ADDIS ABABA UNIVERSITY
SCHOOL OF GRADUATE STUDIES

FURTHER IMPROVEMENT OF THE VOLTAMMETRIC METHOD OF DETERMINATION OF CAFFEINE BY USING 1,4-BENZOQUINONE MODIFIED CARBON PASTE ELECTRODE

ADVISORS: MESFIN REDI (Ph.D)
MERID TESSEMA (Ph.D)
NEGUSSIE WODAJO (Ph.D)

BY

BEWKETU MEHARI

AUGUST 4, 2007

ADDIS ABABA UNIVERSITY

SCHOOL OF GRADUATE STUDIES

FURTHER IMPROVEMENT OF THE VOLTAMMETRIC METHOD OF DETERMINATION OF CAFFEINE BY USING 1,4-BENZOQUINONE MODIFIED CARBON PASTE ELECTRODE

A PROJECT PRESENTED TO THE

DEPARTMENT OF CHEMISTRY

IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE

OF MASTER OF SCIENCE IN CHEMISTRY

ADVISORS: MESFIN REDI (Ph.D)

MERID TESSEMA (Ph.D)

NEGUSSIE WODAJO (Ph.D)

BY

Bewketu Mehari

AUGUST 4, 2007

ADDIS ABABA UNIVERSITY

Acknowledgments

I would like to express my deepest gratitude to my research advisors, Dr. Mesfin Redi, Dr. Merid Tessema and Dr. Negussie Wodajo, for all their unreserved guidance, technical and moral support and encouragement during the entire project work and preparation of the paper.

I would like to acknowledge Ato Tarekegne Birhanu who provided me the caffeine for the experiment.

Above all, I would like to acknowledge the Department of Chemistry, AAU, for giving me the opportunity to work on this MSc. Project.

Table of contents

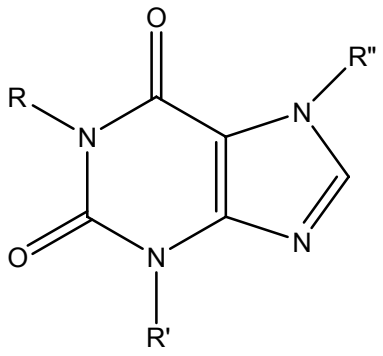
Pages

1. Introduction

1.1. Occurrence and Properties of Caffeine

Caffeine is a naturally occurring alkaloid which belongs to a class of organic compounds called methylxanthines [1]. Chemically, caffeine is 1,3,7-trimethylxanthine [2, 3], meaning it is a xanthine molecule with methyl groups replacing all of the three hydrogens bound to nitrogens in xanthine. The other common members of this class include theophyline and theobromine [1, 2, 4]. Theobromine and theophylline are isomers and caffeine has one more methyl substituent. They all have certain pharmacological properties. Caffeine has stimulating properties to the central nervous and cardiovascular systems [3, 4]. Theobromine and theophylline are analgesic to cardiac muscles and play a role in relaxing the muscles and expansion of coronary artery [4]. Besides, these compounds are considered to be risk factors for asthma, kidney malfunction and cardiovascular diseases [5]. Table 1 shows the structure of xanthine and its naturally occurring methyl derivatives.

Table 1. The structure of xanthine and its naturally occurring N-methyl derivatives [5].

Structure	R	R'	R"	Compound
	H	H	H	Xanthine
	CH_3	CH_3	H	Theophylline
	H	CH_3	CH_3	Theobromine
	CH_3	CH_3	CH_3	Caffeine

Pure caffeine occurs as odorless, white powder with melting point of 278 ^{o}C, and density of 1.2 g/cm^{3} [6]. Caffeine is found naturally in such plants as coffee, tea, and cacao [3, 7, 8]. For the plant, caffeine acts as a natural pesticide against some insects that attempt to feed on the plant [9].

The major natural source of caffeine is the coffee plant. Coffee beans contain relatively large amounts of caffeine [10]. Coffee is originated in the highlands of Ethiopia, and from there spread into the Arabic world and became known in Europe during the early 17th century, at first as a medicine and then as a social drink in the Arab tradition [11]. Now a days, coffee is one of the most popular drinks across the world and the total annual worldwide production of green coffee is estimated to be about six million tons [12] and the coffee industry is second only to oil with an associated revenues of $60 billion worldwide [12, 13]. The roasting of green coffee beans yields the apparently bitter and dark coffee beverage, in which caffeine is responsible for the mild stimulating properties and to a particular proportion of its apparent bitterness [12]. Coffees grown in different parts of the world differ genetically in their composition; as a result the amount of caffeine per gram of coffee bean varies depending on the species of beans, geographic areas, and the preparation processes [14]. The availability of nutrients in soil also affects caffeine content in coffee plants. For example, depletion of phosphorus and potassium caused a 20% decrease and 12% increase, respectively, in caffeine content [15].

1.2. Metabolism of Caffeine

Following intake caffeine is rapidly and almost completely absorbed from the gastrointestinal tract [2, 14, 16, 17] and rapidly distributed in all the body fluids. The peak plasma concentrations of caffeine are usually obtained within few minutes [17]. Caffeine undergoes hepatic metabolism through N-demethylation, acetylation and oxidation, with less than 5% of the caffeine taken being eliminated unchanged in urine [2, 16-18]. Caffeine biotransformation is complex, and 17 urinary metabolites have been detected following caffeine consumption [2]. Five metabolic pathways contribute to caffeine elimination in humans.

About 80% of the caffeine taken is metabolized by 3-N-demethylation to form paraxanthine (1,7-dimethylxanthine), 11% by l-N-demethylation to form theobromine (3,7,-dimethylxanthine), and 4% by 7-N-demethylation to form theophylline (1,3-dimethylxanthine). Formation of the other metabolites; 1,3,7-trlmethyluric acid and 6-amino-5-(N-formylmethylamino)-l,3-dimethyluracil together account for <5% of caffeine elimination [2, 14, 16, 18, 19]. The metabolism is slowed during pregnancy and in women taking oral contraceptives. On the other hand, the metabolism rate of caffeine is greater in smokers than in non-smokers [17].

Of the three main metabolic products of caffeine, theophylline relaxes smooth muscles of the bronchi and has been used as a treatment of asthma [13, 20]. Theobromine increases blood vessel dilation and urine volume [13]. Paraxanthine increases lipolysis, leading to elevated glycerol and free fatty acid in the blood plasma [9].

1.3. Beneficial Effects of Caffeine

Caffeine is a mild stimulant. The stimulatory effect of caffeine usually results in an increased ability for mental activity and muscular work [21]. When taken in a reasonable amount, it reduces a desire for sweets [9] by stimulating the production of adrenal hormones [1, 17, 22] that cause blood sugar to be increased. The weakness, depression, and discomfort from excess of alcohol can be canceled out with black coffee or hypodermic injections of caffeine. Insensibility from hashish or opium is believed to be ended by the use of caffeine medication. Even the dullness and sense of depression from a bit in excess of tobacco is helped by coffee [9].

The other advantageous effects of caffeine include reduced risks of Parkinson’s disease [23, 24], colon cancer [25], diabetes [26], decrease in exercise induced myocardial flow reserve [27] and increase in both sexual motivation and locomotors activity on female mating behavior [28]. Caffeine helps in preventing a positive energy balance and obesity [1]. Caffeine is also an accepted drug for intramuscular applications to treat arterial hypotension [21].

1.4. Toxic Effects of Caffeine

A dose of 50–200 mg of caffeine is generally sufficient for a mild stimulation [16]. Large amounts of caffeine can lead to intoxication, which results in flushing, chills, agitation, irritability, loss of appetite, weakness and tremor [16, 17]. Overdoses of caffeine may result in hypertension, hypotension, tachycardia, vomiting, fever, delusions, hallucinations, seizures, arrhythmia, cardiac arrest, coma and death [16, 17].

Caffeine exerts an acute unfavorable effect on the endothelial function [29] and it is also believed to be a risk factor for rheumatoid arthritis [30]. High levels of caffeine consumption during pregnancy are associated with a higher risk of fetal death [31]. Caffeine is considered to be a risk factor for cardiovascular diseases and may affect behavioral effects of depression and hyperactivity [15]. Caffeine is also believed to be an addictive substance with women being more addictive to it [15].

Caffeine is thought to increase alertness, reduce sleep tendency and produce adverse effects in the central nervous system, such as insomnia. A heightened amount of caffeine intake near bedtime is believed to have harmful effects on sleep like increasing sleep arrival latency, decreasing total sleep time and badly affecting sleep quality. Caffeine intake early in the morning also found to have significant reduction in total sleep time and sleep efficiency [32].

The main metabolite of caffeine, serum paraxanthine, has been used to estimate caffeine intake, and it was found that women with a paraxanthine level greater than 1.845 μg/ml (equivalent to six cups of coffee per day) had almost twice the risk of spontaneous abortion before 140 days' gestation of women with a serum paraxanthine level less than 0.05 μg/ml [31]. Moderate consumption of caffeine is unlikely to increase the risk of spontaneous abortion [33].

Tolerance to the actions of caffeine has been distinguished after its regular consumption [1, 14]. Withdrawal from regular consumption of caffeine has been indicated to result in a variety of symptoms, including: irritability, sleepiness, dysphasia, delirium, nausea, vomiting, rhino rhea, nervousness, restlessness, anxiety, muscle tension, muscle pains and flushed face [8, 15, 34]. Further more, continual caffeine consumption has a negative effect on aortic stiffness and wave reflections [35, 36].

