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นายไชยนรินทร์ พงษ์ประยูร

DEVELOPMENT OF AMPEROMETRIC TECHNIQUE TO DETECT

ANTIOXIDANT CAPACITY IN SERUM

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ไชยนรินทร์ พงษ์ประยูร: การพัฒนาเทคนิคแอมแปโรเมตรีเพื่อตรวจวัดประสิทธิภาพการ
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วิธีแอมแปโรเมตรีที่มีขั้วไฟฟ้าเป็นคาร์บอนพิมพ์สกรีนและค่าศักย์คงที่ 0.15 โวลต์ ถูก
พัฒนาขึ้นเพื่อใช้ตรวจวัดประสิทธิภาพการต้านอนุมูลอิสระในซีรัมได้อย่างง่ายและรวดเร็ว โดยมี
สาร 2,2'-azobis(3-ethylbenzothiazoline)-6-sulfonic acid (ABTS) เป็นตัวสร้างอนุมูลอิสระ
ABTS$^{\cdot+}$ (ABTS cation radical) ถูกสร้างขึ้นจากปฏิกิริยาระหว่างสาร ABTS กับสารโพแทสเซียม
เปอร์ซัลเฟต (K$_2$S$_2$O$_8$) ในสัดส่วนความเข้มข้นโดยโมล 1: 0.25 อนุมูลที่สร้างมีความคงตัวอยู่ได้อย่าง
น้อย 3 วัน เมื่อเก็บในที่มืดที่อุณหภูมิ 4 องศาเซลเซียส

วิธีที่พัฒนาขึ้นถูกใช้เพื่อตรวจวัดประสิทธิภาพการต้านอนุมูลอิสระในซีรัมของคน
ปฏิกิริยาที่เกิดขึ้นระหว่าง ABTS$^{\cdot+}$ กับสารต้านอนุมูลอิสระในตัวอย่างถูกวัดโดยมีสาร Trolox เป็น
สารต้านอนุมูลมาตรฐาน หลังเกิดปฏิกิริยาระหว่าง ABTS$^{\cdot+}$ กับสารต้านอนุมูลอิสระ ABTS$^{\cdot+}$ ที่
เหลือถูกวัดเป็นเวลา 100 วินาที ความเข้มข้นสาร Trolox กับค่าสัญญาณกระแสมีความสัมพันธ์
เชิงเส้นตรงในช่วงความเข้มข้น 0-100 ไมโครโมลาร์ อิเล็กโทรดคาร์บอนพิมพ์สกรีนนี้สามารถใช้วัด
ได้อย่างน้อย 30 ครั้ง โดยมีค่า %RSD   อยู่ที่ 4.9   เปอร์เซนต์ วิธีที่พัฒนาขึ้นสามารถตรวจวัด
ประสิทธิภาพการสารต้านอนุมูลอิสระในซีรัมได้ทั้งผู้ที่มีสุขภาพดีและผู้ป่วย โดยค่าเฉลี่ยสารต้าน
อนุมูลอิสระของผู้ที่มีสุขภาพดีและผู้ป่วยมีค่าเทียบเท่ากับ Trolox 1486 ± 66   และ   1354 ± 85
ไมโครโมลาร์ ตามลำดับ   นอกจากนี้ วิธีนี้ยังสามารถใช้วัดสารต้านอนุมูลอิสระในซีรัมที่เติม α-
tocopherol ได้อีกด้วย

สาขาวิชา   วิศวกรรมชีวเวช   ลายมือชื่อนิสิต _____
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KEYWORDS: ANTIOXIDANT CAPACITY / ABTS$^{\bullet+}$ / POTASSIUM PERSULFATE / AMPEROMETRIC TECHNIQUE / SCREEN-PRINT CARBON PASTE ELECTRODE

CHAINARIN PHONGPRAYOON: DEVELOPMENT OF AMPEROMETRIC TECHNIQUE TO DETECT ANTIOXIDANT CAPACITY IN SERUM. ADVISOR: ASSOC.PROF. PHENSRI THONGNOPNUA, Ph.D., CO-ADVISOR: ASSOC.PROF. MANA SRIYUDTHSAK, D.Eng., 64 pp.

A simple and rapid amperometric method using screen-print carbon paste electrode (SCPE) and bias potential at 0.15V, was developed to determine the antioxidant capacity in serum. The 2,2'-azobis(3-ethylbenzothiazoline)-6-sulfonic acid (ABTS) was used as a free radical generator. ABTS$^{\bullet+}$ (ABTS cation radical) was generated from the reaction between ABTS and potassium persulfate ($K_2S_2O_8$) at a molar ratio of 1: 0.25. The radical was stable at least 3 days when stored at $4^{o}C$ in the dark.

The developed method was used to determine antioxidant capacity in human serum. The reaction of ABTS$^{\bullet+}$ with antioxidant sample was measured, having Trolox as the standard antioxidant. After reaction between ABTS$^{\bullet+}$ and antioxidant sample, the residual ABTS$^{\bullet+}$ was measured for 100 seconds. The relationship between Trolox concentration and current signal was linear at the concentration range of 0-100 μM. SCPE could be used at least thirty times with %RSD of 4.9%. This developed method was successfully used for determining antioxidant capacity of Thai normal subjects and patients. The average antioxidant capacity in sera of normal subjects and patients were determined to be 1486±66 and 1354±85 μM TE, respectively. Moreover, the method could also be used for measuring antioxidant capacity of the spiked serum with α-tocopherol.

Field of Study : Biomedical Engineering   Student's Signature _____

Academic Year : 2010 _____   Advisor's Signature _____

Co-advisor's Signature _____

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LIST OF ABBREVIATIONS

%	percent
μL	microliter
μM	micromolar
°C	degree Celsius
AAPH	2,2'-azobis(2-amidinopropane) dihydrochloride
ABTS	2,2'-azino-bis(3-ethylbenzthiazoline)-6-sulfonic acid
ABTS$^{\bullet+}$	ABTS cation radical
conc	concentration
FRAP	ferric reducing ability of plasma
g	gram
hr	hour(s)
mg	milligram
min	minute(s)
mL	milliliter
mm	millimeter
mM	millimolar
mV	millivolt
nA	nanoampere
nm	nanometer
ORAC	oxygen radical absorbance capacity
R^2	coefficient of determination
RSD	relative standard deviation
SD	standard deviation
SEM	standard error of mean
SOD	superoxide dismutase
SPCE	screen-print carbon paste electrode
TAS	total antioxidant status

TEAC	Trolox equivalent antioxidant capacity
TOSC	total oxyradical scavenging capacity
Trolox	6-hydroxy-2,5,7,8-tetramethychroman-2-carboxylic acid
UV	ultraviolet
V	volt

CHAPTER I

INTRODUCTION

The study of free radicals and antioxidants in food technology and human health has been widely investigated. Free radicals are atoms, molecules, or ions having one or more unpaired electrons in outer orbit (Aruoma, 1994). Free radical may have positive, negative or neutral charge. It is generally unstable and highly reactive due to the presence of unpaired electrons. It can withdraw electron from surrounding compound or molecule to make itself more stable (Goldfarb, 1999). Molecule which losing its electron turn to a new free radical and then returns to its ground state by withdrawing other electron. Thus the chain reaction continues. Free radicals can induce oxidative damage to biomolecules such as protein, lipid and DNA, causing cell injury and death (Fang, Yang, and Wu, 2002). Free radicals are related to several diseases; for example, coronary heart disease, rheumatoid and cancer.

Free radicals can be produced by many ways. They can be produced by external factors such as radiation and air pollution. In human body, free radicals are formed in many processes such as phagocytosis, bio synthesis and metabolism. A 2-5% of total oxygen intake during both rest and exercise is converted to superoxide radicals ($O_2^{\bullet-}$) (Sjodin, Westing, and Apple, 1990). The $O_2^{\bullet-}$ can lead to the production of other damaging radical in reactive oxygen species (ROS) such as hydroxyl radical ($^{\bullet}OH$), peroxyl radical (ROO$^{\bullet}$), hydrogen peroxide (H_2O_2), and Hypochlorite ion (OCl$^-$).

Antioxidant is a substance that can efficiently reduce free radical and prevent oxidation of biological targets by donating its electron. Many publications have been reported that antioxidant related to the protection of non-infectious diseases such as coronary heart disease (Gey and Puska, 1989; Rimm *et al*., 1993), cancer (Peto *et al*., 1981), disease of eye (John and Kenneth, 1999), neuropathology (Stanner *et al*., 2004; Nunomura *et al*., 2006) and Diabetic (Davi, Falco, and Patrono, 2005).

Antioxidant can be defined as any substance that when present at low concentrations compared to the biomolecules, significantly delays or prevents oxidation of biomolecules (Halliwell, 1996). Antioxidants are molecules that can neutralize free radicals by accepting or donating electron to eliminate the unpaired condition. Typically, this means that the antioxidant molecule becomes a free radical in the process of neutralizing a free radical molecule to a non-free-radical molecule. But the antioxidant molecule will usually be a much less reactive than the free radical. The simple reaction between free radical ($R^{\bullet}$) and antioxidant (AH) is shown.

$$R^{\bullet} \ + \ AH \ \rightarrow \ RH \ + \ A^{\bullet} \qquad\qquad (1)$$

Antioxidants are generally divided into three groups (Bartosz, 2003). One is enzymatic antioxidants such as superoxide dismutase (SOD), glutathione Peroxidase (GSH-Px) and catalase. Another group is biological metal chelators in which protein binding with transition metal such as albumin-Cu, ceruloplasmin-Cu, transferin-Fe, ferritin-Fe and myoglobin-Fe. Last group is a broad class of non-specific defense mechanism. This group includes various low-molecular-weight substances such as ascorbic acid (vitamin C), uric acid, α-tocopherols (vitamin E), glutathione, carotenoids, coenzyme Q and bilirubin. The summation of antioxidant activities of low molecular-weight antioxidant is termed as "antioxidant capacity" (Bartosz).

The evaluation of antioxidants properties can be defined in the terms of "antioxidant activity" and "antioxidant capacity". The antioxidant activity corresponds to the rate constant of a single antioxidant against a given free radical such as glutathione peroxidase activity. The antioxidant capacity is the measurement of moles of a given free radical which was scavenged by the antioxidants in the mixture (Mello and Kubota, 2007). Therefore, the sum of low-molecular-weight antioxidants in serum is measured in term of antioxidant capacity.

A variety of methods for antioxidant capacity measurement has been reviewed (Sanchez-Moreno, 2002; Magalhaes et al., 2008; Prieto-Simón et al., 2008). Traditional techniques based on TEAC (Trolox equivalent antioxidant capacity), FRAP (ferric

reducing ability of plasma) and crocin bleaching, could be interfered by color or turbidity of sample. To overcome this weakness, fluorescence-based such as ORAC (oxygen radical absorbance capacity) and chemiluminescence-based have been developed. However, these instrumentations are expensive and required an expert to operate.

In recent years, other innovating technologies such as electrochemical sensor have been developed. These techniques are suitable for less time analyses, based on inexpensive instrumentation and simple operation protocols (Prieto-Simón *et al.,* 2008). Biosensors are sub-group of electrochemical sensors and they have been widely developed to determine antioxidant capacity both in food and biology fields (Mello and Kubota, 2007). Most of biosensors use immobilized enzymes in combination with electrochemical transducer and amperometric detection. These immobilized enzymes are responsible for the direct electron transfer.

Either free radical or free radical generator is required in all methodologies. Antioxidant is measured by means of reaction between antioxidant and free radical, followed by the detection of free radical. Most of amperometric techniques produce $O_2^{\bullet-}$ as free radical in the system. This radical is quite reactive and cannot be oxidized directly on electrode. So, modified electrodes such as superoxide dismutase-based sensor and cytochrome *c*-base sensor have been developed to measure superoxide radical. Moreover, this radical is generated from xanthine / xanthine oxidase reaction in which the xanthine oxidase is very expensive compound.

Objective of This Study

The purpose of this study was to develop amperometric technique, having SCPE as electrode, for antioxidant capacity determination in human sera. The free radical generator which could generate stable free radical was sought. The developed method was evaluated and utilized for determining antioxidant capacity in serum of normal subjects, patients and spiked serum with α-tocopherol.

The Significance of This Study

1. The new, simple and versatile amperometric technique was developed.

2. The developed technique could be utilized for determining antioxidant capacity in human serum.

CHAPTER II

LITURATURE REVIEW

Antioxidant Capacity Determination

Antioxidant capacity is determined in term of the ability to scavenge free radicals. Free radical and its inhibition due to addition of antioxidant sample were measured by several techniques such as spectroscopic technique, gas chromatography and electrochemical biosensor.

2.1 Spectroscopic Technique

2.1.1   Trolox equivalent antioxidant capacity (TEAC) assay

The original TEAC assay was based on the activation of metmyoglobin with hydrogen peroxide in the presence of ABTS to produce $ABTS^{\bullet+}$ which has absorption maxima at 645,734 and 815 nm (Miller, 1993). The presence or absence of antioxidants contributed to the reduction of $ABTS^{\bullet+}$. This was then criticized that the faster reacting antioxidants might reduce ferryl myoglobin radical. Re and co-worker recommended to produce $ABTS^{\bullet+}$ through the reaction between ABTS and $K_2S_2O_8$ (Re *et al*, 1999). This method has been commercialized by Randox Laboratories (San Francisco, USA) as the world's first kit for total antioxidant status (TAS) measurement in serum or plasma.

2.1.2   Ferric reducing ability of plasma (FRAP) assay

FRAP assay involves the reduction of a ferric tripyridyltriazine $(Fe^{3+}$-TPTZ) complex to the ferrous tripyridyltriazine $(Fe^{2+}$-TPTZ) by added antioxidant (Benzie and Strain, 1999). Fe^{2+}-TPTZ has an intensive blue color and can be monitored at 593 nm. The drawback of this method is that Fe^{3+} is not free radical. So, some antioxidant that can effectively reduce free radicals may not be able to efficiently reduce Fe^{3+}. For example, FRAP assay does not measure GSH, an important antioxidant in vivo.

2.1.3 Crocin bleaching (Tubaro *et al.,* 1998)

This prodedure is based on the oxidation of crocin by peroxyl radical produced from 2,2'-azobis(2-amidinopropane) dihydrochloride (AAPH). Crocin has absorbance at 443 nm and was oxidized at the constant rate. Added antioxidant would change rate of oxidation of crocin. Crocin is a natural carotenoid that has been shown an antioxidant property. So, this method has been criticized to measure the ability of an antioxidant in competing against another antioxidant.

2.2 Fluorescent and Luminescent Technique

2.2.1 Oxygen radical absorbance capacity (ORAC) assay

The principle of this assay is based on the intensity of fluorescence decrease of β-PE (beta-phycoerythrin) along time under constant flux of peroxyl radical, produced from decomposition of AAPH. In the presence of antioxidant sample, the decay of fluorescence is inhibited in a period of time (Cao and Prior, 1999). Because of lot-to-lot variability of β-PE, the non-protein fluorescein has been used as the fluorescent target instead (Ou, 2001). The ORAC assay is only method using the area under curve (AUC) for quantification, so this assay considers kinetics of antioxidant action. However, the reaction of peroxyl radical is monitored for more than 30 min (Magalhaes *et al.*, 2008).

