UNIVERSITY OF NAIROBI

FABRICATION AND CHARACTERIZATION OF POLYANILINE-CARBON MODIFIED ELECTRODE (CME) BIOSENSOR FOR ANALYSIS OF BISPHENOL A

BY

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2017.

DECLARATION

I declare that this thesis is my original work and has not been submitted elsewhere for examination, award of degree or publication. Where other people's work or my own work has been used, this has properly been acknowledged and referenced in accordance with the University of Nairobi's requirements.

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DEDICATION

I dedicate this thesis to my mum Domitilla Muthio, my dad Richard Munyao and family members for their steadfast support, financially and morally and their encouragement throughout my studies.

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ABSTRACT

In this work, a biosensor to qualitatively and quantitatively determine the amount of BPA was fabricated, with the aim of analyzing levels of BPA in the environment. It was observed that most drinking bottles in the Kenyan market have a substantial amount of BPA that leaches into drinking water. Monitoring BPA is difficult as various known analytical methods may not be available in a developing country like Kenya. A more versatile and cheaper method is urgently required so as to assess the levels of BPA that the average Kenyan is exposed to with the intention of minimizing the BPA concentration in the environment. The biosensor for determination of Bisphenol A was developed from a bentonite-modified carbon electrode, polyaniline, SLS surfactant and Tyrosinase enzyme. The biosensor obtained was used to determine Bisphenol A in plastic water bottles and feeding baby bottles. Electrochemical methods, including cyclic voltammetry, DPV and square wave voltammetry were used in characterization of the biosensor. Other methods used were UV-VIS and Raman spectroscopy. The pH of the experiments was maintained using a phosphate buffer, and the optimal pH for the operation of the biosensor was obtained as 7.2. An elaborate peak signaling the presence of BPA was found at +0.5V for reduction potential and +0.2V oxidation potential. Under optimized conditions, the detection limit for BPA using the developed biosensor was found to be 2.1 x 10^{-9}M within a concentration range of 0.02-0.2 mM where the response time reached 95% within 15 seconds. The concentrations of BPA in the water from the plastic bottles were found to be 0.030mM, 0.021 mM and 0.035mM for green, pink and blue drinking water bottles respectively and 0.030mM and 0.019mM green and pink feeding baby bottles respectively. We would like to sensitize the government and the people of Kenya against using plastics that have BPA because of the risks associated with the chemical compound. We would also like to recommend that the amount of BPA in other substances like CDs, DVDs, thermal paper and the various medical implements that are known to be manufactured using BPA be established and that the public be likewise alerted on using these substances.

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ABBREVIATIONS AND ACRONYMS

ADHD:	Attention Deficit Hyperactivity Disorder
BPA:	Bisphenol A
BPS	Bisphenol Substitute
BTN:	Bentonite clay
CME:	Carbon Modified Electrode
CV:	Cyclic Voltammetry
DPV:	Deferential Pulse Voltammetry
DMSO	Dimethylsulphoxide
EDC:	Endocrine disrupting chemical
EPA:	Environmental Protection Agency
ER:	Estrogen receptors
ERR:	Estrogen-related receptor
EU:	European Union
FDA:	Food and Drug Administration
FIA:	Flow injection analytical systems
GCE:	Glass Carbon Electrode
HPLC:	High performance liquid chromatography
LOD:	biosensor limit of detection
NTP:	National Toxicology Program
NMR:	Nuclear magnetic resonance
OECD:	Organization of Economic and Cooperative Development
PANI:	Polyaniline
POMA:	Polyaniline (poly2-methoxyaniline)
PPO:	polyphenol oxidase
PVC:	Polyvinyl chloride
SLS	Sodium lauryl sulphate
SWV:	Square Wave Voltammetry
UV-Vis:	Ultra violet-visible
WHO:	World Health Organization

CHAPTER 1: INTRODUCTION

1.1 Background

Bisphenol A (BPA) is a high production volume chemical used in a variety of common consumer products. Most notably, BPA is present in polycarbonate plastics, the epoxy resin liners of aluminum cans, and thermal receipts (Fiege & Heinz-Werner, 2002). It has also been used as an inert ingredient in pesticides, as a fungicide, as an antioxidant, as flame retardant, rubber chemical and polyvinyl chloride stabilizer (Rodriguez-Mozaz *et al.,* 2005). The use of plastics and polythene bags has had its advantages, for example light weight, portability, and ability to fit into any shape required, and therefore has multiple uses. However, concern has been raised about some disadvantages of these substances, like their lack of biodegradability and the materials used in their production, for example Bisphenol A. According to He'lie's-Toussaint et al., (2014) bisphenol A has been used in preparation of plastics, but being a xenoestrogen, it has been found to have adverse effects on the human being, especially on women (He'lie's- Toussaint, 2014). The world production capacity of this compound was 1 million tonnes in 1980s, and more than 2.2 million tonnes in 2009 (National Toxicology Program, 2009). In 2003, U.S. consumption was 856,000 tonnes, 72% of which was used to make polycarbonate plastic and 21% going into epoxy resins (National Toxicology Program, 2009). Products containing Bisphenol A-based plastics have been in commercial use since 1957 (Qiu *et al.,* 2010). In production of epoxy resins, bisphenol A is a key monomer (Sheryl, 2008) and apparently in the commonly used form of polycarbonate plastic (Kroschwitz, 2010).

Known to be estrogenic since the mid-1930s (Dodd and Lawson, 1938), concerns about the use of Bisphenol A in consumer products were regularly reported in the news media in 2008 after several governments issued reports questioning its safety, prompting some retailers to remove products containing it from their shelves (Mita, 2005). Soon after, BPA was established to be estrogenic in MCF-7 human breast cancer cell culture at low concentrations like 2.0-7.0 ppb (Krishnan *et al.,* 1993). Ever since then, several studies have been reported on the estrogenic effects of BPA on humans and animals both in *vitro* and in *vivo* (Christiansen *et al.,* 2000; Mueller 2002; Markey *et al.,* 2005). A 2010 report from the United States Food and Drug Administration (FDA) raised further concerns regarding exposure to fetuses, infants and young children (National Toxicology Program, 2009).

In September 2010, Canada became the first country to declare BPA as a toxic substance (Mittelstaedt, 2010). In the European Union and Canada BPA use was banned in baby

bottles. The first evidence of the estrogenicity of Bisphenol A came from experiments on rats conducted in the 1930s (Erickson, 2008) but it was not until 1997 that adverse effects of low-dose exposure on laboratory animals were first reported. Bisphenol A is an endocrine disruptor that can mimic estrogen and has been shown to cause negative health effects in animal studies. Bisphenol A closely mimics the structure and function of the hormone estrogen with the ability to bind to and activate the same estrogen receptor as the natural hormone (Byrne, 2008). In March 2009 Suffolk County, New York became the first county to pass legislation to do away with baby beverage containers made with Bisphenol A (Vandenberg *et al.,* (2010) and Hunt *et al., (2012)* observed that daily low-dose BPA exposure (measured < 1 ng/mL in maternal serum) significantly disrupted synapsis and recombination between homologous chromosomes at the onset of meiosis, (Vandenberg, 2010).

A study carried out in 2013 found an association between urinary concentrations of BPA and body mass indexes of children and adults aged 6–19 years (Vandenberg *et al,*. 2013). This finding strengthened the association between BPA and obesity.

Furthermore, an article written on 23rd March 2014 showed that, as of 2014, 12 states had banned BPA from children's bottles and feeding containers (Fiorentino, 2014).

The degree at which BPA leaches from polycarbonate bottles into liquid may depend more on the temperature of the liquid or bottle, than the age of the container. BPA has been reported to be detectable in breast milk (Schönfelder *et al.,* 2002). Schönfelder *et al., (2002)* also analyzed BPA content in the blood of pregnant women, in the umbilical blood at birth and in placental tissue and found them to contain BPA at levels within ranges shown to alter development of a foetus.

Information regarding use and effects of Bisphenol A in Kenya is lacking, mainly due to lack of awareness, and also of appropriate methods of its analysis. The analytical methods which have been used commonly for the separation and detection of BPA include: high performance liquid chromatography (HPLC) (Katayama *et al.,* 2001), gas chromatography (GC),or gas chromatography coupled with mass spectrometry (GC-MS) (Pulgar *et al.*, 2000), Liquid Chromatography coupled with mass spectrometry (LC-MS) (Jiménez-Díaz *et al.,* 2010) and capillary electrophoresis (Mei *et al.,* 2011). These methods are specific and highly sensitive, but they are quite expensive, need skilled personnel to operate them, they are also time-consuming, require difficult pre concentration and extraction procedures and thus do not

allow rapid processing of multiple samples and also they are not within the reach of Kenyans. Due to this reason, the sensitive, rapid, and precise methods for determination of phenolic compounds and its derivatives are of growing interest in environmental control and protection (Li *et al.,* 2005; Mita *et al.,* 2007). The recent introduction of sensor and biosensor technologies has overcome the difficulties outlined above. Electrochemical biosensors are considered to be the most attractive and effective techniques for such applications. Electrochemical sensors can operate in turbid media and are more amenable to miniaturization. Moreover, the excellent compatibility of electrochemical sensors with flow injection analytical systems (FIA) increases the potential for assay automation, to which more and more attention has been paid in many fields including food analysis and environmental monitoring(Arya, 2008).

This research aims at developing novel analytical methods for monitoring Bisphenol A using biosensor technology where by enzyme based phenolic biosensor will be fabricated.

With regard to fabrication of enzyme-based phenol biosensors, many examples can be found in the literature. In this case, various sources of Tyrosinase and a wide variety of matrices including; graphite (Sarapuu *et al.,* 2010), carbon paste (Mita *et al.,* 2007), conducting polymers (Dempsey *et al.,* 2004; Li *et al.,* 2006), biopolymers (Tembe *et al.,* 2006), naflon membrane (Zhao *et al.,* 2005) nano particles (Carralero Sanz *et al.,* 2005; Alkasir *et al.,* 2010) and silica sol–gel composite films (Munjal *et al.,* 2002) have been used. The matrices have been used with different combinations of stability and sensitivity, depending on the application.

Some common drawbacks to phenol biosensors in their usage include electrode fouling due to polymerization of radicals, and enzyme inactivation by the generated *o*-quinone. It has been discovered that the electrochemical oxidation of phenolic compounds in general causes the inactivation of electrode surfaces, through deposition of electro polymerized films which are produced when the phenoxy radical attacks the unreacted substrate (Kuramitz *et al.,* 2001).

Therefore the challenges that the developers of biosensors attempt to overcome are minimizing electron fouling and improving Tyrosinase stability. Use of conducting polymers has been found to improve the stability and activity of the Tyrosinase biosensor. The polymers are thereby used as electrode modifiers. They are added onto the electrode surface prior to the immobilization of the enzyme (Chuang *et al.,* 2006; Asav *et al.,* 2009). These enhance the conductivity of the biosensor by assisting in directing electron transfer between

the enzyme's active sites and the electrode. In this study inert adhesive glue was used as cross linking bonding to immobilize the enzyme. Furthermore, during preparation of Tyrosinase enzyme stock solution, bovine serum albumin was used as a protein carrier, and acted as a stabilizing agent in enzymatic reactions, thus enhancing enzyme activity. Biosensors modified with conducting polymers have high conductivity and good stability in air and aqueous solution. (Khanna, 2011)

In this study, a simple, cost effective, easy to use and sensitive electrochemical Tyrosinase biosensor has been synthesized for quantitative detection and determination of bisphenol A. The working electrode was a glassy carbon graphite electrode modified with polyaniline, surfactant and bentonite clay (PANI/ SLS-BTN). The enzyme Tyrosinase was immobilized on top of polymer composites (Try/PANI/ SLS-BTN/GCE) to complete the biosensor. The previous reports on the immobilization methods included covalent linking (Dempsey *et al.*, 2004), Electropolymerization (Chen *et al.,* 2003), polymer entrapment (Sulak *et al.,* 2010), and self-assembled mono layer (SAM) (Ding *et al.,* 2003). It was noted with concern that, some of these immobilizations are relatively complex, time consuming, require the use of solvents that are environmental pollutants, expensive and result in relatively poor stability and bioactivity of Tyrosinase (Mita *et al.,* 2007). In this study, drop coating was used as immobilization method because it needs no skilled personnel, it is precise, simple and reproducible (Matyholo, 2011).

1.2 Problem Statement

Bisphenol A is a toxic substance, whose exposure has been linked to various disorders and diseases for example neurological blastoma especially in children, (Braun *et al.,* 2009) breast and prostate cancer, sexual dysfunction (Li. *et al*., 2010(a)), early maturity, (Fatoki *et al*., 2009), obesity, DNA methylation and disruption of the dopaminergic system. It also causes an increase in hyperactivity and aggression in two-year-old girls due to prenatal exposure. (Braun *et al*., 2009). It has also been associated with oxidative stress and repeated miscarriages (Sui *et al*., 2012), heart disease, diabetes and high levels of liver enzymes (Yin *et al.,* 2011). In addition, Increased BPA levels are associated with decreased sperm quality following environmental and occupational exposure. (Meeker *et al*., 2010), affects testosterone levels in men (Galloway *et al*., 2010) , it is also associated with changes in estrogenic gene expression in adult males (Melzer *et al*., 2011) and increased incidence of coronary heart disease (Melzer *et al*., 2012). Furthermore in 2007, Vom Saal reported that, a growing body of laboratory research on very low doses of BPA levels that fall below the

regulatory safety standards have noted associations with increased rates of breast and prostate cancer, chromosomal abnormalities, brain and behavioral abnormalities, and metabolic disorders as a result of BPA exposure.

Despite the disorders associated with BPA, it is still used in the manufacture of commonly-used commodities, for example polythene bags, plastic bottles, thermal papers, CDs and DVDs and even various substances used in optical and dental purposes. The indication is that many people may be exposed to BPA in unknown levels, just by using these substances.

It has been noted with a lot of concern that analysis of Bisphenol A using various techniques like gas chromatography is wanting. Other methods like NMR, liquid chromatography (LC), both coupled with mass spectrometry (MS) are highly sensitive and specific but they are also expensive, need skilled operators, are time-consuming, require laborious pre-treatment and extraction steps. This means that they are not suitable for routine tests, especially in a developing country like Kenya. It is, therefore difficult to gauge the exposure of the population to BPA. This, together with the fact that there is an increase in cases of people with the aforementioned effects of BPA exposure means that many Kenyans may be exposed to levels of BPA above those that are recommended. A plausible solution to the challenge of relatively easy methods of detection would be the introduction of biosensor technology since these methods have been known to be sensitive, selective and cost effective, highly suitable for routine analysis. This research project has focused on developing simple, low cost and sensitive electrochemical Tyrosinase based biosensor for the determination of BPA.

1.3 Overall objective

The purpose of this study is to fabricate and characterize polyaniline- carbon modified electrode (CME) biosensor for analysis of bisphenol A

1.4 Specific Objectives

i. To characterize polymeric materials of polyaniline using polished glassy carbon graphite electrodes modified with Bentonite clay using cyclic voltammetry (CV), UV-Vis spectroscopy and Raman spectroscopy.

ii. To encapsulate Tyrosinase enzyme into the polymeric materials and optimize conditions for best biosensor responses towards Bisphenol A.

iii. To characterize the bisphenol A biosensor by cyclic voltammetry (CV), differential pulse voltammetry (DPV) and square wave voltammetry (SWV).

iv. To determine the BPA concentration in real samples using the fabricated biosensor by differential pulse voltammetry, square wave voltammetry and cyclic voltammetry.

1.5 Research Justification

Good health in all aspects is a priceless and greatly sort after quality by all human beings. It has direct bearing on the political, social and economic development of any nation, and is the reason why it features in a big way in the Sustainable Development agenda. It is known that globally, there is also a marked increase in breast and prostate cancer cases, cases of sexual dysfunction, including altered hormone levels and erectile problems in men (Yin et al., 2011, Castillo, 1997). Thyroid gland dysfunction cases are also on the increase. Other disorders affecting many people include repeated miscarriages, oxidative stress and inflammation in postmenopausal women.

There has also been scenario where baby girls have developed increased mammary glands and genitalia, including menstruation at an early age. This is an area of concern as it is an anomaly and these girls do not grow properly since they are exposed to issues beyond their mental capacity and also may be regarded by their peers as social misfits which may taint them psychologically **(**Braun *et al.,* 2009**)**.

When a society has a fairly large percentage of its population with such problems, the economy is negatively affected since the capacity of work is reduced either by physical illness or by psychological torture.

BPA is used in the manufacture of various substances that are handled by the ordinary person on a daily basis, for example plastics, CDs, DVDs and polythene bags. Yet BPA is known to trigger the disorders and/or diseases earlier mentioned.

Monitoring BPA is difficult as various known analytical methods may not be viable in a developing country like Kenya. A more versatile and cheaper method is urgently required so as to assess the levels of BPA that the average Kenyan is exposed to with the intention of minimizing the BPA concentration in the environment.

Biosensor technologies have been known to provide cheap alternatives and they also provide for in-situ measurements. Measurements performed in-situ are convenient since they eliminate the need for sample transportation to centralized laboratories and can be used for routine testing. Also based on high enzyme specificity, detailed sample preparation is unnecessary with the benefits of time and economic saving. Currently, biosensor technology has enabled rapid testing of glucose and cholesterol levels in the human body. It is possible to develop similar technologies for the testing of levels of various pollutants in our environment for BPA.

In this research project, polyaniline based nanomaterials using molecular template based techniques were prepared and interrogated for their electro activity and conductivity characteristics. Afterwards the materials were used to encapsulate Tyrosinase enzyme to make the biosensors using carbon electrode modified with bentonite clay. The biosensor responses to various concentrations of BPA were explored. The fabricated biosensor was used to detect and determine BPA concentration in baby bottles and drinking water bottles.

1.6 Significance of the Study

Tyrosinase is a blue copper containing metallo-protein which is binuclear and catalyzes the hydroxylation and oxidation of monophenols and diphenols to *o*-quinone (Dempsey, 2004). The *o*-quinone produced can be reduced electrochemically at an appropriate potential and so the reduction currents obtained serve as good analytical signals for quantitative determination of phenol derivatives. The concentration of the phenols in the solution is directly proportional to reduction current measured. Tyrosinase-based biosensor utilizes the reduction of *o*-quinone at moderately negative potentials, so that the interference from oxidizable species can be prevented (Notsu, 2002).

This research project focuses on the development of Tyrosinase-modified electrode with polyaniline and bentonite clay for BPA detection. The biosensor developed is used to detect bisphenol A in real samples like drinking water bottles and feeding baby bottles. The development of this new biosensor method will provide a breakthrough in the detection and monitoring of BPA and thus save the lives of many people who suffer as a result high levels of accumulated Bisphenol A in their blood and body tissues.

CHAPTER 2: LITERATURE REVIEW

2.1 Introduction

In this chapter, endocrine disrupting compounds and reviews about key findings on effects of BPA on human beings are discussed. The chapter also highlights the scholarly knowledge about BPA including its effects on infants and children, and the reproductive and unreproductive effects in adults. The chapter also describes the scholarly methods that have been used to detect BPA in plastics using biosensors, including the different biosensors that have been used so far especially using Tyrosinase enzyme. This chapter finally gives insights on bentonite clay, Montmorillonite, which was used to modify the indicator electrode in the development of the biosensor.

2.2 Endocrine Disrupting Compounds

The Organization of Economic and Cooperative Development (OECD) has defined Endocrine disrupting compounds (EDCs) as "exogenous substances or mixtures that alter the function(s) of the endocrine systems and consequently causing adverse health effects in an intact organism, its progeny or to (sub) populations"(OECD, 1998). An alternative definition of EDCs by, the Environmental Protection Agency (EPA) is that they are exogenous agents that interfere with the "synthesis, secretion, transport, binding, action, or elimination of natural hormones in the body that are responsible for the maintenance of homeostasis, reproduction, development, and or behavior" (EPA, 1997). There is a wide range of chemical compounds that are capable of disrupting the endocrine systems as shown in table 2.1 (Esplugas, 2007). Bisphenol A is one of the known EDCs in that it can either bind to hormone receptors and mimic or inhibit the action of natural hormones, or it can affect their synthesis and metabolism (Castillo, 1997). For some time, it has been known that the normal operation of the endocrine (hormonal) system can be disrupted by a number of anthropogenic and naturally occurring chemicals, thereby affecting those physiological processes which are under hormonal control. EDCs in literature are commonly differentiated in four categories: natural estrogens (e.g. estrone, 17-β-estradiol and estriol), synthetic estrogens (e.g. ethinylestradiol and tamoxifen), phytoestrogens (e.g. genistein and coumestrol) and xenoestrogens (e.g. Bisphenol A, nonylphenol and DDT) **(**Coille *et al.* 2002**).** Some of these are common raw materials in industries, for example Bisphenol A is used in paper mills, nonylphenol is a detergent, and DDT is a pesticide. EDCs are of many different types and the table 2.1 below illustrates a brief summary of some of most well-known EDCs:

Table 2.1: Endocrine disrupting compounds.

(http://environmentalchristian.wordpress.com/2008/02/10/endocrine-disrupting-compoundsbisphenol-a-phthalate-esters-dioxins and lots of others)

Category	**Name**	**Some Sources**
Alkyl-phenols (surfactants)	Nonylphenol, pentylphenol, octylphenol, nonylphenol mono and diethoxylates	Industrial and municipal effluents
Bisphenolic compounds	Bisphenol A	Polycarbonate plastic and epoxy resins.
Natural hormones/ Synthetic steroids	17-β-estradiol, estrone, testosterone and ethynyl estradiol	Municipal effluent and agricultural runoff
Organ chlorine pesticides	DDT, dieldrin and lindane	Agricultural runoff and atmospheric transport
Organotins	Tributyltin	Shipping harbors used in wood preservative and anti bio fouling agents.
Pesticides	Atrazine, trifluralin and permethrin	Agricultural runoff
Phthalates	Dibutyl phthalate, butyl benzyl phthalate and phthalate esters	Industrial effluent
Phytoestrogens	Isoflavones, ligans and coumestans	Pulp mill effluents
Polybrominated compounds	Polybrominated diphenyl ethers	Flame retardants
Polychlorinated compounds	Polychlorinated dioxins and polychlorinated biphenyls	Insulating fluids and PVC pipes etc.

The word "Estrogen" is used to describe group of chemically similar hormones which include: estrone, estradiol and estriol. Estradiol and estrone forms are produced in the ovaries

in pre-menopausal women, while estriol is produced by the placenta during pregnancy. The estrogenic hormones are responsible for the growth and development of female sexual characteristics and reproduction in both humans and animals (Yin *et al.*, 2011).

Exoestrogens have, in recent times become a contentious issue. For example, epidemiological studies have found significant increase in the incidences of breast, prostate and testicular cancer, which are known to be effects of the presence of exoestrogens in the body. There have also been various cases of decreased sperm count and semen volume and longer times to conception (Yin et al., 2011, Castillo, 1997). Other health effects that have been observed and attributed to EDCs include dermal toxicity, immune toxicity, carcinogenicity, neurobehavioral abnormalities, altered or reduced sexual behavior, attention deficit and hyperactivity disorder, altered thyroid and adrenal cortical function, pathological changes to the spleen and damaged digestive systems amongst others (Fatoki et al., 2009).

2.3 Bisphenol A

Bisphenol A was among the top 50 chemicals produced in manufacturing industries worldwide few years ago (Chang *et al.*, 2002). It is a Phenolic compound with two phenol groups. Figure 2.1 shows Bisphenol A chemical structure.

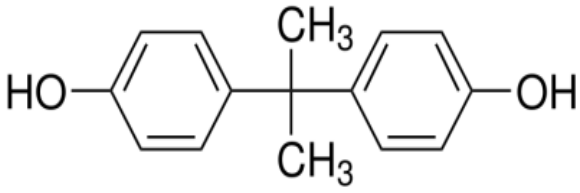


Figure 2.1 Bisphenol A chemical structure. (Willhite, 2008)

Bisphenol A is a typical product of the industrial society produced in large quantities worldwide. More than 80% of BPA is used as a monomer for the production of polycarbonate plastics, epoxy resins, and unsaturated polyester-s Tryene resins (Rodriguez- Mozaz, 2005). As a monomer, BPA is used as coatings on cans, as powder paints, as additives in thermal paper, in dental fillings, and as antioxidants in plastics.

During production of polycarbonate, epoxy, unsaturated polyester, and polysulfone resins bisphenol A is used as the main ingredient (Rufus, 1994). The figure below illustrates the process of synthesis of polycarbonate resins. (Perez, 1998)

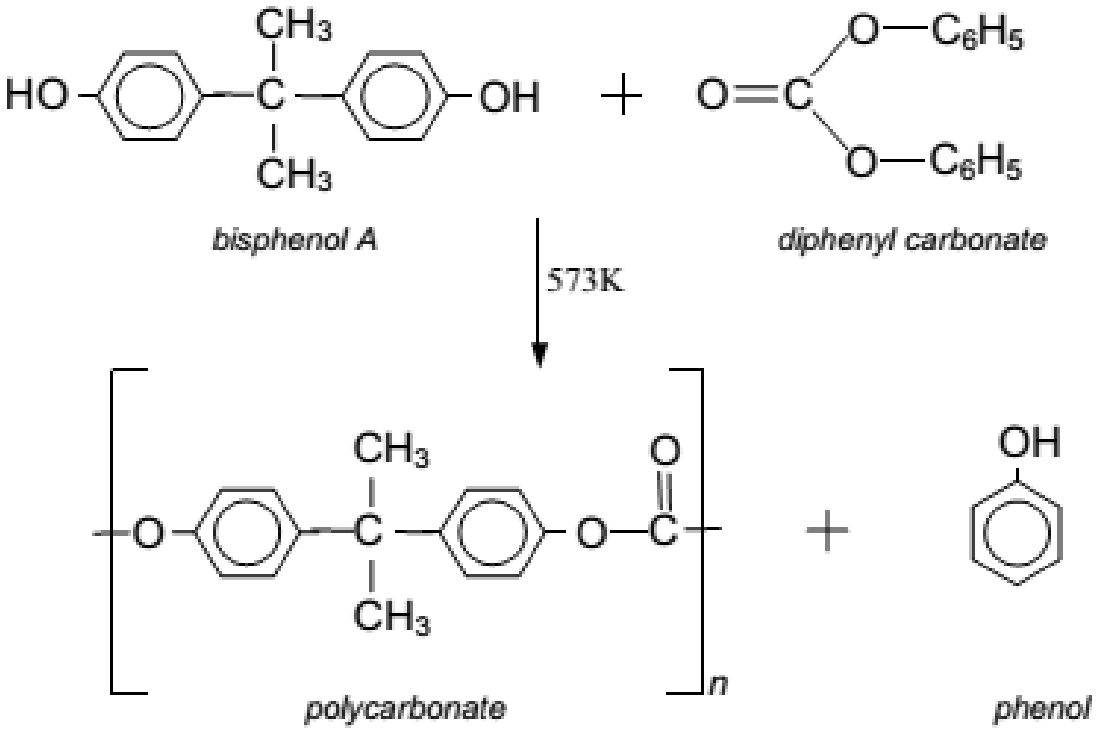


Figure 2.2 Syntheses of Polycarbonate Resins

BPA is used if synthesis of several products. Some of the products synthesized are shown in table 2.2.

Table 2.2 BPA containing products and BPA free products. Date assessed include

Source: (www.shahine.com/omar/BisphenolA.aspx)

Number	Name	Examples	BPAs
	Polyethylene terephthalate (PET)	Soda and water containers, some waterproof packaging.	No
	High-density polyethylene	Milk, detergent, oil bottles. Toys and plastic bags.	No
	Vinyl/polyvinyl chloride (PVC)	Food wraps, vegetable oil bottles and blister packages.	Yes
	Low-density polyethylene	Many plastic bags, shrink wrap and garment bags.	No
	Polypropylene	Refrigerated containers, some bags and most bottle tops.	No
	PolysTryene	Throw away utensils, meat packing and protective packing.	Yes
	Usually layered or mixed plastic.	Acrylic, polycarbonate, polylactic acid, nylon and fiberglass.	May be

Despite its many uses, BPA is known to cause problems to humans by interfering with endogenous hormones in the human body (Castillo, 1997). Table 2.3 shows an estimate of daily Bisphenol A intake, µg/kg body wt/day which was adapted from the National Toxicology Program Expert Panel Report (2008).

Table 2.3: Daily Bisphenol A intake, µg/kg body wt/day in a given population.

Source: National Toxicology Program, U.S. Department of Health and Human Services (*September, 20th 2008 "CERHR Expert Panel Report for Bisphenol A")*

Population	**Daily Bisphenol A intake estimate, µg/kg body wt/day.**
Infant (0–6 months) formula-fed	1–11
Infant (0–6 months) breast-fed	0.2–1
Infant (6–12 months)	1.65–13
Child (1.5–6 years)	0.043–14.7
Adult (general population)	0.008–1.5
Adult (occupational)	0.043–100

2.4 Scholarly Knowledge on BPA

In order to determine the effects of BPA in human beings, a number of effects of BPA in animals have been extensively investigated and the target organs identified in repeat-dose animal studies include intestines, liver and kidney (FAO, 2009).

However, the effects that have elicited a lot of concern have been those that are related to the hormonal activity of BPA and those related to physical, neurological and behavioral development (Takeda, 2009). BPA as a weak oestrogen has a much lower affinity for the oestrogen receptors (ERα and ERβ) than endogenous oestrogen and is rapidly metabolized to BPA-glucuronide which is not hormonally active. More recently, BPA has been shown to bind with high affinity estrogen-related receptor (ERRγ) (figure 2.3), which may be related to its ability to function as reported Endocrine disruptor (Takeda, 2009).

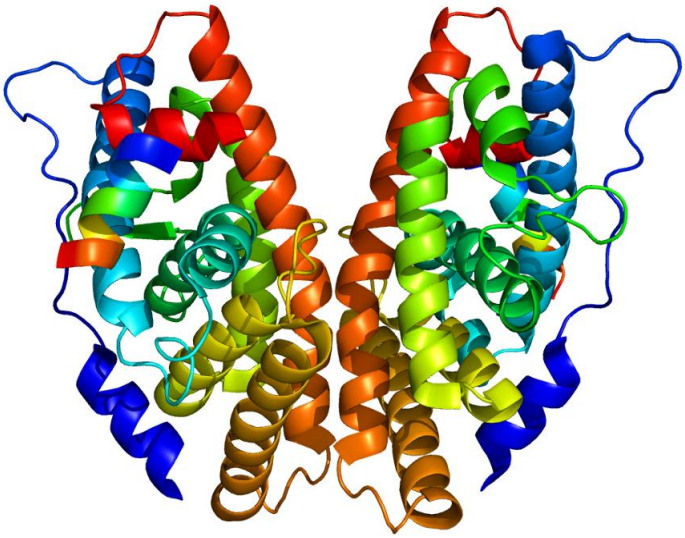


Figure 2.3: ERR-γ found in high concentration in the placenta. (Takeda, 2009).

In 2011 study researchers looked at 455 common plastic products and found that 70% tested positive for estrogenic activity. After the products had been washed or microwaved the proportion rose to 95%, (Ferguson, 2014).

The study observed that, almost all commercially available plastic products which were sampled (even some which were advertised as BPA-free), leached chemicals having endocrine activity (EA). In some cases, BPA-free products released chemicals having more EA than BPA-containing products (Ferguson, 2014).A systematic review published in 2015 found that "based on the current literature, bisphenol substitute, BPS is as hormonally active as BPA, and has endocrine disrupting effects (Rochester, 2015)

Studies carried on rats found that, low doses of BPA during development have persistent effects on brain structure, function and behavior in rats and mice (Vom Saal, 2008). Consequences at the level of neurobehavioral development in mice were found to be caused by long-term low-dose BPA maternal exposure (Palanza & Gioiosa, 2008). According to Ginsberg (2009), neonatal exposure to BPA affected sexually dimorphic brain morphology and neuronal adult phenotypes in mice and altered long-term potentiation in the hippocampus and even nanomolar dosage could induce significant effects on memory processes.

Highly controversial claims were made that BPA could be involved in attention-deficit hyperactivity disorder (ADHD) (Rubin & Soto, 2009). A study on rats prenatally exposed to 40 micro g/kg bw BPA concluded that corticosterone and its actions in the brain are sensitive to the programming effects of BPA (Heindel & Vom Saal, 2009).

Additionally, prenatal and neonatal exposure to BPA in mice showed that, they can potentiate the central dopaminergic systems, resulting in the super sensitivity to the drugs of abuse induced reward effects and hyper locomotion (Newbold *et al*., 2009).

A study carried on 2007 by Panzica, *et al*. showed that, BPA mimics estrogenic activity and has impact on various dopaminergic processes to enhance mesolimbic dopamine activity. A study carried on rats indicated that prenatal and neonatal exposure to low-dose BPA caused deficits in development at dorsolateral striatum through altering the function of dopaminergic receptors (Richter, *et al*., 2007). Bisphenol A has also been shown to bind to the thyroid hormone receptor and perhaps have selective effects on its functions (Palanza & Gioiosa, 2008).

There have been major concerns about BPA effects on triiodothyronine and the present evidence suggests that governing agencies should regulate the use of thyroid-disrupting chemicals especially because such uses relate exposures of pregnant women, neonates and small children to the agents (Patisaul & Polston, 2008).

Bisphenol A also increases cancer risk (Ogiue-Ikeda, *et al.*, 2008). A 2008 review indicated that "perinatal exposure to low doses of BPA alters breast development and increases breast cancer risk (Brown *et al.*, 2009) and that animal experiments and epidemiological data strengthen the hypothesis that fetal exposure to xenoestrogens may be an underlying cause of the increased incidence of breast cancer observed over the last 50 years (Kiguchi *et.al*, 2008).

2.4.1 Studies on reproductive health effects of BPA

Studies linked maternal BPA exposures to an increase in premature births (Chou et al., 2011). Maternal or paternal exposure to BPA during pregnancy was associated with decreased anogenital distance in the sons (Miao et al., 2011) suggesting feminization of male offspring**.** BPA has also been shown to induce neoplastic transformation in human breast epithelial cells (Fernandez & Russo, 2009) and maternal oral exposure. It was also noted that, low concentrations of BPA exposure during lactation increased mammary carcinogenesis in a rodent model and lead to reduced sensitivity to chemotherapy treatment of specific tumors (Jones & Miller, 2008)

It has also been shown that neonatal BPA exposure of 2 μg/kg to mice increased adult prostate weight (Bagchi, 2010). A 2007 in vitro study found that BPA within the range of concentrations currently measured in human serum is associated with permanent increase in prostate size (Richter, *et al.*, 2007).

Furthermore, bisphenol A was found to suppress DNA methylation (Dolinoy *et al*., 2007). A 2009 in vitro study on cytotrophoblasts cells found cytoxic effects in exposure of BPA doses from 0.0002 to 0.2 micrograms per milliliter. The study concluded that, exposure of placental cells to low doses of BPA caused detrimental effects, leading in vivo to adverse pregnancy outcomes such as preeclampsia, intrauterine growth restriction, prematurity and pregnancy loss (Benachour & Aris, 2009).

Studies done on 2010 and 2011 showed that increased BPA levels were associated with decreased sperm quality following environmental **(**Meeker *et al.,* 2010) and occupational **(**Li *et al*., 2011**)** exposure. Higher BPA levels were also associated with poorer sexual function in occupationally or environmentally exposed men, including decreased sexual desire and decreased erection and orgasmic function **(**Li. D *et al*., 2010(a)**).** Several studies indicated that environmental exposures to BPA (i.e. those experienced by typical adults) affected testosterone levels in men **(**Galloway *et al.,* 2010) and were associated with changes in estrogenic gene expression in adult males (Melzer *et al.,* 2011).

