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Multivariate Optimization of Voltammetric Parameters for the Determination of Total Polyphenolic Content in Wine Samples Using an Immobilized Biosensor

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This work was aimed at investigating the changes in the determination of total phenolic (TP) content in wine samples using catechin as a standard. The modification of the glassy carbon electrode (GCE) was carried out using green apple as an enzymatic source of polyphenol oxidase. The experimental variables were optimized using the Box-Behnken design with 3 factors in 15 runs of differential pulse voltammetry (DPV). The design was run in a single block fashion while the order of the experiments was fully randomized to provide greater protection against the effects of lurking variables. Specifically, the three optimized factors included the buffer pH, deposition time (t_d) and scan rate (s_r). Based on the optimized results obtained, we selected the most suitable condition for the determination of the TP content in wine samples 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 with respect to 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 in wine samples, with a higher concentration in red wines than in white wines.

Keywords: Glassy carbon electrode, Box-Behnken design, differential pulse voltammetry, total phenolic content

1. INTRODUCTION

Flavanols are a class of phenolic antioxidants widely distributed throughout the plants and have attracted a huge interest from researchers due to their beneficial effect in human health. Their role is to minimize or prevent the oxidation of other molecules. Oxidation reactions can produce free radicals, which start chain reactions that damage biological cells and results in human diseases such as cancer, cardiovascular diseases, some pathological disorders of gastric and duodenal ulcers, vascular fragility,

viral and bacterial infections [1-3]**.** Flavanols are capable of terminating these chain reactions by removing the free radical intermediates, and inhibit other oxidation reactions by being oxidized themselves [1], resulting in the beneficial effects of wine consumption. However their amounts need to be controlled because of their strict regulations and formation of cloudiness in wines due to these antioxidants and customer demand [4]. Specifically in this work, (+)-catechin is the flavanol of interest, because of the key role it plays in the brewing process by delaying, retarding or preventing the oxidation processes. The differing levels of antioxidant in various wines are due to the differences in raw materials used during the brewing processes. Due to the great importance of polyphenols for wine quality there is a growing interest in the development of selective and sensitive methods for their detection and quantification. There are several techniques that are used for the examination of polyphenolic content and chemical composition of wine, including high-performance liquid chromatography (HPLC) [5-8] and capillary electrophoresis (CE) [9-11] coupled with different detectors, UV–Vis, photo diode array (PDA), mass spectrometry (MS), and electrochemical (EC) detectors. HPLC coupled with UV detection is the preferred method of analysis in the brewery industry [12]. For the purposes of our study the polyphenols oxidase enzyme present in apple was used for the electrochemical measurements of polyphenols (catechin equivalent) in wines [13]. However, it would be difficult to implement in a routine analysis of real samples despite the fact that this method is sensitive and specific. On the other hand Chromatographic methods permit the separation of components present in complex matrices such as beer, to be performed rapidly, and to selectively and sensitively quantify their presence [14]. Nevertheless, chromatographic methods require sample preparation steps, which are tedious and may compromise sample integrity and may introduce sources of error.

The primary goal of this work involves the quantification of flavanols in wines with tissue biosensor. Accordingly the electrochemical behaviour for the determination of catechin is conducted under different experimental conditions, which included the pH of the buffer, deposition time t_d and scan rate s_r. Electrochemical techniques particularly voltammetry provide information, not just on the identity and quantity of a compound, but also on the physicochemical properties, such as redox potential and the number of electrons exchanged during the redox reaction. This information helps in evaluating the antioxidant properties of phenolic compounds and to better understand their reaction mechanisms [15].

