

A Simple Methods for Determination of Methylene Blue using Smartphone-Based as Digital Image Colorimetry

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Abstract

The advancement of smartphones that produce high-resolution photos has made their use even more developed. One application that is currently developing is a digital image produced as colorimetry. Digital image-based colorimetry is an easy and simple determination method and can be an alternative for analyzing dyes quantitatively. In this method, the color response of the digital image is analyzed, namely red-green-blue (RGB) with values from 0 to 255. This data is extracted from digital images using ImageJ 1.52a software for quantitative determination. In this study, 2 standard methods were used, namely external standards and standard addition to evaluating the most suitable method to obtain accurate measurements. In addition, 2 methods were used to determine the relationship of response to methylene blue levels. The 1st method used simple linear regression of each color red, green, or blue (univariate). The 2nd method is used in multivariate linear regression of the 3 colors red, green, and blue. The accuracy of the method was validated using UV-Vis spectrophotometry as the reference method. The results showed that the multivariate models produced very good accuracy, namely precision of up to 99 % compared to the simple regression method with the univariate.

Keywords: Methylene blue, Digital image, Smartphone, Colorimetry, Standard addition

Introduction

Methylene blue (MB) is a cationic dye that is widely applied in textile dyeing and in various other uses such as chemical indicators and biological dye [1,2]. Despite having a variety of uses, MB dye also has drawbacks, such as the increased production of MB waste into the environment [3]. Dyed wastewater has characteristics such as a high concentration of organic matter, poor biodegradability, and greatly affecting the health of microorganisms and causing water pollution [4]. This makes researchers try to find ways to reduce or degrade these dyes by various methods. Laboratory-scale tests were carried out so that a method for determining the reduction in the concentration of the MB substance was also needed.

Several measurement techniques were used to measure MB concentration such as UV-Vis spectrophotometry, mass spectrometry, and electrochemistry [5-7]. However, the development of a fast, simple and inexpensive analytical methodology for MB analysis is needed for applications in remote areas with limited instrumentation. Digital image-based colorimetry is an easy and simple determination method and can be an alternative for analyzing dyes [8]. Firdaus *et al.* [9] mention several advantages of this method, such as the analytical procedure that is environmentally friendly, inexpensive, portable, and simple to analyze various chemical compounds. This method is carried out with the help of an application to measure red-green-blue or RGB color and its relationship with analyte concentration [10].

Several things need to be considered in the digital image-based colorimetry in determining sample concentration [11]. Technically, sampling should be done with preparations such as constant lighting conditions [12]. This is because the light in the environment can be a variable that affects the color of the sample [13]. To overcome this, it is necessary to use methods such as the use of photo studios that have constant and homogeneous lighting. The advantage of digital image-based analysis is that it can minimize

the costs that are usually used from using expensive instruments. In addition, another advantage is that the digital camera on smartphones is used by almost many people so this method is easy and fast [14]. Research on digital image colorimetry on smartphones for chemical analysis has shown great interest in recent years, such as the analysis of metals Hg^{2+} [15], Cu^{2+} [16], paraoxon herbicides [17], methyl parathion pesticides [18], and tetracycline [19].

The external standard method is the most frequently used method in the quantitative analysis [20]. This method uses a series of standards, creates a calibration curve, and then determines an unknown sample from that curve [21]. This method is widely used because the procedure is simpler. However, there are situations where this method approach does not work well. A common problem that can occur in this method is the matrix effect [22,23]. The matrix components can interact with the analyte and change its response in the measurement technique. Meanwhile, in the standard addition method, the standard is added to the sample to correct the 'matrix effect' called spiking [24,25].

Springer *et al.* [8] have used smartphone-based colorimetric methods to determine methylene blue in samples of human and animal dosage forms. This study evaluates operational variables, such as capture devices, angle, and modes of capture to obtain accurate measurements. However, the use of the univariate and multivariate models, and variations of standard methods in the determination of MB as digital image colorimetry, have not been previously reported. In this study, MB was analyzed using smartphone as digital image-based colorimetry. The accuracy of the method was compared to that of a UV-Vis spectrophotometry using the external standard method and standard addition. The digital camera results obtained are processed using ImageJ software to determine the RGB value in the selected area so that it involves thousands of pixels. This research is focused on using these alternative methods to obtain an accurate MB dye concentration value more economically and simply.

