

**APPLICATION OF CATALYSTS AND NANOMATERIALS IN THE DESIGN OF AN
ELECTROCHEMICAL SENSOR FOR OCHRATOXIN A**

A thesis submitted in fulfilment of the requirements for the degree of

MASTERS IN SCIENCE

of

RHODES UNIVERSITY

by

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April 2010

ABSTRACT

Ochratoxin A is the most potent chlorinated derivative of the ochratoxin group, consisting of a 5'-chlorinated dihydroisocoumarin moiety linked by an amide bond to L-phenylalanine. Produced as a secondary fungal metabolite by several species of *Aspergillus* and *Penicillium*, ochratoxin A has been shown to readily contaminate a large variety of commodities including cereals, groundnuts, dried fruit, spices and coffee. This has led to widespread contamination of ochratoxin in wine, beer, milk and meat products. As ochratoxin A is a potent nephrotoxin exhibiting teratogenic and carcinogenic properties, the development of a rapid screening platform for the cost effective control of ochratoxin A content in foodstuffs is therefore required.

The evaluation of metallophthalocyanine and carbon nanotube electrode modification toward the development of a nanostructured biosensor capable of enhancing the electrochemical detection of ochratoxin A in complex media is presented.

Cyclic voltammetry at a glassy carbon electrode allowed for the optimization of detection parameters including pH and type of supporting electrolyte. Britton-Robinson buffer was found to be the most suitable supporting electrolyte in terms of sensitivity and reproducibility obtaining a LOD of 0.28 μM as determined by differential pulse voltammetry. Subsequent analysis determined the dependence of OTA oxidation on pH in acidic media which proceeds with the transfer of two electrons to form a quinone/hydroquinone couple shown to adsorb to the electrode surface. Passivation of the electrode through adsorption of oxidation products was shown to severely limit the detection of OTA upon successive detection cycles.

Comparison of various metallophthalocyanine modifiers showed an increase in sensitivity toward the detection of OTA at phthalocyanine complexes with metal based redox processes. However with the exception of NiPc and CoTCPc complexes, phthalocyanine modification was limited by the increase in deviation of current response and extent of fouling. NiPc modification showed an increase in sensitivity by two fold with fouling characteristics comparable to an unmodified electrode while low improvements in fouling was observed at CoTCPc modified electrodes with sensitivity in detection comparable to an unmodified electrode.

Modification of the electrode with multi- and single walled carbon nanotubes produced a significant increase in sensitivity toward the detection of ochratoxin A. The electrocatalytic activity of nanotube modifiers was attributed to the increase in surface area and to the addition of oxygenated functional groups upon acid treatment as confirmed by Raman spectroscopy. Acid functionalization of the carbon nanotubes for a period of two hours produced the greatest increase in sensitivity obtaining a respective LOD of 0.09 μM and 0.03 μM for analysis of ochratoxin A at multi- and single walled carbon nanotube modified electrodes. Centrifugal purification of carbon nanotubes was deemed necessary to improve the electrocatalytic activity of the nanotube modifiers through the removal of carbonaceous impurities as visualized by atomic force microscopy.

Furthermore, a crude lipase preparation, lipase A, was investigated as a potential biological recognition element for selective detection of ochratoxin A in complex media. Lipase A enabled the hydrolysis of ochratoxin A to the electroactive species ochratoxin α as confirmed by thin layer chromatography and voltammetric analysis. Additional isolation of a pure hydrolase from the lipase A preparation is required prior to utilization within a nanostructured biosensor platform capable of detecting ochratoxin A in complex media.

ACKNOWLEDGEMENTS

I would like to extend my deepest gratitude towards the following persons who enabled the composition and completion of this dissertation:

Dr. Janice Limson, my supervisor, for her invaluable guidance, positive attitude and faith in giving me the freedom to explore various channels during the research process.

The members of the Biosensor Research Group particularly Mr. Ronen Fogel and Mr. Michael Niland for their insightful comments, criticism and encouragement.

My family to whom this dissertation is dedicated to, for all their endless love and support. Thank you for believing in me in every step of the way.

Ms. Amy Taylor for her patience, love and strength over the past three years, for which I am truly grateful.

Many friends for their commitment and good humour throughout the time of my studies. A special thanks goes to Mr. Neville Mclean and Ms. Penny Stopforth for their support and proof reading. Thank you all.

To Rhodes University and the members of the Department of Biochemistry, Microbiology and Biotechnology for their assistance and support.

Prof. Tebello Nyokong for her assistance, support and generous gift of metallophthalocyanines.

Dr. Chamunorwa Togo and Dr. Edith Antunes for their assistance in AFM and Raman spectroscopy.

Mr. Kazuhiko Horikawa and Amano Enzyme Europe Ltd. for their generous gift of Lipase A.

DST/MINTEK Nanotechnology Innovation Centre which without whose funding this dissertation would not have been made possible.

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LIST OF ABBREVIATIONS

In alphabetical order:

A	Amperage
AFM	Atomic force microscopy
ATP	Adenosine-5-triphosphate
au	Arbitrary units
BHQ	Benzohydroquinone
BQ	Benzoquinone
BR	Britton-Robinson
CNT	Carbon nanotube
CoOCPc	Octacarboxylated cobalt phthalocyanine
CoPc	Cobalt phthalocyanine
CoTCPc	Tetracarboxylated cobalt phthalocyanine
CoTSPc	Cobalt tetrasulphonated phthalocyanine
CuPc	Copper phthalocyanine
CuTSPc	Copper tetrasulphonated phthalocyanine
CV	Cyclic voltammetry
Da	Dalton
DNA	Deoxyribonucleic acid
DPV	Differential pulse voltammetry
EC	European Commission
ELISA	Enzyme-linked immunosorbent assay
EU	European Union
F	Farad
FAO	Food and Agricultural Organization of the United Nations
FDA	Food and Drug Administration
FePc	Iron phthalocyanine

g	Gravity
GCE	Glassy carbon electrode
HOMO	Highest occupied molecular orbital state
HPLC	High performance liquid chromatography
IARC	International Agency for Research on Cancer
JECFA	Joint FAO/World Health Organization Expert Committee on Food Additives
LOD	Limit of detection
LOQ	Limit of quantification
LSV	Linear sweep voltammetry
LUMO	Lowest occupied molecular orbital state
M	Molar
MPc	Metallophthalocyanine
MVOC	Myotic volatile organic compounds
MWCNT	Multi walled carbon nanotube
NiPc	Nickel substituted phthalocyanine
NiTPhPyPc	Nickel tetra-4-(pyrrol-1-yl)phenoxyphthalocyanine
OTA	Ochratoxin A
OTB	Ochratoxin B
OTC	Ochratoxin C
OTCT	Ochratoxin catechol product
OTHQ	Ochratoxin hydroquinone product
OTQ	Ochratoxin quinone product
Pc	Phthalocyanine
pKa	Acid dissociation constant
Redox	Reduction/oxidation
RSD	Relative standard deviation
spp	Species

SWCNT	Single walled carbon nanotube
SWV	Square wave voltammetry
TLC	Thin layer chromatography
tRNA	Transfer ribonucleic acid
USD	United states dollar
UV	Ultraviolet radiation
V	Volts
VIS	Visible light
W	Watt
WHO	World Health Organization
ZnPc	Zinc phthalocyanine

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CHAPTER 1

OVERALL INTRODUCTION

1.1 BACKGROUND

Fungi constitute an independent kingdom of the eukarya domain. Organisms of the fungal lineage are recognized as three major groups namely molds, yeasts and mushrooms (Alexopoulos et al., 1996). More than 70 000 species of fungi have been described, all of which share a common mode of nutrition (Hawksworth, 1991). All fungi are heterotrophic in that they are able to synthesis organic material sufficient for growth from preformed organic matter.

Fungi in this manner are primarily saprophytic and have fundamental roles in the cycling and exchange of nutrients within terrestrial and aquatic ecosystems. Saprophytic fungi are typically terrestrial and simply grow into new food sources as the local environment becomes nutrient depleted. To obtain nutrients, decay causing fungi secrete extracellular enzymes into their surroundings to degrade organic material from dead plant or animal tissue (Griffon, 1993). Complete digestion occurs in a stepwise process involving multiple enzymes producing simple soluble units which are able to enter fungal metabolic pathways (Griffon, 1993). Once adsorbed, these compounds are mineralized to carbon dioxide, released as metabolites or liberated as the fungus itself dies and is intern decayed by other microorganisms. In addition to available nutrients, fungi must be exposed to favourable temperature, moisture, aeration, pH and light conditions for growth to take place (Robinson, 1967).

Alternatively fungi are found in dual association with other organisms forming a symbiotic relationship. These interactions can be mutualistic consisting of joint exploitation whereby each partner is a net beneficiary or antagonistic in nature at the expense of the host (Kendrick, 1992). In both cases the fungus benefits nutritionally from the relationship. Fungi which are capable of exploiting living organisms as a nutrient source without contributing to the survival of the host are termed as parasites. This type of symbiotic association draws attention, as the majority of parasitic fungi are pathogenic and as such result in disease of the host (Robinson, 1967). Of these pathogens, obligate fungal parasites grow exclusively in living tissue and establish a delicate symbiotic association to preserve the life span of the host. In this way the host may only show mild symptoms and live indefinitely, although it is parasitized (Frank and Schmid-Hempel, 2008).

In contrast to obligate parasites, parasitic fungi which exist independently from a host are termed facultative. To establish an infection, facultative parasites need only to adapt to conditions within the host in particular to temperature, pH, oxygen and nutrient supply (Herre, 1999). Facultative fungi are not specialised as parasites and often become so virulent as parasites that they result in the death of their host leading to starvation of the fungus (Frank and Schmid-Hempel, 2008). As soil acts as an unlimited reservoir of both obligate and facultative fungal parasites, these pathogens are ubiquitous in the environment.

1.1.1 Epidemiology of Fungi

Pathogenic fungi can cause disease within their host by three major mechanisms (Madigan, et. al, 2000). First, exposure to specific fungal antigens may elicit an immunological response. Upon re-exposure to the same fungi whether through infection or in the local environment, the host may then suffer from an allergic reaction. As an example, a common saprophyte found as leaf mold, *Aspergillus spp.*, produces potent antigens to persons suffering from asthma and other hypersensitivity reactions (Horner, et. al, 1995).

Second, the growth of fungi within the plant or animal host produces disease collectively called mycosis. Mycosis is initiated through fungal infection of surface abrasions, inhalation of fungal spores or by unusual growth of a commensal species (Madigan, et. al, 2000). These fungal infections range in severity from self limiting, superficial lesions to life threatening systemic infections. Superficial mycoses are common and are usually confined to the outer layers of skin, hair and nails. Superficial conditions such as ringworm or athlete's foot are common and are usually spread by individual contact with infected hosts or by contact of contaminated surfaces (Boni and Hazen, 1989). Systemic mycoses involve fungal growth within the host often in response to immunosuppressant factors (Richardson, 2005).

The third fungal disease producing mechanism caused by exposure to toxic fungal metabolites through dietary, respiratory, dermal and other means is collectively called mycotoxicosis (Bennet and Kilch, 2003). Fungi produce biologically active compounds as secondary metabolites to affect a competitive advantage over other micro organisms within an ecological network (Gardiner et al. 2005). Fungal secondary metabolites constitute a broad range of compounds which differ in chemical composition, mode of action and biological role. Thus, the toxic response exerted by these metabolites towards bacterial, plant, insect or mammalian hosts is varied. In human hosts, the

majority of mycotoxicoses result from consumption of contaminated foodstuffs, inhalation of spore-borne toxins and through skin contact with mold infested surfaces (Oancea and Stoia, 2008).

Mycotoxicosis is characterized as non-contagious, non-transferable, non-infectious, and non-traceable to microorganisms other than fungi (Hussein and Brasel, 2001). The symptoms induced are dependent on the type of toxin, 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 (Bennet and Kilch, 2003). The metabolites involved in mycotoxicosis include antibiotics, toxins produced from mushrooms and mycotic volatile organic compounds (MVOC). The remaining 400 known compounds are termed mycotoxins (Bennet and Kilch, 2003).

1.1.2 Mycotoxins

The antibiotic era identified hundreds of fungal metabolites that provided protection in human and animal models against bacterial pathogens without reasonable toxicity (Smith and Henderson, 1991). Screening for antibiotics also produced numerous compounds which were toxic to animals and were therefore removed from commercial consideration (Smith and Henderson, 1991). In subsequent years, research associated with toxic secondary fungal metabolites assumed new significance. This was largely due to an unusual outbreak of turkey disease in the United Kingdom in 1960 during which approximately 100 000 turkey fowls died (Shephard, et. al, 2008). The outbreak was linked to feed containing peanut meal contaminated with secondary fungal metabolites. This realization, lead to a period of extensive research in mycotoxicology in which approximately 400 naturally occurring toxigenic fungal metabolites were recognised in food and feed (Bennet and Kilch, 2003). These toxins were termed as mycotoxins.

Mycotoxins are low molecular weight natural products produced as secondary metabolites of filamentous fungi (Jarvis and Miller, 2005). These metabolites are diverse in chemical structure, biosynthetic origin and toxicological effects, and are only grouped together as they cause disease and death in human beings and other vertebrates (Bennet and Kilch, 2003). A further distinction between mycotoxins and other fungal toxins is based on the affected target and concentration of the metabolite. Hence, fungal toxins which are primarily toxic to invertebrates, plants and other microorganisms are not considered as mycotoxins (Bennet, 1989). Mycotoxins are also considered

distinct from other low molecular weight fungal metabolites, such as alcohols, that are toxic to vertebrates in high concentrations (Bennet, 1989).

The mycotoxins of greatest concern to public health resulting in agricultural and economic loss include the aflatoxins, ochratoxins, citrinin, patulin, zearalenone, fumonisins, trichothecenes and tremorgenic toxins (Richard, 2007). These metabolites are mainly produced by fungal pathogens of major food crops and as a result have the greatest occurrence in food and feed products (Shephard, et. al, 2008).

Table 1.1.2 showing the major food borne mycotoxins, their main producing fungal species and the commodities most frequently contaminated. Adapted from Shephard, et. al, 2008.

Mycotoxin	Fungal species	Food commodity
Aflatoxins	<i>Aspergillus flavus</i> , <i>Aspergillus parasiticus</i>	Maize, wheat, rice, sorghum, nuts, figs, milk, milk products
Ochratoxin A	<i>Aspergillus ochraceus</i> , <i>Penicillium verrucosum</i>	Cereals, dried fruit, spices, wine, coffee
Citrinin	<i>Aspergillus citrinum</i> , <i>Aspergillus niveus</i>	Grain, cheese, sake
Patulin	<i>Penicillium expansum</i>	Apples, apple juice
Zearalenone	<i>Fusarium graminearum</i> , <i>Fusarium culmorum</i>	Cereals, cereal products
Fumonisins	<i>Fusarium verticillioides</i> , <i>Fusarium proliferatum</i>	Maize, maize products, sorghum, asparagus
Deoxynivalenol	<i>Fusarium graminearum</i> , <i>Fusarium culmorum</i>	Cereals, cereal products
T-2 Toxin	<i>Fusarium sporotrichioides</i> , <i>Fusarium poae</i>	Cereals, cereal products

1.1.3 Biological significance of mycotoxigenic fungi

Filamentous fungal species responsible for mycotoxin production are extremely widespread and grow in a variety of substrates under a wide range of environmental conditions. The most active producers of mycotoxins in food crops belong to three genera namely *Aspergillus*, *Fusarium* and *Penicillium* (Sweeney and Dobson, 1998). *Fusarium* species are destructive crop pathogens and typically produce mycotoxins before or immediately after harvesting. *Aspergillus* and *Penicillium* species are more commonly found as contaminants during drying and subsequent storage. Although fungal growth is a prerequisite to mycotoxin contamination, not every strain of a species has the

capacity to produce mycotoxins (Hof and Kupfahl, 2009). In addition, most mycotoxin producing strains are able to synthesise more than one mycotoxin at a time (Hof and Kupfahl, 2009).

The high degree of conservation in mycotoxin production of fungal strains, indicates that mycotoxins may play a key role in the capacity of a fungus to compete for resources or territory within a diverse ecological community (Gardiner et al. 2005). In this regard, mycotoxins are thought to function either as pathogenicity factors in that they damage the host by disturbing physiological growth or as virulence factors in which they facilitate the growth and survival of the fungal strain within a host (Nelson et. al, 1993).

The ecological significance of mycotoxin production is shown in the contrast between fusarium ear rot and gibberella ear rot in maize. In fusarium ear rot, *Fusarium verticilloides* that lack fumonisin production have the same capability to infect maize kernels as fumonisin proficient strains (Munkvold, 2003). As a pathogenicity factor, fumonisin production by *Fusarium verticilloides* provides an advantage over deficient strains by inhibiting fungal growth of competitive species. In contrast to fumonisins, *Fusarium graminearum* strains that lacked trichothecene production due to disruption of the biosynthesis gene are less able to cause gibberella ear rot than their trichothecene proficient counterparts (Munkvold, 2003). Thus, trichothecene production appears to aid plant infection by acting as a virulence factor (Cutler, 1988).

In humans, *Aspergillus fumigatus* causes severe and often fatal disease in immunocompromised individuals. The pathogenicity of *Aspergillus fumigatus* depends not only on the immune status of the host but also on the virulence of the fungal strain (Balloy and Chignard, 2009). All environmental strains of *Aspergillus fumigatus* produce gliotoxin which is reported to inhibit the activity of ciliar epithelial cells of the lung, thereby aiding fungal infection of the airways (Pasqualotto, 2008). As a result, gliotoxin is recognized as a potential virulence factor.

Although mycotoxins may provide a competitive advantage, the extent of mycotoxin production by a fungal species is heavily dependent on nutrient availability and environmental conditions including temperature, humidity, pH and other stress factors (Sweeney and Dobson, 1998).

1.1.4 Prevalence of mycotoxin contamination

The Food and Agricultural Organization of the United Nations (FAO) has estimated that up to 25% of the world's food crops are significantly contaminated with mycotoxins (WHO, 1999). Mycotoxin production by fungal action can occur in the field by plant pathogens such as *Fusarium graminearum*, through fungal infection of stressed plants by *Fusarium verticillioides*, *Aspergillus flavus* and *Aspergillus carbonarius*, through post harvest colonization by *Aspergillus flavus* and *Penicillium verrucosum* or by fungi found in soil and later proliferate during storage of commodities (Logrieco, et. al, 2003). Once a crop becomes infected under field conditions, mycotoxin contamination can accumulate whenever shipping, handling, food processing and subsequent storage practices are conducive to fungal growth (Turner, et. al, 2000).

Crops in tropical and subtropical climates are more susceptible to contamination than those in temperate regions, since the high humidity and ambient temperature in these areas provide optimal conditions for mycotoxin formation (Odoemelam and Osu, 2009). The commodities most frequently contaminated with mycotoxins include wheat, maize, barley, rice, grain, groundnuts, dried fruit, coffee beans and various spices (Kumar, et. al, 2008). Furthermore, crop damage by pests promote fungal growth and subsequent incidence of mycotoxin contamination (St. Leger, et. al, 2000).

Mycotoxin risk assessment of commodities prior to trade has highlighted the divide between developed and developing countries with respect to food safety (Shephard, 2008). Mycotoxin exposure is generally lower in developed countries due to varied diets, the high quality of food standards of commercial suppliers and widely enforced legislation for maximum mycotoxin levels in food products (Shephard, 2008). By contrast, the general population of developing countries tend to consume staple diets, and when not grown on a subsistence basis, food items are obtained from local markets where few regulations governing food quality exist. Food grown in these areas is frequently consumed irrespective of quality due to food scarcity problems. Even if steps were taken to ensure the safety of food products, socio-economic factors including scarcity of food, low literacy levels and poor infrastructure predispose consumers to purchase cheaper commodities with greater incidence of mycotoxin contamination (Sheriff, et. al, 2009).

The most prevalent fungi of cereal crops in Africa and worldwide are the *Fusarium* species, known to produce the mycotoxins fumonisins, trichothecenes, and zearalenone. Doko, et. al, 1996 investigated the natural co-occurrence of Fumonisin and zearalenone from 40 randomly selected

cereal based commodities from parts of Botswana, Kenya, Malawi, Mozambique, South Africa, Tanzania, Uganda, Zambia, and Zimbabwe. High levels of fumonisins were detected in 37 of the 40 samples suggesting a widespread occurrence in eastern and southern Africa. Zearalenone was detected in 5 of the 40 samples with one sample originating from Malawi over the EU regulatory limit.

Storage of improperly dried maize has also contributed to widespread aflatoxin exposure in West Africa (Hell, et. al, 2000). In blood tests carried out in Gambia, Guinea Conakry, Nigeria and Senegal, over 98% of persons tested positive to aflatoxin markers (Bankole and Adebajo, 2003).

Janardhana, et. al, 1999 screened 197 maize grain samples collected from several farms, production plots and regulating markets situated in the Karantaka region of India. Out of the samples screened by enzyme linked immunosorbent assay (ELISA) and thin layer chromatography (TLC), 69 samples were found to be positive for high levels of aflatoxin, ochratoxin, zearalenone, deoxynivalenol and citrinin. The high occurrence of several mycotoxins in the collected samples was accredited to inadequate drying and high ambient temperatures present during grain storage.

Globalization of trade has narrowed the divide between mycotoxin prevalence in developing and developed countries. This is due to the vast amount of commodities originating from developing countries being processed, repackaged and sold as products in developed countries. Certain mycotoxins remain unaffected during the food processing and fermentation process (Bullerman and Bianchinia, 2007). Shephard, et. al, 2003 investigated the level of ochratoxin A in a variety of South African and Italian white and red wines. High performance liquid chromatography (HPLC) analysis confirmed the presence of ochratoxin A in all 24 South African wines tested, with 2 of the 8 Italian wines obtaining ochratoxin A levels above the EU regulatory limit.

For developed and developing countries substandard commodities are often used for the production of animal feed (Fokunang, et. al, 2006). In the feed manufacturing process, various raw materials such as cereal by-products, groundnuts, coconut and oilseed cake are often mixed together resulting in an increased risk of multiple mycotoxin exposure (Lanier, er. al, 2009). In the industrial milling of corn, grain kernels are processed into course grit, fine grit, flour, bran, germ and animal feed flour. Brera, et. al, 2006 evaluated the aflatoxin and zearalenone levels of raw kernels and each of the derived fractions in a European corn milling plant. Although the mycotoxin levels for the raw kernels were below the maximum limit as proposed by the EU commission, set as 2 µg/kg for aflatoxin and 1

mg/kg for zearalenone, the milling process produced a 9 fold increase in aflatoxin and a 4 fold increase in zearalenone levels for the bran, germ and animal feed flour fractions. The increase in mycotoxin levels represents the increased risk of by-product inclusion in animal feed formulations.

To emphasize the extent of mycotoxin contamination within the feed industry, a 2-year survey program was conducted to evaluate various animal feed producers situated throughout Europe, Asia, Oceania the Mediterranean region (Binder, et. al, 2007). HPLC and TLC was used to evaluate a total of 1507 samples sourced directly from European and Mediterranean markets, and a total of 1291 samples originating from Asian-Pacific markets. 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 tested positive for mycotoxin contamination. European and Mediterranean samples had zearalenone and trichothecenes as major contaminants, materials from Asia and the Pacific tended to be contaminated with zearalenone, fumonisins, aflatoxins and trichothecenes.

The prevalence of mycotoxin contamination within animal feed has direct consequences on animal health often leading to economic losses associated with reduced feed intake, feed refusal, poor feed conversion, diminished weight gain, susceptibility to infections and a reduction in fertility (Binder, et. al, 2007). Downstream contamination can also occur through the consumption of mycotoxin contaminated feed by animals used to produce products such as milk, meat and eggs (Shreeve, et. al, 2004). Susceptible animal products are then contaminated by either residual mycotoxins or their toxic biotransformation products. The negative effect on human health by the indirect consumption of mycotoxins in animal products is often intensified in developing countries where animals are more likely to consume lower grade contaminated grains (Wagacha and Muthomi, 2008).

1.1.5 Toxicity of mycotoxins

Mycotoxins of greatest concern to public health include the aflatoxins, ochratoxins, fumonisins, trichothecenes and zearalenone. All of these groups are chemically distinct secondary fungal metabolites with unique biochemical modes of action. The primary source of mycotoxin exposure to the general public is through the ingestion of mycotoxin contaminated food products (Mayer, et. al, 2008). The resultant health impact of mycotoxin exposure is categorized as either acute or chronic. Acute intoxication of humans by mycotoxin exposure is often lethal but rarely observed except in isolated events during war, natural disasters or in communities with poor agricultural practices

(Mayer, et. al, 2008). However, 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. The range of health effects induced by chronic mycotoxin exposure include carcinogenic, genotoxic, teratogenic, immunosuppressive and often organ specific toxic effects (Smith, et. al, 2006).

Diseases caused by aflatoxin consumption are loosely called aflatoxicosis. Acute aflatoxicosis is lethal, while chronic dietary exposure results in immune suppression, cancer and other slow pathological conditions (Qazi and Fayyaz, 2006). Long term dietary exposure to aflatoxin has been associated with human liver carcinomas, which may be compounded by the hepatitis B virus. Approximately 250 000 deaths are caused by liver carcinomas in China and Sub-Saharan Africa annually and are attributed to risk factors such as high daily intake of aflatoxin with the co-occurrence of a high incidence of hepatitis B (Goopman, et. al, 1992). Tumour formation has been linked to the enzymatic conversion of aflatoxin to a reactive epoxide capable of inducing DNA adducts within animal studies. Due to the genotoxic and mutagenic nature of aflatoxin, the International Agency for Research on Cancer has classified aflatoxin as a group I carcinogen (IARC, 1993).

The fumonisins exhibit hepatotoxic and carcinogenic effects in animal models and may play an important role in human carcinogens (Syndenham, et. al, 1991). In the Transkei region of South Africa, the consumption of fumonisin-contaminated maize has been linked to the high occurrence of oesophageal cancer (Shephard, et. al, 2005). Though research is still ongoing to support this association, a similar link between fumonisin consumption and oesophageal cancer has been reported in China and North-eastern Italy, (Zhang, et. al, 1997). The toxic action of fumonisin appears to result from competition with sphingosine, a phospholipid in cell membranes, essential for the proliferation of cells (Tran, et. al, 2003). The International Agency for Research on Cancer has evaluated the cancer risk of fumonisins and classified them as group 2B, a possible human carcinogen (IARC, 2002).

Low dose dietary exposure of agricultural animals to trichothecenes results in feed refusal and decreased weight gain; at higher exposures trichothecenes cause alimentary haemorrhaging, vomiting and diarrhea. In addition to the cytotoxic activity, trichothecenes exert immunosuppressive effects which decrease resistance to microbial infections (Larsen, et. al, 2004). The symptoms produced by trichothecene exposure are initiated by poorly understood metabolic processes related to the inhibition of protein, RNA and DNA synthesis (Rocha, et. al, 2005). Although monogastric

mammals display the greatest susceptibility to these toxins (Barbara, 1996), trichothecenes are not classified as human carcinogens by the International Agency for Research on Cancer.

Zearalenone is a non-steroidal oestrogenic mycotoxin which resembles estradiol, the primary hormone produced by the human ovary (Zinedine, et. al, 2006). The similarity of zearalenone to oestrogen allows for binding to oestrogen receptors in mammalian cells which can lead to infertility, abortion and other reproductive problems (Binder, 2007). Although the actual toxicity is low, the biological potency of zearalenone is high.

Ochratoxin is a potent nephrotoxin which also exhibits immunosuppressive, teratogenic and carcinogenic properties (Varga, et. al, 1996). Several nephropathies affecting animals as well as humans have been attributed to chronic consumption of ochratoxin. In European countries, ochratoxin contamination of pig feed is associated with the high rate of porcine nephropathy (Petzinger and Weidenbach, 2002). Ochratoxin is also implicated as a causative agent in two fatal kidney diseases of humans referred to as Balkan Endemic Nephropathy and Chronic Interstitial Nephropathy (Maaroufi, et. al, 1995). The primary mechanisms attributed to the renal toxicity of ochratoxin include the inhibition of mitochondrial ATP production, inhibition of tRNA synthetase and enhanced lipid peroxidation (Hohler, 1998). Based on the results of carcinogenicity studies and on evidence of effects in humans, the International Agency for Research on Cancer has classified OTA as a group 2B carcinogen (IARC, 1993).

1.1.6 Mycotoxin intervention strategies

Aside from the direct health risk, the economic consequence of mycotoxin contamination is profound (Moss. et. al, 1991). Market losses relating to mycotoxin exposure are attributed to the cost of human health care, veterinary health care, loss of livestock production, loss of forage crops, rejection of feed and regulatory cost. Furthermore the agricultural industry may be forced to increase prices due to rejection of crops, necessary redirection to animal feed lots or whole scale disposal in cases of severe mycotoxin contamination (Binder, er. al, 2007). Consequently, the livestock industry may need to pay higher prices for non-contaminated grains used for animal feed. Developed countries may also infer restrictions in international trade to countries predisposed to mycotoxin contamination. Exports of agricultural commodities from developing countries have already dropped considerably in recent years resulting in major economic losses to producing countries. According to a 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 (Otsuki, et. al 2001). This will result in a projected cost of approximately 670 million USD in trade per year to African countries (Otsuki, et. al 2001).

To offset the agro-economic impact of mycotoxins, countries predisposed to contamination have implemented strategies for the management of mycotoxins from three main fronts: good agricultural practices, legislation and by surveillance and awareness creation (Wagacha and Muthomi, 2008).

Although good agricultural practices may reduce the level of contamination, mycotoxin production by fungal pathogens is often unavoidable. In order to limit the introduction of mycotoxin contaminated commodities into the general food supply, regulatory agencies are used to survey the occurrence and establish regulatory limits of mycotoxin contamination in food and animal feed. Guidelines for establishing regulatory limits are determined by national and international institutions including 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. The regulatory standards set by these institutions 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 and Egmond, 1991).

In 2003, at least 100 countries worldwide had adopted the advisory mycotoxin regulations set by the Joint FAO/World Health Organization Expert Committee on Food Additives (JECFA) (Egmond, et. al, 2007). For the majority of countries, regulatory limits are implemented for aflatoxin, ochratoxin, fumonisins, ergot alkaloids, trichothecenes, zearalenone and patulin. In Africa, 15 countries covering 59 percent of the continents inhabitants have set regulatory limits for mycotoxins (FAO, 2003). The establishment of mycotoxin regulations for the remaining African countries should be viewed in context of local food supply, food safety and available infrastructure. Adoption of regulatory limits for agricultural produce grown on a subsistence basis is normally impractical. In addition, developing countries often adopt higher tolerance standards to accommodate for susceptibility to mycotoxin contamination and for economic development through trade (FAO, 2003).

The discrepancy between international regulatory limits is illustrated in Figure 1.1.6 by the range of regulatory levels enforced for total aflatoxins in food. The most frequently occurring limit of 4 µg/kg

is enforced by 29 countries most of which belong to the EU while another 17 countries spread over most of Africa and Latin America have enforced a higher regulatory limit of 20 µg/kg of foodstuffs (FAO, 2003).

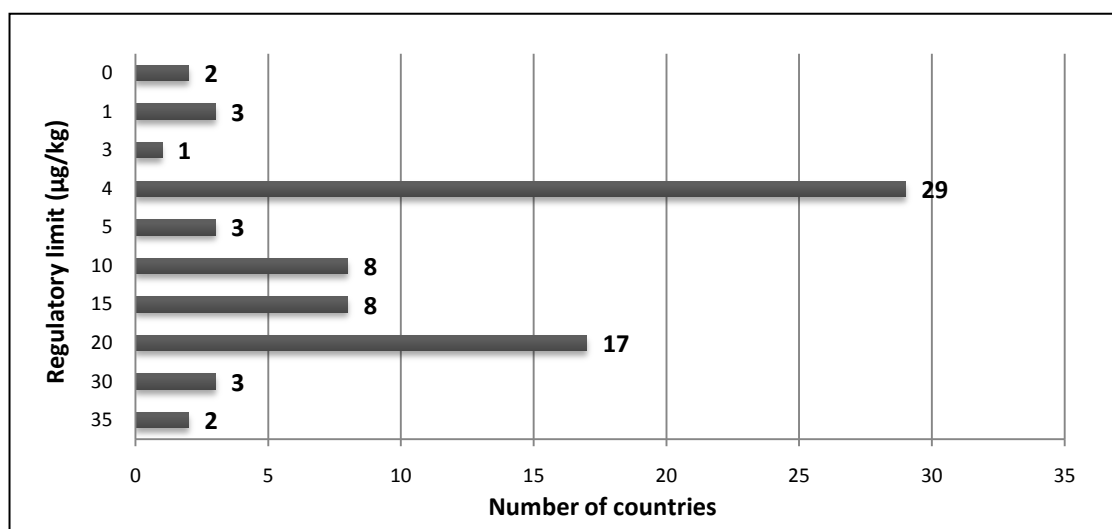


Figure 1.1.6 Worldwide limits for total aflatoxins in human foods. Figure adapted from FAO, 2003.

The inability of exporters in developing countries to comply with regulatory standards is usually not due to high levels of contaminated goods but mainly because of limited infrastructure available for monitoring, testing and certification required to demonstrate compliance with regulations of the importing country (Strosnider, et. al, 2006). Consequently, advisory organisations such as the FAO and WHO have worked closely with developing countries to develop programs which provide technical assistance to alleviate mycotoxin contamination of foods and feeds.

1.1.7 Detection of mycotoxins

The requirement to comply with regulatory limits has prompted the development of analytical methods used for the identification and quantification of mycotoxins in various samples including food grains, animal feed, animal products and other processed goods. 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. However, the difficulty in assessing mycotoxin contamination is due to a multiplicity of factors affecting the field production and level of mycotoxins in agricultural commodities.

Mere isolation and confirmation of mycotoxigenic fungal species does not necessarily indicate the presence of mycotoxins (Hussein and Brasel, 2001). In effect, the degree of mycotoxin production is

dependent on various physical, chemical and biological factors. Physical factors include environmental conditions conducive to fungal colonization and mycotoxin production such as temperature, relative humidity, and insect infestation (Hussein and Brasel, 2001). Chemical factors include the use of fungicides and fertilizers, while biological factors are based on the toxigenic profile of specific fungal species (Hussein and Brasel, 2001). In addition, mycotoxins are produced in trace amounts which are distributed non-homogeneously throughout the field crop (Coker, et. al, 1995). These intrinsic factors emphasize the need for rapid, accurate and sensitive analytical methods capable of identifying and quantifying the level of mycotoxin contamination in various commodities.

Chromatographic based methods are used for the official control and implementation of mycotoxin regulations (Gilbert, 2002). Current quantitative methods used for the determination of regulated mycotoxins incorporate immunoaffinity clean-up with high-performance liquid chromatography separation in combination with a variety of detectors based on fluorescence, ultraviolet light, flame ionisation, electron capture or mass spectrometry (Gilbert, 2002). The quantification of mycotoxins by conventional laboratory based methods provides low limits of detection with specific and unambiguous identification of mycotoxins.

Apart from laboratory based techniques, the development of rapid screening methods for mycotoxins is required for control purposes as well as in situations where in field decisions are necessary such as at granaries, silos and processing plants (Zeng, et. al, 2006). For this purpose, thin-layer chromatography has been used as an alternative to HPLC based methods and has an important role, especially in developing countries, as an inexpensive method for surveillance purposes (Pittet and Rover, 2002). Alternatively, 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 are in development (Micheli, et. al, 2005).

Electrochemical biosensors in particular show promise due to the highly sensitive, accurate and rapid means of analytical detection often associated with the additional benefits of ease of miniaturization, low cost and added portability of analysis (Cigic and Prose, 2009). Electrochemical methods of analyses for mycotoxins have largely focused on detection of aflatoxin while only in recent years has attention been focused on other widespread mycotoxins such as ochratoxin (discussed further in Chapter 2). Substantial scope thus exists for the utilisation of electrochemical sensing technology to enhance the detection of mycotoxins in routine analysis.

1.2 ELECTROCHEMICAL PRINCIPLES AND TECHNIQUES

1.2.1 Electrochemical cell

Electroanalysis of a system requires an electrochemical cell coupled to a potentiostat. The potentiostat is used to apply an appropriate potential required to generate a measurable current produced from electroactive species contained within the electrochemical cell (Kissinger and Heinemann, 1996). Electrochemical cells may comprise of two or three electrodes separated by an electrolyte (Wang, 1994).

For a two electrode cell, the overall chemical reaction taking place within the cell is made up of two independent half reactions. Each half reaction relates to a chemical response to the interfacial potential difference at the corresponding electrode. The half reaction being investigated takes place at the working electrode. To limit electroanalysis to the working electrode, a standardized half reaction is used for the other electrode, called the reference electrode (Kissinger and Heinemann, 1996). The reference electrode maintains a constant potential against which the potential of the working electrode is compared. Common reference electrodes include the standard hydrogen electrode (Pt/H_2), saturated calomel electrode (Hg/HgCl_2) and the silver-silver chloride electrode (Ag/AgCl). In a three electrode cell, an added auxiliary electrode is used to complete the electrochemical circuit by acting as a source or sink of electrons (Kissinger and Heinemann, 1996). Typically an inert metal such as platinum is used for the auxiliary electrode so as to limit the production of species which may interfere with reactions at the working electrode.

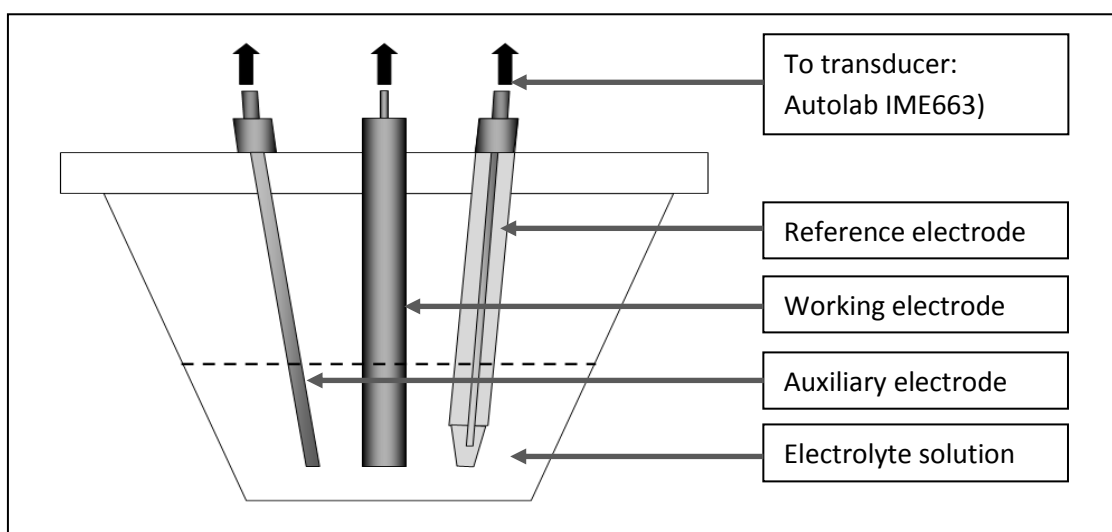


Figure 1.2.1 A three electrode electrochemical cell consisting of a working electrode, auxiliary electrode and a reference electrode. Figure adapted from Kissinger and Heinemann, 1996.

Working electrodes are selected according to the mechanical stability, potential range, electrocatalytic activity and versatility required for analytical applications (Rubinstein, 1995). Typically, the working electrode surface is comprised of a mercury drop, carbonaceous material or inert metals such as gold or platinum. Carbon electrodes are the most common working electrodes in current use due to their low-cost, wide potential range, rich surface chemistry and ease of surface modification. In particular, glassy carbon is extensively used as it is compatible with all common solvents, impermeable to gases and liquids and is easily renewed through surface polishing.

1.2.2 *Mass transport*

Electroanalytical measurements are only taken at a small portion of the phase boundary between the electrode and the bulk solution, the electrode-solution interphase. Since electron transfer occurs at the electrode-solution interface, the analyte of interest must be transported to the electrode for this process to occur. The movement of material from the bulk solution to the electrode surface is called mass transport. Mass transport can occur by three distinct processes namely hydrodynamics, migration and diffusion (Kissinger and Heinemann 1996).

Hydrodynamic mass transport is caused by the movement of solution to the electrode surface by physical stirring of the solution, rotation of the electrode or by forced convection (Brett and Brett, 1993). Movement of the solution transports bulk reactant to the electrode surface while moving electro-generated product away. Migration involves the movement of charged particles to and from the electrode surface due to the influence of an electric field. A negative charge at the vicinity of the electrode surface would attract cations and repel anions. The opposite would be true for a positively charged electrode. Typically the effect of migration is minimised by the addition of a supporting electrolyte which minimises the electric field at the electrode surface.

In a system controlled by diffusion, the direction of motion will take place from a region of higher chemical potential to one of lower chemical potential until a homogenous state is obtained. The movement of solute molecules by diffusion results from the maximization of entropy (Brett and Brett, 1993). In most electroanalytical techniques, conditions are chosen so that transport of the electroactive species is affected by a single mechanism of mass transport, typically that of diffusion.

1.2.3 *Electroanalytical techniques: Cyclic voltammetry*

Electrochemical cells are classified as either galvanic or electrolytic cells. A galvanic cell is one in which reactions occur spontaneously at the electrode surface when the cell circuit is closed. An electrolytic cell is one in which an external voltage is applied to drive electrochemical reactions of interest. Electroanalytical techniques that use galvanic cells are called potentiometric and those that use electrolytic cells are called potentiostatic. Potentiometric techniques include passive measurement of a system in which Nernstian equilibrium is maintained. These static methods involve potentiometric measurements conducted at zero applied current for the evaluation of parameters such as electrolyte pH (Wang, 1994).

Conversely, potentiostatic techniques apply an excitation signal to intentionally disturb a system from equilibrium. Potentiostatic techniques are based on dynamic systems (Wang, 1994). These methods utilize the current response of a system to an applied potential over a period of time to obtain information about the analyte of interest. The current generated is thus proportional to the concentration of the electroactive species. Potentiostatic techniques include linear sweep voltammetry (LSV), cyclic voltammetry (CV), differential pulse voltammetry (DPV), square wave voltammetry (SWV) and amperometric techniques (Wang, 1994).

In cyclic voltammetry, the potential of the working electrode is scanned linearly from an initial potential to a switch potential 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 reduction/oxidation (redox) properties. As a result, cyclic voltammetry is often the first technique used for the determination of redox potentials over the entire working potential range. In addition, the generation of an oxidation product during the forward scan can be analysed during the reduction scan (Kissinger and Heinemann 1996).

A typical cyclic voltammogram is initiated at a potential in which no redox reactions occur. This ensures that the reduced form of redox couple (R) is maintained throughout the bulk solution and the electrode-solution interphase. A linear increase in potential results in the conversion of R into the oxidized form of the redox couple (O) at the electrode surface. During oxidation, the anodic current increases until the surface concentration of R becomes depleted due to its conversion to O. 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. Thus, the surface concentration of R is effectively zero at the switch potential. During the reverse scan for reversible couples, in which O is reduced back to R, the depletion of O results in an accumulation of R at the electrode surface. The reduction of O is accompanied by a cathodic current 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.

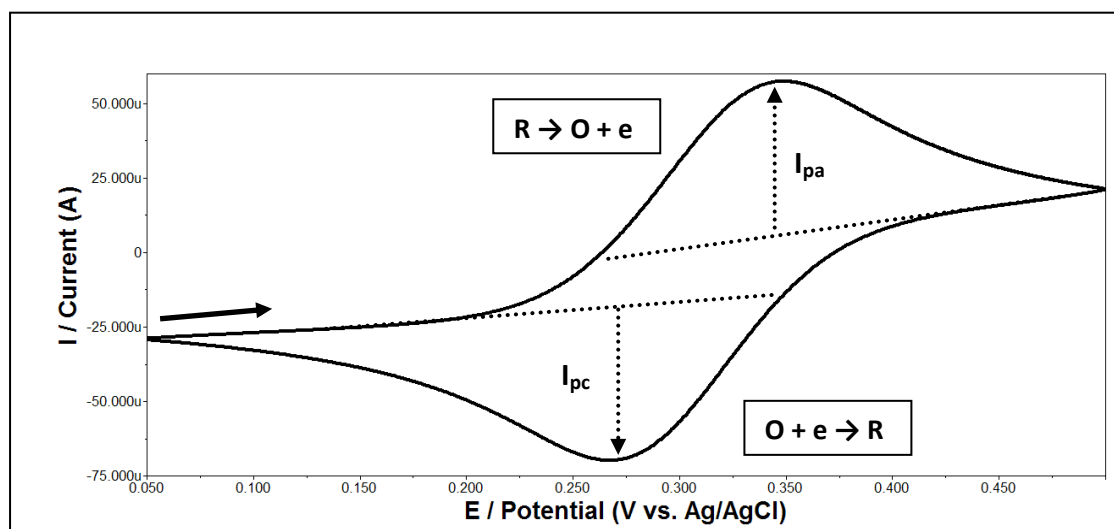


Figure 1.2.3 A typical cyclic voltammogram. I_{pa} is the anodic peak current and I_{pc} is the cathodic peak current. Figure adapted from Kissinger and Heinemann, 1996.

The magnitude of the peak current, peak shape and peak potential obtained from cyclic voltammetry can provide information on the structure and kinetics of electron transfer of the redox couple.

1.2.4 Electroanalytical techniques: Differential Pulse Voltammetry

Differential pulse voltammetry is a potential pulse technique based on the application of a series of short potential pulses superimposed on a linear potential ramp to the working electrode. As each potential pulse has the same amplitude, the potential difference between each pulse is not returned to the same basal potential but is rather in increment to the potential prior to each pulse. In differential pulse voltammetry, the current is sampled before each potential pulse is applied (I_i) and directly before each pulse ends (I_f). The difference in current response ($I_f - I_i$) is plotted against the applied potential to produce the differential pulse voltammograms (Kissinger and Heinemann 1996). As in cyclic voltammetry, the differential current response is proportional to the concentration of the redox species.

Differential pulse voltammetry is used for the detection of trace amounts of analytes. The high degree of sensitivity results from the tendency of the differential process to minimize any contribution from the charging current provided the background current between each pulse remains alike (Kissinger and Heinemann 1996). The influence of electron transfer rate on differential pulse voltammetry also tends to increase peak resolution for kinetically favourable redox reactions while lower currents and broader peaks are obtained for slow reactions.

1.2.5 *Electrochemical kinetics*

A rapid electron transfer between both species of the redox couple and the working electrode is termed a reversible electrochemical reaction. In a reversible couple, the oxidation reaction rate is equivalent to the reduction reaction rate (Kissinger and Heinemann 1996). This results in the regeneration of the electroactive species during the forward and reverse potential scan.

An electrochemical reaction characterised by a fast electron transfer rate follows the Nernstian equation which links the redox potentials to concentration of the electroactive species present at the electrode solution interface. Consequently, if a system follows the Nernstian equation (1.2.5) or an equation derived from it then the electrode reaction is described to be electrochemically reversible (Bard, 1980).

$$E = E_0 + \frac{RT}{nF} \ln \frac{C_O}{C_R} \quad 1.2.5$$

Where E is the electrode potential, E_0 the formal potential, R the Molar gas constant, T the temperature, n the numbers of transferred electrons, F the Faraday constant, C_O the concentration of oxidized redox species and C_R the concentration of reduced redox species.

Derived from the Nernst equation, a reversible couple can be identified from cyclic voltammetry by a peak potential separation ($E_{pa} - E_{pc}$) of $59/n$ mV for all scan rates where E_{pa} is anodic peak potential, E_{pc} is the cathodic peak potential and n is the number of electrons transferred during the redox reaction (Bard, 1980). The magnitude of the peak anodic current and peak cathodic current are equivalent. Furthermore, the peak current increases linearly with the square root of scan rate.

By contrast, an irreversible couple typically exhibits only a single oxidation or reduction peak with no corresponding redox peak, thereby indicating the formation of an electro-inactive product with no regeneration of the initial electroactive species. Typically, irreversible reactions are controlled under charge transfer due to slow electron exchange of the redox species with the working electrode. For an irreversible system, the separation in peak potential is greater than $59/n$ mV and the peak potential is shown to shift toward cathodic potentials with increasing scan rates (Bard, 1980).

A quasi-reversible couple is characterized by the formation of a minor redox peak on the return potential scan. The peak separation potential is greater than that of an electrochemically reversible system and can vary between 80 to 200 mV (Bard, 1980). The current response of a quasi-reversible system is limited by both diffusion and the rate of electron transfer. As a result, the increase in peak current is not proportional to the square root of scan rate.

1.3 CHEMICAL MODIFICATION OF ELECTRODES

Chemical modification of a working electrode surface affords the ability to tailor the kinetics of electron transfer between the electrode and an electroactive species (Wang, 1991). Chemically modified electrodes are prepared by the attachment of a conductive substrate to the surface of a conventional inert electrode. The attached substrate primarily functions as an electrochemical catalyst which mediates the transfer of electrons to and from the electrode surface. Typically, the catalyst forms a complex with the electroactive species during electron transfer and is subsequently regenerated by the redox reaction (Rubinstein, 1995). The increase in electron transfer kinetics effectively reduces the potential required for the redox process as well as provides increased sensitivity of current response (Bard, 1980).

In addition to electrocatalysis, chemical modification can increase electrode stability by providing a protective function against corrosion and solvent effects (Bard, 1980). Another aspect of chemical modification is the characterization of attached films useful for the design of sensor structures or processes.

