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A Dip Test Strip Based on Immobilized Pararosaniline for Detection of Formalin Adulteration in Tofu

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Abstract. A sensitive dip test strip for detection of formalin (HCHO) in tofu samples based on immobilized pararosaniline (PR) has been developed. The test strip has been fabricated by immobilizing PR reagent on filter paper. By using image color analysis (ImageJ) as the average of ΔRGB value as color sensor response, the analytical parameters are obtained from an area of the sensing zone of the test strip using digital output of the color scanner. By using this scanometric method, it is possible to determine HCHO as low as 0.05 mg/L with the concentration range of 2-80 mg/L and the response time of 3 minutes. The reproducibility was found to be 8.04% (RSD) for good recovery. The test strip has been successfully applied to detect formaldehyde in tofu samples, and the results show in good agreement with the Nash method using UV/vis spectrophotometry.

INTRODUCTION

Currently, food adulteration and contamination are significant problems in many countries, particularly in the developing countries [1, 2]. This is due to the fact that in many developing countries, there is a lack of strong regulatory controls, weak infrastructure for food transport, storage and refrigeration as well as increasing consumer demand for fresh food products that have led to an increase in fraudulent practices to increase shelf-life of food products. Food adulteration can have a detrimental impact on the human health, as adulterants can lead to developmental defects, chronic diseases, or even death. Particularly, children are more vulnerable to adulterated and unsafe foods that cause a major mortality of child [3].

Formaldehyde (HCHO) is the simplest member of aldehyde family but a very reactive chemical, where the gaseous form is known as formaldehyde at ambient temperature and a common air pollutant. In its liquid form, formaldehyde known as formalin (35–40% aqueous solution stabilized with methanol) [4]. It is widely used in the manufacture of household products such as paint, furniture laminates and cleaning fluids. It is a proven carcinogen and, therefore, detrimental to public health [5]. Therefore, the use of formalin in food for human consumption is banned around the world, including Indonesia [6]. However, in Indonesia and South-East Asian countries, formalin has been reported to be added fraudulently to foods to extend shelf-life [6, 7]. Therefore, it is necessary to have a method for detection and monitoring of the level of formalin in food as an adulterant.

The official method for the determination of formalin in foodstuffs is based on a colorimetric reaction where sample distillates are mixed with sulfuric acid yielding a purple color if formaldehyde is present. The intensity of the color is proportional to formaldehyde concentration and can be measured by UV spectrophotometer [8]. However, conventional colorimetric methods [9, 10] for the qualitative and quantitative analyses of formalin in food is laborious, since it used wet chemistry where many reagent solutions are involved. In addition, titrations and acetylcholine have been used to detect and quantify relatively the presence of formaldehyde in foods [11, 12]. A pulsed amperometric method [13] and an electronic nose [14] have been reported as alternatives to the conventional methods for the detection of formalin adulteration in seafood. A review of methods for the determination of formalin

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in the diet has recently been published [15]. Currently, a colorimetric-based kit is being used to detect adulteration with formalin [4, 7]. Besides laborious, the drawbacks of this and other colorimetric methods are their poor specificity, selectivity, prolonged analysis times and highly acidic conditions, which together lead to over-reporting as well as false positives [1, 6]. In our view, the most practical and effective technique for detection of formalin in food is a method based on pararosaniline (PR), since PR is one of the specific reagents to detect formalin [17-19].

In this work, a dip test strip based on immobilized PR was developed for the analysis of formalin in tofu, since in many cases of formalin adulteration was found in tofu as one of the most favorite food in Indonesia [6]. Herein, a new method has been designed based on a scanometric method that allows for quantitative measurement of formalin in tofu directly using a dip test strip, without sample preparation and treatment to achieve a considerable improvement in the field applications. Thus, the dip test strip platform became a simple method for detection of harmful organic compounds, such as formalin in food.

