoxins and are used for the official control and implementation of mycotoxin regulations (Gilbert, 2002). Alternative methods such as thin-layer chromatography (TLC) (Prieto-Simón *et al.* 2006; Pittet & Rover, 2002) and a number of rapid, highly sensitive commercial screening methods based on immunochemical techniques (ELISA) (Delmulle *et al.* 2005), non-invasive optical techniques (Kos *et al.* 2003) and electrochemical biosensors (Micheli *et al.* 2005) are currently under development. Table 1.2 provides a summary of currently-available analysis methods and procedures, along with reported limits of detection for prevalent mycotoxins. Although analyses are accurate, substantial scope exists for the utilisation of electrochemical sensing technology to enhance the detection of mycotoxins in routine analysis.

Table 1.2: Biosensors for the analysis and detection of mycotoxins

Analyte	Analysis/Transducer and Bio-recognition Technique	Limit of Detection (nM)	Reference	
Aflatoxins	Amperometry of Enzyme product	1.60	Li <i>et al.</i> (2011 a)	
	EIS of Immunoassay	0.03	Zaijun <i>et al.</i> (2010)	
	Voltammetry of indirect competitive immunoassay	0.19	Tan <i>et al.</i> (2009)	
	QCM of indirect competitive immunoassay	0.03	Jin <i>et al.</i> (2009)	
	Amperometry of enzyme inhibition	15.37	Pohanka <i>et al.</i> (2008)	
	Voltammetry of competitive immunoassay	0.22	Owino <i>et al.</i> (2008)	
	EIS of Immunoassay	3.20 x10 ⁵	Owino <i>et al.</i> (2007)	
	Voltammetry of immunoassay	0.48	Pemberton <i>et al.</i> (2006)	
	Flourescence of competitive immunoassay	0.96	Sapsford <i>et al.</i> (2006)	
	Conductimetry of Antibody inhibition	0.32	Liu <i>et al.</i> (2006)	
	Immunochromatography	0.16	Xiulan <i>et al.</i> (2006)	
	SPR of Antibody inhibition	0.64	van der Gaag <i>et al.</i> (2003)	
	Voltammetry of EIA	0.10	Ammida <i>et al.</i> (2004)	
	Flourescence of immunoaffinity	0.32	Carlson <i>et al.</i> (2000)	
	AFM ₁	Electrochemistry of competitive ELISA	0.12	Parker & Tothill (2009)
		Microelectrode array immunosensor	2.44 x10 ⁴	Parker <i>et al.</i> (2009)
		EIS of EIA	4.57 x10 ⁴	Vig <i>et al.</i> (2009)
		SPFS of inhibition immunoassay	1.83 x10 ³	Wang <i>et al.</i> (2009)
		EIS of Aptamer interaction	3.05	Dinçkaya <i>et al.</i> (2011)
	Sterigmatocystin (AFB Precursor)	Chronoamperometry of enzyme activity	130.00	Yao <i>et al.</i> (2006)
		Chronoamperometry of enzyme activity	9.14	Chen <i>et al.</i> (2009)
		Chronoamperometry of enzyme activity	9.14	Chen <i>et al.</i> (2010)
Ochratoxin A	Voltammetry from Aptamer interaction	0.17	Bonel <i>et al.</i> (2011)	
	Voltammetry from Aptamer interaction	0.27	Barthelmebs <i>et al.</i> (2011 a)	
	Chronoamperometry of enzyme activity	23.85	Alonso-Lomillo <i>et al.</i> (2011)	
	SPR of EIA	0.15	Urusov <i>et al.</i> (2011)	
	Enzyme-Linked Aptamer Assay	2.48	Barthelmebs <i>et al.</i> (2011 b)	
	Competitive indirect ELISA	0.37	Zhang <i>et al.</i> (2011)	
	Chronoamperometry of enzyme activity	0.25	Alonso-Lomillo <i>et al.</i> (2011)	
	EIS/SPR of EIA	0.03	Zamfir <i>et al.</i> (2011)	
	Voltammetry of enzymatic product	0.02	Liu <i>et al.</i> (2009)	
SPR of Antibody inhibition	2.50 x10 ⁻⁴	van der Gaag <i>et al.</i> (2003)		
Citrinin	Electrochemical immunosensor	4.00 x10 ⁻⁴	Arévalo <i>et al.</i> (2011)	
	Indirect competitive ELISA	4.00 x10 ⁻⁴	Li <i>et al.</i> (2011 b)	
	Indirect competitive ELISA	0.04	Duan <i>et al.</i> (2009)	
	Piezoelectric immunosensor	0.10	Qian & Tsai (2008)	
	Indirect competitive ELISA	0.08	Kononenko & Burkin (2007)	
	Indirect competitive EIA	0.26	Dietrich <i>et al.</i> (2001)	
	Indirect enzyme immunoassay	1.04	Abramson <i>et al.</i> (1995)	
	Fumonisin B ₁	SPR of Antibody inhibition	0.36	van der Gaag <i>et al.</i> (2003)
Zearalenone (ZEA)	SPR of Antibody inhibition	0.82	van der Gaag <i>et al.</i> (2003)	
	Amperometry of immunological response	0.82	Panini <i>et al.</i> (2010)	
α and β -Zeralenol (ZEA metabolites)	Whole cell lucifierase production	1.00 (α) 258.0 (β)	Välimaa <i>et al.</i> (2010)	
Trichothecenes	T-2 toxin	ELISA	64.31	Yoshizawa <i>et al.</i> (2004)
	Deoxynivalenol	Immunochromatographic strip	168.74	Xu <i>et al.</i> (2010)
		SPR - Antibody inhibition	1.69	van der Gaag <i>et al.</i> (2003)
		ELISA	269.98	Yoshizawa <i>et al.</i> (2004)
		ELISA	6.70 x10 ⁻⁴	Maragos & McCormick (2000)
		LFD – Immunoassay	0.84	Kolosova <i>et al.</i> (2008)
	Nivalenol	ELISA	256.15	Yoshizawa <i>et al.</i> (2004)

Table Footnotes: TIRE = Total internal reflection ellipsometry, QCM = Quartz Crystal Microbalance, SPR = Surface Plasmon Resonance, optical response, EIS = Electrochemical Impedance Spectroscopy, EIA = enzyme immunosorbent assay, ELISA = Enzyme-linked Immunosorbent Assay, LFD = Lateral Flow Device, SPFS = Surface plasmon-enhanced fluorescence spectroscopy

1.1.2 Citrinin, a prevalent mycotoxin

Citrinin (CIT) [$C_{13}H_{14}O_5$, IUPAC: (3R,4S)-4,6-dihydro-8-hydroxy-3, 4, 5-trimethyl-6-oxo-3H-2-benzopyran-7-carboxylic acid] (Figure 1.1) (Xu *et al.* 2006) is a commonly encountered polyketide mycotoxin first isolated in 1931, as a possible antimicrobial agent. It has been reported to be produced by several species of fungi in diverse geographical locations, under a wide range of environmental conditions on key foodstuffs and to exhibit a range of medical complications in animals and humans (Table 1.1). Although its structure was initially proposed in 1948, subsequent studies were required to properly characterise its stereochemistry and tautomeric configuration in solid state, illustrated in Figure 1.1 below (Hill & Gardella, 1964; Clark *et al.* 2006). Since then, a number of derivatives have been described, including seven metabolites described by Curtis *et al.* (1968) and thermal decomposition products through heating in water to temperatures $>100\text{ }^{\circ}\text{C}$ (Trivedi *et al.* 1993), resulting in a total of sixteen related structures reported by Clark *et al.* (2006). CIT itself is a relatively stable molecule, decomposing by dry heating at temperature $>175\text{ }^{\circ}\text{C}$, and at producing the decomposition products $>100\text{ }^{\circ}\text{C}$ in the presence of water (Xu *et al.* 2006; Trivedi *et al.* 1993; Kitabatake *et al.* 1991).

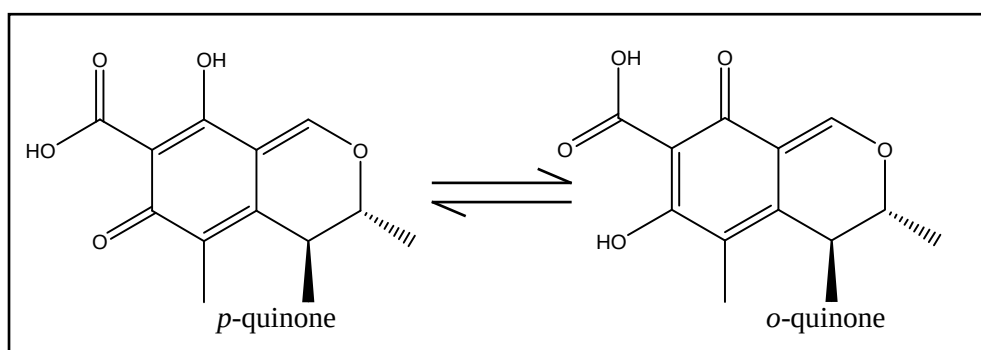


Figure 1.1: Two tautomeric forms of citrinin, found in a dynamic equilibrium in the solid state (Xu *et al.* 2006).

Toxic effects of CIT have been reported (through *in vivo*, *in vitro* and diagnostic studies) for humans (Xu *et al.* 2006, Saito *et al.* 1971), rats (Aleo *et al.* 1991; Friis *et al.* 1969), mice (Gupta *et al.* 1983), rabbits (Hanika *et al.* 1983), pigs (Krogh *et al.* 1973; Friis *et al.* 1969), dogs (Krejci *et al.* 1996; Carlton *et al.* 1974) and poultry (Brown *et al.* 1986; Mehdi *et al.* 1981). Although the exact mechanisms of interaction remain unclear, CIT is primarily reported as a nephrotoxin, causing damage to the proximal tubules of the kidney. CIT-induced nephrotoxicity has been observed both macroscopically (causing structural abortions and influencing renal processes), and biochemically, with regard to disruption of enzymatic processes and electron transport systems.

Macroscopically, it has been reported to induce lesions in mammalian proximal tubules, cause increased hematocrit and renal excretion rates of proteins and glucose, cause depletion of cytoplasmic organelles, mitochondrial pleomorphism, mitochondrial swelling, nuclear fragmentation, cytoplasmic vacuolisation, azotaemia and metabolic acidosis with haemoconcentration and hypokalaemia, many of which induce cell death (Carlton *et al.* 1974; Kanisawa, 1984; Hanika *et al.* 1983; Krejci *et al.* 1996; Kumar *et al.* 2007). On a biochemical basis, CIT has been shown to inhibit enzymatically controlled pathways (such as biosynthetic and metabolic pathways) and cause oxidative stress, wherein CIT has been shown to impede enzymatic antioxidant defences through inhibiting transhydrogenase and GSSG-reductase and to cause alterations in proximal tubule suspensions (Aleo *et al.* 1991; Ribeiro *et al.* 1997) in kidney and liver cells (Kitabatake *et al.* 1993; Ribeiro *et al.* 1997; Da Lozzo *et al.* 1998; Liu *et al.* 2003). CIT has been shown to disrupt the electron transport chain through inhibition of complex I (NADH dehydrogenase) and possibly complex II as a key mechanism of toxicity (Aleo *et al.* 1991; Ribeiro *et al.* 1997). CIT also increased the production of the superoxide anion in the respiratory process which contributed to the oxidative stress and resulting cell death. CIT influences mitochondrial Ca^{+} fluxes and membrane permeability in kidney and liver mitochondria, causing swelling of mitochondria (Da Lozzo *et al.* 1998). Apoptosis of cells has been shown to be induced by CIT, which is expected to arise from the reported transport of cytochrome c from mitochondria to the cytoplasm which activates capsases 3, 6, 7 and 9 which induce apoptosis (Yu *et al.* 2006; Klarić *et al.* 2007). Through these toxic interactions, CIT has been implicated as a causative agent in endemic Balkan nephropathy and yellow rice toxicosis in humans (Saito *et al.* 1971; Bouslimi *et al.* 2008).

In addition to nephrotoxicity, CIT has been reported to be hepatotoxic, to cause chromosomal aborations and to affect bone marrow (Gupta *et al.* 1983; Jeswal 1996; Bouslimi *et al.* 2008; Flajs & Peraica, 2009). Both positive and negative results have been reported on the mutagenicity and genotoxicity of CIT, wherein it has been shown to induce chromosomal aberrations, but not sister chromatid exchange (Jeswal, 1996; Williams *et al.* 2004; Xu *et al.* 2006; Bouslimi *et al.* 2008). Martin *et al.* (1986) showed single stranded breaks in chromosomal DNA at 320 μM . Although Liu *et al.* (2003) reported that no significant DNA strand breakage was caused by CIT at concentrations below 60 μM , Bouslimi *et al.* (2008) showed that CIT caused both single- and double-strand DNA breaks and fragmentation at concentrations as low as 12.4 μM and caused an IC_{50} at 220 μM , a result which was reduced significantly to 24 μM in the presence of ochratoxin A. CIT has furthermore been demonstrated to inhibit RNA and DNA synthesis and also to cause extensive perturbation of the DNA precursor metabolism on a time and concentration dependent basis (Wasternack & Wiesser, 1992; Bouslimi *et al.* 2008). The carcinogenicity of CIT is somewhat contentious as Kanisawa (1984) found no tumour formation after orally treating mice with CIT for 70 weeks, however Arai & Hibino (1983) reported the presence of benign tumours in the kidneys of male rats after 60 and 80 weeks of

treatment. CIT has been reported to be embryocidal and foetotoxic in rats and mice and although no marked morphological aberrations were noted, a marked reduction in the size of foetuses was reported along with a 50% or higher maternal mortality (Reddy *et al.* 1982; Mayura *et al.* 1984; Hood *et al.* 1976).

Although CIT is toxic to many mammalian lines that have been assessed, many of these studies have been carried out *in vivo*, and the results of which have shown marked variability in the levels of cytotoxicity in the different cell lines assessed (Flajs & Peraica, 2009). According to Flajs & Peraica (2009), acute CIT toxicity, reported in relation to the LD₅₀, is dependant of factors including the route of administration, the time of exposure, the type of cells (species) and the cell line (type of cell). This trait is highlighted by comparing studies by Sakai (1955) and Ambrose & DeEds (1945) who assessed the LD₅₀ of CIT in rats through oral treatment (LD₅₀ of 50 mg.kg⁻¹ b.w.) and subcutaneously (LD₅₀ of 67 mg.kg⁻¹ b.w.) respectively. A relevant result of this comparison is that orally consumed CIT (the usual manner in which a person or animal would be exposed CIT) is more toxic than subcutaneously injected CIT. Studies conducted on cultured hepatoma cells have found that doses up to 25 µM were cytostatic, and that concentrations >50 µM were cytotoxic (Lorkowski *et al.* 1980), whilst cell viability assessed via MTT assay for monkey kidney cells was estimated to be >220 µM over a 48 hour period, and an equivalent test on a human embryonic cell line was >120 µM over an equivalent time period (Bouslimi *et al.* 2008). Similar studies conducted on human promyelocytic leukaemia (HL-60) cells and porcine kidney PK15 cells over a 24 hour exposure period found IC₅₀ of CIT to be 50 µmol.L⁻¹ and 68 µmol.L⁻¹ respectively (Klarić *et al.* 2007; Yu *et al.* 2006). Another factor which must be considered with regard to CIT toxicity is that the decomposition product, citrinin H1, formed by heating CIT in the presence of water to temperatures higher than 100 °C, has a cytotoxicity 10-fold that of CIT (Trivedi *et al.* 1993). This is concerning given that the commodities which CIT contaminates are cooked or otherwise processed.

As is detailed above, the toxicity of CIT is noted as detailed above. Toxicity levels or levels of contamination of CIT in foods have however not been stipulated to the best of the knowledge of the author. Furthermore, considering the relative toxicity of CIT, the high rates in which it is produced and encountered around the world, there is relatively limited data available regarding its quantification on foodstuffs. Several methods of analysing it have been described in literature with different sensitivity and accuracy (Table 1.2), based primarily on chromatographic techniques (Abramson *et al.* 1995; Jeswal, 1996; Huwig *et al.* 2001; Bennett & Klich, 2003; Williams *et al.* 2004; Xu *et al.* 2006; Kononenko & Burkin 2007; Krska, & Molineli, 2007; Prieto-Simón *et al.* 2006; Bouslimi *et al.* 2008; Krska *et al.* 2008; Wagacha & Muthomi, 2008; Kumar *et al.* 2008; Arévalo *et al.* 2011). To date, however, no legislation governing its monitoring has been set in place. This is, in

part, due to analytical aspects of CIT not being fully characterised, particularly in aqueous media, and a requirement for an established method of analysis.

The applicability of electroanalysis as a monitoring system, as described through Section 1.2 below, may provide a means of establishing an effective monitoring system for mycotoxins such as CIT. The application of electrochemistry as a transducer system for mycotoxin analyse within a number of biosensor configurations was detailed through Table 1.2. To the best of the knowledge of the author, no direct electroanalytical studies on CIT (studies considering the oxidation or reduction of the CIT molecule) have been reported. The characterisation and assessment of CIT in aqueous media by electroanalysis with the intent of developing a reliable, robust, portable and inexpensive analytical system as such provides the narrower field of research in which this Thesis is situated.

1.2 Electrochemistry: an analytical system for mycotoxins in foods

An overview of electrochemistry and the principles of the methods used through this Thesis have been included in Appendix A, Section A1.

1.2.1 Benefits of the application of electrochemistry in analysis of foods

Many applicable attributes have been cited for the use of electroanalysis as an analytical technique which renders its application in the analysis of food borne toxins promising. Constant developments in electrical and information technology systems allows for the miniaturisation of devices to portable, hand held units that can be used in the field with no influence on the accuracy or sensitivity of responses, allowing for real-time analysis, non-destructive sampling, the easy, low-cost mass-production of most of components which can be disposable (screen printed electrodes) (Bakker & Telting-Diaz, 2002; Ashley, 2003; Farré *et al.* 2005). Further relevant traits include robustness, excellent compatibility with a number of analytical matrices (Ashley, 2003); rapid analysis timeframes and the capability to interlink them with bio-recognition molecules (Mascini & Tombelli, 2008) allows for incorporation of specificity of analysis into the fabrication of sensor arrays which afford simultaneous presentation of both quantitative and qualitative responses to analyte(s).

1.2.2 Drawbacks of the application of electrochemistry in analysis of foods

Fouling, also commonly referred to as poisoning or passivation of the electrode surface, is a process which decreases the sensitivity of an electrode to a given analyte. Fouling primarily occurs through the adsorption of electro-inactive materials to the electrode; effectively hindering further analyses

through impeding access of further analyte to the electrode (Berríos *et al.* 2009). Fouling materials may arise as product(s) of redox reactions (Wang & Tuzhi, 1986) or through adsorption of materials from the analysis medium. As analysis of biological samples is typified by complex analysis matrices (Mascini & Tombelli, 2008), fouling of electrode surfaces poses one of the largest drawbacks of electrochemical analyses of foodstuffs, especially within continuous analysis systems.

Further disadvantages include the generation of false-positives by the presence of electroactive substances present in the matrix (Farré *et al.* 2005). As analytes within a matrix undergo oxidation/reduction at specific potentials, a degree of selectivity is inherent to the electro-analytical processes however, there is a general lack of selectivity noted using electroanalysis as a quantitative technique – hence much research has been focused on the modification of working electrodes (chemical and biological) to impart selectivity (Bakker & Telting-Diaz, 2002; Mascini & Tombelli, 2008; Krska & Molinelli, 2007).

1.2.3 Glassy carbon electrodes

a) Glassy carbon as a working electrode

Working electrodes are selected according to specific analytical requirements: mechanical stability, potential range, electrocatalytic activity and versatility for analytical applications. Although mercury drop electrodes were originally the primary electrode used in electroanalytical studies, a survey of the literature of the past few decades clearly illustrates that solid electrodes and carbon based materials in particular (graphite powder with liquid and solid binders through to carbon fibres, tubes and balls and glassy carbon (GC)) have become the material of choice (Heiduschka *et al.* 1994; McCreery & Cline, 1996; Kissinger & Heineman, 1996; Scholz, 2002; McCreery, 2008). Carbonaceous electrodes have become broadly applied as an electrode material due to their many benefits, including: ease of accessibility; relatively low expense; broad operating potential range; the ease with which they can be cleaned and modified; non-toxicity and a reportedly higher reactivity and lower limits of detection toward many analytes, compared to metal electrodes, particularly towards adsorptive organic compounds (Kissinger & Heineman, 1996; Iotov & Kalcheva, 1998; Weisshaar & Porter, 2001; Scholz, 2002; Zhao *et al.* 2008).

GC in particular is a desirable electrode surface as it retains the aforementioned properties of carbon, but importantly, has characteristically low liquid and gas permeability, high chemical inertness, wide potential range and small pore size (Randin & Yeager, 1975; van der Linden & Dieker, 1980; Heiduschka *et al.* 1994; Kissinger & Heineman, 1996; Dekanski *et al.* 2001 a; Scholz, 2002). These traits allow for its application in a range of media, both aqueous and non-aqueous, as well as in

corrosive media (van der Linden & Dieker, 1980). Due to the versatility of GC electrodes (GCE) in electroanalytical studies, they have been applied in analyses which use large potential sweeps (e.g. voltammetry), small potential perturbations (e.g. electrochemical impedance spectroscopy) as well as potentiostatic techniques (chronoamperometry and chronocoulometry) as hydrostatic electrodes as well as in laminar flow studies (rotating disc electrodes). This flexibility, as well as the reported affinity for responsiveness to organic molecules (van der Linden & Dieker, 1980) makes GCE highly attractive electrode materials for the development of commercial analytical devices for analysis of contaminants in foodstuffs.

b) Origin, structure and function of GCE

GC is typically produced by controlled heating of high molecular-weight carbonaceous polymers such as phenol or formaldehyde resins (or a combination thereof), polyacrylonitrile, polyfurfuryl alcohol, polyvinylidenechloride etc. under an inert atmosphere. The initial heating step is generally carried out under a low pressure, where the resins are gradually heated to 600 – 800 °C to volatilise non-carbon elements. After this, a secondary step is employed to achieve carbonization of the resin material, wherein resin is slowly heated under pressure to temperatures between 1000 and 3000 °C (Jenkins & Kawamura, 1971; Jenkins *et al.* 1972; Kawamura & Jenkins, 1972; van der Linden & Dieker, 1980; Heiduschka *et al.* 1994; Kissinger & Heineman, 1996). Through this procedure, the C-C bonds of the polymer backbone do not break, which limits the size of the graphitic planes (basal and edge planes) in the region of 30 – 70 Å (McCreery, 2008). Although the procedures to this point are relatively uniform, it has been shown that the specifics of the heating procedure (temperature and pressure), as well as the state of the starting material will strongly influence the physical and electrochemical characteristics and resulting analytical stability of the GCE produced (Jenkins & Kawamura, 1971; Kawamura & Jenkins, 1972; van der Linden & Dieker, 1980; Kissinger & Heineman, 1996). GC contains both sp^2 and sp^3 hybridisation bonds of the carbon, depending on the temperature of GCE formation (Randin & Yeager, 1975). Due to the variability introduced by fabrication parameters, GCE have the propensity to vary between producers and batches if conditions are not meticulously monitored and followed. Furthermore, the microstructure of GC is highly disordered at the atomic level (McCreery, 2008), a feature which itself may introduce variability in electrochemical responses.

Jenkins & Kawamura (1971) provided a general description of the internal structure of GC which can be summarised as follows: GC is comprised of a randomly oriented network of highly matted and tangled mesh of graphite like ribbon molecules. The graphite molecules are arranged into ribbon-like chains of stacks of graphite sheets which Kawamura & Jenkins (1972) described as microfibrils, which are cross-linked to adjacent microfibrils by stable carbon-carbon covalent bonds. These inter-fibrillar links are strained and thus are less thermally stable than the bonds within ribbons

(Kawamura & Jenkins, 1972). Oberlin (1989) later illustrated a more detailed structure of GC microstructure by postulating the internal structure to be have spherical symmetry with crumpled sheets of stacked carbon, comprising of layers of planar aromatic carbon ring in sheets, stacked in parallel (Figure 1.2 [A]), which according to Heiduschka *et al.* (1994) completed the model reported by Jenkins & Kawamura (1971) and Kawamura & Jenkins (1972). This model forms the basic structure which is accepted for GCE to date.

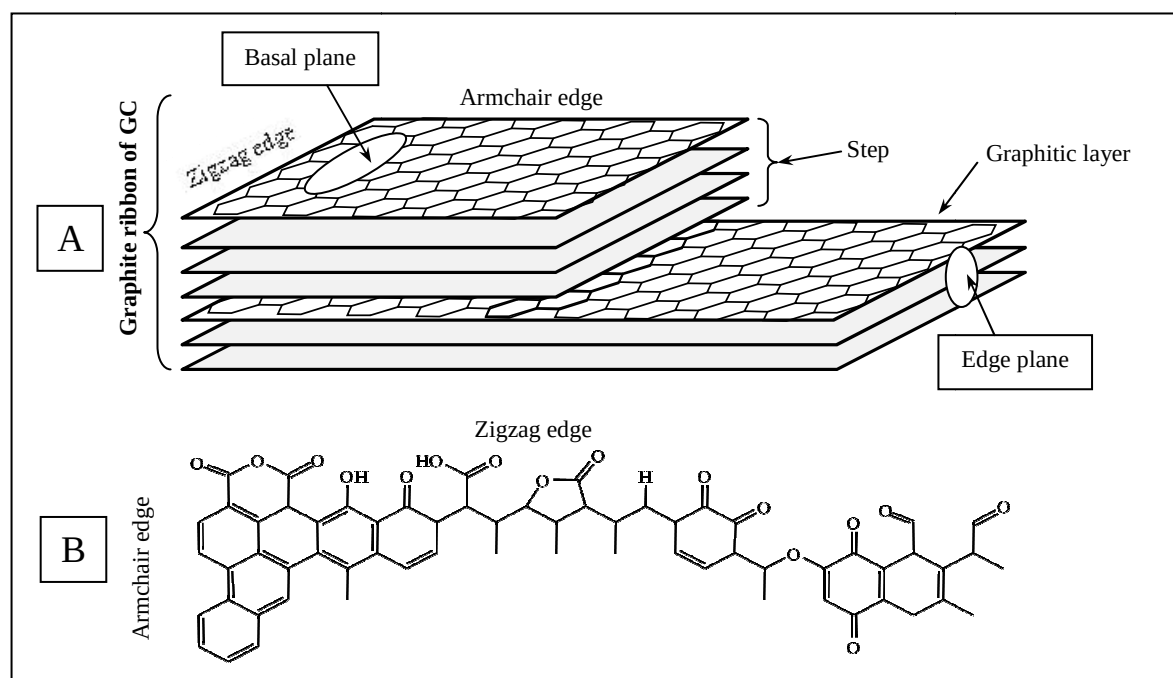


Figure 1.2: Schematic representation of GC surface microstructure of the stacked graphitic sheets of the ribbons of GC [A] (modified from Banks & Compton, 2005; Heiduschka *et al.* 1994) and representative oxide functionalities which form at the edge planes of GCE and oxidised GCE surfaces [B] (modified from Chen & McCreery, 1996; Shen *et al.* 2008; McCreery, 2008).

GC has a density of 1.5 g.cm^{-3} which implies a void volume of 33 %, however, due to pores being small and unconnected GC is impermeable to oil or gas penetration (Kissinger & Heineman, 1996). Typically, GCE is about 1 % as conductive as the basal plane of highly ordered pyrolytic graphite (HOPG) (Kissinger & Heineman, 1996).

Due to the structure of GCE, the solvent-exposed layer is a relatively disordered with basal planes exposed across the length and horizontal planes of graphitic ribbons, and edge plane exposed across the vertical plane (Figure 1.2 [A]). This feature has somewhat divided opinions concerning the mechanisms by which electrochemical activity is attained at GCE. According to McCreery (2008), the edge and basal planes differ greatly in both chemical and electrochemical reactivity. The edge plane of graphite sheets contain a variety of sites (armchair and zigzag, Figure 1.2) as well as a number of possible oxygen containing functionalities (partially represented through Figure 1.2 [B]) in

combination with the basal plane, are considered fundamental to the behaviour of GCE. Although the graphitic ribbons of GCE are largely comprised of basal plane graphite, edge planes are generated at GC as a result of fracturing of the ribbon structure, a process encountered by procedures as simple as polishing of GCE (Rice *et al.* 1989). The influence of edge and basal planes is described by comparison of responses at edge and basal plane HOPG electrodes, which show marked influence on electrochemical responses and provides compelling evidence of the nature of the electrochemical mechanism at GCE (Banks & Compton, 2005; McCreery, 2008).

1.2.4 The influence of GCE surface condition on electrochemical analyses

Application of GCE in routine electrochemical analyses requires that the surface be cleaned before application as many compounds foul the GCE surface when undergoing redox processes, requiring re-generation of the GCE surface. According to Kamau (1988), the nature and reproducibility of the method of electrode preparation as well as the prior history of an electrode treatment (fabrication specific, as well as prior surface treatments) has a major influence on responses gained at that electrode, a feature which is unsurprising, given the variable physico-chemical characteristics of GCE.

Many cleaning protocols have been cited in literature (discussed through Section 1.2.5). Although some procedures, such as polishing of GCE, are routine and necessary, each surface treatment has its own benefits and drawbacks within a given analytical system. The surface condition of an electrode which is obtained from a given cleaning procedure is largely dependent on the mode of action of that procedure (such as oxidant character of the reactive species) and the nature of the carbon material itself (Ray & McCreery, 1999; Kuo & McCreery, 1999; Dekanski *et al.* 2001 a; López-Garzón *et al.* 2003; Terzić *et al.* 2007; Zhao *et al.* 2008). Accordingly, cleaning of GCE will invariably result in electrode surfaces which differ depending on the cleaning procedure(s) used and the way in which they were applied. A further level of variation in surface condition arises through comparison of responses garnered on one surface preparation with a given cleaning procedure to another (different GCE) when cleaned by the same procedure. As each procedure has a different mode of function, the surface of a GCE cannot be reproduced exactly after a cleaning. This trait is highlighted through practices as routine as polishing of GCE discussed below (Section 1.2.5).

Variation in surface characteristics may be attributed to two main factors and the way in which analyte interacts with those factors during analysis. These include morphological or topographical (surface area) variation and surface chemistry variation (functional groups). Both of these are surface confined, but each has far-reaching implications in electrochemical interactions and resulting responses. The interactions of the electrode/electrolyte interface play an integral role in the

electrochemical responses at that surface and should thus be taken into account in any discussion relating to the electrochemical behaviour of that surface (Bleda-Martínez *et al.* 2005; Bleda-Martínez *et al.* 2006). Variation in surface characteristics holds a more pronounced effect on current response when species adsorb on to the electrode than when species are diffusive. This is due to a physical interaction between an adsorbing analyte with the microscopic (as opposed to the geometric surface area) outer Helmholtz plane of the double layer, thereby associating directly with surface morphology and surface chemistry of the electrode (Trasatti & Petrii, 1991; Jarzabek & Borkowska, 1997; Kerner & Pajkossy, 1998; Scholz, 2002; Palasantzas & Backx, 2003).

a) The influence of electrode surface area variability on electrochemical response

As is illustrated by the array of electrochemical processes which are mathematically dependant on the working electrode surface area, it is evident that real surface area is intimately connected with electrochemical responses garnered at an electrode as it provides a basis for the amount of reactant that can be oxidised or reduced to generate a current (Kissinger & Heineman, 1996; Bard & Faulkner, 2001). Although at a macro-scale, GCEs are generally considered to be homogenous surfaces, when considering GCE surface spatial composition at the micro scale, it is clear that this is not the case with regard to both surface topography (surface area) and surface chemistry (functional groups). Bulk differences between GCE can arise through the manufacture process as mentioned in Section 1.2.3 [b]. Due to the ribbon-like structure of GCE matrices, the surface of GCE cannot be atomically planar, and is considered to be a disordered surface (Kissinger & Heineman, 1996). Although the influence of atomic scale roughness on diffusive controlled responses is negligible due to surface area approximating a cross section of the double layer rather than real surface area (Trasatti & Petrii, 1991; Jarzabek & Borkowska, 1997), adsorptive molecules will be strongly influenced by roughness. This is because roughness of the surface in turn influences the amount of analyte adsorbed and hence the resulting recorded current responses upon oxidation or reduction (Kissinger & Heineman, 1996; Bard & Faulkner, 2001).

Pajkossy (2005) separated variations in electrode topography into several classes including *non planar geometry* (shapes visible to the naked eye [>0.1 mm]), which has no influence on electrochemical response, *microscopic roughness* (corrugations, scratches, pits, grooves [10 nm - 100 μ m]) which do influence electrochemical response and *atomic scale roughness* (surface disorder such as steps, kinks, edges and dislocations [<10 nm]) which, although often closely related to microscopic roughness, in practice exerts much greater effect on electrochemical response. Considering the key role of surface area in electroanalysis, researchers have struggled to accurately model and account for its influence in electrochemical responses. A number of theories have attempted to account for the influence of roughness on electrochemical studies. This has most commonly been defined as the

roughness factor (ρ), which is defined by IUPAC as the ratio of the real to macroscopic surface area. $P = 1$ is perfectly smooth, however in rough, solid electrodes such as GCE which are generally reported to range between 1 and 5, the ρ is larger ($1 < \rho < 10$) and $\rho \gg 1$ for porous electrodes (Kissinger & Heineman, 1996; Pajkossy, 1994; Pajkossy, 2005). Attempts have been made to use fractal geometry to model electrode topography and adsorption of molecules to those surfaces (Giona & Giustiniani, 1996; Kerner & Pajkossy, 1998; Huang *et al.* 1999; Mahnke & Mögel 2003; Kim *et al.* 2003; Menshykau *et al.* 2008; Ferrieu *et al.* 2009). Although useful, as it provides a description of the surface without needing to give precise descriptions of surface condition, original research paradigms which were based on electrodes being self-similar fractal surfaces showed results to be highly dependent on the surface fractal dimension (Menshykau *et al.* 2008). Subsequent analyses utilising self-affine fractals and exact mathematical functions found similarly dissatisfactory results due to the complexity of surface characteristics and the degree to which surfaces alter between preparations, resulting in only approximations of character and asymptotic solutions having been found (Menshykau *et al.* 2008). Accounting for ρ in electrochemical responses is as such a challenging problem being central to responses, while difficult to disentangle from responses without supplementary analyses conducted at that electrode. Although this is possible, it is impractical as electrode surfaces will alter with cleaning and resurfacing as described in Section 1.2.5.

b) The influence of chemical variability of an electrode surface

Carbon electrode materials and glassy carbon in particular have been described as having a ‘rich’ surface chemistry (Figure 1.2 [B]) due mainly to the diversity of functional groups which are present, and the varying ratio in which they may be encountered both across a single surface and across multiple preparations of a single surface (Collier & Tougas, 1987; Bowers & Yenser, 1991; Jürgen & Steckhan, 1992; Kissinger & Heineman, 1996; Chen & McCreery, 1996; Bleda-Martínez *et al.* 2006; Shen *et al.* 2008; McCreery, 2008). A basic chemical principle is that certain compounds (i.e. surface confined functional groups) will interact more readily with a given analyte than others and may indeed in some cases impede a given reaction from proceeding. Furthermore, chemical interactions between two molecules are largely governed by the nature of molecules and the state and environment those molecules are in. Due to the variety of surface confined chemistry at GCE electrodes, the surface itself is reactive toward a multitude of possible compounds (Kissinger & Heineman, 1996). This trait makes GCE highly applicable as a general electrode material, however may also introduce variability in response in some cases.

Preparatory processes which are as simple and routine as polishing result in variations in the surface chemistry of GCEs. This has been demonstrated by Hoogvleit *et al.* (1986), wherein polishing of GCE was shown to introduce instability in voltammetric analyses and where the surface composition was

directly related to the reactivity and thus to the resulting voltammetric response at that surface. Kamau (1988) similarly found that the nature and the reproducibility of an electrode preparation as well as the prior history of that electrode influence responses gained at that surface. These serve to highlight the impact of surface condition on electrochemical responses, since, if surface condition is not accurately replicated across multiple preparations the surface chemistry and thus response cannot be uniform.

1.2.5 Modification of GCEs

Modification of GCE has been extensively investigated in order to attain a stable, reproducible surface condition for analyses. This has been driven by factors including carbon electrode microstructure (Bowling *et al.* 1989; Rice *et al.* 1990; Kissinger & Heineman, 1996; Bard & Faulkner, 2001; Ranganathan, & McCreery, 2001, Menshykau *et al.* 2008), electrode cleanliness (Engstrom & Strasser, 1984; Kazee *et al.* 1985; Kiema *et al.* 2003) and functional groups or surface chemistry of an electrode (Evans & Kuwana, 1979; Chen & McCreery, 1996; Kissinger & Heineman, 1996, Chu & Kinoshita, 1997; Swiatkowski *et al.* 2004; Zhao *et al.* 2008) are integral to electrode reactivity.

Commonly applied modifications of GCE can be classed broadly into several categories; physical adsorption, encapsulation, entrapment, covalent attachment, oxidation or reduction reactions and polymer formation (Shen *et al.* 2008; Telesa & Fonseca, 2008). The effects of these are extensive and varied, ranging from increasing the electrode response stability by providing a protective function against corrosion and solvent effects, tailor the kinetics of electron transfer, incorporation of catalytic activity (phthalocyanines, enzymes and nanoparticles), exclusion of particles reaching the surface (use of membrane such as Nafion[®]) to name but a few (Wang, 1991; Kissinger & Heineman, 1996; Bard & Faulkner, 2001; Shen *et al.* 2008). All of these processes result in an influence on the activity of an electrode, and thus are crucial considerations in the design of analytical systems, and tailoring a sensor surface to acquire sensitivity and specificity.

Methodologies which allow for introduction of oxides at GCE as a modification of electrode surface condition include polishing of electrodes (Kazee *et al.* 1985; Kamau *et al.* 1985; Keima *et al.* 2003), laser irradiation (Poon & McCreery, 1986; Bowling *et al.* 1989; Wang *et al.* 1991), vacuum heat treatment (Stutts *et al.* 1983; Fagan *et al.* 1985; Hu *et al.* 1985), gasification by O₂ and CO₂ (Kelemen & Freund, 1988; Perić-Grujić *et al.* 2002), ozone (Zhou & Wipf, 2001; López-Garzón *et al.* 2003), ultrasound (Zhang & Coury, 1993), radio frequency plasma (Evans & Kuwana, 1979; Miller *et al.* 1981) and the most extensively studied over the past three decades, electrochemical oxidation (electro-oxidation) (Engstrom, 1982; Engstrom & Strasser, 1984; Nagaoka & Yoshino, 1986; Wang & Tuzi, 1986; Kepley & Bard 1988; Smyrl *et al.* 1989; Bowers & Yenser, 1990; Giordano *et al.* 1991;

Wang & Martinez, 1990; Bowers *et al.* 1991; Jürgen & Steckhan, 1992; Otero *et al.* 1993; McCreery, *et al.* 1994; Bielby *et al.* 1995; Chu & Kinoshita, 1997; Vettorazzi *et al.* 1998; Dekanski *et al.* 2001 a; López-Garzón *et al.* 2003; Qiao *et al.* 2008; Zhao *et al.* 2008). Three of these processes are considered in more detail for application in this study.

a) Manual oxidation of GCEs through polishing

The most basic of the procedures generally applied to cleaning of GCE electrodes is that of polishing. This is typically carried out by polishing of the electrode with a slurry of an abrasive (e.g. aluminium oxide in a range of particle sizes) in a figure-of-8 motion. The polishing process physically abrades the GCE surface, removing material attached to the surface and a portion of the GC itself and in doing so provides a fresh surface on which subsequent procedures can be performed (Wiesshaar & Kuwana, 1985; Kamau *et al.* 1985; Rice *et al.* 1989; Heiduschka *et al.* 1994; Chen & McCreery, 1996). Unfortunately, polishing breaks down the microstructure of the surface of the electrode (Rice *et al.* 1989) and scratching of the surface has been demonstrated by several high resolution imaging techniques (Engstrom & Strasser, 1984; McDermott *et al.* 1993; Kiema *et al.* 1999; Kiema *et al.* 2003; Shi & Shiu, 2002; Zhao *et al.* 2008; Thiagarajan *et al.* 2009).

Furthermore, polishing results in a wide range of reactions of the GCE surface with air and water, introducing a range of oxide species and resulting in a surface which is generally quite complex (McCreery, 2008). According to Chen & McCreery (1996), polished GCE can have an O/C ratio of up to 20 % oxygen. Functionalities which have been reported to arise from polishing (Figure 1.2 [B]) include hydroxyls, carbonyls, carboxyls and derivatives thereof (quinone, ketone, phenol, carboxylic acid, lactones and acid anhydrides) (Kamau *et al.* 1985; Rice *et al.* 1989; Heiduschka *et al.* 1994; Chen & McCreery, 1996; Zhao *et al.* 2008). As there is no specific control of the formation of the oxides and also as the actual oxide result is dependent on a number of external factors such as the polishing technique and the electrode material itself, the resulting oxide and their proportions vary between studies. As such, even this simple, ubiquitous procedure can induce variation in resulting surfaces after every cleaning procedure (Wiesshaar & Kuwana, 1985; Kamau *et al.* 1985).

b) Chemical Oxidation of GCEs

The application of chemical corrosives, such as acids, has been widely documented through literature in order to oxidise carbon materials. Acid treatment of carbonaceous materials, generally with HNO₃ or H₂SO₄, is generally used to improve the hydrophilicity of surfaces, remove chemical contaminants that may be present, enhance the acid properties and oxidise the surface of carbon materials (Kissinger & Heineman, 1996; Bleda-Martínez *et al.* 2006; McCreery, 2008; Shen *et al.* 2008). Chemical oxidation by means of acid treatment with HNO₃ and H₂SO₄ has been shown to involve the

introduction of carboxylic acid groups at the end caps or at defect sites of carbon nanotubes (Yu *et al.* 2008). The oxidation of GCE surfaces with the use of acid has been used as a cleaning protocol and as a means of introducing oxide species directly to GCE surfaces (Kelemen & Freund, 1988).

Piranha solution is a vigorous chemical oxidant of organic compounds, comprising of 1:3 (v/v) of concentrated H_2SO_4 and H_2O_2 respectively (Carrington *et al.* 2006). The use of piranha solution as an intense chemical oxidant at GCE has been applied as a cleaning step (Carrington *et al.* 2006; Boland *et al.* 2008). After an extensive literature search conducted by the author, it has been concluded that no analytical research has been carried out to date on the exact influence which piranha solution has on a GCE surface, and the resulting morphology of the GCE surface which arises from treatment with piranha solution, or the chemical nature of the resulting surface layer. By inferring the influence of piranha solution on other carbon substrates, as well as the fact that Kelemen & Freund (1988) found HNO_3 oxidised GCE and López-Garzón *et al.* (2003) found similar results for H_2O_2 , it is anticipated that piranha solution too will oxidise GCE and introduce high levels of oxygen species at the electrode surface.

c) Electrochemical oxidation of GCEs

Electrochemical oxidation (electro-oxidation) of GCEs is a process of electrode modification in aqueous solutions which results in the formation of a graphitic oxide film across the surface of the electrode, imparting many beneficial attributes in electroanalytical measurements (Kepley & Bard 1988; Dekanski *et al.* 2001 a; López-Garzón *et al.* 2003; Zhao *et al.* 2008). This has resulted in electro-oxidation as a surface treatment of GCE having been studied fairly extensively (Engstrom, 1982; Nagaoka & Yoshino, 1986; Wang & Tuzi, 1986; Kepley & Bard 1988; Smyrl *et al.* 1989; Bowers & Yenser, 1990; Giordano *et al.* 1990; Wang & Martinez, 1990; Jürgen & Steckhan, 1992; Otero *et al.* 1993; McCreery, *et al.* 1994; Bielby *et al.* 1995; Dekanski *et al.* 2001 a; López-Garzón *et al.* 2003; Zhao *et al.* 2008). The oxidation of GCE requires the application of a working electrode potential to a sufficiently high potential (higher than 1.65 V vs. SCE) (Yang & Lin, 1995) in order to result in the production of an oxide layer at that electrode surface, and involves the corrosion of the underlying carbon surface of the electrode in its formation. This has been shown to be achieved through both application of a static potential for a given period of time or cycling, by altering potential between two points. These, along with parameters such as time of oxidation, oxidation potential (or endpoint of cycle) and solution conditions such as pH and ionic composition, all influence the resultant characteristics and traits of the layer on the GCE (Kepley & Bard 1988; Giordano *et al.* 1990; Jürgen & Steckhan, 1992; Beilby *et al.* 1995; Dekanski *et al.* 2001 a; López-Garzón *et al.* 2003; Zhao *et al.* 2009).

Graphitic oxide is comprised of disordered carbon layers with covalent C-O bonds such that it contains approximately 25% oxygen by weight (Jürgen & Steckhan, 1992). Surface chemical groups include carbonyl and hydroxyl groups in the form of carboxylic acid, quinone, lactone and phenol amongst others (Bowers & Yenser, 1991). Parameters such as reactive species' oxidative capacity, time, temperature and concentration all influence the type and extent of surface groups produced. As the layer breaks down with time, the composition will alter over time (Engstrom, 1982; Jürgen & Steckhan, 1992; Beilby *et al.* 1995). The mechanism of electrochemical oxidation has not been clearly established as it is highly complex (Bowers & Yenser, 1991; Yang & Lin, 1995). A general oxidation mechanism has been noted by Yang & Lin (1995) for the formation of oxides as influenced by the solution (Table 1.3).

Table 1.3: Proposed pathways of graphitic oxide formation according to electrolyte (Yang & Lin, 1995)

Acid Solution		Alkaline solution	
$mC + nH_2O - a_1e^- \rightarrow (m-x)C - OH_{surf} + CO_2$	Eq. 1.1	$C + OH^- - e^- \rightarrow C - H_{ads}$	Eq. 1.4
$(m-x)C - OH_{surf} - nH_2O - b_1e^- \rightarrow qCO_2 + rH^+$	Eq. 1.2	$C - OH_{ads} + OH^- - e^- \rightarrow C + H_2O + 1/2O_2$	Eq. 1.5
$C + O_2 \rightarrow CO_2$	Eq. 1.3	$C + O_2 \rightarrow CO_2$	Eq. 1.6

Electrochemically modified GCEs overcome the influences of underlying GCE surface condition as the resulting surface is a comparatively uniform and relatively thick layer of graphitic oxide which masks underlying GCE condition (Kepley & Bard 1988; Dekanski *et al.* 2001 a). Although a range of oxygen functionalities will arise depending on the conditions of layer formation (allowing for a degree of tailoring of oxide characteristics), it is comparatively chemically homogenous between modifications. Thus it allows for interactions of molecules with it more uniformly, thereby increasing the reproducibility of response.

The improvement to both current stability and response was accredited to the presence or involvement of several possible traits including electron mediation by oxygen containing surface groups (carbonyl and hydroxyl groups including carboxylic acid, phenolic, lactone, and quinoidal groups amongst others), chemisorbed oxygen, graphitic oxide multilayers and the removal of surface impurities (Collier & Tougas, 1987; Kepley & Bard, 1988; Bowers & Yenser, 1991; Jürgen & Steckhan, 1992; Yang & Lin, 1995; Dekanski *et al.* 2001 a). It is improbable that there is a large influence through functionalisation of the electrode surface itself as it has been shown that such anodic treatment will result in the formation of multilayers which coat the electrode surface. As such the resulting surface is of a porous, hydrated electrode rather than a functionalised monolayer surface (Kepley & Bard, 1988).

d) Carbon nanotubes (CNT) and CNT modified GCEs

CNT are nanostructured allotropes of cylindrical mono-atomic thickness sheets of graphite (there are several forms) with dimensions around 1 - 170 nm in diameter and up to several microns in length

(Wildgoose *et al.* 2006). Multi-walled CNT (MWCNT) comprise of multiple sheets of graphite fitted one inside the other as hollow tube, herring bone or bamboo forms (Fernandez & Costa-Garcia, 2008). They have received considerable attention in the fields of material science, electronics, optics and medicine due to their unique structural properties, tensile strength, chemical stability in each of the redox states, electrical and thermal conductivity and rapid reaction rate (Campbell *et al.* 1999; Gavalas & Chaniotakis, 2000; Nugent *et al.* 2001; Baughman *et al.* 2002; Ouyang *et al.* 2002; Tasis *et al.* 2006; Vamvakaki *et al.* 2006; Vamvakaki & Chaniotakis, 2007; Fernandez & Costa-Garcia, 2008).

CNT structure includes both edge and basal planes, as in Figure 1.2 [A] (Wildgoose *et al.* 2006). A thorough comparison of voltammetric responses on MWCNT modified GCE to both edge plane pyrolytic graphite and basal plane pyrolytic graphite electrodes by Dai (2002) found that the electrochemical activity of CNT arises at the edge planes present at defect sites along the surface or at terminal ends of the CNT structure, which can then be transported across the conductive CNT as it is conductive. This has also been corroborated by Gooding (2005), though therein, a further factor of nonlinear diffusion due to the large flux of redox species around the perimeter of the defect site was also considered. In addition, an overlap of diffusion layers may result due to the minor separation distance between adjacent defect sites. The contribution of these two factors allow for the fast rate of mass transport per unit area of the CNT surface resulting in a large current response for redox species with well defined current peaks (Zhao *et al.* 2005).

Although reports as early as Britto *et al.* (1996) applied CNT in electrochemical studies, little work was done on them within electrochemistry thereafter until 2002, where, after a report by Musameh *et al.* (2002), they found widespread application within electrochemistry, primarily within electroanalysis (Wang, 2005; Gooding, 2005; Tasis *et al.* 2006; Wildgoose *et al.* 2006; Vamvakaki *et al.* 2006; Dai *et al.* 2006; Banks *et al.* 2004; Vamvakaki & Chaniotakis, 2007; Fernandez & Costa-Garcia, 2008; Henstridge *et al.* 2010). An extensive array of methodologies for application, including ways of immobilising the CNT at an electrode, co-immobilisation with other materials to tailor response and scanning techniques on those layers have been reported (Henstridge *et al.* 2010). CNT modified electrodes have found extensive application, in particular, within improving a variety of electroanalytical traits to many different and diverse analytes (Zhang *et al.* 2010; Ye *et al.* 2003; Wang *et al.* 2003; Salimi *et al.* 2004; Banks *et al.* 2004; Wang & Musameh, 2004; Wang *et al.* 2004; Deo *et al.* 2005; Gooding, 2005; Tasis *et al.* 2006; Wildgoose *et al.* 2006; Vamvakaki *et al.* 2006; Dai *et al.* 2006; Banks & Compton, 2006; Vamvakaki & Chaniotakis, 2007; Fernandez & Costa-Garcia, 2008; Golikand *et al.* 2009; Henstridge *et al.* 2010; Zhang *et al.* 2010). The electroanalytical benefits of applying CNT at GCE have been reported to include increased sensitivity, lower limits of detection, reduced fouling rates and decreased overpotentials (Dai *et al.* 2006). The decrease of

overpotential associated with CNT modified GCE relative to bare GCE is often reported as evidence for enhanced ‘electrocatalysis’ by the modified electrode surface and remains controversial. Rather, it is likely to be due to the introduction of large numbers of edge-plane-like sites onto the electrode surface compared to the bare electrode substrate (Banks & Compton, 2006; Dai *et al.* 2006) in combination with thin layer like diffusive characteristics (Henstridge *et al.* 2010).

Functionalization of CNT imparts additional chemical versatility by introducing oxide functionalities through removing end caps of MWCNT and introducing nicks in the basal plane (increasing the density of edge plane sites) (Britto *et al.* 1998; Chen *et al.* 2010) which allow for removal of impurities (metal catalyst and carbonaceous impurities), increased hydrophilicity for improved dispersion, increased electronic capacity, polymer formation, subsequent functionalisation (other than oxides) for electrode modification and the incorporation of biomolecules for both spectroscopic and electrochemical sensing applications (Banks *et al.* 2004; Gooding, 2005; Dai *et al.* 2006; Henstridge *et al.* 2010). Chemical oxidation by means of acid treatment is arguably the most common method used to functionalise CNT and involves the introduction of carboxylic acid groups onto the end caps or at defect sites along the CNT surface (Banks *et al.* 2004; Gooding, 2005; Banks & Compton, 2006; Yu *et al.* 2008). This procedure is however random, resulting in CNT segments of varying length (inhomogeneity in CNT characteristics), which when applied to an electrode exacerbates variability already encountered by immobilisation of the CNT.

Physical adsorption (drop dry method) is the simplest most commonly employed of the methods for immobilising CNT at electrodes. This comprises of dispersing the CNT in an organic solvent, often ultrasonicated and then casting an aliquot of known volume onto the working electrode surface and allowing the solvent to evaporate (Henstridge *et al.* 2010). This method leaves a layer of randomly oriented CNT. Although simple, a major drawback of this method involves extremely low inter-electrode reproducibility. Application of CNT modified electrodes have however exhibited high sensitivity toward the detection of hundreds of compounds such as glucose (Salimi *et al.* 2004), uranyle (Golikand *et al.* 2009), uric acid (Ye *et al.* 2003), 2,4,6-trinitrotoluene (Wang *et al.* 2004), nimesulide (Zhang *et al.* 2010), nucleic acids (Wang *et al.* 2003), pesticides (Deo *et al.* 2005), insulin (Wang & Musameh, 2003), dopamine (Henstridge *et al.* 2010), morphine (Salimi *et al.* 2005) and neurotransmitters (Wu *et al.* 2007) (amongst many others, with new reports appearing almost weekly). As the use of CNT as an electrode material is relatively new, significant scope still exists in the understanding and optimization of CNT for use in electroanalytical and bioanalytical sensing.

1.3 Identified knowledge gaps

Agri-toxins, both of biological and anthropogenic origins are found to contaminate foodstuffs for humans and livestock alike, making monitoring of these compounds of extreme analytical importance to mitigate their impact on human and animal health, given their properties of being widespread, often recalcitrant, varied in nature and have an array of potent medical implications. Although chromatographic methods have been identified for the analysis of many of these compounds, severe monitoring shortcomings are present, especially in developing regions, where laboratory based techniques are inaccessible, too expensive and/or there is a lack of facilities and skilled personnel. While biosensor technology is being widely studied, with a number of sensor systems being reported through Table 1.2, the development of new sensor systems which are simple to operate whilst being sensitive, accurate and robust is notoriously expensive and requires extensive research where electrode modifications are involved, especially where enzymatic and cellular systems are employed. Several severe shortcomings remain that require addressing before such technologies find successful application.

Electrochemical analysis of CIT: Although a number of electrochemical biosensor systems were described for mycotoxin analysis (Table 1.2), direct electrochemical analyses of mycotoxins is limited in literature. Reports of direct electroanalysis include aflatoxin B₁ (AFB₁) (Yaacob *et al.* 2008; Hajian & Ensafi, 2009), zearalenone (ZEA) (Ramírez *et al.* 2005; Duca *et al.* 2010), deoxynivalenol (DON) (Molina *et al.* 2008) ochratoxin A (OTA) (Calcutt *et al.* 2001; Oliveira *et al.* 2007; Perrotta *et al.* 2011), with only OTA and ZEA having been reported on at the time that this study was commenced and still no electrochemical analysis of CIT has been reported in literature. Although poorly soluble, reports on the extent of CIT solubility in aqueous media range from completely insoluble (de Carvalho *et al.* 2005) to partially soluble depending on water temperature (Sprenger & Ruoff, 1946). The establishment of an electrochemical analysis system which would allow for the analysis of CIT in aqueous media would provide a simple and user friendly system, avoiding complex solvent extraction procedures. Establishment of an electrochemical method of analysing CIT in aqueous media and subsequent characterisation of CIT as a molecule in aqueous systems would allow development of an inexpensive, fast, specific, sensitive and accurate qualitative and quantitative analytical method for CIT within complex media where co-occurrence is seen.

Electrochemistry at graphitic oxide layers: Although many beneficial characteristics of graphitic oxide at GCE have been reported, the mechanism by which the oxide layer allows for improved electrochemical responses is unknown and thus the elucidation of the mechanisms of interaction and the influences of this on the electrochemical analysis of analytes must be identified to effectively

apply such a system. Due to the poor solubility of CIT in aqueous media, the application of such a layer may allow for adsorption of CIT and in so doing allow for effective electroanalysis of CIT. Application of which may provide a model system with real world application in mind as both the mechanism by which the oxide layer allows for improved electrochemical responses and the effective electroanalysis of CIT could be identified and applied in the monitoring of CIT in environmental samples.

Surface modifications of GCE are employed for many purposes in electrochemistry, however many of the modification procedures do not give reproducible results, which in turn influence current response determination on those electrode modification. No simple technique is currently available which allows for electroanalysts to practically, routinely and effectively account for electrochemical variations as a result of GCE surface condition from the voltammetric results acquired on that electrode. Accordingly the development of a procedure which allows for the normalisation of current response is required.

Analysis of CIT from environmental samples such as food crops and processed food products has been investigated using chromatographic techniques, however the influence of complex media on the detection of CIT with electroanalytical processes has not been investigated. Accordingly, the influence of commonly encountered complex samples are investigated in order to ascertain the interaction of CIT with these samples, and the influence which that has on the development of a sensor for CIT.

1.4 Research aims and objectives

The primary objective of this research was to detail an electroanalysis method for CIT which could rival, or outcompete, currently applied chromatographic techniques. This objective focused on the ability to accurately and reproducibly analyse and assess CIT in aqueous media using voltammetry at GCE. To this end, this research was directed at identifying the traits and characteristics of CIT in aqueous media, identifying the interactions of CIT with the electrode surface and determining the kinetics of oxidation such that these characteristics and properties can be applied toward the development of a methodology to analyse and monitor CIT, and potentially other mycotoxins, from environmental samples.

Achieving these primary objectives entailed several sub-aims and sub-objectives. These aims and objectives include the following areas which are detailed further in the Chapters in which they were addressed in this Thesis:

- a) Assessment and elucidation of the influence of electrode surface condition and the impact of surface condition on the stability of voltammetric responses of CIT.
- b) Characterisation of the surface characteristics of polished and oxidised GCE such that CIT responses at those surfaces can be effectively disentangled, and understood.
- c) Achieving sensitivity and stability in current responses was separated into two sub-sections:
 - i. Detailing a methodology to achieve stability in current responses through accounting for electrode surface variability as a post data acquisition treatment, using capacitance as a reporter of surface characteristics.
 - ii. Apply a pre-data acquisition modification to electrodes (electrochemically generated graphitic oxide) to allow for stability in current responses to be achieved across multiple preparations of that surface.
- d) Through application of electrodes which provide stability in electrochemical responses, the elucidation of the mechanisms and kinetics of CIT oxidation in aqueous media.
- e) To apply the optimised system of analysing CIT to analyses within ‘real world’ (food samples and microbial growth media) samples to provide a practical assessment of the applicability of using electroanalysis as a monitoring system for CIT. This includes determination of the impact of components of such media on the analysis of CIT and elucidation of the procedural requirements for electroanalysis of CIT from food samples and microbial growth media.

1.5 Synopsis of chapters

Chapter 2 details the materials, methodologies, procedures, equipment and experimental considerations which are common through the Thesis.

Chapter 3 provides an overview of the voltammetric behaviour of CIT at unmodified electrodes. This preliminary overview serves to highlight often experienced limitations of electroanalysis in terms of variability over different electrodes.

Chapter 4 examines the oxide layers which result from two procedures examined to stabilise CIT current responses. This Chapter also provides a fundamental investigation of the morphological characteristics, chemical composition and electrochemical traits of those surfaces, as elucidated by an array of techniques including high resolution scanning electron microscopy (HRSEM), x-ray

diffraction (XRD) x-ray photoelectron spectroscopy (XPS), scanning electrochemical microscopy (SECM) and electrochemistry.

Chapter 5 examines and proposes a post data analysis tool for the normalisation of variability in current responses by accounting for relative variations in surface condition.

Chapter 6 utilises surface modifications identified to conduct a comprehensive mechanistic examination and characterisation of the electrochemical behaviour of CIT in aqueous media.

Chapter 7 applies the methods identified which provide stability of CIT current response in aqueous media to a practical assessment of the applicability of using electroanalysis to monitor CIT in complex media (environmental food samples and growth media).

CHAPTER 2

General experimental approach

This Chapter describes general experimental approaches, apparatuses, reagents, preparation methodologies and data treatments applied or followed in the proceeding Chapters. Detailed accounts of specific experimental protocols and methodologies are described in the relevant Chapter.

2.1 Materials

2.1.1 Materials and reagents

All reagents were purchased as technical grade or higher and were used without any further purification. The following were purchased from Sigma Aldrich: methanol (Chromasolv[®]), acetonitrile (chromasolv[®]), n-Hexane (SupraSolv[®]), chloroform, potassium chloride (99+ %), potassium hydroxide, sodium acetate, DL-lactic acid (85 %), $\text{Fe}(\text{CN}_6)^{3-}$, $\text{Fe}(\text{CN}_6)^{4-}$, $[\text{Ru}(\text{NH}_3)_6]^{2+}$, $[\text{Ru}(\text{NH}_3)_6]^{3+}$, 1,2-Benzenedithiol (96 %), β -Mercaptoethanol (98 %), 3-Mercaptopropionic acid (99 %), multi-walled carbon nanotubes (90 % purity) (110 - 170 nm diameters, and 5 - 9 μm lengths) and aluminium oxide (<10 μm diameter, 99.7 % pure) and carbon powder. Boric acid (99 %), glacial acetic acid (97 %), orthophosphoric acid (85 %), disodium hydrogen phosphate dehydrate (99 %), sodium acetate (98 %), citric acid (> 99%), succinic acid (>99 %) and sodium hydroxide were obtained from Merck. The following reagents were obtained from SAARCHM: sodium hydroxide, hydrogen peroxide (100 Vol), nitric acid (55 %) and dimethylformamide (99 %). Sulphuric acid (98 %) was obtained from Holpro Lovasz (South Africa). All ethanol used was absolute (>98 %) and had been dehydrated with molecular sieve (3Å pore size). Water ($d\text{H}_2\text{O}$) was distilled using a MilliQ Millipore system. Nitrogen gas (Instrument grade) was sourced from Afrox[®] South Africa and was passed through a gas-dry filter trap (Chromatography Research Supplies, USA). Snaplock microcentrifuge tubes (1.5 ml) were purchased from Lasec, South Africa.

2.1.2 Analytes

Citrinin (CIT) (isolated from *Penicillium citrinum* and provided freeze dried), Patulin (PAT) (98 %), Ochratoxin A (OTA) (isolated from *Aspergillus ochraceus*), Zearalenone (ZEA), 2,6-dihydroxybenzoic acid (98 %), 5-methylsalicylic acid (98 %), salicylic acid, catechol (> 99 %)

and phenol (> 99 %), 1,2,4-trihydroxybenzene (Triol) (97 %) and 2,4-dimethylaniline (2,4-DMA) (98 %) were all purchased from Sigma Aldrich, South Africa.

Stock solutions of CIT, PAT, OTA, ZEA, 2,6-dihydroxybenzoic acid, 5-methylsalicylic acid and salicylic acid were all prepared in dehydrated absolute methanol at 1 mg.ml⁻¹ and 2,4-dimethylaniline (2,4-DMA) (200 mM) was prepared in anhydrous ethanol and were all stored at – 20 °C prior to use in sealed, non-transparent vessels. Stocks were immediately returned to – 20 °C once sample had been aliquoted for analysis. Triol, catechol and phenol were prepared daily to 100 mM in Milli-Q water as required.

2.1.3 Safety considerations

The analytes assessed, particularly the mycotoxins and 2,4-DMA have varying degrees of toxicity and potentially severe medical implications (Table 1.1). As such each compound was handled with the utmost of care at all times by personnel wearing appropriate protection gear. Solutions and disposables containing the toxins were placed in appropriate biohazard containers and kept in a separate fume hood until disposal by certified waste disposal contractors to the institution.

2.1.4 Buffers and buffer preparation

In each case, buffers were prepared to a given pH $\pm$ 0.002 pH units at room temperature, and prepared fresh every two weeks. Buffers were stored at 4 °C until used. Where applicable, solutions were deoxygenated with nitrogen gas (instrument grade, Afrox) by purging for 5 min prior to analysis.

Sodium acetate (NaOAc) buffer:

Sodium acetate and acetic acid, (glacial) were prepared as 0.2 M stock solutions and titrated against one another until pH 4.0 was reached (Pearse, 1980).

Britton Robinson (BR) buffer:

A stock of double strength (half the prescribed volume) 0.2 M BR buffer, was prepared and stored at 4 °C until used. Once diluted to desired volume, BR buffer contained 0.2 M boric acid, 0.2 M acetic acid and 0.2 M orthophosphoric acid according to Britton & Robinson (1931). pH of buffers were adjusted using 0.5 M NaOH, the volume of the pH adjusted buffer was then corrected by adding Milli-Q water to the correct final concentration.

Citrate buffer:

Solutions of 0.1 M citric acid and 0.2 M dibasic sodium phosphate were titrated against one another until solutions of pH 4.0 and pH 8.0 were reached respectively (Pearse, 1980).

Succinic-lactic acid (SLB) buffer:

A concentrated stock solution was prepared at $\frac{3}{4}$ volume for 0.2 M succinic acid and 0.2 M lactic acid. The solution was then titrated with 0.5 M NaOH until pH 4.0 was reached, and the volume corrected with H₂O.

2.1.5 Functionalisation of multi-walled carbon nanotubes (MWCNT)

MWCNT (20 g) were suspended in 20 ml of H₂SO₄ : HNO₃ (3 : 1 $\frac{v}{v}$) and ultrasonicated (Elmasonic S10H waterbath sonicator, Elma Ultrasonic Technology, Germany) for 2 hours at room temperature. Following sonication, the functionalized MWCNT (fMWCNT) suspension was purified by successive centrifugations at 12 000 *g* for 30 minutes and resuspensions of the pellet in 200 ml of fresh Milli-Q water until the pH of the supernatant stabilised over multiple wash steps to pH 6.0. The pellet was then dried in an oven at 80 °C for application in all studies using fMWCNT. Functionalisation was confirmed through Raman spectroscopy to identify inclusion of oxygen containing functionalities by Mr. Shane Flanagan (Rhodes University, Appendix B).

2.2 Apparatus

2.2.1 Equipment and instrumentation

All electrochemical analyses were carried out using a Potentiostat/Galvanostat 30 (PGSTAT 30) (Eco Chemie, Netherlands). A Voltammetric Analytical stand (VA 663) obtained from Metrohm (Netherlands) was used to control the nitrogen purging. The VA 663 stand was coupled to the PGSTAT 302 via an Autolab IME 663 interface (Swisslab) and controlled via a desktop PC equipped with either: General Purpose Electrochemical software (GPES) Version 4.9 (voltammetry and amperometry); Frequency Response Analyser (FRA) Version 4.9 (Eco Chemie, Netherlands) or NOVA Version 1.5 (Eco Chemie, Netherlands).

A standard three-electrode electrochemical system was employed for all cyclic voltammetric analyses. Working electrodes used include glassy carbon electrode (GCE) (3 mm diameter) (BioAnalytical Systems) (BAS, USA), gold electrode (AuE) (1.5 mm diameter) (BAS) and carbon paste electrodes (CPE) prepared in glass tube (1 mm internal diameter) connected with copper wire. An Ag/AgCl electrode (BAS) saturated in 3 M KCl was used as the reference electrode for aqueous solution analysis, and a silver wire doped with Ag/AgCl was used in solvent analysis. A platinum electrode for VA stand (Metrohm) was used as the auxiliary electrode.

All pH measurements were conducted using a pH 330i pH meter connected to a SenTix 41 pH electrode (WTW, Germany) and calibrated via two point calibration prior to each measurement. Ultrasonication was carried out using an Elmasonic S10H waterbath sonicator (Elma Ultrasonic Technology, Germany).

Electrodes were dried upon chemical functionalization using a PL001 laboratory oven (Prolab Instruments, Switzerland).

Centrifugal purification of MWCNT and cell sedimentation during bioremediation studies was performed using a JA-14 rotor on an Avanti J-E centrifuge (Beckman Coulter, USA). All other small volume centrifugations were performed in microcentrifuge tubes using a microfuge[®] 16 centrifuge (Beckman Coulter, USA).

2.3 Methodology and procedures

2.3.1 Electrochemical methodology

Thorough cleaning of all glassware, including the electrochemical cell was performed prior to use. Initially the glassware was immersed in 5 % v/v nitric acid for 1 hour, then rinsed repeatedly with MilliQ water, followed by an absolute ethanol rinse and finally repeatedly rinsed in MilliQ water to remove any residual ethanol. When not in use, the electrochemical cell was stored immersed in 5 % v/v nitric acid. Prior to electrochemical analysis, the reference electrode was thoroughly rinsed with MilliQ water in order to remove residual KCl. All analyses were conducted at 25 °C. The working solution for analysis consisted of a total volume of 5.0 ml and consisted of the electrolyte solution (buffer) at the total volume minus the volume of analyte assessed required for a given concentration. Accordingly, analysis solutions were prepared by introducing a volume of analyte (stock solution of one of the analytes assessed / extracted samples in absolute methanol/ un-extracted or real world samples). The solution for “conditioning” scans carried out prior to analyte scanning comprised solely of 5.0 ml of the electrolyte used in scanning for the analyte (but with no analyte in solution). All electrolyte solutions were degassed with nitrogen gas (instrument grade, Afrox) for 5 minutes prior to electrochemical analysis.

a) Electrode preparation and cleaning protocols

Glassy carbon electrodes (GCE):

Prior to each scan, all GCE were cleaned by initially rinsing with copious amounts of Milli-Q water followed by absolute ethanol and again with Milli-Q water. The surface of the GCE was then polished for at least 30 seconds in a figure of 8 motion on a Buöhler felt pad (BAS) with an alumina oxide slurry (<10 micron) to a mirror finish, followed by rinsing with absolute ethanol then Milli-Q water. From this point, electrodes were either used directly in electrochemical analysis (as polished GCE, in which case they were sonicated for 1 minute in H₂O before being used), or were modified as is described in Chapter-specific methodology when employed. The cleaning procedure was carried directly before each electrochemical analysis and prior to any modification of the electrode surface.

Gold electrodes (AuE):

An equivalent polishing procedure was followed as that described for GCE above, however after polishing, each AuE were subjected to further cleaning procedures. After polishing, AuE and sonicated in ethanol (60 seconds) and rinsed with Milli-Q water. Thereafter, AuE were chemically etched using fresh Piranha solution for 2 minutes before being rinsed with successive washes of Milli-Q water and ethanol. Electrochemical pretreatment was performed by cycling between

potentials of -0.15 V and +1.60 V (vs. Ag/AgCl) at a scan rate of 0.1 V.s^{-1} in a solution of 0.1 M aqueous H_2SO_4 solution until a stable baseline was achieved (~ 100 cycles), followed by a wash with Milli-Q water. Thereafter AuE were dried under N_2 flow and stored in de-gassed ethanol before being used. The above succession of cleaning stages used in preparing AuEs has been shown to reduce surface roughness and decrease surface inconsistency between cleanings (Carvalho *et al.* 2005).

Carbon paste electrodes (CPE):

Carbon powder (graphite powder, Sigma-Aldrich) was initially ground with a mortar and pestle until the powder became ‘flaky’ and the flakes gained a silver sheen. Mineral oil (Fluka) was then added in a drop wise manner until a thick, dry paste was achieved. This paste was then packed into hollow glass tubes, with an internal diameter of 1 mm. The CPE were electrically connected with copper wire (0.5 mm diameter). Excess oil was pressed from the CPE using paper towelling and compressing the paste with the wire. The cleaning procedure for CPE consisted of plunging the wire connector at the base of the electrode deeper such that a thin layer (which would be the spent surface) was squeezed out the front. This layer was simply wiped off on a paper towel flush to the surface of the glass rod containing the carbon paste, presenting a fresh surface. This cleaning procedure was carried out directly before each electrochemical analysis or modification procedure.

b) Electrode pre-treatment and modification protocols

In each case, the modification or pre-treatment procedures were done so following cleaning of the electrode and prior to use in assessing an analyte. After each of these protocols, electrode conditioning was immediately carried out on each surface modification, the details of which are described in Section 2.3.1 [d].

Electrochemical oxidation of carbon electrodes:

Electrochemical oxidation was carried out according to a slightly modified protocol to that described by Kepley & Bard (1988), though it must be noted that electrochemical modification procedures are numerous and varied within literature. This procedure was carried out on both GCE and CPE and comprised of continuously and successively cycling electrodes in a solution of 0.1 M H_2SO_4 for a total of 20 scans at 200 mV.s^{-1} between potentials of 0.0 V and 1.8 V using cyclic voltammetry. This procedure was followed to generate the electrochemically oxidised base of graphite oxide on the carbon electrodes.

Chemical oxidation of glassy carbon electrodes:

Chemical oxidation was carried out at GCE surfaces only as a modification protocol, as the chemical action of the solution used is extremely vigorous and resulted in physical degradation of CPE. Piranha

solution was prepared immediately before immersion of an electrode from concentrated H_2SO_4 (98 %) and H_2O_2 (100 Vol.) in a ratio of 1:3 v/v . This was performed in a 1.5 ml snaplock microcentrifuge tube sufficient to submerge the tip of the electrode and minimise the possible oxidative effect of vapour on the copper connector at the top of the electrode. Electrodes were submerged for 30 seconds exactly and immediately thereafter gently rinsed with copious amounts of Milli-Q water to avoid damage to the oxide layer formed.

fMWCNT electrode surface modification:

fMWCNT were suspended in DMF (2 mg.ml^{-1}) by sonication for 60 seconds. The same suspension was used in all electrode modifications, wherein, $2.5 \mu\text{l}$ was applied onto the GCE and dried at 70°C , and the entire process repeated twice more, resulting in a final loading of $7.5 \mu\text{l}$ of the fMWCNT suspension.

Self-assembled monolayer (SAM) modification:

SAM modification was carried out at cleaned AuE. The molecules with which SAMs were produced include 1,2-benzenedithiol, cysteaminium chloride, β -mercaptoethanol (98 %), 3-mercaptopropionic acid (99 %) and a mixed SAM of 1,2-benzenedithiol, and 3-mercaptopropionic acid. 1 mM solutions of each were prepared in 20 % ethanol with Milli-Q water. Each solution was de-aerated with N_2 for 20 minutes, thereafter, cleaned gold electrodes were immersed, suspended vertically in solution and left to stand at room temperature in the dark for 18 hours, after which modified electrodes were rinsed and applied directly in analyses.

c) Conditioning of electrodes

Once cleaned or surface modified, all electrodes were conditioned before being used in assessment of an analyte. This process comprised of cycling the electrode under the same potential window used in that study (predominantly LSV) in the specific electrolyte solution used in that study for 15 cycles. This was carried out under identical conditions as to those under which the analyte was to be assessed and immediately before assessment of an analyte, but in the absence of that analyte. This allowed for a stable current baseline to be established for each buffer solution before scanning for the analyte of interest, and was recorded as the background signal for each scan carried out.

2.3.2 Electrochemical scan methods used

Electrochemical impedance spectroscopy (impedance/EIS), cyclic voltammetry (CV), linear sweep voltammetry (LSV), chronoamperometry (amperometry) and chronocoulometry were employed as the sole electroanalytical waveforms through the course of this Thesis. In each case, up to 9 separate GCE

were used, such that global responses across diverse electrodes could be assessed. The GCE were purchased from BAS over several years, thus they would have come from different batches and would provide the variability between GCE that was expected in this study. All scan parameters were carried out a minimum of 3 replicates observation. In each case, the scanning parameters and methods applied through the course of this Thesis have been detailed as part of the Chapter-specific methodology.

2.3.3 Data analysis and interpretation

All data sets comprised of responses reported in triplicate as a minimum. Data sets were initially analysed with application of least of mean squares data treatment for both regression and correlation analyses in the software package Microsoft® Office Excel (2007) unless otherwise stated. Regression analysis results which were applied in further calculations were determined with application of the regression function of Microsoft® Office Excel (2007) “Data analysis” add-in package.

Further data analyses:

Other than the data treatments reported below, which may have been carried out have been detailed within the Chapter specific methodology, relating to the sections in which they were applied. All responses were analyses through determination of the ***mean***, also reported as average, (μ) as well as ***standard deviation from the mean*** (σ) in all data sets (Kitchens, 1998; Rosner, 2000).

Coefficient of variation (C.V.) was calculated according to the ratio of the standard deviation (σ) to the mean (μ) (Rosner, 2000):

$$C. V. = \frac{\sigma}{\mu} \times 100 \quad \text{Eq. 2.1}$$

Significant difference between data points was assessed through conducting ANOVA analyses were conducted using the statistical analysis software package STATISTICA© ver. 8.0 to determine the presence of significant difference of a data set. Significant difference between data points was assessed with application of Fisher least mean squares (Fisher LSD) analysis.

a) Determination of peak height (I_{pa})

Voltammetric scans (Figure 2.1) were interpreted by calculating the peak height (I_p) and peak potential (E_p). In the case of CIT analysis, the primary peak was monitored. A linear front base line was established at the same point in every scan of each analyte for uniformity of data (points may differ between analytes), and I_p acquired as the difference in current response between the peak apex

and the base line, along with E_p , being potential of the apex of that peak. This method was employed across all conditions, electrodes, electrode modifications and analytes assessed.

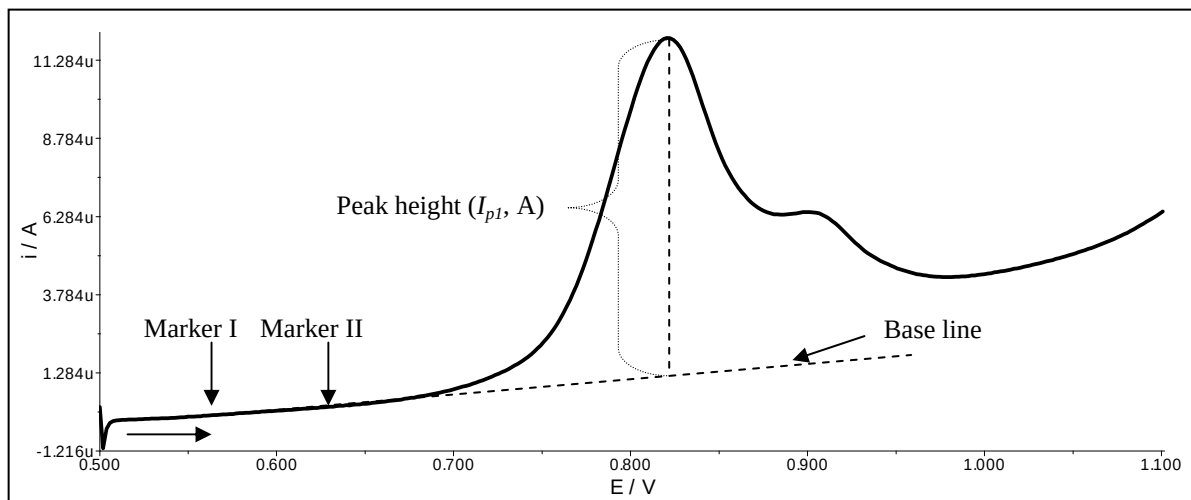


Figure 2.1: Determination of peak height of an LSV scan of CIT at 30 μM in 0.2 M NaOAc buffer pH 4.0 at 0.1 V.s^{-1} vs. Ag/AgCl. Markers specific to CIT set at 0.55 V and 0.60 V respectively.

b) Determination of electrode surface area

AuE surface area determination:

During the cleaning procedure of AuE, the electrodes are cycled in 0.5 M H_2SO_4 from -0.25 V and +1.5 V. This results in the formation of a gold oxide oxidation-reduction couple from gold oxide forming a monolayer on the AuE surface. Monitoring of which through integration of charge (Q) of the area under the gold oxide reduction peak (I_{pc}) at the AuE surface back into gold (Figure 2.2) allows for determination of surface area with application of Eq. 2.5.

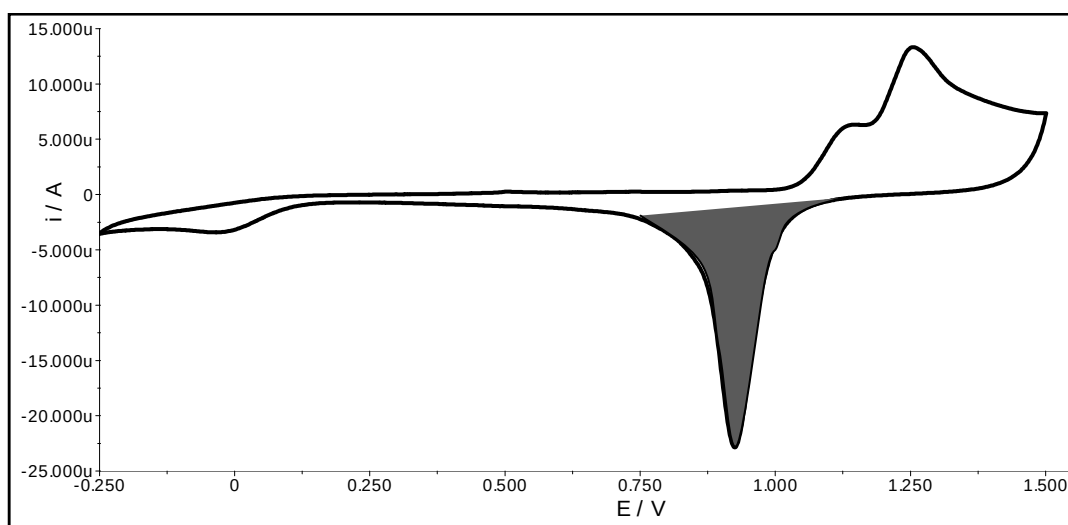


Figure 2.2: CV profile of a clean gold electrode, acquired through cycling by CV in 0.5 M H_2SO_4 between -0.25 V and 1.5 V (approximately 100 cycles) at 0.1 V.s^{-1} vs. Ag/AgCl. Shaded area indicates the area integrated to determine the surface area of the electrode.

The value of the charge required for the reduction of the gold oxide layer is dependent on the crystalline structure of gold and the value of $390 \pm 10 \mu\text{C}.\text{cm}^{-2}$ is a measure of the standard reference charge for polycrystalline Au (Q_{Std}) (Trasatti & Petrii, 1991; Carvalhal *et al.* 2005). Surface area was determined from the ratio between the charge of the gold oxide reduction on the studied electrode surface ($Q_{\text{Au pc}}$) and Q_{Std} .

GCE surface area determination:

Trasatti & Petrii (1991), and Jarzabek & Borkowska (1997) provided comprehensive analyses of experimental methods of determining SA, and highlight the limitations of the various methods of doing so. These can be separated into three main groups: [i] diffusion controlled mass transport of redox probe molecules; [ii] adsorption of probe molecules at an electrode surface or [iii] measurement of interfacial capacitance. However each of the groups proved to determine SA inaccurately or with results which were dependent on the experimental procedure applied.

Such studies are further complicated when attempting to monitor a layer such as graphitic oxide *in situ* at a GCE. The reasons for which include: electrode porosity i.e. extensive internal surface area must be accounted for. As Kepley & Bard (1988) reported poor conductivity of the layer, it was unlikely that the entire oxide layer would contribute to responses, thus only the surface area which was conductive would contribute to electrochemical responses, limiting procedures such as BET analysis which would determine total surface area rather than the electrochemically active surface area. Furthermore, as the graphitic oxide layer is hydrated and degrades over time (Kepley & Bard, 1988), application of *ex situ* procedures may result in alteration of surface area through both dehydration of the layer altering pore structure and time dependant breakdown of the layer. Due to these traits, only electrochemically assessed, *in situ* determination of surface area was considered.

Redox probes such as $[\text{Fe}(\text{CN})_6]^{3-/4-}$ and $[\text{Ru}(\text{NH}_3)_6]^{3+/2+}$ are commonly applied in electrochemical analyses of diffusion controlled mass-transport processes. The limitations of this analytical technique were reviewed by Trasatti & Petrii (1991): essentially, the technique provides, a best, a measure of a cross-sectional area of the diffusion layer of that electrode, not that of the electrode surface area. This diffusion layer forms a ‘smoother’ surface than the real microscopic surface roughness of an electrode, resulting in the analysis tending toward the geometric surface area (0.071 cm^2 for GCEs) and underestimating the real surface area that would interact with adsorbed species, which Menshikau *et al.* (2008) showed posed the greatest influence on electrochemical responses. Further hindering factors of redox probe based determination of surface area include that current yields (I_{pa} and I_{pc}) must be perfectly reversible, requiring exactly controlled electrode characteristics (surface charge density, kinetics, surface chemistry, and surface pK_a) (Trasatti & Petrii, 1991). The application

of adsorptive probe species to measure surface area *in situ* are already precluded, as one cannot ensure that the molecules are effectively removed from the surface (especially true of porous electrodes such as those used in this Thesis). When that electrode would be further used for an analytical experiment as adsorbed contaminants would hinder a desired analyte interacting with the electrode as well as producing interferent signal when scanned, complicating and possibly masking results.

Although each method does hold drawbacks, due to the considerations discussed above, the application of a redox probe and capacitance derived surface area determination methodologies were considered the closest approximation for graphitic oxide layers for determination of real GCE and EOX-GCE surface area.

Within porous layers, it is possible that the influence of bulk mass transport considerations which limited the application of redox probes at solid electrodes may be limited, and possibly allow for more accurate analyses. Due to this the redox probe technique was assessed herein with application of both positively ($\text{Cl}[\text{Ru}(\text{NH}_3)_6]^{2+/3+}$) and negatively ($\text{K}[\text{Fe}(\text{CN})_6]^{3-/4-}$) charged redox probes. Furthermore, as scan rate would influence responses, multiple scan rates were applied, such that the analysis could be reported as a multipoint analysis. Cyclic voltammetry was applied by monitoring the magnitude of I_{pc}/I_{pa} relative to incrementally increased scan rate, of the reversible redox reaction of freshly prepared equimolar (1.0 mM) $\text{K}[\text{Fe}(\text{CN})_6]^{3-/4-}$ or $\text{Cl}[\text{Ru}(\text{NH}_3)_6]^{2+/3+}$, each prepared in 100 mM KCl. Surface area could then be found using the Randles-Sevcik equation for the forward peak of a reversible system (Jarzabek & Borkowska, 1997; Bard & Faulkner, 2001):

$$I_{pa} = (2.69 \times 10^5) n_t^{3/2} A D^{1/2} C_o v^{1/2} \quad \text{Eq. 2.2}$$

Where I_{pa} is the anodic peak current of $\text{K}_3\text{Fe}(\text{CN})_6$ or $\text{Cl}_3[\text{Ru}(\text{NH}_3)_6]$ oxidation, n_t is the number of electrons in the reaction ($n = 1$), D is the diffusion coefficient, ($\text{K}_3\text{Fe}(\text{CN})_6 = 7.6 \times 10^{-6} \text{ cm}^2.\text{s}^{-1}$ and $\text{Cl}_3[\text{Ru}(\text{NH}_3)_6] = 6.0 \times 10^{-6} \text{ cm}^2.\text{s}^{-1}$), A is the surface area, C_o is the concentration of $\text{K}_3\text{Fe}(\text{CN})_6$ ($\text{mol}.\text{cm}^{-3}$) and v is the scan rate ($\text{V}.\text{s}^{-1}$). From a curve of I_{pa} vs. $\sqrt{v}$, the slope was determined and used in determination of A .

Due to the number of parameters which influence capacitance, Trasatti & Petrii (1991) reported that C_{dl} provides a relative measure of change in surface area or as an internal check. As double layer charge develops in relation to surface area, reporting relative change in C_{dl} as a measure of charge acts a proxy of relative change in surface area. Due to the double layer forming across the entire surface which is charged, responses are not limited by reaction kinetics or mass transport consideration of redox molecules. The application of capacitance is described as a central theme through Chapter 5.

c) Assessment of current (I_p) response

Reproducibility, (R) of current response was expressed as:

$$R (\%) = 100 - C.V. \quad \text{Eq. 2.3}$$

Sensitivity of response (S_p) is equivalent to the ratio of peak current response (I_p) to analyte solution concentration (C^o) ($A.M^{-1}$).

$$S_p = \frac{I_p}{C^o} \quad \text{Eq. 2.4}$$

d) Limit of detection

The limit of detection (LOD) is the lowest detectable I_p that is equivalent to three times the standard deviation of the baseline of the blank scan (Kissinger & Heineman, 1996). The LOD can be determined from the gradient, (m) of a linear plot of I_{pl} vs. concentration of the analyte (C^o) and (δ) standard deviation in baseline current according to the equation:

$$LOD = \frac{3\delta}{m} \quad \text{Eq. 2.5}$$

e) Determination of surface coverage/adsorbed material at an electrode, Γ ($mol.cm^{-2}$)

Determination of surface coverage of analyte adsorbed at modified GCE as well as before and after SAM formation at AuE was estimated from the charge (determined as the area under the curve), Q of I_p peaks according to (Kissinger & Heineman, 1996):

$$\Gamma = \frac{Q}{nFA} \quad \text{Eq. 2.6}$$

Where Γ = surface coverage ($mol.cm^{-2}$), F = Faraday constant = $96485.34 C.mol^{-1}$, A = surface area of the electrode (cm^2) and n = number of electrons involved in the reaction.

f) Degree of fouling of electrode surface

The degree of fouling was calculated as a percentage of the original peak current signal according to the equation:

$$\text{Degree of Fouling (\%)} = \frac{P_{a \text{ final}}}{P_{a \text{ Initial}}} \times 100 \quad \text{Eq. 2.7}$$

CHAPTER 3

Preliminary studies evaluating electroanalysis of citrinin at unmodified electrodes

3.1 Introduction

Traditionally, spectroscopic and chromatographic techniques have been used for analysis of mycotoxin contaminated foods (Gilbert, 2002; Pittet & Rover, 2002; Prieto-Simón *et al.* 2006; Turner *et al.* 2007). As described in Chapter 1, electroanalysis offers several advantages in food monitoring processes over these methods, including increased sampling speed, reduced expense, insensitivity to turbidity, possibility for miniaturisation of devices, on site analysis, production of robust and simple to use systems, multiplexing, and mass production at a very low expense (Baeumner, 2003; Meneely *et al.* 2011). The requirement for such analytical systems has been highlighted by reviews concerned with analysis of food-borne toxins (Baeumner, 2002; Prieto-Simón *et al.* 2006; Krska & Molinelli, 2007; Krska *et al.* 2008; Mascini & Tombelli, 2008) and is further evidenced by the range of electrochemical detection systems dedicated to mycotoxin analyses listed in Table 1.2 of Chapter 1. For these reasons, electroanalysis, and amperometric analysis in particular, have been demonstrated to be an efficient and highly suitable means for analysis of food contaminants.

Although many electrochemically transduced sensor systems have, and continue to be described for mycotoxins (Table 1.2), reports of direct analyses of mycotoxins and characterisation of mycotoxins by electrochemical means are limited. Publications detailing electroanalysis of mycotoxins can be found as early as the late eighties for ZEA by Ware *et al.* (1989) and early nineties for altertoxins by Visconti *et al.* (1991), though these studies were linked to chromatographic separation. Direct electrochemical analyses of mycotoxins has only been reported on since the mid-nineties, primarily focused on a group of structurally-related mycotoxins including alternariol, alternariol mono-methyl ether and altertoxin I by Fernández and co-workers (Moressi *et al.* 1997; Holak *et al.* 1997; Ramírez *et al.* 2005). Within a relatively short timeframe, and with relatively few researchers having addressed this field, the possibility for application of direct electroanalysis of mycotoxins has already been made clear, with several reaction media having been used. Electroanalysis usually proceeds with adsorptive preconditioning followed by cyclic, differential pulse or square wave voltammetry, primarily at GCE as shown in Table 3.1. Over the past five years, interest has increased and several studies on prevalent

mycotoxins have been reported. Table 3.1 describes the electroanalytical techniques, electrodes and analysis media which have been applied to the analysis of several mycotoxins.

Table 3.1: Literature review of direct electrochemical analysis of prominent mycotoxins

Analyte	Method ^a	Electrode ^b	Electrolyte ^c	Reference
Aflatoxins	B1, B2, G1 and G2	HPLC linked to DPP	30% ACE with 0.01M TBAB	Holak <i>et al.</i> (1997)
	B1	Adsorptive cathodic SWSV	BR buffer pH 9.0	Yaacob <i>et al.</i> (2008)
	B1 and B2	Adsorptive cathodic SWSV	BR buffer pH 9.0	Hajian & Ensafi (2009)
Ochratoxin A (OTA)	CV	GCE	ACE and in PB (pH 6-8)	Calcutt <i>et al.</i> (2001)
	DPV	GCE	PB (pH 2-12)	Oliveira <i>et al.</i> (2007)
	SWV	GCE	10% ACE 90% 1.0 M HClO ₄	Ramírez <i>et al.</i> (2010)
	CV and SWV	AuE-SAM	PB (pH 4, 7 & 8)	Perrotta <i>et al.</i> (2011)
Zearalenone (ZEA)	Adsorptive SWV	GCE	20% ACE 80% 1.0 M HClO ₄	Ramírez <i>et al.</i> (2005)
	HPLC linked to HV	GCE with MWCNT	methanol:water (60:40)	de Andrés <i>et al.</i> (2008)
	CV and DPV	GCE	DMSO with 0.1 mol/L TBAP	Duca <i>et al.</i> (2010)
Deoxynivalenol	CV and SWV	GCE and CPE	PB (pH 8.0)	Molina <i>et al.</i> (2008)

[a] DPP = differential pulse polarography, SWV = square wave voltammetry, SWSV = square wave stripping voltammetry, CV = cyclic voltammetry, DPV = differential pulse voltammetry, HPLC = high pressure liquid chromatography, HV = Hydrodynamic voltammetry

[b] HMDE = hanging mercury drop electrode, GCE = glassy carbon electrode, AuE-SAM = gold electrode with a cysteamine self assembled monolayer, CPE, carbon paste electrode

[c] ACE = acetonitrile, TBAB = tetrabutylammonium borate (electrolyte), BR = Britton-Robinson buffer, PB = phosphate buffer, DMSO = dimethylsulfoxide, TBAP = tetrabutylammonium perchlorate (electrolyte)

At the time that this study commenced, direct electroanalysis of only OTA (Calcutt *et al.* 2001; Oliveira *et al.* 2007) and ZEA (Ramírez *et al.* 2005) of the highly toxic mycotoxins had been reported on, and only OTA studies considered kinetic parameters of oxidation. Thus, there exists significant scope for further electroanalytical research in this area. Furthermore, no electrochemical analyses have been reported for mycotoxins such as citrinin (CIT), patulin (PAT) as well many of the other mycotoxins listed in Table 1.1. additional scope thus exists for the utilisation of electrochemical sensing technologies to analyse and enhance the detection of mycotoxins in routine analysis, particularly within the paradigm of biosensor development.

Accordingly, this Chapter is directed at assessing the electrochemical activity of CIT at carbonaceous (glassy carbon (GCE) and carbon paste (CPE)) and gold (AuE) electrodes as these materials are routinely used in biosensor applications. Electroanalysis of PAT and ZEA at GCE was also investigated and the results thereof indicated in Appendix C. Although mycotoxins are typically soluble in organic solvents rather than aqueous solutions, if analyses can be carried out in aqueous media, monitoring processes may be made more practical, simple and user-friendly, and thus be more applicable as a monitoring system directed at developing nations, and which is intended to be operated by semi-skilled persons.

3.2 Chapter objectives

The primary objective of this Chapter was to assess the electrochemical response of CIT at different electrodes in aqueous media, serving as a baseline for studies conducted through Chapters 4 - 6. A more detailed electrochemical characterisation of CIT is provided in Chapter 6.

Secondary objectives include:

- a) Voltammetric determination of the peak(s) representative of CIT reduction/oxidation
- b) Assessment of preliminary mechanistic information regarding that electrochemical process and to ascertain under what methodological and scanning parameters CIT can be effectively and reproducibly analysed.
- c) Determination of the source of variability of I_{pl} response and the assessment of the parameters governing the interactions which impart variability, such that stability in response could be obtained.

3.3 Chapter specific methods and materials

General protocols detailing the reagents, analytes, instrumentation and electrochemical procedures which were described in Chapter 2 were followed herein. Specific details on the experiments carried out in this Chapter are illustrated hereafter with reference to their specific sections.

3.3.1 Reagents

A stock solution of 5 mM CIT was prepared in dehydrated absolute methanol and stored at $-20\text{ }^{\circ}\text{C}$ until used in all studies.

3.3.2 Electrochemical procedures and parameters

A three electrode electrochemical cell was utilised with platinum auxiliary electrode and Ag/AgCl reference electrode with the apparatus described through Chapter 2, Section 2.2.1, whilst gold (AuE), glassy carbon (GCE) and carbon paste (CPE) working electrodes were used where appropriate. Bare electrodes (polished and cleaned GCE and AuE and CPE) were used in all the studies with cyclic voltammetry (CV), linear sweep voltammetry (LSV) and chronoamperometry being used as scan forms, with the electrochemical parameters detailed under each subheading below. Working electrode fabrication, cleaning and preparation was followed as described through Chapter 2, Section 2.3.1.

