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*Research Article*

# Electrochemical Enzymatic Biosensor Development by Using Different Electropolymerization Conditions of Polyaniline

Nimet YILDIRIM TİRGİL[a,b*], Zeycan KALKAN [b], Soner ÖZTÜRK [c], Sedat SÜRDEM [d], Mehmet Fatih ÖKTEM [b]

[a] *Department of Biomedical Engineering, Faculty of Engineering and Natural Sciences, Ankara Yıldırım Beyazıt University, Ankara, TURKEY*
[b] *Department of Materials Engineering, Faculty of Engineering and Natural Sciences, Ankara Yıldırım Beyazıt University, Ankara, TURKEY*
[c] *TENMAK, Boron Research Institute, Ankara, TURKEY*
[d] *Department of Environmental Sciences, Graduate School of Natural and Applied Sciences, Gazi University, Ankara, TURKEY*
***** *Corresponding author's e-mail address: nyildirimtirgil@ybu.edu.tr*
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## ABSTRACT

Because of its excellent electrochemical properties, extreme redox performance, and ability to mediate the electron transfer between the electrode surface and the reaction site, polyaniline (PANI) is one of the most ideal and well-known conductive polymers for biosensor design. This research developed an electrochemical enzymatic biosensor system with PANI film-coated screen printed electrodes (SPE) using the one-step direct electropolymerization process. PANI electropolymerization was performed in different acidic solutions, and the effects on the electrodeposition of the potential range, potential scan rate, and cycle number are discussed depending on these acidic solutions. The surface morphologies of films prepared with different processes were characterized by usıng the SEM (scanning electron microscopy) technique. A sensitive and selective catechol biosensor was developed by immobilizing the tyrosinase (Tyr) enzyme into PANI film combined with glutaraldehyde as a cross-linking agent. After optimizing the biosensor performance conditions, the developed biosensor measured catechol in green tea samples.

***Keywords:*** *Conducting Polymers, Polyaniline, Enzymatic Biosensors, Catechol Analysis*

# Farklı Elektropolimerizasyon Koşulları Kullanılarak Hazırlanan Polianilin ile Elektrokimyasal Biyosensör Geliştirilmesi

## ÖZET

Polianilin (PANI), mükemmel elektrokimyasal özellikleri, etkili redoks davranışı ve reaksiyon esnasında elektrot yüzeyine elektron transferine aracılık etme kabiliyeti nedeniyle nedeniyle biyosensör tasarımı için en çok tercih edilen ve bilinen iletken polimerlerden biridir. Bu çalışmada, doğrudan elektropolimerizasyon (tek aşamalı) işlemi kullanılarak hazırlanan PANI film kaplı yüzey baskılı elektrotlar (SPE) ile elektrokimyasal bir enzimatik biyosensör sistemi geliştirilmiştir. Çalışmada, PANI elektropolimerizasyonu farklı asidik çözeltilerde gerçekleştirilmiş ve potansiyel aralığın, potansiyel



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tarama hızının ve döngü sayısının elektrodepozisyona etkileri gözlemlenmiştir. Farklı işlemlerle hazırlanan filmlerin yüzey morfolojileri, taramalı elektron mikroskobu (SEM) tekniği ile karakterize edilmiştir. Glutaraldehit ile çapraz bağlanma tekniği ile tirozinaz (Tyr) enziminin PANI filmine immobilize edilmesiyle oldukça kararlı ve etkili bir katekol biyosensörü hazırlanmıştır. Biyosensör performans koşullarının optimizasyonundan sonra, yeşil çay örneklerinde katekol analizi için geliştirilen biyosensör kullanılmıştır.

***Anahtar Kelimeler:*** *İletken Polimerler, Polianilin,Eenzimatik Biyosensörler, Katekol Aanalizi*

# I. INTRODUCTION

Conductive polymers possess a high conductivity/weight ratio, unique chemical properties [1], well optical and electrical properties, and enable outstanding control of the electrical stimulus [2]. The main feature of a conductive polymer is that there are conjugated (sequentially ordered) double bonds along the polymer's backbone (main chain). In conjugation, the bonds between carbon atoms are arranged in alternating double and single bonds. Each bond contains a strong chemical bond, "sigma" (σ). Moreover, each double bond has a weaker (30%) and less localized "pi" π bond. However, conjugation is insufficient to make the polymer material conductive, and the conductivity can be increased by introducing dopant materials. The task of dopant materials is to increase the number of electrons and "holes" in the material. The location where there is an electron deficiency is called a hole. When such a hole is filled with an electron jumping from a neighboring location, a new hole is created, and as a result of this situation, the charge migrates over a long distance [3]. By doping with different agents, the chemical, physical, and electrical properties of conducting polymers can be changed [4], and by adding antibodies, enzymes, and other biological molecules, these properties can be adapted to the unique needs of their application [2], specifically for biosensor design. The electrochemical polymerization technique is one of the most common methods used to prepare conductive polymers [5]. Electrochemical polymerization occurs by applying an electrical current through electrodes located in a solution containing the monomer, the solvent, and the doping agent. The electrical current causes the monomer to deposit and oxidize on the positively charged working electrode, forming insoluble polymer chains. Electrochemical polymerization only permits the synthesis of a polymer if the monomer may undergo oxidation in the presence of an electrical potential [2]. The electrochemical process leads to its location, thickness, or morphology controlled by applying current or voltage [4].

