JIMMA UNIVERSITY
SCHOOL OF GRADUATE STUDIES
COLLEGE OF NATURAL SCIENCE
DEPARTMENT OF CHEMISTRY

VOLTAMETERIC SENSORFOR DETERMINATION OF CAFFEINE USING NANOHOLE MODIFIED GLASSY CARBON ELECTRODE.

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Voltammetric Sensor for Determination of Caffeine Using Nanohole Modified Glassy Carbon Electrode

By: Mulugeta Tesema Efa

Thesis Submitted to the School of Graduate Studies, Jimma University in Partial Fulfilment of the Requirements for the Degree of Masters of Science in Chemistry

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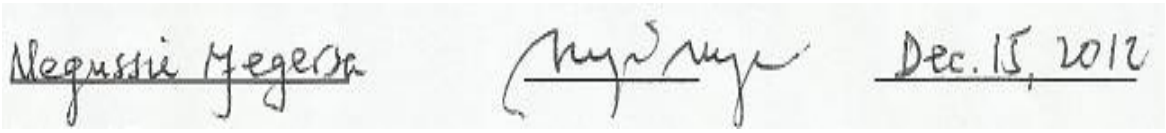

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Lists of Aberration

CME	Chemically modified electrode
CV	Cyclic voltammeters
DCPA	Differential chronoamperometry
EC	Electrochemical cell
GCE	Glassy carbon electrode
AuNPs	Gold nanoparticles
HPLC	High performance liquid chromatography
HQ	Hydroquinone
LOD	Limit of Detection
ME	Mercaptoethnol
NEA	Nanoelectrodes Array
NP	Nanoparticles
PNA	Para nitroaniline
SAMs	Self Assembled Monolayer
TLC	Thin layer chromatography
2D	Two dimensional
3D	Three dimensional
UMEs	Ultra microelectrode

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Abstract

Significant development has been witnessed in electrode surface nanostructuring to improve sensitivity and selectivity in electroanalysis. This work aimed at nanostructuring glassy carbon electrode surface with nanohole using electrochemically nucleated gold nanoparticles as a template. The surface modification procedure developed helps to minimize oxidation potential of caffeine and also to increases mass transport of caffeine to electrode surface thereby increasing sensitivity and selectivity of the modified electrode for determination of caffeine. Randomly nanostructured surface have been fabricated based on sequential electrochemical deposition of gold nanoparticles from H_2SO_4 solution containing $KAuCl_4$ on glassy carbon electrode. To increase the number density of the nucleated nanoparticles at glassy carbon electrode, three rounds of deposition-passivation cycles has been done using chronoamperometry. The growth of nucleated nanoparticles in sequential deposition process was prevented using self assembled monolayer of 2-marcapitoethnol capping agent. Following the nucleation steps, p-nitroaniline film was grafted from its diazonium salt. The nanohole structured surface was fabricated after stripping of the nucleated gold nanoparticles. Nanohole modified electrode showed high electrochemical sensitivity towards oxidation of caffeine due to remarkably increased anodic peak current and decreased over potential of caffeine oxidation. The effect of different experimental parameters was investigated on the peak height of caffeine. The method enables determination of caffeine in the range from 1.0×10^{-7} to 1.0×10^{-3} mol L^{-1}, with limit of detection (LOD = 3δ/slope) of 7.28×10^{-8} mol L^{-1}. The effect of theophylline and theobromine on the determination of caffeine was studied and found to be minimal. Besides, the sensor displayed good stability and reproducibility. The proposed method showed high sensitivity, selectivity and stability in determination of caffeine in coffee

1. Introduction

1. 1 Electroanalysis

Electroanalytical methods have many advantages over other types of chemical analysis methods. The advantages include instrumental simplicity, portability and moderate cost as compared to other analytical methods[1]. The methods are very convenient because measured quantities are obtained as an electrical signal, such as current, potential or charge. This helps the high speed and accuracy of the readings, as well as for atomization of the whole analysis. Electroanalytical methods have found a vast number of applications including environmental studies, pharmaceutical and biological sample analysis. Advance in modern electroanalytical methods has led to substantial increase in the popularity of the methods to new stage and improvements[2].

However; the electroanalysis of a real substances are often challenging at common electrode materials due to overlapping of the signal of a sample in a given complex matrix under the study to specific analytes and passive working electrode which is not enough sensitive to study redox properties of the analytes. If the interferents produce a signal that is indistinguishable from that of the analyte, for such cases, an existing procedure must be modified to differentiate signal of interferents from that of the target analyte[3]. Alternatively, an entirely new analytical method might need to be developed. In either case, the number of variables that must be taken into account usually increases exponentially with the number of species contained in the sample[3].

If we conclude that existing procedure is not applicable for target analysis, consideration must be given to modifications that may overcome the problems[4]. When these modifications achieve their functions without introducing new difficulties, it has to be extensively tested only in the laboratory before its general use. After giving due consideration to existing methods and their modifications, then one may decide that some fits the problem and an entirely new procedure must be developed. So an analytical chemist should be able to the best, sensitive, fast and cheap analytical method for the determination of analyte in a given samples[5].

Nowadays electrochemical analysis based on electrical measurements is widely used in determination of various analytes in different sample matrixes. The most important one is real sample analysis, where the electrochemical behaviour of particular analyte is to be studied in the presence of interferences[6]. One of the most significant examples of a direct determination method relying on an electrochemical phenomenon is methylxanthines (Caffeine, theophylline and theobromine). The anodic oxidation of caffeine has been a subject of numerous studies for selective and rapid determination of caffeine. Electroanalytical methods have recently shown to offer sufficiently selective, sensitive and rapid determination that may be used alone or in conjugation with other analytical methods[7].

The aim of this study was to develop sensitive, simple and reproducible voltammetric sensor for determination of caffeine in coffee.

1. 2 Theoretical Back Ground

1. 2. 1 Nanomateriails for Electroanalysis

The study of nanoscale materials in recent years has been extensive, particularly with respect to metallic NPs[8]. The significance of nanoparticulate materials in electroanalysis are the very specific properties which may be exhibited at the nanoscale[8]. These include enhanced diffusion based on convergent rather than linear diffusion at the smaller NPs, high active surface area, improved selectivity, catalytic activity, higher signal-to-noise ratio and unique optical properties[9].

These unique properties make nanomaterials an appropriate for electroanalytical applications. Enhanced convergent mass transport (3D) to nanoelectrodes facilitates the study of faster electrochemical processes. At the nanoscale, surface can be exposed which are not accessible at the macro scale, in turn giving rise to improved current responses and catalysis. A few commonly used metals for NPs are gold, silver and platinum. The electroanalytical application of such nanomaterials has been found to be quite extensive. This suggested the potential for study of a wide variety of analyte using nanoparticles in electroanalysis[9].

Gold nanoparticles can be very useful in place of bulk electrodes, as they require much less expensive material. Previous work has shown extensive uses of AuNPs in electroanalysis. Recent studies have shown the biocompatibility of AuNPs is highly advantageous, enabling the incorporation of biomolecules such as enzymes and proteins into electrochemical systems[10]. In this work, gold nanoparticles synthesis method was applied to the preparation of nanostructured materials which was used as templates. This method entails synthesizing the desired nanohole material within the glassy carbon electrode by stripping gold nanoparticles that attached on to glassy carbon electrode[11].

Recently, the use of nanoparticles in electroanalysis is an area of research which is continually expanding[12]. Specially, novel nanomaterials with unique physical and chemical properties have been synthesized for electroanalysis in micro and nano-scales to qualitatively, or quantitatively examine the changes of compositions and structures for scientific insights and practical applications[12-14].

Therefore analytical chemists should be aware of the importance of this hot scientific and technical topic and utilize these new materials in improving existing analytical methods and develop new ones.

1. 2 .2 Surface Modification in Electroanalysis

The beauty of electrochemical techniques is to utilize tailor made chemically modified electrode (CME) for sensitive and selective analytical applications which cannot be expected in spectroscopic characterization methods[15]. If we modify the surface of the electrode, somehow we can control how electrode interacts with its environments[15-17]. Surface modification of conventional electrodes are very important in electroanalysis for the enhancement of current responses of the electrode and developing stable and highly target specified interface[17].

The sensitivity and selectivity are the crucial issues for electrode sensor for detecting analyte. Here we have focused on electrode surface modification of nucleated gold nanoparticles as template for determination caffeine[18].

In recent years this uprising into fashion design electrode surfaces, such that the electrode has unique properties, has continued at an even greater rate with exceptional control over the modification process via advances in nanofabrication[19-20].

In general, modified electrodes include electrodes where the surface was deliberately altered to impart functionality distinctly differ from the base electrode. During last decades a large number of different strategies for physical and chemical electrode modification have been developed, aimed at the enhancement in the detection of species under interest. Particularly in biosciences and electrochemical analysis such electrodes became of great important[21-22].

One of the issues raised in the recent research of redox processes taking place at modified electrodes has-been the analysis of changes in the diffusion towards their altered surface[23]. Appearance of novel materials and methods of thin films preparation lead to massive development of chemically modified electrodes with nanoparticles. Such electrodes preparation represents relatively modern approach to electrode systems in addition to thin film of a selected chemical bonded or coated onto the electrode surface. A wide spectrum of their possible applications turned the spotlight of electrochemical research towards the design of electrochemical devices for applications in sensing, energy conversion and storage, molecular electronics etc. So that possible electrode modification are mentioned in this work[24].

1. 2. 2 .1 Micro and Nanoparticles Modified Electrodes

The miniaturisation of electrodes from the macro scale to the micro scale brings many advantages. Consequently, microelectrodes, that are electrodes with at least one dimension of the order of microns, have become a standard tool for both experimental and theoretical electrochemists. Their small geometric area leads to very small currents being drawn at microelectrodes, this decreases ohmic drop as well as renders the counter electrode required when using macro electrodes unnecessary[25, 26].

Furthermore, decreasing the electrode size to nano enhances the rate of mass transport, enabling the study of fast electrode kinetics. All of these effects are exacerbated as electrodes shrink further from the micro scale to the nanoscale, although the nanoscale brings additional complications such as the requirement that migration be considered as the diffusion layer thickness approaches the Debye length. Arrays of microelectrodes have also become common in electroanalysis, as the electrodes modified with nanotubes or nanoparticles and other porous materials due to their showing different behaviour to an isolated electrode[26].

1. 2.2 .1.1 Microelectrodes

The development of ultra small electrodes offers numerous advantages to electrochemistry and analytical chemistry. Electrochemical testing can further be improved by utilizing microelectrodes in place of traditional electrodes. Microelectrodes are defined to be electrodes that have micrometer (μm) dimension; in contrast, traditional electrodes are on the millimetre (mm) scale or larger[27]. Miniaturization of electrodes offers many practical and fundamental advantages, namely, reduction of resistance (ohmic drop), reduction of sample consumption, ability to incorporate many electrodes in a small area, and greater ability to facilitate measurements in low-ionic-strength water samples. The most advantage to using this small sized electrode is in the mass transport uniqueness from the nonlinear diffusion properties accompanying it and this advantage is further amplified when multiple electrodes are utilized in an array. Nonlinear diffusion happens at the boundary of the electrode and from the increased perimeter-to-surface area ratio exhibited by microelectrodes, with respect to larger traditional electrodes, current amplification is accomplished[27].

The application of small-size electrodes was further enhanced by increasing demands from analytical chemistry (e.g. the need for electrodes in miniature cells in detection for high-performance separations or in electrochemical sensors). The term ultra microelectrode is also often used in the literature, but to maintain the terminology consistent, it is preferable to stick to the more logical term, microelectrode. It is now conventionally assumed that a microelectrode has dimensions of tens of micrometers or less down to sub micrometer range.

Microelectrodes of various geometries have been prepared. However This work length cannot systematically cover all practical applications of microelectrodes[27].

1. 2.2 .1.2 Nanoelectrodes

The past many years have seen tremendous growth and increased application of nano electrodes in fundamental electrochemistry, electrochemical analysis, electro catalysis, and many other research areas. Nano electrodes typically refer to voltammetric electrodes with at least one dimension below 100 nm. Most of the outstanding properties of nano

electrodes, such as a small *RC* time constant and fast mass transfer, have been demonstrated on nano electrodes and are, in many cases, more pronounced compared to microelectrodes. Nano electrodes and nano electrode arrays are the natural next step in electrode miniaturization after microelectrodes and their arrays[28]. In recent years significant attention is paid to the use of nanoparticles in many areas of electrochemistry. Underlying this endeavour is an expectation that the changed morphology and electronic structure between the macro and nanoscale could lead to usefully altered electrode reactions and mechanisms. Thus, the use of nanoparticles in electroanalysis became an area of research which is continually expanding now days. Within both the trend towards the miniaturisation of electrodes and the ever increasing progress in preparation and using nanomaterials, a profound development in electroanalysis has been connected with the design and characterisation of electrodes which have at least one dimension on the nanoscale[28].