1.5. Mechanism of pharmacological action of caffeine

Caffeine has many physiological effects, such as gastric acid secretion, diuresis and stimulation of the central nervous and the cardiovascular systems [5, 37, 38]. In addition, caffeine interferes with the uptake and storage of calcium by the sarcoplasmic reticulum [1, 17, 21]. It also increases the respiratory rate and causes bronchodilatation and stimulates lipolysis [17].

The stimulatory action of caffeine involves antagonism of adenosine receptors which are present in brain, blood vessels, kidneys, heart, the gastrointestinal tract and the respiratory hierarchy [11, 14, 17, 23, 24, 39, 40]. Adenosine is an adenine molecule attached to a ribose or deoxyribose sugar molecule. The similarity in chemical structure between the adenine portion of adenosine and the caffeine molecule is the key to how caffeine works [16]. Adenosine, when bound to receptors of nerve cells, slows down nerve cell activity during sleep [40]. Caffeine, being structurally similar to adenosine, has the potential to occupy adenosine receptor sites. When the caffeine molecule binds to the receptors does not cause the cells to slow down; instead, the caffeine blocks the receptors and thereby blocks the regulatory functions of adenosine and produces a stimulatory effect [16]. In addition, the resulting increased nerve activity causes the release of the hormone epinephrine [41], which leads to several effects such as higher heart rate, increased blood pressure, increased blood flow to muscles, decreased blood flow to the skin and inner organs, and release of glucose by the liver [9].

Adenosine also inhibits the release of many neural transmitters including, noradrenalin, dopamine, acetylcholine, and glutamate. By blocking the receptors, caffeine facilitates the release of neurotransmitters [17, 39] and through this mechanism it also promote wakefulness [22]

.

Caffeine enhances muscular performance by lowering the threshold for sarcoplasmic Ca^{2+} release through the ryanodine receptor [21].The cardiovascular effects of caffeine are believed to be the result of multiple mechanisms [16].The possible mechanisms of the cardiovascular effects of caffeine [17] are:

- Antagonistic effects on adenosine receptors
- Inhibition of phosphodiesterases (increase in cyclic nucleotides)
- Activation of the sympathetic nervous system (release of catecholamine from adrenal medulla)
- Stimulation of adrenal cortex (release of corticosteroids)
- Renal effects (diuresis, natriuresis and activation of reninangiotensin aldosterone system).

The adverse physiological effects of caffeine have increased the market for decaffeinated coffee [15, 42] to about 10% of coffee consumption worldwide [43]. Decaffeinated coffee is mainly obtained through industrial decaffeinating process. However, industrial decaffeination usually results in the loss of key flavor compounds from coffee [43]. In a search to overcome this problem, a naturally decaffeinated *Coffea arabica* plant has been discovered in Ethiopia [43]. On the other hand, the possibility of creating tea and coffee (*Coffea arabica*) plants that are naturally deficient in caffeine has been described [44], by cloning the gene encoding caffeine synthase, an enzyme that catalyses the final two steps in the caffeine biosynthesis pathway, from young leaves of tea (*Camellia sinensis*).

All of the above considerations have led to increased interest in the development of a reliable method for the determination of caffeine in both regular and decaffeinated coffee.

1.6. Methods of Analysis of Caffeine

One of the earliest methods of caffeine determination is based upon ultraviolet (UV) spectrophotometery. Caffeine absorbs in the UV region at 276 nm [45- 47]. An aqueous extraction followed by liquid-liquid extraction in to chloroform provides a significantly cleaner solution but still containing interfering compounds [48]. The level of these interfering compounds is commonly reduced by applying column chromatography. In order to avoid these separation procedures, some investigations used mathematical processing to determine caffeine by UV or IR to eliminate the interference. Although this method can improve selectivity it causes the reduction of sensitivity [4, 45].

The more recent methods of caffeine determination generally employ high performance liquid chromatography (HPLC) with UV–spectrophotometeric detection method [49]. Also, other methods such as capillary electrophoresis and thin layer chromatography are used for the separation of caffeine in the analysis of mixtures, combined with several detection methods like UV–spectrophotometery, mass spectrometry and infrared spectrophotomety [4, 5, 12, 50]. However, these methods often require time-consuming sample preparation procedures such as liquid–liquid extraction, solid-phase extraction or the use of more than one chromatographic step [49, 51] and very costly instrumentation and high skilled technician [3, 50, 12].

Among the various methods that have been mentioned for the determination of caffeine one can find biosensors. An amperometry based microbial biosensor has been described for the analysis of caffeine in beverages and fermentation samples by using immobilized whole cells and cell debris of sonicated cells of the isolate [50]. The approach involved a selection strategy for caffeine degrading bacteria that is capable of utilizing caffeine as the only source of carbon and nitrogen from soils and induction of caffeine degrading capacity in the bacteria. In another study, the *in vitro* selection strategy and the construction of allosteric ribozyme sensor has been indicated for the determination of caffeine in solutions [52]. Caffeine responsive ribozymes were converted into fluorescence based riboreporter sensor systems.

Electrochemical methods have rarely been used for the analysis of caffeine mainly due to the very high anodic potential (1.4 V) needed for the oxidation of caffeine which in most cases overlaps with the potential at which the solvent or supporting electrolyte discharges [2, 44]. Several solutions have been proposed, including the use of modified electrodes or unusual combinations of solvent to supporting electrolyte ratios [51, 53].

In recent years, many types of working electrodes by using the various electrochemical techniques have been tested for the determination of caffeine in different materials [13, 51, 53]. The application of common solid electrodes, such as platinum, glassy carbon or graphite, is limited by the useful potential window where the background current due to the oxidation of the solvent or supporting electrolyte is below certain minimum value [5, 13, 53].

Graphite pencil lead electrode as working electrode has been described for the voltammetric determination of caffeine [54]. However, different model of the pencil lead had shown different background current levels which is attributed to difference in composition and roughness of the pencil leads. Hence the selection of the best pencil lead model is a problem associated with commercial pencil leads [48]. In another study, a method that has been described for the detection of caffeine using glassy carbon electrode (GC) by differential pulse voltammetry rendered difficult for quantitative determination due to the volatile methanol from the perchloric acid–methanol (1 + 1) mixture that was used both as a solvent and supporting electrolyte in order to improve the sensitivities and peak separations [51]. A differential pulse voltammetric method based on a Nafion covered glassy carbon electrode has been tested for determination of caffeine in cola beverages and it had shown a detection limit of 0.798 μM [53]. GC electrode modified with a lead-ruthenium pyrochlore oxide in a Nafion matrix has also been used for caffeine determination by squarewave voltammetry with a detection limit of 2 μM [51]. Compared to a bare glassy carbon electrode, the modified electrodes exhibited a significant enhancement of sensitivity and detection limit [51, 53].

The electrochemical determination of caffeine is generally rendered difficult by interference from background currents due to the relatively large positive potentials required for reasonable sensitivity. Hence, the electrochemical methods of determination of caffeine rely on the availability of electrode materials that have the ability to detect compounds that are difficult to oxidize.

In conclusion, the development and application of a sensitive, fast and cost-effective caffeine determination method remains an active area of investigation particularly in food and clinical chemistry, because almost all caffeine originates from dietary sources with coffee and tea being the most popular.

1.7. Mechanism of the Electrochemical Oxidation of Caffeine

The cyclic voltammogram of the oxidation of caffeine is characterized by an anodic peak in the positive going scan and by the absence of any cathodic peak on the reverse scan, indicating that the electrochemical oxidation of caffeine is irreversible [53].

The electrochemical oxidation mechanism of caffeine proceeds by an overall $4e^-$, $4H^+$ process. The first step is a $2e^-$, $2H^+$ oxidation of the C–8 to N–9 bond to give the substituted uric acid followed by an immediate $2e^-$, $2H^+$ oxidation to the 4,5-diol analogue of uric acid, that rapidly fragments [53]. The electrochemical process is shown in Figure 1.

