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Development of a microfluidic paper based with portable system for glucose concentration colorimetric analysis

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Abstract. Diabetes mellitus is a major health problem in Indonesia, with a prevalence of up to 20.4 million people in 2024 according to the International Diabetes Federation (IDF). Early detection of blood sugar is hampered by the expense and limited accessibility of diagnostic tools. Microfluidic paper-based analytical devices (µPADs) are a potential solution because they are inexpensive, portable, and environmentally friendly, in accordance with WHO ASSURED standards. This study aims to develop a µPAD integrated with a portable detection system for colorimetric quantification of glucose concentration using Glucose Oxidase–Peroxidase Aminoantipyrine (GOD-PAP) reagent, which produces a color change proportional to glucose concentration. Initial testing was conducted on phosphate-buffered saline (PBS) and D-glucose solutions as test materials in glucose concentration research. The test materials will be validated using spectrophotometry. Next, the test materials will be applied to µPAD for colorimetric observation using an RGB sensor on a portable device. The quantification results show that the red ratio at 10 minutes using the colorimetric method is highly linear (R^2 = 0.96). Precision validation of the colorimetric method produced an RSD value of less than 5%. The paired t-test yielded a p-value of 0.42 at the 95% confidence level, indicating no significant difference in glucose concentration quantification results obtained with the colorimetric and spectrophotometric methods.

Keywords: µPAD, portable detection system, GOD-PAP, RGB colorimetry, ASSURED

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INTRODUCTION

Diabetes continues to be a serious health problem in Indonesia, with estimates skyrocketing to 28.6 million by 2050. This increase is fuelled by urbanization, sedentary lifestyles, unhealthy diets, and an aging population. One of the main problems is the high number of undiagnosed diabetes cases, with an estimated 15 million people in Indonesia having diabetes without realizing it [1]. Therefore, innovation in accessible, fast, and inexpensive methods for glucose detection is urgently needed to improve early screening, even in remote areas.

Blood glucose is the body's primary energy source, regulated by insulin and glucagon. Blood glucose levels are classified as follows: Fasting Blood Glucose (FBG) (normal < 100 mg/dL), 2-Hour Postprandial Blood Glucose (normal < 140 mg/dL), Random Blood Glucose (≥ 200 mg/dL accompanied by symptoms), and Hemoglobin A1c (HbA1c) (normal < 5.7%). Levels above the normal range indicate prediabetes or diabetes. This classification is vital for early diagnosis, monitoring, and management of diabetes to prevent complications [2].

Conventional clinical diagnostic devices have limitations such as high cost, long analysis time, and the need for routine replacement. In response to this, the WHO established the ASSURED criteria for portable diagnostic devices: Affordable, Sensitive, Specific, User-friendly, Rapid and robust, Equipment-free, and Deliverable to end users [3]. Microfluidic paper-based analytical devices (µPAD) have emerged as a promising technology that meets these criteria due to their low production costs, easy fabrication, minimal sample requirements, and ability to integrate with portable detection systems [4]. Of the various detection methods, colorimetry is most widely used in µPAD due to its simplicity, low cost, and ease of implementation, although detection in whole blood requires blood cell separation.

This study developed a portable ESP32-CAM-based detection system for colorimetric glucose analysis using µPAD. The µPAD was created using permanent markers as an effective and inexpensive hydrophobic barrier. The portable detection system, printed on a wooden plate and equipped with an ESP32-CAM and an LED light, was used for illumination and image capture. ImageJ was used to extract Red, Green, and Blue (RGB) values from enzymatically reacted glucose solutions. Testing was conducted using PBS (Phosphate-Buffered Saline) solutions containing D-glucose as a substitute for human serum, and the samples were then enzymatically tested with GOD-PAP reagents, which are highly reliable. This system is expected to be an innovative solution for affordable, accessible glucose detection, supporting early diabetes screening across Indonesia.

µPAD is a novel analytical tool fabricated from paper-based materials, capable of analyzing small and complex biochemical samples (10^{-9} to 10^{-18} L) in a single analytical process, with microfluidic manipulation capabilities such as transportation, separation, mixing, and partitioning [5]. Given the

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remarkable properties of paper materials, µPAD is an innovative platform for fluid handling and analysis with diverse applications, while offering low cost, ease of fabrication, and independence from specialized equipment.