One of the most known conductive polymer types is Polyaniline (PANI). Due to the discovery of its high conductivity and relatively low cost, PANI has recently captured the scientific community's attention. Researchers are actively discovering its applications, especially in biosensors, due to a range of beneficial properties like direct and simple deposition on the sensor electrode, redox conductivity and polyelectrolyte characteristics, control of thickness, chemical specificities, high surface area, long-term environmental stability [6], ease of synthesis, low cost, and the ability to switch between its conductive and resistive states electrically. PANI can be an influential mediator to electron transfer in redox or enzymatic reactions as a material for sensor and biosensor interfaces. PANI is considered an attractive polymer because it exhibits two redox couples in the correct potential range to allow the transfer of enzyme-polymer charge and thus serves as a mediator of self-contained electron transfer [6]. The various techniques can be used on the PANI surface for the immobilization of desired biomolecules. Control over PANI's shape and dimensions are likely to result in desired physical and electrochemical properties for biosensing application through varying synthesis or processing conditions. Excellent electroactivity can be preserved up to pH 12 in the PANI's significant application zone [7].

The phenolic compounds are widely used in the chemical industry and agriculture and are discharged into the environment. The organism can quickly adsorb these chemicals through skins and mucous membranes. They accumulate in the body due to the difficult removal of phenolic compounds during metabolic processes. Due to these properties, the number of phenolic compounds in natural foods should be determined within quality control. In determining phenolic compounds (HPLC- High-Performance

Liquid Chromatography, GMS-Gas Chromatography-Mass Spectrometry, etc.), traditional analytical methods are used and enzymatic methods use free and immobilized polyphenol oxidase class enzymes [8][9][10]. Electrochemical techniques are among the numerous known methods; their real-time identification of clinical samples is desirable for polyphenol determination [11]. A significant challenge is to build a biosensor for low phenolic compound detection limits, high sensitivity, quick response, efficacy, and simplicity in the tyrosinase enzyme-based amperometric biosensor [12]. It is often known as catechol oxidase or polyphenol oxidase, tyrosinase (Tyr) has two atoms of copper at its active core [13]. This essential enzyme catalyzes oxidation reactions in the presence of molecular oxygen, containing monophenols' hydroxylation into o-dihydroxy phenols and subsequently oxidation-dihydroxy phenols into o-quinones [14].

Due to various favorable features, poly conjugated conducting polymers possess excellent attention to biosensing applications mentioned above. Primarily, PANI film was synthesized from an aniline aqueous solution with various acidic media such as HCl, $H_2SO_4$, and perchloric acids [15]. In this paper, therefore, PANI has been used to increase the analytical efficiency of electrochemical biosensors. An electrochemical biosensor has been generated by deposition of PANI and enzyme trapping by a one-step process. This paper describes an electrochemical biosensor's manufacture and applying the resulting enzyme electrode to voltammetric detect catechol in an aqueous medium.

# II. MATERIALS AND METHODS

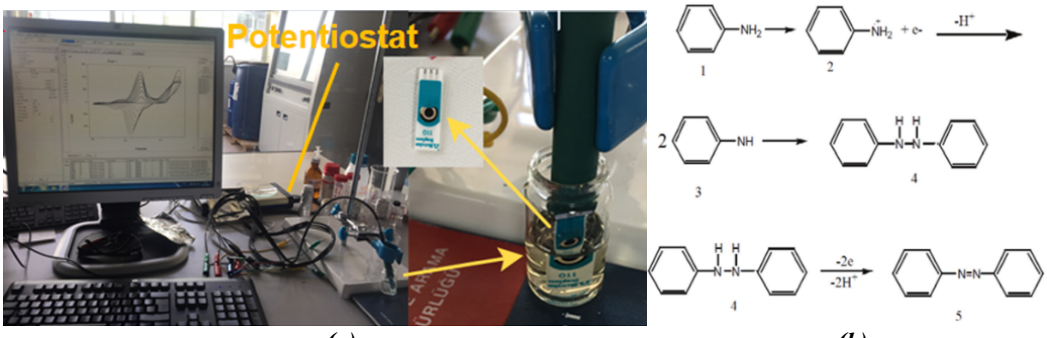
## A. MATERIALS

Tyrosinase from mushroom (Tyr, EC:1.14.18.1), Catechol, Aniline, Perchloric acid ($HClO_4$), Sulfuric acid ($H_2SO_4$), Hydrochloric acid (HCl), and Glutaraldehyde were purchased from Sigma Aldrich. Aniline was purified under vacuum before use. Phosphate buffer was prepared using potassium dihydrogen phosphate and di-potassium hydrogen phosphate. All the chemicals were used under the laboratory grade and MilliQ TKA-Lab pure water was used for the wet process.