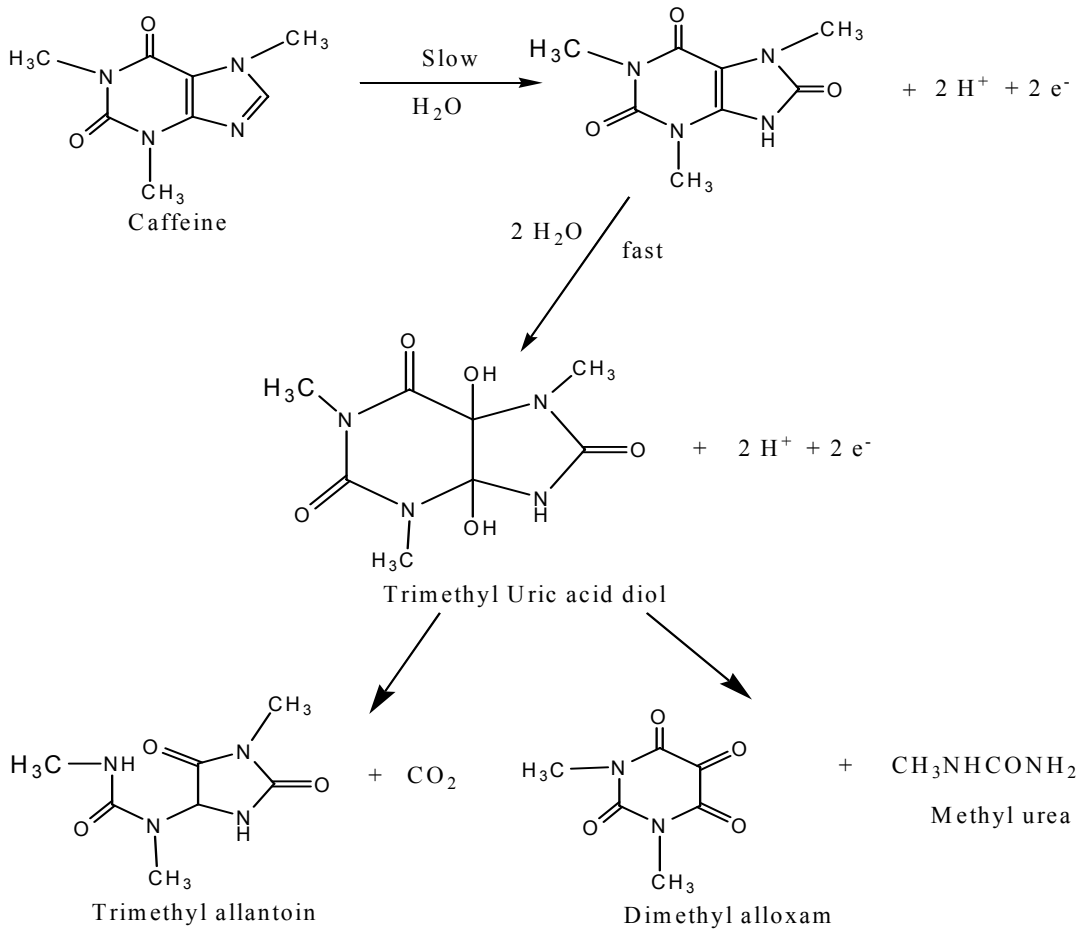


Figure 1. Electrochemical process for the oxidation of caffeine [9]

1.8. Carbon Paste Electrodes

1.8.1. Unmodified Carbon Paste Electrodes

In 1958, R.N. Adams discovered a new type of electrode by using a mixture of carbon powder with a liquid non-electroactive binder and called it as *carbon paste.* His original idea was to develop a dropping carbon electrode (DCE) that could be constructed similarly like the drooping mercury electrode (DME). Although practical experiments with DCE failed, the mixture of carbon powder and a binder prepared in thicker consistency was presented as a new type of electrode material [55].

Binary Mixtures prepared from carbon powder and organic liquid of nonelectrolytic character are known as *bare* or *virgin* or *plain* or *unmodified carbon pastes* [55 - 66]. As nonelectrolytic binders paraffin oils are commonly used. These nonpolar pasting liquids should be chemically inert, insulating, nonvolatile, water immiscible and forming paste mixtures of fine consistency [55]. Silicone oils also represent common type of pasting liquids, especially when the problem of paraffin oils vulnerability in media with organic solvents is considered. Another group of binders is some liquid organophosphates. Their attractive property is a high ion-pairing ability [55]. The electric conductor in carbon pastes is graphite powder with micrometric particles.

Carbon paste electrodes offer an easily renewable surface, low cost and very low background currents especially in the anodic region [55-66]. A disadvantage of carbon pastes is the tendency of the organic liquid binder to dissolve in solutions containing an appreciable fraction of organic solvents. And also, the conventional paste mixtures from spectroscopic graphite powder and paraffin oil suffer from interferents when being polarized cathodically, and consequently they have been used mainly for the determination of easily oxidisable organic compounds [55-66].

1.8.2. Modified Carbon Paste Electrodes

The effort to make use of the favorable mechanical and electrochemical properties of carbon pastes for the preparation of a new design of sensors started at the beginning of the 1980s [55]. Modification of a carbon paste by impregnating the carbon particles with methanolic solution of dimethylglyoxime represents a milestone in the history of carbon paste electrodes. It was a first effort when a classic analytical reagent had served as selective modifier, thus initiating a very successful role of chemically modified carbon paste electrodes in electrochemical analysis [55]. Hand in hand with a rapid development of chemically modified carbon paste electrodes, the favorable mechanical and electrochemical properties of carbon pastes were tested for the preparation of special sensors containing enzymes allowing one to examine some enzymatically catalyzed reactions of biological substances. This way of attaching enzymes to an electrode

material immediately attracted bioanalysts and carbon paste-based enzymatic biosensors had rapidly come to the front [55].

A modified carbon paste is a mixture of powdered graphite, nonelectrolytic liquid binder and a modifying agent. A modifier is usually one substance, but the carbon pastes can also be modified with two or more components. Among modifiers recently used are [55, 67, 68, 69]:

- chemical Compounds and analytical reagents
- ion-exchangers
- clay minerals
- humic substances
- silica
- substrates from living organisms

The preparation of modified carbon paste electrodes is characteristically by means of various alternative procedures. The modifier can be dissolved directly in the binder or admixed mechanically to the paste during its homogenization. It is also possible to soak graphite particles with a solution of a modifier, and after evaporating the solvent use the impregnated carbon powder. Finally, already-prepared pastes can also be modified *in situ* [55-66].

Generally, the main reason to modify an electrode is to obtain a new sensor with desired properties that can impart higher selectivity, sensitivity, or lower detection limit to electroanalytical investigations [55, 70].

1.8.3. 1,4-Benzoquinone Modified Carbon Paste Electrode

Quinones are a class of compounds consisting of conjugated cyclic diketone organic systems. Naturally, quinones function in cellular respiration, photosynthesis, and blood coagulation that occur in certain plants and animals. Their biological functions are mostly related to their electron transfer rates and redox potentials [71].

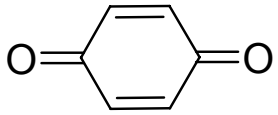


Figure 2. The structure of 1,4-benzoquinone

In aprotic solvents, 1,4-benzoquinone, shown in Figure 2, is reduced in two consecutive one electron steps to form hydroquinone radical anion and hydroquinone dianion [71, 72] as shown by the following general reaction equation:

$$Q + e^- \longrightarrow Q^{\cdot -} \qquad (1)$$

$$Q^{\cdot -} + e^- \longrightarrow Q^{2-} \qquad (2)$$

Benzoquinone and hydroquinone redox couples have been widely used in electrochemical studies [71, 73]. Moreover, benzoquinones have been found important as modifying agents for the preparation of modified carbon paste electrodes. An oligosaccharide dehydrogenase based amperometric sensor by using a benzoquinone bulk modified carbon paste electrode has been used for the determination of low molecular weight saccharides including glucose [74]. The benzoquinone modified carbon paste electrode had shown remarkable advantages from its low noise levels. In another study, the electrochemical behavior of a tetrabromo-p-benzoquinone modified carbon paste electrode has been investigated [73]. The function of the benzoquinone modified electrode for the electrocatalytic oxidation of ascorbic acid, dopamine and uric acid was found to be attractive. In addition, the simultaneous determination of the three

components in the mixture was made possible based on differential pulse voltammetric technique by using the benzoquinone modified carbon paste electrode.

Square wave voltammetry by using 1, 4-benzoquinone modified carbon paste electrode has been described for the determination of caffeine levels in coffee [9]. The linear range for the determination of caffeine by the square wave voltammetric procedure using the modified carbon paste electrode was from 0.5 mM to 8 mM with a detection limit of 5.136 μM caffeine concentration.

In this study attempt was made to improve the detection limit and sensitivity of the square wave voltammetric method of determination of caffeine by using 1,4-benzoquinone modified carbon paste electrode.

2. Objective of the Study

General objective

- To develop a more sensitive, less expensive electrochemical method for the determination of the level of caffeine.

Specific objective

- To improve the sensitivity and detection limit of the square wave voltammetric determination of caffeine by using 1,4-benzoquinone modified carbon paste electrode.

3. Voltammetric Techniques

3.1. Cyclic Voltammetry

Cyclic voltammetry is frequently the first experiment performed in an electroanalytical investigation [71]. A cyclic voltammogram readily shows the presence of species that can undergo redox reactions at the electrode within the applied potential range. The most important informations that can be obtained from a cyclic voltammogram are the magnitudes of the anodic peak current, cathodic peak current, anodic peak potential and cathodic peak potential. For a reversible electrochemical reaction, the formal potential ,E^0, of the couple is approximately centered between the anodic and cathodic peak potentials; the number of electrons transferred in the electrode reaction can be determined from the separation between the peak potentials; the ratio of the anodic and cathodic peak currents is nearly one; the peak currents are directly proportional to the potential scan rate; and the peak potentials are independent of the potential scan rate [75, 76].

3.2. Pulse Voltammetric Techniques

3.2.1. Advantages of Pulse Voltammetric Techniques

The various pulse voltammetric techniques are all based on the difference in the rate of the decay of the double layer charging currents and the faradic currents following a potential step. After the potential is stepped, the charging currents, i_c, decay exponentially with time which is faster than the faradic currents, i_f, decay as a function of the square root of time [75, 76].

Since the current is sampled late in the pulse life, an effective discrimination against the charging current is achieved. Therefore, in pulse voltammetric techniques the measured current is mainly of the faradic current. The discrimination against the charging current that is inherent in these techniques leads to lower detection limits and higher sensitivity

compared to linear sweep techniques, which makes the pulse techniques more suitable for quantitative analysis [75, 76].

3.2.2. Square Wave Voltammetry

Among the various voltammetric techniques, exceptional versatility is found in a method called square wave voltammetry, which was invented by Ramaley and Krause, and developed extensively by the Osteryoungs and their coworkers [75]. Square wave voltammetry can be viewed as combining the best aspects of several pulse voltammetric methods, including the background suppression and sensitivity of differential pulse voltammetry, the diagnostic value of normal pulse voltammetry, and the ability to interrogate products directly in much the manner of reverse pulse voltammetry [75].

