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Voltammetric Quantification of Phytoesterone 1-[5-(1, 3-Benzodioxol-5-yl)-1-oxo-2, 4-pentadienyl] Piperidine

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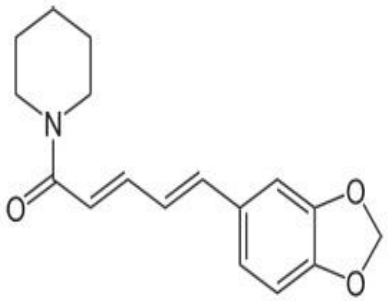
A simple one step electroanalytical method has been developed for the first time for the quantification of antioxidant piperine at glassy carbon based sensor. Piperine is primary bioactive component of pepper which has gained a great deal of attention from researchers all over the world due to its wide range of potential biological applications as an antioxidant and anti-carcinogenic agent. In the present study electrochemical behavior of piperine was investigated using square wave voltammetry. The reaction kinetics was studied and experimental conditions were optimized. The voltammetric studies of piperine at glassy carbon electrode exhibited a well defined cathodic peak for its reduction in Britton-Robinson buffer at pH 7.36. Under optimized experimental condition the square wave reduction peak current was linear over concentration range 8 to 48 µg/mL (R^2=0.995) with limit of detection (LOD) and limit of quantification of 2.4 µg/mL and 8.1µg/mL respectively. Developed method was successfully employed for the analysis of piperine in real samples.

Keywords: Antioxidant, Piperine**,** Phytoesterone, Voltammetry

1. INTRODUCTION

In recent times plant derived derivatives have gained great deal of importance due to their irrefutable potency as phytomedicine. Piperine (1-[5-(1,3-Benzodioxol-5-yl)-1-oxo-2,4-pentadienyl]piperidine) (Scheme I) is a valuable alkaloid extracted from piper nigrum. It is responsible for the pungency of black pepper. Since ancient times it has been used as stimulant, carminative, aphrodisiac, in treating skin diseases, bronchitis and neurological disorders [1]. Piperine also exhibits antioxidant and free radical scavenging properties [2-4]. *In vitro* studies of piperine reveal that it provides protection against oxidative damage by inhibiting or quenching free radicals and reactive oxygen species [5,6].



Scheme I. Structure of Piperine.

Phytoestrogens are natural selective estrogen response modifiers (SERMs) having vast therapeutic potential. In current scenario phytoestrogens are increasingly being researched for management of hormonal and reproductive pathologies. Along with this phytoestrogens also offer the advantages for menopausal symptoms and bone density without carrying the risks of heart disease, coronary artery damage or peripheral vascular issues [24]. Piperine exhibits excellent phytoestrogen properties [25-31].

Several methods have been reported in literature for determination of piperine. Literature survey reveals that, various chromatographic methods such as HPTLC [7-9], HPLC [10, 11] have been reported for the quantification of piperine. Wang et al. have performed simultaneous quantification of piperine along with other compounds by employing HPLC-MS/MS as analytical technique [32]. Ganesh et al. have performed simultaneous estimation of piperine along with gallic acid by using HPLC-MS/MS [33]. However techniques like HPLC, Mass spectroscopy offers advantage of high sensitivity but these methods are quite expensive and require establishment of elaborated lab for performing analysis and other techniques suffers from drawbacks such as poor resolution, lack of sensitivity and reproducibility. Electrochemistry has been proven to be an excellent technique for qualitative as well as quantitative determination of organic molecules in pharmaceutical dosage as well as biological fluid [12-15].It also offers the advantage of shorter analysis time, simple experimental set-up and cost effectiveness. Electrochemical properties and behavior of antioxidant compounds can be effectively understood by their electrochemical characterization under different conditions [16-18]. It has been found that compounds which exhibits less positive oxidation potential or are highly susceptible to electrochemical oxidation possess higher antioxidant properties.

The present work mainly focuses on electrochemical analysis of piperine under optimized experimental conditions and application of developed method for quantification of piperine in real samples.