Materials and methods

Materials, apparatus and software

The materials used in this study were methylene blue ($\text{C}_{16}\text{H}_{18}\text{ClN}_3\text{S}$, Merck) and distilled water. The sample used to confirm the level of methylene blue (MB) was a drug for treating fungal infections of fishes used in the aquarium, which was purchased at the local market (Bandung, Indonesia). The smartphone camera Redmi Note 10 Pro (Xiaomi, China), is used to record images for color viewing based on digital images. The lights used are flashlights from Smartphone Samsung Galaxy M21 Smartphone LED lights (Samsung, Seoul, South Korea). A UV-Vis spectrophotometer (Thermo Fisher Scientific Genesys 10S, Waltham, USA) was used as a reference instrument for quantitative analysis. Quartz cuvettes (1 cm path length) were used in digital image colorimetry and spectrophotometric determinations. ImageJ 1.52a, Excel 2019 (Microsoft Inc.), and Minitab 19 (Minitab Inc.) were used for digital image data processing.

Photographic procedure

The solutions of methylene blue were placed in a quartz cuvette with an optical path of 1 cm on a white background to maintain the same ambient light and photographic conditions. The distance from the flashlight to the sample is 20 cm, while the distance from the camera is 25 cm. Colors were obtained from a representative rectangular area located in each sample. Average RGB values are measured with the ImageJ image processing toolbox. Digital image retrieval and ImageJ processing were carried out in 3 replicates. Further analysis of the RGB data was performed by Excel and Minitab for simple linear regression and partial least squares, respectively. RGB color values are converted to a logarithmic scale to obtain color intensity, following the Lambert-Beer law equation [26].

Reference method and sample analysis

The reference spectrophotometric procedure was carried out using methylene blue in a suitable concentration range. Methylene blue was measured at the maximum absorption wavelength. Analysis of each sample was performed in 3 replicates, and concentrations were calculated from the standard calibration curve. The drug of the fish sample was diluted to a concentration that fell within the range of the calibration curve in a 25 mL volumetric flask. Two calibration methods are used, namely the external standard and standard addition method. The external standard method was made in the methylene blue variation range of 1.0 - 6.0 ppm. Meanwhile, in the standard addition method, 2 mL of the sample solution was used in a 25 mL volumetric flask. Then, 0, 2, 4, 6, 8 and 10 mL of 3 ppm of methylene blue standard were added. The sample concentration was calculated by the linear regression equation.

Results and discussion

Quantification of digital image

The average color values of R, G, and B were analyzed using ImageJ 1.52a software. A certain area of the image is selected as shown in **Figure 1**. The RGB color value selected a certain area consisting of several thousand pixels is important to get accurate results. **Table 1** shows the average R, G, and B color values for various concentrations of methylene blue. To obtain linear regression for further use, such as standard calibration curves, the RGB color values are converted to the intensity obtained using Lambert-Beer's law as follows (1);

$$I_{(R,G,B)} = \text{Log} (A_{0(R,G,B)}/A_{s(R,G,B)}) \quad (1)$$

$I_{(R,G,B)}$ is the effective intensity of the respective colors, and $A_{0(R,G,B)}$ and $A_{s(R,G,B)}$ are the average color values of the blanks and samples, respectively. The digital camera functions as a spectrophotometer that analyzes light from methylene blue. The intensity shown is the sum of the R, G and B color values of the digital image. The logarithmic conversion (intensity) of RGB values to various concentrations of methylene blue is shown in **Table 1**.

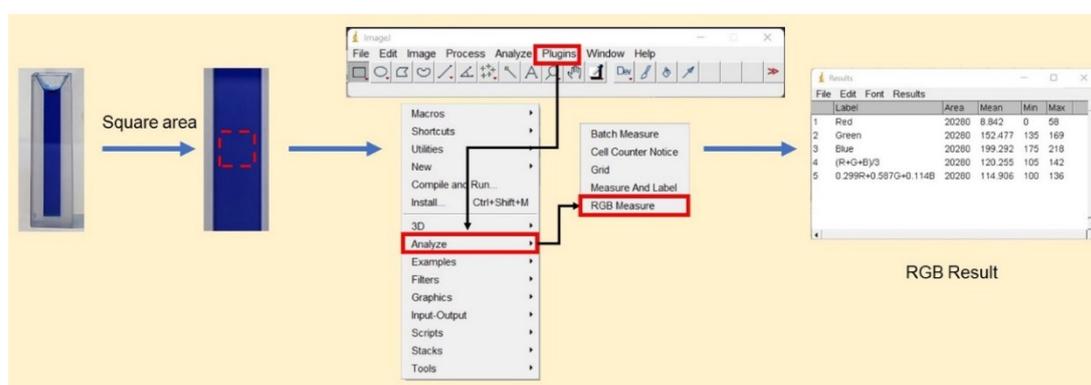


Figure 1 Selecting image area to get RGB values using ImageJ 1.52a software.