Unless otherwise stated, all analyses were conducted in 0.2 M NaOAc buffer at pH 4.0 with CIT dissolved into solution at $20\text{ }\mu\text{M}$ concentration. Voltammetric analyses were performed in a scan range of $+0.5\text{ V}$ to $+1.1\text{ V}$ at 100 mV.s^{-1} vs. Ag/AgCl reference electrode. Where appropriate, a preconditioning procedure of holding the electrode at $+0.4\text{ V}$ for 120 seconds with solution agitation at 1000 rpm was used. Background current (recorded after 15 preconditioning scans in the buffer used without analyte in solution) was subtracted from all voltammetric scans through the course of this Thesis.

a) Scoping analyses for CIT electroactivity at GCE

CIT was analysed at polished GCE using CV at a solution concentration of $50\text{ }\mu\text{M}$ in this section. Initially analyses were conducted in 0.2 M BR buffer, pH 4.0 over a potential range of -0.8 V to $+1.2\text{ V}$ using CV. Both anodic- and cathodic-initiated sweeps were used and included a preconditioning step comprising of holding potential at 0.0 V for 120 seconds with solution agitation at 1000 rpm. Subsequently, a limited scan range of $+0.5\text{ V}$ to $+1.2\text{ V}$ was applied to focus on the representative peaks of CIT. No preconditioning step was applied.

b) Identification of the primary oxidation peak of CIT

Analyses were conducted at polished GCE with CIT at 10 μM via CV. Two potential ranges were assessed, separated between the two CIT peaks (I_{p1} and I_{p2}), the first range was between 0.5 V and 0.87 V, the second was between 0.87 V and 1.1 V. No preconditioning period was used to avoid cross adsorption and formation of possible oxidation products.

c) Analysis of CIT at GCE

Analyses of CIT were carried out using LSV under normal scanning parameters (Section 3.3.2). Three separate GCE were analysed with four scans per electrode, cleaning between each scan, in a single homogenous solution to assess reproducibility of electrochemical responses. No preconditioning hold step was applied.

d) Continuously cycled voltammetric analyses of CIT

Analyses were carried out at GCE and CPE under standard scanning conditions (Section 3.3.2). Five successive, continuously cycled scans were conducted in each voltammetric analyses. Regeneration of GCE surface was investigated using voltammetry by allowing the electrode to stand in agitated CIT solution for time intervals of 1 minute and 5 minutes, respectively, before re-scanning (without cleaning of the working electrode between scans).

e) Assessment of the influence of electrode material on CIT I_{p1} variability

Analysis of CIT at carbon paste electrodes (CPE)

CPE were used for CIT analysis using LSV. CPE were resurfaced after each CIT scan with four scans carried out per electrode in a single solution under standard scanning conditions.

Electroanalysis of CIT at gold electrodes (AuE)

CIT was analysed at cleaned AuEs (Section 2.3.1, Chapter 2) in standard solution conditions (Section 3.3.2) with potential ranges of -0.8 V to +1.1 V and +0.5 V to +1.1 V respectively.

Electroanalysis of CIT at functionalised multi-walled carbon nanotube modified GCE (fMWCNT-GCE)

fMWCNT prepared according to Section 2.1.5, Chapter 2, and deposited on nine separate GCE as described in Section 2.3.1 [b] of Chapter 2 were prepared. Electrodes were resurfaced and scanned on four occasions each ($N = 36$).

f) Assessment of the influence of selected electrolyte characteristics on CIT I_{p1} variability

Detection of CIT in organic solvents

CIT was assessed at GCE relative to a silver wire quasi-reference electrode (Ag-QRE) as the reference electrode and platinum wire as the auxiliary electrode. CV analyses were conducted in absolute dehydrated methanol in a potential range of 0.0 V and 1.1 V at 100 mV.s⁻¹. Lithium perchlorate (LiClO₄) (0.2 M) and triethylammonium phosphate (TEAP) (0.1 M) were employed as electrolytes in separate solutions, each using CIT at 20 μM.

Influence of pH on CIT responses

CIT was assessed at GCE in 0.2 M BR buffer in a pH range of pH 2 – 10, at a concentration of 10 μM. CIT was assessed by CV between +0.5 V to +1.1 V at a scan rate of 100 mV.s⁻¹ without an adsorption step.

g) Assessment of the influence of preconditioning parameters on CIT I_{p1} variability

Influence of adsorption potential on CIT analysis

CIT was assessed at 20 μM using polished GCE in 0.2 M NaOAc, pH 4.0 via LSV in a potential range of 0.5 V to 1.1 V at 100 mV.s⁻¹. The preconditioning step consisted of electrodes being held in solution agitated at 1000 rpm for 60 seconds at potentials of -0.2 V, 0.0 V, 0.2 V, 0.4 V, 0.6 V, 0.8 V and 1.0 V respectively.

Influence of preconditioning duration on CIT electroanalysis

CIT was assessed at 20 μM at polished GCE in 0.2 M NaOAc, pH 4.0 via LSV in a potential range of 0.5 V to 1.1 V at 100 mV.s⁻¹. The preconditioning step consisted of electrodes being held at 0.4 V in solution agitated at 1000 rpm for 0, 10, 30, 60, 90, 120, 150 and 300 seconds respectively.

3.4 Results and discussion

3.4.1 Scoping analyses for CIT electroactivity at GCE

CIT analyses exhibited a strong electrochemical response in aqueous media at GCE (Figure 3.1). Several peaks of interest were present within the scan range and were numbered I_{p1} to I_{p11} according tier sequential formation in the anodic forward scan (black) of Figure 3.1 [A] from the most prominent peak (I_{p1}). For ease of comparison that nomenclature was retained when considering cathodic forward scan (grey) in this section as well as in analyses conducted in Chapter 6. Both scan directions are presented overlain one another for ease of comparison of the peaks.

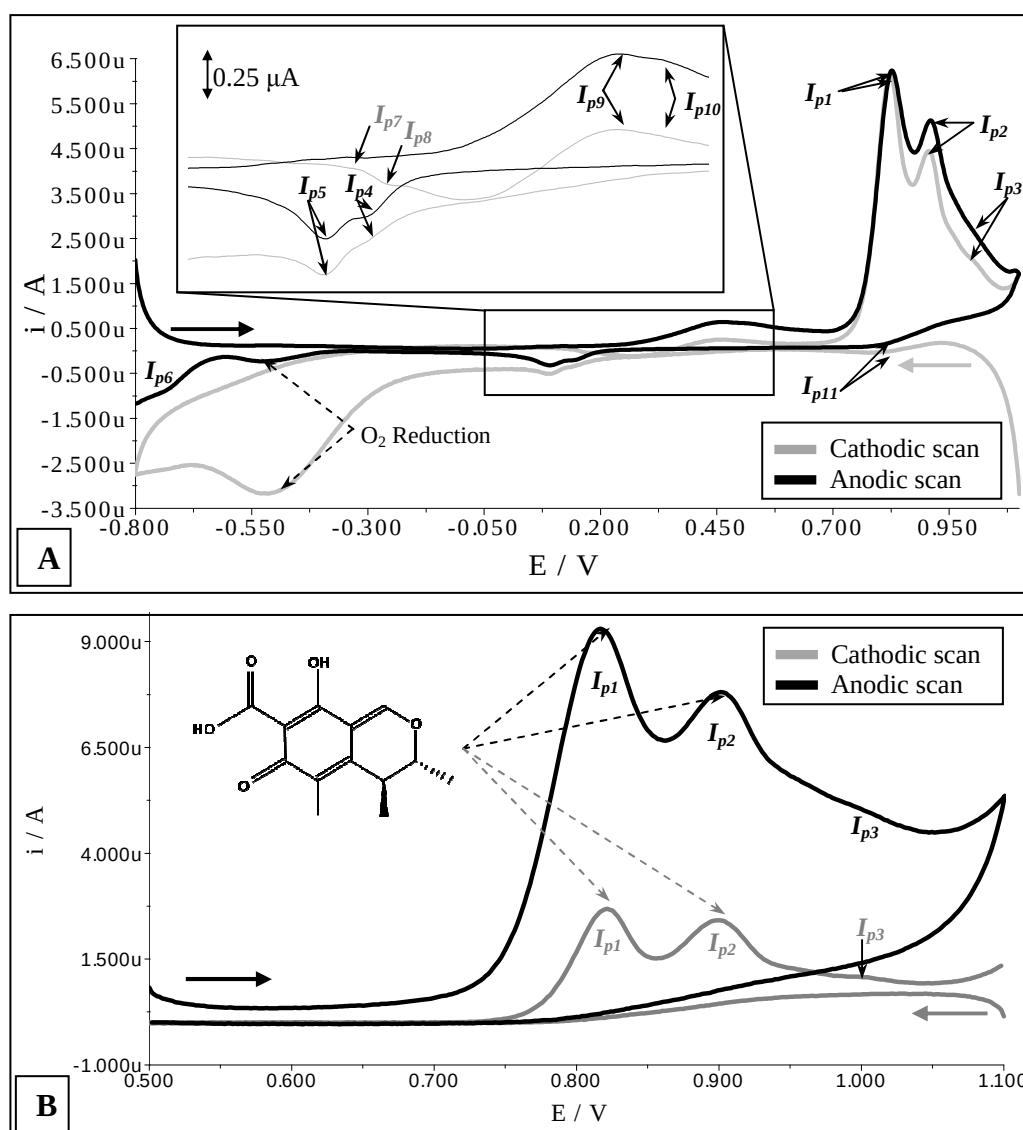


Figure 3.1: Electrochemical profile of 50 μM CIT in 0.2 M BR buffer pH 4.0 on GCE with anodic (black) and cathodic (grey) initiated scans with background current subtracted from all scans. [A] illustrates a scan range of -0.8 V to 1.1 V with annotation of peak from I_{p1} to I_{p10} , and a magnified inset to highlight the peaks from -0.1 V to 0.6 V. [B] illustrates refined scan window of 0.5 V to 1.2 V, with an inset of CIT structure in deaerated solution. In each case background current was subtracted from reported profiles.

As each of the scans of Figure 3.1 were reported with the background signal (buffer solution only) subtracted, responses were assumed to represent the voltammetric reactions associated with CIT. The majority of the peaks numbered in Figure 3.1 were present in both the anodic and cathodic scan directions, though there were noticeable differences in several of the peaks formed. Of particular interest was the absence of I_{p7} and I_{p8} in the initial anodic scan (black trace in Figure 3.1 [A]), which suggested that I_{p7} and I_{p8} are a product of an electrochemical process at potentials more positive than 0.2 V. As these peaks form with successive scans, these peaks are considered to be oxidation products and possibly products of secondary chemical steps, possibly forming a quinone-like couple with I_{p4} and I_{p5} respectively.

I_{p6} was not considered for further analyses as a result of its position (~ -0.75 V), which resulted in it being masked by the oxygen reduction peak as illustrated in the cathodic scan performed in aerated electrolyte (grey trace, Figure 3.1). Scans in aerated solutions were included for parity in analysis of mycotoxins as environmental samples. O_2 was found to have no observable influence on the CIT voltammetric profile bar the aforementioned peak masking. Deaerated solutions were applied through the entirety of reported results in this Thesis, unless otherwise stated.

Although I_{p9} and I_{p10} were initially considered as possible peaks which may represent the oxidation of CIT, these peaks were very limited at lower concentrations (data not shown) which suggests that they may be structurally-related compounds (as described by Clark *et al.*, 2006) present in the CIT standard obtained, present at trace levels. This result thus suggested that one of peaks denoted I_{p1} - I_{p3} arise from CIT electrochemical activity. In the cathodic scan (grey scan) of Figure 3.1 [B], the responses of I_{p1} , I_{p2} and I_{p3} were all relatively small, suggesting that the electrochemical response of CIT must be an oxidative process, as illustrated by larger responses in the anodic scan (black scan) of Figure 3.1 [B]. As the relative change in current response observed in I_{p1} was much larger than I_{p2} or I_{p3} , and as the cathodic scan was initiated at potentials higher than the oxidation potential of I_{p1} , I_{p2} and I_{p3} , it is likely that I_{p2} and I_{p3} are oxidation products and possibly the result of rapidly occurring irreversible chemical steps. I_{p1} was likely to be the source of those oxidation products (considered further through Section 3.4.2). Accordingly, I_{p1} was identified as the focus of analyses in subsequent mechanistic studies.

Upon first inspection it may appear that I_{p1} , I_{p2} and I_{p3} are irreversible faradaic responses due to an absence of defined return peaks in the reductive sweep of CV scans. Though the minor peak labelled I_{p11} at approximately 0.820 V may be interpreted as an indication of a quasi-reversible process, this peak was considered likely to have arisen as a result of the cessation of faradaic reaction from I_{p1} , and was thus not considered to arise as a result of a separate electrochemical reaction. The wave shape of I_{p1} , particularly the sharpness of the leading arm of the peak and the symmetry of the peak, suggest

that it is an electrochemically reversible or quasi-reversible peak (Scholz, 2002). As a reversible or quasi-reversible peak, the absence of a return peak is a clear indication of a homogenous chemical reaction consuming the product generated by the oxidation of CIT (Scholz, 2002).

The primary peak (I_{p1}) was found at $E_{p1} = 0.838 \text{ V} (\pm 0.002 \text{ V})$ and the secondary peak (I_{p2}), $E_{p2} = 0.920 \text{ V} (\pm 0.003 \text{ V})$ (assessed further in section 3.4.3) illustrating peak stability with regard to the potential at which they are found. The sensitivity of response (S_p) of CIT I_{p1} was calculated according to Eq. 2.4 such that $S_{p1} = 0.339 \text{ A.M}^{-1} (\pm 0.068 \text{ A.M}^{-1})$. According to an extensive literature search, this body of work forms the first reported electrochemical analysis of CIT under any conditions. Furthermore, the refined potential window of 0.5 V to 1.1 V was established as the uniform range for voltammetric analysis of CIT.

The diminished I_{p1} response reported in the cathodic scan of Figure 3.1 [B] was likely to be as a result of scanning cathodically, wherein, ramping of the electrode to 1.1V would greatly deplete the concentration of unoxidised CIT localised at the electrode. That feature may have further contributed to the similarity in size of I_{p1} and I_{p2} in Figure 3.1 [B], as production of I_{p2} (by promoting the homogenous chemical reaction mentioned above) may have been favoured due to greater time being allowed for the chemical step to occur before oxidation between 1.100 V and 0.920 V (E_{p2}) than that reported in an anodic scans between 0.838 V (E_{p1}) and 0.920 V (E_{p2}). This thus suggests that I_{p2} arises as a result of a rapid but time dependent chemical step. This finding further illustrates the absence of a peak of I_{p1} in the return scan; as oxidised CIT undergoes a rapid chemical step, it is thus depleted at the electrode surface and hence cannot be reduced in the return scan. The responses may further have been influenced by fouling of the electrode (considered further in Section 3.4.4) arising from oxidation products of the irreversible chemical step (I_{p2}). It is submitted that fouling played a minor role as there was no appreciable shift in peak potential which would be expected as a result of fouling. Due analysis to elucidate these features further was beyond the scope of this initial study thus it was considered further in Chapter 6.

3.4.2 Identification of the primary oxidation peak of CIT

Determination of the waveform related to the primary oxidation of CIT was carried out by shifting the potential window in which scans were conducted, as illustrated in Figure 3.2, and considered in relation to the profile of CIT (Figure 3.1).

When considering the result of Figure 3.2, it is evident that I_{p1} is the primary faradaic product of the oxidation of CIT, as was suggested in Section 3.4.1. The applied potential window of 0.87 V to 1.10 V resulted in lowered current response for I_{p2} and I_{p3} relative to I_{p1} when initiated from 0.50 V,

indicating that I_{p2} as well as I_{p3} are oxidation products and are likely to arise as a result of rapidly occurring irreversible chemical steps post I_{p1} oxidation, suggesting this is an *electron transfer - chemical reaction - electron transfer* (ECE) mechanism.

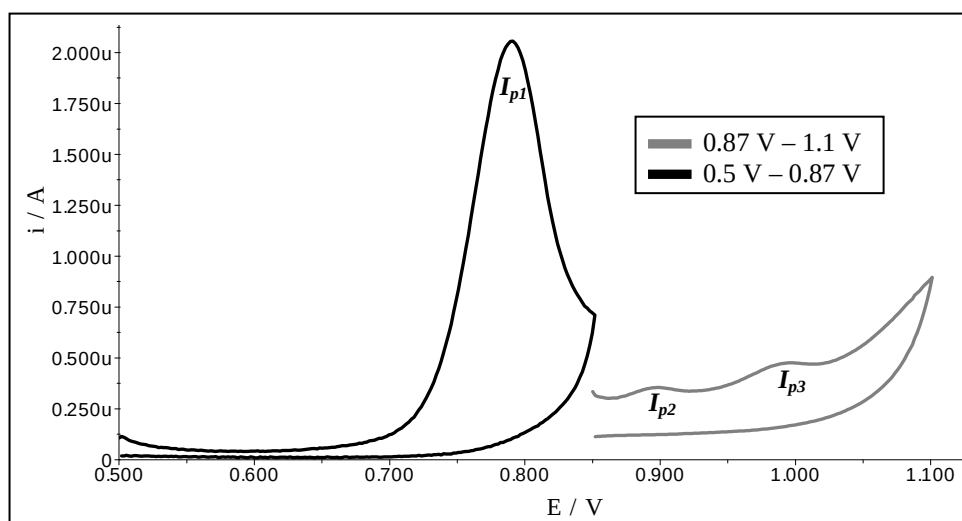


Figure 3.2: CV scans of 20 μM CIT in 0.2 M BR buffer pH 4.0 using GCE electrodes, with scans carried out between 0.5 V and 0.87 V (black) and between 0.87 V and 1.1 V (grey).

3.4.3 Analysis of CIT at GCE

Analysis of CIT electro-oxidation at GCE (Figure 3.3) illustrates that although E_p was highly stable, a large degree of variability was observed in I_p across multiple GCE preparations (polishing and cleaning the GCE between analyses). This influence was evident across *inter*- and *intra*-GCE comparisons of I_p responses, (“*inter*” referring to analysis between different electrodes and “*intra*” analysis at the same electrode). As only oxidative processes were of concern in these analyses, LSV was applied in place of CV. LSV scans were found to provide an equivalent trace of the CIT electrochemical profile observed in CV scans, though with slightly lower variability in I_{p1} and with I_{p3} being more clearly defined (Figure 3.3).

By using coefficient of variation (C.V.) as a relative measure of variability, the deviation from the mean is reported as a percentage of the mean (Rosner, 2000). Thus a measure greater than 10 % indicates reproducibility (R , calculated according to Eq. 2.2) of less than 90 %. Responses with C.V. values $>10\%$ are considered unsatisfactory for analytical significance, since such variability could not be used to determine electrochemical traits or solution concentrations with any confidence.

As is illustrated in Figure 3.3, CIT I_p was highly variable despite the electrode preparation procedure and sample solution being uniform. Direct analysis of CIT I_{p1} across the entire data set (*inter*-GCE variability) of 24 scans (6 GCE each scanned on 4 occasions with cleaning between scans) resulted in an $I_{p1} = 6.77\ \mu\text{A}$ ($\pm 1.36\ \mu\text{A}$), and a resulting C.V. of 20.16 %. As is illustrated in Figure 3.2, this

variability in response was a factor of each of the electrodes applied, wherein, although in some electrodes, variability in I_{p1} responses were lower than the total data set (i.e. C.V. of 13.40 % at GCE 2), none of the electrodes provided responses with sufficient *intra*-GCE I_{p1} response reproducibility, with one set providing a C.V. of 33.50 % at GCE 1.

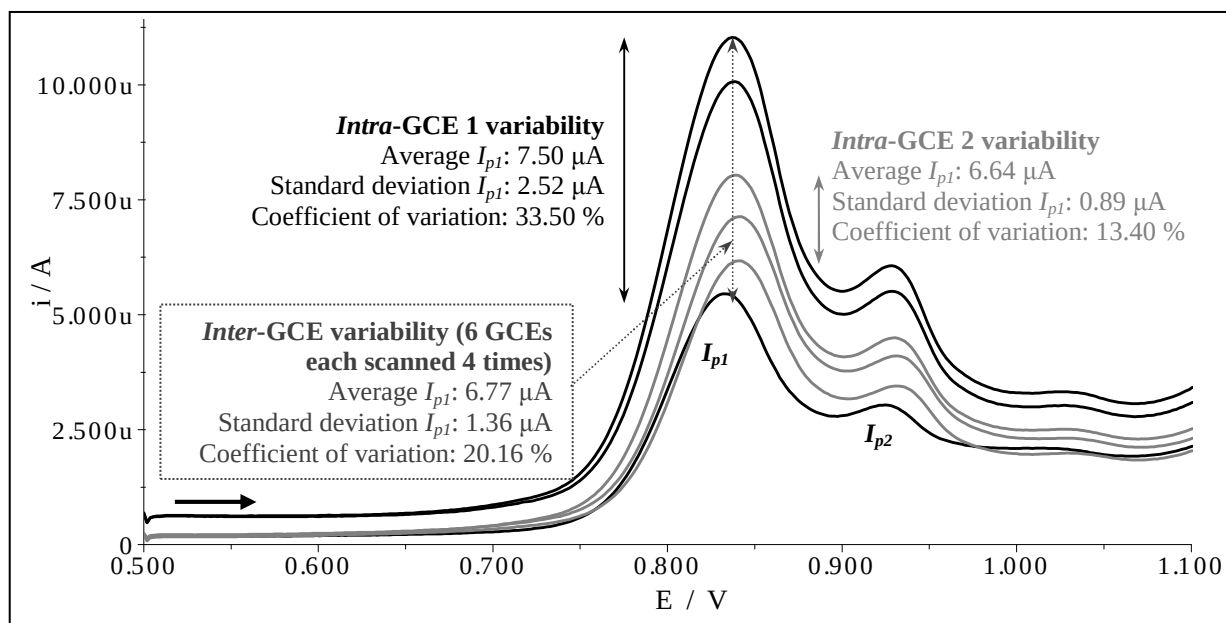


Figure 3.3: Representative LSV scans of CIT showing I_{p1} and I_{p2} variability at GCE 1 (black) and GCE 2 (grey) to illustrate both *inter*- and *intra*-electrode variability. Responses were all recorded in a uniform 20 μ M CIT solution with GEC polished between scans.

As all of the GCE were unmodified in these analyses, low inter-electrode surface heterogeneity was assumed, however, I_{p1} variability remained a troubling feature. Since the scans were carried out under uniform conditions, the contributing factors toward I_{p1} variation could not be environmental in origin (i.e. pH, analyte solution concentration, temperature, etc.). Accordingly, the source of the response irreproducibility was considered to primarily arise from variations associated with the surface of the working electrode(s). This then points to variability in the interaction between the electrode surface and CIT, most likely due to the extent or variation of either a charge-based, or other electrode surface group, interaction with the analyte. Examining routes to normalise or limit such variability is an important feature of the studies in this thesis and is further interrogated in Chapters 4 and 5.

The variability or inconsistency found between I_{p1} responses of CIT was found relatively early on through the course of these studies. Though parameters assessed and discussed hereafter were found to influence electrochemical responses, variability in I_{p1} responses remained present. Furthermore, with higher numbers of scans carried out for a given parameter, the C.V. of I_{p1} responses tended to increase to around 20 %, as mentioned previously. A factor which should, therefore, be borne in mind throughout the remainder of this Chapter is that relative responses within a reported study varying a single analytical parameter (i.e. *intra*-experimental responses) were considered to be representative of

the influence of the studied factor. Thus, intra-experimental comparisons are discussed with relative confidence. However, due to the variability which was known to be present in responses, comparison of I_{p1} responses across separate experimental parameters (inter-experimental responses) were considered and discussed with due caution, as the magnitude of mean I_{p1} variability during a given experiment indicates that I_{p1} responses between studies cannot be generalised. Furthermore, although a directive of this Chapter was assessing means of reducing variability in I_{p1} , the reported standard deviation from mean I_{p1} and resulting C.V. responses were often reduced as compared to the responses illustrated in Figure 3.3. Similarly to inter-experimental generalisations, this is also treated with considered reservation, due to the low sample rate (between 3 and 12 samples per dataset) of each experimental point.

3.4.4 Successive voltammetric analyses of CIT

As variability in the peak current of I_{p1} was evident at GCE in the first scan of voltammetric analyses, successive cycling of GCE in CIT solution was performed to determine whether stability in response could be achieved. As depicted in Figure 3.4, marked decreases in returned I_{p1} responses was observed with successive scans.

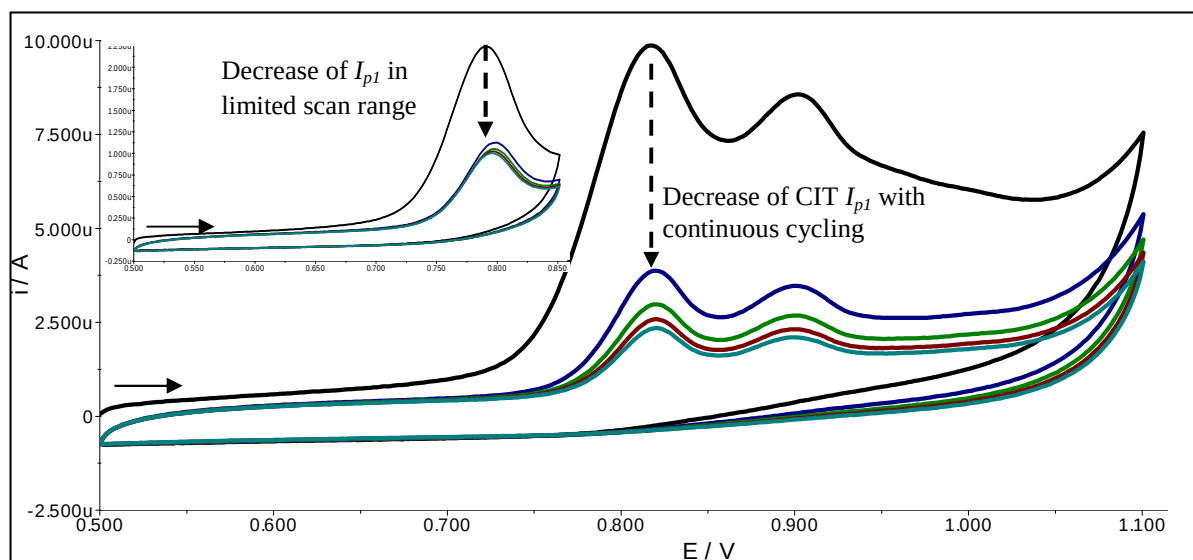


Figure 3.4: Voltammetric profile of 5 successive CV scans. 50 μM CIT in 0.2 M BR buffer pH 4.0 at bare GCE. Inset shows successive scans of I_{p1} only i.e. potential window from 0.5 V to 0.87 V, in a 15 μM CIT solution.

As was indicated in Section 3.1, the reported decrease in I_{p1} is likely to be a result of depletion of analyte at the electrode surface with successive scans. The inset of Figure 3.4, illustrates continuously cycled analyses of CIT with the upper switch potential set at 0.87 V (i.e. prior to onset of I_{p2}). Therein, a similar decrease in I_{p1} was recorded to that reported in the primary set of scans of Figure 3.4. Interestingly, with the absence of oxidation of I_{p2} and I_{p3} , the scans of the inset reach a stable I_{p1} more rapidly than when I_{p2} and I_{p3} are oxidised. This indicates that the products of the electro-oxidation of

I_{p2} and I_{p3} may foul the GCE to a minor extent (Yin *et al.* 2006; Berríos *et al.* 2009). Although electro-oxidation of I_{p1} may contribute to fouling of the GCE, as intimated by a slight shift in E_{p1} between scan 1 and scan 2 in the insert of Figure 3.4, fouling was not considered to be the predominant process affecting successive scans.

Regeneration of I_{p1} current response as a factor of replenished analyte concentration at the GCE surface was assessed through allowing the electrode to stand in solution for at intervals of 1 minute and 5 minutes between scans (without re-polishing the electrode between scans, Figure 3.5). Solutions were agitated between scans to ensure effective analyte replenishment.

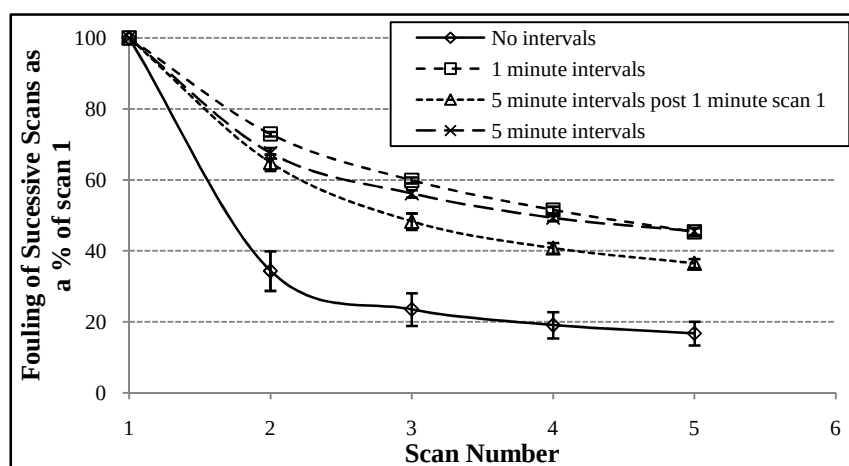


Figure 3.5: Assessment of the possibility of signal regeneration of the electrode during successive scans. I_{p1} responses of 5 successive LSV scans were reported in relation to the time that the electrode was allowed to stand in solution before scanning again, and without being re-polished. Number of scan replicates per data set ≥ 3 and standard deviation was reported from the mean.

As is illustrated through Figure 3.5, responses of repeated cycling with intervals between scans exhibited significantly higher successive I_{p1} responses, compared to responses obtained with continual cycling. Imposing 5 minute intervals allowed I_{p1} responses after 5 scans to reach 45.5 % of the initial peak current, compared to the 16.7 % obtained during continual cycling. These responses illustrate that with sufficient time, the replenishment of analyte at the electrode surface did allow for some recovery in the magnitude of I_{p1} . However, as I_{p1} did not recover to the magnitude of the initial scan, and as the degree by which the magnitude of I_{p1} response decreased with successive time intervals decreased, in combination with depletion of electrolyte at the electrode surface, oxidation products of CIT electroanalysis do foul the GCE to a marginal extent.

3.4.5 Assessment of the influence of electrode material on CIT I_{p1} variability

a) Analysis of CIT at carbon paste electrodes (CPE)

Although strong electroactivity was found at GCE, CPE were assessed as an alternative carbonaceous electrode material. Possessing slightly different surface properties to that of GCE (Kissinger &

Heineman, 1996; Švancara *et al.* 2009), CPE were investigated in order to determine whether CIT I_{p1} variability was confined to GCE as mentioned above or if CIT responses were common to carbonaceous electrodes.

The application of CPE for CIT analysis is reported through Figure 3.6, wherein a similar electrochemical profile to that recorded at GCE was found, though the peaks were not as clearly defined and responses were generally lower than at GCE, the inset of Figure 3.6 reporting $S_{p1} = 0.112$ A.M⁻¹ at CPE compared to $S_{p1} = 0.339$ A.M⁻¹ at GCE. Although peak position was again stable at $E_{p1} = 0.855$ V (± 0.005 V), these were higher than those at GCE ($E_{p1} = 0.838$ V). I_{p1} stability was very similar to the responses at GCE with C.V. of 20.02 % and 20.16 % observed at CPE and GCE respectively. The variability in CIT I_{p1} at CPE was, similarly to GCE, likely to be associated with the electrode surface condition as CPE are by nature a relatively inhomogenous electrode surface with relatively high surface roughness (Švancara *et al.* 2009).

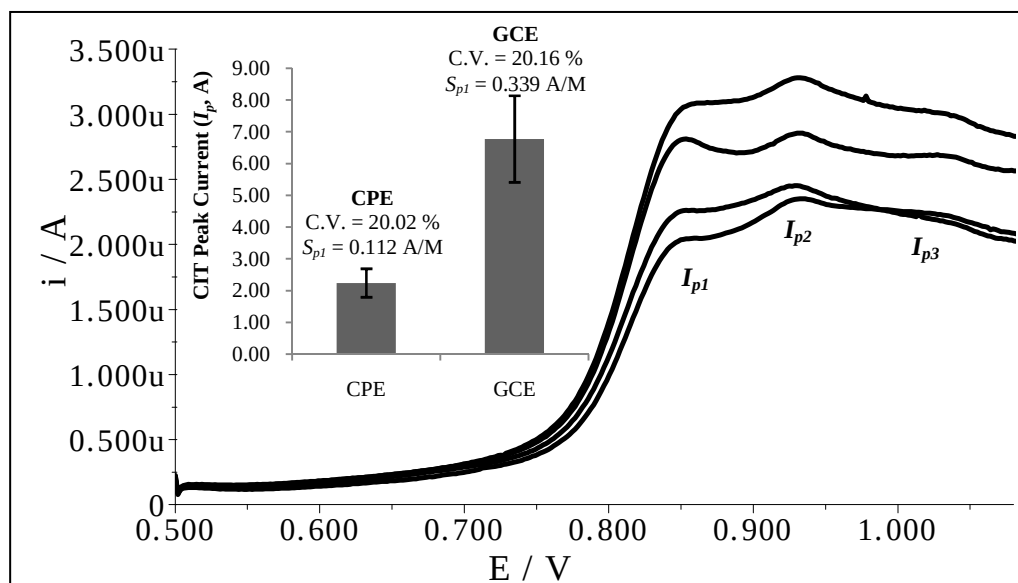


Figure 3.6: LSV scans of 20 μ M CIT at CPE, background current was subtracted from each scan. Inset illustrates the comparison of I_{p1} , C.V. and S_{p1} between GCE and CPE under equivalent conditions, $N \geq 4$ and standard deviation reported from the mean.

b) Analysis of CIT at gold electrodes (AuE)

A gold electrode (AuE) was selected for examination of CIT electroanalysis on the twin assumptions of greater surface homogeneity (as carbonaceous electrode surface heterogeneity was identified as the probable source of the CIT I_{p1} variability) and easily-quantifiable surface area (through measurement of the charge of the gold oxide reduction peak) (Carvalho *et al.* 2005).

As can be seen in Figure 3.7, a minor anodic wave (I_{pA}) at $E_p = 0.850$ V, attributed to CIT oxidation was observed. I_{pA} was poorly defined, with no clear resolution shown for I_{p1} or I_{p2} as observed at GCE

and CPE. As I_{p2} was identified as an oxidation product of CIT analysis, it is possible that it did not interact with the AuE. It was however considered more likely that due to the low current response, peak separation was masked, thus the entire peak was measured as an indicator of CIT I_p . In an effort to address the very low I_{pA} responses at AuE, I_p was reported in relation to electrode surface area. At AuE $I_{pA} = 10.98 \mu\text{A}\cdot\text{cm}^{-2}$ ($\pm 0.95 \mu\text{A}\cdot\text{cm}^{-2}$) with a resulting C.V. of 8.61 % and sensitivity (from Eq. 2.5, modified by accounting for electrode surface area [cm^2]) of $S_{pA} = 0.55 \text{ A}\cdot\text{M}^{-1}\cdot\text{cm}^{-2}$, whilst at GCE $I_{p1} = 95.77 \mu\text{A}\cdot\text{cm}^{-2}$ ($\pm 19.42 \mu\text{A}\cdot\text{cm}^{-2}$) with a resulting C.V. of 20.09 % and $S_{p1} = 4.78 \text{ A}\cdot\text{M}^{-1}\cdot\text{cm}^{-2}$. This translated to AuE producing a mean current response of 11.46 % of that at a GCE for an equivalent concentration of CIT.

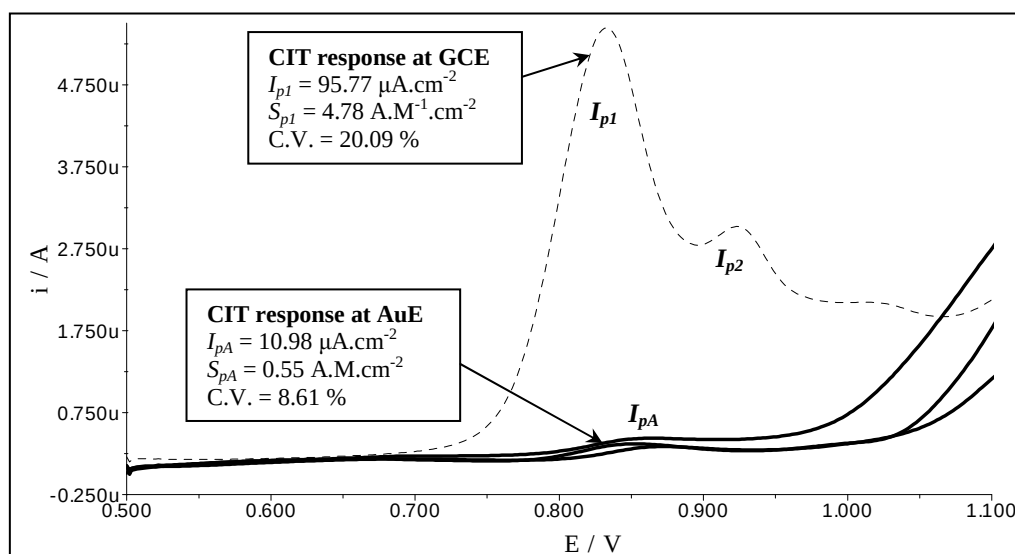


Figure 3.7: Profile of 20 μM CIT in 0.2 M NaOAc pH 4.0 on AuE (solid lines) as compared to GCE (dotted line) under equivalent conditions.

The improvement in the C.V. (8.61 %) of I_p responses at AuE relative to GCE and CPE for CIT electro-oxidation is attributed to the increased uniformity of this surface. The lowered I_p responses at AuE are understood within the reportedly poor oxidation of organic compounds, such as CIT, at this electrode due in part to the complex basic electrochemistry exhibited by the Au surface itself, and furthermore given that oxidation of aromatic compounds is thought to proceed via an ECE system that may not have been possible under these conditions (Burke & Nugent, 1998; Iotov & Kalcheva, 1998). Despite the improvement in C.V., the lowered I_p response and extensive treatment required for AuE limits its application for CIT analysis.

These responses however offered further insight into the interaction of CIT as a molecule at an electrode surface. Although generalised, the assumption that the faradaic response of CIT is influenced by the surface properties of the working electrode is reinforced. It may also suggest that the interaction which allows for greater response at GCE may well be as a result of a charge-based interactions between the electrode and the CIT molecule. This may include both adsorption via

mechanisms such as $\pi=\pi$ stacking or interaction between chemically similar groups present both on GCE not present on AuE (such as surface oxide containing functionalities).

c) Analysis of CIT at functionalised multi-walled carbon nanotube modified GCE (*f*MWCNT-GCE)

A significant body of research detailing the application of MWCNT within electroanalysis was described through the general introduction (Section 1.2.5 [d]). The application of *f*MWCNT to lower variability of electrochemical responses toward CIT was examined.

Representative analyses of CIT electrochemical responses were reported across both multiple *f*MWCNT-modifications of a single GCE (resurfacing a single GCE with *f*MWCNT) and across several GCE (resurfacing across different GCE) (Figure 3.8).

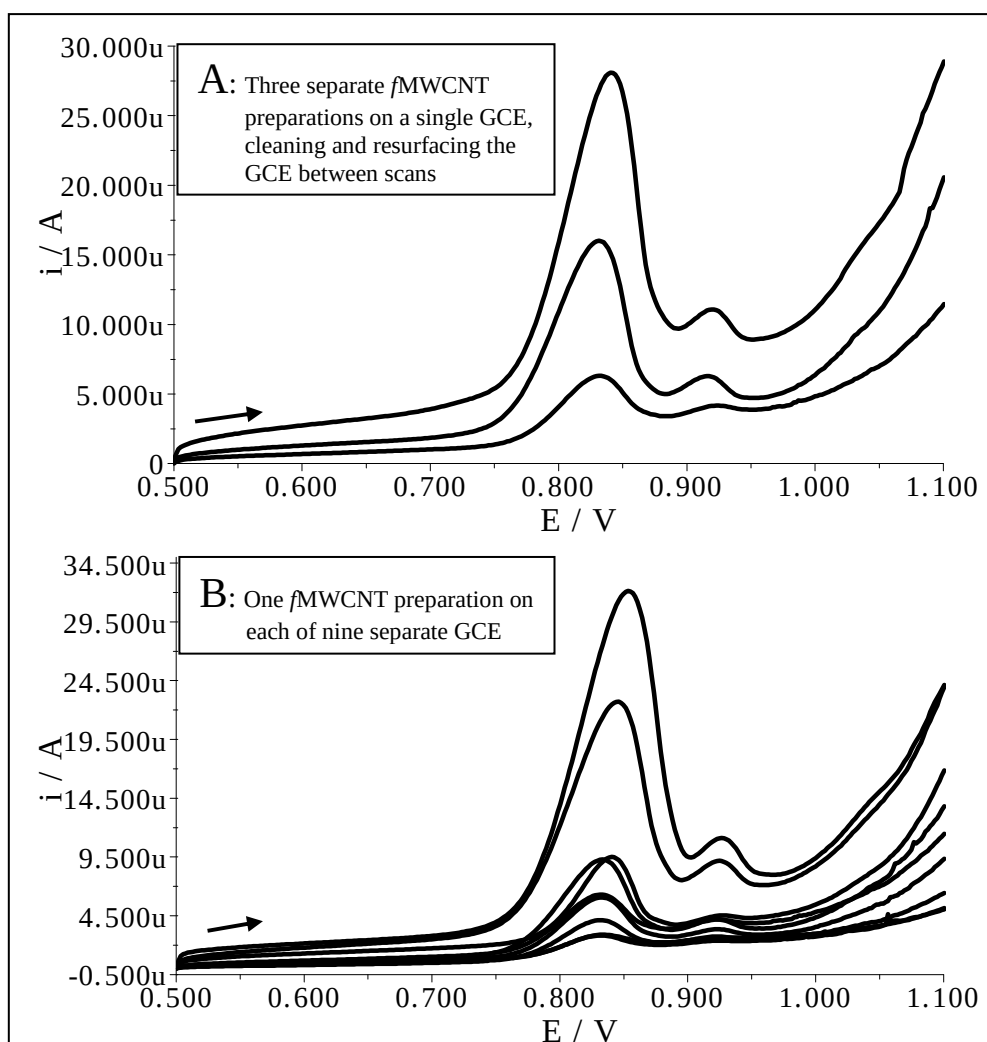


Figure 3.8: Representative scans CIT in a 20 μ M solution at [A] showing a single scan at three equivalent preparations of the same *f*MWCNT-GCE and [B] showing a single scan at each of nine separate *f*MWCNT-GCE.

The electrochemical profile of CIT at f MWCNT-GCE observed through Figure 3.8 was closely comparable to that depicted at bare GCE (Figure 3.2), and indeed exhibited very similar fouling characteristics to those reported in Section 3.4.4. Peak potential (E_{p1}) was relatively stable and no statistically significant lowering of E_{p1} was observed at MWCNT-GCE ($E_{p1} = 0.833 \text{ V} (\pm 0.007 \text{ V})$) relative to GCE ($E_{p1} = 0.84 \text{ V} (\pm 0.004 \text{ V})$), in contrast to that observed in similar studies (Dai *et al.* 2006). Henstridge *et al.* (2010) described that the so called ‘catalytic’ activity of CNT was a factor of thin layer like diffusion within porous layers rather than true catalytic behaviour of the CNT. The absence of a drop in potential may thus be a factor of CIT adsorption at carbonaceous surfaces (adsorption was investigated in more detail in Chapters 5 and 6).

As is clear from scans of Figure 3.8, marked increases in I_{p1} were recorded on several f MWCNT-GCE compared to GCE, however the response of I_{p1} was not consistent across multiple preparations, either at a single electrode or at multiple preparations across multiple GCE. Further investigations into I_{p1} at f MWCNT-GCE were examined and presented in Chapter 5. As the electrochemical profile of I_{p1} response at f MWCNT-GCE was not dissimilar to GCE it indicated that the different surfaces did not introduce aberrant oxidation kinetic procedures or processes relative to one another. As it has been postulated that variability in CIT I_{p1} is a factor of the electrode surface, the extensive variations in I_{p1} at f MWCNT-GCE should reflect a highly inconsistent surface condition.

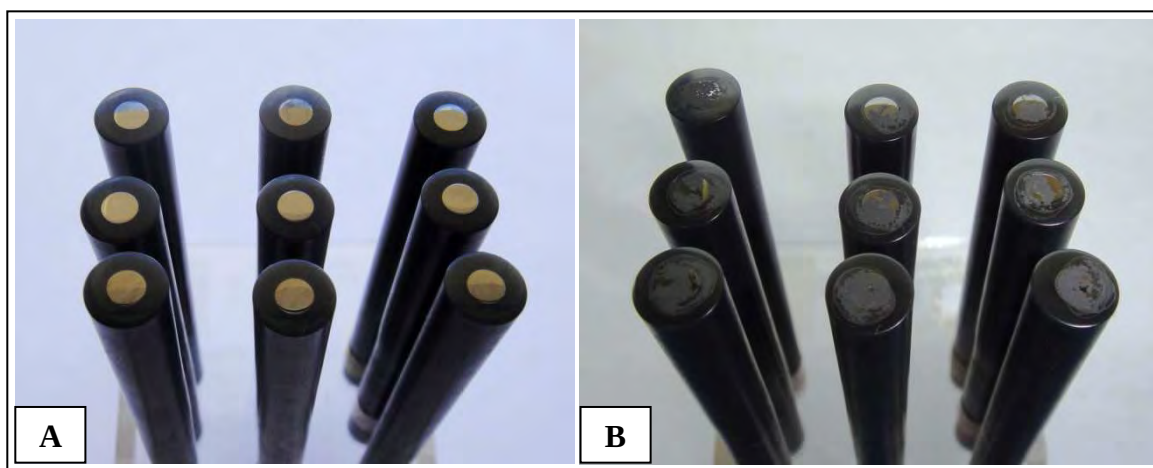


Figure 3.9: Illustration of the variability of GCE surfaces having been modified with f MWCNT [B] as compared to polished GCE [A].

Although specific considerations of oxidised electrode surface condition are considered in detail through Chapter 4 in relation to polished GCE, CNT modified electrode characteristics have been extensively investigated and characterised. Physically adsorbed CNT immobilisation has been applied in the vast majority of reports concerning CNT immobilisation at electrodes. This procedure results in highly matted, porous, and randomly oriented layer(s) and bundles of CNT (Gooding, 2005;

McCreery, 2008; Golikand, 2009) resulting in inhomogeneous surface conditions both across a single preparation and across multiple preparations.

The implications of inhomogeneous surface characteristics on electrochemical responses are investigated further through Chapter 5. In addition to microscopic instability in surface condition with adsorption of f MWCNT, Figure 3.9 provides a macroscopic illustration of variability which is encountered across multiple preparations GCE surfaces with f MWCNT. Variability in CIT peak response is also likely to be aggravated by uneven deposition of f MWCNT at GCE as is clearly depicted in Figure 3.9 [B].

3.4.6 Assessment of the influence of select electrolyte characteristics on CIT I_{p1} variability

a) Analysis of CIT in Organic Solvents

As reported in Table 3.1, organic solvents have been applied for many direct electrochemical analyses of mycotoxins. Although there is consensus in the literature that CIT is poorly soluble in aqueous media, reports on the solubility of CIT in aqueous solvents are conflicting, ranging from completely insoluble (de Carvalho *et al.* 2005) to partially soluble depending on water temperature (Sprenger & Ruoff, 1946). Research reported in this Chapter has shown that CIT is clearly detectable in aqueous buffered solutions. CIT is thus, at least, partially soluble in H_2O . Since the solubility of CIT is likely to affect the way in which it associates with an electrode surface, this section of work was directed at assessing the electrochemical response of CIT in an organic solvent. This is performed to investigate whether the solubility of the CIT molecule could influence the variability of CIT I_{p1} response obtained thus far. These analyses were done using either TEAP or $LiClO_4$ as electrolytes in methanol, a commonly reported solvent of CIT (Xu *et al.* 2006) (Figure 3.10).

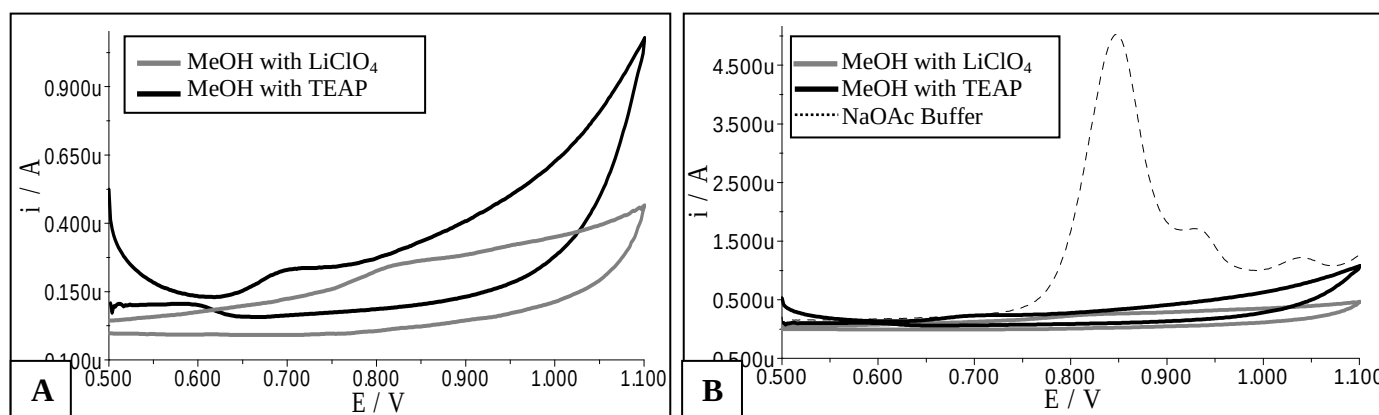


Figure 3.10: CIT analysis at 10 μ M dissolved in methanol (MeOH) with [A] 0.1 M TEAP (black) and 0.2 M $LiClO_4$ (grey) as electrolytes respectively scanned vs. Ag-QRE, as well as [B] inclusion of NaOAc buffer pH 4.0 vs. Ag/AgCl as a comparison.

Although minor anodic responses were observed for CIT electroanalysis in organic solvents in both TEAP and LiClO₄ (Figure 3.10 [A]), these were low in comparison to aqueous solutions, at 1.9 % (LiClO₄) and 3.2 % (TEAP) current response, compared to that obtained in NaOAc buffer at pH 4.0 (Figure 3.10 [B]). Although the influence of organic solvents on the solubility of CIT was anticipated to affect I_p responses, the influence was not anticipated to be as marked as compared to aqueous solutions. It is possible that analyses of CIT in non-aqueous media at more negative potentials, possibly the cathodic region, may have resulted in more defined electrochemical responses. Analyses under such conditions would however have yielded limited diagnostic information within the scope of this study, which is centred on the anodic analysis of CIT in aqueous media. This was due to the practicality of establishing a sensor system which is able to detect target analyte directly from an aqueous extraction, negating the need for any solvents to be used.

An insight which can be gained from I_p responses in dried organic solvents refers to the absence of I_{p2} and I_{p3} in the electrochemical profiles of Figure 3.10. In section 3.4.4, I_{p2} and I_{p3} were described as oxidation products of CIT I_{p1} . The absence of the peaks altogether however indicates that their formation is likely to be a result of an ECE mechanism which may involve H₂O as a participant in formation of those products (Evans, 2008; Costentin *et al.* 2008; Costentin *et al.* 2010), as is discussed further in Chapter 6.

b) Analysis of the influence of pH on CIT analysis

The influence of solution pH was assessed as the interaction of CIT with electrode surface was considered to be a major contributing factor towards variability in current response. As the association of CIT with the electrode is adsorptive (confirmed through Chapters 5 and 6), and was likely to be influenced by the condition electrode surface, pH would theoretically have a marked influence on responses. The results of these analyses are illustrated in Figure 3.11. Given the analytical variability of I_{p1} responses at an unmodified GCE, the results are considered representative of rather than accurate, limiting the use of this pH profile for kinetic determinations. However, they serve a purpose here in identifying a general trend.

Across the pH range investigated (Figure 3.11), responses at pH 4.0 provided the largest I_{p1} responses. The I_{p1} responses were however variable across the entire pH range assessed and were thus considered with due caution. I_{p1} responses were fairly stable at acidic pH from pH 2.0 to pH 4.0, however outside of this range, responses were lower and all except pH 5.0 had larger C.V. responses denoting greater relative variability. This was likely to be as a result of the pK_a of CIT, determination and analysis of which was considered in Chapter 6, Section 6.4.4 [c], as it was beyond the scope of this initial analysis. Herein, it was established that pH 4.0 provided the best apparent pH for analyses, later corroborated through studies reported in Chapter 6, and was thus used for the majority of the rest of

this Thesis. As E_{p1} were relatively stable across the entire pH range assessed, they were considered to provide an accurate response. The slope of the rate of change of E_{p1} in relation to pH was found to be 32 mV.dec^{-1} , a result considered further through Chapter 6, Section 6.4.4 [d].

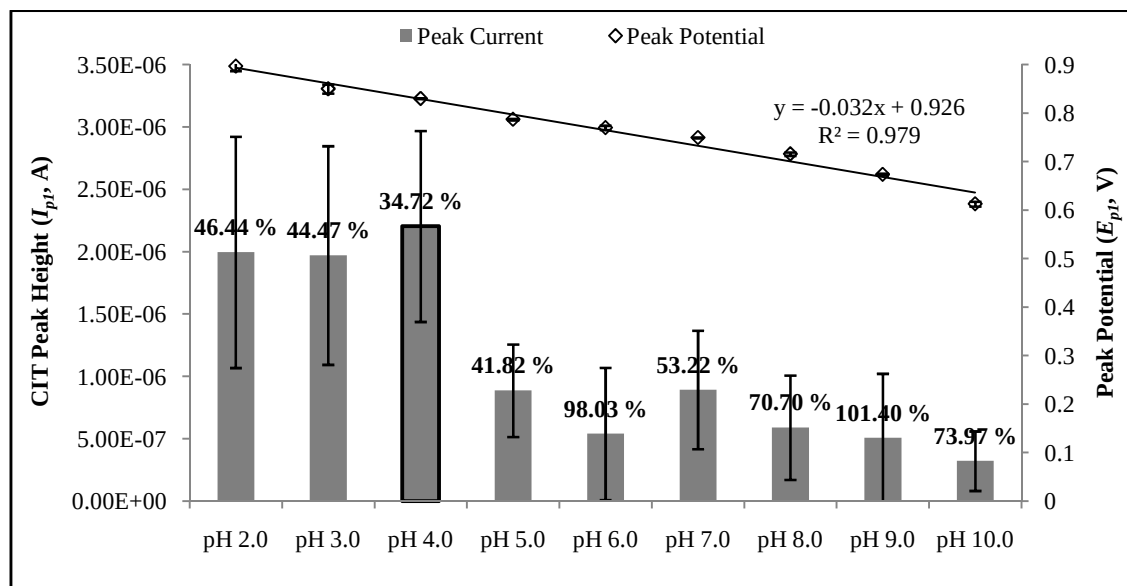


Figure 3.11: Assessment of CIT I_p and E_p responses across pH 2 to pH 10. The C.V. of each I_p response is reported above the respective pH. Scans were conducted in $10 \mu\text{M}$ CIT in 0.2M BR buffer at polished GCE. Number of replicates in each set was ≥ 3 and standard deviation was represented from the mean in each case.

3.4.7 Assessment of the influence of preconditioning parameters on CIT I_{p1} variability

a) Influence of in-scan hold potential on CIT analysis

The application of preconditioning steps, particularly analyte preconcentration at GCE prior to voltammetric analyses has been widely applied for direct electroanalytical studies of mycotoxins (Ramírez *et al.* 2005; Molina *et al.* 2008; Yaacob *et al.* 2008; Hajian & Ensafi, 2009). This study was thus directed at assessing the influence of analyte preconcentration as a factor of the potential at which analyte preconcentration was allowed to occur.

As can be seen in Figure 3.12, the application of conditioning potential has a marked influence on both the magnitude and stability of I_{p1} . The conditioning potentials which provided the highest and most stable responses were at 0.2 V and 0.4 V respectively. This was an expected result when considering the profile of CIT over a broad potential range (Figure 3.1), wherein there is little faradaic activity between 0.35 V and 0.6 V . As the duration of time at which each potential was assessed was uniform, the difference in responses was suggested to be as a result of the potential at which electrodes were preconditioned. As such the relatively limited I_{p1} response which arose with holding at -0.2 V and 0.0 V was probably a factor of the charge at the electrode surface and its influence on the extent of adsorption on the GCE (Weisshaar & Porter, 2001).

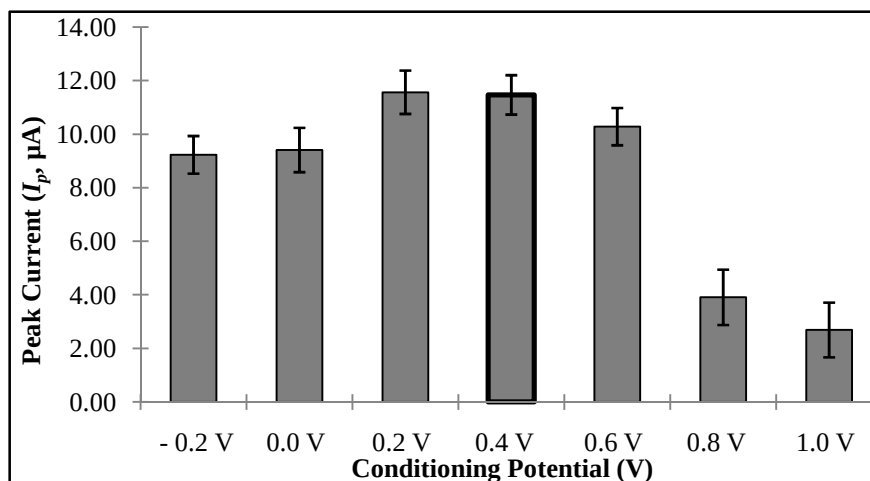


Figure 3.12: Assessment of hold potential effect on I_{p1} . Scans were carried out in 20 μM CIT in 0.2 M NaOAc buffer pH 4.0 on bare GCE at a scan rate of 100 mV/s vs. Ag/AgCl. Hold potentials assessed were -0.2 V, 0.0 V, 0.2 V, 0.4 V, 0.6 V, 0.8 V and 1.0 V, with each held for 60 seconds in a solution agitated at 1000 rpm. Number of scans set ≥ 3 , standard deviation determined from the mean.

A drop in I_{p1} peak height and stability was also seen from 0.6 V upwards. These were potential approaching and subsequently exceeding the oxidation potential of CIT. The limited response could as such be attributed to the presence of oxidised product at the electrode surface as onset of CIT oxidation is evident in the region of 0.7 V (Figure 3.1). A further factor which is likely to contribute to limited I_{p1} responses includes depletion of CIT at the electrode surface due to its oxidation. This is a factor which is progressively amplified as would be expected at 0.8 V and 1.0 V. Accordingly, a potential of 0.4 V was selected as the conditioning potential for analyte preconcentration in future studies as it provided the greatest I_{p1} response and stability.

b) Influence of preconcentration duration (adsorptive duration) on CIT analysis

In a related study to Section 3.4.7 [a], the influence of the duration at which the electrodes were held at a 0.4 V was probed. The resulting I_{p1} height and stability was assessed by preconditioning the electrode for a range of times as is illustrated in Figure 3.13. This was done as an assessment of the extent of analyte preconcentration which CIT at the electrode, and the resulting response gain from that.

As can be seen in Figure 3.13, a distinct influence of increased adsorption time was found, wherein for the first 30 seconds, there is a marked increase in I_{p1} , which then plateaued with further adsorption time. Although it seems that there is a general trend of decrease in resulting peak height from this data set, it must be kept in mind that these points were derived from 3 scans per time set and with the variability that is intrinsic of this scan system (C.V. of 18 %). Due to this the global trends were given more credence than the small degree of variability found between the data points after 30 seconds adsorption. Due to the increase in the current response observed with adsorption being allowed, a 120

second adsorption period was selected for subsequent analyses as it offered a relatively stable response along with a stable potential of response.

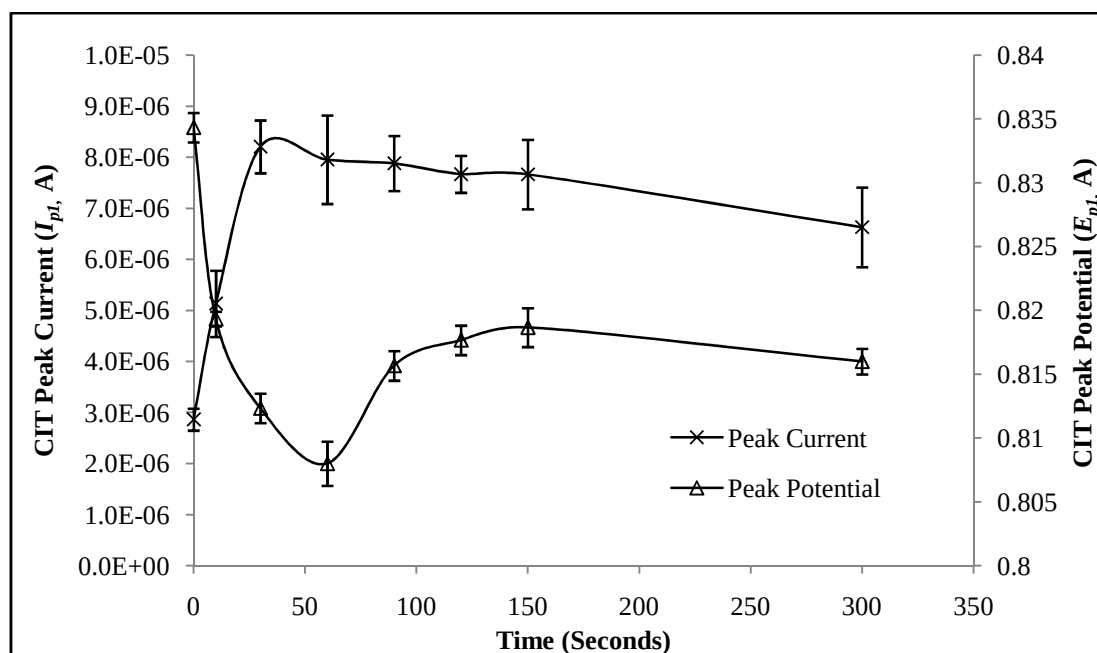


Figure 3.13: Assessment of adsorptive duration on I_{p1} when holding at 0.4 V. Scans were carried out in 20 μ M CIT in 0.2 M NaOAc buffer pH 4.0 at GCE held at 0.4 V for 0, 10, 30, 60, 90, 120, 150 and 300 seconds in a solution agitated at 1000 rpm. Number of scans set ≥ 3 , standard deviation determined from the mean.

3.5 Summary and conclusions

CIT provided a complex, clearly defined electrochemical profile with a prominent irreversible oxidation peak at $E_{p1} = 0.838$ V was shown to be the primary oxidation peak (I_{p1}) of CIT, a result which details the first recorded electrochemical study on CIT (to the best of the knowledge of the author). Although no defined return peak to I_{p1} oxidation was present, is likely to be a reversible or possibly quasi-reversible reaction which is rapidly proceeded by a chemical step which limited the ability for the reductive peak to arise it in voltammetric analyses may make it appear that it is an irreversible oxidation process. The results indicate that CIT oxidation is likely to occur as an ECE mechanism.

It was found that while E_{p1} was stable, I_{p1} was highly variable with regard to inter- and intra-electrode sets. Several parameters were assessed to limit the I_{p1} variability and improve the sensitivity of response. Assessment of the influence of electrode surface condition, through application of AuE and CPE and fMWCNT-GCE, indicated that electroanalysis of CIT yields strong peaks at carbonaceous electrodes. CPE responses resulted in lower sensitivity than GCE responses and provided no

improvement in I_{p1} stability, though those responses in combination with the responses at fMWCNT-GCE and polished GCE did illustrate an affinity of CIT to carbonaceous materials.

Analysis of CIT in organic solvents revealed results which were similar to those found at AuE, indicating that interactions of CIT with electrodes may be based on a surface analyte interaction. Electrochemical procedural parameters including scan direction, scan rate, the use of chronoamperometry as opposed to voltammetry, and also the introduction of preconditioning procedures were assessed as a possible means of stabilising I_{p1} peak responses and increasing sensitivity. Although marginal improvement was found and preferential scanning parameters were defined, further improvement was required to satisfy the statistical analytical reproducibility of direct electroanalysis of CIT.

As variability in the magnitude of I_{p1} was observed across all the analyses conducted it was concluded that the factor governing the variability in I_{p1} was unlikely to be a result of a solution based kinetic consideration. These results cumulatively point to the conclusion that the extent of I_{p1} response gained for CIT is a factor of an electrode confined process of CIT with the electrode surface condition (electrode morphology and surface chemistry). The influence of surface-condition on electrochemical analyses has been widely and extensively considered as a factor in electrochemical analyses (Taylor & Humffray, 1975; Picq *et al.* 1984; Vasquez & Imai, 1985; Gala & Budniok, 1985; Kamau, 1988; Bowling *et al.* 1989; Ponticos & McCreery, 1992; Savitri & Mitra, 1998; Dekanski *et al.* 2001 (a); Weisshaar & Porter, 2001; Braun *et al.* 2003; Kiema *et al.* 2003; Bleda-Martínez *et al.* 2005; Bleda-Martínez *et al.* 2006; Zhao *et al.* 2008; Zhao *et al.* 2009). As CIT provided relatively low I_{p1} at AuE and the greatest inconsistency in I_{p1} at fMWCNT-GCE, which are known to have extensive inhomogeneity between surface preparations, variability was likely to have arisen as a factor of the extent of association of CIT with an electrode (how adsorptive it is at a surface according to surface chemistry and how large the surface area is). Adsorptive behaviour of mycotoxins has been described for several mycotoxins as they require preconditioning (Ramírez *et al.* 2005; Molina *et al.* 2008; Yaacob *et al.* 2008; Hajian & Ensafi, 2009), and was confirmed for CIT through Chapters 5 and 6.

Accordingly, the following Chapters of this Thesis are to be directed to the investigation of carbonaceous electrode surface confined interactions, such that an effective sensor platform could be developed for the accurate and reproducible analysis of CIT and other toxins, and their characterisation.

CHAPTER 4

Characterisation of the physico-chemical characteristics of chemically and electrochemically oxidised GCE surfaces and identification of the mechanisms of improved oxidation characteristics of citrinin at electro-oxidised GCE

4.1 Introduction

Carbonaceous electrodes of various forms, from graphite powder with liquid and solid binders through to carbon fibres, tubes and balls and glassy carbon (GC) are used extensively in electroanalytical applications. Glassy carbon electrodes (GCE), in particular, are widely applied with extensive research on their surface characteristics having been described (Chapter 1, Section 1.2.3). As CIT showed strong electrochemical responses at carbonaceous electrodes (GCE, CPE and fMWCNT), particularly at GCE, as compared to AuE (Chapter 3), GCE was selected as the material of choice for further analyses. As I_{p1} responses for CIT were highly variable at polished GCE (Chapter 3, Section 3.4.3), it was considered necessary to either eliminate or account for variations in electrode surface condition which were considered to cause this variability. Towards this, two approaches were considered: accounting for variability as a post data acquisition analysis (Chapter 5) and secondly, a more conventional approach of pre-data acquisition electrode modification such that uniformity in electrode condition would afford stability in response (Chapter 6 and applied further in Chapter 7). To achieve these aims, two separate GCE modifications were considered, both involving oxidation of the GCE surface, including chemical oxidation by piranha solution (Pi-GCE) and electrochemical oxidation (electro-oxidation) (EOx-GCE). This Chapter is directed at the analysis of the physio-chemical characteristics of those surfaces in relation to polished GCE for their aforementioned use in Chapters 5, 6 and 7.

4.1.1 Polished GCE

Abrasive polishing of GCE using materials such as aluminium oxide, is a key procedure applied in routine electrode preparation for electro-analytical studies and is typically used as the starting point for all GCE surface analysis and modification procedures. Primarily, it is applied to provide a fresh surface on which analysis or pre-analytical modification can be carried out (Wiesshaar & Kuwana, 1985; Kamau *et al.* 1985; Rice *et al.* 1989; Kissinger & Heineman, 1996). Polished GCE have been

imaged via a number of microscopic techniques, including SEM (Engstrom & Strasser, 1984), tapping-mode scanning force microscopy (SFM) (Kiema *et al.* 1999; Kiema *et al.* 2003), STM (McDermott *et al.* 1993; Shi & Shiu, 2002) and AFM (Zhao *et al.* 2008; Thiagarajan *et al.* 2009), which have provided key morphological information into the structure of GCE, as was described through Section 1.2.3 of Chapter 1. The nature of the surface chemistry of GCE has also been studied extensively in literature, and has been found to comprise of a varied combination of surface functionalities (Figure 1.2 [B]), primarily carbonyl and hydroxyl moieties and combinations thereof (Kissinger & Heineman, 1996; Heiduschka *et al.* 1994; Chen & McCreery, 1996; Rice *et al.* 1989; Kamau *et al.* 1985). The diversity of the surface chemistry has, in part, allowed for the broad range of applicability which is characteristic of the GCE, as the complex nature of the surface chemistry allows for the interaction of the electrode with a wide range of analyte molecules and electrolyte solutions. Although a feature which underpins the versatility of GCE in electroanalysis, it also often requires that GCE are stringently prepared in order to attain conformity on surface condition, and thereby, stability of responses derived from these surfaces.

4.1.2 Oxidised GCE

Modification of GCEs has been extensively investigated in order to attain a stable reproducible surface condition for analyses. As was detailed in Chapter 1, Section 1.2.5, GCE modification procedures are numerous and each result in a specific set of traits which are advantageous or detrimental to the system under investigation and allow for electrode surface characteristics to be tailored to analysis of a given analyte.

Anodic electro-oxidation of GCE has been shown to enhance the stability and in some cases magnitude of current response (I_p) of electrochemical analyses at GCE (Cabaniss *et al.* 1985; Thornton *et al.* 1985, Wang & Tuzhi, 1986; Hance & Kuwana, 1987; Kepley & Bard, 1988; Bowers & Yenser, 1991; Jürgen & Steckhan, 1992; Bielby *et al.* 1995). According to Kepley & Bard (1988), an anodic oxidative cycling process carried out between 0 V and 1.8 V at 200 mV.s⁻¹ (vs. Ag-QRE) in 0.1 M H₂SO₄ results in the successive generation of graphite oxide layers at a GCE surface. Although the electro-oxidation procedure applied herein was derived primarily from Kepley & Bard (1988), the electro-oxidation procedure was further combined with reports by Cabaniss *et al.* (1985), Nagaoka & Yoshino (1986), Kepley & Bard (1988), Jürgen & Steckhan, (1992), Yang & Lin (1995), Bielby *et al.* (1995) and Dekanski *et al.* (2001 a). Therein, although both cyclic and potentiometric methodologies were examined in both acid and alkali electrolytes, it was found that, in order to effectively produce an oxide layer, anodic potentials in excess of +1.6 V vs. SCE (Bielby *et al.* 1995) should be applied to GCEs in acidic electrolytes though most procedures include application of anodic potential maxima in the region of +1.8 V (Otero *et al.* 1993).

Although the mechanism graphitic oxide layer formation remain debatable, it is evident that the characteristics of the particular layer produced arise as a result of the cumulative conditions of the parameters used in that electro-oxidation procedure (i.e. time, potential, pH, specific condition of underlying carbon electrode, ions present in the electrolyte, potential, potentiostatic or potentiometric oxidation etc.) (Engstrom, 1982; Nagaoka & Yoshino, 1986; Kepley & Bard, 1988; Giordano *et al.* 1991; Otero *et al.* 1993; Jürgen & Steckhan, 1992; Bielby *et al.* 1995; Dekanski *et al.* 2001 a, López-Garzón, 2003). Engstrom & Strasser (1984) conducted studies into the alteration of the half-wave potential of several analytes, and illustrated that the specific interaction of individual analyte species with the oxide layer determines the applicability of the oxide layer. This feature allows for further tailoring of the layer condition toward CIT analysis. Furthermore, according to Chen & McCreery (1996), the result of a given electrode modification (the resulting reactivity of that electrode) can be traced according to variation in the starting electrode surface condition as modification procedures build on the basal state of the electrode after polishing. As was highlighted by Kepley & Bard (1988), the array of parameters applied in various reports of anodic electro-oxidation alone result in marked variations of the oxide layers which arise from them. Due to this, there is a need to ascertain the characteristics of a surface which results from a modification procedure such that the resulting interaction of molecules with the resulting surface can be characterised. This is needed for Pi-GCE which has not previously been imaged by any means to the best of the knowledge of the author.

4.1.3 Characterisation of oxidised GCE

The chemical composition of graphitic oxide produced by electro-oxidation has been investigated relatively broadly using techniques including X-ray photoelectron spectroscopy (XPS) (Miller *et al.* 1981; Cabaniss *et al.* 1985; Tougas & Collier, 1987; Collier & Tougas, 1987; Kelemen & Freund, 1988; Sullivan *et al.* 2000; Dekanski *et al.* 2001 a; Swiatkowski, *et al.* 2004; Bleda-Martínez *et al.* 2006; Zhao *et al.* 2008), spectroscopic ellipsometry (Kepley & Bard, 1988; Sullivan *et al.* 2000), auger electron spectroscopy (AES) (Dekanski *et al.* 2001 a), mass spectroscopy (MS) (Bowers *et al.* 1991; Sullivan *et al.* 2000; Perić-Grujić *et al.* 2002), UV-vis electroreflectance spectroscopy (Laser & Ariel, 1974), Raman spectroscopy (Bowling *et al.* 1989; McCreery, 2008), infrared (IR) spectroscopy (Giordano *et al.* 1991; Alsmeyer & McCreery, 1991; Yang & Lin, 1995; Yang & Lin, 1994; Ray & McCreery, 1999; Sullivan *et al.* 2000; López-Garzón, 2003; Shen *et al.* 2008), X-ray diffraction (XRD) (Kepley & Bard, 1988) and energy dispersive X-ray spectroscopy (EDX) (Bowers *et al.* 1991; Brodowski *et al.* 2005). Similarly, physical imaging of graphitic oxide has been reported using scanning electron microscopy (SEM) (Engstrom & Strasser 1984; Bowers *et al.* 1991) which apply similar principles to the high resolution SEM (HRSEM) applied in this Chapter, atomic force microscopy (AFM) (Zhao *et al.* 2008; Thiagarajan *et al.* 2009), tapping mode scanning force

microscopy (TMSFM) (Kiema *et al.* 2003) and scanning tunnelling microscopy (STM) (Dekanski *et al.* 2001 a, Shi & Shiu 2002).

As stated previously, the mechanism by which oxides are formed is complex and has only partially been elucidated by Yang & Lin (1995) at EOx-GCE. This lack of clarity in this area is due in part to the feature cited above: the characteristics of graphitic oxide layers are subject to the parameters and conditions of that oxidation process. It has been demonstrated that the chemical composition of an electrode will influence the electroanalytical capabilities of that electrode with several major influences on electrode reactions being suggested. These are: oxide-induced electrocatalytic effects; hydrophobicity; adsorption; double layer and resistivity (Cabaniss *et al.* 1985; Yang & Lin, 1994; Bleda-Martínez *et al.* 2006), each of which will be influenced by the species of, and extent of oxides incorporated, within a given oxide layer. Since each modification method produces a distinct oxide layer, it is necessary to determine the chemical characteristics of those layers such that their interaction with analytes and electrolytes can be elucidated. For those purposes, three characterisation techniques were applied herein. These included EDX, as a deep penetrative technique to assess the characteristics of the entire oxide layer; thereafter, two surface confined techniques were applied including FTIR as a scoping analytical method and also by XPS as a directed characterisation method to identify surface chemical composition.

Although *ex situ* characterisation techniques as described above do provide valuable characterisation techniques, *in situ* analyses are favoured as graphitic oxide is porous and degrade over time (Kepley & Bard, 1988). Electrochemical characterisation studies on EOx-GCE have been conducted by several researchers, including Engstrom (1982), Nagaoka & Yoshino (1986), Kepley & Bard (1988) Bowers *et al.* (1991), Jürgen & Steckhan, (1992) Dekanski *et al.* 2001 and Zhao *et al.* (2008). These form a basis by which the traits of Pi-GCE can be compared to EOx-GCE.

As many of the underlying beneficial characteristics of EOx-GCE rely on either some catalytic-like property of the oxide layer (which is unlikely according to Nagaoka *et al.* 1988) it is likely that these properties arise from an increased reactive surface area under a mechanism similar to that reported by Henstridge *et al.* (2010). The electrode surface functions as a porous layer, where improved responses can be accounted for by a combination of increased surface area and thin-layer-like diffusion within the layer. This assumption is however underpinned by the fact sections of graphitic oxide must be conductive to allow for these characteristics to be explanatory. Although many parameters of graphitic oxide have been characterised, the conductivity of the layer has not yet been well investigated. This is likely to be a factor of the challenges of characterising the conductivity of the layer itself. As a relatively thin and fragile layer, it is difficult to remove from the electrode surface,

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combined with its fragility when dry and degradation over time, *ex situ* responses may differ markedly from the freshly-surfaced EOx-GCE characteristics. Kepley & Bard (1988) have reported that the formation of graphitic oxide results in a loss of conductivity relative to polished GCE, and that the bulk of layers in excess of 25 nm thickness are non-conductive. Little empirical evidence of the conductivity of graphitic oxide layers has been described however. Scanning electrochemical microscopy (SECM) is versatile technique which may be applicable to these analyses, as analysis does not require an electrical connection to an external circuit in order to assess their properties (Bard *et al.* 1989). This property contrasts conventional electroanalytical techniques and, in so doing, allows for unbiased *in situ* measurement of substrates which either cannot be conveniently connected to an external circuit (Bard & Zoski, 2000; Xiong *et al.* 2007), as is likely the case with graphitic oxide. Relative conductivity of the graphitic oxide layer can thus be assessed through the use of approach curves (Kwak & Bard, 1989 a; Kwak & Bard, 1989 b; Bard *et al.* 1992; Borgwarth *et al.* 1995; Bard & Faulkner, 2001; Xiong *et al.* 2007).

Information regarding the principles of analytical instrumentation applied through this Chapter including high resolution scanning electron microscopy (HRSEM), energy dispersive X-ray microanalysis (EDX), fourier transform infrared spectroscopy (FTIR), X-ray photoelectron spectroscopy (XPS) and scanning electrochemical microscopy (SECM) have been included through Appendix A.

In this Chapter, the properties of the oxidised layers formed (Pi-GCE and EOx-GCE) were assessed in relation to polished GCE surfaces through both *ex situ* analytical methods to determine surface morphology (HRSEM) and chemical composition (EDX, FTIR and XPS), as well as through electrochemical analyses as *in situ* characterisation techniques (SECM and voltammetry). These were conducted in order to probe the nature of the two resulting oxide layers in relation to polished GCE, such that responses at the electrodes could be properly understood. This was performed with the intent of generating more sensitive and more stable electrodes for CIT analysis, and thus allowing for application of the surfaces where appropriate in the remainder of the Thesis.

4.2 Chapter objectives

This Chapter was directed at the identification of the surface physio-chemical characteristics of GCE which had been either chemically oxidised using piranha solution (Pi-GCE), or anodically electro-oxidised (EOx-GCE), and assessing the extent to which these properties influence the anodic detection of CIT in relation to unmodified, polished GCE. Due to the primary objective of these studies being the identification of surface characteristics of the oxide layers to account for the

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electrochemical responses of CIT, analyses were directed at surfaces tailored toward the electroanalysis of CIT.

Assessing this primary set of objectives could be separated into secondary objectives as follows:

- a) Assess the responses of CIT at increasing layer thicknesses of graphitic oxide formed at GCE by cyclic oxidation such that the traits of the oxide layer which provided the most preferential responses of CIT could be identified for subsequent characterisation.
- b) Identify the physical nature of the graphitic oxide layers which resulted from the two oxidation procedures through HRSEM and electrochemical analyses in relation to previously identified characteristics of graphitic oxide at GCE.
- c) Determine whether the functional groups present within the graphitic oxide layer influenced the magnitude, position and stability of CIT I_p , through identification of the functional chemistry of and within the graphitic oxide layer in relation to polished GCE
- d) As the electrode modifications were applied in order to improve on electrochemical responses, particularly for CIT, the objectives herein were to illustrate the electrochemical response which arose as a result of the oxide layer themselves and subsequently to identify what traits influences the responses of analytes at those layers.
- e) In order to account for the mechanism which affords improved electrochemical activity, the conductivity of graphitic oxide must be assessed. The objectives herein included the assessment of whether graphitic oxide is conductive and to what extent does conductivity affect the surface area which contributes to electrochemical responses.
- f) The final objective included applying the characteristics determined though the study to outlining a mechanism which described the improved electrochemical responses of CIT at EOx-GCE.

4.3 Chapter specific methods and materials

General protocols, reagents, analytes, methodologies, instrumentation and electrochemical procedures listed in Chapter 2 were followed in this Chapter. which were described in Chapter 2 were followed herein. Specific details on the experiments carried out in this section of the study are illustrated hereafter with reference to their specific sections.

4.3.1 Reagents

A stock solution of 4 mM citrinin (CIT) was prepared with dehydrated absolute methanol and stored at $-20\text{ }^{\circ}\text{C}$ until used, hydroquinone was prepared freshly in milliQ H_2O to a stock concentration of 100 mM immediately prior to use.

4.3.2 Characterisation of electrode surface condition though *ex situ* methodologies

Although electrochemical studies were carried out at standard GCE and AuE purchased from (BioAnalytical Systems) (BAS, USA), HRSEM, EDX, FTIR, XPS and SECM were either conducted at sawn off (5 mm length) GCE or on GCE discs removed from the Teflon sheath by pressure to the rear of the electrode. In each case the glassy carbon (GC) disc of the electrode was connected to a copper connector using carbon paste at the base of the well of the GC disc within the Teflon. Electrochemical responses and internal resistance of the altered GCE were indifferent from that of unaltered GCE. Selection of either sawn off GCE (Figure 4.2 inset) or GC disc was a factor of dimensions applicable to analytical equipment used.

a) High resolution scanning electron microscopy (HRSEM)

High resolution scanning electron microscopy (HRSEM) was employed to visualise the physical morphology of electrode surfaces under each of the conditions employed in this study at the atomic scale. This was carried out on the sawn off GCE described above. Images were recorded for polished, Pi-GCE and EOx-GCE (Chapter 2). Analyses were performed on behalf of the author by Dr R.D. Brimecombe (Mintek, South Africa) using a Nova 230 HRSEM from FEI with an X-ray microanalysis module for energy dispersive microanalysis (EDX) measurements (Appendix A2.1). All visualisations were performed under high vacuum with a high voltage (HV) between 1 and 20 kV. Visualisation detectors used included, initially, a through-lens detector (TLD) to visualise secondary electrons, as well as a back scatter electron (BSE) detector when immersion mode was used, and finally an Everhart-Thornley detector (ETD) which was used to detect secondary electrons. Specific parameters applied in each image are listed as a footnote on the relevant HRSEM images. Detector

selection was based on the quality of the resulting image, and was detailed in the footnote or appropriate figures.

b) Energy dispersive X-ray (EDX) spectroscopy

Spot elemental analysis of oxide layers was carried out on each of the samples assessed by HRSEM through EDX spectroscopy on behalf of the author by Dr R.D. Brimecombe (Mintek, South Africa) using an EDAX Genesis EDX (AMETEK Process and Analytical Instruments) coupled to the FEI Nova 230 HRSEM (Appendix A3.1). The EDAX module was operated using EDAX Genesis v 4.52 Software, performing EDX spectrum collection and quantitative analysis. Background GCE composition was determined on polished GCE, and on oxide layers were assessed by sampling at random points across the GCE surface after both Pi-GCE and on EOx-GCE which had been oxidised for 25, 50, 75 and 100 cycles respectively.

c) Fourier transform infrared (FTIR) spectroscopy

The surface chemistry of polished GCE, Pi-GCE and EOx-GCE was assessed using a PerkinElmer Spectrum 100 FTIR spectrometer (Appendix A4.1) on the sawn off GCE. Data analysis and control of the FTIR was carried out through using the software program Spectrum version 6.3.5 (PerkinElmer, inc., 2009). In each case, the atmospheric background signal determined before analyses was subtracted from all responses. Responses were recorded as an average of 8 scans.

d) X-ray photoelectron spectroscopy (XPS)

The surface chemistry of EOx-GCE and Pi-GCE were assessed relative to polished GCE as a baseline. In each case the inner GC disc plate (Section 4.3.2). This was achieved by applying pressure to the copper wire which protrudes from the rear of the electrode, forcing the GC disc being from the front. XPS analyses were carried out using a Kratos Axis Ultra DLDn (Appendix A5.1), operated by both Dr E.M. Antunes and Dr C. Litwinski of Rhodes University. An Al (monochromatic) anode, equipped with a charge neutraliser was used. Scans were carried out with the following procedure settings: Emission 5 mA, anode (HT); 15kV, operated at a pressure below 5×10^{-9} Torr. Spectra were recorded using a hybrid lens and resolution at 160 eV and pass energy in slot mode. The centre for broad scans was at 520 eV and the width at 1205 eV, with steps at 1 eV and dwell time at 100 ms.

Data Analysis of XPS Spectra

Determination of the area of the peaks was performed through integration of the peak with application of a polynomial curve to fit the baseline of the spectrum with application of NOVA 1.5 software (Eco Chemie, Nederland).

Deconvolution of peaks of the spectra was performed using a continuous probability distribution for normally distributed curves (Eq. 4.1) as was the case for the spectra assessed distribution (Harraway, 1997) with midpoints of the different bonded states applied from published values (Sundberg *et al.* 1989; Nakayama *et al.* 1990; Dekanski *et al.* 2001; Zhou *et al.* 2007). Deconvoluted peaks were modelled with application of the reiterative minima-seeking algorithm, “Solver” of the Microsoft® Office Excel suite, as described has been applied previously by Walsh & Diamond (1995) and Lin *et al.* (2006), with parameters of application in deconvolution of overlapping peaks detailed by Walsh & Diamond (1995). Deconvolution was performed with application of predetermined peak binding energies as is detailed in text for each peak. As multiple deconvoluted peaks overlap in the spectrum, that the area and shape of the modelled peaks were calculated such that total area and shape or modelled peaks equal the peak being deconvoluted, requiring the application of (H) to model the intensity of the overlapping peaks (Walsh & Diamond, 1995) rather than a single peak distribution reported by Harraway (1997).

$$f(x) = H \left(\frac{1}{\sigma\sqrt{2\pi}} \right) e^{-\frac{(x-\mu)^2}{2\sigma^2}} \quad \text{Eq. 4.1}$$

Where, $f(x)$ is the modelled peak intensity at position (x) in the x-axis (Binding Energy, eV), H is the maximal intensity of the modelled peak height (cps) above the base line, σ is the standard deviation and μ is the mean of the modelled peak (eV).

e) Scanning electrochemical microscopy (SECM)

SECM was carried out using a Uniscan Instruments Model 370 scanning electrochemical workstation, monitored and controlled using the software package M370 version 4.11.2339 PAR (Princeton Applied Research, 2011) (Appendix A6.1). Analyses were carried out using a three electrode electrochemical cell, using a 25 μm diameter carbon ultramicroelectrode (UME) insulated with glass which was bevelled to the tip (tip was a disc), a Ag/AgCl reference electrode and a platinum wire auxiliary electrode.

4.3.3 Electrochemical procedures and parameters in modification and characterisation of electrode surface condition

The electrochemical apparatus applied in these studies was a three electrode electrochemical cell using gold (AuE) and glassy carbon (GCE) working electrodes, platinum auxiliary electrode and Ag/AgCl reference electrode. The electrochemical and operational parameters outlined in Chapter 2 with regard to equipment specifications, electrode fabrication, cleaning and modification and scanning procedures were followed as followed.

Unless otherwise stated, all electroanalysis was carried out in a 20 μM CIT solution in 0.2 M sodium acetate buffer (NaOAc) pH 4.0 between 0.5 V and 1.1 V at a scan rate of 100 $\text{mV}\cdot\text{s}^{-1}$ vs. Ag/AgCl.

a) Influence of the extent of electro-oxidation on the electrochemical responses of CIT

CIT responses with regard to the extent of oxidation were derived through varying the number of electro-oxidation cycles (Chapter 2, Section 2.3.1 [b]). Both CIT I_p and E_p were assessed at EOx-GCE using LSV after 0, 1, 5, 10, 15, 20, 25, 30, and 50 oxidation cycles respectively, resurfacing the GCEs after each scan.

b) Voltammetric characterisation of Pi-GCE and EOx-GCE in relation to polished GCE

CV scans of polished GCE, Pi-GCE and EOx-GCE, were conducted between -0.8 V and 1.1 V. Both aerated and de-aerated buffered solutions were assessed. Hydroquinone (0.3 mM) in 0.2 M NaOAc buffer pH 4.0 was also assessed at each surface as a representative of electrochemical response in accordance with Dekanski *et al.* (2001 a).

c) Influence of oxide layer surface chemistry on CIT electrochemical responses through the application of oxide terminating self assembled monolayers (SAMs)

AuE was cleaned and its surface area determined according to Chapter 2. These surfaces were used for formation of SAMs, by making use of the thiol bond with Au surface (Carvalho *et al.* 2005). Thoroughly degassed solutions of 1 mM β -mercaptoethanol (C-OH), cysteamine (C-NH₂), 1,2-benzenedithiol (C-C), 3-mercaptopropionic acid (C-COOH), and a mixed solution of 1:1 (v/v) 0.5 mM 3-mercaptopropionic acid and 0.5 mM 1,2-benzenedithiol were prepared with 80 % dH₂O and 20 % absolute ethanol. These solutions were used in the formation of SAMs, with each solution providing for a single SAM. Cleaned AuE were suspended in one of the solutions for a period of 18 hours to allow formation of the SAM, after which time the analysis of 20 μM CIT at each SAM was carried out.

d) Voltammetry of CIT at EOx-GCE and Pi-GCE in relation to polished GCE

CIT was assessed at each surface in a single 15 μM CIT solution by CV between -0.8 V and 1.1 V. Subsequently, the potential window was narrowed to 0.5 V and 1.1 V and analyses were conducted in a 20 μM CIT solution and assessed for each of the electrode surfaces.

e) Determination of EOx-GCE surface area

Surface area determinations were as described through Chapter 2, Section 2.3.3 [b].

4.3 Results and Discussion

Results in this Chapter are separated into three main sections. The first section is dedicated to determining the parameters of electro-oxidation processes on GCE in relation to CIT responses at the electrodes. The following section included surface investigations of both chemically and electrochemically oxidised GCE. Those studies were directed at identifying surface traits and characteristics, including chemical composition (surface functionalities) and structural morphology (physical appearance and condition) of the layers arising from the two oxidation treatments. The final section assesses the electrochemical responses at the electrodes in relation to one another and suggests a mechanism by which improved electrochemical responses may be garnered at electrodes modified by oxidation through application of the analyses in this Chapter.

4.3.1 Electro-oxidation of glassy carbon electrodes (EOx-GCE)

a) Visual examination of cyclic electro-oxidation of GCE

The surfaces of well polished GCEs are typically described as having a mirror-like shine, however with modification of the electrode surface, the appearance is often altered. Evidence of this effect is represented through Figure 4.1.

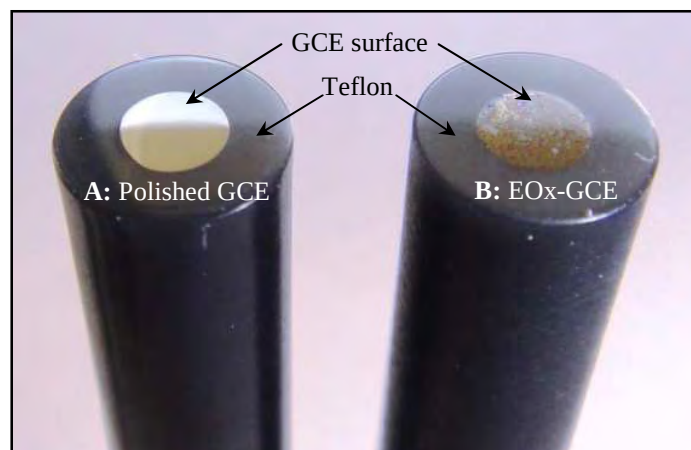


Figure 4.1: Visual change in polished GCE surface [A] in relation to an electrochemically oxidised GCE (EOx-GCE) surface [B].

Once oxidised, the mirror like shine of polished GCE (Figure 4.1 [A]) took on a dulled reflective appearance and also took on a range of colours, including purple, black, yellow, gold, red and green (Figure 4.1 [B]). When dry, the EOx-GCE surface become progressively more opaque and when completely dry, white flakes separated from the surface. Water beaded on the surface of polished GCE, indicating the presence of hydrophobic groups; however after electro-oxidation, beading of water was reduced, indicating the presence of more hydrophilic surface composition at EOx-GCE, a

result in accordance with Bleda-Martínez *et al.* (2006). These responses compare closely with that previously reported (Engstrom & Strasser, 1984; Cabaniss *et al.* 1985; Jürgen & Steckhan, 1992).

b) Influence of the number of electro-oxidation cycles on the electrochemical responses of CIT

As was described through Chapter 3, variability in CIT I_p responses was likely to be a factor of interaction of CIT with inconsistent electrode surface preparations. As the extent of oxidation will influence the amount of graphitic oxide formed (Engstrom & Strasser, 1984; Kepley & Bard, 1988; Sullivan *et al.* 2000) thereby influencing kinetics of responses at EOx-GCE (Vettorazzi *et al.* 1998), the number of oxidation cycles was thought to be likely to influence CIT I_p response. The resulting influences of the extent of oxidation were thus assessed relative to both CIT I_p and E_p as is illustrated in Figure 4.2.

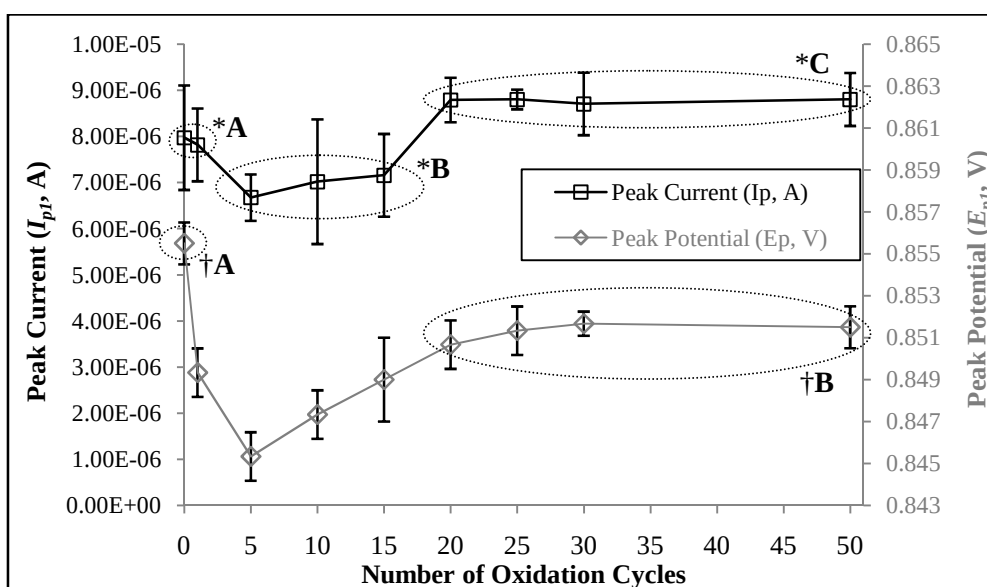


Figure 4.2: Influence of extent of electro-oxidation of GCE on 30 μ M CIT I_p (black squares) and E_p (grey triangles) in 0.2 M NaOAc buffer pH 4.0. Extent of oxidation was controlled through number of oxidation cycles performed, including 0, 1, 5, 10, 15, 20, 25, 30, and 50 successive cycles respectively. Dotted circles represent areas of no significant difference as assessed by ANOVA (Fisher LSD test, $p > 0.05$) Number of scans per data point ≥ 4 , standard deviation was reported from the mean.

As is evident through Figure 4.2, the extent of graphitic oxide formation (by the number of oxidation cycles carried out) indeed influences the response of CIT with regard to both I_{p1} and E_{p1} . Importantly, although both I_p and E_p decreased from initial responses at polished GCE (0 oxidation cycles in Figure 4.2), variability decreased and responses plateaued after 20 oxidation cycles, remaining stable through to 50 cycles. Similar responses were reported by Kepley & Bard (1988) though they stated further that the modification was not permanent as the surface became deactivated after several hours and returned back to its original state, a trait common to EOx-GCE.

The drop in both I_{p1} and E_{p1} after 1 oxidative scan (Figure 4.2) is interesting but as they are largely governed by separate processes, accordingly they were considered separately. Responses were compared by ANOVA using a Fisher LDT test, $p > 0.05$ (Chapter 2, Section 2.3.3) and similar responses grouped together by dotted circles with the symbol [*] for I_{p1} responses and [†] for E_{p1} responses in Figure 4.2. Considering the I_{p1} responses of Figure 4.2, group [*A] was not significantly different from groups [*B] or [*C], however groups [*B] or [*C] were significantly different from one another. The lowered I_{p1} responses with successive formation of oxide layers between 1 and 5 oxidation cycles, followed by an increase in I_{p1} from 15 oxidation cycles and stabilisation of I_{p1} responses after 20 oxidation cycles (group [*C]) may be an indication of a gradual change in the underlying composition of the oxide layer with successive oxidation cycles. Similar traits were reported by Braun *et al.* (2003) for thermal oxidation of GCE wherein a diffusion limitation from oxidation yields a decreasing reaction rate. They proposed that evolution of porosity occurs through the entire layer rather than just the GCE interface, thus gradually the electrochemical responses will improve with improved porosity and thus surface area.

Although the basic physical structure of the layer is known to be porous (Kepley & Bard, 1988) and comprised of graphitic oxide, primarily bearing carbonyl and hydroxyl based moieties (Kepley & Bard, 1988, Yang & Lin, 1995, Sullivan *et al.* 2000), these results may indicate that with more extensive oxidation cycles, the layer may become more organised and more uniform, and in so doing may allow for more effective electrochemical responses. The initial drop in I_{p1} in Figure 4.2 may also be a factor of the layer acting as a diffusion barrier to CIT, a consideration discussed further through Chapter 6 in relation to scan rate studies. Kepley & Bard (1988) showed graphitic oxide layers to be porous and to accumulate analyte, thus with increased layer thickness, the pores would be more numerous, increasing both the internal area of the layer and allowing for more effective accumulation of CIT, thereby accounting for the increase in I_{p1} responses after 15 oxidation cycles, as well as the stabilisation observed after 20 oxidation cycles, possibly due to stabilisation of pore structure after that point (Figure 4.2).