In a nanostructured electrode, a larger portion of atoms is located at the electrode surface as compared to a planar electrode (2D). Nanoparticles modified electrodes possess various advantages over macro electrodes when used for electroanalysis, e.g. such as electro catalysis, higher effective surface area, enhancement of mass transport and control over electrode microenvironment as discussed from the beginning. Overviews of our investigations were carried out in the field of nanoparticles in electroanalytical chemistry.

From the works detailed in this paper, it is clear that metallic nanoparticles modified electrode have much to afford in electroanalysis due to the unique properties of nano particulate materials (e.g. enhanced mass transport, high surface area, improved signal-to-noise ratio)[29]. The unique properties of nano particulate materials can be exploited to enhance the response of electroanalytical techniques[29]. However, according to many authors, at present, much of the work is empirical in nature. Belding and co-workers have compared the behaviour of nanoparticles-modified electrodes with that of conventional unmodified macro electrodes. Here, a conclusion has been made that the voltammetric response from a nanoparticles-modified electrode is substantially different from that expected from a macro electrode[30].

1. 2. 3 Diffusion toward Microelectrodes

As the electrode size decreases the curvature of the diffusion layer increases. Diffusion at microelectrodes is termed convergent diffusion and is generally characterised by voltammetery in which limiting current is observed. Total diffusion limited current is composed of the planar flux (2D) and radial flux diffusion (3D) components.

$$I_{\mathbf{radial}} = i_{\mathbf{planar}} + i_{\mathbf{radial}} \quad (1).$$

For disk, spherical and hemispherical geometry of the electrode, the general expression for the radial component is given by the equation;

$$I_{radial} = arnFdC \quad (2).$$

Where 'r'is electrodes radius and'a' is functions of electrodes geometries. For disk, sphere and hemisphere the' a' values are equal to 4, 4π and 2 π respectively. Such radial diffusion leads to a larger flux at the perimeter of the electrode than at the centre and hence to form a non-uniform current density[31].

Simulation of voltammetric and Chronoamperometric experiments necessitates the solution of Fick's laws of diffusion.

This is most commonly performed numerically using the finite difference or finite element method; however there have been efforts recently to perform simulations using a stochastic approach. Cutress *et al* have modelled diffusion using individual molecules which move in a random walk and are oxidised or reduced when they impact the electrode surface[32]. Using this approach it has been possible to simulate both Chronoamperometric and cyclic Voltammetery. While this method inevitably results in noisy results, Fickian diffusion is only observable for very large numbers of diffusing molecules and so a stochastic model must be used in order to model systems involving very low analyte concentration[32].

1. 2. 4 Electrodes Geometry

Cottrell equation, derived for a planar electrode, can be applied to electrodes of other simple geometries, provided that the temporal and spatial conditions are such that the semi-infinite diffusion to the surface of the electrode is approximately planar. However application spheres of various electrode geometries are applied depending on the problem or task to be solved[33]. Most electrodes are impaired by an edge effect of some sort and therefore do not exhibit uniform accessibility towards diffusing solutes. Only the well defined electrode geometry allows the data collected at the working electrode to be reliably interpreted. The diffusion limited phenomena at a wide variety of different electrode geometries have been frequently studied by several research teams[33]. The electrochemical study of nanoelectrodes and nanoelectrodes arrays is important from a characterization as well as possible application perspective. Around the time that interest in fabrication and evaluation of nanoscale electrodes started, around a decade and a half ago according to some report, imaging technology was not capable of allowing the experimenter to see the nanoelectrodes which had been prepared. It was not possible to see the tips of such electrodes using SEM, thus the electrochemical response was used to infer the dimensions of the surface[34].

Due to the small size, the nanoelectrodes, fast (3D) diffusion field and produce steady state voltammogram (i.e. sigmoid shape).

This voltammogram shape is independent of the nanoelectrodes geometry. Generally the nanoelectrodes critical parameter, radius of the disc for example, is extracted by applying a suitable model for the steady-state current[34]. Fig four illustrated the possible diffusion modes and the corresponding steady-state current (limiting current) equations for hemisphere, inlaid disc and recessed disc electrodes. However, only recent advances in nanofabrication techniques and methodology have enabled the controlled fabrication of such devices.

Some reports have pointed out four main regimes of behaviour affecting diffusion transport to microelectrode arrays [28]. According to the relationship between size of the

individual diffusion layer thicknesses δ, microelectrode size described by their radius r, and the inter centre distance between microelectrodes, d and with increasing diffusion layer thickness. These four regimes are: (i) planar diffusion to each microelectrode as δ<<r, (ii) radial diffusion to each microelectrode as δ = r <d, (iii) a transition zone when r < δ = < d and (iv) planar diffusion to the microelectrode array when δ >>> d. Regime-ii is the optimum one and it corresponds to the case when the response of the whole array is equivalent to that of a single micro disk, times the number of microelectrodes integrating the array according to optimized literature[28].

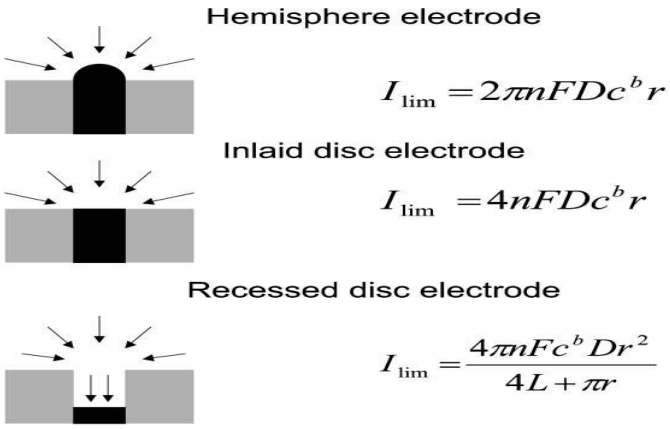


Figure 1 Nanoelectrodes geometries: hemispherical electrode, inlaid disc electrode and recessed disc electrode; diffusion modes to these and the equations for the corresponding steady-state currents[28].

1. 2. 5 Nanoelectrodes Fabrication

Nanoelectrochemistry is a branch of electrochemistry that investigates electrical and electrochemical properties of materials at the nanometre size regime. Nano electrochemistry plays significant role in the fabrication of various sensors, and devices for detecting molecules at very low concentrations[35]. NPs can be synthesized through various chemical, physical, electrochemical, etc. Some of the fabrication processes involved, electrodeposition which offers advantages over competing techniques, in that it requires simpler instrumentation and operating conditions[35].

Furthermore, electrodeposition holds great promise to deposit metals nanoparticles. In advances, in this paper we fabricated nanoscale structures and devices by means of self-assembly monolayer. These techniques, which are collectively referred to as top-down nanofabrication.

1. 2.5.1 Electrochemical Deposition of Metal NPs

Electrode position of metals is a much interested approach with great abilities in electro synthesis of metallic nanoparticles[36]. Metal nano particles (NPS) have been attracting huge attenuation during this decade as discussed above because of their unique properties. In the field of electrochemistry, particularly in electrolysis, metal nanoparticles have been actively utilized as functional nanoparticles. There were some earlier reviews deals with the utilizations gold NPS[36]. Conducting base nano materials for farther modification (such as immune sensing and DNA labelling), the modification of the metal NPS on conducting materials, which means electrode surface, is apparently effective in electrochemical analysis. Therefore some review stressed the application of metal nanoparticles to modify electrode surface have been a main focus in recent works[37], and as well in our work then.

Electrochemical deposition would be a power full method to prepare metal NPS modified electrode. Because of the nucleation and growth of metal NPS occur directly on conducting surface. During the electrolysis the attachment of metal nano particles should be strong enough and the conducting communication between the base conductors - metal NPS should quite smooth[37].

However, the electrolysis reaction does not tend to proceed uniformly, so that careful control of the electrolysis condition would be necessary for preparing metal NPS having size uniformly and controlled density[37]. Electrodeposition can be performed using several techniques, the most commonly employed methods are galvanostatic electrodeposition and potentiostatically electrodeposition. Galvan static electrodeposition involves the flow of a constant current at the working electrode; the potential is varied in order to keep the current constant.

When the deposition is potentiostatically controlled, a constant potential is applied to the working electrode and the current is measured as a function of time. The current passed during an electrochemical deposition can be related to the charge (Q) passed according to Faradays' first lows of equation; $Q = it$ where i is current (in ampere) and t is time (in second)[37].

1.2.5 .1.1 Sequential Deposition of Gold Nanoparticles

Noble metal nanoparticles such as platinum, gold and silver, are especially important because of their excellent electrical and optical properties[38]. In particular, gold metal nanoparticles could be electrochemically deposited via the sequential deposition technique as depicted in figure 2. There are a number of approaches have been developed to prepare AuNPs using different ligands. The synthesis of stable AuNPs, particularly with tunable size and controllable morphology to exploit the excellent size-dependent electronic and optical properties of AuNPs in the fields of electronics and nonlinear optics, the NPs must attach to solid supports. Several methodologies have been used to attach the AuNPs on different substrates, for example the sequential electrodeposition AuNPs[39]. Gold nanoparticles were electrochemically deposited by reduction of the $KAuCl_4$ solution at the electrode surface according to optimized literature[39].

As it was previously reported by Soreta *et al*[39] the sequential deposition approach can be used to increase the particle number density of electro nucleated gold nano particles. In order to control the growth of the gold NP and ensure that new NP are formed during each nucleation round, the fresh gold NP should be insulated with a capping agent(ME).

In this thesis work, a voltammetric sensor for caffeine determination was fabricated based on nanohole modified glassy carbon electrode. The sequential deposition approach developed by Soreta *et al.*[39] were employed in the electrode surface modification procedure.

2 Literature Review

2.1 Caffeine Chemistry and General Information

Caffeine (1,3,7-trimethyxanthine), theophylline (3,7-dimethylxanthine), and theobromine (1, 3-dimethylxanthine) are in the family of alkaloid methylxanthines[41].

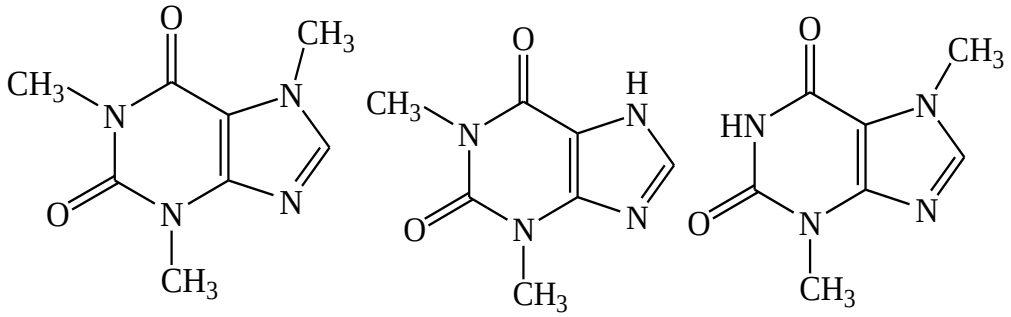


Figure 2 Structure of methylxanthines

Caffeine is an odourless, white solid that has powder form and it is slightly soluble in water due to its moderate polarity. Caffeine is a natural central nervous system stimulant, having the effects of reducing drowsiness and recovering alertness. Since it is widely consumed by humans, caffeine is considered to be the most frequently used psychoactive substance in the world[41, 42].

2 .2 Methods of Caffeine Analysis

There are various methods for determination of caffeine[42, 43]. Some of them are ultraviolet (UV) spectrophotometery, high performance liquid chromatography (HPLC), gravimetric and titrimetric methods. It is not possible to determine caffeine directly in coffee beans by the conventional analytical methods due to the spectral overlap[44,45], so that the derivative analytical methods are relatively needed. Some methodology studies that had involved in the analysis of caffeine in food products were described below[46].

Li *et al*[47] applied UV spectrophotometery a method for the individual determination of caffeine and theobromine in coffee beans . Another methods of caffeine determination were high performance liquid chromatography with UV–spectrophotometeric detection method[48]. Nishitani and Sagesaka[48] developed an improved HPLC analytical method for simulta**n**eously determining caffeine and other catechins as well as other phenolic compounds in coffee. The proposed method provided additional ability to analyze phenolic compounds when compared with former HPLC methods.

Caudle *et al*[49] tried to improve the Association of Official Analytical Chemists (AOAC) official analytical method for analyzing methylxanthines in the coffee. The AOAC method's degree of accuracy and precision was not reliable, especially for caffeine[49]. In this study, the AOAC analytical method only showed recoveries of theobromine and caffeine to be 89.3 and 74.5%, respectively. But Caudle[49] and others successfully changed from an organic extraction to an aqueous extraction and analyzed the samples via reverse-phase HPLC to improve the recoveries of theobromine and caffeine to be 99.6 and 103.4%, respectively. However; this procedure involves relatively long retention times or requires lengthy sample pretreatments.

