CHAPTER IV

RESULTS AND DISCUSSION

In this research, the study and application of a digital image-based colorimetry (DIC) by using a CMOS webcam camera as a detector was established for the determination of aqueous extractable protein in natural rubber (NR) latex and medical latex glove samples. A back-propagation neural network (BPNN) was chosen as a new choice for the protein concentration estimation instead of employing calibration curve construction. Four parameters, namely, background, focusing distance, position of high intensity LEDs and light illumination of LEDs were optimized for fabrication of the DIC lightbox. The results and discussion are presented as followed.

Design and construction of digital image-based colorimeter

Study the effect of background on the color of protein solution with modified Lowry reagent

Optimum conditions giving reproducible and comparable color measurements were studied. In order to correct the optimum background, black (paper) and white (paper and plastic) backgrounds were chosen and elucidated to obtain the real color of sample solutions. The image of a blank solution and 100 $\mu g\ mL^{-1}$ of standard protein solution (after color developing) obtained from black background (Figure 35 (1A, 2A)) indicated a shadow band in sample cell. These figures show darker color than real color of solutions. The images with true color could be obtained when using white background as shown in Figure 35 (1B, 2B, 1C, 2C). In addition, the slope of calibration curve as showed in Figure 36, when using a white paper and white plastic as background for photography is higher than that when using of a black paper as background. Thus, it could be concluded that the sensitivity when using white background is higher than that when using a black background. As a consequence, white background was selected for photography. However, using white paper as background (Figure 35 (1B, 2B) showed the reflected light on the sample cell that can

affect color homogeneity. Therefore, a sample cell holder was constructed by using white plastic (Polytetrafluoroethylene, PTFE) to reduce the side reflection of light. The designed sample cell holder as shown in Figure 37 consists of a compartment for sample cell locating (1 x 1 x 5 cm), sample cell capture (5 x 5 x 5 cm) and base (5 x 8 x 3 cm). The images obtained when using PTFE sample cell holder as a background as demonstrated in Figure 35 (1C, 2C) showed decreasing of reflected light. Using the sample cell holder also provided true and homogenous color of the solutions.

Therefore, the white PTFE sample cell holder was chosen as the optimum background and was applied to the next study.

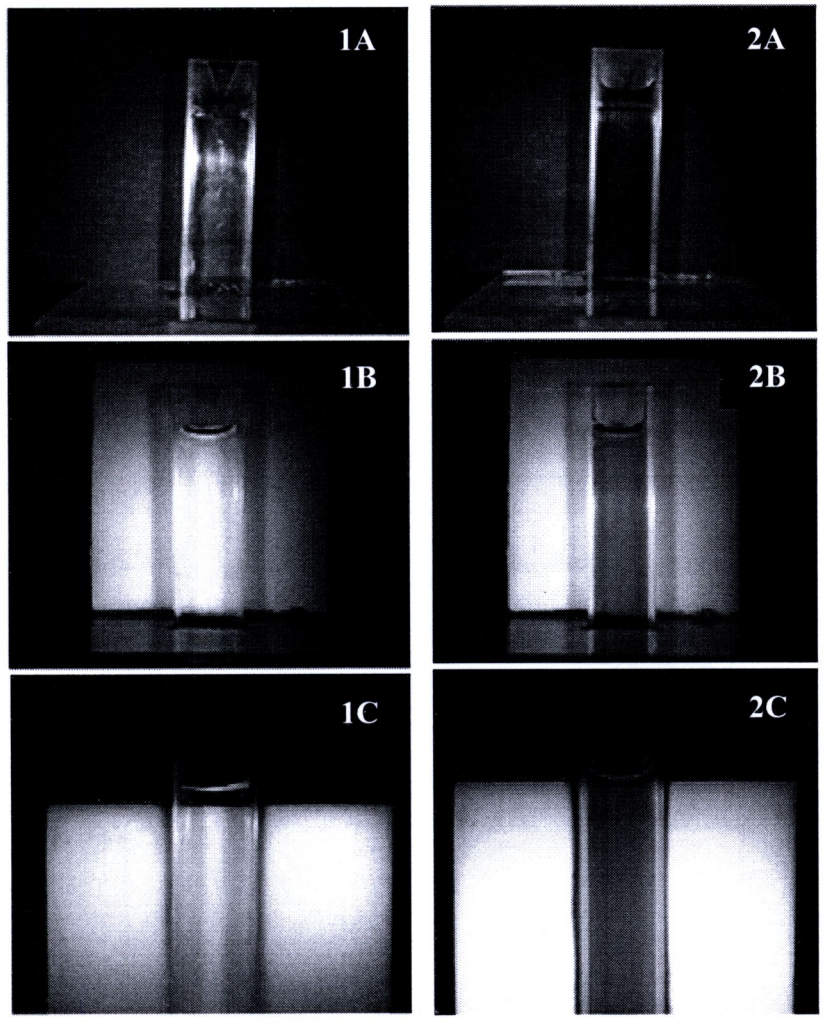

Figure 35 The digital images of blank solution (1) and 100 μg mL^{-1} of protein standard solution (2) captured by using black paper background (A), white paper background (B) and white sample cell holder background (C)

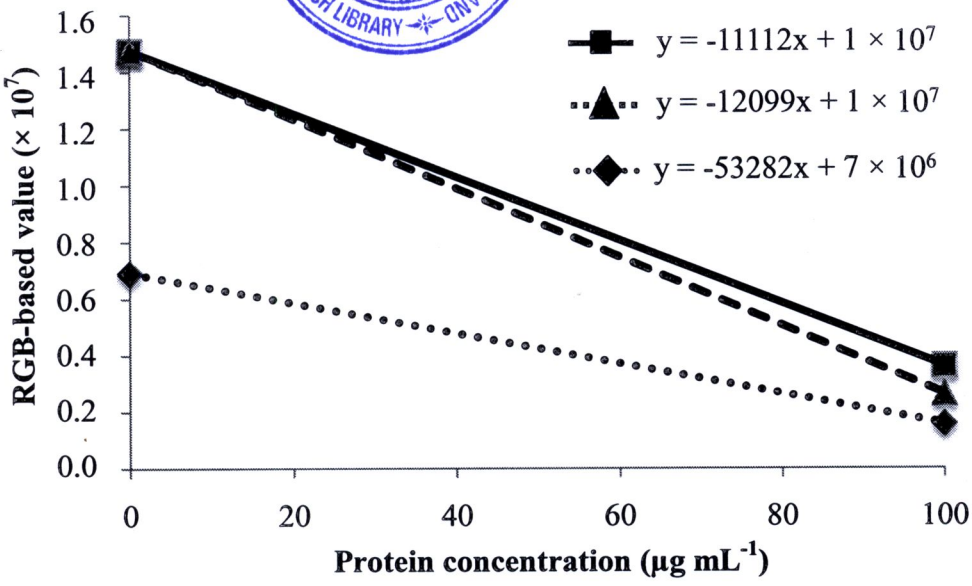

Figure 36 Relationships between RGB-based value and standard protein solutions by using black paper (-◆-), white paper (-■-) and white plastic (-▲-) as a background for photography

