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REGULAR ARTICLE

Electrochemical and spectroscopic studies of the interaction of (+)-epicatechin with bovine serum albumin

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Abstract. The mechanism of the electrochemical oxidation of (+)-epicatechin (epiCAT) in a hydro-alcoholic medium, at a glassy carbon electrode, was studied using cyclic and square wave voltammetric techniques and at different pHs. It proceeds in a cascade mechanism related to the two resorcinol hydroxyl groups and the other three hydroxyl groups in epiCAT which present electroactivity and is pH-dependent. The oxidation of the 3′,4′-dihydroxyl moiety, occurs first at a very low positive potential, and it is a two-electron/two proton reversible reaction. The product obtained from the oxidation of the 3′,4′-dihydroxyl moiety then undergoes a chemical reaction followed by an electrochemical process to give an electroinactive product. The proposed mechanism is an ECE type mechanism. After the addition of bovine serum albumin (BSA) to epiCAT solution, the oxidation peak currents decreased with no peak potential shift and no new peaks appeared. The diffusion coefficients of both free and bound epiCAT were estimated from the cyclic voltammetry data ($D_f = 2.37 \times 10^{-10}$ cm^2s^{-1} and $D_b = 6.28 \times 10^{-11}$ cm^2s^{-1}). An epiCAT-BSA complex is formed with binding constant $K_{app} = 1.8 \times 10^4$, calculated from UV-vis spectroscopy data.

Keywords. (+)-Epicatechin; Bovine serum albumin; UV-vis Spectroscopy; Cyclic voltammetry; Square wave voltammetry; Interactions.

Abbreviations

BSA	Bovine serum albumin
epiCAT	(+)-Epicatechin
SCE	Saturated calomel electrode
CV	Cyclic voltammetry
SWV	Square wave voltammetry

1. Introduction

The development of modern medicine has enabled remarkable advances in the treatment of certain ailments (malaria, cancer, diabetes). Nevertheless, the existing drugs or treatments have not made it possible to eradicate these diseases, which explain the numerous researches in progress aimed at developing new therapeutic agents. In recent decades, natural products such as flavonoids have received significant interest as potential therapeutic agents for the treatment of certain infections such as viral, allergic, platelet and inflammatory diseases; as well as protective effects against chronic diseases.[1–3] In addition, this class of compounds is well-known to promote anti-tumour activities and thus, proves to be useful as chemopreventive agents in human carcinogenesis.[4–7] The polyphenolic compounds, commonly present in higher plants, are also the major chemical constituents of most dietary species. Scheme 1 shows the structure of (+)-epicatechin (epiCAT), the flavanol herein investigated. epiCAT is a major constituent of green tea. Its chemical

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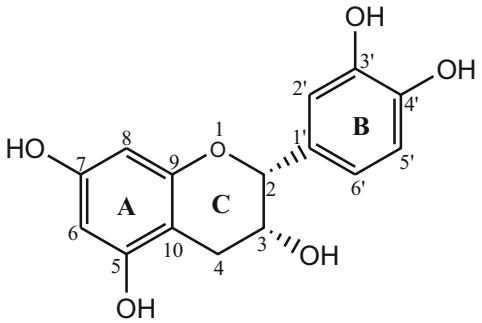


Scheme 1. Chemical structure of epiCAT.

structure displays 5 hydroxyl groups and the antioxidant activities of epiCAT that originate from its favourable one-electron donation capabilities. One of the unique properties of most catechin derivatives is their ability to repair vitamin E.[8–10]

The work described in this paper is part of a process aimed at developing biosensors and studying charge transfers (ions and electrons) between species soluble in aqueous phase and compounds dissolved in the organic phase. The elucidation of interactions between drug molecules and bioactive molecules, such as proteins, DNA and amino acids, has attracted interest in recent years.[11–16] Bovine serum albumin (BSA) has been one of the most extensively studied proteins.[17] The distribution, free concentration and the metabolism of various drugs may be strongly affected by drug-protein interaction in the bloodstream.[17,18] Moreover, the determination and monitoring of drug-protein adducts have important clinical, pharmacological and toxicological implications.[19]

