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Development of Sweat and Saliva Glucose Sensors as Alternative for Non-Invasive Blood Glucose Monitoring

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Abstract. This study aimed to develop a paper-based glucose sensor using artificial sweat and saliva samples, a non-invasive, practical, and affordable concept for measuring glucose levels. The chosen method was the colorimetric method using the Glucose Oxidase (GOx) enzyme, Horseradish Peroxidase (HRP) enzyme, and 3,3′-diaminobenzidine (DAB) chromogen. The wax stamping method was utilized on a piece of filter paper to test the glucose sensor. The test was carried out in several stages, including testing various concentrations of reagents and wax stamping sizes, fabricating the glucose sensors, and testing multiple concentrations of glucose, artificial sweat, and artificial saliva. The RGB method was used to test the resulting color. The data obtained were then validated using UV-Vis Spectrophotometry. The GOx concentration on the glucose sensor affected the color change velocity. The HRP, DAB, and wax stamping size concentration simultaneously indicated a strong correlation with changes in the color intensity that appeared on the glucose sensor. The glucose sensor in this study could measure the glucose concentration of the artificial sweat and saliva samples, respectively, from 0-3000 μM with R^2 = 0.9862 and 0.9987. This study proved a strong correlation between the reagents' concentration and the color intensity on the developed glucose sensor.

Keywords: Colorimetry; Glucose; Non-invasive; Saliva; Sweat

1. Introduction

Diabetes Mellitus (DM) is a global epidemic disease that affects blood glucose levels. According to the 2019 International Diabetes Federation (IDF) Diabetes Atlas, around 463 million adults worldwide, whose ages range from 20-79 years old, suffer from DM. In Indonesia, there are more than 10 million diabetes cases in adults, with a prevalence of 6.2% (IDF, 2019). Self-monitoring blood glucose is important for diabetics, which consists of controlling sugar levels with the necessary diet and physical activity (Gordon, 2019; Meetoo *et al.,* 2018; Dewi *et al.,* 2017).

There are two classifications, namely invasive and non-invasive methods, to measure blood glucose levels. Invasive methods are currently still the most widely used option. This method requires blood extraction from a skin puncture, such as from a fingertip (Ekawita

et al., 2020; Zheng *et al.,* 2018). On the other hand, non-invasive methods will not injure the blood vessels or damage the skin, so this method is painless and can reduce the risk of infection and trauma (Karpova *et al.,* 2019). Respective researchers have developed non-invasive alternative methods using samples of natural body fluids such as tears, saliva, interstitial fluid (ISF), and sweat (Zafar *et al.,* 2022; Lee *et al.,* 2018). However, until now, there is no gold standard for measuring blood glucose levels non-invasively. Sweat can be used as a non-invasive method of sampling due to its accessible readiness, and numerous ways can be applied to stimulate sweat production in several places (Bruen *et al.,* 2017). In addition, the findings in several previous studies have proved a strong correlation between glucose levels occurred in sweat and blood glucose levels with a value of r = 0.76, resulting in the glucose levels in sweat can accurately reflect blood glucose levels (Xiao *et al.,* 2019b; Lee *et al.,* 2018). Of the various types of peripheral fluids, saliva is often considered the "mirror of the body" and has the potential to be used in clinical diagnosis (Gupta *et al.,* 2017). A study by Nadaf *et al.* (2017) proved a good correlation between blood glucose and saliva glucose, with a value of r = 0.715 and p < 0.001.

The limitations of paper-based glucose detection devices in previous studies are still often found. The cost of the device should have been more affordable because of its streamlined design, so that its use could be expanded to all people, especially in areas with inadequate facilities (Vaquer, Barón, and Rica, 2021; Singh *et al.,* 2018). The design concept of the glucose sensor used in this study was a colorimetric technique that used Glucose Oxidase (GOx) and Horseradish Peroxidase (HRP) as enzymes and 3,3'-Diaminobenzidine (DAB) as a chromogen. DAB is one of the hemodynamically and chemically stable chromogenic substrates (Jangi, *et al.,* 2020). The enzymatic method has been used in previous studies because it has several advantages, such as enzymes are not corrosive and can produce specific reactions (Perdani *et al.,* 2020). The enzymatic reaction in the glucose sensor started from a glucose sample that produced gluconic acid and hydrogen peroxide (H_2O_2). Glucose oxidase (GOx) acted as an enzyme that catalyzed this reaction to create the hydrogen peroxide that would oxidize DAB from colorless to brown poly-3,3'-diaminobenzidine [poly(DAB)] (Jangi *et al.,* 2020; Liu *et al.* 2016). Furthermore, the Horseradish Peroxidase enzyme (HRP) then catalyzed the reaction (Wang *et al.,* 2020; Wei *et al.,* 2016). This study used wax stamping as a fabrication method due to its rapidity, cheapness, higher resolution, environmentally friendly and feasible to be repeatedly fabricated (Soum *et al.,* 2019; Santana-Jiménez *et al.,* 2018; He *et al.,* 2015). The wax stamping works as a hydrophobic barrier that prevents the solution from flowing in all directions (Altundemir *et al.,* 2017).

