Koc. J. Sci. Eng., 5(1): (2022) 13-21 https://doi.org/10.34088/kojose.887169

Kocaeli University

Kocaeli Journal of Science and Engineering

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Paper-Based Biosensor System for Fast and Sensitive Phenolic Compounds Detection

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Article Info

Research paper

Received : *February 26, 2021*
Accepted : *October 26, 2021*

Keywords

Biosensors
Food quality
Paper-based sensor
Phenol detection

Abstract

In this original paper, the development of a paper-based, sensitive, low-cost, quantitative and consistent biosensor system was demonstrated for the suitable "naked-eye" detection of phenolic compounds (specifical catechol). The Paper-based system depends on the enzymatic color-changing reaction that observed by using chromogenic agent of 4-AAP (4-aminoantipyrine) which has a specific color changing reaction in the presence of horseradish peroxidase (HRP) enzyme, phenol and H_2O_2. The visual result was associated with the catechol concentration by using image processing software to evaluate the quantitative detection. The developed biosensor system demonstrated a linear detection range from catechol between 2,5 µM to 100 µM with 2.6% to 9.3 % of sd results. The detection limit was also calculated as 2,25 µM. Specificity, selectivity, stability and direct real sample analysis demonstrated the potential applicability of the developed paper-based biosensor system for catechol detection in food samples with minimal investment and an easy-to-use method.

1. Introduction

Phenolic compounds are widely used in food processing, petrochemical engineering, chemical production, printing, dyeing, etc. When these compounds discharge to the environment during or after the processes, they become one of the most harmful pollutants due to phenolic compounds' high toxicity and low biodegradability in the environment [1]. They contaminate agricultural products, poison marine animals, and affect the functions of the human nervous, urinary, and digestive systems. Other than their usage on different production processes, they also existing in many natural sources such as tea, vegetables, fruits, and other plants [2, 3, 4]. For example, catechol (1,2-benzenediol/1,2-dihydroxybenzene) is a critical dihydroxybenzene, and it is recognized as one of the key fragments of tea catechins. Catechol's concentration can be high up to 18-36% weight of the fresh dry tea and it is believed to be the source of many claims made regarding tea's health benefits. Catechol quantification is also essential due to its biological importance such as anti-oxidation, anti-virus activities and affecting of some enzymes. because of its biological roles and environmental significance in such topics as antioxidation, antivirus, toxicity, and carcinogenicity [5, 6, 7]. Therefore, phenolic compound detection is crucial in food quality monitoring and also environmental water pollutants analysis. Several physical and chemical-based analytical methods are available to detect and determine phenolic compounds in various samples. HPLC with fluorimetric detection GC/MS, colorimetric methods or fluorescence excitation-emission matrix (EEM) are the most commonly used ones [8, 9]. Nevertheless, these methods involve complex sample pre-treatment, cost, increasing time, and expert personnel needs. Thus, it is precious to develop a simple, rapid, reliable, and low-cost method to detect phenolic pollutants in environmental and food matrices.

It has been observed that biosensors provide analysis that is less costly, easy, less time consuming, highly sensitive, and selective to various analytes. This has inspired

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to development of easy-to-use, low-cost, and reliable platforms for monitoring several targets with following the "ASSURED" standards ("affordable, sensitive, specific, user-friendly, rapid and robust, equipment-free, and deliverable") by giving results even in the most restricted facilities [10, 11]. Among other biosensor systems, paper-based sensors can accomplish most of the ASSURED criteria' requirements and present some significant advantages such as high surface-to-volume ratio, biocompatibility, low-cost, biodegradability, lightness, and flexibility [10]. The analysis by using paper-based biosensors can be performed with colorimetric and electrochemical transducers, however the colorimetric strategies offer benefits like affectability, soundness, and straightforwardness of location through direct perception rather than the perusers needed for electrochemical gadgets. [12, 13]. However, a significant weakness of colorimetric transduction is the lack of sensitivity and quantitative detection. In the literature, most researchers used a mobile phone with a camera as the detection device, and so the software could be calculated the values by converting images of paper-based systems from the color scale into grayscale [14]. However, they sometimes suffered from adjustable changes in light conditions. Additionally, some people used a scanner to implement testing. However, it was challenging to be portable for on-site detection. Thus, well-standardized smartphone apps can solve these problems and are suited for on-site and real-time detection modes.