2.2.2 Chemiluminescence-based assay

This method was developed from TRAP assay by Metsa-Ketela in 1991 and described in detail by Alho and Leinonen (1999). The principle of this method is that peroxyl radicals produced from AAPH oxidized luminal, leading to the formation of luminal radicals that emit light. Antioxidant in sample inhibits this chemiluminescence for a time that is directly proportional to the antioxidant capacity of sample. The emitted light can be detected by luminometer.

2.3 Gas Chromatography

Winston and his coworkers developed an assay called total oxyradical scavenging capacity (TOSC) assay (Winston *et al.*, 1998). It is based on the oxidation of α-keto-γ-methiolbutyric acid (KMBA) to ethylene by peroxyl radical. The ethylene formation is monitored by gas chromatography. Antioxidant sample partially inhibited ethylene formation by scavenging peroxyl radical. This method is short coming due to long time analysis more than 100 min.

2.4 Electrochemical Biosensor

Most of electrochemical techniques have been produced superoxide radical $(O_2^{\bullet-})$ as free radical in the system. This radical is quite reactive and cannot be oxidized directly on electrode. So, modified electrodes such as superoxide dismutase (SOD)-base sensor and cytochrome *c*-based sensor have been developed to measure superoxide radical.

2.4.1 Cytochrome *c*-based biosensor

The principle is based on evaluating antioxidant capacity by means of the scavenging of superoxide radical $(O_2^{\bullet-})$ which is produced from xanthine / xanthin oxidase reaction (2) (Mello *et al.*, 2006). This sensor is capable to detect superoxide radical.

Xanthine $+ H_2O + O_2 \xrightarrow{XOD}$ Uric acid $+ 2H^+ + O_2^{\bullet-}$

(2)
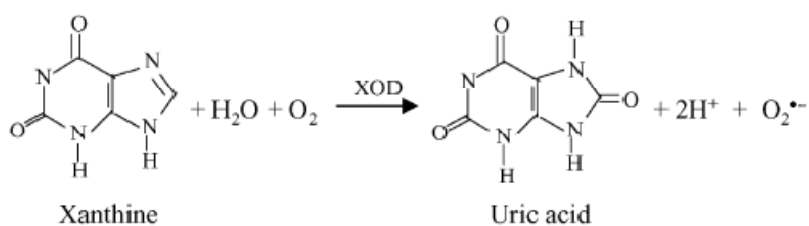
Some superoxide radical is scavenged by antioxidant sample and the leftover superoxide radical is measured by cytochrome *c*-based sensor. The superoxide sensor is based on the reaction between $O_2^{\bullet-}$ and surface immobilized cytochrome *c*, followed by the electron transfer from the cytochrome *c* to the electrode (reaction (3) and (4)). The electron transfer is proportional to the leftover superoxide radical (Tammeveski *et al.*, 1998).

$$Cyt.c[Heme(Fe^{3+})] \; + \; O_2^{\bullet-} \quad \rightarrow \quad Cyt.c[Heme(Fe^{2+})] \; + \; O_2 \qquad (3)$$

$$Cyt.c[Heme(Fe^{2+})] \quad \rightarrow \quad Cyt.c[Heme(Fe^{3+})] \; + \; e^- \qquad (4)$$

The advantage of cytochrome *c*-based sensor is low applied potential used which caused low noise. But cytochrome *c* has peroxidase property so it can reduce hydrogen peroxide, causing false signal. Moreover, xanthine and xanthine oxidase are very expensive compounds. The reviews of cytochrome *c*-based sensor are shown in Table 2.1.

2.4.2 Superoxide dismutase-based sensor

This sensor is used to detect superoxide radical as same as cytochrome *c*-based sensor. Superoxide dismutase immobilized on electrode surface, catalyses the superoxide radical producing molecular oxygen (O_2) and hydrogen peroxide (H_2O_2) (reaction (5)). Generally, the oxidation of H_2O_2 at the electrode surface generates the signal. The advantage of superoxide dismutase-based sensor is the specificity between $O_2^{\bullet-}$ and superoxide dismutase. But the oxidation reaction occurs in a high potential (>0.5 V), which result in interference problems. Moreover, xanthine / xanthine oxidase system is very expensive. The reviews of superoxide dismutase based sensor are shown in Table 2.2.

$$O_2^{\bullet-} + O_2^{\bullet-} + 2H^+ \quad \xrightarrow{\text{SOD}} \quad O_2 + H_2O_2 \qquad (5)$$
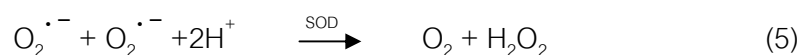
2.4.3 DNA-based sensor

DNA-based sensor is based on the study of DNA damage induced by free radical. One of the most reactive radical species that induce lesions in DNA is $^{\bullet}$OH generated by the Fenton reaction. It can attack both deoxyribose sugar and purine/pyrimidine bases of DNA, causing DNA damage (Jaruga and Dizdaroglu, 1996). With this principle, Mello and his coworker immobilized double stand DNA (ds-DNA) on electrode surface and induced hydroxyl radical to interact guanine base (Mello *et al.*, 2006). The oxidation of guanine base was monitored at potential +1.0V. The antioxidant of plant extracts was evaluated by change of guanine base signal. Liu and coworker developed photogenerated hydroxyl radical system which ds-DNA/(TiO₂)*i*/ITO electrode

were illuminated with UV light (Lui *et al*., 2005). Moreover, antioxidant redox sensors based on DNA modified carbon screen-printed electrodes were also developed by Lui and others (2006).

Table 2.1 Cytochrome *c*-based sensor

No	Immobilization	Transducer	Detection	Detection range	Stability	References
1	SAM-MPA	Amp. (Au) 0.1 V	$O_2^{\cdot-}$	-	5 days	(Gobi and Mizutani, 2000)
2	SAM-MUA:MU	Amp. (Au) 0.13V	$O_2^{\cdot-}$	-	-	(Ge and Lisdat, 2002)
3*	SAM-MUA	Amp. (Au) 0.15V	flavanols, flavonols, flavones, flavonones, isoflavonones	AAE and IC_{50}	7 days	(Ignatov *et al*., 2002)
4	SAM-MUA:MU Multilayer with PASA	Amp. (Au) 0.15V	$O_2^{\cdot-}$	0.4-1.5 μM (superoxide)	1 day / 10 use	(Beissenhirtz *et al*., 2004)
5	SAM-MUA:MU	Amp. (SPE-Au) 0.15V	$O_2^{\cdot-}$	0.3-1.2 μM (superoxide)	-	(Krylov *et al*., 2004)
6	Physical adsorption	Amp. (DDAB-PME) 0.1V	$O_2^{\cdot-}$	0.86-5.93 μM (Hypoxanthine)	30 days	(Guo *et al*., 2005)
7	SAM-MUA:MU	Fluidic chip Amp. 0.15V	cosmetic cream enriched with a green tea extract	• 0.4–1.2 nM for superoxide • 50-1000 mM for hydrogen peroxide • IC_{50} for antioxidant	-	(Krylov, Sczech, and Lisdat, 2007)

SAM = self-assembled monolayers   AAE = ascorbic acid equivalent

MPA = mercaptopropionic acid IC_{50} = 50% inhibition value

MUA:MU = mercaptoundecanoic acid/mercaptoundecanol   PASA = poly(anilinesulfonic acid)

DDAB-PME = didodecyldimethylammonium bromide (DDAB)-modified powder microelectrode

IC_{50} = the antioxidant concentration corresponding to a 50% depression of the superoxide or peroxide sensor signal

* Some of the compounds (trolox, pelargonidine chloride and kaempferol) can be directly oxidized at the electrode. This can interfere with the superoxide measurement. Some of the compounds (alfa-lipoic acid, retinoic acid, hesperidin, *N*-acetyl-L-cystein, melatonin and beta-estradiol) was limited in its scavenging activity to other reactive species and had no scavenging activity against superoxide.

Table 2.2 Superoxide dismutase-based sensor

No	Immobilization	Transducer	Detection	Detection range	Stability	References
1	Physical adsorption (Kappa-carrageenan gel)	Amp. (H_2O_2) 0.65 V	Human kidney tissues	20-2000 μM	≥7 days	(Campanella *et al*., 2000)
2	Physical adsorption (Kappa-carrageenan gel)	Amp. (H_2O_2) 0.65 V	Several fruits and plants	Slope of regression line	-	(Campanella *et al*., 2001)
3	Chemical cross-linking (glutaraldehyde)	Amp. (Pt) 0.5V	$O_2^{\bullet-}$	0-100 μM (hypoxanthine)	-	(Endo *et al*., 2002)
4	SAM (cysteine)	Amp. (Au) 0.3V	$O_2^{\bullet-}$	13-130 nM	7 days	(Ohsaka *et al*., 2002)
5	SAM (cysteine)	Amp. (Au) 0.3V	$O_2^{\bullet-}$	13-130 nM	-	(Tian *et al*., 2002)
6	Physical adsorption (Kappa-carrageenan gel)	Amp. (H_2O_2) 0.65 V	Aromatic herbs, olives and fresh fruit	RAC (relative antioxidant capacity)	-	(Campanella *et al*.,2003)
7	Physical adsorption (Kappa-carrageenan gel)	Amp. (H_2O_2) 0.65 V	White and red wine	RAC	-	(Campanella *et al*., 2004)
8	Silica-PVA Sol-gel encapsulation	Amp. (Au) (-)0.15V	$O_2^{\bullet-}$	0.2-1.6 μM	>20 days	(Di *et al*., 2004)
9	Chemical cross-linking (glutaraldehyde)	Amp. (Pt) 0.65 V	$O_2^{\bullet-}$	20-2000 μM	30 days	(Emregül, 2005)
10	SAM (Cysteine)	Amp. (CFME) +0.25 V, -0.15 V	$O_2^{\bullet-}$	13-105 nmol/L	7 days	(Tian *et al*., 2005)

Published Antioxidant Capacity Value

There were reports of antioxidant capacity of human serum and plasma by various authors using different methods as shown in Table 2.3. As notice, antioxidant capacity values were dependent upon the method principle and free radical used. Trolox which is the analogous cpd of vitamin E has been used as standard antioxidant in which antioxidant capacity values were reported into "Trolox equivalent".

Table 2.3 Total antioxidant capacity of human blood plasma and serum (adapted from Bartosz, 2003: pp. 242)

Samples	Trolox equivalents	Methods
Plasma	316 ± 22 µM	TAS (Randox)
Serum	382 ± 79 µM	Enhanced chemiluminescence
Plasma	429 ± 20 µM	Chemiluminescence
Plasma (heparin)	580 ± 79 µM	TRAP
Plasma (heparin)	830 ± 4 µM	TAS (Randox)
Plasma (heparin)	930 ± 150 µM	FRAP
Plasma	1.017 ± 0.206 mM	FRAP
Plasma	1.071 ± 0.059 mM	H2-DCF-DA/ ABAP
Plasma (heparin)	1.103 ± 0.030 mM	FRAP
Plasma	1.120 ± 0.07 mM	TRAP
Plasma	1.15 ± 0.14 mM	ABAP/ luminol
Plasma (EDTA)	1.155 ± 0.290 mM	H_2-DCF-DA/ ABAP
Serum	1.22 ± 0.11 mM	TAS (Randox)
Plasma (EDTA)	1.272 ± 0.199 mM	ABAP/ luminol
Plasma	1.440 ± 284 mM	ABAP/ luminol
Plasma	1.60 ± 0.18 mM	TAS (Randox)
Serum	1.69 ± 0.20 mM	TAS (Randox)
Plasma (22 ± 5 yr)	1.72 ± 0.20 mM	TAS (Randox)
Plasma (71 ± 4 yr)	1.77 ± 0.13 mM	TAS (Randox)
Serum	1.73 ± 0.20 mM	TAS (Randox)
Plasma (heparin)	1.76 ± 0.19 mM	TAS (Randox)
Plasma (heparin)	1.870 ± 0.010 mM	ORAC
Serum	2.04 ± 0.20 mM	Benzoate oxidation by Fenton reagent
Plasma	2.70 ± 0.50 mM	ABTS$^{\bullet+}$ decolorization
Serum	3.10 ± 0.20 mM	ORAC
Serum	3.38 ± 0.28 mM	ORAC (β-phycoerythrin)
Serum	7.78 ± 0.47 mM	ORAC (fluorescein)

TAS = total antioxidant status, ABAP = 2,2'-azobis(2-amidopropane),

H_2-DCF-DA= 2',7'- dichlorofluorescein diacetate

CHAPTER III

MATERIALS AND METHODS

Materials

1   Chemical Compounds and Reagents

   1.1 Reference standard

   - Trolox (6-hydroxy-2,5,7,8-tetramethychroman-2-carboxylic acid); Fluka, Germany

   1.2 Other chemical compounds

   - ABTS (2,2'-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt), purum, $\geq$ 99.0% (HPLC); Fluka, Germany

   - Potassium persulfate ($K_2S_2O_8$), 99.99%; Sigma –Aldrich, St. Louis, USA

   - AAPH (2,2'-azobis(2-amidinopropane) dihydrochloride); Wako Chemical, USA

   - α-tocopherol, purity 99%; Sigma, St. Louis, USA

   - Sodium chloride (NaCl); Scharlau Chemie S.A., Spain

   - Potassium chloride (KCl); M&B laboratory chemicals, Dagenham England

   - Potassium dihydrogen phosphate (KH_2PO_4); Merck, Germany

   - Di-sodium hydrogen phosphate (Na_2HPO_4); Merck, Germany

2   Apparatus

   2.1 The screen-print carbon paste electrode (SCPE); Bioelectronic research laboratory, Department of Electrical Engineering, Faculty of Engineering, Chulalongkorn University, Thailand.

2.2 The portable potentiostat; Bioelectronic research laboratory, Department of Electrical Engineering, Faculty of Engineering, Chulalongkorn University, Thailand.