Square wave voltammetry is normally carried out at a stationary electrode; and involves the application of the potential waveform shown in Figure 3. As in other forms of pulse voltammetry, the electrode is taken through a series of measurement cycles and the waveform is interpreted as consisting of a staircase scan, each tread of which is superimposed by a symmetrical double pulse, one in the forward direction and one in the reverse. Over many cycles, the wave form is a bipolar square wave superimposed on the staircase, and this view gives to the name of the method [75].

Figure 3 also shows the principal parameters. The square wave is characterized by a pulse height also called amplitude, ΔE_p, measured with respect to the corresponding tread of the staircase, and a pulse width t_p. Alternatively, the pulse width can be expressed in terms of the square wave frequency, *f*, by the formula:

$$f = 1/2t_p \qquad (1)$$

The staircase also called step height or step potential shifts by ΔE_s at the beginning of each cycle; thus the scan rate, ν, can be expressed as:

$$\nu = \Delta E_s/2t_p = f\Delta E_s. \qquad (2)$$

The scan begins at an initial potential, E_i, which can be applied for an arbitrary time to initialize the system as desired [75].

Current samples are taken twice per cycle, at the end of each pulse. The *forward current sample*, i_f, arises from the first pulse per cycle, which is in the direction of the staircase scan. The *reverse current sample*, i_r, is measured at the end of the second pulse, which is in opposite direction. A *difference current* Δi is calculated as $i_f - i_r$. There is diagnostic value in the forward and reverse currents; hence they are preserved separately. Consequently, the result of a single square wave voltammetric run is three voltammograms showing forward, reverse, and difference currents vs. the potential on the corresponding staircase tread [75].

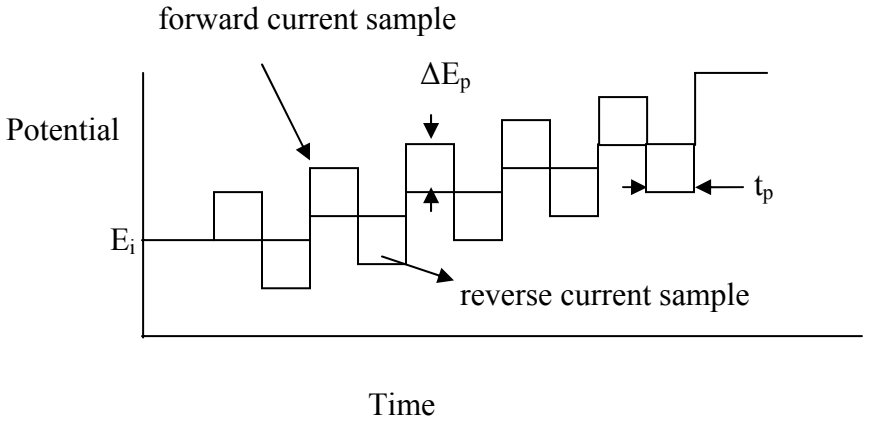


Figure 3. The applied potential wave form in square wave voltammetry

The current is measured at the end of each half cycle, and the current measured on the reverse half–cycle (i_r) is subtracted from the current measured on the forward half-cycle (i_f). This difference current (i_r–i_f) is displayed as a function of the applied potential. The resulting peak shaped voltammogram is symmetrical about the half wave potential, and the peak current is proportional to the concentration [75]. Excellent sensitivity is achieved from the fact that the net current is larger than either the forward or the reverse

components; coupled with the effective discrimination against the charging back ground current, very low detection limits near $1x10^{-8}$ M can be attained [75].

In summery, square wave voltammetry possesses the most important features of both pulse techniques and cyclic voltammetry; hence it is one of the most advanced methods in the family of pulse techniques. For this reason, square wave voltammetry is an exceptionally appealing method for electroanalysis.

3.3. Most Important Parameters in Square Wave Voltammetry

3.3.1. Square Wave Frequency

An increase in square wave frequency results in an increase in the scan rate which in turn increases the peak current [75]. However, at very high frequency, the peak current may become unstable and be obscured by a large residual current. On the other hand very low frequency gives a low but narrow signal, and increases the total analyses time. Hence, the selection of frequency usually requires a compromise among sensitivity, resolution and speed [9].

3.3.2. Square Wave Amplitude

In square wave voltammetry, the peak currents usually increase with increasing amplitude. However, the width of the peak also increases as the square wave amplitude grows larger and normally one refrains from increasing the square wave amplitude, because resolution may be degraded unacceptably [75].

3.3.3. Square Wave Step Potential

The square wave voltammetric peak current usually increases as the step potential increases with an accompanying peak broadening [75]. At higher step heights, too few points are sampled, thus affecting the reproducibility of the detection [9].

4. Experimental part

4.1. Reagents and Solutions

Graphite powder (BDH, UK), paraffin oil (Fulka, Switzerland), Caffeine (Fischer Scientific), NaH_2PO_4, Na_2HPO_4 (Wagtech International Ltd., UK), and NaOH (Lammark chemicals PVT., India), HCl (Riedel-De Haen, Germany), 1,4-benzoquinone (Riedel-De Haen, Germany) were used in the experiment.

Deionised water was used for the preparation of all solutions. For all of the experiments, a mixture of 0.1 M Na_2HPO_4 and 0.1 M NaH_2PO_4 buffer solution was used. Dilute NaOH and dilute HCl solutions were used to adjust the pH of the buffer solution. Stock solution of caffeine was prepared by dissolving 0.5 mg of caffeine in 250 ml of the supporting electrolyte. The required amounts of caffeine working solutions were prepared by diluting the stock solution with the supporting electrolyte.

4.2. Apparatus

The electrochemical experiments were carried out in a three-electrode system containing saturated calomel electrode (SCE) as a reference electrode, platinum foil as a counter electrode and unmodified carbon paste electrode (UCPE) or 1,4-benzoquinone modified carbon paste electrode (1,4-BQMCPE) as working electrode. The experiment and processing of data were made using a BAS 50 W electrochemical analyzer connected to a personal computer.

4.3. Preparation of Working Electrodes

Unmodified carbon paste (100 mg) was prepared by mixing graphite powder with paraffin oil. The composition of the paste was 75 % (w/w) graphite powder and 25 % (w/w) paraffin oil. The mixture was homogenized with mortar and pestle for 30 minutes and allowed to rest for 24 hours. The homogenized paste was packed in to the tip of a

plastic syringe (3 mm diameter, 7 mm deep). A copper wire was inserted from the backside of the syringe to provide electrical contact. Then the surface of the electrode was smoothed against a smooth white paper with a light manual pressure until a shiny surface is emerged.

Modified carbon paste (100 mg) was prepared by mixing graphite powder with 1,4-benzoquinone in paraffin oil. To 10 mg of 1,4-benzoquinone and 70 mg of carbon powder initially mixed with a mortar and pestle for 5 minutes, 23 μL (20 mg) of paraffin oil were added and thoroughly mortared together for 30 minutes. The resulting paste was packed in to the tip of the syringe by extruding a small amount of paste from the tip of the previously prepared unmodified carbon paste electrode.

4.4. Electrochemical measurements

Square wave voltammetric experiments for caffeine solution were conducted with 1,4-benzoquinone modified carbon paste electrode by scanning the potential from –700 mV to 500 mV versus SCE at frequency of 25 Hz, amplitude of 30 mV, step potential of 2 mV. Caffeine determination was made based on the square wave voltammetric peak current of 1,4-BQMCPE after background subtraction.

5. Results and Discussion

5.1. Cyclic Voltammograms of 1,4-BQMCPE and UCPE

The cyclic voltammogram of 1,4-BQMCPE recorded between -0.6 and 0.6 V in the buffer solution showed two peaks at potentials of -0.178 V and 0.042 V as shown in Figure 4. From the figure it is clear that the electrochemical reaction of 1,4-benzoquinone in the bulk of the carbon paste is reversible.

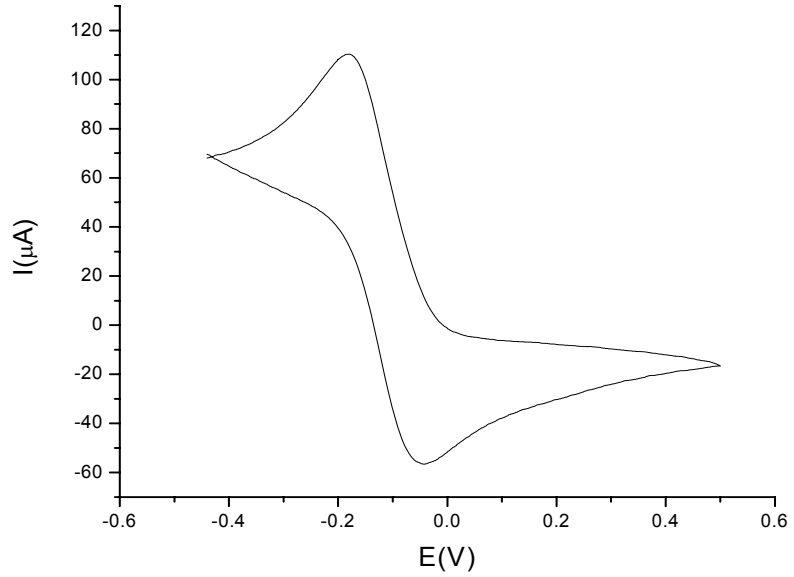


Figure 4. Cyclic voltammogram of 1,4-BQMCPE in 0.1 M Na_2HPO_4/0.1 M NaH_2PO_4 buffer solution at pH 9 and scan rate of 100 mV/s

Figure 5 shows the cyclic voltammogram of the background buffer solution recorded between -0.8 to 1.5 V by using UCPE in which the two peaks are not observed.

