



VOLTAMMETRIC DETERMINATION OF ESTRADIOL IN MILK AND PHARMACEUTICALS BASED ON FUMED-SILICA MODIFIED CARBON PASTE ELECTRODE

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ABSTRACT

In this study, modification of carbon paste electrode (CPE) with a nano-structured substance, fumed silica (FS), was investigated to develop a voltammetric method for the determination of an endocrine disrupting chemical, estradiol (EST). FS prominently increased the voltammetric response of CPE towards EST. The parameters that affect the voltammetric performance of modified CPE were examined to find the optimal conditions. Modified electrode showed a linear response towards EST between the concentration values of 0.1 μM and 15.0 μM EST. Limit of detection value for the method developed was calculated as 0.023 μM . Production reproducibility of the modified electrode was obtained as 3.6 %. The voltammetric behavior of EST was also examined in the presence of different substances to evaluate their interference effects. The modified electrode successfully discriminated the oxidation potential of EST in the presence of different substances. The determination of EST was tested in milk and pharmaceutical samples to evaluate the analytical performance of the method. The recovery analysis of EST in milk samples showed that the modified electrode can be used safely in these samples. The modified electrode also achieved the determination of EST in pharmaceutical samples.

Keywords: Estradiol, Carbon paste electrode, Voltammetry, Fumed silica

1. INTRODUCTION

Endocrine disrupting chemicals (EDCs) can affect the body's endocrine system and produce negative developmental, reproductive and neurological effects on mammals [1, 2]. EDCs can alter the functioning of the endocrine system even at very low concentrations, cause different diseases such as breast and prostate cancer, diabetes and obesity [1, 2]. Estradiol (EST), which is also known as 17 β -estradiol, is a natural oestrogen [3] and also it is an EDC [4]. On the contrary to its negative effects on mammals, EST is widely used in veterinary medicine to heal and to prevent animal infections and also used illegally to fatten animals because of its anabolic effects [5, 6]. As a result of its heavy use in animals, the entrance of EST into food chain is unavoidable. For this reason, it is very important to develop a sensitive, selective and rapid method for the determination of EST.

In recent years, numerous classical analytical methods have been examined for the determination of EST. These methods are high performance liquid chromatography (HPLC) [7, 8], liquid chromatography-mass spectrometry (LC-MS) [9], gas chromatography [10] and immunoassay techniques [11–13]. On the other hand, these methods have several disadvantages since they require use of complicated extraction steps, expensive instruments and long analysis time. Immunoassay techniques can provide high sensitivity for the detection of EST. However, instability of biomolecules in complicated matrixes and the difficulties encountered working with biomolecules restrict the application of these methods [14]. Electrochemical methods with the properties of simple application, fast analysis time and use of inexpensive equipment overcome the certain drawbacks of classical analytical methods. Determination of EST with electroanalytical methods is possible due to the presence of electroactive phenol group in the molecule. In this frame, different studies were performed to prepare different modified electrodes for the determination of EST. Dharuman et al. (2013) prepared an electrode based

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on the modification of indium tin oxide with reduced graphene-gold nano-composite [15]. In a different study, a modified electrode was prepared by using a nano-composite including Pt-nano-clusters and multi-walled carbon nanotubes [16]. Moraes et al. (2015) have used reduced graphene oxide and a metal complex porphyrin to prepare a modified electrode [2]. In our previous study, we have prepared a nanocomposite composed of cysteamine self-assembled gold nanoparticle modified fumed silica decorated graphene nanoribbon [17]. Moreover, different electrochemical sensors were also prepared with a polymeric film bearing poly{1-butyl-3-[3-(N-pyrrole)propyl]imidazole dodecyl sulfonate} [18], molecule imprinted polymer (MIP) based membranes [3,14,19–21], a DNA aptamer [22] and a nanocomposite containing reduced graphene oxide and rhodium nanoparticles [23].

Carbon paste electrodes (CPEs) are preferred to electrochemical applications compared to other electrodes (glassy carbon, pencil graphite etc.) due to their low background current, wide potential range, quick surfacing and low cost. The significant advantage of CPEs over other electrodes is that they allow the preparation of quickly and easily modified electrodes by adding different substances [24]. Therefore, different materials were used to prepare modified CPEs. In this manner, serpentinite [25], gold nanoparticles [26] and carrageenan hydrogel [27] have been used lately as a modifier.