2.3 Heating block; Stuart, UK

2.4 Vortex mixer, Vortex-Genie2; Scientific industries, Germany

2.5 Micropipette; Socorex, Switzerland

Electrochemical measurements were carried out on SCPE using a portable potentiostat, connected to a computer for data collection and analysis (Figure 3.1A and 3.1C). SCPE were fabricated by mixing carbon paste ink with turpentine oil, and then screened onto the PVC (Figure 3.1B). A set of electrode consisted of two identical working electrodes. An insulator layer was covered manually over the conductive track. For preliminary experiment, a working area was 2mm x 5mm (Figure 3.1D).

| A. Schematic of electrochemical sensor setup | B. Sets of electrodes on PVC |
| C. Electrochemical sensor setup | D. A set of electrode, working area 2mm x 5mm |
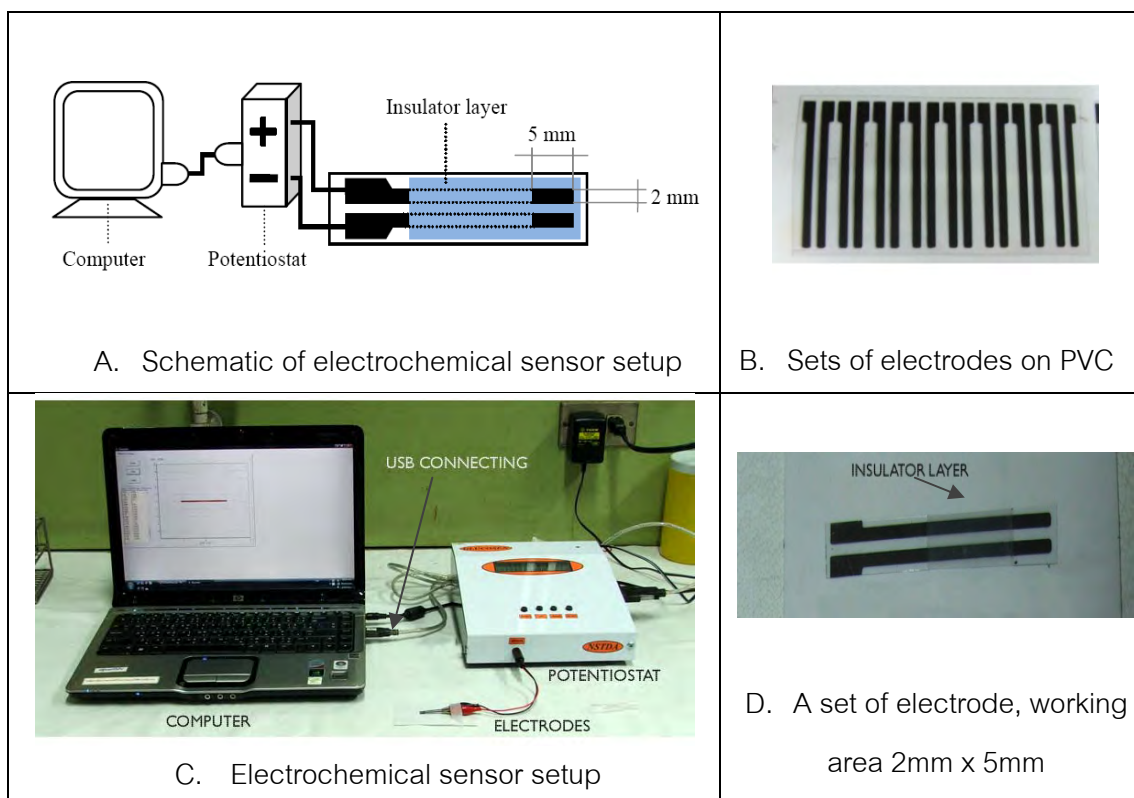
Figure 3.1 Electrochemical measurement setup

3 Human Sera

Human sera were generously supplied from Central Laboratory, Vichaiyut Hospital, Thailand.

For the method of development (method part 2 and 3), human pooled sera were collected from residual specimens. For the application (method part 4), individual leftover sera from blind healthy subjects and patients were used.

Leftover sera of healthy subjects were classified from their normal haematological and biochemical value. Leftover sera of patient were characterized according to the diagnosed pathology and symptoms.

4 Preparation of Solutions

4.1 A stock solution of Trolox (1.0 mM)

A 6.25 mg of Trolox was accurately weighed, dissolved in 1-2 mL of methanol and made up to 25 mL volume with PBS.

4.2 A stock solution of α-tocopherol (10.0 mM)

A 43.07 mg of α-tocopherol was weighed, dissolved and made up to 10 mL volume with PBS. The solution was light protected and used within one week.

4.3 ABTS solution (2.0 mM)

A 2.19 mg of ABTS was accurately weighed and made up to 2 mL volume with PBS. The solution was daily prepared and light protected.

4.4 Potassium persulfate solution (10.0 mM)

A 13.52 mg of $K_2S_2O_8$ was accurately weighed and made up to 5 mL volume with PBS. The solution was used within one month.

4.5 Phosphate buffer saline (PBS), pH 7.4

A 8.0 g NaCl, 0.2 g KCl, 0.2 g KH_2PO_4, and 2.9 g Na_2HPO_4 were accurately weighed and dissolved in purified water, one by one. A solution was made up to 1000 mL.

Methods

Four parts of study were performed in this study. They included

- Determination of the appropriate free radical generator for amperometric technique.

- Development of the method for measuring antioxidant capacity using ABTS$^{\bullet+}$.

- Evaluation of the developed method.

- Application of the developed method to determine antioxidant capacity in serum.

1   Determination of the Appropriate Free Radical Generator for Amperometric Technique.

1.1 Selection of the Proper Free Radical Generator

The crucial point for electrochemical technique is the electroactivity of an analytical substance either by oxidation or reduction under proper condition in assay solution. AAPH and ABTS which have been widely utilized in spectroscopic measurement were selected and compared for their abilities in generating free radical using cyclic voltammetry.

1.1.1   AAPH as free radical generator

AAPH is a water-soluble azo compound which is used extensively as a free radical generator in the determination of antioxidant capacity by spectroscopic technique. Free radical generated from AAPH is peroxyl radical (ROO$^{\bullet}$). The mechanism of generating peroxyl radical is the decomposition of AAPH under incubation period. AAPH is decomposed into one molecular nitrogen (N$_2$) and two carbon-centered radicals (R$^{\bullet}$) which react with oxygen to give two peroxyl radicals (ROO$^{\bullet}$) (equation (6) and (7)). Rate of free radical generation is about 1.36 x 10^{-6} mol/L·sec, at 37°C and neutral pH (Niki, 1990).

(AAPH)

$$2R^{\cdot} + 2O_2 \longrightarrow 2ROO^{\cdot} \qquad (7)$$
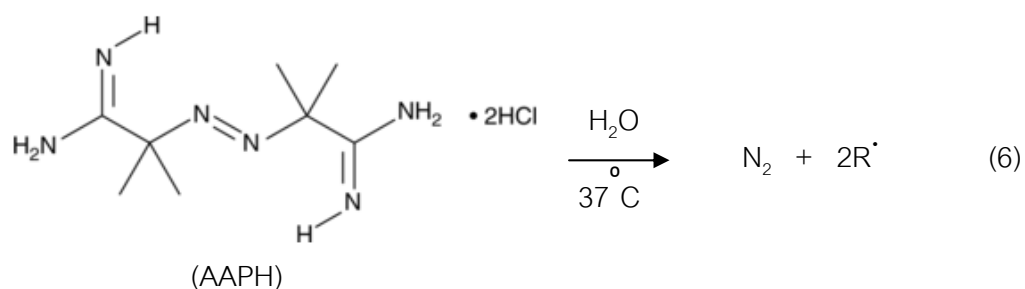
Procedure

A 320 mM AAPH was freshly prepared in PBS. The serial dilutions were made accordingly to 20 mM. These solutions were incubated at $37^{\circ}C$ for 20 min which yielded peroxyl radical in the concentration range of 31.25-500 μM. The calculation of peroxyl radical concentrations is shown in Table 3.1. After incubation, 60 μL of peroxyl radical solution was measured by cyclic voltammetry using SPCE surface area of 2mm x 5mm. The electrochemical measurement was performed at the voltage range of -1.0 to +1.0 V with scan rate of 50 mV/s.

AAPH could be the appropriate free radical generator for amperometric measurement if either oxidation or reduction of peroxyl radical would be observed from cyclic voltammogram.

Table 3.1 The decomposition of AAPH to produce peroxyl radical at 37°C for 20 min.

Initial AAPH (mM)	Peroxyl radical at 37°C, 20 min.* (μM)
320	500
160	250
80	125
40	62.5
20	31.25
* Peroxyl radical = (1.36×10^{-6})(Mole of AAPH)(Time/sec)	

1.1.2   ABTS as free radical generator

ABTS has been used as free radical generator in TEAC assay. The oxidation of ABTS generates a blue/green radical cation, ABTS$^{\bullet+}$, which has absorption maxima at 645,734 and 815 nm (Miller *et al.,* 1993). Several strategies have been used to oxidize ABTS such as enzymatic reaction (Miller *et al.*), chemical reaction (Re *et al.*, 1999) and electrochemical generation (Alonso, Guillen and Barroso, 2003). This study chemically produced ABTS$^{\bullet+}$ by using $K_2S_2O_8$. The ABTS can donate an electron to produce ABTS$^{\bullet+}$ (Figure 3.2). This radical was stable when stored in the dark at room temperature for more than two days (Re *et al.*, 1999).

The scavenging of ABTS$^{\bullet+}$ has been applied to the determination of various compounds, both lipophilic and hydrophilic antioxidant including ascorbic acid, α-tocopherol, uric acid, bilirubin, cysteine, glutathione, and phenolic compound (Bartosz, 2003; Pannala *et al.*, 2001; Walker and Everette, 2009).

ABTS

(2,2'-azinobis-(3-ethylbenzthiazoline-6-sulphonic acid))

Potassium persulfate | -e-
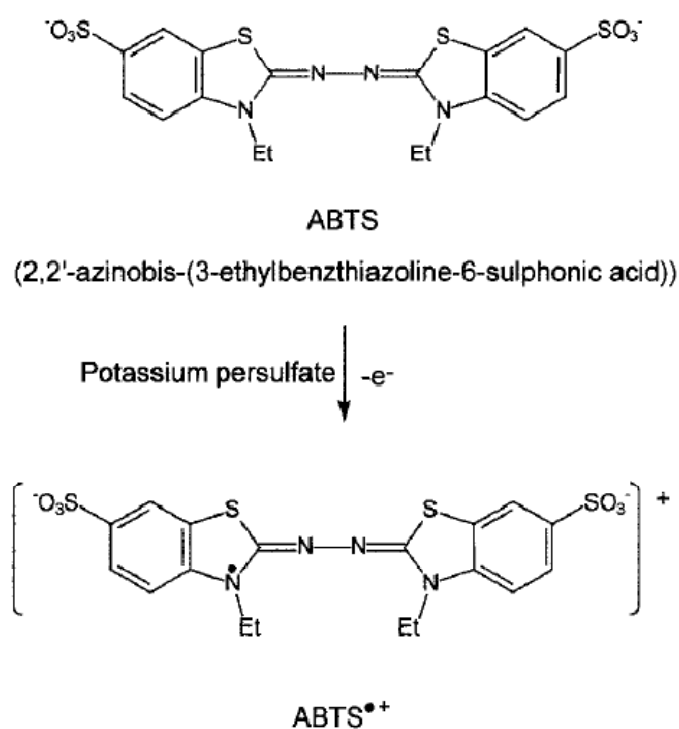
ABTS$^{\bullet+}$

Figure 3.2 The oxidation of ABTS using potassium persulfate (Pannala *et al.*, 2001).

Reaction between ABTS$^{\bullet+}$ and antioxidant has been demonstrated by monophenolic model (Pannala *et al.*, 2001). There is one electron donated from monophenolic compound which reduces ABTS$^{\bullet+}$ to ABTS (Figure 3.3). In contrast, Osman and his co-workers reported that the reaction of ABTS$^{\bullet+}$ with polyphenol lead to degradation of ABTS$^{\bullet+}$. Possible structure of two degradation products (which were purified by reversed-phase chromatography and characterized by UV-visible detection, mass spectrometry, and ^{1}H NMR spectroscopy) are shown in Figure 3.4 (Osman *et al.*, 2006).
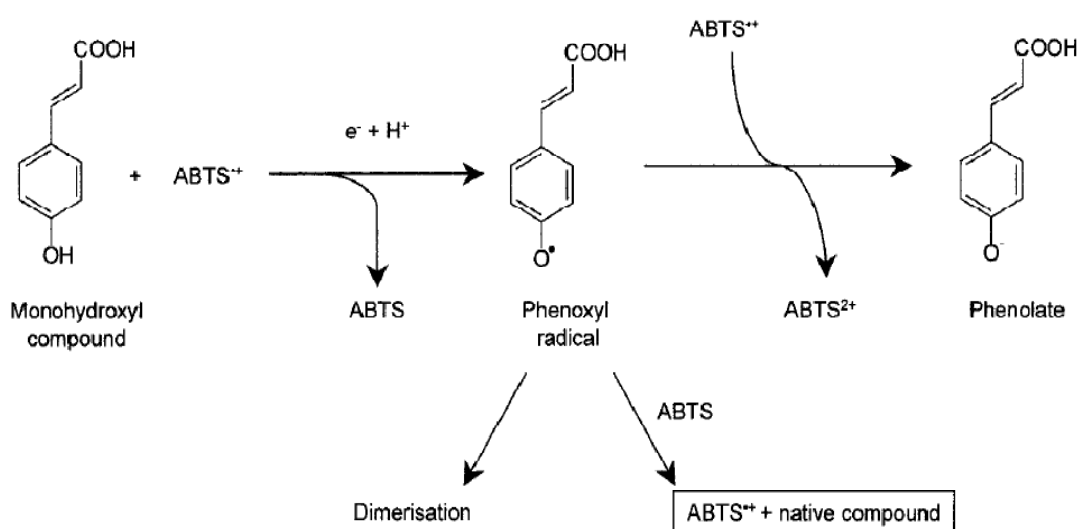
Figure 3.3 Possible mechanism of action of monophenolic compound with ABTS$^{\bullet+}$ (Pannala *et al.*, 2001).
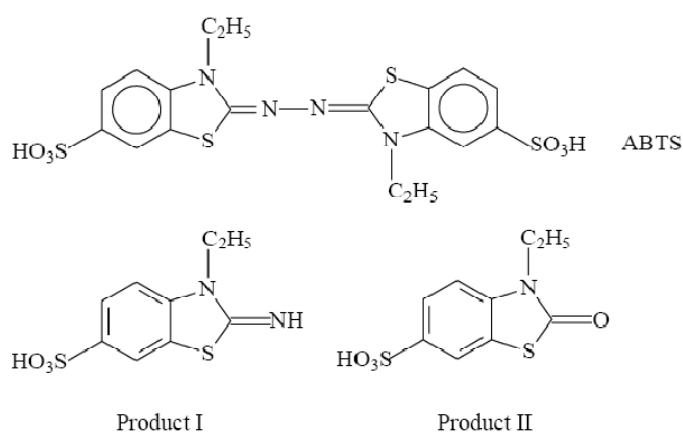
Figure 3.4 Possible structures of degradation product of ABTS$^{\bullet+}$ (Osman *et al.*, 2006).

