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DEVELOPMENT OF FILM-HOLE MODIFIED SCREEN PRINTED CARBON ELECTRODE FOR ELECTROANALYSIS OF CAFFEINE

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JIMMA, ETHIOPIA

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A THESIS SUBMITTED TO JIMMA UNIVERSITY SCHOOL OF GRADUATE STUDIES IN PARTIAL FULFILMENT OF THE REQUUIREMENTS FOR THE DEGREE OF MASTERS OF SCIENCE IN CHEMISTRY (ANALYTICAL CHEMISTRY)

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A Thesis Submitted to Jimma University School of Graduate Studies in Partial fulfillment of the requirements for the Degree of Masters of Science in Chemistry (Analytical Chemistry)

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Abbreviations and Acronyms

BSPCE	Bare screen printed carbon electrode
BDDE	Boron-doped diamond electrode
CAF	Caffeine
CPE	Carbon paste electrode
CA	Chronoamperometry
CE	Counter electrode
CV	Cyclic voltammetry
EPPG	Edge plain pyrolytic graphite
GCE	Glassy carbon electrode
AuNPs	Gold nanoparticles
GPE	Graphite pencil electrode
PNA	Paranitroanniline
RE	Reference electrode
SPCEs	Screen-printed carbon based electrodes
SPEs	Screen-printed electrodes
WE	Working electrode

Abstract

Gold nanoparticles were electrodeposited on the surface of BSPCE using chronoamperometry. A cyclic voltammetric technique was used to graft electropolymerized PNA film on the surface of AuNPs deposited SPCE. The electrodeposited gold nanoparticles were stripped off from electrode surface to create holes on the thin organic film. The modified electrode was characterized using potassium hexacyanoferrate (negative redox probe) and hydroquinone (neutral redox probe). The developed method was then used for the electrochemical determination of caffeine (electropositive analyte). The anodic peak potential for caffeine was shifted to less positive potential with the enhancement of anodic peak current of caffeine at nanohole p-nitroaniline grafted screen printed carbon electrode (nanohole PNA grafted SPCE), which makes it suitable for the determination of caffeine in real sample. Various deposition times of gold nanoparticles were carried out amperommetrically on the surface of BSPCE. The negative potential shift with the increase in the anodic peak current of caffeine was obtained at a 10 s deposition time. Thus, for the cyclic voltammetric study of caffeine, 10 s depositions of gold nanoparticles were used as an optimum deposition time. At an optimized conditions, the oxidation peak current of caffeine was linearly related to the concentration of caffeine in the range of 6 to 16 µM with a correlation coefficient and detection limit (LOD=3ó/Slope) of 0.99943 and 1.92 x10^{-7}M, respectively. Sensor response of caffeine was not affected by possible interfering species as the result obtained from the cyclic voltammetric experiment of the mixture of theophyline and caffeine indicates which showed the selectivity of the modified electrode. The modified electrode has also good reproducibility and stability.

Key words: Screen printed carbon electrode, Cyclic Voltammetry, Chronoamperometry, Gold nanoparticles

1. Introduction

1.1 Background

Caffeine (1,3,7-trimethylxanthine) is a naturally occurring alkaloid belonging to *N* methyl derivatives of xanthine, which is found in tea leaves, coffee beans, cola nuts, cocoa beans and other plants. It is used as a flavoring agent in a variety of beverages, including some soft and energy drinks. Caffeine is also a central nervous system stimulant. In moderate doses, it can increase alertness, reduce fine motor coordination, cause insomnia, headaches, nervousness and dizziness [1 - 3]. However, intense use of caffeine over time can lead to irritability, mutation effects such as inhibition of DNA, anxiety and tremors, among other side effects [4]. It can mobilize calcium from cells, which leads to bone mass loss and is considered as a risk factor for cardiovascular diseases [5, 6].

The development of reliable methods for the evaluation and quantification of caffeine in real samples is an active field of research. In the past, various methods for analyzing caffeine and its analogs have been developed. Amongst the different methods that have been developed such as chromatographic method [7], the electroanalytical methods required less expensive and non sophisticated instrument and also relatively cost effective, portable and easy to operate. In spite of this, electroanalytical methods have rarely been used for the analysis of caffeine except in few recent reports [8]. This is mainly because the oxidation of caffeine occurs at a very high positive potential, which overlaps with the discharge of a background medium [8]. Over the years, many types of working electrodes, modified or unmodified, have been developed and used in various ways in order to improve their performance for voltammetric measurements of caffeine. These include using boron-doped diamond electrodes (BDD) [9], nafion modified BDD [10], cathodically pretreated BDD electrodes [11], 1, 4-benzoquinone or molecularly imprinted polymer modified carbon paste electrodes [12], nafion/carbon nanotube [13, 14], nafion/graphene modified electrodes [15, 16], carbon fiber ultra microelectrodes [17], and polymer modified glassy carbon electrodes (GCE) [18].

On the report appeared on caffeine detection at a nafion modified glassy carbon electrode, the nafion was used to decrease the caffeine oxidation potential, so as not to overlap with oxygen evolution, and increase electrode sensitivity [19]. The benefits of using nafion in electrode modification for more sensitive caffeine detection when carried out in sulphuric acid solution have been attributed mainly to pre-concentration in the nafion polymer layer [19, 20]. A careful selection of the electrode material with the proper potential window is mandatory in order to avoid the overlapping of the electrochemical signal of caffeine with that coming from the discharge of the supporting electrolyte. Therefore, in this work the practical modified electrode was developed to increase sensitivity and to avoid the effect of potential interferents.

1.2. The statement of the problem

The unique chemical and physical properties of nanoparticles make them extremely suitable for designing new and improved sensing devices, especially electrochemical sensors and biosensors. The most common metallic nanoparticles are those obtained from less active metals like gold, palladium and silver. The metal nanoparticles cannot be used as such in modification of electrode surface for determination of caffeine as determination of caffeine requires higher oxidation potentials, which the metal nanoparticles could not resist. Hence, the nanoparticles were used in creating active holes on electropolymerized film that can pre-concentrate caffeine on its surface. Therefore, this work can answer the following questions:

- Is electronucleated gold nanoparticles modified screen-printed carbon electrode selectively improves the determination of caffeine?
- What should be the electropolymerised film type for effective preconcentration of caffeine?
- Which metal nanoparticle and electronucleated under what condition was best in creating active holes for the determination of caffeine?
- What are the optimum conditions under which extraction and determination of caffeine would be possible: pH, deposition time, extraction electrolyte and the appropriate electro analytical method (sweep or potential step)?
- What was the analytical performance of such prepared modified electrode for determination of caffeine as compared to those reported in literature?

1.3. Objectives of the study

General Objective:

The main objective of this research is to develop film-hole modified screen printed carbon electrode for the voltammetric determination of caffeine.

Specific Objective:

The specific objectives of this study were:

- To optimize and select important parameters for electro deposition of gold nanoparticles on screen printed carbon electrode (deposition time) and for electro polymerization of the modifier film
- To optimize electroanalysis parameters for caffeine (pH, scan rate, and deposition time)
- To investigate the selectivity and sensitivity of the modified electrode through interference study.
- To validate the determined caffeine in coffee beans (by comparing the result against standard methods).
- To validate the developed method by studying its reproducibility and stability.

1.4. The Significance of the study

The main significance of the output of this work could be:

- development of simple sensor electrode for quantification of caffeine in coffee beans
- opens up method development for fast screening of caffeine content of coffee beans using portable and disposable sensors.

2. Review of Related Literature

2.1 Caffeine

Caffeine is a central nervous system (CNS) stimulant of the methylxanthine class. It is the world's most widely consumed psychoactive drug. Caffeine is a bitter, white crystalline purine, a methylxanthine alkaloid, and is chemically related to the adenine and guanine bases of deoxyribonucleic acid (DNA) and ribonucleic acid (RNA). It is found in the seeds, nuts, or leaves of a number of plants native to South America and East Asia and confers on them several survival and reproductive benefits. The most well known source of caffeine is the coffee bean beverage drinks such as coffee, tea, and cola [21]. Figure 1, shows the chemical structure of caffeine.

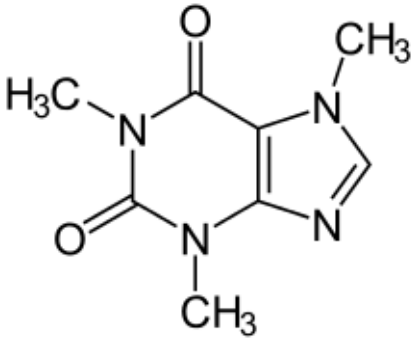


Figure : The molecular structure of caffeine

2.2 Caffeine Analysis

A variety of techniques has been developed for the determination of caffeine in various types of samples. These techniques are mostly based on separation techniques coupled with mass spectrometry (MS) or other spectroscopic analysis [22]. The techniques, which are mainly based on gas chromatography–MS or liquid chromatography–MS [23-25], besides their good sensitivities suffer from many drawbacks such as the bulky equipment and the need of trained personnel for the analysis. Moreover, they are time-consuming and require complex procedures such as sample derivatization, extraction and purification.