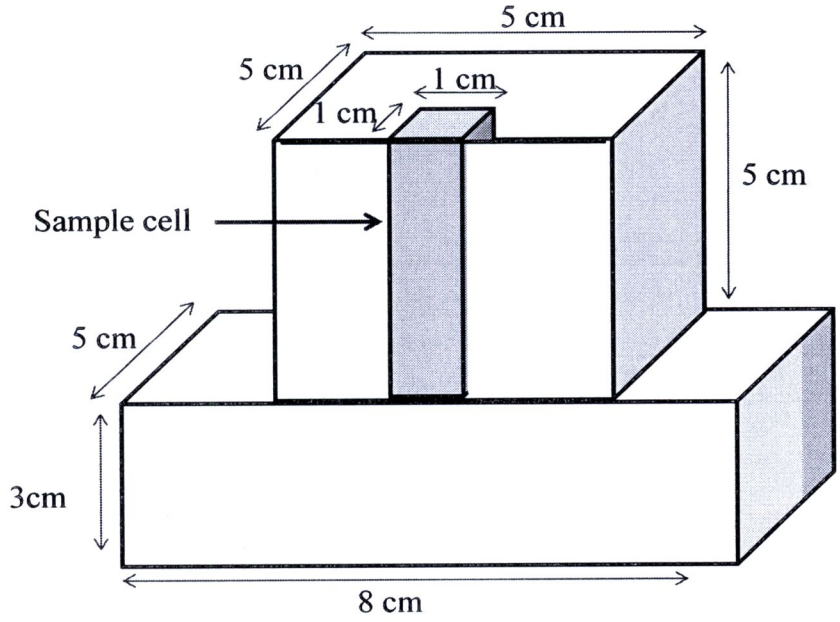

Figure 37 Structure of sample cell holder made of white PTFE

Study the effect of focusing distances on the image size

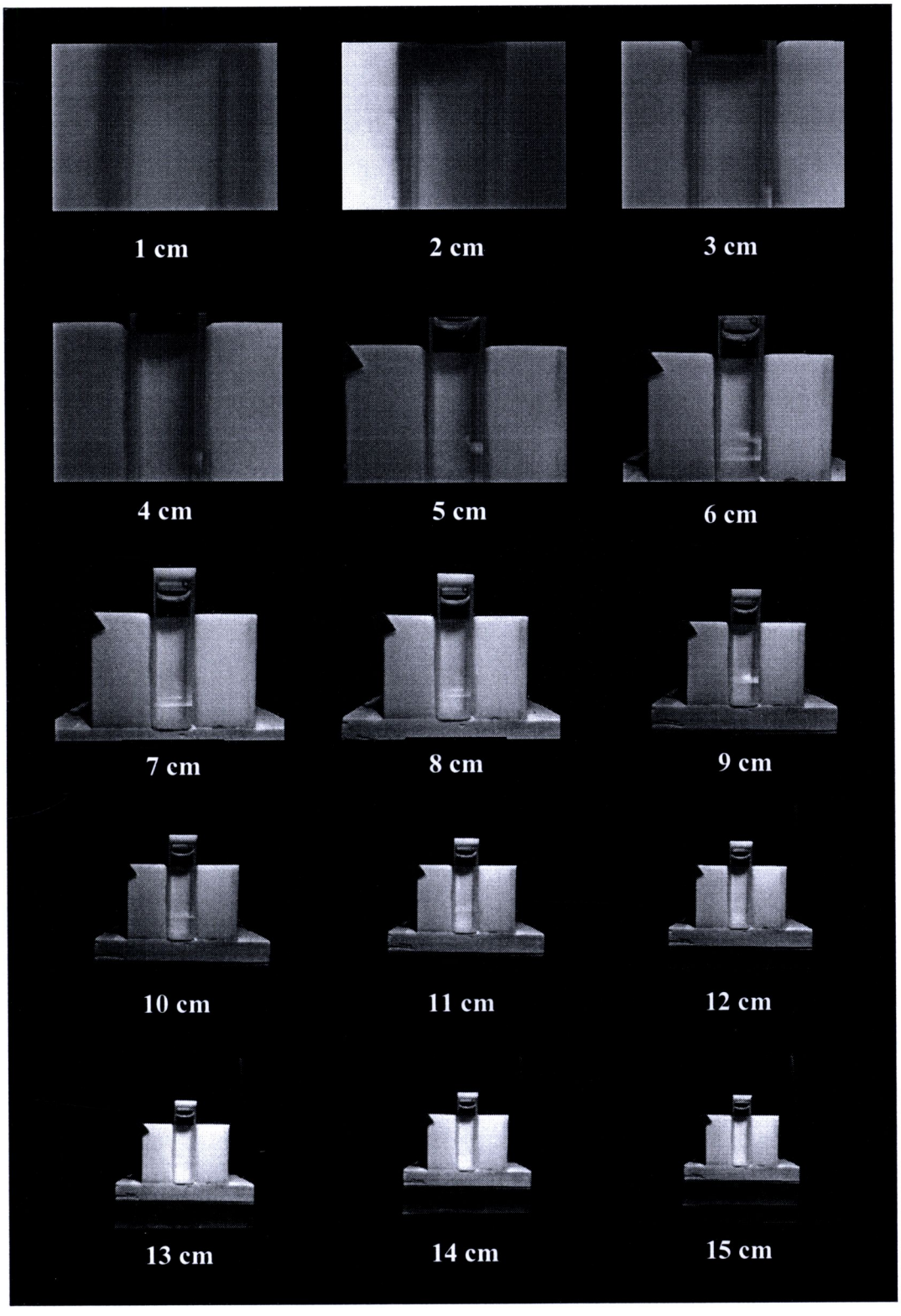

Figure 38 The image sizes at different focusing distances

The focusing distance is important for the quality of an image. The low quality of an image was obtained when the object close to the camera lens while the high resolution of an image was obtained at a suitable distance. Therefore, the optimum of focusing distance from camera lens to sample cell was determined by using automatic focusing of the CMOS webcam camera at the lengths of 1 to 15 cm in order to obtain the highest resolution image and the optimum of a sample cell image size. As can be seen in Figure 38, the images obtained at 1-6 cm focusing distances are blur because these lengths is not the optimum focusing distance of the camera lens. The lengths of 12-15 cm focusing distances are far from the camera lens which giving a low resolution images. The good results were obtained at 7-11 cm of focusing distances. Nevertheless, the focusing distance at 7 cm shows bigger sample cell size than other lengths. It also represented the whole image that is easy to crop for RGB processing. Therefore, the focusing distance at 7 cm was chosen for photography.

Study the effect of the position of high intensity LEDs on color of an image

The light illumination is critical to the homogeneity of the color image while processing. The position of high intensity LEDs was studied in 6 directions (Figure 39). This effect was investigated by measuring the same standard solutions (0 $\mu g\ mL^{-1}$) for all positions. One LED was used in experiment 1, 2 and 3. It was found that the image obtained from 1 and 3 is too bright on a sample cell. These positions provide bright color of the solution causing higher RGB value than the true color. The image obtained from type 2 has a reflected light on the sample cell surface giving the error on RGB value measurement. Consequently, it can be concluded that one LED is not suitable for photography. Then, the two LEDs were used in experiment 4, 5 and 6 for the symmetry light giving from both sides of sample cell. It was found that the image obtained has a shadow in a sample cell and a reflected light on the sample cell surface by capturing in experiment 4 and 5, respectively. The best image was obtained by photographing in the experiment 6 which gave true color of sample cell holder and sample cell. Therefore, the set up of LEDs position as showed in experiment 6 by using two LEDs was chosen as the optimum position. However, the light of LEDs are straight line that cause a shadow at the edge of a sample cell (Figure 39 (6)). Therefore, the angle of two LEDs was studied for obtaining the homogeneous image.