Typically, the colorimetric analysis of phenolic compounds can be accomplished by monitoring the target's oxidative coupling with 4-aminoantipyrine (4-AAP). In detail, the colorless 4-AAP can react with phenolic compounds to procedure a colored quinone imine molecule. To enable the chromogenic reaction, H_2O_2 and enzymes are frequently used to analyze phenol in diverse conditions rapidly. For example, Lin and co-workers described a colorimetric platform with horseradish peroxidase (HRP) for visually detection of phenol [15, 16, 17]. Figure 1 presents the colorimetric reaction of phenolic compounds and 4-AAP that catalysis by HRP in the presence of H_2O_2. While the approach displays a good performance for phenol monitoring, there is still plenty of opportunity to the enhance of the colorimetric method.

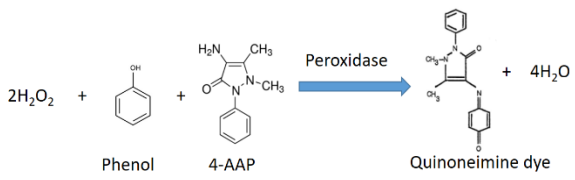

Figure 1. The colorimetric reaction of 4-aminoantipyrine and phenol produced a red color dye in the presence of horseradish peroxidase (HRP) and H_2O_2.

In this work, we demonstrated the establishment of a sensitive, quantitative, low-cost, portable and reliable paper-based biosensor system for the straight “naked-eye” detection of phenolic compounds (specifical catechol). Onto the paper filter material, biocompatible zones were prepared by chitosan modification and enzyme immobilization. The color changing reactions of phenolic compounds were observed by using the chromogenic agent of 4-AAP which has a specific color-changing reaction in the presence of HRP enzyme, phenol and H_2O_2. The visual outcome was corresponded with the catechol focus by the unaided eye and with picture handling programming to assess the quantitative recognition. Thus, catechol analysis, which will be performed using only a smartphone and a prepared paper-based biosensor system without extra equipment, will significantly benefit the time/cost benefit. Accuracy, selectivity, stability, and direct real sample tests showed the promising usage of the created paper-based biosensor framework for catechol recognition in food tests with the insignificant venture and a simple to-utilize technique.

2. Materials and Methods

Reagents and chemicals: RP type II (210 U/mg), chitosan powder (CAS 9012-76-4), Cathecol (1,2-Dihydroxybenzene- CAS 120-80-9), phenol (CAS 108-95-2), 4-nitrophenol (100-02-7), Hydroxyquinone (615-94-1), gallic acid (149-91-7), 4-aminoantipyrine (CAS 83-07-08), acetic acid (CAS 64-19-7), paraffin (CAS 8002-74-2), and # 40 WhatmanTM filter paper (210 µm of thickness and 8 µm of pore size) were ordered from (Sigma-Aldrich, Germany). 100 mM of phosphate buffer solution, distinct pH ranges, was set up from potassium phosphate monobasic and sodium phosphate dibasic, both acquired likewise from Sigma-Aldrich. The working solutions of catechol were prepared in PBS and green tea samples (prepared by regular brewing method).

Detection zone Preparation: Delimited round recognition zones were made utilizing a stamping process (figure 2, A-C), like the philosophy for paper gadgets represented by de Tarso-García et al. [18]. A metal stamp was planned and produced in aluminum to get location zones 6 mm in distance across. The metal stamp incorporates only a metal body and round meager edges which produce the hydrophobic boundaries around the detection zones. During the manufacture, a piece of Whatman paper was submerged into fluid paraffin for ≈2 seconds and put into a non-covered Whatman paper surface. At that point, the aluminum form was warmed at ≈110 °C and squeezed against the paraffin covered paper for ≈5 seconds. This brought about the exchange of paraffin from the upper paper to the non-covered channel paper and encompassed discovery zones with hydrophobic paraffin boundaries. The interaction is meant in Figure 2 with the means of A to C.