Methods for preparing chemically modified electrodes include physical adsorption, covalent attachment and polymer formation (Telesa and Fonseca, 2008). Physical adsorption results from the binding of the modifying species to the electrode surface by weak intermolecular forces. Spontaneous adsorption is achieved through the chemisorption of organic monolayers on metallic or

carbon based electrode surfaces. Stronger covalent attachment is accomplished by the formation of covalent bonds between the substrate and surface groups present on or often functionalized to the electrode (Telesa and Fonseca, 2008). Polymer films can be formed on an electrode surface by thermal, photochemical or electrochemical, polymerization. Polymer modification provides the benefit of high chemical stability, reproducibility and enables the incorporation of additional electroactive groups directly onto the polymer backbone or between multilayer films (Telesa and Fonseca, 2008).

1.3.1 Phthalocyanine and metallophthalocyanine modified electrodes

Phthalocyanines (Pc) are electron rich macrocyclic compounds which are comprised of alternating nitrogen to carbon atom ring structures (Nyokong, 2007). Their transition metal analogues, metallophthalocyanines (MPc), contain a central metal ion namely manganese, iron, cobalt, nickel, copper or zinc within the porphyrin ring structure. The unique photo- and electrochemical properties of phthalocyanines and their metal analogues have lead to extensive commercial use as dyes, catalysts, liquid crystal displays and optical recording materials (Shirota, 2000). For industrial use, additional substituents are often synthesised to the phthalocyanine ring in order to enhance solubility, electroactivity or to allow for coupling to other reagents such as polymers (Nyokong, 2007).

The ability for metallophthalocyanines to exist in a wide range of redox states has resulted in their widespread application for use in electrocatalysis. Depending on the nature of electrocatalysis, metallophthalocyanine species can act as a homogenous or heterogeneous catalyst (Fujiwara and Gushikem, 1999). As a homogenous catalyst, a solution phase complex is formed between the metallophthalocyanine and the species to be catalysed. The complex migrates to the electrode surface to allow for electron transfer to occur which in turn regenerates the metallophthalocyanine. In heterogeneous catalysis, the metallophthalocyanine is chemically modified to the electrode surface and mediates the transfer of electrons between the working electrode and the target species.

The catalytic activity of metallophthalocyanines are strongly dependant on the oxidation state of the central metal, composition of the phthalocyanine ring and the nature of the ligand substituents attached to the ring structure (Nombona and Nyokong, 2009).

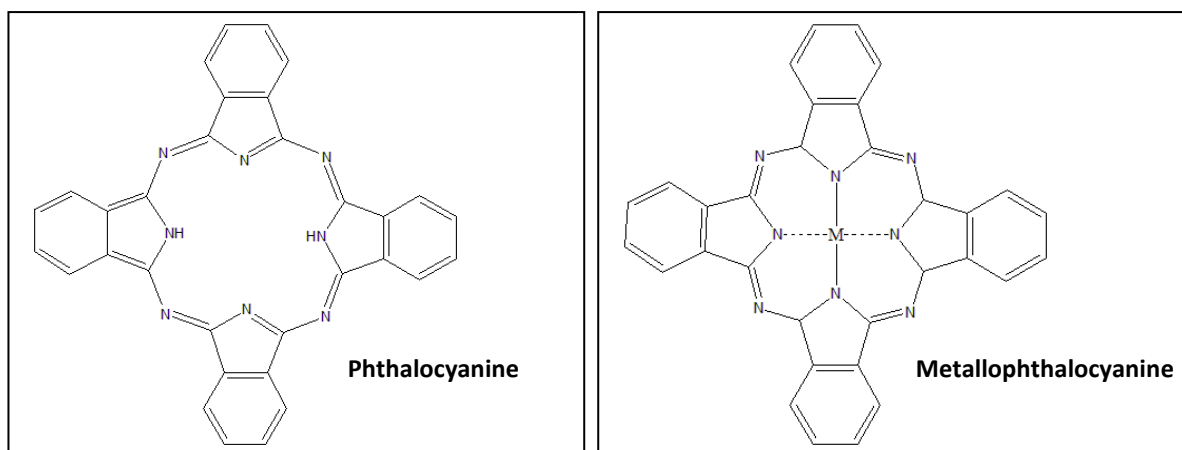


Figure 1.3.1 The molecular structure of a typical phthalocyanine and a metallophthalocyanine. M represents the central metal ion.

The electron transfer process for metallophthalocyanines is mediated by redox reactions based at the central metal or peripheral ring structure. Oxidation reactions centred at the metallophthalocyanine ring are associated with the position of lowest occupied molecular orbital states (LUMO) while reduction reactions are associated with the position of highest occupied molecular orbital states (HOMO) (Zagal, et. al, 2000). HOMO is the molecular orbital which can act as an electron donor, since it contains electrons in the highest energy state. The LUMO is the molecular orbital which can act as an electron acceptor, since it contains electrons in the lowest energy state. Besides ring based electron transfer, redox processes will occur at the central metal provided the metal ion is electroactive and its orbitals lie between the HOMO and LUMO energy states of the metallophthalocyanine ring (Zagal, et. al, 2000).

Metal based redox processes observed for CuPc, FePc and CoPc generally show better electrocatalytic properties than ring based redox reactions observed for ZnPc, NiPc and CuPc complexes (Vasudevan, et. al, 1990). In addition to the central metal and ring structure, the redox potential of metallophthalocyanines is influenced by the electrostatic nature of the peripheral ligand substituents. The occurrence of electrophilic ligand substituents affects a more positive redox potential for metallophthalocyanines while nucleophilic groups shift the potential to more negative values (Koca, et. al, 2007).

Control of the catalytic characteristics through the selective synthesis of various central metal ions, ligand ring and periphery substituents; make metallophthalocyanines strong candidates for the fabrication of electrochemical sensing devices. The recent application of metallophthalocyanine modified electrodes has allowed for the detection of analytes including phenols, thiols,

neurotransmitters, and various organic compounds at reduced overpotentials, fast electron transfer rates and increased current densities.

Metallophthalocyanines as such represent a well established and viable sensor modifier for a range of applications and significant gains can yet be made in utilising metallophthalocyanine modification toward the detection of various sensing targets.

1.3.2 Carbon nanotube modified electrodes

Carbon nanotubes are nanostructured allotropes of carbon that have received considerable attention in the fields of material science, electronics, optics and medicine due to their unique structural properties, tensile strength, chemical stability, electrical and thermal conductivity (Tasis, et. al, 2006). Carbon nanotubes are nanomaterial structures with dimensions close to 1 nm in diameter and up to several microns in length (Wildgoose, et. al, 2006). The two principle forms of carbon nanotubes are single walled (SWCNT) and multi walled carbon nanotubes (MWCNT).

The structure of a SWCNT consists of a one atom thick sheet of graphite wrapped into a seamless cylinder with either open or closed ends (Fernandez and Costa-Garcia, 2008). SWCNTs may be classified as either zigzag, armchair or chiral depending on the configuration of the graphite sheet (Ouyang, et. al, 2002). MWCNTs consist of several concentric cylinders of graphite fitted one inside the other. MWCNT's can exist as several morphological variations including "hollow tube", "herring bone" and "bamboo" forms (Fernandez and Costa-Garcia, 2008). In the hollow tube morphology, the graphite cylinder remains open along its entire length with the graphite sheet forming the cylinder walls parallel to the axis of the nanotube. The internal structure of herringbone MWCNTs are similar to the hollow tube form except that the cylinder walls are tilted at an angle to the nanotube axis. Bamboo like MWCNTs form successive herringbone structures stacked into compartments which close along the length of the nanotube. The conditions and method of synthesis determine the various morphologies of MWCNTs produced, whereby the principle methods of manufacture include arc discharge, laser ablation and chemical vapour deposition (Dai, 2002)

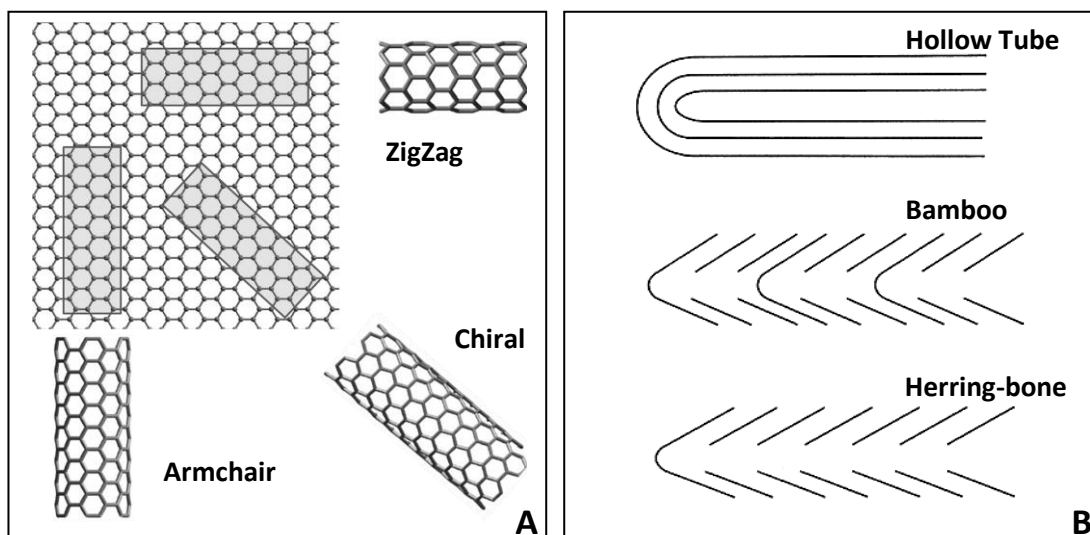


Figure 1.3.2 The various classifications of single walled (A) and multi walled (B) carbon nanotubes. Figure adapted from Meyyappan, 2005.

Two types of graphitic planes can be identified within the MWCNT structure: the basal plane which consists of graphite layers along the nanotube surface and the edge plane where graphite layers are cut perpendicular to the nanotube surface. For most redox species, edge plane pyrolytic graphite electrodes have shown considerably faster electrode kinetics than that of basal plane graphite (Banks, et. al, 2004). Consequently, the electrochemical activity of CNTs is thought to originate from edge planes present at defect sites along the surface or at terminal ends of the carbon nanotube structure (Banks, et. al, 2004).

The electrocatalytic properties of carbon nanotubes result from the combination of defect site electroactivity and nanometre dimensions (Gooding, 2005). More specifically, the minute area of a defect site situated along the carbon nanotube structure results in a nonlinear diffusion of redox species toward the electroactive site. Nonlinear diffusion produces a significant increase in current density due to the large flux of redox species around the perimeter of the defect site. 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 nanotube surface resulting in a large current response for redox species with well defined current peaks (Zhao, et. al, 2005).

Functionalization of carbon nanotubes is used to impart additional chemical versatility essential for the control of solubility, dispersion, polymer formation, electrode modification and the incorporation of biomolecules for both spectroscopic and electrochemical sensing applications.

Chemical oxidation by means of acid treatment is arguably the most common method used to functionalise carbon nanotubes and involves the introduction of carboxylic acid groups onto the end caps or at defect sites along the nanotube surface (Yu, et. al, 2008). The addition of oxygenated species to the nanotube surface subsequently increases the electroactivity and allows for further modification through amide linkages or by esterification (Gooding, 2005).

Another strategy for modifying carbon nanotubes is via action of free radical species generated through chemical or electrochemical reduction (Curulli, et. al, 2006). Aryldiazonium salts are commonly used yielding an aryl free radical species which covalently attaches to the surface of the nanotube. The use of substituted aryl diazonium salts has enabled further functionalization of nanotubes with a variety of chemical substituents including halogens, alkanes, sulphates and carboxyl groups (Bahr and Tour, 2001).

The versatility of functionalization, unique physical and electrocatalytic properties make carbon nanotubes effective nanomaterials for the fabrication of electrochemical sensors. The application of carbon nanotubes as modifiers has attributed the electrode with high chemical stability while retaining surface activity under a wide operational potential window. Consequently carbon nanotube modified electrodes have exhibited high sensitivity toward the detection of numerous compounds including glucose (Salimi, et. al 2004), catechol (Xu, et. al, 2004), uric acid (Ye, et. al, 2003), nucleic acids (Wang, et. al, 2003), pesticides (Deo, et. al, 2005), insulin (Wang and Musameh, 2003), morphine (Salimi, et. al, 2005) and neurotransmitters (Wu , et. al, 2007).

As the use of carbon nanotubes as an electrode material is relatively new, significant scope still exists in the understanding and optimization of nanotubes for use in electroanalytical and bioanalytical sensing. Various techniques such as atomic force microscopy, scanning tunnelling microscopy, transmission electron microscopy, infrared spectroscopy, Raman spectroscopy and x-ray diffraction have been used to address the characterization of nanotubes (Belin and Epron, 2005). The use of atomic force microscopy and Raman spectroscopy were evaluated in this study.

1.3.3 Raman spectroscopy

Raman spectroscopy is an analytical technique based on the scattering of incident light by the interaction of electromagnetic waves with the molecular structure of a particular material. As the

scattered light is characteristic to the chemical bonds present, the information obtained from Raman spectroscopy is used to identify the chemical structure of the scattering material (Kniepp, et. al, 1999). Consequently, Raman spectroscopy is widely used for the characterization of pharmaceuticals (Vankeirsbilck, et. al, 2002), polymers (Glotin and Mandelkern, 1982), thin films (Orendorff, et. al, 2005), semiconductors (McGilp, J.F. 1995) and nanomaterials (Gouadec and Colomban, 2007).

In practice, the scattering of light occurs as a redirection of the electromagnetic wave as it interacts with a physical obstacle. The collision of the electromagnetic wave with the material results in a transfer of energy to the electron cloud of the constituent molecules (Asher, 1993). The transfer of energy excites the molecule from a ground state to a virtual state which is expressed as a distortion in the distribution of the electron cloud (Asher, 1993).

As the energy level of the virtual state is greater than the quantum energy states, the molecule relaxes to a lower energy state through the emission of excess energy in the form of scattered light (Asher, 1993). Depending on the relaxation energy state, the frequency of emission follows Rayleigh, Stokes Raman or anti-Stokes Raman scattering.

The majority of emission takes place as Rayleigh scattering at the same energy and wavelength of the incident light (Smith and Dent, 2005). Rayleigh scattering results from the relaxation of the molecule to the original ground state leading to no net change in energy transfer (Smith and Dent, 2005). Stokes Raman scattering takes place as the molecule returns to a higher energy state resulting in the emission of light with less energy and a longer wavelength than the incident light (Smith and Dent, 2005). Anti-Stokes Raman scattering occurs when the molecule returns to a lower energy state resulting in an increased energy of emission at a shorter wavelength than the incident light (Smith and Dent, 2005).

For a large system of molecules, it is expected that both Stokes and anti-Stokes Raman scattering will occur simultaneously. As the majority of molecules occupy the ground energy state at room temperature, the intensity of Stokes Raman scattering will exceed that of the anti-Stokes Raman scattering (Long, 2002). Thus, Stokes Raman scattering is typically used during Raman spectroscopy whereby the intensity of scattered light is measured as a function of frequency to obtain the Raman spectrum of a particular material (Long, 2002).

For analytical purposes, a laser excitation source is used as the intensity of the laser beam improves the signal to noise ratio to allow for measurement of relatively small Raman shifts (Gooijer and Mank, 1999).

1.3.4 Atomic force microscopy

Atomic force microscopy (AFM) is a scanning probe technique used to analyze the morphology and mechanical properties of materials on the atomic scale (Dufrene, 2003). In most cases, AFM is used to reconstruct the three dimensional topography of nanoscale structures at low cost and within a short period of time. As there is no restriction on the type of sample to be analyzed, AFM is widely used for applications in the semiconductor industry (Decossas, et. al, 2004), material sciences (Wong, et. al, 1997) and the life sciences (Fotiadis, et. al, 2002). In addition to force, specialized AFM probes may be used to measure thermal, magnetic or electrical properties of a surface material (Wickramasinghe, 2000).

The AFM probe consists of a flexible cantilever which terminates at a fine point placed close to the sample to be analysed. As the probe is scanned across the surface, a laser beam is reflected off the back of the cantilever to a position-sensitive photodiode detector. Forces acting between the probe tip and the material surface cause deflection of the cantilever which reflects the laser beam to different points on the photodiode. The resultant plot of the photodiode output versus the position of the probe tip is used to construct a topographic image of the surface material.

Two principle modes of operation exist for the AFM namely static and dynamic mode (Reich, et. al, 2001). In static mode, the probe tip is initially positioned at a distance away from the material surface. As the separation distance is reduced, atomic forces between the two surfaces will begin to act, causing the cantilever to bend toward or away from the material surface due to the action of attractive van der Waals or repulsive electrostatic forces respectively. Beyond a certain point, a strong repulsive force is exerted on the probe tip as the electron orbitals of the probe and surface atoms begin to overlap. Once a preset repulsive force is reached, the probe tip is adjusted to maintain a constant deflection and therefore height above the surface. The constant probe adjustment is used to obtain topographical information of the surface. On completion of the scan, the direction of motion is reversed to return the probe tip to the initial starting position.

In the dynamic mode, the cantilever is set to oscillate as it is moved to and from the surface. The measured parameter is the reduction in the amplitude of oscillation caused by the intermolecular forces between the probe tip and the surface material. The advantage of dynamic mode AFM is improved resolution for relatively rough surfaces with large height differences throughout the material surface (Jalili and Laxminarayana, 2004).

1.4 AIMS AND OBJECTIVES

The aim of this thesis was to develop a nanostructured biosensor capable of enhancing the electrochemical detection of OTA. Electrode modification by metallophthalocyanine and carbon nanotube structures was investigated as a means to improve sensor performance in terms of sensitivity, reproducibility and electrode fouling characteristics. Preliminary selection of a bio-recognition element was investigated as a means of providing selectivity in the detection of OTA in complex media. The required objectives of the thesis were carried out as follows:

Chapter 2 investigated the optimization of electrolyte parameters and assessed the kinetic parameters which govern the sensitive, accurate and reproducible detection of OTA by electrochemical techniques including CV and DPV.

Chapter 3 investigated the electrocatalytic activity of various metallophthalocyanine complexes toward the electrochemical detection of OTA.

Chapter 4 investigated the effect of oxidative functionalization and centrifugal purification on improving the electrocatalytic activity of carbon nanotubes modifiers toward the detection of OTA. Pre-treatment of carbon nanotubes was assessed through AFM, Raman scope, Raman spectroscopy and electrochemical characterization.

Chapter 5 investigated the use of a commercial lipase A preparation as a potential bio-recognition element for the detection of OTA through an electrochemically active OTA hydrolysis product.

CHAPTER 2

ELECTROCHEMICAL CHARACTERIZATION OF OCHRATOXIN A

2.1 INTRODUCTION

Ochratoxins are a group of structurally related mycotoxins produced from several strains of *Penicillium* and *Aspergillus* fungi. Chemically, ochratoxins consist of a dihydroisocoumarin moiety joined by a peptide bond to L-phenylalanine (Harris and Mantle, 2001). The widespread occurrence of filamentous fungi has led to the contamination of OTA in numerous commodities such as maize, grain, coffee, spices and fruit which may lead to downstream contamination in animal fodder, beverages, processed goods and animal products (Hohler, 1998).

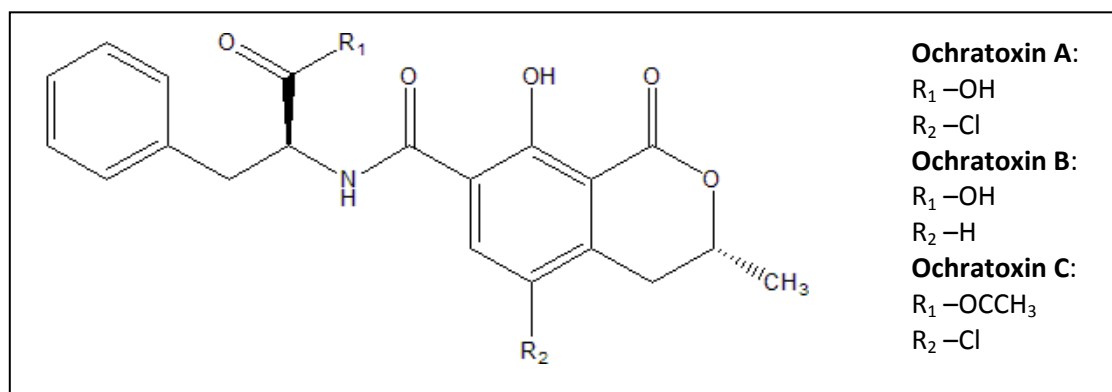


Figure 2.1 The chemical structure of ochratoxin A (OTA), ochratoxin B (OTB) and ochratoxin C (OTC).

Ochratoxin A is the most toxic and commonly occurring ochratoxin consisting of a chloride ion attached to the dihydroisocoumarin moiety (Marquardt and Frohlich 1992). Several analogues including ochratoxin B (OTB) and ochratoxin C (OTC) have been identified since the initial discovery of OTA as a toxic metabolite to animals (O'Brien and Dietrich, 2005). OTB is the non chlorinated analogue of OTA and is considered to be 10 to 20 fold less toxic than OTA *in vivo* (O'Brien and Dietrich, 2005). The ethyl ester of OTA is known as OTC and has similar toxicity as OTA although is rarely found as a natural contaminant (O'Brien and Dietrich, 2005).

Aspergillus ochraceus are the most common producers of OTA in temperate regions with *Penicillium verrucosum* as common producers in more tropical climates (Magan and Aldred, 2007). Other *Aspergillus* species known to produce OTA include *A. auricomus*, *A. carbonarius*, *A. melleus*, *A. niger*, *A. ostianus*, *A. petrakii* and *A. sclerotioru* (Bayman, et. al, 2002). As OTA is thermostable, ingestion of contaminated agricultural products or processed goods thereof typically leads to the

accumulation of OTA in the circulatory system, liver, kidneys and other tissues (Boudra, et. al, 1995) (Zhang, et. al, 2009). Ingestion of OTA had been implicated in a diverse range of toxicological effects including nephrotoxicity, mutagenicity, teratogenicity, neurotoxicity and immunotoxicity (O'Brien and Dietrich, 2005). Considering the long half life of OTA in humans and based on the toxicological studies on animals, OTA has been classified as a potential human carcinogen (class 2B) by the IARC with the JCFAC enforcing a provisional maximum permissible concentration of 5 µg/kg of OTA in raw and processed cereal products thereof. (IARC, 1993)(JECFA, 2007)

The prevalence of OTA exposure in humans and the requirement to comply with regulatory limits has prompted the development of numerous monitoring techniques for the quantitative evaluation and subsequent removal of OTA from food and feed products. Most methods used for the detection of OTA require sample extraction and cleanup typically achieved through liquid-liquid extraction, solid phase extraction or supercritical fluid extraction followed by separation by high performance liquid chromatography, gas chromatography or capillary electrophoresis linked to fluorescent or mass spectroscopy based detection methods (Monaci and Palmisano, 2004).

Recently, electrochemical methods have been investigated in order to provide a highly sensitive, accurate, more portable and less costly means of detection for rapid, onsite analysis of OTA. In addition, minimal sample pretreatment is required for the electrochemical detection of OTA as current approaches in sensor development have moved toward the use of disposable screen printed electrodes designed for single use analysis (Alarcon, et. al, 2006).

Radi, et. al, 2008 developed an ELISA based electrochemical immunosensor on screen printed electrodes for the detection of OTA. A competitive immunoassay between OTA and horseradish-labelled OTA for the antibody modified electrode was used to obtain a limit of detection of 29.72 nM and a detection range of up to 148 nM of OTA. Khan and Dhayal, 2008 immobilised rabbit antibody to a chitosan-polyalanine conduction polymer and measured the interaction of OTA with the immobilised film as a relative change in impedance. The impedimetric immunosensor showed a linear response of up to 24.78 nM OTA concentration with a corresponding detection limit of 2.48 nM. Lu, et. al, 2009 reported a detection limit of 0.02 nM and linear response of up to 250 nM for the determination of OTA in corn samples by an alkaline phosphatase based electrochemical immunosensor. Electrochemical response was obtained by the enzymatic oxidation of 1-naphthyl phosphate which was inversely proportional to the amount bound anti-OTA mouse antibody.

However, fundamental studies on the electrochemical behaviour of OTA are required to extend the sensitivity of detection, to aid the development of novel detection platforms and to assess the feasibility of employing electroanalytical techniques for the detection of OTA in real sample media. Two key publications amongst the limited literature available concerning the electrochemical analysis of OTA have been reported by Calcutt, et. al, 2001 and Oliveira, et. al, 2007.

Calcutt, et. al, 2001 established the voltammetric behaviour of OTA in acetonitrile as well as in phosphate buffer between the pH range of 6 to 8. The oxidation of OTA was shown to occur by an electrochemically irreversible reaction which resulted in the production of a quinone/hydroquinone species as respective oxidation products. Electroanalysis of 4-chlorophenol was carried out as it comprises an electroactive constituent of the dihydroisocoumarin moiety of OTA. A comparison was made between the oxidative behaviour of OTA and 4-chlorophenol in acetonitrile in order to correlate the formation of the respective quinone species to a two electron oxidation process. The electrochemical oxidation of OTA in phosphate buffer was reported to resemble the oxidative behaviour of the deprotonated form of OTA (OTA^-) in acetonitrile in which oxidation of OTA^- was shown to occur through a one electron transfer process. In addition, a diffusion coefficient of $0.48 \times 10^{-5} \text{ cm}^2 \text{ s}^{-1}$ was determined for OTA in phosphate buffer pH 7.

Oliveira, et. al, 2007 investigated the oxidation of OTA in phosphate buffer between the pH range of 2 to 12, thereby extending the electrochemical analysis of OTA in aqueous media. The oxidation of OTA was shown to be pH dependant in acidic media, following a one electron one proton oxidation mechanism to produce quinone/hydroquinone species which readily adsorb to the electrode surface. A diffusion coefficient of $0.37 \times 10^{-5} \text{ cm}^2 \text{ s}^{-1}$ was calculated for OTA in phosphate buffer pH 7. In alkali media, the oxidation of OTA was shown to be independent of electrolyte pH with the oxidation reaction involving only the transfer of a single electron. The detection limit of OTA was evaluated by square wave voltammetry from the OTA and quinone oxidation product peaks as 1.2 μM and 0.3 μM respectively. As reported by Oliveira, et. al, 2007, improvements toward the detection of OTA by electrochemical methods is required in order to evaluate the degree of OTA contamination in complex media.

From the results reported by Calcutt, et. al, 2001 and Oliveira, et. al, 2007, key aspects regarding the electrochemical analysis of OTA still need to be addressed. These include the optimization of electrolyte parameters toward the detection of OTA, clarification of the kinetics of electron transfer during the oxidation of OTA in acidic media, electrochemical analysis of the constituents comprising

OTA particularly 4-chlorophenol and l-phenylalanine, evaluation of fouling characteristics of OTA in relation to the constituents of OTA and the determination of detection limits of OTA in complex media. Furthermore, significant scope exists for the development of chemical modification strategies to allow for electrochemical means of OTA detection to match the sensitivity, selectivity and stability offered by chromatographic and electrophoresis based techniques.

The prospect of electrochemical detection of OTA as well as the investigation of optimal detection parameters and kinetics governing the voltammetric analysis of OTA is investigated in the following chapter.

2.2 AIMS

The overall objective of chapter 2 is to determine the feasibility of using electrochemical techniques for the sensitive, accurate and reproducible detection of OTA by methods of CV and DPV. Detection parameters including the type of supporting electrolyte, potential range, solution pH and limit of detection were investigated for the optimization of subsequent voltammetric analysis. The kinetic parameters governing the detection of OTA and degree of electrode fouling upon successive detection cycles were evaluated to allow for subsequent analysis of detection at chemically modified electrodes. In addition, the electroactive constituents of OTA were identified for subsequent design of the biorecognition element of the OTA biosensor.

2.3 INSTRUMENTATION AND METHODOLOGY

2.3.1 Instrumentation

All Electroanalysis was performed on an Autolab PGSTAT 302 Potentiostat/Galvanostat and analyzed via General Purpose Electrochemical Software (GPES) version 4.9 (Eco Chemie BV, 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).

All pH measurements were conducted using a pH 330i pH meter connected to a SenTix 41 pH electrode (WTW, Germany).

Ultrasonication was carried out using an Elmasonic S10H waterbath sonicator (Elma Ultrasonic Technology, Germany).

2.3.2 Reagents

Stock solutions of ochratoxin A (derived from *Aspergillus ochraceus*, analytical standard, OEKANAL, Sigma Aldrich), 4-chlorophenol (99.9 %, PESTANAL, Sigma Aldrich) and L-phenylalanine (>98 %, Sigma Aldrich) were prepared prior to analysis.

The various electrolytes used were prepared from boric acid (99%, Merck), glacial acetic acid (97 %, Merck), orthophosphoric acid (85 %, Merck), sodium dihydrogen phosphate monohydrate (99 %, Merck), disodium hydrogen phosphate dehydrate (99 %, Merck), sodium acetate (98 %, Merck), glycine (99 %, Sigma Aldrich), hydrochloric acid (32 % Merck), potassium chloride (99 %, Sigma Aldrich).

Stock solutions of 1 mg/ml ochratoxin A were prepared in absolute methanol and stored at – 20 °C prior to use.

Safety Consideration

Ochratoxin A is a nephrotoxic and carcinogenic compound and was handled with the utmost of care. All solutions containing OTA were handled by personnel wearing appropriate protection gear. Solutions containing OTA were placed in an appropriate biohazard container and kept in a separate fume hood until disposal by incineration. Incineration was carried out by private contractors.

2.3.4 Methodologies

Electrochemical apparatus

A standard three electrode cell was utilized for all voltammetric measurements conducted via the Autolab PGSTAT 302. The electrochemical cell consisted of a glassy carbon working electrode, 3mm in diameter, obtained from Bioanalytical Systems (BAS). A Ag/AgCl electrode saturated in 3 mM KCl was used as a reference electrode and a platinum wire as a counter electrode. The three electrodes were placed equidistant from each other in the electrochemical cell. When not in use, the reference electrode was stored in 3M KCl.

Electrochemical parameters

Prior to electrochemical analysis, the reference electrode was thoroughly rinsed with MilliQ water in order to remove residual KCl. The potassium chloride storage solution was also replaced on a two week basis to avoid cross contamination. The platinum counter electrode was washed in 5% nitric acid, followed by thorough rinsing in milliQ water.

All electrolyte solutions were degassed with nitrogen gas (instrument grade, Afrox) for 5 minutes prior to electrochemical analysis. During analysis, a constant stream of nitrogen gas was maintained over the electrochemical cell containing the electrolyte solution. All analyses were conducted at 25 °C. Unless otherwise stated, the working solution used for analysis consisted of a total volume of 5.0 ml.

Electrode pretreatment and cleaning

Cleaning of the GCE prior to analyses consisted of sequential rinsing of the electrode surface with 70 % ethanol, milliQ water, 5 % nitric acid followed by a final thorough rinse in milliQ water. The GCE surface was then polished on Buehler feltpad (BAS) with a concentrated slurry of 99.7% aluminium oxide (<10 micron Sigma-Aldrich) to a mirror finish, then rinsed with milliQ water. The GCE was then sonicated for a period of 2 minutes in milliQ water to remove any residual aluminium oxide. Unless otherwise stated, the cleaning procedure was used directly before each electrochemical analysis. Electrochemical pretreatment of the electrode was performed prior to analysis with pretreatment consisting of successive cycling of the clean electrode in electrolyte solution for 15 CV cycles or until a uniform baseline current was obtained.

Glassware preparation and cleaning

Thorough cleaning of all glassware, including the electrochemical cell was performed prior to use. Initially the glassware was immersed in 5 % nitric acid (Merck), then rinsed repeatedly with MilliQ water, followed by an absolute ethanol (Merck) rinse and finally repeatedly rinsed in MilliQ water to remove any residual ethanol. When not in use, the electrochemical cell was stored in 5% nitric acid. Unless otherwise stated, the above instrumentation, reagents and methodologies were used throughout the entire study.

Chapter specific methodology

2.4.1 Electroanalysis of ochratoxin A

CV analysis of 5 µM OTA was performed in 0.2 M BR buffer pH 5.0 at a bare GCE.

2.4.2 Choice of supporting electrolyte

CV analysis of 5 μM OTA was performed at a bare GCE in 0.2 M glycine hydrochloric acid buffer pH 3, 0.2 M sodium acetate buffer pH 5, 0.2 M sodium phosphate buffer pH 7 and compared to analysis in 0.2 M BR buffer of equivalent pH.

2.4.3 The effect of pH

CV analysis of 5 μM OTA in 0.2 M BR buffer was performed between the range of pH 2 to pH 11 at a bare GCE.

2.4.4 Degree of electrode surface fouling

Repetitive CV scans of 5 μM OTA in 0.2 M BR buffer pH 5 were conducted at a bare GCE without subsequent cleaning of the GCE surface between successive CV cycles.

2.4.5 Mass transport of OTA

CV analysis of 5 μM OTA in 0.2 M BR buffer pH 5 at a bare GCE was assessed for scan rates of 5, 10, 25, 50, 75, 100, 150 and 200 mV/s.

2.4.6 Standard curve for the oxidation of OTA

DPV analysis in 0.2 M BR buffer pH 5 of OTA concentrations of 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 25 and 30 μM was performed at a bare GCE.

2.4.7 Tafel plot analysis of OTA

CV analysis of 5 μM OTA in 0.2 M phosphate buffer pH 7 and BR buffer of pH 1, pH 3, pH 5 and pH 6 were assessed at a bare GCE for scan rates of 5, 10, 25, 50, 75, 100, 150 and 200 mV/s.

2.4.8 Effective surface area determination for GCE

CV analysis of equimolar concentrations of potassium ferricyanide (>99.99 %, Sigma Aldrich) and potassium ferrioxalate (>99.99 %, Sigma Aldrich) was performed in 1 M potassium chloride buffer pH 7 at a bare GCE for scan rates of 5, 10, 25, 50, 75, 100, 150, 175 and 200 mV/s.

2.4.9 OTA electron transfer

CV analysis of 5 μM OTA was assessed in 0.2 M phosphate buffer pH 7 at a bare GCE for scan rates of 5, 10, 25, 50, 75, 100, 150 and 200 mV/s.

2.4.10 Diffusion coefficient for OTA

CV analysis of 5 μM OTA in 0.2 M BR buffer pH 5 at a bare GCE for scan rates of 5, 10, 25, 50, 75, 100, 150 and 200 mV/s.

2.4.11 OTA oxidation mechanism: Correlation with 4-chlorophenol

CVs of 50 μM 4-chlorophenol and 50 mM L-phenylalanine in 0.2 M BR buffer pH 5 were assessed as outlined in general methodologies with the exception that the GCE surface was not cleaned between successive CV scans

2.4.12 OTA oxidation mechanism: pH profile of 4-chlorophenol

CV analysis of 50 μM 4-chlorophenol in 0.2 M BR buffer between the range of pH 2 to pH 10 at a bare GCE.

2.4 RESULTS AND DISCUSSION

2.4.1 Electroanalysis of ochratoxin A

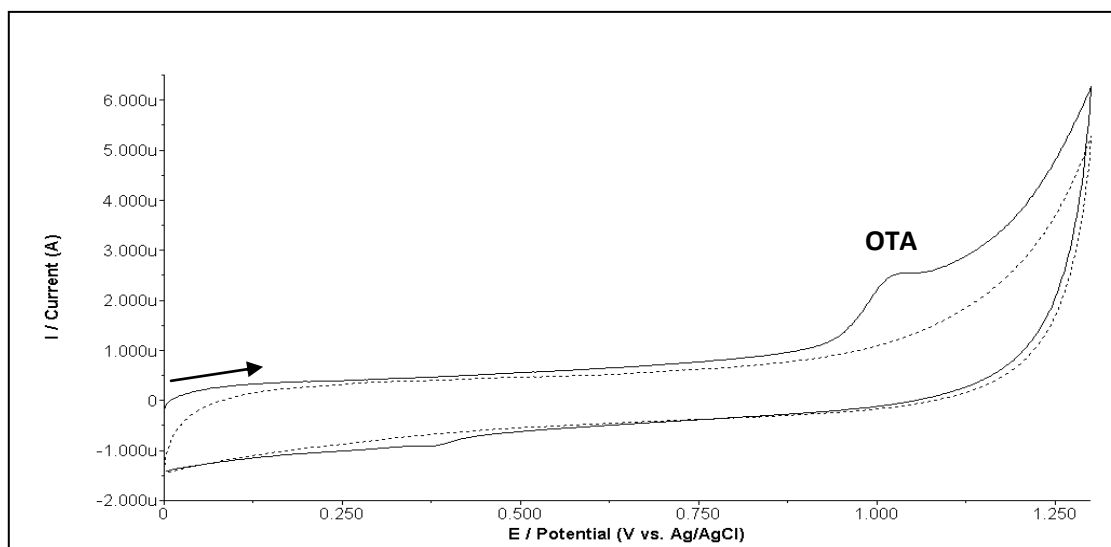


Figure 2.4.1.1 CV obtained of 5 μ M OTA in 0.2 M BR buffer pH 5.0 at a bare GCE. Blank solution is shown by a dotted line. Scan rate 100 mV/s.

The voltammetric analysis of pure ochratoxin A in 0.2 M BR Buffer pH 5.0 yields a single anodic peak at an oxidation potential of 1.04 V. The oxidation potential of the primary peak ranges from 0.80 to 1.20 V depending on the concentration of ochratoxin A and the pH of the electrolyte solution. On the return scan, a single cathodic peak is observed at a reduction potential of 0.38 V in 0.2 M BR Buffer pH 5.0.

A potential difference between the anodic and cathodic ochratoxin A peaks of greater than 59 mV is indicative of an electrochemically irreversible redox process. The irreversibility results from the slow rate of electron exchange between ochratoxin A and the electrode surface. The large separation in peak potential allows for increased diffusion of oxidised product from the electrode surface resulting in a diminished cathodic peak current (Kissinger and Heinemann, 1996). The primary anodic peak was used to measure the concentration of ochratoxin A given the current strength and reproducibility of the oxidation peak.

2.4.2 Choice of supporting electrolyte

Figure 2.4.2.1 shows the current response and oxidation potential for the detection of 5 μM ochratoxin A in 0.2 M glycine hydrochloric acid buffer pH 3 (A), 0.2 M sodium acetate buffer pH 5 (B) and 0.2 M sodium phosphate buffer pH 7 (C) as compared to 0.2 M BR buffer of equivalent pH.

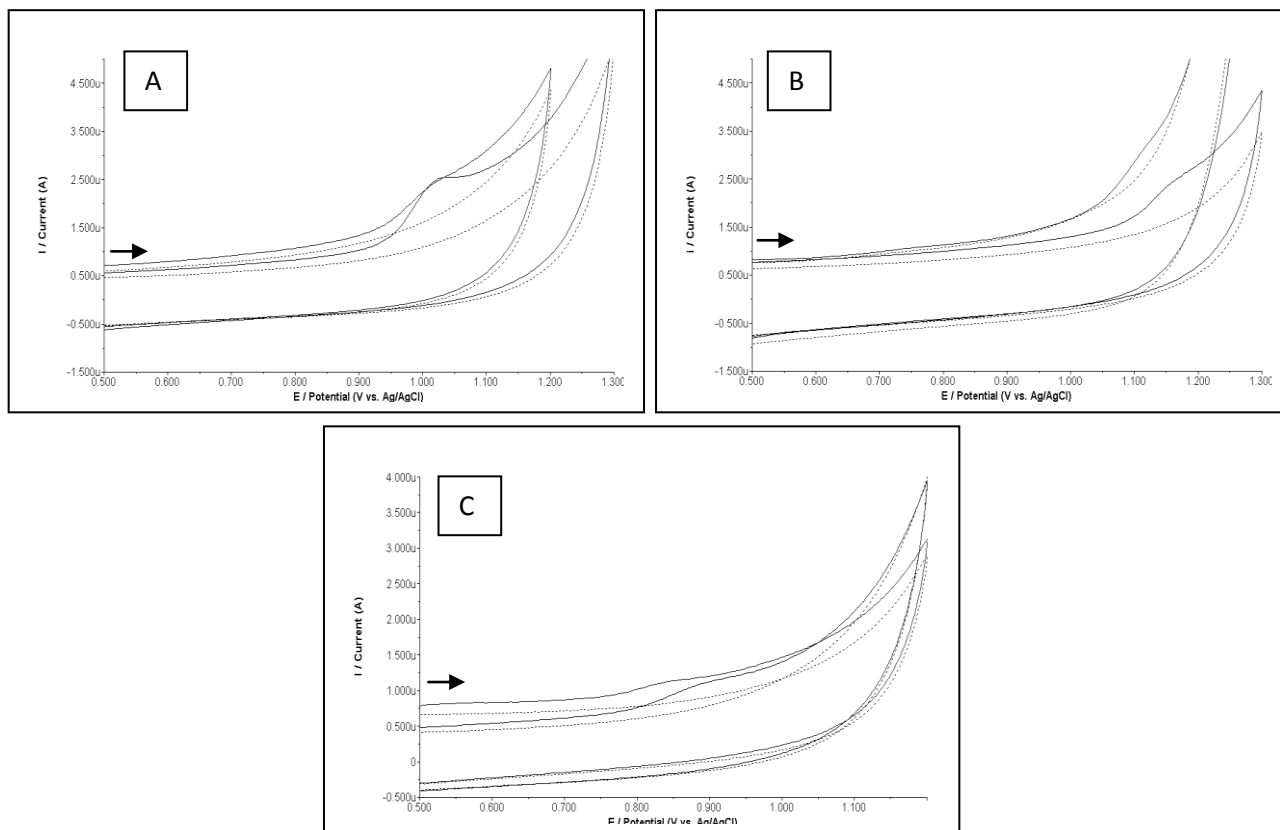


Figure 2.4.2.1 CVs obtained for 5 μM OTA at an unmodified GCE in (A) 0.2 M glycine hydrochloric acid buffer pH 3 and 0.2 M BR buffer pH 3; (B) 0.2 M sodium acetate buffer pH 5 and 0.2 M BR buffer pH 5 and (C) 0.2 M sodium phosphate buffer pH 7 and 0.2 M BR buffer pH 7. Corresponding CV of the blank solutions are shown as dotted lines. Scan Rate 100 mV/s.

A slight negative shift in anodic peak potential was obtained for OTA in 0.2 M glycine-HCl buffer of potential 1.11 V as compared to 1.17 V obtained for OTA in 0.2 M BR buffer pH 3. A near 60% reduction in sensitivity for the detection of OTA was observed for glycine-HCl buffer when compared to BR buffer. This was attributed to a less defined peak current response produced as a result of the limited potential range observed for the glycine-HCl buffer.

Similar anodic peak potentials of 1.01 V and 1.03 V were obtained for the OTA anodic peak in 0.2 M sodium acetate buffer and 0.2 M BR buffer of pH 5 respectively. The magnitude of the current response for ochratoxin A in sodium acetate buffer was near 40% lower than the equivalent response in BR Buffer.

An anodic peak potential of 0.87 V was obtained for the oxidation of OTA in 0.2 M sodium phosphate buffer as compared to the 0.91 V obtained in 0.2 M BR Buffer of pH 7. A decrease of 50% in peak current response for ochratoxin A was observed when sodium phosphate was used as a supporting electrolyte as compared to BR buffer.

In terms of peak current sensitivity and reproducibility, 0.2 M BR buffer was selected as the optimal supporting electrolyte for the detection of ochratoxin A. Table 2.4.2.1 compares the peak current amplitude and reproducibility in response to the OTA anodic peak at the various electrolytes investigated. In addition, BR buffer provides a wide working potential range and broad pH spectrum for the electrochemical analysis of ochratoxin A.

Table 2.4.2.1 compares the peak current response and anodic potential obtained for the oxidation of 5 μ M OTA at an unmodified GCE in the supporting electrolytes examined.

Supporting electrolyte	pH	Ip [μA]	St. Dev. Ip [μA]	Ep [V]	St. Dev. Ep [V]
Glycine-HCl buffer	3	0.208	0.063	1.107	0.009
Britton-Robinson buffer		0.536	0.062	1.169	0.010
Sodium acetate buffer	5	0.535	0.184	1.015	0.005
Britton-Robinson buffer		0.883	0.180	1.029	0.005
Sodium phosphate buffer	7	0.093	0.023	0.867	0.021
Britton-Robinson buffer		0.193	0.014	0.907	0.011

2.4.3 pH profile of ochratoxin A

Figure 2.4.3.1 and 2.4.3.2 shows cyclic voltammograms obtained for 5 μM ochratoxin A in 0.2 M BR buffer between the range of pH 2 to 6 and pH 7 to 11 respectively.

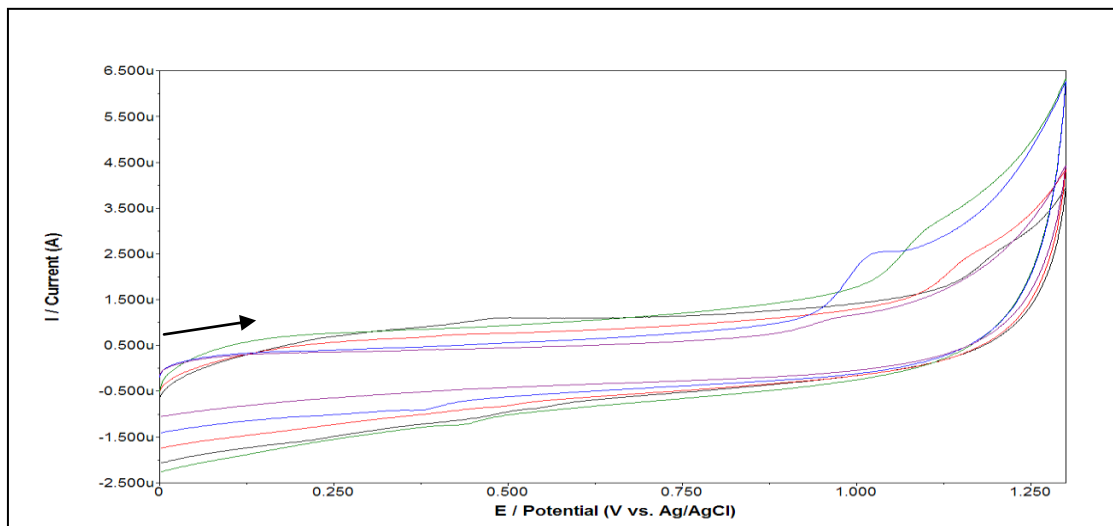


Figure 2.4.3.1 CVs of 5 μM OTA at an unmodified GCE in 0.2 M BR buffer of pH 2 (black), pH 3 (red), pH 4 (green), pH 5 (blue) and pH 6 (magenta). Scan rate 100 mV/s.

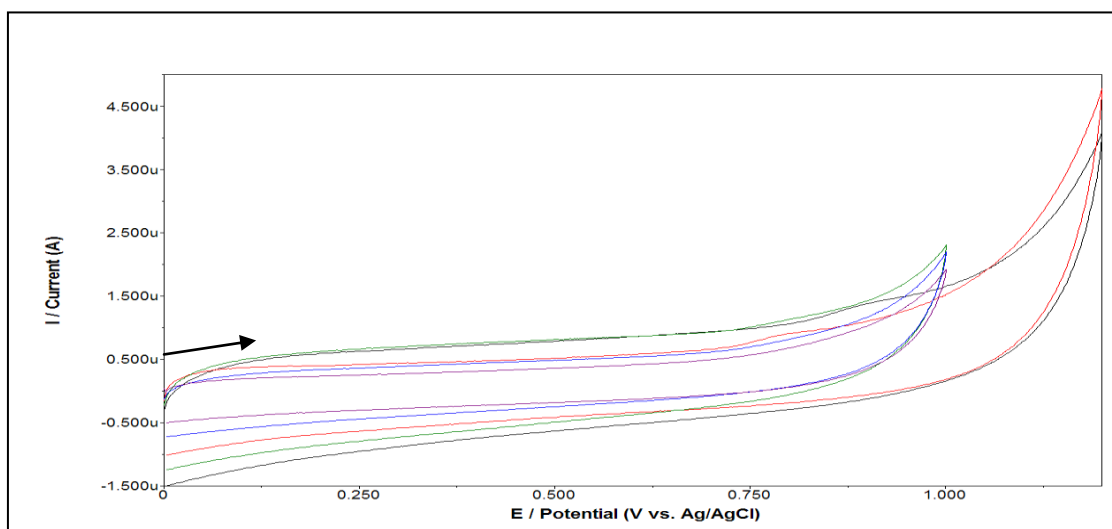


Figure 2.4.3.2 CVs of 5 μM OTA at an unmodified GCE in 0.2 M BR buffer of pH 7 (black), pH 8 (red), pH 9 (green), pH 10 (blue) and pH 11 (magenta). Scan rate 100 mV/s.

Figure 2.4.3.1 and 2.4.3.2 illustrate the role of pH in the detection of ochratoxin A in 0.2 M BR buffer between the pH range of 2 to 11. The change in pH results in a subsequent change in the magnitude of peak current and the position of the anodic peak potential. The cyclic voltammetry profiles of

ochratoxin A show an increase in current response as the pH tends toward less acidic conditions whereby an increase in peak current of 0.41 μA is obtained between the pH range of 2 to 5. A maximum peak current of 0.88 μA was obtained for ochratoxin A at pH 5 in 0.2 M BR buffer.

An increase in pH value toward neutral conditions resulted in a significant decrease in current amplitude of near 75 % between the pH range of 5 to 6. The increase in pH toward more alkaline conditions from pH 6 to pH 11 resulted in a further 37 % decrease in current response.

