CHAPTER 3

Results and Discussion

3.1 Overview of the research

In this research, a system for fast and high throughput screening of antioxidant capacity was developed. The system is based on performing parallel DPPH assay in a well plate and the resulting reaction product was determined by taking a photograph of the well plate and evaluating color intensity by developed program. Calibration graph was plotted between green color intensity and antioxidant concentration (using Trolox as standard) and used for determination of antioxidant capacity of real sample. Firstly, solvents for DPPH assay of antioxidant capacity was optimized to obtain reliable results and fast analysis. In previous report DPPH reaction complete in methanol about 55 min [Brand-Williams, 1995]. Later on two solvent systems were investigated as the medium for DPPH assay such as buffered medium (methanol: 10 mM Tris buffer pH 7.5, 1:1 v/v) has been described in semi-aqueous medium, which occurs firstly through antioxidant deprotonation previous to the electron transfer process [Abderrahim, 2013], and ethanol-buffer solution (ethanol: 10 mM Phosphate-buffered saline pH 7.4, 1:1 v/v) [Friaa, 2006]. In this work, the reaction was observed by considering, slope, linearity and reaction time Therefore, The concentration of DPPH reagent of 350 µM in buffered medium was selected for assay at 10 min, with better sensitivity and shorter analysis time could be achieved. Secondly, the system for antioxidant capacity assay on well plate based on smart phone was developed. The condition for taking photograph were observed in a special design light controlled box that can provide well distribution of light intensity for image capture. Histogram of the intensity of the pictures was examined under various conditions in order to improve sensitivity and reproducibility of the proposed method. Under the optimum condition, analytical characteristics of the procedure were investigated and the proposed system was applied to real sample.

3.2 The study improve sensitivity on reaction of DPPH radical with Trolox

3.2.1 The effect of the solution media on absorption wavelength of DPPH radical with Trolox

The reaction of DPPH radical with Trolox standard antioxidant was studied. The detection was carried out in buffered medium and ethanol-buffer solution. The maximum absorption wavelength was examined from the spectra in the range of 400 to 600 nm as described in section 2.5. The absorption of DPPH in ethanol-phosphate buffer and Tris buffer solutions 50, 100 and 250 μM are depicted in Figure 3.1-3.2. The calibration graph was plotted between absorbance of the maximum absorption wavelength and DPPH concentrations.

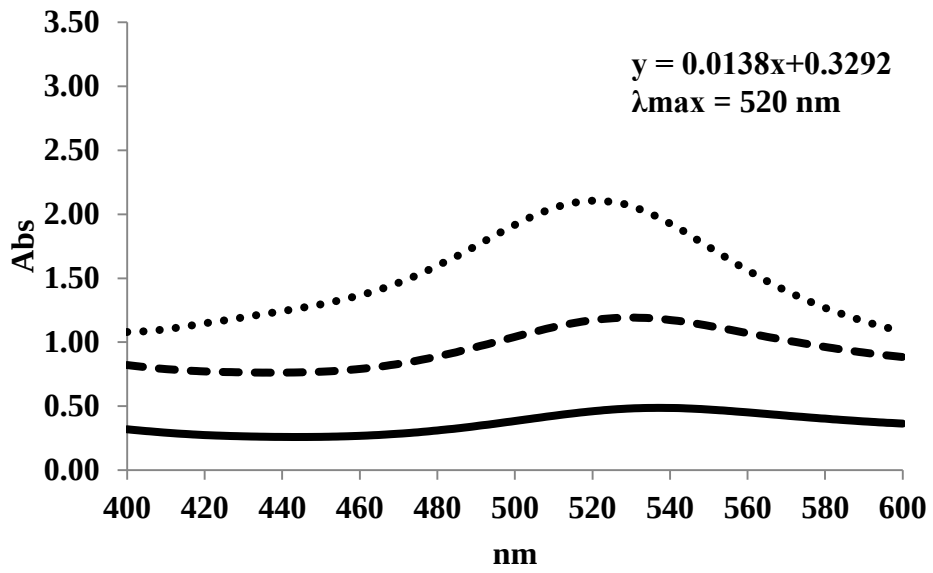

Figure 3.1 Absorption spectra of DPPH in ethanol-phosphate buffer solution; 50, 100 and 250 μM.

