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Research Article

Smart phone assisted paper-based biosensor system for hypoxanthine detection in fish meat samples

Nimet YILDIRIM TİRGİL[1,2*], Bülent ÖZCAN[2], Muhammed Ali BELİBAĞLI[2]

[1]Ankara YıldırımBeyazıt University, Faculty of Engineering and Natural Sciences, Biomedical Engineering, Kecioren, Ankara,06010, Türkiye
[2]Ankara YıldırımBeyazıt University, Faculty of Engineering and Natural Sciences, Materials Engineering, Kecioren, Ankara, 06010 Türkiye

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ABSTRACT

In this work, we present the development of a paper-based, low-cost, sensitive, quantitative and stable platform proper for smartphone-assisted colorimetric detection of the fresh fish product to eliminate people's concerns while consuming seafood. Biocompatible zones were prepared onto the paper filter material by chitosan modification and multi-enzyme immobilization in the developed biosensor system. The visual detected result was connected with the hypoxanthine (Hx) amount by using a smartphone app that can perform image processing to evaluate the quantitative detection. Selectivity, specificity, stability, and direct real sample tests indicated the potential usability of the developed paper-based biosensor system for Hx detection in meat samples with an easy-to-use method. An application for examination of Hx concentration in real fish meat was achieved by placing the paper-based biosensor in contact with fish skin. As a future perspective, the developed method can be practical in developing portable biosensors to assess the fish freshness grade.

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INTRODUCTION

The freshness of fish meat has a critical role in food safety because it is required for high-quality production [1]. Hypoxanthine (Hx) has been identified as one of the main denaturation products of adenosine triphosphate (ATP), which has been found to accumulate shortly after an animal died [2][3]. After a fish dies, the decomposition of ATP (adenosine triphosphate) starts and ADP (adenosine diphosphate), AMP (adenosine 5' phosphate), IMP (inosine 5' phosphate), I (inosine), Hx (hypoxanthine), X (xanthine), and U (uric acid) produced respectively [2].

***Corresponding author.**
*E-mail address: nyildirimtirgil@ybu.edu.tr

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Hypoxanthine quantification can be used as an indicator of fish freshness during this degradation process. Within 24 hours, ATP and AMP drop sharply, IMP rises around 5-24 hours after death, and I and HX rise sharply in the final stages of degradation. The fish began to deteriorate when the Hx concentration exceeded 102 mg/kg, and the fish totally deteriorated when the Hx concentration exceeded 144 mg/kg [1][4].

Many classical methods, such as near-infrared spectroscopy [5], high performance liquid chromatographic (HPLC) [6], chemiluminescence [7], capillary electrophoresis [8], spectrophotometric [9], and electrochemical approaches [10], had been performed for Hx detection with high selectivity and sensitivity. However, most of these strategies require relatively costly hardware and talented individual to perform the tests, which are troublesome to be utilized in on-site applications. Subsequently, it is essential to create helpful strategies for Hx analysis and used to evaluate the fish spoilage level by an untrained person. Meanwhile, several biosensors were developed for the Hx analysis by using mostly colorimetric and electrochemical systems [11][12][13]. These biosensor systems can be applied in the manufacturing of portable biosensors to estimate the fish freshness status and in other applications in which Hx plays an essential role. Colorimetric analysis has acquired much consideration among other biosensor systems as it benefits effortlessness, cost-viability, and visualization. The colorimetric technique had been produced for selective Hx measuring for years [14][15][16]. However, the amount of Hx was determined by measuring the color intensity, which is hard to peruse by the naked eye. Thus, these methods can only be used with specified spectrophotometers for quantitative detections or qualitative detection of Hx. Thus, the laboratory-grade equipment needs to restrict these methods for on-site and real-time application. These methods are established on xanthine oxidase (XOD) that reacts Hx and X to produces H_2O_2, according to the reactions in figure 1.

The generated H_2O_2 is identified using a secondary enzyme; generally, horseradish peroxidase (HRP) and an organic dye, causing a color change, measurable by UV–vis spectroscopy. Few colorimetric methods have been described for HX and X detection with using soluble dyes such 4-aminoantipyrine (A-AAP) and 2,4,6- tribromo-3-hydroxybenzoic acid (TBHBA), 3,3′,5,5′ -tetramethylbenzidine (TMB), or nanorods and gold nanoparticles to measure the enzymatically generated H_2O_2 [16][17][18].

