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SQUARE WAVE VOLTAMMETRIC DETERMINATION OF CAFFEINE IN COFFEE

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DECLARATION

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Table of Contents

Page

Acknowledgements……....i
Table of Contents……...ii
List of Figures……v
List of Tables……....vi
Abstract……....vii

1. Introduction..... 1
1.1. The Coffee Species.....1
1.2. Green Coffee Processing.....2
1.2.1. Dry Processing Method.....3
1.2.2. Wet Processing Method.....4
1.3. Chemical Aspects of Green Coffee.....4
1.3.1. Xanthines.....5
1.3.2. Trigonelline..... 7
1.3.3. Quinic acids and Its Derivatives.....7
1.3.4. Volatile constituents.....9
1.4. Physiological Aspects of Coffee and Caffeine.....9
1.4.1. Mechanism of Action..... 10
1.4.2. Effects on Exocrine Glandular systems.....10
1.4.3. Effects on Endocrine Glandular Systems.....10
1.4.4. Effect on Performance.....11
1.4.5. Beneficial Effects..... 12
1.5. Major Characteristics Coffee Beverage Taste.....13
1.6. Method of Analysis..... 13
1.6.1. Spectroscopic Method..... 14
1.6.2. Chromatographic Method.....14
1.6.3. Voltammetric Method..... 14
1.7. Carbon Electrodes..... 17
1.8. Objective..... 18

2. Theoretical Background..... 20
2.1.Cyclic Voltammetery..... 20
2.2.Pulse Voltammetric Techniques.....21
2.3. Square wave voltammetry.....22
2.4. Choice of Operating Parameters in Square Wave Voltammetry……..……23

2.4.1. Effect of Supporting Electrolyte.....23
2.4.2. Square Wave Frequency.....24
2.4.3. Square Wave Amplitude..... 24
2.4.4. Step Potential…….……24

3. Experimental..... 25
3.1. Reagents and Chemicals.....25
3.2. Apparatus.....25
3.3. Preparation of Working Electrode.....26
3.4. Voltammetric Procedure.....27
3.5 Recovery Study..... 28
3.6. Analysis of Real Samples.....28
3.6.1.Coffee Samples.....28
3.6.2 Sample Preparation.....28
3.6.3. Water Content.....29
4. Results and Discussion.....30
4.1. Cyclic Voltammetric Investigation..... 30
4.2. Square Wave Voltammetric Investigation..... 31
4.2.1 Effect of pH Supporting Electolyte.....31
4.2.2. Effect of Square Wave Frequency.....32
4.2.3. Effect of Square Pulse Amplitude.....35
4.2.4 Effect of Step Potential.....35
4.2.5. Optimum Experimental Condition.....36
4.2.6. Linear Range and Detection limit..... 37
4.2.7. Recovery Study..... 40

4.2.8. Real Sample Analysis……..…….40

5. Conclusion.....42

6. References..... 43

List of Figures

Page

Figure 1. Cross section of coffee cherry..... 3
Figure 2. Structure of xanthines..... 6
Figure 3. Structure of trigoneline..... 8
Figure 4. The structure of chlorogenic acid..... 8
Figure 5. Chemical process for the oxidation of caffeine..... 16
Figure 6. Potential waveform for square voltammetry..... 22
Figure 7. Experimental set up for voltammetric measurment of caffeine.....26
Figure 8. The carbon paste electrode..... 27
Figure 9. Cyclic voltammogram of: (A) 2 mM caffeine concentration and (B) Phosphate buffer pH 6..... 30
Figure 10. Square wave voltammogram of 100 mg/L caffeine from pH 2 to pH 6.…….31
Figure 11. Effect of pH on the peak current…….. 32
Figure 12. Dependence of peak current on the square wave frequency for 50 mg/L of caffeine.....33
Figure 13. Plot of logarithm of square wave peak current versus logarithm of frequency for 50 mg/L caffeine concentration.....34
Figure 14. Square wave voltammograms of 50 mg/L caffeine at various frequencies.....34
Figure 15. Square wave voltammetric peak current for various square wave amplitude.... 35
Figure 16. Square wave voltammetric peak current for various step potentials..... 36
Figure 17. Square wave voltammograms of caffeine for various concentrations.....39
Figure 18. Calibration curve for caffeine with the results of regression line.....39
Figure 19. Standard addition calibration graph for spiked caffeine…….40

List of Tables

Table 1. Solubility of caffeine in water and organic solvents at 25 ^{0}C.....7
Table 2. Optimum experimental conditions for the determination of caffeine by SWV…….36
Table 3. Comparision of CPE developed with other some other methods..... 38
Table 4. Caffeine content in Ethiopian green coffee samples……41

Abstract

Square Wave Voltammetric Determination of Caffeine in Coffee

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A carbon paste electrode was utilized for monitoring caffeine using square wave voltammetry (SWV). This method was applied to determine the caffeine levels in some coffee samples. Optimal experimental conditions for the analysis were found to be as follows: pH value of 6 for the suporting electrolyte, SW frequency of 30 Hz, SW amplitude of 40 mV, and step potential of 7 mV. Having these optimum conditions a linear range was observed with in the concentration of 0 ~ 250 mg/L. For caffeine concentration of 50 mg/L, the detection limit three times the standard deviation in measured concentration (n=6) was found to be (10.67μg/mL). This method was applied for the determination of the caffeine levels in Ethiopian green coffee samples. The caffeine content of Ethiopian green coffee samples ranged from 0.9 - 1.16 % on dry mass basis. The quantities are in good agreement with those reported in the literature for *Coffea arabica*. The results are also in good agreement with values determined by HPLC method.

Key words: Square Wave Voltammetry, Coffee, Caffeine and Carbon Paste Electrode.

Coffea robusta is grown from sea level to 600 m. *Rubustas* lack the acidity and complexity of the best *arabica* coffees, although they often display heavy body [5]. They are used mainly as unnamed additions into the cheaper coffee blends and are very commonly found in restaurants and supermarkets.

Liberica coffee grows as a large strong tree, up to 18 metres in height, with large leathery leaves. The fruits and beans are also large. *Liberica* coffee is grown in Malaysia and in West Africa, but only very small quantities are traded, as demand for its flavour characteristics is low [2].

In addition to genetic variation, environment and method of cultivation add to the diversity. The dozzens of different varities have been given commercial names. Coffee grown in Brazil for example is called Brazils. Coffee grown elsewhere in Latin America, Kenya, Ethiopia and Tanzania is termed milds. Mild coffees are exclusively high quality varieties of *arabica*. Coffee grown in Yemen is called Mocha and that of Indonesia called Java. Most coffee products are mixtures that combine the characteristics of different species and varieties professionally blended to satisfy consumer's tast. Ethiopian *C. arabica* grows exclusively in the region of Keffa, Sidamo, Illubabor, Harrar and Wellega. Ethiopia produces between 180,000 and 200,000 tones of coffee per annum and is the major producer of *arabica* and the second largest coffee producer African country [1].

1.2. Green Coffee Processing

Following harvesting, green coffee is prepared from the cherries (freshly picked coffee fruit) by a relatively complex process of steps. A basic problem to be overcome is to take out the coffee seeds from within the cherry, by removing various covering layers, in the most efficient manner, and to provide the coffee as far as possible as marketable green coffee beans with moisture content less than 10 - 13 percent. The two main processes that have been used for many years are dry process (natural) and wet process (washed). In addition certain cleaning process is adopted together with various grading, and sorting techniques to provide the finished coffees by specification for export.

1.2.1. Dry Processing Method

The dry processing method is the oldest and the simplest. The harvested cherries are subjected to some form of classification and then are dried entirely most usually in the sun for eight to ten days in good weather. This method is adopted for preparation of coffee in most parts of the world. The dried cherry coffee is then subjected to a milling or also called hulling operation to separate out the green beans from the dried husks.

The machine drying of coffee cherry using this method only takes some three days. Clearly sun drying is subject to the varieties of atmospheric conditions, together with possibilities of growth of both desirable and undesirable microorganisms generating substances from the drying pulp that affect subsequent flavor of the coffee brew. Figure 1 shows the cross section of coffee cherry [1].

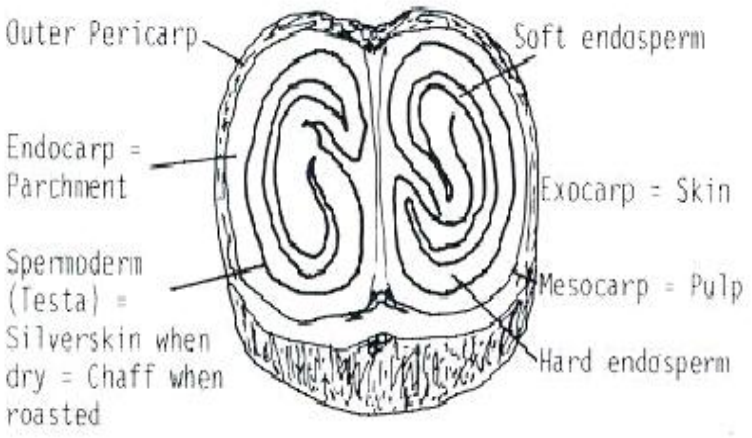

Figure 1. Cross section of coffee cherry.