Figure 2.4.3.3 shows the shift in peak potential for ochratoxin A toward less anodic potentials with an increase in alkalinity. The shift in potential follows the relationship $E_p = 0.063 \text{ pH} + 1.354$ between the pH range of 2 to 7. The slope of the relationship, 63 mV per pH unit, is indicative of an oxidation mechanism for ochratoxin A involving the same number of electrons to protons (Kissinger and Heinemann, 1996). In alkaline conditions above pH 7, the peak potential becomes independent of pH indicating the chemical deprotonation of ochratoxin A. Thus, the acid dissociation constant of OTA was determined as a pKa of 7 as in agreement with results previously reported literature (Marquardt and Frohlich, 1992). The deprotonation of ochratoxin A is supportive of the significant decrease in current response obtained toward physiological pH.

From the above results, pH 5 was selected as the optimal pH for subsequent electrochemical analysis of ochratoxin A given the extent of current response and relative transitional oxidation potential.

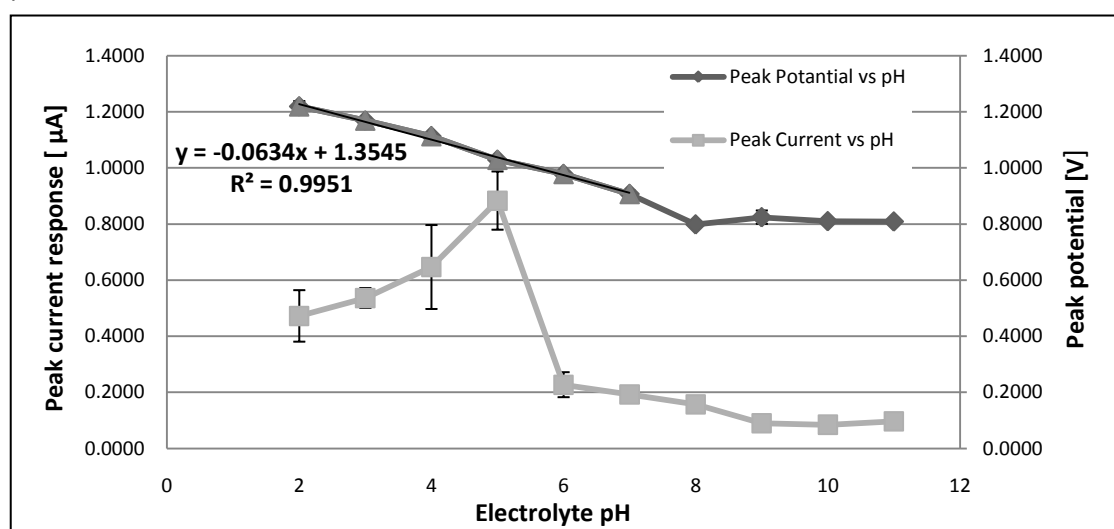


Figure 2.4.3.3 Initial current and potential response of 5 μM Ochratoxin A in 0.2M borate buffer as a function of pH. Number of replicates (3). Error bars show standard deviation from mean. Scan rate 100 mV/s.

2.4.4 Fouling characteristics of ochratoxin A

The degree of fouling was assessed by performing successive cyclic voltammetry scans of OTA in 0.2M BR buffer.

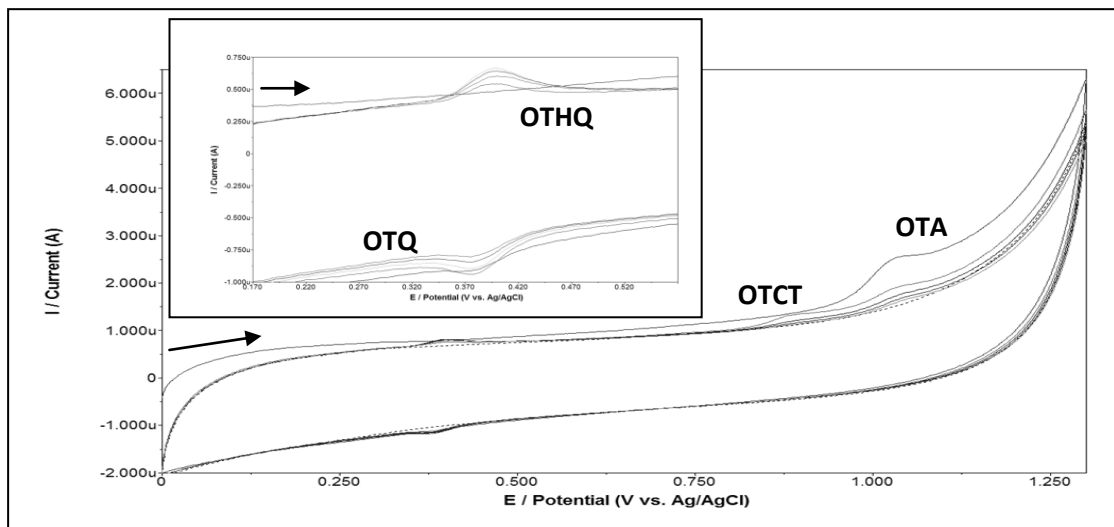


Figure 2.4.4.1 Successive CVs of 5 μ M OTA in 0.2 M BR buffer pH 5 at an unmodified GCE. No regeneration of the electrode surface was done between successive scans. Insert shows the formation of an OTA redox couple. Scan rate 100 mV/s.

Figure 2.4.4.1 illustrates the decrease in peak current amplitude for successive scans of OTA. The peak current decrease can be attributed to passivation of the electrode by the adsorption of oxidised ochratoxin A to the electrode surface. The adsorbed film fouls the electrode surface by preventing the exchange of electrons between the electrode and ochratoxin A present in solution. The extent of surface fouling is shown in Figure 2.4.4.2 as an exponential decrease in peak current amplitude upon successive scans. After five scans in 0.2 M BR buffer pH 5, the number of electroactive sites on the electrode surface was reduced to produce a near 90 % reduction in the current response. A similar decrease to baseline current amplitude was observed for the OTA peak throughout the electrolyte pH range.

Upon oxidation of OTA, the adsorbed film is reduced on the reverse scan of the first cycle to produce a cathodic peak at a potential of 0.37 V. A subsequent CV scan showed the formation of an additional oxidation peak occurring at a potential of 0.39 V, as indicated in Figure 2.4.4.1. A potential difference of 16 mV between the two peak potentials relates to the formation of an electrochemically reversible redox couple.

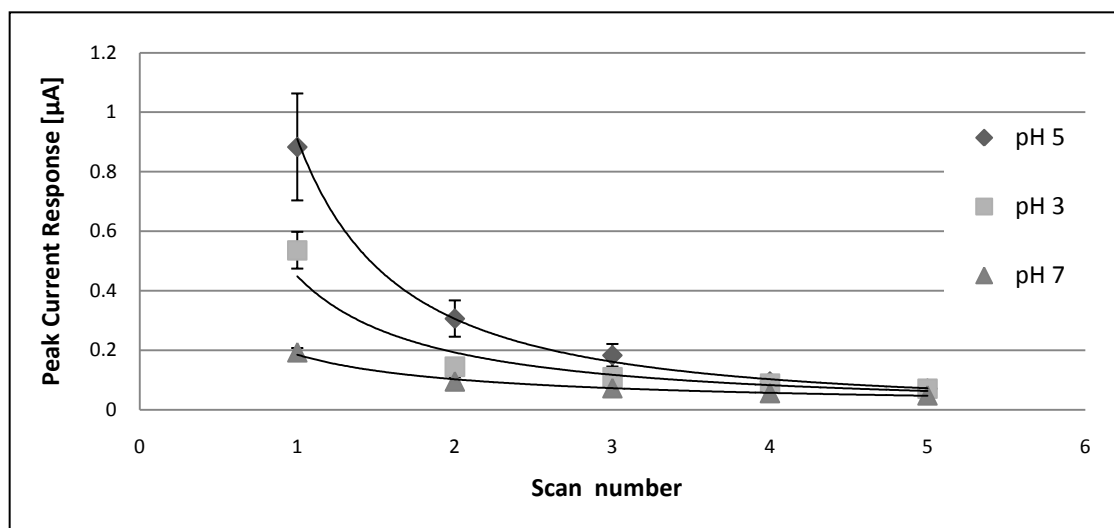


Figure 2.4.4.2 Fouling characteristic for successive CV scans for 5 μ M OTA at an unmodified GCE in 0.2 M BR buffer of pH 3, pH 5 and pH 7. Number of replicates (3). Error bars show standard deviation from mean. Scan rate 100 mV/s.

The peak current magnitude of the reversible couple was substantially less than the ochratoxin A oxidation peak due to the low concentration of the adsorbed oxidation products at the electrode surface. Redox cycling and additional formation through OTA oxidation upon consecutive CV scans resulted in an increase in peak current amplitude for the reversible couple. The reversible redox couple is attributed to the formation of a quinone derivative (OTQ) that is reversibly reduced to form a hydroquinone (OTHQ) species as described by Gillman et al, 1999.

In addition autoxidation of OTHQ is believed to generate OTQ that subsequently reacts with the hydroxide ions in the electrolyte solution to yield an o-hydroquinone that can undergo further oxidation to a catechol species (OTCT) (Gillman, et. al, 1999). The anodic peak observed at a potential of 0.89 V during the second CV scan was attributed to the oxidation of the OTCT at the electrode surface. An irreversible oxidation of the OTCT was observed as no additional cathodic peaks were produced during the reverse CV scan. Additional fouling of the electrode surface by the oxidation of OTCT upon subsequent CV scans could account for the sharp decrease in current response after the initial CV scan.

As shown in Figure 2.4.4.3, OTA oxidation products are weakly adsorbed to the electrode surface toward more alkaline electrolytes. This was observed when no oxidation products were produced upon successive CV scans of OTA in 0.2 M BR buffer of alkaline pH as shown in Figure 2.4.4.3.

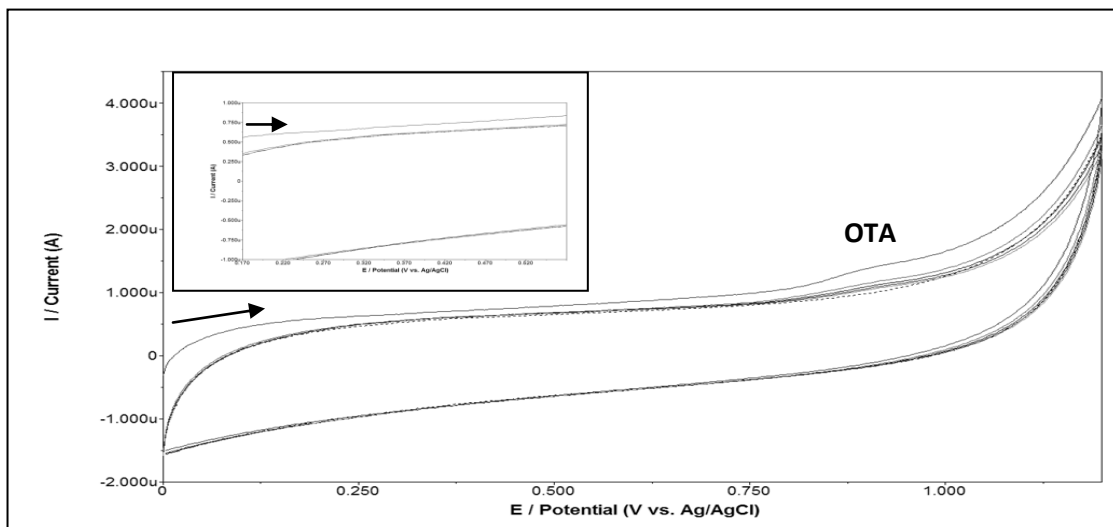


Figure 2.4.4.3 CVs of 5 μM OTA in 0.2 M BR buffer pH 7 at an unmodified GCE. No regeneration of the electrode surface was done between successive scans. Insert shows the formation of an OTA redox couple. Scan rate 100 mV/s.

2.4.5 Mass transport of ochratoxin A

The effect of scan rate on OTA oxidation was determined by recording CV's of 5 μM OTA in 0.2 M BR buffer pH 5 at increasing scan rates of 5, 10, 25, 50, 75, 100, 150 and 200 mV/s.

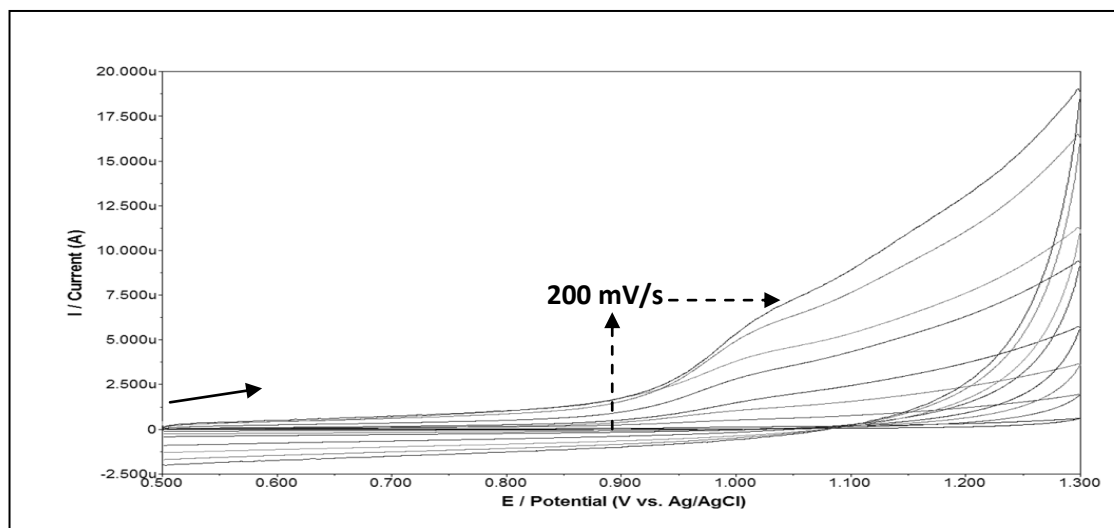


Figure 2.4.5.1 CVs of 5 μM OTA in 0.2 M BR buffer pH 5 for increasing scan rates of 5, 10, 25, 50, 75, 100, 150 and 200 mV/s.

Figure 2.4.5.1 shows the resultant CV's depicting an increase in OTA peak current magnitude for increasing scan rates. The plot of peak current amplitude vs. square root of scan rate, shown in

Figure 2.4.5.2, yields a linear response between the scan rate range of 10 to 150 mV/s. A deviation in linearity was obtained for scan rates below 5 mV/s and above 200 mV/s. Low scan rates tend to introduce convective errors produced from mechanical vibrations of the electrolyte solution over extended analysis times (Strutwolf and Schoeller, 2005). The deviation for in current from linearity at high scan rates high was accredited to kinetic processes as reported by Zare, et. al, 2007.

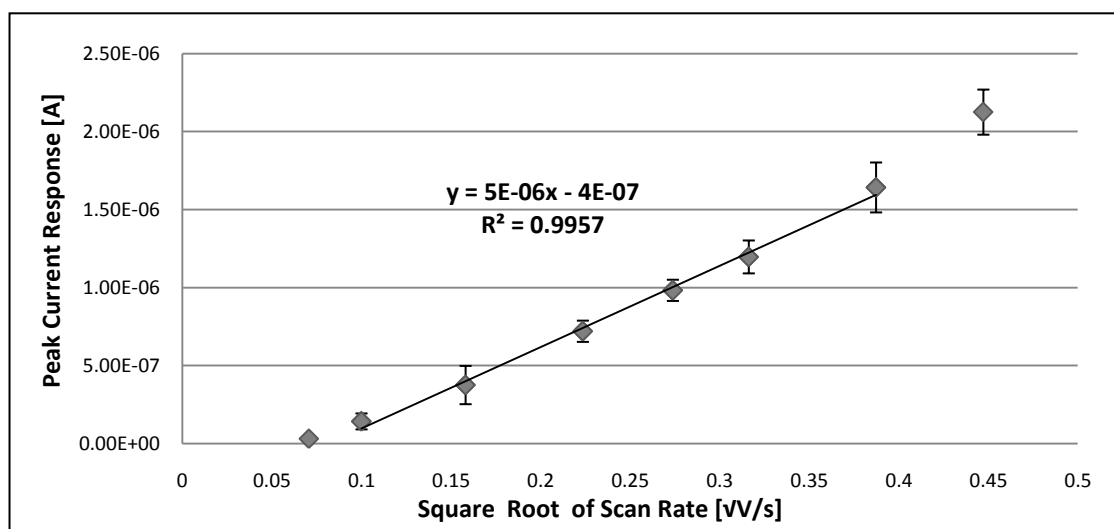


Figure 2.4.5.2 Linear plot of peak current amplitude vs. square root of the scan rate obtained for 5 μM OTA in 0.2 M BR buffer of increasing scan rate. Number of replicates (3). Error bars show standard deviation from mean.

A linear relationship for current amplitude against the square root of the scan rate indicates that mass transport OTA at the electrode solution interphase during the oxidation of OTA is governed by a diffusion controlled process between 10 mV/s and 150 mV/s, in a quiescent solution.

2.4.6 Standard Curve for the oxidation of ochratoxin A

A standard curve was constructed to evaluate the sensitivity, accuracy and reproducibility of detection and therefore quantification of OTA in selected media. DPV was used to establish a standard curve in 0.2 M BR buffer pH 5 for increasing OTA concentrations between 2 and 30 μM . DPV was used as this method provides a negligible contribution of charging current to the background current thereby allowing for greater current sensitivity as compared to CV (Kissinger and Heinemann, 1996). The DPV's shown in Figure 2.4.6.1 depict the increase in peak current response for increasing concentrations of OTA. As shown in Figure 2.4.6.1, DPV produces a well defined current response for 2 μM OTA.

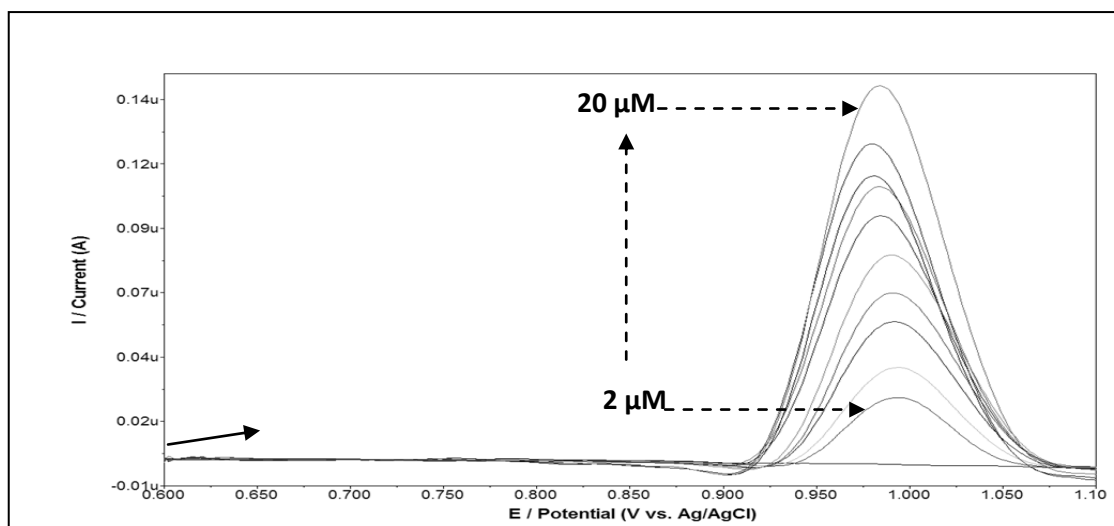


Figure 2.4.6.1 DPVs obtained in 0.2 M BR buffer pH 5 at an unmodified GCE for the oxidation of 2, 4, 6, 8, 10, 12, 14, 16, 18 and 20 μM OTA. Blank solution is shown by a dotted line. Scan rate 5 mV/s.

Figure 2.4.6.2 represents the standard curve of peak current magnitude vs. concentration of OTA obtained from DPV. Linearity in current response was observed for the concentration range of 2 μM to 20 μM in BR buffer pH 5 producing a corresponding correlation coefficient of 0.995. For concentrations above 20 μM , the standard deviation in current response increased to values over 20% effectively reducing the overall reproducibility of response. Thus, the concentration range of 2 μM to 20 μM was used to extrapolate the limit of detection (LOD) defined as:

$$LOD = \frac{3\delta}{S} \quad (2.4.6)$$

Where δ represents the standard deviation in baseline current and S the slope of the standard curve (Almeda, et. al 2009).

The LOD for OTA obtained from DPV in 0.2 M BR buffer pH 5 was calculated as 0.28 μM . Furthermore the limit of quantification (LOQ) for OTA determined as ten times the baseline current multiplied by the inverse of the standard curve slope was calculated as 0.93 μM . The use of BR buffer increased the LOD of OTA to 0.28 μM as compared to the LOD of 1.2 μM obtained by Oliveira et al, 2007 in phosphate buffer.

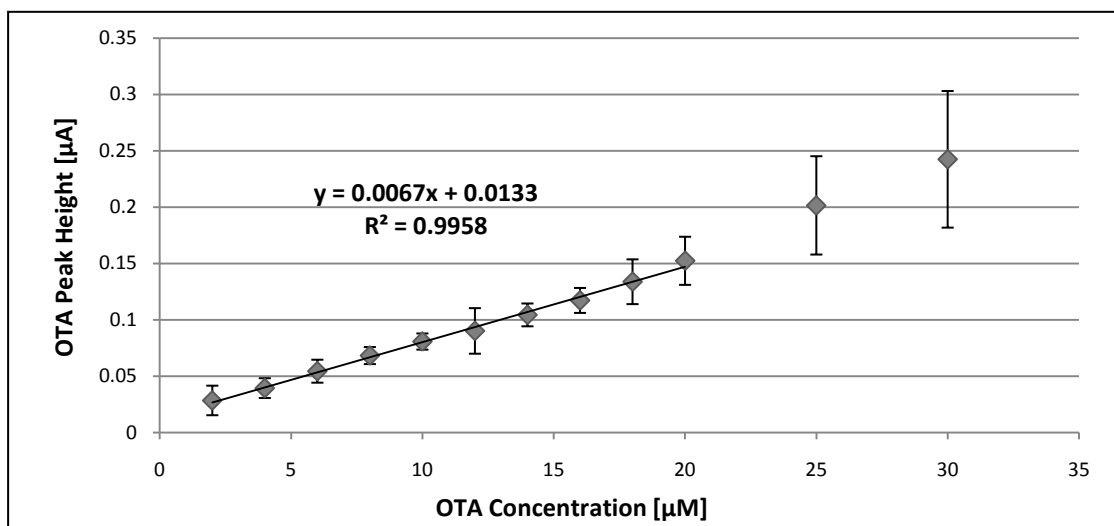


Figure 2.4.6.2 Standard curve obtained from DPV analysis of increasing concentrations of OTA ranging from 2 μM to 30 μM in 0.2 M BR buffer pH 5 at an unmodified GCE. Number of replicates (3). Error bars show standard deviation from mean.

2.4.7 Effective surface area determination for GCE

Ferricyanide redox species undergo a fast outer sphere one electron transfer reaction almost irrespective of the electrode material present and as a result is extensively used in literature for electrode characterisation (Eldin and Abdel-Hady, 2008). For outer sphere electron transfer, ferricyanide does not interact strongly with the electrode surface allowing for redox reactions to occur at a distance of at least a solvent layer from the electrode. This prevents electrode fouling as the concentration of ferricyanide ions at the electrode surface is essentially the same as in the bulk electrolyte solution. The electrochemical reduction of the ferricyanide ion to ferrocyanide is represented as:

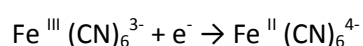


Figure 2.4.7.1 represents the CVs obtained for the oxidation of potassium ferricyanide/ferrocyanide in potassium chloride buffer pH 7 for scan rates ranging between 5 mV/s and 200mV/s.

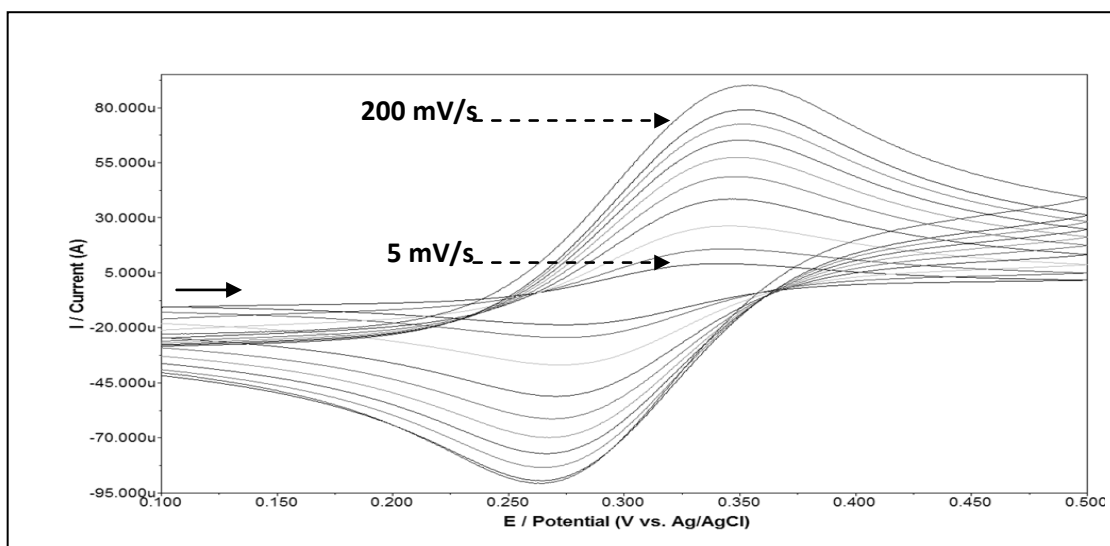


Figure 2.4.7.1 CV of 5 mM potassium ferricyanide/ferrocyanide in 1 M potassium chloride buffer pH 7 at an unmodified GCE measured at a scan rate of 5, 10, 25, 50, 75, 100, 125, 150, 175 and 200 mV/s.

As shown in Figure 2.4.7.1, an anodic peak potential of 0.34 V during the forward scan followed by a cathodic peak potential of 0.27 V during the return scan was observed for the ferricyanide redox system at a scan rate of 100 mV/s. The peak to peak separation in potential of 64 mV is indicative of a fully reversible process present for the redox reaction of ferricyanide. In addition, Figure 2.4.7.2 represents the linear correlation of peak oxidation current and square root of scan rate indicating a diffusion controlled redox process for the ferricyanide couple.

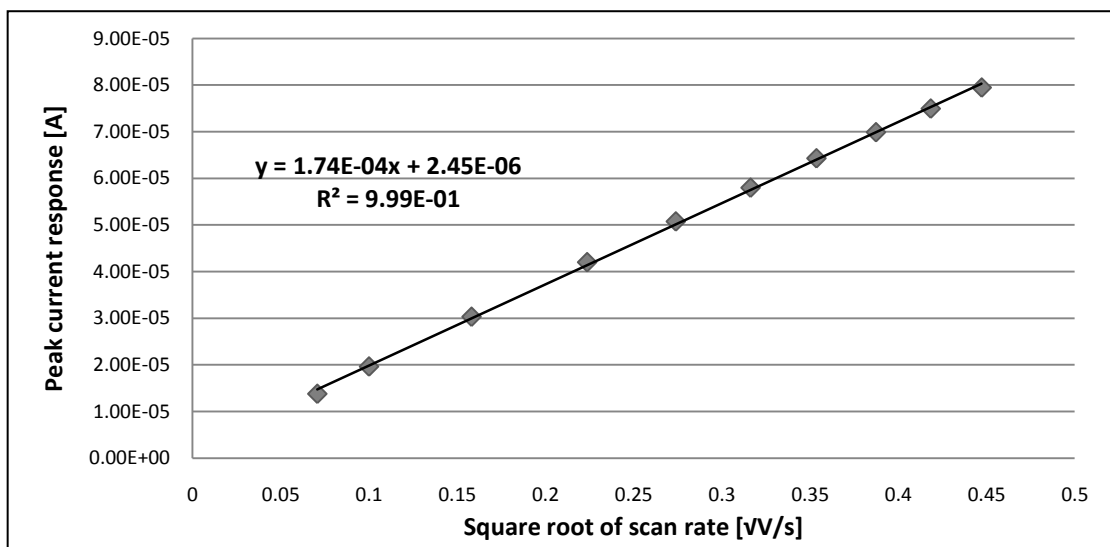


Figure 2.4.7.2 Linear plot of peak current amplitude vs. square root of scan rate obtained for CV analysis of 5 mM potassium ferricyanide in 1 M potassium chloride buffer pH 7 at increasing scan rates. Number of replicates (3). Error bars show standard deviation from mean.

For a diffusion controlled reversible system, the peak current magnitude is defined by the Randles-Sevcik equation:

$$I_p = 2.69 \cdot 10^5 n^{3/2} A D^{1/2} C v^{1/2} \quad (2.4.7.1)$$

Where I_p represents the peak current, n the total number of electrons transferred, A the electrode area, D the diffusion coefficient, C the concentration of ferricyanide and v the scan rate. According to Konopka and McDuffie, the diffusion coefficient for 5 mM potassium ferricyanide in 1 M potassium chloride is approximated as $7.26 \pm 0.11 \times 10^{-6}$.

Thus, calculation of the slope for the linear plot of peak oxidation current vs. the square root of scan rate allowed for the determination of effective surface area for the GCE as 0.048 cm^2 . Determination of the geometric surface area as 0.071 cm^2 allowed for the calculation of surface roughness following the equation:

$$S_R = \frac{A_E}{A_G} \quad (2.4.7.2)$$

Where S_R represents the surface roughness, A_E the effective surface area and A_G the geometric surface area (Jarzabek and Borkowska, 1997). The low value of surface roughness, determined as 0.674 au, was accredited to passivation of the electrode due to oxygen functional groups formed during the electrochemical pre-treatment of the electrode surface.

2.4.8 Tafel analysis of ochratoxin A

The Tafel equation relates the rate of electron transfer across the electrode-solution interphase as a function of overpotential and was first reported as an empirical equation in the form:

$$\eta = a + b \log j \quad (2.4.8.1)$$

Where η represents the overpotential, j the current density and “a” and “b” the characteristic constants of the electrode system (Bockris and Khan, 1993). For a diffusion controlled electrochemical process, a linear region of the η -log j relation is observed and referred to as the Tafel plot (Bockris and Khan, 1993). The slope of the Tafel plot (β) can be used to extract kinetic

parameters of the OTA oxidation mechanism, particularly the number of electrons involved in the rate limiting step of OTA oxidation (n_a) and the transfer coefficient (α) which is a measure of the symmetry of the energy barrier between OTA and the oxidised form of OTA.

In Butler-Volmer kinetics, the energy barrier relates to the potential required to either lower or raise the energy level of the reactant electron in order to favour the oxidation or reduction reaction. The oxidation energy barrier relates to the potential required to sufficiently lower the energy of the reactant electron allowing for the oxidation reaction to be favoured over reduction (Kissinger and Heinemann, 1996). Thus, the product $n_a \cdot \alpha$ acts as a measure of the irreversibility of the reaction and is obtained from the Tafel slope by the following equation:

$$\beta = 2.303 \frac{RT}{n_a \alpha F} \quad (2.4.8.2)$$

Where β represents the Tafel slope, R the Molar gas constant, T the temperature, n_a the number of electrons involved in the rate limiting step, α the transfer coefficient and F the Faraday constant (Bagotski, 2006). For an irreversible diffusion controlled system, the Tafel slope may be determined from the shift in peak oxidation potential upon increasing scan rate. Figure 2.4.8.1 illustrates the cyclic voltammograms for the oxidation of OTA in phosphate buffer pH 7 obtained at increasing scan rates of 5, 10, 25, 50, 75, 100, 150 and 200 mV/s. The increase in peak oxidation current with increasing scan rate is consistent with diffusion controlled mass transport of OTA to the electrode surface. Phosphate buffer pH 7 was selected for the Tafel analysis to allow for comparative analysis to work by Oliveira, et. al, 2007, regarding total electron transfer during the oxidation of OTA.

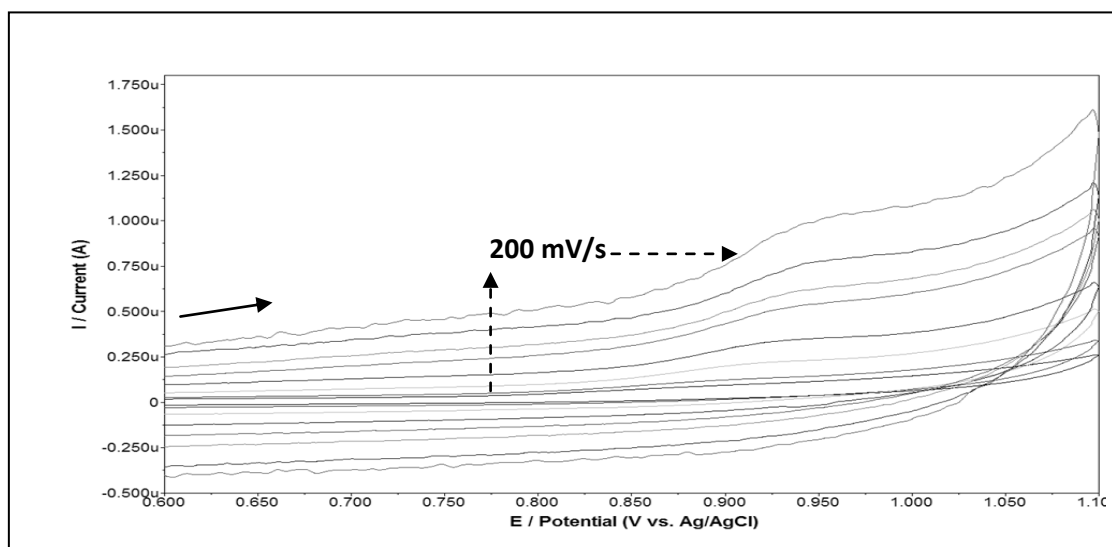


Figure 2.4.8.1 CVs obtained for 5 μ M OTA in 0.2 M phosphate buffer pH 7 at an unmodified GCE for scan rates of 5, 10, 25, 50, 75, 100, 150 and 200 mV/s.

The linear correlation between the shift in peak potential and the logarithmic of scan rate is illustrated in Figure 2.4.8.2 and follows the relation:

$$E_p = \frac{\beta}{2} \log v + \text{constant} \quad (2.4.8.3)$$

Where E_p represents the peak anodic potential (V), v represents the scan rate (V/s) and β represents the Tafel slope (Majidi, et. al, 2006).

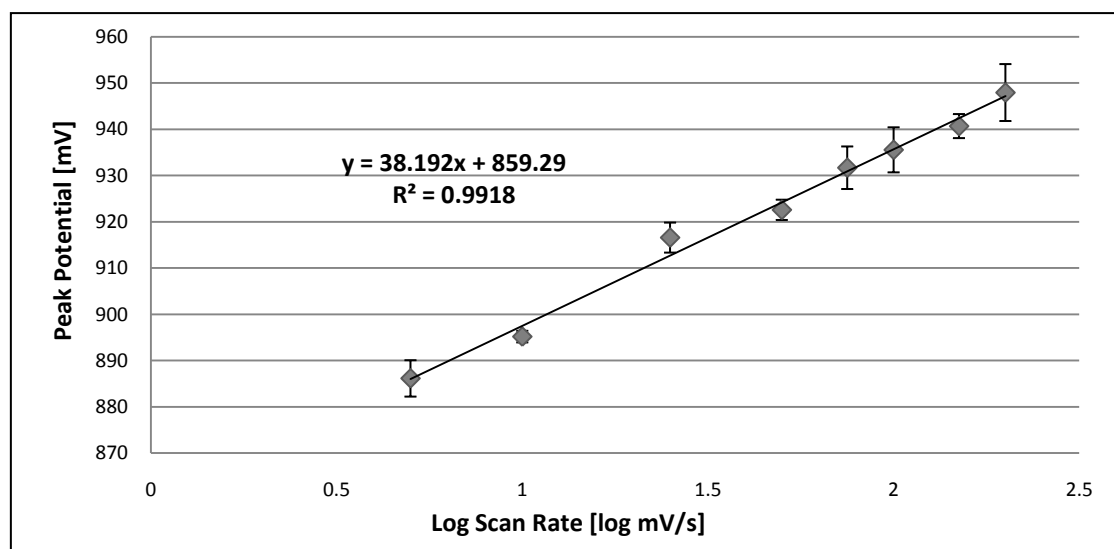


Figure 2.4.8.2 Plot of peak potential against the logarithmic of scan rate obtained for 5 μ M OTA in 0.2 M phosphate buffer pH 5 at an unmodified GCE for the scan rate range of 5 to 200 mV/s. Number of replicates (3). Error bars show standard deviation from mean.

From the plot of peak potential against the logarithmic of scan rate represented in Figure 2.4.8.2, a tafel slope of 76.38 mV/decade with standard deviation of 1.41 mV/decade was determined. A tafel slope of 77 mV/decade typically indicates an initial one electron transfer followed a by slow chemical step (Fletcher, 2009). The product $n_a \cdot \alpha$ for 5 μ M OTA in 0.2 M phosphate buffer pH 7 was calculated as 0.76 au with a standard deviation value of 0.03 au. As a verification of the $n_a \cdot \alpha$ product obtained for 5 μ M OTA in 0.2 M phosphate buffer pH 7, additional CV's of 5 μ M OTA in 0.2 M BR buffer of pH 1, pH 3 and pH 5 were constructed.

Figure 2.4.8.3 shows the plots of peak potential against the logarithmic of scan rate obtained for the oxidation of OTA in BR buffer between the range of pH 1 and pH 5, with corresponding Tafel slope and $n_a \cdot \alpha$ products listed in Table 2.4.8.1.

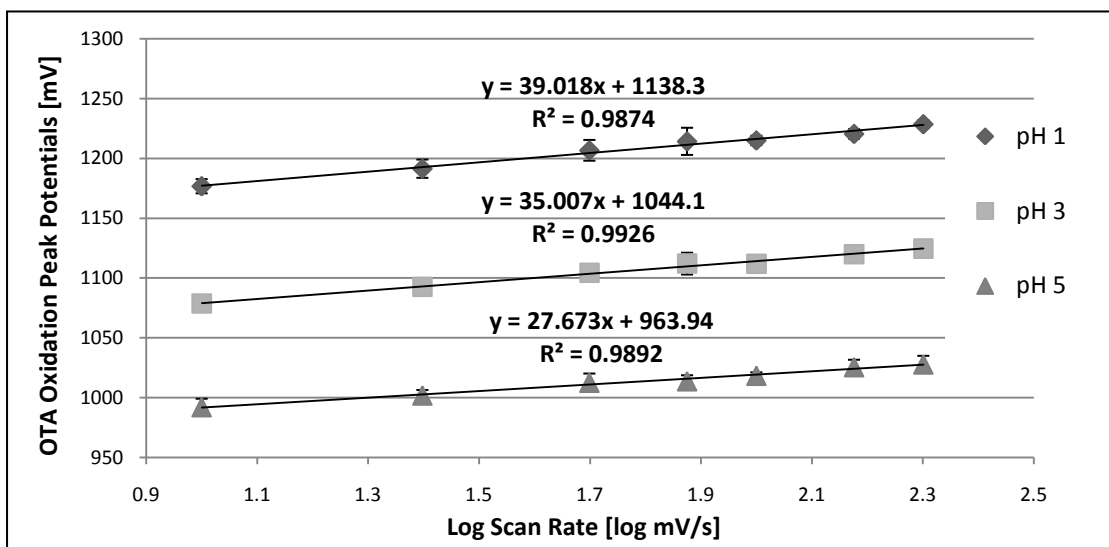


Figure 2.4.8.3 Plot of peak potential against the logarithmic of scan rate obtained for 5 μ M OTA at an unmodified GCE between the scan rate range of 5 to 200 mV/s in 0.2 M BR buffer of pH 1, pH 3, and pH 5. Number of replicates (3). Error bars show standard deviation from mean.

Table 2.4.8.1 summarizes the Tafel slope and $n_a \cdot \alpha$ values obtained from the peak potential against the logarithmic of scan rate plots of 5 μ M OTA in 0.2 M BR buffer of pH 1, pH 3, pH 5 and pH 6.

pH	Avg. Tafel Slope [mV/Decade]	St. Dev. Tafel Slope [mV/Decade]	Avg. $\alpha \cdot n_a$ Values [units]	St. Dev. $\alpha \cdot n_a$ Values [units]
1	78.037	3.948	0.748	0.038
3	70.013	2.702	0.834	0.032
5	55.346	5.535	1.055	0.049

The kinetic parameters of $n_a \cdot \alpha$ obtained for 0.2 M BR buffer range from 0.75 to 1.06 au with a mean value of 0.88 au which is comparable to the $n_a \cdot \alpha$ product obtained for 0.2M phosphate buffer pH 7. The variation of $n_a \cdot \alpha$ parameters across the acidic electrolyte pH range can be accredited to adsorption of OTQ/OTHQ species at the electrode surface during the oxidation of OTA (Corrigan and Evans, 1980).

2.4.9 Electron transfer mechanism of ochratoxin A

Evaluation of the Tafel slope parameters in section 2.4.8, enables the mechanism of electron transfer governing the electrochemical oxidation of OTA to be examined.

From Figure 2.4.8.1, the linear relation of peak current magnitude and square root of scan rate for the oxidation of OTA follows the Randles-Sevcik equation for a diffusion controlled irreversible redox reaction shown as (Bard, 1980):

$$I_p = 2.98 \cdot 10^5 n (\alpha n_a)^{1/2} A D^{1/2} C v^{1/2} \quad (2.4.9.1)$$

Where I_p represents the peak current, n 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, D the diffusion coefficient, C the concentration of OTA and v the scan rate. The kinetic parameter product of $\alpha \cdot n_a$ was calculated in section 2.4.8 from Tafel analysis as 0.76 ± 0.03 au for the equivalent buffer.

According to Oliveira et. al, 2007 the diffusion coefficient for OTA in 0.2 M phosphate buffer pH 7 was approximated as $3.65 \times 10^{-6} \text{ cm}^2 \text{ s}^{-1}$. From Figure 2.4.9.1, sufficient linearity was observed for the correlation between peak current magnitude and square root of scan rate as expressed by a linear correlation coefficient of 0.99. Based on the slope of the plot and by the Randles-Sevcik equation, the total number of electrons transferred was calculated as 2.21 ± 0.17 . Thus, from the peak current response as a function of OTA concentration, scan rate, and other factors as shown below in the Randles-Sevcik equation, a total of two electrons were involved in the oxidation of OTA in 0.2 M phosphate buffer of pH 7. The two electron oxidation mechanism of OTA to OTQ is consistent with the two electron oxidation of common phenolic species to respective quinone/hydroquinone couples through the formation of a phenoxonium cation intermediate (Calcutt, et. al, 2001).

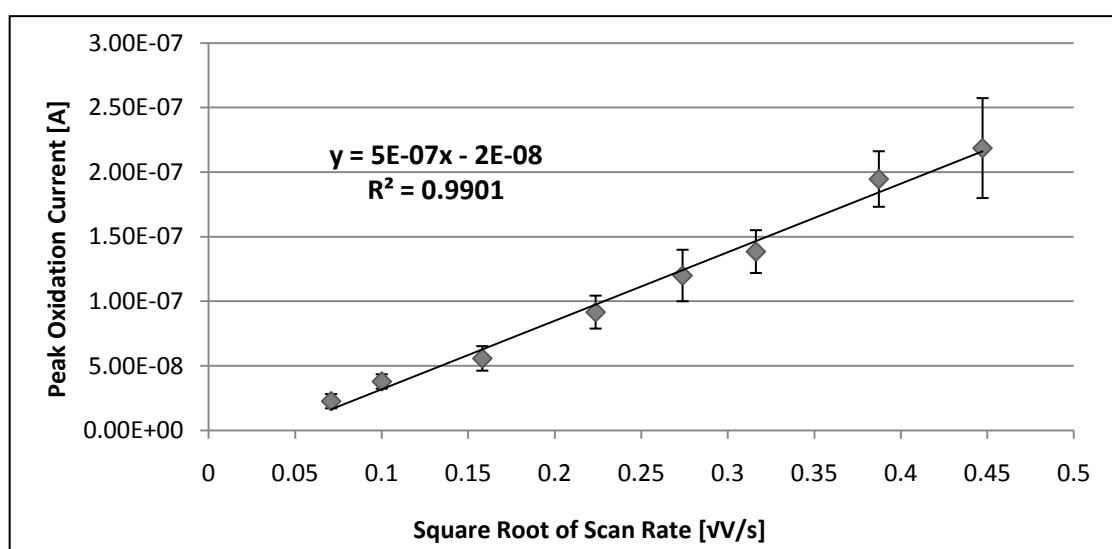


Figure 2.4.9.1 Linear plot of peak current amplitude vs. square root of scan rate obtained for CV analysis of 5 μM OTA in 0.2 M phosphate buffer pH 7 increasing scan rates. Number of replicates (3). Error bars show standard deviation from mean.

To further elucidate the mechanism of electron transfer, the number of electrons involved in the rate limiting step of OTA oxidation was evaluated as shown in Table 2.4.9.1 for 0.2 M BR Buffer between the range of pH 1 to pH 5.

Table 2.4.9.1 summarizes the values of n_a determined from Tafel analysis of 5 μ M OTA in 0.2 M BR buffer of pH 1, pH 3 and pH 5. Transfer coefficient values were assigned to the range of 0.3 to 0.7 au.

pH	α Value:	0.3	0.4	0.5	0.6	0.7
1	E_p vs. Log v	2.49	1.87	1.50	1.25	1.07
3	E_p vs. Log v	2.78	2.08	1.67	1.39	1.19
5	E_p vs. Log v	3.52	2.64	2.11	1.76	1.51

As reported in literature, the transfer coefficient is known to range between values of 0.3 and 0.7 au for several electrochemical reactions (Yoshimori, et. al, 1989). From the Tafel plot analysis in section 2.4.8, the transfer coefficient was approximated between the 0.3 and 0.7 au range in order to evaluate the number of electrons involved in the rate determining step toward oxidation of OTA in 0.2 M BR buffer between the acidic pH range of 1 and 5. Transfer coefficient values of below 0.5 au are typically confined to porous electrodes structures and is dependent on pore size, pore distribution and electrode conductivity (Soderberg, et. al, 2006).

As the glassy carbon electrode surface is a non porous structure highly impermeable to the electrolyte solution, a transfer coefficient tending toward values greater than 0.5 au was used to evaluate the rate determining step as a one electron process. Thus, the two electron oxidation process of OTA consists of two consecutive one electron transfers, as governed by a one electron rate determining step. The one electron rate determining step correlates to the initial one electron transfer followed by a slow chemical step as indicated by the Tafel slope of 77 mV/decade in section 2.4.8

2.4.10 Diffusion coefficient for ochratoxin A

To allow for the theoretical treatment of electrochemical systems, experiments are often designed so that diffusion is the sole form of mass transport. Even in cases where convection and migration occur, diffusion can rarely be ignored as a significant contributor to the overall mass transport of the redox system (Kissinger and Heinemann, 1996). Accordingly, knowledge of the diffusion coefficient for the electrochemical characterization of OTA in various electrolyte solutions is required. Thus, the

diffusion coefficient for OTA in BR buffer was determined since BR buffer was selected as the optimal supporting electrolyte for the detection of OTA. Similar studies were thus conducted for OTA in BR buffer as in section 2.4.9 for phosphate buffer.

The linear dependence of OTA oxidation current on the square root of scan rate, shown in Figure 2.4.10.1, follows the Randles-Sevcik relation for a diffusion controlled irreversible reaction stated in section 2.4.9 by equation 2.4.9.1 with the kinetic product $\alpha.n_a$ for 0.2 M BR buffer pH 5 calculated in section 2.4.7 as 1.055 au.

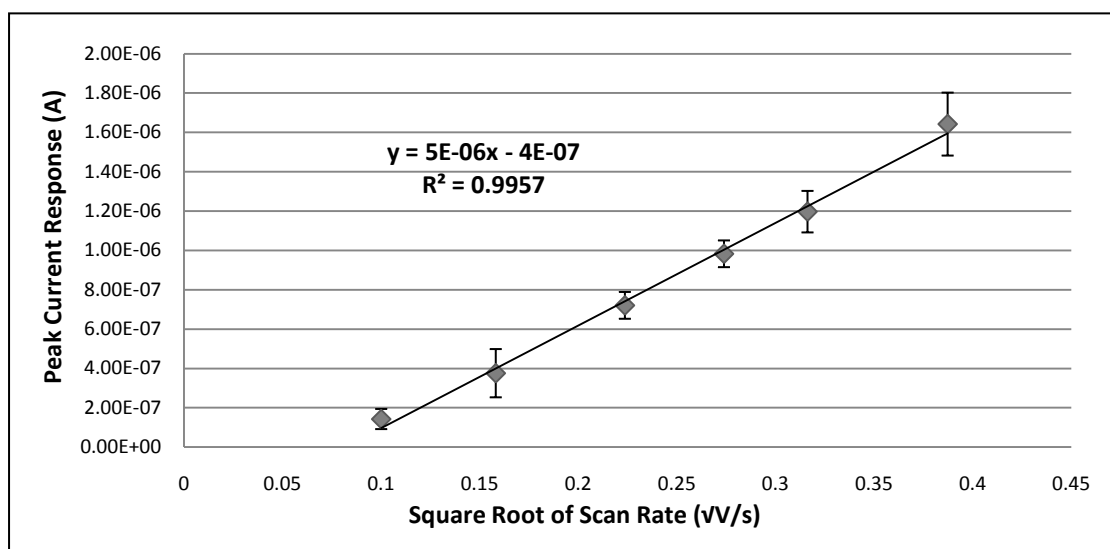


Figure 2.4.10.1 Linear plot of peak current amplitude vs. square root of scan rate obtained for CV analysis of 5 μ M OTA in 0.2 M BR buffer pH 5 increasing scan rates. Number of replicates (3). Error bars show standard deviation from mean.

