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DEVELOPMENT OF ELECTROCHEMICAL DETECTION OF ANTIOXIDANT

IN HERBAL PLANTS

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ในงานวิจัยนี้ขั้วไฟฟ้าคาร์บอนแบบพิมพ์สกรีนดัดแปรด้วยแกรฟีนและพอลิ 3,4-เอทิลีนไดออกซี ไทโอฟีน : พอลิสไตรีนซัลโฟเนต ได้ถูกพัฒนาขึ้นสำหรับนำไปใช้ในการหาปริมาณสารต้านอนุมูลอิสระผ่าน การตรวจวัดอนุมูลอิสระดีพีพีเอช ในขั้นตอนแรกได้ศึกษาหาภาวะที่เหมาะสมสำหรับดัดแปรขั้วไฟฟ้าด้วย เทคนิคอิเล็กโตรสเปรย์ เช่น ปริมาณของพอลิ 3,4-เอทิลีนไดออกซีไทโอฟีน : พอลิสไตรีนซัลโฟเนต, ปริมาณ ของแกรฟีน, อัตราส่วนของพอลิ 3,4-เอทิลีนไดออกซีไทโอฟีน : พอลิสไตรีนซัลโฟเนตและแกรฟีน, เวลาที่ใช้ ในการสเปรย์ นอกจากนี้ได้ศึกษาลักษณะสัณฐานวิทยาของขั้วไฟฟ้าดัดแปรด้วยแกรฟีนและพอลิ 3,4-เอทิลีน ไดออกซีไทโอฟีน : พอลิสไตรีนซัลโฟเนต โดยใช้กล้องจุลทรรศน์อิเล็กตรอนแบบส่องกราด และกล้อง จุลทรรศน์อิเล็กตรอนแบบส่องผ่าน ต่อมาเทคนิคไซคลิกโวลแทมเมตรีได้ถูกนำมาใช้สำหรับตรวจวัดทาง เคมีไฟฟ้าของขั้วไฟฟ้าที่ดัดแปรด้วยแกรฟีนและพอลิ 3,4-เอทิลีนไดออกซีไทโอฟีน : พอลิสไตรีนซัลโฟเนต ด้วยสารละลายมาตรฐานเฟอโรไซยาไนด์ พบว่า ขั้วไฟฟ้าที่ดัดแปรด้วยแกรฟีนและพอลิ 3,4-เอทิลีนไดออกซี ไทโอฟีน : พอลิสไตรีนซัลโฟเนต มีความไวในการตรวจวัดที่สูงกว่าขั้วไฟฟ้าที่ไม่ได้ดัดแปรถึง 3 เท่า นอกจากนี้ค่าความแตกต่างของศักย์ไฟฟ้าของสัญญาณแอโนดิกและแคโทดิกของขั้วไฟฟ้าดัดแปรแกรฟีน และพอลิ 3,4-เอทิลีนไดออกซีไทโอฟีน : พอลิสไตรีนซัลโฟเนตนั้นมีค่าลดลง แสดงให้เห็นว่าขั้วไฟฟ้าที่ดัด แปรด้วยแกรฟีนและพอลิ 3,4-เอทิลีนไดออกซีไทโอฟีน : พอลิสไตรีนซัลโฟเนตนั้นมีความสามารถในการ ส่งผ่านอิเล็กตรอนได้ดีขึ้น หลังจากนั้นนำขั้วไฟฟ้าดัดแปรไปประยุกต์ใช้ในการตรวจวัดสารต้านอนุมูลอิสระ ในสารตัวอย่างจริงด้วยเทคนิคโครโนแอมเพอโรเมตรี โดยใช้ศักย์ไฟฟ้าในการตรวจวัดเท่ากับ 0.20 โวลต์ สำหรับการหาความสัมพันธ์เชิงเส้นตรงได้จากการสร้างกราฟมาตรฐานระหว่างค่ากระแสและความเข้มข้น ของโทรล็อกซึ่งเป็นสารต้านอนุมูลอิสระมาตรฐานอยู่ในช่วง 5-30 ไมโครโมลาร์ มีค่าขีดจำกัดในการตรวจวัด (S/N=3) เท่ากับ 0.59 ไมโครโมลาร์ และขีดจำกัดในการวัดเชิงปริมาณ (S/N=10) เท่ากับ 1.97 ไมโครโมลาร์ สุดท้ายนี้ได้นำระบบขั้วไฟฟ้าที่พัฒนาขึ้นไปใช้ในการหาปริมาณสารต้านอนุมูลอิสระในสารสกัดสมุนไพรและ เครื่องดื่ม โดยผลการทดลองที่ได้มีความสอดคล้องกับผลที่ได้จากสเปกโตรโฟโตเมตรีซึ่งเป็นวิธีมาตรฐาน

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In this work, graphene/poly (3,4-ethylenedioxythiophene): poly (styrenesulfonate) modified screen-printed carbon electrode (G/PEDOT:PSS/SPCE) was developed for the determination of total antioxidant capacity (TAC) via 2,2-diphenyl-1-pycrilhydrazil (DPPH) assay. Initially, the parameters of electrospraying used for electrode fabrication (*e.g.* amount of PEDOT:PSS loading, amount of G loading, ratio of PEDOT:PSS/G and electrospraying time) were optimized. Then, G/PEDOT:PSS/SPCE was characterized by scanning electron microscopy (SEM) and transmission electron microscopy (TEM). Cyclic voltammetry (CV) was performed for electrochemical characterization of G/PEDOT:PSS/SPCE using a standard ferricyanide solution. G/PEDOT:PSS/SPCE showed approximately 3 folds higher current response compared to an unmodified SPCE, verifying that this proposed electrode can increase the electrochemical sensitivity of the system. Moreover, the peak potential difference between anodic and cathodic peaks (ΔE_p) of G/PEDOT:PSS/SPCE substantially decreased, indicating that the G/PEDOT:PSS nanostructure can accelerate the electron transfer kinetics of the system. For the evaluation of TAC in real samples, chronoamperometry was carried out at a detection potential of 0.20 V. The linearity between Trolox concentration and cathodic current response in a range of 5-30 µM was obtained. Limit of detection (LOD) and limit of quantitation (LOQ) using signal-to-noise ratios of three (S/N=3) and ten (S/N=10) were found to be 0.59 µM and 1.97 µM, respectively. Finally, this system was successfully applied for the determination of TAC in herb and herbal beverages and the results correspond well with a conventional UV-Visible spectroscopic method.

Field of Study: Biotechnology	Student's Signature ....
Academic Year: 2014	Advisor's Signature ....
	Co-Advisor's Signature ....
	Co-Advisor's Signature ....

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CONTENTS

LIST OF ABBREVIATIONS

Ag/AgCl	silver/silver chloride
CE	counter electrode
cm	centimeter
CV	cyclic voltammetry
°C	degree celcius
DMF	dimethylformamide
DPPH	2,2-diphenyl-1-picrylhydrazyl
E	potential
EPBS	ethanolic phosphate buffer solution
I_{pc}	cathodic peak current
G	graphene
g	gram
h	hour
HPLC	high performance liquid chromatography
KCl	potassium chloride
kV	kilovolt
LOD	limit of detection
LOQ	limit of quantitation
M	molar
mg	milligram

mM	millimolar
mL	milliliter
mV	millivolt
NaCl	sodium chloride
nm	nanometer
PEDOT:PSS	poly (3,4-ethylenedioxythiophene) : poly (styrenesulfonate)
PVP	polyvinylpyrrolidone
RE	reference electrode
RSD	relative standard deviation
s	second
SD	standard deviation
SEM	scanning electron microscope
SPCE	screen printed carbon electrode
SWV	square wave voltammetry
TAC	total antioxidant capacity
V	volt
WE	working electrode
μA	microampere
μM	micromolar

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CHAPTER I

INTRODUCTION

1.1 Introduction

Free radicals are molecules containing unpaired electrons which are extremely harmful to the human health. They can lead to various diseases such as cancer, cardiovascular disease and inflammatory disorder [1]. The antioxidant neutralizes adverse free radicals by either donating a hydrogen atom or a single electron transfer mechanism [2]. Primary natural sources of antioxidants are vegetables, fruits, and herbs such as vitamin C, vitamin E, carotene and phenolic acid [3]. The intake plants and foods containing an appropriate amount of antioxidant play an essential role as a source of antioxidant [4]. Therefore, researchers have continuously developed various analytical approaches for evaluation of antioxidant capacity in these natural products.

Several techniques have been reported for the determination of total antioxidant capacity (TAC), including high performance liquid chromatography (HPLC) [5], chemiluminescence spectroscopy [6], fluorescence spectroscopy [7] and spectrophotometry [8]. Common spectrophotometric assays have been introduced for the determination of TAC using 2,2'-azino-bis-(3-ethylbenzothiazoline)-6-sulfonic acid (ABTS) [9] and 2,2-diphenyl-1-picrylhydrazyl (DPPH) [10], which react directly and rapidly with most antioxidant compound [11]. Nevertheless, the highly colored or turbid sample is difficult to measure for spectroscopic method. Electrochemistry is an attractive and alternative technique because of its rapid analysis, high sensitivity, minimal power demand, ease of use, portability [5] and this technique is undisturbed by turbidity and color of sample solution [6]. However, conventional electrochemical systems are usually designed to be a small size for portability,

leading to decrease of electrochemical sensitivity. To increase the electrochemical sensitivity, the development of high surface area electrode while maintaining a small size is greatly required.

Nanomaterials, such as metallic nanoparticle, carbon nanotube (CNT), graphene (G), have been used for electrode modification to increase the surface area and electrochemical sensitivity [7-9]. Especially, graphene (G) has been considered as an attractive nanomaterial for electrode modification due to its large surface area, high electrical conductivity, strong mechanical strength and good biocompatibility. Nevertheless, the pure form of G has high tendency to agglomerate to graphite through p–p stacking and van der Waals interactions. To solve this problem, conducting polymers have been used to form the composite with G including polypyrrole (PPy) [12], polyaniline (PANI) [13], and poly (3,4-ethylenedioxythiophene): poly (styrenesulfonate) (PEDOT:PSS) [14]. PEDOT:PSS is selected for this work because it has good conductivity, low redox potential, biocompatibility, high electrochemical and thermal stability [12]. Moreover, the combination between PEDOT:PSS and G can provide excellent electrochemical performance [12]. To further increase the active surface area of electrode, electrospraying fabrication was selected for electrode surface modification. This method produces three dimensional droplet-like nanostructure on electrode surface by applying high voltage electric filed, leading to increase surface area of electrode and enhance the electrochemical sensitivity of the system [16].

Here, an electrochemical sensor fabricated by electrospraying of graphene/poly (3,4-ethylenedioxythiophene) : poly (styrenesulfonate) modified screen-printed carbon electrode (G/PEDOT:PSS/SPCE) coupled with DPPH assay was developed and used for TAC determination. In this system, we measured the cathodic current response of DPPH on G/PEDOT:PSS/SPCE using chronoamperometry.

This novel electrochemical sensor was successfully applied for TAC determination in herb and herbal beverages and the results corresponded well with conventional UV-Vis spectrophotometric results.

1.2 Objective

- To develop a novel electrochemical sensor based on G/PEDOT:PSS/SPCE along with DPPH assay for TAC determination.

- To apply G/PEDOT:PSS/SPCE based electrochemical sensor for the determination of TAC in herb and herbal beverages.

1.3 Scope of the thesis

In this study, we developed a novel electrochemical sensor of G/PEDOT:PSS on SPCE using electrospraying fabrication for the determination of TAC by chronoamperometric detection of DPPH. The parameter such as amount of G loading, amount of PEDOT:PSS loading, ratio of G/PEDOT:PSS and electrospraying time were optimized. Then, the electrochemical behavior and morphology of the developed electrode was investigated. The optimized G/PEDOT:PSS/SPCE was applied to determine TAC in herb and herbal beverages. Finally, the results were compared with conventional UV-Vis spectrophotometry.

CHAPTER II

THEORY AND LITERATURE SURVEY

This chapter introduces an antioxidant, a target analyte of this study. The basic principles and definitions of electrochemical techniques (*i.e.* cyclic voltammetry, square-wave voltammetry and amperometry) are described. The materials used for electrode modification such as graphene, poly (3,4-ethylenedioxythiophene) : poly (styrenesulfonate) and electrospraying fabrication are also explained.

2.1 Antioxidants

An antioxidant may be defined as a substance that can reduce the risk of degenerative diseases arising from oxidation process including cardiovascular disease, cancer, cataract, age-related muscular degeneration and rheumatoid arthritis. The oxidation process normally occurrs from the pollution, temperature, and excessive light [13]. Antioxidants can be classified as preventive and chain-breaking. Preventive antioxidants such as superoxide dismutase, catalase or peroxidase inhibit the formation of reactive oxygen species (ROS). Chain-breaking antioxidants such as vitamin C, vitamin E, uric acid, polyphenols are compounds, scavenging ROS and break radical chain sequence. There are two possible reaction mechanisms which chain-breaking antioxidants can perform (i) hydrogen atom transfer (HAT) (ii) single electron transfer (SET). In natural, antioxidant can be found in food including fruits, vegetables and herbs.

Because the evaluation of individual antioxidant molecules is not practical, all antioxidants in a sample are quantified, called total antioxidant capacity (TAC) [14, 15]. Several methods have been used to assess TAC in food and beverage samples including spectrophotometry [8], chemiluminescence [6], chromatographic separation [16] and fluorescence [7]. Spectrophotometry is based on the reaction between

chromogenic radical and antioxidant. The chromogenic radicals consist of 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) [17] and 2,2-diphenyl-1-picrylhydrazyl (DPPH) [18]. The DPPH assay is widely used owing to its stability, simplicity, rapidity and the reaction concerning only the direct reaction between the radical and an antioxidant [13].

2.1.1 Thai herbs

2.1.1.1 Indian gooseberry

Indian gooseberry (*Phyllanthus emblica* Linn.) or ''Makhaam Pom'' in Thai is widely distributed in tropical and subtropical areas. This fruit contains high content of vitamin C. Moreover, the antioxidants such as, catechol, pyrogallol, ß-carotene, gallic acid and superoxide dismutase enzyme have been also found in this fruit. The extraction of emblica was used as antimicrobial, anti-inflammatory, antioxidant and chemoprotective activities [19].

Figure 2.1 Indian gooseberry (source: http://www.nanahealth.com)

2.1.1.2 Thai blueberry

Thai blueberry (*Antidesma ghaesembilla)*, which is known as "mamao" in Thai is a large black tree and in the Euphobiaceae family. This plant is an alternative for health care, antimicrobial [20] and antioxidant activity, containing the active components such as the phenolic content and the anthocyanin content [21]. Moreover, the dark purple ripe fruits have been developed to prepare various nutritional products, such as wine, jam, and fruit juice.

Figure 2.2 Thai blueberry (source: http://frynn.com)

2.1.1.3 Mulberry

Mulberry (*Morus* species) or "Mon" in Thai is widely grown throughout Thailand. In general, there are three types of mulberry, including white (*Morus alba*), black (*M. nigra*), and red (*M. rubra*). Mulberry fruits have been reported various activities including anti-inflammation [22], antimicrobial [23], antioxidant [24] and neuroprotective effect [25]. These activities obtain from anthocyanin which is a group of phenolic compound [26].

Figure 2.3 Mulberry (source: http://www.termsuk.com)

2.1.1.4 Bamboo grass

Bamboo grass (*Tiliacora triandra* (Colebr.) Diels) has a Thai name "Yanang". It is a native plant of Southeast Asia and widely used in the cuisines of northeast Thailand and Laos. It can be used for detoxication agent, anti-inflammation, anticancer, antibacterial and antipyretic activity [27, 28]. In addition, it also has an antioxidant activity, containing phenolic compound, carotenoid and chlorophyll as a main antioxidant [29].

Figure 2.4 Bamboo grass (source: http://www.oknation.net)

2.1.1.5 Gotu kola

Gotu kola *(Centella asiatica* (L.) Urban) or "Buabok" in Thai is a pharmaceutical plant that has been used as an active ingredient in cosmetic and drug. The *Centella asiatica* extract has pharmacological activities including wound healing, anti-aging [30], anti-inflammation, [31] and anti-cancer effect [32]. The active constituents in the *Centella asiatica* extract are triterpenes namely asiatic acid and asiaticoside [33]. Moreover, high phenolic content has been found in this plant, exhibiting strong association with its antioxidative activity [34, 35]. It is also commonly consumed as a vegetable in salad and juice in several Asian countries.