## B. METHODS

### B. 1. Electropolymerization

The electrochemical measurements were recorded using screen-printed electrodes (SPE) that the SPE three-electrode system consisted of a carbon plate as the counter electrode(CE), carbon plate as the working electrode(WE), and Ag/AgCl as the reference electrode(RE). Electrochemical measurements were carried out using a potentiostat controlled by IviumSoft, software for control, and data acquisition. Fig.1 represents the SPE and potentiostat setup used in this work.




*(a)* *(b)*

***Figure 1. a)*** *The illustration of the SPE and potentiostat setup was used in this work,* ***b)*** *aniline electropolymerization mechanism.*

Electrochemical synthesis is an alternative way to obtain conductive polymers, making the synthetic procedure relatively straightforward. In this work, electropolymerization was carried out; the process of obtaining conductive polymers by the electrochemical method is based on obtaining the Polyaniline (PANI) on the C-SPE electrode was immersed in an aqueous solution containing 0.1 M aniline monomer and $H_2SO_4$, or $HClO_4$ or HCl. In the experiment, the screen printed electrodes (SPE) are scanned within the potential range of −0.5 V to 0.8 V in 0.5 M freshly prepared $H_2SO_4$ solution, 0.4 V to 0.8 V in 0.5 M $HClO_4$ solution, and -0.2 V to 1.0 V in 0.5 M HCl solution, respectively until stable curves of cyclic voltammograms are obtained. Cyclic voltammograms(CVs) were recorded in these different potential ranges in different acidic solutions at a scan rate of 100 mV/s. The current is passed through the solution, and the polymer accumulates on the positively charged working electrode. During the oxidation process to form radical cations that react with other monomers, monomers on the working electrode surface form insoluble polymer chains on the electrode surface. After the aniline's electropolymerization, these PANI modified electrodes were washed with $H_2SO_4$ and distilled water, respectively. The surface activity and conductivity of the electrode are ascertained with $K_3[Fe(CN)_6]$/ $K_4[Fe(CN)_6]$ system and were carried out -0.2 V to 0.8 V in 5 mM $Fe(CN)_6^{3-}$/$Fe(CN)_6^{4-}$ containing 0.1 M KCl solution.

The surface characterization of the polyaniline modified SPEs based on different acidic solutions were evaluated by scanning electron microscope (SPE).

## B. 2. Enzyme immobilization

After the polyaniline modification on the SPEs, enzyme immobilization was performed using glutaraldehyde as a cross-linking agent during Tyr immobilization. For enzyme immobilization, a mixture of 50 µl from 1 mg / mL Tyr in a 50mM phosphate buffer solution (pH 7.0) and 4 µl glutaraldehyde was prepared. Afterward, 8 µL of mixture dispersion was cast onto the SPE surface so that the solvent was allowed to evaporate at room temperature for approximately 1.5 hours.

## B. 3. Catechol biosensor preparation

A sensitive and selective catechol biosensor was developed by immobilizing tyrosinase (Tyr) enzyme into PANI film combined with glutaraldehyde as a cross-linking agent. In Fig. 2, a diagram shows the tyrosinase enzyme's working mechanism and the electrochemical activity of the final product formed by the catechol component's enzymatic reaction. The ultimate product of catechol, o-quinone is an electroactive molecule with electrochemical oxidation and reduction activity. In this mechanism, the enzyme played a key role. An immobilization method in which the enzyme activity was kept at maximum adds value to the biosensor system to be developed.

