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YÜZÜNCÜ YIL UNIVERSITY
INSTITUTE OF NATURAL AND APPLIED SCIENCE
CHEMISTRY DEPARTMENT

DEVELOPMENT A VOLTAMMETRIC METHOD FOR THE SIMULTANEOUS DETERMINATION OF VANILLIN AND CAFFEINE ON A BORON – DOPED DIAMOND ELECTRODE

MASTER THESIS

PREPARING: Hoshyar Saadi ALI
SUPERVISOR: Assoc. Prof. Dr. Yavuz YARDIM

VAN-2016

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ACCEPTANCE AND APPROVAL PAGE

This thesis entitled "**Development a voltammetric method for the simultaneous determination of vanillin and caffiene on a boron–doped diamond electrode**" presented by Hoshyar Saadi ALI under supervision of Assoc. Prof. Yavuz YARDIM in the department of Chemistry has been accepted as a M. Sc. thesis according to Legislations of Graduate Higher Education on 15../09./2016. with unanimity / majority of votes members of jury.

Chair: Prof. Dr. Zühre ŞENTÜRK Signature:

Member: Assoc. Prof. Yavuz YARDIM Signature:

Member: Assist. Prof. Ertuğrul KESKİN Signature:

This thesis has been approved by the committee of The Institute of Natural and Applied Science on 11../09./2016 with decision number 2016/..39..-1

Signature:

Director of Institute

THESIS STATEMENT

All information presented in the thesis obtained in the frame of ethical behavior and academic rules. In addition all kinds of information that does not belong to me have been cited appropriately in the thesis prepared by the thesis writing rules.

Signature

ÖZET

VANİLİN VE KAFEİN'İN BOR KATKILI ELMAS ELEKTROT YÜZEYİNDE EŞ ZAMANLI TAYİNLERİNE YÖNELİK VOLTAMETRİK YÖNTEM GELİŞTİRİLMESİ

ALI, Hoshyar Saadi
Yüksek Lisans Tez: Kimya Bölümü
Danışman: Doç. Dr. Yavuz YARDIM
Ağustos 2016, 97 sayfa

Bu çalışmada, anodik olarak ön işlem görmüş bor katkılı elmas (BDD) elektrot yüzeyinde kafein (CAF) ve vanilin (VAN) 'in eş zamanlı belirlenmesi için dönüşümlü ve adsorptif sıyırma voltametri teknikleri kullanıldı. Pik akım ve pik potansiyeli üzerine pH, tarama hızı, biriktirme parametreleri ve diğer deneysel değişkenler çalışıldı. Kare dalga sıyırma tekniği kullanılarak açık devre koşulları altında, 60 saniye biriktirmeden sonra BDD elektrot yüzeyinde pH 2.5 fosfat tamponunda içinde CAF ve VAN 'in oksidasyon pik potansiyellerini 0.3 V fark olacak şekilde ayırabildi. Gözlenebilme sınırı, VAN için 0.114 μg mL^{-1} (7.49×10^{-7} mol L^{-1}) ve CAF için 0.033 μg mL^{-1} (1.69×10^{-7}mol L^{-1}) idi. Bu metodun pratik uygulanabilirliği, ticari olarak piyasada varolan örnekler ile test edildi. Elde edilen sonuçlar yüksek performanslı sıvı kromatografisi (HPLC) ile kıyaslandı.

Anahtar kelimeler: Kafein, Vanilin, Bor katkılı elmas elektrot, Eş zamanlı belirleme, Kare dalga sıyırma voltametri, Kahve, Kola, Vanilla.

ABSTRACT

DEVELOPMENT A VOLTAMMETRIC METHOD FOR THE SIMULTANEOUS DETERMINATION OF VANILLIN AND CAFFEINE ON A BORON – DOPED DIAMOND ELECTRODE

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M.Sc. Thesis, Chemistry Science
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August 2016, 97 page

Herein, a boron-doped diamond (BDD) electrode that is anodically pretreated was used for the simultaneous determination of vanilline (VAN) and caffeine (CAF) by cyclic and square wave stripping voltammetry. The dependence of peak current and potential on pH, scan rate, accumulation parameters and other experimental variables were studied. By using square-wave stripping mode after 60 s accumulation under open-circuit voltage, the BDD electrode was able to separate the oxidation peak potentials of CAF and VAN present in binary mixtures by about 0.3 V in phosphate buffer, pH 2.5 The limits of detection were 0.114 μg mL^{-1} (7.49×10^{-7} mol L^{-1}) for VAN, and 0.033 μg mL^{-1} (1.69×10^{-7}mol L^{-1}) for CAF. The practical applicability of this methodology was tested in commercially available samples. The results has been compare to high performance liquid chromatography (HPLC) method.

Key words: Caffeine, Vanillin, Boron-doped diamond electrode, Simultaneous determination, Square-wave adsorptive stripping voltammetry, Coffee, Cola, Vanilla.

PREFACE

Foremost, I would like to extend my sincerest thanks and appreciation to those patient souls who helped me accomplish this study; I would like to express my sincere gratitude to my advisor Doç. Dr. Yavuz Yardım for the continuous support of my master study and research, for the patience, motivation, enthusiasm, and immense knowledge. Him guidance helped me in all the time of research and writing of this thesis. I could not have imagined having a better advisor and mentor for my study.

I would like to extremely thank Prof. Dr. Zühre Şentürk for her encouragement, insightful comments, and hard questions in my master study journey.

Last but not the least; special recognition goes out to my family, for their support, encouragement and patience during my pursuit of the master's degree. To my lovely parents, who inspired me and provided constant encouragement during the entire process. To my brothers and sisters, who missed out a lot of time while I sought intellectual enlightenment, finally I would like to thank all of my friends for nonstop encouragements and support.

2016

Hoshyar ALI

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SYMBOLS AND ABBREVIATIONS

Some symbols and abbreviations used in this study are presented below, along with descriptions.

Symbols	Description
Na_2SO_4	Sodium sulfate
CH_3COOH	Acetic acid
H_3PO_4	Phosphoric acid
H_2SO_4	Sulfuric acid
KCI	Potassium chloride
HNO_3	Nitric acid
$LiClO_4$	Lithium perchlorate
Na_2HPO_4	Disodium hydrogen phosphate
NaOH	Sodium hydroxide
H_3BO_3	Boric acid
HCl	Hydrochloric acid
$HClO_4$	Perchloric acid
Hg_2Cl_2	Mercury (I) chloride
KNO_3	Potassium nitrate
L	Liter
mL	Milliliter
µL	Microliter
M	Molarity
µg	Micro gram
V	Volt
mV	Millivolt
°C	Centigrade degrees
Hz	Hertz
S	Second

Log	Logarithm
σ	The total charge density
Φ	The inner potential
ip_c	Cathodic peak current
ip_a	Anodic peak current
ζ	Square-wave period
E	Electrode voltage
E^0	Standard voltage for the redox reaction
R	The gas constant (8.314 J K^{-1} mol^{-1})
T	Temperature (^{0}K)
N	Number of electrons transferred in the electrode reaction
F	Faraday's constant (96 487 coulombs mol^{-1})
Rs	Resolution
A	The selectivity factor for two peaks
N	The column plate number
K	The retention factor
k_2 and k_1	The retention factors of the last and first eluted peaks

Abbreviation	**Description**
VAN	Vanillin
CAF	Caffeine
CV	Cyclic Voltammetry
SWV	Square Wave Voltammetry
GCE	Glassy carbon electrode
CPE	Carbon paste electrode
CFE	Carbon – fiber electrode
DME	The dropping mercury electrode
HMDE	The hanging mercury drop electrode

MFE	Mercury film electrode
LOD	Limit of detection
LOQ	Limit of quantification
DMF	Dimethylformamide
DMSO	Dimethylsulfoxide
TLC	Thin layer chromatography
GC	Gas chromatography
LC	Liquid chromatography
HPLC	High performance liquid chromatography
Ppm	Part per million

1. INTRODUCTION

Vanillin (4-hydroxy-3-methoxybenzaldehyde) (VAN) and Caffeine (1, 3, 7-trimethylxanthine) (CAF) are two worldwide additives and often coexist in many snacks and beverage products (Jiang et al., 2011).

Vanillin (VAN) is an unparalleled, highly prized food and most popular additive as flavor improver. This compound is widely used to contribute to the fragrance of commercial foods such as pudding, ice-cream, custard, cookies, chocolate, and beverages. Vanillin is the main aromatic compound in natural vanilla. The source of vanilla is the bean or pod of the equatorial Vanilla plant, an organ of the orchid family (Sinha et al., 2008).

Molecular Formula: $C_8H_8O_3$ and its systematic name is 4-hydroxy-3-methoxybenzaldehyde. Pure vanillin occurs as white or slightly yellow needle, odor floral, pleasant crystal structure monoclinic melting point: 178-181 ° F, specific gravity: 1.056 at 68.0 ° F. Molar mass 152.15 g mol^{-1}, vapor pressure 2.2 x 10^{-3} mm Hg, water solubility 1 g/100 ml, dissociation constant pKa_1 7.40, pKa_2 11.4 (25°C). Its structural formula is as shown in Figure 1.1 (Kumar et al., 2012).

However, the production of natural vanillin from vanilla pods covers only 0.2% of the market requirement, and its production cost is very high. Vanillin can also be synthesized from low-cost materials such as 2-methoxyphenol, eugenol, and lignin. Synthetic vanillin is used in both food and non-food applications, as a fragrance component in perfumes and cosmetics, and as a flavoring in pharmaceutical preparations. Though synthetic one is cheaper and widely produced, it causes headaches, nausea and vomiting, and could affect liver and kidney functions when large amounts of this flavor enhancer is ingested from the viewpoint of food safety and addiction prevention (Yardım et al., 2013).

In China, according to the “national food safety standards for use of food additives” provisions of GB2760-2011 requirements, all covered by the use of 0 to 6 months of infant formula foods may not add any edible spices. Because the infant body organs of immature, metabolic detoxification ability is rather poor, eat food additives food will increase their daily metabolic burden. Hence, it is necessary to detect and control the content of vanillin (Xinying, 2014).

Caffeine is a naturally occurring alkaloid commonly distributed in natural products found in the leaves, seeds or fruits of over 63 plants species worldwide. Caffeine chemical formula is $C_8H_{10}N_4O_2$ and its systematic name is 1, 3, 5 - trimethylxanthine. Pure caffeine occurs as odorless, white, fleecy masses, glistening needles of powder. Its molecular weight is 194.19 g, melting point is 236°C, point at which caffeine sublimes is 178°C at atmospheric pressure, pH is 6.9 (1% solution), specific gravity is 1.2, volatility is 0.5%, vapor pressure is 760 mm Hg at 178°C, solubility in water is 2.17%, vapor density 6.7. Its structural formula is as shown in Figure 1.1. The widespread occurrence of caffeine in a variety of plants played a major role in the long-standing popularity of caffeine-containing products. The most important sources of caffeine are coffee, tea, guarana, cola nuts and cocoa. The amount of caffeine found in these products varies, the highest amounts are found in guarana (4-7%), followed by tea leaves (3.5%), coffee beans (1.1-2.2%), cola nuts (1.5%) and cocoa beans (0.03%) (Ali et al., 2012).

Caffeine widely used as a flavoring agent in a variety of beverages. CAF may be also considered as the most widely used drug in the world. It is took daily in coffee, tea, cocoa, chocolate, some energy or soft drinks, as well as in drug formulations for the handling of asthma, nasal congestion, headache or to progress athletic ability and ease weight loss. CAF is also characterizing a mild stimulant for central nervous system, muscle, heart and circular systems of the human body (James, 1991).

Caffeine also stimulates the stomach to drain large amounts of acid. This in turn leads to burning in the pits of the stomach and aggravates peptic ulcers of the stomach and duodenum (Wanyika et al., 2010).

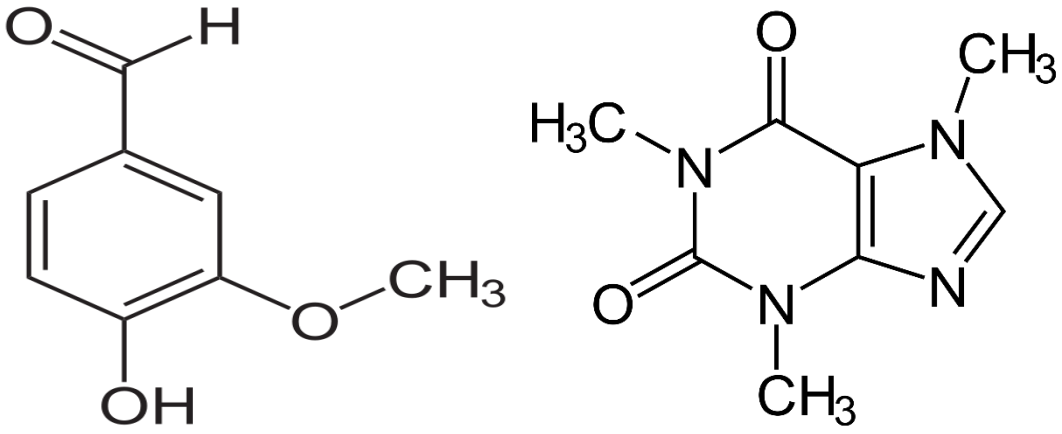
Caffeine is commonly combined with developments in alertness, learning ability and exercise performance when middling consumed. However, drinking large amounts of CAF or taking enough high doses may cause numerous unfavorable symptoms and even possibility reverse effects on health, particulary for infants and children, such as agitation, chills, irritability, loss of appetite, weakness, insomnia, hypertension, gastrointestinal problem, fever, delusions, tachycardia and even death (Reissig et al., 2009; Varnam, 1994).

It has been also reported coma and death in cases of CAF overdose (>200 mg/day) (Suteerapataranon et al., 2009).

The caffeine ingested by insects when they feed on these plant parts act as a natural pesticide that can cripple and kill them (Aklilu et al., 2008).

Simultaneous determination of CAF and VAN in food products is of vital significance and necessity for people, especially for growing children (Mussatto et al., 2011)

Up till now, although there have been plenty of reports about the electrochemical sensors of CAF and VAN (Jiang et al., 2011).



Vanillin Caffeine

Figure 1.1. Structure of vanillin and caffeine.

1.2. Electrochemistry

Electrochemistry is the branch of chemistry concerned with the interrelation of electrical and chemical effects. A large part of this field deals with the study of chemical changes caused by the passage of an electric current and the production of electrical energy by chemical reactions. In fact, the field of electrochemistry encompasses a huge array of different phenomena (e.g., electrophoresis and corrosion), devices (electro chromic displays, electro analytical sensors, batteries, and fuel cells), and technologies (the electroplating of metals and the large-scale production of aluminum and chlorine). While the basic principles of electrochemistry discussed in this text apply to all of these, the main emphasis here is on the application of electrochemical methods to the study of chemical systems.

Scientists make electrochemical measurements on chemical systems for a variety of reasons. They may be interested in obtaining thermodynamic data about a reaction. They may want to generate an unstable intermediate such as a radical ion and study its rate of decay or its spectroscopic properties. They may seek to analyze a solution for trace amounts of metal ions or organic species. In these examples, electrochemical methods are employed as tools in the study of chemical systems in just the way that spectroscopic methods are frequently applied. There are also investigations in which the electrochemical properties of the systems themselves are of primary interest, for example, in the design of a new power source or for the electro synthesis of some product. Many electrochemical methods have been devised. Their application requires an understanding of the fundamental principles of electrode reactions and the electrical properties of electrode-solution interfaces (Bard, 2001).

1.2.1. Electroanalytical techniques

Electroanalytical techniques are interested with the interplay between electricity and chemistry, namely the measurements of electrical quantities, such as current, potential, or charge, and their relationship to chemical parameters. Such use of electrical measurements for analytical purposes has found a vast range of applications, including environmental measurements (1), industrial quality control (2), and biomedical analysis

(3). They have many advantages such as high sensitivity, good selectivity, rapid response, low cost and simplicity. The two principal of electroanalytical measurements are potentiometric and potentiostatic, both types require at least two electrodes (conductors) and a contacting sample (electrolyte) solution, which constitute the electrochemical, the electrode surface is thus a junction between an ionic conductor and an electronic conductor (Figure 1.2) (Wang, 1994).

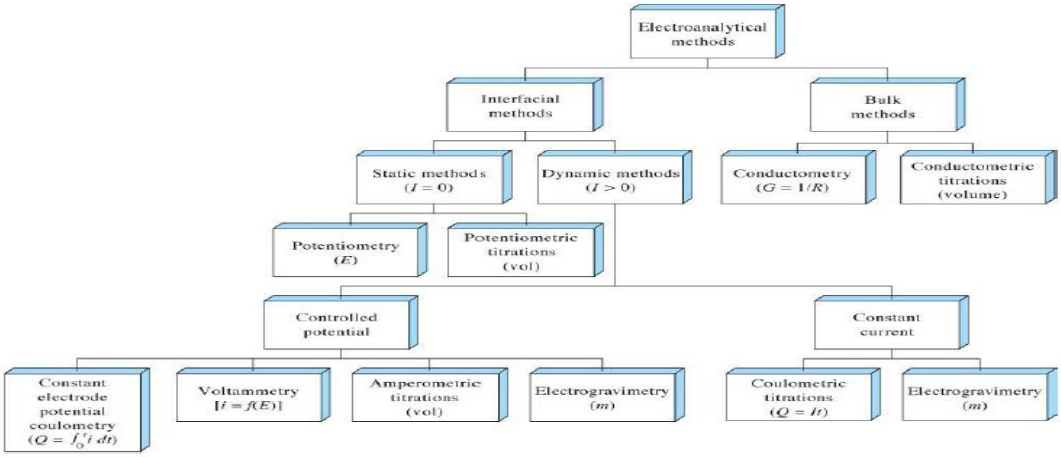


Figure 1.2. Schematic representation of the common electro method (Skoog et al., 1998).

1.2.2. Mass transport – controlled reactions

Mass transport occurs by different modes:

Diffusion- is the motion of species brought about by a concentration gradient (i.e., from high concentration regions to lower concentration regions), aimed at minimizing concentration differences

Convection – transport to the electrode by a total physical movement, such fluid flow occurs with stirring or flow of the solution and with rotation or vibration of the electrode (i.e., forced convection) or due to density gradients (i.e., natural convection).

Migration is the movement of ions through the solution brought about by electrostatics attraction between the ions and the charged electrode (i.e., the charged is carried through the solution by ions according to their transference number) (Figure 1.3) (Wang, 1994).

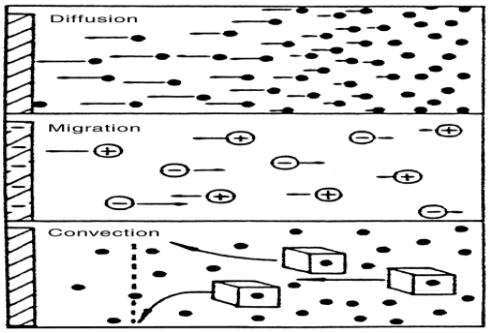


Figure 1.3. The three mode of mass transport.

1.2.3. The electrical double layer

At any electrode immersed in an electrolyte solution, a specific interfacial region is formed. This region is called the double layer. The electrical properties of such a layer are important, since they significantly affect the electrochemical measurements. In an electrical circuit used to measure the current that flows at a particular working electrode (Scholz, 2002). The solution side of the double layer is thought to be made up of several "layers." That closest to the electrode, the inner layer, contains solvent molecules and sometimes other species (ions or molecules) that are said to be specifically adsorbed (Figure 1.4). This inner layer is also called the compact, Helmholtz, or Stern layer.

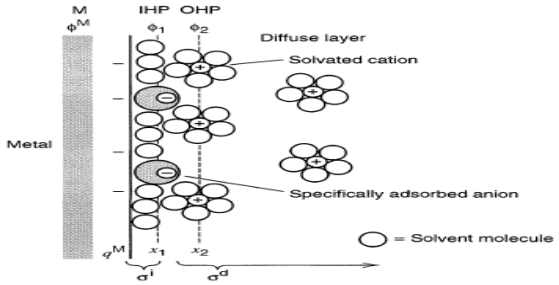


Figure 1.4. Proposed model of the double-layer region under conditions where anions are specifically adsorbed.

The locus of the electrical centers of the specifically adsorbed ions is called the inner Helmholtz plane (IHP), which is at a distance *x*. The total charge density from specifically adsorbed ions in this inner layer is σ *($\mu C/cm^2$).* Solvated ions can approach the metal only to a distance x_2*;* the locus of centers of these nearest solvated ions is called the outer Helmholtz plane (OHP). The interaction of the solvated ions with the

charged metal involves only long-range electrostatic forces, so that their interaction is essentially independent of the chemical properties of the ions. These ions are said to be nonspecifically adsorbed. Because of thermal agitation in the solution, the nonspecifically adsorbed ions are distributed in a threedimensional region called the diffuse layer, which extends from the OHP into the bulk of the solution. The excess charge density in the diffuse layer is <7d, hence the total excess charge density on the solution side of the double layer, is given by

$$\sigma^S = \sigma^i + \sigma^d = -\sigma^M$$

The thickness of the diffuse layer depends on the total ionic concentration in the solution; for concentrations greater than 10^{-2} M, the thickness is less than ~100 A. The potential profile across the double-layer region is shown in Figure 1.5. The structure of the double layer can affect the rates of electrode processes.

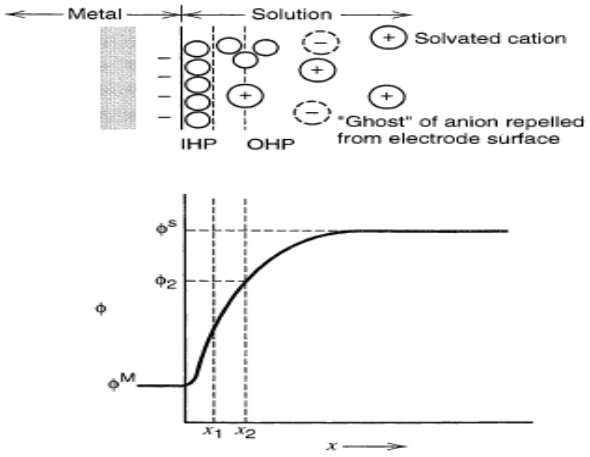


Figure 1.5. Potential profile across the double-layer region in the absence of specific adsorption of ions. The variable ϕ, called the inner potential. (Bard, 2001; Wang, 1994)

1.2.4. Faradaic and nonfaradaic processes

Two types of processes can conduct current across an electrode/solution interface. One kind involves a direct transfer of electrons via oxidation reaction at one electrode and a reduction at the other. Processes of this type are called faradaic processes because they are governed by faradaic laws which state that an amount of

chemical reaction at one electrode is proportional to the current i.e. faradaic current. Electrodes at which faradaic processes occur are sometimes called charge transfer electrodes. Nonfaradaic processes a transitory changes in the structure of the electrode solusion interface e.g adsorption the electrode may be in a potential region that does not facilitate occurrence of a charge transfer reaction. The process is thermodynamically or kinetically unfavorable. To understand the basic difference between a faradaic and a non-faradaic current, imagine an electron travelling down the external circuit to an electrode surface when the electron reaches the solution interface, it can do one of only two things, it can remain at the electrode surface and increase the charge on the double layer which constitutes a non-faradaic current. Alternatively, it can leave the electrode surface and transfer to a species in the solution, thus becoming a part of a faradaic current (Bard et al., 2001; Wang, 1994).

1.2.5. Electrochemical cells

We can study oxidation/reduction equilibria conveniently by measuring the potentials of electrochemical cells in which the two half-reactions making up the equilibrium are participants. For this reason, we must consider some characteristics of electrochemical cells. An electrochemical cell consists of two conductors called electrodes, each of which is immersed in an electrolyte solution. In most of the cells that will be of interest to us, the solutions surrounding the two electrodes are different and must be separated to avoid direct reaction between the reactants. The most common way of avoiding mixing is to insert a salt bridge, such as that shown in Figure 1.6, between the solutions. Conduction of electricity from one electrolyte solution to the other then occurs by migration of potassium ions in the bridge in one direction and chloride ions in the other. However, direct contact between copper metal and silver ions is prevented (Skoog et al., 1998).

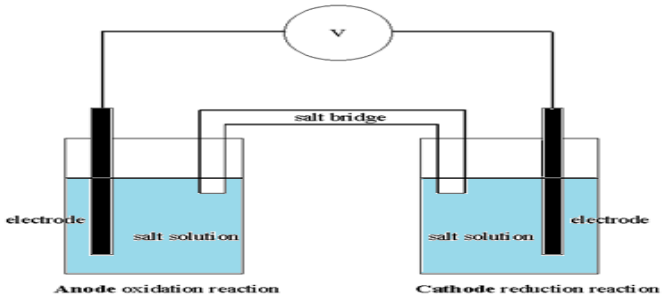


Figure 1.6. A typical two-electrode electrochemical cell.

Three electrode cells (e.g., Figure 1.7) are commonly used in controlled – potential experiments. The cell usually a covered beaker of 5-50 mL volume, and contains the three electrodes (working, reference and auxiliary), which are immersed in the sample solution. While the working electrode is the electrode at which the reaction of interest occurs. The reference electrode provides a stable and reproducible potential (independent of the sample composition) against potential of the working electrode is compared. Such buffering against potential of achieved by a constant composition of both forms its redox couple, for example Ag/AgCl or Hg_2Cl_2. An inert conducting material, such as platinum wire or graphite rod, is usually used as the current – carrying auxiliary electrode the three electrodes, as will as the tube used for budding the deoxygenating gas, are supported in five holes in the cell cover. Complete systems, integrating the three electrode cell, built–in gas control, and magnetic stirrer, along with proper cover, are available commercially (Wang, 2001).

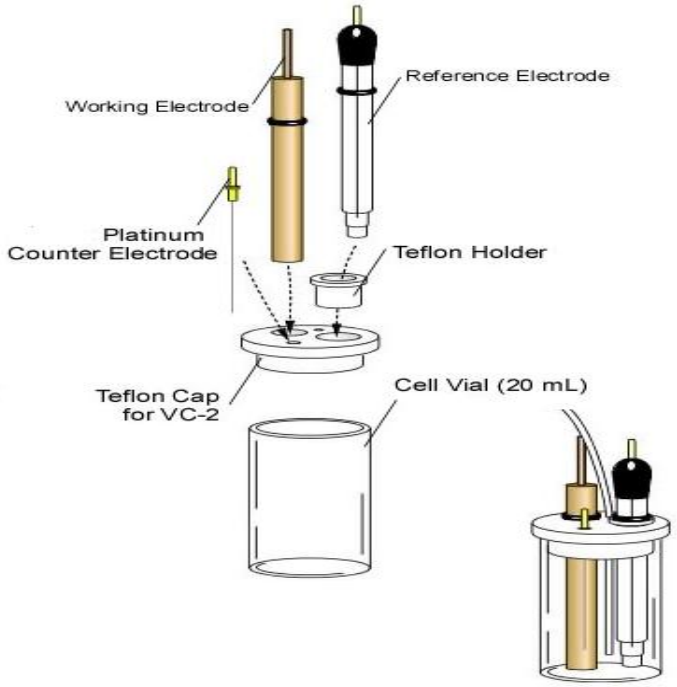


Figure 1.7. Schematic diagram of a cell for voltammetric measurements: w.e., working electrode; r.e., reference electrode; c.e., counter electrode are inserted through holes in the cell cover (ALS Co. Ltd., 2006).