Figure 2.5 Gotu kola (source: http://www.lavitathailand.com)

2.1.2 6-Hydroxy-2, 5,7,8-tetramethylchroman-2-carboxylic acid

6-Hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) is a synthetic compound and a derivative of the vitamin E. Trolox is a powerful antioxidant widely used in biological or biochemical applications [36]. In this work, Trolox was used as a standard antioxidant to determine an analytical performance of our electrochemical system.
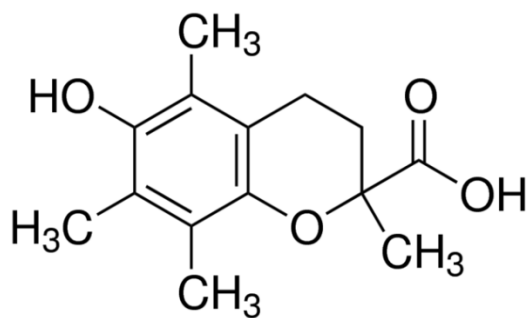
Figure 2.6 A chemical structure of Trolox [37].

Many researchers have studied the determination of antioxidant activities in Thai herbs for medical, pharmaceutical and cosmetic industries.

Aramwit and et al. [26] evaluated the antioxidant activities in mulberry. The results show that purple-colored mulberry fruit extract contained the highest levels of anthocyanin and great antioxidant properties. Anthocyanin content is well correlated with antioxidant activity.

Zainol and *et al.* [34] determined the antioxidant activity and total phenolic compounds in *Centella asiatica* (L.) Urban. The results exhibit that phenolic compounds are the major components in *Centella asiatica* determined by Folin–Ciocalteu method.

Liu and *et al.* [38] measured the antioxidant activity of emblica fruit from six regions in China. The results show that the extracts of emblica fruit had strong antioxidant activity. Therefore, emblica fruit is an alternative source of plant antioxidant with the potential usage in food, cosmetic, and pharmaceutical applications.

2.2 DPPH assay

DPPH is a stable free radical containing an unpaired valence electron at one atom of nitrogen bridge as shown in Figure 2.7 [39]. DPPH is a stable chromogen radical with a deep purple color. Conventional DPPH assay is based on electron donation of antioxidant to neutralize the DPPH radical, which is accompanied by a color change that can be measured at 517 nm [40]. When a DPPH solution (radical form) is mixed with an antioxidant of interest which can donate a hydrogen atom, the reduced form is generated (DPPH) as shown in the reaction presented in Figure 2.8. The violet color is changed to yellow pale color. Non-reacted DPPH can be detected by either spectrometric or electrochemical method.
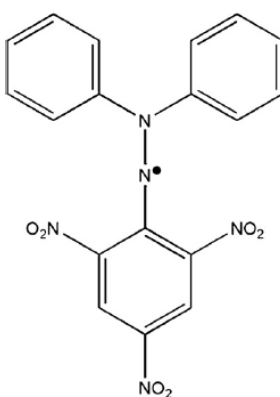
Figure 2.7 A Chemical structure of 2,2-diphenyl-1-picrylhydrazyl radical (DPPH) [41].
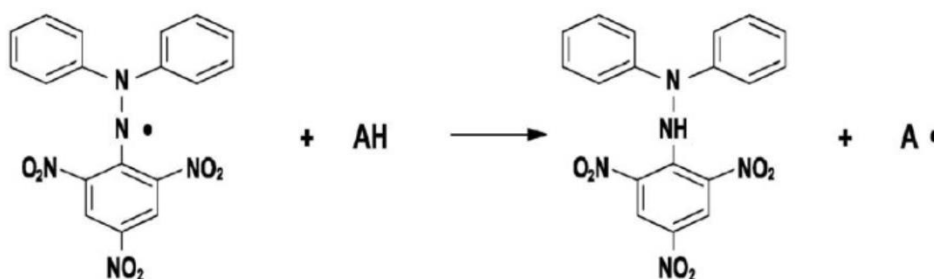
Figure 2.8 Reaction of DPPH free radical with an antioxidant [4].

2.3 Electrochemistry

Electrochemistry is the study of chemical reactions causing an electron flow across the immiscible interface between an electrode and electrolyte solution. This flow of electrons can generate an electric current. This technique can measure electroactive species that oxidation-reduction reaction or redox reaction occurs at the electrode surface. The advantage of this technique includes high sensitivity, portability, small sample volume, and providing both qualitative and quantitative information.

2.3.1 Voltammetry

Voltammetry is a well-established technique that involves the change of potential to a working electrode, followed by measurement of the current relating to the electrochemical reaction of analyte at the working electrode [42]. In the voltammetric measurement, an electrochemical cell has three electrodes system consisting of working electrode (WE), reference electrode (RE), and counter electrode (CE). The electrochemical reaction of analyte can occur at the WE surface. The RE has a stable and well-defined electrochemical potential, and the CE is used to close current circuit in the electrochemical cell.

In this thesis, cyclic voltammetry, square-wave voltammetry, and amperometry were used for electrochemical detection.

2.3.1.1 Cyclic voltammetry

Cyclic voltammetry (CV) is the firstly used technique in voltammetric technique to evaluate the electrochemical behavior of the system. This technique provides information on the thermodynamics of redox process, kinetic of electron transfer reaction, and adsorption processes.

CV is scanned linearly the potential of a stationary WE using a triangular potential waveform (Figure 2.2a). The current resulting from the applied potential is measured during a potential sweep. The result of cyclic voltammetry is plotted between the current versus applied potential as called cyclic voltammogram (Figure 2.9b). The direction of positive scanning potential provides the anodic peak current response (I_{pa}) that is occurred from oxidation reaction. In contrast, the direction of negative scanning potential provides the cathodic peak current response (I_{pc}) that is occurred from reduction reaction.
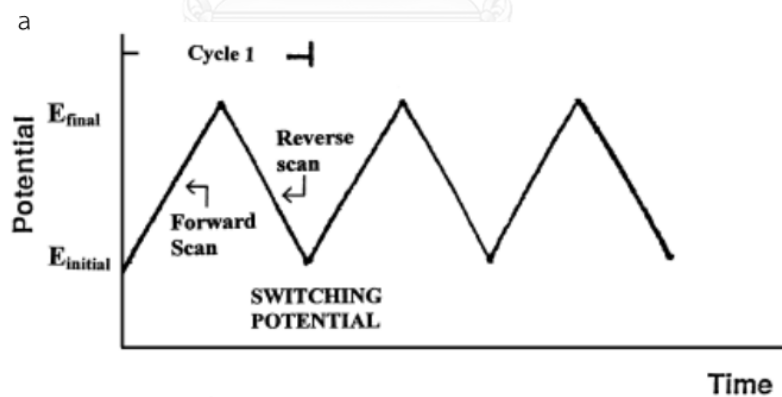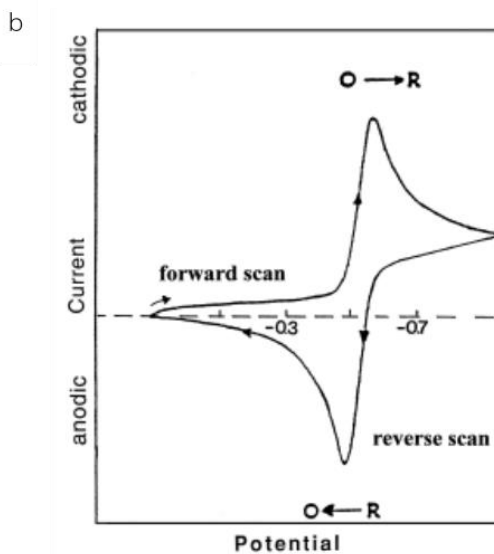
Figure 2.9 (a) Cyclic potential sweep (triangular waveform) and (b) typical cyclic voltammogram for a reversible redox process [43].

Current dependence on the scan rate (at 25°C) is presented by the Randles-Sevick equation:

$$i_p = (2.69 \times 10^5) n^{3/2} A C D^{1/2} V^{1/2} \qquad \text{(equation 2.1)}$$

Where n is the number of electron, A is the electrode area (cm^2), C is the concentration (mol cm^{-3}), D is the diffusion coefficient (cm^{-2} s^{-1}) and V is the scan rates (V s^{-1}).

2.3.1.2 Square-wave voltammetry

Square-wave voltammetry (SWV) is one of an electrochemical techniques that provides well defined peak, good discrimination against background currents, and more sensitive detection of analyte than CV [44]. In each cycle, the current samples were measured twice during square-wave (SW) cycle, once at the end of forward pulse and reverse pulse. The difference between the two measurements is plotted versus scanning potential. The resulting is symmetrical the half-wave potential, and the peak current is proportional to the concentration [43] .

Excellent sensitivity obtains from the fact that the net current is larger than either the forward or the reverse components current. The sensitivity of this method can be increased by enhancing the amplitude or the frequency of the SW. Figure 2.10 shows the excitation signal in SWV.
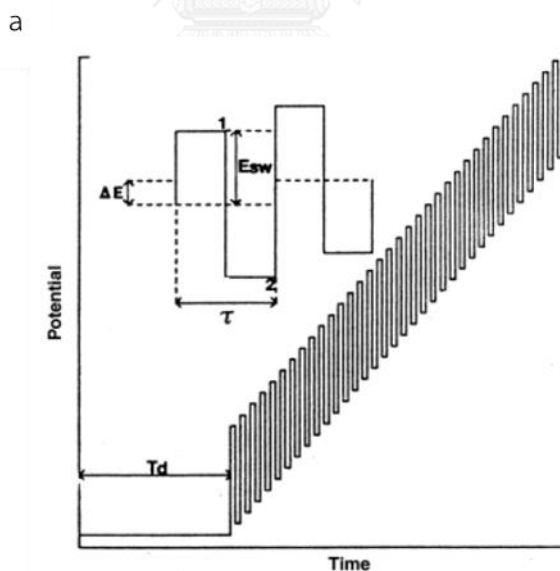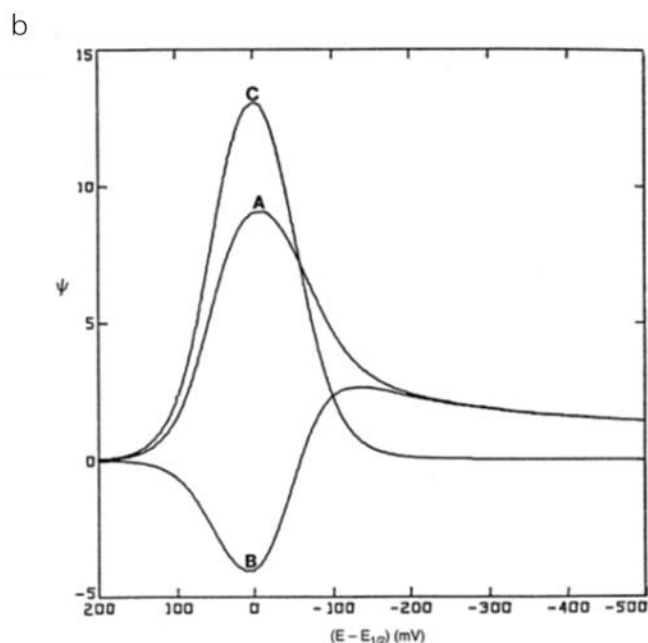
Figure 2.10 (a) Square-wave waveform showing the amplitude, E_{sw} ; step height, ΔE ; square-wave period, τ ; delay time, T_d ; current measurement time, 1 and 2 (b) square-wave voltammogram for reversible electron transfer. Curve A : forward current. Curve B : reverse current. Curve C : net current [43].

2.3.1.3 Amperometry

Amperometry is an electrochemical detection that measures the current response at a constant applied potential as a function of time. The currents at the working electrode were measured resulting from the oxidation or reduction of an electroactive species. Furthermore, the current response is directly proportional to the bulk concentration of the analyte [45].
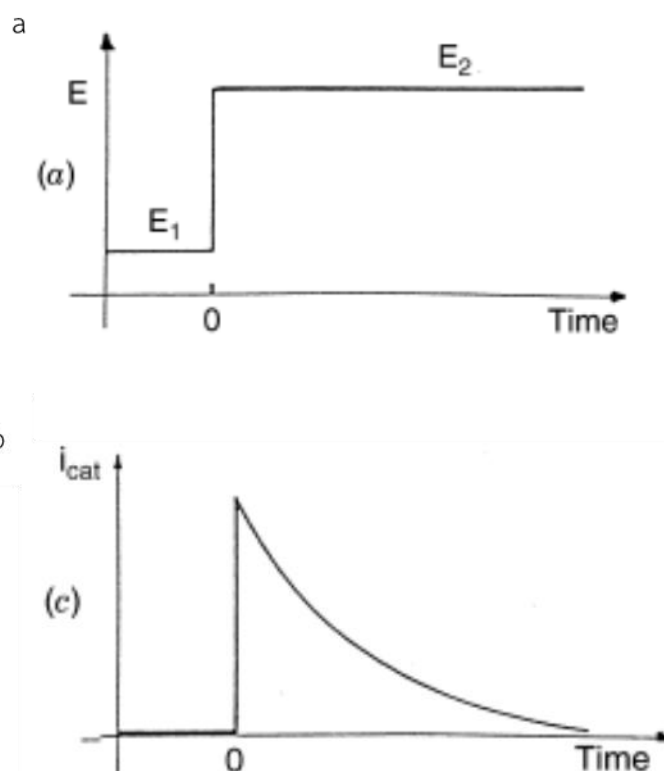
Figure 2.11 (a) Potential-time waveforms and (b) the resulting time-response [43].

Chronoamperometry involves stepping the potential of the working electrode from a value where no faradaic reaction occurs to a potential where the electroactive species at the electrode surface are oxidized or reduced completely. A stationary WE and unstirred solution are used. The resulting current-time dependence is monitored.

There are many reports that use the voltammetry technique for the determination of antioxidant activity as following

Pisoschi and *et al.* [4] monitored the TAC in fruit juices using DPPH free radical along with amperometric method. This method provides an accurate, rapid and sensitive detection. The result obtained from amperometric and spectrophotometric methods were in good agreement.

Amatatongchai and *et al.* [11] presented an amperometric flow injection for the determination of TAC in Thai indigenous vegetable extracts using carbon nanotube modified glassy carbon electrode based (CNT/GCE) with DPPH assay. The system showed a high sensitivity, good precision and applicable to all types of plant extracts.

Milardovic′ and *et al.* [2] proposed the determination of antioxidant activity based on DPPH free radical using glassy carbon electrode by amperometric reduction. The amperometric method showed the good stability and the system was not interfered by turbid sample.

Vasilescu and *et al.* [46] described the determination of the antiradical properties of several oils using a platinum screen-printed working electrode by differential pulse voltammetry. This method is a fast, cheap and satisfactory result in good agreement with conventional HPLC method.

Schulte and *et al.* [47] described an automated electrochemical microtiter plate assay for antioxidant quantification in food samples using a computer-controlled amperometric system. This method can be applied in the quality control unit of the food, agricultural and pharmaceutical industries due to its convenience, time savings and minimization of manual errors.

2.4 Electrode surface modification

Generally, electrochemical system, which consists of WE, RE, and CE, has been designed to be small size for portability, resulting in the decrease of electrochemical sensitivity. For electrochemical detection, WE is the most important part because the reaction of analyte occurs on its surface. To improve the electrochemical sensitivity of the system, carbon-based nanomaterial and conducting polymer were used to modify WE surface.

