CHAPTER 1

INTTRODUCTION

1.1 Overview

Estimation of antioxidant activity is very attractive because antioxidants play a crucial role on the human health to protect many diseases such as cardiovascular diseases. Antioxidant contains in fruits, vegetables, grains, teas, wines and herbs [1]. Therefore the development of several analytical methods for estimation of antioxidant activity has been proceeded for many decades in order to increase sensitivity, shorten analysis time, reduce cost, reagent consumption and sample required for analysis. There are several methods for evaluation of antioxidant in various samples. The chromatographic methods are capable to identify and quantify the contributions of total antioxidant. These methods gave high sensitivity but they involved expensive instrument and sample preparation are complicated. Spectrophotometric methods are widely used for the determination of antioxidant capacities because they are simple, relatively low cost and have convenient operation. However, the spectrophotometric method often suffers from colored and colloidal substances present in sample. Electrochemical methods are particularly appealing for the fast screening of the antioxidant properties of sample, because they combine a relatively simple, low-cost instrumentation, high sensitivity and no effect of colored and colloidal substances in sample. However in previous reports, these methods used expensive reagent.

In this study, flow injection system combined with amperometric detection will be developed for estimation of antioxidant activity. It is based on the detection of decreasing amperometric current from the electrochemical reduction of triiodide reagent. Triiodide is produced by the reaction of iodate with iodide in acidic medium and later on triiodide is undergoes electrochemical reduction on a glassy carbon working electrode at 200 mV versus Ag/AgCl reference electrode [2] producing electrical current. Antioxidant reacts with triiodide leading to the decrease in the electrical current which was directly proportional to antioxidative activity. This approach is similar to the previously reported ferric reducing antioxidant power (FRAP) and cupric reducing antioxidant capacity (CUPRAC) methods [3-4] which could be applied to antioxidant that having reducing property only. The oxidative power of triiodide is in between of Fe(III) and Cu(II) as can be seen from the standard reduction potential (E^0) with respect to SHE at 298 K, 1 M and 1atm as below [5]:

$$Fe(III) + e^- \rightarrow Fe(II) \qquad E^0 = 0.77 \text{ Volt}$$

$$Cu(II) + e^- \rightarrow Cu(I) \qquad E^0 = 0.34 \text{ Volt}$$

$$I_3^- + 2e^- \rightarrow 3I^- \qquad E^0 = 0.54 \text{ Volt}$$

Due to low stability of iodine, this reagent is not widely used in batch method for screening of antioxidative activity. By using flow injection system this problem can be solved. Moreover, this reagent is relatively cheap and electrochemically active at quite low electrical potential, therefore many interferences could be avoided.

1.2 Antioxidant and its important

Oxidation is an essential biological process for energy production in many living organisms. However, this process produces excessive reactive oxygen species (ROS) which include free radicals such as superoxide anion radicals ($O_2^{\cdot}$), hydroxyl radicals

($OH^{\cdot}$) and non free-radical species such as H_2O_2 and single oxygen (O_2). The various forms of activated oxygen which is lead to oxidative stress is now believed to be a key contributing factor in the manifestation chronic disease such cardiovascular disease, hypertension, diabetes mellitus and some forms of cancer. Many fruits and vegetables have many afford protections against some diseases that mainly attributed the antioxidant constituents. Antioxidants are a molecule capable of inhibiting or preventing the oxidation of other molecules. The function of antioxidants has been categorized into two types depending on their mode of action. First, oxidation of biological macromolecules such as nucleic acids, proteins, and lipids as a result of free radical damage (oxidative stress), antioxidants directly involvement in neutralizing free radicals in biological systems and prevent oxidation reactions by being oxidized themselves. Second, excess formation of ROS which a result of many metabolic processes can cause damage and disturb cellular homeostasis. The antioxidant systems involved in the protection against ROS consist of many antioxidant factors including glutathione (GSH), coenzyme Q and several antioxidant enzymes such as superoxide dismutase (SOD), glutathione peroxidase (GSH) and glutathione –S-transferase (GST). Plant, fruits and vegetables are potential sources of natural antioxidant such ascorbic acid (Vitamin C), polyphenols and tocopherols (Vitamin E). The ability of antioxidant to manage oxidation linked diseases has resulted in a strong interest in the development functional food and other dietary strategies for management of oxidation diseases which made estimation of total content of antioxidants very popular. A number of methods have been developed to estimate the efficiency of antioxidants in plant, fruits and vegetables extracts were focused on difference mechanisms of the antioxidant defense system such as

scavenging of radicals, reducing ability of antioxidant and inhibition of lipid peroxidation [6-7].

