

การพัฒนาวิธีตรวจวัดเจนิสเทอีนในเถาวัลย์เปรียง *Derris scandens* ด้วยวิธีทางเคมีไฟฟ้า



บทคัดย่อและแฟ้มข้อมูลฉบับเต็มของวิทยานิพนธ์ตั้งแต่ปีการศึกษา 2554 ที่ให้บริการในคลังปัญญาจุฬาฯ (CUIR)
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METHOD DEVELOPMENT FOR DETERMINATION OF GENISTEIN
IN JEWEL VINE *Derris scandens* BY ELECTROCHEMICAL METHOD

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A Dissertation Submitted in Partial Fulfillment of the Requirements
for the Degree of Doctor of Philosophy Program in Chemistry

Department of Chemistry

Faculty of Science

Chulalongkorn University

Academic Year 2016

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สุรินทร์ยา ไตรภพ : การพัฒนาวิธีตรวจวัดเจนิสเทอินในเถาว์วัลย์เปรียง *Derris scandens* ด้วยวิธีทางเคมีไฟฟ้า (METHOD DEVELOPMENT FOR DETERMINATION OF GENISTEIN IN JEWEL VINE *Derris scandens* BY ELECTROCHEMICAL METHOD) อ. ที่ปรึกษาวิทยานิพนธ์หลัก: ผศ. ดร.สุชาดา จุณนุวัฒน์กุล, อ.ที่ปรึกษาวิทยานิพนธ์ร่วม: ศ. ดร.อรรวรรณ ชัยลภากุล, 76 หน้า.

ในงานวิจัยนี้ได้พัฒนาตัวตรวจวัดทางเคมีไฟฟ้าซึ่งประกอบด้วยระบบที่มีขั้วไฟฟ้าสามชนิด ขั้วไฟฟ้าอ้างอิงซิลเวอร์/ซิลเวอร์คลอไรด์, ขั้วไฟฟ้าช่วยคาร์บอน และขั้วไฟฟ้าใช้งานคาร์บอนเตรียมได้จากการพิมพ์สกรีนหมึกนำไฟฟ้าผ่านรอยฉลบบนแผ่นผ้าตาข่ายลงบนแผ่นพลาสติกที่มีความหนา 0.3 มิลลิเมตร จะได้ตัวตรวจวัดที่มีขนาด 2.5×1.0 เซนติเมตร อุปกรณ์นี้ต้องการสารละลายตัวอย่างเพียง 50 ไมโครลิตร สำหรับการตรวจวัดความเข้มข้นของเจนิสเทอินด้วยเทคนิคสแควร์เวฟ โวลแทมเมทรี ออกซิเดชันพีคของเจนิสเทอินให้สัญญาณได้ดีในสภาวะกรดเกิดขึ้นที่ศักย์ไฟฟ้า 0.6 โวลต์ นอกจากนี้สามารถขยายสัญญาณได้โดยการผสมวัสดุตัดแปรโคบอลต์ททาลไฮยานินและกราฟีนลงไปในหมึกนำไฟฟ้าก่อนพิมพ์สกรีนลงบนแผ่นพลาสติก ภายใต้สภาวะที่เหมาะสมพบช่วงความเป็นเส้นตรง 2.5-150 ไมโครโมลาร์ และขีดจำกัดในการตรวจวัด 1.5 ไมโครโมลาร์ สำหรับตัวตรวจวัดทางเคมีไฟฟ้าที่ตัดแปรด้วยโคบอลต์ททาลไฮยานิน สำหรับตัวตรวจวัดทางเคมีไฟฟ้าที่ตัดแปรด้วยกราฟีนมีช่วงความเป็นเส้นตรง 1-200 ไมโครโมลาร์ และขีดจำกัดในการตรวจวัด 1.0 ไมโครโมลาร์ วิธีที่นำเสนอนี้สามารถนำไปประยุกต์ในการตรวจวัดเจนิสเทอินในเถาว์วัลย์เปรียงซึ่งเป็นยาสมุนไพรไทยที่ออกฤทธิ์ในการต้านการอักเสบได้อย่างน่าพึงพอใจ

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5472867423 : MAJOR CHEMISTRY

KEYWORDS: SCREEN-PRINTED ELECTROCHEMICAL SENSOR / SQUARE WAVE VOLTAMMETRY / GENISTEIN / COBALT(II) PHTHALOCYANINE / GRAPHENE / DERRIS SCANDEN

SURINYA TRAIPOP: METHOD DEVELOPMENT FOR DETERMINATION OF GENISTEIN IN JEWEL VINE *Derris scandens* BY ELECTROCHEMICAL METHOD. ADVISOR: ASST. PROF. SUCHADA CHUANUWATANAKUL, Ph.D., CO-ADVISOR: PROF. ORAWON CHAILAPAKUL, Ph.D., 76 pp.

In this work, the in-house screen-printed electrochemical sensor consisted of three-electrode system was developed for genistein determination. A silver/silver chloride (Ag/AgCl) reference electrode, a carbon counter electrode and a carbon working electrode were prepared onto a 0.3 mm-thick plastic substrate by screen-printing of conductive ink through blocking stencil on a woven mesh. The dimension of the sensor 2.5×1.0 cm was received. Only 50 μ L of sample solution was required on this device for the determination of genistein concentration by square wave voltammetry. The oxidation peak of genistein appeared with good response in acidic media at a potential of 0.6 V. Moreover, the signal was enhanced by mixing modifying material, cobalt(II) phthalocyanine and graphene, into the conductive carbon ink before screen-printing on a plastic substrate. Under optimized conditions, the linear range was found to be 2.5-150 μ M and the detection limit was 1.5 μ M for cobalt(II) phthalocyanine modified electrochemical sensor. For graphene modified electrochemical sensor, the linear range was found to be 1-200 μ M and the detection limit was 1.0 μ M. This proposed method was applied to determine genistein in jewel vine (*Derris scandens*), a Thai herbal medicine with anti-inflammatory activity, with satisfying results.

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Field of Study: Chemistry

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Academic Year: 2016

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ACKNOWLEDGEMENTS

I would like to extend my utmost gratitude to my advisor, Asst. Prof. Dr. Suchada Chuanuwatanakul and my co-advisor, Prof. Dr. Orawon Chailapakul for their guidance, suggestion, and support provided me with throughout the entire course of this investigative study.

I am also deeply grateful to Assoc. Prof. Dr. Vudhichai Parasuk, Assoc. Prof. Dr. Nattaya Ngamrojanavanich and Asst. Prof. Dr. Pakorn Varanusupakul from the Department of Chemistry, Faculty of Science, Chulalongkorn University and Asst. Prof. Dr. Wanida Wonsawat from the Department of Chemistry, Faculty of Science and Technology, Suan Sunandha Rajabhat University, my thesis committee members, forgiving valuable comments and advice.

Acknowledgements are extended to all members of the Electrochemistry and Optical Spectroscopy Research Unit for their helpful and comments during the research work.

The financial supports are gratefully acknowledged from the 90th Anniversary of Chulalongkorn University Fund (Ratchadaphiseksomphot Endowment Fund), the Thailand Research Fund through Research Team Promotion Grant (RTA5780005), and the Electrochemistry and Optical Spectroscopy Research Unit, Department of Chemistry, Faculty of Science, Chulalongkorn University. I am also appreciative to the Department of Chemistry, Faculty of Science, Chulalongkorn University for providing chemicals, laboratory facilities, education, and wonderful experience.

Furthermore, a word of appreciation is extended to my beloved family for everything in my whole life. My special thanks are extended to everyone who helped me to succeed in this dissertation, both during and before my time at Chulalongkorn University.

Without all of these kind supporters, this research undertaking would not have succeeded.

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LIST OF ABBREVIATIONS

%	Percent
V	Potential scan rate
ω	Frequency
μg	Microgram
μL	Microliter
μM	Micromolar
Ag/AgCl	Silver/silver chloride
BR buffer	Britton–Robinson buffer
CoPC	Cobalt(II) phthalocyanine
CV	Cyclic voltammetry
E^0	Formal reduction potential
E_p	Peak potential
$E_{p,a}$	Anodic peak potential
$E_{p,c}$	Cathodic peak potential
E_{step}	Step potential
g	Gram
Hz	Hertz
I_p	Peak current

L	Liter
LOD	Limit of detection
LOQ	Limit of quantitation
M	Molar
mg	Milligram
mg/g	Milligram per gram
mL	Milliliter
mM	Millimolar
°C	Degree Celsius
pH	Potential of hydrogen
PVC	Polyvinyl chloride
R ²	Correlation coefficient
s	Second
SEM	Scanning electron microscope
SPEs	Screen-printed electrodes
SWV	Square wave voltammetry
V	Volt
V/s	Volt per second

CHAPTER I

INTRODUCTION

1.1 Introduction and literature reviews

Derris scandens or jewel vine or well-known in Thai name as “Tao-Wan-Priang” is a member in the Leguminosae family. Its stem is commonly used in Thai traditional medicine as decoction for the treatment bone and joint pain, osteoarthritis [1], expectorant and diuretic. Its root can be used as an insecticide, diuretics and elixirs [2]. Moreover, several properties of this plant were reported in academic journal such as it showed the inhibitory effect of natural killer cell (NK cell) on HIV-infected patients [3], inhibited the activity of α -glucosidase for antidiabetic therapeutics [4, 5], antimigration of cancer cells [6]. Its anti-inflammatory property is interesting in this research because it was found to be the most active traditional Thai medicine to reduce inflammation [7]. Its extract revealed good alleviation of low back pain as same as Diclofenac, a nonsteroidal anti-inflammatory drug [8]. This plant is widely used as alternative traditional medicine, recently.

The anti-inflammatory activity of *Derris scandens* is a result of the active component, genistein [9]. Genistein belongs to the group of isoflavones mainly found in soybean and other legumes [10, 11]. It is a kind of phytoestrogen, whose chemical structure is similar to estrogens, the female sex hormone, naturally occurring in plants. The chemical structure of genistein is illustrated in Figure 1.1. It also has several other potentials such as antioxidant, antitumorigenic, antibacterial, and anti-inflammatory properties [9, 12-14]. Nowadays, many manufactories produce lots of soy-based supplements as a source of genistein. Its concentration varies depending on varieties of soybeans and processing techniques. Knowing the existence and amount of genistein in food and dietary supplements is important for quality control in the process and high profit and safety of consumption. Although no clinical trials have been reported documenting effects of optimum dosage of genistein, a high soy diet containing up to 45 mg of isoflavones per day can cause changes in the menstrual cycle that may help reduce cancer risk [10]. In Asia, population groups who eat soy

foods daily containing 20 mg to 80 mg of genistein have lower rates of breast and prostate cancer than do groups in the West with less genistein in their diets [15].

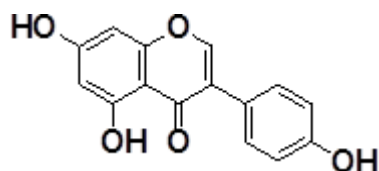


Figure 1.1 Chemical structure of genistein.

Many selective and sensitive methods such as high-performance liquid chromatography (HPLC) combined with different detection systems [16-20], gas chromatography-mass spectrometry (GC-MS) [21, 22] and capillary electrophoresis (CE) [23] have been reported to determine the quantity of genistein. These methods involve complicated process, operate with long analysis times, require the expensive instruments and skilled operating technicians, and are limited to laboratory analysis. Electrochemical techniques are attractive alternatives for the quantification of organic compounds because of their simplicity, high sensitivity and relatively low cost. Moreover, undissolved excipients or colored solutions do not interfere in real sample analysis. For genistein determination by voltammetry, reduction of genistein on a mercury electrode was reported [24, 25]. However, using mercury is well known that it is very harmful. Even a small amount of mercury is toxic to the worker and the environment. Recently, screen-printed electrodes (SPEs) are widely used as economical electrochemical substrates. SPEs are also suitable for on-field analysis because of their advantages, such as simplicity of preparation, disposability, and rapid responses [26, 27]. Carbon ink is commonly screen-printed as a working electrode. Although carbon is less conductive than metal, it provides a wide potential window and it is inexpensive and easy to modify with various modifying materials.

In this work, a simple, rapid and low-cost method for genistein determination by the screen-printed electrochemical sensor is presented which requires only a small amount of sample volume.

1.2 Objectives of the research

This research will develop the simple, rapid and low-cost screen-printed electrochemical sensor for genistein determination in *Derris scandens* with two main goals as follows:

- 1) To develop a simple and rapid electrochemical method for genistein determination
- 2) To apply the developed method for genistein determination in *Derris scandens*

1.3 Scope of the research

The electrochemical sensor was produced by screen-printing technique consisted of three-electrode system (Ag/AgCl reference electrode, carbon working electrode, and carbon counter electrode) for genistein determination. Carbon electrochemical sensor was used to study the electrochemical behavior of genistein by cyclic voltammetry. The effective modifying materials, CoPC, was used to modify the carbon working electrode of the developed sensor and the other sensor was produced with graphene ink as working electrode and counter electrode to improve sensitivity. With the optimized conditions, the developed electrochemical sensor was used to study the analytical performance and detect genistein in *Derris scandens* by square wave voltammetry.

CHAPTER II

THEORY

2.1 Jewel vine, *Derris scandens*

Jewel vine or *Derris scandens* locally Thai called “Thao-Wan-Priang”, is a member in the Leguminosae family. It is an evergreen climbing branched shrub having twining habit. It has a tap root system without aerial roots. The leaves are odd-pinnately compound. Inflorescence is a raceme of pea-like flowers. Fruit is flattened pod, lanceolate, narrow, spindled at both ends as shown in Figure 2.1 [28].

