CHAPTER 1

Introduction

1.1 Overview

High throughput screening (HTS) assays is routinely used in basic biochemical, cell biology and drug discovery research. Assays for HTS did not only require small sample volume, high throughput, and robustness, but also require adequate sensitivity, reproducibility and accuracy in order to discriminate among a very large number of compounds for increase the efficiency of HTS, new screening methods must be faster, cheaper, and more quantitative. Assays need to be miniature to decrease reagent costs and consumption of chemicals. A high-throughput relative DPPH radical scavenging capacity (RDSC) assay was developed and validated [Cheng, 2006]. The development and application of a high throughput RDSC assay using a microplate reader with spectrophotometric detector and 96 well plates for evaluating lipophilic antioxidants is in high demand. This RDSC assay is easy to perform and has acceptable accuracy, precision, and reproducibility. It can be used for screening and investigating the potential natural antioxidants.

Nowadays, smartphone cameras have been applied to field test for colorimetric detection by employing RGB image-processing. Therefore in this research, the development of simple colorimetric detection on well plate for evaluation of antioxidant capacity by using mobile phone for antioxidant capacity assay was carried out. A large numbers of samples, each in small quantity can be analyzed at the same time, providing advantages of rapid, simple, low-cost, low chemical waste and easy operation. Therefore, the developed method was conveniently used in screening the antioxidative properties of local tea sample by DPPH method.

1.2 Antioxidant and its important

Antioxidants can inactivate free radicals and protect our cells from oxidative stress and the damage it causes. Also they can help our immune system defend against bacteria, fungi, viruses, and some cancers [Mandelker, 2008]. There are many other antioxidants that help protect the body. The amount of various antioxidants in food can be determined as "antioxidant activity" which is a measure of how well they inhibit free radicals. There are many different compounds that can act as antioxidants. Some, such as carotenoids (e.g., beta-carotene, lutein, lycopene), can be noticed by the orange-red color found in vegetables containing them. Natural antioxidants such as vitamin C and vitamin E also function as antioxidants. Nowadays, the study on substances that can reduce the severe of free radicals has been increased [Facts about Antioxidants, 2016]. There is increasing interest in antioxidant. Free radicals are produced as a normal part of metabolism but there are some external factors that help to promote the production of free radicals, such as smoking, environmental pollutants, radiation, drugs, pesticides, industrial solvents and ozone. Research has linked oxidative stress to many diseases: arthritis, lung diseases (such as emphysema), heart disease, stroke, ulcers, hypertension, Parkinson's and Alzheimer's diseases, muscular dystrophy, and others [Lobo, 2010]. As a result many diseases have been treated with antioxidants to prevent oxidative damage.

1.3 Method for estimation of antioxidant

To date there are various antioxidant capacity assays. Some procedures are focused on estimates the radical scavenging activity of antioxidant extracts. The methods rely on spectrophotometry and chromatography which involves analytical instruments and other expensive equipment. They are more time-consuming and more difficult experiment for screening antioxidant assays. Generally, indirect methods are used more frequently than direct methods with electron and radical scavenging such as 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay, 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) assay, ferric reducing antioxidant power (FRAP) assay, ferrous oxidation-xylenol orange (FOX) assay, ferric thiocyanate (FTC) assay, and aldehyde/carboxylic acid (ACA) assay [Moon, 2009].

Nowadays there are various antioxidant capacity assays. The widely used DPPH (2,2-diphenyl-1-picrylhydrazyl) assay is based on the premise that a hydrogen donor is

an antioxidant. This colorimetric assay uses the stable DPPH free radicals, which changes from purple to yellow in the presence of antioxidants, and is commonly used as a preliminary study because it reacts directly and rapidly with antioxidant compounds. DPPH free radical reacts with antioxidant (AH) according to equation 1.1 [Moon, 2009, Milardivic, 2006].