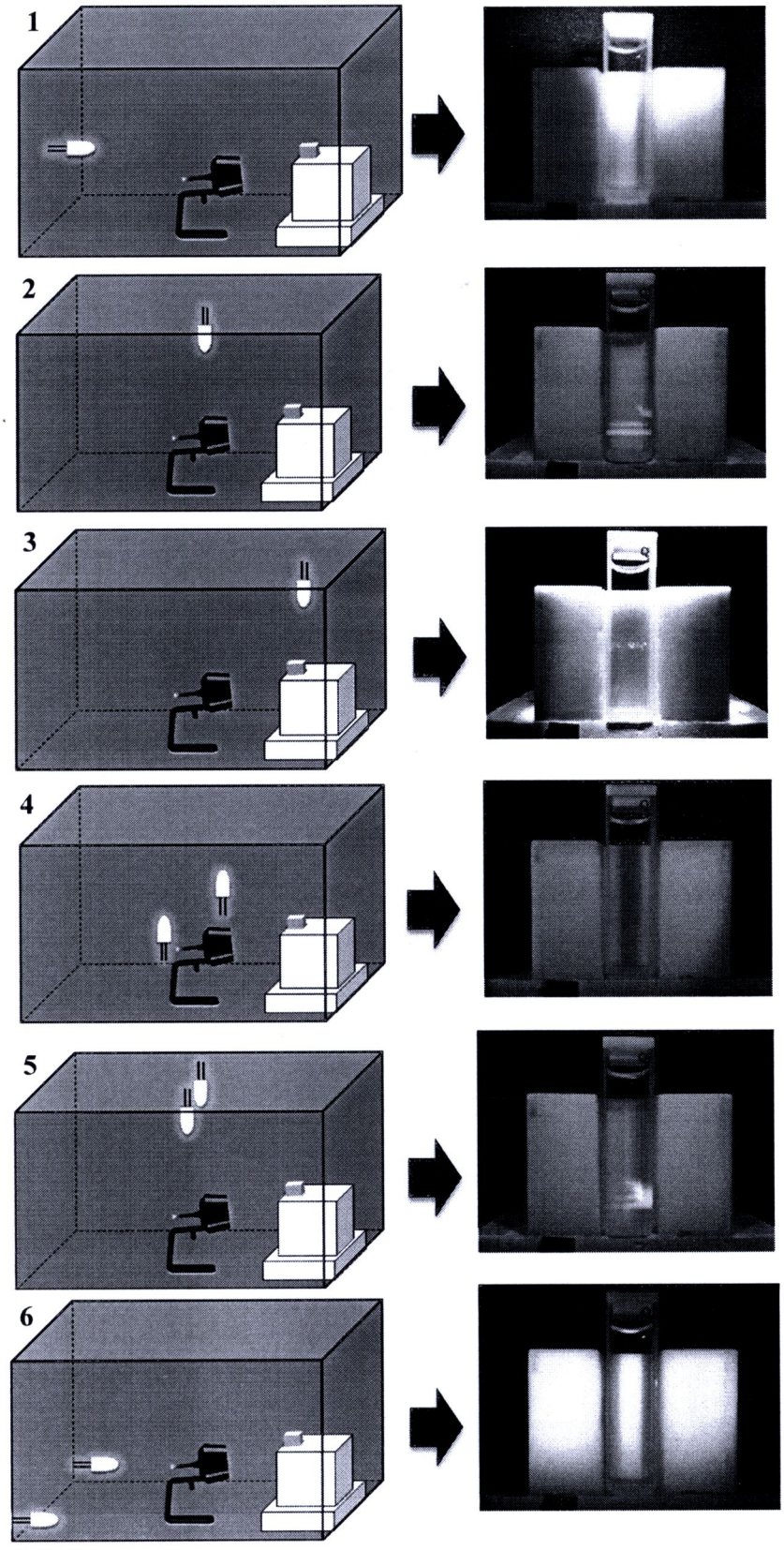

Figure 39 Schematic diagrams and images from different LEDs positions

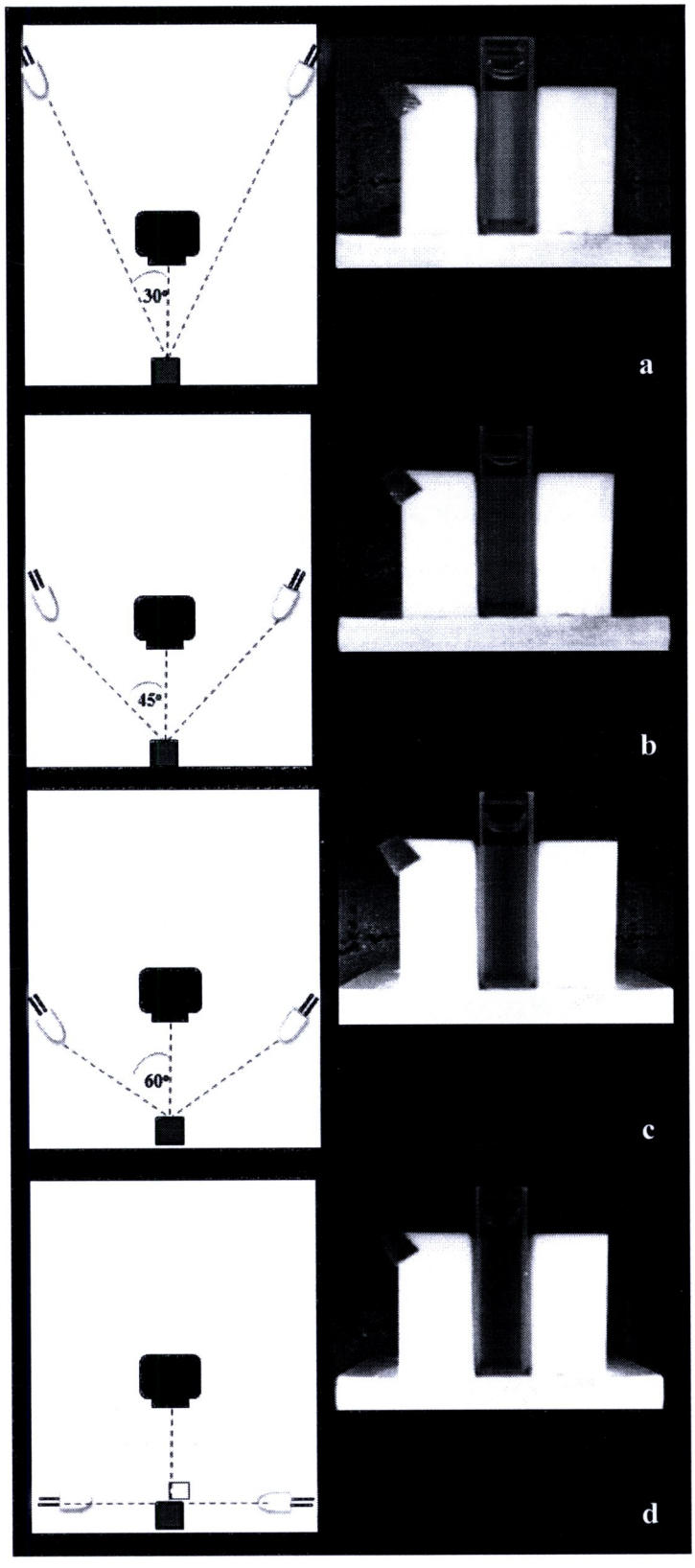

Figure 40 Schematic diagrams and images from different angles of two LEDs at 30(a), 45(b), 60(c), and 90(d) degree

The effect of the angle of two LEDs with the surface of the sample cell was investigated at the angle of 30, 45, 60, and 90 degree as illustrated in Figure 40. A 100 μg mL^{-1} of standard protein solution was transferred to sample cell then the image was taken with CMOS webcam camera. The data was processed by an ANNs written program and visual inspection. The image obtained when setting the angle at 30 degree is not homogeneous and had a bright band at the center and shadow band at both sides of a sample cell. The