This study aimed to design a non-invasive concept to measure the glucose levels in the body using paper-based colorimetric methods. The developed device design is a practical, harmless, and affordable device to simplify the process of routine glucose checks by the entire community. In order to develop the design concept of the device, it is essential to test both the reagents concentration, which is required to measure the glucose levels optimally and the size of wax stamping, which is required to accommodate the optimal reagents and samples. After obtaining the most optimal concentration and size of wax stamping, the research was continued using a paper-based glucose sensor. The glucose sensor was then used to test samples of several concentrations of pure glucose, glucose in sweat, and glucose in saliva using the RGB method. The study ended by validating the RGB findings using UV-Vis Spectrophotometry in order to obtain more accurate and reliable data.

2. Methods

2.1. Materials and equipment

The materials used to measure the glucose level included Glucose Oxidase (GOx), Horseradish Peroxidase (HRP) as the enzyme, a chromogen namely 3,3'-diaminobenzidine (DAB), Chitosan powder as the solute, PBS buffer, glucose, lactic acid, acetic acid, distilled water as the other solution, the PBST solution which composed of 50 mM PBS, 0.9% NaCl, 0.1% Triton X-100, pH 7.4, and other compounds that consisted of NaCl, NH_4Cl, NaOH, KCl, $CaCl_2$, $NaHCO_3$, and Whatman filter paper no.4 whose diameter of 110 mm.

The equipment used to measure the glucose level required some devices consisting of a smartphone with Color Grab™ application (Lookmatix, 2020), several wax stamps, a black box, some hot plates, an oven, an analytical scale with 0.001 mg precision, and the UV-Vis Spectrophotometer.

2.2. Research procedures

2.2.1. Testing with Various Reagent Concentrations

This research used several concentrations, including GOx, HRP, and DAB, to start the test. In each trial of a reagent concentration, the other reagents were used as the constant (identical concentrations). First, a 5 μL solution of GOx and a 5 μL solution of HRP were dropped into the sample reservoir on the filter paper until it dried (20 minutes) before the DAB (5 μL) was added. After the DAB dried, the same concentration of glucose solution (20 μL) was dripped on each reservoir sample. The filter paper was then incubated at room temperature for 20 minutes, followed by taking photographs of the test results with the Color Grab™ application to obtain the RGB values. The test was finalized by recording the results of each reaction to get the most optimal concentrations of GOx, HRP, and DAB.

2.2.2. Testing of Wax Stamping Sizes

After the most optimal GOx, HRP, and DAB concentrations were obtained, the next step was to test various diameters of the sample reservoir in the wax stamping. This step was conducted to get the same volume of samples and reagents in each experiment. The first step was to melt the paraffin at 130°C, followed by heating the wax stamp on the melted hot paraffin to be attached to the wax stamp. Later on, the pattern was formed by pressing the wax stamp on the filter paper for 5 seconds. Following this, the filter paper was preheated in the oven for 10 seconds at 100°C (if the paraffin did not penetrate the back side of the filter paper). After the filter paper was ready, the reagent and glucose were dropped into the sample reservoir and incubated in the filter paper for 20 minutes at room temperature. Finally, the pictures of the resulting color were taken using the Color Grab™ application, and the results were recorded.