2.4.1 Graphene

A novel carbon material namely graphene (G) is a single planar sheet of sp^2-bonded carbon atoms which is densely packed honeycomb two-dimensional lattice [48]. It has become a promising nanomaterial for improving the electrode performance due to its large surface area (theoretical value 2,630 m^2g^{-1}), high electrical conductivity, strong mechanical strength, and efficient direct electrochemistry [49]. It has been widely use in electronic applications as shown in Figure 2.13.
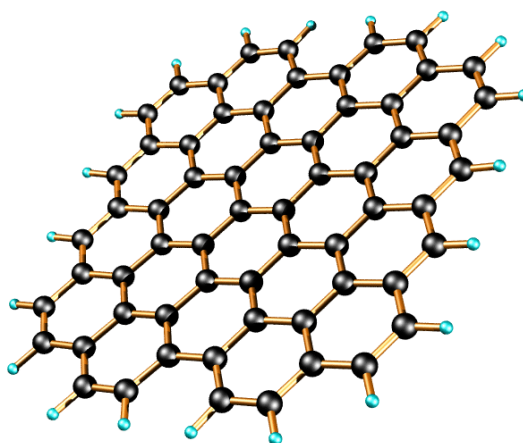
Figure 2.12 A structure of graphene (http://www.serotonin.ucla.edu).
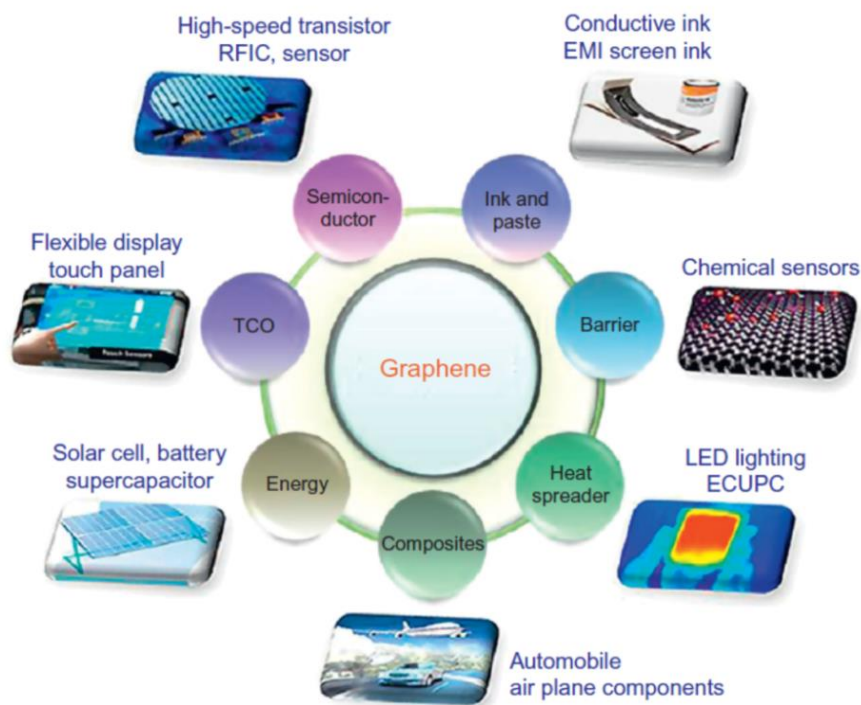
Figure 2.13 Overview of graphene applications [50].

2.4.2 Poly (3,4-ethylenedioxythiophene) : poly (styrenesulfonate)

Conducting polymers (CPs) including polyaniline (PANI) [51], polypyrrole (PPy) [52] and poly (3,4-ethylenedioxythiophene) : poly (styrenesulfonate) (PEDOT:PSS) [53] have been widely used to enhance the sensitivity of electrode as well as improve the distribution of G on electrode surface. Comparing to other conducting polymers, PEDOT:PSS is one of the most attractive conducting polymer, which has high conductivity, low redox potential, high electrochemical, biocompatibility and high stability [12]. The use of PEDOT:PSS hybrid material is beneficial to improve electrical conductivity of the electrochemical system. The structure of PEDOT:PSS is shown in Figure 2.14. The conductivity of PEDOT:PSS have increased steadily, recently reaching 3,065 S cm^{-1} [54].
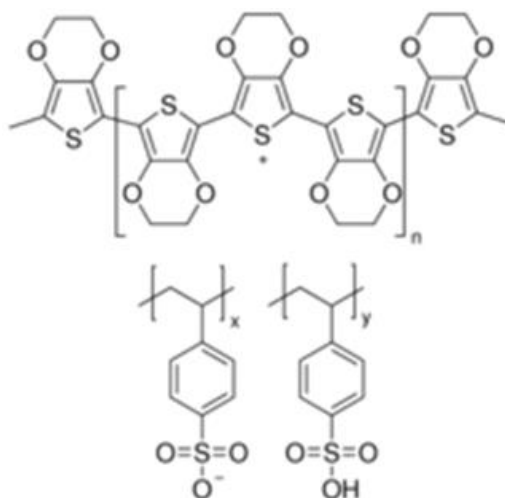
Figure 2.14 A structure of poly (3,4-ethylenedioxythiophene) :

poly (styrenesulfonate) (PEDOT:PSS). (source: http://www.sigmaaldrich.com)

Many researchers used G and conducting polymer as a hybrid material to improve the conductivity of electrode. The modified electrode shows the high conductivity and fast electron transfer kinetic.

Karuwan and *et al.* [12] reported the inkjet-printed G/PEDOT:PSS modified SPCE for the determination of salbutamol in pharmaceutical samples. The G/PEDOT:PSS exhibited the current response of salbutamol which is higher than an unmodified SPCE approximately 30 and 150 times. Moreover, this modified SPCE provides a wide dynamic range, a low detection limit of 1.25 μM.

Wisitsoraat and et al. [55] developed the electrochemical biosensor based on glucose oxidase and G/PEDOT:PSS modified electrode for glucose determination. The results show the high sensitivity, very low detection limits and high stability.

Rodthongkum and *et al.* [56] developed a novel G/PANI/PS modified SPCE for determination of dopamine in human serum and urine by SWV. The G/PANI/PS nanofiber was deposited on the SPCE surface. The current response of G/PANI/PS modified SPCE was higher than unmodified SPCE for 9 times. The modified

electrodes displayed high sensitivity, good selectivity and a wide linear range for dopamine determination in both biological and pharmaceutical samples.

Promphet and *et al.* [57] developed in situ bismuth and G/PANI/PS modified SPCE using electrospinning fabrication for simultaneous determination of lead (Pb^{2+}) and cadmium (Cd^{2+}) in river water samples by square-wave anodic stripping voltammetry (SWASV). The G/PANI/PS nanoporous fiber provides the high surface area, good conductivity and high reproducibility.

Fan and *et al.* [58] developed the G/PANI modified glassy carbon electrode (GCE) for the determination of 4-aminophenol. This modified electrode can be used to determine 4-aminophenol and paracetamol simultaneously with low detection limit and high sensitivity.
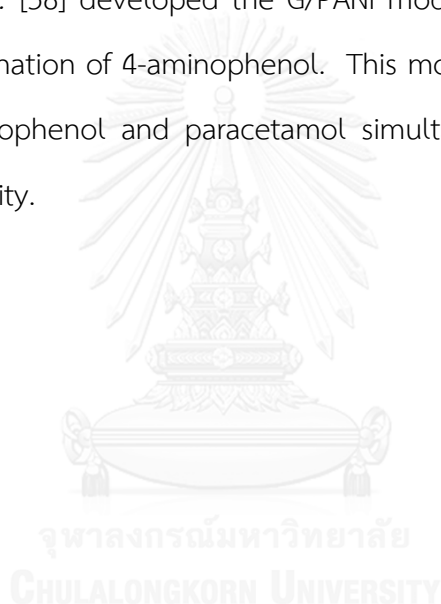
2.5 Electrospraying

Electrospraying was used for electrode modification because it can generate uniform three-dimension droplet-like composite on an electrode surface [59-61]. This method can increase the active surface area of electrode, leading to improve the electrochemical sensitivity of the electrochemical sensor. Generally, electrospraying process consists of high-voltage power supply, syringe pump, ground collector, plastic syringe and stainless-steel needle. The solution of electrode solution was filled into the plastic syringe and then inserted into the syringe pump. After that, a high-voltage power supply was connected between stainless-steel needle and ground collector as shown in Figure 2.15. The reference (RE) and counter (CE) electrodes were covered with aluminum foil to prevent electrode modification from the electrospraying process. Then, the G modifier solution was sprayed onto the working electrode (WE) attached to a ground collector. Finally, the electrospray of G/conducting polymer modified SPCE was obtained.
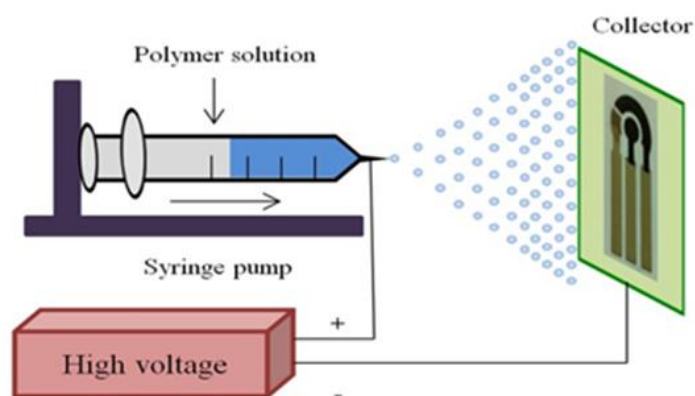
Figure 2.15 Schematic of electrospraying for electrode modification.

For the literature reports, there are few publications that use electrospraying as a method for electrode surface modification as following.

Thammasoontaree *et al.* [59] developed an ultra-performance liquid chromatography (UPLC) coupled with electrosprayed G/PANI modified SPCE for determination of sulfonamides in shrimp. The modified electrode provides a good recovery, high precision, and low limit of detection.

Ruecha *et al.* [60] prepared a G/PVP/PANI nanocomposite using electrospraying for electrode modification and attached cholesterol oxidase as a cholesterol biosensor . This modified electrode increases the current response with a 3-fold compared with an unmodified electrode for standard ferri/ferrocyanide and exhibits a high sensitivity, wide linear range and low limit of detection.

Ruecha et al. [61] modified electrochemical sensor using G/PANI nanocomposite by drop-casting and electrospraying methods for simultaneous detection of Zn(II), Cd(II), and Pb(II). The G/PANI modified electrode exhibited high electrochemical conductivity and increased surface area of electrode.

CHAPTER III

EXPERIMENTAL

3.1 Chemicals and reagents

3.1.1 Graphene (G) nanopowders (SkySpring Nanomaterials Inc, Houston, TX, USA)

3.1.2 Poly (3,4-ethylenedioxythiophene) : poly (styrenesulfonate) (PEDOT:PSS) (Sigma-Aldrich, St. Louis, Mo, USA)

3.1.3 2,2-Diphenyl-1-pycrilhydrazil (DPPH) (Sigma-Aldrich, St. Louis, Mo, USA)

3.1.4 Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) (Sigma-Aldrich, St. Louis, Mo, USA)

3.1.5 Potassium ferricyanide ($K_3[Fe(CN_6)]$) (Sigma-Aldrich, St. Louis, Mo, USA)

3.1.6 N, N-Dimethylformamide (DMF) (Carlo Erba Reagent, Milano, Italy)

3.1.7 Absolute ethanol (C_2H_5OH) (Carlo Erba Reagenti-SDS, Val de Reuil, France)

3.1.8 Potassium dihydrogen phosphate (KH_2PO_4) (Carlo Erba Reagenti-SDS, Val de Reuil, France)

3.1.9 Disodium hydrogen phosphate (Na_2HPO_4) (Merck, Darmstadt, Germany)

3.1.10 Potassium chloride (KCl) (Merck, Darmstadt, Germany)

3.1.11 Sodium chloride (NaCl) (Merck, Darmstadt, Germany)

3.1.12 Carbon ink (Gwent group, Torfean, United Kingdom)

3.1.13 Silver/silver chloride ink (Gwent group, Torfean, United Kingdom)

3.2 Instruments and equipments

3.2.1 High voltage DC module (Gamma high voltage, model UC5-30P/CM/VM (3), Florida, USA)

3.2.2 Syringe pump (New Era Pump, NE300, USA)

3.2.3 Potentiostat e-corder 410 (eDAQ, Australia)

3.2.4 UV-Vis spectrophotometer specord s 100, analytikjena (Germany)

3.2.5 Scanning electron microscope (Japan Electron Optics Laboratory Co., Ltd, Japan)

3.2.6 Transmission electron microscope (Japan Electron Optics Laboratory Co., Ltd, Japan)

3.3 Preparation of solutions

All aqueous solutions were prepared in MilliQ water (R ≥ 18.2 MΩ cm^{-1}). All chemicals and reagents were of analytical grade.

3.3.1 Preparation of 0.1 M potassium chloride solution

Potassium chloride solution (KCl) was used as supporting electrolyte of standard ferricyanide (K$_3$[Fe(CN$_6$)]). 0.1 M KCl was prepared by weighting KCl 1.8640 g and then dissolving in 250 mL of milliQ water.

3.3.2 Preparation of 1 mM potassium ferricyanide

0.0329 g of potassium ferricyanide (K$_3$[Fe(CN$_6$)]) was dissolved in 100 mL of 0.1 M KCl.

3.3.3 Preparation of phosphate buffer solution

1 M phosphate buffer solution (PBS), pH 7.0 was prepared by weight of potassium dihydrogen phosphate (KH$_2$PO$_4$) 0.012 g, disodium hydrogen phosphate

(Na_2HPO_4) 0.072 g, potassium chloride (KCl) 0.01 g and sodium chloride (NaCl) 0.4 g and then dissolved in 100 mL of milliQ water.

3.3.4 Preparation of ethanolic phosphate buffer solution

60% (v/v) absolute ethanol in 0.1 M phosphate buffer solution (EPBS), pH 7.0 was used as supporting electrolyte in all electrochemical measurements. EPBS 60% (v/v) was prepared by mixing 2 mL of 1 M PBS, 6 mL of milliQ water, and 12 mL of absolute ethanol.

3.3.5 Preparation of ethanol-water solution

60% (v/v) ethanol in water was used as solvent for dissolving DPPH, Trolox and five real samples in spectrophotometry for validation method. 60% (v/v) ethanol in water was prepared by pipetting 60 mL of absolute ethanol and 40 mL of milliQ water. Then, two solutions were mixed together.

3.3.6 Preparation of 2,2-diphenyl-1-pycrilhydrazil (DPPH) solution

For electrochemistry, 2.5 mM DPPH was freshly prepared by weight 0.0049 g and then dissolved in 5 mL of 60% (v/v) EPBS. For spectrophotometry, 0.2 mM DPPH was freshly prepared by weight 0.0079 g and then dissolved in 100 mL of ethanol-water solution.

3.3.7 Preparation of Trolox solution

For electrochemistry, the stock of 2 mM of Trolox was freshly prepared by weight 0.0025 g and then dissolved in 5 mL of 60% (v/v) EPBS.

For spectrophotometry, the stock of 100 µM of Trolox was freshly prepared by weight 0.00125 g and then dissolved in 50 mL of ethanol-water.

3.4 Optimization and modification of electrode

3.4.1 Screen-printed carbon electrode fabrication

The screen-printed carbon electrodes (SPCEs) were fabricated on polyvinyl chloride (PVC) substrate. Initially, the silver/silver chloride ink was printed on PVC surface twice to form a reference electrode (RE) and conducting pads (Figure 3.1 step I). Next, the carbon ink was also printed two times onto the same PVC substrate as a working electrode (WE) and counter electrode (CE) (Figure 3.1 step II). In each screen-printing step, the SPCEs were dried in an oven at 55 °C for 1 h to evaporate the solvent [62].

Step I. Ag/AgCl ink screen-printed
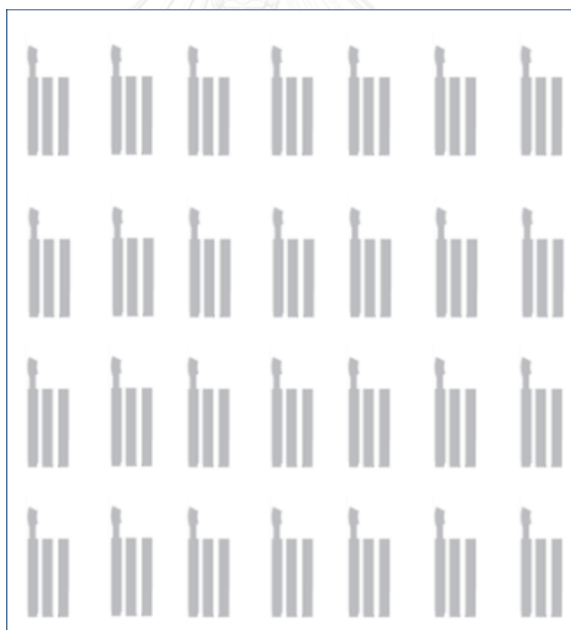
Step II. Carbon ink screen-printed

Figure 3.1 Screen-printed carbon electrodes using a manual screen-printing technique.

3.4.2 Optimization of the modified electrode composition

3.4.2.1 Influence of PEDOT:PSS loading

Amount of PEDOT:PSS loading was studied and optimized. 4 mg/mL of G loading was fixed for optimization of PEDOT:PSS loading. The amount of PEDOT:PSS in the range of 0, 2, 4, 6, 8, 10 and 12 mg was dissolved in 1 mL of DMF and then sonicated for 12 h at room temperature. G in PEDOT:PSS was obtained and mixed by vortex at room temperature prior to electrospraying fabrication.