Glassy carbon electrode (GCE) has been found to be one of the most suitable electrodes for organic molecules. It is generally resistant to solvents, unlike most metals, and shows low background currents [16**].** However the exposure of the electrode to measuring solution causes the surface of the electrode to be scratched, thus reducing the electrode lifetime and therefore raise a need for new electrodes, whereas with the use of a immobilized biosensor, the electrode surface gets covered with the paste and the surface is renewed after every run thus minimizing electrode surface decay. The use of electrochemical biosensor methods such as Cyclic Voltammetry (CV) and Differential Pulse Voltammetry (DPV) is an attractive alternative to normal voltammetry, which uses a unmodified working electrode. In addition, biosensors have high sensitivity and selectivity towards their target molecules in solution provided selected parameters are well optimized. They are also cost effective because the surface of the working electrode is coated with an enzyme protecting it from the sample

matrix as a result increasing the life time of the electrode. The enzyme polyphenol oxidase has been found to be present in a number of different plant tissues including, apple [13], banana [13], mushroom [17], avocado [17], quince [18], and potato [17, 19, 20]. Literature studies revealed that for the determination of catechol related compounds, potato and apple were considered to be the best biosensors, resulting in the best responses among the evaluated tissues. [13].

For this purpose multivariate optimization of experimental parameters for the determination of polyphenolic compounds in wine samples was utilised. Specifically, the Box–Behnken experimental design was used to achieve optimum conditions for the concentration of catechin. The three factors included pH of the measuring buffer, deposition time t_d of the analyte to electrode paste and the scan rate s_r. Pareto charts and partial least squares (PLS) analysis were used to relate response variables to the explanatory variables. In addition, electrochemical oxidation/reduction of catechin was studied with cylic voltammetry (CV). Finally, the concentration of the TP was determined using the optimised conditions within the potential range of -0.2 V to 0.6 V.

2. EXPERIMENTAL

2.1. Apparatus

All analytical measurements were performed with a 797 VA Computrace from Metrohm (Herisau, Switzerland). This is a three electrode system of a 3mm diameter rotating disc electrode (GCE), reference electrode made of Ag/AgCl (saturated AgCl, 3 M KCl), and the auxiliary electrode that is made of platinum wire. Scans were evaluated with the Metrohm 797 VA potentiostat. A pH meter, Crison Micro pH 2000, from Crison Instruments (South Africa (SA) was used to adjust the pH of the buffer solutions. All working solutions and the buffer were prepared with deionized water from a water purification system called Aqua Max^{TM} Basic 360 Series from Trilab (Durban, SA). The electrochemical buffers together with the samples were stored in the fridge at 4 °C. The software provided with the equipment enabled automatic peak evaluation (current signal) and estimation of the concentration in a standard addition mode. All samples were allowed to reach room temperature prior to the commencement of any voltammetric measurements.

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 of 99.9% purity was obtained from AFROX (Durban, SA). Alumina powder ≤ 3μm 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 (2010,

from Du Toitskloof Cellar SA), Pinotage (2009, from Swartland Winery SA) and Baronne (2009, from Nederburg SA) were purchased at a local Supermarket in Durban, SA.

2.3. Methods

2.3.1. Preparation of the carbon paste electrode

Because of the several measurements to be performed with the GCE, its surface was going to be contaminated with the product of the electrode redox processes and to overcome this, 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. Subsequently, its activity was 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 supporting electrolyte.

The carbon paste of approximately 1.7 g comprised of 40 % graphite powder, 40 % of Nujol and 20 % of ground green apple. 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. Therefore the polishing step with aluminium oxide was repeated only once a day.

2.3.2.Preparation of buffer

According to the structural design of our experiment shown in table 1, it was necessary to prepare three separate buffer solutions with different pH values. The phosphate buffers were prepared by weighing approximately 0.8 g of $NaH_2PO_4.2H_2O$ and 5.2 g of Na_2HPO_4 then dissolving into a 500 ml volumetric flask and adjusted with 0.1 M HCl or 0.1 M NaOH to the desired pH and the final volume with deionized water. All buffer solutions were stored in the fridge at 4 ^{0}C.

2.3.3. Preparation of standard

The method of standard addition was used utilized for the determination of catechin with a DPV mode. A 1ppm stock solution of (+)-Catechin was prepared by weighing adequate volume and dissolving in deionized water in a volumetric flasks. Thereafter, an adequate volume of the prepared stock solution was diluted to make a 12 ppb standard was used for the subsequent optimization of all experimental parameters.