Spectroscopic techniques are also very popular for caffeine detection due to the easy accessibility of spectrophotometers in general analytical laboratories. These techniques, based on UV–Vis, IR and NMR analysis [26, 27], show lower sensitivities as compared to chromatographic techniques and are based on time-consuming protocols.

In contrast, electrochemical techniques are known to be rapid, sensitive, cost effective, simple to use and accurate [28-33]. Since caffeine is an electro active molecule [34-36], it can be easily oxidized using common electrode surfaces (i.e. glassy carbon (GC) [37], edge plain pyrolytic graphite (EPPG) [38], carbon paste electrode (CPE) [39], graphite pencil electrode (GPE) [40] and boron doped diamond electrode (BDDE) [41]. Given its high oxidation potential (around 1.4 to 1.5 V Vs Ag/AgCl electrode on carbon surfaces), a careful selection of the electrode material with the proper potential window is mandatory in order to avoid the overlapping of the electrochemical signal of caffeine with that coming from the discharge of the supporting electrolyte. Recent efforts have been focused on the improvement of analytical performance by using different chemically modified electrodes [42] in order to enhance both the selectivity and sensitivity of caffeine detection [43, 44]. This is one of the active research areas that needs further investigation to develop practical modified electrodes with high sensitivity and avoids the effect of potential interferents.

2.3. Screen-printed Electrode

Screen-printing is an alternative attractive approach for the preparation of electrochemical sensors. The advantages of screen-printing for electrode fabrication are the low-cost, disposability, minimal activation requirements, scope for mass production, between-sensor reproducibility, flexibility with respect to the choice of the supporting materials and electrode geometry and potential for effective bulk or surface modification [45-47]**.**

Screen-printed electrodes (figure 2) are now replacing conventional electrodes due to their advantages; being disposable to reduce the lengthy electrode cleaning and conditioning procedures, which make the analysis rapid. Screen printed electrodes based on graphite are potentially capable for the electrochemical determination of caffeine. Therefore, the aim of this work was to assess the utility of graphite based screen-printed electrodes for the rapid voltammetric assay of caffeine in coffee.

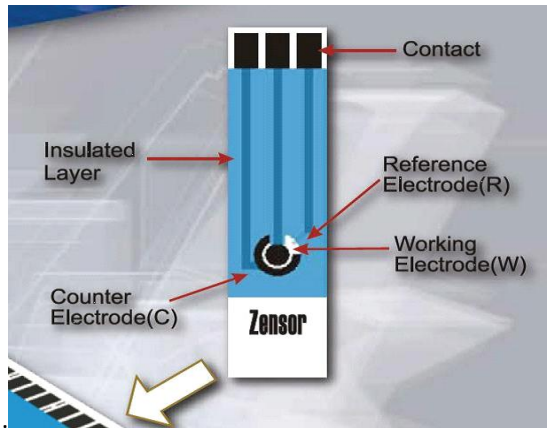


Figure : Screen-printed electrode [48]

Advantages and disadvantages of Screen-printed electrodes (SPEs)

SPEs are especially recommended in the large-scale production of electrodes with easy use and portability properties, which have been studied by Hart, Banks and Wang [49-52]. In addition, these miniaturized screen-printed electrodes are suitable for working with sample micro volumes, and are disposable [53, 54]**.** These electrodes are disposable and are meant to be replaced if fouling occurs. Each new electrode affords a clean and reproducible surface. Their main advantage over conventional electrodes is that the problems with surface fouling can be eliminated, as SPEs are intended to be employed only once and then replaced by new ones from the same batch. Among numerous variants of SPEs, screen-printed carbon based electrodes (SPCEs) have gained great attention because of their easy-to-make modification of the surface by immobilizing the reagent of choice onto the electrode surface or by adding such a substance into the carbon ink yet before the electrodes (usually in a series) are machined.

The main limitation of SPEs appears to be on the content of organic solvents in the buffer solutions either used for the batch voltammetric methods or in the mobile phase used in liquid flow methods [55]**.** Organic solvents can be responsible for the dissolution of insulate inks and consequently the decrease of limit of detection and sensitivity [56, 57]**.** Naturally, the composition of the mobile phase must be compatible with the material of the detection cells housing SPEs in liquid flow methods so that their dissolution can be prevented.

2.4. Screen-printing technology

The use of screen-printing technology in the serial production of disposable low-cost screen-printed electrodes (SPEs) for the electrochemical determination of a wide range of substances is currently undergoing widespread growth [58-62]. Screen-printing techniques offer high-volume production of inexpensive, highly reproducible and reliable sensors, providing precise control over the SPEs dimensions, excellent uniformity, high reproducibility and the potential for mass production [63].

A SPE consists of a chemically inert substrate on which the three electrodes, namely the working, reference and counter electrodes (WE, RE and CE), respectively, are printed through screen-printing methodology [64]. Different substrates can be employed in SPE devices and the extensive range of modifications to SPEs opens numerous fields of applications [65]. Particularly, the use of these electrodes on the analytical detection of pharmaceuticals in a wide range of samples can provide important advantages, such as no extensive sample processing, low detection limits, simplicity, low cost, portability and potential for miniaturization [66].

2.5. **Voltammetric techniques**

Voltammetry is an electroanalytical technique based on the measurement of current flowing through an electrode dipped in solution containing electro active compounds while a potential is imposed upon it [67]**.** It is typically performed using a three electrode potentiostat, which accurately controls the applied potential. The redox reaction takes places at working electrode, because the working electrode is where the reaction of interest is taking place. The working electrodes are usually solid (platinum, gold or carbon). If the working electrode is formed by drop of mercury, the analytical technique is called polarography [68]. The second electrode is a reference electrode, which maintains a constant potential throughout the experiments and the third electrode the counter electrode, which complete the electrical circuit. The counter electrode also known as the auxiliary electrode, is often much larger than working electrode to minimize current density at the electrode surface [68]**.** The common characteristic of all technique is that they involve the application of a potential (E) to an electrode and monitoring of the resulting current (I) flowing through electrochemical cell [69]**.**

The analytical advantage of various voltammetric techniques include excellent sensitivity with very large useful linear concentration range for both inorganic and organic species (10^{-12} to 10^{-1}), a large number of useful solvents and electrolytes, a wide range of temperature, rapid analysis times (in seconds), simultaneous determination of analytes, the ability to determine kinetics and mechanistic parameters, a well developed theory and thus the ability to reasonably estimate the values of unknown parameters, and the case with which different potential wave-forms can be generated and small current measured. The use of the voltammetric techniques is the basis of the comprehension of the laws concerning several electrochemical phenomena and has a great importance in several technological fields like: research of corrosion-proof materials, research of new electrodic process for chemical industries; for example, millions of tons of aluminum, chlorine, soda are produced by means of electrochemical reactions and production of new type of batteries that can store rapidly great quantity of energy. It is also used as quantitative analysis of trace metals those of oxidizable or reducible chemicals at g/L levels or less [70]**.**

2.5.1. Cyclic Voltammetry

Cyclic voltammetry is a potential controlled reversal electroanalytical technique in which a sweep potential is imposed on the working electrode and the current response is measured. A potential sweep is applied backwards and forwards between two limits, the starting potential and the switching potential. Cyclic voltammetry is the most widely used technique for acquiring qualitative informations about electrochemical reactions [71]. Figure 3, shows the potential-time stimulation signal of the cyclic voltammetry.

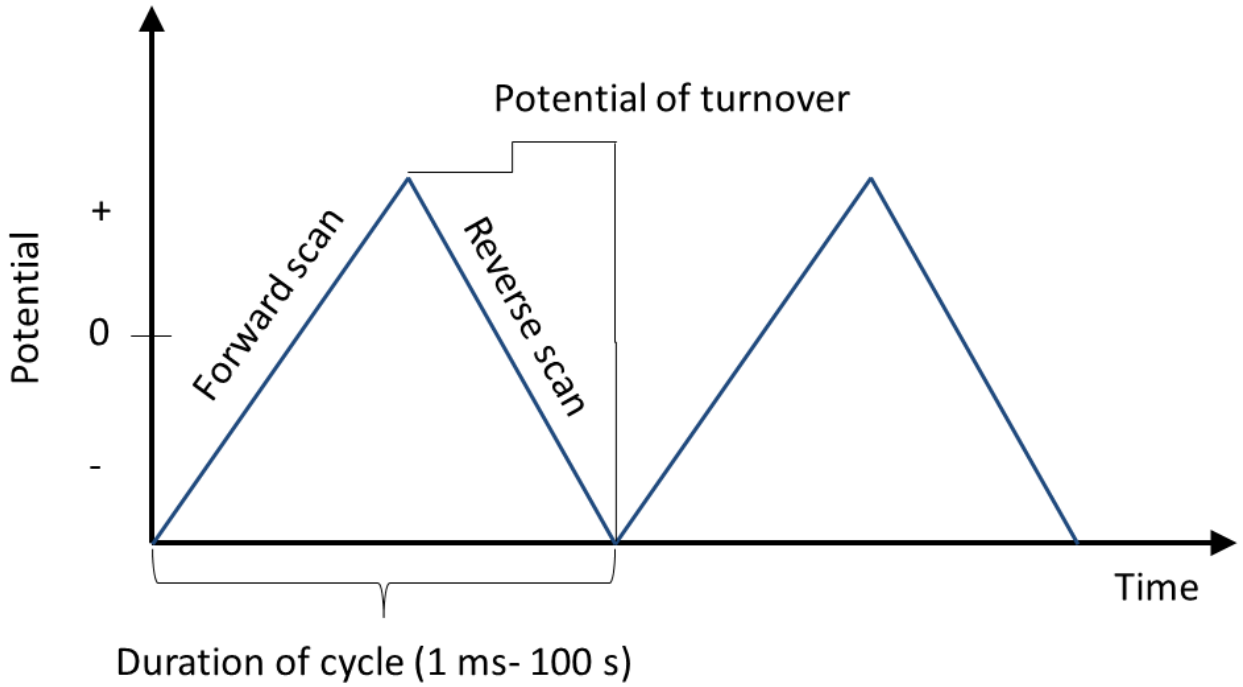


Figure : Potential-time Stimulation Signal of the cyclic voltammetry [72]