Meanwhile, traditional colorimetric-based detection methods which rely on spectrophotometers cannot realize on-site portable analysis. However, developing portable and mobile devices for real-time testing is essential for Hx detection in some exceptional conditions. Nowadays, in some example studies, smartphones were used as detection tools that can avoid the use of expensive and immense instrumentation. Moreover, smartphones' ubiquitous accessibility and comprehensive availability make detections achievable in inaccessible or undeveloped regions [19][5]. Therefore, here in this work, a smartphone-assisted colorimetric paper-based biosensor system was developed for Hx's reliable, precise and real-time determination.

In this work, we aimed to prepare a paper-based, sensitive, quantitative, low-cost, and reliable platform appropriate for smartphone-assisted Hx detection for fish freshness to eliminate people's concerns while consuming seafood. During the paper based biosensor preparation steps, biocompatible zones were prepared by chitosan modification and multi-enzyme immobilization onto the paper filter material. The color-changing reaction of chromogenic compounds that enzymatically reacts by HRP was observed in the existence of H_2O_2 produced from the enzymatic reaction of Hx by the enzyme of XOD. The visual result was processed with a smartphone application and connected with the Hx concentration to evaluate the quantitative detection. Selectivity, specificity, stability, and direct real sample tests were performed to present the possible applicability of the developed paper-based biosensor system for Hx analysis in real samples. An application for examination of Hx in real fish meat was achieved by placing the paper biosensor in contact with fish skin. As a future perspective, the developed method can be applied to develop a portable

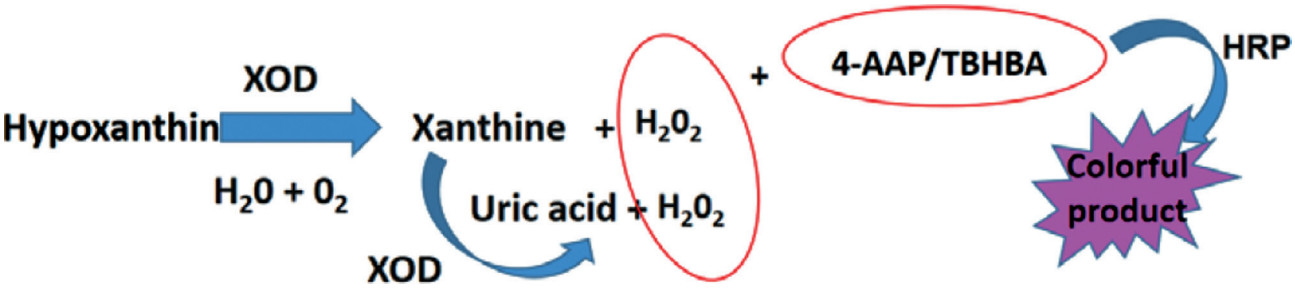

Figure 1. The enzymatic reaction of Hx to uric acid and colorimetric reaction of 4-AAP/TBHBA and H_2O_2 produced a colorful product in the presence of horseradish peroxidase (HRP).

biosensors device for the assessment of the fish freshness grade wherever needed.

MATERIALS AND METHODS

Reagents and chemicals: HRP type II (210 U/mg), Xanthine Oxidase from bovine milk (50UN- EC Number 232-657-6), chitosan powder (CAS 9012-76-4), 4-aminoantipyrine (CAS 83-07-08), 4,6- tribromo-3-hydroxybenzoic acid (14348-40-4), Hypoxanthine (68-94-0), acetic acid (CAS 64-19-7), paraffin (CAS 8002-74-2), glucose (CAS 50-99-7), lactic acid (CAS 50-21-5), uric acid (CAS 69-93-2), and ascorbic acid (CAS 50-81-7) and #40 Whatman™ filter paper were purchased from (Sigma-Aldrich, Germany). Phosphate buffer solution (PBS) at 100 mM, different pH ranges, was prepared from sodium phosphate dibasic and potassium phosphate monobasic, both also obtained from Sigma-Aldrich. For the real sample testing part, anchovy fishes were bought from local grocery stores and stored at 4°C for 2 days before testing of Hx.

Detection zone Preparation: Surrounded round detection zones were formed by adapting a stamping process from the methodology of the paper devices fabrication published by de Tarso-García et al. [20]. A metal stamp was designed using an aluminum circular rode to achieve detection zones about 6 mm in diameter. The metal stamp includes just a metal body and thin circular edges, producing hydrophobic barriers around the detection zones. A # 40 Whatman™ filter paper was submerged into liquid paraffin for ≈2 seconds during the detection zones fabrication and put into a blank Whatman paper surface. Then, the stamp was heated at ≈110°C and pressed onto the paraffin-covered paper for about 5 seconds. This caused the paraffin transfer from the upper filter paper to the blank filter paper and produce surrounded zones with hydrophobic paraffin barriers. In Figure 2, with the steps of A to C, the procedure is signified.