3.4.2.2 Influence of G loading

Amount of G loading was studied and optimized in the range of 0, 2, 4, 6, 8 and 10 mg. Different G amount was dissolved in 1 mL of DMF and sonicated for 24 h at room temperature. 10 mg/mL of PEDOT:PSS loading was fixed for optimization of G loading. The influence of amount of G and PEDOT:PSS loading was investigated by SWV using 2.5 mM of DPPH in 60% (v/v) EPBS.
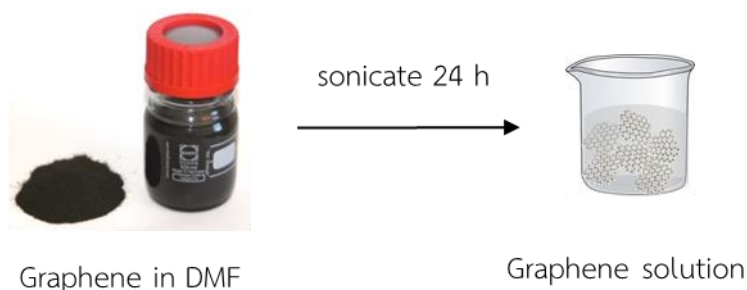
Figure 3.2 Preparation of G solution

3.4.2.3 Influence ratio of PEDOT:PSS/G

The ratio between PEDOT:PSS and G was studied and optimized in the range of 1:1, 2:1, 3:1, 4:1 and 5:1 (v/v) (different volumes of the PEDOT:PSS ranging from 500, 667, 750, 800 and 833 µL). The solution of PEDOT:PSS and G was mixed by vortex at room temperature. Influence ratio on DPPH detection was performed by SWV using 2.5 mM of DPPH in 60% (v/v) EPBS.

3.4.2.4 Influence of electrospraying time

The influence of electrospraying time on the electrochemical detection of DPPH was optimized via SWV. The different spraying times in the electrospraying fabrication at 2, 4, 6, 8 and 10 minutes were studied with the same condition.

3.4.3 Electrospraying fabrication of screen-printed carbon electrode

3.4.3.1 Preparation of G/PEDOT:PSS solution

The solution of G and PEDOT:PSS were prepared by following method. Firstly, G nanopowder (0.04 g) was dispersed in 10 mL of DMF and sonicated for 24 h at room temperature. Next, PEDOT:PSS (0.1 g) was dissolved in 10 mL of DMF and sonicated for 12 h at room temperature. After that, the solution of G and PEPOT:PSS was well mixed together and filled onto a plastic syringe No.26 prior to use in the electrospraying process.

3.4.3.2. An electrospraying process

The WE surface of SPCE was modified by electrospraying. Initially, the G and PEDOT:PSS solutions were filled into a plastic syringe. The RE and CE were covered by aluminum foil to prevent electrode modification from electrospraying process. For electrospraying system, an applied voltage of 7.5 kV, a flow rate of 1.0 mL/h, and a distance between electrode and needle of 5 cm were used to generate droplet-like nanostructure on the SPCE surface.

3.5 Physical characterization

The morphology of G/PEDOT:PSS and distribution of G on SPCE surface were investigated using a JSM-6400 field emission scanning electron microscrope (SEM) and a JEM-2100 tramsmission electron microscope (TEM).

3.6 Electrochemical characterization

All electrochemical measurements were carried out using a potentiostat with e-corder 410 (eDAQ). A three electrode system was used throughout the experiment. The developed G/PEDOT:PSS/SPCE was used as a working electrode (WE) with 3.5 mm in diameter. Carbon and Ag/AgCl electrodes were used as the counter electrode (CE) and reference electrode (RE), respectively. The SPCE is shown in Figure 3.3
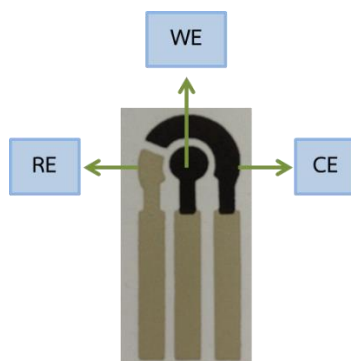
Figure 3.3 Screen-printed carbon electrode using manual screen printing technique (RE : reference electrode, WE : working electrode (modified electrode), CE : counter electrode).

3.6.1 Cyclic voltammetric procedures

Cyclic voltammetry (CV) was performed to compare the electrochemical performance between the unmodified SPCE and G/PEDOT:PSS/SPCE. The electrochemical behaviors of ferricyanide and DPPH were used to distinguish the electrode performance. Cyclic voltammetric conditions used were scan rate of 250 mV/s and scanning potential range of -0.2 to 1.2 V vs. Ag/AgCl.

3.6.2 Square wave voltammetry procedure

For the electrode optimization, square wave voltammetry (SWV) was performed for electrochemical characterization of G/PEDOT:PSS/SPCE. The SWV conditions includes (i) a potential range from 0 to +0.6 V, (ii) a pulse amplitude of 25 mV, (iii) a square wave frequency of 15 Hz, and (iv) a step height of 1 mV.

3.6.3 Amperometry procedure

3.6.3.1 Optimization of amperometric detection potential

In this research, chronoamperometry was used for indirect determination of TAC via DPPH assay. An important parameter in chronoamperometry is a detection potential optimized by using hydrodynamic voltammetry. The detection potential in a range from -0.1 to 0.4 V vs. Ag/AgCl was studied and scanned from 0 to 100 s. The current response obtained from 6 mM of DPPH in 60% (v/v) EPBS was compared to background current of 60% (v/v) EPBS. The amperometric currents were recorded at a steady state current of 40 s.

3.7 The analytical performance of G/PEDOT:PSS modified SPCE

3.7.1 Calibration curve

Calibration curves of TAC in the presence of DPPH were conducted on G/PEDOT:PSS modified SPCE. Trolox was used as a standard antioxidant. The mixture of 40 µL of 6 mM DPPH in 60% (v/v) EPBS and 40 µL of different concentration of Trolox were dropped on G/PEDOT:PSS/SPCE and then measured by optimal amperometric conditions using sampling time at 40 s. The linear calibration was obtained from the plot of the decreased DPPH current after adding Trolox and concentration of Trolox in a range of 5-30 µM.

3.7.2 Limit of detection

The limit of detection (LOD) was calculated from LOD = $3S_b/m$ [63], where S_b is the standard deviation of the background (measured at least seven electrodes of the background) and m is the slope of the calibration curve.

3.7.3 Limit of quantitation

The limit of quantitation (LOQ) was calculated from LOD = $10S_b/m$ [63], where S_b is the standard deviation of the background (measured at least seven electrodes of the background) and m is the slope of the calibration curve.

3.7.4 Repeatability

The repeatability of G/PEDOT:PSS modified SPCE was measured within the same day at least seven times without the replacement of the electrode. The three concentrations of Trolox (10, 20, 30 µM represent the low, medium, and high level, respectively) from the calibration curve were chosen for testing the repeatability. The percentage of relative standard deviation (%RSD) was calculated from

%RSD = (standard deviation/mean) ×100

3.7.5 Reproducibility

The reproducibility of G/PEDOT:PSS/SPCE was tested by measuring at least seven electrodes of each concentration. The three concentrations of Trolox (10, 20, 30 µM represent the low, medium, and high level, respectively) from the calibration curve were chosen for studying of reproducibility.

3.8 Real sample analysis

3.8.1 Preparation of plant extraction

Indian gooseberry was purchased from the Kanchanaburi province in Thailand while herbal drinks (Thai blueberry, Mulberry, Bamboo grass and Gotu kola) were purchased from local supermarkets in Thailand. Botanical names of these herbs are *Phyllanthus emblica* L. (Indian gooseberry), *Antidesma ghaesembilla* Gaertn. (Thai blueberry), *Antidesma ghaesembilla* Gaertn. (Mulberry), *Tiliacora triandra* (Colebr.) Diels (Bamboo grass), *Centella asiatica* (L.) Urban (Gotu kola).

For antioxidant extraction, fresh Indian gooseberries were grinded by a grinder. Powder (10 g) was extracted for 24 h in 100 mL of ethanol and then filtered through 0.45 μm filter paper. After that, ethanol was removed using a rotary evaporator at 40°C. The crude plant extracts were freeze-dried and then stored at 4°C prior to analysis as described previously [38, 47].

For analysis, the 10 mg of crude extracts were dissolved in 10 mL of 60% (v/v) EPBS. Next, sample solutions were diluted (1,000 times) prior to analysis.

3.9 UV-Vis Spectrophotometry

In this research, UV-Vis spectrophotometry was used as a standard method. This method was adapted from the method of Milardovic′ and *et al*. [2]. Firstly, UV-Visible spectra of 0.2 mM DPPH solution was obtained by scanning the wavelength in a range of 200-900 nm. The maximum absorption was found to be 523 nm [64].

The 0.2 mM DPPH solution, standard Trolox and five real samples were dissolved in ethanol-water. The calibration curve was constructed from the plot between the decrease of DPPH absorbance at 523 nm after adding standard Trolox and concentration of Trolox in a range of 5-30 µM.

For real samples analysis, diluted real samples in the presence of DPPH were mixed for 10 s and the absorbance was measured at 523 nm. The ethanol-water solution was used as a blank.
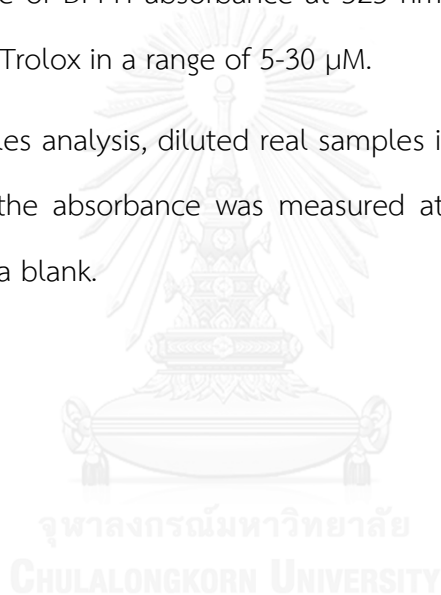
CHAPTER IV

RESULTS AND DISCUSSION

This chapter presents the results of G/PEDOT:PSS modified SPCE and its application for antioxidant evaluation. The results are separated into 6 parts including (1) optimization of the electrode modification, (2) electrochemical performance of modified electrode, (3) characterization of electrode morphology, (4) analytical performance, (5) optimization of amperometry, and (6) real sample analysis.

4.1 Optimization of the electrode modification

There are several factors affectting the performance of G/PEDOT:PSS/SPCE. These factors including amount of PEDOT:PSS loading, amount of G loading, PEDOT:PSS and G ratio, and electrospraying time were studied and optimized. The optimized electrodes were tested towards the electrochemical detection of DPPH by using SWV.

4.1.1 Amount of PEDOT:PSS loading

An effect of amount of PEDOT:PSS loading on the electrochemical sensitivity of G/PEDOT:PSS/SPCE was studied. The amount of PEDOT:PSS loading ranging from 0 to 12 mg/mL was used. Figure 4.1a and 4.1b show that the cathodic peak currents of 2.5 mM DPPH gradually increased from 2 to 10 mg/mL, and the current is highest at 10 mg/mL of PEDOT:PSS loading. Above 10 mg/mL of PEDOT:PSS loading, the cathodic peak current start decreasing. This is probably due to the limited solubility of this polymer. Thus, a 10 mg/mL of PEDOT:PSS loading was chosen as an optimum condition.

Figure 4.1 (a) SW voltammograms of 2.5 mM DPPH in 0.1 M EPBS (pH 7.0) measured on a G/PEDPT:PSS/SPCE with different amounts of PEDOT:PSS loaded. (b) cathodic peak current (I_{pc}) obtained from Figure 4.1(a). SWV conditions: scanning potential range of 0 to +0.6 V with a pulse amplitude of 25 mV, a square wave frequency of 15 Hz, and a step height of 1 mV.

4.1.2 Influence of G loading

An effect of G loading on the electrochemical sensitivity of G/PEDOT:PSS/SPCE was investigated in a range of 0-10 mg/mL. As shown in Figure 4.2a and 4.2b, the cathodic peak currents increase when the amount of G loading increase from 0 to 4 mg/mL. The results indicated that the composition of G and PEDOT:PSS can increase the electrochemical electricity of electrode. Nevertheless, the cathodic peak currents decrease when G loadings above 4 mg/mL. The decrease in current responses was probably caused by the agglomeration of G [56]. Therefore, 4 mg/mL of G loading was chosen as an optimal G concentration for further experiments.

Figure 4.2 (a) SW voltammograms of 2.5 mM DPPH in 0.1 M EPBS (pH 7.0) measured on a G/PEDPT:PSS/SPCE with different amounts of G loaded. (b) cathodic peak current (I_{pc}) obtained from Figure 4.2(a). SWV conditions: scanning potential range of 0 to +0.6 V with a pulse amplitude of 25 mV, a square wave frequency of 15 Hz, and a step height of 1 mV.

