

# Digital Photometric Determination of Protein Using Biuret, Bradford and Bicinchoninic Acid Reagents

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The concentration of dissolved protein was determined separately with biuret, bicinchoninic acid and Bradford reagents using conventional absorption spectrophotometry and digital photometry. The latter involved color analysis of the digital photographs using computer software (RGB analysis of image colors). Accuracy and precision of the digital photographic and conventional spectrophotometric methods were compared using t-test and F-test. No significant differences were observed in determining protein concentration at 95 % confidence level based on RGB-derived parameters, namely % luminance,  $a^*$ , and  $b^*$ ; similar results were obtained based on spectrophotometric and digital photometric methods. Results using the digital photometric method were accurate and repeatable. Comparison of average protein concentrations obtained using the spectrophotometric and digital photometric methods of a known solution showed that a fabricated light box gave a higher precision than an assembled set-up. The results

indicate the potential use of digital photometry, using a simple digital camera with free-access software and a fabricated light box, as an alternative to absorption spectrophotometry in the accurate determination of soluble protein concentration.

## KEYWORDS

Bicinchoninic acid, Biuret, Bradford, Digital photometry, Protein determination

## INTRODUCTION

Determination of protein in solution (Scopes 1987) usually involves the use of an absorption spectrophotometer in order to measure the concentration of a colored complex of protein and dye such as biuret, Coomassie Blue G-250 dye (Bradford assay) and bicinchoninic acid. Alternatively,  $\text{Cu}^+$  produced by oxidation of peptide nitrogen(s) reduces Folin-Ciocalteu reagent and forms a blue complex (Lowry assay). However, color measurement of the protein-dye complex may also be done by taking photographs of the colored solution using a digital camera followed by color analysis of the photographs using free-access computer software. An example of this software, which is available from the internet, uses RGB (red green blue) analysis of image colors and allows determination of RGB values of a given area of digital color photographs (Byers 2006).

Some applications of digital photometric analysis include estimation of red wine quality based on wine color (Almela et al.

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1995), study of insect and plant colors (Byers 2006), analysis of total dissolved organic matter (Hislop et al. 2010) and determination of degree of ripeness of papaya (Bron and Jacomino 2006, Calegario et al. 1997, Domingo et al. 2012).

The present paper deals with the application of digital photometry and color analysis in determining dissolved protein concentration based on biuret, Bradford and bicinchoninic acid assays in comparison with absorption spectrophotometry. The experimental results showed that the digital photometric method

using a simple digital camera, a fabricated light box and free-access software for RGB analysis of color images gave accurate and reproducible soluble protein concentrations which were not statistically different from those obtained using the conventional spectrophotometric method.

## MATERIALS AND METHODS

### Reagents and Equipment

Reagent grade chemicals from Sigma-Aldrich and J.T. Baker were used in the present study including bovine serum albumin (BSA), crude egg albumin, Coomassie Brilliant Blue G-250 (CBBG) and sodium bicinchoninate. Spectrophotometric analysis was done with a Shimadzu UV Mini 1240 Spectrophotometer and digital photographic data were gathered using a 12 megapixel Olympus FE-46 digital camera. The software RGB Analysis of Image Colors was used for color analysis of the photographs.