2. EXPERIMENTAL

2.1 Reagents and Chemicals

Piperine 97 % purity was procured from Sigma Aldrich, USA. Stock solution for piperine was prepared in methanol.1M KCl solution was used as supporting electrolyte. Britton-Robinson (B-R)

buffer was prepared in double distilled water. All chemicals used were of analytical grade and were used as received. Square wave voltammetry and cyclic voltammetry were employed as electroanalytical technique.

2.2 Apparatus

In order to carry out electrochemical measurements AUTOLAB (Eco-chemie B.V., Utreht, The Netherlands) potentiostat-galvanostat with NOVA 1.10 computrance software was used. Glassy carbon electrode was used as working electrode, Ag/AgCl as reference (3.0 M KCl) and platinum was employed as an auxillary electrode. For pH-metric measurements Decible DB-1011 digital pH meter fitted with a glass electrode and saturated calomel electrode as reference electrode was employed.

2.3 Preparation of real samples

In order to carry out real sample analysis 1.0 g of pepper sample was taken and it was finely ground in a mortar pestle. After that sample was transferred to 100 mL volumetric flask and 25 mL pure methanol was added to it. The resulting mixture was shaken vigorously for 40 minutes followed by ultra-sonication for 2 hours. Finally volume was made up to 100 mL with pure methanol and solution was transferred to centrifuge tubes. The sample was then centrifuged at 3000 rpm for 20 minutes and allowed to settle for 2 minutes. The supernatant was transferred and preserved for further analysis.

2.4 Polishing of glassy carbon electrode

Alumina powder with particle size ranging from 0.05 to 0.1 μm was used for polishing the surface of glassy carbon electrode. Further it was washed with double distilled water and ethanol respectively followed by ultrasonication in double distilled water and ethanol several times and this process was continued until mirror like finish was obtained.

3. RESULTS AND DISCUSSION

3.1 Effect of pH

Britton-Robinson (B-R) buffer (pH range 2.3-9.1) was employed to study the effect of pH on the electrochemical reaction of piperine on the surface of glassy carbon.Fig.1(A) represents cyclic voltammogram for plot between current and potential at different pH. Fig.1 (B) displays the relationship between peak current and pH. From the Fig.1(B) it is clear that reduction peak current for piperine increased with increasing pH until it reached the maxima at 7.36 and then decreased with further increase in pH. Hence pH 7.36 was for used further electrochemical analysis. When graph was

plotted between pH (2.3-7.6) and potential a straight line was obtained with R^2=0.987, a negative shift in peak potential was observed which is suggestive of involvement of protons in the electrode process (equation 1).

According to classical nernstain equation when graph is plotted between potential and pH and the absolute value of slope is equal to the theoretical value of 59mV/pH, then it suggests that equal number of electron and protons are involved in electrode process. In the current scenario value of slope was found to be 0.059 which is equal to theoretical value of 59 mV/ (classical nernstain one electron one proton process) (Fig.1C), suggesting that number of protons and electrons involved in electrochemical process of piperine are equal [19, 23, 28].

SWV; E/V (Ag/AgCl) = 0.059 pH + 1.049 R^2 = 0.987 (1)

