AN INVESTIGATION OF THE VOLTAMMETRIC BEHAVIOUR OF ANTIOXIDANTS IN FLAVONOIDS

By

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Submitted in fulfilment of the requirements for the degree of Master of Applied Science in Chemistry in the Faculty of Applied Sciences at the Durban University of Technology

April 2020

DECLARATION

I, Lee-Ann Ramsarup, hereby declare that the work presented in this thesis entitle “An Investigation of the Voltammetric Behaviour of Antioxidants in Flavonoids” has not been submitted to any other University or Institution for any degree or examination. This is my own work and where the work of others was used, it was to the best of my knowledge accurately reported and duly acknowledged.

Signature of Lee-Ann Ramsarup (Student) Date

Signature of Prof Krishna Bisetty (Supervisor) Date

Signature of Dr Suvardhan Kanchi (Co- Supervisor) Date

DEDICATION

This work is dedicated to my late uncle, Gayson Samuel. I am thankful for his presence and support throughout my life. His memory lives on forever.

ACKNOWLEDGMENTS

All that I have and all that I am is because of my Lord and Saviour Jesus Christ. All honour and glory to Him for giving me the strength and faith to see this project to completion.

I wish to express my sincere appreciation to my Supervisor, Prof Krishna Bisetty for his assistance and constant motivation throughout my research.

I extend my heartfelt appreciation to my Co-Supervisor, Dr Suvardhan Kanchi for his time, support and direction during this journey.

Special thanks to Dr Myalo I Sabela (Lecturer) and Nokukhanya Xhakaza (Technician) for assisting me with their time and experience during the experimental phase of the research work.

I would also like to thank the management and fellow colleagues at Metrohm South Africa for their unwavering support and understanding.

To my parents, Pat and Ashok Ramsarup, my brother Karl and Narintha, I am thankful for your prayers and support. I have learned to be strong and courageous because of you.

I am eternally thankful to my husband, Dr Viren Chunilall and my son Anvir. Thank you both for being supportive, encouraging and understanding throughout this process. Your dedication and love for me is priceless. Thank you for the inspiration to make each day memorable.

ABSTRACT

The two case studies in this work involve the development and fabrication of an electrochemical biosensor using various enzymes for the evaluation of the electrochemical responses, relating to the total phenolic (TP) content and the antioxidant activities in wine and tea samples respectively. The modification of the glassy carbon electrode (GCE) was carried out using green apple as an enzymatic source of polyphenol oxidase and laccase enzyme. The experimental variables were optimized using the Box-Behnken experimental design as a predictive model, for a better understanding of the parameters and their interaction responses with each other during an electrochemical analysis. This multivariate optimization method is based on a factorial design, where the three most influential factors include the electrolyte pH, the deposition time (t_d) and the scan rate (s_r). The design was run in a single block fashion while random order of experiment was selected to provide greater protection against the effects of outlying variables. The optimized results obtained yielded the most suitable conditions for the determination of the TP content in wine samples. They were selected as follows: phosphate buffer of pH 7.65 as supporting electrolyte, t_d 29.8 s and s_r 25.0 mV/s respectively. The method was optimized for the current signal at a deposition potential of 0.2 V and within an oxidation potential of -0.2 V to 0.6 V. Good analytical responses were obtained with apple sensors for the detection of TP content in wine samples, with a higher concentration in red wines than in white wines.

Differential pulse voltammetry (DPV) and cyclic voltammetry (CV) were used to establish and interpret the redox mechanisms of flavonoids present in alcoholic and non-alcoholic beverages. The sensor responses were evaluated by first, investigating the changes in the total phenolic (TP) content in wine samples using catechin as a standard. Thereafter, the electrochemical behaviour of rutin and ascorbic acid, antioxidant capacities (trolox reagents) were established in tea samples, yielding a positive linear correlation between the trolox equivalent antioxidant capacities (TEAC) and TP content (R^2 = 0.9812 ± 0.012). DPV was applied to the laccase modified GCE, and the experimental results indicate that this sensor shows good reducing properties. The scavenging ability of 2,2'-Azino-*bis*(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) a diammonium salt, was assessed in the sample extracts, which yielded half-maximal effective concentration (EC_{50}) values of 10.80 µg/ml and 11.62 µg/ml for ascorbic acid and rutin respectively. These findings indicated that the experimental design was a convenient method to evaluate the statistical significance of the optimised parameters, and the positive linearity for the TEAC and the TP content confirms the robustness of this methodology.

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ACRONYMS

AA	antioxidant activity
AAPH	2,2-azobis 2-amidopropane dihydrochloride
ABTS	2,2'-Azino-*bis*(3-ethylbenzothiazoline-6-sulfonic acid)
Au	Gold
BBD	Box Behnken design
CNTs	carbon nanotubes
CP	Conducting polymers
CV	cyclic voltammetry
Cyt-C	Cytochrome C
DMSO	dimethyl sulphoxide
DPPH	1, 1 diphenyl 2, picryl hydrazyl
DPV	differential pulse voltammetry
EC_{50}	effective concentration
ECD	electrochemical detection
E*pa*	Anodic peak potential
E*pc*	Cathodic peak potential
FAD	Flavin-adenine dinucleotide
FIA	Flame Ionization Analysis
GOD	Glucose Oxidase
GCE	glassy carbon electrode
H_2O_2	hydrogen peroxide
HMDE	hanging mercury drop
HPLC	High performance Liquid chromatography
HRP	Horseradish Peroxidase

HX	hypoxanthine
*IC*50	50 % inhibition
LDL	Low density lipoprotein
NBT	Nitro Blue tetrazolium
NMR	nuclear magnetic resonance
ORAC	Oxygen Radical Absorbance Capacity
PB	Phosphate buffer
PANi	Polyaniline
PVC	polyvinylchloride
PVS	polyvinylsulfonic acid
ROS	Reactive Oxygen Species
SAM	Self assembled monolayer
SOD	Superoxide dismutase
s_r	scan rate
t_d	deposition time
TE	Trolox Equivalent
TEAC	trolox equivalent antioxidant capacities
TP	Total phenolic content
XOD	Xanthine oxidase

RESEARCH OUTPUTS

The findings of this dissertation resulted in one published manuscript:

K. Bisetty, M. I. Sabela, S. Khulu, M. Xhakaza and L. Ramsarup, "Multivariate Optimization of Voltammetric Parameters for the Determination of Total Polyphenolic Content in Wine Samples Using an Immobilized Biosensor", *International Journal of Electrochemical Science*, **2011**, 6: 3631 – 3643.

CHAPTER 1

INTRODUCTION

This chapter provides an overview of flavonoids with particular emphasis on the antioxidant activities in alcoholic and non-alcoholic beverages using electrochemical techniques. The aim and objectives of this work are also presented followed by a brief outline of this thesis.

1.1 Flavonoids

Flavonoids are phenolic substances widely found in nature and are known for their health benefits when consumed. They occur in processed and non-processed food items and form part of a class of polyphenols generally containing two or more aromatic rings, a phenyl ring- A and a heterocyclic ring- C connected through a C-C bridge to an aromatic- B ring as shown below in Fig 1.1.

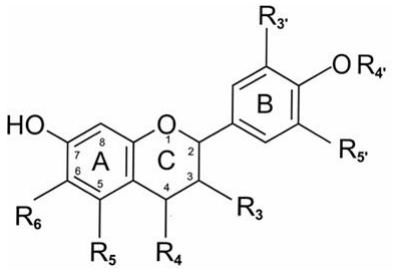

Figure 1. 1: General structure and numbering pattern for common food flavonoids (Beecher 2003).

Flavonoids classified as flavonols, flavanones, flavones, isoflavones, flavonols and anthocyanidins are based on their differences in molecular backbone structure (Beecher 2003; Harborne, Marby and Marby 2013). The configuration and the total number of hydroxyl groups greatly influences the mechanism of the antioxidant activity.

The flavone apigenin and quercetin flavonols are present in many fruits and vegetables, such as apples, berries and broccoli. Naringenin is a flavanone with citrus properties. Catechin and other flavanol polyphenols are found in green tea. Anthocyanidins are mainly responsible for the deep colors found in grapes and red wine. Genistein belonging to the isoflavone subclass

is found in legumes. The flavonoid thought to be most consumed is catechin, which is the main focus of this research.

1.2 Antioxidants

Energy-producing processes in cells generate oxy radicals and Reactive Oxygen Species (ROS). Antioxidants are substances that inhibit oxidation and guard the body against the damaging effects of free radicals. Enzymes such as catalase, glutathione peroxidase and superoxide dismutase as well as non-enzymatic counterparts such as vitamin C, vitamin E and glutathione are naturally occurring defence mechanisms within the body. We are required to increase our uptake of antioxidant-rich foods as the antioxidants produced in the body are not enough to fight off all free radicals produced (Patel 2008). Oxidation constitutes one of the main factors in the deterioration of food products as well.

In recent studies free radical scavenging activity of flavones, anthocyanins and particularly flavonols have been outlined through a variety of *in vitro* models (Afanasev *et al.* 1989; Formica and Regelson 1995; Pietri *et al.* 1997; Dobsak *et al.* 1999; Duthie and Dobson 1999; Kerr, Suleiman and Halestrap 1999; Cui *et al.* 2002; Pataki *et al.* 2002; Yamashiro *et al.* 2003; Mahesh and Menon 2004). Flavonoids have the capacity to act as antioxidants. Based on their structure, oligomeric compounds exhibit a higher number of target sites for free radicals produced from semiquinone radicals (Robak and Gryglewski 1988; Bors and Michel 1999; Bors, Michel and Stettmaier 2000; Rohdewald 2002).

Displaying good antioxidant potentials in *in vitro* cell culture and being single electron donors, flavonoids are a derivative of conjugated ring structures with hydroxyl groups incorporating antioxidant behavior by scavenging superoxide anions (Husain, Cillard and Cillard 1987), lipid peroxyl radicals (Fuchs *et al.* 1989; Lotito and Frei 2004) and singlet oxygen constituents (Wang and Goodman 1999).

Flavonoids also have the ability to enhance intracellular antioxidant defence against free radicals with the rapid generation of antioxidative enzymes (Kandaswami and Middleton 1994; Wei, Peng and Lau 1997; Bayeta and Lau 2000; Lewis 2017). Flavonoids are involved in enzyme inhibition particularly responsible for superoxide anion generation such as protein kinase C (Ursini *et al.* 1994) and xanthine oxidase (Hanasaki, Ogawa and Fukui 1994). Additionally enzymes involved in ROS production such as lipoxygenase, cyclooxygenase,

glutathione S-transferase, mitochondrial succinoxidase, microsomal monooxygenase and NADH oxidase all undergo flavonoid inhibition (Korkina and Afanas' Ev 1996; Brown *et al.* 1998).

Flavones and Catechins are found to be the most powerful flavonoids in exhibiting antioxidant behaviour and are known for protecting the body against ROS by direct scavenging of free radicals. As a result of their thermodynamic properties and lower redox potentials (Jovanovic *et al.* 1994), flavonoids are able to reduce extremely oxidizing free radicals such as peroxyl, hydroxyl, alkoxyl and superoxide radicals by hydrogen atom donation: where, R$^{\bullet}$ represents hydroxyl, peroxyl, alkoxyl and radicals and superoxide anion (Torel, Cillard and Cillard 1986; Husain, Cillard and Cillard 1987; Robak and Gryglewski 1988) with redox potentials between 2.13-1.0 V (Buettner 1993). The aroxyl radical may react with a second radical, acquiring a stable quinone structure during the oxidation process, as seen in Fig 1.2.

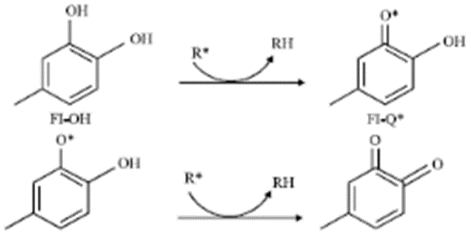

Figure 1. 2: Scavenging of ROS (R*) by flavonoids (Torel, Cillard and Cillard 1986; Husain, Cillard and Cillard 1987; Robak and Gryglewski 1988).

Flavonoids stabilize the ROS by reacting with a reactive compound of the radical and due to the high reactivity of the hydroxyl group of the flavonoids, radicals are rendered inactive. Epicatechin and rutin are also powerful radical scavengers (Nijveldt *et al.* 2001).

The antioxidant activity of polyphenolic compounds has extensively been researched and evaluated due to flavonoid scavenging ability by several researchers as can be seen in the review by Lotito and co-workers (Lotito and Frei 2006). It is noted that a correlation between antioxidant properties and the total polyphenolic content exists (Javanmardi *et al.* 2003).

Flavanols present in tea and wine products are responsible for terminating chain reactions and removing free radical intermediates. Other oxidation reactions are inhibited as flavanols undergo oxidation themselves (Bartolomé, Pena-Neira and Gómez-Cordovés 2000). There are strict guidelines enforced in the beverage industry, especially when wines are fortified as

excessive amounts of flavanols can result in cloudiness, thus affecting the quality of the wine (Zhao *et al.* 2010). Accordingly, there is a growing interest in developing sensitive and selective methods for quantification and detection of polyphenolic compounds. Several techniques have been used in the detection and characterization of antioxidant activities of polyphenols including, electrochemical detection (ECD) (Novak *et al.* 2008), nuclear magnetic resonance (NMR) (Pawlowska, Oleszek and Braca 2008), (Christophoridou and Dais 2009), and high performance liquid chromatography (HPLC) (Gambelli and Santaroni 2004; Aguirre *et al.* 2010; Wirth *et al.* 2010; Šeruga, Novak and Jakobek 2011) coupled with UV detector is the ideal and preferred method in the brewing industry. (+)-Catechin has been chosen for this study due to its key role in retarding oxidation processes and brewing processes (Mojica *et al.* 2007).

1.3 Problem Statement

Limitations of analysis from method to method exist based on previous studies, involving highly expensive instrumentation, with low detection, low sensitivity and low selectivity, which has led to the study and investigation of simple, fast, sensitive and cost-effective electroanalytical methods for the characterization of antioxidants and modification of biosensors.

1.4 Aim and Objectives

This study is aimed at the development of an electrochemical biosensor for the rapid assessment of antioxidant activity in flavonoids present in tea and wine samples.

The objective of this study is to:

1. Develop a multivariate optimization method (pH of the buffer, deposition time and scan rate) for the electrochemical detection of polyphenols.

2. Modify a glassy carbon electrode with apple paste and nujol oil for the detection of total phenolic content in wine samples

3. Fabricate a laccase/nujol oil/graphite immobilized electrochemical biosensor for the evaluation of antioxidants in tea samples

4. Evaluate the performance and sensitivity of the developed electrochemical biosensors

1.5 Thesis Outline

An overview of flavonoids with emphasis on antioxidant activity in alcoholic and non-alcoholic beverages is covered in chapter 1, further chapters in this work are presented as follows:

Chapter 2 describes the literature review of flavonoids, specifically focused on Catechin structure represented in Fig 2.1 and antioxidant properties shown in Fig 2.2. Electrochemical biosensors with emphasis on HRP modification processes can be seen in Fig 2.3 and 2.4 followed by electroanalytical techniques.

Chapter 3 outlines theoretical principles involved in this study, including cyclic voltammetry, differential pulse voltammetry with a graphical representation of a typical DPV for catechin in Fig 3.4 and amperometry. A focus on experimental design (multivariate optimization method) including a Box-Behnken design in Fig 3.6.

Chapter 4 presents Case Study 1 on the Multivariate Optimization procedure implemented for the determination of total polyphenolic content in wine samples using an apple paste modified glassy carbon electrode. Optimised voltammetric parameters using a Box Behnken experimental design, including the materials, instrumentation, electrode modification and real sample preparation with results and discussion will be discussed in this chapter.

Chapter 5 presents Case Study 2 whereby, a glassy carbon electrode modified with laccase is used for the quantification of antioxidant capacity in tea using ascorbic acid and rutin as Trolox equivalent antioxidant standards. Biosensor fabrication, including the materials, instrumentation, experimental optimization and real sample preparation with results and discussion will be covered in this chapter.

Chapter 6 provides a brief summary of case study 1 and case study 2 with concluding remarks.

CHAPTER 2

LITERATURE REVIEW

This chapter provides an overview of flavonoids, namely catechins, mainly aimed at determining the antioxidant activity in polyphenolic compounds by various electrochemical sensing methods. A review on electrochemical biosensors is also presented in relation to fabrication methods employed in this work using enzyme and polymer technology.

2.1 Catechin

Catechin belongs to the flavonoid subclass flavanols with tea flavanols being classified within the same compound group. Typically characterised as being colourless and water-soluble, tea flavanols are readily oxidised in basic solutions and highly reactive with various constituents (Larger, Jones and Dacombe 1998).

Catechin has been researched for its prevalence in traditional herbal remedies. Catechins and epicatechins can be found in cocoa (Kwik-Uribe and Bektash 2008) which is found to be in high concentration followed by prunes, peaches, vinegar and green tea (Galvez *et al.* 1994; Quinde-Axtell and Baik 2006; Pacheco-Palencia, Mertens-Talcott and Talcott 2008). (-)-Epicatechin and (+)-catechin are naturally occurring polyphenols in argan and acai oil (Cheng and Crisosto 1995). Barley grain is rich in this antioxidant; however, it contributes to dough discoloration in baking processes (Kielhorn and Thorngate 1999). A standard cup of tea contains between 100 to 2000 mg of phenolic compounds, typically consisting of catechins, flavonols and flavones, thearubigins and theaflavins polyphenols range from 100 to 2000 mg per capita per day (Lindsay 1999). Catechins in green tea consist of catechin, epicatechin, epigallocatechin, epicatechin gallate, epigallocatechin gallate representing between 80–90%. Flavonols consisting of kaempferol, quercetin and myricetin glycosides represent less than 10 % of total flavonoids (Balentine, Wiseman and Bouwens 1997). During the production of black tea, these polyphenolic components form complex condensation products (theaflavins, thearubigins).