Fumed-silica (FS) is a nano-sized and synthetic silicone oxide product. It is fabricated via high-temperature flame hydrolysis technique, which involves the conversion of silicon tetra chloride (SiCl_4) to gas phase using an oxy hydrogen flame. In the gas phase, SiCl_4 reacts with water to form silica (SiO_2) and hydrochloric acid [28]. The surface of FS is covered with highly reactive silanol groups which are available for chemical reactions. Due to its high surface area and non-porous structure, FS has started to be used in cosmetics, paint and coating industries in recent years [29]. FS was also used in electrochemical studies. For example, Kaushik et al. (2009) have prepared a nano-composite using FS and chitosan to determine ochratoxin-A [30]. In our previous studies, we have used FS as a support material for the preparation of different catalysts, which were used in the preparation of modified electrodes [17,29]. These studies revealed that FS was a very promising modifier to prepare modified electrodes.

In this study, a simple and very efficient modified CPE was prepared to determine EST in food and pharmaceutical samples. FS was used as a modifier and it increased tremendously the voltammetric response of CPE towards EST. A voltammetric method was developed by optimizing experimental variables such as the percentage of FS, pH and accumulation time. Analytical performance of the method developed was very satisfactory since it has improved analytical parameters such as wide linear range and low detection limit. The effects of some possible interfering substance on the voltammetric behaviour of EST were examined. Finally, determination of EST in milk and pharmaceutical samples was tested. The results showed that FS modified CPE was very efficient in the fast and cheap determination of EST.

2. MATERIALS AND METHODS

2.1. Reagents

17 β -estradiol (99%), fumed silica (powder, 14 nm), sodium carbonate (anhydrous, 99.5%), potassium ferricyanide (99%) and hexamine ruthenium (III) chloride (98%) were purchased from Sigma–Aldrich. Aqueous solutions were prepared with ultrapure water (Sartorius, Germany). Stock solutions of EST were prepared by using a mixture of acetonitrile;ultra-pure water (1:1, v/v) and stored at +4°C. Phosphate buffered solutions (PBS) were prepared from 0.1 M KH_2PO_4 (99%, Fluka) solutions. Milk samples was purchased from a local market. Estrofem and Trisequens (Nove Nordisk Sağlık Ürünleri Tic. Ltd. Şti., İstanbul, Turkey) was purchased from a local pharmacy.

2.2. Instruments

Electrochemical measurements were performed with a conventional three-electrode electrochemical cell. CPE and FS modified CPE were used as working electrodes. A home-made Pt-wire electrode and a saturated calomel electrode (SCE, Gamry Instruments, USA) were employed as an auxiliary and reference electrode, respectively. Differential pulse voltammetry (DPV) measurements were performed with an AUTOLAB PGSTAT204 (Netherlands) model potentiostat. Electrochemical impedance spectroscopy (EIS) measurements were performed with a CHI 660D Electrochemical Workstation equipped CHI model 680 Amp Booster (Shanghai Chenhua Co., China).

2.3. Preparation of Nitric Acid Treated FS (NT-FS)

Preparation of NT-FS was carried out as previously described in the literature [7]. Briefly, 50 mg FS was added into 4.0 M HNO₃ aqueous solution and magnetically stirred for 4 hours. After that, the solid phase was filtered and washed with copies of ultra-pure water. Finally, the solid phase was transferred into a vacuum oven and dried at 65 °C under vacuum (600 mbar).

2.4. Preparation of Modified Electrodes

To prepare CPE, firstly, graphite powder and paraffin oil were mixed in a mass ratio of 70:30 and mixed up to obtain a homogenized paste. After that, the homogenized mixture was packed into a glass electrode holder (internal diameter: 3.0 mm. Length: 13 cm) and polished to obtain a smooth surface. The electrical contact was obtained with a copper wire. The modified CPE was fabricated in the same way by adding different amounts of NT-FS (5 – 12.5 wt% respect to CPE) into the paste structure. The electrodes were denoted as CPE(5NT-FS), CPE(7.5NT-FS), CPE(10NT-FS) and CPE(12.5NT-FS) according to their NT-FS content. A weighing paper was used for smoothing the surface of the electrodes. When the electrodes are not used, they are kept at room temperature.

2.5. Preparation of Real Samples

Milk and pharmaceutical samples were examined for the determination of EST. Milk samples were obtained from a local market. Initially, 3.0 mL milk sample was mixed with 6.0 mL of acetonitrile and 150 µL acetic acid. Acetic acid was used to precipitate proteins found in milk sample. After that, the mixture was vortexed for 2 min and waited in a dark place for 15 min. Next, the mixture was centrifuged 5000 rpm for 15 min. The resulting liquid phase was diluted to 10 mL with ultra-pure water.

The pharmaceutical samples were prepared as follows. Two different drugs containing EST were used in the studies. Firstly, ten tablets of each pharmaceutical were grounded and a known amount was weighed. Second, the sample was treated with 50 mL acetonitrile and the mixture was filtered. After that, final volume was diluted to 100 mL with ultra-pure water. Standard addition method was used to determine the amount of EST.