From the plot in Figure 2.4.10.1, the Randles-Sevcik slope was determined from linear regression as $5.22 \pm 0.17 \times 10^{-6} \text{ A/(V/s)}^{1/2}$. From the measured slope, the diffusion coefficient of OTA in 0.2 M BR buffer pH 5 was determined as $1.25 \pm 0.27 \times 10^{-5} \text{ cm}^2/\text{s}$. The calculated diffusion coefficient for OTA in BR buffer is near three times the respective values of $3.65 \times 10^{-6} \text{ cm}^2/\text{s}$ and $0.48 \times 10^{-5} \text{ cm}^2/\text{s}$ reported by Oliveira et. al. and Calcutt et. al. for OTA in 0.2 M phosphate buffer pH7.

The deviation in diffusion coefficient from values reported in literature was accredited to an increase in the Randles-Sevcik slope produced from a more pronounced current response obtained for OTA in BR buffer as compared to that of phosphate buffer. Additional deviation may result from the second order dependence of the diffusion coefficient on peak current magnitude as any variability in the measured Randles-Sevcik slope would be amplified in the determination of diffusion coefficient from CV techniques.

2.4.11 Ochratoxin A oxidation mechanism: Correlation with 4-chlorophenol

To gain an understanding of the voltammetric behaviour of OTA, a correlation study between the dihydroisocoumarin and phenylalanine constituents of OTA was performed. The dihydroisocoumarin moiety was represented by the 4-chlorophenol species.

Figure 2.4.11.1 represents the successive CV cycles obtained for 50 μM 4-chlorophenol in 0.2 M BR buffer pH 5. CV of 4-chlorophenol in 0.2 M BR buffer pH 5 revealed a single anodic peak on the forward scan of the first cycle with a peak oxidation potential of 0.79 V at a scan rate of 100mV/s. The oxidation potential of the anodic peak was dependant on the concentration of 4-chlorophenol and on electrolyte pH.

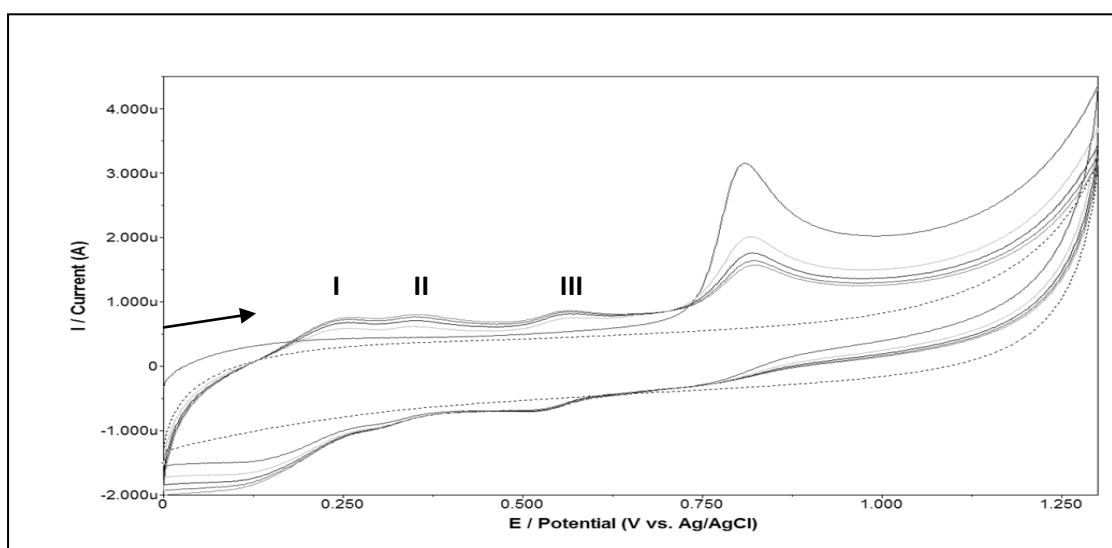


Figure 2.4.11.1 Successive CVs of 50 μM 4-chlorophenol in 0.2 M BR buffer pH 5 at an unmodified GCE. No regeneration of the electrode surface was done between successive scans. Scan rate 100 mV/s.

On the reverse scan, three cathodic peaks are observed for the 4-chlorophenol oxidation products at reduction potentials of 0.17V, 0.33V and 0.54 V respectively. The small peak current magnitude of the cathodic peaks as compared to the anodic peak coupled with a peak potential separation greater than 59 mV between the anodic peak and cathodic peaks is consistent with an irreversible redox system.

On the forward scan of the subsequent CV cycle, additional anodic peaks were observed for the 4-chlorophenol oxidation products at respective anodic potentials of 0.23 V, 0.34 V and 0.56 V corresponding to the formation of three distinct reversible redox couples.

The reversible couples occurring at the anodic potentials of 0.23 V, 0.34 V and 0.56 V were labelled as the primary (I), secondary (II) and tertiary (III) oxidation couples respectively. Similar peaks were observed by Calcutt, et. al, 2001 during the oxidation of 4-chlorophenol in acetonitrile solution and were identified as a benzoquinone (BQ)/benzohydroquinone (BHQ) couple. Thus, oxidation of OTA and 4-chlorophenol results in the formation of their respective quinone/hydroquinone species.

The high degree of reversibility of the primary and secondary couples is illustrated by the peak separation differences of 20 mV and 10 mV respectively, while a peak potential difference of 60 mV exhibited by the tertiary couple indicates slightly slower electron transfer rates.

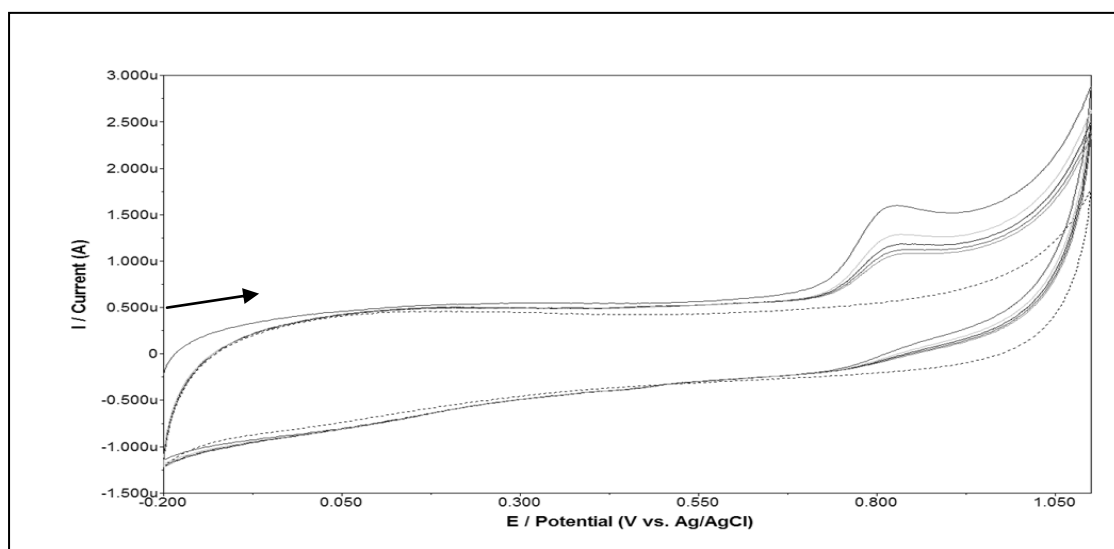


Figure 2.4.11.2 Successive CVs of 50 mM phenylalanine in 0.2 M BR buffer pH 5 at an unmodified GCE. No regeneration of the electrode surface was done between successive scans. Scan rate 100 mV/s.

Figure 2.4.11.2 illustrates the CV analysis of the phenylalanine constituent of OTA in BR buffer pH 5. Due to the partial electroactivity of phenylalanine, a tenfold increase in concentration of phenylalanine was used, as compared to 4-chlorophenol, to allow for a comparative analysis. The forward scan of the first CV cycle of phenylalanine produced a single anodic peak at 0.82 V with no cathodic peaks observed on the return scan. No additional peaks were observed other than the phenylalanine oxidation peak upon subsequent CV cycles.

Fouling of the electrode surface upon successive CV scans results in the decrease in peak current magnitude for the 4-chlorophenol anodic peak, as shown in Figure 2.4.11.1. After five successive scans in 0.2 M BR buffer pH 5, the extent of electrode fouling is shown by the 77 % reduction in 4-chlorophenol peak current magnitude. Partial fouling was observed for the phenylalanine anodic peak as a 55 % reduction in peak current magnitude upon 5 successive CV cycles.

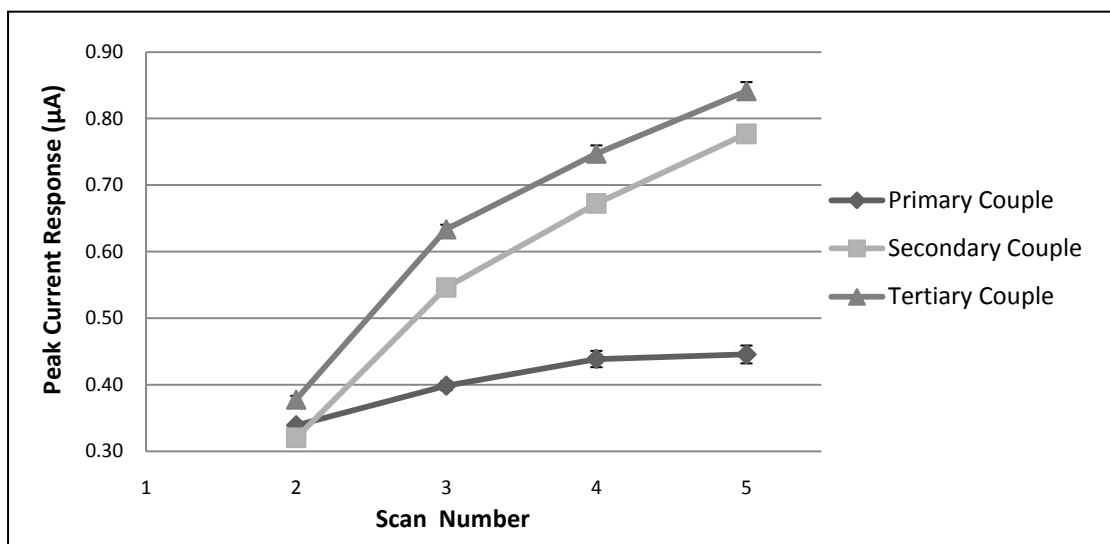


Figure 2.4.11.3 Fouling characteristics of the primary (0.23 V), secondary (0.34 V) and tertiary (0.56 V) couples produced through successive CV scans for 50 μM 4-chlorophenol in 0.2 M BR buffer pH 5. Number of replicates (3). Error bars show standard deviation from mean. Scan rate 100 mV/s.

Fouling of the electrode occurs through the adsorption of oxidised 4-chlorophenol to the electrode surface whereby both the formation and redox cycling of oxidised product contributes to electrode passivation. As shown in Figure 2.4.11.3, the increase in peak current magnitude for adsorbed species indicates the accumulation of oxidised product on the electrode surface upon successive CV cycles.

The cyclic voltammetric behaviour of 4-chlorophenol and phenylalanine is similar to that observed for OTA in terms of electrochemically irreversible reactions and high anodic potentials required for oxidation. In both redox species, the oxidation of 4-chlorophenol and phenylalanine is observed at a 200 mV less anodic potential than that of OTA oxidation in 0.2 M BR buffer pH 5. Similar fouling profiles are observed for 4-chlorophenol and OTA as both redox species produced oxidation products which accumulate as a film on the electrode resulting in surface passivation. The oxidation of OTA appears to foul the electrode surface to a greater extent than that of 4-chlorophenol which may be due to the adsorption of an electrochemically inactive species formed through the oxidation of OTCT. Two additional reversible couples are observed for the oxidation of 4-chlorophenol as compared to OTA in 0.2 M BR buffer.

The additional couples are attributed to oxidation of 4-chlorophenol to benzoquinone or 4-chlorocatechol which in turn oxidise through phenolic ring opening to chloromaleic acid or malic acid respectively (Johnson, et. al, 1999). Malic acid either oxidises to acetic acid or forms a reversible couple with succinic acid while oxidation of chloromaleic acid is likely to produce acetic acid

(Johnson, et. al, 1999). Hence, the decrease in fouling observed for 4-chlorophenol compared to OTA may be due to the eventual electrolysis of 4-chlorophenol to low molecular weight carboxylic acid species which diffuse from the electrode surface to the bulk electrolyte solution.

From the above results, the phenylalanine moiety was not considered as the electroactive constituent of OTA due to the partial electroactivity and absence of electroactive oxidation products present for the phenylalanine species. The similarity in voltammetric behaviour between OTA and 4-chlorophenol indicates that the electroactivity of OTA is considered to originate from the 4-chlorophenol species of the OTA dihydroisocoumarin moiety.

2.4.12 Ochratoxin A oxidation mechanism: pH profile of 4-chlorophenol

To further correlate the electrochemical behaviour of OTA with 4-chlorophenol, the effect of pH on the electrochemical oxidation of 4-chlorophenol was evaluated in 0.2 M BR buffer between the pH range of 2 to 10.

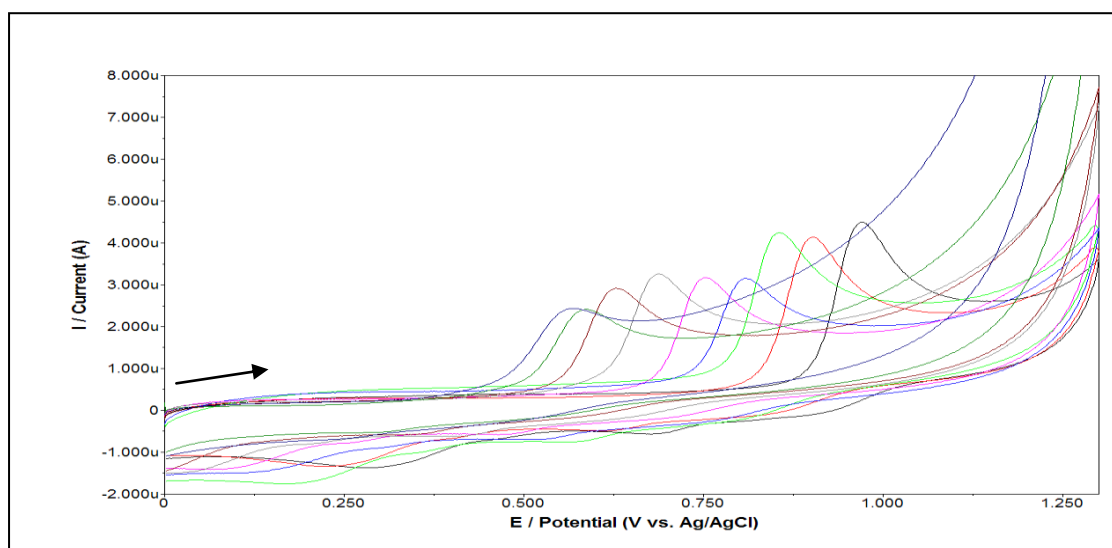


Figure 2.4.12.1 CVs of 50 μ M 4-chlorophenol at an unmodified GCE in 0.2 M BR buffer of pH 2 (black), pH 3 (light red), pH 4 (light green), pH 5 (light blue), pH 6 (light magenta), pH 7 (grey), pH 8 (red), pH 9 (green) and pH 10 (blue). Scan rate 100 mV/s.

As shown in Figure 2.4.12.1, the magnitude of peak oxidation current for 4-chlorophenol is observed to decrease with increasing alkalinity. An average peak current maxima of 2.90 μ A was obtained for 4-chlorophenol under acidic conditions between pH 2 and pH 4. An increase in pH toward less acidic conditions resulted in a 30% decrease in current magnitude between pH 4 and pH 5. Toward more alkaline conditions from pH 6 to pH 11, a further 50% decrease in current response was noted.

Figure 2.4.12.2 illustrates the linear shift in oxidation peak potential toward less anodic potentials upon increasing pH. The shift in potential follows the relationship $E_p = -0.052 \text{ pH} + 1.063$ with a correlation coefficient of 0.993 across the pH range tested. The slope of peak potential to pH correlates to an oxidation mechanism of 4-chlorophenol involving the transfer of an equal number of electrons to protons (Calcutt, et. al, 2001).

Similar to the pH profile observed for OTA, the current amplitude for 4-chlorophenol is greatest toward the more acidic pH range. However, a gradual decrease in peak current amplitude was observed for 4-chlorophenol with increasing alkalinity dissimilar to the rapid decrease in current amplitude observed for OTA at physiological pH.

The slope of peak potential to pH was consistent throughout the pH range of 2 to 10 showing that the mechanism of oxidation remained consistent. This is different from OTA as the peak potential of OTA was only dependant on pH between the pH range of 2 to 7. The difference in oxidation potential between 4-chlorophenol and OTA was accredited to the dissimilar pKa values between OTA and 4-chlorophenol reported as 7.0 and 9.4 at 25 °C respectively (Xiao, et. al, 1996).

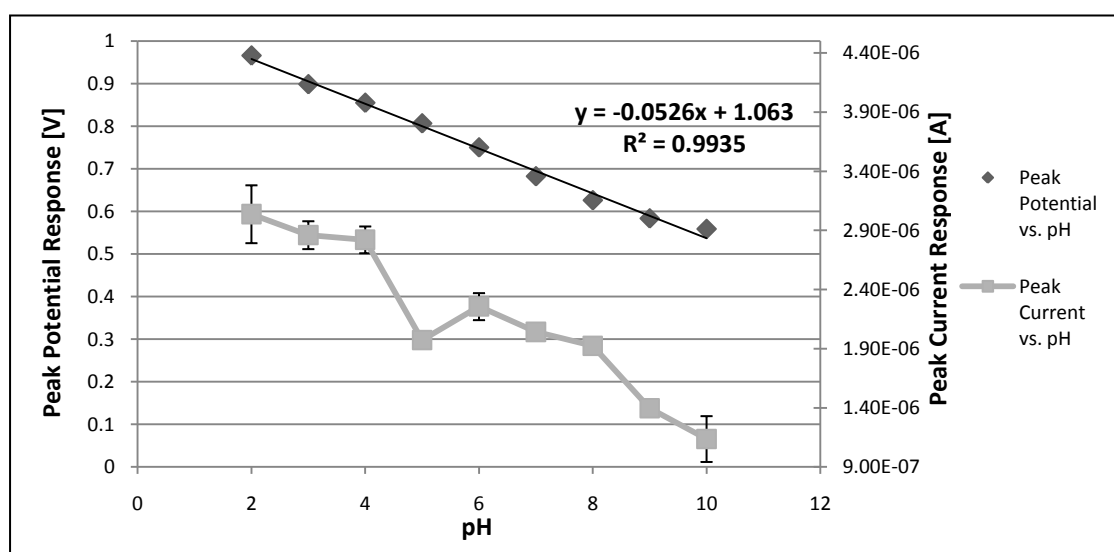


Figure 2.4.12.2 Initial current and potential response of 50 μM 4-chlorophenol in 0.2M BR buffer as a function of pH. Number of replicates (3). Error bars show standard deviation from mean. Scan rate 100 mV/s.

2.5 CONCLUSIONS

The aforementioned chapter proves the potential of voltammetric methods as a means for the sensitive, accurate and less costly detection of OTA as supported by findings previously reported by Oliveira et al, 2007. With regard to work published by Oliveira et al, 2007 further information was provided on the detection of OTA through the use of BR buffer as an optimal supporting electrolyte. BR buffer was shown to provide a wider potential range with a broad pH spectrum toward the detection of OTA. Although the use of BR buffer increased the LOD of OTA to 0.28 μM as compared to the LOD of 1.2 μM obtained by Oliveira et al, 2007 in phosphate buffer, additional improvements in detection sensitivity are required in order to meet the regulatory limit of OTA set as 0.01 μM (5 $\mu\text{g/kg}$) in human food (FAO, 2003).

Analysis of OTA in BR buffer throughout the pH range of 2 to 11 showed a maximum current response at pH 5, followed by a rapid decrease in sensitivity toward more alkaline pH. Although a greater sensitivity in detection of OTA was obtained in acidic media, significant passivation of the electrode surface was shown to occur upon successive detection cycles. Fouling of the electrode was accredited to adsorption of OTQ/OTHQ species as supported by similar results obtained by Calcutt et al, 2001. CV analysis showed a decrease in adsorption of oxidation products in alkaline media, which may be exploited as a means of surface regeneration during consecutive electrochemical analysis of OTA.

Tafel analysis was used to evaluate the electron transfer mechanism governing the oxidation of OTA in acidic media, approximated as a two electron and two proton transfer process. The mechanism of electron transfer was found to be governed by a one electron rate determining step which may indicate the formation of a phenoxonium cation intermediate prior to the formation of OTQ. Evaluation of the mechanism of electron transfer enabled the determination of a diffusion coefficient of $1.25 \pm 0.27 \times 10^{-5} \text{ cm}^2/\text{s}$ for the oxidation of OTA in BR buffer pH 5.

In addition, a comparison between the voltammetric behaviour between OTA and 4-chlorophenol was made. The similarity in electron transfer kinetics and fouling characteristics between the two species suggests that the electroactive nature of OTA originates from the dihydroisocoumarin moiety and more specifically from the oxidation of the 4-chlorophenol substituent of the dihydroisocoumarin moiety of OTA. This is of significant importance to biosensor design as

biotransformation of the dihydroisocoumarin moiety could be used as a potential means of achieving selectivity in the detection of OTA.

CHAPTER 3

DETECTION OF OCHRATOXIN A AT METALLOPHTHALOCYANINE MODIFIED ELECTRODES

3.1 INTRODUCTION

The electrochemical detection of organic compounds including amines, alcohols, phenols, ketones, thiols and organic halides is typically limited by slow electron transfer rates at conventional electrodes and often complicated by irreversible passivation of the electrode surface.

In order to overcome these limitations, a wide range of electrode pretreatment and modification approaches have been used to enhance electron transfer kinetics and to minimize fouling of the electrode surface upon successive detection cycles (Wang, 1991). Chemical modification of electrode surfaces with macrocyclic compounds such as porphyrins, phthalocyanines and metallophthalocyanines has been shown to exhibit substantial electrocatalytic activity toward a wide variety of redox processes (Vasudevan, et. al, 1998). The electrocatalysis of MPc complexes, characterised by a shift in potential to lower values and or enhanced detection currents, is afforded by the mediation in electron transfer between the electrode surface and the MPc metal centre or peripheral ring structure (Mafatle and Nyokong, 1997). Furthermore, MPc modification is reported to decrease electrode fouling characteristics as a result of added steric hindrance to oxidation products at the electrode surface (Ureta-Zanarta, et. al, 2003).

Although limited research is available regarding the electrochemical detection of OTA, the chemical literature contains numerous reports on the voltammetric detection of 4-chlorophenol previously identified as the electroactive substituent of OTA in section 2.11 and section 2.12. Chlorophenols constitute a major class of organic pollutants which readily contaminate the ecosystem and bio-accumulate in the food chain (Johnson, et. al, 1999). It is for this reason that highly sensitive and robust means of environmental monitoring is required, often leading to the use of electrochemical techniques. As detection of chlorophenols at unmodified electrodes is complicated by the high degree of electrode fouling, the use of chemical modification of electrodes has been investigated to enhance the sensitivity and reproducibility of phenolic detection (Rodgers, et. al, 1999) (Pigani, et. al, 2007).

In particular, MPc modification has been shown to reduce surface passivation and act as electrocatalysts toward the oxidation of phenols and chlorophenols. As reported by Mafatle and Nyokong, 1997, modification of a GCE with CoPc increased both the sensitivity and stability of the modified GCE toward the detection of phenol, 2-chlorophenol and 4-chlorophenol. Obirai, et. al, 2004 reported a lower oxidation potential and enhanced oxidation current for the detection of 4-chlorophenol at a poly-NiTPhPyPc modified vitreous carbon electrode. Peeters, et. al, 2007 investigated the oxidation of 4-chlorophenol at a CoTSPc and CuTSPc modified gold electrode showing a resultant decrease in surface fouling characteristics.

Hence, the similarity in voltammetric behaviour between OTA and 4-chlorophenol coupled to the electrocatalytic activity of MPc complexes toward the electrochemical detection of chlorophenols posed the use of MPc modification as a means to enhance sensitivity and improved fouling characteristics observed during the electrochemical detection of OTA.

3.2 AIMS

The purpose of this section is to enhance the detection of OTA through the chemical modification of MPc complexes to the electrode surface. To achieve the overall outcome, the following aims were addressed:

Evaluation of the voltammetric behaviour of MPc complexes in the selected supporting electrolyte followed by the identification of central metal and ring based redox processes which may be catalytic toward the oxidation of OTA.

Determination of a potential mechanism governing the oxidation of OTA at MPc modified electrodes with particular reference to the established oxidation of 4-chlorophenol at CoPc modified electrodes.

Comparison of various MPc modifiers in terms of sensitivity, reproducibility and electrocatalytic activity toward the detection of OTA. Further evaluation of the fouling characteristics of OTA at various MPc modified electrodes in terms of electrode stability and resistance to fouling upon successive OTA detection cycles.

3.3 METHODOLOGY

3.3.1 Instrumentation

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

3.3.2 Reagents

The MPc complexes of Cobalt(II) phthalocyanine (97 %, Sigma Aldrich), Magnesium(II) phthalocyanine (85 % Sigma Aldrich), Nickel(II) phthalocyanine (85 %, Sigma Aldrich), manganese(II) phthalocyanine (85 %, Sigma Aldrich), zinc(II) phthalocyanine (97 %, Sigma Aldrich), cobalt tetracarboxylated phthalocyanine (Nyokong, Rhodes University), cobalt octocarboxylated phthalocyanine (Nyokong, Rhodes University) and 29H, 32H-phthalocyanine (98 %, Sigma Aldrich) were evaluated for electrocatalysis toward the detection of OTA.

MPc modification to GCE

MPc stock solutions of 1 mg/ml were prepared in dimethylformamide (DMF) (Merck) and dispersed by sonication for a period of 15 minutes. Physical adsorption of the dispersed MPc solutions was performed by drop coating the GCE surface with 10 μ l of the MPc solution followed by drying at 70 °C for a period of 30 minutes. Once dried, the modified electrode was rinsed with DMF followed by thorough rinsing with milliQ water to remove any residual DMF. Chemical modification of the GCE with MPc complexes was repeated prior to each electrochemical analysis. GCE cleaning and polishing, as outlined in chapter 2, was performed prior to each chemical modification step.

3.3.3 Methodologies

3.4.1 Electrochemical characterization of MPc complexes

CVs of 1 mg/ml MgPc modified GCE in 0.2 M BR buffer pH 5 was assessed as outlined in section 2.3 with the exception that the modified GCE surface was not cleaned between subsequent CV scans.

3.4.2 Electrochemical detection of OTA by MPc modified electrodes

CV analysis of 5 μ M OTA in 0.2 M BR buffer pH 5 at a 1 mg/ml CoPc modified GCE and compared to equivalent analysis at a bare GCE.

3.4.3 OTA electrocatalysis comparison of metallophthalocyanines

CVs of 5 μ M OTA in 0.2 M BR buffer pH 5 were evaluated for bare GCEs and GCEs modified with 1 mg/ml of CoPc, MgPc, NiPc, CuPc, ZnPc, CoTCPC, CoOCPc and unmetallated Pc complexes.

3.4.4 MPc modified GCE fouling profile

Successive CVs of 5 μ M OTA in 0.2 M BR buffer pH 5 were evaluated for GCEs modified with 1 mg/ml of CoPc, MgPc, NiPc, CuPc, ZnPc, CoTCPC, CoOCPc and unmetallated Pc complexes as outlined in section 2.3 with the exception that the modified GCEs were not cleaned between subsequent CV scans.

3.4 RESULTS AND DISCUSSION

3.4.1 Electrochemical characterization of MPc complexes

The voltammetric behaviour of MPc layers modified at the surface of a GCE was assessed by CV in 0.2 M BR buffer. The voltammograms obtained for the CoPc modified GCE was selected to illustrate the redox behaviour of an MPc modified electrode.

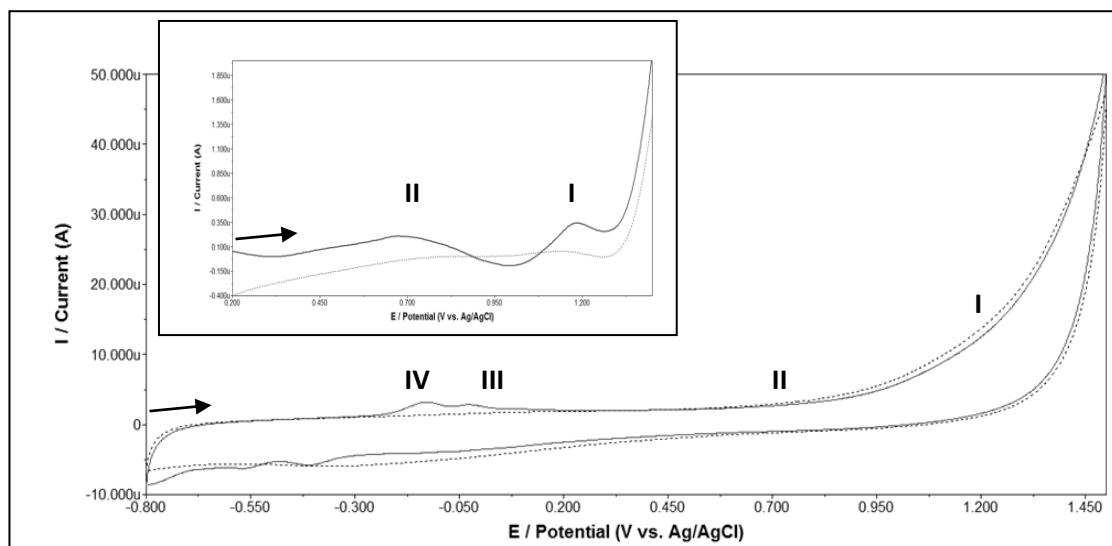


Figure 3.4.1.1 CV of 1 mg/ml CoPc modified GCE in 0.2 M BR buffer pH 5. Response of unmodified GCE shown by dotted line. Insert shows the exponential baseline correction curves obtained for the CoPc modified GCE initial CV scan (solid line) and the unmodified GCE CV scan (dotted line) between the potentials 0.2 V and 1.3 V. Scan Rate 100 mV/s.

Four redox processes were identified for the CoPc modified GCE, with corresponding anodic redox peaks labelled as I, II, III and IV at the respective potentials of 1.18 V, 0.71 V, -0.02V and -0.14 V.

Based on the electrochemical mechanism of MPc complexes in aqueous media, the redox process labelled as I and II are assigned to the redox reactions $M^{3+}Pc^{1-}/M^{3+}Pc^{2-}$ and $M^{3+}Pc^{2-}/M^{2+}Pc^{2-}$ respectively (Leznoff and Lever, 1993). Toward more cathodic potentials the redox processes III and IV are assigned to the redox reactions $M^{2+}Pc^{2-}/M^{1+}Pc^{2-}$ and $M^{1+}Pc^{2-}/M^{1+}Pc^{3-}$. (Leznoff and Lever, 1993). Consequently, the redox reactions I and IV correspond to oxidation of the peripheral CoPc ring structure while reactions II and III correspond to oxidation of the transition metal substituent of the CoPc layer modified to the GCE surface.

As shown in Figure 3.4.1.1, cathodic peak potentials of -0.41 V and -0.57 V were observed for the redox reactions III and IV on the return CV scan. The peak separation distance of 0.39 V and 0.43 V

observed for the $\text{Co}^{2+}\text{Pc}^{2-}/\text{Co}^{1+}\text{Pc}^{2-}$ and $\text{Co}^{1+}\text{Pc}^{2-}/\text{Co}^{1+}\text{Pc}^{3-}$ reactions is indicative of an electrochemically reversible reaction with fast electron transfer kinetics. No corresponding cathodic peaks were observed for the $\text{Co}^{3+}\text{Pc}^{1-}/\text{Co}^{3+}\text{Pc}^{2-}$ and $\text{Co}^{3+}\text{Pc}^{2-}/\text{Co}^{2+}\text{Pc}^{2-}$ reactions on the return scan indicating an irreversible redox process governed by slow electron transfer.

Furthermore, successive CV cycles were shown to reduce the peak current magnitude for both redox couples I and II. The decrease in peak current magnitude is typical to ring oxidation and is accredited to decomposition of the peripheral CoPc ring structure during electrochemical oxidation as confirmed by the irreversible nature of reaction II (Koca, et. al, 2006).

3.4.2 Electrochemical detection of OTA by MPc modified electrodes

The CoPc modified GCE was selected to illustrate the mechanism for the catalytic oxidation of OTA by MPc modified electrodes. Figure 3.4.2.1 shows the oxidation of OTA by a CoPc modified GCE as compared to an unmodified GCE in BR buffer pH 5. Dotted lines represent baseline CV scans for CoPc modified and unmodified GCE obtained after 10 successive CV cycles in 0.2 M BR buffer pH 5.

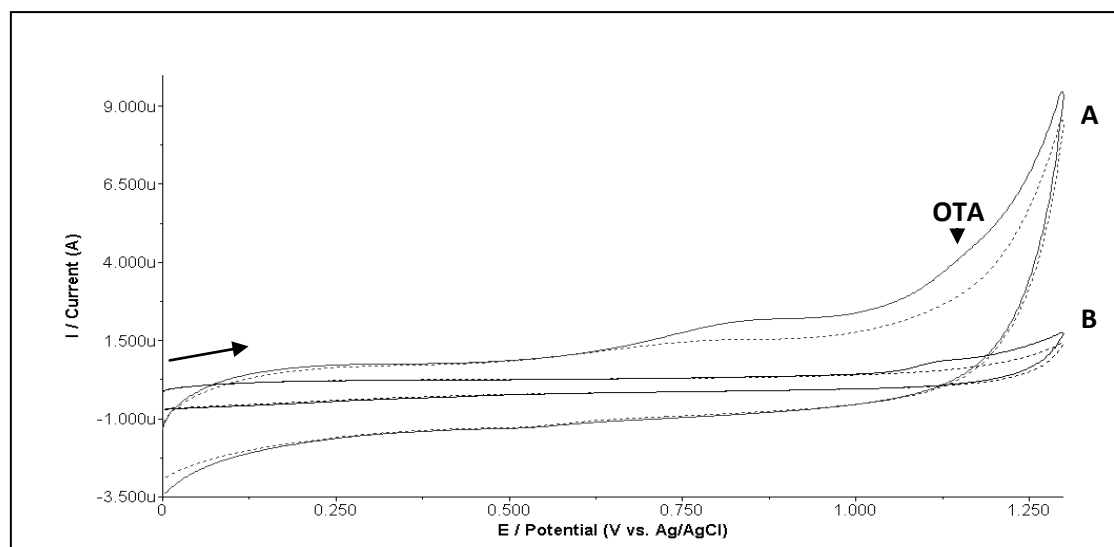


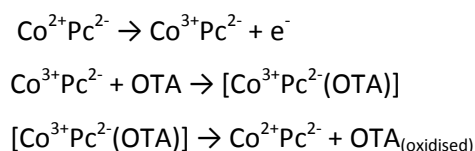
Figure 3.4.2.1 CV of 5 μM OTA in 0.2 M BR buffer pH 5 at a 1 mg/ml CoPc modified (A) and unmodified GCE (B). CVs of blank solutions for 1 mg/ml CoPc modified and unmodified shown as dotted lines. Scan rate 100 mV/s.

Oxidation of 5 μM OTA at the CoPc modified GCE was observed at an anodic peak potential of 1.19 V which is near 50 mV more positive than oxidation of OTA observed for the unmodified GCE at a peak potential of 1.14 V. The shift in oxidation potential toward more anodic potentials may reflect the formation of adducts between solution phase OTA and the adsorbed CoPc species.

An average peak current magnitude of $0.71 \pm 0.33 \mu\text{A}$ was obtained for oxidation of OTA at the CoPc modified GCE, showing a two fold increase in sensitivity as compared to the unmodified GCE. Compared to the voltammetric analysis of OTA in section 2.1, a decrease in the current response for OTA oxidation was noted for the unmodified GCE and accredited to differences in electrode conditioning.

Due to the similarity in the voltammetric behaviour between OTA and 4-chlorophenol, as shown in section 2.11, the mechanism of oxidation for OTA at a CoPc modified GCE is thought to correspond to the oxidation of 4-chlorophenol at an equivalent electrode. Thus, in order to account for the increase in sensitivity, the oxidation of OTA was compared to that of 4-chlorophenol at a substituted CoPc modified electrode as described by Peeters et al, 2007.

The oxidation of 4-chlorophenol by the substituted CoPc modified electrode is stated to involve the $\text{Co}^{3+}\text{Pc}^{2-}/\text{Co}^{2+}\text{Pc}^{2-}$ redox couple. Since the oxidation potential of the $\text{Co}^{3+}\text{Pc}^{2-}/\text{Co}^{2+}\text{Pc}^{2-}$ couple is observed at 0.71 V which is lower than the oxidation potential of OTA at the CoPc modified GCE, then oxidation of the $\text{Co}^{2+}\text{Pc}^{2-}$ to $\text{Co}^{3+}\text{Pc}^{2-}$ should have occurred before that of OTA. Thus, the suggested mechanism through which the electrocatalytic oxidation of OTA operates at the CoPc modified GCE may be represented as:



Whereby, electrochemical oxidation of $\text{Co}^{2+}\text{Pc}^{2-}$ to $\text{Co}^{3+}\text{Pc}^{2-}$ allows for formation of the $\text{OTA-Co}^{3+}\text{Pc}^{2-}$ complex. Formation of the $\text{OTA-Co}^{3+}\text{Pc}^{2-}$ complex assists in electron transfer to the electrode surface followed by a subsequent regeneration of the $\text{Co}^{2+}\text{Pc}^{2-}$ species. The increase in electron transfer results in an increase in sensitivity of the modified GCE toward the detection of OTA.

The increase in sensitivity by modification of the GCE with CoPc species came at the expense of a marked increase in standard deviation for OTA current response which was observed to increase from 5 % for the unmodified GCE to 46 % for the CoPc modified GCE. The increase in standard deviation in current response was attributed to an uneven distribution of the CoPc structures on the GCE surface due to the use of physical adsorption as the primary method of electrode modification (Telesa and Fonseca, 2008).

3.4.3 OTA electrocatalysis comparison of metallophthalocyanines

Various MPc complexes modified to a GCE were assessed for electrocatalytic properties toward the oxidation of OTA in 0.2 M BR buffer of pH 5. The GCE was modified with equivalent amounts of CoPc, MgPc, NiPc, CuPc, ZnPc, CoTCPC, CoOCPc and unmetallated Pc structures.

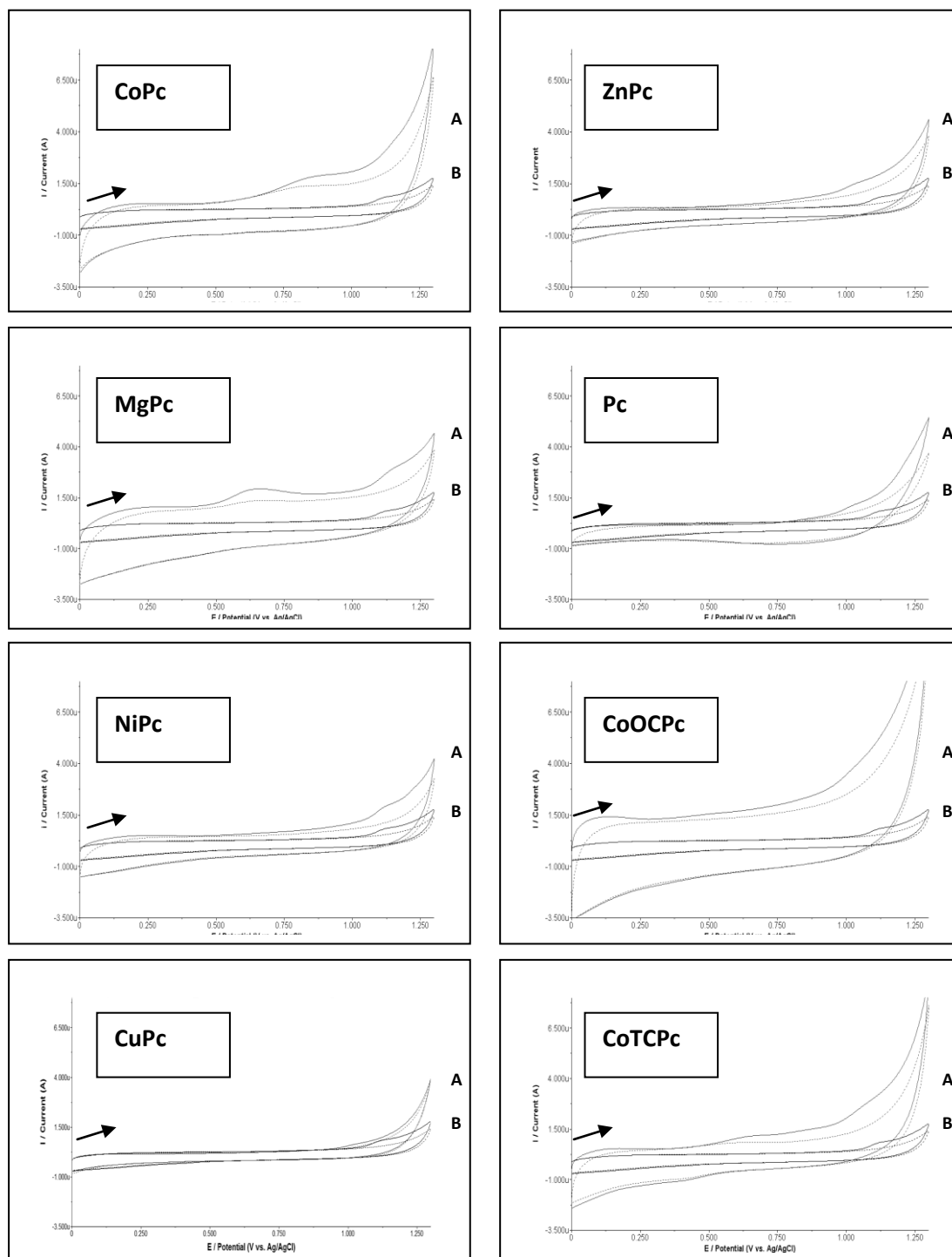


Figure 3.4.3.1 illustrates the CVs obtained for the oxidation of 5 μ M OTA in 0.2 M BR buffer pH 5 at various 1 mg/ml MPc modified GCE (A) as compared to an unmodified GCE (B). CVs of blank solutions for MPc modified and unmodified shown as dotted lines. Scan rate 100 mV/s.

As shown by the baseline voltammograms in Figure 3.4.3.1, each modified MPc layer contains redox couples which have specific anodic and cathodic peak potentials involved in electrocatalysis. Figure 3.4.3.2 summarizes the peak current response and corresponding standard deviation observed for the OTA oxidation peak at various MPc modified electrodes.

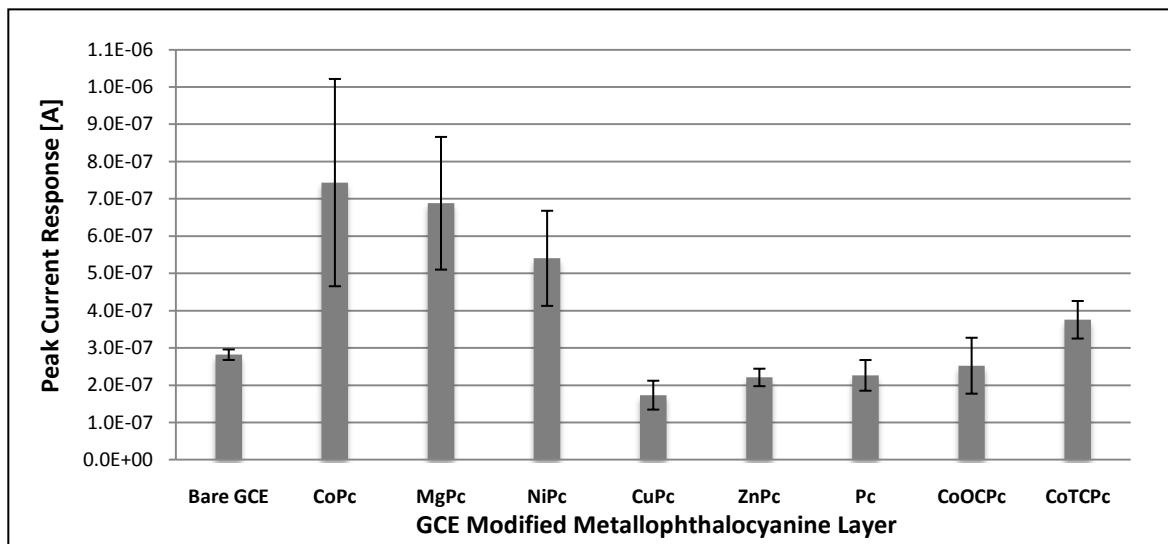


Figure 3.4.3.2 Bar graph comparing the peak current response for 5 μ M OTA in 0.2 M BR buffer pH 5 at various 1 mg/ml MPc modified GCEs. Number of replicates (3). Error bars show standard deviation from mean.

A marked increase in sensitivity for the detection of OTA was observed for the CoPc modified GCE producing a two fold increase in current response for the oxidation of OTA as compared to the unmodified GCE. The sensitivity in detection of OTA for the modified GCE was found to increase with MPc complexes as follows: CoPc > MgPc > NiPc > CoTCPc > CoOCPc > Pc > ZnPc > CuPc.

The redox properties of MPc complexes are expected to occur at the central transitional metal for CoPc, CuPc, CoTCPc and CoOCPc species whilst ring based processes occur for MgPc, NiPc, ZnPc and unmetallated Pc (Chebotareva and Nyokong, 1997). MPc complexes with metal based oxidation processes typically show better catalytic activity towards the oxidation of compounds hence the good catalytic activity of CoPc towards the oxidation of OTA (Vasudevan, et. al, 1990).

The current response observed for CoTCPc was comparable to the unmodified GCE while CoOCPc, CuPc, Pc and ZnPc complexes appeared to passivate the GCE surface as seen by a reduction in current response. As no catalytic activity was observed for the ZnPc and unmetallated Pc, ring based processes are not likely to be involved in the catalytic oxidation of OTA using MPc complexes. The high catalytic activity of NiPc and MgPc toward the oxidation of OTA is the exception as only ring based redox processes are known for these two MPc complexes. Similar improvements in terms of

electrode stability and electrocatalysis have been reported toward the oxidation of chlorophenols at NiPc modified electrodes with the electrocatalytic activity linked to the formation of O-Ni-O architecture of the NiPc film although the exact mechanism of activity has not yet been established (Agboola and Nyokong, 2007) (Obirai, et. al, 2005). Thus, it is suggested that the nickel substituent of the NiPc modified electrode may play a role in the catalytic oxidation of OTA.

In addition to the gain in sensitivity for OTA detection, the MPc modified GCE's were evaluated by the reproducibility of current response and the influence on anodic potential required for the oxidation of OTA.

For each measured oxidation of OTA, a new MPc modified GCE was fabricated in order to evaluate the reproducibility of the MPc layer toward the oxidation of OTA. The standard deviation in OTA peak current magnitude, shown in Figure 3.4.3.2, was used to evaluate the reproducibility of current response as tabulated with the relative standard deviation in Table 3.4.3.1.

Table 3.4.3.1 summarizes the amplitude of peak current and corresponding standard deviation in response obtained for 5 μ M OTA in 0.2 M BR buffer pH 5 at various 1 mg/ml MPc modified GCEs. Number of replicates (3).

Modified Metallophthalocyanine Layer	Average Peak Current Response [μA]	Standard Deviation in Peak Current Response [μA]	Relative Deviation in Peak Current Response [%]
Bare GCE	0.282	0.014	5.01
CoPc	0.743	0.278	37.40
MgPc	0.688	0.178	25.87
NiPc	0.540	0.128	23.61
CuPc	0.173	0.039	22.36
ZnPc	0.221	0.023	10.61
Pc	0.226	0.041	18.20
CoOCPc	0.252	0.075	29.76
CoTCPc	0.375	0.050	13.42

Electrode modification of the ZnPc layer produced the most reproducible surface obtaining a relative standard deviation of 10.61 % which is relatively near the value of 5.01 % obtained for the unmodified GCE. From Table 3.4.3.1, the general trend in reproducibility of OTA oxidation current response obtained for the MPc modified GCE's is as follows: ZnPc > CoTCPc > Pc > CuPc > NiPc > MgPc > CoOCPc > CoPc.

