A Simple and Cost-effective Microfluidic Paper-Based Biosensor Analytical Device and its Application for Hypoxanthine Detection in Meat Samples

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Abstract

In this paper, we combined lab-on-paper technology with biosensor techniques to fabricate a new analytical tool for hypoxanthine detection. The combination of these two technologies produces a quick, selective, and cost-effective analytical metrology for detection of hypoxanthine in meat samples. The paper-based colorimetric biosensor was developed based on dienzyme catalytic reactions. In presence of hypoxanthine, xanthine oxidase (XOD) catalyzes to form hydrogen peroxide (H2O2). Then H2O2 couples with o-dianisidine in the presence of horseradish peroxidase (HRP), resulting in a brown color. The color intensity of the detection zone on the paper was imaged using a scanner. For quantitative analysis of hypoxanthine, the images of the colorimetric results were analyzed with ImageJ software using a blue histogram. Under optimum conditions, the developed paper-based biosensor was found to detect hypoxanthine with a detection limit of 1.8 mg L−1 and a quantitative limit of 6.1 mg L−1. The proposed assay exhibited a linear dynamic in the range of 5–40 mg L−1. The analysis time was 5 min for triplicate measurement. This method was further evaluated by measuring the recovery of hypoxanthine added to meat samples. Finally, this method was applied to detect hypoxanthine in fresh and processed meat samples, and the results were validated against spectrophotometric detection, showing good accuracy. This simple method is cost-effective and requires no advanced instruments, offering an alternative to conventional methods.

Keywords: Microfluidic paper-based analytical device, Biosensor, Hypoxanthine, Meat sample.

Introduction

Meat freshness is an indicator of product quality. After fish die, adenosine triphosphate degrades into adenosine di-phosphate, adenosine 5′phosphate, inosine 5′ phosphate, inosine, hypoxanthine, xanthine, and uric acid, respectively (Andrade et al. 2014; Lougovois et al. 2003; Mora et al. 2010). Hypoxanthine imparts a bitter "off" taste, and this compound increases with prolonged storage (Devi et al. 2013; Hernández-Cázares et al. 2010; Lawal and Adeloju 2012a). When hypoxanthine concentration exceeds 102 mg kg−1, the fish begins to deteriorate, and when it exceeds 144 mg kg−1, the fish is completely deteriorated (Chen et al. 2017). Thus, hypoxanthine concentration detection has been used to evaluate fish freshness in the food industry. The quantification of hypoxanthine in food samples is mainly performed using complex instruments, such as high-performance liquid chromatography (Czauderna and Kowalczyk 2000; Farthing et al. 2007; Mei et al. 1996), spectrophotometric (Roberts et al. 1991; Giacomello and Salernot 1977; Johnson et al. 1997), and electrochemistry techniques (Hu et al. 2000; Si et al. 2018; Wang et al. 2012). Classical high-performance liquid chromatography has been widely used to detect hypoxan-thine concentration in various types of food samples. Although high-performance liquid chromatography pro-vides high sensitivity, it requires expensive instruments, is time consuming, requires sample pretreatment, and relies on highly skilled personnel. Such equipments constitute major disadvantages. A simple, cost-effective, and selec-tive hypoxanthine detection method has considerable value to control the quality

of meat. Biosensor techniques offer a rapid, selective, and cost-effective analytical method (Caygill et al. 2010; Mehrotra 2016; Roda et al. 2016). Biosensors have been developed for various uses in diagnostic (Metkar and Girigoswami 2019; Rebelo et al. 2019), the food industry (Lv et al. 2018; Ye et al. 2019), and the environment (Zai-Hui et al. 2018; He et al. 2018; Peng et al. 2019). In recent years, biosensors have been used extensively to monitor hypoxanthine for meat quantity evaluation. The most significant electrochemical platforms used for hypoxanthine detection include the voltammetric (Pierini et al. 2018), amperometric (Gonzalez et al. 1991), and potentiometric (Lawal and Adeloju 2012b) types. Until now, there have been few reports about colorimetric biosensors that can achieve hypoxanthine detection, such as those based on the etching of gold nanorods in the pres-ence of xanthine oxidase (XOD) and Fe2+ (Chen et al. 2017), which results in a color change, that can be observed with the naked eye. Lab-on-paper or microfluidic paper-based analytical devices (μPADs) offer great potential for analytical tools due to their low cost, low sample and re-agent consumption, easy fabrication, and good probity (Granica et al. 2018; Zhu et al. 2019). To date, there have been no reports about the use of μPADs for hypoxanthine analysis in meat samples.