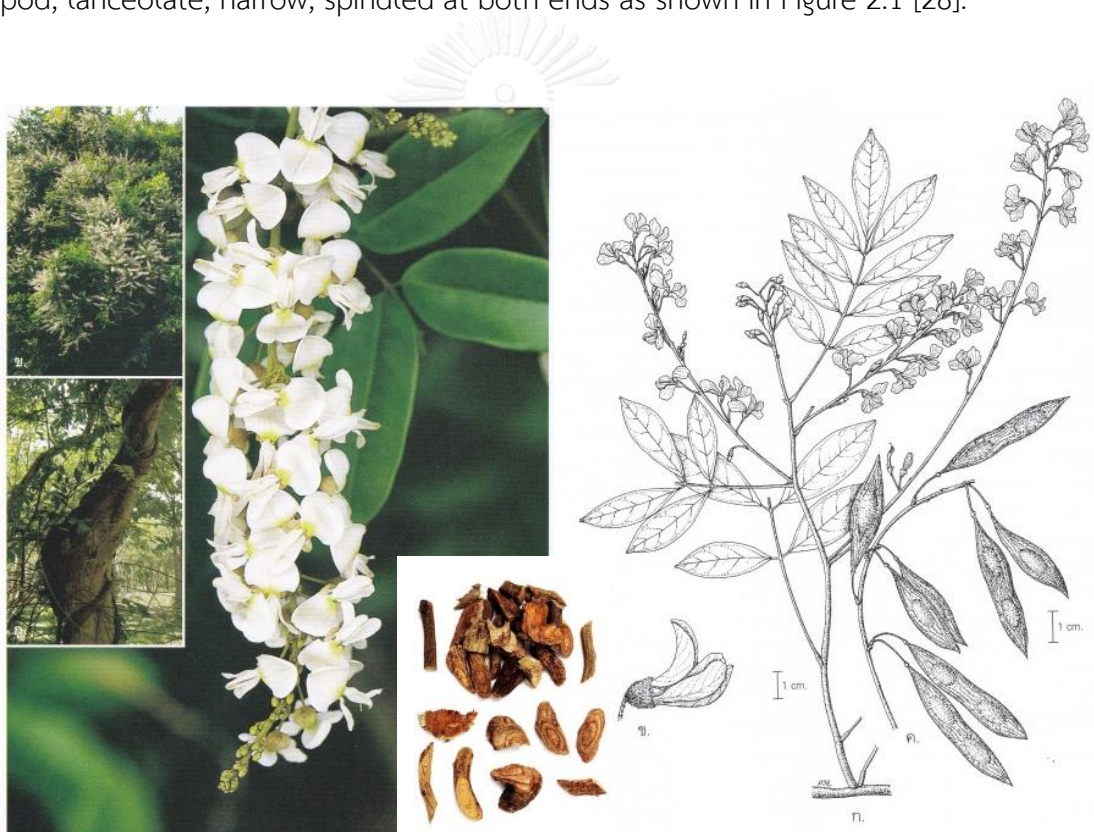


Figure 2.1 Jewel vine [28]

This plant is widely distributed throughout Indian subcontinent and Southeast Asia, including Thailand. Its stem is commonly used in Thai traditional medicine as decoction for the treatment bone and joint pain, osteoarthritis [1], expectorant and diuretic. Its root can be used as an insecticide, diuretics and elixirs [2]. Moreover, several properties of this plant were reported in academic journal such as it showed the inhibitory effect of natural killer cell (NK cell) on HIV-infected patients [3], inhibited the activity of α -glucosidase for antidiabetic therapeutics [4, 5], antimigration of cancer cells [6].

Derris scandens extract revealed good alleviation of low back pain as same as Diclofenac, a nonsteroidal anti-inflammatory drug. The clinical study reported the benefit of treatment of patients with low back pain with the 50% ethanol extract of *Derris scandens* at a dose of 600 mg/day and with diclofenac at a dose of 75 mg/day for seven days were comparable without no side effects from both treatments [8]. The safety of *Derris scandens* and its effects on immune system have been investigated in 12 healthy volunteers. The volunteers received 400 mg/day of *Derris scandens* hydroalcoholic extract for 2 months. The results suggested that the *Derris scandens* extract was safe and could induce the secretion of cytokines that might help in modulating immune responses [29].

Its anti-inflammatory property is interesting in this research because it was found to be the most active traditional Thai medicine to reduce inflammation. In 2003, Laupattarakasem group had been studied the anti-inflammatory ability in various Thai medicinal plants [7]. They found that *Derris scandens* extract show the most effective activity. Then, they found 4 effective compounds from the aqueous extract of *Derris scandens* stem including genistein and its glycoside, 7-O- α -rhamno(1 \rightarrow 6)- β -glucosylgenistein, and two isoprenyl derivatives 5,7,4'-trihydroxy-6,5'-di-prenyl isoflavone and scandenin (Figure 2.2). Although the isoprenylated compounds show the most effective to inhibit releasing inflammatory agent, its concentration in the extract are quite low. HPLC analysis showed that the genistein accounted for most of the activity of the total extract. Moreover, antioxidant studies showed that genistein and showed activity comparable to standard antioxidants without cytotoxicity [9].

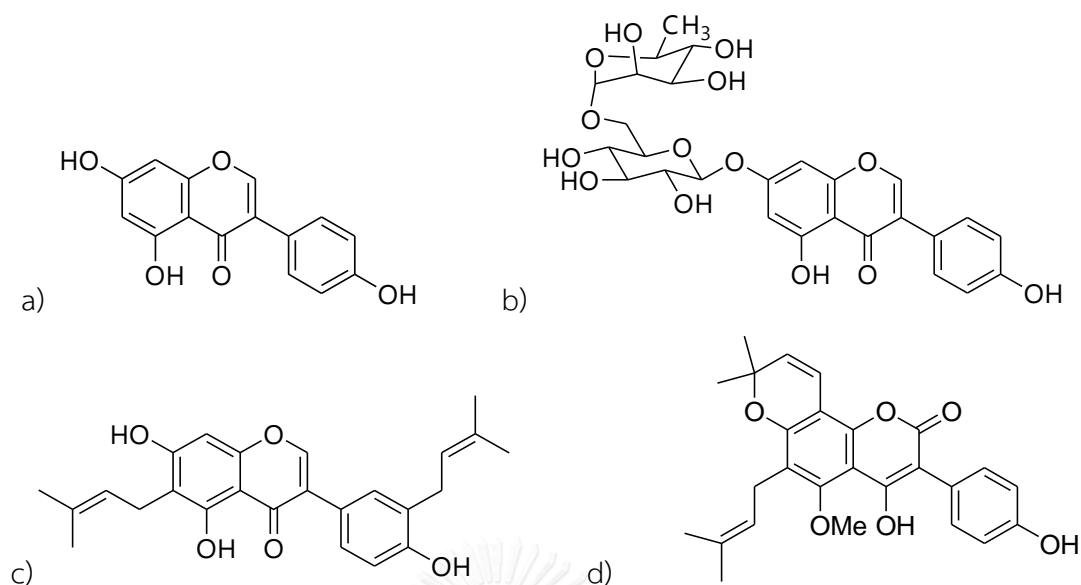


Figure 2.2 Structure of effective compounds found in *Derris scandens* extract
 a) genistein, b) 7-O- α -rhamno(1 \rightarrow 6)- β -glucosylgenistein, c) 5,7,4'-trihydroxy-6,5'-di-prenylisoflavone, and d) scandenin.

2.2 Genistein

Genistein (5,7-dihydroxy-3-(4-hydroxyphenyl)chromen-4-one; $C_{15}H_{10}O_5$) belongs to the group of isoflavones mainly found in soybean and other legumes [10, 11]. It is a kind of phytoestrogen, whose chemical structure is similar to estrogens, the female sex hormone, naturally occurring in plants. It also has several other potentials such as antioxidant, antitumorigenic, antibacterial, and anti-inflammatory properties [9, 12-14].

The disadvantage of genistein is not exactly clear, some research reported that genistein can be used without any defect but some research reported the overdose of genistein can stimulate growth of cancer. The perinatal dietary administration of genistein at concentrations up to 250 mg/kg diet did not result in toxic effects in rat [30]. However, dietary genistein more than 250 mg/kg increased tumor size in ovariectomized athymic nude mice implanted with human breast cancer [31]. Although no clinical trials have been reported documenting effects of optimum dosage of genistein for human, a high soy diet containing up to 45 mg of isoflavones per day

can cause changes in the menstrual cycle that may help reduce cancer risk [10]. In Asia, population groups who eat soy foods daily containing 20 mg to 80 mg of genistein have lower rates of breast and prostate cancer than do groups in the West with less genistein in their diets [15]. Therefore, the determination of genistein is considerable to control appropriate amount of consuming.

2.2.1 Determination of genistein

There are many selective and sensitive methods have been reported to determine the quantity of genistein such as gas chromatography mass-spectrometry (GC-MS), capillary electrophoresis (CE) and high-performance liquid chromatography (HPLC) combined with different detection systems [32]. These methods involve complicated process, operate with long analysis times, require the expensive instruments and skilled operating technicians, and are limited to laboratory analysis. The electrochemical technique is an attractive alternative method for quantification because of its simplicity, fast analysis and relatively low cost. Moreover, undissolved excipients or colored solutions do not interfere in real sample analysis.

In 2010, Liu and coworker studied different macroporous resins as the adsorption materials for enrichment and separation of genistein and apigenin from extracts of pigeon pea roots. Reversed-phase HPLC with UV detector was used to determine the contents of analytes. ADS-5 resin, non-polar polystyrene with large surface area and large pore size, showed the maximum effective with about 10 enrichment factor for both analytes [32].

The electrochemical technique for genistein determination was proposed in 1999. Müllner and Sontag described determination of two phytoestrogens: daidzein and genistein, in soy based food and supplements by high performance liquid chromatography coupled with coulometric electrode array detection. Eight modified palladium electrodes were applied various potentials with increments of 60 mV. This method provided linearity in the range of 5-400 $\mu\text{g/L}$ and the detection limits were 0.9 $\mu\text{g/L}$ and 0.95 $\mu\text{g/L}$ for daidzein and genistein, respectively [33].

A single sweep polarography with dropping mercury electrode was used by Zhang and coworker in 2001 [24]. They found reduction peak of genistein occurred at -1.17 V and proposed its reduction mechanism as shown in Figure 2.3.

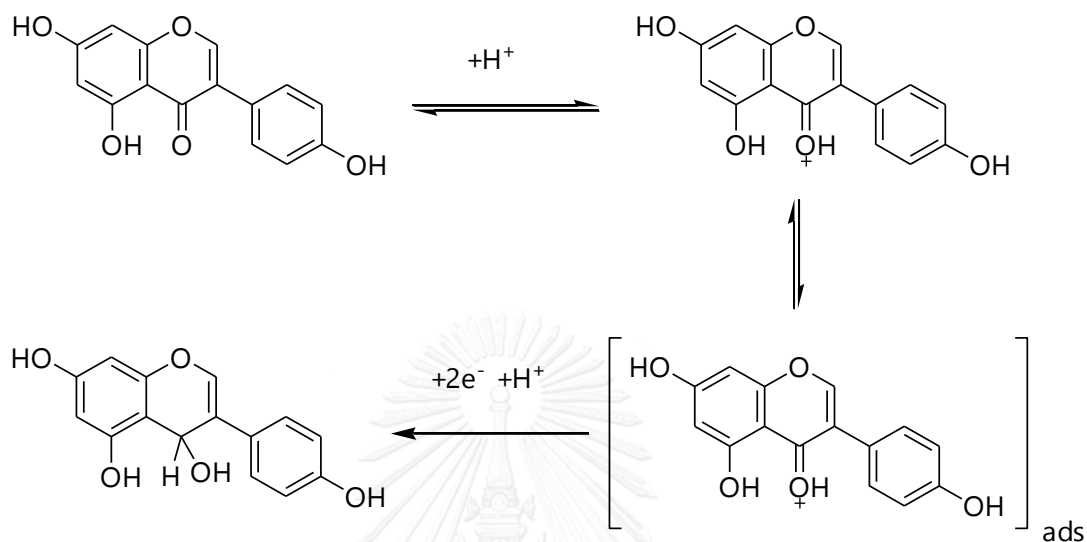


Figure 2.3 Reduction mechanism of genistein

In 2010, Fogliatto et al. proposed square-wave voltammetry to determine genistein in soy flours and soy based supplements. Cetyltrimethyl ammonium bromide (CTAB), a cationic surfactant, was introduced into buffer solution to improve sensitivity. The linear relationship between peak current and genistein concentration of this methodology was in the range of 1.14×10^{-7} to 1.09×10^{-6} mol/L with detection limit and quantification limit of 3.43×10^{-8} and 1.14×10^{-7} mol/L, respectively [25]. However, using mercury electrode is well known that it is very harmful. Mercury can volatile to the atmosphere. Small amount of mercury can be toxic to worker and the environment. So, it is better to avoid using this electrode.

In the same year, Saracino and Raggi proposed HPLC with coulometric detection to determine amounts of genistein, daidzein and glycitein in plasma of postmenopausal women. C18 reversed phase column was selected to separate the analytes with appropriate resolution and run time. Two working electrodes of porous graphite were used for screening interfering substances (-0.200 V) and detection ($+0.500$ V). Clozapine was suitable internal standard with relatively short retention time. Moreover, solid phase extraction (SPE) was used to clean up plasma sample before analysis. The LOD and LOQ for genistein was 0.15 ng/mL and 0.5 ng/mL, respectively [34].

Nurmi and coworker proposed high performance liquid chromatography with coulometric electrode array detector (HPLC-CEAD) to evaluate the amount of isoflavones in soy based supplements. After hydrolysis with β -glucuronidase and hydrochloric acid, aglycones were separated and determined by HPLC-CEAD. Eight electrode pairs were applied at potentials from 200 to 750 mV. Genistein was retained in HPLC column for 44.22 minutes and detected at 510 mV [35].

Peñalvo et al. developed a simple method to analyse total isoflavones in soy products. Four different extraction procedures were tested to optimize the sample pre-treatment. The best extraction was performed by using 1 M HCl in 80% ethanol with 1 hour incubation at 80°C . Then the extracts were analyzed by HPLC-CEAD with four electrode pairs [36].

Capillary electrophoresis with electrochemical detection (CE-ED) was used to determine daidzein and genistein in soy products by Peng et al. Prior to separation and analysis with carbon disk electrode, samples were extracted with 10 mL of 70% ethanol for 2 hours in an ultrasonic bath. Recovery with 96% of genistein was received from this procedure [23].

2.3 Electrochemical techniques

Electrochemistry is a branch of chemistry concerned with the interrelation of electrical and chemical effects. A large part of this field deals with the study of chemical changes caused by the passage of an electric current and the production of electrical energy by chemical reactions. Electroanalytical techniques are the equipment of scientist for studying an analyte by measuring the potential and/or current in an electrochemical cell containing the analyte. These methods can be broken down into several categories depending on which aspects of the cell are controlled and which are measured. The electrochemical techniques which are used in this work are described as follow.

2.3.1 Cyclic voltammetry (CV)

Cyclic voltammetry is the most popular electrochemical method. It provides both qualitative and quantitative information as well as a fast and reliable characterization tool. It is generally used to study the electrochemical properties of an analyte in solution. In a cyclic voltammetry experiment, the working electrode potential is ramped linearly versus time like linear sweep voltammetry. This ramping is known as the experiment's scan rate. Cyclic voltammetry consists of scanning linearly the potential of a stationary working electrode, using a triangular potential waveform, as shown in Figure 2.4 (left). The voltage is swept between two values of potential at a fixed rate. Depending on the information sought, single or multiple cycles can be used. During the potential sweep, the potentiostat measures the current resulting from the applied potential. The resulting current-potential plot is termed a cyclic voltammogram. Cyclic voltammetric measurements were characterized by a peak potential, E_p , the potential at which the current reaches its maximum value. Whereas, I_p is the value that is called the peak current. The $I_{p,a}$ and $E_{p,a}$ are the anodic peak current and anodic peak potential, respectively. The $I_{p,c}$ and $E_{p,c}$ are the cathodic peak current and cathodic peak potential, respectively (Figure 2.4 (right)).