2.3.4. Preparation of samples

Wine samples were filtered through a Teflon disc filter (0.45µm and 9mm 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 standard in the measuring cell containing the wine sample.

2.3.5. Procedure for optimization of experimental parameters

The carbon paste electrode prepared as described in section 2.3.1 was used to perform electrochemical measurements at different experimental factors. The Box–Behnken design generates the three experimental factors (pH, t_d and s_r) randomly for 15 levels as show in table 1. 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.

Table 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)	*[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

2.4. 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

3. RESULTS AND DISCUSSION

3.1. Optimisation of experimental procedure

In order to understand the relationship between variables and their relevance to the actual determination of catechin in wine samples, a Box-Behnken design was created to study the effects of 3 factors in 15 runs [21]. Three replicates were measured for each sample and the average response was used to attain the optimum parameters as shown in table 1. The scatter plot in figure 1 allows for the visualization of multivariate data in a three dimensional space having the values of one variable determining the position on the horizontal axis and the value of the other variable determining the position on the vertical axis shows that the experiment was well distributed.

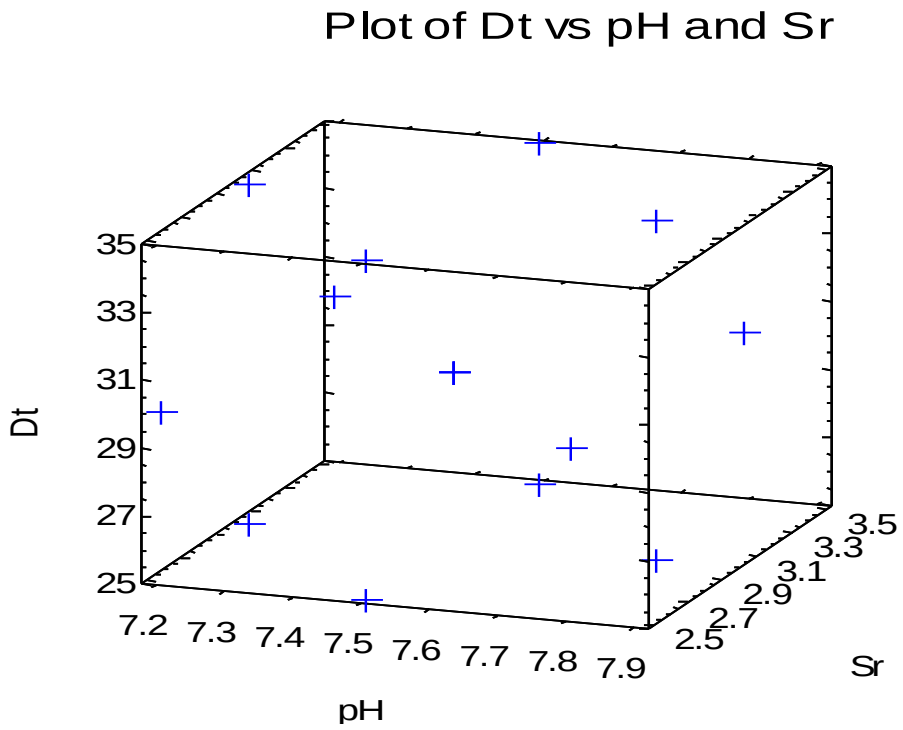


Figure 1. 3D Scatter plot of variables t_d versus pH and s_r used for experimental design. t_d is the deposition time, pH is the ionic strength of the phosphate buffer and s_r is the scan rate.

The experiments were randomized in order to provide protection against the factor that may affect the measured result, but are not of primary interest. In this work, the multivariate analysis approach enabled the identification of interaction effects between the experimental parameters. The influence of deposition time (t_d) on the cathodic voltammetric responses obtained was investigated with a 12 ppb catechin standard. This variable t_d, affects the current signal which is proportional to the concentration; hence its optimization is very crucial. The pH of a buffer is the most critical variable that determines the $E_{1/2}$ in any electrochemical methods, yielded and optimum value of 7.65. The pareto chart in Figure 2 shows the interaction between the two variables A and B, indicating a favourable matrix effect of the parameters in a decreasing order of importance [21].

