DEVELOPMENT OF FLOW INJECTION ANALYSIS SYSTEM FOR THE DETERMINATION OF ANTIOXIDANT CAPACITY IN SOME NATURAL EXTRACTS

NOOKRAI MRAZEK

MASTER OF SCIENCE

PROGRAM IN APPLIED CHEMISTRY

MAE FAH LUANG UNIVERSITY

2011

DEVELOPMENT OF FLOW INJECTION ANALYSIS SYSTEM FOR THE DETERMINATION OF ANTIOXIDANT CAPACITY IN SOME NATURAL EXTRACTS

NOOKRAI MRAZEK

THIS THESIS IS A PARTIAL FULFILLMENT OF
THE REQUIREMENTS FOR THE DEGREE OF
MASTER OF SCIENCE
PROGRAM IN APPLIED CHEMISTRY

MAE FAH LUANG UNIVERSITY

2011

DEVELOPMENT OF FLOW INJECTION ANALYSIS SYSTEM FOR THE DETERMINATION OF ANTIOXIDANT CAPACITY IN SOME NATURAL EXTRACTS

NOOKRAI MRAZEK

THIS THESIS HAS BEEN APPROVED

TO BE A PARTIAL FULFILLMENT OF THE REQUIREMENTS

FOR THE DEGREE OF MASTER OF SCIENCE

PROGRAM IN APPLIED CHEMISTRY

2011

EXAMINING COMMITTEE

Yuthapong Udnan .....CHAIRPERSON

(Asst. Prof. Dr. Yuthapong Udnan)

.....COMMITTEE

(Asst. Prof. Dr. Siripat Suteerapataranon)

S. Deachat .....COMMITTEE

(Dr. Suwanna Deachathai)

Kanchana Watla-iad .....COMMITTEE

(Dr. Kanchana Watla-iad)

ACKNOWLEDGEMENTS

I wish to acknowledge my adviser, Asst. Prof. Dr. Siripat Suteerapataranon, for her valuable advice, criticisms, analyzing the results, and encouragement throughout my study. I would also like to acknowledge my co-adviser, Dr. Suwanna Deachathai, for her valuable guidance and assistance during conducting samples collection, analyzing the results, and processing the context of the manuscript. I would also like to thank my thesis committee members and Dr. Kanchana Watla-iad for their time and effort in reading and making suggestions for improving this thesis.

I would like to acknowledge the Scientific & Technological Instruments Center, Mae Fah Luang University, Chiang Rai, Thailand, for all laboratory facilities. The author is also grateful Mr. Trinop Promgool, the master degree student in Applied Chemistry, for his help and assistance during conducting samples collection.

Finally, special and important persons who have understood and encouraged me to carry on my study successfully are my parents, and my family. I would link to thank them for everything, which are the source of inspiration and motivation to complete the thesis.

Nookrai Mrazek

Thesis Title	Development of flow injection analysis system for the determination of antioxidant capacity in some natural extracts
Author	Nookrai Mrazek
Degree	Master of Science (Applied Chemistry)
Supervisory Committee	Asst. Prof. Dr. Siripat Suteerapataranon Dr. Suwanna Deachathai

ABSTRACT

A flow injection (FI) spectrophotometric system was developed for the determination of antioxidant capacity. The analysis is based on the color disappearance due to the scavenging of 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical by antioxidant compounds. Butylated hydroxy toluene (BHT) and ascorbic acid were used as antioxidant standards. The proposed FI system was a single-line system consisted of a peristaltic pump, a 6-port injection valve with a 50 μL sample loop, a reaction coil and a visible spectrophotometer (wavelength = 520 nm). $DPPH^{\bullet}$ reagent was pumped through the reaction coil and detector. A standard/sample was injected into the $DPPH^{\bullet}$ stream and mixed at the reaction coil. The product zone was finally detected in the spectrophotometer. Effects of the $DPPH^{\bullet}$ concentration (0.025-0.150 mM), $DPPH^{\bullet}$ flow rate (0.5-2.0 mL/min), and reaction coil length (50-500 cm) were studied. The optimum conditions were $DPPH^{\bullet}$ concentration 0.075 mM, $DPPH^{\bullet}$ flow rate 1 mL/min and reaction coil length 200 cm. The optimized system provided the linear range of 0.5-15.0 mM BHT and 0.010-0.300 mM ascorbic acid with correlation coefficient (R^2) of 0.9994 and 0.9995, respectively. %RSD (n=10) was less than 5%. Detection limit and quantitation limit were 0.252 mM and 0.409 mM for BHT and 0.008 mM and 0.017 mM for ascorbic acid, respectively. Sample throughput was 20 samples/hr. Validation of the FI method using BHT and ascorbic acid as standards was performed by comparing the antioxidant capacity

obtained by the FI method with the batch method. The results obtained by the FI method using ascorbic acid as standard agreed with those obtained by the batch method (*t*-test, confidence level 95%). However, the variance of the results obtained by the FI method using BHT as standard was significantly different from those obtained by using the batch method. The proposed FI method was probably able to apply to the analysis of fast reacting antioxidants. Using the FI-spectrophotometric system with ascorbic acid as standard the antioxidant capacity found in the natural sample extracts, vitamin E, commercial antioxidant product and commercial herbal products were 0.02-17.38 mM AAE, 0.01 mM AAE, 0.03 mM AAE, and 0.02-0.19 mM AAE respectively.

Keywords: 2,2-diphenyl-1-picrylhydrazyl (DPPH)/Antioxidant capacity/BHT/Ascorbic acid/Natural extracts/Flow injection analysis

TABLE OF CONTENTS

TABLE OF CONTENTS (continued)

LIST OF TABLES

LIST OF FIGURES

LIST OF FIGURES (continued)

CHAPTER 1

INTRODUCTION

1.1 Background

There is recent evidence that free radicals induce oxidative damage to biomolecules. This damage has been implicated in aging and in several human pathologies such as cancer, atherosclerosis, rheumatoid arthritis and other diseases (Huang, Ou & Prior, 2005; Kaur & Kapoor, 2001; Tang, Zhang & Geng, 2005). Natural products such as fruits and vegetables have aroused considerable interest recently because of their potential beneficial effects on human health. It has been reported that they have antiviral, anti-allergic, antiplatelet, anti-inflammatory, anti-tumor and antioxidant activities (Valko et al., 2007). Companies have been established to extract antioxidant from natural products, pack these extracts and sell to the public. For these companies there is a critical need for a quick and simple analysis method for quality control of finished product and of raw materials being extracted. Labeling legislation in the developed countries is now requiring the label to include the actual concentrations of active ingredients.

Over the past two decades, several methods have been used to determine the antioxidant activity in natural products, such as the thiobarbituric acid reactive substances (TBARS) assay (Aqil, Ahmad & Mehmood, 2006), trolox equivalent antioxidant capacity (TEAC) assay (Iveković, Milardvić, Roboz & Grabarić, 2005), total radical-trapping antioxidant parameter (TRAP) assay (Miller, Rigelhof, Marguart, Prakash & Kanter, 2000), and 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical assay (Brand-Williams, Cuvelier & Berset, 1995; Magalhães, Santos, Segundo, Reis & Lima, 2009; Megalhães, Segundo, Reis & Lima, 2006; Molyneux, 2004; Niederländer, Van Beek, Bartasiute & Koleva, 2008). TBARS assay is commonly used for the detection of lipid peroxidation. Free radical damage to lipids results in the production of malondialdehyde (MDA), which reacts with 2-thiobarbituric acid (TBA) under conditions of high

temperature and acidity generating a chromogen that can be measured either spectrophotometrically or spectrofluorometrically. The TBARS assay has been used to quantify oxidative damage (lipid peroxidation) in fish liver and gonad samples (Oakes & Kraak, 2003). TEAC assay is a widely used in vitro assay for the determination of antioxidant activity of pure compounds (Böhm & Schlesier, 2004; Megalhães et al., 2009). The TEAC assay is based on the measurement of the decrease in the color intensity of 2,2'-azinobis (3-ethylbenzothiazoline-6)-sulfonic acid ($ABTS^{\bullet+}$) radical by antioxidant, expressed relative to Trolox. TEAC assay has been broadly applied in assaying food samples (Re et al., 1999; Huang et al., 2005). An advantage of the TEAC assay is that it is operationally simple. However, the disadvantage of this assay is the ABTS radical is not found in mammalian biology and thus represents a nonphysiological radical (Huang et al., 2005). Also, the TEAC values may not be the same for slow reactions, and it may take a long time to reach the endpoint. The evaluation of antioxidant capacity using TEAC can be troublesome or even impossible, but it can be used to provide a ranking order of antioxidants (Arts, Haenen, Voss & Bast, 2004; Phipps, Sharaf & Butterweck, 2007). TRAP assay determines the overall ability of an antioxidant to trap free radicals (Iveković et al., 2005; Kumaraswamy & Satish, 2008; Miller et al., 2000) by monitoring the ability of antioxidant species to interfere with the reaction between peroxyl radicals generated by AAPH [2,2'-azobis(2-amidinopropane)dihydrochloride] and a target probe such as fluorescence (*R*)-phycoerythrin or oxygen uptake (Phipps et al., 2007). The TRAP assay was designed and is often used for measurement of in vivo antioxidant capacity in serum or plasma because it measures nonenzymatic antioxidants such as glutathione, ascorbic acid, α-tocopherol, and β-carotene but the assay is relatively complex and time consuming and requires a high degree of expertise and experience (Phipps et al., 2007). The $DPPH^{\bullet}$ assay is based on the reduction by antioxidants of the purple DPPH radical to the corresponding pale yellow and measured colorimetrically at 520 nm (Molyneux, 2004).

Among these methods, $DPPH^{\bullet}$ assays have been most widely used. The $DPPH^{\bullet}$ assays is relatively simple and stable and the $DPPH^{\bullet}$ is available commercially in high purity. The DPPH radical decolourisation method is strongly consistent and correlates with total phenolic content in the samples. Literatures suggested that the $DPPH^{\bullet}$ assay was an easy and accurate method with regard to measuring the antioxidant capacity of fruit and vegetable juices or extracts (Huang et al., 2005; Sánchez-Moreno, 2002). However, the batch $DPPH^{\bullet}$ assay has disadvantages, for example, it

uses high amounts of reagent, it is time consuming and tedious, and requires strict adherence to reaction time limits (Prior, Wu & Schaich, 2005).

A simple analytical tool such as flow injection technique has been well known for reducing reagent and time consumption. Flow injection analysis (FIA) is an approach to chemical analysis that is accomplished by injecting a plug of sample into a flowing carrier stream. As the injected zone moves downstream, the sample solution disperses into the reagent, causing the reaction to occur. A flow through detector placed downstream records the desired physical parameter such as colorimetric absorbance or fluorescence (Tang et al., 2005). The FIA can provide good reproducibility and rapid analysis of colorimetric methods. FIA methods typical reduce reagent consumption and waste and do not require completely color development which means the analysis can be done faster than batch methods. The $DPPH^{\bullet}$ batch method is a colorimetric method that may be adaptable to the FIA technique. Simple routine FIA colorimetric methods have been developed for the $ABTS^{\bullet+}$ assay but have not been developed for the $DPPH^{\bullet}$ assay. However, the flow-based methods based on the $DPPH^{\bullet}$ assay that have been developed are FIA with electron spin resonance (ESR) detector, and sequential injection analysis (SIA) and multisyringe flow injection analysis (MSFIA) using colorimetric detectors. These flow-based methods are generally more complicated, and require more expensive equipments.

Therefore, the aim of this work was to develop a simple FI-spectrophotometric system using a single line for the determination of antioxidant capacity based on $DPPH^{\bullet}$ assay in some natural product samples. The optimization of the FI system was carried out. Butylated hydroxy toluene (BHT) and ascorbic acid were used as standard. The antioxidant capacity was calculated as butylated hydroxy toluene equivalent (BHTE) and ascorbic acid equivalent (AAE). The proposed system was employed to determine the antioxidant capacity in some crude natural product extracts and Thai herb product samples. The comparison between the results obtained by the proposed system and those by the original batch method was performed.

1.2 Objectives

1.2.1 To develop a FI-spectrophotometric system for the determination of antioxidant capacity using DPPH method

1.2.2 To find the optimum conditions for the determination of antioxidant capacity by the developed FIA system

1.2.3 To determine antioxidant capacity in some natural product extracts using the developed FIA and compare the results with those obtained by the standard batch method

1.3 Scope of this study

A simple FI-spectrophotometric system was proposed for the determination of antioxidant capacity. The $DPPH^{\cdot}$ spectrophotometric assay was applied. The proposed method was used to determine the antioxidant capacity in ethanol extracts of dried herbal teas, vitamin E, a commercial antioxidant product and natural products extracted from some parts (leaves, branches, stems, twigs, roots) of *Oroxylum indicum* and *Camellia sinensis* var. *assamica* using acetone, hexane and methanol as the extractants.

1.4 Benefits

An analytical method which is rapid and cost-effective, and consumes less reagents and less time, for determination of antioxidant activity in some natural product extracts was developed. This method would be valuable to a nutraceutical company that extracts or packages natural products. This method allows for quick testing of raw ingredient for acceptance into production. Being able to test and release product quickly helps to increase production levels and efficiency.

1.5 Definitions of key terms

FIA	Flow injection analysis
SIA	Sequential injection analysis
MSFIA	Multisyringe flow rate injection analysis
$DPPH^{\bullet}$	2,2-diphenyl-1-picrylhydrazyl radical
ORAC	Oxygen radical absorption capacity
TBARS	Thiobarbituric acid reactive substances
TEAC	Trolox equivalent antioxidant capacity
$ABTS^{\bullet+}$	2,2′-azinobis-(3-ethylbenzothiazoline-6-sulphonic acid)
TRAP	Total radical-trapping antioxidant parameter
ESR	Electron spin resonance
HPLC	High performance liquid chromatography
BHT	Butylated hydroxy toluene
F_{calc}	Calculated F values
F_{table}	Critical values of F for a two-tailed test ($P = 0.05$)
AAE	Ascorbic acid equivalent
BHTE	Butylated hydroxy toluene equivalent

CHAPTER 2

LITERATURE REVIEW

2.1 Antioxidant and antioxidant capacity

Antioxidants are molecules which can safely interact with free radicals and terminate the chain reaction before vital molecules are damaged. These antioxidant molecules neutralize free radicals by H^+ ion donation or by electron transfer reactions (Pala & Tabakçoğlu, 2007). The free radicals that antioxidants react with are atoms or groups of atoms with an odd (unpaired) number of electrons and can be formed when oxygen interacts with certain molecules. Once formed these highly reactive radicals can start a chain reaction, like dominoes. For human health their chief danger comes from the damage they can do when they react with important cellular components such as DNA, or the cell membrane. Cells may function poorly or die if this occurs. To prevent free radical damage the body has a defense system of antioxidants. Although there are several enzyme systems within the human body that scavenge free radicals, the principle micronutrient (vitamin) antioxidants are vitamin E, beta-carotene, vitamin C, and polyphenols. Antioxidant compounds like phenolic acids, polyphenols and flavonoids also scavenge free radicals such as peroxide, hydroperoxide or lipid peroxyl and thus inhibit the oxidative mechanisms that lead to degenerative diseases (Scartezzini & Speroni, 2000). Many natural products such as fruits and vegetables contain significant levels of antioxidants. The natural products have aroused considerable interest recently because of their potential beneficial effects on human health. They have been reported to have antiviral, anti-allergic, antiplatelet, anti-inflammatory, anti-tumor and antioxidant activities (Valko et al., 2007). Antioxidants are also widely used as ingredients in dietary supplements in the hope of maintaining health and preventing diseases such as cancer and coronary heart disease (Huang et al., 2005; Kaur & Kapoor, 2001; Tang et al., 2005). There are other synthetic organic compounds (such as BHT) that have been developed for specific uses to

other synthetic organic compounds (such as BHT) that have been developed for specific uses to reduce oxidation in a variety of products. In addition to these uses of natural antioxidants in medicine, these compounds have many industrial uses, such as preservatives in food and cosmetics and preventing the degradation of rubber and gasoline (Niederländer et al., 2008).

Although initial studies suggested that antioxidant supplements might promote health, later large clinical trials did not detect any benefit and suggested instead that excess supplementation may be harmful (Aqil et al., 2006). Further studies have been proposed to investigate the effects and whether they are beneficial or harmful. Antioxidant capacity thus has been measured in order to evaluate the beneficial of antioxidant including natural and synthetic ones (Moure et al., 2001). Antioxidant capacity is a measure of the ability of antioxidant compounds, extracts or organic chemicals to react with and neutralize radicals or peroxides or oxidizing agents. There are many complex naturally occurring compounds that have some antioxidant capacity and this makes measurement of total antioxidant capacity more difficult. Some compounds have low antioxidant capacity or react very slowly such as phenols. Other compounds have high capacity and can quickly neutralize radicals. Most of the antioxidant compounds in a typical diet are derived from plant sources and belong to various classes of compounds with a wide variety of physical and chemical properties. Some natural products contain compounds that range from low to very high capacity. Those products with high antioxidant capacity are more beneficial to human health.

2.2 Techniques for the determination of antioxidant capacity

Over the past two decades, several techniques have been developed to determine the antioxidant capacity in natural products, such as electron spin resonance (ESR) (Ukeda, Adachi & Sawamura, 2002), high-performance liquid chromatography (HPLC) (Kosar, Dorman, Bachmayer, Baser & Hiltunen, 2003), chemiluminescence (CL) (Costin, Barnett, Lewis & McGillivery, 2003; Prior et al., 2005), electrochemistry (Chen, Gorton & Åkesson, 2002), spectrofluorimetry (Tang et al., 2005), and spectrophotometry (Prior et al., 2005). Table 2.1 identifies the techniques, their advantages and disadvantages, type of sample and reference.