For this reason, this work presents a new paper-based bio-sensor (μPAD biosensor) for hypoxanthine detection in meat samples (Fig. 1). The concept for the design of colorimetric μPAD biosensor for hypoxanthine analysis is based on the catalysis of hypoxanthine by XOD into hydrogen peroxide (H2O2) as shown in Eq. 1.

$$\textit{Hypoxanthine} + O_2 + H_2O \xrightarrow{\text{XOD}} \text{Uric acid} + H_2O_2 \qquad (1)$$

The product of the first reaction, H2O2 reacts with o-dianisidine by horseradish peroxidase (HRP) in the second reaction to produce a brown color (Eq. 2).

$$\underset{\text{(Colorless)}}{H_2O_2 + \text{Reduced o-dianisidine}} \xrightarrow{\text{HRP}} \underset{\text{(Brown color)}}{\text{Oxidized o-dianisidine}} + H_2O \qquad (2)$$

The color reaction of hypoxanthine is introduced on the μPAD biosensor. The resulting change in color on the μPAD biosensor is subsequently imaged using a scanner, followed by analysis with ImageJ software using a blue histogram to get mean color intensity. The μPAD biosen-sor's characteristics, including precision, accuracy, sensi-tivity, linear range, detection limit (LOD), quantification limit (LOQ), selectivity, and stability, are then studied. Finally, the developed μPAD biosensor is used to detect hypoxanthine concentrations in meat samples, and the an-alytical results are compared with a reference method, such as a spectrophotometry assay.

Experiment

Reagents and Materials

All chemicals used in this study were of analytical grade. Hypoxanthine, XOD (EC 1.17.3.2, 1.0–2.0 units/mg), HRP (EC 1.11.1.7, 250–330 units/mg), and o-dianisidine were ob-tained from Sigma-Aldrich (St. Louis, MO, USA). Sodium dihydrogen orthophosphate and disodium hydrogen ortho-phosphate were obtained from Ajax Finechem (Seven Hills, New South Wales, Australia). Polylactic acid was supplied by Nature Work (Blair, NE, USA). Dichloromethane was pur-chased from RCI Labscan (Pathumwan, Bangkok, Thailand). Patterned screening block was obtained from Chaiyaboon Brothers Co., Ltd. (Lam Luk Ka, Pathum Thani, Thailand). Whatman chromatography paper No. 4 (25 × 25 cm pure cellulose paper) was purchased from GE Healthcare (Little, Buckinghamshire, UK).

Apparatus

A Canoscan lide 120 scanner was supplied by Canon (Ha Noi, Vietnam), and a UV-1601 UV-visible spectrophotometer was obtained from Shimadzu (Kyoto, Japan). A VCX 130 PB Vibra-cell ultrasonic homogenizer was purchased from Sonics & Materials Inc. (Newtown, CT, USA).

Fabrication of the μPAD Biosensor

The fabrication process of the μPAD biosensor is shown in Fig. 1. The pattern was first designed using Adobe Illustrator. Figure 1 a shows a paper-based pattern. The device is tree shaped and consists of two separate zones: a sample zone and a detection zone in triplicate, which was used to create the patterned screen. The screen was made from a 100-mesh polyester fabric on a wooden frame. The patterned screen was placed onto a sheet of chromatogra-phy paper. After that, 10 mL of 10% (w/v) polylactic acid solution was applied over the screen. The patterned paper was ready to use after drying.

Colorimetric Assay of Hypoxanthine on the μPAD Biosensor

The colorimetric assay of hypoxanthine on the μPAD bio-sensor is shown in Fig. 1b. Hypoxanthine was measured with the created μPAD biosensor. Briefly, 25 μL of stan-dard solution or sample was dropped onto the sample zone of the μPAD biosensor. After that, 3 μL of 0.5 mg mL−1 XOD was dropped onto the detection zone to react with hypoxanthine for 2 min. Next, 3 μL of a mixed solution of containing 0.01 mg mL−1 of HRP and 3 mM of o-dianisidine (1:1) was dropped onto the detection zone and incubated for 3 min. Subsequently, a brown solution was obtained on the detection zone. The color changes on the paper were measured by a scanner and analyzed using ImageJ to obtain intensity value. Briefly, μPADs were scanned and the image was 2550 × 3507 pixels and was saved as a TIFF image. Then the JPEG image was opened with ImageJ software. Then the second step was done by selecting a circular icon for selecting the reader area of detection zone on the μPAD which set the specific position in circular (90 width × 90 height). Next step, the cropped images were analyzed to obtain the RGB channel intensi-ties. The mean value in blue channel of histogram was measured. The mean intensity value of each detection zone was obtained by subtracting the intensity from that of the background. Then these values were used for construction of calibration standard curve or calculation of hypoxan-thine concentration in real samples.