Interestingly, the oxide layer (after 20 oxidation cycles) does not hinder the oxidation reaction as compared to unmodified GCE, even though in Figure 4.2, lower I_{p1} responses were initially observed with the introduction of graphitic oxide. Rather, stable I_{p1} and E_{p1} responses are observed in the presence of graphitic oxide at the electrode surface, with [†A] being significantly different from all other responses across the data set. This trait indicates that the layer stabilised the CIT oxidation process to some extent, possibly as a function of mass transport being more thin-layer like, as is the case for CNTs at electrodes acting as a porous surface, described by Henstridge *et al.* (2010). After an

initial, substantial, drop in E_{pl} from polished GCE with the introduction of graphitic oxide, the E_{pl} increased with successive cycles until it too stabilised at 20 oxidation cycles onward (group [†B]).

Although slight, comparison of the E_{pl} of responses at EOx-GCE after 20 oxidation cycles with an $E_{pl} = 0.851 \text{ V} (\pm 0.001 \text{ V})$ to polished GCE (Chapter 3, Section 3.4.1) with $E_{pl} = 0.838 \text{ V} (\pm 0.002 \text{ V})$ revealed that the graphitic oxide layer did shift oxidation to slightly higher potentials, however it also increased the stability of peak potential responses. Although the oxide layer does influence the kinetics of oxidation to an extent, this influence is minor and the improvement in the I_{pl} responses compared to a polished GCE outweigh any negative impact of shifting E_{pl} to slightly higher potentials. Due to the factors outlined above and the stability in both I_{pl} and E_{pl} responses for CIT oxidation at EOx-GCE after 20 oxidation cycles, that point was selected as the standard for electro-oxidation of GCE for the specific analysis of CIT.

4.3.2 Elucidation of polished GCE, Pi-GCE and EOx-GCE surface morphology using high resolution scanning electron microscopy (HRSEM)

a) HRSEM of polished GCE

Typically, GCE are polished to a mirror shine (Figure 4.1 [A]), at which point the surface is generally considered ‘smooth’ and uniform. Although polished GCE have been imaged via a number of microscopic techniques, as was reported through the introduction to this Chapter, the results represented in this section form the first HRSEM examinations of GCE to the best of the knowledge of the author and allow for detailed microscopic surface characterisation. Representative images of HRSEM of GCE are presented in Figure 4.3.

At low magnification, Figure 4.3 [A] and [B], as in Figure 4.1 [A], GCE appears to be a smooth and homogenous surface, especially when compared to conductive tape and Teflon coating. Analysis of high magnifications of GCE surfaces Figure 4.3 [C] and [D] however clearly illustrate the marked influence of polishing on GCE where inhomogeneity of the surface morphology is clearly visible. Furthermore, subsequent polishes of a GCE (Figure 4.3 [D]) show that there is a change in the surface condition with each polishing. The variability in the extent of abrasion by the polishing procedure between polishes (Wiesshaar & Kuwana, 1985; Kamau *et al.* 1985; Rice *et al.* 1989) will result in differences in effective surface area between polishing procedures.

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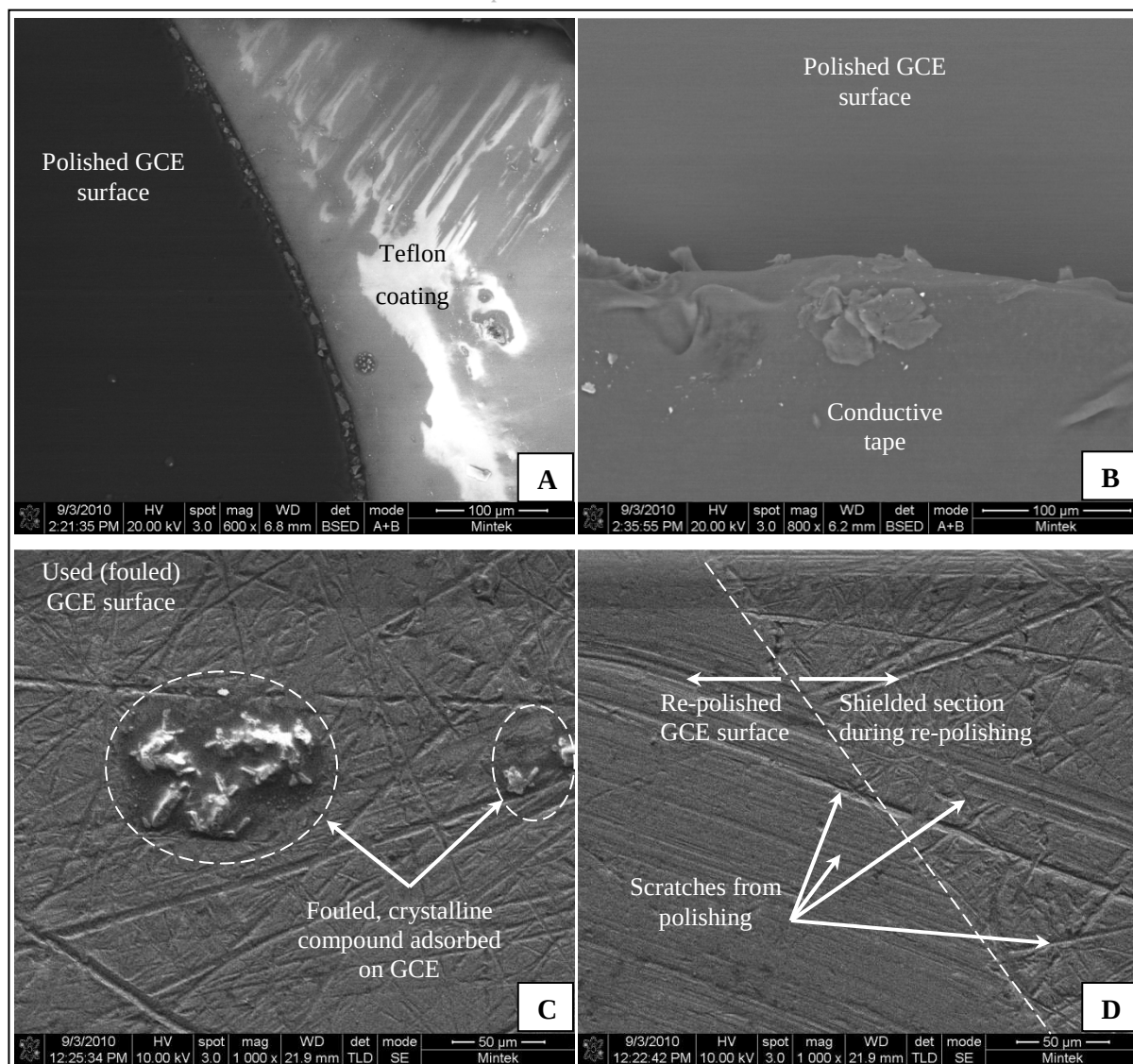


Figure 4.3: HRSEM images of polished GCE. Low magnification images [A] and [B] compared the GCE surface to the Teflon coating and conductive tape respectively. High magnification images illustrate the need for polishing as a result of GCE fouling by material adsorbed after electroanalysis [C] and the influence of polishing on the previously fouled surface as an abrasive process [D]. Magnification, detector, mode and scale of the images are included as footnote in each image.

It is not expected that the physical characteristics of bulk GCE will influence the result of polishing (i.e. densities and hardness of GCE are relatively spatially uniform), though variability with regard to functionalities of the surface has been noted (Collier & Tougas, 1987; Yang & Lin, 1994; Kissinger & Heineman, 1996; Chen & McCreery, 1996; Ray *et al.* 1999). These findings agree closely with those of literature (Kamau *et al.* 1985; Wiesshaar & Kuwana, 1985; Kissinger & Heineman, 1996; Kiema *et al.* 2003; Zhao *et al.* 2008) and the influence which polishing has been reported to have on polished GCE.

While the presence of micropores ranging from 10 Å to 20 Å have been reported by several researchers (Shi & Shiu, 2002; Kissinger & Heineman, 1996; Nagaoka & Yoshino, 1986) these pores

were not observable at the magnifications of the images in Figure 4.3. Shi & Shiu (2002) also reported on the presence of microcrystallites (approximately 25 nm in diameter) present on GCEs imaged via STM. Although not clearly highlighted through the images of Figure 4.3, the roughness evident in [C] and [D] could be influenced by these feature, though since Kissinger & Heineman (1996) reported the roughness factor of GCE to range from 1.5 to 3.5, it is likely to be more influenced by the amorphous macromolecular structure of GCE (combination of basal planes and edge planes exposed by abrasion during polishing).

Although polishing procedure clearly induces variability in the GCE surface state (Figure 4.3 [D]) its application is a necessity due to it providing a fresh, clean electrode surface after fouling of an electrode, the necessity for which has been highlighted by Figure 4.3 [C]. Although Figure 4.3 [C] illustrates fouling of the GCE surface in the form of adsorbed crystalline matter, subsequent polishing removes that material as well as amorphous material present at the fouled surface. Analysis of the influence of polishing of GCE can be made on a generalised basis; however, factors such as the particle size of the aluminium oxide, the force used during polishing, the duration of polishing, the motion used for polishing, the type of surface or felt pad polished on and the consistency of the slurry, to name but a few factors, will each influence the resulting surface from a polishing procedure, resulting in different surface characteristics (chemical and morphological) of GCE after each polishing (Wiesshaar & Kuwana, 1985; Kamau *et al.* 1985).

Although generally considered to result in a uniform surface preparation, the images of Figure 4.3 show that the resulting surface is clearly different at the micro-scale for each polishing. Kamau (1988) stated that the prior history of an electrode treatment has a major influence on responses gained at that electrode as well as on the specifics of subsequent modifications of that surface. As polishing produces inconsistencies in surface area between procedures, when more complex cleaning and pre-treatment procedures are used on those electrodes, this underlying variability may be amplified. Accordingly, electrode modifications which introduce homogeneity to the GCE surface were preferentially selected. Modification of GCE by formation of graphitic oxide has been shown to introduce stability in electrochemical response (Wang & Tuzhi, 1986; Kiema *et al.* 2003), as compared to polished GCE. Two means of forming graphitic oxide at GCE were analysed hereafter, including chemical oxidation by piranha solution and anodic electro-oxidation.

b) HRSEM of chemically (piranha solution) oxidised GCE (Pi-GCE)

Although application of piranha solution at GCE has been reported by Carrington *et al.* (2006) and Berríos *et al.* (2008), to the best of the knowledge of the author, Figure 4.4 provides the first observation of the impact of piranha solution on GCE morphology.

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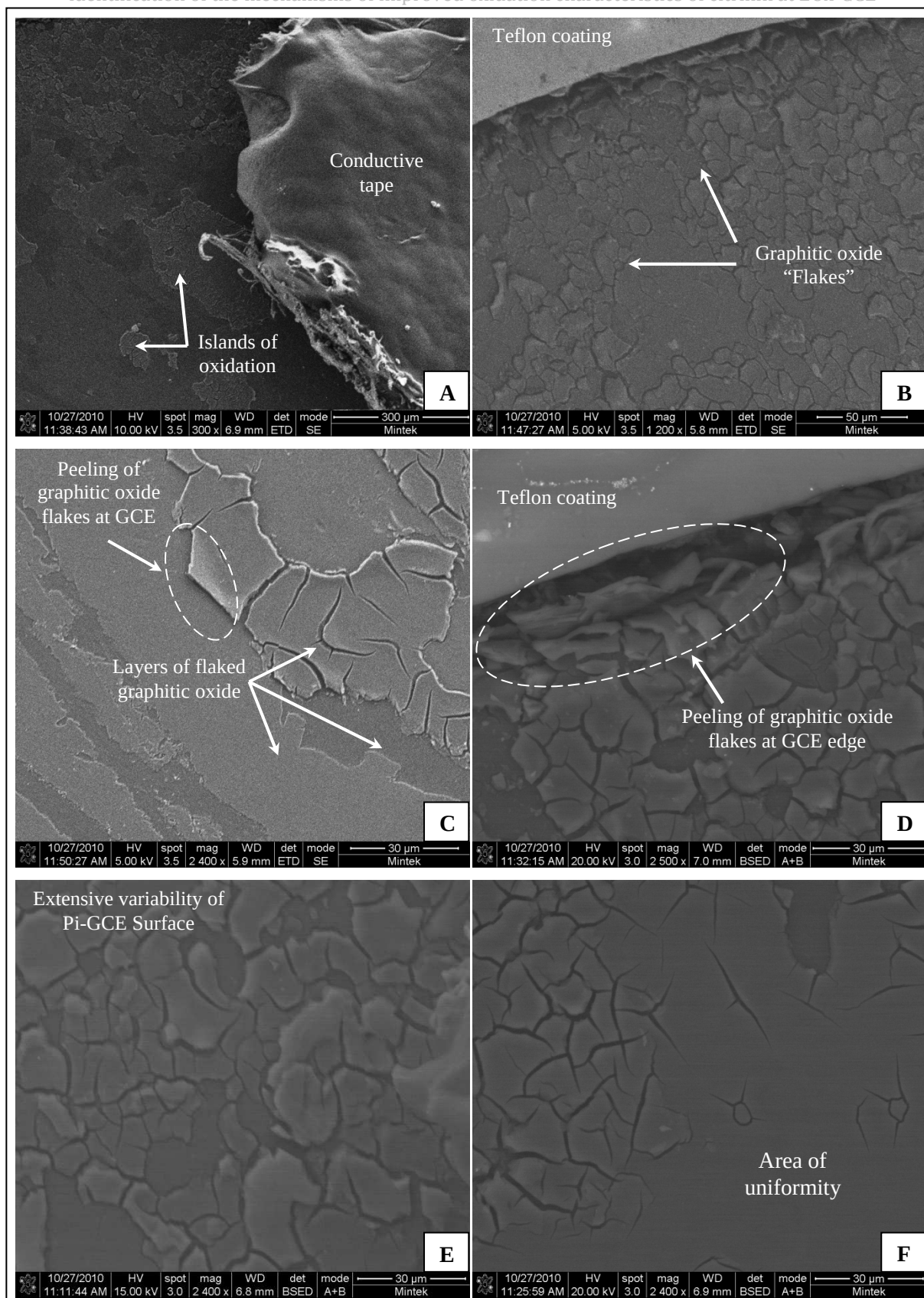


Figure 4.4: HRSEM images of Pi-GCE surfaces. Figure [A] illustrates low magnification of GCE as compared to conductive tape. Figures [B] – [F] illustrate higher magnifications with different modes and detectors, illustrating the formation and subsequent flaking of graphitic oxide from the GCE and resulting inhomogeneity of the surface. Specific magnification, detector (det), mode and scale of the figures are included as a footnote in each image, refer to Section 4.3.2 [a] for further explanation of the footnote.

Comparing HRSEM images of polished GCE (Figure 4.3) to those of Pi-GCE (Figure 4.4), it is clear that the application of piranha solution as a chemical oxidant has a visible and substantial effect on the GCE surface. Even when viewed under low magnification, Pi-GCE (Figure 4.4 [A]) showed extensive inconsistency in surface morphology with apparent formation of islands or patches of oxidation of the GCE surface as compared to polished GCE (Figure 4.3 [A] and [B]). Furthermore, the areas of oxidation appear to have different depths on the Pi-GCE surface which suggests etching, layer ablation/exfoliation or inconsistent layering on the GCE. This inconsistency is clearly visible under higher magnifications (Figure 4.4 [B] – [F]) wherein, rather than extensive etching of the GCE surface, it appears as a cracked, flaking layer which resembles the cracking of dried mud on the GCE surface. When examining Figure 4.4 [C] and [D], it appears that the islands initially suggested from Figure 4.4 [A] result from delamination of layers from the GCE surface by flaking of the graphitic oxide film. This phenomenon results not only in highly variable surface areas between modifications, but also that the thickness of the formed graphitic oxide layer will be neither laterally uniform nor thick due to this delamination. This is characteristic of the aggressive nature of oxidation of piranha solution. These parameters combine to result in what is clearly a highly variable surface (intra-oxidation variability) as well as across repeated oxidation procedures (inter-oxidation variability).

Layer ablation accounts for the formation of ‘flakes’ which separate from the GCE. The ‘flakes’ appear to be of a similar composition to the graphitic oxide formed by electro-oxidation. This is assessed further through surface chemical analysis detailed in Section 4.3.3 of this Chapter. As piranha solution is an aggressive oxidant, acting indiscriminately on a surface, it is not surprising that the GCE did not result in a uniform mat of graphitic oxide, though areas of uniform thickness are evident (e.g. Figure 4.4 [F]).

An important point to note with regard to this process is that due to indiscriminate oxidation by piranha solution, the underlying polished surface condition was unlikely to hold influence on the resulting oxide layer formed. One parameter which may bear a slight influence on initial oxidation is that of the presence of scratches at the electrode surface may provide sites at which the piranha solution can act more vigorously. Beyond that, however, it is proposed that polishing is unlikely to hold much influence: if oxidation did progress from scratch lines, one would expect more extensive straight lines in the morphology of the oxide layer. As these are not present in Figure 4.4 [B] - [F], it can be concluded that the flakes arise independently of GEC surface structure after polishing.

The impact of the presence of these flakes within the constraints of electrochemical analysis could plausibly explain I_p variability which is highlighted in Figure 5.5, Chapter 5. As is clearly evident from the images of Figure 4.4, there is no homogeneity in the electrode surface condition when

chemically oxidised. From consideration of images [B] and [C] of Figure 4.4, it can be surmised that the flakes observed on the GCE surface arise as a result of an oxidative process at the GCE surface rather than deposition from solution. This can be stated as the flakes firstly do not form on the Teflon coating, and secondly, as is highlighted in image [C] of Figure 4.4, the flakes peel away from the electrode surface at the edges where there would be more surface area exposed to the piranha solution, which would account for more oxidative activity at those areas.

c) HRSEM of electro-oxidised GCE (EOx-GCE)

The images of EOx-GCE (Figure 4.5) describe a surface which is distinct from both polished GCE and Pi-GCE surfaces, highlighted by a prominent and extensive layer (in contrast to un-oxidised GCE and the Teflon coat by Figure 4.5 [A] and [B]). The EOx-GCE graphitic oxide layer is a fairly inconsistent surface, with many surface features best described as “ridges”, “bumps” and “valleys” when viewed at magnifications of Figure 4.5 [A] – [E]. The imperfections of the EOx-GCE layer is however more uniform compared to Pi-GCE (Figure 4.4) and is a marked improvement as compared to polished GCE (Figure 4.3) as scratches from polishing are completely masked by the graphitic oxide layer. The deformations of the EOx-GCE are effectively macro-scale, non-planar features, described as ‘shape’ of the electrode surface according to the notation detailed by Pajkossy (2005). Pajkossy (2005) further described such surface features as having a lesser influence on responses than electrode surface roughness, due primarily to the influence of scale in altering surface area of a macroplanar electrode surface. Other than the ‘shape’ imperfections, the EOx-GCE layer seemed to form relatively uniformly and continuously across the GCE surface. Physically, the is considered relatively uniform and to present characteristics typical of similarly produced graphitic oxide layers (Bowers *et al.* 1991; Heiduschka *et al.* 1994; Dekanski *et al.* 2001; Kiema *et al.* 2003; Engstrom & Strasser 1984). A further feature evident from the images of Figure 4.5 is the removal of impurities from direct contact with the electrode surface, illustrated through Figure 4.5 [A] and [B], a result corroborated by studies by Kiema *et al.* (2003).

Shi & Shiu (2002) viewed EOx-GCE produced by both electro-oxidation using STM. Although imaged at higher magnification than that reported in Figure 4.5 [F], they found that the extent of oxidation influenced the uniformity of the layer at the nano-scale, wherein through an increase in oxidation extent, a proportional increase in the voids/etch pits in the oxide layer was found. Although not clearly visible through the images of Figure 4.5, this result, along with the findings of Engstrom & Strasser (1984), Kepley & Bard (1988), Nagaoka *et al.* (1988), Bowers & Yenser (1991) and Dekanski *et al.* (2001 a) suggest that the EOx-GCE layer is porous, and may allow for inclusion of molecules such as CIT within the oxide layer.

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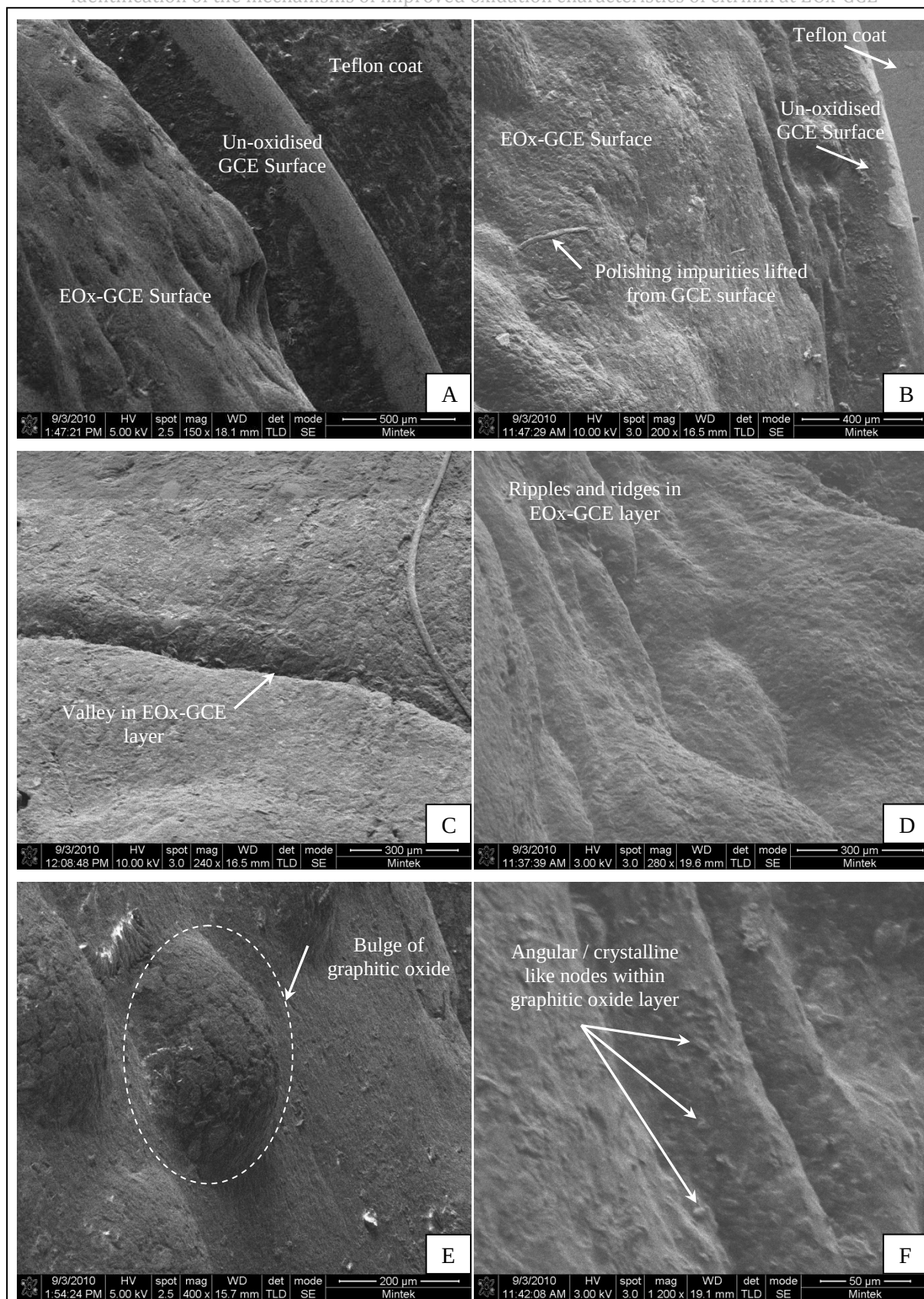


Figure 4.5: HRSEM images of EOx-GCE surfaces. Figures [A] and [B] illustrate low magnification views, contrasting the graphitic oxide with un-oxidised GCE surface and Teflon coat. Figures [C] – [F] show images of increasing magnification illustrating the topography of the graphitic oxide layer. Specific magnification, detector, mode and scale of the figures above are included as a footnote in each image which are further explained in Section 4.3.2 [a].

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A further trait which was found to be characteristic of EOx-GCE is the presence of light spots which occupy approximately 5 % of the imaged surface, illustrated through Figure 4.6. These features have been reported previously by Bowers *et al.* (1991), who used SEM, wherein they were demonstrated to be primarily surface related and to occur predominantly on GCE which had been oxidised in solutions of H_2SO_4 (as was the case in this study). Although not commented on, the SEM images reported by Engstrom & Strasser (1984) illustrate similar light spots as those seen in Figure 4.6.

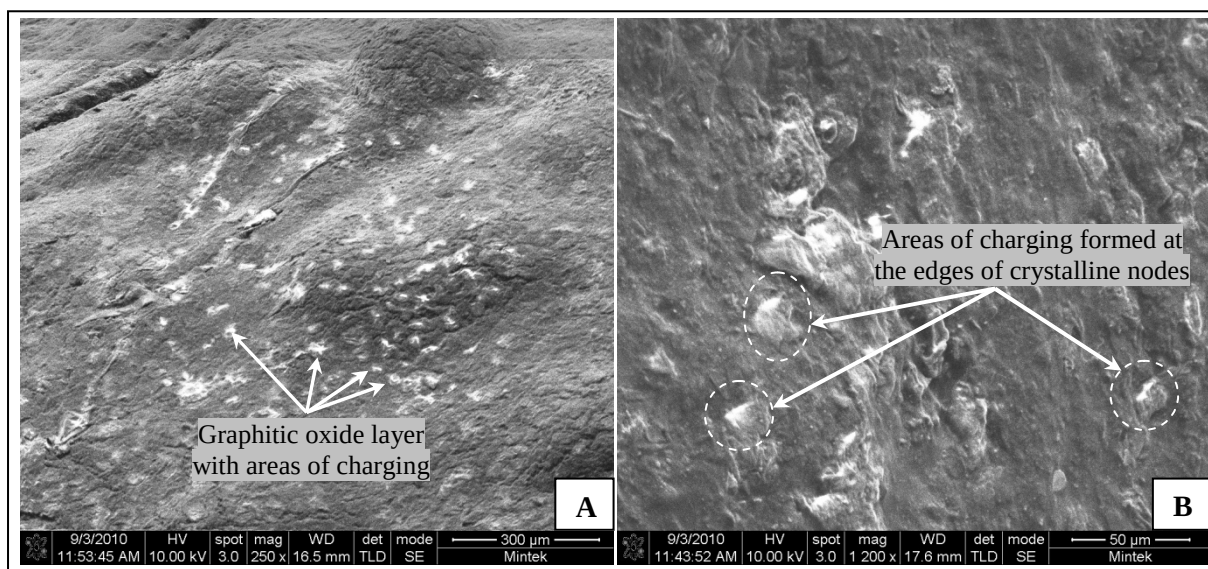


Figure 4.6: HRSEM images of areas of charging at nodes within the graphitic oxide layer of EOx-GCE at low [A] and high magnification [B] respectively. Specific magnification, detector, mode and scale of the figures above are included as a footnote in each image.

The presence of the light spots are considered to be a function of the imaging of surfaces with electron beams, as this feature is not evident in images recorded using AFM (Thiagarajan *et al.* 2009), STM (Shi & Shiu, 2002) or SFM (Kiema *et al.* 1999) on EOx-GCE. Due to this, the light spots are likely to be areas of charging as a result of lower conductivity or possibly an insulatory nature in these regions. Accordingly; henceforth, the light spots are termed areas of charging. Bowers *et al.* (1991), when conducting spatially resolved X-ray compositional mapping of the areas of charging found a significantly higher proportion of sulphur in the areas of charging compared to the darker regions.

In addition to not being visible under imaging techniques other than SEM, these areas of charging were not present in either polished GCE or Pi-GCE surfaces, even though H_2SO_4 was present in the piranha solution used in chemical oxidation. Accordingly, their occurrence is likely to have arisen through an electrochemically linked oxidation mechanism, possibly involving S or the accumulation of S from H_2SO_4 . Considering the surface coverage of charging reported from this study, and by Bowers *et al.* (1991), in comparison with the relatively low concentration of areas of charging which can be seen in SEM images reported by Engstrom & Strasser (1984) (who applied an chronoamperometric oxidation at 1.75 V for 5 minutes), it seems that cyclic oxidation in particular

drives the formation of the areas of charging. As there to no marked differences in electrochemical activity between the two potentiometrically and cyclically formed graphitic oxide, it is unlikely that the areas of charging have much of an influence on electrochemical responses. As such it is the position of the author that many of the traits garnered from oxide layers are a factor of the bulk layer (i.e. dark areas of Figure 4.6).

It is additionally noted that the areas of charging form at the edges of raised nodes through the extent of the oxide surface as is shown in Figure 4.6 [B]. These areas of charging are not present on all of the nodes (Figure 4.5 [F]), though when a charging spot is seen, they are only present on the nodes. This result is particularly interesting when considering the process which may account for the presence of these charging spots as the layer is produced through a cycling process of oxidation and reduction, ending at 0 V after a reductive sweep. Although the presence of nodes on oxide layers has been reported by several researchers, they are more apparent when visualised by STM and SEM than in AFM images (Bowers *et al.* 1991; Shi & Shiu, 2002; Thiagarajan *et al.* 2009). Thiagarajan *et al.* 2009 carried out AFM analysis of EOx-GCE using a very similar methodology to that applied in this study, and described a highly comparative set of features to those reported herein. The nodes themselves seemed to have defined, straight edges and seem to arise almost as a result of crystallisation of parts of the oxide layer where there is greater charge density (as seen by the appearance of areas of charging). From both this study and the studies cited (Bowers *et al.* 1991 and Engstrom & Strasser, 1984), it is submitted that the crystalline nature of the areas of charging may amplify the insulator nature of graphitic oxide which was suggested by Kepley & Bard (1988). As the areas of charging had no marked influence in electrochemical analyses at these layers, they were not studied in greater detail. The matter of conductivity of the layer as a whole was however considered further through Section 4.3.4 [e] below.

d) Subsection conclusions

To the best of the knowledge of the author, the images presented in this study are the first HRSEM images of EOx-GCE and polished GCE and furthermore are the first morphological representations of the influence of piranha solution on GCE (Pi-GCE). The morphology of each of the surfaces were distinct from one another. Both electro-oxidation and chemical oxidation were illustrated to generate a surface (assumed to be graphitic oxide at this point), distinct from the abrasive scratches evident at polished GCE. EOx-GCE produced a comparatively uniform and thick oxide layer as compared to the flaked surface of Pi-GCE. The layer was, however, not completely homogenous at its surface. This result however indicates that the improved electrochemical responses (as has been reported in literature) will be a factor of the internal composition of the layer, rather than the outer surface morphology.

4.3.3 Elucidation of the chemical composition of polished, Pi-GCE and EOx-GCE surfaces

a) Energy dispersive X-ray spectroscopy (EDX)

Several points across the graphitic oxide layer produced at a GCE were randomly assessed for analysis by EDX *in situ*. This study was carried out as a qualitative analysis of the chemical composition of the layer in conjunction with HRSEM analyses. A representative spectrum, Figure 4.7, is shown below, with comparative sections of spectra compared through Figure 4.8 and the results from those studies tabulated in Table 4.1 below.

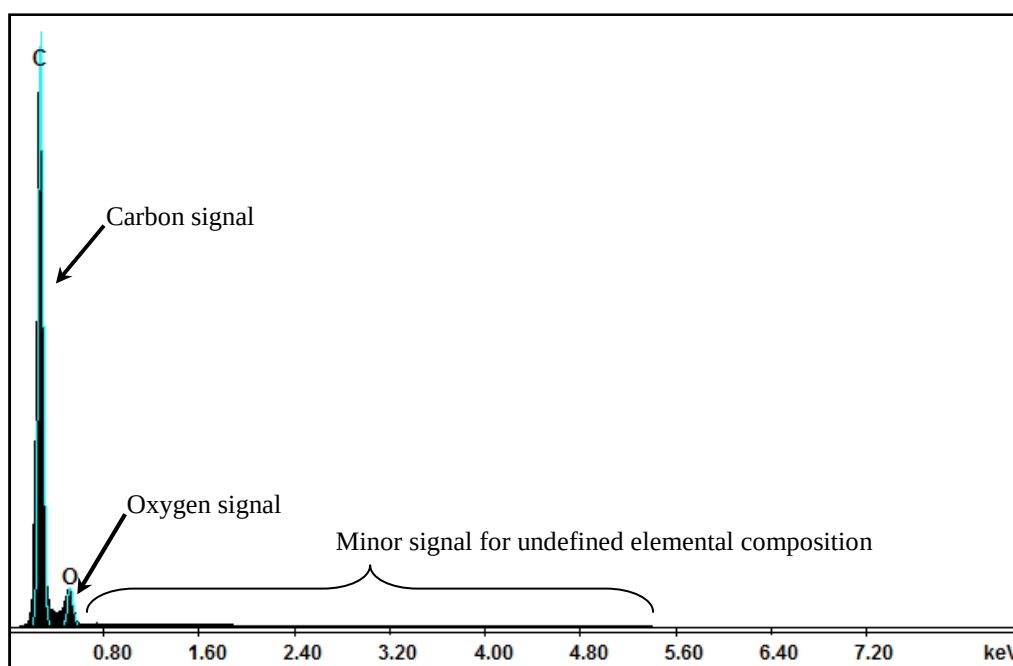


Figure 4.7: Representative full EDX spectrum acquired at EOx-GCE (75 cycles).

Figure 4.7 illustrates a representative elemental spectrum of an EOx-GCE surface. This result indicates that in addition to the carbon signal, there is a defined presence of oxygen species at each of the surfaces assessed with little indication of other elemental components in the layer. While Bowers *et al.* (1991) conducted spatially resolved X-ray compositional mapping of EOx-GCE (also formed in H_2SO_4) and reported on the presence of S within the areas of charging discussed above, little signal indicating other elemental components of oxide layers has been found in this study. As has been relatively well documented by Nagaoka & Yoshino (1986), oxide layers can take up protons, and as the layer is porous (Picq *et al.* 1984; Nagaoka & Yoshino, 1986), the layer may accumulate solvated ions, accounting for the presence of the minor, undefined signals within the spectrum.

A comparison of the chemical elemental composition of the surface with different oxide modifications is illustrated in Figure 4.8. Although the introduction of oxygen-containing functionalities has been widely reported for graphitic oxide layers formed by electro-oxidation, this result provides the first

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known empirical evidence confirming that chemical oxidation via piranha solution introduces oxygen containing functionalities at GCE, as was expected. A difference in the extent of oxidation is apparent for each of the oxidation procedures assessed, with a gradual increase in the extent of oxygen from polished GCE through to 100 oxidation cycles for EOx-GCE. The absolute % of carbon: oxygen for each of the spectra of polished GCE, EOx-GCE and Pi-GCE are represented in Table 4.1.

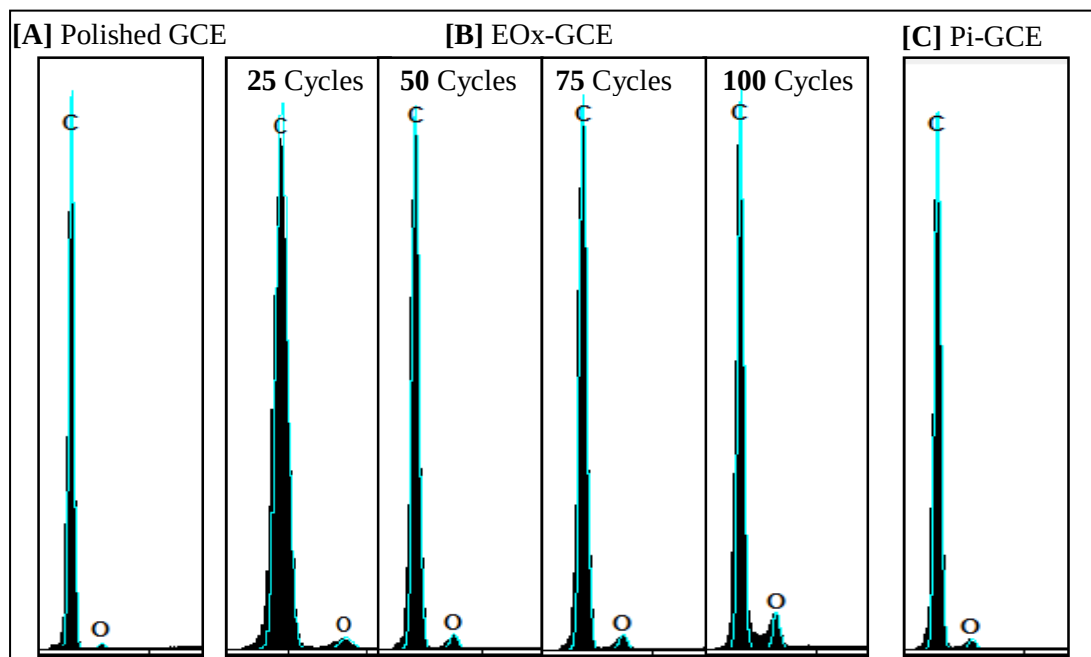


Figure 4.8: Comparative EDX spectra of the C and O signal region of [A] polished GCE, [B] EOx-GCE with oxidation cycles numbering 25, 50, 75 and 100 scans respectively and [C] Pi-GCE.

As is evident in Table 4.1 below, there is an increase in the extent of oxygen present at GCE surfaces with modification through both oxidation procedures.

Table 4.1: Carbon and oxygen composition of polished GCE, EOx-GCE and Pi-GCE as determined by EDX.

GCE Surface	Surface Modification	Elemental Composition	
		% C	% O
Polished GCE	Polishing	98.11	1.89
Pi-GCE	Piranha Solution	96.46	3.54
EOx-GCE	25 Cycles	96.28	3.72
	50 Cycles	93.39	4.61
	75 Cycles	95.18	4.82
	100 Cycles	90.32	9.68

With regard to the comparison of the two procedures, the % of O present are nearly equivalent for chemically oxidised and electrochemically oxidised GCE after 25 cycles. The similarity of these results is of interest when considering the thickness and morphology of the oxide layers relative to one another as was evident from HRSEM imaging (Figures 4.4 and 4.5). Comparison of these suggests some visual evidence that the EOx-GCE oxide layer is thicker than a Pi-GCE oxide layer, confirmed by the finding that the oxide layers flake off at the Pi-GCE, keeping that oxide layer

thinner. Due to this, it is suggested that the EOx-GCE oxide layer is likely to be more porous and hydrated (as was suggested by Kepley & Bard, 1988) than Pi-GCE.

Table 4.1 shows that there is an increase in the extent of oxidation for EOx-GCE with more extensive oxidation. This is a result which compared favourably with that which has been reported in literature, as it has been suggested that successive layers form with each oxidation cycle (Kepley & Bard, 1988). The values of % O determined in this study compare favourably with those reported by Dekanski *et al.* (2001 a) who analysed the elemental composition of graphitic oxide formed at GCE by anodic polarisation in H₂SO₄. They report % O between 2.1 and 4.7 % of the oxide layer using auger electron spectroscopy (AES), responses which align with those reported through this study in Table 4.1.

Kepley & Bard (1988) reported that very little unoxidized carbon would be present in an electrochemically activated layer (25 % O assessed by XPS). Despite its use within similar experiments in literature, EDX nevertheless carries limited qualitative and quantitative capabilities in relation to XPS analyses. These results were thus considered with due caution and illustrate the requirement for more detailed analyses through XPS, as was carried out in Section 4.3.3 [c]. As EDX responses were collected as a result of deep penetrative techniques (electron beam penetration in EDX), rather than a shallow technique such as XPS (Marmioli *et al.* 2004), the C component of the result will comprise of C determined from the bulk electrode material in addition to the oxide layer as it was assessed in situ. Due to this, it was considered necessary to further investigate the chemical composition of the electrode surfaces using XPS and FTIR as surface confined techniques to assess the surface condition of the oxide layer with little influence from the underlying bulk GCE itself.

b) Fourier transform infrared (FTIR) spectroscopy

Fourier Transform Infrared (FTIR) spectroscopy provides a relatively experimentally simple technique by which the chemical composition of a surface or film can be determined with greater sensitivity than normal IR (Lide, 2005). FTIR was used as the initial scoping technique, providing cursory information to assess the influence of chemical and electro-oxidation of GCE respectively. In these studies, a broad spectrum of 4000 cm⁻¹ to 600 cm⁻¹ was assessed for GCE, Pi-GCE and EOx-GCE respectively, responses of which have been illustrated relative to one another in Figure 4.9. The atmospheric background signal was subtracted from each of the spectra depicted.

Several studies have applied IR spectroscopy methods to probe GCE surface condition (Giordano *et al.* 1991; Yang & Lin, 1994; Yang & Lin, 1995; Sullivan *et al.* 2000; López-Garzón, 2003; Swiatkowski *et al.* 2004; Shen *et al.* 2008). FTIR spectra of EOx-GCE are, however, typically complex, and are reported to be poorly resolved (Yang & Lin, 1995). The FTIR spectra of Figure 4.9

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do suggest differences between the two oxidations treatments relative both to one another and to polished GCE. The signal strength acquired for analyses were however low, with maximum transmittance signal from all the surfaces of $\sim 10\%$. This limited response was found with both front on analysis (PerkinElmer Spectrum 100 FTIR spectrometer) illustrated in Figure 4.9 and with oblique angle analysis (FTIR module connected to Thermo Electron Corporation – Nicolet 5700 FT Raman spectrophotometer) (data not shown). Due to the weak signal response by both apparatuses, the spectra were assessed with due caution and were used as scoping studies to identify complexity and possible trends. Accordingly, identification of surface chemical functionalities from the FTIR scans were not specifically annotated at any of the surfaces assessed.

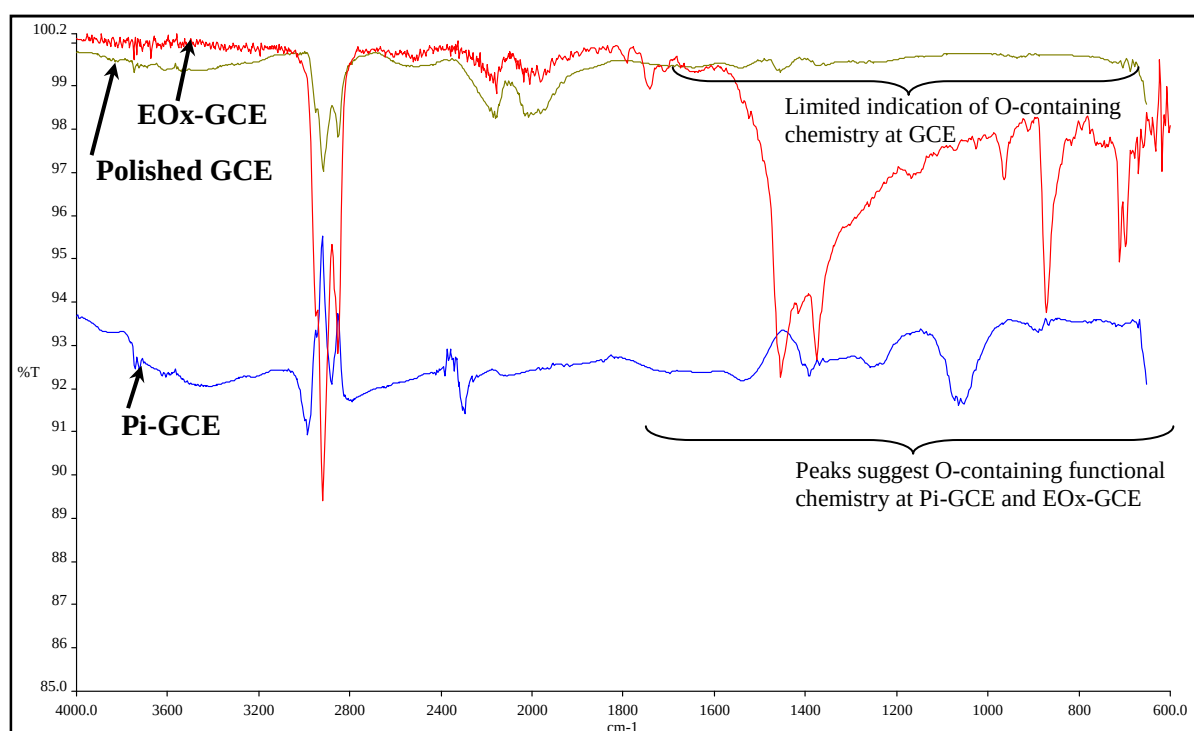


Figure 4.9: FTIR spectra of polished GCE (brown) as compared to Pi-GCE (blue) and EOx-GCE (red) with atmospheric background signal subtracted from each analysis.

From the results of Figure 4.9, it can be suggested that many oxygen containing functionalities are present at both Pi-GCE (as supported by EDX in this study) and EOx-GCE, due to the presence of many peaks in the wavenumber range from 1850 cm^{-1} to 600 cm^{-1} (Yang & Lin, 1994; Yang & Lin, 1995; Sullivan *et al.* 2000; López-Garzón, 2003; Zhou *et al.* 2007; Shen *et al.* 2008). This range also includes carbonyl moieties (1300 cm^{-1} to 1700 cm^{-1}), which are anticipated to have been present in significant quantities at the oxidised electrode surfaces (Yang & Lin, 1994). This is further confirmed by the limited presence of peaks in this region at polished GCE, suggesting that the oxidative processes introduce extensive oxygen containing functionalities at GCE in accordance with EDX responses. As the FTIR profiles at each of the surface are distinct from one another, the surfaces are likely to be distinctive in their chemical composition. Although a difference between the two

oxidation treatments can be noted from these responses, it is likely that the differences are subtle, as the bulk elemental compositions as determined by EDX analyses suggested very similar proportions of C and O for Pi-GCE and EOx-GCE which were assessed in these studies. Peak assignments presented through several publications which assessed oxidised GCE have been tabulated (Table 4.2) as an indication of the likely surface chemistry which would be expected at the surfaces assessed. Identification of the traits of the GEC preparations assessed through this study required greater resolution of analysis, thus XPS analyses were carried out as discussed below.

Table 4.2: Summary of FTIR peak position to functionalities and groups or GCE and oxides at GCE reported in literature

Group or Functionality	FTIR Peak Assignment Regions (cm ⁻¹)
(v) C-O of ethers and carboxyls	1000 - 1300
(δ) C-OH of alcohols and carboxyls	1049 - 1276, 1160 - 1200
(v) C-OH	1000 - 1220
(v) C-OH of phenols	1660 - 1720, 2800 - 3600
(v) COOH	1120 - 1200, 1665 - 1760, 1722, 2500 - 3300
(v) C=O of acid anhydrides	980 - 1300, 1590 - 1600, 1740 - 1850, 1778, 1779
(v) C=O of lactones	1160 - 1370, 1675 - 1790, 1710 - 1850, 1780, 1700
(v) C=O of carboxyl	1527 - 1712, 1696, 2050 - 2080, 1720, 1729
(v) C=O of quinones	1550 - 1680, 1668, 1678, 1673
(v) C=O of aromatic ketone	1696
CO _{ads}	2045, 2056
(v) C-C of aromatic rings	1585 - 1600, 1611
(δ) C-H	1550
(v) C-H	2600 - 3000, 2995
(v) C=H	3042
(v) O-H	3200 - 3670

Functionalities were separated according to bond stretching (v) and bending in plane (δ). Table was compiled from values reported by Giordano *et al.* (1991), Yang & Lin (1994), Yang & Lin (1995), Sullivan *et al.* (2000), Wang *et al.* (2001); López-Garzón, (2003), Swiatkowski *et al.* 2004; Lide (2005) and Shen *et al.* (2008).

c) X-ray photoelectron spectroscopy (XPS)

Since FTIR analyses did not allow for sufficient resolution in responses to accurately identify subtle differences in chemical composition, XPS analyses were conducted on the surfaces. As XPS analyses are confined to the upper surfaces of the layer, approximately 50 Å according to Sullivan *et al.* (2000), the results depicted as a result of these analyses assume XPS responses arise solely from the graphitic oxide layer in Pi-GCE and EOx-GCE. This allowed for the underlying bulk GCE matrix not to influence responses with regard to the C content of the samples, as was the case in EDX analyses.

The XPS spectra were quantified according to peak position and intensity, whereby peak position denotes the elemental composition of a sample and peak intensity denotes the relative amount within the sample. Figure 4.10 illustrates a comparison of XPS spectra recorded between binding energies of

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0 eV and 1000 eV at polished GCE, Pi-GCE and EOx-GCE. Subsequent analyses provide focused analyses of peaks such that their properties could be further analysed.

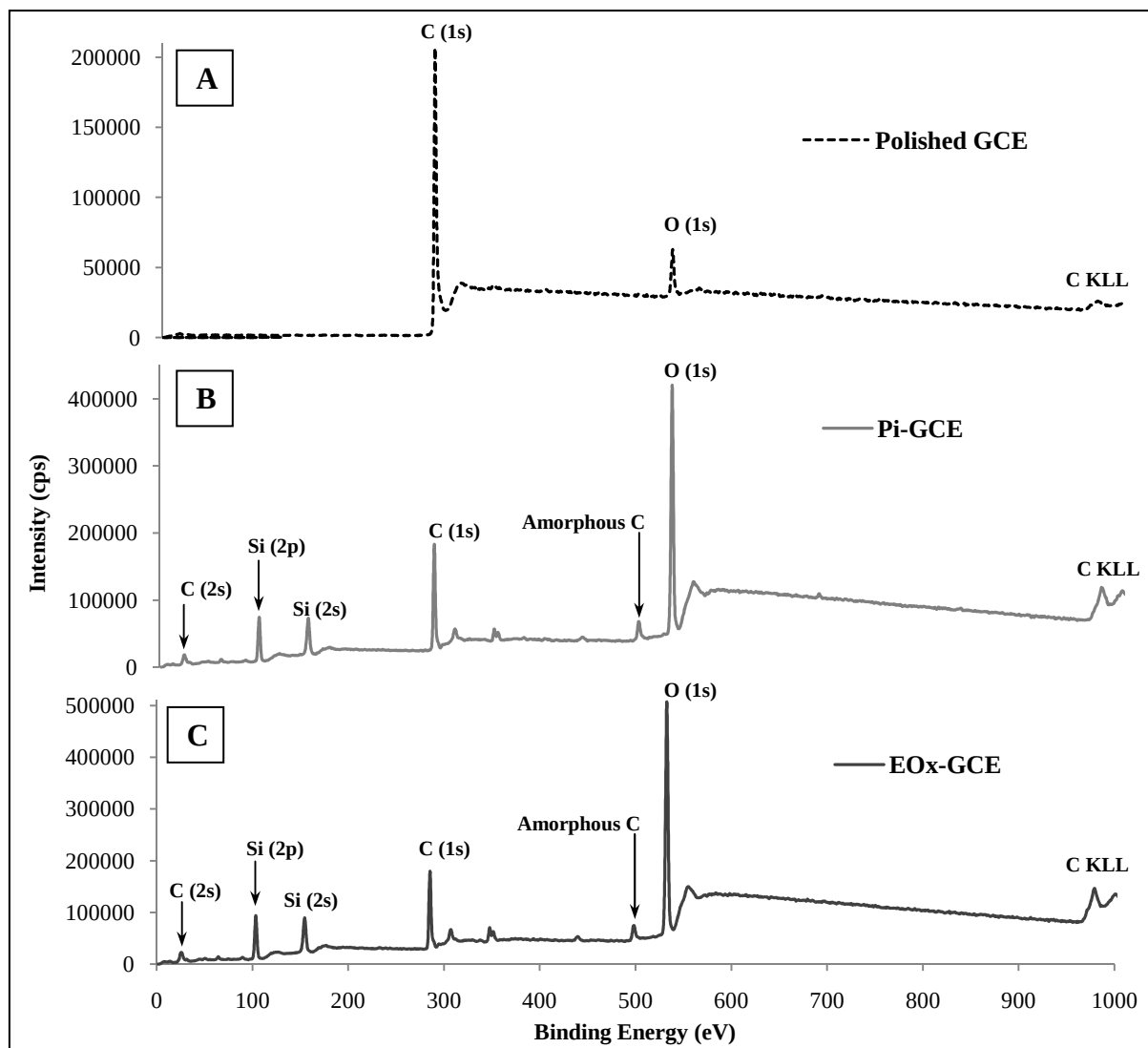


Figure 4.10: Chemical composition of polished GCE [A], Pi-GCE [B] and EOx-GCE [C] determined via XPS analysis reporting binding energies of 0 eV to 1000 eV.

As is evident in Figure 4.10, the spectrum of the polished GCE disc resulted in three broad peaks, characteristic of XPS spectra of GCE, which included a peak at 285 eV (indicative of C [1s]), at 533 eV (indicative of O [1s]) and at 984 eV (C KLL, which represents the energy of the electrons ejected from the C atoms due to the filling of (1s) state or K shell by an electron from the L shell with the ejection of an electron from an L shell) (Miller *et al.* 1981; Kamau *et al.* 1985; Collier & Tougas, 1987; Tougas & Collier, 1987; Kelemen & Freund, 1988; Sundberg *et al.* 1989; Alexander & Jones 1994; Dekanski *et al.* 2001 a; Jovanović *et al.* 2005; Zhou *et al.* 2007; Actis *et al.* 2008). These peaks were further present in the oxidised GCE spectra with equivalent peak position for both C (1s) and O (1s) and C KLL. Although C (1s) and O (1s) were found separately at equivalent binding energies

between the electrode surfaces assessed, the characteristics of the C (1s) and O (1s) peaks in the spectra collated at Pi-GCE and EOx-GCE oxide layers differed greatly from polished GCE, which, along with the array of subsequent peaks detailed in Figure 4.10 and highlighted in Figure 4.11, illustrate that the oxidation processes altered the chemical composition of the GCE surface, results which correlate with EDX and FTIR analyses.

Through analysis of spectra of Figure 4.10, it is evident that the relative abundance of C illustrated by [C (1s)] decreased with a corresponding increase in oxygen illustrated by O (1s) as a result of the oxidative processes (Dekanski *et al.* 2001; Zhou *et al.* 2007). This characteristic was described via a ratio of the area of the O (1s) / C (1s), reported as a % abundance of 18.90 % at polished GCE, 27.67 % at Pi-GCE and 33.47 % at EOx-GCE (Table 4.3). These responses clearly illustrate firstly an extensive increase in the abundance of oxygen containing groups at the surface of the oxidised electrodes and secondly that this occurs to a slightly greater extent when oxidised under electro-oxidation than chemical oxidation, as was also suggested by EDX analyses (Section 4.3.3 [a]). The % O of the EOx-GCE as well as the Pi-GCE layers were slightly higher than the 25 % O reported by Dekanski *et al.* (2001), who applied chronoamperometric anodic anodisation rather than voltammetric anaodisation as was applied herein, suggesting that cyclic oxidation may afford more extensive oxidation of the surface.

Other than the increase in O containing functionalities demonstrated in Figure 4.10, further alterations in the surface composition of the oxidised GCE in relation to the polished GCE are highlighted when measuring a narrower range of binding energies (Figure 4.11). Although distinct from polished GCE, the peaks formed at Pi-GCE and EOx-GCE are very similar to one another, suggesting subtle differences in chemical species and abundance. The two Si peaks at 105 eV and 156 eV (Bartella *et al.* 1987) were due to the presence of glass cover slips used to mound Pi-GCE and EOx-GCE, whereas the polished GCE was not mounted on a glass cover slip. A further set of peaks of interest include the presence of S (3s) at 15 eV (Susac *et al.* 2007) and the S group between 162 eV and 171 eV (Quijada & Vázquez, 2005; Médard & Morin, 2009), denoting the incorporation of S containing functionalities into the graphitic oxide layer, a result in accordance with spatially resolved X-ray compositional mapping studies carried out by Bowers *et al.* (1991). Although H₂SO₄ was present in each of the oxidation solutions, it is interesting that both procedures resulted in peaks of similar binding energies. The intensities of response at Pi-GCE were however lower than those at EOx-GCE, which may suggest that S peaks are as a result of S being present in pores in the graphitic oxide layer. As it is thought that the Pi-GCE is less porous, and would thus be less hydrated, it would thus have accumulated less S, which may account for these responses. This is a scenario which is corroborated by comparison of electrochemical responses between Pi-GCE and EOx-GCE (Figure 4.14 [C]).

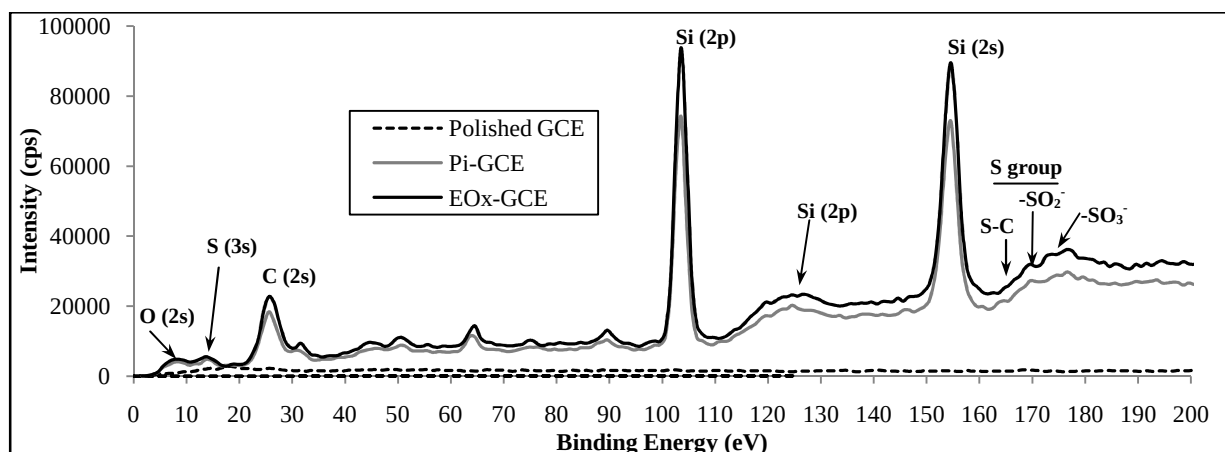


Figure 4.11: Chemical composition of polished GCE [dotted line], Pi-GCE [grey line] and EOx-GCE [black line] determined via XPS analysis within a focused binding energy range of 0 eV to 200 eV.

In order to further assess the both the nature of the oxide layers and to differentiate between the Pi-GCE and EOx-GCE surface chemistry, further investigation of the spectra was undertaken through deconvolution of peaks to reveal information pertaining to the types of carbon-oxygen groups present (Dekanski *et al.* 2001; Zhou *et al.* 2007). Deconvolution of the spectra was performed through application of a Gaussian distribution (Sprenger & Anderson, 1991) as was described in Section 4.3.3 [d], using Eq. 4.1. in particular, deconvolution allowed for identification of the oxide composition of the surfaces studied herein through analysis of the spectra of C (1s) (Kamau *et al.* 1985; Sundberg *et al.* 1989; Nakayama *et al.* 1990; Xie & Sherwood, 1990; Dekanski *et al.* 2001; Zhou *et al.* 2007; Jovanović *et al.* 2005) as well as the O (1s) (Xie & Sherwood, 1990; Zielke *et al.* 1996; Zhou *et al.* 2007). To this extent, Figures 4.12 and 4.13 illustrate comparisons of the peaks from each surface treatment and subsequent deconvolution of each of those peaks for both C (1s) and O (1s) spectra respectively such that the oxide functionalities could be identified.

The C (1s) spectrum was deconvoluted through application of the five peak model described by Nakayama *et al.* (1990), Sundberg *et al.* (1989), Dekanski *et al.* (2001 a) and Zhou *et al.* (2007) which denotes graphitic C at peak 1 (284.6 eV), C in phenol, ether or alcohol groups at peak 2 (286.0 - 286.3 eV), C in quinone or carbonyl at peak 3 (287.3 – 287.6 eV), C in carboxyls or ester groups at peak 4 (288.8 – 289.1 eV) and peak 5 pertaining to C in carbonate groups or as adsorbed CO and CO₂ at (290.5 – 291.2 eV). The resulting spectra were reported for each of the surfaces assessed through Figure 4.12.

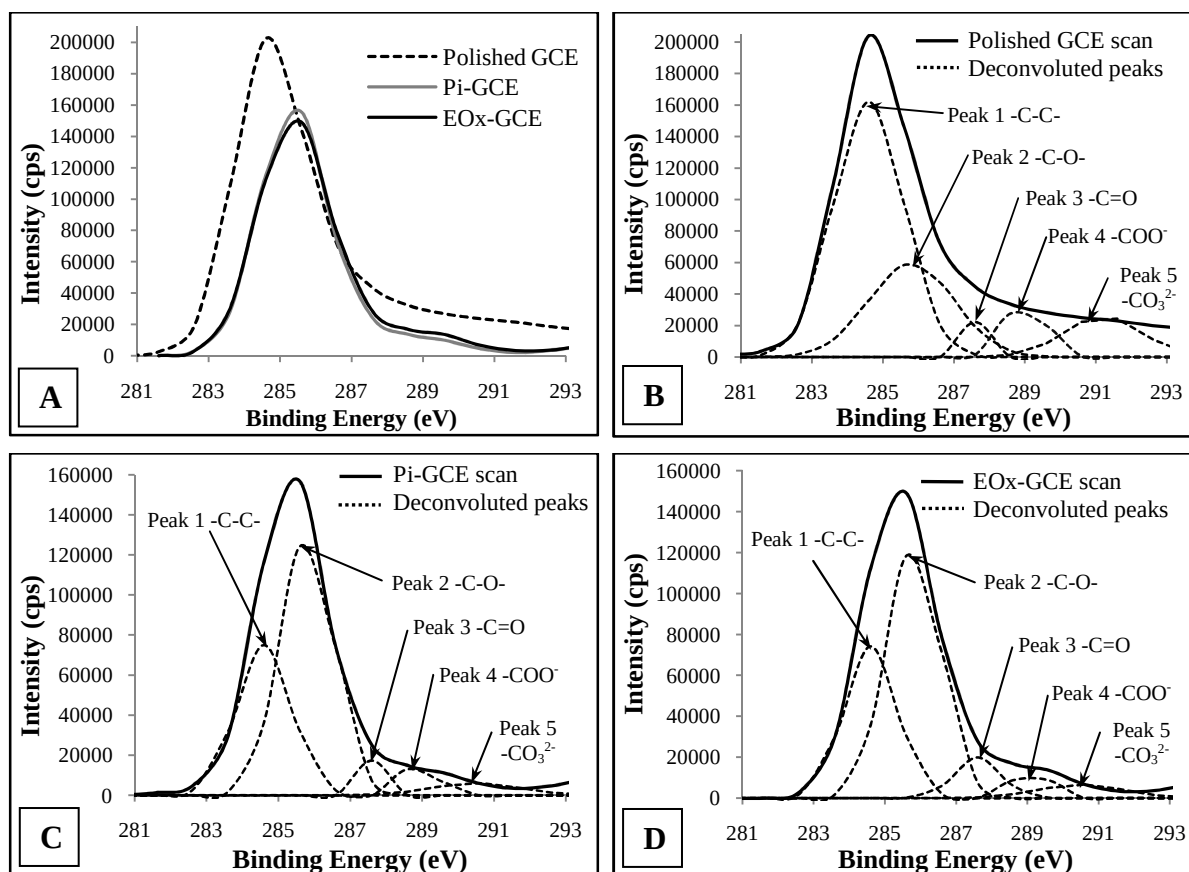


Figure 4.12: XPS analysis comparing the C (1s) spectra of polished GCE (dotted line), Pi-GCE (grey line) and EOx-GCE (black line) [A] and subsequent deconvolution of polished GCE [B], Pi-GCE [C] and EOx-GCE [D], each with the recorded spectrum as a black line and the deconvoluted peaks as dashed lines.

In Figure 4.1, it is evident that the polished GCE (Figure 4.12 [B]) has a pronounced graphitic C (peak 1), along with lower oxide responses (peaks 2-5) this response is in accordance with reports of polished GCE detailed by Dekanski *et al.* (2001). The scans of both Pi-GCE and EOx-GCE however describe extensive binding of C in several forms to O (Figure 4.12 [C] and [D]), with a phenolic peak which was larger than that reported by both Dekanski *et al.* (2001) and Zhou *et al.* (2007), indicating more extensive oxidation than those studies though they compared favourably with responses reported by Xie & Sherwood (1990). This response suggests that a majority of surface C is bound to O in accordance with Kepley & Bard (1988) for GCE surfaces anodically cycled in H_2SO_4 .

Of further interest was the similarity in the spectra of Pi-GCE and EOx-GCE, considering the variability in final surface characteristics encountered across various electro-oxidation procedures. These features were further detailed through deconvolution of the O (1s) spectrum (Figure 4.13) and the assessment of relative abundance of the oxide groups from each spectra as area of the peaks detailed through Table 4.3. Figure 4.13 detailed the results of a five peak deconvolution of the O (1s) spectrum as described by Zielke *et al.* (1996) and Zhou *et al.* (2007). These peak positions included peak 1 at 531.0 – 531.9 eV, showing O of the carbonyl of quinone functionalities, peak 2 at

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532.3 - 532.8 eV describing O in the carbonyl of esters, anhydrides and hydroxyl functionalities, peak 3 found at 533.1 – 533.8 eV related to O of non-carbonyl groups in esters and anhydrides, peak 4 at 534.3 – 535.4 eV is O of carboxyl groups and peak 5 at 536.0 – 536.5 eV describes O of adsorbed water or oxygen.

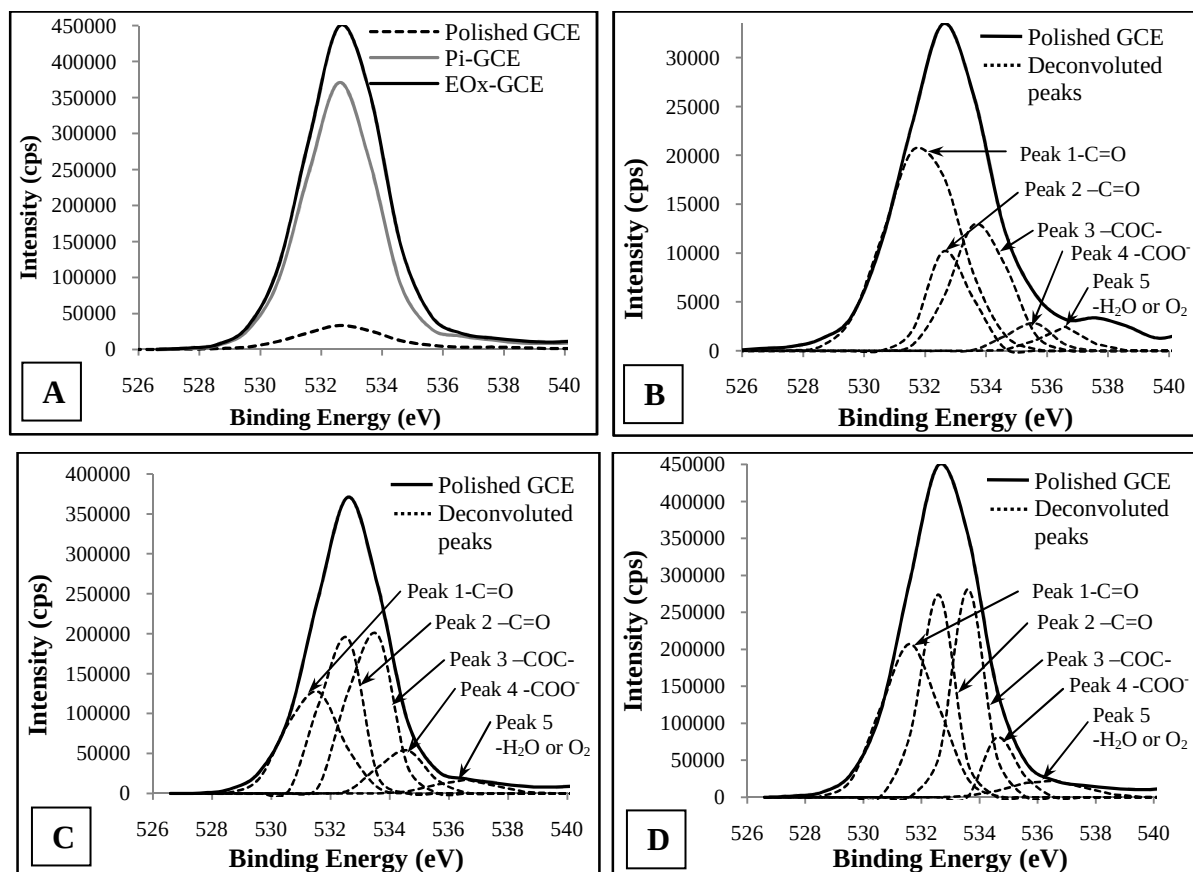


Figure 4.13: XPS analyses comparing the O (1s) spectra of polished GCE (dotted line), Pi-GCE (grey line) and EOx-GCE (black line) [A] and subsequent deconvolution of polished GCE [B], Pi-GCE [C] and EOx-GCE [D] each with the recorded spectrum as a black line and the deconvoluted peaks as dashed lines.

Table 4.3: Summary of peak areas of deconvoluted O (1s) and C (1s) spectra recorded at polished GCE, Pi-GCE and EOx-GCE

		Polished GCE		Pi-GCE		EOx-GCE	
		C(1s)	O(1s)	C(1s)	O(1s)	C(1s)	O(1s)
% of Max C (1s) or O (1s)		100.00	8.62	62.90	80.58	64.52	100.00
Total O (1s) / C (1s) (%)		18.90		27.67		33.47	
% of different functional groups in respective C(1s) and O (1s) spectra to total area	Peak 1	63.26	54.17	36.14	27.13	33.81	36.76
	Peak 2	28.18	15.13	61.14	30.06	56.47	29.58
	Peak 3	3.49	25.19	4.34	33.55	7.84	28.60
	Peak 4	7.85	4.31	4.90	9.81	4.23	7.99
	Peak 5	11.15	3.63	4.67	3.82	4.76	5.05
C_{ox}/C_{gr}		0.625		1.947		2.027	

C (1s) and O (1s) responses were reported from the spectra of Figure 4.12 and Figure 4.13 respectively. The % Max C (1s) or O (1s) was reported as % of the the area of total unconvoluted peak area of the maximum area of that spectrum respectively. The % functional groups were calculated as the % area of the deconvoluted peaks in the area of the unconvoluted spectrum peak respectively. C_{gr} = graphitic C (Peak 1), C_{ox} = Carbon bonded to different functional groups (Peaks 1 - 4) of the C (1s) spectrum.

Relation of the deconvoluted O (1s) and C (1s) peaks of Pi-GCE and EOx-GCE suggests that both modifications provide complex surface chemistry of graphitic oxide layers in relation to polished GCE in accordance with that which was previously stated in this Chapter. This feature can be highlighted through the ratio of C_{gr} (graphitic C, peak 1)/ C_{ox} (Carbon bonded to different O functional groups, peaks 1 - 4) in Table 4.3, which illustrates the increase in oxygen functionalities bound to the electrode surface from 0.625 at polished GCE to 1.947 and 2.027 at Pi-GCE and EOx-GCE respectively. This response, along with the % of max O (1s) and C (1s) in Table 4.3 illustrates that EOx-GCE have a higher % O than Pi-GE (as was found by EDX and suggested by FTIR) and both EOx-GCE and Pi-GCE have significantly higher % O than polished GCE. Through consideration of both Figures 4.12 and 4.13 and the summarised responses of Table 4.3, the oxide layers comprise of similar ratios of primarily hydroxyl, carbonyl and carboxyl groups and include functionalities such as phenols, ethers, alcohols, quinones, esters and anhydrides. These responses correlate with both XPS studies at EOx-GCE by Dekanski *et al.* (2001 a) and Zhou *et al.* (2007). Although the complexity of the oxide layers suggested by FTIR is corroborated herein, however, unlike that suggested by FTIR studies, which were considered with due caution, the EOx-GCE layer does not appear significantly more complex than Pi-GCE. The increase in C=O, COO⁻ at both oxide layers in relation to polished GCE suggest an increased hydrophilicity as was reported in Section 4.3.1 [a] and reported by Bleda-Martínez *et al.* (2006). These responses provide a specific indication of the chemical composition of the oxide layers produced by the specific electro-oxidation procedure applied herein and also that of piranha solution oxidised GCE which had not previously been assessed.

d) Subsection conclusions

Similarly to morphological characterisation by HRSEM (Section 4.3.2), the characterisation of the three surfaces by EDX, FTIR and XPS suggested that the oxidised surfaces were chemically distinct from polished GCE, comprising significantly higher % O. Unlike HRSEM analyses however, chemical characterisation of the surfaces, and XPS analyses in particular, revealed that although slight differences were evident between the two oxide layers, they were relatively similar to one another. In accordance with cited literature, these results firstly confirmed that both chemical and electrochemical oxidation resulted in the formation of graphitic oxide, and that the moieties comprising of those layers were largely associated with hydroxyl, carbonyl or combinations thereof. An implication of interest however is that due to the similarity of chemical composition it is unlikely that the variability seen in I_{p1} responses is as a result of interaction of the analyte with chemical functionalities within the oxide layer as the slight variation in composition does not account for the extent of variability in CIT I_{p1} at Pi-GCE (Chapter 5) and stability of I_{p1} at EOx-GCE (Section 4.3.4). This suggests that variability or stability in I_{p1} responses is as a result of a physical nature of the electrode, rather than interactions with its chemical composition.

4.3.4 Electrochemical investigation and characterisation of EOx-GCE and Pi-GCE

The following section assesses several electrochemical traits relating to graphitic oxide layers. Firstly, the voltammetric background and faradaic (hydroquinone as a reporter) signals at polished GCE, Pi-GCE and EOx-GCE, respectively, are compared to assess the relative responses at the surfaces. Thereafter, CIT responses at each surface are assessed, along with the influence of surface chemical composition on CIT response as investigated through Self Assembled Monolayers (SAMs), at AuE terminating in some of the functional chemical groups identified in Section 4.3.3. Thereafter, physical characteristics including surface area and conductivity of the EOx-GCE layer were assessed. Application of the responses derived through those studies allow for a proposed mechanism by which these electrochemical responses are garnered from graphitic oxide layers to be proposed.

a) Voltammetric characterisation of EOx-GCE and Pi-GCE

As the morphology and chemistry of electrodes have been shown to influence the electroactivity of electrodes to specific analytes (Chapter 1, Section 1.2.4), the following study is an investigation of the voltammetric response which arises through the presence of the oxide layer itself at GCE. As this Thesis is centred on electrochemical and in particular voltammetric analyses, the voltammetric characterisation of the surface oxide layer is integral to understanding responses at those surfaces.

Figure 4.14 provides an illustration of the voltammetric responses garnered at GCE, Pi-GCE and EOx-GCE both in the electrolyte only (0.2 M NaOAc buffer pH 4.0 which had been deaerated and aerated, respectively) as well as responses to 0.3 mM hydroquinone as a representative redox molecule to illustrate both oxidative and reductive processes.

As is evident in Figure 4.14, importantly, the potential region between 0.3 V and 1.1 V is devoid of faradaic current from the oxide layers themselves. These surface modifications thus provide a region in which the analytes of interest in this Thesis (CIT, as well as 2,4-DMA and Triol in Chapter 5) will have no apparent underlying interfering faradaic processes which may influence the signal from of those analytes. As such, these layers provide a stable region for the voltammetric analysis of the analytes of interest. Three major observations were drawn from studies represented in Figure 4.14. The first is that a marked increase in the background current occurs for both oxide layers, compared to polished GCE (Figure 4.14 [B]), a result highlighted by the separation of the forward and reverse scans (double layer current, I_{dl}) at 0.6 V therein. This increase was more extensive at EOx-GCE than Pi-GCE. The procedure of oxidising GCE thus has a marked influence on the resulting electrochemical profile at that electrode. Jürgen & Steckhan (1992) reported increases in I_{dl} as high as 10 – 32 times between polished and EOx-GCE, though the extent of these layers seemed to be dependent on the composition of the anodisation solution and the extent of oxidation. This trait was

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discussed further in relation to surface area determination (Section 4.3.4 [d]). The second parameter is that although more pronounced at EOx-GCE, a broad set of peaks became more prominent at both Pi-GCE and EOx-GCE as compared to polished GCE in the region of +0.2 V, a feature attributed to the oxidation procedures applied. The final observation is that the presence of oxide layers corresponds to increased faradaic responses of hydroquinone (Figure 4.14 [A]) at each of the layers, suggesting a positive influence for current response at EOx-GCE and Pi-GCE.

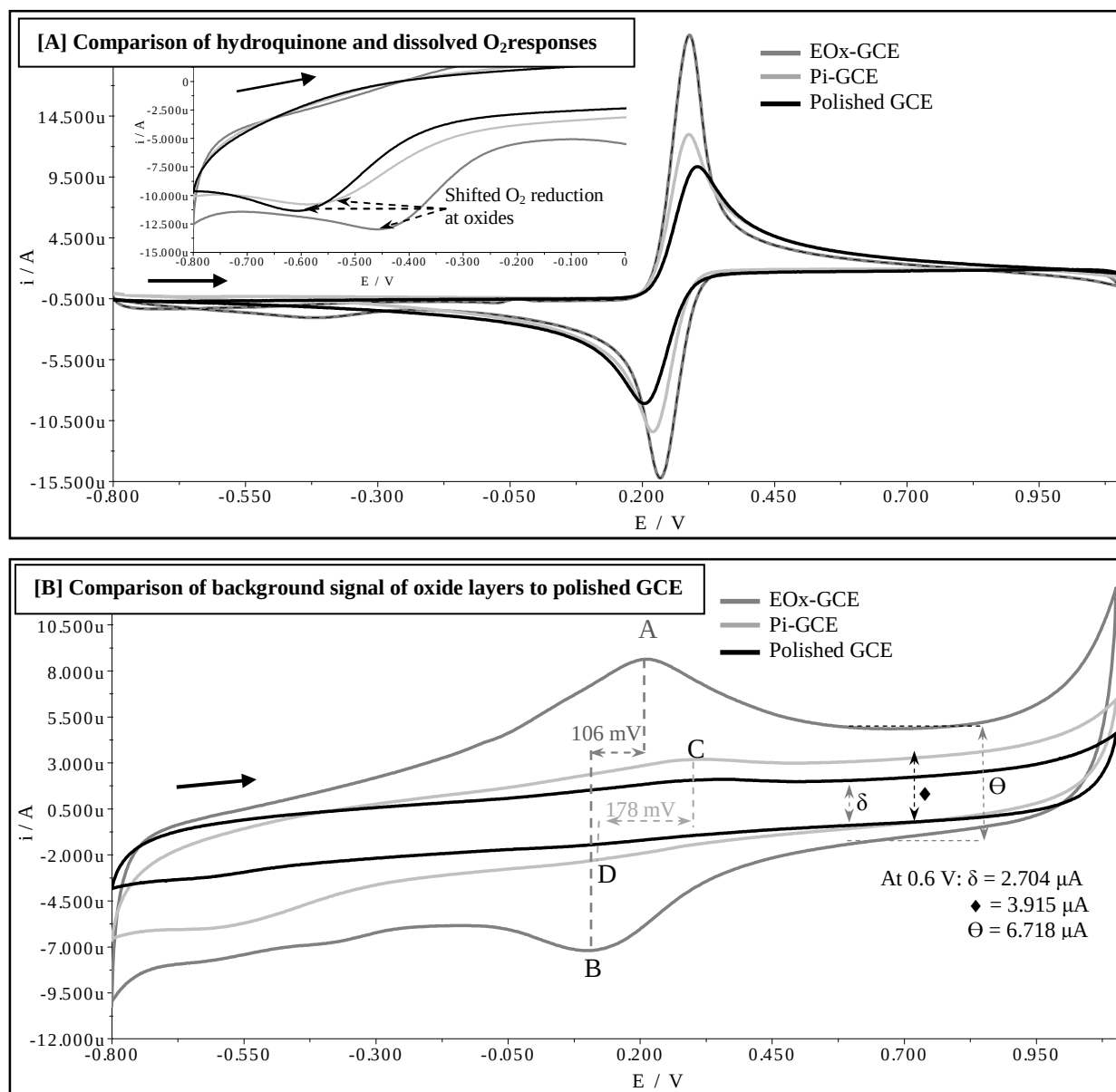


Figure 4.14: CV scans illustrating electrochemical responses at polished GCE (black) Pi-GCE (light grey) and EOx-GCE (dark grey) with comparison of [A] 0.3 mM hydroquinone with background current subtracted from each scan and an inset illustrating the O₂ reduction peak in aerated electrolyte at each surface. [B] provides a comparison of background responses at each surface in degassed 0.2 M NaOAc buffer pH 4.0 in the absence of hydroquinone.

The E_p of the peaks of the surface-associated couple were highlighted in Figure 4.14 [B], wherein the couple of the EOx-GCE (dark grey), was recorded with the anodic peak (A) at E_{pa} of 0.240 V and the cathodic peak (B) at E_{pc} of 0.134 V, resulting in a peak separation ($\Delta E_p = E_{pa} - E_{pc}$) of 0.106 V. The couple of the Pi-GCE was recorded with the anodic peak (C) at E_{pa} of 0.253 V and the cathodic peak (D) at E_{pc} of 0.075 V, which is slightly broader than EOx-GCE, resulting in a ΔE_p of 0.178 V. The presence of these peaks are commonly reported for EOx-GCE, and are typically attributed to redox reactions of oxygen functionalities (notably, o-quinone) formed during the oxidation procedure within the oxide layer (Engstrom, 1982; Hu *et al.* 1985; Nagaoka & Yoshino, 1986; Nagaoka *et al.* 1988; Kepley & Bard, 1988; Zhao *et al.* 2008). Hu *et al.* (1985), a hypothesis reported as recently as Zhao *et al.* (2008). Credence for this has been derived from characteristics including that the couple closely resembles the quinone redox process as a reversible two electrode system (Kissinger & Heineman, 1996) and that it is unlikely to be an oxide functionality such as carbonyl, because redox procedures of these functionalities are generally followed by irreversible chemical reactions in aqueous media (Nagaoka *et al.* 1988).

The surface-associated couple which arises as a result of oxidation in the region of +0.2 V is stable when scanned continuously and reversible according to Kissinger & Heineman (1996) (peak separation of EOx-GCE ΔE_p of 0.106 V, Figure 4.14 [B]). The presence of quinonic species was suggested at EOx-GCE through FTIR and XPS analyses. Thirdly, oxygen functionalities, such as catechol and quinone, have been applied as activations of GCE surfaces, wherein they have been shown to act as mediators, resulting in more effective electron transfer and greater responses (Kissinger & Heineman, 1996), which itself is a trait widely reported for graphitic oxide layers, improving both oxidation and reduction potentials for a number of analysed molecules, and demonstrated herein for oxygen reduction, and hydroquinone (Kepley & Bard, 1988; Dekanski *et al.* 2001; Zhao *et al.* 2008). It must be noted however that the mechanism by which this happens is not understood for graphitic oxide layers and is a point of conjecture which to date has been the topic of limited discussion.

As can be seen by comparing the deaerated and aerated electrolyte solution scans of (Figure 4.14 [B] and the inset of Figure 4.14 [A]), similarly to the responses reported for hydroquinone oxidation and reduction (which had a lower peak separation), the oxygen peak shifts toward more anodic potentials at EOx-GCE than at Pi-GCE suggesting that the oxide layer lowers the half wave potential. Similar responses have been reported on for EOx-GCE by Engstrom (1982), Engstrom & Strasser (1984), Nagaoka *et al.* (1986); Nagaoka & Yoshino (1986), Kepley & Bard (1988), Jürgen & Steckhan (1992) and Zhao *et al.* (2008).

Although morphological characterisation by HRSEM showed that Pi-GCE, EOx-GCE and polished GCE are distinct from one another, chemical characterisation by EDX, FTIR and XPS have showed that Pi-GCE and EOx-GCE were relatively similar to one another as compared to the responses at polished GCE. The voltammetric responses at those layers however suggest that Pi-GCE behaves more closely to polished GCE than EOx-GCE. This response stands to corroborate the finding reported in Section 4.3.3 [d] wherein it was suggested that I_{pl} responses arise as a result of a physical nature of the electrode, wherein, as the Pi-GCE appears to be less porous than the EOx-GCE, resulting in it acting as a poorer accumulator of analyte than the EOx-GCE layer and thus does not afford the same level of improved electrochemical response offered by EOx-GCE. This was illustrated through the position of the O_2 reduction peak, the relative magnitude of hydroquinone oxidation and reduction peaks, as well as the profile of the background signal recorded in buffer, including the position and size of the surface-associated couple (Figure 4.14).

b) Voltammetry of CIT on EOx-GCE and Pi-GCE

In this section, the application of oxide layers as an electrode modification was specifically targeted for the analysis of CIT. is investigated and illustrated through Figure 4.15.

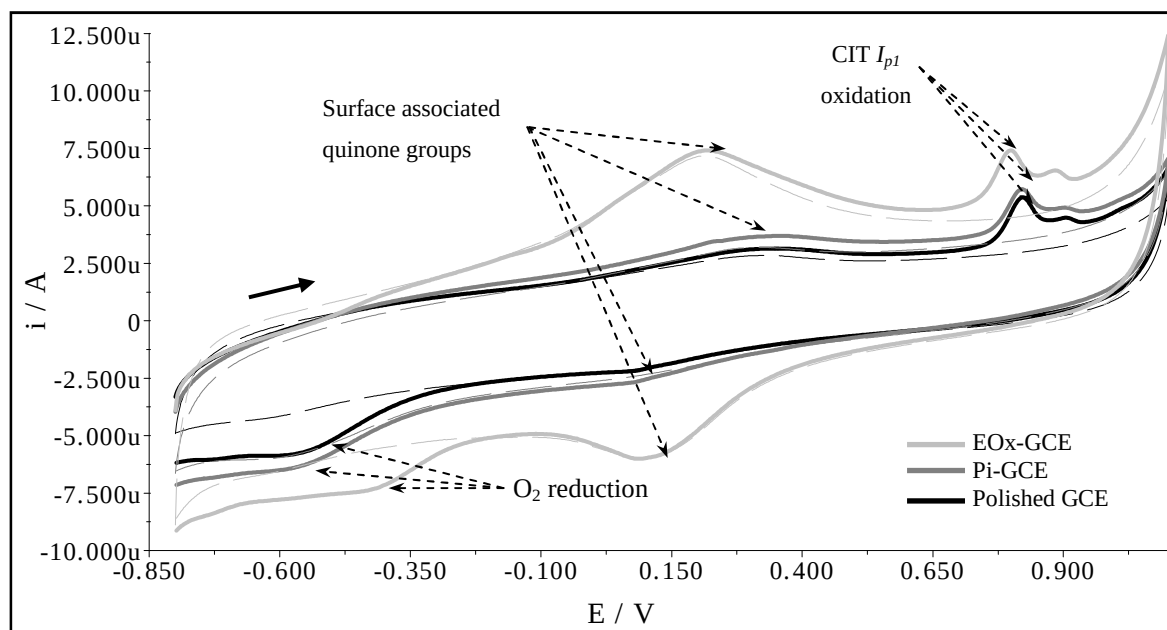


Figure 4.15: CV scans of 15 μ M CIT in 0.2 M NaOAc buffer pH 4.0 at EOx-GCE (light grey), Pi-GCE (grey) and polished GCE (black), each with background current represented as dashed lines. All scans were carried out between -0.8 V and 1.1 V at 100 mV/s vs. Ag/AgCl.

As is evident from Figure 4.15, CIT provides defined electrochemical responses at graphitic oxide layers (Pi-GCE and EOx-GCE) which are not drastically different from polished GCE with regard to signal profile. As was found for hydroquinone analysis at each of the surfaces (Figure 4.14), CIT I_p responses were however not equivalent as the surfacing of GCE with graphitic oxide allows for the

oxidation of CIT with equivalent or improved I_p and E_p responses relative to polished GCE, with EOx-GCE provided sharper peak signals and lower E_p in relation to both Pi-GCE and polished GCE (Figure 4.17).

As responses at EOx-GCE in Figure 4.15 suggested improvements of CIT responses, Figure 4.16 illustrates a more focussed study in a narrow potential window across several electrodes, each resurfaced on several occasions such that both sensitivity and reproducibility of response could be assessed.

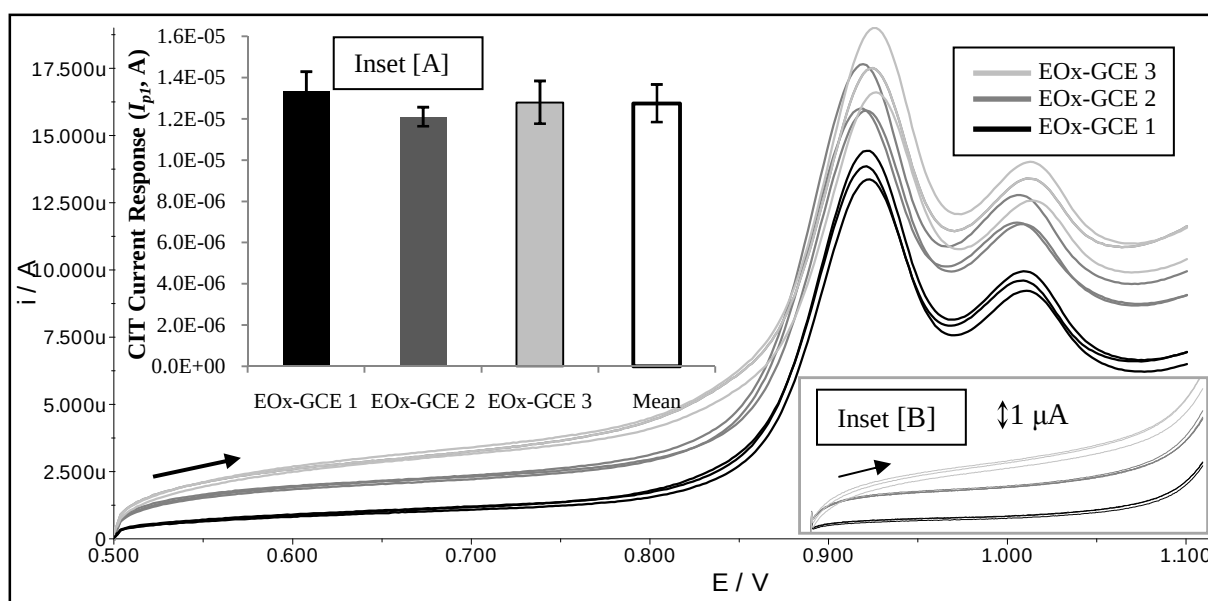


Figure 4.16: Voltammetric responses of 20 μ M CIT at EOx-GCE across multiple electrodes each resurfaced on three occasions, polishing between scans. Inset [A] illustrates the mean I_p at each EOx-GCE and the mean of all responses across all the EOx-GCE, each with standard deviation reported from the mean ($N \geq 3$). Inset [B] illustrates the background currents of each scan of the main figure. All scans were carried out in 0.2 M NaOAc buffer pH 1.0 between 0.5 V and 1.1 V at a scan rate of 100 mV/s vs. Ag/AgCl.

Electroanalysis of CIT at EOx-GCE provides highly reproducible I_{p1} responses with regard to both *inter*- and *intra*-electrode reproducibility as highlighted through inset A of Figure 4.16. This was possible even though each electrode possessed a characteristic background signal which may have appeared to suggest variability in I_{p1} response as illustrated both through the faradaic responses to CIT of Figure 4.16 as well as the background current of those scans illustrated through inset B of Figure 4.16.

Figure 4.17 illustrates a comparison of CIT I_{p1} and E_{p1} responses at oxidised and un-oxidised GCE and CPE as an assessment of electrochemical responses of CIT at each of the surfaces assessed. CIT I_{p1} responses reported at CPE in Figure 4.17 illustrate that although better reproducibility was found on un-oxidised surfaces (Bare CPE) than EOx-CPE, the magnitude of these responses was very limited. Whilst EOx-CPE offered greatly improved I_{p1} responses on occasion, these responses were

highly variable as compared to analyses at GCE. Accordingly, CPE were abandoned, and all subsequent analyses were conducted at GCE.

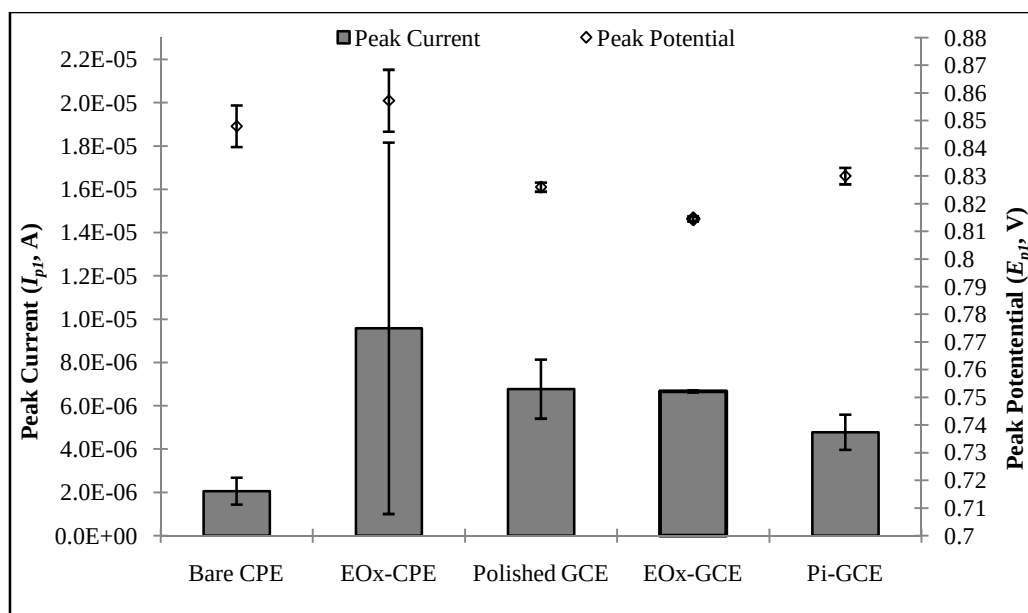


Figure 4.17: Comparison of summarised CIT I_{p1} and E_{p1} responses at oxidised (EOx-CPE and EOx-GCE) and un-oxidised (Bare CPE and Polished GCE) electrodes. Number of scans per set ≥ 3 with standard deviation reported from the mean.

The responses of EOx-GCE of Figure 4.17 described relatively high and importantly very stable I_{p1} responses ($6.67 \mu\text{A} \pm 0.05 \mu\text{A}$) as well as the lowest E_{p1} ($0.815 \text{ V} \pm 0.001 \text{ V}$). Although I_{p1} was not significantly larger at EOx-GCE than polished GCE, this response illustrates that the influence of the presence of graphitic oxide at the GCE which was shown to act as a diffusion barrier to CIT through Section 3.4.1 [b] holds no significant impact when assessed under this regime. The limited I_{p1} response at Pi-GCE in relation to polished GCE and EOx-GCE suggests that this was not the case at Pi-GCE, possible as a result of the limited porosity of that layer which may hinder responses more than at EOx-GCE. Along with providing stable I_{p1} responses at EOx-GCE variations in E_{p1} these peaks were found at lower potentials than at either polished GCE or Pi-GCE, suggesting that there was no negative impact of the EOx-GCE on the kinetics of CIT oxidation. Accordingly, CIT oxidation at EOx-GCE surfaces by LSV was selected as the standard conditions under which CIT was to be analysed and the oxidation mechanism elucidated.

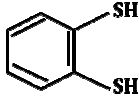
c) Assessment of the interaction of CIT with electrode surface chemistry through analyses at Self Assembled Monolayers (SAMs) terminating in oxide functionalities

The influence of electrode surface chemistry on I_{p1} responses of an analyte is a factor which governs a large portion of electrochemical responses (Bleda-Martínez *et al.* 2005; Bleda-Martínez *et al.* 2006). As such, it should be investigated to ascertain which surface-analyte interactions, if any, affect electrochemical response. Oxygen functionalities, such as catechol and quinone in particular have

been shown to activate GCE surfaces, and act as mediators, resulting in more effective electron transfer and greater responses, improving both oxidation and reduction potentials for a number of analysed molecules (Nagaoka *et al.* 1986; Kissinger & Heineman, 1996).

The following study investigated the interaction of CIT with specific oxide functionalities. This was performed by modifying a gold electrode (AuE) with conductive SAMs terminating in directed oxide functionalities identified through Section 4.3.3 [c]. Although it was demonstrated that the use of AuE did result in limited peak formation (Chapter 3, Section 3.4.5 [B]), the application of gold surfaces does allow for the uniform modification of the electrode with the formation of self assembled monolayers (SAMs) with known terminal functionalities. Short chain components (Table 4.4) were applied in order to minimise passivation of electron tunnelling that occurs with the use of long chained ordered SAMs (Wang *et al.* 1999). The terminal functionalities applied included hydroxyl (β -mercaptoethanol, [R-OH]) and carboxyl (3-mercaptopropionic acid, [R-COOH]) as representatives of oxide functionalities which make up a majority of the surface chemistry of the oxide layer. The SAMs containing R-COOH and R-OH would be negatively charged, as such a further SAM terminating in a positively charged molecule, in the form of amine (NH_2) as cysteamine, was included. Aromatic carbon (C-C) was included as a comparative hydrophobic edge plane equivalent for carbon electrodes, whilst -OH and -COOH would act as proxy for oxidised carbon at edge plain sites as active sites are oxidised after surface fracturing and exposure of an edge plain (Heiduschka *et al.* 1994). A further assessment that was included comprising of a mixed SAM of 1,2-benzenedithiol and 3-mercaptopropionic acid. The results of the LSV scans conducted at each of the surfaces are shown in Figure 4.18.

Table 4.4: Structures of SAMs used as chemical and charge proxies of graphitic oxide

			
β -mercaptoethanol (M-OH)	cysteamine (NH_2)	3-mercaptopropionic acid (M-COOH)	1,2-benzenedithiol (C-C)

Through Figure 4.18, it is clear that although a CIT peak was at 0.860 V (in the region previously reported for CIT oxidation), the signal was weak, similarly to that reported at AuE analyses in Chapter 3 (Section 3.4.5 [b]). As the SAMs were prepared in accordance with slightly modified protocols which have been widely applied in literature (Zhang & Wang, 2002; Campuzano *et al.* 2005; Carvalhal *et al.* 2005; Tichoniuk *et al.* 2008), they were assumed to be present at the AuE in this study and to be relatively uniform.