1.3 Method for estimation of antioxidant

1.3.1 Chromatographic methods

Chromatographic methods such as high performance liquid chromatography and gas chromatography offered advantages including, automatic operation, small amounts of sample required and high sensitivity to identify and quantify the substances contributing to total antioxidant. Some reports on the determination of antioxidant by chromatographic methods are summarized in Table 1.1.

Table 1.1 Summary of some chromatographic methods for the determination of antioxidant

Methods	Sample	Analytes	LOD (ppm)	Linear range (ppm)	Ref.
HPLC	Red Wines	Gallic acid, (+)-catechin, (-)-epicatechin,caffeic acid, p-coumaric, and quercetin	Gallic acid= 1.3, (+)-catechin= 0.6, (-)-epicatechin= 0.4, caffeic acid= 0.3, p-coumaric= 0.6,and quercetin =0.5	Gallic acid = 1-100, (+)-catechin = 1-125, (-)-epicatechin = 1-100, caffeic acid = 1-100, p-coumaric = 1-100, and quercetin = 1-100	[8]
HPLC	White wines	Caftaric, procyanidin B1, (+)-catechin, procyanidin B2, (-)-epicatechin, rutin, myricetin, trans-resveratrol, Quercetin, Apigenin and Kaempferol	Caftaric= 0.1, procyanidin B1= 0.05, (+)-catechin= 0.1, procyanidin B2= 0.05, (-)-epicatechin= 0.1, rutin= 0.3, myricetin= 0.025, trans-resveratrol= 0.02, Quercetin= 0.2, Apigenin= 0.05 and Kaempferol= 0.04	Caftaric= 0.3-100, procyanidin B1= 0.5-100, (+)-catechin= 0.2-70, procyanidin B2= 0.5-100, (-)-epicatechin= 0.2-60, rutin= 0.2-46, myricetin= 0.06-15, trans-resveratrol= 0.04-5.2, Quercetin= 0.3-30, Apigenin= 0.17-0.20 and Kaempferol= 0.13-15	[9]

Table 1.1 Continued.

Methods	Sample	Analytes	LOD	Linear range (ppm)	Ref.
Reverse – phase HPLC	Cooking oil, margarine, butter and cheese	Propyl gallate, tertiary butyl hydroquinone, butylated hydroxyanisole and butylated hydroxytoluene.	Propyl gallate= 0.3 ppm, tertiary butyl hydroquinone = 0.5 ppm, butylated hydroxyanisole= 0.5 ppm and butylated hydroxytoluene. = 0.5 ppm	All the analytes =1-300	[10]
GC-MS	Water and soil	3,5-di-tert-butyl-4-hydroxy-toluene, 3,5-di-tert-butyl-4-hydroxybenzaldehyde and 3,5-di-tert-butyl-4-hydroxyphenylethane	3,5-di-tert-butyl-4-hydroxy-toluene= 5 ppt and 3,5-di-tert-butyl-4-hydroxybenzaldehyde = 16 ppt	-	[11]

Table 1.1 Continued.

Methods	Sample	Analytes	LOD	Linear range (ppm)	Ref.
LC-MS	Cosmetic Products	Butylated hydroxyanisol, butylated hydroxytoluene, α-tocopherol and α- tocopherol acetate	Butylated hydroxyanisol= 99 ng/g, butylated hydroxytoluene= 87 ng/g, α-tocopherol= 142 ng/g and α- tocopherol acetate= 15.3 ng/g	butylated hydroxyanisol= 200-20000 ng/g, butylated hydroxytoluene= 200-20000 ng/g, α-tocopherol= 200-20000 ng/g and α- tocopherol acetate= 20-2000 ng/g	[12]
HPLC	Multi-vitamin samples	Kaempferol, retinol, retinyl acetate, cholecalciferol, γ-tocopherol and α-tocopherol	Kaempferol= 1.7 nM, retinol= 30.64 nM, retinyl acetate= 26.02 nM, cholecalciferol= 205.72 nM, γ-tocopherol= 55.23 nM, α-tocopherol= 62.26 nM	Kaempferol, retinol, retinyl acetate, γ-tocopherol and α-tocopherol = 100-10000 nM and cholecalciferol=400-40000 nM	[13]