Several analytical methods have been used to study these interactions in the literature, such as gel electrophoresis, footprinting technique, X-ray crystallography,[20,21] structural modelling[22] and spectroscopic techniques, especially fluorescence spectroscopy.[23,24] These different methods have limitations, in particular the cost of the devices, the complexity of the analyses, the availability of reagents and the characterization of the interaction between polyphenolic compounds and proteins. UV-vis spectroscopy[25–28] and electrochemical methods[28–32] have been herein used to investigate the drug-protein interactions between epiCAT and BSA, since they exhibit some advantages including direct monitoring, high sensitivity and simplicity. Compared to spectroscopic methods, electrochemical assays are simple, easily implemented, low-cost and show fast response for the characterization of the binding properties of organic compounds to proteins in solution.[32] Electrochemical methods are also useful tools to determine the electrochemical redox response of biomolecules in biological samples.[33] In this work, electrochemical methods and UV-vis spectroscopy were used to elucidate the interaction between epiCAT and BSA and a mechanism proposed in hydro-alcoholic medium.

2. Experimental

2.1 *Chemicals and reagents*

(+)-Epicatechin (epiCAT) was obtained from the CH_2Cl_2-MeOH (1:1) extract of *Detarium microcarpum* using a procedure described elsewhere[34] and all the other reagents were Merck analytical grade. Stock solutions of 50 μM epiCAT were prepared in ethanol and kept in a refrigerator at 10 °C. These solutions were then diluted to the convenient concentrations with buffer supporting electrolyte. BSA was purchased from Merck and used as received without further purification. Stock solutions of BSA were daily prepared by dissolving appropriate amounts in double-distilled water.

2.2 *Apparatus*

Electrochemical data were recorded using a μ-Autolab potenstiostat connected to a computer equipped with the GPES electrochemical analysis software (Eco-Chemie, Netherlands) and to a standard three-electrode cell. A glassy carbon electrode (GCE, 3 mm diameter) was used as working electrode, a platinum (Pt) wire served as counter electrode and the reference electrode was a saturated calomel reference electrode (SCE). The working electrode was polished with 0.3 μm alumina slurry on a polishing cloth, sonicated in deionized water, rinsed with water-acetone and dried before use. Solutions containing ethanol and K_2HPO_4/KH_2PO_4 (both 0.1 M) 1:1 (v/v) or KCl (0.1 M), with pH adjusted with KOH or HCl, were used as supporting electrolyte. epiCAT was added directly to the cell after obtaining cyclic and square wave voltammograms at the electrode immersed in the epiCAT-free solution. The solutions were purged with nitrogen for eight minutes before recording the voltammograms, and the experiments were carried out at room temperature. Visible spectra were recorded in solution on GENESYS 10S UV-vis spectrophotometer.

3. Results and Discussion

It has been shown that the antioxidant activity of flavonoids resides in the aromatic OH groups.[35] The structure of epiCAT presents 5 functional OH groups, which can undergo oxidation or reduction.

3.1 *Electrochemical study of epiCAT in the absence of BSA*

Figure 1A shows cyclic voltammograms (CV) at GCE immersed in hydro-alcoholic medium at pH 7.04, (a) in the absence of epiCAT and (b) presence of 50 µM epiCAT at scan rate 100 mVs^{-1}. Figure 1A(b) shows two distinct peaks (I/I′ and II). The peak pair I/I′ is well-developed and quasi-reversible, while the second, II, located at a more negative potential is irreversible. When the electrode was scanned within the potential range from -0.4 V to 0.6 V (redox system I/I′) and -0.4 V to -1.2 V (system II, Figure 1B inset), peak II disappeared with this restriction, while the behaviour of system I/I′ did not change (Figure 1B). This indicates that the electroactive species that generates system II is obtained from process I/I′. A similar voltammetric profile was observed by Rafiee and Nematollahi, and suggests a reaction between the quinone that results from the oxidation of epiCat and ethanol present in the hydro-alcoholic medium.[36]