2.5.2. Chronoamperometry (CA)

Chronoamperometry is an electrochemical technique in which the potential of the working electrode is stepped and the resulting current from faradaic processes occurring at the electrode (caused by the potential step) is monitored as a function of time. Limited information about the identity of the electrolyzed species can be obtained from the ratio of the peak oxidation current versus the peak reduction current. However, as with all pulsed techniques, chronoamperometry generates high charging currents, which decay exponentially with time as any RC circuit. The Faradaic current, which is due to electron transfer events and is most often the current component of interest decays as described in the Cottrell equation, which defines the current-time dependence for linear diffusion control:

$I = nFACD^{1/2}\pi^{-1/2}t^{-1/2,}$

where n, F, D, A, C are number of electrons transferred, Faradic constant, diffusion coefficient(cm^2s^{-1}), electrode area(cm^2) and concentration ($molm^{-3}$), respectively.

In most electrochemical cells, this decay is much slower than the charging decay cells with no supporting electrolyte are notable exceptions. Most commonly investigated with a three electrode system. Since the current is integrated over relatively longer time intervals, chronoamperometry gives a better signal to noise ratio in comparison to other amperometric technique [73, 74, 75].

2.6. Surface modification in electroanalysis

Exploitation of nanomaterials and nanoparticles in electroanalysis is an area of research that is continually growing. Surface modification of conventional electrodes for enhanced current response is very important in developing a stable and highly target specific interface [76]. Sensitivity and selectivity are the crucial issues for the development of sensors for detecting electroactive molecules. The aim of this research is to provide an overview of the recent works in this field including advantages and disadvantages of surface modification by metal nanoparticles (gold). Here, this study is focused on the benefits of electrode surface modification as shown by sensor performance in terms of voltammetric response to the caffeine using gold nanoparticles.

Metal nanoparticles have wide applications in different kinds of electroanalytical methods and can be used to construct novel and improved sensing devices, particularly electrochemical sensors and biosensors. Owing to their small size (in order of 1- 100 nm), metal nanoparticles exhibit unique chemical, physical and electronic properties. They can absorb biomolecules strongly and play an important role in the modification of electrodes to improve their electrocatalytic activities. Large surface area of deposited metal nanoparticles permits improvement of analytical performance in terms of low detection limits and short deposition time. Transition metal nanomaterials possess high catalytic activity and facilitate electron transfer for many electrochemical reactions. A wide variety of metallic nanoparticles has been studied to assess the applications of these materials in electroanalysis. Screen-printed electrodes are planar devices with plastic substrates that are coated with layers of electroconductive insulating inks at controlled thickness. Such an electrode modified with silver nanoparticles by using electrochemical deposition has been utilized successfully [77].

3. Materials and Methods

3.1. Chemicals and Reagents

Caffeine reagent ($C_8N_4O_2H_{10}$, anhydrous powder 99%), theophylline ($C_8N_4O_2H_8$, hydrous powder 99%), theobromine ($C_8N_4O_2H_8$, hydrous powder, 99%), potassium tetrachloroaurate ($KAuCl_4$, 99.995%), were used for this study.

Para-nitro aniline ($C_6H_6N_2O_2$, 99%), sodium nitrite ($NaNO_2$, 96%), hydrochloric acid (HCl, 37%), Sulphuric acid (H_2SO_4, 98%), hydroquinone, ($C_6H_6O_2$, 99%), potassium nitrate (KNO_3, 99%), potassium chloride (KCl, 995), potassium hexacyanoferrate ($K_3Fe(CN)_6$, 97%) chloroform ($CHCl_3$), calcium carbonate ($CaCO_3$, 99%), sodium sulphate (Na_2SO_4, 99%) and Sodium hydroxide (NaOH, 99.3%) were used. Distilled water was used to prepare all solutions.

3.2. Apparatus and Instruments

The study was carried out in a three electrodes setup with screen-printed carbon electrode (SPCE) or the modified screen-printed carbon electrode as the working electrode, a carbon ring as the counter electrode and a silver contact as a pseudo-reference electrode. A Cyclic Voltammetery and Amperometric measurements were performed using Epsilon electrochemical analyzer, (Bioanalytical Systems, Inc. USA model) to run all experiments. The volumetric flasks, sonicators, grinders, micropipettes, pH meter, and beakers were also used.

3.3. Experimental Procedures

3.3.1. pH Measurement

The pH measurements were done with the pH meter (K06043, Hanna model) and calibrated with the standard buffer solution for each activity (buffers solution of pH 4.01, 6.8, and 9.2).

3.3.2. Electrode preparation

According to the procedures reported in the literature [78], the pre-treating procedures for the SPCE include soaking, sonicating and electrochemical conditioning. For soaking 3 $mmolL^{-1}$ of NaOH was prepared in distilled water. Then, the SPCE was soaked in this solution for at least 30 min rinsed properly with distilled water for several times and dried in air. After it was dried, sonication took place for 3 min to remove lastly the absorbed molecules and it was then rinsed appropriately with distilled water, dried in air and prepared for the electrochemical conditioning. For electrochemical conditioning purpose, 1M KNO_3 was used. Then, the potential scanning from 1.0 V to 1.8 V in 1 M KNO_3 took place for at least six complete scans at 100 mV/s to decrease the background current due to carbon oxidation and a reproducible cyclic voltammogram was obtained.

3.3.3. Surface Modification of the Electrode

Gold nanoparticles were electrodeposited over the surface of BSPCE employing the procedures developed in the literature [79]. This was done by mixing 50 µL of 10 $mmolL^{-1}$ $KAuCl_4$ in 5 mL of 0.5 $molL^{-1}$ H_2SO_4 using micropipette. Then, chronoamperometry was run by applying 0 V stepped from 1.1 V for various deposition times to be selected based on analytical signal of caffeine on the modified SPCE.

3.3.4. Electropolymerization of p-nitroaniline Film

p-Nitroaniline film was electropolymerized on AuNPs deposited/SPCE from 3 $mmolL^{-1}$ of p-nitroaniline diazonium salt solution that was prepared according to literature [80]. Briefly, solutions of 3 $mmolL^{-1}$ of p-nitroaniline and 0.1 $molL^{-1}$ sodium nitrite in 0.5 $molL^{-1}$ of hydrochloric acid were prepared separately in a volumetric flask and stored at 4ºC for 1 hr. Then 400 μL of 0.1 $molL^{-1}$ $NaNO_2$ was added to 20 mL of 3 $mmolL^{-1}$ paranitroanniline (PNA) under stirring at room temperature. Cyclic voltammetry was used to graft the film on AuNPs deposited/SPCE by scanning the potential from 0.6 to -0.2 V for five cycles at a scan rate of 100 mVs^{-1}.

3.3.5. Stripping of Gold Nanoparticles

Gold nanoparticles were stripped off from PNA grafted AuNPs deposited/SPCE using CV by scanning the potential from 0 to 1400 mV for three cyclic scans at a scan rate of 0.1 Vs^{-1} in 1 mol L^{-1} KCl solution.

3.3.6. Preparation of Caffeine Solutions

Standard stock solution of caffeine (0.02 $molL^{-1}$) was prepared in 0.01 $molL^{-1}$ H_2SO_4 and stored at 4 °C.

3.3.7. Caffeine Extraction Using Sulphuric Acid

Caffeine was extracted from raw coffee, which was milled and ground with an analytical grinder (Emmericher-Rhein Nr 5181). The grounded bean was sieved with a mesh size of 500 µm. Extracting solvents suitable for electroanalytical purpose such as hot water and dilute H_2SO_4 solution were tested.