2.2.3. Fabrication of the Glucose Sensor

The fabricating process of the paper-based glucose sensors began by preparing the filter paper in a round shape, then printing a hydrophobic barrier with paraffin and anticipating the complete absorption. Subsequently, the Chitosan was prepared in acetate solution (5 μL) to be dropped on the sample reservoir until it dried at room temperature. Afterward, the other materials, such as GOx, HRP, and DAB, were dropped on the sample reservoir and allowed the sensor to dry further at room temperature. Lastly, the PBST solution was sent to the sample reservoir until it dried at room temperature.
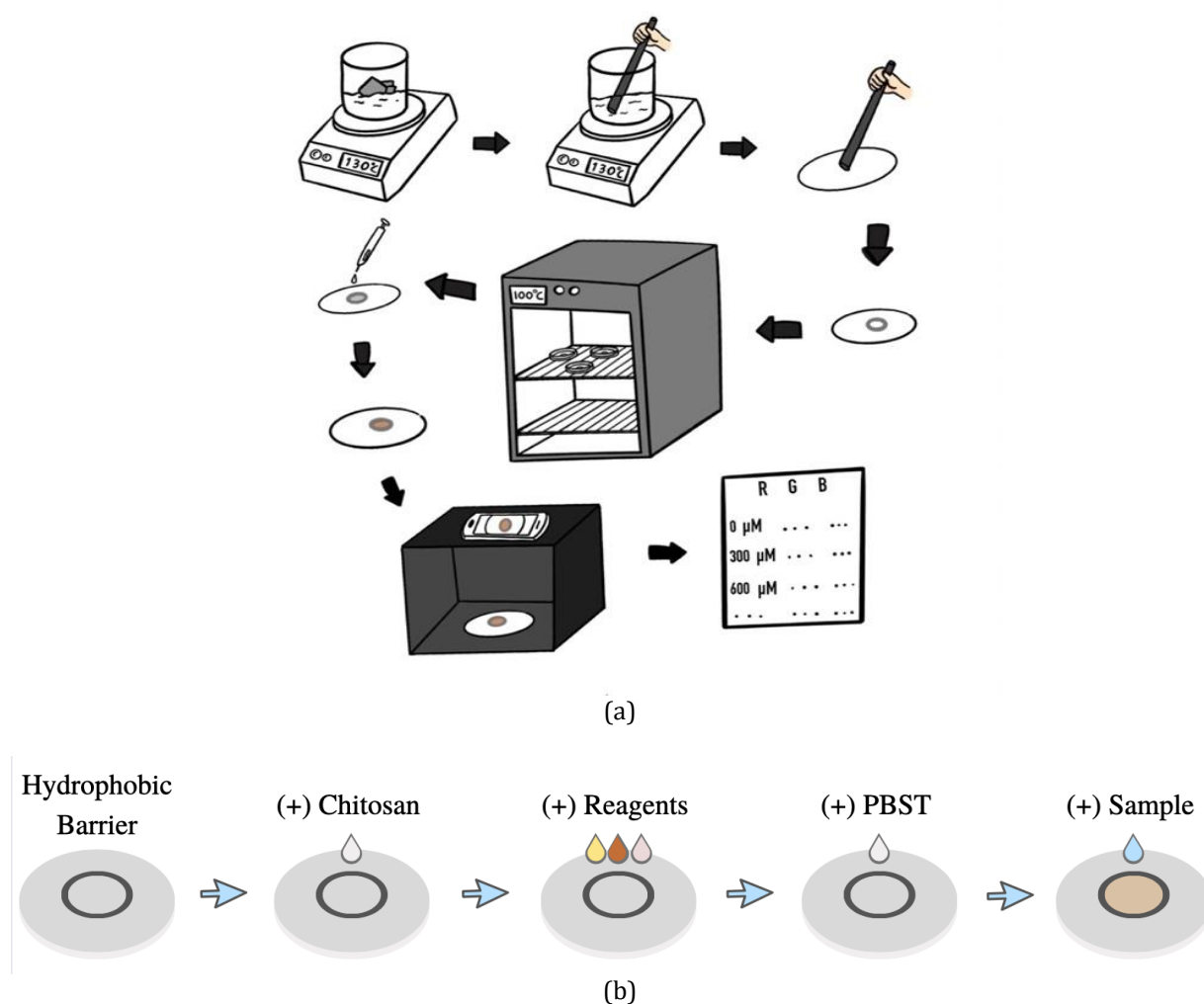
(a)

(b)

Figure 1 Paper-Based Glucose Sensor Fabrication Method (a) and Dropping Process on Glucose Sensor (b)

<u>2.2.4. Measurements with Various Concentrations of Glucose, Artificial Sweat, and Artificial Saliva</u>