1.2.6. Voltammetry

The term voltammetry refers to a group of electroanalytical methods in which we acquire information about the analyte by measuring current in an electrochemical cell as a function of applied potential (Figure 1.8). We obtain this information under conditions that promote polarization of a small indicator or working electrode. When current proportional to analyte concentration is monitored at a fixed potential, the technique is called amperometry. To enhance polarization, working electrodes in voltammetry and amperometry have surface areas of a few square millimeters at the most and in some applications, a few square micrometers or less. Voltammetry is widely used by inorganic, physical, and biological chemists for fundamental studies of oxidation and reduction processes in various media, adsorption processes on surfaces, and electron transfer mechanisms at chemically modified electrode surfaces (Skoog et al., 1998).

Analytical advantages of the various voltammetric techniques include excellent sensitivity with a very large useful linear concentration range for both inorganic and organic species, a large number of useful solvents and electrolytes, a wide range of temperatures, rapid analysis times (seconds), simultaneous determination of several analytes, the ability to determine kinetic and mechanistic parameters, a well-developed theory and thus the ability to reasonably estimate the values of unknown parameters, and the ease with which different potential waveforms can be generated and small currents 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 thecnological fields, like:

- Research of corrosion proof materials (corrosion is a consequence of a series of electrochemical reactions)
- Research of new electrodic processes for chemical industries (in fact, for example, million of tons of aluminium, chlorine, soda are produced by means of electrochemical reactions)
- Production of new type of batteries that can store rapidly a great quantities of energy (Protti, 2001).

One of the most important application of voltammetry is the quantitative analysis of trace of metals (or, anyway, of those reducible or oxidizable chemicals) at g/L levels or less. This introduction deals with the quali–quantitative aspects of the voltammetric analysis of trace of heavy metals and of organic substances in solution.

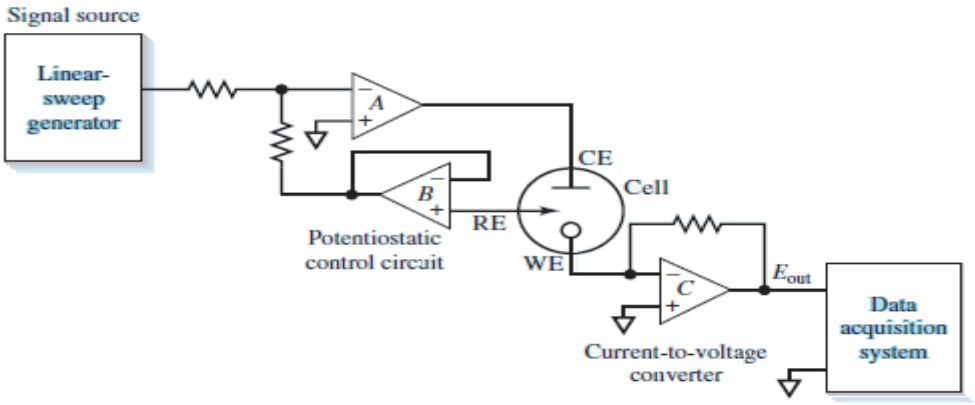


Figure 1.8. The three-electrode cell has a working electrode (WE), reference electrode (RE), and a counter electrode (CE) (Skoog et al., 1998)

1.2.6.1. Excitation signals in voltammetry

In voltammetry, a variable potential excitation signal is impressed on a working electrode in an electrochemical cell. This excitation signal produces a characteristic current response, which is the measurable quantity. The waveforms of four of the most common excitation signals used in voltammetry are shown in Figure 1.9. The classical voltammetric excitation signal is the linear scan shown in Figure 1.9 (upper of the Figure) in which the voltage applied to the cell increases linearly (usually over a 2 to 3 V range) as a function of time. The current in the cell is then recorded as a function of time and thus as a function of the applied voltage. In amperometry, current is recorded at fixed applied voltage (Skoog et al., 1998).

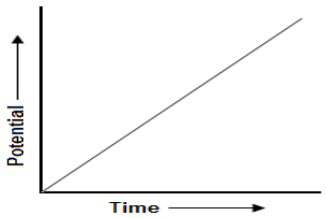
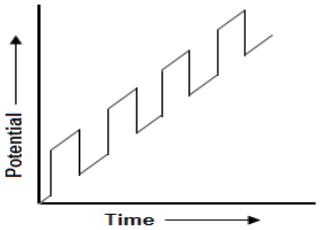
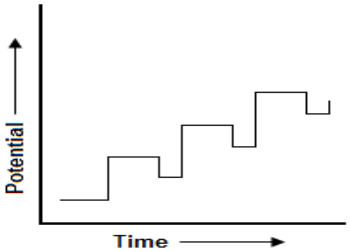
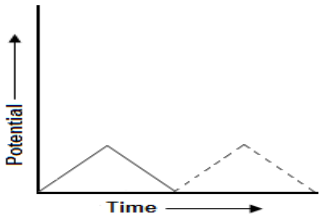
Name	Wave form	Type Voltammetry
Polarography		Polarography Hydrodynamic Voltammetry
Differential pulse		Differential pulse Voltammetry
Square Wave		Square Wave Voltammetry
Triangular		Cyclic Voltammetry

Figure 1.9. Voltage versus time excitation signals used in voltammetry.

1.2.7. Cyclic voltammetry

Cyclic voltammetry is very frequently used because it offers a wealth of experimental information and insights into both the kinetic and thermodynamic details of many chemical systems. Because of significant advances in the theoretical understanding of the technique today, even complex chemical systems such as electrodes modified with film or particulate deposits may be studied quantitatively by cyclic voltammetry. In early electrochemical work, measurements were usually undertaken under equilibrium conditions (potentiometry) where extremely accurate measurements of thermodynamic properties are possible. However, it was soon realised that the time dependence of signals can provide useful kinetic data. Many early voltammetric studies were conducted on solid electrodes made from metals such as gold or platinum. However, the complexity of the chemical processes at the interface between solid metals and aqueous electrolytes inhibited the rapid development of novel transient methods (Scholz, 2001). Cyclic voltammetry consists of scanning linearly the potential of the stationary working electrode (in an unstirred solution) depend using a triangular wave form (Figure 1.10), and during the potential sweep the potentiostat measures the resulting current. Single or multiple cycles can be used depending on the information sought. The resulting plot of current versus potential is termed a cyclic voltammogram, cyclic voltammogram can be divided into three types, reversible, (Figure 1.10), quasi reversible, and irreversible. The characteristic peaks in the cyclic voltammogram are caused by the formation of the diffusion layer near the electrode surface.

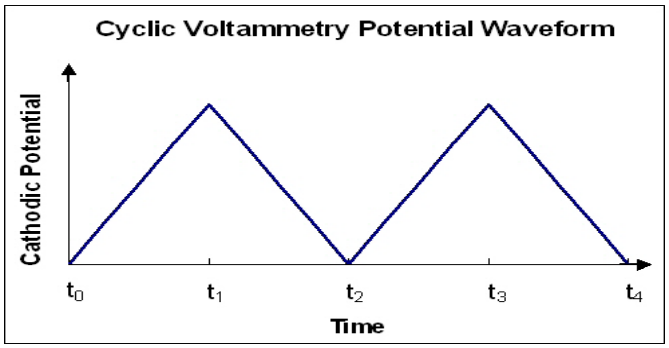


Figure 1.10. Potential-time excitation signal in cyclic voltammetric experiment.

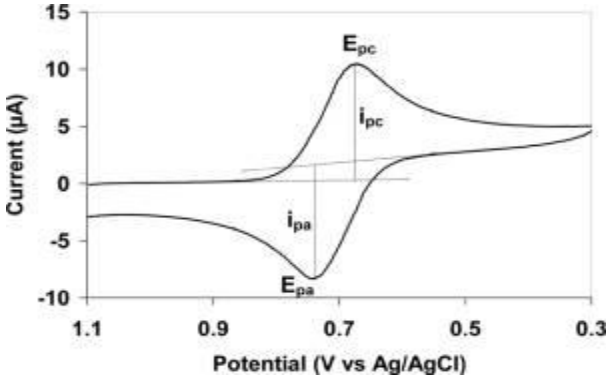


Figure 1.11. Typical cyclic voltammogram, where ip_c and ip_a show the peak cathodic and anodic current respectively for a reversible reaction.

1.2.8. Square wave voltammetry, SWV

Square-wave voltammetry (SWV) is one of the four main voltammetric techniques. Introduction by modern computer-controlled electroanalytical devices such as Autolab and µAutolab (both EcoChemie, Utrecht), BAS 100 A (Bioanalytical Systems), and PAR Model 384 B (Princeton Applied Research) The implementation of SWV prospered in the last decade, first because of the diffused use of the instruments mentioned above, second because of a well-developed theory, and finally, and the most important, because of its high sensitivity to surface-confined electrode reactions (Scholz, 2010). This technique represents a further development of the preceding one. A rapid step scanning of potential is applied to the electrode and, moreover on each step is superimposed a high frequency square wave (20–100 Hz). The current is sampled two times at the end of the two half waves. If the amplitude of the wave is very little and the redox system is reversible, during the first half wave the electro active compound can be reduced (or oxidised), while, in the second half wave, at the contrary, it can be oxidised (or reduced) (Figure 1.12). The two current are then summed up and so, the sensitivity is increased (Protti, 2001). Many advantages for square wave voltammetry such as high speed and very low detection limit near $1x10^{-8}$ M can be achieved. From the rapid scanning capability and the reversal nature of the square wave voltammetry, kinetic studies can be undertaken. As a comparison between square wave and differential pulse voltammetry for reversible and irreversible cases, results indicate that the square wave

currents are higher than the differential pulse response with 4 and 3.3 times respectively (Figure 1.13) (Wang, 1994).

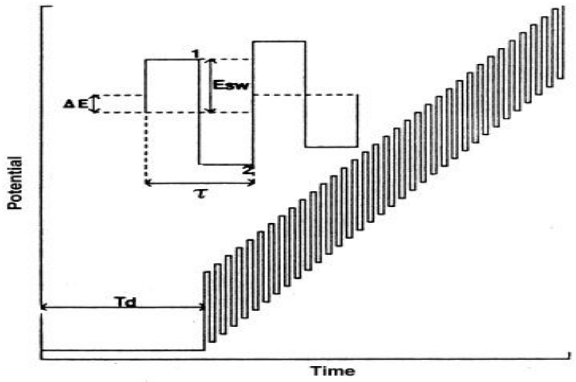


Figure 1.12. Square–wave waveform showing the amplitude (E_{sw}), step height (ΔE), square-wave period (ζ), delay time (T), and current measurement times (1 , 2).

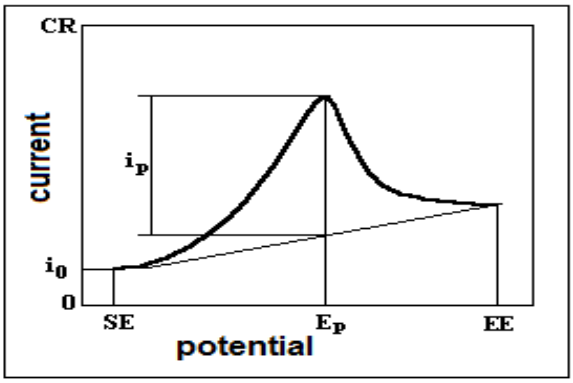


Figure 1. 13. Square–wave voltammetry for reversible electron transfer. (Protti, 2001).

1.2.9. Stripping voltammetry

Stripping technique is one of the extreme important and critical electrochemical technique for measuring trace metals and organic samples. The term stripping is used to a group of procedures based on two steps as follows:

1- The electrochemical precipitation or accumulation of the investigated analyte at the electrode surface under the controlled potential.

2- The stripping or dissolution of the investigated analyte from the electrode surface by the voltammetric techniques (stripping=measurement step).

As a common characteristic all stripping methods include a preconcentration step (deposition step) but they called different names because of the using different analysis (stripping) step. The sensitivity of voltammetric methods can be greatly enhanced by the use of a preconcentration, or accumulation step in which the compound is accumulated at the electrode by either a faradaic (anodic, cathodic or potentiometric) or non-faradaic (adsorption) process (Figure 1.14).

Electroanalytical stripping techniques are the best known analytical method which can boast the following advantages:

1- Low detection and determination limits.

2- High sensitivity.

3- Relative simplicity, rapidity.

4- Wide spectrum of the analyte (especially drug compounds).

5- Insignificant effect of the matrix (from endogenous substances in biological media or from excipients in pharmaceutical dosage forms).

6- Low cost of equipment (Kubiak et al., 2001; Ozkan, 2015; Kissenger and Heinemann, 1996).

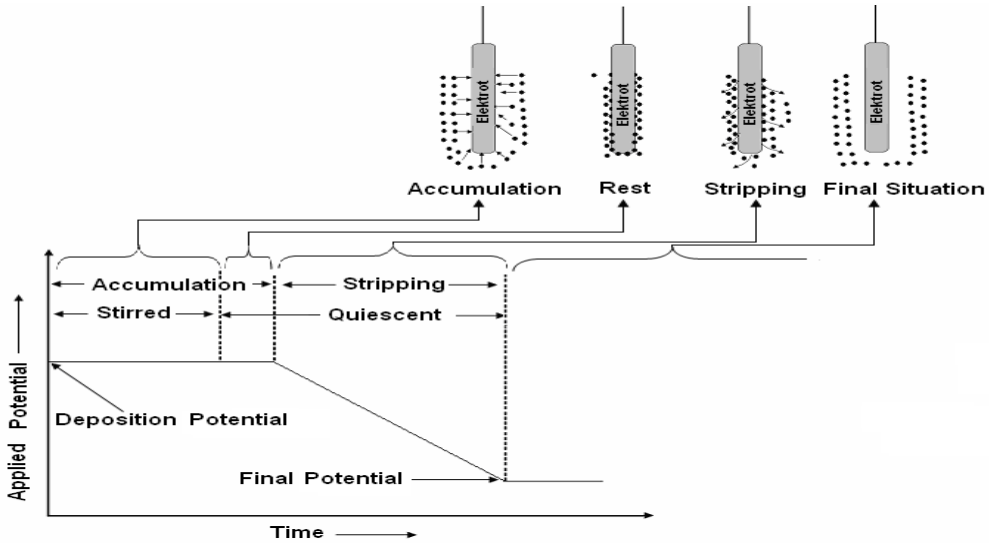


Figure 1.14. Events in the voltage-time plot and the electrode surface in the stripping analysis.

Stripping voltammetry is used fundamentally in trace analysis. The limit of detection depends on the factor of proportion between the activity of the accumulated substance and the bulk concentration of the analyte. This factor is a constant in the situation of a chemical accumulation, but for electrochemical accumulation it depends

on the electrode potential. The factor of proportion between the extreme stripping current and the analyte concentration is seldom known exactly, actually it is frequently neglected. For the analysis it suffices to establish the linear relationship experimentally. The slope of this relationship may vary from one sample to another because of different influences of the matrix. In this case the concentration of the analyte is determined by the method of standard additions (Scholz, 1992).

1.2.10. The supporting electrolyte and solvent used in voltammetry

Electrochemical measurements were carried out in a solvent-supporting electrolyte system. Very pure of the solvent and supporting electrolyte. It is important in the voltammetric measurement. It must be determined before the measurement to be made of the solvent and supporting electrolyte, the solvent to be selected electrochemical inertness, conductivity, resolving power, chemical inertness, viscosity, dielectric constant, easy availability, knowledge of the properties of cheapness and easy to be purified is required. In addition the solvent should not react with the analyte and should be electrochemically inert over a wide voltage range. Water is the most commonly used solvent is generally sufficient to be treated twice. From organic solvent dimethylformamide (DMF), dimethylsulfoxide (DMSO), acetonitrile and methanol are frequently used solvent. In some embodiments mixtures of solvents are also used. The toxicity of DMF, and has drawbacks such as entering undesirable reactions. Thus DMSO and acetonitrile are more suitable solvent by DMF. DMSO is the only downside narrow operating range. Very pure commercially available and used without the need for any purification;

a) Providing a solution conductivity,

b) Control of the pH value (protection of electroactivity in the voltage range of organic materials and inorganic materials to be hydrolyzed)

c) Obtaining a well-developed and well-separated peaks of some complex formation

d) Be shifted to a more negative voltage peak of hydrogen and must be added to the destruction of the catalytic effect of hydrogen peak (Heyrovsk and Zuman, 1968).

Supporting electrolyte composition can influence the selectivity of the voltammetric measurements. Ideally electrolyte, well-defined for the simultaneous

determination of designated species and should give well-separated peaks. Due to the sensitivity of certain impurities in the supporting electrolyte voltammetric techniques can affect the accuracy of the transaction. Therefore, supporting electrolytes should be prepared from fairly high purity reagents and should not be reduced or oxidated easily. Supporting electrolyte concentration, all other the concentration of electroactive species must be large. In general, the solution prepared in concentrations from 0.1-1 M. These concentration values provide minimum contamination and high conductivity (Wang, 2000).

1.2.11. Electrode profile in the mixed solution

The intermediate surface of the stirred solution between a working electrodes immersed in solution composed of heterogeneous due to exchange electrons. Depending on the stirring system and it has the shape of three movements. Moving (stirred) of heterogeneous composition of the system (Figure 1.15).

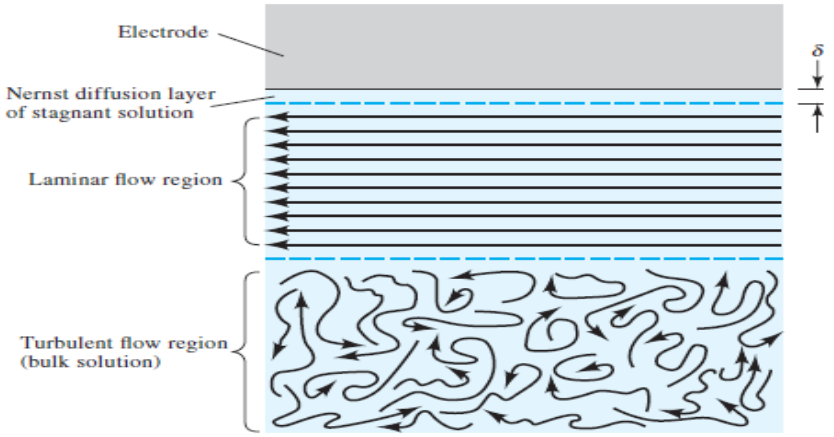


Figure 1.15. Flow patterns and regions of interest near the working electrode in hydrodynamic voltammetry (Skoog et al., 1998).

Turbulent flow region: moving away from the electrode is observed in the solution stack.

Laminar flow zone: when approaching the electrode surface layers to be parallel to one another are shifted out of the electrode surface.

Nernst diffusion layer: too close to the electrode surface (δ cm) laminar flow is approximately zero. This region ‘dead zone’ is also called, the reason for the formation

of stagnant zones are formed on the surface electrode with strong gravity and friction thin film solution.

1.2.12. Electrode voltage-analyte concentration Faradaic relations and operations

The aim of electroanalytical experiments is controlled applied voltage, target current which is proportional to the analyte concentration is to obtain a response. Electrode-electrolyte interface during the redox reactions occurring in the flow are transmitted by direct transfer of electrons. During the transmission of current between the solution and the electrode surface, oxidation of one of the electrodes and the other reduction reaction occurs. This reaction in general;

$O + ne^- = R$

O and R, respectively, is shown by the oxidized redox couple and the reaction expressed by the reduced form. In systems that are controlled by the rules of thermodynamics, electrode voltage (E), the concentration of electroactive species at the electrode surface (C^o and C), used to detect according to the Nernst equation.

$E = E^0 + (2.3RT / nF) \text{ Log } C^0 / C$

E^0 = standard voltage for the redox reaction

R = the gas constant (8.314 J K^{-1} mol^{-1})

T = temperature (^0K)

n= number of electrons transferred in the electrode reaction

F = Faraday's constant (96 487 coulombs mol^{-1})

The amount of chemical substances in an electrode that is directly proportional to the flux representing these types of operations "Faradaic processes" in the current formed in this way is called Faradaic currents.

The substance to be analyzed and the concentration of the product only as a function of the distance from the electrode surface and varies in the Nernst layer (Bard, 1980; Wang, 2000).

1.2.13. Reference electrodes used in voltammetry

The ideal reference electrode has a potential that is accurately known, constant, and completely insensitive to the composition of the analyte solution. In addition, this electrode should be rugged, easy to assemble, and should maintain a constant potential while passing minimal currents.

The most commonly used electrodes comparison are:

Calomel Reference Electrodes: Calomel reference electrodes consist of mercury in contact with a solution that is saturated with mercury (I) chloride (calomel) and that also contains a known concentration of potassium chloride. Calomel half-cells can be represented as follows:

$Hg|Hg_2Cl_2$ (sat'd), KCl (*X*m) ||

where *X* represents the molar concentration of potassium chloride in the solution. The electrode potential for this half-cell is determined by the reaction

$Hg_2Cl_2(s) + 2e^- \rightleftharpoons 2\,Hg(l) + 2Cl^-(aq)$

And depends on the chloride concentration. Thus, the KCl concentration must be specified in describing the electrode. The "saturated" in a saturated calomel electrode refers to the KCl concentration and not the calomel concentration. All calomel electrodes are saturated with Hg_2Cl_2 (calomel). Notice the Hg—Hg bond in the structure. There is considerable evidence that a similar type of bonding occurs in aqueous solution, and so mercury (I) is represented as Hg_2.

Silver/Silver Chloride Reference Electrodes: The most widely marketed reference electrode system consists of a silver electrode immersed in a solution of potassium chloride that has been saturated with silver chloride:

Ag|AgCl (sat'd), KCl (sat'd)||

The electrode potential is determined by the half-reaction

$AgCl(s) + e^- \rightleftharpoons Ag(s) + Cl^-$

Normally, this electrode is prepared with either a saturated or a 3.5 M potassium chloride solution. Figure 1.16 shows a commercial model of this electrode, which is little more than a piece of glass tubing that has a narrow opening at the bottom connected to a Vycor plug for making contact with the analyte solution. The tube

contains a silver wire coated with a layer of silver chloride that is immersed in a potassium chloride solution saturated with silver chloride.

Silver/silver chloride electrodes have the advantage that they can be used at temperatures greater than 60°C, while calomel electrodes cannot. On the other hand, mercury (II) ions react with fewer sample components than do silver ions (which can react with proteins, for example). Such reactions can lead to plugging of the junction between the electrode and the analyte solution. At 25°C, the potential of the saturated calomel electrode versus the standard hydrogen electrode is 0.244 V. For the saturated silver/silver chloride electrode, it is 0.199 V (Skoog et al., 1998).

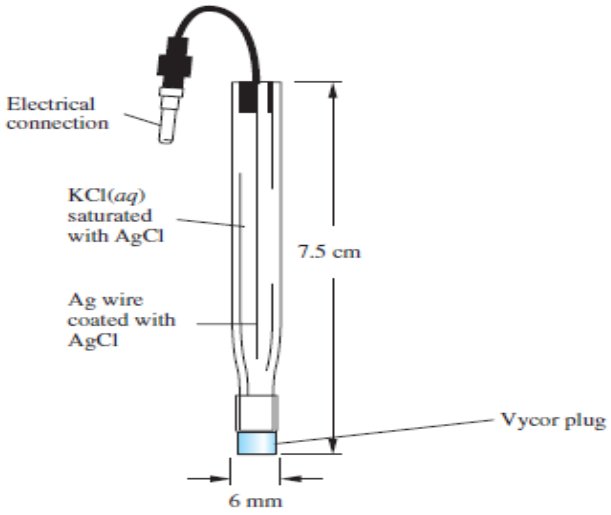


Figure 1.16. Diagram of a silver/silver chloride electrode (Skoog et al., 1998).

1.2.14. Working electrodes used in voltammetry

The performance of the voltammetric procedure is strongly influenced by the material of the working electrode. The working electrode should provide high signal to noise characteristics, as a reproducible response thus, its selection depends primarily on two factors: the redox behavior of the target analyte and the background current over the potential region required for the measurement. Other consideration include the potential window, electrical conductivity, surface reproducibility, mechanical properties, cost availability, and toxicity, a range of materials have found application as working electrodes for electroanalysis. The most popular are those involving mercury, carbon, or noble metals (especially platinum and gold) (Figure 1.17).

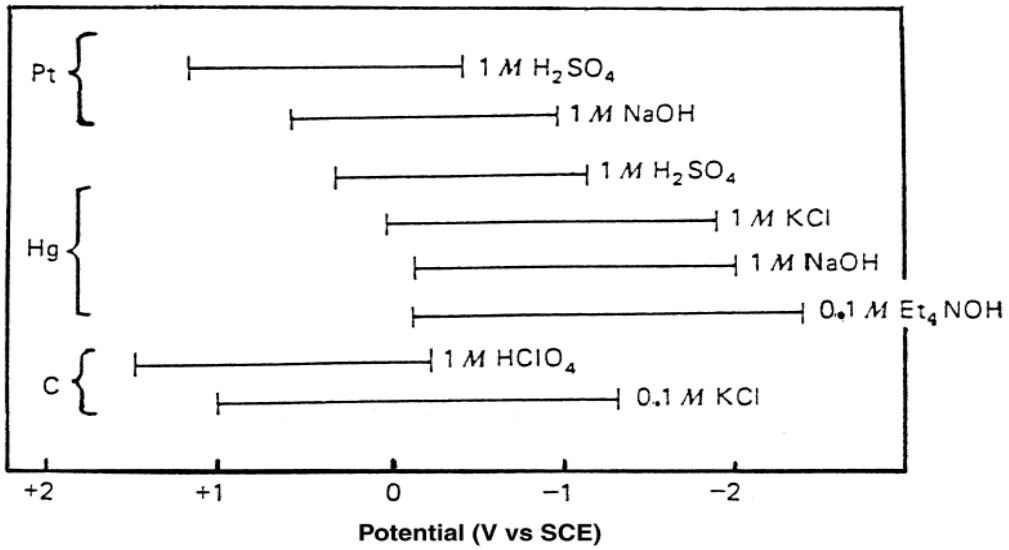


Figure 1.17. Accessible potential window of platinum, mercury and carbon electrode in various supporting electrolytes (Skoog et al., 1998).