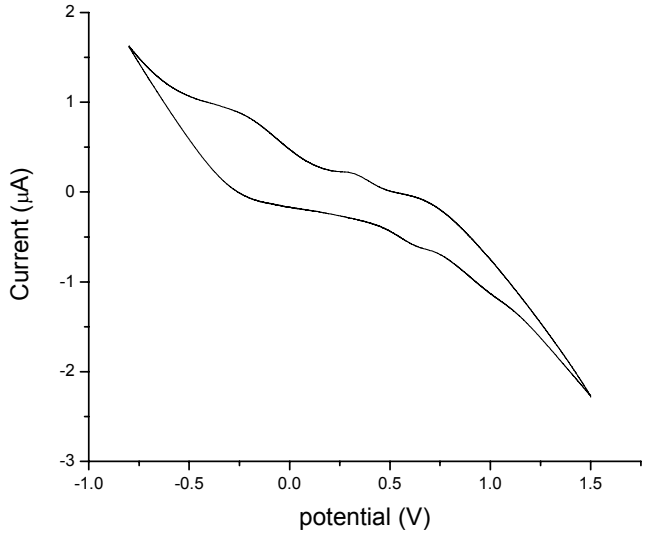


Figure 5. Cyclic voltammogram of UCPE in 0.1 M Na_2HPO_4/0.1 M NaH_2PO_4 buffer solution at pH 9 and scan rate of 100 mV/s

From observations of Figure 5 it was concluded that, the background buffer solution has no any interfering signal during the electrochemical measurements on the 1,4-BQMCPE in the potential range -0.8 to 1.5 V.

5.2. Effect of pH of the Buffer Solution

The effect of pH on the squarewave voltametric response of 1,4-BQMCPE was studied in the pH range of 2 up to 12. Figure 6 shows the voltammograms.

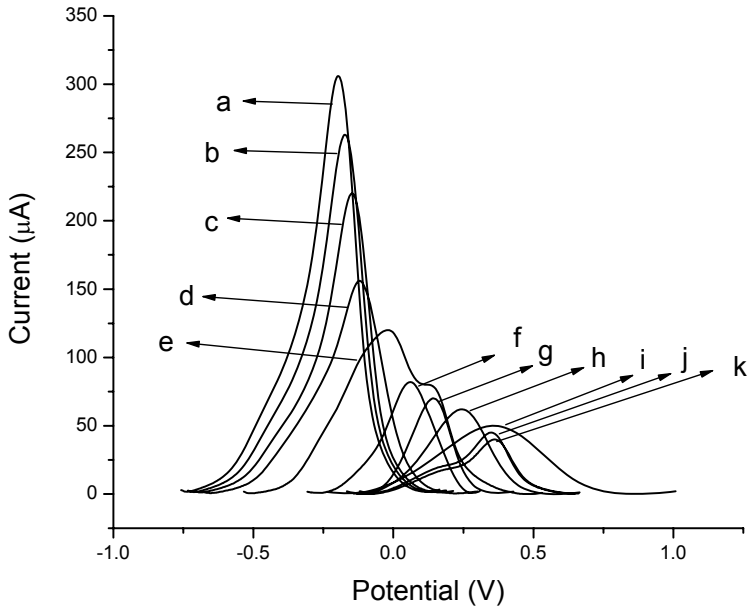


Figure 6. Squarewave voltammograms of 1,4-BQMCPE at different pH values: a)12 b) 11 c) 10 d) 9 e) 8 f) 7 g) 6 h) 5 i) 4 j) 3 k) 2.

The effect of pH on the peak current and the peak potential of 1,4-benzoquinone for the average values of three measurements by using three separate electrodes are shown in Figure 6 and 7 respectively.

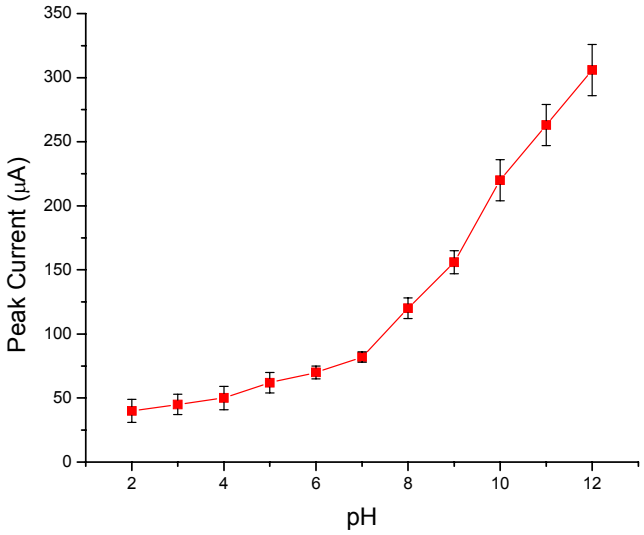


Figure 7. Variation of the peak current of 1,4-BQMCPE with pH

As can be seen from Figure 7, the peak current of 1,4-benzoquinone increases gradually with increasing pH up to pH 7 and then rises sharply from pH 7 up to pH 12. The figure also shows the uncertainties involved in the measurements by using error bars. The highest uncertainties were observed during the measurement of the voltammetric peak currents of the modified electrode at higher pH values especially pH 10 and upwards.

Figure 8 shows the effect of pH on the peak potential of 1,4-BQMCPE . It is observed that the peak potential of 1,4-benzoquinone shifts towards more negative potentials as the pH increases. Larger uncertainty values in the measurement of pH effects on peak potentials were observed for both smaller ($pH \leq 4$) and higher pH values ($pH \geq 10$).

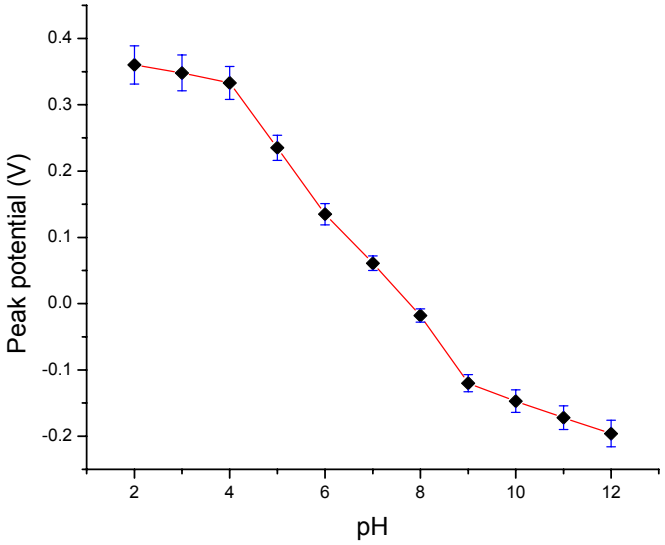


Figure 8. Variation of the peak potential of 1,4- BQMCPE with pH

In the experiment conducted, the electrode was not stable at higher pH values, especially above pH 10, and the results were not reproducible. Therefore, we used pH 9 through out the experiment.

5.3. Effect of Square Wave Frequency

The effect of square wave frequency on the peak current of 1,4-BQMCPE was studied by varying the square wave frequency from 10 Hz to 50 Hz at step potential of 2 mV and amplitude of 30 mV in the buffer solution. The effect of square wave frequency on peak current of 1,4-benzoquinone is shown in Figure 9.

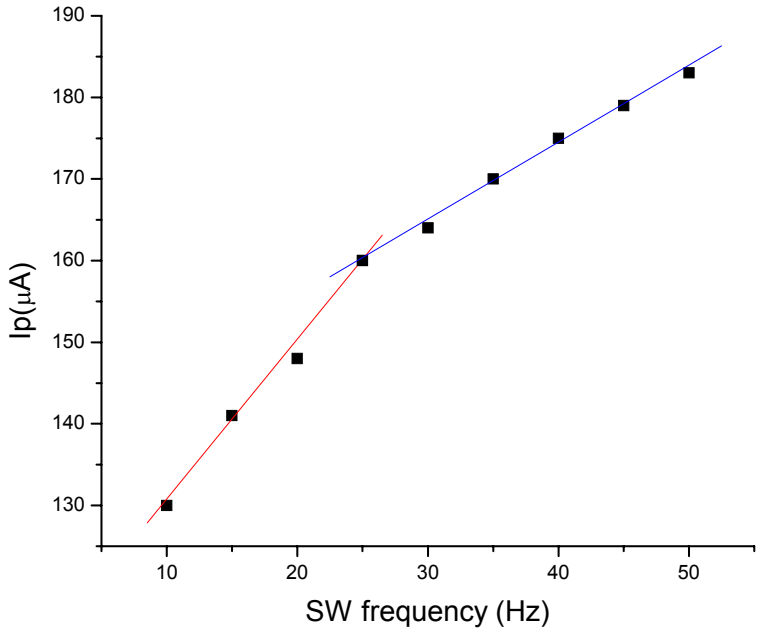


Figure 9. Dependence of peak current of 1,4-BQMCPE on the square wave frequency

As can be seen from Figure 9, the magnitude of the peak current increases linearly with increasing the square wave frequency, however the slop changes to a lower value after frequency of 25 Hz. These indicate that, the dependence of the peak current on frequency is being distorted due to an accompanying peak broadening. In addition, in the experiment conducted the peak current was unstable at higher frequencies and which affects the reproducibility of the measurement. As a result, 25 Hz was chosen as the square wave frequency for the subsequent experiments.