$$DPPH^{\bullet} + AH \rightarrow DPPH\text{-}H + A^{\bullet} \qquad 1.1$$

The method is based on the spectrophotometric measurement of $DPPH^{\bullet}$ concentration changes resulting from the reaction of $DPPH^{\bullet}$ with an antioxidant. It is a rapid, simple, inexpensive and widely used method. The method has been developed to determine the antioxidant assays based on Trolox equivalent antioxidant capacity (TEAC) as well. Antioxidant causes a change in absorbance of DPPH. This change is compared to the change induced by Trolox, the reference standard which is a commercial water-soluble vitamin E and the antioxidant capacity of the sample is expressed in micromoles of Trolox equivalents (TE) per 100 g of sample or Trolox units per 100 g due to doubts concerning the direct determination of DPPH obtained from calibration curve. DPPH rapid assay was carried out in buffer medium (methanol: 10 mM Tris buffer pH 7.5, 1:1 v/v) to reduce the reaction time to 10 min [Abderrahim, 2013].

Another common method for assay of antioxidative activity is the ABTS method. The absorbance of the reaction at 734 nm of this assay which changes from blue to green. These two radicals ($ABTS^{\bullet}$ and $DPPH^{\bullet}$) may be neutralized either by direct reduction electron transfer or by radical quenching hydrogen atom transfer. DPPH method is better than ABTS method because it is more selective with OH-group and have better access to the radical site and can show higher apparent antioxidant activity [Del Carlo, 2004].

The FRAP method based on the reduction of Fe^{3+} to Fe^{2+} occurs in the presence of 2,4,6-trypyridyl-s-triazine complex under acidic conditions, forming a blue color analyzed by a spectrophotometer. The absorbance of the reaction was measured at 593 nm [Antolovich, 2002]. The FRAP assay is a fast, simple and rapid. In contrast, FRAP cannot detect compounds that act by hydrogen transfer, particularly thiols and proteins.

The FOX and FTC method, this assay with the same mechanisms associated. The difference is that a ferric ion formed by an oxidant from a ferrous ion by monitoring the formation of ferric thiocyanate complex forming a blue to purple color. The absorbance analyzed by a spectrophotometer at 500 nm [Moon, 2009]. This assay is simple and highly reproducible. But also the results are overestimated or not reliable by using a spectrophotometer.

The ACA method is used for studying oxidation phenomena of food. The proposed mechanisms of the reduction from alkylaldehyde to alkylcarboxylic acid monitored by gas chromatography [Moon, 2009]. The method is easy but sample must be lipid soluble.

Although many methods used spectrophotometry for screening antioxidant activity but the methods must also be faster, cheaper and appropriate for performing by colorimetric detection. Therefore, the developed method was conveniently used by employing DPPH assay. The analytical methods for antioxidative assay with electron and radical scavenging are summarized in Table 1.1

Table 1.1 Methods for antioxidative assay associated with electron and radical scavenging.

Assay	Method	Mechanism	Ref.
DPPH (2,2-diphenyl-1-picrylhydrazyl)	Spectrophotometry	Scavenging activity	[Moon, 2009]
ABTS (2,20-azinobis(3-ethylbenzothiazoline-6-sulfonic acid)	Spectrophotometry	Scavenging activity	[Moon, 2009]
FRAP (ferric reducing antioxidant power)	Spectrophotometry	Reducing power	[Antolovich, 2002]
FOX (ferrous oxidation-xylenol)	Spectrophotometry	Lipid peroxidation inhibition	[Moon, 2009]
FTC (ferric thiocyanate)	Spectrophotometry	Lipid peroxidation inhibition	[Moon, 2009]
ACA (aldehyde/carboxylic acid)	Gas chromatography	Slow oxidation phenomena	[Moon, 2009]

1.4 Colorimetric analysis

1.4.1 Camera based colorimetric analysis

To date, photographic method have been applied to field test for colorimetric detection by using cameras of digital, video and mobile phones and including scanner which is easy operation and fast analysis. Image processing is popularly employed, RGB that consists of red (R), green (G) and blue (B), the intensities initial from 0 to 255. White is represented by (255, 255, 255) and black by (0,0,0) as shown in Figure 1.1.