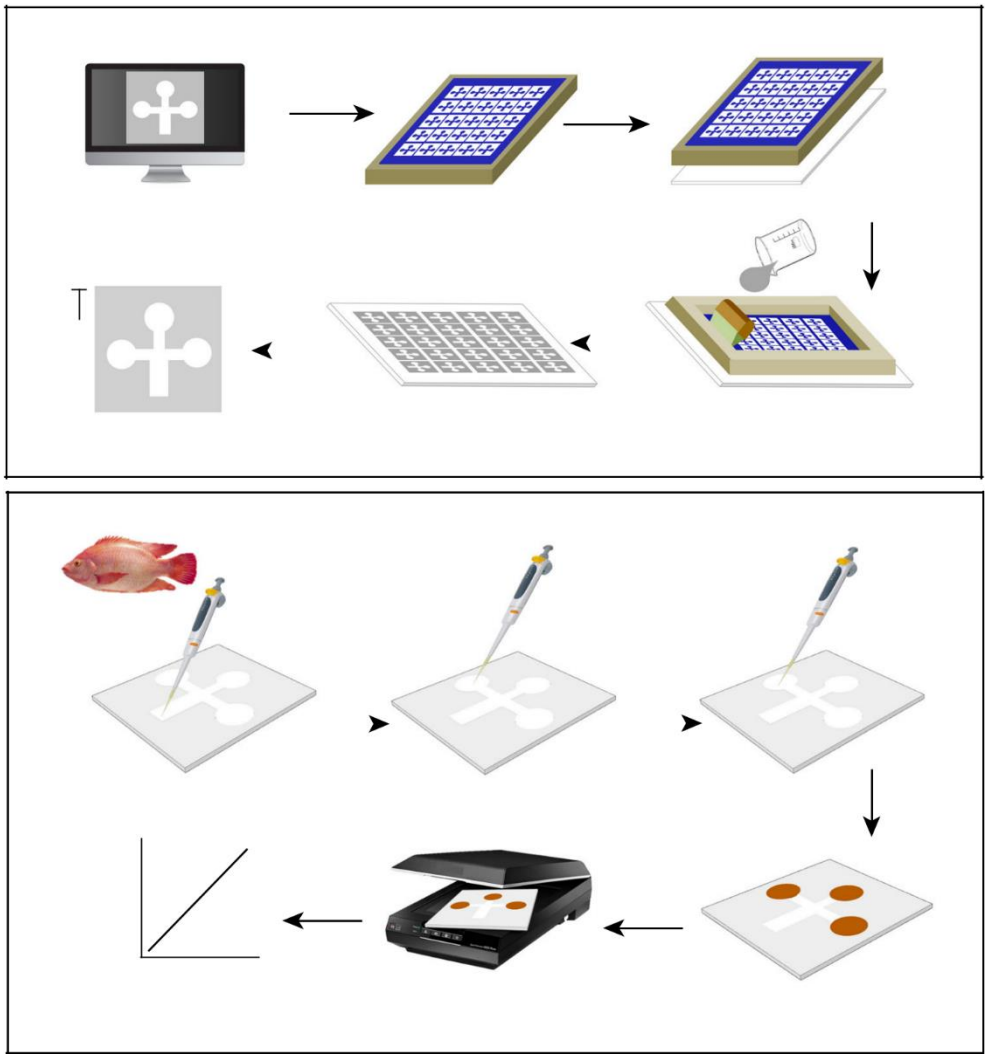

Fig. 1 a Schematic diagram of screen-printing for fabrication of μPAD biosensor and b schematic diagram of colorimetric assay of hypoxanthine on a μPAD biosensor

Optimization of Hypoxanthine μPAD Biosensor

To evaluate the optimum conditions of the μPAD biosensor, XOD and HRP concentration, pH value of XOD and HRP, reaction time, and concentration of o-dianisidine were studied to obtain high analytical signals. Only the investigated param-eter was varied; all other parameters were fixed for detection of hypoxanthine 100 mg L−1.

Evaluation of Analytical Performance of μPAD Biosensor

The following parameters were studied to evaluate the μPAD biosensor's performance: precision, accuracy, linear range, detection limit, limit of quantification, selectivity, and stability of the μPAD biosensor.