5.4. Effect of Square Wave Amplitude

The effect of square wave amplitude on the peak current of 1,4-BQMCE was studied by varying the square wave amplitude from 10 mV to 50 mV at frequency of 25 Hz and step potential of 2 mV. The peak current for 1,4-benzoquinone as a function of the square wave amplitude is shown in Figure 10.

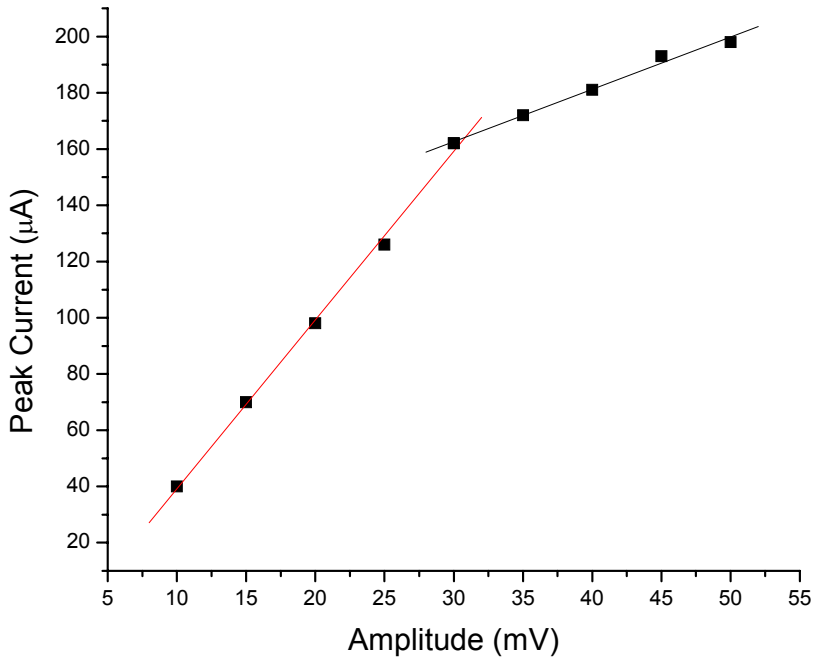


Figure 10. Square wave voltammetric peak currents of 1,4-BQMCPE at various square wave amplitude.

Upon increasing the square wave amplitude a linear increase in the peak current was observed accompanied by peak broadening in particular when the amplitude was greater than 30 mV. Hence, 30 mV was chosen as the square wave amplitude for the subsequent experiment.

5.5. Effect of Square Wave Step Potential

The effect of square wave step potential on the peak current of 1,4-benzoquinone was studied by varying the step potential from 1 mV to 10 mV at frequency of 25 Hz and amplitude of 30 mV. The peak current for 1,4-benzoquinone as a function of the square wave step potential is shown in Figure 11.

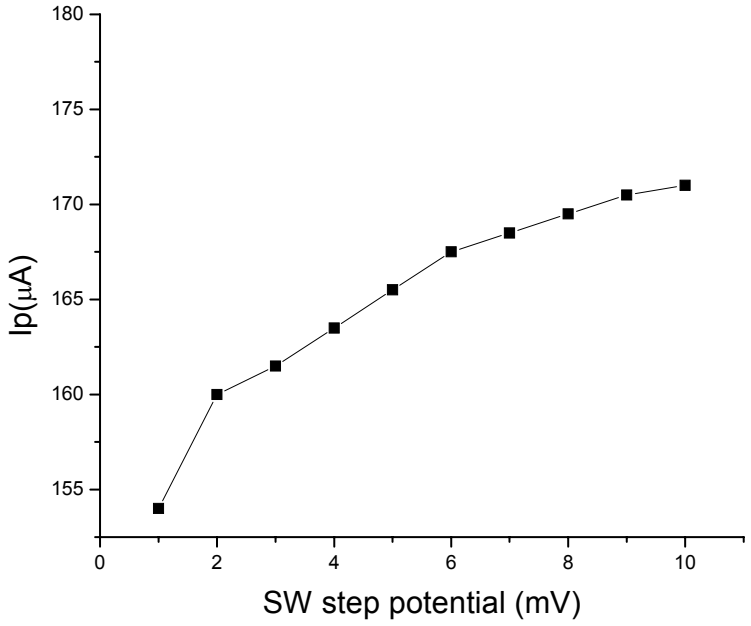


Figure 11. Square wave voltammetric peak current of 1,4-BQMCPE at various step potentials.

The peak current increases as the square wave step potential increases, however, accompanied by peak broadening and affecting the reproducibility of the detection. Hence, 2 mV was chosen as the square wave step potential for the subsequent experiment.

5.6. Effect of Modifier Content

The effect of the amount of 1,4-benzoquinone in the carbon paste on the voltammetric response of the modified carbon paste electrode was studied by varying the amount of 1,4-benzoquinone. The responses are shown in Figure 12.

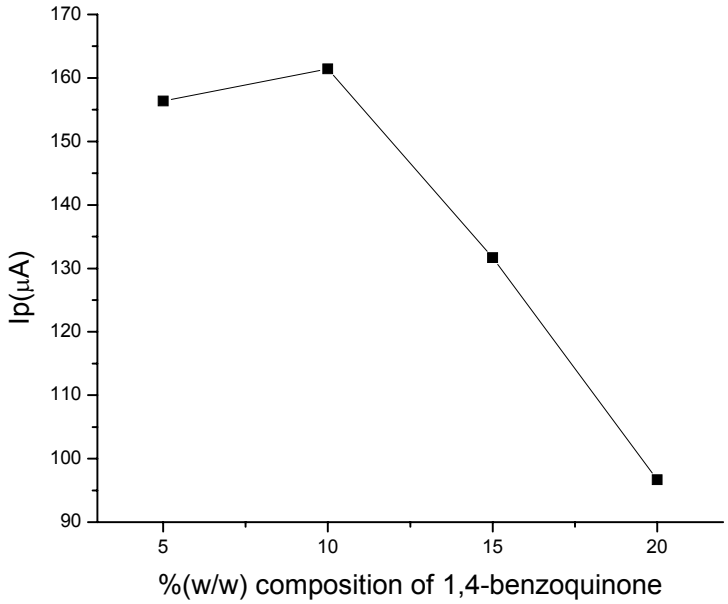


Figure 12. The effect of amount of 1,4-benzoquinone in the 1,4-BQMCPE electrode on the squarewave voltammetric peak current.

The maximum peak current was observed at 10 % (w/w) composition of 1,4-benzoquinone in the carbon paste. For amounts higher than 10 % (w/w) the peak currents decreased significantly. This may be due to a decrease in the graphite content in the paste and, consequently reduction of the electric conductivity of the electrode [55]. The best composition for the electrode was found to be, 10 % (w/w) 1,4-benzoquinone, 20 % (w/w) paraffin oil, and 70 % (w/w) graphite powder. The optimum parameters used for the experiment are summarized in Table 2.

Table 2: Optimum values of the experimental parameters.

Parameters	**Optimum values**
pH of the supporting electrolyte	9
Square wave frequency (Hz)	25
Square wave amplitude (mV)	30
Square wave step potential (mV)	2
Composition of 1,4-BQMCPE % (w/w)	10

6. Square Wave Investigation

The square wave voltammogram of 1,4–benzoquinone modified carbon paste electrode, under the optimum experimental parameters of Table 3, in the absence of caffeine is shown in Figure 13.

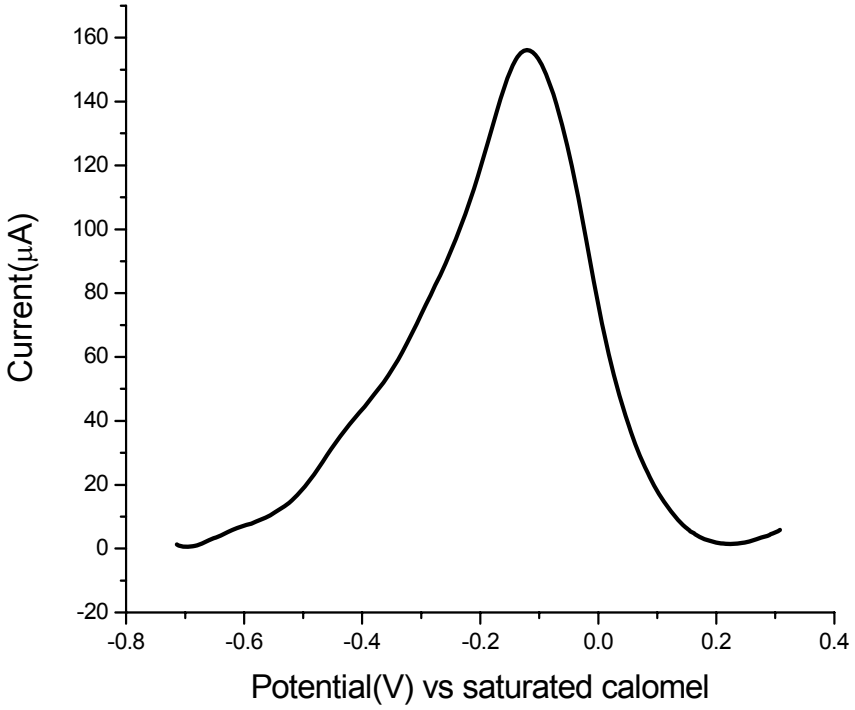


Figure 13. Square wave voltammogram of 1,4-BQMCPE

In the presence of caffeine within the solution, the square wave voltammogram of 1,4-benzoquinone displays a distinct reduction of the peak current. Further increase in the concentration of caffeine resulted in a successive decrease of 1,4-benzoquinone redox peak. The reason for the reduction of the peak current with increase in caffeine concentration is not well understood. Figure 14 shows the square wave voltammograms obtained by using the modified electrode for different caffeine concentrations.

