CHAPTER III

RESEARCH METHODOLOGY

This chapter describes the fabrication of digital image-based colorimeter (DIC) for the determination of aqueous extractable protein in natural rubber (NR) latex and medical latex gloves. This chapter covers the experimental details in the order of following;

1. Design and fabrication of digital image-based colorimeter

1.1 Investigate the effect of background on the color of the solution of proteins with modified Lowry reagent.

1.2 Elucidate the effect of focusing distance on the image size.

1.3 Study the effect of the position of high intensity light emitting diodes (LEDs) on color of images.

1.4 Study the effect of the LEDs illumination on the brightness and the darkness of images.

2. Design color processing software.

3. Application to natural rubber latex and medical latex gloves proteins assay.

Instrument and apparatus

1. Ultraviolet-Visible Spectrophotometer, V-650 Series spectrophotometer, Japan.
2. Centrifuge, Hettich zentrifugen D-7200 Tuttlingen, Germany
3. Vortex mixer, Vortex-genie G-560E Scientific Industries, U.S.A.
4. pH-meter, SG20 Mettler Toledo, Switzerland.
5. Digital lux meter, LX-802, Nicety, China.
6. Analytical balance (4 digits), Sartorius, Germany.
7. Deionized water machine, Elga, England.
8. CMOS webcam camera, Microsoft LifeCam HD-5000, U.S.A.
9. Laptop, Lenovo G640, Lenovo, China.

Chemicals

1. Albumin from hen egg white (crude), Fluka, Netherland.
2. Potassium chloride, KCl (74.56 g mol^{-1}), AR grade, Carlo ERBA, Italy.
3. Disodium hydrogen orthophosphate, Na_2HPO_4 (141.96 g mol^{-1}), AR grade, Carlo ERBA, Italy.
4. Potassium dihydrogen phosphate, KH_2PO_4 (136.09 g mol^{-1}), AR grade, Merck, Germany.
5. 65% Nitric acid, HNO_3 (63.01 g mol^{-1}), AR grade, RCl Labscan Limited, Thailand.
6. Sodium hydroxide, NaOH (40.00 g mol^{-1}), AR grade, RCl Labscan Limited, Thailand.
7. Sodium tartrate, $C_4H_8Na_2O_8$ (230.08 g mol^{-1}), AR grade, LOBA Chemie, India.
8. Sodium chloride, NaCl (58.44 g mol^{-1}), AR grade, Merck, Germany.
9. Sodium deoxycholate (DOC), $C_{24}H_{39}O_4Na$ (414.55 g mol^{-1}), AR grade, Fluka, Switzerland.
10. Copper sulphate pentahydrate, $CuSO_4.5H_2O$ (249.68 g mol^{-1}), AR grade, Carlo ERBA, Italy.
11. 2 M Folin- Ciocalteu , Sigma-Aldrich, Switzerland.
12. Trichloroacetic (TCA), $C_2HCl_3O_2$ (163.39 g mol^{-1}), AR grade, Sigma-Aldrich, Germany.
13. Phosphotungstic acid (PTA), $H_3O_{40}PW_{12}.XH_2O$ (2880.05 g mol^{-1}), AR grade, Fluka, Switzerland.
14. Deionized (DI) water

Preparation of standard solutions and reagents

1. Standard solution of protein [2]

The stock protein standard solution at the concentration of 1 mg mL^{-1} was prepared by dissolving 100 mg of albumin from hen egg white in 100 mL phosphate buffer for 2 h at room temperature in a polypropylene container. Filter the solution through a low protein binding filter paper (0.45 μm) and determine the absorbance at 280 nm using a UV-Vis spectrophotometer. Divide the absorbance by 0.64 to calculate

the actual concentration of the albumin stock solution. Store the standard protein solution at 4°C. The solution is stable for 7 days.

2. Phosphate buffer saline [91]

Phosphate buffer saline pH 7.4 was used for the extraction process which were prepared by dissolving 8.0 g of NaCl, 0.20 g of KCl, 1.44 g of Na_2HPO_4, and 0.24 g of KH_2PO_4 in 750 mL of DI water. Then, the pH of the solution was adjusted to 7.4 using concentrated H_2SO_4 or NaOH and finally the volume was adjusted to 800 mL with DI water.