Chitosan, Enzymes, and chromogenic agents immobilization onto the detection zones: Chitosan was used as supporting material for the immobilization of XOD/HRP enzyme and 4-AAP/TBHBA chromogenic agents onto the prepared detection zones. The detection zones were firstly modified with chitosan solutions at 0.25%, 0.5%, and 1% (m/v) concentrations in acetic acid at 2% (v/v). After the

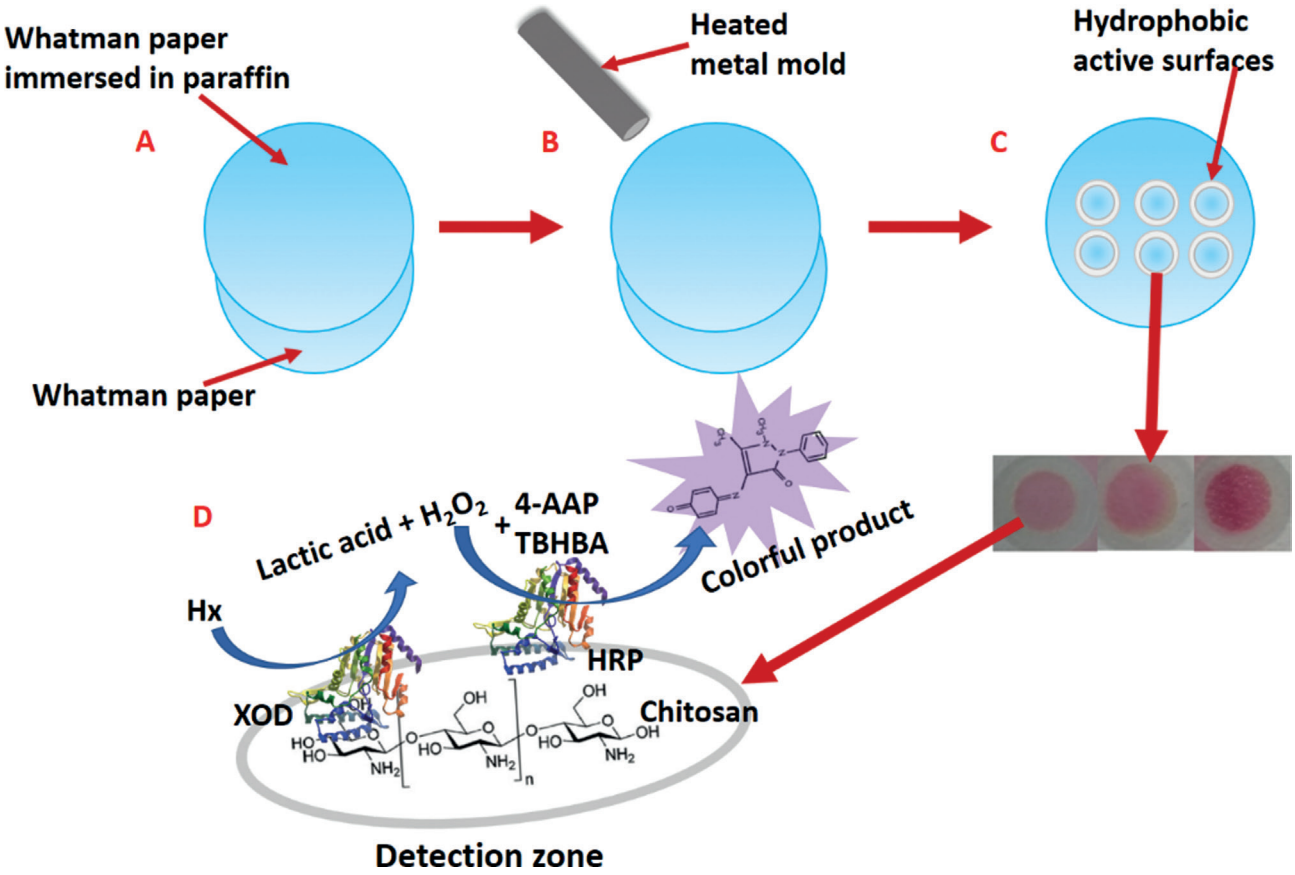

Figure 2. Demonstration of the biosensor system's production steps. (A) to (C); the preparation of the paper-based detection zones. (D); chemical and enzymatic modification of the detection zone is presented.

chitosan modification, the XOD (50 U/mL) and HRP (250 U/mL) enzymes were dissolved 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 conditions. The chromogenic agents were prepared with 0,1 M of 4-AAP and 5 mg/mL of TBHBA in PBS. In Figure 2, D paper-based biosensor system' preparatıon steps were presented by adding all of the reagents sequentially. 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 ready-to-use paper-based biosensor systems were kept at 4°C by protecting them from light contact.