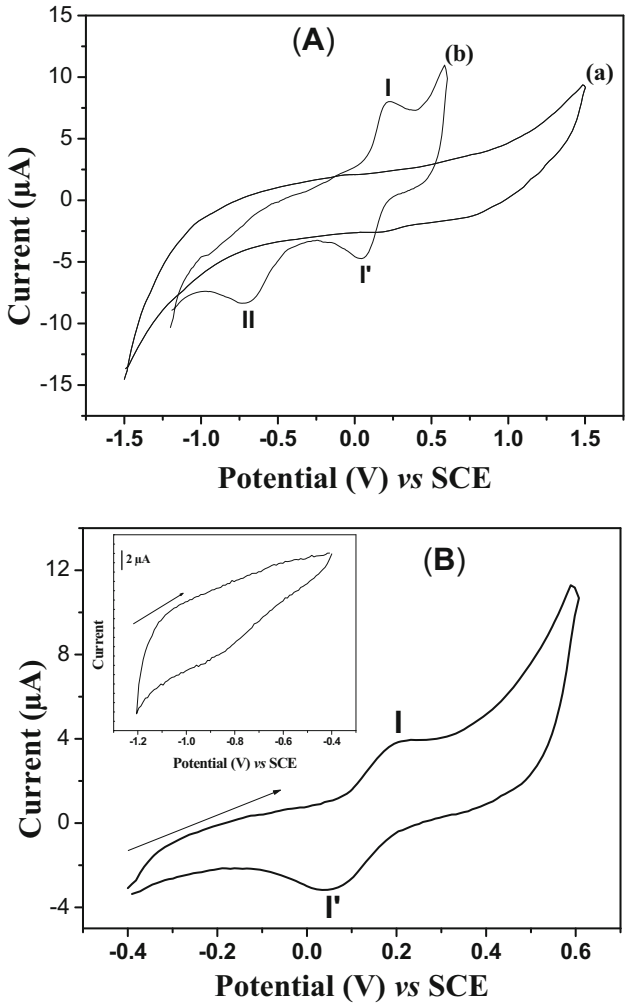
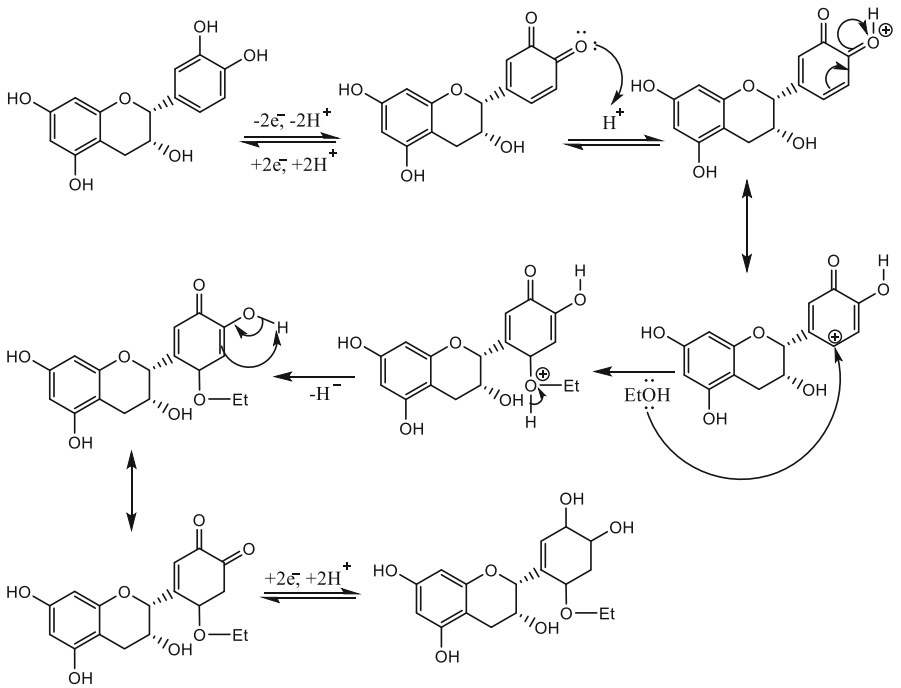


Figure 1. Cyclic voltammograms (**A**) (**a**) in the absence of and (**b**) in the presence of 50 µM epiCAT and (**B**) effect of the resting potential on the net epiCAT CV peaks I, I′ and II at pH 7.04 in 0.1 M phosphate buffer/hydro-alcoholic medium at GCE. Scan rate 100 mVs^{-1}.

Both systems are affected by the scan rate (Figure 2) at a constant pH. The peak current increases with the scan rate. This scan rate-dependence could also indicate an electrochemical-chemical-electrochemical (ECE) reaction mechanism, consistent with a chemical rearrangement following the oxidation. The oxidation peak current is proportional to the square root of the scan rate from 10 to 100 mVs^{-1} (Figure 2 inset). This indicates that the electrochemical process is controlled by diffusion.[37] Upon increasing the scan rate, there occurs a peak potential shift towards more positive values, as well as a visible increase in the current densities. This phenomenon is typical of an ECE mechanism[38] as shown in Scheme 2.