2.6. Voltammetric Measurements

Voltammetric measurements were performed in an electrochemical cell filled with 10 mL of 0.1 M phosphate buffered solution for the detection of EST. Differential pulse voltammograms were recorded in the potential values between +0.2 V and +0.8 V. The pulse amplitude and scan rate were selected as 50 mV and 10 mV s⁻¹, respectively. The surface of the modified electrode was renewed at every measurement and electrode surface was polished with a weighing paper.

3. RESULTS AND DISCUSSIONS

3.1. Evaluation of Voltammetric Performance of the Modified Electrode

Voltammetric performances of CPE and CPE(12.5NT-FS) were examined via adsorptive differential pulse voltammetry measurements in phosphate buffered solution containing 5.0 μM EST (Fig. 1). A well-defined peak indicating the oxidation of EST was observed at +0.553 V with CPE. This peak slightly shifted to the potential value of +0.532 V in the case of CPE(12.5NT-FS). The oxidation peak current value of EST increased prominently when the CPE(12.5NT-FS) was used instead of CPE. This result reveals that the presence of 12.5 % NT-FS in CPE structure increased the sensitivity of CPE. This may arise from the molecular interaction between silanol (Si-OH) groups found on NT-FS surface and EST. A voltammetric method was developed by using CPE(12.5NT-FS) as a working electrode.

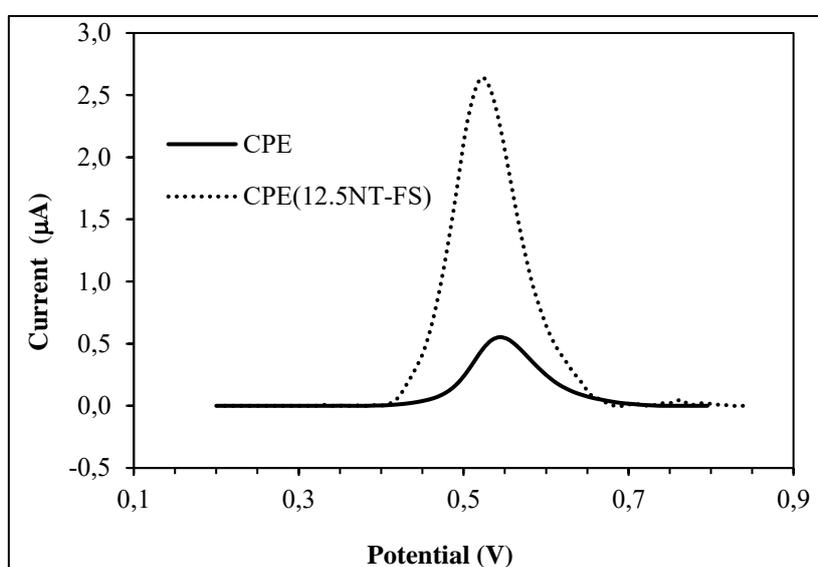


Figure 1. Baseline-corrected differential pulse voltammograms of CPE and CPE(12.5NT-FS) obtained in 0.1 M phosphate buffered solution (pH 6.0) containing 5.0 μM EST. Accumulation time: 60 s, stirring rate: 250 rpm.

3.2. Characterization of the Modified Electrode

Cyclic voltammetry (CV) measurements were performed in KCl solution containing the negatively-charged redox couple ($\text{Fe}(\text{CN})_6^{4-/3-}$) and positively-charged redox probe ($\text{Ru}(\text{NH}_3)_6^{3+}$) for the electrochemical characterization of CPE(12.5NT-FS) (Fig. 2). Cyclic voltammograms of 5.0 mM $\text{Fe}(\text{CN})_6^{3-/4-}$ obtained with bare CPE and CPE(12.5NT-FS) are shown in Figure 2a. A well-defined oxidation peak was observed at +0.304 V with bare CPE, which belongs to the oxidation of $\text{Fe}(\text{CN})_6^{4-}$. This peak was observed at the potential value of +0.258 V for CPE(12.5NT-FS). As seen, a prominent shift (46 mV) was observed with CPE(12.5NT-FS) in the oxidation potential of $\text{Fe}(\text{CN})_6^{4-}$. In the reverse scans, the reduction of $\text{Fe}(\text{CN})_6^{3-}$ was observed at the potential values of +0.102 V and +0.114 V for CPE and CPE(12.5NT-FS), respectively. The peak separation values, ΔE_p ($E_{pa} - E_{pc}$), were calculated as +0.202 and +0.144 V for CPE and CPE(12.5NT-FS), respectively. The decrease in the peak separation values in the presence of the modified electrode and the increase in the peak currents indicate that the presence of NT-FS as a modifier increases the electron transfer rate, which contributes to the electrochemical performance at the electrode surface.