MATERIAL AND METHOD

Chemical and Material

All reagents were used as purchased without further purification. Pararosaniline hydrochloride was supplied from Sigma (UK). Hydrochloric acid (HCl 37% pa), sulfuric acid solution (H_2SO_4) and methanol (96%), were obtained from BDH-Merck (UK). A standardized stock solution of HCHO (0.1 M) was prepared by diluting 0.8 ml of 37.5% HCHO (Merck, UK) to 100 ml with water, and was stored in a refrigerator [20]. A calibration curve was prepared at eleven concentrations: 1, 2, 4, 8, 10, 20, 30, 40, 50, 80 and 100 mg/L using double distilled water. The Pararosaniline reagent (PR) was prepared by solving 30 mg of PR in 10 ml water to make concentration 3000 mg/L. A similar procedure was applied for other concentrations of the PR reagent. Then, the reagent was acidified by adding 2 ml of acid mixture, consist of 1:1 volume ratio of HCl (4 M) and H_2SO_4 (1 M). All reagents and inorganic salts were of analytical grade and made using double distillate water.

Strip Preparation

To prepare the test strip, the white opaque acrylic sheet was cut into little sheets (5.0 x 0.5cm with ± 0.1 cm thickness), and each sheet had detection zone of 0.5 x 0.5 cm (Fig. 1). Then, a piece of transparency double tape (3 M) was attached to the detection zone. The immobilized PR on filter paper (CAT No.1095.093, Merck, UK) as a sensitive membrane for HCHO was then placed in the detection zone of 0.5 x 0.5 cm. The immobilization of PR was performed on the clean filter paper using adsorption method. The PR reagent was transferred onto the filter paper (0.5 x 0.5 cm) by added drop wise into the paper and dried for 30 minutes at ambient temperature to obtain the PR membrane. Afterward, the membrane was stuck gently onto the sensing zone to construct HCHO sensor according to the design as a test strip (Fig. 1). Finally, the test strip was ready to be used as a HCHO sensor or stored in a dry container for further use.

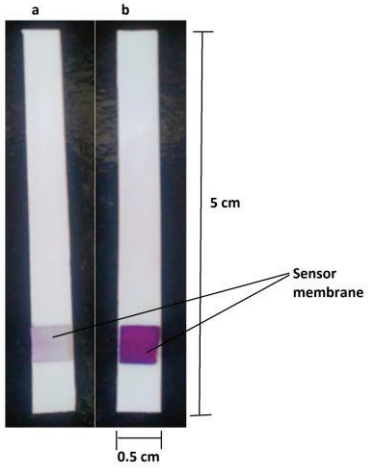


FIGURE 1. The design of the dip test strip for formalin detection in tofu; (a) before and (b) after reaction with HCHO

Optimization of the Test Strip

The optimization study of the sensor reagent was performed on the concentration of PR used, volume of reagent transferred and pH of the sample tested. Here, the fresh solutions of the acidified PR reagent with the solution mixture of HCl and H_2SO_4 at various concentrations (1000-5000 mg/L) were tested towards 30 mg/L HCHO. The various volumes (2-6 µL) of transferred reagents were tested on the sensing membrane (0.5 x 0.5 cm with ± 5 mm thickness). The pH of the sample tested was also performed in acid pH (3 – 6) using acetate buffer by mixing between 0.1 M acetic acid and 0.1 M sodium acetate in the desired proportions to get the required pH, since mostly tofu sales in the acid condition in order to help coagulation process of tofu using acetic acid [21, 22].

Sample Preparation

A tofu sample was cut into small pieces (4 to 5 mm), accurately weighed (12.5 g) and placed into a 50 ml centrifuge tube containing 25.00 ml of water, the extraction solvent. The extraction of HCHO from food sample was adopted from a method of Wang *et al.* [23]. Sample tubes were capped and shaken at 240 rpm for 15 min (IKA Labortechnik, Germany) to extract the HCHO.

Measurement Procedure

After the introduction of HCHO solution in the tofu sample to the sensing zone of the test strip, the color change was captured by image scanned using a flatbed scanner (CanoScan, LIDE 110, Japan) at 300 dpi resolution. The scanned images were then analyzed with ImageJ program (https://imagej.nih.gov/ij/). Herein, the color intensity values of sensors were given as Δ mean RGB, by subtracting the intensity value of mean red, green, and blue (RGB) after the application of sample from the intensity value of mean RGB without the sample (control). All of the experiments were carried out in triplicate.