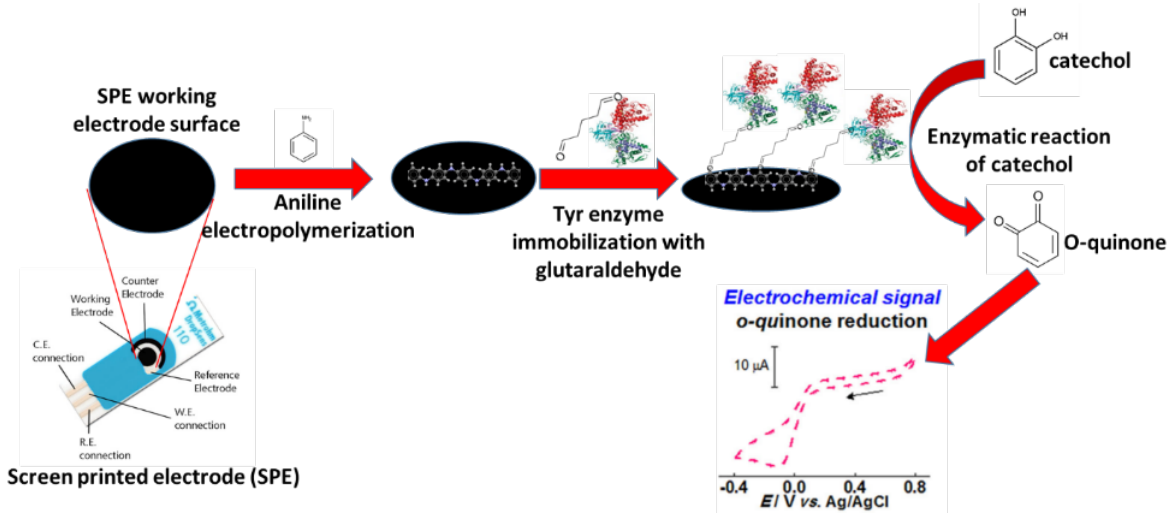



***Figure 2.*** *The diagram shows the tyrosinase enzyme's working mechanism and the electrochemical activity of the final product formed by the catechol component's enzymatic reaction of the catechol component* [16].

The biosensor was performed to detect catechol by voltammetric measurements at the applied potential range of -0.9 V to 0.8 V in the steady-state condition in different amounts of catechol 10, 25, 50, 100, 200, 300 μM, respectively in 50 mM pH buffer solution. To obtain optimum conditions the enzymatic biosensor system was tested in different pH solutions of PBS (pH 6 to 8), including 200 μM of catechol. The developed enzymatic biosensor system's selectivity performance was tested with different phenolic compounds for biosensor selectivity observation in addition to these experiments. 200 μM of catechol, gallic acid, hydroquinone, n-nitrophenol were tested, and percentage signals were calculated depending on the biosensor signal with 200 μM of catechol, respectively.

After optimizing the working conditions of the developed biosensor system to calculate the potential matrix effect of real samples on the biosensor performance, we evaluated spiked green tea samples containing different concentrations of catechol (50 μM,100 μM, and 200 μM).

# III. RESULTS AND DISCUSSION

## A. RESULTS

### A. 1. Electropolymerization of aniline in different acidic solutions

SPEs were immersed in the solution of 0.1 M aniline and different acidic solutions of 0.5 M $H_2SO_4$, 0.5 M $HClO_4$, and 0.5 M HCl solution. SPEs were scanned between the potential range of −0.5 V to 0.8 for $H_2SO_4$ solution, 0.4 V to 0.8 V for $HClO_4$ solution, and -0.2 V to 1.0 V for HCl solution until stable curves of cyclic voltammograms (CVs) are obtained as shown in Fig.3. (a)in HCl solution (b) $H_2SO_4$ solution (c) in $HClO_4$. Comparing the CVs of these in different solutions, it is found that both anodic and cathodic peak currents increased abruptly in the case of aniline monomer in different acidic mediums, especially for $HClO_4$, which demonstrates that it expands the electron transfer rate for PANI formed onto SPE.

After the electropolymerization, the electrode's surface activity and conductivity are ascertained by performing cyclic voltammograms between -0.2 V and 0.8 V in the solution of 0.1 M KCl containing 5 mM $Fe(CN)_6^{3-}/Fe(CN)_6^{4-}$ redox molecule. As shown in Fig.2(d), electrode surface conductivity analysis with $Fe(CN)_6^{3-}/Fe(CN)_6^{4-}$ redox molecule results indicate that electrode surface conductivity after electropolymerization of aniline in HClO4solution has the highest voltammogram peak height, which is the evidence of high surface conductivity and this green curve coming from HClO4 is the best. Blue curve coming from HCl shows that the Oxidation peak was shifted more positive potential range, showing that the electrode surface was disrupted. So we quit testing HCl after this point. The more conductive the surface becomes, the better the $Fe(CN)_6^{3-}/Fe(CN)_6^{4-}$ redox molecule oxidation and reduction peaks.

The surface morphology of the bare and polyaniline modified SPEs based on different acidic solutions was characterized by SEM studies (Figure 4a–e). Depending on the SEM images for different acidic solutions, Figure (4, a) represented bare SPE as a homogeneous surface, Figure (4,b) as not dense but homogeneous, Figure (4,c) the surface is very dense, nonhomogeneous, and quite dense, so it affects electrode surface conductivity, Figure (4,d) $HClO_4$ based modification represented the densest and most homogeneous surface and Figure (4,e)the results indicate that enzyme particles attached to the surface. Morphology permits the Tyrosinase enzyme uniformly entrapped on the surface, and the porous structure disappeared utilizing the Tyrosinase enzyme.