Accordingly, the increase in sensitivity for the detection of OTA is indirectly proportional to the reproducibility of current response obtained for the various MPc modified GCE's. The indirect association is substantiated by the high sensitivity and poor reproducibility obtained for the CoPc modified GCE. The poor reproducibility in current response derives from surface irregularities on the GCE surface produced from unordered stacking of the modified MPc layer during physical adsorption (Sivanesana and John, 2009).

The ability of the MPc layers to act as electron mediators was evaluated by the reduction in anodic potential required for the oxidation of OTA. Figure 3.4.3.3 summarizes the anodic potentials observed for OTA oxidation at the MPc modified GCE's whereby the unmetallated Pc produced the least anodic OTA oxidation potential of 1.02 V as compared to the unmodified GCE OTA oxidation potential of 1.12 V.

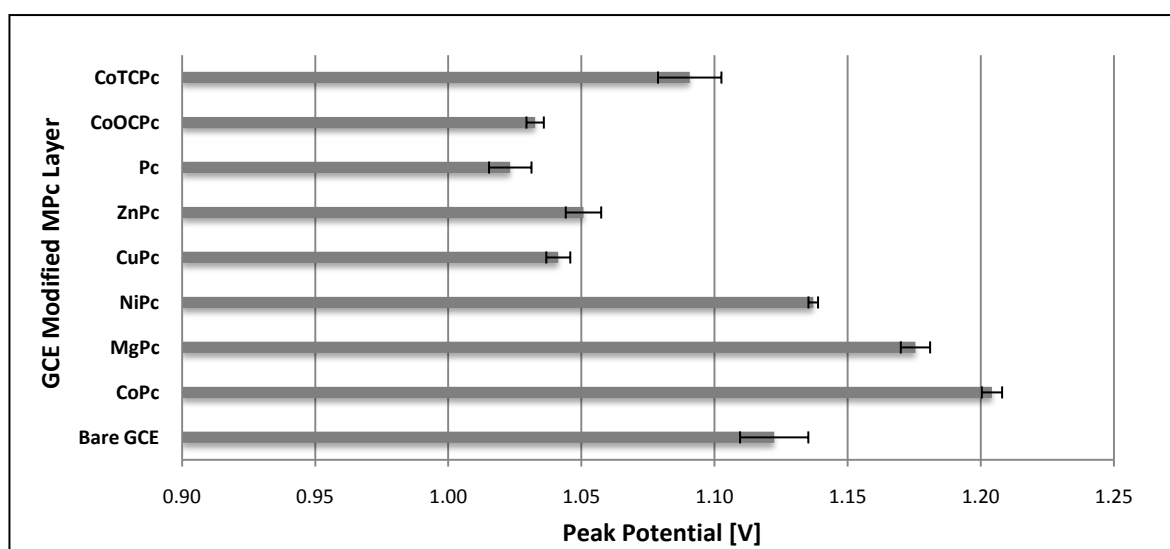


Figure 3.4.3.3 Bar graph comparing the peak anodic potential of 5 μ M OTA in 0.2 M BR buffer pH 5 at various 1 mg/ml MPc modified GCEs. Number of replicates (3). Error bars show standard deviation from mean.

With regard to the shift in potential toward the oxidation of OTA, the degree of electrocatalysis was observed to increase for the MPc modified GCE's as follows: Pc > CoOCPc > CuPc > ZnPc > CoTCPc > NiPc > MgPc > CoPc. The ability of the MPc layers to act as electron mediators follows the trend in reproducibility of OTA current response.

The NiPc modified GCE showed a 1.91 fold increase in sensitivity with a RSD of 23.61 % and an equivalent OTA oxidation potential of 1.13 V equivalent to that of the unmodified GCE, while the CoTCPc modified produced a 1.33 fold increase in sensitivity with a RSD of 13.42 % and an OTA

oxidation potential of 1.09 V representing a 30 mV reduction in OTA oxidation potential as compared the unmodified GCE.

From the above results, the electrochemical behaviour of OTA depends on the molecular structure of the MPc film layers modified to the GCE surface. As evaluated from the degree of sensitivity, reproducibility and electron transfer capability, both the NiPc and CoTCPc complexes represent viable modification layers toward the electrocatalytic detection of OTA in 0.2 M BR buffer pH 5.

3.4.4 Metallophthalocyanine modified GCE fouling profile

The stability of the MPc modified GCEs were assessed through the variation in current response with successive CV scans towards the oxidation of OTA as shown in Figure 3.4.4.1. As observed for the unmodified GCE, a decrease in current response with successive CV scans was observed for the various MPc modified GCE's. The decrease in current response was attributed to fouling of the electrode surface by OTA oxidation products as stated in section 2.4 and section 2.11. However, the magnitude of OTA oxidation current for the CoPc, MgPc, NiPc and CoTCPc modified GCE's was observed to stabilise above that of the unmodified GCE after five successive CV scans.

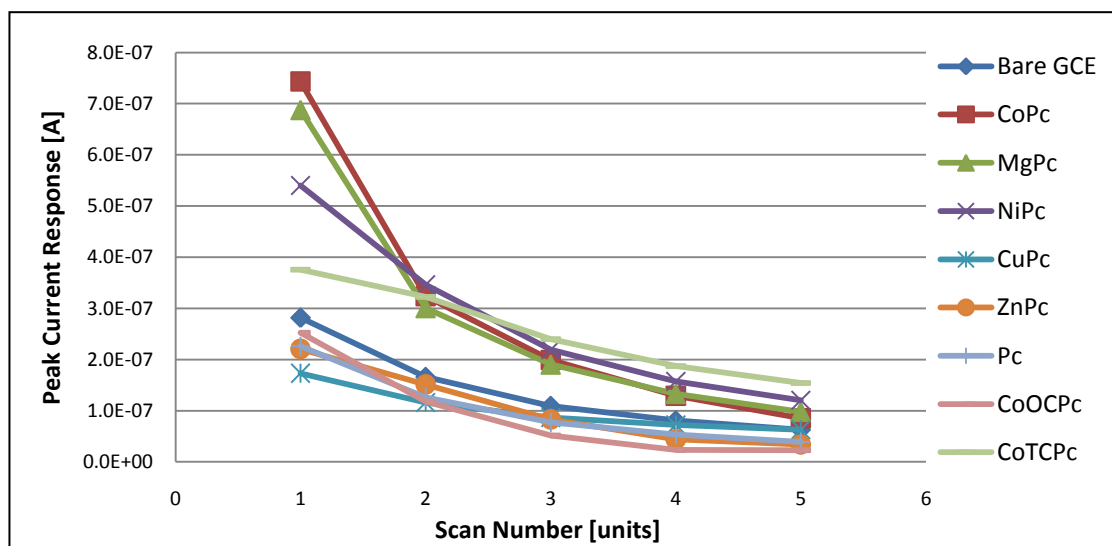


Figure 3.4.4.1 The variation in current magnitude evaluated for bare GCE and CoPc, MgPc, NiPc, CuPc, ZnPc, CoTCPc, CoOCPc and nonmetallated Pc modified GCE's.

Thus, modification of the GCE by CoPc, MgPc, NiPc and CoTCPc layers increased the sensitivity for detection of OTA even after continuous fouling of the electrode surface. The largest increase in sensitivity toward the oxidation of OTA was observed for the NiPc and CoTCPc layers providing a

respective 1.91 and 2.44 fold increase in current response as compared to the unmodified GCE after five successive CV scans. The sensitivity in detection for OTA after five successive CV scans was found to increase with MPc complexes as follows: CoTCPc > NiPc > MgPc > CoPc > CuPc > Pc > ZnPc > CoOCPc.

In addition to an increase in sensitivity, the degree of fouling upon successive CV scans is required to evaluate the effect of MPc modification on the stability of the electrode. The degree of fouling was evaluated as rate of decrease in current response with increasing scan number. Table 3.4.4.1 shows the percentage of sensitivity retained for the detection of OTA per successive CV scans with respect to the initial OTA oxidation peak.

Table 3.4.4.1 summarizes the relative current response of 5 μ M OTA in 0.2 M BR buffer pH 5 at GCEs modified with the respective 1 mg/ml MPc layers. Current response reported as a relative percentage of the initial current response obtained for OTA. No of replicates (3).

Scan No.	Bare GCE	CoPc	MgPc	NiPc	CuPc	ZnPc	Pc	CoOCPc	CoTCPc
1	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00
2	58.89	43.55	43.71	64.03	67.03	68.55	55.71	46.77	85.84
3	38.77	26.81	27.67	40.54	50.01	37.64	34.01	20.40	63.85
4	28.67	17.36	19.33	29.09	41.78	20.04	23.51	9.35	49.87
5	22.38	11.48	14.25	22.31	36.17	15.44	17.14	9.06	41.01

From Table 3.4.4.1., the gain in sensitivity of detection for OTA by the CoPc and MgPc modified GCE's is offset by the large degree of fouling observed upon successive CV cycles. The extent of fouling is represented by the respective retention in sensitivity of 11.48 % and 14.25 % observed for the CoPc and MgPc modified GCE's as compared to the unmodified GCE which retained 22.38 % sensitivity toward the detection of OTA after five successive CV scans.

The NiPc modified GCE retained 22.31 % sensitivity in OTA detection which is comparable to that of the unmodified GCE. Low susceptibility to surface fouling was observed for the CuPc and CoTCPc modified GCE obtaining a respective 36.17 % and 41.01 % retention in sensitivity after five successive CV scans. Thus, the degree of fouling was observed to increase for the MPc modified GCE's as follows: CoOCPc > CoPc > MgPc > ZnPc > Pc > NiPc > CuPc > CoTCPc.

A comparison of the sensitivity gain and fouling resistance exhibited by the MPc modified GCE's after successive CV scans showed that the CoTCPc and NiPc layers offered the greatest stability toward the detection of OTA in 0.2 M BR buffer pH 5.

3.5 CONCLUSION

The identification of 4-chlorophenol as an electroactive substituent of OTA in section 2.11 as well as information on the oxidation of 4-chlorophenol at various MPc modified electrodes suggested that the mechanism of OTA oxidation at the CoPc modified GCE involved oxidation of the central metal ion of the CoPc followed by the oxidation of OTA and subsequent regeneration of the CoPc modifier.

The mechanism of OTA oxidation was supported by the electrocatalytic activity of metal based MPcs toward the detection of OTA. With the exception of NiPc and MgPc, ring based MPc complexes typically passivated the electrode toward the oxidation of OTA. Although metal based MPcs produced a near two fold increase in detection sensitivity, significant deviation in current response upon MPc modification was observed. Irreproducibility in the detection of OTA was attributed to the unordered stacking of the MPc complexes to the electrode surface during physical adsorption.

The effect of MPc modification on fouling of the electrode surface as a result of OTA oxidation was evaluated in terms of stability and sensitivity in detection after five successive CV cycles. Voltammetric analysis showed that MPc modification generally had no effect on the extent of fouling at the electrode surface although low susceptibility to surface fouling was observed for the modification of CuPc and CoTCPc.

Thus, depending on the molecular structure of the MPc complexes modified to the GCE surface, a sufficient gain in detection sensitivity, fouling resistance and cathodic shift in peak potential was obtained toward the oxidation of OTA in aqueous media. Of the MPcs evaluated, NiPC and CoTCPc showed the greatest potential toward enhancing the sensitivity, reproducibility and fouling resistance toward the detection of OTA. Whereby NiPc modification increased the sensitivity of OTA detection by 1.92 fold and retained 22.31 % sensitivity in OTA detection comparable to that of the unmodified GCE. Low susceptibility to surface fouling was observed for the CoTCPc modified GCE obtaining a 41.01 % retention in sensitivity after five successive CV scans with a marginal 1.33 fold increase in detection sensitivity toward OTA.

CHAPTER 4

DETECTION OF OCHRATOXIN A AT CARBON NANOTUBE MODIFIED ELECTRODES

4.1 INTRODUCTION

The versatile structural, mechanical and electronic properties of carbon nanotubes have attracted considerable interest since their rediscovery by Iijima in 1991. These unique properties along with high conductivity, chemical and thermal stability has established CNTs as one of the key electrode materials for use in electroanalytical and bioanalytical applications (Baughman, et. al, 2002).

Chemical modification of CNTs to electrode surfaces has proved the ability of CNTs to mediate fast electron transfer kinetics for a wide range of electroactive species (Gong, et. al, 2005). The reduction in redox potentials and ability to resist fouling of CNT modified electrodes is also well documented (Wang, et. al, 2003). The nanometer dimensions and large surface area to weight ratio inherent to CNTs provides an ideal platform for immobilization of biomolecules extending the use of CNT modification in biosensor design and development (Gustavo, et. al, 2007). The application of CNTs as films, pastes and membranes offers a versatile support for the immobilisation of biomolecules within biosensor design (Gustavo, et. al, 2007). Electrical contact between the electrode and modified CNT enables the entire structure to be used as a mediator for electron transfer between the biological recognition component and the electrode surface.

The initial use of pristine CNTs in the design of biosensors was limited due to the absence of functional groups and inherent insolubility in aqueous media (Jeykumari and Narayanan, 2009). However, extensive research in CNT functionalization to enhance solubilisation and dispersibility by means of acid treatment, surfactants, polymerization and sidewall functionalization has largely overcome this limitation (Tasis, et. al, 2006). Oxidation of CNTs by acid treatment is arguably the most widely used method of CNT functionalization as the addition of oxygenated species has the added benefit of increasing CNT electroactivity (Tasis, et. al, 2006).

An additional limiting factor in the use of nanotubes for the development of electrochemical sensors is the high level of impurities present in commercially available CNTs. Common contaminants include metal catalysts, graphite, amorphous carbon and carbon nanoparticles produced during CNT

synthesis (Arepalli, et. al, 2004). As impurities increase variability in electrode composition and invariably sensor response, numerous methods of CNT purification have been developed including acid oxidation, gas phase oxidation, electrochemical oxidation, microfiltration and chromatographic based techniques (Hou, et. al, 2008).

Although oxidative treatment is widely used to etch away carbonaceous impurities and metal particles, the efficiency and duration of purification has engendered considerable debate in literature due to the successive degradation of CNT structure upon chemical oxidation (Hou, et. al, 2008). An alternative approach to oxidative treatment is the recent use of centrifugal purification which provides a rapid and highly efficient removal of graphite and other carbonaceous impurities without causing damage to the CNT structure (Arnold, et. al, 2005).

Considering the extensive use of CNT modification in sensor development, the electrocatalytic activity of MWCNT and SWCNT modified GCEs were assessed for electrocatalytic activity toward the detection of OTA. The effect of acid functionalization and subsequent centrifugal purification of the CNT structures on the sensitivity and reproducibility of detection was evaluated by AFM, Raman spectroscopy, optical microscopy and voltammetric techniques.

4.2 AIMS

The purpose of this chapter is to evaluate the use of MWCNT and SWCNT modification to enhance the detection of OTA. Assessment of the effect of acid functionalization coupled to centrifugal purification of the CNT structures on the detection of OTA was also investigated. To achieve the overall object, the following aims were addressed:

Characterization of the electrocatalytic potential of CNT modifiers in terms of available surface area and catalytic functional groups afforded to the electrode upon modification.

The electrocatalytic activity of MWCNT and SWCNT modification was evaluated toward the detection of OTA in terms of sensitivity, reproducibility and stability of current response.

The effect of various acid functionalization times on MWCNT and SWCNT modifiers toward the electrocatalysis of OTA was evaluated.

The effect of centrifugal purification on the acid functionalized MWCNT and SWCNT modifiers was evaluated in terms of relative electrocatalytic activity toward the detection of OTA.

The electrocatalytic activity of MWCNT and SWCNT supernatant and pellet fractions toward the detection of OTA was evaluated.

The relative purity and effect of physical adsorption on the morphological conformation of purified MWCNT and SWCNT fractions was investigated through the use of AFM and Raman scope analysis.

The electrocatalytic activity of CNT modified electrodes toward the detection of OTA was assessed in complex media.

4.3 METHODOLOGY

General methods performed in this section are outlined in chapter 2 with specific methodology outlined below:

4.3.1 Instrumentation

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

Centrifugal purification was performed using a JA-14 rotor on an Avanti J-E centrifuge (Beckman Coulter, United States of America).

Atomic force microscopy (AFM) was recorded in non-contact mode in air with a CP-11 scanning probe microscope (Veeco Instrumentals, Carl Zeiss, South Africa) at a scan rate of 1 Hz.

Raman spectroscopy was recorded using a Bruker RAM II FT-Raman spectrometer with an excitation Nd:YAG laser (1064 nm) source. The Raman Scope coupled to the Raman spectrometer was used for optical microscopy (40X magnification).

4.3.2 Reagents

MWCNT (90 %, 110-170 nm diameter, 5-9 μm length, Sigma Aldrich) and SWCNT (8 %, 0.9-1.2 nm diameter, 10-30 μm length, Sigma Aldrich) were used as modifier for the chemical modification of the GCE surface. Potassium chloride (99.9 %, Sigma Aldrich) was used for Raman Spectroscopy analysis of the MWCNT/SWCNT fractions. Ninhydrin (99%, Merck) was used for staining of phenylalanine during TLC analysis. . Apple juice was obtained from a local commercial supplier.

Oxidative pretreatment of MWCNT/SWCNT

Pristine SWCNT and MWCNT samples were suspended in a 20 ml acidic solution of H_2SO_4 (98 %, Merck): HNO_3 (55%, Merck) (3:1 v/v) and sonicated for preset functionalization times of 2, 4, 6, 8 and 24 hours. Once sonicated, the functionalized MWCNT/SWCNT suspension was diluted to a total volume of 200 ml with milliQ water.

Centrifugal purification of MWCNT/SWCNT

Centrifugal purification of the MWCNTs/SWCNTs was performed directly after oxidative pretreatment so as to prevent any further degradation of the CNT structures. Purification of the functionalized MWCNT/SWCNT suspension was performed by centrifugation at 12 000 g for a period of 30 minutes. The resultant acidic supernatant was discarded. The collected pellet was resuspended in milliQ water and re-centrifuged. Centrifugation was repeated until the aqueous phase was at a neutral pH. The supernatant fractions were collected after each successive centrifugation and pooled to form the MWCNT or SWCNT supernatant fraction. The final pellet fraction was labelled as the MWCNT or SWCNT pellet fraction. The collected fractions were subsequently dried and resuspended in 20 ml milliQ water prior to use.

MWCNT/SWCNT surface modification

MWCNT/SWCNT stock solutions of 1 mg/ml were prepared in DPF and dispersed by sonication for a period of 15 minutes. The dispersed CNTs (10 μ l) were drop coated to the GCE surface and dried at 70 °C for a period of 30 minutes. The CNT modified GCEs were then rinsed with DMF followed by thorough rinsing with milliQ prior to electrochemical analysis.

4.3.3 Methodologies

Specific methodology for this chapter is outlined further:

4.4.1 Electrochemical characterization of carbon nanotubes

CVs of 1 mg/ml purified MWCNT/SWCNT modified GCEs in 0.2 M BR buffer pH 5 was assessed as outlined in general methodologies with the exception that the modified GCE surface was not cleaned between subsequent CV scans.

4.4.2 Electrocatalysis of carbon nanotube modified electrode toward ochratoxin A

CV analysis of 5 μ M OTA in 0.2 M BR buffer was assessed at a GCE modified with 1 mg/ml purified MWCNT/SWCNT functionalized for 2 hours.

4.4.3 Acid functionalization of MWCNT

CV analysis of 5 μM OTA in 0.2 M BR buffer was compared between GCEs modified with 1 mg/ml purified MWCNT fractions functionalized for a period of 2, 4, 6, 8, and 24 hours.

4.4.4 Acid functionalization of SWCNT

CV analysis of 5 μM OTA in 0.2 M BR buffer was compared between GCEs modified with 1 mg/ml purified SWCNT fractions functionalized for a period of 2, 4, 6, 8, and 24 hours.

4.4.5 AFM characterization of carbon nanotube modifiers

AFM analysis was performed on standard glass microscope slides modified with 1 mg/ml purified MWCNT/SWCNT fractions functionalized for a period of 2,4,6,8 and 24 hours.

4.4.6 Raman scope characterization of carbon nanotube modifiers

Raman Scope analysis was performed on standard glass microscope slides modified with 1 mg/ml purified MWCNT/SWCNT fractions functionalized for a period of 2,4,6,8 and 24 hours.

4.4.7 Raman spec characterization of carbon nanotube modifiers

Purified MWCNT/SWCNT fractions (1 mg/ml) functionalized for a period of 2, 4, 6, 8 and 24 hours were mixed with 0.5 wt% with KCl prior to Raman spectroscopy analysis at a laser power of 100 mW averaged for 1024 scans.

4.4.8 Electrocatalysis of carbon nanotube modified electrode in complex media

DPV analysis of 5 μM OTA in apple juice:0.2 M BR buffer (1:2 v/v) of pH 5.5 was performed at a bare GCE and at GCEs modified with 1 mg/ml purified MWCNT/SWCNT fractions functionalized for a period of 2 hours.

4.4 RESULTS AND DISCUSSION

4.4.1 Electrochemical characterization of carbon nanotubes

Carbon nanotube modification offers an improvement over conventional electrodes in electroanalytical performance due to the inherent nanomolecular dimensions, mechanical stability and unique electronic properties of nanotubes (Gooding, 2005). The electroanalytical properties afforded by CNT electrode modification are sensitive to structural variations in the CNT layer namely due to the length, diameter and helicity of the carbon atoms which constitute the nanotube wall (Gooding, 2005). Figure 4.4.1.1 represents typical CV's obtained for MWCNT modified GCE in 0.2 M BR buffer pH 5 as compared to the voltammograms obtained for an unmodified GCE. CNTs acid functionalized for two hours were used to modify the relevant GCE.

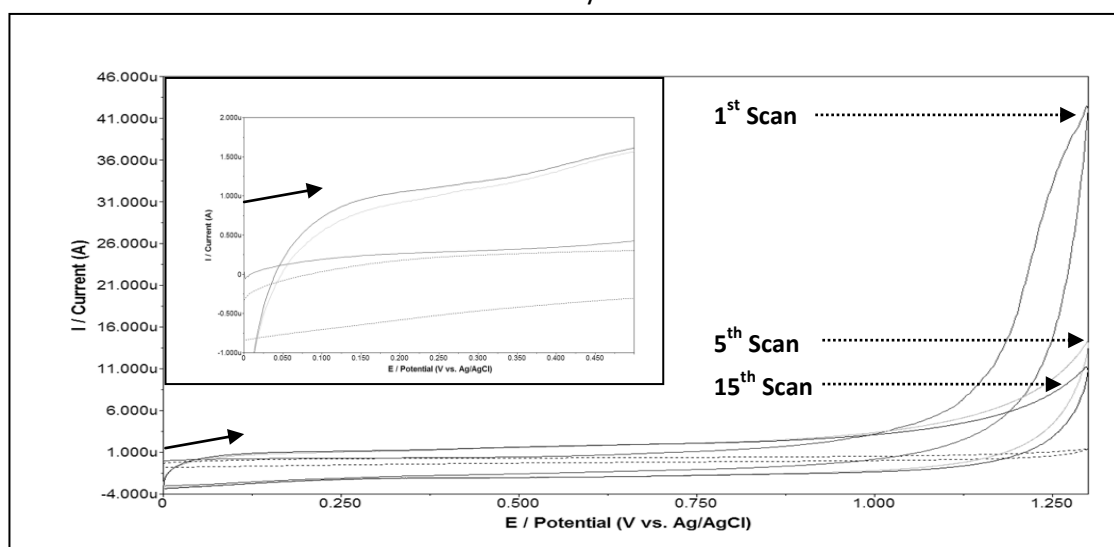


Figure 4.4.1.1 Initial CV cycle, 5th successive cycle and 15th successive cycle shown for 1 mg/ml 2 hour acid functionalized MWCNT modified GCE in 0.2 M BR buffer pH 5. Insert shows the presence of oxide species at a potential of 0.2 V. Response of unmodified GCE shown by dotted line. Scan Rate 100 mV/s.

As shown in the insert of Figure 4.4.1.1, the baseline charging current for the MWCNT modified GCE is 1.67 times larger than that of the unmodified GCE. The difference in baseline current is due to the increase in electroactive surface area afforded by CNT modification which leads to an increase in the number of electrolyte ions attracted to the electrode surface due to electrostatic interactions. As the applied potential is insufficient to allow for electron transfer, a capacitive layer is generated between the surface bound CNT's and solution phase ions of opposite charge (Emmenegger, et. al, 2003).

The capacitance generated between the two layers is termed the double layer capacitance. As the surface morphology of the CNT layer is of a porous and non uniform nature, nanotube modification increases the surface area available for charging of the double layer which increases the charging current observed for CNT modified GCE's as compared to an unmodified GCE (Emmenegger, et. al, 2003). The dependence of double layer capacitance on surface morphology allows for the characterisation of accessible surface area defined as the surface area of the CNT modification layer available for electron exchange with redox species within the electrode solution interphase.

In order to give an indication of the electroactive surface area available for the CNT modified GCE, the double layer capacitance (C_{dl}) of the modified electrode was calculated from the voltammetric charge of the CV scan according to the equation:

$$C_{dl} = \frac{q_a + q_c}{2 A \Delta V} \quad (4.4.1)$$

Where q_a represents the anodic voltammetric charge, q_c the cathodic voltammetric charge, A the electrode surface area and ΔV the potential range of the CV. The anodic charge (q_a) and cathodic charge (q_c) were selected at potential of 0.75 V in order to represent the baseline charging current for each electrode surface (Kim, et. al, 2006).

The average double layer capacitance of the unmodified GCE was determined as $0.46 \pm 0.07 \mu\text{F}/\text{mm}^2$ at a scan rate of 100 mV/s which is comparable to values of capacitance varying between 0.02 to 0.2 $\mu\text{F}/\text{mm}^2$ found in literature for GCE's (Li, et. al , 2002). Modification of the GCE surface by the MWCNT layer effectively increased the average double layer capacitance to $1.98 \pm 1.14 \mu\text{F}/\text{mm}^2$. The increase in double layer capacitance represented an increase in available surface area achieved through modification of the electrode by the non uniform and highly permeable MWCNT film. Considering the loading mass of the CNT layer, measured as 10 μg , and surface area of the unmodified electrode, a specific capacitance of $0.95 \pm 0.55 \mu\text{F}/\text{g}$ was calculated for the MWCNT film.

Similarly from Figure 4.4.1.2, an average double layer capacitance of $18.61 \pm 5.76 \mu\text{F}/\text{mm}^2$ was obtained for the SWCNT modified GCE which is near 40 fold greater than the unmodified GCE. The specific capacitance of the SWCNT layer was determined as $8.93 \pm 2.75 \mu\text{F}/\text{g}$. The large increase in double layer capacitance reflects the large increase in surface area achieved through SWCNT electrode modification. As identical loading concentrations of the MWCNT and SWCNT films were

used, the difference in capacitance between the two layers was accredited to the smaller dimensions of SWCNTs which in turn increased the degree of surface irregularity (Li, et. al , 2002).

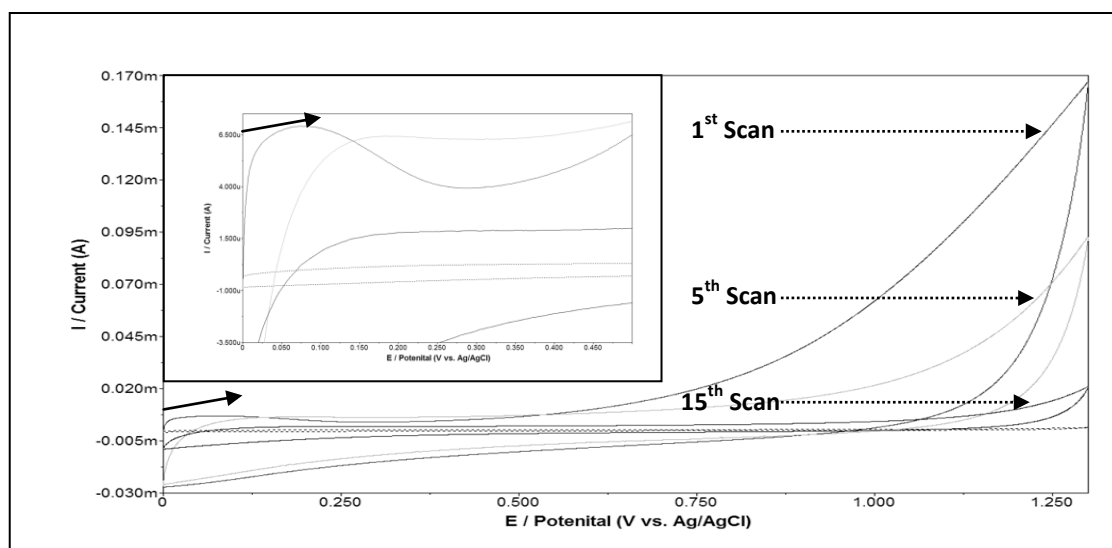


Figure 4.4.1.2 Initial CV cycle, 5th successive cycle and 15th successive cycle shown for 1 mg/ml 2 hour acid functionalized SWCNT modified GCE in 0.2 M BR buffer pH 5. Insert shows the presence of oxide species at a potential of 0.2 V. Response of unmodified GCE shown by dotted line. Scan Rate 100 mV/s.

In addition to an increase in the surface area achieved through CNT modification, acid functionalization of CNTs is used to further increase the electroactivity of the modified CNT surface. Acid functionalization creates oxygenated functional groups on the CNT film present as defect sites within the nanotube wall. The additional oxide species present on the modification layer are highly electroactive having electronic properties analogous to edge plane sites of pyrolytic graphite (Banks, et. al, 2004). Oxygen containing groups also increase the hydrophilic nature of the CNT surface which facilitates surface affinity towards polar molecules present in the electrolyte solution (Li and Zhang, 2006). The contribution of these two factors allows for fast electron transfer rates per unit area of the nanotube surface resulting in an increase in sensitivity toward the electrochemical detection of solution species with more defined redox peaks.

The presence of oxygenated species on the CNT modified GCE was characterised by the formation of redox peaks at a potential of 0.2 V in 0.2 M BR buffer pH 5. The peaks originate from redox reactions of -COOH, -OH and -C=O functional groups introduced onto the CNT surface during acid functionalization (Yu, et. al, 2008). Hence, the peak height observed for the oxygenated species was used to evaluate the degree of oxygenated species present on the nanotube surface (Chen, et. al, 2002).

Form Figure 4.4.1.1 and Figure 4.4.1.2, an average peak current of $2.42 \pm 1.76 \mu\text{A}$ was observed for the MWCNT modified GCE oxygenated species as compared to an average $5.57 \pm 1.40 \mu\text{A}$ peak current for the SWCNT modified GCE. The 2.30 times increase in functional groups for the SWCNT modified GCE as compared to the MWCNT layer is indicative of the high density of SWCNT packing on the modified GCE surface. No functional group redox peaks were observed for the unmodified GCE confirming the effect of acid functionalization on the CNT surfaces.

Faradaic processes of the oxygenated functional groups contribute to the pronounced effective capacitance observed for the CNT layers (Shanmugam and Gedankan, 2006). However, this contribution accounts for less than one third of the observed effective capacitance (Li, et. al, 2002). The double layer capacitance is still the major component of effective capacitance and as such is used to characterise the accessible surface area of the CNT modified electrodes. The large effective capacitance of the SWCNT modified GCE is due to the high density of electroactive functional groups present on the large surface area afforded by the permeable and irregular arrangement of SWCNT's on the GCE surface.

4.4.2 Electrocatalysis of carbon nanotube modified electrodes toward ochratoxin A

The electrocatalytic activity of CNT modified GCEs was assessed for the voltammetric oxidation of OTA in BR buffer pH 5. MWCNTs and SWCNTs acid functionalized for two hours were used to modify the relevant GCEs.

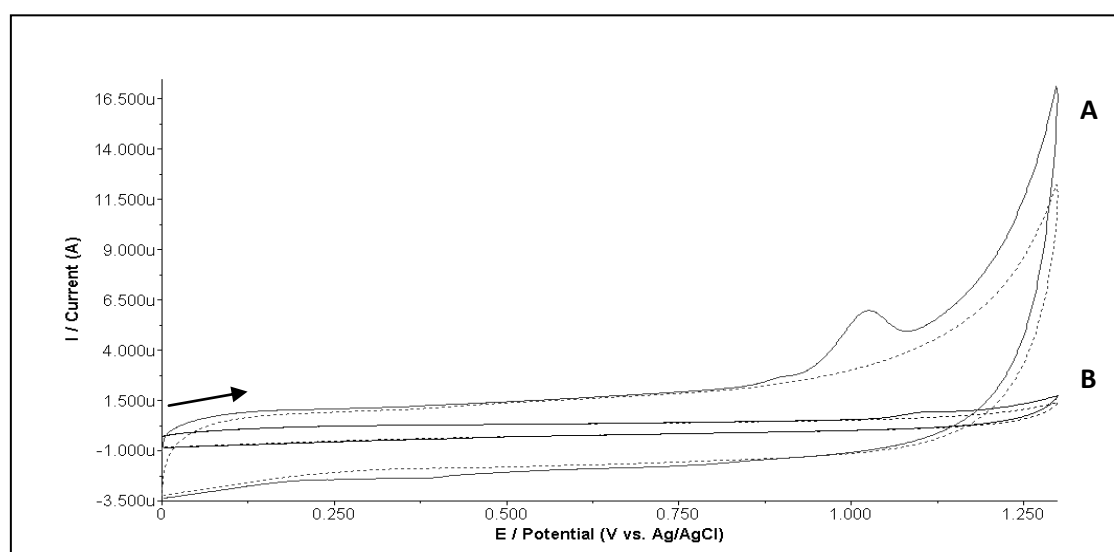


Figure 4.4.2.1 CVs obtained for the oxidation of $5 \mu\text{M}$ OTA at 2 hour functionalized MWCNT modified (A) and unmodified (B) GCE in 0.2 M BR buffer pH 5. Blank solutions for the MWCNT modified and unmodified GCE are shown as dotted lines. Scan rate 100 mV/s .

From Figure 4.4.2.1, a marked increase in peak current magnitude was observed for the MWCNT modified GCE in relation to the unmodified electrode. MWCNT modification of the GCE produced an average OTA oxidation current of $2.42 \pm 0.78 \mu\text{A}$ which equates to an 8.78 fold increase in sensitivity for the detection of OTA as compared to the unmodified GCE.

The increase in sensitivity observed for the MWCNT modified GCE was accredited to enhanced electron transfer kinetics between OTA and the oxygenated functional groups along the edge plane defect sites of the MWCNT surface (Banks and Compton, 2005). Furthermore the increase in effective capacitance, as shown in section 4.1, corresponds to the large surface area afforded by MWCNT modification which increases the density of functional group exposed to OTA in solution (Emmenegger, et. al, 2003).

However, detection of OTA by the MWCNT modified GCE is less reproducible than equivalent analysis at an unmodified GCE, given the substantial increase in standard deviation of current response observed for OTA. The reduction in reproducibility of OTA detection is associated with the non uniform morphology of MWCNT deposition during modification of the GCE surface by physical adsorption.

The increase in relative deviation of 57.94 % for the effective capacitance of the MWCNT modified GCE as compared to the relative deviation in capacitance of 14.85 % observed for the unmodified GCE reflects the difference in surface morphologies obtained for the MWCNT modification layers. Impurities present as varying amounts of graphite, amorphous carbon and carbon coated metal particles may also contribute to the irreproducibility in OTA detection (Vigolo, et. al, 2010).

As shown in Figure 4.4.2.2, an average peak current magnitude of $5.57 \pm 1.62 \mu\text{A}$ for OTA was observed at the SWCNT modified GCE which relates to a 20.18 fold increase in sensitivity toward the detection of OTA as compared to an unmodified GCE.

The gain in sensitivity for SWCNT modification in relation to MWCNT modification was accredited to the significant increase in surface area afforded by high packing density and high surface area to volume ratio of the nanotubes comprising the SWCNT modified GCE (Li, et. al, 2002). The measured effective capacitance for the SWCNT and MWCNT modified GCEs discussed in section 4.1 relates to the difference in surface area for the respective modification layers.

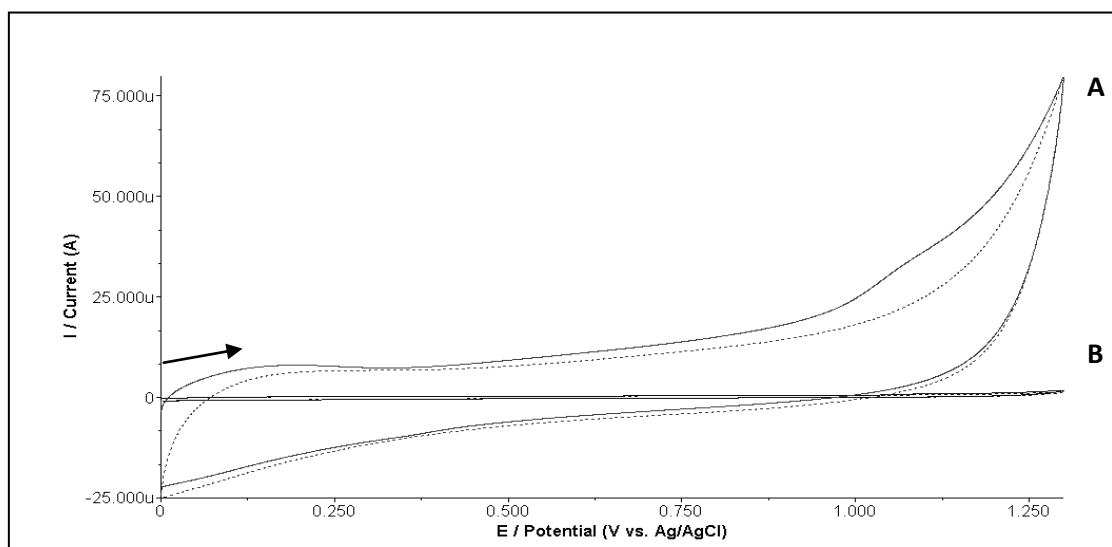


Figure 4.4.2.2 CVs obtained for the oxidation of 5 μM OTA at 2 hour functionalized SWCNT modified (A) and unmodified (B) GCE in 0.2 M BR buffer pH 5. Blank solutions for the SWCNT modified and unmodified GCE are shown as dotted lines. Scan rate 100 mV/s.

Furthermore, the increase in sensitivity per increase in capacitance of the MWCNT modified GCE was 13.87 times greater than the SWCNT modification layer leading to the assumption that the density of oxygenated functional groups on the SWCNT layer was significantly greater than that of the MWCNT layer thereby contributing to the additional gain in sensitivity toward the detection of OTA.

4.4.3 Acid functionalization of MWCNTs

The effect of variation in acid functionalization times on the electrocatalytic activity of MWCNT modified GCE's for the detection of OTA in 0.2 M BR buffer pH 5 was investigated. Acid functionalization times of 0, 2, 4, 6, 8, and 24 hours were evaluated for the MWCNT modification layers. Figure 4.4.3.1 represents the CVs obtained for the oxidation of OTA in BR buffer pH 5 at GCEs modified with MWCNT layers exposed to acid functionalization times of 0, 2, 4, 6, 8 and 24 hours.

The corresponding DPVs, shown in the insert of Figure 4.4.3.1, demonstrate the change in peak current magnitude for the anodic OTA peak upon increasing functionalization times. From Figure 4.4.3.1, the MWCNT layer exposed to a functionalization time of two hours displayed the greatest increase in sensitivity toward the detection of OTA obtaining an extrapolated LOD of 0.09 μM . The 25.56 % increase in RSD when compared to an unmodified GCE was accredited to the morphological variation in surface area introduced during physical adsorption of the MWCNT layer to the GCE

surface. In addition, an electrocatalytic shift in potential of 0.102 ± 0.013 V was observed for the two hour functionalized MWCNT layer when compared to an unmodified GCE.

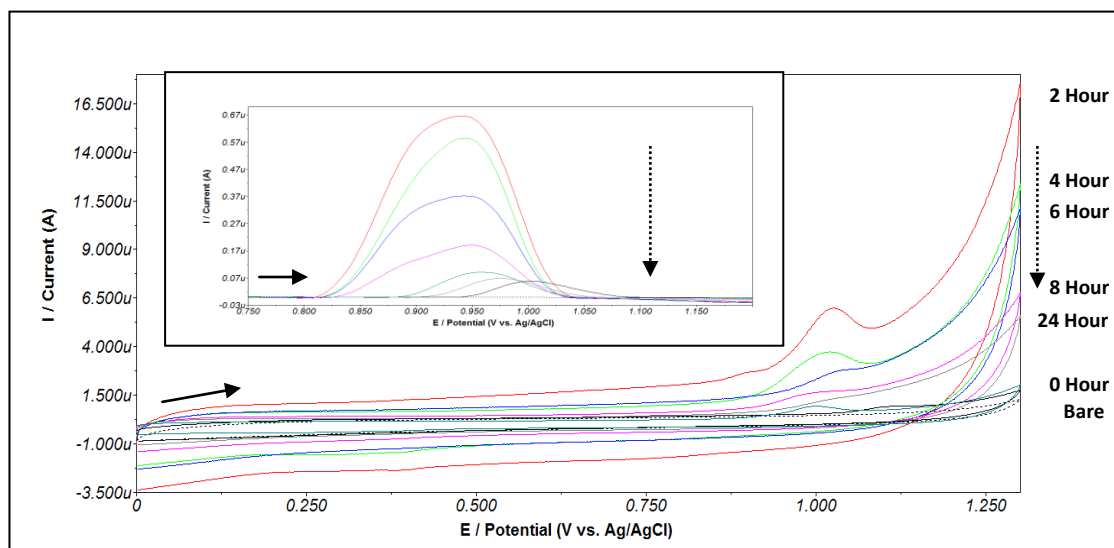


Figure 4.4.3.1 CVs of unmodified GCE (black), 0 (cyan), 2 (red), 4 (green), 6 (blue), 8 (magenta) and 24 (grey) hour functionalized MWCNT modified GCEs in solution of 5 μ M OTA in 0.2 M BR buffer pH 5. Scan rate 100mV/s. Unmodified GCE in solution of 0.2 M BR buffer pH 5 shown as dotted line. Insert shows corresponding DPVs at 5 mV/s scan rate.

Figure 4.4.3.2 summarizes the peak current response and corresponding standard deviation observed for the oxidation of OTA at various functionalization times of the MWCNT modified GCEs.

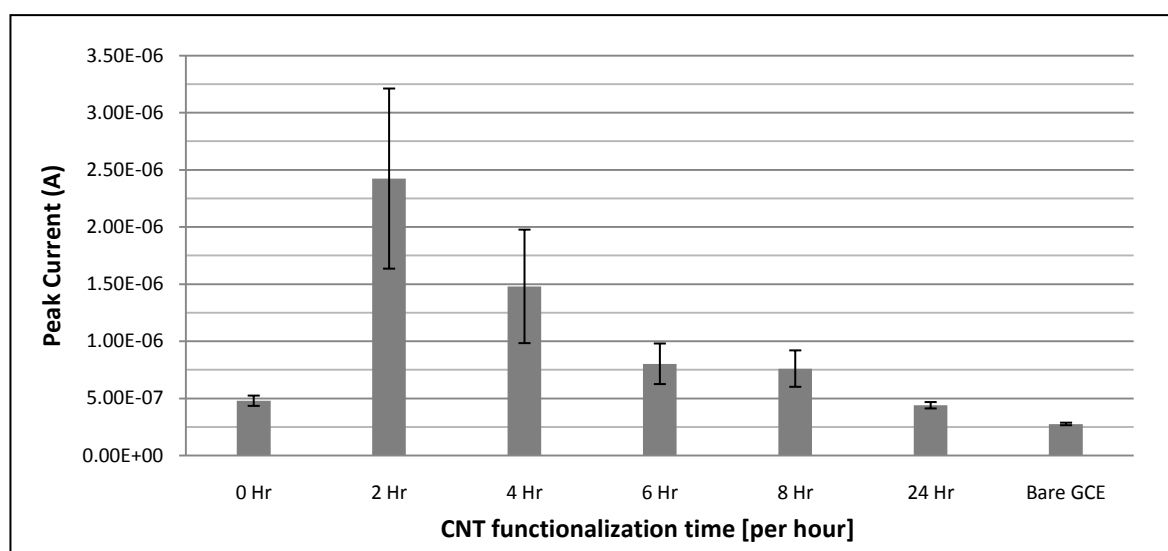


Figure 4.4.3.2 Bar graph of peak current amplitude for the oxidation of 5 μ M OTA in 0.2 M BR buffer pH 5 at 1 mg/ml MWCNT modified GCE of increasing acid functionalization times. Number of replicates (3). Error bars show standard deviation from mean.

The OTA oxidation peak observed for the various MWCNT layers showed an exponential reduction in peak current magnitude upon increasing functionalization times with the peak current response of the MWCNT layer acid functionalized for 24 hours being comparable to that of an unmodified GCE. Compared to the unmodified GCE, MWCNT modification of the GCE resulted in a shift in OTA oxidation peak potential toward less anodic potentials, as shown in Figure 4.4.3.3.

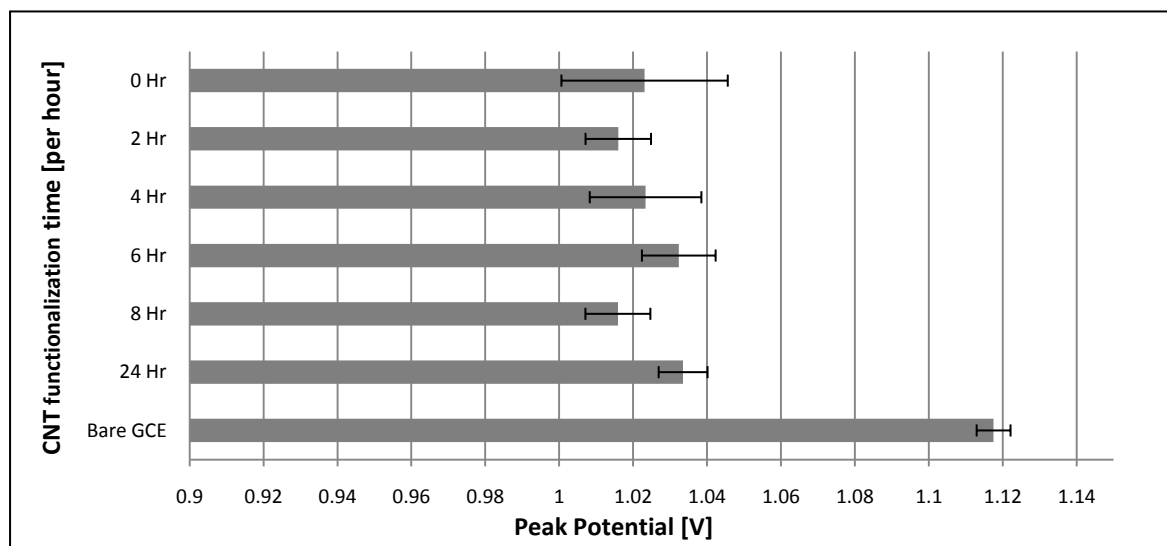


Figure 4.4.3.3 Bar graph of anodic potential for the 5 μ M OTA peak in 0.2 M BR buffer pH 5 at 1 mg/ml MWCNT modified GCE of increasing acid functionalization times. Number of replicates (3). Error bars show standard deviation from mean.

As shown in section 4.2, the electrocatalytic activity of the MWCNT modification layers toward the oxidation of OTA can be attributed to both the increase in surface area of the modified GCE and the presence of defect sites on the MWCNT surface. Defect sites are introduced during acid treatment through oxidative damage to the basal plane graphene layers situated at the nanotube ends and sidewall thereby producing edge plane like defect sites on the nanotube surface (Wildgoose, et. al, 2006). Acid functionalization increases the number of oxygenated functional groups particularly hydroxyl, quinonyl and carboxylate species present on the MWCNT surface by the formation of defect sites within the nanotube structure (Wildgoose, et. al, 2006). Thus, functional groups oxidized to the defect site ends act as preferential molecular adsorption sites and as electroactive sites available for the oxidation of OTA. However, continued acidification results in degradation of the CNT nanomolecular structure leading to disruption of the intrinsic electronic properties and electrical conductivity exhibited by the nanotube layer (Luong et. al, 2005).

From the above results, a functionalization time of two hours was sufficient to introduce oxygenated functional groups present as defect sites on the nanotube surface without excessive damage to the MWCNT structure. Although the density of functional groups within the defect sites increased with

acid treatment, functionalization times beyond two hours resulted in a reduction in sensitivity toward the detection of OTA due to successive degradation of the MWCNT structure. Structural degradation of MWCNTs is shown to reduce electrical conductivity and surface area to volume ratio inherent to the MWCNT modification layer.

The reduction in surface area available toward the oxidation of OTA is verified by the decline in effective capacitance observed for the MWCNT modified GCEs upon increasing functionalization time, as shown in Figure 4.4.3.4.

Degradation of the MWCNT structure to graphitic or amorphous allotropes of carbon alters the mechanism of electron transfer from edge plane like behaviour of the defect sites to that of basal plane graphite which reduces the overall electrocatalytic nature of the MWCNT layer toward the oxidation of OTA.