Various glucose concentrations in distilled water at concentrations of 0 μM, 300 μM, 600 μM, 900 μM, 1200 μM, 1500 μM, 1800 μM, 2100 μM, 2400 μM, 2700 μM, and 3000 μM were prepared. Furthermore, the glucose solution was dropped onto different sensor reservoirs. While experimenting, the results of each reaction were recorded. Afterward, artificial sweat was prepared with 20 g/L NaCl, 17.5 g/L NH$_4$Cl, 5 g/L acetic acids, and 15 g/L lactic acid. Furthermore, 0.1 mM NaOH solution was added to the artificial sweat solution to make the sweat produce a pH of 6.0 (Xiao *et al.*, 2019a). The artificial saliva with 36 grams of NaCl, 1.6 grams of KCl, 0.96 grams of CaCl$_2$, 0.8 grams of NaHCO$_3$, and 400 ccs of equilibrated water was made using HCl until it reached a pH of 7 (Ramadhani, Sulistiyani, and Hidayati, 2017). Then, pure glucose was added to the artificial sweat and saliva in various concentrations. Accordingly, artificial sweat and saliva with various glucose concentrations were then dropped into different sample reservoirs. With that, the sensors were incubated at room temperature for 20 minutes, then the sample pictures of the color were taken to get the RGB value. The RGB results of each reaction were recorded to be validated with UV-Vis Spectrophotometry as an additional test. Before the test, the reagents and samples were mixed with PBS and dropped into a micro cuvette. Lastly, the absorbance results of each example were recorded.

2.2.5. Evaluation of the Experimental Results

The RGB value indicated the color intensity that appeared due to the glucose concentration in the sample. The RGB value obtained from each glucose sensor was described in a comparison graph. From these data, the concentration and volume of reagents that provided the most optimal measurement of glucose levels were determined. The wax stamping size was then also selected to obtain the optimal reagents and the samples of the artificial sweat and saliva.

3.   Results and Discussion

3.1. The Relation Between Reagent Concentration and Color Change Intensity

There were three variations of concentrations for each of the reagents used in this test. GOx and HRP had concentrations of 0.05 U/µL, 0.1 U/µL, and 0.2 U/µL. Meanwhile, 3,3′-diaminobenzidine (DAB) had concentrations of 10,000 µM, 12,500 µM, and 15,000 µM. The test of various concentrations of GOx showed no significant relationship between various concentrations of GOx and the intensity of color change. However, GOx concentrations could affect the speed of the color change. The higher the GOx concentration, the faster the color change occurred. This finding was in line with the function of the enzyme glucose oxidase as a catalyst for the oxidation of glucose to gluconic acid and hydrogen peroxide (Yee *et al.*, 2019; Lee *et al.*, 2018). Therefore, the GOx concentration of 0.2 U/µL was the concentration that could cause a fast color change.

On the other hand, GOx concentrations did not affect the color intensity. Several factors could affect the yield of the GOx reaction, such as time, temperature, pH, and concentration of glucose samples (Xiong *et al.*, 2017). In this test, each reaction was carried out with the same time duration (20 minutes), temperature (room temperature), pH (pH 7), and the same concentration of glucose samples (2400 M). This factor caused the absence of a significant difference in the results of color intensity in all variations of GOx concentration. Therefore, the concentration of GOx needed to measure glucose levels optimally was 0.05 U/µL. In the test of various concentrations of HRP, the test results proved that 0.2 U/µL was the most optimal concentration.

Nevertheless, the concentration of 0.05 U/µL was in line with the reference journal. The concentration of GOx and HRP must have a ratio of 1:1 to give a better result (Jin *et al.*, 2020). The DAB test result showed that the most optimal concentration of DAB was 10,000 µM. However, 10,000 µM was not proportional to the mole ratio between glucose concentration and DAB, which was 1:4. Thus, the researcher did a calculation and obtained the best concentration of DAB for 0-3000 µM of glucose, which was 15,000 µM.

3.2. Stamping Wax Size was Required to Get the Optimal Volume of Reagents and Samples

This study tested three different wax stamping diameter sizes, which were 5.5 mm, 8 mm, and 10 mm, using the RGB method. Based on the results of RGB value analysis, the most optimal wax stamping diameter was 5.5 mm. However, in this study, the wax stamping size with a diameter of 5.5 mm did not accommodate the entire volume of reagents and samples as it caused leakage. Therefore, the diameter of the wax stamping optimal for use in this study was 8 mm. The findings of this study were consistent with previous studies that predicted dimensions could affect the results in paper-based microfluidic devices. According to the study results, the error on paper-based sensor devices with a circular area of more than 25 mm^2 was less than 10%, while the device error was 20% with a circular area of more than 15 mm^2 (Catalan-Cario *et al.*, 2020). The area of a circle used in this sensor was 50.24 mm^2 (circle diameter = 8 mm). With this area of the circle, the total error was less than 10% and would be considered an optimal wax stamping size.