Table 2.1 Some analytical techniques for the determination of antioxidant capacity

Techniques	Type of sample	Advantages	Disadvantages	Reference
ESR	1 Green tea 2 Oolong tea 3 Coffee and 4 Red wine	1 No color interference 2 Specific and sensitive for detecting radicals 3 May not properly evaluate slow reacting antioxidants	1 Expensive instruments 2 Requires a complicated procedure including washing 3 High level of technician skills and training for analysis	Ukeda et al., 2002
HPLC	1 Herbal products 2 Beverages 3 Plant products	1 HPLC can be combined with many different assay 2 Useful in finding what compound antioxidants are present	1 Complicated in operation 2 Requires standardization with known antioxidant compounds 3 Not suitable for total antioxidant capacity	Kosar et al., 2003
Chemilumi-nescence	1 Wines 2 Individual antioxidants compounds	1 No color interference 2 Very low detection limit 3 High sensitivity 4 Adaptable to automation	1 Poor in selectivity 2 Selection of emitter is critical 3 More specific to individual oxidation compounds	Costin et al., 2003; Prior et al., 2005
Electro-chemical (voltammetry)	1 Milk 2 Dairy products	1 Fast analysis 2 No color interference	1 Electrochemistry has poor reproducibility 2 Complicated cell detector system 3 Not robust in method	Chen et al., 2002; Tang et al., 2005
Spectro-fluorimetry	1 Food products	1 Simple 2 Selective and sensitive 3 inexpensive fluorometers detector	1 Extended reaction times 2 Temperature sensitive 3 Measures primarily hydroxyl radical	Tang et al., 2005
UV-Visible Spectrophotometry	1 Food samples 2 Herbal and Plant extracts	1 UV-Visible spectrometer is a simple detector and system not expensive 2 Easy to operate 3 Extensive training not required	1 Requires a colorimetric antioxidant reaction 2 Colored sample interferences 3 Limited detection level	Prior et al., 2005

2.3 Spectrophotometric detection methods

The spectrophotometric systems are low in cost, simple to use and can achieve high sample analysis rates. Several spectrophotometric assays have been frequently used to estimate antioxidant capacities in fresh fruits and vegetables and their products including trolox equivalent antioxidant capacity (TEAC) assay (Arnao, 2000; Arts et al., 2004; Böhm & Schlesier, 2004; Ivekovi ć et al., 2005; Leong & Shui, 2002; Magalhães et al., 2009), 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay (Böhm & Schlesier, 2004; Brand-Williams et al., 1995; Magalhães et al., 2006; Magalhães et al., 2009; Molyneux, 2004), ferric reducing antioxidant power (FRAP) assay (Guo et al., 2003; Thaipong, Boonprakop, Crosbyb, Cisneros-Zevallosc & Byrne, 2006; Phipps et al., 2007), oxygen radical absorption capacity (ORAC) assay (Phipps et al., 2007; Thaipong et al., 2006), thiobarbituric acid reactive substances (TBARS) assay (Aqil et al., 2006; Böhm & Schlesier, 2004), total radical-trapping antioxidant parameter (TRAP) assay (Böhm & Schlesier, 2004; Miller et al., 2000; Phipps et al., 2007), folin-ciocalteu (F-C) or total phenolics assay (Phipps et al., 2007). Antioxidants can be very simple compounds or very complex compounds. The methods that have been developed target specific types of reactions. Some methods measure H^+ transfer, others measure electron transfer (see Table 2.2). Many modifications to these methods have been made to improve their ability to predict antioxidant capacity in specific matrices. At present there is no method that can give total antioxidant levels in all matrices. Table 2.2 summarizes the spectrophotometric methods available for measurement of antioxidants. The table identifies the concept advantages and disadvantages of each method.

Table 2.2 Summary of the spectrophotometric methods for the determination of total antioxidants

Method	Concept	Type of sample	Advantages	Disadvantages	Reference
TEAC	Based on the formation of an $ABTS^{+-}$ colored radical which is then used to react with sample antioxidant	1 Sorghum and Sorghum products 2 Natural product 3 Food products	1 Inexpensive and easy to use 2 Stable to pH hence can be used to study pH effect on activity 3 Fast reaction (30 minute) total antioxidant value can be estimated 4 Suitable for ranking of antioxidant	1 Extra step to generate free radical from ABTS salt necessary 2 Generated unstable free radical for long periods of time 3 Not standardized, hence hard to compare values across laboratory 4 Reaction with non antioxidants may bias results	Awika, Rooney, Wu, Prior & Cisneros-Zevallos, 2003; Phipps et al., 2007; Re et al., 1999
FRAP	Based on an electron transfer with an organic ferric salt. It only measures the reducing capability based on the ferric ion.	1 Beverages	1 Can be used for quick screening of redox status 2 Simple and inexpensive 3 No specialized equipment is required	1 Measures only the reducing capability based on ferric iron, which is not relevant to antioxidant activity mechanistically and physiologically 2 It does not measure H^{+} transfer reactions 3 Results are time dependent on reaction rates of different antioxidants	Phipps et al., 2007; Re et al., 1999
TBARS	TBARS measures peroxidative damage to lipids that occurs with free radicals. It measures the level of MDA that results from lipid damage	1 Fats and oils and other lipids	1 Standard method for lipid peroxidation 2 Simple spectrophotometer detection 3 Mainly applies to meat and fish analysis	1 Limited to lipid oxidation 2 In direct measurement of antioxidant activity 3 Can be problematic because of possible side reactions with non oxidants	Böhm & Schlesier, 2004; Oakes & Kraak, 2003

Table 2.2 (Continued)

Method	Concept	Type of sample	Advantages	Disadvantages	Reference
ORAC	Measurement of antioxidant inhibition of peroxyl radical oxidation	1 Food products 2 Beverages	1 Uses biologically relevant free radicals 2 Standardized: allows for data comparison across laboratories 3 Integrates both degree and time of antioxidant reaction 4 Method can be applied for both fast and slow reacting antioxidants	1 Normally requires use of expensive equipment 2 Data variability can be large across equipment 3 pH sensitive 4 Original method does not measure lipophilic antioxidants 5 Temperature control is critical 6 Assay may take up to 1 hour to complete reaction	Awika et al., 2003; Phipps et al., 2007; Thaipong et al., 2006
TRAP	Measure the ability of antioxidants to reduce the reaction of a peroxyl radical and a target probe	1 Primarily been used in plasma and serum	1 Designed specifically for in vivo antioxidant capacity in serum or plasma 2 Covers both slow and fast reacting antioxidants	1 Time consuming and complex 2 High level of expertise required 3 Results expressed in many different ways which make it difficult to compare lab results	Phipps et al., 2007; Prior et al., 2005; Evelson et al., 2001
Folin-Ciocalteu (F-C)	The F-C method is an electron transfer based assay in which a molybdate reagent reacts with phenols to form a blue product	1 Botanical samples	1 Simple and sensitive 2 Useful in characterizing botanied samples 3 Only simple spectrophotometric equipment required	1 Reaction is slow and pH dependent 2 Assay has not been standardized and results can vary widely depending or test parameters 3 Many compounds interfere with the method	Phipps et al., 2007; Prior et al., 2005

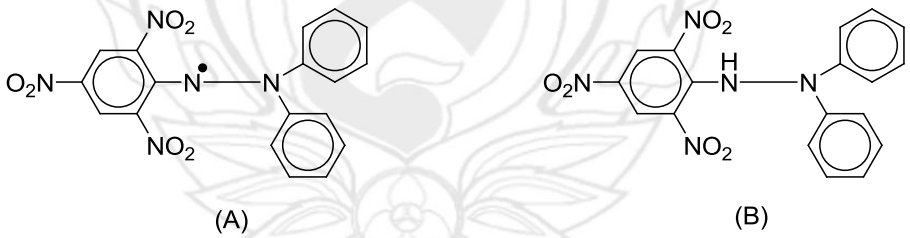
Table 2.2 (Continued)

Method	Concept	Type of sample	Advantages	Disadvantages	Reference
DPPH	Based on a direct reaction of a stable radical with antioxidants. The reaction can be electron or H^+ transfer	1 Food products 2 Plant samples 3 Herbs and beverages	1 Inexpensive and easy to use 2 Stable free radical that is ready to use 3 High correlation with ORAC 4 True radical antioxidant reaction 5 Reacts rapidly with most antioxidants	1 Color interference may lead to underestimation of activity 2 Sensitive to pH 3 Not standardized 4 Measurement of slow reacting antioxidants can extend the time of analysis 5 Timing of reactions is critical for reproducibility	Awika et al., 2003; Böhm & Schlesier, 2004; Prior et al., 2005; Lee, Koo & Min, 2004; Milardović, Ivekovic & Grabarić, 2006

2.4 DPPH• assay

The DPPH• assay is based on the reduction by antioxidants of the purple DPPH radical to the corresponding pale yellow and measured colorimetrically at 520 nm (Molyneux, 2004). Among those methods listed in table 2.2, DPPH• assays have been most widely used because it is relatively simple and stable and the DPPH• is available commercially in high purity. The DPPH radical decolourisation method is strongly consistent and correlates with total phenolic content in the samples. In addition, literatures suggested that the DPPH• assay was an easy and accurate method with regard to measuring the antioxidant capacity of fruit and vegetable juices or extracts (Huang et al., 2005; Sánchez-Moreno, 2002). Therefore, this work was interested in using the DPPH• assay for the determination of antioxidant capacity.

The molecule of 2,2-diphenyl-1-picryl-hydrazyl (Figure 2.1 (A)) is characterized as a stable free radical by virtue of the delocalization of the spare electron over the molecule as a whole, so that the molecules do not dimerise, as would be the case with most other free radicals. The delocalization also gives rise to the deep violet colour, characterized by an absorption band in ethanol solution centred at about 520 nm.



From Molyneux, P. (2004). The use of the stable free radical diphenylpicrylhydrazyl (DPPH) for estimating antioxidant activity. **Journal of the Science of Technology, 26**(2), 211-219.

Figure 2.1 Chemical structures of (A) 2,2-diphenyl-1-picryl-hydrazyl radical and (B) diphenylpicrylhydrazine (nonradical)

When a solution of $DPPH^{\bullet}$ is mixed with that of a substance that can donate a hydrogen atom, then this gives rise to the reduced form (Figure 2.1 (B)) with the loss of this violet colour (although there would be expected to be a residual pale yellow colour from the picryl group still present). Representing the DPPH radical by $DPPH^{\bullet}$ and the donor molecule by AH, the primary reaction is

$$DPPH^{\bullet} + AH \longrightarrow DPPH_2 + A^{\bullet} \quad [1]$$

Violet — yellow

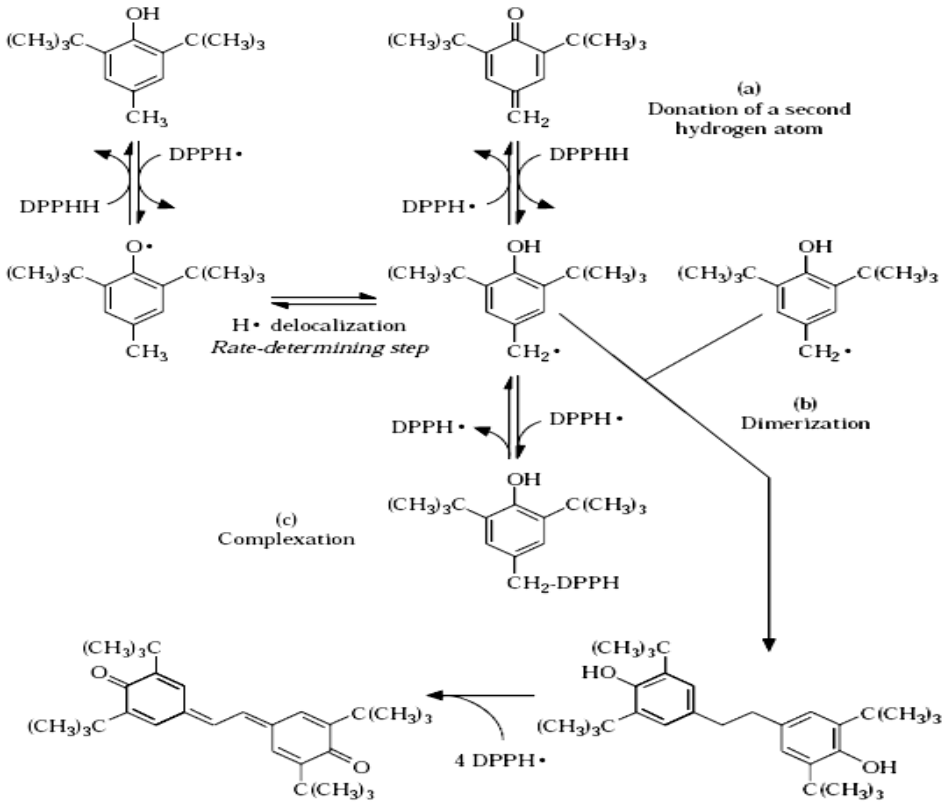
where $DPPH_2$ is the reduced form and $A^{\bullet}$ is free radical produced in this first step. This latter radical will then undergo further reactions which control the overall stoichiometry, that is, the number of molecules of DPPH reduced (decolorised) by one molecule of the reductant.

The reaction [1] is therefore intended to provide the link with the reactions taking place in an oxidizing system, such as the autoxidation of a lipid or other unsaturated substance; the $DPPH^{\bullet}$ is thus intended to represent the free radicals formed in the system whose activity is to be suppressed by the substance AH (Molyneux, 2004). In this work, we used butylated hydroxy toluene (BHT) and ascorbic acid as the antioxidant standards because BHT reacts slowly and ascorbic acid reacts very fast. This gives a range of reaction rates that covers the fast and slow rates of antioxidant compounds.

2.4.1 Reaction between $DPPH^{\bullet}$ and BHT

It must be emphasized that the stoichiometric value does not explain all the aspects of antioxidant efficiency. BHT appears to be a strong antioxidant because 1 mol of BHT reduces about 3 mol of $DPPH^{\bullet}$ (1.8 ration at 30 min and 2.8 at 300 min), even though it reacts very slowly (plateau reached after 5 h at 20 °C) (Figure 2.2). BHT follows a pseudo-second order kinetic model with a reaction order of 0.4 for BHT and 1.5 for DPPH which indicates the reaction rate is more dependent on DPPH concentration (Bondet, Brand-williams & Berset, 1997). The second order rate constant for this reaction is 0.3 (±0.14) x 10^{-5} $\mu M^{-1}s^{-1}$. The rate of reaction is dependent on both the reaction rate of the the initial step as well as the reaction rates of the possible second reaction steps (Mishra, Ojha & Chaudhury, 2012). BHT reacts similarly to polyphenols in their

antioxidant reaction with multiple hydrogen transfers and longer reaction time (Awika et al., 2003).



From Bondet, V., Brand-Williams, W. & Berset, C. (1997). Kinetics and mechanisms of antioxidant activity using the DPPH$^{\cdot}$ free radical method. **Lebensm Wiss u Technol-Food Science and Technology**, **30**, 609-615.

Figure 2.2 Proposed mechanism for BHT/ DPPH$^{\cdot}$ reaction

2.4.2 Reaction between DPPH$^{\cdot}$ and ascorbic acid

Ascorbic acid is an antioxidant compound found in many natural products. Ascorbic acid and isoascorbic acid each reduce nearly two DPPH$^{\cdot}$ molecules as shown in Figure 2.3 (Brand-Williams et al., 1995). Ascorbic acid reaction rate is very fast and gives similar response as fast reacting antioxidant compounds. This reaction is simpler and less complicated than the BHT

reaction. The reaction is second order with the rate constant (k_2) of 2.25 (±0.04) x 10^{-5} $\mu M^{-1} s^{-1}$ (Mishra et al., 2012). The reaction rate of ascorbic acid is not significantly affected by differing concentrations of $DPPH^{\bullet}$ or ascorbic acid.

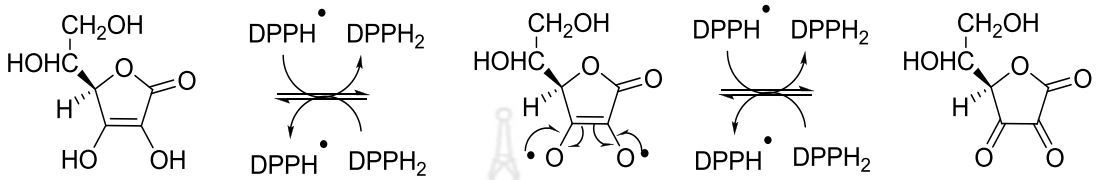


Figure 2.3 Proposed mechanism for ascorbic acid/$DPPH^{\bullet}$ reaction

As mentioned above, the $DPPH^{\bullet}$ assay was of interest due to its consistency and simplicity. However, the batch $DPPH^{\bullet}$ assay has disadvantages, for example, it is time consuming and tedious, uses high amounts of reagent, and requires strict adherence to reaction time limits (Prior et al., 2005). Thus, developing a method to overcome these drawbacks was the aim of this work.

2.5 Flow-based method

Basically the definition of flow-based method is the injection, reaction and detection of an analyte in a continuous flowing stream. Flow-based analytical techniques fall into one of four categories: air-segmented, unsegmented continuous flow, flow injection analysis, and sequential injection analysis (Fang, 1993). In air-segmented and some earlier unsegmented flow systems, samples are aspirated into a liquid moving stream at a fixed flow rate for a specific period of time. An operational feature of a segmented analyzer is that air bubbles are used to segment the sample and reagent mixtures to facilitate mixing and minimize dispersion. Flow injection analysis involves the rapid injection of a sample into a continuously flowing unsegmented carrier stream. One or more reagent solutions are continuously merged with the carrier prior to detection. The injected sample zone undergoes dispersion and is mixed with the carrier and reagent solutions. The resultant product is transported through a flow-through detector for measurement and then to

waste. The dispersion or dilution of the sample zone can be controlled and adapted to the required analysis, by the optimization of several factors including the injected sample volume, the flow rate of carrier and reagent streams, the reaction coil length, and the inner diameter of the tubing (Zagatto, Van Staden, Maniasso, Stefan & Marshall, 2002).