Color Intensity Measurement Procedure: The obtained color change results were analyzed using a smartphone app, 'ON Color Measure,' to process the data with the RGB model [21][22]. The smartphone apps for color intensity analysis is going to be an exciting field on biosensor application that some of the recent works already tested their validation and verification [23]. The paper device's photo was taken directly (without using any workstation) with the same smartphone every time by using the same height (15 cm.) and resolution (4032x2268). After the photo was taken, the RGB values of the detection zones were read using the 'ON Color Measure' app with selecting the mid-point of the spots, as seen in figure 3. In the RGB model, red (R), green (G), and blue (B) light are detected together in several ways to produce an extensive array of colors. The model's name comes from the initials of the three additive primary colors, red, green, and blue. Each color is characterized on a scale from 0 to 225 when 0 signifies no contribution to the color, and 225 signifies the R+G+B components' maximum contribution. Typically, in this model, the white color is indicated as the highest influence of the R+G+B components, and the color black is the minimum value. As the enzymatic reaction generates a color change from a colorless substrate to a colorful product, during the detection, the colorless initial substrate was recognized as the highest value for all of the R+G+B parts, and the created color was characterized as a decrease of this RGB value. Thus, lower substrate (Hx) amount is defined as high RGB values, and higher concentrations formed low RGB values (Figure 3). The values for R+G+B parts were measured to accomplish a precise explanation, and the mean value was used at each experimental part of this work. In the calibration curves, an opposite relationship among mean values of RGB components and Hx amount was observed in buffer solution and fish samples. As seen in figure 3, we prepared each paper-based device with six detection zones and tested them for each experimental part. A repeating number of experiments were 6 for each Hx concentration and other optimization steps.

Biosensor development and performance optimization: After preparation of the detection zones and immobilization of the enzymes+4-AAP/ TBHBA onto them, a

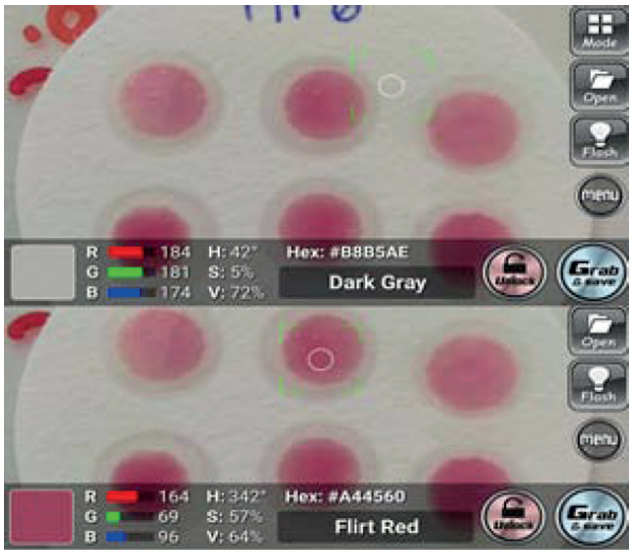

Figure 3. A smartphone app, 'ON Color Measure,' analysis process with the RGB model.

fixed concentration of Hx was injected onto the sensor surface and incubated at different temperatures (room temp., 30°C and 40°C were tested) for different time length (10, 20, 30 and 45 minutes were tested). For the selectivity tests, some frequently presenting species such as glucose, lactic acid(LA), uric acid (UA), and ascorbic acid (AA), which were selected as possible interferences to estimate the selectivity of the prepared multicolor Hx sensor, were analyzed by the developed biosensor system. Afterward, diverse concentrations of Hx solutions were added to the detection zones in optimum conditions to determine the calibration curve and limit of detection of the developed biosensor system. Lastly, to examine the stability feature of the developed biosensor system, the colorimetric results for a Hx solution of 100 μg/ml were recorded over more than 30 days.