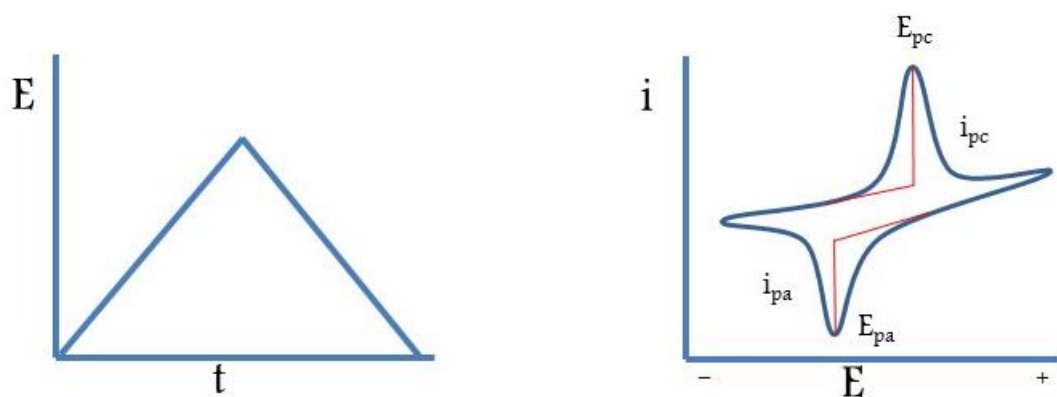


Figure 2.4 Cyclic voltammetric excitation signal (left) and cyclic voltammogram of a reversible reaction (right).

The peak current for a reversible couple is given by the Randles-Sevcik equation below:

$$I_p = 2.69 \times 10^5 n^{3/2} A C_O^* D_O^{1/2} \nu^{1/2} \quad (2.1)$$

For irreversible processes, the individual peaks are reduced in size and widely separated. The peak current given by

$$I_p = (2.99 \times 10^5) [(1-\alpha)n\alpha]^{1/2} A C_O^* D_O^{1/2} \nu^{1/2} \quad (2.2)$$

where n_α is the number of electrons transferred in the rate determining step, α is the transfer coefficient, A is the electrode area (cm^2), C_O^* is the bulk concentration of the analyte (M); and D_O is the analyte's diffusion coefficient (cm^2/s).

2.3.2 Square wave voltammetry (SWV)

Square wave voltammetry is an extremely useful technique for measuring trace levels of organic and inorganic species. The sensitivities of square wave voltammetry are better than those of normal pulse voltammetry. Figure 2.5 shows the potential waveform and the voltammogram of square wave voltammetry. The working electrode is applied with this potential waveform and measured the current twice during each square wave cycle, once at the end of forward pulse and once at the end of the backward pulse.

There are two advantages to measuring the different current. First, it increases the discrimination against the charging current, since any residual charging current is subtracted out. Second, the shape of the current response is a symmetric peak, rather than the sigmoidal curve typically found for normal pulse voltammetry.

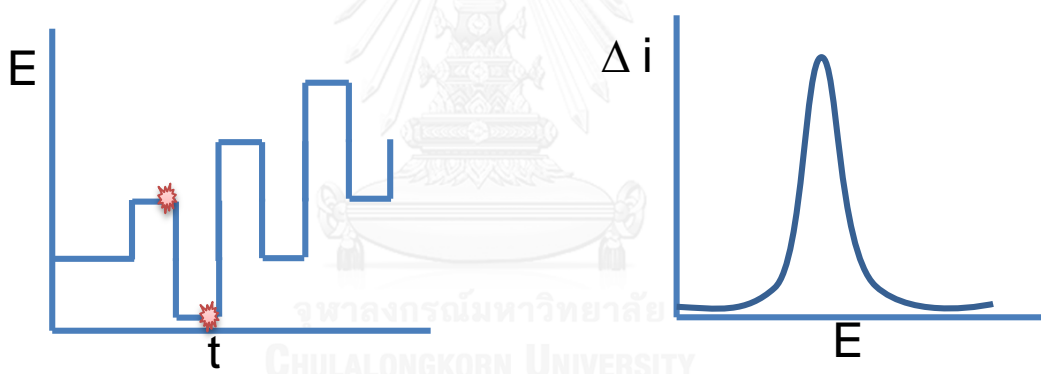


Figure 2.5 Square wave voltammetric excitation signal (left) and square wave voltammogram (right).

2.4 Electrodes

The voltammetric experiment is performed with three electrodes that consists of a working electrode, a reference electrode and a counter electrode. Normally, the electrode provides the interface across which a charge can be transferred. At the convenient applied potential, the reduction or oxidation of electroactive species occurs at the surface of a working electrode, results in the mass transport of new material to the electrode surface and the generation of a current.

2.4.1 Working electrode

Working electrode is the electrode that occurs the interesting reaction. In the voltammetric method, the small surface area of the working electrode is used for enhancing polarization and minimizing depletion of the analyte. The electrode performance seriously depends on the material of the electrode. The ideal working electrode should give a high signal-to-noise ratio of the interesting analytes, a reproducible electrode surface, a wide potential window, high electrical conductivity, low cost, low toxicity and availability. Chemically modified electrodes are employed for the analysis of both organic and inorganic samples.

2.4.2 Reference electrode

Reference electrode is an electrode which has a stable and well-known electrode potential. The potential applied to the working electrode is measured within the context of a known potential, which is in turn obtained from the reference electrode. The high stability of the electrode potential is usually reached by employing a redox system with constant (buffered or saturated) concentrations of each participants of the redox reaction. The most widely used is the silver/silver chloride reference electrode because it is simple, low-cost, stable and non-toxic.

2.4.3 Counter electrode

Counter electrode or auxiliary electrode normally used to minimize errors from the cell resistance while controlling the potential of working electrode. This is an electrode used in a three electrode electrochemical cell for voltammetric analysis or other reactions in which an electric current is expected to flow. The counter electrode, along with the working electrode, provides circuit over which current is either applied or measured. The potential of the counter electrode is usually not measured and is adjusted to balance the reaction occurring at the working electrode. The counter electrode often has a surface area much larger than the working electrode to ensure that the half-reaction at the counter electrode can occur fast enough so as not to limit the process at the working electrode. It is often fabricated from electrochemically inert materials such as gold, platinum, or carbon.

2.4.4 Screen-printed electrodes (SPEs)

Screen-printed electrode is a kind of remarkable electrodes. Recently, SPEs are widely used as the economical electrochemical substrates. SPEs are also suitable for on-field analysis because of their advantages, such as simplicity of preparation, disposability, and rapid responses [26, 27]. Moreover, it can be fabricated on several types of substrates. Especially, small amount of sample can be used. SPEs are produced by printing different conductive inks on various types of chemically inert substrates through the blocking stencil on a woven mesh, and a roller or squeegee is moved across the screen stencil to force or pump ink or other printable materials past the threads of the woven mesh in the open areas. Ag/AgCl is the mostly used material as a pseudo reference electrode. Carbon ink is commonly screen-printed as a working electrode. Although carbon is less conductive than metal, it provides a wide potential window and it is inexpensive and easy to modify with various modifying materials.

2.5 Modifying materials

High sensitivity is important for quantification. Determination of organic compounds is not as easy as metal ions. There are not only slower electron transfer but also adduct generation during the process. This research propose two effective electrodes modified with effective modifying materials, cobalt(II) phthalocyanine and graphene for genistein determination.

2.5.1 Cobalt(II) phthalocyanine (CoPC)

Phthalocyanines is a large macrocyclic molecules. It is a planar aromatic macrocycles consisting of four isoindole units presenting an 18 π -electron aromatic cloud delocalized over an arrangement of alternated carbon and nitrogen atoms. It has an aromatic ring system that acts both as an electron donor and acceptor. It can form highly conjugated planar complexes with various metal ions such as cobalt. The center of metal ions can be inserted in the place of hydrogens present in non-metallated phthalocyanine. Phthalocyanines and their metal derivatives are highly conjugated planar complexes (Figure 2.6). They are widely used as dyes or pigments for a long time. They are also very useful for the electronic device and applied in the fields of conductive polymers, chemical sensors and electrochromism. Because of the large conjugated π system, excellent photoelectric characteristics, high thermal and chemical stability, and most importantly the easy functionalization of phthalocyanines render them the ideal organic semiconductor materials.

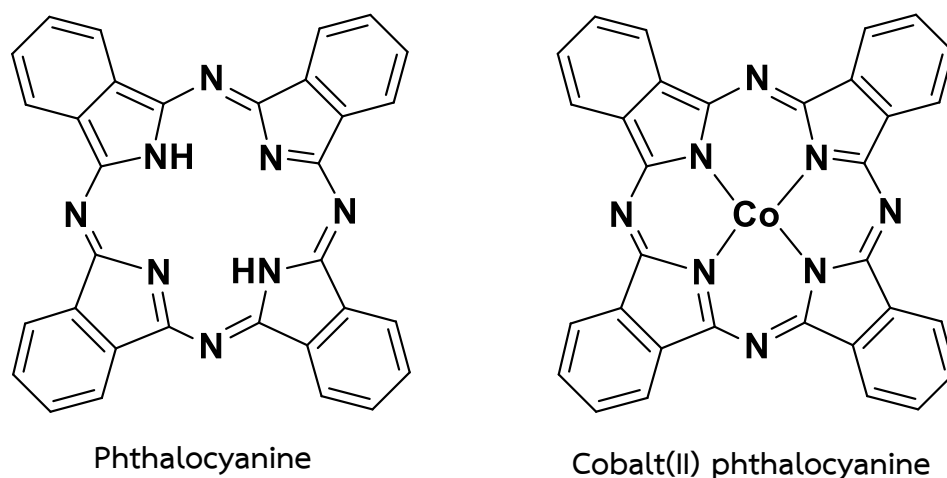


Figure 2.6 Structure of phthalocyanine and cobalt(II) phthalocyanine

For the electrochemical detection of phenolic compounds, it can be oxidized and its electrooxidation is known to form dimeric and/or polymeric oxidation products. These by-products can adsorb and deactivate the electrode surface. Therefore, to address this problem, many researchers proposed CoPC as a very effective complex for the determination of phenols [37, 38]. In 1998, Mafatle employed CoPC and its derivative in electrocatalytic detection of phenolic compounds [39]. The results shown that CoPC deposited onto the glassy carbon electrode (GCE) improved the stability of the electrode, by reducing electrode fouling caused by the electrooxidation products of the phenolic compounds. Moreover, the role of the central metal in the catalytic process was studied. The comparison of the electrocatalytic activity of some of the first row transition metal phthalocyanines for the detection of mono-substituted phenolic compounds shown the following trend:



In 2008, Wael and Adriaens reported on the electrocatalytic oxidation of hydroxide using the different central metal ion phthalocyanines and porphyrins immobilized on gold electrodes [40]. The electrocatalytic activity of cobalt was found to be the greatest among the present series of the investigated metal. A possible mechanism for the enhanced reactivity of cobalt ion macrocycles towards the oxygen evolution was also given. It is stated that the electrocatalytic activity towards an adsorbate involves several aspects, such as the coordination state of the central metal ion, the nature of the ligand, the stability of the complexes, the number of d electrons, the energy of orbitals and the strength of the bonding between the central metal ion and the axial ligand.

2.5.2 Graphene

Graphite is well known as the two dimensional sp^2 hybridized forms of carbon found in pencil lead so far. It is a layered material formed by stacks of graphene sheets held together by weak van der Waals forces as illustrated in Figure 2.7. The weak interaction between the sheets allows them to slide relatively easily across one another and gives pencils their writing ability.

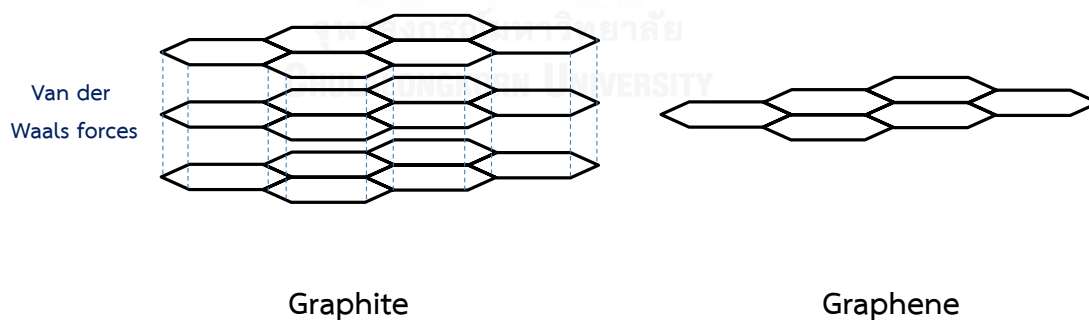


Figure 2.7 Graphite and graphene structure

In 2004, Geim and Novoselov extracted single-atom-thick crystallites from bulk graphite very simply [41]. They pulled graphene layers from graphite by a process called either micromechanical cleavage or the Scotch tape technique. They do not revealed only isolation of graphene but also its extraordinary properties. Graphene, fundamentally one single layer of graphite, is a layer of sp^2 bonded carbon atoms arranged in a honeycomb (hexagonal) lattice with each atom forming 3 bonds with each of its nearest neighbors. Its bonds and electron configuration are the reasons for the extraordinary properties of graphene, which include a very large surface area [at 2630 m^2/g , it is double that of single-walled carbon nanotubes], a tunable band gap, room-temperature Hall effect, high mechanical strength (200 times greater than steel), and high elasticity and thermal conductivity [42]. It has been a rapidly rising star on the horizon of materials science and condensed-matter physics ever since.

Graphene is an ideal material for electrochemistry because of its very large 2-D electrical conductivity, large surface area and low cost [43]. Shang and co-workers firstly reported multilayer graphene nanoflake films on silicon substrates for electrochemical sensing in 2008 [44]. Fast electron-transfer kinetics for the ferro/ferricyanide redox system and excellent electrocatalytic activity for simultaneously determining dopamine, ascorbic acid and uric acid were demonstrated.

CHAPTER III

EXPERIMENT

This part describes the information of the instruments and chemicals, the electrochemical sensor fabrication, and electrochemical measurement which were used in this work.

3.1 Instruments and apparatus

The instruments and apparatus used in this research are listed in Table 3.1. The electrochemical behaviors of genistein were measured by a potentiostat. The scanning electron microscope (SEM) was used to inspect in-depth details of the working electrode surface.

Table 3.1 List of the instruments and apparatus used in this research

Instruments and apparatus	Suppliers
Analytical balance	Mettler Toledo, Switzerland
AutoLab PG 30 potentiostat/galvanostat	Metrohm Autolab B.V., The Netherlands
Centrifuge	Cole Parmer, USA
Faraday cage	Copperplate, custom made
Hot air oven	Memmert, USA
Micropipette and tips	Eppendorf, Germany
Milli-Q ultrapure water purification system	Millipore, USA
pH meter, 827 pH Lab	Metrohm, Switzerland
Polyvinylchloride (PVC) substrate	Yingyong plastic glass, Thailand
Scanning electron microscope, JSM-5410LV	JEOL, Japan
Screen printing frames	Silkcut LP., Thailand
Screen printing squeegees	Chaiyaboon, Thailand

Instruments and apparatus	Suppliers
Ultrasonic bath	Elma Schmidbauer GmbH, Germany
Vortex mixer, VTX-3000L	LMS, Japan

3.2 Chemicals and reagents

All reagents were analytical reagent grade (Table 3.2) and prepared using ultrapure water (resistivity $\geq 18.2 \text{ M}\Omega\cdot\text{cm}$). The conductive carbon ink, carbon graphene ink and silver/silver chloride ink were used for electrochemical sensor fabrication and CoPC was used to modify the working electrode. Jewel vine tea powder was purchased from the local market for sample analysis.