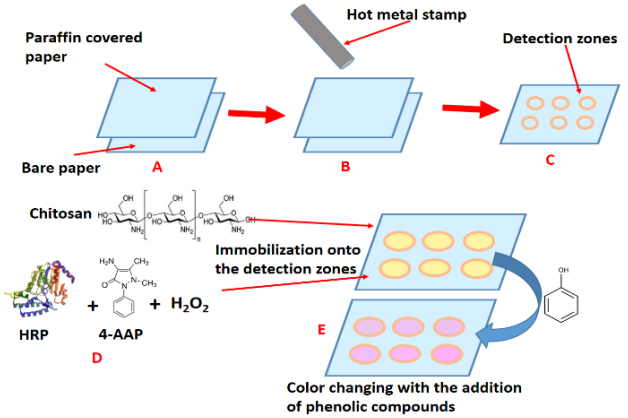

Figure 2. Portrayal of the paper-based sensor manufacture steps. (A) to (C); the advancement of the detection zone. (D) to (E); chemical and enzymatic modification of detection zone is presented.

Chitosan, Enzyme and chromogenic agent immobilization onto the detection zones: Chitosan was used as supporting material for the immobilization of HRP enzyme and 4-AAP chromogenic agent onto the prepared detection zones. The detection zones were firstly modified with chitosan solutions at 0.25%, 0.5%, 1%, and 2% (m/v) concentrations in acetic acid at 2% (v/v). After the chitosan modification the HRP (250 U/mL) enzyme was prepared in PBS at pH 6.0, 6.5, 7.0, 7,5, and 8.0 to immobilize onto the paper surfaces. Different pH values were prepared to investigate the enzymatic reaction efficiency in these pH conditions. The chromogenic agent, 4-AAP solution of 0,1 M, was prepared within PBS (100 mM, Ph 7.2). Paper-based biosensor framework was set up by consecutively adding the entirety of the reagents as introduced in Figure 2,D–E. 6 uL of chitosan solution, 3 uL of the enzyme solution and 3 uL of chromophore solution were added to the detection zones step by step and allowed to dry at room temperature. The prepared paper materials for the developed biosensor system were stored at 4°C not exposed to direct light. H_2O_2, the color-changing reaction indicator, was tested both by immobilizing onto the detection zones and adding to the phenolic compounds solutions. To optimize the H_2O_2 amount, the different percent composition of H_2O_2 solutions (0.05 % to 0.01 %) were mixed with 4-AAP or samples then injected onto the detection zones.

Color Detection Procedure: The obtained color change results were analyzed using a smartphone app, 'ON Color Measure,' to process the information with the RGB model [19, 20]. The smart phone apps for color intensity analysis is going to be an exciting filed on biosensor application that some of the recent works alrady tested their validation and verification [21]. In the RGB model red (R), green (G), and blue (B) light are added together in various ways to generate a broad array of colors. The name of the model comes from the initials of the three additive primary colors, red, green, and blue. Each tone is described on a scale from 0 to 225 when 0 addresses no commitment to the tone, and 225 addresses the R+G+B parts' top-level input. Ordinarily, in this model, the white tone is shown as the top level augmentation of the R+G+B parts, and the dark tone is the minimum value. Since the enzymatic response creates a color change from the white substrate to a colorful product, during the detection, the white introductory substrate was perceived as the maximum value for the entirety of the R+G+B parts, and the product color intensity was addressed with a decrease of these RGB values. Thus, lower phenolic compounds amount described as high RGB values, and higher concentrations formed low RGB values (Fig. 3). To accomplish a precise explanation, the total distribution of the R+G+B prices were measured, and the average values were used at each experimental part of this work. In the calibration curves an opposite relationship between mean RGB values and phenolic compounds concentration were observed in PBS and tea samples.

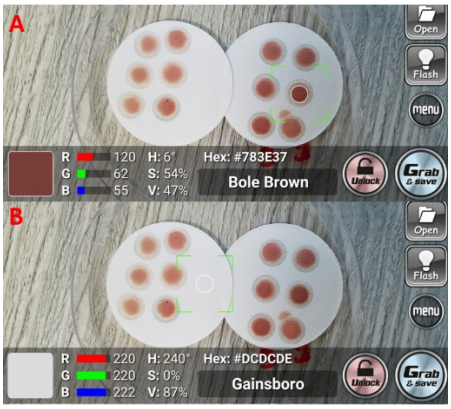

Figure 3. A smartphone app, 'ON Color Measure,' analysis process with the RGB model.