1.2.2. Wet Processing Method

This process is more sophisticated than the dry process, and by general consent leads to a better quality coffee and command higher price. In this method the cherry is squeezed in a pulping machine, which removes the outer fleshy material (mesocarp and exocarp) leaving a bean covered in mucilage. This mucilage is fermented and dispersed.

Pulping involves the removal of the outer red skin (exocarp) and the white fleshy pulp (mesocarp) and the separation of the pulp and beans. During the pulping stage, it is recommended that the cherries should be kept under water for 12-24 hours. Then the cherries are drawn to hoppers of the pulpers with water. The waste pulp is drawn out at the rear of the machine. The separated pulped beans then pass in short water washing channels for flotation and separation by means of sluice gates.

The second stage, fermentation is necessary to remove any residual adhering pulp and importantly to remove a very adhesive mucilaginous layer leaving only the parchment enclosing the green beans. Fermentation in large scale practice is a batch process taking place in rectangular sectioned concrete tanks filled with water. Natural enzymes in the mucilage and bacteria in the environment work together to break down the mucilage. The time of fermentation is 24 hours on average. Finally, drying the wet parchment coffee from which mucilage has been removed is accomplished by either sun drying or by hot air drying in a suitable mechanical dryer. The thin parchment (husk) is removed by hulling, as in the dry process [1].

1.3. Chemical Aspects of Green Coffee

The various constituents in coffee are xanthines, trigoneline, chlorogenic acids, free amines and amino acids, carbohydrates, lipids, quenic acids and its derivatives and volatile constituents [1].

1.3.1. Xanthines

Caffeine (1,3,7- trimethyl xanthine), is the major xanthine in green coffee. Xanthines are alkaloids. Alkaloids are classes of naturally occurring compounds of nitrogen and having the property of an organic amine base. Smaller quantities of theobromine (3,7-dimethyl xanthine) and traces of theophylline (1,3-dimethyl xanthine) have also been determined. They are all known to have mild stimulations to a varying extent; cause stimulation of the central nervous system and the skeletal muscle. Caffeine is the most powerful in this respect [1].

The caffeine content of green coffee varies depending on the species of beans, where they are grown and the way they are processed. Environmental and agricultural factors are considerably less important than genetic variation in controlling the caffeine content of green coffee. Reviews of extensive studies of caffeine content of green coffee beans in % on dry mass basis show that [6];

Coffee arabica 0.6 - 1.6%
Coffee liberica 1.4 - 1.8%
Coffee robusta 1.2 - 2.4%

It is also known that a naturally decaffeinated coffee was discovered in Ethiopia by a Brazilian research group in June 2004 [7]. Even though *robusta* has higher caffeine content, it lacks many of the flavoring found in *arabica*, so it is most often used for instant coffees. Pure caffeine occurs as odorless, long white, silky crystal with a melting pt. 238^0c, sublimation pt. 178 ^{o}C, and density 1.2 g/cm^3. The structure of caffeine and other xanthines are given in Figure 2.

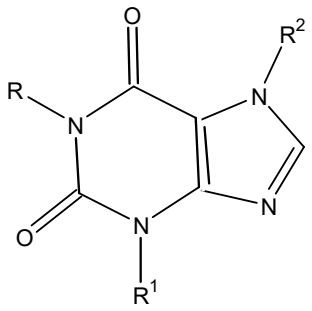

Figure 2. Structure of xanthines

R	R^1	R^2	Compound
CH_3	CH_3	H	Theophylline
H	CH_3	CH_3	Theobromine
CH_3	CH_3	CH_3	Caffeine

Solubility of Caffeine in Water and Organic Solvents

Caffeine is moderately soluble in water and wide range of organic solvents as shown in Table 1.

Table 1. Solubility of caffeine in water and organic solvents at 25 ºC [7].

Solvent	Solubility g/100 g Solvent
Water	2.13
Ethanol	1.88
Methanol	1.14
Acetone	2.32
Benzene	0.91
Carbon tetrachloride	0.09
Chloroform	12.9
Dichloromethane	9

1.3.2. Trigonelline

Trigonelline is the N-methyl betaine of pyridine-3-carboxilic acid. It is found in all commercial green coffees [6]. Roasting causes progressive destruction. The degradation products include vitamin B, (nicotinic acid), nicotine amide and a range of aroma volatiles, which includes pyridines and pyroles. The structure of trigoneline is shown in Figure 3.

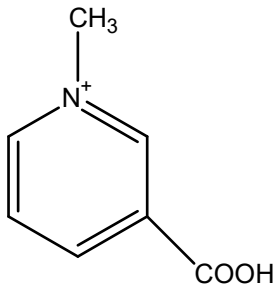

Figure 3. Structure of trigoneline

1.3.3. Quinic acids and Its Derivatives

Quinic acid occurs free and in esterified form. The esters known as chlorogenic acids form a quantitatively important fraction of green and roasted coffee beans. The solubility of chlorogenic acids is 4% at 25 ºC in water; having higher solubility in hot water. It is readily soluble in ethanol and acetone, with very slight solubility in acetyl acetate. Figure 4 shows the structure of chlorogenic acid [6].

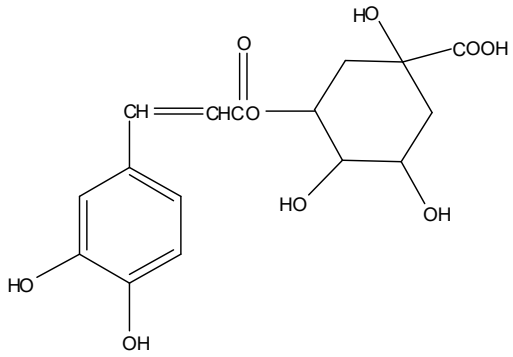

Figure 4. The structure of chlorogenic acid

1.3.4. Volatile Constituents

The characteristics odor of coffee has been attributed to a group of methoxy pyrazines, aliphatic hydrocarbons, carbonyls, acids, alcohols, and thiols; furans, pyrrols, pyridines and qunolines; phenols, aromatic amines have also been detected [8].

1.4. Physiological Aspects of Coffee and Caffeine

The effects of coffee consumption are associated with compounds, which in dietary terms are found only, or almost only in coffee beverage. Scientific attention has been focused upon caffeine and the chlorogenic acids. Caffeine is much more studied because of its known pharmacological actions.

Depending on the techniques of preparation a cup of coffee (150 mL) contains about 100 mg caffeine with a range from half this value for weak beverage to about double this value for typical espresso. Typical coffee brews contain some 30-120 mg total chlorogenic acids per 100 mL [8].

In humans, caffeine appears in all tissue fluids about five minutes after application, and peak blood plasma levels are reached after about 20 to 30 minutes. Caffeine is almost entirely metabolized, and only about 1% is excreted via urine in its non-metabolized form. The main metabolites belong to the classes of demethylated xanthines, uric acids and uracid derivatives of caffeine. The half life of caffeine in plasma has been reported to be about three to six hours but varies widely between individuals. The lathal dose (LD_{50}) for humans has been reported to be 60 mg/kg [9]. With no evidence of the harmful effects, up to 300 mg/day (3 cups of coffee) is considered moderate with a vast majority of adult population [10].

1.4.1. Mechanism of Action

Caffeine is thought to act on the brain by blocking adenosine receptors. Adenosine when bound to receptors of nerve cells; slows down nerve cell activity; this happens, among other times, during sleep. The caffeine molecule being similar to adenosine, binds to the same receptors but does not cause the cell to slow down; instead the caffeine blocks the receptors and there by the adenosine action. The resulting increased nerve activity causes the release of the hormone epinephrine [11], which in turn leads to several effects such as higher heart rate, increased blood pressure, increased blood flow to muscles, and release of glucose by the liver. In addition, caffeine similar to amphetamines increases the level of the neurotransmitter dopamine in the brain [12]. Dopamine is critical to the way the brain controls our movements, a reduction in its concentration within the brain is associated with Parkinson's disease [13].

1.4.2. Effects on Exocrine Glandular Systems

Caffeine and other xanthines increase secretion by exocrine glandular systems. Both acid and pepsin production increases after moderate dose of caffeine. Decaffeinated coffee also has shown only slightly smaller effects than those of normal coffee. It is also found that each is about twice as potent as equivalent of pure caffeine. This indicates that substances in coffee other than caffeine are important for gastric acid secretion [14].

1.4.3. Effects on Endocrine Glandular Systems

Caffeine as well as other methyl xanthines have stimulating effect on the endocrine secretion by different glands. The best known effect in this respect is probably the increase in circulating cathcolamines and rennin [10]. Stimulation of insulin and parathyroid hormone has been reported for theophiline only.

1.4.4. Effect on Performance

It is noted that caffeine has shown performance improvement that might decrease due to fatigue. Researchers on intelligence test after caffeine intake found improvement for types of performance, which depend on intellectual speed in which the task has been routinized through previous practice [15, 16].

1.4.5. Beneficial Effects

Scientific evidences continue to show that not only is coffee drinking in moderation safe, and no threat to health, it can also have some benefits [17]. Research findings show that moderate amounts of caffeine can help improve alertness and concentration [16].

Coffee and Bronchial Asthma

Caffeine has long been known to help asthmatics and many have found regular consumption of coffee to assist in moderating attacks. Scientifically this has been supported by two large studies in the USA and in Italy where three or more cups of coffee per day were associated in a dose related manner with reduced prevalence of asthma [17].