Reduction properties of tea catechins are dependent on their ability to delocalize free electrons in addition to the presence of the B ring catechol moiety.

Most flavonoids occur structurally with a sugar molecule attached. However tea catechins do not resemble this feature. Structurally catechin in Fig 2.1 possesses two aromatic rings, namely A and B, along with a heterocyclic C -ring as many polyphenols exist. The A and B ring bear similarity to the resorcinol moiety and catechol moiety respectively. Carbons 2 and 3 possess two chiral centers yielding four diastereoisomers, two in *Trans* configuration and the other two in *Cis* configuration. Catechin isomers are in *Trans* configuration and epicatechin are in *Cis* configuration.

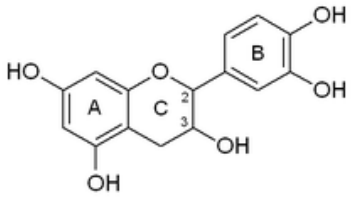

Figure 2. 1: Structure of catechin (Shankar, Ganapathy and Srivastava 2007).

Compared with other flavonoids, the antioxidant potential of catechins is low which can be related to their chemical structure based on the presence of the B ring catechol moiety and the double bond activation on the C ring by a hydroxyl group (Tournaire *et al.* 1993).

2.1.1 Antioxidant properties of catechins

Catechins are able to scavenge not only free radicals but also generate free radicals, thus representing their powerful effects as a result of combined mechanisms (Valko *et al.* 2007; Oliveira-Marques *et al.* 2009). The antioxidant potency of catechins is influenced through direct mechanisms such as ROS and chelating metal ions (Braicu *et al.* 2013) and indirect mechanisms whereby antioxidant enzymes are induced and or pro-oxidant enzymes are inhibited thus generating phase II detoxification enzymes as seen in Fig 2.2 (Cabrera et al., 2006).

Catechin diastereoisomers bear similar chemical structure containing phenolic hydroxyl groups which are involved in free radical stabilization, thus leading to direct antioxidant activity of catechins and their free radical scavenging properties (Fraga *et al.* 2010). The cycle of new radical generation is broken as the phenolic -OH groups of tea catechins react with nitrogen and oxygen species in a termination reaction as depicted in Fig 2.2. Free radicals are reduced as the aromatic group maintains stability through expected aroxyl radicals during catechin single electron donation of the phenolic hydroxyl group (Bors *et al.* 1990; Fan, Sang and Jiang

2017). The antioxidant radical is generated after reaction with the initial reactive species. This radical is stabilised by the interaction of -OH groups with the π –electrons in the aromatic ring (Parr and Bolwell 2000). Literature studies have revealed that the antioxidant potential of phenolic components is dependent on the arrangement of -OH groups along with the number and extent of conjugation in the structure, with the number of -OH groups in the molecule directly related to the antioxidant activity of phenolic components (Rice-evans *et al.* 1995), (Cao, Sofic and Prior 1997).

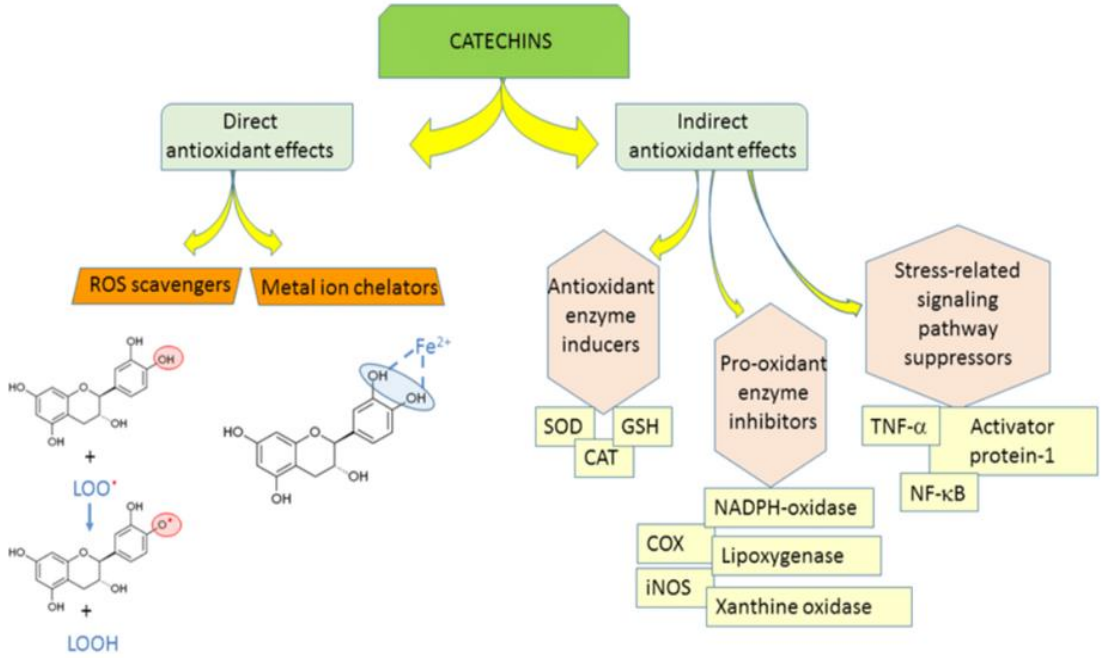

Figure 2. 2: Antioxidant properties of catechins.

Direct effects- ROS-reactive oxygen species, Metal ion chelators. Indirect effects- Antioxidant enzyme inducers SOD-superoxide dismutase. Pro-oxidant enzyme inhibitors NADPH-oxidase – nicotinamide adenine dinucleotide phosphate oxidase. Stress-related signalling suppressors TNF-α-tumor necrosis factor-alpha (Youn *et al.* 2006).

Catechins are able to stop radical chain reactions, thus preventing cellular lipids from oxidising. Phenolic compounds are also able to chelate metal ions which are responsible for free radical production (Fraga *et al.* 2010). This ability increases the antioxidant capacity of phenolic compounds. Hydroxyl groups present in the catechin molecule can act as chelation sites as depicted in Fig 2.2. Catechins are able to regulate antioxidant enzymes (Meng, Velalar and Ruan 2008; Rodríguez-Ramiro *et al.* 2011), protein synthesis and are responsible for signalling

pathway suppressors (Fan, Sang and Jiang 2017). The presence of catechins in drinking water reveals an increase in superoxide (SOD) and catalase (CAT) which is important in scavenging ROS (Khan *et al.* 1992). Catechins consumed in green tea are also responsible for the suppression of many oxidative stress-related pathways responsible for inflammation processes (Fan, Sang and Jiang 2017).

Electrochemical applications reveal that oxidation mechanisms for catechin are pH-dependent and irreversible (Janeiro and Brett 2004). Extensive research is focused on the therapeutic benefit of catechin in green tea as they are known to inhibit cancer cell formation and growth in the body. Specifically in this work, (+)-catechin has been selected as the flavanol of interest because it serves an important function in the brewing process by slowing down the rate of oxidation processes. Electrochemical techniques are often applied for oxidation-reduction processes using cyclic voltammetry.

2.2 Antioxidant Evaluation Models

Various models and methods for assessing antioxidant activity have been developed over the years and can be classified into two groups. Models can be based on assays used to measure oxygen-free radical scavenging as well as those that are based on inhibition of low-density lipoprotein oxidation. Some of the most widely used techniques will be discussed below (Antolovich *et al.* 2002; Sánchez-Moreno 2002; Dotan, Lichtenberg and Pinchuk 2004).

2.2.1 DPPH method (1, 1-diphenyl 2, picryl hydrazyl)

The most widely known technique used for the screening of antioxidant activity in flavonoids is the 1, 1-diphenyl 2, picryl hydrazyl (DPPH) method. The evaluation involves the radical scavenging ability of the antioxidant between its reactions with the stable DPPH radical. The analysis involves the reduction of a methyl alcohol solution followed by the measurement of absorbance decrease of DPPH. This decrease of DPPH absorbance is proportional to the concentration of the free radical scavenging ability of the antioxidant which is expressed as EC_{50} value (Navarro *et al.* 1993; Vani *et al.* 1997; Sanchez, Larrauri and Saura-Calixto 1999). The EC_{50} value represents the actual antioxidant concentration required to decrease the initial DPPH concentration by 50%. Antioxidants can be progressively fast, intermediate or slow based on previous research (Brand-Williams, Cuvelier and Berset 1995; Sánchez-Moreno, Larrauri and Saura-Calixto 1998).

2.2.2 Superoxide radical scavenging activity

Superoxide radical scavenging activity is based on spectrophotometric detection in the presence of light and Nitro Blue tetrazolium (NBT). Reduction of NBT is a favored method. The method involves the generation of superoxide radical by riboflavin oxidation in the presence of light, followed by the reduction of NBT. The superoxide radical reduces NBT resulting in the formation of a blue colored complex that is measured at a wavelength of 560 nm. The EC_{50} value is measured in relation to the antioxidant capacity to inhibit the colored complex to 50 %. Another detection method has been reported whereby a colorimetric reaction is involved. Oxidation of hydroxylamine results in the detection of the superoxide radical, yielding nitrite (Robak and Gryglewski 1988; Babu, Shylesh and Padikkala 2001).

2.2.3 Conjugated diene assay

This method is widely used for the detection and monitoring of Low-density lipoprotein (LDL) oxidation utilizing conjugated diene measurement in terms of quantification. A UV-Vis technique is employed based on the conversion of double bonds during linoleic acid oxidation into conjugated double bonds. Characterization of the conjugated diene is quite strong at an absorption of 234 nm, and the antioxidant activity is expressed as Inhibitory concentration (IC_{50}) (Bostwick *et al.* 2000; Tiwari 2001; Vaya and Aviram 2001).

2.2.4 ABTS (2,2-azinobis(3-ethylbenzothiazoline-6-sulfonate) Method

Antioxidant activity of flavonoids in wines is usually monitored by measuring their ability to scavenge the radical cation 2,2-azino-bis (3-ethylbenzothiazoline-6-sulfonate) (ABTS+) which has a characteristic colour at 645nm,743nm and 815 nm (Kanner *et al.* 1994; Rice-Evans and Miller 1994; Vinson and Hontz 1995; Simonetti, Pietta and Testolin 1997). This method is useful for the ability to distinguish between additives, physical and chemical effects.

2.2.5 Hydroxyl radical scavenging activity

This method is based on the generation of hydroxyl radicals by using the Fenton reaction, including the Fe^{3+}/ascorbate/EDTA/H_2O_2 system. Scavenging of the hydroxyl radical is measured in the presence of the antioxidant. In a typical reaction, the hydroxyl radicals formed in the oxidation process is reacted with dimethyl sulphoxide (DMSO) yielding formaldehyde. In the presence of Nash reagent (2 M ammonium acetate with 0.05 M acetic acid and 0.02M

acetyl acetone in distilled water), formaldehyde produces an intense yellow colour which is then measured at an absorbance of 412 nm (Babu, Shylesh and Padikkala 2001).

2.2.6 Xanthine oxidase method

Xanthine oxidase (XOD) plays a significant role as a biological source of superoxide radicals. The enzyme is responsible for the production of uric acid by oxidation of hypoxanthine and xanthine. Re-oxidation of XOD produces superoxide radicals and hydrogen peroxide. Inhibition of XOD is measured by the decrease in the production of uric acid by spectrophotometric methods.

2.2.7 Cytochrome C test

This method is very similar to the XOD method. A cytochrome reduction method where superoxide anions are assayed and measured by spectrophotometric means, is described by (McCord and Fridovich 1969). The conversion of xanthine to uric acid yields superoxide radicals which then reduce ferri-Cyt C to ferro-Cyt C. The Scavenger activity is based on the decrease in the reduction of ferri-Cyt C (Ho *et al.* 1999).

2.2.8 Oxygen Radical Absorbance Capacity (ORAC)

This method was developed to test the antioxidant power of chemical substances and food products. This test enables us to determine the antioxidant ability within a product or chemical to protect against the free radical formation. The test involves the use of Trolox (a water-soluble analogue of Vitamin E) as a standard which is then used to determine Trolox Equivalent (TE). Free radicals generated are based on using (2,2-azobis 2-amidopropane dihydrochloride) AAPH whilst measuring the decrease in fluorescence. Antioxidant activity is expressed as Trolox Equivalent Antioxidant power which is proportional to ORAC value (Frei *et al.* 1990; Cao, Alessio and Cutler 1993; Prior *et al.* 1998).

2.3 Enzymes

Enzymes are large complex macromolecules or catalysts usually consisting of protein. Enzymes used in biosensors involve oxidation or reduction by electrochemical detection and are highly specific when it comes to particular substrates (Campaña *et al.* 2019). Enzymes can be classified into six categories, namely oxidoreductases which are responsible for catalysing oxidation/reduction reactions and Transferases are involved in transfer reactions. Hydrolases

are important for hydrolysing substrates into two parts, whereas Lyases cleave a substrate to a molecule. Isomerases are responsible for converting one isomer into another and Ligases join two or more molecules together (Robinson 2015). The formation of an enzyme-substrate complex during an enzymatic reaction can be seen below:

$$E + S \longrightarrow ES \qquad \text{- (Eq 2.1)}$$

Enzyme Substrate Enzyme-Substrate Complex

This is a simple physical phenomenon of binding involving weak forces between E and S. Generally, this is an easy, fast step in any enzyme action. Enzymes use a special site called an active site to form an ES complex which is relatively little in comparison to the entire capacity of the enzyme (Bugg 2012).

Stability of enzymes is one of the important considerations for their biotechnological applications (Klibanov 1979; Gupta 1991). In order to increase the stability of enzymes and thus to improve the process economy, various attempts have been made (Minagawa *et al.* 1998). Different immobilization methods can be used to increase enzyme stability.

2.3.1 Cytochrome C

Cytochrome c (Cyt c) plays a pivotal role in the mitochondrial respiratory chain (Wang *et al.* 2002). Cyt c is a heme-containing metalloprotein existing between the inner and outer membranes of mitochondria. Electron transfer processes and the oxidation-reduction reaction of Cyt c is of major interest due to their involvement in biosensor modification (Xiang *et al.* 2007). Significant research has gone into the investigation of Cyt c focusing on the rate of electron–transfer reaction on biosensors (Tarlov and Bowden 1991; Collinson, Bowden and Tarlov 1992; Cooper, Greenough and McNeil 1993; Feng *et al.* 1995; Hawkridge and Taniguchi 1995) which has been established that is dependent on the length of modifiers as well as the alignment of protein structure (Kuznetsov, Byzova and Shumakovich 1994; Terrettaz *et al.* 1996; Feng 1997). There seems to be an influential relationship between protein structure, reaction intermediates immobilized on Cyt c and electrolyte solutions (Jin *et al.* 1997). Due to slow electron transfer of Cyt c at bare electrodes, modification of the electrode surface is applied to increase the electron transfer rate. One of the major issues with regard to the fabrication of modified electrodes with Cyt c is in the lack of control on the thickness and uniformity of the modifying component. Conducting polymers (CP) and carbon nanotubes

(CNTs) exhibit immense suitability and sensitivity in the Cyt c immobilization process (So *et al.* 2005; Manesh *et al.* 2006; Xiang *et al.* 2008). Self-assembled monolayer (SAM) modified biosensors have been proven to provide favourable physical and chemical properties of electrode surface for Cyt c binding thereby improving sensitivity (Dickerson *et al.* 1971; Laviron 1979; Dai, Liu and Ju 2004). Superoxide dismutase (SOD) and hypoxanthine (HX) have been used in immobilization procedures with Cyt c (Gobi and Mizutani 2000).

2.3.2. Laccase

Laccase belongs to a group of enzymes called polyphenol oxidases and is usually called multi-copper oxidases as they contain copper atoms in the catalytic centre (Brijwani, Rigdon and Vadlani 2010). Laccase enzymes are polymeric and usually contain a type 1, type 2 and type 3 copper centre. These enzymes are usually found in fungi and plants as well as in bacteria and insects (Messerschmidt and Huber 1990; Baldrian 2006; Kunamneni *et al.* 2007). During oxidation, a single electron is lost thus forming a free-radical which generally undergoes a further oxidation reaction which may include hydration, polymerization or disproportionation (Sharma, Goel and Capalash 2007). There are various industries that benefit from laccase applications. The food industry, including the baking industry, is heavily invested in the use of laccase enzyme in their dough-making the process to improve texture and flavour. The wine and beer industry also relies on the addition of laccase during the production phase to overcome flavour changes and haze formation attributed to protein precipitation that arise from concentrations of polyphenols present in these beverages (Mathiasen 1995). Laccase is also capable of assisting in bioremediation of water and wastewater in the food industry by engaging in processes that restore the environment back to its original form (Thassitou and Arvanitoyannis 2001).