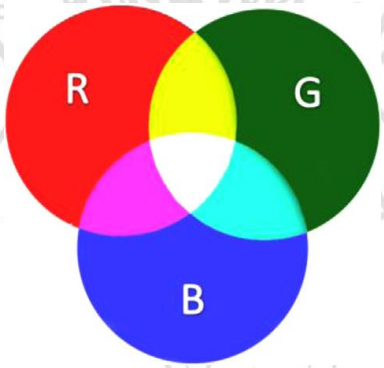

Figure 1.1 Diagram of RGB system [Grudpan, 2015]

Applications of digital camera used for image capture for analysis of some foods and beverages are summarized in Table 1.2. Alimelli et al. described the use of computer screen photoassisted technique (CSPT) combination with webcam camera to capture images as shown in Figure 1.2 [Alimelli, 2007]. The colorimetric information by selecting the areas of interests in a picture was obtained as signals of the RGB intensity by matrix laboratory (MATLAB). The method was demonstrated for determining of complexing agent in of red wine (total anthocyanins and polyphenols).

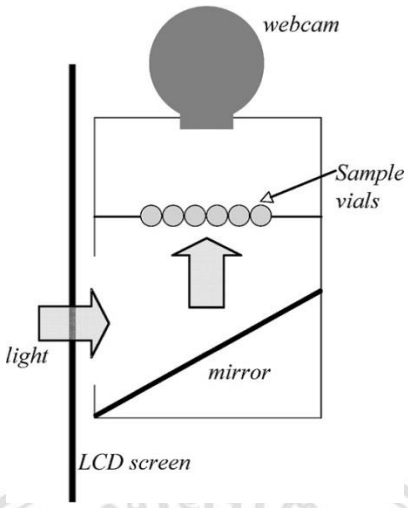

Figure 1.2 Schematic of the set-up for wine quality measurements. Up to six samples could be monitored simultaneously [Alimelli, 2007]

The method for observing images of webcam camera by RGB values for determination of total acidity in red wines using acid–base titration was developed [Torres, 2011]. Anthocyanines change theirs color by absorbing electromagnetic radiation in the visible region yielding various color depending on the pH. Figure 1.3 shows diagram of the photographic system. Wongwilai et al. described the use of webcam camera based on lab on chip for determination of acid – based in vinegar samples to observe by calibration graph of acetic acid concentration [Wongwilai, 2010].

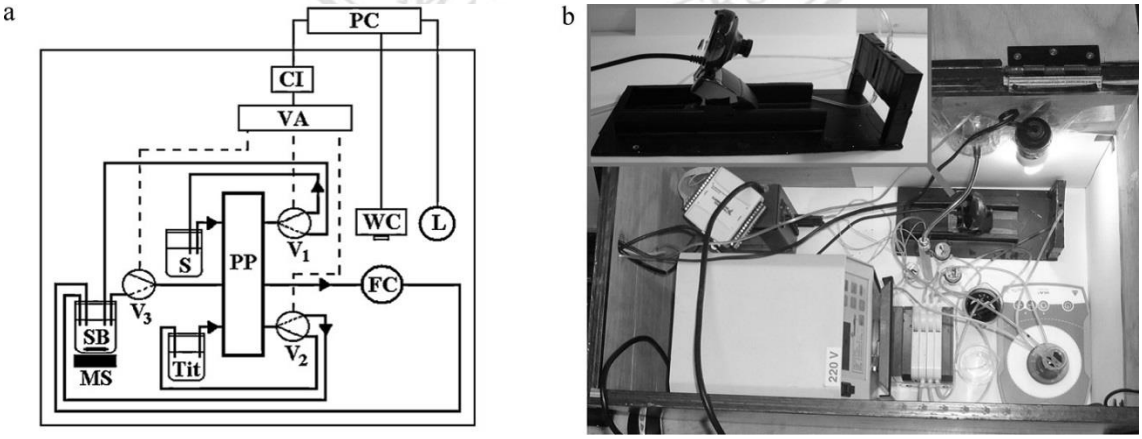

Figure 1.3 Schematic diagram (a) and photograph (b) of the system proposed for determination of total acidity in red wines by using digital image-based titrations. [Torres, 2011]