image obtained when setting the angle at 60 and 90 degrees has a shadow band at the center and bright band at both sides of a sample cell. The best image was obtained when setting the angle at 45 degree. The homogeneous image was received in this angle which could be described as the plot between RGB value and the number of iterations as showed in Figure 41. It was found that each R, G and B value shows no significant difference when reading for 3,000 times. Therefore, the angle of the two LEDs setting at 45 degree with respect to a sample cell surface was chosen for the next study.

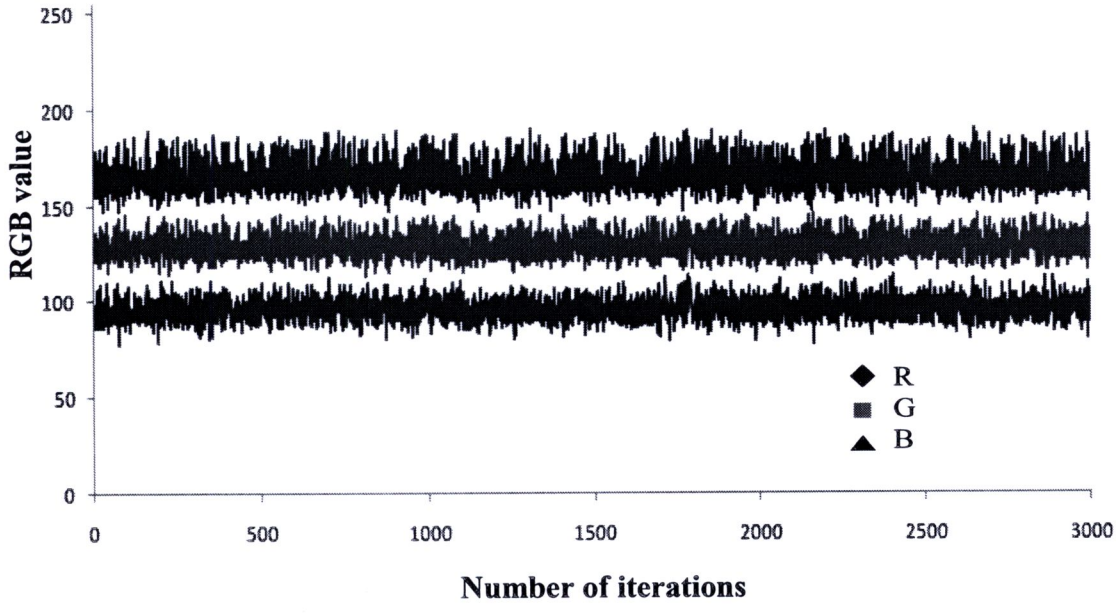

Figure 41 Relationships between RGB value and the number of iterations of 100 μg mL^{-1} of standard protein solution when setting the angle at 45 degree