Studies frequently used in voltammetry electrode can be classified as follows;

1- Mercury electrodes

2- Solid electrodes

3- Chemically modified electrodes

4- Microelectrodes

1.2.14.1. Mercury electrodes

Mercury is widely used in the practice of electroanalytical chemistry both for working electrodes and for reference electrodes (in the latter case usually as an electrode of the second kind). The use of mercury is nearly ideal for working electrode construction for several reasons mercury has a large liquid range (-38.9 to 356.9 C^0 at the normal pressure) and electrodes of various shapes may be easily prepared either in pure form or by deposition of mercury on the conducting support. The surface of such electrodes is highly uniform and reproducible if the mercury is clean (Kissinger, 1996). Disadvantages of the use of mercury are its limited anodic range (due to the oxidation of mercury) and its toxicity.

There are several types of mercury

1-the dropping mercury electrode (DME)

2-the hanging mercury drop electrode (HDE)

3-mercury film electrode (MFE)

DME used in polarographic and electrocapillary studies, HM is widely used as a working electrode for the cyclic voltammetry and stripping techniques, MFE particularly widely used in the stripping methods (Wang, 2000).

1.2.14.2. Solid electrodes

The limited anodic potential range of mercury electrodes has precluded their utility for monitoring oxidizable compounds solid electrodes is preferred because allowing of a wider voltage anodic study to examine the electrochemical properties of the compound can be oxidized. There are various solid working electrodes made of different materials such as carbon, platinum, gold, silver and copper can be used as rotating discs and stationary electrodes are designed as vibratory solid electrodes. Analysis depending on the analyte, the biggest problem is the necessity of creating a homogeneous surface of solid electrode for each new analysis. The use of such electrodes requires precise electrode pretreatment and polishing to obtain reproducible results the nature of these pretreatment steps depends on the material involved. Mechanical polishing (to a smoth finish) and potential cucling are commonly used metal while various chemical, electrochemical, or thermal surface procedure are added for activating carbon based electrodes (Wang, 2000), solid electrodes can be classified as follows in terms of common usage;

1 -Metal electrodes

2- Carbon electrodes

1.2.14.2.1. Metal electrodes

Noble metals are usually used in making metal electrodes. The most common use has gold and platinum electrodes. Such electrodes offer a very favorable electron-transfer kinetics and a large anodic potential range, in contrast, the low hydrogen overvoltage at these electrodes limits the cathodic potential window (to the – 0.2 to – 0.5) region, depending upon the pH). The surface problem is less severe in nonaqueouse

media where noble metal are often an ideal choice. Compared to platinum electrode, gold ones are more inert, and hence are less prone to the formation of stable oxide films or surface contamination. Gold electrode are also widely used as substrates for self-assembled organosulfur monolayers or for stripping measurements of trace metal, other metals, such as copper, nikel, or silver have been used as electrode materials in connection with specific applications, such as the detection of amino acids or carbohydrates in alkaline medium (copper and nickel) and of cyanide or sulfur compounds (silver) unlike platinum or gold electrode, the copper electrode offers a stable response for carbohydrates at constant potential (Wang, 2000).

1.2.14.2.2. Carbon electrode

Solid electrodes based on carbon are currently in widespred use in electroanalysis, primarily because of their broad potential window, low background current, rich surface, low cost, chemical inertness, and suitability for various sensing and application. Carbon is easily contaminated by organic compounds due to the high surface activity. Depending on various functional groups of active oxygen comprises groups of the aromatic ring in the graphite powder (hydrogen, hydroxyl, carboxyl groups and even quinones) may form bonds in the carbon surface. This carbon material surface due to the presence of many different functional groups may be attached (Wang, 2000). Carbon electrodes; glassy carbon, carbon paste, screen printed carbon can be analyzed as five sub-headings, including carbon-fiber and graphite pencil. Carbon atoms in the Figure 1.18 shows the sequence graphite powder.

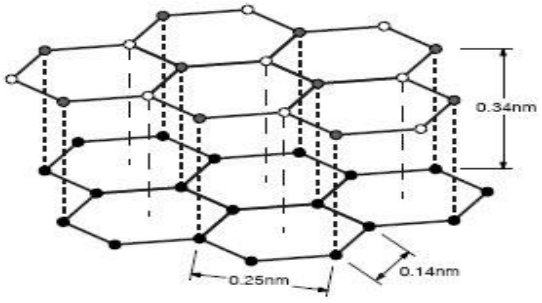


Figure 1.18. Arrangement of the carbon atoms in graphite powder.

Glassy carbon electrode (GCE):

Glassy carbon electrode also known as vitreous glassy carbon has been the subject of intense electroanalytical research in the past 10 years because of its excellent mechanical and electrical properties, wide potential window, chemical inertness (solvent resistance), and relatively reproducible performance. The electrode body made of inert material into glassy carbon electrode surface is obtained by compressing only 1-2 mm position. The glassy carbon material or the phenol-formaldehyde polymers with controlled temperature program (300-1200 ° C) or polyacrylonitrile is obtained by the 1000-3000 ° C temperature range suffered the carbonization under pressure. High-density, small pores containing an amorphous structure. Likewise it consists of interconnected thin graphite (Figure 1.19). Surface pre-treatment to be applied to the glassy carbon electrode will increase the reproducibility and analytical performance by allowing the activation of the electrode. Carbon paste electrodes based on physical strength is more due to the response electrode surface is much smoother and more uniform repeatable. As with other metal ions to be deposited on the carbon electrode negative voltage and enables various polymers to be coated (Wang, 2000).

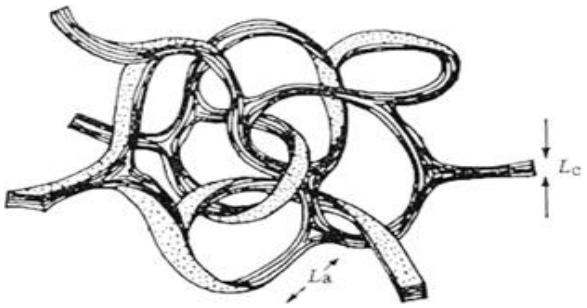


Figure 1.19. The structure of the amorphous glassy carbon electrode.

Carbon past electrode (CPE):

Carbon paste electrode (CPE) belong to a special group of heterogeneous carbon electrodes. CPEs are represented by carbon paste, i.e. a mixture prepared from carbon (graphite) powder and a suitable liquid binder packed into a suitably designed electrode body (Figure 1.20). Due to numerous advantageous properties and characteristics, these electrodes are widely used mainly for voltammetric measurements; however, carbon paste-based sensors are also applicable in amperometry, coulometry, and potentiometry. In general, the reason why CPEs are still popular can be seen mainly in the fact that

carbon pastes are easily obtainable at minimal costs and are especially suitable for preparing an electrode material with desired composition, and hence, with pre-determined properties. Electrodes made in this way are usually thought to be used as highly selective sensors for both inorganic and organic electrochemistry (Svancara et al., 1999).

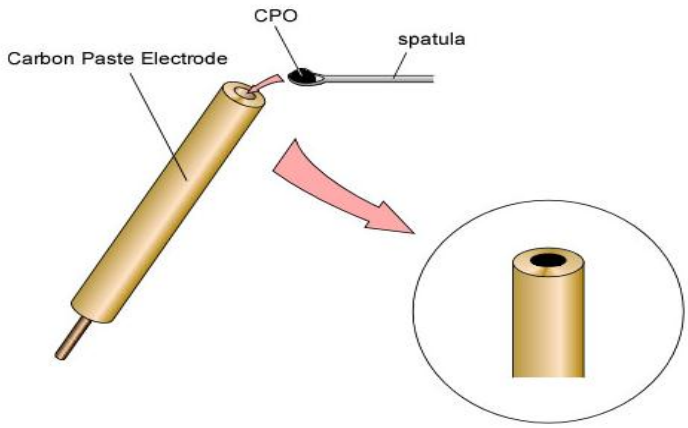


Figure 1.20. Carbon paste electrodes (Als Co., Ltd., 2006).

Carbon – fiber electrode:

Growing interest ultramikro electrodes in electrochemical analysis led to the use of carbon fibers in a wide range. Such materials, high-temperature-resistant polymer compounds produced by bonding depositing catalytic pyrolysis textile chemical gas. Different carbon fiber microstructure are available, depending upon the manufacturing process (Figure 1.21). The can be classified into three broad categories, namely, low-, medium-, and high-modulate types. The last type is most suitable for electrochemical studies because of its well-ordered graphite-like structure and low porosity and the most electroanalytical application will be done on fibers of 5-20 μm diameter that provide the coveted radical diffusion (Wang, 2000).

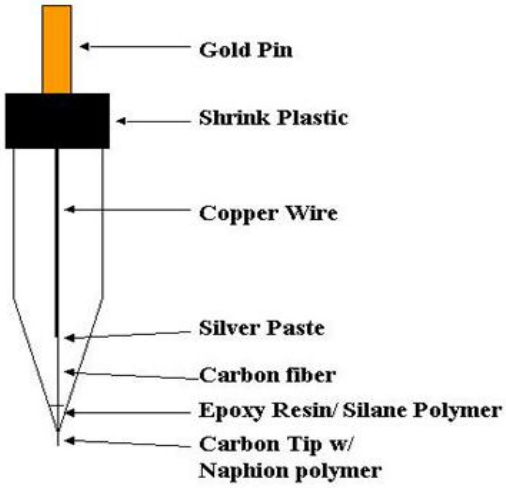


Figure 1.21. Carbon fiber electrode diameter ranging up to 7-30 μm.

Boron-doped diamond electrode:

The era of diamond electrodes started in the eighties by isolated studies of Japanese researchers who suggested the ion-implanted diamond electrodes and Russians suggesting semi-conducting diamond electrodes for photoelectrochemistry. Since then, a tremendous progress could be limited in applications ranging from electrosynthesis, electroanalysis, use in Li-ion batteries, fuel cells, to diamond-based biosensors. During these years it was well established that conductive diamond thin films are in many ways ideal as electrode materials (Figure 1.22). The highest popularity have gained polycrystalline, borond oped diamond (BDD) thin films introduced in 1992 by Fujishima. The first studies conducted with BDD electrodes (BDDE) a year later outlined their suitability for electrosynthesis, electroanalysis, and electrochemical waste treatment. The number of papers devoted to these topics has exceeded 400. Simultaneously, the continuous fundamental research on diamond materials recognized them as potential wide band gap semi-conductors with good electronic, mechanical and chemical properties. Intensive research, especially in the last five years, was focused on the use of diamond-based electronic devices in biosensing, optoelectronics, acoustic, quantum computing and other advanced technologies. Nevertheless, the applications of today, the use of boron-doped diamond (BDD) as a new carbon electrode material is well established for several electrochemical applications, especially electroanalytical ones (Peckova et al., 2009), It is well established that BDD electrodes have several advantages compared with other carbon electrode such as glassy carbon (GC), pyrolytic

graphite (PG), highly oriented pyrolytic graphite (HOPG), and platinum etc., because (BDD) has several superior electrochemical properties such as a wide working potential window in aqueous solutions (up to 3 V), low and stable background current, negligible adsorption of organic compounds and relative insensitivity to dissolved oxygen the extreme robustness and high resistance to corrosion even in strong acidic media, good electroactivity toward certain organic and inorganic species all of which deactivate the surface of other conventional electrodes recommend BDD as an excellent electrode material for several applications, especially in the feld of electroanalytical chemistry. These felds of study have grown considerably in the past decade. Recent studies reported in the literature have shown that several inorganic, organic and biomolecules can be satisfactorily determined with the use of BDD electrodes (Yardım et al., 2014; Laranjeira et al., 2011; Park et al., 2005; Banda, 2012).

Figure 1.22. Boron-doped diamond electrode.

1.2.14.3. Chemically modified electrodes

The attachment of a chemical reagent on the electrode surface, modifying the surface of electrodes to provide some control over how the electrode interacts with its environment has been one of the most active areas of research interest in electrochemistry within the last 30 years. Whereas once the performance of an electrode was limited to the solution it was placed into, the material from which the electrode was made and the potential applied to the surface, the ability to chemically modify electrodes has provided a powerful route to tuning their performance. This has been particularly important to electroanalytical chemistry, where modification has provided

routes to providing selectivity, resisting fouling, concentrating species, improving electrocatalytic properties and limiting access of interferences in a complex sample, such as a biological fluid, but has also had major impact for research into energy conversion and storage, corrosion protection, molecular electronics, electrochromic devices and fundamental research into phenomena that influence electrochemical processes (Alkire et al., 2009; Wang, 2000).

1.2.14.4. Microelectrodes

The term of microelectrodes is reserved here for electrodes with at least dimension not greater then 25 μm. There is a growing interest in the miniaturization in analytical chemistry superiority in the application of these electrodes, such dimension present obvious analytical advantages, including the exploration of microscopic domains, the measurement of regional concentration profile, the determination of capillary electrophoresis and be counted in the microflow system in use in the analysis of very small sample volumes The microelectrodes are usually made of a material inert pressurized from a rod attached to a small flat conductive disk. As this printed circuit board or may be an inert metal such as gold, pyrolytic graphite, glassy carbon, a metal may be coated with a mercury film (Figure 1.23). Some specific analysis and semiconductors such as indium tin oxide may be also used (Wang, 2000).

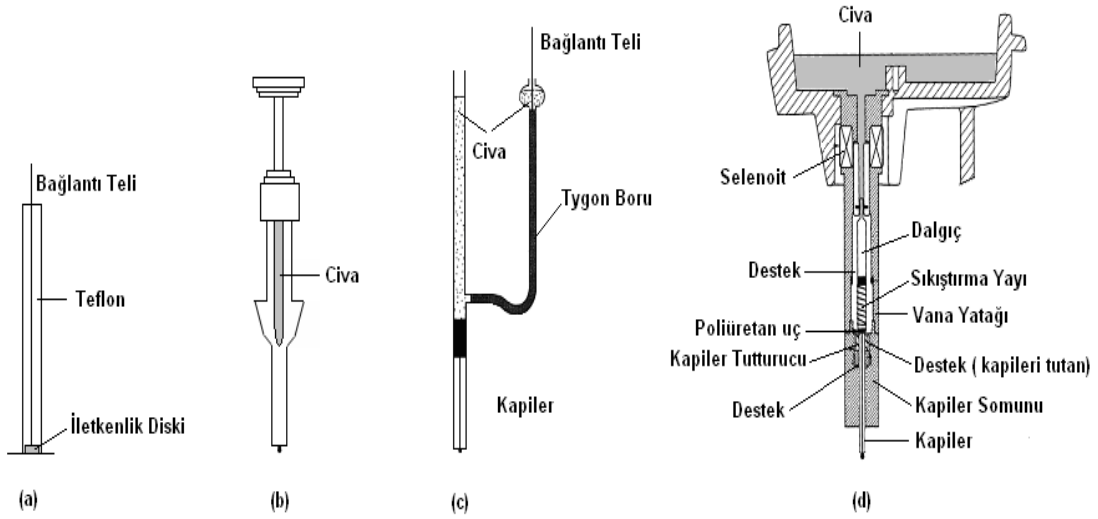


Figure 1.23. Some common types of microelectrodes (a) disc electrode, (b) hanging mercury drop electrode, (c) the dropping mercury electrode, (d) stationary mercury drop electrode (Skoog et al., 1998).

1.3. Chromatography

The word of chromatography is derived from two Greek words–chroma means color and grapheine write chromatography literally mean color writing. Chromatography is usually introduced as a technique for separating and/or identifying the components in a mixture. The basic principle is that components in a mixture have different tendencies to adsorb onto a surface or dissolve in a solvent. It is a powerful method in industry, where it is used on a large scale to separate and purify the intermediates and products in various syntheses. There are several different types of chromatography currently in use–ie paper chromatography; thin layer chromatography (TLC); gas chromatography (GC); liquid chromatography (LC); high performance liquid chromatography (HPLC); ion exchange chromatography; and gel permeation or gel filtration chromatography (Figure 1.24). Chromatography usually consist of a mobile phase and a stationary phase. the mobile phase usually refers to the mixture of the substance to be separated dissolved in a liquid or a gas. The stationary phase is a porous solid matrix through which the sample contained in the mobile phase percolates the interaction between the mobile and the stationary phase result in the separation of the compounds from the mixture the interaction include the physico chemical principles such as the adsorption, ion–exchange, partition, molecular exclusion (Harvey, 2000).

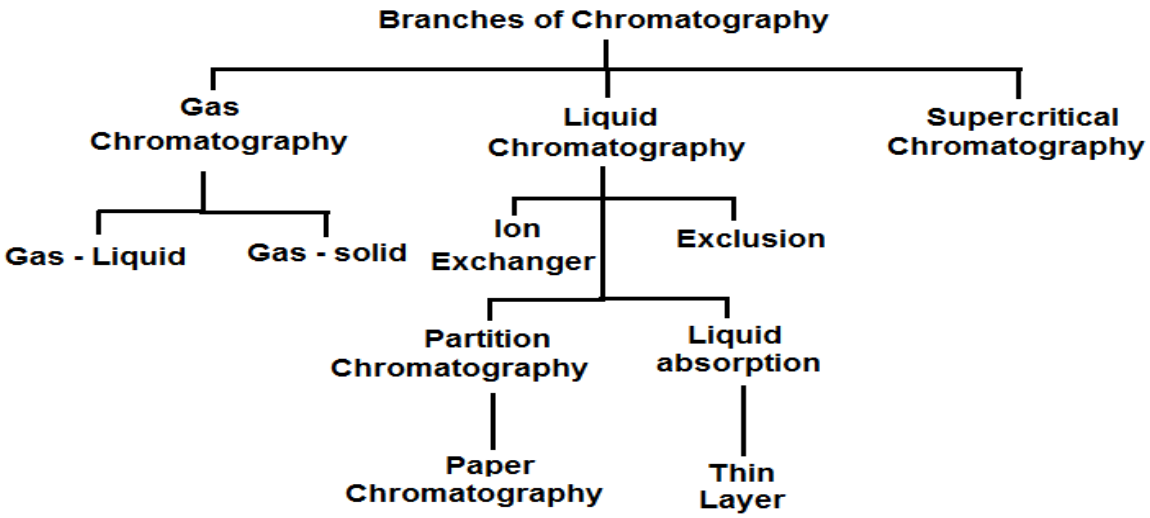


Figure 1.24. Categories of chromatography and their relationship to each other.

1.3.1. Practical Aspects of HPLC Theory

In order to understand HPLC and to utilize its practical applications effectively, some basic concepts of chromatographic theory are presented here.

1.3.2. Chromatographic principles

Resolution:

A common goal of HPLC method development is to achieve adequate resolution of the least-well separated peaks (Rs ≥ 1) and it is the measure of the given separation. Resolution can be described as a function of three independent factors according to equation;

$$Rs = \frac{1}{4}(\alpha - 1) \bullet \left(\sqrt{N}\right) \bullet \left(\frac{k}{1+k}\right)$$

Where α is the selectivity factor for two peaks, N is the column plate number, and k is the retention factor. To obtain optimum resolution of two peaks in the shortest time, all three of these separation factors must be optimised (Kenkel, 2002).

Peak Capacity:

The peak capacity (n_c) is defined as the maximum number of component peaks that can be packed side-by-side into the available separation space, with just enough resolution (Rs) (usually considered to be unity) between neighbours to satisfy analytical goals.

Isocratic Elution:

The theoretical peak capacity in isocratic elution is described according to the folowing equation;

$$n_c = 1 + \frac{\sqrt{N}}{4} \ln \frac{1+k_2}{1+k_1}$$

Where k_2 and k_1 are the retention factors of the last and first eluted peaks, respectively. Isocratic elution is preferred for samples containing less than 10 components or when the gradient baseline impedes trace analysis. Often the peak capacity of isocratic elution with a mobile phase of fixed composition, is limited to yield sufficient resolution of complex samples of wide range in retention ($k_2/k_1 > 15$) (Harvey, 2000).

Gradient Elution:

Gradient elution is another means by which complex samples can be separated, where the mobile phase composition is changed during the chromatographic run, either stepwise or continuously, and can be employed to overcome the limitations in peak capacity and subsequently handle multi-component samples (more than 10 compounds). The peak capacity in gradient elution (where the run time may be increased with no determination to band broadening as in an isocratic system) is described according to the tquation.

$$n_c = 1 + \frac{\sqrt{N}}{4}\frac{1 + k_{G2}}{1 + k_{G1}}$$

Where k_{G2} and k_{G1} are the retention factors of the last and first eluted peaks, respectively. In gradient elution, the separation performance is described by column peak capacity.

Multidimensional HPLC (MDLC):

A more powerful means of increasing the peak capacity and also gaining selectivity can be achieved by incorporating more than one separation dimension, referred to as multidimensional separation, for HPLC this generally implies two dimensions. In the expanded two-dimensional separation space, offering an additional separation step that ideally presents a different retention mechanism to that of the first dimension, the probability that two species will elute with exactly the same retention time in both separation dimensions decreases compared to the one-dimensional separation. Multidimensional HPLC has been comprehensively reviewed by numerous research groups (Holler, 1998).

1.3.3. High performance liquid chromatography

High performance liquid chromatography (High Pressure Liquid Chromatography) or (HPLC) is a chromatographic technique. High Performance Liquid Chromatography (HPLC) was developed in the late 1960s and early 1970s. Today, it is widely applied for separations and purifications in a variety of areas including pharmaceuticals, biotechnology, environmental, polymer and food industries analysis. The reasons for the popularity of HPLC include sensitivity, separation ability of non-volatile compounds, and its widespread applicability to substances that are of strong interest to industry, science, and the public at large. Some of these substances of interest include, amino acids, proteins, nucleic acids, hydrocarbons, carbohydrates, drugs, terpenoids, pesticides, antibiotics, steroids, metal-organic species, and others too numerous to mention (Holler., 1998). In all chromatographic techniques, the sample is transported or carried by a mobile phase. This mobile phase may be a gas, liquid, or supercritical fluid depending upon the chosen technique. With HPLC, as the name suggests, the mobile phase is a liquid. The mobile phase and sample are forced through a column containing the stationary phase. The mobile phase and stationary phase are the soul of the chromatographic technique. Both must be carefully chosen to achieve separation of the components of the mixture of interest. The phases are chosen so that some components are retained longer on the stationary phase while others are transported out of the column more quickly. Species that have a strong affinity for the stationary phase are retained longer while those with a stronger affinity for the mobile phase are eluted more quickly. HPLC allows the analyst to take advantage of differences, even very slight differences, to separate components of mixtures. This is doing by choosing an appropriate column and mobile phase combination. Reversed phase HPLC consists of a nonpolar, usually hydrocarbon, stationary phase with a polar mobile phase. Examples of the stationary phase would be the use of a C8 or C18 column. This column consists of an eight or eighteen carbon hydrocarbon chain bonded onto a solid support, such as silica. The mobile phase would be polar solvents such as methanol, water, or a combination thereof. Reversed phase HPLC is the most popular

liquid chromatography technique. It has wide applicability to a diverse group of compounds. Once the stationary phase is chosen, the mobile phase may be altered to increase or improve separation. One may vary the composition of the mobile 26 phase by making it more or less polar, adjusting pH, adding ionic salts, or adding surfactants. A mobile phase consisting of purely water has been explored because of the desirable characteristics it may present. A 100% aqueous mobile phase offers the advantages of being a very inexpensive and non-toxic eluent. However, reproducibility of C18 and C8 stationary phases, the most common columns used for reversed-phase HPLC, decreases when the eluent is more than 98% aqueous. This problem has been explained as a collapsing of the stationary phase hydrocarbon chain when exposed to pure water. If the organic composition of the mobile phase was too low, the stationary phase would collapse onto itself in a low-energy conformation (Doshi et al., 2002) further explored the application of aqueous mobile phases to reversed-phase stationary phases for the analysis of starch syrups. One of the variables examined was pH. The 100% water mobile phase was adjusted to pH values of 6.5 and 2.0 using 0.005 M sulfuric acid, and pH 10.0 using triethlyamine. It was noted that the low pH, 2.0, had no significant effect upon retention times or resolution. However, slightly improved resolution was observed with the higher pH, 10.0, but this slight improvement was determined not to be valuable enough to risk degradation of the column at high pH (Classens et al., 1984).

1.3.3.1. Instrumentation

As shown in the Figure 1.25, HPLC instrumentation involve a pump, injector, column, detector and integrator or acquisition and display system. The heart of the system is the column where separation take place. Since the stationary phase may be consistsed of micron-sized porous particles, a high-pressure pump is required to move the mobile phase through the column. The chromatographic process begins by injecting the solute into the injector at the end of the column. Separation of components take place as the analytes and mobile phase are pumped through the column. Ultimately, each component elutes from the column as a peak on the data display. Detection of the eluting components is important, and the method used for detection is dependent upon the detector used. The restraint of the detector to each component is displayed on a chart

recorder or computer screen and is known as a chromatogram. To collect, store and analyze the chromatographic data, integrators and other data-processing equipment are frequently used (Parris, 1984).

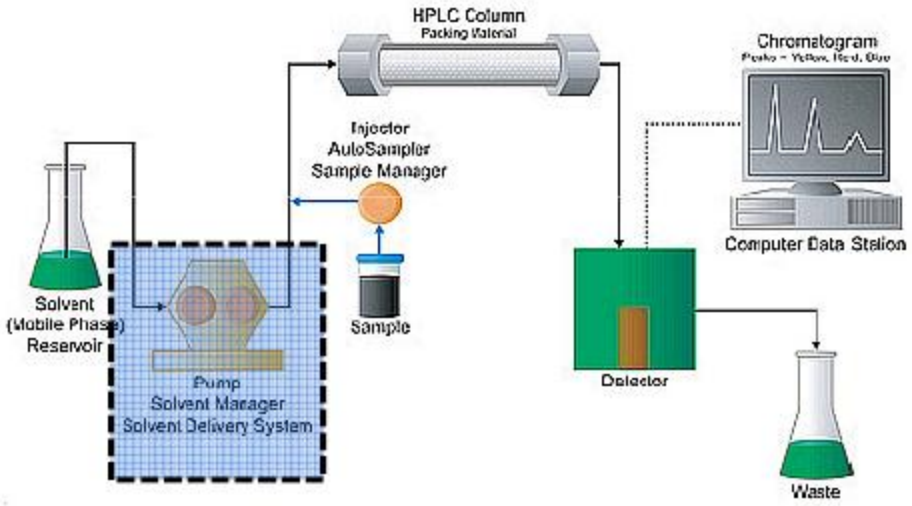


Figure 1.25. Block diagram showing components of a typical apparatus for HPLC.

1.3.3.2. Mobile phase and reservoir

The mobile phase in HPLC refers to the solvent being continuously applied to the column or stationary phase and its acts as a carrier to the sample solution. The type and assembly of the mobile phase affects the separation of the components. Different solvents are used for different kindes of HPLC. For normal-phase HPLC, the solvent is commonly nonpolar, and, in reverse-phase HPLC, the solvent is normally a mixture of water and a polar organic solvent. The purity of solvents and inorganic salts used to make the mobile phase is essential. A general rule of thumb is to use the highest purity of solvent that is available and practical depending on the particular application. The most popular solvent reservoirs are as simple as glass bottles with tubing connecting them to the pump inlet (Holler, 1989).