Other methods such as capillary electrophoresis and thin layer chromatography were used for separation of caffeine in the analysis of mixtures, combined with several detection methods like, Nuclear magnetic resonance (NMR) spectroscopy, mass spectrometry and infrared spectrophotometer[50]. Chen *et al*[51] investigated the feasibility of using near infrared (NIR) spectroscopy as a fast method which is non-destructive and less time consuming than other frequently used analytical methods for estimating the content of caffeine and total polyphenols in green coffee. An amperometric based microbial biosensor has been described for the analysis of caffeine in beverages and fermentation samples by using immobilized whole cells and cell fragment of sonicated cells of the isolate[52]. The approach involved a selection strategy for caffeine degrading bacteria that is capable of utilizing caffeine as the only source of carbon and nitrogen from soils and it is an induction of caffeine degrading capacity in the bacteria. In another study, the *in vitro* selection strategy and the construction of allosteric ribosome sensor has been indicated for the determination of caffeine in solutions[52].

However; all the methods mentioned above have their own disadvantages, often require time-consuming sample preparation procedures such as liquid–liquid extraction, solid-phase extraction or the use of more than one chromatographic step[53], and very costly instrumentation and needs high skilled technician and more complicated than electro analytical methods[54].

This opens opportunities for electrochemical methods to be employed for caffeine analysis. However; only a few papers dealing with an electroanalysis of caffeine at more common electrode materials had appeared[55]. This is because the oxidation of caffeine occurs at a very high positive potential, and may overlap with electrochemical reactions limiting potential window from the anodic side. This often gives low reproducible analysis[55-57].To avoid the overlapping of the oxidation peak of caffeine with the electrolyte, several types of electrodes have been examined using electrochemical techniques. From these electrodes carbon-fibber microelectrodes[58], modified electrodes[59], micro array electrodes[60], screen-printed electrodes[61] , carbon paste electrodes[62-64], and graphite pencil electrodes (GPE) have been tested for the determination of caffeine in different materials. However the application of such common solid electrodes, limited by the useful potential window where the background current due to the oxidation of the solvent or supporting electrolyte is below certain minimum value[65, 66].

For the electrochemical determination of caffeine, nafion ruthenium oxide pyrochlore chemically modified electrodes have been used according to Eder *et al*[67]. Indirect voltammetric method was used for the determination of caffeine in coffee using 1, 4 benzoquinone modified carbon paste electrode was reported by Aklilu *et al*[68]. Carbon paste electrodes represent one of the most types of working electrode[62]. Carbon paste electrodes have many advantages such as easily renewable surface, low cost, and have very low background currents especially in the anodic region as reported by Svancara et al[69]. One of the disadvantages of carbon paste electrodes is the tendency of organic binder to dissolve in solutions containing organic solvents.

Graphite pencil lead electrode as working electrode has been described for the voltammetric determination of caffeine[70]. However, different model of the pencil lead had shown different background current levels which is attributed to difference in composition and roughness of the pencil leads. Hence the selection of the best pencil lead model is a problem associated with commercial pencil leads[71]. Spatarua *et al*[72] improved the separation of caffeine peak by employing a boron doped diamond (BDD) electrode, which has wider potential window on the anodic side than the common carbon paste electrode (CPE) or the glassy carbon electrodes.

In another study, a method that has been described for the detection of caffeine using glassy carbon electrode by differential pulse voltammetry rendered difficult for quantitative determination due to the volatile methanol from the per-choleric acid–methanol (1:1) mixture that was used both as a solvent and supporting electrolyte in order to improve the sensitivities and peak separations[73]. A differential pulse Voltammetric method based on a nafion covered glassy carbon electrode has been tested for determination of caffeine in cola beverages, but it had shown poor detection limit of 0.798 μM[74]. GCE modified with a lead-ruthenium pyrochlore oxide in a nafion matrix has also been used for caffeine determination by square wave voltammetry with a detection limit of 2 μM[75], while Anil Kiran[76] used a molecularly imprinted polymer as sensor for detecting caffeine.

Polymer-modified electrodes (PMEs) have received considerable attention in recent years due to their good stability, reproducibility, increased active sites, homogeneity in electrochemical deposition and strong adherence to the electrode surface[77]. However, among the electroanalytical methods recently reported for the determination of caffeine , a single work was published based on electro polymerized polymer-modified electrode[78] which could be because of the high interfering background current at its oxidative potential[79]. It could be concluded that, compared to a conventional electrode, the modified electrodes exhibited a significant enhancement of sensitivity and low detection limit. However; these various surface modifications are still not sufficiently for enhancing electrode sensitivity to minimizing oxidation of caffeine.

Nanotechnology is a rapidly evolving field that constantly requires new combinations of techniques and methods that can aid in resolving challenging chemical issues. Nanoparticles modified surface have proved to be an excellent tool for the preparation of nano-structured materials and which received considerable attention in electroanalytical applications. Nanomateriails have been widely used in analytical chemistry as chemical sensor materials. Therefore electroanalytical application of such nanomaterials has been found to be quit extensive which clearly indicate that a large scope for further study in the area of electrochemistry. So that, electrochemical methods for determination of caffeine rely on the availability of electrode materials that have the ability to detect compounds that are difficult to oxidize[78, 79].

Hence, we planned to develop nanohole-modified glassy electrode that lowers the oxidation potential of caffeine for its determination without a significant influence from background current.

Even though many electrochemical methods have been used surface modified electrodes for the detection of caffeine, no investigation has been reported on the electroanalysis of caffeine by nanhole modified electrodes glassy carbon electrode.

Generally, the main reason to modify an electrode is to obtain a new sensor with desired properties that can impart higher selectivity, sensitivity, or lower detection limit to electroanalytical determination caffeine particularly in food and clinical chemistry.

2.3 Statement of the Problems

The electrochemical determinations of caffeine at the more common electrode materials are generally not feasible because the oxidation of caffeine occurs at a very positive potential, thus overlapping with the discharge of the background medium. This is why there have been numerous studies aiming to develop reliable methods for determination of caffeine. Recently randomly nanoarrayed electrodes have been widely used in electrochemistry as chemical sensor. So that present work is directed to a nanoarrayed electrodes fabrication for selective the determination of caffeine by increasing electrode sensitivity, thereby minimizing over oxidation potentials of caffeine[80]. The present work can answer the following questions;

- ❖ What electrode nano modification strategy can be used to lower oxidation potential of caffeine?
- ❖ How electrochemical selective detection of caffeine is possible?

2.4. Hypothesis

Incontrast to bulk electrode, nanostructured electrode have incredible potential as electrochemical sensor exhibiting enhanced performance. These differences are caused by changing conditions of the mass transport from the bulk of solution toward the electrode.

- ✓ Nanohole modified glassy carbon electrode can minimize over oxidation potentials of caffeine by increasing mass transport of caffeine toward the surface of the electrode.

2.5. Objectives of the Study

2. 5 .1. General Objective

- ❖ To develop highly sensitive, less expensive and fast electrochemical sensor for the determination of caffeine in real sample (coffee).

2. 5 .2 Specific Objectives

- ✓ To modify GCE with random nanohole arrays.
- ✓ To investigate the electrochemical behaviour of caffeine at nanohole modified glassy carbon electrode by cyclic voltammeter.
- ✓ To study the influence of supporting electrolytes, pH, concentration, scans rate, and interference on current response of Au /PNA/GCE form the oxidation behaviour of caffeine.
- ✓ To study the reproducibility and stability of randomly fabricated nanoarrayed electrode.
- ✓ To apply nanohole modified electrode to the determination of caffeine in the coffee.
- ✓ To compare the sensitivity and detection limit of cyclic voltammetric determination of caffeine using nanohole modified GCE with that of bare GCE.

3. Materials and Methods

3 .1 Chemicals and Reagents

Caffeine reagent ($C_8N_4O_2H_{10}$, anhydrous powder 99%, Aldrich), theophylline ($C_8N_4O_2H_8$, hydrous powder 99%, Aldrich), theobromine ($C_8N_4O_2H_8$, hydrous powder, 99%, Aldrich), potassiumteterachloroaurate ($KAuCl_4$, 99.995%, Aldrich), were purchased from sigma Aldrich (all are Germany).

Para-nitro aniline ($C_6H_6N_2O_2$, 99%, Kiran), sodium nitrite ($NaNO_2$, 96%, Nice), hydrochloric acid (HCl, 37%, Riedel dehaen), hexamine ruthenium chloride ($Ru(NH_3)_6Cl_3$, 98%, Aldrich), sulphuric acid (H_2SO_4, 98%, Merck), hydroquinone, ($C_6H_6O_2$, 99%, Kiran), 2-mercaptoethanol ($HSCH_2CH_2OH$, 99%, Aldrich), sodium per chlorate ($NaClO_4$, 98%+ , Sigma), potassium nitrate (KNO_3, 99%, NICE), potassium chloride (KCl, 995, Finkem), potassium hexacyanoferrate ($K_3Fe(CN)_6$, 97%, Labmerk) chloroform ($CHCl_3$), calcium carbonate ($CaCO_3$, 99% NICE), sodium sulphate (Na_2SO_4, 99%, Finkem) and Sodium hydroxide (NaOH, 99.3%, Eines) were used. Distilled water was used to prepare all solutions. All chemicals were analytical grade and used without further purification.

3.2 Experimental Procedures

3.2.1. Electrochemical Instrument

Cyclic Voltammetery and Amperometric measurement were performed using Epsilon electrochemical analyzer, (Bioanalytical Systems, Inc. USA model), containing three electrode system Ag/AgCl as reference electrode, platinum as counter electrode and glassy carbon electrode (3 mm diameter) as working electrode.

3.2.2 pH Measurement

The pH measurements has been done with the pH meter (K06043, Hanna model) and calibrated with the standard buffer solution for each activities (buffers powder of pH 2, 4, 9.2, Nice)

3.2.3 Electrode Pre-treatment

Prior to modification, GC electrode (3 mm diameter) was polished with a polishing paper and micro cloth (BAS, Bioanalytical Systems USA) and then further it was polished to the mirror finishing with alumina slurries of 3 μm (BAS, USA) and rinsed thoroughly with distilled water. The electrode was then conditioned to diminish background current due to oxidation of the electrode. It was used immediately following the cleaning and conditioning steps to deposit nanoparticles based on literature[39].

3.2. 4 Surface Modification of the Electrode

Fabrication of the electrode has been done in three-steps (scheme 1)[39]. These were; (i) electrochemical deposition of gold nanoparticles (ii) Grafting of p-nitroaniline film and (iii) Stripping of gold nanoparticles.

(i) Electrochemical Deposition of Gold Nanoparticles

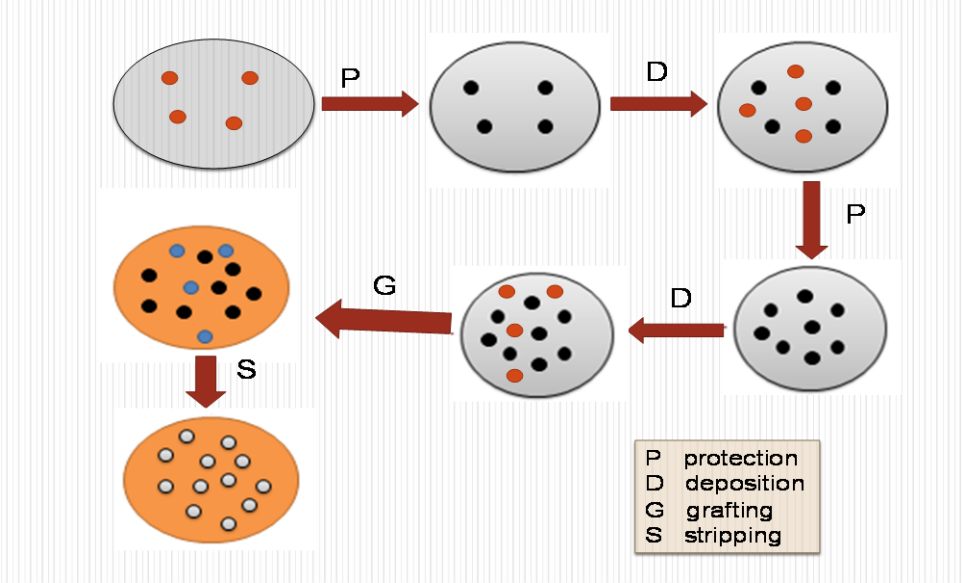
Gold nanoparticles were electrodeposited from solution of 0.1 mmol L^{-1} $KAuCl_4$ in 0.5 mol L^{-1} H_2SO_4 using chronoamperometry by applying 0 V stepped to 1.1 V for 5s[39]. In each deposition step SAM of ME was used, by maintaining AuNPs deposited electrode upside down in the tip of micro pipette for 2 hr at room temperature.

(ii) Grafting of p-nitroaniline Film

p-nitroaniline film was grafted on GC electrode from 3 mmol L^{-1} of p-nitroaniline diazonium salt solution that was prepared according to literature[81, 82]. Briefly solution of 3 mmol L^{-1} of p-nitroaniline and 0.1 mol L^{-1} sodium nitrite in 0.5 mol $L^{-1}$of hydrochloric acid were kept separately in an ice jacketed beaker for 1 hr. Then 400 μL of 0.1 mol L^{-1} $NaNO_2$ was added to 20 mL of 3 mmol L^{-1} paranitoaniline (PNA) under stirring at room temperature and then CV was used to graft the film on bare GCE and AuNPs deposited GC electrode from 0.6 to -0.2 V for 3 cycles at scan rate of 100 mVs^{-1}.