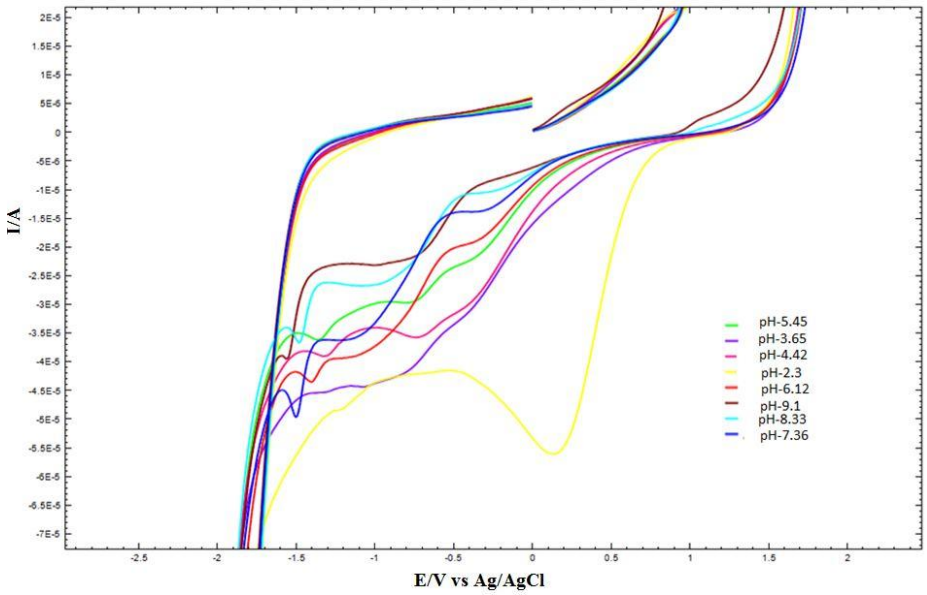


Figure 1(A): Influence of pH on peak current and peak potential of piperine, scan rate 0.1V $s^{-1,}$ B-R Buffer (pH range 2.3-9.1)

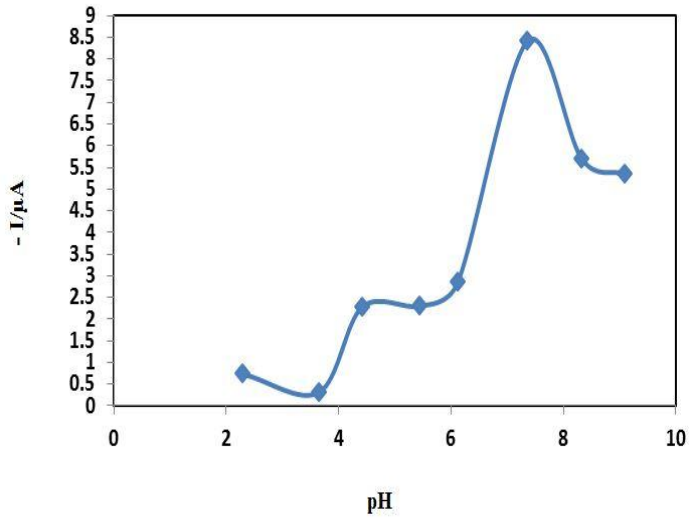


Figure 1(B): Plot of pH vs peak current (I/A)

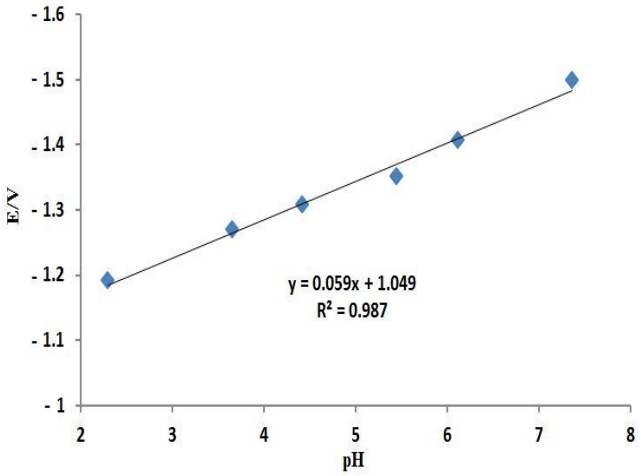


Figure 1(C): Plot of pH vs peak potential (E/V)

3.2 Effect of scan rate on the peak current of piperine

The reaction kinetics of piperine on surface of glassy carbon electrode was studied by employing cyclic voltammetry as electroanalytical technique. Fig.2A represents the effect of scan rate on peak current and peak potential. The cyclic voltammogram of 80µg/mL piperine in B-R buffer (pH-7.36), 1 M KCl were recorded with scan rate ranging from 100-300 mV/s. From Fig.2B it is clear that reduction peak current for piperine increased with increase of scan rate and log of peak current had a linear relationship with log of scan rate with regression equation;

$$\log I\,(\mu A) = 1.099 + \log 0.953\ v\ (mV/s)\ (R^2 = 0.991) \qquad (2)$$

For diffusion controlled processes slope close to 0.5 is expected whereas for adsorption controlled processes slope close to 1 is expected [20-22]. From equation (2) value of slope was found to be 0.953 which indicates that reduction of piperine on glassy carbon electrode is predominantly adsorption controlled.