An important breakthrough in laboratory automation methods was achieved when the traditional concept of equilibrium measurements was abandoned and assays based on non-equilibrium conditions, were introduced (Ruzicka & Hansen, 1975). These methods are markedly advantageous when compared to batch procedures since they improve determination rate as well as precision and accuracy, due to the reduction of human intervention. A marked decrease in the reagent consumption and waste production is also achieved, concepts that are normally associated to the perspectives of "Green Chemistry" (Rocha, Nóbrega & Filho, 2001). The chemistry is similar to manual methods but automated, reduces technician time and is not complicated.

Flow injection analysis has been the most commonly uesd flow technique in automatic determination due to its simple handling, economical instrumentation, easy miniaturisation, speed of analysis and accuracy. However, some limitations can be pointed out to FIA like: constant flow of reagents which results in increase reagent consumption and waste production; and low automation degree for some reactions. To overcome these limitations a new concept named sequential injection analysis (SIA) based on the guidelines provided by FIA but using a microprocessoor based control of samples and reagents was proposed. The concept of Sequential Injection Analysis (SI) was "born" in 1990 by J. Ruzicka and G.D. Marshall. At that time, Flow Injection Analysis was becoming a widely accepted and rapidly evolving technique in most academic analytical chemistry laboratories.

When compared with FIA, sequential injection analysis allows considerable saving of reagent and a significant decrease on the chemical waste produced, since just the required volumes of sample and reagent are used. However, sample analysis rate is lower than standard FIA. SIA requires extensive software and microprocessor contol of all aspects of sample and reagent handling. Also, the use of syringe pumps requires a refill cycle and they have poor precision for low sample volumes (Global FIA, 2008). The FIA technique was selected because of its simplicity as well as fast individual sample analysis time.

2.5.1 Principle of flow injection analysis

In the mid-1970s a new and analytically important stage of development was initiated when a new flow analysis technique named flow injection analysis (FIA) was developed in Denmark by Ruzicka and Hansen (1975). This proved to be a useful and efficient instrumental method for carrying out analytical determination. Ruzicka and Hansen initially defined FIA as: "A new concept of continuous flow analysis based on injecting the sample into a rapidly flowing carrier stream which has not been segmented by air." After some years of developing FIA techniques (1988) the FIA concept was revised and changed to: "Information gathering from a concentration gradient formed from an injected, well defined zone fluid, dispersed into a continuous unsegmented flow stream of carrier"

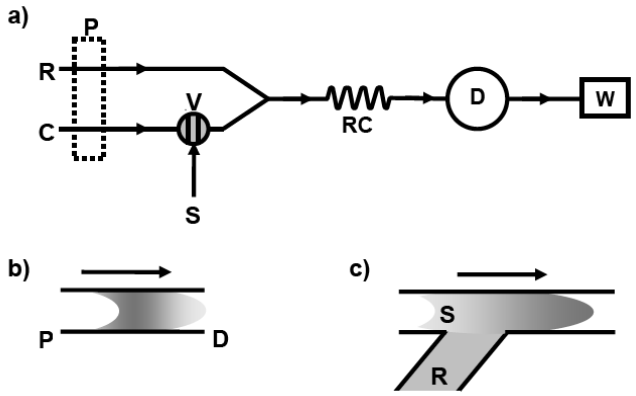
Three principles constitute the basic of FIA:

2.5.1.1 Reproducible sample injection or insertion in a flowing carrier stream

2.5.1.2 Controlled dispersion of the sample zone

2.5.1.3 Reproducible timing of its movement, from the injection point to the detection system.

A typical FIA manifold and the dispersion pattern achieved in the flow conduits are depicted in Figure 2.4



From Neves, M. S. A. C. (2006). **Flow systems for the trace determination of phosphate in waters based on the spectrophotometric vanadomolybdate method**. Master's Thesis. Environmental Diagnostics, Cranfield University: United Kingdom.

Figure 2.4 a) Schematic representation of a flow injection analysis basic manifold. b) Dispersion pattern inside the tubes. c) Reagent is added through a confluence point. R-reagent; C-carrier; P-peristaltic pump; S-sample or standard solution; V-injection valve; RC-reaction coil; D-detection system; W-waste

A simple FIA manifold consists of a pump (P), used to propel one or several flowing streams solutions (including the carrier stream) at a constant rate; an injection valve (V), to introduce a well-defined volume of a sample into the carrier stream; a confluence point, located after the injection port where the reagent is confluence point, located after the injection port where the reagent is continuously added; a coil, usually referred to as reactor (RC), along which the sample disperse and react with components of the carrier and/or reagent streams; and finally, a flow through detector (D) that monitors the products of reaction and transduces the signal generated to a recorder or a microprocessor.

Different types of samples treatments such as dilution, gas diffusion, dialysis, and extraction, can be performed in the flow injection systems by adding the appropriate device to the manifold.

2.5.2 Flow-based spectroscopic methods for antioxidant determination

Visible and UV spectrophotometers are by far the most widely used type of detectors and these are frequently used in flow methods. This is due to several factors:

2.5.2.1 They are easily applied. A conventional batch spectrophotometer can simply be converted into a flow-through spectrophotometer just by replacing the conventional cuvette with a flow- through cell.

2.5.2.2 It does not demand qualified personnel.

2.5.2.3 When using an auto sample with the spectrometer, technician time can be greatly reduced.

2.5.2.4 An increase in the degree of precision and sample throughput are generally achieved.

2.5.2.5 Detection limits are usually lower than the conventional methods since the noise contribution due to human handling of the sample is eliminated.

These are the main reasons why we are interested in using UV/Visible spectrometry for the analysis of antioxidants. Most laboratories have UV/Visible spectrometers and they are simple to operate.

It is known that in spectrophotometry, according to the Beer-Lambert's law the absorbance signal (A) is proportional to absorptivity (a), which is wavelength dependent, the light path length (b) and the concentration of analyte (c):

$$A = abc$$

Therefore, the increase in the optical path length and/or the change in the wavelength lead to enhanced method sensitivity. However, flow based spectrophotometric systems present their own drawbacks. As previously explained, the formation of concentration gradients is one of the flow analysis characteristics. When this phenomenon occurs a refractive index gradient can result, known as the "schlieren" effect. This makes measurements more complicated and affects sensitivity and repeatability of the method (Rocha & Nóbrega, 1997).

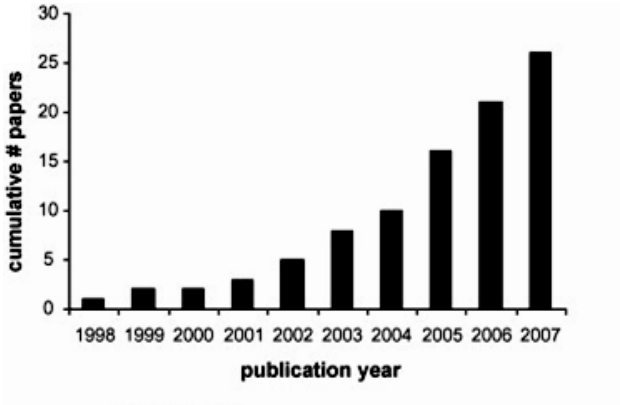
Under good mixing conditions, the "schlieren" effect becomes less pronounced and a continuous concentration gradient is established along the processed sample (Rocha & Nóbrega, 1997).

Length and design and diameter of mixing coils can minimize this effect. Matrix matching of sample and carrier streams also minimizes this effect (Fang, 1993).

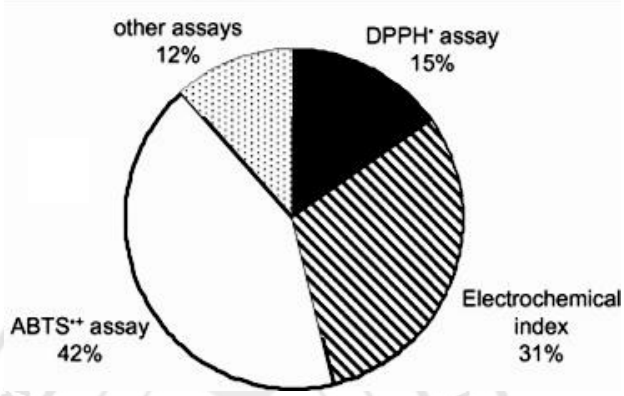
Zagatto et al. (1990) proposed an instrumental method for "schlieren" compensation based on measurements at two wavelengths. A dual-wavelength beam passes through the flowing sample, the emergent light is dispersed and the resulting monochromatic beams are measured simultaneously by separate detectors, the difference in absorbances constituting the measurement basic. Real-time subtraction of the wavelength-independent noise is then achieved (Zagatto, Arruda, Jacintho & Mattos, 1990). In order to be able to do this kind of correction, a diode-array spectrophotometer is needed.

There are other types of detectors that can be used with flow based systems for antioxidant analysis, detectors such as ESR, CL, Potentiometry, Amperometry and Biamperometry. These other detectors are generally more expensive and complicate to operate. A literature review of method for the determination of antioxidant activity was done and is summarized in Table 2.3. As depicted in Figure 2.5 (a), the number of publications devoted to this subject has grown significantly, especially in the past 3 years. Moreover, different assays have been automated (Figure 2.5 (b)). In fact, more than half of the proposed applications are based on the utilization of colored, radical species ($ABTS^{\bullet+}$ or $DPPH^{\bullet}$) that mimic the reactive nitrogen species (RNS)/reactive oxygen speies (ROS) found in vivo. Other methods aimed the determination of "total reducing capacity", for which the amperometrtic determination of an "electrochemical index" accounts for about 31% of the flow systems reported. Considering this division, an overview is presented in the next sections. One of the most common methods for assessing the antioxidant capacity is the $ABTS^{\bullet+}$ or TEAC (trolox equivalent antioxidant capacity) assay.

(a)



(b)



From Magalhães, L. M., Santos, M., Segundo, M.A., Reis, S. & Lima, J. L. F. C. (2009). Flow injection based methods for fast screening of antioxidant capacity. **Talanta**, **77**, 1559-1566.

Figure 2.5 (a) Cumulative distribution of papers dealing with automatic flow based determination of total antioxidant capacity per publication year. (b) Distribution of the same papers regarding assay type. Papers from 2008 are not included

Table 2.3 Summary of the flow-based methods developed for the determination of scavenging activity

Flow method	Assay	Generation of reactive species	Detection system	Type of sample	Determination rate (h^{-1})	RSD (%)	Reference
FIA	$ABTS^{\bullet+}$	Chemical ($K_2S_2O_8$)	Vis (734 nm)	Beer, coffee, cola, juices, tea	30	<1.7	Pellegrini, Del Rio, Colombi, Bianchi & Bighenti, 2003
FIA	$ABTS^{\bullet+}$	Chemical ($K_2S_2O_8$)	Vis (n. g.)	Mouthrinse, white wine and plasma	-	<2.7	Bompadre, Leone, Politi & Battino, 2004
FIA	$ABTS^{\bullet+}$	Enzymatic (H_2O_2 + HRP)	Vis (414 nm)	Honey and wine	120	<5	Labrinea & Georgiou, 2005
FIA	$ABTS^{\bullet+}$	Electrochemical (in-line)	Vis (734 nm)	Coffee, red wine, tea	32	<1.95	Ivekovic et al., 2005
FIA	$ABTS^{\bullet+}$	Enzymatic (H_2O_2 + HRP, in-line)	Biamperometry	Juices, tea, wine	42	n.g.	Milardovic, Kerekovic, Derrico & Rumenjak, 2007
FIA	$ABTS^{\bullet+}$	Enzymatic (glucose + GOD/ H_2O_2 + HRP, in-line)	Biamperometry	Spirits and wine	-	n.g.	Milardovic, Kerekovic & Rumenjak, 2007
SIA	$ABTS^{\bullet+}$	Chemical ($K_2S_2O_8$)	Vis (734 nm)	Beer, juices, milk, tea, yoghurt	9-20	n.g.	Lima, Toth & Rangel, 2005
SIA	$ABTS^{\bullet+}$	Chemical ($K_2S_2O_8$)	Vis (734 nm)	Wine	15 or 42	<2.4	Pinto, Saraiva, Reis & Lima, 2005
MSFIA	$ABTS^{\bullet+}$	Enzymatic (H_2O_2 + HRP)	Vis (734 nm)	Beer, juices, tea, wine	12 or 18	<3.1	Magalhães, Segundo, Reis et al., 2007
FIA	DPPH	-	ESR	Coffee, red wine, tea	13	<3.2	Ukeda et al., 2002

Table 2.3 (Continued)

Flow method	Assay	Generation of reactive species	Detection system	Type of sample	Determination rate (h^{-1})	RSD (%)	Reference
SIA	DPPH	-	Vis (525 nm)	Herbal and mushroom extracts	45	<1.8	Polášek, Skála, Opletal & Jahodář, 2004
MSFIA	DPPH	-	Vis (517 nm)	Beers, juices, tea, wines	13	<1.0	Magalhães et al., 2006
MSFIA	DPPH	-	Vis (517 nm)	n.a.	14	n.g.	Magalhães, Segundo, Siquet et al., 2007
FIA	Total phenolics	-	CL	Wine	120	<0.8	Costin et al., 2003
FIA	CL-antioxidant capacity	-	CL	Olive oil	180	<2.8	Minioti & Georgiou, 2008
FIA	Reduction of Fe (III) complex	-	Potentiometry	Fruit extract, herbal infusion and tea	100	<1.8	Shpigun, Arharova, Brainina & Ivanova, 2006
MSFIA	Folin-Ciocalteu reducing capacity	-	Vis (750 nm)	Beer, juices, tea, wine	12	<1.3	Magalhães, Segundo, Reis et al., 2007; Magalhães et al., 2006
FIA	Ampero-metric reducing capacity	-	Amperometry (+0.4 V)	Wine	<90	n.g.	Mannino, Brenna, Buratti & Cosio, 1998
FIA	Ampero-metric reducing capacity	-	Amperometry (+0.5 V)	Olive oil	90	<3.5	Mannino, Buratti, Cosio & Pellegrini, 1999

Table 2.3 (Continued)

Flow method	Assay	Generation of reactive species	Detection system	Type of sample	Determination rate (h^{-1})	RSD (%)	Reference
FIA	Ampero-metric reducing capacity	-	Amperometry (+0.5 V)	Vegetable extracts	60	<3.5	Buratti, Pellegrini, Brenna & Mannino, 2001
FIA	Ampero-metric reducing capacity	-	Amperometry (+0.5 V)	Herbal extracts	60	n.g.	Cosio, Buratti, Mannino & Benedetti, 2006
FIA	Ampero-metric reducing capacity	-	Amperometry (+0.5 V)	Honey, propolis and royal jelly	n.g.	<2.5	Buratti, Benedetti & Cosio, 2007

Note. FIA, flow injection analysis; SIA, sequential injection analysis; MSFIA, multisyringe flow rate injection analysis; ESR, electron spin resonance spectrometry; Vis, spectrometry; HRP, horseradish peroxidase; GOD, glucose oxidase; CL, chemiluminescence; n.g., not given; n.a., not applicable.

CHAPTER 3

METHODOLOGY

3.1 Chemicals and equipments

All chemicals are analytical reagent grade and used without further purified, otherwise stated, as follows:

1. Ethanol, ≥99% (Merck, Darmstadt, Germany)
2. 2,2-diphenyl-1-picrylhydrazyl radical (DPPH$^{\bullet}$), (Sigma-Aldrich Chemie GmbH, Steinheim, Germany)
3. L(+)-Ascorbic acid, (POCH SA, Sowińskiego 11, Poland)
4. Butylated hydroxy toluene (BHT), ≥99% (Fluka Analytical, Spain)

Equipments employed in this work are listed as follows:

1. Analytical balance (Sartorius model BT2245, Germany)
2. A peristaltic pump (REGLO Analog MS-2/6, Ismatec, Switzerland)
3. A pump tube (Silicone tubing, 1.65 mm i.d., Glattbrugg, Switzerland)
4. A 6-port injection valve (V-450, UpChurch Scientific, Washington, USA)
5. A polymer tubing (PFA tube, UpChurch Scientific, Washington, USA)
6. A spectrophotometer (Spekol 1200, Analytik Jena AG, Germany)
7. Aspect Plus program (Carl Zeiss Jena GmbH, Germany)

3.2 Preparation of reagents

3.2.1 Ascorbic acid solutions

Ascorbic acid stock solution (1 mM) was prepared by weighing 0.0176 g of ascorbic acid,

dissolving and diluting with ethanol to 100 mL in a volumetric flask. Working standard solutions (0.010-0.300 mM) were prepared freshly by dilution of the stock solution in ethanol.

3.2.2 BHT solutions

BHT stock solution (50 mM) was prepared by weighing 1.1018 g of BHT, dissolving and diluting with ethanol to 100 mL in a volumetric flask. Working standard solutions (0.50-15 mM) were prepared daily by dilution of stock solution in ethanol.

3.2.3 DPPH$^{\cdot}$ solution

DPPH$^{\cdot}$ stock solution (1 mM) was prepared by weighing 0.0394 g of DPPH$^{\cdot}$, dissolving and diluting with ethanol in a 100 mL volumetric flask. The stock solution of DPPH$^{\cdot}$ was stored at 4°C. This solution was stable for a week when protected from light in a brown bottle and refrigerated. For the analysis using the batch method, a DPPH$^{\cdot}$ solution (0.050 mM) in ethanol was prepared.