2.4.2 Non-reproductive health effects of BPA Studies

Some of the most concerning and compelling evidence linking BPA exposure to effects in humans is the effects of developmental exposures on behavior. A 2009 study reported that prenatal exposure was associated with an increase in hyperactivity and aggression in two-year-old girls **(**Braun *et al.,* 2009**)**. In a follow-up assessment of this cohort of children, average maternal BPA levels were associated with an increase in anxiety and hyperactivity, and poorer emotional control and inhibition in three-year-old girls **(**Braun *et al.,* 2011b**).** These results suggested that the behavior of BPA-exposed girls was masculinized. This is perhaps most revealing when considered in the context of animal studies, which have indicated that BPA can masculinize behaviors of female rodents, and may feminize the behaviors of male rodents (Adewale *et al.,* 2011**).**

In vitro studies have suggested that BPA can promote the growth of neuro blastoma cells (Zhuh, 2009).

Maternal BPA levels also influenced newborn hormone levels that are associated with lipid metabolism (Chou *et al.,* 2011). These results were consistent with a study in mice documenting disruption of glucose homeostasis in mothers and male offspring as a function of increased BPA exposure **(**Magdalena *et al.,* 2010). These studies indicated that, the offspring may be at risk for diabetes or obesity later in life. Additionally, studies done in

2012 indicated that, BPA activates the human pregnane X receptor (Sui *et al.,* 2012), which is involved in lipid homeostasis in addition to steroid and xenobiotic chemical metabolism.
Studies also showed that BPA could affect other endocrine parameters in addition to reproductive hormones and possibly metabolic homeostasis, for example higher BPA levels were associated with decreased thyroid hormone levels in adults (Meeker *et al*., 2011**).**
Early prenatal exposure was associated with an increase in child wheeze at six months of age **(**Spanier *et al*., 2012**).**
Additionally, BPA exposure was also found to influence the developing immune system.
BPA levels were associated with antibody titers to a common pathogen (cytomegalovirus). This relationship was found to be more common in individuals younger than 18 years old **(**Clayton *et al*., 2011). Studies conducted in 2007 showed that, for young children the average BPA exposure level is 42.98 ng/kg/day based on the environmental levels (Vandenberg *et al*.2007).
Furthermore, the first study showing an association between urinary BPA levels and heart disease was published in 2008 by Lang *et al.,* where by Individuals with higher BPA exposures were more likely to report cardiovascular diseases. In 2010, another cross-sectional study representative of the US population found that, higher BPA levels were associated with an increased incidence of coronary heart disease (Melzer *et al.,* 2010). This study was followed by a longitudinal study, in which BPA exposures were measured in adults free of coronary heart disease, and these individuals were then followed for 10 years (Melzer *et al.,* 2012). Individuals with higher urinary BPA levels at time zero were more likely to develop coronary heart disease at the end of the study compared to their counter parts with low urinary BPA concentrations at time zero. This study thus addressed the issue of causation, and suggested that BPA exposures could cause heart disease and refuted the suggestion that heart disease was caused by increase in BPA exposure. This study was similar to what was reported in 2008 **(**Lang *et al.,* 2008). A 2014 Korean study pointed out a strong correlation between hypertension and the BPA used in the plastic lining of canned drinks (Lee, 2014)

Studies of additional adult populations indicated that increased urinary BPA levels were associated with obesity in adults (Carwile and Michels, 2011). It was noted that obesity increased with BPA exposure, which merited concern among scientists and public health officials (Gore, 2007). A 2008 review of available studies concluded that "perinatal BPA exposure acts to exert persistent effects on body weight and adiposity (Byrne, 2008). Another 2003 review also concluded that "Eliminating exposures to BPA and improving

nutrition during development offer the potential for reducing obesity and associated diseases (O'Connor & Chapin, 2003).

A 2007 review concluded that BPA, like other xenoestrogens, should be considered as a player within the nervous system that can regulate or alter its functions through multiple pathways (Okada, 2008). BPA, at the reference safe limit for human exposure, was found to impact intestinal permeability and may represent a risk factor in female offspring for developing severe colonic inflammation in adulthood (Braniste, *et al.*, 2009)

2.5 Actions taken by various states due to issues concerning BPA

Given the negative health effects associated with BPA, a number of states took various actions in an effort to reduce BPA in the environment and regulatory bodies have determined safety levels for humans (Ginsberg, 2009).

In 2007, a consensus statement by 38 experts on Bisphenol A concluded that average levels in people are above those that cause harm to them. This was proved by using many animals in laboratory experiments (Vom Saal, 2007)

A panel convened by the U.S. National Institutes of Health determined that there was "some concern" about BPA's effects on fetal and infant brain development and behavior. The concern over the effect of BPA on infants was also heightened by the fact that infants and children are estimated to have the highest daily intake of BPA. In 2008 the U.S. National Toxicology Program (NTP) agreed with the panel, expressing "some concern for effects on the brain, behavior, and prostate gland in fetuses, infants, and children at current human exposures to Bisphenol A," and "minimal concern for effects on the mammary gland and an earlier age for puberty for females in fetuses, infants, and children at current human exposures to Bisphenol A. In January 2008 the FDA expressed the same level of concern.

The NTP had "*negligible"* concern that exposure of Bisphenol A to pregnant women will result in fetal or neonatal mortality, birth defects, or reduced birth weight and growth in their offspring (Erickson, 2008).

According to national conference of State Legislatures, laws were passed in several countries with regards to manufacturing and distribution of bottles containing BPA.

In U.S., Food and Drug Administration expressed concern about the potential effects of BPA on the brain, and in prostate gland of fetuses, infants and children. In July 2011, the Administration announced that it would no longer allow BPA in baby bottles and children's

drinking cups. The Administration did not restrict their use in other consumer products. (Noonan, 2011)

In 2011, California passed a bill that prohibited the manufactures from selling or distributing bottles or cups which contained BPA at a detectable level above 0.1 parts per billion if the containers were designed to be used by children three years of age or younger (Ann, 2011). The state required the manufactures to replace BPA in these products with the least toxic alternative and prohibited them from replacing BPA with certain carcinogens or reproductive toxicants. These restrictions took effect on July 1, 2013

In January 2011, the Massachusetts Department of Public Health revised its hazardous substances administrative regulations that declared children's reusable food or beverage containers containing bisphenol A banned hazardous substances. The ban covered those containers that were manufactured on or after January 7, 2011, or sold at retail on or after July 1, 2011. Previously, the Department had issued a public health advisory in August 2009 warning parents of infants and young children to avoid storing infant formula or breast milk in plastic bottles containing BPA (Noonan, 2011)

In Washington senate law was passed in March 2010. The law prohibited the manufacture, sale or distribution of empty bottles, cups or other food or beverage containers that contained BPA (West, 2010). Metal cans were exempted from this ban. The law also prohibited the manufacture, sale or distribution of empty sports bottles of 64 ounces or less that contained BPA after July 1, 2012.

In Columbia law was passed in March 2011 which prohibited manufacture, sale or distribution of bottles, cups or containers made from BPA if they were designed to be filled with food or liquids. The restrictions took effect as from July 1, 2011 (Noonan, 2011),

Canada banned BPA in baby bottles in August 2008 (Cao *at al.,* 2010) While Chicago banned the sale of baby bottles containing BPA in May 2009 (Forman, 2009)

Suffolk County, New York banned baby bottles containing BPA in April 2009 (Forman, 2009). On 24 March 2014, the French Senate unanimously approved a proposition of law to ban BPA from baby bottles (Audran, 2014). The National Assembly (Lower House) approved the text on 23 June 2010, and it has been applicable law since 2 July 2010 (Liao , 2012). On 12 October 2011, the French National Assembly voted a law forbidding the use of Bisphenol A in products aimed at less than 3-year-old children (Monde, L. (2011) and 2014 for all food containers (Audran, 2014).

As of 2014, 12 states had banned BPA from children's bottles and feeding containers (Tavernisef, 2014).

2.6 Studies on Bisphenol Substitute (BPS)

Concerns over the health effects of BPA has encouraged plastics manufacturers to produce "BPA-free" products, with a label 'BPA-free' in products such as water bottles and food containers. However, bisphenol S (BPS) is closely identical in structure to BPA. Currently several studies have described the dangers of BPS such as reproductive health issues and cancer (Chen *et al*., 2016). In addition, researchers from Health Canada, a federal department responsible for helping citizens maintain and improve their health found another health concern associated with BPS in that, it triggered the formation of fat cells. The formation of the fat cells has lead to health issues like obesity and diabetes (Kossman, 2016). Other studies showed that BPS caused severe reproductive defects including germline apoptosis, embryonic lethality asthma and birth defects (Chen *et al*., 2016).

BPS shares a similar structure and versatility to BPA and has been used in numerous products from currency to thermal receipt paper. Widespread human exposure to BPS was confirmed in an analysis of urine samples taken in the U.S., Japan, China, and five other Asian countries (Liao, 2012). Researchers found BPS in all the receipt paper, 87 % of the paper currency and 52 % of recycled paper they tested. The study also found that people may have been absorbing 19 times more BPS through their skin than the amount of BPA they absorbed, when it was more widely used (Liao, 2012).

2.7 Biosensors

Traditional methods such as high performance liquid chromatography (HPLC), gas chromatography (GC) and NMR were developed for detection of BPA in food substances so as to reduce BPA intake in humans. These methods were found to be costly, needed expensive and sophisticated equipments, required skilled personnel and they are time consuming for routine tests. They are also unaffordable by the common Kenyan and hence there was a need to develop biosensor system. (Daniel *et al*., 2001).

Biosensors are analytical devices which are used for the detection of an analyte that combines a biological component with a physicochemical detector. They were developed for several decentralized analytical applications and are useful tools in medicine, biotechnology, genetic engineering, food quality control, military, agriculture, environmental monitoring and other practical fields (Rodriguez-Mozazet *et al*.,2005).

The concept of a biosensor dates back to 1962 when Clark and Lyons described the development of the first "enzyme sensor" (Zhang *et al.*, 2002). Since then, various biosensors have been developed to detect a wide range of biochemical parameters and there has been incredible activities witnessed in this area of biosensors.

Zhang *et al.* (2002) reported five features of systematic description of a biosensor, namely; the detected or measured parameter, the working principle of the transducer, the physical and chemical or biochemical model, the application and the technology and materials for sensor fabrication. Figure 2.4 shows schematic representation of a biosensor.

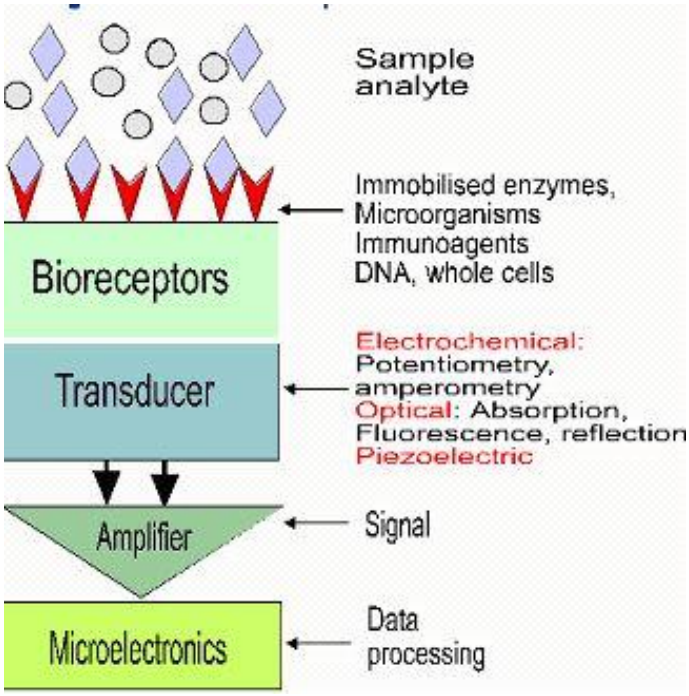


Figure 2.4: Schematic Representation of a Biosensor (Source:dipcia.unica.it/superf

The transducers may consist of a pair of electrodes, an interface layer incorporating the biological recognition molecules and a protective coating. Traditional electrode systems for measurements of concentrations of ions in liquids and dissolved gas partial pressures contain only a working electrode (usually a noble metal wire) and an electrically stable reference electrode, such as Ag/AgCl, though sometimes a counter electrode is included. A simple electrical or chemical modification may improve specific electrode properties (Zhang *et al*.2002). The biological materials used can be enzymes, antibodies, receptors, DNA or micro-organisms. The biochemical response is converted into optical and optic signal transducing elements (electrode, optical, piezo crystal etc.) which are then amplified, measured and decoded by an appropriate electronic unit. For a biosensor to be used it has to

be characterized. To characterize a biosensor, a number of parameters, for example linearity, sensitivity, selectivity, stability and response time have to be considered properties (Zhang *et al*.2002)..

Linearity refers to the maximum linear value of sensor calibration curve while sensitivity is the value of electrode response per substrate concentration. Selectivity implies the interference of chemicals must be minimized to obtain correct results and time response, the necessary time to have 95% of the response.

2.8 Types of Biosensors

There are several types of biosensors that have been used, for example electrochemical, amperometric, potentiometric and conductimetric biosensors. Other types of biosensors which are used in health care include: Glucose biosensors, immunosensors, DNA and enzyme biosensors.

2.8.1 Electrochemical biosensors

The fundamental principle of this class of biosensors is that, it has many chemical reactions that produce or consume ions or electrons. These chemical reactions in sequence cause some changes in electrical properties of the reaction solution and these changes can be sensed out and used as a measuring parameter. Depending on the electrochemical parameter measured, electrochemical biosensors can further be classified into amperometric, potentiometric, and conductimetric or impedimetric biosensors (Daniel *et al*., 2001).

2.8.2 Amperometric biosensor

Amperometric biosensors are used to measure the current produced during the oxidation or reduction of a product or reactant at a constant applied potential. These sensors are noticed to have fast response time and good sensitivity (Apetrei, M. (2011). However, the excellent specificity of the biological component can be compromised by the partial selectivity of the electrode. Due to the lack of specificity, ample sample preparation, separation or some compensation for interfacing signals is usually required (Zhi-Da *et al.*, 2014).

2.8.3 Potentiometric biosensor

Potentiometric biosensors use a gas sensing electrode as the physical transducer or an ion-selective electrode to relate electrical potentials to the concentration of analyte. These biosensors have large dynamic ranges, are selective and non-destructive. Potentiometry is

hardly used as a detection method in biosensors where enzymes are immobilized in an electrodeposited polymer layer. However, some experiments were demonstrated using amperometric detection by PPy (Polypyrrole) based electrode (Gerard *et al.* 2002). In these experiments, there were difficulties encountered regarding the immobilization of enzymes in the electrodeposited polymer layer, that is, the mechanism of the entrapment and dynamics effects on biosensors (Gerard *et al.* 2002).

2.8.4 Conductimetric Biosensors

These biosensors focus on measuring the changes in the conductance of the biological component occurring between a pair of metal electrodes. During electrochemical reactions, ions or electrons are either produced or consumed and this usually causes an overall change in the conductivity of the solution (Wang *et al.* 2006). This change produced is measured and calibrated to a proper scale. The advantage of Conductimetric biosensors is that they have planar conductimetric electrodes which are suitable for miniaturization and large scale production, they do not need a reference electrode, the applied voltage can be effectively small to reduce substantially the sensor's power consumption and large spectrum of analyte of different nature can be determined on the basis of various reactions and mechanisms (Wang *et al.* 2006). However, they need skilled personnel to use them and also they are expensive for routine tests.

2.8.5 Biosensors used in health care

Biosensors in health care are used for selective determination of various blood analytes like glucose, urea, lactate, uric acid and cholesterol. The determination of the blood analytes helps in screening and treatment of a number of diseases. Such biosensors include DNA, glucose and enzyme biosensors, and immunosensors.

2.8.5.1 DNA biosensors

DNA biosensors are commonly used and they have a number of applications in; clinical diagnosis of inherited diseases, rapid detection of pathogenic infections, and screening of DNA colonies required in molecular biology. Conducting polymers are used for this DNA biosensor development. Immobilization of DNA on a conducting polymer matrix facilitates the detection of a signal (amperometric or potentiometric) generated as a result of interaction of proteins or drugs with DNA (Shibiao *et al*., 2015).

2.8.5.2 Glucose biosensors

Glucose biosensors are commonly used for detection of glucose level in the blood. Elevated levels of glucose in the blood can lead to diabetes mellitus. The biosensor has been developed as a useful tool to manage diabetes and maintain normal blood glucose level in human body. Maintenance of the glucose level helps to avoid the long term undesirable consequences of elevated blood glucose, including neuropathies, blindness and failure of key body organs such as kidney (Shibiao *et al*., 2015).

2.8.5.3 Immunosensors

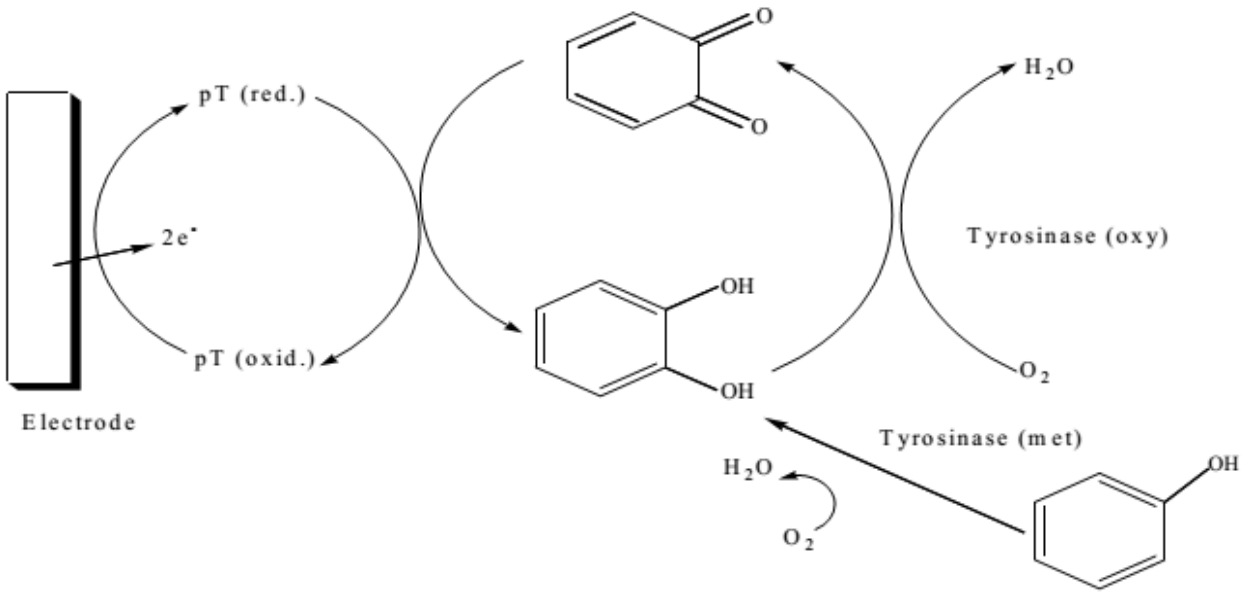
Immunosensors have brought about effective combination of immunochemistry and electrochemistry in an analytical device. Quite a number of immunosensors have been developed based on conducting polymers. The use of conducting polymer for reversible immunosensors is a unique approach based on pulsed electrochemical detection developed by (Ybarra, 2012).This approach has been employed for the detection of organochlorine pesticides including PCBs, atrazines and chlorinated phenols. Porter and colleagues in 2000 investigated electroplated conducting polymers as antibody receptor in immunosensors (Porter *et al*., 2000).

2.8.5.4 Enzyme biosensors

In the measuring of phenolic compounds, electrochemical methods based on enzymes have been commonly used because of their advantages like good selectivity, long-term stability and potential for efficiency and automation (Matyholo, 2011). An enzyme biosensor is derived from a combination of a transducer with a thin enzymatic layer, which usually measures the concentration of a substrate. The enzymatic reaction transforms the substrate into a reaction product that is detectable by the electrode (Narli, 2006). The concentration of any substance can be measured so long as its presence affects the rate of an enzymatic reaction which is especially true for enzyme inhibitors. The signal that is current or potential measured is proportional to the rate-limiting step in the overall reaction (Narli, 2006).The enzyme is entrapped to a conducting polymer through immobilization. Immobilization of an enzyme helps in increasing the stability of the enzyme. The use of free enzyme is limited because of its instability and rapid inactivation (Matyholo, 2011) and thus immobilization of an enzyme provides multiple and repetitive use by increasing stability of enzyme (Narli, 2006). When free enzyme in solution is compared to the immobilized enzyme, the immobilized enzyme is more stable and resistant to various environmental changes (Matyholo, 2011). Enzyme immobilization also prevents contamination and protects the

enzyme against changes in pH, temperature and ionic strength in the bulk solvent (Narli, 2006). In this study Tyrosinase enzyme was used as the bio component and conducting polymers as the matrix. Tyrosinase based biosensor was developed for simple and affective analytical methods for determination of phenolic compounds due to its high sensitivity, effectiveness, and simplicity (Zhao, 2009). Scheme 2.1 shows the proposed processes that take place at biosensor layers, whereby monophenol is oxidized to diphenol. The process is catalyzed by Tyrosinase enzyme with the presence of oxygen molecule.

Scheme 2.1: Proposed processes at biosensor layer Redrawn from (Dempsey, 2004)



2.8.6 Enzyme Immobilization

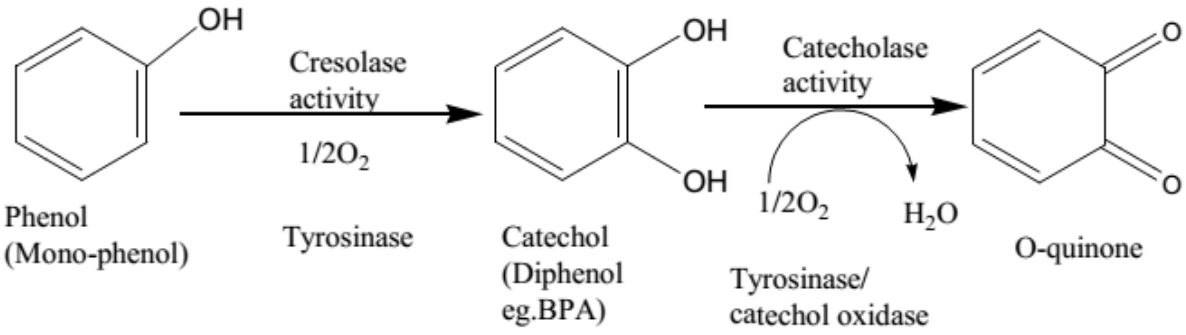
Most applications of enzyme normally require immobilization to allow the enzyme to be used repeatedly. Immobilization of enzyme has been identified by many authors as important step in fabrication of enzyme biosensors because it ensures intimate contact between the enzyme and the underlying transducer and it also prevents the enzyme from being washed off the electrode when analysis is performed in aqueous samples (Faria *et al.*, 2007). The immobilization techniques that have been reported in literature include; covalent linking (Biegunski *et al.* 2006), polymer entrapment (Chen *et al.* 2003), drop-coating (Zhuo *et al.* 2006; Wang *et al.* 2009; Alarcon *et al.* 2010) and electro-polymerization (Arslan *et al.* 2005). Some of these immobilization methods are relatively complex and some result to poor stability and bioactivity. Enzyme activity can be reduced by immobilization of biological

components and environmental conditions (Alarcon *et al.* 2010). The methodology used for immobilization, surface area of electrode, porosity, hydrophilic character of immobilizing matrix and reaction conditions is very critical for the activity of immobilized enzyme due to its sensitivity. For phenol biosensors, enzyme immobilization has been done on different support surfaces such as glassy carbon electrode modified with electrodeposited gold nanoparticles (Carralero Sanz *et al.* 2005), boron-doped diamond electrode (Notsu *et al.* 2002), carbon fiber electrode (Kuramitz *et al.* 2001), electrode modified with single walled nanotubes (Zhao *et al.* 2005) and electrode modified with poly(thionine) film (Dempsey *et al.* 2004). However, some of these immobilization support surfaces and methods are somewhat difficult and do not present an excellent enzyme stability. Studies have shown that conducting polymers are suitable material for the entrapment of enzyme and they reveal conducting polymer material serves as promising matrix for enzyme immobilization and stability (Gerard *et al.* 2002). Electrochemically synthesized conducting polymers allow direct deposition of the polymer film on the electrode surface and at the same time trapping the enzyme. Conducting polymers have the ability to transfer electric charge created by the biochemical reaction in the electronic circuit.

2.8.7 Tyrosinase Enzyme

Tyrosinase is a blue copper protein with two copper atoms in active centers. Tyrosinase can be considered as a polyphenol oxidase (PPO) (Vicentini, 2013). This enzyme catalyzes two consecutive oxidation reactions: first, hydroxylation of monophenols into *o*-diphenols (monooxygenase, cresolase, monophenolase or hydroxylase activity) and also the two-electron oxidation of diphenols (e.g. catechol, BPA etc.) to *o*-quinones (catecholase or diphenolase activity) with molecular oxygen and formation of water (Bartlett, 2008). Tyrosinase has been extensively used in biosensor construction for the determination of phenols (Fiorentino, 2014) . The *o*-quinones generated are reduced electrochemically at appropriate potentials thus reduction currents obtained serve as analytical signals which are proportional to the concentrations of phenols or phenol derivatives (Notsu, 2002). The Tyrosinase mechanism of mediated phenol oxidation is shown in scheme 2.2 and equations for oxidation of phenol structure 2.1:

Scheme 2.2: Tyrosinase mechanism mediated phenol oxidation. (Khan 2007; Ramsden et al.2010)



$$phenol + \frac{1}{2}O_2 \xrightarrow{monoooxygenase} O-diphenol \qquad \text{(Equation 2.1)}$$

$$O-diphenol + \frac{1}{2}O_2 \xrightarrow{catecholase} O-quinone + H_2O \qquad \text{(Equation 2.2)}$$

Figure 2.5 shows some structures of Tyrosinase enzyme showing the two copper ions active site bonded together. ***(Matoba et al., 2006)***

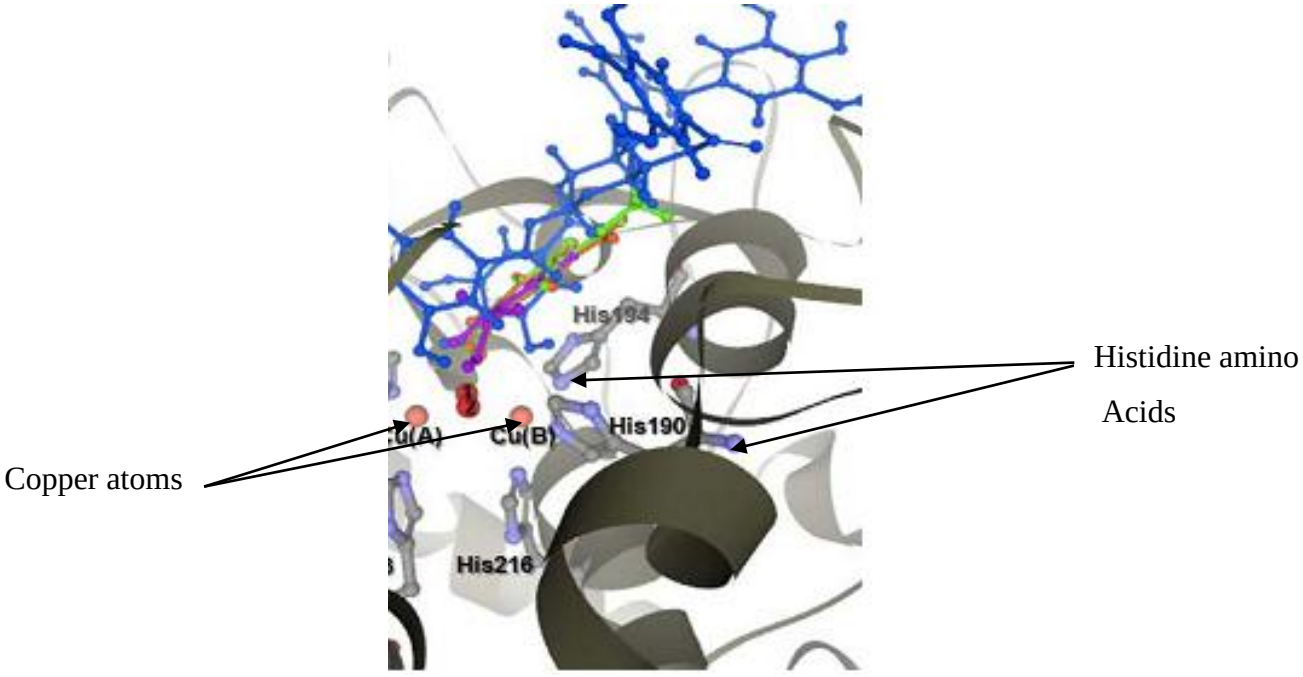
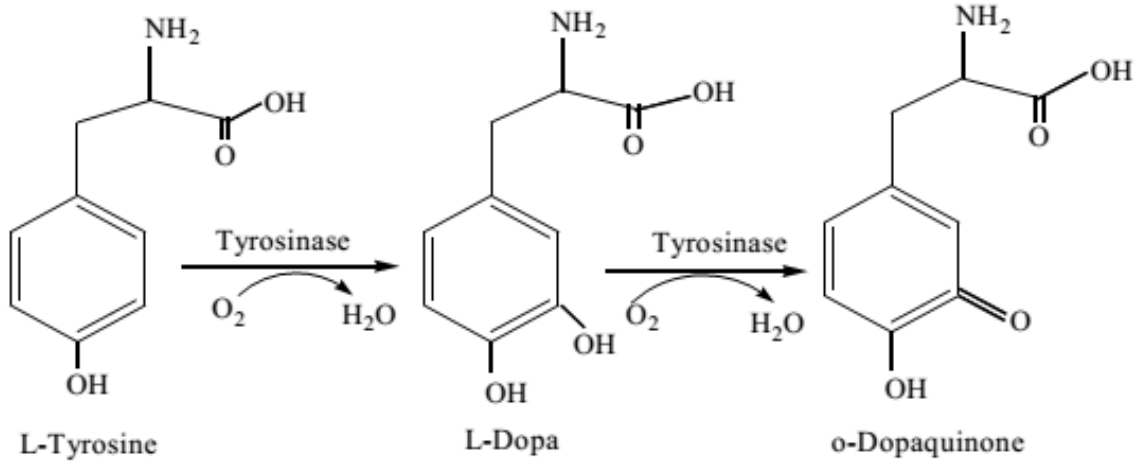


Figure 2.5: Tyrosinase enzyme Structure showing bio-nuclear copper (Cu) active site (Matoba et al., 2006)

2.8.8: Catalytic cycle of Tyrosinase

Tyrosinase catalytic cycle is composed of three different enzymatic forms involved in its reactions. The type three copper centers of Tyrosinase are found in three redox forms throughout the catalytic reaction. First, the deoxy form [Cu (I)-Cu (I)] is a reduced species; it binds with oxygen to give the oxy form [Cu (II)-O_2 2-Cu (II)] (Permyakov, 2009). Within the oxy form, molecular oxygen is bound as peroxide in a μ-η 2: η 2 side-on bridging mode, which weakens the O–O bond and makes it active. The [Cu (II)-Cu (II)] form is understood as an inactive enzymatic form, where Cu (II) ions are normally bridged to a small ligands, such as a water molecule or hydroxide ion (Matoba *et al.*, 2006). Tyrosinase is commonly used in catalyzation and synthesis of melanin through the hydroxylation of L-Tryosine to dihydroxy L-phenylalanine (L-DOPA) which is followed by oxidation of L-DOPA to o-dopaquinone mechanism (Rani *et al*., 2007). This is shown in the scheme 2.3.

Scheme 2.3: L-Tryosine oxidation to o-dopaquinone mechanism. (Faria et al., 2007)



The produced o-dopaquinone is unstable so it polymerizes and precipitates into melanin (Narli, 2006). The cresolase activity of Tyrosinase is important because it synthesizes L-DOPA. According to Nihei (2004), Tyrosinase is mainly employed for biosynthesis of the large biological pigment, melanin responsible for skin, eye, inner ear and hair melanization. Tyrosinase also causes browning in fruits, mushrooms and vegetables. Tyrosinase has widespread applications in medical and industrial fields. For example, Tyrosinase may play a

role in neuromelanin formation in the human brain; it could be central to dopamine neurotoxicity and contributes to the neuro degeneration associated with Parkinson's disease (Narli, 2006). Patients who suffer from Parkinson's disease show major decrease in the concentration of dopamine found in the large area of the brain (Rani *et al.,* 2007). Shortage of neurotransmitter dopamine can lead to neurological disorder. Neurological disorder is a disease which normally affects old people and is manifested in forms of tremor, rigidity, slowness of speech and lastly dementia. (Munjal *et al*., 2002). Also, the production of epidermal hyper pigmentation of melanin causes some dermatological disorders such as melasma, freckles, ephelide, senility and lentignes (Narli, 2006). L-dopa has been approved as a drug for curing Parkinson's disease and the mentioned drug has been in the market since 1967 (Faria *et al.,* 2007).

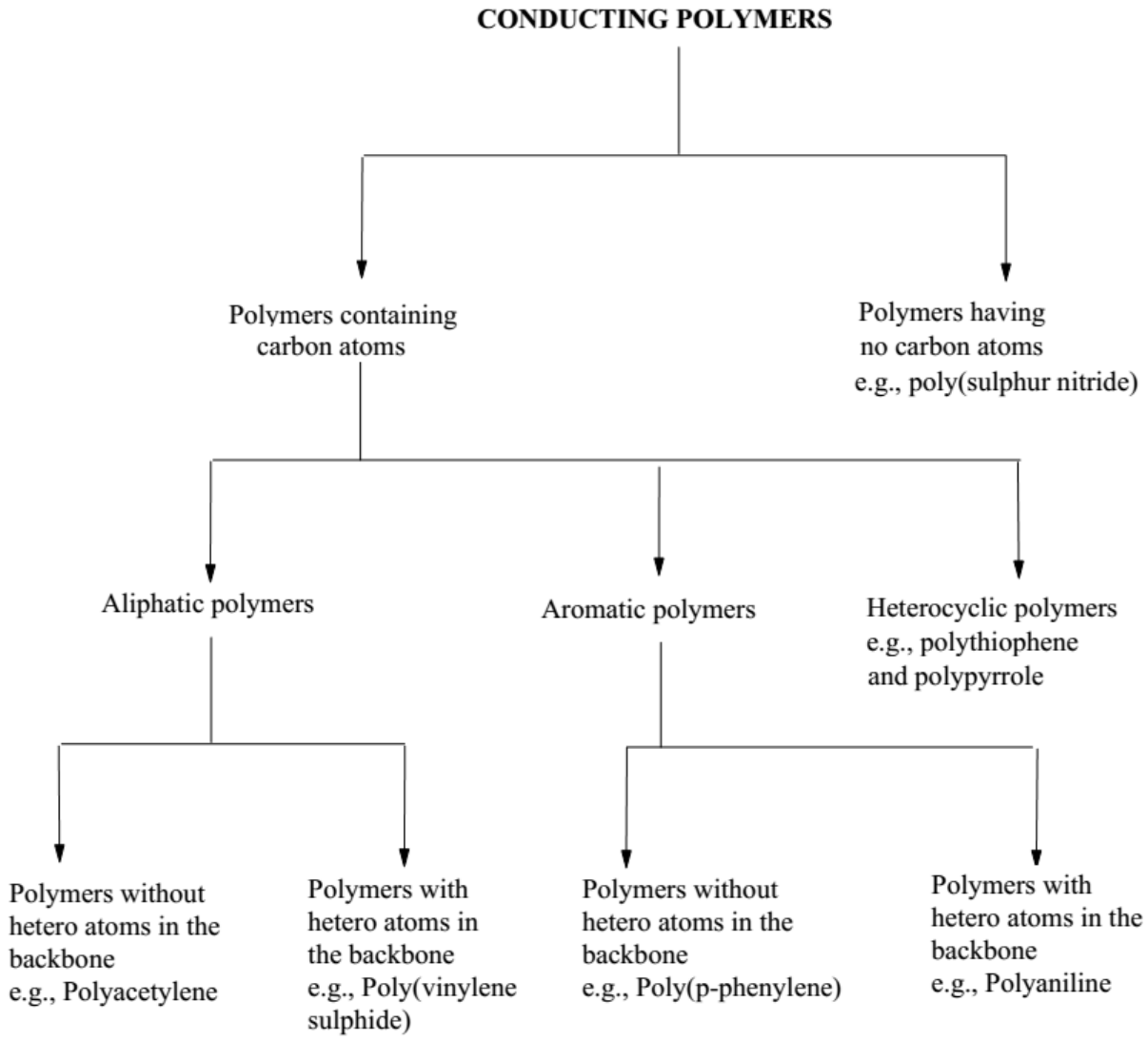
Industrially, Tyrosinase is utilized in determination of phenols and its derivatives. (Fenoll *et al.,* 2001). Tyrosinase influences the precipitation of phenols, which then make it possible to filter it out from surface water and industrial discharge sources. The enzyme has also been used as a sensor to detect the concentration of phenols in waste water (Zhao *et al.,* 2005). Tyrosinase based biosensor utilizes reduction of quinone at negative potential to prevent phenol polymerization as well as interferences from other oxidizable species (Sidek, 2005). The mechanism in scheme 2.2 represents the oxidation phenolic substrate (mono-phenol) by polyphenol oxidize (Tyrosinase) produces a dihydroxybenzene (catechols) which is further oxidized to o-quinone in the presence of molecular oxygen. O-quinone is usually unstable and therefore it undergoes a non-enzymatic polymerization to form pigments called melanins (Matoba *et al.,* 2006).