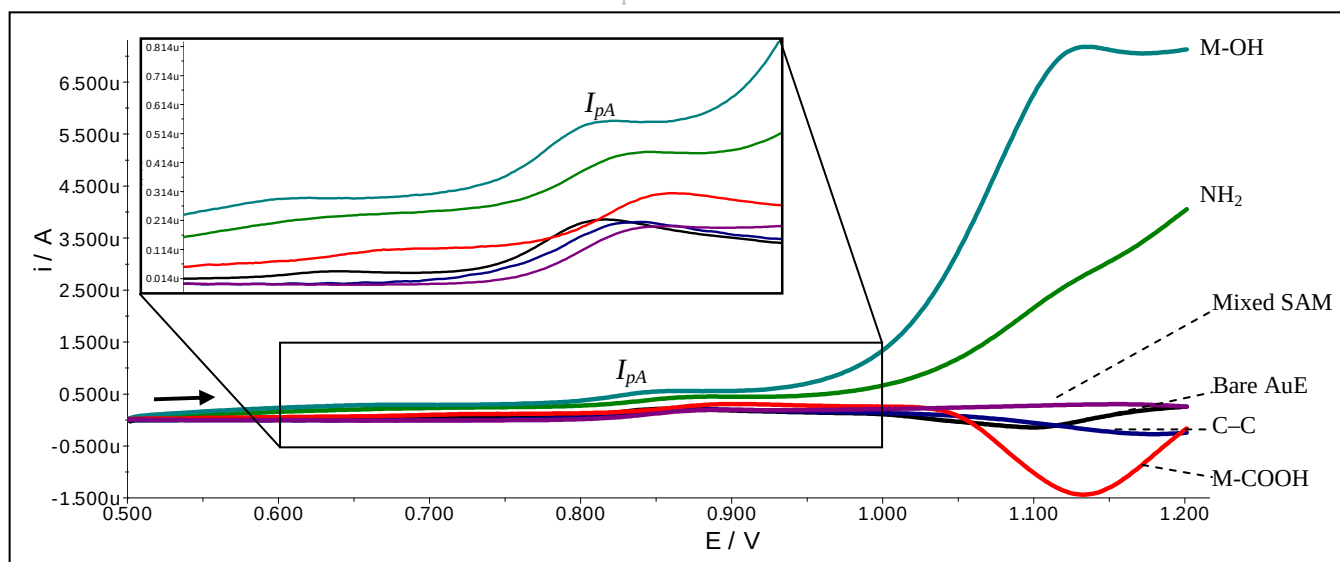


Figure 4.18: representative LSV scans of CIT at SAMs of β -Mercaptoethanol [M-OH] (Cyan), Cysteamine [NH₂] (Green), 1,2-Benzenedithiol [C-C] (Blue), 3-Mercaptopropionic acid [M-COOH] (Red), mixed SAM [1:1 M-COOH and C-C] (Purple) and bare AuE (Black), each in 20 μ M CIT in 0.2 M NaOAc buffer pH 4.0 with background signal subtracted.

The calculated responses at each of the surfaces assessed were summarised through Table 4.5. Although responses were specific to the surface assessed, the SAM responses were of approximately the same magnitude as that of bare AuE. When considered in relation to CIT responses pwe geometric area of the electrodes, at polished GCE [$I_{p1} = 95.77 \mu\text{A}\cdot\text{cm}^{-2} (\pm 19.42 \mu\text{A}\cdot\text{cm}^{-2})$] and EOx-GCE [$I_{p1} = 124.35 \mu\text{A}\cdot\text{cm}^{-2} (\pm 6.82 \mu\text{A}\cdot\text{cm}^{-2})$], responses at AuE were 3.18 % of polished GCE and 0.16 % of those at EOx-GCE. As all of the CIT I_p responses at SAM modified AuE were within a similar range to the responses of bare AuE (Table 4.5), it is clear that the responses at AuE and SAM electrodes were highly diminished as compared to carbonaceous electrodes.

As limited responses were recorded at each of the SAM surfaces, the significance of the result is found in that there appears to be no marked beneficial electrochemical responses which can be derived from charge-based, or chemical-based, interaction of CIT with surface functionalities. This study may have benefitted from interrogation of different dispersals of SAMs, though as the results were so poor in relation to carbon based and in particular oxidised GCE responses for CIT oxidation, these analyses were deemed superfluous. Accordingly, as no marked positive responses could be derived from the SAMs assessed, it was suggested that the interaction of CIT with GCE oxide layers which allows for improved electrochemical responses is likely to be a result of an interaction with the bulk structure of the graphitic oxide layer rather than an interaction based on the chemical composition of that structure. In accordance with this suggestion, the diminished responses at SAMs may have been a result of the comparatively homogenous and flat surface structure of SAMs as compared to GCE or EOx-GCE. This feature may thus allow CIT detection to be enhanced at GCE and EOx-GCE because the *porous surface structure* of the oxide layer may allow better

adsorption of CIT. These responses did not negate the possibility that CIT associated with the surface structure on a charge or chemical associated interaction. Having a SAM terminated in a group capable of H-bonding did not limit the response gained at bare AuE (which itself will have surface oxides (Carvalho *et al.* 2005)), but having an aromatic terminated SAM did. That feature suggests that H-bonding may be involved in these interactions. The relative increase in surface oxo-groups at GCE and EOx-GCE relative to the SAMS assessed may allow for more H-bonding between surface groups and CIT, and thus allow for improved adsorption and/or electron transfer, and hence voltammetric response. Thus the improved electrochemical characteristics observed at EOx-GCE are likely to arise as a result of improved adsorption of CIT at GCE and EOx-GCE as a result of greater surface area of oxo-groups, hence pointing to a physical basis of interaction with the graphitic oxide layer rather than a chemically based interaction.

Table 4.5: CIT peak responses recorded at AuE and SAM modified AuE.

AuE Surface		Peak Potential (E_{pl} , V)	Peak Current (I_{pl} , μA)	$i_{pl} \cdot cm^{-2}$ (μA)
Bare AuE	μ	0.861	0.20	3.05
	σ	0.008	0.04	0.93
	C.V.	0.87	21.27	30.48
M-OH	μ	0.860	0.25	6.02
	σ	0.005	0.03	1.25
	C.V.	0.54	13.20	20.72
M-COOH	μ	0.861	0.17	1.48
	σ	0.035	0.02	1.24
	C.V.	4.02	10.05	83.85
C-C	μ	0.917	0.15	2.93
	σ	0.057	0.055	1.81
	C.V.	6.21	34.94	61.73
C-NH ₂	μ	0.874	0.14	2.05
	σ	0.011	0.01	0.48
	C.V.	1.21	9.63	23.12
Mixed SAM	μ	0.888	0.24	3.89
	σ	0.004	0.05	1.24
	C.V.	0.41	18.86	31.83

μ = mean, σ = standard deviation from the mean, C.V. = coefficient of variation. Number scans per set = 3.

d) Voltammetric determination of oxide layer surface area

As was described through the general methodology (Chapter 2, Section 2.3.3 [b]), the determination of surface area of GCE is hampered by a number of factors. Factors particularly pertinent to interpreting surface area at oxidised GCE include the interaction of charged species of the electrode surface with electrolyte and electrolyte pH (Trasatti & Petrii, 1991). The determination of surface area is however necessary for effective quantification of responses at the electrodes. The determination of

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the surface area of GCE as well as EOx-GCE was conducted by measuring the I_{pa} of both positively ($[\text{Ru}(\text{NH}_3)_6]^{2+/3+}$) and negatively ($[\text{K}[\text{Fe}(\text{CN})_6]^{3-/4-}]$) charged redox probes at increasing voltammetric scan rates.

Figure 4.19 [A] shows representative responses at increasing scan rate and Figure 4.19 [B] shows a representative plot of resulting I_{pa} in relation to scan rate, from which surface area was calculated using Eq. 2.2 (Chapter 2), the results of which are reported in Table 4.6. As was illustrated in Figure 4.14 [B], a progressive increase in the extent of the charging current was measured at an E_p of 0.6 V from polished GCE to Pi-GCE and on to EOx-GCE. This feature was applied as a secondary means of analysing surface area measuring double layer capacitance (C_{dl}) in Farads (F), according to Eq. 5.10 as is discussed through Chapter 5 as a central theme.

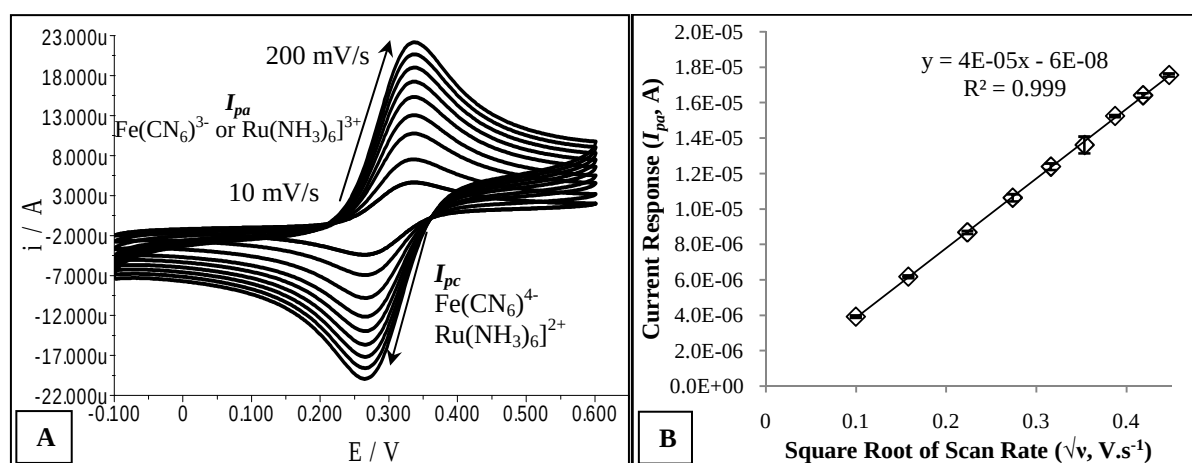


Figure 4.19: Determination of EOx-GCE surface area in relation to I_{pa} of equimolar (1.0 mM solutions) of $\text{K}_3[\text{Fe}(\text{CN})_6]$ and $\text{K}_3[\text{Fe}(\text{CN})_6]$ or $[\text{Ru}(\text{NH}_3)_6]\text{Cl}_3$ and $[\text{Ru}(\text{NH}_3)_6]\text{Cl}_2$ respectively. [A] illustrates representative peak profiles of the $\text{Fe}(\text{CN})_6^{3-/4-}$ couple with [B] illustrating the relationship between $\text{Ru}(\text{NH}_3)_6^{3+}$ I_{pa} and square root of scan rate respectively, $n \geq 3$ scans per data point and standard deviation was reported from the mean.

Table 4.6: Summary of surface area determination at polished GCE EOx-GCE

Electrode surface	Electrode surface area (10^{-2} cm^2)	
	$\text{Fe}(\text{CN})_6^{3-/4-}$	$\text{Ru}(\text{NH}_3)_6^{2+/3+}$
Polished GCE	6.52 (± 0.07)	5.11 (± 0.03)
EOx-GCE	6.21 (± 0.12)	5.96 (± 0.04)
% Increase in surface area from polished GCE	-4.70 %	16.53 %

In Table 4.6, either a decrease or an increase in apparent surface area, depending on probe molecules of opposing charge. This was expected in relation to Trasatti & Petrii (1991) and Kellomäki (1985) as the application of the $[\text{Fe}(\text{CN})_6]^{3-/4-}$ and $[\text{Ru}(\text{NH}_3)_6]^{3+/2+}$ redox couples allowed for an assessment of response relative to the charge of the layer. As $[\text{Fe}(\text{CN})_6]^{3-/4-}$ is negatively charged, the drop in apparent surface area from polished GCE to EOx-GCE suggests that the response of $[\text{Fe}(\text{CN})_6]^{3-/4-}$ oxidation was likely to be governed by electrostatic repulsion between the molecule and an increase

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of electronegativity with oxidation of the layer. An increase in negative surface charge is supported by analysis which resulted in a 16.53 % apparent increase in surface area recorded between polished GCE and EOx-GCE when using positively charged $[\text{Ru}(\text{NH}_3)_6]^{3+/2+}$.

Oxide layers as porous electrodes include a further set of conditions which require consideration as they usually show a strong dependence on the applied sweep rate in voltammetric analysis, wherein at high scan rates some less accessible surfaces may not be accounted for as a result of slowed mass transport through the oxide layer (Boggio *et al.* 1987). Furthermore, as the mechanism governing charging of electrodes is influenced by pH through surface proton exchange, the state of charge is strongly dependent on pH (Daghetti *et al.* 1983). As this is not the case (linearity in Figure 4.19 [B]), it is likely that the EOx-GCE measured response are not acting as porous layers but rather as rough layers at the electrode surface which are overlaid and connected to graphitic oxide which accumulates CIT, but may not participate in electrochemical reactions.

Analyses of C_{dl} responses, determined via Eq. 5.10, at polished GCE, Pi-GCE and EOx-GCE at 0.6 V were carried out and responses summarised through Table 4.7. The increases reported thus describe a significant increase in apparent surface area at both Pi-GCE and EOx-GCE from that of polished GCE. As the entire electroactive area is accounted for by virtue of the measurement itself, the result is proposed to be near to the maximum possible relative surface area.

Table 4.7: Summary of surface area determination at Pi-GCE and EOx-GCE in relation to polished GCE

	Polished GCE	Pi-GCE	EOx-GCE
mean Capacitance (C_{dl})	5.14 μF ($\pm 2.02 \mu\text{F}$)	6.82 μF ($\pm 1.55 \mu\text{F}$)	12.40 μF ($\pm 2.13 \mu\text{F}$)
% Increase in surface area from polished GCE	-	32.73 % (± 7.44 %)	140.84 % (± 24.20 %)

Due to the complexities associated with determining the actual surface area of the electroactive portion of EOx-GCE, although the response quoted by $[\text{Ru}(\text{NH}_3)_6]^{3+/2+}$ provides an increase in surface area, it is proposed that the response would be near to the lower limit of increase in surface area. The relative increase reported by C_{dl} is not influenced by charge of a probe molecule or by the ability of molecules to access pores within the oxide layer (Kellomäki, 1985; Trasatti & Petrii, 1991). Accordingly, EOx-GCE provides an increase in electroactive surface area of between 16.53 % and 140.84 % from polished GCE, with the extent of surface area being dependant on the specific interactions of the molecule associating with that layer.

e) Assessment of graphitic oxide conductivity through scanning electrochemical microscopy (SECM)

The responses of Figure 4.20 describe the reduction of $K_4[Fe(CN)_6]$ (R) as represented by, I_{pc} of Figure 4.19 [A]. According to Bard & Faulkner (2001), when immersed in solution and far from a substrate, unhindered current response ($R \rightarrow O$) of an approach curve at an ultramicroelectrode (UME) probe tip (I_T) results in the establishment of a steady-state current ($I_{T,\infty}$) represented by Eq. 4.2.

$$I_{T,\infty} = 4nFD_0C_0a \quad \text{Eq. 4.2}$$

Where n is the number of electrons, F is Faradays constant, D_0 is the diffusion coefficient C_0 is concentration of the redox species and a is the radius of the tip of the probe UME.

According to the theory of approach curve analyses (Kwak & Bard, 1989 a; Kwak & Bard, 1989 b; Bard *et al.* 1992; Borgwarth *et al.* 1995; Bard & Faulkner, 2001), deviation of I_T from $I_{T,\infty}$ provides information pertaining to the characteristics of the substrate. As $I_{T,\infty}$ relies on conversion of R to O (in this study), as the UME comes in closer proximity with a substrate, the diffusion of R to the UME surface will be influenced. If the substrate is conductive, as the UME approaches contact with it, conductivity of the substrate allows for the apparent surface area of the UME to be increased and thus the amount of R being reduced increases (current proceeds to more negative values in this case), resulting in $I_T > I_{T,\infty}$. If the substrate is insulatory however, with closer proximity of the UME to the substrate mass transport of R to the UME tip is decreased and thus $I_T < I_{T,\infty}$ (current proceeds to less negative values in this case). Figure 4.20 illustrates the SECM approach curves conducted at GCE and EOx-GCE in relation to Teflon coating as a negative control representing an insulator material.

In Figure 4.20, a positive response of conductivity (increase in I_T , more negative values relative to $I_{T,\infty}$) was recorded with an approach curve to polished GCE with $\Delta I_T = -3.42 \times 10^{-8}$ A. Similarly, a negative control response was recorded by approach curve to Teflon as an insulatory substrate providing a $\Delta I_T = +1.12 \times 10^{-8}$ A. The approach curve to EOx-GCE subsequently provided a slight increase in current response with $\Delta I_T = -5.30 \times 10^{-9}$ A. Although a limited response in comparison to polished GCE, the increase in signal described attenuated conductivity of the graphitic oxide layer. As is evident between the -115 μm and -125 μm region of the EOx-GCE approach curve, I_T swapped from increasing relatively rapidly (conductivity) to decreasing again sharply. This indicates a layer of accumulated $[Fe(CN)_6]^{4-}$ close to the GCE or within the oxide layer. Although graphitic oxide is porous and allows accumulation of analyte through the entire body of the oxide layer (Kepley & Bard, 1988), layering of graphitic oxide as suggested by Dekanski *et al.* (2001) may not allow an electrical connection with increasing separation from the GCE surface due to the formation of functional groups

not only at the edges of the layers but also in the interlayer spaces (Dekanski *et al.* 2001). The rapid drop in I_T may also have been impacted by the probe entering and compacting graphitic oxide, resulting in it becoming insulatory at the electrode surface. This response would suggest that the conductivity of graphitic oxide itself is limited, but in its porous, hydrated form, the graphitic oxide may act as a scaffold which could be charged and allow for apparent conductivity of a limited separation from the GCE.

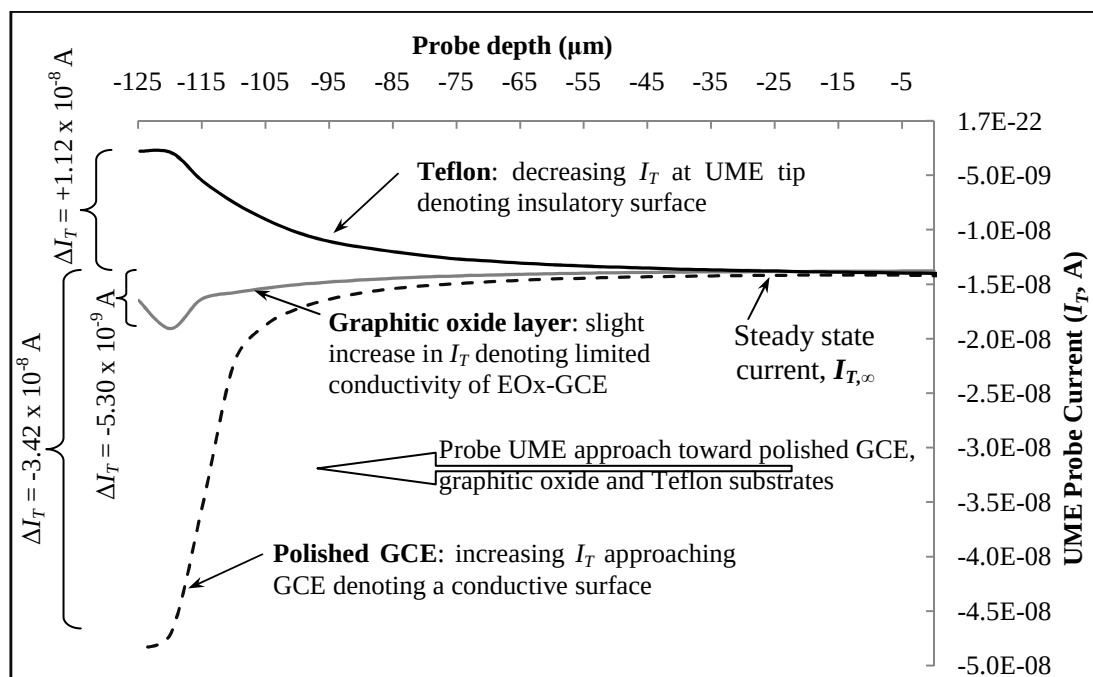


Figure 4.20: Determination of EOx-GCE conductivity through approach curve analyses by SECM using a 25 μm UME with a 1.0 mM solution of $\text{K}_3[\text{Fe}(\text{CN})_6]$. Polished GCE (dashed line) was included as a conductive positive control and Teflon (black line) included as an insulator as a negative control relative to EOx-GCE (grey line).

Figure 4.21 provides an illustration of the reproducibility of conductivity of EOx-GCE across multiple graphitic oxide layers through multiple approach curves, each carried out at resurfaced EOx-GCE.

As illustrated through Figure 4.21, although slight deviation in the mean magnitude of ΔI_T from $I_{T,\infty}$ was $4.67 \times 10^{-9} \text{ A}$, $\pm 3.72 \times 10^{-10} \text{ A}$ (C.V. = 7.95 %), the conformity of UME current responses across three separate polished and resurfaced EOx-GCE describes a uniform electrode condition at an apparently uniform separation from the GCE. The result of which is that deviations in surface condition reported by HRSEM bear little influence on the electrochemical response of the layer. These characteristics are discussed further through Section (f) below. These results, as such, concur with that reported by Kepley & Bard (1988) in that graphitic oxide does not convey conductivity over large separations from the electrode surface. SECM analyses herein however illustrated that graphitic oxide did convey conductivity with limited separation from the electrode surface. Furthermore, the

extent of that conductivity, or the separation from the electrode to which conductivity is observed appears relatively uniform as illustrated in Figure 4.21.

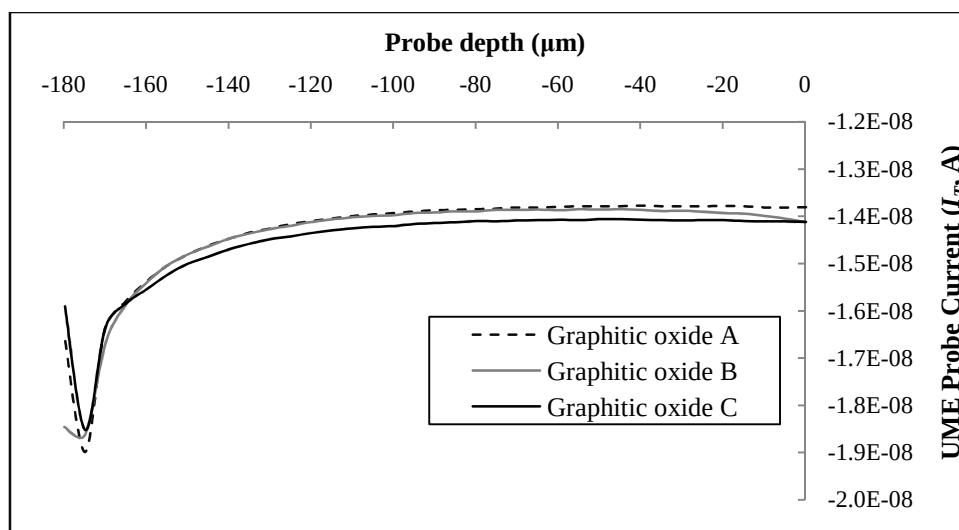


Figure 4.21: Assessment of the uniformity of EOx-GCE graphitic oxide layer conductivity through approach curve analyses by SECM using a 25 μm UME with a 1.0 mM solution of $\text{K}_4[\text{Fe}(\text{CN})_6]$ at three separate preparations of EOx-GCE.

f) Towards the mechanism by which EOx-GCE affords improved electrochemical responses

Through Chapter 3 and herein it was shown that although CIT responses are influenced by electrode surface characteristics that relationship was likely not to extend further than physical association of CIT with the electrode surface. Fagan *et al.* (1985) reported that the presence of oxide functional groups is not necessary for the rapid electro-oxidation of ascorbic acid, and Nagaoka *et al.* (1986) showed that the electrochemical behaviour of adsorbed quinone is very different to that of what would be surface-confined quinone (those of the oxide layer) with regard to oxygen reduction. Although these results are not necessarily transferable to all analytes, these two studies, along with the study by Nagaoka *et al.* (1988) and through analyses conducted at SAM modified AuE herein, illustrate that the influence of oxides may not be as marked as previously anticipated for CIT analysis. Rather, these results indicate that the structure of the oxide layer as an entity produces many of the beneficial traits which have been reported for graphitic oxide layers.

The mechanism by which graphitic oxide affords improved electrochemical characteristics was thus proposed herein according to a structural basis. As it has been established that graphitic oxide is both porous and hydrated (Kepley & Bard, 1988), it was submitted that the mechanism by which improved electrochemical traits are imparted by graphitic oxide are likely to be a factor of the electrode acting as a porous, analyte trapping electrode, similar to that described by Henstridge *et al.* (2010). A schematic representation of the mechanism described has been included through Figure 4.22.

Graphitic oxide has been shown to accumulate electroactive species through adsorption studies of analytes (Nagaoka & Yoshino, 1986), by secondary mass ion spectroscopy (Picq *et al.* 1984) and later through both CIT adsorption (Chapters 5 and 6) and also 2,4-dimethylaniline (DMA) adsorption (Chapter 5).

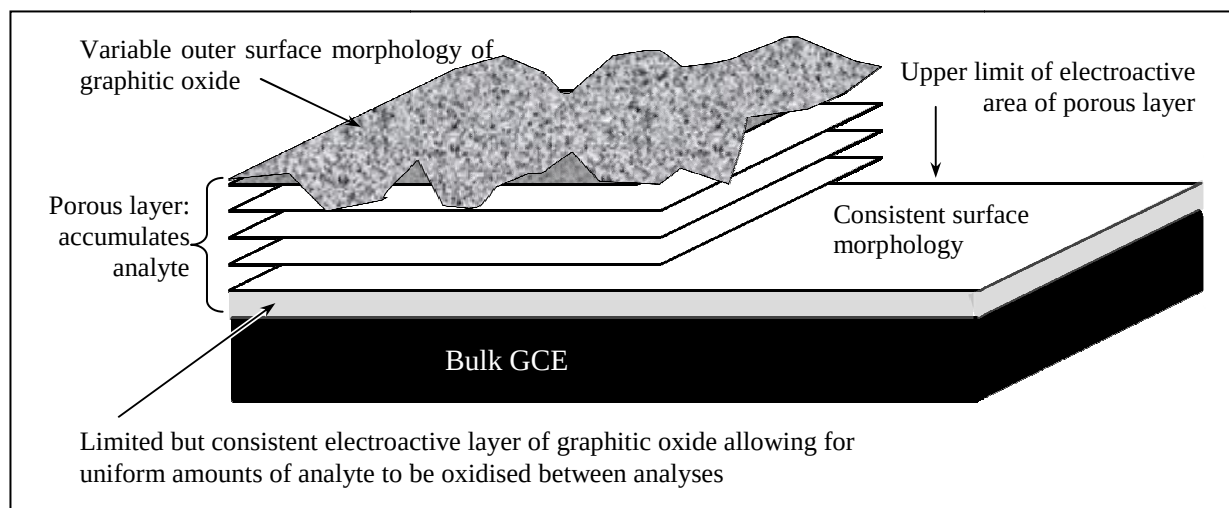


Figure 4.22: Schematic describing the mechanism by which graphitic oxide allows for improved electrochemical characteristics.

As was described in Section 4.3.4 (d), there is an increase in surface area with inclusion of graphitic oxide, as such the reported porosity of the graphitic oxide layer does influence electrochemical responses through altering surface area. HRSEM analysis revealed that the upper surface of the layer was not completely uniform, but was a relatively thick layer. Importantly, through Section 4.3.4 (e), it was shown that only defined, reproducible segments (probably that which is in direct or very close contact with the GCE surface) of the total oxide layer will be conductive. Thus only that limited layer which is conductive will contribute to both the electrochemically active surface area and the I_p responses. As a result of these properties, the graphitic oxide layer accumulates analyte at the electrode surface. As a reproducible layer within the bulk of the oxide layer is electrochemically active, the surface area and the amount of analyte adsorbed and subsequently oxidised between analyses and preparations is stabilised. In so doing, this allows for both larger I_p responses and, importantly, accounts for the stability in response which was observed for CIT analyses through Section 4.3.4 (b), even though the surface morphology of graphitic oxide layers as imaged by HRSEM analyses appeared relatively rough. Furthermore, due to the porous nature of the electrode, it is proposed that the reduced potentials (catalytic like behaviour) observed when comparing hydroquinone and oxygen reduction potentials of EOx-GCE to Pi-GCE and polished GCE will arise through thin layer like mass transport, as described by Henstridge *et al.* (2010) for porous electrodes.

4.4 Summary and Conclusions

This chapter assessed the oxide layers which were formed at GCE by both chemical (Pi-GCE) and electro-chemical (EOx-GCE) oxidation. These layers were characterised by assessing morphological (HRSEM) and chemical (EDX, XPS and FTIR) characteristics as well as a range of electrochemically determined traits and characteristics.

HRSEM analysis of polished GCE, Pi-GCE and EOx-GCE provided the first HRSEM images of these surfaces to the best of the knowledge of the author. These images illustrated distinct traits for each surface. Variability in GCE surface condition from scratching of the surface as a result of polishing was clearly illustrated. Due to the random nature of the polishing procedure, the images illustrated that GCE surface morphology changes each time it is polished. Pi-GCE surfaces were highly variable morphologically. Although graphitic oxide was formed, the aggressive nature of piranha solution caused the resulting oxide layer to crack, separate and flake from the GCE surface. EOx-GCE produced a comparatively uniform, thick oxide surface which also had ridges and bumps across its surface. At a microscopic scale the surface was further revealed to be relatively rough due to the formation of crystalline like nodes. Due to the angular appearance of the nodes, they were postulated to have arisen as a result of the site localised dehydration of the graphitic oxide layer. Although nodes at EOx-GCE have been reported previously, these images provide resolution and detail previously not visible, including that the nodes are crystalline like and that light spots (areas of charging) form at the edge of some of these nodes, possibly a result of a pronounced insulatory nature of the nodes. Thus although improved as compared to Pi-GCE and polished GCE, EOx-GCE essentially remain an morphologically inhomogenous outer surface which could not account for the stability offered in CIT analysis at EOx-GCE, a parameter which has a topic of contention in literature since the early 1980's.

Chemical characterisation of the surfaces revealed that although oxygen functionalities were present at polished GCE. Both oxidative procedures however resulted in an increase in oxygen containing functionalities and a drop in the extent of carbon at the surface. Thereby these studies confirmed the formation of graphitic oxide at both Pi-GCE and EOx-GCE and provided the first indication of the influence of piranha solution on GCE surface chemistry. Pi-GCE and EOx-GCE shared common functional chemistry, though relative levels of each did differ, indicating that similar oxidation processes may have occurred. Surface functionalities were shown to be predominated by hydroxyl and carbonyl containing functionalities. Due to the similarity in surface functionalities, it was unlikely that surface chemistry of Pi-GCE was influencing the variability of CIT I_{pl} response. The absence of any improved electrochemical characteristics in analyses of CIT responses at oxide terminating SAMs served to corroborate this. The SAM studies further suggested that the improved electrochemical

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responses are likely to be as a result of a physical interaction of the analyte, and that this interaction was likely to be associated with H-bonding of CIT to the graphitic oxide layer.

Voltammetric analysis showed that the graphitic oxide layers related closely to literature (Nagaoka *et al.* 1986; Kepley & Bard, 1988; Nagaoka *et al.* 1988; Dekanski *et al.* 2001). The Pi-GCE was shown to have limited porosity as compared to EOx-GCE, resulting in voltammetric responses which more closely resembled those of polished GCE than the EOx-GCE. These traits were transferred to analysis of hydroquinone as a representative probe, which showed that although minor improvements in peak current and peak separation were possible, improved characteristics were limited, but which provides a suitable surface for analyses conducted in Chapter 5. EOx-GCE provides markedly improved electrochemical responses toward CIT and hydroquinone and importantly allowed for greater sensitivity of CIT analysis as well as highly stable I_p response. In so doing EOx-GCE provides an appropriate surface for studies conducted through Chapters 6 and 7.

Through showing that the EOx-GCE graphitic oxide layer does provide an increased surface area and that a small, but uniform layer which is likely to be in direct contact with the GCE surface is conductive and thus electroactive. This feature, in connection with the finding that chemical composition is likely to play little influence of the mechanism of improved electrochemical function gained by graphitic oxide, a general mechanism was proposed which accounts for the improved electrochemical characteristics offered by EOx-GCE. This is based on the interaction of analyte with the oxide layer on a physical basis rather than a chemical basis, wherein analyte is accumulated within the bulk of the oxide layer, but as only analyte within the electroactive region of the oxide layer is oxidised, the amount oxidised is rendered more uniform, and as the surface area is larger, the I_{p1} response is larger. In so doing, this study is of relevance not just to CIT but also to similar analytes where variability is a concern as it describes the electrochemical mechanism of function of EOx-GCE.

CHAPTER 5

Towards Normalising Variability in Current Response at Polished, Oxidised and Multi-walled Carbon Nanotube Modified Glassy Carbon Electrodes Using Double Layer Capacitance

5.1 Introduction

Carbonaceous electrodes have been widely reported as having inconsistencies in surface condition through variability in both composition (chemistry) and structure (morphology). Such variability was reported on GCE through Chapter 4, as well as extensive reports in literature (Kazee *et al.* 1985; Kamau *et al.* 1985; Hu *et al.* 1985; Kamau, 1988; Heiduschka *et al.* 1994; Zhao *et al.* 2008; Zhao *et al.* 2009), carbon paste electrodes (CPE) (Švancara *et al.* 2009) and carbon nanotube (CNT) modified electrodes (Gooding, 2005; Gooding *et al.* 2007). Within applied electrochemical analysis and sensor fabrication and application, key factors include reproducibility and accuracy of response. The influence of electrode surface condition on electrochemical response has been widely cited, wherein heterogeneity in surface condition may impart variability in electrochemical response (Taylor & Humffray, 1975; Picq *et al.* 1984; Vasquez & Imai, 1985; Gala & Budniok, 1985; Kamau, 1988; Bowling *et al.* 1989; Ponticos & McCreery, 1992; Savitri & Mitra, 1998; Dekanski *et al.* 2001; Weisshaar & Porter, 2001; Braun *et al.* 2003; Kiema *et al.* 2003; Bleda-Martínez *et al.* 2005; Bleda-Martínez *et al.* 2006; Zhao *et al.* 2008; Zhao *et al.* 2009).

Although many examples of surface treatments allow for reproducible faradaic responses at GCE, (as cited in Chapter 1 and Chapter 4), in many cases variability in electrode surface condition cannot be avoided, resulting in inconsistent capacitance and faradaic responses between electrodes (Gala & Budniok, 1985; Bleda-Martínez *et al.* 2006). This may arise through heterogeneity across multiple surface preparations at single electrode surfaces as well as heterogeneity across multiple electrode surfaces. Scenarios which may result in inconsistent capacitance and faradaic responses between electrodes include; screen printed electrodes, micro electrodes where minute variations in surface condition affect responses, continuous analysis systems which may result in electrodes being fouled or physically altered during prolonged application and other industrial processes where pristine

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electrode surfaces cannot be guaranteed. Although electrode surface characteristics are integral to accurate quantification of the traits and characteristics of electrode responses and should be taken into account in any discussion relating to the electrochemical behaviour of an electrode surface, this is rarely the case (Gala & Budniok, 1985; Bleda-Martínez *et al.* 2006; Zhao *et al.* 2009). Thus, although traditional electrode preparatory methods exist which minimise I_p variability, alternative approaches may need to be considered for situations where pristine electrode condition cannot be guaranteed, or where micro-variations in electrode condition have pronounced influences on electroanalytical responses, such as in the case of adsorptive molecules. Analyses at oxidised GCE are problematic as methods such as standard addition are not possible due to current responses of compounds such as CIT being retarded in subsequent scans if electrodes had not been freshly prepared. Through Chapter 6 of this Thesis, this was shown to be a combined result of localised depletion of analyte at the electrode post scanning and limited replenishment of analyte at the electrode surface due to the presence of an oxide layer. This Chapter thus aims to explore a new means of attaining uniformity in I_p but as a post data-acquisition procedure, such that a methodology which can potentially normalise variability in current responses due to varying surface conditions can be established.

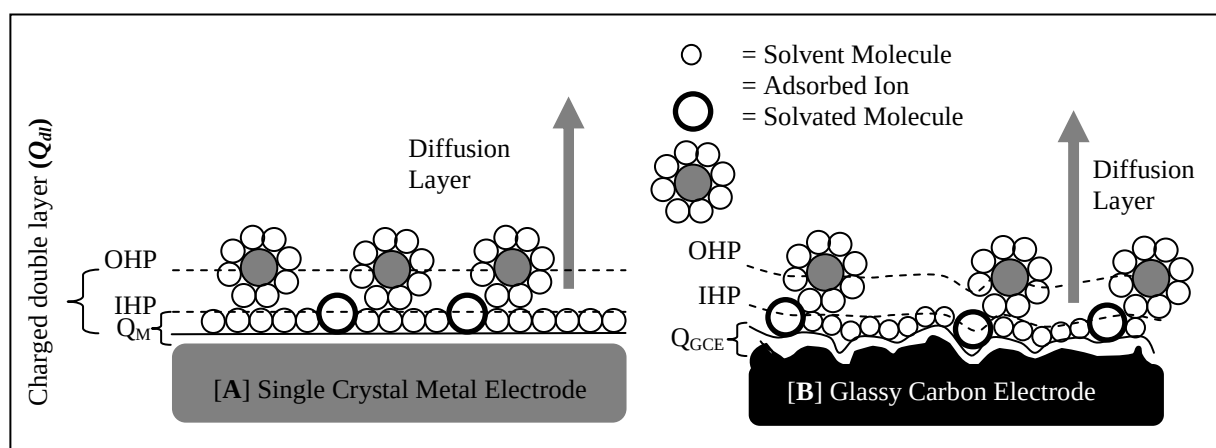
Many methods of experimentally determining individual parameters of electrode surface condition (morphology and chemistry) have been described as was cited through Chapter 4 of this Thesis. These are typically experimentally taxing, time consuming and impractical for integration into routine electroanalysis studies. This is well described for surface area determination where, although essential for quantification of faradaic peak current (I_p) responses, experimentally determined surface areas are subject not only to the method used, but also to the conditions of that assay, resulting in it being extremely difficult to accurately and practically determine the surface area of carbonaceous electrodes through these methods (Trasatti & Petrii, 1991; Jarzabek & Borkowska, 1997). Furthermore, electrode responses are multi-variant systems which may alter according to the conditions at an electrode surface which *ex situ* analyses may not accurately report (Ragiosha & Bondarenko, 2005).

A practical means of rapidly monitoring the relative variation in surface condition between analyses and a simple means of quantifying and relating this to responses is thus required and is the focus of the investigations herein. Several desirable traits for such a method would include the following:

- 1) the analysis method would be able to account for minimal variations in both morphological and chemical traits of an electrode.
- 2) the analysis procedure would be *in situ* and preferentially based on an electrochemical technique such that traits associated with that system in particular could be accounted for.
- 3) the responses would be relatively easily, rapidly and reproducibly accessed.

- 4) the analysis itself would not influence the state of the electrode surface such that subsequent electrochemical analyses at that surface would not be affected.
- 5) alteration in the condition of the electrode surface would alter the measured term to that same extent that electrochemical responses may be altered.

A term which fulfils all of these desirable traits and characteristics is measurement of the capacitance arising from the charge / discharge of the electrode double layer, “double layer capacitance” (C_{dl}) (Bard & Faulkner, 2001). The application C_{dl} to I_p is a relatively complex avenue by variability in I_p could be addressed, however application of conventional methods such as standard addition of analyte are not appropriate in such instances. The adsorptive nature of analytes such as CIT and 2,4-DMA render such a method inappropriate because they are adsorptive and form monolayers, which render analyses problematic as they are a result of electrode surface confined interactions. Due to this responses are not directly linked to bulk solution concentration. Thus, addition of standards of known concentration vs. unknown electrochemical surface area would involve making specific inferences from plateau-response regions, which is an inaccurate analysis.



Scheme 5.1: Representation of the electric double layer at [A] an atomically smooth and chemically uniform single crystal metal electrode and [B] morphologically and chemically inhomogeneous GCE. OHP represents the outer Helmholtz plane, IHP represents the Inner Helmholtz plane, Q_M is the charge of the metal, Q_{GCE} is the charge of the GCE (Bard & Faulkner, 2001; Kissinger & Heineman, 1996).

The double layer is a charged interfacial region which arises between an electrode surface and a solution it is immersed. This is as a result of attractive and repulsive forces of charged molecules present at the working electrode surface and those in solution (Scheme 5.1) (Kissinger & Heineman, 1996; Scholz, 2002; Bard & Faulkner, 2001). Charging or discharging of the double layer creates a measurable flow of current to be recorded and thus acts as the intermediary between a chemical reaction of an analyte and an electrical response (Scholz, 2002). It can be separated into several component layers which comprise the electrode solution interface (Scheme 5.1). The layer closest to the electrode surface is termed the inner Helmholtz plane (IHP), Stern or compact layer, and is comprised of adsorbed solvent molecules and anions, with the layer passing through the middle of the

adsorbed anions. The next layer is referred to as the outer Helmholtz plane (OHP) and is comprised of solvated ions in contact with the IHP, with the layer passing through the centre of the nearest ions to the inner layer (Bard & Faulkner, 2001).

The properties of the double layer are important as they have a significant effect on electrochemical measurements. Within the context of this study, a key point is that the properties of a double layer will arise as a direct result of the conditions at an electrode surface.

Although some single crystal electrodes and basal plane highly ordered pyrolytic graphite (HOPG) electrodes are atomically smooth (Kissinger & Heineman, 1996), producing planar double layer traits as seen in Scheme 5.1 [A], double layer structure is more varied at normal solid electrodes Scheme 5.1 [B] such as GCE. The double layer can be monitored through observation of the charge (Q) (as the integration of current) of the electric double layer (Bard & Faulkner, 2001). The measure of the ability of a body to store charge is termed capacitance (C) (Scholz, 2002; Bard & Faulkner, 2001; Kissinger *et al.* 1996). As the electric double layer is a charged layer, it acts as a capacitor (Scholz, 2002), referred to as double layer capacitance (C_{dl}). C_{dl} can be described by the equation:

$$Q = C_{dl}A (E - E_{pzc}) \quad \text{Eq. 5.1}$$

Wherein, Q is charge associated with the formation /dissipation of C_{dl} , C_{dl} is the double layer capacitance per unit area (A) E is the applied potential to the working electrode and E_{pzc} is the electrode potential of zero charge (Wang, 1994).

Although it is widely accepted that C_{dl} alters in relation to surface area, which was shown to be highly variable at polished GCE and Pi-GCE, it has been demonstrated that C_{dl} does not only depend on the state of the carbon electrode but on the treatment and resulting condition of that electrode, with particular influence being reported for surface chemistry and in particular surface oxides (Pajkossy, 1994; Kerner & Pajkossy, 1998; Heiduschka *et al.* 1994; Fuertes *et al.* 2004; Bleda-Martínez *et al.* 2005; Gooding, 2005). The heterogeneity of GCE surface condition (both morphology and chemistry) thus influences Q_{GCE} (charge of the GCE surface), through variations in the type/density of surface functional groups present at the electrode and the surface area over which the double layer forms, influencing E_{pzc} of Eq. 5.1. Other electrode confined factors include the potential at which the electrode is held (Eq. 5.1), porosity, pore structure, conductivity of a porous layer, as well as solution based factors such as dielectric constants and dimensions of ions also affect C_{dl} (Gala & Budniok, 1985; Pajkossy, 1994; Chu & Kinoshita, 1997; Kerner & Pajkossy, 1998; Palasantzas & Backx, 2003; Kim *et al.* 2003; Lozano-Castelló *et al.* 2003; Moulton *et al.* 2004; Pajkossy, 2005; Bleda-Martínez *et*

al. 2005; Albina *et al.* 2006; Centeno & Stoecki, 2006; Seo & Park, 2010; Li-xiang & Feng, 2011). As the double layer forms as a result of the surface condition, and is intimately associated with the surface characteristics, it has the possibility to be applied as an *in situ* probe to measure minute changes in surface conditions (Ragoisha & Bondarenko, 2005). The key benefit thereof is that monitoring the double layer allows for a representative measure of the same traits which propagate faradaic current response to be measured under the same conditions as those which result in a faradaic response (Scholz, 2002; Bard & Faulkner, 2001; Kissinger & Heineman, 1996). In so doing variability in I_p could possibly be modulated by the C_{dl} of that electrode as a post data analysis treatment.

As a well described principle, there are many experimental procedures, and importantly *in situ* electrochemical techniques to monitor C_{dl} of an electrode. These most commonly involve measurement of C_{dl} by electrochemical impedance spectroscopy (EIS) (Heiduschka *et al.* 1994; Pajkossy, 1994; Pajkossy, 2005; Freger & Bason, 2007; Ates, 2011). EIS effectively produces a response signal derived from multiple frequencies (ω) within a small scale potential perturbation range, which allows for the EIS to accurately report on responses as a multi-point analysis method (Bard & Faulkner, 2001; Scholz, 2002) and is commonly applied in the study of electrode and interfacial properties of modified electrodes (Heiduschka *et al.* 1994; Zhang *et al.* 2010). Although cyclic voltammetry has been used to determine capacitance (Jiang & Kucernak, 2000; Zheng *et al.* 2010), linear sweep voltammetry (LSV) has not typically been used. As was demonstrated by Bard & Faulkner (2001) and reported on in the methodology section, the same principles apply in LSV scans.

Although faradaic responses are also influenced by parameters such as analyte specific rate constants (diffusion or adsorption) and solution concentration (Bard & Faulkner, 2001), the traits of an electrode surface condition, notably surface area, strongly influence both faradaic response and C_{dl} . Accordingly, monitoring of one parameter (C_{dl}) may aid in elucidating the responses of the other (I_p) due to common parameters being monitored, application of which is a central theme in this Chapter.

The influence of mass transport at GCE has been considered previously by Hance & Kuwana (1987) through application of the molecule 1,2,4-trihydroxybenzene (Triol) (Figure 5.1).

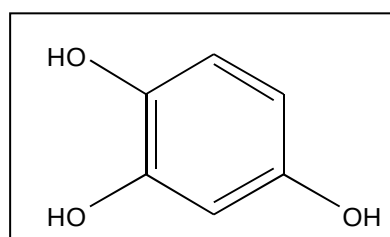


Figure 5.1. Structure of 1,2,4-trihydroxybenzene (Triol).

Triol is a neutrally-charged organic molecule which produces defined, reversible redox peaks at both polished GCE and oxidised GCE (Hance & Kuwana, 1987). As well as being structurally comparable to CIT, oxidation of Triol, as in CIT (described in Chapter 6) proceeds via the loss of electrons from hydroxyl moieties. Importantly, however, Triol has been shown to exhibit either predominantly adsorptive or diffusive mass transport kinetics as a function of solution concentration (Hance & Kuwana, 1987). Due to its tuneable mass-transport, it is an idea reference molecule to probe the interrelatedness of C_{dl} to I_p under separate mass transport regimes. This property allows for Triol to be applied within this study as a reference molecule for the investigation for the relative influence of mass transport considerations.

Aniline based recalcitrant toxins are commonly encountered in watercourses. Although the application of anilines in agricultural and industrial practices is economically necessary, it poses a threat to people and the environment as many of these compounds are both toxic and recalcitrant. 2,4-dimethylaniline (2,4-DMA) (Figure 5.2) is one such commonly encountered aniline which is highly stable and recalcitrant (Brimecombe *et al.* 2006).

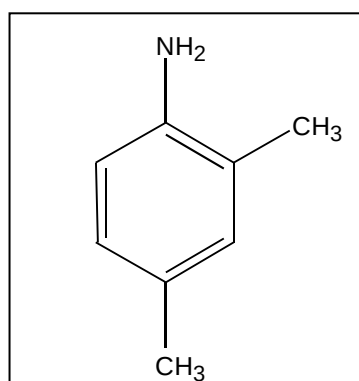


Figure 5.2: Structural formula of 2,4-dimethylaniline (2,4-DMA)

The prevalence of 2,4-DMA in the greater environment arises from a number of direct sources as well as degradation products from a number of parent compounds. These include its use as a precursor in the production of many chemicals such as dyes and pharmaceuticals (Brimecombe *et al.* 2006) and fuels (Rozkov *et al.* 1999). Furthermore it is also commonly found as a breakdown product of a number of these compounds such as the acaricide amitraz (Brimecombe *et al.* 2006; Rozkov *et al.* 1999). This has resulted in it being a commonly encountered contaminant in industrial waste waters which are a major source of this toxin. In addition to this, it has been shown to be transferred to humans when contaminated products such as milk, honey, eggs and meat are consumed (Kamel *et al.* 2007; Leníček *et al.* 2006). 2,4-DMA is teratogenic, mutagenic, genotoxic and oncogenic (Osano *et al.* 2002 a, Osano *et al.* 2002 b). Accordingly the determination and subsequent removal of 2,4-DMA

from a given environment is essential in order to limit perpetuation of adverse affects in that environment over time.

The application of voltammetrically determined C_{dl} as an easily accessible, representative, measure of electrode specific surface condition is proposed. In so doing, this study aims to apply C_{dl} of an electrode to the normalisation of I_p of adsorbed analytes on that electrode. For these purposes, electroanalysis of CIT, 2,4-DMA and Triol are investigated to interrogate this theory.

5.2 Chapter objectives

This Chapter was directed at the identification of a practically viable means of monitoring the relative variation of electrode surface condition between analyses, both rapidly and with relative ease, such that variability in I_p response as a result of surface variability can be accounted for, and stability in responses achieved. This Chapter aims to apply C_{dl} to acts as an *in situ* thin layer probe to monitor and account for relative variations in surface condition of electrodes within a set of analyses. Through relation of I_p to C_{dl} , we aim to normalise variability in I_p recorded across multiple electrodes by making each I_p response a factor of that electrodes surface condition. Thus, although traditional electrode preparatory methods exist which minimise I_p variability, this paper aims to provide a new means of attaining uniformity in I_p as a post data-acquisition procedure, such that a methodology can be established for situations where pristine electrode condition cannot be guaranteed.

Secondary objectives which are considered in relation to the primary aims include:

- a) The objective of this section is to assess the application of voltammetrically determined C_{dl} as an easily accessible measure of electrode specific surface condition. This requires that the accuracy of LSV-determined C_{dl} be validated in relation to C_{dl} determined through either EIS or chronoamperometry.
- b) Assess whether it is possible to relate I_p determined at an electrode to the C_{dl} as a measure of surface condition and ascertain whether there is uniformity in the extent to which one varies in relation to the other as a result in inconsistency across multiple electrode preparations.
- c) Determine whether I_p which varies between electrode preparations relative to the surface condition of that electrode can be normalised by accounting for that surface condition through application of C_{dl} and under what analyte mass-transport regime does C_{dl} account for I_p . The application of Triol whose mass transport is governed by solution concentration can be used to describe the influence of diffusive and adsorptive based processes on mediation by C_{dl} .
- d) Assess whether of application of C_{dl} may mediate I_p variability of chemically distinct analyte molecules (CIT and 2,4-DMA). A further objective includes probing whether any traits are

Chapter 5

Normalising I_p Variability at Carbonaceous Electrodes Using C_{dl}

transferrable across markedly different electrode surface conditions through using three different carbonaceous electrodes (GCE, Pi-GCE and fMWCNT-GCE).

- e) Identify whether mediation of I_p by C_{dl} provides a practical means of assessing solution concentration. The objective herein is thus to assess whether confidence in resulting concentration can be attained from initially variable I_p responses.

5.3 Chapter specific methods and materials

5.3.1 Analytes

Stock solution of CIT (5 mM) was prepared in anhydrous methanol and stored at -20 °C until used and Triol (100 mM) was prepared fresh prior to use, in Milli-Q water.

5.3.2 Electrochemical methodology

A three electrode electrochemical cell was utilised with glassy carbon electrodes (GCE) working electrodes, Ag/AgCl reference electrode and platinum auxiliary electrode were applied according to the parameters and procedures described through Chapter 2 with regard to equipment and electrode operation, preparation and modification as well as electrochemical procedures outlined.

Cleaning protocols, as detailed in Chapter 2, were followed relating to electrode cleaning (polishing) and electrochemical pre-treatment (15 conditioning scans in buffer with the final scan recorded as the background signal) and modification including chemical oxidation (Pi-GCE) or physical adsorption of functionalised multi-walled carbon nanotubes (fMWCNT-GCE). In each study, between 3 and 9 separate, labelled GCE electrodes (which were purchased over several years) were used such that more generalised responses across electrodes could be assessed.

The electrochemical scanning procedures and parameters listed in Chapter 2 were followed in this Chapter. Unless otherwise stated all analyses were carried out in 0.2 M sodium acetate (NaOAc) buffer at pH 4.00 with CIT at 20 μ M. Analyses were all conducted at a scan rate of at 0.1 V/s with all potentials reported relative to Ag/AgCl, (unless otherwise stated). Electrode pre-treatment procedures were followed stringently such that the variation in results between preparations could not be a factor of inaccurate application of the preconditioning steps. All parameters assessed within these studies were carried out on a minimum of 3 replicates per electrode used per study conducted.

The following section details the scanning parameters of the methods used, followed by the methods of determining C_{dl} from each method.

a) Electrochemical scanning parameters

Linear Sweep Voltammetry (LSV): Unless otherwise stated, CIT was scanned between 0.5 V and 1.1 V. Where applicable, an adsorption step was employed prior to scanning. This entailed holding the potential at 0.4 V for 120 seconds with solution agitation at 1000 rpm, unless otherwise stated. Concentration-dependent CIT analyses applied a slightly varied protocol, including a 30 second

adsorption period at 1, 3, 5, 10, 20 and 30 μM CIT in order to emphasise the influence of solution concentration.

Triol was measured in both 0.2 M NaOAc (pH 4.0) and 1.0 M H_2SO_4 at solution concentrations of 0.05 mM and 0.80 mM in both electrolytes to determine the effect of diffusion and adsorption as these conditions have been shown to induce adsorptive and diffusive behaviours respectively (Hance & Kuwana, 1987). The potential window for Triol analysis was between 0.0 V and 0.5 V.

2,4-DMA was measured at a concentration of 30 μM , between 0.2 V and 1.0 V. A 30 second adsorption step at 0.45 V was used prior to scanning.

b) Electrochemical determination of C_{dl}

C_{dl} was determined before analyte was introduced into solution (buffer only), and subsequently also determined in solution containing the analyte. C_{dl} was determined by LSV method on every electrode preparation throughout the course of the study. The steady state ‘capacitance points’ (Potential β , Figure 5.5) used were CIT = 0.6 V, 2,4-DMA = 0.4 V and Triol = 0.03 V, to remove interference from faradaic responses at each analyte.

Cross-validation of C_{dl} determined by LSV was performed on Pi-GCE surfaces only, relative to C_{dl} determined via EIS and τ Methods described hereafter. The following three precautions were observed to obtain uniformity in recorded C_{dl} :

- 1) C_{dl} was determined by each method at each electrode preparation prior to analysis of analyte such that C_{dl} could be compared to one another in relation to I_p of that electrode.
- 2) Given the influence of potential on C_{dl} (Bard & Faulkner, 2001), C_{dl} was determined at 0.6 V for each method.
- 3) C_{dl} determination was assumed not to affect the state of the electrode surface, thereby I_p was not altered by recording C_{dl} .

Capacitance Determination by EIS: Redox probes were not used to avoid influence of adsorbed redox probe species on subsequent analyses, as graphitic oxide is porous (Kepley & Bard, 1988). EIS analyses were carried out immediately after pre-treatment in NaOAc buffer both with and without analyte in solution at 20 μM at 0.6 V between a frequency range of 0.1 and 10000 Hz with a standby potential of 0.05 V. A modified Randles equivalent circuit (Scheme 5.2 [B]) (Bard & Faulkner, 2001) was used, with the Warburg element removed due to the absence of diffusive impedance (absence of a redox probe) and C_{dl} was replaced by the constant phase element (Scheme 5.2 [B]) (CPE, Q) (Wu & Zhang 1995; Scholz, 2002; Akinbulu *et al.* 2010).



Scheme 5.2: Equivalent circuit used in EIS measurements. A) Randles equivalent circuit without Warburg impedance and B) Modified Randles equivalent circuit with C_{dl} replaced with a CPE element (Q).

Wherein R_s is solution resistance (Ω), R_p is polarisation resistance (Ω), W is Warburg impedance, C_{dl} is capacitance of the double layer (F) and Q is a constant phase element that models pseudocapacitance of the interfacial double layer. Data fittings and simulation were performed using Nova software (Eco Chemie, The Netherlands). The modulus of the pseudocapacitance is presented and referred to as Q_{dl} .

Capacitance Determination from Step Chronoamperometry (τ Method): The τ method was used to determine C_{dl} derived from an exponentially decaying current response after a potential step in chronoamperometry (Bard & Faulkner, 2001), as in Figure 5.3. A standby potential of 0.4 V was used, two steps were applied, the first at 0.55 V for 0.2 seconds, followed by a step to 0.6 V for 0.3 seconds, sampling I at 50 μ s intervals (Figure 5.3).

According to Bard & Faulkner (2001), the behaviour of current (I) in relation to time (t), with a potential step of magnitude E is:

$$i = \left(\frac{E}{R_s} \right) e^{-\frac{t}{R_s C_d}} \quad \text{Eq. 5.2}$$

Where R_s is solution resistance (Ω) and C_{dl} is double layer capacitance (F). Furthermore, the result of a potential step input is an exponentially decaying current with time constant (Bard & Faulkner, 2001):

$$\tau = R_s C_d \quad \text{Eq. 5.3}$$

At an exponentially decaying current, the current charging the double layer will drop to 37% of its initial apex value at $t = \tau$ and further to 5% at $t = 3\tau$. The positions of τ and 3τ relative to the curve are illustrated in Fig. 1. Maximum current was recorded and the time (t) at τ and 3τ were determined at 37% and 5% of that maximum current respectively. For the purposes of clarity, the following explanation illustrates τ only, however the same process was also followed for 3τ , and the final result divided by 3 to find τ . The two resulting C_{dl} values were averaged then averaged as the reported τC_{dl} . The following equation was applied as a basis (Bard & Faulkner, 2001):

$$i_{step}(t) = \left(\frac{\Delta E_{step}}{R_s} \right) e^{-\frac{t}{R_s C_{dl}}} \quad \text{Eq. 5.4}$$

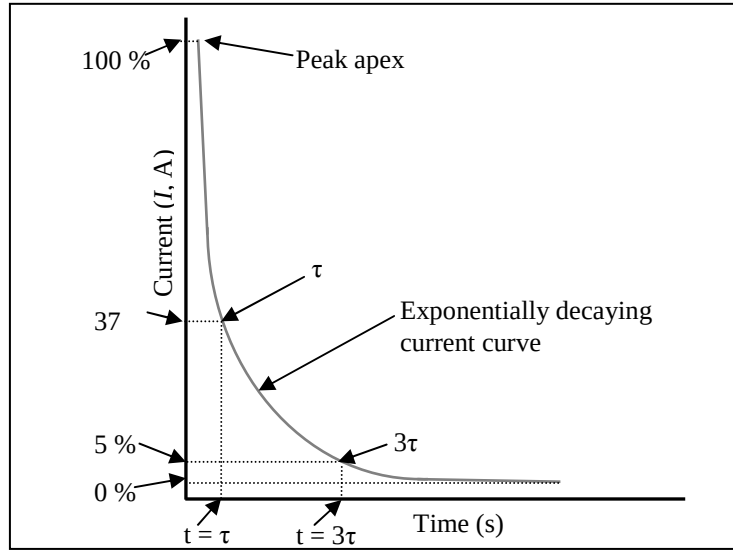


Figure 5.3. Determination of time at τ and 3τ from an exponentially decaying current after a potential step in a chronoamperogram.

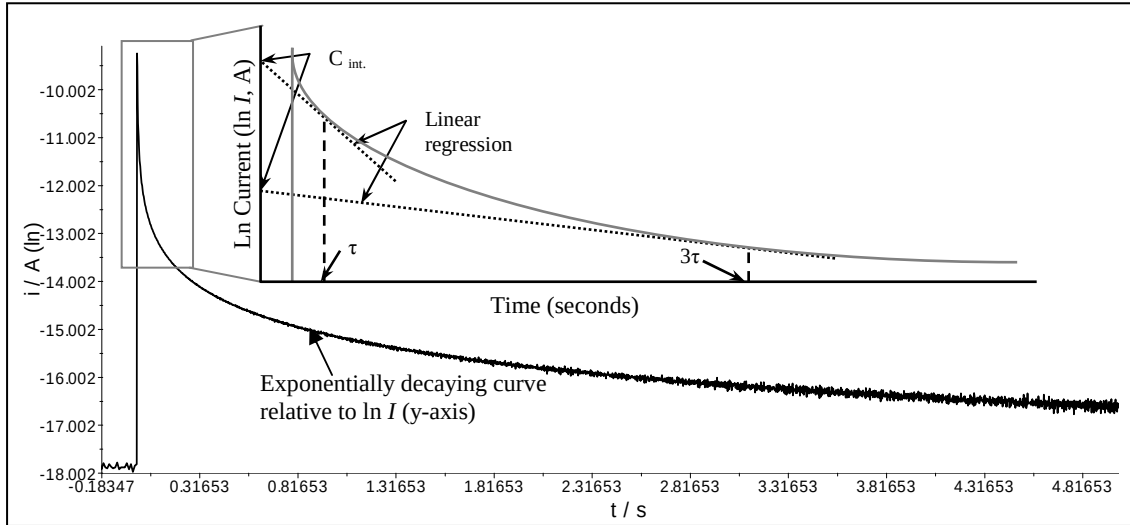


Figure 5.4. Determination of $C_{int.}$ at τ and 3τ relative to the $\ln$ current (I, A) (y-axis) of the exponentially decaying current response in a chronoamperogram. Insert illustrated the wider scope of the magnified section.

By rewriting Eq. 5.4 as the natural logarithm ($\ln$) (Paul & Leddy, 1995) C_{dl} can be isolated:

$$\ln[i_{step}(t)] = \ln\left(\frac{\Delta E_{step}}{R_s}\right) - \frac{t}{R_s C_{dl}} \quad \text{Eq. 5.5}$$

With the y-axis ($\ln I$) and rewriting Eq. 5.5 as $y = mx + c$, and substituting $\tau = t$ (Eq. 5.3) then:

$$\ln[i_{step}(t)] = \left(\frac{-1}{\tau}\right)t + \ln\left(\frac{\Delta E_{step}}{R_s}\right) \quad \text{Eq. 5.6}$$

By linear regression at points τ and 3τ to $\ln I_p$ (A) of the chronoamperogram (Figure 5.4), which had a gradient of $(-1/\tau)$, the y-intercept (C intercept [$C_{int.}$]) of the equation was found (Figure 5.4).

As the $C_{int.} = \ln\left(\frac{\Delta E_{step}}{R_s}\right)$ from Eq. 5.6, the following was derived:

$$R_s = \frac{\Delta E_{step}}{e^{C_{int.}}} \quad \text{Eq. 5.7}$$

Thereafter by substituting the R_s value back into Eq. 5.3, the following can be derived:

$$C_{dl} = \tau \frac{e^{C_{int.}}}{\Delta E_{step}} \quad \text{Eq. 5.8}$$

Capacitance Determination from LSV: As described by Bard & Faulkner (2001), an LSV scan initiates from a point at charge (q) = 0 and time (t) = 0 described by Eq. 5.9:

$$i = \nu C_{dl} \left[1 - e^{\frac{-t}{R_s C_{dl}}} \right] \quad \text{Eq. 5.9}$$

Under a voltage ramp at a scan rate (ν), current (I) is generated, associated with charging of the double layer (i.e. is non-faradaic), increasing to a steady-state where the time constant $R_s C_{dl}$ is small compared to ν (Bard & Faulkner, 2001; Zheng *et al.* 2010), resulting in the bracketed portion of Eq. 5.9 rapidly tending to 1, thus at a sufficiently large potential difference (where charging current is at steady state):

$$i = \nu C_{dl} \quad \text{Eq. 5.10}$$

Where I is the change in current from $t = 0$ (point α) to the steady-state (potential β of Figure 5.5), ν is the scanning rate ($V.s^{-1}$), C_{dl} is the double-layer capacitance ($F.cm^{-2}$). Figure 5.5 depicts the determination of C_{dl} by this methodology.

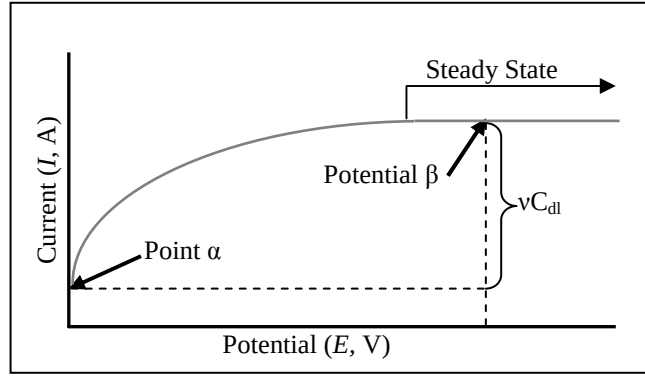


Figure 5.5: Determination of C_{dl} from the non faradaic portion of an LSV scan (modified from Bard & Faulkner, 2001).

5.3.3 Data Analysis

a) Determination of coefficient of variation (C.V.)

All data points were recorded in triplicate as a minimum. C.V. (Eq. 2.3, Chapter 2) was calculated via the ratio of the standard deviation (σ) to the mean (μ) (Rosner, 2000), though herein it was also calculated according to:

$$C.V. = \mu(\theta_1, \theta_2, \theta_3 \dots \theta_n) \quad \text{Eq. 5.11}$$

$$\theta = \left(\frac{I_{p,n} - I_{p \text{ mod },n}}{I_{p,n}} \right) \times 100 \% \quad \text{Eq. 5.12}$$

Where

Determination and explanation of $I_{p \text{ mod }}$ is described in section 5.4.5.

b) Regression analysis of data

As data was not normally distributed, a nonparametric regression model was required. Accordingly, Kendall-Theil Robust Line Fit (Kendall-Theil) (Sokal & Rohlf, 1995; Helsel & Hirsch, 2002), also referred to as the Theil-Sen or Sen slope estimator, was used to determine the relationship between I_p and C_{dl} , as has been applied by several studies (Akritas *et al.* 1995; Peng *et al.* 2008; Lavignini *et al.* 2011) via the software programme KTRLine ver. 1.0 (Granato, 2006). The method allows for robust determination of regression slope through determining median slope (m), which allows for regression to be insensitive to outliers. It is preferred in situations which may have skewed responses through application of methods compared to regression analyses commonly applied to parametric data sets such as the least mean squares regression (Theil, 1950 (a) (b) (c); Sen, 1968; Kitchens, 1998; Rosner, 2000).

c) Correlation analysis of data

When using the Kendal-theil regression, a nonparametric correlation coefficient is required, thus the Spearman's r_s (Kitchens, 1998; Rosner, 2000), as applied by several studies (Clemett *et al.* 1999; Meissner *et al.* 2000; Edmonds *et al.* 2008; Inagaki *et al.* 2009) was calculated for relationship between I_p and C_{dl} and on each of the surfaces assessed using STATISTICA© ver. 8.0. Spearman's r_s is an ordinary correlation coefficient which is based on ranks assigned to x 's and y 's separately rather than the actual scores (Rosner, 2000; Kitchens, 1998). These analyses were performed on all data sets unless otherwise stated.

5.4 Results and discussion

Three distinct surface preparations were examined through this section such that extensive variability in responses at these surface conditions could be assessed. These surfaces included polished GCE, Pi-GCE (piranha solution oxidised) and fMWCNT-GCE (functionalised multi-walled carbon nanotube), each containing similarities in their characteristics to the other two electrodes, but being sufficiently varied in form, composition and structure to allow for a broad scope of possible electrode surfaces on which such a process could be applied for subsequent analyses of CIT I_{p1} . Further investigations were directed at assessing the practical application of the procedure as a method and identification of areas where such an application would be beneficial within electroanalytical studies.

5.4.1 Electrochemical analysis of CIT at polished GCE, Pi-GCE and fMWCNT-GCE

Citrinin (CIT) current responses (I_p) at the carbonaceous electrodes assessed for this study, including polished GCE (GCE) (Figure 3.4), functionalised multi-walled carbon nanotube modified GCE (fMWCNT-GCE) (Figure 3.16) and at piranha solution oxidised GCE (Pi-GCE) (Figure 5.6) produced a common two-peak electrochemical profile. Representative current profiles at Pi-GCE (Figure 5.6) illustrate very similar traits to those reported at GCE and fMWCNT-GCE. Very stable peak potentials were found at each surface with $E_{p1} = 0.838 \text{ V} (\pm 0.002 \text{ V})$ at polished GCE, $E_{p1} = 0.830 \text{ V} (\pm 0.003 \text{ V})$ Pi-GCE and $E_{p1} = 0.833 \text{ V} (\pm 0.006 \text{ V})$ at fMWCNT-GCE, but also illustrated marked variability in I_{p1} responses. The extent of I_{p1} variability was collated in Figure 5.7 as a summary of the I_{p1} responses from each data set (GCE, Pi-GCE and fMWCNT-GCE). Therein, the variability encountered is highlighted through the coefficient of variation (C.V.) as it allows for the relative comparison of different sets of data (Rosner, 2000), with C.V. >10 % describing extensive inter-category variability.

Although the difference between mean I_{p1} responses at each surface was not large, extensive variability was encountered, with C.V. responses for CIT I_{p1} analyses being 20.16 %, 22.87 % and 101.92 % for GCE, Pi-GCE and fMWCNT-GCE respectively, (Figure 5.7). The extent of that variability between electrodes is highlighted through Table 5.1, which represents responses from each fMWCNT-GCE electrode set as the most variable data set.

As shown in Figure 5.6, for Pi-GCE, while each scan initiates from a common origin, the charging current, as well as subsequent I_{p1} rapidly diverge between measurements. The variability followed no sequential change with consecutive analyses and was thus deemed to be specific to individual electrode surface condition for each preparation. As highlighted by the inset of Figure 5.6, a correlation between the magnitude of charging current and resulting I_{p1} was evident, wherein the

extent of charging current seemed to pre-empt the magnitude of I_{p1} for each electrode preparation. This, along with the fact that all the scans were carried out under uniform conditions in a single solution, indicate that I_{p1} variation for CIT had likely arisen as a factor of the electrode surface condition, indicated by charging current. This trait was also observed at GCE (Figure 3.4) and at fMWCNT-GCE (Figure 3.16 [A]).

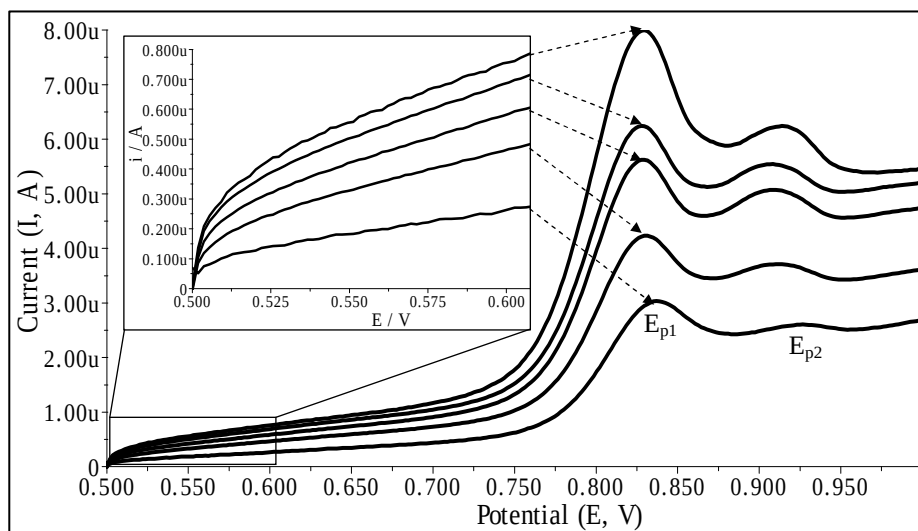


Figure 5.6: Representative scans of CIT peak profile and I_{p1} variability in a single 20 μM solution at separate Pi-GCE. Each scan was recorded on a freshly prepared Pi-GCE. Insert shows a magnification of the 0.5 V to 0.6 V range to illustrate the relationship between charging current and subsequent faradaic current from CIT oxidation at each surface.

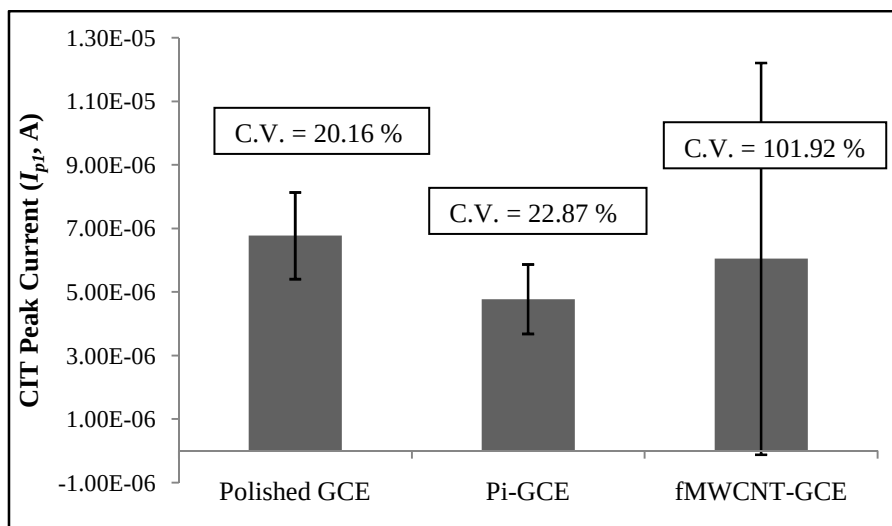


Figure 5.7: Averaged CIT I_{p1} responses in 20 μM CIT solution at polished GCE ($n=24$), Pi-GCE ($n=18$) and fMWCNT-GCE ($n = 36$), along with the standard deviation and coefficient of variation (C.V.) of each.

Although the C.V. results were lower within separate electrode sets (across several preparation of a given surface condition of one GCE, data not shown) for each of the surface preparations, each electrode produced variable responses. As well as being discussed further in relation to Pi-GCE in

section 5.4.3, this was highlighted by f MWCNT-GCE responses through Table 5.1, wherein each electrode resulted in C.V. of greater than 10% variability, and only one of the nine electrode sets had C.V. below 20%. Although greatly improved I_p responses were found at some f MWCNT-GCE as compared to polished GCE, the extent of the variability effectively excluded the application of physically adsorbed f MWCNT under normal analysis applications for CIT measurement. As described in Chapter 3, the instability in f MWCNT-GCE responses arises from it being extremely difficult to accurately control the amount, distribution and orientation of f MWCNT at the electrode surface and that the f MWCNT effectively acts as a highly conductive, porous electrode which has the ability to extensively alter C_{dl} between preparations (Henstridge *et al.* 2010). The application of f MWCNT thus provides a highly complex and variable set of conditions which depend on a number of parameters and provides a good test of whether charging current can predict subsequent I_{p1} responses at that electrode surface.

Table 5.1: Summarised data of representative CIT analysis at each f MWCNT-GCE set assessed.

f MWCNT-GCE:	1	2	3	4	5	6	7	8	9	Average
μ (μ A)	2.72	19.60	3.00	6.19	9.53	4.18	2.96	3.29	2.95	6.05
σ	0.53	8.93	1.13	2.31	4.50	2.39	0.71	0.98	0.72	6.16
C.V. (%)	19.64	45.58	37.72	37.31	47.20	57.12	23.94	29.80	24.34	101.92

μ = mean, σ = standard deviation from the mean, C.V. = coefficient of variation calculated as per Eq. 2.3. Responses were reported as averages of all scans from all nine GCEs assessed. Scans were carried out in 20 μ M CIT in 0.2 M NaOAc, pH 4.0, on GCE modified with f MWCNT.

As all scanning parameters were identical for all analyses performed, and no evidence was found for the surfaces themselves affecting kinetics of response, the variability could not be attributed to factors such as non-uniform oxidation kinetics or environmental/solution based factors and therefore it is a more probable inference that this arose as a result of deviation of the electrode surface condition between preparations. As surface condition of each of the surfaces assessed has been described as being variable through the course of this Thesis and through reported studies herein, and because the interaction of CIT with the specific traits of an electrode was expected to hold a marked influence on I_{p1} responses, deviation in surface preparation was investigated as the cause of I_{p1} variability and as the parameter which needed to be accounted for in order to normalise I_{p1} responses.

5.4.2 Cross validation of C_{dl} determined through LSV with C_{dl} determined by EIS and τ methods

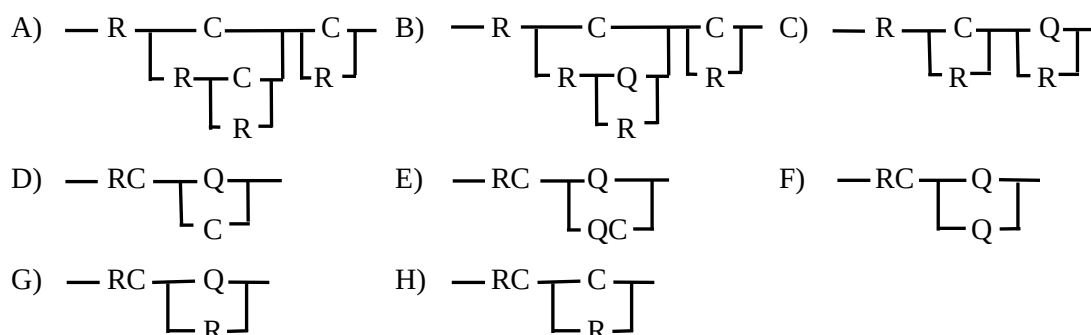
Although cyclic voltammetry has been used to determine capacitance (Jiang & Kucernak, 2000; Zheng *et al.* 2010), linear sweep voltammetry (LSV) has not typically been used. As was demonstrated by Bard & Faulkner (2001) and reported on in the methodology section, the same principles apply in a LSV scan. The following section details two further methods of determining C_{dl} (EIS and step chronoamperometry referred to as the τ method) for cross validation of the LSV method

with the intention of providing a practically and experimentally simple means of determining C_{dl} . C_{dl} was determined by each method on each electrode preparation such that results of each method could be compared directly to one another for each electrode surface such that LSV derived C_{dl} could be corroborated as a method.

a) Determination of C_{dl} by EIS

According to K'Owino & Sadik (2005), impedance changes can be quantified according to an [RC] equivalent circuit, which is generally considered to represent non-faradaic impedance. They found, as was the case in this study (discussed below), that the [RC] circuit modelled response does not compare to experimental impedance.

It is well documented that impedance at solid electrodes is not purely capacitive in the absence of faradaic reactions, and further that deviation of capacitive behaviour is strongly dependent on the state of disorder of the electrode surface (the smoother and cleaner an electrode, the more capacitive it will be) (Pajkossy, 1994; Heiduschka *et al.* 1994; Kerner & Pajkossy, 1998). The chemical oxidation procedure applied to GCEs in this study resulted in a marked influence on both chemical and morphological condition of the GCE (Pi-GCE analyses of Chapter 4). Accounting for this requires a more complex equivalent circuit to accommodate these changes in surface properties, or the application of a distributed capacitor in the form of a constant phase element (CPE) [Q]. As it was desirable to retain the application of a pure capacitive element [C] in the equivalent circuit for accuracy of reported C_{dl} responses, several variants of equivalent circuits were analysed (Scheme 5.3) which included both [C] and [Q] as well as (RC) elements to represent a layer at the electrode. Further analysis of circuits which provided some degree of conformity of modelled data to experimental data are represented graphically in Figure 5.8. The “closeness of fit” of circuits which did provide some degree of fit are compared in Table 5.2 by reporting on χ^2 values as a measure of the closest fit.



Scheme 5.3: Circuit diagrams assessed which did not result in valid equivalent circuit responses.

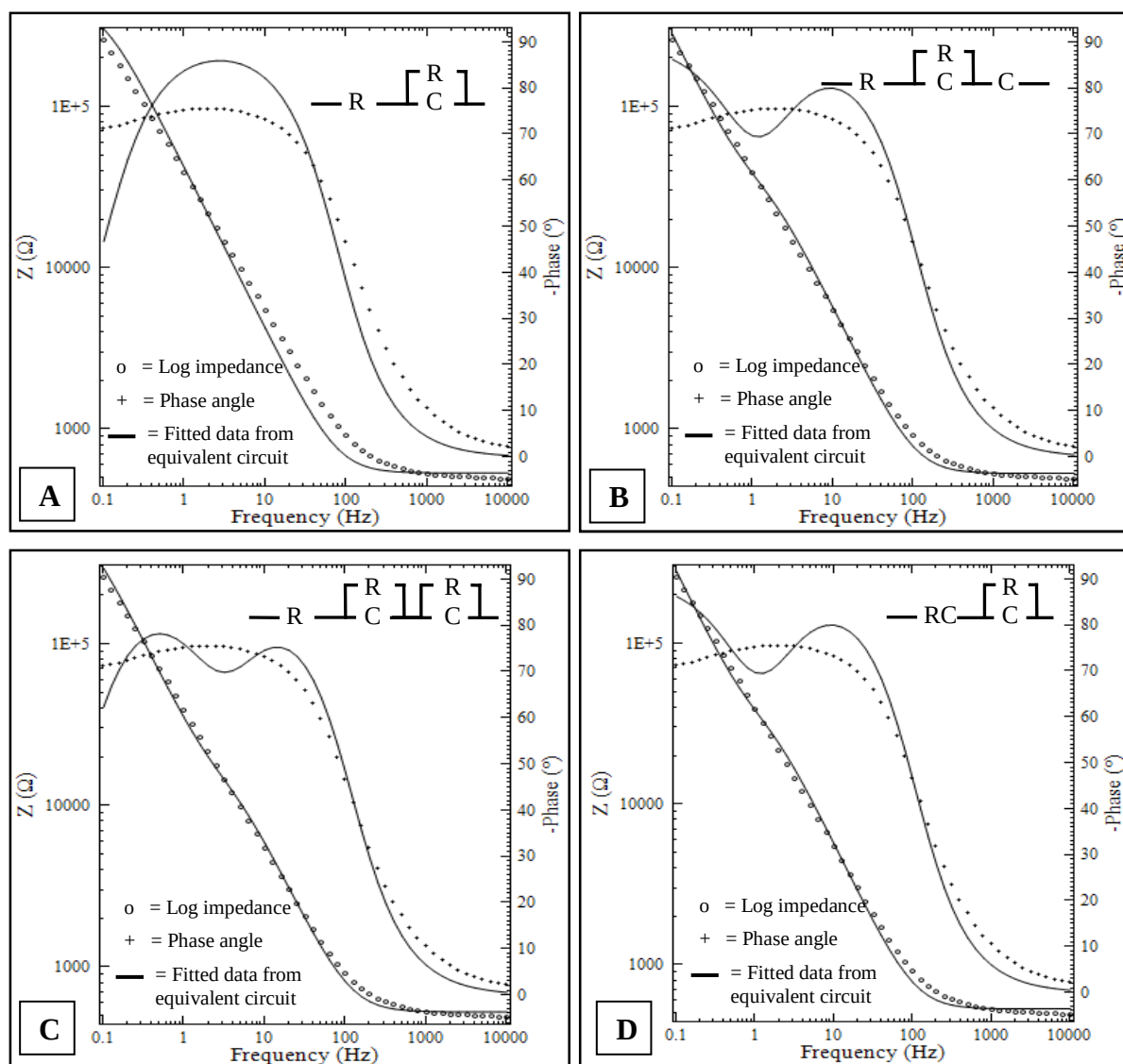


Figure 5.8: Representative Bode plots of EIS data from Pi-GCE with data fit from [A]: [R(RC)], [B]: [R(RC)C], [C] [R(RC)(RC)] and [D] [RC(RC)].

Table 5.2: Measure of the goodness of fit of equivalent circuits assessed as depicted by χ^2 analyses.

Equivalent Circuit	[R(RQ)]	[R(RC)]	[R(RC)C]	[RC(RC)]	[R(RC)(RC)]
$\mu \chi^2$	0.05	2.31	0.67	0.86	0.42
$\sigma \chi^2$	0.03	0.48	0.17	0.23	0.11

μ = mean, σ = standard deviation from the mean

As is evident from the bode plots (Figure 5.8) and Table 5.2, showing χ^2 results across the entire data set for each equivalent circuit, the equivalent circuits which include a pure capacitor provide very poor fits of modelled to experimental data. Due to the presence of graphitic oxide at the electrode surface after modification, the electrode is considered to be both rough and an imperfect capacitor. As such, it acts as a distributed capacitor, making the application of C_{dl} as a purely capacitive circuit element invalid Heiduschka *et al.* (1994). With such a result, capacitive behaviour of the impedance spectrum $Z(\omega)$ is generally approximated with the implementation of a constant phase element (CPE)

in place of C_{dl} as an empirical representation of electrode surface inhomogeneities as it represents deviation from ideal capacitive behaviour (Pajkossy, 1994; Wu & Zhang, 1995; Scholz, 2002, Yang *et al.* 2005; Akinbulu *et al.* 2010). This application correlated closely with that reported by Heiduschka *et al.* (1994), wherein they too assessed EIS of oxidised GCE and required the application of a CPE in place of C_{dl} . The impedance spectrum of a CPE ($Z_{CPE} [\omega]$) can be represented as follows:

$$Z_{CPE}(\omega) = \left(\frac{1}{\sigma}\right)(i\omega)^{-\alpha} \quad \text{Eq. 5.13}$$

Wherein ω is the frequency range, i is the imaginary unit ($-1^{1/2}$), and the CPE coefficient σ determined from data analysis (with a unit $s^{\alpha}.\Omega^{-1}$) and the CPE exponent α (generally between 0.7 and 0.9 at solid electrodes) are positive constants. Therein C_{dl} , is proportional to Y_0 (the pre-exponential parameter of CPE) of a purely capacitive surface, as described by the closeness of the exponent n to 1 (Neves *et al.* 2006; Özcan *et al.* 2008). As modelled n values were shown to be very similar to one-another $0.87 (\pm 0.01)$ from equivalent circuit analyses of EIS data, although Y_0 is not equal to C_{dl} , Y_0 approximates C_{dl} in this instance. Y_0 (henceforth Q_{dl}) was thus used to report on EIS derived C_{dl} . An example of the modelled fit of the equivalent circuit to experimental data with application of a CPE is illustrated through Figure 5.9, plotted as both Bode and Nyquist plots.

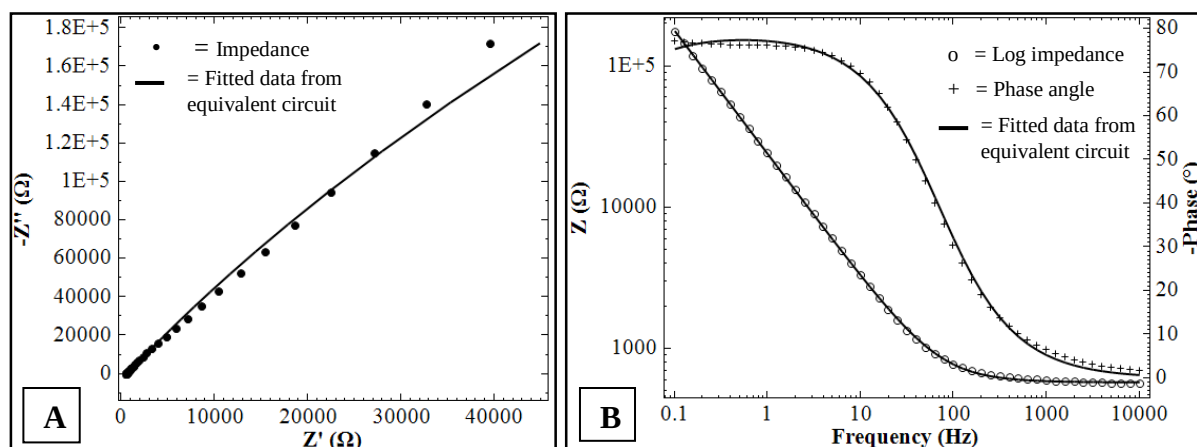


Figure 5.9: Representative EIS spectrum of Pi-GCE in buffer solution with fitted data represented on [A] Nyquist and [B] Bode plots respectively.

As is evident from Figure 5.9, the use of the CPE (Scheme 5.2 [B]) to model capacitive behaviour provides a modelled response which is close to the experimental data, and was much more accurate than circuits which applied a pure capacitor (Table 5.2). Due to these results, the equivalent circuit of Scheme 5.2 [B] was applied in EIS analyses of all data at Pi-GCE. The relatively straight line illustrated by the Nyquist plot of the entire impedance spectrum (Figure 5.9 [A]) is similar to that described by Heiduschka *et al.* (1994) as representative of a response of a rough electrode acting as a

distributed capacitor. As no defined semicircle was found in the impedance spectrum (Figure 5.9), the Randles equivalent circuit was applied as it approximates linear, low frequency, mass transfer controlled impedance responses (Bard & Faulkner, 2001).

b) Corroboration of LSV Derived C_{dl} with τC_{dl} and EIS Q_{dl}

Unlike EIS analyses, C_{dl} could be determined directly from the LSV and τ methods respectively as was described through the methodology section. C_{dl} determined via the LSV method was cross validated against τC_{dl} and Q_{dl} by Spearman correlation (r_s) and Kendall-Theil regression analyses, comparing CIT I_{p1} to capacitance (C_{dl} or Q_{dl}) determined by each method for the surface that correlated to each I_{p1} response (Figure 5.10). Furthermore, a direct comparison of LSV- and τ -derived C_{dl} to Q_{dl} as an established method Bard & Faulkner (2001) (Figure 5.11) was made. C_{dl} was determined in the absence of the analyte (buffer only) in all reported responses herein due to the influence of CIT on recorded capacitance as discussed in section 5.4.3.

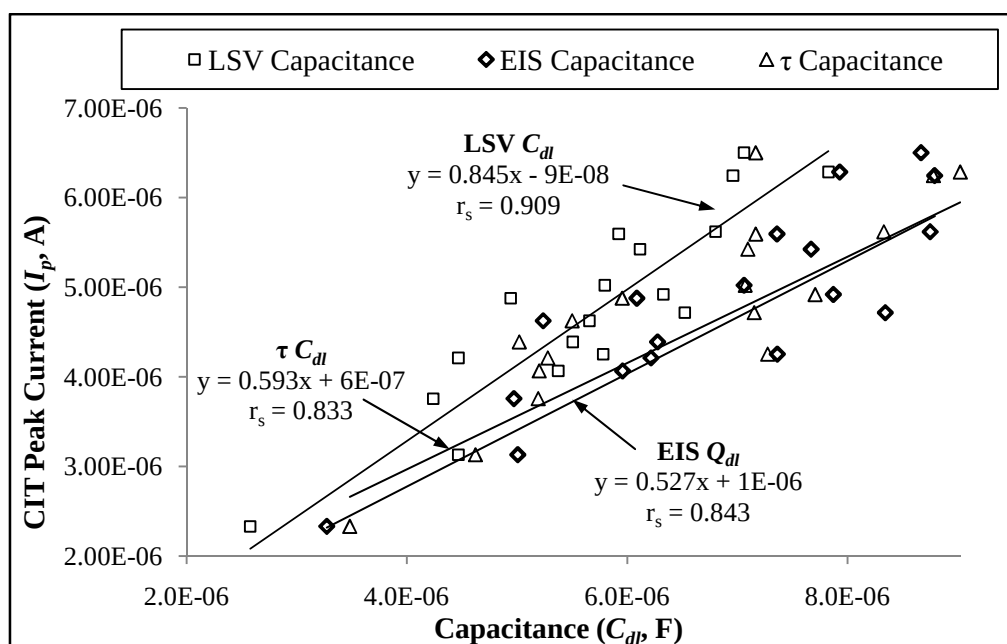


Figure 5.10: Scatter plots relating CIT I_{p1} to C_{dl} of each electrode determined through EIS (diamond), LSV method (square) and the τ method (triangle). Each method of C_{dl} determination was carried out on each of six replicates performed on three Pi-GCE ($n = 18$).

As stated in the Methodology of this Chapter, Kendall-Theil regression (slope) and Spearman's correlation (r_s) were used for the aforementioned reasons. In Figure 5.10, good linear relationships between C_{dl} and I_{p1} were found for all methods of determining capacitance (LSV, EIS and τ) with $r_s > 0.83$ for all methods. The responses were comprised of near proportional contributions of C_{dl} and I_{p1} relative to one another. That relationship was common across the entire range assessed; suggesting

that C_{dl} acts as an indicator of I_p of an electrode and that the relationship is valid across a broad range of C_{dl} and I_{p1} responses.

Some variability was found between the three methods of assessing C_{dl} given that recorded electrode traits are influenced by the method and conditions of analysis (Trasatti & Petrii, 1991). Some variability may also have arisen from Q_{dl} responses effectively being pseudo- C_{dl} responses. Furthermore, as each electrode was assessed by EIS, then τ and finally by the LSV method, some time-dependent degradation of the graphitic oxide layer may have occurred (Kepley & Bard, 1998) to influence C_{dl} through an expected combination of reduced surface area and altered state of the oxide surface chemistry (Bleda-Martínez *et al.* 2005; Seo & Park, 2010; Li-xiang & Feng, 2011). Considering this, it can be inferred that C_{dl} responses are relatively similar and that the measure of C_{dl} is representative across the three methods assessed.

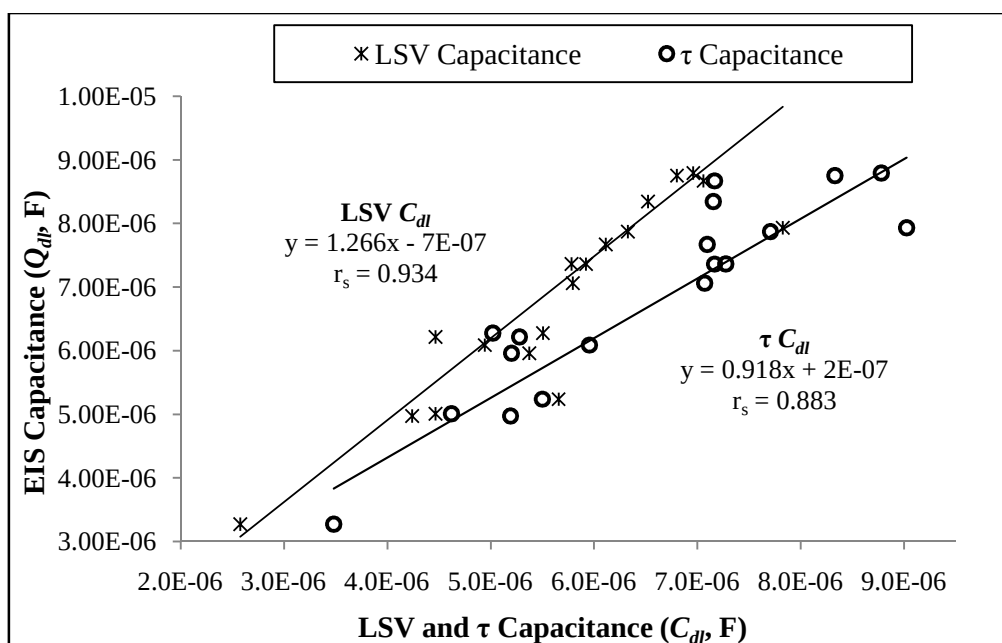


Figure 5.11: Scatter plot relating C_{dl} determined through EIS (y-axis) to C_{dl} determined by LSV (square markers) and τ (triangle markers) methods respectively.

The relative similarity of C_{dl} determined by the LSV and τ methods were compared to one another in relation to Q_{dl} as a well established and widely applied multiple point analyses method Figure 5.11 (Kissinger & Heineman, 1996; Scholz, 2002). This relation allowed for a direct comparison of capacitance responses determined at each electrode by each of the methods rather than relating the responses to another variable. Therein, equivalence of response between the methods would be described by the proportionality of each of LSV and τC_{dl} to EIS Q_{dl} . That would be represented by a slope with gradient (m) = 1. As is evident in Figure 5.12, the relation of LSV to EIS has a close proportionality ($m = 1.266$), which along with a very strong correlation of $r_s = 0.934$, indicating that

LSV assessed C_{dl} tracks well with EIS-derived C_{dl} derived measurements. Due to the ease and rapidity of determination of C_{dl} by LSV in combination with the ease of data analysis in relation to EIS and τ methodologies and the relative similarity of recorded responses over the entire data set across each of the methods assessed, the LSV method was assumed to be validated and used throughout the remainder of the Chapter as the method for measurement of C_{dl} .

5.4.3 Relation of the C_{dl} of an electrode surface to I_p recorded at that surface (intra-electrode responses)

In the introduction to this Chapter (Section 5.1), common factors were suggested to influence both I_p response at a given surface and the C_{dl} of that surface as a factor a surface condition. This relationship was described in relation to the electric double layer and proposed to be recorded through monitoring of the double layer by relating C_{dl} to I_p of an electrode. This relationship was considered briefly through Figure 5.10, which suggested a near proportional relationship of C_{dl} to I_p over a broad range of responses. In order to effectively probe that relationship of C_{dl} to I_{p1} , further studies were conducted relating both parameters over multiple electrodes of a Pi-GCE as a representative single surface preparation (Figure 5.12) and thereafter across multiple preparations of each of the surface preparations (GCE, Pi-GCE and fMWCNT-GCE) (Figure 5.13).