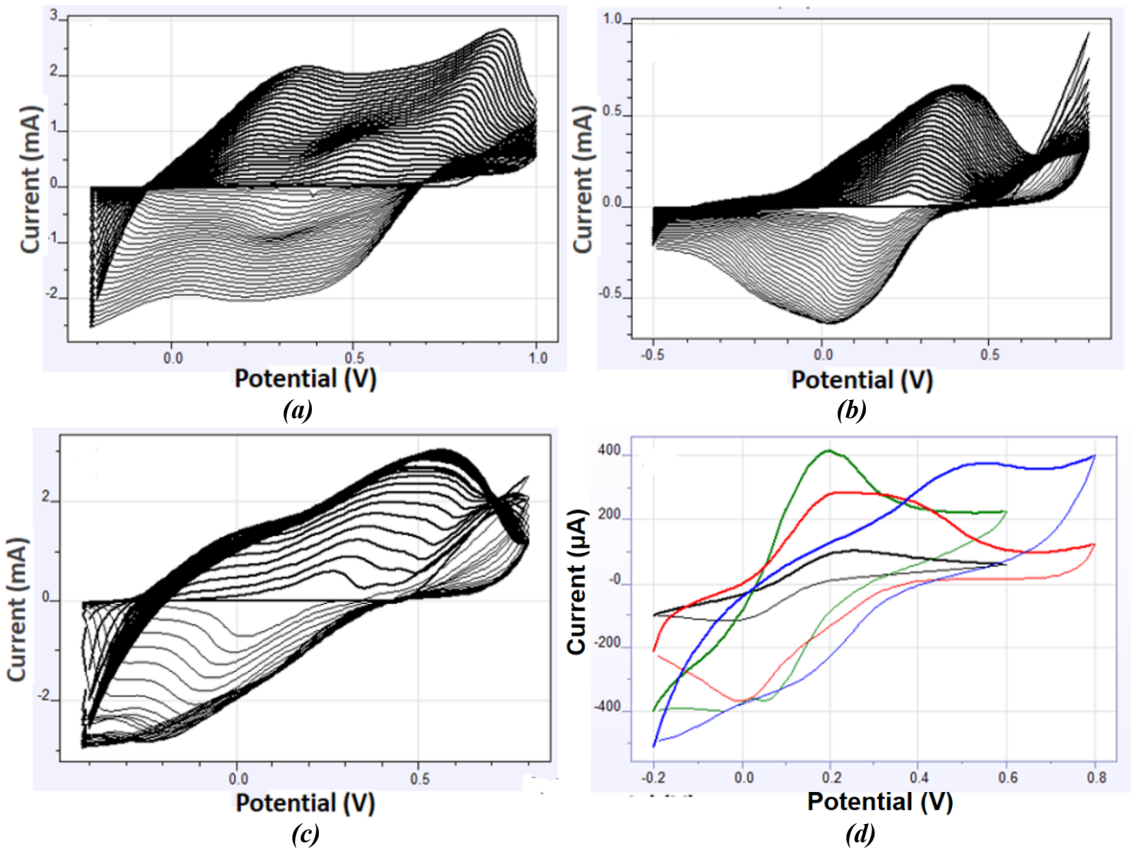



***Figure 3.*** *The polymerization solutions contain of 0.1 M aniline monomer dissolved in 0.5 M different supporting electrolytes. Aniline electropolymerization* ***(a)****in HCl solution* ***(b)*** *$H_2SO_4$ solution* ***(c)****in $HClO_4$ solution* ***(d)****Electrode surface conductivity analysis with 5 mM Potassium $Fe(CN)_6^{3-}/Fe(CN)_6^{4-}$ redox moleculein KCl solution (Black: bare SPE, Blue: HCl electropolymerization, Red: $H_2SO_4$ electropolimerization, Green: $HClO_4$ electropolimerization).*