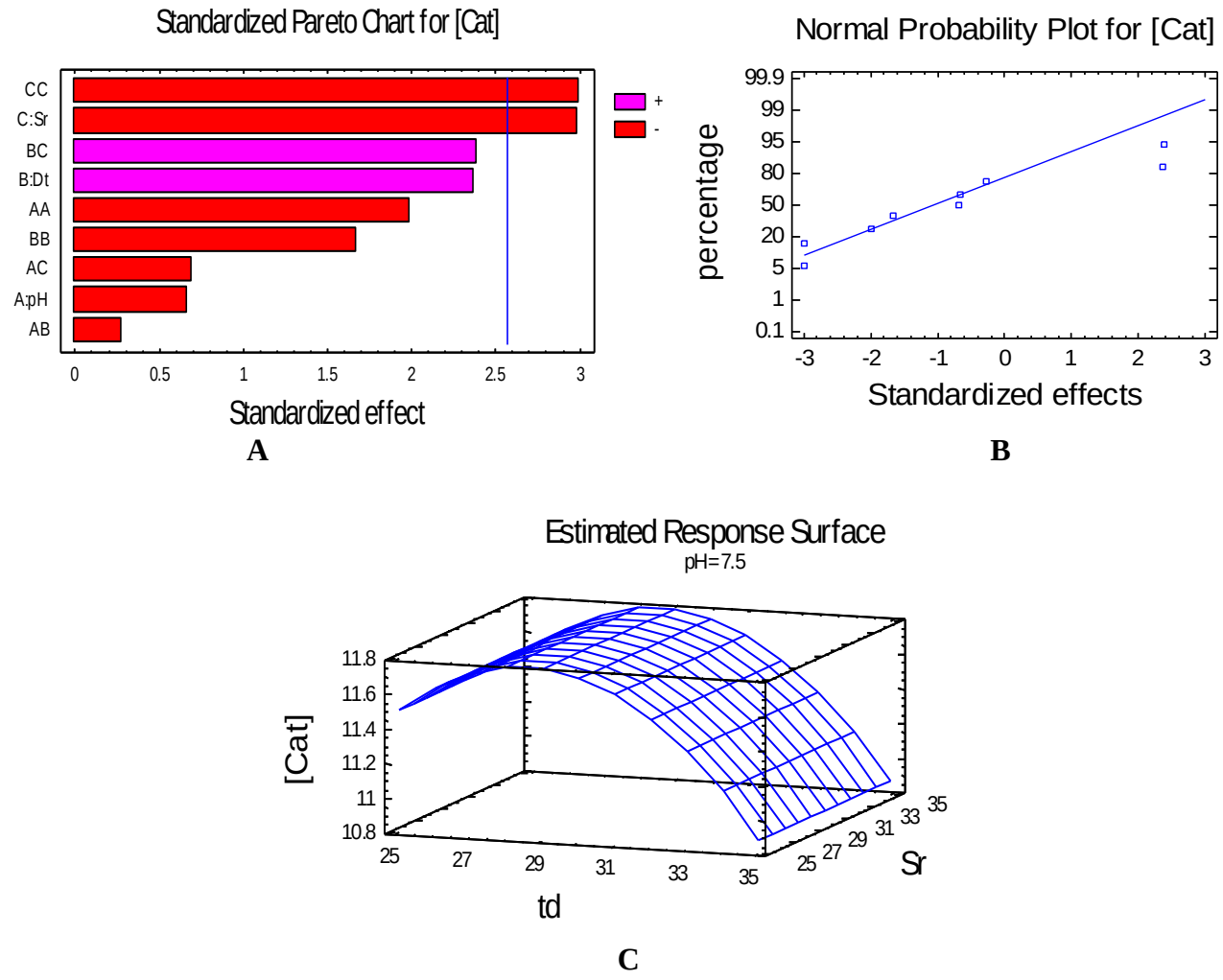


Figure 2. **A**. Pareto Charts shows the matrix of selected variables against the standardization effect. **B**. Matrix are ranked according to the order of importance **C.** the response surface plot shows the distribution of [Cat] as a function of t_d and S_r at constant pH=7.5 after elimination of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