4.1.3 Influence of ratio of PEDOT:PSS and G

An appropriate ratio of PEDOT:PSS and G was investigated as shown in Figure 4.3a and 4.3b. The ratio of PEDOT:PSS and G from 1:1 to 5:1 (v/v) was studied. When the ratio of PEDOT:PSS and G was increased from 1:1 to 2:1 (v/v), the cathodic peak currents slightly increased. The cathodic peak currents also decreased when the ratio of PEDOT:PSS and G was changed in the range from 3:1 to 5:1. Thus, the ratio of PEDOT:PSS and G at 2:1 (v/v) was selected for further experiments.
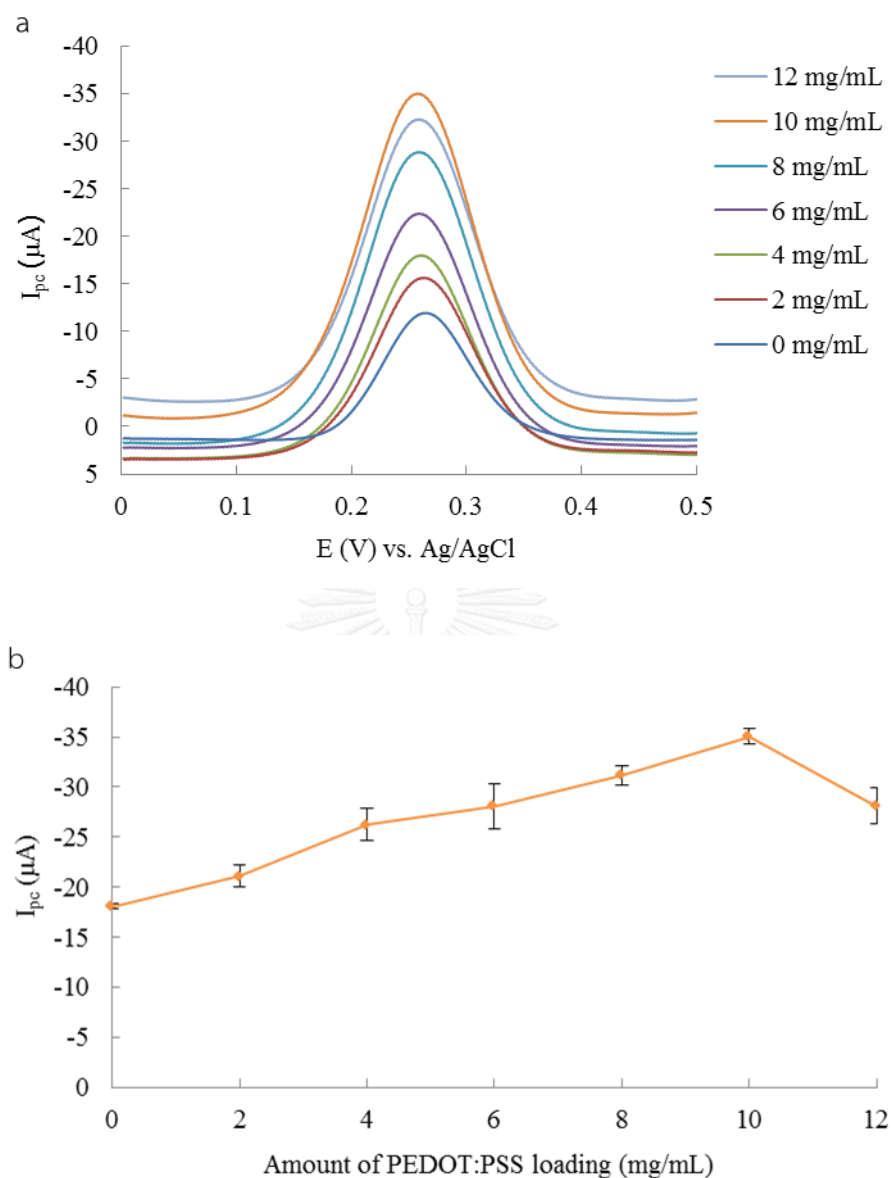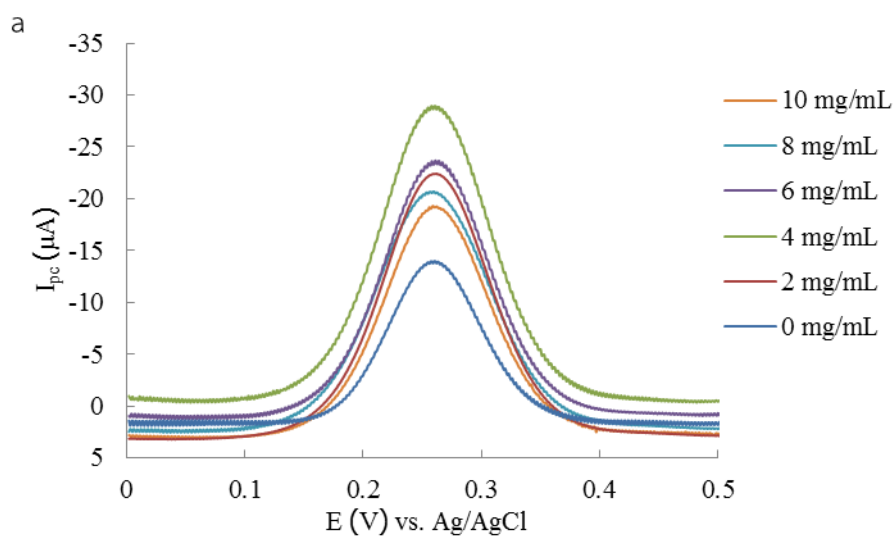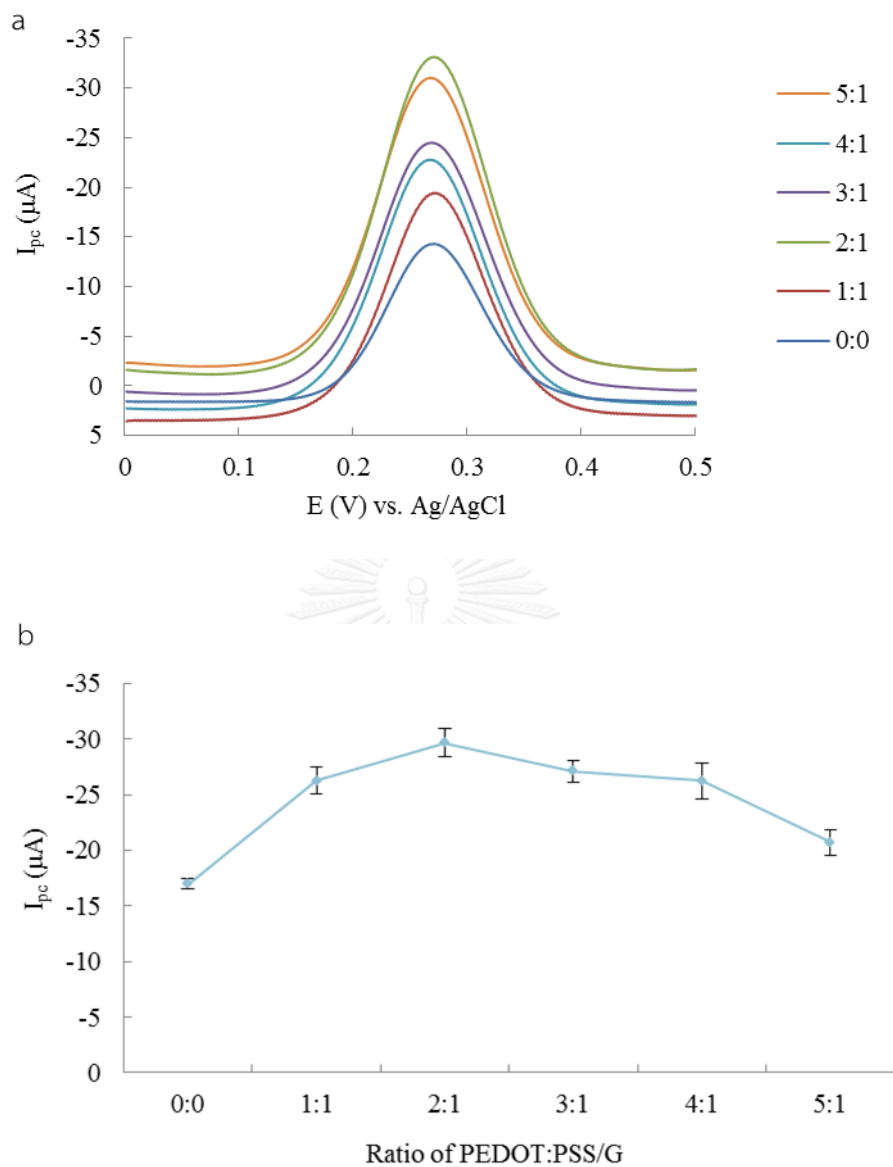
Figure 4.3 (a) SW voltammograms of 2.5 mM DPPH in 0.1 M EPBS (pH 7.0) measured on a G/PEDPT:PSS/SPCE with different ratios of PEDOT:PSS and G loaded. (b) cathodic peak current (I_{pc}) obtained from Figure 4.3(a). SWV conditions: scanning potential range of 0 to +0.6 V with a pulse amplitude of 25 mV, a square wave frequency of 15 Hz, and a step height of 1 mV.

4.1.4 Influence of electrospraying time

The electrochemical sensitivity of modified electrode at different electrospraying time in a range of 2 to 12 minutes was investigated as shown in Figure 4.4a and 4.4b. When the spraying time increased from 2 to 6 minutes, the cathodic peak currents increased. At 6 minutes, the cathodic peak currents start decreasing. The decrease of current was probably caused by electrode fouling due to excess thickness of the electrode surface.
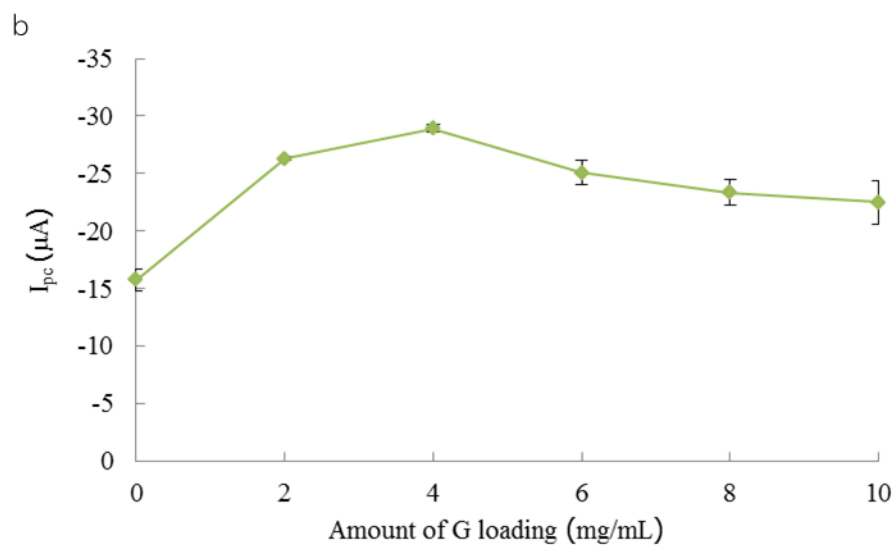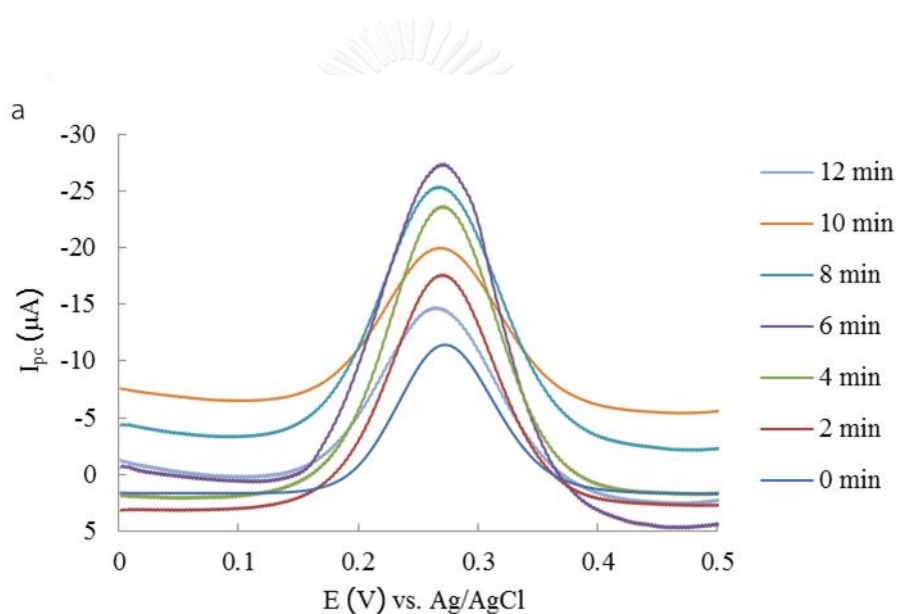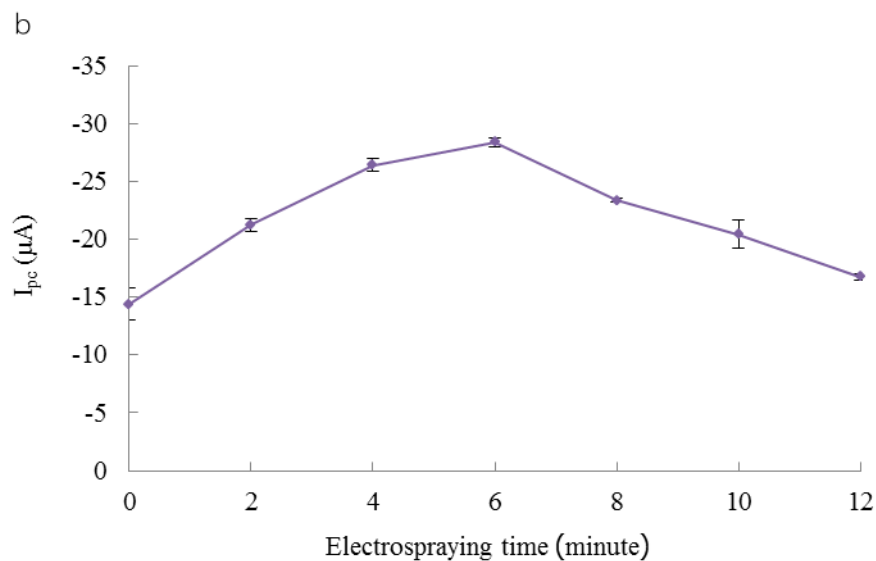
Figure 4.4 (a) SW voltammograms of 2.5 mM DPPH in 0.1 M EPBS (pH 7.0) measured on a G/PEDPT:PSS/SPCE with different spraying time. (b) cathodic peak current (I_{pc}) obtained from Figure 4.4(a). SWV conditions: scanning potential range of 0 to +0.6 V with a pulse amplitude of 25 mV, a square wave frequency of 15 Hz, and a step height of 1 mV.

In summary, 10 mg/mL of PEDOT:PSS, 4 mg/mL of G, ratio of PEDOT:PSS and G at 2:1 (v/v) and 6 minutes electrospraying time were used as the optimal conditions for electrospraying.

4.2 Physical characterization of modified electrode

The morphology of electrosprayed G/PEDOT:PSS/SPCE was characterized by scanning electron microscopy (SEM) and transmission electron microscopy (TEM), respectively. A SEM image of G/PEDOT:PSS/SPCE (Figure 4.5a) displays the uniform 3D droplet-like structure of G/PEDOT:PSS on the surface of electrode. Moreover, a TEM image of G/PEDOT:PSS (4.5b) shows the ultra-thin sheet of G, indicating that G is well dispersed without the aggregation. Moreover, the electron diffraction pattern of G as shown in the Figure 4.5c is matched with the previous work [60].
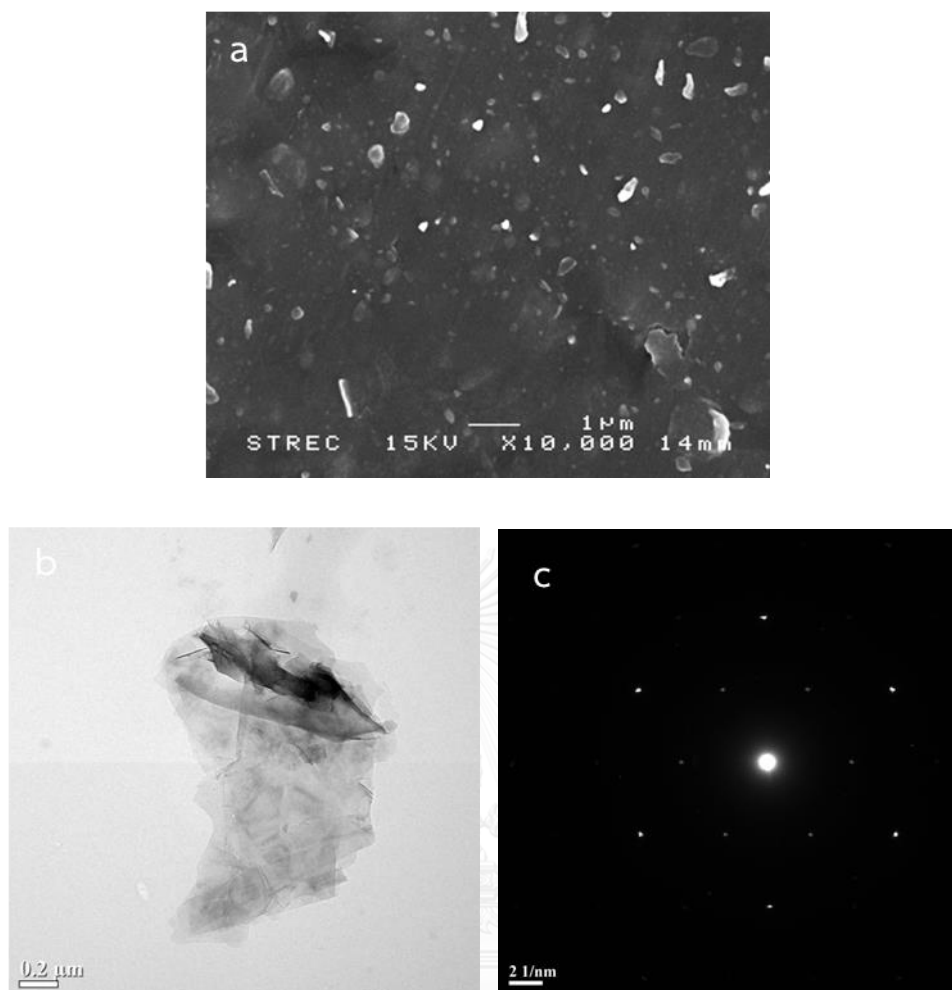
Figure 4.5 (a) SEM image of the G/PEDOT:PSS modified electrode with 10,000× magnification, (b) TEM image of G disperse in nanocomposite, and (c) electron diffraction pattern of G dispersed in the nanocomposite.

4.3 Electrochemical characterization of modified electrode

4.3.1 Electrochemical measurement of ferricyanide solution

Cyclic voltammetry of 1 mM ferricyanide in 0.1 M KCl was performed for electrochemical characterization of the unmodified SPCE (blue line), PEDOT:PSS modified SPCE (red line) and G/PEDOT:PSS modified SPCE (green line) as shown in Figure 4.6. Table 4.1 shows the cathodic current response (I_{pc}) and and potential difference values (ΔE_p) of unmodified SPCE, PEDOT:PSS/SPCE and G/PEDOT:PSS/SPCE.