Reduced risk of Parkinson's Disease

Parkinson's disease occurs when neurons degenerate (lose the ability to function normally) in a part of the brain. As these neurons degenerate, dopamine levels fall, and the balance between dopamine and other neurotransmitters, such as acetylcholine, is thrown off. This neurotransmitter imbalance affects the way muscles work and leads to movement problems. The incidence of Parkinson's disease was found to be lower in those who drank coffee. A naturally occurring xanthine in the brain called adenosine is used as a neurotransmitter at some synapses. When adenosine receptors are blocked, levels of the neurotransmitter

dopamine increase. Caffeine may protect against Parkinson's disease by blocking adenosine receptors, thus increasing the amount of dopamine in the brain [13].

Antioxidant Substances in Coffee

The antioxidant activity of coffee results from polyphenols whose main components are chlorogenic acids. The ingestion of a cup of coffee produces significant increase (5.5%) in plasma antioxidant capacity [18]. The comparison of antioxidative capacity of coffee with that of other popular beverages such as wines, cocoa and tea known to contain antioxidants has showed relatively high activity of coffee [19].

Reduced Risk of Stone Formation (Calculi)

A group of researchers reported caffeinated coffee was found to be significantly more effective than water in helping patients avoid kidney stones [17].

Reduced Risk of Colon Cancer

There is some convincing evidence for a protective effect of coffee against the development of colon or colorectal cancers. Controlled study conducted in Sweden showed that coffee consumption appears to be protective against colon cancer and tea against rectal tumors. The conclusion was that frequent eating increases, whereas high coffee intake decreases the excretion of bile acids, which are suspected to be carcinogenic to the colon. Thus frequent coffee intake may counterbalance the effect of frequent eating. On the flip side, it is clear that coffee is not for every one. It may raise cholesterol levels in some people and may contribute to artery clogging. But most recent large studies show no significant adverse effect on most healthy people. Although, heart patients and those at risk for Osteoporosis are advised to limit or avoid coffee [17].

1.5. Major Characteristics of Coffee Beverage Taste

Acidity - The sensation of dryness in the back and under the edges of the mouth. This is a desirable quality and not to be confused with sour (which is considered a bad quality of coffee). Acidity creates a lively, bright taste without which the coffee would taste flat.

Aroma – Without aroma, one could only taste sweet, sour, bitter and salty. This is where one gets the subtle differences such as floral, nutty or fruity.

Body – The way the coffee feels in ones mouth, its viscosity or heaviness.

It has been demonstrated that there is no simple relationship between body and viscosity. Experiments suggest complex interactions between bitterness receptor sites, astringent phenols that could bind to them, and salivary proteins.

Flavor – This is the overall perception of the three characteristics above. Flavor can be rich (full bodied), complex (multi-flavored), or balanced (no one characteristic over powers the others). Flavor is a summary of acidity, body and aroma when combined provide a sensation of aromatic and taste compounds perceived by the senses of smell and taste.

1.6. Method of Analysis

The original method adopted was based on gravimetric measurements of caffeine in chloroform extract [6].

1.6.1. Spectroscopic Method

Another early method was based primarily upon UV spectrometry. Caffeine absorbs strongly in the ultraviolet region at 276 nm in chloroform. An aqueous extraction followed by liquid - liquid extraction in to chloroform layer provides significantly cleaner but still containing interfering compounds that absorb at 276 nm. The level of these interfering components may be reduced by column chromatography employing alkaline and acid celite columns in series. Despite all these clean up procedures direct measurement of absorption at 276 nm is still subject to background interference. This may be overcome by background correction [20, 21].

1.6.2. Chromatographic Method

More recent methods generally employ high performance liquid chromatography, HPLC. Caffeine is very amenable to liquid chromatography using reversed phase systems and as it contains strong ultraviolet chromophores, can be readily detected by UV detector. A wide range of methods have been published, all using very similar chromatographic conditions but differing in sample preparation procedures [22-26].

1.6.3. Voltammetric Method

The chromatographic method is rather costly compared to electro analytical methods. In spite of these the electroanalytical methods have rarely been used for the analysis of caffeine except in few recent reports. This is because the oxidation of caffeine occurs at very high positive potential, (1.4 V), which overlaps with the discharge of the background electrolyte [27-30].

In recent years many types of working electrodes have been developed [29]. The application of solid electrodes is limited by the useful potential window, where the

background current is below certain minimum value. The background current is due to the oxidation or reduction of the solvent or supporting electrolyte that results in a current of few microamperes.

In aqueous solution, the anodic potential window is limited by oxidation of water and interaction of the electrode with water or the supporting electrolyte. The oxidation of water causes serious problem in basic solution. For noble metallic electrodes like platinum or gold the useful anodic limit extends to only +1.0 V.

Two types of working electrodes have been applied for the voltammetric determination of caffeine. The first one is nafion/lead ruthenium oxide pyrochlore chemically modified electrode. Nafion modified electrodes are known cation exchangers. It was widely used for preconcentrating and determining trace concentrations of electroactive cations and the ruthenium oxide served as electrocatalyst. It has very good selectivity and high detection limit except its cost [28].

The second one is simple graphite pencil lead electrode with 0.5 mm diameter of pentel type 6H model. The use of pencil lead electrode as working electrode has been shown in some application [29, 31, 32]. However, different model of the pencil leads showed different background current few displaying minimum residual currents. The difference in residual current is attributed to difference in composition and roughness of the pencil leads. Pencil leads are composed of graphite, polymeric binder, and other additives (eg. clays). This along with the different porosities can influence the redox activity of the analyte. Even with the same model but different production batch number graphite pencil lead show significant variation in their response. Hence the selection of best pencil lead model is a problem associated with commercial pencil leads.

Based up on cyclic voltammetery, coulometry and chromatographic measurements the over all mechanisms of oxidation of caffeine involves four protons and four electrons [27, 30]. The mechanism proceeds by two electron potential controlling oxidation of carbon - nitrogen double bond to give dimethyl substituted uric acid at 1.4 V. This intermediate is

more readily oxidized than the parent compound immediately further oxidized in two electron process to uric acid 4,5, diol. This primary electrochemical product is extremely unstable as a result of steric factors so that it rapidly fragments to give the dimethyl substituted alloxan, allantoin and methyl urea. The electrochemical process is shown in Figure 5.

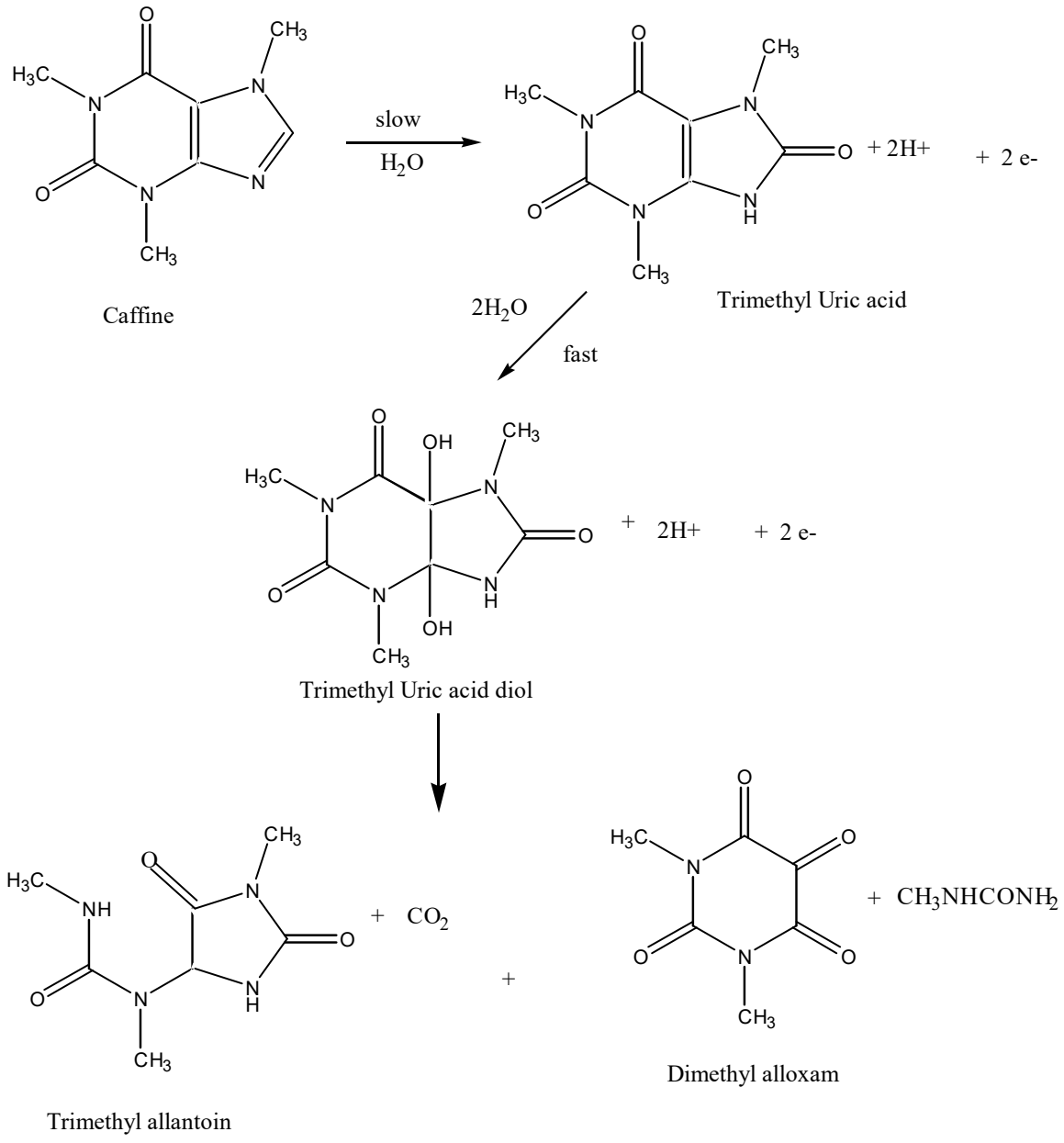

Figure 5. Electrochemical process for the oxidation of caffeine.