3. Modified Lowry Reagents [2]

3.1 Alkaline tartrate solution

Alkaline tartrate solution was prepared by dissolving 2.22 g of Na_2CO_3, 0.44 g of NaOH and 0.18 g of sodium tartrate ($C_4H_8Na_2O_8$) in DI water and adjusting to a volume of 100 mL.

3.2 Copper sulfate solution

Copper sulfate solution was prepared by dissolving 7.0 g of $CuSO_4.5H_2O$ in DI water and adjusting to a volume of 100 mL.

3.3 Alkaline copper tartrate solution

Alkaline copper tartrate solution was freshly prepared by mixing 1.0 mL of copper sulfate solution and 150 mL of alkaline tartrate solution.

3.4 50% (v/v) Folin-Ciocalteu reagent

Folin-Ciocalteu reagent at the concentration of 50% (v/v) was prepared by diluting 25 mL of Folin-Ciocalteu reagent with 25 mL of DI water.

3.5 0.15% (w/v) Sodium deoxycholate (DOC)

0.15% (w/v) of DOC solution was prepared by dissolving 0.15 g of $C_{24}H_{39}O_4Na$ in DI water and adjusting to a volume of 100 mL.

3.6 72% (w/v) Trichoroacetic acid (TCA)

72% (w/v) of TCA solution was prepared by dissolving 7.2 g of $C_2HCl_3O_2$ in DI water and adjusting to a volume of 10 mL.

3.7 72% (w/v) Phosphotungstic acid (PTA)

72% (w/v) of PTA solution was prepared by dissolving 7.2 g of $H_3O_{40}PW_{12}$ in DI water and adjusting to a volume of 10 mL.

3.8 0.2 M Sodium hydroxide solution

0.2 M Sodium hydroxide solution was prepared by dissolving 2.0 g of NaOH in DI water and adjusting to a volume of 250 mL.

Extraction and assay procedure [2]

1. Extraction procedure

A 0.5 g of high ammonium NR latex or medical latex gloves (sampling 9 pieces from each brand and cutting into small pieces, at approximate size of 0.5 cm^2) was taken into a 15 mL centrifuge tube. Then, 5 mL of phosphate buffer saline pH 7.4 was added. The specimen was extracted by shaking using vortex mixture at room temperature in the middle level for 120 minutes. Then the extraction solution was centrifuged at 2500 rpm for 15 minutes. Consequently, the extract was filtered through a low protein binding filter paper (0.45 μm) into a 15 mL centrifuge tube to collect the supernatant liquid.

2. Acid precipitation

A 1 mL of each solution, the extraction buffer (blank), standard protein solution, and the sample extract was placed into a 15 mL centrifuge tube. A 0.1 mL of 0.15% (w/v) DOC solution was added and throughly mixed. The mixture was left for 10 minutes. Then, 0.2 mL of a freshly prepared solution of 1:1 TCA and PTA was added in order to precipitate the proteins followed by mixing and standing for 30 minutes. After that, the solution was centrifuged at 5000 rpm for 20 minutes. The supernatant liquid was decanted and redissolved the precipitates by using 1 mL of 0.1 M NaOH.

3. Color developing and measuring

A 2.5 mL of alkaline copper tartrate solution and 0.3 mL of 50% (v/v) folin-ciocalteu reagent were taken into the protein extract from acid precipitation process. The solutions were transferred into the sample cell and waited for 30 min. Then, the solutions the absorbance at the 750 nm wavelength and the RGB values by UV-Vis spectrophotometer and DIC detector were measured, respectively.