Square wave voltammetry (SWV) offers a higher analysis speed, lower consumption of electroactive species, and reduced inhibition of the electrode surface[38,40] and unifies the advantages of CV and pulse voltammetry techniques. SWV results (Figure 3) were compared to those obtained at the same pH using CV (Figure 1). Similar results to CV were obtained, that is two distinct peaks (I/I′ and II). A great advantage of the SWV method is the possibility to see during a scan if the electron transfer reaction is reversible or not. The quasireversibility of the oxidation peak I is clearly shown for the pH studied. The second reduction peak II is produced by the I/I′ system (Figure 3).

In epiCAT chemical structure, there is conjugation between rings A and B. The 3 OH group's activity is enhanced by an electron-donating effect of the hydroxyl groups at positions 5 and 7. The resulting intermediate is stabilised by the electron-donating effect of the hydroxyl groups in ring B, and the C-3 hydroxyl group can also form intermolecular hydrogen bonds. The oxidation of epiCAT proceeds in a cascade mechanism related to the substituent groups in ring B which all present electroactivity and whose oxidation potentials are identified with peak I. On ring B the oxidation of the 3′,4′-dihydroxyl electron-donating groups, occurs first at very low positive potentials and is a two-electron reversible reaction.[36]



Scheme 2. Proposed mechanism for the oxidation of epiCAT in a hydro-alcoholic medium.

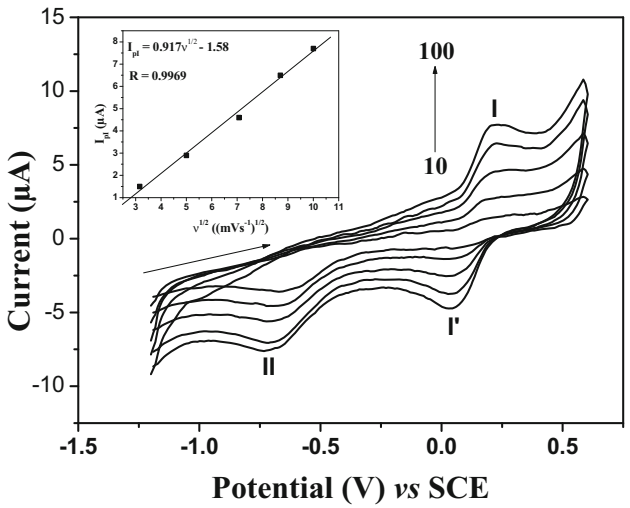


Figure 2. Cyclic voltammograms of 50 μM epiCAT at pH 7.04 in 0.1 M phosphate buffer/hydro-alcoholic medium at GCE for different scan rates: 10, 25, 50, 75 and 100 mVs^{-1}. Inset I_{p1} against $v^{1/2}$.

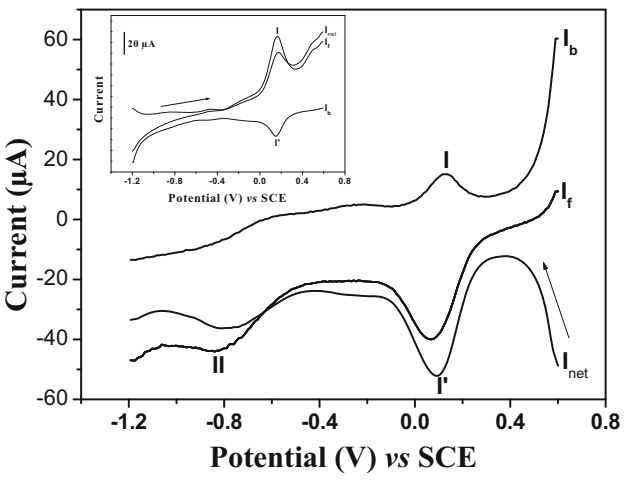


Figure 3. Square wave voltammograms of 50 μM epiCAT at pH 7.04 in hydro-alcoholic medium at GCE: I_{net}, total current; I_f, forward current; I_b, backward current. Frequency 50 Hz and amplitude 50 mV.