(iii) Stripping of Gold Nanoparticles

Gold nanoparticles was stripped from P-nitroaniline grafted AuNPs deposited GC electrode using CV from 0 mV to 1400 mV for eight cyclic scans at a scan rate of $0.1Vs^{-1}$ in 0.1 mol L^{-1} KCl solution[39].



Scheme 1 Nanoarrayed electrode fabrication steps

3. 2. 5 Preparation Caffeine Solutions

Standard stock solution of caffeine (2.0×10^{-2} mol L^{-1}) was prepared in deionised water and diluted with in 0.01mol L^{-1} H_2SO_4 and stored at 4 °C. The pH of the solution was adjusted either by 0.1 mol L^{-1} H_2SO_4 or NaOH[83].

3.2.5.1 Caffeine Extraction Using Sulphuric Acid

Caffeine was extracted from raw coffee by taking 20 g coffee bean 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. Four gram of the powder was added into 150 mL beaker, and then dissolved in 100 mL of 6 mol L^{-1} H_2SO_4[84].

The mixture was diluted with 50 mL of 0.1mol L^{-1} H_2SO_4 to the mark after 4 min, 5 min, and 10 min acid digestion and filtered, and then ready for analysis by adjusting the pH of the solution to 1.92 by 0.1mol L^{-1} NaOH. Following caffeine also extracted from roasted coffee in the same manner.

Caffeine was also extracted from brewed coffee by dissolving four grams portion of roasted grounded coffee in 100 mL of boiling water. The brewed coffee was immediately cooled to room temperature in an ice bath, after which sample were stored at 4^0C, and then it was diluted to 50 mL with 0.1 mol L^{-1} H_2S0_4 (pH 1.92) and filtered through a dry filter paper for analysis[85]. In all cases, working standards of caffeine 50, 150, 200, 250, 300 and 350 µL was successive ejected in to 5 mL of each sample solution for the determination of caffeine

3.2.5.2 Caffeine Extraction Using Chloroform

Four grams of ground 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[86]. The residue was allowed to cool and settle down, and then the solution was filtered. 100 mL of coffee extract solution was mixed with two grams of sodium carbonate. These reacts with some of the substances in the coffee extract and make them precipitate and after decantation transferred to empty Erlenmeyer flask and then 35 mL of chloroform was added to it . The mixture was vigorously swirled for 5 minutes and the chloroform water mixture was filtered. Chloroform solution of caffeine remaining on the filter paper was transferred in to 50 mL Erlenmeyer flask. To this solution 0.25 g of anhydrous sodium sulphate was added in order to remove the last traces of water[87]. Chloroform was removed to dryness under reduced pressure (rotary vapour) at temperature of 40^0c. The recovered mass was dissolved in 0.1mol L^{-1} H_2S0_4. Working standards of caffeine 50, 150, 200, 250, 300 and 350 µL was successive ejected in to 5 mL of sample solution for the determination of caffeine [87.]

Determination of Melting Point of the Isolated Caffeine

0.0014 g of residue of caffeine was added in to a melting point tube[87]. Melting point was measured by pressing the open end of the tube down on the caffeine, turning the tub right-side-up and tapping the tube on the bench until the solid falls to the bottom of the tube.

3.2.5.3 UV/Vis Spectrophotometeric Determination of Caffeine

An electrochemical method analysis of caffeine was compared with UV/Vis Spectrophotometery (T80PG instrument, CTD). For this purpose 1000 ppm caffeine was prepared by dissolving 200 mg of caffeine standard in 200 mL of distilled water. Then 10.0 mL of 0.01 mol L^{-1} hydrochloric acid and 2 mL 0.01mol L^{-1} basic lead acetate solution was added before topping to the mark. Working standards was prepared by pipetting 10, 20, 30, 40, 50 and 100 µL aliquots of the stock standard solution in to 1mLof sample solution[88].

Caffeine was extracted for UV/Vis analysis based on procedure described in literature[88]. Briefly, four gram of raw coffee was accurately weighed and dissolved in boiled water and made to the net volume of 20 mL with distilled water as sample solution. 20 mL this sample solution were pipetted to 250 mL flask and then 10 mL of 0.01 mol L^{-1} hydrochloric acid and 2 mL of 0.01 mol L^{-1} basic lead acetate solution were added . Finally the solution was made to mark with distilled water and filtered to clarify. 50 mL filtered solution was again added to 100 mL flask, and then 0.2 mL of 4.5 mol L^{-1} sulphuric acid was added. Finally the solution was made to mark with distilled water.

4. Results and Discussion

4. 1 Cyclic Voltammetry of $KAuCl_4$

Based on the literature[39] , gold nanoparticles were deposited from 0.1 mmol L^{-1} $KAuCl_4$ in 0.5 mol L^{-1} aqueous H_2SO_4. As shown in Figure 3 AuNPs was oxidized at 1.2 V indicated pure deposition AuNPs. The cathodic peak observed around 0.6 V showed the reduction of AuNPs. In addition, deposition of AuNPs were checked by running CV of electrode only in supporting electrolyte after depositing gold on GCE. The appearance of sharp cathodic peak at 0.955V (Inset), is an indication of deposited AuNPs and shows good agreement with previous report[39].

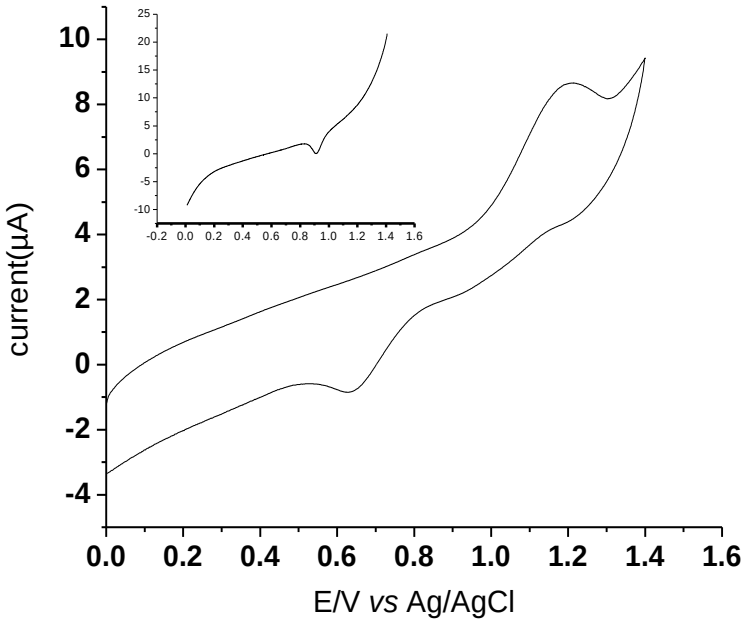


Figure 3 Cyclic voltammogram of 0.1 mmol L^- $KAuCl_4$ in 0.5mol L^{-1} H_2SO_4 at bare GC electrode. Inset, reduction of a gold oxide layer at scan rate100 mVs^{-1}.

4.2 Fabrication of Nanostructured Electrode

4.2.1 Chronoamperometric Deposition of Gold nanoparticles

Next to cyclic voltammetry, gold nanoparticles was electrodeposited using chronoamperometry by applying 0 V stepped to 1.1 V for 5s from solution 0.1 mmol $L^{-1}KAuCl_4$ in 0.5 mol L^{-1} H_2SO_4[39]. To increase number nanoparticles (avoid secondary nucleation) a sequential deposition technique using self assembling monolayer of 2-mercaptoethanol was used as described in procedure.

4.2.2 Grafting of P-nitroaniline Diazonium Film

Surface grafting of bare and AuNPs modified GC electrodes with P-nitroaniline (NPA) moiety was performed using CV for 3 cycles from - 0.2 V to 0.6V, at scan rate of 100 mVs^{-1}[89]. As can be seen from Figure 4 redox peak of the p-nitroaniline film at AuNPs deposited GC electrode, decrease in the subsequent cycles indicate monolayer coverage of p-nitroaniline films on the electrodes surface. However on bare GCE there is no significant monolayer coverage p-nitroaniline film. The film formation on the surface of the electrode was confirmed using common redox probes such as $K_3Fe(CN)_6$, $Ru(NH_3)_6Cl_3$ and hydroquinone (HQ)[89].

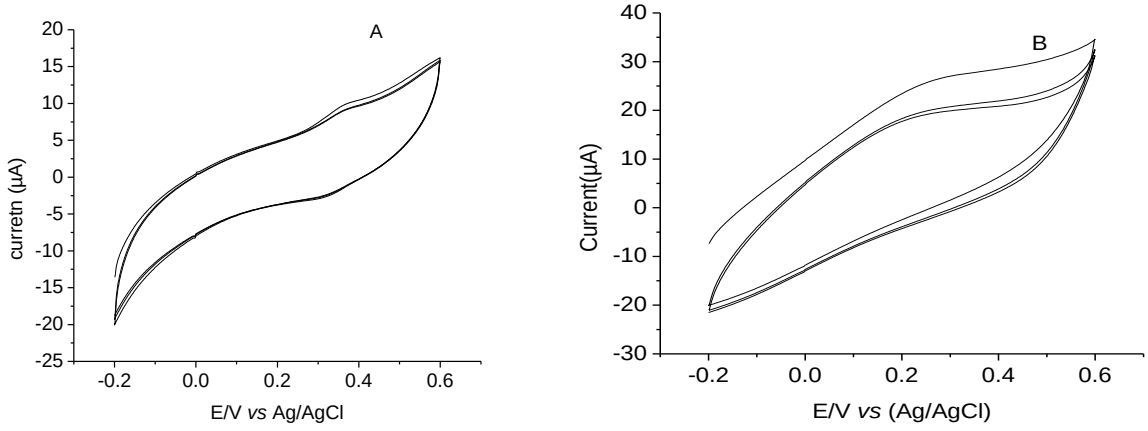


Figure 4 Cyclic voltammogram of 3 mmol L^{-1} PNA in 0.5 mol L^{-1} HCl on bare GCE (A) and nanohole/GCE (B) after addition of 0.1 mol L^{-1} $NaNO_2$ on AuNPs deposited GC (V = 100 mVs^{-1})

4.2.3 Stripping of Gold Nanoparticles

After grafting NPA film on GCE, the next step was to produce nanohole. This was achieved by stripping the nucleated AuNPs using CV from 0 mV to 1400 mV with eight cyclic scans at a scan rate of 0.1 Vs^{-1}. As shown in Figure 5 the anodic peak current observed in the first cycle around 950 mV is due to oxidation of AuNPs and its magnitude decreased in the successive cycles indicate complete removal AuNPs. The electrode fabricated following the gold nanoparticles stripping step is referred as nanohole modified electrode throughout this document[39].

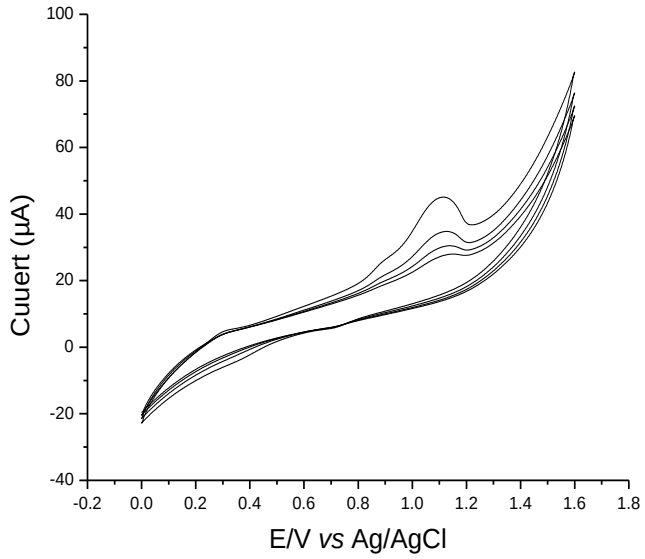


Figure 5 Cyclic voltammogram of gold nanoparticles nucleated nitro phenyl modified GC in 0.1 mol L^{-1} KCl (V= 100 mVs^{-1})

4. 3. Electrochemical Characterization the Fabricated Electrode

The prepared nanohole modified electrode was characterized using cyclic voltammetery using hydroquinone, hexamine ruthenium chloride and potassium hexacyanoferrate probes.

4. 3. 1 Cyclic Voltammogram of $K_3Fe(CN)_6$

The characterizations of nanoarrayed electrode were further investigated using cyclic Voltammetery. As shown in Figure 6 pair of well-defined redox peaks current for $K_3Fe(CN)_6$ probe were observed at the bare GCE, but after the modification of the surface with nanostructure, the anodic and cathodic current peaks were almost disappeared, showing that PNA acted as a blocking layer for electron and mass transfer that hindered the diffusion ferric cyanide toward the electrode surface. This is due to the electrostatic repulsive interaction effect between PNA and $K_3Fe(CN)_6$[90].