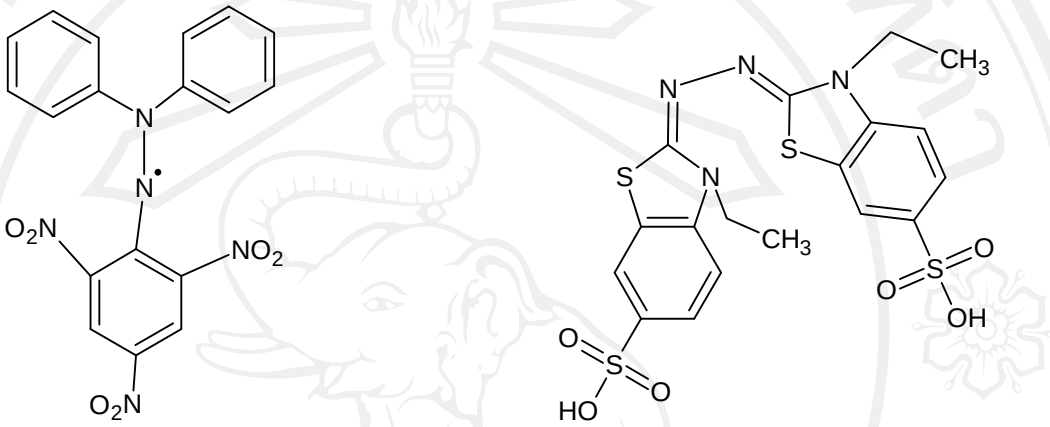
Although chromatographic methods have many advantages, they also have several drawbacks such as expensive instrument, long operation time and require many steps of sample preparation.

1.3.2 Methods based on the neutralization of free radical

Many antioxidant assays are based on ability of the antioxidant to neutralize or quench free radicals. The two free radicals that have been most commonly used as reagent for assessing antioxidant activity are 1,1 – diphenyl-2-picrylhydrazyl (DPPH) and 2,2'-azinobis(3-ethyl-benzothiazoline-6-sulfonic acid) (ABTS).

The DPPH free radical is a stable radical with one electron delocalized over the molecule. This delocalization gives a deep purple color with absorption maximum at 517 nm in an ethanol solution. When an antioxidant capable of donating hydrogen reacts with the DPPH radical it gives rise to a nonradical reduced form of DPPH which has a yellow color. The decrease in the absorption is measured spectrophotometrically and compared with an ethanol control to calculate the DPPH free radical scavenging activity. The antioxidant assay based on the ABTS is similar to the DPPH assay. ABTS can react with hydroxyl, peroxyl, alkoxyl and inorganic radicals to form a relatively stable radical cation with absorption maximum at 734 nm. The structure of DPPH and ABTS, two most commonly used indicators in antioxidant assays based on free radicals scavenging are shown in Figure 1.1 [6] and some reports based on these reactions are summarized in Table 1.2. Although these methods are a simple and fast, the reagent used are expensive. The antioxidant capacity in these assays depends on the chemical structure of the antioxidant. The reduction in the

DPPH radical is dependent on the number of hydroxyl groups present in the antioxidant.



1,1 – diphenyl-2-picrylhydrazyl (DPPH) radical

2,2'-azinobis(3-ethyl-benzothiazoline-6-sulfonic acid) (ABTS)

Figure 1.1 DPPH and ABTS structures.

Table 1.2 Summary of some methods based on the neutralization of free radical

Method	Sample	Standard	Linear range	Ref
ABTS and DPPH assay	Guava fruit extracts	Trolox	25-600 µM for ABTS assay and 25-800 µM for DPPH assay	[14]
ABTS and DPPH assay	Commercial food in United State	Ascorbic acid	10-100 ppm for ABTS and DPPH assay	[15]
DPPH assay	Potatoes	Ascorbic acid	0-400 ppm	[16]
ABTS and DPPH assay	Acerola *(Malpighia emarginata DC.)* fruits and derivatives	Trolox	40-200 µM for ABTS and DPPH assay	[17]
DPPH assay	Vegetable juices	Ascorbic acid	0-1000 µM	[18]
ABTS assay	Date palm *(Phoenix dactylifera)* fruits	Trolox	0-15 µM	[19]
ABTS assay	Sweet potato genotypes	Trolox	0.500 µM	[20]