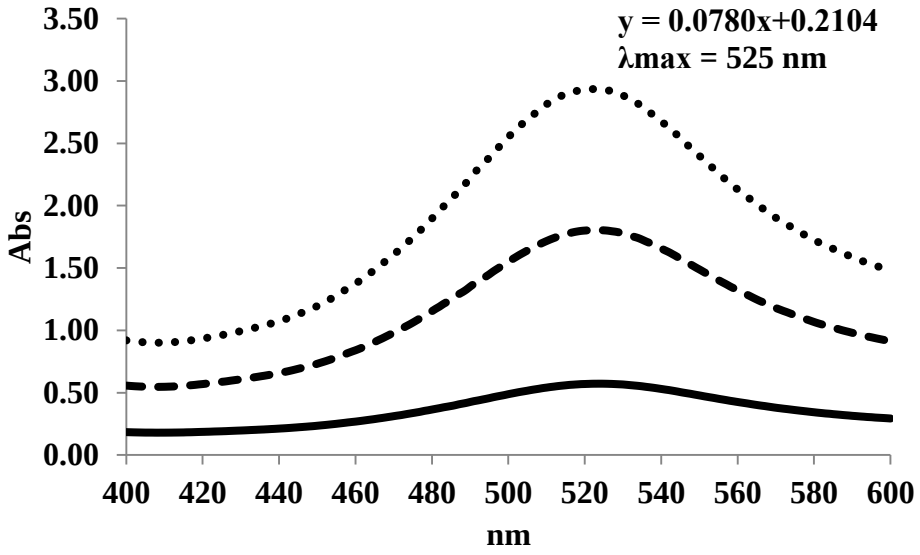

Figure 3.2 Absorption spectra of DPPH in Tris buffered medium; 50, 100 and 250 μM.

The absorption spectra as shown in Figure 3.1 and 3.2 illustrated the effect of solution medium on spectra of dot line 50 μM, dashed line 100 μM and solid line 250 μM DPPH radical. The maximum absorption wavelengths in ethanol-phosphate buffer and Tris buffer solutions are at 525 and 520 respectively. The calibration equations of DPPH in ethanol-phosphate buffer solution is y = 0.0138x + 0.3292 and in Tris buffer medium is y = 0.0780x + 0.2104. The slope of calibration in Tris buffer medium is higher sensitivity than in the ethanol-buffer solution. Therefore, Tris buffered medium is more appropriate for DPPH assay.

3.2.2 The effect of the solution media on reaction kinetic of DPPH radical with Trolox

The reaction time between DPPH radical (300 μM) and Trolox standard concentrations 10 and 70 μM in Tris buffer medium and ethanol-buffer solution was investigated in the range of 0-60 min.

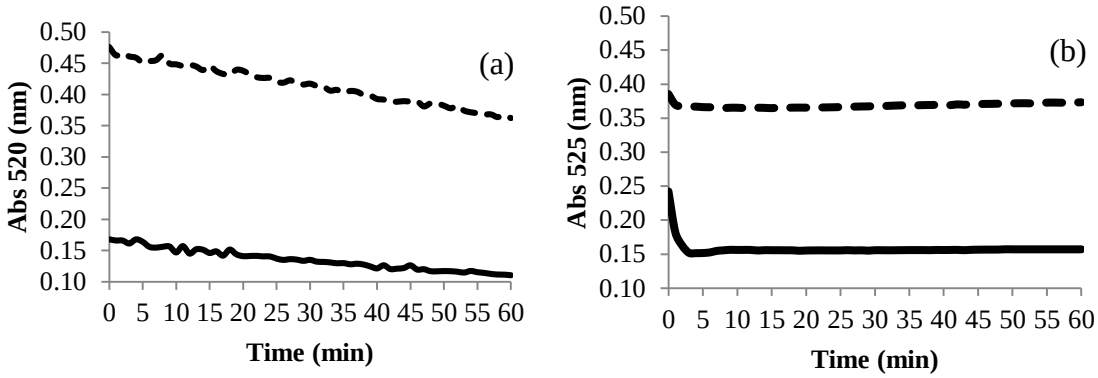

Figure 3.3 Absorbance-Time plot indicating reaction kinetic between DPPH radical (350 μM) and Trolox standard concentrations, dashed line 10 and solid line 70 μM in (a) ethanol-phosphate buffer and (b) Tris buffer solutions.

As shown in Figure 3.3, Tris buffered medium is more appropriate for DPPH assay. Reaction was completed within 10 min. Moreover, absorbance at the maximum absorption wavelength of 525 nm in buffer medium was higher than that of in ethanol-buffer solution. Therefore, better sensitivity and shorter analysis time could be achieved.

3.2.3 Concentration-response curve for DPPH

Concentrations-response curve for the absorbance at 525 nm of DPPH with various concentrations of DPPH was studied in the range of 50-400 μM in Tris buffer medium. The result is shown in Figure 3.4.