3.3 Samples and preparation

There were 4 types of samples: natural extract (12 samples; No. 1-12), vitamin E gel cap (1 sample; No. 13), commercial antioxidant product (1 sample; No. 14) and dried herbal tea (13 samples; No. 15-27) samples, as shown in Table 3.1. The natural extract samples were the extracts of some parts of *Oroxylum indicum* (Indian trumpet flower) and *Camellia sinensis var. assamica* (tea) plants. Acetone ($(CH_3)_2CO$), hexane (C_6H_{14}) and methanol (CH_3OH) were used as the extractant. A part of plant (3 kg) was chopped and soaked at room temperature for 7 days in the extractant (8 L). Removal of the solvent from each extract under reduced pressure gave dark green viscous extracts. To prepare solutions for analysis, the extract, commercial antioxidant product and vitamin E gel cap were weighed (6.10 mg each) and diluted in absolute ethanol (1 mL). For the samples no.15-27, 0.15 g of each sample was extracted with 25 mL absolute ethanol by shaking for 15 minutes after which they were filtered and ready for analysis of antioxidant capacity.

Table 3.1 List of samples

No.	Sample	Type
1	*Oroxylum indicum*	Leaf hexane extract
2	*Oroxylum indicum*	Leaf methanolic extract
3	*Oroxylum indicum*	Leaf acetone extract
4	*Oroxylum indicum*	Branch acetone extract
5	*Oroxylum indicum*	Stem acetone extract
6	*Oroxylum indicum*	Stem methanolic extract
7	*Oroxylum indicum*	Stem hexane extract
8	*Oroxylum indicum*	Twig methanolic extract
9	*Oroxylum indicum*	Root acetone extract
10	*Oroxylum indicum*	Root methanolic extract
11	*Camellia sinensis var. assamica*	Leaf methanolic extract
12	*Camellia sinensis var. assamica*	Leaf hexane extract
13	Vitamin E	Commercial gel cup (Select™, Canada)
14	Exo	Commercial antioxidant product
15	*Thumbergia laurifolia* Linn	Commercial dried herb
16	*Stevia rebaudiana* Bertoni	Commercial dried herb
17	*Coscinium fenestratum* (Gaertn.) Colebr	Commercial dried herb
18	*Leptochloa chinensis* (L.) Nees	Commercial dried herb
19	*Centella asiatica* Urban	Commercial dried herb
20	*Glycyrrhiza glabra* Linn	Commercial dried herb
21	*Rhinacanthus nasutus* (L.) Kurz	Commercial dried herb
22	*Prunus cerasoides* D.Don	Commercial dried herb
23	*Moringa oleifera* Lam	Commercial dried herb
24	*Aegel marmelos* Corr	Commercial dried herb
25	*Senna alexandrina* P. Miller	Commercial dried herb
26	*Cymbopogon citratus* Stapf	Commercial dried herb
27	*Phyllanthus amarus* Schum & Thon	Commercial dried herb

3.4 Reference method

A 6.1 mg of the extract sample was diluted to 1 mL with absolute ethanol. An aliquot (50 μL) of the diluted extract and 3 mL of 0.05 mM $DPPH^{\cdot}$ ethanolic solution were mixed and incubated at room temperature for 30 minutes. The absorbance of the solution was measured at 517 nm. Absorption measurements were performed in triplicate (Mahabusarakam, Deachathai, Phongpaichit, Jansakul & Taylor, 2004). Samples were diluted as necessary to ensure the results are within the calibration range. Ascorbic acid and BHT were used as standard. In order to simplify the determination of antioxidant capacity, the sample extracts were reported as equivalent to ascorbic acid and BHT.

3.5 Flow injection setup

Flow injection-spectrophotometric system proposed in this work was a single line system (Figure 3.1), which meant that a sample was injected into a reagent stream and the product zone was propelled through a reaction coil and a detector, respectively. The FI instrumentation employed the following items.

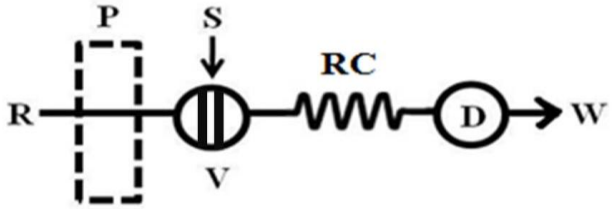


Figure 3.1 Schematic diagram of a single-line FI system. R-reagent $DPPH^{\cdot}$ solution; P-peristaltic pump; S-sample or standard solution; V-injection valve; RC-reaction coil; D-detector; W-waste

3.5.1 Liquid delivery system

A peristaltic pump (Figure 3.2) was used as the propelling unit in the FIA setup. A pump tube was used and substituted whenever malfuctions (perceivable in the form of deviation from the usual analytical signal) due to variation of flow rates or lost of elasticity were detected. The pumped volume was calibrated by measuring the volume of water in a graduated cylinder over a measured time interval.

Figure 3.2 The peristaltic pump

3.5.2 Injection valve

Sample and standard injections in the FI set-up were made using a 6-port injection valve (Figure 3.3).

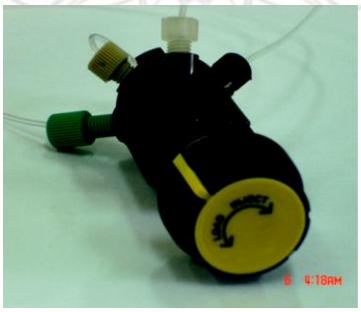
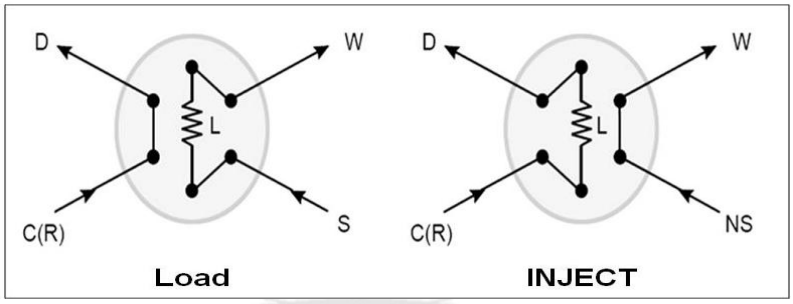


Figure 3.3 The 6-port injection valve

It is a rotary valve operated within two stages: load and injet (Figure 3.4). During the first stage, sample is aspirated into the sample loop with pre-determined volume, while the carrier (or reagent) stream is pumped through the manifold. When the cavity is filled with the sample, the valve is switched to the injection mode. The sample is completely swept by the carrier stream out of the volumetric cavity and brought into the manifold as a discrete plug.



From Neves, M. S. A. C. (2006). **Flow systems for the trace determination of phosphate in waters based on the spectrophotometric vanadomolybdate method**. Master's Thesis. Environmental Diagnostics, Cranfield University: United Kingdom.

Figure 3.4 Schematic diagram of a 6-port rotary injection valve (S-sample; NS-new sample; C-carrier; D-reaction coil and detector; L-sample loop; W-waste)

3.5.3 Reaction coil

The reaction coil is a length of polymer tubing with an internal diameter of 0.040 inches. The reaction coil was wound around a 2 cm round core as shown in the Figure 3.5. The length of coil impacts reaction time and mixing of the reagents and sample. The lengths were varied to determine optimum length for the $DPPH^{\cdot}$ chemistry.

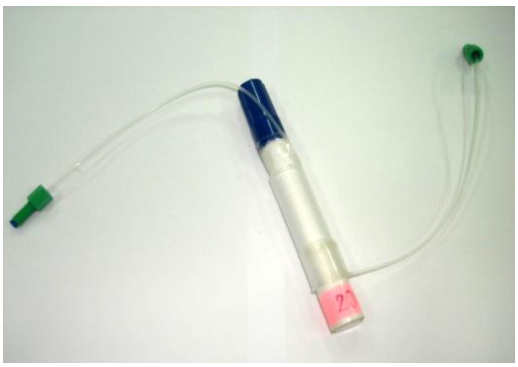


Figure 3.5 FIA reaction coil

3.5.4 Detector

A spectrophotometer was used as the detector. It was controlled and the absorbance data was collected using the Aspect Plus program. It was linked through a PC desktop via RS232 port for controlling (Figure 3.6).

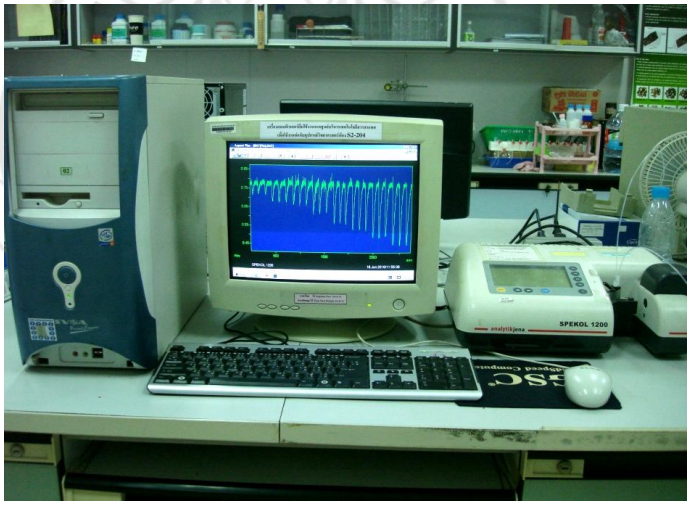


Figure 3.6 The spectrophotometer used in this work

The FI-spectrophotometric setup used for this study is shown in Figure 3.7. The flow manifold was designed to allow direct introduction of the sample with a simple configuration. In this arrangement, the $DPPH^{\bullet}$ solution as a reagent (R) was pumped into the system and the standard or

sample solution (S) (50 μL) was then injected into the reagent stream. These solutions merged in a confluence and the resulting solution further mixed while passsing though the reaction coil. The product zone was then pumped to the detector, and measured at wavelength of 520 nm.

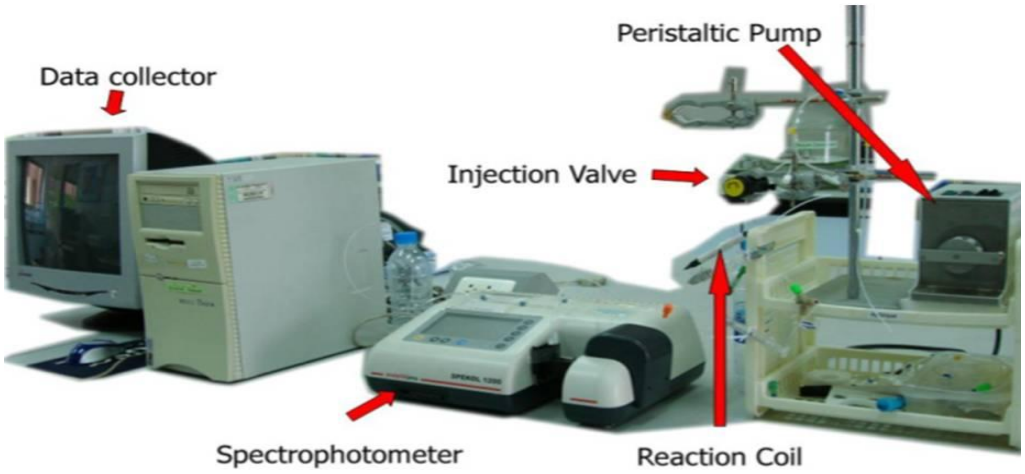


Figure 3.7 The developed FI-spectrophotometric system

3.6 Optimization of the developed FI spectrophotometric method

After setting up a preliminary manifold configuration, based on the reference method, parameters were optimized on a trial basis to allow determination over the expected range. Subsequently, parameters were manipulated in order to maximize sensitivity, and to minimize reagent consumption. The optimization process that was used for this method consists in varying, within a certain interval, the parameter that is being optimized, while keeping the other factors fixed.

3.6.1 Linear range

To study the linear range for BHT and ascorbic acid standards, 0.025-50 mM BHT and 0.005-1 mM ascorbic acid standards were employed. $DPPH^{\bullet}$ concentration (0.075 mM), flow rate (1 mL min^{-1}) and reaction coil length (200 cm) were kept constant. Calibration curves of BHT and ascorbic acid standards were constructed.

3.6.2 DPPH$^{\bullet}$ concentration

DPPH$^{\bullet}$ concentrations range of above and below the concentration used in the reference method (0.5 mM), were varied. The DPPH$^{\bullet}$ concentration in the ranges of 0.025-0.3 mM and 0.025-0.15 mM were used for BHT and ascorbic acid standards, respectively. The sensitivity, i.e. slope, of the calibration curve obtained for each DPPH$^{\bullet}$ concentration was plotted against DPPH$^{\bullet}$ concentration to select the optimum concentration. Once the optimum DPPH$^{\bullet}$ concentration was determined, the linearity of the BHT and ascorbic acid standards was confirmed.

3.6.3 Flow rate

Flow rate is an important factor to sample throughput (Tang et al., 2005). Higher flow rates may increase the sensitivity (slope) and linearity (R^2) of calibration graphs due to the increase in the degree of dispersion and mixing. Higher flow rates can reduce the analysis time but may increase the reagent consumption (Suteerapataranon & Pudta, 2008). The optimum flow rate depends on the chemistry of the reaction, the rate of the reaction and the rate of dispersion of sample into the DPPH$^{\bullet}$ reagent. In this experiment, the flow rates of DPPH$^{\bullet}$ reagent studied were 0.5, 0.6, 0.8, 1.0, 1.5 and 2.0 ml min^{-1}. The reaction coil length of 200 cm, and the DPPH$^{\bullet}$ concentration of 0.075 mM were used.

3.6.4 Reaction coil length

Reaction coil length plays a major role in determining the characteristics of any FIA system and significantly interacts with other factors; tubing internal diameter, system geometry, flow rate, and temperature-related variables. These factors play their part in determining the amount of dispersion that a sample plug undergoes as it travels through the reaction coil and FIA system. The reaction coil length also directly impacts the amount of reaction time (Fang, 1993). The reaction coil used in this work was a piece of PFA tubings (0.030 cm i.d.). For BHT standard, the reaction coil length studied were 100, 200, 300 and 500 cm, and for the ascorbic acid standard the coil lengths studied were 50, 100, 200, 300 and 500 cm.

3.7 Validation of the FI system

The optimized system was then characterized in terms of detection and quantification limits, sample throughput and repeatability. Limit of detection, defined as the analyte concentration (C_{LD}) which produces a signal (X_{LD}) that can be statistically distinguished from the blank signal. The value of X_{LD} is given by the equation (3.1),

$$X_{LD} = \bar{X}_b + 3S_b \quad (3.1)$$

where $\bar{X}_b$ is the mean of ten blank measures and S_b is the respective standard deviation. The minimum detectable concentration can then be calculated by interpolation in the calibration curve, assumed to be constant for low concentrations. The limit of quantification is defined as the analyte concentration (C_{LQ}) which produces a signal (X_{LQ}), with a mathematical expression based on statistical processing of blanks (equation (3.2)).

$$X_{LQ} = \bar{X}_b + 10S_b \quad (3.2)$$

The repeatability of the developed method was assessed by the determination of the relative standard deviation (RSD) values (expressed as percentage), corresponding to several consecutive determinations of natural product extract samples. Natural product samples with different analyte concentrations were injected to provide a reliable estimate of the repeatability of the method along the application range (Currie, 1995).

3.8 Determination of antioxidant capacity in some natural product samples and method validation

All samples were analyzed using the FI system as well as the standard method. The sets of sample analysis data from the two methods were statistically compared using the *Q* Test, *F* Test and *t* test at 95% confidence level for determining significance of differences in results.

CHAPTER 4

RESULTS AND DISCUSSION

4.1 Wavelength selection

The measured absorbance spectrum of $DPPH^{\bullet}$ reagent is shown in Figure 4.1. The $DPPH^{\bullet}$ spectrum shows two main absorption maximums, one in the UV range at about 328 nm and one at 520 nm. The absorption at the UV wavelength of 328 nm is primarily due to carbon to carbon double bonds in the DPPH compound and the absorption at the visible wavelength of 520 nm is due to the purple color of the DPPH radical (Hristea et al., 2002). The UV absorption maximum at 328 nm is not suitable for measuring the $DPPH^{\bullet}$ reaction because the ethanol sample extracts are likely to contain dissolved double bonded carbon compounds that would interfere with the analysis. The visible wavelength maximum of 520 nm is suitable for most sample extract analysis except for highly colored extracts. The method that has been developed to determine the antioxidant capacity of some natural product utilizes the stable 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical. The odd electron in the DPPH free radical gives a strong absorption maximum at 520 nm and is purple in color. The color turns from purple to yellow as the molar absorptivity of the DPPH radical at 520 nm reduces from 9660 to 1640 L mol^{-1} cm^{-1} when the odd electron of DPPH radical becomes paired with a hydrogen from a free radical scavenging antioxidant to form the reduced DPPH-H. The resulting decolorization is stoichiometric with respect to number of electrons captured (Prakash, 2001). Through this work, the wavelength of 520 nm was used for measuring the absorbance because it was the wavelength at maximum absorption. This maximum absorbance wavelength gives best sensitivity, lowest DL and highest absorbance for standards and samples (Blauch, 2009).

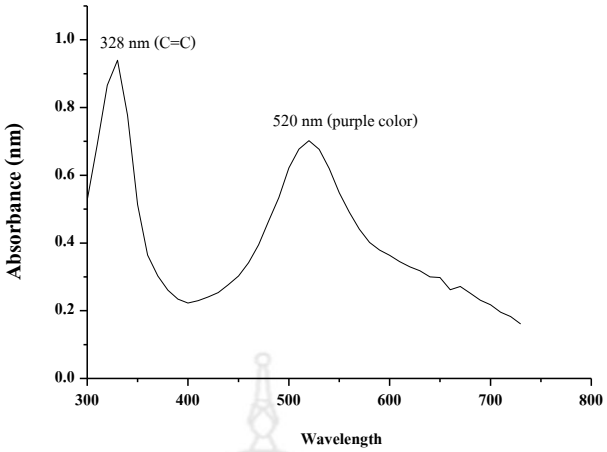


Figure 4.1 Absorption spectrum of DPPH$^{\bullet}$ reagent

4.2 Study of dynamic range

4.2.1 BHT

Figure 4.2 shows the FIA gram of BHT standard. The absorbance at the baseline of unreacted DPPH is approximately 0.8. The reaction of DPPH$^{\bullet}$ and antioxidant reduces the purple color absorbance and the peaks show a reduction in absorbance with increasing concentration of BHT. Figure 4.3 shows the plot of peak height versus BHT concentration. The plot is not linear to concentration. This was because the reaction is both time and concentration dependent. Using this FIA method, we have reduced time of reaction from batch method of 30 minutes to approximately 3 minutes. We can also see from this graph that at very low concentrations, the reaction is very fast in comparison to high concentration. At high concentrations of BHT, 30-50 mM, the reduction in absorbance leveled off indicating the DPPH$^{\bullet}$ was totally consumed. Also, the waste stream was completely yellow with no purple DPPH$^{\bullet}$ showing. Because of the curve line observed we plotted the square root of BHT concentration versus peak height and were able to obtain a more linear curve. From this plot, the optimum linear range is 0.500 mM to 10 mM ($y = 0.0651x + 0.0135$, $R^2 = 0.9994$) (as shown in Figure 4.4). We selected the BHT concentration range of 0.500 to 10 mM for the analysis.

