CHAPTER 2

Experimental

2.1 Chemicals

1. 2,2-diphenyl-1-picrylhydrazyl : $C_{18}H_{12}N_5O_6$ (Sigma-Aldrich, USA)
2. 6-Hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid : $C_{14}H_{18}O_4$ (Sigma-Aldrich, USA)
3. Sodium chloride : NaCl (Sigma-Aldrich, USA)
4. Sodium phosphate dibasic dodecahydrate : $HNa_2\ PO_4 \cdot 12H_2O$ (Ajax Finechem, New Zealand)
5. Potassium phosphate monobasic : KH_2PO_4 (Fisher, USA)
6. Potassium chloride : KCl (Ajax Finechem, New Zealand)
7. Tris(hydroxymethyl)aminomethane : $C_4H_{11}NO_3$ (BHD, England)
8. Sodium hydroxide : NaOH (Merck, USA)
9. Hydrochloric acid 37%: HCl (RCl Labscan, Thailand)
10. Ethanol 99.9 %: CH_3CH_2OH (QREC, Newzealand)
11. Methanol 99.9 %: CH_4O (RCI Labscan, Thailand)

2.2 Materials and instruments

1. Spectrophotometer (Genesys 10S UV-Vis, China)
2. 3D printing (Flashforge 3D printer dreamer, China)
3. Chromatography paper Whatman No.1 (Sigma-Aldrich, USA)
4. Black acrylic plastic (Market,Thailand)
5. Light emitting diode (LED) daylight 6500K, (TOSHIBA, China)
6. Photo professional foldable flash diffuser soft box (Generic, China)
7. Smartphone iPhone 5S A1530 (Apple Inc., China)
8. Canon pixma MG 3500 (Canon, Singapore)
9. Electronic multichannel pipette (RAININ, USA)

2.3 Software

1. Microsoft Excel 2007 (Microsoft, USA)
2. Yamera Application (AppMadang, USA)
3. Adobe Photoshop CS5 (Adobe, USA)
4. Homemade Program (Computer Science, Thailand)

2.4 Preparation of reagents

All chemicals used in this research are analytical reagent grade. All solutions were prepared by dissolving in deionized (DI) water obtained from a Millipore water purification system (Millipore, Sweden).

2.4.1 Standard solution of DPPH and Trolox

The stock standard solution of 2,2-diphenyl-1-picrylhydrazyl (DPPH) 1 mM was prepared by dissolving 0.0197 g in 50 mL of methanol and solutions of 50-400 µM DPPH were prepared by diluting the stock solution with methanol-Tris buffered solution (methanol : 10 mM Tris buffer pH 7.5, 1:1 v/v).

Standard solution of 6-Hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (Trolox) 0.4 mM was prepared by dissolving 0.0500 g in 50 mL of DI water and 0-220 µM Trolox solutions were prepared by diluting the stock solution in DI water.

2.4.2 Reagent solutions

2.4.2.1 Buffered medium (methanol : 10 mM Tris buffer pH 7.5, 1:1 v/v)

Buffered medium : Tris buffer 10 mM was prepared by dissolving 1.2114 g of Tris(hydroxymethyl)aminomethane in 800 mL of DI water and adjusted to pH 7.5 by adding 0.1 M NaOH and adjusting the final volume to 1000 mL. Finally add methanol (methanol: 10 mM/l Tris buffer pH 7.5, 1:1 v/v).

2.4.2.2 Ethanol-buffer solution (ethanol: 10 mM Phosphate-buffered saline pH 7.4, 1:1 v/v)

Ethanol-buffer solution : Phosphate-buffered saline (PBS) 10 mM was prepared by dissolving 3.6175 g of Na_2HPO_4, 0.2475 g of KH_2PO_4, 8.0063 g of NaCl,

0.2017 g of KCl. Start with 800 mL of distilled water to dissolve all salts. Adjust the pH to 7.4 with 0.1 M HCl and 0.1 M NaOH. Add deionized water to a total volume of 1000 mL. A final concentration of 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, 137 mM NaCl, and 2.7 mM KCl. Finally add ethanol to the solution (ethanol: 10 mm/l Phosphate-buffered saline pH 7.4, 1:1 v/v).