Optimization procedures

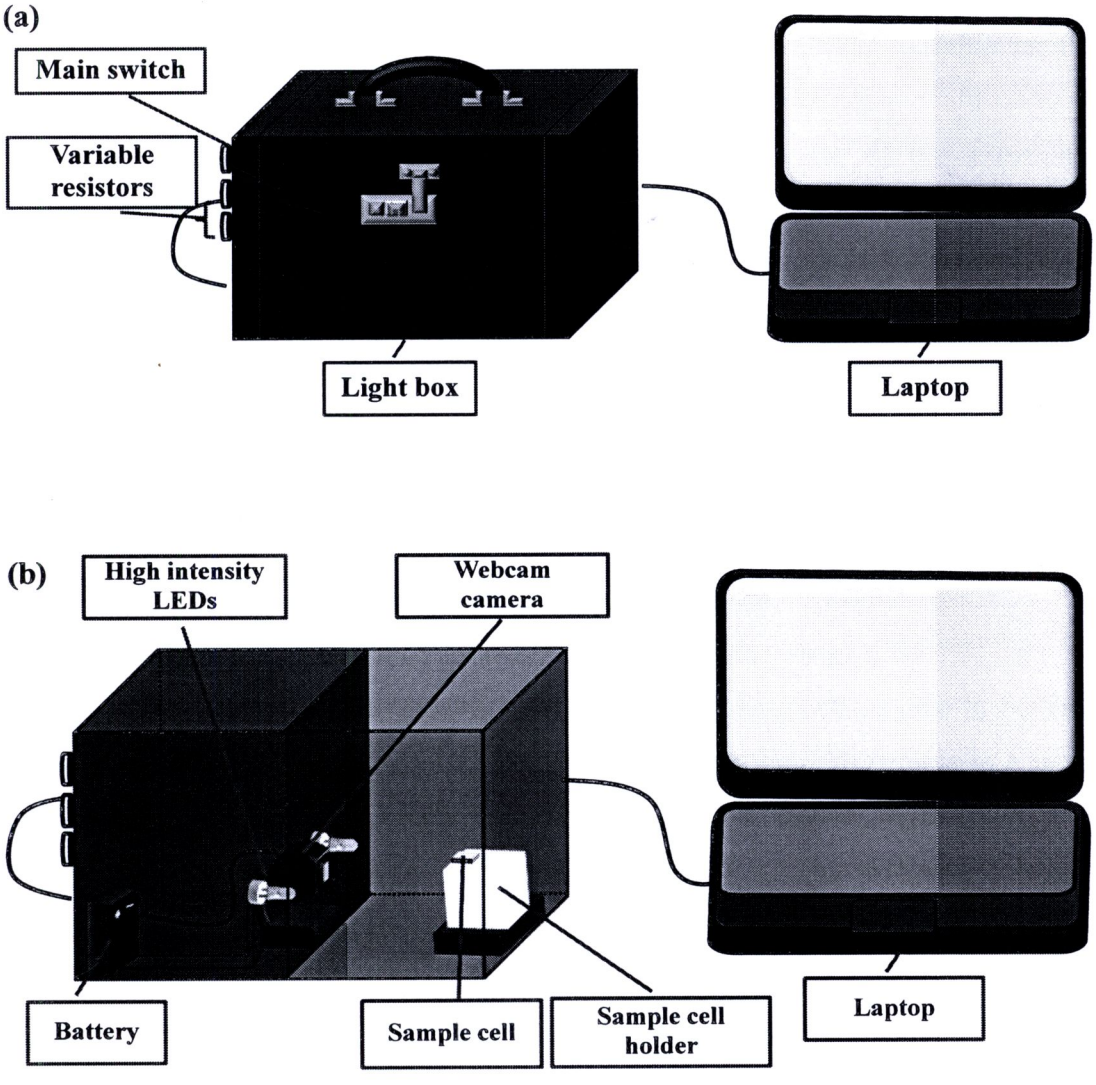

Figure 29 Schematic diagram of a DIC system (a) outside view and (b) inside view

Design and fabrication of digital image-based colorimeter

A schematic diagram of the system assembled for digital images acquisition is depicted in Figure 29. A light box was made of a 30 × 20 × 20 cm (width × height × depth) black cardboard to protect the system from outside light. The CMOS webcam camera (Microsoft HD-5000, China) connected with laptop (Lenovo G640, China) was secured on the center floor of the box and in front of the sample cell (quartz cuvette, 10 mm Hellma, U.S.A.) which was located in a sample cell holder made by a white plastic. Two white high intensity LEDs (Thailand) were placed at the camera

backside to give constant light intensity throughout the experiment. The LEDs driver were powered by a 4.5 V DC from 3 AA dry cell batteries (Panasonic rechargeable HHR-3LVT, China) and the illumination was adjusted by 10 kΩ variable resistors (Thailand).