Measurements were also performed with a positively-charged redox probe ($\text{Ru}(\text{NH}_3)_6^{3+}$) (Fig. 2b). A prominent improvement in the electrochemical behaviour of $\text{Ru}(\text{NH}_3)_6^{3+}$ was observed with CPE(12.5NT-FS) compared to bare CPE. These results verify that the electron transfer rate of CPE increased prominently in the presence of NT-FS as a modifier.

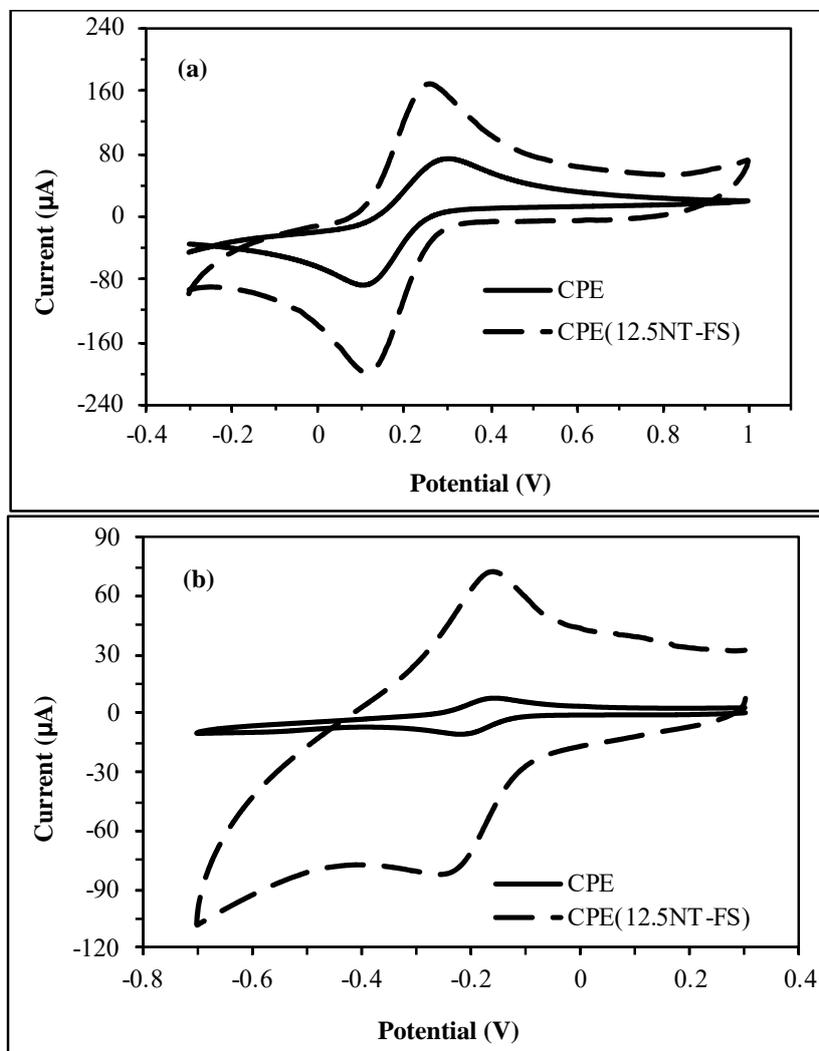


Figure 2. Cyclic voltammograms of CPE and CPE(12.5NT-FS) obtained in 0.1 M KCl solutions containing 5.0 mM $\text{Fe}(\text{CN})_6^{4-/3-}$ (a) and 1.0 mM $\text{Ru}(\text{NH}_3)_6^{3+}$ (b). Scan rate: 50 mV s^{-1} .

3.3. Electrochemical Behaviour of EST at Modified Electrode

Cyclic voltammetry measurements were performed with CPE(12.5NT-FS) in 0.1 M PBS (pH 6) to determine electrochemical behaviour of EST. There was no meaningful electroactivity in the studied potential range in the absence of EST (Fig. 3a). An oxidation peak was appeared at +0.595 V after addition of 50.0 μM EST into the measurement solution (Fig. 3b). No reduction peak was seen in the reverse scan. This result indicates that the electrochemical oxidation of EST on CPE(12.5NT-FS) is not reversible.