According to another study, Santana-Jimenez *et al.* used wax stamping with a diameter of 3 mm. With this diameter, the amount of enzyme solution, reagent, and sample dripped was 5µL (Santana-Jimenez *et al.,* 2018), and the area of the wax stamping was 7 mm². Therefore, the ratio between the amount of dripped volume (5 µL) and the circle area (7 mm²) in the Santana-Jimenez study was 1:1.4. In this study, the optimal diameter was 8 mm with a circular area of 50 mm². Thus, we obtained a comparison between the amount of volume that dripped (35 µL) and the area of the circle (50 mm²) in this research was approximately 1:1.4. From the results of these calculations, the diameter size of this study was in accordance with the diameter of the reference journal.

3.3. The Correlation of Glucose Concentration in Samples and Intensity of Color Change

<u>3.1.1. Pure Glucose</u>

Based on the test results, there was a strong correlation between various concentrations of pure glucose and the intensity of the color change. Glucose concentrations used in this study were 0 µM, 300 µM, 600 µM, 900 µM, 1200 µM, 1500 µM, 1800 µM, 2100 µM, 2400 µM, 2700 µM, and 3000 µM. There were variants in color intensity from low to high as the glucose concentration increased. It was possible to see the difference in color intensity with the naked eye. Based on the results of Pearson correlation analysis, there was a strong negative correlation between glucose concentration and RGB value with the correlation coefficients respectively -0.9964, -0.9900, and -0.9811 (R^2 = 0.9928, 0.9801, and 0.9626). The three correlation coefficients illustrated that the higher the glucose concentration, the lower the RGB value. This correlation finding is in accordance with several previous studies, which show that the increase in glucose concentration resulted in increasing color intensity and decreasing RGB colorimetric value. (Dominguez *et al.,* 2017; Gabriel *et al.,* 2017). With this strong correlation, this prototype could optimally identify glucose concentrations from 0 to 3000 µM. With this range of glucose concentrations, diabetic patients have the possibility to use this prototype to measure glucose levels with sweat and saliva.

<u>3.3.2. Artificial Sweat</u>

This study utilized 11 glucose concentration variations in artificial sweat. It was the same as in the pure glucose test. The test results using the RGB method showed a strong negative correlation between the glucose concentration in artificial sweat and the three RGB values. The researchers succeeded in proving this correlation with the Spearman correlation coefficient of -0.9931 (R^2 = 0.9862) for RGB-R, and the Pearson correlation coefficient, respectively, for RGB-G and RGB-B were -0.9469 and -0.9048 (R^2 = 0.8966 and 0.8187). The linear regression equation of the RGB-R data calibration curve is y = -0.011x + 189.664 (figure 3a). The equation was made based on RGB-R data because the RGB-R values provided the most visible difference and significantly contributed to color changes caused by enzyme catalytic reactions (Xiao *et al.,* 2019a). The findings in this study were in accordance with the findings by Xiao *et al.*, who found something similar, which had a negative linear relationship between the RGB-R values and 0 to 1500 uM glucose concentrations in artificial sweat. Therefore, paper-based sensors can be relied upon to detect glucose in sweat (Xiao *et al.,* 2019a).

The glucose level in sweat is 10-1110 µM (Xiao *et al.,* 2019b). The sweat glucose level of normal people is 60-110 µM, while the sweat glucose level of diabetic patients is 10-1000 µM (Bruen *et al.,* 2017). The glucose sensor in this study could detect glucose levels in artificial sweat with a concentration of 0 to 3000 µM. Therefore, it could detect sweat glucose levels in normal people to diabetic patients. In addition, the difference in color intensity could be seen visually. The higher the glucose concentration in artificial sweat, the more pronounced the brown color on the glucose sensor (Figure 2). This color changed

because the glucose in the artificial sweat samples activated a reagent-chromogen system reaction that resulted in an increased color intensity along with an increase in glucose concentration. Based on these findings, the strong correlation between glucose concentration in artificial sweat and color intensity could be the basis for the design concept of a glucose sensor using paper-based colorimetric methods.
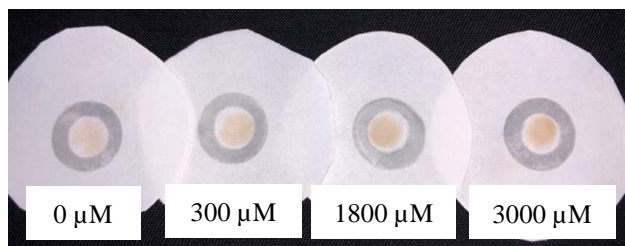

| 0 µM | 300 µM | 1800 µM | 3000 µM |

Figure 2 Color Differences in Four Different Concentrations of Glucose in Artificial Sweat