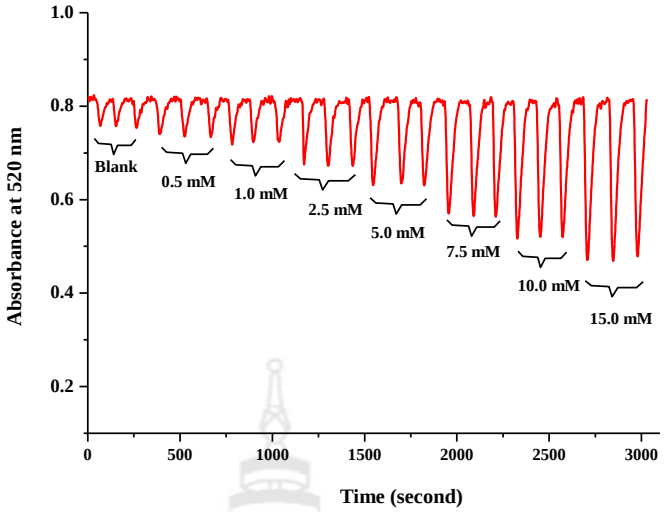


Figure 4.2 FIA gram of BHT standard (triplicate injections)

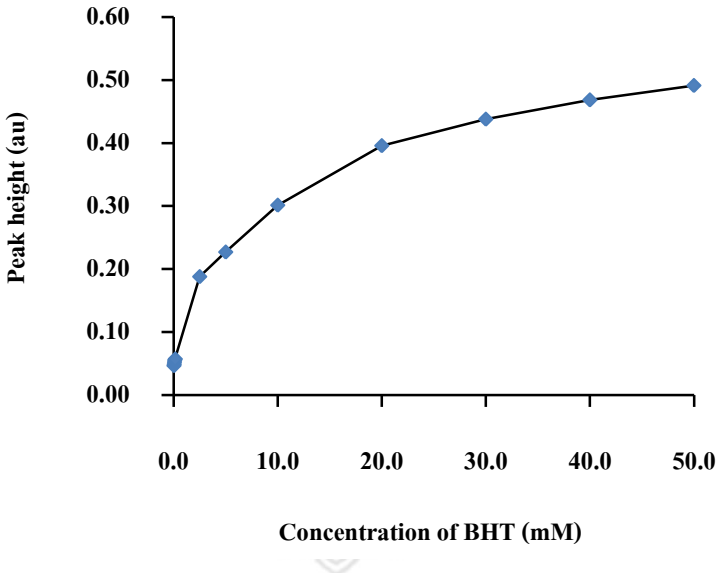


Figure 4.3 A plot of peak height of DPPH$^{\bullet}$ versus concentration of BHT (mM) (Conditions: concentration of DPPH$^{\bullet}$ 0.05 mM, sample volume 50 µL, flow rate 0.8 mL/min, reaction coil length 200 nm, wavelength 520 nm)

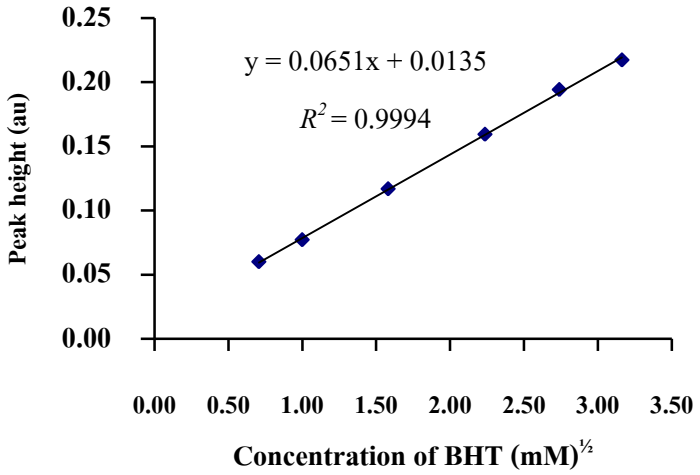


Figure 4.4 Calibration graph of BHT standards (0.5-10 mM) (Conditions: concentration of DPPH$^{\bullet}$ 0.05 mM, sample volume 50 µL, flow rate 0.8 mL/min, reaction coil length 200 nm, wavelength 520 nm)

4.2.2 Ascorbic acid

The FIA gram of ascorbic acid standards (0.010-0.300 mM) with triplicate injections is shown in Figure 4.5. The plot of peak height versus ascorbic acid concentration (Figure 4.6) shows linearity up to 0.30 mM. Above 0.30 mM up to 1.00 mM the curve levels off. At ascorbic acid concentrations above 0.60 mM the absorbance was quite constant because the DPPH$^{\bullet}$ reagent was completely consumed. The linear range was found to be 0.010-0.300 mM (y = 1.6125x - 0.0027, R^2 = 0.9995) (Figure 4.7). The calibration curve of ascorbic acid is different from that of BHT in that the curve of ascorbic acid plots peak height against ascorbic acid concentration is linear while that of BHT uses the square root function. This is because the ascorbic acid reacts very fast whereas the BHT reacts slowly. The FIA reaction time of 3 minutes allows for a complete reaction of ascorbic acid with the DPPH$^{\bullet}$. The reaction between ascorbic acid and DPPH$^{\bullet}$ simply occurs by hydrogen transferring from each OH group, and no other complex reactions are taking place. These are the reason why the concentration range of the ascorbic acid is lower than that of BHT.

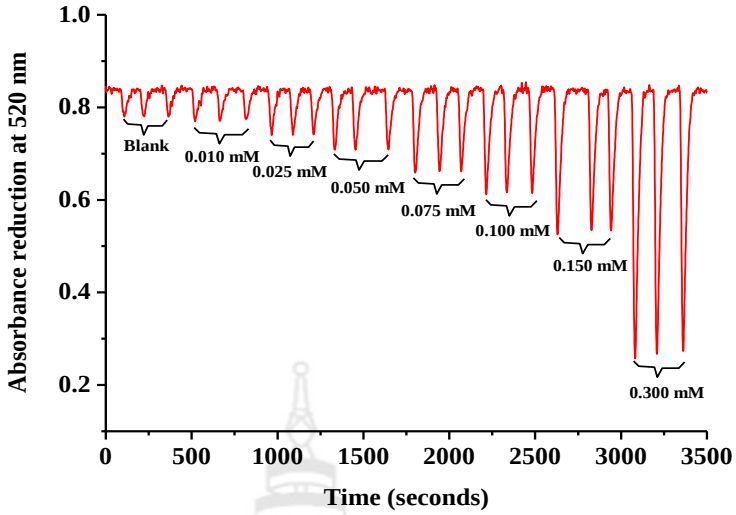


Figure 4.5 FIA gram of ascorbic acid standard (0.010-0.300 mM)

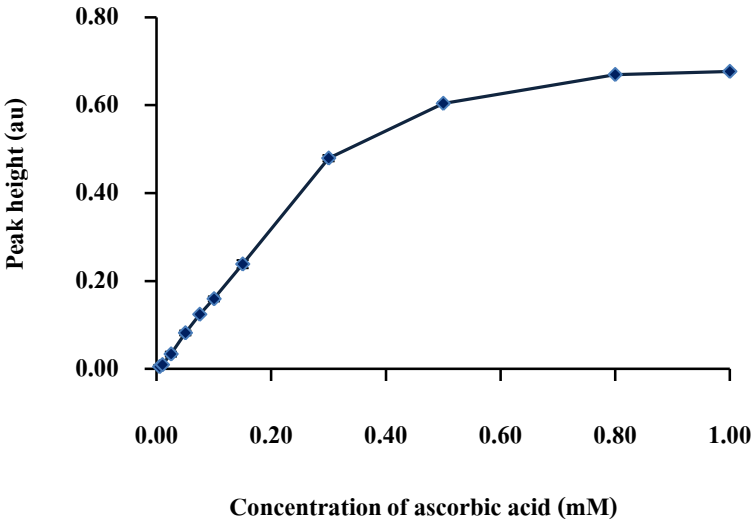


Figure 4.6 A plot of peak height against concentration of ascorbic acid standards (0.010-1.000 mM) (Conditions: concentration of $DPPH^{\bullet}$ 0.075 mM, sample volume 50 μL, flow rate 1.0 mL/min, reaction coil length 200 nm, wavelength 520 nm)

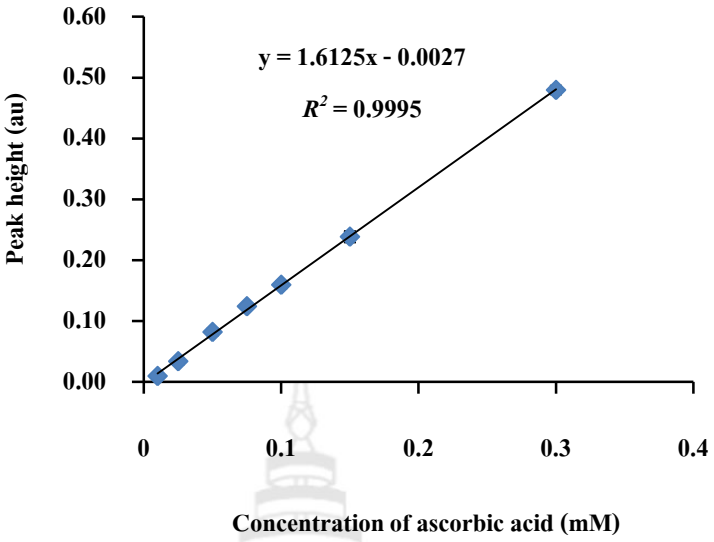


Figure 4.7 Calibration graph of ascorbic acid standards (0.010-0.300 mM) (Conditions: concentration of DPPH$^{\bullet}$ 0.075 mM, sample volume 50 µL, flow rate 1.0 mL/min, reaction coil length 200 nm, wavelength 520 nm)

4.3 Optimization of the FI-spectrophotometric method using BHT as standard

4.3.1 Effect of DPPH$^{\bullet}$ concentration

Figure 4.8 illustrates the effect of the DPPH$^{\bullet}$ concentration on the sensitivity (i.e. the slope) of the BHT calibration graph. Three replications were performed for each DPPH$^{\bullet}$ concentration. The sensitivity increased as the DPPH$^{\bullet}$ concentration increased through the range of 0.025-0.150 mM, because, the higher concentration of DPPH$^{\bullet}$ provided more DPPH$^{\bullet}$ to react with BHT. Nevertheless, at concentrations higher than 0.150 mM, a problem occurs with absorbance readings being too high and beyond the linear response range of the detector. The concentration of DPPH radical solution 0.100 mM and 0.150 mM also produced noisy signals and gave lower R^2 values (0.9979 and 0.9942) and higher standard deviations (0.0032 and 0.0059) due to the high absorbance readings. Figure 4.9 shows a repeatability comparison of sensitivity of the 0.05 and 0.075 mM DPPH$^{\bullet}$ solution. This testing was done over several days. From the repeatability tests, the concentration of 0.075 mM was confirmed for use in the FIA method. The reasons for this selection are; (i) sensitivity obtained by using 0.075 mM DPPH$^{\bullet}$ was better than using 0.05 mM

DPPH$^{\bullet}$, (ii) using 0.075 mM DPPH$^{\bullet}$ (%RSD = 1.87%) provided lower %RSD than using 0.05 mM DPPH$^{\bullet}$ (%RSD = 3.30%), and (iii) the baseline absorbance of 0.075 mM DPPH$^{\bullet}$ was below 1.0. The optimum concentration of DPPH$^{\bullet}$ was determined to be 0.075 mM. At this concentration the R^2 value was very good and the SD was the lowest. The concentration of DPPH$^{\bullet}$ 0.075 mM is well within the optimum detector linear measurement. In this work, the DPPH$^{\bullet}$ concentration of 0.075 mM was used to study the other effects.

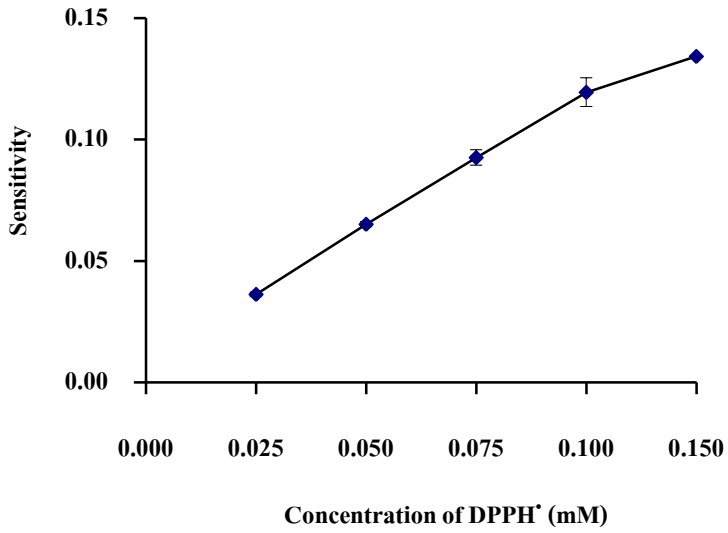


Figure 4.8 Effect of DPPH$^{\bullet}$ concentration on the sensitivities of the BHT standard curves (Conditions: sample volume 50 μL, flow rate 1.0 mL/min, reaction coil length 200 nm, wavelength 520 nm)

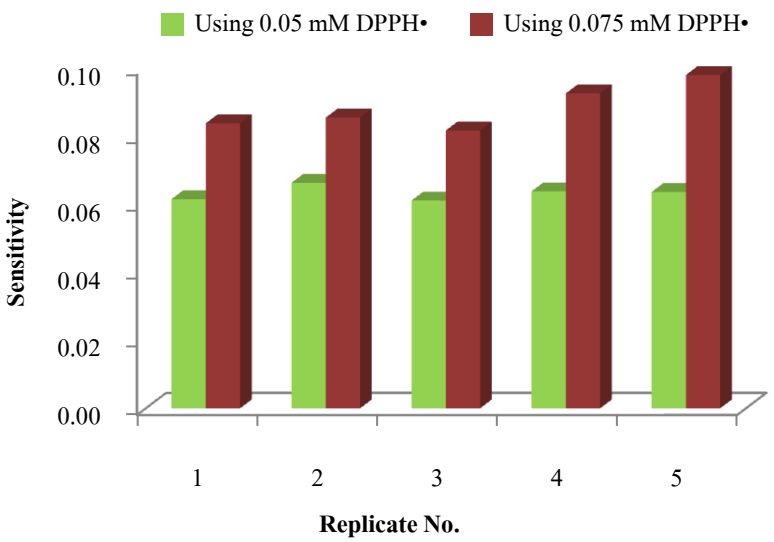


Figure 4.9 Comparison sensitivity of concentration of DPPH$^{\cdot}$ between 0.05 mM and 0.075 mM (Conditions: sample volume 50 µL, flow rate 1.0 mL/min, reaction coil length 200 nm, wavelength 520 nm)

4.3.2 Effect of DPPH$^{\cdot}$ flow rate

As expected, the sensitivity decreased when the DPPH$^{\cdot}$ flow rate was increased (Figure 4.10) because the reaction time between the DPPH$^{\cdot}$ and the antioxidant was reduced. BHT is a slow reacting antioxidant and a reduction in reaction time will reduce sensitivity. At very low flow rates, the correlation coefficient was lower and highly variable. This effect may be due to a change in dispersion and mixing and also some degassing was occurring. Both the ethanol and samples contain dissolved gas from the air. Changes in temperature or pressure can allow these dissolved gases to form bubbles. The pulsing of the pump causes pressure changes in the reaction coil and at low flow rates; there is more time for small gas bubbles to form. An increase in temperature will also have the same effect. The flow rate of 1.0 mL/min was chosen because the sensitivity was best and the correlation coefficient was acceptable.