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

A 100 μg mL^{-1} of standard protein solution and blank solution obtained after color developing were pictured by using black and white paper as background. The light position was controlled at 45 degree with respect to a sample cell surface. The light illumination was controlled at 350 lux by using variable resistors. The focusing distance from camera lens to the sample cell surface was set at 7.0 cm. The solution was transferred to sample cell then taking an image with CMOS webcam camera. The data was processed by an ANNs written program and visual inspection.

Evaluating the effect of focusing distance on the image size

The focusing distances from camera lens to sample cell surface in the range of 1 to 15 cm were adjusted by controlling the light position with respect to a sample cell surface at 45 degree and the light illumination at 350 lux. A blank solution was transferred to sample cell then taking an image with CMOS webcam camera at different distances. The data was processed by visual inspection.

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

The light positions were varied in 6 types as shown in Figure 30 by controlling the light illumination at 350 lux and the focusing distance from camera lens to sample cell surface at 7.0 cm. A blank solution was transferred to sample cell then taking an image with CMOS webcam camera. The data was processed by visual inspection. From the optimal position, the angle of light of LEDs with respect to a sample cell surface was studied at 30, 45, 60, and 90 degree as shown in Figure 31. A 100 μg mL^{-1} of standard protein solution after color developing was transferred to sample cell then an image was taken with CMOS webcam camera. The data was processed by an ANNs written program and visual inspection.