Biosensor performance optimization: After preparation of the detection zones and immobilization of the enzyme+4-AAP onto them, a fixed concentration (50 µM) of phenolic compounds including catechol, phenol, N-nitrophenol, Hydroxyquinone, and gallic acid were added to the zones. Afterward, different concentrations of catechol (the most sensitive and selective target for the system) solutions were added to the detection zones in optimum conditions to determine the calibration curve and limit of detection of the developed biosensor system. To research the stability of the created biosensor framework, the colorimetric reaction for a catechol test of 50 µM was recorded over 30 days.

Real sample testing: By using the optimum performance conditions for the developed biosensor system, real tea samples were tested. 10 µM, 50 µM, and 100 µM concentrations of catechol spiked onto the teas samples, and catechol detection was performed by the developed biosensor system. Percentage of recovery and cv (coefficient of variation) results were calculated for each concentration using three independent experiments.

3. Results and Discussion

Manufacture and Optimization of the Paper-Based Sensor: The created detection zones were covered with chitosan to accomplish an appropriate spot for analysis. The chitosan was utilized in concentrations from 0.25 to 2 % (mass to volume), which framed the meager film above the cellulose substrate of paper with a thickness reliant on the concentration. Figure 4 shows the morphology got for a paper-based biosensor framework before and after chitosan adjustment. Scanning electron microscopy (SEM) exposed the commonplace porous fibrillar design of the clear cellulose substrate and a chitosan film covering the cellulose filaments and obstructing the porous regions. It is found in figure 4 that the most uniform modification was accomplished with a 1% concentration of chitosan (Figure 4, D), which is likewise thick enough to cover the entire paper surface. After the enzyme immobilization onto the chitosan-covered paper surface, we got SEM images of Figure 4, F, representing the successful enzyme entrapping on chitosan layers.

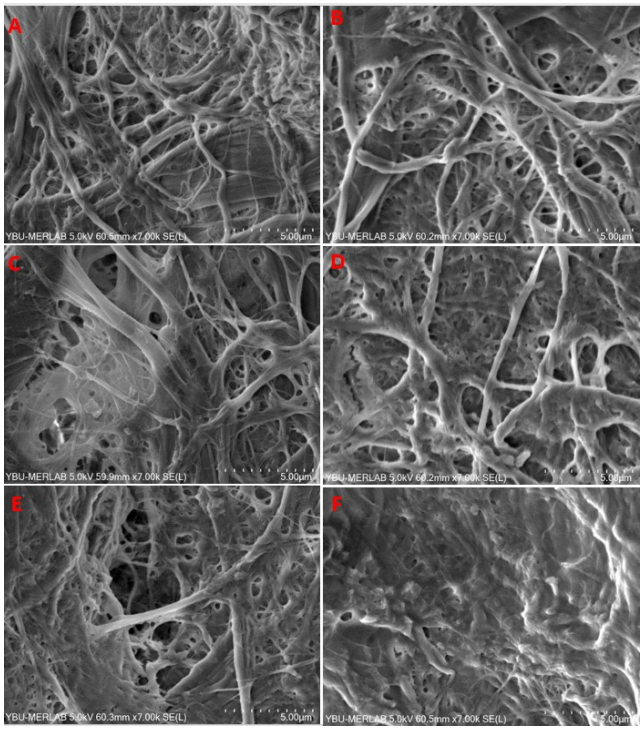

Figure 4. SEM images of modified papers with different chitosan concentrations; A) Blank paper, B) 0.25 %, C) 0.5 %, D) 1 %, E) 2 % and F) enzyme immobilized paper surface with % 2 concentration of chitosan (scale bars are 5.0 µm for each figure).

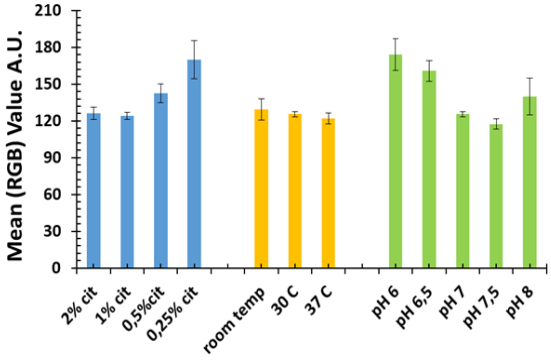

Figure 5. Impact of the concentration of chitosan solutions, detection temperature, and pH over the mean RGB values for the concentration of 50 µM of catechol.