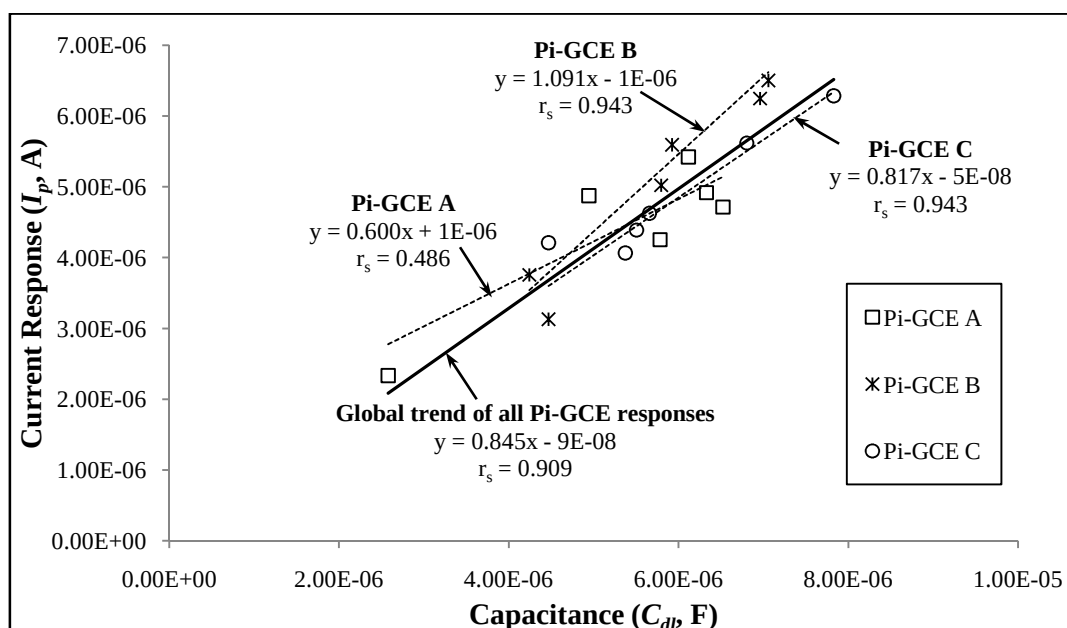


Figure 5.12: Scatter chart of C_{dl} and I_{p1} for each Pi-GCE A, B and C with six scans per electrode ($n = 18$).

As these results were collected using three separate GCEs (labelled Pi-GCE A, B and C), each being polished and re-oxidised after each CIT scan (on six occasions), responses were reported for separate Pi-GCEs as well as the global trend for all of the Pi-GCE responses. A key result herein is that although each electrode reported on a trend which seemed to be specific to that Pi-GCE, those

responses fell within a range that was common across all of the electrodes assessed. This finding is illustrated through Figure 5.12, as the intra-electrode r_s results are generally greater than those of inter-electrode results. This result illustrates that there is intrinsic variability between electrodes, but that the electrodes follow a similar trend to one another. Furthermore, separate electrodes do not group the data points, but rather are spread out across the trends. The result of this finding is that the magnitude of C_{dl} and I_{p1} remain relative to one another over a broad range of responses, and that all that finding is true across multiple electrodes which seem to follow a common trend. As such although each electrode may have an underlying specific trend, those responses were within a common trend across several electrodes and all of the electrodes recorded responses through the entire trend, thus a correlation over the entire trend (solid line, Figure 5.12) describes a relationship between C_{dl} and I_{p1} which is not confined to any single electrode or surface condition.

These results as such report on a broad range of response for each electrode, which is indicative of highly varied surface conditions as reported through Chapter 4. This finding is significant with regard to relating C_{dl} to I_{p1} as it illustrates that C_{dl} relates to I_{p1} over a broad range of surface conditions for each electrode, but that each of the electrodes report on a correlation within a common spread of C_{dl} to I_{p1} about the global trend (solid trend line, Figure 5.12). The inferred implication of this result is that relation of C_{dl} to I_{p1} appears to be a valid method of tracking I_{p1} as a factor of surface conditions over a number of electrode surfaces, making the method applicable with variability over multiple electrodes rather than variability within results from a single electrode.

Due to these responses, only the global trends of entire data sets were reported on for the remainder of this Chapter as although not reported for each analysis, common responses were found across each surface assessed.

To test how generalisable the discovered linear relationship between C_{dl} and I_{p1} was, the relationship was investigated and related across GCE, Pi-GCE and fMWCNT-GCE (Figure 5.13). Although common electrode traits were retained by each of the surfaces being carbonaceous electrodes (similarities in chemical functional groups), differences in surface condition are marked. These include that although having a variable surface area due to polishing, GCE is considered a solid electrode (Kissinger & Heineman, 1996) whereas Pi-GCE (graphitic oxide, Chapter 4, Kepley & Bard, 1988) and fMWCNT-GCE are effectively porous electrodes (Dumitrescu *et al.* 2007; Henstridge *et al.* 2010) which greatly alters surface area considerations between electrodes. Differences between Pi-GCE and fMWCNT-GCE further include that although both are porous, fMWCNT will be conductive and have a lower C:O ratio than graphitic oxide (Kepley & Bard, 1988, Wang, 2005). The extent of variability was illustrated through the images of polished and oxidised

GCE in Chapter 4 and fMWCNT deposits in Appendix B, as well as in Chapter 3, Figure 3.10, which illustrated variability in distribution of fMWCNT on GCE prepared by physical adsorption. The result of this variability is highlighted through the spread of C_{dl} responses which arose at each surface and were pronounced at fMWCNT (Figure 5.13).

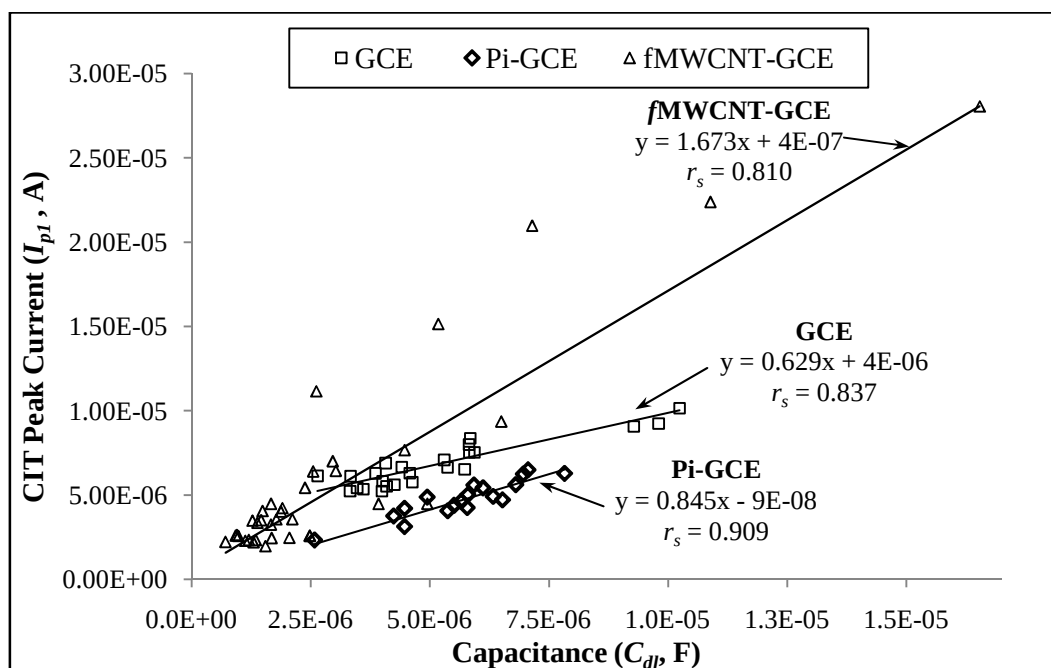


Figure 5.13: Scatter plot of C_{dl} and I_{p1} on GCE (square) ($n = 24$), Pi-GCE (diamond) ($n = 18$) and fMWCNT-GCE (triangle) ($n = 36$). All analyses were carried out in 0.2 M NaOAc, pH 4.0, 20 μ M CIT, C_{dl} was determined in NaOAc only (no CIT in solution).

As is evident in Figure 5.13, there is very high variability in both C_{dl} and I_p at each surface. Strong correlation was however found between C_{dl} to I_p across each of the surfaces assessed with r_s values of 0.837, 0.909 and 0.810 for GCE, Pi-GCE and fMWCNT-GCE respectively (Figure 5.13). The line representing the correlation of fMWCNT-GCE appears to neglect the weighting of the higher values. It should be noted however that these values were not excluded from the analysis and the apparent low trend was due to the atypical linear regressional model used herein, wherein weighting was over the entire population, at the cost of more obvious linearity. Considering the C.V. of I_{p1} for each of the surfaces (20.16 %, 22.87 % and 101.92 %), the high r_s values suggest a marked relationship between C_{dl} and I_p across distinct surface conditions. As conserved traits were identified at each surface, the relationship between C_{dl} and I_p is specific to each surface as demonstrated by differences in ‘m’ and ‘c’ values of the trends. As the trends are robust across all surface condition however, it suggests that although the relationship between C_{dl} and I_p may be surface specific, the fact that the different surfaces alter the relationship indicates that both are sensitive to the specifics of a given surface and thus are representative of that surface. This indicated that C_{dl} may be applicable to tracking variability across multiple electrodes of a single preparation, as well as being applicable across varied surfaces.

Pi-GCE in particular produced more consistent C_{dl} responses as they were more tightly grouped. These responses along with the comparatively lower I_{p1} in relation to polished GCE may have arisen as a result of delamination of the graphitic oxide as reported in Chapter 4 (Section 4.3.2 [b]), which would have limited the extent of electrochemical interactions but in so doing would have resulted in the observed responses.

Each of the surfaces assessed (Figure 5.13) provided distinct traits of C_{dl} to I_{p1} reminiscent of the variety of factors which interplay to result in the results of both C_{dl} and I_{p1} in each case. It is important to note that the relationship of C_{dl} to I_{p1} as indicated the closeness of the gradient to 1 should not necessarily be proportional because that would require I_p to relate directly to C_{dl} . That scenario is unlikely as factors including electroactivity in relation to molecule size and orientation at the electrode surface have no relation to C_{dl} of that electrode.

5.4.4 Influence of analyte adsorption and mass transport characteristics on C_{dl} and I_p using both CIT and 1,2,4-benzenetriol (Triol) as a model systems

Considering the influence of surface condition on the relationship of C_{dl} to I_{p1} (Figure 5.13), it was evident that the specific interactions with the characteristics of an electrode surface will strongly influence the magnitude of both C_{dl} and I_{p1} . It was thus considered prudent to investigate parameters which may affect this interaction. Due to the number of parameters which interplay in the relationship between C_{dl} and I_{p1} , it can be considered a dynamic system with many parameters. A complete study of all of these parameters would be extensive and does not lend benefit within the confines of this thesis. Accordingly only the influences of adsorption of analyte on the C_{dl} response and of mass transport on I_{p1} response were considered as part of this study as they covered a number of the parameters and sufficed to identify major parameters which needed to be considered. These studies were carried out by using CIT as an analyte in considering the influence on adsorption of analyte on recorded C_{dl} and through applying Triol to assess the influence of mass transport on I_{p1} as Hance & Kuwana (1987) described its mass transport characteristics to be controlled by solution concentration in aqueous media, being adsorptive at 0.05 mM and diffusive at 0.08 mM. The electrochemical profile of Triol was reported in Appendix C.

Adsorption of CIT was illustrated through representative EIS analyses. The presence of CIT in solution results in a shift in EIS spectra (Figure 5.14). As the presence of CIT in solution was the only factor which differed between the analyses therein, it is submitted that the deviation of the EIS spectrum was as a result of influence of CIT at the electrode surface. Data was modelled according to a modified Randles equivalent circuit (Scheme 5.2 [B]), the mean responses of which were tabulated

for each electrode in buffer and CIT solutions respectively through Table 5.3. Therein, the relatively small difference in solution resistance (R_s) between the two solutions ($594 \Omega \pm 74 \Omega$ and $567 \Omega \pm 51 \Omega$ for CIT and Buffer solutions respectively) suggest that the presence of CIT in solution had little influence on solution resistance, however when considering the difference between mean polarisation resistance (R_p) and the CPE coefficient (Q_{dl}) marked deviation was evident relative to their standard deviations. Özcan *et al.* (2008) reported on similar results for the adsorption of methionine. As only the circuit elements which considered interaction with the electrode surface and the double layer in particular showed marked deviation, it was inferred that the CIT interacts at the electrode similarly to methionine, and is adsorptive (as was discussed in detail at EOx-GCE in Chapter 6, Section 6.4.2).

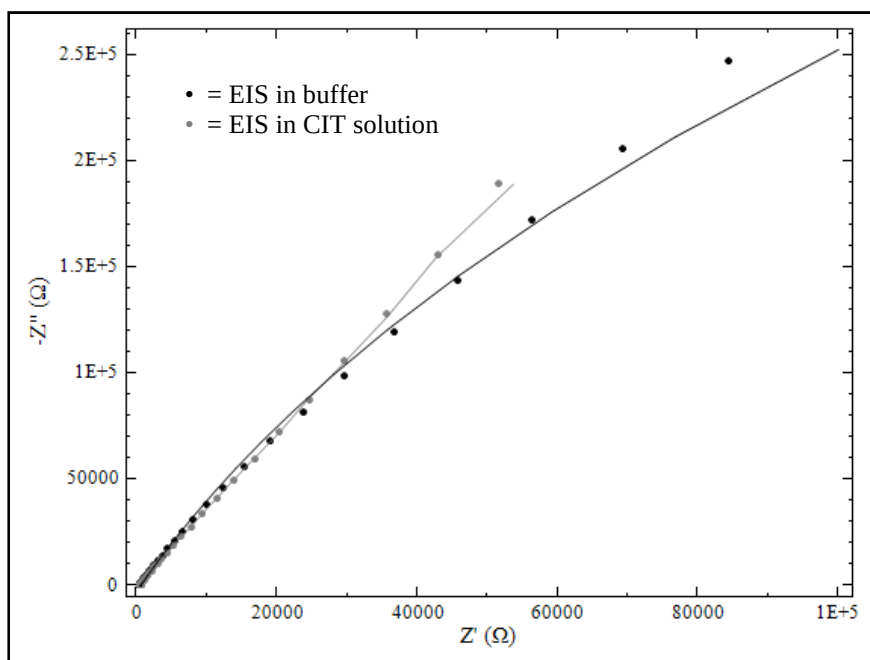


Figure 5.14: Representative Nyquist plot comparison of EIS in buffer (black markers) and CIT (grey markers) solutions on Pi-GCE, each with modelled data from the equivalent circuit (Scheme 5.2 [B]).

Table 5.3: Summarised EIS data determined in buffer and in CIT solution

	Mean R_s (Ω)	Mean R_p (Ω)	Mean Q_{dl} (F)
CIT Solution	594 ± 74 (C.V. = 12.46 %)	$1.72 \times 10^6 \pm 5.76 \times 10^5$ (C.V. 33.50 %)	$5.76 \times 10^{-6} \pm 1.29 \times 10^{-6}$ (C.V. = 22.37 %)
Buffer	567 ± 51 (C.V. = 8.94 %)	$2.60 \times 10^6 \pm 2.22 \times 10^6$ (C.V. = 85.30 %)	$6.82 \times 10^{-6} \pm 1.55 \times 10^{-6}$ (C.V. = 22.70 %)

Reported mean values were determined from all Pi-GCE determined from equivalent circuit of Scheme 5.2[B]

Further indication of the influence of CIT on C_{dl} was assessed through multiple electrode analyses as described by relating C_{dl} from each electrode in both buffer and CIT solution to I_p . This was carried out using both LSV (C_{dl}) and EIS (Q_{dl}) derived capacitance through Figure 5.15.

In both LSV [A] and EIS [B] responses of Figure 5.15, the capacitance responses on each electrode were decreased in the presence of CIT, compared to responses recorded in buffer only. As there are many avenues through which the presence of CIT in solution could have influenced capacitance, it would be speculative to assign a mechanism underpinning this result. It can, however, be said that the presence of CIT influenced the recorded charge at the electrode, thus, CIT may result in the neutralisation of charge through binding to surface groups or through a process such as coating of the electrode. In both cases, the retardation of charge build up or retention due to the adsorption of CIT is an underlying feature.

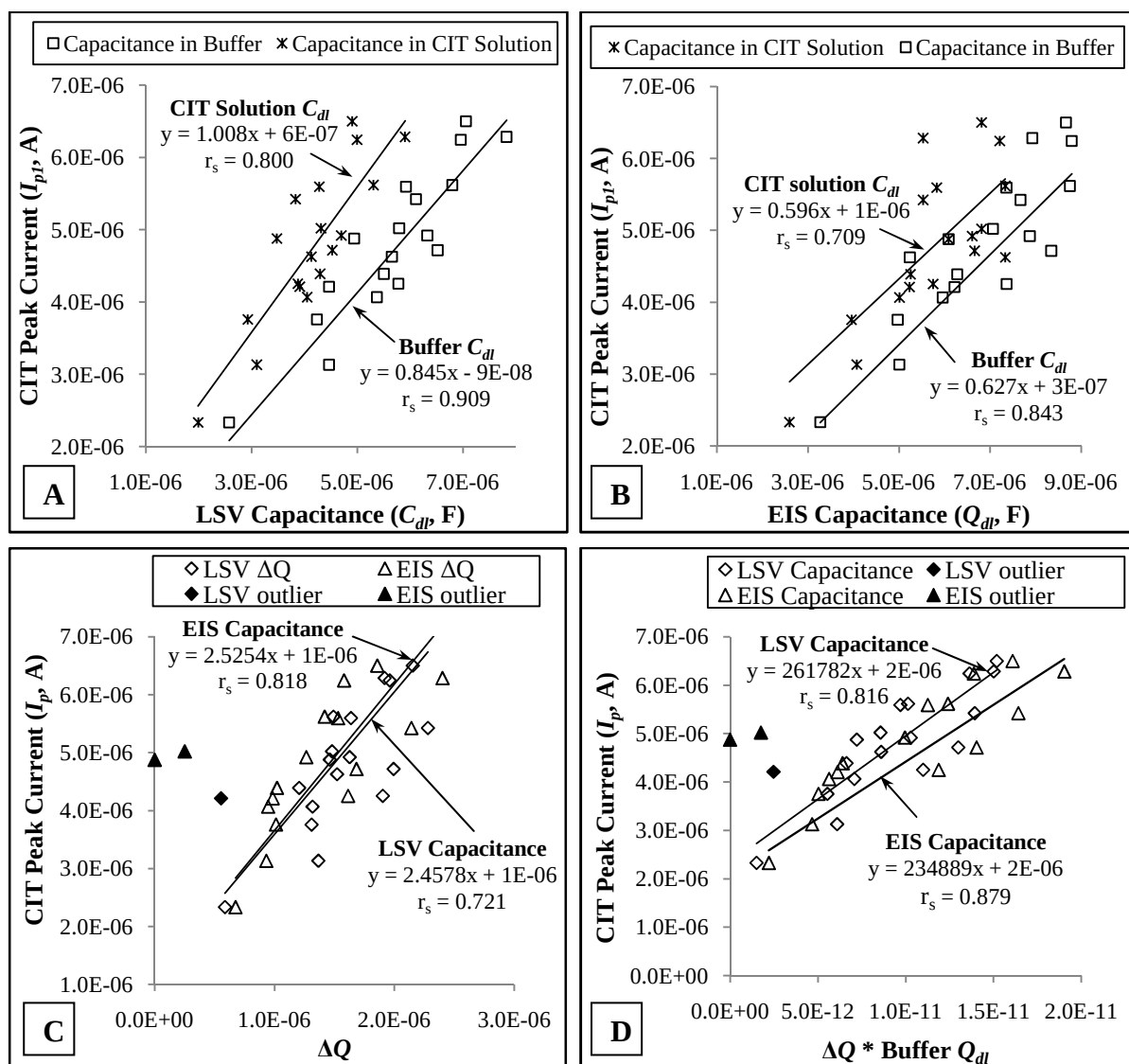


Figure 5.15: Comparison of I_{p1} to the C_{dl} determined in buffer solution (no CIT in solution) and in a 20 μM CIT solution. C_{dl} was determined from LSV [A] and Q_{dl} was determined by EIS [B]. Further relation of I_{p1} was to ΔQ (buffer Q_{dl} – CIT solution Q_{dl}) [C] and ΔQ multiplied by Q_{dl} [D]. Responses were recorded across three separate Pi-GCE, each scanned six times and re-surfaced after each scan ($n = 18$).

Although adsorption is discussed further through Chapter 6, further evidence of adsorption of CIT at graphitic oxide is found through Figure 5.15 [B] and [D]. In particular Figure 5.15 [B] suggests that CIT adsorbs as a result of the change in charge (ΔQ) between CIT solution determined C_{dl} and buffer determined C_{dl} for LSV analyses, and Q_{dl} for EIS studies alike (referred to as Q_{buffer} for both LSV and EIS analyses for simplicity of explanation in this explanation). As CIT was the only factor which altered, the ΔQ ($Q_{buffer} - Q_{CIT\ solution}$) suggests that CIT is adsorptive and further that $\Delta Q \propto \Gamma_{CIT}$ (amount of CIT adsorbed at the electrode), as inferred by Hance & Kuwana (1987) in similar studies. There are several implications of this finding as suggested by (Hance & Kuwana, 1987), including firstly that Q_{buffer} is representative of surface area of the GCE. Secondly, this provides further evidence that CIT is adsorptive. Thirdly it suggests that as $\Delta Q \propto \Gamma_{CIT}$, ΔQ is also proportional to I_p .

Figure 5.15 [D] represents $\Delta Q \times Q_{buffer}$, which, according to the postulated findings above is an equivalent representation of $\Gamma_{CIT} \times$ surface area. When plotted against I_p , r_s values increased which reinforce the assertion that Q_{buffer} reports on electrode surface area. These responses were described by Bard & Faulkner (2001) according to the following equation for an irreversible reaction of adsorbed O at an electrode:

$$I_p = \frac{\alpha F^2 A v \Gamma_0}{2.718 RT} \quad \text{Eq. 5.14}$$

Wherein $A = Q_{buffer}$ and $\Gamma_0 = \Delta Q$ in Figure 5.15 [D].

From these results, it can thus be stated that CIT adsorbs at Pi-GCE and negatively affects recorded C_{dl} . Due to that influence, only C_{dl} recorded in buffer was reported on henceforth such that an accurate representation of the double layer and thus surface condition could be recorded.

Although the interaction of analyte was described to influence C_{dl} through adsorption of CIT, the extent of interaction of analyte with an electrode surface condition forms a further key underlying feature with regard to mass transport and whether the stability of I_p responses may be influenced by adsorptive or diffusive based I_p responses. Triol has been shown to exhibit either diffusive or adsorptive mass transport kinetics as a factor of concentration in aqueous media, where at 0.80 mM it is primarily diffusion controlled and at 0.05 mM it is primarily adsorption controlled (Hance & Kuwana, 1987).

Figure 5.16 illustrates the graphed relation of Triol I_p under adsorptive and diffusive controlled processes in relation to C_{dl} . Therein, the magnitude and stability of I_p were both affected by concentration and thus by mass transport. Under adsorptive control (0.05 mM) Triol, I_p responses

were unstable, with a mean response of $3.44 \mu\text{A} \pm 0.48 \mu\text{A}$ (C.V. of 16.14 %). These responses suggested similar traits to those of CIT, where Triol I_p alters in relation to electrode C_{dl} ($r_s = 0.853$). This result is significant in itself as it indicates that the correlation of I_p and C_{dl} is not confined to CIT and may be broadly applicable. Under diffusive control (0.8 mM) however responses were dissimilar. Mean I_p were comparatively large and stable at $28.79 \mu\text{A} \pm 1.77 \mu\text{A}$ (C.V. of 6.14 %), along with a profound lack of linearity between I_p and C_{dl} ($r_s = 0.483$).

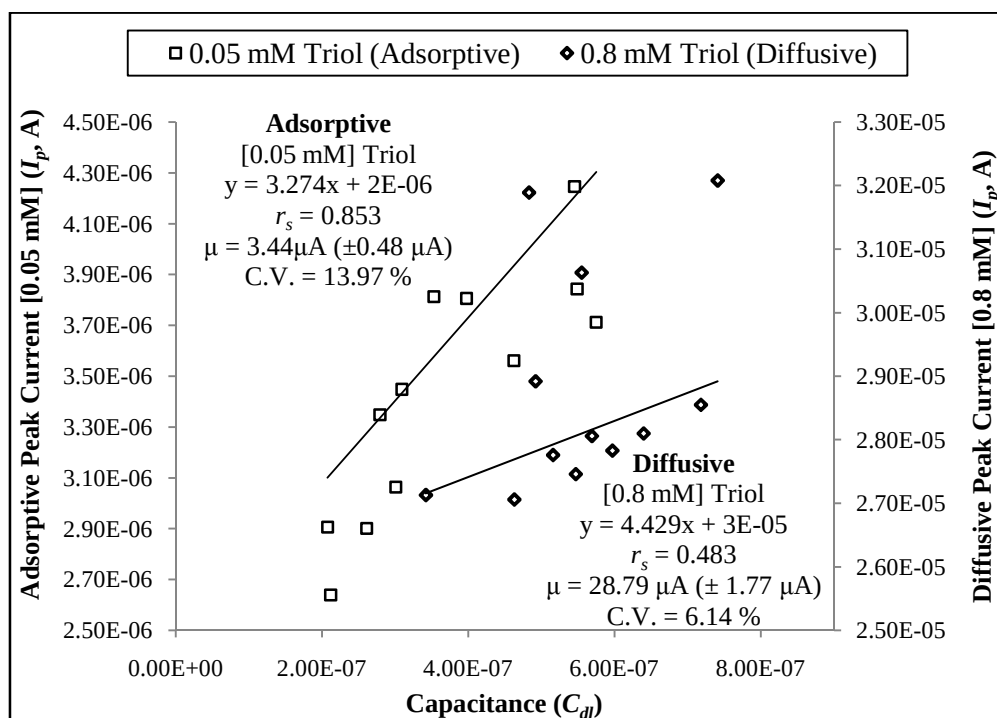


Figure 5.16: Scatter plot comparing Triol I_{p1} to electrode C_{dl} under adsorptive controlled [0.05 mM] (square, primary y-axis) and diffusive [0.8 mM] (diamond, secondary y-axis) mass transport kinetics. All analyses were carried out at Pi-GCE in 0.2 M NaOAc buffer, pH 4.0. $n = 12$ per concentration, μ = mean, C.V. = coefficient of variation (Eq. 2.3).

These results illustrate that under adsorptive control, anodic I_p of Triol tracks relative to C_{dl} , as was the case with CIT, but does not under diffusive control. This is as a result of I_p contribution from diffusive current being a factor of the cross-sectional diffusive area of an electrode, which is representative of geometric surface area, rather than real surface area (Trasatti & Petrii, 1991), and thus cannot be accurately accounted for by C_{dl} . Under adsorptive mass transport, however, the molecules associate directly with the electrode surface and can be predicted by individual C_{dl} for each electrode. This result suggests that this method may only be applied to adsorptive molecules.

5.4.5 Application of C_{dl} toward the normalisation of CIT I_{p1}

Normalising of I_{p1} with C_{dl} was assessed across GCE, Pi-GCE and fMWCNT-GCE and reported on according to three data treatment methods (Table 5.4). Method [A] made sole use of direct peak

height analysis from LSV scans. Methods [B] and [C] involved making I_p a factor of surface condition using C_{dl} . In method [B] this was performed by dividing I_p by the C_{dl} of each electrode. This was performed as a proof-of-concept data treatment in order to display a general decrease in relative variability when applying C_{dl} to I_p measurements. As a more accurate method of normalising current response, method [C] was ultimately employed, wherein the slope from Kendall-Theil regression analysis of I_{p1} and C_{dl} was applied. The resulting straight line equation was used to calculate modelled I_p ($I_{p \text{ mod}}$) for each measured C_{dl} . The mean (μ) of each method was determined directly from I_p [A], I_p / C_{dl} [B] and $I_{p \text{ mod}}$ [C]. C.V. was calculated according to Eq. 2.3 for [A] and [B] and Eq. 5.11 and Eq. 5.12 for [C].

Table 5.4: Normalisation of I_{p1} by application of C_{dl} at GCE, Pi-GCE and fMWCNT-GCE

Surface:	GCE			Pi-GCE			fMWCNT-GCE		
Method:	[A]	[B]	[C]	[A]	[B]	[C]	[A]	[B]	[C]
μ	6.77 μA	14.03 A/F	6.82 A/F	4.78 μA	0.84 A/F	4.71 A/F	6.05 μA	21.10 A/F	5.45 A/F
σ	1.36	2.94	0.49	1.09	0.08	0.40	6.16	7.10	1.55
C.V. (%)	20.16	20.93	7.18	22.87	9.99	8.47	101.92	33.63	25.79

μ = mean, σ = standard deviation from the mean, C.V. = coefficient of variation

Method [A]: Direct analysis of I_{p1} from LSV scans, μ and σ calculated from measured I_{p1} , C.V. calculated according to Eq. 2.3

Method [B]: Calculated as I_{p1} / C_{dl} of each electrode, μ and σ of resulting I_{p1} / C_{dl} , C.V. calculated according to Eq. 2.3

Method [C]: Calculated $I_{p \text{ mod}}$ using C_{dl} of each electrode from Kendall-Theil regression slope of I_{p1} and C_{dl} , μ = mean of $I_{p \text{ mod}}$, σ = averaged difference between I_p and $I_{p \text{ mod}}$ of each electrode, C.V. calculated according to Eq. 5.11 and 5.12

Application of these three data treatments allowed for the sequential reduction of C.V. to approximately a third of the starting value from method [A] to [C] for each surface assessed. This included reduction of C.V. from 20.16 % to 7.18 % for GCE, 22.87 % to 8.47 % for Pi-GCE and 101.92 % to 25.79 % for fMWCNT-GCE.

5.4.6 Towards practical application of the relationship of C_{dl} to I_p in determination of solution concentration

Normalising of CIT I_p response (method [C]) was assessed for its application within a simple method to determine analyte solution concentration from I_p . Kendall-Theil regression analyses allowed for the trend of the data sets reported as $I_{p \text{ mod}}$ to be assessed. These responses were plotted for each concentration assessed as is depicted in Figure 5.17, with the regression trend for each concentration plotted in the same colour as the I_p and C_{dl} responses for each concentration. Included therein was a representation of the maximum and minimum slopes from that regression slope. Those slopes were calculated by substituting the maximum and minimum slopes from Kendall-theil analysis into the equation for each line, which were determined as the 95 % confidence interval from the slope. In so doing the spread of data was highlighted. As is evident however, responses in 20 μM CIT as well as some of those observed in 10 μM CIT fall within the 95 % confidence interval range of CIT solution

responses at 30 μM . The implication of this result is that due to I_{p1} responses of discrete solution concentration overlapping, solution concentrations cannot be identified directly from those I_{p1} responses as they cannot be assigned to a given solution concentration with confidence.

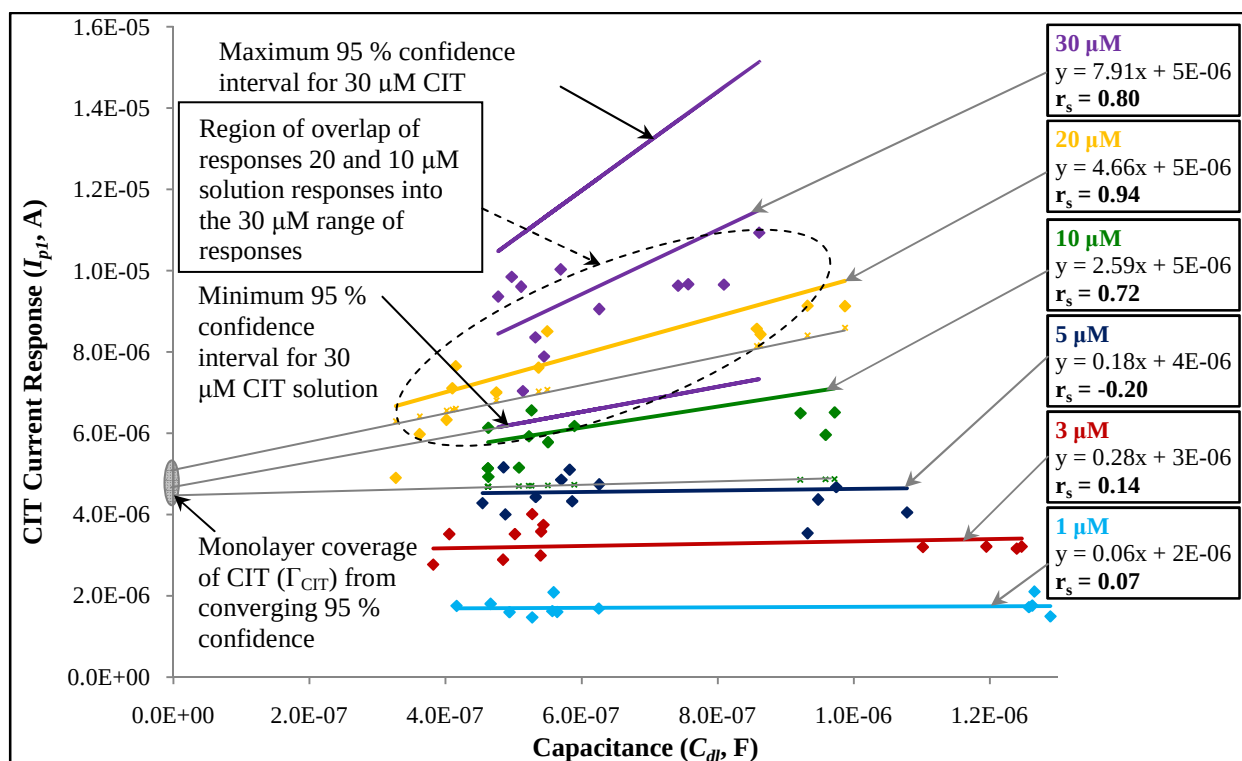


Figure 5.17: Scatter plot of C_{dl} to CIT I_p along with Kendall-Theil regression trends at 1 μM (light blue), 3 μM (red), 5 μM (dark blue), 10 μM (green), 20 μM (yellow) and 30 μM (purple). A 30 second adsorption period was used rather than 120 seconds in order to accentuate the influence of solution concentration, $N = 12$ per set.

Through Figure 5.17 it became evident that the Kendall-Theil regression slopes ($I_{p \text{ mod}}$) determined from relating of I_p to C_{dl} , follow distinct gradients according to concentration. The r_s values of each concentration of Figure 5.17 describe the validity of the slope from each concentration as they represent the correlation between I_p and C_{dl} at that concentration. Strong correlations were found at 10 μM or higher ($r_s = 0.72$ at 10 μM , $r_s = 0.94$ at 20 μM and $r_s = 0.80$ at 30 μM), whilst a lack of correlation was found below 10 μM ($r_s = 0.07$ at 1 μM , $r_s = 0.14$ at 3 μM and $r_s = 0. -0.20$ at 5 μM).

Investigation of the application of regression slope ($I_{p \text{ mod}}$) of the data sets was assessed through Figure 5.18. Linear regression of the concentration range of 10 μM to 30 μM illustrated strong linearity with an R^2 of 0.984, which thus allowed for solution concentration to be determined by this method *within this range*. The lack of correlation at low concentration could arise as a result of the formation of sub-monolayer(s) of analyte, resulting in I_p being less dependent on surface area and thus not represented by C_{dl} of that surface. The implication therein is that although the method allows for the determination of concentration from initially variable I_p , those responses must be formed as a result of near or greater than monolayer coverage of the electrode surface such that the entire surface of the electrode,

and thus the total measure of C_{dl} would be relative to the recorded I_p . Accordingly, Figure 5.19 provides a closely comparable response, possessing, a two part concentration curve becomes evident with linearity between 1 μ M and 5 μ M (black markers) as well as linearity between 10 μ M and 30 μ M (grey markers), each with strong correlation and linearity with $R^2 = 0.991$ and $R^2 = 0.999$ respectively. Although the linearity of response was strong therein however, the overlap of the standard deviations across relatively widely dispersed concentrations illustrates the requirement for the methodology described above.

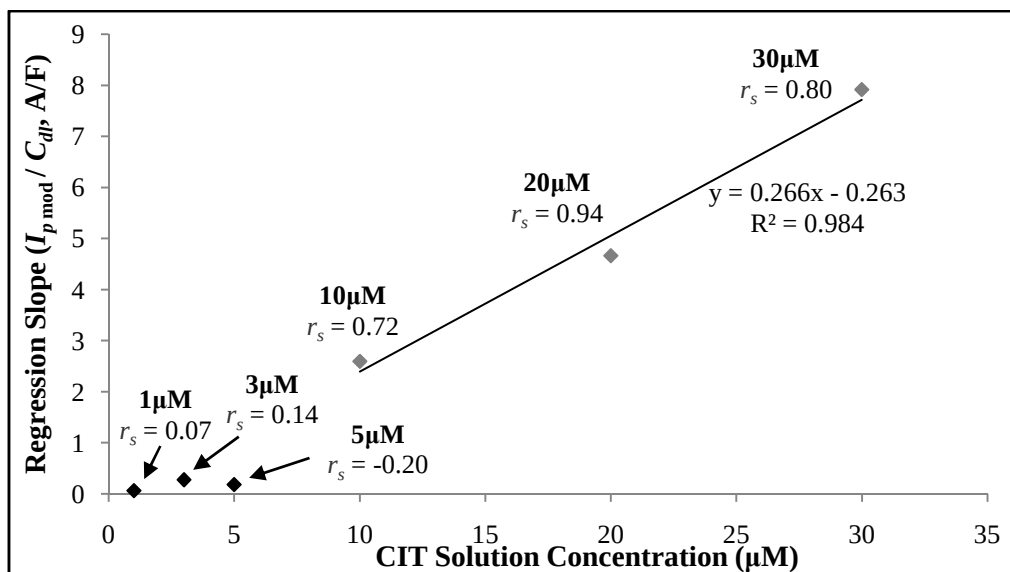


Figure 5.18: CIT $I_{p \text{ mod}}$ regression slope as compared to CIT solutions concentrations of 1, 3 and 5 μ M (black markers) and 10, 20 and 30 μ M (grey markers) in 0.2 M NaOAc buffer, N = 12 per set. A 30 second adsorption period was used rather than 120 seconds in order to accentuate the influence of solution concentration.

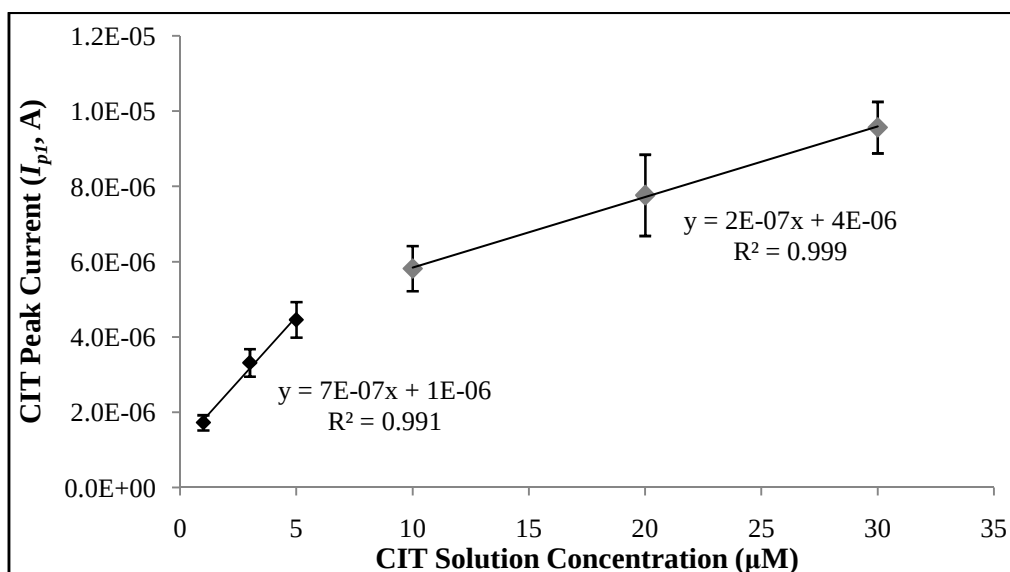


Figure 5.19: CIT I_{p1} responses as compared to corresponding CIT solutions of 1, 3 and 5 μ M (black markers) and 10, 20 and 30 μ M (grey markers) in, N = 12 per set. A 30 second adsorption period was used rather than 120 seconds in order to accentuate the influence of solution concentration.

5.4.7 Application of C_{dl} as a mediator of 2,4-dimethylaniline (2,4-DMA) as a structurally and chemically distinct molecule to CIT

Although the application of C_{dl} as a system to account for I_p variability was demonstrated in relation to CIT, in order to probe the applicability of this methodology for adsorptive analytes further this was conducted through assessment of a structurally and chemically distinct aromatic environmental toxin which relies on the oxidation of an amine rather than hydroxyl as is the case in CIT oxidation (discussed through Chapter 6) in the form of 2,4-dimethylaniline (2,4-DMA). The electrochemical profile of 2,4-DMA in aqueous media is reported in Appendix C and furthermore, its characteristics have been well described, having been shown to be adsorptive, allowing for it to act as a reference molecule (Brimecombe, 2006; Brimecombe *et al.* 2006; Brimecombe & Limson, 2007). In so doing this allowed for the investigation of the influence of surface chemistry on a distinctly different, but adsorptive molecule from CIT, the results of which are summarised in Table 5.5 across GCE, Pi-GCE and fMWCNT-GCE.

Table 5.5: Application of C_{dl} to the normalisation of 2,4-DMA I_p at GCE, Pi-GCE and fMWCNT-GCE.

Surface:	GCE			Pi-GCE			fMWCNT-GCE		
Method:	[A]	[B]	[C]	[A]	[B]	[C]	[A]	[B]	[C]
μ	0.82 μ A	0.09 A/F	0.92 A/F	1.36 μ A	0.12 A/F	1.34 A/F	0.75 μ A	0.13 A/F	0.63 A/F
σ	0.22	0.02	0.12	0.11	0.01	0.06	0.25	0.08	0.17
C.V. (%)	27.01	17.40	16.39	8.37	7.64	4.67	34.00	63.99	19.46

μ = mean, σ = standard deviation from the mean, C.V. = coefficient of variation

Method [A]: Direct analysis of I_{p1} from LSV scans, μ and σ calculated from measured I_{p1} , C.V. calculated according to Eq. 2.3

Method [B]: Calculated as I_{p1} / C_{dl} of each electrode, μ and σ of resulting I_{p1} / C_{dl} , C.V. calculated according to Eq. 2.3

Method [C]: Calculated $I_{p \text{ mod}}$ using C_{dl} of each electrode from Kendall-Theil regression slope of I_{p1} and C_{dl} , μ = mean of $I_{p \text{ mod}}$, σ = averaged difference between I_p and $I_{p \text{ mod}}$ of each electrode, C.V. calculated according to Eq. 5.11 and 5.12

As in CIT analyses, C.V. were improved with application of the C_{dl} for 2,4-DMA to approximately half the starting C.V. rather than a third of the initial C.V. As a further validation of the application of Method [C], an increase in C.V. was seen from method [A] to method [B] of fMWCNT-GCE, however with application of Method [C], the C.V. was corrected substantially, reinforcing its application. Although application of C_{dl} was not quite as effective as in CIT analyses, these results do further probe the breadth of application of using C_{dl} as a normaliser of variability in I_p response. This result, along with those of CIT illustrates that C_{dl} can be used to predict I_p of highly variable and diverse carbonaceous electrode conditions for two distinctly different toxins, at distinct surface conditions.

5.5 Summary and conclusions

Anodic electrochemical detection of adsorptive analytes including CIT, 2,4-DMA and Triol at GCEs with heterogeneous surface conditions (variable surface morphology and/or chemistry assessed herein at polished GCE, Pi-GCE and fMWCNT-GCE) result in highly variable, and thus analytically inaccurate, I_p responses across multiple electrode preparations (re-surfacing of an electrode). As C_{dl} and I_p are similarly influenced by electrode surface properties and processes, it is plausible that I_p recorded at an electrode could be normalised by the C_{dl} of that electrode as a representative measure of that electrode's surface condition. Probing that hypothesis through comparing the I_p of CIT, Triol and 2,4-DMA to the C_{dl} of an electrode showed strong linear correlation across each of the surfaces assessed. That correlation described conserved trends of responses within which responses across multiple preparations were found. These results suggested that variations in measured C_{dl} are representative of the processes that give rise to the variation of I_p . As the correlation of I_p to C_{dl} was maintained over several surface modifications, multiple preparations of those surfaces and across multiple electrodes, the link between I_p and C_{dl} is considered broadly applicable. The correlation was, as anticipated, significantly reduced under either diffusive mass transport kinetics and in response to sub-monolayer surface coverage of analyte, due to the independence of these mass-transport states to electrode surface area, and hence, C_{dl} . Furthermore, as C_{dl} acts as a representative measure of the surface condition, adsorbed analyte reduced recorded C_{dl} , requiring that C_{dl} be recorded in the absence of analyte in order to attain a representative measure of the surface condition. Measurement of C_{dl} from LSV as an *in situ* procedure was cross validated using EIS and τ methods and illustrated similar results with regard to both magnitude and stability of response, providing a comparatively rapid and simple means of assessing C_{dl} . By normalising I_p as a factor of C_{dl} , conformity in I_p responses can be identified, allowing for variability in I_p to be reduced to approximately a third of starting C.V. in CIT and analysis and half of the initial C.V. in 2,4-DMA analysis. As a scoping study into the applicability of using C_{dl} to mediate I_p as a general method, correlation and subsequent stabilisation of I_p responses of 2,4-DMA as a chemically and structurally diverse analyte molecule suggested relatively broad applicability. Application of regression slope from such analyses may allow for practical application in measuring current response as a factor of bulk solution concentration, as discrete solution concentrations can be identified from seemingly variable I_p responses, warranting further interrogation of these findings.

CHAPTER 6

Voltammetric analysis and characterisation of citrinin at electrochemically oxidised glassy carbon electrodes

6.1 Introduction

Chapters 3, 4 and 5 reported that analytical reproducibility of CIT I_{p1} responses at several carbonaceous surfaces was problematic as a result of variability in the properties of the electrode surface condition, restricting both accurate analysis of the analyte and also limiting the confidence of any characterisation of the electrochemical behaviour thereof at the surfaces investigated. Chapter 5 proposed a novel means of normalising variability as a post data acquisition methodology. Although a method with promise, it had limited scope for elucidation of CIT mechanistic behaviour. Modification of GCE using electro-oxidation provided consistent electrochemical responses for CIT oxidation (Chapter 4, Section 4.3.4), a result which was proposed to be as a result of the accumulation of CIT in the graphitic oxide layer and oxidation according to a mechanism proposed in Section 4.3.4 [f]. The stability afforded by electro-oxidised GCE (EOx-GCE) allows for direct electroanalysis of CIT with responses of suitable stability to allow for the elucidation and interrogation of a method by which CIT can be directly and accurately assayed in aqueous media.

While Chapter 1, Section 1.1.2 provided a review of literature available on CIT, these studies focused largely on identifying the structure of CIT and the associated compounds, chromatographic analysis and investigations of CIT toxicity. Studies concerned with the characterisation of CIT were however limited. Chromatographic analyses of CIT in organic solvents have shown that CIT is sensitive to pH and the solvents used (Vail & Homann, 1990; Wilkes & Sutherland, 1998; Xu *et al.* 2006). Thermal decomposition of CIT has been reported at 175 °C under dry heating (Kitabatake *et al.* 1991) as well as the formation of a more toxic compound, citrinin H1, formed under heating at temperatures in excess of 100 °C in water, and degradation into several further toxic compounds in excess of 140 °C (Trivedi *et al.* 1993). Beyond the traits listed above, little research has been conducted regarding the stability of CIT and reports pertaining to its analysis or its characterisation are very limited, with no directed studies considering these aspects in aqueous media having been considered. In order to better understand the molecule and tailor analytical systems towards its analysis, further characterisation of CIT in aqueous media is required. Due to this, there is a relatively broad knowledge gap which must

be addressed in order to better understand the CIT molecule such that it can be effectively analysed from environmental samples.

As electroanalytical studies have not previously been conducted on CIT, no information is available regarding the kinetics of CIT oxidation, a feature which will prove highly beneficial in the development of analytical systems for CIT in environmental samples. Accordingly, the central directive of this Chapter is the electrochemical characterisation of the traits and behaviour of CIT oxidation in aqueous media at EOx-GCE.

6.2 Chapter objectives

This Chapter was directed at the investigation of the characteristics of CIT electroanalysis in aqueous media, and assessing what procedural and environmental parameters influence those responses. Ascertaining this primary objective included several secondary objectives, described as follows:

- a) Examine whether EOx-GCE provide reproducible, sensitive and accurate responses for CIT oxidation in aqueous media at EOx-GCE. This includes determining whether responses acquired at EOx-GCE are representative of CIT oxidation at polished GCE and assessing the parameters under which CIT could be effectively and analysed.
- b) Identify and assess the accumulation and adsorption characteristics of CIT at EOx-GCE into the oxide layer and further to identify the extent of adsorption of CIT within the graphitic oxide layer.
- c) Examine the influence of repreted voltammetric analyses of CIT at EOx-GCE as well as to ascertain whether limitation of I_{pl} with successive scans can be mitigated through solution and scanning parameters.
- d) Determine whether the ionic composition of aqueous electrolytes influences CIT responses at EOx-GCE. This included assessment of pH and ionic composition at different concentrations of CIT.
- e) Determining characteristics parameters, limit of detection and information regarding the mass transport and oxidation kinetics of CIT:
 1. Ascertain what the influences of scan rate on CIT oxidation are and apply these studies in the determination of the characteristics of the CIT oxidation reaction mechanism.
 2. Identify a scheme of the mechanistic pathway of oxidation of CIT in aqueous media
 3. Subsequent objectives of this section include determining the source of electrons in oxidation through relation of peak profiles to analogues of CIT and the identification of the kinetics of that mechanism.

6.3 Chapter specific methods and materials

The procedures, reagents and equipment detailed in Chapter 2 were followed herein unless otherwise stated. Specific details on the experiments carried out in Chapter are illustrated hereafter with reference to their specific sections.

6.3.1 Analytes

Stock solutions of 5 mM CIT was prepared with dehydrated absolute methanol and stored at $-20\text{ }^{\circ}\text{C}$ until used. Stocks of 100 mM solutions of 2,6-dihydroxybenzoic acid (98 %), 5-methylsalicylic acid (98 %), salicylic acid, catechol (>99 %) and phenol (>99 %), were prepared in anhydrous ethanol prior to analysis.

6.3.2 Buffer Solutions

Each buffer was prepared and stored as described in Section 2.1.4 of Chapter 2. Buffers applied herein included Britton Robinson (BR) buffer and citrate-phosphate buffer, each prepared at pH 4.0 and at pH 8.0 as well as sodium acetate (NaOAc) buffer and succinic acid-lactic acid buffer, each prepared at pH 4.0.

6.3.3 Electrochemical procedures and parameters

A three electrode electrochemical cell was utilised glassy carbon (GCE) or carbon paste (CPE) working electrodes with a platinum auxiliary electrode and Ag/AgCl reference electrode in all studies according to the set up and parameters described through Chapter 2. Electrodes were prepared, cleaned and modified as was detailed through Section 2.3.1 of Chapter 2. Any deviations in electrochemical scanning procedures reported in Section 2.3.2 of Chapter 2 are detailed hereafter.

Electro-oxidation of GCE, and in some cases of CPE, (Chapter 2, Section 2.3.1 [b]) was applied at all electrodes unless otherwise stated and the final scan of electrochemical conditioning (Section 2.3.1 [c]) was recorded as the background signal.

Unless otherwise stated, voltammetric scans of CIT were carried out by LSV in a potential range of 0.5 V to 1.1 V after conditioning in buffer for 15 scans, scanned anodically at $0.1\text{ V}\cdot\text{s}^{-1}$ vs. Ag/AgCl. Standard CIT solution conditions included a solution concentration of $20\text{ }\mu\text{M}$ in 0.2 M NaOAc, pH 4.0. In-scan preconditioning included an adsorption period of 120 seconds, with the electrode held at a potential of 0.4 V (Chapter 3), for 120 seconds with solution agitation at 2000 rpm (Chapter 3).

a) Voltammetric analysis of CIT at EOx-GCE

Voltammetric analysis of CIT was carried out via CV under standard solution conditions in a scan range of -0.8 V to 1.1 V, scanned by both anodic (-0.8 V to 1.1 V) and cathodic (1.1 V to -0.8 V) initiated scan sweeps respectively under both cathodic and anodic initiated scans.

b) Adsorption of CIT at EOx-GCE

Optimisation of duration of adsorption for voltammetric analysis of CIT by LSV

LSV scans were carried out under standard voltammetric conditions. Adsorption of CIT at EOx-GCE was assessed by altering the duration of adsorption in solution stirred at 2000 rpm for time intervals of 0, 10, 20, 30, 60, 90, 120 and 150 seconds, respectively, prior to analysis with potential held at 0.4 V.

Characteristics of the adsorbed layer of CIT at polished GCE and EOx-GCE

The dimensions of the CIT molecule on arbitrarily designated X, Y and Z axes were determined in Å using Discover Studio Visualiser (2007). The theoretical amount of CIT adsorbed at the electrode (M) was determined according to the maxima that each face of the CIT molecule could perfectly cover the geometric surface area of GCE. The surface areas of CIT were calculated according to the formula:

$$A = L \times B \quad \text{Eq. 6.1}$$

Where A = surface area (cm^2), L is length and B is breadth. By converting the surface area of each face of a CIT molecule ($\text{\AA}^2$), Moles per cm^2 was calculated using Avogadro's constant (6.02×10^{23} molecules.mol $^{-1}$). This result was then related to the geometric surface area of a GCE according to the equation:

$$A = \pi r^2 \quad \text{Eq. 6.2}$$

Where r is the radius of a GCE (1.5 mm). Measured, electroactive, surface-confined CIT at both polished GCE and EOx-GCE (Γ_{CIT} , M.cm ^{2}) was calculated from LSV scans via Eq. 2.6 (Chapter 2). Q_{CIT} was calculated directly from LSV by integrating the area under the CIT peak using as a function of GPES software (Kissinger & Heineman, 1996). The number of layers of CIT adsorbed at the electrode was calculated according to each possible orientation of CIT by dividing measured Γ_{CIT} by the theoretical maxima calculated for monolayer coverage.

c) Influence of Continuous Voltammetric Cycling on the Analysis of CIT

Continuously cycled voltammetric analyses of CIT at EOx-GCE Responses were assessed through measurement of I_{p1} obtained from successive LSV scans without cleaning the electrode between scans (Wang & Hutchins, 1985; Berríos *et al.* 2009). Recovery of the electrode surface was assessed by including 10 minutes time intervals between successive scans with the electrode left standing in quiescent CIT solution to assess whether there was recovery of the primary peak.

Influence of pH on continuously cycled voltammetric analyses

The pH-dependent studies were carried out in 20 μM CIT in 0.2 M BR buffer at pH 1.0 through to pH 10.0. Standard LSV scanning conditions were used, with 5 successive scans per pH unit carried out in triplicate. The extent of suppression of I_{p1} was calculated according to Eq. 2.7.

Influence of the extent of electrochemical oxidation of GCE on I_{p1} responses in continuously cycled voltammetric analyses

LSV scans were carried out on GCE which had been oxidised according, with I_{p1} assessed for GCE which had been oxidised for 1, 5, 10, 15, 20, 25, 30, and 50 oxidation cycles respectively.

d) Influence of pH on the analysis of CIT at EOx-GCE

CIT Peak Profile Shift as a Function of pH

LSV analysis of 20 μM CIT was carried out in 0.2 M BR buffer between a range of pH 1.0 and pH 10.0. All results were determined in single solutions for each respective pH.

Stability of CIT as a Function of pH

Replicates of LSV analysis of 20 μM CIT were carried out at pHs 7, 8 and 9 in 0.2 M BR buffer. After allowing the solution to stand for 120 minutes, the CIT solution at pH 8.0 was shifted to pH 5.0 with acetic acid, and scanned again immediately to assess the influence of pH on CIT stability. The 15 conditioning scans were conducted at both pH 8 and pH 5 in respective sets to assess their influence.

Determination of CIT pKa

CIT pKa was calculated according to the ionisation of oxygen moieties identified in Figure 6.3 in aqueous solution according to Hilal *et al.*, (1995), using the software programme SPARC 4.2 (2009).

Peak profile of CIT at targeted pH range

LSV scans carried out between pH 1.0 and pH 7.0 in 0.2 M BR buffer. Furthermore, both 120 second and 0 second adsorption sets were carried out for analyses of 20 μM CIT in aqueous and 25 % methanol solutions.

e) Effect of supporting electrolyte and conditioning pH on CIT analysis

Assessment of supporting electrolyte as a function of ionic composition and conditioning pH

LSV analysis of 20 μM was CIT carried out in 0.2 M NaOAc buffer pH 4.0, 0.2 M SLB buffer pH 4.0, 0.2 M BR buffer at pH 4.0 and pH 8.0 and 0.2 M citrate-phosphate buffer at pH 4.0 and pH 8.0. All voltammetric analyses of CIT were carried out at pH 4.0 in the respective buffers. Analyses were conducted on both polished GCE and EOx-GCE.

f) Influence of CIT solution concentration on voltammetry at EOx-GCE

Standard concentration curve for CIT oxidation in aqueous solution

LSV analysis was carried out in 0.2 M NaOAc buffer, pH 4.0 with CIT concentrations altered by successive spikes of CIT stock (5 mM), resulting in solution concentrations of: 0.80 μM , 1.60 μM , 2.39 μM , 3.99 μM , 5.98 μM , 7.96 μM , 11.93 μM , 15.90 μM , 19.85 μM , 23.97 μM , 31.67 μM , 39.50 μM and 78.24 μM respectively. In each measurement, a scan rate of 150 mV.s^{-1} was employed.

Relation of solution concentration to adsorbed amount of CIT at EOx-GCE

The relationship between solution concentration and I_p was assessed by determining Γ_{CIT} at the electrode surface, as determined by area under the CIT peak according to Eq. 2.6.

Modelling adsorption of CIT as a factor of concentration

The Γ_{CIT} was further modelled according to the Langmuir isotherm (Bard & Faulkner, 2001) in relation to solution concentration. Modelling was performed using Microsoft® Office's "Solver", a reiterative minima-seeking algorithm for non-linear curve-fitting, as has been previously performed by (Lin *et al.* 2006) and application and the various adjustable parameters explained by Walsh & Diamond (1995). The responses herein were compared to the Γ_{CIT} using the Langmuir equation (Masel, 1996):

$$\Gamma = \Gamma_{\text{max}} \frac{Kc}{1 + Kc} \quad \text{Eq. 6.3}$$

Where K = Langmuir equilibrium constant, C = solution concentration (M), Γ = amount adsorbed (M.cm^{-2}) and Γ_{max} = maximum amount adsorbed (M.cm^{-2}) as C increases and $I_p = I_{\text{ads}} + I_{\text{diff}}$. I_{ads} (current response (A) derived from adsorptive mass transport) was reported herein as the response modelled by the Langmuir equation (Eq. 6.3) and I_{diff} (current response (A) derived from diffusive mass transport) is the response modelled according to the Randles-Sevcik equation (Eq. 6.4).

$$I_{p1} = (2.99 \times 10^5) n_t (\alpha \cdot n_a)^{1/2} A D^{1/2} C^0 \nu^{1/2} \quad \text{Eq. 6.4}$$

Where, at 298 K, (n_t) represents the total number of electrons transferred, (α) the transfer coefficient, (n_a) the number of electrons transferred during the rate limiting step, (A) the electrode surface area (cm^2), (D) the diffusion coefficient ($\text{cm}^2.\text{s}^{-1}$), (C°) the concentration of CIT ($\text{mol}.\text{cm}^{-3}$) and (ν) the scan rate ($\text{V}.\text{s}^{-1}$).

Determining the voltammetric limit of detection (LOD) of CIT at EOx-GCE

LOD was determined through measurement using linear sweep voltammetry (LSV) and application of Eq. 2.5.

g) Influence of scan rate on the electrochemical characteristics of CIT at EOx-GCE

Scan rate studies in aqueous solution as an adsorptive process

LSV analysis of 20 μM CIT was carried out in 0.2 M pH 4.0 NaOAc buffer at scan rates of 1, 10, 25, 50, 75, 100, 150, 200, 250 and 300 $\text{mV}.\text{s}^{-1}$ respectively, with preconditioning including allowing for adsorption to occur (120 seconds) for adsorptive responses.

Scan rate studies in aqueous solution as a diffusive process

Similarly, LSV analysis of 20 μM CIT was carried out in 0.2 M pH 4.0 NaOAc buffer at scan rates of 10, 25, 50, 75, 100, 150 and 200 $\text{mV}.\text{s}^{-1}$ respectively. Unlike the previous section, scans were carried out immediately as the EOx-GCE was immersed in CIT solution, such that any response would be a factor of diffusive response.

Assessment of the influence of scan rate on CIT mass transport and adsorption

Data determined under both adsorptive and diffusive processes above was assessed and plotted as indicated in text in Section 6.4.7.

h) Assessment of CIT oxidation reaction mechanism in aqueous media at EOx-GCE

LSV analysis of CIT was carried out in 0.2 M pH 4.0 NaOAc buffer at scan rates of 10, 25, 50, 75, 100, 150 and 200 $\text{mV}.\text{s}^{-1}$ respectively both *with* (adsorptive control) and *without* (diffusive control) pre-accumulation step applied.

Determination of the number of electrons transferred per molecule of CIT oxidised (n_t)

The number of electrons involved in the oxidation (n_t) of CIT was calculated via two separate methodologies including application of potential difference of the breadth of the peak at the half peak height ($\Delta E_{p1, 1/2}$) according to Eq. 6.5 (Bard & Faulkner, 2001; Scholz, 2002). Secondly, n_t was determined through relation of a plot of $\ln$ scan rate ($\ln \nu$, $\text{V}.\text{s}^{-1}$) and peak potential (E_{p1} , $\text{V}.\text{s}^{-1}$), according to Eq. 6.6 (Bard & Faulkner, 2001; Santos & Sequeria, 2010). Each of the analyses were

performed at scan rates of 10, 25, 50, 75, 100, 150 and 200 mV.s^{-1} respectively, as is detailed through text in Section 6.4.8 [a].

i) CIT oxidation mechanism

Correlation of CIT voltammetry with analogues

The source of electrons from the CIT molecule was assessed electrochemically through assessing the responses of structural analogues of CIT such that electrochemical responses of combinations of the structure of CIT could be assessed. These analyses were all conducted in solutions at 50 μM analyte concentration in 0.2 M NaOAc buffer pH 4.0, at a scan rate of 100 mV.s^{-1} from 0.2 V to 1.2 V at EOx-GCE with a 120 second adsorption period. Background currents were subtracted from scans in each case. Molecules assessed were: 2,6-dihydroxybenzoic Acid (2,6-DHBA), salicylic Acid (SCA), 5-methylsalicylic Acid (5-MSA), phenol (PHE) and catechol (CAT).

6.4 Results and discussion

6.4.1 Voltammetric analysis of CIT at electrochemically oxidised (EOx) GCE and CPE

a) Wide potential range scans of CIT on EOx-GCE

This section was conducted as a comparative analysis of CIT response at EOx-GCE (Figures 6.1 and 6.2 for anodically and cathodically initiated scan sets respectively) as compared to responses at polished GCE (Chapter 3, Section 3.4.1). In this study CIT was assessed at 20 μM as opposed to 50 μM in Chapter 3.

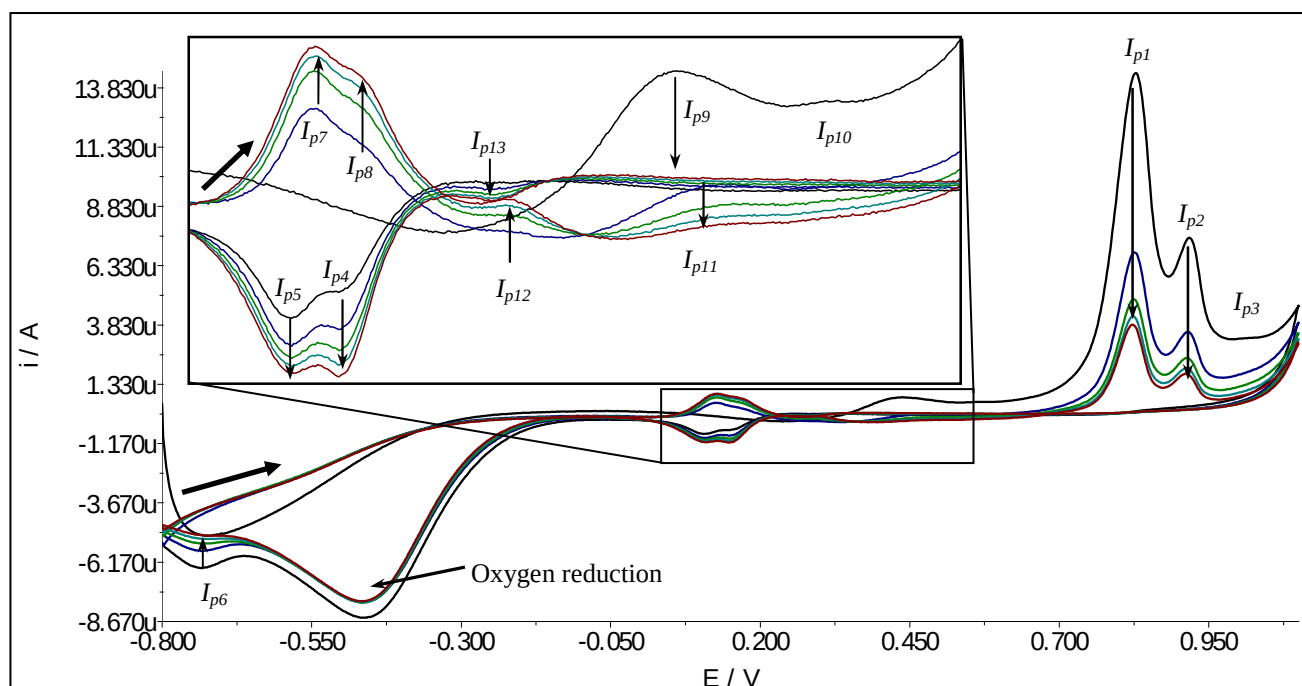


Figure 6.1: Successive anodic CV scans of 20 μM CIT in 0.2 M NaOAc buffer pH 4.0 at an EOx-GCE with background current subtracted. Scan 1 (black) scan 2 (blue) scan 3 (green) scan 4 (cyan) and scan 5 (red). The inset is a magnification of a section of the same scan for clarity. Solution was not de-oxygenated.

As is evident from Figures 6.1 and 6.2, there is an extensive set of faradaic peaks which arise through the oxidation of CIT at EOx-GCE. The nomenclature detailed through Chapter 3, Section 3.4.1 [c] for CIT responses at polished GCE was retained for comparison. I_{p1} to I_{p10} were conserved between the two surface preparations and furthermore, according to Torriero *et al.* (2004), the formation I_{p1} without a complementary reduction peak in the reductive scan is typical for a fast irreversible chemical reaction couple to the charge transfer. As detailed through Figures 6.1 and 6.2, several further peaks were evident at EOx-GCE in the initial scan, reported as I_{p11} to I_{p14} . Of the previously unrecorded peaks, I_{p12} and I_{p14} were evident in both Figures 6.1 and 6.2. These peaks were found at 0.307 V and 0.300 V respectively which both increased in magnitude with successive, continuously cycled scans. They were, as such, expected to be oxidation products of CIT appearing at EOx-GCE

rather than polished GCE. Retention of oxidation products at or within the graphitic oxide layer of the EOx-GCE may have allowed for more complex EC or ECE reactions to ensue to account for their presence. It is also likely that that they are oxidation products which were retained at the electrode and thus became more detectable. Such a process may account for the formation of I_{p11} (Figure 6.1) and I_{p14} (Figure 6.2), with the direction of the scan preferentially resulting in the formation of an O or R species respectively.

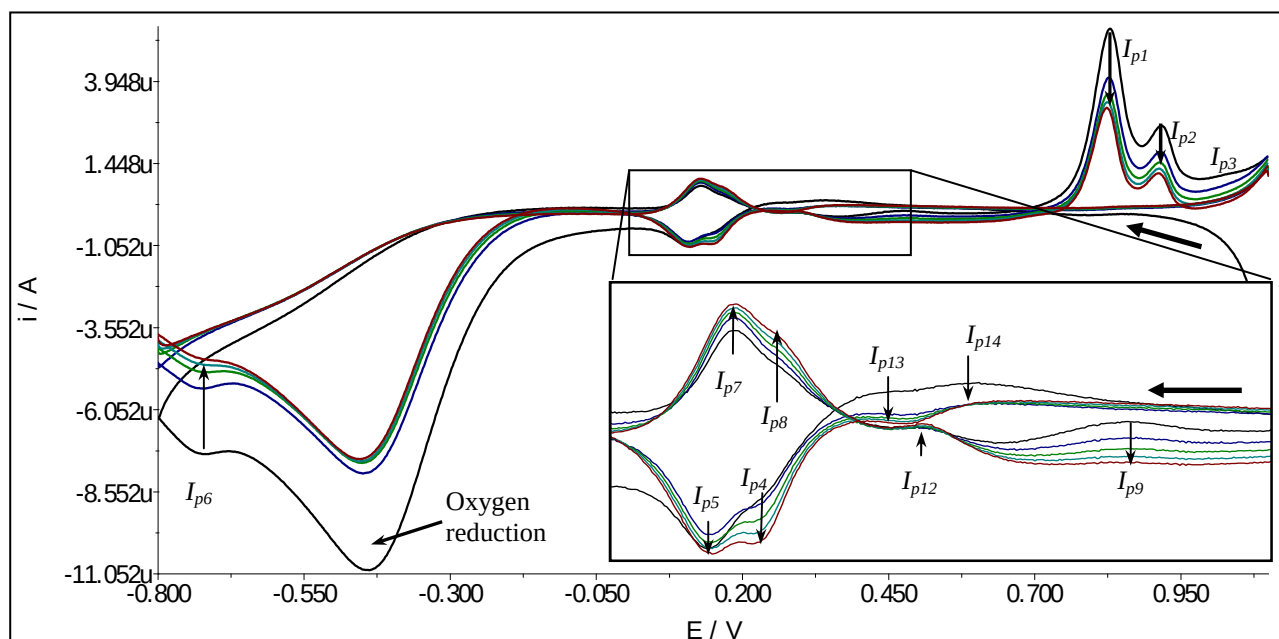


Figure 6.2: Successive cathodic CV scans of 20 μM CIT in 0.2 M NaOAc buffer pH 4.0 at an EOx-GCE with background current having been subtracted. Scan 1 (black) scan 2 (blue) scan 3 (green) scan 4 (cyan) and scan 5 (red). The inset is a magnification of a section of the same scan for clarity. Solution was not de-oxygenated.

Apart from I_{p11} to I_{p14} , the electrochemical profile of EOx-GCE is largely indifferent from polished GCE, and the slight variations which are present (I_{p11} to I_{p14}) are likely to be as a result of the retention of oxidised species at or within the graphitic oxide layer. This observation has several implications, the first of which is that the graphitic oxide layer does not mediate any apparent catalytic activity with regard to analysis of CIT e.g. decrease of E_{p1} . Secondly, the similarities in the electrochemical profiles suggests that there is no marked difference in the mechanisms of oxidation and reduction at each of the surfaces assessed, and thus that oxidative processes of CIT in general appear to be undergone relatively uniformly at the carbonaceous electrodes assessed in aqueous media. The increased responses of CIT electroanalysis at EOx-GCE are thus also likely to be as a result of the mechanism reported in Section 4.3.4 (f) of Chapter 4. Thirdly, as the profiles of CIT at polished and oxidised GCE are largely indifferent, I_{p1} was retained as the primary peak for all subsequent analyses. Finally, EOx-GCE provided an electrode surface which is representative of the electrochemical processes which govern oxidation of CIT at electrodes, and thus, along with the stability in I_p , EOx-GCE

provides a suitable surface for both general electroanalysis and determination of the electrochemical oxidation mechanisms of CIT.

The responses reported through this section are further corroborated by the findings of Section 4.3.4 [b], which demonstrated that EOx-GCE provides stability in I_{p1} across multiple electrodes as well as multiple preparations of single electrodes. Furthermore, that study showed that EOx-GCE does so with far greater efficiency than at Pi-GCE, polished GCE bare, CPE and EOx-CPE within the focused analysis window of 0.5 V to 1.1 V, as was applied through Chapter 3.

6.4.2 Adsorption of CIT at EOx-GCE

a) Optimisation of duration of adsorptive preconditioning of CIT at EOx-GCE

Adsorption of organic molecules into graphitic oxide has been previously investigated by several researchers and has proved highly effective as an analysis surface (Engstrom, 1982; Nagaoka & Yoshino, 1986; Hance & Kuwana, 1987; Kepley & Bard, 1988). CIT is poorly soluble in aqueous media (de Carvalho *et al.* 2005) and according to Kissinger & Heineman (1996), generally, the more hydrophobic a molecule is, the stronger it adsorbs at graphitic surfaces. Adsorption or accumulation of CIT as a factor of deposition time was assessed by measuring I_{p1} and E_{p1} responses after increasing exposure time of the electrode in agitated CIT solution, the results of which are reported in Figure 6.3.

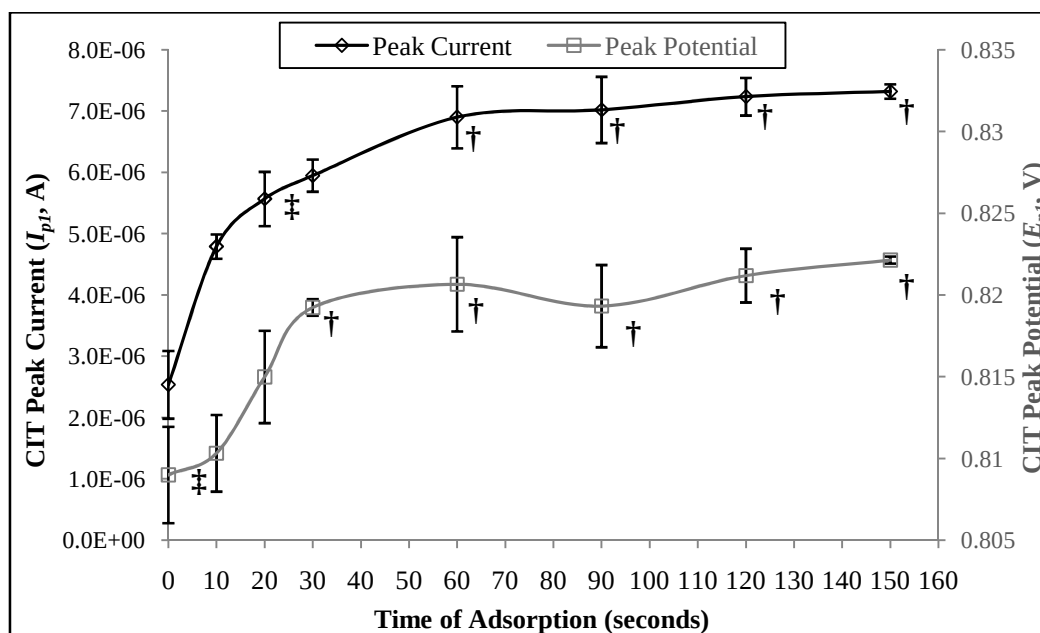


Figure 6.3: Assessment of the influence of adsorptive preconditioning at EOx-GCE on CIT I_{p1} (black) and E_{p1} (grey). CIT responses were recorded after 0, 10, 20, 30, 60, 90, 120 and 150 seconds held at a potential of 0.4 V with solution agitated at 2000 rpm. $N \geq 4$ per data point with standard deviation reported from the mean. [†] represents no significant difference between several data points and [‡] represents no significant difference between adjacent data points assessed by Fisher LSD test ($p > 0.05$) in ANOVA.

The responses of Figure 6.3 describe an increase in I_{p1} with an increase in deposition time, increasing from $2.54 \mu\text{A}$ ($\pm 0.55 \mu\text{A}$) at 0 seconds to $6.90 \mu\text{A}$ ($\pm 0.51 \mu\text{A}$) at 60 seconds, a response more than twice that at 0 seconds. As the response at 0 seconds was also lower than that of a polished GCE ($6.77 \mu\text{A} \pm 1.36 \mu\text{A}$; Figure 6.3) this response indicates that the graphitic oxide layer of EOx-GCE initially acts as a diffusion barrier to CIT. Thereafter, the gradual time-dependent increase in I_{p1} to 60 seconds suggests that the layer may accumulate CIT, but that the tailing rate of increase in I_{p1} between 0 seconds and 60 seconds suggests that this rate of accumulation is dependent on a concentration gradient between the interior of the graphitic oxide layer and the bulk solution that rapidly equilibrates. This hypothesis is corroborated by I_{p1} responses plateauing from 60 to 150 seconds, with no significant difference in that region observed as assessed by ANOVA using a Fisher LSD test with $p > 0.05$.

Along with the increase observed in I_{p1} in Figure 6.3, E_{p1} increased from an initial response of 0.809 V ($\pm 0.003 \text{ V}$) to 0.819 V ($\pm 0.001 \text{ V}$) at 30 seconds, an increase of 10 mV , which is relatively small, after which time, the E_{p1} responses also plateaued with no significant difference in responses according to the ANOVA analysis. When considered together, the relatively sharp increases in I_{p1} and E_{p1} observed until 60 and 30 seconds respectively may indicate different states of adsorption or possibly different orientations of CIT adsorbed at the electrode, and may further be a factor of the amount of CIT adsorbed at the electrode (Γ_{CIT}). From these results it can be concluded that CIT accumulates at the electrode surface with an increase in adsorption time, however the extent of adsorption of CIT to the electrode material requires further probing.

Through EIS studies in Chapter 5 it was suggested that CIT adsorbs at Pi-GCE, as evidenced by a drop in Q_{dl} upon exposure of the electrode to CIT as well as relation of ΔQ_{dl} to CIT I_{p1} through Section 5.4.4. As Pi-GCE and EOx-GCE are of similar chemical composition (Chapter 4, Section 4.3.3), it is probable that adsorption of CIT within the graphitic oxide layers would be similar. Accumulation and adsorption within the oxide layer is assessed and discussed further below as well as in Section 6.4.6. Under the assumption that adsorption does occur, it is submitted that the accumulation within the oxide layer governs the improved responses at graphitic oxide (described through Chapter 4, Section 4.3.4 [f]). With increased time of exposure, the oxide layer in the vicinity of the electrode becomes saturated with CIT, allowing for reproducible responses and accounting for the plateau in I_p displayed in Figure 6.3. As the extent of accumulation, and thus by extension, saturation and thus maximal I_p response, was achieved after 60 seconds with relative stability, 120 seconds was selected as a standard condition for analysis of CIT to reduce possible variability which might be encountered under potentially hampered mass transport into the layer in complex media.

b) Characteristics of the Adsorbed Layer of CIT at EOx-GCE

In order to ascertain the extent of adsorption of CIT at EOx-GCE, it was modelled according to maximum coverage (perfect stacking of the molecule based on the dimensions of the molecule itself) on a GCE surface. The dimensions of the CIT molecule were determined as illustrated in Figure 6.4, as $X = 9.12 \text{ \AA}$, $Y = 6.62 \text{ \AA}$ and $Z = 3.68 \text{ \AA}$.

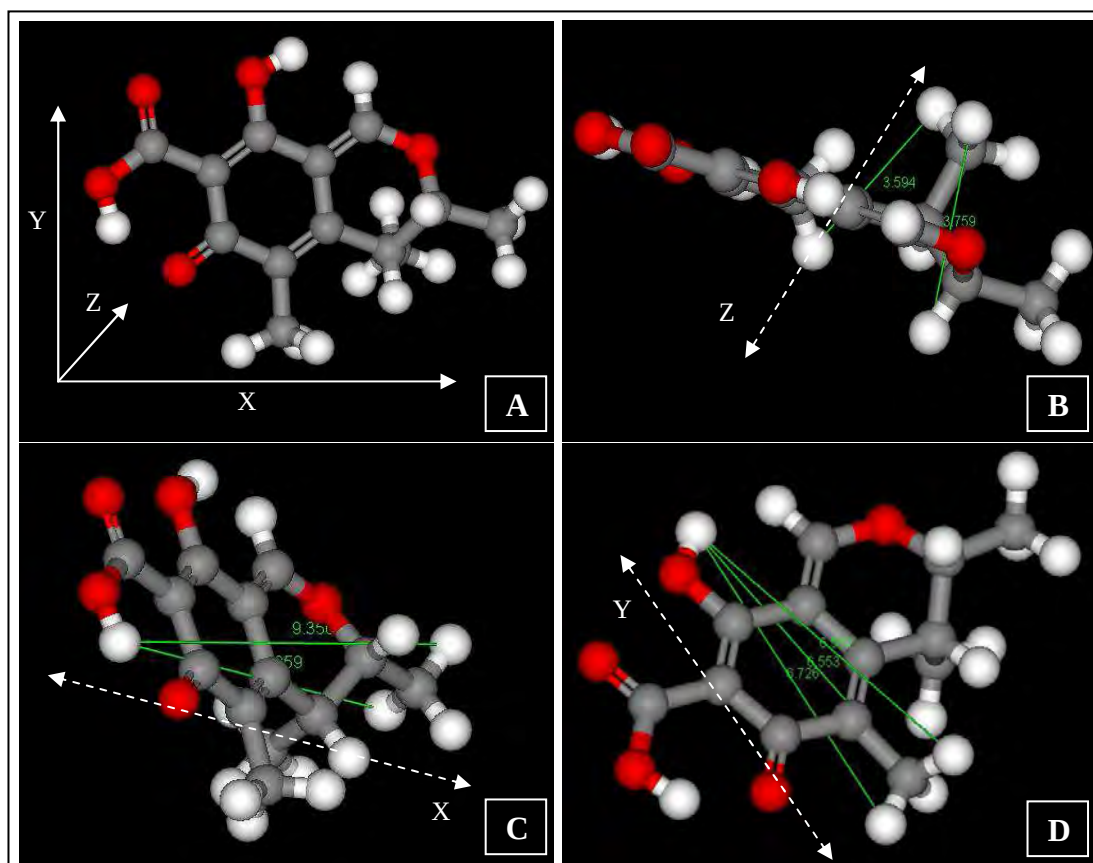


Figure 6.4: 3D structure of CIT modelled with Discovery Studio Visualiser (2007) relative to X, Y and Z axis [A] wherein grey represents Carbon, red represents Oxygen and white represents Hydrogen. Molecular dimensions for CIT along the Z axis [B], X axis [C] and the Y axis [D], the orientation of each indicated by dotted while line. Green lines indicate distances across the molecule along each axis, measured in $\text{\AA}$.

Theoretical maxima of surface coverage were calculated for CIT orienting in one of three possible planes in Figure 6.4; XY [Figure 6.4 A], XZ [Figure 6.4 B] or YZ [Figure 6.4 C]. The dimensions of length and breadth of the CIT molecule (theoretical maxima of monolayer coverage in moles of $\text{CIT} \cdot \text{cm}^{-2}$) were calculated by averaging distances across the faces of the CIT molecule, the results of which are summarised in Table 6.1.

The responses of Table 6.1 assume perfect organisation and stacking of CIT molecules at the electrode i.e. without any gaps left on the GCE surface. This is an unlikely scenario considering the variable surface condition of GCE. Furthermore, graphitic oxide is present at the electrode surface, the porous structure of which will influence the possible amount and orientation of CIT which could

adsorb. Finally, multilayers were considered to form which would result in a packing surface area greater or equal to the geometric surface area of GCE. Under these assumptions of condition, it is possible to determine “ideal” values against which experimentally determined values can be compared.

Table 6.1: Dimensions of the CIT molecule and resulting theoretical maxima of Moles of CIT present at a geometric surface area of a GCE.

		Surface Area of CIT molecule face ($\text{\AA}^2$)	Moles of CIT per cm^2	Moles of CIT on electrode surface ^[A]
Molecule Plane	XY	60.28	2.76×10^{-10}	1.95×10^{-11}
	XZ	33.48	4.96×10^{-10}	3.51×10^{-11}
	YZ	24.34	6.82×10^{-10}	4.82×10^{-11}

Relative surface areas of faces of the CIT molecule (according to XZ, XY and YZ axes of Figure 6.4) were calculated according to Eq. 6.1. Moles of CIT per cm^2 were calculated by accounting for Avogadro’s constant, (Section 6.3.3 [b]).
[A] = calculated from geometric surface area of 0.0707 cm^2 , Eq. 6.2.

The calculated theoretical maxima for adsorption of CIT were related to a measured amount of adsorbed CIT on GCE and EOx-GCE, determined according to Eq. 2.6. The results of that analysis, along with the determined number of layers for each adsorptive plane of CIT (Table 6.1) are tabulated below in Table 6.2, with multilayers highlighted in bold. Several factors pertaining to the adsorption of CIT in each orientation at carbonaceous electrodes were investigated therein, including surface condition (by assessing adsorption at both GCE and EOx-GCE), CIT solution concentration (1, 20 and $300 \mu\text{M}$) as well as with and without an adsorptive preconditioning step (as in Section 6.4.2 [a]).

Importantly, responses at EOx-GCE were more consistent than at polished GCE as identified by the deviations reported for each category of polished GCE as compared to EOx-GCE in Table 6.2. The influences of these deviations are reflected through the variability in I_{p1} reported in Figure 6.3. The uniformity of Γ_{CIT} at EOx-GCE thus acts to corroborate the assertion that this electrode configuration affords uniformity in the amount of CIT oxidised affording stability in response, and such that the oxidation characteristics of CIT are uniform and can be determined with accuracy.

Through relation of the responses of Table 6.2, it is evident that each of the parameters assessed influence the amount of CIT adsorbed (Γ_{CIT}) at the electrode surfaces. The inclusion of an adsorption time of 120 seconds results in a significant increase in Γ_{CIT} . Secondly, the solution concentration of CIT has a marked influence, with higher Γ_{CIT} corresponding to higher solution concentrations. Interestingly, the Γ_{CIT} at polished GCE ($\Gamma_{\text{CIT GCE}}$) was higher than at EOx-GCE ($\Gamma_{\text{CIT EOx-GCE}}$) (as reported by the column $\Gamma_{\text{CIT EOx-GCE}} / \Gamma_{\text{CIT GCE}}$) in each category assessed other than at $300 \mu\text{M}$ which had a response of 1.30. This suggests that Γ_{CIT} is limited by the rate of accumulation of CIT into the oxide layer which was noted to be a time-dependent factor in Section 6.4.2 [a]. These responses suggest that the oxide layer limits the rate of accumulation of CIT by hindering the rate of diffusion,

but given sufficient time and/or solution concentration, Γ_{CIT} will be consistently higher at EOx-GCE than at polished GCE. As such EOx-GCE is likely to allow more efficient compartmentalisation of CIT within the porous structure of the graphitic oxide within the conductive zone from the electrode. As a similar response was encountered herein by Γ_{CIT} being larger at higher concentration, the process governing both accumulation of CIT into the oxide layer and adsorption in the oxide layer is suggested to be a factor of the concentration gradient into the oxide layer.

Table 6.2: Indicating the amount of CIT adsorbed at both oxidised and polished GCE at concentrations of 1 μ M, 20 μ M and 300 μ M. Number of resulting layers determined from adsorbed concentration and theoretical maximum adsorption are also indicated.

	[CIT] (M.L ⁻¹)	Q_{CIT} ($\times 10^{-6}$ C)	Γ_{CIT} on GCE ($\times 10^{-9}$ M/cm ²)	Moles of CIT Adsorbed ($\times 10^{-11}$ M)	Number of Layers of CIT	$\Gamma_{CIT Ads} /$ $\Gamma_{CIT No Ads}$	$\Gamma_{CIT EOx-GCE}$ $/ \Gamma_{CIT GCE}$
EOx-GCE	Adsorption	300 μ M	9.32 (± 0.23)	1.37 (± 0.03)	9.66 (± 0.24) XY 4.96 (± 0.12) XZ 2.75 (± 0.07) YZ 2.00 (± 0.05)	1.28	1.30
		20 μ M	6.00 (± 0.03)	0.44 (± 0.51)	3.11 (± 0.04) XY 1.60 (± 0.02) XZ 0.89 (± 0.01) YZ 0.64 (± 0.01)	4.55	0.97
		1 μ M	2.42 (± 0.10)	0.18 (± 0.01)	1.25 (± 0.10) XY 0.64 (± 0.05) XZ 0.36 (± 0.03) YZ 0.26 (± 0.03)	9.51	0.48
	No adsorption	300 μ M	7.29 (± 0.47)	1.07 (± 0.07)	7.55 (± 0.48) XY 3.88 (± 0.25) XZ 2.15 (± 0.14) YZ 1.57 (± 0.10)		0.94
		20 μ M	1.32 (± 0.04)	0.09 (± 0.001)	0.68 (± 0.04) XY 0.35 (± 0.02) XZ 0.20 (± 0.01) YZ 0.14 (± 0.01)		0.78
		1 μ M	0.25 (± 0.02)	0.02 (± 0.003)	0.13 (± 0.02) XY 0.07 (± 0.01) XZ 0.04 (± 0.01) YZ 0.03 (± 0.00)		0.80
Polished GCE	Adsorption	300 μ M	7.19 (± 0.60)	1.05 (± 0.09)	7.45 (± 0.62) XY 3.83 (± 0.32) XZ 2.13 (± 0.18) YZ 1.55 (± 0.13)	0.93	
		20 μ M	6.19 (± 1.22)	0.45 (± 0.18)	3.21 (± 1.26) XY 1.65 (± 0.65) XZ 0.91 (± 0.36) YZ 0.67 (± 0.26)	3.64	
		1 μ M	5.05 (± 0.18)	0.37 (± 0.03)	2.62 (± 0.19) XY 1.34 (± 0.10) XZ 0.75 (± 0.05) YZ 0.54 (± 0.04)	15.96	
	No Adsorption	300 μ M	7.75 (± 1.05)	1.14 (± 0.15)	8.03 (± 1.08) XY 4.12 (± 0.56) XZ 2.29 (± 0.31) YZ 1.67 (± 0.22)		
		20 μ M	1.70 (± 0.27)	0.13 (± 0.04)	0.88 (± 0.28) XY 0.45 (± 0.15) XZ 0.25 (± 0.08) YZ 0.18 (± 0.06)		
		1 μ M	0.32 (± 0.02)	0.02 (± 0.002)	0.16 (± 0.01) XY 0.08 (± 0.01) XZ 0.05 (± 0.00) YZ 0.03 (± 0.00)		

Adsorption refers to an adsorptive preconditioning step in analysis of maintaining the electrode in CIT solution for 120 seconds prior to analysis, **No adsorption** refers to the absence of this step. $\Gamma_{CIT Ads}$ refers to the amount of CIT at the electrode under adsorptive preconditioning and $\Gamma_{CIT No Ads}$ refers to the amount of CIT at the electrode in the absence of adsorptive preconditioning. Numbers in bold indicate multilayers.

The column ‘Number of Layers of CIT’ in Table 6.2 described that CIT adsorbs at carbonaceous electrodes and forms multilayers as compared to theoretical maxima at both polished GCE and EOx-GCE (Table 6.1). Bold responses represent multilayer formation. CIT multilayer formation at both surfaces suggests CIT accumulates onto itself, possibly in the XY orientation via π - π interactions (Yajima *et al.* 2003), possibly allowing oxidation to proceed through a process such as electron hopping (Kissinger & Heineman, 1996). Although this mechanism may also influence CIT oxidation at EOx-GCE, CIT will not be packed densely and will not form true multilayers. Thus CIT oxidation is likely to rely on the thin layer of conductivity of graphitic oxide detailed in Section 4.3.4 [e], Chapter 4.

6.4.3 Influence of Continuous Voltammetric Cycling on the Analysis of CIT at EOxGCE

a) Continuously cycled voltammetric analyses of CIT at EOx-GCE

The influence of continuous cycling at EOx-GCE was assessed by conducting multiple, successive LSV scans without replenishing the electrode surface between scans (Berríos *et al.* 2009). A typical response is illustrated in Figure 6.5.

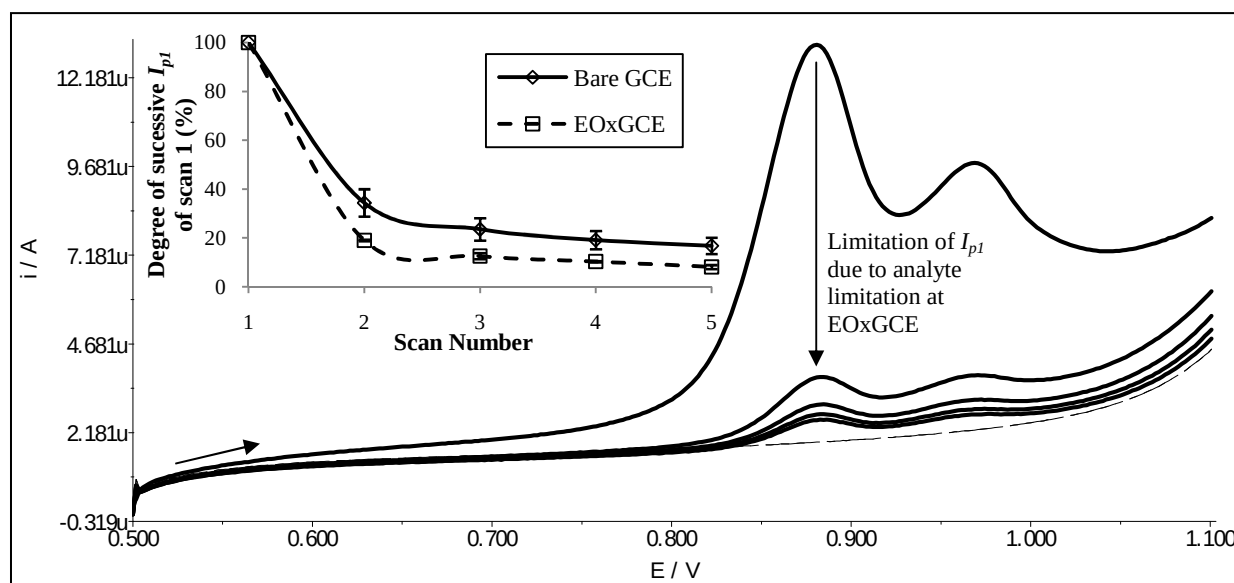


Figure 6.5: Successive LSV analyses of CIT at EOx-GCE in 20 μ M CIT in 0.2 M BR buffer pH 2.0 (black lines) in relation to the background current (dashed line). Inset shows the summarised extent of I_{p1} of successive scans on EOx-GCE as compared to polished GCE as a function of a % of the initial scan of each set (Eq. 2.7). Standard deviation was reported from the mean, ≥ 3 replicates per set.

As is evident in Figure 6.5, there is a marked decrease in I_{p1} across successive scans, similarly to that reported in Chapter 3, Section 3.4.4, wherein the response was attributed primarily to depletion of analyte at the GCE surface and fouling of the GCE surface to a lesser extent. This effect was observed to a similar extent on EOx-CPE (data not shown). It was furthermore reported by Torriero *et al* (2004) for the oxidation of salicyclic acid on aqueous media, a close structural analogue to CIT and was

illustrated in this Chapter to exhibit very similar electrochemical responses to CIT. The inset of Figure 6.5 draws a comparison of I_{p1} responses acquired across continuous cycling at EOx-GCE with polished GCE (Chapter 3, Section 3.4.4) under equivalent scanning and solution conditions. As indicated by the inset, I_{p1} was suppressed more extensively with successive scans at EOx-GCE compared to polished GCE, with almost complete hindrance of the formation of I_{p1} occurring after the first scan. The I_{p1} of scan 2 was 19.01 % of scan 1, and there was furthermore negligible difference in I_{p1} response between scans 2 and 5. This was likely to be owing to the graphitic oxide layer entrapping product/s (oligomeric or polymeric) of oxidation (Berríos *et al.* 2009), limiting transport of oxidation products away from the surface as well as limiting import of analyte to the electrode surface. This was a feature similar to the way CIT accumulation has been shown to be hampered (Section 6.4.2) and as was suggested in Section 6.4.1.

Recovery of I_{p1} response after scanning at EOx-GCE was assessed by allowing the electrode to stand in a solution of 20 μM CIT, and scanning at 10 minute intervals as an extended period without cleaning the electrode to allow for import of un-oxidised analyte if possible. The result of this analysis is illustrated in Figure 6.6.

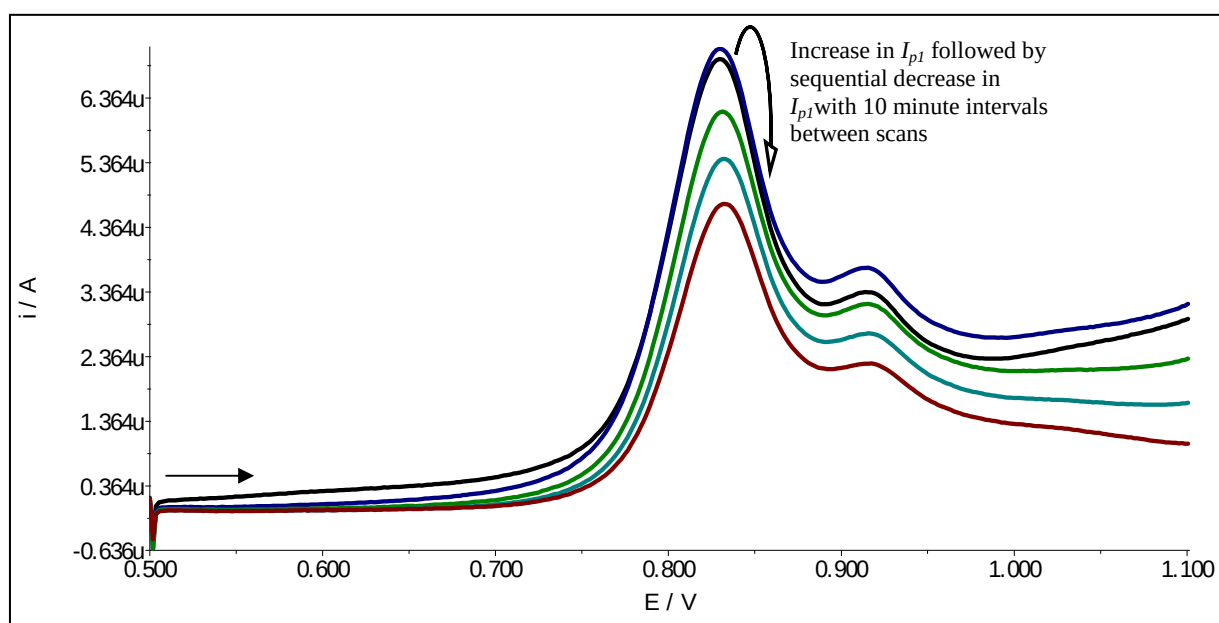


Figure 6.6: CIT I_{p1} response with analysis at 10 minute intervals. Successive anodic LSVs of 20 μM CIT in 0.2 M NaOAc buffer pH 2.0 at EOxGCE, where scans were carried out at 10 minute intervals from 0 minute (black) 10 minute (blue) 20 minute (green) 30 minute (cyan) and 40 minute (red). Background current was subtracted from each scan. Scans were carried out between 0.5 V and 1.1 V at a scan rate of 150 $\text{mV}\cdot\text{s}^{-1}$ vs. Ag/AgCl.