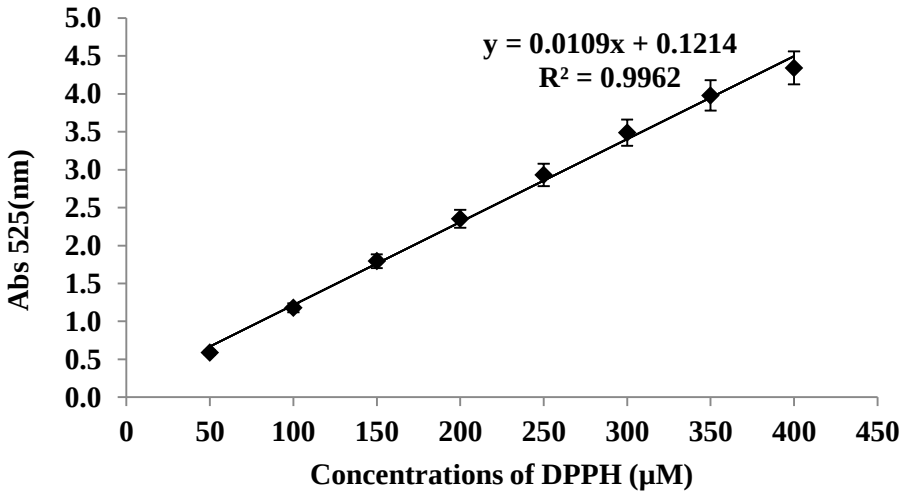

Figure 3.4 Calibration graph of DPPH concentrations for estimation of antioxidant activity

The initial concentration of DPPH radical was selected from a graph plotting between absorbance at 525 nm and DPPH radical concentration as shown in Figure 3.5. The maximum concentration that still gave linear relationship was chosen, therefore 350 µM DPPH initial concentration for determination of antioxidant activity. The reaction of Trolox with DPPH radical will reduce absorbance of DPPH radical proportionally.

3.3 Colorimetric analytical system based on camera detection

3.3.1 Study of interior wall in the photographic unit

The white and black papers as back screen of interior wall in the box were studied as described in section 2.10.1. An image histogram is a type of histogram that acts as a graphical representation of the tonal distribution in a digital image. It plots the number of pixels for each tonal value. By looking at the histogram for a specific image will be able to judge the entire tonal distribution at a glance. The horizontal axis of the graph represents a particular brightness value, while the vertical axis represents the number of pixels in that particular tone. The left side of the horizontal axis represents the black and dark areas, the middle represents medium grey and the right hand side represents light and pure white areas. The histogram in Figure 3.5 (a) is a very bright

image and Figure 3.5 (b) with few dark areas on the left side. The corresponding photograph to these histograms are shown in Figure 3.5(c) and 3.5(d), respectively.The result in the graph indicated better light distribution of white paper interior then the black one. Therefore it was selected to be used as a back screen. An image histograms can be analyzed for peaks and valleys of the light is not well distribution throughout the photograph. The narrow peak and appearing on the right side of histrogram (white image) is preferred in this work.

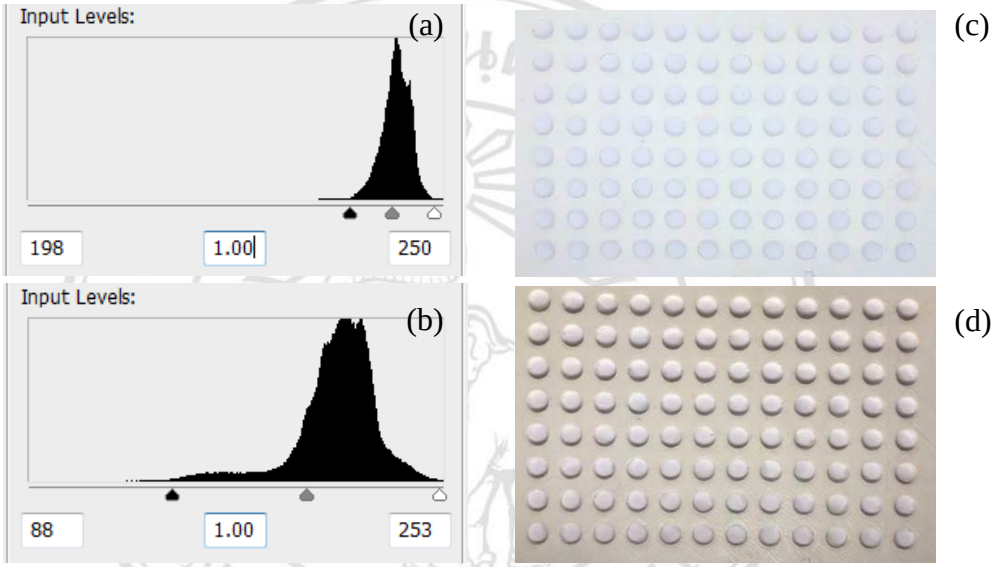

Figure 3.5 Histogram showing tonal distribution of a digital image of interior in the box with (a) white paper (b) black paper on interior wall of the box, and photographs corresponded to the histograms (c) white paper (d) black. For more details see text.