Previous research demonstrated that the pH dependence of hydroxyflavane antioxidant activity is mainly due to an increased radical scavenging ability of the flavonoids upon their deprotonation. Because deprotonation generally enhances the antioxidant action of the hydroxyflavanes and because only the ionisation potential, and not the bond dissociation energies of the flavanol, becomes significantly lower upon deprotonation, it can be concluded that electron donation is the dominant mechanism of antioxidant action of the flavanol after deprotonation. Upon deprotonation the radical prowling capacity increases because the electron and not proton donation becomes easier. The implication is that not only the ease of radical prowling but also the mechanism of antioxidant activity may change upon deprotonation, and electron donation may be more important for flavonoid antioxidant action at physiological pH.[41] The influence of pH on the square wave voltammograms at GCE

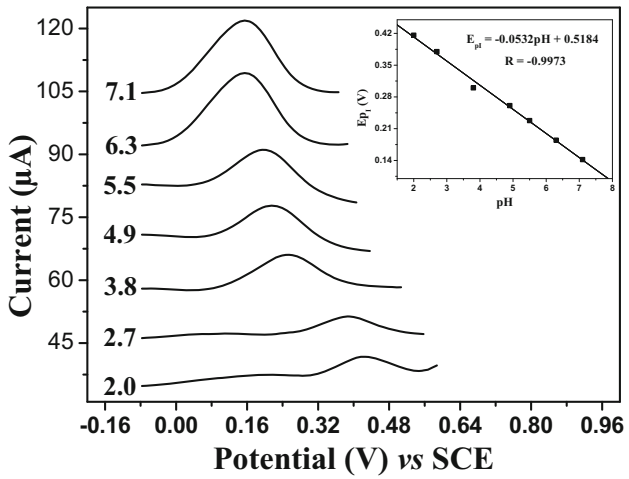


Figure 4. Square wave voltammograms of 50 µM epiCAT at pH 7.04 in a hydro-alcoholic medium at GCE at different pHs. Frequency 50 Hz and amplitude 50 mV.

immersed in 50 µM epiCAT in hydro-alcoholic medium and 100 mVs^{-1} is shown in Figure 4. Observation from the aforementioned figure depicts that the relative heights and positions of the voltammetric peaks are indeed strongly sensitive to the pH.

The potential of peak I markedly decreased with increase in the solution pH and the dependence of anodic peak I potential (E_{pI}) on the solution pH generates a straight line (Figure 4 inset), whose slope is 53.20 mV/pH unit (R = 0.9973). This is typical of phenolic systems in aqueous media[39] and depicts an electrochemical reaction followed by deprotonation involving the same number of electrons as protons.

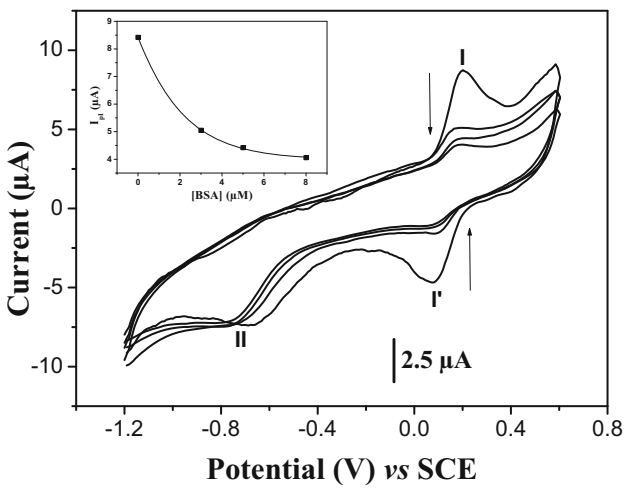


Figure 5. Cyclic voltammograms of 50 µM epiCAT at pH 7.04 in 0.1 M phosphate buffer/hydro-alcoholic medium at GCE for different concentrations of BSA: 0, 3, 5, 8 µM. Scan rate 100 mVs^{-1}. Inset I_{pI} against [BSA].

Table 1. Effect of added BSA on the anodic and cathodic peak currents of epiCAT.

[epiCAT] (µM)	BSA [µM]	I_{paI} (µA)	I_{pcI} (µA)
50	0	8.42	− 4.58
50	3	5.05	− 1.53
50	5	4.42	− 1.12
50	8	4.06	− 0.92

3.2 *Electrochemical study of epiCAT in the presence of BSA*

The addition of BSA decreased the peak currents of epiCAT and there was no peak potential shift (Figure 5). The relevant electrochemical data for the different BSA concentrations are presented in Table 1.