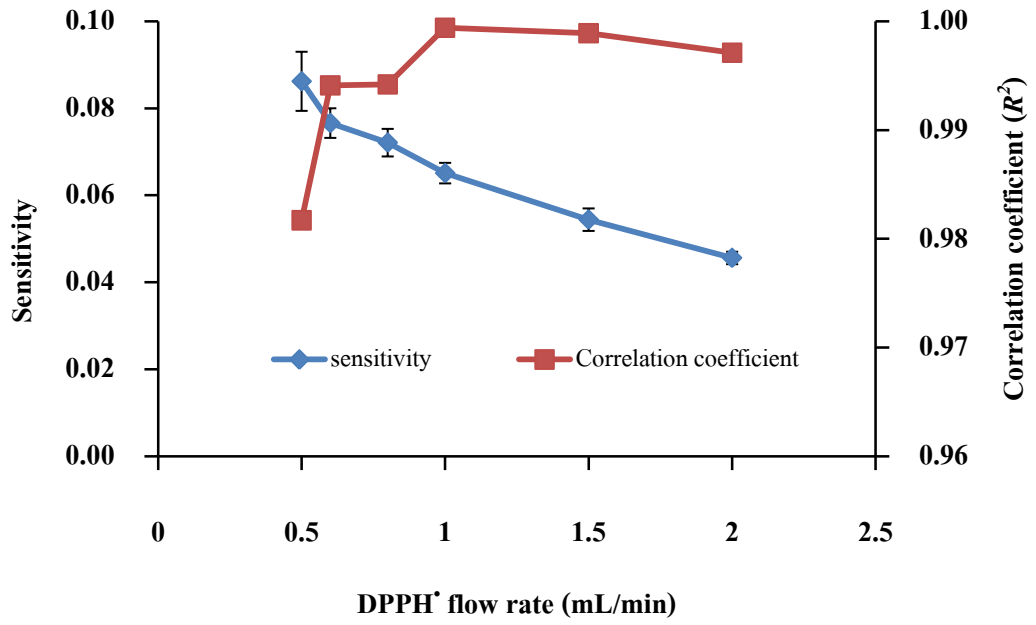


Figure 4.10 Effect of DPPH$^{\bullet}$ flow rate on the correlation coefficients and sensitivities of BHT standard curves (Conditions: concentration of DPPH$^{\bullet}$ 0.075 mM, sample volume 50 µL, reaction coil length 200 nm, wavelength 520 nm)

4.3.3 Effect of reaction coil length

Figure 4.11 shows the effect of reaction coil length on the sensitivities of BHT standards. The results showed that the sensitivity increased when the reaction coil length was increased because the rate of the reaction between BHT and DPPH$^{\bullet}$ is so slow that time required for the reaction to complete is very long. The sensitivity of the standard curves obtained by using 100 cm coil was lower than those using a longer coil. The 100 cm reaction coil produced noisy signal probably due to incomplete mixing and dispersion of sample into the DPPH$^{\bullet}$. As well, there may have been insufficient reaction time which will increase variability. The sensitivities of standard curves obtained by using 300 and 500 cm coil were higher than those using a shorter coil. However, both the 300 and 500 cm reduces the through put of samples and significantly increases analysis time, and reagent consumption. Although the coil length of 200 cm did not improve the sensitivity, it was selected because it provided shorter analysis time, good sensitivity (y = 0.0927x - 0.0544) and the best correlation coefficient (R^2 = 0.9994).

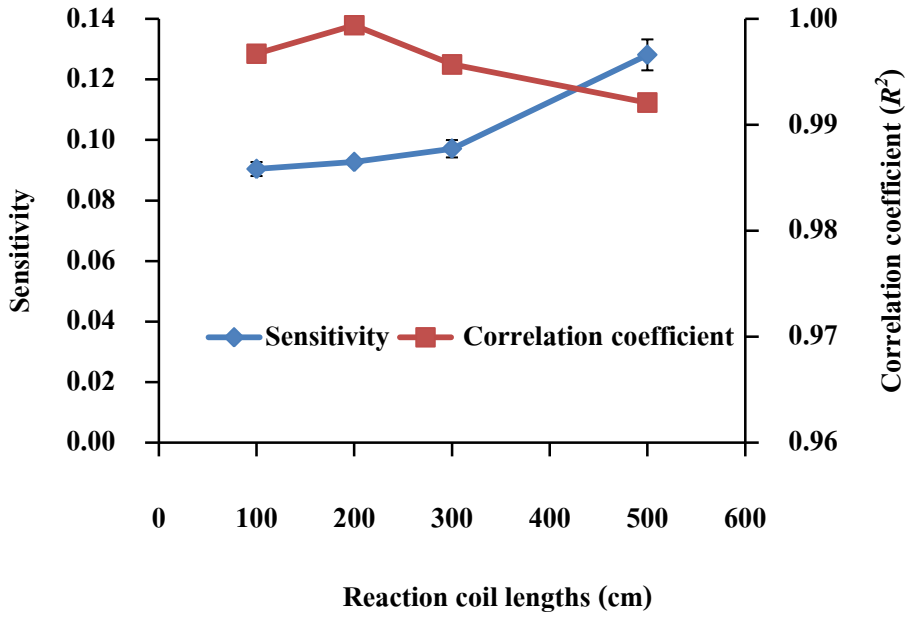


Figure 4.11 Effect of the reaction coil length on the sensitivities of BHT standard curves (Conditions: concentration of DPPH$^{\bullet}$ 0.075 mM, sample volume 50 µL, flow rate 1.0 mL/min, wavelength 520 nm)

The optimized conditions for the proposed FI-spectrophotometric system for the determination of antioxidation capacity using BHT as the equivalent standards were: DPPH$^{\bullet}$ concentration 0.075 mM, DPPH$^{\bullet}$ flow rate 1.0 mL/min, reaction coil length 200 cm and sample volume 50 µL.

4.4 Optimization of the FI-spectrophotometric method using ascorbic acid as standard

4.4.1 Effect of DPPH$^{\bullet}$ concentration

Figure 4.12 illustrates the effect of the DPPH$^{\bullet}$ concentration on the sensitivity of the calibration graph. Three replications were performed for each DPPH$^{\bullet}$ concentration. The sensitivity increased as the DPPH$^{\bullet}$ concentration increased through the range of 0.025-0.075 mM, because, the higher concentration of DPPH$^{\bullet}$ provided, the more DPPH$^{\bullet}$ to react with ascorbic acid. Nevertheless, at concentrations higher than 0.075 mM, the sensitivity decreased dramatically. This is

because the higher DPPH$^{\cdot}$ concentration gave too high absorbance that resulted in an increase in signal noise and reduced instrument sensitivity. Consequently, the noise signal interfered the absorbance measurement. In this work, the DPPH$^{\cdot}$ concentration of 0.075 mM gave a very good R^2 value (0.9995) and the lowest SD (0.0179) and was selected to study the other effects.

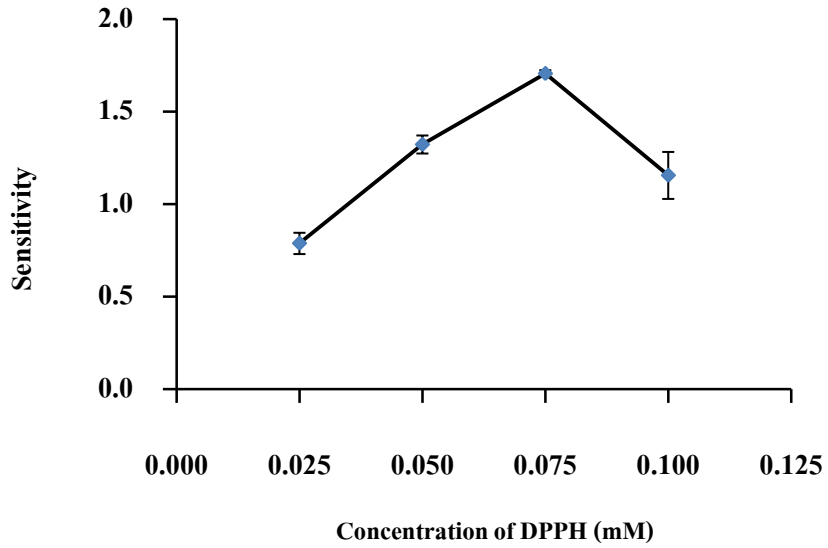


Figure 4.12 Effect of DPPH$^{\cdot}$ concentration on the sensitivities of the ascorbic acid standard curves (Conditions: sample volume 50 μL, flow rate 1.0 mL/min, reaction coil length 200 nm, wavelength 520 nm)

4.4.2 Effect of DPPH$^{\cdot}$ flow rate

Similar to BHT, the sensitivity decreased when the DPPH$^{\cdot}$ flow rate was increased (Figure 4.13) because the reaction time between the DPPH$^{\cdot}$ and the antioxidant was reduced. At high flow rates there may be insufficient time to allow for proper dispersion and mixing of standard in the DPPH$^{\cdot}$. The flow rate of 1.0 mL/min was chosen because the correlation coefficient was the highest and the sensitivity was acceptable.

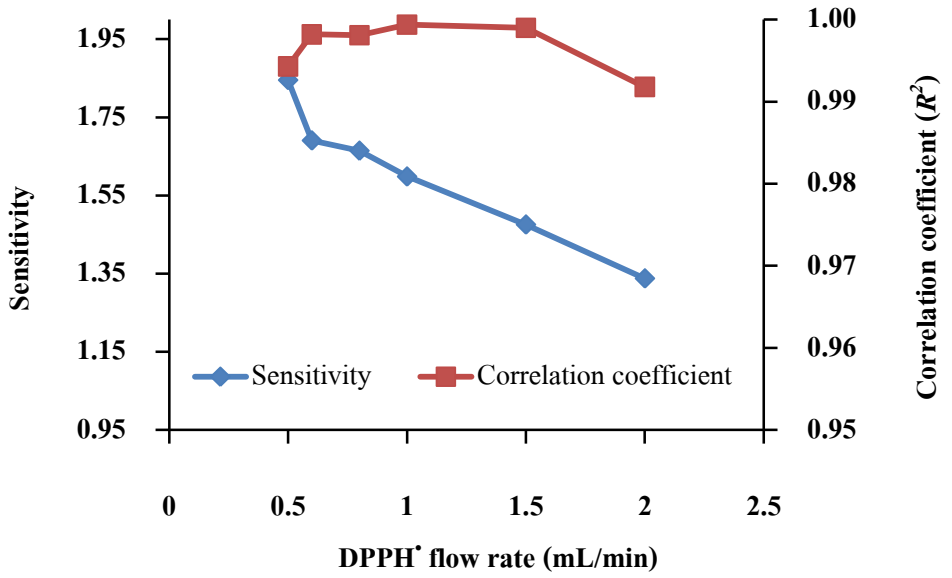


Figure 4.13 Effect of DPPH• flow rate on the correlation coefficients and sensitivities of ascorbic acid standard curves (Conditions: concentration of DPPH• 0.075 mM, sample volume 50 µL, reaction coil length 200 nm, wavelength 520 nm)

4.4.3 Effect of reaction coil length

It was found that the sensitivity decreased when the reaction coil length was increased (Figure 4.14) because the rate of the reaction between ascorbic acid and DPPH• is so fast that time required for the reaction to complete is short. The sensitivities of the standard curves obtained by using 50 and 100 cm coil were higher than those using a longer coil. However, both the 50 and 100 cm reaction coils produced noisy signal probably due to incomplete mixing and dispersion of sample into the DPPH•. As well, there may have been insufficient reaction time which will increase variability. The length of the reaction coil affects reaction time, mixing time, and sample through put. If the reaction coil is too long, increased dispersion results in further dilution and broadening of the peak occurs and results in lower sensitivity, but if it is too short incomplete mixing may occur. The results are similar to the study of effect of reaction coil length on BHT standard curve. Although the coil length of 200 cm did not improve the sensitivity, it was selected because it provided shorter analysis time, acceptable sensitivity (y = 1.744x-0.0189) and correlation coefficient (R^2 = 0.9993).

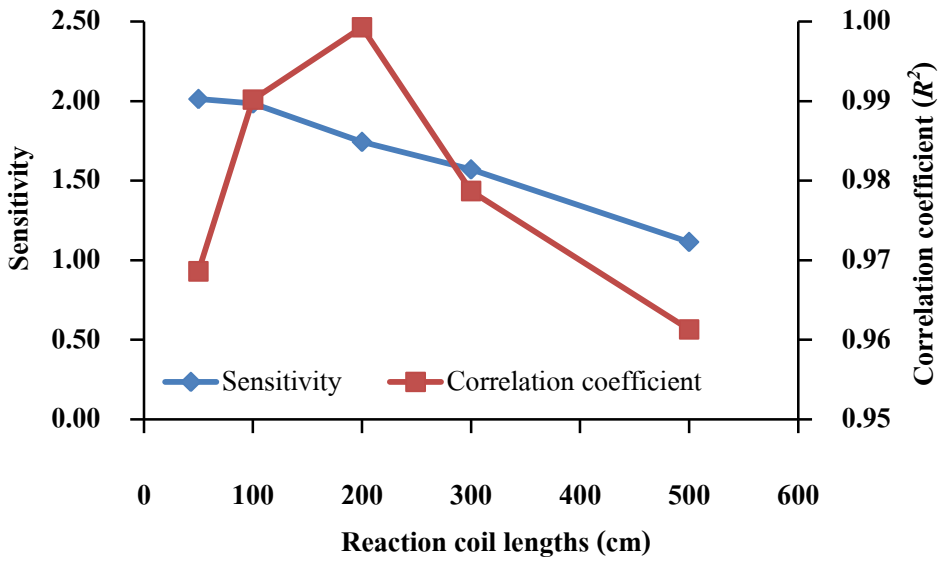


Figure 4.14 Effect of the reaction coil length on the sensitivities of ascorbic acid standard curves (Conditions: concentration of DPPH$^{\bullet}$ 0.075 mM, sample volume 50 µL, flow rate 1.0 mL/min, wavelength 520 nm)

The optimized conditions for the proposed FI-spectrophotometric system for the determination of antioxidation capacity using ascorbic acid as the equivalent standards were: DPPH$^{\bullet}$ concentration 0.075 mM, DPPH$^{\bullet}$ flow rate 1 mL/min, reaction coil length 200 cm and sample volume 50 µL.

4.5 Validation of the FI-spectrophotometric method

4.5.1 Validation using BHT standards

To further validate the optimized FI method, the BHT standards were analyzed 10 times. The average, SD, and %RSD of peak height were calculated and shown in Table 4.1. The %RSD values of less than 5 indicating good stability. Figure 4.15 shows the calibration curve of BHT using the average signal (n=10). The calibration gave a regression equation of y = 0.0859x-0.038 with R^2 of 0.9994 indicates excellent linearity. Table 4.2 compares the calibration characteristics of the FI and batch methods using BHT as standard. The standard deviation of the 10 blank readings were multiplied by 3 and 10 and then converted to mM BHT to calculate the LOD and

LOQ. The %RSD obtained by the FI method was higher than that by the manual method. This might be because of the difference in the measuring method. For the FI method, each measurement was performed by injecting the standard/sample solution into the reagent stream and resulting in the product zone for measurement. The mixing in the tubing was expected to be reproducible but it could produce uncertainty indeed. In addition, the noise signal in the FI system could affect the standard/sample signal. On the other hand, for the batch method, a standard/sample was mixed with the reagent manually and let stand for 30 min before measurement. To perform each measurement, the solution was poured into a cuvette. Thus, there was no effect from mixing. However, the FI method gave %RSD of less than 5%, which showed good precision. The LOD (0.252 mM) and LOQ (0.409 mM) obtained by the FI method were higher than those obtained by the batch method. This is probably explained by the noisy blank signal and the uncertainty during mixing process in the FI system. The SD of blank signal was obtained by injecting blank 10 times. Whereas obtaining blank absorbance 10 times by the batch method was done by reading the absorbance of the same solution 10 times. Therefore, the 3SD and 10SD values were high for the FI method. Moreover, the BHT calibration equation used for determining antioxidant capacity might not be applicable due to the insufficient reaction time in the FI system.

Table 4.1 Study of repeatability of BHT measurement

Concentration of BHT (mM)	Peak height (n=10)		
	Average	SD	%RSD
Blank	0.0726	0.0017	2.33
0.5	0.0981	0.0035	3.57
1.0	0.1210	0.0060	4.98
2.5	0.1679	0.0059	3.54
5.0	0.2263	0.0044	1.97
7.5	0.2661	0.0039	1.45
10.0	0.3085	0.0040	1.31
15.0	0.3691	0.0060	1.63

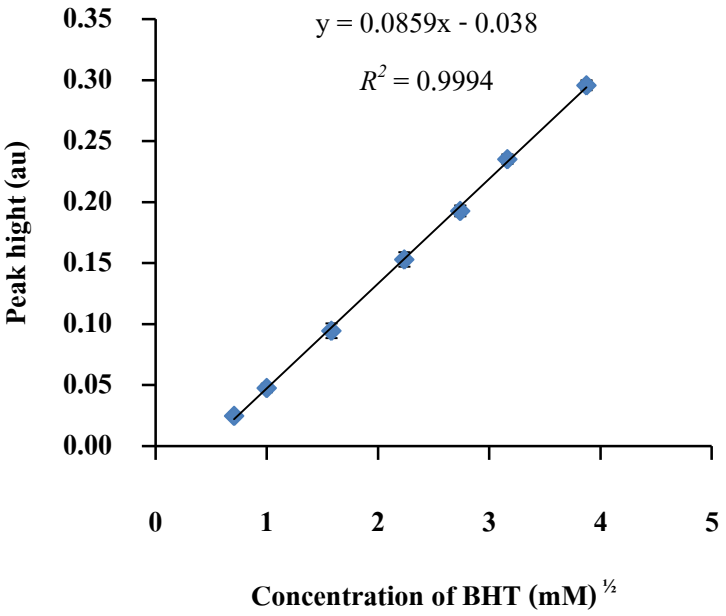


Figure 4.15 Standard curve of BHT using mean peak height (n=10) (Conditions: concentration of DPPH$^{\bullet}$ 0.075 mM, sample volume 50 µL, flow rate 1.0 mL/min, reaction coil length 200 cm, wavelength 520 nm)

Table 4.2 Characteristics of the FI and the batch methods using BHT standard

	FI method	**Batch method**
Linear range (mM)	0.5-15	0.5-7.5
Linear equation	y=0.0859x-0.038	y=0.1533x-0.0182
Correlation coefficient (R^2)	0.9994	0.9999
RSD (%)	1.31-4.98	0.03-1.39
LOD (mM BHTE)	0.252	0.022
LOQ (mM BHTE)	0.409	0.046
Time to analysis (minutes/sample)	3	30

4.5.2 Validation using ascorbic acid standards

The study was performed similarly to the BHT standard. Figure 4.16 shows the calibration curve of ascorbic acid using the average signal (n=10). The calibration gave a regression equation of y = 1.6027x - 0.0064 with R^2 value of 0.9995 indicates good linearity and was used to calculate the LOD and LOQ. The repeatability study results are shown in Table 4.3. The %RSD of less than 5 indicating good stability. Table 4.4 compares the calibration characteristics of the FI and batch methods using ascorbic acid as standard. The %RSD of the FI method is slightly higher than that of the batch method. A possible explanation for the higher RSD, similarly to using BHT as standard, is that for FIA, the standards were injected multiple times whereas for the batch method the cuvette containing the standard was just read multiple times (same sample). The injection step can add to results variability. However, the LOD and LOQ were essentially identical although the blank signal in the FI method produced high SD value. The values of LOD and LOQ could rather be influenced by the linear equation. The equation, which was obtained by measuring the product of reaction between ascorbic acid and $DPPH^{\cdot}$, was the result of the reaction. It might be implied that ascorbic acid probably reacted completely with the $DPPH^{\cdot}$ in the 3 min FI analysis time and results would be expected to be comparable to the 30 min batch time.