3.3.8. Caffeine Extraction Using Chloroform

For standard analytical method for determination of caffeine in coffee beans, chloroform was used to extract caffeine from coffee beans. 4g of grounded raw coffee was added into 250 mL Erlenmeyer flask fitted with a stopper, and then 250 mL of boiled distilled water was added while stirring. The residue was allowed to cool and settle down, and then the solution was filtered. 100 mL of coffee extract solution was mixed with 2g of sodium carbonate. These reacts with some of the substances in the coffee extract and make them precipitate and were transferred to separatory funnel and then 35 mL of chloroform was added to it. The mixture was vigorously swirled for 5 minutes and the chloroform water mixture was separated. The chloroform solution of caffeine collected from the separatory funnel was transferred to 150 mL Erlenmeyer flask. To this solution some amounts of anhydrous sodium sulphate was added in order to remove the last traces of water. Chloroform was removed to dryness under reduced pressure (rotary vapour) at temperature of 45ºC. The recovered mass was then dissolved in distilled water. Pipetting 50, 150, 200, 250, 300 and 350 µL aliquots of the stock standard solution in to 5 mL of sample solution prepared working standards.

3.3.9. Real Sample Analysis

The proposed method was exploited for the determination of caffeine in commercially available coffee beans. For Voltammetric analysis, 4g of raw powder coffee was added into 150 mL beaker, and then dissolved in 100 mL of 6 $molL^{-1}$ H_2SO_4 [81]. The mixture was diluted with 50 mL of 0.1 $molL^{-1}$ H_2SO_4 to the mark after acid digestion and filtered, and then the pH of the solution adjusted to 1.90 by 0.1 $molL^{-1}$ NaOH. Cyclic Voltammetric determination of caffeine at the modified electrode was done by standard addition method. Working standards of caffeine , 6 $\mu molL^{-1}$, 8 $\mu molL^{-1}$, 10 $\mu molL^{-1}$, 12 $\mu molL^{-1}$, and 16 $\mu molL^{-1}$ were separately prepared from 1 mM Stock solution of caffeine standard and diluted to the mark by 0.01 M H_2SO_4. 5 mL of each standard solution was added into three 50.0 mL volumetric flasks containing 3 mL of the sample solution for the determination of caffeine.

For Uv-Vis spectrophotometric determination of caffeine in coffee, first 4g of powdered raw coffee was dissolved in 250 mL of boiled distilled water. Then, the solution was cooled to room temperature and caffeine was extracted by chloroform. The caffeine was collected after removing the chloroform by using rotavapor at 45ºC [81]. For this purpose, 1000 ppm caffeine was prepared by dissolving 200 mg of caffeine standard in 200 mL of distilled water. Working standards of caffeine 10, 20, 30, 40, 50 and 100 ppm of was ejected to 1 mL of sample solution for determination of caffeine [82].

4. Results and Discussion

4.1. Electrodeposition of AuNPs on SPCE

Electrochemical deposition process of gold nanoparticles includes the nucleation and growth of crystals. Gold nanoparticles were electrodeposited over the surface of bare screen printed carbon electrode (BSPCEs) (Figure 4) employing the procedures developed in the literature [79]. Briefly, 50 μL of 10 mM $KAuCl_4$ solution was mixed with 5 mL of 0.5 M H_2SO_4 and dropped on the surface of bare screen printed carbon electrode (BSPCE), and a constant potential of 1.1 V was applied for 10 s and amperommetric experiment was run. After gold nanoparticles deposition, the electrode surface was generously rinsed with distilled water and dried in air at room temperature before electropolymerization of PNA to be taken place.

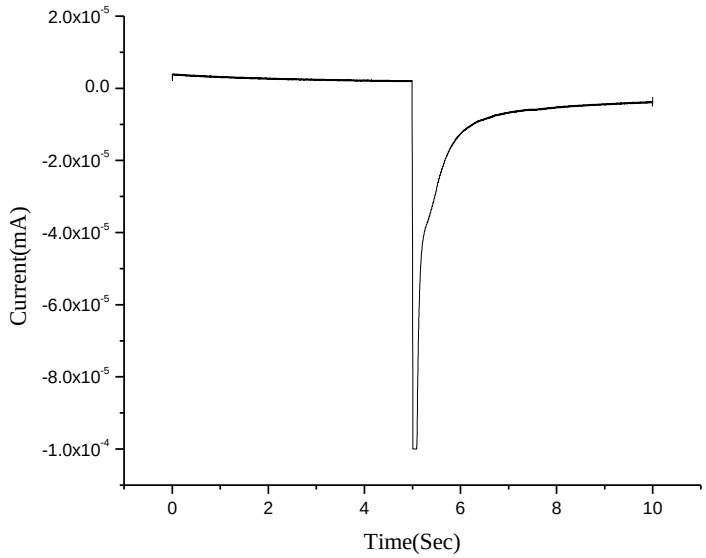


Figure : Chronoamperogram of 10 mM of $KAuCl_4$ in 0.5 M H_2SO_4 at BSPCE.

4.2. Electropolymmerization of PNA film

After chronoamperommetric deposition of AuNPs on a bare screen-printed carbon electrode, 3 mM of p-nitroaniline (PNA) in 0.1 M $NaNO_2$ was electropolymerized on the surface of AuNPs deposited SPCE. This was done by scanning of the potential from –0.2 V to 0.6 V for five cyclic scans (Figure 5) at a scan rate of 0.1 V s^{-1} using cyclic voltammetric technique. The formation of p-nitroaniline (PNA) film (negative layer) on the surface of AuNPs deposited SPCE was checked by running the cyclic voltammetry of $K_3Fe(CN)_6$ and hydroquinone (HQ).

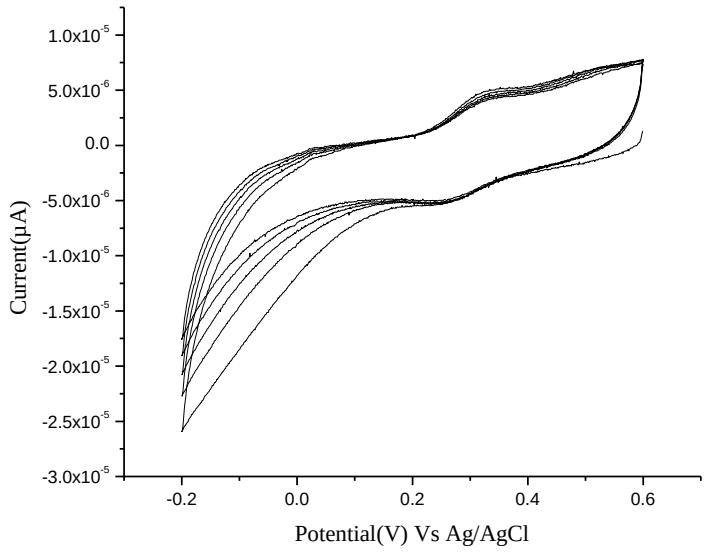


Figure : Cyclic voltammogram of electropolymerization of 10 mM PNA in 0.1 M $NaNO_2$ on the surface of AuNPs deposited/ SPCE.

4.3. Stripping off AuNPs

After depositing AuNPs on a bare SPCE from a solution of 10 mM $KAuCl_4$ and coating with PNA film from a solution of 3 mM PNA, the deposited AuNPs was stripped off in 1 M KCl to produce a nanohole which in turn used to convert a planar diffusion of electroactive species to the electrode into a radial diffusion of the electroactive species towards the electrode. This was done by scanning of the potential from 0 to 1.4 V with three cyclic scans at a scan rate of 100 mV/s. As can be seen from the graph below (Figure 6) the magnitude of the oxidation peak current was decreased from the first cycle to the third cycle, which indicates the complete removal of the deposited AuNPs from the electrode.

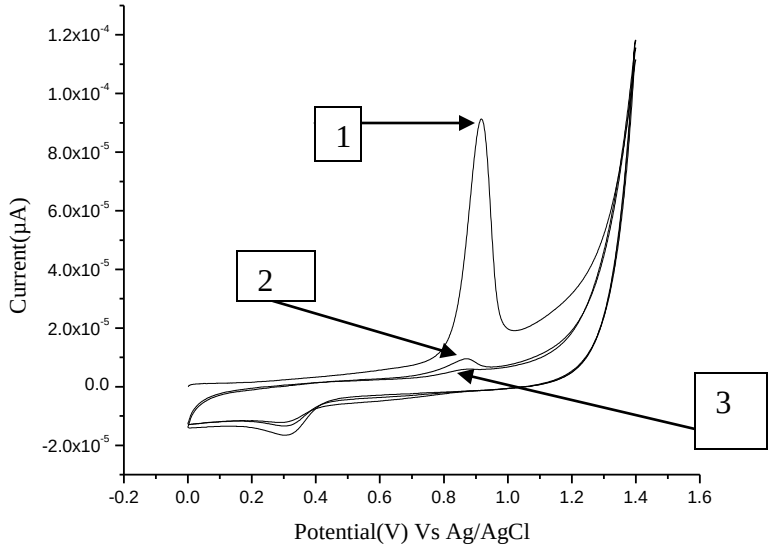


Figure : Cyclic voltammograms of Stripping off AuNPs in 1 M KCl at three cyclic scans (cycle 1, cycle2, and cycle3).