These results show that the current responses of PEDOT:PSS and G/PEDOT:PSS modified SPCE increase approximately 2 times and 3 times compared to unmodified SPCE, respectively. Interestingly, a significant decrease of potential difference values (ΔE_p) between anodic and cathodic peak potential of G/PEDOT:PSS/SPCE is found to be 0.1 V, compared to ΔE_p of unmodified SPCE (ΔE_p=1.0 V), verifying that G/PEDOT:PSS nanocomposite can also enhance the electron transfer kinetics of this system.
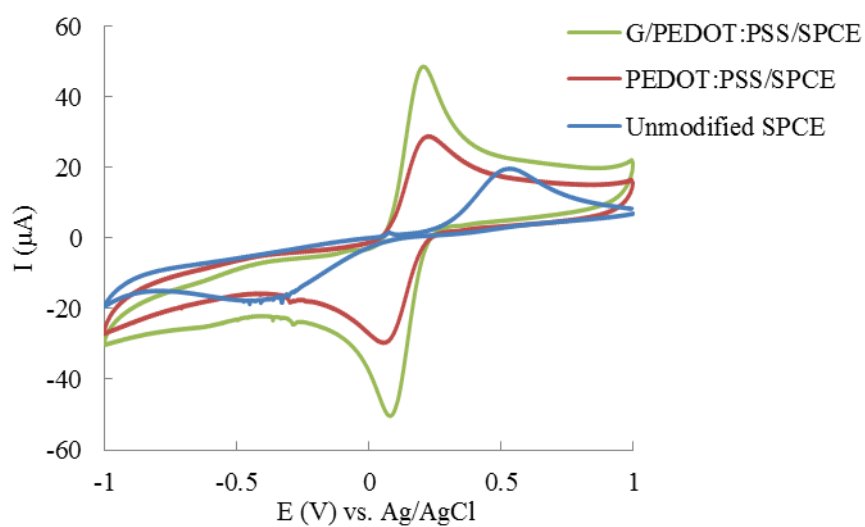
Figure 4.6 Cyclic voltammograms of 1 mM ferricyanide in 0.1 M KCl with scan rate of 250 mV/s measured on unmodified SPCE (blue line), PEDOT:PSS modified electrode (red line) and G/PEDOT:PSS modified electrode (green line).

Table 4.1 The cathodic current response (I_{pc}) and potential difference values (ΔE_p) of unmodified SPCE, PEDOT:PSS/SPCE and G/PEDOT:PSS/SPCE

Type of SPCE	I_{pc} (µA)	ΔE_p (V)
Unmodified SPCE	15.13±0.44	1.00
PEDOT:PSS/SPCE	29.08±1.76	0.13
G/PEDOT:PSS/SPCE	49.29±1.95	0.13

4.3.2 Electrochemical measurement of DPPH free radical

From the result of ferricyanide standard solution, the current response of G/PEDOT:PSS modified SPCE is the highest when compared with unmodified SPCE. Thus, G/PEDOT:PSS/SPCE was chosen for further determination of TAC. In this study, the TAC was investigated based on DPPH free radical.

Figure 4.7 shows the cyclic voltammograms of DPPH solution measured on the unmodified SPCE (blue line) and G/PEDOT:PSS/SPCE (green line). Two reversible redox couples of DPPH radical were observed (1st couple E_{pc}= 0.22 V and E_{pa}= 0.31 V and the 2nd couple E_{pc}= 0.66 V and E_{pa}= 0.74 V). The current response of G/PEDOT:PSS/SPCE is approximately 2 times higher than unmodified SPCE. These results verify that G/PEDOT:PSS can be an alternative electrochemical sensor for DPPH detection.
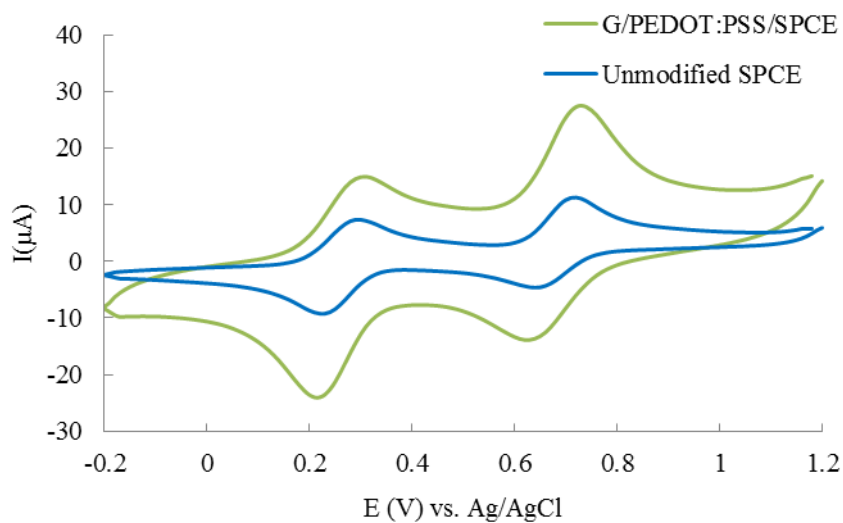
Figure 4.7 Cyclic voltammograms of 2.5 mM DPPH in 0.1 M EPBS solution (pH 7.0) with scan rate of 625 mV/s measured on unmodified SPCE (blue line) and G/PEDOT:PSS modified electrode (green line).
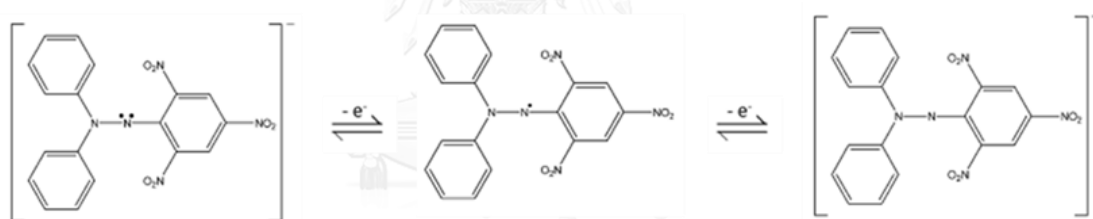
Figure 4.8 Reversible, one-electron reduction (left) and oxidation (right) of DPPH [65].

Figure 4.9 shows the square wave voltammograms of DPPH solution measured on the unmodified SPCE (blue line) and G/PEDOT:PSS/SPCE (green line). The current response of G/PEDOT:PSS/SPCE is higher than unmodified SPCE approximately 2 times.
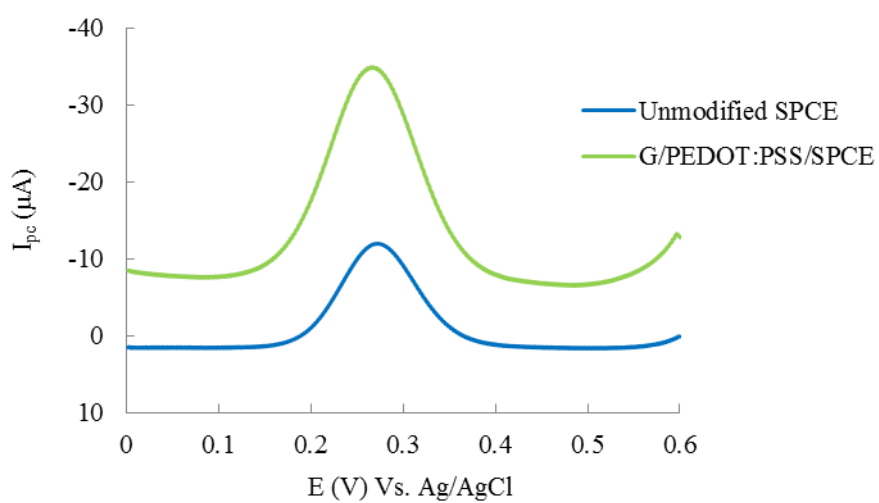
Figure 4.9 SW voltammograms of 2.5 mM DPPH in 0.1 M EPBS solution (pH 7.0) measured on unmodified SPCE (blue line), G/PEDOT:PSS/SPCE (green line). SWV conditions: scanning potential range of 0 to +0.6 V with a pulse amplitude of 25 mV, a square wave frequency of 15 Hz, and a step height of 1 mV.
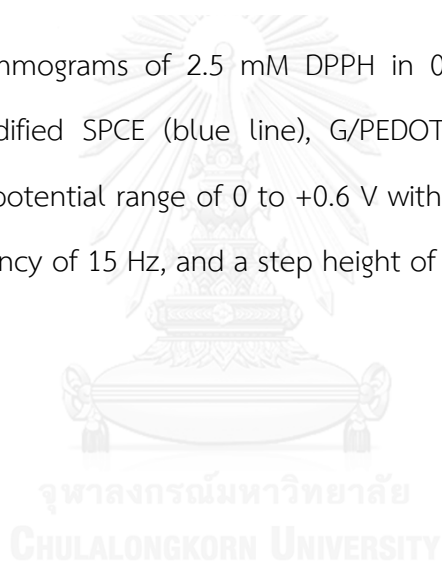
4.3.3 The performance of the G/PEDOT:PSS/SPCE

To study mass transfer process on the G/PEDOT:PSS/SPCE, the relationship between anodic and cathodic peak current and varying scan rate in a range of 100-400 mVs^{-1} was investigated as shown in Figure 4.10a. Both anodic and cathodic currents increased with scan rate. Figure 4.10b shows the anodic and cathodic current responses are directly proportional to square root of the scan rate ($V^{1/2}$), indicating that the mass transport of DPPH radical on G/PEDOT:PSS/SPCE is diffusion-controlled process, related to the Randle-Sevcik equation (4.1)

$$i_p = (2.69 \times 10^5) n^{3/2} A C D^{1/2} V^{1/2} \qquad \text{(equation 4.1)}$$

Where n is the number of electrons appearing in half-reaction for the redox couple, F is Faraday constant (96,485 C/mol), A is the electrode area (cm^2), V is scan rate (V/s), D is the analyte's diffusion coefficient (cm^2/s) , R = 8.314 J/mol K, and T is the absolute temperature (K).

b
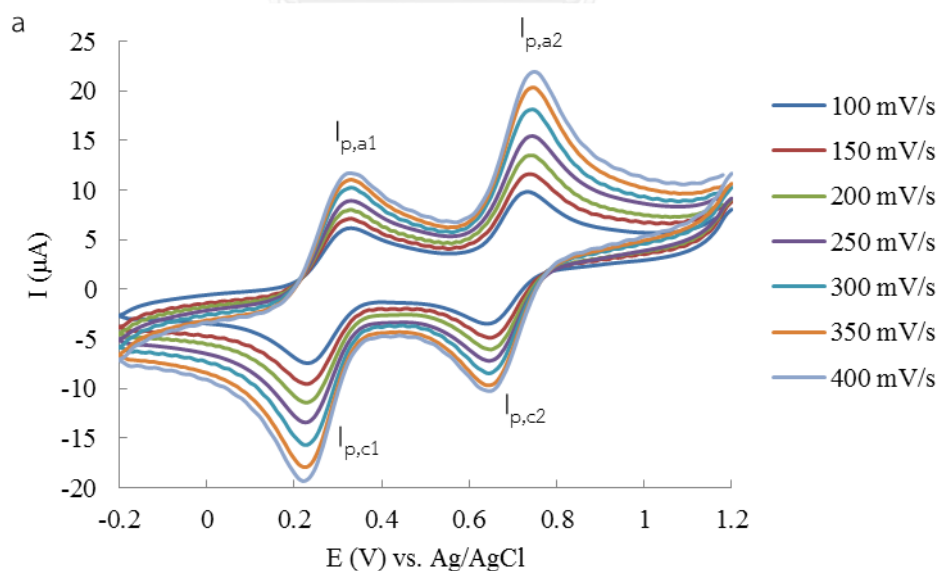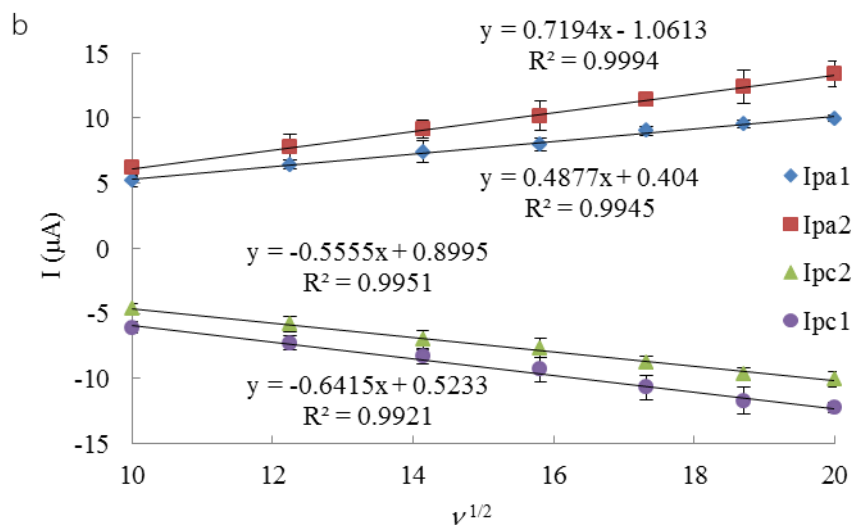
Figure 4.10 (a) Cyclic voltammogram of 2.5 mM DPPH in 0.1 M EPBS solution (pH 7.0) measured on the G/PEDOT:PSS-modified electrode at scan rate of 100, 150, 200, 250, 300, 350 and 400 mV/s and (b) relationship between the anodic and cathodic peak currents (μA) and (scan rate)$^{1/2}$.

4.4 Optimization of electrochemical parameters for DPPH detection

Chronoamperometry was used for indirect electrochemical determination of TAC via DPPH assay. The sensitivity and selectivity of the system can be adjusted by the selection of the detection potential. To optimize the detection potential in the chronoamperometric detection of DPPH, the hydrodynamic voltammetry of 6 mM DPPH versus background solution was performed. The effect of detection potential on the cathodic current of DPPH in a range of -0.1 V to 0.4 V vs. Ag/AgCl was studied. Figure 4.11 shows a hydrodynamic voltammogram measured on G/PEDOT:PSS/SPCE for DPPH (blue line) versus 0.1 M EPBS (green line). When the detection potential is changed from -0.1 V to 0.2 V, the cathodic current response drastically decreases. The cathodic current response reached a plateau when the detection potential is lower than 0.2 V. Figure 4.11b shows the S/B ratios which were calculated from Figure 4.11a at each point to obtain the optimal detection. Thus, the detection

potential of 0.2 V vs. Ag/AgCl was chosen as optimal detection potential for cathodic

chronoamperometric detection of DPPH solution.
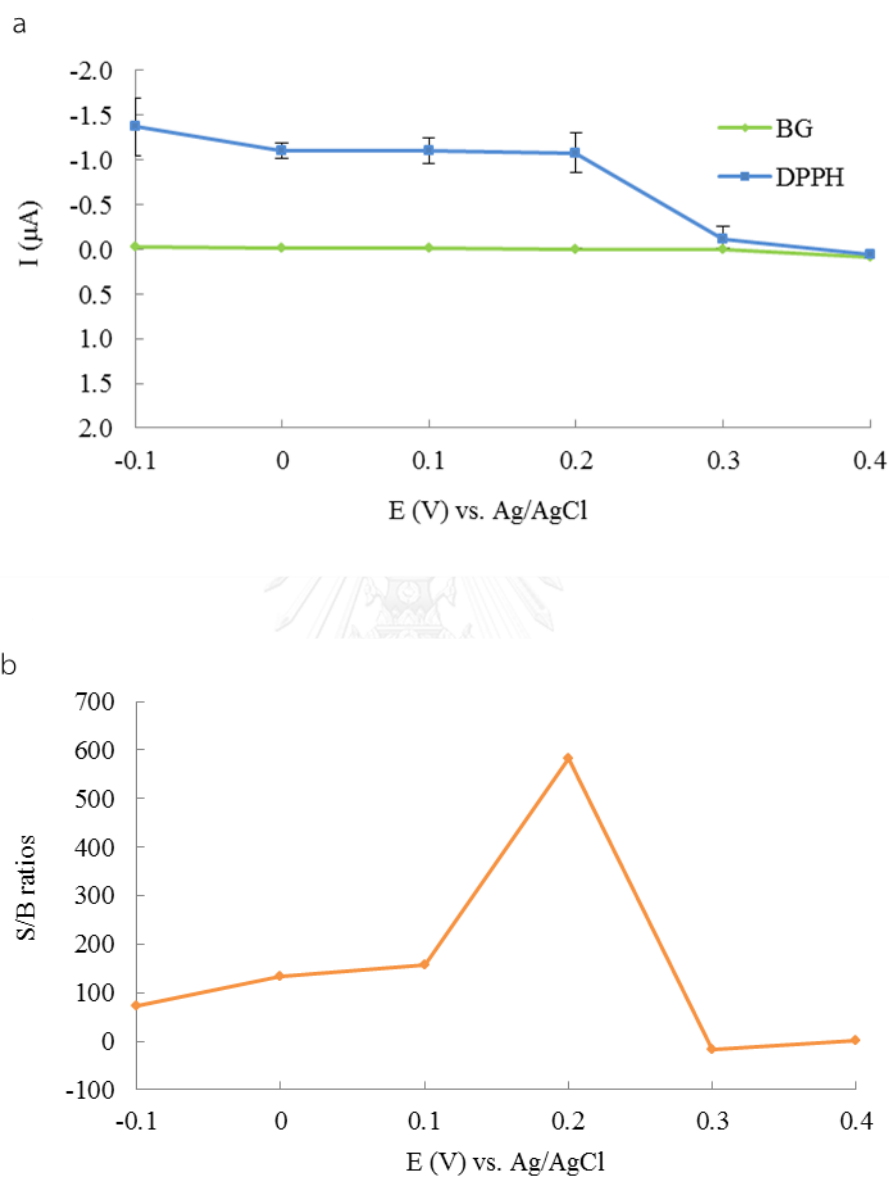
Figure 4.11 (a) Hydrodynamic voltammogram and (b) signal-to-background (S/B) ratio of 6 mM DPPH (blue line) and background (green line) at a 40 s sampling time measured on G/PEDOT:PSS/SPCE.