Real sample testing: Real fish samples were tested using the optimum performance conditions for the developed biosensor system. The prepared paper systems with immobilized enzymes and chromogenic agents were located onto the fish skin (figure 10). After incubating them at room temperature for 20 minutes, RGB values of the reacted paper biosensor surface were measured with a smartphone app. Using the linear dose response curve, the Hx amount of the fish surfaces was calculated for at least three independent detection zones. In this part of the study, the fish were bought from local grocery stores and stored at 4°C for 2 days before testing of Hx. We aimed to detect the freshness of fish samples stored for a short time after harvesting.

RESULTS AND DISCUSSION

Manufacturing the Paper-Based Biosensor and Optimization of the Parameters: The fabricated detection

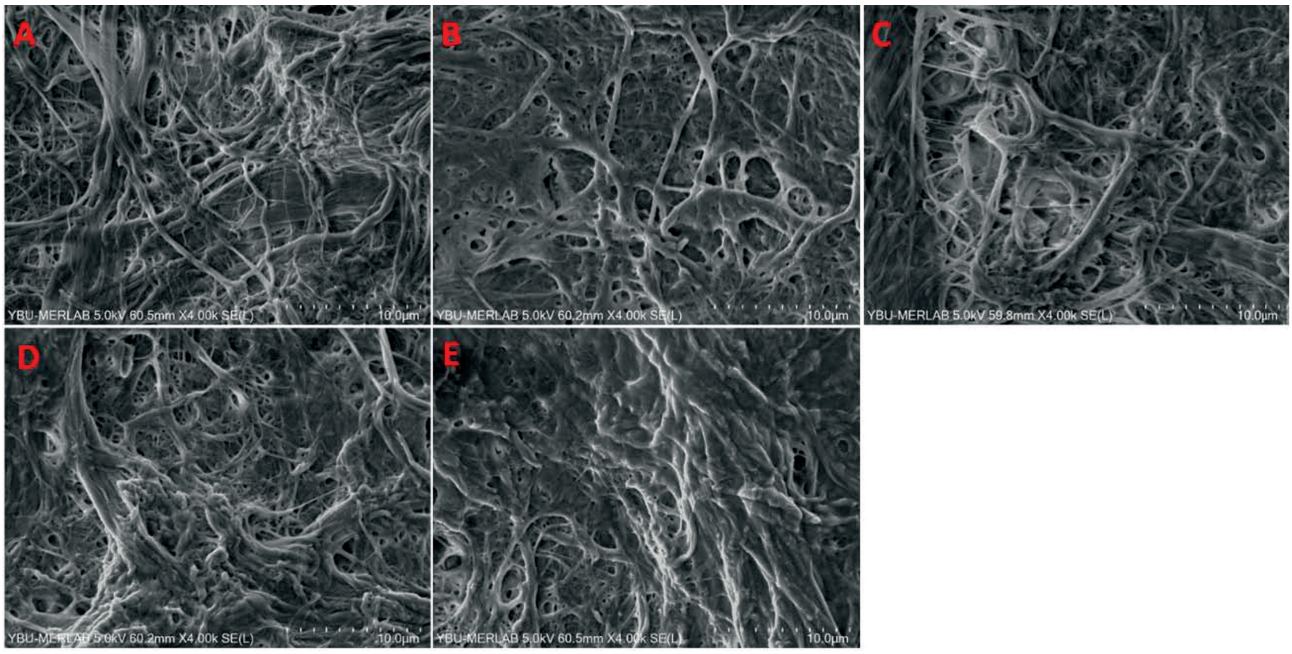

Figure 4. SEM images of modified paper surfaces with different chitosan concentrations; A) Blank paper, B) 0.25%, C) 0.5%, D) 1% and E) enzyme immobilized paper surface with% 1 concentration of chitosan.

zones were covered with chitosan to achieve a suitable immobilization layer for enzymes. The chitosan was used in concentrations between 0.25 and 2% (m/v), which formed a thin covering layer on the cellulose material of paper with a thickness reliant on the chitosan amounts. In figure 4, the morphology obtained for the blank paper surface and chitosan/enzyme immobilized paper-based biosensor system surfaces were demonstrated. Scanning electron microscopy (SEM) images were presented the characteristic porous fibrillary structure of the blank paper (cellulose) surface. A chitosan film covered cellulose fibers by forming the porous areas. It is seen in figure 4 that the most uniform modification was achieved with a 1% concentration of chitosan (Figure 4, D), which is also dense enough to cover the whole paper surface. After the enzyme immobilization onto the chitosan-covered paper surface, we got SEM images of Figure 4, E, representing the successful enzyme entrapping on chitosan layers.