Table 3.2 List of the chemicals used in this research

Instruments and apparatus	Suppliers
Boric acid (H_3BO_3)	Merck, Germany
Carbon graphene ink	Gwent, UK
Carbon ink, Electrodag PF-407C	Acheson, USA
Cobalt(II) phthalocyanine ($\text{C}_{32}\text{H}_{16}\text{CoN}_8$)	Sigma Aldrich, Switzerland
Ethanol	Merck, Germany
Genistein ($\text{C}_{15}\text{H}_{10}\text{O}_5$)	Sigma Aldrich, Switzerland
Potassium hexacyanoferrate(III) ($\text{K}_3[\text{Fe}(\text{CN})_6]$)	Merck, Germany
Potassium hexacyanoferrate(II) trihydrate ($\text{K}_4[\text{Fe}(\text{CN})_6]\cdot 3\text{H}_2\text{O}$)	Merck, Germany
Silver/silver chloride paste	Gwent Group, UK
Sodium hydroxide (NaOH)	Merck, Germany
Thao Wan Praing Tea	Abhibhubejhr herbal products, Thailand

All acid solutions used for supporting electrolyte are listed in Table 3.3.

Table 3.3 List of the acid solutions used in this research

Name	Formula	Mw. (g/mole)	Assay (%)	Density (g/cm ³)	Company	Country
Acetic acid	CH ₃ COOH	60.05	100	1.05	Merck	Germany
Hydrochloric acid	HCl	36.46	37	1.19	Merck	Germany
Nitric acid	HNO ₃	63.01	65	1.39	Merck	Germany
Perchloric acid	HClO ₄	100.46	70-72	1.68	Merck	Germany
Phosphoric acid	H ₃ PO ₄	98.00	85	1.71	Merck	Germany
Sulfuric acid	H ₂ SO ₄	98.08	95-97	1.84	Merck	Germany

3.3 Chemical preparations

3.3.1 1.0 mM stock standard genistein preparation

A 1.0 mM standard genistein stock solution was prepared by dissolving 6.76 mg genistein standard powder in ethanol. The stock standard solution should be stored in darkness at 4°C until used.

3.3.2 Working standard solution

The working solution was freshly prepared by appropriate diluting the stock solution and mixing standard solution with 2.0 M H₂SO₄ supporting electrolyte in 1:3 volume ratio before determining the genistein content by the electrochemical measurement.

3.3.3 0.5 M Britton–Robinson buffer (BR buffer)

H₃BO₃ was weighed for 7.72875 g, then mixed with 8.43 mL H₃PO₄ and 7.15 mL CH₃COOH in the total amount of 250 mL aqueous solution. The mixture was adjusted to desired pH with 2.5 M NaOH.

3.3.4 Supporting electrolyte: 2 M H₂SO₄

The stock 10 M H₂SO₄ was prepared by diluting 5.55 mL concentrated H₂SO₄ with ultrapure water to 10 mL. Five mL of stock solution was transferred to 25 mL volumetric flask to prepare 2 M H₂SO₄.

3.3.5 Sample preparation

For sample preparation, 0.01 g of jewel vine powder was accurately weighed and extracted twice in 1.0 mL ethanol by centrifugation at 6,000 rpm for 15 minutes. The supernatant of sample solution was mixed with 2.0 M H₂SO₄ supporting electrolyte in 1:3 volume ratio before determining the genistein content by the electrochemical measurement.

3.4 Electrochemical sensor fabrication

The electrochemical sensor with three-electrode system was fabricated in our laboratory by screen-printing technique [45]. Fabrication process of screen-printed electrochemical sensor is summarized in Figure 3.1.



Figure 3.1 Step of screen-printed electrochemical sensor fabrication.

First, the PVC plastic substrate (0.3 mm-thick) was cut to the proper size 12×14 cm, and then was cleaned with ethanol. Second, the screen-printing technique was started by applying Ag/AgCl ink through the blocking stencil, which its pattern shown in Figure 3.2, over the cleaned substrate. The reference electrode and conductive track were obtained by this process. Printed ink was dried in an oven at 55°C for 1 hour before performing the next printing step.

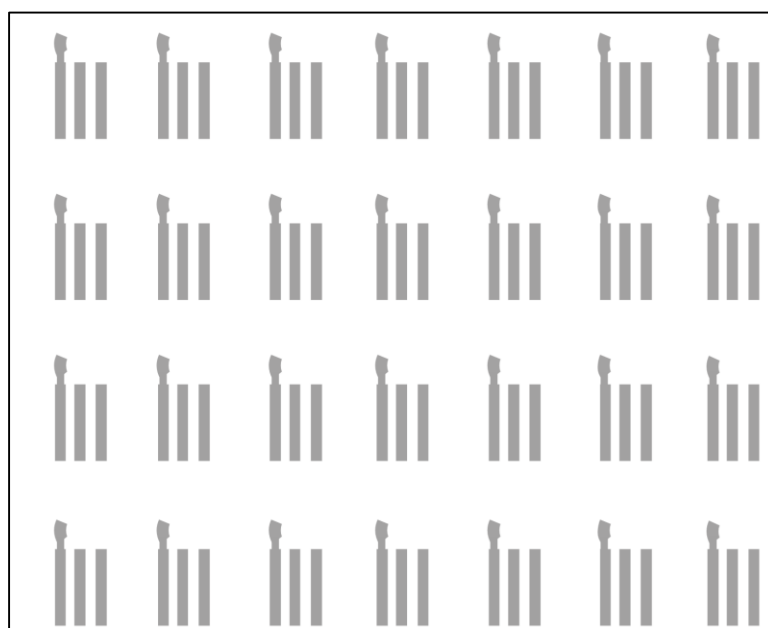


Figure 3.2 The pattern of the blocking stencil for screen-printing by Ag/AgCl ink.

Third, the carbon ink or carbon graphene ink was also printed by the same technique onto the same substrate for the working and counter electrodes as shown in Figure 3.3. Moreover, the carbon electrode was easily modified by directly thoroughly mixing 0.5 g carbon ink with 0.03 g CoPC before screen-printing onto the substrate to fabricate the CoPC modified carbon electrode. The screen-printed electrodes were dried in an oven at 55°C for 1 hour after each printing step.



Figure 3.3 The pattern of the blocking stencil for screen-printing by carbon ink.

Finally, the plastic sticker insulator with a semi-circular hole was stuck on the obtained screen-printed electrodes to control the active surface area and prevent electrical short circuit. The 1.5x2.5 cm electrochemical sensor with Ag/AgCl reference electrode, carbon working electrode, and carbon counter electrode was received as illustrated in Figure 3.4.

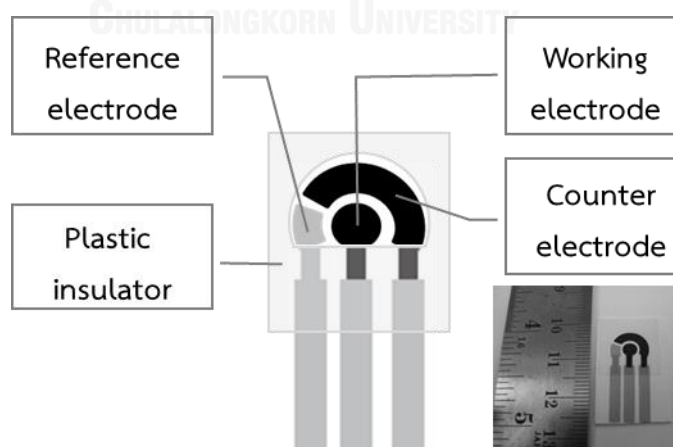


Figure 3.4 The in-house screen-printed three electrode electrochemical sensor.

3.5 Electrochemical measurement procedure

A 50 μL of the working solution was dropped on the electrochemical sensor. The electrochemical measurement was performed by cyclic voltammetry (CV) and square wave voltammetry (SWV) with appropriate parameters (Table 3.4-3.6). All electrochemical measurements were carried out at room temperature in a Faraday cage to prevent electrical noise.

Table 3.4 The cyclic voltammetry parameters for carbon electrochemical sensor

Parameters	Set value
Start potential	-1.30 V
Upper vertex potential	1.30 V
Lower vertex potential	-1.30 V
Initial direction	Up
Potential interval	0.01 V
Scan rate	0.10 V/s

Table 3.5 The square wave voltammetry parameters for CoPC-modified electrochemical sensor

Parameters	Optimal value
Frequency	150 Hz
Step potential	0.01 V
Amplitude	0.05 V
Deposition potential	0.50 V
Deposition time	3 s

Table 3.6 The square wave voltammetry parameters for graphene-modified electrochemical sensor

Parameters	Optimal value
Frequency	250 Hz
Step potential	0.01 V
Amplitude	0.07 V

3.6 The analytical performance

3.6.1 Calibration and linearity

Genistein standard solutions in the concentration range of 1.0–300.0 μM was analyzed by square wave voltammetry using the screen-printed electrochemical sensors under the optimized conditions. The average peak currents of triplicate measurements were used to plot the calibration curves which linear range can be obtained.

3.6.2 Limit of detection (LOD) and limit of quantification (LOQ)

The concentration of genistein in real sample was determined by standard addition calibration method. The limit of detection (LOD) and limit of quantification (LOQ) were calculated from $3S_{bl}/S$ and $10S_{bl}/S$, respectively, where S_{bl} is standard deviation of blank ($n=10$), and S is sensitivity of detection obtained from slope of the calibration curve.

CHAPTER IV

RESULTS AND DISCUSSION

4.1 Carbon electrochemical sensor

The electrochemical sensor with carbon working electrode was prior used to study the electrochemical behavior of genistein and the suitable voltammetric method for genistein determination.

4.1.1 Electrochemical behavior of genistein

The redox reactions of 0.25 mM genistein in 0.5 M BR buffer at pH 2 and 8 on a carbon electrochemical sensor were investigated by cyclic voltammetry using a scanning potential from -1.3 to +1.3 V with potential scan rate of 0.1 V/s.

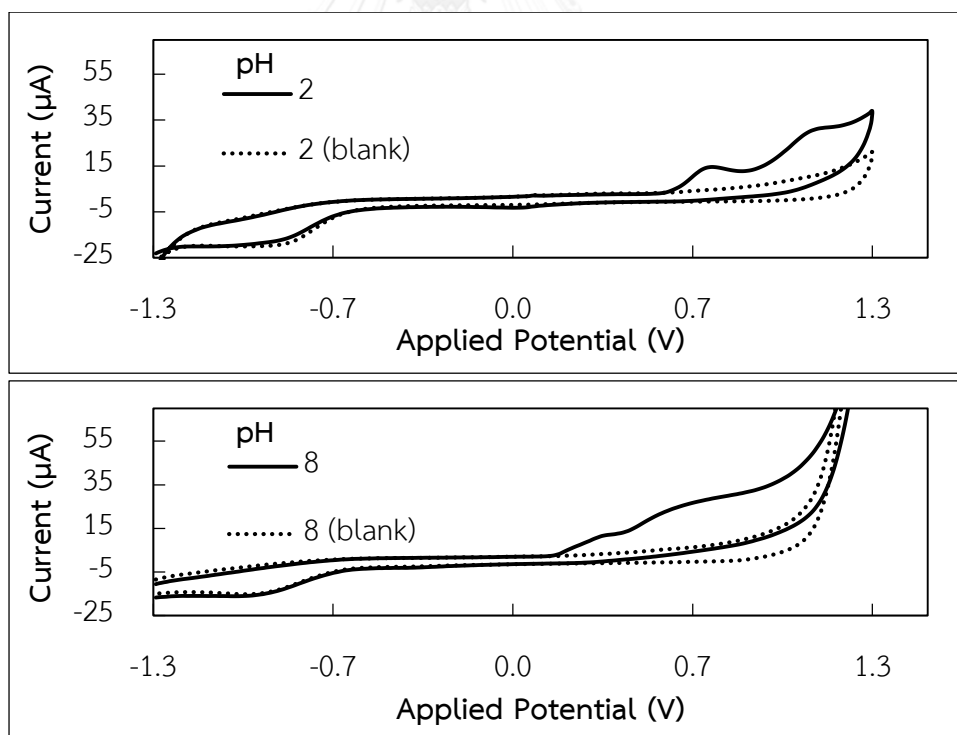


Figure 4.1 Typical cyclic voltammograms of 0.25 mM genistein in 0.5 M BR buffer at pH 2 and 8 on carbon electrochemical sensor.

The typical voltammograms of genistein shown in Figure 4.1 presented that there were two peaks obviously observed at positive applied potential without any reduction peak in acidic medium (pH 2). For basic medium (pH 8), undefined peak shape and worse resolution were observed although the oxidation peak appeared at the lower positive potential. This result corresponds to the previous report [46]. They proposed the mechanism of genistein redox reaction via two steps of oxidation as illustrated in Figure 4.2. First step is oxidation of 4'-OH with one electron and one proton transfer followed by the oxidation of the groups 5,7-di-OH with two electrons and two protons transfer in the reaction.

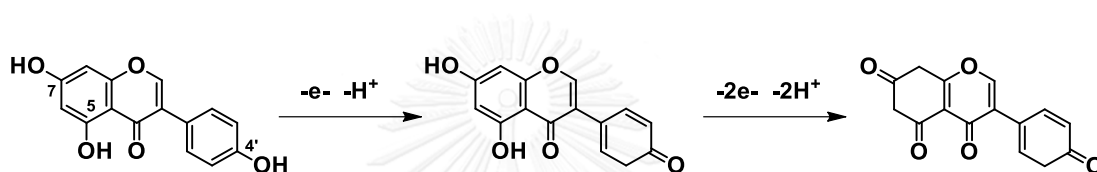


Figure 4.2 Oxidation mechanism of genistein [46].

The influence of the pH on the oxidation of genistein was also investigated over a pH range of 2-8 in the BR buffer. The results in Figure 4.3 shown that increasing pH of the solution caused decreasing value of both peak potential and peak current. In the case of basic medium, genistein can be deprotonated to be more negative molecule. The deprotonated form was transferred to the electrode surface easier by the electrostatic force leading to shift of peak potential to less positive potential. However, this form was unstable and its electron did not only transfer to the electrode surface but also other molecules in the solution resulting in the lower sensitivity. The first oxidation of genistein was observed in various pH range (Figure 4.4 and Figure 4.5) because of its intense intensity and more stable (narrow error bar).

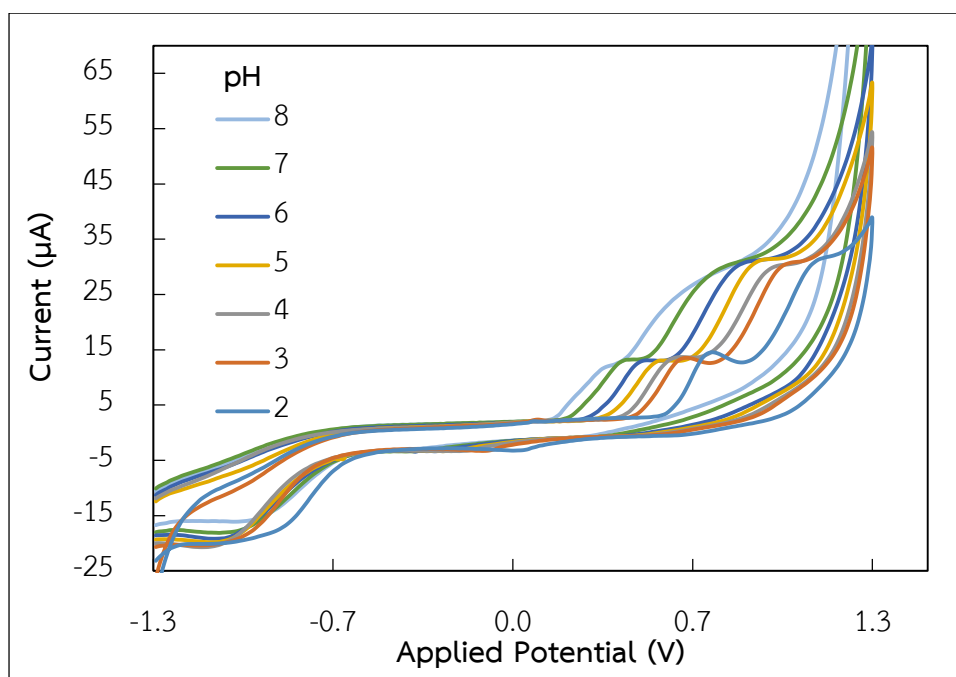


Figure 4.3 Cyclic voltammograms of 0.25 mM genistein in various pH (2-8) of 0.5 M BR buffer on carbon electrochemical sensor.