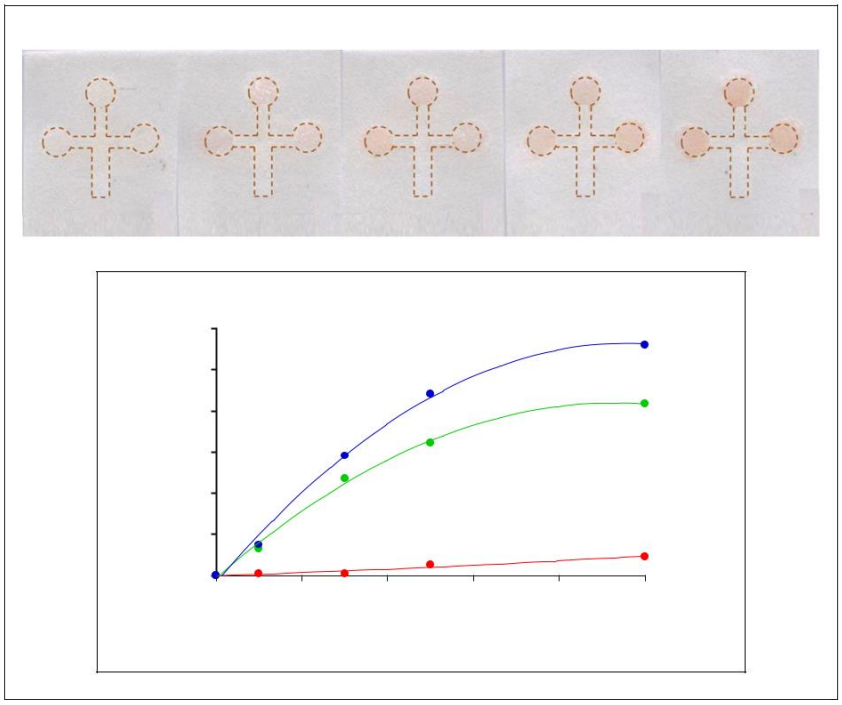

Fig. 2 a Color change in the μPAD biosensor using various concentrations of hypoxanthine from 0 to 100 mg L−1 and b RGB intensity values results

Analysis of Hypoxanthine in Meat Samples

To validate the proposed method, the developed μPAD biosensor was used to detect hypoxanthine in nine meat samples including fresh and processed meat such as pork, chicken, fish meat, and fish sauce. The extraction method was carried out according to the procedure described in Devi et al. (2013). All solid meat samples (1 g of each sample) were minced, mixed with 3 mL of chilled dis-tilled water, and homogenized using an ultrasonic homog-enizer for 10 min. The homogenous mixture was diluted with 7 mL chilled distilled water. The solution was then centrifuged, and supernatant was filtered using 0.45 μm of filter paper for further analysis of hypoxanthine using the μPAD biosensor. In the case of fish sauce, the sample was only diluted with 0.1 M phosphate buffer with a pH of 7.00.

Colorimetric Assay of Hypoxanthine Using Spectrophotometric Method as a Reference Method

To detect hypoxanthine using the colorimetric spectrophoto-metric method, 30 μL of extracted sample solution was added to a microcentrifuge tube. Then, a mixture of 30 μL of 0.5 mg mL−1 XOD, 15 μL of 0.01 mg mL−1 HRP, and 15 μL of 3 mM o-dianisidine was added to the above solution and incubated for 3 min. The absorption of this solution was then measured at 430 nm wavelength using a UV-visible spectrophotometer.

Results and Discussion

Response of the μPAD Biosensor During Hypoxanthine Detection

Hypoxanthine detection was performed using the μPAD biosensor. The hypoxanthine standard solution was dropped onto the sample zone and reached the detection zone by capillary penetration, resulting in enzymatic reac-tions. In the presence of water and oxygen, hypoxanthine

reacts with XOD to product H2O2 in the detection zone. The H2O2 then reduces o-dianisidine to oxidized o-dianisidine by reacting with HRP, resulting in a color change in the detection zone from colorless to brown within 5 min. As can be seen in Fig. 2a, the gradual increase in color intensity corresponds to the increasing hypoxanthine concentration. Figure 2b shows the results of RGB values. It is seen that the blue intensity analysis was the best sensitive among the RGB color. Thus, a blue histogram was used for analysis of color intensity value.