3.3.2 Study focal length of the mobile phone camera

Effect of distance between the mobile phone camera and sample holder (well plate) was studied as described in section 2.10.2. The peak area of from histogram of the well plate, inside light controlled box was considered. Area under the graph is evaluated by Adobe Photoshop CS5. The horizontal axis is the brightness levels at the 256 level (gray level), with values ranging from 0-255 when the level is low (on the left) represents a little brighter or more presence a black color. The high level (on the right) refers to a very bright or the presence of a white color. The vertical axis of the graph shows the number of pixels in each gray level, which is a relative value. The length of the mobile phone camera and well plate at 18 cm as shown in Figure 2.1

number 1 and distance between the behind well of the box and well plate at 3.5 cm as shown in Figure 2.1 number 2 was chosen because they gave smaller peak area (well light distribution) and peak position area on the right side (white color) as shown in Figure 3.6.

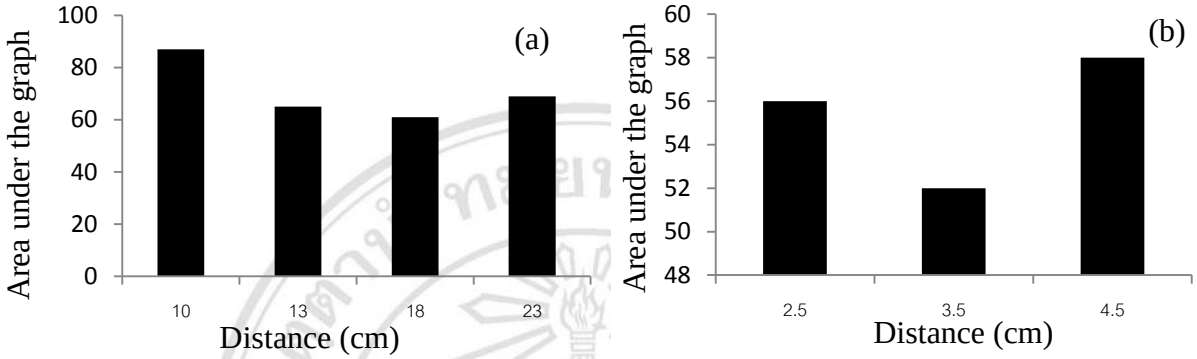

Figure 3.6 Peak area under the graph of tonal histogram at various distance between the mobile phone camera and well plate (a) 10-23 cm and at various distances between behind wall of the box and well plate (b) 2.5-4.5 cm.

3.3.3 Lighting control of photographic unit

The LED light source covered with soft box as described in section 2.10.3. was used to control illumination in the measuring box. The image histograms of picture taken under light control conditions are shown in Figure 3.7, (a) LED without filter and (b) LED with soft box covered. Figure 3.8(b) showed narrower peak shape and lower area under the image histogram, indicating that it provided a smooth photo with less shadow. Therefore the soft box covered LED was selected to improve sensitivity and precision of the detection. The conresponded photography of the histrograms (a) and (b) are shown in Figure 3.7 (c) and 3.7(d), respectively.