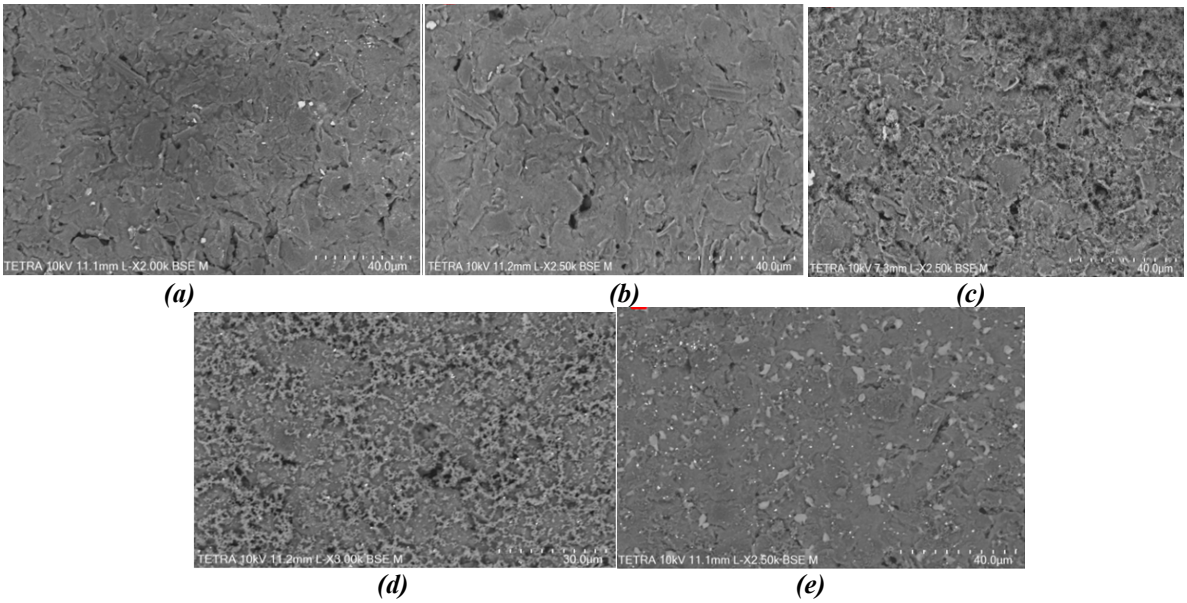



***Figure 4.*** *SEM images aniline electropolymerizatıon in acidic solutions****(a)*** *bare SPE* **(b)** $H_2SO_4$ ***(c)*** *HCl* ***(d)*** *$HClO_4$* ***(e)*** *Enzyme immobilized SPE electrodes after aniline electropolymerization.*

## A. 2. Biosensor Performance development

### A.2.1. pH Optimization

200 µM concentration of catechol was tested using different acidic solution-based polyaniline modified and enzyme immobilized SPEs in different pH solutions of PBS including pH 6, pH 6.5, pH 7, pH 7.5, and pH 8. As represented in figure 5, for both acidic solutions based on electropolymerized SPE, the optimum pHs were observed as pH 6.5.

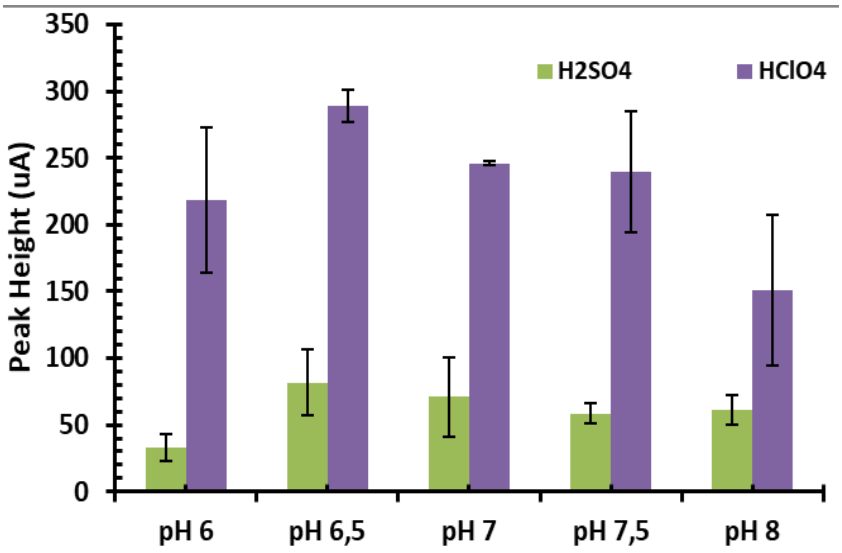



***Figure 5.*** *The effect of the detection solution's pH on the biosensor's performance was evaluated by incubating the electrode in 200 µM catechol solution prepared at the PBS with the pH range of 6–8.*

### A.2.2. Dose-response curve

Different concentrations of catechol (10,25,50,100,200,300 µM) were added onto 50 mM pH 6.5 buffer solution, and the voltammetric measurements at the applied potential range of -0.9 V to 0.8 V were performed. The reduction peak for the O-quinone product of the enzymatic reaction of catechol was observed, and the peak heights were calculated for each concentration. The linear range was identified for both acidic solutions based on aniline modified SPEs between 10 µM and 300 µM where the $HClO_4$ based enzymatic biosensor system had higher signals and therefore sensitive response as shown in Fig.6.

### A.2.3. Biosensor specificity

To determine the specificity of the developed enzymatic biosensor for analyzing catechol, different phenolic compounds such as catechol, gallic acid, hydroquinone, n-nitrophenol were evaluated at the concentration level of 200 µM. The biosensor system's responses for these compounds were compared with the results of 200 µM of catechol detection. The results in Fig. 7 clearly show that non-specific phenolic compounds do not generate any significant peak at the voltage where O-quinone's reduction peak is located. Therefore, the developed biosensor system is quite specific for only catechol detection.