The great decrease in CV peak currents could be attributed to the diffusion of epiCAT bound to the slowly diffusing BSA with large molecular weight. These current changes are therefore linked to the diffusion of an equilibrium mixture of free and bound epiCAT to the electrode. This result is in accordance with the observations of Zhao *et al.*[14] in the study of the interaction of camptothecin and homologous proteins; human serum albumin (HAS). The authors give two possible mechanisms to explain the peak decrease. One is that, in the case of this study, BSA interacts with epiCAT to form an electrochemically inactive complex, which blocks the electron transfer between epiCAT and the electrode. The other explanation is the competitive adsorption of BSA at the GCE surface which blocks the electron transfer process. These results indicated that the interaction between epiCAT and BSA occurred and a new electrochemically inactive epiCAT-BSA complex was formed, lowering the free epiCAT concentration.

3.3 *UV-vis spectroscopy of epiCAT in the presence of BSA*

The interaction of epiCAT and BSA was also studied by UV-vis spectroscopy. Figure 6 shows the UV-vis absorption spectra of BSA (Figure 6a), and epiCAT in the absence (Figure 6b) and in the presence of BSA (Figure 6c) in a hydro-alcoholic medium.

The UV-vis spectrum of epiCAT showed an intense band II at λ_{max} 275 nm[42] and a less intense band I at λ_{max} 430 nm (Figure 6b). Band I is related to the absorbance of ring B whereas band II is related to the π-π^* transition absorbance in ring A. When epiCAT

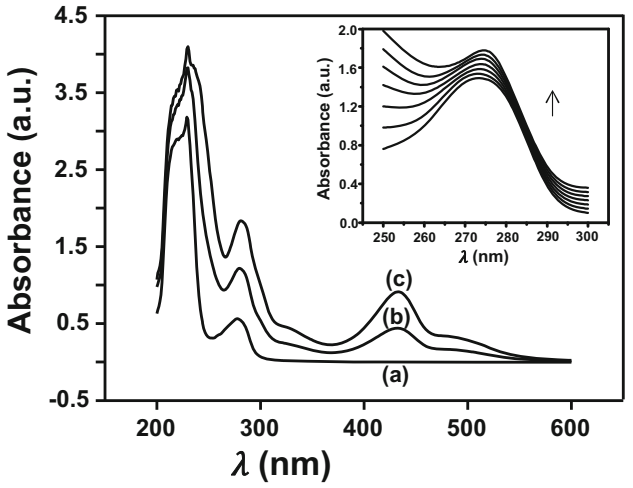


Figure 6. Absorption spectra of **a** BSA (10 μM), **b** epiCAT (50 μM) and **c** epiCAT (50 μM)/BSA (10 μM) mixture at pH 7.04 in hydro-alcoholic medium. Inset: Absorption spectra of BSA (50 μM) in the presence of epiCAT (0, 8, 16, 24, 32, 40, and 48 μM).

and BSA are mixed, the spectrum is significantly different from that of epiCAT. Band I show a stronger absorbance at a higher wavelength, red shift and displays hypochromicity. The changes in the absorption spectrum indicate that there is a binding interaction between epiCAT and BSA, which induces the conformational change of BSA. This suggests that epiCAT and BSA could form an epiCAT-BSA complex. Figure 6 inset shows the absorption spectra of BSA in the presence of increasing concentrations of epiCAT. It was found that as the epiCAT concentration increases, the intensity of the band at 275 nm increases significantly with a blue shift. This increase in intensity could be attributed to the formation of the ground state complex between BSA and epiCAT. epiCAT showed the highest absorbance in ethanol but in all other solvents, the nature of the spectra was very similar. Also, the effect of the amount of ethanol on BSA was checked and no significant changes in the absorption spectra upon addition of ethanol were observed.

Table 2. Effect of added epiCAT on the epiCAT-BSA absorbance.

[BSA] (μM)	[epiCAT] (μM)	Absorbance (a.u.)
50	0	1.4
50	8	1.49
50	16	1.55
50	24	1.61
50	32	1.65
50	40	1.7
50	48	1.75

3.4 *Determination of the binding constant (K_{app}) and diffusion coefficients*

The relevant UV-vis spectroscopic data for different epiCAT concentrations are presented in Table 2. From the data in Table 2, 1/(A - A_0) as a function of 1/[epiCAT] was plotted (Figure 7).