Figure 6.6 illustrates that although the electrode surface is passivated with successive scans, the rate of drop of I_{p1} with successive scans is markedly slower than in Figure 6.5, dropping to 69.23 % after 5 scans as opposed to 19.01 % after the first scan. This result thus demonstrates that there is recovery of

the electrode surface, similar to that reported in Section 3.4.4. The charging current preceding I_{p1} also dropped with successive scans, further corroborating that analyte was not being replenished at the electrode surface. This response suggests that the oxide layer limits detachment of oxidation product, and subsequent replenishment of un-oxidised analyte at the EOxGCE surface relative to polished GCE. It was anticipated that the electrode was fouled to an extent, similarly to that reported in Chapter 3. The primary factor limiting I_{p1} response of continuously cycled scans was however anticipated to be the limitation of both oxidised product leaving the electrode surface and replenishment of analyte at the electrode surface prior to scanning. Although present, the rate of recovery of EOx-GCE was considered insufficient to warrant further investigation toward application within routine analysis conditions in the opinion of the author. An interesting result however is that scan 2, which was carried out after 10 minutes of standing in solution, shows a marginally higher I_{p1} response than scan 1 at 0 minutes, a result which was not observed at polished GCE (Section 3.4.4).

b) Influence of pH on continuously cycled voltammetric analyses of CIT

The influence of solution conditions such as pH in which electrochemical oxidation is carried out has been shown to influence the characteristics of the resulting oxide layer (Jürgen & Steckhan, 1992). Solution pH can furthermore influence the condition of the surface chemistry at an electrode through e.g. influencing the extent of protonation of surface groups (McMurry, 2000). The influence pH on continuously cycled voltammetric analyses of CIT at EOx-GCE is illustrated through Figure 6.7. Therein, little deviation in the extent of fouling across the entire pH range assessed was observed as compared to responses reported in Figure 6.5. These results were also true for pH 2, 4, 6, 8 and 10, not represented for clarity therein.

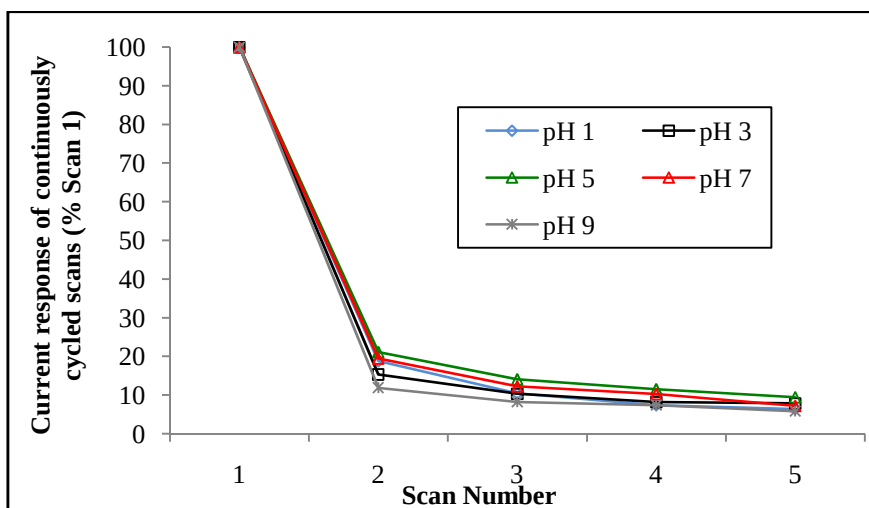


Figure 6.7: Influence of pH on continuously cycled voltammetric analyses of CIT. Successive LSV scans were conducted in 20 μM CIT in 0.2 M BR buffer at a scan rate of 150 mV.s^{-1} vs. Ag/AgCl at EOx-GCE. Although fouling was assessed for each pH unit between pH 1 and pH 10, only alternate pH results are illustrated herein for clarity. $N \geq 3$ replicates per set, error bars removed for clarity.

Through comparing fouling in BR buffer (Figure 6.7), wherein I_{p1} dropped to 18.80 % at scan 2 at pH 4.0, to fouling in NaOAc buffer of equivalent pH, I_{p1} dropped to 18.69 % at scan 2 after 20 oxidation cycles (Figure 6.8), it is evident that under similar physical conditions, there is no significant influence of ionic composition on I_{p1} responses of continuously cycled voltammetric analyses.

c) Influence of the extent of electrochemical oxidation of GCE on continuously cycled voltammetric analyses of CIT

As the magnitude of CIT current responses in continuously cycled voltammetric analyses was observed to be limited under all of the conditions assessed in this study at EOx-GCE; the oxide layer itself was expected to be implicated. As the number of oxidation cycles was shown to influence CIT I_{p1} (Chapter 4, Section 4.3.1 [b]), and as the amount of cycling influences the thickness of the graphitic oxide layer (Hance & Kuwana, 1987), and thus the import and export of species into and out of the oxide layer (Section 6.4.2), the influence of the number of oxidation cycles on fouling was assessed as represented in Figure 6.8.

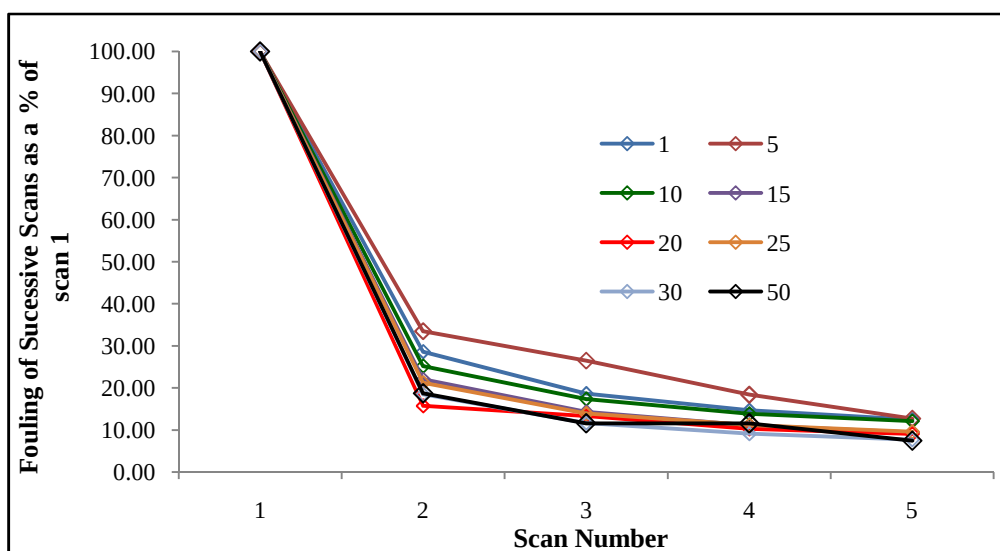


Figure 6.8: Influence of the number of electrochemical oxidation cycles on continuously cycled CIT responses. Successive anodic LSVs of 20 μ M CIT in 0.2 M NaOAc buffer, pH 4.0 after 1, 5, 10, 15, 20, 25, 30 and 50 anodic oxidation cycles in H_2SO_4 respectively. Scans were carried out between 0.5 V and 1.1 V at a scan rate of 150 $mV.s^{-1}$ vs. Ag/AgCl, $n \geq 3$ replicates per set.

When considering the results of Figure 6.8, although limitation of the magnitude of I_{p1} with increasing numbers of continuous cycles is extensive across all of the oxidation cycle parameters assessed, variability in the extent of fouling according to the extent of oxidation was observed. When relating these results to those of Figure 4.2 (Chapter 4, Section 4.3.1 [b]), which described I_{p1} and E_{p1} at varying levels of oxidation, it becomes evident that the extent of limitation of I_{p1} shown herein follows a similar pattern as the I_{p1} response of Figure 4.2 with an initial drop in the extent of fouling, followed by a gradual increase in the extent of fouling, with the extent of fouling plateauing after 30

oxidation cycles. Accordingly, these responses provide further evidence that graphitic oxide hinders CIT oxidation products from exiting the oxide layer and being replenished by un-oxidised CIT given that an increase in oxide layer thickness (with more extensive cycling) results in an increased degree of limitation of I_{p1} with successive scans.

Accordingly, from these results, (Figure 6.7 and 6.8) it was determined that pH and ionic composition of the solution have no noticeable influence on the extent of limitation of I_{p1} with successive scans. Although it was influenced to a small degree by several parameters, none illustrated any significant minimisation on the extent of suppression of limitation of I_{p1} . Thus it is considered unavoidable that I_{p1} will be suppressed with successive scans at EOxGCE under the conditions investigated here and only the initial scan of analyses were applied in analytical studies henceforth.

6.4.4 Influence of pH on Analysis of CIT at EOx-GCE

a) CIT peak profile shift as a function of pH

The influence of solution pH on the electrochemical analysis of CIT in 0.2 M BR buffer between the ranges of pH 1 to pH 10 was assessed and the responses summarised in Figure 6.9.

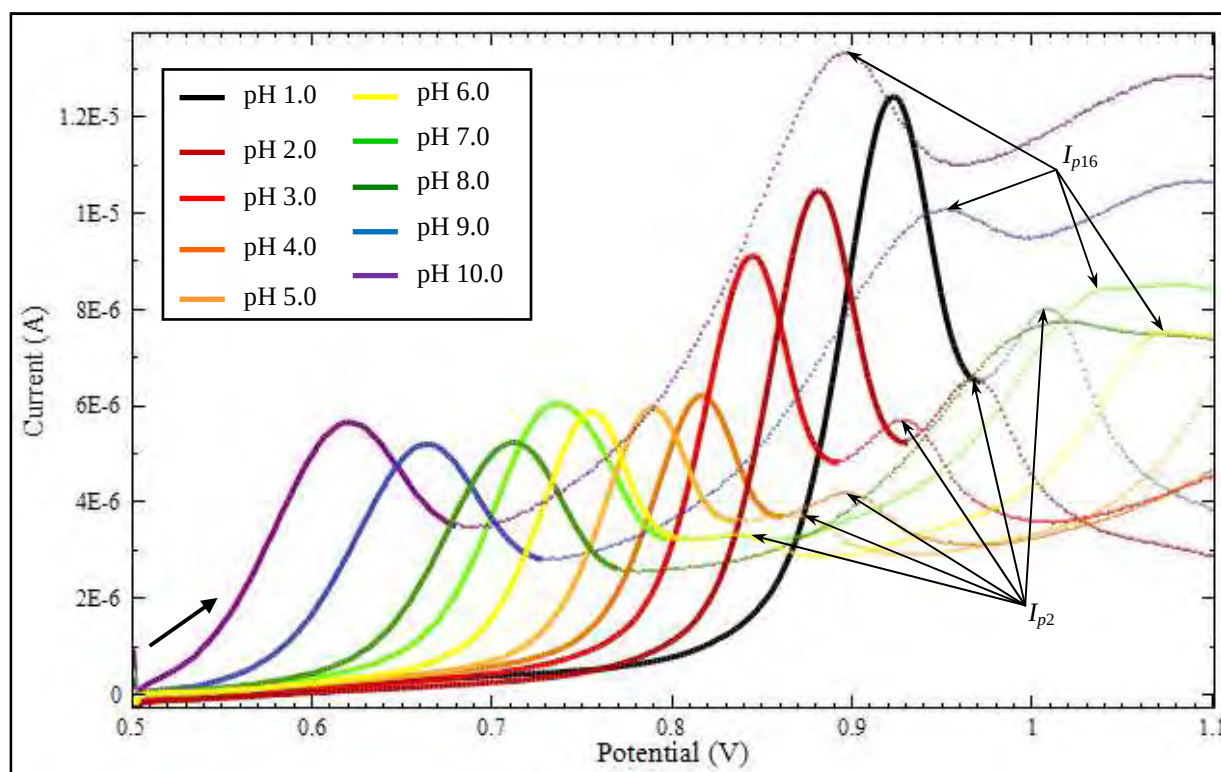


Figure 6.9: Peak profile of CIT according to solution pH. LSV scans of 20 μM CIT in 0.2 M BR buffer pH 1.0 to pH 10.0 (right to left) at EOxGCE. Each scan had its respective base line subtracted. In each case, the scan was represented as a dotted line post I_{p1} for clarity across intersecting scans)

The change in solution pH results in a shift in both the magnitude of I_{p1} and the position of E_{p1} . The LSV profiles of CIT show a larger I_{p1} current response and more defined peaks (both I_{p1} and I_{p2}) at acidic pHs with a decrease in I_{p1} from pH 1.0 to pH 4.0. Thereafter current response plateaued through to the alkaline pH range.

Stable I_{p1} responses (as evidenced by a low coefficient of variation in current response) were observed between pH 1.0 and pH 6.0. However between pH 7.0 and pH 10.0 instability in I_{p1} was observed, with an increase in the coefficient of variation (state figure) observed further as a shift in the CIT peak profile over time when a single solution was repeatedly assessed at freshly resurfaced EOx-GCE, (discussed further below in section 6.4.4 [b]). These results suggest that CIT is stable at acidic pH and unstable at alkali pH.

The breadth (potential breadth at half peak height) of the peaks between pH 7.0 and pH 10.0 increased relative to those of pH 1.0 to pH 6.0 (Figure 6.9). I_{p2} shifted uniformly with I_{p1} toward less anodic potentials with an increase in alkalinity and did not intersect (Figure 6.10). The broadening of I_{p1} and a drop in I_{p2} resulted in I_{p2} no longer being visible in scans above pH 7.0 are likely to be as a result of a pH-dependant mechanism. A subsequent peak, denoted I_{p16} was observed at pH values greater than pH 6.0 and is discussed further below.

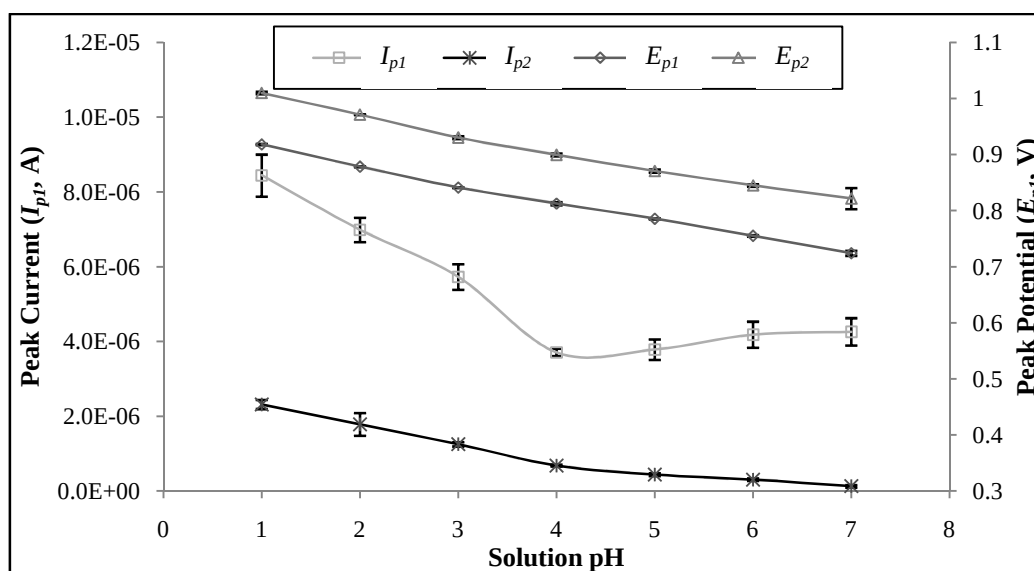


Figure 6.10: Current and potential response of I_{p1} and I_{p2} in 20 μM CIT in 0.2M BR buffer in relation to pH 1 to pH 7, scan rate 150 $\text{mV}\cdot\text{s}^{-1}$.

b) Stability of CIT I_{p1} as a Function of pH

The time dependant effect of alkali solution on CIT peak profiles are illustrated in Figure 6.11 at pH 7 (A), pH 8 (B) and pH 9 (C). The influence of alkali media on the profile of CIT is evident through a

shift in E_p at all alkali pH values examined. This shift was evident within 5 minutes at pH 7.0, 30 seconds at pH 8.0 and without a detectable delay at pH 9.0 and above.

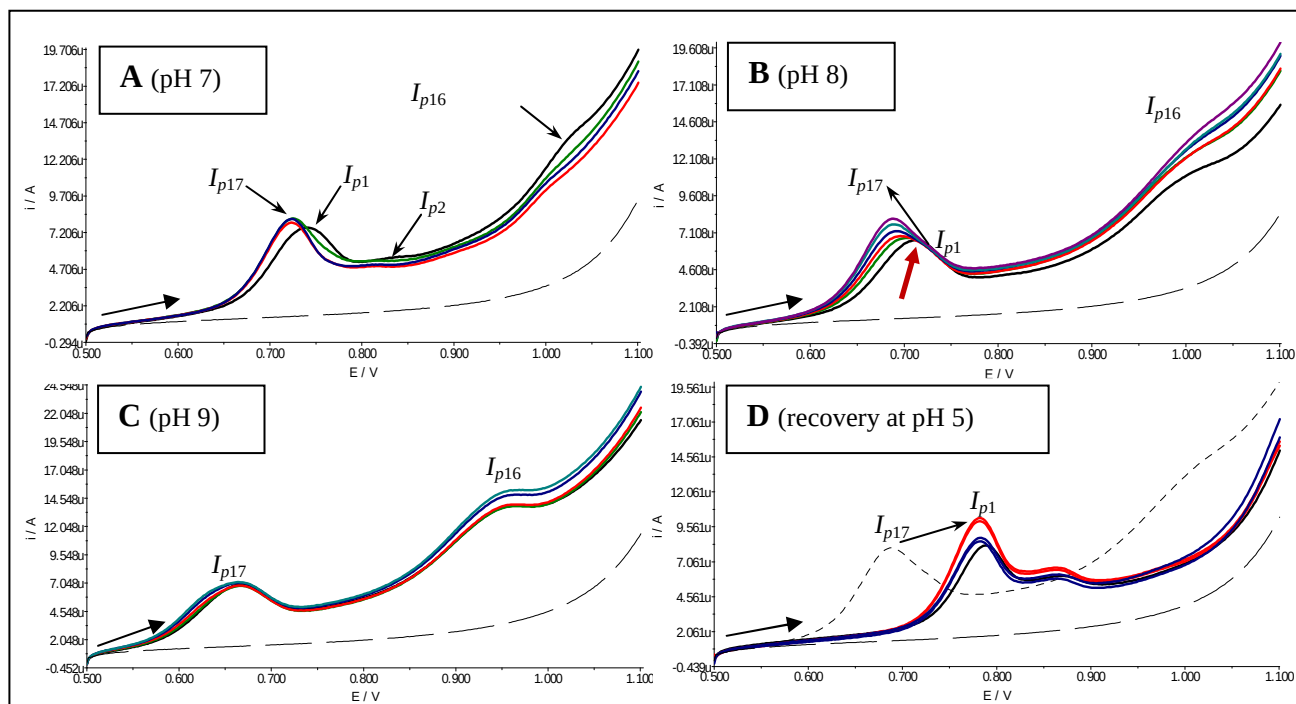


Figure 6.11: Influence of alkali pH on CIT profile. LSV scans of 20 μM CIT in 0.2 M BR at EOx-GCE. Successive scans in a single solution with cleaning and resurfacing of each electrode between scans: at pH 7.0 [A], pH 8.0 [B] and pH 9.0 [C], each with scan 1 (black) scan 2 (green) scan 3 (light red) scan 4 (blue) scan 5 (cyan) and buffer (dashed line). [D] illustrates a scan at pH 8.0 (dotted line, scan 6 of B) after which the solution pH was dropped to pH 5.0 and scans were conducted on cleaned and re-oxidised GCE after conditioning scans being carried out at pH 5.0 (blue) and pH 8.0 (light red) as compared to a scan in fresh solution at pH 5.0 (conditioned at pH 5.0) (black) and blank (dashed line).

At pH 7 (Figure 6.11 [A]) an initial scan (black line) E_{p1} shifted upon re-scanning in the same solution (at freshly prepared surface), towards less positive potential values. The peak is denoted here as I_{p17} . This behaviour was evident from pH 6.0 to pH 10.0 following time-dependent scanning. Also at these pH conditions an additional peak became prominent with increased alkalinity and denoted as I_{p16} , and indicated in Figures 6.11 A, B and C. As is evident from Figures 6.11 [B] and [C] (scans carried out in progressively more alkaline solution), the shift to or development of I_{p16} and I_{p17} from I_{p1} and I_{p2} became more rapid with increased alkalinity. While this behaviour could be attributed to the shift in I_{p1} towards more negative E_p and accumulating at the electrode, this hypothesis was dismissed when considering the evidence of Figure 6.11 [B]. The red arrow of Figure 6.11 [B] indicates a point which would be the apex of I_{p1} (black line) and is common to all the scans of and does not shift. Rather, a development of a subsequent peak which arises from the leading shoulder of I_{p1} is evident, forming I_{p17} . Fig 6.12 C shows a broad peak for I_{p17} at pH 9.0, the development or rather presence of both I_{p16} and I_{p17} arose almost instantaneously once CIT was introduced and remained stable in that from.

The development of I_{p16} and I_{p17} could arise via several processes including hydrolysis, nucleophilic attack and protonation or deprotonation (Ebbing & Gammon, 1999), each of which would probably be associated with the –OH moiety of CIT. Although it was considered possible that there may be a change in the electrode surface chemistry as the pH extends to more alkali conditions, (influencing the adsorbed nature of the CIT at alkali pH (Ebbing & Gammon, 1999)), it was considered unlikely to generate such a pronounced response. A more likely explanation was derived from Figure 6.11 [D] which examines the effect of in situ alteration of pH (from pH 8 to pH 5). Here, following retention of CIT in solution for a 120 minute period at pH 8, the pH was shifted to an acidic pH (5.0) and a CV scanned. In Figure 6.11[D] the peak observed at pH 8 (indicated here as a dotted line) is observed to shift from that typical of a solution at pH 8.0 to one typical of pH 5.0, wherein, both I_{p1} and I_{p2} were completely regenerated and I_{p16} and I_{p17} were not present. Accordingly the process is reversible with no permanent effect on the CIT molecule, and is thus likely to be a solution-analyte interaction. Due to this, it is submitted that the influence which alkali pH has on the molecule is most likely not nucleophilic attack and is thus likely to be either deprotonation or an influence on the isomer (Figure 1.1, Chapter 1) which is reversible. The influence of the pH at which an electrode is conditioned on CIT I_p response is assessed further through Section 6.4.5.

This result is significant as it indicates that CIT is stable as a function of pH over the time scales assessed i.e. in excess of one hour. Secondly, with respect to practical application in the detection of CIT in environmental samples and biosensor development, an alkaline sample containing CIT may be altered to a desirable pH and then analysed with confidence that a representative amount of analyte would be in a suitable form.

c) Determination of CIT pK_a

The pK_a of CIT has been reported to be 2.2 to 2.3 by Quilliam (2001), however no mention was made of the means of calculating those values. Herein, the pK_a of CIT was calculated via computer modelling to interrogate reported findings. This was performed using the freely available computer modelling software package, SPARC 4.2, as was described by Hilal *et al.* (1995) and validated by Liao & Nicklaus, (2009) against eight other modelling programs and experimentally derived data for 197 pharmaceutical substances and determined in that study to be among the most accurate examined. Analyses were conducted according to the ionisable moieties labelled in Figure 6.12. If group 1 (hydroxyl) is ionised, the calculated acid disassociation constant of CIT is determined as a pK_a of 3.13 (Table 6.3). Ionisation of group 2 (carboxyl) as indicated by Figure 6.12 may influence the development of I_{p16} and I_{p17} as these become evident once pH exceeds pH 6.0.

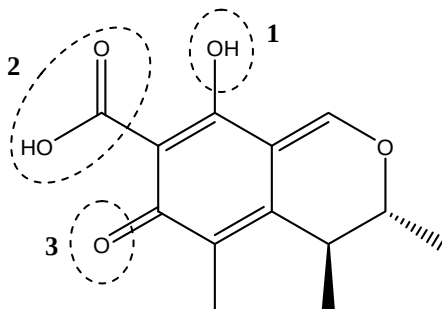


Figure 6.12: Structure of CIT indicating groups which could be ionised as 1, 2 and 3.

Table 6.3: Resulting pKa values as calculated according to ionised groups listed in Figure 7.16.

Ionised Group Position	CIT pK _a Result
1	3.13
2	5.02
3	-3.6

As the previously reported pK_a CIT differed to those determined herein, the inflection point as a result of pH change was assessed as a supplementary analysis (Figure 6.13).

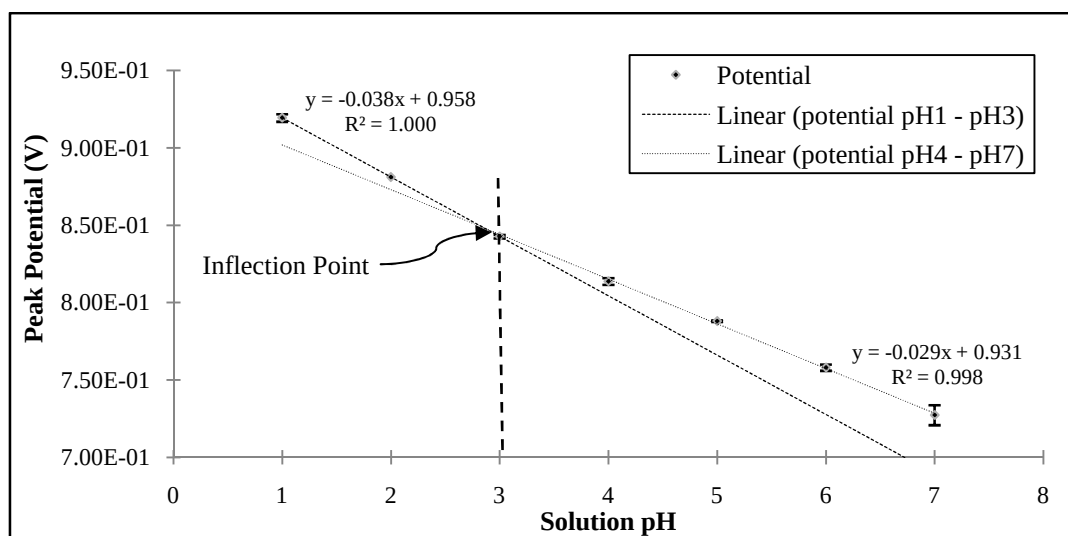


Figure 6.13: Inflection point of the potentials determined from CIT peak responses between pH 1 and pH 7 for 20 μ M CIT in 0.2M BR buffer. Number of replicates in each set was ≥ 3 with standard deviation calculated from the mean of data. Background current subtracted values are illustrated.

In Figure 6.13, an inflection point very near to a value of pH 3.13 indicated by SPARC 4.2 was found. Torreiro *et al.* (2004) similarly reported an inflection point in this region for the oxidation of salicylic acid which is structurally very similar to CIT and holds a very similar pK_a of 2.97, arising from two separate slopes (55 mV.dec^{-1} from pH 1-3 and 26 mV.dec^{-1} from pH 3-7) being determined as a function of pH. Due to this the value predicted by SPARC 4.2 was considered to be accurate. The variation in the expected pK_a of a hydroxyl bound to an aromatic ring (phenol) may have been shifted

as a function of the furan ring or other substituents attached to the benzene ring which may contribute to the acidity of the hydroxyl moiety.

d) Peak profile of CIT at targeted pH range

As the preceding sections showed, the analysis of CIT at alkali pHs induced a complicated set of peak characteristics with regard to both I_p and E_p . Accordingly the following section includes the results of acidic pH only as they provided consistently stable results over extended time periods. Figure 6.14 illustrates the I_p and E_p trends of I_{p1} from the data illustrated in Figure 6.9 from pH 1.0 to pH 7.0.

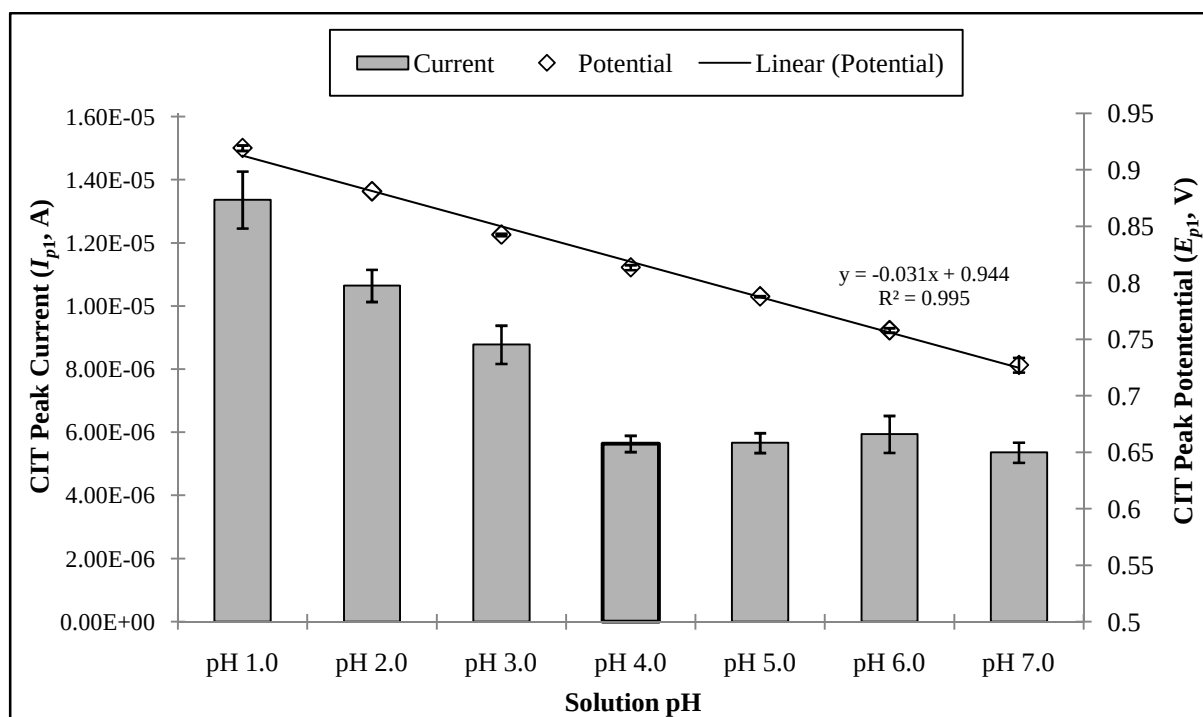


Figure 6.14: Assessment of CIT at a selected pH range with regard to I_{p1} and E_{p1} response of 20 μ M CIT in 0.2M BR buffer in relation to pH 1 to pH 7. Number of replicates in each set was ≥ 3 . Error bars show standard deviation from mean of data for which all scans had their background current subtracted.

A maximum current response of 13.34 μ A was obtained at pH 1.0. This translates to a sensitivity (Eq. 2.4) of $S_{p1} = 0.667 \text{ A.M}^{-1}$, a significantly larger response than that reported for polished GCE in Chapter 3, Section 3.4.1 [c]. The magnitude of I_{p1} , and thus S_{p1} , was influenced by pH. With increasing alkalinity, a sharp drop in I_{p1} was observed from pH 1.0 (assigned here as 100% peak height) to pH 4.0 (43.14 % of peak at pH 1.0), after which responses plateaued through to pH 7.0. This provides a resulting shift in I_{p1} which closely resembled the responses reported by Torriero *et al.* (2004) in pH dependent electroanalysis of salicylic acid, wherein a drop in I_p was reported to pH 4.0, however thereafter with increasing alkalinity to pH 12.0 little variation in I_p was recorded. The drop in I_{p1} from pH 1.0 to pH 4.0 is likely to have been as a result of the influence of the ionisation state (with $\text{pK}_{a1} = 3.13$). Reproducibility of results was well within acceptable levels, with C.V. below 10 % across all of the responses between pH 1.0 and pH 7.0.

The shift in potential of the trend of Figure 6.14 followed the relationship $E_p = 0.031$ V per pH unit across the range of pH 1.0 to pH 7.0. The stability of the slope ($R^2 = 0.995$) over the region suggests that only one moiety is involved in this region, and was likely to be that of the hydroxyl moiety. This assumption was based on the slope of the relationship, 31 mV.dec^{-1} , being indicative of an oxidation mechanism for CIT involving an uneven number of electrons to protons (Kissinger & Heineman, 1996), which is consistent for a phenol moiety oxidation as a 1 H^+ and 2e^- process, as was reported for oxidation of salicylic acid in particular (Torriero *et al.* 2004; Costentin *et al.* 2008; Evans, 2008; Costentin *et al.* 2010). Furthermore, it indicates that electron transfer is rapid. The influence of the hydroxyl moiety was further considered through Section 6.4.9 of this Chapter as the source of electrons in electro-oxidation.

In Chapter 3, Section 3.4.6 [b], which illustrated the pH profile of CIT at polished GCE, a very similar slope of 32 mV.dec^{-1} was recorded. The similarity of the responses at polished and EOx-GCE suggest that the mechanisms governing the oxidation of CIT at polished GCE are the same of those assessed herein at EOx-GCE, and thus that the determination of the oxidation mechanism of CIT at EOx-GCE are representative of CIT oxidation, and are not limited to this surface.

The pH at which analyses were conducted was selected on a basis of the results reported above as well as that the body of this research was intended for the development of biosensor systems. The implication of including interactions with biological components is that they are pH sensitive in almost all cases. Thus analyses of CIT were assessed as close to physiological pH as possible to allowing for characterisation of CIT at a pH at which the sensor configuration will be used, as is further described through Chapter 7 of this Thesis. Under these parameters, pH 4.0 (highlighted with a black border in Figure 6.14) was selected as the optimal pH for subsequent electrochemical analysis of CIT as it is at a point where both I_p and E_p were stable.

e) CIT E_{p1} profile under altered solubility conditions relative to pH

The trends for the shift in CIT E_{p1} per decade analysed over a range of pH 2.0 to pH 6.0 was carried out both with and without allowing for adsorption to occur, as well as under increased solubility conditions. As CIT has been reported to be sparingly soluble under aqueous condition (Sprenger & Ruoff, 1946; de Carvalho *et al.* 2005; Xu *et al.* 2006), but soluble in methanol (Xu *et al.* 2006), increasing the organic solvent presence in solution was anticipated to allow for CIT to be more soluble i.e. to increase the diffusion coefficient. The influence of anticipated increased diffusive traits of CIT is illustrated in Figure 6.15.

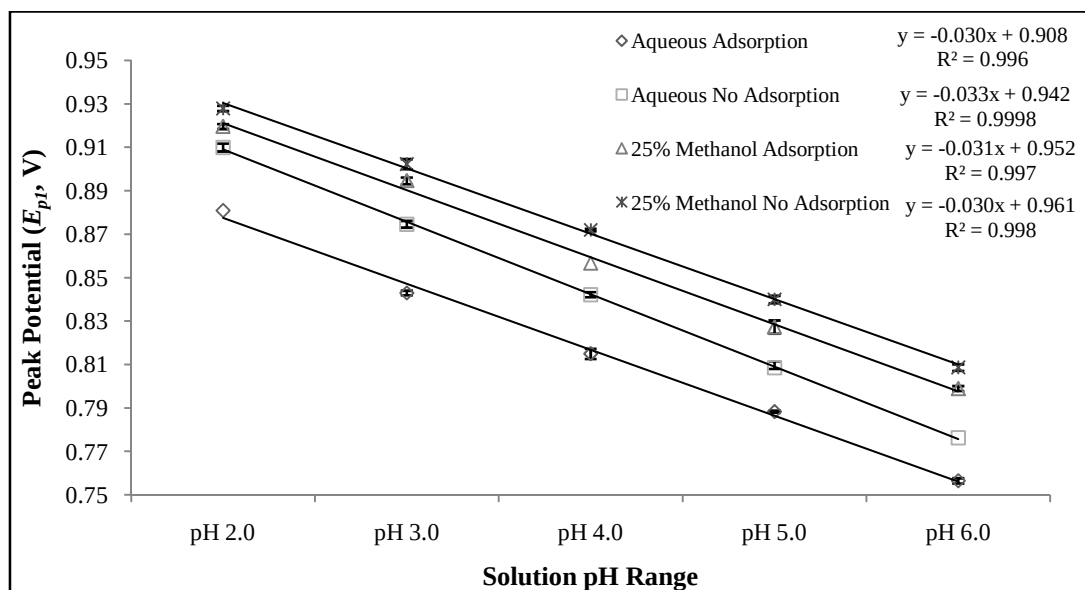


Figure 6.15: Potential response of 20 μM CIT in 0.2M BR buffer across pH 2 to pH 6 following adsorption (120 seconds in agitated solution) and no adsorption in both aqueous and 25% methanol solutions. Number of replicates in each set was ≥ 3 . Error bars show standard deviation from mean of data with background current subtracted for all scans.

As is evident in Figure 6.15, the slopes across all of the conditions assessed are relatively equivalent, at 30 – 33 mV per pH unit, and each have high R^2 values at 0.996, 0.999, 0.997 and 0.998 for aqueous solution with and without adsorption and 25% methanol with and without adsorption respectively, indicating that in each case there is high mechanistic conformity across the entire pH range. Accordingly, the oxidation mechanism for CIT involves the transfer of an uneven number of electrons to protons, regardless of the solvent and mass transport conditions present through this thesis.

6.4.5 Effect of supporting electrolyte and conditioning pH on CIT analysis

Four different electrolytes were analysed in order to ascertain the effect of ionic composition as well as conditioning pH on CIT analysis at both polished GCE and EOx-GCE (Table 6.4). The effect of pH in different electrolytes was assessed by conditioning electrodes at either pH 4.0 or pH 8.0 prior to scanning, but in all cases CIT scans were conducted at pH 4.0 due to the CIT instability at alkali pH.

No visible impact was observed on the surface of the GCE or the oxide layer as a result of alteration of the electrolytes. Far lower variability at EOx-GCE was observed again as compared to polished GCE. As shown in Table 6.4, in all cases, bar that of analysis in BR where the electrode was conditioned at pH 8.0, C.V. results at polished GCE were in excess of 10 % which places these outside of a usable range. Accordingly, the presence of the oxide layer itself results in a normalisation of results, without which analysis could not be accurately carried out.

Table 6.4: CIT I_p and E_p responses and their reproducibility in: 0.2 M BR (pH 4.0 and pH 8.0), Phosphate-Citrate (pH 4.0 and pH 8.0), NaOAc (pH 4.0) and SLB (pH 4.0) as determined at both polished GCE and EOx-GCE.

Buffer	Electrode Surface	Conditioning pH	μI_p [μ A]	σI_p [μ A]	C.V.	μE_p [V]	σE_p [V]
BR	Polished GCE	4	5.86	0.61	10.44	0.848	0.002
	EOx-GCE	4	5.81	0.10 †	1.70	0.842	0.000
	Polished GCE	8	7.52	0.38	4.99	0.842	0.001
	EOx-GCE	8	5.82	0.04 †	0.70	0.836	0.001
Phosphate-Citrate	Polished GCE	4	5.24	0.62	11.78	0.838	0.002
	EOx-GCE	4	7.73 ‡	0.36 †	4.61	0.830	0.001
	Polished GCE	8	8.23	1.03	12.52	0.832	0.001
	EOx-GCE	8	8.54 ‡	0.37 †	4.30	0.823	0.00
NaOAc	Polished GCE	4	6.14	1.13	18.39	0.836	0.001
	EOx-GCE	4	6.14	0.25 †	4.09	0.829	0.001
SLB	Polished GCE	4	6.84	0.74	10.83	0.840	0.001
	EOx-GCE	4	6.40	0.23 †	3.53	0.833	0.000

BR is Britton-Robinson buffer, NaOAc is sodium acetate buffer and SLB is succinic acid-lactic acid buffer. μ = mean, σ = standard deviation from the mean, C.V. = coefficient of variation calculated as per Eq. 2.1. ‡ represents higher I_{pl} compared to polished GCE and † represents more stable I_{pl} as compared to polished GCE

As is evident in Table 6.4 at polished GCE, the difference in E_p between scans acclimatised at pH 4.0 NaOAc, SLB and phosphate-citrate were insignificant ($0.839 \text{ V} \pm 0.006 \text{ V}$). However, the responses acquired in BR buffer were found at more positive E_p than responses observed in NaOAc and SLB. The E_p responses returned on EOx-GCE were consistently at less positive potentials than the corresponding polished GCEs for both pH 4.0 and pH 8.0 conditioning steps ($0.832 \text{ V} \pm 0.007 \text{ V}$). Furthermore, the ratio of that difference between polished GCE and EOx-GCE was equivalent across all the electrolyte solutions. This indicates that although the electrolytes have an effect on the magnitude of the result itself, this effect is a factor of a solution or solution electrode interfacial mechanism, rather than a modification of the surface itself by the electrolyte. There is no marked influence of the electrolyte on the surface oxide layer in each case as the responses are proportional to their respective polished GCE sets. This trait was also evident in electrodes conditioned at pH 8.0, though E_p responses were at more cathodic potentials relative to those of pH 4 conditioned sets, as anticipated by Section 6.4.4 [a].

From the values in Table 6.4 the shift in E_p to less negative potentials on EOx-GCE as compared to polished GCE at pH 4.0 could be attributed to the accumulation of CIT by the oxide layer, possibly allowing for the oxide layer of stacked CIT to act as an electron shuttle (Kissinger & Heineman, 1996). A second, more likely, possibility is that of an alteration in the charge of the surface allowing for better adsorption kinetics for CIT, in so doing reducing the activation energy required to induce oxidation of CIT. This suggests the presence of a micro-environmental

influence at the electrode surface associated with the pK_a of the electrode in relation to the solution pH (Ebbing & Gammon, 1999).

CIT I_p responses at polished GCE and EOx-GCE conditioned at pH 4.0 were not significantly different from one another across each electrolyte assessed, other than responses in phosphate-citrate buffer, which was higher relative to the other sets. This indicates that ionic composition does not have a marked influence on I_p response.

The difference in the influence of electrolyte composition on CIT E_p and I_p point to that fact that the mechanism(s) which influence the improvements of heterogeneous electron transfer kinetics is/are different to those which govern I_p . In terms of I_p sensitivity and reproducibility, studies in 0.2 M phosphate-citrate buffer provided the highest response, however the other buffers were only slightly less effective. As the BR and NaOAc were closely comparable to one another and offered stability over a large pH range, they were selected as the primary buffers used in subsequent analysis.

6.4.6 Influence of CIT solution concentration on voltammetry at EOx-GCE

a) Influence of CIT concentration on I_p at EOx-GCE

The influence of CIT solution concentration on I_p was assessed by analyses in solution with graduated increases of CIT concentration. A representative profile of those responses is illustrated in Figure 6.16 showing the increase in I_p response for increasing concentrations of CIT for both I_{p1} and I_{p2} .

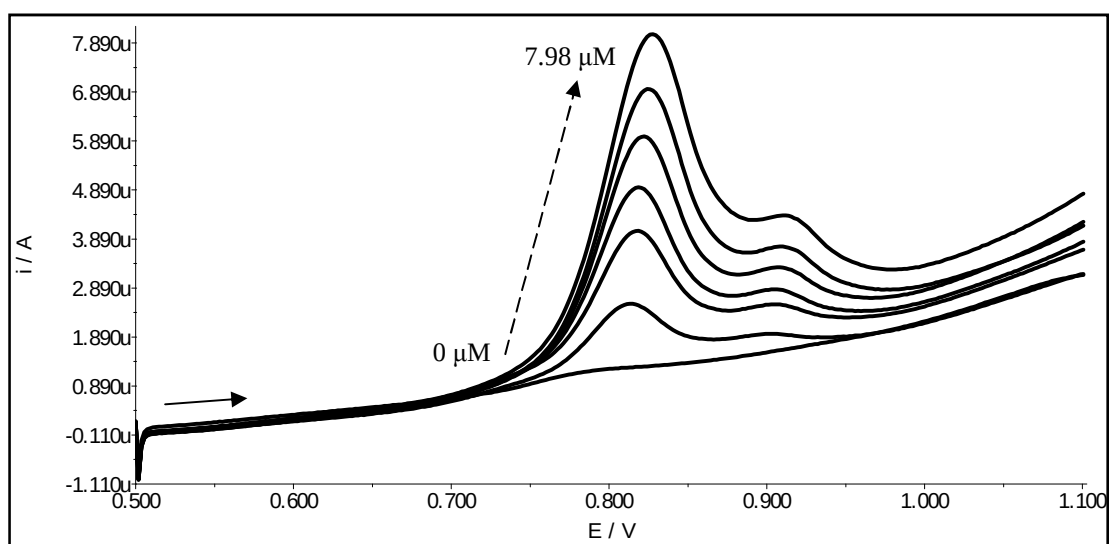


Figure 6.16: Solution concentration dependent peak profile of CIT. LSVs obtained in 0.2 M NaOAc buffer pH 4.0 at EOx-GCE from 0.80 μM to 7.98 μM , each scan with respective background currents subtracted.

Figure 6.17 represents the standard curve of I_p and concentration. As limited linearity was found with regard to the dependence of I_p on solution concentration, I_p at EOx-GCE is largely non-proportional to the solution concentration. This response is likely to be a factor of the concentration independent rate of accumulation and adsorption of CIT described in Section 6.4.2 [b]. Linearity observed at higher concentrations is consistent with diffusional current found at higher concentrations.

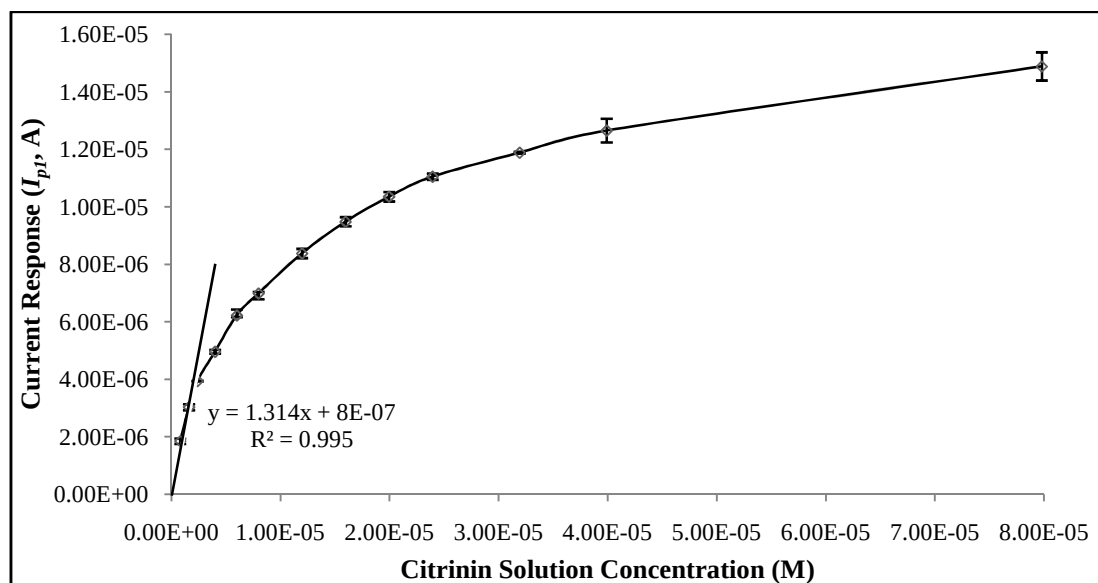


Figure 6.17: Standard curve obtained from LSV analysis of increasing concentrations of CIT ranging from 0.80 μ M to 78.24 μ M in 0.2 M NaOAc buffer pH 4.0 at an EOx-GCE. Three replicates were carried out per concentration, error bars show standard deviation from mean.

b) Relation of solution concentration to amount of adsorbed CIT at EOx-GCE

As CIT has been shown to be adsorptive at EOx-GCE in Section 6.4.2, the amount of CIT adsorbed (Γ_{CIT}) (Eq. 2.6) was related to CIT solution concentration, Figure 6.18.

As can be seen in Figure 6.18 [A], linearity was again limited when relating solution concentration to Γ_{CIT} , which suggests that bulk concentration influences the amount of CIT present at EOx-GCE and thus the rate of accumulation of CIT into the layer, in keeping with Section 6.4.2. At lower solution concentrations there is a relatively uniform increase of moles of CIT adsorbed (Figure 6.18 [A]) which corroborates the assertion that the amount of CIT which adsorbs is dependent on solution concentration. Secondly, the rate of accumulation of CIT into the oxide layer is dependent on concentration, accounting for the rapid increase in amount adsorbed at low concentration (where the layer will not be saturated) and the tapering response at higher concentration, where the graphitic oxide layer becomes saturated more rapidly. These responses thus indicate a concentration dependant process of CIT accumulation into the oxide layer, limited by saturated of the oxide layer, thereby limiting representative responses at higher solution concentrations.

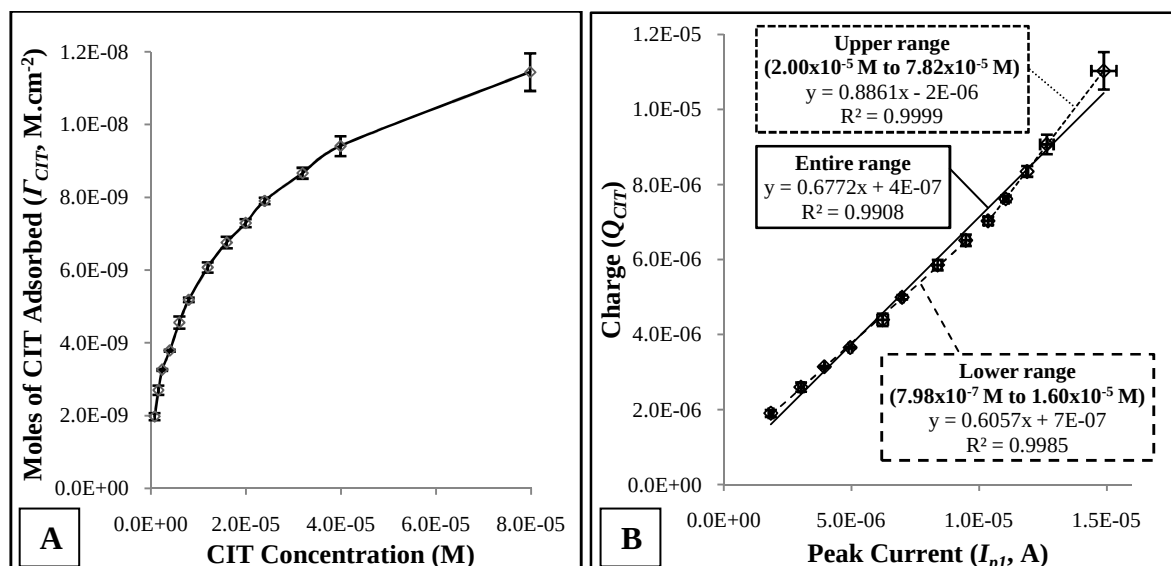


Figure 6.18: Representation of the dependence of [A] the amount of CIT adsorbed (Γ_{CIT}) at EOx-GCE in relation to the concentration of CIT in solution ranging from 0.80 μ M to 78.24 μ M and [B] Q_{CIT} on (I_{pl}), each in 0.2 M NaOAc buffer pH 4.0. $N \geq 3$ per concentration, error bars show standard deviation from mean.

Figure 6.18 [B] relating I_{pl} to Q_{CIT} provides strong linearity across the entire range assessed. This response suggests a predictable rate of change where responses alter in relation to concentration with a rate limiting step being the rate of accumulation into the oxide layer. This was assessed further by relating experimental data to modelled responses below. A slight deviation was evident in the “upper range” labelled in Figure 6.18 [B], which was likely as a result of area having a more limited influence on I_{pl} at higher concentrations as a result of diffusive I_p exerting a greater contribution to I_{pl} , as is assessed further through Section 6.4.7 [c].

c) Modelling current response of CIT as a factor of concentration, and the influence of mass transport of CIT

Adsorption of CIT at EOx-GCE was modelled according to the Langmuir isotherm (Eq. 6.3) as it relates the coverage or adsorption of molecules on a surface to concentration of a medium above the surface at a fixed temperature as is described by Kissinger & Heineman, (1996) and Masel (1996). Electroanalytical studies which have applied the Langmuir isotherm to model responses include Maeda *et al.* (1997), Pérez *et al.* (2002) and Zachek *et al.* (2008). Figure 6.19 illustrates a comparison of experimentally determined CIT adsorbed at the electrode (Γ_{CIT}) (black line with diamond) as compared to modelled responses of CIT (Grey lines and markers). The modelled responses include firstly, data modelled as an adsorptive system with application of the Langmuir equation (Eq. 6.3), the current response of which was reported as I_{ads} (grey line, triangle markers). Secondly, data was modelled as a diffusive system with application of the Randles-Sevcik equation (Eq. 6.4), the current response of which was reported as I_{diff} (grey line, star markers). Finally, a combination of adsorptive

(I_{ads}) and diffusive (I_{diff}) modelled responses was reported as a combined modelled response of $I_p = I_{ads} + I_{diff}$, reported in Figure 6.19 (grey line with square markers).

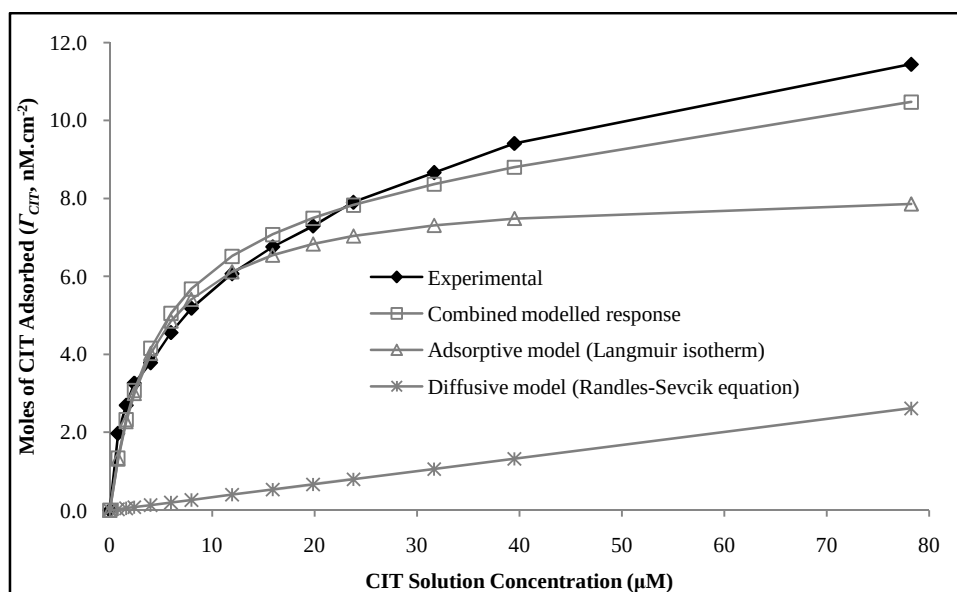


Figure 6.19: Comparison of experimental data (black) to data adsorptive response modelled using the Langmuir isotherm (grey with triangle marker, Eq. 6.3), diffusive response modelled using the Randles-Sevcik equation (grey with star marker, Eq. 6.4) and a combination of the two modelled responses (grey with square marker, adsorptive + diffusive response). Modelled data points were calculated using Solver in Microsoft Excel.

Although a purely adsorptive modelled response (Figure 6.19, grey line with triangle markers) provides a response which largely describes the experimentally determined responses at lower concentrations, at higher concentrations, a poor fit to experimental data was encountered. When considering diffusive modelled response (Figure 6.19, grey line with star markers), the opposite influence was observed, wherein, at low concentration, there was no apparent resemblance to the experimental data, but at higher concentration the diffusive modelled data more closely represented the experimentally determined response.

These responses thus primarily indicate that the experimentally determined responses arise as a result of a complex system comprising of both diffusive and adsorptive predominated mass transport regimes which alter in relation to solution concentration. This feature is expanded upon in Section 6.4.7. If the modelled adsorptive response is combined with a modelled diffusive response, the resulting accumulative response closely compares with the experimentally determined responses, and thus illustrates that both adsorptive and diffusive components contribute to recorded I_{p1} ($I_{p1} = I_{ads} + I_{diff}$), with the contribution of I_{ads} to CIT I_{p1} being greater than that of I_{diff} .

d) Limit of detection (LOD) and limit of quantification (LOQ) of CIT at EOx-GCE

As the entire concentration range assessed in Figure 6.17 was not linear, only the low concentration range of 0.80 μM to 2.39 μM (which did show linearity) was applied in determination of LOD, as is illustrated in Figure 6.17. LOD was determined according to Eq. 2.5 and was found to be 20 nM for LSV analysis of CIT in 0.2 M NaOAc buffer, pH 4.0. Table 6.5 provides a comparison of LODs reported in literature determined by several analytical techniques.

Table 6.5: Reported procedures and detection limits of CIT

Analytical Technique	Analytical Procedure	LOD	Reference
Colourimetric analysis	FD	320.24 nM	Trantham & Wilson (1984)
		Not reported	Vázquez <i>et al.</i> (1997)
Chromatographic analyses	TLC	96.07 nM	Gimeno & Martins (1983)
	TLC	48.04 nM	Gimeno (1984)
	TLC	800.59 nM	Jackson & Ceigler (1978)
	HPLC-UV	3203.36 nM	Vail & Homann (1990)
	HPLC-UV	1280.94 nM	Phillips <i>et al.</i> (1980)
	HPLC-FD	32.02 nM	Orti <i>et al.</i> (1986)
	HPLC-FD	32.02 nM	Vail & Homann (1990)
	HPLC-FD	90.00 nM	Franco <i>et al.</i> (1996)
	HPLC-FD	3.20 nM	Meister (2004)
	HPLC-FD	288.21 nM	Dohnal <i>et al.</i> (2010)

1) Biosensor systems and detection limits for CIT were reported through Table 1.2 (Chapter 1)

2) TLC = Thin layer chromatography; HPLC = High pressure liquid chromatography; UV = Ultra violet detection; FD = Fluorescence detection.

Although some of the enzyme-linked assays and the HPLC-FD analysis described by Meister (2004) afforded lower LOD than that which was reported for electrochemical analysis of CIT, the LOD of 20 nM reported herein provides a result among the most sensitive yet determined. Furthermore, the method reported herein provides a procedure which is less time consuming and far simpler than the chromatographic and enzyme linked techniques. Accordingly the LOD reported for electrochemical analyses of CIT provides a highly competitive analytical technique.

As limited peak resolution in I_{p1} was experimentally determined at very low concentrations, the limit of quantification (experimentally determined limit of detection of CIT) was investigated. This was assessed using LSV as well as differential pulse voltammetry (DPV) and square wave voltammetry (SWV), the scans of which are reported in Appendix D. DPV was used as it provides negligible contribution of charging current to the background current and in so doing allows for potentially greater current sensitivity as compared to LSV (Kissinger & Heineman, 1996). Although no discernable peaks were found in this concentration range using SWV, the results of DPV and LSV scans at concentrations of 0.16 μM to 16 nM did provide measurable I_p responses. Although both scan forms were able to detect CIT at low concentrations (Appendix D), limited resolution was observed at concentrations lower than 80 nM when assessed by LSV, a response higher than that reported by the calculated LOD (20 nM). As well as providing increased resolution of peaks at low concentration than

LSV, DPV responses provided defined and reproducible I_p responses at concentrations as low as 16 nM, as reported in Appendix D.

6.4.7 Influence of scan rate on the electrochemical characteristics of CIT at EOx-GCE

a) Scan rate studies in aqueous solution as an adsorptive process

Figure 6.20 shows the effect of scan rate in the range of 1 mV.s^{-1} to 300 mV.s^{-1} on I_{p1} which had been recorded with the application of adsorptive preconditioning (holding the EOx-GCE in solution at 0.4 V for 120 seconds) to ensure adsorption occurred. Although the magnitude of I_{p1} was much lower at slower scan rates than that which was previously reported at 100 mV.s^{-1} , there were no subsequent peaks formed within the profile and the proportion of I_{p1} to I_{p2} did not vary with alteration of scan rate. This response stands to corroborate the finding that I_{p1} is the primary oxidation peak and that I_{p2} forms as an oxidation product of I_{p1} , further illustrating the presence of an ECE mechanism.

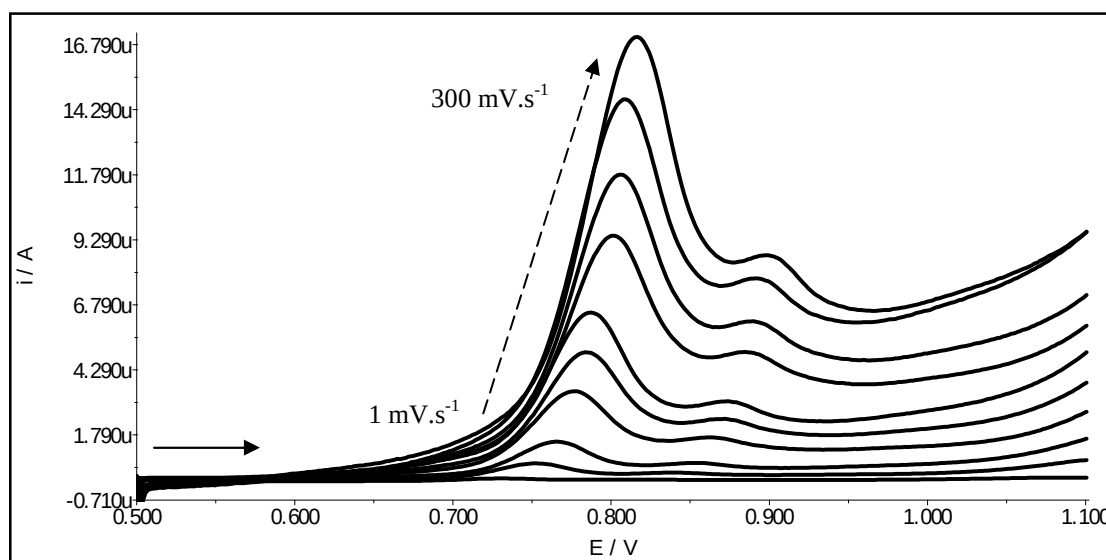


Figure 6.20: Representative peak profile of 20 μM CIT in 0.2 M NaOAc buffer pH 4.0 as a function of scan rate. LSV scans each with background current subtracted acquired under increasing scan rates of 1, 10, 25, 50, 75, 100, 150, 200, 250 and 300 mV.s^{-1} vs. Ag/AgCl after 120 seconds adsorption in each case.

The influence of scan rate was reported graphically as plots of I_{p1} in relation to square root of scan rate ($v^{-1/2}$) (Figure 6.21 [A]) and scan rate (v) (Figure 6.21 [B]).

In Figure 6.21 [A], at v exceeding 75 mV.s^{-1} , a linear relationship ($R^2 = 0.996$) was observed between I_{p1} and $v^{-1/2}$, indicative of an electrode process that is diffusion-controlled at v greater than 100 mV.s^{-1} , (Bard & Faulkner, 2001), similarly to analyses at high concentration (Section 6.4.6 (c)).

In Figure 6.21 [B] the entire range is linear when I_{p1} was plotted against v which suggested an adsorptive process (in accordance with Section 6.4.2) controlled by kinetics (Bard & Faulkner, 2001).

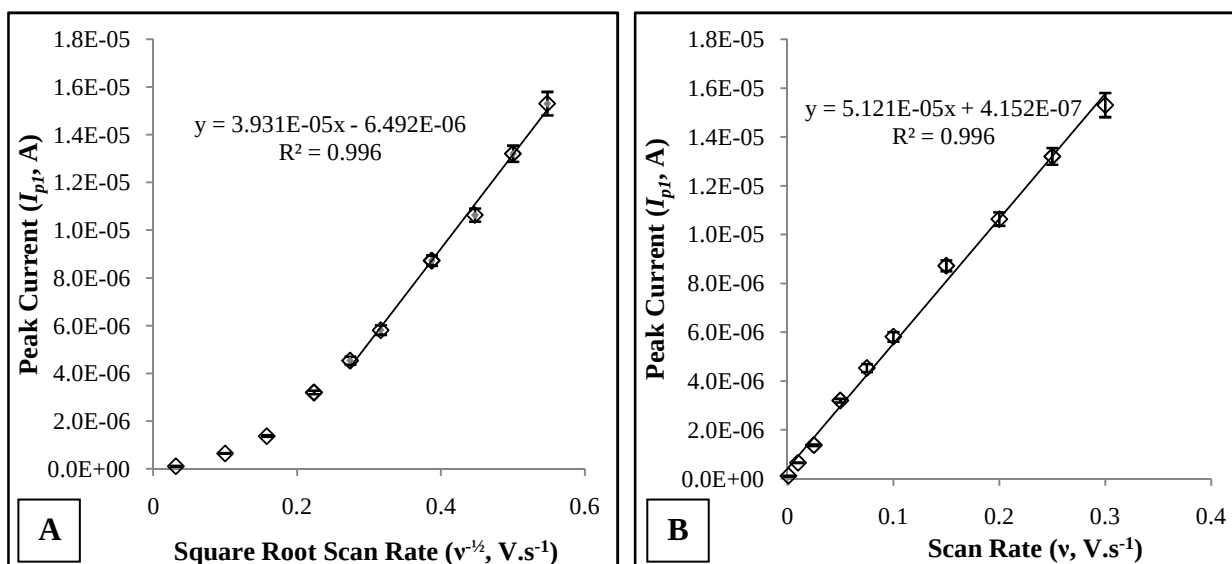


Figure 6.21: Linear plot of I_{p1} (with background currents subtracted) vs. [A] square root of the scan rate and [B] scan rate obtained for 20 μ M CIT in 0.2 M NaOAc buffer of increasing scan rate under adsorptive control (adsorptive preconditioning for 120 seconds). $N \geq 3$ scans per scan rate, error bars show standard deviation from mean.

b) Scan rate studies in aqueous solution as a diffusive process

Section 6.4.6 (c) illustrated that CIT I_{p1} arises as a result of combined I_{ads} and I_{diff} , though primarily due to I_{ads} . As a result of the complexity associated with the electroanalysis of CIT, CIT was assessed as a primarily diffusive controlled (I_{diff}) process by precluding any adsorptive periods in analysis such that accumulation and adsorption would be limited. The results of these analyses were reported as graphs of I_{p1} in relation to $v^{-1/2}$ (Figure 6.22 [A]) and v (Figure 6.22 [B]).

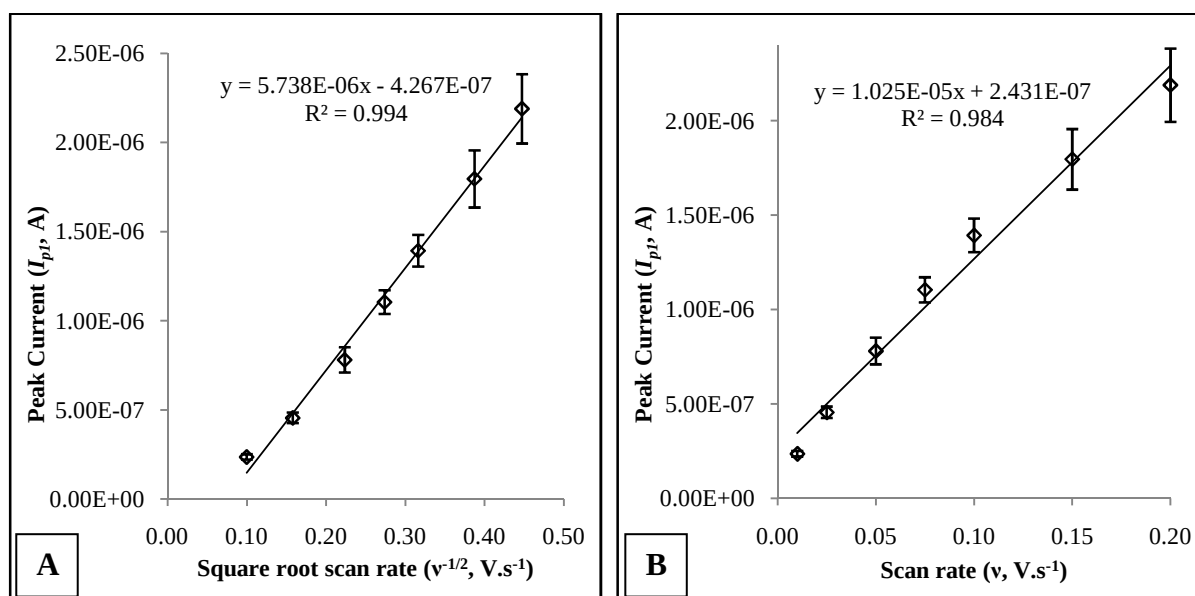


Figure 6.22: Linear plot of I_{p1} (with background currents subtracted) vs. [A] square root of the scan rate and [B] scan rate obtained for 20 μ M CIT in 0.2 M NaOAc buffer at scan rates of 10, 25, 50, 75, 100, 150 and 200 $mV.s^{-1}$ performed under diffusive control. $N \geq 3$ scans per scan rate, error bars show standard deviation from mean.

Figure 6.22 shows a linear range for CIT response between scan rates of 10 and 200 mV.s^{-1} , particularly in Figure 6.22 [A] with linearity in relation to the square root of scan rate ($R^2 = 0.994$), indicating that it can be assessed as a diffusion controlled process. Although linearity was observed in both [A] and [B] of Figure 6.22, Figure 6.22 [A], illustrates linearity with a greater correlation than when compared to scan rate directly ($R^2 = 0.984$). These responses suggest a primarily diffusion controlled process under these conditions, though similarity between the two processes suggests both diffusive and adsorptive processes may be involved.

c) Assessment of the influence of scan rate on CIT mass transport and adsorption

Due to the closeness of R^2 for Figure 6.22 [A] and [B], a further data analysis was performed to conclusively determine the predominant form of mass transport under adsorptive and diffusive control. Information pertaining to the characteristics of mass transport occurring at the surface of an electrode can be derived from assessing the slope (m) derived from a plot of $\log I_{p1}$ (A) vs. $\log \nu$ (V.s^{-1}) (Figure 6.23) (Laviron *et al.* 1980; Malinauskas *et al.* 2000; Bard & Faulkner, 2001; Lawrence & Wang, 2006; Hegde *et al.* 2008; Zidan *et al.* 2010; Okumura *et al.* 2011).

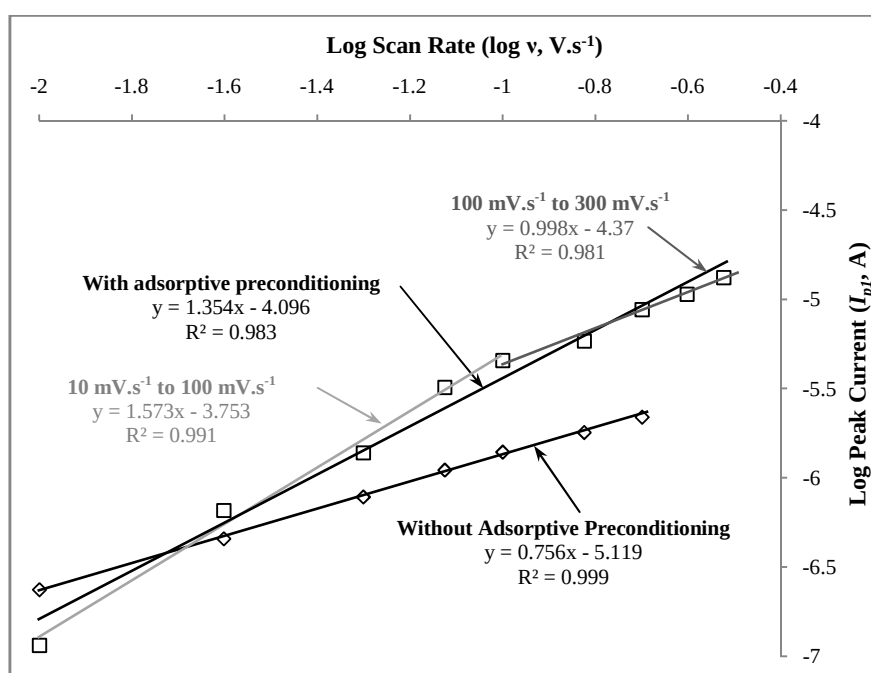


Figure 6.23: The relationship between log scan rate, ν and $\log I_{p1}$ for the oxidation of 20 μM CIT at EOx-GCE in 0.2 M NaOAc buffer pH 4.0. The data set labelled adsorptive process was attained with a 120 second adsorptive preconditioning period whilst the diffusive set was scanned without a preconditioning period.

A linear relationship between $\log I_{p1}$ and $\log \nu$ with a gradient (m) near the value of 0.50 describes a system which is purely diffusion-controlled (Hegde *et al.* 2008; Zidan *et al.* 2010), an m value of 1.0 or higher describes an ideally adsorptive system (Zare *et al.* 2005; Okumura *et al.* 2011) and values between 0.5 and 1.0 describe a transient zone of combined adsorptive and diffusive behaviour

(Malinauskas *et al.* 2000; Lawrence & Wang, 2006). The slopes of Figure 6.23 include analyses conducted both with an adsorptive preconditioning step (square markers) and without adsorptive preconditioning (diamond markers), which entailed scanning as soon as the electrode was introduced into CIT solution. The slope of the adsorptive set ($m = 1.354$) is indicative of a strongly adsorptive or kinetic controlled process (Zare *et al.* 2005; Okumura *et al.* 2011), and supports the findings of Section 6.4.2. Within this data set, two separate trends were evident (greyscale ranges) depending on scan rate and which has an inflection point at $\sim 100 \text{ mV.s}^{-1}$. The slope of the slower scan rate range was $m = 1.573$, which describes strongly adsorptive, kinetically-controlled, behaviour (Zare *et al.* 2005; Okumura *et al.* 2011). At scan rates higher than 100 mV.s^{-1} , a slope of 0.998 was found, which again shows adsorptive behaviour to predominate (Zare *et al.* 2005; Okumura *et al.* 2011). Similarly, the slope of the set determined in the absence of an adsorptive step ($m = 0.756$) suggests a system controlled by both adsorption and diffusion (Malinauskas *et al.* 2000; Lawrence & Wang, 2006), reinforcing observations of Section 6.4.7 [b]; ultimately, suggesting more complexity in the process controlling mass transport. These responses correlate closely with that reported for the absence of a linear relationship between I_{pl} and square root of scan rate ($v^{1/2}$) in Figure 6.20 [a]. This result suggests that the electrochemical processes for CIT are complex and that mass transport mechanisms differ with scan rate as well as solution concentration (Section 6.4.6 [c]), a response assessed further through Section 6.4.7 [d].

Figure 6.24 comprises of two plots which provide useful information pertaining to the reaction mechanism of CIT. According to Hegde *et al.* (2008) and Kissinger & Heineman (1996), the dependence of the current function ($I_{pl} \cdot v^{-1/2}$) on log scan rate ($\log v$) provides further information pertaining to the adsorptive processes at the electrode surfaces, the response of which is illustrated in Figure 6.24 [A]. Therein, an increase in current function with increase in v indicates an adsorptive process. As is evident in Figure 6.24 [A], under primarily adsorptive conditions (with adsorptive preconditioning), several separate stages of increase in current function is present, suggesting a multistage adsorption process (Murali *et al.* 2002; Hegde *et al.* 2008). When adsorptive preconditioning was not included (placing the system primarily under diffusive control), the extent of the increases was significantly lower (20-fold less) and responses remained more uniform across the entire scan range assessed, suggesting a more limited adsorption process occurring than under conditions considered to be under predominantly adsorptive mass transport control.

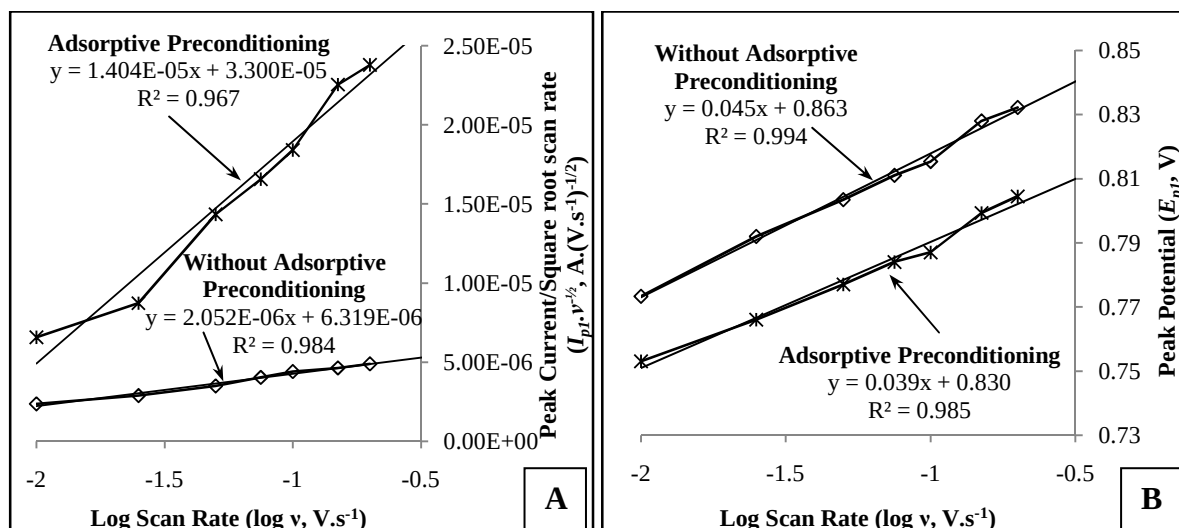


Figure 6.24: Figures detailing the dependence of log scan rate ($\log v$) with the current function (peak height/square root scan rate [$I_{p1} \cdot v^{-1/2}$]) [A] and peak potential (E_p) [B], for the oxidation of 20 μM CIT at EOx-GCE in 0.2 M NaOAc buffer pH 4.0. The data sets labelled adsorptive process were attained with a 120 second adsorptive preconditioning period.

Hegde *et al.* (2008) suggested that the influence of scan rate on peak potential (E_p) could be assessed by relating log scan rate ($\log v$) to E_{p1} (Figure 6.24 [B]) which provides information pertaining to the reaction mechanism. Therein, E_p is dependent on scan rate with linear plots of E_p vs $\log v$ with correlation coefficients of $R^2 = 0.985$ (adsorptive preconditioning) and $R^2 = 0.994$ (without adsorptive preconditioning). According to Hegde *et al.* (2008), such a response is consistent with an EC reaction where the electrode confined oxidation reaction is coupled to a subsequent irreversible chemical step. The lower peak potentials of the adsorbed analyses illustrates that oxidation of adsorbed compounds is more kinetically favourable, compared to the diffusive analyses (i.e. without adsorptive preconditioning in Figure 6.24 [B]). As the two trends exhibit nearly equal gradients 45 and 39 mV for diffusive and adsorptive analyses, respectively), this indicates that although kinetic differences occur, the mechanism by which oxidation of CIT proceeds is likely to be conserved under both adsorptive and diffusive conditions.

6.4.8 Assessment of CIT oxidation reaction mechanism in aqueous media at EOx-GCE

Although a further set of analyses at 20 μM was performed in solutions of 25 % methanol (Appendix E), insignificant differences were encountered as compared to the purely aqueous systems reported previously, and thus those analyses were not reported on herein.

a) Determination of the number of electrons transferred (n_e) in oxidation of CIT

When assessing peak potential under varying electrolyte pH (Section 6.4.4 [e]), gradients ranging between 30 to 33 mV.dec^{-1} were found under each of the solution conditions assessed, including CIT under both adsorptive and diffusive control assessed in both aqueous media and 25% methanol, which

are indicative of an oxidation mechanism for CIT involving an uneven number of electrons to protons and often represent a $2e^-$ (n_t) and $1H^+$ transfer (Costentin *et al.* 2010). This is a result which further illustrates uniformity on the oxidation mechanism of CIT under the conditions investigated in this study. It should be noted that n_t reported herein refers to I_{p1} , and illustrates the E component of the $E_rC_iE_r$ reaction mechanism reported on through this Chapter.

Due to I_{p1} being considered to arise from a reversible electrochemical process (due to wave shape, Chapter 3, Section 3.4.4), calculation of n_t was achieved through determination of the magnitude of the difference in potential at half peak height ($\Delta E_{p, \frac{1}{2}}$), and calculating n_t according to Eq. 6.5 (Bard & Faulkner, 2001).

$$\Delta E_{p, \frac{1}{2}} = 3.53 \frac{RT}{n_t F} = \frac{90.6}{n_t} \quad \text{Eq. 6.5}$$

These analyses were carried out on CIT I_{p1} responses, scanned at scan rates of 10, 25, 50, 75, 100, 150 and 200 mV.s^{-1} , and in each case allowing for both adsorptive preconditioning (primarily kinetically controlled) and without adsorptive preconditioning (primarily diffusively controlled). The resulting averaged n_t responses across scan ranges assessed for adsorptive preconditioning was $n_t = 1.401 \pm 0.083$, and was for $n_t = 1.540 \pm 0.130$ for without adsorptive preconditioning. As, in both cases, n_t calculated in this manner was consistently greater than 1, n_t was determined to be $2e^-$. This thus further illustrates uniformity in the mechanism of oxidation of CIT.

A further analysis was performed to corroborate n_t calculated above. This was performed through relation of peak potential (E_{p1} , V) and the natural logarithm ($\ln$) of scan rate (ν , V.s^{-1}), as was applied by Santos & Sequeria (2010). This scenario is detailed by Bard & Faulkner (2001) for the case of a reversible electrochemical (E_r) followed by an irreversible chemical step (C_i) in an EC mechanism assessed by linear sweep voltammetry, with relation to the dimensionless kinetic parameter and λ , as is the case in these analyses (Eq. 6.6).

$$E_p = E_{\frac{1}{2}} - \frac{RT}{n_t F} 0.780 + \frac{RT}{2n_t F} \ln \lambda \quad \text{Eq. 6.6}$$

Under these conditions, if I_{p1} is a result of an E_rC_i mechanism, the peak will shift towards more negative potentials at a rate of approximately $\frac{30}{n_t}$ mV per decade of increase in scan rate (ν , V.s^{-1}) at 298 K (Bard and Faulkner, 2001; Scholz, 2002). Figure 6.25 illustrates the relation of E_{p1} (V) and $\ln \nu$ (V.s^{-1}) for adsorbed and diffusively analysed CIT I_{p1} .

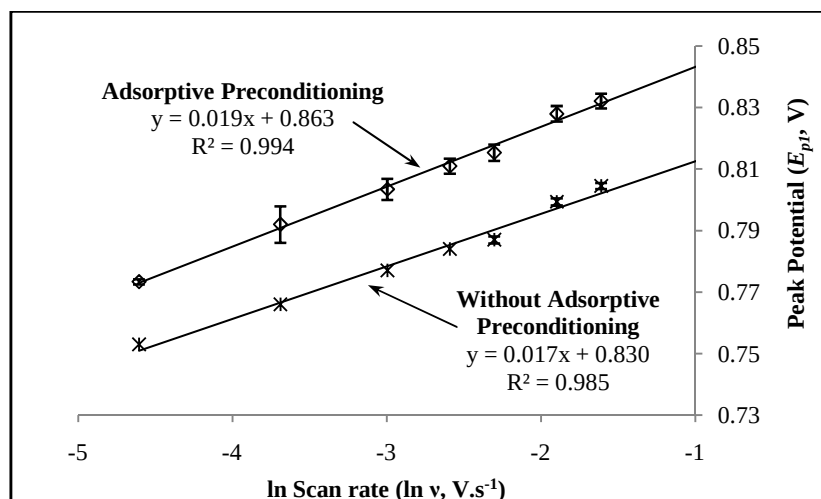


Figure 6.25: Figures detailing the dependence of natural log of scan rate ($\ln v$, $V.s^{-1}$) with peak potential (E_{p1} , $V.s^{-1}$). The data sets labelled adsorptive preconditioning were attained with a 120 second accumulation period prior to scanning.

In accordance with a shift of E_{p1} at a rate of approximately $\frac{30}{n_t}$ mV per decade, gradients of 19 $mV.dec^{-1}$ and 17 $mV.dec^{-1}$ were recorded for adsorptive and diffusive analyses respectively in Figure 6.25. . As the gradients correspond with the assumption presented, these results firstly provide further evidence that the oxidation mechanism of CIT I_{p1} is an E_r reaction followed by a rapid C_i process. This method of analysis determined n_t to be 1.58 and 1.76 electrons, for adsorptive and diffusive mass transport regimens respectively, confirming that a 2 electron transfer electrochemical process occurs during the generation of I_{p1} . The presence of a $2e^-$ process has been previously reported a closely structurally related compound which exhibits very similar electrochemical properties in the form of salicylic acid (Torriero *et al.*, 2004).

The author notes that more detailed kinetic analyses are likely to be yielded through digital simulations of the reaction mechanism, which couldn't be performed for this study due to technical limitations. Doing so would reveal information beyond the relatively simple E_rC_i mechanism outlined for I_{p1} oxidation herein, likely illustrating a ECECE mechanism, and providing information regarding the generation of I_{p2} and I_{p3} . As these peaks were not the focus of this Thesis however these analyses were not included herein and are a consideration intended for future supplementary studies.

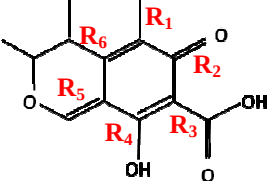
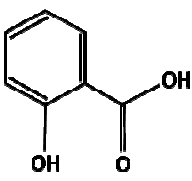
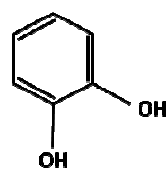
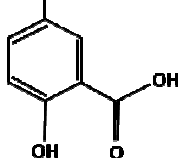
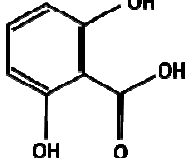
6.4.9 CIT oxidation mechanism

a) Correlation of CIT voltammetry with analogues

The source of electrons involved during the oxidation of CIT was investigated through comparison of voltammetric profiles of CIT with analogues of CIT. As CIT had not previously been studied

electrochemically, the analogues were selected according to their structural similarity to CIT, with specific focus on the hydroxyl bearing ring highlighted in relation to CIT, as shown in Table 6.6.

Table 6.6: Structures of CIT and its analogues assessed in this study

		
Citrinin (CIT)	Salicylic Acid (SCA)	Catechol (CAT)
		
5-Methylsalicylic Acid (5-MSA)	2,6-Dihydroxybenzoic Acid (2,6-DHBA)	Phenol (PHE)

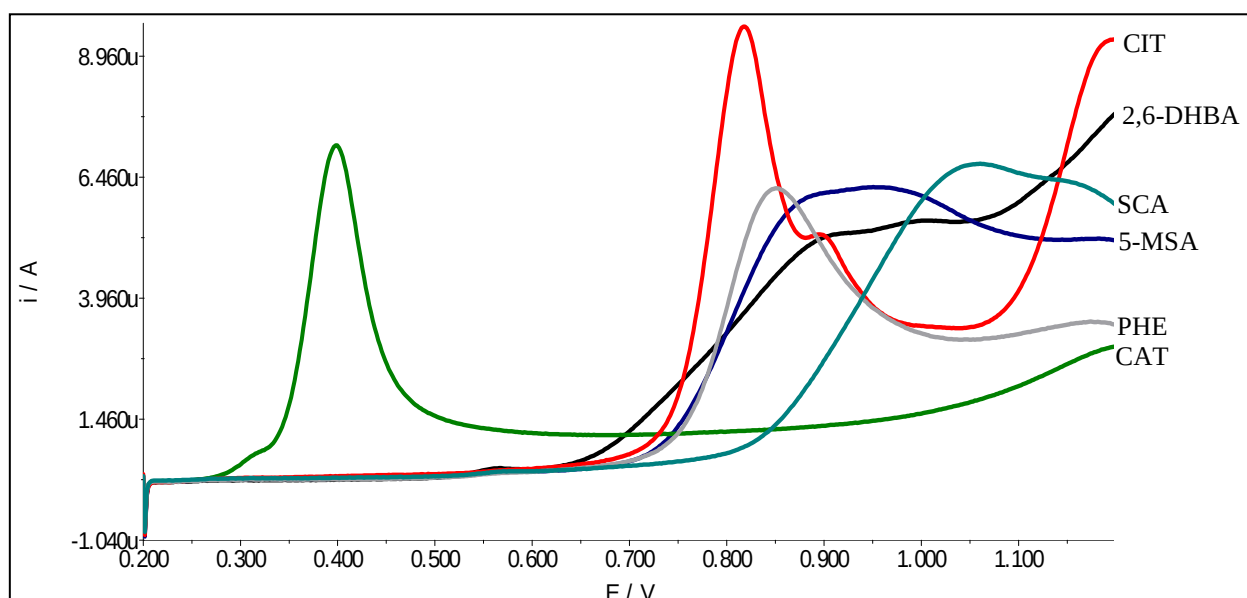


Figure 6.26: LSV scans of 50 μM CIT (CIT, red), 2,6-dihydroxybenzoic Acid (2,6-DHBA, black), Salicylic Acid (SCA, cyan), 5-Methylsalicylic Acid (5-MSA, blue), Phenol (PHE, grey) and Catechol (CAT, green). Scan rate $100 \text{ mV}\cdot\text{s}^{-1}$ from 0.2 V to 1.2 V at EOxGCE with 120 second adsorption period in 0.2 M NaOAc buffer pH 4.0. Background currents were subtracted from scans in each case.

Electrochemical analyses of the analogues of CIT were reported in relation to the electrochemical profile of CIT in Figure 6.26, each conducted under equivalent scanning conditions in 0.2 M NaOAc buffer, pH 4.0. The electrochemical profiles of the CIT analogues revealed that compounds which comprised of both hydroxyl and carboxyl moieties (2,6-DHBA [black], SCA [cyan] and 5-MSA [blue]) each produced a twin peak profile. In the absence of the carboxyl group at position R_3 , the double peak profile was lost (PHE, [grey] and CAT, [green]), and only a single peak associated with the hydroxyl moieties was encountered. Of further key importance was the finding that when both the

carboxyl and hydroxyl moieties were present, the primary peak was likely to be that of the hydroxyl moiety (Torriero *et al.* 2004). The peaks of 2,6-DHBA (black) and 5-MSA (blue) are very similar to one another and are lower than SCA (cyan), which is a more simple molecule. This suggests that the carboxyl group is involved in the EC process in aqueous media. When side groups are present in positions R₁ and R₂, the electron holding capability of the carboxyl is reduced as there is a more even distribution of charge across the molecule, and the position of I_{p1} shifts closer to that reported for CIT with greater structural similarity to CIT.

PHE and CAT provide marked resemblances to the CIT peak. In particular the PHE peak, is arises at a similar E_p . As no carboxyl group was present on either of these molecules, the electron contributing group is likely to be that of the hydroxyl group. This finding was corroborated by studies by Torriero *et al.* (2004) who showed that the I_{p1} of SCA was also as a result of oxidation of the and hydroxyl moiety.

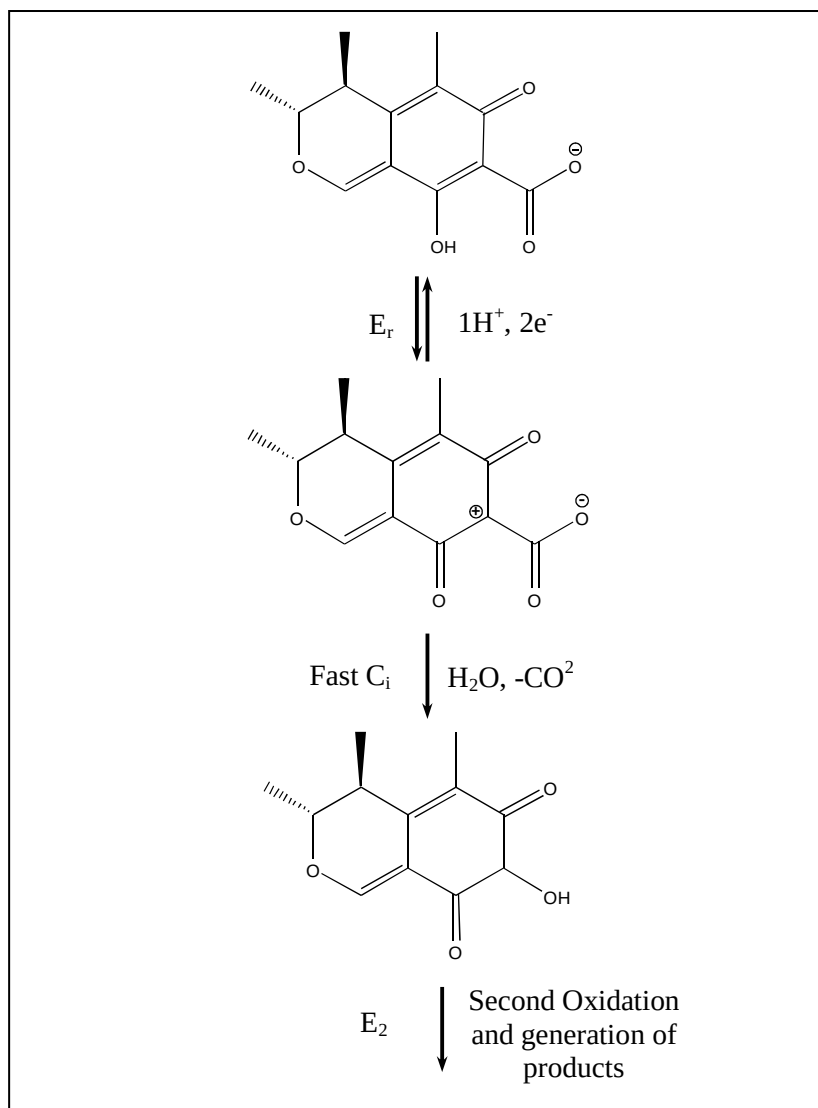
Accordingly, when CIT is oxidised the mechanism plausibly involves the oxidation of the hydroxyl group at position R₄, and the potential at which this group is oxidised is governed by the substituent's surrounding the hydroxyl group on the ring. This finding correlates well with the finding that the oxidation of CIT is a 2e⁻.

b) Proposed scheme of CIT oxidation in aqueous media

In Section 6.4.9 (a), both hydroxyl and carboxyl moieties are implicated in the position of I_{p1} subsequent generation of I_{p2} . Oxidation of CIT I_{p1} was shown to involve an uneven number of protons and electrons (Section 6.4.4 (d)), later illustrated to be 2e⁻ (Section 6.4.8 [a]) and hence 1H⁺. As has been illustrated by several studies in this Chapter, the oxidation mechanism of I_{p1} is likely to proceed via a rapid, reversible electrochemical step (E_r), followed by a rapid irreversible chemical step (C_i). According to these identified parameters, the following reaction scheme is proposed with further mechanistic studies being required for elucidation of the E and C pathways, as it denotes to I_{p2} , (Section 6.4.8 [a]).

In Scheme 6.1, the oxidation of CIT is proposed to initiate with a reversible electrochemical step, involving 1H⁺ and 2e⁻, followed by a rapidly occurring irreversible chemical step through interaction with H₂O. The interaction of CIT with H₂O has previously been reported by Trivedi *et al.* (1993) in the formation of CIT H1. Although the final structure of CIT H1 differs slightly from the structures reported in Scheme 6.1, the reaction scheme reported by Trivedi *et al.* (1993) illustrates the decarboxylation of CIT and incorporation of further hydroxyls in the presence of H₂O. The reaction of

the oxidised form of CIT with H_2O is proposed to occur more readily than the proposed reduction of oxidised CIT, thereby accounting for the absence of a return peak in CV analyses.



Scheme 6.1: Chemical oxidation mechanism of CIT at pH 4.0 in aqueous media

6.5 Summary and conclusions

The mechanism of mass transport, oxidation mechanism and electrochemical kinetic assessment of CIT oxidation was voltammetrically analysed at EOx-GCE. The use of EOx-GCE as electrode surface was selected, as this surface modification provided a clearly defined and stable I_p response. As no major variations in the CIT peak profile, or pH-dependent peak potential shift, were encountered at EOx-GCE in relation to polished GCE, the mechanism of oxidation at EOx-GCE was deemed uniform, and allowed for identification of electrochemical parameters of CIT oxidation.

Through assessments of the adsorptive traits of CIT across several studies, it was shown that CIT both accumulates into the oxide layer and adsorbs to graphitic oxide, as greater than monolayer coverage of the analyte at the electrode surface was found to occur. Although the accumulation of CIT into the oxide layer was suggested to aid in the improved analytical characteristics of CIT, this process was shown to be a rate limiting procedure, wherein accumulation of CIT into the oxide layer hindered transport to the electrode surface. The presence of the oxide layer also resulted in a greatly extended replenishment time of unoxidised CIT at the oxide layer (relative to unmodified GCEs), and may also have contributed to a higher degree of fouling. Although parameters including the extent of oxidation and solution pH were investigated to mitigate these findings, little improvement was found.