The impact of the chitosan amounts, pH values, and detection temperature over the colorimetric results was examined before catechol calibration. The detections were performed with a fixed concentration of Hx (100 μg/ml), which is within the testing range for Hx in real meat samples [24][25][26]. Figure 5 displays the mean value for RGB components for the same Hx amount under different conditions.

It is seen from figure 4 that when the chitosan concentration increases, the means of RGB values decrease, which represents enhance the activity of Hx's enzymatic reaction. This is because of the modification with higher chitosan concentrations to create a biocompatible environment for the enzymatic assay. Thus, the optimum chitosan percentage for the enzyme immobilization onto the detection zones was concluded as 1%. In addition to the chitosan percentage, 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 (with using the optimum chitosan concentration). As it was estimated, the maximum activity was observed at pH 7.0, which is generally the optimum pH value for biological molecules.

Detection temperature and time length are other essential conditions for the maximum enzymatic activity, 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. The detection rime length was also tested for 10 minutes to 45 minutes. As seen from figure 6, even the maximum activity observed at 37°C, even in the room temperature enzyme, had acceptable activity and closed RGB values to the higher temperatures. Thus we concluded to perform the biosensor system at room temperature, which helps to real-time and on-site detection of real samples. We observed the highest and equal signal with 20, 30, and 45 minutes for the enzymatic reaction time lengths evaluation. Thus we concluded to use the developed biosensor system with 20 minutes reaction time length.

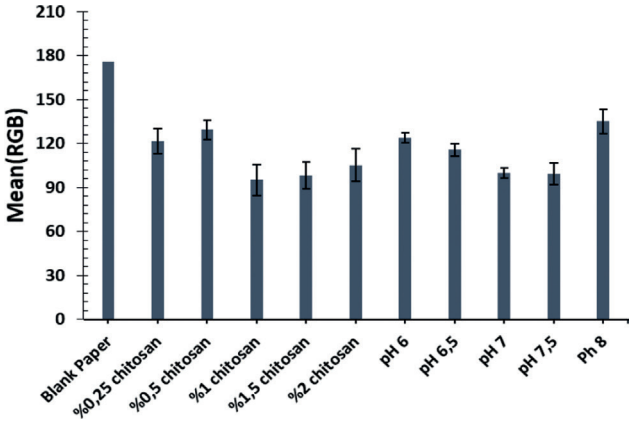

Figure 5. Impact of chitosan amount and pH value of the solutions over the mean value for RGB components of a fixed concentration of 100 µg/ml Hx.

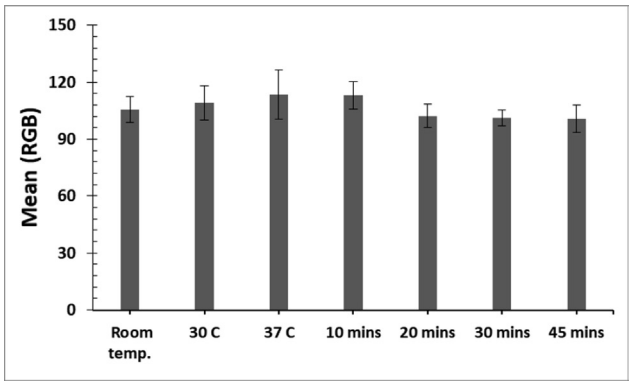

Figure 6. Effect of detection temperature and time length over the mean value for RGB components of a fixed concentration of 100 µg/ml Hx.

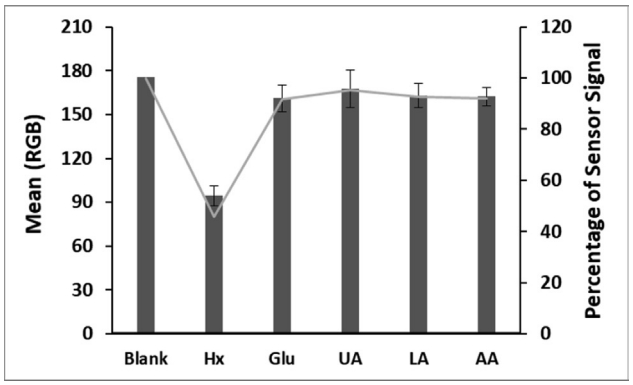

Figure 7. The selectivity test results for the developed biosensor system with mean RGB values for Hx and different biological compounds (each was applied on 100 µg/ml concentrations).