### Preparation of Sample and Stock Solutions

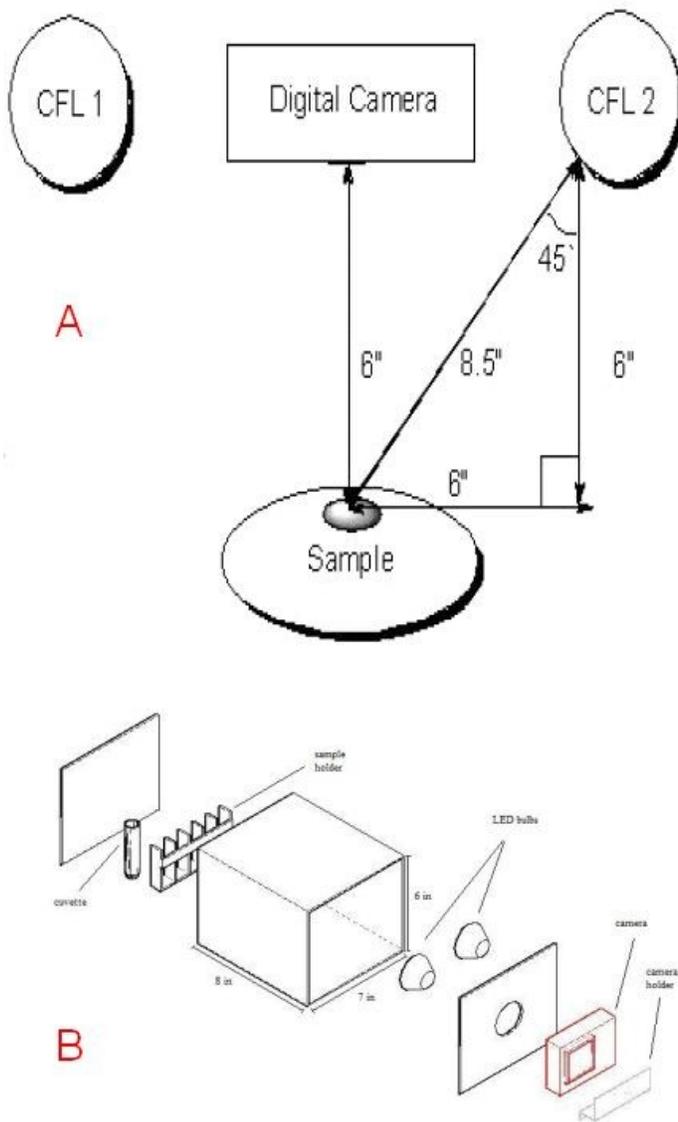
The sample for Biuret assay was prepared as 30 mg/mL egg albumin solution. The sample for Bradford and BCA assays was prepared as 1 mg/mL egg albumin solution. Stock solutions were prepared with BSA concentrations of 1, 10 and 20 mg/mL.

### Preparation of Protein Assay Reagents

**Biuret reagent.** Cupric sulfate pentahydrate (1.5 g) and sodium potassium tartrate (6 g) were weighed and dissolved in 500 mL distilled water. To the resulting solution were added 300 mL of 10 % NaOH with constant stirring. The resulting mixture was then transferred quantitatively into a 1 L volumetric flask and diluted to mark with distilled water.

**Bradford reagent.** Coomassie Brilliant Blue G-250 (CBBG), 100 mg, was dissolved in 50 mL 95 % ethanol. To the resulting solution were added 100 mL of 85 % (w/v) phosphoric acid and stirred overnight for complete dissolution. The resulting mixture was then transferred quantitatively into a 1 L volumetric flask and diluted to mark with distilled water. The reagent used for the day's work was filtered through Whatman no.1 filter paper prior to use.

**BCA reagent.** Reagent A: Sodium bicinchoninate (1 g), sodium carbonate (2 g), sodium tartrate (0.16 g), NaOH (0.4 g) and sodium bicarbonate (0.95 g) were dissolved in 50 mL distilled water. The resulting solution was transferred quantitatively into a 100 mL volumetric flask and diluted to mark with distilled



**Figure 1.** Schematic diagrams of (a) assembled set-up and (b) light box.

water. The pH of the resulting mixture was adjusted to 11.25 using 10 M NaOH.

Reagent B: Cupric sulfate pentahydrate (0.4 g) was dissolved in 5 mL distilled water. The resulting solution was transferred quantitatively into a 10 mL volumetric flask and diluted to mark with distilled water. For the standard working solution (SWR), 100 volumes of reagent A were added to 2 volumes of reagent B.

### Protein Assays

Published procedures for biuret, Bradford and BCA protein assays were followed with minor modifications (Bradford 1976, Gornall et al. 1949, Sedmak and Grossberg 1977, Smith et al. 1985, Spector 1978, Stoscheck 1990).