Phosphate-Buffered Saline (PBS) is a widely used buffer solution in biological and biochemical research due to its ability to maintain stable pH and osmolarity, thereby mimicking human physiological conditions [6]. The primary advantage of PBS lies in its stability, which preserves the integrity of biological samples during long-term storage [7] as well as its compatibility with various analytical techniques, without interfering with the biological activity of the samples [8].

The liquid retention volume within porous media, such as Whatman No. 1 filter paper, is determined by the hydrophilic surface area, the medium's thickness, and its porosity, as described in Equation (1).

$$V = A \cdot h \cdot \phi \tag{1}$$

The absorption volume of paper (V) is calculated based on the hydrophilic zone area (A), paper thickness (h), and porosity (ϕ). This formula ensures that the dispensed reagent volume can be adequately retained, preventing leakage or premature drying before reaching the detection zone.

The GOD-PAP method (Glucose Oxidase–Peroxidase Aminoantypyrine) is a semi-automated analytical technique for glucose determination. In this method, glucose oxidase (GOD) catalyzes the oxidation of β-D-glucose into gluconic acid and hydrogen peroxide (H_2O_2). The generated H_2O_2 subsequently reacts in the presence of peroxidase, which catalyzes the reaction between H_2O_2, 4-aminoantipyrine, and phenol to form a colored quinoneimine compound, as shown in equations (2) and (3). This chromogenic product exhibits a characteristic color measurable colorimetrically at 505 nm. The color intensity is directly proportional to the glucose concentration in the sample, and absorbance values are compared against standard solutions treated under identical conditions [9].

$$Glucose + O_2 + H_2O \xrightarrow{Glucose\ Oxidase} Gluconic\ Acid + H_2O_2 \tag{2}$$

$$2H_2O_2 + 4 - AAP + Phenol \xrightarrow{Peroxidase} Red\ Quinoneimine\ Dye + 4H_2O \tag{3}$$

The reaction catalyzed by peroxidase produces quinoneimine, a red-colored compound. The intensity of this red color is directly proportional to the glucose concentration in the sample.

Portable µPAD-based detection systems have emerged as innovative solutions in point-of-care (POC) diagnostics, combining ease of use, low cost, and high portability. These platforms employ paper as the primary substrate, where hydrophilic microchannels are defined and bounded by hydrophobic barriers to direct passive sample flow without the need for external pumps [10]. The main advantage of this system is its ability to perform complex biochemical analyses using simple, affordable devices, making it well-suited for remote areas or healthcare facilities with limited resources.

Various glucose testing methods have been established, including spectrophotometry, electrochemistry, and polarimetry. However, colorimetry—based on enzymatic reactions (commonly involving glucose oxidase) that yield color changes proportional to glucose concentration—stands out due to its simplicity and cost-effectiveness. While traditional spectrophotometric biochemical analyzers are accurate, they are also complex and prone to certain errors. As an alternative, image-based methods have recently gained attention. These approaches capture images of glucose solutions after reaction and extract quantitative features from variations in color intensity.

Recent studies, such as Cai et al. (2024), developed an origami µPAD for blood glucose detection by reading colorimetric results with a smartphone camera. The reaction zone color was produced by the enzymatic oxidation of glucose catalyzed by glucose oxidase (GOx), horseradish peroxidase (HRP), and potassium iodide (KI), forming a yellow-brown complex. The smartphone camera captured the resulting color, and RGB intensity values were extracted using a dedicated application. Instead of using raw R, G, or B values, researchers transformed them into standardized color ratios to obtain more stable and accurate results. In their study, the intensity ratio used was G/(R+G+B), which demonstrated improved stability, accuracy, and practicality for portable smartphone-based detection systems [11].

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METHODOLOGY

This study aims to develop a microfluidic Paper-Based Analytical Device with an integrated portable detection system to quantitatively measure glucose concentration in Phosphate-Buffered Saline and D-Glucose test media using colorimetry. The initial stage of the study involves validating the sample using spectrophotometry. This was done to determine the true value of samples prepared using a 100 mg/dL standard glucose.

The µPAD pattern was designed in 2 dimensions using a hydrophobic plotting technique, a fast and cost-effective method. The fabrication process involved creating patterns on single-layer Whatman No. 1 paper using a 3D-printed template and permanent markers. This hydrophobic pattern serves to channel the reagent to the outlet zone (reaction), minimizing the use of reagents and samples in microliter volumes, thereby increasing the efficiency of the analysis.

Colorimetric measurements were performed using a detection system equipped with ESP32-CAM to capture images of the color reaction in real time. The device design includes a closed chamber with controlled lighting to ensure imaging consistency. The resulting images are stored and processed in ImageJ for color intensity analysis using the RGB (Red, Green, Blue) components. The RGB values are then converted into ratios for glucose quantification.