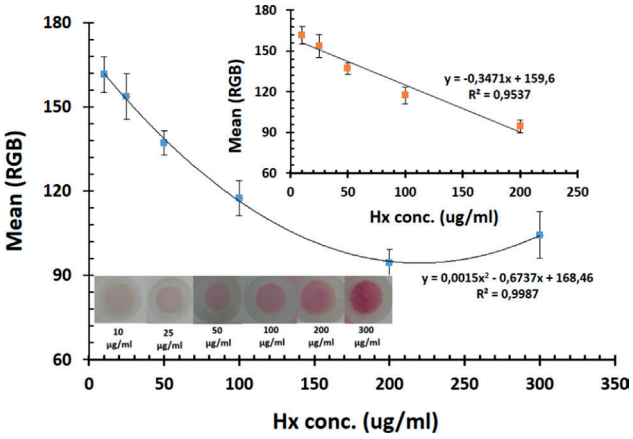

Figure 8. Calibration curve achieved for Hx detection in pH 7.0 PBS solution with inset figures showing the linear range and naked-eye visual scale obtained for Hx detection.

Biosensor performance conditions: After preparation of the detection zones and immobilization of the enzyme and 4-AAP/TBHBA onto the paper surfaces on the optimized conditions, a fixed concentration of Hx and such other related molecules including glucose, lactic acid(LA), uric acid (UA), and ascorbic acid (AA) were added to the zones. In addition to these 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, Hx, glucose, lactic acid(LA), uric acid (UA), and ascorbic acid (AA) generates% of RGB values of 45.7, 91.6, 95.1, 92.7 and 92.2 depends on the blank experiment values (figure 7). The results represent that the developed biosensor system is highly selective for Hx than the other compounds. Thus the developed biosensor system could be used for selective Hx detection on real meat samples.

Afterward, different concentrations of Hx solutions were injected onto the detection zones, prepared with optimized conditions to determine the calibration curve and limit of detection of the developed biosensor system. Figure 8 shows the linear response results (RGB values) achieved for the prepared biosensor system after increasing the Hx amounts from 10 µg/ml to 300 µg/ml with 4.2% to 8.3% of sd (standard deviation) results.

The measured RGB mean values of the developed biosensor syste m stored under optimized conditions were analyzed over 30 days, as presented in Figure 9. The paper-based biosensor systems were prepared with and without immobilization of 4-AAP/TBHBA onto the

detection zones. The stability was performed with storage of the developed paper-based biosensor system at 4°C and in dark conditions by protecting from direct light exposure. It is seen from Figure 9 that immobilization without 4-AAP/TBHBA gave more stable results than immobilization with 4-AAP/TBHBA. This is because of the non-specific reaction between 4-AAP/TBHBA even without H_2O_2 over time. The average deviation for immobilization without 4-AAP/TBHBA was 12,6%, and 29,2% for the immobilization with 4-AAP/TBHBA comparing the measured RGB values for the last day with the initial (first day) values. Thus, we concluded that the paper-based biosensor systems should be prepared without immobilizing 4-AAP/TBHBA during the

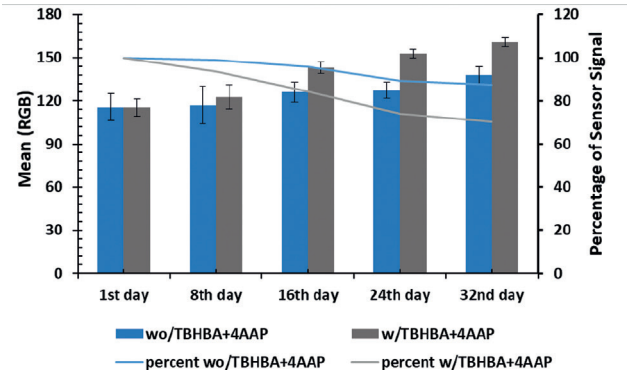

Figure 9. Stability assessment for developed paper-based biosensor system stored at 4°C in dark conditions over 30 days.