The effect of the LEDs illumination

The light illumination of high intensity LEDs affects the color homogeneity of an image. Besides, the illumination is important for data processing by ANNs. Therefore, every image must be captured under the same condition for reproducible and comparable results. In order to study the illumination, two white high intensity LEDs were used for providing constant light while capturing pictures. The illumination was experimented between 0-700 lux by using lux meter at the position as illustrated in Figure 32. A blank solution and 100 $\mu g\ mL^{-1}$ of standard protein solution were tested under the same condition. The results were shown in Figure 42. The RGB values were increased with increasing the light illumination up to 200 lux after which the RGB values became constant which could be attributed to the low light intensity which is not enough for the camera aperture. When increasing the light illumination until sufficient enough or more for the camera aperture, the light was controlled by automatic adjusting of the camera aperture causing the constant RGB values and the true color image. In addition, as can be seen in Figure 42(B), decreasing in RGB value was observed when the LEDs illumination was applied between 20 - 60 lux. It could be due to the fact that these pictures were blur at 20 - 60 lux because they blue and black spots were noticed in these pictures. Consequently, when black spots were randomly chosen and read more than blue spots, the average of RGB value was low. However, this phenomena did not affect to the RGB values when increasing the light illumination greater than 200 lux. Therefore, the light illumination at 300 lux was selected for further image capturing.

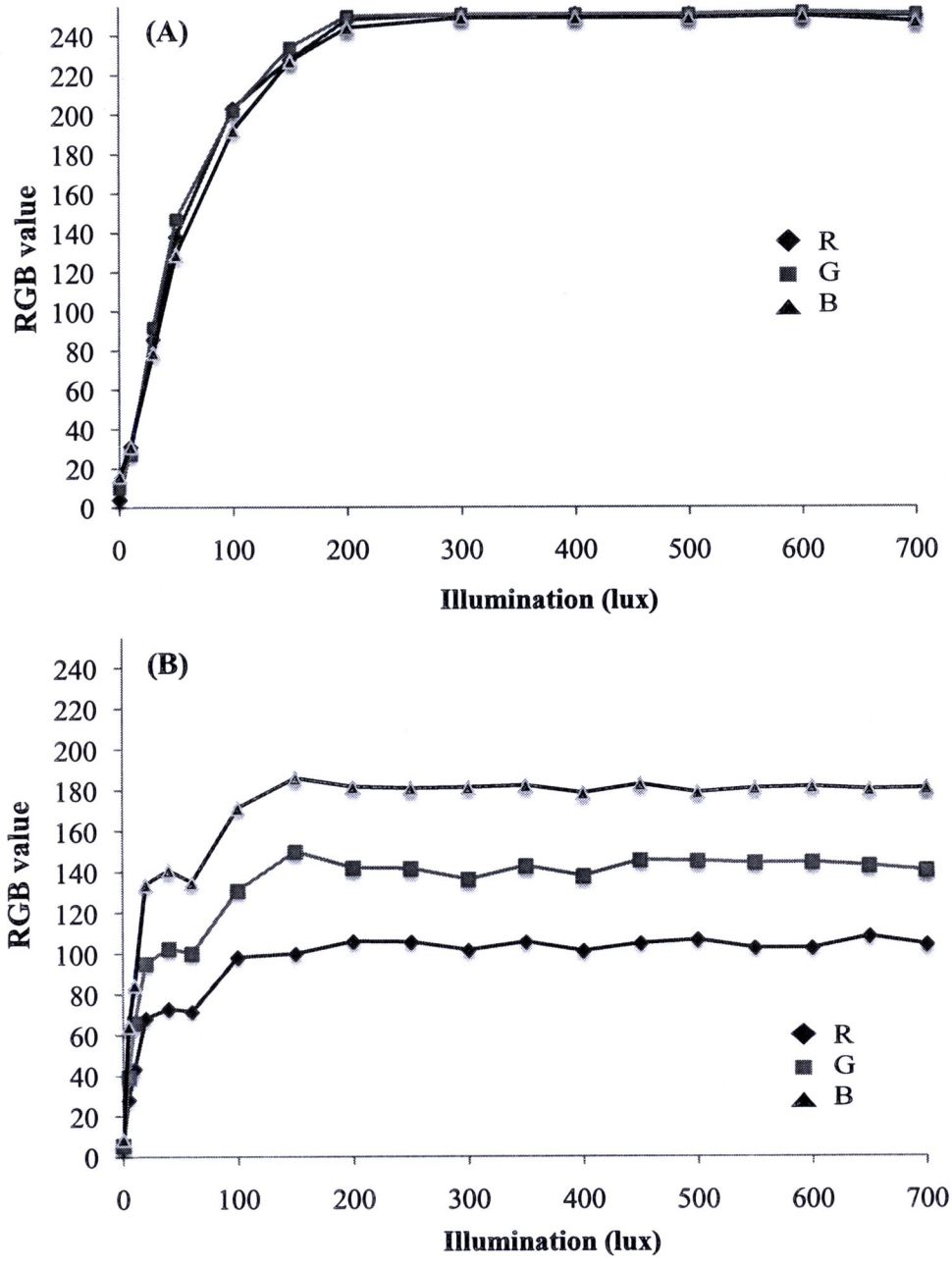

Figure 42 Relationships between RGB value and the light illuminations from blank solution (A) and 100 μg mL^{-1} of protein standard solution (B)

Design color processing software

The prediction performance of the written program was validated by the accuracy and precision. The accuracy of protein concentration prediction from the DIC-ANNs method was verified by using the means squared error (MSE) as shown in Table 4. It was found that the average MSE from protein standard solutions testing at

1, 2, 5 and 8 μg mL^{-1} using method 1 was lower than that from method 2 and 3. The lowest MSE indicated that accuracy of method 1 was higher than the other methods. Method 1 used the average of RGB-based value that was in wider range (0-16777215) than the other methods causing less error values when processing and providing low MSE. As compared to method 2 and 3 which used the average and mode number of R, G and B value (0-255), when these numbers were converted to RGB-based values, the high difference value attributed to high MSE was obtained. Therefore, method 1 was chosen for the prediction of extractable protein in NR latex and medical latex gloves.