The colorimetric method will be compared with spectrophotometry 721 as a standard reference. The validation process includes a linearity test to evaluate the relationship between glucose concentration and the resulting color ratio, a precision test measuring repeatability through six test repetitions, and a paired t-test comparing colorimetric and spectrophotometric results to assess statistical equivalence.

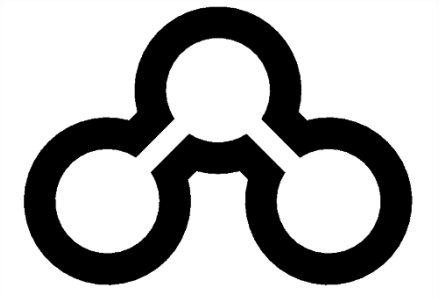

Figure 1. µPAD design

The µPAD design was developed using Autodesk Inventor 2023 software to ensure optimal accuracy and efficiency of reagent flow to the detection zone. The µPAD consists of two main parts: a hydrophilic area as a medium for holding reagents and samples and where chemical reactions occur, and a hydrophobic barrier that forms a pattern to limit the hydrophilic area according to the design and prevent channel leakage. The hydrophobic zoning is tailored to the reaction requirements, including one inlet zone for reagent dripping, connected via a microchannel to two outlet zones where glucose will be dripped for reaction. Glucose sample dripping will be performed once the reagent has moistened the outlet zone. These zones are specifically divided into the inlet zone (the reagent-receiving area), the outlet zone (the detection area), and the microchannel (the connecting channel that regulates fluid flow). This design pattern is applied to all concentrations. µPAD fabrication is carried out on Whatman no.1 paper using a plotting technique with the help of a 3D-printed template pattern, as an alternative when a wax printer is not available. The fabrication stages include placing the template pattern for plotting, then drawing with a permanent marker to create a hydrophobic zone.

The µPAD-based portable detection system has become an innovative solution in point-of-care diagnostics because it combines ease of use, low cost, and high portability. This platform uses colorimetry for detection. The colorimetric detection technique involves observing color changes that occur in the µPAD. The main advantage of this system is its ability to capture images using simple, affordable devices, making it ideal for applications in remote areas or health facilities with limited resources.

The device is fabricated using plywood and wood glue as base materials, making it easy to produce and cost-effective. The entire interior surface of the device is painted black to create a dark environment, preventing light reflections that can interfere with the accuracy of RGB-based colorimetric measurements, and ensuring consistent lighting and stable image-capture conditions. This modular design features sides that open for easy sample placement, optimizing lighting in a closed space, and is easy to replicate, making it a portable and cost-effective solution for µPAD analysis. Furthermore, sample and reagent application on the µPAD is performed using a 10 µL micropipette to ensure measurement precision and

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efficiency, in accordance with the dimensions of the µPAD microfluidic channels.

In preparing the test samples, this study will use five glucose levels: 60 mg/dL, 120 mg/dL, 180 mg/dL, 240 mg/dL, and 300 mg/dL. These concentrations were chosen because they represent the general range of glucose concentrations in humans. Preparing these concentrations requires several steps.

The first step is to prepare a 1000 mg/dl stock volume. The stock solution will serve as the master solution, used as the basis for preparing all lower-concentration glucose standard solutions by dilution. Dilution to a target volume of 5 mL at the desired concentration is shown in Table 1.