The equilibrium constant for the epiCAT-BSA complex formation is given by Equation (1). Values of the apparent association constant, K_{app}, were obtained from the BSA absorption band at 275 nm according to the Benesi and Hildebrand equation[43]:

$$\frac{1}{A - A_0} = \frac{1}{A_c - A_0} + \frac{1}{K_{app}(A_c - A_0)[epiCAT]} \quad (1)$$

where A_0 is the absorbance of BSA in the absence of epiCAT and A_c is the recorded absorbance at 275 nm for BSA at different epiCAT concentrations. The double reciprocal plot of 1/(A - A_0) against 1/[epiCAT] is linear and the apparent association constant (K_{app}) was determined and found to be 1.8×10^4 (R= 0.9977). The value of K_{app} is considerably high, thereby indicating the formation of a stable complex between BSA and epiCAT.

Diffusion coefficients were determined using the Randles-Ševčik equation.[37] For a quasi-reversible anodic electrochemical process, the peak current in cyclic voltammetry can be given by:

$$I_p = (2.99 \times 10^5) n(\alpha n_a)^{1/2} A C_0 D^{1/2} v^{1/2} \quad (2)$$

where I_p is the peak current, n is the number of electrons involved in the oxidation, αn_a is a parameter reflecting the irreversibility of the oxidation, A is the

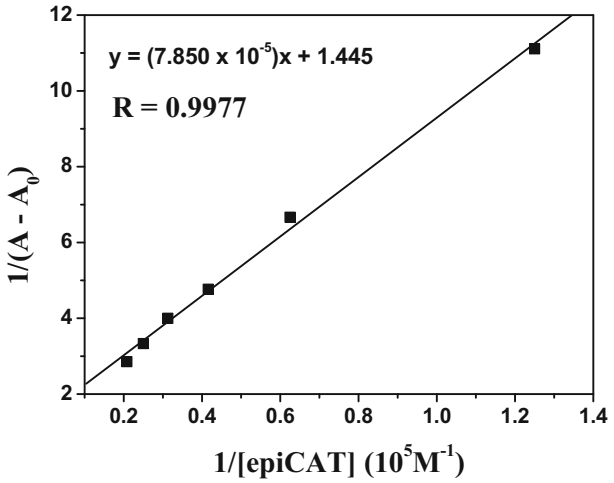


Figure 7. Evolution of 1/(A - A_0) as function of 1/[epiCAT].

area of the electrode (7 x 10^{-2} cm^2), C_0 is the substrate concentration (50 μM), *v* is the potential scan rate, and D is the diffusion coefficient of the substrate (epiCAT). From Figure 2 inset, the diffusion coefficient of epiCAT was calculated and found to be D_f = 2.37 x 10^{-10} cm^2s^{-1}.

In the case of complexation, it can be written:

$$I = B[D_f^{1/2}C_{epiCAT}^0 - (D_f^{1/2} - D_b^{1/2})C_b] \quad (3)$$

where B represents all the constants, C^0_{epiCAT} is the initial epiCAT concentration, C_b is the epiCAT-BSA complex concentration and D_f and D_b are the diffusion coefficients of the epiCAT and epiCAT-BSA complex respectively.

Moreover, the current I_0 in the absence of BSA is given by:

$$I_0 = BD_f^{1/2}C_{epiCAT}^0 \quad (4)$$

and the value obtained for this current is I_0 = 8.42 μA (Table 1). At maximum epiCAT/BSA interaction (depicted by the lowest current recorded in Figure 5 inset), it was assumed that the C_b = [BSA]. Based on this approximation and using Equation 4, the diffusion coefficient of the epiCAT-BSA complex was calculated and found to be D_b = 6.28 x 10^{-11} cm^2s^{-1}.

4. Conclusions

The data obtained herein is consistent with the available literature, and suggest that voltammetric studies may provide a convenient method for studying the electrochemical oxidation mechanisms of epicatechin type compounds in aqueous media. Also, epicatechin can bind in intercalation mode at neutral pH to bovine serum albumin. The interaction between epicatechin and bovine serum albumin resulted in a decrease of the epicatechin peak current with increasing bovine serum albumin concentration. UV-vis spectroscopy was also used to investigate the later interaction. The UV-vis spectroscopic method was applied to evaluate the binding constant of the epicatechin-bovine serum albumin complex. The binding constant indicated that not only was the binding force between epicatechin and bovine serum albumin strong but also that the binding process was spontaneous. The results of this study could play an important role in the pharmacological and/or clinical research on epicatechin based drugs.

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