### Determination of Protein Concentration Using Digital Photometric Method

**Experimental set-ups.** The assembled digital photometric set-up for the initial experiments is shown in Figure 1(a). A white sheet of paper was placed on a table at the center of which was placed a transparent container. Two 15-watt fluorescent lights were placed 21.59 cm away from the transparent container on both sides of the container. Four mL each of the BSA standard were placed on the container. The camera was positioned using a tripod 15.24 cm directly above the flat surface. The room light was switched off and the two fluorescent lights were switched on. Using a Sony Super SteadyShot digital camera with 7.2 megapixels in a no-flash

setting, several pictures of each of the BSA standards were taken. Then the pictures were uploaded onto a computer.

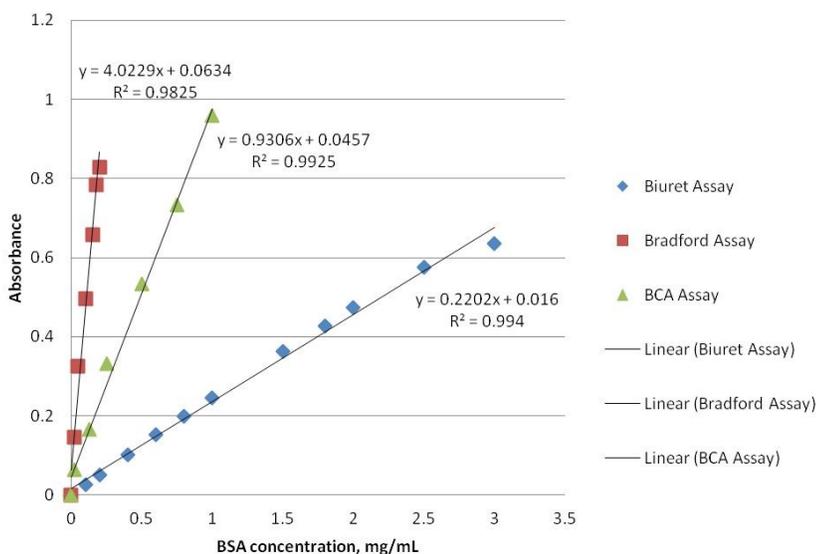
A light box made of plywood was fabricated based on the design of Suzuki et al. (2006); on one side of the box were mounted a digital camera and two white-color LEDs (0.6 watt each) with electric power supplied from a wall socket. On the other side of the box was mounted a sample holder. The schematic diagram of the light box is shown in Figure 1(b).

**Optimization of the Set-up.** Optimization was done by using  $\text{KMnO}_4$  solutions. First a stock solution of 0.01 M was prepared. A mass of 0.395 g of  $\text{KMnO}_4$  was weighed and dissolved in 100 mL distilled water. After all of the solids dissolved, the resulting solution was transferred quantitatively into a 250 mL volumetric flask and diluted to mark using distilled water. Standard solutions were prepared from the stock solution, mixed using a vortex mixer and allowed to stand for 2 min. The absorbance was then read at 525 nm in a spectrophotometer; three trials were performed. The absorbances obtained were averaged and plotted against the concentration of standard  $\text{KMnO}_4$  solutions in order to generate a standard curve.