2.3.3 Horseradish Peroxidase (HRP)

Horseradish is a rich source of peroxidase stemming from the roots of the perennial herb called (Armoracia *rusticana*) and belonging to a large family of isoenzymes. Isoenzymes have distinct physical, kinetic and chemical properties; therefore, differing in molecular forms with differences in their amino acid sequence (Welinder 1979) however, the isoenzyme C (HRP C) is most predominant. Commercial application for plant peroxidases is well recognized in immunodetection and biosensors. HRP displays stability, specificity and sensitivity in the reaction and toward analyte detection.

A large number of procedures have been developed for the detection of H_2O_2 using HRP modification procedures. The reduction mechanism of peroxide molecule at HRP-modified electrode is seen in Fig 2.3. HRP is useful for H_2O_2 reduction and other peroxides related to the dairy, pharmaceutical and environmental industries (Somasundrum, Kirtikara and Tanticharoen 1996) extending to the monitoring and control of these peroxides. HRP is a popular enzyme used in medical research due to its sensitivity in immunoassay and nucleic acid detection. General techniques involve amperometric sensors used in combination with Flow injection analysis (FIA) systems for the detection of various analytes such as glucose, amino acids, lactate and cholesterol (Pandey, Glazier and Weetall 1993).

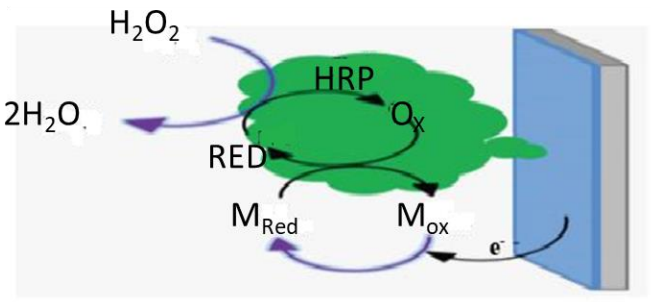

Figure 2. 3 Mechanism of direct electro-enzymatic reduction of a peroxide molecule at HRP-modified electrode (Saleh Ahammad 2013).

2.4. Polymers

Polymers are found to be synthetic or natural with the most widely researched naturally occurring polymers being deoxyribonucleic acid (DNA) and ribonucleic acid (RNA). Polymers have gained a great deal of attention with respect to electrode modification in recent years. Inorganic polymers have often been selected for this process as they are inexpensive, stable, possess great physical strength, less prone to microbial contamination and are themselves inert (Deshpande and Amalnerkar 1993). Conducting polymers, ion-exchange and redox polymers are generally used in the electrode immobilization modification process (Brown 2018).

Polyaniline (PANi) is a conducting polymer and has been researched for its synthesis and electro-activity properties based on oxidation states (Salaneck *et al.* 1986; Genies *et al.* 1990). Conducting polymers such as PANi, polyacetylene and polypyrrole possess semiconducting properties and is thus favoured in electronic devices, catalysis and membrane construction (Mishra 2018). Redox behaviour of conducting polymers is an important electrochemical property that is studied in aqueous and non-aqueous solutions (Watanabe *et al.* 1989).

PANi chains have a structure that contains nitrogen groups with alternating phenyl rings providing an ordered structure for polyconjugation. Polyconjugation is ensured as a result of monomer units whereby PANi chains contain a greater number of para-substituted aniline fragments linked in a head-to-tail configuration (Hagiwara, Demura and Iwata 1987). Furthermore, should the configuration also change whereby the ortho or meta substituent components co-polymerize there will be a noted decrease in the conductivity.

2.5 Biosensors

A sensor can be defined as a device that measures or detects a physical property and responds to the signal by a recording or sound. The measurable signal is observed as a result of a transducer. The principle of developing a biosensor is based on the interaction between the analyte or substance of interest and the biological material immobilized onto the surface of a transducer, which then, in turn generates a concentration-dependent signal. Biological materials could be in the form of enzymes, cells or antibiotics (Koncki 2007). There are three overall types of sensors: physical, chemical and biosensors. Biosensors are widely used in the medical field for diagnosis of biological fluids, measuring concentrations of drugs in blood and assessing the immune status of the body (Alcock *et al.* 1992). Biotechnological applications of biosensors include optimization of technological processes and their stage by stage monitoring (Luong, Bouvrette and Male 1997). Biosensor analysers can provide a rapid and effective analysis of contamination in natural objects or products during ecological monitoring.

Previous analytical techniques in the chemical field have often relied on photometric transducers such as spectroscopic and colorimetric methods. In recent times more sensors are being developed by electrochemical means due to their ease of use, simplistic construction and cost. Transducers can be subdivided into four main groups: Electrochemical, Optical, Piezo-Electric and Thermal. For the purposes of this study, the electrochemical transducers will be emphasised.

2.5.1 Electrochemical Transducers

In this setup, measurement of the potential or emf of a cell at a zero current is monitored. The potential is proportional to the logarithm of the concentration of the substance being analysed. Another mode that falls into this group is called voltammetric or amperometric. A potential is

applied to the cell until oxidation or reduction of the substance occurs; the observed current rise or fall is indicated. This peak height is directly proportional to the concentration of the electroactive material. If the oxidation/reduction potential of the analyte is known, the potential may be stepped accordingly to the known value, and the current value is then observed, this mode is known as amperometric. Amperometric sensors are known for their selectivity to the species being analysed however, in most cases, three modes of oxidation reactions occur namely, first, second and third generation. The first-generation refers to oxygen electrode-based sensor, second-generation sensors are mediator-based, whilst the third-generation electrodes are directly coupled to enzymes.

The glucose sensor is a classic example of an enzyme-based amperometric sensor that was developed in 1962 using a dissolved oxygen electrode (Clark Jr and Lyons 1962). The first operational glucose biosensor was developed in 1967 based on amperometric measurement of oxygen depletion in an immobilized glucose oxidase (GOD) gel (Updike and Hicks 1967). The oxidation mechanism of a glucose molecule at HRP- modified electrode is shown in Fig 2.4.

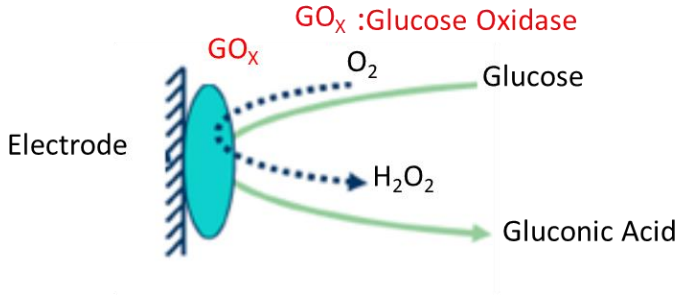

Figure 2. 4: Mechanism of direct electro-enzymatic oxidation of a glucose molecule at HRP-modified electrode (Xu *et al.* 2014).

The resulting high electrode over-potential and interferences led to the replacement of O_2 with other oxidising agents that were reversible, had controllable concentrations and the appropriate oxidation potentials. Mediators were used for this purpose, mainly based on iron or, in the case of the glucose sensor, ferrocene complexes (Cass *et al.* 1984). Free ions are subject to hydrolysis and precipitation hence do not make good mediators. As per reaction below:

GOOX + glucose → GOR + gluconolactone -(Eq 2.2)

GOR + 2Fc + →GOOX + 2 Fe -(Eq 2.3)

Fc ↔ Fc + + e- (measured) -(Eq 2.4)

Where Fc is ferrocene carboxylate

Oxidation of glucose to gluconolactone is carried out by Flavin-adenine dinucleotide (FAD) which is a component of glucose oxidase. This is the converted to $FADH_2$. The latter is then re-oxidised to FAD by the Fe^+ mediator followed by re-oxidation of Fe to Fe^{2+} at the electrode. The hydrogen peroxide produced in this reaction is then determined by an amperometric measurement, whereby the current flowing through measures the glucose concentration. The over-potential is reduced, and there is less interference. Mediators should be independent of pH, should be stable, non-toxic, and react rapidly with chosen enzymes. In certain instances, modification of electrode surfaces is required due to protein denaturing. Third generation electrodes utilize compounds that are not electroactive. When wiring an enzyme directly to the electrode surface, this relates to rapid electron transfer and high current densities.

Consisting of a counter electrode, a reference electrode and the working electrode (containing protein film, electrode material and insulator). A suitable electrolyte solution along with immobilization technique is employed followed by electrochemical detection using amperometry, DPV and or CV. Electrochemical equipment required for developing biosensors includes a three-electrode system.

2.6 Immobilization methods

Immobilization is a process involving attachment of a biological component onto a transducer. Immobilization is utilized to enhance enzyme favourability within an electrochemical process. This procedure ensures that the modified sensor can be used over prolonged periods, thus controlling reaction intermediates during reactions (Tosa *et al.* 1966; Hernandez and Fernandez-Lafuente 2011). The five main methods include; Adsorption, microencapsulation, entrapment, cross-linking and covalent bonding. Proper immobilization greatly improves the lifespan of the biosensor.

Adsorption involves minimal preparation, and it is the simplest of immobilization methods. Adsorption can further be broken down into two groups that are physisorption and chemisorption.

Weak bonding via van der Waals bond formation usually takes place in physisorption. However, chemisorption involves much stronger covalent bonds (Volesky 2001). Substrates including alumina, cellulose, silica and clay can easily absorb enzymes onto their surfaces with no reagents or clean up required. This technique can be used over short term experimental procedures as their typical lifespan is a day. The enzyme can be easily removed under optimised

parameters from the supporting material and regenerated once again when required by electrochemical deposition. The enzyme may also undergo various changes due to the influence of pH and this will need to be optimised during method development (Lehninger *et al.* 2005).

Microencapsulation was adopted in early biosensor fabrication as in the first glucose biosensor modified oxygen electrode. Biomaterial adheres to a membrane enabling close contact between the enzyme and transducer. Cellulose acetate, polycarbonate and collagen are often used as membrane sources. The main positives to using this technique are that the membrane remains stable in changing parameters such as pH, ionic strength and substrate concentration. The modified electrode is also reliable and has a high degree of specificity (Park and Chang 2000). The typical working life of the biosensor is approximately five days.

Entrapment involves an irreversible polymerization process whereby a monomer solution is mixed with a biomaterial allowing for the enzyme to become entrapped onto the gel matrix (Subramanian *et al.* 1999). There are disadvantages associated with this technique in which enzyme activity can be lost through the porous gel and as a result, cause slow reaction times to occur (Sheldon 2007). Polyacrylamide and polyaniline are often used as conducting polymers as they have stability of approximately 28 days.

Cross-linking is a technique that was developed to overcome the enzyme activity loss presented in the entrapment method. This method does not require a supporting constituent. Enzymes are chemically bonded to supporting materials using bifunctional agents such as glutaraldehyde, hexamethyl diisocyanate and 1,5-dinitro-2, 4-difluorobenzene (Sheldon 2007). The mechanical strength of the biosensor is compromised with some degree of damage to the enzyme. Stability in terms of the life span of this biosensor is quite good, ranging from 3- 12 months.

The approach to designing a biosensor using covalent bonding involves creating a bond between a functional group, the enzyme and the substrate. The nucleophilic groups in amino acids are the most suitable groups for coupling. Reactions during this process must be performed at low temperatures and pH levels, in doing this, the enzyme in the active site is protected.

2.7 Electroanalytical detection of polyphenolic compounds

Literature studies on the antioxidant properties of polyphenolic compounds revealed greater advantages using electroanalytical techniques. During electrochemical processes, antioxidants

present in polyphenolic compounds undergo various reactions, including electron transfer, oxidation and reduction. The rate of electrode reaction, oxidation potentials and electron transfer can be determined electrochemically to provide rapid evaluation of the antioxidant activity. Cyclic voltammetry, amperometry and square wave voltammetry have often been applied to investigate antioxidant properties in wine and tea polyphenols. Electrochemical factors such as scan rate, deposition time, electrolyte choice and pH are amongst the parameters that impact on antioxidant behaviour (Kilmartin 2001). Many *in vitro* and *in vivo* procedures exist for the measurement of the total antioxidant activity of biological substances.

Electrochemical oxidation has been effectively used for the detection of radical scavenging in polyphenolic compounds which in turn has been associated with antioxidant power (Chevion, Roberts and Chevion 2000). Cyclic voltammetry (CV) studies have also been confirmed for the ability to quantify the low-molecular-weight antioxidant capacity of the tissue, blood plasma and plant extracts (Chevion, Roberts and Chevion 2000; Bortolomeazzi *et al.* 2007; Yakovleva *et al.* 2007). The CV is thus employed as a technique to determine the redox properties of polyphenols. For example, the oxidative mechanism for ascorbic acid has been studied at length, and the general outcome is that AA undergoes an irreversible reaction (Dryhurst G. 1982). The initial step during this oxidation process involves the electron transfer of a deprotonated anion followed by the oxidation of the single electron to a radical anion. The radical anion undergoes a rapid, irreversible single-electron oxidation process to form dehydroascorbic acid. Cyclic voltammetry with a modified GCE working electrode has been used to screen the total antioxidant capacity and flavonoid content in onions (Pisoschi, Danet and Kalinowski 2009). Previous studies have also been able to assess the property similarities between catechin and juglone using CV to establish electrochemical potentials of both analytes.

CHAPTER 3

THEORETICAL PRINCIPLES

This chapter provides an overview of theoretical principles underpinning the experimental methodologies employed in this work, including a discussion on experimental design.

3.1 Cyclic Voltammetry

Cyclic Voltammetry (CV) is a technique of high importance in the electroanalytical realm. Information provided can be useful for defining reaction intermediates, obtaining stability of a reaction and determine redox processes. Additionally, the location of redox potentials of electroactive species can be assessed as described by Randles (Randles 1948).

The overall technique is based on varying applied voltage in either a forward or reverse direction whilst monitoring current. Typical CV experiments involve a stationary working electrode which is subjected to a triangular potential sweep, in a three-electrode arrangement in order to lower ohmic resistance. A separate reference electrode is used, and the potential of the working electrode is measured relative to this reference electrode. During a measurement, the voltage is applied between two values V_1 and V_2 at a fixed scan rate, as the voltage reaches V_2 the scan is reversed, and the voltage is swept back to V_1. The current stabilised at the electrode surface area is measured, and referred to as current density. A CV is the plot of current density against applied potential. Peak heights and width are characteristic and dependant on electrolyte solution, electrode material and scan rates. Typically a cyclic voltammogram is a time-dependent function of a number of physical and chemical factors. A simple CV response for a reversible redox system can be seen in Fig. 3.1.

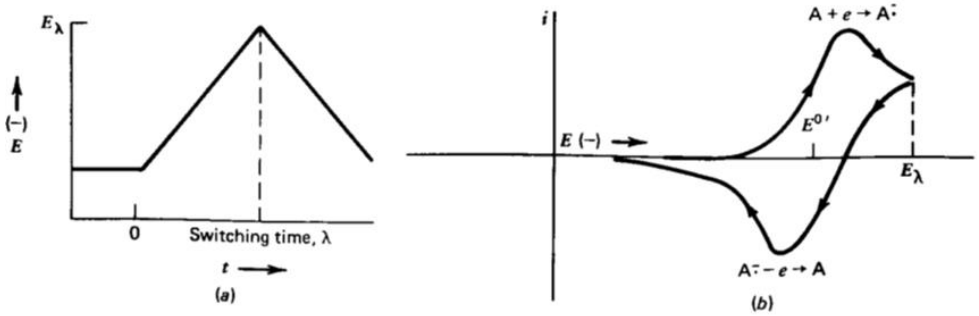

Figure 3. 1: **a**) Potential-time excitation signal of a cyclic potential sweep **b**) Typical cyclic voltammogram (Li, Peng and Peng 2003).

During a reversible CV oxidation reaction for catechin, the potential is swept linearly from -0.2V to 0.6 V, after which the scan direction is reversed (at +0.6 V), and the potential is swept linearly back to - 0.2 V. The arrows in Figure 3.2 indicate scan directions.

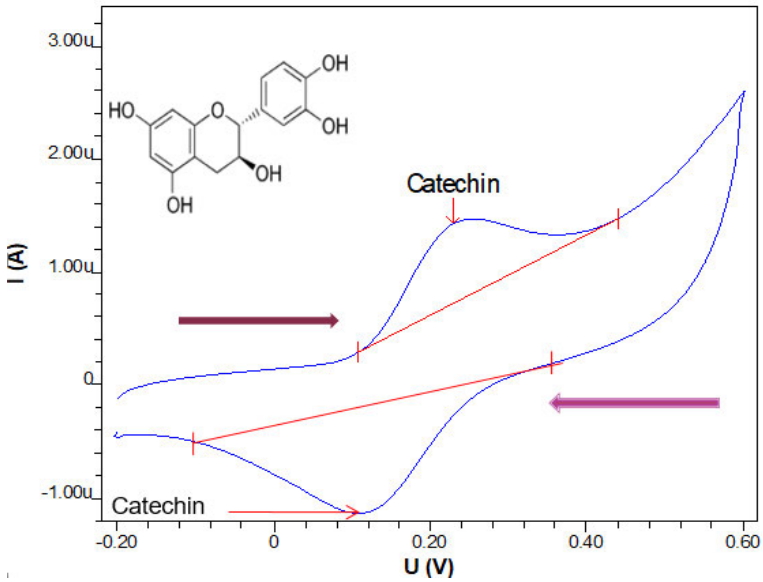

Figure 3. 2: A typical cyclic voltammogram for the oxidation of catechin. Arrows indicate the scan direction: the start potential is - 0.20 V.