Lou et al. has developed nondestructive assessment of antioxidant activity in stages of mulberry fruits by using chlorophyll fluorescence and RGB photographic

system [Wongwilai, 2010]. This methods monitored by using a pulse-amplitude modulation (MINIPAM) fluorometer and Canon EOS 50D camera, respectively for monitoring. Color images were analyzed by using Image J program. The results from the methods were compared with chemical parameters (total flavonoid, total phenols, ABTS and DPPH radical scavenging activities and sugars). The results indicated good correlation of chlorophyll fluorescence and RGB method. Sun has developed method for determination of protein content of rice samples by using digital image processing RGB signals and detected by a scanner[Sun, 2008]. The results showed good correlation with Kjeldahl method. The method is comfortable for application in quality evaluation of rice samples.

Most modern mobile phones include a camera, which is typically designed with a signal processor or complementary metal oxide-semiconductor (CMOS) sensor. Its quality is equal to digital cameras and easy to transfer data by image sharing such as wifi or Bluetooth, that can be developed to use in routine analysis. Coskun et al. have developed allergen testing tool, termed iTube by images processing on a cellphone and analyzed by colorimetric assays for detection of allergens in food samples. Smart application can digitally processed within 1 s. This automated device can be useful for public health as shown in Figure 1.4 [Coskun, 2013].

Figure 1.4 Schematic diagram of allergen testing tool [Coskun, 2013]

Table 1.2 Summary of camera applications used for image capture and analysis of some foods and beverages.

Analyte	Device	Color system	Application	Range	Precision	Ref.
Allergens	Smartphone App.	RGB	Food	1-25 mg/L		[Coskun, 2013]
Total flavonoid; Total phenols; Radical scavenging activities; Sugars	Digital camera	RGB	Mulberry	Total phenol 185-344 mg GAE/100 mg		[Lou, 2012]
Acidity (Total)	WebCam	RGB	Red wine		1%	[Torres, 2011]
Acetic acid	WebCam	RGB	Vinegar	3.1-6.3%	5.60%	[Wongwilai, 2010]
Protein	Scanner	RGB	Rice	5-13 g/100 g		[Sun, 2008]
Total polyphenols; Anthocyanins	Webcam	RGB	Red wine	500-3000 mg/L 100-300 mg/L	5%	[Alimelli, 2007]

1.4.2 Colorimetric analysis on well plate platform

Simple colorimetric methods that have been developed by monitoring on well plate platform are summarized in Table 1.3. The method has a problem about the effect of lens and light reflection of solutions and shadow causing from illumination that reduces the homogeneity of the image. These effects of lighting can be reduced by supplying with the necessary items for a particular purpose such as rectangular cells, lateral lighting, diffusing screens and cylindrical test tubes [Capitán-Vallvey, 2015].

Nowadays, there is the developed method that used CSPT technique based on spectral fingerprinting by webcam camera for the evaluation of a prospective enzyme-linked immunosorbent assay (ELISA), used for medical diagnostics of patients suffering from chronic inflammatory disorders in body of human and blood [Filippini, 2005]. Paper based diagnostics specificity of ELISA has been monitored through the detection of rabbit IgG and the HIV-1 envelope antigen gp41 by webcam camera. [Cheng, 2010]. System of a new 96-well plate paper-based ELISA for an enzyme-substrate by AuNPs conjugated with IgG antibodies and images analysis by a digital camera and windows or android tablet is shown in Figure 1.5 [Murdock, 2013]. The method has developed for analysis of glucose, creatinine, triglycerides, total cholesterol and total protein in blood samples, based on colorimetric detection on 64-microwell plate by scanner and processed the results by software program designed, as shown in Figure 1.6 [Medeiros de Morais, 2014]