The precision of the DIC-ANNs was verified by using the relative standard error (%RSE). The %RSE is the standard error expressed as a fraction of the estimate value and is usually displayed as a percentage. The high %RSE is subject to high sampling error. As shown in Figure 43, the %RSE was low when reading the RGB values up to 3000 times giving the high precision of the measuring. Therefore, the numbers of iteration for 3000 times were selected for the RGB values reading in the written program.

The reproducibility of this work using method 1 for data processing was verified by the relative standard error (% RSD) in protein determination carried out between days for 11 replications. The % RSD of protein standard solutions at 2, 5 and 8 μg mL^{-1} was 0. Moreover, it is not necessary to re-construct calibration curve for individual analysis because the precision between days defined as % RSD was relatively low. Therefore, this method showed good reproducibility for protein determination at the concentration more than 2 μg mL^{-1}. Nevertheless, protein concentration at 1 μg mL^{-1} could be detected by proposed method because the MSE at this concentration was relatively low. As a consequence, the minimum level of protein determination by proposed method was at 1 μg mL^{-1}. According to the study, it was found that the maximum level of protein determination by proposed method was at the concentration of 100 μg mL^{-1}.

Table 4 Composition of prediction set and means squared error (MSE) of the standard proteins solution

	Method 1		Method 2		Method 3	
Actual[a]	Found[a]	MSE	Found[a]	MSE	Found[a]	MSE
1.00	1.18	0.149	1.36	0.231	1.56	0.248
2.00	2.00	0.000	2.18	0.149	2.27	0.198
5.00	5.00	0.000	4.91	0.183	5.00	0.182
8.00	8.00	0.000	7.72	0.198	7.63	0.231
Mean of MSE		0.037		0.190		0.215

[a]Concentration of protein (μg mL^{-1}), (n=11)

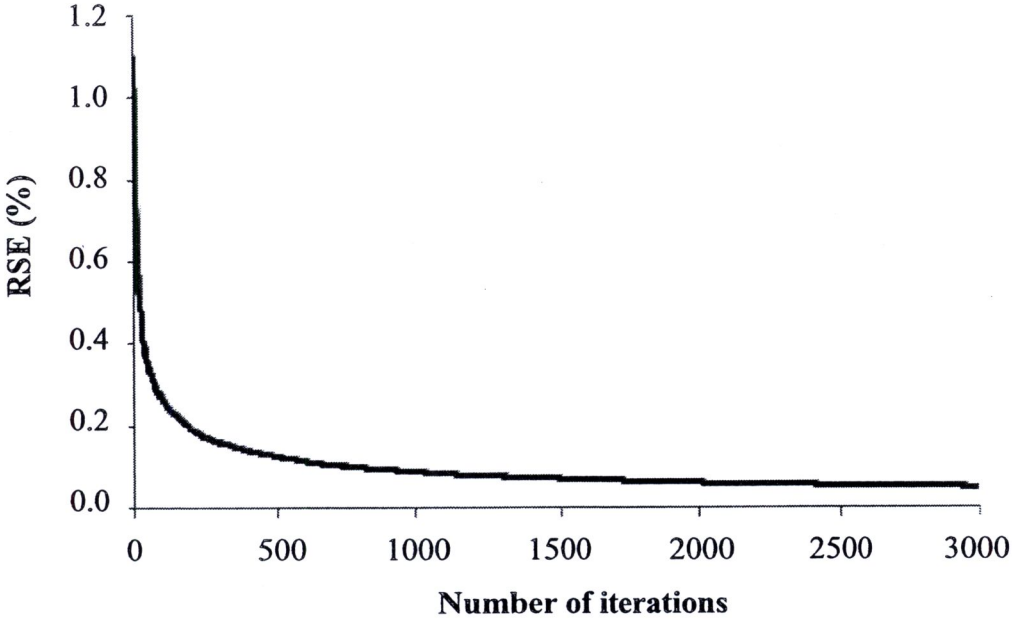

Figure 43 Plots of %RSE as a function of the number of iterations for standard protein solution (5 μg mL^{-1})

Application to protein assay in NR latex and medical latex gloves

As demonstrated in Figures 44 and 45, RGB value decreased with increasing standard protein concentration whereas the color of the solution increased. This relation was described with the principle of RGB color model when additive color mixing begins with black (0, 0, 0) and ends with white (255, 255, 255) [78]. When RGB-based values were potted in accordance with protein concentration (Figure 46), the decreasing in the slope of calibration curve was observed. Therefore, the sensitivity using RGB-based value was higher than R, G and B value. The images of aqueous extractable protein in NR latex and latex gloves samples were shown in Figure 47. All images were processed by using digital image-based colorimetry coupled with artificial neural network (DIC-ANNs).

The results from the determination of aqueous extractable protein in NR latex and medical latex gloves samples were given in Table 5 and 6, respectively. The results obtained by the DIC-ANNs and spectrophotometry show good agreement with no statistical difference at 95% confidence level by applying the t-test.