It is observed by interpreting the forward scan, that between -0.2 V and -1.5 V no current is passed as there is insufficient driving force (overpotential) for the oxidation of species A to B. As the potential increases, the rate of oxidation from A to B increases. The measured current increases exponentially since the electron transfer kinetics proceeds rapidly rendering the species electrochemically reversible. The concentrations of species A and B at the electrode surface obey the Nernst equation Eq 3.1:

Nernst equation: $E = E0 + RT/nF \ln [Ox] / [red.]$ (Eq 3.1)

where:

E is the potential of the cell

E0 is the standard potential

N is the number of electrons

F is the Faraday constant

For CV half-cell reactions the equation becomes E0 = Epa +Epc /2 where Epa and Epc are anodic and cathodic peak potentials respectively.

At an overpotential of 0.18 V concentrations of species A and B at the electrode surface are equal. On reversing the potential, concentrations of species A and B continue to obey Nernst equation, scanning in a negative direction a peak is observed at + 0.2 V. This being associated with the reduction of species B to A, depleting species B at the electrode surface.

Further interpretation of a CV is usually performed with the measurement of the peak current in a forward scan followed by the peak to peak separation of both reverse and forward scans. Ideally in a one-electron reversible redox system, the peak to peak separation should be 57 mV (at 25 ^{o}C). However, this value is often greater between 59-60 mV in standard reversible systems.

The Randles-Sevcik equation is generally applied for the determination of peak current for a reversible *n* electron redox species equation 3.2:

$$ip = 2.69 \times 10^5 \, n^{3/2} \, Ade^{1/2} \, Cv^{1/2} \quad \text{(Eq 3.2)}$$

Where *ip* represents peak current (A) associated with the forward scan and *n* is the number of electrons for the redox couple, appearing in half-reaction. *A* denotes the area of the electrode (cm^2) whilst *D* is the diffusion coefficient (cm^2/s). *C* is the symbol for concentration (mol/cm^3), and *v* is scan rate (V/s). The Randles-Sevcik equation is used to plot the peak current versus the square root of the scan rate. From the slope of the linear regression, the diffusion coefficient of the electrons within polymers and sensor material can be estimated.

An important factor when utilising cyclic voltammetric analysis is to establish whether the electron transfer rate occurring is reversible or irreversible in nature. The wave shape represented in a forward scan in an irreversible redox system will appear with a distinct difference to that of a reversible voltammogram. The difference between half-peak current and peak current can be used to quantify the voltammogram.

3.2 Differential Pulse Voltammetry

Differential pulse voltammetry (DPV) has been employed over the years to increase the speed and sensitivity of measurement. This technique is often selected as it is well suited for reversible as well as irreversible systems. Similarly to that of CV, a staircase-shaped increasing potential is applied between the working electrode and the reference electrode. Small square wave pulses with a constant potential are applied between 5 and 100 milliseconds after which a final potential is changed, and reached and then the pulse is repeated. Traditionally voltammetry includes all methods whereby current-potential measurements are made at stationary electrodes, including GCE and hanging mercury drop (HMDE).

The value of the current before each pulse, and at the end of the pulse is measured and the difference is plotted against potential (Kissinger and Heineman 1996). The current change in the region of half-wave potential is at its greatest, thus leading to a further reduction of the capacitive current contribution, which directly increases the sensitivity. A typical DPV voltammogram can be seen in Fig 3.3.

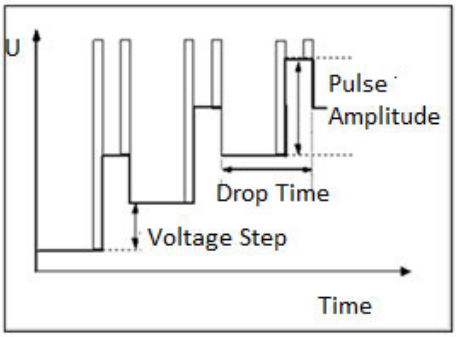

Figure 3. 3: A typical DPV measurement process whereby the current is measured twice at each mercury drop, before each pulse and at the end of the pulse time (Scholz 2015).

DPV is a technique of choice used for achieving a high degree of sensitivity and precision for the detection of trace amounts of analytes by minimizing the effect of the charging current and

extracting faradaic current. A Differential pulse voltammogram can provide crucial information regarding reversible and irreversible reaction characteristics. In general reversible reactions are characterised by symmetrical peaks, and irreversible reactions characterised by asymmetrical peaks. The detection limits are found in the region of 10^{-8} M as peak current is proportional to the concentration of the analyte (Kissinger and Heineman 1996). The voltammogram in Fig 3.4 reveals a definitive half-wave potential peak at +0.18 V for catchin determination.

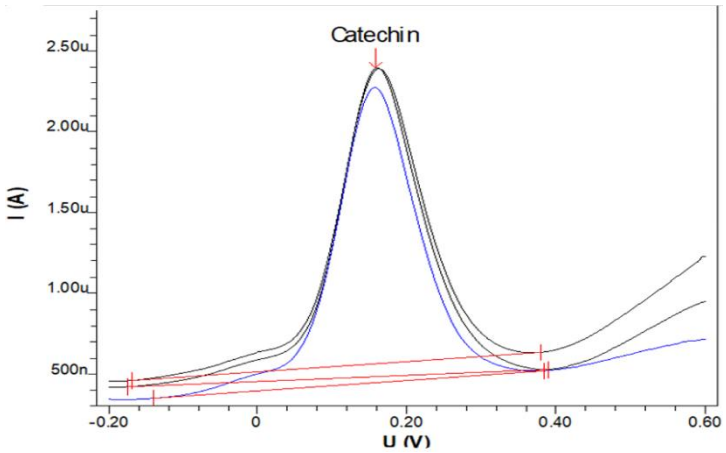

Figure 3. 4: Typical current vs potential curve for DPV.

3.3 Amperometry

Amperometry is a technique employed to provide quantitative information regarding analyte species unlike certain non-electrochemical techniques including atomic absorption spectroscopy and X-ray fluorescence. During the measurement, a fixed potential is applied upon a working electrode, thereby producing an initial current. This transient current is related to the activity of redox species at the electrode interface. The value for electrode potential is carefully selected to ensure that only the specific ion is reduced. The advantage of employing amperometry, is that good detection limits can be achieved, and selectivity is often easily attained through optimization of detection potential. Good linear range and reproducibility under well-defined and controlled conditions are also evident with this technique. Fig 3.5a represents a fixed potential, and a typical amperogram involves a plot of current generated as a function of time, as seen in Fig 3.5b.

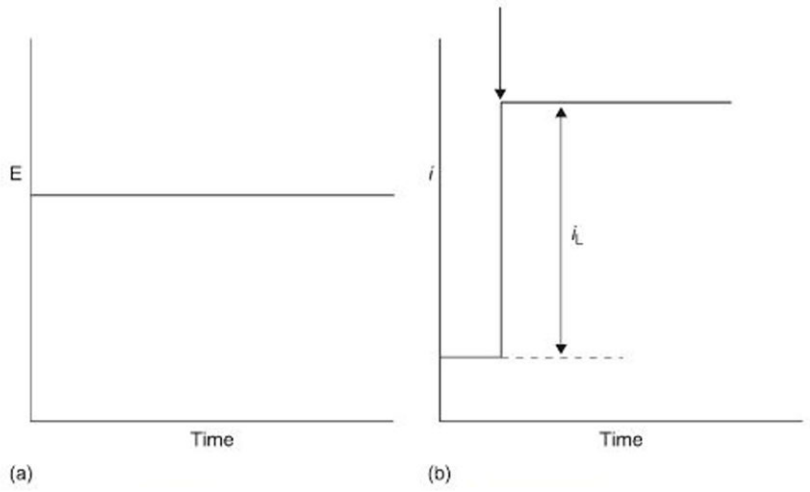

Figure 3. 5: **a**) Represents a fixed potential input **b**) A typical amperogram response to analyte addition (Honeychurch 2012).

3.4 Experimental Design

Chemometric tools using mathematical models are being applied for the optimization of analytical methods in recent years with great success. The main advantages being that the number of experimental analysis being performed is drastically reduced, thereby ensuring lower reagent usage and less time spent in a laboratory. Introducing mathematical models for the evaluation of statistical data enables the end-user to investigate interaction properties and effects of chosen methodological parameters involved in analytical techniques.

Multivariate optimization methods encompass a factorial design strategy for which all stages of all variables are altered consecutively. Single variable optimization methods and procedures may lack in providing robust statistical data. Interaction effects of a single variable are, in most cases, reliant on the level of the other variables included in the optimization procedure. In order to establish substantial effects presented in an electrochemical or analytical system, the first process in designing a multivariate optimization procedure requires a full factorial design considering all factors. Step two involves a more in-depth look at the optimum operating conditions by implementing a multi-layered experimental design in the form of Doehlert matrix (DM), central composite designs (CCD) or a Box-Behnken design (BBD) (Massart *et al.* 1998; Box, Hunter and Hunter 2005; Neto, Scarminio and Bruns 2006).

Methods for optimization and implementing factorial design have been researched in the analytical field. Major advantages to applying these techniques are in the reduction of laboratory analysis leading to a more cost-effective and faster approach to research. The main goal of utilizing such methods is in the application of several factors included in a mathematical model to evaluate statistical data. The Box Behnken design (BBD) is a multivariate method that is built on a 3 level incomplete complex design (Massart *et al.* 1998; Box, Hunter and Hunter 2005; Neto, Scarminio and Bruns 2006). This particular method was developed to lower or limit the size of a sample as each parameter is introduced whereby the coefficients or constants are approximated in a second degree least-squares polynomial.

Typically a Box-Behnken design comprises of a two or a three-variable model with the corresponding block of samples and is evaluated on the number of parameters selected that must be rotatable (Box and Behnken 1960). Rotatability is met if the variance of the response predicted is a function of the distance from the centre (Ferreira *et al.* 2003) of the cube representation as in Fig 3.6. BBD evaluation is useful in the examination of a set of quantitative experimental parameters as it is a response surface technique (Box and Behnken 1960).

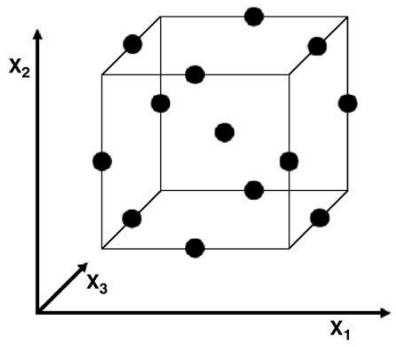

Figure 3. 6: Structure of a three-factor Box–Behnken design (Box and Behnken 1960).

CHAPTER 4

CASE STUDY 1: MULTIVARIATE OPTIMIZATION

This chapter deals with the optimization of parameters such as pH, scan rates and deposition time followed by a methodological study to assess the sensitivity of the electrochemical biosensor for the determination of antioxidant activity in wine samples. Modification of a glassy carbon electrode (GCE) using green apple as the enzymatic substrate was used for the determination of Total phenolic content in wine samples The Box Behnken factorial design was applied thereby investigating optimised variables.

4.1 Introduction

Antioxidants have been the topic of interest for many years, and studies have revealed that flavanols have a beneficial effect on human health. The role of these polyphenolic antioxidants aiding in minimising and preventing oxidation of other molecules. Moderate consumption of wines, specifically red wine, has been shown to reduce and or prevent disorders of gastric ulcers, cardiovascular diseases and cancers associated with ageing (Stanner *et al.* 2004). Polyphenolic compounds found in wines are involved in terminating oxidation chain reaction processes in the body by removing the free radical intermediates that are formed. They play a role in acting as metal-chelating agents, inhibitors of oxidative enzymes and free-radical scavengers, thus providing protection against the above-mentioned diseases (Rice-Evans, Miller and Paganga 1997). The interest in evaluating the antioxidant activity of wine in relation to their phenolic content goes beyond immediate health benefits as flavanol amounts must be controlled in the winemaking process as they contribute to the overall quality of wines.

The flavanol of interest in this work specifically is (+)-catechin, and this is due to its effects in the brewing process as it retards and prevents oxidation processes. Raw materials during brewing processes affect organoleptic characteristic, namely colour and sharpness with grape variety leading to varying levels of antioxidants present. Recent publications describing the correlation between antioxidant properties of both white and red wines have been documented

with their polyphenolic content which peaks the interest in evaluating sensitive and selective methods for detection and quantification (Alonso *et al.* 2002).

A number of techniques have been used for the assessment of polyphenolic content and chemical constituents of wine. These include high-performance liquid chromatography (HPLC) (Gambelli and Santaroni 2004; Aguirre *et al.* 2010; Wirth *et al.* 2010; Šeruga, Novak and Jakobek 2011) and capillary electrophoresis (CE) (Arce *et al.* 1998; Sun *et al.* 2008; Peres *et al.* 2009) in combination with various detectors such as photodiode array (PDA), electrochemical (EC), UV–Vis and mass spectrometry (MS). The brewing industry prefers HPLC coupled with a UV detector as a method of choice (Mojica *et al.* 2007).

This research involves the use of polyphenol oxidase present in green apple to conduct electrochemical measurements of polyphenols (catechin equivalent) in wines (Eggins *et al.* 1997). There is a difficulty associated with evaluating real samples with this method, although it does provide specificity and sensitivity. Chromatographic methods yield desirable separation of components in protein containing-matrices such as beer, in a rapid manner with the ability to quantify with selectivity and sensitivity required (Maoela *et al.* 2009). Despite these positives, chromatographic methods are known to necessitate long-winded sample preparation processes that usually introduce sources of error.

The main focus of this research is to utilize a modified tissue biosensor for the quantification of flavanols in wine samples. The process involves the study of electrochemical behaviour for the determination of catechin conducted under varied experimental conditions, those including pH of the buffer, scan rate (s_r) and deposition time (td). Voltammetry is one of the electrochemical techniques that impart qualitative and quantitative information of compounds in addition to their physicochemical properties such as the number of electrons exchanged during redox reaction and potential. The data provided from this electrochemical technique assists in assessing antioxidant properties of phenolic compounds which allows for the interpretation of reaction mechanisms (Novak *et al.* 2008).

The selection of electrodes for voltammetric applications is based on the nature of the samples and the physical properties of the constructed electrode. Particularly for organic molecules, glassy carbon electrode (GCE) has been found to be one of the most satisfactory electrodes. This is due to it being highly resistant to solvents in comparison to other metals, and it also reveals low

background currents (Kilmartin, Zou and Waterhouse 2001). It has been found that the electrode surface is etched over a period of time with constant exposure to the electrolyte solution. This occurrence gradually reduces the electrode lifespan and signal output with no rejuvenation option available, hence the alternative use of an immobilized biosensor. In this process, the electrode surface is modified with a paste or gel and the electrode surface is regenerated after each analysis run, thereby reducing electrode surface spoil. Cyclic Voltammetry (CV) and Differential Pulse Voltammetry (DPV) are techniques suitable for the evaluation of electrochemical properties of compounds using modified biosensors. This is due to biosensors posing high selectivity and sensitivity toward their target molecules in supporting electrolyte, as long as the chosen parameters are highly optimized. Biosensor modification has proven to be a cost-effective solution to increase the working life of the electrode. In most cases there is an enzyme actively protecting the electrode surface from sample matrices. Literature studies have shown that polyphenol oxidase is present in many plant tissues, with potato and apple yielding the best responses for the determination of catechol related compounds (Eggins *et al.* 1997).

The implementation of statistical design to evaluate the validation, traceability and optimization process during all stages in an experiment results in an effective method protocol (Niewiara, Baś and Kubiak 2007). The experimental design is further geared to improve upon the data process for method development and validation (Swartz and Krull 1999; Husakova *et al.* 2005). During the validation process, performance parameters including accuracy and precision should be investigated to ensure that the method optimization is robust (Green 1996) within a well-defined mathematical model as the experimental design is used to substantiate changes in factor values in association to observed responses. Various chemometric strategies exist for the optimization of electroanalytical methods, those including, first-order models (Plackett-Burman design) and higher-order models (Central composite design, Box-Behnken design and Doehlert Matrix), (Tarley *et al.* 2009).

In order to optimize experimental parameters for the determination of polyphenolic compounds in wine samples, the Box-Behnken multivariate experimental design was effected to derive optimum conditions for the determination of the concentration of catechin using three factors. These include pH of the measuring buffer, deposition time (t_d) of the analyte to electrode paste and the scan rate (s_r). In order to relate response variables to evaluative variables, partial least squares (PLS) and Pareto charts were used. Additionally, electrochemical oxidation/reduction

reactions of catechin were studied with (CV), with the concentration of the TP being determined using the optimised conditions within the potential range of -0.2 V to 0.6 V.

4.2. Experimental

4.2.1 Apparatus

Electrochemical experiments were carried out using a 797 VA Computrace from Metrohm (Herisau, Switzerland). Cyclic voltammograms (CV) and Differential Pulse voltammograms (DPV) were monitored and measured with the 797 VA Computrace and the 797 VA software. An electrochemical cell with a conventional three-electrode setup was used consisting of a 3 mm diameter rotating disc glassy carbon (GC) working electrodes, a platinum wire counter electrode, and an Ag/AgCl (Saturated AgCl, 3 M KCl) reference electrode. A 781 pH/Ion meter coupled with 801 stirrer supplied by Metrohm (Herisau, Switzerland) was used for the pH adjustment of the buffer solutions. All working solutions were prepared with deionised water from an Aqua Max ^{TM} basic 360 Series from Trilab (Durban, SA) water purification system.