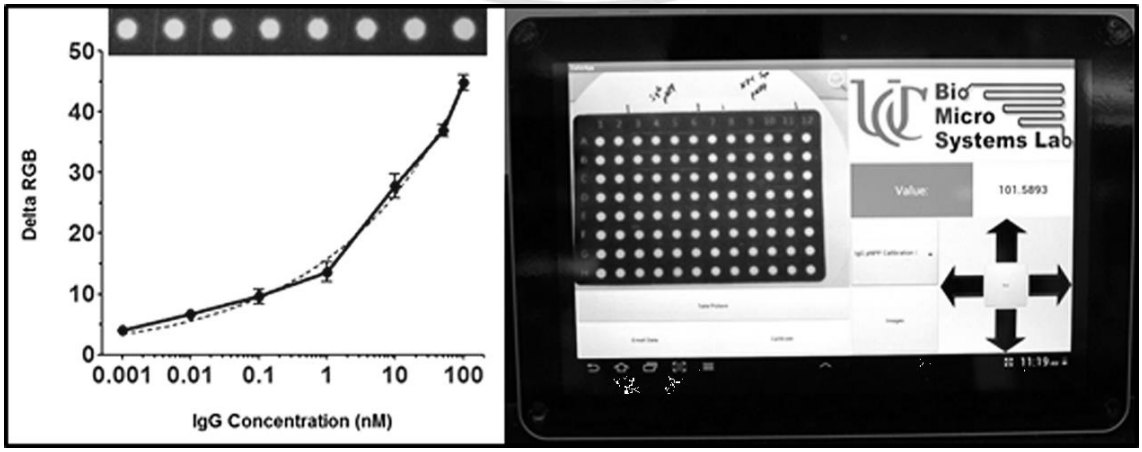

Figure 1.5 Image analysis and quantification of IgG image paper-based ELISAs results with Windows- and Android-based tablets [Murdock, 2013]

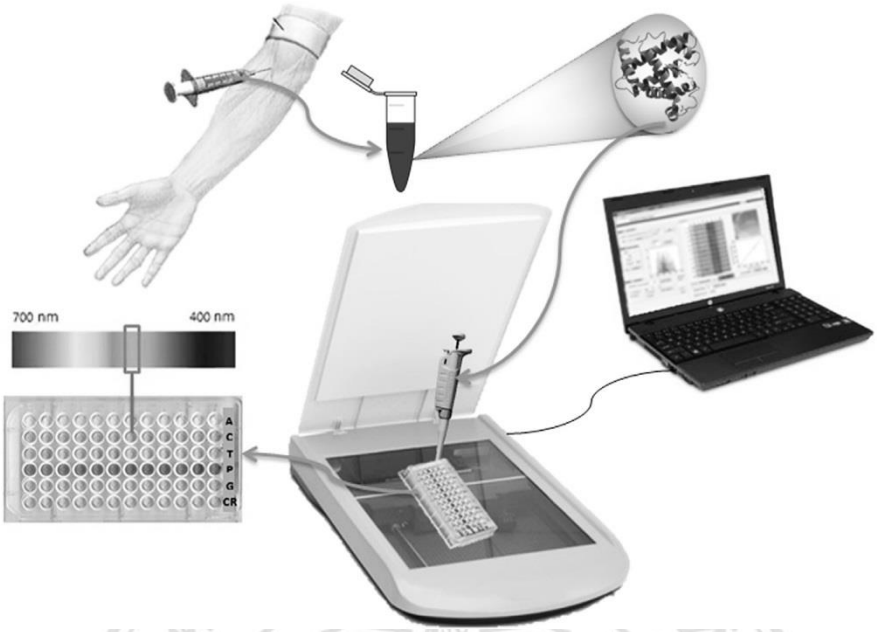

Figure 1.6 Experimental arrangement to collect and analyze images for determination of the concentration of glucose, triglycerides, creatinine, total cholesterol and total protein in blood serum [Lelis, 2014]

Recently, after 2011 smart phone was widely used as colorimetric analysis and performance of a built in camera of smart phone was improved with ISO parameters such as automatic focus, automatic white balance, automatic sensitivity. The systems based on Android and IOS devices are applied in diverse applications as shown in Table 1.4. The iPhone 5s has an "A7 chip" which is designed with a signal processor that is quality equal to DSLR cameras. A built in camera called "iSight" total 8 megapixels with f/2.2 aperture. [Choodum, 2013]. Therefore in this work iPhone5S was used. Photograph of the well plate was taken and the image was analyzed for color intensity by the developed software.