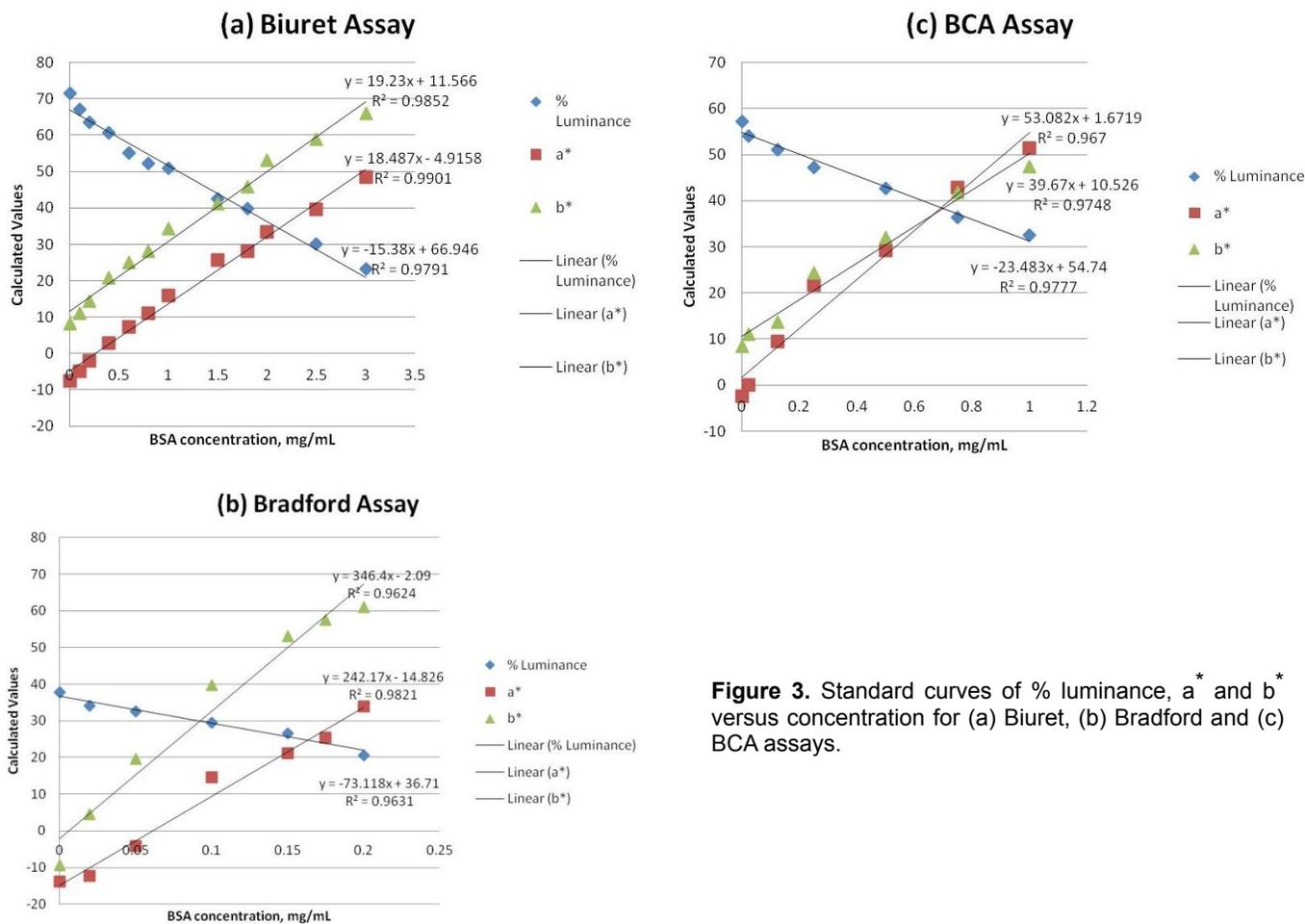
The initial dimensions of the box were 20.32 x 17.78 x 15.24 (length x width x height, all in cm) and were based on angle of view of the camera and camera-to-sample distance (Suzuki et al. 2006). The length of the box was made either longer (22.86 cm) or shorter (17.78 cm). The linearity of the standard curve generated from each of the boxes was evaluated and compared with the ideal correlation value of 1.00 using t-test at 95 % confidence level. The box that gave no significant difference from the ideal value was used for the rest of the experiments. A test for accuracy was performed on two permanganate solutions of known concentration, namely  $2.5 \times 10^{-4}$  and  $3.5 \times 10^{-4}$  M. The sample solutions were analyzed in three trials using spectrophotometry and digital photometry.