4.2.2 Reagents and Chemicals

All analytical grade reagents were used. (+)-Catechin hydrate (C1788) was obtained from Sigma (Durban, SA), Sodium phosphate dibasic (Na_2HPO_4) and Sodium dihydrogen phosphate dihydrate ($NaH_2PO_4.2H_2O$) were obtained from Capital Lab Supplies (Durban, SA). Nujol (Mineral oil) and Graphite powder (282863) < 20μm were purchased from Aldrich (SA). Sodium Hydroxide (NaOH) and 32% Hydrochloric Acid (HCl) supplied by MERCK (Durban, SA). Nitrogen gas of 99.9% purity was obtained from AFROX (Durban, SA). Alumina powder ≤ 3 μm was supplied by Metrohm (Durban, SA). Green apple was used for the preparation of the biosensor surface (paste) and the four wine samples Sauvignon Blanc (2010, bottled by Swartland Winery SA), Sauvignon Blanc from Du Toitskloof Cellar SA), Pinotage (2009, from Swartland Winery SA) and Baronne (2009, from Nederburg SA) were purchased at a local liquor store in Durban, SA.

4.3. Methods

4.3.1. Preparation of carbon paste electrodes

The GCE requires a cleaning procedure prior to modification. During several measurements, the electrode surface is contaminated with the product of the electrode redox process. In order to overcome this contamination, prior to modification, the surface of the working electrode

(GCE) was cleaned by polishing for 10s with alumina paste (mixture of alumina and water) on a polishing cloth followed by rinsing with high-purity water and dried with nitrogen., its activity was subsequently regenerated by electrochemically cleaning, scanning 5–10 cycles in the potential range between -0.5 and 1.0 V with a scan rate of 50 mV s^{-1} in the presence of a phosphate buffer solution.

Carbon paste with an approximate mass of 1.7 g comprised of 40 % graphite powder, 40 % of Nujol and 20 % of ground green apple was prepared. This paste was incorporated onto a chemically and electrochemically cleaned surface of GCE before electroanalytical measurements were performed. The electrode surface was renewed by incorporating a new paste for every scan performed, and the measuring solution was purged with nitrogen prior to analysis. However, it should be noted that the immobilization of the material can often be a problem due to the insufficient thickness of the tissues to maintain mechanical stability resulting in slow responses because of the long diffusion path between the test solution and the detector surface of the electrode. The polishing step with aluminium oxide was, therefore repeated once daily.

4.3.2. Preparation of phosphate buffer supporting electrolyte

The phosphate buffer solution was prepared by weighing suitable masses of $NaH_2PO_4.2H_2O$ and Na_2HPO_4 in a 500 ml volumetric flask followed by a pH adjustment using a 0.1 M HCl or 0.1 M NaOH to the desired pH. For the purpose of the multivariate design, as shown in Table 4.1, it was necessary to prepare three separate buffer solutions with varied pH values. All buffer solutions were stored in a fridge at 4 ^{o}C.

4.3.3. Preparation of the catechin standard

From a 1000 ppm (+)-catechin standard, a 1ppm, 10 ppm and 12 ppb stock solution of (+)-catechin was prepared by weighing an adequate volume and diluting in deionized water in volumetric flasks. These standards were used for the optimization of all intended experimental parameters.

4.3.4 Preparation of real samples

All wine samples were filtered through a Teflon disc filter (0.45 μm and 9 mm diameter). White wines were diluted 100 times, whilst red wines were diluted 1000 times with the phosphate buffer of pH 7.6 before electrochemical measurements were conducted. All sample measurements were done in triplicate, and the analytes were quantified by addition of the catechin standard in the measuring cell containing the wine sample.

4.3.5 Procedure for optimization of experimental parameters in Box–Behnken design

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A Box-Behnken design with fifteen levels, three independent factors (pH, t_d and s_r) and three replicates at the central point was applied to this study. The carbon paste electrode was prepared as described in section 4.3.1 and used to perform electrochemical measurements at varied independent factors. The experiment was fully randomized to ensure that confounding variables were lowered, this being attained by levelling the three independent variables that were not accounted for in the optimization design. Table 4.1 shows the experimental design of the three factors pH, t_d and s_r. The method was optimized with respect to the current signal which is proportional to the concentration of catechin within an oxidation potential range of -0.2 V to 0.6 V. Analysis of variance (ANOVA), Pareto charts, interaction plots and surface responses were performed to evaluate the statistical and correlation data of the responses of the executed model. The design structure enables the full evaluation of the responses in relation to simultaneous factor variations on all levels studied. This includes the optimization of the experimental conditions for the determination of polyphenolic content in wine samples, using a 12 ppb stock catechin standard at a pH ranging from 7.2 to 7.8.

Table 4. 1: Shows the structure of the 3 factors, 15 levels of Box-Behnken experimental design and response values *Average of three replicate.

ID	pH	t_d(s)	S_r (mV s^{-1})	*[Cat](mg/l)
1	7.5	35	2.5	8.69
2	7.5	30	3.0	12.96
3	7.8	30	3.5	11.62
4	7.5	25	3.5	10.84
5	7.8	25	3.0	11.33
6	7.2	30	3.5	10.34
7	7.2	25	3.0	11.20
8	7.5	35	3.5	11.82
9	7.5	30	3.0	11.72
10	7.5	25	2.5	11.27
11	7.2	30	2.5	8.93
12	7.8	30	2.5	11.24
13	7.8	35	3.0	10.69
14	7.2	35	3.0	10.97
15	7.5	30	3.0	12.73

4.4 Data Evaluation

STATGRAPHICS *Plus* version 5.1 and Microsoft excel® 2007 were used for data evaluation and preparation of the experimental design. Peak evaluation was performed with 797 PC Software 1.3 ® 2008.

4.5 Results and discussion

4.5.1 Optimization of experimental procedure

The relationship between variables and their bearing on the actual determination of catechin in wine samples can be understood by applying a Box Behnken design. The Box-Behnken design was created to analyse the effects of 3 factors in 15 runs (Martinez-Gomez *et al.* 2005). As shown in Table 4.1, to obtain the optimum parameters, three replicates were measured for each sample with the average response being recorded. To establish even distribution of the experiment a scatter plot in Fig 4.1 presents the multivariate data in a three-dimensional space displaying values of one variable determined in the position on the horizontal axis and the other variable determined in the position on the vertical axis.

Plot of Dt vs pH and Sr

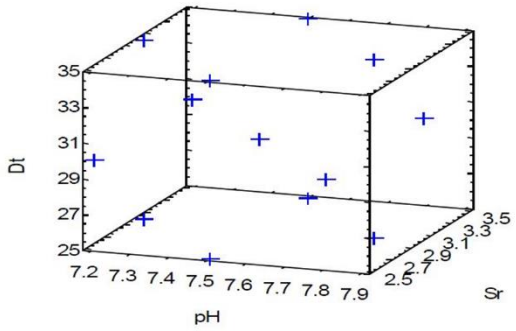


Figure 4. 1: 3D Scatter plot of variables td versus pH and sr used for the experimental design. t_d is the deposition time, pH is the ionic strength of the phosphate buffer and s_r is the scan rate.

Undesirable factors that are not of prime concern and may affect the measured result was eliminated by randomized experiments. During this study, the implementation of the multivariate analysis approach permitted the identification of interaction effects between the experimental parameters. A 12 ppb catechin standard was used to investigate the cathodic voltammetric responses influenced by deposition time (t_d). The optimization of the variable t_d is pivotal as deposition time affects the current signal, which is proportional to concentration. The next crucial variable is the pH of a buffer as this variable determines the half-wave potential ($E_{1/2}$) in all electrochemical methods. In the case of this study, the optimum value yielded for the pH of the buffer was 7.65. A graphical representation of the interaction between two variables A and B can be seen in the Pareto chart in Fig 4.2. The Pareto chart reveals a favourable matrix effect of the parameters in decreasing order of importance (Martinez-Gomez *et al.* 2005).

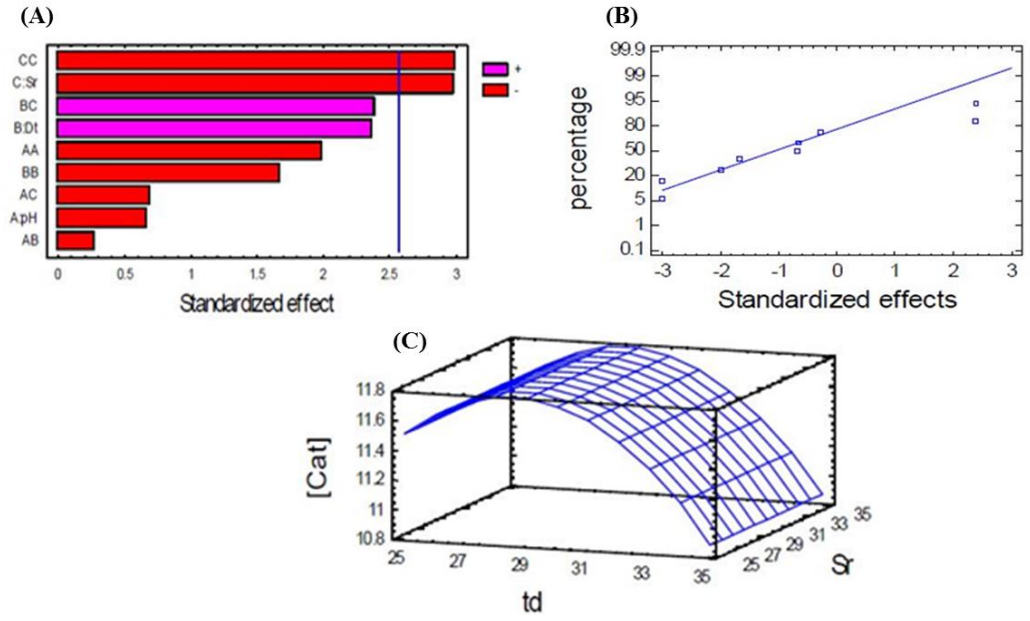


Figure 4. 2: (**A**) Pareto Chart shows the matrix of selected variables against the standardization effect (**B**) Matrix are ranked according to the order of importance (**C)** the response surface plot shows the distribution of [Cat] as a function of td and Sr at constant pH 7.5 after elimination of C:C, C:Sr and B:C.

The coefficient of determination depicted as the R-Squared statistic indicates that the fitted model explains 85.124 % of the variability in [Cat], (Fig 4.2A). Significant effects are presented on the vertical line plot and the length of each bar denoted is proportional to the standardized effect (Fig 4.2B). In the standardised Pareto chart, bars of matrixes C:C and C:Sr extend further than the line depicting effects that are statistically significant at the 95% confidence level. A closer look at the linear effect of the Scan rate on the concentration of catechin values reveals a great significance thus resulting in the elimination of the matrixes C:C, C:Sr, and B:C prior to the optimisation/standardization of the experimental design. The maximum catechin response obtained when td= 30s as presented in Fig 4.2C of the response surface plot. Within the preselected pH of 7.5, s_r is shown to have no effect on the concentration of catechin. This is as a result of the increase in deposition time which ultimately can cause an increase in solubility thereby increasing the current response. Mutual concession between pH of the buffer and td is necessary in certain experimental models. Table 4.2 depicts the optimized electrochemical parameters utilized from using standard solutions for the qualitative measurements using CV and the quantification of Total Phenolic (TP) content in different wine samples using DPV.

Table 4. 2: Shows the list of the optimised parameters that were used for determination of the concentration the total polyphenolic compounds in wine samples.

Factors	Low	High	Optimum
pH	7.2	7.8	7.65
t_d	25.0	35	29.77
s_r	25.0	35	25.0

4.5.2 Electrochemical oxidation/reduction of polyphenols (biosensor)

The choice of electrolyte solution or buffer has a significant effect on the half-wave potential therefore the $E_{1/2}$ potential can be reported with respect to the buffer used to perform electroanalytical measurements. For this study phosphate buffer with pH 7.6 was used. Immobilization of the material can be extremely difficult due to the extended diffusion path between the buffer solution and the detector surface. For this reason, the tissue material should be of an adequate thickness to maintain chemical and mechanical stability. It was imperative to keep the solution unstirred during the experimental measurement which results in the occurrence of mass transport by diffusion due to the concentration gradients created around the immobilised biosensor surface.

Adsorption of the analyte onto the modified electrode was reduced as the working electrode was immersed into the buffer solution, followed by a direct CV measurement at a scan rate of 25 mV/s in the potential range, from -0.2 to 0.6 V. The electroactive nature of the polyphenols are well documented, as the existence of the hydroxyl groups attached to the aromatic rings, undergo electrochemical oxidation (Janeiro and Brett 2004). The oxidation mechanism of catechin progresses in successive steps in relation to the catechol moiety (B-ring) and the 3'-hydroxyl groups. Fig 4.3 describes the observed oxidation process when the potential of the working electrode is more positive than that of a redox couple present in solution.

The anodic peak E*pa* is observed at 0.219 V. In contrast, the cathodic peak E*pc* is at 0.128 V, occurring as the working electrode potential moves toward becoming more negative than the reduction potential of a redox couple. The Epa value ranges between -0.15 and 0.25 V, with the main peak due to the oxidation of the catechol moiety, the 3',4'-dihydroxyl electron-donating groups at the B ring, which appears first at a very low positive potential**.** The voltammetric responses of catechin is in accordance with literature (Janeiro and Brett 2004).

The determined potential of $E^{0'}$= 0.18 V, for a reversible couple found between *Epa* and *Epc* denoted by $E^{0'}$ = (*Epa* + *Epc*)/2. The observed Epa peak at 0.22 V, suggests that catechin has a relatively high antioxidant activity, when compared to other flavonoids (Simić *et al.* 2007).

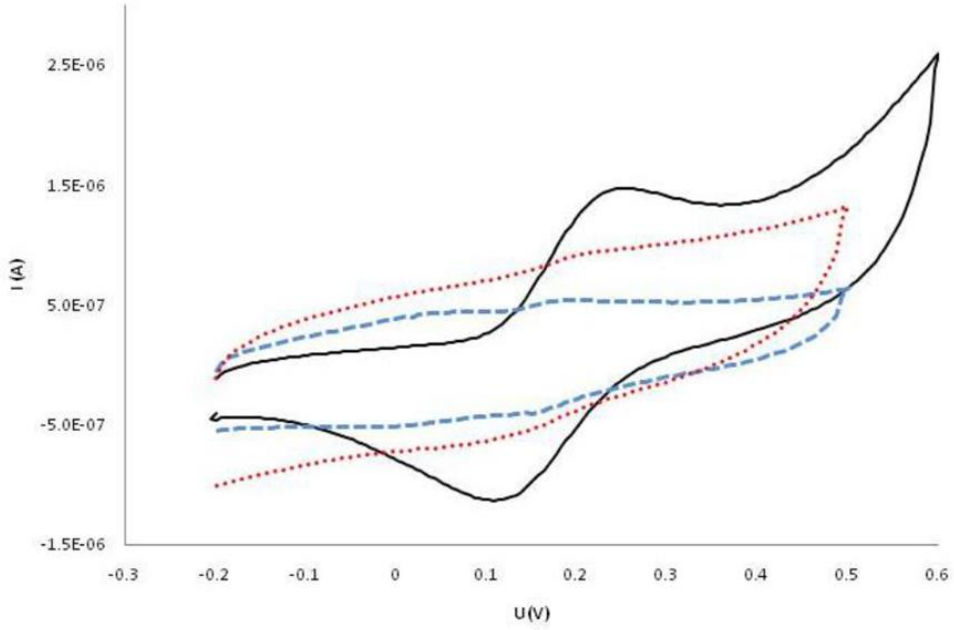
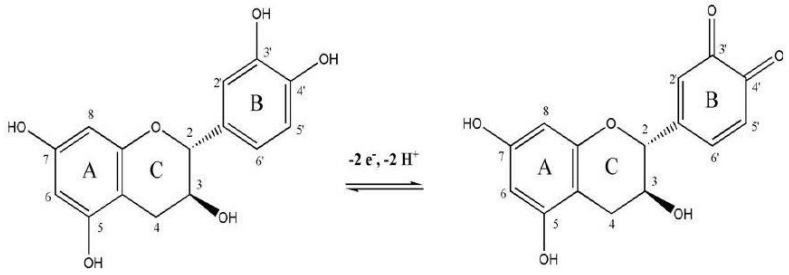


Figure 4. 3: Cyclic voltammogramms of (+)-catechin within -0.20 V to 0.60 V. ••••Bare glassy carbon electrode in a buffer, ----GCE modified with apple paste in a buffer and — oxidation/reduction of catechin standard at the modified GCE.

This can be attributed to the (+)- catechin molecule having several -OH functional groups attached to all three rings (A5, A7, B3', B4' and C3). Although all hydroxyl groups can undergo electrochemical oxidation, the catechol (B-ring) is more easily oxidizable than the resorcinol (A-ring), and glycoside (C-ring), as shown in Scheme 4.1, (Janeiro and Brett 2004; Maoela *et al.* 2009; Šeruga, Novak and Jakobek 2011). The two hydroxyl substituents of the B-ring are oxidised consecutively forming a quinone as seen in Scheme 4.1.



Scheme 4.1: Oxidation/reduction scheme of catechin under optimum conditions; pH of electrolyte 7.65, t_d of 29.77s and S_r of 25.0mV/s. (Janeiro and Brett 2004; Maoela *et al.* 2009; Šeruga, Novak and Jakobek 2011).

Various electrode materials can be modified to study the oxidative/reductive mechanisms, however GCE is the most suitable for organic molecules. One of the major properties is that it is highly solvent resistant and exhibits a low background current, unlike most metals. Peak current values were used to calculate the limit of detection (LOD) and the limit of quantification (LOQ) with the following equations: LOD = 3 s/m, and LOQ = 10 s/m, where s represents the standard deviation of the peak currents (n=5) and m is the slope of the calibration curve (Yilmaz *et al.* 2008), as shown in Fig 4.4.