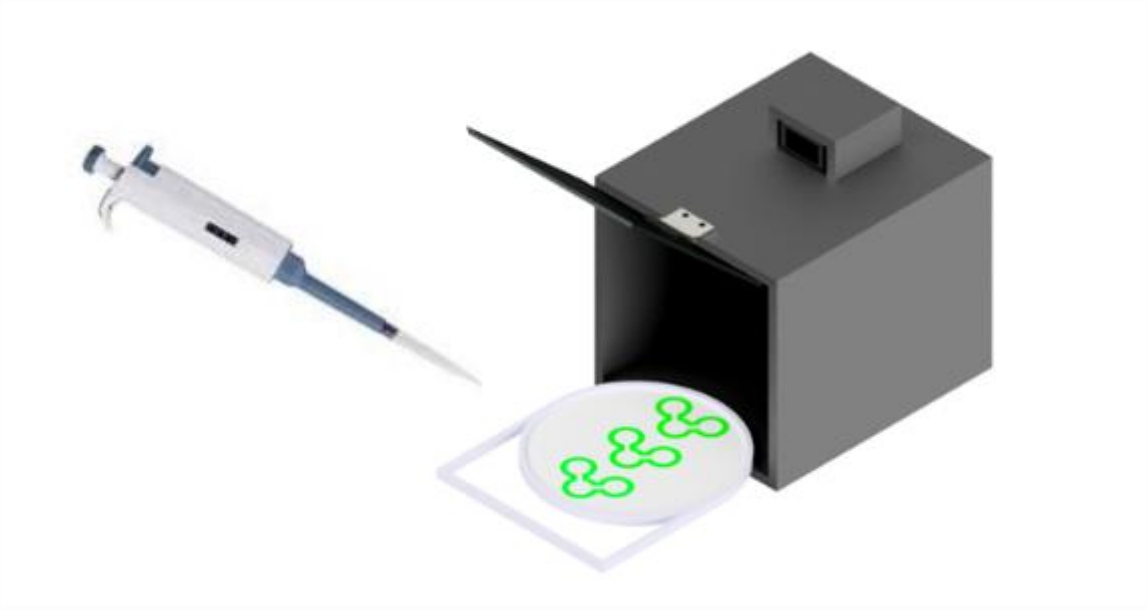

Figure 2. Portable detection system

Table 1. Sample Composition

Concentration target (mg/dL)	Stock volume 1000 mg/dL (mL)	PBS Volume (mL)	Total Volume (mL)
60	0.3	4.70	5
120	0.6	4.40	5
180	0.9	4.10	5
240	1.2	3.80	5
300	1.5	3.50	5

Glucose concentration was analyzed using the µPAD design via image analysis. RGB values were determined by adding 4×10 µL of reagent solution to the inlet zone. Next, 10 µL of glucose solution was added to both outlet zones after the reagent had moistened them. Color intensity analysis was performed at 1 minute, 5 minutes, and 10 minutes after the reagent met the glucose sample. The µPAD image was captured with an ESP32-CAM, saved as a JPEG, and then analyzed in RGB using ImageJ, following the procedure below.

1. Open the ImageJ program, click the file menu→open (select the image to be analyzed) → ok.

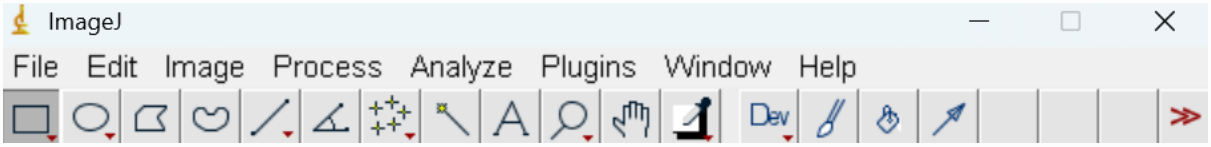

Figure 3. Image J Software

e-ISSN 2623-0313
p-ISSN 3032-1972

2. Select the region of interest at the bottom of the ImageJ menu, and set the area to be analyzed.

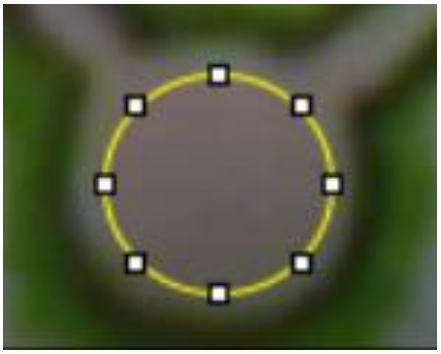

Figure 4. Region of Interest

3. To retrieve RGB data, click plugins→analyze→RGB measure. The RGB measurement results will be displayed in ImageJ as shown in the image.

Results

File Edit Font Results

	Label	Area	Mean	Min	Max
1	Red	2248	106.189	91	121
2	Green	2248	96.193	86	107
3	Blue	2248	93.680	77	104
4	(R+G+B)/3	2248	98.650	86	111
5	0.299R+0.587G+0.114B	2248	98.966	87	111

Figure 5. RGB measure

4. The next RGB value is recorded and applied to all glucose concentrations.
5. Reaction test observations are carried out in 6 detection zones for each glucose concentration.
6. After obtaining the total RGB values, each sample RGB is averaged.
7. The average values of the RGB are plotted on a graph to see which one produces the best linearity and precision.
8. The color intensity is analyzed from the RGB values obtained, mainly by examining the ratio between the RGB components.

$$R = \frac{R}{R+G+B} \quad (4)$$

$$G = \frac{G}{R+G+B} \quad (5)$$

$$B = \frac{B}{R+G+B} \quad (6)$$

9. After the color intensity is obtained, a standard curve is constructed by plotting the RGB ratio intensity (y-axis) against the measured glucose concentration (x-axis). From this standard curve relationship, a regression equation can be determined.
10. Glucose quantification can be determined if the ratio of one color aspect has the highest linearity among the others.
11. The best ratio will be entered into the regression equation that has been obtained, where the ratio value is entered into the y variable, and then the x variable is the calculated glucose concentration value.