The impact of the concentration of chitosan, pH ranges, and detection temperature over the colorimetric reaction was examined before catechol detection. The tests were directed with a sample concentration of 50 µM, which is inside the recognition range for catechol in food tests. Figure 5 shows the distinctions in the mean RGB values for the equivalent catechol concentration under various conditions.

It is seen from figure 5 that when the chitosan concentration increases, the means of RGB values decrease, which represents enhancing the activity of catechol's enzymatic reaction. The improved colorimetric reaction can be qualified initially to the surrounded detection zones, which expanded the entrapped enzyme concentration in the detection zone with a homogeny profile. In addition, the change with higher chitosan concentration gave to establishing a biocompatible climate for the enzymatic assay, given the chitosan's high affinity for proteins. Thus, the optimum chitosan percentage for the enzyme immobilization onto the detection zones was concluded as 1%.

Detection temperature is another essential condition for the maximum enzymatic activity, which is generally observed at 37 °C for most enzymes. Thus, we tested 37 °C, 30 °C, and room temperature (≈22 °C) to observe the temperature effect on the developed biosensor system performance. As seen from figure 5, even the maximum activity observed at 37 °C, even at the room temperature enzyme, had acceptable activity and closed RGB values to the higher temperatures. Thus, performing of biosensor system at room temperature was concluded, which helps to perform real-time and on-site detection of real samples.

In addition to the chitosan percentage and detection temperature, the pH values for the immobilized enzyme solution were tested with the range of pH 6.0, 6.5, 7.0, 7,5, and 8.0. As it was estimated, the maximum activity was observed at pH 7.5, which is generally the optimum pH value for biological molecules.

After the immobilization condition optimization, which was performed by the addition of 0.03 % of H_2O_2 solution directly onto the 4-AAP, the percentage amount of

H_2O_2 and the additional way for the H_2O_2 was evaluated (results are represented in Figure 6). Firstly, 0.05 %, 0.03 %, and 0.01 % of H_2O_2 were added onto the 50 µM of catechol and injected onto the detection zones, prepared using optimum conditions. In a second way, 0.05 %, 0.03 %, and 0.01 % of H_2O_2 were added to the 4-AAP solution during the immobilization. The results for the additional way of the H_2O_2 solutions were quite the same for the freshly used biosensor systems, but when we stored them ready to use the paper-based device, H_2O_2 and 4-AAP reacted by time in the presence of HRP enzyme (stability tests also represents this results). Thus we concluded to add H_2O_2 after immobilization and freshly before catechol testing on the developed biosensor system.

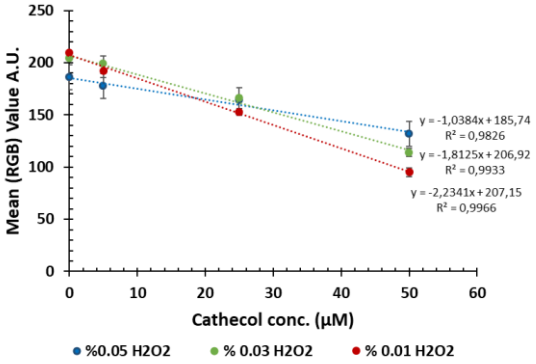

Figure 6. Effect of H_2O_2 amount on the developed paper-based biosensor system with different concentrations of catechol.

On the other hand, different H_2O_2 percentages gave different biosensor responses with different catechol concentrations. When the concentration ratios of catechol that range from 0 to 50 µM were tested, the test by using 0.01 % of H_2O_2 solution represented the most linear and distinguished results depending on the catechol concentrations (Fig. 6). The access amount of H_2O_2 generated undesired color changing not related to the catechol but also a non-specific reaction between 4-AAP and H_2O_2. Thus, we concluded to use 0.01 % of H_2O_2 freshly and after immobilization of all molecules onto the paper device.