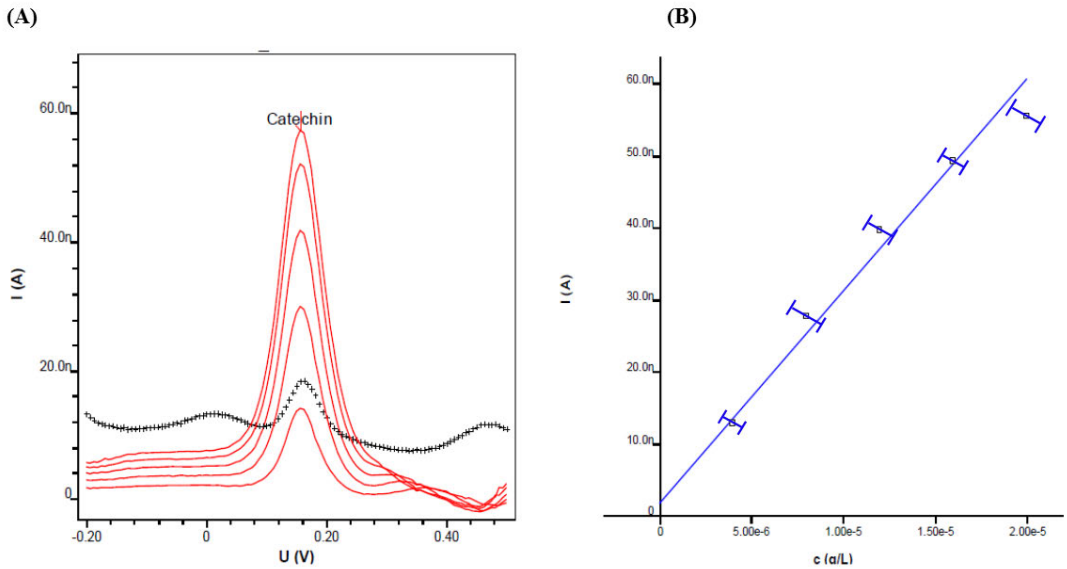


Figure 4. 4: **(A-B)** Differential Pulse Voltammogram (DPV) of catechin standards 40, 80, 120, 160 and 200 ppb obtained using optimised parameters from experimental design section 2.3.5. pH of electrolyte 7.65, td of 29.77s and sr of 25.0mV/s. **** blank and — catechin standards from 40 to 200ppb.

The $E_{1/2}$ of the polyphenolic compound remains constant as observed in Fig 4.4A as the concentration increases. Calibration data corresponding to Fig 4.4B yields a linear response with a correlation coefficient (R^2) of 0.98820. The linear dynamic range was found to be between 5.00 e $^{-6}$ µg/L – 2.00 e $^{-5}$ µg/L. The lowest detectable LOD concentration of the standards with green apple paste was found to be 1.76 ppb and lowest quantifiable concentration LOQ was 5.86 ppb (shown in Table 4.3).

Table 4. 3: Statistics of the calibration plot and limit of detection at optimum conditions.

Analyte	**Y.reg/offset**	**Slope**	**Mean dev**	**Corr. Coeff**	**LOD (ug/l)**	**LOQ (ug/l)**
Catechin	1.717e-009	2.944e-006	2.295e-009	0.98820	1.76 ±0.32	5.860 ±0.58

The ability to achieve this level of sensitivity is related to the redox process occurring between the polyphenolic constituent in the electrolyte solution and the electron transfer mechanism taking place at the junction of the modified working electrode. The increased sensitivity is also attributed to oxidative processes at the phenolic B–Ring of catechol. It is not uncommon to expect the varied TP content values in wine samples as was reported in this study. The variety of branded wines, grape variants, colours, regions, seasonal changes and wine making protocols yield multifarious phenolic content and antioxidant activities (Eggins *et al.* 1997; Gambelli and Santaroni 2004; Staško *et al.* 2006; Peres *et al.* 2009; Šeruga, Novak and Jakobek 2011).

Standard addition is employed to provide a reasonable level of accuracy for the concentration of TP content in wine samples. This technique also can evaluate the sample matrix effects. A typical DPV for the wine sample and catechin standard is presented in Fig 4.5 below. A blank consisting of only the electrolyte solution was therefore measured before performing two additions factoring in the presence or absence of the electroactive species present in the buffer. The measured current signal obtained for the blank was thus subtracted from standards and samples thereafter.

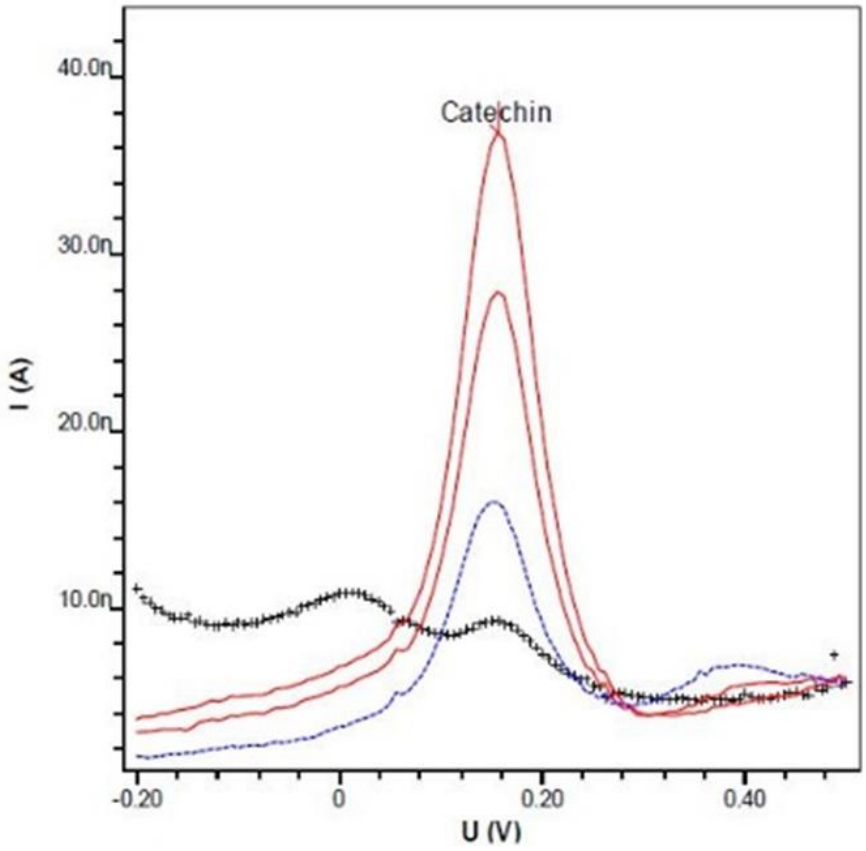


Figure 4. 5: DPV of the red wine (Baronne) sample. **** blank (electrolyte and paste), - --- (sample) peak due to Total Phenolic content appears at 0.18 V in the first scan. — (addition 1 and 2) Overlaid peaks due to addition of catechin standard.

Catechin equivalent values for both red and white wines produced notable differences and is tabulated in Table 4.4. In most studies the main reasoning for this variance often results from processes in wine making.

Table 4. 4: Table of results obtained from wine samples using DPV at optimum conditions (n=3).

Wine Samples	**Color**	**Catechin Equivalent(mg/l)**	**Mdn (mg/l)**	**RSD%**
Swartland	white	58.54	59.04	1.72
Du Toitskloof	white	59.38	59.20	2.33
Nederburg	red	612.67	598.20	4.74
Swartland	red	1033.53	1033.80	1.40

Both red and white grapes go through a different process to achieve the same outcome which is fermented wine. Red grapes are crushed with their skins and fruit in the process to result in red wine while white grapes are converted into a juice without the skin and flesh of the fruit and then produced into wine for consumption. The measured values obtained are representative of the total phenolic concentration using catechin equivalent concentration. Techniques involving entrapment utilize irreversible polymerization processes and often have the disadvantage of slow reaction times. This is caused by enzyme activity loss through the porous gel matrix. On the positive side the electrode has a much longer lifespan when compared with with adsorption immobilization processes as in the case of the green apple modified sensor which had to be renewed after every analysis run. The reported methods are sensitive due to the modified electrode surfaces using nanomaterials however the usage of apple paste in our study makes the sensor cost effective and eco-friendly.

4.6 Conclusion

The voltammetric behaviour of antioxidants was investigated by various electrode modification and fabrication techniques. It was evident from the study that parameters such as scan rate (s_r) electrolyte pH and deposition time (t_d) had to be sufficiently optimised before the electroanalytical analysis. This was necessary to ensure reproducibility and accuracy of the results. To understand the interaction of electrochemical parameters the predicted Box-Behnken experimental design proved proficient, as experimental data obtained in the optimization study are comparable with the estimated values presented on the surface response models. It was observed that immobilization of enzymes and tissue materials with adequate thickness ensure mechanical stability yielding results for phenolic content in red and white wines. A LOD of 1.76 ppb was obtained for catechin indicating that green apple immobilised paste is quite sensitive. Glassy Carbon Electrode modified with green apple paste is a cost

effective, eco-friendly option when compared to more expensive nanomaterials used in newer methodologies. The actual measured content of the catechin (equivalent) in white and red wines was found to be approximately, 60 ppm and 500-1000 ppm respectively, which is acceptable for control purposes in the brewery industry. The chosen experimental design can be similarly applied to various other analytical methods and techniques.

CHAPTER 5

CASE STUDY 2: A GLASSY CARBON ELECTRODE MODIFIED WITH LACCASE FOR THE QUANTIFICATION OF ANTIOXIDANT CAPACITY IN TEA

This chapter investigates and evaluates the antioxidant properties in polyphenolic compounds found in tea by electrochemical techniques using a modified laccase biosensor. Rutin and ascorbic acid were used to determine antioxidant capacities based on an antioxidant standard Trolox. *In vitro* photometric method using 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid (ABTS) as a mediator to assess laccase activity is employed and applied to this study.

5.1 Introduction

Herbal teas in particular, green and rooibos varieties are being investigated for their health benefits, particularly for the role they play in the prevention and reduction of an array of diseases in the human body, such as cancer, heart disease and diabetes (Weisburger and Chung 2002; Borges *et al.* 2016; Lorenzo and Munekata 2016; Nash and Ward 2017; Williamson 2017; Xie *et al.* 2017).

Previous research indicates that rooibos and many herbal variants are more accurately referred to as tisanes as they are not derived from the Camellia sinensis species of the tea plant , however both teas and tisanes are being recognized for their polyphenol content thought to conciliate tremendous health benefits (Cleverdon *et al.* 2018). Polyphenols exhibit great antioxidant activity due to their reducing properties (Wu 2002). The polyphenol outline in both teas and tisanes is significantly varied thus leading to multiple biological processes including antioxidant, antifibrotic, hypolipidemic, hypocholesterolemia, anti-obesity, antiviral, antimutagenic and neuroprotective effects (Scalbert, Johnson and Saltmarsh 2005; Preedy 2012).

Green or red varieties of Rooibos originate from the Aspalathus linearis species, while green tea is derived from the Camellia sinensis species (McAlpine and Ward 2016). Rooibos in

particular has sparked much interest as it does not contain caffeine while green tea on the other hand stems from the Camellia sinensis plant. It contains around 30–50 mg of caffeine per serving which is insignificant compared to coffee.

Rooibos and green tea varieties comprise of vitamins, amino acids, proteins, caffeine, volatile compounds, polyphenols, and several undefined constituents (Cabrera, Giménez and López 2003). The core structure of polyphenolic compounds includes one or more aromatic rings containing hydroxide substituents and or functional groups. Combined, a group of phenolic compounds fall into a class of flavonoids called catechins (Lima *et al.* 2009; Ashihara *et al.* 2010). The following primary catechins are found in green tea; (-) epigallocatechin gallate, (-)epigallocatechin, (-)epicatechin gallate, epicatechin and catechin (Ho *et al.* 1999; Nagle, Ferreira and Zhou 2006).

Analytical methods have been employed for the evaluation of antioxidant compounds in plasma, pharmaceutical formulations, fruit juices, wines and teas involving techniques of separation, such as high-performance liquid chromatography with electrochemical detection (HPLC-ECD) (Kilmartin 2001), potentiometric (Amini *et al.* 2001), gas chromatography mass spec (GC-MS) (Wojdyło, Oszmiański and Czemerys 2007) and voltammetry (Alemu, Abegaz and Bezabih 2007; Zhang, Xu and Zheng 2007; Yilmaz *et al.* 2008; Šeruga, Novak and Jakobek 2011). Many of the analytical techniques are costly and require tedious pre-treatment steps, additionally large volumes of eluent and reagents are often wasted in the process.

Electrochemical techniques involving fabricated electrodes are used to study redox mechanisms, as they are more sensitive to the bare electrodes. Biosensors effectively are based on the interaction between a substance of interest and the biological material immobilized onto its surface. The concentration of the analyte is proportional to the signal output, for quantitative evaluation. The choice of the biological substance used in the immobilization procedure may contribute to the minimal pre-treatment of a cost effective more sensitive, selective and reproducible sensor. The present work uses an in-house laccase modified biosensor for the electrochemical investigation of the antioxidant properties of herbal tea samples.

Laccase Trametes versicolor enzyme was used for this study. The laccase molecule is a glycoprotein being characterised by four copper atoms in each monomer, distributed in three redox centres forming a trinuclear copper ‘cluster’ with a molecular mass between 50-100 kDa

(Gomes and Rebelo 2003). Belonging to the family of multi copper oxidases they are produced by microorganisms, higher order plants, fungal species including insects and bacteria (Gianfreda, Xu and Bollag 1999; Di Fusco *et al.* 2010). These enzymes are responsible for acceleration of oxidation reactions of phenolic species followed by the reduction of oxygen to water. A pertinent characteristic in the formation of laccase leads to greater enzyme stability attributed to molecular bonding within the carbohydrate moiety (Piette, Ludwig and Adams 1962). These enzymes tend to display a broad specificity for reducing substrates such as polyphenols, aromatic diamines and aminophenols, with the oxidation process leading to the development of free radicals with the transfer of a single electron transfer to laccase. Following on, the oxidation of the free radical by laccase produces polymers or quinones arising from a phenol (Solomon, Baldwin and Lowery 1992; Xu 1996; Rosatto *et al.* 2001).

Applications utilizing laccase enzyme are diverse and found in pulp bleaching, removal of phenolics from wine, organic synthesis, effluent decolouration, detergents and medical components (Wong and Yu 1999; Kunamneni *et al.* 2007). Immobilization and modification procedures on various working supports have been studied, using laccase from several origins (Durán *et al.* 2002).

Various electrochemical methods exist for the evaluation and detection of the antioxidant activity, including the development of chromatographic, fluorimetric and photometric methods. Photometric methods utilizing the synthetic radicals, ABTS [2, 2'-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)] and DPPH* [2,2-diphenyl-1-picrylhydrazil], are often used as a comparative antioxidant measure against the standard antioxidant Trolox, to evaluate the TEAC value (Trolox equivalent antioxidant activity), (Miller *et al.* 1993; Brand-Williams, Cuvelier and Berset 1995; Fukumoto and Mazza 2000; Pulido, Bravo and Saura-Calixto 2000). Further research has shown that ABTS is a favourable mediator used in the evaluation of laccase activity in fungal cultures, as it displays an efficient electron transfer between the laccase and the working surface area to generate a stable chromophoric cation radical of ABTS (Gil and Rebelo 2010). The well established mechanism for ABTS, involves two consecutive single-electron oxidations by the laccase enzyme whereby, the electrolyte matrix, the electrode composition and the pH directly impacts on the stability of $ABTS^{+}$ and $ABTS^{2+}$ (Staško *et al.* 2006).

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This study utilizes electrochemical methodology to assess the antioxidant properties of tea samples, with a modified laccase sensor, due to their potent antioxidant activity. Green tea and rooibos tea varieties were evaluated as rich sources of antioxidants, with rutin and ascorbic acid as Trolox compounds. On the otherhand, spectrophotometric procedures were selected to assess the antiradical capacity of the ABTS free radical by the reduction process.

5.2. Experimental

5.2.1 Apparatus

All analytical measurements were performed with a 797 VA Computrace from Metrohm (Herisau, Switzerland) including a three-electrode system consisting of a 3mm diameter rotating disc electrode (GCE), an auxiliary electrode made of platinum wire and a reference electrode made of Ag/AgCl (saturated AgCl, 3 M KCl). Voltammograms were evaluated with the Metrohm 797 VA Computrace. A Metrohm 781 pH/Ion meter coupled with 801 stirrer was used for pH adjustment of electrolyte solutions. The Aqua Max^{TM} Basic 360 Series water purification system supplied by Trilab (Durban, SA) was used to prepare all working solutions. Samples and electrolyte solutions were stored in the refrigerator at a temperature of 4 °C. Spectroscopic measurements were performed with a Varian 50 UV/Visible spectrophotometer with a 1.0 cm path length cell. The integrated software within the spectrophotometer allowed for automatic peak evaluation (current signal), absorbance and estimation of the concentration in a standard addition mode. All analytical measurements were performed at room temperature.