Quantification of glucose by spectrophotometry using a filter at a wavelength of 505 nm, as shown in Table 2.

e-ISSN 2623-0313
p-ISSN 3032-1972

Table 2. Spectrophotometry testing procedure

Dispense	Blank	Standard	Sample
Reagent 1	1 ml	1 ml	1 ml
Distilled water	10 µl	-	-
Standard	-	10 µl	-
Sample	-	-	10 µl

$$Glucose\ mg/dl = \frac{A_x}{A_s} \times ntration\ of\ Standard \tag{7}$$

$$R^2 = \frac{[n\sum(x_i\,y_i) - \sum x_i \sum y_i]^2}{[n\sum x_i^2 - (\sum x_i)^2][n\sum y_i^2 - (\sum y_i)^2]} \tag{8}$$

$$D = \sqrt{\frac{\sum_{i=1}^{n}(x_i - \bar{x})^2}{n-1}} \tag{9}$$

$$RSD = \frac{SD}{\bar{x}} \times 100\% \tag{10}$$

Absorbance readings of the standard and sample were taken against the reagent blank, and the reaction showed an increase in absorbance with increasing concentration. Concentration calculations using the spectrophotometry method followed equation (7).

Validation was performed by comparing µPAD color-intensity readings with spectrophotometer absorbance readings. In this experiment, the glucose concentration curve will be shown using color intensity for the colorimetric method, and the glucose concentration will be shown as the absorbance value.

The correlation coefficient R^2 is used to indicate the accuracy of the average of all coordinate points on the standard curve relative to the linear line obtained from the regression equation. The closer the correlation coefficient R^2 is to 1, the better the linear model explains the data.

Precision is indicated by the standard deviation, and the relative standard deviation is used to determine the accuracy of the sample solution measurement data obtained using equations (9) and (10).

In measuring precision, RSD indicates the method's relative precision. According to Minister of Health Regulation No. 43/2013, the acceptable maximum RSD value is 5% [12].

This study also used a paired t-test to assess whether there was a statistically significant difference between the glucose levels measured by the two methods. In statistical analysis, a p-value greater than 0.05 indicates no significant difference between the two methods, whereas a p-value less than or equal to 0.05 indicates a significant difference.

RESULT AND DISCUSSION

The µPAD design uses a micropipette to dispense 40 µL of reagent and 20 µL of glucose sample into two outlet zones, aiming to optimize the hydrophilic zone area and pattern for spatial efficiency. The dimensions of the µPAD are optimally determined for the inlet zone (reagent dispensing), outlet zone (glucose dispensing), and microchannel. The inlet zone is assumed to have a diameter of 10 mm, designed to hold a total of 40 µL (four 10 µL drops) and ensure flow to the outlet zone. The reaction zone is also set to 10 mm in diameter to ensure sufficient volume for easy quantification of color changes. The microchannel is designed with a length of 5.2 mm and a width of 2 mm to prevent fluid drying and ensure the required flow rate. The selected µPAD pattern is a '∨' shape (with a 90° angle) due to its space efficiency and suitability for the requirements of one inlet zone and two outlet zones. This final design, with 10 mm-diameter inlet and outlet zones and a 5.2 mm x 2.00 mm microchannel, is expected to retain the liquid without leakage or premature drying.

In fabricating the portable detection system, this study utilizes ESP32-CAM to capture images of the reaction test results. The detection system design will follow the design in Figure 2. The fabrication results of the portable detection system are shown in Figure 7.

e-ISSN 2623-0313
p-ISSN 3032-1972

In the spectrophotometric method, the calculated glucose value is obtained by first measuring the absorbance of the 100 mg/dL glucose standard and entering it into equation (7). The absorbance value of the 100 mg/dL glucose standard is 0.01 at a wavelength of 505 nm, as shown in Table 3.