1.7. Carbon Electrodes

Solid electrodes based on carbon are currently in wide spread use in voltammetric analysis, primarily because of their broad potential window, low background current, low cost and chemical inertness. The slow kinetics of carbon oxidations lead to a wide useful potential range particularly in the positive direction. The most popular carbon electrodes materials are glassy carbon, graphite pencil, carbon fiber and carbon paste electrodes.

The Carbon Paste Electrode

The carbon paste electrode was invented by Adams at the end of the 1950s. It is a mixture of powdered graphite with various water immiscible organic pasting liquids. Practical consideration of low volatility, purity and expense narrow the choice to few liquids. This includes Nujol (mineral oil), paraffin oil and silcone grease. The paste composition strongly affects the electrode reactivity; with increase in pasting liquid content decreasing the electron transfer rates, as well as the background current contribution. The optimum composition is reported to be 70 to 80% by weight of graphite powder [33].

The electrode surface should be smoothed by rubbing the electrode several times slowly across a smooth paper surface. Like any mechanical procedure one develops a methodological and reproducible way of smoothing the carbon pate surface. It has been widely applied in electrochemistry mainly as a substitute for noble metals. It is advantageous over dry graphite because the latter has higher residual current [34]. Carbon paste electrodes have practically zero residual current over the entire anodic potential range. This is because there is no oxidation current due to dissolution of oxygen or desorption of hydrogen.

Carbon paste electrodes possess many advantages. It has wider accessible potential window than common metallic electrodes, which means more redox reactions can be observed at carbon paste electrodes than at metallic electrodes. In addition it is inexpensive, easy to prepare and renew [35].

1.8. Objective of the Study

Coffee is the world most popular drink next to tea. The coffee beverage is consumed by about one third of the world population. In terms of commercial importance coffee is worth about 60 billion dollar worldwide making coffee the second most valuable commodity in the world after petroleum. The active ingredient that makes coffee valuable to human is caffeine.

One of the requirements of a good coffee is weather it carries a reasonable satisfying quantity of the stimulant caffeine. The other criterion is, it should give delightful, distinctive taste and flavor on roasting and brewing. Both the amount of caffeine and aroma of coffee vary based on the species of coffee and the place it is grown. Therefore the determination of the level of caffeine in green coffee grown in different parts of Ethiopia is relevant for the coffee exporters.

The Ethiopian Standard and Quality Authority (ESQA), has set rough guideline for the amount of caffeine in regular green coffee of about 1% on dry mass basis [19]. The ESQA has adopted the guideline from the International Standard Organization, ISO.

On the other hand there are people sensitive to caffeine even to 10 mg per cup. This increases consumption of decaffeinated coffee. The market for decaffeinated coffee is about 10 % of coffee consumption worldwide. Decaffeinated coffee contains 5 up to 10 % of original amount of caffeine present in regular coffee. The industrial decaffeinating process causes loss of key flavoring compounds. The decaffeinated coffee is more expensive than regular coffee because of industrial cost.

There have been many attempts for searching of naturally decaffeinated coffee since 1987. Brazillian scientists reported success in 2004 from Ethiopia. Following the report for the discovery of naturally decaffeinated coffee, responsible institutions in Ethiopia have begun their own search for similar coffee tree. Thus, less expensive validated voltammetric

method for the determination of caffeine in green coffee can also help the agricultural researchers involved in this area.

General objective:

- To develop less expensive voltammetric method and study the level of caffeine in green coffee grown in Ethiopia.

Specific objective:

- The Specific objectives of these study include:
  - Development of a suitable method for square wave voltammetric determination of caffeine by using carbon paste electrode.
  - Analysis of the level of caffeine in coffee grown in different regions of Ethiopia.

2. Theoretical Background

2.1. Cyclic Voltammetery

Cyclic voltammeter (CV) is an elctroanlytical technique reported in 1938 and described theoretically in 1948 by Randles and Sevcik. It has grown enormously in its popularity over the past few decades so much so that obtaining CV is often the first experiment performed by the elctrochemist giving invaluable information as to the presence of electroactive species in solution. Cyclic voltammetery enables rapid observation of redox behavior of an analyte over the entire potential range available. CV is, thus an essential technique for initial electrochemical studies of new systems and has also proven very useful in obtaining mechanistic information about fairly complicated electrode reactions. Although CV is particularly useful in qualitative studies of electrode processes, it is less well suited for quantitative measurement of potentials or currents [36].

Its merits are, thus, largely in qualitative or diagnostic experiments. Quantitative measurements (of rates or concentration) are best obtained by employing step or pulse techniques. For an irreversible process i.e. with sluggish electron transfer at the electrode surface, the peak current is given by [37].

$$i_p=(2.99X10^5)n\,(\alpha n_a)^{1/2}\,AC^*\,D^{1/2}\,\nu^{1/2} \qquad (1)$$

Where n- is the overall electron transfer, n_a - the number of electrons in the rate determining step, α - is the transfer coefficient, A - the electrode area, C^* - bulk concentration, D - diffusion coefficients of the electroactive species, and ν - scan rate.

For many organic compounds redox reactions are accompanied by chemical reactions that precede or follow the redox process. Such electrochemical reaction mechanisms are commonly classified by using the letters E and C, where E stands for electron transfer at the electrode and C for solution reaction coupled to electrode reaction. The occurrence of

the chemical reaction, directly affects the available surface concentration of electroactive species.

A fairly complex process is an electrochemical reaction coupled with chemical reaction of the type ECE. In this mechanism the chemical step being interposed between electron transfer steps.

$$A \pm ne^- \rightleftharpoons B$$

$$B + Z \longrightarrow C \qquad (2)$$

$$C \pm ne^- \rightleftharpoons D$$

Where Z is the solvent

If the product of the chemical reaction is more readily oxidized than the reactant the forward electron transfer step becomes irreversible. If further the product of the electrochemical reaction (C) is relatively unstable; the second peak cannot be detected and the process is treated as a simple irreversible process. The oxidation of caffeine shows this type of mechanism (Figure 5).

2.2. Pulse Voltammetric Techniques

Pulse voltammetric technique introduced by Barker and Jenkin is aimed at lowering the detection limit of voltammetric measurements. The basis of all pulse techniques is the difference in the rate of the decay of the charging and the faradic currents following a potential step (or "pulse"). The charging current decays exponentially, whereas the faradic current (for a diffusion-controlled current) decays as a function of $1/(t)^{1/2}$, that is, the rate of decay of the charging current is considerably faster than the faradic current. Therefore, after pulse application the measured current consists solely of the faradic current; that is, measuring the current at the end of a potential pulse allows discrimination between the faradic and charging currents [38].

2.3. Square Wave Voltammetry

Square wave voltammetry, SWV, is large amplitude differential technique in which a waveform composed of symmetrical square wave is superimposed on a base staircase potential. The square wave signal is defined by its frequency f, the amplitude E_{sw}, and scan increment ΔE. The square wave amplitude is one half of the peak to peak amplitude and the potential increment ΔE is the step height of the stair case waveform. Figure 6 shows the potential time waveform of SWV.

Current is sampled twice during each square wave cycle, once at the end of the forward pulse and once at the end of the reverse pulse. Since the square wave modulation amplitude is very large, the reverse pulse causes the reverse reaction of the forward pulse (the product). The difference between the two measurements is plotted versus the base staircase potential. Excellent sensitivity is achieved from the fact that the net current is larger than either the forward or reverse component; compiled with the effective discrimination against the charging background current [39].