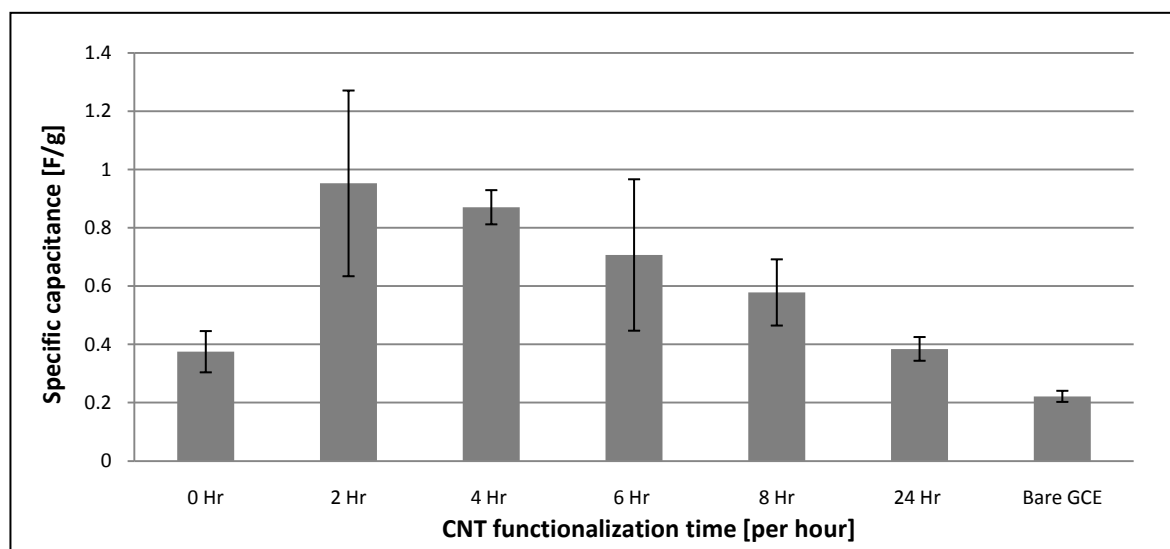


Figure 4.4.3.4 Bar graph of specific capacitance measured for the unmodified and 1 mg/ml MWCNT modified GCEs of increasing acid functionalization times in 0.2 M BR buffer pH 5. Number of replicates (3). Error bars show standard deviation from mean.

4.4.4 Acid functionalization of SWCNTs

The effect of acid treatment on the electroactivity of SWCNT modified GCEs toward the detection of OTA in 0.2 M BR buffer pH 5 was assessed for functionalization times of 0, 2, 4, 6, 8, and 24 hours. Centrifugal purification of the SWCNT sample was performed on completion of the respective acid functionalization step to produce a SWCNT pellet and supernatant fraction. As SWCNT layers are of lower molecular weight and are more susceptible to structural degradation by acid treatment than

equivalent MWCNT layers, the electrocatalytic behaviour of both the SWCNT pellet and supernatant fractions were investigated toward the oxidation of OTA.

Figure 4.4.4.1 illustrates the CVs and corresponding DPVs obtained for the oxidation of OTA in BR buffer pH 5 at GCEs modified with SWCNT pellet fractions.

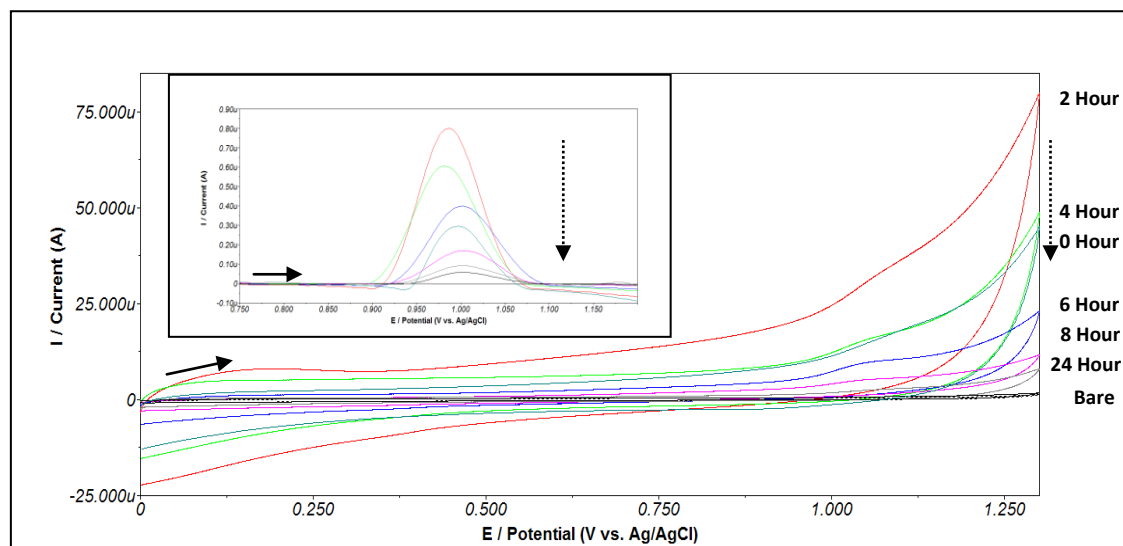


Figure 4.4.4.1 CVs of unmodified GCE (black), 0 (cyan), 2 (red), 4 (green), 6 (blue), 8 (magenta) and 24 (grey) hour functionalized SWCNT **pellet** modified GCEs in solution of 5 μM OTA in 0.2 M BR buffer pH 5. Scan rate 100mV/s. CV of unmodified GCE in 0.2 M BR buffer shown as dotted line. Insert shows corresponding DPVs at 5 mV/s scan rate.

The CVs and corresponding DPVs for SWCNT supernatant fractions of increasing acid functionalization times are shown in Figure 4.4.4.2 for OTA concentration and buffer conditions equivalent to the SWCNT pellet modified GCEs in Figure 4.4.4.1.

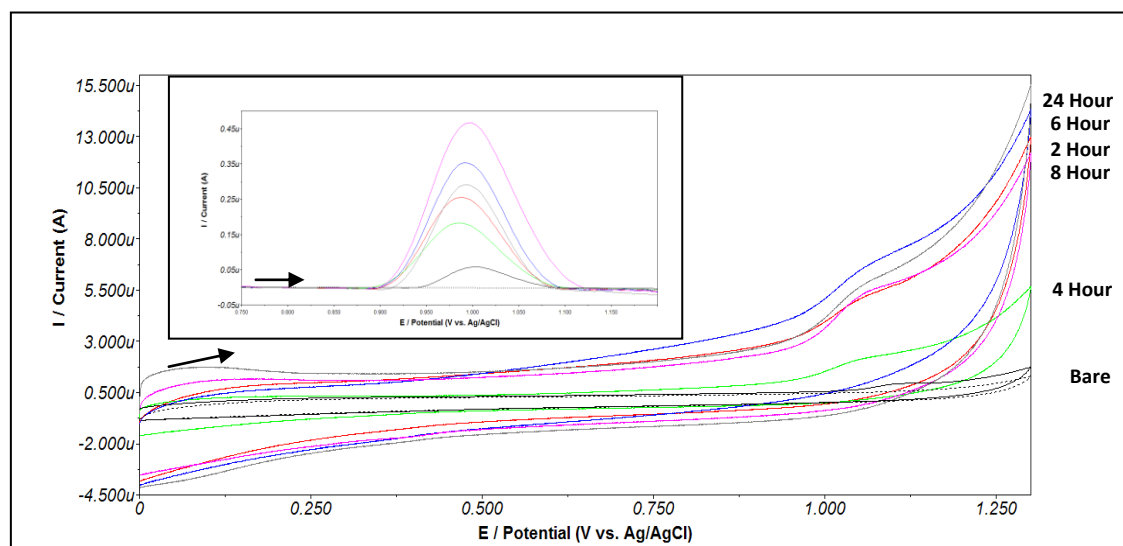


Figure 4.4.4.2 CVs of unmodified GCE (black), 2 (red), 4 (green), 6 (blue), 8 (magenta) and 24 (grey) hour functionalized SWCNT **supernatant** modified GCEs in solution of 5 μM OTA in 0.2 M BR buffer pH 5. CV of unmodified GCE in 0.2 M BR buffer shown as dotted line. Scan rate 100mV/s. Insert shows corresponding DPVs at 5 mV/s scan rate.

As observed for the MWCNT modified GCEs, the 2 hour functionalized SWCNT pellet showed the greatest increase in sensitivity toward the detection of OTA for the SWCNT modified GCEs in 0.2 M BR buffer pH 5, obtaining an extrapolated LOD of 0.03 μM . A successive decrease in peak current response was observed for the SWCNT pellet modified GCEs upon increased acid functionalization times, with the peak current magnitude of the 24 hour functionalised SWCNT pellet being comparable to that of an unmodified GCE. The sensitivity in detection of OTA for the SWCNT supernatant modified GCEs was shown to increase with functionalization time, with the greatest increase in sensitivity observed for the eight hour functionalized supernatant fraction obtaining an average OTA peak current magnitude of $2.02 \pm 1.52 \mu\text{A}$. The increase in sensitivity afforded by the eight hour functionalized supernatant fraction equates to an extrapolated LOD of 0.10 μM .

Figure 4.4.4.3 summarizes the peak current magnitude and corresponding deviation observed for the oxidation of OTA at SWCNT pellet and supernatant modified GCEs of respective acid functionalization times.

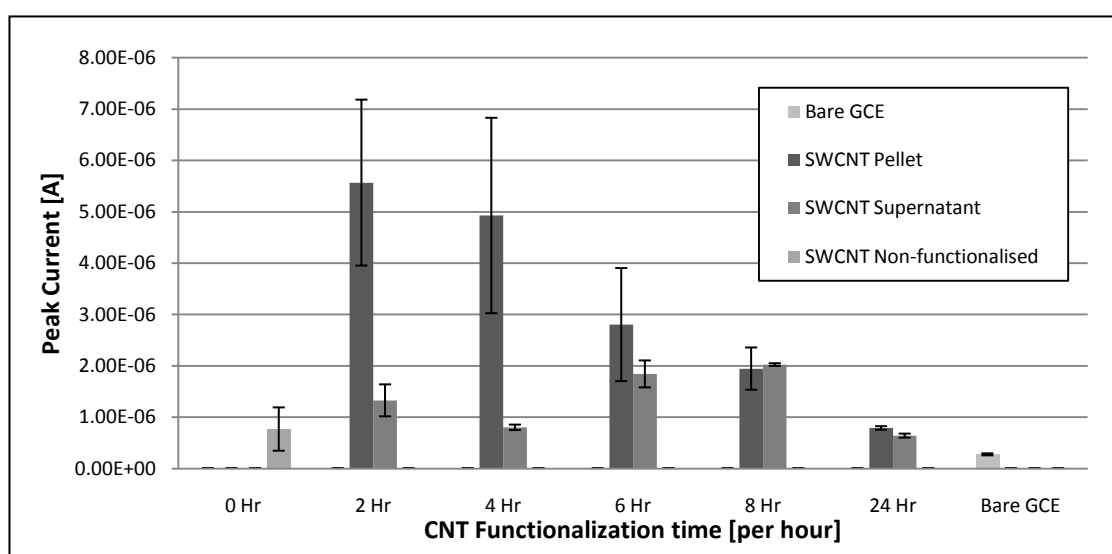


Figure 4.4.4.3 Bar graph comparing the peak current amplitude of 5 μM OTA in 0.2 M BR buffer pH 5 between 1 mg/ml SWCNT pellet and supernatant modified GCE of increasing acid functionalization times. Number of replicates (3). Error bars show standard deviation from mean.

As shown in Figure 4.4.4.4, a similar shift in peak potential was observed for both the SWCNT pellet and supernatant fractions throughout the acid functionalization range thereby indicating a lower electrocatalytic activity toward the oxidation of OTA for the SWCNT fractions as compared to equivalent MWCNT fractions.

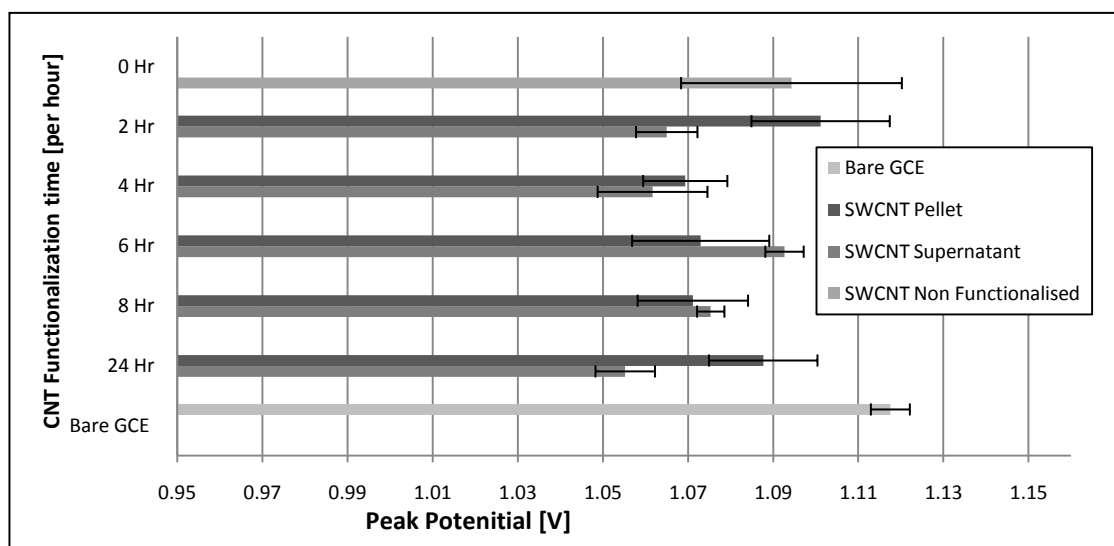


Figure 4.4.4.4 Bar graph comparing the anodic potential of the 5 μ M OTA peak in 0.2 M BR buffer pH 5 between 1 mg/ml SWCNT pellet and supernatant modified GCE of increasing acid functionalization times. Number of replicates (3). Error bars show standard deviation from mean.

The 20 fold increase in sensitivity observed for the two hour functionalized SWCNT pellet fraction as compared to an unmodified GCE, was attributed to both the electroactivity of oxygenated functional groups present at defect sites along the nanotube surface and the high surface area to volume ratio of the SWCNT layer afforded by preservation of the SWCNT structural integrity during acid treatment. The effect of structural degradation with increased acid functionalization on the modified SWCNTs was observed by the reduction in sensitivity toward the detection of OTA for pellet fractions of functionalization times beyond two hours.

At prolonged functionalization times, defect formation along the nanotube surface results in the eventual cleavage of the nanotube structure and liberation of carbonaceous impurities particularly amorphous or graphitic allotropes of carbon (Luong et. al, 2005). Due to the low molecular weight and hydrophilic nature of these carbon allotropes, centrifugal purification allows for the preferential retention of the carbonaceous impurities in the SWCNT supernatant fractions (Nepal, et. al, 2005). To confirm the low extent of oxidative damage to the SWCNT structure of the 2 hour pellet fraction, a comparison was made between the 2 hour supernatant and 24 hour pellet SWCNT effective capacitance values which are 1.09 ± 0.31 F/g and 0.92 ± 0.11 F/g respectively.

The similarity in effective capacitance indicates that the composition of carbonaceous material in the 2 hour supernatant fraction is comparable to the 24 hour pellet fraction which consists primarily of carbonaceous impurities due to complete degradation of the SWCNT structure. The low value of

effective capacitance indicates an absence of shortened nanotubes retained within the 2 hour supernatant fraction which shows an overall preservation of SWCNT integrity.

The reduction in sensitivity toward the detection of OTA upon increased functionalization times for the SWCNT pellet fractions was attributed to both the accumulation of carbonaceous impurities and to the shortening of the SWCNT structure which effectively reduced the centrifugal purification efficiency. Consequently, the decrease in molecular weight for carbonaceous material upon acid treatment resulted in the convergence of OTA detection sensitivity between SWCNT pellet and supernatant fractions due to an equal retention of shortened SWCNT and carbonaceous impurities present between both 8 hour functionalized fractions. A similar observation was made regarding the convergence of effective capacitance values between the SWCNT pellet and supernatant fractions for increased acid functionalization time, as shown in Figure 4.4.4.4.

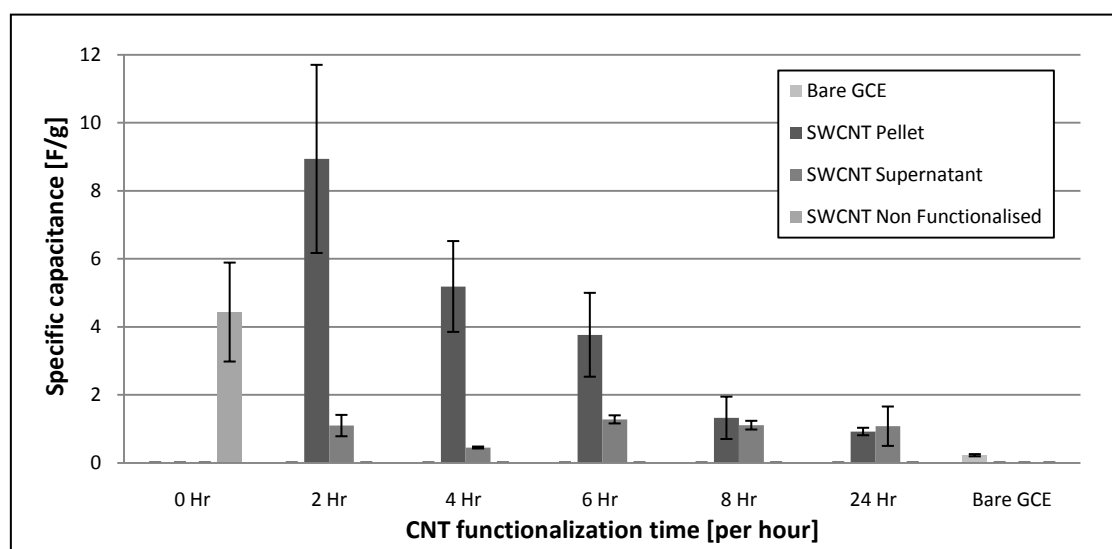


Figure 4.4.4.5 Bar graph comparing specific capacitance between 1 mg/ml SWCNT pellet and supernatant modified GCE of increasing acid functionalization times measured in 0.2 M BR buffer pH 5. Number of replicates (3). Error bars show standard deviation from mean.

In contrast to the reduction in sensitivity toward the detection of OTA, accumulation of carbonaceous impurities within the SWCNT fractions produced a marginal change in OTA peak oxidation potential between the pellet and supernatant SWCNT modified GCEs. The observed shift in OTA peak oxidation potential toward less anodic potentials for the SWCNT fractions may indicate the electrocatalytic activity of carbonaceous impurities toward the oxidation of OTA.

4.4.5 AFM characterization of carbon nanotube modifiers

An investigation on the surface morphology of acid functionalised MWCNT and SWCNT layers adsorbed to a glass substrate was carried out using atomic force microscopy (AFM) (Marshall, 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 functionalization procedure (Nepal, et. al, 2005). Separate AFM analysis was performed on the CNT pellet and supernatant fractions produced from centrifugal purification on completion of acid treatment. MWCNT supernatant fractions were excluded from analysis due to the low yield of carbonaceous material produced from the centrifugal purification procedure. AFM scans obtained for 6 hour acid functionalised MWCNT and SWCNT pellet fractions are illustrated in Figure 4.4.5.1.

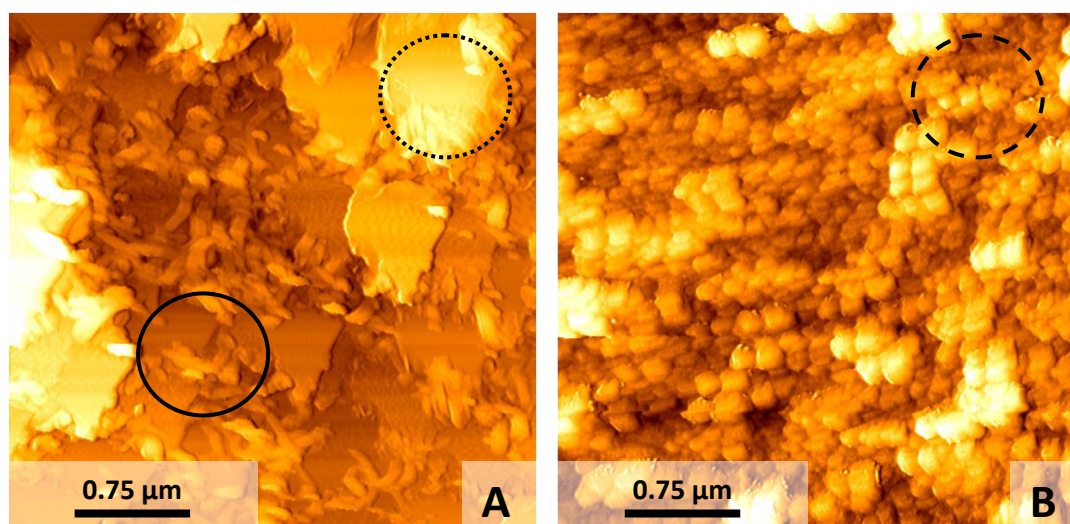


Figure 4.4.5.1 AFM images of 1 mg/ml MWCNT (A) and SWCNT (B) pellet in DMF and dried to a glass silica substrate at 60 °C. Images correspond to 6 hour acid functionalized CNT fractions. Insert shows individual MWCNTs (solid), SWCNT bundles (dashed) and carbonaceous sheets (dotted).

The porous, non uniform surface morphology observed for the 6 hour functionalized SWCNT and MWCNT layers 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 could not 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.

For the SWCNT modified surface, a more uniform layering of SWCNT bundles were observed with no apparent resolution of individual nanotubes and sheet like carbonaceous impurities. The large amount of graphitic or amorphous carbon produced during acid treatment of SWCNTs is suspected to coat the nanotube surface and assist in nanotube bundling (Harutyunyan, et. al, 2002). Increased SWCNT binding through the incorporation of carbonaceous impurities could account for the higher packing density of the SWCNT modification layer as compared to the equivalent MWCNT surface.

Additional AFM analysis was performed on the acid functionalized SWCNT fractions in order to evaluate the efficiency of the centrifugal purification procedure on the CNT fractions and thereby account for the catalytic behaviour of functionalized CNT modified electrodes toward the oxidation of OTA. The acid functionalization times of 2, 4, 6, and 8 hours used for AFM analysis were chosen to investigate the full range of variation in surface composition for the adsorbed CNT fractions. Due to the high interconnectivity of the CNT network, additional AFM analysis was performed on areas of low nanotube density in order to resolve individual CNT structures. The regions of high and low carbonaceous density obtained for glass surfaces modified by SWCNT pellet fractions exposed to acid functionalization times of 2, 4, 6 and 8 hours are shown in Figure 4.4.5.2.

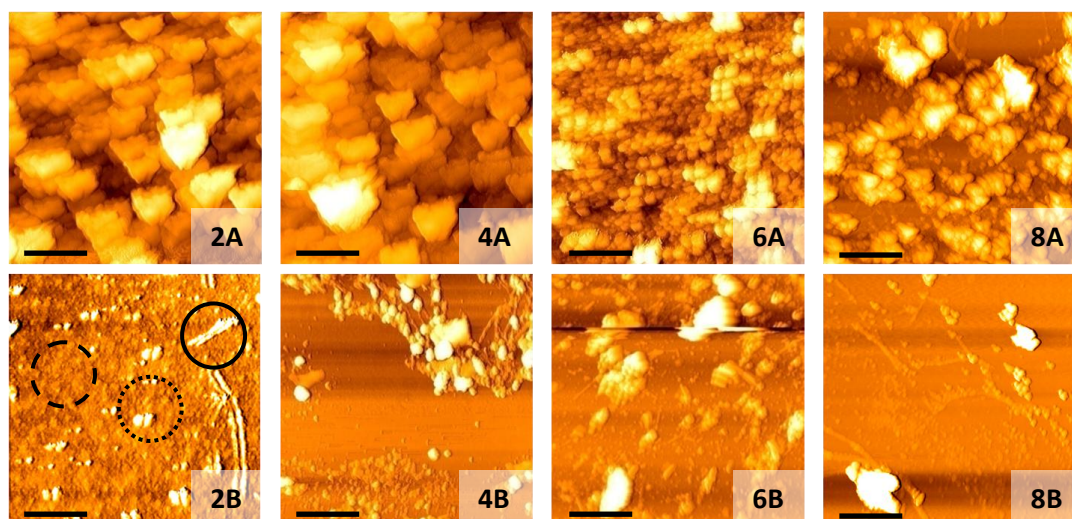


Figure 4.4.5.2 AFM images of 1 mg/ml SWCNT pellets in DMF dried on glass at 60°C. Images correspond to SWCNT dense regions of functionalization time 2 (2A), 4 (4A), 6 (6A) and 8 (8A) hours, and to SWCNT sparse regions of functionalization time 2 (2B), 4 (4B), 6 (6B) and 8 (8B) hours. Scale bars represent 0.75 μm . Insert shows individual SWCNTs (solid), carbonaceous clumps (dotted) and noncrystalline carbon (dashed).

AFM analysis of the dense SWCNT regions showed the formation of uniform, tightly layered SWCNT clumps which appear to reduce in size upon increased acid functionalization times with a large degree of structural degradation observed for the 8 hour SWCNT fraction. The gradual decrease in surface porosity upon increased acid functionalization times could account for the difference in catalytic behaviour observed for the SWCNT fractions toward the oxidation of OTA.

The dispersed SWCNT regions allowed for the identification of individual nanotubes of 40 nm in size and of carbonaceous impurities which were shown to range from fine noncrystalline carbon to larger graphitic clumps of comparable size to the individual SWCNTs. The large nanotube size observed for the individual SWCNT, as compared to the MWCNT size, could indicate twisting of multiple SWCNTs into a singular tubular structure (Zhang, et. al, 2000). The fine structure of carbonaceous impurities in Figure 4.4.5.3 (8A), confirms the near complete degradation of SWCNTs upon acid treatment and would account for the ability of carbonaceous impurities to bind to individual nanotubes and thereby assist in nanotube bundle formation.

Figure 4.4.5.3 shows the regions of high and low carbonaceous density for SWCNT supernatant fractions exposed to acid functionalization times of 2, 4, 6, and 8 hours and subsequently modified to a glass surface for AFM analysis.

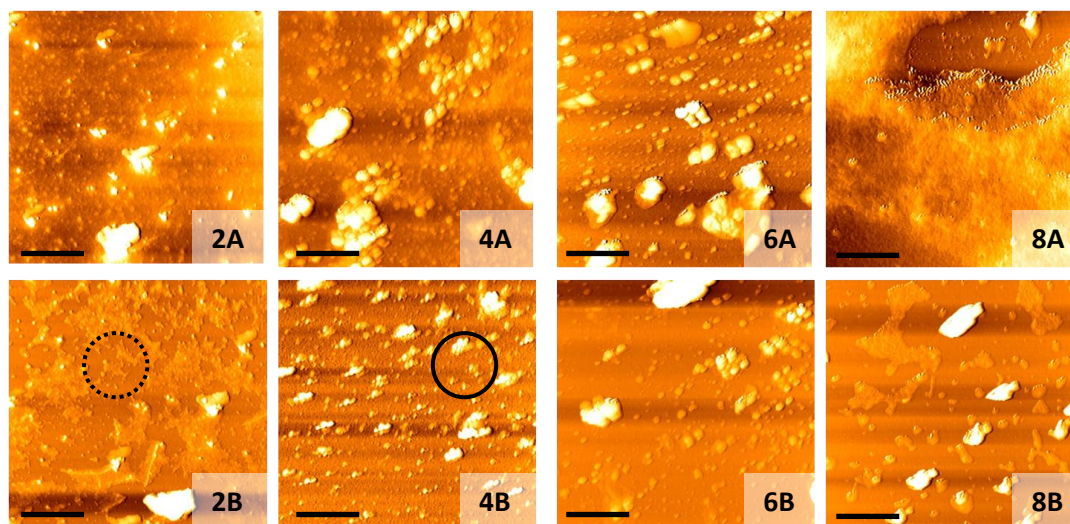


Figure 4.4.5.3 AFM images of 1 mg/ml SWCNT supernatants in DMF dried on glass at 60°C. Images correspond to SWCNT dense regions of functionalization time 2 (2A), 4 (4A), 6 (6A) and 8 (8A) hours, and to SWCNT sparse regions of functionalization time 2 (2B), 4 (4B), 6 (6B) and 8 (8B) hours. Scale bars represent 0.75 μm . Insert shows carbonaceous clumps (solid) and noncrystalline carbon impurities (dotted).

Unlike the densely packed nanotube bundles of the SWCNT pellet fractions, AFM analysis of the SWCNT supernatant dense regions show a reduced amount of SWCNT bundles identified amongst an inter-dispersed layer of carbonaceous impurities characterised as clumps of sizes below that of individual SWCNTs. For acid functionalization times ranging from 2 to 6 hours, the increase in nanotube bundle retention observed for the carbonaceous dense regions supports the observed increase in electrocatalytic activity of the SWCNT supernatant fractions toward the oxidation of OTA.

The increase in nanotube bundle retention for the supernatant fractions is attributed to the reduction in bundle size as a result of acid treatment. Prolonged acid treatment resulted in significant degradation of the nanotube bundles and subsequent retention of carbonaceous impurities within the SWCNT supernatant fractions. As near complete degradation of the nanotube bundles were observed for the 8 hour functionalized fraction, the electrocatalytic activity of the supernatant surface was attributed to an increase in surface area afforded by the adsorption of graphite and amorphous carbon impurities.

Comparison of the surface morphology between SWCNT pellet and supernatant fractions revealed the necessity for nanotube purification after acid treatment as the inclusion of carbonaceous impurities resulted in an overall reduction in sensitivity for the detection of OTA. Furthermore, dispersion of individual nanotubes from CNT bundles would allow for a significant gain in sensitivity toward the detection of OTA through increased exposure of oxygenated functional groups and available surface area.

4.4.6 Raman Scope characterization of carbon nanotube modifiers

The macroscopic morphology of physically adsorbed MWCNT and SWCNT 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 functionalization 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. Separate Raman scope analysis was performed on SWCNT pellet and supernatant fractions in order to evaluate the removal efficiency of carbonaceous impurities from the SWCNT pellet achieved through centrifugal purification. The MWCNT supernatant fraction was excluded from analysis due to the low retention of carbonaceous material. Figure 4.4.6.1 illustrates the Raman scope images obtained for acid functionalized MWCNT pellet fractions physically adsorbed to a glass silica substrate.

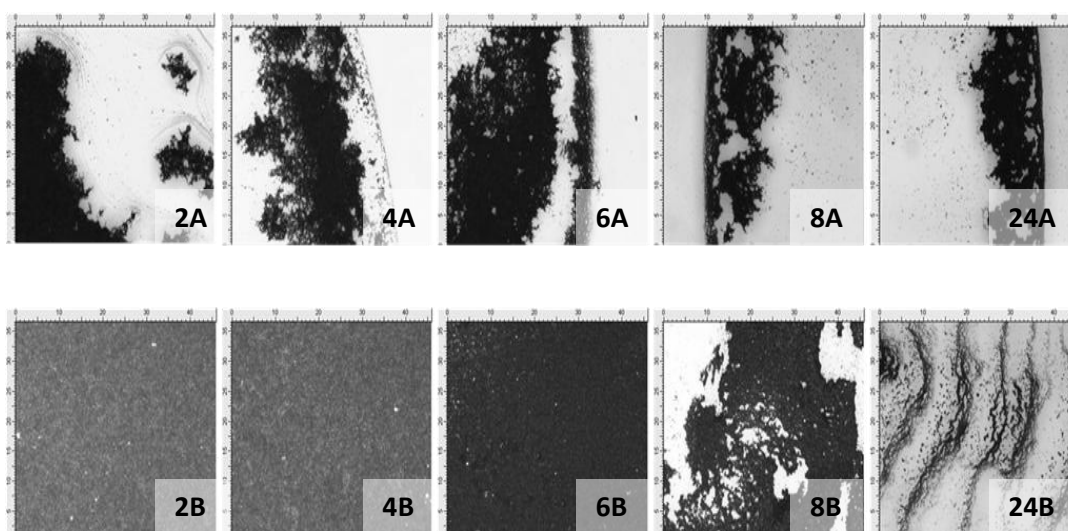


Figure 4.4.6.1 Raman scope images of 1 mg/ml MWCNT fractions in DMF dried on glass at 60°C. Images correspond to high density MWCNT regions of functionalization time 2 (2A), 4 (4A), 6 (6A), 8 (8A) and 24 (24A) hours, and to low density MWCNT regions of functionalization time 2 (2B), 4 (4B), 6 (6B), 8 (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 functionalization 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 functionalization times beyond 8 hours as observed by the irregularity of layer deposition within the centre of the adsorbed fractions shown in Figure 4.4.6.1 (8B) and (24B).

Figure 4.4.6.2 illustrates the Raman scope analysis obtained for SWCNT pellet fractions of increasing acid functionalization times physically adsorbed to the surface of a glass substrate.

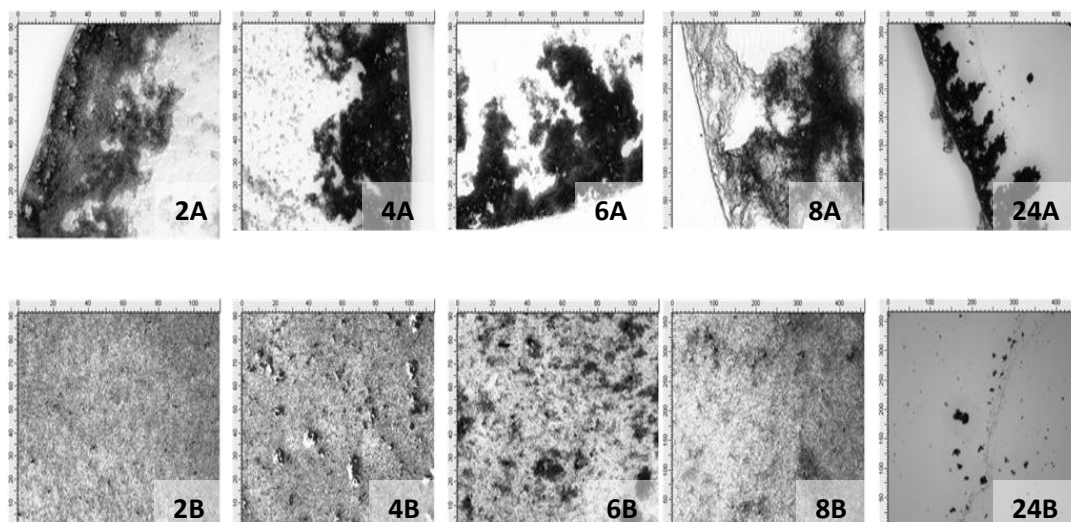


Figure 4.4.6.2 Raman scope images of 1 mg/ml SWCNT **pellet** fractions in DMF dried on glass at 60°C. Images correspond to high density SWCNT regions of functionalization time 2 (2A), 4 (4A), 6 (6A), 8 (8A) and 24 (24A) hours, and to low density SWCNT regions of functionalization time 2 (2B), 4 (4B), 6 (6B), 8 (8B) and 24 (24B) hours. Scale shown in μm .

Similar surface morphologies were observed for the SWCNT and MWCNT pellet fractions of equivalent functionalization time as shown by the concentration of high density SWCNT aggregates at the centre and periphery regions of the adsorbed pellet fraction. Compared to the MWCNT pellet fractions, a reduction in SWCNT aggregate size was observed and accredited to the small nanotube dimensions and high susceptibility to oxidative degradation inherent to SWCNTs.

From Figure 4.4.6.2, the high density SWCNT aggregates were shown to diminish in size upon acid treatment leading to an accumulation of carbonaceous impurities within the SWCNT pellet fractions. Poor purification efficiency for the 8 hour functionalized pellet fraction resulted in considerable retention of carbonaceous impurities within the peripheral SWCNT aggregates. No distinction in impurity retention between the high density SWCNT aggregates for increasing functionalization times could be resolved from Raman scope analysis. Figure 4.4.6.3 shows Raman scope images obtained for the glass substrate surfaces modified with SWCNT supernatant fractions of increasing acid functionalization times.

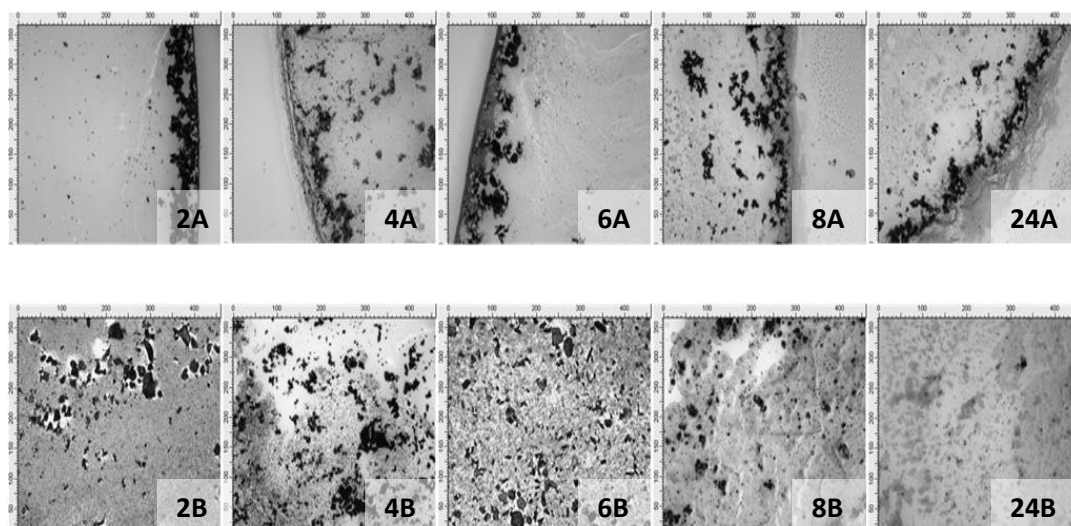


Figure 4.4.6.3 Raman scope images of 1 mg/ml SWCNT **supernatant** fractions in DMF dried on glass at 60°C. Images correspond to high density SWCNT regions of functionalization time 2 (2A), 4 (4A), 6 (6A), 8 (8A) and 24 (24A) hours, and to low density SWCNT regions of functionalization time 2 (2B), 4 (4B), 6 (6B), 8 (8B) and 24 (24B) hours. Scale shown in μm .

A significant reduction in aggregate density was observed for high density SWCNT supernatant fractions due to the preferential retention of low molecular weight carbonaceous material within the supernatant fraction. Dispersion of carbonaceous material observed for SWCNT supernatant fractions at prolonged functionalization times was observed and attributed to the reduction in SWCNT aggregates available for the adsorption and retention of carbonaceous impurities.

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 electrocatalysis. The large degree of carbonaceous impurities retained within the supernatant fractions of the functionalized CNTs also illustrates the necessity for CNT purification, particularly for the SWCNT fractions due to their low nanotube purity and high susceptibility to oxidative degradation upon acid treatment. 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.

4.4.7 Raman spectroscopy characterization of carbon nanotube modifiers

Raman spectroscopy was used to determine the effect of acid treatment on the physical and electronic properties of CNTs (Belin and Epron, 2005). Determination of the Raman spectra allowed for the efficacy of acid functionalization and centrifugal purification of MWCNT and SWCNT 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 4.4.7.1 depicts the Raman spectra obtained for the MWCNT pellet fraction at increasing acid functionalization times.

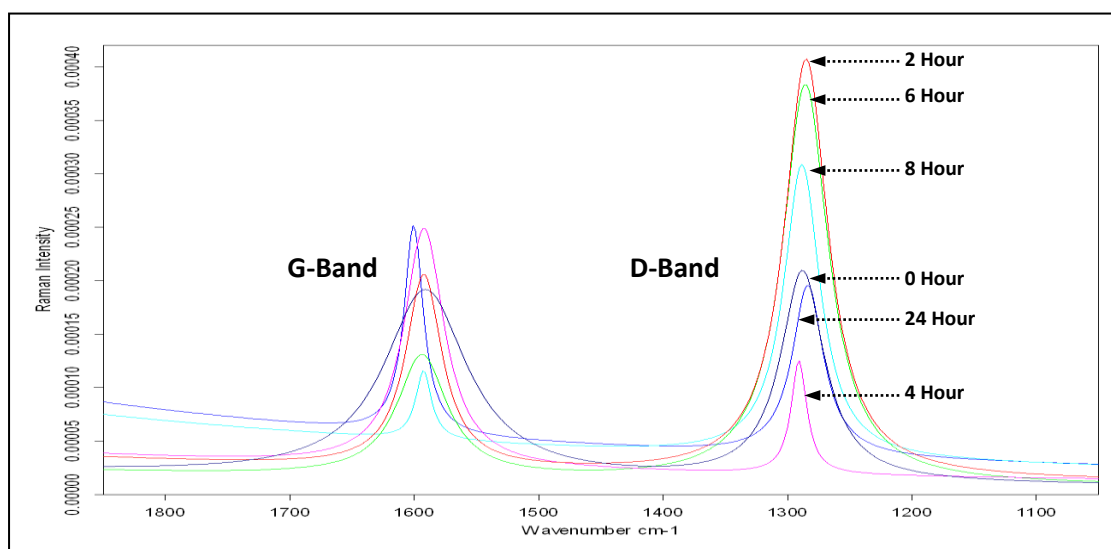


Figure 4.4.7.1 Raman Spectrum of MWCNT fractions of 0 (black), 2 (red), 4 (magenta), 6 (green), 8 (cyan) and 24 (blue) hour functionalization times mixed with 0.5 wt% in KCL taken at 100mW averaged for 1024 scans.

The 2 hour functionalized MWCNT 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 functionalization 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.

The D-band and G-band intensities obtained from Raman spectroscopy analysis of 2, 4, 6, 8, and 24 hour acid functionalized SWCNT pellet fractions are shown in Figure 4.4.7.2.

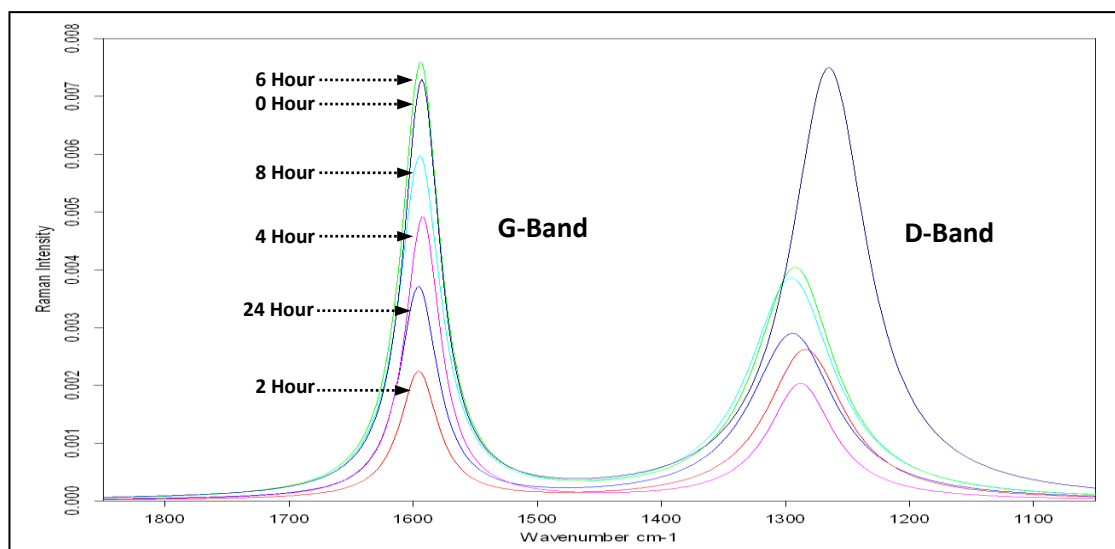


Figure 4.4.7.2 Raman Spectrum of SWCNT pellet fractions of 0 (black), 2 (red), 4 (magenta), 6 (green), 8 (cyan) and 24 (blue) hour functionalization times mixed with 0.5 wt% in KCL taken at 100mW averaged for 1024 scans.

A decrease in G-band intensity was observed between the pristine SWCNT fraction and the 2 hour functionalized SWCNT pellet fraction, followed by a successive increase in G-band intensity for up to the 6 hour functionalized fraction. A similar increase in G-band intensity was observed for the SWCNT supernatant fraction between the functionalization range from 2 to 8 hours as shown in Figure 4.4.7.3.

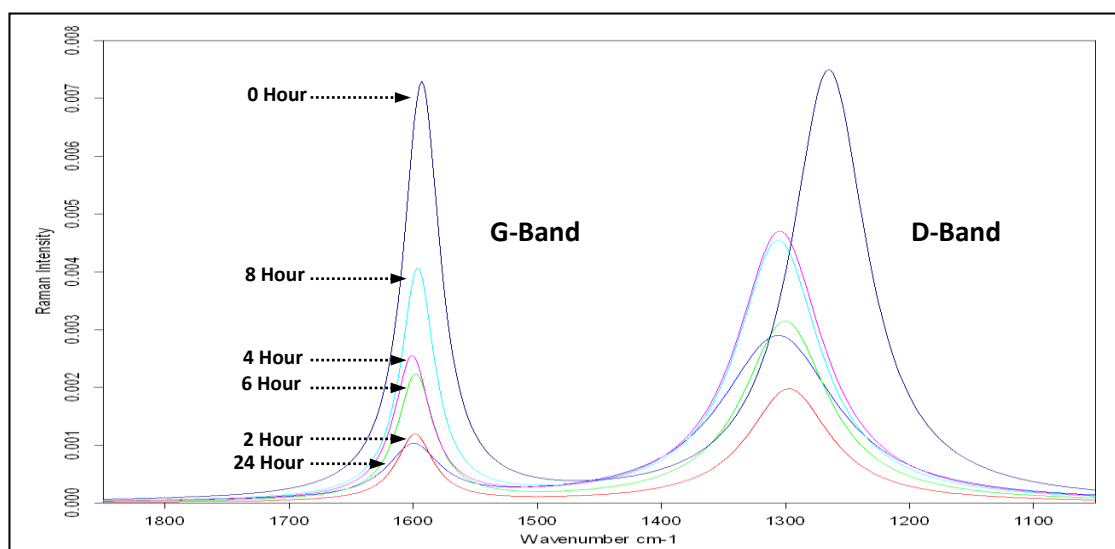


Figure 4.4.7.3 Raman Spectrum of SWCNT supernatant fractions of 0 (black), 2 (red), 4 (magenta), 6 (green), 8 (cyan) and 24 (blue) hour functionalization times mixed with 0.5 wt% in KCL taken at 100mW averaged for 1024 scans.

The D-band intensities for both the SWCNT pellet and supernatant fractions were shown to increase upon increasing functionalization times, relative to the 2 hour functionalized fractions. The SWCNT

pellet fractions generally show lower D-band intensities than that of the supernatant fractions, with the lowest D-band intensities observed for the 2 and 4 hour functionalized pellet fractions. A near 2 fold decrease in D-band intensity was observed for the SWCNT pellet and supernatant fractions functionalized for 2 hours as compared to the pristine SWCNT sample.

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 4.4.7.4 summarizes the D/G band intensities determined for the MWCNT and SWCNT fractions of increasing functionalization times

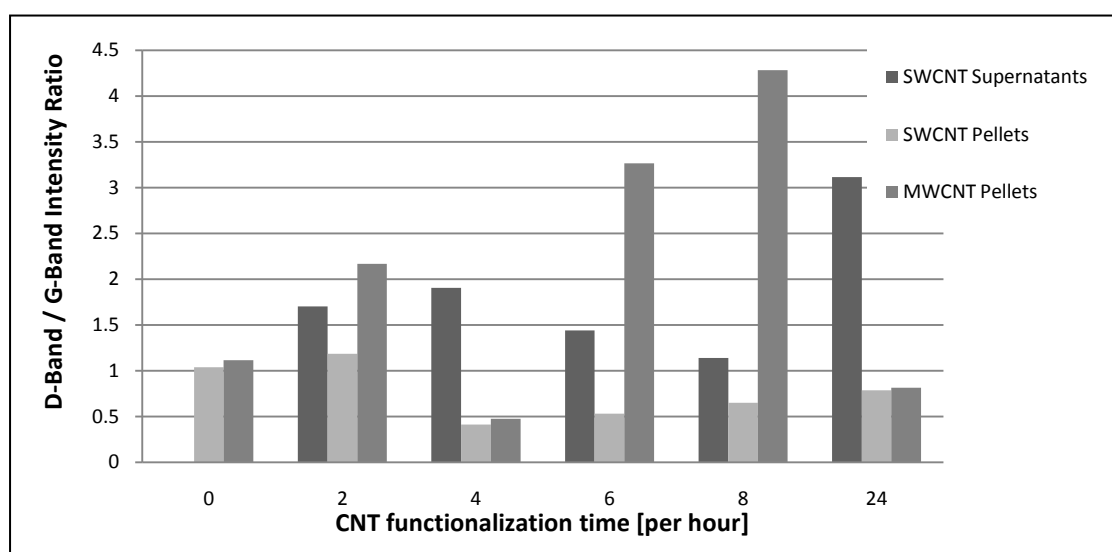


Figure 4.4.7.4 Bar graph comparing the D-band/G-band intensities for MWCNT , SWCNT pellet and SWCNT supernatant fractions of functionalization times 0, 2, 4, 6, 8 and 24 hours. Nanotube fractions were mixed 0.5 wt% in KCL taken at 100mW averaged for 1024 scans.

For the MWCNT fractions for functionalization 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.