**Digital Photometry.** Digital photographic data were gathered using a 12 megapixel Olympus digital camera (model FE-46). An aliquot (1.5 mL) of each standard solution was placed in a cuvette. The digital camera settings were ISO 100, aperture value f3.5, shutter speed 1/125 and spot focus. The flash option of the camera was turned off so that only light coming from the LED lamps reached the photographed solution.

**RGB Determination using RGB Analysis of Image Colors.** Color analysis of the still photographs was done using the RGB Analysis of Image Colors software; this was downloaded from the Internet with JavaScript and Java applet



**Figure 2.** Standard curves for spectrophotometric protein determination using Biuret, Bradford and BCA assays.



**Figure 3.** Standard curves of % luminance,  $a^*$  and  $b^*$  versus concentration for (a) Biuret, (b) Bradford and (c) BCA assays.

which loads a digital image and analyzes the red, green and blue (RGB) components of the pixels in any rectangular area of relatively uniform color (Byers 2006). Percent luminance as well as  $a^*$  and  $b^*$  values were also calculated (Suzuki et al. 2006).

The calculated  $a^*$  and  $b^*$  values of the standard solutions with the same BSA concentrations were averaged and plotted against the BSA concentration. The equation of the line was obtained by linear regression and each sample concentration was interpolated using the sample  $a^*$  and  $b^*$  values.

### Validation of Accuracy

Solutions with known concentration were prepared using stock solutions of BSA and subjected to Biuret, BCA and Bradford assays. The solutions were analyzed using visible spectrophotometry and digital photometry. Different methods of calculating concentration from RGB values were performed; the calculated concentrations were compared with known BSA concentrations.

### Test for Repeatability

Digital photometric determination of protein concentration was repeated in order to assess experimental reproducibility. The set-up was reassembled and a new set of standards was prepared. The analysis was performed on the same day by the same analyst and average concentrations obtained from the duplicate experiments were compared.

### RESULTS AND DISCUSSION

Accuracy of the results was determined using linear regression analysis. The concentrations of sample permanganate solutions were calculated from % luminance,  $a^*$  and  $b^*$  plots and the experimentally determined concentrations were compared with theoretical values using t-test at 95 % confidence level; the value of  $t_{tab}$  was 4.303.

It can be seen in Table 1 that the measured concentrations were not significantly different from theoretical values at 95 %

confidence level. Spectrophotometric analysis and the different methods for calculating permanganate concentration based on digital photometry were found to be accurate.

### Biuret Assay

The biuret reagent changes from blue to violet in the presence of proteins. The violet color is due to the complex formed from the cupric ions ( $\text{Cu}^{2+}$ ) and four nitrogen atoms of peptide chains (Gornall et al. 1949). The colored complex is most sensitive at 540 nm; at this wavelength absorbance is proportional to the concentration of proteins. The standard curve generated from spectrophotometric analysis is shown in Figure 2.

The concentrations of sample solutions were determined using linear regression analysis based on average absorbances of sample solutions and the linear equation of the calibration curve. The computed concentrations of sample solutions are presented in Table 2.

### Determination of protein concentration using % luminance

The same standard and sample solutions previously used were also used in the digital photometry. The standard curve for the digital photometric analysis using the % luminance equation is shown in Figure 3. The RGB values of the sample solutions were used to calculate % luminance and the concentrations of sample solutions.

### Determination of protein concentration using $a^*$ and $b^*$ plots

The  $a^*$  and  $b^*$  values, which were obtained from RGB values of the standard solutions, were found to increase with increasing concentration. Negative  $a^*$  values indicate green while positive values indicate red; on the other hand, negative  $b^*$  values indicate blue and positive values indicate yellow (Suzuki et al. 2006). The concentrations from 0 – 0.20 mg/mL showed negative  $a^*$  values, which means that the green component of these solutions was higher than the red component. The  $a^*$  and  $b^*$  values of the sample solutions were averaged and used to calculate concentrations of sample solutions.

Based on the results, no significant difference was observed at 95 % confidence level between the concentrations obtained using a spectrophotometer, on the one

hand, and  $a^*$  and  $b^*$  plots (digital photometry), on the other hand. Moreover, concentrations obtained from  $a^*$  and  $b^*$  plots were not significantly different from each other.