In this study, the enzyme Tyrosinase was used as the biological recognition element while the electro synthesized conducting polymer and polyaniline doped with a surfactant SLS was used as mediator to shuttle electrons between the active site of the enzyme and the surface of the electrode. Modified Glassy carbon electrode was used as the signal transducing element. The biosensor was completed by immobilizing Tyrosinase enzyme by drop coating method into a carbon modified electrode using bentonite clay.

2.9 Historical background of conducting Polymers

Conducting polymers are organic compounds with extended π-orbital systems which are responsible for electron movement from one end of the polymer to the other (Janata, 2002). Conducting polymers have been used in different biosensors fabrication in several fields like in health care which includes medical diagnosis, for example glucose, fructose, lactate, ethanol, cholesterol, etc. Immunosensors used in medical diagnostics and environmental sensors, DNA sensors used for detection of various genetic disorders and lastly polyphenol oxidase sensors used in environmental monitoring to control pollution and detection of hazardous chemicals (Stenger-Smith, 1998). Common classes of organic conductive polymers includes polyacetylene, polypyrrole (Jansson, 1980), polythiophene, polyterthiophene (Waltman, 1983), polyaniline and polyfluorine.

Scheme 2.4: Classification of conducting polymers (Rerum, 2007)



Conducting polymers have come out as a potential tool for electrochemical sensors due to their straightforward preparation methods, unique properties, and stability in air (Diaz and Logan, 1980). Conducting polymers have been applied as energy storage devices, electrochemical devices, chemical sensors and electro-catalysts in biosensors. They are a promising group of compounds, which are widely applied in chemical sensors and a variety of other applications (Diaz *et al.,* 1980). Conducting polymers contain π-electron backbone responsible for their unusual electronic properties such as electrical conductivity, low energy optical transitions, low ionization potential and high electron affinity. The extended π-conjugated systems of the conducting polymers have single and double bonds alternating along the polymer chain (Gerard *et al.,* 2002).

Conducting polymers show almost no conductivity in neutral (uncharged) state but become electrically conductive upon partial oxidation or reduction, a process commonly referred to as doping (Oudard *et al.,* 1988). It is possible to change the required electronic and mechanical properties of conducting polymers by chemical modeling and synthesis. Conducting polymers can be synthesized either electrochemically (Sulak *et al.,* 2010) or by chemical oxidative polymerization methods (Storrier *et al.,* 1994). Chemical synthesis is favored because conducting polymers can be synthesized in large quantities which are attractive from a practical point of view. However, electrochemical synthesis of polymers has the advantage of being more pure than the chemical synthesis (Sulak *et al.,* 2010). Chemical synthesis allows scale-up of the polymers, which is currently not possible with electrochemical synthesis. However, electrochemical polymerization is the most preferred general method for preparing conducting polymers because of its relatively straightforward synthesis procedure, simplicity and reproducibility (Sulak *et al.,* 2010).

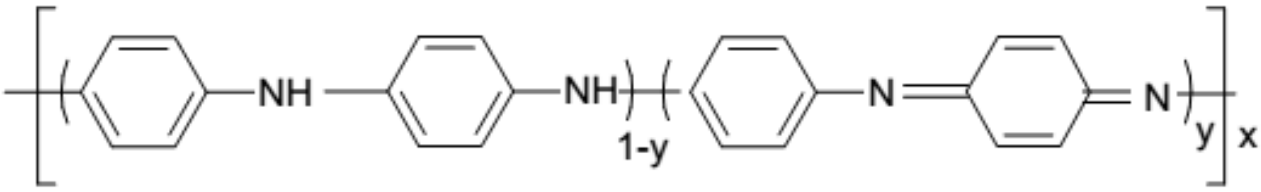
The common technique used in electrochemical polymerization is oxidative coupling. This technique involves oxidation of monomers to form a cation radical followed by coupling to form di-cations and the repetition leads to the polymer formation *(*Rerum, *2007)*. Electrochemical polymerization can be carried out galvanostatically (constant current), potentiostatically (constant potential) or by potential scanning/cycling or sweeping methods.

The thickness of the film can be controlled by varying either the potential or current with time. When electrochemical polymerization is used, the monomers at the working electrode surface undergo oxidation to form radical cations that react with other monomers or radical

cations, forming insoluble polymer chains on the electrode surface *(*Rerum, *2007)*. Among the conducting polymers, polyaniline and polypyrrole are mostly used conducting polymers worldwide because of their desirable electrical conductivity, environmental stability, low cost of production, ease of synthesis and favorable physiochemical properties. These polymers preserve extensive interest because of several technological applications in different fields such as sensors and biosensor, rechargeable batteries, microelectronics devices and corrosion protection (Nabid *et al.,* 2008). Among the organic, aromatic conducting polymers, polyaniline (PANI) is unique due to its electrical properties which are controlled by both oxidation state and protonation. However, these polymers are mechanically weak, low processable and also its application is limited due to its poor solvent solubility (Conklin *et al.*, 1995; Mavundla *et al.,* 2008).

2.9.1 Polyaniline

Polyaniline (PANI) is the oldest known organic electro conducting polymer, since it was used



for textile coloring one century ago (Sayed & Dinesan, 1991; Wallace *et al.*, 2009).

The base form of polyaniline has the following generalized composition where y represents the oxidation states of the polymer (MacDiarmid, 1986).

Fig 2.6: Structure of polyaniline (Mac Diarmid, 1986)

Great interest in research of polyaniline has been connected to discovery of its conductivity in the form of emeraldine salt and existence of different oxidation forms (Inzelt, 2008) as cited in Wallace *et al.*, 2009)

2.9.2 Oxidation states of Polyaniline

Unlike other known electro-conducting polymers, polyaniline can exist, depending on degree of oxidation, in different forms, namely: leucoemeraldine, emeraldine and pernigraniline. Leucoemeraldine, e.g. Leucoemeraldine base, refers to the fully reduced form; emeraldine, e.g. Emeraldine base, is half-oxidized, while pernigraniline, e.g. Pernigraniline base is the completely oxidized form of polyaniline. The only conducting form of polyaniline is

emeraldine salt, obtained by doping or protonation of emeraldine base (Fedorko *et al.*, 2010; MacDiarmid *et al.*, 1987, Pron & Rannou, 2002).

The unique feature of the mentioned polyaniline forms is ease of its mutual conversions by both chemical and electrochemical reactions as it can be seen in Fig. 2.7. (Gospodinova & Terlemezyan, 1998; Kang *et al.*, 1998; Stejskal *et al.*, 1996).

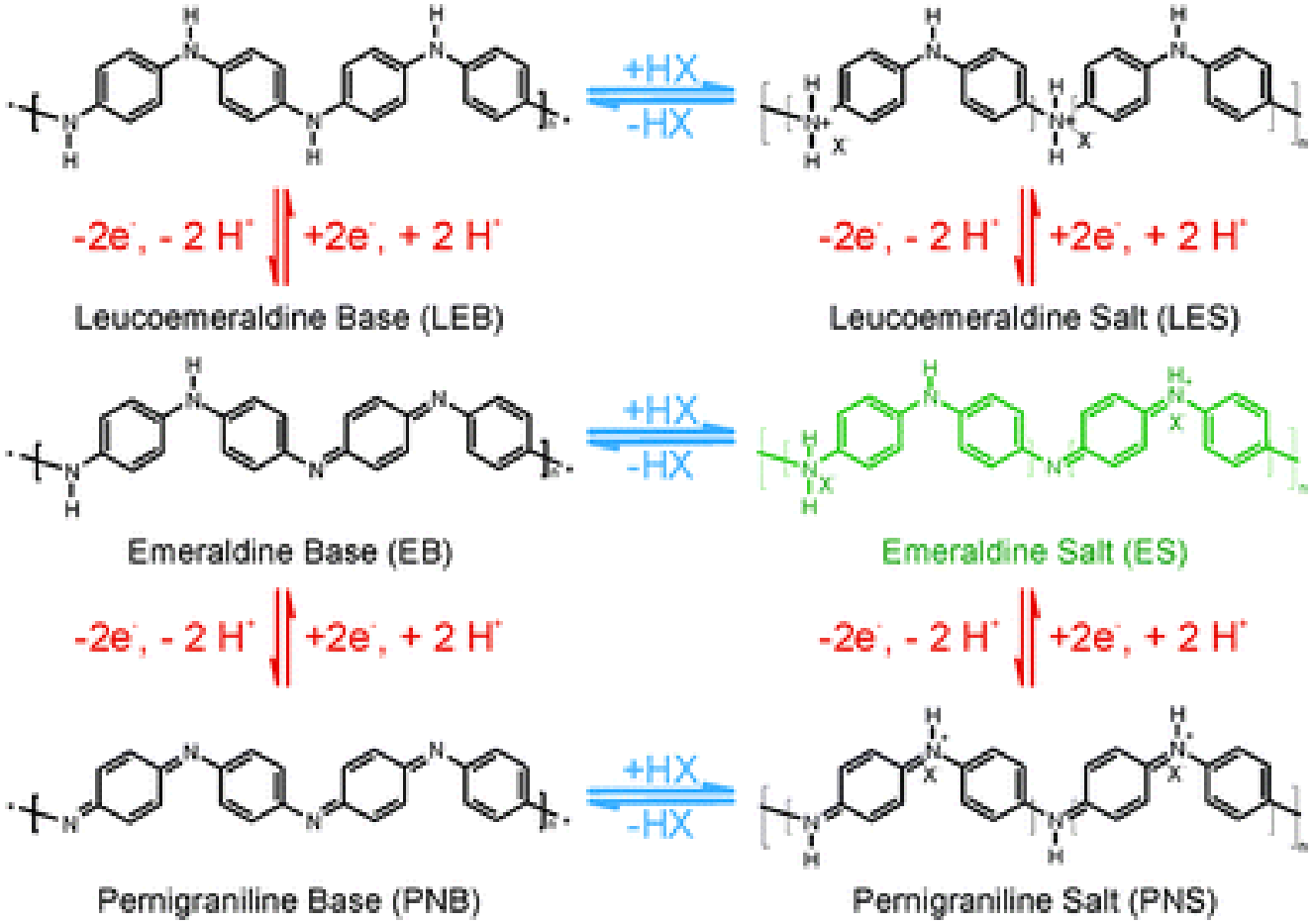


Fig. 2.7: Different forms of Polyaniline.

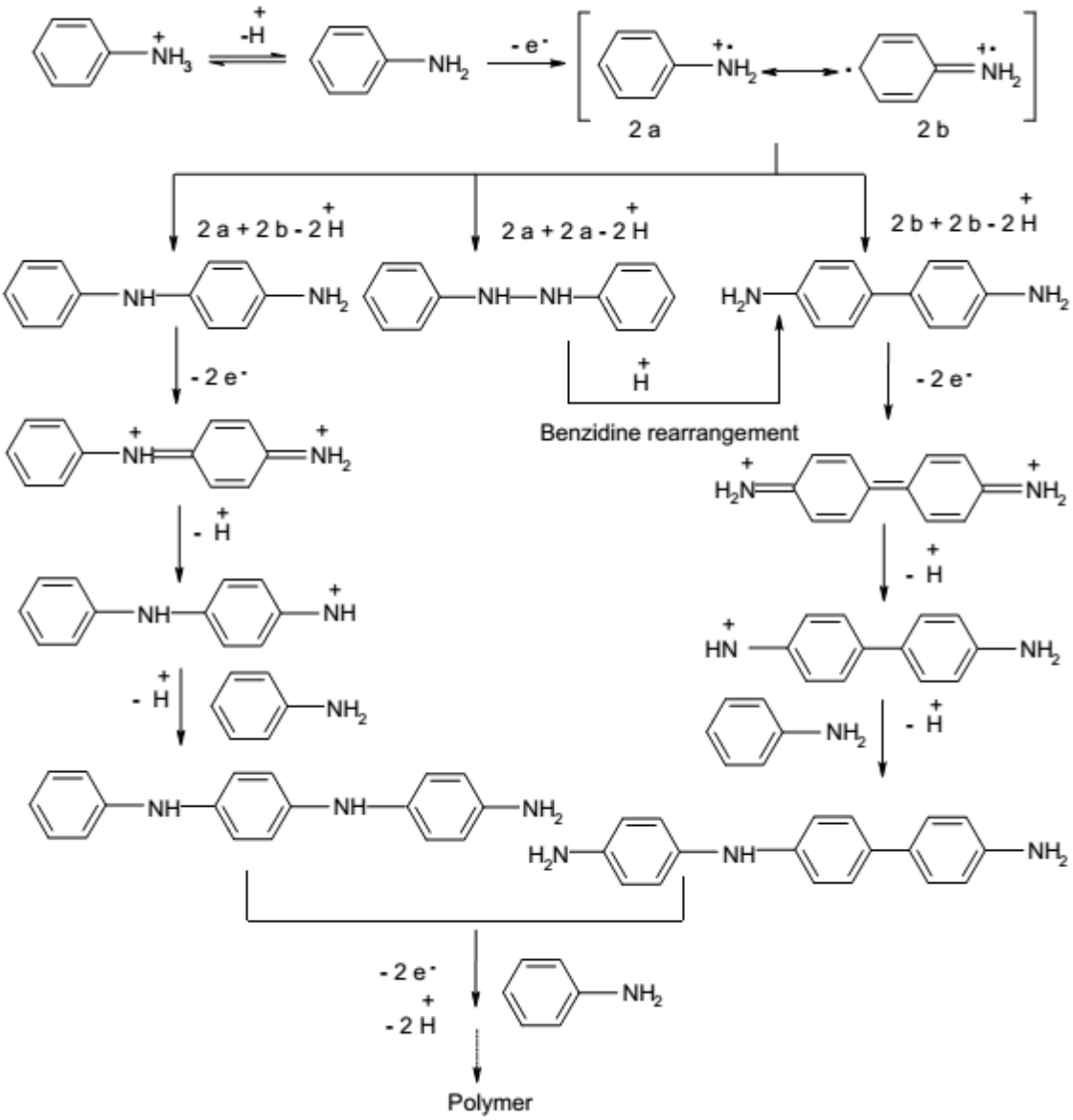
Apart from the changes in oxidation levels, all the transitions among polyaniline forms are manifested by color and conductivity changes (Stejskal *et al.*, 1996). The conducting protonated emeraldine in the form of green emeraldine salt is obtained as a product of electrochemical polymerization of aniline in acidic electrolytes. This can be easily transformed by further oxidation to fully oxidized dark blue pernigraniline salt, which can be treated by alkali to form violet pernigraniline. Emeraldine salt can also be reduced to transparent leucoemeraldine, or can be transformed by alkali to blue non conducting

emeraldine. The two blue forms of polyaniline, pernigraniline salt and emeraldine have different shades of blue (Stejskal *et al.*,1996). Reduction of emeraldine salt to leucoemeraldine and oxidation to pernigraniline states are followed by decrease in conductivity (Stejskal *et al.*, 2010).

2.9.3 Electropolymerization Mechanism of Aniline

Aniline can be polymerized electrochemically in organic or acidic aqueous media. Electropolymerization of aniline to polyaniline in aqueous H_2SO_4 was first reported in 1962 (Mohilner, 1962). The polymerization of aniline is reported as a bimolecular reaction involving a radical cation intermediate (scheme 2.5) The reaction produces benzidine and 4-amino diphenylamine in different proportions depending on the pH of the medium as the major intermediate species during the aniline polymerization Wei *et al.*, . (1989) reported a significant increase in the rate of polymerization of aniline when a small amount of the dimeric species was added as the initiators. Mohilner, (1962) inferred that the oxidation of aniline to form a primary radical cation is the rate-limiting step in electrochemical polymerization of aniline. It has been shown that a seed film of polyaniline significantly enhances the rate of polymerization even at potentials as low as +0.55 V using double potential step experiments (Sasaki, 1986). It was also proposed that the polymer chain ends incorporate neutral aniline monomer by radical cation mechanism.

Scheme 2.5: Mechanism for the Electro polymerization of aniline. (Wei et al., 1989)



2.9.4 Nanostructured Polyaniline

Synthesis of nanostructured PANI, especially as nanofibers, can improve polyaniline electrical, thermal and mechanical stabilities (Kinimura, 1988). The nanofibers can have a high impact in electronic devices and molecular sensors due to their extremely high surface area, synthetic versatility and low-cost. The conventional synthesis of polyaniline, based on the oxidative polymerization of aniline in the presence of a strong acid used as dopant, typically results in an irregular granular morphology (Fedorko *et al.,* 2010).

2.10 Glassy carbon Electrode

Glassy carbon electrode has been used due to its specific surface orientation. It's most important properties include high temperature resistance, low density, low electrical resistance, low friction, low thermal resistance, extreme resistance to chemical attack and impermeability to gases and liquids. (Sobkowiak *et al.,* 1995). It is usually employed as an electrode material for the fabrication of sensors (Thiago, 2002). Glassy carbon pastes, carbon paste etc. electrodes when modified are termed as chemically modified electrodes. Chemically modified electrodes have been employed for the analysis of organic molecules for example viz, Paracetamol, aspirin, caffeine, phenol, catechol, resorcinol, hydroquinone, dopamine, L-dopa, epinephrine, nor epinephrine, methyl parathion, ethyl parathion, venlafaxine, desvenlafaxine, imipramine, trimipramine, desipramine as well as metal ions like bismuth and antimony (Sanghavi *et al., 2013*)

2.11 Bentonite Clay

Bentonite clay is also called *montmorillonite* clay. It has high tendency to absorb and retain water, and it swells. This property makes it desirable for applications in modifying electrode to obtain desirable results (Bard *et al.,* 1986). Bentonite has a density of 1.25g/cm3 which is comparable to other clay minerals from different parts of the world (Bard *et al.,* 1986). It has a solvent retention capacity of 22.5% and 4.8% for water and organic solvents respectively. Its moisture content is 8.5%. It swells by a factor of 1.7 and 1.4 in water and organic solvents respectively (Orata and segor,2000).

Bentonite carries a relatively strong negative ionic charge. The negative charge is compensated by adsorbing a cation (either sodium or calcium) to the interior of the molecule, this is what makes it either sodium bentonite/ or calcium bentonite clay. $Mg^{2+}/Al^{3+}/Fe^{2+}$ substitution in the clay layers gives rise to interlayer cations which neutralize the clay charge. These weakly bound cations of Ca^{2+}, Na^{+} and Mg^{2+} have Lewis acid properties (Szyjnkarczuk *et al.,* 1994). The cations absorb water, enlarging the pore size of

the clay resulting in a source of Brønsted acidity due to polarization of the hydration sphere. Bentonite clay when used with an enzyme to analyze a substance, pre-concentrates the analyte thus giving better results. In addition to the fact that *montmorillonite* clay is cheap, it has a well-defined layered structures, flexible adsorption properties, higher thermal and chemical stability and potential as catalysts and/or catalyst support (Orata and segor, 2000).

Furthermore, bentonite is a clay mineral and clay minerals are phyllosilicates (layer silicates) that dominantly make up the fine grained fraction of soil and sediments (Grim, 1968). The basic building block of phyllosilicates structure is Alumino silicate layer comprising of a silica tetrahedral sheet and alumina octahedral structure joined together in ratio 2: 1(Grim, 1968) and (Bard *et al.,* 1986). The clay has a particle size diameter smaller than 2 micrometer (Grim, 1968).

In addition Bentonite is a Montmorillonite clay with principal intercalated cations being alkaline earth ions, alkali metal ions and a fixed amount of other components like Al, Fe and SiO_2. These cations are exchangeable and because the layer lattice structure is expandable, the ions and large molecules can penetrate between the sheets resulting in increased basal spacing. The ability of large organic molecules to penetrate and orient themselves along the plane of the silicate sheet has been reported by Theng (1974) and Solomon *et al*., (1968).

2.12 Research Motivation

Several research projects have been conducted on the determination of BPA in plastics using techniques like gas chromatography (Olmo *et al.,* 1997), high performance liquid chromatography (Ngamna, 2005 and Omer *et al.,* 2016), nano graphene-based Tyrosinase biosensor (Bankim & Srivastava, 2010) and aptamer-based biosensor and methods like Nuclear magnetic resonance (NMR) Spectroscopy) (Sung, 2002). Although many research groups have worked on BPA, polymerization of aniline was not the objective and preparation of a biosensor using SLS surfactant, Tyrosinase enzyme and carbon modified electrode using Bentonite clay have remained unexplored. Thus, in this work biosensor from structured polyaniline was synthesized and used in the detection of Bisphenol A. The ultimate aim was to develop a BPA biosensor gadget to assess the BPA level in various plastic containers and to map the way forward into eliminating or minimizing BPA in the environment. The results of this study will help to come up with publications and presentations at workshops, and also a policy brief to the relevant government ministries will be made.

2.13 Theoretical Framework

In this project, the bio catalytic selectivity of Tyrosinase towards its analyte for analysis of Bisphenol A using bentonite modified electrode was harnessed. Most body reactions are catalyzed by enzymes without which they would be very slow and ineffective. It is possible to mimic the conditions required for enzyme functionality to our benefit in fabrication of enzyme catalyzed electro analytical devices. The key is to perform the enzymatic reactions on an electrode by tethering the enzyme to the electrode, simulating the best conditions for the reaction to occur and providing the substrate for the enzyme to work on. In the work, the possibility of preparing polyaniline using structure directing materials is explored.

Various authors have shown that the presence of surfactant molecules during polymerization can lead to production of polymer structures at the nano-scale level. Clarks and Lyon in 1968 showed that an electrode surface can be modified with any suitable material including conducting polymers with the latter maintaining its activity. It has also been shown that nano-scaled polymeric materials display better catalytic activity (Grennan, 2006). The reduction in particle size of the polymeric materials to nanometer scale imparts unique properties to make them suitable for chemical and physical sensing and can enhance sensor properties (OhS and Ims 2002).

It has also been reported that the use of conducting polymer materials in the encapsulation of enzymes in the biosensor fabrication has several other advantages (Viswanath, 2007). They help in overcoming over potential problems related to the analyte detection, provide a suitable micro-environment for the biocatalyst immobilization, localize the biocatalyst close to the electrochemical interface thus wading off interferences, and are characterized by a distributed array of catalytic sites which enhance electron exchange between the enzyme and the electrode (Chen, 2006). Two methods for the preparation of the nano-scaled polymers have been identified. These are the template and the non-template methods. These polymers are prepared from their requisite monomers through either the chemical or electrochemical oxidative polymerization processes (Malinauskas, 2005). Once the polymers are prepared their electrochemical activity can be interrogated using various electrochemical techniques.

Electro active polymers will display well-formed reductive and oxidative waves. Their electronic transitions are shown by use of spectroscopic techniques namely UV-VIS and Raman spectroscopy which are characterized by formation of new bands associated with the formation of the polaron or bipolaron on bands (Fernandes, 2005). Once the biosensor is formed using suitable techniques e.g. electrostatic, dip-dry, and drop-dry or spin coating techniques, biosensor responses are followed amperometrically where a current response corresponding to the concentration of the analyte is monitored (Iwuoha, 2003).

CHAPTER 3: METHODOLOGY

3.1 Introduction

Preparation of polyaniline (PANI) polymer using 0.1 M sulphuric acid as supporting electrolyte and also as doping agent was done and the resulting polymer was characterized both spectroscopically and electrochemically using cyclic, square wave and differential voltammetric modes UV-Vis and Raman spectroscopy methods.

The polymeric biosensor was then prepared and characterized by encapsulating the Tyrosinase enzyme onto the polymeric materials and optimizing conditions for a best biosensor response towards Bisphenol A. The Tyrosinase enzyme used throughout the experiment was freshly extracted from white button mushrooms (*Agaricus bisporus).* Determination of BPA in drinking water bottles and feeding baby bottles was done using the constructed biosensor.

3.2 Reagents and Materials

The reagents used for this study were as follows: Disodium hydrogen phosphate ($Na_2HPO_4.7H_2O$) from BHD, Potassium dihydrogen phosphate ($KH_2PO_4.7H_2O$) from BHD, Aniline from (Aldrich 99%), bentonite clay (Athi River Mining Company), Sodium lauryl sulphate (SLS) (Kobian) ,Ammonium Per sulphate (APS) (Kobian), Sulphuric (VI) acid (Kobian), Tris – HCl buffer (Kobian), sodium chloride (Kobian), resin DEAE Sepharose (DE-52)(Griffin chemicals), Bisphenol A (BPA) (sigma Aldrich chemicals), Tyrosinase enzyme (from white button mushrooms), Dimethyl sulphoxide (DMSO) (BHD) and distilled water from University of Nairobi.

All reagents were of analytical grade and were used as obtained except aniline which was triply distilled and purged with nitrogen before use and the Tyrosinase enzyme which was extracted from white button mushroom and then purified. Deionized water was used as a reagent for aqueous solution preparations.

3.3 Preparation of working solutions

3.3.1 Preparation of aniline

Aniline was triply distilled to achieve purity and stored under nitrogen. To make 0.1M aniline, 0.93ml of distilled aniline was placed into a 100ml volumetric flask and 0.1M sulphuric acid added to the mark.

3.3.2 Preparation of phosphate buffer solution

Phosphate buffer solution of 0.1 M was prepared by dissolving 7.098 g of disodium hydrogen phosphate ($Na_{2S}HPO_4.7H_2O$) and 6.8046 g of potassium dihydrogen phosphate ($KH_2PO_4.7H_2O$) separately in 500 mL deionized water, then mixing the salt solutions according to Sørensen equation (Kuhlmann, 2006) to obtain the required pH range (4.2 to 10.2). The pH meter was calibrated with standard solutions. The pH was controlled using sulphuric (IV) acid and Sodium hydroxide. The phosphate buffer solution was stored in refrigerator at 4ºC for not more than 2 weeks to maintain the pH.

3.3.3 Extraction and purification of Tyrosinase enzyme

Extraction of mushroom Tyrosinase was done by the method of Kamahldin *et al*., (2004) with few modifications. The sliced mushrooms were homogenized using a blender. Enzyme extraction was prepared with 30 mL of cold 50 mM Tris- HCl buffer (pH 5.8) for 25g of frozen mushroom. The homogenate was stirred for 30 minutes in cold conditions then centrifuged at 13500 rpm for 30 minutes at a temperature of 4^0C and the supernatant collected. The supernatant was used as a source of enzyme.

The purification of Tyrosinase was performed by the method of Kamahldin *et al*., (2004) with minor modifications. Crude enzyme extract was purified by salt precipitation (using Ammonium Sulphate), dialysis and ion exchange chromatography. These procedures were employed in series so as to obtain the enzyme in its purest form.

Ammonium sulphate precipitation was done in an ice bath using finely ground ammonium sulphate. The powder was weighed and added slowly to the extract by constant stirring to ensure complete solubility and to make 35% saturated solution. The solution was centrifuged at 13500 rpm for 30 minutes at a temperature of 4°C. Ammonium sulphate precipitation procedure was repeated to make 35% – 70% saturated solution and the precipitates were collected and dissolved in 15 mL of 50 mM Tris- HCl .The precipitate was dialyzed against 50 mM Tris- HCl buffer (pH 5.8) for 24 hours by replacing the 50 mM Tris- HCl buffer thrice at regular intervals. The dialyzed fraction was then loaded into a DE-52 column and eluted with Tris- HCl pH 5.8. The proteins were then, eluted from the column using 10 ml of 150mM NaCl solution. The collected protein fractions at 150mM salt eluent were pooled and concentrated by lyophylization (the sample was transferred into reagent bottles and freeze dried at -80^0C). The lyophilized sample was then reconstituted with 10ml of 0.1M phosphate buffer pH 7.2. The obtained enzyme sample was used for construction of biosensor without further purification. The enzyme was immobilized into surface of bare glassy carbon and

Poly aniline- bentonite (PANI-BTN) with inert adhesive glue by drop- coating method. The enzyme was kept refrigerated at a temperature of 4^0C for two weeks.

3.3.4 Preparation of Bentonite clay

Thick slurry was prepared by putting 0.05ml of electrochemically inert adhesive onto a watch glass and adding 0. 01g of bentonite clay. Using a stirring rod, the two were mixed to form thick slurry which was applied to bare glass carbon electrode by drop coating method. The mixture was spread on the surface of polished carbon electrode up to a thickness of about 0.6mm and left to air-dry for 12 hours while covered with cotton cloth to avoid contamination. The surface area of the bentonite modified electrode was approximately 0.60 mm^2

3.3.5 Preparation of 0.01M Bisphenol A stock solution

Stock solution of 0.01 M Bisphenol A was prepared by weighing out 0.05705 g Bisphenol A and dissolving in 25 mL ethanol-water mixture in the ratio 2:3 and sonicating for 3 minutes. A blank solution was also prepared by mixing ethanol and water in the ratio 2:3 and sonicating it for 3 minutes.

3.3.6 Preparation of surfactant

Sodium lauryl sulphate (SLS) was prepared by weighing out 0.1225 g of SLS and dissolving it in 50 mL of distilled water. 20 ml of 0.005M Sodium lauryl sulphate was added to 80 ml of 0.1 M aniline in 0.1Msulphuric acid.

3.4 Measurement and Instrumentation

Electrochemical measurements were performed using an Auto Lab. PGSTAT 12 potentiostat with a three-electrode system consisting of platinum wire as the counter electrode, Ag/AgCl (saturated 4.0 M KCl) as the reference electrode and glassy carbon electrode (bare glassy carbon electrode, Tyrosinase/ glassy carbon electrode, Try/ Polyaniline-bentonite/GCE and Try/ Polyaniline-BTN/ SLS/GCE as the working electrode.

The bare glassy carbon electrode was polished with sand paper and on glass for final polishing then the electrode was rinsed with distilled water. The auxiliary electrode was cleaned by rinsing with copious amount of distilled water for several minutes and the same was done to Ag/AgCl electrode. All potentials were quoted with respect to the Ag/AgCl electrode.

The experiments were carried out at controlled room temperature of 25°C. All electrochemical measurements were carried out in phosphate buffer solution pH (4.2 to 10.2). UV-Vis spectroscopy was performed using Shimadzu DUV-3700 PC scanning spectrometer while Raman spectroscopy was done using Perkin-Elmer Lambda-20 (Olympus BX 51) spectrometer with a 785 nm Laser source. A motorized microscope stage sample holder and a charge-coupled device (CCD) detector were used. Instrument parameters were as follows: X50 objectives, 30 μm spot size, and 60 second acquisition time and baseline correction were performed for all measurements.

3.5 Biosensor Construction

Glass carbon electrode was first drop coated with *Montmorillonite* clay (bentonite clay) and allowed to dry at room temperature over night in a protected environment to prevent contamination from dust. Aniline mixed with SLS was then polymerized by electrodeposition method on the bentonite modified electrode. After electro polymerization, the electrode was washed with deionized water on the sides to remove the excess monomer from the electrode surface. 5 μL of Tyrosinase enzyme was mixed with 0.5mL of 0.1 M phosphate buffer solution in a vial then 5 μL of this mixture was mixed with an electrochemically inert adhesive and drop-coated on the surface of GCE modified with PANI/ SLS- BTN. The electrode was allowed to dry at room temperature overnight in a dust-free environment. Tyrosinase/ polyaniline /sls – bentonite (PANI/ SLS - BTN)/ GCE (biosensor) was then characterized in 0.1 M phosphate buffer, pH 7.2 by cyclic voltammetry, Square wave voltammetry and differential pulse voltammetry in the potential range between -1.2V to +1.2 V. The biosensor was stored in 0.1 M phosphate buffer at 4°C when not in use.

3.6 Electrochemical techniques used.

3.6.1 Cyclic voltammetry (CV)

This is an electrolytic method widely used in many areas of chemistry that utilizes microelectrodes and an unstirred solution so that measured current is limited by analyte diffusion at the electrode surface. It has wide applications in the study of redox processes, electrochemical properties of analytes in solution, and for understanding reaction intermediates. This technique utilizes three or two electrode electrochemical cell systems in the measurement of current resulting from application of potential. The potential is usually measured between the working electrode and the reference electrode and the current is measured between the working electrode and the auxiliary electrode.

The technique works by varying some applied potential at a working electrode at some scan rate in both forward and reverse directions while monitoring the current. For example, the first scan can be negative to the switching potential, in which case if the scan is reversed; it runs in the positive direction. A partial cycle, full cycle, or a series of cycles can be performed depending on the analysis targeted. Important parameters are usually obtained from cyclic voltamogramms for analysis of redox properties and properties of an electro active sample. The parameters include cathodic and anodic peak potentials (Epc and Epa respectively) as well as cathodic and anodic peak currents (i_{pc} and i_{pa} respectively). Information about the sample under investigation can be obtained from the peak parameters. This includes whether the electrochemical process showed by the sample is reversible, irreversible or quasi-reversible ***(***Andrienko, 2008***)***. A typical cyclic voltamogramm illustrating the peak parameters, that is, E_{pa} ,E_{pc}, i_{pa} and I_{pc} as stated above is shown in figure 3.1 below.

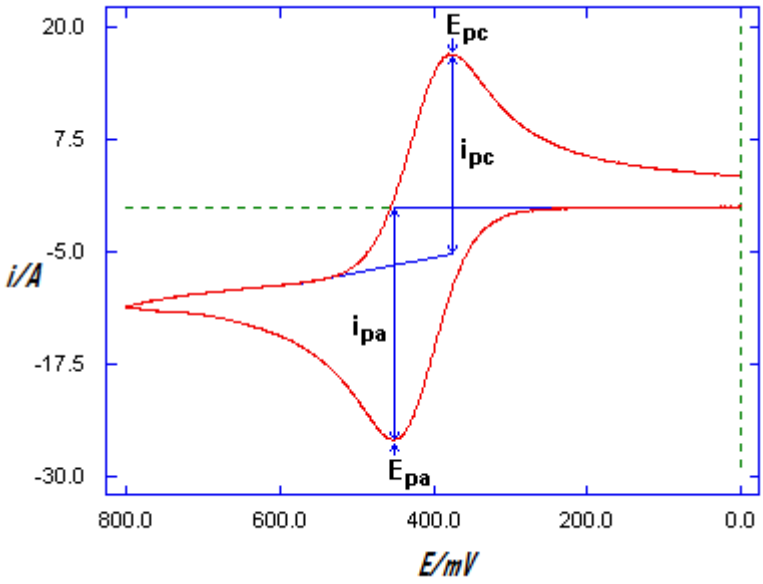


Figure 3.1 Cyclic voltammogram showing the basic peak parameters.