Table 1 Average RGB color value and effective intensity of methylene blue.

Concentration of methylene blue (ppm)	Average color value ^a			Effective intensity ^a		
	A _R	A _G	A _B	I _R	I _G	I _B
0.0	154.5	156.5	149.3	0	0	0
1.0	142.2	153.0	152.2	0.0359	0.0099	-0.0084
2.0	126.0	150.2	153.3	0.0884	0.0179	-0.0115
3.0	103.9	142.9	153.4	0.1725	0.0394	-0.0118
4.0	95.2	140.1	155.6	0.2103	0.0482	-0.0178
5.0	83.0	137.1	157.1	0.2700	0.0574	-0.0219
6.0	73.9	134.3	158.4	0.3205	0.0666	-0.0256

^a data was shown in the mean with 3 times of testing.

Analytical performance and application

The accuracy of the digital image method was evaluated using 2 standard methods. In this study, 2 types of standards were used, namely the external standard method and the standard addition method.

External standard method

The standard variation of methylene blue concentration of 1.0 - 6.0 ppm was used as the external standard. **Figure 2(a)** shows a digital image of methylene blue in various concentrations. Then, a linear relationship was made between the response of RGB color intensity vs MB concentration. Thus, the

resulting calibration curve $y = ax + b$ is obtained, where y represents the intensity of the red, green, and blue colors of the digital image and x is the concentration of MB. **Figure 2(b)** shows that the slope value is 0.0554; 0.0116, and -0.0039 for R, G, and B, respectively. The 3 colors have very good linearity with a correlation coefficient (R^2) above 0.9 with red having the highest R^2 of 0.9928. In addition, these results show that red has the highest sensitivity compared to green and blue. So that for univariate calculations, red will be used as a response to determine sample levels.

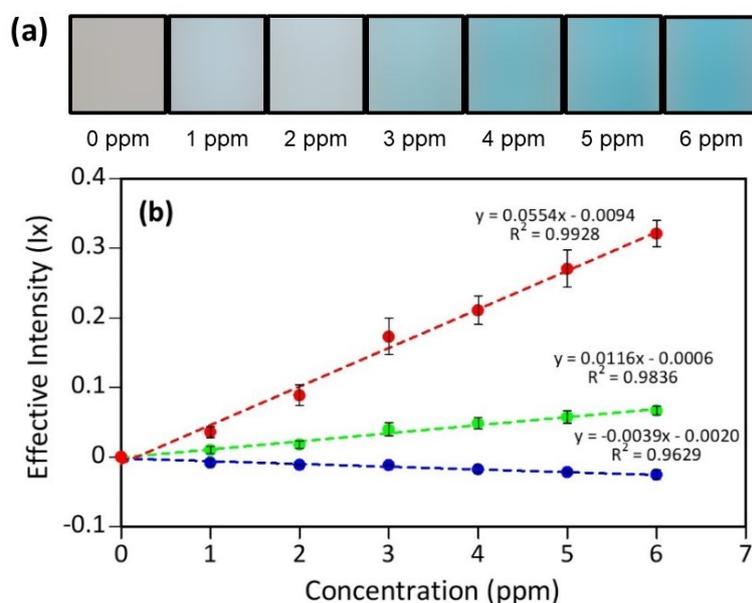


Figure 2 Digital image of methylene blue in various concentrations (a) and calibration curves of the responses of each red, green, and blue color using an external standard method (b).