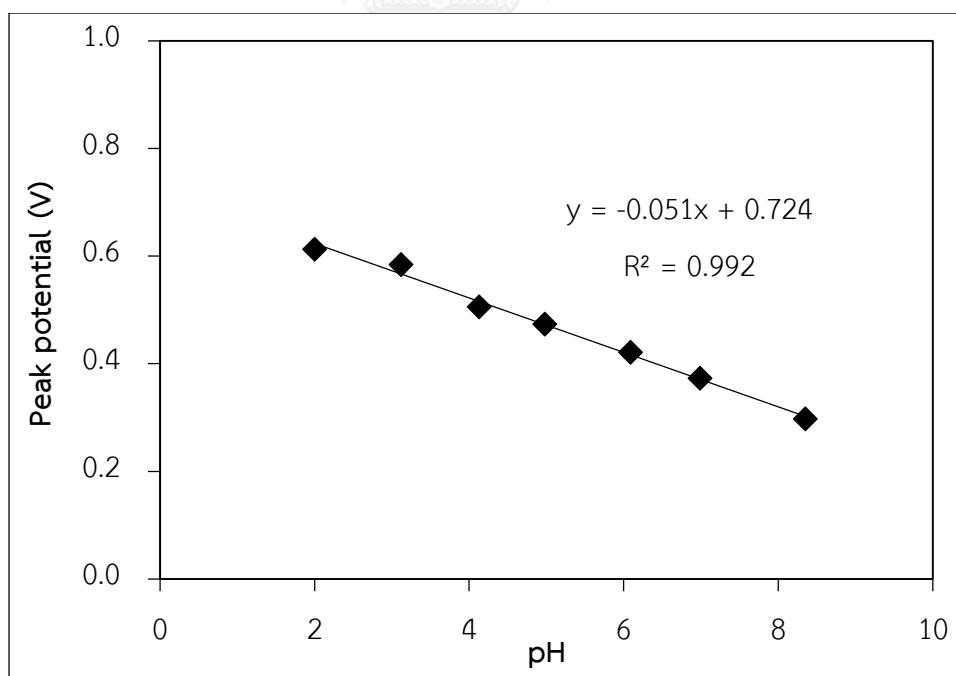


Figure 4.4 Variation of peak potential of 0.25 mM genistein at various pH on carbon electrochemical sensor.

The peak potential shifted to more negative values when the pH of the medium was increased. This suggested that protons participate in the oxidation process. The slope of -0.051 V was obtained from the plot of oxidation peak potential (E_p) vs. pH (Figure 4.4). The slope nearby -0.059 V at room temperature indicates an equal number of electrons and protons being involved in the redox reaction by solving the Equation 4.1. The proportion of m and n is the ratio of proton and electron participating in the reaction. This result corresponds to the previous mentioned oxidation mechanism of genistein.

$$E_p = E^0 - \frac{(2.303mRT)}{nF} pH = E^0 - \frac{(0.059m)}{n} pH \quad (4.1)$$

The similar trend was observed with the peak current as shown in Figure 4.5. The peak current decreased when the pH value was increased. The signal of genistein in acidic medium showed higher sensitivity than in basic medium. So, acidic medium should be the choice for supporting electrolyte.

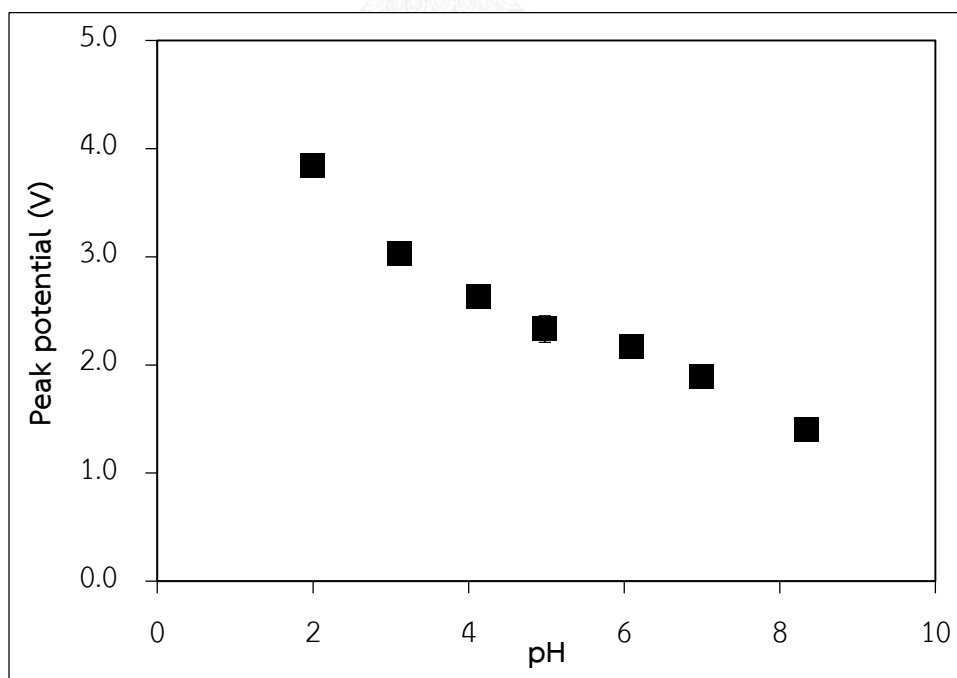


Figure 4.5 Variation of peak current of 0.25 mM genistein at various pH on carbon electrochemical sensor.

4.1.2 Voltammetric method for determination of genistein

High sensitivity is required for quantification. Suitable method must be investigated. Therefore, 3 types of voltammetric methods (linear sweep, square wave, and differential pulse voltammetry) were performed in this purpose. The scan rate was set at 0.1 V/s to measure the diluted genistein standard solution (3 μM). As the results shown in Figure 4.6, square wave and differential pulse voltammetry provided 2 obviously observed oxidation peaks of genistein at about 0.4 and 0.7 V. Although, linear sweep voltammetry provided higher current signal after applied potential to 0.4 V, there was no identifiable oxidation peak. Consequently, square wave voltammetry with highest observed genistein oxidation peak was selected for further determination of genistein.

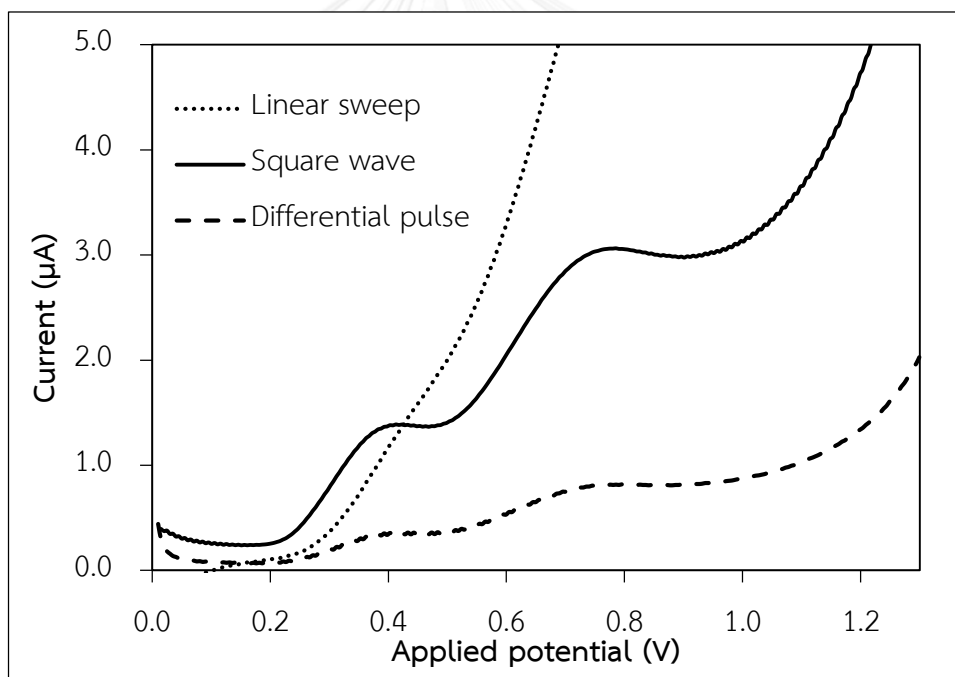


Figure 4.6 Voltammograms of 3 μM genistein at pH 7 on carbon electrochemical sensor obtained by linear sweep (.....), square wave (—), and differential pulse (- - -) voltammetry.

4.2 CoPC-modified carbon electrochemical sensor

The effective modifying material for phenolic compounds, CoPC, was proposed to improve sensitivity of genistein. This material was reported to improve the stability of the electrode by reducing electrode poisoning caused by the adsorption of the electrooxidation products.

4.2.1 The amount of CoPC modifying material

The carbon electrode was modified by thoroughly mixing CoPC and carbon ink in the screen-printing procedure. Effect of the amount of CoPC was shown in Figure 4.7. The signal of genistein increased when the amount of CoPC was increased from 0 to 6% w/w. Increasing amount of CoPC more than 6% w/w resulted in lower signal and precision. It may be because of difficulty to mix high amount of CoPC thoroughly with carbon ink resulting heterogeneous mixtures. Therefore, 6% w/w of CoPC was added to the carbon ink to modify the carbon electrode with highest efficiency of genistein signal improvement.

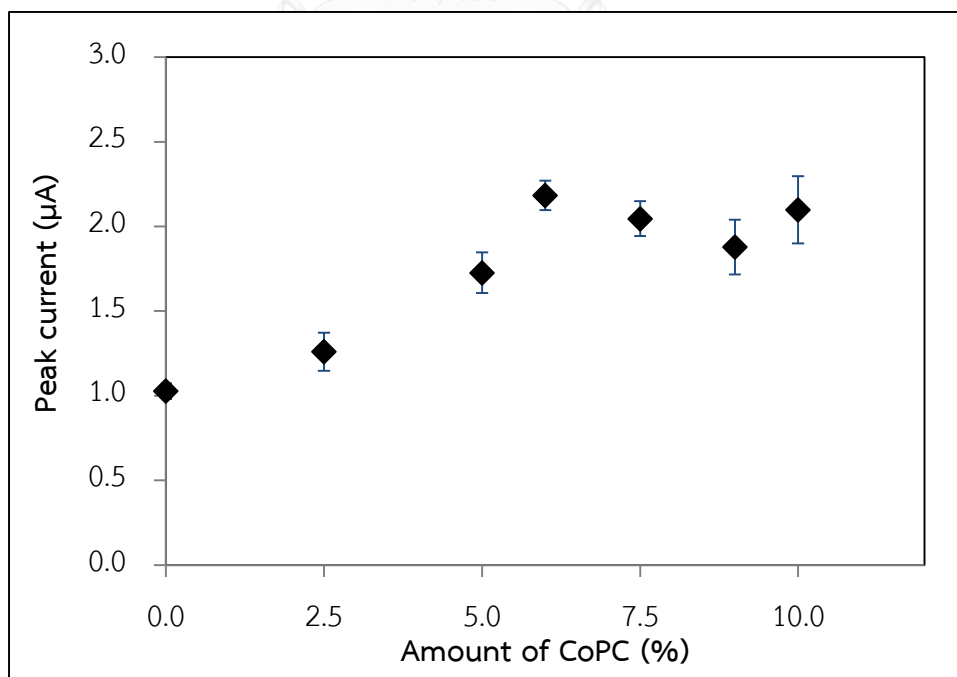


Figure 4.7 Effect of the amount of CoPC in carbon ink on SWV signal of ... M genistein.

To compare the electrochemical sensitivity of a CoPC-modified carbon sensor with a bare sensor, square wave voltammetry was carried out in a 0.25 mM genistein solution. The first step of genistein oxidation was the choice for quantitative analysis because it provided higher sensitivity than the second one, and it also required lower potential that reduced risks of interferences. Consequently, only oxidation of 4'-OH at about 0.6 V was focused. The results in Figure 4.8 showed that CoPC can improve signal of genistein oxidation without peak potential shift.

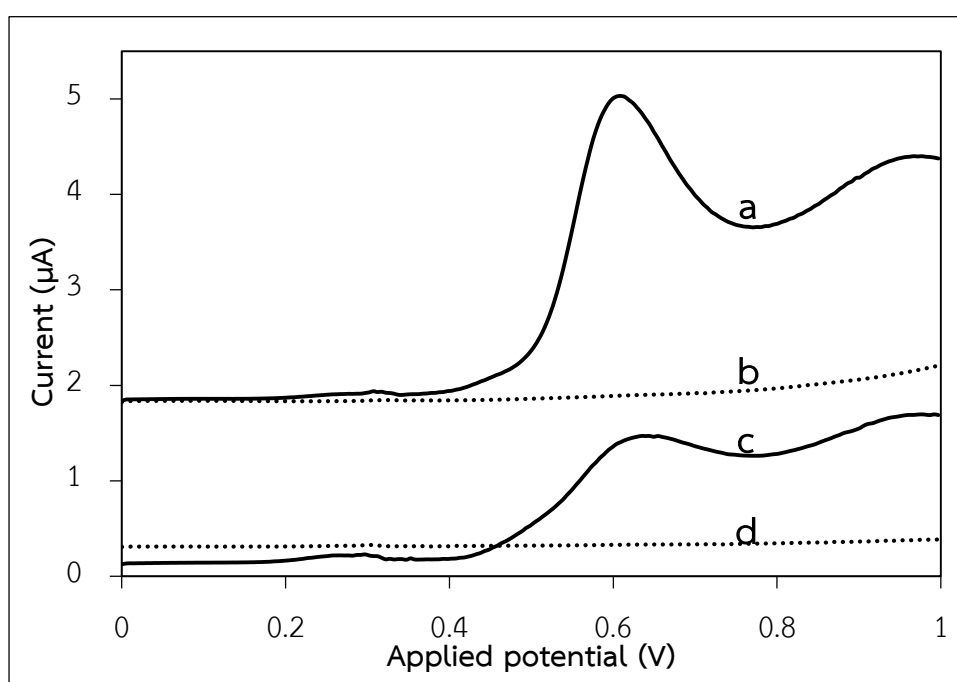


Figure 4.8 Typical square wave voltammograms of 0.25 mM genistein on a CoPC-modified carbon electrochemical sensor (a) and on a bare carbon electrochemical sensor (c) with their blank voltammograms (b and d, respectively).