1.3.3.3. Pumping systems

The pump delivers (meters) solvent for the LC system. The requirements for liquid chromatographic pumps include (1) the generation of pressures of up to 6000 psi (lb/in2), (2) pulse-free output, (3) flow rates ranging from 0.1 to 10 mL/min, (4) flow reproducibilities of 0.5% relative or better, and (5) resistance to corrosion by a variety of solvents. Two major types of pumps are used in HPLC instruments: the screw-driven syringe type and the reciprocating pump. Reciprocating types are used in almost all commercial instruments. Figure 1.26, explains the operating principles of the reciprocating pump. This device consists of a small cylindrical chamber that is full of and then emptied by the backand- forth movement of a piston. The pumping movement produces a pulsed flow that must be thereafter damped because the pulses evidence as baseline noise on the chromatogram. Modern HPLC instruments use dual pump heads or elliptical cams to reduce such pulsations. Advantages of reciprocating pumps include small internal volume (35 to 400 mL), high output pressure (up to 10,000 psi), ready adaptation to gradient elution, and constant flow rates, which are largely independent of column back-pressure and solvent viscosity (Meyer, 2004; Skoog, et al., 1996).

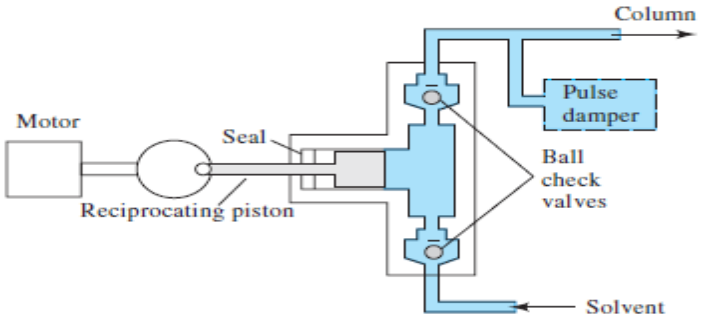


Figure 1.26. A reciprocating pump for HPLC.

1.3.3.4. Injectors

The sample is introduced to the head of the column as a narrow band, with minimum disturbance to the column bed. This should be done without stopping solvent flow to the column, using high pressure sample loop valves of stainless steel. In the fill position, the sample loop is filled at atmospheric pressure. When the valve is actuated, the sample in the loop is swept into the column (Figure 1.27) (Holler, 1989).

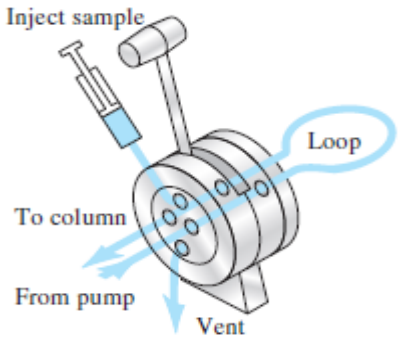


Figure 1.27. A sampling loop for liquid chromatography.

1.3.3.5. Columns for HPLC

Considered the heart of the chromatograph the column's stationary phase separates the sample components of interest using various physical and chemical parameters. Liquid chromatographic columns are usually constructed from stainless steel tubing, although glass and polymer tubing, such as polyetheretherketone (PEEK), are sometimes used. In addition, stainless steel columns lined with glass or PEEK are also available. Most columns range in length from 5 to 25 cm and have inside diameters of 3 to 5 mm. Straight columns are invariably used. The most common particle size of packings is 3 or 5 mm. commonly used columns are 10 or 15 cm long, 4.6 mm in inside diameter, and packed with 5-mm particles. Columns of this type provide 40,000 to 70,000 plates/m. In the 1980s, microcolumns became available with inside diameters of 1 to 4.6 mm and lengths of 3 to 7.5 cm. These columns, which are packed with 3- or 5-mm particles, contain as many as 100,000 plates/m and have the advantage of speed and minimal solvent consumption. This latter property is of considerable importance because the high-purity solvents required for liquid chromatography are expensive to purchase and to dispose of after use. Figure 1.28, illustrates the speed with which a separation can be performed on a microbore column. In this example, MS/MS was used to monitor the separation of rosuvastatin from human plasma components on a column that was 5 cm in length with an inside diameter of 1.0 mm. The column was packed with 3-mm particles. Less than 3 minutes were required for the separation (Fifield and Kealey, 2000).

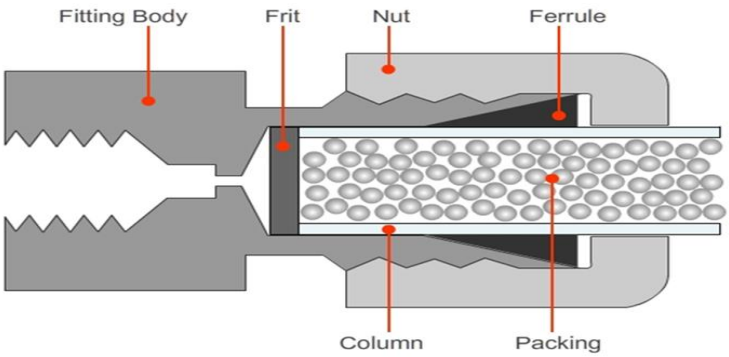


Figure 1.28. The column for HPLC

1.3.3.6. HPLC detectors

A major instrumentation requirement in modern LC is a sensitive detector for continuously monitoring the column effluent. Unfortunately, the sensing of LC bands can be difficult, since the physical properties of both mobile phase and solutes are often quite similar. Various approaches to the problem of detection have been pursued in the development of modern LC;

• Differential measurement of a bulk or general property of both sample and solvent.

• Measurement of a sample property that is not possessed by the mobile phase.

• Detection after eliminating the mobile phase.

A variety of detectors have been developed for LC based on one of these approaches. Thus, the detector used will depend on the nature of the sample. The most widely used detectors for liquid chromatography are based on absorption of ultraviolet or visible radiation. Both photometers and spectrophotometers, specifically designed for use with chromatographic columns, are available from commercial sources. Photometers often make use of the 254 nm and 280 nm lines from a mercury source because many organic functional groups absorb in the region. Deuterium sources or tungsten-filament sources with interference filters also provide a simple means of detecting absorbing species. Some modern instruments are equipped with filter wheels that contain several interference filters, which can be rapidly switched into place. Spectrophotometric detectors are considerably more versatile than photometers and are also widely used in high-performance instruments (Figure 1.29). Modern instruments use diode-array

detectors that can display an entire spectrum as an analyte exits the column (Mcmahon, 2007; Skoog et al., 1996; Snyder et al., 1979).

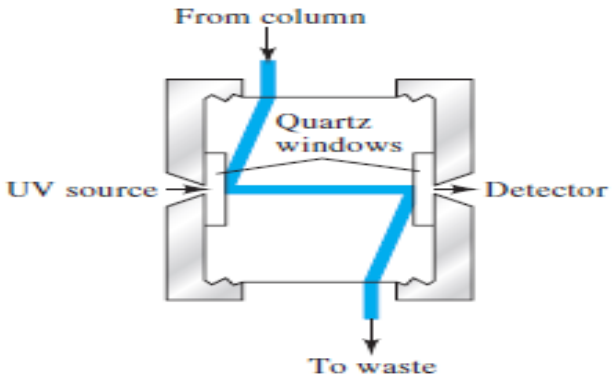


Figure 1.29. A UV-visible absorption detector for HP.

2. LITERATURE REVIEWS

Determination of vanillin and caffeine by different method

Veni et al. (2013), high performance thin layer chromatography method was used for determination of vanillin in food products. Aluminium plates precoated with silica gel 60 GF 254 were used as stationary phase and a mixture of methanol-water-glacial acetic acid in the ratio of 20:05:02 (v/v) as mobile phase. Determination was carried out by scanning the developed spots using a densitometer in absorbance mode at 275 nm. The Rf value of vanillin was 0.84. Linearity was observed in the concentration range of 200-1000 ng/spot.

Sujalmi et al. (2014), high performance liquid chromatographic (HPLC) method was used for the separation, identification and quantification of vanillin in ethanolic extracts of cured vanilla. The vanillin was separated on C18 column using a mobile phase gradient of methanol-acidified water (10-90), detection at 280 nm. The HPLC technique allows a more accurate means of determining the vanillin content of vanilla than the spectrophotometric method.

Guo-lan et al. (2009), a gas chromatography method was uesd for determination of vanillin content in milk powder the flame ionization detector (FID) has been established. The extraction efficiency of different solvents has been investigated at the stage of pretreatment. Then,optimization of separation conditions,such as capillary columns with different polarity,carrier gas speed of flow,column temperature,is carried out.Thus the good separation effect of peaks is obtained.The standard curve equation is y = 8539099x-2189 with a correlation coefficient of 0.9995,and the detection limit is 0.003 mg g^{-1}.The recovery ranges from 93.8 % to 99.4 % with an average value of 96.8 %,RSD = 2.5 %(n=5).The established method is applied to determine vanillin in three brand milk powder,and the results show that the method is accurate and satisfactory.

Sanchez et al. (1990), a spectrophotometric was used for the co-occurrent determination of vanillin (4- hydroxy-3-methoxybenzaldehyde) and syringaldehyde (4-hydroxy-3, 5-di methoxy benzaldehyde). The method reduces the mutual interference between vanillin and syringaldehyde and let the determination of these compounds without a previous extraction step. The precision of the method, expressed as the relative standard deviation, is better than 4%. All spectrophotometric measurements were carried out on a Shimadzu UV-240 Graphicord recording spectrophotometer in 1-cm quartz cells. The instrument parameters were: slit-width, 2 nm; scan speed, 3 nm s^{-1}, and recorder chart speed, 10 nm cm^{-1}. First derivative ultraviolet (UV) spectra were obtained with a Shimadzu attachment (optional programhnterface, Model OPI-2) giving from first to fourth derivatives, and AA values of 1, 2 and 4 nm.

Ruiz et al. (1990), Vanillin (4-hydroxy-3-methoxybenzaldehydea) and p-hydroxybenzaldehyde were determined by spectrophotometric derivative measurements in vanilla bean extracts. The method tolerates p-hydroxybenzaldehyde to vanillin ratios of 3: l (w/w) with relative error below 5% in the determination of vanillin. This method compares favorably with the official AOAC spectrophotometric procedure. Relative errors of 0.70% and RSD's of 1.0% are obtained.

Chowdhury et al. (2012), high-Performance Liquid Chromatography was used for the determination of caffeine. Chromatographic determination was execution on a reversed phase C18 column (4.5 mm x 250 mm; 5 μm particle size) using a mixture of water and methanol (60:40) as mobile phase at a flow rate of 1ml/min with UV detection at 272 nm. The method was also used in determination caffeine content in five commercial brands available in Bangladeshi market. The method was linear in the range between 12 – 28 μg/ml, showed good correlation coefficient (R^2 = 0.9992) and good accuracy study (97.35 %-100.02%). The method was found to specific for caffeine in presence of common excipients or in presence of paracetamol in combination dosage form. Hence it can be employed for routine analysis of caffeine both in bulk and commercial formulations and in combination dosage form with paracetamol.

Nour et al. (2010), High-Performance Liquid Chromatography was used for the determination of caffeine.in the Different brands of soft and energy carbonated drinks available on the Romanian market . HPLC with a diode array UV-VIS detector at 217 nm. The mobile phase consisted of potassium dihydrogen orthophosphate buffer (0.02

mol/L, pH 4.3) and acetonitrile (88:12, v/v). the column was a reverse phase C18 and The caffeine contents in energy drink samples ranged from 16.82 mg/100 mL to 39.48 mg/100 mL while the carbonated soft drink group showed caffeine content in the range of 9.79 –14.38 mg/100 mL.

Mohammed et al. (2012), ten brands of beverages (soft and energy drinks) consumed in Basrah/Iraq, were analyzed for its pH, trace minerals and caffeine contents. The beverages included (O_2, Boom Boom, Wild Tiger, Power horse, Kalaschnikow, Pit bull, Pepsi, Mountain dew, Riviere and Barbican). Caffeine was determined by using shimadzu gas chromatography. Caffeine is separated on a micro C18 column, using a mobile phase of 25% acetic acid, 70% water and 5% isopropyl alcohol at pH 3.0 and UV detection at 254 nm. Beverages are filtered through 0.45 micron filters and injected directly into the chromatograph. Caffeine is eluted in approximately 7 min.

Amos-Tautua et al. (2013), UV spectrophotometric method was used for Quantitative analysis of caffeine, this study was performed to determine the pH and levels of caffeine in eight brands of carbonated and energy drinks available in local market in Yenagoa, Nigeria. Carbon tetrachloride used as the extracted solvent. Results showed that the pH of the beverages were slightly acidic ranging from 5.92-6.44. The minimum caffeine level was observed in the carbonated soft drink Coca Cola (43.71±0.55 ppm), while the energy drink, Red Bull sample showed the highest caffeine content (58.31±0.35 ppm). The carbonated soft drinks showed caffeine levels in the range of 43.71 and 45.83 ppm with average concentration of 44.52 ppm, whereas in the energy drinks it ranged from 47.56 to 58.31 ppm with a mean concentration of 52.24 ppm. The caffeine content in all the beverage samples analyzed in this study are well below the maximum allowable limits set by the US Food and Drugs Administration.

Ogah et al. (2012), determined the caffeine content of various brands of cocoa and coffee-based beverages in Lagos, Nigeria. Ten brands of these products were purchased from various shops in the Lagos metropolis. Caffeine was carefully extracted from each product and analyzed by UV/vis spectrophotometric methods. The results show that the caffeine content of the cocoa-based products ranged from 1.8 to 3.1 mg/g of product while classic and decaffeinated coffee contained 8.4 and 2.3 mg/g of product

respectively. These amounts are considered low and safe for healthy adults when compared with established safety levels of the US Food and Drugs Administration.

Liu et al. (2015), the electrolytic manganese dioxide - graphene (EMDG) composite was used for determination of vanillin in neutral solutions. Vanillin produces an anodic peak at this electrode at about 0.67 V. The electrocatalytic behavior was moreover exploited as a sensitive detection planner for the vanillin determination by differential-pulse voltammetry (DPV). Under optimized conditions, the anodic peak current increased linearly with the vanillin concentration over the range from 1.0×10^{-7} to 4.5×10^{-5} mol L^{-1}. The detection limit was calculated as 3.2×10^{-8} mol L^{-1} (S/N=3). The proposed method was used to the detection of biscuits and chocolates samples with satisfactory results.

Luo and Liu (2012), poly (acid chrome blue K) modified glassy carbon electrode (GCE) was applied for the determination of vanillin. This modified electrode presented excellent electrocatalytic restraint to vanillin with the increase of the electrochemical restraint. Under the optimal conditions a good linear voltammetric response could be obtained over the range of $1.0\times10^{-7}\sim7.0\times10^{-5}$ mol dm^{-3} and the detection limit was found as 3.2×10^{-8} mol dm^{-3} (3σ). The suggested method was successfully used to biscuits and dark-chocolate samples with satisfactory results.

Yardım et al. (2011), the boron-doped diamond electrode was applied for detecting of vanillin. Using square-wave stripping voltammetry, the detecting of vanillin was carried out in phosphate buffer, pH 2.5 at +1.14 V (vs. Ag/AgCl) (a pre-concentration step being carried out at zero potential condition for 60 s). A linear calibration curve was obtained in the concentration range of 0.5–15.0 μgmL^{-1} ($3.3 - 10^{-6}$-9.8 - 10^{-5} mol L^{-1}) with a detection limit of 0.024 μgmL^{-1} ($1.6 -10^{-7}$ mol L^{-1}). As an example, the practical application of the suggested method was tested for the detection of this flavouring agent in commercial pudding powder of Keshkule (Turkish milk pudding with almond flour).

Chethana et al. (2012), the Lysine modified carbon paste electrode (LCPE) was used for the determination of vanillin. The Lysine modified carbon paste electrode showed excellent electrochemical catalytic activity towards vanillin. The

electrochemical attitude of vanillin at the surface of LCPE has been analyzed with respect to sweep rate and pH of the solution. The oxidation peak of vanillin increased significantly compared to that of carbon paste electrode (CPE). An analytical procedure based on differential pulse voltammetry (DPV) has been optimized with a detection limit of 2.88 μM (S/N = 3).

Jinyun Peng et al. (2012), the graphene film-coated glassy carbon electrode (GCE) was used for determination of vanillin Under the optimized conditions, the oxidation peak current was proportionate to vanillin concentration in the range of 6.0×10^{-7} to 4.8×10^{-5}mol L^{-1} with the correlation coefficient of 0.9996 and the detection limit of 5.6×10^{-8} mol L^{-1} (S/N = 3). The results indicated that graphene modified GCE displayed efficiently electro catalytic oxidation for vanillin with relatively high sensitivity and stability. The proposed method was successfully used to biscuits samples with satisfactory results.

Xinying (2014), a poly valine modified electrode was prepared by cyclic voltammetry. This method is simple, highly sensitive and shows good reproducibility. It was found that in pH 7.0 phosphate buffer solution, the oxidation peak current is linearly propertional to its concentration of vanillin in a range of 9.60×10^{-8} ~6.61×10^{-5} mol L^{-1}, with a limit of detection of 1.00×10-8 mol L^{-1}. The method has been demonstrated in the finding of vanillin in infant formula samples.

Bettazzi et al. (2006), a disposable electrochemical sensor was applied for the determination of vanillin. An analytical procedure based on square-wave voltammetry (SWV) was optimised and a detection limit of 0.4 M for vanillin was found. A relative standard deviation of 2% was calculated for a vanillin concentration of 0.1 M. The method was applied to the detecting of vanillin in natural concentrated vanilla extracts and in final products such as yoghurt and compote.

Ague et al. (1999), the electrochemical behavior of cylindrical carbon fiber microelectrodes (CFMEs), 8 mm in length, Square-wave voltammetry (SWV) in ethyl acetate has been used to develop a method for the detecting of the food additive vanillin. A linear calibration graph was obtained in the 1.0 x 10^{-5} to 7.0 x 10^{-4} mol L^{-1}concentration range with a slope of (7.1±0.1) x 10^{3} μA l mol^{-1}A limit of detection of 4.2 X 10^{-6} mol L^{-1} vanillin, and a relative standard deviation of 2.0% for a concentration

level of 5.0x 10^{-4} mol L^{-1}(n=10) were obtained. The SWV method was applied to the determination of vanillin in dehydrated pudding powder.

Li et al. (2015), the Ag-Pd/GO electrod was applied for the detecting of vanillin, and the fabricated sensor shows a wide detection range of 0.02–45 mmol dm^{-3}, low detection limit of 5 nmol dm^{-3} the practical applicability of the suggested method, some food products such as cookie, pastry, jelly and chocolate were used as matrix models to detecting vanillin, further, the RSD is lower than 4.6%, revealing a good precision. The recoveries ranging from 98.8% to 103.5% are satisfactory, which suggests that the proposed method could be suitable and reliable for sensing vanillin in real samples.

Shang et al. (2013), the AuPd–graphene hybrid based electrode was used for detecting of vanillin Under the optimal conditions, the peak current was proportional to the concentration of vanillin in the ranges of 0.1-7 and 10-40μM. the detection limit was 20 nM. The sensitivities were 1.60 and 0.170 mA mM^{-1} cm^{-2}, respectively. The electrode was successfully used to the detecting of vanillin in vanilla bean, vanilla tea and biscuit samples.

Guo-lan (2009), a Nafion–ruthenium oxide pyrochlore chemicallymodified electrode is used for the r determination of vanillin content in milk powder by gas chromatography with flame ionization detector (FID) has been established.The extraction efficiency of different solvents has been investigated at the stage of pretreatment.Then,optimization of separation conditions,such as capillary columns with different polarity,carrier gas speed of flow,column temperature,is carried out.Thus the good separation effect of peaks is obtained.The standard curve equation is y = 8539099 x-2189 with a correlation coefficient of 0.9995,and the detection limit is 0.003 mg.g-1.The recovery ranges from 93.8 % to 99.4 % with an average value of 96.8 %,RSD = 2.5 %(n=5).The established method is applied to determine vanillin in tree brand milk powder,and the results indicate that the method is accurate and satisfactory.

Deng et al., (2015), the graphene–polyvinylpyrrolidone composite film modified acetylene black paste electrode (GR–PVP/ABPE) was applied for determination of vanillin. In 0.1 M H3PO4 solution, the oxidation peak current of vanillin increased significantly at GR–PVP/ABPE compared with bare ABPE, PVP/ABPE and GR/ABPE. Under the optimal experimental conditions, the oxidation peak current was proportional to vanillin concentration in the range of 0.02–2.0 μM, 2.0–40 μM and 40–100 μM. The

detection limit was 10 nM. This sensor was used successfully for vanillin determination in various food samples.

Ziyatdinova et al. (2016), Glassy carbon electrode (GCE) modified with carbon nanofibers (CNF) and surfactants has been tested for the determination of vanillin in Britton–Robinson buffer (pH=2.0). Cationic cetylpyridium bromide (CPB), nonionic Triton X100, and anionic sodium dodecylsulfate surfactants under different concentrations have been tested asmodifier of CNF/GCE. The best form of CVs and voltammetric characteristics of vanillin have been obtained on CPB (0.5 mmol dm-3)/CNF/GCE Differential pulse voltammetry was used for the quantification determination of vanillin. The linear dynamic ranges of the vanillin determination are 0.50–75.0and 75.0–750 lmol dm-3 with the limits of detection and quantification 0.14 and 0.46 lmol dm-3 of vanillin, respectively. The developed approach has been applied for the vanillin quantification in foodstuff (vanilla sugar, vanilla pods, and cream milk powder).

Zhao et al., (2014), an arginine (Arg) functionalized graphene (Arg-G) nanocomposite was used for the determination of vanillin. The Arg-G modified glassy carbon electrode (GCE) exhibied excellent sensitivity properties by cyclic voltammetry (CV) and differential pulse voltammetry (DPV). Under the optimized conditions, the oxidation peak current was proportionate to vanillin concentration in the range of 2.0×10^{-6} to 7.0 × 10−5 mol L^{-1} with the detection limit of 1.0×10^{-6} mol L−1 (S/N = 3) and the correlation coefficient of 0.9986. Furthermore, the sensor could be applied for the detection of vanillin in real food samples with satisfactory results.

Zen et al. (1998), a Nafion–ruthenium oxide pyrochlore chemically modified electrode is applied for the detecting of caffeine in drinks by square-wave voltammetry. Compared to a bare glassy carbon electrode, the chemically modified electrode shows a marked enhancement of the current response. A linear calibration curve was received over the 5–200 mM range in 0.05 M $HClO_4$ solution with a detection limit (3s) of 2 mM. The results of 15 successive repetitive measurement–regeneration cycles exhibited a relative standard deviation of 2.7% for 10 mM caffeine. Thus, the electrode regeneration gives a good reproducible surface. Quantitative analysis was execution by the standard addition method for caffeine content in tea, coffee, decaffeinated coffee, and cola drinks.

Svítková et al. (2012), a boron doped diamond electrode was applied for deecting of caffeine. It was found by cyclic voltammetry that caffeine provided highly reproducible and well-defined irreversible oxidation peak, at very positive potential of +1.55 V vs. Ag/AgCl electrode. The effect of pH and scan rate on the voltammetric response of caffeine oxidation were studied to select the optimum experimental conditions. Linear response of peak current on the concentration in the range from 4×10^{-7} to 2.5×10^{-5} mol L^{-1}, good repeatability (RSD of 2.1 %) and the detection limit of 1.5×10^{-7} mol L^{-1} without any chemical modifications and electrochemical surface pretreatment were observed by differential pulse voltammetry in 0.4 mol L^{-1} perchloric acid. The proposed method was successfully applied for the caffeine determination in commercially available beverage samples, with results in a close statistical agreement to these declared by manufacturer.

Tadesse et al. (2013), a carbon paste electrode was modulated with anthraquinone. Cyclic voltammetry (CV) was used to study the properties of the modified electrode toward the oxidation of caffeine (CAF). Compared to the unmodulated electrode, the AQMCPE showed excellent catalytic activity for the oxidation of caffeine. SWV was applied to plot the calibration curve and there was a good linear relationship between anodic peak current and CAF concentration in the range $2.0 \times 10^{-6} - 8.0 \times 10^{-4}$ M, with the correlation coefficient of 0.998 and a detection limit of 1.43×10^{-7} M. The application of the modulated electrode for the detecting of CAF in pharmaceutical formulation showed good recovery with reproducible results.

Zen et al. (1996), a Nafion/ruthenium oxide pyrochlore chemically modified electrode was used for the co-occurrent detecting of caffeine and acetaminophen in drug formulations by square-wave voltammetry. Linear calibration curves are obtained in 0.05 M perchloric acid, the supporting electrolyte, over 10-250 and 5-250 μM for caffeine and acetaminophen, respectively. The detection limits (30) are 2.2 and 1.2 μM for caffeine and acetaminophen, respectively. The practical analytical utility is illustrated by selective measurements of caffeine and acetaminophen in several commercially available drugs, without any preliminary treatment.

Goyal et al. (2011), an edge plane pyrolytic graphite electrode (EPPGE) was used for the simultaneous determination of acetyl salicylic acid (ASA) and caffeine (CAF) by square-wave voltammetry. At pH 7.2, two well defined peaks with peak

potential (Ep) of ~1225 mV and ~1335 mV were appeared for the oxidation of ASA and CAF, respectively. Anodic peaks for ASA and CAF exhibited a systematic increase in peak current with increase in concentration in the range 0.02–100 μMwith sensitivity of 0.16 and 0.17 μA/μM, respectively. Detection limits (3σ/slope) for ASA and CAF were found to be 0.01 and 0.008 μM, respectively whereas, the limit of quantifications were found to be 0.032 and 0.026 μM, respectively. The proposed sensor has been utilized for the simultaneous determination of ASP and CAF in human urine samples, pharmaceutical preparations and coffee beverages.

Lourenc et al. (2009), a boron-doped diamond (BDD) electrode was used for the single or co-occurrent detection of caffeine and paracetamol in aqueous media (acetate buffer, pH 4.5) by using squarewave voltammetry (SWV) or differential pulse voltammetry (DPV). Using DPV with the cathodically pre-treated BDD electrode, a separation of about 550 mV between the peak oxidation potentials of paracetamol and caffeine present in binary mixtures was obtained. The calibration curves for the simultaneous determination of paracetamol and caffeine showed an excellent linear response, ranging from 5.0×10^{-7} mol L^{-1} to 8.3×10^{-5} mol L^{-1} for both compounds. The detection limits for the simultaneous determination of caffeine and paracetamol were 4.9×10^{-7} mol L^{-1} and 3.5×10−8 mol L^{-1}, respectively. The suggest method was successfully used in the simultaneous determination of caffeine and paracetamol in several pharmaceutical formulations (tablets), with results similar to those obtained using a high-performance liquid chromatography method (at 95% confidence level).