Solution conditions were found to have a marked influence on the voltammetric profile of CIT analysis. In pH studies, a rapid drop in I_{p1} was encountered from pH 1.0 through to pH 3.0, however between pH 4.0 and pH 6.0, the peak profile was stable. Levelling of I_{p1} responses above pH 3.0 was likely to be due to the pKa of CIT being determined at 3.13 for ionisation of the carboxyl moiety. Analyses at alkali pH revealed that CIT is sensitive to a reversible process which altered the peak profile of CIT wherein I_{p1} was masked by the formation of I_{p17} . This procedure was likely to be arise as a result of either extensive deprotonation of the molecule or a shift toward an isomeric form. This trait limited analyses to acidic pH, with pH 4.0 being primarily applied due to the stability of responses and to maintain parity with environmental samples detailed in Chapter 7. This study also, illustrated that CIT is not irreversibly affected by pH and thus CIT present in an alkali medium can be accurately determined following titration to an acidic pH. The shift of E_{p1} with pH was found to be relatively stable, suggesting that a conserved oxidation mechanism, irrespective of the solvent and electrode conditions applied in this study. The resulting gradients of the pH study, approximately 30 mV.dec⁻¹, suggested further that the oxidation mechanism was likely to be an uneven number of protons and electrons, and furthermore likely to be a $2e^-$, $1H^+$ process. This process was also found to be independent of the adsorptive and diffusive mass transport methods investigated in this study. Hence, the oxidation mechanism of CIT appears to be relatively uniform in aqueous media.

Supporting electrolyte was found to influence CIT slightly, with electrolyte-specific electrochemical responses of CIT. As the BR and NaOAc were closely comparable to one another and offered stability over a large pH range with regard to both E_{p1} and I_{p1} , they were selected for further analyses.

Establishment of a concentration curve illustrated that the extent of accumulation plateaus at higher concentrations due to saturation of the oxide layer by the analyte, slowing the rate of uptake and amount of CIT oxidised in relation to solution concentration and thus resulted in a curved concentration profile. From those analyses, an LOD of 20 nM was determined (from LSV analyses), though with application of DPV an LOQ of 16 nM was established, a response which is highly competitive in relation to many of the commercially applied chromatographic LODs, especially as this provides a more rapid, less expensive and less time consuming technique.

Investigation of the oxidation reaction mechanism of CIT was carried out through scan rate studies. Further evidence describing the mixed diffusive-adsorptive characteristics of CIT were illustrated and as it was demonstrated that analyses could be carried out under primarily diffusive control, it was used as a comparative scheme to corroborate results responses determined under adsorptive control. The analysis of E_p in relation to pH range revealed that the oxidation mechanism for CIT involved an uneven number of electrons to protons, and was later illustrated $n_e = 2e^-$ which was accompanied by transfer of $1H^+$ per molecule oxidised. According to the parameters identified, a reaction scheme for the initial stages of CIT oxidation (I_{p1}) was proposed, involving both hydroxyl and carboxyl moieties of the CIT molecule, which appears to be confirmed through the voltammetry of structural analogues of the hydroxyl/carboxyl-bearing ring on CIT.

CHAPTER 7

Electroanalysis of Citrinin in Complex Media and Environmental Samples

7.1 Introduction

As stated in Chapter 1, the presence of mycotoxins in foods is generally considered to be unavoidable due to the prominence of fungi on foods through growth, storage of raw crops, processing of those crops well as storage of processed foods. As was further detailed through Chapter 1, although prevalent in developed nations, developing nations are at greater risk of mycotoxin ingestion. As was further detailed in Chapter 1 Section 1.1.1, when combined with typically poor medical treatment in developing countries, the impacts of mycotoxin exposure are marked and affect both persons and animals consuming those foods, having far reaching medical and socioeconomic impacts. As a result, specific attention has recently been paid to monitoring of crops and foodstuffs such that the presence of toxins can be assessed prior to consumption and appropriate steps taken to ensure that the crop can be produced as a toxin-free product.

Various methods for the detection of mycotoxins and CIT in particular have been proposed in literature for analysis of both solids and liquid, of which reversed-phase high performance liquid chromatography (RP-HPLC) with ultraviolet (UV) detection is the most widely used (Xu *et al.* 2006). Other reported methods include HPLC with fluorescence detection (FD), gas chromatography (GC), thin layer chromatography (TLC), capillary electrophoresis (CE) with either pre-column or post-column derivatisation, flame ionisation detection (FID), or electron-capture detection (ECD) and mass spectrometry (MS) (Scott *et al.* 1972; Placinta *et al.* 1999; Krska & Molinelli, 2007; Krska *et al.* 2008).

In HPLC analyses, although FD has been reported for CIT, analysis of mycotoxins in general by FD requires that detectable fluorescence of the mycotoxin occurs. HPLC with electrochemical (EC) detection has been shown to provide increased sensitivity toward the detection of some phenolic compounds (Ruana & Urbe, 1993; Pocurull *et al.* 1996). Furthermore, electrochemical detection (EC), unlike UV detection, does not allow for the use of gradient elution or the use of all liquid chromatography (LC) solvents (Ruana & Urbe, 1993; Angelino & Gennaro, 1997). Due to the fouling behaviour detailed for CIT through this Thesis, it is furthermore unlikely that HPLC-EC could be readily applied in analysis of such mycotoxins.

Literature reveals that reverse phase high-performance liquid chromatography (RP-HPLC) is the most commonly used method for the analysis of CIT from environmental samples. Detectors applied in these analyses were mainly UV or FD and an acidic mobile phase was generally used (Jackson & Ceigler 1978; Phillips *et al.* 1980; Gimeno & Martins 1983; Vail & Homann 1990; Franco *et al.* 1996; Reinhard & Zimmerli 1999; Vázquez *et al.* 2001; Meister, 2004; Xu *et al.* 2006; Mossini & Kemmelmeier, 2008; Dohnal *et al.* 2010). Due to the reported stability of response as well as equipment availability, UV detection was selected for analysis of CIT (as a validation in these studies), similar to that reported by Vail & Homann (1990) and Philips *et al.* (1980).

While highly sensitive and selective, HPLC is not a rapid technique and is relatively expensive. It requires extensive sample pre-treatment and highly pure samples and solvents. This, in combination with being laboratory confined and requiring skilled personnel to operate the equipment (which is, itself, costly), making this a relatively expensive technique. The electrochemical approach suggested in this Thesis is thus investigated as a platform for the establishment of a methodology which can be applied in the field as a low expense diagnostic tool to quantify CIT contamination and allow for exposure to be reduced.

Electroanalytical, and bio-electroanalytical systems, have often been afforded the distinction of allowing for direct analysis in complex media such as real world samples with both specificity and sensitivity combined with minimal, and in some cases, no sample preparation (Baeumner *et al.* 2003; Velasco-Garcia & Mottram, 2003; Rodriguez-Mozaz *et al.* 2004; Krska & Molinelli, 2007; Krska *et al.* 2008). These properties have a further marked benefit of allowing for a broad range of sample matrices to be assessed with little resulting influence on the analytical system, thereby affording broad application in a variety of sample matrices with relative ease (Velasco-Garcia & Mottram, 2003; Krska *et al.* 2008). Although a marked benefit with regard to practical application, the possible influence of the constituents of environmental media on electroanalysis of the target analyte must be assessed to determine the extent of the applicability of electroanalysis within those detection matrices. In this case, this entails assessment of electroanalysis as a viable system for monitoring of CIT and other such toxins in agricultural commodities. Mycotoxins have indeed been shown to adsorb to a number of substrates which may be encountered in real world samples (Huwig *et al.* 2001).

Penicillium citrinum (*P. citrinum*) is a commonly encountered fungal species, several strains of which have been reported to produce citrinin as a secondary metabolite (Deshpande & Sarje, 1966; Davis *et al.* 1975; Vining, 1990; Vázquez *et al.* 2001; Mossini & Kemmelmeier, 2008; Gao *et al.* 2011), the characteristic for which the strain was named. Several strains have also been demonstrated to produce

other biocidal compounds such as ochratoxin A (Rosa *et al.* 2006) and penicillic acid (Delgado *et al.* 2011). The occurrence of *P. citrinum* is relatively widely reported and has been isolated in soils (Vining, 1990), from hypersaline aqueous environments (Cantrell *et al.* 2006) and importantly with regard to these studies, from crops such as rice, wheat and maize (Davis *et al.* 1975; Jackson & Ciegler, 1978; Comerio *et al.* 1998; Boca *et al.* 2002) and poultry feeds (Rosa *et al.* 2006). *P. citrinum* has been widely cultured under laboratory conditions in a number of both solid (agar gels) and liquid (broth media) substrates (Deshpande & Sarje, 1966; Jackson & Ciegler, 1978; Davis *et al.* 1975; Comerio *et al.* 1998; Vázquez *et al.* 2001; Mossini & Kemmelmeier, 2008; Gao *et al.* 2011). These attributes render this species an attractive choice as a case study for the detection of CIT in complex media, as it combines time-dependent production of CIT with the time-dependent change in media constituents as a result of the fermentation.

Through the course of this Thesis, the electrochemical analysis of CIT has been investigated at carbon and modified carbon electrodes. As was illustrated in Chapter 6, the application of EOx-GCE allowed for highly reproducible and sensitive analysis of CIT in buffered solutions. Analysis of compounds in environmental samples is however more complex due to the influence of the numerous compounds in the detection matrix which may impede, or mask, analysis of the compound of interest. The research reported in this Chapter, as such, sought to investigate the electrochemical analysis of CIT within environmental samples and culture media and further investigates interactions which CIT may have with co-interferents present in these solutions.

7.2 Chapter Objectives

This Chapter is directed at investigating the conditions under which the electroanalysis of CIT could be practically applied toward the analysis of real world samples. The overarching aims of this Chapter thus included investigation of the electrochemical detection characteristics of CIT in environmental samples as complex media and to ascertain whether detection can proceed directly (with no sample preparation) in aqueous solution, to retain the simplicity and low analytical expense of the analysis system. In order to develop a successful monitoring protocol for CIT detection under an array of varied solution conditions, as would be expected of environmental samples, the following objectives needed to be addressed:

- a) Assess the effects of separate components likely to be encountered in food(s) samples (proteins, lipids and carbohydrates) and determine the influence of these parameters on the

electrochemical behaviour of CIT and its analysis directly in aqueous media. This was achieved by the evaluation of the effects of:

1. The influence of a range of components likely to be encountered in food/environmental samples in a range of complexities,
 2. The influence of solutions of ground grains (maize flour and rice flour) on the analysis of CIT in aqueous solution.
 3. Culture media, as the culture of *P. citrinum* would influence the composition of media over time as well as produce CIT.
- b) Produce CIT through culture of *P. citrinum* as an example of a complex matrix
- c) Ascertain whether extraction of CIT from environmental samples into an organic phase by liquid-liquid extraction will offer improved detection of CIT. This objective included the evaluation of:
1. HPLC analysis of extracted samples of *P. citrinum* culture media.
 2. Electroanalysis of extracted samples of *P. citrinum* culture media for comparison to HPLC responses.

7.3 Chapter specific methods and materials

The procedures, reagents and equipment detailed in Chapter 2 were followed herein unless otherwise stated. Specific details on the experiments carried out in Chapter are illustrated hereafter with reference to their specific sections.

7.3.1 Analyte, reagents and media components

A stock solution of 5 mM Citrinin (CIT) was prepared with dehydrated absolute methanol and stored at $-20\text{ }^{\circ}\text{C}$ until used. Sulphuric acid (98 %) was obtained from Holpro Lovasz (South Africa). Sodium hydrogen carbonate was obtained from Saarchem[®]. Ammonia solution (25 %), D (+) glucose, n-hexane (SupraSolv[®]), chloroform, potato dextrose agar (PDA), nutrient broth (NB) and yeast extract were each sourced from Merck whilst L-lysine ($\geq 98\%$), albumin from bovine serum (BSA), Bradford reagent, acetonitrile ($> 99.9\%$), mineral oil and methanol ($> 99.9\%$) were purchased from Sigma-Aldrich. In order to assess representative samples likely to be encountered in real samples analysis, fructose, xylitol, starch, olive oil (virgin), sunflower oil, ground maize flour and rice flour were all purchased from a local retail outlet.

7.3.2 Electrochemical procedures and parameters

A three electrode electrochemical cell was utilised with a glassy carbon (GCE) working electrodes, a platinum auxiliary electrode and Ag/AgCl reference electrode was applied in all electrochemical studies with the apparatus described through Chapter 2, Section 2.2.1. Electro-oxidation of GCE, (Chapter 2, Section 2.3.1 [b]) was applied at all electrodes unless otherwise stated and the final scan of electrochemical conditioning (Section 2.3.1 [c]) was recorded as the background signal.

Electrochemical conditioning (Section 2.3.1 [c]) was carried out in the respective analysis medium (buffered solutions not containing lipids, carbohydrates, proteins etc., and in the absence of CIT), and the final scan recorded as the background signal. All analyses unless otherwise indicated were carried out in 0.2 M NaOAc buffer pH 4.0 with CIT diluted into solution at 20 μM final concentration. In each case, samples were analysed via LSV from 0.5 V to 1.1 V at $0.1\text{ V}\cdot\text{s}^{-1}$, with in-scan preconditioning included as an adsorption period of 120 seconds at 0.4 V included (Chapter 3), for 120 seconds with solution agitation at 2000 rpm (Chapter 3).

Analyses of environmental samples (spiked rice and maize flour as well as fungal culture media) were assessed in a 1 : 3 v/v 0.8 M NaOAc buffer : sample medium. As media in which fungi or bacteria are cultured alter in composition over the culture period due both to uptake of components by the cultured

fungus and the release of metabolites into solution (Madigan *et al.* 2000), electrochemical conditioning for analyses in culture media was conducted in 1:3 v/v 0.8 M NaOAc buffer : minimal salt medium (MSM) as a stable reference to establishing a background signal.

7.3.3 Voltammetric assay of CIT in complex media

a) Voltammetric assay of possible interferents in real world samples

Constituents of media were separated into groups according to their chemistry. Proteins (ammonia, yeast extract, L-lysine and albumin from bovine serum [BSA]), carbohydrates (glucose, fructose, xylitol and starch) and lipids (mineral oil, olive oil and sunflower oil) were selected. Media were each prepared at 1 mg.ml⁻¹ in 0.2 M NaOAc, with the exception of ammonia (which formed ammonium hydroxide in the aqueous media) which was prepared at 3.41 μ g.ml⁻¹. The lower amount of ammonia was selected as a result of higher concentrations masking the electrochemical profile of CIT.

b) EOx-GCE surface-confined impact of complex media on CIT responses

GCEs which had been electro-oxidised, but not electrochemically conditioned, were dipped in solutions containing L-lysine, fructose and maize flour solution (Section 7.3.2 [c]) for 30 seconds (referred to as a doped EOx-GCE) and then rinsed with milliQ H₂O. Thereafter, electrodes were electrochemically conditioned in 0.2 M NaOAc buffer and then immediately thereafter responses at the doped EOx-GCE electrode was assessed in a solution of 20 μ M CIT in 0.2 M NaOAc buffer. This was performed in order to assess the impact of media constituents bound or adsorbed, to the EOx-GCE surface.

c) Analysis of CIT in spiked real world samples

Maize and rice were assessed as detection matrices due to the high consumption of these products. To that extent, 10 grams of maize flour and rice flour were soaked in 50 ml of 0.2 M NaOAc buffer, pH 4.0, overnight at 4 °C. The supernatant of each was collected and used as the analysis medium, and were referred to as maize flour and rice flour solution, respectively. CIT was spiked into solution at 20 μ M and 40 μ M concentrations and voltammetric analyses were conducted directly on media with no sample pre-treatment as in section 7.3.2 [a].

d) Analysis of CIT from Penicillium citrinum culture media

CIT was assessed both directly in the culture media as well as in solutions which were extracted from the culture media (Section 7.3.4 [b]). For direct analysis, 3 ml of culture media were assessed with 1 ml of 0.8 M NaOAc buffer pH 4.0 (i.e. 1 : 3 v/v 0.8 M NaOAc buffer : sample medium, as in Section 7.3.1). Background currents were recorded in MSM media in an equivalent ratio with 0.8 M NaOAc

buffer, pH 4.0. As the constituents of the media would alter throughout the culturing of *P. citrinum*, MSM was used as a uniform baseline.

Analyses of extracts from media were performed by spiking 400 μl of extracted samples into 0.2 M NaOAc buffer, pH 4.0, to a final volume of 4.0 ml. Responses were reported in relation a concentration curve established from the same samples applied in the establishment of the HPLC concentration curve (Section 7.3.5 [c]) at concentrations of 1, 3, 5, 10, 20 and 30 μM respectively, each of which was performed in triplicate as a minimum.

7.3.4 Spectrophotometric analyses

Spectroscopic analyses including UV/Vis and fluorescence spectroscopy, were carried out with a Synergy Mx monochromator-based multi-mode microplate reader with equipment control and data analysis performed using Gen5 (Version 1.10) data analysis software (BioTek instruments, inc).

a) Fluorescence analysis of CIT

The solution-phase impact of media components on CIT was assessed by spiking solutions containing L-lysine, yeast extract or fructose (each at 1 $\text{mg}\cdot\text{ml}^{-1}$) or maize meal solution (Section 7.3.4), each in 0.2 M NaOAc buffer pH 4.0 with CIT and assessing the resulting fluorescence spectra. Samples were analysed in 200 μl aliquots in a 96-well polystyrene cell culture microplate with a flat $\mu\text{Clear}^{\circledR}$ bottom (Greiner bio-one). Samples were measured in triplicate with excitation at $\lambda = 330\text{ nm}$ and emission at $\lambda = 500\text{ nm}$ for endpoint analyses (Trantham & Wilson, 1984; Vail & Homann, 1990; Vázquez *et al.* 1997), as well as with excitation at 330 nm and measured emission spectrum of 400 nm to 600 nm with 5 nm intervals. Spectral emission analyses were performed to determine possible shift in CIT fluorescence peaks due to complexation with components of media examined.

b) Assessment of total protein concentration in media via Bradford assay

In order to identify the total protein content of the complex media which were assessed, Bradford assays were conducted (Bradford, 1976). As media were made in both 0.2 M NaOAc buffer pH 4.0 and minimal salts medium (MSM) (described in Section 7.3.4), reference standard concentration curves were established in both of the media. The standard concentration curves against which samples were compared were established using bovine serum albumin (BSA) at 0, 0.01, 0.05, 0.1, 0.2, 0.4, 0.6, 0.8, 1.0, 1.2 and 1.4 $\text{mg}\cdot\text{ml}^{-1}$. Bradford assays were carried out with absorbance measured at $\lambda = 595\text{ nm}$, with protein content reported as a colour change from red to blue as a result of complexation of protein with the dye, Brilliant Blue G (Compton & Jones, 1985). In each assay, 10 μl of protein-bearing solution were added to 240 μl Bradford reagent, allowed to stand at 25 $^{\circ}\text{C}$ for 5 minutes before recording absorbance.

7.3.5 Culturing of *Penicillium citrinum*

A vacuum-dried culture of *Penicillium citrinum* (*P. citrinum*), strain designation Sp. 465, which had been characterised as a CIT producing strain was purchased from the Deutsche Sammlung von Microorganismen und Zellkulturen (DSMZ) in Germany. Due to this it was assumed to be a pure strain and to produce CIT. Thus, no microscopy was needed to confirm the morphology of the isolate. The strain was rehydrated with nutrient broth (NB) and then sub-cultured on potato dextrose agar (PDA) plates as well as PDA slants as solid media. The PDA plates were used in all subsequent inoculations of media assessed and the PDA slants were refrigerated and stored as stock cultures.

a) Preparation of media for culture of *P. citrinum*

In order to apply growth conditions and thus produce subsequent analysis media which would be representative of real world samples, liquid culture media were prepared from ground grains directly rather than purchased media. As was described by Abbasi & Mortazavipour (2011), minimal salts medium was applied as the base for liquid growth media, such that trace elements and micronutrients would be made available to the culture fungi. The minimal salts medium consisted of (per litre of MilliQ water) 1.0 g of K_2HPO_4 , 0.6 g of NaH_2PO_4 , 0.2 g of $MgSO_4 \cdot 7H_2O$, 0.2 g of KCl, 2 mg of yeast extract, and 1 ml of a trace element solution which comprised 0.05 mg of H_3BO_3 , 0.2 mg of $CaSO_4$, 0.1 mg of $CoSO_4$, 0.2 mg of $CuSO_4$, 3 mg of $FeSO_4$, 0.02 mg of $MnCl_2$, 0.1 mg of $NaMoO_4 \cdot 2H_2O$, 0.02 mg of $NiCl_2$, and 0.03 mg of $ZnSO_4 \cdot 7H_2O$ (Park, *et al.* 1999).

To the minimal salts medium, ground maize meal and ground rice flour were added as the nutrient sources for culture of *P. citrinum*. Fischer & Kane (1967) described a procedure for production of cornmeal agar, which was slightly altered for the production of the maize meal and rice flour liquid media. Herein, maize broth (MB) and rice broth (RB) were prepared by blending 50.0 g of maize meal or rice flour, respectively, with 800 ml of MSM and leaving the solution to stand in a refrigerator for 18 hours at 4 °C. The media were then heated and stirred constantly with a magnetic stirrer at 60 °C for 1 hour to allow α - and β -amylases in the grains to degrade starches to simple sugars (Apar & Özbek, 2004) which are more readily bio-available. The media were then passed through a fine sieve, filtered through cheesecloth and made up to final volumes of 1 L with MilliQ water. Media were then transferred into 500 ml Erlenmeyer flasks, each containing 100 ml of media sealed and then autoclaved.

b) Fungal culture conditions and sampling regimes

Spores of sub-cultured *P. citrinum* from PDA plates were inoculated into the MB and RB respectively using an inoculation loop by aseptic technique. The flasks were incubated at 25 °C on a rotary shaker (Labcon) at 100 rpm similar to that reported by Gao *et al.* (2011) who reported on a limited time scale

culture of *P. citrinum*. This fungal culture was allowed to proceed for 144 hours, with samples extracted aseptically from each of the media at 12 hour intervals for analysis of CIT production by electrochemistry and HPLC. Controls assessed included MSM medium without a nutrient source, as well as MB and RB which had not been inoculated with *P. citrinum*. Controls were incubated in parallel with the culture media and were aseptically sampled at 24 hour intervals. All media and inoculants were prepared in triplicate separately sampled to provide triplicate assessments.

7.3.6 HPLC analysis of CIT in complex media

a) Apparatus and analytical parameters applied in HPLC analyses

The chromatographic system used in this study was a Prominence modular HPLC (Shimadzu, Japan). The HPLC apparatus comprised of a CMB-20A communications bus module, a SIL-20A auto sampler, with chromatography conducted via a LC-20AB liquid chromatograph and UV detection performed using an SPC-M20A diode array detector. Data acquisition was achieved through application of a PC with LC-solution software (Shimadzu).

Chromatographic conditions for the detection of CIT were conducted according to a slightly modified protocol to that reported by Vail & Hamann (1990) and entailed reverse phase HPLC analysis. Separation of samples and isolation of CIT was achieved using a 5 μm LC-18 analytical column (Supelco, USA) with dimensions of 10 cm x 4.6 mm, as similar C_{18} columns have been reported for HPLC analyses of CIT by Phillips *et al.* (1980), Vail & Homann (1990), Franco *et al.* (1996), Dohnal *et al.* 2010 and Tokuşoğlu *et al.* (2010). Samples (20 μL injections) were loaded onto the column for all experiments. In accordance with the procedure reported by Vail & Homann (1990), isocratic elution was applied using a mobile phase comprising of acetonitrile-water-acetic acid (40:59:1) and tetrabutylammonium phosphate (0.025 M), which was degassed under vacuum and filtered through a 0.45 μm nylon membrane filter (Whatman, USA). A flow rate of 1 $\text{mL}\cdot\text{min}^{-1}$ of mobile phase was used for elution, rather than 2 $\text{mL}\cdot\text{min}^{-1}$ as was reported by Vail & Homann (1990). UV detection was carried out with the photodiode array detector at with analyses were performed at $\lambda = 330 \text{ nm}$.

b) Sample preparation and extraction of CIT from culture media

In literature, CIT extraction protocols are available from solid substrates such as fungal cultures grown on grains, milled grains and cheese (Franco *et al.* 1996; Vázquez *et al.* 1997; Meister, 2004; Dohnal *et al.* 2010). As culture media were liquid, liquid-liquid extraction (LLE) was however required. Vail & Homann (1990) applied a crude LLE with a preparatory pre-column prior to HPLC analyses, and Mossini & Kemmelmeier (2008) applied a relatively crude LLE using chloroform. However, a more stringent extraction was desired to effectively remove organic material from the

sample. As was reported by Wilkes & Sutherland (1998), this is possible for CIT through alternating CIT between organic and aqueous phases as a result of pH adjustment of the aqueous fraction. The application of double step chloroform extraction of CIT has been reported by Vázquez *et al.* (2001) as well as Dohnal *et al.* (2010).

The LLE procedure applied was adapted from Dohnal *et al.* (2010), and entailed extraction from 4 ml of samples from culture media. 4 ml of acetonitrile was added to the media sample, and acidified it with 20 µl concentrated sulphuric acid. Next, the suspension was extracted on an orbital shaker for 1 min with 5 ml of *n*-Hexane and the aqueous layer retained. This step was repeated two further times. To the aqueous layer, 1 ml of chloroform was added, and the solution shaken for 20 minutes, after which the chloroform fraction was retained after centrifugation at 2000 rpm for 2 minutes, and the step repeated two further times. The chloroform fractions were pooled, 2.5 ml of 5 % sodium bicarbonate was added and the solution extracted for 10 minutes, a step which was repeated twice. The aqueous fractions were pooled and filtered through 0.20 µm syringe filter (GVS, Italy) to remove any remaining debris, and were then acidified to pH 1.5 with concentrated HCl and allowed to stand for 20 minutes. The aqueous phase was then extracted to 4 ml of chloroform, centrifuged at 2000 rpm for 2 minutes, and the samples dried at 40 °C in a sealed vessel with silica crystals. The dried samples were then re-dissolved in 500 µl of methanol, filtered through a 0.20 µm syringe filter (GVS, Italy) which was then flushed with a further 500 µl of methanol to bring the total volume to 1 ml. These samples were then applied directly in HPLC (20 µl per analysis) and electroanalysis (400 µl in 4 ml of 0.2 M NaOAc buffer).

c) Media assessed, standards and concentration curves

HPLC analysis was conducted on the maize broth (MB) *P. citrinum* culture only, as a representative case study. Maize broth inoculated with *P. citrinum* were assessed for the production of CIT along with controls of the cell culture which included MSM (no nutrient source) and MB which had not been inoculated with *P. citrinum*. In each case, an internal standard was assessed by spiking a 100 µl extract with 2 µl of CIT stock solution (5 mM in MeOH), resulting in a 100 µM final concentration which was sampled in 20 µl aliquots.

Pure samples of CIT were spiked into MSM at concentrations of 0, 1, 5, 10, 20, 50 and 100 µM. These solutions were then extracted as was reported above and the resulting media retained for establishment of concentration curves which would report directly on the concentration of CIT after the extraction procedure. This was deemed necessary as a result of the wide range of extraction efficiencies reported through different extraction procedures, ranging from 60 % to 100 % efficiency (Phillips *et al.* 1980; Meister, 2004; Dohnal *et al.* 2010).

7.4 Results and Discussion

7.4.1 Voltammetric assay of possible interferents in real world samples

It is widely known that the components of complex matrices have the ability to impact on analytical studies by interfering with or masking measurements. Components likely to be found in such complex media, were thus analysed separately with regard to their impact on CIT electroanalysis, the results of which are illustrated through Figure 7.1 with each set compared to CIT responses in NaOAc buffer as a comparison of unhindered electrochemical response (control). These are summarised in Figure 7.2, reporting on alterations of peak potential (E_p) as well as peak current (I_{p1}) of CIT in the presence of food component examples. While pre-analytical conditioning of the electrode took place in pure buffered NaOAc, all reported controls were performed by scanning of the media in the absence of CIT after conditioning of the electrode to establish a baseline response of the background media, and in so doing illustrate that reported peaks were that of CIT. The only samples containing discernible peaks within the detection window of CIT was yeast extract. Yeast extract, L-lysine, NH_3 , and starch detection matrices each resulted in elevated baseline currents, but (with the exception of yeast extract solution, did not possess discernible peaks.

The media assessed were compiled according to three categories which would be encountered in crop samples: proteins, carbohydrates and lipids. Each category comprised several examples of either varying complexity or composition. Protein components included ammonia (dissociated in aqueous media as ammonium hydroxide) as a simple proxy for amines and amino groups of proteins, L-Lysine as a commonly encountered amino acid (protein monomer), bovine serum albumin (BSA) as a large, soluble, protein and yeast extract powder as a complex medium comprising or multiple protein components of varying solubilities. The examples of carbohydrates included fructose and glucose simple monosaccharides, xylitol as a further naturally occurring sugar alcohol and, finally, starch as a complex carbohydrate. The final group, lipids, comprised of olive oil as a fruit derived oil, sunflower oil as a seed-derived oil and mineral oil as extracted oil from fossil fuels.

As illustrated Figures 7.1 and Figure 7.2, inclusion of the proxies for the constituents of real world samples all impacted on the voltammetric responses of CIT, affecting both I_{p1} and E_{p1} to varying degrees. This response indicates that electroanalysis of CIT will be impacted by the components of food-based matrices assessed. Furthermore, the influences are likely to arise from a combination of impacts both directly on the CIT molecule (e.g. complexation in solution) and the electrode surface (electrode passivation by adsorbing interferents). The influences of the media assessed are discussed below according to the groupings of proteins, carbohydrates and lipids separately.

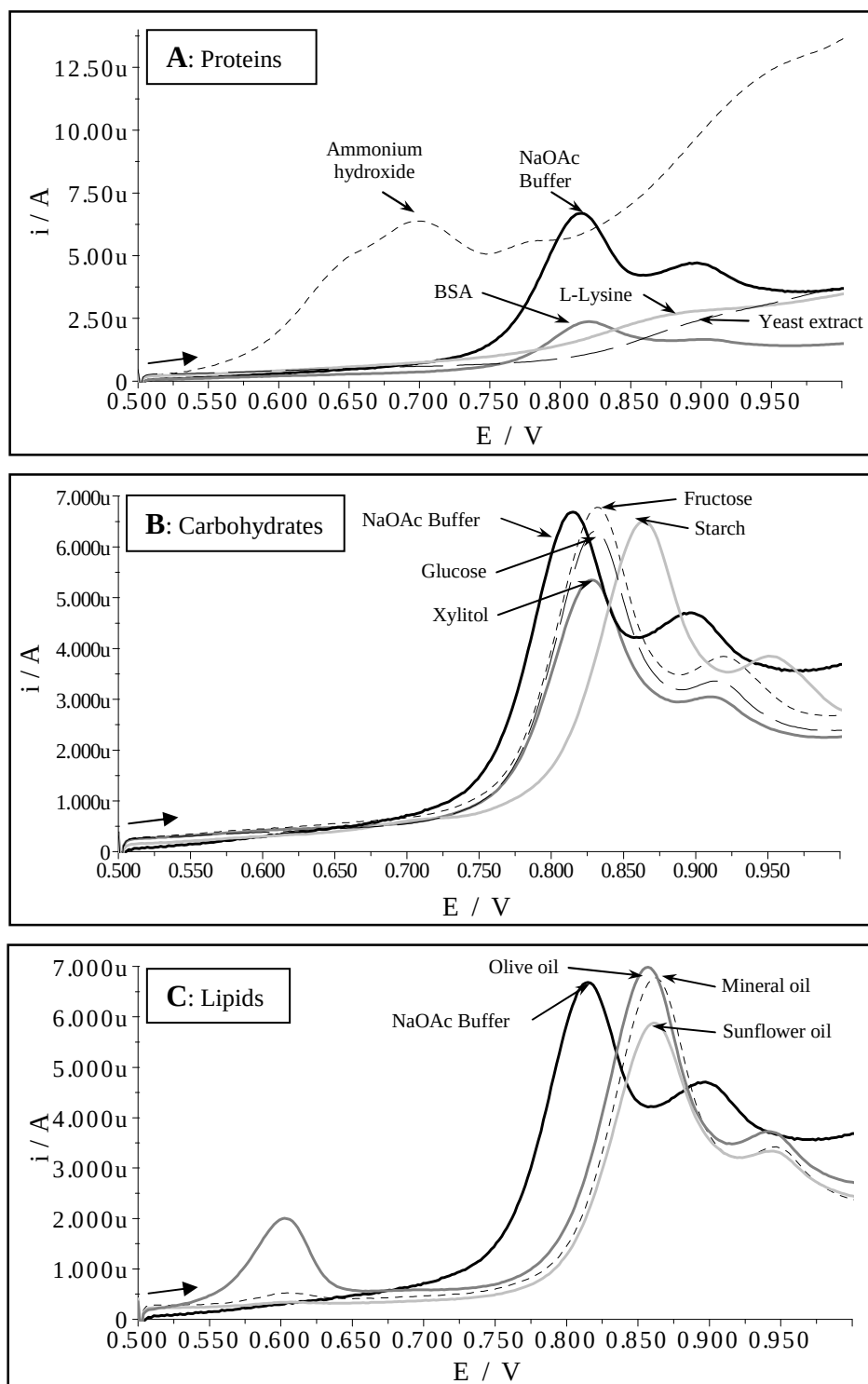


Figure 7.1: Voltammetric assessment of the influence of environmental media on CIT responses. LSV scans of 20 μM CIT in 0.2 M NaOAc buffer in solutions containing [A] proteins (ammonium hydroxide, L-lysine, bovine serum albumin (BSA) and yeast extract), [B] shows responses in solutions containing carbohydrates (fructose, glucose, xylitol and starch), [C] shows solutions containing lipids (mineral oil, sunflower oil and olive oil). In each case responses were reported with reference to CIT in buffer (NaOAc) as a control. All components were present in solution at 1 $\text{mg} \cdot \text{ml}^{-1}$, though ammonia was spiked at 3.41 $\mu\text{g} \cdot \text{ml}^{-1}$. Background current was subtracted from each respective scan.

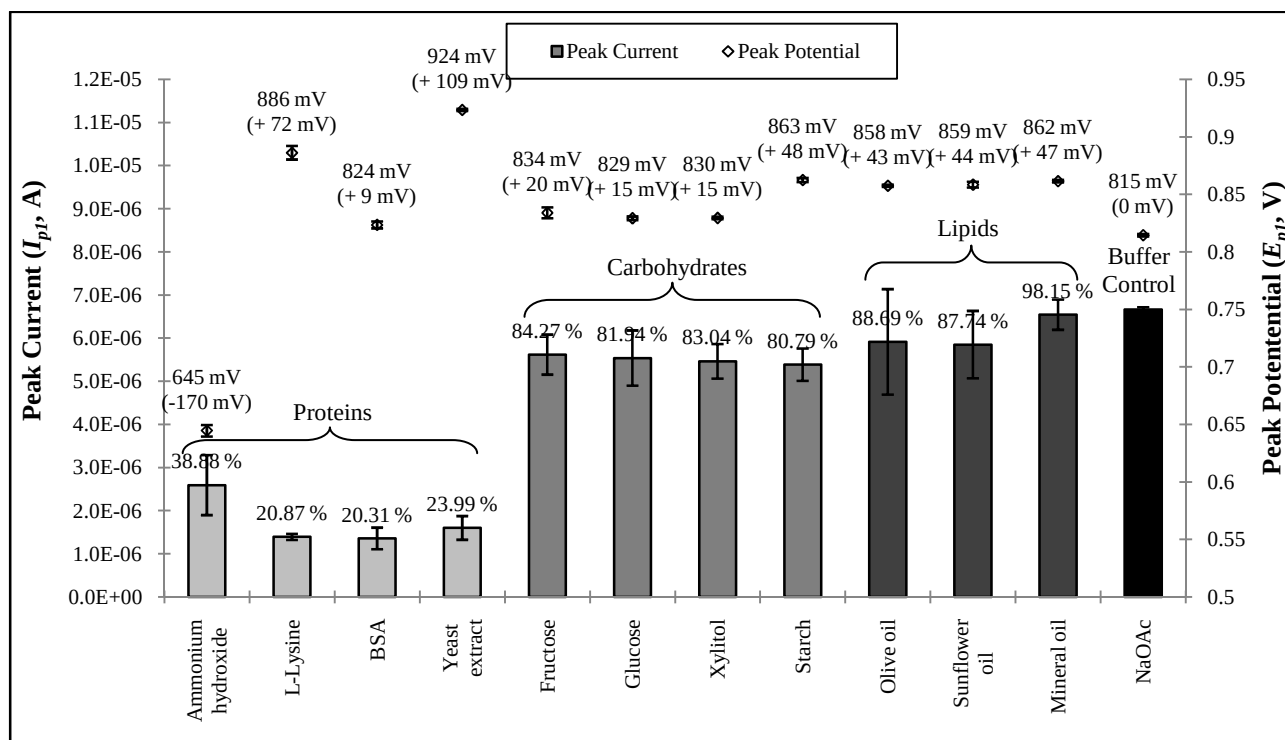


Figure 7.2: Summarised CIT I_{p1} (bars) and E_{p1} (diamond markers) responses of CIT assessed under solutions containing ammonium hydroxide, L-lysine, bovine serum albumin (BSA) and yeast extract (protein solutions, light grey); fructose, glucose, xylitol and starch (carbohydrate solutions, medium grey) and mineral oil, sunflower oil and olive oil (lipids solutions, dark grey). Responses were reported with reference to CIT in buffer only (black) as a control with the % of I_{p1} of the control reported above the column for each solution and both the actual E_{p1} and the change in E_{p1} in relation to the control (black column) was reported above the diamond marker for each medium respectively. Error bars report standard deviation from the mean, $n \geq 4$.

Although CIT responses were affected by each of the components assessed, the components which resulted in the greatest influence on both I_{p1} and E_{p1} were the protein group, as reported in Figure 7.1 [A]. As is evident therein, the electrochemical profiles of CIT were altered significantly in the presence of each of the ‘protein’ solutions. These included a complex of varied proteins (yeast extract), a single type of proteins (BSA), representative subunits of proteins (the amino acid L-Lysine) and ammonium hydroxide as an amine terminating compound. As I_{p1} responses ranged from 20.31 % to 38.88 % (Figure 7.2) of the standard, the extent of repression by each of the protein media assessed is pronounced. Furthermore, as each of the media assessed showed marked alteration in both I_{p1} and E_{p1} , these responses suggest firstly that CIT complexes with proteins, and secondly that this complexation is non-specific with regard to the type of protein, therefore likely to affect all samples containing proteins, even ammonium hydroxide (as the simplest amine proxy) which sufficiently influenced responses. E_{p1} responses were also affected by proteins in solution, shifting the CIT peak to potentials both more positive (by 9 mV, 72 mV and 109 mV for BSA L-lysine and yeast extract respectively) and more negative potentials (170 mV for ammonium hydroxide), a response likely to be supplemented by the ammonium hydroxide shifting the pH to more alkali conditions. The shift to

more positive potentials by true protein solutions was expected with a result suggesting complexation of CIT to proteins and possible fouling of the electrode by CIT-protein complexes. The responses in ammonium hydroxide were however of interest as this was included as a representative proxy of amine groups in proteins. As a relatively simple molecule, the shift in both I_{p1} and E_{p1} was likely to be as a result of a charge-based interaction with CIT in solution. As a shift in I_{p1} and E_{p1} was recorded in each of the protein solutions assessed, it is proposed that the association of CIT with proteins is with amine groups of the proteins in agreement with negative charge at this pH.

Huwig *et al.* (2001) provide a review on the detoxification of feed with a range of adsorbents. Of particular interest with regard to this study were the applications of yeast and products from yeast, with particular detoxification found through the use of cell walls which harbour proteins. Park (1993) reported that ammonia has been used in chemical detoxification of feeds contaminated with mycotoxins, suggesting a capacity for the complexation or, at least, interaction of CIT with ammonium hydroxide in solution.

A likely site of complexation is with the carboxyl moiety of the CIT molecule with amine groups. As this interaction is in the ortho- position to the hydroxyl moiety (which was proposed to be the source of electrons in electro-oxidation through Section 6.4.9), the carboxy-amine interaction may limit the ability of the oxidation pathway presented in Chapter 5 to proceed, and thus limited the electrochemical oxidation of CIT when interacting with a protein.

In contrast to protein analyses, carbohydrate analyses provided a relatively stable set of CIT responses with regard to both CIT I_{p1} and E_{p1} , as illustrated by Figure 7.1 [B] and Figure 7.2. In Figure 7.1 [B], the distinctive twin peak profile of CIT in NaOAc buffer solution was evident, which along with the relatively small positive shift in E_{p1} (approximately 15 mV) suggested that there was minimal influence on the kinetics of oxidation of CIT by carbohydrates. Dampening of I_{p1} to ~85 % of the buffer control was observed, indicating a difference in current response which was statistically significant, but of relatively limited impact, compared to protein-influenced responses. Starch, as the most complex of the carbohydrates assessed, resulted in the greatest relative shift in both I_{p1} and E_{p1} (of the carbohydrates). These responses indicate that carbohydrates impart little influence with regard to complexation of CIT, and the dampening of responses were more likely to be as a result of fouling of the electrode than interaction of CIT with the molecule.

Similar responses to the buffer control were encountered in solutions containing lipids, with no significant difference in I_{p1} although a positive shift in E_{p1} of 43 to 47 mV was encountered. These responses describe what is expected to be a slight fouling of the EOx-GCE surface, hindering the

oxidative process slightly and thereby shifting E_{p1} to more positive potentials. There was a relatively large standard deviation in I_{p1} responses which suggest that there may have been some inclusion of CIT into micelles or oil droplets.

7.4.2 Assessment of CIT complexation to components of media

Although Section 7.4.1 illustrated that complexation of CIT with constituents likely to be encountered in samples of agricultural commodities, it did not illustrate whether the reduced signal was as a result of complexation in solution (solution-phase binding of CIT to solute or suspended molecules) or fouling of the electrode by those media components. The following section was thus directed at an attempt to separate the two parameters for representative samples of proteins and carbohydrates as they presented the greatest impact in Section 7.4.1.

a) EOx-GCE surface confined impact of complex media on CIT responses

The influence of complex media on CIT was assessed through dipping EOx-GCE into solutions containing representative media of proteins (the amino acid L-lysine), carbohydrates (fructose) as the simplest of the complex media, as well as a more complex sample of media extracted from maize flour, each referred to as a doped EOx-GCE. These samples were then analysed in 20 μ M CIT in 0.2 M NaOAc buffer thereafter and compared to EOx-GCE responses at un-doped EOx-GCE as a control response. In so doing, solution based interactions of CIT with the components assessed could be avoided, and any recorded influence could be attributed to predominantly surface-based interactions. The electrochemical responses of CIT at each of the doped EOx-GCE are reported through Figure 7.3.

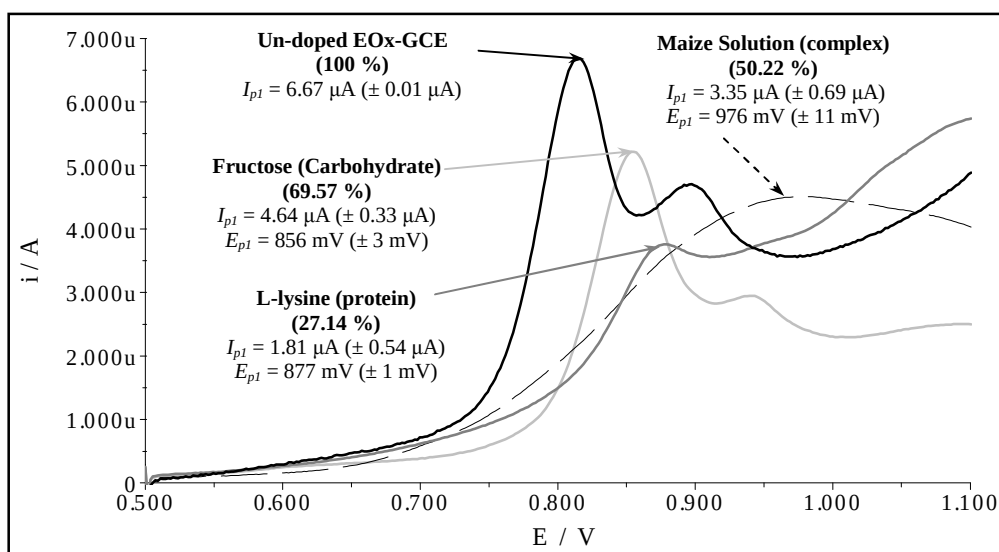


Figure 7.3: Representative scans of voltammetric assessment of the surface confined influence of complex media on responses in 20 μ M CIT 0.2 M NaOAc buffer pH 4.0. EOx-GCE were doped in solution containing fructose (light grey), L-lysine (dark grey) and maize solution (dashed line) and were compared to responses in NaOAc at un-doped EOx-GCE (black). Background current was subtracted from each respective scan.

As is evident in Figure 7.3, each of the media-doped EOx-GCE (where EOx-GCE were dipped into complex media and then assessed in 20 μ M CIT in 0.2 M NaOAc buffer only) resulted in a shift in both the I_{p1} and E_{p1} of CIT, compared to an un-doped EOx-GCE also assessed in buffered solution (black). As was anticipated from Section 7.4.1, greater shifts were encountered in protein media (L-lysine) than carbohydrate (fructose) corroborating the finding that proteins affect responses to a greater extent than carbohydrates. L-lysine doped EOx-GCE, however again resulted in a more pronounced influence, dropping the CIT response to 27.14 % of the un-doped electrodes compared to fructose-doped electrode which had a resulting response of 69.57 % (Figure 7.3).

As the extent of current suppression by fructose-doped EOx-GCE was larger than that reported for fructose in solution (Section 7.4.1), it is most likely that carbohydrates influence CIT I_{p1} through complexation with the electrode surface rather than via a binding event with the CIT molecule to any great extent. Although complexation of proteins to the electrode is also likely to impact on response, it is difficult to detangle the extent of electrode bound and solution bound interaction of proteins with CIT, though both scenarios strongly affect CIT I_{p1} . The broad and suppressed CIT I_{p1} response of CIT I_{p1} in maize solution suggests a marked surface interaction and resulting influence of complex media containing an array of proteins and carbohydrates on CIT I_{p1} .

b) Solution phase complexation of CIT to components in media

The electrochemical studies conducted through both Section 7.4.1 and 7.4.2 [a] suggested that complexation of CIT with components of complex media may also occur in solution with proteins as a pre-electroanalysis interaction. As CIT has been demonstrated to exhibit fluorescence with excitation at 330 nm and emission at 500 nm (Trantham & Wilson, 1984; Vail & Homann, 1990; Vázquez *et al.* 1997), this medium was assessed as it afforded the ability to monitor possible solution based interactions of CIT. According to Moerner & Fromm (2003) fluorescence spectroscopy provides a powerful technique to explore the nanoscale behavior of single molecules in complex local environments in solution phase. Moerner & Fromm (2003) however further detailed several experimental limitations of the applicability of fluorescence analysis in media containing samples of biological origin, chief among which include background signals which from fluorescence generated by residues or structures within protein or carbohydrate samples, may mask the signal of the analyte of interest (Homchaudhuri & Swaminathan, 2001; Moerner & Fromm, 2003).

The analysis of the influence of complex media on CIT in solution was reported in Figure 7.4 and illustrates a range of responses in each of the media assessed. As solution phase complexation was assessed in these analyses, the magnitude of the fluorescence peak of CIT at $\lambda = 550$ nm was of interest, wherein a decrease in response of 1000 RFU or more indicated complexation.

Carbohydrates resulted in CIT fluorescence responses (dark grey columns) in the region of the responses in buffered solution only (black bar, no proteins or carbohydrates in solution, but spiked with CIT as a control), with a significant difference only being encountered in the starch sample. These responses were further corroborated by the spectra reported through Figure 7.5 [B] where fluorescence responses closely followed the profile of CIT in buffer only. These responses corroborate the suggestions of Sections 7.4.1 and 7.4.2 [a] that there is minimal solution based interaction between CIT and carbohydrates and that the suppression of I_{p1} in voltammetric analyses in carbohydrate solutions is likely to be as a result of increasing surface complexity through interaction with the electrode. Lipids were excluded from these analyses on the basis of limited interaction with CIT (Figure 7.2) and incompatibility with this analytical technique.

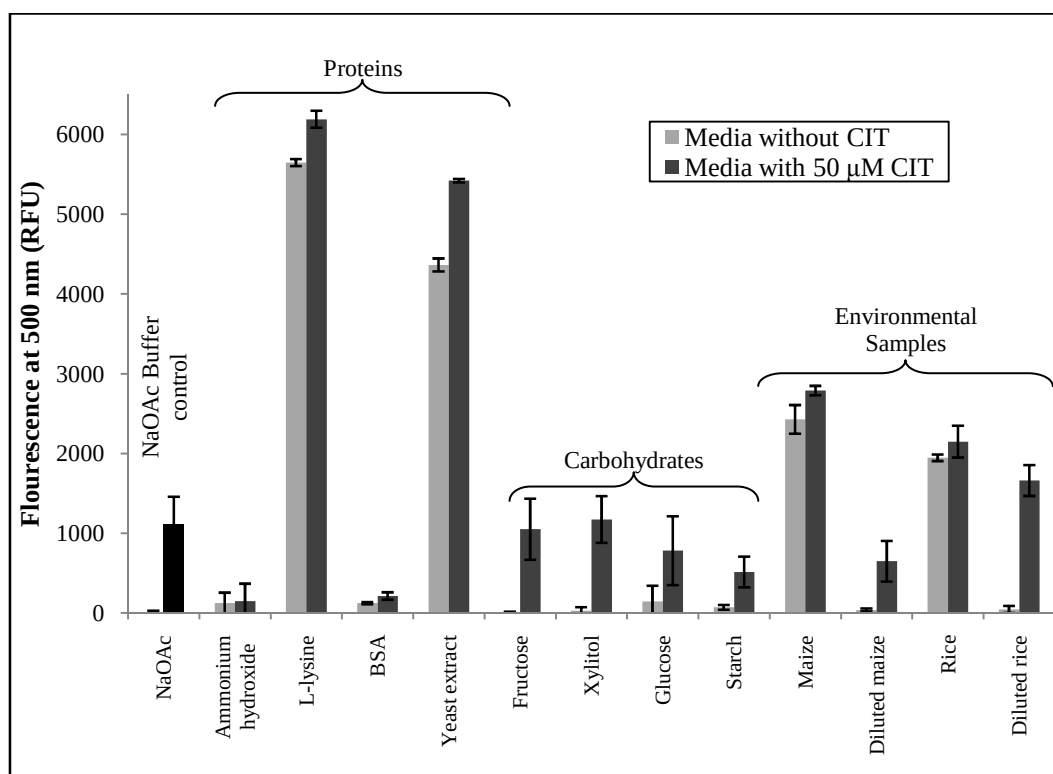


Figure 7.4: Averaged fluorescence responses attained in media both without (light grey) and with CIT at 50 μ M (dark grey) assessed in media containing ammonium hydroxide, L-lysine, bovine serum albumin (BSA) and yeast extract (protein solutions); fructose, glucose, xylitol and starch (carbohydrate solutions); maize and rice solutions as well as 100x dilutions (with 0.2 M NaOAc buffer) of maize and rice solutions respectively. Responses were reported with reference to CIT in NaOAc buffer only. Error bars report standard deviation from the mean, $n = 3$ per set.

It was anticipated that proteins in solution would complex with CIT and thus limit the fluorescence response. As is evident in Figure 7.4 however, both L-lysine and yeast extract media resulted in extensive fluorescence responses both in the absence and presence of CIT. These responses were as a result of the CIT fluorescence peak being overshadowed by the background fluorescence of the

protein in the media. These responses were illustrated through Figure 7.5 [A], wherein the averaged fluorescence spectra of L-lysine, yeast extract and maize solution both with and without CIT in solution is compared to equivalent analyses in NaOAc buffer. In, L-lysine responses, the peaks evident in the region of 420 nm have been previously reported to be characteristics of luminescence of L-lysine (Homchaudhuri & Swaminathan, 2001). Peaks in the same 420 nm region were observed in yeast extract, and as Homchaudhuri & Swaminathan (2001) also reported that amino acids including L-arginine, L-serine, L-glutamate and L-isoleucine the issued no peaks in that region thus they are likely also to be as a result of L-lysine in yeast extract, and to a lower extent, in maize solution.

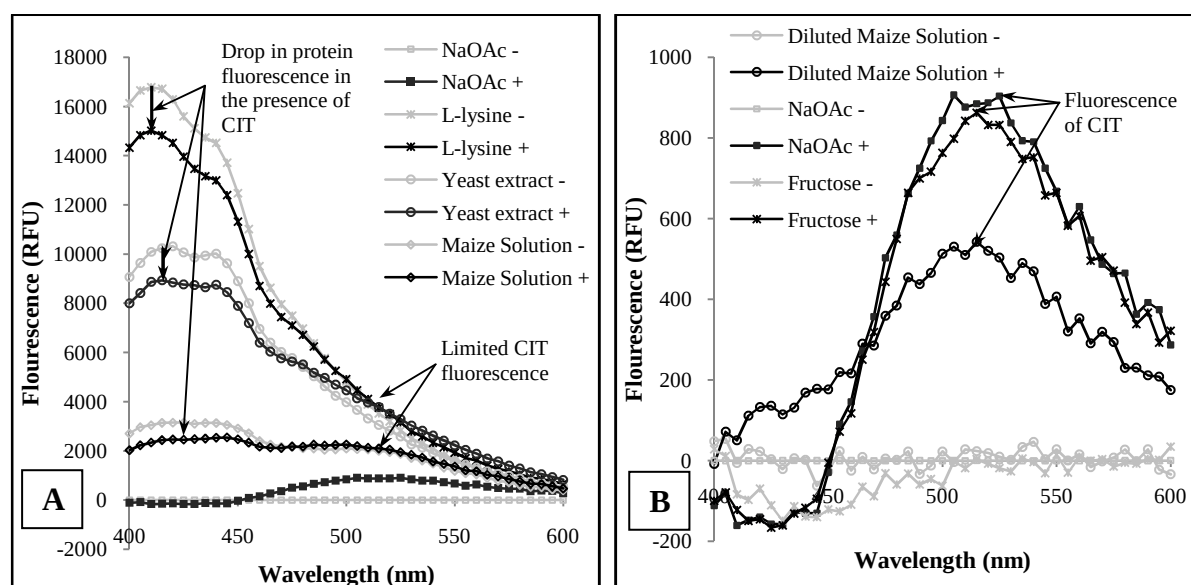


Figure 7.5: Averaged fluorescence emission spectra attained in media both without (light grey designated “-” in the key) and with CIT at 50 μ M (dark grey designated “+” in the key) assessed in [A] media containing L-lysine, yeast extract and maize solution and [B] media containing fructose and maize solution diluted 100-fold. Responses were reported with reference to fluorescence in NaOAc buffer both with and without CIT. Spectra reported as averages of 3 equivalent analyses.

Due to the large shoulder of fluorescence from proteins, CIT fluorescence responses were masked and thus the L-lysine and yeast extract media could not be applied in assessing CIT fluorescence directly. Complexation of these media with CIT in solution could however be identified from monitoring fluorescence of the proteins themselves (at approximately 420 nm in Figure 7.5 [A]), as has similarly been assessed for BSA binding to various molecules (Homchaudhuri & Swaminathan, 2001; Wang *et al.* 2006; Yang *et al.* 2009). Therein, fluorescence was lowered when CIT was in solution, suggesting that when bound to CIT, protein fluorescence is limited. This suggests that CIT binds to amino acid residues in L-lysine, yeast extract and maize solution respectively. The fluorescence responses of BSA and ammonium hydroxide media (Figure 7.4) both demonstrate a markedly lower response of CIT fluorescence in relation to NaOAc buffer as neither produced a protein fluorescence peak in the region of 420 nm. In these cases (BSA and ammonium hydroxide), the lack of a significant difference

between CIT spiked and unspiked fluorescence responses provides evidence of the complexation of CIT in solution. These responses correlate closely with literature of several studies which monitor the interactions of BSA with a variety of molecules in solution by fluorescence spectroscopy have been reported (Wang *et al.* 2006; Yang *et al.* 2009). The non-specific complexation of CIT with proteins other than antibodies raised against it for biosensor configurations has been described by Liu *et al.* (2011), who showed specific conjugation of CIT to BSA and by Li *et al.* (2011) who showed conjugation of CIT to keyhole limpet hemocyanin.

Further corroboration that protein content in media affects CIT responses can be identified through considering the relative responses of maize and rice solutions (environmental samples). Similar to L-lysine and yeast extract responses in Figure 7.4 and Figure 7.5 [A], maize and rice solutions resulted in large, protein-originating fluorescent signals which masked the fluorescence of CIT. Through comparison of those responses to equivalent analyses conducted in maize and rice solution which had each been diluted 100-fold (Figure 7.4 and Figure 7.5 [B]) illustrated that with a dilution of protein of maize broth (MB) in solution (as illustrated further in Section 7.4.3), fluorescence of CIT could be detected. These results thus served to confirm that CIT interacts with a range of proteins in solution.

c) Implications of the complexation of CIT to components in media

With regard to electroanalysis of CIT, the implication of its complexation with proteins is that solutions which contain proteins will negatively impact on detection sensitivity of CIT, distorting recorded responses. As the protein assessments made herein were of soluble proteins, these responses report only on a portion of the possible protein interactions that could be expected in a food sample as the entire sample will not be solubilised. The implication thereof is under-representation of actual CIT samples. Quantification of the extent and range of proteins, both soluble and insoluble, with which CIT interacts will however be an extensive body of research which is beyond the scope of the research of this Thesis. This study however highlights the importance of the consideration of protein-CIT interactions in all analytical procedures in order to accurately account for the level of contamination of a sample.

The results of Sections 7.4.1 and 7.4.2 suggest binding and complexation of CIT to a range of proteins of varying complexity. Considering these responses and the reports in literature detailing conjugation to other proteins such as hemocyanin (Li *et al.* 2011), BSA (Liu *et al.* 2011) and glucose oxidase (Abramson *et al.* 1995), conjugation of CIT to simple amino acids to large, highly varied and complex proteins appears to occur readily and with apparent non-specificity. As was detailed through Chapter 1, Table 1.2, several immunoassay based bio-recognition systems towards CIT have been developed.

Although the studies each reported on low cross reactivity of the antibodies with other analytes, the influence of non specific interaction of protein components in aqueous samples was not considered (Abramson *et al.* 1995; Dietrich *et al.* 2001; Kononenko & Burkin, 2007; Duan *et al.* 2009; Li *et al.* 2011; Arévalo *et al.* 2011). Although each of these studies did assesses real world samples which would contain proteins, each use liquid extraction to the organic phase and thus would have limited the concentration of protein in the analysis solutions. Due to the likely non-specific interaction of CIT with proteins, it is the position of the author that biosensor systems which are centred on the application of a protein bio-recognition system such as an antibody or an enzyme will likely be negatively influenced by non-specific binding in complex media when direct aqueous measurements are employed.

Analysis of CIT in aqueous systems has been a central theme through this Thesis such that an analysis system could be established without the requirement for organic solvents and extractions. This was in order to simplify the process and render it usable by a layperson and easily applicable to field analyses. As this would be unlikely with a protein based system due to non-specific binding of proteins, it is submitted that nucleic acid based sensor systems (such as aptamers) would be better suited as biorecognition agents for analysis in aqueous media. Studies considering the development of such a sensor systems have already been assessed for a closely related mycotoxin in the form of ochratoxin A, thereby establishing the a basis for future studies in this regard (Cruz-Aguado & Penner, 2008; Kuang *et al.* 2010; Barthelmebs *et al.* 2011 (a) (b); Bonel *et al.* 2011; De Girolamo *et al.* 2011; Yang *et al.* 2011).

7.4.3 Direct electroanalysis of aqueous real world samples

Figure 7.6 reports on the electroanalysis of CIT spiked into solutions of ground maize and rice soaked in 0.2 M NaOAc buffer overnight and Figure 7.7 provides the summarised responses of those analyses.

As is evident in Figure 7.6, direct analysis of CIT in aqueous maize and rice solutions (grey scans) resulted in greatly altered electrochemical profiles from responses in NaOAc buffer. No clear formation of the two peak profile characteristics of CIT in 0.2 M NaOAc buffer was observed (black scans of Figure 7.1) in undiluted maize broth (MB) or rice broth (RB). The responses of CIT in the maize and rice solutions both closely compared with the profiles reported for solutions containing protein, as was illustrated in Figure 7.1 [A]. Although there was a slight difference between the two solutions themselves, no apparent influence could be found with increasing the solution concentration of CIT from 20 μ M to 40 μ M (Figure 7.7). Through diluting RB and MB 100-fold and then spiking

with CIT however, strong electrochemical responses characteristic of CIT oxidation were evident (Figure 7.6), as well as an increase in I_{p1} corresponding to an increase in solution of CIT from 20 μM to 40 μM (Figure 7.7) in both media.

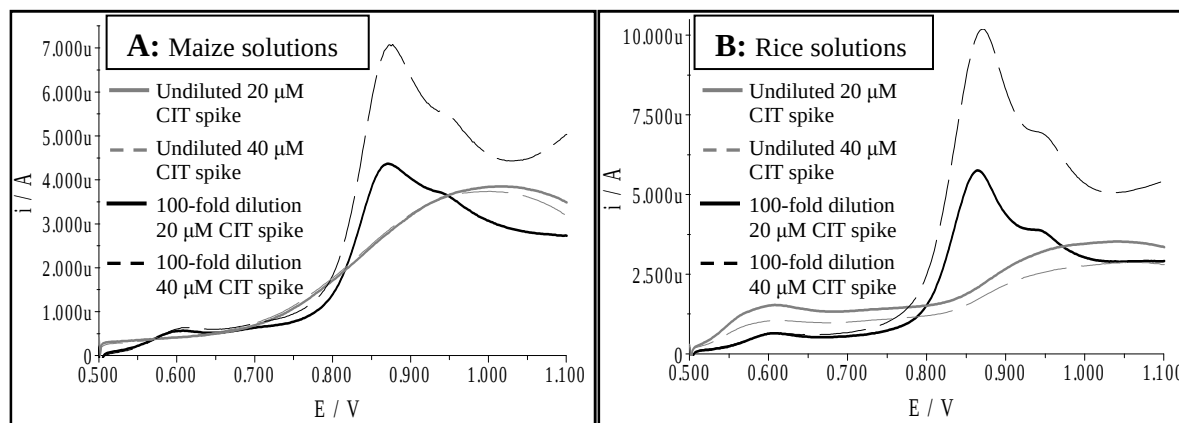


Figure 7.6: Representative LSV profiles of aqueous real world samples spiked with CIT. Figure [A] illustrates analyses in maize solution and [B] illustrates analyses in rice solution, each prepared by soaking ground maize and rice respectively at 0.2 g.ml⁻¹ in 0.2 M NaOAc buffer overnight. Solutions were assessed directly when spiked with CIT at 20 μM (solid grey) and 40 μM (dashed grey) as well as fresh MB and RB solutions which had been diluted 100-fold with 0.2 M NaOAc buffer dilution spiked with CIT at 20 μM (solid black) and 40 μM (dashed black). Scans were reported with respective background currents subtracted.

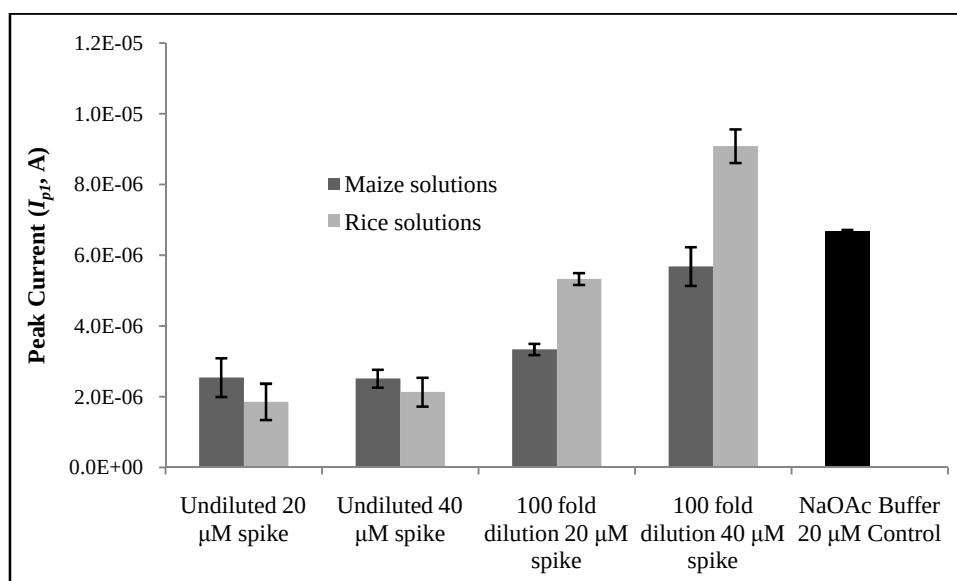


Figure 7.7: Averaged I_{p1} of CIT responses in spiked maize (dark grey) and rice (light grey) solutions respectively. Standard deviation reported from mean, $N \geq 3$ per solution assessed.

In Figure 7.7, although CIT I_{p1} increased with an increase in dilution, neither RB nor MB showed I_{p1} comparable to the control. Diluted RB showed better recovery of CIT than MB however there was no significant difference for either of the two when undiluted.

In order to confirm that the curtailed responses were as a result of proteins in solution, total protein concentration was determined via Bradford assay (Bradford, 1976) in relation to a BSA standard concentration curve with a resulting equation from linear regression of $y = 0.510x$ ($R^2 = 0.994$). From these analyses it was found that the total protein content of maize solution was 0.281 mg.ml^{-1} and rice solution was 0.152 mg.ml^{-1} , which accounts for the limited CIT responses when assessed directly in those media. Bradford assays of the 100-fold dilutions of each of the media illustrated that protein concentration dropped significantly to below the detectable limit of the assay, a result which accounts for the resulting prominence of the CIT I_{p1} peak in these analyses as proteins no longer affected the response.

7.4.4 Culture of *Penicillium citrinum*

P. citrinum strain Sp. 465 (DSMZ) has been characterised as a CIT producing strain. Its application within this study was assessed for several reasons. These included firstly that it allowed for the continuous assessment of CIT within complex aqueous media through direct monitoring of the culture media. Secondly, as the cultured fungus would consume nutrients including proteins, carbohydrates and minerals from solution during the growth stage, and subsequently release a range of secretions into the growth medium, the bulk composition of the medium would alter with time, yielding an altering analysis medium which could allow for insight to be gained into more effective analysis of CIT. Furthermore, as *Penicillium* species have been widely investigated from investigating its culture is well documented and easily achieved (Arai *et al.* 1989; Diggins 1999; Antinori *et al.* 2006).

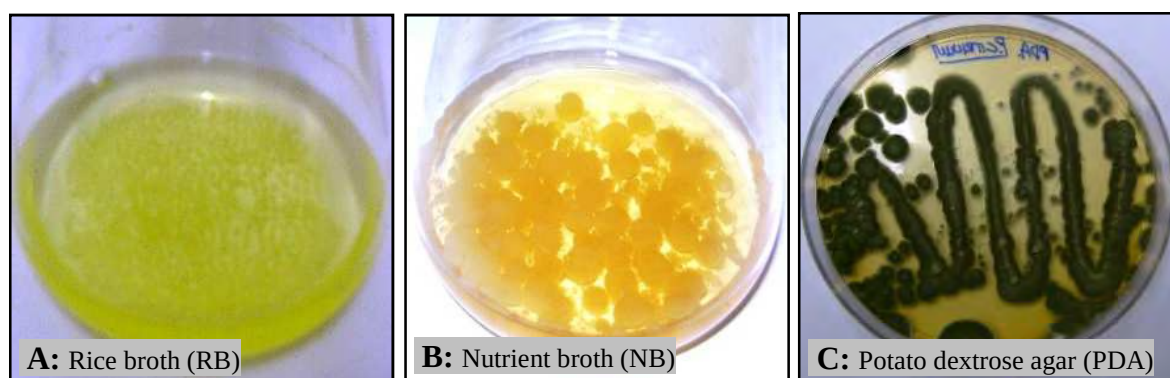


Figure 7.8: Culture of *P.citrinum* on potato dextrose agar (PDA) [A] as well as in nutrient broth (NB) [B] and maize broth (MB) [C]. PDA plates and growth media were incubated at 25 °C for seven days with the growth media continuously agitated on a rotary shaker at 100 rpm.

The culture of *P. citrinum* in this study was carried out using batch flasks (500 ml Erlenmeyer flasks), which allowed for the media to be sampled aseptically at 12 hour intervals. Corresponding with the environmental samples assessed through Section 7.4.3, the media which were applied were produced from ground maize (corn) and ground rice. Figure 7.8 provides images comparing the morphology of

P. citrinum when cultured on a solid medium, potato dextrose agar (PDA) as compared to a liquid media including nutrient broth (NB) and maize broth (MB).

The morphology of *P. citrinum* cultured in each of the liquid culture media as well as PDA (solid culture medium) illustrated in Figure 7.8 are clearly distinct from one another, a result typical of fungi cultured under various media (Madigan & Martinco, 2000). In broth media, *P. citrinum* hyphae cultured in globular balls which were white and smooth, though the balls were distinctly larger in NB (Figure 7.8 [B]) than in RB (Figure 7.8 [A]), where balls remained smaller and presented with a rougher appearance than NB. As illustrated in Figure 7.9, *P. citrinum* cultured in MB presented with an indistinguishable morphology to that of RB. When cultured on PDA (Figure 7.8 [C]), *P. citrinum* presented with a dense blue-green felt of conidiophores, which tended to white in the centre of colonies, where it was older. These growth characteristics are typical of *Penicillium* sp. and has been reported for *P. citrinum* by Vázquez *et al.* (2001).

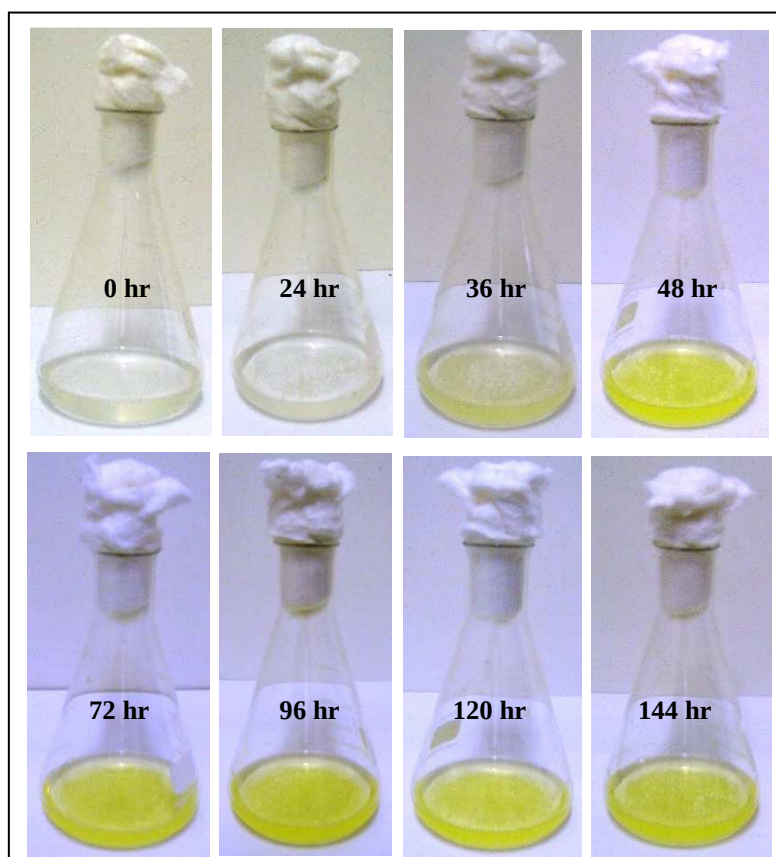


Figure 7.9: Representative photographs of the culture of *P. citrinum* in maize broth (MB) over a seven day period. Flasks were incubated at 25 °C on a rotary shaker at 100 rpm.

Figure 7.9 illustrates the progressive change in the appearance of the growth medium (MB) with the culture of *P. citrinum*. As there was no visible distinction between MB and RB cultures, only MB was illustrated in Figure 7.9. Therein, from an initially cloudy growth medium (0 hr), balls of *P. citrinum*

mycelium were evident within 24 hours and reached a stable approximate size by 48 hours of culture. A distinct change was observed in the culture medium, wherein after 24 hours the medium had become clear, and by 36 hours the production of a yellow substance became evident. As the yellow pigment was not observed in the absence of *P. citrinum*, it was likely to be a metabolic product of the culture fungi. With extended culture, media took on a deeper yellow pigment until the culture was ceased at 144 hours with no further marked changes in the cultured media.

7.4.5 Analysis of CIT produced through culture of *Penicillium citrinum* by HPLC and electroanalysis as a comparative case study of CIT analysis in complex media

a) Direct electroanalysis of CIT in aqueous *P. citrinum* culture media

Similar to the analyses conducted in Section 7.4.3, electroanalysis of CIT in aqueous culture media was assessed to determine whether altering constituents of those media would allow for direct electroanalysis of CIT. A slightly different ratio of sample to buffer was applied herein (1:3 %v 0.8 M NaOAc buffer : sample medium), however solution pH was seen to shift no more than 0.02 units from pH 4.0. In order to account for the influence of protein content within the media, Figure 7.10 provides a measure of total protein content in solution as well as fluorescence analysis of CIT and rice broth (RB). It should be noted that MB and RB are distinct from maize and rice solutions reported on previously in this Chapter (MB and RB are growth media heated during preparation, Section 7.3.4).

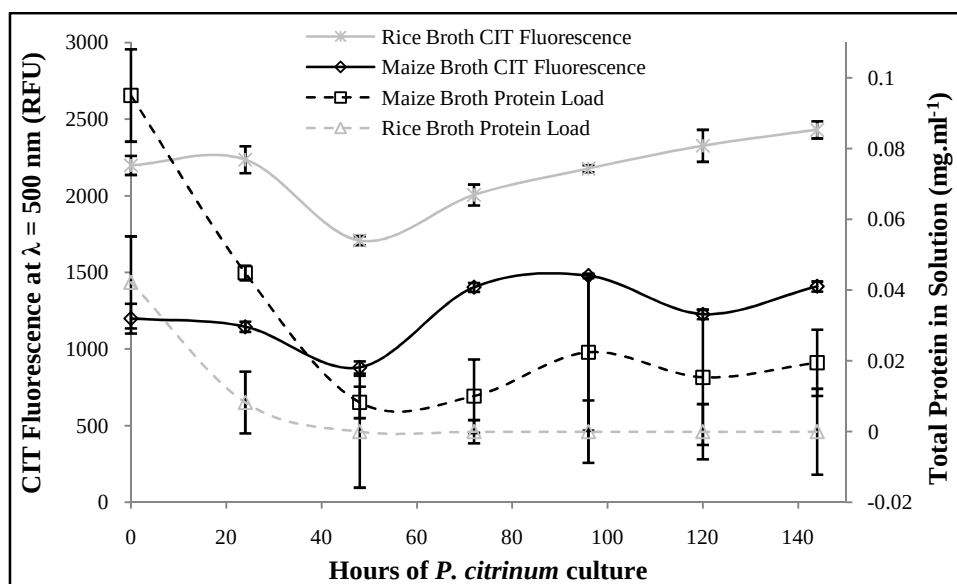


Figure 7.10: Summarised spectroscopic analyses reporting emission fluorescence ($\lambda = 500$ nm) for CIT analysis (solid lines) and total protein concentration through Bradford assay (absorbance $\lambda = 595$ nm) in maize broth (black lines) and rice broth (grey lines). Standard deviation is reported from the mean response ($n \geq 3$).

Total protein content of MB and RB media was determined through Bradford assay similarly to Section 7.4.3, though the standard curve herein was established in MSM from 0.01 to 1.0 mg.ml⁻¹

with a resulting equation from regression of $y = 0.565x$ and an $R^2 = 0.998$. As expected, Figure 7.10 illustrates a gradual decrease in total protein load in both MB and RB (dashed lines) from the point of inoculation at 0 hours through to approximately 48 hours. Thereafter the residual protein level stabilised (RB protein load was below detectable limit, thus designated 0). A correlation is evident when relating CIT measured by fluorescence (solid lines of Figure 7.10) to the determined protein load of MB and RB. As was found in Section 7.4.2 [b], fluorescence with emission at $\lambda = 500$ nm in the presence of proteins is hindered by fluorescent interference from the proteins. The drop in protein load in solution in both MB and RB from 0 to 48 hours was mirrored by a drop in fluorescence signal in Figure 7.10. Once the protein load stabilised, the apparent CIT concentration (as measured by fluorescence) in both MB and RB appeared to increase. This suggests the production of CIT by *P. citrinum*. As proteins were likely to be in solution and as the shoulder of protein fluorescence responses were not completely lost, inaccuracy of the response was however expected, thus they were considered with due caution. This factor may impact on fluorescence analyses applying HPLC-FD.

Direct electroanalysis of CIT in equivalent aqueous samples of both MB and RB was assessed, the responses of which were reported through Figure 7.11 with reference to both I_{p1} and E_{p1} at each sampling point. Through Figure 7.11, it is evident that I_{p1} responses (bars) were again hampered by the presence of proteins in solution in both MB and RB. For example, at 0 hours for each of the media (Figure 7.11), relatively large I_{p1} responses were encountered, however these were not CIT oxidation responses, as was described in Figure 7.4, and were likely to be as a result of the proteins in solution in the media. Furthermore, the decrease over time in total protein (shown in Figure 7.10) did not allow for CIT produced by the cultured *P. citrinum* to be detected conclusively in either of the media (MB in Figure 7.11 [A] and RB in Figure 7.11 [B]) over the timescale assessed.

These results definitively illustrate the requirement for a level of sample pretreatment as an unavoidable requirement to remove proteins and other particles from solution which may be negatively influence electroanalysis of CIT.

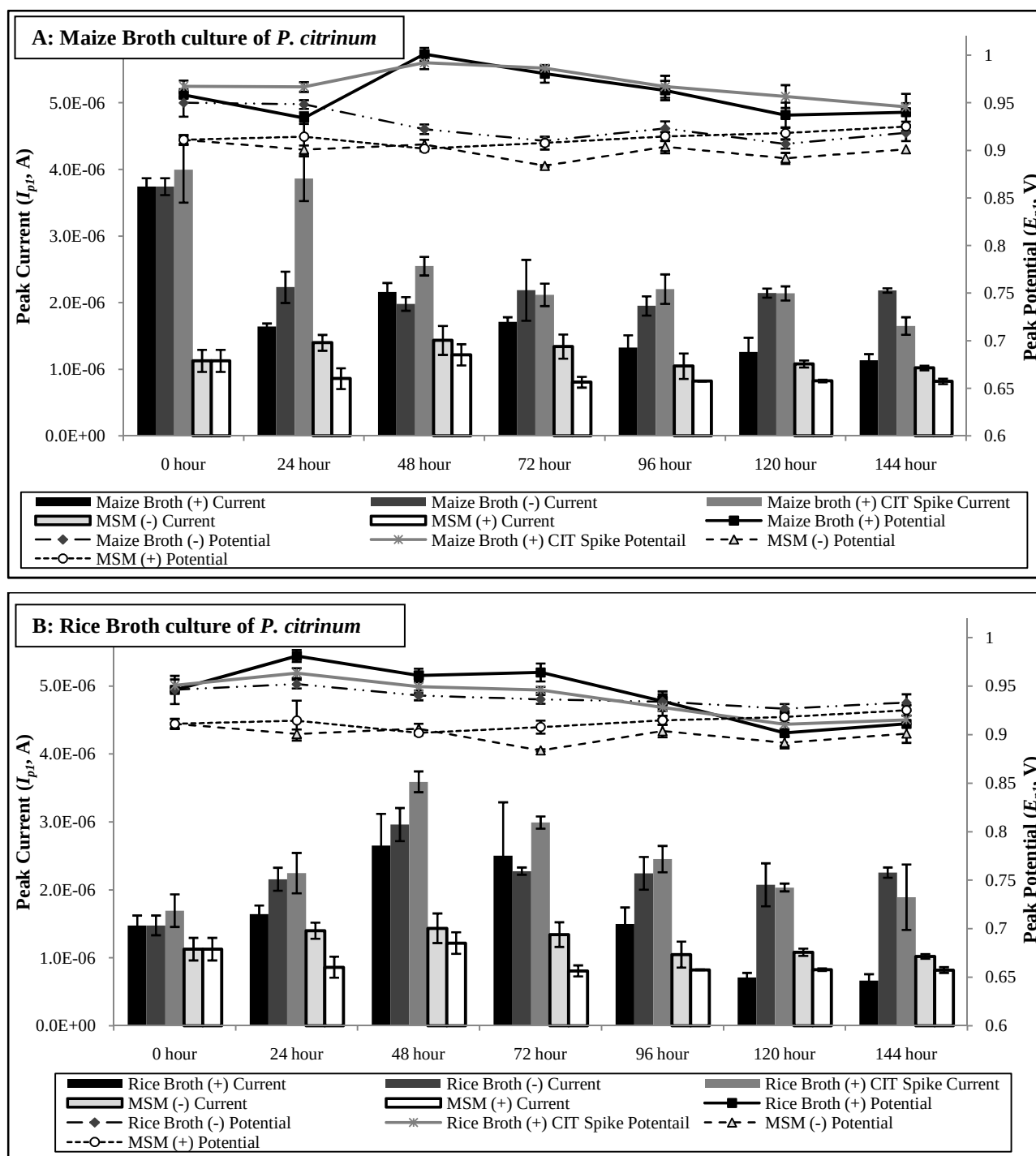


Figure 7.11: Summarised aqueous electrochemical analysis of CIT production by *P. citrinum* cultured in [A] maize broth and [B] rice broth culture media with samples recorded in 24 hour intervals. Media designated (+) in the key were inoculated with *P. citrinum* and those designated (-) were not inoculated. In each, I_{p1} (columns) and E_{p1} (lines) responses were recorded directly in culture media at 1:3 % culture medium to 0.8 M NaOAc buffer pH 4.0. standard deviation was reported from the mean and $N \geq 3$ per set.

b) Analysis of CIT extracted from maize broth in which *P. citrinum* was cultured

In literature to date, to the best of the knowledge of the author, all analyses regarding the detection of CIT, including those cited through the Introduction of this Chapter for HPLC analyses and through Table 1.2 as well as in Section 1.1.2 of Chapter 1, have been performed on CIT after being extracted

into, or prepared in organic solvents; predominantly methanol (aqueous phase buffers were applied in several studies though CIT was introduced as solubilised in a solvent).

Extraction and sample cleanup is a prerequisite for HPLC analyses, however extraction of CIT from the aqueous culture media into an organic solvent may further aid in disassociating CIT from protein(s), and thus may allow for it to be detected more effectively from complex media. Furthermore, extraction may allow for sampled CIT to be concentrated into a lower volume, and thus allow for detection of CIT at a lower apparent concentration.

According to a survey by Majors (2002), liquid-liquid extraction (LLE) was applied to around 40 % of all sample preparation procedures and included extractions from diverse matrices which include petroleum products, natural products, body fluids and fruits, grains and vegetables. Pre-extraction of CIT from a number of foodstuffs has been extensively reported (Phillips *et al.* 1980; Vail & Homann, 1990; Wilkes & Sutherland, 1998; Meister 2004; Dohnal *et al.* 2010). Fluorescence analysis of CIT culture media (Figure 7.10) suggested a possibly higher production level of CIT in MB and, importantly, also showed higher protein loads in MB solution than RB. These features illustrate that MB is the more complex of the two growth media, and efficient extraction of which will establish the extraction procedure as being more universally applicable. The products of LLE extraction on samples of MB are illustrated through Figure 7.12.

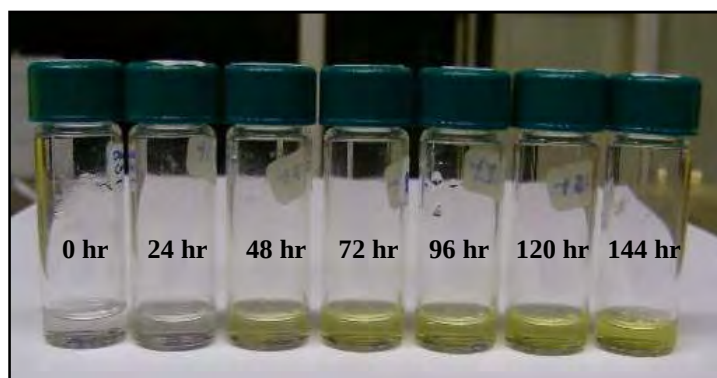


Figure 7.12: Photograph of daily samples of MB culture media extracted into methanol by LLE.

In order to draw a comparison between electroanalysis of CIT and chromatographic analysis of CIT, the extracted samples were analysed using both electrochemistry at EOx-GCE and by HPLC.

c) Analysis of CIT extracted from maize broth by HPLC

Samples of pure CIT dissolved in methanol were prepared for chromatographic analyses under the protocol presented herein. Figure 7.13 illustrates a representative HPLC-UV profile of CIT, with retention time for CIT of 4.56 minutes. This profile resembles the profile reported by Vail & Homann

(1990) (on which this procedure was based), although the retention that study was slightly shorter at 3.86 minutes. Phillips *et al.* (1980) also reported a retention time of 4.25 minutes for CIT with application a similar mobile phase parameters. The CIT peak was well defined and stable with respect to time, and thus afforded a viable system for analysis of CIT which compared relatively closely with that reported in literature.

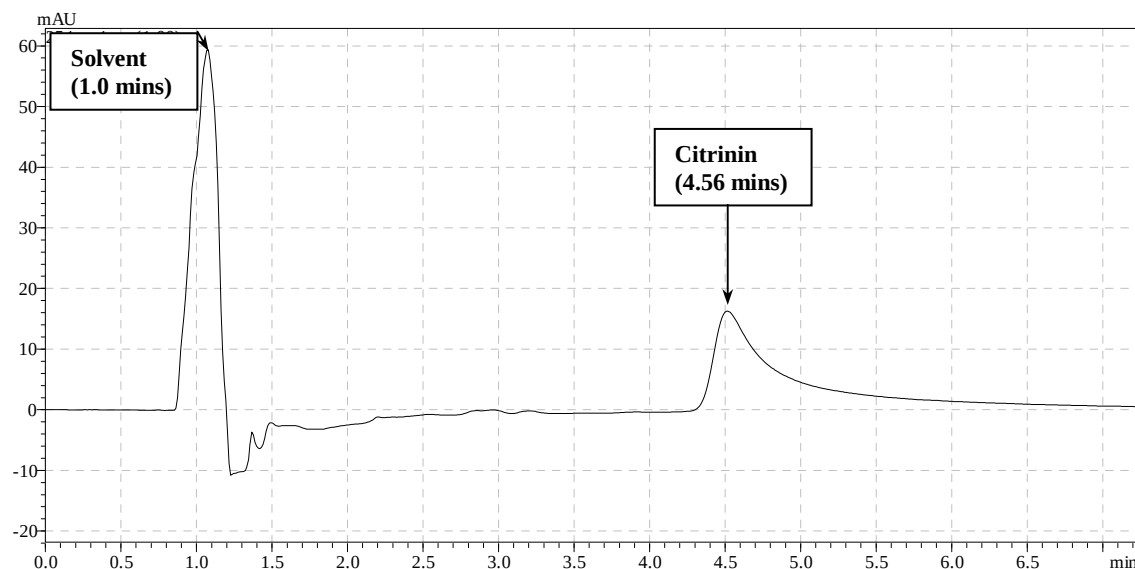


Figure 7.13: HPLC chromatogram of sample standard of 100 μ M CIT in methanol. Analysis was conducted with UV absorbance at $\lambda = 330$ nm, mobile phase flow rate 1 ml.min⁻¹.

Figure 7.14 shows an absorbance analyses which were recorded in parallel with of CIT absorbance as reported in Figure 7.13 above as confirmation analyses of CIT identity.

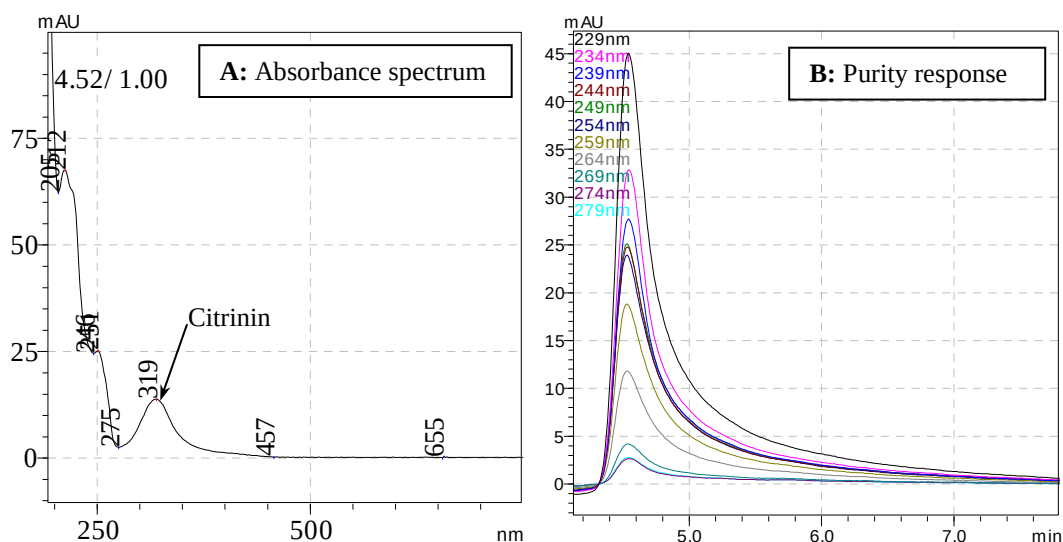


Figure 7.14: Absorbance profile of CIT recorded during chromatography at the apex of the CIT peak, reporting an absorbance spectrum of $\lambda = 200$ nm to $\lambda = 800$ nm [A] and a purity spectrum at wavelengths from 229 nm to 279 nm [B].

Figure 7.14 [A] is an absorbance spectrum ($\lambda = 200 \text{ nm}$ to $\lambda = 800 \text{ nm}$), wherein the CIT peak was reported at 319 nm rather than 330 nm, as reported by Vail & Homann (1990) and Phillips *et al.* (1980). Figure 7.14 [B] provides a purity analysis response determined by absorbance within set wavelengths from $\lambda = 229 \text{ nm}$ to $\lambda = 279 \text{ nm}$ in 5 nm intervals, each recorded in parallel. The purity of response reported on shorted wavelengths and illustrated the presence of particulate matter such as proteins and cell debris remaining in the extracted sample. These two absorbance spectra were recorded in parallel with chromatographic analysis and were used in conjunction with the chromatogram as a qualitative tool to confirm the identity of CIT during analysis of extracted culture media.

A standard concentration curve was prepared at concentrations ranging from 0 to 50 μM . The concentration curve included samples of pure CIT solubilised in methanol, referred to henceforth as “CIT standard” and an equivalent concentration range of CIT which was spiked into, and then extracted from growth media for determination of extraction efficiency, responses of which are referred to henceforth as “extracted CIT standard”. The samples were each were assessed by HPLC and concentration curves established, the results of which are illustrated in Figure 7.15.

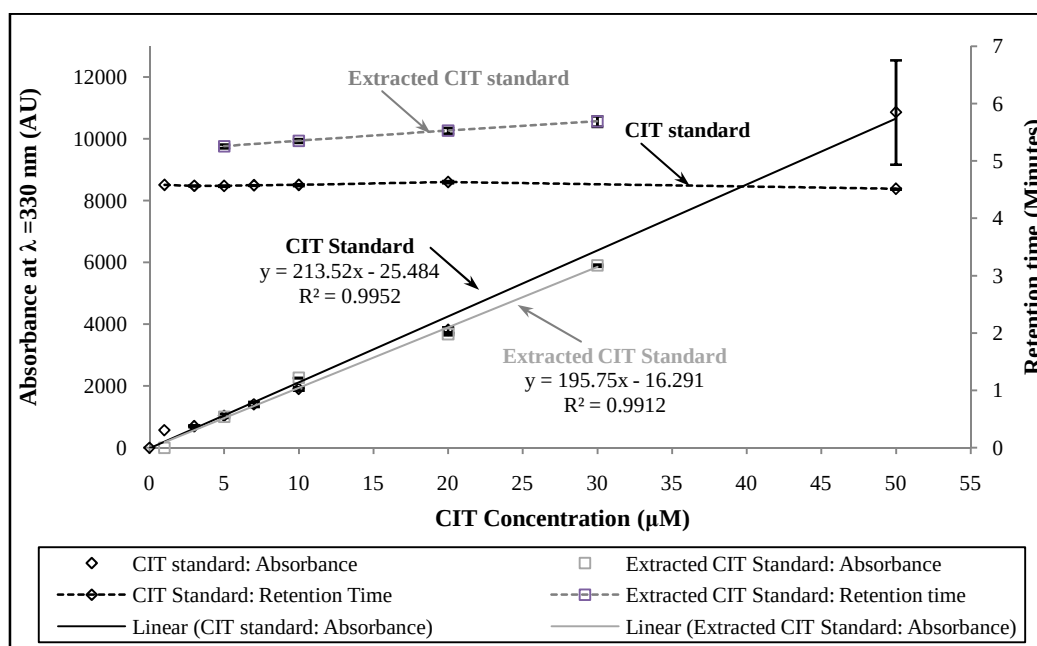


Figure 7.15: CIT concentration curves assessed by HPLC-UV (retention time 4.56 mins), including “CIT Standard” (black data sets) and “Extracted CIT Standard” (grey data sets), reporting absorbance peak height (solid lines) as well as retention time (dashed lines) at each concentration. Standard deviation was reported from the mean with $N \geq 3$ per data point.

Figure 7.15 illustrates that chromatographic responses were stable with regard to absorbance peak height (concentration) but retention time of the extracted CIT standard solution responses were shifted slightly with increasing CIT concentration, whilst the CIT standard solution analyses maintained

stable retention time responses. This result suggests that the extraction procedure may alter the CIT molecule (e.g. ionisation state), and is a parameter which should be considered in analysis of environmental samples as the retention time may shift as a result of either the extraction procedure or the sample from which CIT was extracted.

An average extraction efficiency of 91.75 % was determined over the entire concentration range assessed in Figure 7.15 (extracted curve [grey] to the standard concentration curve [black]). This is a relatively strong response in relation to extraction efficiencies reported in literature which range from 76.5 % - 104.95 % recovery efficiency, depending on the medium from which CIT was extracted and the procedure applied (Phillips *et al.* 1980; Vail & Homann, 1990; Meister *et al.* 2004; Dohnal *et al.* 2010). A further factor of note is that although at higher concentrations, extraction efficiencies were nearly comparable to standard CIT responses, at low concentration, CIT was not detected when extracted, as highlighted by the absence of a response at 1 μM solution concentration, whereas at the same concentration, clear responses were found in the standard curve. This response suggests that the extraction procedure will not recover CIT at concentration below 1 μM .

From the linear section of the curve of extracted CIT standards in Figure 7.15, the LOD of HPLC analysis of CIT was calculated with application of Eq. 2.6 (Chapter 2) to be 582 nM. This is a result which is improved as compared to HPLC-UV LOD responses reported by Vail & Homann (1990) (LOD = 3203.36 nM) and Phillips *et al.* (1980) (LOD = 1280.94 nM). This result was however lower than the limit of quantification (LOQ) observed through experimental analyses as, at concentrations lower than 1 μM (within the range reported by Vail & Homann (1990) and Phillips *et al.* (1980)), CIT could not be detected (Figure 7.15), accordingly, the LOQ of extracted CIT by this HPLC method was established as a range between 1 and 5 μM .

Samples of MB culture media which had been extracted were assessed by HPLC-UV, the results of which are illustrated through Figure 7.16. These analyses were designed to assess whether CIT which was produced by *P. citrinum* cultured in the media could be detected. The media assessed included MB which had not been inoculated with *P. citrinum* “Maize Broth (-)” as a negative control as CIT would not be produced and MB which had been inoculated with *P. citrinum* “Maize Broth (+)”, as well as MSM as a further control solution. Each of the media were assessed with a set of internal standards which entailed spiking of pure CIT into the extracted media to a final solution concentration of 100 μM , referred to as “spiked” media henceforth to avoid repetition.

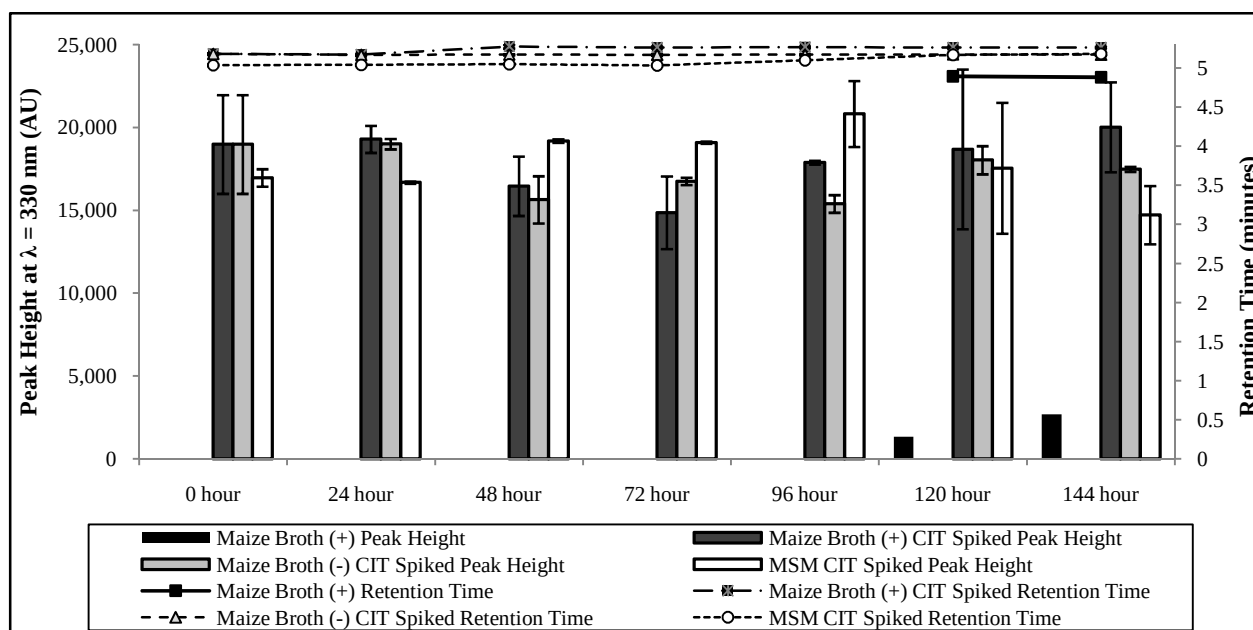


Figure 7.16: HPLC responses of CIT peak height and retention time responses in maize broth which was inoculated with *P. citrinum* (Maize broth (+)) as well as negative controls of maize broth not inoculated with *P. citrinum* (Maize broth (-)) and MSM, as well as internal standard spikes of CIT (100 μ M) in each medium. Although Maize broth (-), MSM (-) and MSM (+) were assessed, no CIT peak was recorded thus they were excluded from the key for clarity. Error bars report standard deviation from the mean, $N \geq 3$ per data point.