Table 4.3 Study of repeatability of ascorbic acid measurement

Concentration of ascorbic acid	Peak height (n=10)		
(mM)	Average	SD	%RSD
Blank	0.0783	0.0021	2.65
0.010	0.0928	0.0045	4.89
0.025	0.1148	0.0033	2.87
0.050	0.1465	0.0055	3.73
0.075	0.1907	0.0016	0.82
0.100	0.2305	0.0048	2.07
0.150	0.3115	0.0081	2.59
0.300	0.5546	0.0159	2.86

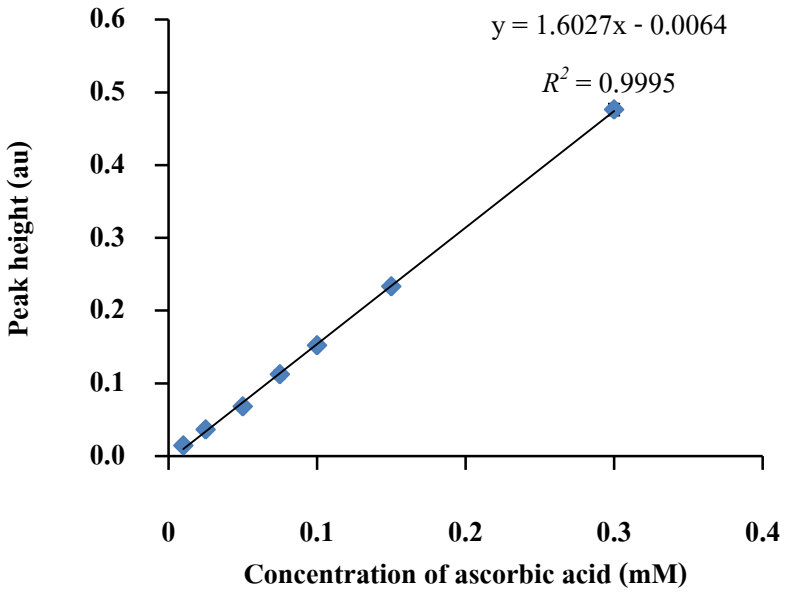


Figure 4.16 Standard curve of ascorbic acid (mM) with peak height (Conditions: concentration of DPPH$^{\bullet}$ 0.075 mM, sample volume 50 µL, flow rate 1.0 mL/min, wavelength 520 nm)

Table 4.4 Characteristics of the FI and the batch methods using ascorbic acid standard

	FI method	**Batch method**
Linear range (mM)	0.01-0.30	0.01-0.30
Linear equation	y=1.6027x-0.0064	y=0.4045x-0.0014
Correlation coefficient (R^2)	0.9995	0.9951
RSD (%)	0.82-4.89	0.01-0.41
LOD (mM AAE)	0.008	0.007
LOQ (mM AAE)	0.017	0.022
Time to analysis (minutes/sample)	3	30

4.6 Sample analysis

The optimized FI-spectrophotometric system was employed to determine the antioxidant capacity of the natural product samples. The absorbance of each sample was calculated to mM using the regression equations generated for BHT and ascorbic acid. All samples were injected and analyzed multiple times to give an SD value for the sample. The results obtained were compared to those obtained by using the standard batch method. The calibration curves were constructed daily.

4.6.1 Sample analysis using BHT calibration

The variance of the antioxidant capacity of the samples obtained by the FI method were significantly different from those obtained by the standard batch method (Table 4.5), using *F*-test (F_{calc} = 50-24,000, F_{table} = 39.00, P = 0.05). There were only 6 samples (*Oroxylum indicum*; leaf methanolic extract, leaf acetone extract, stem acetone extract, root acetone extract, leaf hexane extract and dried herb (*Prunus cerasoides* D.Don) provided the F_{calc} less than the F_{table}. Therefore, the comparison of the method using t test could not be applied. Figure 4.17 shows the correlation between the sample analysis results obtained by batch and FIA methods. It shows the poor correlation and the FI method using BHT as standard gave positive bias for total antioxidant capacity determination. This probably indicates that the samples may not contain slow reacting antioxidants, but primarily fast reacting only. As reported by (Bondet et al., 1997), the reaction of BHT and $DPPH^{\cdot}$ follows pseudo-second order and the reaction rate is dependent of $DPPH^{\cdot}$ concentration. The rate is also dependent of the initial and second-step reaction rate (Mishra, et al., 2012). Consequently, the BHT calibration might not be suitable for determining total antioxidant capacity.

Table 4.5 Comparison of the antioxidant capacity of the samples obtained by the proposed FI and the batch standard method using BHT as standard

Sample No.	Antioxidation capacity using BHT (mM BHTE ± SD*)	
	FIA	Batch
1	1.469 ± 0.125	0.095 ± 0.006
2	7.284 ± 0.005	1.105 ± 0.005
3	171.476 ± 0.100	13.705 ± 0.048
4	82.623 ± 0.365	2.748 ± 0.007
5	198.172 ± 0.054	37.679 ± 0.011
6	4.604 ± 0.069	0.204 ± 0.009
7	0.797 ± 0.051	0.011 ± 0.001
8	26.705 ± 0.229	2.896 ± 0.013
9	315.661 ± 0.025	64.833 ± 0.023
10	9.017 ± 0.190	2.995 ± 0.012
11	736.796 ± 0.245	153.545 ± 0.025
12	292.667 ± 0.032	48.797 ± 0.018
13	0.041 ± 0.004	0.025 ± 0.009
14	0.548 ± 0.039	0.051 ± 0.005
15	1.293 ± 0.131	0.084 ± 0.005
16	13.277 ± 0.310	0.335 ± 0.003
17	0.479 ± 0.048	0.046 ± 0.001
18	1.740 ± 0.093	0.086 ± 0.004
19	0.382 ± 0.034	0.037 ± 0.001
20	8.378 ± 0.061	0.268 ± 0.001
21	0.168 ± 0.023	0.028 ± 0.001
22	16.915 ± 0.005	0.478 ± 0.002
23	0.622 ± 0.012	0.050 ± 0.001
24	0.277 ± 0.065	0.035 ± 0.001
25	0.730 ± 0.089	0.051 ± 0.006
26	0.334 ± 0.041	0.036 ± 0.001
27	8.215 ± 0.432	0.259 ± 0.009

Note. * n = 3

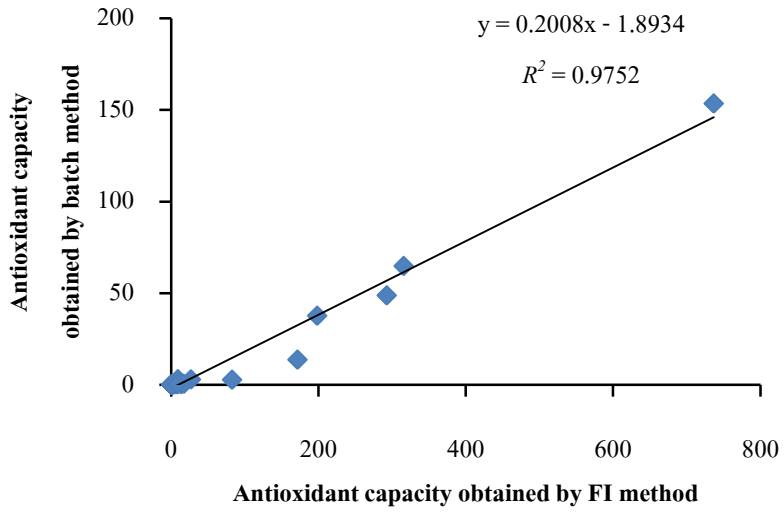


Figure 4.17 Correlation of the sample analysis results obtained by the standard batch and the FI methods using BHT as standard

4.6.2 Sample analysis using ascorbic acid calibration

It was found that the antioxidation capacity obtained by the FI-spectrophotometric method agreed with those obtained by the standard method (Table 4.6) using *t* test at 95% confidence level, paired sample *P* value = 0.0522. The regression equation y = 1.0606x + 0.1077, R^2 = 0.9933 (Figure 4.18) indicates almost a 1 to 1 relationship between the two methods. It could be assumed that the antioxidants in these samples reacted with the $DPPH^{\cdot}$ could be analyzed by the FI method using ascorbic acid as standard. The antioxidants could probably be classified as fast reacting antioxidants.

Table 4.6 Comparison of the antioxidant capacity of the samples obtained by the proposed FI and the batch standard method using ascorbic acid as standard and stand deviation

Sample No.	Antioxidation capacity using ascorbic acid (mM AAE ± SD*)	
	FIA	Batch
1	0.041 ± 0.003	0.129 ± 0.003
2	0.124 ± 0.001	0.534 ± 0.001
3	2.744 ± 0.001	2.772 ± 0.004
4	1.342 ± 0.003	1.578 ± 0.005
5	2.990 ± 0.001	3.526 ± 0.004
6	0.093 ± 0.001	0.174 ± 0.003
7	0.023 ± 0.002	0.063 ± 0.002
8	0.514 ± 0.003	0.826 ± 0.002
9	4.304 ± 0.001	4.379 ± 0.004
10	0.141 ± 0.002	0.839 ± 0.002
11	17.381 ± 0.004	18.222 ± 0.001
12	5.439 ± 0.001	7.091 ± 0.004
13	0.009 ± 0.001	0.010 ± 0.001
14	0.035 ± 0.001	0.038 ± 0.005
15	0.054 ± 0.003	0.065 ± 0.004
16	0.172 ± 0.002	0.187 ± 0.001
17	0.033 ± 0.002	0.033 ± 0.001
18	0.062 ± 0.002	0.067 ± 0.003
19	0.029 ± 0.001	0.024 ± 0.001
20	0.136 ± 0.001	0.161 ± 0.001
21	0.019 ± 0.001	0.014 ± 0.001
22	0.194 ± 0.001	0.235 ± 0.001
23	0.037 ± 0.001	0.037 ± 0.001
24	0.025 ± 0.003	0.022 ± 0.001
25	0.040 ± 0.002	0.039 ± 0.006
26	0.027 ± 0.002	0.023 ± 0.001
27	0.135 ± 0.004	0.158 ± 0.001

Note. * n = 3

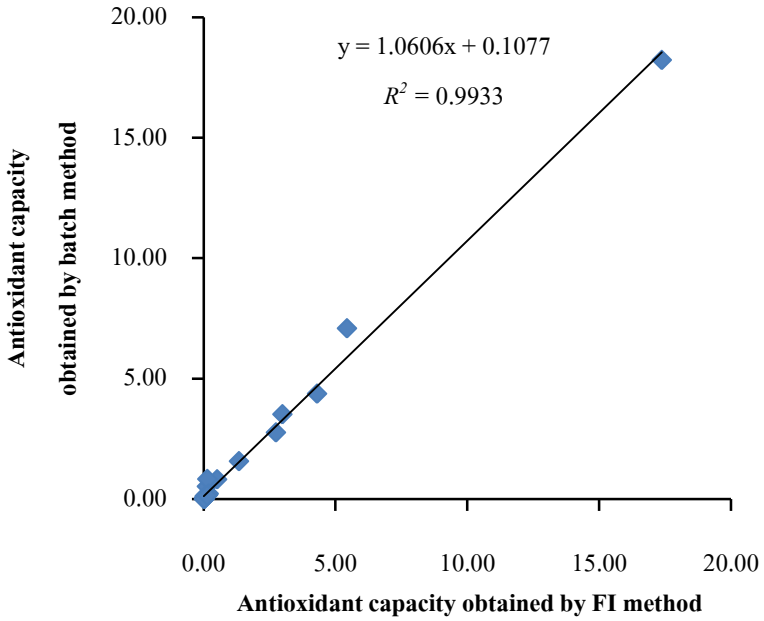


Figure 4.18 Correlation of the sample analysis results obtained by the standard batch and the FI methods using ascorbic acid as standard

CHAPTER 5

CONCLUSIONS

A flow injection spectrophotometric system for the determination of antioxidant capacity based on $DPPH^{\bullet}$ was set up. The developed system utilizes low cost instrumentation and is not complicated in its application to antioxidant analysis. Ascorbic acid and BHT were used as calibration standards.

In determining the optimum conditions for the method, variations in the parameters will result in changes to sensitivity, detection limit, and sample through put as well as correlation coefficient. For $DPPH^{\bullet}$ concentration, higher levels of $DPPH^{\bullet}$ would exceed the linear range of the spectrometer detector and result in lower sensitivity. Lower levels could result in insufficient $DPPH^{\bullet}$ being available to complete the reaction. For $DPPH^{\bullet}$ flow rate, sensitivity decreased at higher flow rates, and the correlation coefficient was decreased at both high and low flow rates. Sample analysis rate is reduced at lower flow rates. For reaction coil length, correlation coefficient was lower for both short and long coil lengths and sample analysis rate decreases with increasing length. Using ascorbic acid, sensitivity decreased with increasing coil length. For BHT, the opposite occurs with sensitivity increasing with increasing coil length due to slow-rate reaction. The optimized method parameters for both ascorbic acid and BHT are $DPPH^{\bullet}$ concentration of 0.075 mM, wavelength 520 nm, flow rate 1 ml/min, reaction coil length 200 cm, flow cell path length 1 cm, sample volume 50 μL. Using these parameters and ascorbic acid as the standard, the LOD was 0.008 mM AAE, the LOQ was 0.017 mM AAE. The linear range for ascorbic acid standards was 0.010-0.300 mM ($y = 1.6027x-0.0064$, $R^2 = 0.9995$). Using BHT as standard the LOD was 0.252 mM, the LOQ was 0.409 mM. The linear range for BHT standards was 0.500-15 mM ($y = 0.0859x-0.038$, $R^2 = 0.9994$). The optimized conditions provided the best match of sensitivity and correlation and sample analysis rate.

The optimized FI system was used to analyze some natural product extracts and samples. These same samples were also analyzed using the standard batch method. The correlation of the samples analyzed to the manual batch method using ascorbic acid as standard was excellent ($y = 1.0606x + 0.1077$, $R^2 = 0.9933$), whereas the correlation using BHT as the standard was poor ($y = 0.2008x - 1.8934$, $R^2 = 0.9752$). This indicates that the herbal samples analyzed may contain primarily intermediate to fast reacting antioxidants. For samples containing primarily fast reacting antioxidants, the ascorbic acid standard calibration, thus, was applicable. Other types of sample matrices would need to be confirmed by comparison to the standard batch method and then select appropriate standard compounds to use. As the optimized parameters are the same for both fast and slow reacting standards, these same parameters would likely be acceptable for other standards.

The manual method requires approximately 32 minutes from start of sample analysis to reporting data. Using the optimized FI system sample analysis is completed in 3 minutes. Sample analysis rates were 20 samples/hour for the FI method and 15 samples/hour for the batch method. The batch method is critically time dependent. Each sample must be timed so that the reaction time is 30 minutes. The batch method also requires accurate pipetting of both reagent and sample. Volume of reagent used is similar between the batch method and the FI method. For the FI method the reaction time is short and is set by the reaction coil length. No pipetting of reagent or sample is required, and the sample is injected through a 50 μL sample loop. This reduces technician time and labor costs and allows for routine rapid analysis. Technician time is less for the FI method and could be reduced even further by using an auto sampler. This is important in a nutraceutical production environment for quality control of batched and finished products. In a production environment, the amount of time that a mixed product batch is on hold for quality control testing is critical for plant production. A long hold time reduces the amount of final product that can be packaged.

In addition, future testing should be done using an auto sampler. This will help to confirm the potential for an increase in sample throughput to approximately 30 to 45 per hour. This would double the output of the manual method of 15 per hour. Employing other FI techniques such as stopped flow may show improvement of the BHT calibration and sample analysis. Different techniques may allow for more time for the BHT reaction to go to completion. For the determination of antioxidant capacity using $DPPH^{\bullet}$ assay and BHT as standard for measurement of capacity by IC_{50}

there were no reviews or papers available using FIA techniques. A review of available methods for determination of antioxidant capacity shows that Trolox standards are routinely used for $DPPH^{\bullet}$ assay (Pisoschi, Cheregi & Danet, 2009). Confirmation of acceptability of other standards such as Trolox could be investigated.

REFERENCES

REFERENCES

Aqil, F., Ahmad, I. & Mehmood, Z. (2006). Antioxidant and free radical scavenging properties of twelve traditionally used indian medicinal plants. **Turkish Journal of Biology, 30**, 177-183.

Arnao, M. B. (2000). Some methodological problems in the determination of antioxidant activity using chromogen radicals: a practical case. **Trends in Food Science & Technology, 11**, 419-421.

Arts, M. J. T. J., Haenen, G. R. M. M., Voss, H-P. & Bast, A. (2004). Antioxidant capacity of reaction products limits the applicability of the trolox equivalent antioxidant capacity (TEAC) assay. **Food and Chemical Toxicology, 42**, 45-49.

Awika, J. M., Rooney, L. W., Wu, X., Reior, R. L. & Cisneros-Zevallos. (2003). Sceening method to measure antioxidant activity of sorghum (Sorghum bicolor) and sorghum products. **Agricultural Food Chemistry, 51**, 6657-6662.