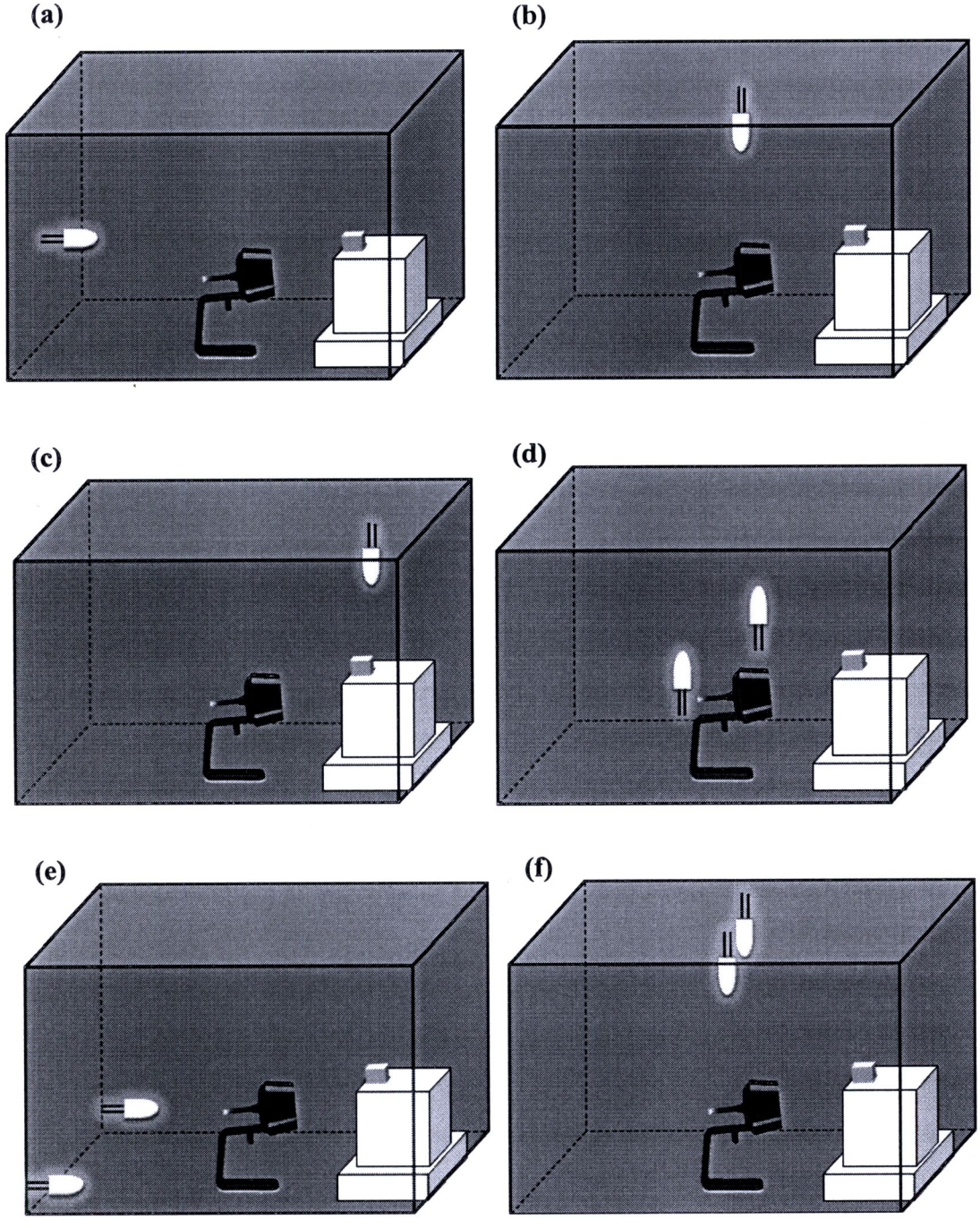

Figure 30 Schematic diagrams of different the LEDs positions

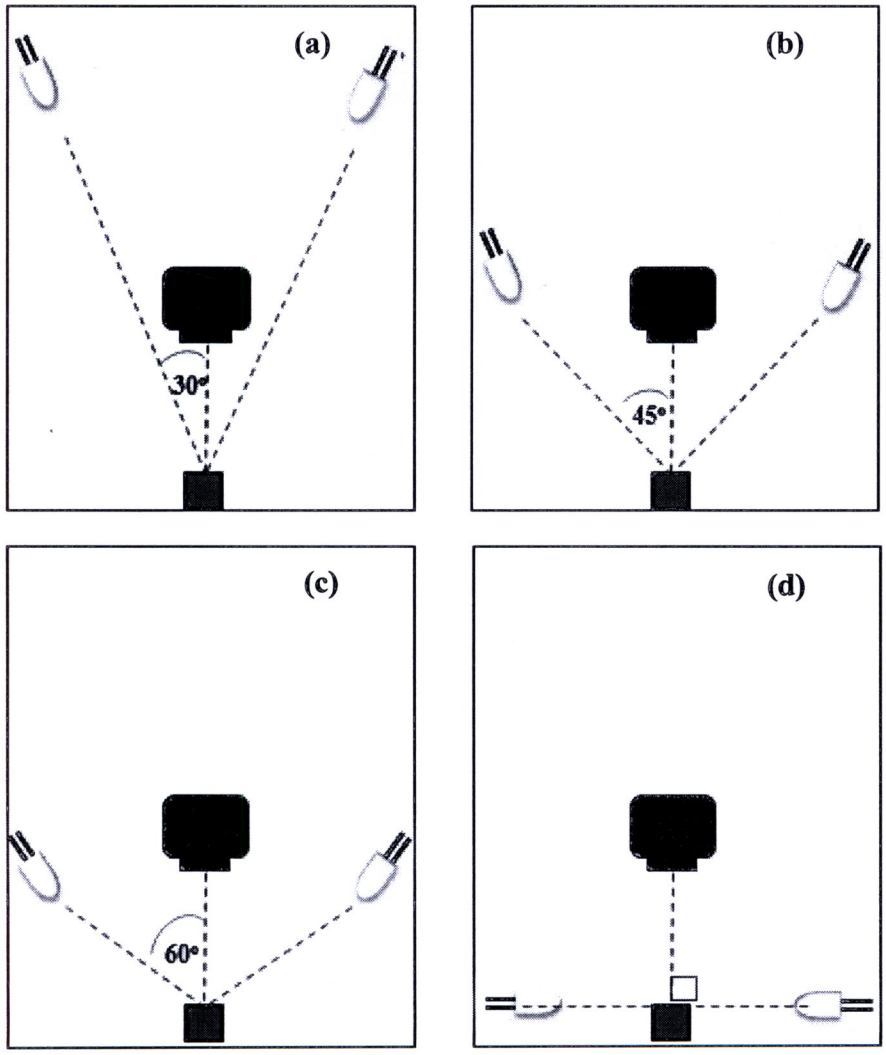

Figure 31 Schematic diagrams of the angle of LEDs at (a) 30, (b) 45, (c) 60 and (d) 90 degree with respect to a sample cell surface

Study the effect of the LEDs illumination on brightness and darkness of an image

The illumination was varied and tested between 0-700 lux by adjusting a 10 kΩ variable resistors. The light box was set as shown in Figure 32 by controlling the light position at 45 degree with respect to a sample cell surface and the focusing distance from camera lens to sample cell surface at 7.0 cm. Color product of 100 μg mL^{-1} of standard protein solution and blank solution were transferred to sample cell then taking an image with CMOS webcam camera. The data was processed by an ANNs written program and visual inspection.