4.4. The Electrochemical Characterization of PNA Grafted AuNPs deposited SPCE

P-nitroaniline (negative polymer) coated on the AuNPs deposited SPCE forms a negative layers on the electrode and therefore it attracts positive analytes and repels negative analytes. In this study, $K_3Fe(CN)_6$ (negative probe) and hydroquinone(neutral probe) were used to characterize the modified electrode.

4.4.1. Cyclic Voltammetry of $K_3Fe(CN)_6$

The formation of p-nitroaniline film on AuNPs deposited SPCE was proved by running cyclic voltammetry of $K_3Fe(CN)_6$ at p-nitroaniline grafted screen printed carbon electrode. As can be seen from Figure 7, the redox peak current of $K_3Fe(CN)_6$ at nanohole grafted SPCE decreased compared to the bare SPCE due to the partial coating of SPCE by PNA film. But the redox peak current of $K_3Fe(CN)_6$ totally disappeared at PNA grafted SPCE because of the complete insullation of the active site of the electrode by PNA film.

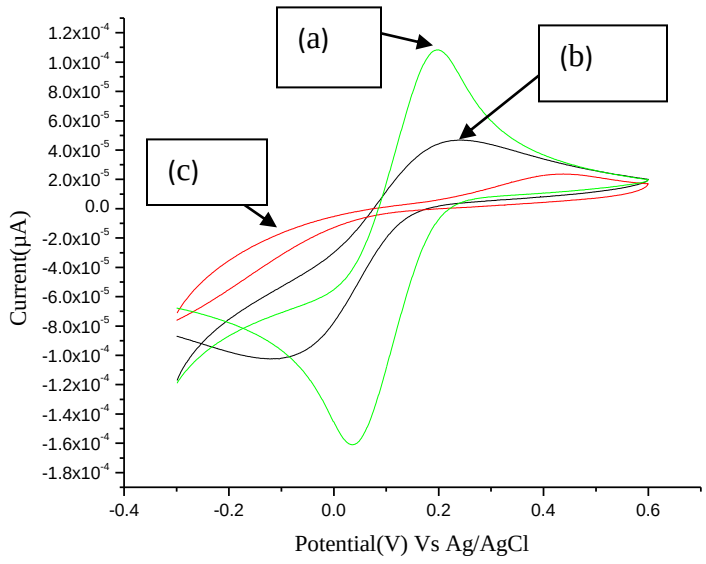


Figure : Cyclic voltammograms of 10 mM $K_3Fe(CN)_6$ in 1 M KNO_3: at (a) BSPCE ;(b) nanohole PNA grafted SPCE; and (c) PNA grafted SPCE. In all cases, the scan rate is 0.1 V/s.

4.4.2. Cyclic Voltammetry of Hydroquinone

Figure 8 shows the Cyclic voltammogram of HQ at nanohole PNA grafted SPCE (**Figure 8, a**) and PNA grafted SPCE (**Figure 8, b**). At nanohole PNA grafted SPCE, the redox peak current of HQ was enhanced due to three dimensional diffusion (radial diffusion) of the electroactive species towards the nanohole electrode. However, at p-nitroaniline grafted screen-printed carbon electrode the redox peak current of HQ was decreased, this is due to the coverage of the active sites of the electrode by p-nitroaniline film.

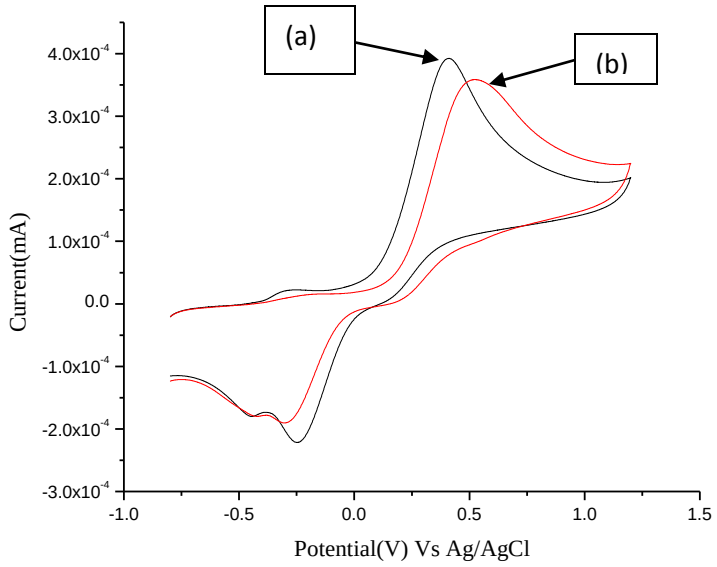


Figure : Cyclic Voltammogram of 10 mM HQ in 1M KNO_3 at: (a) nanohole PNA grafted SPCE; and (b) PNA grafted SPCE. The scan rate is 0.1 V/s.

4.5. The Electrochemical Behavior of Caffeine at SPCE

The electrochemical behavior of caffeine at SPCE was investigated using cyclic voltammetry. Because caffeine is an electropositive analyte, its oxidation peak current was enhanced at nanohole PNA grafted SPCE in relative to BSPCE. This is because of the electrostatic attraction between negatively charged PNA film and positively charged caffeine analyte in addition to the three dimensional (radial) diffusion of an electroactive species toward a nanohole electrode. Figure 9, shows the cyclic voltammograms obtained for caffeine (0.02M) at the BSPCE (a) and nanohole PNA grafted SPCE(b), in 0.01 mol L^{-1} H_2SO_4 (pH 1.90) with a scan rate of 50 mVs^{-1}. The electrochemical response of caffeine showed one broad anodic peak potential at about 1426 mV versus Ag/AgCl at BSPCE (Figure 9, a). Under the same conditions, relatively a sharp peak at 1392 mV (Figure 9, b) has been obtained at nanohole PNA grafted SPCE. The potential shift towards less positive direction accompanied by the remarkable oxidation peak current enhancement at the modified SPCE was a clear evidence of the catalytic effect of the modified SPCE toward caffeine oxidation. The absence of any reduction peak in the reverse scan indicates that the electrochemical oxidation of caffeine is irreversible in nature.

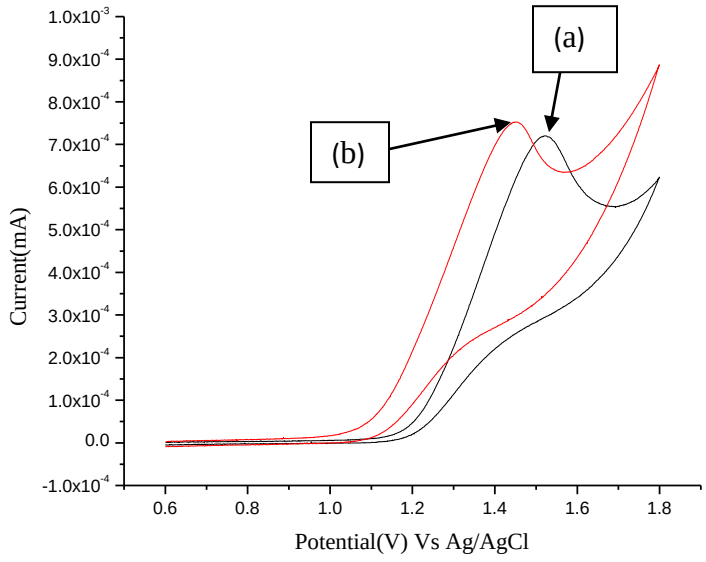


Figure : Cyclic Voltammograms of 0.02 M stand CAF in 0.01 M H_2SO_4 (pH 1.90) at: bare SPCE (a), Nanohole PNA grafted SPCE (b). The scan rate is 50 mV/s.

4.6. The Optimization of Experimental Parameters

4.6.1. Effect of Concentration

The relationship between oxidation peak currents and caffeine concentrations was studied within the range of $1x10^{-7}$ to $1x10^{-3}$ $molL^{-1}$. The study of the effect of concentration at optimized conditions on nanohole PNA grafted SPCE, showed that there was a linear relationship between the peak current and concentration. As can be seen from the Figure 10, the redox peak current of caffeine was increased with the increase in concentration.

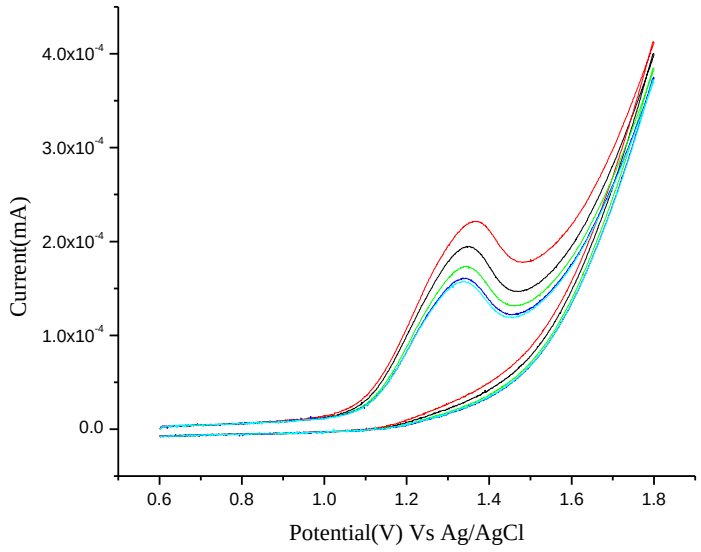


Figure : Cyclic voltammograms of different concentrations of caffeine at nanohole PNA grafted SPCE in 0.01 M H_2SO_4 : (a) 1×10^{-7} , (b) 1×10^{-6}, (c) 1×10^{-5}, (d) 1×10^{-4}, and (e) 1×10^{-3} mol L^{-1} at a scan rat of 50 mVs^{-1} .