From spectrophotometric measurements, glucose concentrations were close to the expected values. Thus, the glucose samples used in spectrophotometry can also be used in colorimetry.

The results of glucose quantification using the colorimetric method began with measuring the RGB (Red, Green, Blue) values of digital images in ImageJ. Further data processing aimed to find the ratio values of each color intensity following equations (4); (5); (6) as shown in Table 4.

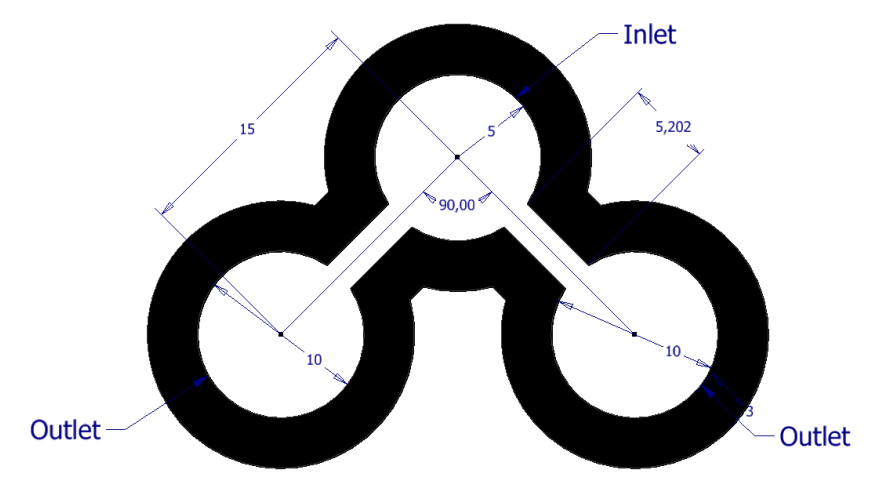

Figure 6. µPAD final design

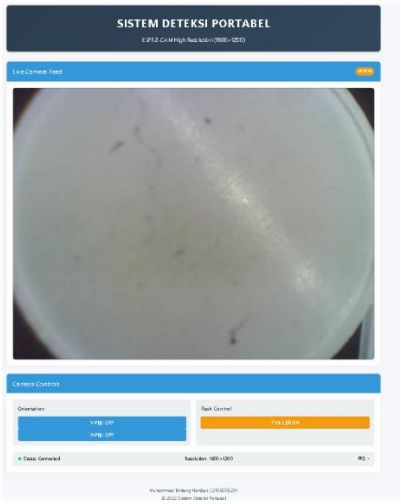

Figure 7. Portable detection system

Table 3. Spectrophotometry glucose quantification

No	Glucose Sample (mg/dL)	Absorbance	Quantified glucose
1	60	0.006	60
2	120	0.012	120
3	180	0.018	180
4	240	0.024	240
5	300	0.035	350

e-ISSN 2623-0313
p-ISSN 3032-1972

Table 4. RGB ratio value

Minute	Concentration (mg/dL)	Ratio R	Ratio G	Ratio B
	60	0.355	0.330	0.315
	120	0.363	0.333	0.304
1	180	0.364	0.328	0.308
	240	0.365	0.330	0.305
	300	0.371	0.332	0.297
	60	0.365	0.330	0.305
	120	0.374	0.332	0.294
5	180	0.373	0.330	0.297
	240	0.380	0.337	0.283
	300	0.390	0.338	0.273
	60	0.364	0.331	0.305
	120	0.373	0.332	0.295
10	180	0.376	0.329	0.297
	240	0.383	0.333	0.284
	300	0.394	0.336	0.269

The data from the ratio will then be plotted on a graph to determine its linearity.