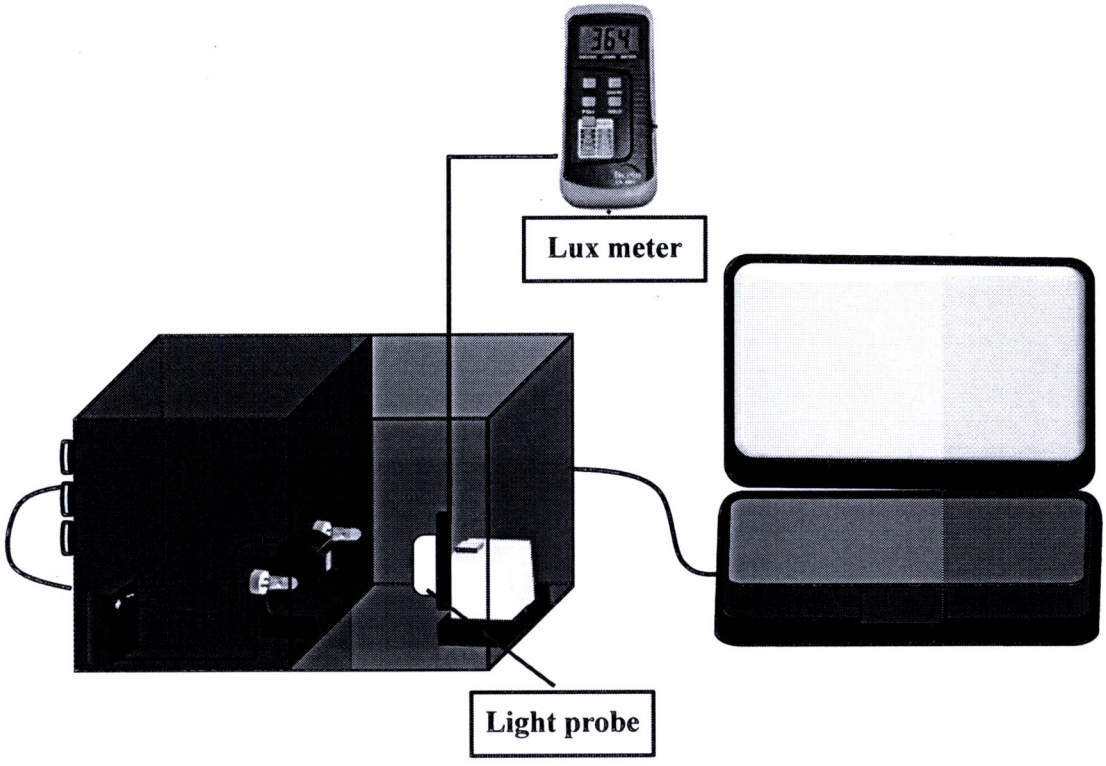

Figure 32 Schematic diagrams for the LEDs illumination study

Design color processing software

The protein standard solutions and sample solutions were captured and stored as "jpeg" (24-bit) compressed files and 1280 pixels x 720 pixels of dimension by software written in Visual Basic version 6.0 as shown in Figure 33. In the region selection, all images were delimited by user to give a homogenous area for data processing as shown in Figure 34. Then these selected images were processed with the software by random learning for 3,000 times in each image to get the average of red, green and blue (RGB) value [8]. For protein estimation, the feed-forward ANN which known as the back propagation neural network (BPNN) was selected for color processing. This BPNN consists of three components: an input layer, a hidden layer and an output layer. For the determination of protein, BPNN methodology involves RGB learning of 11 concentrations of proteins in the range of 0-10 $\mu g\ mL^{-1}$. All images of standard protein concentrations and blank were processed by input the RGB values (each color representing different intervals from 0 to 255) into database. Then the written software predicted the amount of protein residue in NR latex and latex

gloves by comparing the RGB value of the sample image with the nearest RGB value of the protein standard solution in the database.