Statistical Analysis

The detection of HCHO on the tofu samples were compared with the values measured by the Nash method [24], using one-way ANOVA test [25]. The Nash method is based on a reaction between HCHO and a diketone in the presence of ammonium acetate and the reaction is also known as the Hantzsch reaction, and measurement was performed using UV/vis spectrophotometry at the maximum wavelength (415 nm). In addition, the ANOVA test was also used to compare the test strip results using the sample preparation and direct detection of HCHO on tofu sample without sample preparation toward the Nash method. The direct detection of HCHO on tofu sample performed just by dipping the test strip into the tofu sample since tofu texture is soft and can be inserted with the test strip easily. Then, it was left at the optimum time for reaction with HCHO inside the tofu. Correlation analysis was used to quantify the association between results obtained using the different methods [25].

RESULTS AND DISCUSSION

Test Strip Fabrication

The fabrication of HCHO sensor in the form of the test strip was performed by absorption of the acidified PR solution using HCl and H_2SO_4, onto the filter paper as sensor membrane on the strip. As expected, the slightly pink sensor membrane was changed into purple color just after the application of 30 mg/L HCHO solution at optimum reaction time as given in Fig. 1. The color change can be typically observed by naked eye. However, for the determination of HCHO concentration, the color intensity of the sensor membrane must be calculated by measuring their Δ mean RGB values after the sample introduction. The reaction between PR and HCHO is presented in a scheme as given in Fig. 2.

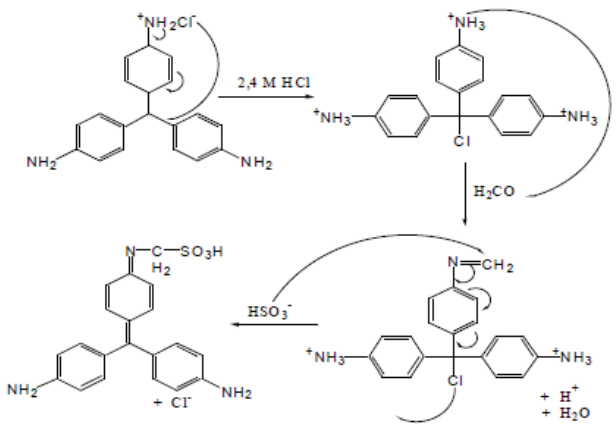
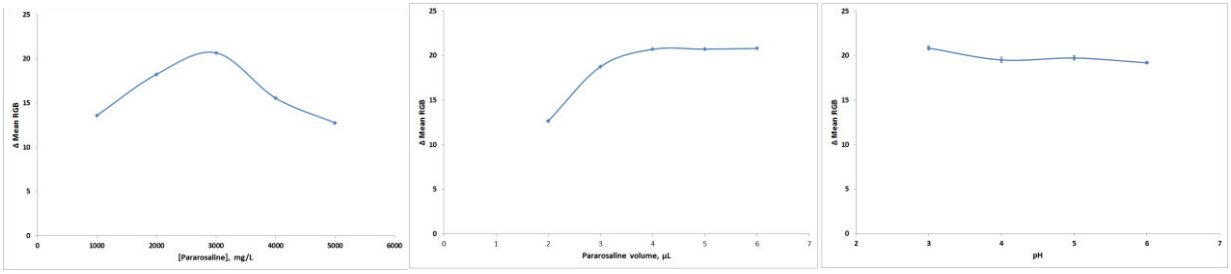