In addition to univariate calculations, in this study, the concentration calculation will also be tested using the partial least squares method. In this case, calculations were carried out to determine the effect of 3 variables R, G, and B on the concentration of methylene blue. Simple linear regression calculated with Excel 2019 (Microsoft Inc.) provides the easiest calculations as is usually done with standard calibration curves in UV-Vis spectrophotometry. On the other hand, the partial least squares calculated using Minitab 19 software (Minitab Inc.) is a better calculation method by treating RGB data as multivariate which results in a new linear regression [9], which is shown in Eq. (2). Regression multivariate is a technique used to measure the extent to which various independent variables and various dependent variables are linearly related. The resulting equation has correlation coefficient of 0.954.

$$[\text{Methylene Blue}] = 0.14 + 26.00 I_R - 65.00 I_G - 23.00 I_B \quad (2)$$

Standard addition method

The standard addition method is usually used to eliminate the matrix effect [27]. In this study, a variation of the standard amount was added to the analyte and diluted to the same volume. The resulting RGB values were then determined for all solutions and plotted to obtain a regression curve. In this case, the y-axis is the color response and the x-axis is expressed in terms of the standard volume of methylene blue analyte added. **Figure 3(a)** shows a digital image of the sample on various additions of 3 ppm methylene blue standard. The regression line is extrapolated to a point on the x-axis where $y = 0$ (**Figure 3(b)**). This negative intercept on the x-axis corresponds to the amount of analyte in the test sample [28]. In this method, the intensity of the red color is used in response to the addition of standard methylene blue. However, the correlation coefficient obtained is less good than the external standard method.

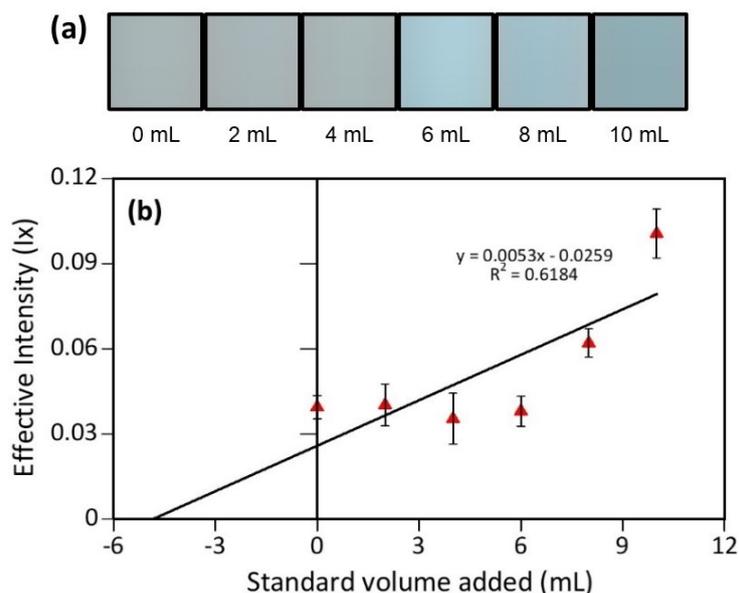


Figure 2 Digital image of the sample on various additions of 3 ppm methylene blue standard (a) and linear regression for red color response (b).

Method accuracy

Measurement accuracy is the close agreement between the value of the quantity measured and the actual value of the quantity. Accuracy studies imply an evaluation of the intermediate precision and correctness of the measurements expressed in bias with respect to the reference value (London *et al.* 2017). To validate the proposed method, in this study we carried out a recovery experiment of a real sample, which has its own sample matrix, using the external standard method and standard addition method. The drug for treating fungal infections of fishes used in the aquarium, which was purchased at the local market in Bandung, Indonesia was selected as the sample. The concentration of methylene blue was determined from the intensity of the red color from the external standard curve equation (**Figure 2**) for univariate and Eq. (2) using the intensity of red, green, and blue for multivariate. In addition, the standard addition method using the red intensity response was also used.

Using a UV-Vis spectrophotometer, the maximum wavelength of methylene blue was found. It is known from **Figure 4(a)** that methylene blue shows a maximum wavelength of 665 nm which is used to determine the absorbance of the sample. Then, the concentration variation is also used to create an external standard curve in **Figure 4(b)**. For the standard addition method, a plot between absorbance and standard volume was also plotted in **Figure 4(c)**. The results of the accuracy test are shown in **Table 2**. The accuracy, calculated using Eqs. (3) and (4), is calculated as relative error (RE) and precision value [29]. RE is a measure of precision used to describe accuracy [30]. Specifically, how accurate a measurement is compared to the true value.

$$\text{RE (\%)} = (C_{\text{true}} - C_{\text{obs}}) / C_{\text{true}} \times 100 \% \quad (3)$$

$$\text{Precision (\%)} = C_{\text{obs}} / C_{\text{true}} \times 100 \% \quad (4)$$

where C_{true} is the concentration of methylene blue calculated from a UV-Vis spectrophotometer and C_{obs} is the concentration of methylene blue calculated using a digital image.