<u>Procedure</u>

ABTS$^{\bullet+}$ was generated by mixing 2.0 mM ABTS solution with 0.5 mM $K_2S_2O_8$ solution in the volume ratio of 1:1. The mixture solution was kept in the dark at room temperature for 12 hr before measuring the free radical. ABTS$^{\bullet+}$ solution was also diluted with PBS to make the difference amount which equal to ABTS in concentration range of 31.25-500 μM. The same procedure of measuring peroxyl radical was used. ABTS could be the appropriate free radical generator for amperometric measurement if either oxidation or reduction of ABTS$^{\bullet+}$ would be observed from cyclic voltammogram.

1.2 Selection of Appropriate Bias Potential according to the Aforementioned Process in 1.1.2

Amperometric method need bias potential for measurement. To determine bias potential, free radicals were measured in cyclic voltammetry (as follow in 1.1). Anodic and cathodic peaks of ABTS$^{\bullet+}$ on cyclic voltammogram could be approximately defined the bias potential of ABTS$^{\bullet+}$. However, bias potential could effect to current response in amperometric method. Therefore, selected bias potential should be confirmed by amperometric measurement.

<u>Procedure</u>

ABTS$^{\bullet+}$ solutions were prepared in the difference amount according to 1.1.2. Then ABTS$^{\bullet+}$ solutions were measured by amperometry which various bias potentials (0.10, 0.15, 0.20, and 0.25 V) were applied. A 60 μL of ABTS$^{\bullet+}$ solution was dropped on the electrode at 10th second and measured for 110 seconds. The relationship between ABTS concentrations and current signals were plotted. To reduce the variation of data, moving average (n=5) of current signals were used.

Cyclic voltammogram exhibited anodic peak of ABTS$^{\bullet+}$ at potential of 0.15V. So, this experiment was compared current signal between bias potential of 0.15V and others. The appropriate bias potential should give enough current; however, bias potential should be low to avoid interference.

1.3 Determination of the Optimum Condition for producing stable ABTS$^{\bullet+}$.

The optimum condition for producing stable ABTS$^{\bullet+}$ was determined in terms of high production of ABTS$^{\bullet+}$ and long life time storage without degradation. The proper concentration of $K_2S_2O_8$ used and the storage temperature for free radical were then determined.

 1.3.1 Determination of the proper ratio of $K_2S_2O_8$ for the production of ABTS$^{\bullet+}$

 Procedure

 A 1.0 mM ABTS solution was oxidized with $K_2S_2O_8$ aqueous solution at different concentration range of 0.10-0.45 mM and stood in the dark at 4°C for more than 12 hr. ABTS$^{\bullet+}$ from the mixture was measured by amperometric technique at bias potential of 0.15V. The measurement was repeated four times for each concentration ratio. The concentration ratio of ABTS and $K_2S_2O_8$ which could produce the maximum current signal of ABTS$^{\bullet+}$ will be determined.

 1.3.2 Determination of the storage time and temperature for ABTS$^{\bullet+}$

 The appropriate storage time and temperature for ABTS$^{\bullet+}$ were determined as following:

 Procedure

 A 1.0 mM ABTS and 0.25 mM $K_2S_2O_8$ were mixed for generating ABTS$^{\bullet+}$. There are two set of experiments; one was kept ABTS$^{\bullet+}$ in the dark and stored at room temperature, another was kept in the dark and stored at 4°C. The resulting ABTS$^{\bullet+}$ solution was then used for determining the current signal by amperometry consequently up to 5 days. Three replications were performed each measurement.

 The appropriate storage time for ABTS$^{\bullet+}$ would cause the stable current of current signal.

1.4 The Relationship between ABTS Concentration and Current Signal.

A series of $ABTS^{\bullet+}$ amounts were analyzed by amperometric technique according to the following developed condition. These $ABTS^{\bullet+}$ amounts were generated from ABTS in the concentration of 10, 20, 40, 60, 80,100, 200, 400, 600, 800, and 1000 μM. The pattern of linear relationship between ABTS in the concentration and current was determined.

2 Development of the Method for Measuring Antioxidant Capacity Using $ABTS^{\bullet+}$.

From the previous section, $ABTS^{\bullet+}$ had been proven to be able to use in amperometric technique. This section would describe the development of the method by determining the appropriate amount of $ABTS^{\bullet+}$, the dilution of serum and the appropriate reaction time.

2.1 Determination of the Appropriate Amount of $ABTS^{\bullet+}$ for Reacting with Antioxidant

The amount of $ABTS^{\bullet+}$ used could affect current signal and antioxidant in sample. Therefore, the appropriate amount of $ABTS^{\bullet+}$ is needed for method development.

Procedure

Three concentrations of ABTS were studied. They were 500, 1000 and 2000 μM. At these three concentrations, the calibration curve for $ABTS^{\bullet+}$ was constructed having Trolox as standard antioxidant. The range concentrations of Trolox that supposed to be completely reacted with $ABTS^{\bullet+}$ were used.

In the mean time, antioxidant capacity in human sera was determined along these ABTS concentrations. To represent average value of antioxidant capacity, this part of study used human pooled sera which prepared from leftover sera of five healthy human. Pooled sera were diluted with PBS in the series that supposed to be completely reacted with $ABTS^{\bullet+}$. The concentrations of Trolox and the dilutions of sera were as shown in table 3.2.

Table 3.2 Trolox concentration and sera proportion for determining appropriate amount of ABTS$^{\bullet+}$

ABTS (µM)	Antioxidant	
500	Trolox concentrations (µM)	25, 50,75, 100, 125, 150, 175 and 200
1000		50, 100, 150, 200, 250, 300, 350 and 400
2000		100, 200, 300, 400, 500, 600, 700 and 800
500	Pooled human serum dilutions	1:160, 1:80, 1:40, 1:27, 1:20, 1:16 and 1:13
1000		1:80, 1:40, 1:20, 1:13, 1:10, 1:8 and 1:6.7
2000		1:40, 1:20, 1:10, 1:6.7, 1:5, 1:4 and 1:3.3

ABTS$^{\bullet+}$ solution was mixed with either Trolox or human sera solution in the volume ratio of 1:1 in test tube. Then 60 µL of mixture solution was dropped on the electrodes connected to the amperometric measurement. The current signal of amperometry was measured for 110 seconds. Moving average (n=5) of current signal at 100th second were calculated and reported. The appropriate amount of ABTS used should have the following performance.

-   Trolox standard curve which had a linear relationship.
-   Current signals of leftover ABTS$^{\bullet+}$ were high enough to measure and clearly seen the changes.

2.2 Determination of the Serum Dilution

Serum would be diluted before measurement to reduce the effect of other substances in serum such as protein and albumin. The dilution of serum also improved the efficiency of electrode. In addition, the dilution of serum affected the residual ABTS$^{\bullet+}$ which produced current signal. So, the residual ABTS$^{\bullet+}$ should be more enough and produced current signal which in range of the calibration curve.

Procedure

This study also used human pooled serum. The concentration of ABTS as mentioned in 2.1 was used. Pooled serum was diluted in range of 1:10 to 1:100

A pooled serum was diluted in range 1:10 to 1:100 and analyzed according to the developed method. Three replications were performed in each serum sample. The current signals of $ABTS^{\bullet+}$ were observed.

2.3 Determination of an Appropriate Reaction Time

A rapid measurement is one of the goals of this development. This study would determine optimum reaction time between $ABTS^{\bullet+}$ and antioxidant in serum. Most TEAC values reported in the literature were measured at time intervals between 1 and 6 min (Ivekovic et al., 2005). Therefore, reaction times were studied both less and more than 6 min.

Procedure

This study also used human pooled serum. The concentration of ABTS and the dilution of serum as mentioned in 2.1 were used. Pooled serum was diluted in range of 1:200 to 1:25 to compare reaction time in different dilution.

$ABTS^{\bullet+}$ was reacted with diluted sera and incubated for 30 minutes. Current signal of $ABTS^{\bullet+}$ was measured immediately after mixed and every 10 minutes until the end of incubation. The optimum reaction time should be the time that obtained good correlation between serum dilution and current.

3 Evaluation of the Developed Method

The developed method from 2.1, 2.2 and 2.3 was evaluated in term of linearity, accuracy, precision and the precision of electrode.

3.1 Determination of Linear Range of the Method

Procedure

A series of standard Trolox concentration (20, 40, 60, 80 and 100 μM) were analyzed according to the developed method. Six replications were performed by three different electrodes (two replications per an electrode). The pattern of linear relationship of the mentioned concentration was determined by regression equation.

3.2 Accuracy and Precision of the Developed Method

3.2.1 Intra-day accuracy and precision

Three concentration of standard Trolox which represent low, medium and high concentration in the calibration curve were used. They were 25, 50 and 75 μM of Trolox in PBS. Six replications of these standard solutions were analyzed according to the developed method along with the calibration curve.

The accuracy of analytical method can be described in term of %bias as shown in equation (10).

$$\%bias = [(analyzed\ conc. - true\ conc.)/true\ conc.] \times 100 \qquad (8)$$

For the acceptable accuracy, the percentage of bias obtained from the analysis of standard Trolox should be within ± 10%.

The precision of analytical method were determined in the term of %RSD of Trolox concentration. The method was considered to be precise if the %RSD was not more than 10% for all mentioned concentration.

      3.2.2   Inter-day accuracy and precision

The procedure for inter-day analysis was followed to procedure of intra-day but one replication of standard Trolox was analyzed on three different days. Also, the precision were determined with the same criteria as the intra-day procedure.

3.3 The Replicated Use of Electrode

This study tested the precision of an electrode according. Thirty replications of one serum sample were analyzed using the same electrode. The precision of an electrode could be determined in term of %RSD of current signal. The electrode was considered to be precise if the %RSD did not more than 10%.

4   The Determination of Antioxidant Capacity in Human Serum.

According to the developed method, the measurement of antioxidant capacity in human serum was follow by this procedure.

1. $ABTS^{\bullet+}$ was generated by reacting 500 µM ABTS solution with 0.125 µM $K_2S_2O_8$ solution (molar ratio of 1:0.25). The mixture solution was kept in the dark at 4 ^{o}C for 12 hr before use. The solution was used within 3 days.

2. The amperometric measurement was performed on SCPE, with electrical contact of 2mm x 5mm. The bias potential of 0.15V was applied to SCPE by potentiostat.

3. Serum sample was diluted with PBS into dilution 1:25.

4. Both $ABTS^{\bullet+}$ solution and diluted serum were mixed in volume ratio of 1:1. Then 60 µL of the mixture was immediately dropped on SCPE.

5. The current signals were collected for 110 sec. the moving average current of 100th sec were calculated and reported. Each sample was measured 3 times.

6. After finish the measurement, the electrode was rinsed with PBS before next measurement.

4.1 Determination of Antioxidant Capacity in Human Serum

Antioxidant capacity in leftover human sera were determined using the developed method. There were sera of 30 normal subjects (15 males and 15 females), aged range 13 to 59 (33 ±12 years) and 5 patients with different pathology. Normal subjects were characterized from their normal hematological and biochemical values. Patients were classified according to the diagnosed pathology/symptoms.

<u>Procedure</u>

Individual serum samples were diluted with PBS in the volume ratio of 1:25 and analyzed following the developed method. The current response of ABTS$^{\bullet+}$ to Trolox solutions were plot as calibration curve for determining antioxidant capacity of serum sample in term of Trolox equivalent (TE). The calibration for TE was as following equation (9).

$$\text{Trolox Equivalent } (\mu M) \; = \; \frac{[\text{measured current} - \text{intercept}] \times \text{serum dilution}}{\text{slope of regression line}} \qquad (9)$$

Antioxidant capacity in patients was supposed to be different from normal subjects. The method should be able to discriminate between normal and low antioxidant capacity in these two groups.

4.2 Determination of Antioxidant Capacity in Spiked Serum with α-Tocopherol.

This method was applied to measure antioxidant in spiked serum which had antioxidant capacity more than normal value. α-tocopherol is one of the important antioxidants in human. To simulate high antioxidant capacity in serum, standard α-tocopherol solutions (1000, 1500 and 2500 μM) were added to serum and analyzed antioxidant capacity in this spiked serum by the developed method.

CHAPTER IV

RESULTS AND DISCUSSION

1   Determination of the Appropriate Free Radical Generators for Amperometric
    Technique.

1.1 Selection of the Proper Free Radical Generator
1.1.1   AAPH as free radical generator

To investigate electroactivity of peroxyl radical

Peroxyl radical produced from the decomposition of AAPH, could exhibit neither oxidation nor reduction peak. Every concentrations of peroxyl radical showed the same pattern and were similar to base line (Figure 4.1). Therefore, peroxyl radical did not have electroactivity under this condition.
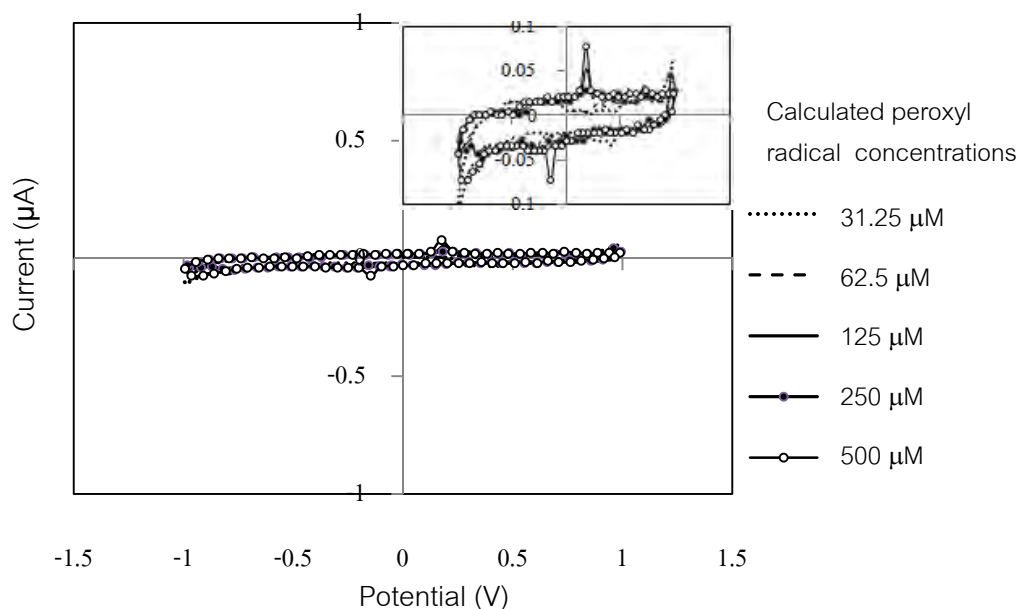

Figure 4.1 Cyclic voltammogram of peroxyl radical in PBS, scan rate 50 mV/s. This cyclic voltammograms was enlarged to see the detail at above-right-corner.
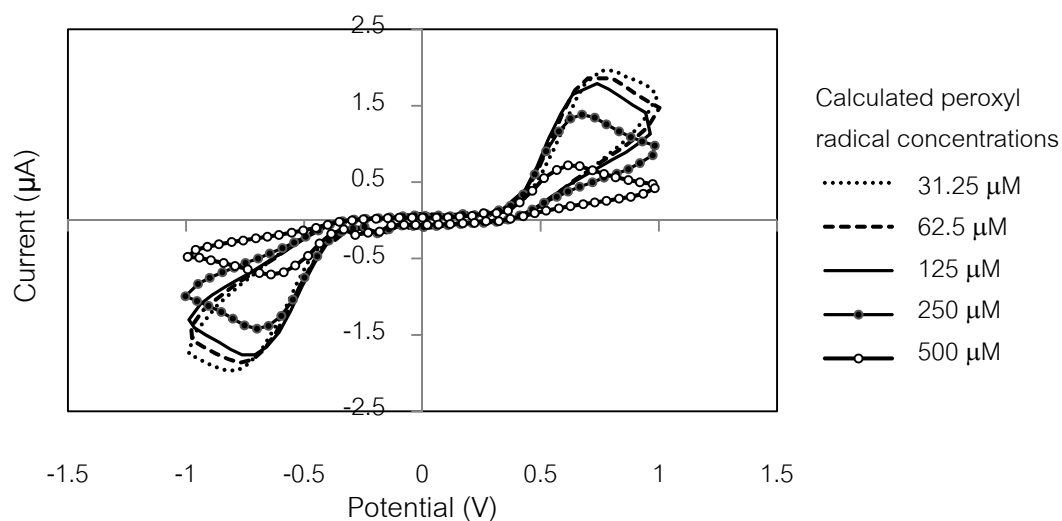
Figure 4.2 Cyclic voltammogram of Trolox after reacting with peroxyl radical.