Fig.2C illustrates that peak current (I_p) for piperine increased linearly with scan rate (v) which indicates that reduction of piperine at glassy carbon electrode is adsorption controlled process [23]. Linear regression equation can be expressed as follows.

$$I\,(\mu A) = 0.012 + 0.062\ v\ (mV/s) \quad (R^2 = 0.989) \qquad (3)$$

From Fig. 2D it is clear that in scan rate range of 100-300 mV/s the cathodic peak potential for piperine increased linearly with napierian logarithm of scan rate (ln v) and regression equation was found to be ;

$$E_p\ (V) = 1.464 + 0.026 \ln v \quad (R^2 = 0.991) \qquad (4)$$

The reaction of piperine was found to be irreversible process and linear relation between E_p and Napierian logarithm of scan rate (ln v) followed the equation (5, 6) [23].

$$E_p = E^{\theta'} - \frac{RT}{\alpha nF}\left[0.780 + \ln\left(\frac{D^{1/2}}{k^0}\right) + \ln\left(\frac{\alpha nF\nu}{RT}\right)^{1/2}\right] \quad \textbf{(5)}$$

$$= K + \frac{RT}{2\alpha nF}\ln\ \nu \quad \textbf{(6)}$$

Where $E^{\Theta,}$ is the formal redox potential,

α is electron transfer coefficient,

R is gas constant,

F is Faraday constant,

k^0 is standard heterogeneous rate constant,

n is number of electrons involved

For the above equation slope would be equal to RT/2αnF

Hence the value of 'n' (number of electrons involved) was found to be 0.99, and thus one electron was involved in the electrode reaction of piperine (Scheme II). As described in section 3.1 number of protons and electron involved in electroxidation of piperine is equal. [23, 27]