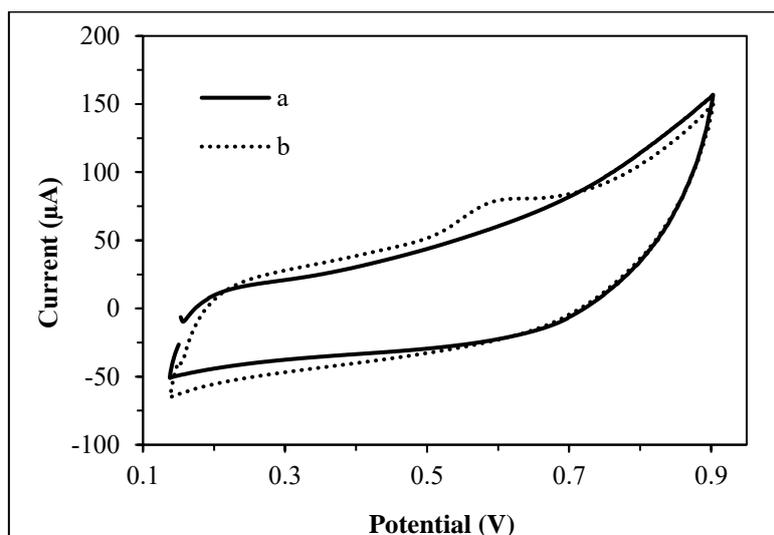


Figure 3. Cyclic voltammograms of CPE(12.5NT-FS) obtained in 0.1 M PBS (pH 6.0) in the absence (a) and presence of 50.0 μM EST (b). Accumulation time: 60 s, stirring rate: 250 rpm.

3.4. The Effects of NT-FS Amount on the Voltammetric Response of the Electrode

A series of modified electrodes were prepared by mixing graphite powder with different amounts of NT-FS (5%, 7.5%, 10% and 12.5%) to evaluate the effects of NT-FS amount on the voltammetric response of the modified electrode. The oxidation peak current values of 5.0 μM EST are shown in Figure 4. As seen, the oxidation peak current of EST gradually increased by the increase of NT-FS amount in paste structure. The voltammetric response of bare CPE towards EST increased 6 times in the presence of 12.5% NT-FS. After this value, further increase in NT-FS content led to paste preparation problems. Therefore, this value was selected as the optimal value.

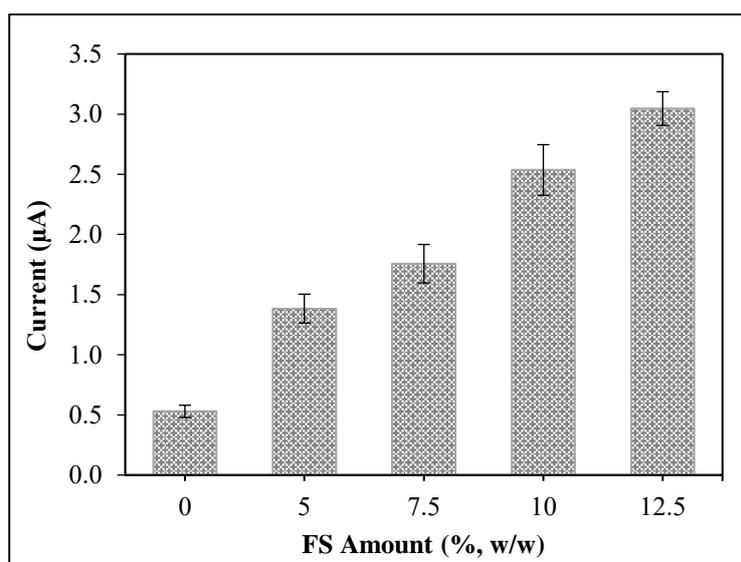


Figure 4. The oxidation peak current values of 5.0 μM EST obtained in 0.1 M PBS (pH 6.0) with modified CPEs containing different amounts of NT-FS. Accumulation time: 60 s, stirring rate: 250 rpm.

3.5. Optimization of Measurement Conditions

Differential pulse voltammetry measurements at different pH values were conducted to evaluate the effect of pH on the voltammetric response of the CPE(12.5NT-FS). The differential pulse voltammograms, which were obtained in PBS having different pH values, were given in Figure 5. As can be seen from Figure 5, the oxidation of EST on CPE(12.5NT-FS) took place at all the pH values studied. The intensity of the peak started to increase by the increase of pH. It reached to its highest value at pH 6.0. After this value, further increase of pH led to a dramatic decrease in the intensity of the oxidation peak. Therefore, optimal value was selected as 6.0. A linear relationship was observed between the oxidation potential of EST and pH, having an equation of $E \text{ (V)} = -0.0471 \text{ pH} + 0.824$ ($R^2: 0.9868$). The slope (-0.0471) of this line is very close to the theoretical value of -0.0592 , which showing the presence of same number of protons and electrons in the redox reaction of EST.

Another parameter that was examined was the accumulation time. To investigate this parameter, CPE(12.5NT-FS) was immersed into EST solution, waited different time periods and differential pulse voltammetry measurements were performed (Fig. 6). The oxidation peak current of EST gradually increased by the increase of accumulation time. This increase continued up to 240 s. After this time, a slight decrease in the oxidation peak current was observed. Therefore, the optimum accumulation time was chosen as 240 s and used in the subsequent experiments.