CV also gives an insight into how fast the electron transfer process is, relative to other processes such as diffusion. For instance, if the electron transfer is fast relative to the diffusion of electro active species from the bulk solution of the surface of the electrode, the reaction is said to be electrochemically reversible..

3.6.2 Deferential pulse voltammetry

Deferential pulse voltammetry is comparable to normal pulse voltammetry (NPV) in the sense that the potential is also scanned with a series of pulses. However, it differs from NPV because each potential pulse is fixed, of small amplitude (10 to 100 mV), and is superimposed on a slowly changing base potential (Shahrokhian, 2007). In this study, the three electrode system is used in which current is measured at two points for each pulse, the first point, just before the application of the pulse and the second at the end of the pulse. These sampling points are selected to allow for the decay of the charging current. The difference between current measurements at these points for each pulse is determined and plotted against the base potential. Pre-concentration time used was 5s, scan rate was 20 mV s^{-1} and pulse amplitude of 50 mV) was used.

3.6.3 Square wave voltammetry

Square wave voltammetry (SWV) is a form of linear potential sweep voltammetry which has found numerous applications in various fields such as medicine. When first reported by Barker in 1957, the working electrode utilized was primarily a dropping mercury electrode (DME). Currently, Square wave voltammetry is used as technique in analytical applications and fundamental studies of electrode mechanisms. SWV technique is one of the most sensitive ways for the direct evaluation of concentrations. It is widely used for several analyses, especially on pharmaceutical compounds.

3.7 Non-electrochemical techniques.

3.7.1 Ultra violet-visible (UV-Vis) spectroscopy

UV-Vis spectroscopy probes the electronic transitions of molecules that absorb light in the ultraviolet and visible region of the electromagnetic spectrum and is considered to be a reliable and accurate analytical technique for the qualitative as well as the quantitative analysis of samples. When sample molecules are exposed to light having an energy that matches a possible electronic transition within the molecule, some of the light energy will be absorbed as the electron is promoted to a higher energy orbital. An optical spectrometer records the wavelengths at which absorption occurs, together with the degree of absorption at each wavelength. The resulting spectrum is presented as a graph of absorbance versus wavelength (Harvey *et.al* 2001) .Various kinds of electronic excitation may occur in organic molecules by absorbing the energies available in the 200 to 900 nm spectrum. As a rule,

energetically favored electron promotion will be from the highest occupied molecular orbital to the lowest unoccupied molecular orbital, and the resulting species is called an excited state. In this study, the UV-Vis spectrum was recorded with a PC-driven Shimadzu DUV-3700 PC scanning spectrometer (resolution 0.1 nm). Spectro electrochemical experiments were made in a quartz cuvette of 1 cm path length. After electro deposition of doped or undoped PANI on glassy carbon electrode (25 cycles four times) the polymer was dissolved in 4 mL dimethylsulphoxide (DMSO) solution. The DMSO solution turned into dark-green color in the case of PANI while it turned blue when PANI was dedoped using 1M ammonia solution. Any colour change observed was representing the switching between reduced and oxidized states. The spectra observed were recorded in the region between 300-900 nm.

3.7.2 Raman Spectroscopy

Raman spectroscopy is a scattering technique and is based on the Raman Effect discovered by C V. Raman in 1928, which is the inelastic scattering of photons by molecules (Brame *et al.,* 1976). Raman scattering is the process of radiation of scattered light by dipoles induced in the molecule by the incident light and modulated by the vibrations of the molecules. When a sample is irradiated by monochromatic light from a laser source, the Rayleigh scattering has the highest probability. In this scattering process neither loss nor gain of energy matters, since the scattered light has the same frequency as the radiation source. However, a small fraction of the scattered light also exhibits shifts in frequency corresponding to the sample's vibrational transitions. Lines shifted to frequencies lower than the source frequency are produced by ground-state molecules and are called Stokes lines (Gustavo *et al*., 2007).

On the other hand the slightly weaker lines at higher frequency are due to molecules in excited vibration state, which are called anti-Stokes lines. Since most molecules are in their vibrational ground state at ambient temperature the intensity of Stokes lines are higher than the anti-Stoke lines (Gustavo *et al*., 2007). This is the reason why only the Stokes lines are recorded as the Raman spectrum. The main limitation of the Raman spectroscopy is the fluorescence. Fluorescence occurs if molecules have electronic energy levels that can be excited by the monochromatic laser source Ferraro *et al.,* 1994). The only way to prevent superimposing by fluorescence is by shifting the Raman excitation wavelength into the near-IR, which has insufficient energy to excite electronic states. Certain Raman lines increase in intensity and are strongly enhanced if the exciting laser frequency coincides with that of an electronic absorption of the scattering molecule (Ferraro *et al.,* 1994). In this study, Raman

spectra were measured on Perkin-Elmer Lambda-20 spectrometer equipped with liquid nitrogen cooled CCD camera detector at a resolution of 2 cm^{-1}. Samples were illuminated with 514.5 and 647.1 nm laser light from an Ar^+ and Kr^+-ion lasers Coherent Innova 70, respectively. The Raman spectra obtained were slightly smoothed and baseline-corrected.

3.8 Detection and Investigation of Bisphenol A

The electrochemical behaviour of BPA was investigated on bare GCE, Try/PANI/GCE, PANI- BTN/GCE and Try/ PANI/SLS-BTN /GCE in 0.1 M phosphate buffer at pH ranging 4.2 to 10.2 using CV, SWV and DPV within a potential range of -1.2V to +1.2 V in the presence of oxygen. Aliquots of the 0.01 M stock solution of BPA were injected into phosphate buffer in increasing concentrations and their voltamogramms were recorded. The CV, SWV and DPV experiments were performed in unstirred solutions.

3.9 Detection and determination of BPA concentration in drinking water bottles and baby bottles using fabricated biosensor.

Detection and determination of BPA concentration in two types of plastic feeding baby bottles and in three types of drinking water bottles was investigated using the fabricated biosensor (Try/PANI/ SLS -BTN/GCE). The determination of the BPA was achieved by directly using cyclic voltammetry, square wave voltammetry and differential pulse voltammetry. This was done by putting a fixed volume of water in the plastic bottles at known temperature and maintaining the temperature between 50^0C- 95^0C for five minutes then investigating the presence and concentration of BPA in the water in 0.1 M phosphate buffer at pH of 7.2. The temperature was varied for water in each bottle. The concentration of BPA in drinking water bottles and feeding baby bottles was determined by comparing the calibration curves with the previously obtained calibration curves when 0.1 M BPA in 0.1 M phosphate buffer at pH of 7.2.

CHAPTER 4: RESULTS AND DISCUSSIONS

4.1 Introduction

Electrochemical polymerization of monomer is achieved by employing cyclic voltammetry technique, the potential window used is dependent on the type of monomer used in a particular study whether it's substituted or not and also on the type of electrode used as well as the solvent used. The scan rate, at which the polymerization process is performed, is very significant because it determines the compatibility and stability of the film on the surface of the electrode. To confirm polymerization, the same peak characteristics observed during polymerization should be present during characterization in absence of the monomer.
In this study, this is performed at different scan rates where the peak current is expected to increase with the increase in scan rate.

This chapter discusses a number of techniques which include: cyclic voltammetry, differential pulse voltammetry, square wave voltammetry, ultra-visible spectrometry and Raman spectroscopy that were used to characterize the polymer film deposited onto the surface of the electrode. The techniques above were employed to confirm successful polymerization and that the polymer film was successfully deposited onto the surface of glassy carbon electrode. The methods also proved the success of dopant employed by observing the redox peaks which were both in the absence and presence of the dopant.

In this study, cyclic voltammetry was used to give evidence that BPA has the potential to polymerize onto the surface of glassy carbon electrode leading to shielding of the electrode surface and thus preventing electron transfer from the electrode surface to the electrolyte which would cause electrode fouling. This fouling problem is controlled by modifying the electrode surface before detection takes place. Clearly labeled graphs are shown to represent the results obtained from polymerization and the characterization study of polyaniline.
The solubility of the electropolymerized polyaniline was determined in various solvents. Sulphuric (VI) acid doped PANI was found to be soluble in dimethylsulphoxide (DMSO) and insoluble in other solvents like acetone, chloroform, methanol, m-cresol benzene, and water.

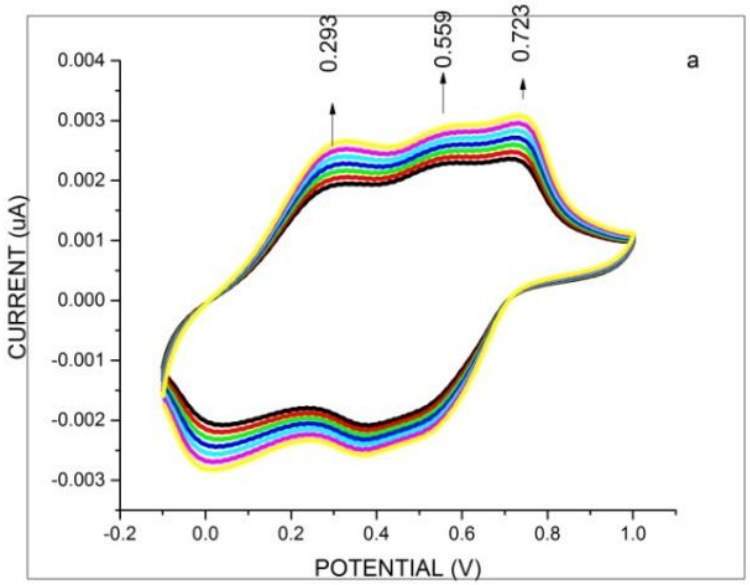
4.1.1 Characterization of bentonite clay and electrochemical synthesis of PANI using bare GCE, bentonite modified GCE and surfactant SLS.

The bentonite clay that was used in this study was characterized by AAS – Varian spectra 10 using the pellet-making technique and it had particle size of 150 micrometers. The bentonite was found in a solid matrix made up of different oxides with different composition (Mbui *et al.*, 2014) as show in the table 4.1.

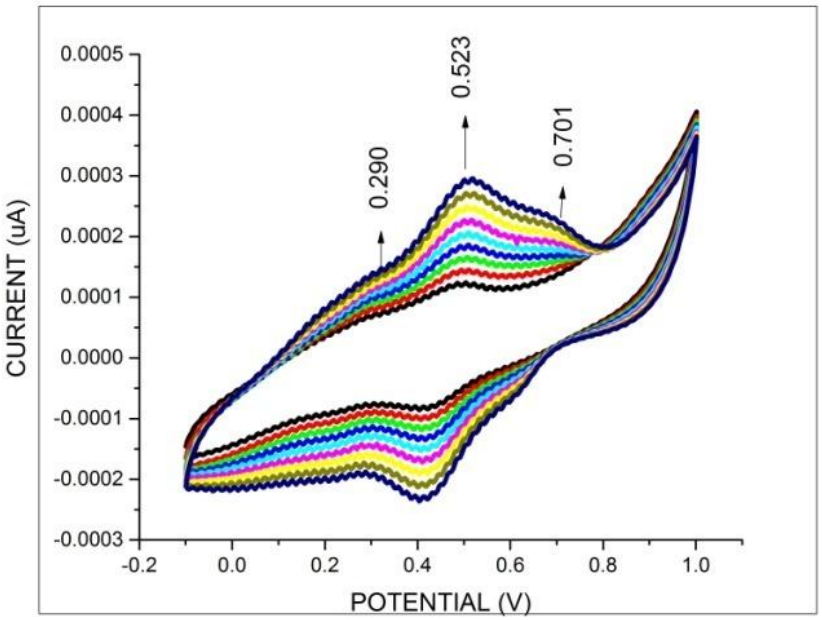
Table 4.1: Bentonite composition

Solid Matrix	SiO_2	Al_2O_3	CaO	MgO	Na_2O	K_2O	TiO_2	MnO	Fe_2O_3	LOI
Bentonite clay	47.21	14.4	3.27	0.29	1.71	1.00	0.62	0.03	10.93	2.40

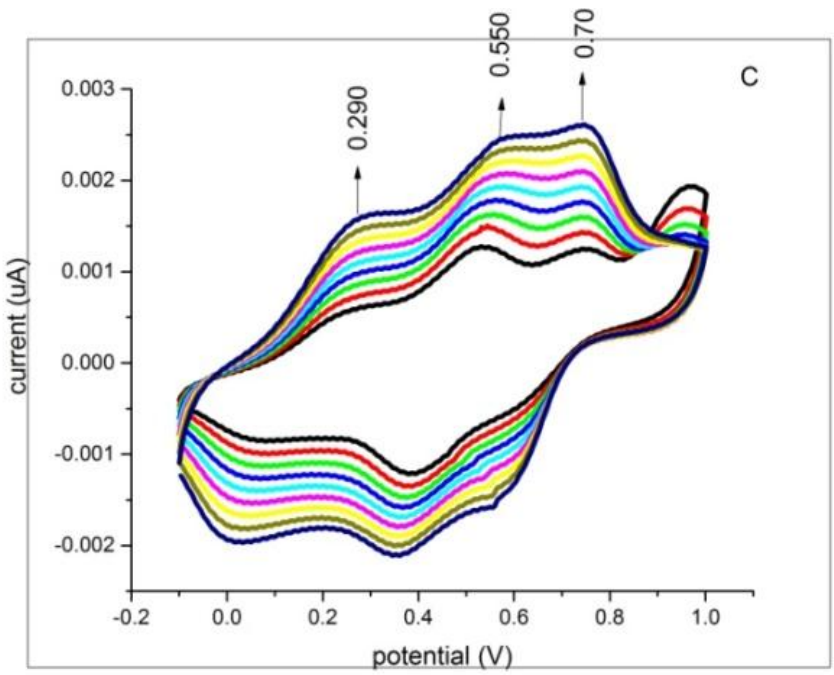
After characterization of bentonite clay, electrochemical polymerization of the monomer aniline on bare GCE surface, bentonite modified GCE , aniline with sodium lauryl sulphate (SLS) on bare GCE surface and aniline with SLS using bentonite modified GCE was achieved by cycling the potential repeatedly between -0.1V and +0.1 V at a scan rate of 40 mVs^{-1}. The color of the polymer film formed on all the surfaces was dark green. The cyclic voltammograms for the electro deposition of PANI films are as shown in Figure 4.1 (a), (b), (c) and (d) .



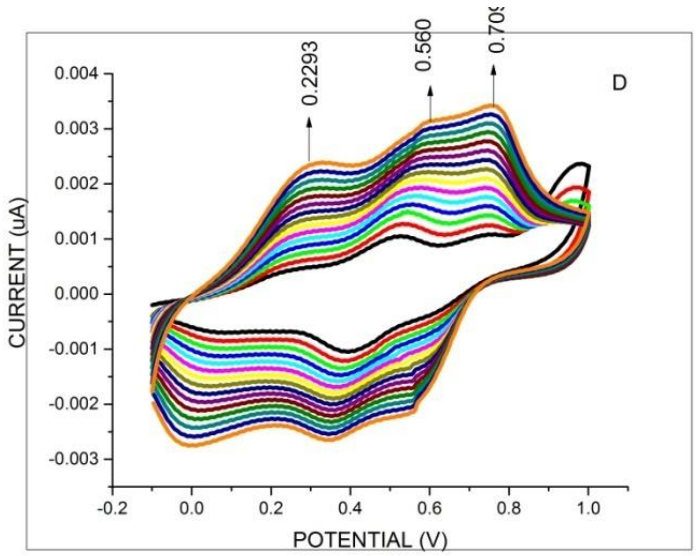
(a)


0.0005
0.0004
0.0003
0.0002
0.0001
0.0000
-0.0001
-0.0002
-0.0003
CURRENT (uA)
0.290
0.523
0.701
-0.2
0.0
0.2
0.4
0.6
0.8
1.0
POTENTIAL (V)

(b)


0.003
0.002
0.001
0.000
-0.001
-0.002
current (uA)
0.290
0.550
0.70
C
-0.2
0.0
0.2
0.4
0.6
0.8
1.0
potential (V)

(c)



(d)

Figure 4.1: Electrochemical synthesis of PANI using 0.1M aniline in 0.1 M H_2SO_4 at scan rate of 40 mVs^{-1} using : (a) bare GCE, (b) bentonite modified electrode,(c) 0.005mM surfactant and bare GCE (d) 0.005mM surfactant using bentonite modified electrode.

Bentonite clay was used in this experiment to study the effect of the host matrix on the redox behavior of PANI. The electrolyte media contained 0.1M aniline in 0.1M sulphuric acid solution (figure 4.1 (a) and (b)) while for figure 4.1 (c) and (d) the electrolyte media contained 0.1M aniline in 0.1M sulphuric acid solution and 0.005M SLS . The sulphuric acid was used as supporting electrolyte and also as doping agent for the resulting polymer.

Two pairs of redox peaks centered at approximately +0.2 V and + 0.5V, corresponding to the transition from leucoemeraldine to emeraldine and emeraldine to pernigraniline states of aniline respectively were observed. The appearance of another pair of redox peaks observed at approximately +0.7 V for all the voltammograms is much more complex and can be attributed to many different intermediates and degradation products (cross-linked polymer, benzoquinone) formed during the electrochemical preparation of the polymers (Songa *et al.*, 2009).

In the cases where bentonite was used, figure 4.1(b) and (d), the cyclic voltammograms had better defined peaks than when bare GCE was used. The reduction and oxidation peaks occurred at +0.22/ +0.01V, +0.56V/ +0.41V and +0.7V/ +0.6V respectively for 0.005mM surfactant using bentonite modified electrode, figure 4.1(d) and for bentonite modified electrode, figure 4.1 (b) the reduction and oxidation peaks occurred at +0.29V/ +0.24V, +0.52V/ +0.46V and +0.70V/ +0.61V respectively.

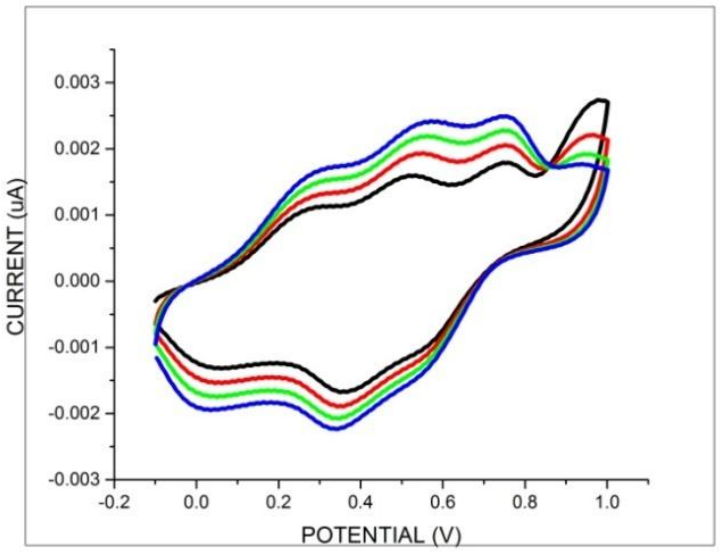
The improved redox response of aniline when bentonite modified electrode was used can be attributed to the pre-concentration of the aniline molecules as a result of being trapped in the octahedral layers in the bentonite (Mbui *et al.,* 2014).

Polyaniline has various oxidation states that are both pH and potential dependent. It is generally agreed (Abdiryim, 2005) that polyaniline has three different fundamental forms: leucoemeraldine (LE: fully reduced), emeraldine base (EB: half oxidized), and pernigraniline (PN: fully oxidized). The only electrically conducting one is, however, the emeraldine salt form (ES: half oxidized), which is protonated form of EB. Refer to figure 2.7 discussed earlier. In this study the emeraldine salt form was formed.

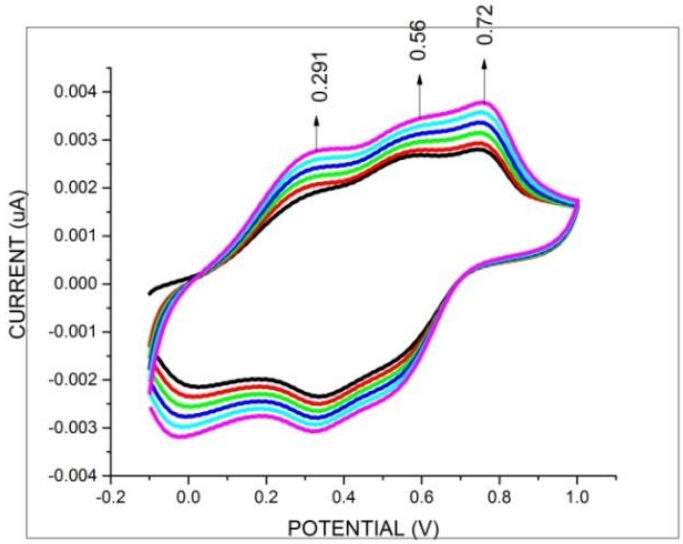
4.1.2 Electro polymerization Mechanism of Aniline

Electrodeposition of polyaniline films onto the GCE surface proceeds via radical cation mechanism. In this procedure, the monomers at the working electrode surface undergo oxidation to form radical cations that react with other monomers or radical cations, forming insoluble polymer chains on electrode surface (as was shown in scheme 2.5 in this document) The reaction produces benzidine and 4- amino diphenylamine in different proportions depending on the pH of the medium as the major intermediate species during the aniline polymerization (Johnson *et al.,* 1996 and Hong *et al., 2005*). Wei *et al.,* (1989) reported a significant increase in the rate of polymerization of aniline when a small amount of the dimeric species was added as the initiators.

For electro polymerization, the potential is cycled repeatedly to allow uniform distribution of the film on the surface of electrode. The thinner the potential film on GCE surface, the better the electron transfer (Matyholo, 2011). This was evidenced by characterization of polyaniline using different film thickness of polyaniline in bare electrode as shown in fig. 4.2 (a-d)


CURRENT (uA)
POTENTIAL (V)
0.003
0.002
0.001
0.000
-0.001
-0.002
-0.003
-0.2
0.0
0.2
0.4
0.6
0.8
1.0

(a)


0.291
0.56
0.72
CURRENT (uA)
POTENTIAL (V)
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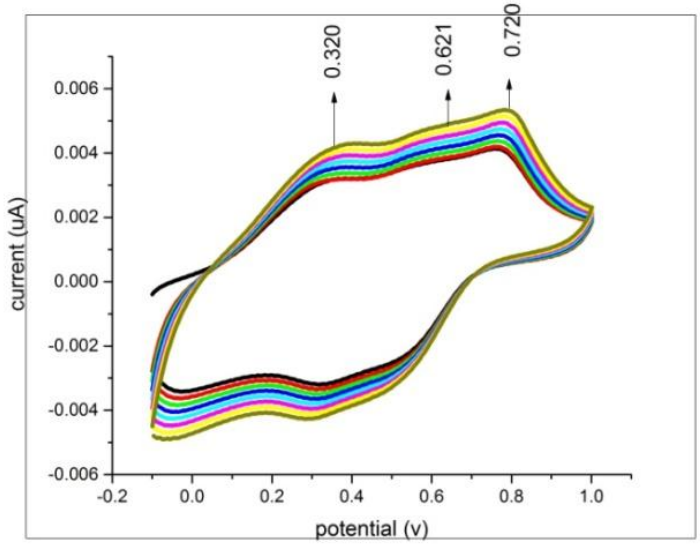
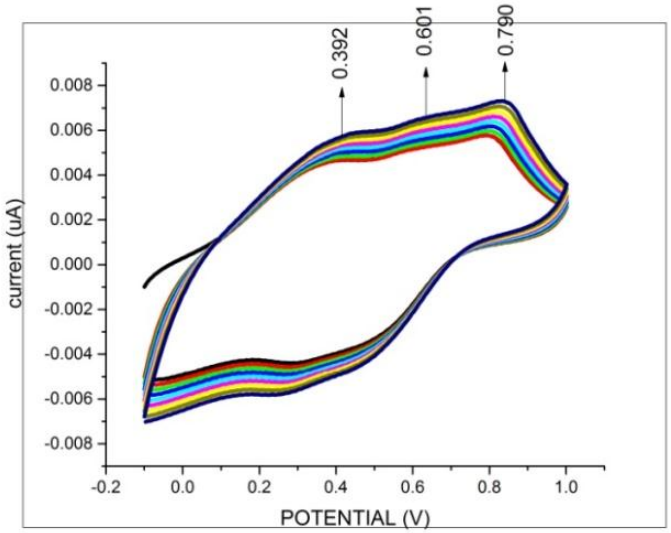
0.003
0.002
0.001
0.000
-0.001
-0.002
-0.003
-0.004
-0.2
0.0
0.2
0.4
0.6
0.8
1.0

(b)


0.320
0.621
0.720
current (uA)
potential (v)
0.006
0.004
0.002
0.000
-0.002
-0.004
-0.006
-0.2
0.0
0.2
0.4
0.6
0.8
1.0

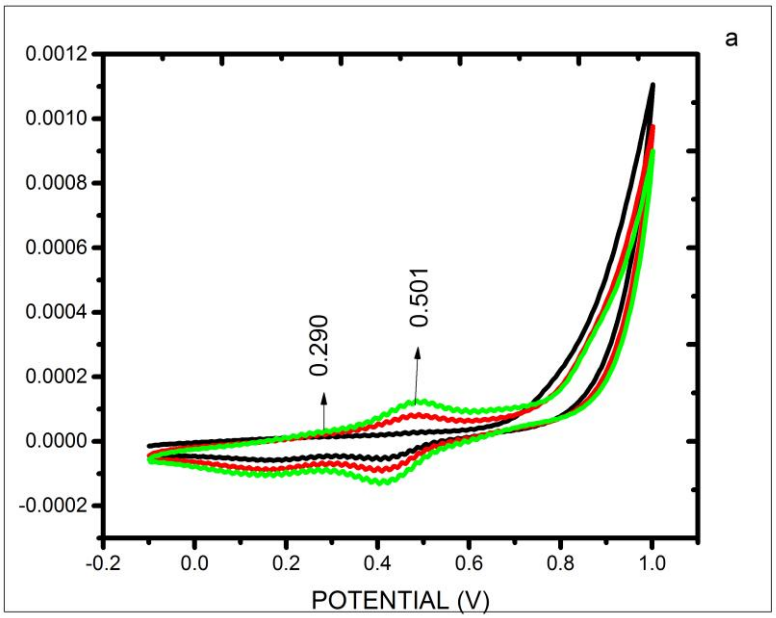
(c)



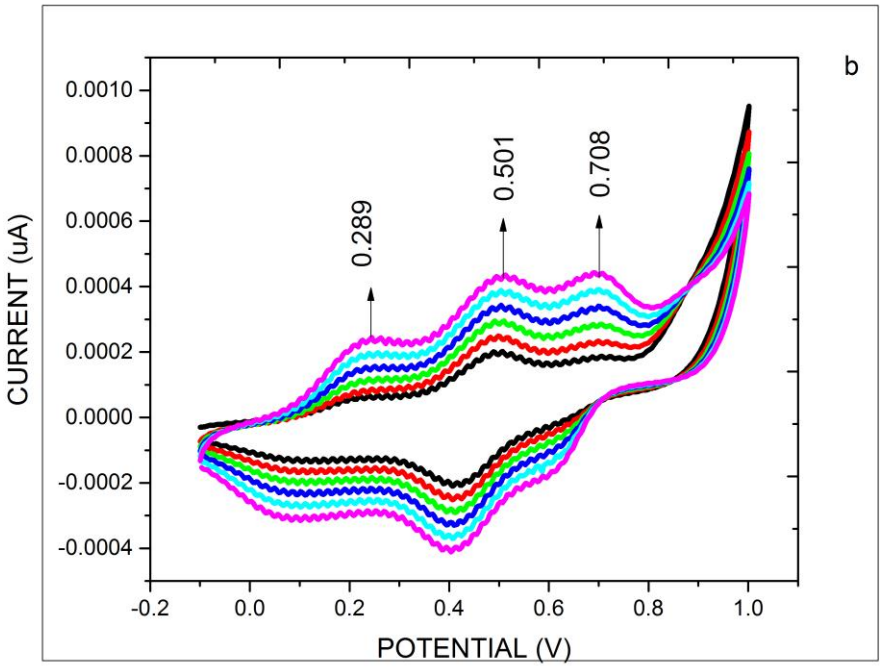
(d)

Fig. 4.2: Characterization of polyaniline in 0.1 M H_2SO_4 at scan rate 40 mV s- at different cycles(a) 4 cycles, (b) 6 cycles, (c) 8 cycles and (d) 10 cycles using bare GCE.

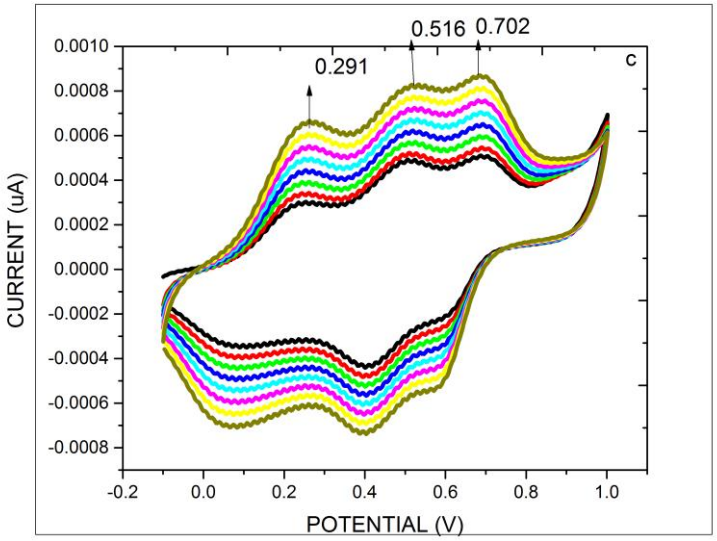
From the voltamogramms obtained, the oxidation peaks are more enhanced when an electrode with thin film of polyaniline is used than when the film is thicker for both bare GCE and GCE modified with bentonite clay, figure 4.3 (a-d). These results were similar to those obtained by Matyholo (2011) and Songa *et al*., (2009). From the results obtained it is also observed that GCE modified with bentonite clay has more enhanced peaks than bare GCE. It is probable that bentonite clay pre-concentrates PANI and all the other reagents onto the working electrode hence giving more elaborate signals. Similar observations have been obtained by Mbui *et al.,* (2014), who observed that electroactive species in water were concentrated by bentonite giving more elaborate peaks.


a
CURRENT (uA)
POTENTIAL (V)
0.290
0.501
0.0012
0.0010
0.0008
0.0006
0.0004
0.0002
0.0000
-0.0002
-0.2
0.0
0.2
0.4
0.6
0.8
1.0

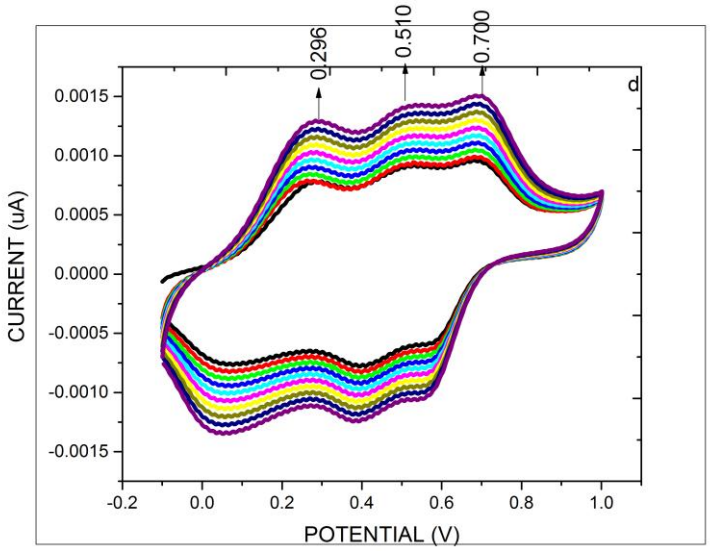
(a)


b
CURRENT (uA)
POTENTIAL (V)
0.289
0.501
0.708
0.0010
0.0008
0.0006
0.0004
0.0002
0.0000
-0.0002
-0.0004
-0.2
0.0
0.2
0.4
0.6
0.8
1.0

(b)



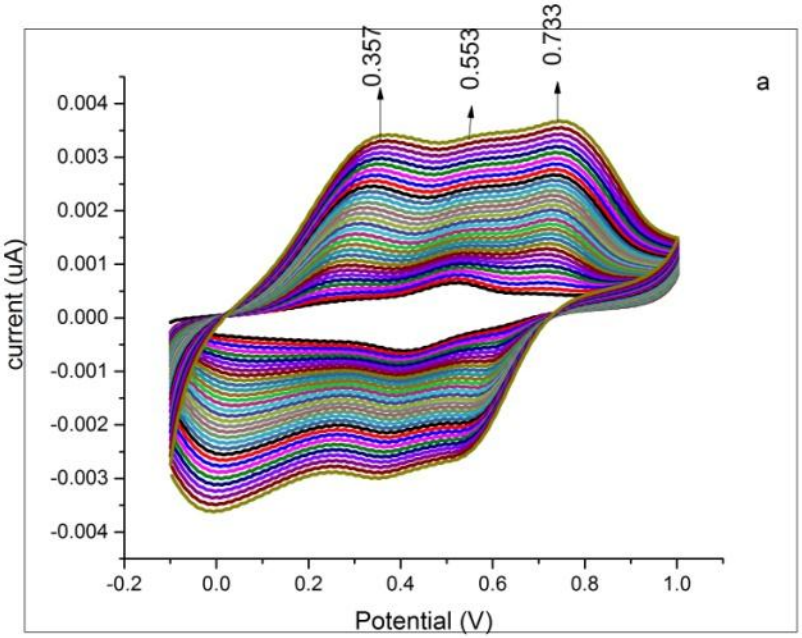
(c)



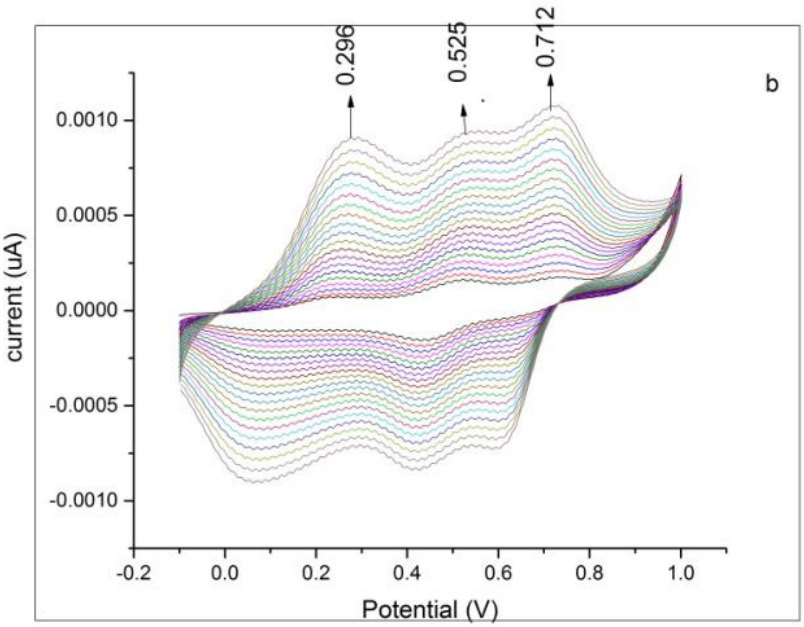
(d)

Fig. 4.3: Characterization of PANI in 0.1 M H_2SO_4 at scan rate 40 mV s- at different cycles(a) 4 cycles, (b) 6 cycles, (c) 8 cycles and (d) 10 cycles using GCE modified with bentonite clay.