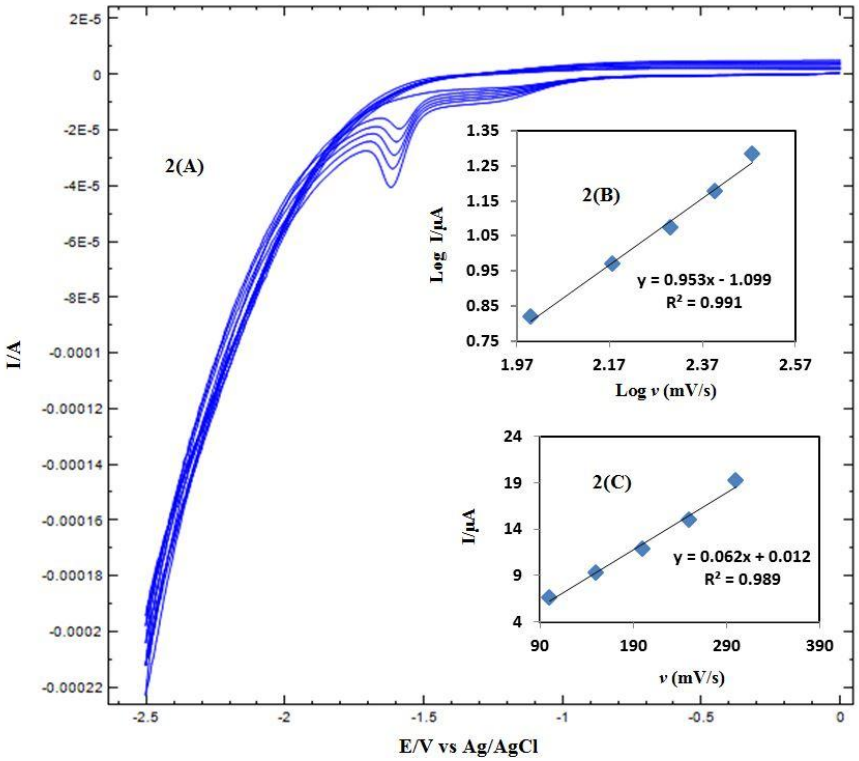


Figure 2. (A): Cyclic voltammograms of Piperine at glassy carbon electrode in B-R buffer (pH 7.36) at different scan rates (a-f): (a) Blank (b) 100 mVs^{-1}(c) 150 mVs^{-1} (d) 200 mVs^{-1} (e) 250 mVs^{-1} (f) 300 mV s^{-1}. **(B):** Inset: (A) Plot of log scan rate (ν/mVs^{-1}) versus log current (I/A) **(C):** Inset: Plot between the peak current (I/A) and scan rate (ν/ mVs^{-1}).

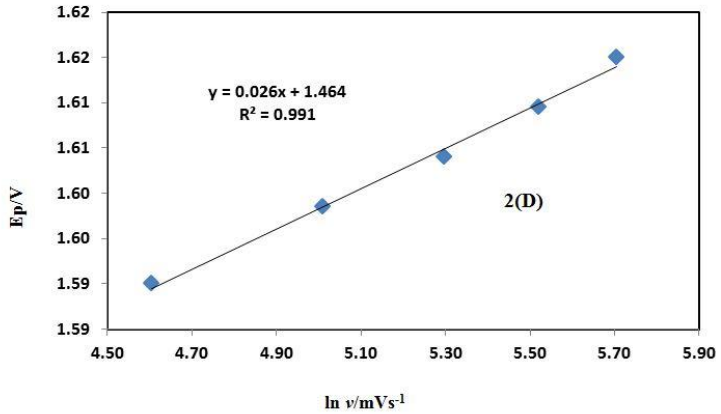
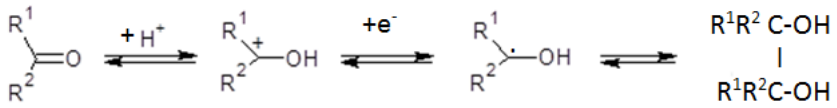


Figure 2. (D): Plot of Napierian logarithm of scan rate (ln *v*/ mVs^{-1}) versus peak potential (E_p/V)

Reaction Mechanism

In general the first electrochemical step is a reversible transfer of an electron from electrode to the carbonyl compound after protonation which further results in formation of free radical alcohol, which further undergoes dimerization to form di alcohol (Scheme II) [29].



Scheme II. Reaction Mechanism

3.3 Validation of proposed method

3.3.1. Calibration curve and Limit of detection

The square wave voltammogram for piperine were recorded at bare GCE in concentration range of 8 to 48µg/mL under optimized electrochemical conditions. The dependence of cathodic peak current on concentration of piperine was investigated by employing square wave voltammetry as electroanalytical technique. It was observed that cathodic peak current increased with increase in concentration of piperine over a calibration curve range of 8 to 48µg/mL with regression of R^2=0.998 (Fig.3);

$I/\mu A = 0.055\ (\mu g/mL) + 1.681\ (R^2=0.998)$ (7)

3.3.2 Limit of detection (LOD) and Quantification (LOQ)

The limit of detection and limit of quantification were calculated by using following equations:

LOD = 3S/m

LOQ = 10 S/m

Where 'S' represents the standard deviation of intercept and 'm' represents mean of slope of calibration curve. The data of three calibration curves were used for calculating standard deviation of intercepts and mean of slope [27, 28].

The limit of detection (LOD) and limit of quantification (LOQ) for piperine were found to be 2.4 µg/mL and 8.1 µg/mL respectively.