5.2.2 Reagents and chemicals

All analytical grade reagents were used for this study. Rutin trihydrate (78095) and ABTS were obtained from Sigma (Durban, South Africa SA). Sodium phosphate dibasic (Na_2HPO_4) and Sodium dihydrogen phosphate dihydrate ($NaH_2PO_4.2H_2O$) were obtained from Capital Lab Supplies (Durban, SA). Nujol (Mineral oil) and Graphite powder (282863) < 20µm were purchased from Aldrich (SA). Sodium hydroxide (NaOH), potassium persulphate ($K_2S_2O_8$) and Ascorbic Acid was supplied by MERCK (Durban, SA). Nitrogen gas of 99.9% purity was obtained from AFROX (Durban, SA). Alumina powder ≤ 3µm was supplied by Metrohm (Durban, SA). Trametes versicolor laccase (51639) was supplied by Sigma (Durban, SA) and used for the modification preparation of the biosensor surface. Green tea and Rooibos tea, were purchased at a local supermarket in Durban, SA.

5.3. Methods

5.3.1. Fabrication of laccase/graphite/GCE

Enzyme immobilization can be performed by a variety of methods such as adsorption, entrapment, crosslinking and covalent bonding. In this instant, adsorption of the enzyme onto a solid state electrode is carried out. Approximately 0.5 g paste composed of laccase (0.2g), nujol (0.1g) and graphite (0.2g) was immobilized on the polished surface of the GCE. Alumina Al_2O_3 was used to manually clean the glassy carbon electrode followed by an electrochemical cleaning procedure scanning within a -1.0 to +1.0V range. A 40 mM laccase standard was prepared from which 2 ml was used for film deposition on the paste for duration of 10 min. This process was performed to ensure maximum electrical contact between the modified laccase GCE and the measuring solution.

5.3.2. Preparation of phosphate buffer

Phosphate buffer solution was prepared by weighing suitable masses of $NaH_2PO_4.2H_2O$ and Na_2HPO_4 in a 500 ml volumetric flask followed by a pH adjustment using a 0.1 M HCl or 0.1 M NaOH to the desired pH. All buffer solutions were stored in a fridge at 4 ^oC.

5.3.3. Preparation of rutin and ascorbic acid standards

1 mM stock solutions of ascorbic acid and rutin were prepared by dissolving an equal mass in the methanol: buffer (50:50 v/v) ratio. Working standard solutions were prepared from these stock solutions by dilution with the buffer. The standard addition curve was prepared by using 10.0 mg l^{-1} solutions of ascorbic acid and rutin. Total Antioxidant values are expressed in terms of rutin and ascorbic acid equivalent per mass, as they were used as reference compounds in this study.

5.3.4 Extraction of phenolic content from herbal tea samples

Herbal tea extracts from samples were obtained by brewing approximately 2.0 g of tea sample with 100 ml of hot water in a vacuum flask at 65 ^oC for 30 min while placed on the rotary hot plate. This process ensures maximum yield and minimal loss through evaporation since the analytes are volatile. After extraction, the obtained aqueous herbal tea extracts were subsequently analysed for their total phenolic contents and radical scavenging capacity by electrochemical methods and ABTS radical scavenging methods.

5.3.5 Oxidation of phenols by ABTS

In terms of hydrogen donating ability or free radical scavenging ability, the free radical scavenging activity was measured using the stable ABTS. Rutin and ascorbic acid standards were prepared separately and mixed with 2.0 ml of a 25 mM ABTS enzyme followed by incubation at a temperature of 25°C for 30 min thus allowing the reaction to reach equilibrium. Absorbance measurements for both reaction mixtures was performed with a double beam UV-Visible spectrophotometer at respective wavelengths. The λ_{max} of rutin and ascorbic acid standards were determined before absorbance measurements. Taking the absorbance of the blank into account, a calibration curve was plotted for absorbance of Trolox (rutin and ascorbic acid). Various volumes of the tea extracts between (0.1 and 1 ml) with 2 ml of ABTS and phosphate buffer were added in a vial up to a total volume of 3.5 ml. Before absorbance measurements, the reaction mixtures were placed in a dark area for 5 min. Absorbance measurements were recorded at 734 nm and analysed in triplicate. A calibration plot represented as EC_{50}, (which is the concentration of sample required to scavenge 50 % of ABTS) value was determined.

5.3.6 Electrochemical method

In the electrochemical cell, a 15 ml volume of supporting electrolyte and 1 ml of the standard solution was added by micro-pipette. The solution was purged with pure nitrogen prior to electrochemical measurements. During CV measurements the solution was stirred. Measurements were run from -1.00 to +1.00 V at the laccase immobilized GCE with a scan rate of 100 mV s^{-1}. The same procedure was followed for sample analysis. The standard addition method was applied adding successive aliquots of 10 µL as 10 µM ascorbic and rutin standard solutions to the electrochemical cell. The DPV was recorded at a scan rate of 5 mV s^{-1} and a pulse amplitude of 50 mV with the potential ranging from -200 mV to +600 mV. After the stirrer was stopped, the potential was scanned towards a positive potential. All measurements were carried out at room temperature (23 ± 3 ^{o}C).

5.4 Data evaluation

Nova Software 1.3 ® 2008, along with the VA 797 Computrace software was used for peak and electrochemical evaluation. Statistical data between Trolox Equivalent Antioxidant Capacity (TEAC) of the extracts, antioxidant activity AA, and TP content were evaluated by analysis of variance (ANOVA). STATGRAPHICS Plus version 5.1 and Microsoft excel® 2010 was used for Grubb's test for outlier identification.

5.5 Results and discussion

5.5.1 Optimization of experimental procedure

Immobilization of the enzyme onto the surface of the electrode remains to be one of the most important factors in attaining performance of a laccase biosensor. To achieve greater sensitivity and reproducibility an alternative approach to electrode modification was sought. The immobilization technique involves cross-linking whereby the enzyme is chemically bonded or fixed to a solid support enzyme. This method is most useful for stabilizing adsorbed biomaterials. Poor electronic coupling between electrode and enzyme can occur due to denaturation, in which the biochemical properties of laccase can be affected (Shleev *et al.* 2005). Fig 5.1 reveals that the voltammetric responses (I_p) and the peak potentials are influenced strongly, when the concentration of buffer is not sufficient to maintain the pH at the surface of the working electrode, despite the appearance of new peaks (Yilmaz *et al.* 2008).

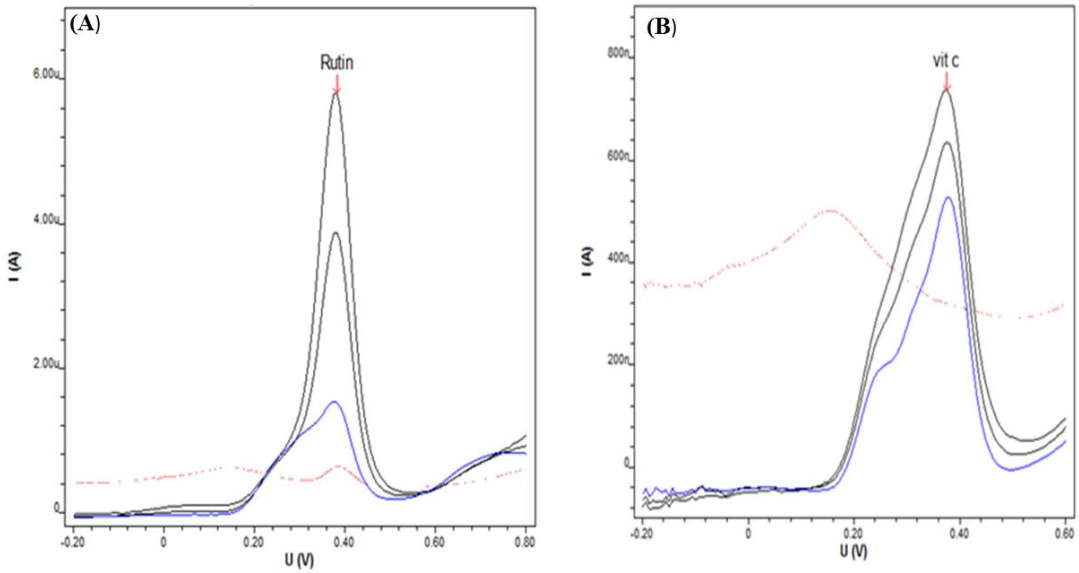


Figure 5. 1: Shows DP voltammogram of recorded at scan rate 5 $mV.s^{-1}$, pulse amplitude 50 mV, initial potential -200 mV and final potential +600 mV(**A)** Rutin and (**B**) Ascorbic acid equivalent Green tea. ---blank, ——sample, — standard addition 1 and 2.

Electrode potential values are dependent upon the redox behaviour of the compounds showing their oxidative ability. As can be seen in Fig 5.1 as the concentration of extracts are added to the vessel, reducing abilities of the herbal tea is increased, resulting in superimposed voltammograms. Experimental conditions are well optimised as can be seen in the presence of the current signal (I_p) of the samples. The laccase enzyme which is of relatively large molecular size can affect the rate of diffusion to the transducer hence optimisation of the current response signal was of importance. A sensitivity of approximately 1.5 uA/5 µM was established for the immobilized laccase electrode. The total phenolic content was determined by Differential Pulse Voltammetry, whereby the current is proportional to the concentration as can be seen in Table 5.1. Continuous renewal or rejuvenation of the electrode surface between determinations was required.

Table 5. 1: Differential Pulse Voltammetry (DPV) results for teas samples (n = 3). Comparison of Total Phenolic (TP) contents and Antioxidant Activities (AA).

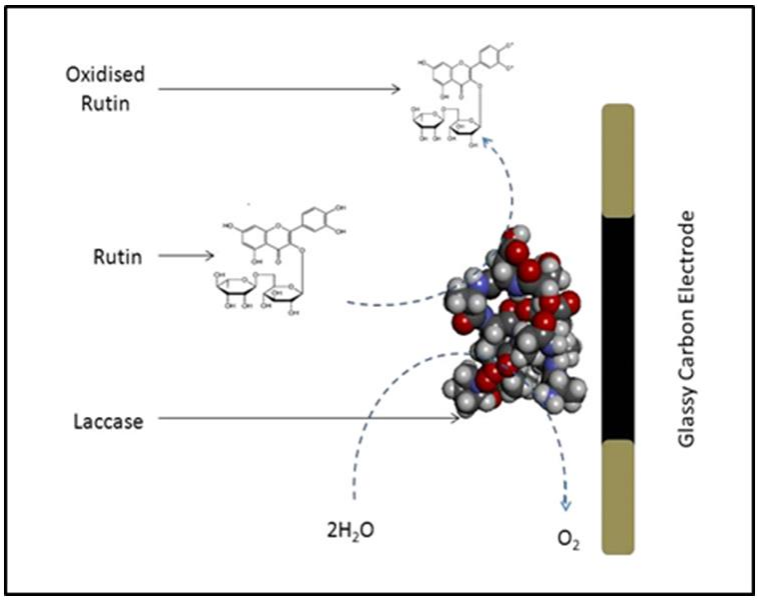
Sample	**Analytes**	TP $_{biosensor}$ **(mg/g)**	**Corr. R^2**
Rooibos	Rutin	5.282± 0.23	0.9976
	Ascorbic Acid	2.253± 0.34	0.9952
Green Tea	Rutin	7.282± 0.18	0.9976
	Ascorbic Acid	5.35± 0.09	0.9993

In Table 5.1, rutin represents the polyphenolic content and ascorbic acid represents phenolic equivalent content. The polyphenolic content appears to be greater in quantity in the evaluated tea samples than phenols.

5.5.2 Electrochemical oxidation/reduction of polyphenols (biosensor)

A modified laccase biosensor was used for the determination of phenolic content in tea samples as the laccase enzyme exhibits good electron transfer kinetics. During the reaction mechanism, laccase oxidises the substrate resulting in a loss of a single electron thus usually forming an oxygen centred free radical, which may undergo further enzyme-catalysed oxidation or otherwise a non-enzymatic reaction such as hydration, disproportionation or polymerization (Javanmardi *et al.* 2003). The oxidation mechanism of rutin results in the

formation of the conjugate base which is pictorially depicted in Scheme 5.1. The corresponding cyclic voltammogram is shown in Fig 5.2B.



Scheme 5.1: Image of the mechanism of rutin oxidation by immobilized laccase on the GCE surface.

The formation of the conjugate base as seen in Scheme 5.1 is attributed to the deprotonation of antioxidant molecules taking place at ±0.45 V. Laccase enzymes have a molecular mass of 60-90 k Da and contain between 15-30 % carbohydrate. Laccase enzymes contain four copper ions per molecule that typically undergo one electron oxidation of the related phenolic compound followed by reduction of oxygen to water (Durán *et al.* 2002). During substrate oxidation by laccase four electrons from the substrate compound are taken while the Cu^{2+} of its active center undergoes reduction to Cu^{+}. Transferred electrons to produce water returns the reduced laccase to rest status. The insertion of low molecular weight organic compounds or mediators are used to facilitate oxidation of non-phenolic compounds by the highly active radicals besides laccase. The prediction of redox mechanisms and phenol determination is based on the mediated electron transfer during enzyme application. The oxidation of the reducing substrates involves the free radical formation upon the transfer of a one electron laccase.

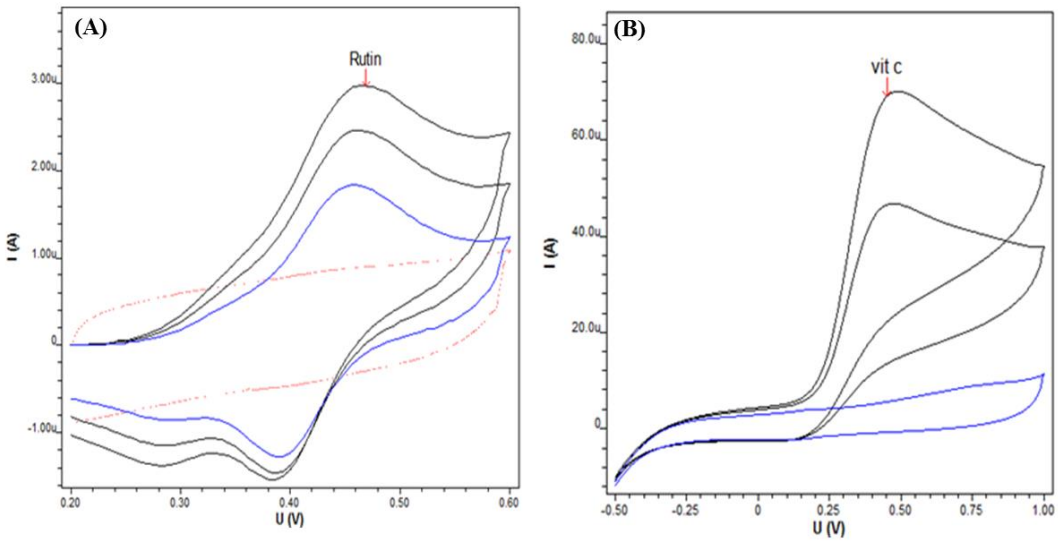


Figure 5. 2: **(A)** Rutin and (**B**) Ascorbic Acid obtained from a Green tea sample. CVs of a laccase modified biosensor in 0.1 M phosphate buffer at pH 5.5; Scan rate 100 mVs^{-1} in potential range of (-0.2 - 1.0 V).

The cyclic voltammograms of rutin and ascorbic acid at the surface of the modified laccase biosensor at a pH 5.5 is shown in Fig 5.2. The electrocatalytic oxidation of ascorbic acid can be expressed by the following reaction:

$$C_6H_8O_6 \rightarrow C_6H_6O_6 + 2H^+ + 2\,e^- \quad \text{(Eq 5.1)}$$

(Ascorbic Acid) (Dehydroascorbic Acid)

The oxidation of ascorbic acid involves two electrons and two protons leading to the production of dehydroascorbic acid. Following at a pH lower than 5.5 is an irreversible reaction (Yilmaz *et al.* 2008). During this experiment it was found that ascorbic acid has a reduction peak at $E_{1/2}$ = E_{pa} = 0.45 V and is irreversible while rutin has an $E_{1/2}$ calculated to be 0.43 V and an E_{pa} and E_{pc} at 0.46 and 0.39 V respectively. In the presence of polyphenol oxidase, compounds containing the catechol group are known to be oxidised forming 1.2-benzoquinone, the reaction in this case can be reversible (Wojdyło, Oszmiański and Czemerys 2007).

5.5.3 Trolox equivalent antioxidant capacities (TEAC) and EC_{50}

In the presence of potassium persulphate $K_2S_2O_8$, the ABTS cation radical was produced at an absorbance of 734 nm. This reaction was also responsible for a visible deep purple colour that was stable at room temperature. The existence of phenol in samples was tested by initially mixing ABTS radical solution with a sample extract, resulting in a positive colour change observed after a few minutes of standing in a dark environment. Absorbance measurements were read after a few minutes and performed in triplicate. Antioxidant activities are shown in Fig 5.3.