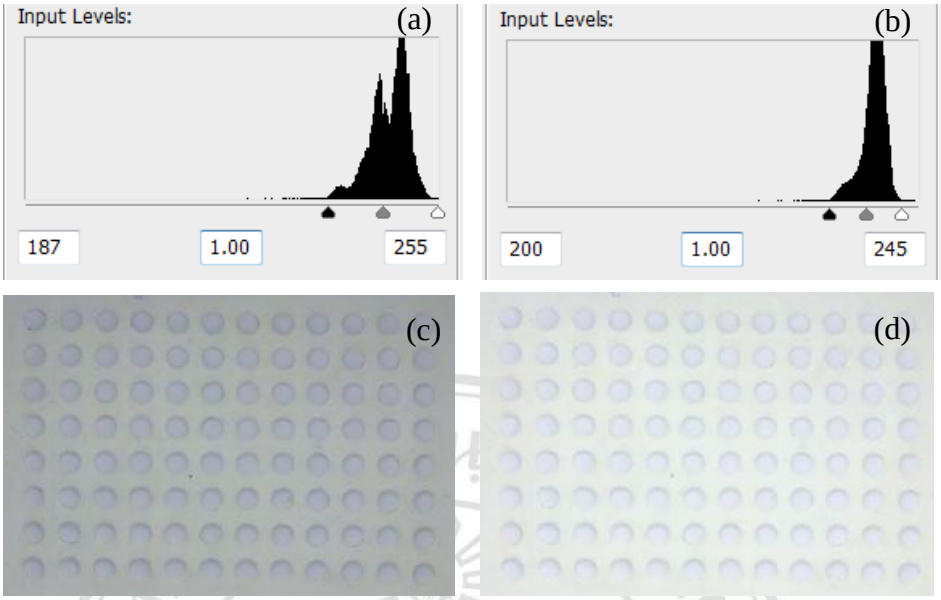

Figure 3.7 Image histograms of different lighting control procedures (a) LED without filter (b) LED covered with soft box. The conresponded photography of (a) and (b) cases (c) and (d).

3.3.4 ISO mode of smart phone camera

The camera of iPhone 5 used Yamera application on the smart phone to fix ISO parameter. The detection on red, green, blue papers at 9 points distributed around the well plate as shown in figure 3.8 (a) was carried out by using different ISO modes. The RGB values obtained from 9 points were used to calculate RSD that shows the precision of measurement from different wells of the well plate. Effect of ISO parameter on precision is shown in Figure 3.8(b). All ISO modes provide RSD less than 5% for all RGB values, with HDR mode give lower RSD.

(a) (b)

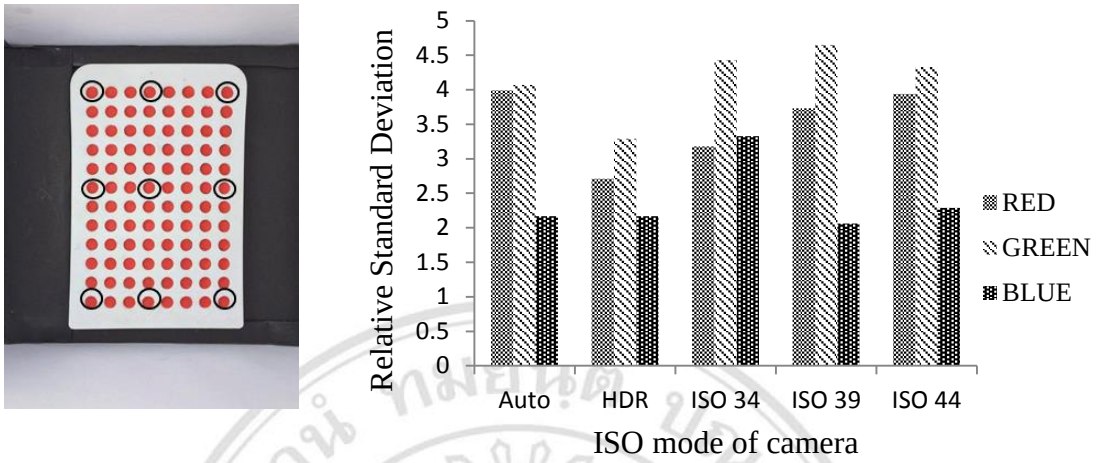

Figure 3.8 (a) 9 detection points on well plate and (b) Relative standard deviation of color intensities on the detection of red green blue papers of different positions of well plate by using various ISO modes: Auto, HDR, ISO 34-39.

3.3.5 Colors sensitivity of pantone papers

The method photographic with be used for analysis of various substances that have different colors and color intensity. Therefore, paper pantone colors were printed to compare performance of camera to detect different colors i.e. purple, blue, green, yellow, orange and red as described in section 2.10.5. A The pantone colors with different color intensities is depicted in Figure 3.9. Photographs of the pantone were taken using different ISO mode. The RGB values obtained from photograph of different colors and color intensities were used to plot calibration graphs. Linearity, R^2 of graphs were exanimated and summarized in Figure 3.9. The results as shown in Figure 3.9 indicated that Auto, HDR, ISO34 and ISO44 modes gave non-linear color intensity for some colors. However, ISO39 mode gave high linearity to all colors. The ISO 39 mode was selected for improve sensitivity of colorimetric detection.