FIGURE 2. Reaction scheme of PR towards HCHO that required acid condition

Optimization of the Test Strip

In order to determine the optimum concentration of PR used for the sensor membrane, several studies were performed to obtain the best parameters (high color intensity and rapid response time) for the sensor membrane towards 30 mg/L HCHO. Firstly, the optimum concentration of the PR was searched by measuring color intensity of sensor using various concentration of the PR starting from 1000 to 5000 mg/L 48 after addition of HCl (4 M) and H_2SO_4 (1 M). Figure 3(a) shows that PR concentration at 3000 mg/L gave the highest intensity among others; hence it was selected as the concentration for optimizing the concentration of volume of PR transferred to the membrane. Secondly, the optimum volume of the PR was 4 µL. Since, this volume (4 µL) revealed that the PR at 3000 mg/L exhibited the highest color intensity among others, as it can be seen in Fig. 3(b). Therefore, the PR at 3000 mg/L with 4 µl is used to immobilize the PR on the sensor membrane. Lastly, the optimum pH for the sample was to be at acid condition (pH 3-6) where the highest color intensity change was found at pH 3 towards 30 mg/L HCHO as shown in Fig. 3(c). However, the differences in another pH (4-6) in term Δ mean RGB as the test strip response towards HCHO was not significant and only slightly reduced in the strip response. Therefore, the direct reaction of the test strip without buffer solution toward tofu sample is possible, since the tofu sample marketed was also in acid condition [21, 22]. Herein, we also detected HCHO in tofu directly just by dipping the test strip on tofu sample and left for 3 min for the dip test strip to react with HCHO and the results showed similar response toward similar concentration of HCHO (30 mg/L).



(a) **(b)** **(c)**

FIGURE 3. Effect of experimental parameter of the test strip toward HCHO (30 mg/L); (a) PR concentration; (b) volume of reagent; (c) pH

Response Time

The response time of the test strip as HCHO sensor was studied using 30 mg/L HCHO solution. The sensor response was recorded every minute until stable color intensity value was obtained. The response time of the test strip was constant at 3 min after addition of HCHO solution, as shown in Fig. 4. Therefore, this response time at 3 min was used as reaction time to complete full-color change of the test strip for further measurements.

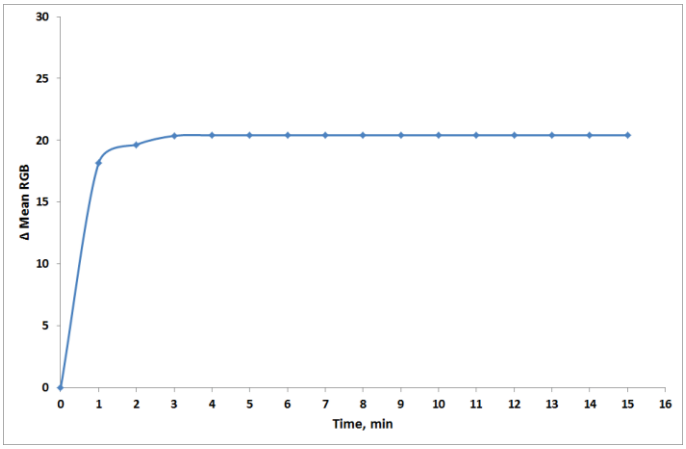


FIGURE 4. The response time of the test strip toward HCHO (30 mg/L) on the test strip response where pH 3 gave the optimum sensor response. The each data was recorded every 1 minute

Formaldehyde Measurement

As described earlier, HCHO solution was used as a standard for determining formalin in tofu sample, since it is already known that formalin has been used as an adulterant in tofu [6]. The calibration curve was constructed by plotting concentration of HCHO introduced to the test strip vs. its color intensity change (Δ mean RGB), as depicted in Fig. 5, using a matrix free calibration curve and matrix matched calibrations curve. It can be seen that range of the sensor response is linear towards HCHO in the concentration range of 2 – 80 mg/L with a coefficient of correlation of 0.999 for both curves. Therefore, in this case, the matrix did not affect the test strip response towards HCHO since the test strip response is much affected by the acid condition of the reaction toward HCHO in both cases as given in Fig. 2. The sensitivity of the test strip is described as the slope of the linear curve which was calculated to be 0.199 Δ mean RGB/mg/L HCHO ($\alpha = 0.05$, $n = 3$). The detection limit (LOD) of the test strip, which is defined as the concentration of sample generating a signal equal to the blank signal plus three times its standard deviation ($n = 5$) was calculated to be 0.05 mg/L, while for the limit of quantitation (LOQ) of the test strip (10 SD of blank signal), was found to be 0.16 mg/L. The LOQ of the test strip method much lower and meets the acceptable residue levels of HCHO in food, such as vegetable (63 mg/kg) and seafood (10 mg/kg for crustaceans and 60 mg/kg for Gadidae fish) [26]. The reproducibility of the test strip response was tested toward 30 mg/L of HCHO and has relative standard deviation (RSD) value lower than 1 %, which indicates the developed method has excellent reproducibility [27].