(a)

$$y = -0.011x + 189.664$$
$$R^2 = 0.9862$$

$$y = 0.00006x + 0.025$$
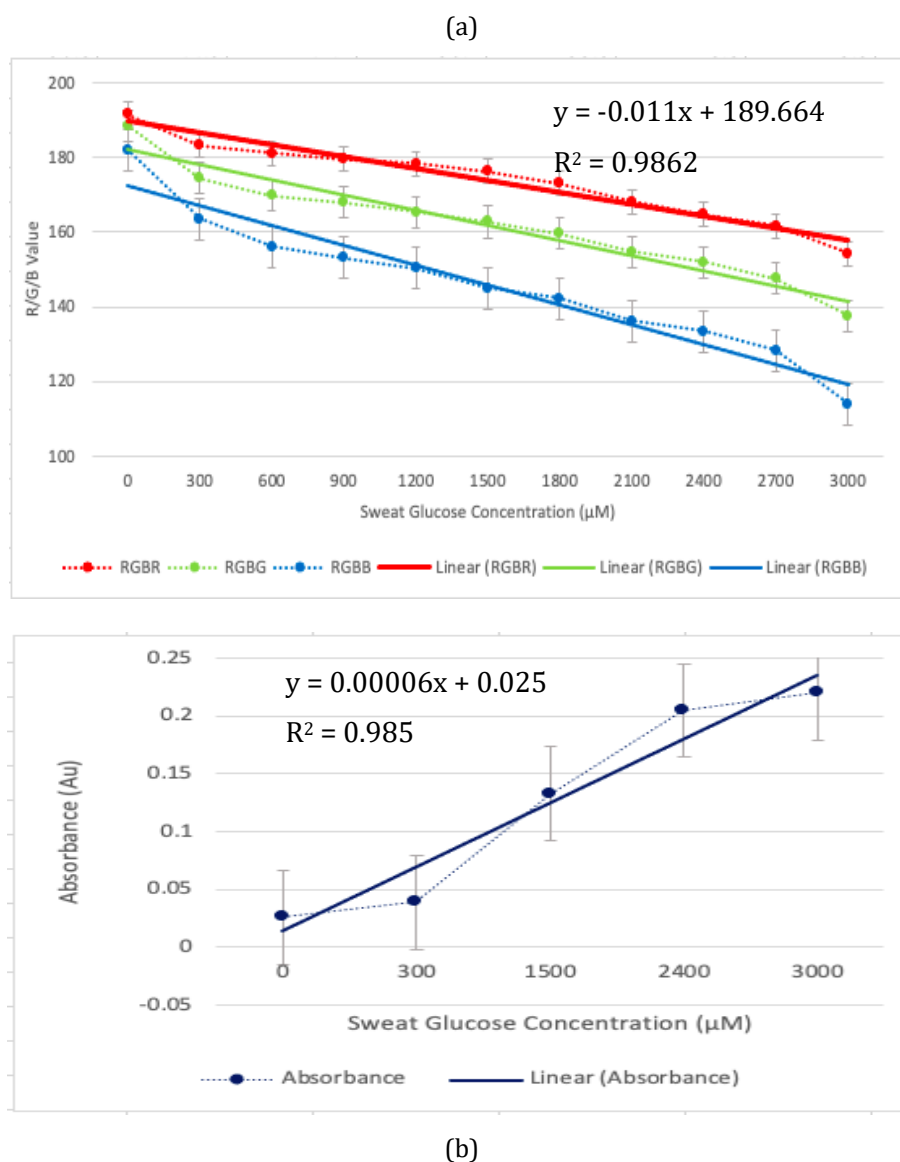$$R^2 = 0.985$$

(b)

Figure 3 Linear Regression Graph of Artificial Sweat RGB Test (a) and Linear Regression Graph of Artificial Sweat Spectrophotometric UV-Vis Test (b)