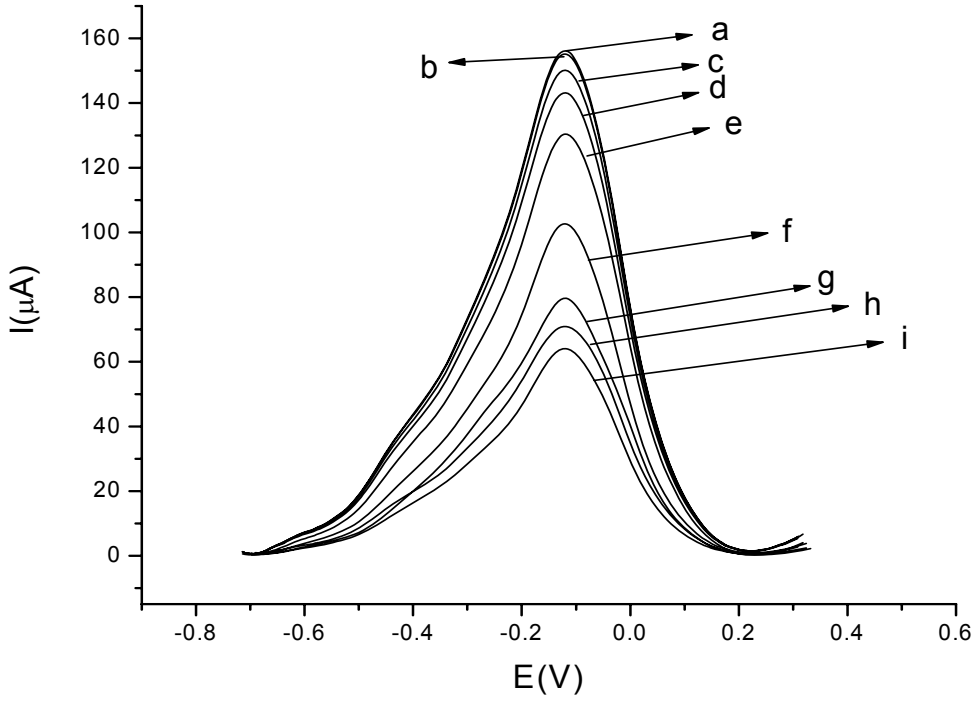


Figure 14. Square wave voltammogram of 1,4–BQMCPE at different caffeine concentrations: a) 0 b) 0.3 c) 0.5 d) 1 e) 2 f) 4 g) 6 h)18 i) 10 μM caffeine.

7. Linear Range and Detection Limit

The square wave voltammetric peak current of 1,4–BQMCPE againest 0 up to 10 μM concentrations of caffeine recorded based on the optimum experimental conditions shown in Table 3 are shown in Figure 15.

As can be seen from the figure, the peak currents above caffeine concentration of 6 μM lie outside the straight line. Therefore the linear range extends only from 0 to 6 μM caffeine concentration.

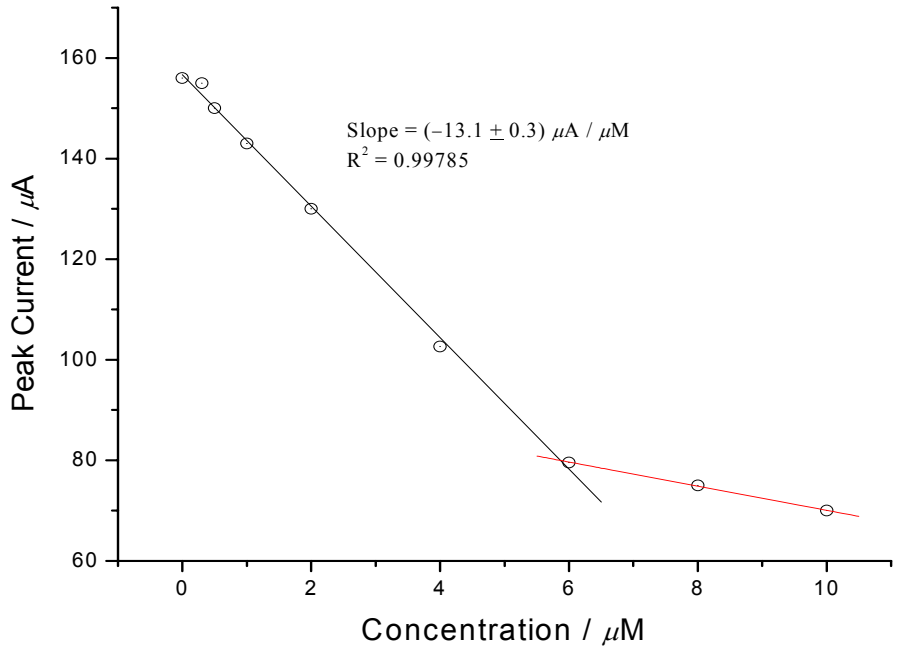


Figure 15. Plot of the square wave voltammetric peak currents of 1,4-BQMCPE for 0 up to 10 μM caffeine concentrations.

A calibration curve was plotted based on the average voltammetric peak currents of three measurements performed by using three separate 1,4-benzoquinone modified carbon paste electrodes in the range 0 to 6 μM concentration of caffeine. Figure 16 shows the calibration curve of the square wave peak currents of 1,4-BQMCPE against the concentrations of caffeine.

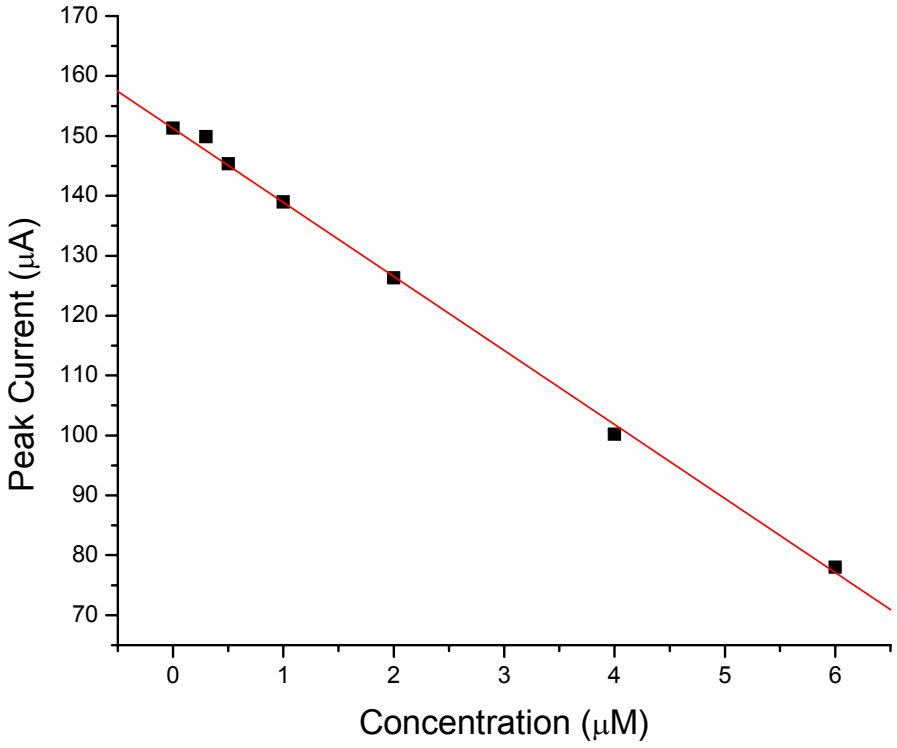


Figure 17. Calibration curve for the square wave voltammetric responses of 1,4-BQMCPE for the caffeine concentration range 0-6 μM: Slope = −12.4±1.0 μA/μM, R = 0.9994.

The detection limit, which is three times the standard deviation for three separate serious of measurements using one modified electrode for each was found to be 30 nM. And the sensitivity of the method as shown by the slope of the above calibration curve is 12.4 μA/μM. The result obtained in this study is almost three orders of magnitude better than the previously reported [9] detection limit of 5.136 μM for caffeine determination based on squarewave voltammetry by using 1,4-benzoquinone modified carbon paste electrode.

8. Conclusion

The method presented in these study is a simple and fast procedure for the determination of caffeine based on square wave voltammetric analysis by using 1,4-benzoquinone modified carbon paste electrode. The linear working range was much lower and the detection limit was greatly improved to allow a sensitive detection of caffeine. The very low detection limit and its high sensitivity suggest that the modified carbon paste electrode can act as a useful electrode material for the development of electrochemical sensor for caffeine.