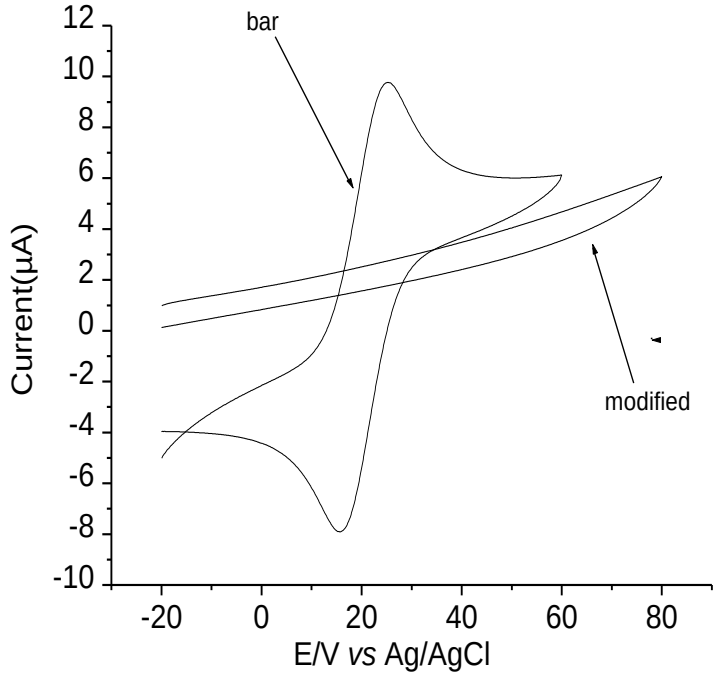


Figure 6 Cyclic voltammogram of 10 mmol L^{-1} Fe $(CN)_6^{-3}$ on bare and NP modified GCE (electrolyte 0.1 mol L^{-1} KCl (V = 50 mVs^{-1}).

4.3.2 Cyclic Voltammogram of Hexamine Ruthenium Chloride

Redox peak current of $Ru(NH_3)_6^{3+}$ at nanohole modified glassy carbon electrode was compared to at PNA grafted AuNPs deposited GCE[91]. The current response increased at nanohole modified electrode due to the electrostatic interaction attractions between negatively charged film with $Ru(NH_3)_6^{+3}$ and also increased mass transfer contribution to the electrode due to their large surface area than film grafted on gold nanoparticles modified GCE(Figure7)[91].

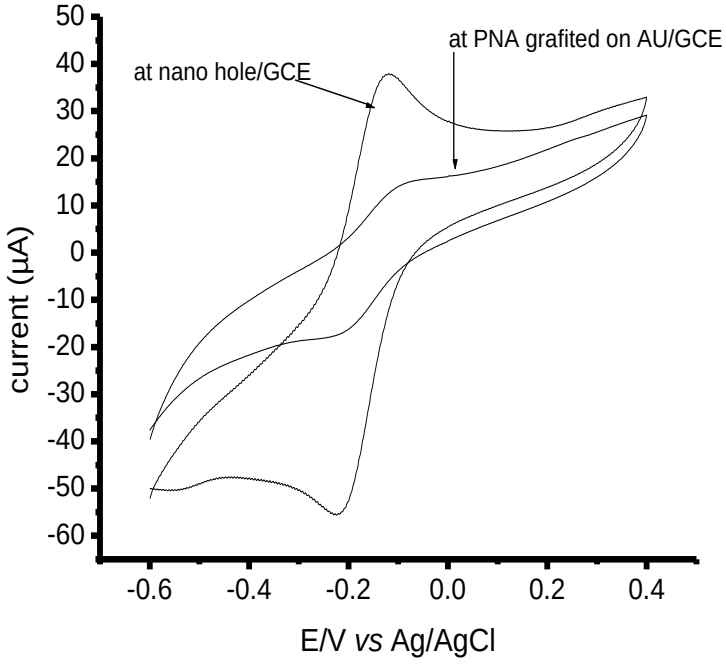


Figure 7 Cyclic voltammogram of 1 mmol L^{-1} $Ru(NH_3)_6^{+3}$ in 0.1 mol L^{-1} KNO_3 at nanohole modified /GCE and PNA grafted on AuNPs

4.3.3 Cyclic Voltammogram of Hydroquinone

The cyclic voltammogram presented in Figure 8 is the voltammetric response of HQ solution at nanohole modified GCE, bare GCE and nitro phenyl modified GCE[92].

However, the minimized redox potential separations observed, at nanohole modified glassy carbon electrode than bare and PNA modified bare electrode is due to improved enhanced mass transport[93].

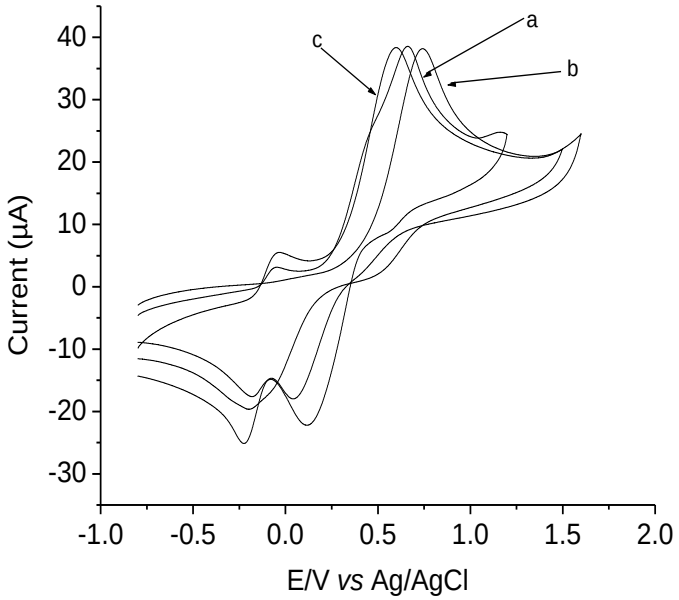


Figure 8 Cyclic voltammogram of 10 mmol L^{-1} HQ in 0.1 mol L^{-1} $NaClO_4$ on (a) nanohole modified GCE, (b) bare GCE and (c) PNA/ GCE (V = 100 mVs^{-1}).

4.4 Voltammetric Behaviour of Caffeine at Nanohole Modified GCE

Figure 9 shows cyclic voltammogram of 2.0×10^{-5} mol L^{-1} caffeine in 0.01mol L^{-1} H_2SO_4 at nanohole modified GCE (pH 1.92). From 800 mV to 1600 mV potential window no oxidation peak current of caffeine was observed at the bare GCE, but an anodic peak of

caffeine was observed around 1490 mV at nanohole/GCE, this shows the modified electrode decrease over potential oxidation of caffeine, due to enhanced mass transport of caffeine to the surface the electrode. The result shows the proposed method displayed good feasibility for determination of caffeine as compared to previous report[94].

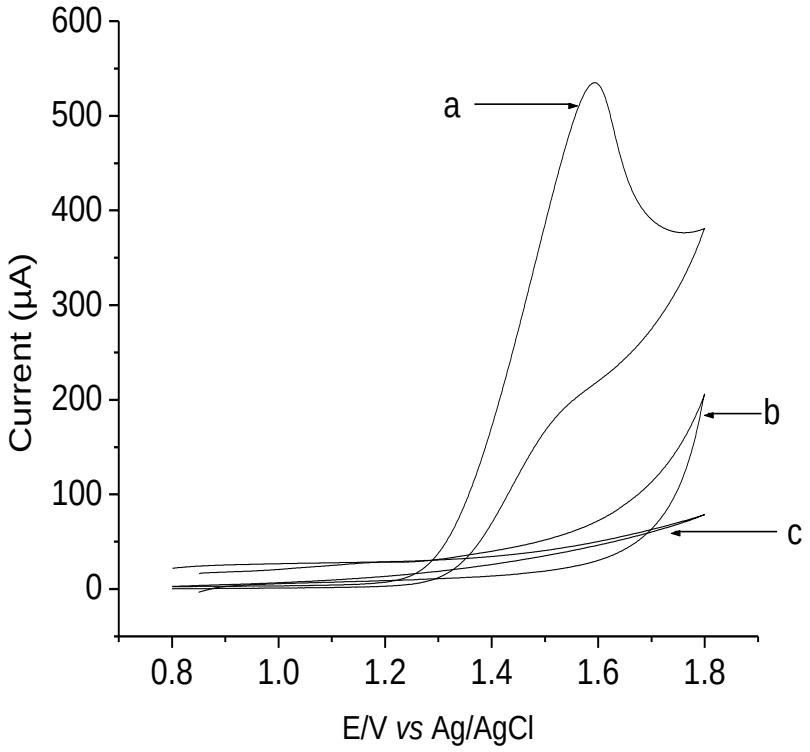
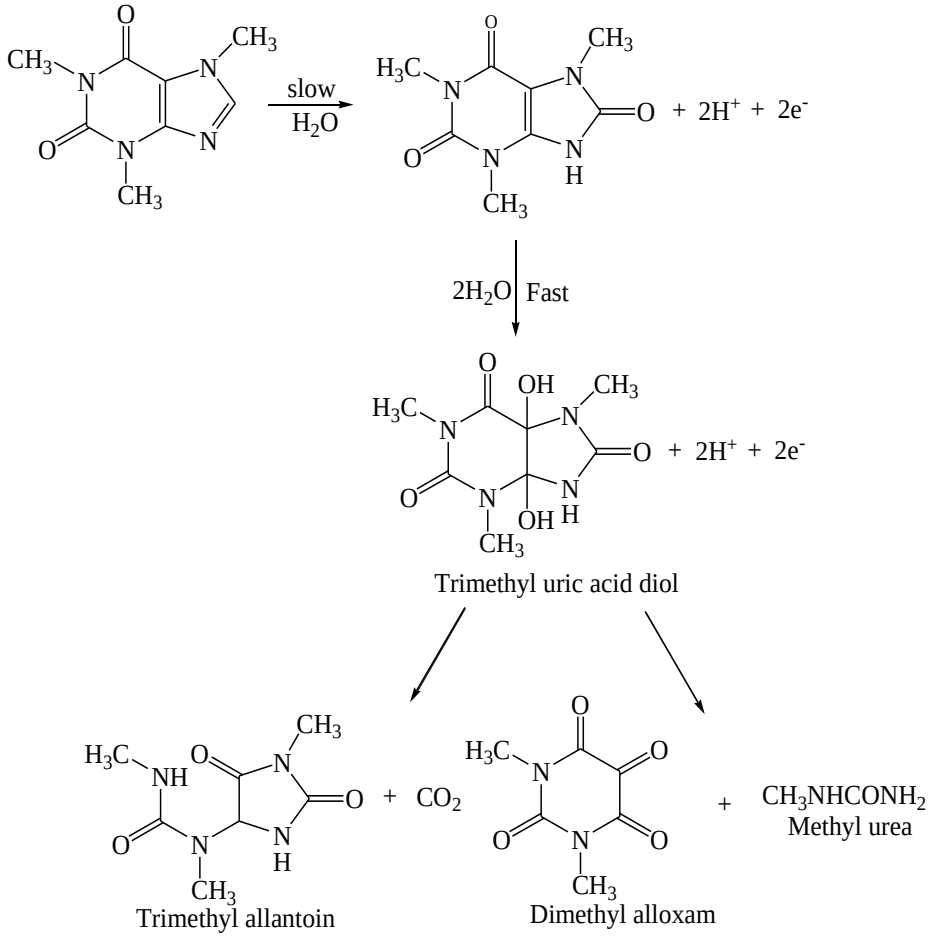


Figure 9 Cyclic voltammogram 2.0×10^{-5} mol L^{-1} Caffeine in 0.01mmol L^{-1} H_2SO_4 recorded at the nano hole /GCE (a), only in supporting electrolyte (b) and at bar GCE in the presence of caffeine (c), (all at scan rate of 50 mVs^{-1}).

Caffeine undergo irreversible electrochemical reaction[95]. The electrochemical oxidation mechanism of caffeine proceeds by an overall $4e^-$, $4H^+$ process as shown scheme 3. The first step, a 2e, $2H^+$ oxidation of the C-8 to N-9 bond to give the substituted uric acid, is followed by an immediate 2e, $2H^+$ oxidation to the 4, 5-diol analogue of uric acid, that rapidly fragments[95].



Scheme 2 Mechanism for electrochemical oxidation of caffeine

4.5 Optimization of Experimental Parameters

4. 5. 1 Effect of Supporting Electrolyte

Various types of electrolyte solutions, including acetic, sulphuric, nitric and hydrochloric acid were tested as supporting electrolytes[96]. This because electrochemical behaviour of caffeine is can be affected by supporting electrolyte solutions[96]. As shown in Figure 10 out of these electrolytes, sulphuric acid solution is the most suitable medium yielding the best peak separation for caffeine oxidation from the background currents. So, sulphuric acid solution is used as supporting electrolyte for caffeine analysis throughout this work.