1.3.3 Spectrophotometric methods

Spectrophotometric methods are widely used for the determination of antioxidant capacities because they involve simple procedure and lower cost of instrument and operation. They are mainly based on reaction between a chromogenic compound and antioxidant. After reaction, the residual concentrations of chromogenic compound or the complex formed by the reaction are determined spectrophotometrically or colorimetrically. Ferrous tartrate and Folin–Ciocalteu (F–C) are widely used reagents for the spectrophotometric determination of total phenolic and polyphenolic antioxidants in many samples. Ferrous tartrate method based on trihydroxyphenols antioxidant reacts with ferrous tartrate solution to form an intense purple color that measured the absorbance at 540 nm. Folin–Ciocalteu reaction involves oxidation in alkaline solution of phenols by the yellow molybdotungstophosphoric heteropolyanion reagent and colorimetric measurement of the resultant molybdotungstophosphate blue. These blue pigments have a maximum absorption depending on the qualitative and quantitative composition of reducing substance which depending on the pH of solutions [21-22]. The methods based on reducing power of antioxidant such as ferric reducing antioxidant power (FRAP) is also widely used. The method involve the reduction of Fe(III)-ligand with antioxidant to form Fe(II)-ligand and monitored the absorbance change at maximum wavelength [3]. Cupric reducing antioxidant capacity method (CUPRAC) utilized the copper(II)-neocuproine [Cu(II)-Nc] reagent as the chromogenic oxidizing agent. The Cu(II)-Nc solution is reduced by antioxidant come to the highly colored Cu(I)-Nc chelate showing maximum absorption at 450 nm [4]. And Ceric ion reducing antioxidant capacity (CERAC) is based on the reduction of Cerium(IV) sulfate with antioxidant in

dilute sulfuric acid at room temperature. The absorbance of unreacted Ce(IV) is measured at 320 nm [23]. The summary of some spectrophotometric methods for the estimation of antioxidant activity are shown in Table 1.3. However, the methods with spectrophotometric detection would encounter the serious problem with interferences from colored and colloidal substances in sample.

Table 1.3 The summary of some spectrophotometric methods for the estimation of antioxidant activity

Methodology	Sample	Standard	Linear range	Ref
Polyphenol content was determined using ferrous tartrate and Folin-Ciocaltue assay. And the effect of different extracting solvents was investigated.	Black tea and mate tea	Gallic acid	5-50 ppm	[21]
A new time-temperature-alkali-alcohol combination with Folin-Ciocaltue method used estimation of phenolic content.	Plant methanol extracts	Caffeic acid	20-80 ppm	[22]
A ferric reducting antioxidant power (FRAP) assay evaluated the antioxidant potential of aqueous acetone extracts of raw and dry sample	Moth bean	Trolox	100-2000 µM	[24]
Antioxidant potential was determined by ferric reducting antioxidant power (FRAP) assay. And the possible effects of different preparation methods were evaluated.	Green and black tea	Ascorbic acid	0-1000 µM	[25]

Table 1.3 Continued

Methodology	Sample	Standard	Linear range	Ref
A Cupric reducing antioxidant capacity (CUPRAC) method evaluated antioxidant content. And the effect of different extracting solvents was investigated.	Sumac (*Rhus Coriaria L.*)	butylated hydroxyanisol, butylated hydroxytoluene, tocopherol and trolox	10-30 ppm	[26]
Important and representative commercial wines available in China was investigated the antioxidant capacity by Cupric reducing antioxidant capacity (CUPRAC) method	Selected China wines	Trolox	50-500 M	[27]

1.3.4 Electrochemical methods

Electrochemical methods were studied including potentiometric, amperometric and voltammetric methods. Potentiometric method is based on the change of potential signal measurements the composition change of a redox-reagent solution which was proportional to logarithm of concentration of redox-reagent solution. Amperometric method is based on measurement of electrical current on an electrode at fixed potential which was proportional to the concentration of electroactive species. Voltammetry is obtained by measuring the current as the potential is varied. Electrochemical methods give a simple, low-cost instrumentation and no effect of colored and colloidal substances in sample. However in previous reports, these methods used expensive reagent and complicated operation. Some electrochemical methods for the estimation of antioxidant activity are summarized in Table 1.4.

Table 1.4 The summary of some electrochemical methods for the estimation of antioxidant activity

Methodology	Sample	Standard	Linear range	Ref
The total antioxidant capacity was estimated by the development of potentiometric method using an iodine-modified platinum electrode.	Hips, hop cones and lemon juice	Ascorbic acid	0.05-3 mM	[28]
Total polyphenol content was determined by differential pulse voltammetry (DPV) method. The total polyphenol was carried out the oxidation peak potentials on glassy carbon electrode working at0.440 V vs SCE reference electrode	Red Wine	Catechin	1-15 ppm	[8]
The antioxidant activity was evaluated by a new method based on the amperometric reduction of DPPH on a glassy carbon electrode at 140 mV vs Hg_2Cl_2/ 3M KCl .	Tea, wine and some beverages	Trolox	0-30 μM	[29]