By adding standard Trolox at 1000 μM into peroxyl radical solutions, oxidation and reduction peaks of Trolox were exhibited in Figure 4.2. The more amount of peroxyl radical was used, the low current signal of Trolox was observed.

Trolox had electroactivity which its oxidation potential on glassy carbon electrode (electrolyte: acetic acid + acetonitrille solution, 90:10 v/v) was around 0.6 V (Malyszko and Karbarz, 2006). Trolox was decreased when reacting with free radical. Therefore, there was existing peroxyl radical in the solutions but they did not have electroactivity. So AAPH could not be the proper free radical generator for amperometric measurement.

1.1.2   ABTS as free radical generator

To investigate electroactivity of ABTS$^{\bullet+}$

As demonstrated in Figure 4.3, a 1.0 mM ABTS alone exhibited low oxidation peak, while $K_2S_2O_8$ could not display any oxidation peak. Both anodic and cathodic peak of ABTS$^{\bullet+}$ at ±0.15V confirmed the reversible electroactivity of ABTS$^{\bullet+}$. Therefore, this study selected ABTS as a free radical generator.
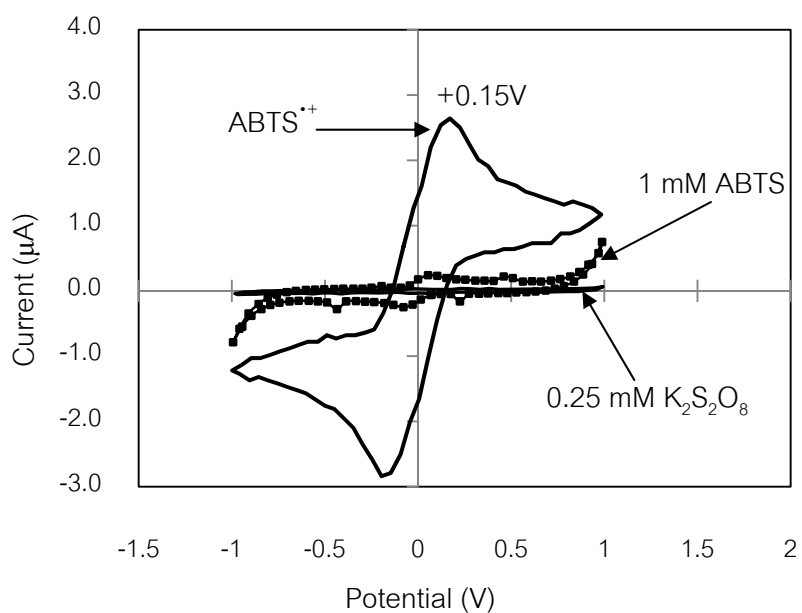
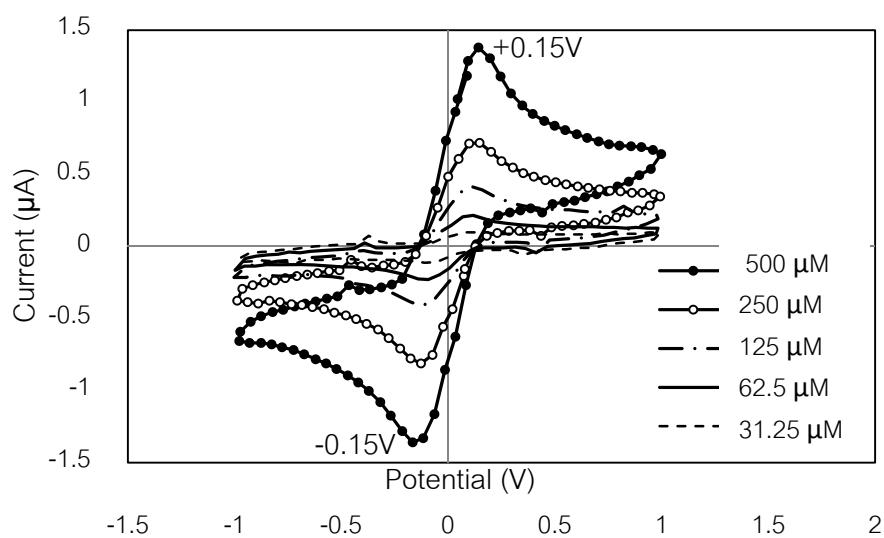Figure 4.3 Cyclic voltammogram of ABTS$^{\bullet+}$, ABTS and $K_2S_2O_8$ in PBS, pH 7.4

Figure 4.4 Cyclic voltammogram of different amount of ABTS$^{\bullet+}$.

Cyclic voltammogram of different amount of ABTS$^{\bullet+}$ is shown in Figure 4.4. The current signal was proportional to amount of ABTS$^{\bullet+}$ in both anodic and cathodic part of voltammograms especially at the potential of $\pm 0.15V$.

ABTS$^{\bullet+}$ could be measured by amperometric method while peroxyl radical could not. These results reflect the mechanism of the chemical reaction between antioxidant and these free radicals. Peroxyl radical is based on hydrogen atom transfer (HAT) reaction but ABTS$^{\bullet+}$ is based on electron transfer (ET) reaction. Since amperometric technique was directly measured electron in the reaction; therefore, amperometric technique could be used to measure ABTS$^{\bullet+}$.

1.2 Selection of Appropriate Bias Potential for Amperometric Measurement of ABTS$^{\bullet+}$

Amperogram of ABTS$^{\bullet+}$ which produced from ABTS in concentration range of 31.25-1000 μM is demonstrated in Figure 4.5. When ABTS$^{\bullet+}$ solutions were dropped on the electrode at 10th second, the current signals were rapidly increased. The current signals were collected until they were stable. Based on the data, current signals more than 100th second were close to current signal at 100th second. To reduce the variation of data, moving average (n=5) of current signals at 100th second was calculated and reported.
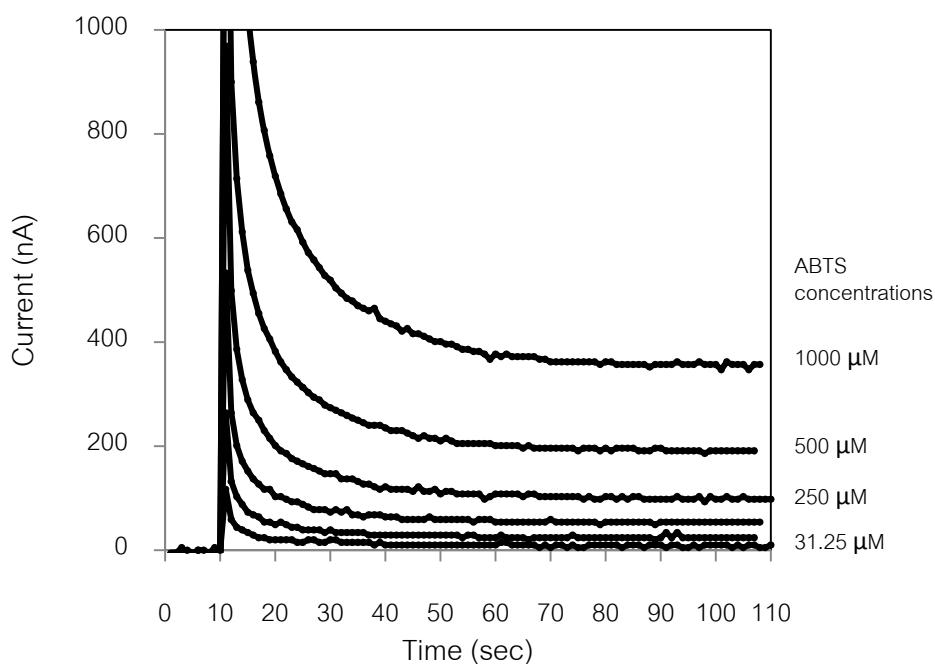
Figure 4.5 Amperogram of ABTS$^{\bullet+}$, bias potential of 0.15V

At bias potential of 0.15V and using ABTS 1000 μM, the current of ABTS$^{\bullet+}$ was 358 nA. The lower ABTS concentrations, the lower current signals were observed.

Once bias potentials were varying between 0.10, and 0.25 V, current signals were changed too. Relationship between ABTS concentrations and current signals with different bias potential could be plotted (Figure 4.6). Using potential 0.10V, the current signal was high enough to measure (ABTS 1000 μM yielded current 226 nA). The higher potential at 0.15, 0.20, and 0.25V, yielded current 358, 386 and 422 nA, respectively.

Bias potential of 0.15V yielded more current than potential of 0.10V about 50%, while potential of 0.20 and 0.25V yielded more current than potential of 0.15V only 10%. Moreover, at the higher potential, the more possible interference could present. So potential of 0.15V was chosen as a bias potential for ABTS$^{\bullet+}$ in amperometric technique.

Figure 4.6 Relationship plot between ABTS concentration and current signal using different bias potentials.

1.3 Determination of the Optimum Condition for ABTS$^{\bullet+}$ Production

    1.3.1   Determination of the proper ratio of $K_2S_2O_8$ for the production of ABTS$^{\bullet+}$

    According to Figure 4.7, the concentration of $K_2S_2O_8$ solution at 0.25 mM could generate the highest amount of ABTS$^{\bullet+}$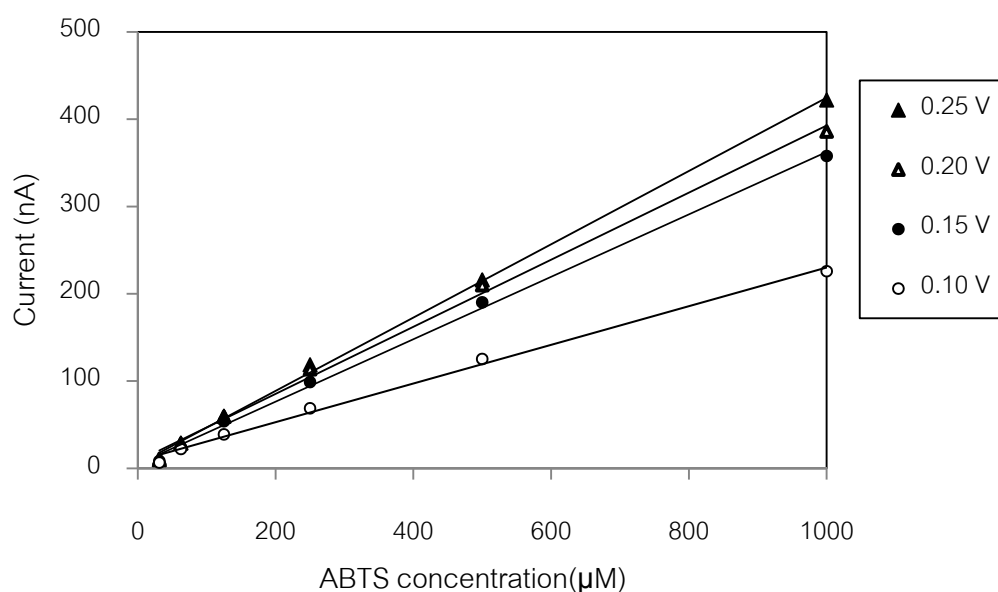 from 1.0 mM ABTS solution, such that the highest current signal was observed. Therefore, a molar ratio between ABTS and $K_2S_2O_8$ at 1:0.25 could produce the maximum amount of ABTS$^{\bullet+}$.