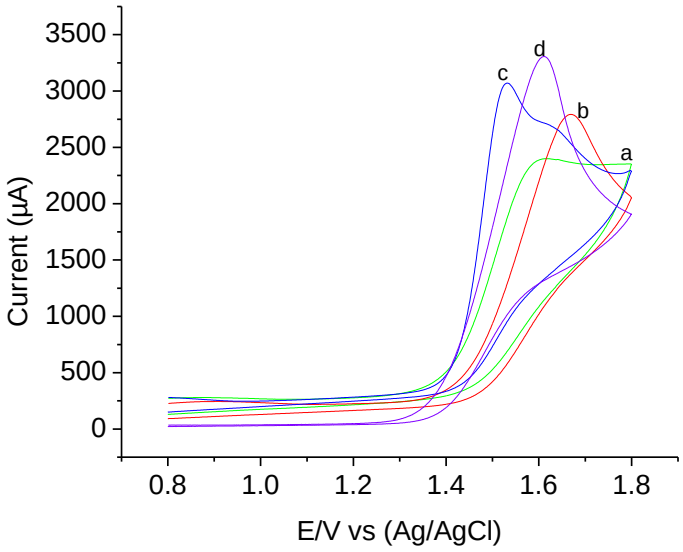


Figure 10 Cyclic voltagram of the nanohole modified/GCE in the presence of $2x10^{-5}$ mol L^{-1} caffeine in 0.01mol L^{-1} different supporting electrolytes: CH_3COO (a), HCl (b), HNO_3 (c) and H_2SO_4 (d) at scan rat of 50 mVs^{-1}

4.5. 2 Effect of pH

The effects of pH of supporting electrolyte on the oxidation peak current of caffeine were studied from pH range of 0.8 to 7.5 in 0.01mol L^{-1} H_2SO_4. As shown in Figure 11 magnitude of the peak current decreased at higher pH values and the decrease in anodic peak current is more pronounced as the pH increased further[96]. When the pH values were raised to neutral (pH 7.0), the oxidative peak current of caffeine faded away and the oxidative peak current gradually decreased. The sharpness of the peaks also decreases along with increasing the pH of the solution[96].

This observation could be due to dominant hydroxyl ions at high pH that repelled by the negative film modify the electrode surface, this minimize diffusion of the caffeine to the electrode surface and also due to the electrostatic attraction of caffeine with electrode surface since it was protonated. To remove unreacted species from its surface, electrodes

was conditioned by cycling the potential between - 0.2 and 1.2 V (10 cycles, 0.1 Vs^{-1} in the supporting electrolyte solution).

At pH 0.8 where maximum peak current was obtained with narrow potential window, however; this voltammogram suffers from poor back ground current. The one at pH 1.92 showed the next maximum peak current with wide potential window, thus used throughout the analytical determination of caffeine in this work[96].

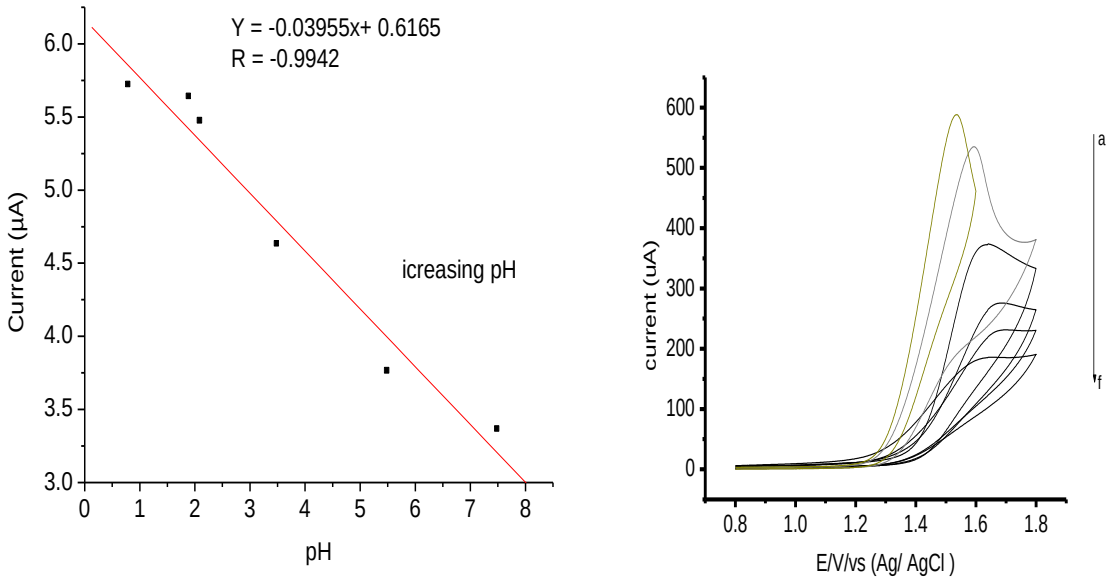


Figure 11The cyclic voltammogram of 1.0×10^{-3} mol L^{-1} caffeine at nano hole /GCE in 0.01mol L^{-1} H_2SO_4 at different pH values (a → f): 0.8, 1.9, 2.1, 3.5, 5.5 and 7.5

4. 5. 3 Effect of Concentration

The relationship between oxidation peak currents and caffeine concentrations was studied within the range of $1x10^{-7}$ to $1x10^{-3}$ mol L^{-1}. A s can be seen from Figure 12, oxidation peak current of caffeine increase with increase in concentration[97]. Regression linear equation of peak current vs concentration is i_p (µA) = 0.11 x + 0.130 (µM), and correlation coefficient R for the equation is 0.998 .

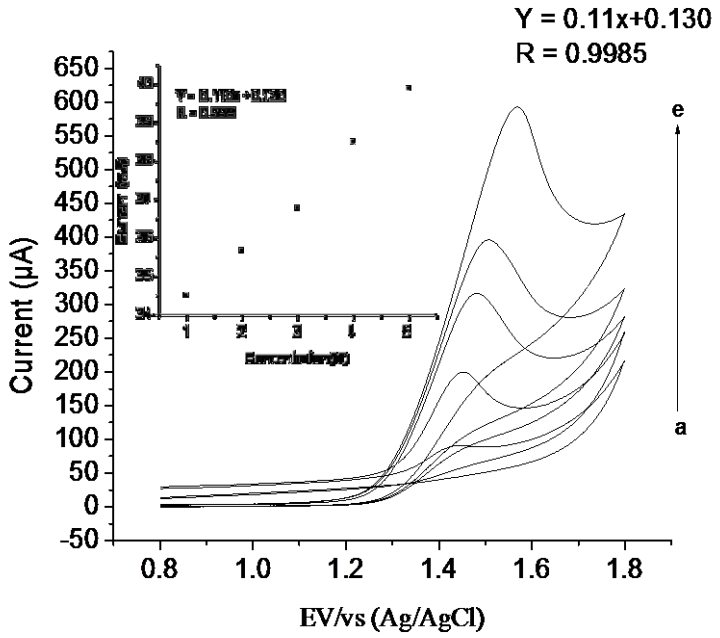


Figure 12 Cyclic voltammogram gram of different concentrations caffeine at nanohole GCE in 0.010 mol L^{-1} H_2SO_4 (a) 1×10^{-7}, (b) 1×10^{-6}, (c) 1×10^{-5}, (d) 1×10^{-4},(e) 1×10^{-3} mol L^{-1} at scan rat of 50 mV s^{-1}.

4. 5. 4 Effect of Scan rate

In order to investigate whether the oxidation caffeine at the nanohole/GCE is diffusion control or not, the effect scan rate has to be studied. The scan rate study was done both in low and high scan rate ranges. The effect of scan rate on the oxidative peak current of caffeine was studied within the ranges of 0.03 to10 Vs^{-1}. As shown in Figure 13, oxidation peak currents of caffeine at low scan rate (0.03 – 1 V s^{-1}) is gradually increased as the scan rates increased from 0.03 to 1 Vs^{-1}. The best linear fit (R = 0.997, Figure 14) was observed for plot of peak current vs to the square root of the scan rate within this range, indicating electrode reaction is diffusion controlled[98].

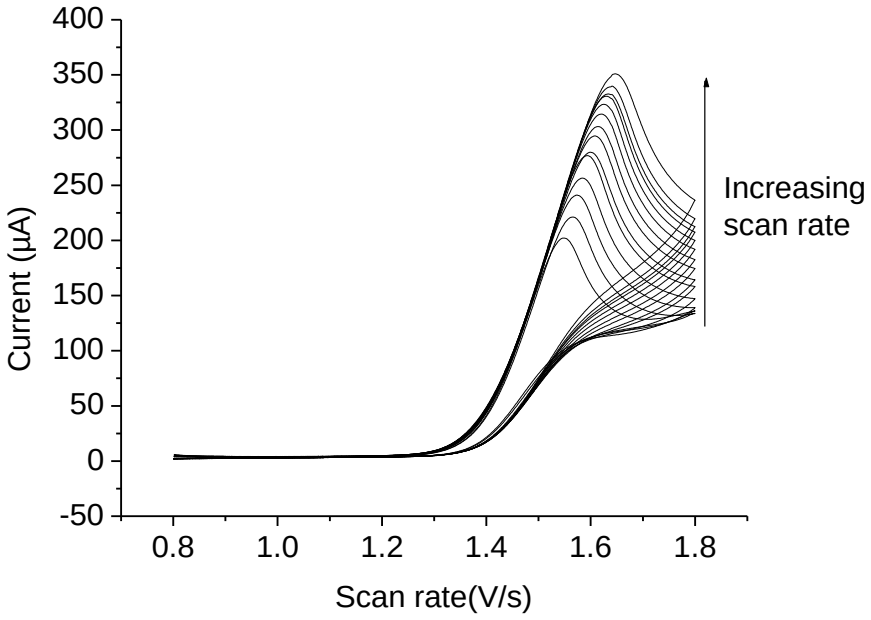


Figure 13 Cyclic voltammogram of caffeine at different scan rate of: 0.03.0.05, 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8 and 1Vs^{-1}

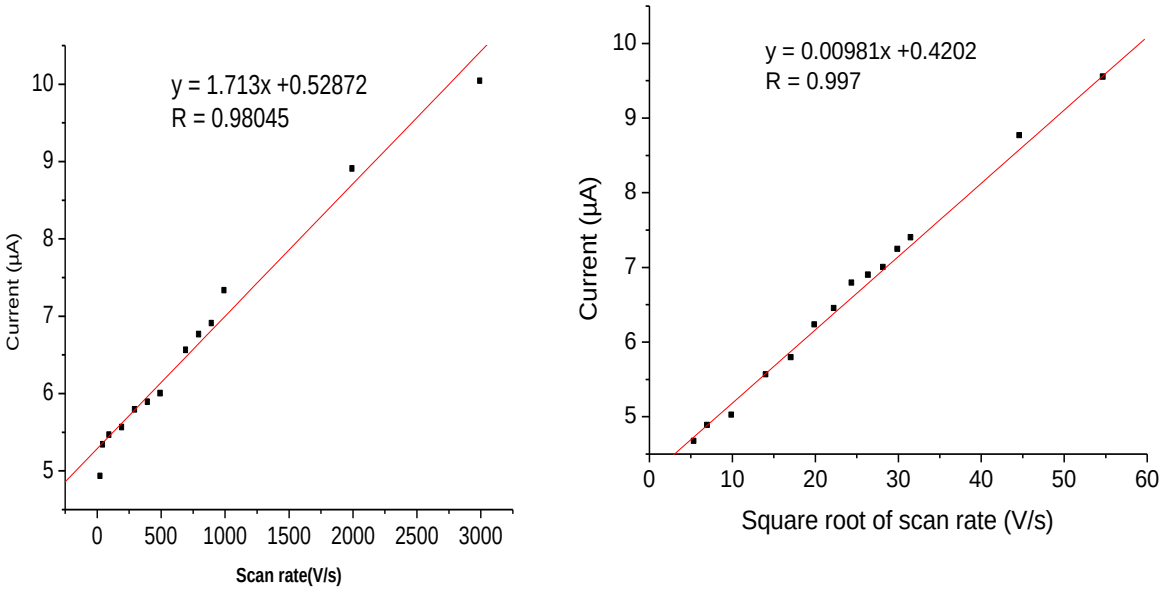


Figure 14 Calibration curve caffeine at of low scan rate.

From 1 to 10 Vs^{-1} oxidation peak potentials increases and even becomes more stable with further increase of scan rates.

This observation indicated that absorption processes occurred onto nano hole modified GCE surface[98]. This can be rationalized by considering the size of the diffusion layer and the time taken to record the scan. Form this we concluded that electrochemical oxidation of caffeine on the surface of nanohole/GCE is not a fully diffusion-controlled process; rather it is adsorption reaction, and the peak current increases linearly with increasing scan rate. So absorption current increases more rapid than diffusion current[98].