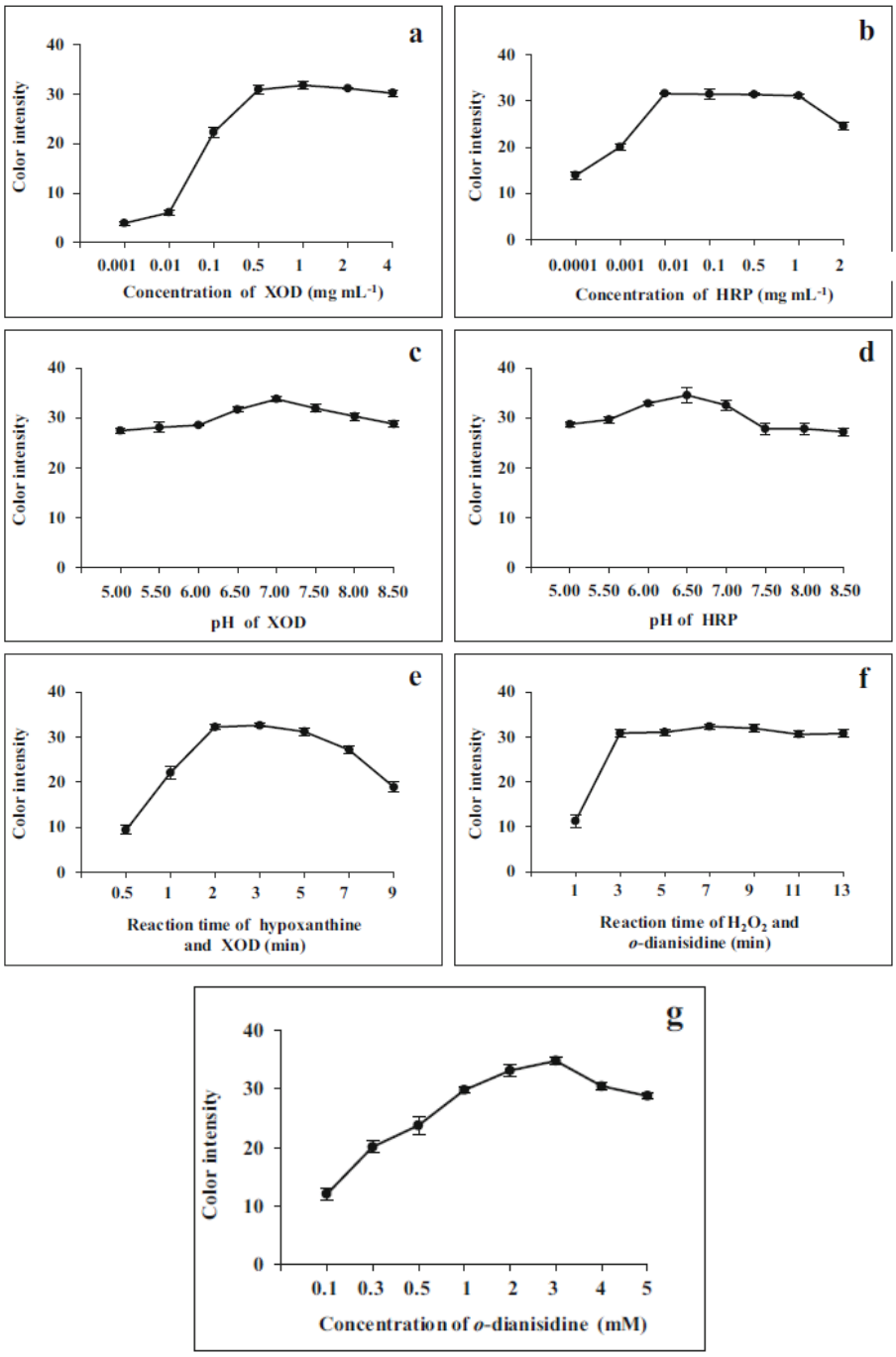

Fig. 3 Optimum conditions of hypoxanthine detection using the μPAD biosensor device. a Concentration of XOD. b Concentration of HRP. c Effect of pH value of XOD. d Effect of pH value of HRP. e Reaction time of hypoxanthine reacting with XOD. f Reaction time of H2O2 reacting with o-dianisidine. g Concentration of o-dianisidine

Optimum Hypoxanthine Detection Conditions Using the µPAD Biosensor

Effects of XOD and HRP Concentrations

Because XOD and HRP concentrations may affect biosensor performance, the effects of XOD and HRP were examined in the range of 0.001 to 4 mg mL−1 and 0.0001 to 2 mg mL−1, respectively. As seen in Fig. 3a, when XOD concentration increased from 0.001 to 0.5 mg L−1, the biosensor's response increased due to increasing enzymatic reactions. However, XOD concentration higher than 0.5 mg L−1 yielded no signif-icant response change because all substrate molecules were bound to enzymes. As such, an optimized XOD concentration of 0.5 mg mL−1 was selected to achieve the best response. The optimization of HRP concentration was also investigated, and the results are shown in Fig. 3b. Color intensity increased with increased HRP concentration, but no significant change was obtained when the concentration of HRP exceeded 0.01 mg mL−1. Hence, the concentration of 0.01 mg mL−1 HRP was chosen as optimal.