4.2.2 Modified surface electrode morphology

The surface morphologies of the bare carbon electrode and the CoPC-modified carbon electrode was shown in Figure 4.9. These SEM images clearly reveal the difference of the electrode surface. Micro rod crystals of CoPC were dispersed thoroughly over the surface of the modified electrode (Figure 4.9 b and d). This shows the improvement of surface area of the modified electrode leading to electron transfer and sensitivity are also improved.

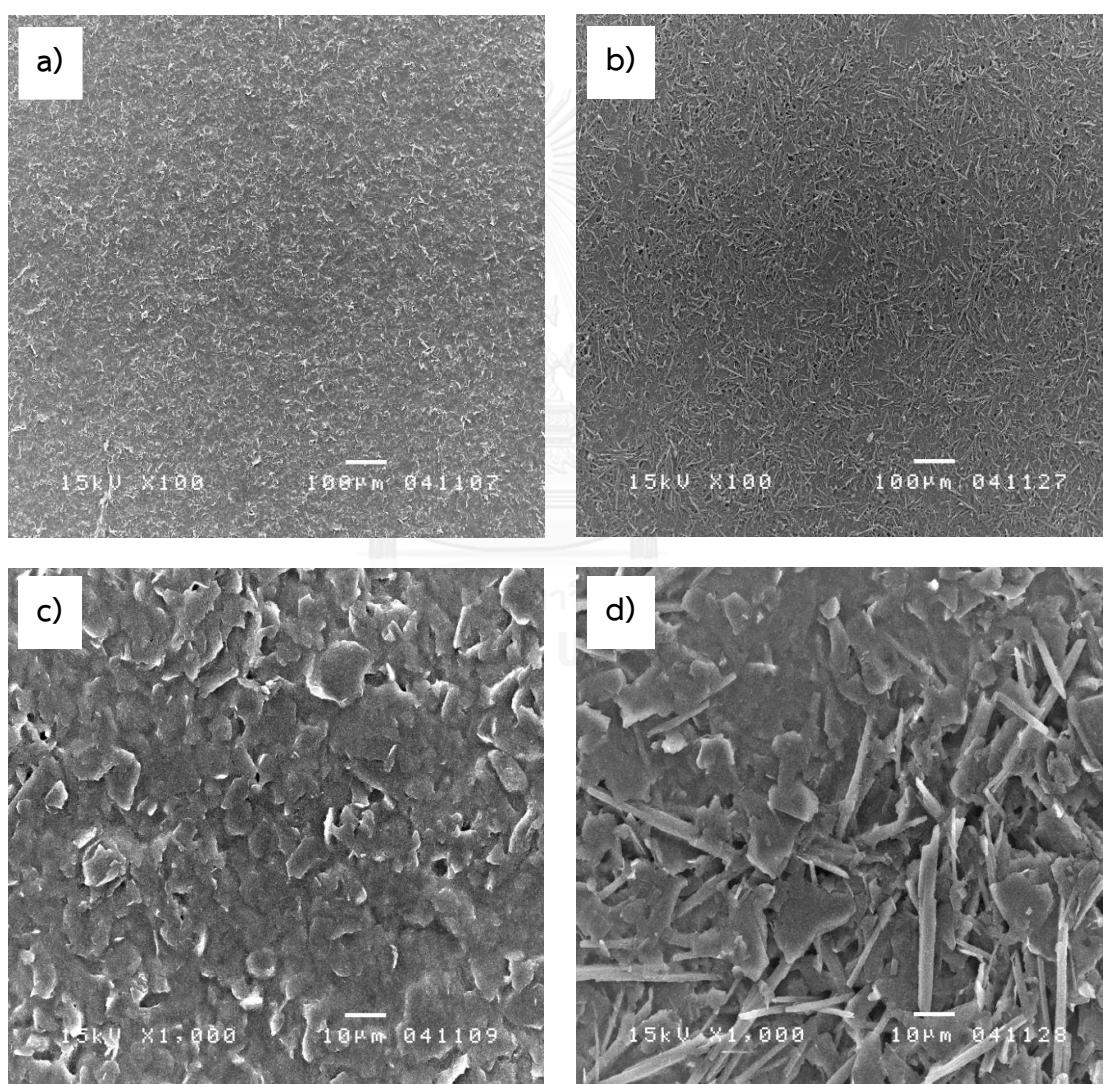


Figure 4.9 SEM images of the surface of bare electrode with a magnitude of a) 100x and c) 1,000x compared with the surface of CoPC modified electrode with a magnitude of b) 100x and d) 1,000x.

The effect of the potential scan rate (\mathbf{V}) on the peak current (I_p) was investigated with ferro/ferricyanide redox system by CV as shown in Figure 4.10. It is well known that the peak current is proportional to the square root of scan rate ($\mathbf{V}^{1/2}$) and the redox process is controlled by diffusion. In this case, the effective surface area of the CoPC-modified carbon electrode can be calculated by slope of the plot and Equation 4.2. The effective surface area was found to be 157 cm^2 .

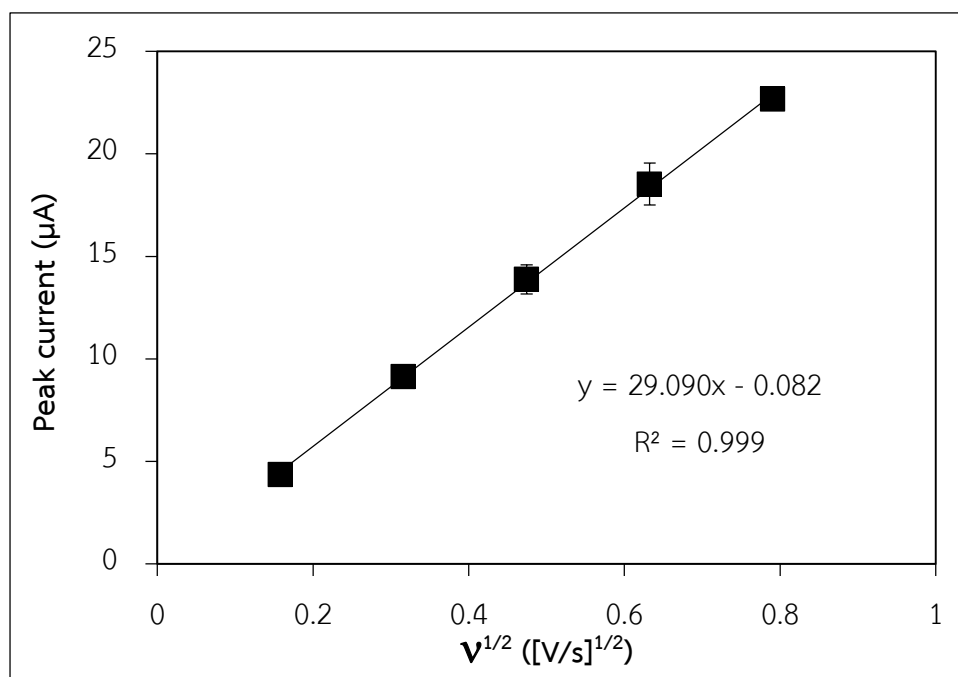


Figure 4.10 The dependence of peak current of 0.25 mM ferro/ferricyanide redox system on the potential scan rate.

$$I_p = 2.69 \times 10^5 n^{3/2} A C_O^* D_O^{1/2} \mathbf{V}^{1/2} \quad (4.2)$$

when,

n is the number of electrons transferred; $n=1$,

A is the electrode area (cm^2),

C_O^* is the bulk concentration of the analyte (M); $C_O^* = 0.25 \times 10^{-3}$ M,

D_O is the analyte's diffusion coefficient (cm^2/s); $D_O = 7.60 \times 10^{-6}$ cm^2/s .

The effect of the potential scan rate on the peak current was also investigated with genistein standard solution as shown in Figure 4.11. The peak current of genistein is proportional to the square root of scan rate with a correlation coefficient (R^2 value) of 0.999. This case indicates that the oxidation of genistein at the CoPC-modified carbon electrode is controlled by diffusion instead of adsorption. The result corresponds to the using of CoPC was reported reducing adsorption by the oxidative products of phenolic compounds.

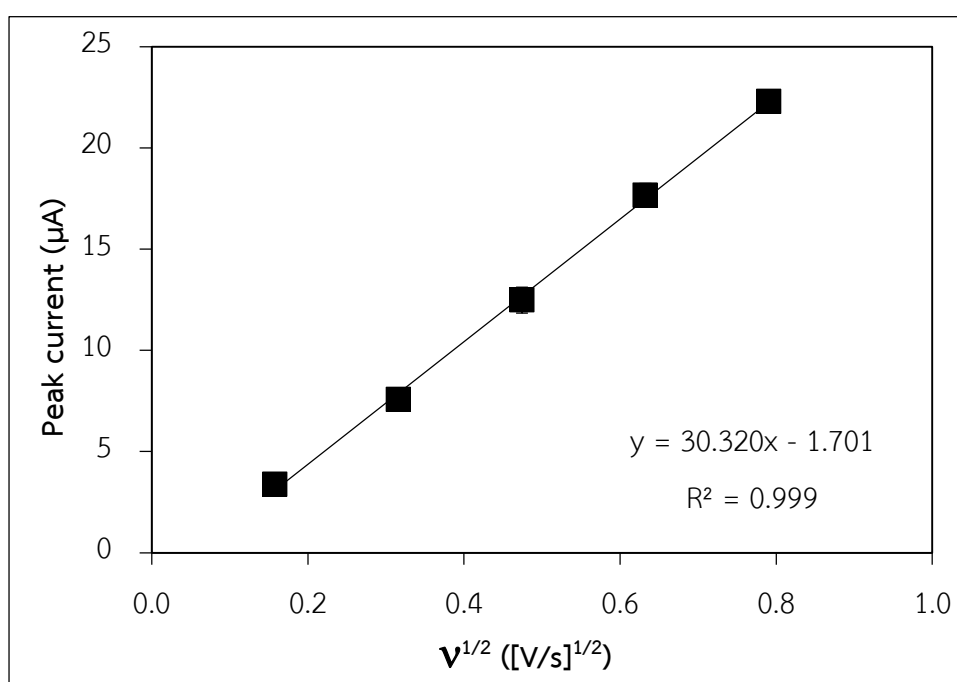


Figure 4.11 The dependence of peak current of 0.25 mM genistein on the potential scan rate.

For an irreversible oxidation system with one-electron reaction, the peak current is a function of scan rate as given in the Equation 4.3 [47, 48].

$$I_p = (2.99 \times 10^5)[(1-\alpha)n_\alpha]^{1/2}AC_0^*D_0^{1/2}v^{1/2} \quad (4.3)$$

when,

α is the transfer coefficient,

n_α is the number of electrons transferred up to, and including the rate determining step,

A is the electrode area (cm^2); $A = 157 \text{ cm}^2$,

C_0^* is the bulk concentration of the analyte (M); $C_0^* = 0.25 \times 10^{-3} \text{ M}$,

D_0 is the analyte's diffusion coefficient (cm^2/sec).

The diffusion coefficient of genistein on CoPC-modified carbon electrode could be also calculated from this equation when the term $(1-\alpha)n_\alpha$ was known. This term can be calculated from the slope of the peak potential (E_p) vs $\ln(v)$ following relationship in the Equation 4.4.

$$E_p = K + \left[\frac{RT}{2(1-\alpha)n_\alpha F} \right] \ln(v) = K + \left(\frac{0.0128}{(1-\alpha)n_\alpha} \right) \ln(v) \quad (4.4)$$

where

$$K = E_p + \left[\frac{RT}{(1-\alpha)n_\alpha F} \right] \left[0.78 + \frac{1}{2} \ln \left(\frac{(1-\alpha)n_\alpha F D_0}{k^0{}^2 RT} \right) \right]$$

The slope from the plot of E_p vs $\ln(v)$ was 0.0135 as shown in Figure 4.12. Consequently, the term $(1-\alpha)n_\alpha$ and the diffusion coefficient of genistein on CoPC-modified carbon electrode were found to be 0.948 and $7.0 \times 10^{-6} \text{ cm}^2/\text{s}$, respectively.

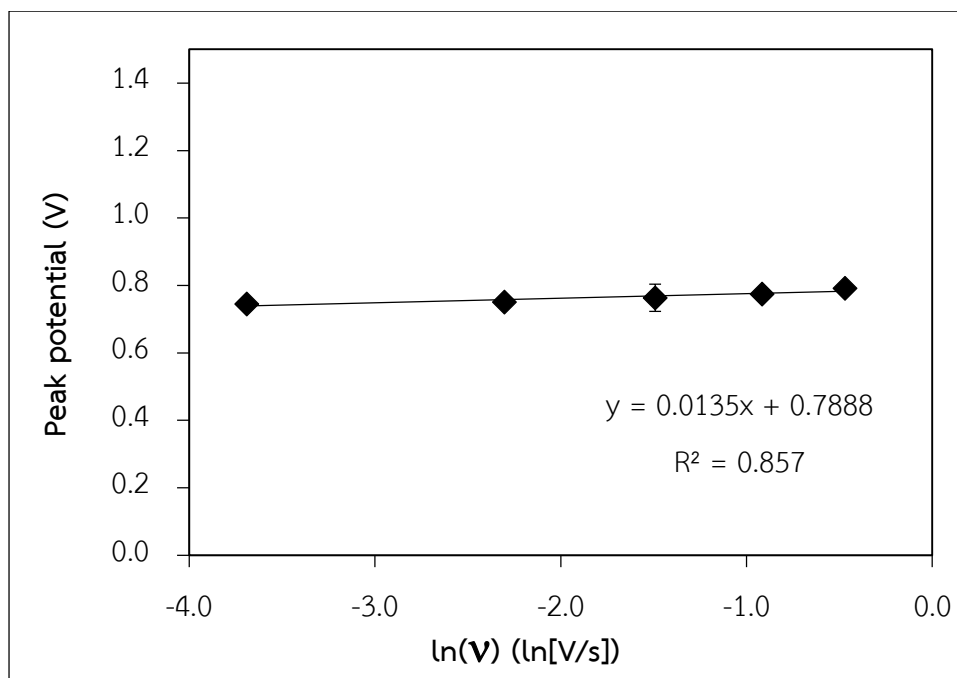


Figure 4.12 The dependence of peak potential of 0.25 mM genistein on the natural logarithm of potential scan rate.

4.2.3 Supporting electrolyte for genistein determination on CoPC-modified carbon electrochemical sensor

The peak current decreased when the pH value was increased as described in Section 4.1.1. The signal of genistein in acidic medium showed higher sensitivity than in basic medium. Using BR buffer as supporting electrolyte was limited the strongest acid at pH 2. Accordingly, genistein oxidation was demonstrated in various types of acids at the concentration of 0.01 M. Genistein in H_2SO_4 showed the highest current with appropriate potential because of acid strength and more proton generation as shown in Figure 4.13. Therefore, this acid was selected as a supporting electrolyte for genistein determination. A suitable concentration of H_2SO_4 was also evaluated. Stronger acid gave sharper and higher genistein peak at about 0.6 V. For this reason, 2 M of H_2SO_4 was selected with a good sensitivity.