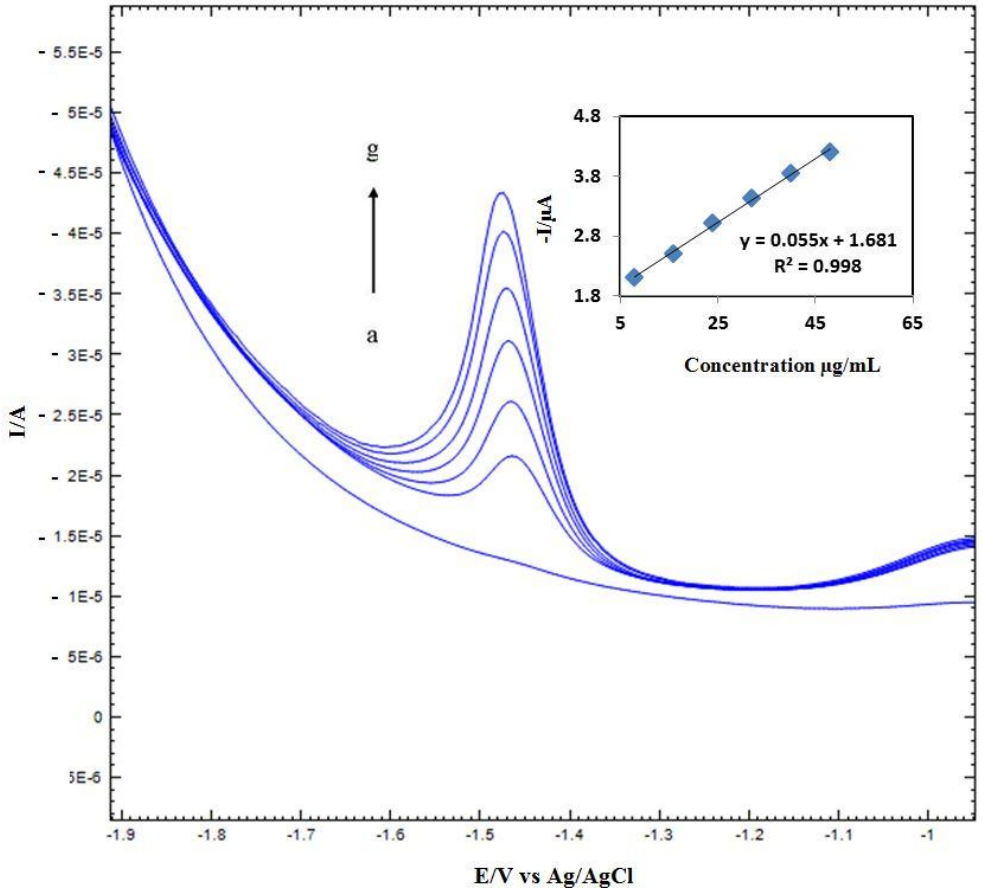


Figure 3. Linearity of squarewave voltammetric peak current of Piperine at glassy carbon electrode at different concentrations (pH 7.36): (a) Blank (b) 8 µg/mL (c) 16 µg/mL (d) 24 µg/mL (e) 32 µg/mL (f) 40 µg/mL (g) 48 µg/mL. Inset: Plot of Current vs. Concentrations of piperine B-R buffer (pH 7.36).

3.3.2. Reproducibility

In order to investigate the reproducibility of developed sensor, a reproducibility experiment was carried out by repeating the determination of specific known concentration (40µg/mL) of piperine multiple times. Reproducibility experiment was carried out as per Guidance for Industry Bioanalytical Method Validation according to which "reproducibility of the method is assessed by replicate measurements using the assay, including quality controls". For this purpose known concentration of

piperine (40µg/mL) were run for six consecutive times and relative standard deviation was calculated. According to guidance for Industry Bioanalytical Method Validation the relative standard deviation should be less than 15 %.[34] The relative standard deviation for current response for six successive measurements of piperine was found to be 2.53 %, illustrating remarkable reproducibility of developed method (Table 1) [27].