Effects of pH Value of XOD and HRP

The value of pH affects the function of the enzyme. Therefore, the pH value of XOD was investigated by using 0.1 M phos-phate buffer ranging from 5.00 to 8.50. The results shown in Fig. 3c indicate that color intensity decreases from pH 7.00 to 8.50. Thus, pH 7.00 was used as the optimal pH value for hypoxanthine detection. The effect of pH value on HRP ac-tivity was also optimized by investigating pH ranges from 5.00 to 8.50 in 0.1 M phosphate buffer. As shown in Fig. 3d, the intensity increased with increased pH value. The color intensity reached peak value at pH 6.50. Hence, the pH of HRP was set at 6.50.

Reaction Time

Reaction time is an important factor that influences the reac-tion of Eqs. 1 and 2. Therefore, reaction times were studied. Figure 3e shows the results of reaction time during the hydro-lysis of hypoxanthine when catalyzed by XOD to generate H2O2, the substrate of the second reaction. Color intensity increased as the reaction time increased from 1 to 2 min and decreased as the reaction time increased from 2 to 9 min. Thus, 2 min was chosen as the optimum reaction time between hypoxanthine and XOD. The H2O2 generated from the first reaction is used to oxidize o-dianisidine in secondary reaction with HRP, resulting in a color change to brown. Therefore, the reaction time of this reaction was optimized. In Fig. 3f, color intensity reached maximum at 3 min and remained constant from 3 to 13 min. Thus, 3 min was selected as the reaction time for the catalysis reaction of HRP, as shown in Eq. 2.

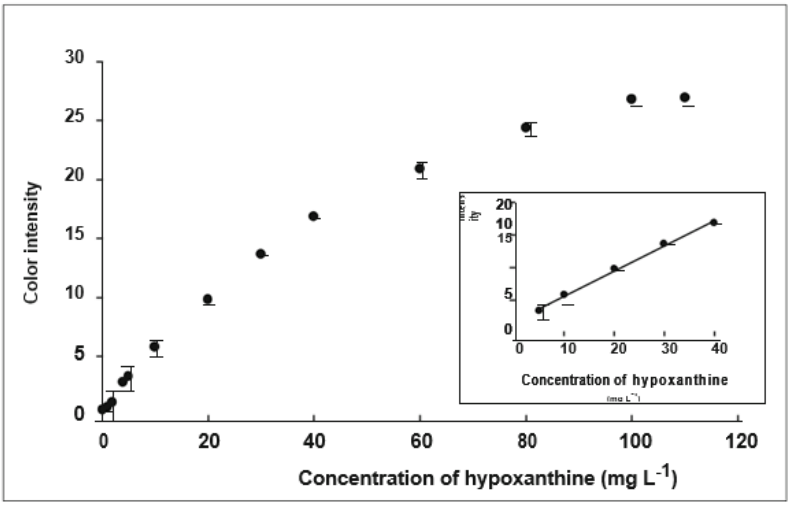

Fig. 4 Calibration curve for hypoxanthine under optimal conditions. Inset: Linear range of the calibration curve for 5–40 mg L−1

Concentration of o-Dianisidine

The concentration of o-dianisidine has a significant influence on color reaction. Thus, the concentration of o-dianisidine was optimized, and the results are shown in Fig. 3g. From Fig. 3g, it is observed that the highest color signal was obtained at 3 mM. Therefore, 3 mM of o-dianisidine was used for the next experiment.