The R-Squared statistic indicates that the fitted model explains 85.124 % of the variability in [Cat]. The length of each bar is proportional to the standardized effect and the vertical line plot designates the significant effects. In this case, bars of matrixes C:C and C:Sr extend beyond the line depicting effects that are statistically significant at the 95% confidence level. The linear effect of the Scan rate on the concentration of catechin values was found to be significant. Therefore the matrixes C:C, C:Sr, and B:C were eliminated prior to the optimisation/standardization of the experimental design. The response surface plot in Figure 2C reveals a maximum catechin response obtained when t_d = 30s while s_r has no effect on [cat] within the selected range at a constant pH of 7.5. This is due to the fact that when the deposition time increases, it can cause an increase in solubility, thus increasing the current response. In some instances, a compromise between pH of the buffer and t_d is necessary. Optimized electrochemical parameters shown in table 2, using the standard solutions were utilized for

the qualitative measurements using CV and the quantification of Total Phenolic (TP) content in different wine samples using DPV.

Table 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

3.2. Electrochemical Oxidation/Reduction of polyphenols (biosensor)

In this work it was also evident that the buffer had a great impact on the $E_{1/2}$ potential. Hence, the $E_{1/2}$ can be reported with respect to the buffer used to perform electroanalytical measurements, in this case the phosphate buffer with pH 7.6. However the immobilization of the material can be a problem because of the long diffusion path between the test solution and the detector surface, thus the tissue should be of a sufficient thickness to maintain mechanical and chemical stability. During the experimental measurement, the solution was unstirred; therefore mass transport can occur only by diffusion due to the concentration gradients created around the electrode immobilised biosensor surface.

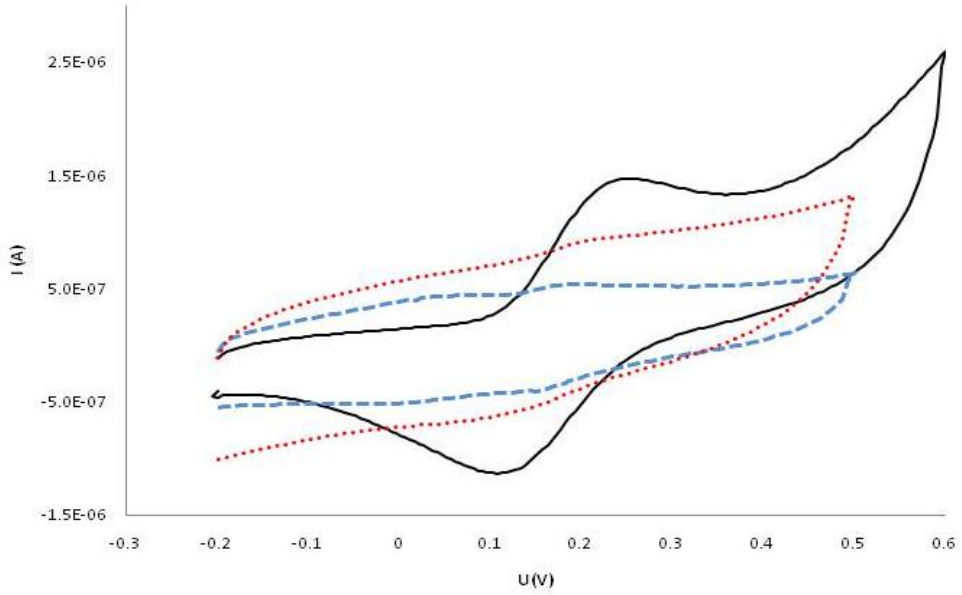
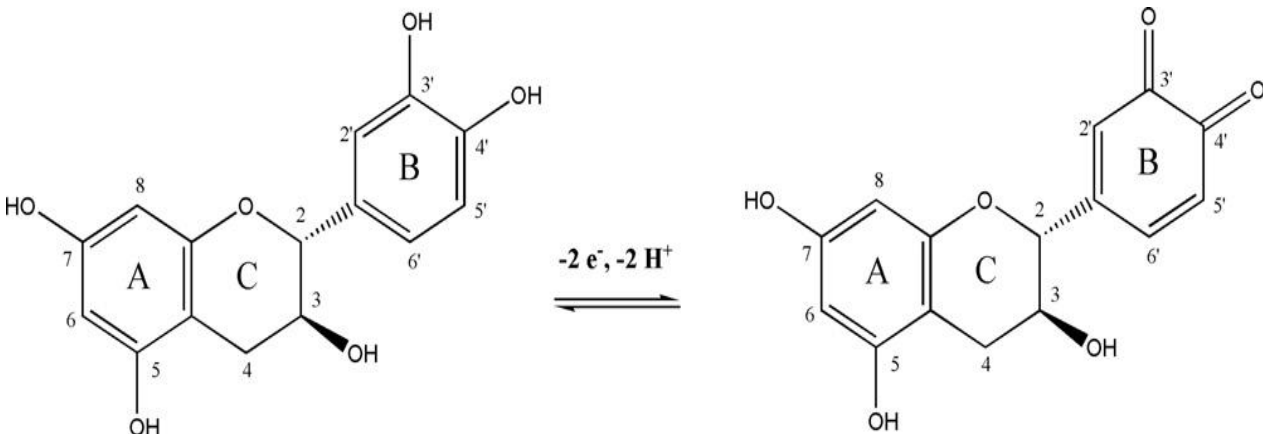