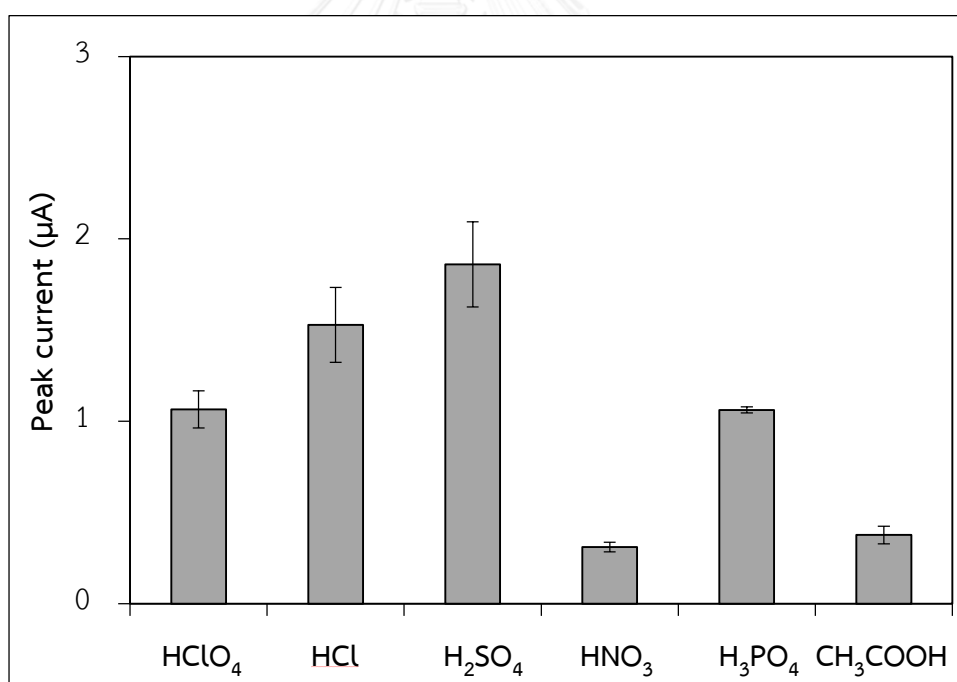


Figure 4.13 Effect of acid types (0.01 M) on the SWV signal of 0.25 mM genistein in 0.1 M various acid on CoPC-modified carbon electrochemical sensor.

4.2.4 SWV parameters optimization on CoPC-modified carbon electrochemical sensor

SWV parameters were optimized for the genistein determination including frequency, step potential, amplitude, deposition potential and deposition time.

For frequency, the parameter was optimized as shown in Figure 4.14 and Figure 4.15 in the range of 20-200 Hz. The genistein signal was enhanced and shifted slightly to more positive potentials when the frequency was increased. The increasing of this parameter affected to the increasing of scan rate. The scan rate (ν) was defined by the step potential (E_{step}) and the frequency (ω) in Equation 4.5.

$$\nu = E_{\text{step}} \cdot \omega \quad (4.5)$$

The higher scan rate provoked a higher peak current of genistein. The peak current of genistein was enhanced and stable after frequency reach 150 Hz.

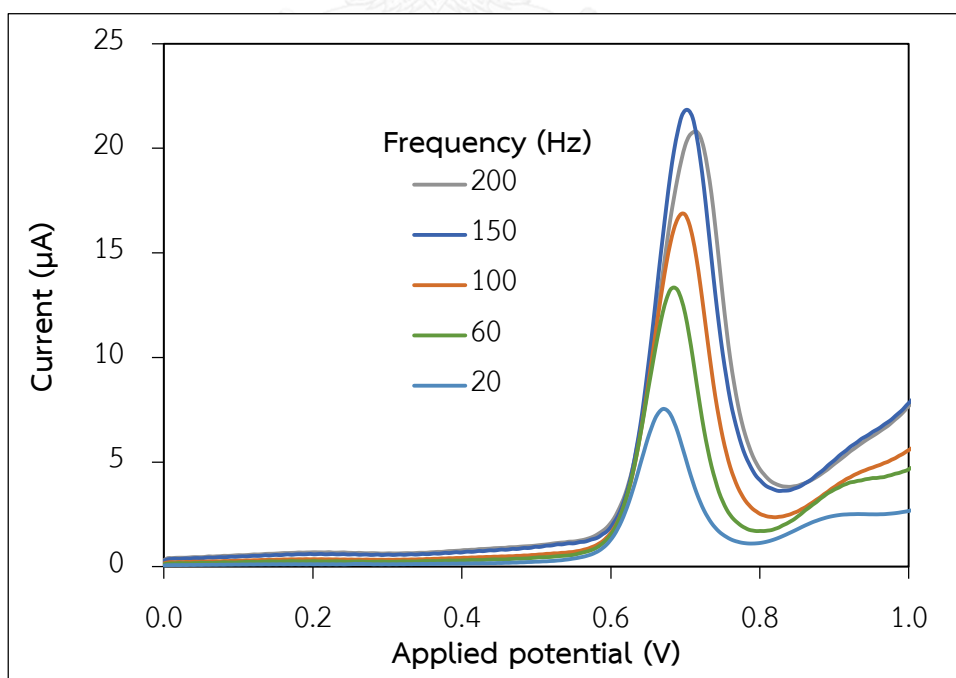


Figure 4.14 Square wave voltammograms of 0.25 mM genistein in 2 M H_2SO_4 on CoPC-modified carbon electrochemical sensor in variation of frequency.

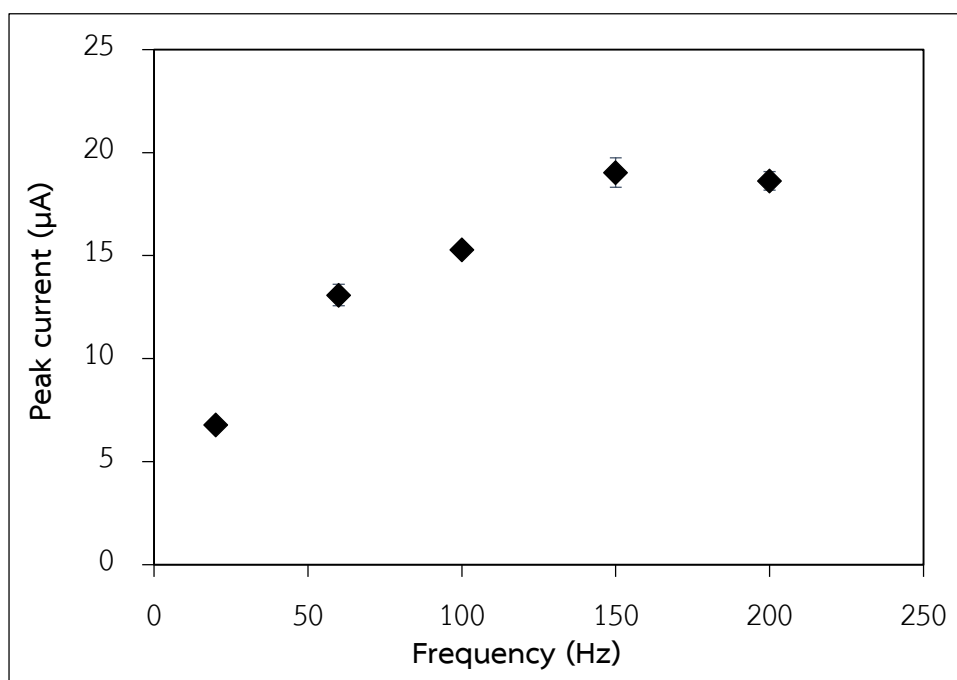


Figure 4.15 Effect of frequency on the SWV signal of 0.25 mM genistein in 2 M H₂SO₄ on CoPC-modified carbon.

For step potential, the parameter was optimized as shown in Figure 4.16 and Figure 4.17 in the range of 0.001-0.012 V. The increasing of this parameter also affected to the increasing of scan rate (Equation 4.5). The same result as in the effect of frequency was observed. The genistein signal was enhanced and shifted slightly to more positive potentials when the step potential was increased because of higher scan rate. The peak current of genistein was enhanced and stable after step potential reach 0.010 V.

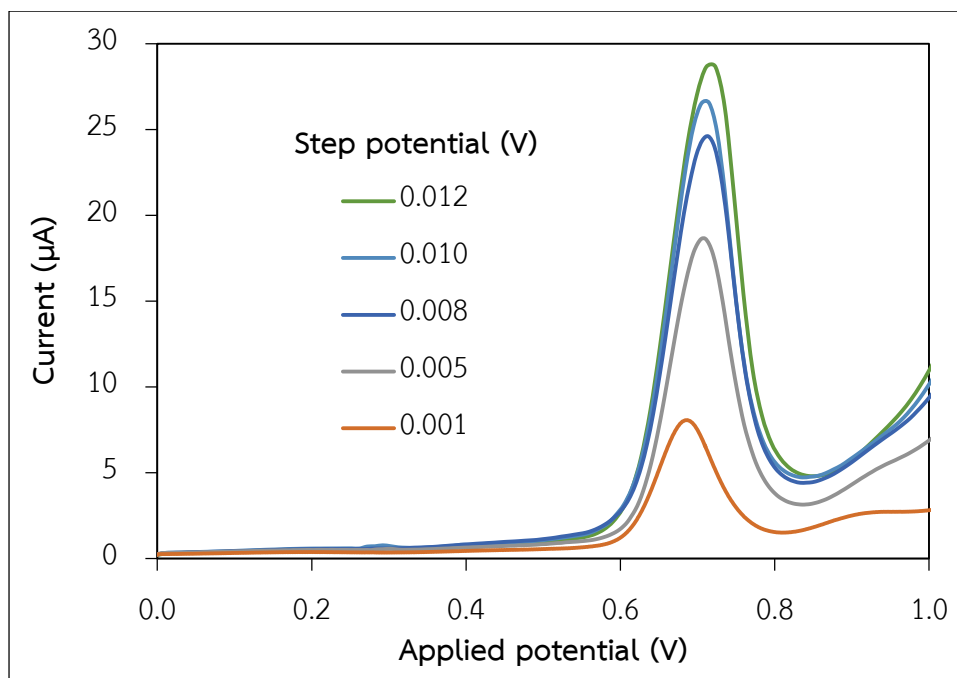


Figure 4.16 Square wave voltammograms of 0.25 mM genistein in 2 M H₂SO₄ on CoPC-modified carbon electrochemical sensor in variation of step potential.

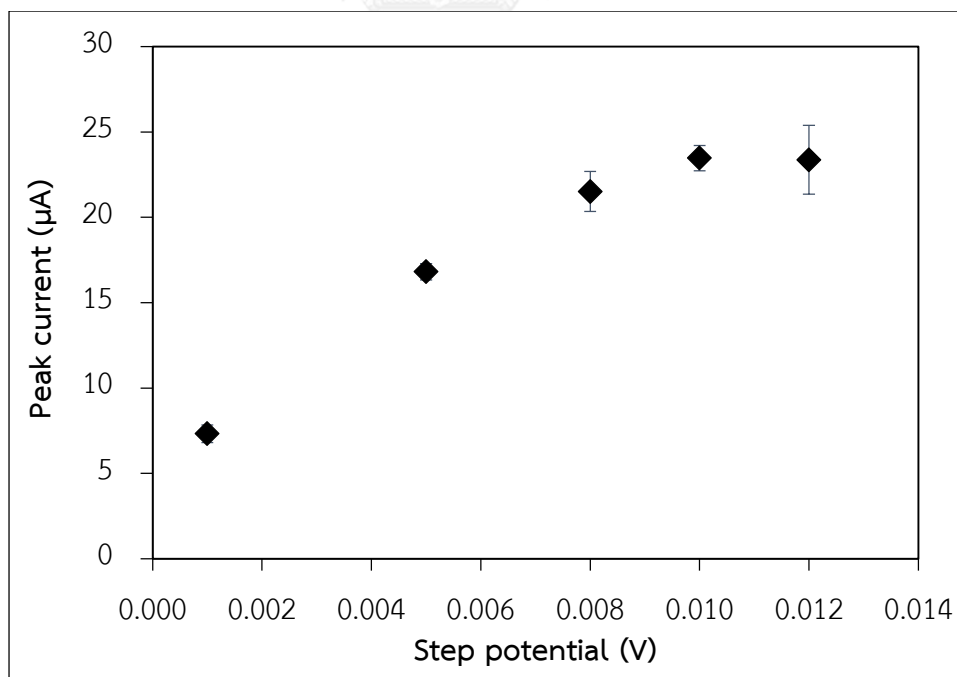


Figure 4.17 Effect of step potential on the SWV signal of 0.25 mM genistein in 2 M H₂SO₄ on CoPC-modified carbon electrochemical sensor.

In the case of amplitude (Figure 4.18), increasing of this parameter affected to increase the peak current of genistein and the peak potential was shifted to less positive because of the larger difference between the current before and after the application of the wave. Amplitude was study in the range of 0.01-0.10 V and found the suitable signal at 0.05 V (Figure 4.19).

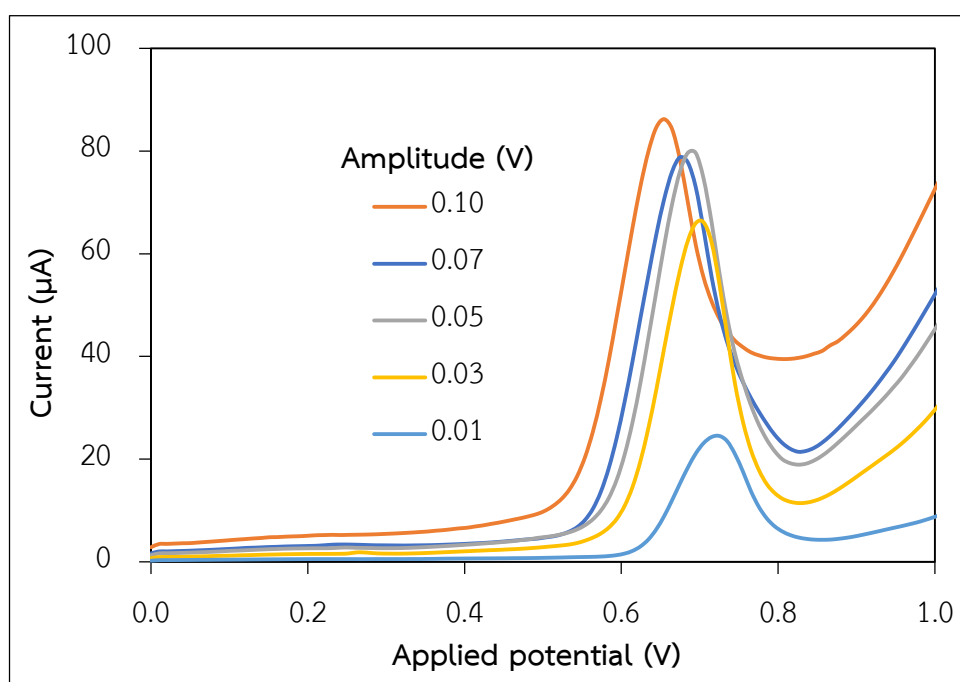


Figure 4.18 Square wave voltammograms of 0.25 mM genistein in 2 M H₂SO₄ on CoPC-modified carbon electrochemical sensor in variation of amplitude.