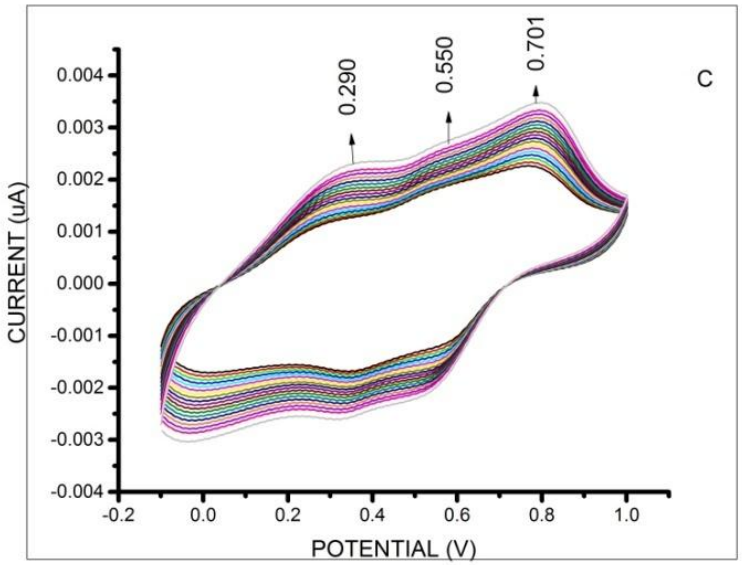
Multi scan voltammetry resulted in an increase in the redox peaks which indicated the formation of conducting polymer on GCE surface (fig 4.4). Multi scan voltammetry also provides further evidence that the GCE surface is conductive


0.357
0.553
0.733
a
0.004
0.003
0.002
0.001
0.000
-0.001
-0.002
-0.003
-0.004
current (uA)
-0.2
0.0
0.2
0.4
0.6
0.8
1.0
Potential (V)

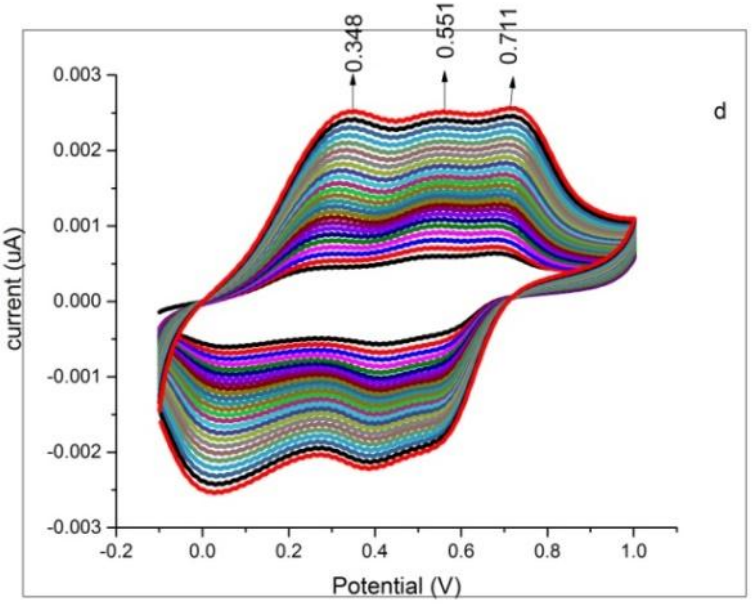
(a)


0.296
0.525
0.712
b
0.0010
0.0005
0.0000
-0.0005
-0.0010
current (uA)
-0.2
0.0
0.2
0.4
0.6
0.8
1.0
Potential (V)

(b)



(c)



(d)

Figure 4.4: Multi scan voltammetry of PANI using 0.1 M aniline and 0.1 M H2SO4 at scan rate 40 mV s-1 (a) using bare carbon electrode, (b) using bentonite modified electrode,(c) using 0.005mM surfactant on bare GCE (d) 0.005mM surfactant using bentonite modified electrode

In all the cases the cyclic voltammograms both with and without surfactant had peaks with both the oxidation and reduction peaks. The reduction and oxidation peaks for PANI on bare GCE occurred at approximately +0.36 V/ +0.01V, +0.55 V/ +0.39V and +0.73 V/+0.60V

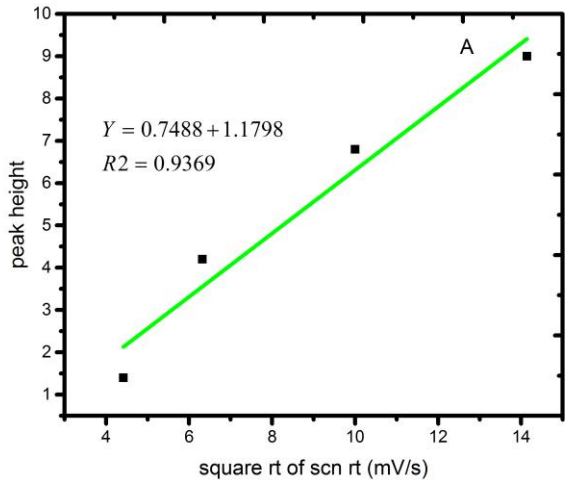
respectively figure 4.4 (a). The reduction and oxidation peaks for PANI using bentonite modified electrode were, + 0.29V/+0.14V, +0.53V/ +0.40V and + 0.73V/ +0.61V respectively while for PANI using bentonite modified electrode and surfactant, reduction and oxidation peaks occurred at +0.348V /+0.10V , +0.551V/ +0.42V and +0.710V/ +0.60V.

From the results obtained, the reduction peaks in cyclic voltammograms obtained when bentonite modified electrode was used are sharp and more enhanced figure 4.4 (b and d) because aniline is concentrated on the surface of electrode by adsorption in the clay film due to soaking of the clay modified electrode in the aniline solution (Fitch *et al.*, 1990). From the results obtained, the bentonite host matrix which is characterized by the presence of octahedral and tetrahedral plates (Bard *et al*., 1990) probably suppressed or inhibited the follow-up chemical reaction observed in the bare carbon case (Huang, 2010). This improved redox response of aniline in the bentonite modified electrode. The enhanced response with bentonite-modified electrodes can also be attributed to the pre-concentration of the aniline molecules as a result of being trapped in the octahedral layers in the bentonite. The alignments of aniline in the clay montmorrilonite matrix not only enhanced the electrochemical signal, but also lead to reduction in Gibbs free energy resulting from entropic effects as a result of the realignment of the redox functional groups in the bentonite host matrix. (Mbui *et al.*, 2014) and (Orata *et al.,* 2014). This is not surprising given the selective and complexation properties of clay Montmorillonite.

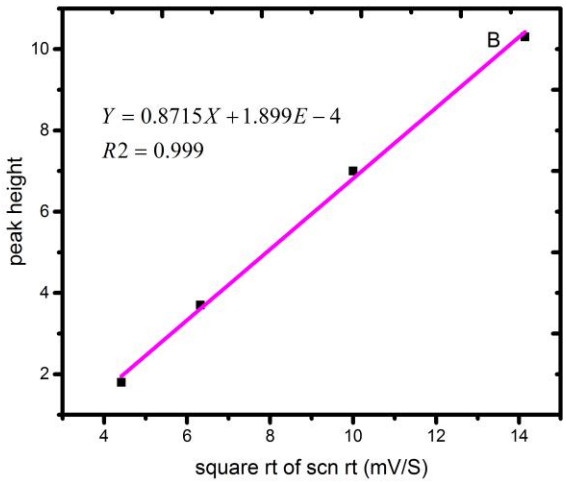
In addition, the relatively well defined peaks are a suggestion of an improvement in electron transfer kinetics probably resulting from electro catalysis by the electrode modification material, bentonite (Orata and Segor,2000). The enhanced redox features can also be attributed to the nature of the electrolyte media, the clay Montmorillonite has face to face (stacked) rather than edge to face (house of cards) orientation as had been observed previously by other authors from X-ray diffraction measurements (Fitch *et al.,* 1990) and this orientation helps in pre-concentrating the analyte. The well defined current peaks can also be attributed to electro catalysis by metal ions in the bentonite (Bard *at al*., 1986). The ratio of the peak currents obtained at the clay-modified and bare electrode would in general be an indicator of the conductivity of the clay film.

To assess the effect of scan rate on the peak height, and therefore to determine the nature of the reaction, scan rate dependence studies were conducted. A plot of anodic peak height

versus square root of scan rate for polyaniline on GCE and on bentonite modified electrode in 0.1 M sulphuric acid yielded to a linear plot (fig. 4.5 (a and b). The observation showed that the reaction is diffusion-controlled. From the linear calibration graphs obtained in figure 4.6, bentonite modified electrode had a higher correlation coefficient (R^2) of 0.99 while bare GCE had R^2 value of 0.93.



(a)



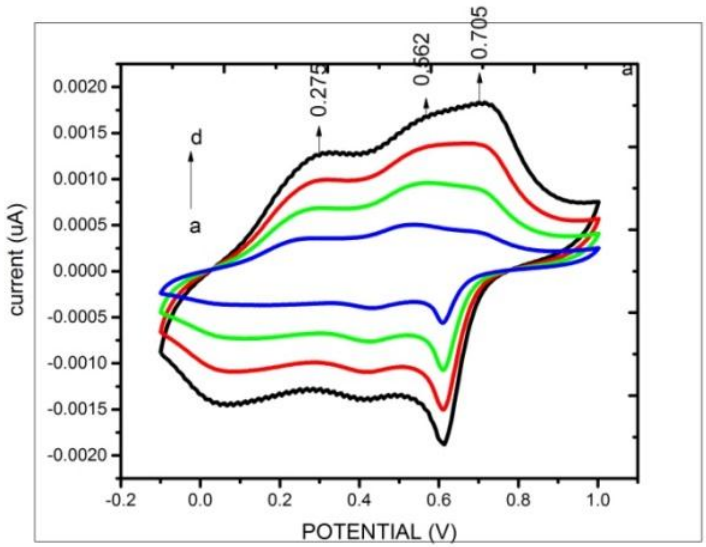
(b)

Fig 4.5: Peak height vs. square root of scan rate (200mV, 100mV, 40 mV, 20 mV) using (a) bare GCE (b) bentonite modified carbon

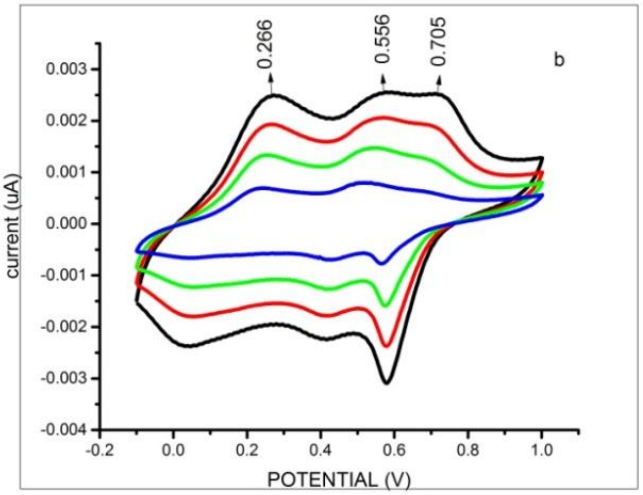
4.1.3 Electrochemical characterization of the PANI and PANI-SLS using GCE and Bentonite modified electrodes by cyclic voltammetry

Electrodeposited PANI and PANI- SLS film was further subjected to characterization by cyclic voltammetry in 0.1 M sulphuric acid at various scan rates. Characterization of the

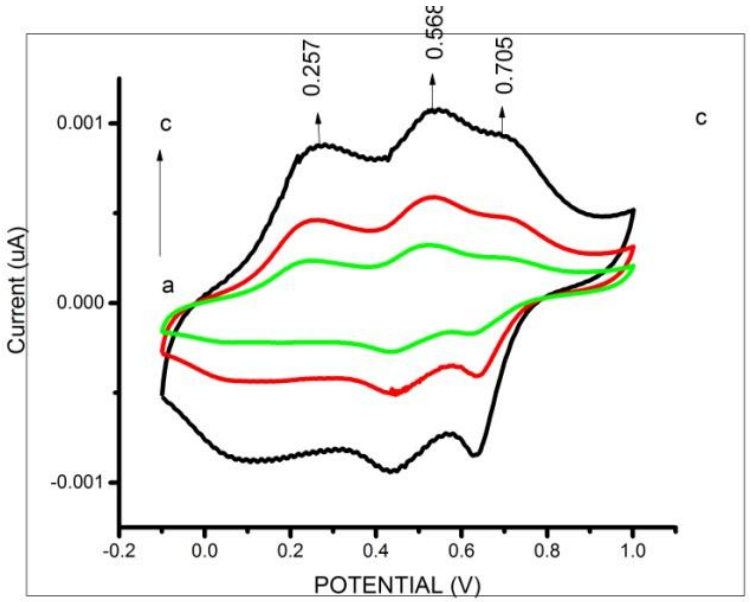
polymer was performed to study the position of the redox peak couples in order to investigate if polymerization took place. To confirm polymerization, the same peak characteristics observed during polymerization should be present during characterization of the polymer (Zhang, 2006). Cyclic voltammetric characterization of polymer films in 0.1 M H_2SO_4 (Figure 4.6) showed three distinct redox pairs, which proved that the PANI and PANI- SLS films are electroactive and showed fast reversible electrochemistry. The results obtained were similar to those obtained by Mathebe *et al.*, (2004) who observed that there were three pairs of redox peaks observed for PANI centered at approximately +0.2 V, +0.5V and +0.7V. The +0.2V peak corresponded to the transformation of leucoemeraldine base to emeraldine salt, the +0.7 V peak was as a result of degradation products and the +0.5 V peak corresponded to the transformation of emeraldine salt to pernigraniline salt.



(a)



(b)



(c)

Fig: 4.6: Cyclic voltammograms for electrochemical characterization of (a) PANI (b) PANI – SLS at scan rates of 10, 20, 30 and40mV s^{-1} (a-d) (c) PANI in bentonite modified electrode GCE/PANI-BTN at scan rates 10, 20, and 40 m V s^{-1}in 0.1 M H_2SO_4 solution

It was observed that the peak heights increased with increase in scan rates for GCE/PANI, GCE/PANI-SLS and GCE/PANI-BTN which confirmed that the polymer was electroactive and diffusion of electrons was occurring along the polymer chain. The anodic peak heights showed a linear dependence on the scan rate indicating that a thin film of surface bound conducting electroactive polymer was observed, undertaking fast reversible electron transfer reaction (Mathebe *et al.*, 2004).

The fact that peak height increased with an increasing scan rate also indicated good adherence of the polymer onto the GC electrode surface, increased peak current separation, and also shift of peak potential slightly with the anodic peak to positive and the cathodic peak to negative potential directions. This is because the charging and discharging of the electro-active conducting polymer is rate determining. As the scan rate increases the peak current of PANI increased linearly, indicating an adherent film on the glassy carbon electrode, this was further confirmed by a straight line graph obtained by plotting peak height vs. square root of scan rate as presented in Figure 4.5.

The results of characterization obtained in this study confirmed successful polymerization and that PANI and PANI-SLS were successfully attached onto the glassy carbon electrode surface and also proves the success of the dopant used.

4.1.4 Characterization of PANI, PANI- SLS and PANI –BTN films using UV-Vis spectroscopy

UV-Vis spectroscopy is a technique commonly used to give qualitative indication of the intrinsic redox states of conducting polymers. In this study, UV-Vis spectroscopy was used to give an indication of the redox state of PANI, PANI- SLS and PANI-bentonite and to study the changes that occur as a result of doping.

The electro polymerized PANI and PANI-SLS films were dissolved in DMSO and subjected to UV-Vis analysis. The UV-Vis spectra results are shown in Figure 4.7 below. Differences in the positions and size of absorption bands are observed when UV-Vis spectrum of PANI is compared with that of PANI-SLS. The UV-Vis spectrum of both PANI and PANI-SLS film showed a broad peak in the region ca. 600 nm and a small peak in the region ca. 401 nm. The appearance of the quinoid π-π* transition band at ca. 600 nm in the spectra is indicative of incomplete doping of PANI during electro polymerization (Songa *et al.*, 2009). The peak displayed at ca. 310 nm, for the PANI samples corresponds to n-π* transitions of aniline. The strong band at ca. 600 nm is characteristic of emeraldine base.

The absorption band at about 401 nm was attributed to the π-π* transition within benzoid segment that is, excitation of the nitrogen atom in benzoid segment. The features observed for PANI and PANI- SLS were found to be similar to those of polyaniline and its derivatives indicating the immobilization of the polymer on the electrode surface. Increase of the voltage lead to the absorption peak intensity also to increases due to the regular arrangement of monomer units in electrochemical polymerization.

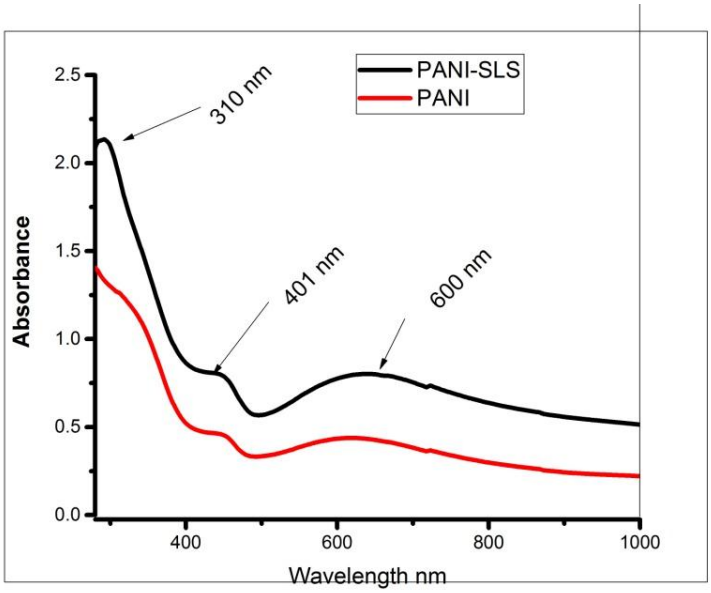


Figure 4.7: UV-Vis spectroscopy of PANI and PANI- SLS

When the spectra of PANI, PANI-SLS, PANI-BTN and PANI-SLS BTN were compared, there was a peak observed at 401nm and another broad peak was observed at 600nm for PANI-SLS BTN. For PANI-BTN two peaks were observed again at 401nm and 600nm fig. 4.8. The spectra of PANI-BTN and PANI-SLS BTN had higher absorbance compared to those of PANI and PANI-SLS. These results agreed with Beer Lambert's law of concentration, where the absorbance is directly related to concentration. This means that the higher absorbance values correspond to higher concentration and since the concentration used was the same, this further agreed with the observation made earlier that bentonite pre-concentrates PANI and the electrolyte into the electrode giving spectra with higher absorbance. There were no peaks observed for 0.1 M sulphuric acid alone. The results obtained are shown in figure *4.8 below*.

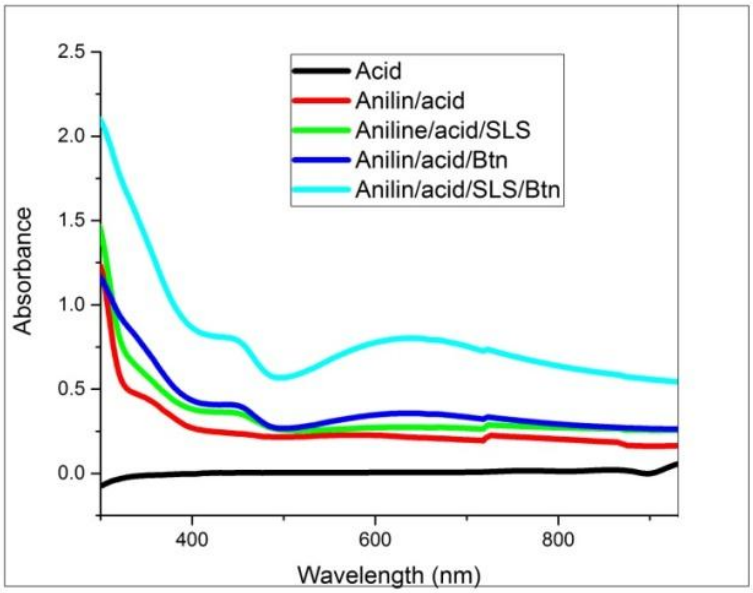


Figure 4.8: UV-Vis spectroscopy of, acid, PANI- acid, PANI/acid/ SLS and PANI/acid/ SLS/BTN

Characterization of PANI, PANI- SLS, PANI-BTN and PANI (dedoped) films using UV-Vis spectroscopy gave the results shown in figure 4.9 below. The results obtained showed that the absorbance peak of bentonite was noticeably higher than when PANI was deposited in bare GCE.

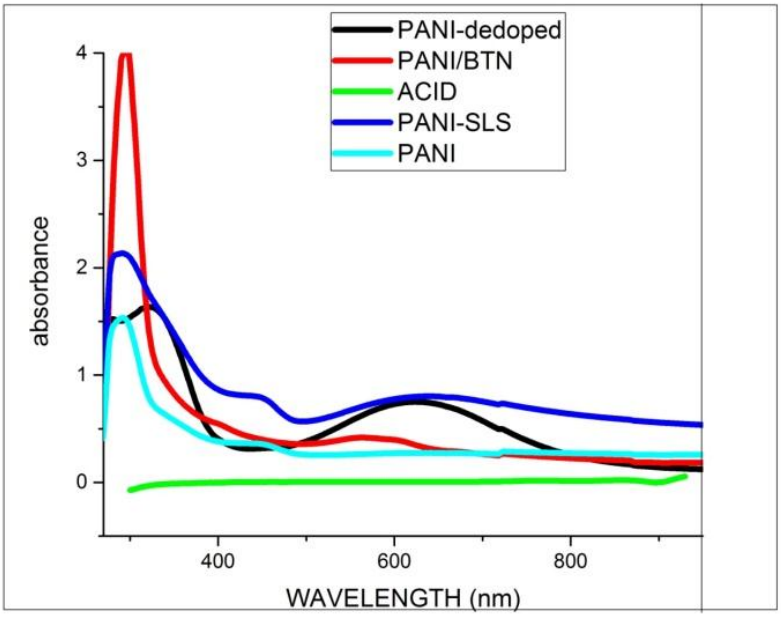


Figure 4.9: Characterization of PANI, PANI- SLS, PANI-BTN and PANI (dedoped) films using UV-Vis spectroscopy.

4.1.5 Characterization of PANI, PANI- SLS and PANI –BTN films using Raman spectroscopy.

Raman spectroscopy has been extensively employed for the investigation of electronic and chemical structures of PANI Forms. Raman spectra of EB and PB were obtained with several excitation laser lines and some of these spectra are presented in *Figure 4.10(a) and b.*

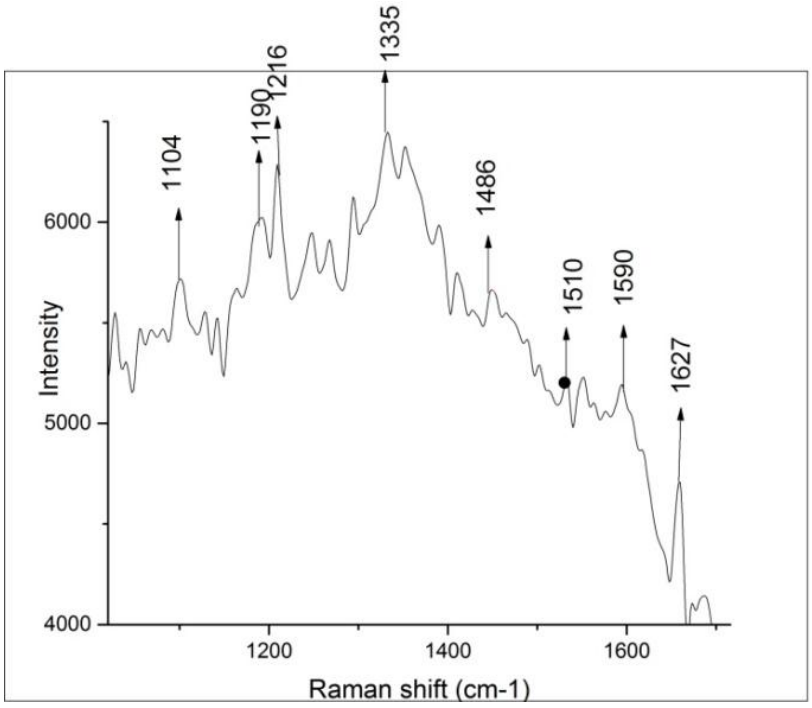


Figure 4.10(a): Raman spectrum of polyaniline PANI

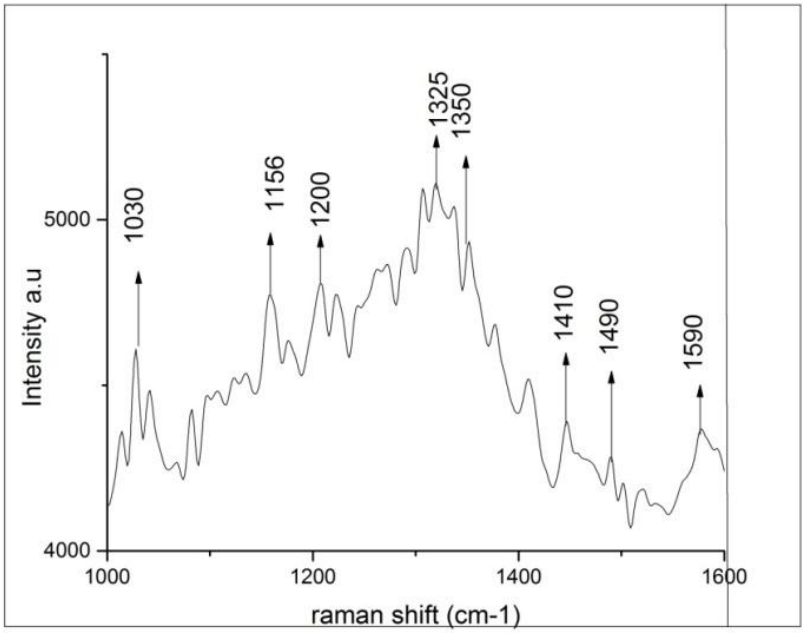


Figure 4.10(b) Raman spectrum of polyaniline modified with bentonite (PANI-BTN)

From the spectra obtained, there is a characteristic band at the 1156 cm^{-1} which is LB form due to βCH of benzene rings (Brivio, 1983). Another band is observed at 1200 cm^{-1} represents C–N bonds. C- N band at 1490 cm^{-1} shows a clear shift when the excitation photon energy is changed (Nascimento, 2007). Quinoid C-C at 1600 cm^{-1}, present a frequency shift. Raman spectra obtained with the 640 nm line, recorded for the reversible emeraldine – leucoemeraldine transition which are dominated by the (C-C) band at 1590 cm^{-1}, the imine (C=N) and δ(NH) vibration band with the maximum at 1490 cm^{-1}, the semibenzenoid polaronic (C~N+•) band at 1350 cm^{-1}, and the band of CH in plane bending vibration below 1200 cm^{-1}.

A small absorption band was observed at 1135cm^{-1} in PANI –BTN which results from C-H bending of quinoid/ benzoid ring. A band observed at 1590 in PANI was attributed by C-C stretching of benzenoid ring. Three Raman bands are observed at 1156, 1410 and 1590 which are characteristic of quinoid structure of PANI-BTN. These results were similar to those obtained by Nascimento (2007).

The protonated semiquinone radical structure vibrations resonate with the near-infrared excitation radiation so the Raman bands of semiquinone polaronic ν(C~N+•) stretching modes are enhanced and thus any changes due to the method of electro polymerization should be easier to follow. The polaronic band position and intensity depends on: first, conjugation length of π-electrons in the chain causing shift to a lower energy and an intensity increase for a more extended conjugation. Secondly, degree of protonation of the chain - bands due to

protonated and less protonated forms in the leucoemeraldine-emeraldine transition overlap and the latter is situated at a higher energy. Furthermore, symmetric (C~N+•~C) and non-symmetric (C~N+•-C) polaronic bonds that means equivalent and non-equivalent neighbour polaronic bonds, i.e. the bonds in the inner and the outer sites of the conjugated chain segment, respectively. The symmetric polaronic vibration band is situated at a lower energy than the non-symmetric one and a ratio of intensities of the two contributions should increase with conjugation length. (Boyer,1999).

Raman study on the kinetics of electrochemical degradation of PANI showed that, holding the PANI electrode at potential +0.8 V vs. Ag/AgCl caused no significant qualitative modifications in the Raman spectra except for the observed changes in the relative intensities of some peaks. The behavior concerns the overlapping ν(CC) stretching bands in quinoid and semibenzenoid rings, with Raman bands at 1595 and 1630 cm^{-1}, respectively (Mazeikiene, 2008).

4.2 Biosensor construction, characterization and application

The surface of the working electrode was first modified by electrodeposition of polyaniline followed by drop coating of electrode with thin slurry (0.002ml) of bentonite clay. Thin film of polyaniline, having an oxidative peak current of 0.0008 mA on the cyclic voltammogram was used as the host matrix. The resultant voltammogram is as shown in figure 4.11 below. After drying, the polyaniline- bentonite modified electrode was then transferred to a solution containing 0.001M BPA in 0.1M phosphate buffer.

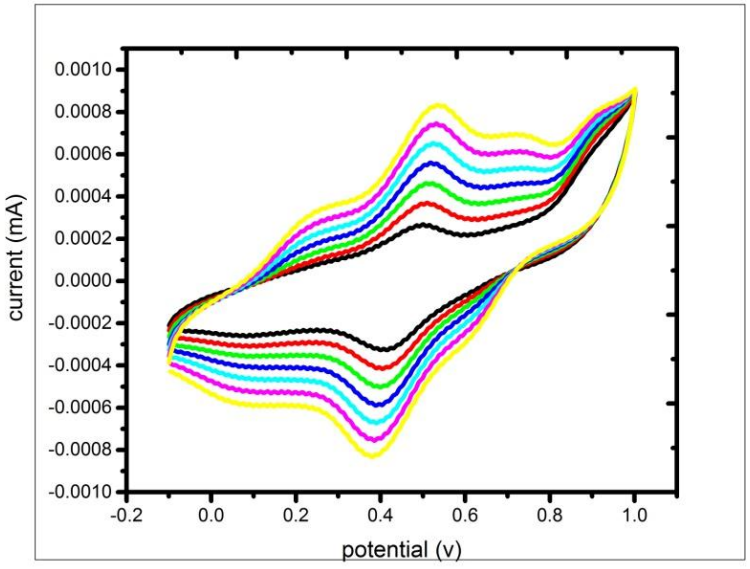
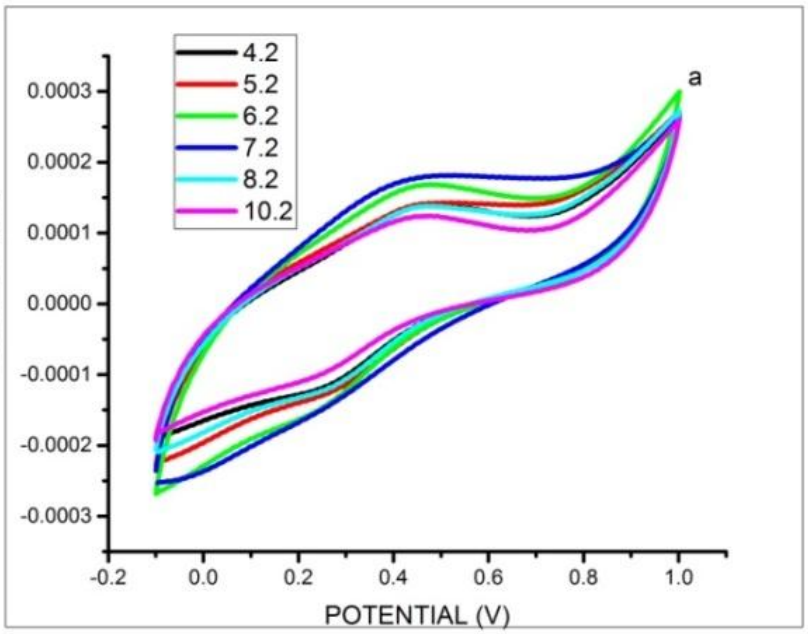


Figure 4.11 Cyclic voltammogram of GCE/ PANI- BTN scan rate 40mV

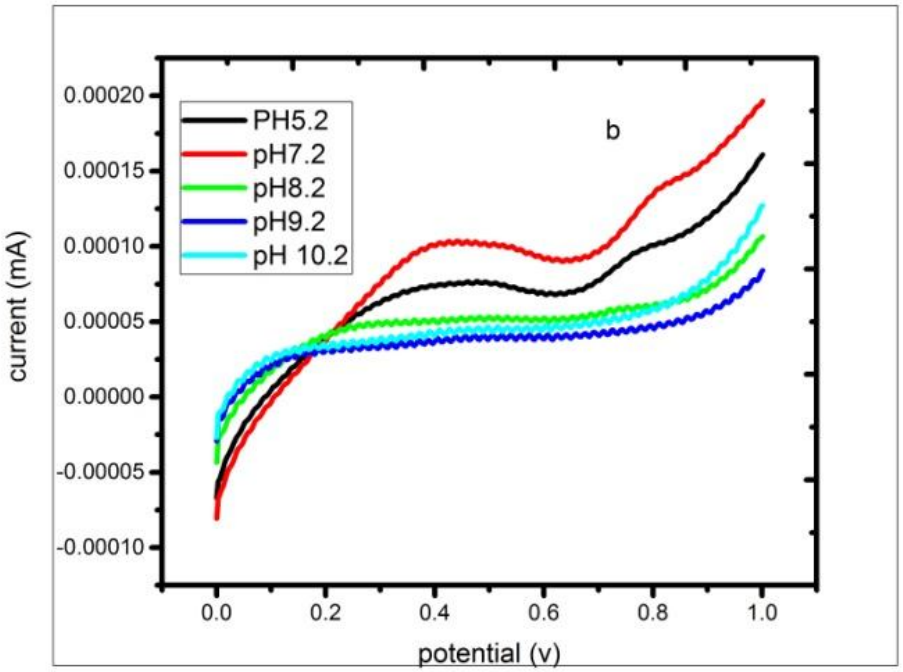
Tyrosinase based (Try/PANI-BTN /GCE) biosensor was then constructed, evaluated and optimized for detection of BPA. It was placed into a three-electrode electrochemical cell into which the analytes and samples of interest were injected and determined. The electrodes were connected to the computer-controlled potentiostat and the determination of the BPA achieved directly using cyclic voltammetry, square wave voltammetry and differential pulse voltammetry. The response of the immobilized Tyrosinase enzyme to its substrate BPA was a measure of its activity. The detection of the BPA was then carried out in the presence of a fixed concentration of BPA.