Figure 3. Cyclic voltammogramms of (+)-catechin within -0.20 V to 0.60 V. ••••Bare glassy carbon electrode in a buffer, ----GCE modified with apple paste in a buffer and — oxidation/reduction of catechin standard at the modified GCE.

Cyclic voltammograms were recorded immediately after the working electrode was immersed in the solution minimizing the adsorption of the analyte on the electrode at a scan rate of 25 mV/s in the potential range from -0.2 to 0.6 V. Polyphenols are well known to be electroactive, due to the presence of hydroxyl groups attached to the aromatic rings, which undergo electrochemical oxidation reactions [22]. The oxidation mechanism of catechin proceeds in sequential steps, related to the catechol moiety (B-ring) and 3'- hydroxyl group. When the potential of the working electrode is more positive than that of a redox couple present in the solution, the anodic peak E_{pa} that corresponds to a reversible process is observed at 0.219 V shown in figure 3 and the cathodic peak E_{pc} at 0.128 V as the working electrode potential becomes more negative than the reduction potential of a redox couple.

The main peak is characterized by E_{pa} value ranging between -0.15 and 0.25 V. This peak is due to the oxidation of the catechol moiety, 3',4'-dihydroxyl electron-donating groups at ring B, occurs first at a very low positive potential. This voltammetric behaviour of catechin is in close agreement with those reported in literature [22]. Since this is a reversible couple the formal potential for $E^{0'}$= 0.18 V is centered between E_{pa} and E_{pc} denoted by $E^{0'} = (E_{pa} + E_{pc})/2$. Moreover the E_{pa} peak observed at 0.22 V illustrates that catechin has a relatively high antioxidant activity [23]. The (+)-catechin molecule has several OH functional groups attached to all three rings (A5, A7, B3', B4' and C3). All the hydroxyl groups can be electrochemically oxidized but the catechol (B-ring) is most easily oxidizable than the resorcinol (A-ring), and glycoside (C-ring), hence the postulated mechanism is as shown in scheme 1 [5, 14, 22]. The two hydroxyl substituents of the B-ring are oxidised sequentially forming a quinone as below.



Scheme 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.