4.6.2. The Effect of Scan Rate

The effect of scan rate on the oxidation peak potential and oxidation peak current of caffeine at nanohole PNA grafted SPCE was examined by cyclic voltammetry by changing the scan rate from 16 – 100 mVs^{-1}. Figure 11,shows the cyclic voltammograms of 0.02 M caffeine in 0.01 M H_2SO_4 (pH 1.90) at a scan rate ranging between 16 and 100 mVs^{-1}.The electrode reaction was irreversible as shown from the lack of a reduction peak in the cyclic voltammogram. This was also further confirmed by the shift of peak potential toward more positive value with increasing scan rate. As indicated in the Figure 11, the peak current increased with increase of scan rate and due to excellent peak response, a scan rate of 50 mVs^{-1} was chosen for subsequent determinations. The peak current of caffeine showed linear dependence with the scan rate, as shown in the Figure.

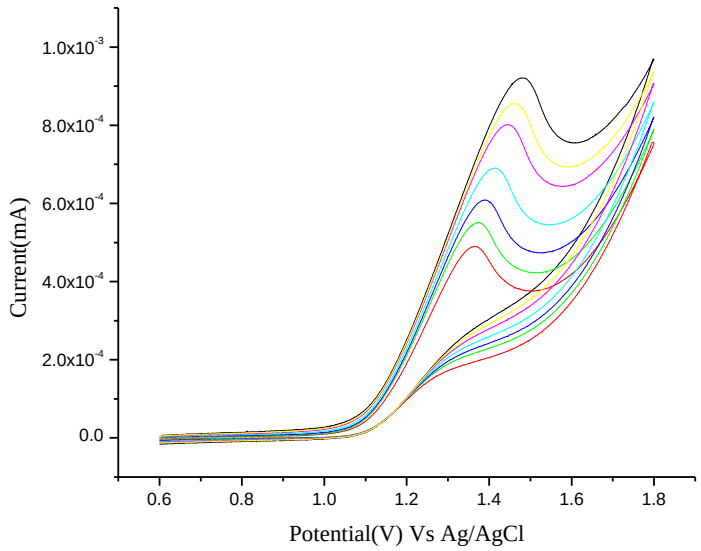


Figure : Cyclic voltammograms of 0.02 M CAF in 0.01 M H_2SO_4 at nanohole PNA grafted SPCE at different scan rates of 16, 25, 36, 49, 64, 81 and 100 mV/s.

4.6.3. The Effect of pH

The effect of pH of supporting electrolyte on the oxidation peak potential of caffeine was studied from pH range of 1.90 to 7.5 in 0.01 mol L^{-1} H_2SO_4. The pH value of an electrolyte solution is an important factor that affects the oxidation reduction behavior of an electroactive species [83]. Therefore, the effect of variation of pH for the oxidation of 0.02 M caffeine was studied. In this work, cyclic voltammetry was used to investigate the effect of pH ranging from 1.90 to 7.5 using sulphuric acid solution (0.01M) as supporting electrolyte, at a scan rate of 50 mVs^{-1}. 0.1M NaOH and 0.1M H_2SO_4 were used for adjusting the pH of the solution. As shown in the Figure 12, the anodic peak potential of caffeine shifted towards negative potential with the enhancement of the anodic peak current as the pH decreases from 7.5 to 1.90.

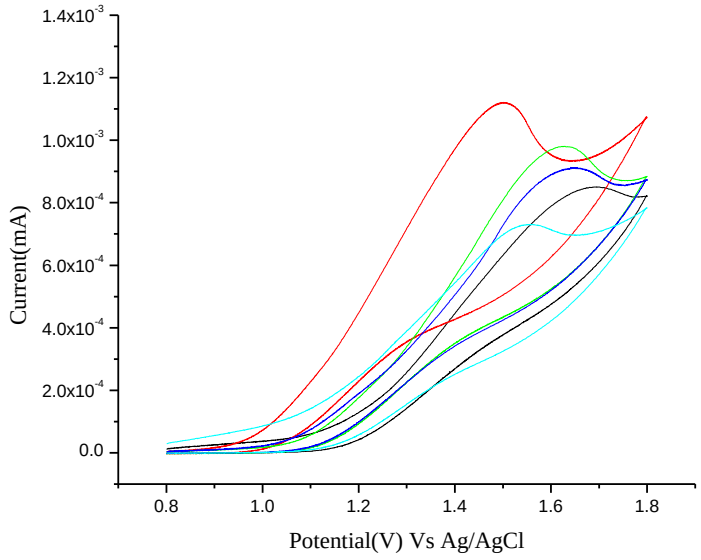
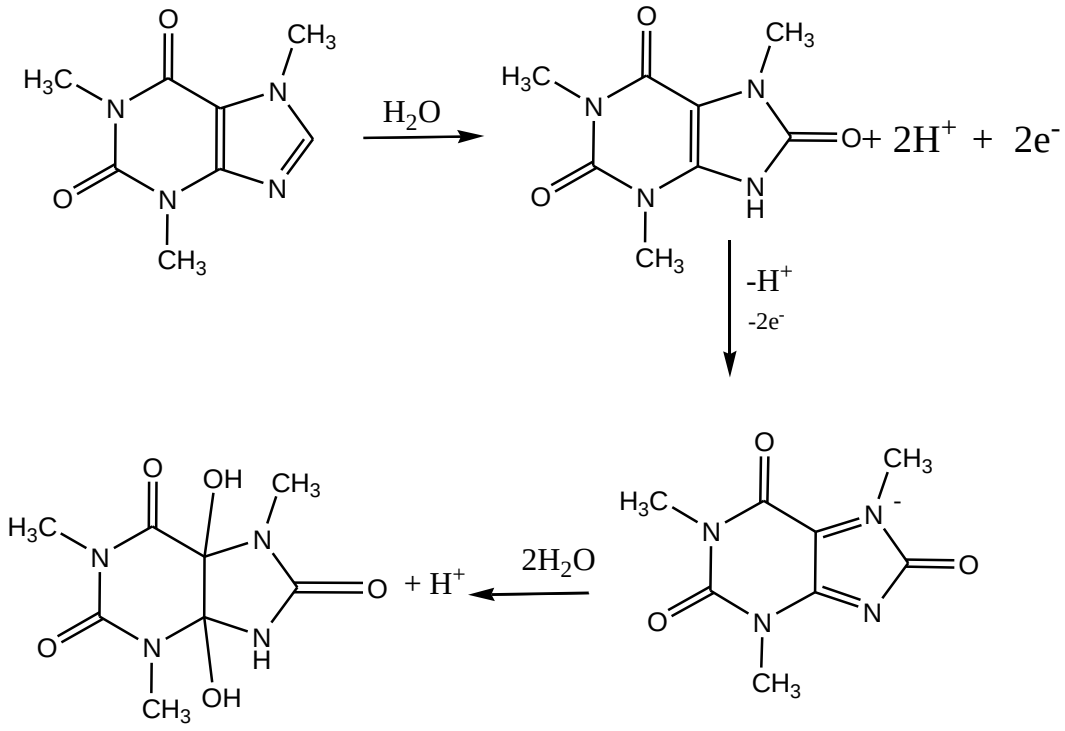


Figure : Cyclic voltammograms of 0.02 M CAF in 0.01 M H_2SO_4 at nanohole PNA grafted SPCE at different pH values of 1.90, 2.1, 3.5, 5.5, and 7.5

This shift of anodic peak potential towards negative with the enhancement of the anodic peak current was evidence that reflects the involvement of protons in the electrode process. The linear relationship between pH and anodic peak potential shows that equal number of protons and electrons are involved in the oxidation process of caffeine within the studied pH range. Hence, the overall process involves four protons and four electrons as suggested by Dryhurst and Hansen [84].

The first step is a $2H^+$, 2e− oxidation of the C-8 to N-9 bond to give the substituted uric acid. This is followed by an immediate $2H^+$, 2e− oxidation to the 4, 5-diol analog of uric acid, which rapidly fragments, (scheme 1).