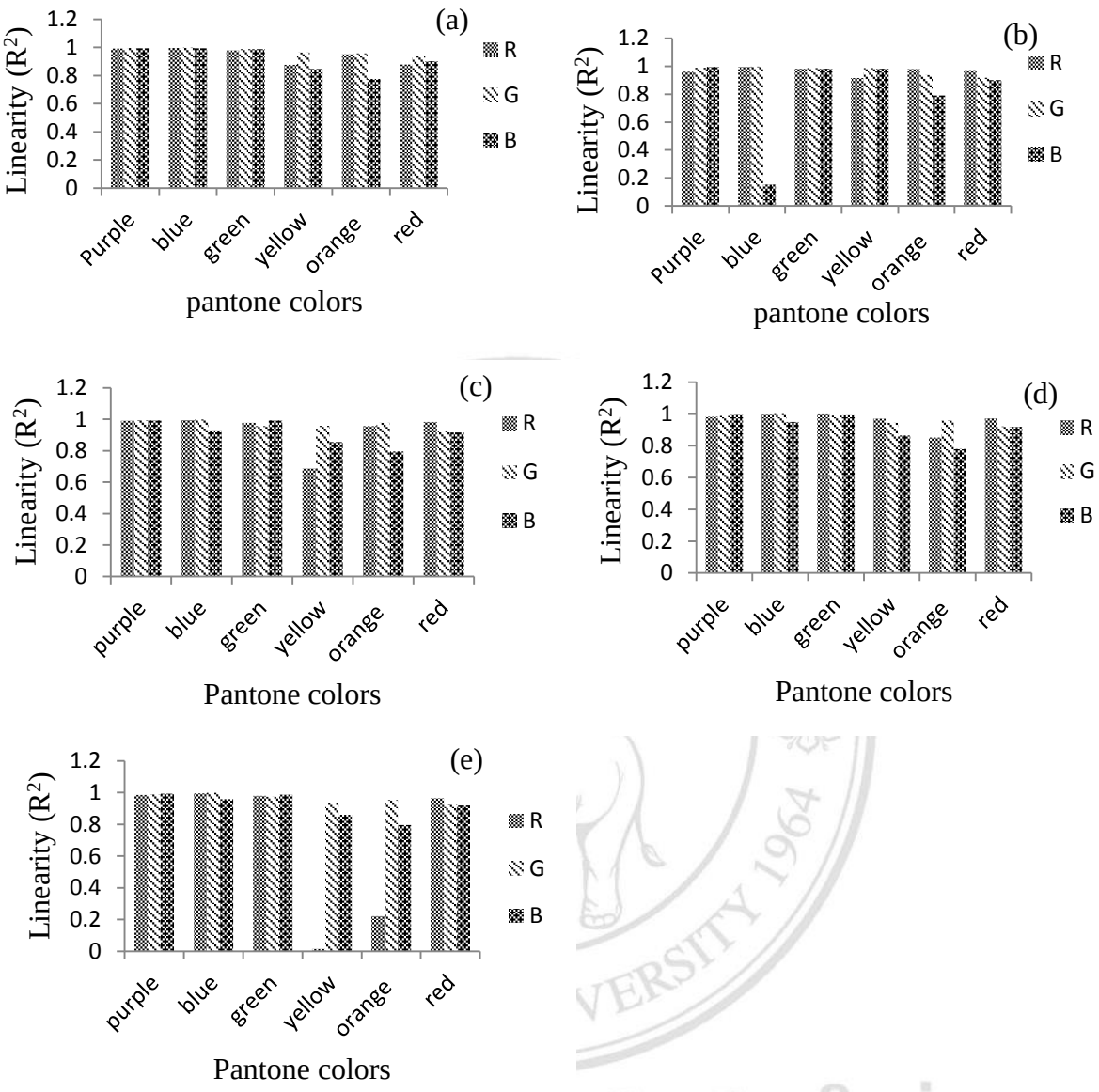

Figure 3.9 Linearity of pantone colors, purple, blue, green, yellow, orange, and red by fixed ISO modes: Auto (a), HDR (b), ISO34 (c), ISO39 (d), ISO44 (e).

3.3.6 Effect of the reaction time

The concentration of DPPH reagent of 350 µM was selected for assay. The DPPH reagent and Trolox standard 20 µL each were added in to the well plate. The reaction time between DPPH radical and Trolox standard 0-70 µM was studied in the range 3-30 min. The color of image product were observed for the intensity of red(R), green(G), blue(B) values by the developed computer program. The data were transferred to an Excel Software (Version 2007). Intensity of green color indicating

reaction kinetic, gave high sensitivity and linearity. The reaction was completed within 7-20 min. Therefore, green intensity was selected to measure the product in this developed method. The developed system provided fast and easy operation method for determining antioxidative capacity assay.

Table 3.1 Effect of the reaction time on antioxidative assay.