Glassy carbon has been found to be the most suitable for organic molecules. It is generally resistant to solvents, unlike most metals, and shows low background currents. The LOD and LOQ values were calculated on the peak current using the relevant equations: LOD = 3 s/m, and LOQ = 10 s/m, where s is the standard deviation of the peak currents (n=5) and m is the slope of the calibration curve [24]. On a closer inspection of figure 4A, it can also be observed that there is no change in the

$E_{1/2}$ of the polyphenolic as the concentration was increased and the calibration data (in figure 4B) obtained obey the linear regression equation with a correlation coefficient (R^2) of *0.98820*.

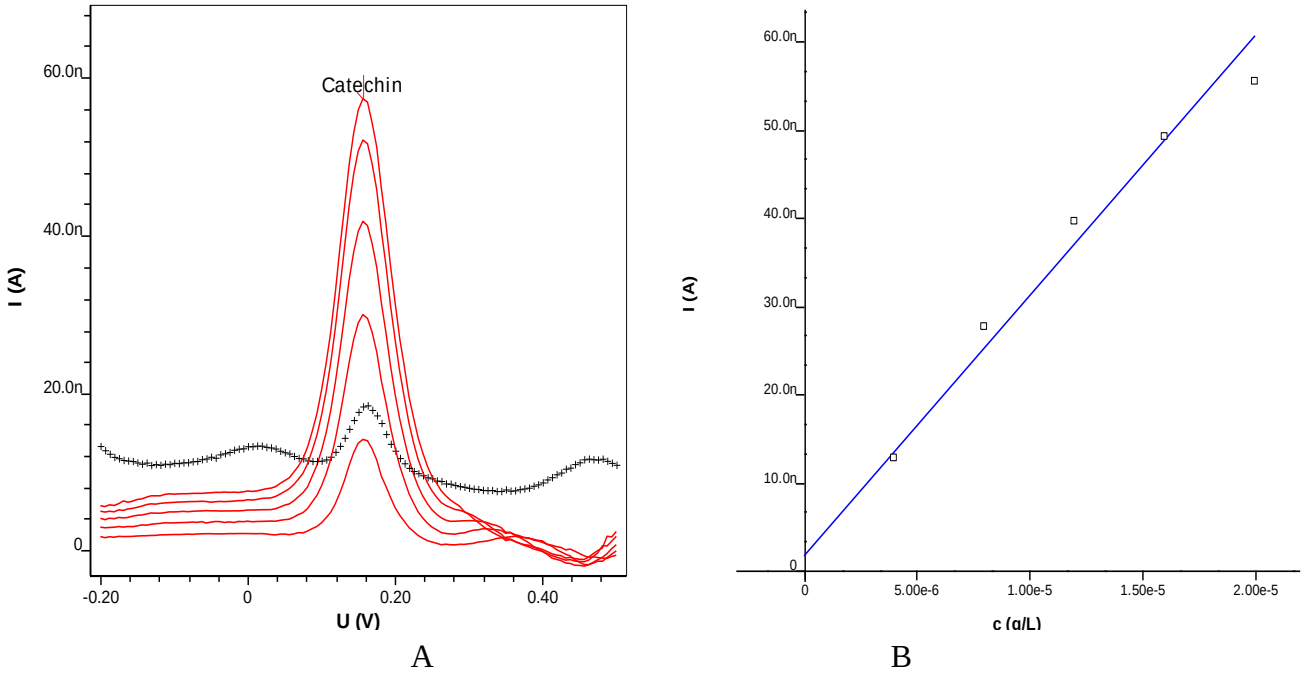


Figure 4. Differential Pulse Voltammogram (DPV) of catechin standards 40, 80, 120, 160 and 200 ppb obtained using optimised parameters from experimental design section 2.3.5. pH of electrolyte 7.65, t_d of 29.77s and S_r of 25.0mV/s. **** blank and — catechin standards from 40 to 200ppb.

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 3).