Table 1. Reproducibility experiment for piperine (40 µg/mL) at GCE for six consecutive runs, scan rate 100 mVs^{-1}B-R buffer pH -7.36

Current in Micro Ampere(µA)	Average Current in Micro Ampere (µA)	%RSD
2.22 2.29 2.26 2.35 2.37 3.34	2.34[a]	2.53[b]

a - Average of current for six replicate readings

b - % RSD for Six replicate readings

3.3.3. Precision of developed method

According to Guidance for Industry Bioanalytical Method Validation "precision of method may be defined as the closeness of individual measures of an analyte when the procedure is applied repeatedly to multiple aliquots of a single homogeneous volume of biological matrix. A minimum of three concentrations in the range of expected study sample concentrations is recommended. The precision determined at each concentration level should not exceed 15% of the coefficient of variation (CV)". [34]

Table 2. Precision experiment at GCE at three different concentration (16, 32, 40 µg/mL) level of piperine at three different time interval, scan rate 100 mVs^{-1} , B-R buffer pH-7.36

Intra-day repeatability		
Concentration (µg/mL)	Average Current in Micro Ampere (µA)	%CV
16	1.02[a]	2.42[b]
32	1.91[a]	3.68[b]
40	2.32[a]	2.80[b]

a - Average of three replicates readings at each concentration

b - % CV of three replicates readings at each concentration

In order to assess precision of developed method precision stability experiment were carried out by analyzing series of three different concentrations of piperine (16, 32, 40 µg/mL) at three different time interval in a day. The coefficient of variance (%CV) for the peak currents in SWV based

on three replicates for concentration of 16 µg/mL was found to be (2.42%), for concentration of 32µg/mL (3.68 %), for concentration of 40µg/mL (2.80 %), which suggests that the developed method exhibits excellent precision for quantification of piperine. (Table 2) [27].