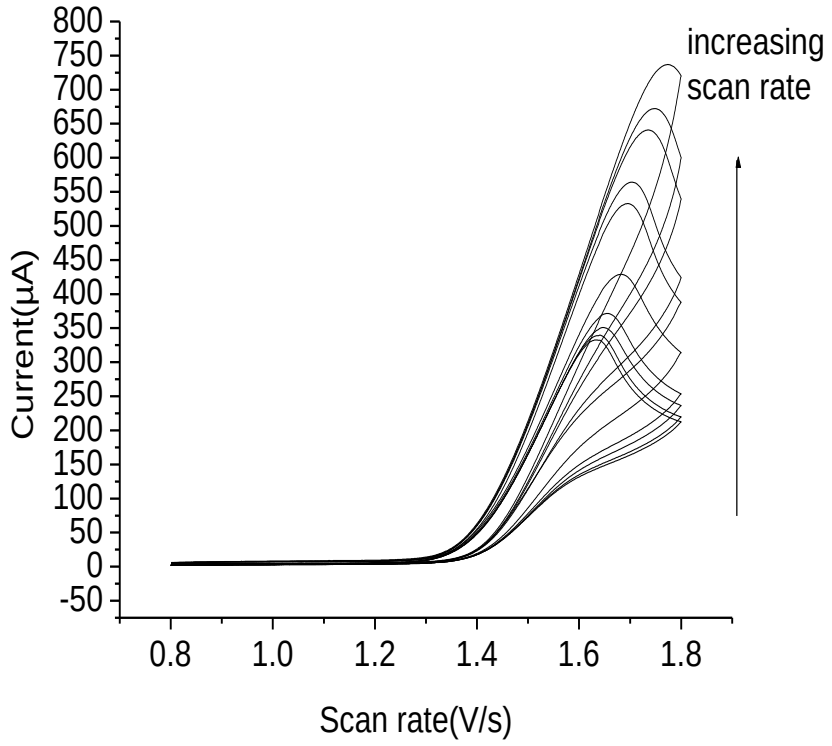


Figure 15 Cyclic voltammogram of caffeine at high scan rate of: 1, 2, 3, 4, 5, 6, 7, 8, and 10 Vs^{-1}

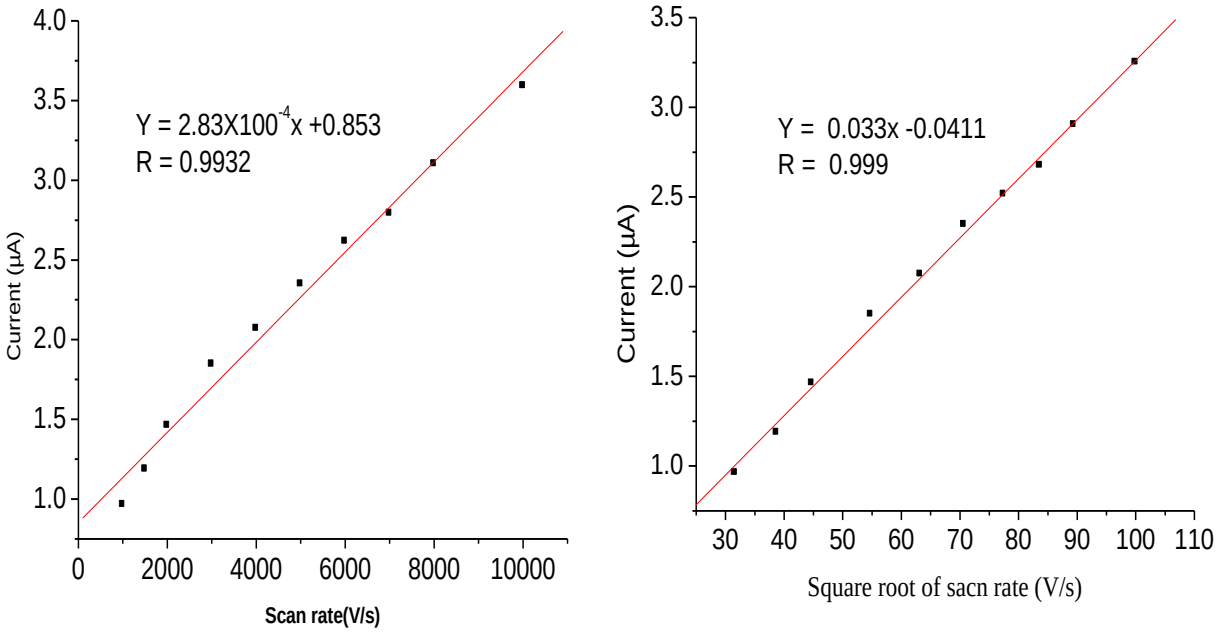


Figure 16 Calibration curve caffeine at high scan rat of: 1, 2, 3, 4, 5, 6, 7, 8, and 10 Vs^{-1}

4. 5.5 Interferences Study

The interference of some compounds that have chemical structure closer to caffeine[99] and those that co-exist with caffeine in different samples were evaluated at the nanohole modified glassy carbon sensor. The selected compounds for this test were theophylline and theobromine. The test was performed in 0.01 mol L^{-1} H_2SO_4 solution. The influence of interfering species is shown below; theobromine oxidized at 1335 mV (Figure 17) and theophylline oxidized at 1355 mV (Figure 18) on the determination of caffeine at nanohole/GCE (a) and bar GCE(b)[99].

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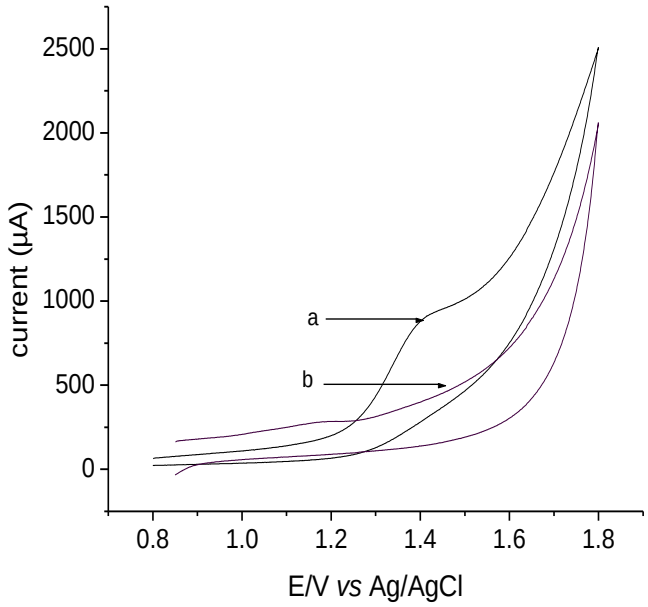

Figure 17 Cyclic voltammogram of theobromine at nanohole/GCE (a) and bare (b).

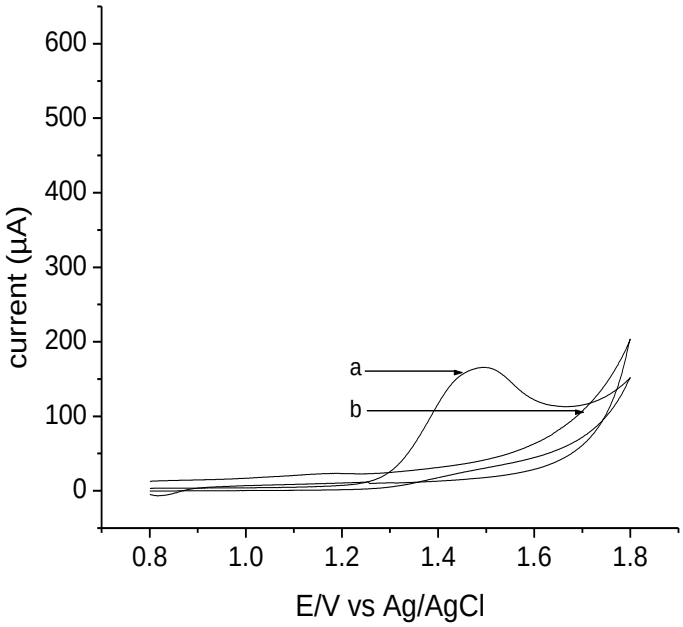

Figure 18 Cyclic voltammogram of theophylline at nanohole/GCE (a) and bare GCE (b).

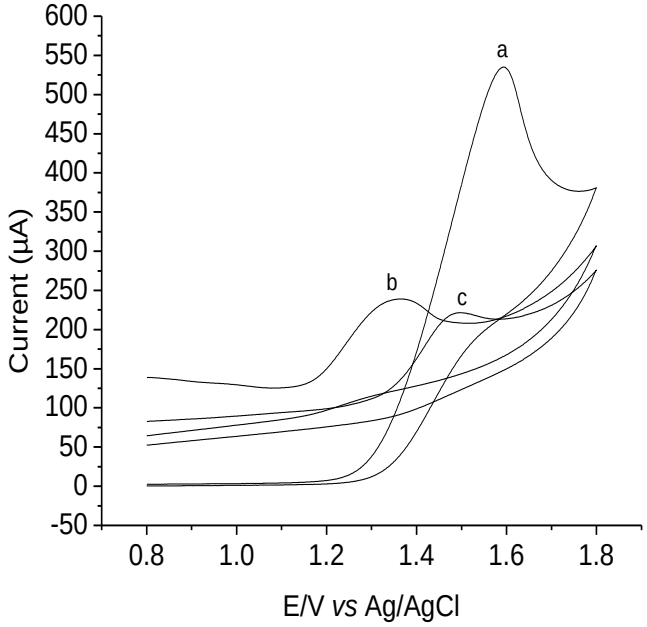

Figure 19 Cyclic voltamogram of caffeine (a), theophylline (b), and theobromine (c) in 0.01mol L^{-1} H_2S0_4 at scan rat of 50 mVs^{-1}

The experimental results showed that the presence of these interferents did not significantly influence the determination of caffeine in the coffee since they oxidized at lower potential of caffeine at nanohole modified GCE.

Even when a fixed amount of caffeine mixed with theobromine and theophylline, under the same experimental conditions, the results showed two anodic response for both interferents and caffeine[100]. The following potential were obtained from mixture of theobromine and caffeine 1328 mV and 1486 mV (Figure 20), theophylline and caffeine 1348 and caffeine 1470 mV were obtained (Figure 21) respectively. These indicated that nanohole/GCE had good selectivity for determination of caffeine in the presence of these two interferents[101].

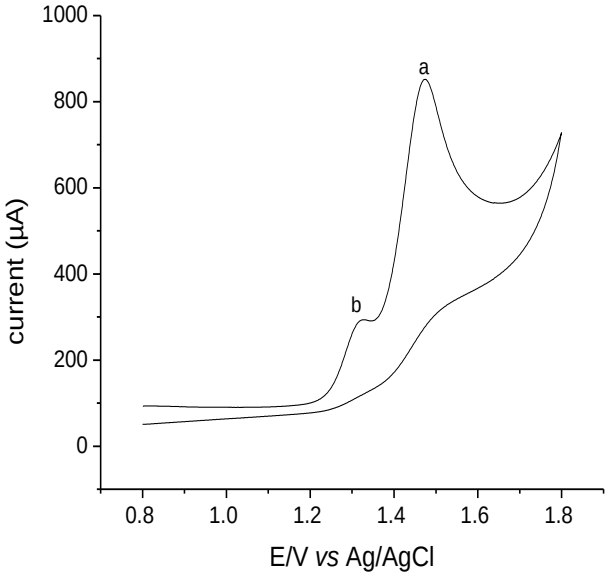

Figure 20 Cyclic voltammogram of a mixture of caffeine ($2{\cdot}0 \times 10^{-3}$ mol L^{-1}) and theobromine ($2{\cdot}0 \times 10^{-3}$ mol L^{-1}) (a) caffeine (b) theobromine peaks.

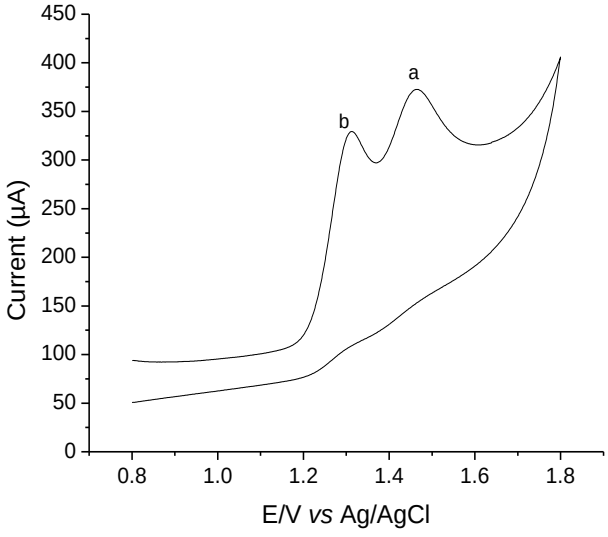

Figure 21 Cyclic voltammogram of mixture of caffeine ($2{\cdot}0 \times 10^{-3}$ mol L^{-1}) and theophylline ($2{\cdot}0 \times 10^{-3}$ mol L^{-1}) caffeine and (b) theophylline peaks

4. 6 Reproducibility and Stability of the Modified Electrode

Stability and reproducibility are the two vital characteristics for the modified electrode. Five GC electrodes were modified based on optimized conditions and then oxidation peak current of $2{\cdot}0 \times 10^{-3}$ mol L^{-1} caffeine was measured in each case. The peak current kept almost unchanged. The RSD of the peak current was 4.3% (n = 5), revealing good reproducibility of the modified electrode[101].