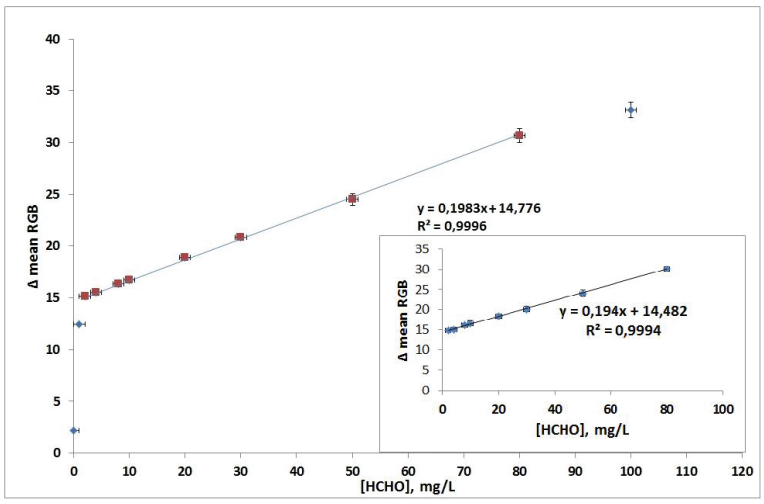


FIGURE 5. Calibration of the test strip toward HCHO, where the linear response (r = 0.999) was achieved in the range 2 – 80 mg/L, and inset show the calibration curve for matrix matched calibrations curve in the same range

Selectivity

To evaluate the interference of the test strip in the detection of HCHO, the influence of a potentially interfering substance, i.e. acetic acid, sugar (sucrose) and salt, on the formalin determination in simulated tofu sample consist of 30 mg/L formalin concentration was examined. Different concentration (30, 300 and 3000 mg/L in water) of interfering substances and their effects on color intensity changes in the sensor were investigated. Interfering signal, calculated by subtracting color intensity change of interfering substance added-sample with that of the original formalin sample only, and divided by color intensity change of original sample was found less than 5% at ratio 1:100 for acetic acid, sugar, and salt, as depicted in Fig. 6. This finding indicated that acetic acid, sugar, and salt at a concentration ratio of 1:100 did not interfere greatly with formalin measurement in the test strip.

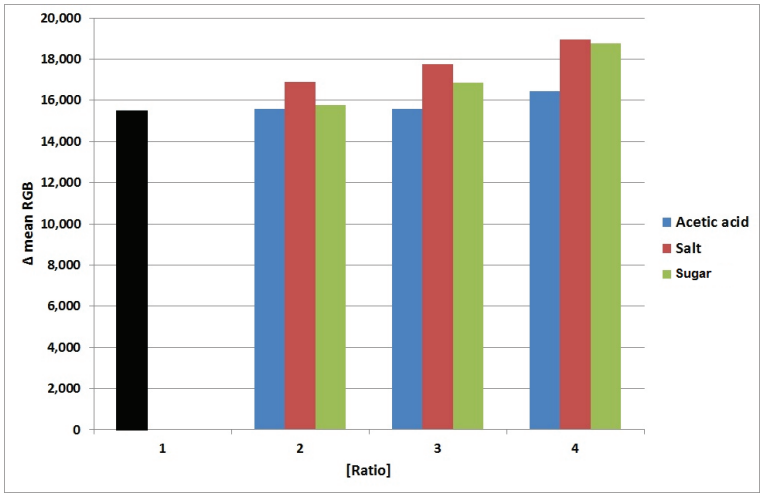


FIGURE 6. The different concentration ratio of 1(1:0), 2(1:1), 3(1:10) and 4(1:100) of interfering substances, and their effects on the color intensity (Δ mean RGB) changes of the test strip

Recovery

To evaluate the performance of the test strip for HCHO detection, a simulated sample containing 30 mg/L HCHO was analyzed using the test strip by standard addition method, following recovery test suggested by Huber [28]. After individual addition of a series solution containing 39, 44, and 49 mg/L HCHO to represent 30, 45, and 60% of initial HCHO concentration, the recovery of HCHO was found to be in 99.87, 97.31 and 91.79% respectively for each series of addition. Since the test strip used disposable, and then this finding suggested that the test strip has good accuracy and great potential for determining formalin in tofu samples.