The last step of this research was validating the RGB test results using the UV-Vis Spectrophotometry method. First, we determined the appropriate wavelength of the

reaction between the reagent-chromogen and glucose in the sample. The peak absorbance value of the reaction was at a wavelength of 419 nm. Then, we proceeded with testing artificial sweat samples with five glucose concentration variations (0, 300 µM, 1500 µM, 2400 µM, and 3000 µM) at a wavelength of 419 nm. Based on the results, there was a strong positive correlation between glucose concentration in artificial sweat and light absorbance at 419 nm with a Spearman correlation coefficient of 0.993 (R^2 = 0.985) and a linear regression equation y = 0.00006x + 0.025 (Figure 3b). The positive correlation explains that the higher the glucose concentration in artificial sweat, the higher the light absorbance value at a wavelength of 419 nm. In comparison, the negative correlation in the RGB equation illustrates that the higher the glucose concentration, the lower the RGB value. This study proved that the RGB method was valid. Therefore, the RGB approach can be applied to the design concept of detecting glucose in sweating paper-based colorimetry.

3.3.3. Artificial Saliva

In the test of artificial saliva samples with RGB, the researcher made the saliva glucose concentration in 11 concentration variations as in the pure glucose test. Based on the experimental results, a strong correlation was found between various glucose concentrations in artificial saliva and the intensity of the color change. The higher the sample concentrations, the higher the intensity of the color formed. In previous research from Xiao *et al.*, the linear regression equation y = -0.19x + 231.68 was obtained with a value of R^2 = 0.9896. This value indicated that the R (red) value provided a good linear relationship (Xiao *et al.*, 2019a). In this study, we obtained a linear regression equation from the calibration curve of R(red) data on RGB, namely y = -0.015x + 192.686, with a value of R^2 = 0.991 (Figure 5a). This value indicates a good correlation between the intensity of the color produced and various glucose concentrations in artificial saliva. This correlation was even better than the previous research conducted by reference journals.

The high correlation proves that the prototype can significantly measure saliva glucose concentrations from 0-3000 µM. The average saliva concentration in normal people is 8-210 µM. While in diabetes patients, the saliva glucose concentration was 100-700 µM (Siddiqui *et al.,* 2018). Therefore, this prototype can detect salivary glucose levels in people with diabetes. Color gradations from the artificial saliva glucose test results with concentrations of 0, 300 µM, 1800 µM, and 3000 µM can also be seen with the naked eye (Figure 4).
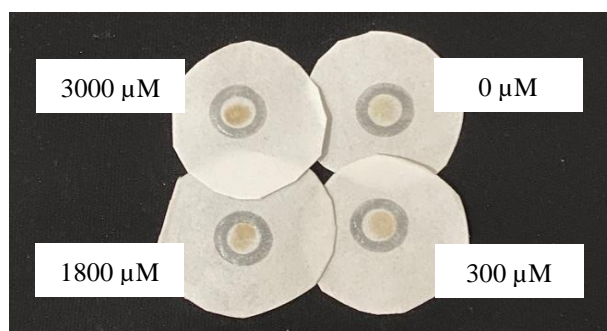

Figure 4 Color Differences in Four Different Concentrations of Glucose in Artificial Saliva

After testing with RGB, UV-Vis spectrophotometry was used to validate all the results obtained using an artificial saliva sample that consisted of 5 glucose concentration variations (0, 300 µM, 1500 µM, 2400 µM, and 3000 µM). With a wavelength of 419 nm, the trial results showed a strong correlation between the various glucose concentrations in the artificial saliva and the resulting color intensity. All data from this experiment were made