Table 1 Recovery of hypoxanthine in meat samples using the μPAD biosensor

Sample	Concentration of hypoxanthine (mg L^{-1})			Recovery (%)
	Original	Spiked	Found	
Pork	8.45 ± 0.11	15	22.92 ± 0.37	96.5 ± 2.5
		20	27.05 ± 0.50	93.0 ± 2.5
Nile tilapia fish	0	15	15.71 ± 0.65	104.7 ± 4.3
		20	20.41 ± 0.75	102.0 ± 3.8
Chicken	10.23 ± 0.38	15	25.24 ± 0.50	100.1 ± 3.3
		20	29.16 ± 0.96	94.6 ± 4.8
Fish sauce	6.22 ± 0.88	15	20.55 ± 0.56	95.5 ± 3.8
		20	24.06 ± 0.38	89.2 ± 1.9

Analytical Performance of the μPAD Biosensor

The performance of the μPAD biosensor was verified using optimum conditions based on the above parameters. The developed μPAD biosensor exhibited a linear range of 5–40 mg L−1, as shown in Fig. 4. The linear regression equation was y = 0.3869x + 1.749 with a correlation coef-ficient of 0.9957. A low detection limit of 1.8 mg L−1 was evaluated using a signal-to-noise ratio of 3, which was corresponding to 18 mg kg−1 in the meat sample (Nardo et al. 2016). The quantification limit was 6.1 mg L−1 with an acceptable precision (% RSD = 4.75) and accuracy (% recovery = 95.8) (Taverniers et al. 2004). To evaluate the precision of the μPAD biosensor, intra-μPAD biosensor repeatability and inter-μPAD biosensor reproducibility were studied using the same concentration of hypoxanthine (50 mg L−1) expressed as RSD (n = 7). The results showed that % RSD repeatability and reproducibility were 3.5 and 4.1, respectively, indicating good precision (Taverniers et al. 2004). The accuracy of the μPAD biosensor was evaluated through a recovery test. Four real samples (pork, fish, chicken, and fish sauce) were spiked with three dif-ferent concentrations of hypoxanthine. As shown in Table 1, recovery values ranged between 89.2 and 104.7%, indicating good accuracy of the developed μPAD biosensor (Taverniers et al. 2004). These results showed the capability of the developed μPAD biosensor to detect hypoxanthine in real samples without clean-up and pre-concentration sample steps.

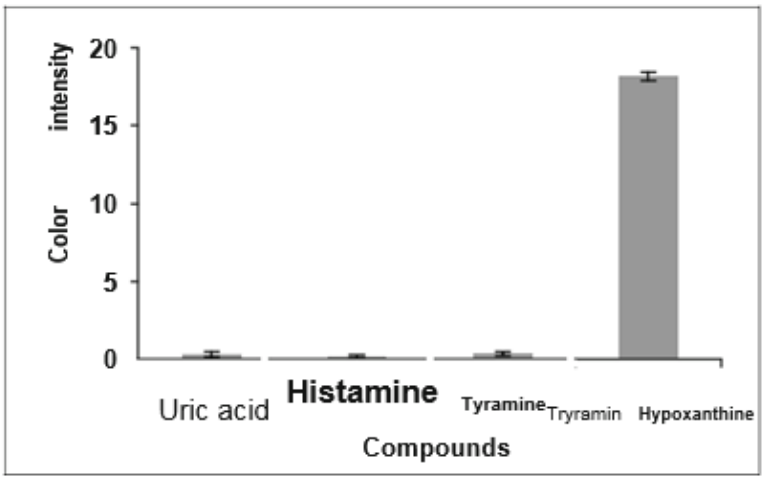

Fig. 5 Selectivity test with uric acid, histamine, and tyramine (50 mg L−1) using the developed µPAD biosensor.

The Selectivity and Stability of the µPAD Biosensor

The selectivity of the µPAD biosensor toward hypoxan-thine was studied by testing with uric acid, histamine, and tyramine in the same concentration as hypoxanthine (50 mg L−1). Minimal color intensity was observed with uric acid, histamine, and tyramine, but significant color intensity was shown with hypoxanthine (Fig. 5). These results indicated very good selectivity for the analytical device even in the presence of other compounds in real samples. The long-time stability of µPAD biosensor was also studied. The µPAD biosensor was stored at 4 °C in a refrigerator and tested every week by measuring the re-sponse to 50 mg L−1 hypoxanthine. The long-term stability of this µPAD biosensor showed that the response yielded about 96% of the initial response after 2 weeks. The reduc-tion in response may be caused to the loss of enzyme ac-tivity of XOD. After 4 weeks, the µPAD biosensor re-sponse still retains 89% which may due to the short life time of XOD (Albelda et al. 2017).