Time (min)	Sensitivity			Linearity (R^2)		
	R	G	B	R	G	B
3	0.3922	0.3681	0.3146	0.3440	0.9830	0.0480
5	0.3452	0.3467	0.1251	0.9790	0.9775	0.7123
7	0.3086	0.3997	0.0689	0.9380	0.9900	0.1825
9	0.3715	0.3859	0.5227	0.9215	0.9981	0.5227
10	0.3415	0.3911	0.0463	0.9573	0.9950	0.2401
20	0.3043	0.3833	0.0463	0.9260	0.9973	0.1401
30	0.3278	0.3873	0.0167	0.9704	0.8622	0.0201

3.3.7 Effect of DPPH volume

DPPH volume affected on sensitivity of analysis as described in section 2.10.7. The DPPH reagent of 10-25 µL was added in to the well plate, with 20 µL of various concentrations of Trolox standard (0-70 µM) and calibration graphs were plotted to determine sensitivity (Slope) and linearity (R^2) as sumerized in Table 3.2. The volume of 20 µL gave high sensitivity and good linearity. This volume give well dispersion of solution, good mixing and homogeneous color distribution on the paper in the well.

Table 3.2 Effect of DPPH volume on antioxidative assay.

Volume (µL)	Sensitivity (Unit/µM)	Linearity (R^2)
10	0.335	0.991
15	0.354	0.994
20	0.519	0.995
25	0.487	0.975

The optimum conditions were summarized in Table 3.3

Table 3.3 The optimum condition for antioxidant capacity array by DPPH method.

Parameter	Studied range	Selected condition
Back screen	White and Black interior	White
Light control	No filter and cover with soft box	cover with soft box
Focal length (cm)	10, 13, 18, 23	18
Mode of smart phone camera	Auto, HDR, ISO34, ISO39, ISO 44	ISO 39
Color to be used	Red, Green, Blue	Green
Reaction time (min)	3-30	9-20
Concentration of DPPH (μM)	50-400	350
Volume of DPPH (μL)	10-25	20
Volume of sample/Standard (μL)	10-25	20

3.4 Analytical characteristics of the procedure

Using optimum conditions as summarized, a calibration graph plotting between green color intensity and Trolox standard antioxidant concentration was used for antioxidative assay. It was found to be linear in the range of 0-130 μM for both the spectrophotometric and the proposed methods, as shown in Figure 3.11(a)-(d), The linear range of calibration graph was selected to create the calibration graph for sample analysis. The calibration equation of spectrophotometric method, $y = - 0.0057x + 1.5844$, $R^2 = 0.9979$ and the proposed method, $y = 0.6278x + 153.03$, $R^2 = 0.9955$ with detection limit of 9.3 μM were achieved as shown in Table 3.4. The developed method presented wide linear range and good linearity.