The obtained concentrations (using Biuret assay) from % luminance were also compared with concentrations obtained from  $a^*$  and  $b^*$  plots. No significant difference was observed between concentrations obtained from % luminance and  $a^*$  and  $b^*$  plots at 95 % confidence level (Table 2).

### Bradford Assay

The Bradford assay involves binding of Coomassie Brilliant Blue G-250 dye with protein resulting in the blue form of the dye which has an absorption maximum at a wavelength of 595 nm. Moreover, it is important to mix the tubes using a vortex mixer in order to disperse the protein-dye aggregates which may interfere in the analysis. The standard curve using spectrophotometric analysis is shown in Figure 2.

RGB values of sample solutions were used to calculate % luminance and then concentrations of the sample solutions.

Based on the results, no significant differences were observed between concentrations of the samples obtained from spectrophotometric analysis and % luminance plots at 95 % confidence level.

The  $a^*$  and  $b^*$  values of the sample solutions were calculated and then concentrations of sample solutions.

Based on results of the t-test, no significant differences were observed between concentrations obtained from spectrophotometric analysis and  $a^*$  and  $b^*$  plots at 95 %

**Table 1.** Test for accuracy of experimental methods for measuring permanganate concentration using % luminance,  $a^*$  and  $b^*$  plots.

Method		$\text{KMnO}_4$ Concentration (mmol/L)	Standard Deviation	$t_{calc}$	Significant Difference?
Spectrophotometry	Sample 1	0.240	0.004	1.333	No
	Sample 2	0.335	0.005	1.263	No
% Luminance	Sample 1	0.229	0.016	0.708	No
	Sample 2	0.335	0.016	0.704	No
$a^*$	Sample 1	0.226	0.017	0.691	No
	Sample 2	0.330	0.016	0.708	No
$b^*$	Sample 1	0.237	0.020	0.283	No
	Sample 2	0.352	0.021	0.000	No

confidence level. Similarly, no significant differences were observed at 95% confidence level between concentrations obtained from the a\* and b\* plots, as well as between concentrations obtained from % luminance, on one hand, and a\* and b\* plots, on the other hand (Table 2).

### BCA Assay

The BCA (bicinchoninic acid) assay involves a color change from green to purple in the presence of proteins. The purple-colored complex is due to the chelation of two molecules of BCA to the cuprous ion. The complex formed absorbs strongly at a wavelength of 562 nm. Incubation at a higher temperature increases the sensitivity of the assay by assisting in the formation of the complex. The standard curve using spectrophotometric analysis is shown in Figure 2.

RGB values of the sample solutions were used to calculate % luminance and then solution concentrations of the sample solutions. Using linear regression analysis, an equation of the straight line in Figure 3 was computed and concentrations of sample solutions were determined. Table 2 shows the calculated concentrations of sample solutions based on absorbance and luminance.

Based on the results, no significant differences were observed between concentrations of the samples obtained from absorbance and % luminance plots.

The calculated a\* and b\* values were then averaged and by using linear regression analysis, the concentrations of the sample solutions were determined.

Based on results of the t-test, no significant differences were observed between the concentrations obtained from spectrophotometric data and a\* and b\* plots at 95 % confidence level (Table 2). Similarly, no significant differences were observed between concentrations obtained from the a\* and b\* plots at 95 % confidence level.

The obtained concentrations from % luminance were also compared with those obtained from a\* and b\* plots. Based on results of the t-test, no significant differences were obtained between the concentrations obtained from % luminance and a\* and b\* plots at 95 % confidence level (Table 2).

### Validation of Accuracy

This was done to check the validity of the different methods performed. All the standard curves previously used were also employed for the validation of accuracy. The protein concentrations were obtained using linear regression analysis.

The obtained concentrations were then compared with the known BSA concentration using t-test at 95 % confidence level; the value of  $t_{tab}$  was 4.303.