Detection of Hypoxanthine in Meat Samples

To test the performance of µPAD biosensor in real sample applications, nine meat samples were chosen and analytical results were compared with that spectrophotometric meth-od. All analytical results of two methods were tested with a linear regression and statistic t test. Figure 6 shows the results that an excellent correlation coefficient (r = 0.9983) was obtained between the hypoxanthine concen-trations by these two methods. The mean values of the two methods were also compared using a statistic t test at 95% confidence. The results showed that calculated t values for all samples were less than the t critical value, so it can be concluded that there is no statistically significant difference between the methodologies. These observations expressed accuracy of the present µPAD biosensor. Moreover, Nile tilapia fish (Oreochromis niloticus) was chosen as the sample to test the performance of the devel-oped µPAD biosensor for monitoring fish freshness. After the death of the fish, the amount of hypoxanthine in-creased, affecting the quality of the fish. Thus, the devel-oped µPAD biosensor was used to detect hypoxanthine concentration in Nile tilapia fish at various times ranging from 1 to 20 h after death. Hypoxanthine level increased as the storage time increased, as shown in Fig. 7. After 20 h, hypoxanthine concentration was very high, meaning that the Nile tilapia was completely deteriorated. These results showed that the developed µPAD biosensor has potential applications for evaluating meat freshness.

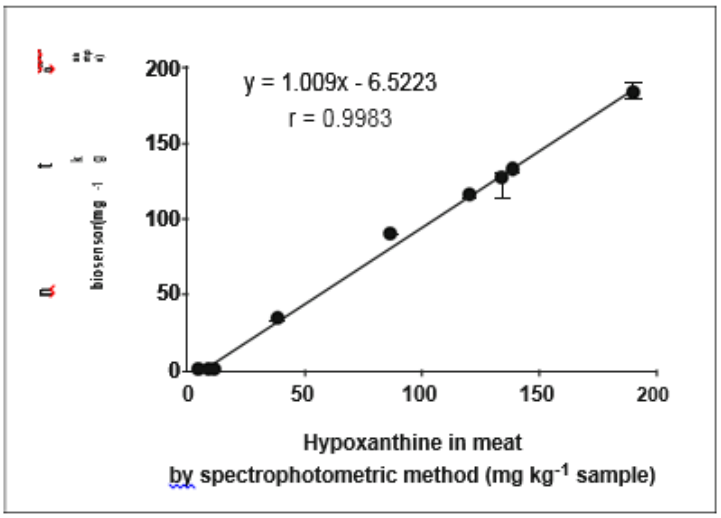

Fig. 6 Correlation curve between hypoxanthine concentrations as detected by standard spectrophotometric (X-axis) method and the present μPAD biosensor r (Y-axis)

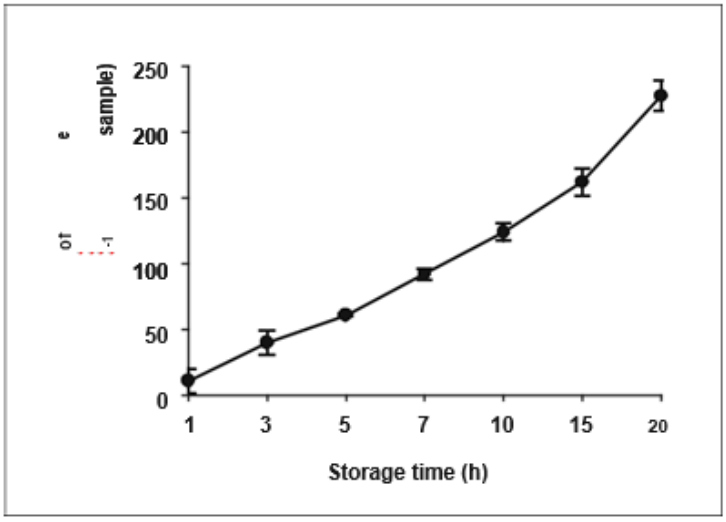

Fig. 7 Detection of hypoxanthine in Nile tilapia fish sample during storage for 20 h at room temperature

Conclusion

In conclusion, we have successfully developed a new col-orimetric biosensor method and microfluidic paper-based analytical device to create a simple, selective, and cost-effective μPAD biosensor for hypoxanthine detection in meat samples. This colorimetric μPAD biosensor was based on multienzyme reactions on paper that produced a brown color. Color intensity was recorded using a scan-ner and exported to ImageJ software to create average intensity. The μPAD biosensor was found to be highly selective toward hypoxanthine and shows 89.2–104.7% recovery of hypoxanthine from meat samples. The limit of detection of the proposed μPAD biosensor for hypo-xanthine detection was 1.8 mg L−1, which is lower than the previous works (Nguyen and Luong 1993). A test using our μPAD biosensor, which can measure hypoxan-thine samples, requires just 5 min to complete and re-quires no special equipment. Thus, this μPAD biosensor provides a rapid, cheap, and simple method of μ analysis.

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