4.3 Optimization pH study of Tyrosinase enzyme

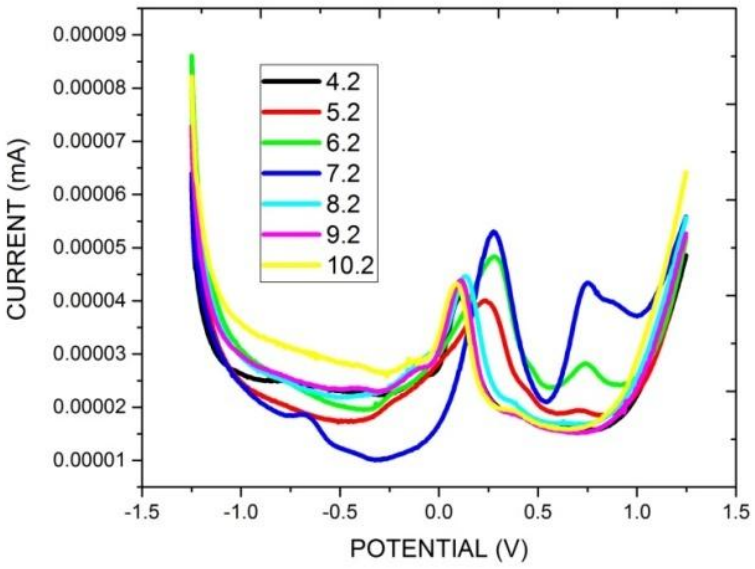
Cyclic, linear sweep and differential pulse voltammetry techniques were employed for the optimization and activation of Tyrosinase using biosensor (Try/PANI-BTN/GCE) at potential range of -1.0 V to + 1.0 V. This study was performed to investigate the outcomes of Tyrosinase response to pH when immobilized on GCE / PANI-BTN surface examined in 0.1 M phosphate buffer pH range 4.2 to 10.2 with fixed BPA concentration of 0.001M BPA. The pH study was performed to find the optimum activation pH of Tyrosinase enzyme. Figure 4.12 below shows Results for Tyrosinase enzyme pH optimization in Try/PANI- BTN/GCE in 0.1 M phosphate buffer of pH 4.2- 10.2 at fixed concentration of 0.001 M BPA of 120.0 µM in 100ml phosphate buffer using Cyclic, linear sweep and differential pulse voltammetry of Try/PANI-BTN/GCE.


4.2
5.2
6.2
7.2
8.2
10.2
a
CURRENT (mA)
POTENTIAL (V)
0.0003
0.0002
0.0001
0.0000
-0.0001
-0.0002
-0.0003
-0.2
0.0
0.2
0.4
0.6
0.8
1.0

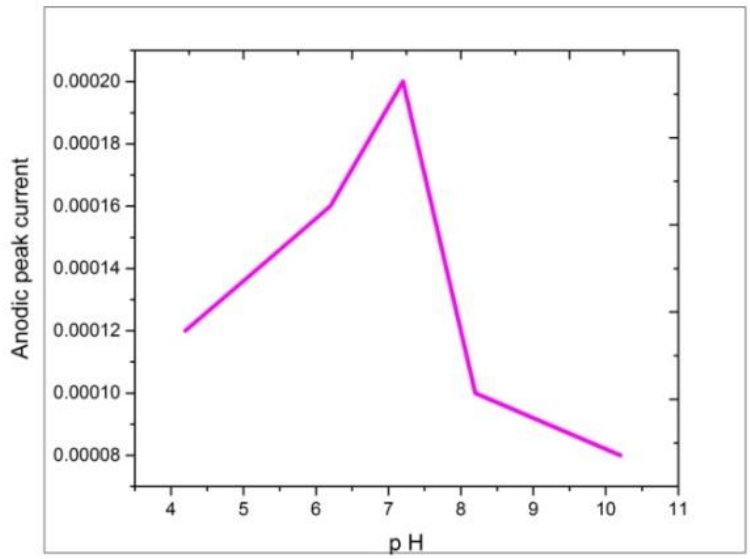
(a)


PH5.2
pH7.2
pH8.2
pH9.2
pH 10.2
b
current (mA)
potential (v)
0.00020
0.00015
0.00010
0.00005
0.00000
-0.00005
-0.00010
0.0
0.2
0.4
0.6
0.8
1.0

(b)



(c)



(d)

Figure 4.12: Tyrosinase enzyme pH optimization inTry/PANI- BTN/GCE using (a) cyclic voltammetry (b) linear sweep voltammetry (c) Differential pulse voltammetry (d) linear calibration curve.

The cathodic peak current was observed to increase with increasing pH solution from 4.2 until it reached 7.2 but when the pH value of the solution exceeded 7.2, the cathodic peak current decreased rapidly with each increment of pH solution until 10.2. Therefore the optimized pH measurement for Tyrosinase under the experimental conditions was found to be 7.2, which is similar to the value reported by *Akyilmaz et al.,* (2010) for the free Tyrosinase enzyme and Khan *et al.,* (2007) who also reported the pH range of Tyrosinase as being

between 4.5 and 7.0. This proves that Tyrosinase enzyme sustains its activation in neutral medium. This may also indicate the optimum pH value of Tyrosinase enzyme was not affected by the immobilization procedure used in the preparation of the biosensor.

This behavior indicates that the Tyrosinase activity increases from pH 4.2 up to 7.2 and gradually decreases when pH values exceed 7.2 most probably because the enzyme gets denatured at high pH values. It was also observed that the potential shifted with increase in pH, which was an indication that protons take part in the redox process of BPA on Try/PANI-BTN- SLS /GCE modified electrode. The reason for this behavior could be the reduction of *o*-quinone that requires H^+ in acidic medium, H^+ concentration is higher than that of the nitrogen atoms of the polymer and phenol hydroxyl of BPA are protonated in the form of $–NH_3^+$ and $–OH_2^+$ (Kuramitz *et al.,* 2001). In theory, the optimum pH of Tyrosinase is approximately pH 6.50 and *Li et al (2005)* reported that this value is even higher when Tyrosinase is immobilized on modified surface.

4.4 Electrochemical polymerization of BPA on bare glassy carbon electrode surface.

The electrochemical behavior of BPA on bare glassy carbon electrode was investigated by cyclic voltammetry (CV), square wave voltammetry (SWV) and differential pulse voltammetry (DPV) by successive additions of aliquots of 0.001 M BPA into the 0.1M phosphate buffer p H 7.2 which was used as the supporting electrolyte and the results are shown in Figure 4.13. The BPA was used as a substrate to establish the capability of bare glassy carbon electrode, the optimal conditions, detection limit, dynamic range, and stability. In this study, the response was monitored by peak current signal, which is proportional to the BPA concentration (Mita, 2005).

The electrochemical polymerization of BPA on glassy carbon electrode was investigated by addition of 120 µL (1.2×10^{-5} M) of 0.001 M BPA in 80 mL phosphate buffer pH 4.2 to 10.2. The potential was cycled five times between -0. 1V and +1.0 V at a scan rate of 40 mV s^{-1}. Only one oxidation peak was observed at potential +0.2 V (c) and two reduction peaks at + 0.13V (b) and + 0.8V (a).

The peak current was observed to decrease as the number of cycles increased. This behavior confirmed findings by Kuramitz *et al., (*2001) that polymerization of BPA on bare glassy carbon electrode leads to electron transfer blockage causing electrode fouling. The same behavior was observed for all pH values used ranging from pH 4.2 to 10.2. This study indicated that there was need for electrode modification which is used to eliminate fouling.

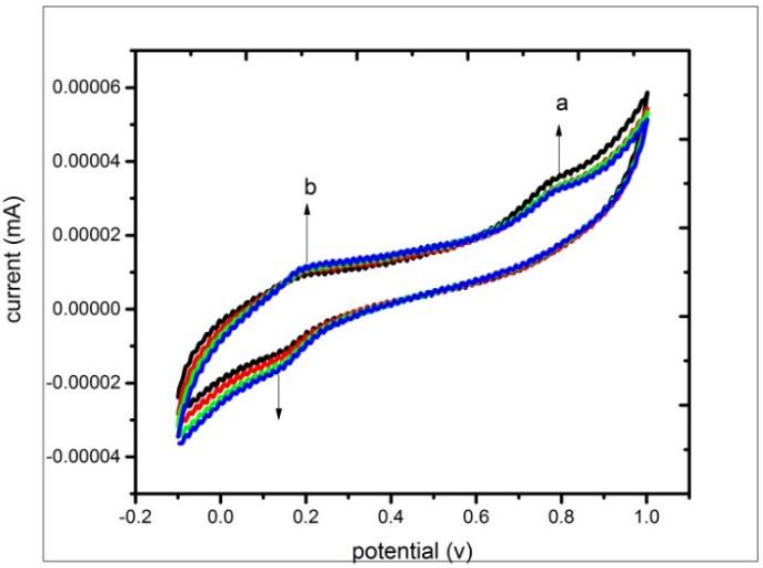
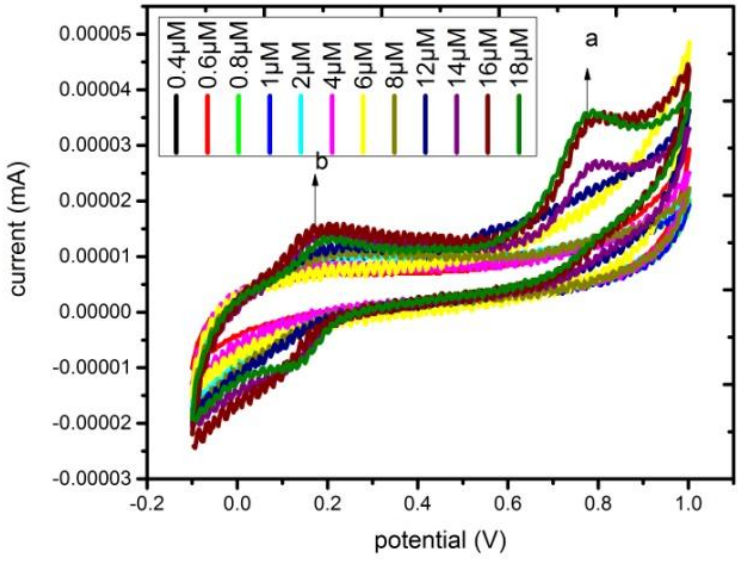


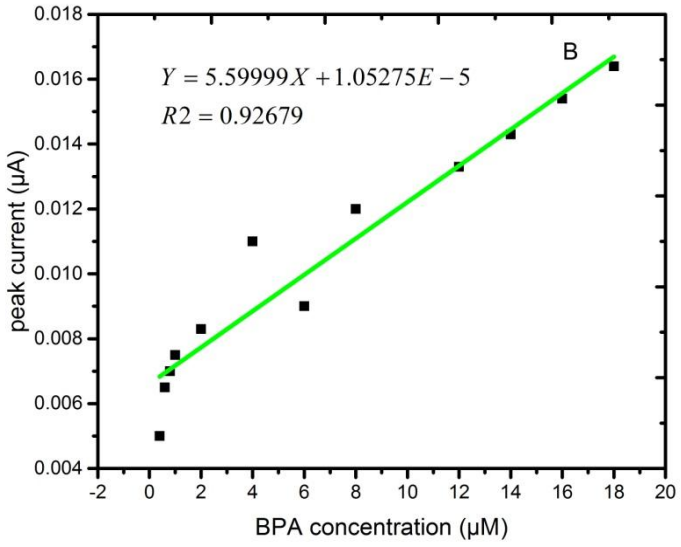
Figure 4.13: Electropolymerization of 120 µL of 0.001 M BPA in 80 mL phosphate buffer pH 7.2 on bare glassy carbon electrode.

The above experiment was repeated for different concentrations of BPA (0.4 μM - 18 μM BPA) in 80 mL phosphate buffer pH 7.2. As in the experiment above, only one oxidation peak was observed at a potential of +0. 19 V and two reduction peaks at potential of +0.23 V and +0.80V. The peak current was observed to increase as the concentration of BPA increased (Figure 4.14 (a)

Two oxidation peaks were observed at +0.1 V and +0.71 V (*Figure 4.14 (a).* The increase in peak currents with increase in BPA concentrations was also observed in the two oxidation peaks.



(a)



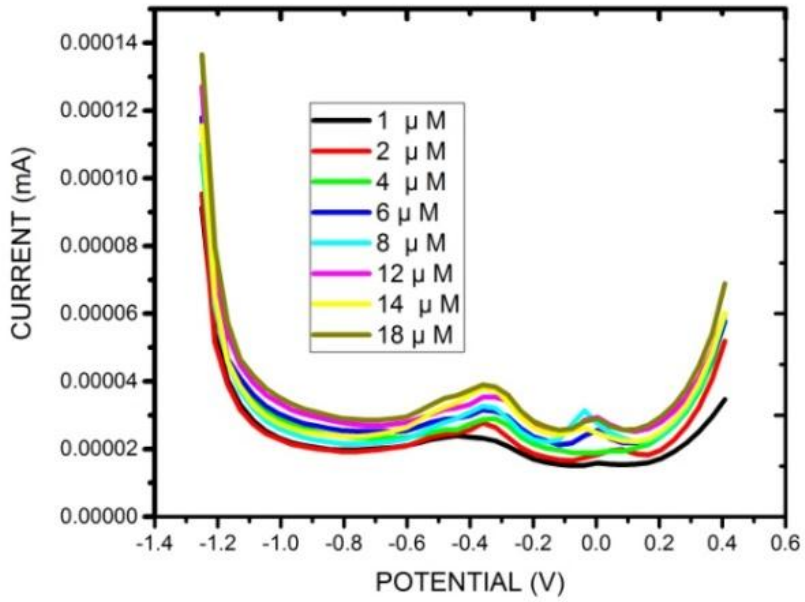
(b)

Figure 4.14: Electropolymerization of 120 µL BPA in 80 mL phosphate buffer pH 7.2 on bare glassy carbon electrode using different concentrations ,Voltammetry of 0.001 BPA on bare GCE using (a) cyclic voltammetry (b) Linear calibration curve of anodic peak current verses BPA concentration.

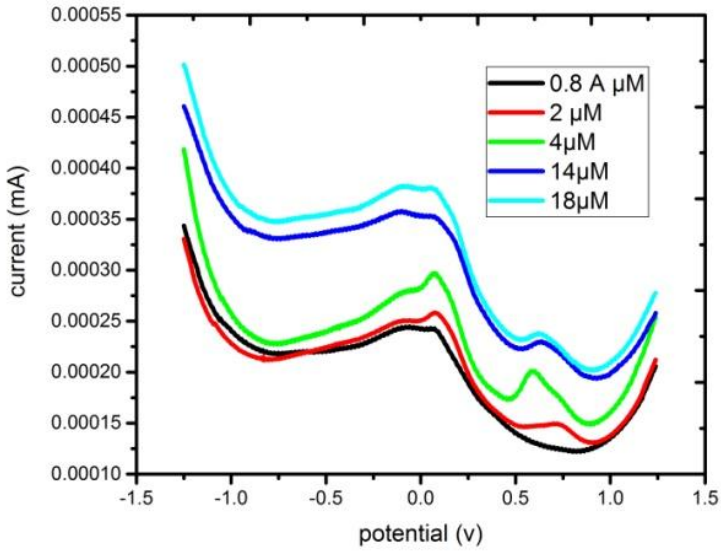
The two oxidation peaks observed in the cyclic voltammogram in figure 4.14 (a) above are as a result of oxidative polymerization of phenolic compounds (BPA). The +0.1 V peak corresponds to oxidation of monophenol to diphenol while +0.71 V peak corresponds to further oxidation of diphenol to quinone. The formed quinone on electrode surface gets reduced at appropriate potential and converted into signal (Kartsonaki, 2012). Electrochemical oxidation of BPA led to deposition of electropolymerized film blocking electron transfer causing electrode fouling making bare GCE difficult to be used for detection of BPA (Notsu *et al.*, 2002). Bare GCE was found to have a potential for detection of BPA but the problem was fouling caused by oxidative polymerization of BPA on GCE surface. A conducting polymer is required to intervene as it is known to work as a mediator for shuttling of electrons from the GCE surface to substrate binder and biological element which can be enzyme, DNA or antibody. The biological element used in this study is an enzyme which is incorporated within the construction of the biosensor.

Electrochemical polymerization of BPA on glassy carbon electrode was also achieved by cycling the potential repeatedly between -1.5 V and +1.5 V at a scan rate of 4 mV s^{-1} using square wave voltammetry and potential between -1.4V to + 0.6V using differential pulse voltammetry.

Figure 4.15 (b) Illustrates the DPV results for the electrochemical behavior of BPA at the bare glassy carbon electrode as a result of the successive additions of aliquots of BPA into 0.1 M phosphate buffer. The DPV results showed two reduction peaks centered at +0.05 V and -0.55 V. The reduction peak currents were observed to increase with increase in BPA concentrations. The reduction peak centered at +0.5 V and the peak current was observed to be increasing with increasing concentrations up to 18.0 µM. For the SWV, the reduction peaks were centered at +0.55 V and + 0.05V Figure 4.15 (a). The peak current also increased with increase in BPA concentrations up to 18.0 µM (Figure 4.15 a and b). DPV and SWV modes of measurements were used because they gave more defined peaks compared to cyclic voltammetry as shown in figure 4.15 (a) and (b) below.



(a)



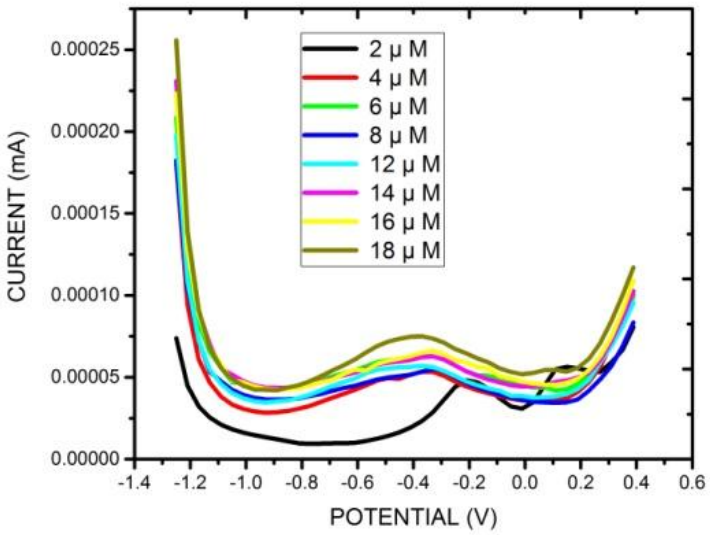
(b)

Figure 4.15: Electropolymerization of 120 µL BPA in 80 mL phosphate buffer pH 7.2 on bare glassy carbon electrode using different concentrations ,Voltammetry of 0.001 BPA on bare GCE using (a) square wave voltammetry and (b) Differential pause voltammetry

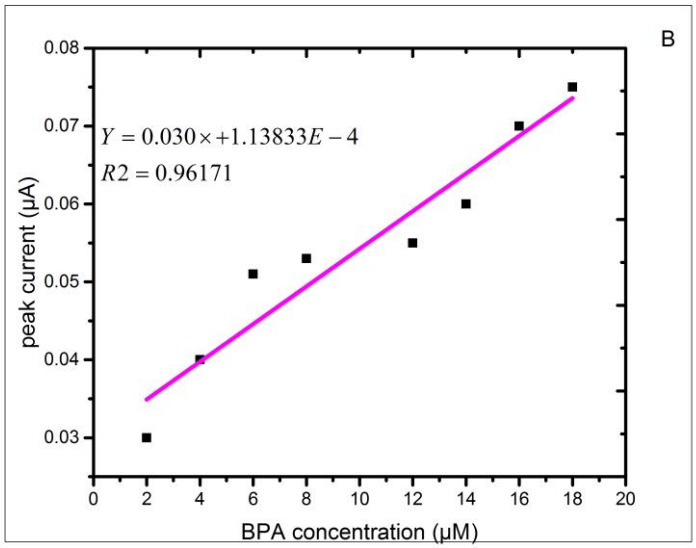
The DPV results obtained were found to be similar to those obtained by CV. In conclusion, the inactivation of electrode surface depends on the adsorptivity of the species of bisphenol A to the electrode, which is stronger in neutral medium than in alkaline medium (Krishnan *et al.*, 1993).

4.5 Electrochemical detection of BPA using Tyrosinase modified glassy carbon electrode.

The electrochemical behavior of BPA on glassy carbon electrode modified with Tyrosinase enzyme (Try/GCE) was investigated using DPV by successive additions of aliquots of 0.001M BPA into the phosphate buffer and the results obtained are shown in Figure 4.16 below. The study was performed at potential range similar to that of bare carbon electrode figure 4.13 and a pH of 7.2, 0.1M phosphate buffer. Tyrosinase enzyme was immobilized onto the surface of bare glassy carbon by drop coating method and the DPV results obtained showed a reduction peak centered at -0.5 V Figure 4.16.



(a)



(b)

Figure 4.16 (a) Differential pulse voltammograms showing the electrochemical behavior of various concentrations of BPA at the surface of Tyrosinase glassy carbon electrode (Try/GCE) (b) linear calibration curve for anodic peak current verses BPA concentration.

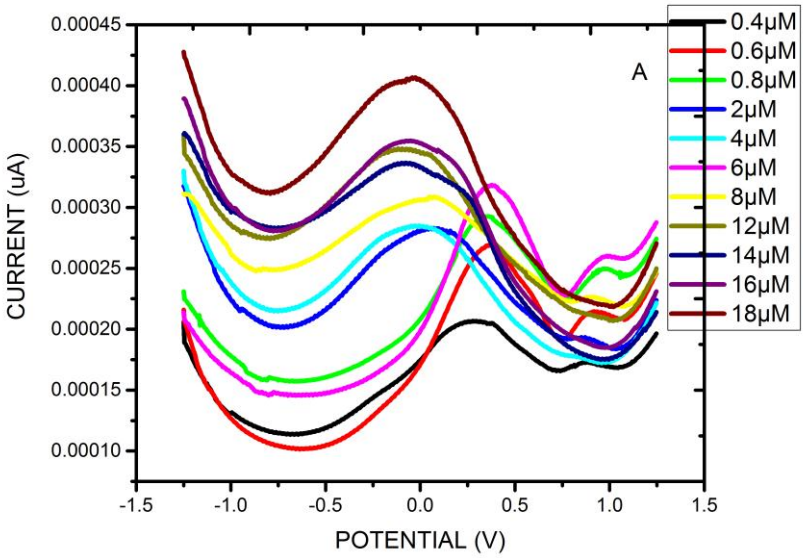
The reduction peaks were observed to increase with increase in BPA concentrations up to 18.0 µM. A defined reduction peak was observed at +0.19 V (Figure 4.16 (a). The increase in peak current with increase in BPA concentrations was also observed in oxidation peak. The Try/GCE showed good response on BPA detection but it did not last long because Tyrosinase was directly immobilized on bare GCE without the template. After few cycles were done,

there was no evidence if the Tyrosinase was still on the GCE surface and hence there was need for a polymer and a host matrix to work as mediator.

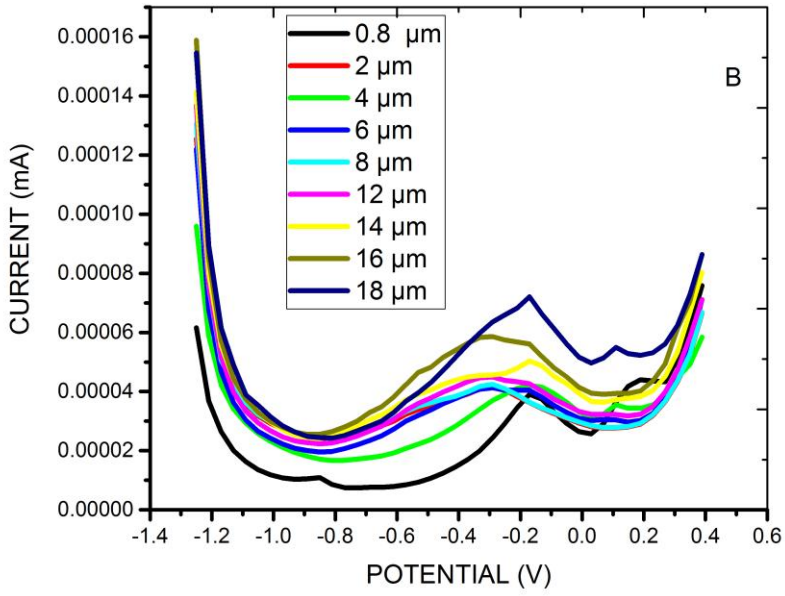
Phenolic compounds are identified to cause inactivation for glassy carbon electrode surface since they are oxidized electrochemically, through the deposition of electropolymerized films which are created when phenoxy radical attacks an unreacted substrate (Kuramitz *et al.,* 2001). This polymerized film of BPA on the Try/GCE surface prevents the electron transfer between the electrode surface and the enzyme, Kuramitz *et al* (2001) in their report supported this behavior. Inactivation of electrode surface is more easily induced in neutral and acidic medium than in alkaline medium. Similar observations were made when bare GCE was used. The same behavior was also observed in reduction peaks. The DPV results obtained were found to be similar to those obtained by CV but DPV was used because it gave more defined and clearer peaks (figure 4.16a). Figure 4.16 (b) illustrates linear calibration curve for the response of Try/GCE to additions of aliquots of 0.001 M BPA. From the results obtained, the Try/GCE showed promising response in detection of BPA.

4.6 Electrochemical detection of BPA using the biosensor (Try/PANI-BTN/SLS/GCE)

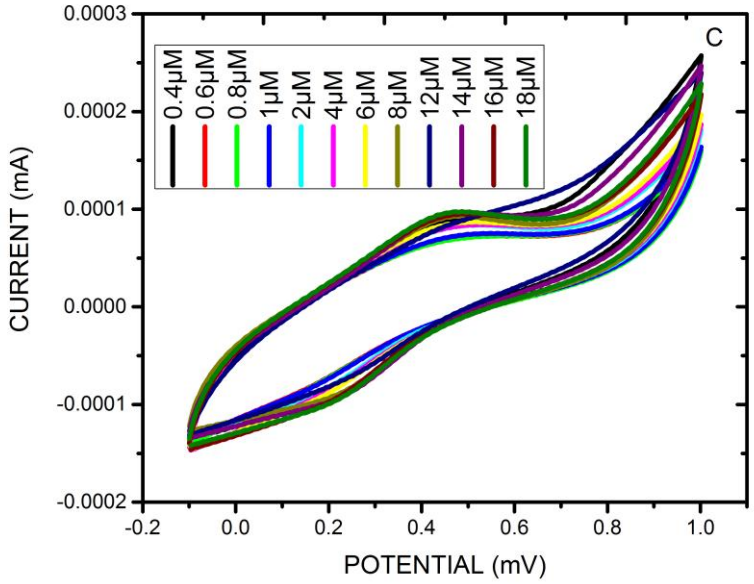
Electrochemical detection of BPA was done using SWV, DPV and CV. Figure 4.17 (a) below displays SWV plots for the biosensor (Try/PANI-BTN/GCE) response to different concentrations of BPA into 0.1 M phosphate buffer p H 7.2. The SWV results showed one reduction peak centered at +0.5 V and the peak slightly moved to the negative potential by approximately 0.1V with increase in concentration. The slight shift in peak potentials with change in concentration could be explained by Nernst's equation which points out to the fact that the e.m.f of a cell changes with the activity of the species being oxidized and the species being reduced. The DPV results also showed one reduction peak at -0.1 V. The reduction peak at -0.1 V showed increase in peak currents with increase in BPA concentrations which is supported by the increase shown in the DPV and SWV results presented in the Figure 4.18 below. Results obtained in this study compares well with those obtained by Matyholo (2011)


A
CURRENT (uA)
POTENTIAL (V)
0.4μM
0.6μM
0.8μM
2μM
4μM
6μM
8μM
12μM
14μM
16μM
18μM

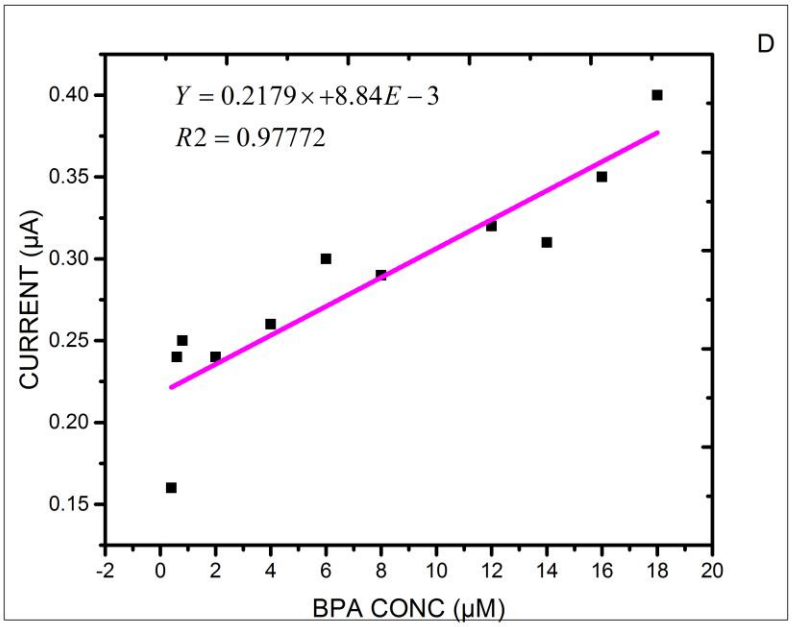
(a)


B
CURRENT (mA)
POTENTIAL (V)
0.8 μm
2 μm
4 μm
6 μm
8 μm
12 μm
14 μm
16 μm
18 μm

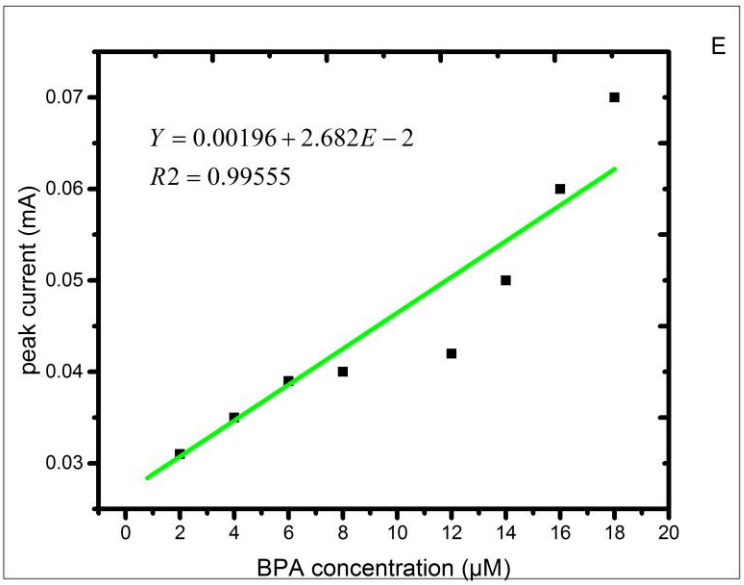
(b)


C
0.4μM
0.6μM
0.8μM
1μM
2μM
4μM
6μM
8μM
12μM
14μM
16μM
18μM
CURRENT (mA)
POTENTIAL (mV)
0.0003
0.0002
0.0001
0.0000
-0.0001
-0.0002
-0.2
0.0
0.2
0.4
0.6
0.8
1.0

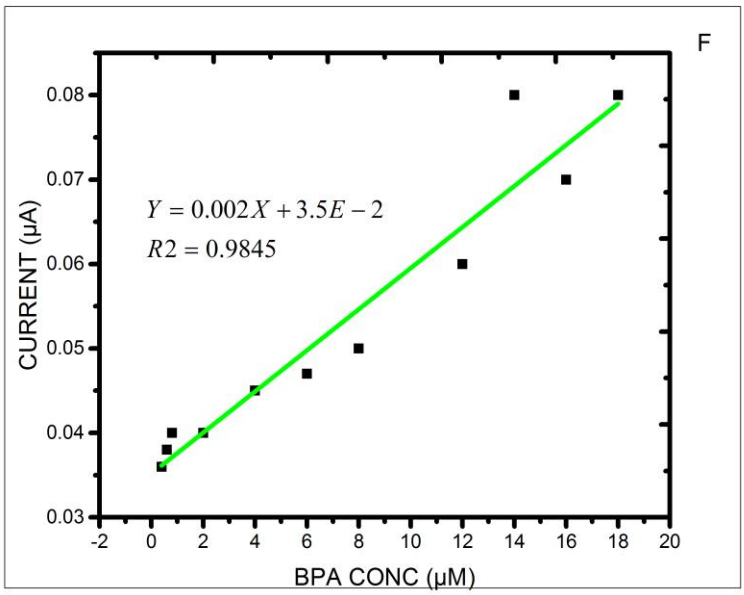
(c)


D
$Y = 0.2179 \times +8.84E-3$
$R2 = 0.97772$
CURRENT (μA)
BPA CONC (μM)
0.40
0.35
0.30
0.25
0.20
0.15
-2
0
2
4
6
8
10
12
14
16
18
20

(d)



(e)



(f)

Figure 4.17: Square wave voltammetry (a) SWV reduction (b) DPV (c) CV (d) SWV Linear calibration curve reduction peak (e) DPV Linear calibration curve of reduction peak (f) CV Linear calibration curve of reduction peak results for the biosensor in response to various concentrations of BPA.

The behavior observed for the peak at -0.1 V from the results obtained, is the expected behavior in the presence of Tyrosinase enzyme, which electrochemically reduces the diphenol to *o*-quinone at relatively low potentials. The bentonite modified biosensor showed good response to detection of BPA (figure 4.17 (a, b and c)), when compared to a surface

without modification (try/ GCE) (figure 4.16 (a))and bare GCE (figure 4.14 (a)). The good response to BPA when biosensor modified with bentonite is used is shown by the well developed peaks observed in fig 4.17 a, b and c. Linear fit calibration graphs of reduction peak potentials verses BPA concentration was obtained with correlation coefficient (R^2) between 0.97-0.99. This improved response of BPA to bentonite modified biosensor can be attributed to the pre-concentration of the BPA molecules as a result of being trapped in the octahedral layers in the bentonite. The alignments of aniline in the clay montmorrilonite matrix not only enhanced the electrochemical signal, but also led to reduction in Gibbs free energy resulting from entropic effects as a result of the realignment of the redox functional groups in the bentonite host matrix (Mbui *et al.,* (2014).
The surfactant used also influenced the rate of polymer formation, particle size, distribution, morphology and homogeneity (Armes *et al.,* 1990) leading to formation of enhanced peaks. The results obtained collaborate with those observed by Mbui *et al.,* (2014).

Based on these results, the detection limit for BPA which is given by; 3 x standard deviation of the blank)/Sensitivity was calculated to be 2.1 x 10^{-9}M within a concentration range of 0.4 -18.0 μM BPA. Sensitivity in this case is defined as the slope of the calibration curve. The detection limit value obtained in this study was compared to literature and it was found to be similar to the value reported by Matyholo *et al* (2011) which was 1.9 x 10^{-8} M at wide range of 1.0 x 10^{-16} μM estimated for Try/PDMA-PSS biosensor used to investigate the effect of BPA on activity of Tyrosinase enzyme. The detection limit value obtained in this study was higher but very close to those reported in literature for other techniques such as Elisa, HPLC-MS, GC-MS and LC-MS.

The results obtained of SWV and DPV figure 4.17 (a and b) also showed that the bentonite modified biosensor exhibited high sensitivity, high catalytic activity and high conductivity when compared to bare GCE and Try/GCE, mainly because of the good conducting polymer material PANI and host material BTN, deposited on bare GCE surface prior to the immobilization of Tyrosinase enzyme. Results obtained from Try/PANI-BTN biosensor for determination of bisphenol A by the method discussed, were compared with other method for BPA determination, and the results obtained are tabulated in table 4.1 in this document. The application of developed polymeric Tyrosinase biosensor for determination of BPA compound in water was considered to be the best method due to short response time, cheap fabrication materials, easy storage when not in use and portability visa-vis chromatographic

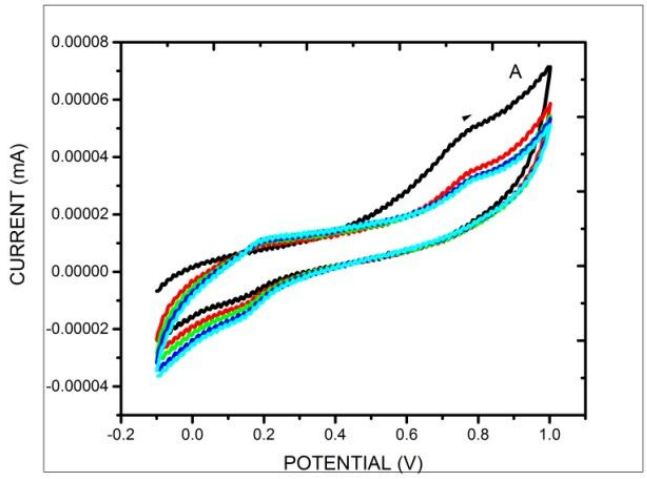
methods which are deemed to be expensive, need skilled operators, time consuming sample pretreatment steps and long response time.