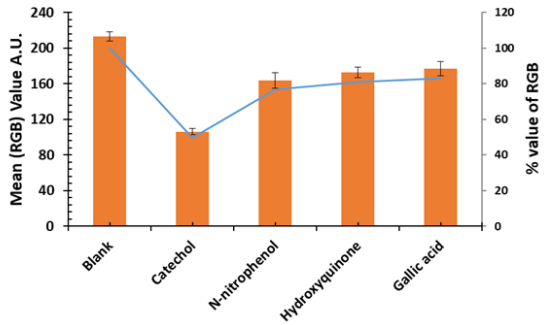

Figure 7. Selectivity analysis for the manufactured biosensor framework with mean colorimetric RGB values for catechol and diverse phenolic compounds (each was applied on 50 µM concentrations).

After preparation of the detection zones and immobilization of the enzyme and 4-AAP onto the paper surfaces on the optimized conditions, a fixed concentration (50 µM) of phenolic compounds including catechol, phenol, N-nitrophenol, Hydroxyquinone, and gallic acid were added to the zones with including 0.01 % of H_2O_2. In addition to these phenolic compounds, the blank solution, which includes only BPS, was tested. After measuring the RGB values of the color changed detection zones with the 'ON Color Measure' app, catechol, N-nitrophenol, Hydroxiquinone, and gallic acid generates % of RGB values of 49.9, 76.8, 80.8, and 83.1 depends on the blank experiment values (Fig. 7). The results represent that the developed biosensor system is not 100 percent selective for catechol, but the system also has more sensitivity and selectivity for catechol than the other phenolic compounds. Thus the developed biosensor system could be used for sensitive catechol detection and has potential usage for total phenolic compound detection on real food samples.

Afterward, different concentrations of catechol (the most sensitive and selective target for the system) solutions were added to the detection zones, which were prepared with optimized conditions, to determine the calibration curve and limit of detection of the developed biosensor system. Figure 8 shows the linear response (RGB values) obtained for the developed paper-based biosensor system after increasing the catechol amounts from 2,5 µM to 100 µM with 2.6% to 9.3 % of sd results. The detection limit was also calculated as 2,25 µM using the 3 times Standard Deviation value SD± that prepared most of the biosensor-related works [22, 23, 24, 25]. In addition to these measure responses, the color-changing for the different catechol concentrations represented naked eye observation between 0 µM and 200 µM.

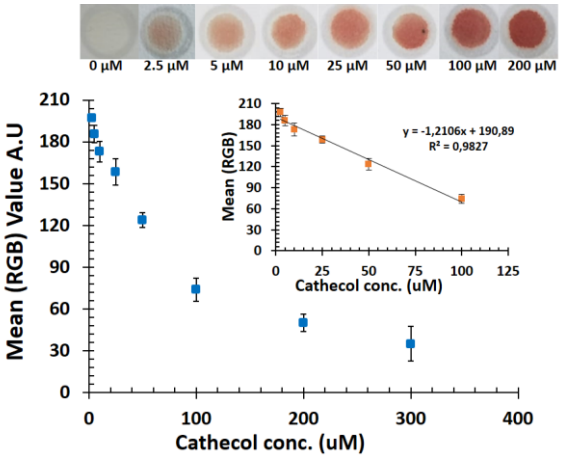

Figure 8. Calibration curve acquired for catechol detection in PBS with an inset figure representing the linear range and naked-eye visual scale achieved for catechol detection.

The RGB profile of the created biosensor framework stored under standard conditions was examined for 5 weeks, as demonstrated in Figure 9. The paper-based biosensor systems were prepared with and without immobilization of H_2O_2 onto the detection zones. The stability was accomplished with storage in the dark environment and +4 °C conditions, which is effortlessly accessible with a standard fridge. It is clearly seen from Figure 9 that immobilization without H_2O_2 gave more stable results than immobilization with H_2O_2. This is because the enzyme denaturation with acidic H_2O_2 and the non-specific reaction between 4-AAP and H_2O_2, even without phenolic compounds. The average deviation for immobilization without H_2O_2 was 6.32 %, and 21,2 % for the immobilization with H_2O_2 comparing the measured RGB values of the last day's results with the first day. Thus, we concluded that the paper-based biosensor systems should be prepared without immobilization of H_2O_2 during the initial preparation process, and the H_2O_2 solution should be added to the phenolic compound included solution just before the analysis. This proposes the practicality of the paper-based biosensor in restricted supply conditions within an acceptable performance over a period of time.