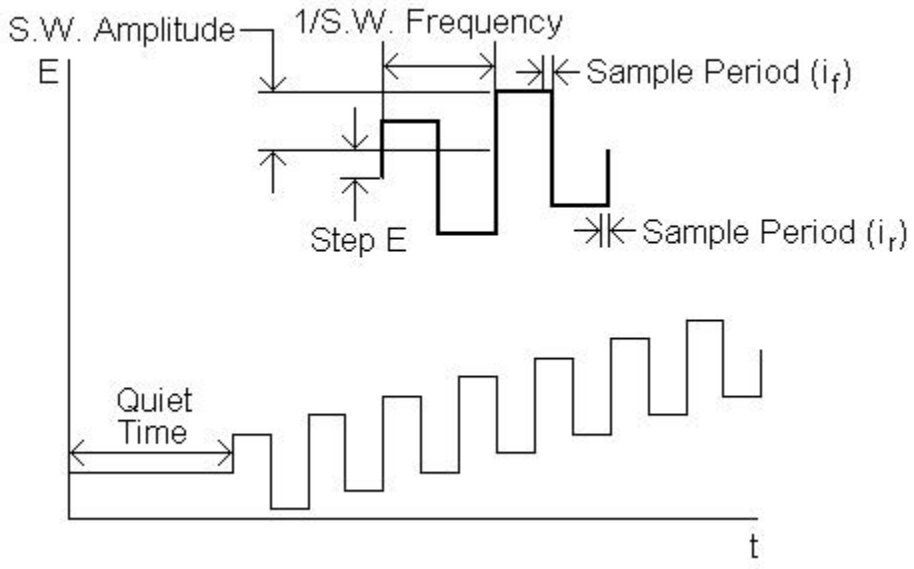

Figure 6. Potential waveform for square wave voltammetry.

The net peak current for irreversible system is given by [38, 40]:

$$i_p = \text{const}\ \alpha\ n^2 \Delta E\ E_{sw}\ (fD)^{1/2} C^* \qquad (3)$$

Where: ΔE - step potential, f – square wave frequency, and E_{sw}- square wave amplitude. Other symbols have the same meaning as in equation (1).

The effective scan rate is given by $f\Delta E$. Kinetic parameters can also benefit from the rapid scanning and the reversal nature of square wave voltammetry. This method possesses both pulse techniques and cyclic voltammery hence it is one of the most advanced members in the family of pulse techniques [41]. For this reason, SWV is an exceptionally appealing method for analytical usage [42-44], as well as for mechanistic or kinetic studies [45-47] of the electrode mechanism that has already been widely verified experimentally.

2.4. Choice of Operating Parameters in Square Wave Voltammetry

2.4.1. Effect of Supporting Electrolyte

The main role of supporting electrolyte in an electrochemical cell is to provide the sources of ions to conduct current across the cell and to dissolve the electroactive species. Electrolytic media of high conductivity are desirable since they prevent internal heating due to I^2R power dissipation and permit relative error free control of electrode potential. The solution must contain a sufficient concentration of the conducting species so that a large ohmic drop is prevented, migration current is suppressed, and constancy of activity and diffusion coefficient is maintained. When acids or bases are formed during the redox process the electrolyte may be chosen to serve as buffer. Buffer systems such as acetate or phosphate are used when pH control is essential.

Care must be taken in that the electrolyte chosen is truly inert in the potential range of the experiment, not reacting with the electrode. Solution conditions in particular pH have profound effects [48].

2.4.2. Square Wave Frequency

The choice of frequency is one the most important instrumental variable that requires due consideration. It can be seen from equation 3, that the peak current increases as frequency, f, increases. However the peak broadening or half peak width also increases with increasing frequency. It can also increase the residual current resulting in unstable current [49, 50]. On the other hand very slow frequency gives a low but narrow signal, and increases the total analysis time. Hence, selection of frequency usually requires a compromise among sensitivity, resolution, and speed.

2.4.3. Square Wave Amplitude

It can be seen from equation 3 that i_p increases as pulse amplitude increases. However, the peak width also increases with increasing pulse amplitude. Therefore, the optimum pulse amplitude must be found to maximize sensitivity, i_p and resolution (small peak width).

2.4.4. Step Potential

The value of step potential should be less than the pulse amplitude. The net current increases as step potential increases, however, accompanied by peak broadening and loss of peak symmetry. Therefore, the optimum step potential must be found to maximize sensitivity and better peak symmetry.

3. Experimental

3.1. Reagents and Chemicals

Carbon powder and Nujol oils (Fulka, Switzerlad), Caffeine (Evans, UK), NaH_2PO_4, Na_2HPO_4, H_3PO_4 and anhydrous Na_2SO_4 (Wagtech Intrernational Ltd., UK.), Chloroform (Ridel-de Haen, Germany), MgO (BDH, UK) were used in the experiments.

A stock solution of caffeine (5 mg/mL) was made in deionized water. Working standard solutions of lower concentration were prepared fresh by dilution of stock solution in water. The pH of the solution was varied between 2 and 10, by mixing 0.1M Na_2HPO_4, 0.1M NaH_2PO_4 and 0.1M H_3PO_4 to get required buffer. All solutions were prepared from deionized water.

3.2. Apparatus

The electrochemical behavior of caffeine at carbon past electrode has been studied by using voltammeteric methods. The experimentation and processing of data were made using a BAS – 50W electrochemical analyzer (Bioanalytical-System) connected to a personal computer.

An electrochemical cell with three electrode system, carbon paste electrodes as the working electrode, platinum as auxiliary electrode and Ag/AgCl as reference electrodes were used for the measurements. A magnetic stirrer with a Teflon coated stirring bar was used for homogenization. The measurements were made by stirring the solution for 30 second and waiting for 15 second quit time. The measurements were repeated for each solution until a good reproducibility of the result was obtained. Standard solution was added to the electrochemical cell with micropipette and disposable tips. Figure 7 shows the experimental set up employed for voltammetric measurements.

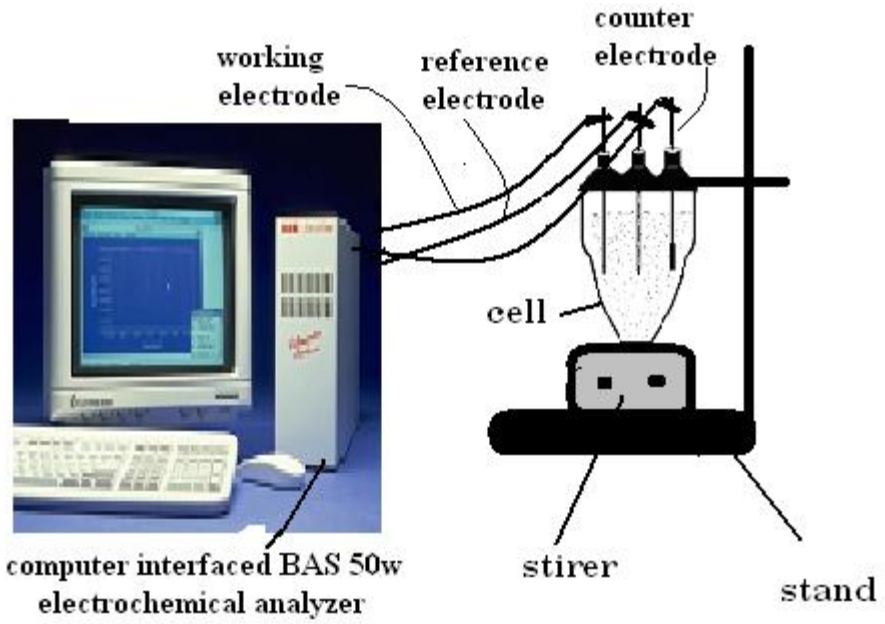

Figure 7. Experimental set up for voltammetric measurment of caffeine.

3.3. Preparation of the Working Electrode

The carbon paste electrode was prepared by the same method as reported previously [51]. It was prepared by adding 0.36 mL nujol (0.843 g/ml) to 1g of carbon powder and thoroughly hand mixing in mortar and pestle for 30 min. The CPE body was fabricated from 1mL plastic syringe of 3 mm outer diameter. The carbon paste electrodes were prepared by pressing the carbon paste into the well of a plastic syringe electrode body and electrical contact was made in the center of the rod with carbon paste through a copper wire. Then their surface was smoothed against a smooth white paper while as light manual pressure was applied to the plastic syringe until a shiny surface is emerged.

A single electrode surface could be used for 8 up to 10 measurements without a decrease in reproducibility. Thereafter the carbon paste electrode was renewed by extruding a small amount of paste from the tip of the electrode. The diagram for carbon paste electrode is shown in Figure 8.

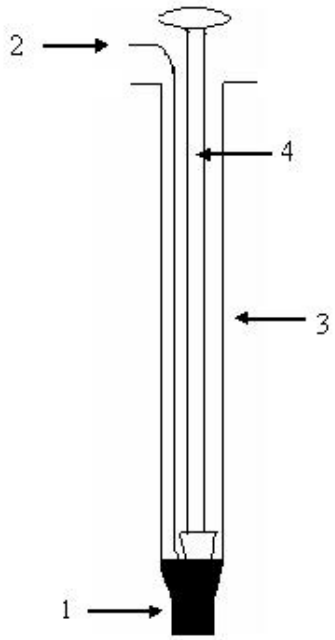

Figure 8. The carbon paste electrode: (1) paste, (2) copper wire, (3) syringe body and (4) piston.

3.4. Voltammetric Procedure

Cyclic voltammograms (CVs) of caffeine solution were run with carbon paste electrode. The CVs were recorded between 0 and 1.8 volt with scan rate of 200 mV/s. Square wave voltammetery was carried out to ascertain best conditions for sensitivity and quantization. Several experimental parameters such as pH of solution, square wave frequency, step potential and square wave amplitude were investigated to develop a procedure for the voltammetric determination of caffeine.