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

Sample	Calculated Hx concentration (µg/ml)	% sd
1	8,5	7,47
2	193,8	5,88
3	43,6	7,30
4	112,8	9,96

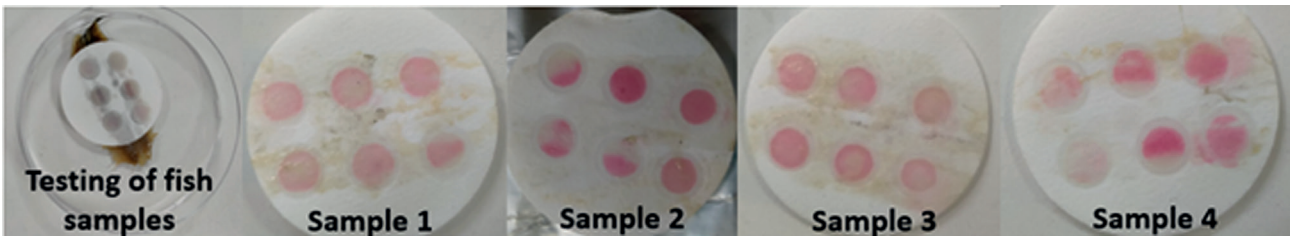

Figure 10. Naked eye detectible color changing for real fist sample testing with developed paper-based biosensor system.

Table 2. Comparison of some analytical characteristics of the developed Hx biosensor with those previously reported.

Detection System	Biosensor system	Detection range	Stability/ Reusability	Real sample testing	Ref.
Colorimetric Sensor	Paper-Based Enzyme Biosensor	4 to 35 µM (5.4 to 47.6 µg/ml)	60 days at -20°C, 10 days at 4°C.	Fish samples without extraction	[11]
Colorimetric Sensor	Microfluidic Paper-Based Biosensor	5–40 µg/ml	4 weeks at 4°C	Different meat samples with extraction	[13]
Electrochemical Sensor	Enzymatic biosensor	6-30 µM (8.2 to 40.8 µg/ml)	NA	Fish samples with extraction	[12]
Electrochemical Sensor	Conductive polymer based enzymatic biosensor	5 µM to 5 mM (6.8 to 6800 µg/ml)	45 days at 4°C	Fish samples with extraction	[27]
Electrochemical Sensor	Polypyrrole film based enzymatic system	25 µM to 10 mM (34 to 13000 µg/ml)	30 days at 4°C	Fish samples with extraction	[28]
Electrochemical Sensor	carbon nanotubes and graphene modified enzyme biosensor	5–50 Mm (6.8 to 68 µg/ml)	NA	Extracted human serum samples	[29]
Paper based colorimetric Sensor	Enzymatic system	10 µg/ml to 300 µg/ml	32 days	Fish sample without extraction	This work

initial preparation process for the long time usage, and the 4-AAP/TBHBA solution should be added to the zones just before the analysis.

Detection of Hx in Real Fish Meat Samples: To demonstrate the paper-based biosensor system's applicability on real samples, the quantitative Hx concentration detection in real fish samples were made. The prepared paper systems with immobilized enzymes and chromogenic agents were located onto the fish skin (figure 10). After incubating them at room temperature for 20 minutes, RGB values of the reacted paper biosensor surface were measured with a smartphone app. Using the linear dose response curve, the Hx amount of the fish surfaces was calculated for at least six independent detection zones. The achieving results for% of sd from 5.88 to 9.96% (for at least three independent experiments) were obtained, as shown in Table 1. Figure 10 also represents the naked eye detectible color changing for real fist sample testing. With these Hx analysis results, we can conclude that all of the fish samples stored at 4 C for 2 days started to generate Hx and spoilage.

To sum up the overall efficiency of the developed biosensor system, compared to other reported works for Hx detection, our results demonstrate better sensitivity, linear detection range, and stability efficiency than most. Additionally, compared to their previous system's cost, the developed paper-based biosensor system serves more efficient usage for real samples over one month of storage (Table 2).

CONCLUSION

In conclusion, during this work, a smartphone-supported paper-based biosensor system was developed for Hx detection in fish samples which is critical for evaluating the freshness level. Specificity, selectivity, stability, and direct real fish sample tests showed the successful application of the developed paper-based biosensor system for Hx with minimal investment and an easy-to-use method. The linear response results achieved for the prepared biosensor system after increasing the Hx amounts from 10 µg/ml to 300 µg/ml with 4.2% to 8.3% of sd (standard deviation) results. An application for examination of Hx concentration in real fish meat was achieved by placing the paper-based biosensor in contact with fish skin. Additionally, we can conclude that this approach can be visually observed with the "naked eye," without exterior analyzers or additional necessities, which is highly convenient for zones with restricted resources. From a future perspective, the developed biosensor system can be applied to fabricate a portable analyzing tool to assess the fish spoilage grade for on-site and real-time detections.

CONFLICT OF INTEREST

No conflict of interest was declared by the authors.

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