Table 2 The test results to validate the method (n = 3).

Method	CDIC Smartphone (ppm)	C _{UV-Vis Spectrophotometry} (ppm)	RE (%)	Precision (%)
Univariate = Red				
External standard	84.1 ± 1.2	92.1	8.8	91.2
Standard addition	95.3 ± 2.5	87.2	9.3	109.3
Multivariate				
External standard	91.2 ± 0.5	92.1	1.0	99.0

From the proposed digital image-based colorimetric method, the univariate method gave 91 % precision for the external standard method and 109 % for the standard addition method. These results show that there is not much difference between digital images from the 2 methods. Moreover, multivariate results give 99 % precision. Therefore, this method has satisfactory accuracy.

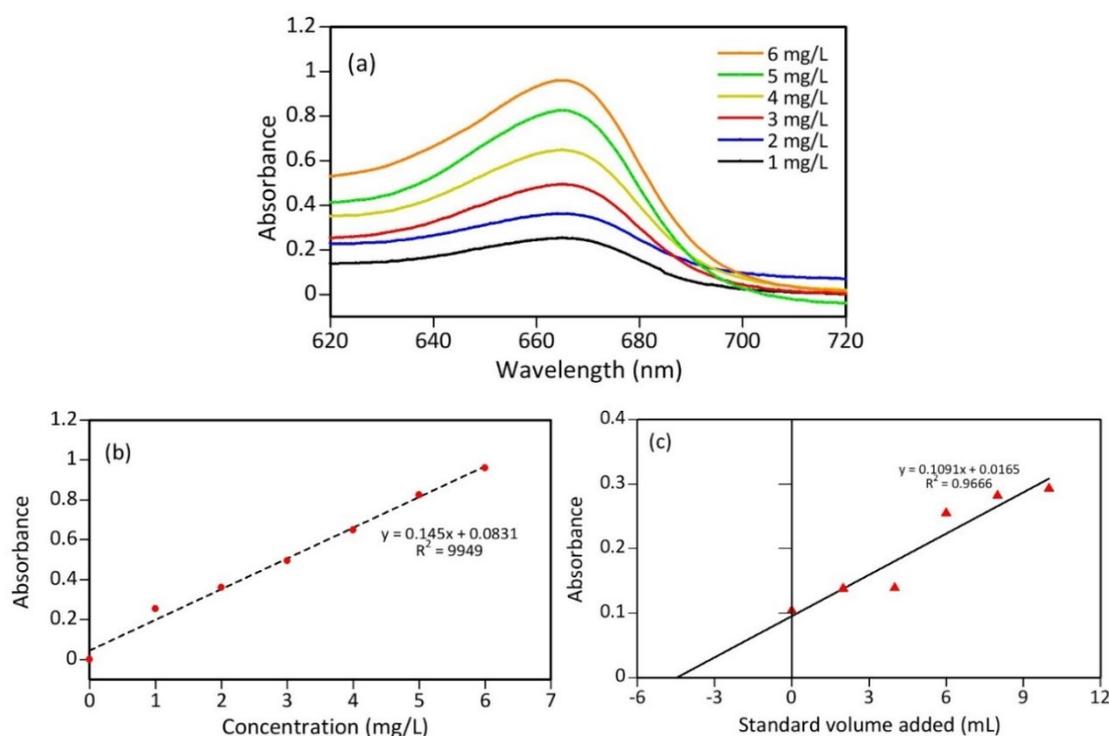


Figure 4 (a) Spectra of methylene blue, (b) Calibration curve of the external standard method, and (c) linear regression of various additions of 3 ppm methylene blue standard.

Conclusions

A simple, fast, and inexpensive colorimetric method for quantitative analysis of methylene blue content has been proposed using a smartphone camera. This method shows good accuracy with simple equipment without sophisticated instruments. The univariate models for external standard and standard addition methods using simple linear regression showed up to 90 % precision for fish drug samples. While the multivariate models for the external standard method give more precision results up to 99 % compared to the UV-Vis spectrophotometry which uses an expensive instrument.

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