    To investigate the decreasing ABTS$^{\bullet+}$, ABTS$^{\bullet+}$ solutions were also measured by cyclic voltammetry. At fixed ABTS concentration of 1.0 mM, the generated ABTS$^{\bullet+}$ were increased, when more concentration of $K_2S_2O_8$ were used. Until the molar ratio was 1: 0.25, the current response was highest (Figure 4.8A). When using $K_2S_2O_8$ more than 0.25 mM, ABTS$^{\bullet+}$ were decreased and other substances could be generated (Figure 4.8B). There was other peak occurred at the potential of 0.45V. Moreover, a little green precipitate was found in these solutions. These substances could be one of the substances that have been reported by Pannala and Osman (Pannala *et al.*, 2001; Osman *et al.*, 2006). See more details in 1.1.2-chapter III.
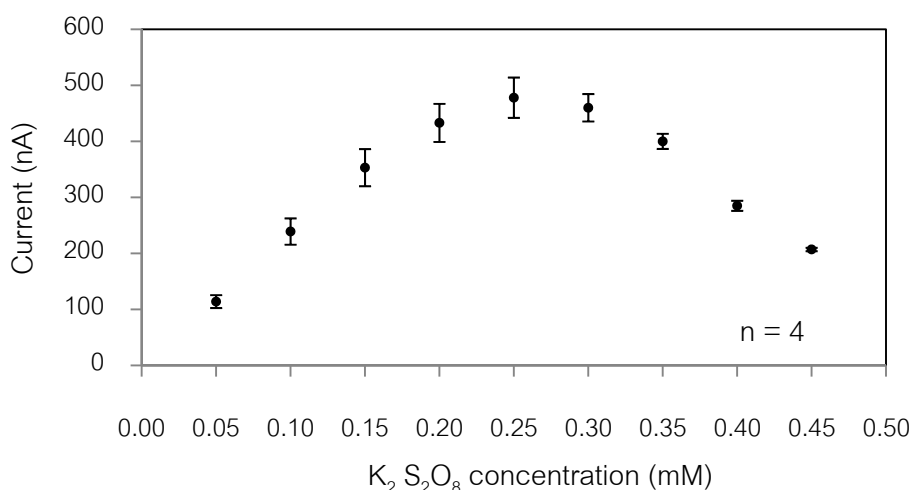
Figure 4.7 Current responses of ABTS$^{\bullet+}$ which generated from different concentration of $K_2S_2O_8$ and fixed ABTS concentration at 1.0 mM.
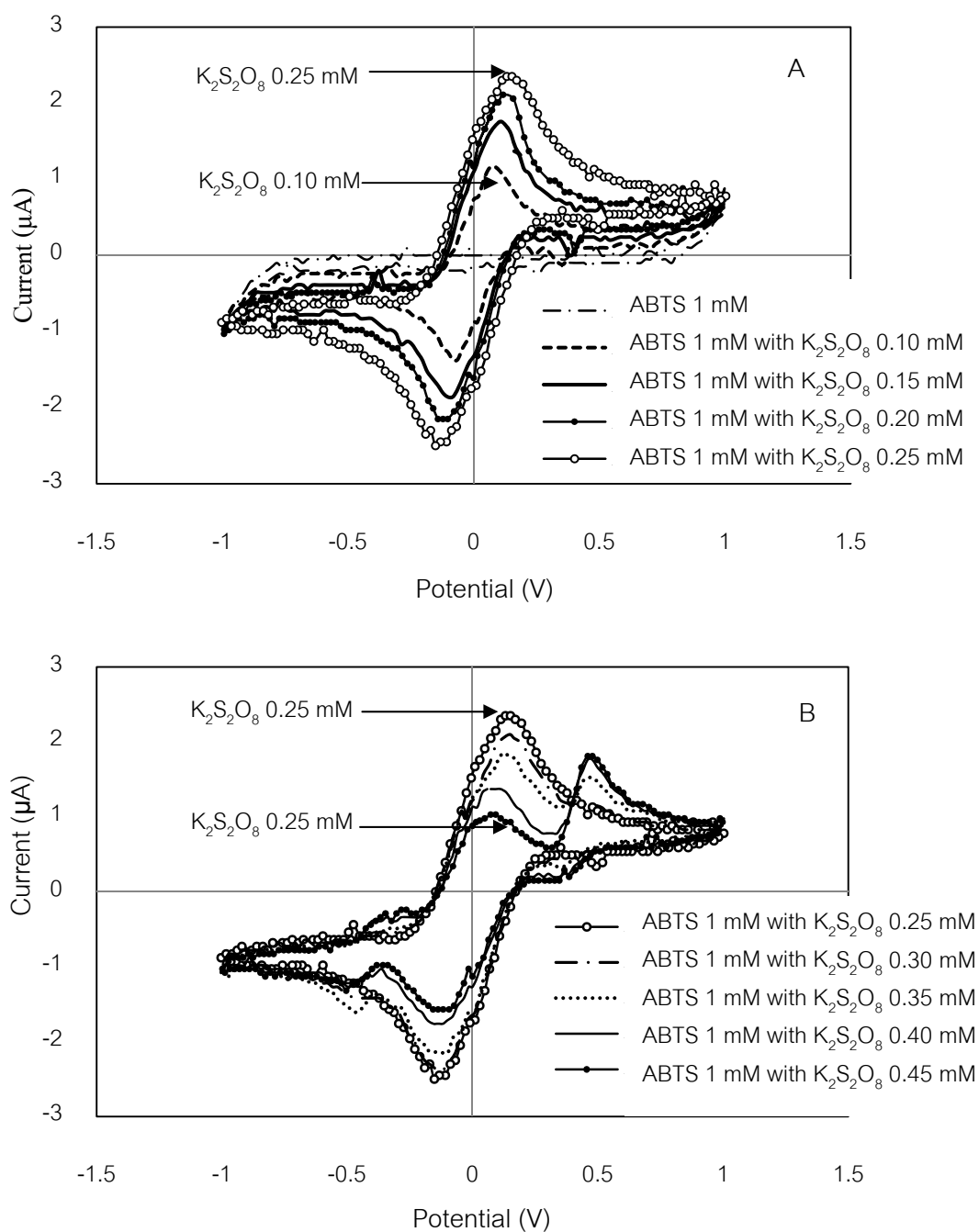
Figure 4.8 Cyclic voltammograms of ABTS$^{\bullet+}$ which were generated by different concentration of $K_2S_2O_8$. (A) Using $K_2S_2O_8$ 0.1-0.25 mM, (B) Using $K_2S_2O_8$ 0.25-0.45 mM.

Previous report for the production of ABTS$^{\bullet+}$, was using $K_2S_2O_8$ at a molar ratio of 1:0.35 (Re *et al.*, 1999). In this study, it was found that this ratio contributed low amount of ABTS$^{\bullet+}$ than the ratio of 1:0.25. As the result, ABTS$^{\bullet+}$ was supposed to be produced by ABTS/$K_2S_2O_8$ at a molar ratio of 1:0.25 which produced the highest ABTS$^{\bullet+}$.

1.3.2   Determination of the storage time and temperature of ABTS$^{•+}$

Stored at room temperature

Figure 4.9 shows current signals of generated ABTS$^{•+}$ (after mixed with $K_2S_2O_8$), when stored in the dark at room temperature for five days. The current was rapidly increased and reached maximum current (approximately500 nA) within 90 min as shown in small picture. After that, the current was slowly decreased and stable for 2-3 days. The stable current was approximately 270 nA.
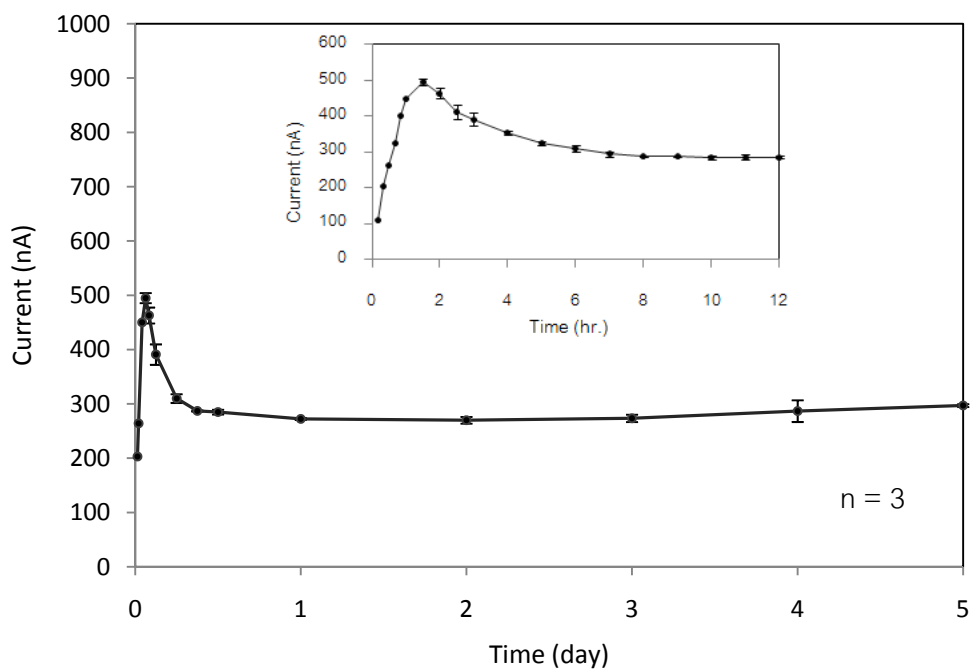
Figure 4.9 Current signal of ABTS$^{•+}$ versus time diagram when keep in dark at room temperature for 5 days after oxidation. The current signal in early 12 hr was demonstrated in small picture.

Stored at 4 ⁰C

The stability of ABTS$^{\bullet+}$ was improved when storing in the dark at 4$^{\mathbf{o}}$C. The satisfactory result is shown in figure 4.10. Oxidation of ABTS by $K_2S_2O_8$ occurred immediately, but the current was not maximal and stable until 12 hours was passed. The radical form of ABTS was stable at 4$^{\mathbf{o}}$C in the dark at least 3 days with only slightly decrease of current signal.

Temperature could affect the kinetic of ABTS$^{\bullet+}$. When stored at high temperature, the reaction between ABTS and $K_2S_2O_8$ was fast forward. The reaction of ABTS$^{\bullet+}$ was rapid both production and degradation. When stored at low temperature, the activity of ABTS$^{\bullet+}$ was slow which resulting in increase lifespan and stability. So ABTS$^{\bullet+}$ solution should be stored in the dark at 4$^{\mathbf{o}}$C.
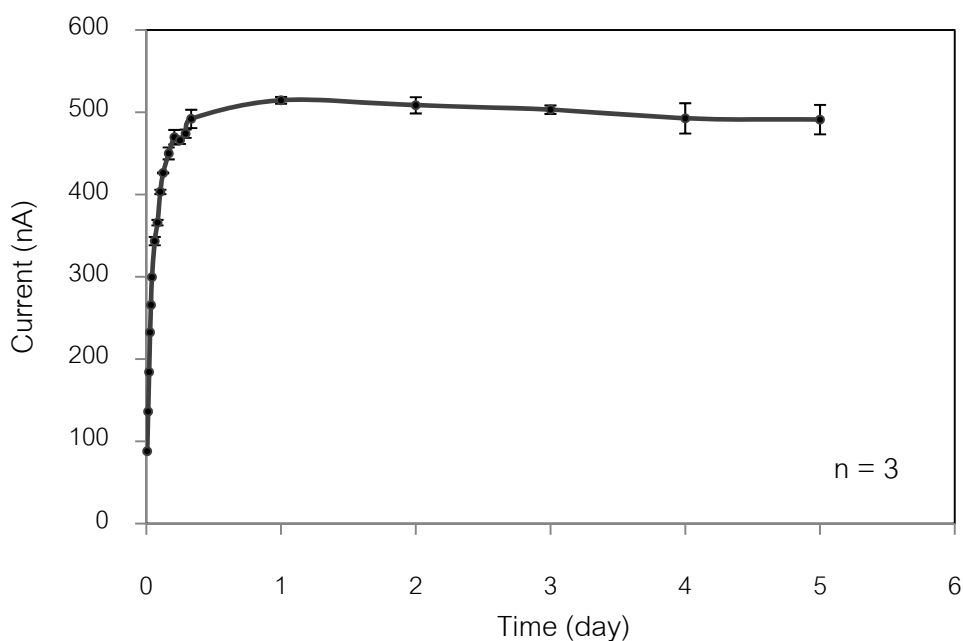
Figure 4.10 Current signal of ABTS$^{\bullet+}$ versus time diagram when keep in dark at 4$^{\mathbf{o}}$C for 5 days.

1.4 The Relationship between ABTS$^{\bullet+}$ Concentration and Responded Current.

The relationship between concentration of ABTS and current could be explained as the linear pattern with the linear range of 10-1000 µM (Figure 4.11). The representative regression line equation was: Current [nA] = 0.43Conc.[µM] + 3.35[nA] in which the coefficient of determination (R^2) = 0.9995.
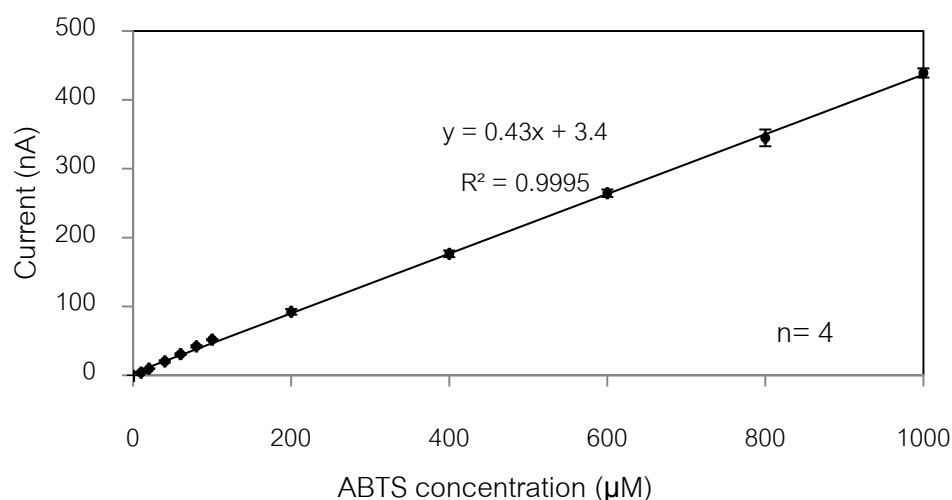
Figure 4.11 The linear relationship between ABTS concentration and current.

For the concentration of ABTS more than 1000 µM, the green precipitate was observed in solution. Hence, at fix molar ratio of ABTS/$K_2S_2O_8$ (1:0.25), the concentration of ABTS should not more than 1000 µM. Therefore, the condition used in developed amperometric method for antioxidant capacity would be as follows:

- Free radical generator was ABTS which produced ABTS$^{\bullet+}$ by reacting with $K_2S_2O_8$ at a molar ratio of 1:0.25. ABTS should not more than 1000 µM.
- ABTS$^{\bullet+}$ was prepared for 12 hours before use and could be stored in the dark at 4°C at least 3 days.
- Electrode surface area was 2mm x 5mm with analytical volume of 60 µL.
- Bias potential in amperometric method was 0.15V.
- Measurement time was 100 seconds.

2   Development of the Method for Antioxidant Capacity Measurement Using ABTS[•+].

2.1 Determination of Appropriate Amount of ABTS[•+] for Reacting with Antioxidant

The concentration of ABTS was studied. When using ABTS 500 and 1000 µM, the relationship between current and Trolox concentration could be plotted as linear pattern. However, there was non-linear line when using ABTS concentration of 2000 µM (Figure 4.12). Therefore, the linearity of Trolox standard curve when using different concentration of ABTS was as follows;

- ABTS 500 µM : 0-175 µM of Trolox

- ABTS 1000 µM : 100-400 µM of Trolox

- ABTS 2000 µM : 200-800 µM of Trolox

According to Figure 4.12, the standard curve of ABTS at 500 µM was the most linear. Although the current signal was low but it was enough to see the changes. Moreover, low volume of reagent was required. So the ABTS concentration of 500 µM was selected.
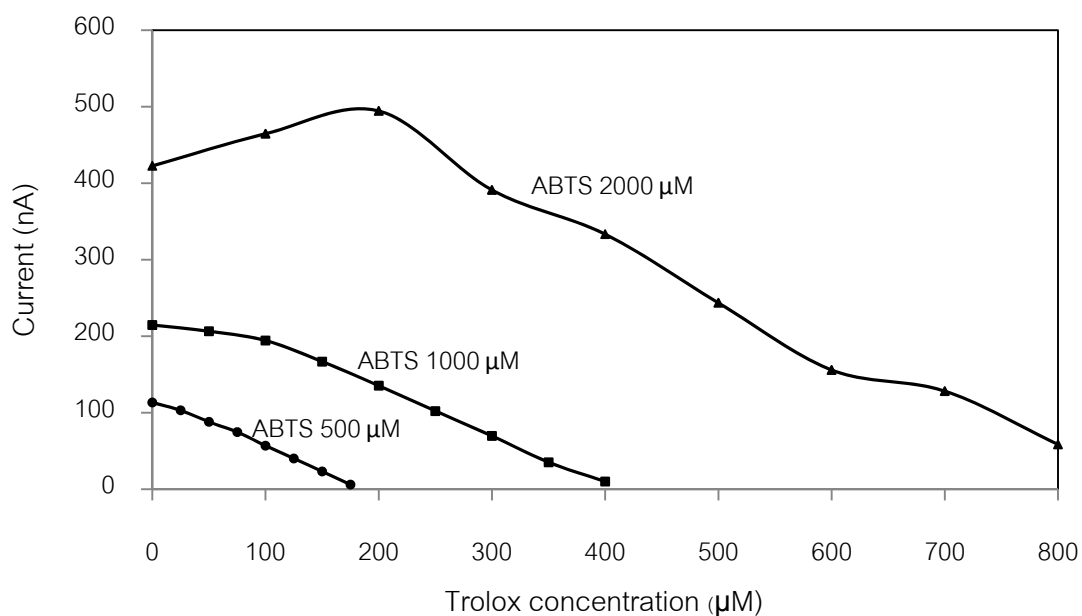
Figure 4.12 Trolox standard curves using ABTS concentration of 500, 1000 and 2000 µM

Current signals of measuring the diluted serum were shown in Table 4.1. There was relationship between ABTS concentration and serum dilution. ABTS concentration of 500 µM was supposed to be reacted with serum in dilution range of 1:160 to 1:13, while ABTS concentration of 1000 and 2000 µM was supposed to be reacted with serum in lower dilution. The dilution of serum would be determined in next experiment.