2.5 The study improve sensitivity on reaction of DPPH radical with Trolox in batch method

The study was conducted to find out how to improve the method to be rapid. The assay was carried out in buffered medium and ethanol-buffer solution. The maximum absorption wavelength of 50, 100 and 250 μM concentrations DPPH in ethanol-buffer solvent and buffered medium solution was studied in the range of 400 to 600 nm. The effect of the solution media on reaction kinetic of DPPH radical with Trolox standard concentrations 10,70 μM was studied in the range of 0-60 min. The concentrations-response curve with concentrations of DPPH in the range of 50-400 μM in Tris buffered medium were studied.

2.6 Analytical procedure of a simple colorimetric detection system on well plate

A simple colorimetric detection system was applied for evaluation of antioxidative activity by DPPH method in local tea samples. A piece of 5 mm diameter sliced by punched chromatographic paper was placed in each well for absorbing the solution. Then the standard DPPH solution (350 μM) was added into the well plate, 20 μL each well, followed by 20 μL of standard antioxidant or sample solution were mixed on chromatographic paper. The paper available at low cost, biocompatible, and it provides a clear RGB signal when taking a photograph. The color was changed from purple to yellow and at 9 min the more stable reduced form of DPPH was produced. Photograph of the well plate was taken by smart phone (iPhone 5S) as described in the appendix A. It was analyzed for color intensity by the developed software as described in section 2.9. Antioxidant activity of the sample can be evaluated from the calibration graph and reported as Trolox equivalent concentration.

2.7 Batch spectrophotometric procedure

Standard solution of DPPH (50-400 μM) was prepared in buffered medium (methanol: 10 mM Tris buffer pH 7.5, 1:1 v/v). In order to find the best DPPH concentration to be used the DPPH solution 1.5 mL was added and followed by 1.5 mL of Trolox was added 1.5 mL in the range of concentrations 10-130 μM and the absorbance was recorded. The maximum absorption wavelength of DPPH radical at 525 nm was observed in Tris buffer medium. The standard method was carried out using a spectrophotometer for comparison of the results with a simple colorimetric detection system for antioxidant capacity assay.

2.8 Apparatus and instrument setup

2.8.1 Photographic unit

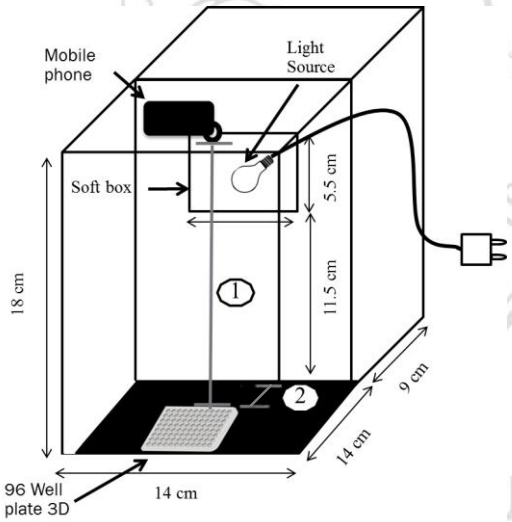

Figure 2.1 Photographic system in a light controlled box.

The design of colorimetric analyzer consisted of 2 boxes of dimensions of 14 × 14 × 18 cm and 9 × 14 × 18, respectively as shown in Figure 2.1. The boxes were made from black acrylic to protect light enter into the detection unit with white paper on its interior wall and black paper under the well plate for preventing light reflection. The well plate was made by 3D printing with white ABS plastic. Light intensity in the box was controlled with light emitting diode (LED), white light (6500K of color

temperature) covered with photo professional foldable flash diffuser soft box, using as broad bulb spectrum light sources placing out of the detection box.

The smart phone camera was set at optimized exposure conditions to obtain the best determination performances on Yamera Application (App), i.e., automatic, high dynamic range (HDR), automatic sensitivity (where the ISO speed was set within 34-44), and used in single image mode. The images appeared pixel of the histogram. The iPhone 5s was used with a built in camera of 8 megapixels resolution with f/2.2 aperture.