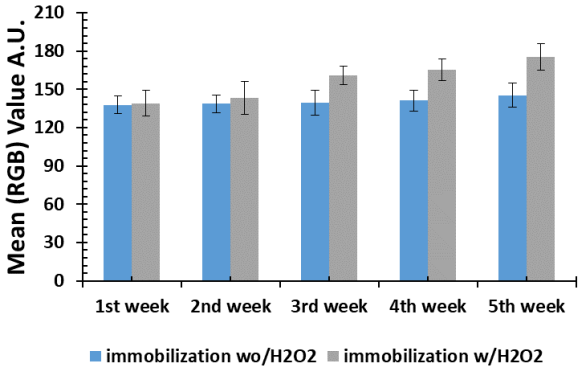

Figure 9. Stability test for fabricated paper-based sensors stored at 4 °C in dark conditions over 30 days.

Detection of Catechol in Tea Samples: To demonstrate the paper-based sensor's applicability for monitoring real samples, the detection of catechol in tea samples was performed. By using the optimum performance conditions for the developed biosensor system, real tea samples were tested. 10 µM, 50 µM, and 100 µM concentrations of catechol spiked onto the tea samples prepared by brewing in hot water. These different catechol concentrations were detected by the developed biosensor system, and the accuracy was evaluated through recovery percentages, achieving results from 92 to 114% (for three independent experiments), as shown in Table 1. also represents the calibration curves for catechol detection in BPS and tea samples, representing the closeness of the results with PBS and complex matrix.

Table 1. Results for catechol determination in real samples by using the developed paper-based biosensor system.

Added	Found (ave. value)	Recovery %	cv %
10	15,23	152,30	8,20
50	52,59	105,18	12,16
100	88,38	88,38	7,11

4. Conclusions

Compared to other reported works for catechol detection, our results demonstrate better sensitivity, wider linear detection range and better stability efficiency than most of them. Additionally, comparing the cost of them precious system, the developed paper-based biosensor system serves more efficient usage for real samples over 5 weeks of storage (Table 2). Additionally, we can conclude that this approach can be visually observed with the "naked eye," without the need for exterior analyzers or additional necessities, which according to the ASSURED criteria, is highly convenient for zones with restricted resources.

Table 2. Comparison of some analytical characteristics of the proposed methods with those of previously reported

Detection System	Biosensor system	Detection range	Stability/ Reusability	Real sample testing	Ref.
Colorimetric Sensor	Polymer modified enzyme based system	2.5 µM to 50 µM	1 months	Wine and fruit juice samples	[26]
Colorimetric Sensor	Non-enzymatic system	0.87 µM to 56 µM	Not Specified	Water and plasma samples	[27]
Electrochemical impedance spectroscopy (EIS)	PANI modified enzymatic system	1.0 µM to 100 µM	5 months	Wastewater samples	[28]
Amperometric Sensor	PANI copolymer based enzymatic system	5.0 µM to 80 µM	over 120 times	Not Specified	[29]
Potentiometric Sensor	Polypyrrole film based enzymatic system	1 µM to 16 µM	1 month	Not Specified	[30]

Table 2. (Cont.) Comparison of some analytical characteristics of the proposed methods with those of previously reported

Detection System	Biosensor system	Detection range	Stability/ Reusability	Real sample testing	Ref.
Cyclic voltammetry Sensor	Carbon nanofibers used enzymatic system	1 µM to 310 µM	30 days	Water samples	[31]
Fluorescent Sensor	Carbon dots based enzymatic system	2,66 µM to 341 µM	Not Specified	Not Specified	[32]
Fluorescent Sensor	carbon quantum dots based non-enzymatic system	0.002 µM to 0.05 µM	Not Specified	River water samples	[33]
Paper based colorimetric Sensor	4-AAP based enzymatic system	2,5 µM to 100 µM	5 weeks	Green tea sample	This work

Declaration of Ethical Standards

The author(s) of this article declare that the materials and methods used in this study do not require ethical committee permission and/or legal-special permission.

Conflict of Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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