Based on the results, there were no significant differences

**Table 2.** Comparison of protein concentrations obtained from spectrophotometry, % luminance, a\* and b\* plots for Biuret, Bradford and BCA assays.

Sample*	Spectrophotometry		% Luminance		Significant Difference?	a*		Significant Difference?	b*		Significant Difference?	
	Mean Concentration (mg/mL)	Std. dev	Mean Concentration (mg/mL)	Std. dev		Mean Concentration (mg/mL)	Std. dev		Mean Concentration (mg/mL)	Std. dev		
<b>Biuret Assay</b>												
1	2.449	0.011	2.404	0.036	No	2.399	0.052	No	2.440	0.037	No	
2	0.971	0.005	1.058	0.074	No	1.043	0.100	No	1.015	0.151	No	
<b>Bradford Assay</b>												
1	0.068	0.004	0.084	0.027	No	0.060	0.009	No	0.074	0.009	No	
2	0.096	0.006	0.104	0.008	No	0.094	0.003	No	0.099	0.003	No	
<b>BCA Assay</b>												
1	0.527	0.024	0.512	0.044	No	0.505	0.014	No	0.531	0.037	No	
2	0.819	0.021	0.827	0.031	No	0.808	0.039	No	0.752	0.058	No	

\*Egg albumin

**Table 3.** Validation of accuracy for measuring protein concentration using spectrophotometry and digital photometry based on Biuret, Bradford and BCA assays.

Method	Biuret assay		Bradford assay		BCA assay		Biuret assay		Bradford assay		BCA assay	
	BSA Mean Conc (mg/mL)	Standard Deviation	BSA Mean Conc (mg/mL)	Standard Deviation	BSA Mean Conc (mg/mL)	Standard Deviation	$t_{calc}$	Significant Difference?	$t_{calc}$	Significant Difference?	$t_{calc}$	Significant Difference?
Spectrophotometry	2.048	0.031	0.108	0.006	0.507	0.005	2.682	No	2.309	No	2.425	No
% Luminance	2.055	0.035	0.113	0.008	0.511	0.007	2.722	No	2.815	No	2.722	No
a*	2.069	0.042	0.106	0.004	0.508	0.005	2.846	No	2.598	No	2.771	No
b*	2.040	0.025	0.110	0.006	0.512	0.009	2.771	No	2.887	No	2.309	No

between the theoretical value and the concentrations obtained from spectrophotometric analysis and digital photometry at 95 % confidence level.

### Test for repeatability

To test for reproducibility of results, two separate experiments, with three trials for each, were conducted on the three protein assays using the same experimental set-up and camera settings and different standard and sample solutions. The value of  $t_{tab}$  was 2.776 at 95 % confidence level.

Based on the results, no significant differences were observed from the two experiments for the three protein assays at 95 % confidence level (Table 4).

**Comparison of results using assembled digital photometric set-up and light box.** The concentrations obtained for Biuret assay using the assembled photometric set-up were compared with those obtained using the light box based on t-test at 95 % confidence level. The value of  $t_{tab}$  was 2.776.

Based on the results, there were no significant differences between the concentrations obtained using the two experimental set-ups at 95 % confidence level. Comparison of the standard deviations was also done using F-test in order to determine if there was a significant difference between the standard deviations. F-test was done at 95 % confidence level; the value of  $F_{tab}$  was 19.00.

Significant differences were observed between the standard deviations using the assembled set-up and light box. This means that the results using the light box were more reproducible; the precision of protein determination was increased using the fabricated light box.

### SUMMARY AND CONCLUSION

The digital photometric method of determining protein concentration in solution was compared with the conventional spectrophotometric method using the biuret, Bradford and BCA assays. This method involves color analysis of digital images taken of protein-dye solutions using the RGB Analysis of Image Colors software.

A fabricated light box was compared with an assembled set-up for collecting the digital images in terms of precision and accuracy of the results. Solutions of potassium permanganate were used for optimizing light box dimensions and the following digital camera settings were used for photographic data gathering: ISO 100, aperture value f3.5, shutter speed 1/125 and spot focus.