2.8.2 96-well plate 3D design

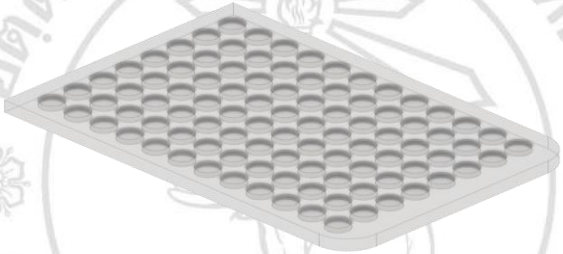

Figure 2.2 96-well plate 3D design

Photographic taken on a normal 96-well plate has problems about effect of lens, light reflection of solutions and shadow causing from illumination that reduces the homogeneity of the image. In order to solve their problems a new design of 96-well plate was made by using a homemade software. A with 96-well plate 3D design dimensions of 12 × 8 cm with diameter of circle well of 6 mm and depth of 2 mm for fixing position of solution in the well plate. A more shallow well plate reduces shadow in the photograph. The design 3D well plate was printed by 3D printer using a white ABS plastic that minimizes light reflection.

2.8.3 Lab on paper

Chromatographic paper, smooth surface, 0.18 mm thick (Whatman No.1) was used to reduce the effect of light reflection from the solution. A piece of 5 mm paper was made by a punched tool. A piece of paper was placed in each well of a 96 well

plate for absorbing the solution, and acts as a medium for reaction to take place, producing color on the paper.

2.9 Computer program for antioxidative assay

The main objective of this work is to detect the RGB color of the input image of a microtiter plate with 96 sample wells. All input images are collected from the designed reader system with different light levels in order to study the effect of light intensity on RGB detection. However, the gained image is in RGB color format, the different light conditions may cause the worsen quality of original image. The computer program was developed for evaluating RGB color intensity of the image. The procedure of the program consists of different steps as shown in Figure 2.3. The image obtained from the designed photographic system of section 2.8.1 was input into program. Image segmentation is an important and critical step that leads to the analysis of the processed image data. First step, region of interest plate segmentation, it needs to convert the input image to a binary image. The area of white color pixel is related to the region of a desired plate and conversely black pixel is related to background or environment of the plate in the image. Thus, the region of the interest microtiter plate is cropped from an input image as shown in Figure 2.3 In the second step, edge detection and image morphological operations such as dilation and erosion are performed to detect spot regions. The distance between two closest columns or rows is computed and applied as parameter for centroids estimation using circular hough transform as shown in Figure 2.2 In the last step, the average RGB color intensity of 9 coordinate pixels around each center of spot is computed. The values of R, G and B color intensity were then summarized in excel table.

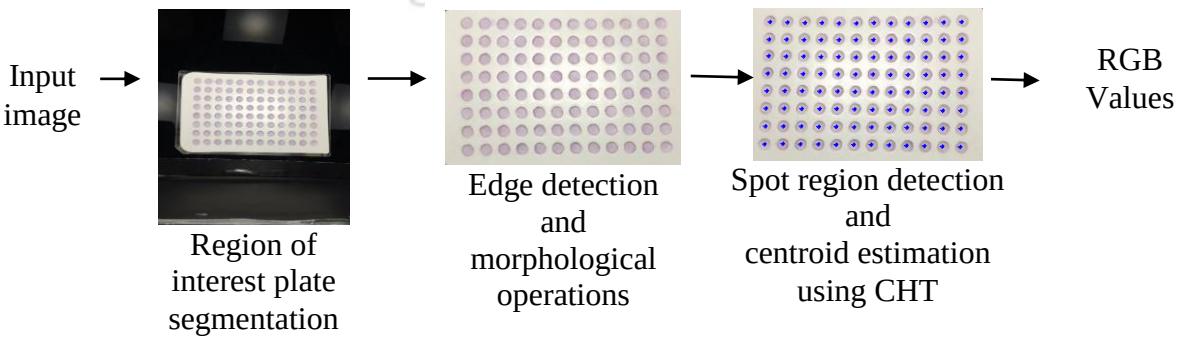

Figure 2.3 Procedure of computer program for antioxidative assay. First step, Input Image and region of interest plate segmentation. Second step, edge detection and image morphological operations. Last step, average RGB color intensity and read color information.