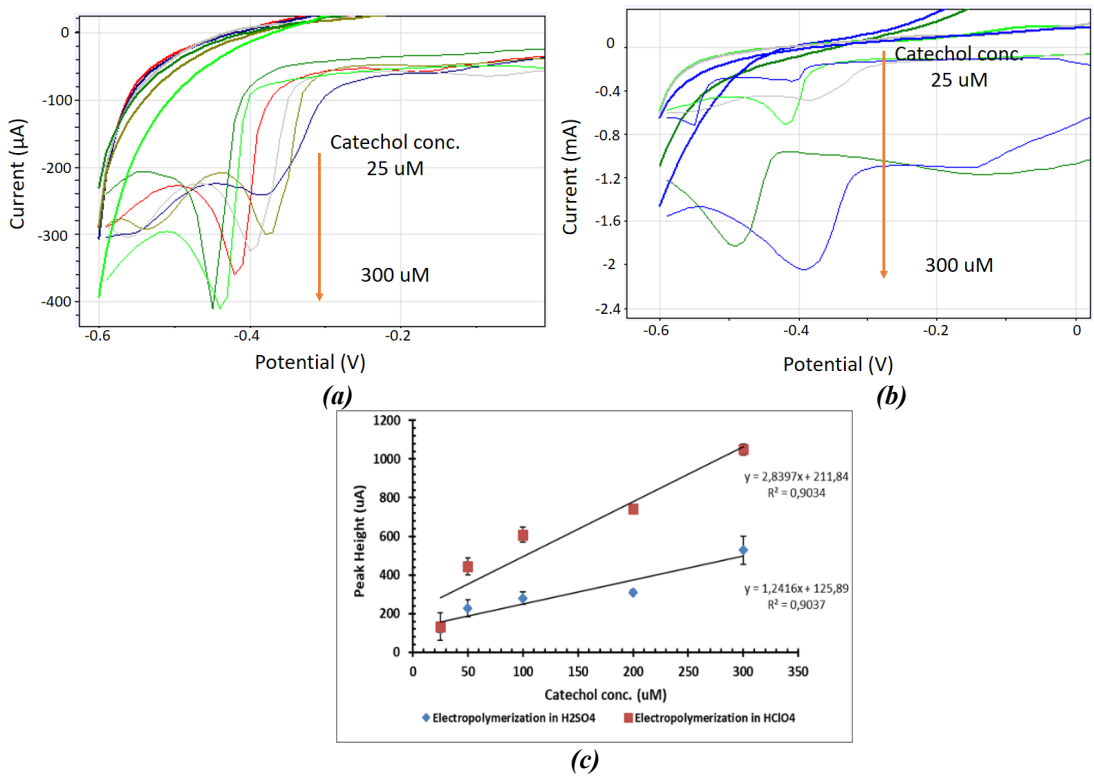



***Figure 6.*** *Catechol detection(25,50,100,200,300 µM)* in $H_2SO_4$ (a) and $HClO_4$ *(b) polymerization, and the dose response curves for these polymerization conditions (c).*

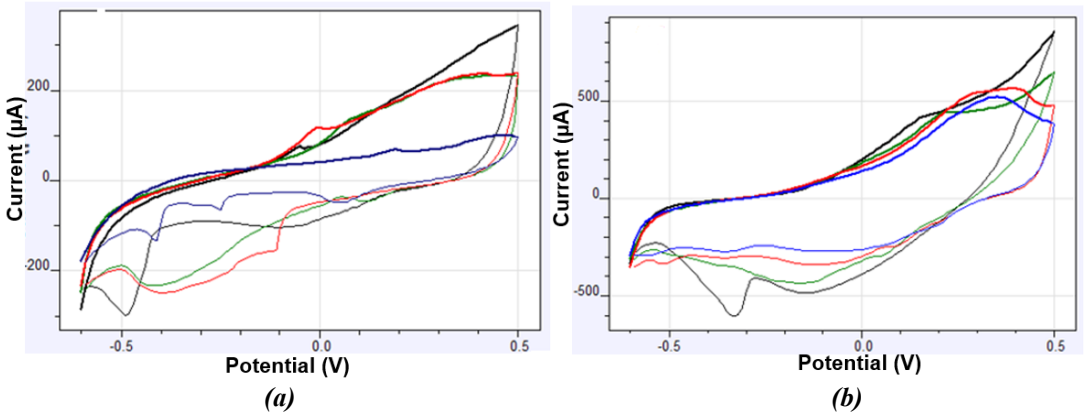



***Figure 7. (a)*** *$H_2SO_4$ aniline electropolymerized SPE* ***(b)*** *$HClO_4$ aniline electropolymerized SPE.(Black: catechol, Blue: gallic acid, Green: N-nitrophenol, Red: Hydroquinone )*

## A.3. Real Sample Testing

To demonstrate the biosensor's ability to detect catechol in tea samples under the optimized conditions. 50 µM,100 µM, and 200 µM spiked tea samples were tested with developed enzymatic biosensor systems in Figure 8. The results are briefly presented in Table 1. The percentage recovery results of all measured samples were between 93% and 110%, and the parallel tests showed sd% deviation results between 2% and 9%. These results showed that the potential interference from the background composition of real samples was within 16% and is considered acceptable for food quality monitoring tests.