D-band/G-bands obtained for the SWCNT pellets show a sharp decrease in intensity beyond the 2 hour functionalized fraction with a further gradual increase in band intensity upon increased functionalization times. With the exception of the 24 hour functionalized fraction, an opposite trend was observed for the SWCNT supernatant fractions with a gradual reduction in D-band/G-band intensity obtained for functionalization times beyond that of 4 hours. The large D-band/G-band intensity of the 24 hour SWCNT supernatant fraction was accredited to its relatively low G-band

intensity. The decrease in D-band intensity from the pristine MWCNT fraction to the 2 hour functionalized fraction was attributed to the removal of carbonaceous impurities initially present in the pristine sample. A more significant decrease in D-band intensity was observed between the pristine and 2 hour functionalised SWCNT fraction, which shows the considerable amount of carbonaceous impurities initially present in the SWCNT sample as compared to the equivalent MWCNT sample. The removal of impurities present in the pristine CNT samples also demonstrates the importance of an initial purification step.

The low D-band intensities for the 2 and 4 hour SWCNT pellet fractions indicate the low degree of amorphous carbon present. The increase in D-band intensity for functionalization times beyond that of 4 hours indicates the increase in amorphous carbon present as a result of CNT degradation. The increase in SWCNT pellet fraction G-band intensity indicates that the structural integrity for the majority of CNTs is maintained up to the 6 hour point. After functionalization times of 6 hours, the linear decrease in G-band intensity would then correspond to a significant breakdown in CNT structure.

This is verified by the large amount of amorphous carbon impurities present in the SWCNT pellet and supernatant fractions for functionalization times beyond 6 hours, as shown by AFM analysis in section 4.6. Furthermore, the extent of structural degradation is shown by the similarity in G band intensity between the 8 hour functionalized SWCNT pellet and supernatant fractions due to the equal retention of SWCNT bundles within the SWCNT fractions as a result of the reduction in SWCNT bundle size upon acid treatment.

The similarity in G-band intensity and gradual decrease in D-band intensity observed for the functionalized MWCNT fractions for functionalization 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 functionalization of the outer MWCNT graphene layers thereby resulted in an increase in D-band/G-band intensity (Datsyuka, et. al, 2008). The increase in D-band/G-band intensity for the 2 hour functionalized SWCNT pellet fraction was accredited to both the functionalization of the outer SWCNT bundles as well as the removal of carbonaceous impurities present in the pristine SWCNT sample (Yu, et. al, 2006).

The reduction in D-band/G-band intensities for the SWCNT pellet fractions beyond a functionalization time of 4 hours was due to the structural degradation of the SWCNTs as shown by the reduction in G-band intensity and the resultant retention of carbonaceous impurities within the collected fractions. A preferential retention of carbonaceous impurities within the SWCNT supernatant fractions as compared to the equivalent pellet fractions was observed as a similarity in G-band intensity with a comparable increase in D-band intensity for the supernatant fractions upon acid treatment. From the above results, acid treatment times up to 4 hours allowed for the functionalization of CNTs without significant degradation of the overall CNT structure and resultant formation of carbonaceous impurities within the CNT pellet fractions, which could account for the decrease in OTA detection sensitivity for functionalization times beyond that of 4 hours.

4.4.8 Voltammetric analysis of OTA in complex media

DPV was used to assess the voltammetric behaviour of OTA in complex media consisting of a commercial apple juice solution. Minimal sample pretreatment was performed on the commercial apple juice other than a 1:2 (v/v) dilution with 0.5 M BR buffer to achieve a final solution pH of 5.5. A standard curve was established from DPV analysis of OTA between the concentration range of 5 μM to 25 μM in order to evaluate the effect of OTA analysis in buffered apple juice solution on the sensitivity and reproducibility of OTA detection. Figure 4.4.8.1 shows the DPVs obtained for an unmodified GCE in buffered apple juice solution of pH 5.5 for the oxidation of OTA between the concentration range of 5 to 25 μM .

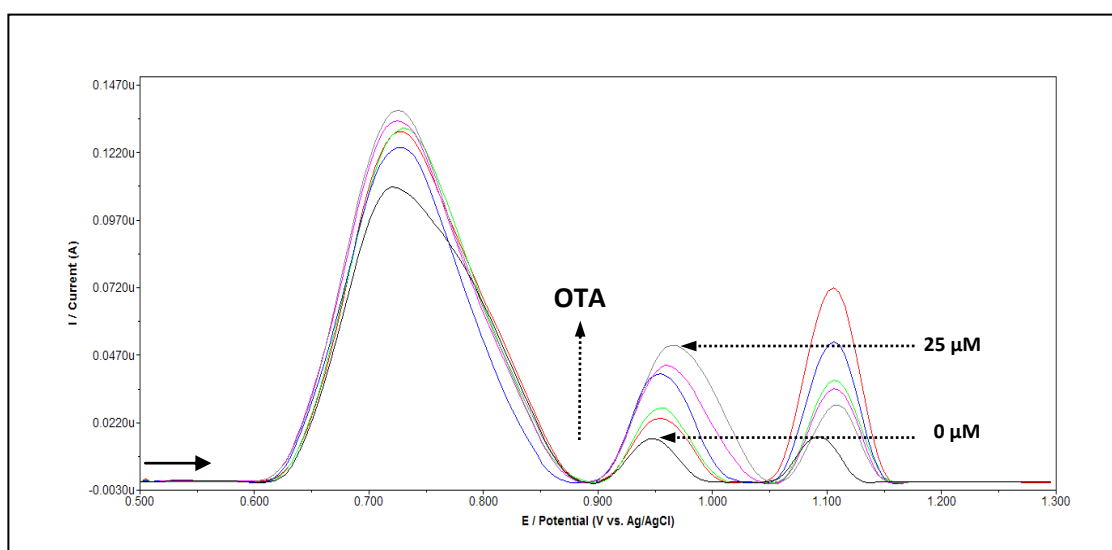


Figure 4.4.8.1 DPVs obtained in apple juice:0.2 M BR buffer pH 5.5 (1:2 v/v) at an unmodified GCE for the oxidation of 0 (black), 5 (red), 10 (green), 15 (blue), 20 (magenta) and 25 (grey) μM OTA. Scan rate 5 mV/s.

Initial voltammetric analysis of buffered apple juice solution pH 5.5 produced three distinctive anodic peaks between the potential range of 0.6 V and 1.2 V respectively. The anodic peak identified at a potential of 0.95 V may interfere with the detection of OTA as the interferent peak is a mere 0.05 V more cathodic than the OTA oxidation peak observed in 0.2 M BR buffer pH 5.5. The peak observed at a potential of 0.95 V showed a linear increase in peak current magnitude with a corresponding shift in peak potential to more cathodic potentials upon successive additions of OTA. The anodic peaks observed at potentials of 0.73 V and 1.11 V were assumed to be produced from the oxidation of interferent species due to the non linear increase in peak current magnitude upon successive additions of OTA. The interferent peaks were assumed to originate from the oxidation of electroactive phenolic or flavonoid constituents initially present in the commercial apple juice (Gamache, et. al, 1993).

The standard curve of peak current magnitude vs. concentration of OTA in buffered apple juice solution for an unmodified GCE is shown in Figure 4.4.8.4. From the standard curve, the LOD of OTA at an unmodified GCE was determined as 2.46 μM .

The difference in sensitivity in the detection of OTA between buffered apple juice solution and BR buffer is illustrated by the peak current magnitude obtained for the oxidation of 5 μM OTA in buffered apple juice solution of $0.095 \pm 0.032 \mu\text{A}$ compared to the peak current of $0.613 \pm 0.221 \mu\text{A}$ obtained in 0.5 M BR buffer of equivalent pH. The decrease in current strength relates to a decrease in relative sensitivity of 85 % for the detection of OTA in buffered apple juice solution compared to 0.5 M BR buffer of equivalent pH. The reduction in sensitivity was attributed to fouling of the electrode surface through the oxidation of apple juice interferents (Naczka and Shahidi, 2006). A reduction in sensitivity due to low ionic strength was dismissed due to dilution of the apple juice with 0.5 M BR buffer during pretreatment.

In addition to an unmodified GCE, DPV analysis was investigated at GCEs modified with 2 hour functionalized MWCNT and SWCNT modifiers in order to evaluate the effect of CNT modification toward the detection of OTA in complex media as shown in Figure 4.4.8.2 and Figure 4.4.8.3.

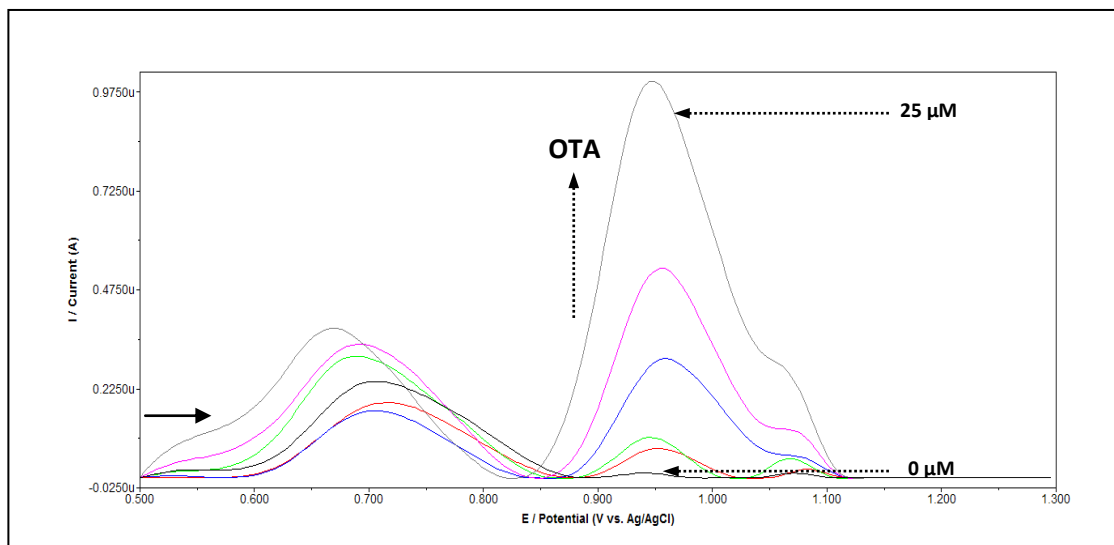


Figure 4.4.8.2 DPVs obtained in apple juice:0.2 M BR buffer pH 5.5 (1:2 v/v) at a 2 hour acid functionalized MWCNT GCE for the oxidation of 0 (black), 5 (red), 10 (green), 15 (blue), 20 (magenta) and 25 (grey) μM OTA. Scan rate 5 mV/s.

An anodic peak potential of 0.95 V was observed for the oxidation of OTA at the MWCNT modified GCE which is comparable to the oxidation potential of OTA at an unmodified GCE. DPV at the MWCNT modified GCE was used to construct a standard curve for OTA between the concentration range of 5 μM to 25 μM , as shown in Figure 4.4.8.4.

The slope of the standard curve produced from MWCNT modification of the GCE was 32 times greater than the slope obtained for OTA analysis at an unmodified GCE reflecting the large amount of electroactive sites present at the MWCNT modification layer. The increase in peak current magnitude afforded by MWCNT modification resulted in a LOD of 0.08 μM for the detection of OTA in buffered apple juice solution of pH 5.5. MWCNT modification produced a 32 times increase in sensitivity compared to equivalent DPV analysis by an unmodified GCE of OTA in buffered apple juice.

Although MWCNT modification produced an increase in sensitivity toward the detection of OTA as compared to an unmodified GCE, a significant decrease in sensitivity was observed for the equivalent DPV analysis of OTA at the SWCNT modified GCE relative to the MWCNT modified GCE. The reduction in sensitivity was attributed to interference from apple juice constituents and not the difference in surface morphology of the MWCNT layers as confirmed by the similarity in reproducibility of current response between both media.

Figure 4.4.8.3 shows the successive increase in peak current magnitude for consecutive additions of OTA with no significant change in the oxidation potential evaluated for the OTA oxidation peak at 0.95 V for the 2 hour functionalized SWCNT modified GCE.

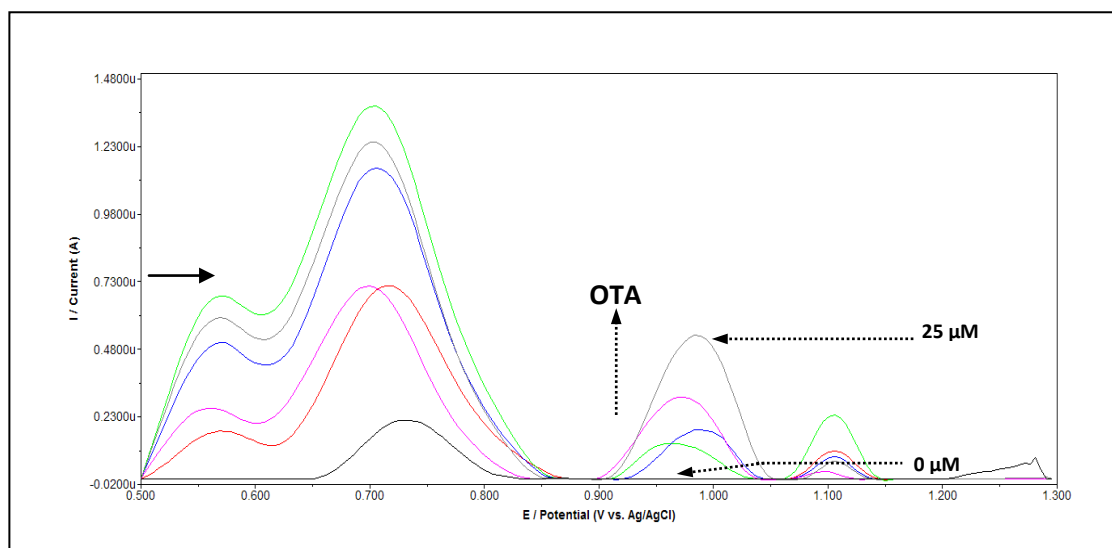


Figure 4.4.8.3 DPVs obtained in apple juice:0.2 M BR buffer pH 5.5 (1:2 v/v) at a 2 hour acid functionalized SWCNT GCE for the oxidation of 0 (black), 5 (red), 10 (green), 15 (blue), 20 (magenta) and 25 (grey) OTA. Scan rate 5 mV/s.

DPV analysis of OTA in buffered apple juice produced an 18 times increase in detection sensitivity as compared to analysis at an unmodified GCE as shown by the LOD of 0.14 μM determined for the SWCNT modified GCE toward the detection of OTA. The LOD determined for OTA at a SWCNT modified GCE in buffered apple juice represents a 95 % decrease in relative sensitivity when compared to equivalent analysis of OTA at SWCNT modified GCEs in 0.5 M BR buffer pH 5.5. As SWCNT modification produced an average 4 fold increase in peak current magnitude for the interferent peak of 0.73 V anodic potential as compared to the MWCNT modified GCE, the reduction in sensitivity toward the detection of OTA was accredited to the preferential electroactivity of SWCNT modified GCEs toward the interferent constituents present in the buffered apple juice solution and to associated co-adsorbent effects.

Figure 4.4.8.4 summarizes the peak current response of increasing concentrations OTA in buffered apple juice solution of pH 5.5 at an unmodified, MWCNT modified and SWCNT modified GCE.

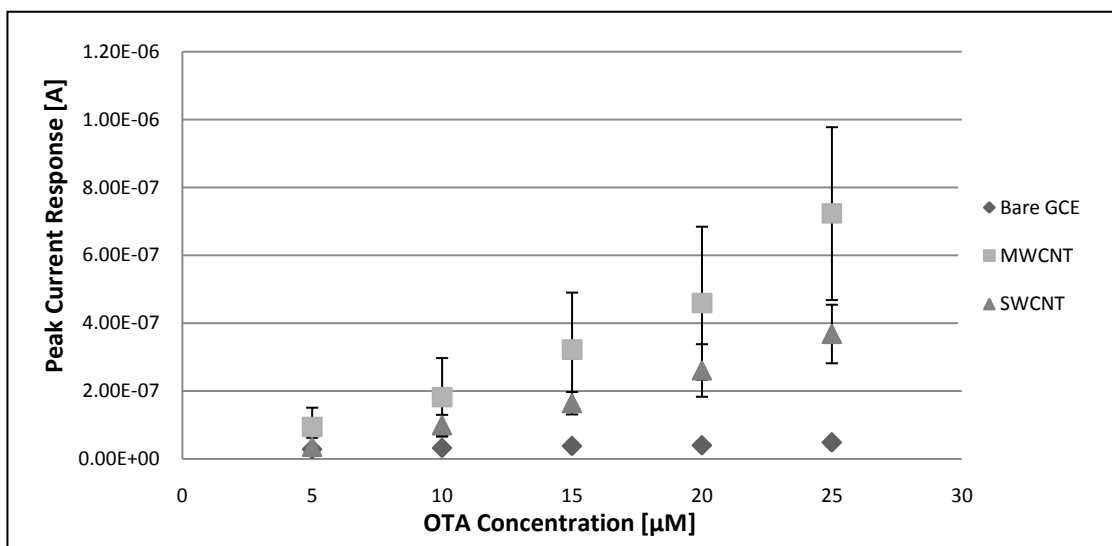


Figure 4.4.8.4 The peak current response of increasing concentrations OTA in apple juice:0.2 M BR buffer pH 5.5 (1:2 v/v) at an unmodified, 2 hour acid functionalized MWCNT and 2 hour functionalized SWCNT GCE.

While further optimization is required for the detection of OTA in commercial apple juice, the linear increase in sensitivity toward the detection of OTA by MWCNT and SWCNT modified GCEs indicates the potential of CNT modification for the electrochemical analysis of OTA in complex media with minimal sample preparation. The presence of interferent species observed at the anodic potential of the OTA oxidation peak raises the importance of the need for specificity in biosensor design and development.

4.5 CONCLUSION

Acid treatment of the MWCNT/SWCNT modifiers produced a significant increase in sensitivity toward the detection of OTA although progress must be made towards limiting the corresponding increase in deviation of current response. To address the low degree of reproducibility, centrifugal purification was performed and evaluated through AFM, Raman spectroscopy and Raman Scope microscopy of the functionalized CNT fractions.

The significant increase in sensitivity toward the detection of OTA was attributed to both the increase in surface area afforded by CNT modification and to the presence of electrocatalytic oxygenated species functionalized to the CNT surface upon acid treatment. The increase in detection sensitivity afforded by SWCNT modification over MWNCT modification was thus attributed to the inherent susceptibility of SWCNTs to oxidative functionalization and higher bundle density upon immobilization. Prolonged acid treatment was shown to decrease the electrocatalytic activity of the MWCNT/SWCNT fractions towards the oxidation of OTA due to degradation of the tubular CNT structure and to the accumulation of carbonaceous impurities within the functionalized CNT fraction. Thus, acid treatment should ideally be restricted to functionalization times of two hours or less with further characterization deemed necessary to identify the optimal treatment time.

The low reproducibility in detection was accredited to the large degree of carbonaceous impurities within the MWCNT/SWCNT fractions as well as to the highly porous and non uniform morphology of CNT bundles modified to the substrate surface through physical adsorption, as confirmed by AFM and Raman Scope analysis. Centrifugal purification of the functionalized MWCNT/SWCNT fractions was shown to sufficiently retain carbonaceous impurities within the supernatant fractions as confirmed by Raman spectroscopy analysis. The efficiency of purification became limited toward prolonged acid treatment times due to the similarity in density between carbonaceous impurities and overoxidised/degraded CNTs. The retention of carbonaceous impurities along with degraded CNTs resulted in a convergence of electroactivity between the CNT pellet and supernatant fractions for functionalization times beyond 4 hours.

As the electrocatalytic activity of the CNT modified GCEs toward the detection of OTA was established experimentally, the effect of CNT modification on the detection of OTA in complex media was evaluated. Subsequent detection in buffered apple juice at CNT modified electrodes produced a substantial increase in sensitivity relative to equivalent detection at an unmodified GCE.

Compared to the MWCNT modified GCE, a decrease in detection sensitivity toward OTA was observed for the SWCNT modified GCE which was attributed to co-adsorption effects of phenolic interferent species to the SWCNT modification surface. Thus, evaluation of SWCNT and MWCNT modification toward the detection of OTA needs to be evaluated and optimized for the required detection matrix and may require the inclusion of a specificity factor.

CHAPTER 5

BIORECOGNITION OF OCHRATOXIN A

5.1 INTRODUCTION

The prevalence of OTA contamination of agricultural products is heavily reliant on pre-harvest and post-harvest conditions conducive to fungal growth (Magan and Aldred, 2005). Environmental conditions prior to harvest including temperature, humidity, insect damage, fungicide use and ecology of fungal species all contribute to the level of fungal contamination and potential production of OTA (Amezqueta, et. al, 2009). As additional contamination can occur during post-harvest storage, transport, processing and packaging, consistent monitoring of OTA contamination is required throughout all important stages of the production chain. Considering the toxicity of OTA, sensitive and reliable methods of detection are required in order to minimise the entry of OTA into the public food supply (Amezqueta, et. al, 2009).

Traditional methods of analysis of OTA in complex biological and environmental media have been achieved through combinations of sample pretreatment and electrophoretic or chromatographic methods which are relatively expensive, time consuming and do not allow for onsite monitoring (Monaci and Palmisano, 2004). An alternative approach to laboratory based techniques is the use of electrochemical sensors which may provide a rapid, sensitive and onsite method for the quantification of OTA (Kaushik, et. al, 2008). Although detection is made possible through direct electrochemical analysis, the incorporation of a biological recognition element is required in order to provide the necessary selectivity of quantification of OTA in complex media.

In biosensor construction, selectivity is achieved through use of various biological components including enzymes, nucleic acid, antibodies or whole cells either in solution or immobilised to the sensor surface (Tothill, 2001). The identification and inclusion of bio-recognition elements is frequently one of the more challenging tasks in biosensor design and development. Although limited research towards the use of biosensor based detection of mycotoxins is available, developments toward the detoxification of OTA in contaminated foodstuffs has identified several biological components capable of providing selectivity through the biotransformation of OTA (Varga, et. al, 2005).

Several publications on the isolation of filamentous fungi capable of degrading OTA are available, one such report by Abrunhosa et al 2002 showed the isolation of 51 strains of filamentous fungi from grapes, with ability to degrade more than 80 % of OTA added to a culture medium with black aspergilli strains of *A. clavatus*, *A. ochraceus*, *A. versicolor* and *A. wentii* shown to be the most effective. Verga et al 2000 evaluated more than 70 *Aspergillus* species with *A. fumigatus* and several black *Aspergillus* species shown to detoxify OTA. The pathway of OTA degradation was suspected to involve a carboxypeptidase secreted by *A. niger* capable of hydrolysing the peptide bond of OTA to form OT α and phenylalanine (Pitout, 1969). Stander et al 2000, screened 23 commercially available hydrolyses for the ability to degrade OTA to nontoxic compounds with a crude lipase preparation derived from *A. niger* proved to substantially hydrolyse OTA to OT α and phenylalanine.

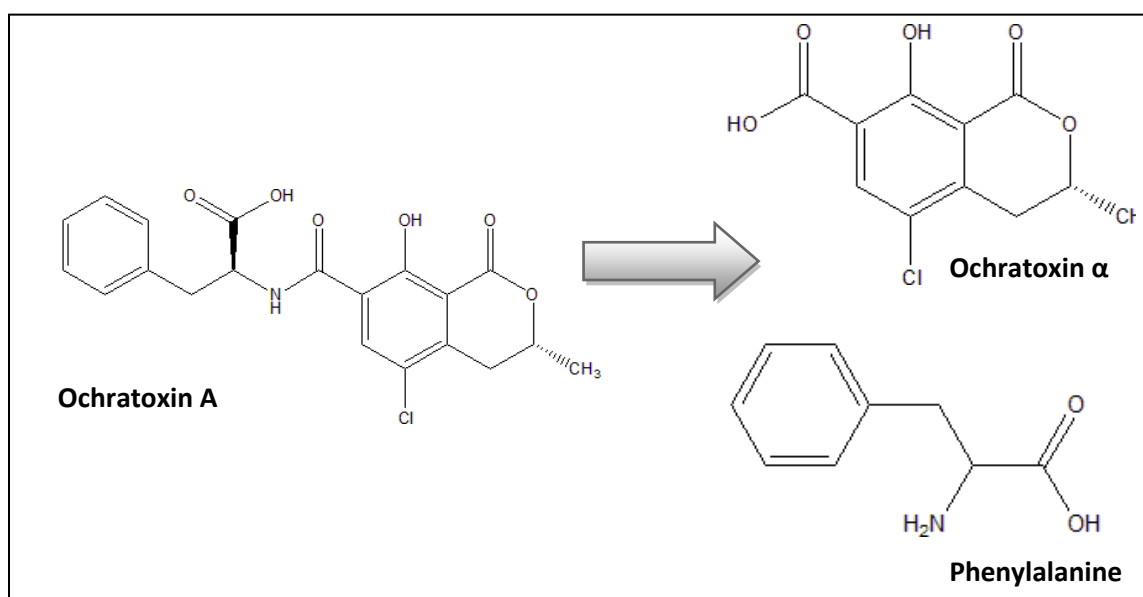


Figure 5.1 The hydrolysis of ochratoxin A to ochratoxin α and L-phenylalanine.

Due to the stability and ease of immobilization offered by enzymatic recognition elements, the identification of a commercially available enzyme with hydrolytic activity towards OTA is of great benefit toward the development of an OTA biosensor. As hydrolysis of OTA to OT α conserved the electroactive 4-chlorophenol substituent of OTA, identification of lipase A as a possible biological recognition element for the electrochemical detection of OTA was investigated.

5.2 AIMS

As a target enzyme was set to be identified for the development of a biosensor for OTA, the overall objective of this chapter was thus to investigate the use of a commercial lipase A preparation as a preliminary biological recognition element aimed toward the detection of OTA in complex media. The potential of lipase A as a selectivity element was assessed as follows:

Evaluation of the activity of crude lipase A preparation by determination of the Michaelis-Menton kinetics of lipase A toward the hydrolysis of p-nitrophenyl myristate.

Evaluation of the hydrolytic activity of crude lipase A preparation toward OTA as assessed by fluorescence spectroscopy.

Evaluation of the hydrolytic activity of crude lipase A preparation toward OTA through the quantification of OTA hydrolysis and resultant formation of OT α through DPV.

The conformation of lipase A induced hydrolysis of OTA to OT α and identification of potential means of lipase A purification through TLC analysis.

5.3 METHODOLOGY

General electrochemical methods performed in this section are outlined in chapter 2 with specific methodology outlined below:

5.3.1 Instrumentation

UV-Vis spectrophotometry measurements were performed on a PowerWaveX spectrophotometer (BIO TEK Instrumentals Inc, United States of America) and analysed by KCJunior software (BIO TEK Instrumentals Inc, United States of America).

Fluorescence spectrophotometry was measured using a F-2500 fluorescence spectrophotometer (HITACHI, Japan) and analyzed by FL Solutions software (HITACHI, Japan).

TLC Plates were visualized by a UVIPRO Chemi gel doc system (UVITEC, United Kingdom) and analyzed by UVIPRO software (UVITEC, United Kingdom).

5.3.2 Reagents

Lipase A "Amano" preparation (Amano Enzyme Inc, Japan) and p-nitrophenyl myristate (Sigma Aldrich) were used for the evaluation of OTA hydrolysis.

Lipase A hydrolysis of ochratoxin A

Enzymatic hydrolysis of OTA was performed by the addition of 30 μ M OTA to solutions of lipase A prepared prior to use in 0.2 M phosphate buffer pH 6 at 40 °C. The hydrolysis time and lipase A concentration was set as required.

Acid hydrolysis of ochratoxin A

Chemical hydrolysis of 300 μ M OTA was produced by heating OTA in hydrochloric acid under reflux for a period of 48 hours.

Fluorescence spectroscopy of ochratoxin A hydrolysis

Fluorescence spectrophotometry was used to evaluate the natural fluorescence of OTA in 0.2 M phosphate buffer pH 6. An excitation wavelength of 340 nm and corresponding maximum emission wavelength of 448 nm was determined for OTA analysis by fluorescence spectroscopy.

Molecular exclusion chromatography of lipase A

A glass column was packed with sephadex G-25 (35-140 μ m, 150 ml, GE Healthcare, United Kingdom) and conditioned with a 0.2 M phosphate buffer pH 6 mobile phase at 4 °C. A lipase A solution (10 mg/ml) was transferred onto the column and eluted with 0.2 M phosphate buffer pH 6. Fractions (1.5 ml) of the eluant were collected and analysed for protein content by UV-VIS spectroscopy at an absorbance wavelength of 260 nm and 280 nm.

Ultrafiltration of lipase A

A 10 mg/ml lipase A solution (5 ml) in 0.2 M phosphate buffer pH 6 at 4 °C was concentrated by ultra filtration on a Macrosep centrifugal device Pall, United States of America) at 5000 g. The resultant pellet fraction was resuspended and re-centrifuged for 10 consecutive runs at 5000 g in 4 °C. The final retained fraction was resuspended in 0.2 M phosphate buffer pH 6 at 4 °C and labelled as lipase A_T. A subsequent Ultrafiltration step was performed on lipase A_T with the collected retained and eluted fractions labelled as lipase A_R and lipase A_E respectively.

Thin layer chromatography of hydrolyzed ochratoxin A

For the TLC analysis of OT α , 100 μ M OTA, 1mg/ml lipase A_T, lipase A_R hydrolyzed OTA, lipase A_E hydrolyzed, lipase A_T hydrolyzed OTA and acid hydrolyzed OTA samples were analyzed on Aluminium TLC plates (Silica gel 60, Merck) developed with toluene/ethyl acetate/formic acid (5:4:1). The TLC plate was visualized under a 366 nm UV light. For TLC analysis of l-phenylalanine, 100 μ M OTA, 1mg/ml lipase A_T, lipase A_R hydrolyzed OTA, lipase A_E hydrolyzed, lipase A_T hydrolyzed OTA and acid hydrolyzed OTA samples were analyzed on Aluminium TLC plates (Silica gel 60, Merck) developed with butanol/acetic acid/milliQ (5:2:3). The TLC plate was visualized by staining in 0.5% ninhydrin at 110 °C for 5 min.

5.3.3 Methodologies

5.4.1 Enzyme kinetics of lipase A toward p-nitrophenyl myristate

The hydrolytic activity of lipase A was evaluated through the hydrolysis of p-nitrophenyl myristate (pNM) in 0.2 M phosphate buffer at an optimum pH and temperature of pH 6 and 40 °C. Reaction rate was determined from the gradient of the curves of product concentration versus time. Product concentration was determined spectrophotometrically at 405 nm.

5.4.2 Fluorescence Spectroscopy detection of ochratoxin A hydrolysis by lipase A

A standard curve for the fluorometric detection of OTA was constructed by analysis of the emission spectra of OTA (448 nm) measured at an excitation wavelength of 340 nm in 0.2 M phosphate buffer pH 6 for OTA concentrations 1, 5, 10, 20, 30, 40 and 50 µM. Similar fluorescent analysis was performed to detect the decrease in OTA emission spectra upon hydrolysis by 1 mg/ml lipase A.

5.4.3 Electrochemical detection of ochratoxin A hydrolysis by Lipase A

DPV analysis was performed on 12.5 µM OTA solutions in 0.2 M phosphate buffer pH 6.5 at 40 °C hydrolyzed by 0.5 mg/ml lipase A. DPV measurements were taken at 10 minute intervals over the 120 minute hydrolysis reaction. DPV analysis was also performed on 12.5 µM OTA solutions in 0.2 M phosphate buffer pH 6.5 hydrolyzed at 40 °C in 0.1, 0.5, 1, 2 and 5 mg/ml standard additions of lipase A.

5.4.4 Thin layer chromatography of ochratoxin A hydrolysis products

DPV analysis was used to assess the voltammetric profiles of 30 µM OTA hydrolyzed by lipase AT, lipase AE, lipase AR and HCl reflux in 0.2 M phosphate buffer pH 6. DPV analysis was correlated to OTA, OTα and p-phenylalanine bands identified by TLC.

5.4 RESULTS AND DISCUSSION

5.4.1 Enzyme kinetics of lipase A toward p-nitrophenyl myristate

Enzymatic hydrolysis of OTA was investigated as a viable means to achieve substrate specificity toward the electrochemical detection of OTA in complex media. Stander et. al, 2000 investigated the hydrolytic activity of over 23 commercially available hydrolyases to degrade OTA into nontoxic components. Of the 23 enzymes tested, only a lipase preparation derived from *Aspergillus niger*, Lipase A, could substantially hydrolyze OTA into OT α and phenylalanine, as confirmed by HPLC with fluorescence detection. As the electroactive 4-chlorophenol substituent of OTA is retained within the chemical structure of OT α , lipase A hydrolysis was selected as a premise for the electrochemical detection of OTA in complex media.

Multiple protein bands were observed for SDS-PAGE electrophoresis of the lipase A preparation. Stander et al, 2000 reported a single step purification of the preparation by anion exchange chromatography to a pure enzyme fraction capable of hydrolyzing OTA into OT α and phenylalanine. The pure enzyme fraction was subsequently identified as a lipase through the cleavage of p-nitrophenyl palmitate, a true lipase substrate (Pancreach and Baratti, 1996). Although a purification factor of 300 was reported by Stander et al, 2000, the crude lipase A preparation was used for preliminary development of the OTA biosensor platform.

To evaluate the hydrolytic activity of the commercial lipase A obtained, the steady state kinetic behaviour of the lipase preparation was evaluated through the hydrolysis of p-nitrophenyl myristate (pNM) in 0.2 M phosphate buffer at an optimum pH and temperature of pH 6 and 40 °C respectively (Hasan, et. al, 2009). The pNM substrate was selected to provide consistency in kinetic work previously reported by Stander, et. al, 2000. The optimal conditions for lipase A hydrolytic activity is shown in Table 5.4.1.1 .

Table 5.4.1.1. Optimal conditions for lipase A hydrolysis adapted from Amano Enzyme Inc.

pH Stability	2 - 9
Optimum pH	4.5 - 6.5
Thermostability	0°C - 50°C
Optimum temperature	40°C - 45°C

Figure 5.4.1.1 illustrates a standard curve indicating the generation of p-nitrophenol released from pNM between the concentration range of 0.001 mM and 0.5 mM in 0.2 M phosphate buffer pH 6 as monitored by UV-VIS spectroscopy at an absorbance of 405 nm.

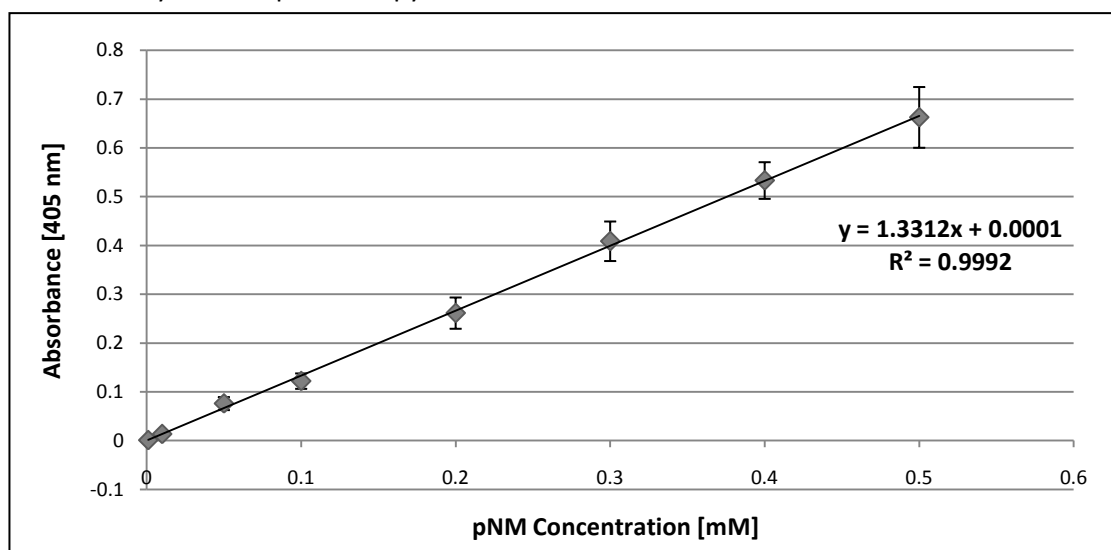


Figure 5.4.1.1 Standard curve obtained from UV-VIS analysis for increasing concentrations of pNM ranging from 0.001 mM to 0.5 mM in 0.2 M phosphate buffer pH 6 measured at an absorbance of 405 nm. Number of replicates (3). Error bars show standard deviation from mean.

Linearity in the quantification of pNM in phosphate buffer was observed producing a correlation coefficient of 0.999 au. From the slope of the standard curve, a molar extinction coefficient (ϵ_{pNM}) of $2262 \text{ M}^{-1} \text{ cm}^{-1}$ was determined for pNM in 0.2 M phosphate buffer pH 6.

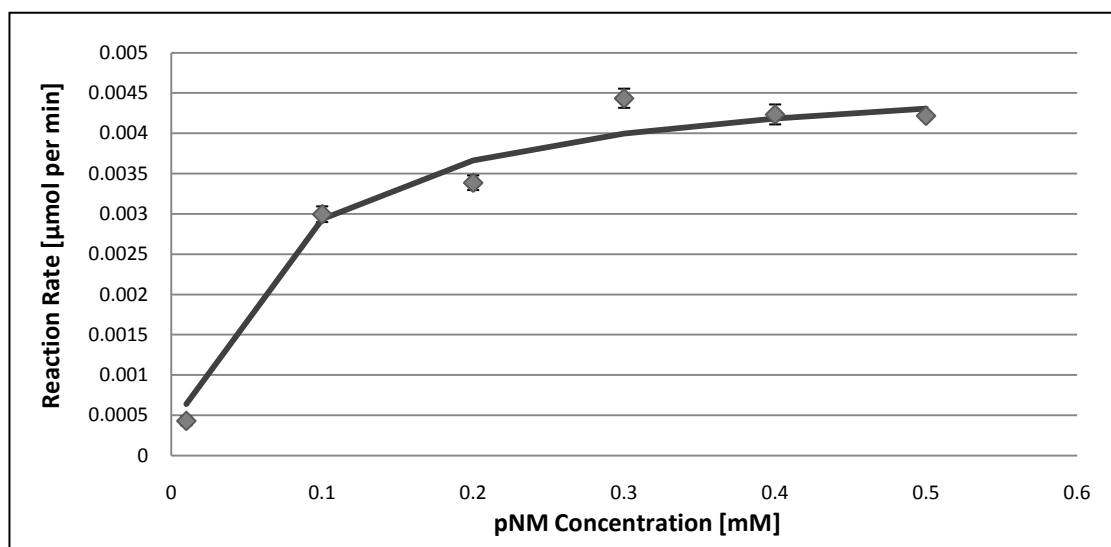


Figure 5.4.1.2 The rate of pNM hydrolysis measured at a substrate concentration of 0.01 mM to 0.5 mM for a fixed lipase A concentration of 0.01 mg/ml in 0.2 M phosphate buffer pH 6. The release of p-nitrophenol from pNM was measured spectrophotometrically at 405 nm at 40 °C. Number of replicates (3). Error bars show standard deviation from mean.

From Figure 5.4.1.2, the rate of pNM hydrolysis followed Michaelis-Menton kinetics between the pNM concentration range of 0.01 mM and 0.5 mM as observed from the least squares nonlinear regression fitting of the Michaelis-Menton equation stated as:

$$V_0 = \frac{V_{\max (app)} [S]}{K_{m(app)} + [S]} \quad (5.4.1.1)$$

Where V_0 represents the initial rate of reaction, $V_{\max (app)}$ the maximum rate of reaction, $K_{m(app)}$ the Michaelis constant and S the pNM concentration (Campbell and Farrel, 2006). The kinetic parameters of $V_{\max (app)}$ and $K_{m(app)}$ were calculated from the regression lines of best fit as 5.06 nmol.min⁻¹ and 76.28 μ M respectively.

The kinetic parameter of $K_{m(app)}$ is near 100 fold lower than the K_m value of 7.37 mM reported for the hydrolysis of pNM by a low molecular weight lipase purified from *Aspergillus niger*, which implies a high affinity of lipase A to the pNM substrate (Shu, et. al, 2007). The high affinity to pNM confirms the lipase activity of the lipase A preparation. The term “apparent” was assigned to the calculated kinetic parameters due to cumulative reaction rates of multiple enzymes present within the lipase A preparation. Approximation of $V_{\max}$ at a pNM concentration of 0.5 mM allowed for the determination of turnover number, $k_{cat(app)}$ defined as (Campbell and Farrel, 2006):

$$k_{cat(app)} = \frac{V_{\max (app)}}{[E_t]} \quad (5.4.1.2)$$

Where E_t represents the total amount of lipase A. The value of $K_{cat(app)}$ was determined as 3.74 μ mol.min⁻¹.mg⁻¹ from the linear plot of maximum reaction rate and total amount of lipase A, as shown in Figure 5.4.1.3. The value of $K_{cat(app)}$ is significantly greater than the turnover number of lipase A reported for the hydrolysis of various p-nitrophenyl esters in aqueous media (Furutani, et. al, 1995). The high value of $K_{cat(app)}$ was accredited to the optimal conditions selected for the hydrolysis of pNM in 0.2 M phosphate buffer.

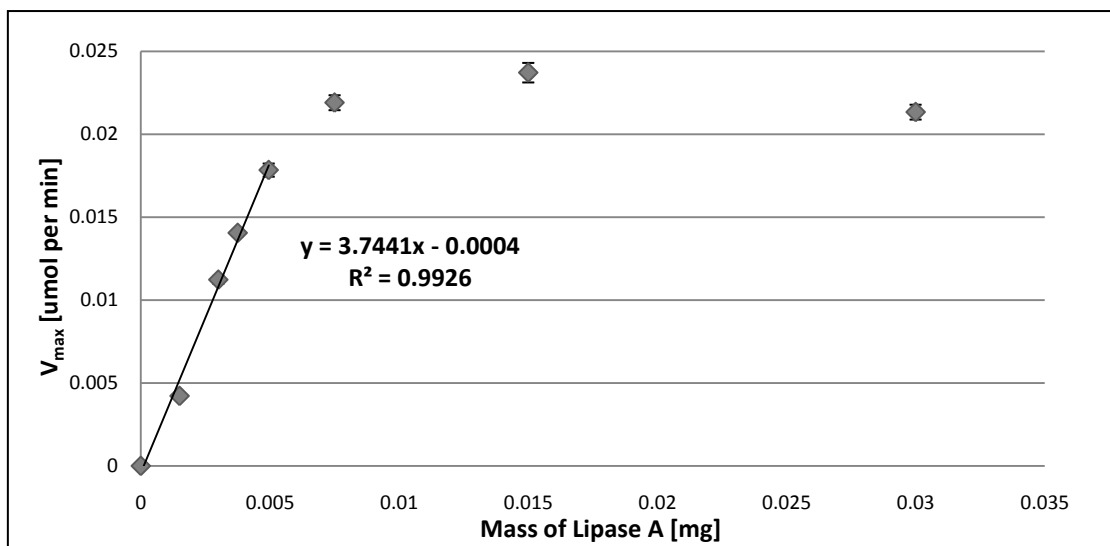


Figure 5.4.1.3 Linear plot of maximum reaction rate vs. mass of lipase A determined from the rate of pNM hydrolysis measured at a fixed pNM concentration of 0.5 mM in 0.2 M phosphate buffer pH 6. The release of p-nitrophenol from pNM was measured spectrophotometrically at 405 nm at 40 °C. Number of replicates (3). Error bars show standard deviation from mean.

5.4.2 Fluorescence Spectroscopy detection of ochratoxin A hydrolysis by lipase A

Fluorescence spectroscopy has become one of the most sensitive, robust and commonly available detection methods used for the routine analysis of OTA in complex media. The sensitivity in fluorometric detection is afforded by the high intensity of fluorescence and large separation in absorption and emission spectra inherent to OTA (Cigic and Prosen, 2009). The fluorescent nature of OTA originates from the isocoumarin moiety which exhibits an excitation absorbance at 330 nm with a corresponding fluorescence between the emission spectra of 460 nm and 480 nm (Frenette, et. al 2008). To evaluate the sensitivity and reproducibility in detection of OTA by fluorescence spectroscopy, a standard curve was constructed for the OTA concentration range of 1 μ M to 50 μ M.

The emission spectra observed for the fluorometric detection of OTA produced two separate fluorescent peaks at emission wavelengths of 336 nm and 448 nm accredited to the excitation wavelength and to the OTA fluorophore respectively. An increase in emission intensity at 448 nm was observed for successive additions of OTA, producing a linear increase in emission intensity between the OTA concentration range of 1 μ M to 40 μ M.

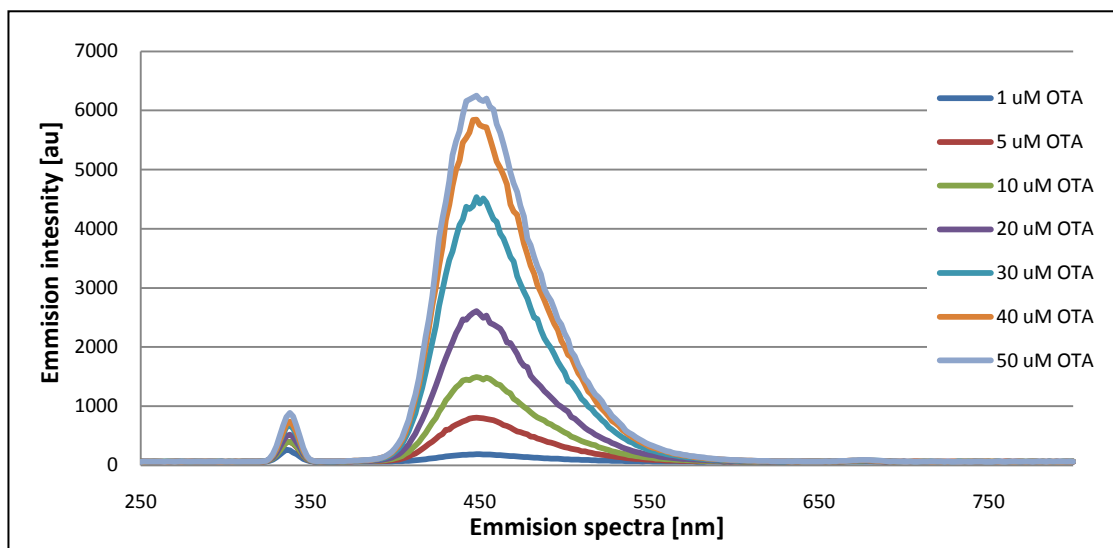


Figure 5.4.2.1 The emission spectra measured at an excitation wavelength of 340 nm in 0.2 M phosphate buffer pH 6 at OTA concentrations of 1, 5, 10, 20, 30, 40 and 50 μM.

As shown by the standard curve in Figure 5.4.2.2, the linearity in emission intensity is illustrated by the correlation coefficient of 0.995. From the slope of the standard curve, a LOD of 0.04 μM was determined for the fluorescence analysis of OTA in 0.2 M phosphate buffer pH 6. The LOD determined from fluorescence relates to a 10 fold increase in sensitivity when compared to an LOD of 0.28 μM obtained from DPV analysis of OTA, as shown in section 2.6.

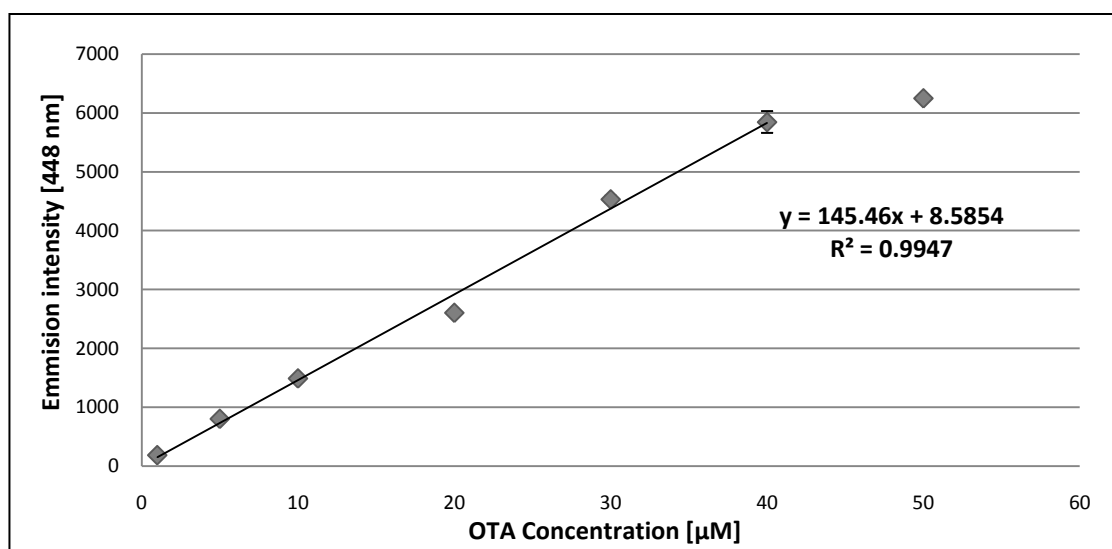


Figure 5.4.2.2 Standard curve obtained from fluorescent analysis for increasing concentrations of OTA ranging from 1 μM to 50 μM in 0.2 M phosphate buffer pH 6 measured at an excitation wavelength of 340 nm. Number of replicates (3). Error bars show standard deviation from mean.