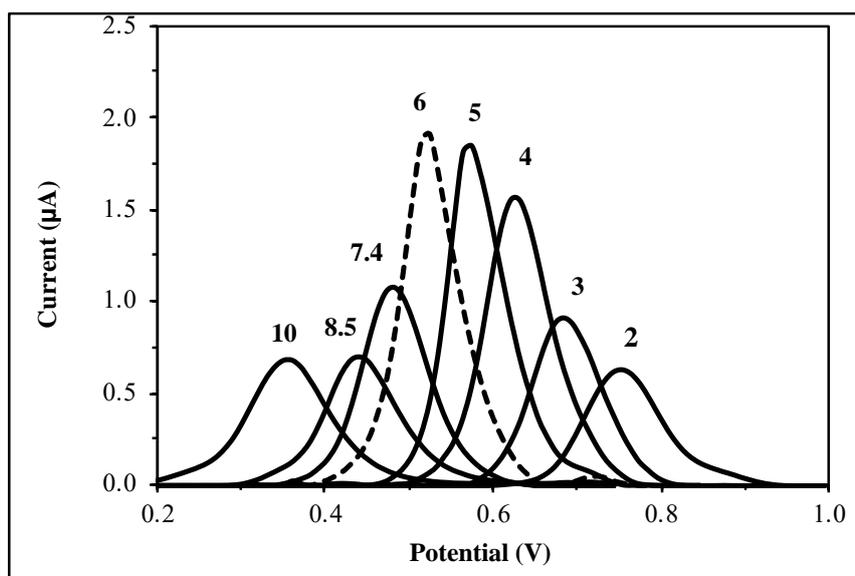


Figure 5. Baseline-corrected differential pulse voltammograms of 5.0 μM EST obtained with CPE(12.5NT-FS) in PBS having different pH values. Accumulation time: 60 s, stirring rate: 250 rpm

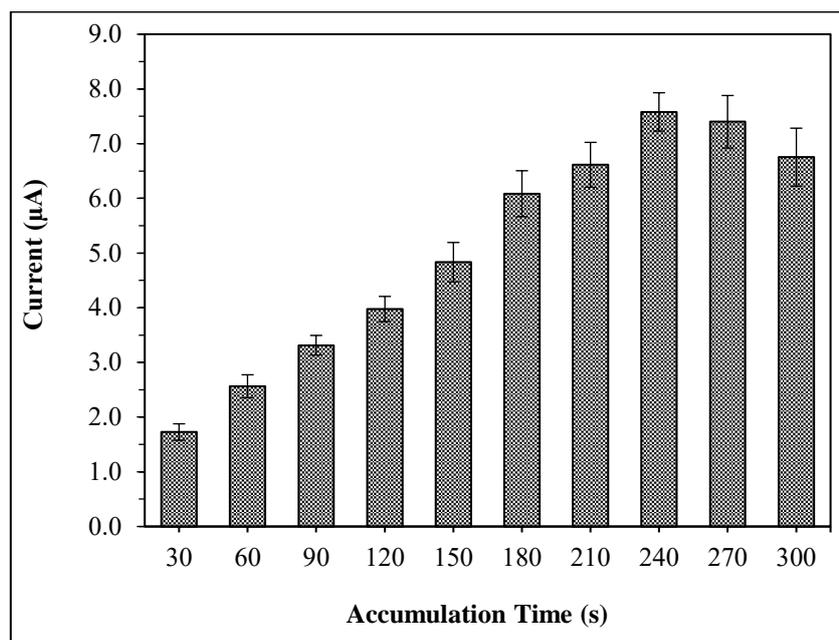


Figure 6. The effects of accumulation time on the oxidation peak currents of 5.0 μM EST in 0.1 M PBS (pH: 6.0).

3.6. Evaluation of the Effects of Possible Interfering Substances

The effects of different species that might be found in real samples on the voltammetric response of CPE(12.5NT-FS) were investigated to determine their interfering effects. No meaningful change was observed in the voltammetric response of 5.0 μM EST in the presence of excess amounts of ascorbic acid (1000 μM), uric acid (100 μM), potassium chloride (2000 μM), calcium chloride (2000 μM), glucose (2000 μM), urea (2000 μM), citric acid (2000 μM) and o-nitrophenol (25 μM). These results indicate that CPE(12.5NT-FS) allows the selective determination of EST in the presence of different substances.