Table 1.3 Methods for colorimetric analysis on well plate platform

Analyte	Support	Device	Application	LOD	Ref.
Glucose; Creatinine; Triglycerides; Total cholesterol; Total protein	ELISA microplate	Scanner	Blood serum	16.2 mg/dL 51.7mg/dL 0.12 mg/dL 41.5 mg/dL 10.62 g/dL	[Lelis, 2014]
Neuropeptide Y	Paper based well plate	Smartphone Tablet	Saliva	0.001-10 µM	[Murdock, 2013]
Human IgG	Paper based well plate	Scanner		10^1-10^4 fM	[Cheng, 2010]
Anti-neutrophil cytoplasm antibodies	Enzyme-linked immunosorbent assay (ELISA)	Webcam	Chronic inflammatory disorders		[Filippini, 2005]

Table 1.4 Summary of methods for colorimetric analysis based on smartphone camera.

Analyte	System	Mobile phone	Application	Linear rage	Precision	LOD	Ref.
PSA	IOS	iPhone 4/4s	Whole blood	0.9-60 ng/mL	7%	0.4 ng/mL	[Barbosa, 2015]
Phosphorus	Android	Nokia X Dual SIMRM-980	Soil	0.0–1.0 mg/L	<2%	0.01 mg/L	[Moonrungsee, 2015]
Cortisol	Android	Samsung Galaxy Note 1	Saliva	0.01-10 ng/mL		0.01 ng/mL	[Choi, 2014]
Cholesterol Total	IOS	iPhone	Blood	140-400 mg/dL	3.90%		[Oncescu, 2014]
b-D-Galactosidase	IOS	iPhone 4S		0.7-12 nM		0.7 nM	[Thom, 2014]
pH; NO_2^-	Android	Samsung Galaxy SII		4-9 pH u.; 0.52-100 mg/L	0.167 (MSE) 0.51%	0.52 mg/L	[Lopez-Ruiz, 2014]

Table 1.4 (continued)

Analyte	System	Mobile phone	Application	Linear rage	Precision	LOD	Ref.
pH; Protein; Glucose	IOS; Android	iPhone 5; Samsung I5500 Galaxy 5	Urine	5-9 pH u. <100 mg/dL <300 mg/dL		1.66/1.21 pH u. 41/33 mg/L 92/69 mg/L	[Yetisen, 2014]
Vitamin D	IOS	iPhone	Blood	0-110 nM		10 nM	[Lee, 2014]
Hg(II)	Android	Samsung Galaxy S II	Tap water; Natural water	0-5 µM		3.5 µg/L	[Wei, 2014]
pH; chlorine; alkalinity	IOS	iPhone 4S	Water	6.5-8.5 u pH; 0.5-6- mg/L; <200 mg/L			[Schaefer, 2014]

Table 1.4 (continued)

Analyte	System	Mobile phone	Application	Linear rage	Precistion	LOD	Ref.
DBAE; L-proline	Android	Samsung Galaxy S (i9000)		0.1-5 mM; 0.1-10 mM		0.1 mM; 0.1 mM	[Delaney, 2013]
pH	IOS	iPhone	Sweat, saliva	3.0-9.0 pH u.		0.2 pH u.	[Oncoscu, 2013]
2,4,6-Trinitrotoluene	IOS	iPhone	Soil extracts	10–500 mg/L	2.1–7.4%	3.8mg/L	[Choodum, 2013]
Chlorine	Android	Samsung Galaxy S GT-I9000	Water	0.3-1.0 mg/L	7%		[Sumriddetchkajorn, 2013]
O_2 gas	Android	HTC, Desire HD		0-100%		1.50%	[Lopez-Ruiz, 2012]
pH	Android	Samsung Galaxy		1.0-11.0 pH u.			[Chang, 2012]

1.8 Research objectives

The objectives of this research are listed as follows:

1. To develop RGB color sensor detection system on well plate for high throughput screening of antioxidant capacity by DPPH method.

2. To apply the developed system for antioxidant assay of tea samples.