Habibi et al. (2012), a simple and sensitive electrochemical detection of caffeine (CAF) using a single-walled carbon nanotubes on carbon-ceramic electrode (SWCNT/CCE) is reported. CAF was oxidized at the surface of the modified electrode to produce an anodic peak at 1.38 V versus the saturated calomel electrode in 0.01 mol/L, pH 1.7 H_2SO_4 solution in cyclic voltammetry. The experimental parameters, namely, type of electrolyte, pH value, and amount of SWCNTs casted, were optimized. Using the optimum conditions, the anodic peak current in differential pulse voltammetry was linear with CAF concentration in the range of 2.5×10^{-7} - 1.0×10^{-4} mol/L. The detection limit was 1.2×10^{-7} mol/L (S/N = 3). The modified electrode exhibited good stability and can be easily regenerated. The relative standard deviation

of the peak current obtained for a 5.0×10^{-5} mol/L CAF solution was 3.0%. The method was successfully used for the determination of CAF in some practical samples.

Švorc et al. (2012), a boron-doped diamond (BDD) electrode was applied for the detection of caffeine using cyclic voltammetry (CV) and differential pulse voltammetry (DPV). Linear response of peak current on the concentration in the range from $4 \text{-} 10^{-7}$ to $2.5 \text{-} 10^{-5}$ M, good repeatability (RSD of 2.1%) and detection limit of $1.5 \text{x} 10^{-7}$ M without any chemical modifications and electrochemical surface pretreatment were evaluated. The effect of possible interferents appeared to be negligible which evidently proved very good selectivity. The proposed method was successfully applied for the caffeine determination in commercially available beverage samples, with results in a close statistical agreement to those declared by manufacturer and HPLC used as independent method.

Yardım et al. (2013), a boron-doped diamond (BDD) electrode was used for the co-occurrent determination of caffeine (CAF) and chlorogenic acid (CGA) by cyclic and adsorptive stripping voltammetry. The dependence of peak current and potential on pH, scan rate, accumulation parameters and other experimental variables were studied. By using square-wave stripping mode after 60 s accumulation under open-circuit voltage, the BDD electrode was able to separate the oxidation peak potentials of CAF and CGA present in binary mixtures by about 0.4 V in Britton-Robinson buffer at pH 1.0. The limits of detection were 0.107 μg mL^{-1} (5.51×10^{-7} M) for CAF, and 0.448 μg mL^{-1} (1.26×10^{-6} M) for CGA. The practical applicability of this methodology was tested in commercially available beverage samples.

Lourenca et al. (2010), a boron-doped diamond electrode was used for the simultaneous anodic detection of ascorbic acid (AA) and caffeine (CAF) by differential pulse voltammetry. Linear calibration curves (r = 0.999) were obtained from 1.9×10^{-5} to $2.1 \times 10^{-}4$ mol L^{-1} for AA and from 9.7×10^{-6} to 1.1×10^{-4} mol L^{-1} for CAF, with detection limits of 19 mmol L^{-1} and 7.0 mmol L^{-1}, respectively. This method was successfully applied for the detecting of AA and CAF in pharmaceutical formulations, with results equal to those obtained using a HPLC reference method.

Raj and John (2013), the electrocatalytic activity of electrochemically reduced GO (ERGO) modified electrode (ERGO) was used for detecting of four substantial purine derivatives, uric acid (UA), xanthine (XN), hypoxanthine (HXN) and caffeine

(CAF) at physiological pH. Further, it successfully separates the voltammetric signals of the four purine derivatives in a mixture and subsequently used for the simultaneous determination of them. Selective determination one of the purine derivative in attend of low concentrations other three purine derivatives was also understand at the present modified electrode. Using differential pulse voltammetry, detection limits of 8.8×10^{-8} M, 1.1×10^{-7} M, 3.2×10^{-7}M and 4.3×10^{-7}M were obtained for uric acid, xanthine, hypoxanthine and caffeine, sequential. The practical application of the modified electrode was pretended by altogether determining the concentrations of UA, XN, HXN and CAF in human blood plasma and urine samples.

Jiang et al. (2014), a nitrogen-doped graphene/carbon nanotubes (NGR–NCNTs) nanocomposite was used for the simultaneous determination of Caffeine (CAF) and Vanillin (VAN) determination was showed by cyclic Voltammetry (CV) and Square wave voltammetry (SWV). ENGR–NCNTs/GCE exhibited a wide linearity of 0.06–50 μM for CAF and 0.01–10 μM for VAN with detection limits of 0.02 μM and 3,3 10^{-3}μM, respectively moreover, Under optimal condition, the application of the proposed sensor in food products . was proven to be practical and reliable. The desirable results show that the ENGR–NCNTs nanocomposite has promising potential in electrocatalytic biosensor application.

Suw Young Ly et al. (2009), a simple commercial graphite pencil electrode(GPE) was applied for monitoring caffeine using the square wave anodic stripping voltammetry (SWASV) method. Optimal experimental conditions for the analysis were found to be as follows: pH value of 9 for the medium; deposition potential of 0.0V; deposition time of 120 s; SW frequency of 25Hz SW amplitude of 45 mV,andsteppotential of 6mV.Given these optimum conditions, a linear range was observed within the concentration of 0-500mgL^{-1}. At caffeine concentrations of 50.0, 250.0, and 500.0mg L^{-1}, the relative standard deviations in measured concentrations (n¼12) were 0.19, 0.09, and 0.11%, respectively. The detection limit was found to be 9.2mgL^{-1}.

Brunetti et al. (2006), a simple differential pulse voltammetric method based on a Nafion-covered glassy carbon electrode was developed for the quantitative determination of caffeine in cola beverages. The modified electrode exhibits a clear improvement of the current response compared to that observed at a bare glassy carbon

electrode. The method allows quantifying the analyte in the $9.95\text{-}10^{-7}$ – $1.06\text{-}10^{-5}$ M range, with a detection limit of7.98-10-7 M in 0.1 M sulfuric acidsolutions. The developed procedure was tested by recovery studies. A glassy carbon electrode (GCE) modified with multiwall carbon nanotubes (MWCNT) was used for a Simultaneous determination of ascorbic acid (AA) and caffeine (CAF) and it has been achieved by square wave voltammetry (SWV) The electrodes could be used for the determination of AA and CAF in a wide concentration range 10–500 -M, respectively, whereas the detectionlimit has been found to be 1.0×10^{-2} -M and 3.52×10^{-3} _M respectively. In view of high sensitivity for the detection of the drugs, the technique has been used for the reliable determination of AA and CAFin tea leaves, coffee, cold drink (mountain dew), pharmaceutical preparations and urine samples (Gupta et al., 2013).

Sun et al. (2011), a Nafion–Gr modified glassy carbonelectrode (Nafion–Gr/GCE) was apllied for determination of caffeine. Nafion–Gr modified glassy carbonelectrode were investigated by cyclic voltammetry and differential pulse voltammetry.The results that the showed Nafion–Gr/GCE exhibited excellent electrocatalytic activity to caffeine. Such electrocatalytic behavior of Gr is attributed to its unique physical and chemical properties, e.g., Caffeine can be effectively accumulated at Nafion–Gr/GCE and produce a sensitive anodic peak.subtle electronic characteristics and strong adsorptive capability. This electrochemical sensor showed an excellent performance for detecting caffeine with a detection limit of 1.2×10^{-7}M (S/N = 3), a satisfied recovery from 98.6% to 102.0%. and a reproducibilityof 5.2% relative standard deviation, thesensor shows great promise for simple and sensitive determination of caffeine.

3. MATERIAL METHODS

3.1. The tools and equipment used in the experiment

Electrochemical analyzer	TYPE III μautolab with GPES 4.9 software
Electrochemical cell assembly	
a)Working Electrode	BDD working electrode (Scientific Ltd.; Ø: 3mm, diameter)
b)Reference electrode	Ag/AgCl (3 mol L^{-1} NaCl) (Model RE-1 BAS, USA),
c)The auxiliary electrodes	platinum wire(MF 1032,BAS)
Electrochemical test cells	10 - ml septum-capped single standard glass cell (MR1208)
c)Mixing system	Magnetic stirrer (ARE heating and magnetic Stirrer), magnet (Spinb VM micro)
pH metre	: WTW, inolab pH 720
Automatic micro pipettes	: Eppendrof
Centrifuge	: Cooled Hermle Z320
HPLC	Agilent 1100 autosampler system, with a diode-array detector set at 207 nm
Chromatographic column	A nucleosil C18 (250 mm × 4.6 m, 5 μm)

Buffer containers in various sizes (pyrex®), different sizes balloonjojette, pipette, measure, test tubes, beakers, flasks and glass tubes.

3.2. The chemicals used in the experiments

Name of chemicals	Manufacturer Name
Caffeine	Sigma
Vanillin	Sigma
Acetic acid (100%)	Merck
Phosphoric acid (85%)	Merck
Nitric acid (63%)	Merck
Sodium hydroxide	Merck
HCl	Merck
Iron ІІІ	Merck
Mangnisum Nitral	Merck
Sodium Chloride	Merck
Zinic Nitrate	Merck
Copper Nitrate	Merck
Potasium Nitrate	Merck
Calicum Nitrate	Merck
Sucarous	Sigma
Fructus	Sigma
Glucose	Sigma
Chlorogenic acid	Sigma
Citric acid	Sigma
Ascorbic acid	Sigma
Ethanol	Merck
H_2SO_4	Merck
$NaH_2PO_4.2H_2O$	Merck
$Na_2HPO_4.7H_2O$	Merck

All studies Milli-Q water (Millipore) was used. Experimental studies at room temperature (25 ± 0.5 ° C) were carried out.

3.3. Preparation of buffer and solution

In the preparation of all buffers Milli-Q water (Millipore) was used. After the buffer solution prepared in Pyrex® glass bottle and stored in the refrigerator. Stock solutions of 1.0 mg mL^{-1} VAN and 1.0 mg mL^{-1} CAF were prepared by dissolving in water/ethanol mixture (50:50 v/v) and water, respectively. On the day of the experiment working solutions were prepared by diluting with a selected supporting electrolyte.

Preparation of 0.1 M acetate buffer solution (pH 4.7);

0.1 M acetate buffer solution was prepared by taking 5.75 mL (%100) CH_3COOH and mixed with 900 ml of water, 0.1 M NaOH was used to adjusted pH 4.7 and completed to 1 liter with water.

Preparation of 0.1 M Britton- Robinson (BR) buffer solution (pH 2 to 7);

0.1 M Britton- Robinson (BR) buffer solution prepared by taking 6.83 g H_3BO_3, 6.74 mL H_3PO_4 (%85) and 5.72 mL CH_3COOH (%100) all of them were mixed with 900 mL of water, 0.1 M NaOH and HCl were used to adjusted pH 2 to 7 and completed to 1 liter with water.

Preparation of 0.1 M phosphate buffer solution (pH 2.5);

0.1 M phosphate buffer solution prepared by taking 6.74 mL H_3PO_4 (%85) and mixed with 900 ml of water, 0.1 M NaOH was used to adjusted pH 4.7 and completed to 1 liter with water.

Preparation of 0.1 M phosphate buffer solution (pH 7.4);

0.1 M phosphate buffer solution prepared by taking 3.12 g $NaH_2PO_4.2H_2O$ and 21.44 g $Na_2HPO_4.7H_2O$ dissolved both of them in 900 mL water, 0.1 M NaOH was used to adjusted pH 7.4 and completed to 1 liter with water.

3.4. Voltammetric procedure

The general procedure adopted for obtaining adsorptive stripping voltammograms was as follows: The required aliquots of the VAN and/or CAF working solutions were placed into the voltammetric cell containing selected supporting electrolyte (phosphate buffer) at a desired pH (pH 2.5). The previously treated electrode was placed in the cell, and a selected accumulation potential (open-circuit) was then applied for a selected preconcentration period (60 s) at a stirring speed of 500 rpm.

After an equilibration time of 10 s, the voltammogram was recorded, while the potential was scanned from +0.2 to +1.8 V using SW modulation with 14 mV scan increment, 70 mV pulse amplitude, and a frequency of 50 Hz successive measurements were carried out by repeating the above assay protocol on the working electrode. All measurements were performed in triplicate.

3.5. Treatment of the commercial samples

Samples of commercial vanilla, cola and instant coffee were purchased from the local supermarket, and briefly stored at room temperature until submitted to the sample preparation procedure. All samples examined were analyzed shortly after opening. Vanilla solutions were prepared by dissolving 1 g of vanilla powder in 50 mL of water/ethanol mixture (50:50 v/v). The sample solution was placed in an ultrasonic bath for 30 min to complete dissolution, then the mixture has been waited on the heater (70 ^{o}C) at about 2 hours. An adequate volume of the supernatant solution was transferred to a voltammetric cell already containing 10 mL of the above mentioned supporting electrolyte and analyzed in the day of preparation. Coffee solutions were prepared by dissolving 1 g of coffee powder in 25 mL of water/ethanol mixture (50:50 v/v). The sample solution was placed in an ultrasonic bath for 30 min to complete dissolution, then the mixture has been waited on the heater (70 ^{o}C) at about 2 hours. An adequate volume of the supernatant solution was transferred to a voltammetric cell already containing 10 mL of the above mentioned supporting electrolyte and analyzed in the day of preparation. In the case of the sample of cola beverage, the content of three bottles was mixed thoroughly. After sonical elimination of gas, 150 μL aliquots of the samples (without any previous dilution or extraction) were transferred into the voltammetric cell containing 10 mL of 0.1 M PBS (pH 2.5) Quantifications for samples were performed by means of the standard addition method.

3.6. HPLC experiment

HPLC studies were performed using an Agilent 1100 autosampler system with C18 nucleosil column (250 mm × 4.6 mm, 5 μm) and with a diode-array detector set at 207 nm.

For HPLC experiments, the mobile phase was acetonitrile/water mixture (25:75 v/v) adjusted to pH 2.5 with H_3PO_4 (85 %) and NaOH (1 M). All solutions were filtered using a vacuum filtration system through 0.45-μm membrane filters from Agilent Technologies.

For purposes of comparison, a high-performance liquid chromatography (HPLC) method was also used for the determination of VAN and CAF in the commercial samples. Standart solution of VAN, CAF, the mixture of VAN and CAF was prepared as 50 μg mL^{-1} in mobile phase and later this solution was injected into the column in the range of 1-60 μL (0.05- 3.0 μg) . Calibration curves were obtained by plotting the compound amounts vs. the peak area. Analysis of the samples, the adequate volumes of the samples transferred into the HPLC vials and they were diluted with the mobile phase. Quantifications for samples were performed by means of the calibration curve.

4. RESULTS AND DISCUSSION

The oxidation behavior of these compounds was first studied by CV without an accumulation step (t_{acc}=0 s) obtained with anodically pretreated BDD electrode. Figure 4.1, 4.2 and 4.3 show the CV curves of single component of 50 µg mL^{-1} VAN, 50 µg mL^{-1} CAF, and 50 µg $mL^{-1}$of VAN and CAF, respectively. All CV curves were obtained in 0.1 M phosphate buffer solution (pH 2.5) solution recorded within the potential window from 0 and +1.8 V at a scan rate of 100 mV s^{-1}.As can be seen, both compounds were oxidized on anodically pretreated BDD electrode yielding single oxidation process at +1.15 V and +1.51 V, respectively, without presence of any cathodic peak on the reverse scan. It is clear that the electrochemical reactions of these compounds at the BDD electrode are irreversible.

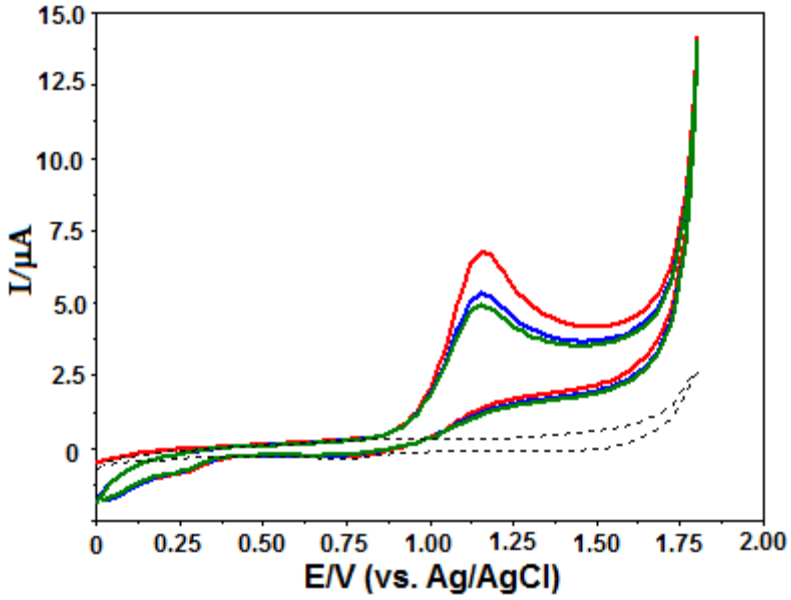


Figure 4.1. The repetitive cyclic voltammograms of 50 µg mL^{-1} VAN in 0.1 M phosphate buffer solution at pH 2.5. Electrode, anodically pretreated BDD; scan rate, 100 mV s^{-1}. Dashed lines represent background current.

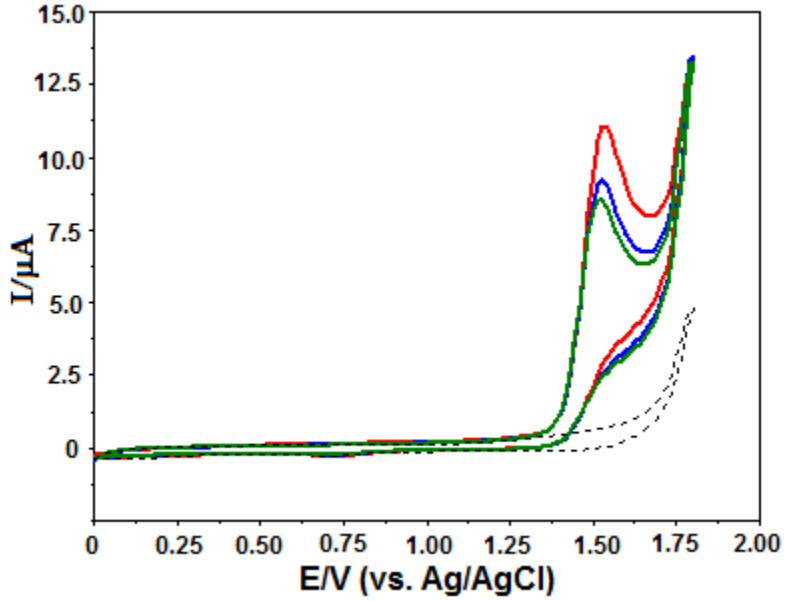


Figure 4.2. The repetitive cyclic voltammograms of 50 μg mL^{-1} CAF in 0.1 M phosphate buffer solution at pH 2.5. Electrode, anodically pretreated BDD; scan rate, 100 mV s^{-1}. Dashed lines represent background current.

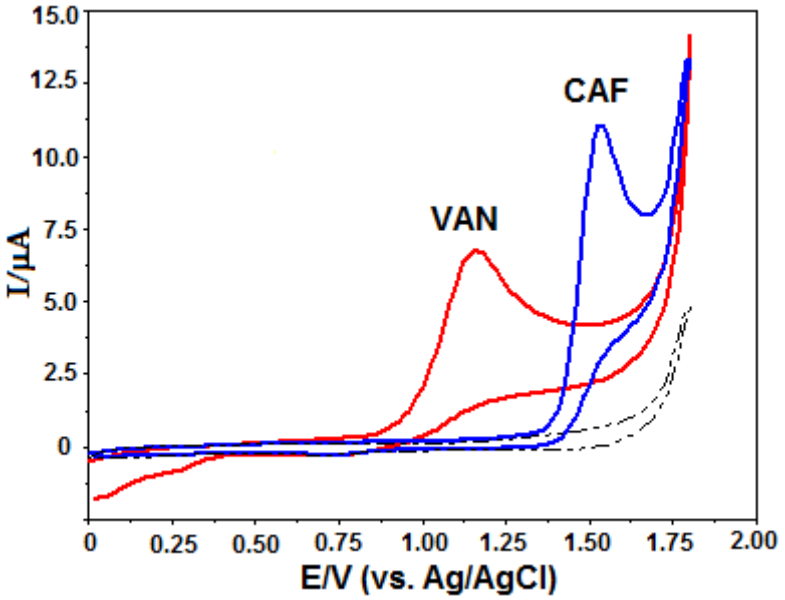


Figure 4.3. The cyclic voltammograms of 50 μg mL^{-1} VAN and CAF in 0.1 M phosphate buffer solution at pH 2.5. Electrode, anodically pretreated BDD; scan rate, 100 mV s^{-1}. Dashed lines represent background current.

Next, the effect of scan rate on the voltammetric response of CAF and VAN oxidation was investigated under the above experimental conditions. The oxidation peak currents increased with a positive shift in the potential when the scan rate increased, a typical characteristic of irreversible electrochemical reactions. The current response (Ip) was linearly proportional to the square root of scan rate ($v^{1/2}$) within the range 10-400 mV s^{-1} (Figure 4.4 and 4.5) according to the relationships:

I (μA) = 0.448$v^{1/2}$ (mV s^{-1}) + 0.172 (n=8, r = 0.993 (for VAN)

I (μA) = 0.682 $v^{1/2}$ (mV s^{-1}) + 0.504 (n=8,r = 0.993 (for CAF)

All these facts pointed toward the diffusion-controlled nature of the electrode process for both compounds. However, the plot of Ip versus v was also linear expressed by the equations:

I(μA) = 0.018ν (mV s^{-1}) + 1.95 (n = 8, r = 0.985) (for VAN)

I(μA) = 0.027ν (mV s^{-1}) + 3.82 (n = 8, r = 0.966) (for CAF)

This demonstrates that in spite of there being a diffusive process, there is also adsorption component (more effective for VAN) in the electrochemical process. This phenomenon may be attributed to the partial involvement of the diffusive compound molecules in the electrode reaction of the adsorbed ones. Thus, the electrochemical process has a mixed control for both compounds.

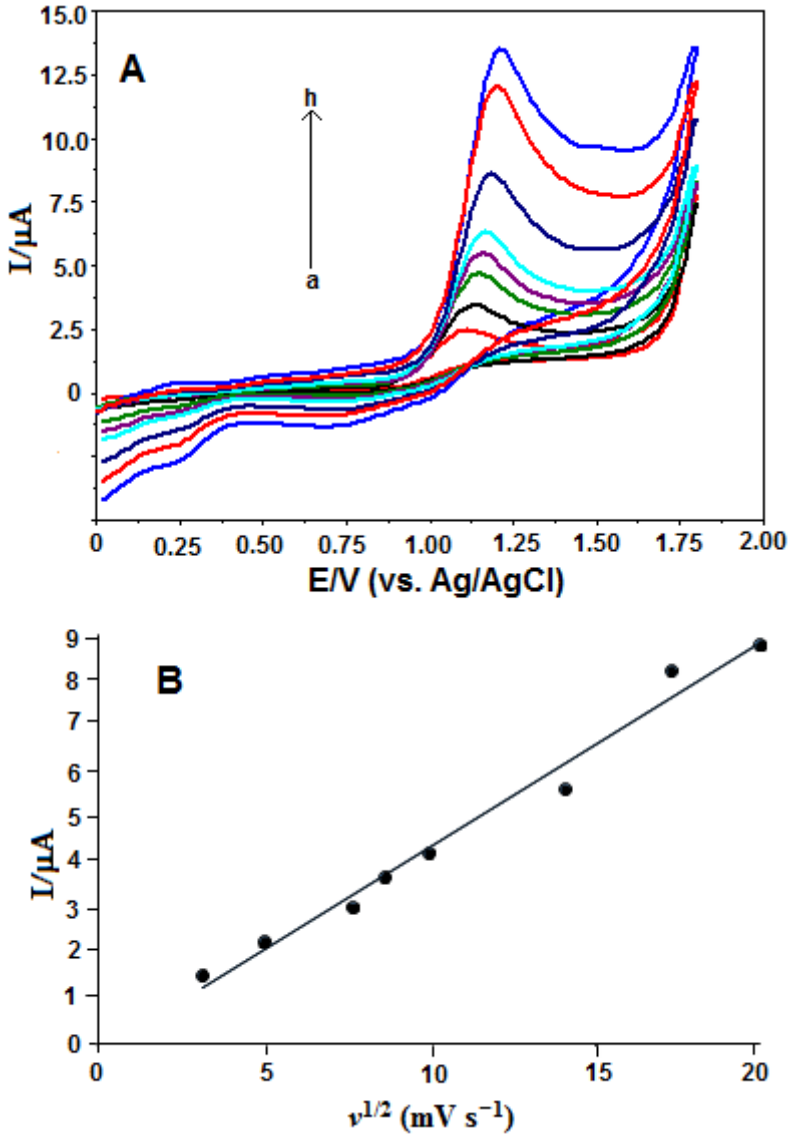


Figure 4.4. A) The cyclic voltammograms of 50 µg mL^{-1} VAN in 0.1 M phosphate buffer solution at pH 2.5with scan rate ranging from (a) 10, (b) 25, (c) 50, (d) 75, (e) 100, (f) 200, (g) 300 and (h) 400 mV s^{-1}.(B) The corresponding plot of $v^{1/2}$ vs I. Electrode, anodically pretreated BDD.