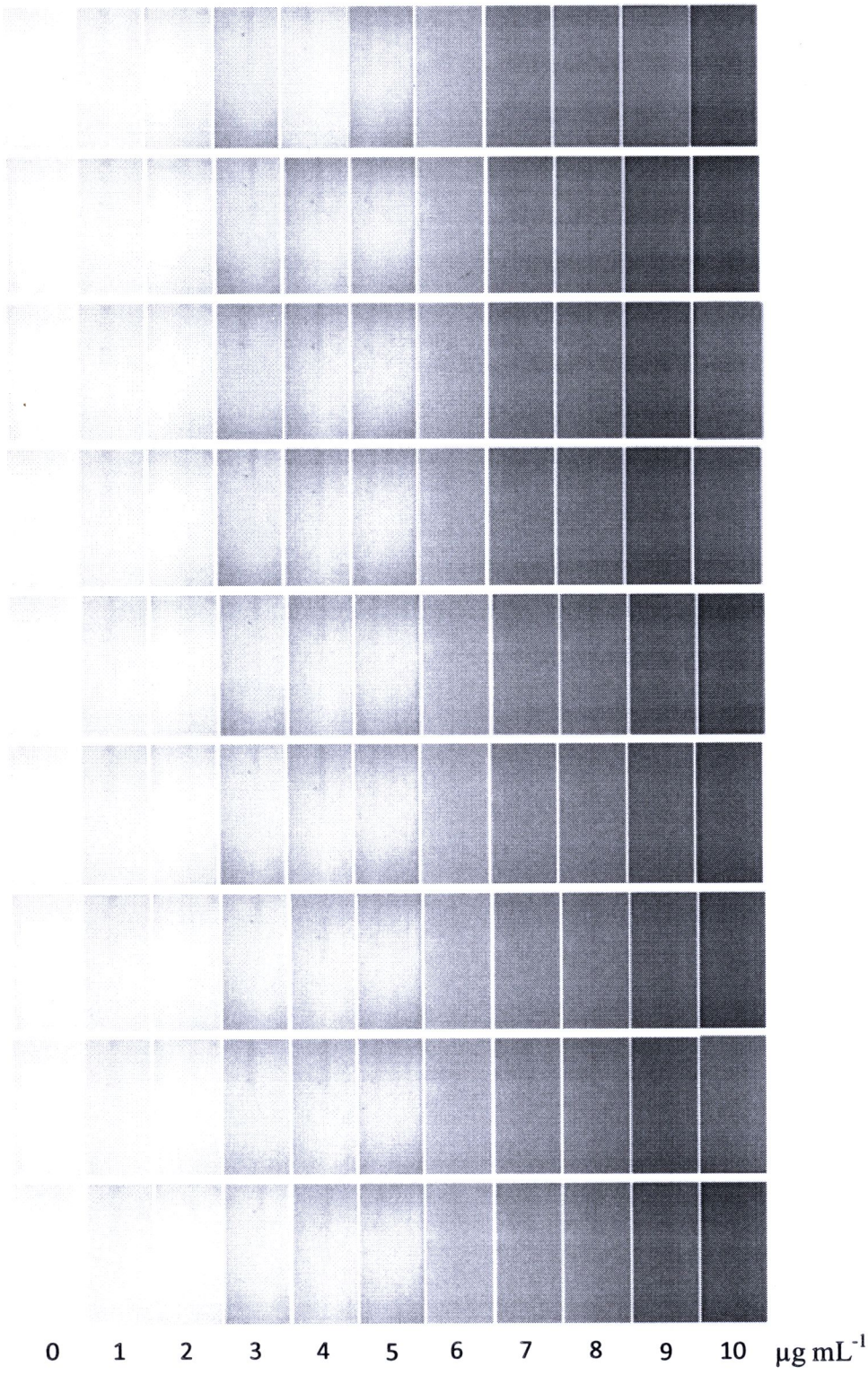

Figure 44 Digital images of the standard proteins solutions (0-10 μg mL^{-1}) with Modified Lowry reagent (n=9)

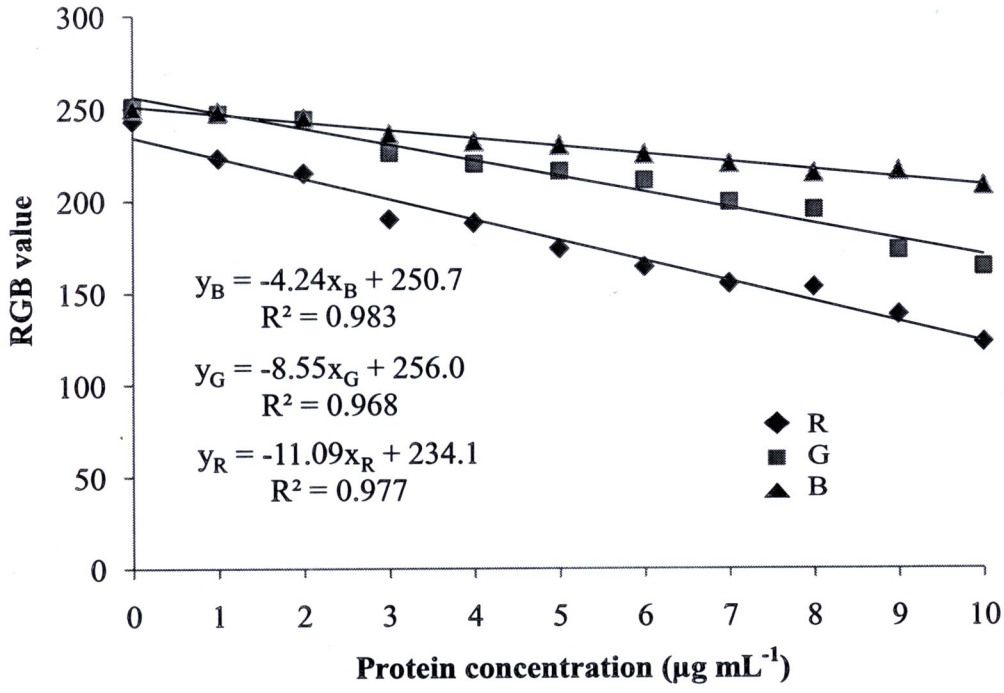

Figure 45 Plots of relationships between RGB value and the concentration of protein standard solution