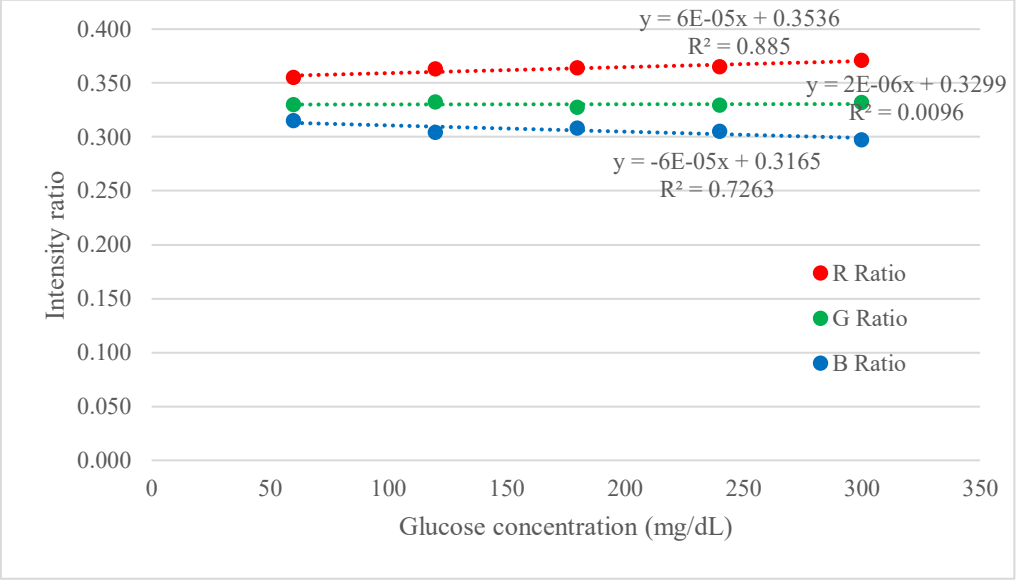

Figure 8. One-minute ratio graph

e-ISSN 2623-0313
p-ISSN 3032-1972

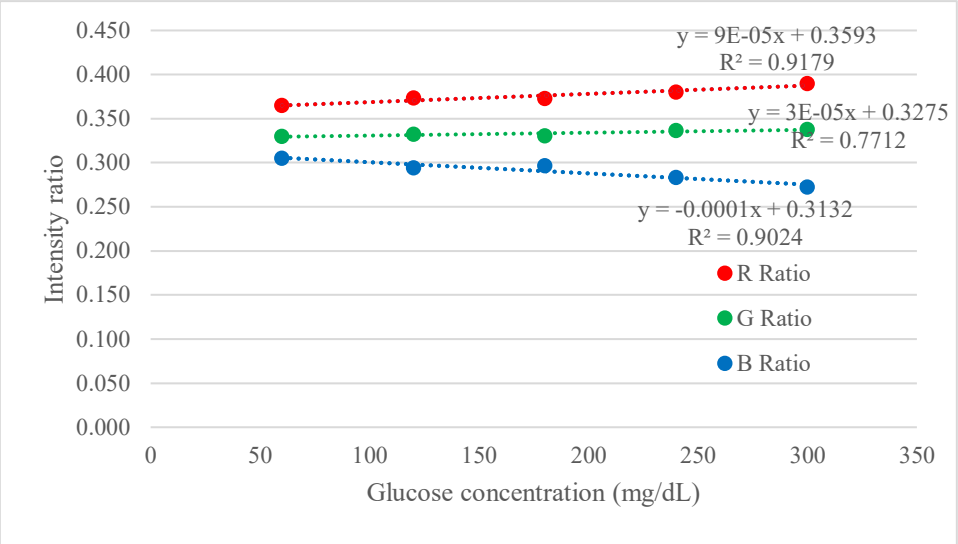

Figure 9. Five-minute ratio graph

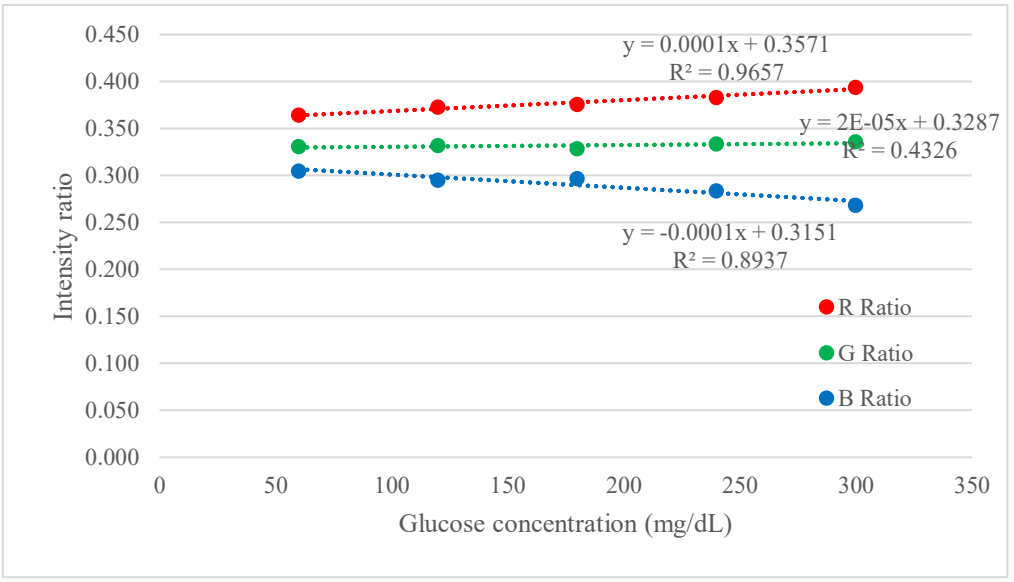

Figure 10. Ten-minute ratio graph

The sample selection is presented in Table 5. The sample selection is also followed by a linearity calculation.