3.7. Determination of Analytical Parameters

To determine linear range and detection limit of the proposed method, DPV measurements were performed with CPE(12.5NT-FS) at optimized conditions and peak currents were recorded. The oxidation peak currents of EST increased linearly between the concentration values of 0.1 μM and 15.0 μM (Fig. 7a). The calibration plot displayed a good linearity and a wide working range with a linear equation of $I (\mu\text{A}) = 1.26 C_{\text{EST}} (\mu\text{M}) + 0.33$ ($R^2=0.996$). The limit of detection (LOD) value and limit of quantification (LOQ) were calculated as 0.023 μM and 0.077 μM, respectively, taking the signal/noise (S/N) ratio 3 and 10, respectively. It is important to note that the measurements were repeated with bare CPE at the same conditions, but the oxidation peak currents of EST obtained with bare CPE did not show linearity in the studied concentration range (Fig. 7b). These results show that NT-FS has a prominent effect on the performance of CPE.

The fabrication reproducibility of CPE(12.5NT-FS) was evaluated by measuring 5.0 μM EST with ten different fresh electrodes. Relative standard deviation of the measurements was calculated as 3.6%, indicating high production reproducibility of CPE(12.5NT-FS).

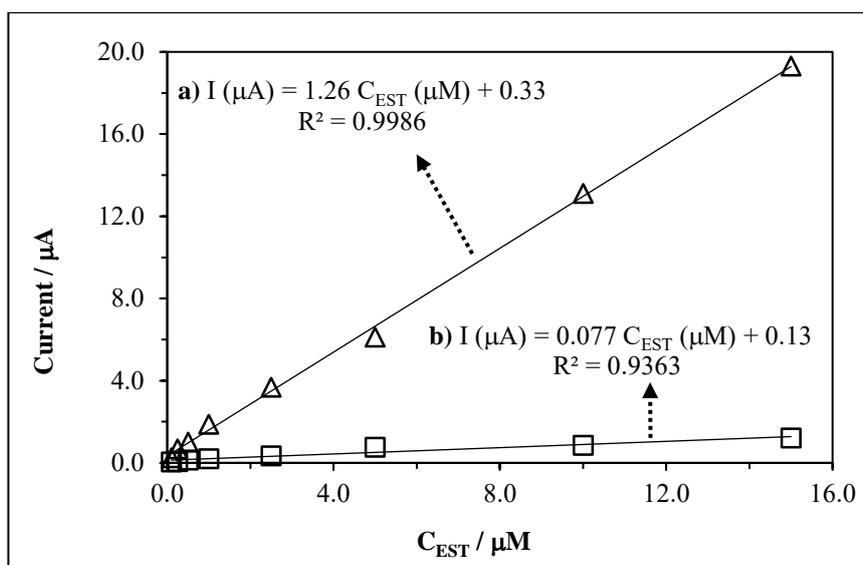


Figure 7. The calibration curves for EST obtained in 0.1 M PBS (pH: 6.0) by CPE(12.5NT-FS) (a) and CPE (b) electrodes. Accumulation time: 240 s, stirring rate: 250 rpm.

3.8. Investigation of the Performance of CPE(12.5NT-FS) in Real Samples

The performance of CPE(12.5NT-FS) in real samples is very important from analytical chemistry. Therefore, determination of EST with CPE(12.5NT-FS) was examined in milk and pharmaceutical samples. Firstly, milk samples were prepared and measurements were performed. Differential pulse voltammogram of fifty-times diluted milk sample was given in Figure 8. As can be seen from the figure, there was no noticeable peak at the oxidation potential value of EST, indicating that there was no EST in the original milk sample. To evaluate the performance of CPE(12.5NT-FS), EST-spiked milk samples were prepared and measurements were repeated (Fig. 9). A well-defined oxidation peak was observed at +0.57 V, which corresponds to the oxidation of EST (Fig. 9, dotted line). The intensity of this peak gradually increased with the increase of EST concentration in the measurement medium (Fig. 9, straight lines). Another oxidation peak was also observed at +0.36 V, which may correspond to oxidation of an electroactive substance that found in the milk sample. The presence of this peak did not prevent the selective determination of EST. These results show that CPE(12.5NT-FS) can be used in the determination of EST in milk samples. The results obtained during the recovery studies were given in Table 1. The recovery values are in the range of 92.47 % and 100.04 %, indicating high recovery of EST.

The applicability of the method was also tested in two different pharmaceutical samples. The EST content of pharmaceutical samples were determined with the proposed method by using standard addition method. The amount of EST found in pharmaceutical samples were determined as 2.051 mg tablet⁻¹ and 2.087 mg tablet⁻¹. The EST content of the pharmaceutical samples studied here was indicated as 2.00 mg tablet⁻¹ by the producer companies. As can be seen, the values obtained with our proposed method were comparable with that of the reported values. These results revealed that the proposed method can also be used in the determination of EST in pharmaceutical samples.

Table 1. Results of recovery analysis of EST in milk samples.