***Table 1.*** *Catechol spiked green tea samples result with developed polyaniline based enzymatic biosensor system.*

| Aniline Electropolymerization in $HClO_4$ | | | | Aniline Electropolymerization in $H_2SO_4$ | | | |
|---|---|---|---|---|---|---|---|
| **Added** | **Found** | **Recovery** *(%)* | **sd** *(%)* | **Added** | **Found** | **Recovery** *(%)* | **sd** *(%)* |
| **50** | 55,28455 | 110,5691 | 5,942074 | **50** | 55,84989 | 111,6998 | 18,57475 |
| **100** | 93,73984 | 93,73984 | 2,192579 | **100** | 103,3113 | 103,3113 | 11,24118 |
| **200** | 205,6911 | 102,8455 | 9,198137 | **200** | 221,4128 | 110,7064 | 14,9494 |

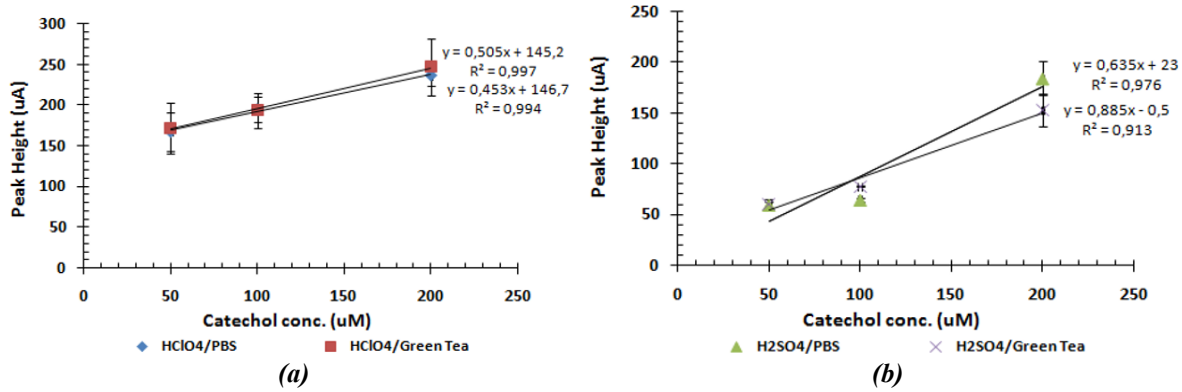



***Figure 8.*** *Catechol spiked green tea samples' testing results with developed polyaniline-based enzymatic biosensor system* ***(a)*** *$HClO_4$ solution,* ***(b)*** *$H_2SO_4$ solution.*

## B. DISCUSSION

In this work, we modified SPEs with conductive PANI films using different acidic solution-based electropolymerization of aniline. SPEs were scanned within the potential range of −0.5 V to 0.8 for $H_2SO_4$ solution, 0.4 V to 0.8 V for $HClO_4$ solution, and -0.2 V to 1.0 V for HCl solution until steady curves of cyclic voltammograms (CVs) were obtained. Comparing the CVs of these in different solutions, it is found that the $HClO_4$ solution increases the electron transfer rate for PANI formed onto SPE. Electrode surface conductivity analysis with $Fe(CN)_6^{3-}/Fe(CN)_6^{4-}$ redox molecule results also support this conclusion, which has the highest voltammogram peak height with $HClO_4$ based electropolymerization. On the SEM images of SPEs with PANI modification in different acidic solutions, we can see the significant influence of $HClO_4$ based modification. Hence the SPE surface with $HClO_4$ based modification lead to a more uniform and dense PANI film.

After the electropolymerization steps, a Tyr enzyme-based biosensor system was developed for catechol detection. Tyr enzyme was immobilized onto the PANI modified SPE surface, which was also observed by SEM images. The biosensor systems' linear ranges were identified for all acidic solutions based on electropolymerization between 10 µM and 300 µM, where the $HClO_4$ based enzymatic biosensor system had higher signals and, therefore, sensitive response. The phenolic component in natural foods was tested in the linear range of ≈5 µM to 1≈65 µM with similar systems [17][18][19]. Considering these references, we aimed to make determinations at similar intervals to the developed biosensor system. The biosensor system was also having quite a specificity for catechol detection compared to other phenolic compounds. Additionally, the developed enzymatic biosensor system was successfully tested with real tea samples with 93% to 110% recovery and 2% and 9% sd% deviation results. Thus, these results exhibited that the potential interference from the background of real samples was within 16% and is considered acceptable for food quality monitoring tests.

# IV. CONCLUSION

In view of its excellent electrochemical properties, polyaniline (PANI) is one of the most favored and well-known conductive polymers for biosensor design. Using the direct electropolymerization (one-step) method, this paper developed an electrochemical enzymatic biosensor system with PANI film-coated screen-printed electrodes (SPE). Electropolymerization of PANI was performed in various acidic solutions. SEM characterized the film surface morphologies prepared with different processes. A stable and efficient catechol biosensor was developed by immobilizing tyrosinase (Tyr) enzymes into the PANI film in combination with cross-linking with glutaraldehyde. The developed biosensor was used to detect catechol in green tea samples after optimization of biosensor performance conditions. Using the portable potentiostat and SPEs, this developed enzymatic biosensor system has the potential for on-site analysis of catechol detection in real samples. Thus, the study's future perspective could be developing a portable, easy-to-use prototype product for real sample testing.

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