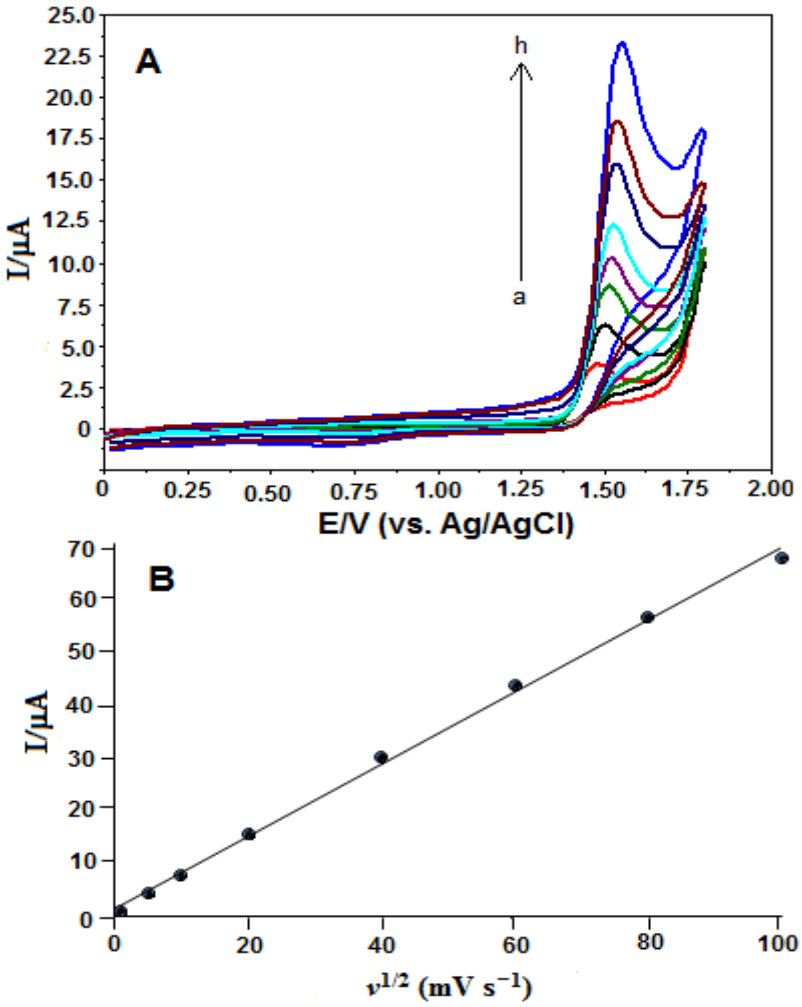


Figure 4.5. A) The cyclic voltammograms of 50 µg mL^{-1}CAF in 0.1 M phosphate buffer solution at pH 2.5with scan rate ranging from (a) 10, (b) 25, (c) 50, (d) 75, (e) 100, (f) 200, (g) 300 and (h) 400 mV s^{-1}.(B) The corresponding plotof $v^{1/2}$ vs I. Electrode, anodically pretreated BDD.

The electrochemical response of BDD electrode is strongly affected by the type of pretreatment applied to the surface before experiments. Thus, this effect is big importance in the case of electroanalytical studies. Although BDD electrodes are known to be resistant to fouling, a preliminary conclusion indicated that slight fouling occurred at BDD electrode without pretreatment during CAF and VAN oxidation, and thus a way to restore the initial activity of the BDD electrode surface was necessary. To test the effect of pretreatment procedure on the improved electrochemical response of the BDD electrode for the simultaneus determination of CAF and VAN, the electrode was either

anodically or cathodically pretreated and its stripping voltammetric signal was assessed (Figure 4.6). BDD electrode was treated by a cathodic cleaning (-1.8V for 60 s) and an anodic cleaning (+1.8V for 60 s). In order to decrease the background current, the acidic media of 0.5 M H_2SO_4 was used for both electrochemical cleanings. Anodically pretreated BDD electrode presented more positive oxidation peak potential and lower current intensity for both compounds than those obtained on the other procedures. On the other hand, anodically pretreated BDD presented a best peak definition and more intense current signal than that on cathodically pretreated one. Additionally, highest reproducibility of the measurements was obtained with the anodic activation procedure. Consequently, the anodic pretreatment was chosen. This pretreatment, which was repeated daily before starting the voltammetric measurements, was always preceded by a 30 s anodic pretreatment, which cleaned the electrode surface by oxidizing any adsorbed contaminant.

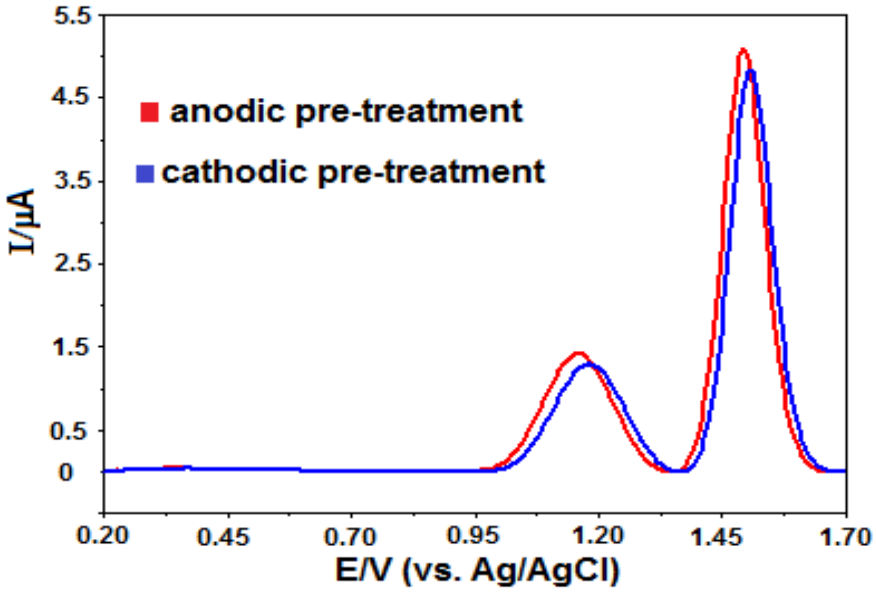


Figure 4.6. SW stripping voltammogramsof 10 μg mL^{-1} VAN and CAF in 0.1 M PBS pH 2.5 solution at the different cleaning procedures. Electrode, BDD; pre-concentration period, 60 s at open-circuit condition; SWV parameters: frequency, 50 Hz; scan increment, 8 mV; pulse amplitude, 30 mV.

The observed responses suggested the possibility of setting up method to determine VAN and CAF by square wave form. Among the stripping wave forms, the SW modulation combines good sensitivity with high speed, and reduces problems with

poisoning of the electrode surface. As a consequence, further work was dedicated towards studying the influence of nature and acidity of the supporting electrolyte upon the SW response, after performing an accumulation step at BDD electrode.

The effect of solution pH on the electrochemical response 10 µg mL^{-1} vanillin at boron-doped diamond electrode was investigated with Britton-Robinson solution in the pH range 2.0-7.0 by SW stripping voltammtry, with an open-circuit mode at 60 s. As can be seen from the Figure 4.7., decreasing the solution acidity resulted in a decrease of the peak heights from pH 2 to 5 but on increasing pH > 5.0 then the peak high was increased. For a solution with pH between 2.0 and 7.0, vanillin oxidation peak potential was displaced to less positive values (from +1.105 at pH 2.0 to +0.652 at pH 7.0). Figure 4.8 depicts the SW stripping voltammograms in various supporting electrolytes. Using 0.1 M phosphate buffer pH 2.5, acetate buffer pH 4.8 and phosphate buffer 7.4, anodic peak potentials of +1.136, 1.081 and 0.652 V respectively, with the different of the anodic peak currents (2.01, 0.867, and 2.63µA).

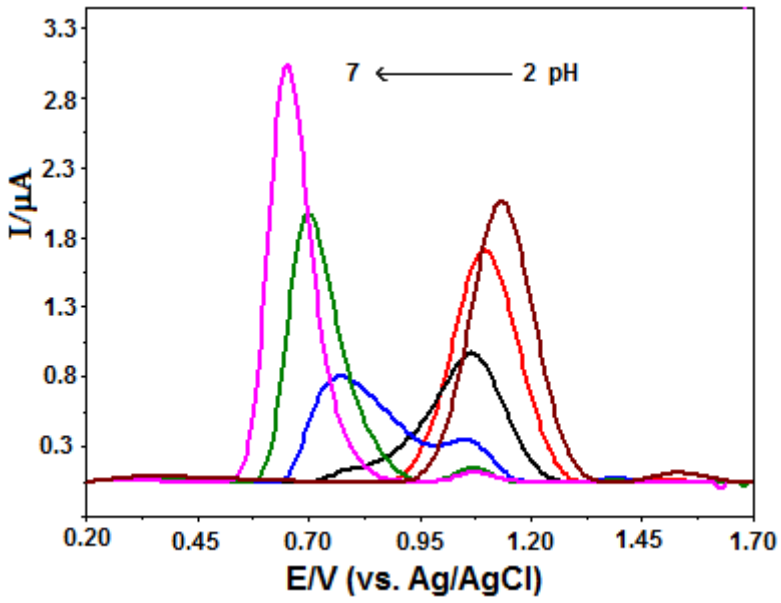


Figure 4.7. SW stripping voltammograms of 10µg mL^{-1} VAN solutions in Britton-Robinson buffer pH 2-7. Electrode, BDD; pre-concentration period, 60 s at open-circuit condition; SWV parameters: frequency, 50 Hz; scan increment, 8 mV; pulse amplitude, 30 mV.

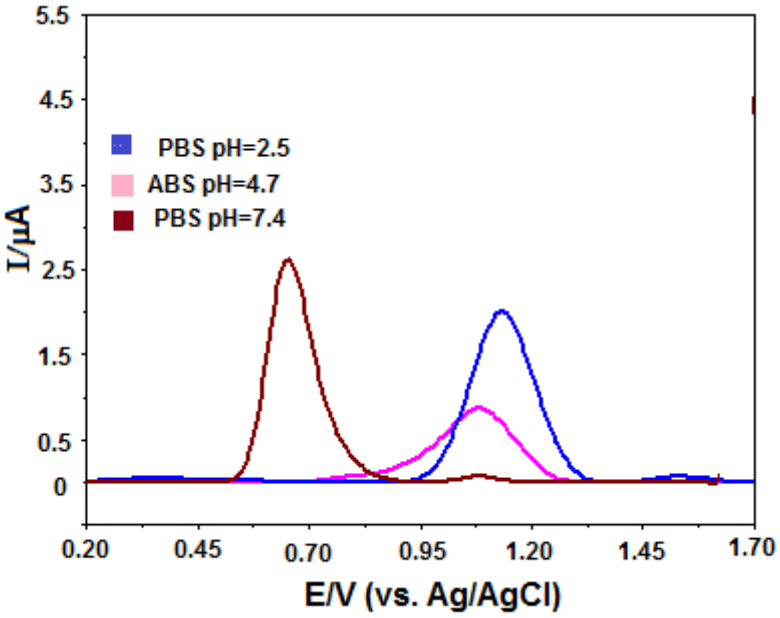


Figure 4.8. SW stripping voltammograms of 10 µg mL^{-1} VAN in various supporting electrolytes at different pH values. Electrode, BDD; pre-concentration period, 60 s at open-circuit condition; SWV parameters: frequency, 50 Hz; scan increment, 8 mV; pulse amplitude, 30 mV.

The effect of solution pH on the electrochemical response 10 µg mL^{-1} CAF at boron-doped diamond electrode was investigated with Britton-Robinson solution in the pH range 2.0-7.0 by SW stripping voltammetry, with an open-circuit mode at 60 s. As can be seen from the figure, decreasing the solution acidity (pH from 2 to 7) resulted in a decrease of the peak heights from 5.347 to 2.109 µA (Figure 4.9). Figure 4.10. Depicts the SW stripping voltammograms in various supporting electrolytes. Using 0.1 M phosphate buffer pH 2.5, acetate buffer pH 4.7 and phosphate buffer 7.4, anodic peak potentials of CAF +1.493, 1.446 and 1.442 V were obtained the anodic peak currents with different degrees (5.77, 4.83 and 2.23µA).

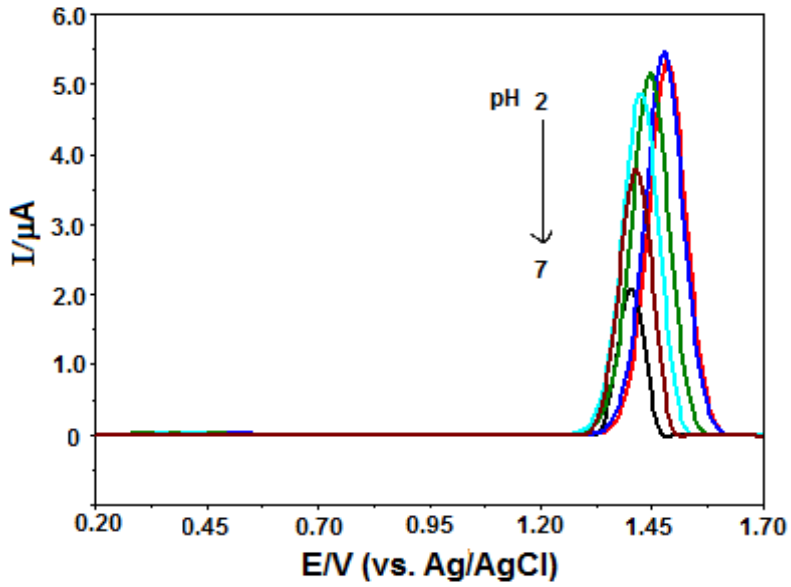


Figure 4.9. SW stripping voltammograms of 10 µg mL^{-1} CAFsolutions in Britton-Robinson buffer pH 2-7. Electrode, BDD; pre-concentration period, 60 s at open-circuit condition; SWV parameters: frequency, 50 Hz; scan increment, 8 mV; pulse amplitude, 30 mV.

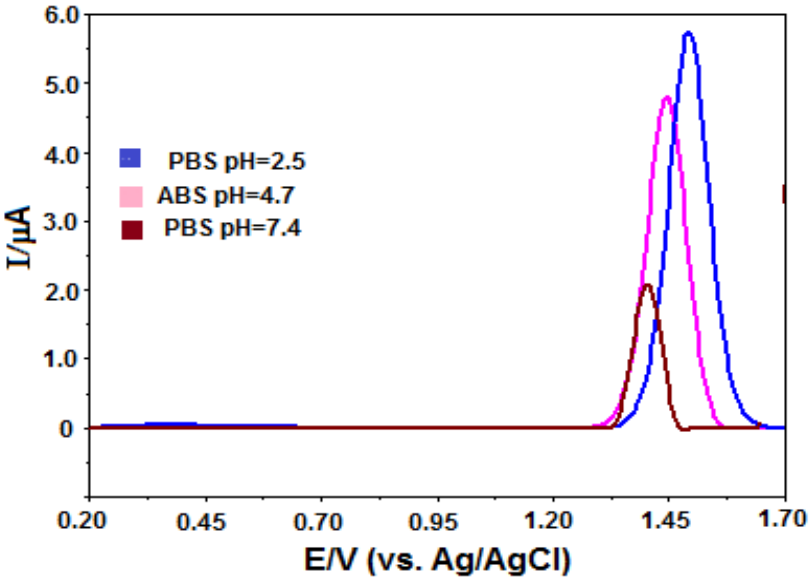


Figure 4.10. SW stripping voltammograms of 10 µg mL^{-1} CAF in various supporting electrolytes at different pH values. Electrode, BDD; pre-concentration period, 60 s at open-circuit condition; SWV parameters: frequency, 50 Hz; scan increment, 8 mV; pulse amplitude, 30 mV.

Figure 4.11. shows the SW stripping voltammograms for both VAN and CAF in the 0.1 M phosphate buffer pH 2.5 and Britton-Robinson solution in the pH 7.0 the anodic peak currents of VAN and CAF are 2.01 µA and 5.77 µA respectively, in the phosphate buffer pH 2.5 the anodic peak currents of VAN and CAF are 2.992 µA and 2.109 µA respectively, in the Britton-Robinson solution in the pH 7.0. According to the obtained results, the 0.1 M phosphate buffer solution at pH 2.5 are the most suitable media for the simultaneuos determination of VAN and CAF, relativelly yielding the high peak current, better peak shape, and also the best background signal, which was chosen for further experiments and development of the methodology.

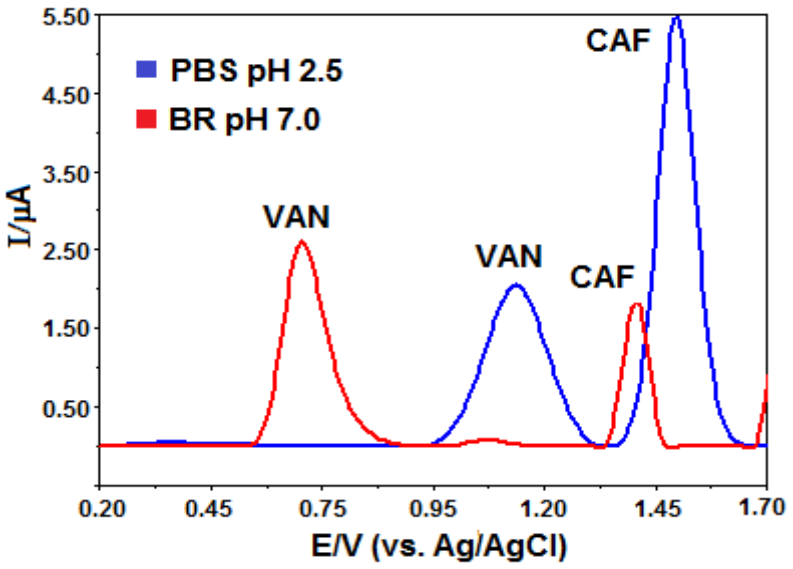


Figure 4 11. SW stripping voltammograms of 10 µg mL^{-1} CAF and 10 µg mL^{-1} VAN in the phosphate buffer pH 2.5 and the Britton-Robinson solution in the pH 7.0. Electrode, BDD; pre-concentration period, 60 s at open-circuit condition; SWV parameters: frequency, 50 Hz; scan increment, 8 mV; pulse amplitude, 30 mV.

To identify the adsorptive character of the compounds on BDD electrode, the effect of accumulation potential (E_{acc}) and time (t_{acc}) was investigated simultaneouslyfor10 µg mL^{-1} CAF or 10µg mL^{-1} VAN in phosphate buffer solution (pH 2.5) within the potential range +0.2 to +1.7 V (data not presented). Keeping accumulation potential at open-circuit condition at a concentrations of 10 µg mL^{-1}, the oxidation peaks current of VAN and CAF gradually increased with increasing

accumulation time from 0 to 60 s, and reached the maximum peak current response at 60 s. Further increasing the accumulation time (60-360 s), there is no significant increase in the current response. This phenomenon is probably due to the saturated adsorption of VAN and CAF on the BDD electrode surface, and no changeis observed when increasing the accumulation time. Therefore, an accumulation time of 60 s was used for each voltammetric measurement of VAN and CAF. Keeping the accumulation time as 60 s at a concentration of 10 μg mL^{-1}, the accumulation potential was investigated in either at open-circuit condition or over the potential range from +0.1 to +0.5 V with an accumulation time of 60 s.The results prove that varying accumulation potential shows the influence on the increase of RES oxidation current. The peak current reached its maximum at an accumulation potential of 0.3 to 0.4 V and at open-circuit condition. Herein, open-circuit condition is chosen as the optimum accumulation potential since the best baseline was obtained.

Further work was dedicated towards studying the effect of SW parameters, such as frequency (*f*), pulse amplitude (*a)* and scan increment (ΔE_s) for 10μg mL^{-1} VAN and CAF were evaluated under the optimum experimental conditions.When the *f* changed from 25 to 125 Hz (ΔE_s= 8 mV, *a*= 30 mV) the peak current increased linearly, however the background current and noise were also increased at *f* values higher than 50 Hz. Thus, *f*= 50 Hz was selected for all subsequent experiments. The influence of *a* was studied in the range from 20 to 80 mV (remaining parameters: ΔE_s= 8 mV, *f* = 50 Hz). The peak currents of VAN and CAF rapidly increased until *a* = 80 mV. However, the best peak morphology and sharper one was obtained at 70 mV, since the peak became wider and deformed at higher values of *a.* Hence, *a*=70 mV was chosen for all following experiments. When the $\Delta E_s$was changed from 6 to 16 mV, and the remaining parameters were constant (*f* = 50 Hz, *a* = 70 mV), the recorded signal increased until the value of 14 mV followed by slowlier increase from 14 to 16 mV. In addition, at higher values of 14 mV, an increase in ΔE_s resulted in a broading in the voltammograms were observed. The ΔE_s of 14 mV was chosen for next experiments.

Having characterized the response of both VAN and CAF on the anodically pretreated BDD electrode, well-defined peaks presented a good peak-potential separation (more than 0.35 V), using phosphate buffer solution at pH 2.5, and optimized accumulation time of 60 s at open-circuit voltage. To further investigate the

voltammetric response when VAN and CAF co-exist, SW-AdSV behavior of VAN (or CAF) was tested in the presence of a large excess of CAF (or VAN).

Initially, for the individual determination of VAN, its concentration was varied in the range 1.0-100.0 μg mL^{-1} ($6.57x10^{-6}$ – $6.57x10^{-4}$ mol L^{-1}) in solutions containing CAF at the fixed concentration of 10 μg mL^{-1} ($5.15x10^{-5}$ mol L^{-1}) (Figure 4.12). Similarly, keeping the concentration of VAN was constant at 10 μg mL^{-1} ($6.57x10^{-5}$ mol L^{-1}), the determination of CAF was accomplished in the concentration range 0.25-100.0 μg mL^{-1}($1.29x10^{-6}$ – $5.15x10^{-4}$ mol L^{-1}) (Figure 4.13). As seen from Figure 4.12 and 4.13, stripping current for VAN and CAF increased regularly as their corresponding concentration was increased, respectively, while the one of the compound kept at constant concentration did not change. The analytical curves shown in Figure 4.12 B and 4.13 B depict linear responses for both compounds according to the regression equations:

I (μA) = 0.201C (μg mL^{-1}) + 0.026 (r = 0.999, n = 8) (for VAN)

I(μA) = 0.409 C (μg mL^{-1}) + 0.674 (r = 0.998, n = 10) (for CAF)

Where I is the adsorptive stripping peak current, C concentration of compounds.

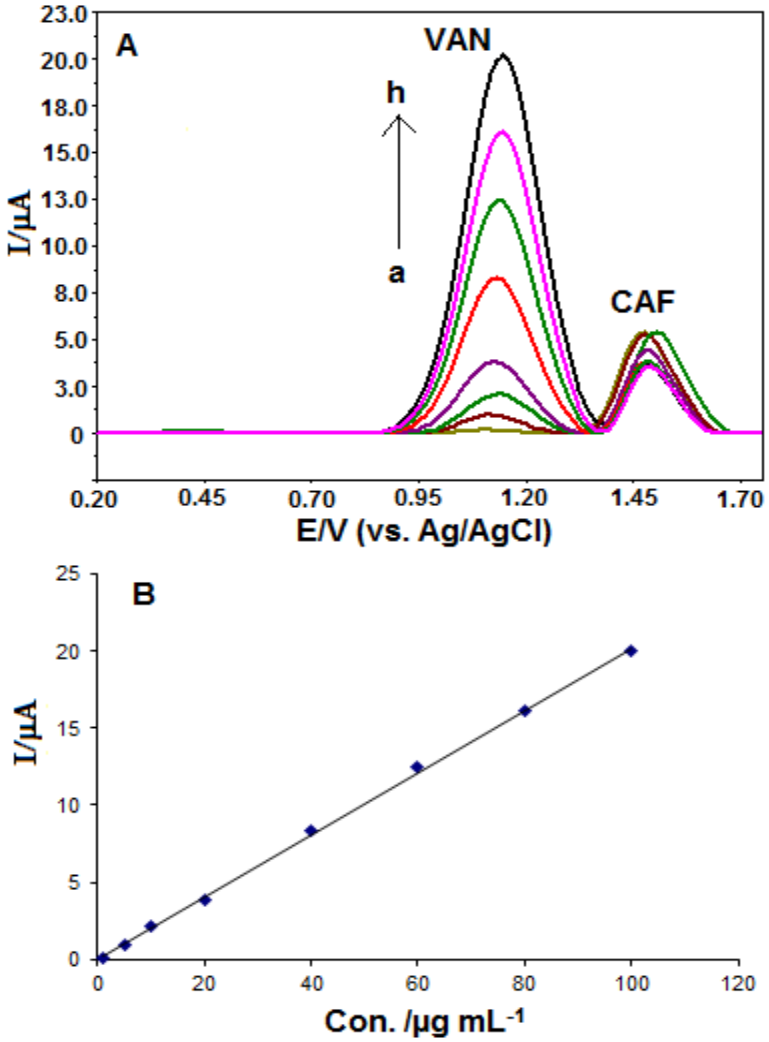


Figure 4.12. SW voltammograms obtained at the BDD electrode for VAN at different concentrations in the presence of 10 µg mL^{-1} CAF: (a) 1.0, (b) 5.0, (c) 10.0, (d) 20.0, (e) 40.0, (f) 60.0, (g) 80.0 and (h) 100.0 µg mL^{-1}. (B) The corresponding calibration curve of VAN. Pre-concentration period, 60 s at open-circuit condition; SWV parameters: frequency, 50 Hz; scan increment, 14 mV; pulse amplitude, 70 mV.

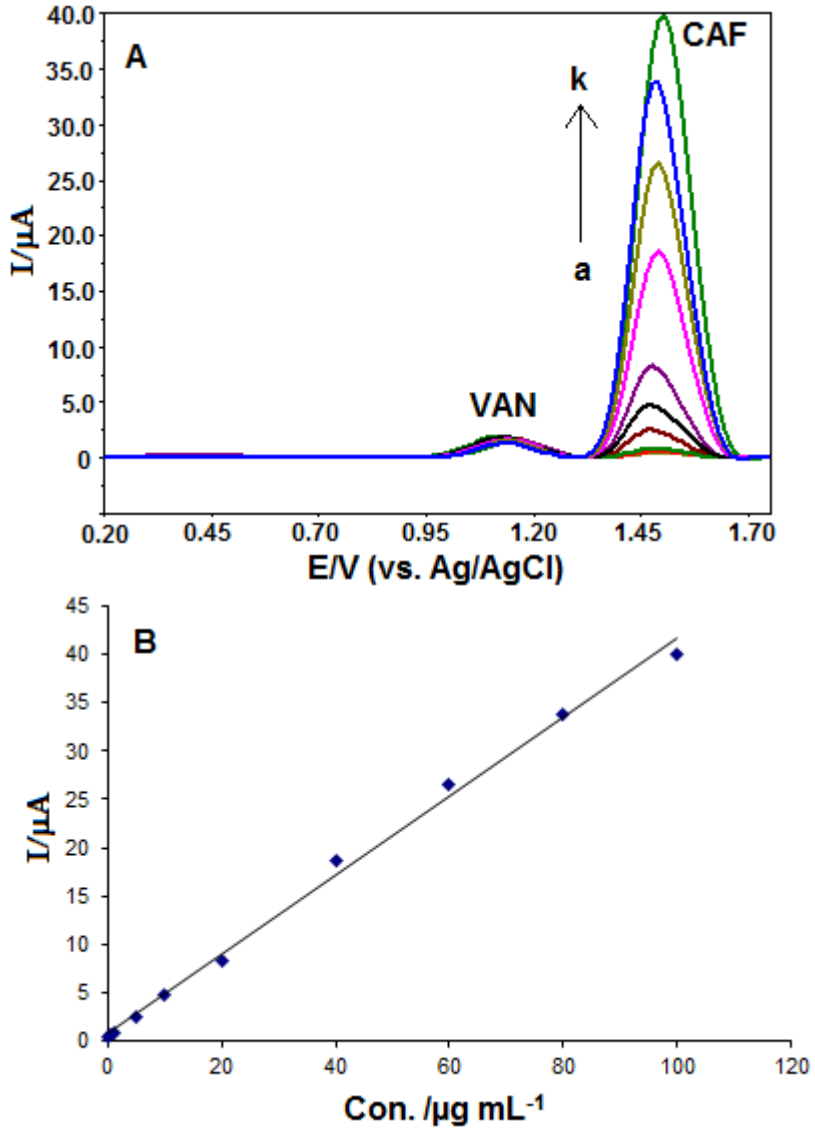


Figure 4.13. SW voltammograms obtained at the BDD electrode for CAF at different concentrations in the presence of 10 µg mL^{-1} VAN: (a) 0.25, (b) 0.5, (c) 1.0, (d) 5.0, (e) 10.0, (f) 20.0, (g) 40.0, (h) 60.0, (l) 80.0 and (k) 100.0 µg mL^{-1}. (B) The corresponding calibration curve of CAF. Other operating conditions as indicated in Figure 4.12.