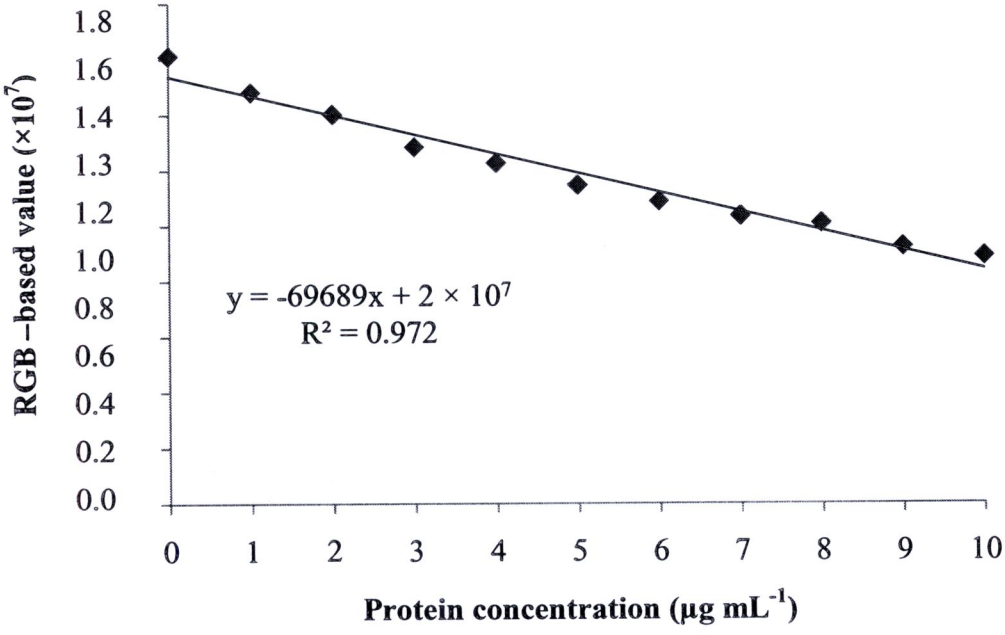

Figure 46 Plots of relationships between RGB-based value and the concentration of protein standard solution

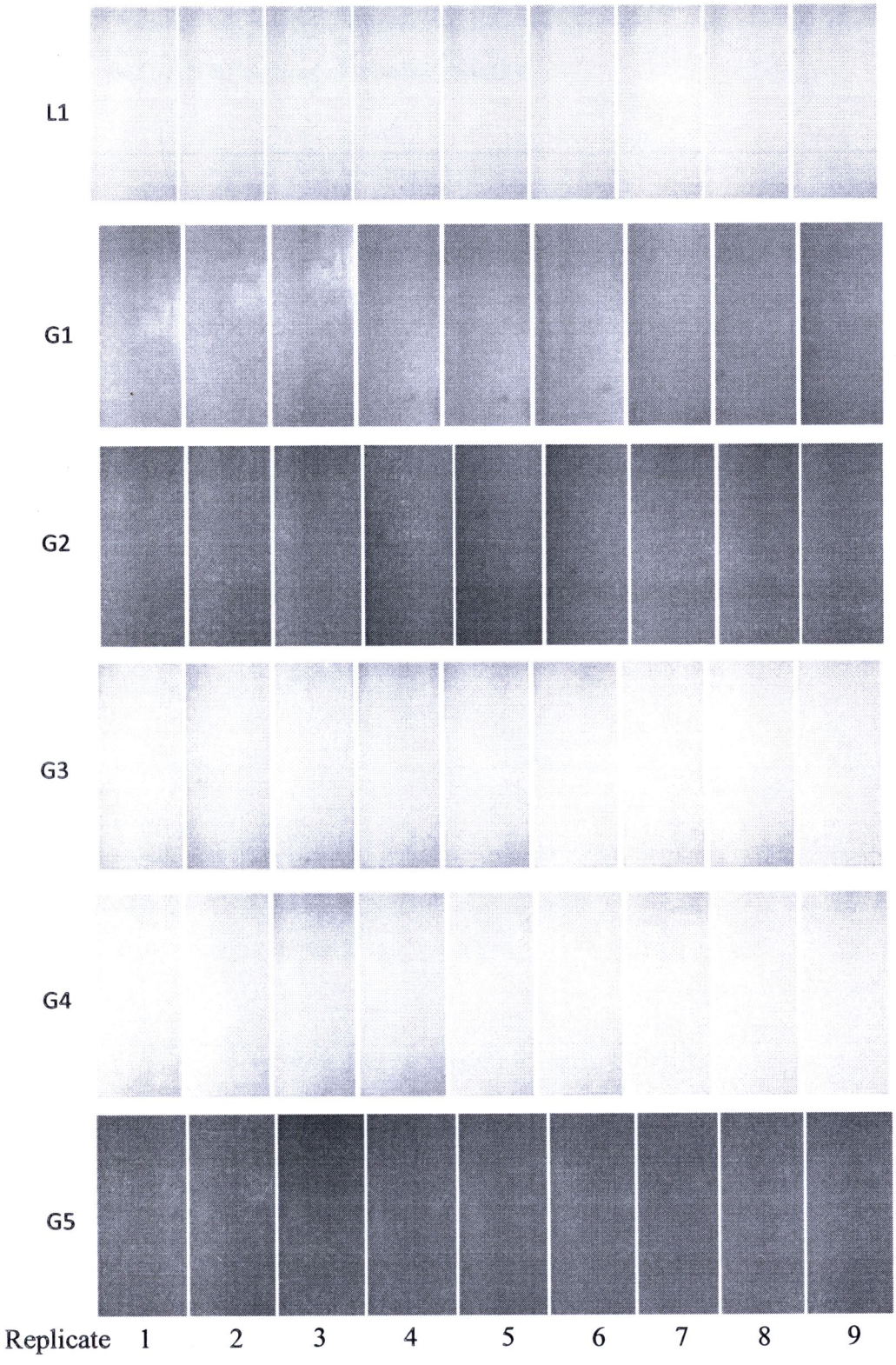

Figure 47 Digital images of the protein in the samples with modified Lowry reagent (n=9). NR latex (L1), Pro gloves (G1), Hycare (G2), Sempermed (G3), GPO (G4), and Saf-gard (G5)

Table 5 Results of protein determination in NR latex sample by the proposed DIC-ANNs and spectrophotometry

Method	N	Mean of protein concentration in NR latex ($\mu g\ g^{-1}$)	SD	RSD (%)
DIC-ANNs	9	50.5	1.81	3.58
Spectrophotometry	9	62.3	2.29	3.67

Table 6 Results of proteins assay (μg/glove) in medical latex glove samples determined by the proposed DIC-ANNs compared with spectrophotometry

Samples trade name	Method			
	DIC-ANNs[a]	RSD (%)	Spectrophotometry[a]	RSD (%)
Pro gloves	231.3±24.8	10.7	238.3±5.1	2.1
Hycare	326.9±26.3	8.0	333.4±9.6	2.8
Sempermed	188.0±21.9	11.7	196.0±5.3	2.7
GPO	131.8±18.9	14.3	144.6±7.9	5.5
Saf-gard	433.1±15.0	3.5	447.1±13.2	2.9

[a] Mean value ± standard deviation (n =9)

RSD (%) = relative standard deviation