4.5 Analytical performance of this system

The analytical performance of G/PEDOT:PSS/SPCE was studied. Figure 4.12a shows the chronoamperogram of Trolox in the range of 0-30 µM with 6 mM DPPH. The calibration curve was plotted between standard Trolox concentrations and current response (ΔI) recorded at 40 seconds as shown in Figure 4.12b. The ΔI values were obtained from subtraction the analytical signal of current background ($\Delta I = I_{DPPH} - I_{DPPH+Trolox}$). The linear range was obtained in a range of 5-30 µM with a correlation coefficient (R^2) of 0.9923 for Trolox. The limit of detection (LOD, S/N=3) and the limit of quantitation (LOQ, S/N=10) were found to be 0.59 µM and 1.97 µM, respectively.

a
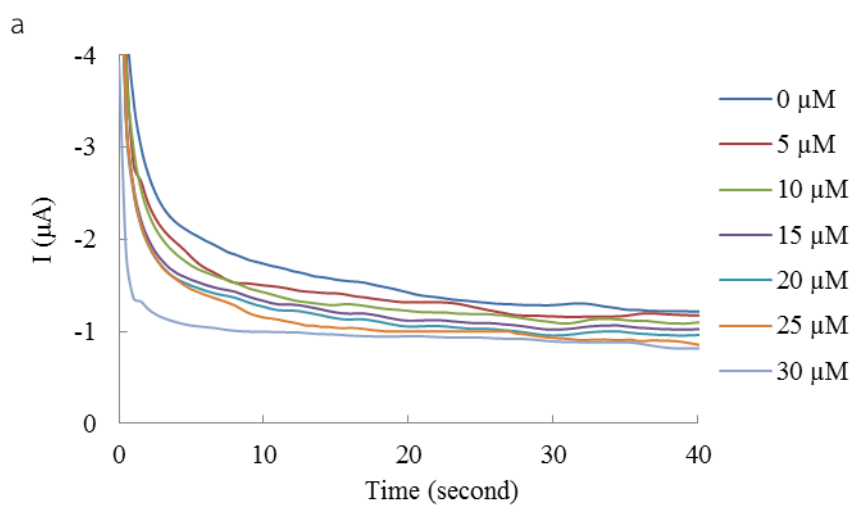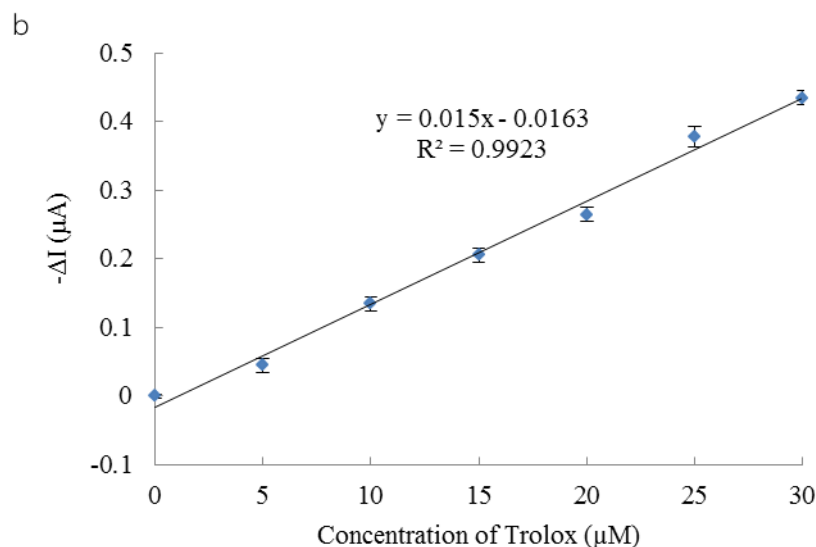
Figure 4.12 (a) Chronoamperogram and (b) calibration curve of Trolox in the concentration range of 5 to 30 µM in the EPBS solution, pH 7.0 measured on G/PEDOT:PSS/SPCE.

4.6 Reproducibility and repeatability of the modified electrode

The reproducibility of G/PEDOT:PSS/SPCE was investigated by measuring the chronoamperometric detection using the three concentration of Trolox (10, 20, 30 µM represent the low, medium, and high level, respectively). The reproducibility of G/PEDOT:PSS/SPCE was obtained from 7 electrodes as shown in Figure 4.13. Table 4.2 shows the relative standard deviation (RSD) of 3 concentrations (10, 20 and 30 µM), which were found to be 1.87, 2.13 and 2.90, respectively.

The repeatability of G/PEDOT:PSS/SPCE was obtained from 7 different measurements as shown in Figure 4.14. Table 4.3 shows the relative standard deviation (RSD) of 3 concentrations (10, 20 and 30 µM), which were found to be 2.83, 2.78 and 2.27, respectively.
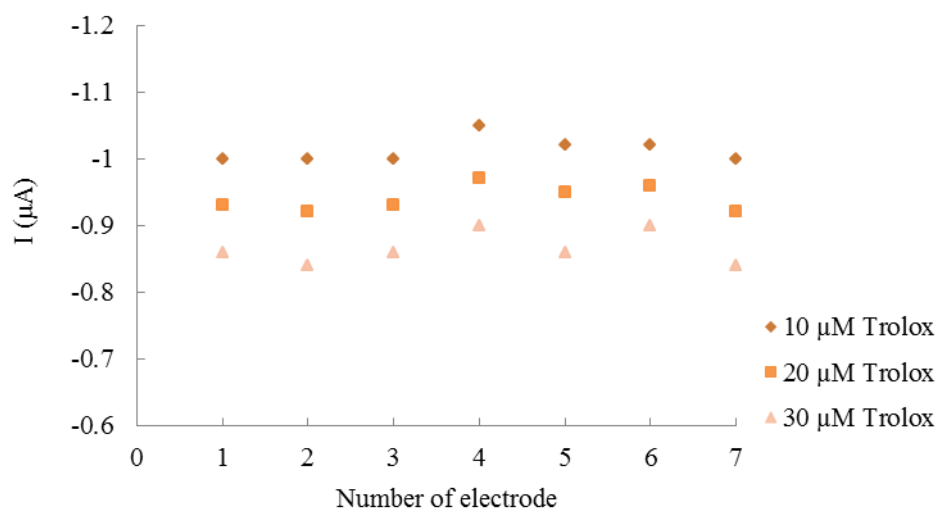
Figure 4.13 The current response of G/PEDOT:PSS/SPCE using the 3 concentrations of Trolox (10, 20, 30 μM represent the low, medium, and high level, respectively) from the calibration curve in presence of 6 mM DPPH by 7 different electrodes.

Table 4.2 The percentage of relative standard deviation (%RSD) of the G/PEDOT:PSS/SPCE by 7 difference electrodes.

Trolox concentration (μM)	%RSD (n=7)
10	1.87
20	2.13
30	2.90
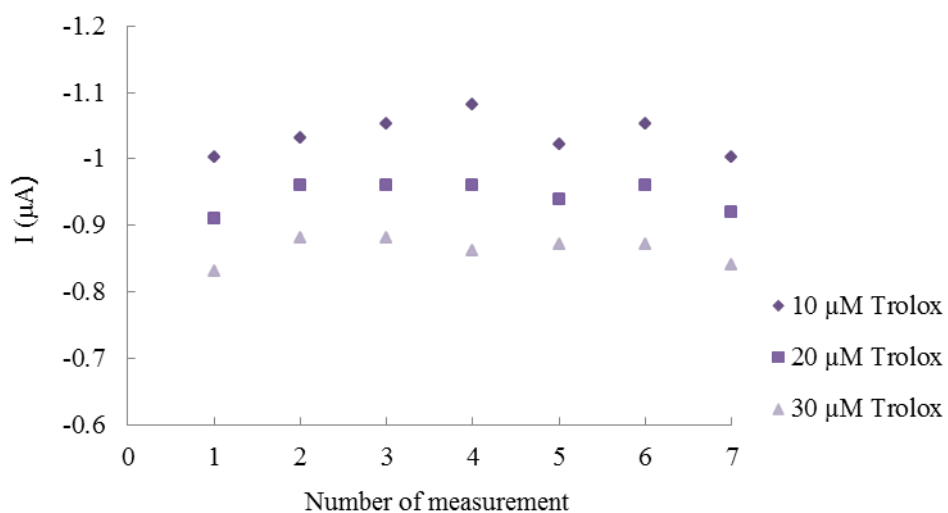
Figure 4.14 The current response of G/PEDOT:PSS/SPCE using the 3 concentrations of Trolox (10, 20, 30 µM represent the low, medium, and high level, respectively) from the calibration curve in presence of 6 mM DPPH by 7 different measurements.

Table 4.3 The percentage of relative standard deviation (%RSD) of the G/PEDOT:PSS/SPCE by 7different measurements.

Trolox concentration (µM)	%RSD (n=7)
10	2.83
20	2.78
30	2.27

4.7 Real sample analyses

   G/PEDOT:PSS/SPCE was used to evaluate the TAC in five Thai herbs, including extraction plant and herbal drinks. Prior to analysis, dried herb sample was prepared using ethanolic extraction to separate antioxidants from a dried plant matrix. TAC of herbal beverages were directly determined without any sample preparation. In case of dried plant, TAC value was expressed as mg Trolox equivalent per gram of dry plant (mg of Trolox g^{-1} of sample). Each sample was measured 3 times (n=3). The analytical results obtained from this system were compared with the conventional UV-Vis spectrophotometry as shown in Table 4.4. A paired t-test at 95% confidential interval was used to compare between these methods. It was found that there is no significant difference from the results that obtained from G/PEDOT:PSS/SPCE and conventional UV-Vis spectrophotometry. Obviously, these results demonstrate that G/PEDOT:PSS/SPCE was successfully applied for determination of TAC in both herb and herbal beverages with the satisfactory results.

Table 4.4 TAC values of five Thai herbs obtained from our method versus a conventional UV-Vis spectrophotometry (spectrometric, [26]).

Sample	Type of sample	Found TAC value	
		Our proposed method	Spectrophotometric method
Indian gooseberry	Dried plant	513.29±3.39 mg/g*	508.30±4.03 mg/g*
Thai blueberry	Beverage	5.25±0.15 g/L	5.00±0.15 g/L
Mulberry	Beverage	3.69±0.14 g/L	3.56±0.13 g/L
Bamboo grass	Beverage	3.89±0.12 g/L	3.75±0.14 g/L
Guto kola	Beverage	4.65±0.15 g/L	4.49±0.09 g/L

*mg of Trolox/g of sample

CHAPTER V

CONCLUSIONS

5.1 Conclusions

We successfully developed an electrochemical sensor using electrosprayed G/PEDOT:PSS/SPCE coupled with DPPH assay for the evaluation of TAC in herbs and herbal beverages.

The optimal conditions for electrode modification consisted of 10 mg/mL, of PEDOT:PSS 4 mg/mL of G, the ratio PEDOT:PSS and G at 2:1 (v/v), 6 minutes of electrospraying time. Optimal electrospraying conditions included 7.5 kV of applied voltage, 1 mL/h of flow rate, and 5 cm of needle to ground collector.

The optimization of chronoamperometric detection potential was found to be 0.2 V vs. Ag/AgCl. G/PEDOT:PSS/SPCE provides higher current response approximately 2 times for DPPH and 3 times for standard ferricyanide greater than an unmodified SPCE. The calibration curve was obtained in a linear range of 5-30 μM with a correlation coefficient (R^2) of 0.9923 for Trolox as a standard antioxidant. The limits of detection (LOD) and quantitation (LOQ) were found to be 0.59 μM and 1.97 μM, respectively. Moreover, this electrochemical system showed a good repeatability and reproducibility.

To validate this developed system, G/PEDOT:PSS/SPCE was applied to evaluate TAC in herb and herbal beverage samples and compared with a conventional UV-Vis spectrophotometry. The results obtained from two methods were in a good agreement.

5.2 Suggestion for future application

G/PEDOT:PSS/SPCE might be an alternative system for TAC in various application fields, such as medical diagnosis, pharmaceutical product, environmental monitoring and food quality control.

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APPENDIX

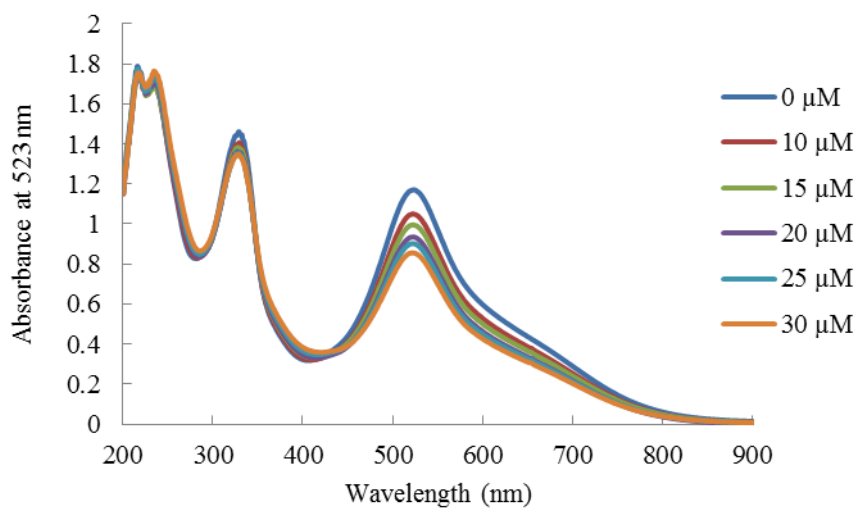Figure A.15 The UV–Vis spectra of 0.2 mM DPPH in ethanol-water after the addition of Trolox in the range of 10 to 30 μM.
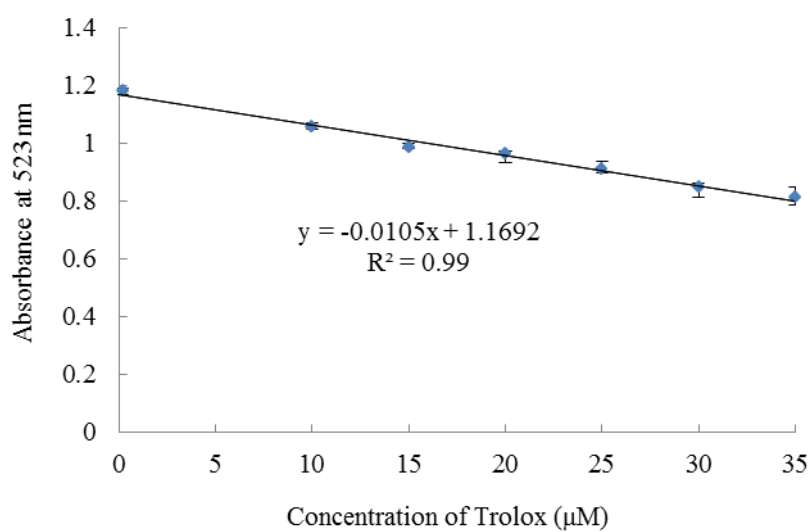