The sensitivity of the proposed method was checked in terms of the limit of detection (LOD) values. LOD was calculated using the following equations:

LOD = 3*s*/*m*;

Where s is the standard deviation of the peak current (three runs) of the lowest concentration of the related linearity range, and m the slope of the related calibration plot.

From the data obtained by the analytical curves, LOD was achieved as 0.223 μg mL^{-1} (1.47×10^{-6} mol L^{-1}) for VAN, and 0.059 μg mL^{-1} (3.04×10^{-7} mol L^{-1}) for CAF. The obtained results showed that neither VAN nor CAF interfere with the oxidation signals of each other, indicating that their responses are independent.

After this initial study, VAN and CAF were determined by simultaneously changing their equal concentrations. Figure 4.14A shows the SW voltammograms obtained for solutions containing VAN and CAF in the phosphate buffer solution at pH 2.5. The calibration curves for VAN and CAF (Figure 4.14B) present a good linear response in the concentration range 1.0 μg mL^{-1} to 100.0 μg mL^{-1}. Using the peak currents at potentials of + 1.14 and +1.50 V, the respective calibration equations are:

I (μA) = 0.140 C (μg mL^{-1}) + 0.386 (r = 0.994, n = 8) (for VAN)

I(μA) = 0.288 C (μg mL^{-1}) + 1.353 (r = 0.996, n = 8) (for CAF)

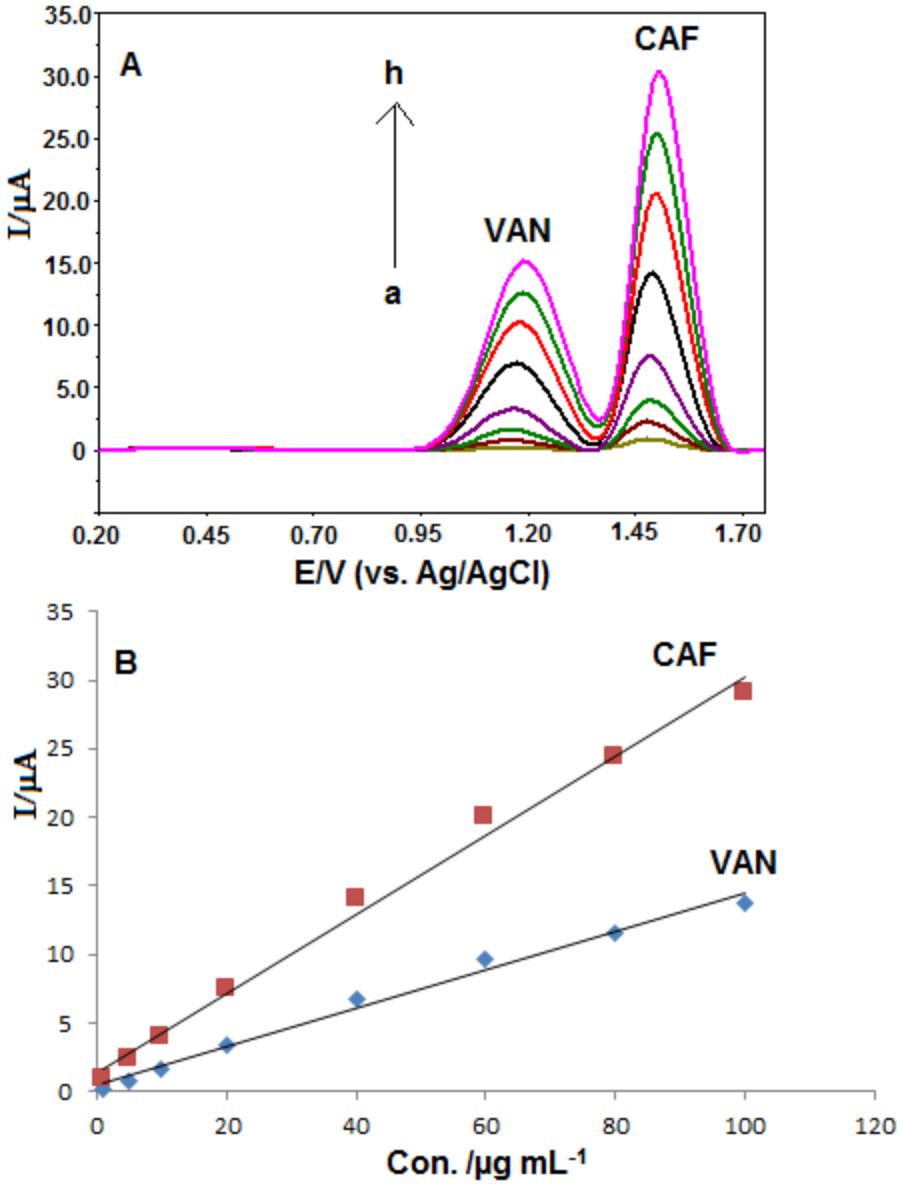


Figure 4.14. SW voltammograms obtained at the BDD electrode for VAN and CAF at equal concentrations in phosphate buffer solution at pH 2.5: (a) 1.0, (b) 5.0, (c) 10.0, (d) 20.0, (e) 40.0, (f) 60.0, (g) 80.0 and (h) 100.0 µg mL^{-1}. (B) The corresponding calibration curve of VAN and CAF. Other operating conditions as indicated in Figure 4.12.

From these plots LOD was calculated as 0.234 µg mL^{-1} (1.54×10^{-6} mol L^{-1}) for VAN while it was found to be 0.071 µg mL^{-1} (3.66×10^{-7} mol L^{-1}) for CAF, respectively. These regression equations show that the sensitivity of anodically pretreated BDD electrode towards CAF is nearly 3 times higher than that towards VAN.

The precision was determined by successive measurements (n=10) of a 1 μg mL^{-1} VAN and CAF solution; corresponding relative standard deviations (RSD) of 5.67% and 2.99% were obtained. Further, inter-day precision was examined by measuring the current response for three consecutive days for the same concentration of VAN and CAF and the RSDs were found to be 8.46% and 4.54, respectively, which are acceptable for practical applications.

The effect of some possible interfering inorganic ions and organic compounds were investigated by addition of the compounds to a solution containing 10 μg mL^{-1}VAN and CAF in the phosphate buffer at pH 2.5. The tolerance limit was defined as the maximum concentration of potential interfering substance that causes a relative error less than ±5% for the simultaneuos determination of VAN and CAF. The interference of some metal ions was examined. At about 10-fold excess, Fe^{3+}, Mg^{2+}, K^{+}, Na^{+}, Ca^{2+}, Cu^{2+} and Zn^{2+} did not significantly influence the height of the peak currents at BDD electrode under the selected experiment conditions. The effects of different organic compounds were also examined, no substancial change was observed for 10μg mL^{-1} VAN and CAF in the presence of at about 10-fold excess glucose, fructose, sucrose, salicylic acid and citric acid. Chlorogenic acid (CGA) is a prominent polyphenol compound found in considerably amounts in coffee beans and varying forms of coffee, so it is important that examined its interferences. Under the experimental conditions the corresponding voltammograms of 10 μg mL^{-1} VAN, CAF and CGA were recorded, and no substancial change was observed (Figure 4.15).

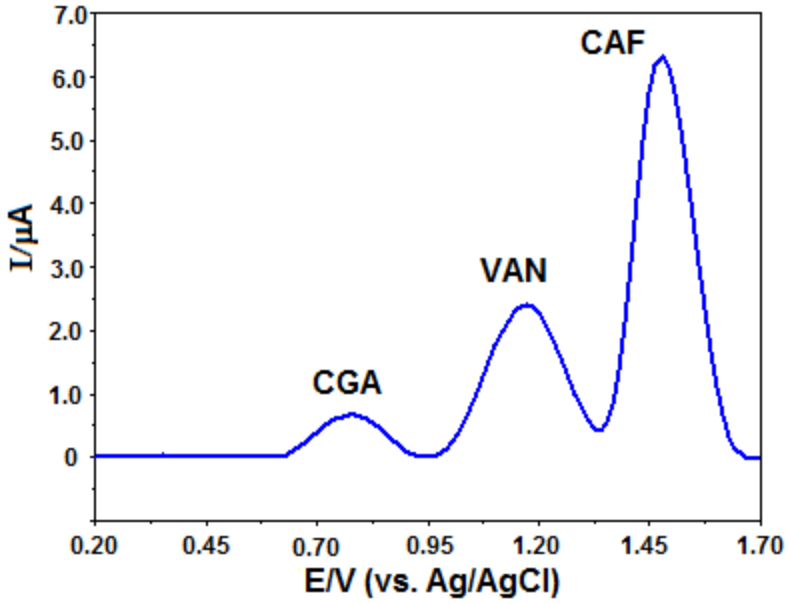


Figure 4.15. SW voltammograms obtained at the BDD electrode for 10 μg mL^{-1} VAN, CAF and CGA in phosphate buffer solution at pH 2.5. Other operating conditions as indicated in Figure 4.12.

In order to confirm the performance of the proposed methodology in real samples, its applicability was tested in three different samples of commercial products (vanilla sugar, cola and instant coffee with milk) frequently consumed in Turkey. Following the sample preparation, that is quick and easy, the voltammetric procedure under the experimental conditions was carried out.

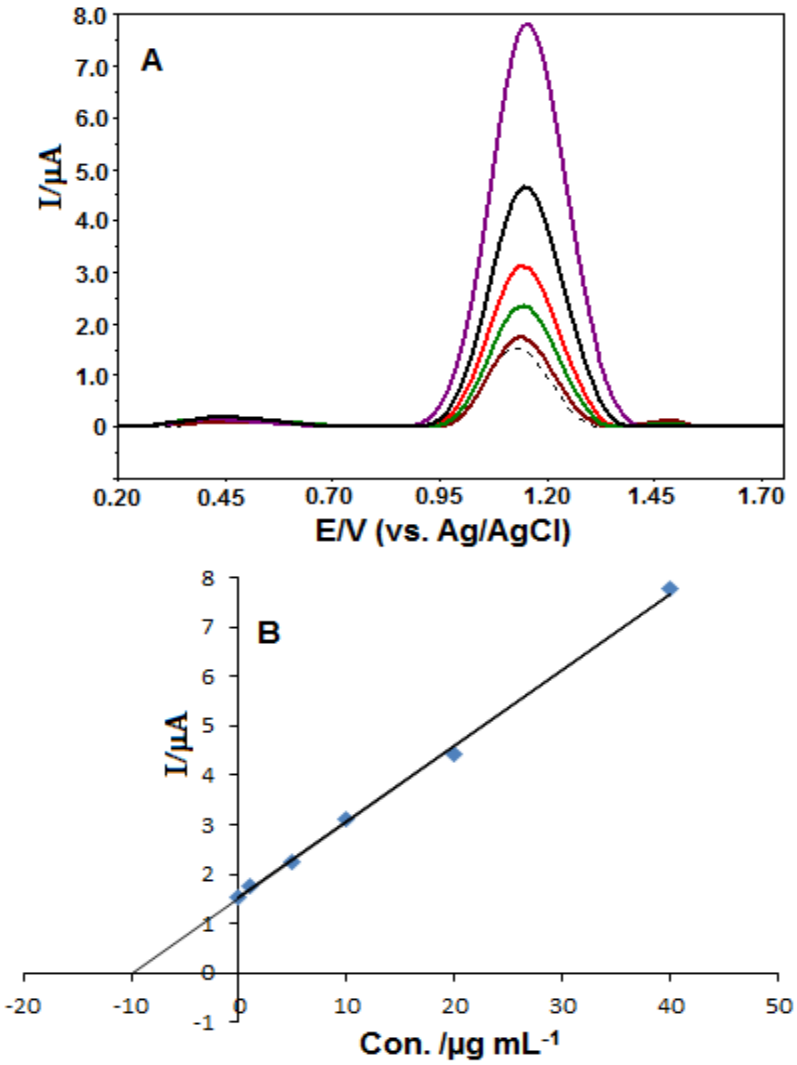


Figure 4.16. SW voltammograms obtained for the determination of VAN in spiked sample. The sample spiked at a VAN levels of (a) 1.0, (b) 5.0, (c) 10.0, (d) 15.0 and (e) 30.0 µg mL^{-1}. (B) The standart addition curve of VAN. Dashed lines represent the diluted sample of the vanilla sugar. Other operating conditions as indicated in Figure 4.12.

The practical application of the present system is tested by measuring the concentration of VAN in the samples of commercial vanilla sugar product. After the extraction and purification process which explained in section 3.5, it was taken appropriate volumes of the final extract and transferred 10 mL of 0.1 M phosphate buffer solution at pH 2.5. Quantifications were performed by means of the standard addition method and the average of triplicate measurements. Under the optimum

experimental condition, the SW voltammogram for the sample of vanilla sugar is shown in Fig. 4.16. The results are summarized in Table 1.

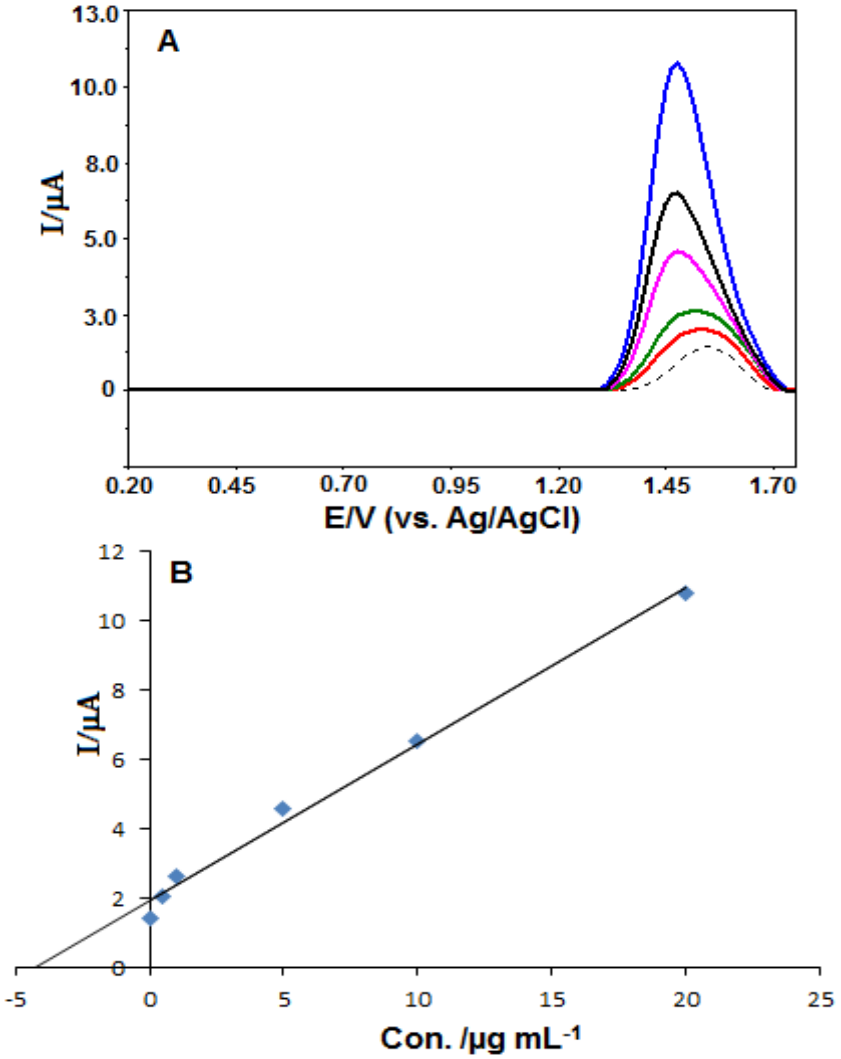


Figure 4.17. SW voltammograms obtained for the determination of CAF in spiked sample. The sample spiked at a CAF levels of (a) 0.5, (b) 1.0, (c) 5.0, (d) 10.0 and (e) 20.0 µg mL^{-1}. (B) The standart addition curve of CAF. Dashed lines represent the diluted sample of the sample of cola beverage. Other operating conditions as indicated in Figure 4.12.

Following the sample preparation (section 3. 5), that is quick and easy, the voltammetric procedure under the experimental conditions was carried out. After sonical elimination of gas, the adequate aliquots of the samples (without any previous dilution or extraction) were transferred into the voltammetric cell containing 10 mL of 0.1 M phosphate buffer solution at pH 2.5. Representative voltammograms of cola

sample is shown in Fig. 4.17. The peaks appeared at +1.65 V can be assigned to the oxidation of CAF, since multiple standard additions of CAF exhibited a concomitant increase in the peak currents without any distortion of the peak potential. Quantifications were performed by means of the standard addition method and the average of triplicate measurements. The results are summarized in Table 1.

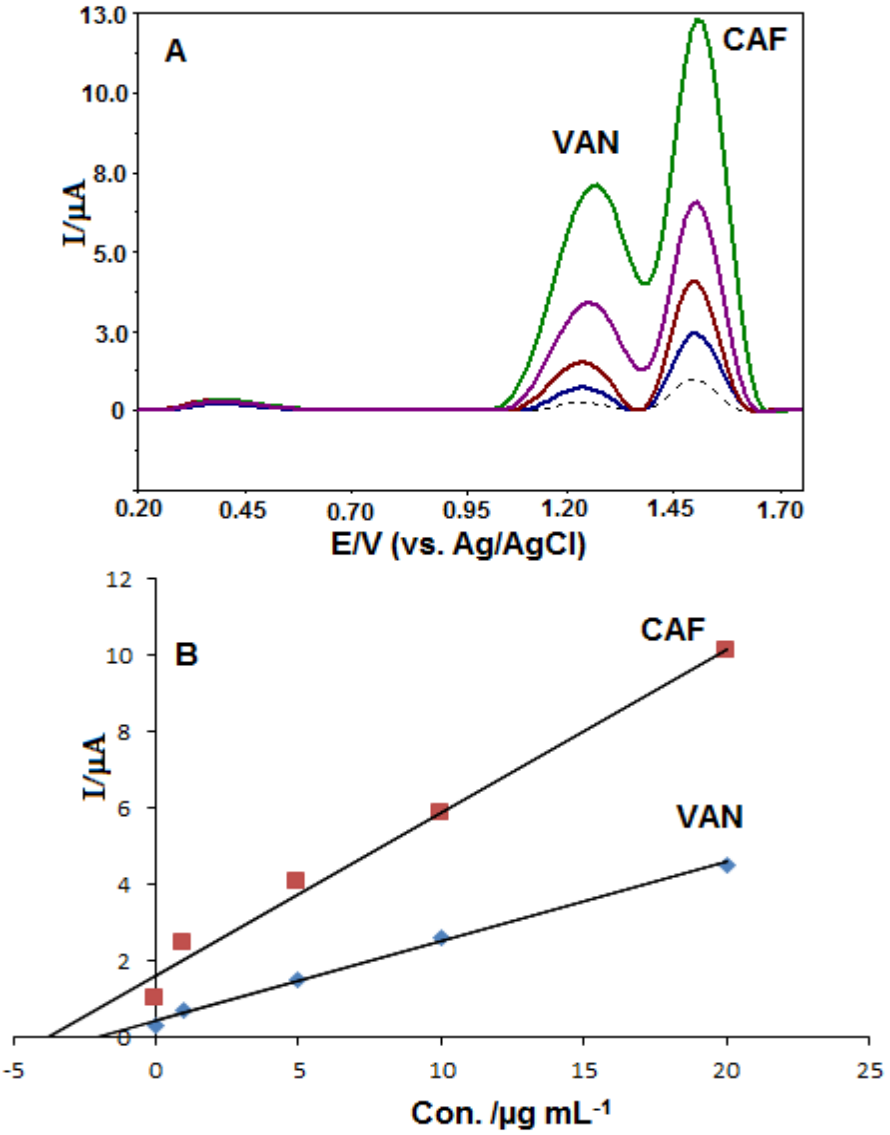


Figure 4.18. SW voltammograms obtained for the determinations of VAN and CAF in spiked sample. The sample spiked at a CAF levels of (a) 1.0, (b) 5.0, (c) 10.0 and (d) 20.0 µg mL^{-1}. (B) The standart addition curves of VAN and CAF. Dashed lines represent the diluted sample of the sample of inistant coffee. Other operating conditions as indicated in Figure 4.11.

Representative voltammograms of the instant coffe with milk samples are shown in Figure 6.18. The well-defined oxidation peaks at about +1.23 and +1.51 V were observed after transference of the sample of coffee with milk, with peak heights proportional to the sample addition. The peak appeared at +1.23 V (Fig.17) can be assigned to the oxidation of VAN, since multiple standard additions of VAN exhibited a concomitant increase in the peak currents without any distortion of the peak potential. The peaks appeared at +1.51 V (Fig.4.18) can be assigned to the oxidation of CAF, since multiple standard additions of CAF exhibited a concomitant increase in the peak currents without any distortion of the peak potential. The nominal content of the instant coffe with amounts were calculated from the corresponding regression equations of previously plotted calibration curves. The results are summarized in Table 1.

Comparison by HPLC

To prove the reliability of data obtained, the results obtained by the voltammetric method were compared with HPLC analysis. Firstly, various concentrations of VAN were analyzed using HPLC and the peak area was calculated. A well-defined peak is obtained at Rt~8.687 min in the standard sample of VAN as shown in Figure 4.19A. A calibration curve was obtained by plotting the peak area of the analyte peaks against the analyte concentration (Figure 4.19B). The quantitation of VAN in the vanilla sugar sample was calculated related calibration curve. The variation of peak area with added VAN amount (Q, μg) was linear and obeyed the following equality:

$S = 5767\ Q + 69.53$

Where S is peak area having correlation coefficient 0.999. Figure 4.20 shows a typical HPLC chromatogram of the vanilla sugar samples. The results are summarized in Table 1.

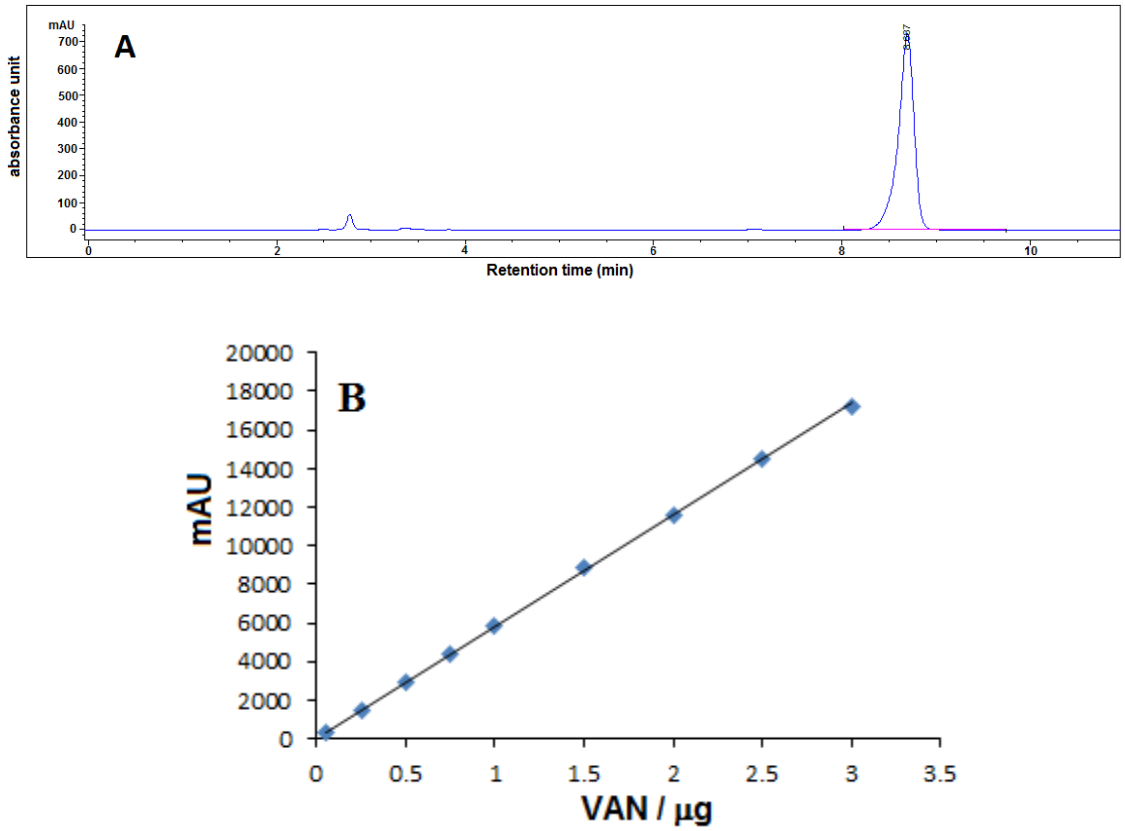


Figure 4.19. The standard solution chromatograms (A) and calibration plot of VAN (t_R=8.687) at different concentrations (0.05–3.0 µg) (B). Chromatographic conditions: mobile phase; acetonitrile/water mixture (25:75 v/v) pH 2.5 with H_3PO_4, diode-array detector set at 207 nm, flow rate, 1 ml min^{-1}.