Table 1.4 Continued

Methodology	Sample	Standard	Linear range	Ref
Free radical scavenging activity of *Geshoidin* was carried out based on the electrochemical reduction of oxygen by differential pulse voltammetric method. The proportional decrease of the oxygen peak current corresponding to concentration of the *Geshoidin*.	*Tella*	*Geshoidin*	1-100 µM	[30]
Butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT) was determined by square-wave voltammetry (SWV). The determination of these antioxidants was carried out the oxidation peak potentials of BHA and BHT on boron-dope-dimond working electrode at 0.65 V and 0.93 V vs for BHA and BHT, respectively.	Mayonnaise and magarine	Butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT)	0.6-10 µM for both	[31]
The total antioxidant capacity was investigated by the biamperomtric method using the redox couple DPPH/DPPH$^{\cdot}$.	Commercial fruit juice	Trolox	5-30 µM	[32]

1.3.5 Flow analysis methods

Flow injection analysis (FIA) is based on the injection of a liquid sample into a moving continuous carrier stream of a suitable liquid. The injected sample forms a zone, which is then transported toward a flow through detector that continuously records as a signal such as absorbance, potential or other physical parameters.

Flow injection-based techniques offer many advantages including the reproducible detection of the product, minimizing chemical consumption compared to conventional batch methods, and increasing sample throughput. Moreover, due to being the closed-system, the contamination during the measurement from the environment can be avoided. FI-method allows the more flexible equipments as they can be coupled with a variety of detectors. Some analytical methods combine with the flow injection system for estimation of antioxidant are summarized in Table 1.5.

Table 1.5 Some analytical methods based on with the flow injection system for estimation of antioxidant

Technique	Methodology	Sample	Standard	Linear range	Ref
FIA-poten	Total antioxidant activity was measured by flow injection potentiometric utilizing the transient negative signal when the composition change of a $[Fe(CN)_6]^{3-}/[Fe(CN)_6]^{4-}$ redox-reagent solution	Green and black tea infusion, herbal infusion and fresh fruit extracts	Ascorbic acid	0.01-0.1 mM and 0.1-1.0 mM	[33]
FIA-biamp	Determination of total antioxidant capacity by biamperometric measurement $ABTS^{+}$/ABTS redox couple using Interdigitated gold electrode (IDE)	Alcoholic beverages	Trolox	20-2000 μM	[34]

Table 1.5 Continued

Technique	Methodology	Sample	Standard	Linear range	Ref
FIA-amp	Flow injection amperometric system operating with disposable pencil graphite electrode was evaluated total phenolic content and antioxidant power. The total phenolic content and antioxidant power was evaluated by measuring the current resulted from the oxidation of DPPH at the established potentials +0.8 and 0.5 V, respectively	Tea infusions	Caffeic acid for total phenolic content, Trolox for antioxidant power	1-20 ppm	[35]
FIA-amp	Determination of total antioxidant power was evaluated by direct flow injection system with amperometric detection operating of caffeic acid at a glassy carbon electrode potential of +0.5 V (vs. Ag/AgCl)	Olive oils	Caffeic acid	0.5-10 ppm	[36]

Table 1.5 Continued

Technique	Methodology	Sample	Standard	Linear range	Ref
FIA-multi pluse-amp	The couple of multiple pulse amperometric detection at boron-doped diamond electrode and flow injection analysis was developed for the simultaneous determination of butylated hydrosyanisole (BHA) and butylated hydroxytoluene (BHT)	Commercial mayonnaises	butylated hydrosyanisole (BHA) and butylated **hydroxytoluene** (BHT)	0.050-3.0 μM for BHA and 0.70-70 μM for BHT	[37]
FIA-amp	Tannin content is quantified by the reduction current of residual ferricyanide, which is proportional to the remaining quantity of ferricyanide	Tea beverages	Tannic acid	10-50 ppm and 100-500 ppm	[38]

Table 1.5 Continued

Technique	Methodology	Sample	Standard	Linear range	Ref
FIA-amp	The total phenolic content was determined by the development of electrochemical flow system using graphite lead pencil as an electrode and home-made flow injection cell	Tea infusion	Trolox	1-20 ppm	[39]
FIA-biamp	The total antioxidant capacity was assessed by flow injection biamperometric method using a DPPH/DPPH couple	Commercial Fruit Juices	Trolox	5 - 30 µM	[40]
FIA-amp	The flavonoids was determine by flow injection amperometric method using Boron – Doped Diamond as a working electrode.	Green tea	rutin	0.01-0.25 mM	[41]

Note: poten = potentiometry, biamp = biamperometry, amp = amperometry

1.4 Research objectives

The aims of this research are listed as follows:

1.4.1 To develop a flow injection-amperometric method for estimation of antioxidant activity based on the reaction of antioxidant and triiodide ion.

1.4.2 To apply the developed method for the estimation of antioxidant activity in real samples.