9. References

1. M. W-Plantenga, K. Diepvens, A.M.C.P. Joosen, S. B-Parent, A. Tremblay, Physiology & Behavior xx (2006) xxx.
2. John O. Miners and Donald J. Birkett, Gen. Pharmac. 27 (1996) 245.
3. Jyh-Myng Zen, Yuan-Shih Ting, Analytica Chimica Acta 342 (1997) 175.
4. Yan-Li Wei, Chuan Dong, Shao-Min Shuang, Dian-Sheng Liu, Spectrochimica Acta Part A 61 (2005) 2584.
5. N. Spa*f*tarua, B.V. Sarada, D. A. Tryk, A. Fujishima, Electroanalysis 14 (2002) 721.
6. M. Mumin, K. Akhter, Z. Abedin, Z. Hossain , Malaysian Journal of chemistry 8 (2006) 45.
7. L. Frost, P. Vestergaard, Am J Clin Nutr 81 (2005) 578.
8. S. Gokulakrishnan, K. Chandraraj, S.N. Gummadi, Enzyme and Microbial Technology 37 (2005) 225.
9. M. Aklilu, M.Sc. Thesis (2006) Addis Ababa University.
10. I. Pavel, A. Szeghalmi, D. Moigno, S. Cinta, W. Kiefer, Biopolymers (Biospectroscopy) 72 (2003) 25.
11. M. Ortha,b, B. Amannb, N. Ratnarajc, P.N. Patsalosc, J.C. Rothwella, Clinical Neurophysiology 116 (2005) 308.
12. A. Pizzariello, J.S Vorc, M. Stredansky, S. Miertus, J Sci Food Agric 79 (1999) 1136.
13. P. Saba, W.A. Brown, S. Omanovic, Materials Chemistry and Physics xxx (2006) xxx.
14. H.G. Mandel, Food and Chemical Toxicology 40 (2002) 1231.
15. S. Gokulakrishnan, K. Chandraraj, S.N. Gummadi, Enzyme and Microbial Technology 37 (2005) 225.
16. S. Kerrigan, T. Lindsey, Forensic Science International 153 (2005) 67.
17. M-L. Nurminen, L. Niittynen, R. Korpela and H. Vapaatalo, European Journal of Clinical Nutrition 53 (1999) 831.
18. K.M. Madyastha, G.R. Sridhar, Biochemical and Biophysical Research Communications 249 (1998) 178.

19. E. Begas, E. Kouvaras, A. Tsakalof, S. Papakosta, E. K. Asprodini, Biomed. Chromatogr. 21 (2007) 190.
20. M. Stredansky, A. Pizzariello, S. Miertus, J. Svorc, Analytical Biochemistry 285 (2000) 225.
21. Z. Textor, M. Beer, M. Anetseder, H. Ko-Stler, E. Kagerbauer, W. Kenn, D. Hahn, N. Roewer, Muscle Nerve 28 (2003) 353.
22. R. J.S-Pascualb, M. V-Floresa, R. Ma Camposa, Sleep Medicine xx (2006) 1.
23. A. Ascherio, S.M. Zhang, M.A. Hernan, I. Kawachi, G.A. Colditz, F. E. Speizer, W.C. Willett, Ann Neurol 50 (2001) 56.
24. E.D. Louis, E.C. Jurewicz, L. Applegate, J.A. Luchsinger, P. F-Litvak, M. Parides, Movement Disorders 19 (2004) 499.
25. A. Tavani, A. Pregnolato, C.L. Vecchia, E. Negri, R. Talamini, S. Franceschi, Int. J. Cancer 73 (1997) 193.
26. J. Greenberg, K. Axen, R. Schnoll, C. Boozer, International Journal of Obesity 29 (2005) 1121.
27. M. Namdar, P. Koepfli, R. Grathwoh, P.T. Siegrist, M. Klainguti, T. Schepis, R. Delaloye, C.A. Wyss, S.P. Fleischmann, O. Gaemperli, P.A. Kaufmann, JACC 47 (2006) 405.
28. F.A. Guarraci, A. Benson, Pharmacology, Biochemistry and Behavior 82 (2005) 522.
29. C.M. Papamichael, K.A. Aznaouridis, E.N. Karatzis, K.N. Karatzi, K.S. Stamatelopoulos, G. Vamvakou, J. P. Lekakis, M.E. Mavrikakis, Clinical Science 109 (2005) 55.
30. T.R. Mikuls, J.R. Cerhan, L.A. Criswell, L. Merlino, A.S. Mudano, M. Burma, A.R. Folsom, K.G. Saag, Arthritis & Rheumatism 46 (2002) 83.
31. B.H. Bech, E.A. Nohr, M. Vaeth, T.B. Henriksen, J. Olsen, Am J Epidemiol 162 (2005) 983.
32. M. S-Ortunoa, N. Mooreb, J. Taillarda, C.Valtata, D. Legerc, B. Bioulaca, P.Philipa, Sleep Medicine 6 (2005) 247.

33. M. Klebanoff, R. Levine, Rebeccadersimonian, S.C.D.,Johnd. Clemens, A. Wilkins, The New England Journal of Medicine 341(2007) 1639.
34. C. P. O'Brien, J. Bergman, Food and chemical toxicology 40 (2002) 1257.
35. Vlachopoulos et al, Am J Clin Nutr 81 (2005) 1307.
36. E. Karatzisa, T.G. Papaioannou, K. Aznaouridis, International Journal of Cardiology 98 (2005) 425.
37. Qian-Li Zhang, H-Zhen Lian, W-Han Wang, H-Yuan Chen, Journal of Chromatography A, 1098 (2005) 172.
38. A. Krisko, M. Kveder, G. Pifat, Clinica Chimica Acta 355 (2005) 47.
39. J. Tchekalarovaa, H. Kubovab, P. Mares, B r a i n r e s e a r c h x x (2 0 0 6) x x x.
40. Z.L-Huang, W-Min Qu, N.Eguachi, Nature neuroscience 8 (2005) 858.
41. W.C. Winkelmayer, M.J. Stampfer, JAMA 294 (2005) 2330.
42. A.S-Maria, A. Lopez, M.M. Diaz, A.I. Ortiz, C. Caballo, Teratogenesis, Carcinogenesis, and Mutagenesis 21 (2001) 207.
43. K.H. Dausmann, J. Glos, J.U. Ganzhorn, G. Heldmaier, Nature 429 (2004) 826.|
44. M. Kato, K. Mizuno, A. Crozier, T. Fujimura, H. Ashihara, Nature 406 (2000) 956.
45. D. Chuan , W. Yan-Li, S. Shao-Min, Spectrochimica Acta Part A 59 (2003) 1469.
46. Q. Chen, J. Zhao, X. Huang, H. Zhang, M. Liu, Microchemical Journal 83 (2006) 42.
47. D.K. Singh, A. Sahu, Analytical Biochemistry 349 (2006) 176.
48. Y. Getinet, M.Sc. Thesis (2005) Addis Ababa University.
49. A. Weimann, M. Sabroe, H.E. Poulsen, J. Mass Spectrom. 40 (2005) 307–316.
50. V.R.S. Babu, S. Patra , N.G. Karanth , M.A. Kumarb, M.S. Thakur, Analytica Chimica Acta 582 (2007) 329.
51. J-Myng Zen, Y-Shih Ting, Ying Shih, Analyst, May 123 (1998) 1145.
52. A. Ferguson, R.M. Boomer, M. Kurz, S.C. Keene, J.L. Diener, Nucleic Acids Research 32 (2004) 1756.
53. B. Brunetti, E. Desimoni, P. Casati, Electroanalysis 19 (2007) 385.
54. Ly,-SY, Jung-Ys, Kim-Mh, Han-Ik, Jung-Ww, Kim-Hs, Microchim-Acta. 146 (2004) 207

55. I. Svancara, K. Vytras, J. Barek, J. Zima, Critical Reviews in Analytical Chemistry 31 (2001) 311.
56. A. Safavi, N. Maleki, O. Moradlou, F. Tajabadi, Analytical Biochemistry 359 (2006) 224.
57. G. Shul, J. S-Plenet, L. Gaillon , M. Opallo, Electrochemistry Communications 8 (2006) 1111.
58. S.F.Wang , H.Y. Xiong, Q.X. Zeng, Electrochemistry Communications 9 (2007) 807.
59. K. Schachl, H. Alemu, K. Kalcher, J. Jeozkova, I. Svancara, K. Vytoras, Analyst, 122 (1997) 985.
60. G. Shul, J. Sirieix-Plenet, L. Gaillon, M. Opallo, Electrochemistry Communications 8 (2006) 1111.
61. D. Moscone, D. D.Ottavi, D. Compagnone, G. Palleschi, Anal.Chem. 73 (2001) 2529.
62. M.E. Lozano-Chaves, J.M. Palacios-Santander, L.M. Cubillana-Aguilera, I. Naranjo-Rodriguez, J.L. H-Hidalgo-de-Cisneros, Sensors and Actuators B 115 (2006) 575.
63. R.M. Kotkar, P.B. Desai, A.K. Srivastava, Sensors and Actuators B xxx (2006) xxx.
64. R.M. Kotkar, A.K. Srivastava, Sensors and Actuators B 119 (2006) 524.
65. H.R. Zare, N. Nasirizadeh, S-Mahdi Golabi , M. Namazian, Sensors and Actuators B 114 (2006) 610.
66. A. Safavi, N. Maleki, O. Moradlou, F. Tajabadi, Analytical Biochemistry 359 (2006) 224.
67. C. Mousty, Applied Clay Science 27 (2004) 159.
68. Z. Navra-tilova, P. Kula, Electroanalysis 837 (2003) 15.
69. A. Walcarius, Analytica Chimica Acta 384 (1999) 1.
70. J-Myng Zen, A.S. Kumar, D-Mung Tsai, Electroanalysis 1073 (2003) 15.
71. J.E. Heffner, J.C. Raber, O.A. Moe, C.T. Wigal, JChemEd.chem.wisc.edu 75 (1998) 365.
72. M. Salas, B. Gordillo, F.J. Gonzalez, Arkivoc (vi) (2005) 172.
73. H.R. Zare, N. Nasirizadeh, M.M. Ardakani, Journal of Electroanalytical Chemistry 577 (2005) 25.
74. M. Tessema, T. Ruzgas, L. Gorton and T. Ikeda, Analytica Chimica Acta 310 (1995)161.

75. A.J. Bard, Electrochemical Methods 3rd ed. (2000).

76. P. Delahay, New Instrumental Methods in Electrochemistry (1980).