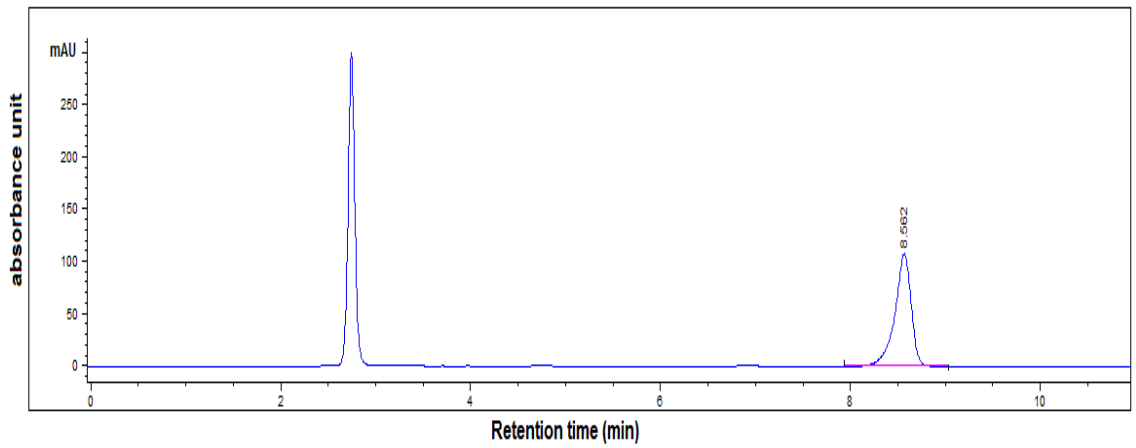


Figure 4.20. Typical chromatogram of VAN in the vanilla sugar samples. Other operating conditions as indicated in Figure 4.19.

Secondly, various concentrations of CAF were analyzed using HPLC and the peak area was calculated. A well-defined peak is obtained at Rt~5.378 min in the standard sample of CAF as shown in Figure 4.21A. A calibration curve was obtained by plotting the peak area of the analyte peaks against the analyte concentration (Figure 4.21B). The quantitation of CAF in the cola was calculated related calibration curve. The variation of peak area with added CAF amount (Q, µg) was linear and obeyed the following equality:

$S = 7545\ Q + 407.1$

Where S is peak area having correlation coefficient 0.998. Figure 4.22 shows a typical HPLC chromatogram of the cola samples. The results are summarized in Table 1.

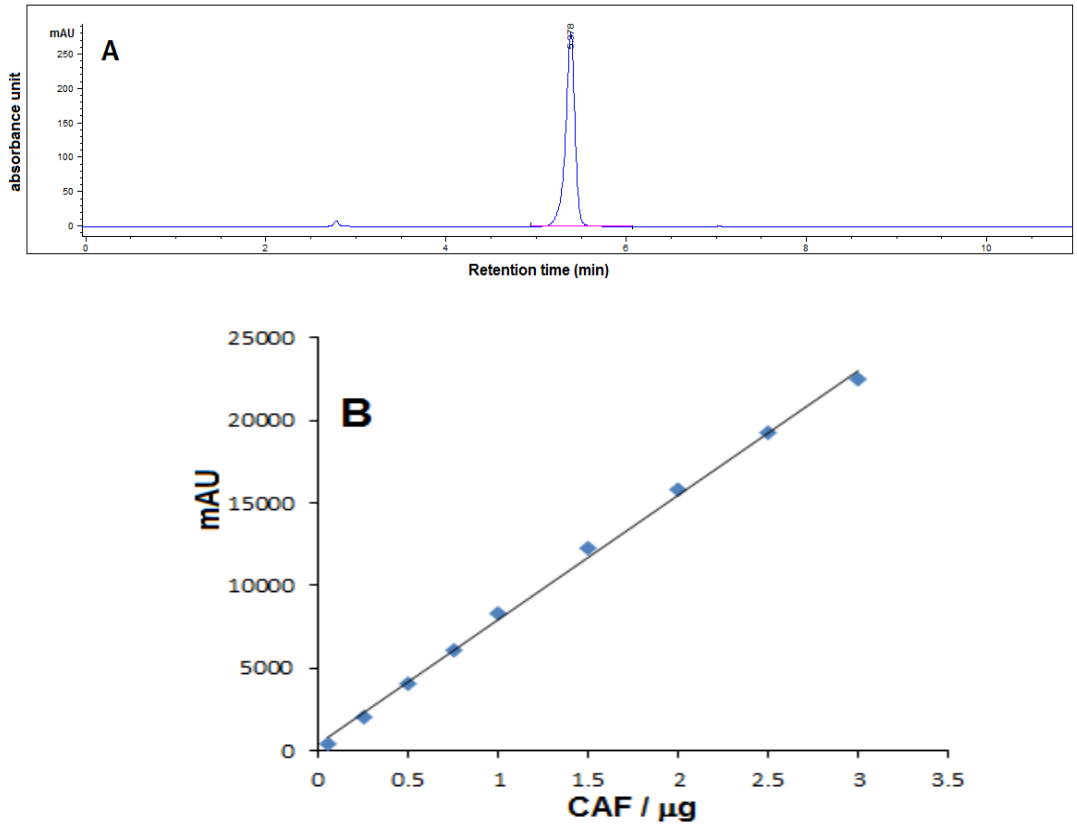


Figure 4.21. The standard solution chromatograms (A) and calibration plot of CAF (t_R=5.378) at different concentrations (0.05–3.0 µg) (B). Other operating conditions as indicated in Figure 4.19.

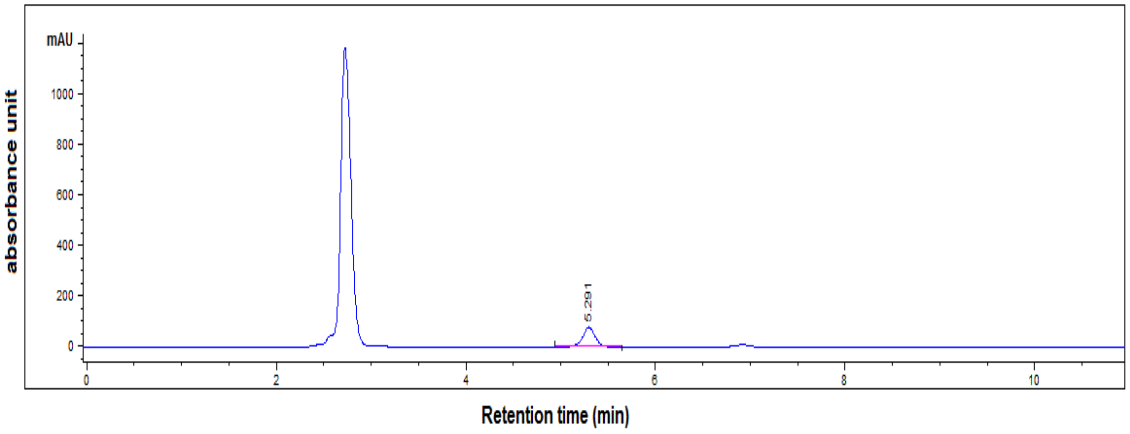


Figure 4.22. Typical chromatogram of CAF in the cola samples. Other operating conditions as indicated in Figure 4.19.

Lastly, various concentrations of CAF and VAN were analyzed using HPLC and the peak areas were calculated. Two well-defined peaks are obtained at Rt~5.269 min (for CAF) and Rt~8.461 min (for VAN) in the standard samples of CAF and VAN as shown in Figure 4.23A. The calibration curves were obtained by plotting the peak areas of the analyte peaks against the analytes concentrations (Figure 4.23B). The quantitations of CAF and VAN in the instant coffee with milk were calculated related calibration curves. The variation of peak area with added CAF and VAN amount (Q, µg) were linear and obeyed the following equalities:

S = 365.4 Q + 415.1 (r=0.998, for CAF)

S = 272.8 Q + 21.9 (r=0.999, for VAN)

Figure 4.24 shows a typical HPLC chromatogram of the instant coffee with milk samples. The results are summarized in Table 1.

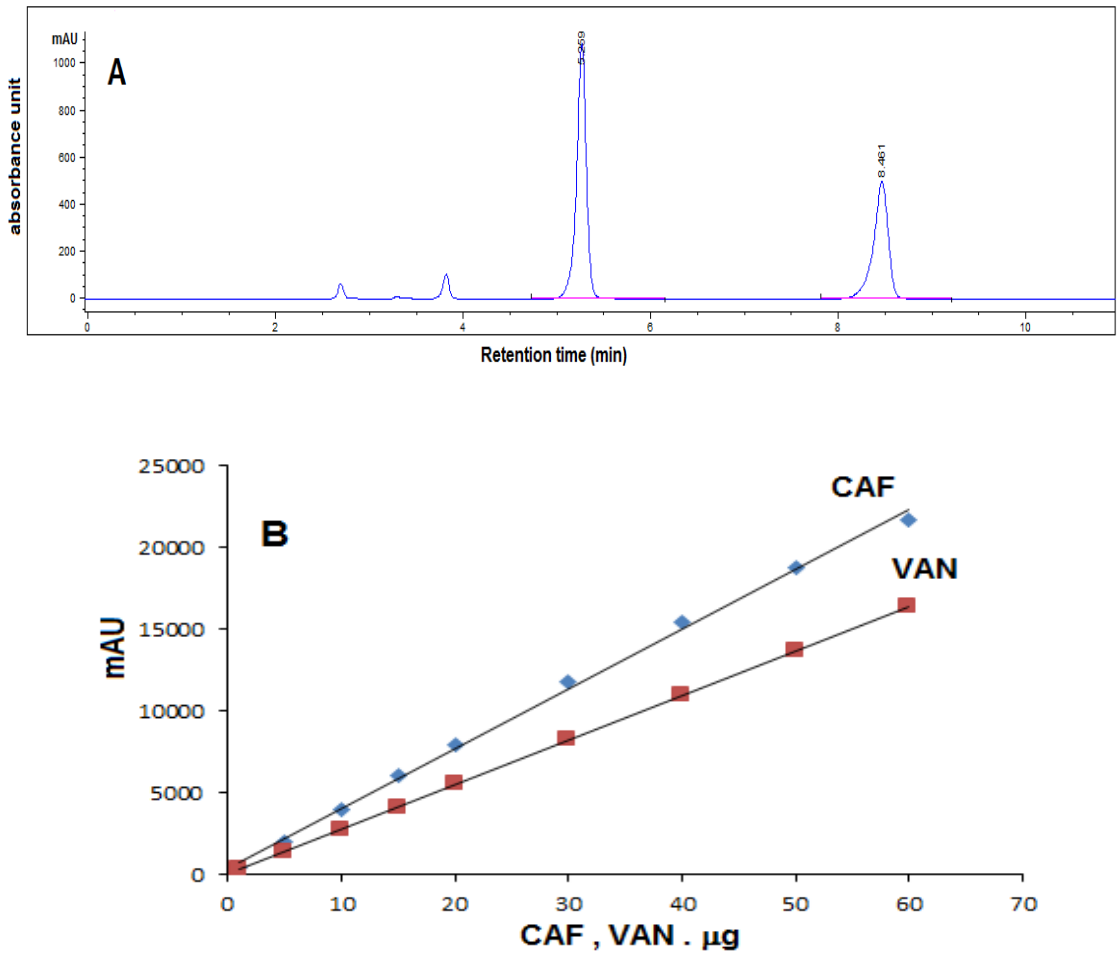


Figure 4.23. The standard simultaneous solution chromatograms (A) and calibration plot of CAF (t_R=5.269) and VAN (t_R=8.461) at different concentrations (0.05–3.0 µg) (B). Other operating conditions as indicated in Figure 4.19.

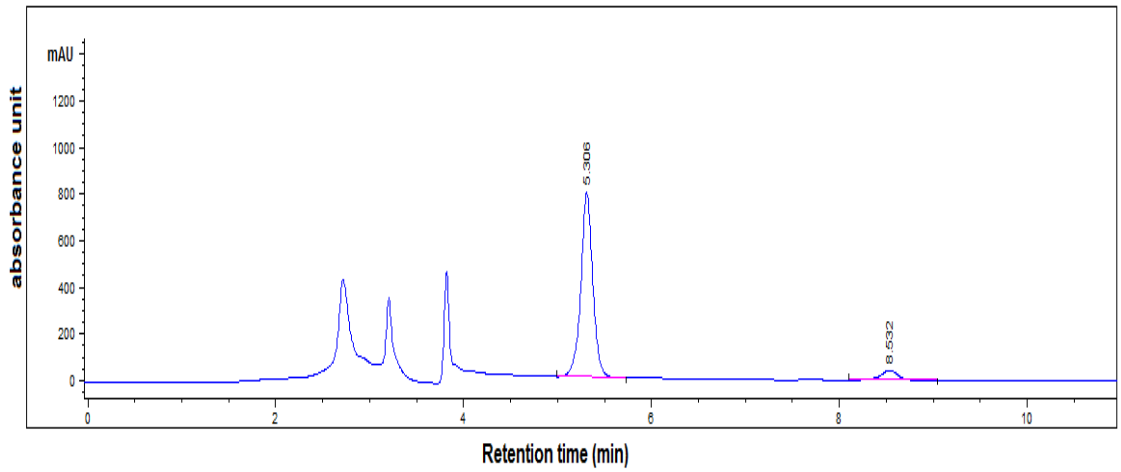


Figure 4.24. Typical chromatogram of CAF and VAN in the instant coffee with milk samples. Other operating conditions as indicated in Figure 4.18.

Table 4.1. Comparison results of the analysis of CAF and VAN content in the commercial samples by using SW-AdSV (proposed) and HPLC (comparative) methods.

Samples	**Analyte**	**SW-AdSV**	**HPLC**	E_1[a] **(%)**
Vanilla	VAN[b,c]	32.65± 1.22	31.88±0.68	2.42
Cola	CAF[b,d]	106.25± 2.83	111.32±1.09	-4.55
Coffee	VAN[b,c]	0.272±0.014	0.259±0.006	5.79
	CAF[b,c]	2.58±0.11	2.49±0.05	3.61

[a]Relative error (%) = [(voltammetric value-HPLC value)/HPLC value] × 100
[b]Mean ± SD (n = 3), [c]mg g^{-1}, [d]mg L^{-1}

5. CONCLUSION

The obtained results allow concluding that SWV along with a anodically pre-treated BDD electrode can be used with some benefits for the quantitative determination of VAN and CAF. With the good sensitivity, excellent detection limit and wide linear range, the proposed method provides a possibility for simultaneous detection of VAN and CAF in the food samples. As it is quite common, the here proposed electroanalytical method has the advantages of being considerably less time-consuming and less expensive than other analytical methods that also apply to the determination of these substances, especially HPLC.

The developed method is about 15 times less sensitive and have much high detection limits as compared to the previously reported at nitrogen-doped graphene/carbon nanotubes (Jiang et al., 2014). Consequently, the above-presented method provides a fast, sensitive and simple approach to the determination of capsaicin in the commercial pepper products.

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APPENDIX INDEX

GENİŞLETİLMİŞ ÖZET

1. ÖZET

Bu çalışmada, anodik olarak ön işlem görmüş bor katkılı elmas (BDD) elektrot yüzeyinde kafein (CAF) ve vanilin (VAN) 'in eş zamanlı belirlenmesi için dönüşümlü ve adsorptif sıyırma voltametri teknikleri kullanıldı. Pik akım ve pik potansiyeli üzerine pH, tarama hızı, biriktirme parametreleri ve diğer deneysel değişkenler çalışıldı. Kare dalga sıyırma tekniği kullanılarak açık devre koşulları altında, 60 saniye biriktirmeden sonra BDD elektrot yüzeyinde pH 2.5 fosfat tamponunda içinde CAF ve VAN 'in oksidasyon pik potansiyellerini 0.3 V fark olacak şekilde ayırabildi. Gözlenebilme sınırı, VAN için 0.114 μg mL^{-1} (7.49×10^{-7} mol L^{-1}) ve CAF için 0.033 μg mL^{-1} (1.69×10^{-7}mol L^{-1}) idi. Bu metodun pratik uygulanabilirliği, ticari olarak piyasada varolan örnekler ile test edildi. Elde edilen sonuçlar yüksek performanslı sıvı kromatografisi (HPLC) ile kıyaslandı.

Anahtar kelimeler: Kafein, Vanilin, Bor katkılı elmas elektrot, Eş zamanlı belirleme, Kare dalga sıyırma voltametri, Kahve, Kola, Vanilla.

2. MATERYAL VE YÖNTEM

2.1. Voltametrik analiz

Anodik bölgede eş zamanlı tayinleri yapılması planlanan CAF ve VAN'nin uygun çözelti ortamında BDD elektrot üzerindeki elektrokimyasal ve adsorptif davranışları dönüşümlü voltametri tekniği ile araştırılmıştır. Duyarlı bir teknik olan kare dalga voltametri tekniği ile destek elektrolitinin türü ve pH'sının, biriktirme gerilimi ve süresinin, kare-dalga formu değişkenlerinin (frekans, puls amplitüd, gerilim adımı) elektrot yanıtına etkisi incelenmiş ve eşzamanlı tayine olanak tanıyacak en iyi deney koşulları belirlenmiştir. En iyi deney koşullarında bileşiklerin eş zamanlı tayini için aletsel performans özellikleri çalışma aralığı, kesinlik (tekrarlanabilirlik) ve duyarlılık (gözlenebilme sınırı, LOD) belirlenmiştir.

2.2. Kromatografik Analiz

HPLC çalışmaları, 207 nm'de diod-array dedektör ve C18 nükleosil kolon (250 mm × 4.6 mm, 5 μm) ile, Agilent 1100 autosampler sistem kullanılarak yapıldı. HPLC deneyleri için mobil faz; NaOH (1 M) ve H_3PO_4 (85 %) içeren pH 2.5'te (25:75 v/v) asetonitril-su karışımı kullanıldı. Tüm çözeltiler Agilent Teknolojilerinden 0.45-μm membran fitler boyunca bir vakum filtrasyon sistem kullanılarak süzüldü. Yüksek performansı sıvı kromatoğrafisi ayrıca ticari örneklerde VAN ve CAF belirlemek için kullanıldı. CAF ve VAN'ın standart çözeltisinden VAN ve CAF karışımı 50 μg mL^{-1} mobil faz olarak hazırlandı ve 1-60 μL (0.05- 3.0 μg) aralığında kolona enjekte edildi. Kaliprasyon eğrisi, pik alanında bileşen miktarlarının grafiği ile elde edildi.

2.2.Örneklerin Numulerinin Hazırlanması

Ticari olarak vanilin, kola ve kahve yerel marketlerden temin edildi ve presedür uygulanana dek oda sıcaklığında depolandı. Tüm örnekler açılıktan kısa süre sonra analiz edildi. Vanilya çözeltisi (50:50 v/v) 50 ml etanol-su karışımında 1 g vanilya çözülerek hazırlandı. Çözünmenin tamamlanmaması için örnek çözelti 30 dakika ultrasonik banyoda muamele edildi ve daha sonra 70 ^{o}C'de 2 saat bekletildi. Yeterli miktardaki çözelti alınarak yukarda bahsedilen destek elektrot hücresini alındı ve o gün analizi yapıldı. Kahve çözeltisi (50:50 v/v) 25 ml etanol-su karışımında 1 g kahve tozu çözülerek hazırlandı. Vanilya çözeltisinde olduğu gibi 30 dakika ultrasonik banyo ve 70^{0}C sıcaklıkta bekletilip, destek elktrot ile analizi yapıldı. Kola numunesinde gazın sonikal ayrılmasından sonra örnekten 150 µL (herhangi ön seyreltme veya ekstraksiyon olmaksızın) 0.1 M PBS (pH 2.5)'deb 10 ml içeren voltametrik hücreye alındı ve analiz gerçekleştirildi.

3. SONUÇ

Bu çalışmada, anodik olarak ön işlem görmüş BDD elektrot yüzeyinde kafein (CAF) ve vanilin (VAN) 'in eş zamanlı belirlenmesi için dönüşümlü ve adsorptif sıyırma voltametri teknikleri kullanılarak yöntem geliştirilmiştir. Elektrokimyasal yöntem geliştirmede ilk olarak dönüşümlü voltametri (CV) tekniği kullanılarak 50 µg mL^{-1} VAN ve CAF'nin 0.1 M fosfat tamponu çözeltisinde (pH 2.5), 100 mV s^{-1} tarama hızında, 0 ile +1.8 V potansiyel aralığında CV grafikleri elde edildmiştir. Bileşiklerin her ikiside anodik ön işlem görmüş BDD elektrot üzerinde VAN +1.15 V'da ve CAF ise +1.51 V'da yüksetgenme sinyali vermiştir. CV tekniği ile 10-400 mV s^{-1} aralığındaki hız taraması sonuçlarında her iki bileşiğinde difüzyon kontrollü olmasının yanı sıra adsorptif karakter taşıdıkları bulunmuştur. Anodik ve katodik ön işlem çalışmalarında ise anodik ön işlem görmüş BDD elektrodun ilgili analitlere karşı daha iyi sonuçlar verdiği gözlemlenmiştir. Asitliğin VAN ve CAF'nin yükseltgenme sinyalleri üzerine etkisi ayrı ayrı olarak Britton-Robinson (pH 2-7) tamponu, pH 2.5'de 0.1 M fosfat tamponu, pH 4.8'de asetat tamponu ve pH 7,4'de fosfat tamponu çözeltileri kullanılarak araştırılmıştır. Elde edilen bulgular pik akmı, pik potansiyeli ve pik morfolojisi açısından değerlendirilmiş ve en iyi sonuçlar pH 2.5 0.1 M fosfat tampon

çözeltisinde elde edilmiş çalışmanın bundan sonrasına ilgili ortamda devam edilmiştir. BDD elektrot üzerinde adsorptif karekteri olan bileşiklerin en iyi biriktirme potansiyeli (E_{acc}) ve zamanı (t_{acc}) niceliklerini belirlemek için, E_{acc} açık devre ve +0.1-+0.5 V potansiyel aralıklarında, t_{acc} ise 0-360 s aralıklarında araştırılmıştır. En iyi sonuçlar açık devre potansisyeli ve 60 s elde edildiğinden çalışmanın bundan sonraki bölümüne bu parametreler ile devam edilmiştir. Kare dalga parametrelerinin optimizasyonu için frekans (25-125 Hz), puls adımı (6-16 mV) ve amplitüd (20-80 mV) çalışmaları yapıldı. Optimmum parametreler olarak frekans 50 Hz, puls adımı 14 mV ve amplitüd 70 mV olarak belirlendi.

Optimum deney koşulları altında 10 μg mL^{-1} ($5.15x10^{-5}$ mol L^{-1}) CAF ortamada sabit tutularak VAN derişimi 1.0-100.0 μg mL^{-1} ($6.57x10^{-6}$ – $6.57x10^{-4}$ mol L^{-1}) aralığında atrırıldı. İlgili derişim arlığında doğrusalık sağlanmış ve elde edilen denklem; I (μA) = 0.201C (μg mL^{-1}) + 0.026 (r = 0.999, n = 8) şekildedir. Benzer şekilde, VAN 10 μg mL^{-1} ($6.57x10^{-5}$ mol L^{-1}) konsantrasyonunu sabit tutularak CAF derişimi 0.25-100μg mL^{-1}($1.29x10^{-6}$ – $5.15x10^{-4}$ mol L^{-1}) aralığında artırıldı. İlgili derişim arlığında doğrusalık sağlanmış ve elde edilen denklem; I(μA) = 0.409 C (μg mL^{-1}) + 0.674 (r = 0.998, n = 10) şekildedir. Analitik eğrileri ile elde edilen verilerden, gözlem sınırı (LOD) VAN için 0.223 μg mL^{-1} (1.47×10^{-6} mol L^{-1}) olarak ve CAF için 0.059 μg mL^{-1} (3.04×10^{-7} mol L^{-1}) olarak bulundu. Bu ilk çalışmadan sonra, VAN ve CAF'ın eş zamanlı olarak tayini eşit konsantrasyonlarını değiştirerek belirlendi. VAN ve CAF için kalibrasyon eğrileri 1.0 μg mL^{-1} ve 100.0 μg mL^{-1} konsantrasyon aralığında doğrusal bir aralık vermektedir. +1.14 V (VAN) ve +1.50 V (CAF) potansiyellerde ilgili kalibrasyon denklemleri şunlardır: I (μA) = 0.140 C (μg mL^{-1}) + 0.386 (r = 0.994, n = 8) (VAN), I(μA) = 0.288 C (μg mL^{-1}) + 1.353 (r = 0.996, n = 8) (CAF). Bu verilerle, LOD VAN için 0.234 μg mL^{-1} (1.54×10^{-6} mol L^{-1}) ve CAF için 0.071 μg mL^{-1} (3.66×10^{-7} mol L^{-1}) olarak hesaplanmıştır. 1 μg mL^{-1} VAN ve CAF için gün içi bağıl standart sapma (% RSD) (n=10) sırasıyla %5.67 ve %2.99 günler arası ise (ardışık üç gün) %8.46 ile %4.54 olarak bulunmuştur. Seçicilik çalışması VAN ve CAF içeren ortama Fe^{3+}, Mg^{2+}, K^{+}, Na^{+}, Ca^{2+}, Cu^{2+}, Zn^{2+}, glükoz, fruktoz, sukroz, salisilik asit, sitrik asit, klorojenik asit ilavesi ile yapılmış herhangi bir girişim etkisine rastlanılmamıştır.

Gerçek örneklerde, önerilen yöntemin performansını göstermek için, Türkiye'de sıklıkla tüketilen ticari ürünlerin üç farklı örneği (vanilya şekeri, kola ve sütlü hazır

kahve) üzerine uygulanabilirliği test edilmiştir. Elde edilen verilerin güvenilirliğini kanıtlamak için, voltametrik yöntemle elde edilen sonuçlar, yüksek performanslı sıvı kromatografisi (HPLC) analizi ile karşılaştırıldı. HPLC analizinde VAN'in için alıkonma zamanı (Rt) 8.687 dakikada elde edildi. CAF için ise Rt=5.378 dakikada elde edildi. CAF ve VAN 'ın ikili karışımları da HPLC kullanılarak analiz edilmiştir. Kalibrasyon eğrileri, analitlerin konsantrasyonlarına karşın pik alanından elde edildi. Elde edilen sonuçlar aşgıdaki tabloda verilmiştir.

Çizelge 1. Voltametrik ve HPLC yöntemleri kullanılarak ticari örneklerde CAF ve VAN içeriği analizlerini karşılaştırması sonucu.

Örnekler	**Analit**	**Voltametri**	**HPLC**	E_1[a] **(%)**
Vanilya	VAN[b,c]	32.65± 1.22	31.88±0.68	2.42
Kola	CAF[b,d]	106.25± 2.83	111.32±1.09	-4.55
Kahve	VAN[b,c]	0.272±0.014	0.259±0.006	5.79
	CAF[b,c]	2.58±0.11	2.49±0.05	3.61

[a]Bağıl hata (%) = [(voltametrik değer-HPLC değer)/HPLC değer] × 100
[b]ortalama ± SD (n = 3), [c]mg g^{-1}, [d]mg L^{-1}

Elde edilen sonuçlar, anodik olarak ön işlem görmüş BDD elektrot ve kare dalga voltametrisi tekniği ile CAF ve VAN eş zamanlı belirlenmesi için bazı önemli yararları ile kullanılabileceğini göstermektedir. Yüksek seçicilik, iyi gözlenebilme sınırı ve geniş doğrusal aralık, önerilen yöntemin yiyecek örneklerinde CAF ve VAN'ın eş zamanlı doğru bir şekilde belirlenmesini sağlamaktadır.

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