Table 4.1 Current signal of measuring the diluted serum, using ABTS concentration of 500, 1000 and 2000 µM

ABTS concentrations	Serum dilution	Current signal (nA)
500 µM	1: 160	108
	1: 80	95
	1: 40	70
	1:27	54
	1: 20	42
	1: 16	30
	1:13	23
1000 µM	1:80	180
	1:40	155
	1:20	115
	1:13	82
	1:10	57
	1:8	40
	1:6.7	24
2000 µM	1:40	364
	1:20	372
	1:10	341
	1:6.7	283
	1:5	255
	1:4	200
	1:3.3	172

2.2 Determination of Sample Dilution

Current signal of ABTS$^{\bullet+}$ when reacted with diluted serum are shown in Figure 4.13. The outline was linear when dilution of serum was in range 1:100 to 1:20, otherwise, the outline changes to curve when dilution was lower than 1:20. The more diluted sera exhibited higher current than the less diluted samples. It is explained that as the serum was diluted, the antioxidant capacity in serum was also diluted resulting the more leftover of ABTS$^{\bullet+}$, inducing the higher current.

This experiment fixed ABTS concentration at 500 µM. The dilution of sample should be more than 1:20 to sustain the higher measured current. However, the proper dilution of serum was 1:25 which provided current of ABTS$^{\bullet+}$ to be 50-60 nA. This current value was on a middle of calibration range. If antioxidant capacity in serum was more or lower than this average value, the currents were still in range of calibration cureve. The current of serum dilution was confirmed by thirty replications measurement of one sample. The current was 51 ± 4.9 nA, while the range of calibration curve was 10-130 nA. Therefore, serum dilution of 1:25 was selected to measure the antioxidant in an individual serum.
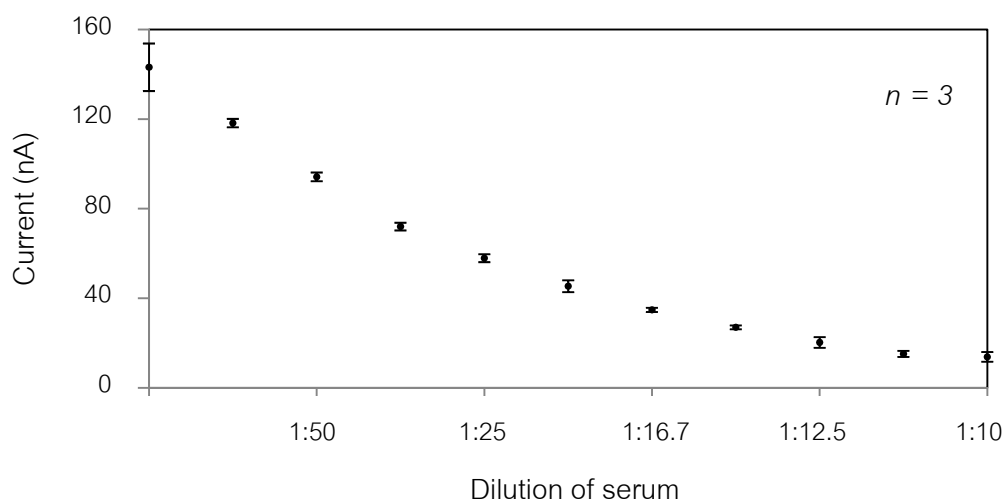
Figure 4.13 Current signals of diluted serum

2.3 Determination of an Appropriate Reaction Time

Rate of reaction between serum and ABTS$^{\bullet+}$ was fast in early 10 min but did not fully reach the equilibrium point within 30 min (Figure 4.14). Reaction rate at 20-30 min was slow especially of serum with low dilution. From data of Figure 4.14, antioxidant capacity of diluted serum was calculated in term of TE and plotted as shown in Figure 4.15. The immediate measurement obtained a good correlation between serum dilution and current.
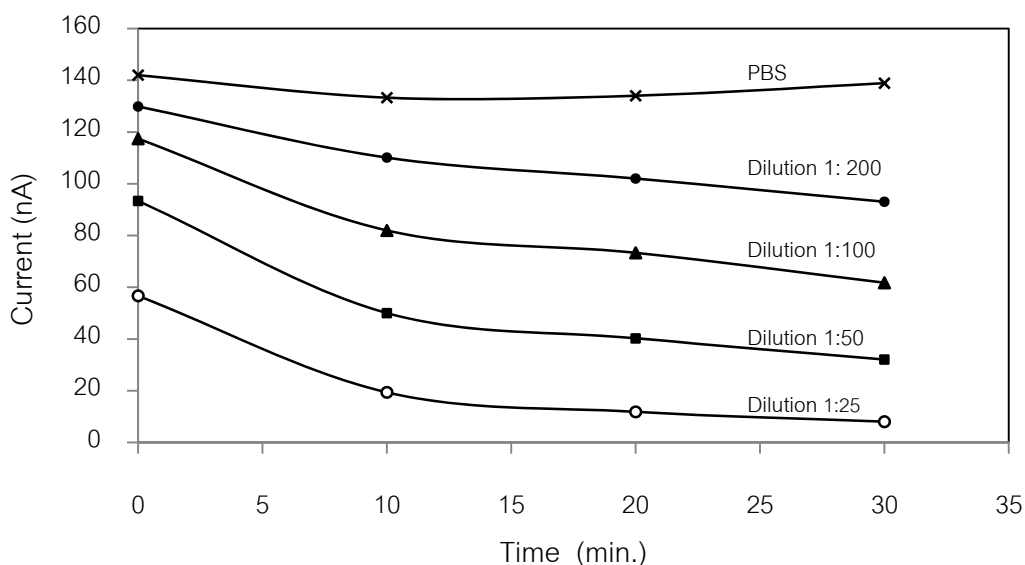
Figure 4.14 Rate of reaction between ABTS$^{\bullet+}$ and diluted serum
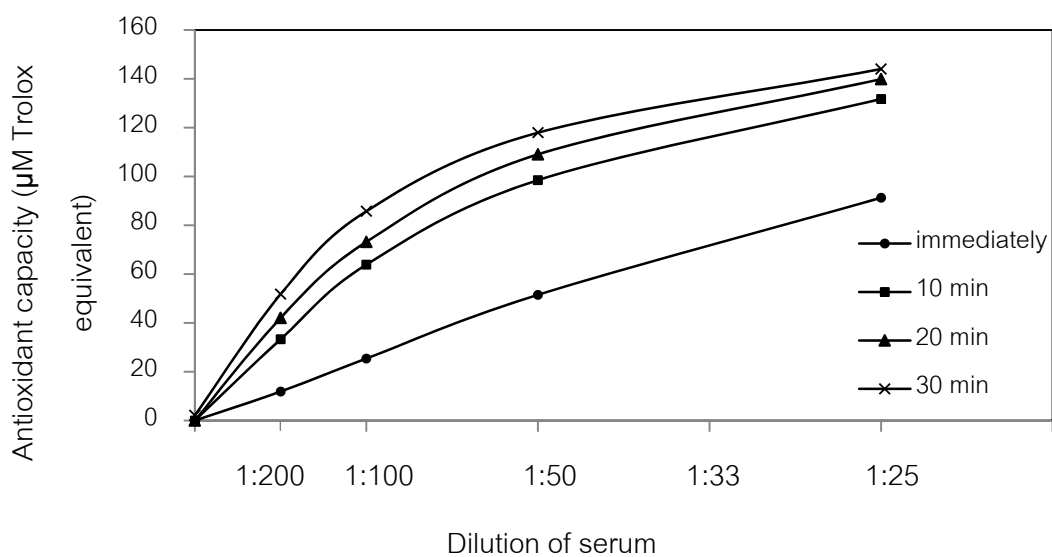
Figure 4.15 Antioxidant in diluted serum when measured at the different time.

Most TEAC values reported in the literature were measured at time intervals between 1 and 6 min (Ivekovic et al., 2005). Immediately measurement in this study showed that it was obtained a good correlation between current and serum dilution. Therefore, immediately measurement could indicate the different value of antioxidant capacity in serum.

Immediately measurement would result in lower antioxidant capacity than it should be. However, immediately measurement represented the capacity of fast reaction antioxidant. $ABTS^{\bullet+}$ reacts instantaneously with several antioxidants, such as Trolox, ascorbic acid, uric acid, cysteine, glutathione and bilirubin. In contrast, the $ABTS^{\bullet+}$ reacts slowly with albumin (Romay et al., 1996). The slow degradation of $ABTS^{\bullet+}$ at above 10 min might be due to the protein content of the serum samples.

The conditions that were found in method of development were as following:

- The appropriate concentration of ABTS was 500 μM.

- The proper dilution of serum was 1:25.

- The appropriate reaction time was immediately measurement.

3   Evaluation of the Developed Method

3.1 Determination of Linearity of the Method

The relationship between concentration of Trolox and current for $ABTS^{\bullet+}$ (500 μM) could be explained as the linear pattern with the linear range of 0-100 μM (Figure 4.16). Regression line has an equation of Current [nA] = -1.2Conc.[μM] + 134 with R² = 0.9966. To confirm the linear pattern, the %RSD of slope, intercept and R^2 were determined to be 3.80, 2.09 and 0.145, respectively (Table 4.2). All of the %RSD were within the limit range.

The serum was supposed to be diluted 1:25 before measurement, so the measurement range of antioxidant capacity in serum should be 0-2500 μM of Trolox equivalent.
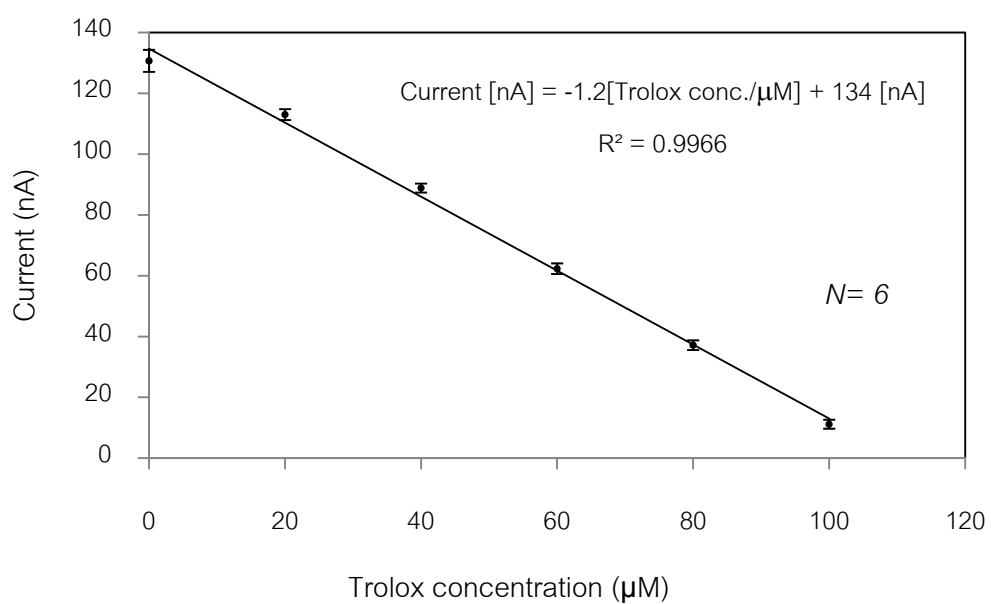
Figure 4.16 Calibration curve of Trolox.

Table 4.2 The current signal of antioxidant calibration curve

N	Slope	Intercept	R^2
1	-1.26	138	0.9978
2	-1.27	138	0.9974
3	-1.15	130	0.9940
4	-1.24	135	0.9957
5	-1.18	134	0.9964
6	-1.19	134	0.9950
Mean	-1.22	135	0.9961
SD	0.05	2.8	0.0014
%RSD	3.80	2.1	0.1450

3.2 Accuracy and Precision of the Developed Method

3.2.1 Intra-day accuracy and precision

% biases in the same day of Trolox in PBS were ranged from -9.20 to +9.60 for the three Trolox concentrations (Table 4.3). Percentages of relative standard deviation in the same day were between 3.8 and 7.7 (Table 4.4). Therefore, this method for standard Trolox analysis was accurate and precise enough for applicable use in the same day.

3.2.2 Inter-day accuracy and precision

% biases at three difference days of Trolox in PBS were ranged from -23.20 to +8.0 (Table 4.5). Percentages of relative standard deviation in inter-day precision were between 0.9 and 17.1 (Table 4.6).

Both accuracy and precision of intra-day were within the limit range (±10% of %bias and 10% of %RSD). However, precision of inter-day were not acceptable at 25.0 μM which had %RSD of 17.1. It was possible that when low concentration of Trolox was analyzed, the current of ABTS$^{\bullet+}$ solution was high and had more variation.