in the form of a calibration curve and obtained a linear regression equation y = 0.0006x + 0.025 with a value of R^2 = 0.9831 (Figure 5b). This value indicated a good correlation between the absorbance value and the various glucose concentrations inartificial saliva samples. Similar to the study on sweat, the results of the data obtained in the assay with RGB and UV-Vis spectrophotometry gave inversely correlated results. The higher the glucose concentration, the lower the RGB value, while the absorbance value of the UV-Vis spectrophotometric test was lower. The results of this trial have proven to validate the results obtained in research with RGB.
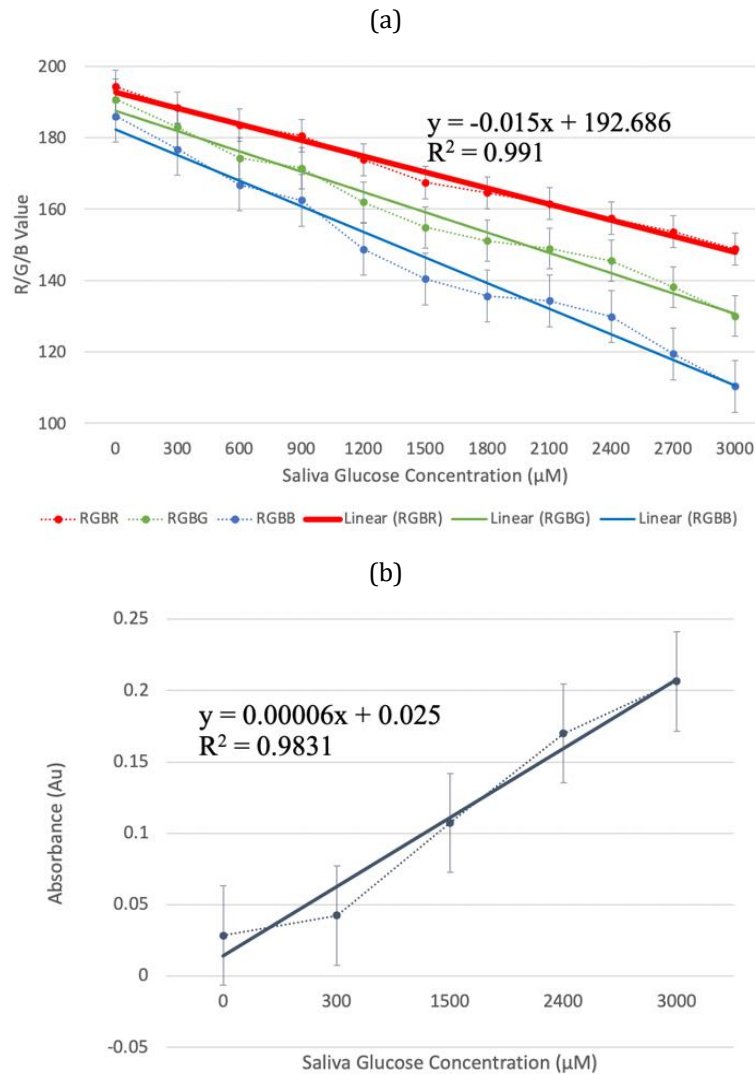
(a)

(b)

Figure 5 Linear Regression Graph of the RGB Test of Artificial Saliva (a) and Linear Regression Graph of the UV-Vis Spectrophotometric Test of Artificial Saliva (b)

3.3.4. Sensor Sensitivity and Accuracy

The smartphone device used in this study would help glucose detection be more practical. Therefore, there are several things to note. First, taking pictures will be different if you do not follow the black box instructions correctly. In addition, the RGB value in the application will change if the user uses the zoom-in/out feature, which will reduce the validity of reading the glucose concentration results in the sample. Therefore, adjustments are needed to the black box to help provide similar image results for each type of smartphone used.

In addition to using a black box, wax stamping could also affect the sensitivity and accuracy of the glucose sensor. The first was about the successful penetration of paraffin on the filter paper. In our research, the paraffin occasionally did not penetrate the back of the filter paper, reducing the sensitivity and accuracy of the results. The second was the possibility of paraffin entering the sample reservoir circle during the wax stamping process. Paraffin droplets that accidentally dropped into the sample reservoir could also reduce the accuracy of the glucose sensor. Therefore, it is more advisable to use a more guaranteed method in terms of shape and print results, such as wax printing or beeswax printing which are useful for biological detection (Nunut *et al.*, 2020).

4. Conclusions

This study proved a highly correlated relationship between the concentration of Horseradish Peroxidase (HRP) as an enzyme and 3,3'-diaminobenzidine (DAB) as a chromogen with the color change intensity by the colorimetric method. Glucose Oxidase (GOx) affected the speed of color changes on the glucose sensor. The size of the hydrophobic barrier can also affect the intensity of the resulting color. The size of the wax stamping that gave optimal results had a ratio of 1:1.4 between the area of the circle formed by wax stamping and the volume of liquid that was dropped. In addition, this study proved a strong correlation between various glucose concentrations and color intensity. The solutions used in this research consist of variations of pure glucose solutions, artificial sweat, and saliva solutions concentrations. R^2 values proved a strong correlation, 0.9928 for pure glucose, 0.9862 for glucose in artificial sweat, and 0.991 for artificial saliva. The glucose sensor in this study has been proven to measure glucose levels with a concentration of 0 to 3000 μM optimally, including normal glucose levels and glucose levels in diabetic patients in sweat and saliva. This finding could be the basis for developing a non-invasive glucose level detection device in sweat and saliva.

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