Table 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	5.860

The key to this high sensitivity is due to the fact that electron transfer occurs at the intersection of the immobilised working electrode with the reactive polyphenolic compound present at the measuring solution. In addition, polyphenolic oxidase increases the sensitivity with its ability to facilitate oxidation at the catecholic B-ring. It is evident in this work, very different values of the Total Phenolic content in wine samples have been reported, but this is not unexpected because different brands of wines are produced from very different grapes by different technologies [5, 8, 10, 13, 25].

We have used the standard addition method to determine the TP content in wines samples. Two additions were performed while taking into consideration the effect of any other electroactive species present in the blank (electrolyte) solution. Hence the current signal for the blank solution was subtracted from that of the samples and standards. The significant different of catechin equivalent between red and white wines observed in table 4 can be attributed to the differences in wine making processes**.**

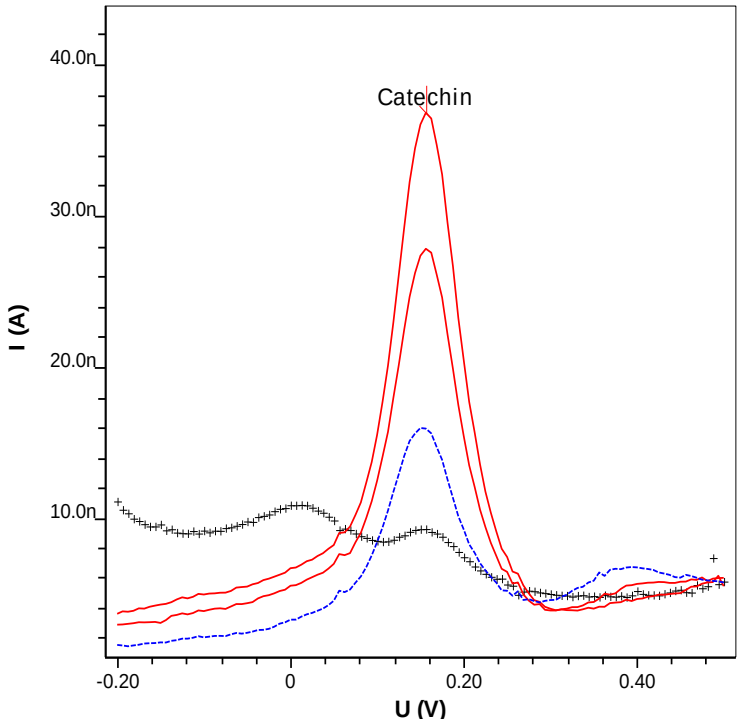


Figure 5. DP voltammogramm of the red wine (Baronne) sample. **** blank (electrolyte and paste), ---- (sample) Peak due to Total phenolic content appears at 0.18 V in the first scan. — (addition 1 and 2) Overlaid peaks due to addition of catechin standard.

Table 4. Table of results obtained from wine samples using DPV at optimum conditions. **Number of replicates n=3*

Wine Samples	Color	Catechin Equivalent(mg/L)	Mdn (mg/L)	RSD%
Swarland	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

Basically, this is sensible because the red wine is the outcome of the crushed grape while the white wine is an outcome of the grape juice (no skin or meat of the fruit). The values obtained represent an overall total flavanol concentration measured in units of catechin equivalents.

4. CONCLUSION

In this study it was evident that parameters such as pH of the buffer, the scan rate and the deposition time should be adequately optimized before electroanalytical determination of the analytes in order to improve the quality of the results. The predicted Box-Behnken experimental design is useful for the understanding of the levels of factors and interactions among the studied factors. The experimental responses obtained in the optimization conditions are in agreement with the estimated values based on the response surface models. This approach can easily be transferred to other analytical techniques. Moreover the immobilization of tissue materials with sufficient thickness, revealed mechanical stability which proved to be very effective for the determination of the phenolic content in both red and white wines. The LOD of 1.76 ppb and LOQ of 5.86 ppb (RSd 2.5%) obtained for catechin suggests the highly sensitive nature of the immobilized paste. The content of the catechin (equivalent) in white and red wines is approximately, 60 ppm and 500-1000 ppm respectively, which is satisfactory for control purposes in the brewery industry.

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