Appropriate amounts of caffeine working solutions were placed in a cell containing the supporting electrolyte solution at the selected pH. The electrode was kept in the cell and the solution was stirred at 100 rpm for 30 seconds. The stirring was stopped and after a 15 second rest period anodic sweep was carried out at initial potential of 0 V and final potential of 1.6 V. The pH of the solution was varied between 2 and 10 to get the best

measurement condition. The instrumental parameters: square wave frequency, square wave amplitude, step potential were also varied to get best sensitivity for the analytical measurements.

3.5 Recovery Study

To evaluate the extraction procedure of caffeine using chloroform solution, 1 mL of 1.25 mg/mL caffeine was added to 25 mL volumetric flask and filled with distilled water. The caffeine was extracted with 50 mL chloroform twice. After removing the chloroform, the residue was dissolved in phosphate buffer pH 6, and the concentration of caffeine was determined by standard addition method. That is after measuring the response of the sample, the concentration of the analyte was deliberately increased by adding a certain volume of a standard solution. The response was measured and this procedure was repeated three times. The unknown concentration was determined by extrapolation of the regression line to the negative x- axis and taking the absolute value of the x-intercept.

3.6. Analysis of Real Samples

3.6.1. Coffee Samples

The coffee samples were export standard Ethiopian green coffee beans supplied by Ethiopian Coffee Commerce, either sun dried or washed. The samples were Jima (washed) Bench-Maji (Sun dried), Godre (Sun dried), Wellega (Washed), Mena – Angetu (Sun dried) and Wenago (Sun dried).

3.6.2 Sample Preparation

The coffee bean was milled and ground with an analytical grinder suitable for grinding green coffee bean. The ground was sieved in woven wire clothe with a mesh size of 500 μm. The caffeine occurs as a potassium and caffeine chlorogenate. The salt is scarcely

decomposed by dry chloroform, but is easily decomposed by the presence of water and then gives up its caffeine. A 0.25 g of ground coffee and 0.1 g MgO were added into a 125 mL Erlenmeyer flask fitted with a stopper. 50 mL of distilled water was added to the flask and boiled for 20 minute in a hot plate [52].

After allowing the residue to settle, the solution was filtered while hot. For removal of interferents; liquid - liquid extraction using chloroform was performed in to 250 mL separatory funnel. The extraction was performed with 100 mL of chloroform twice by gentle swirling of the mixture to mix the layers and carefully inverting the funnels several times. This was done for about 10 minutes. The extracts were mixed and passed through 0.25 g anhydrous Na_2SO_4.

The chloroform was removed to dryness by rotatory evaporator until 10 mL was left, then transferred to vials and allowed to dryness in the hood. The residue was dissolved in 5 mL hot supporting electrolyte and transferred to 25 mL volumetric flask. Dissolving was repeated three times and diluted with the buffer to the mark. The concentration of caffeine was determined using standard addition method.

3.6.3. Water Content

The water content was determined based on the loss in mass at 105 ^oC until constant mass [53]. 5 g of test portion was placed in glass dish and heated in an oven capable of being controlled at 105 ^oC. After drying for 8 hours in the oven the loss in mass, principally water and very small quantities of volatile matter was considered as water content.

4. Results and Discussion

4.1. Cyclic Voltammetric Investigation

Cyclic voltammograms of caffeine at carbon paste electrode in 0.1M NaH_2PO_4 and 0.1M Na_2HPO_4 at pH 6 are shown in Figure 9.

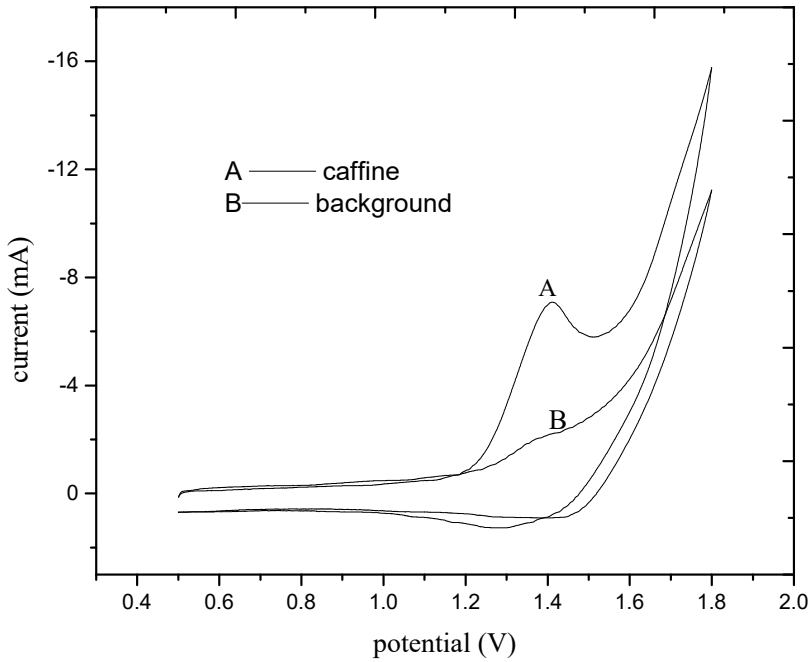

Figure 9. Cyclic voltammogram of: (A) 2 mM caffeine concentration and (B) Phosphate buffer at pH 6.

As can be seen in Figure 9, caffeine is an electroactive compound because it shows an oxidation peak current at 1.40 V. In the absence of caffeine no such peak was observed. Since no reduction current was observed in the reverse scan, it is possible to conclude that the electrochemical oxidation of caffeine is irreversible.

4.2. Square Wave Voltammetric Investigation

4.2.1 Effect of pH of Supporting Electrolyte

The pH of the medium used for measurement has a profound effect on the voltammetric response. This important variable was studied for caffeine at carbon paste electrode by square wave voltammetry in the pH range 2 to 10. The oxidation current increases from pH 2 to pH 6. Above pH 6 a measurable background current close to the oxidation potential of caffeine was observed. This made the measurement of the peak current of caffeine difficult to extract from the background. The background current is expected from the oxidation of hydroxide ions to form oxygen. Hence the optimum pH 6 was selected for the measurements. The square wave voltammogram dependence of peak current on the pH of the solution from pH 2 – pH 6 (from top to bottom) is shown in Figure 10. The effect of the pH of supporting electrolyte on peak current of caffeine is shown in Figure 11. For peak currents above pH 6 the values were obtained after subtracting background current for the electrolyte from the peak current measured for the given concentration of caffeine. This causes uncertainty since the measured background current can not be reproducible.

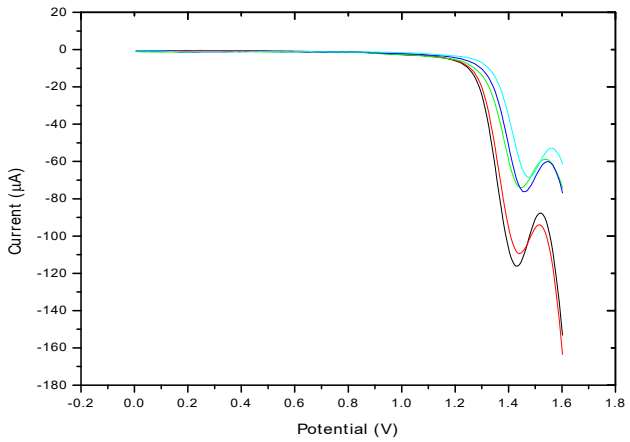

Figure 10. Square wave voltammogram of 100 mg/L caffeine from pH 2 to pH 6.

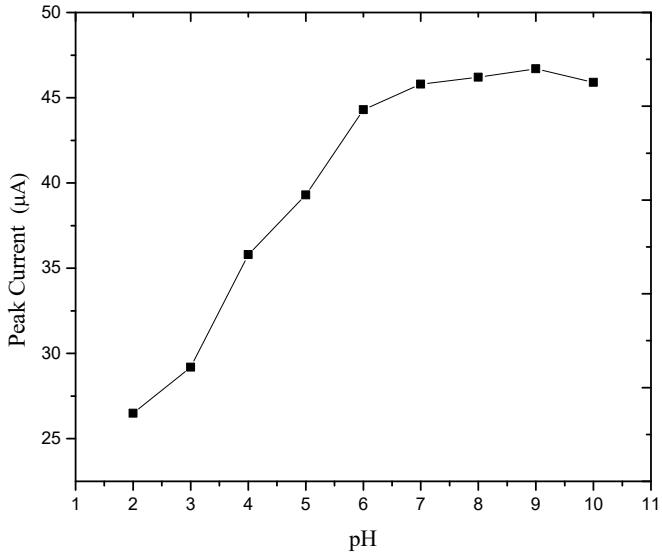

Figure 11. Effect of pH on the peak current.

4.2.2. Effect of Square Wave Frequency

The effect of frequency on the peak current was studied varying the square wave frequency from 5 Hz to 45 Hz at step potential of 4 mV and amplitude of 20 mV for 50 mg/L solution. The peak current increases, reaches almost a maximum at 40 Hz accompanied by peak broadening. The effect of square wave frequency on peak current is shown in Figure 12. In practice owing to greater noise and peak broadening an optimum frequency of 30 Hz was chosen for subsequent experiments.