HPLC analyses conducted on samples extracted from MB (Figure 7.16) revealed that CIT was produced by the cultured *P. citrinum* and that it was detectable from 120 hours of cell culture (black bars). These responses were confirmed as CIT through application of both the CIT fingerprint analysis (Figure 7.14) and retention time or chromatographic analysis (Figure 7.13). The absence of any peaks in the Maize Broth (-) (un-inoculated with *P. citrinum*) and MSM media (which had no C or N and thus did not allow cell growth, thereby eliminating production of CIT), which removed from the key of Figure 7.16 for clarity, illustrated that the CIT recorded in Figure 7.16 was as a metabolic product of the *P. citrinum* and that it was produced through fermentation in the MB. Although the internal standards included media spiked with CIT to a final concentration of 100 μ M, the average of the calculated spiked concentrations across the entire culture period were determined to be 91.33 μ M, 88.61 μ M and 92.20 μ M for spiked MSM, spiked Maize broth (+) and spiked Maize Broth (-) respectively. Considering the slight suppression in response reported for extracted samples in Figure 7.15, these responses were considered accurate, and the slightly lowered response likely to be as a result of residual protein. This result is significant as it illustrates that the extraction procedure applied is effective and removed almost all apparent contaminants from the starting sample, which, as confirmed by the presence of the black columns, allows for the detection of CIT from complex media.

The retention time of the produced CIT (black markers and line of Figure 7.16) was at a lower time (4.88 minutes) than the internal standards, which comprised of media which had been spiked with CIT, which were at approximately 5.20 minutes and compared more closely with the retention time

recorded for responses of “CIT standard” reported in Figure 7.13 (4.56 minutes). This response is interesting as the “extracted CIT standard” curve (Figure 7.15) was also at longer retention times than CIT which was produced in Maize broth (+), referred to henceforth as “produced CIT”. The deviation which was present in retention time between analyses of “CIT standard” solutions and “produced CIT” solutions indicates however that although the extraction procedure is effective, parameter(s) which could not be identified directly influenced responses are affecting the analyses. Possible explanations for the improved response include that the culturing of *P. citrinum* lowered the solution concentration of substances such as proteins (Figure 7.10) and possibly trace elements which may otherwise have interacted with CIT to increase retention time. As the retention time did not align completely with analyses in “CIT standard” solution, it is likely that the extraction procedure did not completely remove protein from solution. A further possibility is that CIT was possibly not disassociating from protein effectively, or association of CIT with a protein may alter the ionisation state of CIT. This illustrates that although promising, the extraction procedure should be further investigated to more efficiently extract CIT from complex media. This discussion is continued in relation to electrochemical responses of extracted CIT.

This result provides a conclusive response by an established methodology detailing the detection of CIT from complex media.

d) Analysis of CIT extracted from maize broth by electrochemistry at EOx-GCE

Figure 7.17 illustrates electrochemical responses to “CIT standard” and “extracted CIT” samples from the culture of *P. citrinum* in maize broth (MB). Electroanalysis reported through Figure 7.17 was carried out on the same solutions which were assessed by HPLC analysis, and thereby allows for a comparison of the methods. Electroanalysis was carried out at EOx-GCE, as was performed in Chapter 6, and was confirmed as being CIT through relation of peak profile as well as peak potential to CIT spiked into solution as an internal standard.

As can be seen in Figure 7.17, limited responses were recorded on the absence of CIT, unlike that which was recorded in Figure 7.11 [A]. This response illustrates that the extraction procedure was effective and removed the components of the media (e.g. proteins) which were generating current response in Figure 7.11 [A], and thus allowed for an accurate analysis of CIT which had been extracted from growth media.

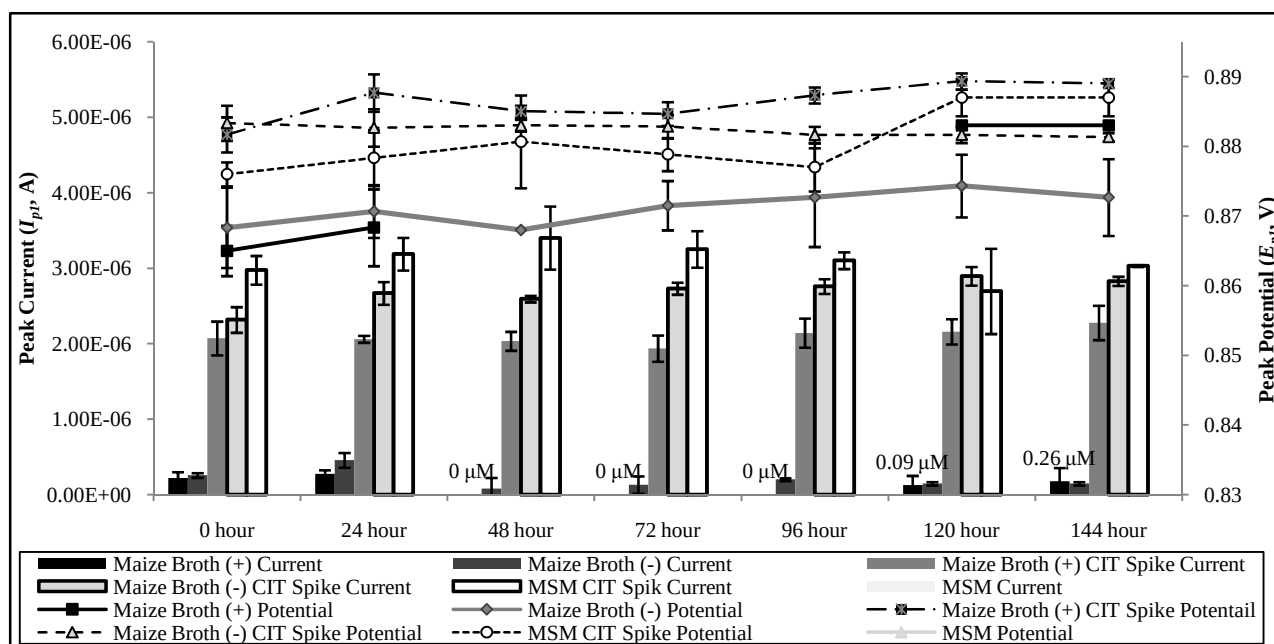


Figure 7.17: Electroanalysis of CIT extracted from maize broth inoculated with *P. citrinum* at EOx-GCE, including negative controls of un-inoculated maize broth and MSM as well as internal standard spike s of CIT in each medium. Error bars report standard deviation from the mean, $N \geq 3$ per data point.

Through the presence of black bars in Figure 7.17 (Maize Broth (+), which had been inoculated with *P. citrinum*) at 120 and 144 hours respectively, indicates that electroanalysis was able to detect CIT after 120 hours, as was similarly found under HPLC analyses (Figure 7.16). Although responses were recorded at 0 and 24 hours in the same sample set, these responses were not a result of CIT production and were likely to be a response to residual proteins in solution which were not extracted by the LLE, as was reported for Figure 7.11 [A]. This was evidenced by the peak profile as well as the E_{p1} (black markers) at 0 and 24 hours being lower than the responses at 120 and 144 hours of “produced CIT” (CIT which had been produced by culture of *P. citrinum* inoculated into MB), as well as both I_{p1} and E_{p1} being in and in the range of analyses conducted in Maize Broth (-), a negative control of MB which had not been inoculated with *P. citrinum*. Although no CIT responses were found in these samples in HPLC analyses, electroanalysis revealed the formation of peaks in extracted solutions which remained relatively stable in both I_{p1} (medium grey columns) and E_{p1} (solid grey line). As this set of peaks was also evident in Maize Broth (+) (black bar and line) at 0 and 24 hours, but disappeared thereafter with extended culture, this result lends credence to the proposal that CIT is not efficiently separated from the components in complex media.

These responses are further corroborated by the responses of recorded toward the internal control solutions of media spiked with CIT to a final concentration of 20 μM , referred to as “CIT spike” in Figure 7.17 to avoid repetition. Therein, I_{p1} response of CIT spiked into solutions of Maize Broth (-) “Maize Broth (-) CIT Spike” retained a stable but suppressed I_{p1} response through the entire culture period, a result likely to be as a result of the residual proteins in solution. I_{p1} responses in “Maize

Broth (+) CIT Spike” solutions, which were inoculated with *P. citrinum*, and thus allowed for removal of proteins from solution by the *P. citrinum* and production of CIT, showed a gradual increase in CIT I_{p1} response over the culture period as protein levels in solution dropped. This response continued until CIT I_{p1} responses in “Maize Broth (+) CIT Spike” solutions were equivalent to “MSM CIT Spike” solutions which was void of a majority of the complexities of environmental samples, and thus allowed for it to maintain larger and stable CIT I_{p1} responses.

The spiked CIT responses (20 μ M) in MSM, Maize Broth (-) and Maize Broth (+) solutions were slightly suppressed in relation to the standard curve of “extracted CIT samples”, suggesting further, as was the case in HPLC analysis, that an influence of either the CIT extraction procedure or residual components of solution affected CIT response. Furthermore, electrochemical responses in “extracted CIT solution” resulted in an altered peak profile (data not shown), wherein the development of I_{p2} was limited in comparison to typical responses in CIT standard solution (in NaOAc buffer, Chapter 6).

As it was suggested through Chapter 6 that I_{p2} formed as a result of a chemical step following an initial oxidation of CIT, the suppression of its formation may allude to a retained influence of the association of CIT with particles (most likely proteins) in complex media which, as was suggested in Section 7.4.5 [c] above, may be an indication that the LLE extraction procedure does not liberate CIT in its native form. Similarly, the shifted responses may be as a result of the ionisation /coupled state of CIT. This influence is further suggested through Figure 7.18 which provides comparisons of I_{p1} and E_{p1} responses of “extracted CIT standard” to “CIT standard” solutions, similarly to that reported in Figure 7.15. The “CIT standard” solution responses reported in Figure 7.18 were modified from Section 6.4.6 [a]).

Although HPLC analyses conducted at $\lambda = 330$ nm were observed to be comparable between “CIT standard” and “extracted CIT standard” standard samples respectively, Figure 7.18 describes marked differences in both I_{p1} and E_{p1} responses under electrochemical analysis. Importantly however, electroanalysis was able to detect CIT at concentrations lower than 1 μ M in extracted solutions, which was not possible through HPLC analyses, indicating greater sensitivity of the electroanalysis system at low solution concentration than was possible with HPLC analyses. The calculated LOD (according to Eq. 2.6, Chapter 2) of electrochemical analysis of extracted CIT was determined to be 20 nM, a response equivalent to the LOD determined in Chapter 6, Section 6.4.6 for analysis in buffered media. This concentration is significantly lower than that determined for HPLC analyses in this Chapter. Although that value is in excess of the LOQ determined for electroanalysis of CIT, as 90 nM was detected at 120 hours in Figure 7.17, the LOQ was not extensively lower than the LOD, and 90 nM was established as the LOQ.

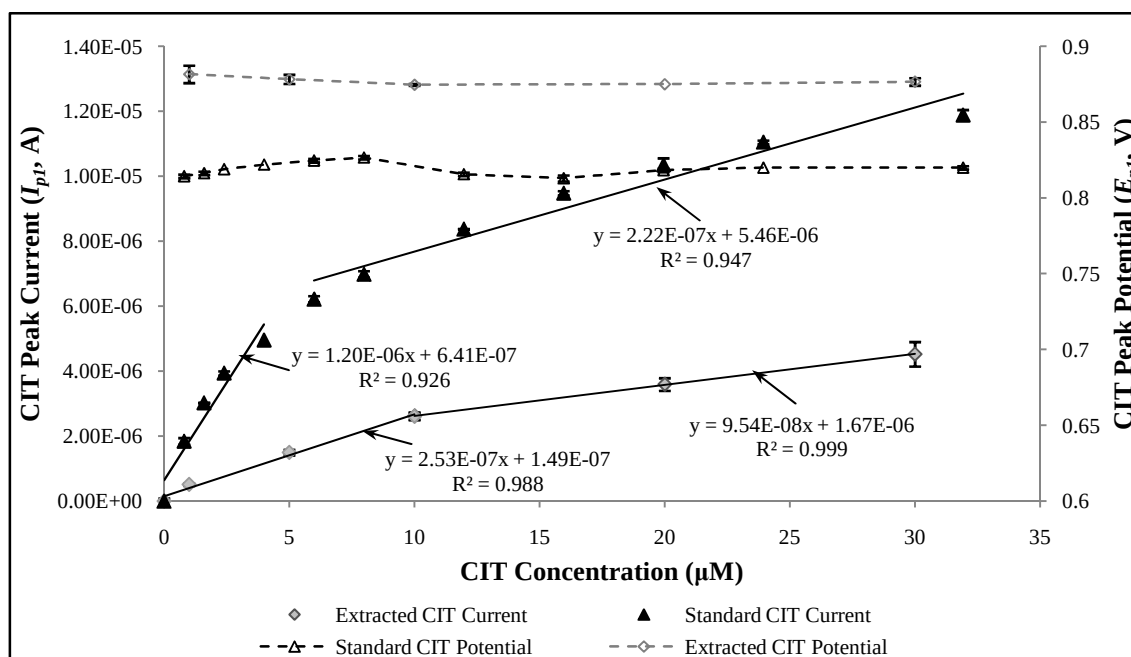


Figure 7.18: Electrochemical concentration curve responses (I_{p1} and E_{p1}) of CIT in MSM which was extracted (grey) in relation to unextracted CIT (black), each assessed in 0.2 M NaOAc buffer, pH 4.0. Error bars report standard deviation from the mean, $N \geq 3$ per data point.

At high concentrations, the electro-analytical responses were greatly suppressed in relation to the responses or CIT standard solutions, suggesting that the upper limit of detection is reached before that of the HPLC method. As the detection mechanism of each of the analytical systems rely on different processes, it stands to reason that an influence on moieties which are electrochemically active (-OH as described through Chapter 6, Section 6.4.9) may not have been similarly reflected in HPLC responses. As the I_{p1} responses were suppressed, E_{p1} responses were shifted to more positive potentials and I_{p2} was not formed as readily, the hydroxyl may not be readily accessible for oxidation.

7.5 Summary and Conclusions

The electroanalysis of CIT in components of environmental samples illustrated that the CIT associates with components of media. This was evident to an extent for carbohydrates in solution as a result of surface complexation of carbohydrates on the electrode rather than solution phase interaction of CIT with the carbohydrates. CIT was however found to interact both strongly and non-specifically with proteins and amino acids. These interactions were shown to occur both in solution and by complexation of proteins to the electrode surface. These features result in direct electroanalysis of CIT in media containing these components being unfeasible a result which prescribed the requirement for application of an extraction procedure to isolate CIT.

With extraction from media, CIT can be detected by electroanalysis. This, response confirms that electroanalysis can be applied in environmental analysis of CIT. The responses provides a comparable result to those acquired using HPLC as electroanalysis was able to detect CIT within the same range (120 hours) that was possible with HPLC analyses. This response is key within this study as it describes that electroanalysis with EOx-GCE can be used to detect CIT from complex media. This result was significant and highly pleasing as it demonstrated that electroanalysis, as a comparatively inexpensive, on site and simpler technique, provided responses in the same range as those of HPLC as an established technique, and thus provides an alternative which is likely to be of great benefit toward analysis of CIT and possibly other food borne toxins. Electroanalysis provided a lower LOD (20 nM) and an LOQ (90 nM) than was found for HPLC (582 nM). In so doing this technique provided an alternative methodology to HPLC analyses. Due to the required sample cleanup procedures however, this technique did not make analysis much faster or indeed that much simpler than HPLC analyses. In illustrating these results however, there is a clear indication of the possibility for further developments in this area of research.

As electroanalysis responses in extracted media were suppressed due to the presence of low levels of protein in solution, the LLE extraction procedure did not remove all of the protein from solution. Accordingly, although the LLE procedure provided a marked improvement in the responses gained in complex media (Figure 7.17 as compared to Figure 7.11 [A]), and affords the ability to apply electroanalysis towards detection of CIT in real world samples, it is likely that the responses will be markedly improved by a refinement to the extraction.

Further marked implications with regard to the interactions of CIT with proteins include that these analyses only considered the influenced of soluble proteins, however as CIT appears to interact non-specifically with proteins; CIT may attach to insoluble proteins, which would thus not be extracted according to the procedures applied herein and thus may result in recorded CIT under representing the CIT load of a sample. This interaction further limits the applicability of protein based bio-recognition elements (such as antibodies or enzymes) which may be applied in a biosensor as CIT will attach non-specifically to a range of proteins and thus may result in inaccurate responses with such systems. It is likely that bio-recognition systems such as aptamers would provide a more suitable platforms for development of biosensor systems for molecules such as CIT.

It is suggested that application of solid phase extraction (SPE) where CIT is bound to a column matrix to separate it from media with gradual elution to more efficiently purify it will provide a more effective extraction procedure. SPE is capable of extracting and concentrating polar and non-polar compounds, irrespective of pKa through interaction between the polymeric skeleton of material within

a column and the functional groups of the target molecule (Hennion, 1999; Bagheri & Mohammadi, 2003). Materials which can be used for HPLC include silica microcolumns (C18), porous graphitic carbons, polyurethane foams and polymetric resins with a number of elution steps which can be tailored (Coquart & Hennion, 1992; Angelino & Gennaro, 1997). Further benefits of such an extraction include that LLE often require more sample preparation time, larger workload for the analyst as well an increased risk of error (Knutsson *et al.* 1996; Berhanu *et al.* 2006). Such a study would undoubtedly improve greatly in the responses gained for CIT from complex media, particularly if responses were continued to be assessed by both HPLC and electroanalysis as comparison of responses have proven to provide highly beneficial insights into detection of CIT. This would however constitute a potentially vast study which is supplementary to the framework of the current Thesis and thus is not to be conducted herein.

CHAPTER 8

Conclusions and future recommendations

8.1 Conclusions

This thesis was directed at the establishment of a stable electroanalytical procedure for detection of the mycotoxin citrinin (CIT) in foodstuffs. This analyte, being adsorptive, provided an ideal target to probe, at a fundamental level the nature of the electrode interactions which can often lead to inconsistent analytical responses as well as the mechanisms or methods to overcome such instability. Regarding the knowledge gaps identified through Chapter 1, Section 1.3, the following research outcomes are summarised.

This Thesis provided the first electrochemical assessment of the mycotoxin CIT. CIT was shown to be strongly electrochemically active at carbonaceous electrode, particularly GCE, with an irreversible primary oxidation peak at $E_{p1} = 0.838$ V at pH 4.0 in 0.2 M NaOAc buffer vs. Ag/AgCl. Although E_{p1} was stable, I_{p1} was highly variable (C.V. = 20.16 %). This variability was identified to arise primarily as a result of the interaction of CIT with inconsistent surface area of electrodes after resurfacing. In order to interrogate this, fundamental analyses into the condition of electrode surfaces following a range of treatments was performed such that a means of attaining I_p stability in molecules such as CIT could be identified.

8.1.1 Fundamental studies

a) GCE surface characterisation

Chemical, electrochemical and morphological investigations of polished GCE in relation to two types of oxidised GCE surfaces, those prepared by chemical oxidation by application of piranha solution (Pi-GCE) and those induced electrochemically at anodic potentials (EOx-GCE) were performed with the aim of relating electrochemical responses of CIT to electrode surface characteristics. These studies provided the first investigations of the impacts and resulting electrode characteristics of piranha solution as an oxidant of GCE (Pi-GCE). Furthermore, they provided a detailed assessment of the characteristics of EOx-GCE which arose as a result of the specific electro-oxidation applied as it was derived specifically in relation to the oxidation of CIT.

Both oxidation procedures were shown to introduce graphitic oxide at GCE as illustrated through EDX, XPS and FTIR analyses. Although XPS highlighted slight differences in oxide composition were present between Pi-GCE and EOx-GCE oxide layers (total % O of 27.67 % and 33.47 % at Pi-GCE and EOx-GCE respectively), these did not account for the marked differences observed in the stability of CIT I_{p1} responses at each electrode (C.V. = 22.87 % at Pi-GCE and C.V. = 0.73 % at EOx-GCE). These responses led to the conclusion that CIT responses are governed primarily by interaction of CIT with morphological rather than chemical characteristics of the electrode surface. Morphological analyses of the GCE surfaces revealed that although the oxidation procedures masked the high level of scratching of polished GCE, neither Pi-GCE nor EOx-GCE were completely smooth surfaces, and Pi-GCE in particular presented irregular surfaces as a result of defoliation of the oxide layer. These findings lead to the conclusion that electrochemical responses are as a result of a mechanism within the oxide layer at the GCE, rather than the outer surface imaged by HRSEM.

Although several theories have been proposed to account for the improved electrochemical traits observed at EOx-GCE, each has been shown to be deficient in accounting for all observed behaviour. The studies conducted herein proposed the application of a structural approach to describing the mechanism. As it was shown that graphitic oxide is poorly conductive, as assessed through SECM, only a slight increase in electrode surface area which contributes to electrochemical responses is evident following surface modification, as was illustrated through electrochemical surface area determination. This small increase combination with the graphitic oxide layer being thick results in the boundary of the electrochemically active portion of the oxide layer (that which may contribute to propagating a redox reaction) being within the oxide layer. This feature, in combination with the accumulation of analyte within the oxide layer due to porosity of the layer, resulted in the electrode configuration being deemed to effectively behave as a rough electrode with analyte encapsulated at its surface, thereby allowing for thin layer like diffusive characteristics to take place. Furthermore, as the electrochemically active surface area of the electrode is within the oxide layer, and conductivity of the graphitic oxide is stable, the electrochemically active surface area remains stable across multiple electrode preparations and is independent of the outer morphological appearance of the graphitic oxide layer.

These studies may thus provide an explanation which could be relatively widely applied within electroanalysis for other molecules which may suffer from variability in response as a result of being adsorptive and being influenced by inconsistent surface preparation between analyses. In particular, this study established a methodology by which CIT could be accurately and reproducibly analysed in applied studies, including the characterisation of CIT in aqueous media and electroanalysis in complex media.

b) Accounting for variability in electrode surface characteristics

Although electrode modifications such as oxidation of GCE are typically applied in electroanalysis to tailor electroanalysis and stabilise I_p responses, these studies undertook a novel approach to attaining stability in I_p . This was conducted for application in scenarios where modification of electrodes prior to analysis are not feasible, where uniformity of electrode surface condition cannot be guaranteed, or where electrode surface condition may alter during analyses (such as under continuous analysis systems). Application of the double layer capacitance (C_{dl}) of an electrode as a thin layer in-situ probe of the state of the electrode (particularly its surface area) was assessed as it could be determined from a voltammetric scan without extensive analytical burden. These analyses showed that C_{dl} tracked I_{p1} responses proportionally, a set of responses which was true across structurally diverse molecules (CIT, Triol and 2,4-dimethylaniline) and across surfaces which were intentionally selected to have heterogeneous surface characteristics to introduce variability in I_{p1} and which were distinct from one another (polished GCE, Pi-GCE and fMWCNT-GCE). The methodology thus provided for a relatively broadly applicable system and established a basis for the methodology as a general means of accounting for variability within a data set. This was highlighted by the methodology dropping CIT C.V. to a third of starting value across polished GCE (C.V. = 7.18 %), Pi-GCE (C.V = 8.47 %) and fMWCNT-GCE (C.V. = 25.79 %).

These studies showed that the proposed application of C_{dl} could thus be applied in the identification of discrete solution concentrations of analyte as a practical application of the relationship between C_{dl} and I_p where this was previously not possible. These analyses applied regression slopes providing a measure across a data set, resulting in a number of measurements needing to be taken at a given solution concentration. Identified parameters which pertained to the application of the methodology, include that the analyte of interest be adsorptive to allow for C_{dl} (as a surface confined measure) to account for the magnitude of I_p response. As C_{dl} is a measure of the double layer charge across the entire surface, accuracy of analysis requires monolayer coverage of analyte at the electrode surface. Though these parameters limited further application of this methodology in electroanalysis of CIT within the confines of this Thesis, this study provides a platform for a novel approach to accounting for variability in I_{p1} which has potential for extensive further study and application within the field of electroanalysis.

8.1.2 Applied studies

a) Electrochemical characterisation of CIT in aqueous media

Application of EOx-GCE, as illustrated through fundamental analyses, allowed for stability of CIT I_{p1} , and thus the electrochemical characterisation of CIT. As the oxidation kinetics of CIT at polished

GCE (identified through E_{p1} responses) were shown to be near equivalent to EOx-GCE, EOx-GCE provided a surface at which the kinetics of CIT oxidation and characteristic behavioural traits in aqueous media could be determined as representative responses of the kinetics of oxidation at polished GCE. Electroanalysis of CIT was shown to be influenced by solution conditions including ionic composition, CIT concentration and pH, each of which described the sensitivity with which analyses can be conducted at EOx-GCE, and further the versatility of the surface in electroanalysis. The pK_a of CIT was determined to be 3.13, a result which corresponded to a pH dependent decrease in CIT I_{p1} from pH 1.0 to pH 4.0, but stability in response thereafter to pH 7.0. Analyses at alkali pH resulted in a reversible process described as either deprotonation or a shift toward an isomeric form which limited analyses to acidic solutions. As the process was reversible however, CIT can be assessed from samples which may be alkali if buffered to suitable acidic pH.

An LOD for CIT of 20 nM was determined, a result which is highly competitive in relation to chromatographic methodologies. The kinetics of CIT oxidation were shown to be relatively uniform in aqueous media. Mass transport was determined to comprise largely of adsorptive and diffusive processes, depending largely on analyte concentration and the presence of a preconcentrative pre-analytical step, but was shown to be independent of the electrode and electrolyte conditions investigated in this study. Although focused on I_{p1} , the mechanism of CIT oxidation was identified as an E_rC_i process, proceeding by the transfer of $2e^-$ and $1H^+$ per molecule of CIT, and the chemical step likely involving H_2O addition to the cationic intermediate. A reaction scheme for the mechanism of oxidation was proposed, involving the hydroxyl and carboxyl moieties of CIT has been proposed and peak presence and behaviour was demonstrated to be consistent with structural analogues of the carboxyl/hydroxyl- bearing ring of CIT.

b) Analysis of CIT from environmental samples

The electroanalysis of CIT in complex media was assessed at EOx-GCE. Those analyses revealed that the direct electroanalysis of CIT in aqueous media is hindered by the individual components of foodstuffs. Although carbohydrates from foodstuffs impact on CIT I_{p1} primarily through complexation with the electrode surface, it was shown that CIT associates non-specifically with proteins, a finding which limits the ability to detect CIT directly in media without sample cleanup.

Following liquid-liquid extraction (LLE) of CIT from *P. citrinum* growth media, analyses illustrated that electroanalysis of CIT from complex media is possible. Corroborating analysis of the same LLE extracted samples of CIT by HPLC-UV, revealed that CIT could be detected via electroanalysis detected within the same time period as was achieved through HPLC analysis. Furthermore, the voltammetric procedure provided a lower LOD (20 nM) and LOQ of 90 nM after extraction of CIT.

Both responses are lower than those for CIT detection than was found for HPLC (582 nM). Thus, electroanalysis provides a methodology which can be applied in the monitoring of food samples with accuracy and sensitivity at a level which is comparable to currently employed chromatographic techniques. Furthermore, due to the versatility of the EOx-GCE, this Thesis provides a basis by which further mycotoxins could be assessed via electroanalysis.

8.2 Future recommendations

8.2.1 Electroanalysis at EOx-GCE

Through these studies, detailed investigations of the characteristics of EOx-GCE provided a theory detailing the mechanism of function of this GCE surface modification. These analyses focussed largely on adsorptive molecules, as such further studies directed at diffusive mass transport based analyses may further develop the theory proposed herein.

As efficiency of the layer was shown to be largely limited by the conductivity of graphitic oxide, studies directed at improving the conductivity of the oxide layer may greatly improve sensitivity of response. Possible avenues for this include incorporation of CNT into the oxide layer.

These studies illustrated that electro-oxidation of GCE modification is highly effective in the electroanalysis of several molecules, of which both CIT was particularly sensitive to surface condition. Many further examples of analytes assessed at EOx-GCE are reported in literature (e.g. hydroquinone and 2,3-dicyanohydroquinone (Kepley & Bard, 1988; Dekanski *et al.* 2001 (a)); N,N-dimethyl-l-naphthylamine and N,N,N',N'-tetramethyl-l,l'-naphthidine (Otero *et al.* 1993) as well as catechol and ascorbic acid (Engstrom & Strasser, 1984)). As such this further application of this surface may afford the electroanalysis of a range of other mycotoxins and adsorptive molecules.

8.2.2 Application of C_{dl} to account for electrode surface condition

This Thesis provided an initial investigation into the application of C_{dl} to account for electrode surface condition, thereby identifying a selection of the potential parameters which may affect its application and warrants continued research and development. Significant potential for the application of such a methodology can be found in analyses which concern the application of nanomaterials such as CNT and fullerenes at electrode surfaces. Although benefits of their application is widespread through literature, and their utilisation in the construction of electrode surfaces is promising, their application in practical analyses has been limited by variability in response due to difficulties with regard to their

immobilisation at electrode surfaces, a factor which may be overcome with analyses via methods proposed here.

Further possible areas of research which may find application of this method include ultra micro electrodes, where due to the small electrode surface area, minute alterations have the potential to greatly influence responses. In line detection systems may also be applicable in systems where electrodes may gradually alter in condition as a result of continued analyses

8.2.3 Citrinin

This research provided a basis for establishing electroanalysis as a methodology of analysing CIT in complex media. Although great stability of electroanalysis was reported in buffered solution (Chapter 6), sensitivity of response was hampered by the components of environmental samples, particularly proteins, requiring the application of extraction procedures.

Through improvement of the extraction procedure, the efficiency by which CIT can be detected by electroanalysis may be enhanced. It is suggested that solid phase extraction procedures be investigated such that CIT can be bound to a column and then eluted to a purer sample. The sample size of media and components of media which influence CIT response should be broadened to improve on the applicability of electroanalysis of CIT.

Although this study provides strong evidence for direct electroanalysis of CIT, further developments in specificity and sensitivity of analysis may arise through development of biosensor systems for CIT. As CIT binds non-specifically to proteins, it is submitted that protein based biorecognition elements could be subject to non-specificity in response. As a result it is proposed that the application of Aptamer based biorecognition systems be developed.

Although a mechanism of CIT oxidation was proposed in Chapter 6 of this Thesis, further digital simulation of the ECE process would provide valuable information pertaining to subsequent peak generation, validation of the proposed mechanism, and would likely yield a greater understanding of the kinetics of CIT oxidation.

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Appendix A

Principles of Analytical Instrumentation

A1. Electrochemistry and electroanalysis

A1.1 Principles of electrochemistry

Electrochemistry is an analytical discipline associated with the study and analysis of electric potential (E ; measured in volts, V), current (I ; measured in amps, A), the integral of current, charge (Q ; measured in coulombs, C) and resistivity (R ; measured in ohms, Ω) (Bard & Faulkner, 2001). Through alteration one of those measureable parameters (e.g. potential) away from the pre-existing equilibrium and measuring the response of the system with another parameter (e.g. current), dynamic electroanalytical measurements may be performed. Typically, faradaic reactions are measured as a result of chemical reactions of *reduction* or *oxidation* (redox) of analyte in solution at the electrode surface, the specifics of which provide information on the analyte (Bard & Faulkner, 2001).

Electrochemical cells may be classified as either *galvanic*, wherein reactions occur spontaneously at the electrode surface at open circuit potential or *electrolytic* which required an imparted alteration of the equilibrium between electrodes immersed in an electrolyte solution (the electrochemical cell) in order to instigate a response (Wang, 1994). Electroanalytical techniques from these cell configurations are classed according to which properties of a cell are measured and those which are altered. Voltammetric systems measure current response relative to a linear change in potential, amperometric systems and coulometric systems measure current response and charge response respectively relative to a set potential (alteration garnered by stepping potential or altering solution condition), potentiometric systems measure potential shifts relative to set current and impedimetric systems measure impedance or admittance according to small amplitude potential perturbation relative to frequency (Bard & Faulkner, 2001).

Electrochemical cells, as applied in this Thesis, are comprised of three electrodes immersed in an electrolyte solution. Control of electrode potential is afforded by a potentiostat, which affords manipulation of the parameters mentioned above (Figure A1.1). The reaction being investigated (redox of a target analyte) takes place at the working electrode, the potential of which is altered in relation to a second electrode at a constant potential, called the reference electrode. An auxiliary electrode completes the circuit by acting as a source or sink of electrons to/from the working electrode

(Bard & Faulkner, 2001). Materials and characteristics of each of the electrodes as well as those of the electrolyte will alter the parameters of an electrochemical cell and allows tailoring of systems towards given analytical procedures. Figure A1 described the equipment setup which was applied in the electrochemical analyses conducted through this Thesis.

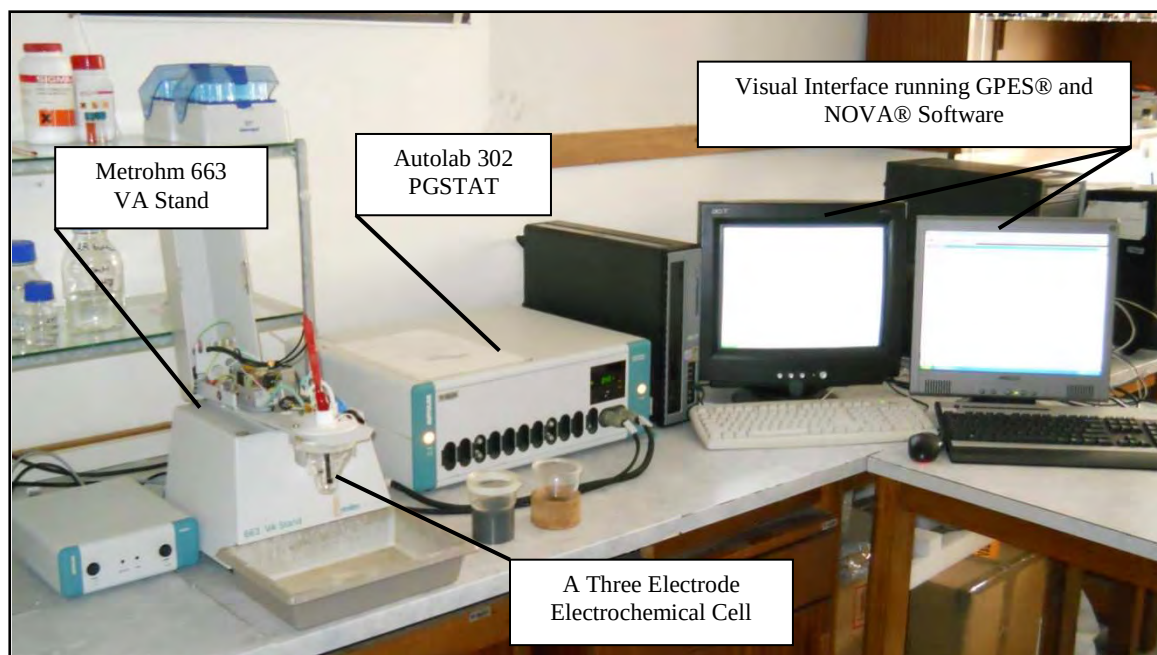
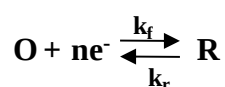


Figure A1.1: The equipment setup of the Autolab PGSTAT 302 Potentiostat/Galvanostat attached to a Metrohm Voltammetric Analytical stand (VA 663) with computer interface operating GPES software.

Potential and charge can be explained by the classic generic-compound reversible Nernstian reaction at the electrode-electrolyte interface at fixed potential (Kissinger & Heineman, 1996):



Scheme A1.1

Where $n\text{e}^-$ represents a stoichiometric addition of n electrons to the oxidised form **O** to generate the reduced form of the compound, **R**. The addition/release of electrons to **O** by the electrode is measured as current entering/leaving the electrode, thus, measurement of the current measures the proportional conversion rate of $\text{O} \rightarrow \text{R}$ and vice versa (Kissinger and Heineman, 1996). The rate of conversion of **O** to **R** depicted by the rate constants, k_f for the forward reaction and k_r for the reverse reaction are potential-dependent (Kissinger & Heinemann, 1996).

Alteration of potential of the working electrode drives the reaction and alters the equilibrium of $k_f:k_r$, determining which reaction becomes prevalent. The potentials at which oxidation/reduction occur for

a given compound are indicative of that reaction. For the purposes of oxidation, less positive potentials (oxidation is shown as positive by convention) indicate more favourable electron donation to the electrode by the analyte. The opposite is true for analyte reduction (Bard and Faulkner, 2001). As these reactions occur at the electrode-solution interface, the amount of current (peak current, I_p , Figure A1.2) is dependent of the number of reactions, which is driven by the transport of the reactant of the favoured reaction to the interface (mass transport). Thus, while I_p is an indication of the concentration of target analyte at or near the electrode surface, its magnitude (in either the reduction direction, I_{pc} or oxidation direction, I_{pa}) is also indicative of the rate of electron transfer during the redox process for reversible systems (Kissinger & Heinemann, 1996).

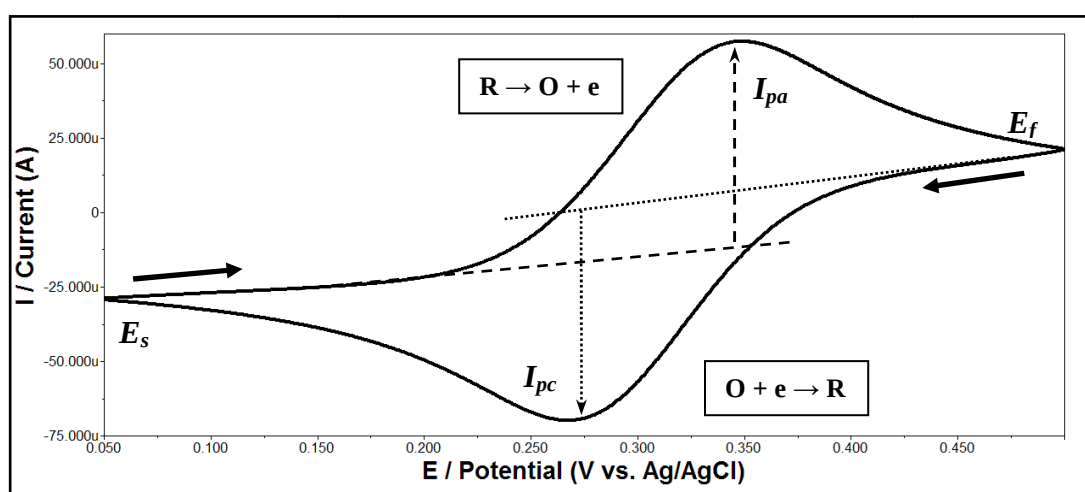


Figure A1.2: A typical cyclic voltammogram and the determination of peak current response. I_{pa} is the anodic peak current and I_{pc} is the cathodic peak current. Figure adapted from Kissinger & Heineman, 1996.

The peak heights, their position and shape yield a significant amount of information regarding the structure and behaviour of the target analyte as well as which process they conform to. Understanding the traits of the chemical reaction which the target analyte conforms to is fundamental in understanding the behaviour of the analyte at the electrode surface. Three processes are known to occur, namely *reversible*, *quasi-reversible* and *irreversible*.

Reversible processes (as in Figure A1.2), follow the Randles-Sevcik equation (Eq. 4.2), with complete regeneration of reactant and oxidation (I_{pa}) and reduction (I_{pc}) products are equal and k_f and k_r are fast and equal. A reversible couple can be identified from cyclic voltammetry by a peak potential separation ($E_{pa} - E_{pc}$) (Figure A1.2) of $0.059 \text{ V} \cdot n^{-1}$ (n is the number of electrons transferred during the redox reaction) and I_p is proportional to the square root of scan rate (Bard & Faulkner, 2001).

Non-reversible process occur when are redox process where the charge transfer step occurs at a slow rate such that only I_{pa} or I_{pc} is observed (irreversible), or with a weak return peak in some cases, but I_{pa}

$\neq I_{pc}$, (quasi-reversible). This is due to heterogeneous rate constants in k_f or k_r , resulting in the oxidation and reduction processes never occurring at equilibrium. The separation in peak potential is greater than 0.059 V.n^{-1} and generally shifted toward cathodic potentials with increasing scan rates (Bard & Faulkner, 2001). I_p of a quasi-reversible system is limited by both diffusion and the rate of electron transfer and thus increase in I_p is not proportional to the square root of scan rate.

A1.2 Electrochemical techniques

a) *Potentiodynamic methods: Voltammetry*

In cyclic voltammetry (CV), as seen in Figure A1.2, a potential difference is applied to the working electrode linearly at a set rate from an initial/starting potential (E_s) to a switch potential (E_f) where the voltage is scanned back to the starting value. The change in current during the forward and reverse scan is monitored as a function of the change in potential. During the potential scan, the analyte of interest is oxidised or reduced depending on its redox properties. As a result CV is often the first technique used for the determination of redox characteristics over the entire working potential range as behavioural kinetics and thermodynamics of analytes within chemical systems are highlighted (Noel & Vasu, 1990). A typical CV voltammogram is initiated at a potential in which no redox reactions occur. This ensures that the initial form of redox couple (R), in this case, is maintained throughout the bulk solution and the electrode-solution interphase. As the voltage is increased sufficiently positive of the redox potential, any R that reaches the electrode surface through diffusion is instantaneously oxidised to O. During the reverse (cathodic scan) for reversible couples, in which O is reduced back to R, O is depleted at the electrode surface and reverted back to R which accumulates at the electrode surface. The reduction of O is accompanied by a cathodic current (I_{pc}) which increases until the concentration of O becomes depleted at the electrode surface. In effect, the production of an anodic current peak during the oxidation cycle enables the formation of a cathodic current peak during the reduction cycle. A linear sweep voltammogram (LSV) would be equivalent to that shown in Figure A1.2, but would only represent one direction (either anodic or cathodic) of the potential sweeps shown therein. Linear sweep voltammetry (LSV) is operated according to the same principles, however in the case of LSV, the voltage of the working electrode is also scanned linearly at a set rate from an initial/starting potential, however rather than a switch potential, it is scanned to an end point as a one way process (represents one half of the CV process). The magnitude of the peak current, peak shape, peak separation and peak potential obtained from cyclic voltammetry can provide information on the structure and kinetics of electron transfer of the redox couple.

b) Potentiostatic method: Amperometry

The direct proportionality between the concentration of the target analyte and the magnitude of resultant current due to a constant potential being maintained between the working and auxiliary electrodes is the basis behind amperometry (Bagotsky, 2006; Bard & Faulkner, 2001; Kissinger & Heineman, 1996). This process as such applies a square wave voltage signal as a step from a voltage where no faradaic process occurs at the electrode (no current recorded) to a point where the faradaic process is initiated ($R \rightarrow O + e^-$ for an oxidative process) and current is recorded over time to a point where the surface concentration is effectively zero (Bagotsky, 2006; Kissinger & Heineman, 1996). At this point the system will be in equilibrium, and the current response will be in steady state relative to time unless there is a factor influencing it, such as diffusion of fresh electrolyte to the surface, change in solution concentration or a determined change in voltage. While linear diffusion of analyte to the electrode results in an exponential decay of current (as in the classical Cottrell Equation), convection of the solution through stirring (classed as hydrodynamic analyses) permits more stable current-time behaviour for diffusive systems (as in the classical Levich equation, for a rotating disc electrode undergoing a Faradaic process) (Heineman and Kissinger, 1996).

c) Galvanostatic method: Chronocoulometry

Chronocoulometry is based on the same methodology as that of chronoamperometry, however in the case of chronocoulometry, the current response is integrated such that the recorded response in charge (Q) (Kissinger & Heineman, 1996). As was described by Faraday, the amount of material deposited or evolved from an electrode is directly proportional to current (I) and time (t), and thus the quantity of Q that passes through a solution (Scholz, 2002). According to this, measurement of Q provides an excellent tool for both qualitative and quantitative analysis of redox reactions and can provide information such as the amount of material oxidised or reduced (if M and n are known) (Scholz, 2002; Bagotsky, 2006; Kissinger & Heineman, 1996).

d) Small-amplitude potential perturbation: Electrochemical impedance spectroscopy (EIS)

The methods reported above rely on large scale variations in potential (sweeps or steps) to induce faradaic responses far from the point where the electrode potential would be at equilibrium with regard to reactants. In contrast to these, the measurement of impedance relies on small scale perturbations (mV) at a steady state with the purpose of assessing the way in which the system follows the perturbation as a measure of resistive (R) and capacitive (C) components of layers at the electrode-electrolyte interface. By this, the porosity, passivation properties as well as probing electrochemical and mass transport mechanisms of redox and electrolytic species can be assessed for a given system (Bagotsky, 2006; Bard & Faulkner, 2001; Kissinger & Heineman, 1996). Since this is

carried out at, or near to equilibrium potential, there is very little flux in the system. Due to this, there is little influence of the i - E response curve, which in turn makes the assessment of kinetic factors far simpler. As the system is in steady-state during the analysis period, this system allows for precise measurements because the results can be averaged over multiple measurements over a term (time or frequency) (Bard & Faulkner, 2001). Furthermore, this allows for responses to be treated theoretically according to simplified i - E characteristics (Bard & Faulkner, 2001).

EIS as a method is based on the application of small amplitude changes in potential in a sinusoid wave which is determined by the frequency (rate of change of potential) and amplitude (extent of change of potential) which are independent factors of one another (Kissinger & Heineman, 1996). The R and C components are adjusted relative to the characteristics of the cell and the frequency applied until balance is achieved (Kissinger & Heineman, 1996). As has been described previously, when voltage is applied across a cell, a flow of electrons is generated as current. In the case of an impedimetric cell system, impedance (Z) is measured from a sinusoid potential (which is time dependent), resulting in a sinusoid current response of equivalent frequency when the other factors comprising the system are linear (resistor, capacitor and inductor). Depending on the extent of the composition of the resistive, capacitive and inductive elements of that system, the resulting sinusoidal current response from the applied sinusoidal potential, will differ from that sinusoidal potential (although at the same frequency) according to a phase shift or phase angle (Θ) where $\Theta = 0^\circ$ for a perfect resistor and $\Theta = 90^\circ$ for a perfect capacitor (Kissinger & Heineman, 1996).

According to Scholz (2002) the application of impedance allows for the investigation and elucidation of the electrical properties of materials and interfaces at conductive electrodes, as well as identifying electrode kinetics and interactions in complex electrochemical systems. This is possible as changes in an electrochemical system cause shifts in the steady state such as diffusion coefficients, charge transfer characteristics including resistance and number of electrons, reaction rate constants and double layer capacitance (Scholz, 2002). In so doing impedance provides a powerful technique for the characterisation of electrochemical systems and also provides a basis for targeted modification of the parameters of a system which may allow for improved electroanalytical characteristics.

A2. High resolution scanning electron microscopy (HRSEM)

Scanning electron microscopy (SEM) has been used for a huge number of imaging applications since Knolls work in 1935, with many different biological samples, chemical compounds and formations having been effectively analysed over the years. Since 1935 however, significant advancements have

been made in the precision of electron micrographs. This has allowed more detailed images with atomic resolution (Le Paven-Thivet *et al.* 2000). Such electron microscopes are now called high resolution scanning electron microscopes (HRSEM). Typical SEMs work by recording the scatter of electrons which have interacted with a sample after having been emitted from a high energy beam of electrons emitted in a vacuum. HRSEM, utilises a high electron beam thermionically emitted electron gun (zirconium oxide in this instance) to scan a sample in a raster mode. Resolution is attained through the electron beam being focussed by lasers and specialised lenses to a point as small as 0.5 nm in diameter (Nova, 2009). Through repeated random scattering and absorption with the surface atoms of the sample, the electrons lose energy and produce a signal that is converted into an image that represents the distribution of the current from the specimen. This procedure allows for HRSEM to obtain information regarding the topographical and compositional features of a sample, all visualised as high resolution images as small as 1 nm (Nova, 2009).



Figure A2.1: The equipment setup of the FEI Nova 230 high resolution scanning electron microscope (HRSEM) with an EDAX X-ray microanalysis module for energy dispersive microanalysis (EDX) measurements used in this study. Image courtesy of Dr. R.D. Brimecombe.

Interaction of electrons with the sample result in several types of electron scatter, including secondary electrons (SE), back-scattered electrons (BSE), characteristic X-rays, light, specimen current and transmitted electrons (TE). BSE are incident electrons (from the primary electron beam) which are scattered or deflected inside the specimen being visualised by multiple elastic collisions, resulting in the electrons escaping from the specimens surface (Gauvin *et al.* 2006). SE's originate from valence electrons which are ejected from the imaged material after inelastic collisions with the primary electron beam (Gauvin *et al.* 2006). The scatter from each form of electrons provides different

information regarding to the sample and are used independently to render images of the sample surface (Bazzola & Russel, 1999).

With equipment similar to that used in this study (Figure A2.1), Stevens *et al.* (2008) compared sub-micron images of crystals with higher resolution than images of the same samples visualised with atomic force microscopy (AFM). Furthermore, nanoparticles such as Sn and SiO₂ (Gauvin *et al.* 2006), Ti thin films (Le Paven-Thivet *et al.* 2000), CNTs (Gauvin *et al.* 2006; Mashazi *et al.* 2011), titanium doped tungsten disulphide (Scharf *et al.* 2009) and mesoporous silica epoxies (Romanes *et al.* 2008).

A3. Energy dispersive X-ray microanalysis (EDX) linked to HRSEM

Not long after X-rays were discovered, it became evident that specific X-rays was closely linked to the atomic structure of the chemical element from which it was derived, and further that each chemical element possessed a unique signature when stimulated to emit X-rays (Bertin, 1975; Noran, 1999). Classically X-ray analyses consisted of exciting a sample with a beam of X-rays, and monitoring X-ray emission when those components revert to their ground state. The distinctive X-ray signal emitted was measured through processes such as separation into component wavelengths by a diffraction crystal (wavelength-dispersive spectrometer) (Noran, 1999). Subsequently, with the advent of electron microscopes, it was found that excitation by a beam of electrons would instigate a release of element-specific X-rays similar to those generated by X-ray excitation (Suter *et al.* 1976). For EDX, X-rays are generated by the electron beam (of the HRSEM) ejecting an electron from an inner shell of a sample atom which results in a vacancy which must be filled by a valence electron from a higher-energy shell of that atom. By dropping to a lower energy level, that electron releases energy in the form of electromagnetic radiation which is exactly equal to the energy difference between the two electronic levels involved, which is specific to the element. Since this energy difference is fairly large for inner shells, the radiation appears as X-rays which are characteristics of the element (Noran, 1999).

In this study, energy dispersive X-ray microanalysis (EDX) was conducted using an EDAX module connected to a FEI Nova 230 HRSEM (Figure A2.1). X-rays emitted from a sample after excitation from the HRSEM electron beam were analysed by the EDAX detector positioned at 30 ° with respect to the sample stage. Major benefits derived from EDX analyses include that several elements may be detected at exactly the same point and under exactly the same experimental conditions (König, 1976; Suter *et al.* 1976). As an HRSEM electron beam is used, the area of the sample point can be as that

imaged under HRSEM. This allows for one to focus on a given portion of a sample and determine the chemical composition of a defined portion of that sample which may be as small as 40 nm diameter (König, 1976; Brunner *et al.* 2000).

A4. Fourier transform infrared (FTIR) spectroscopy

Infrared (IR) spectroscopy is generally referred to as the workhorse of analytical chemistry due to its ease of application, the specificity and accuracy of results and the range of samples which can be assessed using this technique. Infrared spectroscopy operates on the basis of monitoring the frequency of vibrations of bonds between nuclei (Ebbing & Gammon, 1999). As molecules absorb IR radiation, the bonds between each set of atoms holds a specific vibrational frequency, and when irradiated by IR with the same frequency, the bond is caused to vibrate more strongly, thereby specifically identifying each bond. This allows easy identification of the atoms that the bond holds together (Ebbing & Gammon, 1999). When assessing a sample, a spectrum of frequencies units are emitted in a spectrum which will be distinctive for molecule(s) according to the size and position of the peaks formed. In so doing, this technique offers information regarding the chemical composition and also information about the structure of the molecule.

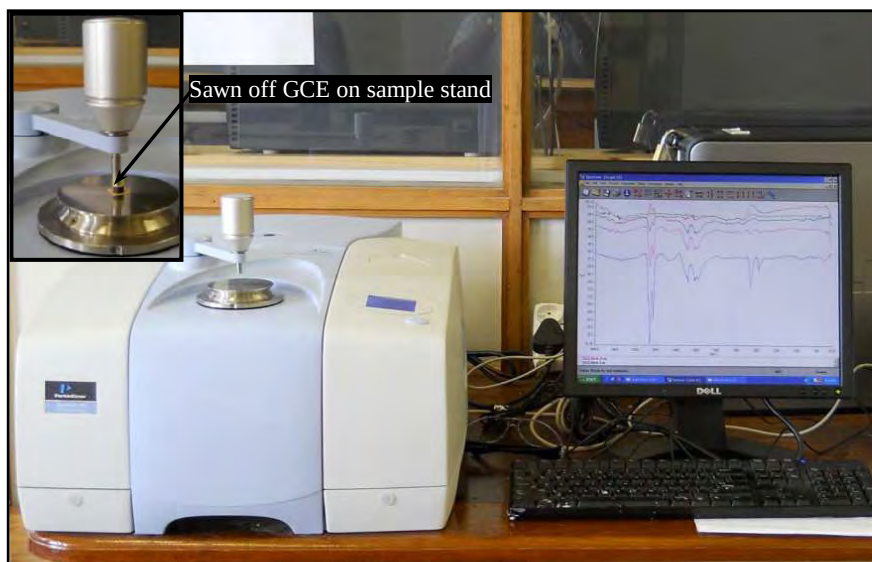


Figure A4.1: The equipment setup of the PerkinElmer Spectrum 100 FTIR spectrometer used in this study. Inset Showing Sawn off GCE on the Sample stand of the FTIR apparatus.

As opposed to normal IR which is generally associated with measuring a small spectrum of wavelengths at a time, Fourier transform infrared spectroscopy (FTIR) is an IR technique in which the sample is irradiated and the entire IR spectrum is recorded at once. Furthermore unlike normal IR which are generally offer information based on absorbance, FTIR spectra offer information for

absorption, emission, photoconductivity or Raman scattering, in so doing offer more detail than standard IR and will often also afford greater sensitivity.

Many FTIR studies which have investigated films and in particular film based GCE modifications have been published (Zhang & Lin, 1997; Wang *et al.* 2001 a; Wang *et al.* 2001 b). Importantly however, specific studies on graphitic oxide have been carried out by researchers including (Shen *et al.* 2008; Lopez-Garzon *et al.* 2003; Sullivan *et al.* 2000; Yang & Lin, 1995; Yang & Lin 1994).

A5. X-ray Photoelectron Spectroscopy (XPS)

X-ray photoelectron spectroscopy (XPS), also known as electron spectroscopy for chemical analysis (ESCA), is a quantitative technique which is based on the photoelectric effect outlined by Einstein in 1905. According to Nefedov (1988), the concept of the photon was used to describe the ejection of electrons from a surface when photons impinge on it according to Einstein's photoeffect equation:

$$h\nu = E_b + E_{kin} \quad \text{Eq. A5.1}$$

Where $h\nu$ is the quantum energy, E_b is the binding energy of the electron in matter and E_{kin} is the kinetic energy of the ejected electron. The technique can be summarised as a measurement of the kinetic energy of inner valence electrons which have been ejected from materials being assessed by incident X-rays (photons of known energy [$h\nu$]), which then allows one to determine E_b , which is a sensitive characteristic of chemical bonds and is closely related to the energy of ionisation (Nefedov, 1988; Ebbing & Gammon, 1999).

Analysis of the energy of the photoelectrons leaving a sample allows for the generation of a spectrum of peaks in which both the binding energy and area of the peak can be used to determine the composition of the materials of the surface. Furthermore, the shape of each peak and the binding energy provide information regarding the chemical state of the emitting atom as they will be slightly altered depending on the state of the atom (Nefedov, 1988). In so doing the technique allows for analysis of the chemical composition and nature of a broad range of samples (except helium and hydrogen) to a depth profile of approximately 50 Å, including the elemental composition, the chemical and electronic states and the empirical formula of a material (Nefedov, 1988; Sullivan *et al.* 2000).



Figure A5.1: The equipment setup of the Kratos Axis Ultra DLD XPS used in this study.

A6. Scanning Electrochemical Microscopy (SECM)

The scanning electrochemical microscope (SECM) is a scanning probe techniques based on measurement of current at the tip of an ultramicroelectrode (UME) which was introduced in 1989 by Bard and co-workers (Bard *et al.* 1989). SECM allows for the chemistry near interfaces to be examined at high resolution through detecting electrochemical responses reactions that occur at the tip of a UME as it is scanned in close proximity to a substrate (Bard & Faulkner, 2001). In so doing this technique allows for one to obtain information pertaining to surface chemistry, conductivity, reaction rates and topography through a feedback current (Bard & Zoski, 2000).

In addition to imaging which is achieved by rastering (scanning in the $x - y$ plane) the UME above a substrate, other modes of operation include surface conditioning which allows one to alter substrate surface by controlling the tip at different potentials and durations and approach curve mode which allows for scanning in the x , y and z planes whilst while the probe and possibly substrate potentials are controlled and currents are measured (Bard & Faulkner, 2001). Through these procedures numerous studies have been conducted with applications including studies of localised and directed nano-modifications of surfaces such as with corrosion and deposition of metals, biological analyses (high resolution studies on photosynthesis) and studies on dyes, photoelectric coatings, semiconductor materials and electron transfer kinetics (Kwak & Bard, 1989 A; Kwak & Bard, 1989 B; Bard *et al.* 1992; Borgwarth *et al.* 1995; Ratcliff *et al.* 1996; Bard & Zoski, 2000; Xiong *et al.* 2007).

Appendix B

Characterisation of fMWCNT

These studies were conducted and compiled by Mr. Shane Flanagan of Rhodes University as part of his MSc Thesis studies and included the characterisation of the acid functionalized fMWCNT used in this study. This appendix represents an abridged report of that section of the MSc Thesis.

B1. AFM characterisation of fMWCNT

An investigation on the surface morphology of acid functionalised MWCNT in relation to similarly oxidised SWCNT layers adsorbed to a glass substrate was carried out using atomic force microscopy (AFM) (Marchall *et al.* 2006). Centrifugal purification was used as a robust method to separate the CNT fractions from impurities and protic ions produced from the acid functionalisation procedure (Nepal *et al.* 2005). AFM scans obtained for 6 hour acid functionalised MWCNT pellet fraction is illustrated in Figure B1.

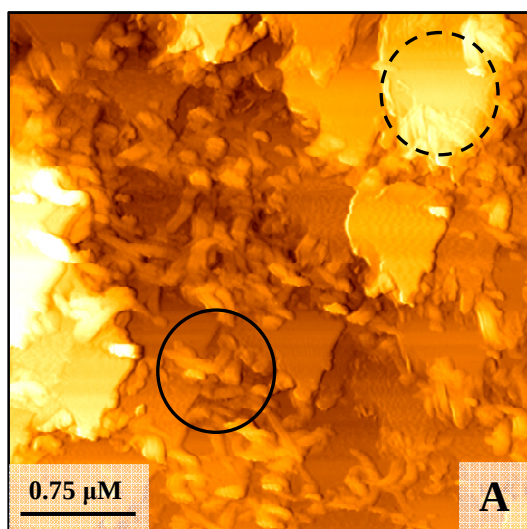


Figure B1: AFM images of 1 mg/ml MWCNT pellet in DMF and dried to a glass silica substrate at 60 °C. Images correspond to 6 hour acid functionalised CNT fractions. Insert shows individual MWCNTs (solid), and carbonaceous sheets (dotted).

The porous, irregular, surface morphology observed for the 6 hour functionalised MWCNT layer was attributed to a high packing density of nanotubes formed within an interconnected network of carbonaceous material (Dumitrescu *et al.* 2007). Although the composition of the carbonaceous network cannot be determined by AFM, no metallic catalysts were assumed to be present as all metal

particles would have been oxidised during acid treatment and removed by the centrifugal purification procedure (Zhang *et al.* 2000). Thus, the carbonaceous network was assumed to contain primarily CNT, graphite, amorphous carbon and charged surface groups produced from acid treatment (Wildgoose *et al.* 2006). For the MWCNT modified surface, individual nanotubes of 70 nm in size could be visualized and were shown to aggregate into bundle like structures incorporating multiple MWCNTs. Layering of the MWCNT bundles and carbonaceous impurities, identified as flat sheet like structures, result in indiscernible macroscopic clumps of variable height which were observed to scatter throughout the MWCNT modified surface.

B2. Raman Scope characterization of fMWCNT

The macroscopic morphology of physically adsorbed MWCNT layers were investigated by Raman Scope microscopy set to a 20x magnification level. A glass substrate was used as the modification surface for the CNT layers exposed to acid functionalisation times of 2, 4, 6, 8 and 24 hours.

Physical adsorption of the CNT fractions produced an uneven coverage of carbonaceous material bound to the surface of the glass substrate in which regions of high carbonaceous density were typically observed at the centre and along the peripheral regions of the adsorbed CNT fraction. Raman scope analysis was thus performed at the high density regions in order to assess the change in surface morphology of the CNT layers upon acid treatment. The MWCNT supernatant fraction was excluded from analysis due to the low retention of carbonaceous material, thus all analyses reported from the pellet. Figure B2 illustrates the Raman scope images obtained for acid functionalized MWCNT pellet fractions physically adsorbed to a glass silica substrate.

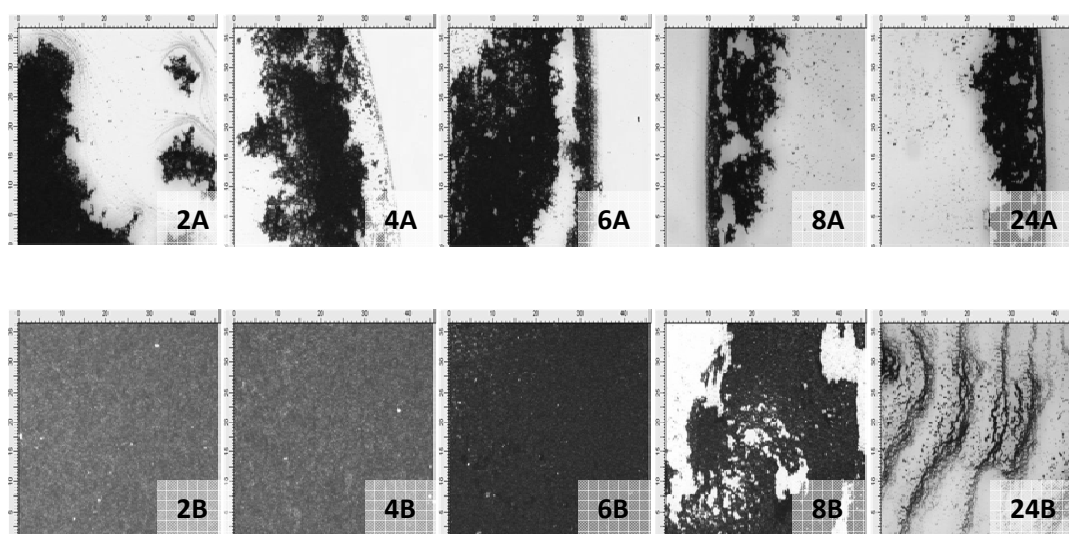


Figure B2: Raman scope images of 1 mg/ml MWCNT fractions in DMF dried on glass at 60 °C. Images correspond to high density MWCNT regions of functionalisation time 2 (2A), 4 (4A), 6 (6A), 8 (8A) and 24 (24A) hours, and to low density MWCNT regions of functionalisation time 2 (2B), 4 (4B), 6 (6B), 8 (8B) and 24 (24B) hours. Scale shown in μm .

A non uniform surface coverage of carbonaceous material was observed for MWCNT pellet fractions physically adsorbed to the glass substrate surface. The surface morphology of the adsorbed fractions comprised of carbonaceous impurities dispersed along the substrate surface or bound to an interconnected network of MWCNT aggregates of varying density. Low density aggregates were primarily comprised of carbonaceous dispersions while uniform carbonaceous layers and macroscopic carbonaceous structures were observed for high density MWCNT aggregates formed at the centre and periphery of the adsorbed fractions respectively. A reduction in aggregate density was observed for increased functionalisation times and was accredited to oxidative degradation of MWCNTs during acid treatment and the removal of carbonaceous impurities upon centrifugal purification.

A comparison of the peripheral macroscopic structures between the 2 hour and 24 hour functionalized MWCNT fractions shows the extent of MWCNT degradation coupled to the removal of carbonaceous impurities. Furthermore, significant degradation of the MWCNT aggregates was observed for functionalisation times beyond 8 hours as observed by the irregularity of layer deposition within the centre of the adsorbed fractions shown in Figure B2 (8B) and (24B).

From the above results, further progress must be made toward the modification of surfaces by uniform layers of high purity CNTs in order to effectively increase the electroactive surface area available for electroanalysis. The large degree of carbonaceous impurities retained within the supernatant fractions of the functionalized CNTs also illustrates the necessity for CNT purification. In addition, poor purification efficiency was observed upon increased acid treatment times due to the successive accumulation of carbonaceous impurities through structural degradation of the CNT fractions. To increase the efficiency of purification, the effect of centrifugation at higher rotational speeds or for extended periods of time would need to be investigated.

B3. Raman spectroscopy characterisation of fMWCNT

Raman spectroscopy was used to determine the effect of acid treatment on the physical and electronic properties of CNTs (Belin & Epron, 2005). Determination of the Raman spectra allowed for the efficacy of acid functionalisation and centrifugal purification of MWCNT fractions to be evaluated through analysis of the G-band and D-band spectra inherent to CNTs. The in plane bond stretch mode G-Band between 1500 cm^{-1} and 1600 cm^{-1} is indicative of the nanotube structural integrity while the disorder induced D-band around 1300 cm^{-1} relates to both defect sites within the nanotube structure and the presence of carbonaceous impurities (Burghard, 2005; Delhaes *et al.* 2006).

Figure B3 depicts the Raman spectra obtained for the MWCNT pellet fraction at increasing acid functionalisation times. Therein, the 2 hour fMWCNT fraction showed a rapid increase in D band intensity with a minimal change in G band intensity as compared to the pristine MWCNT sample. For functionalisation times beyond 4 hours, acid treatment resulted in a rapid decrease in D band intensities and a corresponding decrease in G band intensities with the exception of the 24 hour functionalized fraction.

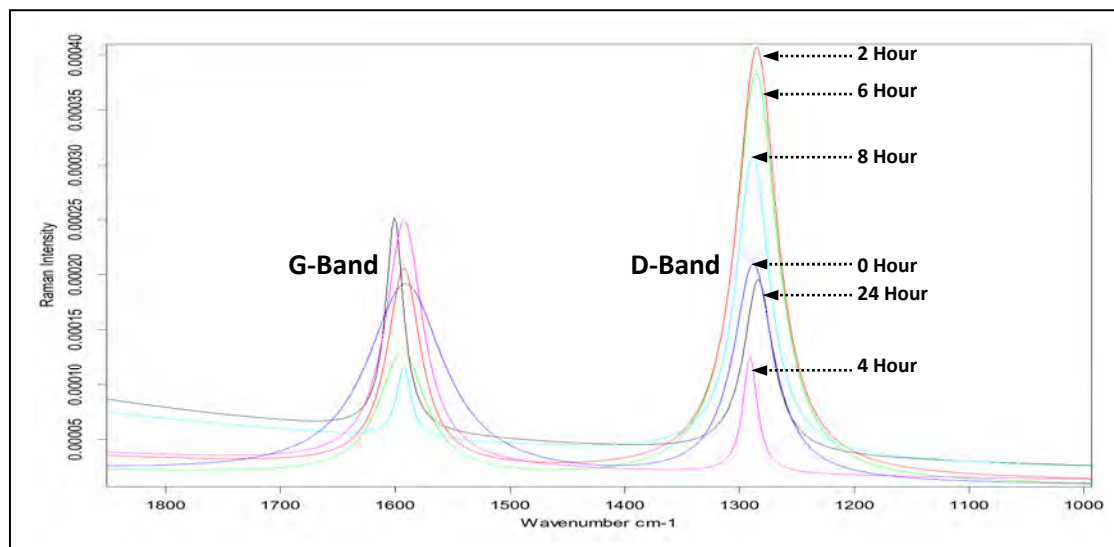


Figure B3: Raman Spectrum of MWCNT fractions of 0 (blue), 2 (red), 4 (pink), 6 (green), 8 (cyan) and 24 (black) hour functionalisation times mixed with 0.5 wt% in KCL taken at 100mW averaged for 1024 scans.

The ratio between the D-band and G-band is used as an indicator of CNT purity, whereby a high D/G band ratio is typically indicative of a high quantity of structural defects (Burghard, 2005; Delhaes *et al.* 2006). Figure 4 summarizes the D/G band intensities determined for the MWCNT fractions of increasing functionalisation times.

For the MWCNT fractions for functionalisation times from 2 to 8 hours, a near linear increase in D-band/G-band intensity was observed, with the exception of the 4 hour functionalized fraction, as a result of an increase in D-band intensity with corresponding decrease in G-band intensity. The 24 hour MWCNT fraction showed a lower D-band/G-band intensity ratio than that of the pristine MWCNT sample. The decrease in D-band intensity from the pristine MWCNT fraction to the 2 hour functionalised fraction was attributed to the removal of carbonaceous impurities initially present in the pristine sample. The removal of impurities present in the pristine CNT samples demonstrates the importance of an initial purification step.

The similarity in G-band intensity and gradual decrease in D-band intensity observed for the fMWCNT fractions for functionalisation times between 2 to 8 hours denotes the overall maintenance

of MWCNT structural integrity with successive exfoliation and removal of amorphous carbon impurities from the MWCNTs upon acid treatment. Oxygenated functionalisation of the outer MWCNT graphene layers thereby resulted in an increase in D-band/G-band intensity (Datsyuka *et al.* 2008).

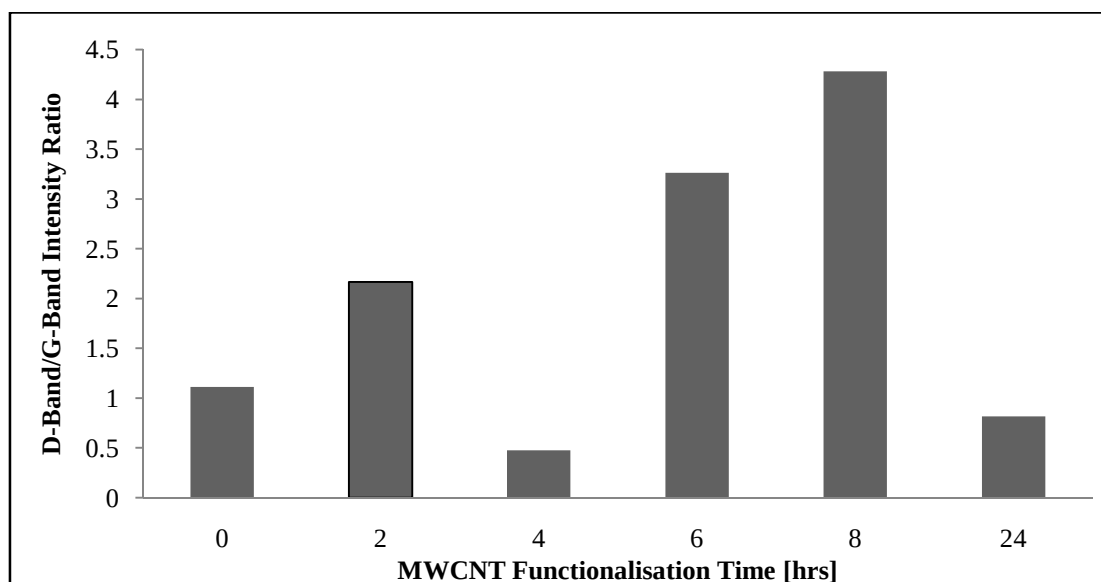


Figure B4: Bar graph comparing the D-band/G-band intensities for MWCNT which had been functionalised for 0, 2, 4, 6, 8 and 24 hours respectively. CNT fractions were mixed 0.5 wt% in KCL taken at 100mW averaged for 1024 scans.

From the above results, acid treatment times up to 4 hours allowed for the functionalisation of CNTs without significant degradation of the overall CNT structure and resultant formation of carbonaceous impurities within the CNT pellet fractions, Strong electrochemical responses were observed with GCE surfaced with 2 hour functionalised MWCNT, thus they were applied in further studies.

Appendix C

Voltammetric analysis of 1,2,4-trihydroxybenzene (Triol) and 2,4-dimethylaniline (2,4-DMA)

C1. Methodology

Stock solutions of 100 mM 2,4-DMA and Triol were prepared with absolute ethanol and H₂O respectively and stored at – 20 °C until used.

C1.1 Electrochemical procedures and parameters

An equivalent apparatus and set of experimental conditions to that detailed in Section 5.3 of Chapter 5 was applied herein. Triol was however assessed within a potential range of 0 V to 0.6 V and 2,4-DMA was assessed within a range of 0.2 V to 1.0 V. Concentrations and electrolytes applied were detailed in relation to each figure.

C2. Analysis of Triol at Pi-GCE using LSV

Figures C1 and C2 are representative figures of the electrochemical signal of Triol in 0.2 M NaOAc buffer and 1.0 M H₂SO₄ as assessed at both 0.05 mM and 0.8 mM. Although CV scans are not, Triol yields reversible peaks under each of the conditions assessed.

The peak profile of Triol (Figure C1) is sensitive to concentration as well as ionic composition of the electrolyte. In 0.2 M NaOAc buffer pH 4.0, Triol has a characteristic peak at an $E_p = 0.20$ V and $I_p = 27.82$ μ A at 0.8 mM. The peak shifts to $E_p = 0.15$ V and $I_p = 4.03$ μ A when concentration is reduced to 0.05 mM. When scanned in 1.0 M H₂SO₄, the peak is found at significantly higher potentials of $E_p = 0.41$ V and an $I_p = 2.18$ μ A at 0.05 mM and an $E_p = 0.42$ V and an I_p of 28.27 μ A at 0.8 mM.

Appendix C

Voltammetric screening of select mycotoxins

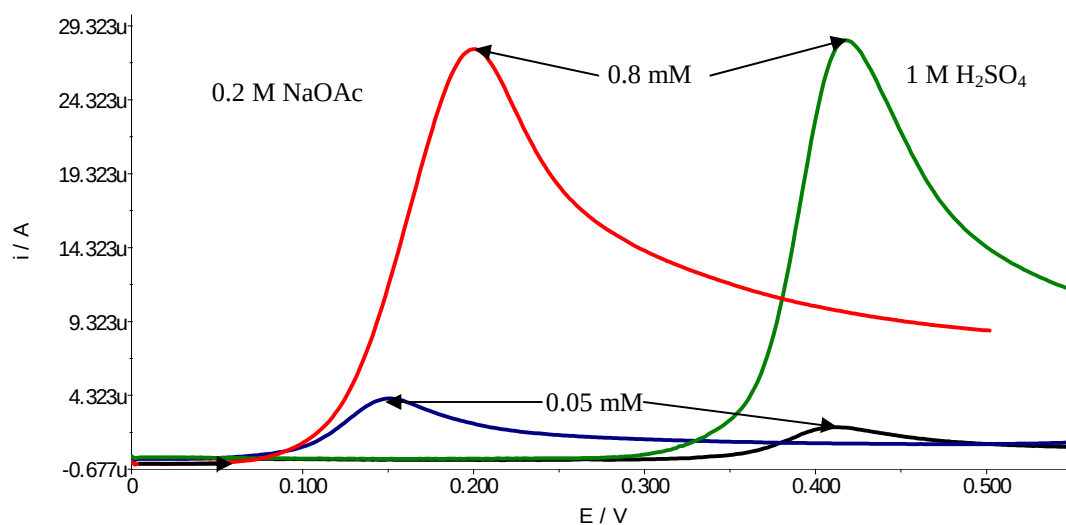


Figure C1: Electrochemical profile of Triol at 0.05 mM and 0.8 mM in both 0.2 M NaOAc, pH 4.0 and 1.0 M H₂SO₄. Scanned at 100 mV.s⁻¹ vs. Ag/AgCl. Background current was subtracted for each of the scans.

Figure C2 provides representative responses of the absence of fouling with continuous cycling of scans without resurfacing the electrode.

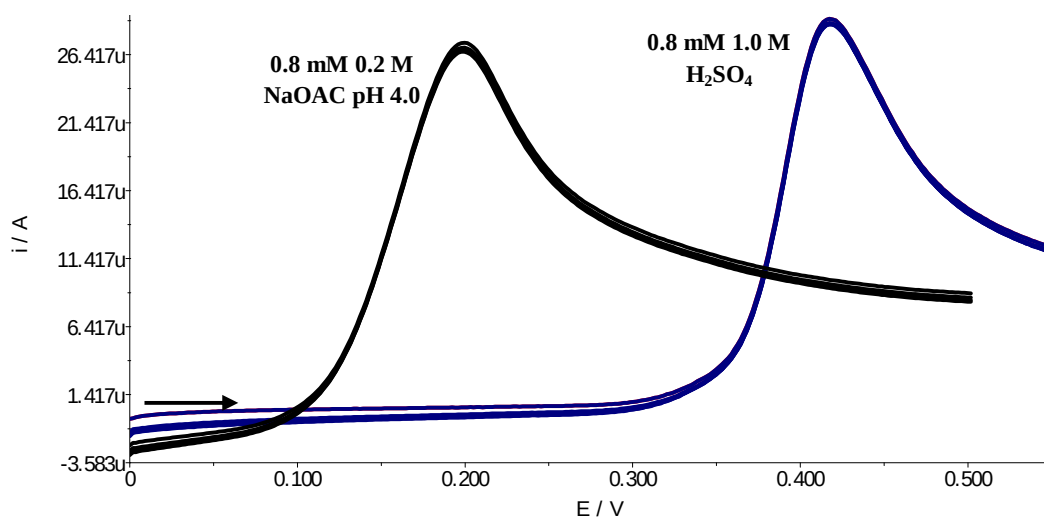


Figure C2: Absence of fouling Triol in 0.8 mM 0.2 M NaOAc pH 4.0 and 1.0 M H₂SO₄. Scans were carried out at 100 mV.s⁻¹ vs. Ag/AgCl.

The peak profile is stable, and the electrode is not fouled by successive oxidation sweeps at a single electrode (Figure C2). The peak profiles do also differ slightly with regard to the shape of the peak, as, at more acidic pHs, it is sharper and more clearly defined than in when assessed in 0.2 M NaOAc, pH 4.0.

Table C1: I_p responses of Triol at 0.05 mM and 0.8 mM in 0.2 M NaOAc, pH 4.0, and 1.0 M H₂SO₄.

	0.2 M NaOAc Buffer Triol Current Response		1.0 M H ₂ SO ₄ Buffer Triol Current Response	
	0.05 mM Triol	0.8 mM Triol	0.05 mM Triol	0.8 mM Triol
Average	3.19×10^{-6}	2.25×10^{-6}	2.78×10^{-5}	2.68×10^{-5}
Standard Deviation	2.57×10^{-7}	1.94×10^{-7}	9.49×10^{-7}	1.08×10^{-6}
C.V. (%)	8.04	8.64	3.41	4.03

Unlike the CIT data sets, the I_p responses of each of the parameters yield stable responses when assessed directly, as is illustrated by the extent of C.V. variation. Accordingly, this provides an extremely stable platform as a molecule against which Triol can be compared.

C3. Analysis of 2,4-DMA at polished GCE using LSV

The voltammetric profile of 2,4-DMA as assessed by LSV in 0.2 M NaOAc buffer is shown in Figure C1, Electrochemical analysis of 2,4-DMA has been carried out previously by Brimecombe et al. (2006 a) and Brimecombe and Limson (2006 b), which form the basis on which this molecule is applied in this study.

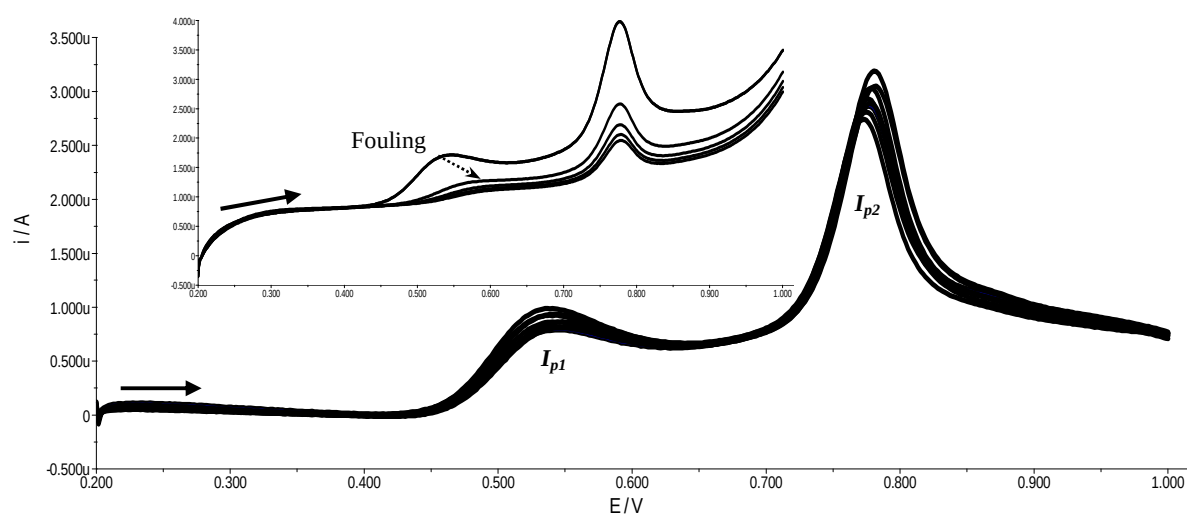


Figure C3: Peak profile of 30µM 2,4-DMA in 0.2 M NaOAc pH 4.0 determined with 3 separate scans on 4 different polished GCE, background current was subtracted from each of the scans. Insert illustrates fouling of 2,4-DMA through 5 consecutive scans in 30µM 2,4-DMA in 0.2 M NaOAc, pH 4.0. All scans were carried out at 100 mV.s⁻¹ vs. Ag/AgCl.

2,4-DMA has a distinctive peak profile consisting of two peaks at an E_p of 0.547 V ($\pm$ 0.008 V) and 0.777 V ($\pm$ 0.002 V) for I_{p1} and I_{p2} respectively. The peak designated I_{p1} (Figure C3) was used for all analysis as it was found that the I_{p2} arises as a result of the oxidation of 2,4-DMA resulting in a cation intermediate which in turn reacts with unoxidised 2,4-DMA to form tetramethylbenzidine (TMB) (Brimecombe and Limson, 2006 b). When scanned in buffer, the peak profile is fairly reproducible,

with an average current response of 8.76×10^{-7} A ($\pm 8.45 \times 10^{-8}$ A), which has a C.V. of 9.65 %. As is seen in the insert, 2,4-DMA does foul rather extensively, and as such the first scan of each scan set was used as a measure of 2,4-DMA concentration.

C4. Analysis of 2,4-DMA at Pi-GCE and EOx-GCE in relation to polished GCE

Assessment of more complex surface chemistry was carried out through oxidising the GCE surface with both piranha solution, however a further oxidation method was also applied in the form of electro-oxidation of the GCE. Although not represented individually herein, as was done in Figure C3, the resulting peak profiles of 2,4-DMA on Pi-GCE and EOx-GCE surface are similar to that of polished GCE, as too were the rates of fouling. There are however slight variations in the profile of each, the degree of which is illustrated in Figure C4. Therein the resulting 2,4-DMA profiles on each surface is illustrated. The analysed results of these analyses are summarised in tabulated far as is illustrated in Table C2 below.

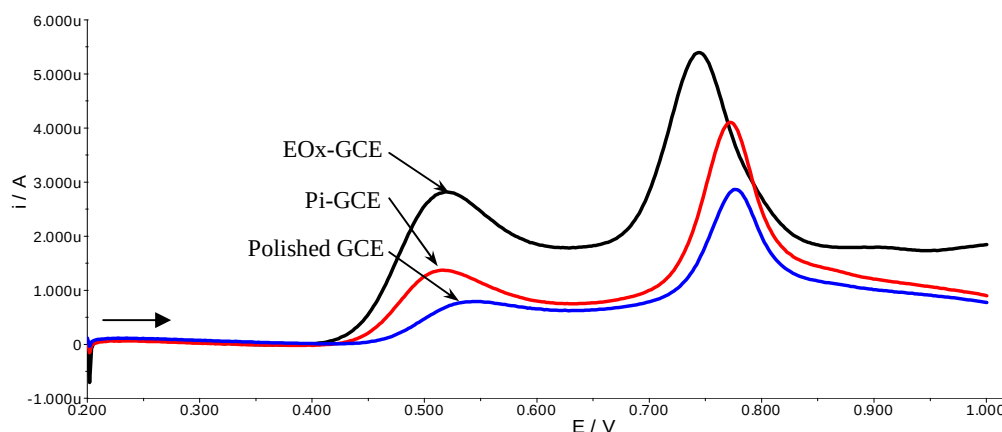


Figure C4: Comparison of peak profiles of 30 μ M 2,4-DMA in 0.2 M NaOAc pH 4.0 determined on bare GCE, Pi-GCE and EOx-GCE. Background current was subtracted from each of the scans and all scans were carried out at 100 mV.s⁻¹ vs. Ag/AgCl.

Table C2: Summarised I_{p1} responses of 2,4-DMA analysis at polished GCE, Pi-GCE EOx-GCE. N $\geq$ 3 per electrode set assessed.

	Polished GCE	Pi-GCE	EOx-GCE
Average	8.76×10^{-7}	1.36×10^{-6}	2.90×10^{-6}
Standard deviation	8.45×10^{-8}	1.14×10^{-7}	1.59×10^{-7}
Coefficient of Variation (%)	9.65	8.35	5.48

Appendix D

Limit of CIT Quantification

D1. Methodology

The electrochemical apparatus and methodologies reported through Chapter 2 with regard to operation and preparation of electrodes and scanning conditions and parameters were followed in this Abstract.

LOQ (limit of quantification) was assessed using LSV, differential pulse voltammetry (DPV) and square wave voltammetry (SWV). All scans were carried out in 0.2 M NaOAc buffer pH 4.0 at EOx-GCE at solution concentrations of 1.59×10^{-8} M, 7.98×10^{-8} M, 8.70×10^{-8} M and 1.595×10^{-7} M and a 120 second adsorption period was applied in each scan.

D2. Results

The figures below illustrate the electrochemical detection of CIT by LSV and DPV respectively. They were conducted with the intent of determining the LOQ of CIT, a limit which was set at 15.98 nM (pale grey scan). Although on occasion lower LOQ were found, responses were reproducibly recorded at 15.98 nM, thus it was established as the LOQ.

Appendix D
Voltammetric screening of select mycotoxins

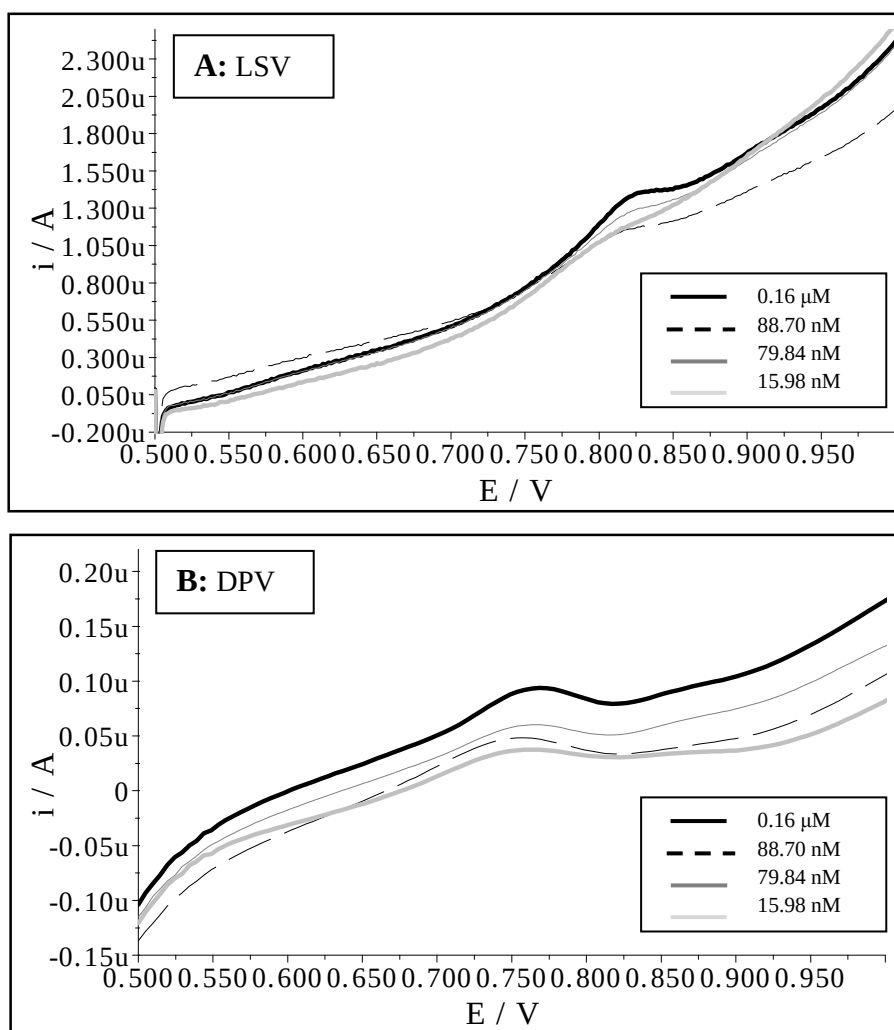


Figure D1: LSV [A] and DPV [B] scans obtained in 0.2 M NaOAc buffer pH 4.0 at EOx-GCE for the oxidation of 0.16 μM (black), 79.84 μM (dark grey), 88.70 μM (dashed line) and 15.98 μM (light grey), each having had their respective background currents subtracted.

Appendix E

Influence of the scan rate on CIT analyses in 25% methanol

E1 Methodology

Stock solution 5 mM CIT was applied. The electrochemical apparatus and methodologies reported through Chapter 2 and Chapter 6 with regard to operation and preparation of electrodes and scanning conditions and parameters were followed in this Abstract.

An equivalent study to that detailed in aqueous solution as a diffusive process of Chapter 6 was carried out herein, though, the CIT solution was comprised of 25% methanol. The responses were reported in Figure E1 by plotting both I_{p1} vs. $\sqrt{\nu}$ [A], as well as $\log I_{p1}$ vs. $\log \nu$ [B].

E2 Results and discussion

As a complex adsorptive process was identified for CIT oxidation, the influence of increased solubility on the mass transport characteristics of CIT at EOx-GCE was assessed by increasing the organic solvent in the buffer solution to 25 %, the results of which are reported through Figure E1.

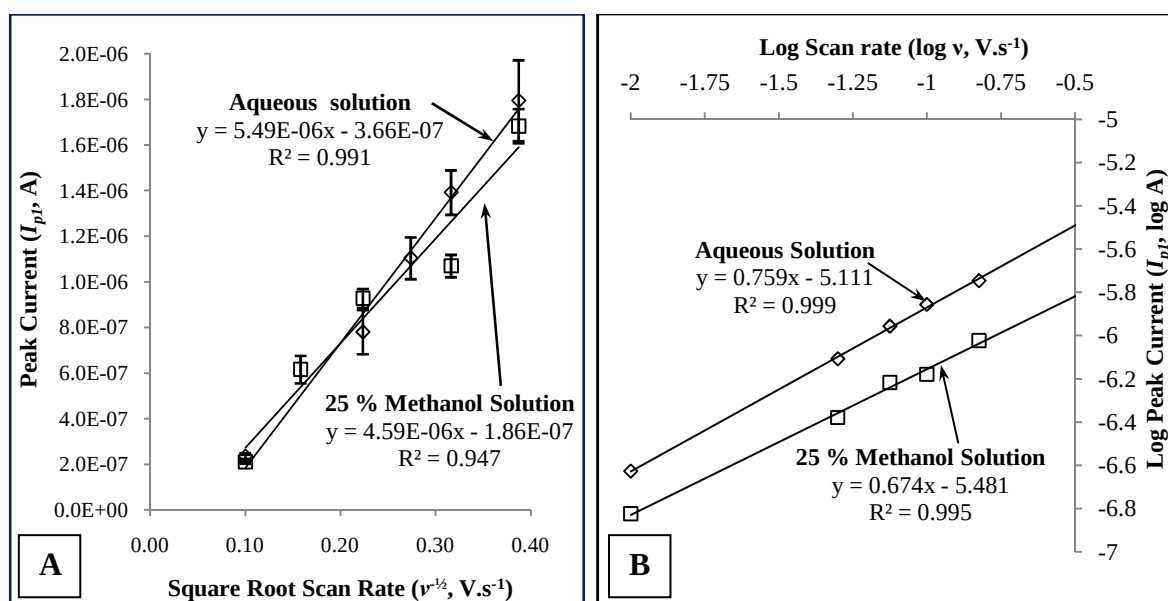


Figure E1: Responses of 20 μM CIT in 0.2 M NaOAc buffer with 25 % methanol at pH 4.0 at scan rates of 10, 50, 75, 100 and 150 mV.s^{-1} . I_{p1} was monitored for aqueous solution (grey) and 25% methanol (black) in relation to square root scan rate ($\nu^{1/2}$) [A] as well as $\log I_{p1}$ vs. $\log \nu$ [B]. $N \geq 3$ scans per scan rate, error bars show standard deviation from mean

As is evident in Figure E1, very little difference was found when relating responses found in aqueous media (Chapter 6, Section 6.4.7) and under increased solvation of CIT. This was true for both I_{p1} vs. $v^{-1/2}$ [A] and I_{p1} vs. $\log v$ [B], with minimal deviation across both the m values and R^2 values. According to these responses, although slightly lower I_p responses were recorded for CIT in 25 % methanol solutions, there was no apparent influence on the mechanism or extent of mass transport incurred by increasing the solubility of CIT.

This effect was assessed further through plotting measured responses (solid lines) in aqueous (black) and 25% methanol (grey) solutions against modelled responses for diffusive processes through applying the Randles-Sevcik equation (Eq. 6.4), as was described in Chapter 6, Section 6.4.7.

The results of the analyses were illustrated in Figure E2, in relation to both scan rate [A] and square root scan rate [B], with each comparing experimentally determined responses (solid lines) to modelled responses (markers) for aqueous media (black) and 25 % methanol (grey). Therein, there was again little relative variation between both modelled and experimentally determined responses when related to both scan rate and square root scan rate. These responses, along with the responses of Figure E1 suggest that although adsorptive processes are present, the extent of solubility of CIT in solution has little influence on the adsorptive properties of CIT at EOx-GCE and secondly has no apparent influence on the extent of diffusion control of the oxidation process.

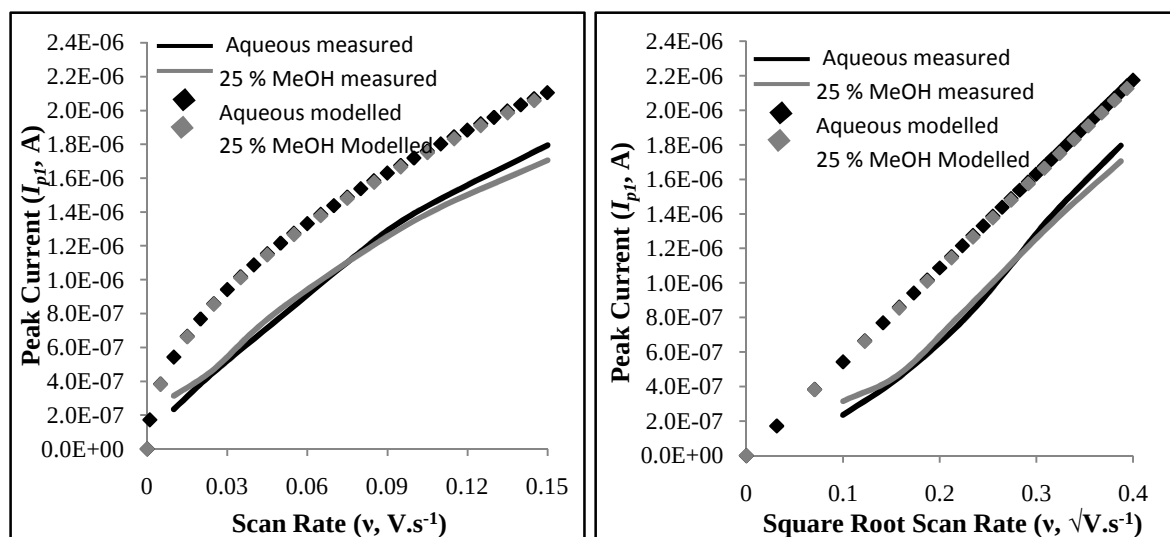


Figure E2: Plots of measured peak current (I_{p1}) against scan rate (v) [A] and square root of scan rate ($v^{1/2}$) [B] of 20 μ M CIT in both aqueous (black) (0.2 M NaOAc buffer at pH 4.0) and 25% methanol (grey) solutions (solid lines) as compared to diffusive controlled modelled data (marker points) for each set.