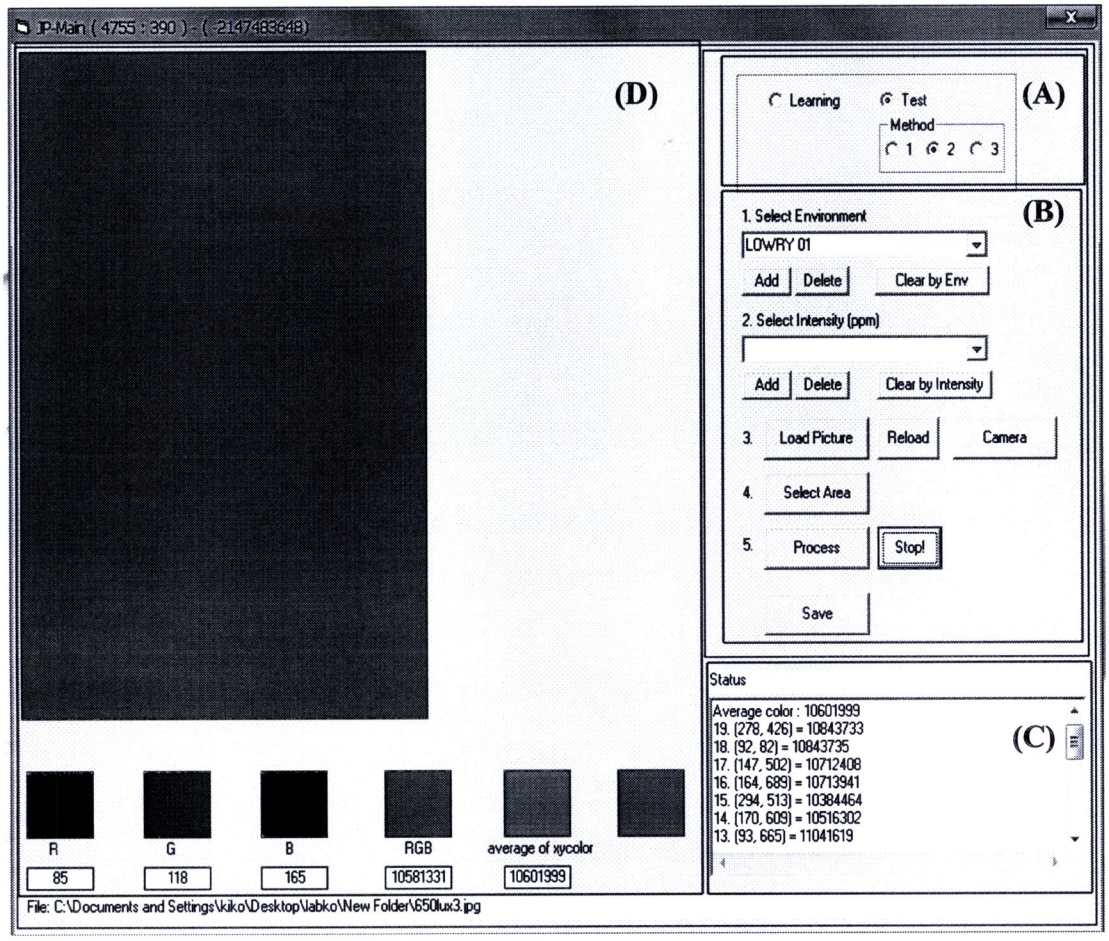

Figure 33 Display of the ANNs written program; (A) learning or testing mode, (B) controlling mode, (C) running mode and (D) monitoring mode.