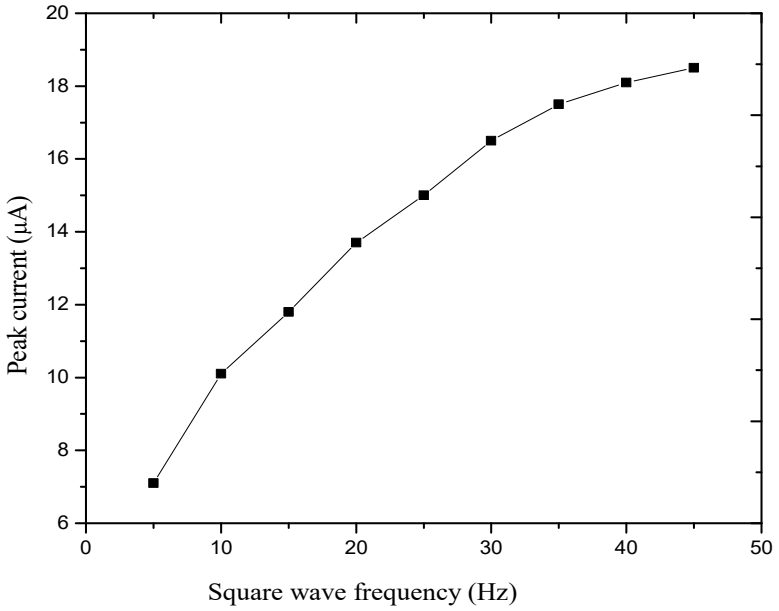

Figure 12. Dependence of peak current on the square wave frequency for 50 mg/L of caffeine.

Square wave frequency has also a diagnostic value, as it indicates whether the electrochemical process is diffusion or adsorptive at the electrode surface. This can be evaluated by taking the logarithm of equation 3.and substituting the values in to this equation. The plot of log i_p versus log f is linear with slope 0.5 as shown in Figure 13. This confirms that the electrode process is diffusion controlled, which is in agreement with previously reported results for caffeine oxidation at both chemically modified and unmodified electrodes [23, 24].

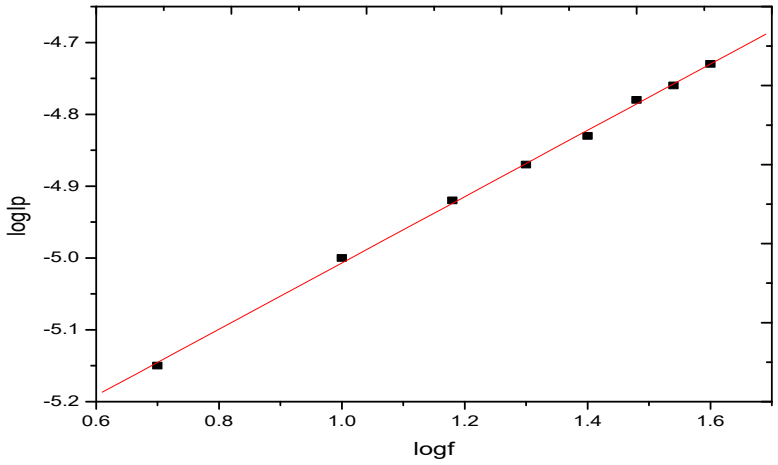

Figure 13. Plot of logarithm of square wave peak current versus logarithm of frequency for 50 mg/L caffeine concentration.

The square wave voltammograms for the dependence of peak current on the square wave frequency is shown in Figure 14, where the above graphs were obtained. The dependence of the peak current on the square wave frequency (Figure 12) and the logarithm of square wave peak current on the logarithm of square wave frequency (Figure 13) have been extracted from these results.

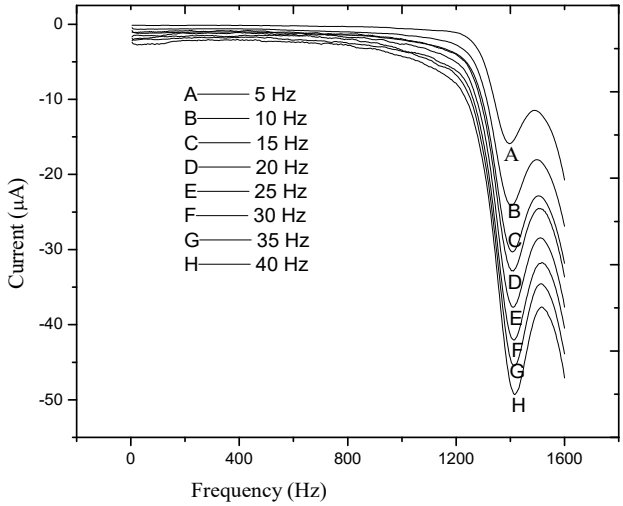

Figure 14. Square wave voltammograms of 50 mg/L caffeine at various frequencies.

4.2.3. Effect of Square Wave Pulse Amplitude

The effect of pulse amplitude was studied by varying the amplitude from 5 mV to 45 mV at frequency of 30 Hz and step potential of 4 mV. A similar pattern was observed upon increasing the pulse amplitude in which the peak current increased linearly accompanied by peak broadening. The peak current for caffeine as a function of the square wave amplitude is illustrated in Figure 15. As seen in the Figure, the responses tend to level off at around 40 mV. Thus pulse amplitude of 40 mV was chosen for the measurement.

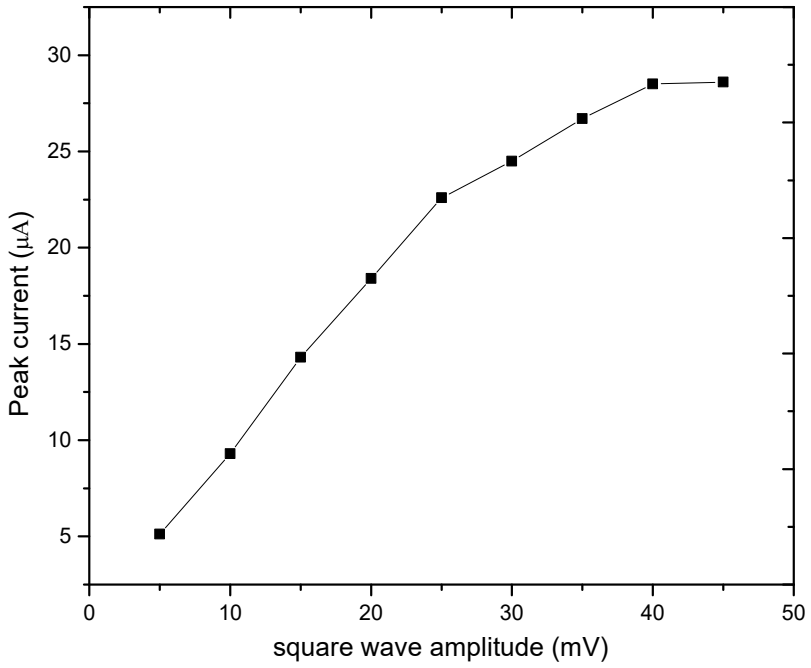

Figure 15. Square wave voltammetric peak currents at various square wave amplitude.

4.2.4 Effect of Step Potential

The effect of step potential was studied by varying the potential from 2 to 10 mV at frequency of 30 Hz and amplitude 40 mV. In a similar pattern to the pulse amplitude a

linear increase in peak current was observed upon increasing the step potential. However for potentials above 8 mV the responses level off also accompanied by peak broadening. The peak current for caffeine as a function of the step potential is illustrated in Figure 16. As a compromise between increase in peak current and peak broadening, a step potential of 7 mV was chosen for subsequent measurements.

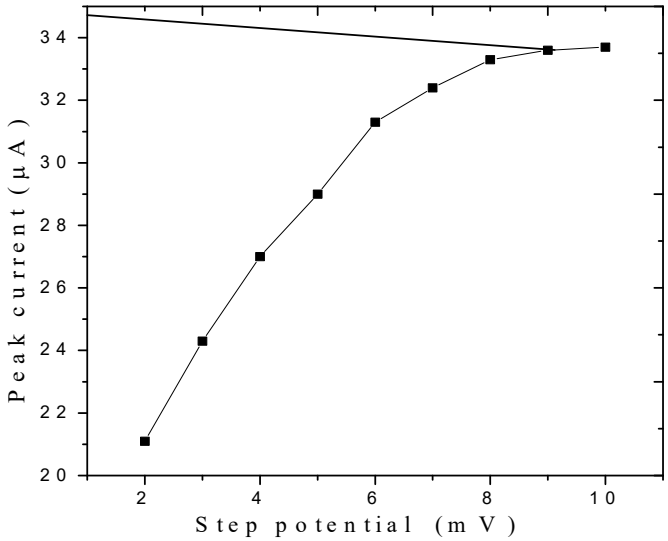

Figure 16. Square wave voltammetric peak current at various step potentials.

4.2.5. Optimum Experimental Conditions

Effects of pH and instrumental parameters have been studied to obtain optimum conditions for the determination of caffeine. The optimum parameters used for the determination are summarized in Table 2.

Table 2. Optimum experimental conditions for the determination of caffeine by SWV.