Table 4.2: Analytical parameters for the determination of bisphenol A

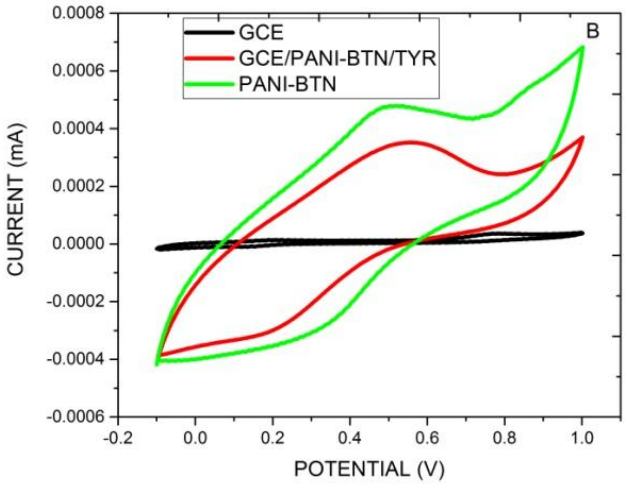
Technique	**BPA Samples Type**	**Limiting detection**	**Retention Time**	**References**
GC-MS	Water samples	2.6 x 10^{-9} M	8 minutes	(del Olmo *et al.* 1997)
GC	Wastewater samples	2.6 x 10^{-11} M	7.02 minutes	(Vílchez *et al.* 2001)
Elisa	Real water samples	4.4 x 10^{-10} M	6 minutes	(Zhao *et al.* 2002)
Try-SWNTs sensor	5 µM phenol + 0.1 M phosphate buffer	2 x 10^{-8} M	20 seconds	(Zhao *et al.* 2005)
LC-MS/MS	Placenta tissue samples	8.74 x 10^{-10} M	10.5 minutes	(Jiménez-Díaz *et al.* 2010
Try/Teflon membrane biosensor	2.5 µM +5 mM phosphate buffer	9.5 x 10^{-8} M	1 minute	(Akyilmaz *et al.* 2010)
HPLC-MS W	Water samples	2.5 x 10^{-9} M	15 minutes	(Jiang *et al.* 2011)
Try/PDMA-PSS biosensor	0.001 M BPA + 0.1 M phosphate buffer	1.9 x 10^{-8} M	12 seconds	(Matyholo et al., 2011).
Try/SLS/PANI-BTN biosensor	0.001 M BPA + 0.1 M phosphate buffer.	2.1 x 10^{-9}M	15 seconds	(Lucia *et al;*, 2017)

4.7 Voltammetric characterization of the biosensor

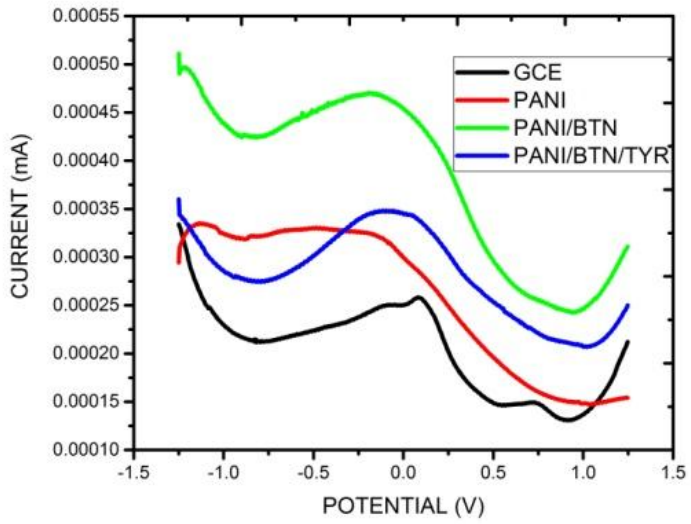
Electrochemical characterization and optimization of biosensor response (Try/ SLS/ PANI-BTN /GCE) was achieved by cyclic voltammetry, differential pause voltammetry and square wave voltammetry in 0.1 M phosphate buffer , pH 7.2 at a potential range of -0.1V to +1.0 V and a scan rate of 40 mV s^{-1}. The results which were obtained are shown in Figure 4.18 Cyclic voltammogram for bare GCE and (b) cyclic voltamogramm showing comparison of the oxidation and reduction peaks of bare GCE, PANI-BTN and GCE/PANI-BTN/TRY (Figure 4.18 (a and b) . Bare GCE showed a pair of reduction peaks at +0.22 V and +0.80 V, and oxidation peak at +0.18 V figure 4.18 a. A pair of redox peaks was observed for PANI-BTN at +0.56 V reductions and at +0.32 V oxidation peak figure 4.18 b. In GCE/PANI-BTN/TRY a pair of redox peaks, one reduction peak at approximately +0.58 V and oxidation peak at approximately + 0.28 V at lower peak current than PANI-BTN but higher than the bare GCE.



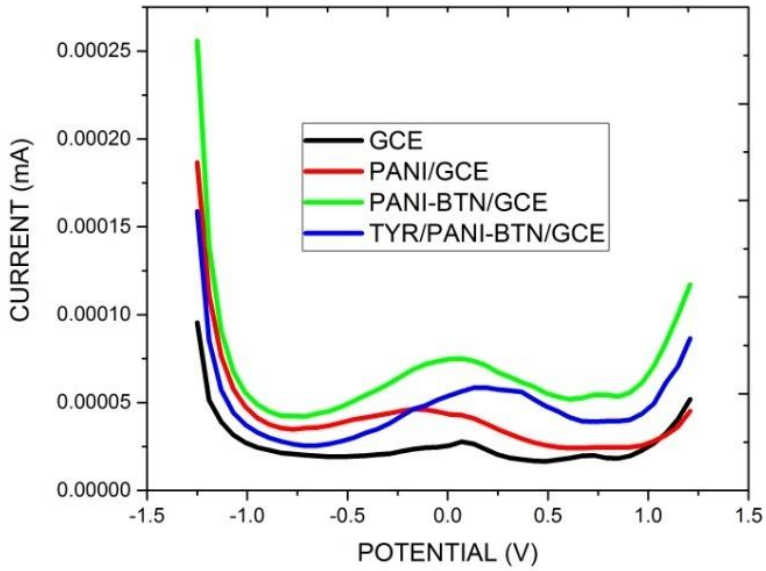
(a)



(b)



(c)



(d)

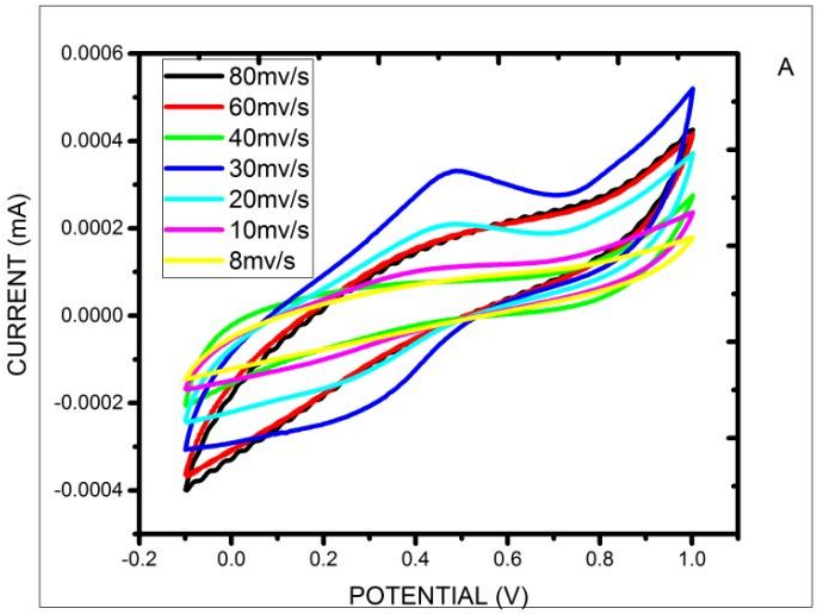
Figure 4.18: Results for electrochemical characterization of (a) cyclic voltammetry for bare GCE (b) cyclic voltammetry(c) square wave voltammetry (d) differential pause voltammetry for GCE, PANI-BTN/ GCE and Try/PANI-BTN/GCE voltammetry in 0.1 M phosphate buffer pH 7.2.

The increase in reduction peaks current of PANI-BTN/GCE, figure 4.18 (b-d) confirmed successful electro deposition of different layers onto electrode surface. On the other hand, the decrease in peak current of biosensor Try/PANI-BTN/GCE figure 4.18 (b-d) is influenced by, the presence of the enzyme Tyrosinase and indicates a stronger enzyme binding and higher catalytic activity on modified GCE surface leading to improvement in electrochemical behavior (Li *et al.*, 2005). Thus the inert adhesive glue used for attaching the enzyme may have acted as an enzyme cross-linker and led to the successful incorporation of Tyrosinase to the modified GCE surface.

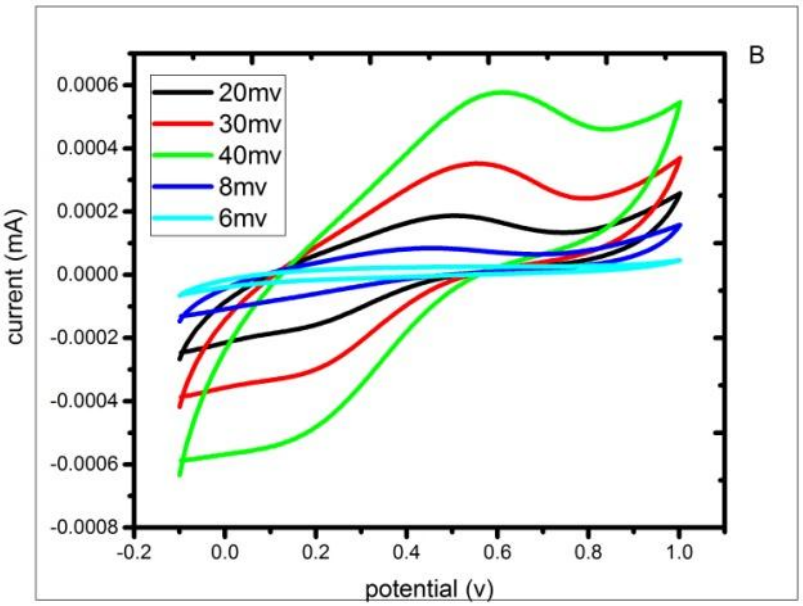
The square wave and differential pulse voltammetry results obtained for the biosensor, bare GCE, PANI-BTN and TRY/PANI-BTN/GCE are also shown in Figure 4.18 (c) and (d) respectively. In square wave voltammetry, one reduction peak was observed for bare GCE at +0.21 V, PANI-BTN/GCE at -0.22 V and one reduction peak was observed for Try/PANI-BTN/GCE at approximately -0.15 V. For differential pulse voltammetry, one reduction peak was observed for bare GCE at +0.20 V, PANI-BTN/GCE at -0.23 V and one broad reduction peak was observed for Try/PANI-BTN/GCE at approximately -0.10 V.

From the cyclic voltammetry, the peak current for PANI-BTN was observed to be higher than that of the biosensor with a peak current of 0.51 µA. the peak current of Try/PANI- BTN/ GCE decreased to 0.38 µA with a shift to more negative potential influenced by the presence of Tyrosinase immobilized on modified GCE electrode. This suggests that the detection of BPA using the biosensor (Try/PANI-BTN/GCE) can be achieved at much lower potentials without any interference from the immobilized PANI-BTN.

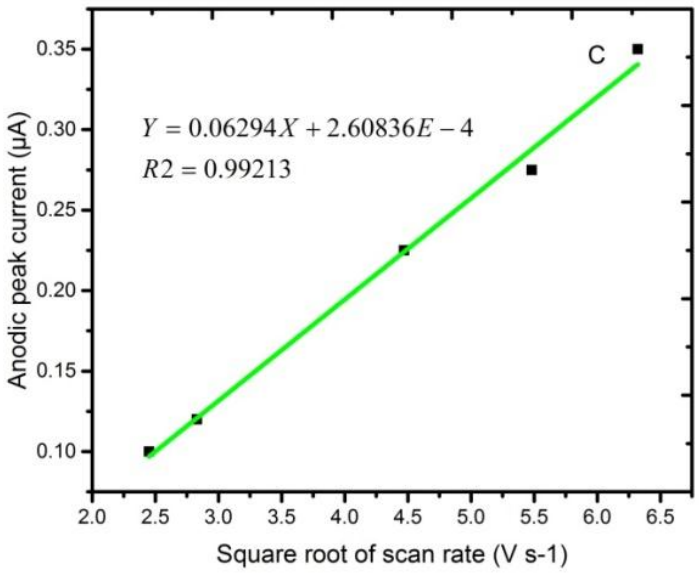
Figure 4.19 (a) and (b) shows cyclic voltammograms for electrochemical characterization of the biosensor (Try/ PANI-BTN /GCE) which was performed in the absence of analyte at different scan rates.


A
80mv/s
60mv/s
40mv/s
30mv/s
20mv/s
10mv/s
8mv/s
CURRENT (mA)
POTENTIAL (V)
0.0006
0.0004
0.0002
0.0000
-0.0002
-0.0004
-0.2
0.0
0.2
0.4
0.6
0.8
1.0

(a)


B
20mv
30mv
40mv
8mv
6mv
current (mA)
potential (v)
0.0006
0.0004
0.0002
0.0000
-0.0002
-0.0004
-0.0006
-0.0008
-0.2
0.0
0.2
0.4
0.6
0.8
1.0

(b)



(C)

Figure 4.19: Cyclic voltammogram for electrochemical characterization of biosensor (a) at scan rates 8,10,20,30,40,60,80 mV s-1, (b) at scan rates 6, 8,20,30,40 mV s-1 and (c) Linear plot of peak current against square root of scan rates in 0.1 M phosphate buffer solution containing 0.001 M BPA.

A pair redox peak was observed for (Try/ PANI-BTN /GCE) at approximately +0.59 V reduction and at +0.21V oxidation peak. The peak currents of the paired redox peak were observed to increase with increase in scan rate from 6-30 mV s^{-1}. This behavior confirmed that there was good electron transfer between the electrode surfaces and the electrolyte, but the oxidation peak reduced and disappeared at high scan rates. This meant that the BPA oxidation reaction occurs at a low scan rate. From the CV plot, a linear calibration curve was plotted to explore the relationship of cathodic and anodic peak current (*Ipc* and *I pa*) against square root of scan rate (υ), the linearity showed the characteristic of surface bound or adsorbed species conducting electro-active polymer, undergoing fast reversible electron transfer reaction (Matyholo, 2011). The surface concentration of PANI – BTN film on the surface of GCE, *T* PANI* can be estimated from plot of I_P (peak current) against *v* (scan rate) in accordance to Laviron's equation as shown below.

$$I_p = n^2 F^2 T^*_{PANI-BTN} \frac{v}{4RT} \qquad \text{Equation (3.1)}$$

and

$$Q = nFT^{*}_{PANI-BTN}\,A \qquad \text{Equation (3.2)}$$

Laviron's equation can therefore be described as:

$$T^{*}_{PANI-BTN} = \frac{Q}{nFA} \qquad \text{Equation (3.3)}$$

Where *n* represents number of electrons (2), *F* is the faraday constant (96.500 C mole^{-1}), T*$_{\text{PANI- BTN}}$ is the surface concentration of PANI-BTN film (mol cm^{-2}), *A* is the surface area of the electrode (0.0707cm^{-2}), *v* is the scan rate (V s^{-1}), *Ip* is the peak current (A) and *Q* is the quantity of charge (C) calculated by *A*/υ, where *A* represents area of oxidation peak from CV for the first segment obtained from CV integration with scan rate 0.006 V s^{-1}. The surface concentration (*T**PANI-BTN) of the adsorbed electro active species evaluated from the slope was estimated to be 1.0210 x 10 $^{-8}$ mol cm^{-2}. The surface concentration value obtained in this study was compared to literature and it was similar to the value reported by Mathebe *et al.*, in (2004) as 1.85 x 10 $^{-7}$ mol cm^{-2} for PANI film, Songa *et al* (2009) with surface concentration of 1.2478 x 10 $^{-8}$ mol cm^{-2} for PANI-FcPF6 and Matyholo (2011) with surface concentration of 1.0218 x 10 $^{-7}$ mol cm^{-2} for (*T**PDMA-PSS) using glassy carbon electrode.

These results gave evidence that PANI-BTN has indeed similar properties with PANI as reported in literature. The peak current was found to be increasing with increase in scan rate. This indicates that the peak current is diffusion controlled (Mathebe *et al.*, 2004). The diffusion coefficient, D_e was calculated from a CV plot of *Ip* against υ in Figure 4.19 (c) using the Randle Sevcik equation shown in equation 4 below.

$$I_p/v^{1/2} = (2.69x10^5)n^{3/2}AD^{1/2}C \qquad \text{Equation (3.4)}$$

C is the concentration of analyte in (mol cm^{-3}), *n* is number of electrons transferred in the redox reaction (2), *A* is the surface area of glassy carbon electrode = 0.0707 cm^2, *v* is the scan rate (V s $^{-1}$), *Ip* is the peak current (μA), *D* the diffusion coefficient of the electroactive species (cm^2 s^{-1}) and slope = 0.06294 obtained from the plot of *Ip* against υ$^{1/2}$ with correlation coefficient (R^2) of 0.99213 shown in the linear calibration plot above Figure 4.19 (c).

The value of D was estimated to be 8.401 x 10^{-8} cm^2 s^{-1} and it was similar to the D value reported by Mathebe *et al* which was 8.68 x 10^{-9} cm^2 s^{-1} for PANI film, Songa et al which was 2.41 x 10^{-8} cm^2 s^{-1} for PDMA-PSS in gold electrode (Mathebe *et al.,* 2004; Songa *et al.,* 2009) and Matyholo 8.424 x $10^{-7}cm^2$ s^{-1} (Matyholo, 2011). The D value depends on the electro-deposition conditions used and the homogeneity of the film. The D value obtained was higher than the reported ones in literature, which may be due to the presence of the bentonite clay and SLS used.

As stated earlier, bentonite leads to pre-concentration of the aniline molecules as a result of being trapped in the octahedral layers in the bentonite and this increased the conductivity of PANI-BTN film, resulting in increase in Diffusion coefficient electron value. These results confirmed high conductivity of polymeric film influenced by bentonite thus resulting to faster electron transfer.

4.8 Detection and determination of BPA concentration in drinking water bottles and baby bottles using fabricated biosensor.

This section presents the results of detection and determination of the concentration of bisphenol A by Try/PANI-BTN/ SLS/GCE biosensor employing cyclic voltammetry, square wave voltammetry and differential pulse voltammetry monitoring the interaction of Tyrosinase and the bisphenol A in three types of plastic drinking water bottles and two types of feeding baby bottles purchased from a local supermarket.

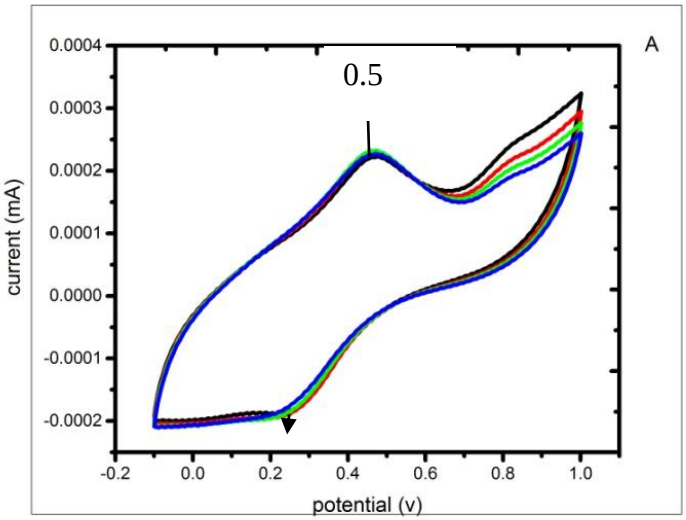
4.8.1 Detection and determination of BPA concentration in drinking water bottles.

Three different types of commonly used drinking water bottles of different colours: Pink water bottle, blue and green was investigated for the presence of BPA using the biosensor and cyclic voltammetry, square wave voltammetry and differential pulse voltammetry modes.

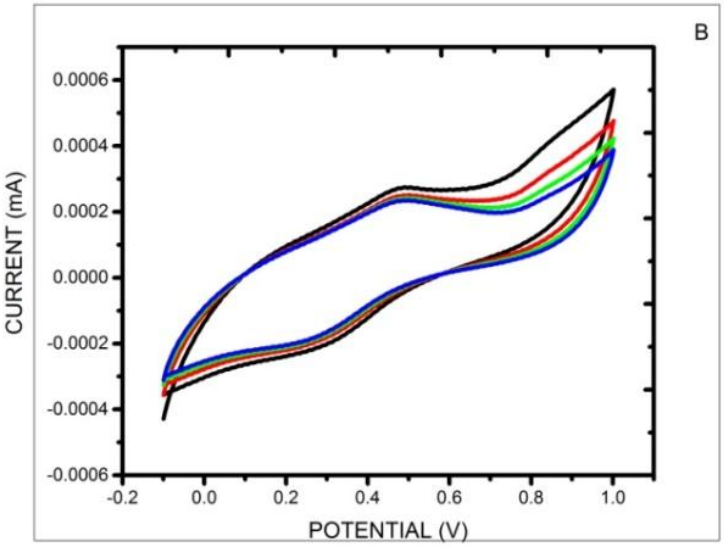
The electrochemical detection of BPA in drinking water bottles was investigated using the biosensor. This was done by addition of 40 ml of water at temperatures of 50° C, 60°, C 70° C and 95° C then the temperature maintained in the bottles for five minutes and cooled to room temperature. The cooled water was then added to 40 ml of 0.1M phosphate buffer pH 7.2.

Detection of BPA in blue drinking water bottle was done by cyclic voltammetry. It was achieved by cycling the potential repeatedly between -0. 1V and +1.0 V at a scan rate of 40 mV s^{-1} for 4 voltammetric cycles. Only one reduction peak was observed at potential of +0.50

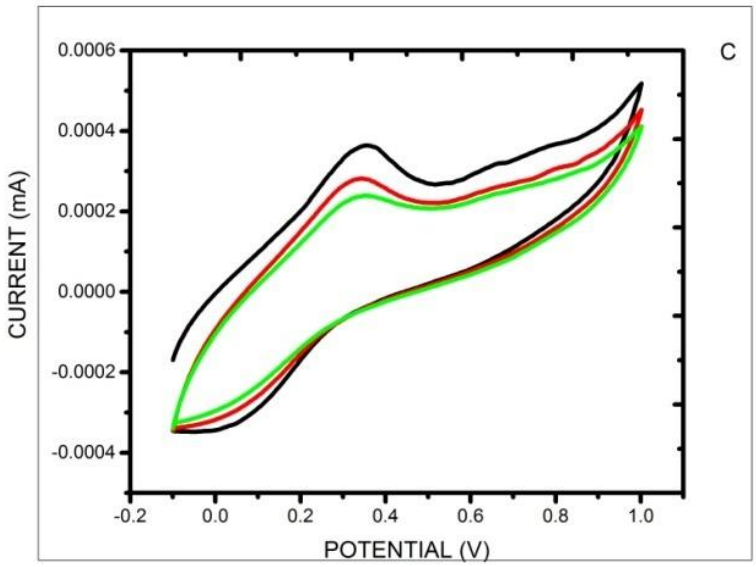
V and one oxidation peak at +0.20 V figures 4.20 (a-d). The peak current was observed to increase as the number of cycles increased. This behavior was also observed in determination of BPA figure 4.4 (d) which was discussed earlier where the peak current increased with increase in the number of scans. In addition, one reduction peak at +0.50V and one oxidation peak at +0.20 V was also observed when 0.001M of BPA was used as substrate and biosensor used to determine its presence figure 4.17(c). From these results it can be concluded that when water at a temperature of 50° - 95° C was put in the drinking water bottles, approximately 0.001M BPA leached into the water. The results obtained were shown in figure 4.20 (a) - (c) below.



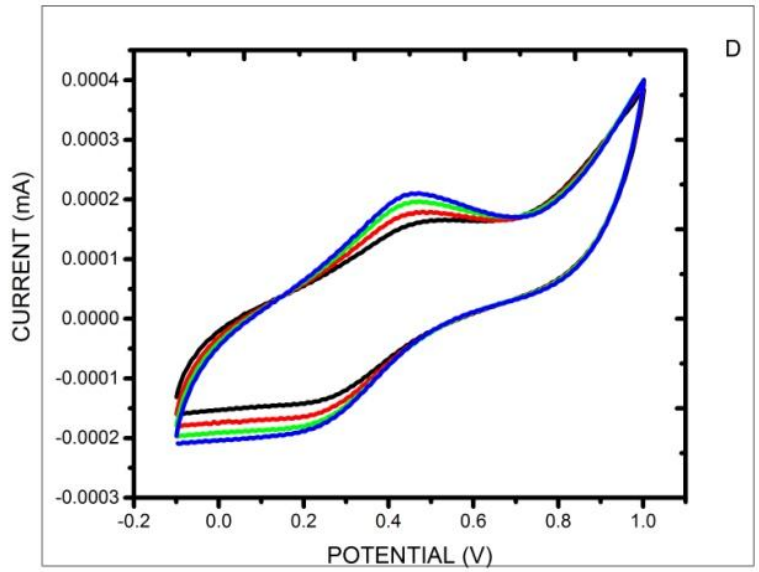
(a)



(b)



(c)

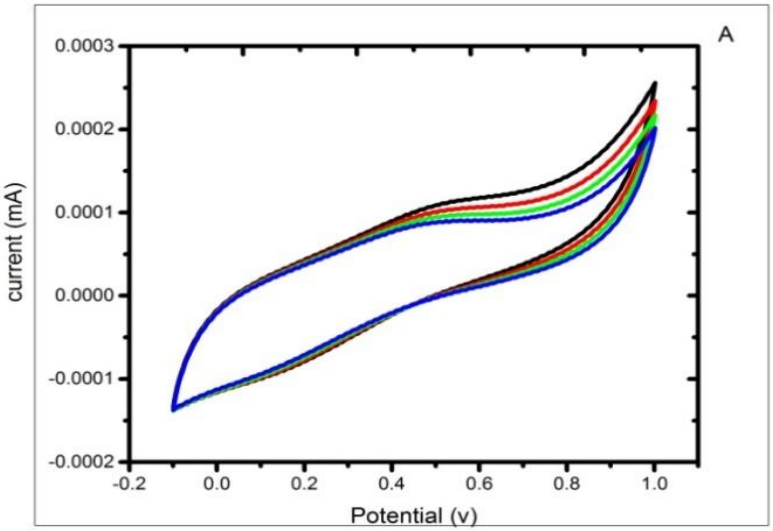


(d)

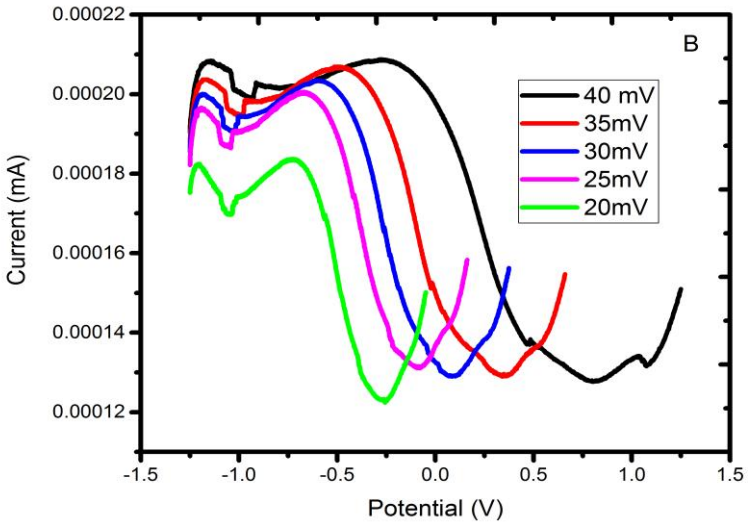
Figure 4.20: cyclic voltammetry of 40ml water in blue drinking water bottle in 40ml 0.1M PBS and pH 7.2 at temperatures of (a) 95 ^{0}C, (b) 70 ^{0}C using biosensor and (c) 80 ^{o}C using biosensor at different scan rates (d) Water in blue drinking water bottle in 0.1 M PBS left for 5 days at RTP

Water in pink drinking water bottles which had been at a temperature of 70 ^{0}c then cooled and 0.1M phosphate buffer at a P H of 7.2 added to it were cycled at different scan rates. The results obtained were as shown in figure 4.21 b. From the results obtained using cyclic voltammetry, only one reduction peak was observed at +0.5 V and one reduction peak at + 0.2V. Linear calibration curve was drawn figure 4.21 (c) and it gave a linear response of R^2

= 0.9924 with a detection limit of 2.148 x 10 $^{-8}$M. The value obtained is very similar to that obtained when 0.001 M BPA in 0.1 M Phosphate buffer P H 7.2 was determined using the biosensor which was 2.1 x 10 $^{-9}$M .



(a)



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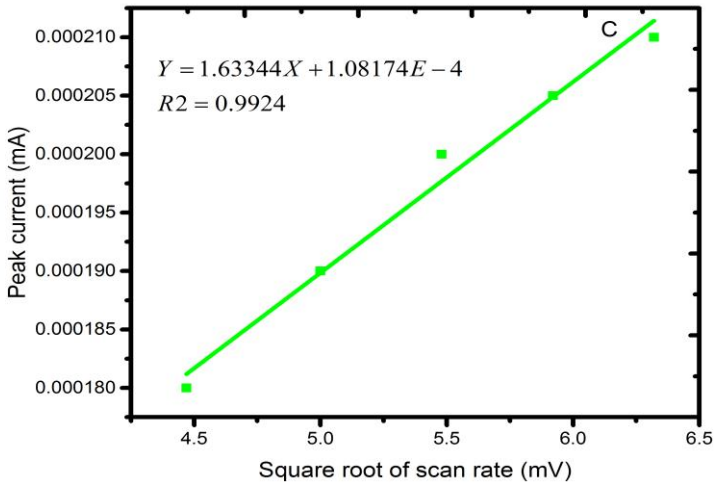
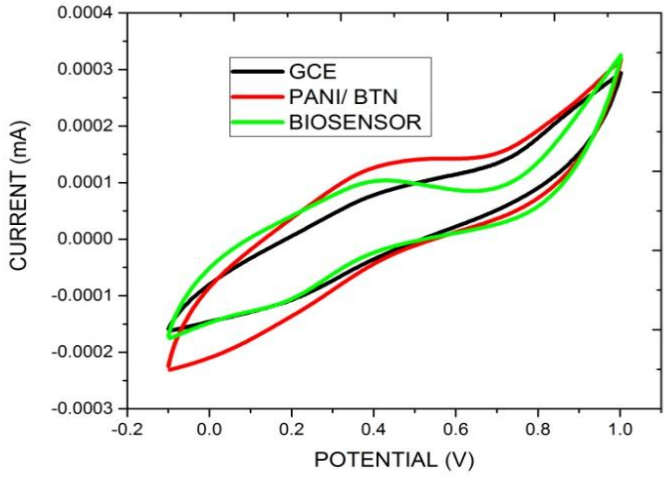


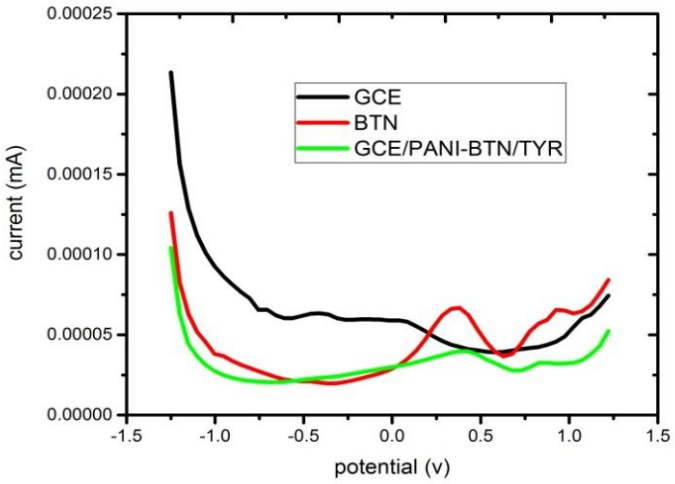
(c)

Figure 4.21: Water in pink drinking water bottle heated to 70 0c then cooled and mixed with 0.1 M PBS buffer at different scan rates (a) CV (b) SWV (c) SWV linear graph peak current verses scan rate.

BPA was also investigated in pink drinking water bottle using the biosensor and the results were compared to those obtained when GCE and PANI-BTN were used. The results obtained were shown in figure 4.22 (a). From the results obtained only one reduction peak at +0.48 V was observed using the biosensor and oxidation peak was obtained at approximately + 0.22 V with the biosensor. The reduction peak obtained using biosensor was at a lower current compared to when PANI-BTN was used. This was due to presence of the enzyme Tyrosinase and hence stronger enzyme binding and higher catalytic activity on bentonite modified GCE surface which lead to improvement in electrochemical behavior (Li *et al.*, 2005). There was no peak obtained when bare GCE was used. Similar results were obtained in voltammetric characterization of biosensor using 0.01M BPA in 0.1M phosphate buffer PH 7.2 figure 4.18.



(a)



(b)

Figure 4.22: (a) CV of water in pink drinking water bottle heated to 70 ^{0}C cooled and mixed with 0.1 M PBS buffer at a scan rate of 40 mVs^{-1} using GCE/ PANI-BTN and biosensor (b) DPV of water in green water bottle.

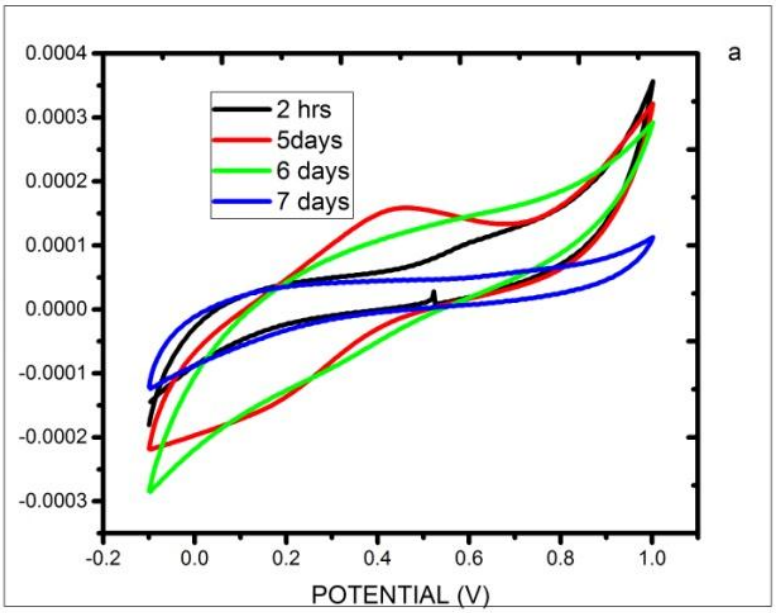
Detection and determination of BPA was also done using green drinking water bottle in which tap water was left in the plastic bottle starting from two hours for two weeks. 40 ml of the water was taken and mixed with 40 ml of 0.1 M PBS and the results obtained are shown in figure 4.23. From the results obtained, one reduction and oxidation peaks were obtained at +0.49V and +0.23 V respectively at day 5. Past day five there was no further increase in the peak current. This could have been attributed to high concentration of BPA in water, BPA was the substrate and hence its high concentration in water occupied the active sites of the Tyrosinase enzyme leading to no further increase of the peak current figure 4.23 (a and e).