Scheme : Mechanisms of the Electrochemical Oxidation of Caffeine [85]

The effect of variation of pH on the oxidation peak current of the electrocatalytic oxidation of caffeine at nanohole PNA grafted SPCE was also studied. The anodic peak current decreased linearly with increase in solution pH from 1.90 to 7.5 (Figure 12). However, better shape of cyclic voltammogram and excellent peak response were obtained at pH 1.90. So, based on this fact the pH 1.90 was used as a suitable medium throughout this work

4.6.4. The effect of deposition times

The composition and crystalline structure of the electrode material can be controlled by adjusting the electrodeposition parameters [86, 87]. For example, the thickness of the deposited films (gold nanoparticles layer) is proportional to the time of electrodeposition and affects the current response of the electrode. This implies that the thickness of the gold nanoparticles layer increased gradually with time, which caused a decrease of the current response. For this purpose, three deposition times of AuNPs on a BSPCE were studied by repeatedly changing the deposition times from 5s to 15s on three different SPCEs. Two rounds of deposition (10s) exhibited the highest oxidation peak current of caffeine (Figure 13). Thus, because of this, the deposition time of 10 s was chosen as an optimum condition for the subsequent experimental measurements in this work.

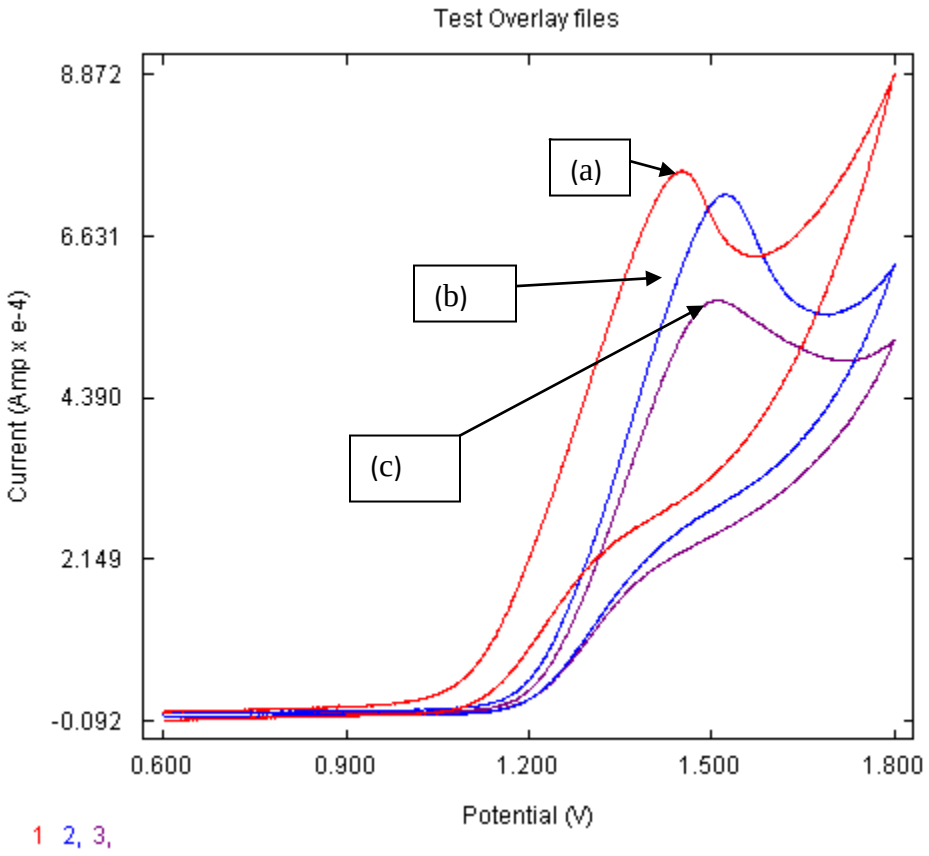


Figure : Cyclic Voltammograms of 0.02 M CAF at different deposition times: (a).10 s (b). 5 s (c).15 s

4.7. The studies of Interferences

For the study of the selectivity and sensitivity of the developed method, the effects of the common coexisting species in coffee (in this particular work: theobromine and theophyline) which overlap with the redox peak current of caffeine were studied.. These interferences have similar chemical structure with caffeine [88]. Figure 14 and Figure 15 show the electrochemical response of theobromine and theophyline both at BSPCE and nanohole PNA grafted SPCE, respectively.

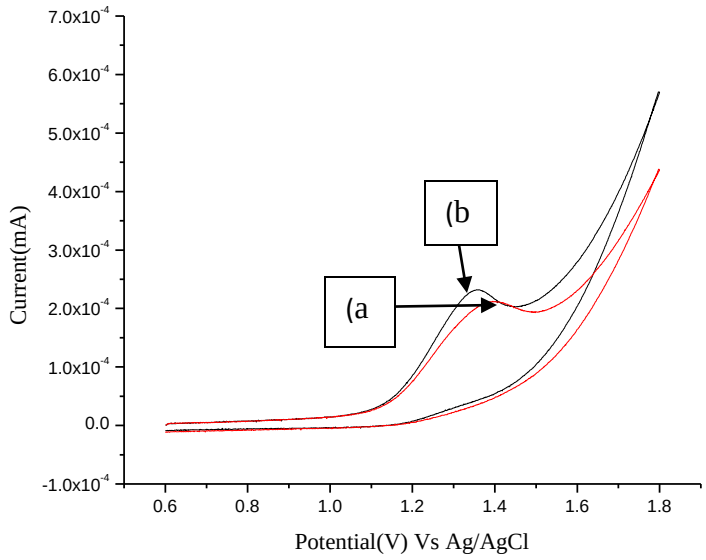


Figure : Cyclic voltammograms of Theobromine at: (1) BSPCE (a) and (2) Nanohole PNA grafted SPCE (b)

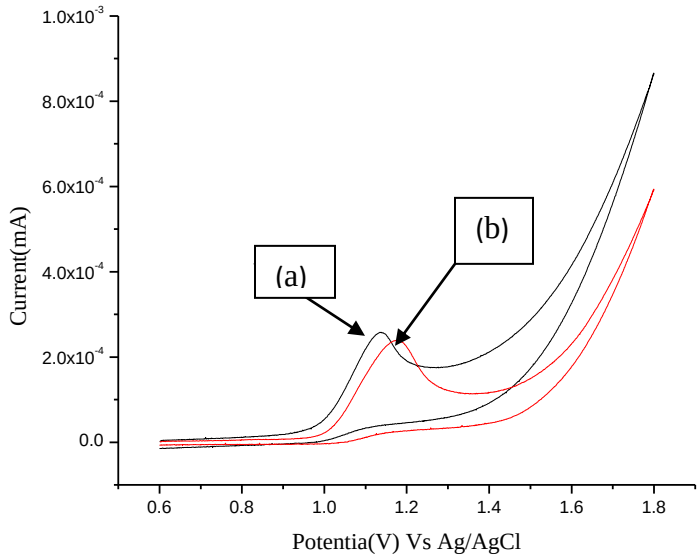


Figure : Cyclic voltammograms of Theophyline at: 1/Nanohole PNA grafted SPCE (a); and 2/ BSPCE (b)

The experimental result of the cyclic voltammetry of the mixture of theophyline and caffeine indicates the possibility of the determination of caffeine in real samples even in the presence of interferences. This is due to, the lower oxidation potential of theophyline than caffeine at nanohole PNA grafted SPCE. The following anodic oxidation potentials were recorded from the cyclic voltammetric experiment of the mixture of theophyline and caffeine at nanohole PNA grafted SPCE: 1152 mV and 1361 mV, respectively, Figure 16. This indicates that nanohole PNA grafted SPCE has good selectivity for the determination of caffeine in the presence of theophyline [89].

However, from the cyclic voltammetric result of the mixture of theobromine and caffeine, the oxidation peak current of theobromine overlaps with that of caffeine (Figure 17).This indicates that theobromine and caffeine oxidize at the same potential.

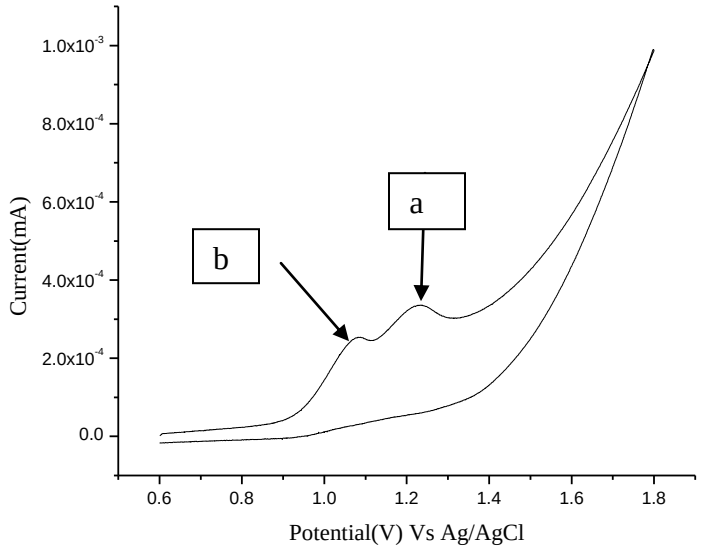


Figure : Cyclic voltammogram of mixtures of CAF (a) and theophyline (b) at Nanohole PNA grafted SPCE