Stability

In this work, various storage conditions were applied for stability test of the test strip. It was separately stored in a well-capped cabinet at room (25 ºC) and in a chiller at 4 ºC temperature. Then, the sensor response to added 30 mg/L HCHO was measured by using ImageJ program every day, until 15% decrease of the initial response was obtained. It was found that after 7 days the sensor response was observed to decrease more than 15% when it was stored at room temperature. Thus, it can be noted that stability of test strip was only maintained for a week at room temperature. In an attempt to increase the stability of the strip, it was also stored at a chilled temperature. The sensor response towards HCHO solution was found to be stable during 16 days storage at a chilled temperature. After this period, the strip response decreased more than 15%, suggesting that stability of the test strip was preserved up to two weeks in a chiller.

Application towards HCHO in Tofu Samples

In order to demonstrate the practical use of the test strip, various tofu samples collected from the traditional market in Jember were tested using the matrix matched calibration curve. Formalin (HCHO) in tofu samples was calculated and expressed in mg/L both using sample preparation and direct detection, just by dipping of the test strip on tofu sample. As a comparison, the Nash method [24] using UV/vis spectrophotometer at the maximum wavelength (415 nm) was applied for HCHO determination in tofu sample. The results obtained show that the test strip, both with sample preparation and direct dipping of the test strip in good agreement with the Nash method as depicted in Table 1. Since the calculated values of t were less than t table with a value of 2.776 (df = 4 and α = 0.05). Moreover, HCHO concentrations detected by the test strip and using the Nash method were highly correlated, the coefficient of correlation (r) was found to be 0.999. This is indicating the practical use of the dip test strip for the determination of HCHO in tofu sample.

TABLE 1. The results of formalin detection in tofu samples

Tofu sample	Test strip with sample preparation (mg/l)	Test strip by dipping detection (mg/L)	Nash method (mg/L)
A	63,097	63,069	65,329
B	80,321	80,280	79,875
C	43,897	43,890	46,325
D	78,403	78,418	79,875
E	27,103	27,068	29,375

Here, when sample preparation is used, since the amount of tofu (12.5 g) is cut and mixed with water (25.00 ml), then the dilution factor should be added in the calculation of concentration HCHO found in the sample. So the effect of water addition could be count as dilution factor. While in direct dipping used, the concentration of HCHO found was directly reflect HCHO content in tofu sample.

It can also be noted that the amount of reagent used (4 µL) is reduced in the developed method, while the larger volume of reagent (3 mL) is used in the Nash method or other spectrophotometry based method for HCHO analysis.

Moreover, the test strip developed was simpler, low-cost, and suitable for field application than that of spectrophotometric method for the determination of HCHO in tofu.

CONCLUSIONS

A simple dip test strip for detection of HCHO in tofu was developed based on PR reagent immobilized onto filter paper. This test has two important characteristics. First, the concentration of HCHO can be detected using the scanometric method with color image analysis for color change of the strip. Secondly, all the reagents needed for the reaction take place were immobilized in the sensing membrane. By using this scanometric method, the HCHO has been determined in the concentration range of 2-80 mg/L, with limit detection of 0.05 mg/L. It has good reproducibility (RSD = 8.04%) and the rapid response time of 3 minutes. The test strip has been applied successfully to detect HCHO in tofu samples, and the results show in good agreement with the Nash method. Thus, the test strip is rapid, very simple to operate, reproducible, low-cost and reliable as a simple formalin sensor for field application. In addition, it can be used as an alternative method for measuring HCHO in tofu samples directly without sample preparation, just by dipping the test strip onto the tofu sample.

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