2.10 Optimization of Antioxidative Assay

2.10.1 Back screen of photographic unit

The white and black papers as back screen of interior in the box were studied to increase smooth distribution in illumination of the light and improved precision on antioxidant capacity assay. The histogram of image that acts as a graphical representation of the tonal distribution in a digital image is evaluated by Adobe Photoshop CS5.

2.10.2 Focal length of the mobile phone camera

Effect of distance between the mobile phone camera and well plate was studied in the range of 10-23 cm as described in Figure 2.1 as shown in number 1 and effect of distance between behind the box wall and well plate of 2.5-4.5 cm as shown in number 2 was also investigated.

2.10.3 Lighting control

The LED light source covers with soft box as described in Figure 2.1 was used to produce different light conditions. The histogram of lighting control without filter showed broader peak than with soft box covered light source.

2.10.4 Performance of smart phone camera

The ISO parameter was used to improve sensitivity and reduce effect of shadow of the picture as comparison with Auto and HDR mode. The camera of smart phone used Yamera (App.) on smart phone to fix ISO.

2.10.5 Study sensitivity of colors

Paper pantone colors were printed to compare performance of camera on determination of color intensity of various colors, e.g., purple, blue, green, yellow, orange and red, and to confirm linearity with Adobe photoshop CS5 program and take a picture by fix modes Auto, HDR, ISO34, ISO39, and ISO44.

2.10.6 Effect of the reaction time

In this method, timing should be controlled precisely in order to obtain accurate result. Using Optimum condition of taking photograph in a light controlled box was used for study effect of the reaction time on antioxidative assay. It was studied in the range 3-30 min. The reaction time between DPPH radical and Trolox standard was studied in buffered medium.

2.10.7 Effect of DPPH volume

DPPH volume will affect on senstivity of analysis by various concentration of Trolox standard. So the volume of DPPH was studied in the reage of 10-25 μL by using multichannel micropipette to control volume and dropping solution on chromatography paper in each well for absorbing the solution.

2.11 Analytical characteristics of the procedure

2.11.1 Calibration curves and limit of detection

Using optimum conditions, a calibration graph plotting between green intensity or absorbance and Trolox standard antioxidant concentration was used constructed for antioxidative assay. It was found to be linear in the range 0-130 μM for both the proposed method and spectrophotometric method. The limit of detection (LOD) was calculated based on the criteria, LOD = 3SD of intercept of calibration graph/slope of the calibration graph as described in the appendix B.

2.11.2 Precision study

The precision of proposed method was examined by analysis of 10 µM Trolox for 11 replicates. Stability of the developed method was examined by repeatedly inter-day analysis of 10 µM Trolox for 3 replicates for 3 days. The percentage of relative standard deviation (%RSD) value was used to evaluate the precision which calculated from the equation 2.1.

$$\%RSD = \frac{S.D.}{\bar{x}} \times 100 \qquad 2.1$$

When; S.D. = Standard deviation

$\bar{x}$ = Mean value

2.11.3 Accuracy study

The sample solutions were measured for antioxidative activity by using theproposed method and compared with the batch spectrophotometric method. The results obtained from both the methods were not significantly different, according to examination the t-test at 95% confidence level as described in the appendix E.

2.12 Preparation of sample

2.12.1 Application of the method to real samples

The ready to drink tea samples were prepared by diluting with DI water and cooled to ambient temperature before the analysis. Sample were kept for analysis within 1–2 weeks of preparation.

2.12.2 Sample analysis

Trolox equivalents (TE) can be obtained from calibration curve. The antioxidant capacity of the sample is expressed in micromolar of Trolox equivalents (TE) per 1 milliliter of sample which calculated as described in the appendix D.