3.3.4. Analysis of real samples

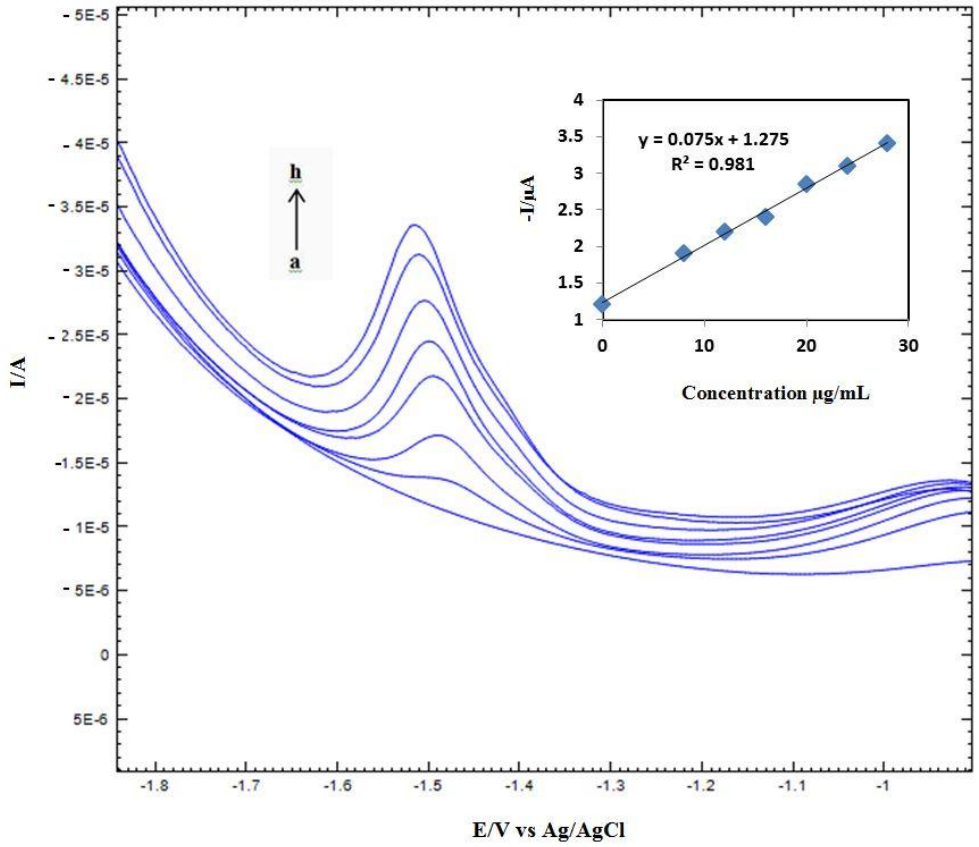


Figure 4. Square-wave voltammograms for determination of piperine in real sample (black pepper) by Standard addition method (a) blank, (b) sample (c) 8 µg/mL (d) 12 µg/mL (e) 16 µg/mL (f) 20 µg/mL (g) 24 µg/mL (h) 28 µg/mL of piperine standard added. Inset: Plot of Current vs. Concentrations of piperine B-R buffer (pH 7.36).

Real sample analysis for piperine was performed in black pepper. Sample preparation has been described in section 2.3. Standard addition method was employed for the analysis of real samples. "The standard addition method is commonly used to determine the concentration of an analyte that is in a complex matrix such as biological fluids, soil samples, etc. The reason for using the standard addition method is that the matrix may contain other components that interfere with the analyte signal causing inaccuracy in the determined concentration. The change in instrument response between the sample and the spiked samples is assumed to be due only to change in analyte concentration". (Standard Addition Methods-Analytical sensors).

The procedure adapted was as follows:

First of all sample was splitted into even aliquots (7 aliquots) in separate volumetric flask of same volume (2mL). The first flask was then diluted to volume with methanol. Then a standard containing the analyte (piperine1000µg/mL) was added in increasing volumes (200µL-700 µL) to the subsequent flasks and each flask was diluted to volume with methanol. The instrument response was then measured for all of the diluted solutions and the data was plotted with concentration in the x-axis and instrument response in the y-axis. Linear regression was performed and the slope (m) and y-intercept (b) of the calibration curve were used to calculate the concentration of analyte (piperine) in the real sample. (Figure 4)

$C_x = b.C_s/m.V_x$ (8)

C_x=Concentration of the sample, C_s = Concentration of the standard, V_x = Volume of the sample aliquot, m = Slope of linear regression, b = Intercept of linear regression

3.3.5 Interference analysis (Selectivity)

In order to investigate selectivity of developed method an interference analysis was carried out in presence of some common interfering inorganic ions and organic compounds. The investigation was performed in presence of 20µg/mL piperine. For this purpose percent recovery was calculated for analyte (piperine) in the presence of interferent by using following formula:

$$\% \text{ Recovery} = \frac{\text{Current response for analyte in presence of interferent}}{\text{Current response for analyte alone}} \times 100$$

Results indicated that examined compounds and ions had no significant influence in the detection of piperine. In the presence of these interferents recovery of piperine changed in range of 97-102 %, indicating excellent selectivity of developed method [23,27]. The obtained results are listed in Table 3

Table 3. Interference Analysis (Selectivity) of different species for the determination of piperine at bare GCE

Interferent	Interferent Concentration (µg/mL)	Recovery%
Inorganic ions (Na^{+},K^{+},SO_4^{2-},Ca^{2+},NH^{4+},CO_3^{2-})	40	104.27
Glucose	20	99.14
Fructose	20	100.34
Vanillin	20	102.73
Capsaicin	20	103.07
Cinnemaldehyde	20	97.02

4. CONCLUSION

Plants are considered to be most important sources of natural therapeutics. In the current scenario in order to improve quality and longetivity of life, focus is on the development of plant based medicinal preparations. It is of great importance to develop simple, sensitive and cost effective methods for determination and quantification of these phytomedicnes. Electroanalysis is a well suited approach in context of evaluation of antioxidant properties of plant based products because antioxidant mechanism *in vitro* system is based on electron transfer during the redox reaction. The present work emphasizes on development of simple, convenient, and accurate voltammetric method for quantification of piperine on glassy carbon electrode. Also there was no need for any precipitation, evaporation, or extraction prior to analysis. Developed method was successfully employed for quantification of piperine in real sample.

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