$$y = -0.0105x + 1.1692$$
$$R^2 = 0.99$$

Figure A.16 Calibration curve of 0.2 mM DPPH in ethanol-water after the addition of Trolox in the range of 10 to 30 μM.
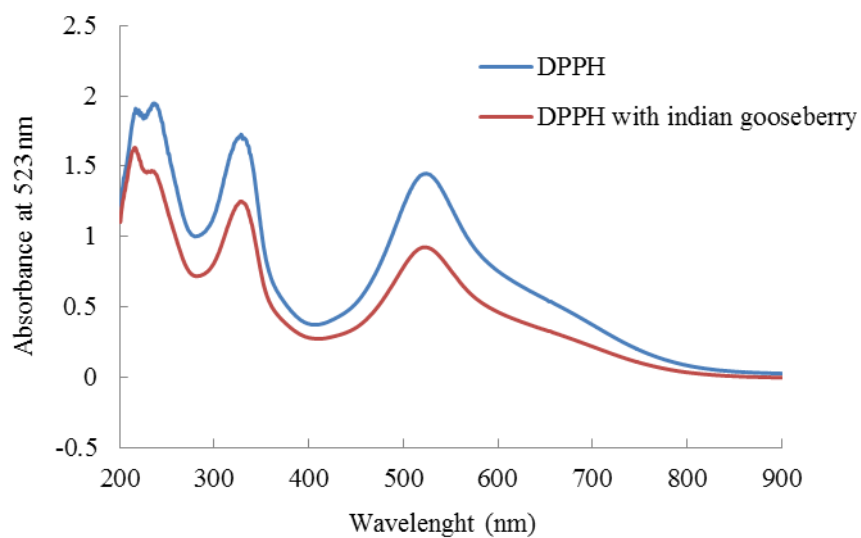
Figure A.17 The UV–Vis spectra of 0.2 mM DPPH in ethanol-water after the addition of indian gooseberry at 1000X dilution.
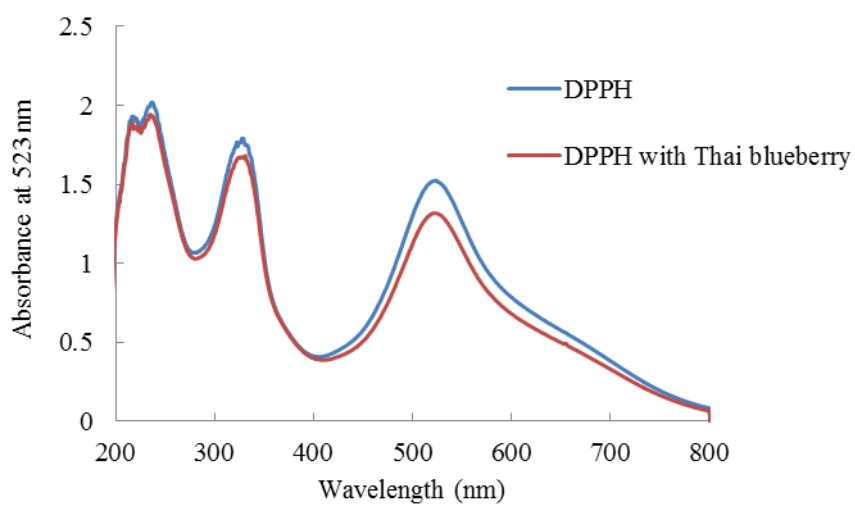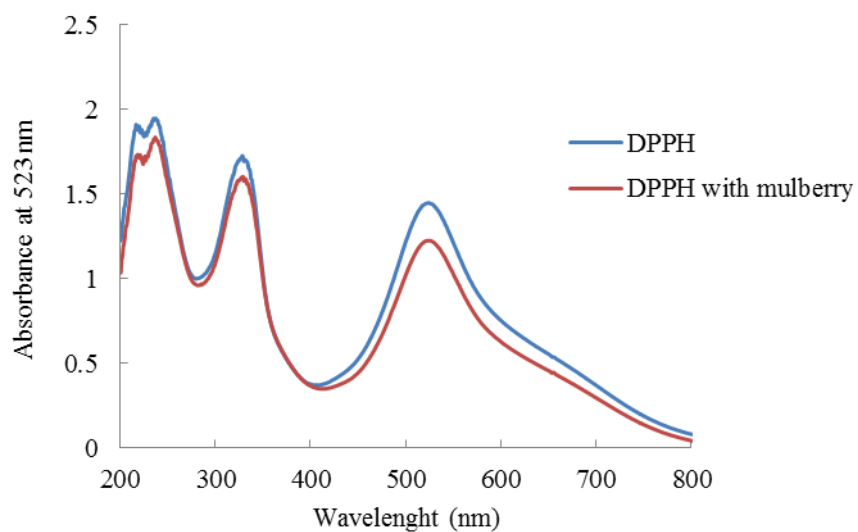

Figure A.18 The UV–Vis spectra of 0.2 mM DPPH in ethanol-water after the addition of Thai blueberry at 1000X dilution.

Figure A.19 The UV–Vis spectra of 0.2 mM DPPH in ethanol-water after the addition of mulberry at 1000X dilution.
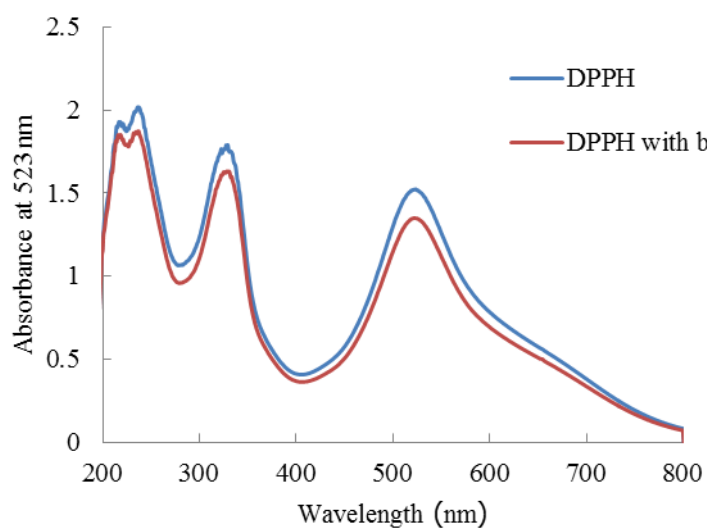
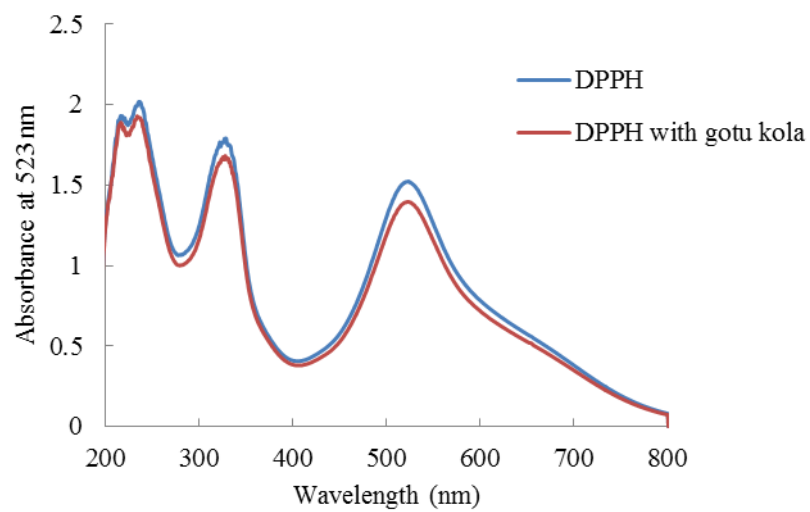Figure A.20 The UV–Vis spectra of 0.2 mM DPPH in ethanol-water after the addition of bamboo grass at 1000X dilution.
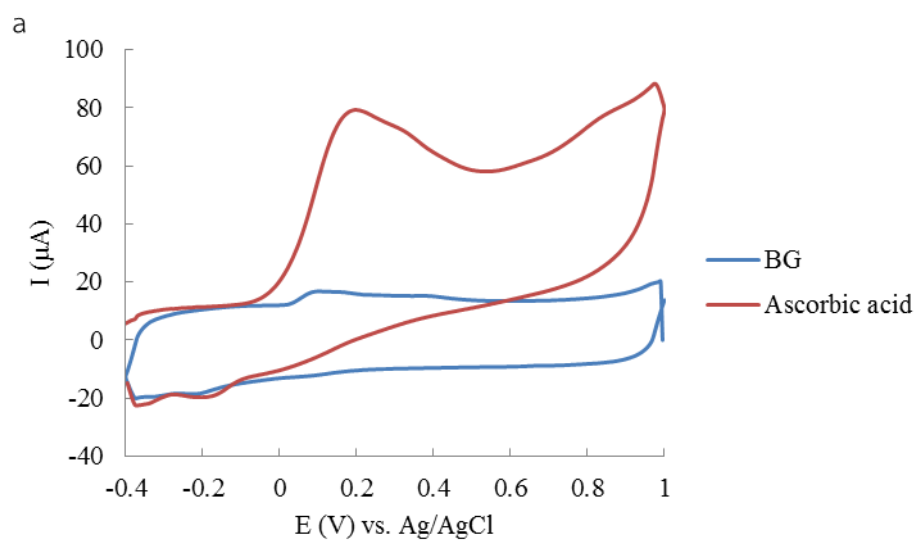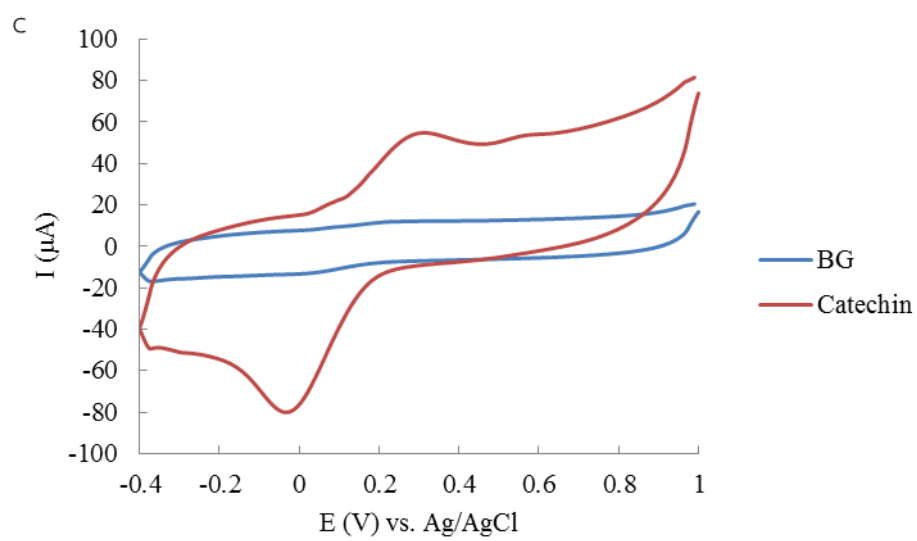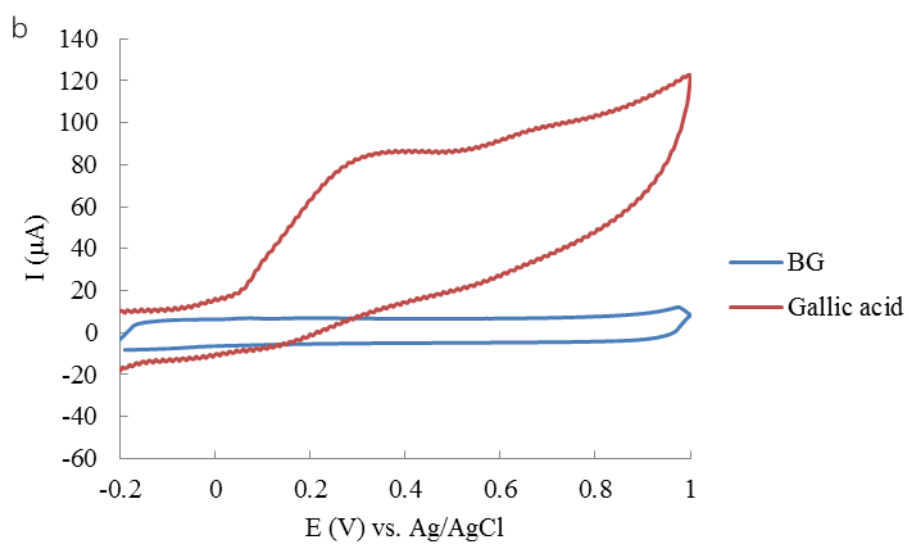
Figure A.21 The UV–Vis spectra of 0.2 mM DPPH in ethanol-water after the addition of gotu kola at 1000X dilution.
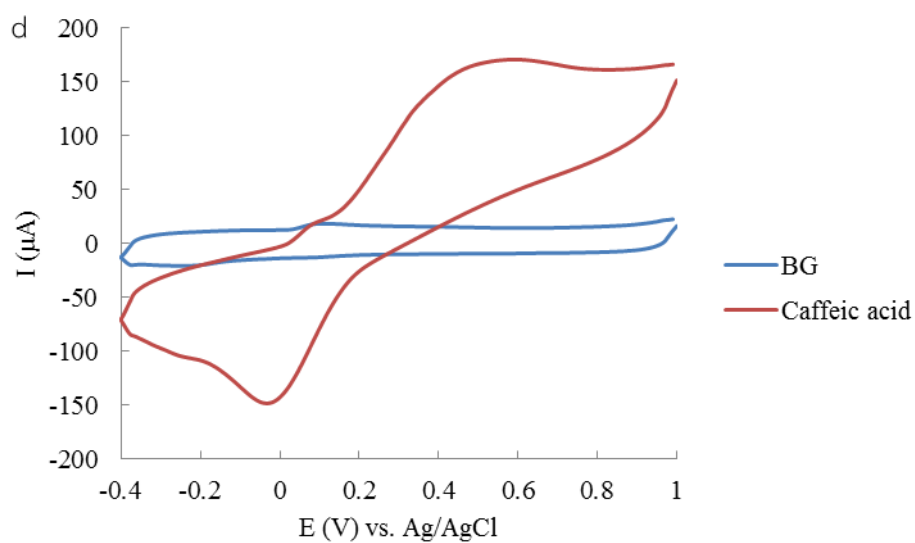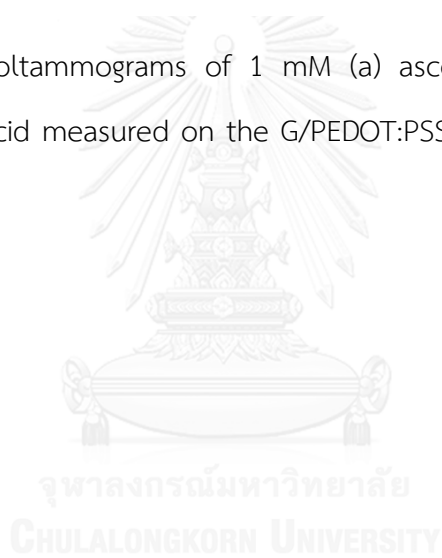
Figure A.22 Cyclic voltammograms of 1 mM (a) ascorbic acid (b) gallic acid (c) catechin (d) caffeic acid measured on the G/PEDOT:PSS/SPCE in the 0.1 M EPBS, pH 7.0.

VITA

Ruksuda Tirawattanakoson was born in December 6, 1989 in Bangkok, Thailand. She graduated in Bachelor Degree of Science program in Biochemistry from Chulalongkorn University, Bangkok, Thailand (2008-2012) and expect graduate in Master Degree of Science program in Biotechnology from Chulalongkorn University, Bangkok, Thailand in 2015.

Poster presentation

Tirawattanakoson, R., Rattanarat, P., Ngamrojanavanich, N., Rodthongkum, N., Chailapakul, O. "Graphene-conducting polymer nanocomposite modified electrochemical sensor for free radical scavenger screening of ascorbic acid" International Bioscience Conference, September 29-30, 2014, Phuket, Thailand.

Proceeding

Tirawattanakoson, R., Rattanarat, P., Ngamrojanavanich, N., Rodthongkum, N., Chailapakul, O. Graphene-conducting polymer nanocomposite modified electrochemical sensor for free radical scavenger screening of ascorbic acid. International Bioscience Conference 2014. 2014: 151-154.