Sstability of the nanohole modified GC electrode toward oxidation of caffeine was tested. After first day analysis, the nanohole/GCE was stored in 0.01 mol L^{-1} supporting electrolyte at 4°C for three days. The modified GC electrode was scanned repeatedly in supporting electrolyte until the steady current was attained. Then, the stabilized electrode was used to run CV of caffeine and the measurements were recorded. The peak current showed almost no change. Each measurement preceded by linear scanning of the electrode. Again stored in 0.01 mol L^{-1} supporting electrolyte at 4°C for 7 days , and then next for 15 days and the peak current was recorded without no more change and again we extended the storage time to 20 days in supporting electrolyte. The calculated mean current for 45 duration was demonstrating the stability of the modified electrode[101]. The peak current of caffeine retained 97% of its initial response current after 45 days. This shows the modified electrode has long term current response.

Table1. The peak current recorded after 45 days storage of electrode in supporting

Current recoded at first day		Current recoded after 45 days storage of electrode in 0.01 mol L^{-1} H_2SO_4	
Average Peak current	Peak potential	Average Peak current	Peak potential
2.773	1489	2.560	1486
2.808	1490	2.558	1485

2.808	1490	2.600	1487

4.7 Amperometric Response of Caffeine

Chronoamperometric experiments were carried out to study limit of detection (LOD) of nanohole/GC in the presence of caffeine (Figure 22). Chronoamperometric method was preferred as the cyclic Voltammetery lacked the requisite sensitivity at the low concentrations monitored[102]. The chronoamperometric curve of the sensor in sulphuric acid solution containing various concentrations of caffeine was obtained using optimized potential (1.5V) by CV.

There was a linear relation between peak current and caffeine concentration over the range of 2 μ mol L^{-1} to16 μ mol L^{-1} with limit of detection (LOD = 3δ/slope) of 7.28 × 10^{-8} mol L^{-1}. Current response of caffeine increase with caffeine concentration at nanohole modified GCE

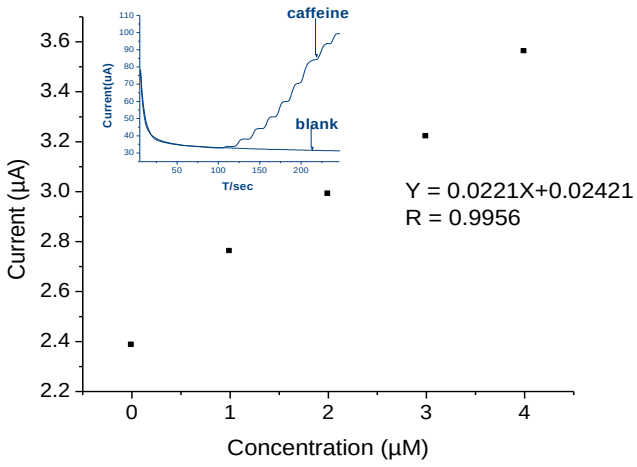

Figure 22 Amperometric response of different concentration of caffeine: 2 μ mol L^{-1}, *4μ* mol L^{-1}, *6 μ* mol L^{-1}, *8μ* mol L^{-1}, *10 μ* mol L^{-1}, *12μ* mol L^{-1}, *14μ* mol L^{-1}, *and 16 μ* mol L^{-1}

4.8 Real Sample Analysis

It was hoped that complete extraction of caffeine from coffee could be effected by acid digestion procedure. This extraction procedure has advantage that, the method takes less time than the others, has ability to penetrate through the coffee beans than other solvents, and environmental it is not pollutant. Three different coffee samples were individually analyzed. The samples were prepared as reported in the experimental section and analyzed by using the standard addition method (by analyzing the sample as received after five standard additions). The highest amount of caffeine in samples analyzed was found in raw coffee sample while the lowest was found in roasted and brewed coffee sample. The resulting caffeine content, reported in Table 3 and are in good agreement with pervious literature[103].

4.8. 1 Influence of Extraction Time

Preliminary experiments were performed to optimize the best time of caffeine extraction from coffee. As shown in Figure 23 the voltammetric peak current recorded at nanohole modified glassy electrode decreased with time extraction. The significant difference in peak current of caffeine recorded at different time of extractions is due to high protonation reactions could affect the caffeine. So, 4 min was optimized for extraction of caffeine from coffee throughout this work[103]

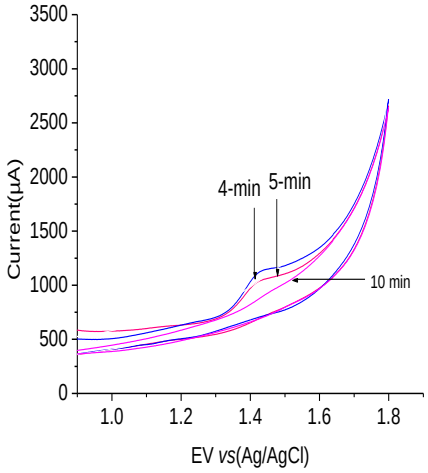

Figure 23 Cyclic voltammogram of raw coffee bean at different time of extraction.

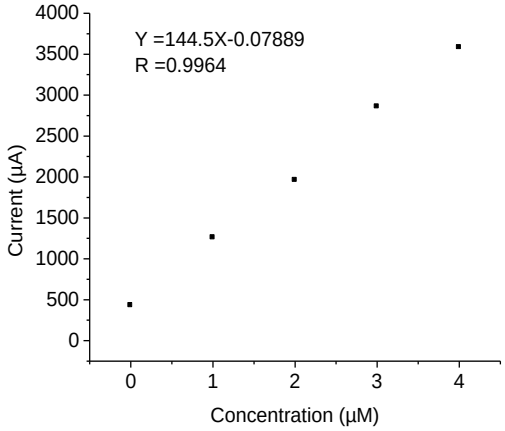

Figure24 Calibration curve of raw coffee for determination of caffeine.

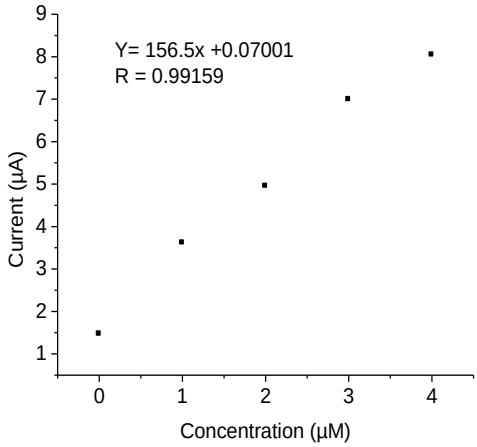

Figure 25 Calibration curve of roasted coffee for determination of caffeine.

Table 2 Concentration of caffeine obtained by cyclic voltammetric analysis

Coffee samples	w/w %
raw coffee	0.39
roasted coffee	0.32
brewed coffee	0.28

4.8. 2 Analysis of Chloroform ($CHCl_3$) Extracts of coffee

Extraction of caffeine from coffee was achieved by using chloroform as an extracting solvent. However the result indicated that, the concentration of caffeine obtained by chloroform extraction is much lower than those obtained by acid digestion method (Table 3)[87]. This is because as it is known caffeine has no hydrogen atoms than those found on methyl group. The four nitrogen atoms have a lone pair of electron that forms polar hydrogen bonds which tends to increase solubility of caffeine in acidified water than chloroform and in turn decrease yield of chloroform extraction[87].

Melting point measurements are usually used to characterize purity of extracts. Pure caffeine melts at 238 ° C[104]. The melting points of the $CHCl_3$ extracts were within the range of materials 232 – 238^0c showing slightly lower melting point than pure caffeine.

The slight disagreement with the pure caffeine melting point could be due to presence of impurities in the extracted product.

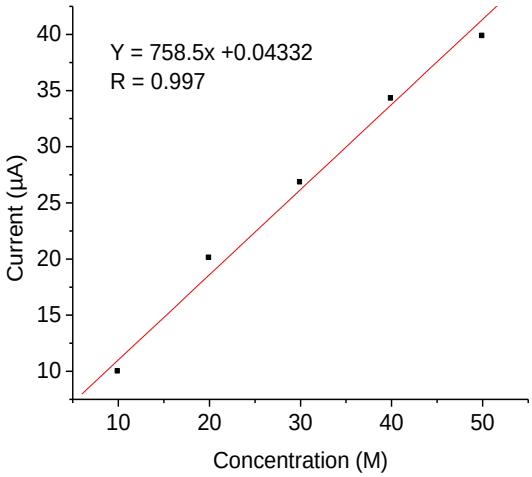

Figure 26 calibration curve for Caffeine as obtained from cyclic voltammetric method for determination of caffeine from recovered mass.

Table 3 Concentration caffeine obtained from raw coffee by different procedures

Procedures	w/w %
Acid digestion (normal procedure)	0.390
Chloroform extraction (controlled procedure)	0.333

4.8.3 Analysis of UV/Vis spectrophotometer result

For validation purpose, the result of electrochemical method was compared with the one obtained by UV/Vis spectrophotometer [103]. The absorbance of the working standards and samples were measured at 272 nm. The caffeine concentration of the samples were

calculated from linear regression equation of absorbance versus standard concentration, as it can be seen from calibration curve (Figure 27) with LOD 0.1µg/m[103]. The amounts of caffeine obtained by UV/Vis spectrophotometeric method were lower than those obtained by electrochemical method which used to achieve lower LOD (Table 5).This suggests that the present method is more sensitive than UV/Vis spectrophotometeric method for determination of caffeine.

Table 4.Measured absorbance values of standard solution

Caffeine Standard (ppm)	Absorbance
10	1.1703
20	1.372
30	1.555
40	1.735
50	1.899
100	2.811

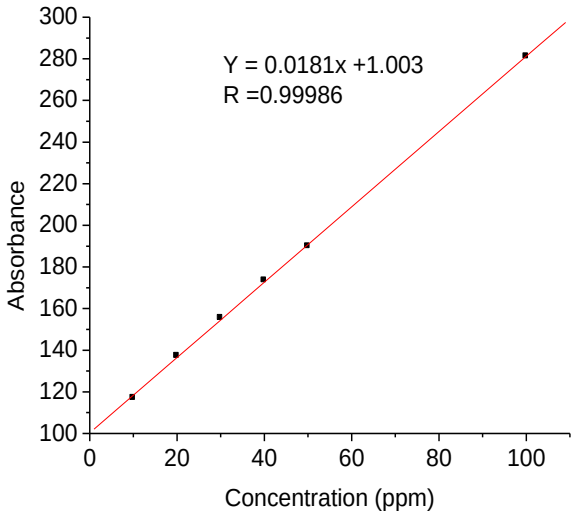

Figure 27 Caffeine calibration curve for UV/ Vis Spectrophotometeric method

Table 5 Concentration caffeine obtained from raw coffee by different methods

Methods	w/w %
Cylicvoltametry	0.39
UV/VIS Spectrophotometeric method (n = 3)	0.36

Caffeine determination using different methods are possible. Here under this, a comparison of different parameters found during caffeine detection as detailed in the corresponding references given.

Table 6 Comparison between the newly developed caffeine sensors and other reported methods

Methods	**Analytica range(mol L^{-1})**	**Limits of detections (mol L^{-1})**	Ref
Nanohole **modified GCE**	1×10^{-7}– 1×1^{-3}	7.28×10^{-8}	This work
Utilization of electrochemical methods in determination of trace elements in beverages	$4 \times 10^{-7} – 2.5 \times 10^{-5}$	1.5×10^{-7}	64
Voltammetric sensor for caffeine based on glassy carbon electrode modified with Nafion and graphene oxide	$4.0\times10^{-7} – 8.0\times10^{-5}$	2.0×10^{-7}	96

Amperometric detection of three purine alkaloids following their separation by micellar ectrokinetic capillary chromatography	4.0×10^{-6}– 5.0×10^{-4}	2.0×10^{-6}	104

As it is indicated in the table 6, nanohole modified glassy carbon electrode detection of caffeine is the most sensitive method than the rest of the methods.

.

5. Conclusions

This study demonstrated a new caffeine voltammetric sensor at nanohole modified glassy carbon electrode, showing good sensitivity and selectivity for the detection of caffeine and thereby minimized oxidation of caffeine as indicted in table 6. Nanohole modified glassy carbon electrode exhibited significant advantages of wide linear range and low detection limit for caffeine compared with previous works. Besides, the modified electrode showed good reproducibility and stability, and also showed two different peak current selectively for caffeine and interferents mixture siumoltanisuly and this offered a good possibility for extending the technique in routine analysis of caffeine.

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