Table 4.3 The intra-day accuracy of analysis of standard Trolox in PBS

Trolox concentration (μM)	%Bias (n=6)						Mean
	1	2	3	4	5	6	
25.0	-9.2	+4.0	+8.0	+9.6	-4.4	-5.2	+0.5
50.0	-2.4	+5.4	-3.6	+3.6	+2.4	+5.0	+1.7
75.0	+6.3	+0.3	+3.2	-6.1	+6.0	+2.4	+2.0

Table 4.4 The intra-day precision of analysis of standard Trolox in PBS

Trolox concentration (µM)	Analyzed Trolox concentration (n=6)						Mean	SD	%RSD
	1	2	3	4	5	6			
25.0	22.7	26.0	27.0	27.4	23.9	23.7	25.1	1.9	7.7
50.0	48.8	52.7	48.2	51.8	51.2	52.5	50.9	1.9	3.8
75.0	79.7	75.2	77.4	70.4	79.5	76.8	76.5	3.4	4.5

Table 4.5 The inter-day accuracy of analysis of standard Trolox in PBS

Trolox concentration (µM)	%Bias (3 days)			Mean
	1	2	3	
25.0	+8.0	-9.6	-23.2	-8.3
50.0	+3.6	+5.2	+3.4	+4.1
75.0	+3.2	+6.8	-2.3	+2.6

Table 4.6 The inter-day precision of analysis of standard Trolox in PBS

Trolox concentration (µM)	Analyzed Trolox concentration (3 days)			Mean	SD	%RSD
	1	2	3			
25.0	27.0	22.6	19.2	22.9	3.9	17.1
50.0	51.8	52.6	51.7	52.0	0.5	0.9
75.0	77.4	80.1	73.3	76.9	3.4	4.5

3.3 The Replicated Use of Electrode

Figure 4.17 shows current of thirty replications of single electrode. The current signals were stable at the beginning and slightly decreased at the end. %RSD of thirty current signals was 4.9 and could be decreased to 4.5 and 2.1 if an electrode was used only twenty and ten times, respectively (Table 4.7). As a result, this SCPE could be used at least thirty times with acceptable precision.
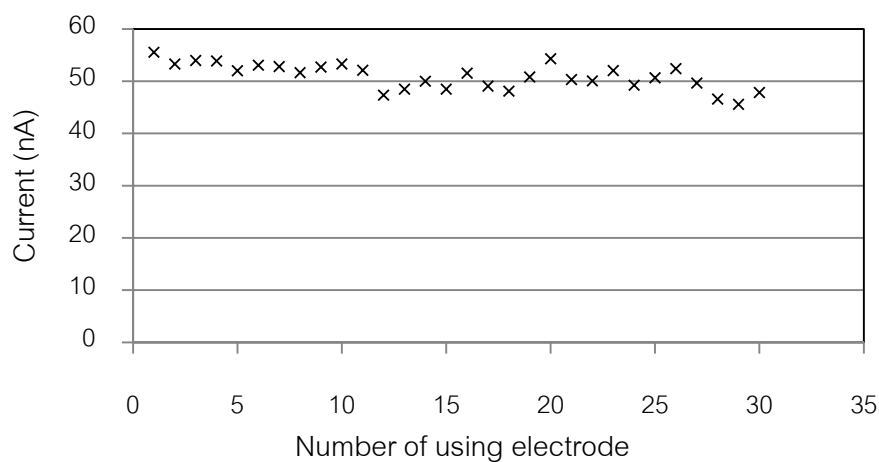
Figure 4.17 Current signal of replicated use of electrode

Table 4.7 The precision of observed current signal after using the same electrode

Number of using electrode (number of times)	Mean of current signal (nA)	S.D.	% RSD
10	53.2	1.1	2.1
20	51.6	2.3	4.5
30	50.9	2.5	4.9

4   Application of the Developed Method to Determine Antioxidant Capacity in Human
    Serum.

4.1 Antioxidant Capacity of Normal Subjects and Patients

Using the developed method, antioxidant capacity of normal subjects and patients are shown in Table 4.8 and 4.9, respectively. The mean(SD) values of antioxidant capacity in normal subjects and patients were determined to be 1486 ± 65 and 1354 ± 85 µM TE, respectively.

The developed method was successfully used for determining antioxidant capacity of Thai normal subjects (n=30) and patients (n=5). The mean value of antioxidant capacity in patients was lower than normal subjects. Therefore, the method was able to discriminate antioxidant capacity in these two groups which were normal and low antioxidant capacity value.

Table 4.8 Antioxidant capacity of normal subjects

No.	Gender	Age (years)	Current signal (nA)	Trolox equivalent (μM)
1	Male	15	63	1480
2	Male	20	58	1569
3	Male	25	65	1425
4	Male	27	67	1398
5	Male	28	62	1494
6	Male	31	59	1555
7	Male	38	59	1549
8	Male	39	63	1466
9	Male	39	58	1576
10	Male	40	58	1576
11	Male	45	63	1466
12	Male	46	70	1329
13	Male	47	65	1432
14	Male	56	66	1412
15	Male	59	61	1507
16	Female	13	63	1466
17	Female	18	64	1453
18	Female	19	57	1590
19	Female	19	62	1494
20	Female	21	65	1439
21	Female	25	60	1535
22	Female	27	64	1459
23	Female	28	60	1542
24	Female	29	67	1391
25	Female	32	60	1542
26	Female	32	59	1549
27	Female	39	66	1418
28	Female	40	59	1549
29	Female	44	64	1459
30	Female	49	63	1466
Mean ± SD		33 ± 12	62 ± 3	1486 ± 65

Table 4.9 Antioxidant capacity of patients

No.	Gender	Ages (years)	Current signal (nA)	Trolox equivalent (µM)
1	Male	42	69	1350
2	Female	51	67	1384
3	Male	71	70	1322
4	Female	74	74	1240
5	Female	77	63	1473
Mean± SD		63 ±16	69 ± 4	1354 ± 85

There were reports of antioxidant capacity value in human serum and plasma using TAS kit (Total antioxidant status by Randox). The principle of TAS was based on ABTS free radical and spectrophotometric detection. Mean value of antioxidant capacity in normal subjects measured by this developed method was closed to TAS method (Table 4.10). While antioxidant capacity in patient group was lower than normal group. Therefore, this amperometric technique could be utilized for determining antioxidant capacity in human serum as same as other technique.

Table 4.10 Published antioxidant capacity based on TAS method

Samples	Trolox equivalents (µM)		Refference
	Normal group	Pathological group	
Serum	1220 ± 110	1360± 220	Farriol *et al.,* 2001
Serum	1600 ± 180	141$_0$ ± 280	Rocha-Pereira *et al.,* 2001
serum	1690 ± 200	1050 ± 260	Dasgupta *et al.,* 1997
Serum	1730 ± 200	1580 ±100	Mahle *et al.,* 1997
Plasma (heparin)	1760 ± 190	1520 ± 140	van Bakel *et al.,* 2000
Serum	**1486 ± 65**	**1353 ± 85**	**This thesis**

4.2 Determination of Antioxidant Capacity in Spiked Serum with α-Tocopherol

A human serum with spiked α-tocopherol had antioxidant capacity higher than the original serum. Antioxidant capacity in serum and spike serum with α-tocopherol 1000, 1500 and 2500 μM were determined to be 1631, 2095, 2406 and 2988, respectively (Figure 4.18).
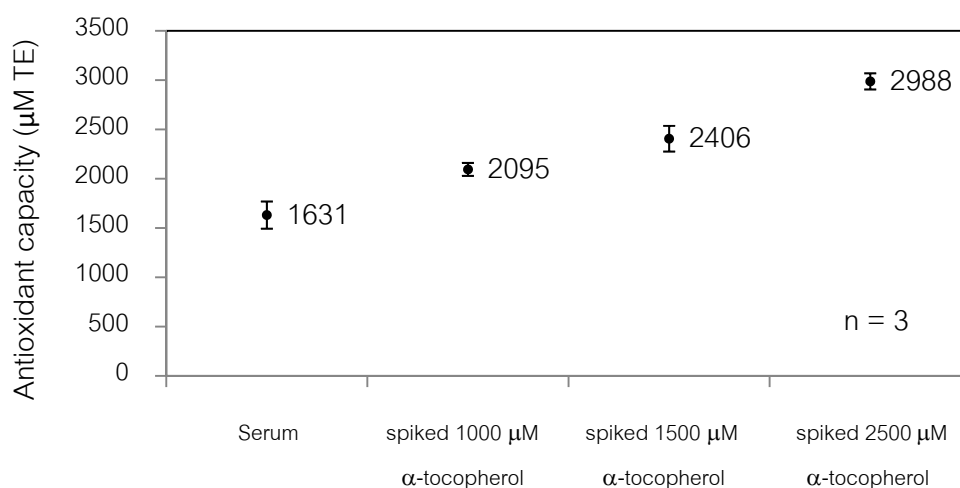
Figure 4.18 Chart of antioxidant capacity in serum with spiked α-tocopherol

The developed method could be determined of antioxidant capacity in various statuses of human sera. The method was sensitive enough to indicate the normal, low or high value of antioxidant capacity in serum. Therefore, the developed method will be utilized to determine antioxidant status of normal subjects, patient and people with supplemental antioxidant.

CHAPTER V

CONCLUSION

The new, simple and versatile amperometric technique for determining antioxidant capacity in human serum was developed, using ABTS as free radical generator. $ABTS^{\bullet+}$ exhibited electroactivity which was measured at bias potential of 0.15V. The advantages of this method were less time of analysis, no problem of sample color and did not use of enzyme.

$ABTS^{\bullet+}$ was produced from oxidation of ABTS with $K_2S_2O_8$ at the molar ratio of 1:0.25. This ratio could produce maximal $ABTS^{\bullet+}$ which was stable when stored in the dark at $4^{\circ}C$ at least 3 days.

The reaction used $ABTS^{\bullet+}$ which was generated from ABTS concentration of 500 μM, while serum was supposed to be diluted with PBS 1:25 before measurement. The linear range of Trolox calibration curve was 0-100 μM. Because serum was diluted before measurement, so the determination range of antioxidant capacity in serum should be 0-2500 μM TE. The reaction between $ABTS^{\bullet+}$ and diluted serum was immediately measured by SCPE connected to potentiostat. The measuring time was 100 seconds.

A SCPE could be used at least thirty times with acceptable precision. It was over expected from the proposal on disposable use of electrode. The intra-day and inter-day precision of method was precise enough to indicate value.

This amperometric method was successfully used for determining antioxidant capacity in normal subject and patient. Moreover, the antioxidant capacity in normal subject using this developed method was closed to the values of different techniques that have been reported. Therefore, amperometric technique could be utilized for the study of antioxidant capacity in serum as well as other techniques. This method was also able to measure antioxidant capacity in spiked serum with α-tocopherol. Therefore,

the developed method was sensitive enough to indicate the normal, low and high value of antioxidant in serum.

This study was an innovation. This amperometric method did not use enzyme in any method. Moreover, SCPE which could be produced as mass production was used. So, it was a low cost instrument for determining antioxidant capacity. $ABTS^{\bullet+}$ was measured by amperometric technique. So, it was no sample's color and turbidity problem. This innovation may be utilized for routine check-up of antioxidant capacity in human serum.

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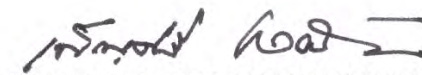APPENDICES

Appendix i

วันที่ 15 สิงหาคม พ.ศ. 2553

เรื่อง ขอซีรั่มเหลือใช้ของผู้ป่วยเพื่อนำไปในงานวิจัยของนิสิต

เรียน หัวหน้าห้องปฏิบัติการกลาง โรงพยาบาลวิชัยยุทธ

เนื่องด้วย นายไชยนรินทร์ พงษ์ประยูร นิสิตปริญญาโท หลักสูตรวิศวกรรมชีวเวช บัณฑิตวิทยาลัย จุฬาลงกรณ์มหาวิทยาลัย ได้ทำงานวิจัยเกี่ยวข้องกับการตรวจวัดประสิทธิภาพสารต้านอนุมูลอิสระในซีรั่ม มี ความจำเป็นต้องใช้ซีรั่มในงานวิจัยดังกล่าว จึงใคร่ขอความอนุเคราะห์ซีรั่มที่เหลือใช้จากการวิเคราะห์และ ข้อมูลด้านสุขภาพของผู้ป่วยที่มาใช้บริการจากห้องปฏิบัติการกลาง รพ.วิชัยยุทธ จำนวน 25-30 ตัวอย่าง โดย ใช้ตัวอย่างละไม่เกิน 5 มิลลิลิตร ทั้งนี้ข้อมูลด้านสุขภาพและผลการวิเคราะห์ทุกอย่างของผู้ป่วยทุกรายจะถูก ปิดเป็นความลับ และไม่ระบุว่าเป็นของผู้ป่วยรายใด

จึงเรียนมาเพื่อขอความอนุเคราะห์ และขอขอบพระคุณเป็นอย่างสูงมา ณ ที่นี้ด้วย

ขอแสดงความนับถือ
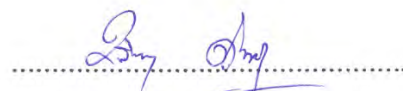
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(รองศาสตราจารย์ ดร.เพ็ญศรี ทองนพเนื้อ)
อาจารย์ที่ปรึกษาวิทยานิพนธ์

หัวหน้าห้องปฏิบัติการกลาง โรงพยาบาลวิชัยยุทธ

☑ ให้ความอนุเคราะห์

☐ ไม่ให้ความอนุเคราะห์

☐ อื่น ๆ ....

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(ทนพญ.มัลลิกา ดำรงวิริยะกุล)
หัวหน้าห้องปฏิบัติการกลาง รพ.วิชัยยุทธ

Appendix ii

Table A1. The current signals of ABTS$^{\cdot+}$ by amperometric measurement with different
bias potential.

Bias potential (V)	ABTS$^{\cdot+}$ concentration (µM)					
	31.25	62.5	125	250	500	1000
0.10	7	22	39	69	125	226
0.15	8	24	54	99	190	358
0.20	11	28	59	114	210	386
0.25	10	29	60	119	216	422

Appendix iii

Table A2. Current signal of ABTS$^{\bullet+}$ when reacted with diluted serum (from 2.2 determination of sample dilution)

Serum dilution	Current (nA) (n=3)
1:100	118 ± 1.9
1:50	94 ± 1.9
1:33	72 ± 1.7
1:25	58 ± 1.8
1:20	45 ± 2.6
1:17	35 ± 0.9
1:14	27 ± 0.9
1:13	20 ± 2.4
1:11	15 ± 1.3
1:10	14 ± 2.2

Appendix iv

Table A3. The current signal of Trolox calibration curve.

N	Current for ABTS$^{•+}$ (nA)					
	Trolox 0 µM	Trolox 20 µM	Trolox 40 µM	Trolox 60 µM	Trolox 80 µM	Trolox 100 µM
1	135	113	91	61	37	10
2	134	114	90	62	36	9
3	125	110	87	63	37	12
4	131	115	88	60	35	11
5	130	112	89	65	39	13
6	129	114	88	63	39	12

VITA

Mr. Chainarin Phongprayoon was born on March 24, 1982 in Rachaburi, Thailand. He had received his Bachelor of Science in Medical Technology with second class honors in 2005 from Faculty of Allied Health Sciences, Chulalongkorn University, Thailand. After his graduation, he has worked in Central Laboratory, Vichaiyut Hospital, Bangkok, Thailand.