RGB values were obtained from the color images of the colored solutions and used to calculate % luminance,  $a^*$  and  $b^*$  values. Concentrations of sample solutions were obtained from standard curves based on the RGB-based parameters using linear regression analysis and then compared with those obtained from spectrophotometric analysis using t-test. Based on the results, the concentrations obtained from spectrophotometric and digital photometric analyses were not significantly different at 95 % confidence level. This result was observed for the biuret, Bradford and BCA protein assays. The concentrations obtained from digital photometry using % luminance,  $a^*$  and  $b^*$  showed no significant differences at 95 % confidence level for the three protein assays.

Validation of experimental accuracy was also done for the

**Table 4.** Evaluation of reproducibility for measuring protein concentration using Biuret, Bradford and BCA assays.

Method	Experiment 1		Experiment 2		$t_{calc}$	Significant Difference?
	Mean Concentration* (mg/mL)	Standard Deviation	Mean Concentration* (mg/mL)	Standard Deviation		
<b>Biuret Assay</b>						
Spectrophotometry	2.449	0.011	2.451	0.012	0.213	No
% Luminance	2.404	0.036	2.414	0.027	0.385	No
$a^*$	2.399	0.052	2.407	0.038	0.215	No
$b^*$	2.440	0.037	2.411	0.023	1.153	No
<b>Bradford assay</b>						
Spectrophotometry	0.068	0.004	0.072	0.006	0.961	No
% Luminance	0.084	0.027	0.091	0.025	0.329	No
$a^*$	0.060	0.009	0.063	0.006	0.480	No
$b^*$	0.074	0.009	0.071	0.008	0.432	No
<b>BCA Assay</b>						
Spectrophotometry	0.527	0.024	0.534	0.026	0.343	No
% Luminance	0.512	0.044	0.521	0.041	0.259	No
$a^*$	0.505	0.014	0.501	0.016	0.326	No
$b^*$	0.531	0.037	0.523	0.039	0.258	No

\*Egg albumin solution

visible spectrophotometric and digital photometric methods using a protein solution of known concentration. The results showed that both methods were accurate at 95 % confidence level; this was observed for the three protein assays. However, because the limits of detection and linearity were not determined in the present study, conclusions regarding accuracy can only be made within the range of actual protein concentrations used in the study.

Based on results of t-test, the concentrations obtained from digital photometry using the assembled digital camera set-up and fabricated light box did not show any significant difference at 95 % confidence level. However, the results of F-test show that the precision of protein determination was greater using the light box. The digital cameras used were different using the two methodologies; one with 12 megapixels (MP) was used with the light box and another one with 7.5 MP was used with the assembled set-up. This was probably the main reason for the higher precision obtained with the light box as earlier studies on the same box with two different cameras had shown (J. dR. Vedad and E.J. del Rosario, unpublished observations). However, the effects of different light source intensities for the light box and assembled set-up on the results of color image analysis are probably insignificant because these intensity differences are 'corrected for' in the calculation of RGB values. In comparison, for another color model, e.g. HSI based on hue (H), saturation (S) and intensity (I), color intensity coming from the object (as measured by the camera) is directly dependent on light source intensity. Nevertheless, a fabricated light box is preferable to an assembled set up for greater ease in performing the experiments.

The results of the present study show that digital photometry, using a relatively cheap digital camera, a simple fabricated light box and free-access software, can be used as an alternative to conventional (visible) absorption spectrophotometry in accurately measuring soluble protein concentration. The potential impact of this novel methodology is greater in under-developed countries, as well as in remote places of fairly developed ones, where price differences are substantial for a spectrophotometer and a simple digital camera.

#### CONFLICT OF INTEREST STATEMENT

The authors declare that there is no conflict of interest arising from this study.

#### CONTRIBUTIONS OF INDIVIDUAL AUTHORS

This paper is based on the B.S. Chemistry theses of A.A.

Yanos and M.N. Bautista which were done under the supervision of M.R.N. Angelia and E.J. del Rosario as thesis advisers.

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