Sample	Added (μM)	Detected (μM)	% Recovery
MS-A	0.0	Not detected	---
	50.0	50.02	100.04
MS-B	0.0	Not detected	---
	100	92.47	92.47

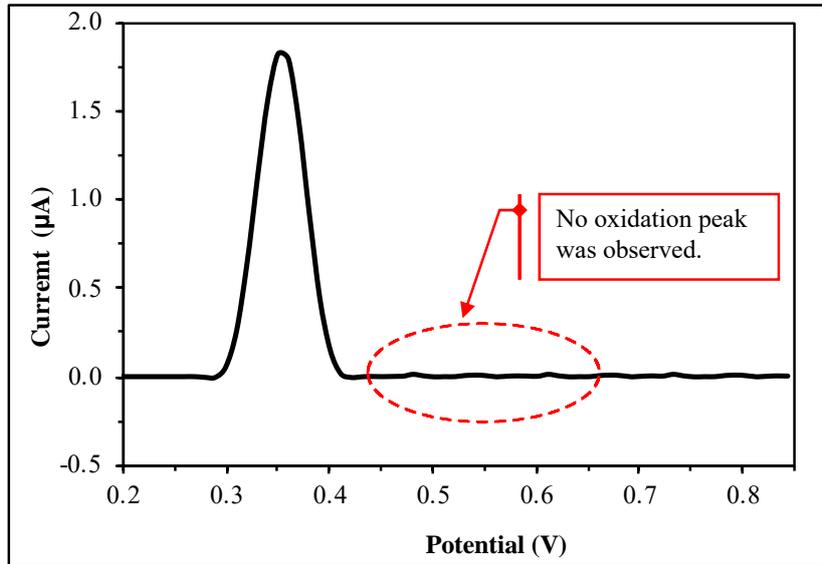


Figure 8. Baseline-corrected DPV of fifty-times diluted milk sample obtained in 0.1 M PBS (pH: 6) with CPE(12.5NT-FS). Accumulation time: 240 s, stirring rate: 250 rpm.

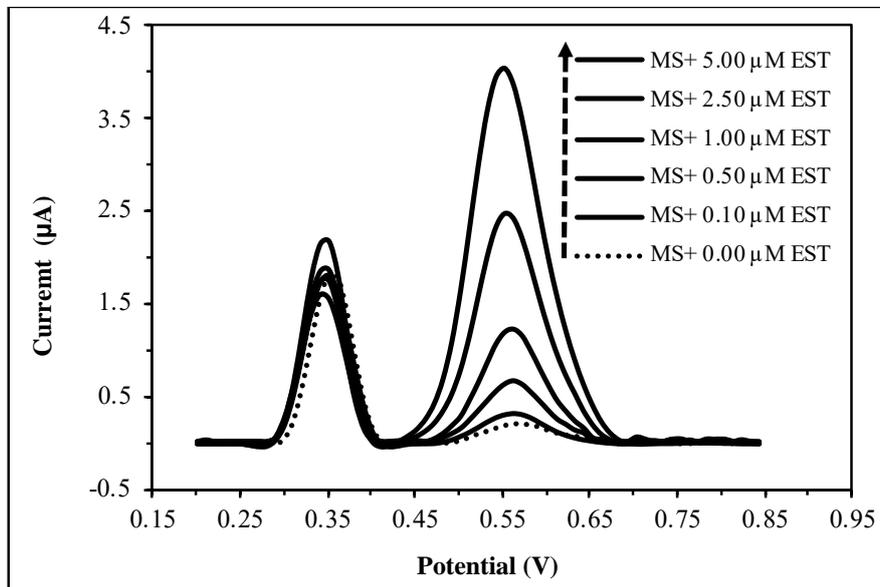


Figure 9. Baseline-corrected DPVs of fifty-times diluted EST-spiked milk sample obtained in 0.1 M PBS (pH: 6) with CPE(12.5NT-FS) in the absence (dotted line) and presence (straight lines) of increasing concentrations of EST. Accumulation time: 240 s, stirring rate: 250 rpm.

4. CONCLUSIONS

In this study, a voltammetric method was developed for the determination of EST in milk and pharmaceutical samples. This new method was based on the modification of CPE with FS, which was a nanosized silicon oxide substance. Electrochemical characterization studies revealed that the presence of FS in CPE increased prominently the electron transfer rate of redox species of $\text{Fe}(\text{CN})_6^{4-/3-}$ and $\text{Ru}(\text{NH}_3)_6^{3+}$. The oxidation peak current of EST obtained with CPE(12.5NT-FS) was 6-times higher than that of obtained with CPE. The CPE(12.5NT-FS) has the properties of wide linear range, low detection limit, short analysis time and log-term stability. The determination of EST in milk and pharmaceutical samples successfully achieved with the method proposed here.

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