Parameters	Optimum values
pH of supporting electrolyte	6
Square wave frequency (HZ)	30
Square wave amplitude (mV)	40
Step potential (mV)	7
Initial potential (mV)	0

4.2.6. Linear Range and Detection limit

The analytical utility of a given procedure depends on obtaining well defined dependence of current responses on concentration. The dependence of the voltammetric signal on the concentration of caffeine and the inherent sensitivity of the method are illustrated by the square wave voltammetry for different concentrations of caffeine. According to the optimum experimental conditions (Table 2), and the procedure described in section 3. 4, the relationship between the square wave voltammetric peak current and the caffeine concentration was examined. A calibration curve was constructed by measuring the peak current for each calibration standard until three consecutive identical readings were obtained. Figures 17 and 18 show square wave voltammogram and calibration graph for the dependence of the square wave peak current on the concentration of caffeine respectively. The current response for caffeine was linear in the concentration range 50 mg/L - 250 mg/L. The linear response equation for the dependence of peak current (i_p) and caffeine concentration (mg/L) was as follows:

i_p (μA) = 1.35 E-5 + 3.5 E-7 x (mg/L),

and the correlation coefficent was (R= 0.993). The detection limit, concentration equal to three times the standard deviation of the peak current for six determination of 50 mg/L of caffeine was found to be 10.67 µg/mL. The relative standard deviation of the results was 3.65 µg/mL for six successive determination of 50 mg/L caffeine. Table 4 shows the comparison of linear range and detection limit between CPE and other methods. Although the detection limit of carbon paste electrode is slightly lower than the HPLC method the low cost of electrode and instrumental equipments make the voltammetric methods advantageous over the HPLC method. The nafion ruthenium pyrochlore chemically modified electrode has also lower detection limit the electrocatalyst ruthenium oxide is very expensive. Therefore, the method developed is a reasonable alternative for the determination of caffeine in regular and decaffeinated coffee.

Table 4. Comparison between CPE and other methods.

Method [Ref]	**Detection limit**	**Linear range**
CPE developed	10.67 µg/mL	50-250 mg/L
HPLC method [25]	0.3 µg/mL	0- 300 mg/L
Nafion CME method [28]	0.4 µg/mL	0-100 mg/L

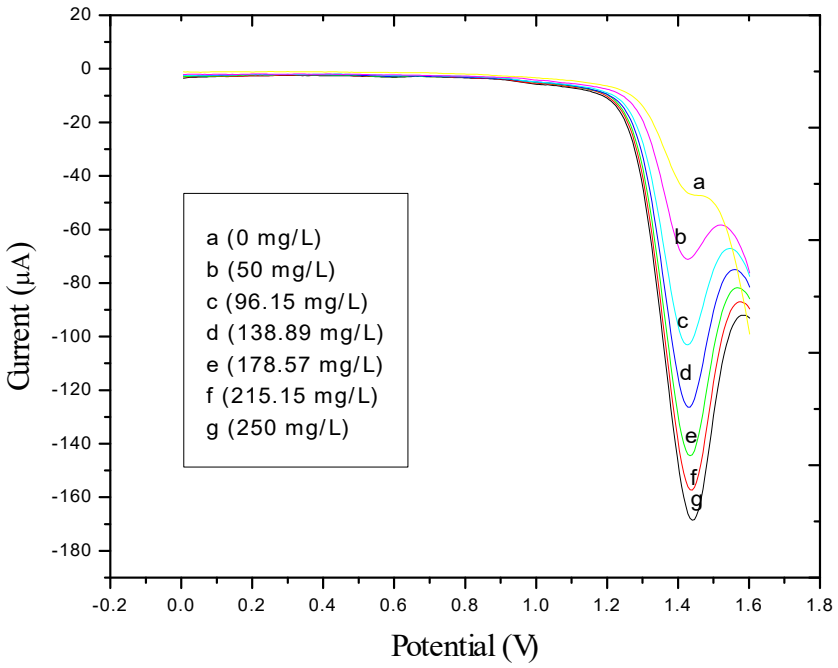

Figure 17. Square wave voltammograms of caffeine for various concentrations.

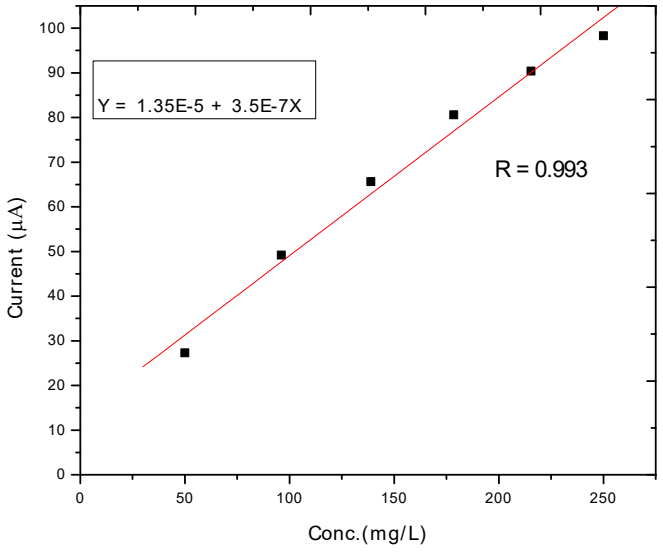

Figure 18. Calibration curve for caffeine with the results of regression line.

4.2.7. Recovery Study

The results for recovery of the method determined by standard addition method for caffeine spiked in to distilled water as described in section 3.5 was found to be 93%. Figure 19 shows standard addition calibration graph for caffeine spiked in water. From the results it is evident that the method is very effective for the determination of caffeine.

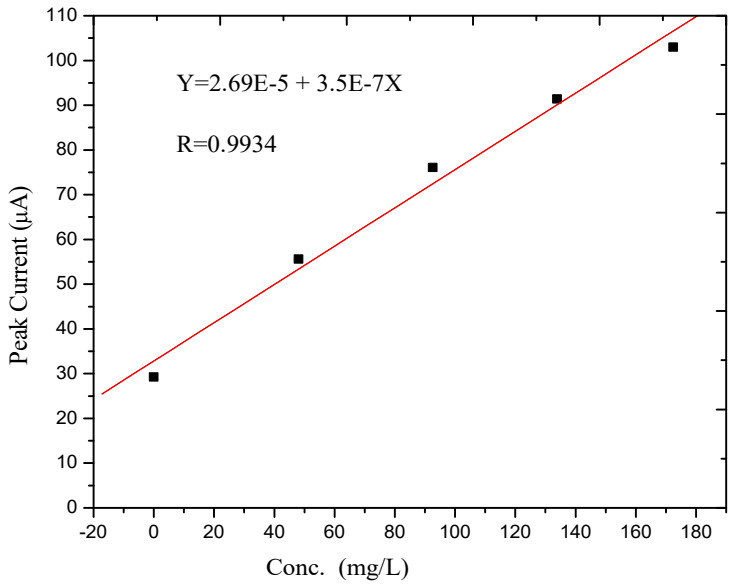

Figure 19. Standard addition calibration graph for spiked caffeine.

4.2.8. Real Sample Analysis

The method developed was applied for the determination of caffeine in Ethiopian green coffee samples. The coffee samples were taken from different part of Ethiopia, which are known for large scale coffee cultivation. The extraction of caffeine was performed by the procedure described in section 3.6.2. The water content of green coffee samples as determined using procedure 3.6.3 ranges from 9% - 10%. The results are with in the acceptable ranges of the moisture content for export green coffee samples, 8 – 12.5%, recommended by the ISO [52]. By means of standard addition method, the quantity of caffeine in the given sample were determined, the results are given in Table 3. The

precision of the method was determined by extracting a single green coffee sample three times. The relative standard deviation was found to be 2.7%. The quantities of caffeine in the samples on wet mass basis were compared with the HPLC method determined in organic chemistry laboratory within the department (personal communication). The result obtained was found to be in close agreement (< 5 % average variation) with that method.

Table 3. Caffeine content of Ethiopian green coffee samples

Sample area	**Caffeine content in % on dry mass basis**	
	by SWV	**by HPLC**
Jimma	1.16	1.17
Bench- Maji	1.02	0.89
Mezenger (Godere)	1.08	0.92
Wellega	1.10	1.22
Bale (Mena-Angetu)	1.10	1.11
Wenago	0.89	0.88

The close agreement between the two methods confirms suitable applicability of this method for the determination of caffeine level for various coffee samples. The level of caffeine determined ranges from 0.9% - 1.16% on dry mass basis indicates that the caffeine levels of export standard Ethiopian green coffee samples are with in the range of the guide lines set by ISO, which is about 1%. The values are also in good agreement with those reported in the literature, 1.2 % for *C. arabica* from Brazil [25].

5. Conclusion

The optimum conditions for the determination of caffeine with square wave voltammetry utilizing carbon paste electrode as the working electrode were found as follows: square wave frequency 30 Hz, square wave amplitude 40 mV, and step potential 7 mV in 0.1M phosphate buffer pH 6.0. The square wave peak current exhibits linear range between 0 - 250 mg/L of caffeine. The detection limit was also determined to be 10.67 μg/mL. This method was applied for the determination of caffeine levels in Ethiopian green coffee samples. The caffeine content ranged from 0.9 - 1.16 % on dry mass basis. The quantities are in good agreement with those reported in the literature for *Coffea arabica*.

The results are also in good agreement with those determined by HPLC method. Thus the SWV method with carbon paste electrode is a useful technique for monitoring caffeine in coffee.

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