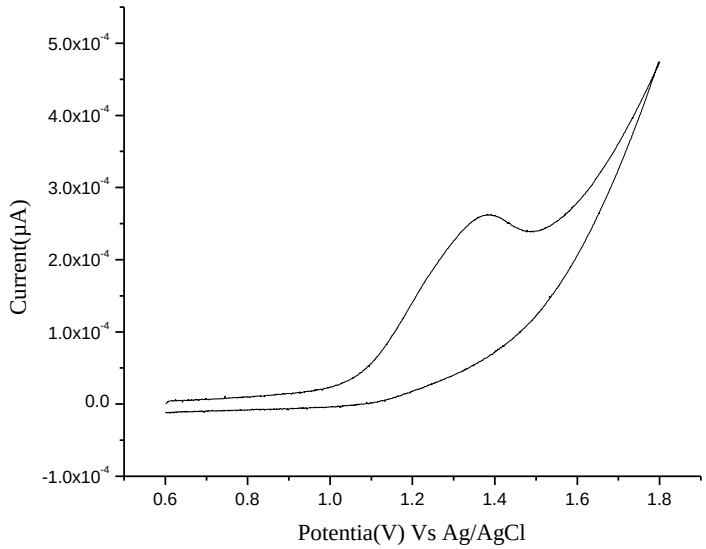


Figure 17: Cyclic voltammogram of mixtures of CAF and theobromine at Nanohole PNA grafted SPCE

4.8. Uv-Vis Spectrophotometric Determination of Caffeine

For Uv/vis spectrophotometric determination of caffeine in raw coffee beans, the caffeine was extracted according to the procedures mentioned in the experimental section. The extracted caffeine was collected after removing the solvent using rotavapor and dried**.** The melting point of the collected caffeine was measured to be 234 ºC**,** which shows the purity of the extracted caffeine.

For validation purpose, the cyclic voltametric result of caffeine was compared with Uv-Vis spectrophotometric method [81]. The absorbance of working standards and samples were measured at 272 nm. The caffeine concentration of the sample was calculated from the calibration curve by extrapolation of the regression line to the x-axis. The amount of caffeine obtained by UV-Vis spectrophotometeric method is lower than that obtained by electrochemical method which gives lower LOD. This suggests that the present method is more sensitive than UV-Vis spectrophotometric method for determination of caffeine.

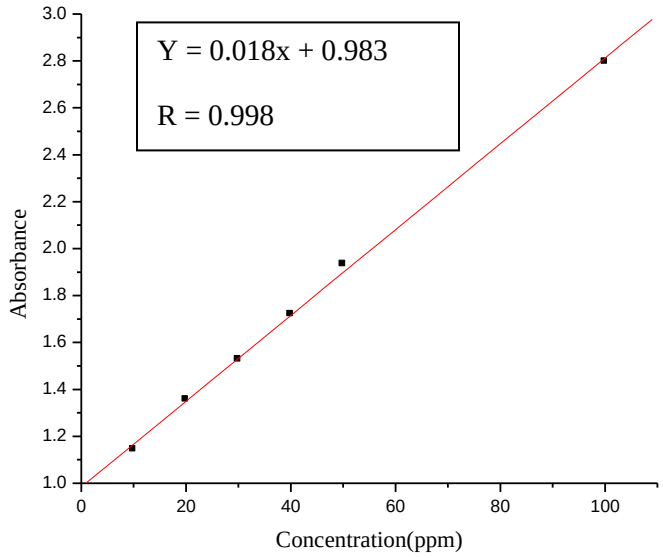


Figure 18: Caffeine calibration curve for UV/ Vis Spectrophotometeric method

4.9. Cyclic Voltammetric Determination of Caffeine at nanohole PNA grafted SPCE

Cyclic Voltammetric determination of caffeine at nanohole PNA grafted SPCE was done by standard addition method. Working standards of caffeine ,6 $\mu molL^{-1}$, 8 $\mu molL^{-1}$, 10 $\mu molL^{-1}$, 12 $\mu molL^{-1}$, and 16 $\mu molL^{-1}$ were separately prepared from 1 mM Stock solution of caffeine standard and diluted to the mark by 0.01 M H_2SO_4. 5 mL of each standard solution was added into three 50.0 mL volumetric flasks containing 3 mL of the sample solution for the determination of caffeine.

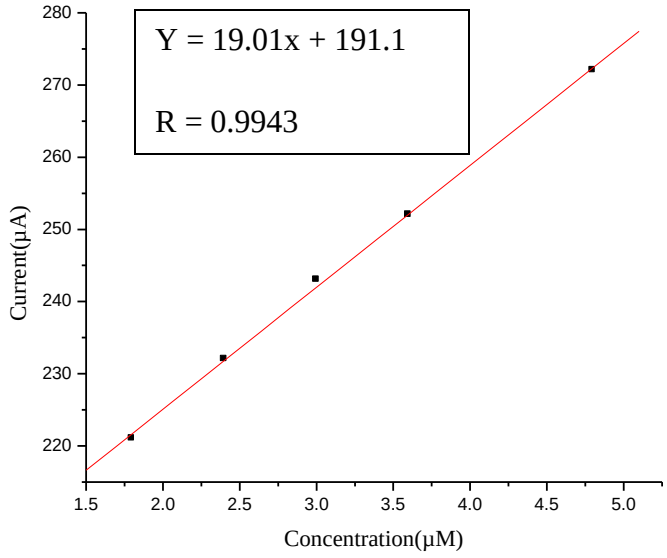


Figure 19: Calibration curve for Caffeine as obtained from cyclic voltammetric method for determination of caffeine from recovered mass.

4.10. Analytical applications

4.10.1. Real sample analysis

The applicability of the developed method for the determination of caffeine in real sample was investigated by cyclic voltammetry. The cyclic voltammetric detected value of caffeine was compared with the Uv-Vis spectrophotometric detected value in Table 1. From the experimental results, it is found that the result obtained using nanohole PNA grafted SPCE was in a good agreement with that obtained by Uv-Vis spectrophotometric method.

Table1: Concentration of caffeine obtained from raw coffee by different methods

Methods	**w\w%**
Cyclic Voltammetry	0.38
Uv/vis Spectrophotometry	0.36

4.10. 2. Reproducibility and Stability of the Modified Electrode

The reproducibility and stability of the fabricated electrode was tested by cyclic voltammetry at optimized parameters. For the study of the reproducibility of the modified electrode three different nanohole PNA grafted SPCE were prepared at three different days at the same conditions and the oxidation peak current responses were recorded**.** The relative standard deviation (RSD) obtained for three successive determination of 0.02 M caffeine at nanohole PNA grafted SPCE was 6.2%, which showed that the electrode has good reproducibility. The stability of the modified electrode was also investigated by storing the electrode in air, at room temperature and in the dark for 25 days, in the laboratory. After 25 days storage of the electrode, the oxidation peak current of caffeine was recorded and it retained 98 % of its original response indicating that the modified electrode was stable and has long duration times.

Table2: Comparison of the analytical performance of nanohole PNA grafted SPCE for determination of caffeine with previously reported electrodes.

Electrode	**Modifier**	**Technique**	**Linear Conc. Range(µM)**	**LOD(µM)**	**Sample**	**Reference**
Glassy Carbon Electrode(GCE)	Lignin	DPV	6 - 100	0.837	Coffee	[90]
Glassy Carbon Electrode(GCE)	Gold/p-nitroaniline	CV	2 - 16	0.0728	Coffee	[91]
Screen printed Carbon Electrode(SPCE)	nafion/Graphene	DP/Ads.SV	1 - 10	0.021	Coffee	[92]
Screen printed Carbon Electrode(SPCE)	Gold/p-nitroaniline	CV	6 - 16	0.192	Coffee	This Work

5. Conclusion

This work demonstrates the ability of nanohole PNA grafted SPCE for the electrochemical determination of caffeine at lower oxidation potential with the enhancements of its oxidation peak current. From the results obtained in the cyclic voltammetric experiments, the oxidation of caffeine at the surface of the modified screen printed carbon electrode occurs at a potential less positive than at a bare screen printed carbon electrode (Figure 9). Under the optimized experimental conditions, the anodic peak current of caffeine was proportional to its concentration in the range 1.0 x 10^{-7} M to 1.0 × 10^{-3} M using cyclic voltammetry with a detection limit of 1.92 × 10^{-7} $molL^{-1}$(LOD=3ó/Slope) and a correlation coefficient of 0.99943. Low detection limit, good reproducibility and stability indicated the applicability of the developed method for the electrochemical determination of caffeine. Moreover, simple preparation, good sensitivity and selectivity, low cost, and rapid analysis time make the developed method suitable for the electrochemical investigation and routine determination of caffeine in real samples such as raw coffee beans.

5.1. Recommendation

The fabricated nanohole PNA grafted SPCE was found to have good performances for selective determination of caffeine (electropositive analyte). This suggests that the developed method might be used as a strategy for the selective determination of other electroactive positively charged analytes in the presence of anionic interferences.

6. References

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