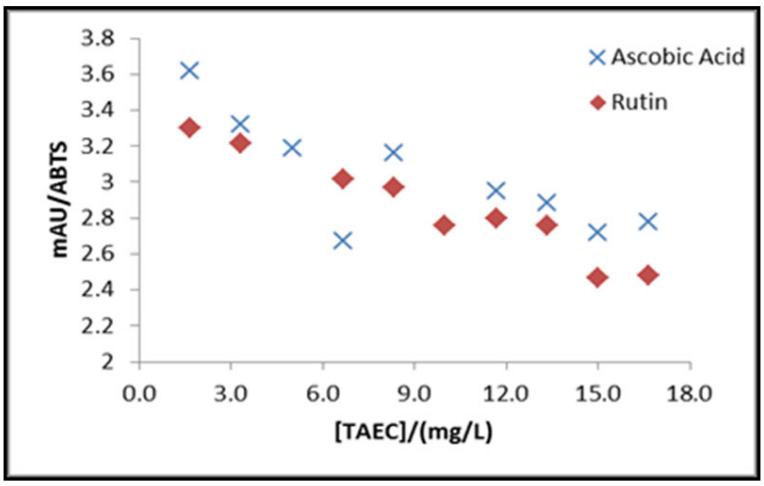


Figure 5. 3: Total Antioxidant TEAC obtained with ABTS method.

The activity study showed a reasonable correlation of the phenolic content which was due to increasing the concentration correlation sample extract by varying the volume. This method involves the non-enzymatic generation of the free radical, with increasing its stability in the presence of antioxidants. During protonation, the conjugate base that forms possesses an additional double bond, thus increases the number of π electrons to the cation. This causes an increased delocalization of the electrons, resulting in significant shifts of the absorption wavelength of the phenolic compound. ABTS present in the reaction mixture, accepts an electron donated by an antioxidant compound present in the sample resulting in colour loss as can be seen in Fig 5.3. A linear model representing the relationship between Total Phenolic content and TEAC obtained with the modified laccase biosensor were fitted and shown in Fig 5.4 with the corresponding analysis of variance in Table 5.2.

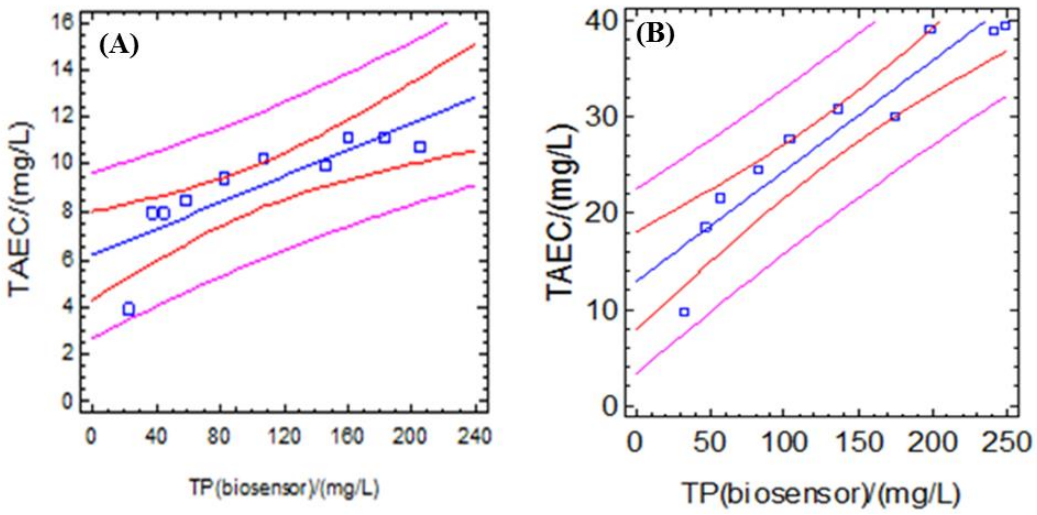


Figure 5. 4: Comparison between TPC and TEAC obtained with a modified laccase biosensor electrode with (**A**) Rooibos and (**B**) Green tea samples.

For both Rooibos and Green tea samples depicted in Table 5.2, P-values calculated from ANOVA were evaluated to be less than 0.01, therefore at the 99,9 % confidence level it can be said that there is a statistically significant relationship between Total Phenolic content and TEAC. The correlation coefficients for Rooibos and Green tea respectively 0.8219 and 0.9410 indicates that a stronger relationship between variables exists in Green tea. The model as fitted is explained in the R-Squared statistic, 69.21 % and 88.55 % of the variability for both Rooibos and Green tea respectively. A higher Standard Error of Estimate for this model of 3.52 was attained for Green tea than that of Rooibos 1.29.

Table 5. 2: Analysis of Variance for the model of Trolox Equivalent Antioxidant Content (TEAC) and Total Phenolic (TP) content.

Sample	Source	Sum of Squares	Df	F-Ratio	P-Value	Correlation Coefficient	R-squared (%)	Standard Error of Est
Rooibos	Model	30.01	1	17.98	0.0028	0.8319	69.21	1.292
	Residual	13.35	8					
	Total (Corr.)	43.36	9					
Green tea	Model	765.16	1	61.87	0.0004	0.9410	88.55	3.517
	Residual	98.94	8					
	Total (Corr.)	864.15	9					

For the evaluation of this relationship as seen in Fig 5.4, the data was subjected to the Grubbs test for outlier identification. Out of 10 points in each series, a single point was identified as an outlier and eliminated, therefore only 9 points were retained. Further to this study the effective concentration of antioxidants as depicted in Table 5.3 and the ability to scavenge 50 % of the ABTS radical (EC_{50}) for Rooibos and Green tea extracts was calculated to be 10.80 µg/ml and 11.62 µg/ml, as opposed to that of 13.31 ug/ml and 15.23 ug/ml for rutin and ascorbic acid respectively as they are well-known antioxidants.

Table 5. 3: Antioxidant Activities (AA) and EC50 determined using ABTS free radical scavenging assay.

Sample	AA_{ABTS} (mmol/L)	AA_{ABTS} EC_{50} (µg/ml)
Green Tea	35.43	11.62
Rooibos	41.65	10.80

The study of the two compounds revealed a difference in free radical scavenging activity with rutin yielding a greater value to ascorbic acid. This behavioural difference can be attributed to the structure of the diphenols on the B-ring of Rutin which have a higher ability to quench free radicals in solution. This is achieved by donation of hydrogen of -OH groups and the ability to stabilize the respective radical forms due to electron delocalization resulting in the formation of double bonds depicted in Scheme 5.1. Antioxidant activity of plant extracts can be extended beyond phenolic compounds. Table 5.3 reveals that both samples have been oxidized in the presence of ABTS by spectroscopic evaluation. The initial absorbance measurement of Rooibos tea samples was lower than that of the Green tea sample. Both samples exhibited good inhibition ABTS oxidation effect after 5 min of storage in the dark environment.

5.6 Conclusion

A modified laccase biosensor was used to determine antioxidant activities in herbal tea products. Spectrophotochemical and electrochemical techniques were applied to assess Trolox Equivalent Antioxidant Capacities (TEAC) and TP content. A linear correlation between total phenolic content and antioxidant capacities yielded an (R^2 = 0.9812 ± 0.012) at a pH of 5.5 with Rutin and Ascorbic Acid as reference solutions. This value reveals that phenolic compounds are directly responsible for antioxidant activities of these beverages.

In comparison to bare electrodes, modified sensors exhibit greater sensitivity. The laccase-based sensor methodology produced by adsorption immobilization exhibited superior sensitivity thus providing reproducibility in the detection of Total Phenolic Content. The goal of this study was to fabricate an alternative sensor with the hope of attaining significantly better sensitivity and reproducibility. The approximated sensitivity of the laccase immobilized electrode was established at around 1.5 uA/5 µM. The LOD obtained was 5.0 (µM) and 2.0 (µM) for Rutin and Ascorbic Acid respectively indicating that the laccase paste immobilization is sensitive and selective. A significant factor involved a constant regeneration of electrode surface between runs and is as such reasonable to deduce that choice of substrate and immobilization methodology directly influence the functionality of the biosensor.

CHAPTER 6

SUMMARY OF THE TWO CASE STUDIES

6.1 Summary of Case Study 1

Here we successfully fabricated a biosensor using polyphenol oxidase enzyme present in apple tissue for the quantification of flavanols in wines. The objective of determining flavanol content in wines is multi focused due to their significant health benefits, inhibition properties and free radical scavenging abilities. The logical undertaking involved the evaluation of electrochemical behaviour for the determination of (+)-catechin conducted under varied experimental conditions. The selected pH range of the electrolyte solution, scan rate (s_r) and deposition time (t_d) can significantly affect peak heights, peak potential, wave characteristics and reversibility of reaction intermediates during electrochemical responses. After establishing that the above mentioned factors play a key role in the experimental procedure, a further step was taken to ensure that the parameters pH, (s_r) and (t_d) were optimised for the determination of polyphenolic compounds in wine samples. Various strategies exist for the implementation of statistical design and in this case the higher order Box-Behnken multivariate experimental design was utilised to attain optimum conditions for the determination of concentration of (+)-catechin. Plackett-Burman design was considered as a screening design method where factors are not established. In the case of this study the three variables required for optimization were already selected. Partial least squares (PLS) and Pareto charts were applied to indicate response variables and explanatory variables in the analysis.

The choice of electrodes were based on the robustness with the ability to withstand many immobilization procedures and resistivity to physical conditions. A GCE was chosen as it is easily available, ready for modification and easy to maintain for the duration of an electrochemical experiment. Green apple tissue was easily adsorbed onto the support material made of nujol and alumina mixture. Due to the adsorption procedure the electrode did exhibit low efficiency and required electrochemical regeneration in a 5-10 scan cycle in a potential range between -0.5 and 1.0 V with a scan rate of 50 mV s^{-1} in the presence of the electrolyte buffer to enable enzyme activation after each analysis run. The poor response are as a result of the weak bonds present at the surface of the electrode and the support material immobilized with the enzyme.

According to the experimental optimization protocol the experiments were fully randomized to ensure that only measured values of interest are assessed. The three factors of interest were run at 15 levels with three replicates measured for each sample. A scatter plot revealed the three dimensional view of the experimental design using pH, scan rate and deposition time. The multivariate approach enabled the recognition of effects involved in interaction between the experimental parameters. A 12 ppb catechin standard was used to establish the effect of deposition time (t_d) on the cathodic voltammetric responses. The pH of electrolyte was an especially important variable as it directly influences the $E_{1/2}$ value in any electrochemical method. The optimum pH was obtained at a value of 7.65. The use of a Pareto chart to establish interactions between two variables A and B indicate statistically that the linear effect of the scan rate on the concentration of catechin values was found to be of great significance. From this plot the affected matrices were eliminated and only the optimized electrochemical parameters were then used for the qualitative analysis using CV and DPV for the quantification of TP content in wine samples within the potential range of -0.2 V to 0.6 V.

During electrochemical oxidation the optimized electrolyte pH was observed at 7.6, with the main peak ranging between -0.15 and 0.25 V which is due to the oxidation at the catechol ring. This *Epa* peak observed at 0.22 V also suggests that catechin displays high antioxidant activity. The lowest detectable concentration LOD of the standards with polyphenol oxidase enzyme was found to be 1.76 ppb and LOQ was found to be 5.86 ppb. Catechin equivalent values measured in mg/l were found to be much higher in red wines as compared to white wines and is acceptable due to the crushing and juicing process during the wine making process.

6.2 Summary of Case Study 2

Here we successfully fabricated a laccase modified biosensor with increased sensitivity and reproducibility for the evaluation of antioxidant properties in polyphenolic compounds found in tisanes and herbal teas. The main reason for delving into this study is to establish the known interdependence between antioxidant activity and polyphenolic content. The first step in the process was to establish which type of electrode, enzyme substrate and electrochemical method would be most beneficial and efficient in evaluating the phenolic content in tea samples. As was used in the previous case study, a glassy carbon electrode was selected as it is most suited for organic constituents.

After careful assessment, laccase enzyme was selected, and it produced a steady and stable electrochemical response to polyphenolic compounds. This was achieved due to their efficient unrestricted oxidation capacity and covalently-linked carbohydrate functional group structure that aids in greater stability of the enzyme. The choice of laccase enzyme for immobilization also provided a more cost effective, sensitive electrode that required minimal pre-treatment. Adsorption immobilization was used with the addition of nujol, graphite and laccase followed by electrochemical procedure. Laccase film was then electrochemically deposited for ten minutes to ensure that a sufficient electrical connection is achieved between the electrolyte solution and electrode surface and a uniformed film thickness is attained.

The optimization procedure required careful performance evaluation of the laccase modified electrode. This was performed by DPV to assess that denaturing of the enzyme was not present within the immobilised electrode. It was established that the optimum pH of the electrolyte solution is 5.5. Rutin and Ascorbic Acid were used in this study as working standards and reference compounds respectively. Based on the redox behaviour of tea samples it was observed that there is an increase in reducing abilities as the concentration during standard addition within the cell increases which leads to over layed or superimposed voltammograms.

All measurements were performed in triplicate with current signal present and response indicated that the parameters selected for the analysis were optimised. This was especially significant due to the large size of the laccase enzyme which due to its size can hamper direct immobilization of the multi film on the surface from an aqueous medium. The main focus was to enhance signal quality and sensitivity with the sensitivity of the laccase modified electrode estimated around 1.5 uA/5 μM.

To quantify the TP content in tea samples, DPV was used whereby measured current values are proportional to the concentration of analyte. In this study it was evaluated that rutin, which represents polyphenolic content, was present in higher quantity in rooibos and green tea samples as compared to ascorbic acid, a phenolic equivalent content. The CV was utilised to study redox reactions and the voltammograms obtained indicate that ascorbic acid is irreversible with a reduction peak at $E_{1/2} = Epa = 0.45$ V, which in some cases is reversible as a result of the oxidation of catechol groups found in certain compounds in the presence of polyphenol oxidase. The $E_{1/2}$ value of rutin was measured at 0.43 V which relates to scavenging

capacity. A contributing factor to free radical scavenging capacity is based on the elevated reactivity of -OH constituents that interact in conjugate acid formation.

A variety of electrochemical and photometric methods are employed for the analysis and detection of antioxidant activity and in this study the synthetic radical ABTS was used. ABTS reveals rapid electron transfer kinetics between laccase and the surface of the electrode thus represents a suitable mediator. Trolox equivalent antioxidant content TEAC with ABTS method and TP content acquired with the modified laccase sensor were fitted with subsequent results generated from ANOVA for both green tea and rooibos samples. The p-Values generated for green tea and rooibos are 0.0004 and 0.0028 respectively both being less than 0.01. This indicated that at 99 % confidence level a statistically consequential relationship between Total phenolic content and Trolox equivalent antioxidant content exists. The correlation coefficient for rutin was higher than that of rooibos indicates a greater dependence on variables attained in green tea. Additional values for effective concentration of antioxidants EC_{50}, able to scavenge 50 % of ABTS radical was calculated to be 10.80 μg/ml and 11.62 μg/ml for Rooibos and Green tea respectively. The obtained values for rutin and ascorbic acid is 13.31 ug/ml and 15.23 ug/ml respectively.

Rutin and ascorbic acid were seen to reveal a contrast in free radical scavenging activity with rutin displaying a higher activity than ascorbic acid. The varied behaviour can be attributed to structural differences with rutin possessing a B ring easily able to stabilise radicals through H^+ donation.

6.3 Conclusions

The present work involved the development of a sensitive and reproducible methodology to evaluate the antioxidant activity of wines and teas, using immobilized based biosensors. An effective mediator with support should be selected in the immobilization procedure to ensure that a selective yet sensitive sensor is fabricated and that it can be applied to electrochemical parameters. Factors such as pH of the electrolyte, scan rate and deposition time require optimization. The Box-Behnken experimental design is suitable for evaluating these critical parameters before the electroanalytical determination. Box-Behnken factorial design reveals levels of factors and surface responses which can be adapted to other analytical techniques.

Electrochemical and spectrophotochemical methods are useful in determining antioxidant activities in beverages. A positive linear correlation between the Total Phenolic content and Trolox Equivalent Antioxidant content in tea samples indicate that phenolic compounds are responsible for antioxidant activities in these samples. Enzyme based immobilization techniques are favourable due to their high stability and sensitivity. Entrapment, adsorption and intermolecular cross-linking enzymes through covalent bonds improves stability and efficiency of probes. Although the modified electrodes were cost effective, they still required rejuvenation after each sample analysis and further studies will have to be conducted to ascertain if a better immobilization process will enable a greater stability and increase lifespan.

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APPENDIX

Table S1: Comparison of Analytical Responses of the developed biosensors with present sensor.

Modified Electrode	**Analyte**	**Response LOD**	**Citation**
GCE/Graphene Nanosheets	Quercetin	0,0039 (µM)	(Saber-Tehrani *et al.* 2013)
Graphite Disc Electrode/MWCNTs and Parafin	Quercetin	0.0048 (µM)	(Jin *et al.* 2006)
GCE/MWCNTs dispersed in Polyacrylic Acid	Quercetin	0.0075 (µM)	(Gutiérrez *et al.* 2010)
GCE/MWCNTs dispersed in Polyethyleneimine	Quercetin	0.0089 (µM)	(Gutiérrez *et al.* 2010)
GCE/Graphite powder, nujol and green apple tissue	Catechin	1.76 (µg/L)	This work

Table S2: Comparison of Analytical Responses of the developed biosensors with present sensor.

Modified Electrode	**Analyte**	**Response LOD**	**Citation**
Screen Pinted Electrode/MWCNTs/Si/Laccase	Catecholamine	0.42 (µM)	(Li *et al.* 2012)
Pt Electrode modified with Laccase	Catecholamine	0.2 (µM)	(Quan and Shin 2004)
Laccase crosslinked Bovine Serum Albumin with and Glutaraldehyde	Catechol	7.0 (µM)	(Sarika, Rekha and Narasimha Murthy 2015)
GCE/Graphite and Laccasse	Rutin and Ascorbic Acid	5.0 (µM) and 2.0 (µM)	This work