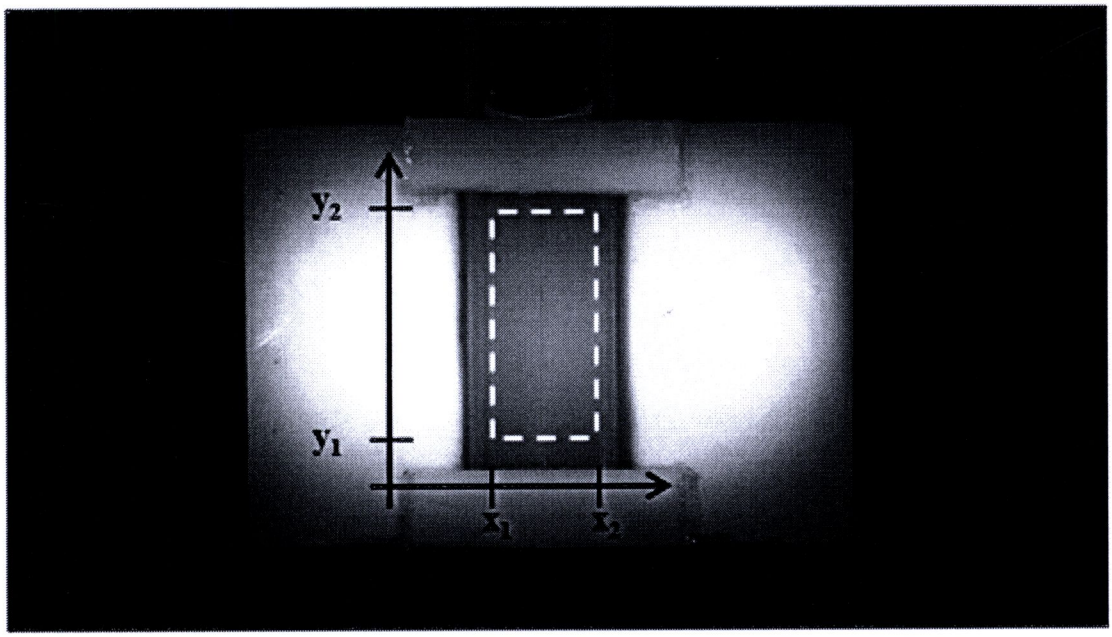

Figure 34 Delimited region of a digital image. x_1, x_2, y_1 and y_2 represent the coordinates of the delimited region

Operating of the written program (Figure 30) is divided into the following four modes:

(A) Learning or testing mode

This mode is the selection of data training or testing. Learning mode is inputting and keeping the RGB-based value as calculated in equation 1 into the database. Testing mode was studied by three different methods;

1. Method 1 uses the average of RGB-based value of the reading for 3,000 times.

2. Method 2 uses the average of R, G and B by reading for 3,000 times then converting to RGB-based value.

3. Method 3 uses the mode number of R, G and B by reading for 3,000 times then converting to RGB-based value.

$$\text{RGB-based value} = R \times 256^2 + G \times 256 + B \quad (1)$$

Where: R is the decimal value for red, G for green and B for blue.

Many electronic devices use the RGB color model, which gets its name from the three primary colors, red, green and blue. A six-digit hexadecimal number represents a given color by assigning two digits for each of the primary colors. For example, FF0000 is bright red, because red's contribution is FF, the largest two-digit hexadecimal number. The digits for green and blue are all zeros, meaning that both are excluded. Hexadecimal is a concise way to write the binary values which is normally used in the computers system. RGB are often written as three decimal numbers and converted them to a single decimal number or RGB-based value as described in the equation 1. For example, 486DD3 can be written as "211, 109, 72" then these numbers were pluged into the RGB-based value that is $211 \times 256^2 + 109 \times 256 + 72 = 4746707$.

(B) Controlling mode

Controlling mode consists of the following five steps:

1. Select environment
2. Select intensity
3. Take, load or reload an image
4. Select area
5. Process, stop or save

(C) Running mode

Running mode shows the number of iteration, the position of random point and the RGB-based value while data processing.

(D) Monitoring mode

Monitoring mode shows the color of the image, R, G, B value and the RGB-based value.

Application to extractable protein assay in NR latex and medical latex gloves

NR latex (high ammonia latex, 60% of dry rubber content (DRC)) was purchased from Thai Rubber Latex Corporation (Thailand) Public Company Limited. Medical latex gloves samples were random selected from 5 different brands: Pro gloves (DR. Boo, Chonburi Thailand), Hycare (Hycare International, Songkla Thailand), Sempermed (Siam Sempermed, Songkla Thailand), GPO (Siam Sempermed, Songkla Thailand), Saf-gard (Union Glove, Singburi Thailand). NR latex

samples were kept in PE bottles at room temperature. Medical latex gloves samples were kept in PE bag at room temperature. Protein assay in NR latex and medical latex gloves samples were tested following the extraction and assay procedure [2].