Table 5. Red, Green, Blue Ratio

Minute	R Ratio	G Ratio	B Ratio
1	88%	0.9%	72%
5	91%	77%	90%
10	96%	35%	86%

The R ratio at the 10th minute is the best linearity. Next, glucose quantification by colorimetry is calculated by substituting the R ratio value into variable y; then x is the calculated glucose result. In Table 5, the R ratio at the 10th minute is the best linearity with the regression equation y = 0.0001x + 0.3571.

Table 6. Colorimetric glucose quantification

No	Glucose Sample (mg/dL)	R Ratio	Quantified glucose (mg/dL)
1	60	0.364	64.0730
2	120	0.373	139.2717

e-ISSN 2623-0313
p-ISSN 3032-1972

No	Glucose Sample (mg/dL)	R Ratio	Quantified glucose (mg/dL)
3	180	0.376	160.8032
4	240	0.383	222.5172
5	300	0.396	327.0036

Table 7. RGB ratio RSD

No	Glucose Concentration	RSD (R Ratio at 10 Minutes) %
1	60 mg/dl	1.92%
2	120 mg/dl	2.01%
3	180 mg/dl	2.13%
4	240 mg/dl	2.09%
5	300 mg/dl	2.53%

Table 8. Glucose quantified by colorimetry and spectrophotometry

No	Colorimetry	Spectrophotometry
1	64.0730	60
2	139.2717	120
3	160.8032	180
4	222.5172	240
5	327.0036	350

The red channel (R) ratio at the 10th minute showed the best linearity with glucose concentration, with a coefficient of determination (R^2) of 0.96 and a regression equation of $y = 0.0001x + 0.3571$. This indicates that changes in color intensity in the R channel significantly represent an increase in glucose concentration in the sample. Therefore, the R ratio at the 10th minute was selected as the primary parameter for glucose calculation using the µPAD-based colorimetric method. For comparison, the spectrophotometric method used as the reference showed very high linearity ($R^2 = 0.98$), confirming that the colorimetric results were comparable to the standard method. Thus, the R ratio at the optimal time can serve as the basis for quantitative glucose measurement using a digital color reading system. The precision values for the colorimetric method are shown in Table 7.

The %RSD ranged from 1.92% to 2.53%, indicating high precision (a %RSD < 5% is considered good in bioanalytical methods). The lowest %RSD value (1.92%) at 60 mg/dL indicated the best reproducibility, while the highest was at 300 mg/dL (2.53%). An increase in glucose concentration did not significantly increase %RSD, indicating that the measurement method was stable within the tested range.

The paired t-test in this study used glucose concentration values derived from measurements using the colorimetric and spectrophotometric methods at 505 nm.

Using a significance level (α) of 0.05 (which means a confidence level of 95%), the critical t-value for a two-tailed test with 4 degrees of freedom is 2.776. Since the absolute value of the t-count (|-0.893|) is smaller than the critical t-value (2.776), or in other words, the p-value (0.42) is greater than α (0.05). Based on the paired t-test results, there is no statistically significant difference between the colorimetric and spectrophotometric measurements at the 95% confidence level. This shows that both methods yield consistent results on the test data.

CONCLUSION

The linear regression analysis showed correlation coefficients of 96% for the colorimetric method and 98% for the spectrophotometric method. Precision testing showed that the overall relative standard deviation (RSD) was less than 5%, indicating good repeatability of the measurements. Furthermore, the paired *t*-test yielded a p-value of 0.42 at the 95% confidence level, indicating no significant difference between glucose quantification results obtained using the colorimetric and spectrophotometric methods.

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AUTHOR CONTRIBUTION

Muhammad Bintang Herdian: Conceptualization, Methodology, Validation, Data curation, Visualization, Writing - original draft. Ridho Irwansyah: Resources, Project administration, Supervision, Writing - review.

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