BPA was determined in water contained in the same green bottle on the third day using DPV and CV. The results obtained for DPV showed one reduction peak at +0.1 V and one oxidation peak at +0.5V. The results obtained for CV were as shown in figure 4.23 (c) below. From the results obtained, one reduction peak was obtained at +0.5V with a shifts to the positive potential. Table 4.3 shows Shift in potentials of water at 70 ^{0}C in pink drinking water bottle in fig 4.23 a.

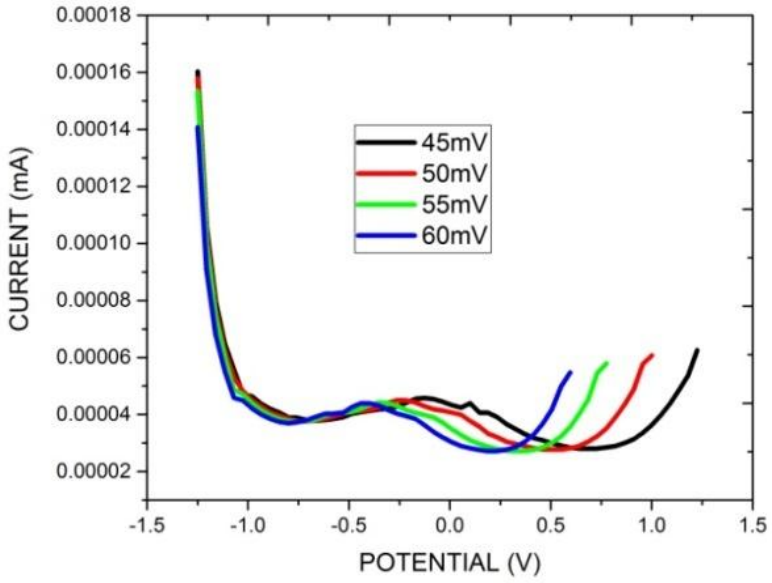
Table 4.3: Shift in potentials of water at 70 0C in pink drinking water bottle.

Surface	**GCE**	**PANI/BTN**	**BIOSENSOR**
Reduction peak Potential (mV)	No peak observed	+0.4	+0.5

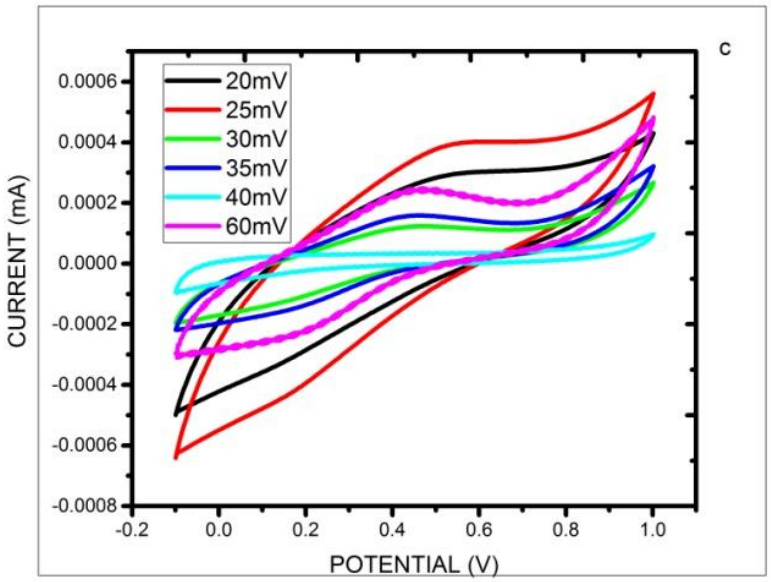
In addition, CV was also conducted for tap water at room temperature after it had been left to stay in the green bottle for five days at a scan rate of 40mV/s. 4.23 (d). The results obtained showed one reduction peak at approximately +0.51V and one oxidation peak at +0.22V. Figure 4.23 (e) shows linear calibration curve of DPV with R^2 value of 0.999. This value is similar to the value obtained when the biosensor was used to determine the presence of known concentration of BPA which was used as substrate fig 4.17 (e) whose R^2 value was 0.995. The detection limit for BPA in the green water bottle by the developed biosensor was calculated and found to be 0.03 mM.


a
2 hrs
5days
6 days
7 days
CURRENT (mA)
POTENTIAL (V)
0.0004
0.0003
0.0002
0.0001
0.0000
-0.0001
-0.0002
-0.0003
-0.2
0.0
0.2
0.4
0.6
0.8
1.0

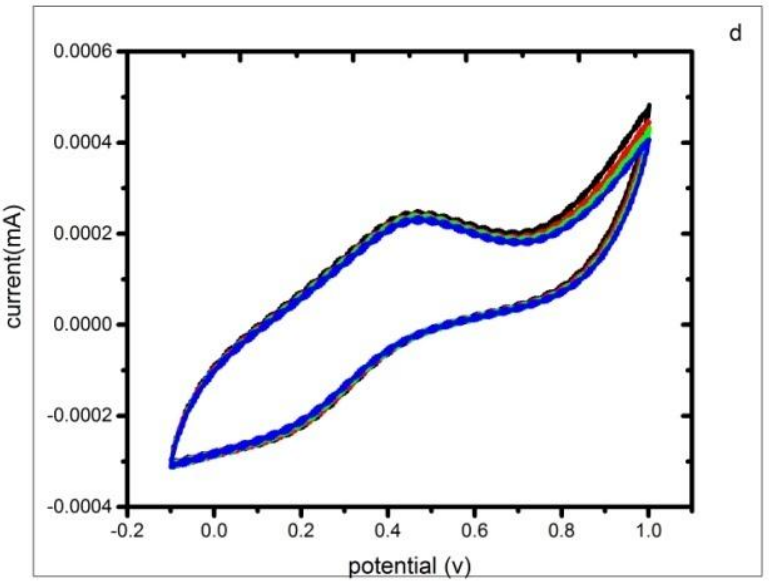
(a)


45mV
50mV
55mV
60mV
CURRENT (mA)
POTENTIAL (V)
0.00018
0.00016
0.00014
0.00012
0.00010
0.00008
0.00006
0.00004
0.00002
-1.5
-1.0
-0.5
0.0
0.5
1.0
1.5

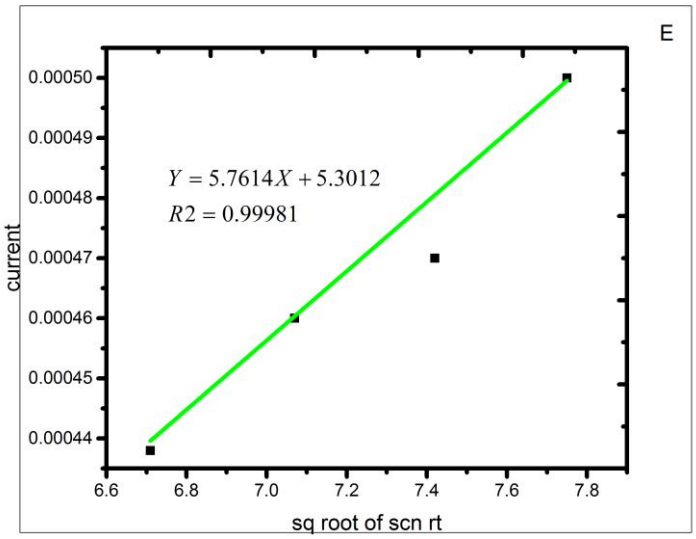
(b)


c
20mV
25mV
30mV
35mV
40mV
60mV
CURRENT (mA)
POTENTIAL (V)
0.0006
0.0004
0.0002
0.0000
-0.0002
-0.0004
-0.0006
-0.0008
-0.2
0.0
0.2
0.4
0.6
0.8
1.0

(c)


d
current(mA)
potential (v)
0.0006
0.0004
0.0002
0.0000
-0.0002
-0.0004
-0.2
0.0
0.2
0.4
0.6
0.8
1.0

(d)



(e)

Figure 4.23 (a) Tap water at room temp using biosensor between 2 hours- 7 days (b) DPV of tap water after 3 days in green bottle at different scan rates (C) Tap water at room temp using biosensor at day three at different scan rates (d) tap water at room temp using biosensor at day 5 at a scan rate of 40 mV/s (e) peak current verses square root of scan rate DPV of tap water after 3 days in green bottle

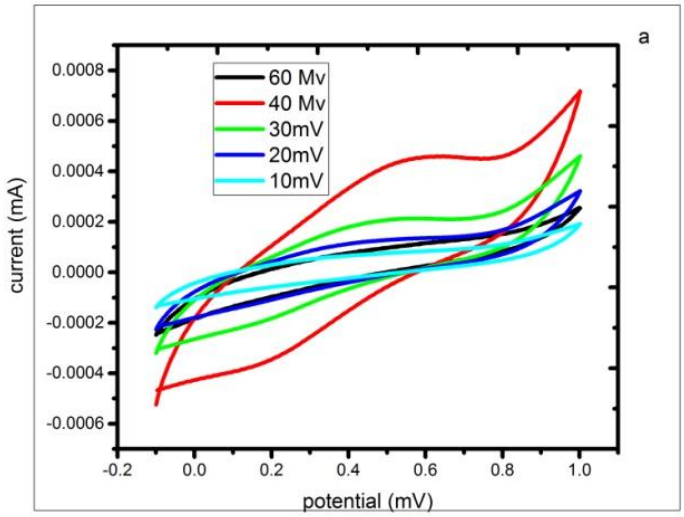
4.8.2 Detection of BPA in baby bottles.

Two different types of feeding baby bottles (green and pink) were investigated for the presence of BPA using cyclic voltammetry, square wave voltammetry and differential pulse voltammetry. The electrochemical detection of BPA in feeding baby bottles was investigated using the biosensor constructed. This was done by addition of 40 ml of previously heated water at temperatures of 50° C, 60° C, 70° C and 95°C then the temperature maintained for five minutes and cooled to room temperature to 40 ml, 0.1M phosphate buffer pH 7.2.

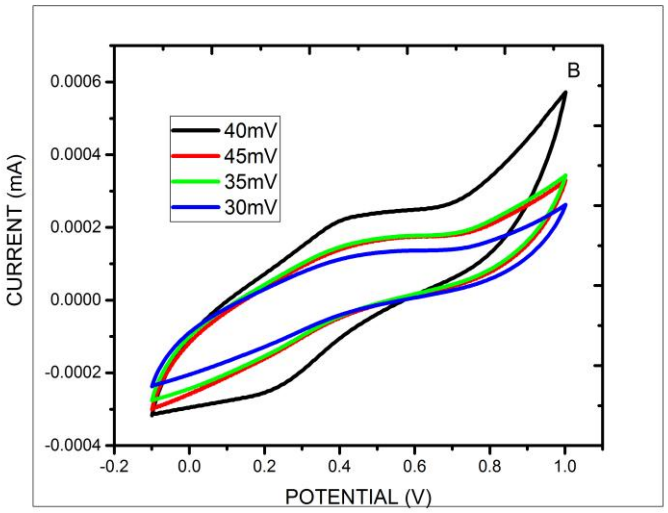
Cyclic voltammetry for green feeding baby bottle was achieved by cycling the potential repeatedly between -0. 1 and +1.0 V at scan rates of 10, 20, 30, 40, and 60 mV s^{-1}. Only one reduction peak was observed at potential of +0.50 V at a current of 0.0004 mA and one oxidation peak at + 0.21V at a scan rate of 40 mV s-1. The peak current increased with increase in scan rate and decreased at high scan rate, figure 4.26 (a) and (b) below. This behavior was also observed in determination of BPA in figure 4.17 (c). A linear plot of peak current verses square root of scan rate (figure 4.26 (c)) shows a linear relationship with a correlation coefficient of 0.953 obeying the equation:

I_p = 1.5733 µA L mol^{-1} x [BPA] + 7.4908. (Equation 3.5)

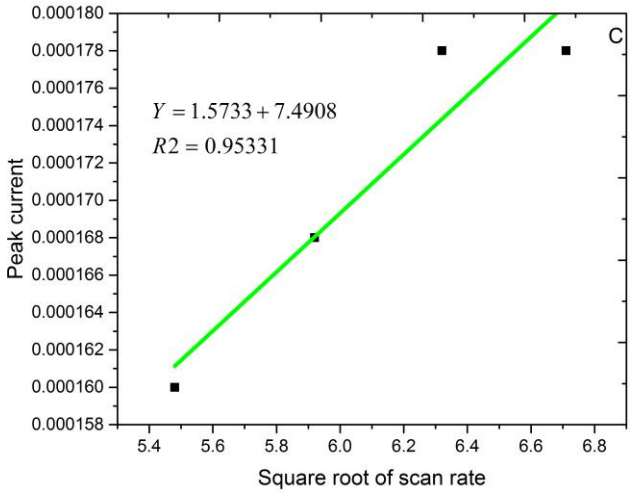
The formula 3σ/slope was employed to calculate detection limit, where σ is the standard deviation of the blank. Under the optimized conditions, the detection limit of water in green feeding baby bottle was 3.112 x 10^{-10} M. The detection limit was found to be in good agreement with those reported in literature by Del Olmol *et al.*, 1997 which was 2.6 x 10^{-9} M for BPA determination in water samples using GC- MS technique, (Jiang *et al.*2011) which was 2.5 x 10^{-9} M for determination of BPA in water samples using HPLC-MS technique and this study where detection value when 0.001M BPA in 0.1M phosphate buffer was determined it was 3.1 x 10^{-8}M· .



(a)



(b)

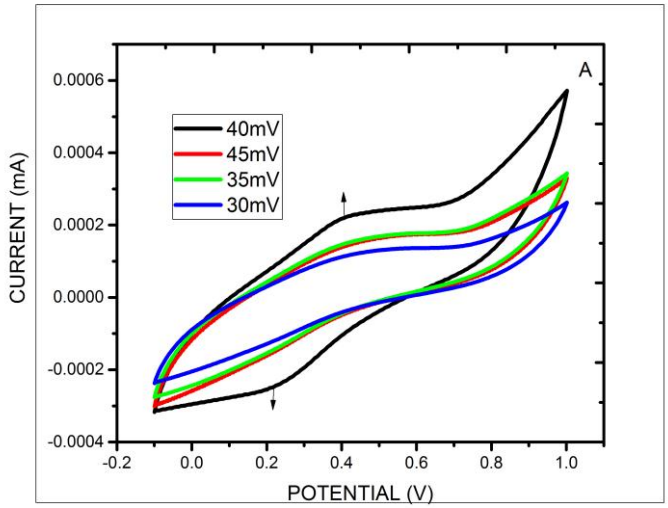


(c)

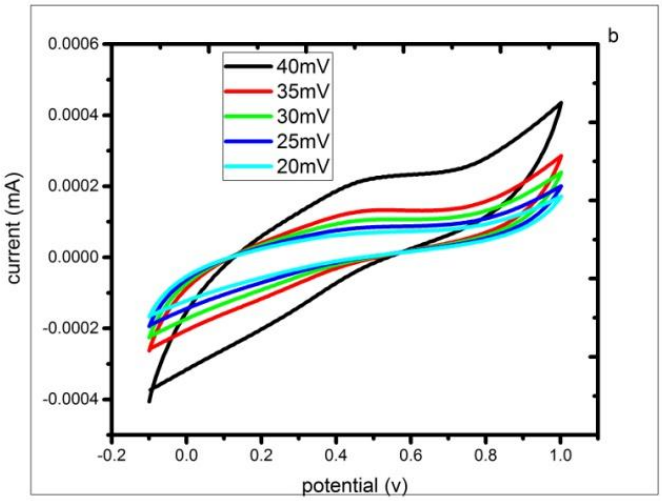
Figure: 4.26 (a) CV of water at 70 ºC in green baby bottle in 0.1m PBS pH 7.2 using biosensor at different scan rate (b) water at 95 ºC in green baby bottle 0.1m PBS pH 7.2 using biosensor at different scan rates. (c) Peak current verses square root of scan rate

A linear calibration of CV of water at 95 ºC in green baby bottle 0.1m PBS pH 7.2 using biosensor at different scan rates shows increase in peak current with increase in scan rate.

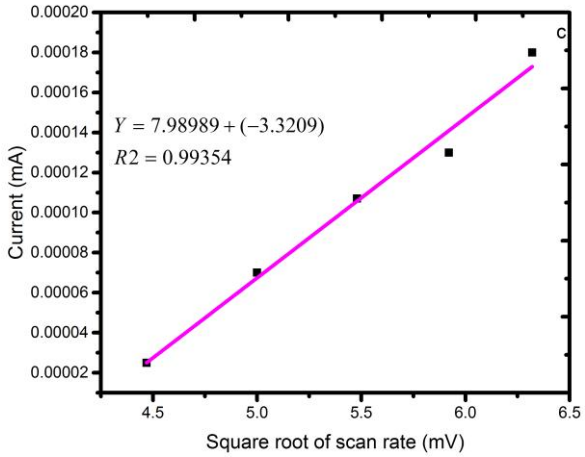
When BPA in pink baby bottle was determined using the biosensor at different scan rates, a pair of redox peaks were obtained. One reduction peak at +0.5V and one reduction peak was obtained at +0.22V. Clearer and more defined peaks were observed when the water was subjected to higher temperatures as shown in Figure: 4.27 (a) and (b) below. The correlation coefficient obtained when linear calibration curve of pink baby bottle at 70 ^{0}C was 0.993 and this value was in agreement with the previous studies done on determination of known concentration of BPA using biosensor fig 4.17 (c). The calculated detection limit of BPA in the pink baby bottle using the calibration curve figure 4.27 (c) was 1.977 x 10 $^{-8}$M.



(a)



(b)



(c)

Figure 4.27: Water in a pink baby bottle at a temperature of (a) 80 ^{0}C and (b) 70 ^{0}C using biosensor at different scan rates (c) linear plot of peak current verses scan rate of pink baby bottle at 70 ^{0}C

4.8.3 Comparison for the water bottles and the baby bottles.

A comparison was done for all the water bottles and the baby bottles using cyclic voltammetry, square wave voltammetry and differential pulse voltammetry. For cyclic voltammetry, this was achieved by cycling the potential repeatedly between -0. 1V and +1.0 V at a scan rate of 40 mV s^{-1}. The results obtained were as shown in figure 4.28 below**.** From the results obtained, one reduction peak was observed at + 0.48V with a shift to the positive potential. One oxidation peak was observed at +0.23V. The CV results obtained were found to be similar to those obtained earlier on voltammetric characterization of biosensor using a concentration of 0.002M BPA in 0.1M PBS at a pH 7.2. From the results obtained, some BPA leaked into the water from plastic bottles when they were subjected to high temperatures.

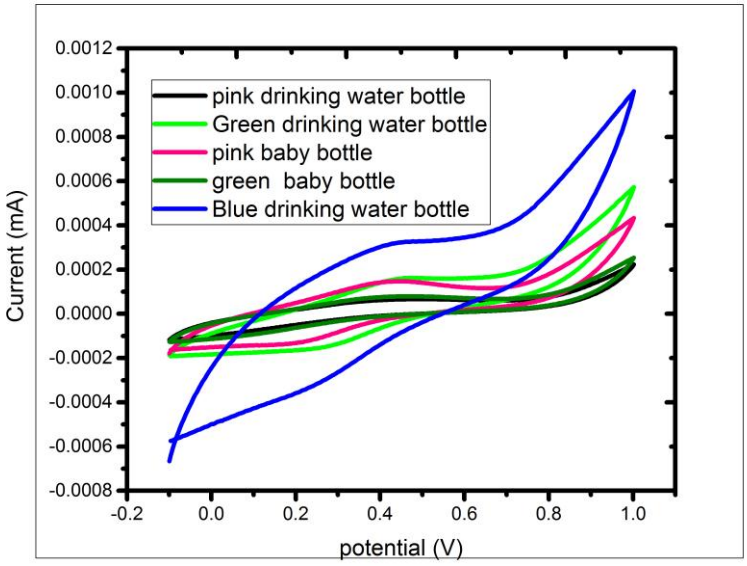
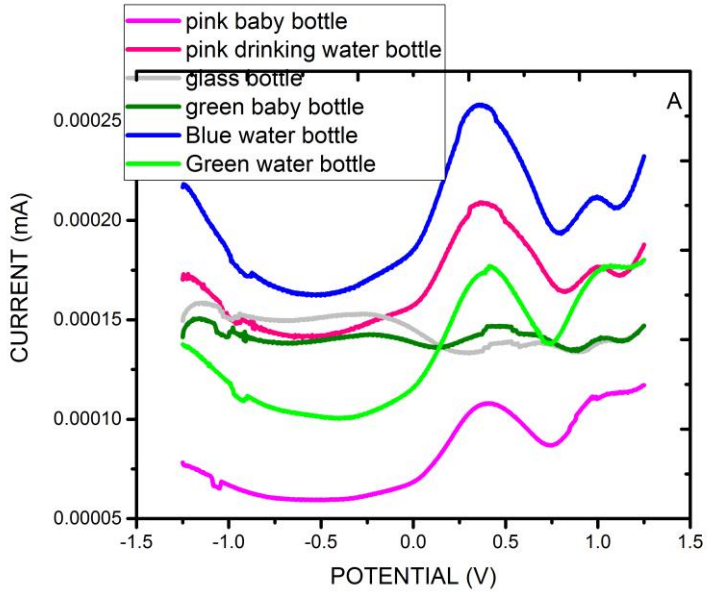


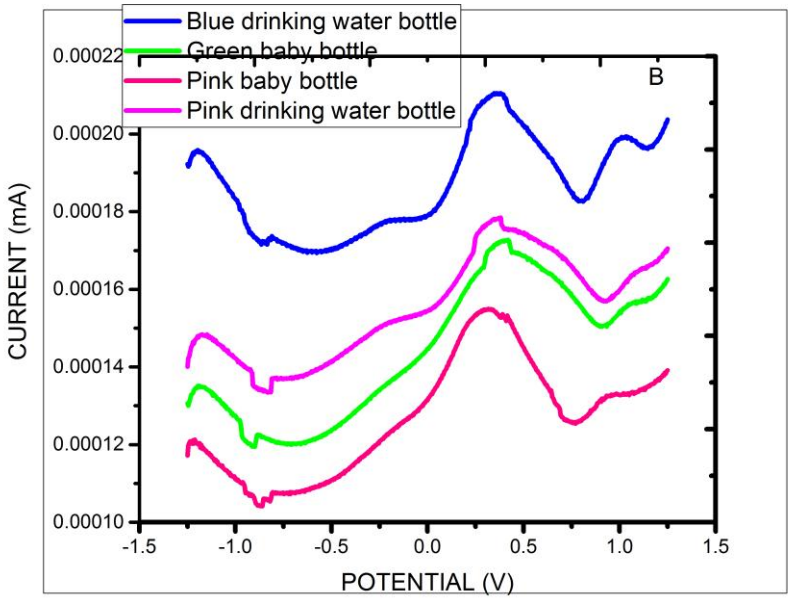
Figure: 4.28: Comparison of water in water bottles and baby bottle at a temperature of 50 ^{0} c then water left to cool and mixed with 0.1 M PBS buffer.

Figure 4.29 (a) and (b) illustrates the SWV results for the electrochemical behavior of BPA using the biosensor as a result of addition of water at a temperature of 70^{0}C and 50 ^{0}C into different bottles then letting it to cool in the bottles. 40 ml of the water was then added into 40 ml of 0.1 M phosphate buffer. The study was performed at a potential range of -1.2V to +1.2 V. The SWV results for water at a temperature of 70^{0} C showed one reduction peak centered at +0.50V while at a temperature of 50 ^{0}C the reduction peak centered at +0.49 V.

There was no peak observed when tap water was put in glass flask and 0.1M PBS buffer added was investigated. This showed specificity of the biosensor in detecting BPA since BPA is not used to make glass .This results obtained are similar to those observed when CV was used and those obtained by Matyholo *et al* (2011).



(a)



(b)

Figure: 4.29: SWV of different drinking water bottles and feeding baby at a temperature of (a) 70˚ C (b) 50 ˚C using biosensor (GCE/ PANI- BTN/SLS/TRY).

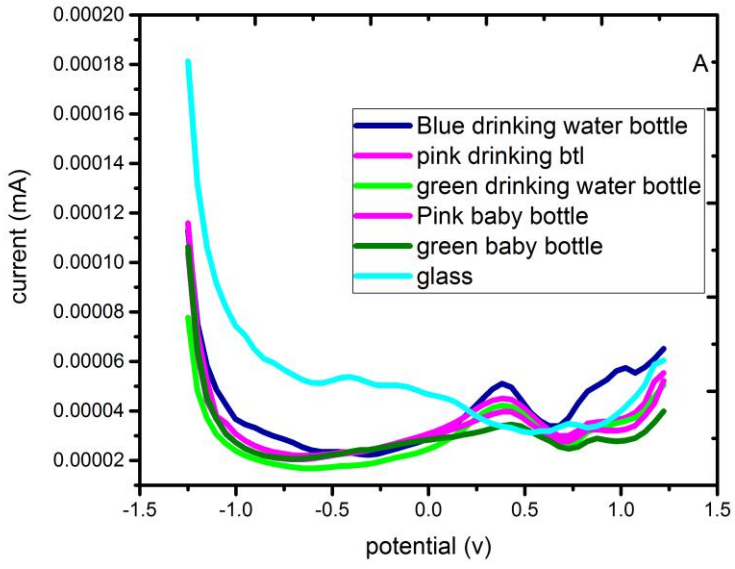
The results for peak current for square wave voltammetry (figure 4.29 (a))using the five bottles used in this study at a temperature of 70˚ C using biosensor were plotted as shown in table 4.4 below. From the results obtained, the blue water bottle had the highest peak current

of 0.00025(m A). These results indicated that the blue water bottle had the highest amount of BPA concentration that leached in the water.

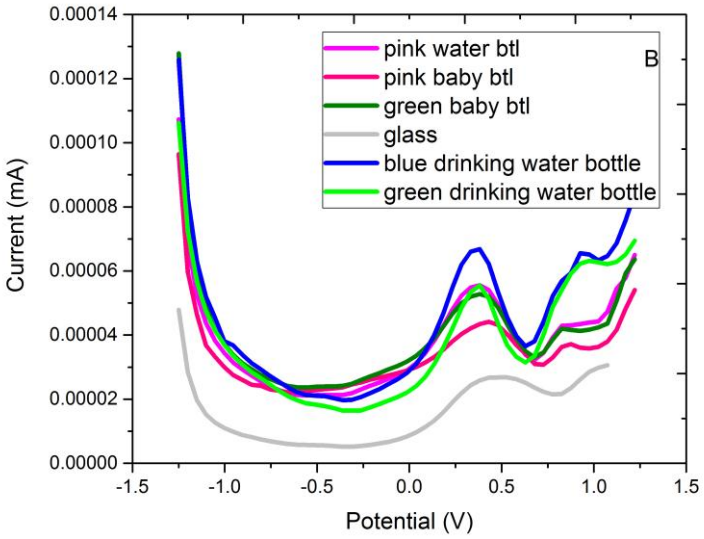
Table 4.4: peak current for square wave voltammetry (figure 4.29 (a)) for different bottles using biosensor.

Type of bottle	**Peak current (mA)**
Pink baby bottle	0.00012
Pink drinking water bottle	0.00022
Green baby bottle	0.00015
Blue water bottle	0.00025
Green water bottle	0.00018

Figure 4.30 (a) and (b) illustrates the DPV results for the electrochemical behavior of BPA in drinking water bottles and feeding baby bottles using (a) GCE/ PANI-BTN/SLS/TRY (b) PANI-BTN at a temperature of 70° C. The study was performed at a potential range of -1.2V to 1.2 V.



(a)



(b)

Figure: 4.30: DPV of different drinking water bottles using (a) PANI-BTN (b) GCE/ PANI-BTN/SLS/TRY (biosensor).

The DPV results for both GCE/ PANI-BTN/SLS/TRY and PANI-BTN showed one reduction peak centered at +0.49 V with slightly shift in peak potentials to more positive potentials. The peak current of GCE/ PANI- BTN/SLS/TRY is higher than that of PANI-BTN The increase in reduction peaks current of PANI-BTN confirms successful electrodeposition of different layers onto electrode surface.

From the study this results were in agreement with other studies which reported the presence of plasticizer residues in water stored in bottles (Tokunaga *et al.*, 2008). This could be attributed to the migration of BPA from the bottle material to the water since bottle quality may vary depending on the raw material and the technology used in bottle production (Amiridou *et al.*, 2011). Cross contamination during analytical procedure due to wide BPA in plastic industry could be another cause.

From this study, it can be concluded that, exposure to Bisphenol A can occur through ingestion of contaminated water and food. The ester bonds in BPA-based polymers found in drinking water bottles, feeding baby bottles, e.t.c are subject to hydrolysis. Heat and repeated washing of polycarbonate products have been shown to result in an increase in the rate of leaching of BPA (Vom-Saal *et al.*, 2008) and therefore it leaches into food and drinks from their storage containers.

CHAPTER 5: CONCLUSION AND RECOMMENDATIONS

5.1 Conclusion

In this work, a biosensor for determination of Bisphenol A (hereafter referred to as BPA) was constructed using glassy carbon electrode (GCE), bentonite (BTN) Sodium lauryl sulphate (SLS) and Tyrosinase (TRY). The bentonite and the enzyme were drop-coated on the GCE using an electrochemically inert adhesive. Biosensor characterization and application was performed using various methods such as cyclic voltammetry, square wave voltammetry and differential pulse voltammetry. The optimum activation pH for Tyrosinase enzyme was obtained at pH 7.2 proving that Tyrosinase worked best at neutral pH.

Detection and quantification of BPA was achieved by the developed Try/PANI-BTN /SLS/GCE biosensor. However bare glassy carbon electrode was identified to be able to detect BPA but the down fall was fouling meaning BPA polymerized on GCE surface blocking electron transfer process and the detection potential at which it was responding was very wide. The detection using of BPA Try/ PANI-BTN/SLS/GCE biosensor was the most preferred due to much lower detection limit of 0.02mM than those obtained in bare electrode. Tyrosinase based biosensor, Try/PANI-BTN/ SLS/ GCE showed good bio electrochemical response to BPA detection with the characteristics of a usual enzyme-catalyzed reaction.

The biosensor constructed was used to detect BPA in drinking water bottles and feeding baby bottles using the above named methods, by adding water containing zero BPA to the bottles at varying temperatures and time intervals and assessing the presence of BPA in the water at the end of the experiment. A control was set up by placing the water in glass bottles at similar conditions. When the results obtained of detection of BPA using the sample bottles were compared to those obtained using different concentrations of BPA the results showed that the drinking water bottles and feeding baby bottles had detectable amounts of BPA which included, 0.030mM, 0.021 mM and 0.035mM for green, pink and blue drinking water bottles respectively and 0.019mM and 0.030 mM green and pink feeding baby bottles respectively that had leached into the water at elevated temperatures from 50°C. The results also showed that when water at room temperature was left in drinking water bottle, after 3 days at optimum conditions some concentration of BPA also leaked to the water.

Under the optimum conditions (25°C, pH 7.2, 0.4 V), the Try/ SLS/PANI-BTN /GCE displayed rapid response, good sensitivity, good stability and reproducibility.

The aim of the study was to synthesize a novel nanostructured biosensor which is , highly sensitive, cheap and fast response using polyaniline, surfactant (SLS) and carbon modified electrode with bentonite clay (BTN) for quantitative determination, analysis and characterization of bisphenol A. The biosensor developed was to be used for detect BPA in drinking water bottles and in feeding baby bottles and the developed Try/ PANI/ SLS-BTN/GCE biosensor certified the study aim. The fabrication material cost was very low and preparation of this biosensor was less time consuming and it produced reliable results which are reproducible. The availability of the fabrication material and low cost of Try/ PANI-BTN/SLS /GCE biosensor makes it affordable when compared to other methods.

5.2: Recommendations.

The biosensor parameters should be investigated further and optimized in order to achieve lower detection limits. The applicability of the developed biosensor should be determined by analyzing more real samples in the market.

Researchers could adopt the methodology and optimization parameters investigated in this work to obtain a portable BPA detector for analysis of samples in the market.

The results obtained from this study could be used as a baseline to monitor and eliminate the use of BPA-containing substances.

Using the results from real samples obtained in this work, the government could sensitize industries on use of BPA in plastic bottles and establish laws that eliminate use of BPA-containing substances. The members of the public should be sensitized on the dangers of using substances made from BPA and advised to use containers that are BPA-free.

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APPENDIXES

Appendix 1: Calculations involved for preparation of aniline

Molar weight of aniline = 93.13 g

% purity (minimum assay) of aniline = 98.5%

Density of aniline = 1.0217 g/ ml

If 1 mole of aniline = 93.139 with % purity of 98.5%

Thus 100%

(100%/98.5%) × 93.13 = 94. 5482 g

1Mole of aniline contains 94. 5482 g

Thus 0.1 moles will contain 94. 5482 g× 0.1 =9. 45482 g

1mole of aniline has a density of 1.0217 g/ml

9. 45482 g

(9. 45482 g/1.0217 g/ml) × 1 = 9.254 ml

9.254 ml are in 1000 ml

? 100

9.254 ml × (100/1000) **= 0.93ml**.

Appendix 2: Definition of Significant Terms

Bisphenol A: commonly abbreviated as **BPA**, is an organic compound with two phenol functional groups. It is used to make polycarbonate plastics found in numerous commercial products, including laptops, cell phones, baby bottles, water main pipes, and laboratory and hospital equipment for example dental fillings sealants, food containers and epoxy resins.

Biosensor: this is an analytical device, used for the detection of an analyte that combines a biological component with a physicochemical detector.

Cyclic voltammetry: This is an electrolytic method that utilizes microelectrodes and an unstirred solution so that measured current is limited by analyte diffusion at the electrode surface (Arya, 2008).

Differential pulse voltammetry: This is an electrochemical technique in which potential is scanned with a series of pulses. Each potential pulse is fixed, of small amplitude (10 to 100 mV), and is superimposed on a slowly changing base potential (Bernhardt, 2006).

Endocrine disruptor: a substance that possesses properties which are suspected to cause endocrine disruption in an intact organism (Propper, 2005).

Estrogens: Estrogens are hormones that are important for sexual and reproductive development, mainly in women. They are also referred to as female sex hormones. The term "estrogen" refers to all of the chemically similar hormones in this group, which are estrone, estradiol (primary in women of reproductive age) and estriol.

Endocrine disrupting compounds: exogenous substances or mixtures that alter the function(s) of the endocrine systems and consequently causing adverse health effects in an intact organism, its progeny or to (sub) populations (OECD, 1998).)

Glassy carbon electrode: This is polished electrodes used for the analysis of organic molecules.

Monitoring gadget: this is a device that will be used to check or observe the levels of Bisphenol A **.**

Polyaniline: Polyaniline (PANI) is a conducting polymer easily processed by melt or solution process, and is environmentally and thermally stable.

Polymeric: from the word polymer, these are substances of compounds having the same elements combined in the same proportion but different molecular weights.

Square wave voltammetry: This is a form of linear potential sweep voltammetry which has found numerous applications in various fields such as medicine and in sensors.

Tyrosinase enzyme: This is a blue copper protein with two copper atoms in active centers, which can be considered as a polyphenol oxidase (PPO) (Viventini, 2013).