The sensitive and highly reproducible detection of OTA by fluorescence spectroscopy allowed for the evaluation of OTA hydrolysis through the action of lipase A. Figure 5.4.2.3 illustrates the emission spectra measured at an excitation wavelength of 340 nm for the hydrolysis of 12.5 μ M OTA in 0.2 M phosphate buffer pH 6 for a period of 120 minutes. The hydrolysis of OTA was initiated by the addition of 0.5 mg/ml lipase A at a hydrolysis time of 0 minutes.

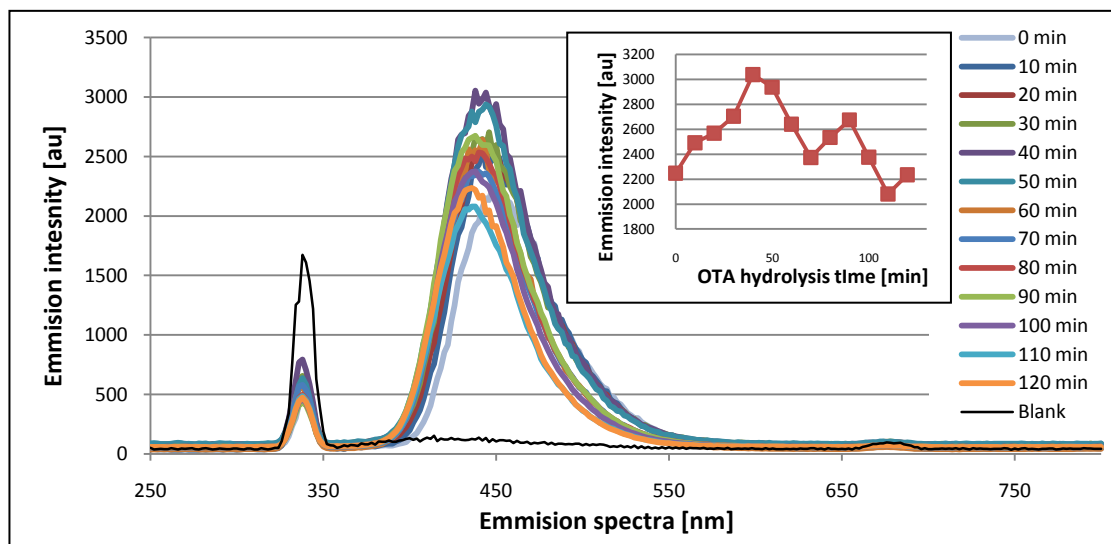


Figure 5.4.2.3 The emission spectra measured at an excitation wavelength of 340 nm at 10 minute intervals for a period of 120 minutes in 0.2 M phosphate buffer pH 6 for 12.5 μ M OTA in solution with 0.5 mg/ml lipase A.

As shown by the insert in Figure 5.4.2.3, no significant decrease in emission intensity was observed for the OTA fluorescent peak upon the addition of lipase A. As OT α exhibits a fluorescent peak at an emission wavelength of 430 nm, the fluctuation in emission intensity observed for OTA was attributed to the similarity in emission spectra between the OTA and OT α fluorescent peaks whereby the decrease in OTA emission intensity was offset by the increase in OT α emission intensity upon OTA hydrolysis. Although the fluctuation in intensity prevented the direct quantification of OTA hydrolysis, a linear shift in peak emission wavelength from 448 nm to 436 nm was observed for the enzymatic conversion of OTA to OT α as shown in Figure 5.4.2.4.

A prolonged hydrolysis time of 48 hours resulted in the formation of a fluorescent peak at an emission wavelength of 436 nm. Hence, with the assumption that the fluorescent peak of OT α occurs at an emission wavelength of 436 nm in 0.2 M phosphate buffer pH 6 then near complete hydrolysis of OTA would have occurred at a hydrolysis time of 110 minutes for the addition of 0.5 mg/ml lipase A.

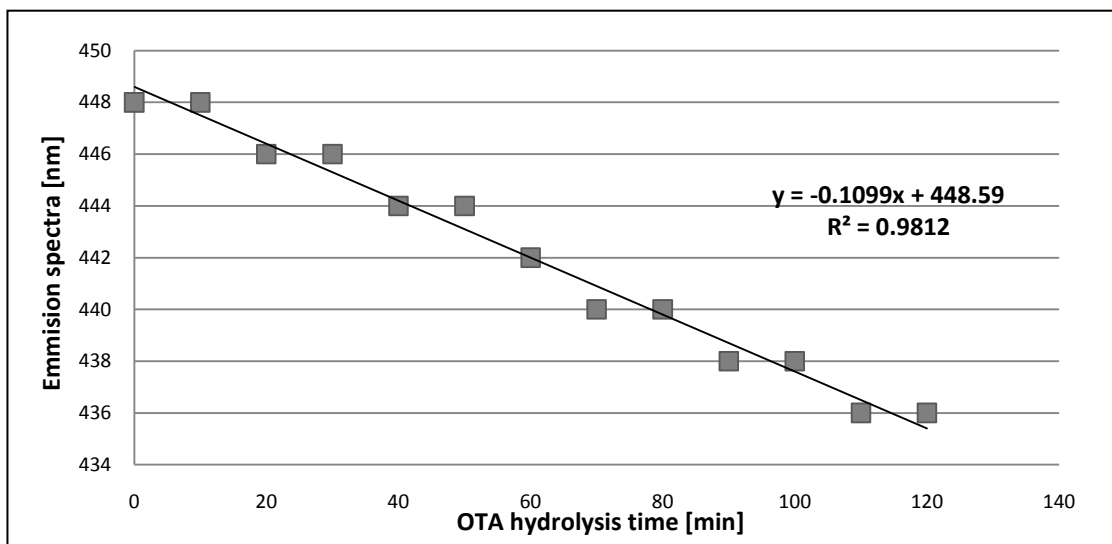


Figure 5.4.2.4 The peak emission wavelength measured at an excitation wavelength of 340 nm at 10 minute intervals for a period of 120 minutes in 0.2 M phosphate buffer pH 6 for 12.5 μ M OTA in solution with 0.5 mg/ml lipase A.

With the assumption that complete hydrolysis of OTA to OT α would result in a shift in emission wavelength from 448 nm to 436 nm as confirmed by the 48 hour hydrolysis of OTA by lipase A, then from Figure 5.4.2.4, near complete hydrolysis of 12.5 μ M OTA would have occurred at a hydrolysis time of 110 minutes. The rate of OTA hydrolysis by the action of lipase A correlates to a 16 times slower hydrolysis rate than analysis reported by Stander et. al, 2000. The increase in reaction rate reported by Stander et. al, 2000 was attributed to a purification of the lipase A preparation prior to the evaluation of hydrolysis rate toward OTA.

5.4.3 Electrochemical detection of ochratoxin A hydrolysis by Lipase A

The use of DPV toward the specific detection and quantification of lipase A induced hydrolysis of OTA to OT α was investigated. DPV was selected to provide a quantitative method of analysis supportive of the qualitative fluorescent determination of OTA hydrolysis reported in section 5.2. The large differential between the analytical signal and background charging current inherent to DPV afforded the sensitivity and selectivity required for the quantification of OTA hydrolysis. Figure 5.4.3.1 illustrates DPVs obtained for the hydrolysis of 12.5 μ M OTA in 0.2 M phosphate buffer pH 6.5 measured at 20 minute intervals for a period of 110 minutes at 40 °C. The hydrolysis of OTA was initiated by the addition of 0.5 mg/ml Lipase A at a hydrolysis time of 0 minutes.

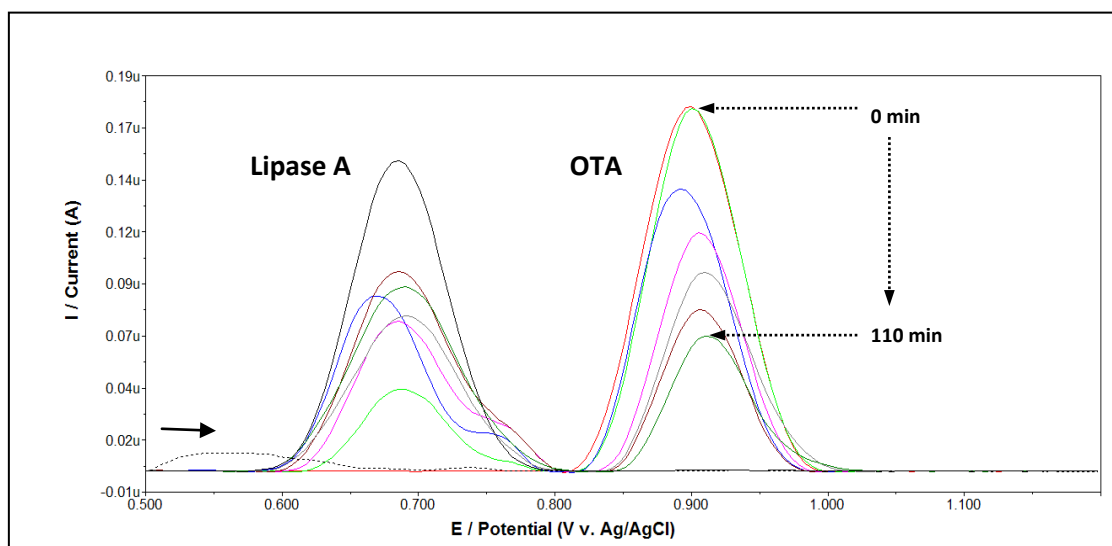


Figure 5.4.3.1 DPV analysis of 12.5 μM OTA in a 0.5 mg/ml lipase A solution of 0.2 M phosphate buffer pH 6 at 40 $^{\circ}\text{C}$ measured at 20 minute intervals for a period of 100 minutes. DPV scans correspond to hydrolysis times of 0, 10, 30, 50, 70, 90 and 110 minutes. Blank solution is shown by a dotted line. Scan rate 5 mV/s.

DPV analysis showed the formation of a single anodic peak of potential 0.90 V corresponding to the oxidation of OTA prior to the addition of lipase A. Followed by the formation of the primary OTA peak, a secondary peak of potential 0.69 V was observed upon the immediate addition of lipase A. Subsequent hydrolysis resulted in a linear decrease in peak current amplitude for the OTA oxidation peak as shown in Figure 5.4.3.2. A correlation coefficient of 0.967 indicates the linearity in current response. Figure 5.4.3.2 depicts the decrease in current amplitude of the OTA anodic peak resulting from Lipase A induced hydrolysis in 0.2 M phosphate buffer pH 6 for a period of 120 minutes at 40 $^{\circ}\text{C}$.

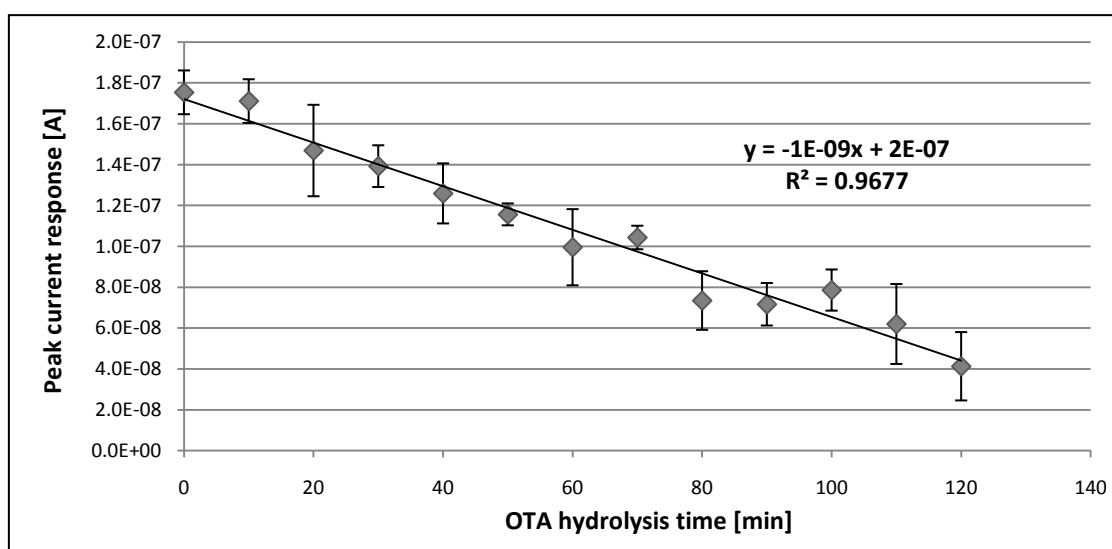


Figure 5.4.3.2 The peak current response for OTA measured by the DPV analysis of 12.5 μM OTA in a 0.5 mg/ml lipase A solution of 0.2 M phosphate buffer pH 6 at 40 $^{\circ}\text{C}$.

Resolution of the OTA anodic peak and linearity in current response expressed by DPV analysis enabled both the verification and quantification of OTA hydrolysis by lipase A. Extrapolation of the linear relation between current response and hydrolysis time to baseline current proved near complete degradation of OTA at a hydrolysis time of 150 minutes which relates to a slower hydrolysis rate than previously assumed from fluorescence spectroscopy in section 5.2. The hydrolysis rate of OTA in 0.2 M phosphate buffer pH 6.5 at 40 °C was determined from DPV analysis as 0.28 U/g. The slow rate of OTA hydrolysis was attributed to both the low purity of the lipase A preparation and to possible interference in enzymatic binding due to the chloride group present on the isocoumarin moiety of OTA.

DPV analysis of the lipase A standard solution containing an OTA concentration of 0 μ M showed the formation of an anodic peak of identical potential to the secondary anodic peak observed during the hydrolysis of OTA. Considering the low protein content of the lipase A preparation, determined as 11.62 wt% by Bradford assay, the secondary anodic peak of potential 0.69 V was identified as an electroactive interferent present in the lipase A preparation.

As no additional peaks were observed during OTA hydrolysis, the broad lipase A interferent peak exhibiting an average peak width of 85 mV was suspected to mask the OT α peak formed during the hydrolysis of OTA. Isolation of a pure sample of OT α would assist in evaluation of the OT α peak.

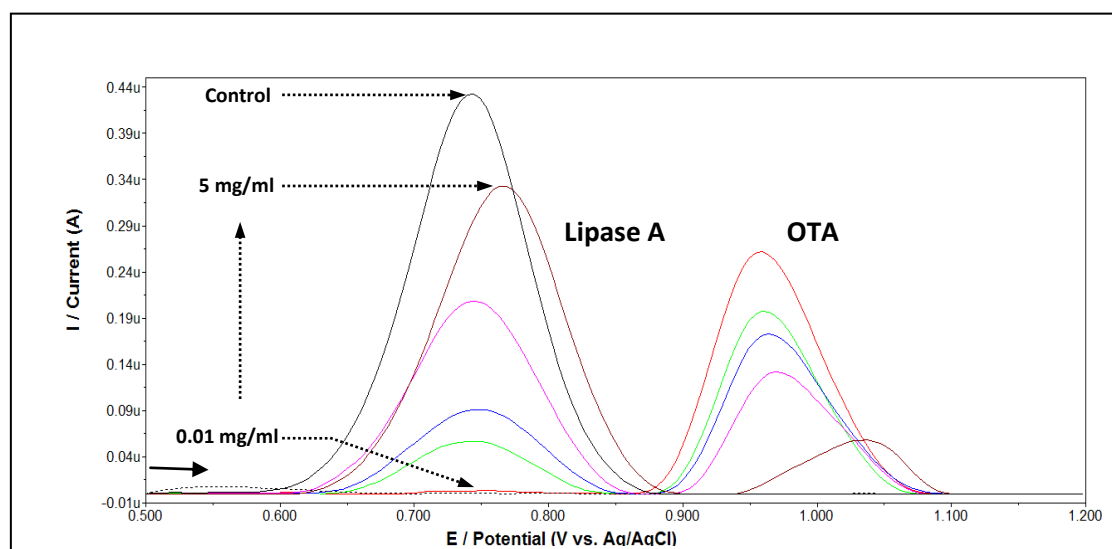


Figure 5.4.3.3 DPVs obtained for the hydrolysis of 12.5 μ M OTA in 0.2 M phosphate buffer pH 6 at 40 °C for lipase A concentrations of 0.01, 0.5, 1.2 and 5 mg/ml. Control solution containing 5 mg/ml lipase with no OTA is shown. DPVs were measured at a hydrolysis time of 30 minutes. Blank solution is shown by a dotted line. Scan rate 5 mV/s.

As shown in Figure 5.4.3.3, the linear increase in current amplitude observed for the secondary anodic peak upon standard additions of lipase A confirmed the origin of the interferent species to be within the lipase A preparation. Standard additions of lipase A was shown to increase the OTA hydrolysis rate, whereby a lipase A concentration of 5 mg/ml produced a 95 % reduction in peak current amplitude of the OTA peak after a hydrolysis time of 30 minutes. The decrease in current amplitude per increase in lipase A concentration is shown in Figure 5.4.3.4.

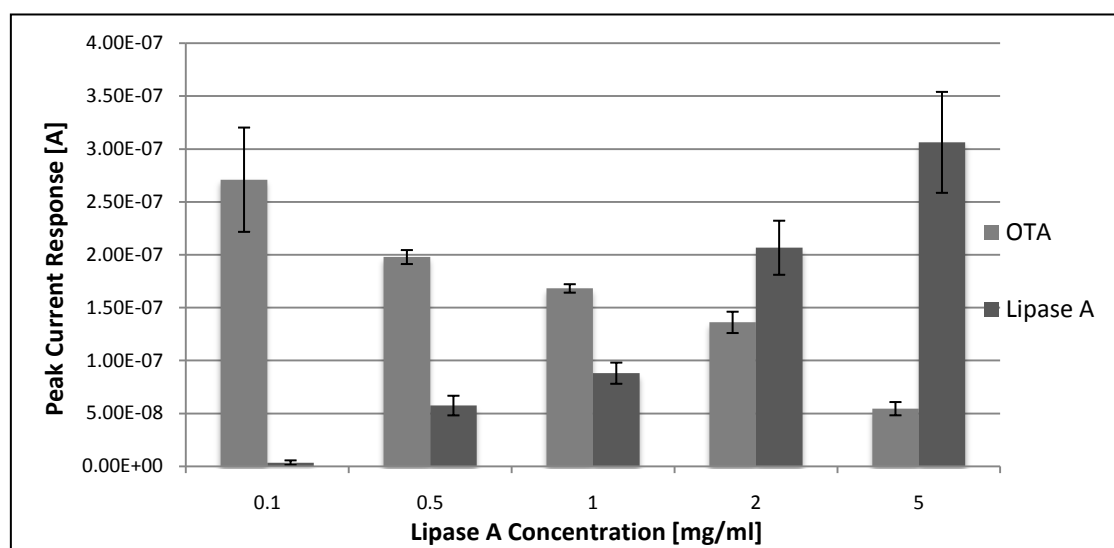


Figure 5.4.3.4 Bar graph comparing the peak current amplitude of the OTA and lipase A observed from DPVs analysis obtained from the hydrolysis of 12.5 μ M OTA in 0.2 M phosphate buffer pH 6 at 40 °C for lipase A concentrations of 0.01, 0.5, 1, 2 and 5 mg/ml. DPVs were measured at a hydrolysis time of 30 minutes.

Although a lipase A concentration of 5 mg/ml produced a near full hydrolysis of OTA to OT α within 30 minutes, the corresponding increase in lipase A interferent peak current amplitude prevented resolution of the OT α peak. Limiting the concentration of lipase A to 0.01 mg/ml in order to reduce the current amplitude of the interferent peak produced no corresponding resolution of the OT α peak due both to the large half peak potential of the interferent peak and slow rate of OTA hydrolysis by lipase A.

5.4.4 Thin layer chromatography of ochratoxin A hydrolysis products

The incorporation of lipase A within the sensor platform as an element of selectivity toward the detection of OTA, required the removal of interferent species capable of masking the OT α peak.

Molecular exclusion chromatography was investigated as a means to achieve separation between interferent species and the hydrolytic enzyme component of the lipase A preparation. As interferent

species are typically of low molecular weight, an exclusion column of Sephadex G-25 was selected to effectively desalt out the high molecular weight enzyme fraction from the interferent species within the lipase A preparation. Upon elution from the exclusion column, the protein content of each fraction was determined from UV-VIS spectroscopy by the relation:

$$C_T = 1.55 A_{280} - 0.76 A_{260} \quad (5.4.1)$$

Where C_T represents the total protein concentration, A_{280} the absorbance reading at 280 nm and A_{260} the absorbance reading at 260 nm (Wilson and Walker, 2000). The 280 nm and 260 nm wavelengths correspond to the absorption maxima of protein and nucleic acid interferents respectively. Figure 5.4.4.1 illustrates the total protein concentration of each eluted fraction determined from UV-VIS spectroscopy and by the relation 5.4.1.

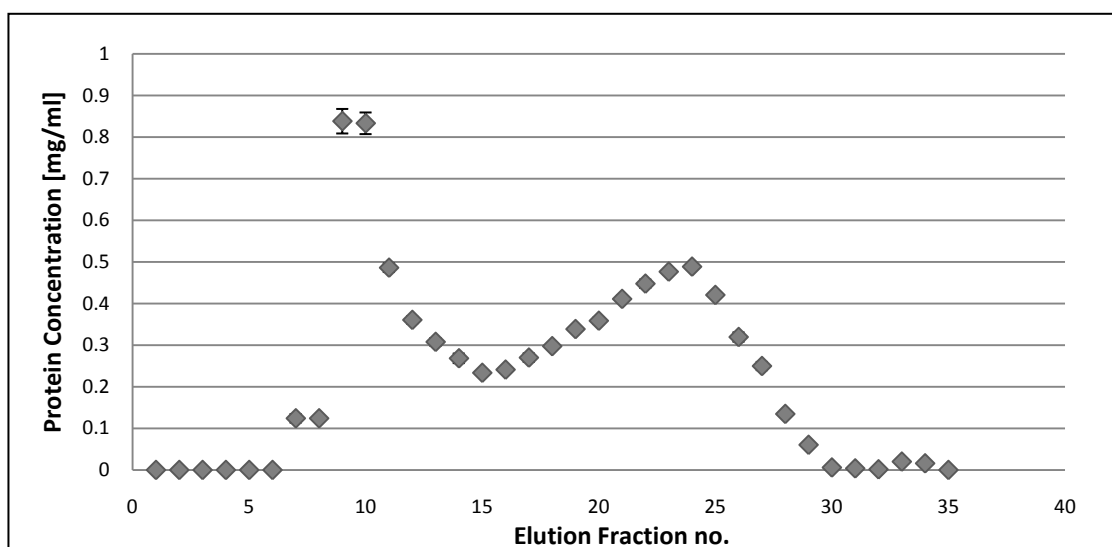


Figure 5.4.4.1 Elution profile for 10 mg/ml lipase A eluted through a sephadex G-25 molecular exclusion column and measured by UV-VIS spectroscopy at an absorbance wavelength of 280 nm and 260 nm to determine protein concentration. A 0.2 M phosphate buffer pH 6 at 4 °C was used as the mobile phase.

As shown in Figure 5.4.4.1, the majority of proteins were excluded from the column and subsequently eluted with the void volume as in agreement with the reported 15 to 100 kDa molecular weight range of proteins present within the lipase A preparation (Stander, et. al, 2000).

Once eluted, the fractions numbered 8 to 14 were pooled and labelled as Fraction A. The pooled fraction was then assessed for total protein concentration by Bradford assay and for the presence of interferent species by DPV analysis. Similar analysis was done on fractions 15 to 21 and for fractions 22 to 30 labelled as Fraction B and Fraction C respectively. Bradford assay of the pooled fractions

showed the elution of 52 %wt. of total protein in Fraction A followed by 25 %wt. in Fraction B with Fraction C containing the remaining 23 % of eluted protein. Figure 5.4.4.2 represents the DPVs observed for the respective pooled fractions of lipase A in 0.2 M phosphate buffer of pH 6.5.

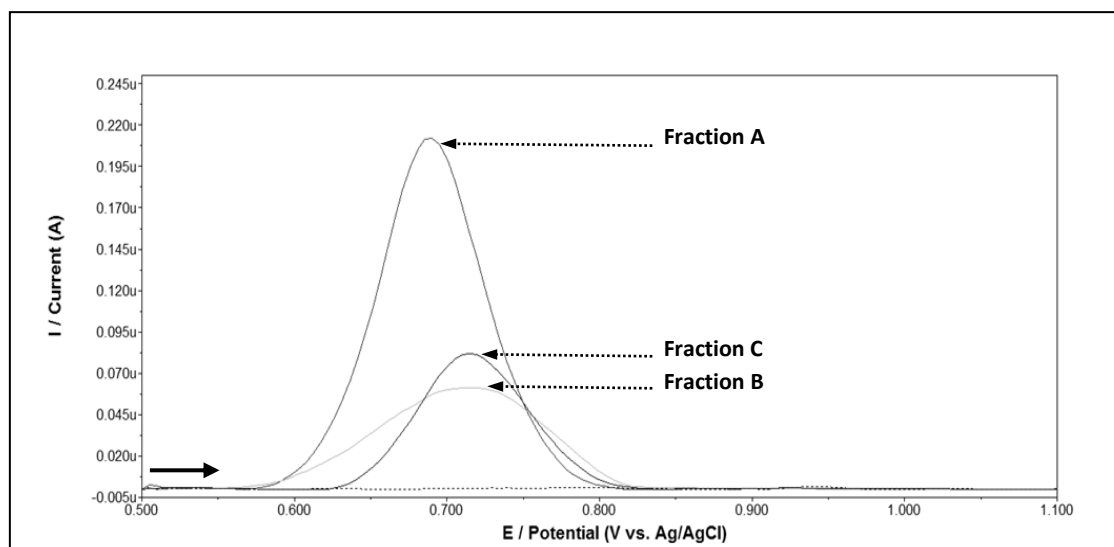


Figure 5.4.4.2 DPV analysis of the purified lipase Fraction A, lipase Fraction B, lipase Fraction C fractions in 0.2 M phosphate buffer pH 6.5. Blank solution is shown by a dotted line. Scan rate 5 mV/s.

For DPV analysis shown in Figure 5.4.4.2, an anodic peak of potential 0.69 V, 0.71 V and 0.71 V were observed for lipase fractions A, B and C respectively. As all three anodic peaks of the pooled protein fractions were within the potential range of the interferent peak observed in Figure 5.4.3.3, the retention of interferent species was assumed to be confined to the protein fraction of the crude preparation. The direct correlation between peak current amplitude and protein concentration of the pooled fractions could indicate partial binding of the interferent species to protein structures within the lipase A preparation.

Ultrafiltration was employed in order to determine whether partially bound interferent species could be removed from the protein structures of the lipase A preparation. After ten successive centrifugal purification cycles, the remaining protein fraction labelled as lipase A_T was analysed by DPV for hydrolytic activity toward OTA and for the retention of interferent species. A subsequent purification step of lipase A_T was used to produce lipase A_R and lipase A_E from the retained and eluted fractions respectively. Further DPV analysis was performed on lipase A_R and lipase A_E in order to evaluate the efficiency in separation of the interferent species from the retained protein fractions. DPV analysis of the purified fractions is shown in Figure 5.4.4.3.

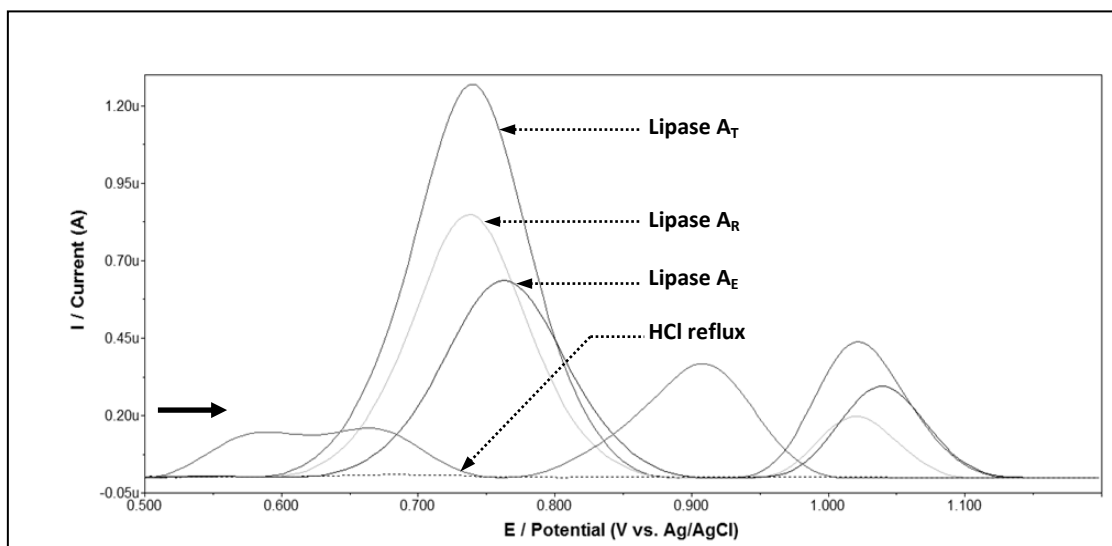


Figure 5.4.4.3 DPV analysis of OTA solutions hydrolyzed by the purified lipase A_T, lipase A_R, lipase A_E and HCL reflux fractions in 0.2 M phosphate buffer pH 6 at 40 °C. DPVs were measured at a hydrolysis time of 48 hours. Blank solution is shown by a dotted line. Scan rate 5 mV/s.

DPV analysis of the purified fractions showed the formation of the primary OTA peak at a potential of 1.02 V and a secondary anodic peak of potential 0.73 V corresponding to oxidation of the interferent species retained within the lipase A_T fraction. The retention of interferent species within the lipase A_T fraction after ten successive purification cycles further supports the possible binding of interferent species to available protein structures within the lipase A preparation. As most lipases are reported as acidic glycoproteins, the interferent species may be present as a collection of glycan chains bound to the polypeptide backbone of protein structures within the lipase A preparation (Krishna and Karanth, 2002) (Martens and Frankenberger, 1991). As interferent species were retained within both the lipase A_R and lipase A_E fractions, chemical hydrolysis of OTA by acid reflux was investigated as an alternative means to identify the OTα peak without the presence of interferent species.

The hydrolytic cleavage of OTA to OTα and phenylalanine by HCl reflux is well documented (van der Merwe, 1965). Thus, HCl hydrolysis of OTA was used to produce OTα for DPV analysis and subsequent identification of the OTα peak. As shown in Figure 5.4.4.3, the HCl hydrolysis fraction produced three distinct peaks of anodic potentials 0.90 V, 0.67 V and 0.58 V between the potential range of 0.5 V and 1.2V respectively. The anodic peaks at 0.90 V and 0.58 V were separate to the anodic peaks observed for the lipase hydrolysed fractions while the anodic peak at 0.67 V is similar to the oxidation potential of the lipase A interferent species. Thus, in order to identify the OTα peak from the three distinct anodic peaks, thin layer chromatography (TLC) was used to correlate the anodic peaks of the HCl hydrolysis fraction to those of the lipase hydrolysed fractions.

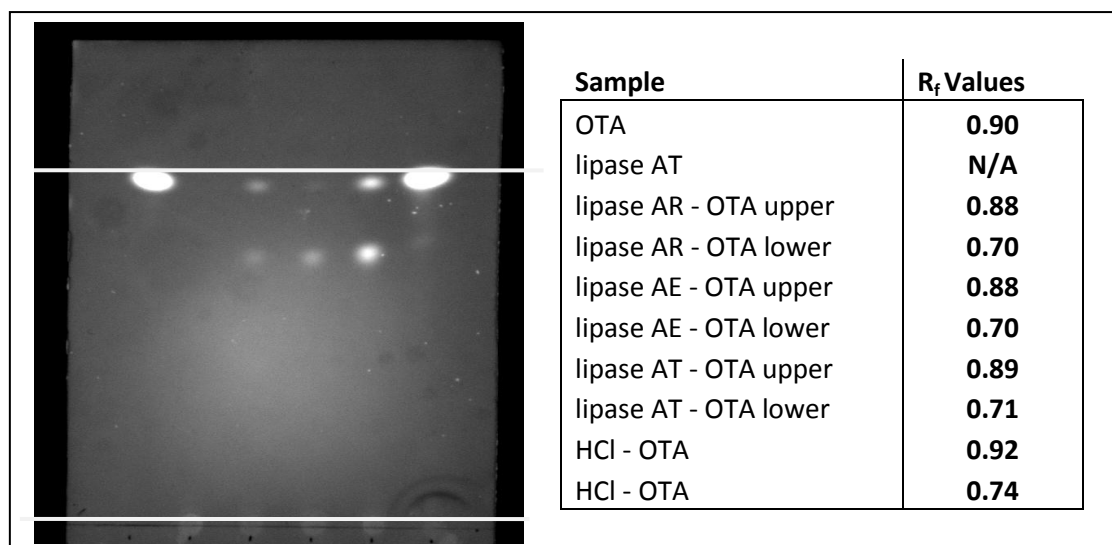


Figure 5.4.4.4 illustrates the TLC plate developed in toluene/ethyl acetate/formic acid (5:4:1) for the UV visualization of 100 μ M OTA, lipase A_T, 30 μ M OTA hydrolyzed by lipase A_R, 30 μ M OTA hydrolyzed by lipase A_E, 30 μ M OTA hydrolyzed by lipase A_T and 100 μ M OTA hydrolyzed by HCl reflux. The TLC plate was visualized under a 366 nm UV light.

From TLC analysis, a single band corresponding to OTA was observed at an R_f value of 0.90 au. The OTA band was identified by the natural blue fluorescence of OTA visualized under UV light. A similar band was identified for the lipase A_T, lipase A_R, Lipase A_E and HCl hydrolysis lanes at R_f values of 0.89, 0.88, 0.88 and 0.92 au respectively. The blue fluorescent band observed for the lipase A fractions was identified as OTA as confirmed by the decrease in intensity as a result of OTA hydrolysis shown by the relative decrease in peak current amplitude of the OTA peak in Figure 5.4.4.2. As no corresponding OTA peak was observed during DPV analysis of the HCl hydrolysis fraction, the 0.92 R_f band observed for the HCl hydrolysis fraction was accredited to the formation of a methylated ester of OTA (Li, et. al, 1998).

An additional band exhibiting a purple fluorescence was identified for the lipase A_T, lipase A_R, Lipase A_E and HCl hydrolysis lanes at R_f values of 0.71, 0.70, 0.70 and 0.74 au respectively. The purple fluorescent band of lower R_f value than OTA was attributed to OT α . The R_f values determined for TLC analysis of OTA and OT α correlate with values found in literature (Felice, et. al, 2008). As a further confirmation of lipase A and HCl reflux induced hydrolysis of OTA, an additional TLC analysis step was performed toward the detection of phenylalanine for the respective hydrolysis fractions.

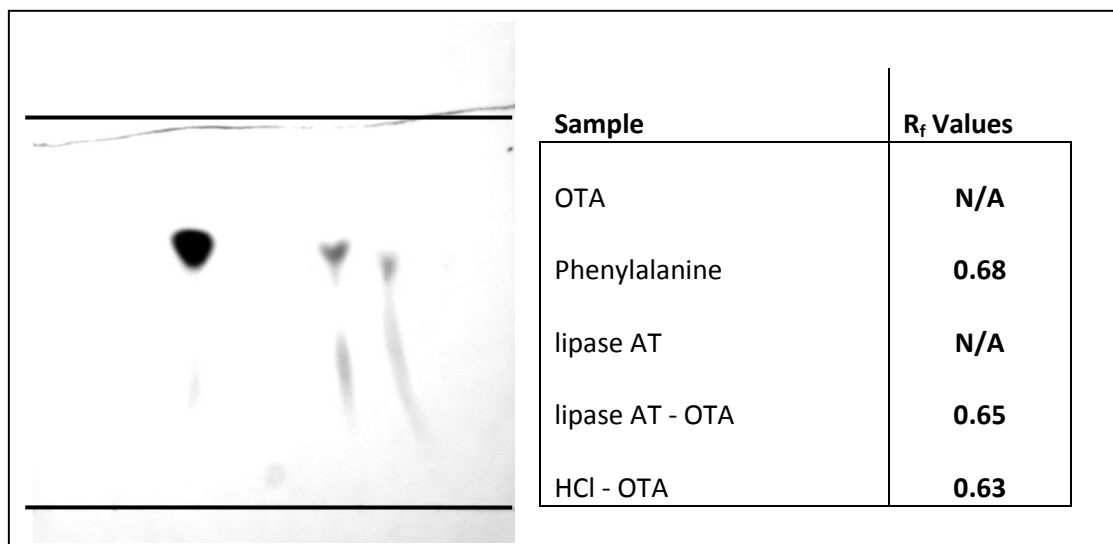


Figure 5.4.5.5 illustrates the TLC plate developed in butanol/acetic acid/milliQ (5:2:3) for the visualization of 100 μ M OTA, lipase A_T, 100 μ M phenylalanine, 30 μ M OTA hydrolyzed by lipase A_T and 100 μ M OTA hydrolyzed by HCl reflux. The TLC plate was visualized by staining in 0.5% ninhydrin at 110 °C for 5 min.

From Figure 5.4.5.5, phenylalanine was identified as a ninhydrin positive reddish brown band at an R_f value of 0.68 au (Ganther, 2001). An identical band was observed for the lipase A_T and HCl hydrolysis fractions at R_f values of 0.65 and 0.63, thereby verifying the hydrolysis of OTA to OT α and phenylalanine for the respective fractions.

Confirmation of OTA hydrolysis to OT α by the lipase A and HCl hydrolysis fractions allowed for the identification of the OT α peak from the three distinct peaks observed for the DPV analysis of the HCl hydrolysis fraction. The anodic peaks at potentials of 0.90 V and 0.58 V were not attributed to the oxidation of OT α as these peaks were separate to the anodic peaks observed for the lipase hydrolyzed fractions. Consequently, the anodic peak of potential 0.67 V was recognized as the OT α oxidation peak which was observed to coincide with the oxidation potential of the lipase A interferent peak. The anodic peaks of potentials 0.90 V and 0.58 V may be accredited to the oxidation of the methylated ester of OTA and a methylated form of OT α although additional analysis is required.

Although the presence of interferent species within the lipase A preparation effectively masked the detection of the OT α peak produced upon lipase A induced hydrolysis of OTA, the hydrolytic activity of lipase A toward OTA still presents a potentially viable means to achieve substrate specificity toward the electrochemical detection of OTA in complex media.

Confirmation of OTA hydrolysis to an electrochemically active OT α through the hydrolytic action of lipase A exemplifies the need for adequate separation of interferent species bound to the crude enzymatic preparation prior to the use of lipase A as a selectivity element within the OTA sensor platform.

5.5 CONCLUSION

The hydrolytic activity of lipase A toward OTA was evaluated by means of DPV, fluorescence spectroscopy and thin layer chromatography. Preliminary analysis of the Michaelis-Menton kinetics of lipase A induced hydrolysis of pNM was used to evaluate optimal environmental conditions toward lipase A activity. The optimal parameters for hydrolytic activity are summarized in Table 5.4.1.1.

The hydrolysis of pNM by lipase A showed typical Michaelis-Menton kinetics between the pNM concentration range of 0.01 and 0.5 mM. Evaluation of the K_m and V_{max} values of 5.06 nmol.min⁻¹ and 76.28 μ M respectively showed the high affinity of the lipase A preparation toward pNM. The high enzymatic efficiency toward pNM hydrolysis showed that the crude preparation was a true lipase as in agreement with Stander, et. al, 2000.

Owing to the natural fluorescence of OTA, the hydrolysis of OTA by lipase A was initially investigated by fluorescence spectroscopy with optimal excitation and emission wavelengths evaluated for the detection of OTA in phosphate buffer as 336 nm and 448 nm respectively. Fluorescence spectroscopy provided a highly sensitive means of detection obtaining a LOD of 0.04 μ M, although only qualitative analysis on the hydrolysis rate of lipase A toward OTA could be made as a result of overlap in emission spectra between OTA and OT α .

Quantification of OTA hydrolysis was achieved through the electrochemical detection of OTA by DPV whereby a rate of 0.28 U/g was achieved for the hydrolysis of OTA in 0.2 M phosphate buffer pH 6.5 at 40 °C. The slow rate of hydrolysis was attributed to the low purity of the lipase A preparation and to possible hindrance in enzymatic binding due to the chloride group present on the isocoumarin moiety of OTA. Although the hydrolysis of OTA could be determined through DPV analysis, the crude lipase A preparation did not provide specificity toward the detection of OTA as a result of interferent species masking the OT α peak.

Two purification methods were investigated in order to remove the electroactive interferent species namely Sephadex G-25 molecular exclusion chromatography and ultrafiltration through a 10 kDa macrosep centrifugation device. Subsequent DPV analysis showed the retention of interferent species primarily to proteinated fractions leading to the assumption that the interferent species may be present as constituent of glycoproteins within the lipase A preparation.

TLC confirmation of OTA hydrolysis to an electrochemically active OT α species through the hydrolytic action of lipase A exemplifies the need for adequate separation of interferent species bound to the crude enzymatic preparation prior to the use of lipase A as a selectivity element within the OTA sensor platform. The use of lipase A as selectivity element represents a first step towards the development of an enzyme based electrochemical biosensor toward the detection of OTA in complex media.

CHAPTER 6

OVERALL CONCLUSIONS AND RECOMMENDATIONS FOR FUTURE WORK

6.1 CONCLUSIONS

The thesis examined the use of MPC and CNT modifiers as a preliminary platform to enhance the electrochemical detection of OTA with the identification of a prospective biological recognition element sought to develop a preliminary nanostructured biosensor for the detection of OTA in complex media. Fundamental studies on the ideal pre-treatment procedures of CNTs were established and investigated for use in the electrochemical OTA sensor.

The electrochemical analysis of OTA in a wide range of supporting electrolytes was evaluated with the selection of BR buffer as an optimal supporting electrolyte in terms of the sensitivity and reproducibility in the detection of OTA. Electrochemical detection of OTA in BR buffer was shown to be diffusion controlled obtaining a diffusion coefficient of $1.25 \pm 0.27 \times 10^{-5} \text{ cm}^2/\text{s}$ in 0.2 M BR buffer pH 5. Linearity in current response for the detection of OTA in BR buffer was observed for the OTA concentration range of 2 μM to 20 μM producing a LOD of 0.28 μM .

Subsequent analysis determined the dependence of OTA oxidation on pH with pH 5 evaluated as the optimal electrolyte pH in terms of current response. Evaluation of the kinetic parameters of OTA by Tafel plot analysis showed a transfer of two electrons and two protons during the oxidation of OTA in acid media. In alkaline media, deprotonation of OTA was observed leading to the oxidation of OTA becoming pH independent and involving the transfer of only one electron resulting in a significant decrease in detection sensitivity.

Oxidation of OTA at the electrode surface occurs by means of an irreversible process producing quinone/hydroquinone couple which adsorbs to the electrode surface in acidic pH. Passivation of the electrode through adsorption of these oxidation products severely limits the detection of OTA upon successive CV cycles. Similar fouling profiles were observed for the electrochemical detection of 4-chlorophenol which was identified as the electroactive constituent of OTA. The difference in oxidation potential between OTA and 4-chlorophenol was subsequently used as a basis for the use of lipase A induced hydrolysis of OTA as a specificity element in the preliminary biosensor platform.

Chemical modification of the electrode surface was investigated to enhance the electrocatalytic detection of OTA in terms of sensitivity, reproducibility and resistance to surface fouling. Modification of the electrode surface with MPc complexes with metal based redox processes produced a marginal increase in sensitivity towards the detection of OTA while with the exception of NiPc, ring based MPc complexes showed no electrocatalytic activity toward the oxidation of OTA. Comparison of the electrocatalytic activity of the various MPc modifiers toward the oxidation of OTA identified NiPc modification to increase the sensitivity of OTA detection by 1.92 fold with susceptibility to fouling being comparative to an unmodified GCE. Low susceptibility to surface fouling was only observed for the CoTCPc modified GCE obtaining a 41.01 % retention in sensitivity compared to the 22.38 % retention in sensitivity by the unmodified GCE after five successive detection cycles. The improvement in fouling characteristics was attributed to steric hindrance of the CoTCPc modified electrode toward the adsorption of quinone/hydroquinone species produced upon OTA oxidation.

Modification of the electrode surface with acid functionalized MWCNTs and SWCNTs produced a significant increase in sensitivity toward the detection of OTA although at the expense of increased deviation in current response. From capacitive measurements, the favourable electrocatalytic activity of CNTs was attributed to both the addition of oxygenated functional groups and to the increase in available surface area afforded by the highly porous CNT network. The gain in surface area appeared to have a greater influence on sensitivity of detection as illustrated by the catalytic activity of SWCNT modification toward the oxidation of OTA compared to MWCNT modification with inherently lower surface area to weight ratios. AFM and Raman scope analysis was used to confirm the decrease in reproducibility of detection to the irregular surface morphology of the physically adsorbed CNTs and to the large degree of carbonaceous materials retained within the CNT fractions after centrifugal purification.

Centrifugal purification of the CNTs was deemed necessary in order to improve the electrocatalytic activity of the CNTs toward the detection of OTA through the removal of carbonaceous impurities initially present in commercial pristine CNT fractions or produced from acid treatment of the CNT fractions. Raman spectroscopy of the purified fractions showed the interplay between the addition of electroactive functional groups to the CNT surface and degradation of the CNT structure upon acid treatment.

A functionalization time of 2 hours was shown to substantially enhance the detection response obtaining a respective LOD of 0.03 μM and 0.09 μM for the oxidation of OTA at SWCNT and MWCNT modified electrodes. CNTs exposed to acid functionalization times beyond 4 hours showed a substantial decrease in detection sensitivity due to the degradation of the CNT structure and resultant retention of amorphous carbon within the purified CNT fraction. In addition, the reduction in nanotube length upon extended functionalization times limited the efficiency of centrifugal purification due to the similarity in density between carbonaceous impurities and the degraded CNTs. Compared to unmodified GCEs, modification of the electrode surface with 2 hour functionalized SWCNTs increased the sensitivity of detection to near the regulatory limit of 0.01 μM set for OTA in human food (FAO, 2003). Additional improvements in uniform surface coverage of CNTs modified to the electrode surface should enable the regulatory limit to be met by electrochemical means of OTA detection.

A crude lipase preparation *Aspergillus niger*, lipase A, was investigated as a potential biological recognition element for selective detection of OTA in complex media. Michaelis-Menton analysis of the hydrolytic cleavage of pNM was used to evaluate the optimal operating conditions of lipase A, listed in Table 5.4.1.1, toward the hydrolysis of OTA. Although fluorescence spectroscopy could not quantify the hydrolysis of OTA by lipase A, a qualitative analysis was made through the shift in emission wavelength from 448 nm to 436 nm during the cleavage of OTA to OT α . The blueshift in emission spectra upon OTA hydrolysis was observed to correspond to the natural fluorescence of OTA and OT α bands evaluated during TLC analysis.

The rate of OTA hydrolysis was determined as 0.28 U/g from DPV analysis of the change in peak current amplitude of OTA upon the addition of lipase A. DPV analysis confirmed the presence of an interferent peak originating from the crude lipase A preparation and was shown to mask the OT α peak. Subsequent attempts to remove the interferent species by molecular exclusion chromatography and through ultrafiltration were met with minimal success. Although retention of the interferent species limited the use of the crude lipase A preparation as a biological recognition element for the electrochemical detection of OTA, the hydrolytic activity of lipase A toward OTA still presents a viable means to achieve substrate specificity through the electrochemical detection of OT α in complex media.

From the above conclusions, the electrochemical detection of OTA can be enhanced in terms of sensitivity, reproducibility and stability through the selection of optimal electrolyte parameters and through electrode modification of MPc and CNT complexes following acid functionalization and centrifugal purification pre-treatment. Selectivity in detection of OTA was achieved through Lipase A induced hydrolysis although additional purification of the crude lipase A preparation is required prior to utilization within the preliminary biosensor platform.

6.2 FUTURE RECOMMENDATIONS

Fouling of the electrode during the electrochemical analysis of OTA can be addressed through the use of screen printed electrodes. The effect of CNT and MPc modification on screen printed electrodes toward the sensitivity and reproducibility of OTA detection will need to be evaluated. Considerations toward the correct disposal and possible re-use of nanostructured screen printed electrodes should be made.

Developments toward the purification of commercial CNTs prior to use in electroanalysis are required. The success of centrifugal purification in separating carbonaceous impurities from the CNT modifiers may be extended through the use of surfactants to increase the dispersion of CNTs in aqueous media. Initial centrifugation may also be employed to firstly remove the carbonaceous impurities produced from CNT synthesis, followed by a combination of sonication and ultracentrifugation to unbundle the CNTs and remove any remaining impurities. The use of mild functionalization techniques which maintain the tubular structure of CNTs while allowing the addition of electroactive functional groups will need to be evaluated.

Identification of electroactive species present in the crude lipase A preparation needs to be addressed in order to adapt the purification protocol used for the isolation of the lipase A enzyme fractions. Preparative gel electrophoresis followed by screening of lipases capable of hydrolyzing OTA may be employed for the isolation of enzymes to be used within the preliminary biosensor construct. Additional screening of peptidases capable of hydrolyzing OTA to OT α at a faster rate than lipase A could be assessed. Alternatively the use of synthetic oligonucleotides for the specific binding of OTA may be adapted for use on the nanostructured sensor platform (Cruz-Aguado and Penner, 2008).

The respective 9.72 fold and 3.26 fold increase in detection limits of OTA afforded by SWCNT and MWCNT modification over unmodified GCEs illustrates the potential of CNT incorporation within sensor design. Given the varying production mechanisms and low purity of as-produced CNTs, additional fundamental studies on CNT pre-treatment and uniform modification to the electrode surface is required.

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