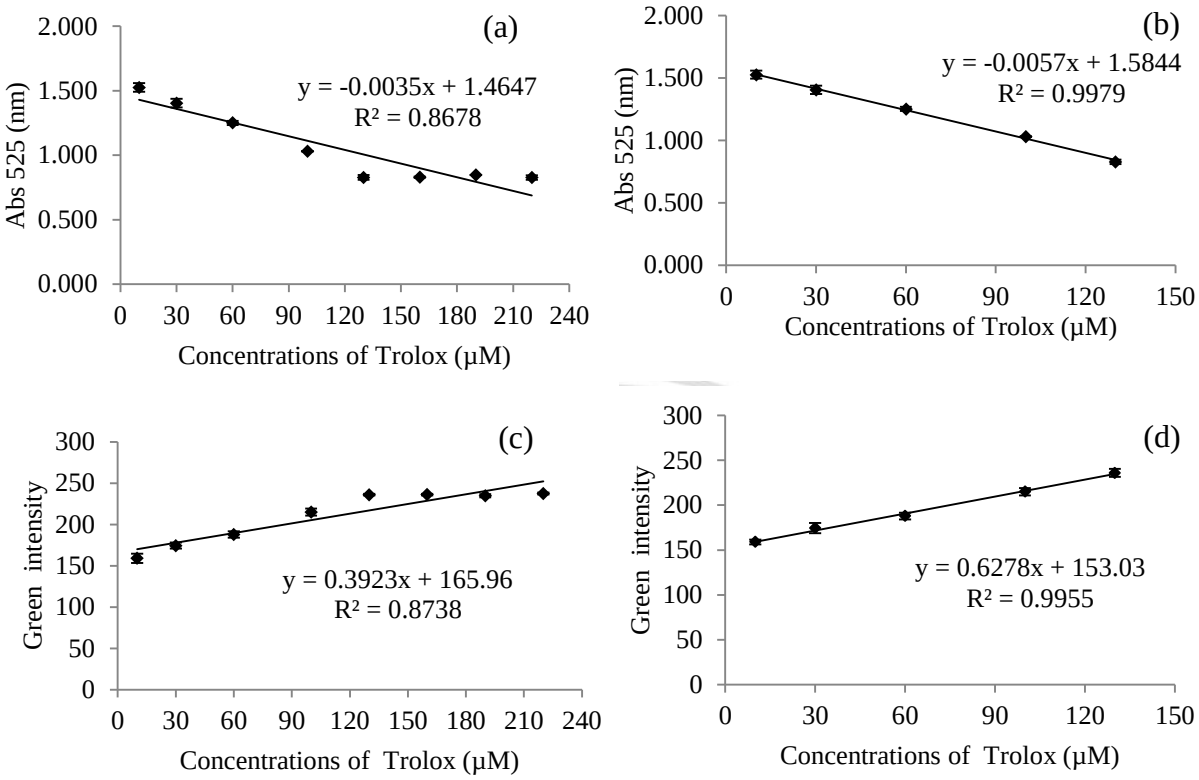

Figure 3.10 Dynamic range of curve of spectrophotometric and the proposed methods (a) and (c) and linear range of methods (b) and (d), respectively.

Table 3.4 Calibration data for antioxidative assay of spectrophotometric and the proposed methods.

Method	Regression	R^2	Linear range (µM)	LOD (µM)
Spectrophotometry	y = -0.0057x + 1.5844	0.9979	0-130	6.3
Proposed	y = 0.6278 x + 153.03	0.9955	0-130	9.3

3.4.1 Precision study

Precision of the colorimetric detection was studied by measuring 10 µM Trolox for 11 replicates. The developed system provided high precision for antioxidant capacity assay by considering from %RSD obtained which is 3.5%.

3.4.2 Stability of proposed method

The percentage of relative standard deviation (%RSD) values were used for evaluating the precision which calculated from the equation 2.1 in section 2.13.2. Moreover, the stability of the colorimetric analysis was investigated by measuring antioxidant capacity of 10 µM Trolox for 11 replicates in different days. %RSD was 1.3, indicating high inter-day precision for the analysis of 3 days, showing good stability of the system for continuous analysis.

Table 3.5 The intensity of color green obtained for antioxidant capacity of 10 µM Trolox for 11 replicates.

Antioxidant capacity (n=11)	Intensity of green color
1	155
2	165
3	164
4	160
5	169
6	167
7	159
8	152
9	166
10	161
11	169
Mean	163
SD	5.7
%RSD	3.5

3.4.3 Real sample analysis

The analytical application to real sample was investigated for tea samples by using the proposed colorimetric detection system and compared with the batch spectrophotometric method. Local tea samples were prepared as described in section 2.12.1. Trolox equivalents (TE) can be obtained from calibration curve plotting between absorbance and concentration of Trolox standard (10-130 µM) for batch spectrophotometric method, and plotting between green intensity and concentration of Trolox standard (10-130 µM) for proposed method. TE was calculated by using

calibration curve. The results are presented in Table 3.4. Considering from t-test (as described in the appendix E), the results indicated that the proposed method is reliable for antioxidant capacity assay of tea samples. The results obtained from both the methods were not significantly different, according to examination the t-test at 95% confidence level.($t_{calculated}$ = -0.25 and $t_{tableated}$ = 2.09).

Table 3.6 Trolox equivalent concentration antioxidant capacity of tea samples obtained by the proposed method comparing with the spectrophotometric method.

Sample no.	Trolox equivalent concentration (mmol/mL), (n=3)	
	Spectrophotometric method	Proposed method
1	2.26 ± 0.01	3.66 ± 0.35
2	4.11 ± 0.00	4.87 ± 0.17
3	7.54 ± 0.00	7.58 ± 0.46
4	9.95 ± 0.01	7.38 ± 0.60
5	6.14 ± 0.00	6.38 ± 0.35
6	4.20 ± 0.00	4.67 ± 0.52
7	6.31 ± 0.00	6.88 ± 0.70
8	3.07 ± 0.00	3.16 ± 0.30
9	4.39 ± 0.00	4.47 ± 0.35
10	5.25 ± 0.00	6.08 ± 0.63
11	6.85 ± 0.00	6.88 ± 0.63
12	3.30 ± 0.01	3.66 ± 0.35
13	5.49 ± 0.00	6.08 ± 0.46
14	2.51 ± 0.01	3.66 ± 0.46
15	7.82 ± 0.00	7.58 ± 0.43
16	7.12 ± 0.01	7.08 ± 0.63
17	ND	ND
18	2.58 ± 0.00	2.25 ± 0.43
19	5.59 ± 0.01	6.98 ± 0.76
20	5.97 ± 0.00	6.08 ± 0.8

ND = None detected

The results obtained from the proposed method correlated well with the spectrophotometric method, as indicated by correlation equation, y = 1.1411x-1.0234, r^2 = 0.9539