Blauch, D. N. (2009). **Spectrophotometry Absorbance Spectrum**. Retrieved August 29, 2011, from http://www.chm.davidson.edu/vce/spectrophotometry/AbsorbanceSpectrum.html

Böhm, V. & Schlesier, K. (2004). Methods to evaluate the antioxidant activity. **Production Practices and Quality Assessment of Food Crops, 3**, 55-71.

Bondet, V., Brand-Williams, W. & Berset, C. (1997). Kinetics and mechanisms of antioxidant activity using the DPPH$^{\cdot}$ free radical method. **Lebensm Wiss u Technol - Food Science and Technology, 30**, 609-615.

Bompadre, S., Leone, L., Polity, A. & Battino, M. (2004). Improved FIA-ABTS method for antioxidant capacity determination in different biological samples. **Free Radical Research, 38**(8), 831-838.

Brand-Williams, W., Cuvelier, M. E. & Berset, C. (1995). Use of a free radical method to evaluate antioxidant activity. **Lebensm Wiss u Technol - Food Science and Technology, 28**, 25-30.

Buratti, S., Benedetti, S. & Cosio, M. S. (2007). Evaluation of the antioxidant power of honey, propolis and royal jelly by amperometric flow injection analysis. **Talanta, 71**, 1387-1392.

Buratti, S., Pellegrini, N., Brenna, O. V. & Mannino, S. (2001). Rapid electrochemical method for the evaluation of the antioxidant power of some lipophilic food extracts. **Journal of Agricultural Food Chemistry, 49**, 5136-5141.

Chen, J., Gorton, L. & Åkesson, B. (2002). Electrochemical studies on antioxidants in bovine milk. **Analytica Chimica Acta, 474**, 137-146.

Cosio, M. S., Buratti, S., Mannino, S. & Benedetti, S. (2006). Use of an electrochemical method to evaluate the antioxidant activity of herb extracts from the Labiatae family. **Food Chemistry, 97**, 725-731.

Costin, J. W., Barnett, N. W., Lewis, S. W. & McGillivery, D. J. (2003). Monitiring the total phenolic/antioxidant levels in wine using flow injection analysis with acidic potassium permanganate chemiluminescence detection. **Analytical Chimica Acta, 499**, 47-56.

Currie, L. A. (1995). Nomenclature in evaluation of analytical methods including detection and quantification capabilities. **International Union of Pure and Applied Chemistry, 67**(10), 1699-1723.

Evelson, P., Travacio, M., Repetto, M., Escobar, J., Llesuy, S. & Lissi[†], E. A. (2001). Evaluation of total reactive antioxidant potential (TRAP) of tissue homogenates and their cytosols. **Archives of Biochemistry and Biophysics, 388**(2), 261-266.

Fang, Z. (1993). Introduction. In **Flow Injection Separation and Preconcentration** (pp. 1-18). New York: VCH Publishers.

Global FIA, Inc. (2008). **Sequential Injection Analysis**. Retrieved August 29, 2011, from http://www.globalfia.com/index.php?option=com_content&view=article&id=54&Itemid=86

Guo, C., Yang, J., Wei, J., Li, Y., Xu, J. & Jiang, Y. (2003). Antioxidant activities of peel, pulp and seed fractions of common fruits as determined by FRAP assay. **Nutrition Research, 23**, 1719-1726.

Hristea, E. N., Hillebrand, M., Caproiu, M. T., Caldararu, H., Constantinescu, T. & Balaban, A. T. (2002). Scavenging the hydroxyl radical by 2, 2-diphenyl-1-picrylhydrazyl. **ARKIVOC, 2**, 123-132.

Huang, D., Ou, B. & Prior, R. L. (2005). The chemistry behind antioxidant capacity assay. **Journal of Agricultural and Food Chemistry, 53**(6), 1841-1856.

Iveković, D., Milardović, S., Roboz, M. & Grabarić, B. S. (2005). Evaluation of the antioxidant activity by flow injection analysis method with electrochemically generated ABTS radical cation. **Analyst, 130**, 708-714.

Kaur, C. & Kapoor, H. C. (2001). Antioxidant in fruits and vegetables-the millennium's health. **International Journal of Food Science and Technology, 36**, 703-725.

Kosar, M., Dorman, H. J. D., Bachmayer, O., Baser, K. H. C. & Hiltunen, R. (2003). An improved on-line HPLC-DPPH$^{\bullet}$ method for the screening of free radical scavenging compounds in water extracts of lamiaceae plants. **Chemistry of Natural Compounds, 39** (2), 161-166.

Kumaraswamy, M. V. & Satish, S. (2008). Antioxidant and anti-lipoxygenase activity of Thespesia lampas Dalz & Gibs. **Advances in Biological Research, 2**(3-4), 56-59.

Labrinea, E. P. & Georgiou, C. A. (2005). Rapid, fully automated flow injection antioxidant capacity assay. **Agricultural Food Chemistry, 53**, 4341-4346.

Lee, J., Koo, N. & Min, D. B. (2004). Reactive oxygen species, aging, and antioxidative nutraceuticals. **Comprehensive Reviews of Food Science and Food Safety, 3**, 21-33.

Leong, L. P. & Shui, G. (2002). An investigation of antioxidant capacity of fruits in Singapore Markets. **Food Chemistry, 76**, 69-75.

Lima, M. J. R., Tóth, I. V. & Rangel, A. O. S. S. (2005). A new approach for the sequential injection spectrophotometric determination of the total antioxidant activity. **Talanta, 68**, 207-213.

Magalhães, L. M., Santos, M., Segundo, M. A., Reis, S. & Lima, J. L. F. C. (2009). Flow injection based methods for fast screening of antioxidant capacity. **Talanta, 77**, 1559-1566.

Magalhães, L. M., Segundo, M. A., Reis, S. & Lima, J. L. F. C. (2006). Automatic method for determination of total antioxidant capacity using 2,2-diphenyl-1-picrylhydrazyl assay. **Analytica Chimica Acta, 558**, 310-318.

Magalhães, L. M., Segundo, M. A., Reis, S., Lima, J. L. F. C., Toth, I. V. & Rangel, A. O. S. S (2007). Automatic flow system for sequential determination of $ABTS^{*+}$ scavenging capacity and Folin-Ciocalteu index: a comparative study in food products. **Analytica Chimica Acta**, 592, 193-210.

Magalhães, L. M., Segundo, M. A., Siquet, C., Reis, S. & Lima, J. L. F. C. (2007). Multi-syringe flow injection system for the determination of the scavenging capacity of the diphenylpicrylhydrazyl radical in methanol and ethanolic media. **Microchimica Acta, 157**, 113-118.

Mahabusarakam, W., Deachathai, S., Phongpaichit, S., Jansakul, C. & Taylor,W. C. (2004). A benzyl and isoflavone derivatives from derris scandens benth. **Phytochemistry, 65**, 1185-1191.

Mannino, S., Brenna, O., Buratti, S. & Cosio, M. S. (1998). A new method for the evaluation of the "antioxidant power" of wines. **Electroanalysis, 10**, 908-912.

Mannino, S., Buratti, S., Cosio, M. S. & Pellegrini, N. (1999). Evaluation of the "antioxidant power" of olive oils based on a FIA system with amperometric detection. **Analyst**, 124, 1115-1118.

Milardovic, S., Kereković, I., Derrico, R. & Rumenjak, V. (2007). A novel method for flow injection analysis of total antioxidant capacity using enzymatically produced $ABTS^{\bullet+}$ and biamperometric detector containing interdigitated electrode. **Talanta, 71**, 213-220.

Milardovic, S., Kereković, I. & Rumenjak, V. (2007). A flow injection biamperometric method for determination of total antioxidant capacity of alcoholic beverages using bienzymatically produced $ABTS^{\bullet+}$. **Food Chemistry, 105**, 1688-1694.

Milardovic´, S., Ivekovic, D., & Grabaric´, B. S. (2006). A novel amperometric method for antioxidant activity determination using DPPH free radical. **Bioelectrochemistry, 68**, 175-180.

Miller, H. E., Rigelhof, F., Marguart, L., Prakash, A. & Kanter, M. (2000). Antioxidant content of whole grain breakfast cereals, fruits and vegetables. **Journal of the American College of Nutrition, 19**(3), 3125-3195.

Minioti, K. S. & Georgiou, C. A. (2008). High throughput flow injection bioluminometric method for olive oil antioxidant capacity. **Food Chemistry, 109**, 455-461.

Mishra, K., Ojha, H. & Chaudhury, N. K. (2012). Estimation of antiradical properties of antioxidants using $DPPH^{\bullet}$ assay: A critical review and results. **Food chemistry, 130**, 1036-1043.

Molyneux, P. (2004). The use of the stable free radical diphenylpicrylhydrazyl (DPPH) for estimating antioxidant activity. **Journal of the Science of Technology, 26**(2), 211-219.

Moure, A., Cruz, J. M., Franco, D., Domínguez, J. M., Sineiro, J., Domínguez, H., Núñez, M. J. & Parajó, J. C. (2001). Natural antioxidants from residual sources. **Food Chemistry**, 72, 145-171.

Neves, M. S. A. C. (2006). **Flow systems for the trace determination of phosphate in waters based on the spectrophotometric vanadomolybdate method**. Master's Thesis. Environmental Diagnostics, Cranfield University: United Kingdom.

Niederländer, H. A. G., Van Beek, T. A., Bartasiute, A. & Koleva, I. I. (2008). Antioxidant activity assays on-line with liquid chromatography. **Journal of Chromatography A, 1210**, 121-134.

Oakes, K. D. & Kraak, G. J. V. D. (2003). Utility of the TBARS assay in detecting oxidative stress in white sucker (Catostomus commersoni) populations exposed to pulp mill effluent. **Aquatic Toxicology, 63**, 447-463.

Pala, F. S. & Tabakçoğlu, K. (2007). Free radicals: Our enemies or friends?. **Advances in Molecular Biology, 1**, 63-69.

Pellegrini, N., Del Rio, D., Colombi, B., Bianchi, M. & Brighenti, F. (2003). Application of the 2, 2'- azinobis (3-ethylbenzothiazoline-6-sulfonic acid) radical cation assay to a flow injection system for the evaluation of antioxidant activity of some pure compounds and beverages. **Journal of Agricultural Food Chemistry, 51**, 260-264.

Phipps S. M., Sharaf M. H. M. & Butterweck V. (2007). Assessing antioxidant activity in botanicals and other dietary supplements, **Pharmacopeial Forum, 33**, 810-814.

Pinto, P. C. A. G., Saraiva, M. L. M. F. S., Reis, S. & Lima, J. L. F. C. (2005). Automatic sequential determination of the hydrogen peroxide scavenging activity and evaluation of the antioxidant potential by the 2,2-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) radical cation assay in wines by sequential injection analysis. **Analytica Chimica Acta, 531**, 25-32.

Pisoschi, A. M., Chergi, M. C. & Danet, A. F. (2009). Total antioxidant capacity of some commercial fruit juices: electrochemical and spectrophotometrical approaches. **Molecules, 14**, 480-493.

Polášek, M., Skála. P., Opletal, L. & Jahodář, L. (2004). Rapid automated assay of anti-oxidation/radical-scavenging activity of natural substances by sequential injection technique (SIA) using spectrophotometric detection. **Analytical and Bioanalytical Chemistry, 379**, 754-758.

Prakash, A. (2001). Antioxidant activity. **Medallion Laboratories Analytical progress, 19**, 1-6.

Prior, R. L., Wu, X. & Schaich, K. (2005). Standardized methods for the determination of antioxidant capacity and phenolics in foods and dietary supplements. **Journal of Agricultural and Food Chemistry, 53**, 4290-4302.

Re, R., Pellegrini, N., Proteggente, A., Pannala, A., Yang, M. & Rice-Evans, C. (1999). Antioxidant activity applying an improved ABTS radical cation decolorization assay. **Free Radical Biology & Medicine, 26**, 1231-1237.

Rocha, F. R. P. & Nóbrega, J. A. (1997). Overcoming the schlieren effect in flow injection spectrophotometry by introduction of large sample volumes. Determination of chloride in the electrolyte of lead-acid batteries. **Journal of the Brazilian Chemical Society, 8**(6), 625-629.

Rocha, F. R. P., Nóbrega, J. A. & Filho, O. F. (2001). Flow analysis strategies to greener analytical chemistry. **Green Chem, 3**, 216-220.

Ruzicka, J. & Hansen, E. H. (1975). Flow injection analyses part I; a new concept of fast continous flow analysis. **Analytica Chimica Acta, 78**, 145-157.

Ruzicka, J. & Marshall, G. D. (1990). Sequential injection: a new concept for chemical sensors, process analysis and laboratory assays. **Analytica Chimica Acta, 237**(2), 329-343.

Sánchez-Moreno, C. (2002). Method used to evaluate the free radical scavenging activity in foods and biological systems. **Food Science and Technology International, 8**(3), 121-137.

Scartezzini, P. & Speroni, E. (2000). Review on some plants of Indian traditional medicine with antioxidant activity. **Journal Ethnopharmacol, 71**, 23-43.

Shpigun, L. K., Arharova. M. A., Brainina, K. Z. & Ivanova, A. V. (2006). Flow injection potentiometric determination of total antioxidant activity of plant extracts. **Analytica Chimica Acta, 573**, 419-426.

Suteerapataranon, S. & Pudta, D. (2008). Development of flow injection analysis-spectrophotometry for rapid determination of total polyphenols in tea extracts. **Journal of Flow Injection Analysis, 25**(1), 61-64.

Tang, B., Zhang, L. & Geng, Y. (2005). Determination of the antioxidant capacity of different food natural products with a new developed flow injection spectrofluorimetry detecting hydroxyl radicals. **Talanta, 65**, 769-775.

Thaipong, K., Boonprakob, U., Crosbyb, K., Cisneros-Zevallosc, L. & Byrnec, D. H. (2006). Comparison of ABTS, DPPH, FRAP, and ORAC assays for estimating antioxidant activity from guava fruit extracts. **Journal of Food Composition and Analysis, 19**, 669-675.

Ukeda, H., Adachi, Y. & Sawamura, M. (2002). Flow injection analysis of DPPH radical based on electron spin resonance. **Talanta, 58**, 1279-1283.

Valko, M, Leibfritz, D., Moncol, J., Cronin, M. T. D., Mazur, M. & Telser, J. (2007). Free radicals and antioxidants in normal physiological functions and human disease. **The International Journal of Biochemistry & Cell Biology, 39**, 44-84.

Zagatto, E. A. G., Arruda, M. A. Z., Jacintho, A. O. & Mattos, I. L. (1990). Compensation of the Schlieren effect in flow injection analysis by using dual wavelength spectrophotometry. **Analytica Chimica Acta, 234**, 153-160.

Zagatto, E. A. G., VAN Staden, J. F. Maniasso, N., Stefan, R. I. & Marshall, G. D. (2002). Information essential for characterizing a flow-based analytical system. **Pure and Applied Chemistry, 74**(4), 585-592.

CURRICULUM VITAE

CURRICULUM VITAE

NAME	Mrs. Nookrai Mrazek
DATE OF BIRTH	15 October 1983
ADDRESS	300/81 Koolpuntvill Soi 9, Moo 9, Robwieng, Muang, Chiang Rai, 57000

EDUCATIONAL BACKGROUND

2006	Bachelor of Science Major in Chemistry Valaya Alongkorn Rajabhat University under the Royal Patronage

WORK EXPERIENCE

2006-2008	Laboratory Analyst IQA Laboratory, Co., Ltd., Bangkok
2004-2005	Laboratory Technician, University work experience, Thaidono Gengen Chemical Co.,Ltd., Rayong
2003	Quality Control Technician, Thai Nisca Co., Ltd., Phra Nakhon Si Ayutthaya
2002	Quality Control Technician, Mitsui High-Tec (Thailand) Co., Ltd., Phra Nakhon Si Ayutthaya
2001	Production, U.Yong Industry Co., Ltd., Samut Songkhram

PUBLICATION

Nookrai Mrazek, Kanchana Watla-iad, Suwanna Deachathai & Siripat Suteerapataranon. Rapid antioxidant capacity screening in herbal extracts using a simple flow injection-spectrophotometric system. **Food Chemistry**, Accepted 21 October 2011.

PRESENTATIONS

Oral presentation

Nookrai Mrazek, Kanchana Watla-iad, Suwanna Deachathai & Siripat Suteerapataranon. (2009, October 15-17). Development of flow injection analysis system for the determination of antioxidant capacity based on 2,2-diphenyl-1-picrylhydrazyl assay. In **The 35th Congress on science on Science and Technology of Thailand** (p. 118). The Tide Resort (Bangsaen Beach), Chonburi, Thailand.

Poster presentations

Nookrai Mrazek, Kanchana Watla-iad, Suwanna Deachathai & Siripat Suteerapataranon. (2010, January 21-23). A simple flow injection-spectrophotometric system for the determination of antioxidant capacity based on DPPH assay. In **The Pure and Applied Chemistry International Conference** (p. 158). Sunee Grand Hotel and Convention Center, Ubon Ratchathani, Thailand.

Nookrai Mrazek, Kanchana Watla-iad, Suwanna Deachathai & Siripat Suteerapataranon. (2009, April 23-24). Development of flow injection analysis system for the determination of antioxidant activity in some natural product extracts. In **Research for better life quality: Symposium on flow based analysis** (p. 5). Chiang Mai University, Chiang Mai, Thailand.

Nookrai Mrazek, Kanchana Watla-iad, Suwanna Deachathai & Siripat Suteerapataranon. (2009, January 14-16). Development of flow injection analysis system for the determination of antioxidant activity in some natural product extracts. In **The Pure and Applied Chemistry International Conference** (p. 132). Naresuan University, Phitsanulok, Thailand.

Nookrai Mrazek, Kanchana Watla-iad & Siripat Suteerapataranon. (2008, October 31-November 2). Optimization of phosphate analysis in meat products injection analysis using vanadomolydate method. In **The 34th Congress on science on Science and Technology of Thailand** (p. 137). Queen Sirikit National Convention Center, Bangkok, Thailand.