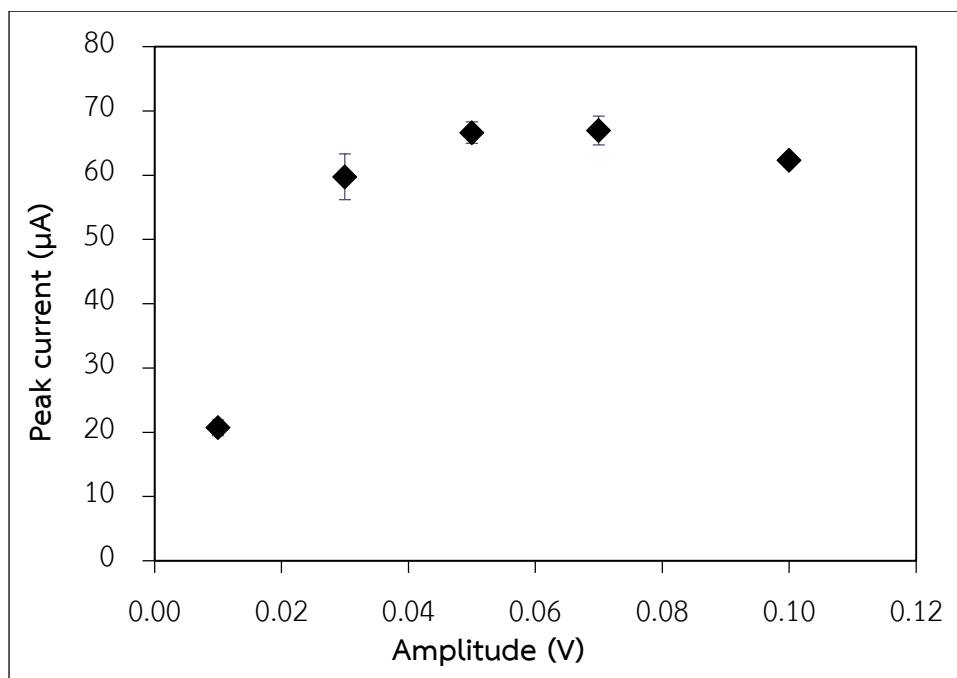


Figure 4.19 Effect of amplitude on the SWV signal of 0.25 mM genistein in 2 M H_2SO_4 on CoPC-modified carbon electrochemical sensor.

Fast deposition was also studied to accumulate genistein on the electrode surface for more sensitivity. Deposition potential in the range of -0.5 to 0.7 V and deposition time in the range of 0-90 s were evaluated (Figure 4.20 and Figure 4.23). Positive potential caused the electrode surface to be positive charge. Genistein was transferred and accumulated onto the electrode surface by electrostatic movement of negative species in the solution to the positive electrode surface. The high concentration of genistein accumulated on the electrode surface resulted the high measured current. However, potential was deposited over 0.5 V caused the pre-oxidation of genistein partially occurred before measurement. The current of genistein was decreased when the potential was deposited more than 0.5 V. With the optimum deposition potential, genistein signal was improved within 3 s.

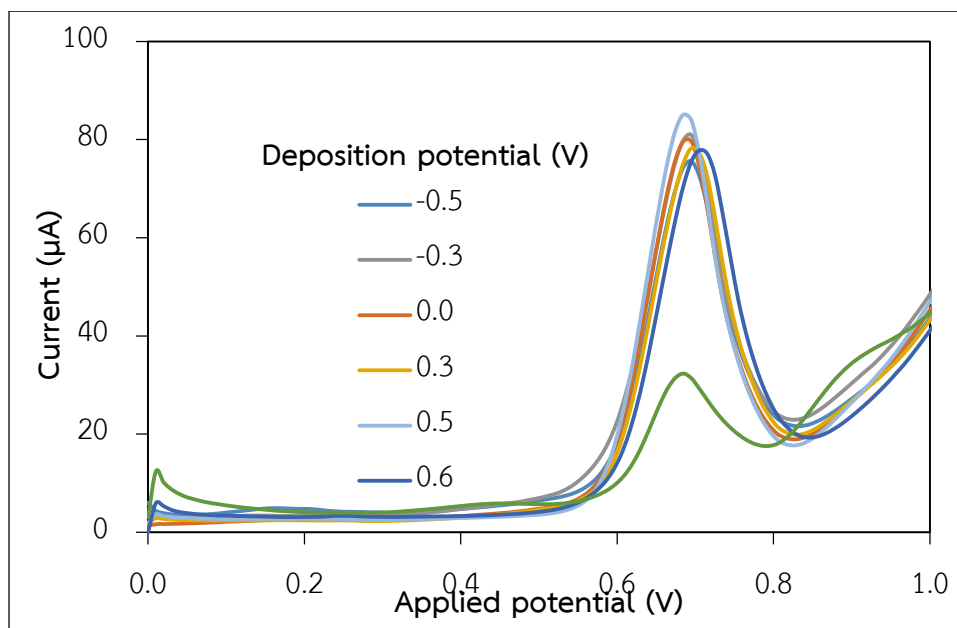


Figure 4.20 Square wave voltammograms of 0.25 mM genistein in 2 M H_2SO_4 on CoPC-modified carbon electrochemical sensor in variation of deposition potential.

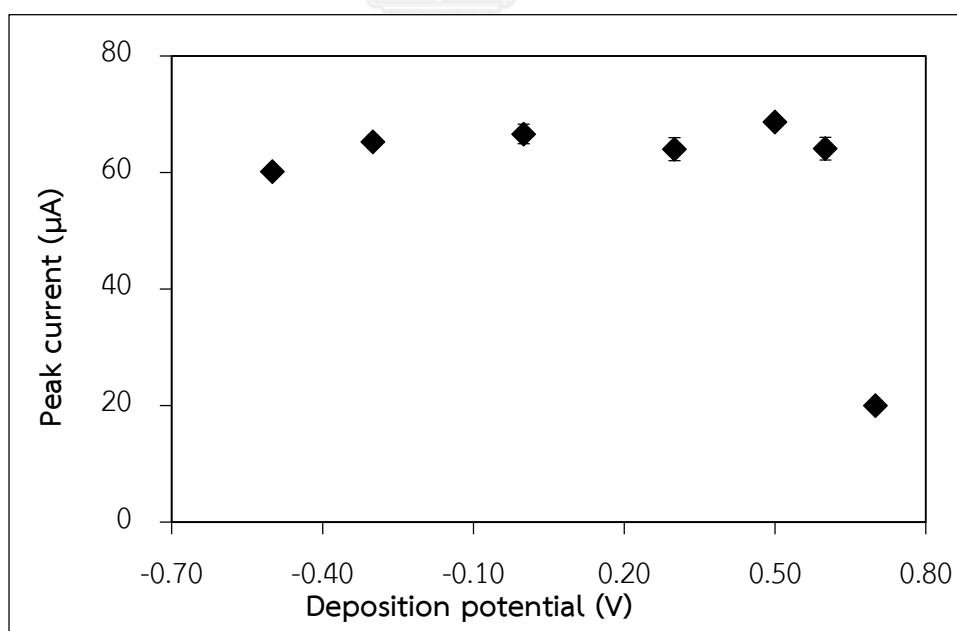


Figure 4.21 Effect of deposition potential on the SWV signal of 0.25 mM genistein in 2 M H_2SO_4 on CoPC-modified carbon electrochemical sensor.

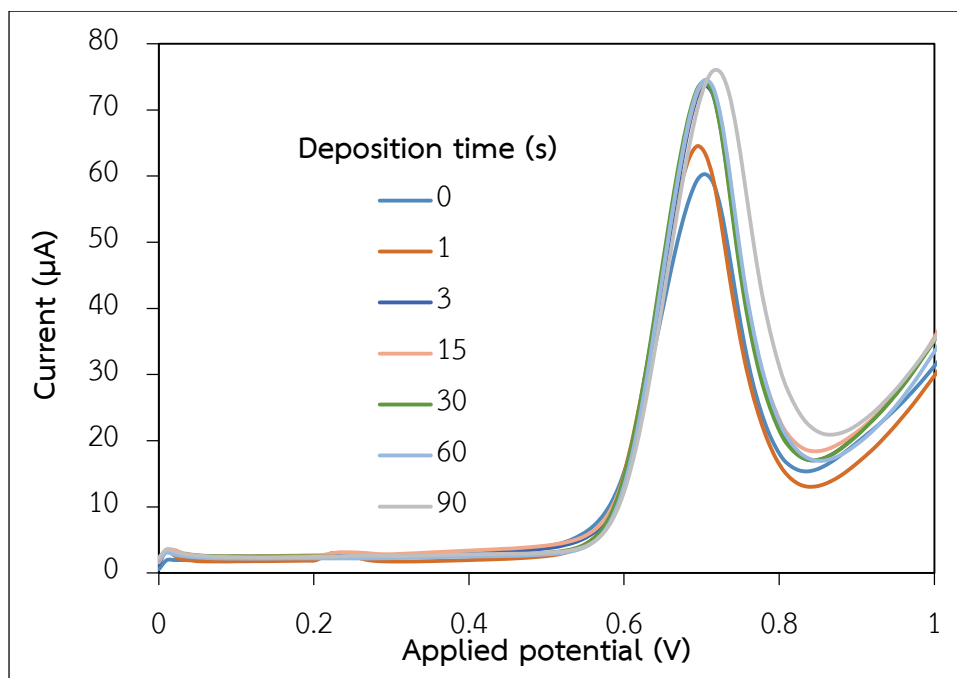


Figure 4.22 Square wave voltammograms of 0.25 mM genistein in 2 M H₂SO₄ on CoPC-modified carbon electrochemical sensor in variation of deposition time.

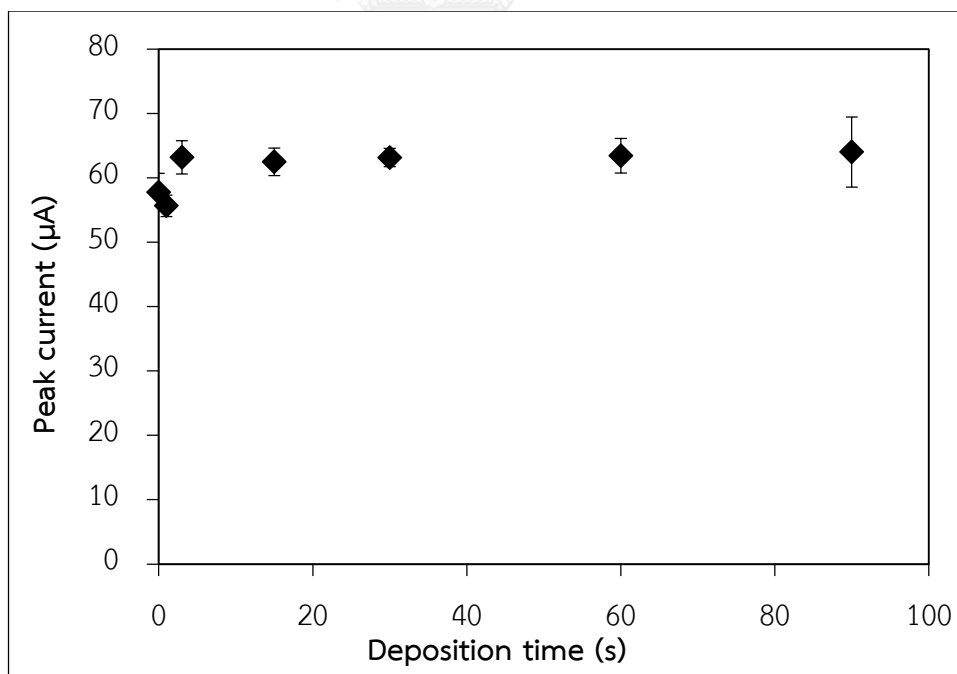


Figure 4.23 Effect of deposition time on the SWV signal of 0.25 mM genistein in 2 M H₂SO₄ on CoPC-modified carbon electrochemical sensor.

The optimal values of the studied SWV parameters for genistein determination on CoPC-modified carbon electrochemical sensor are reported in Table 4.1.

Table 4.1 The optimized parameters of SWV for genistein determination on CoPC-modified carbon electrochemical sensor.

Parameters	Optimized range	Optimal value
Frequency	20 - 200 Hz	150 Hz
Step potential	0.001 - 0.012 V	0.010 V
Amplitude	0.01 - 0.10 V	0.05 V
Deposition potential	-0.5 - 0.7 V	0.50 V
Deposition time	0 - 90 s	3 s

4.2.5 The analytical performance on CoPC-modified carbon electrochemical sensor

With the optimum conditions, concentration of genistein was detected by SWV. The peak current was linearly proportional to the concentration of genistein over the range of 2.5-150 μM with a correlation coefficient (R^2 value) of 0.998 as shown in Figure 4.24 and Figure 4.25. From these data, the limit of detection (LOD) and limit of quantitation (LOQ) were calculated to be 1.5 μM and 5.0 μM , respectively. The precision of this method was calculated as percent relative standard deviation (%RSD) and found to be 3.33%.

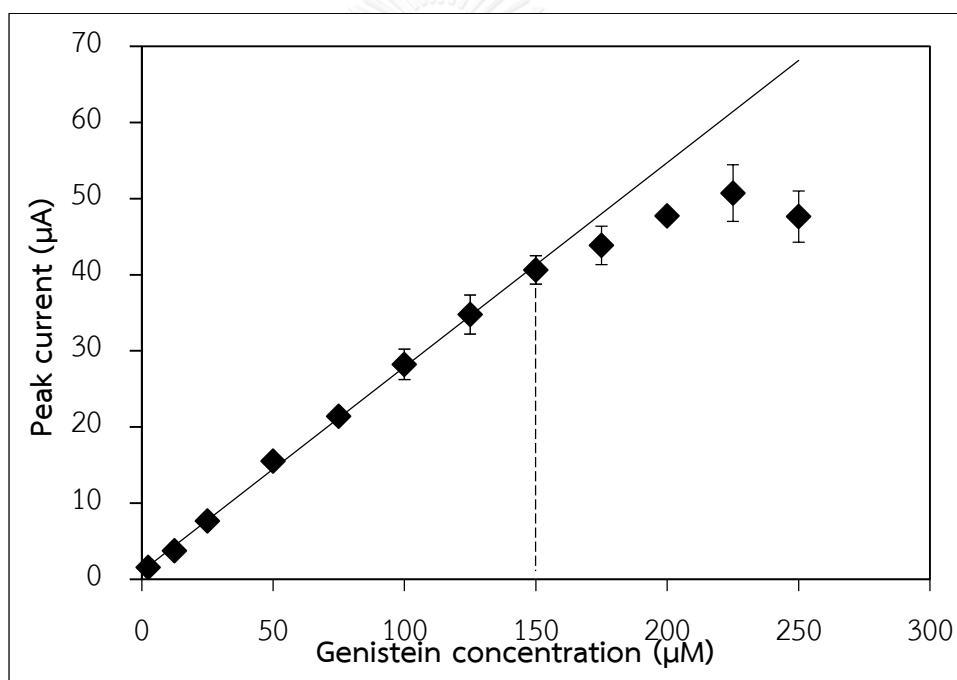


Figure 4.24 The relation of peak current and concentration of standard genistein on CoPC-modified carbon electrochemical sensor.

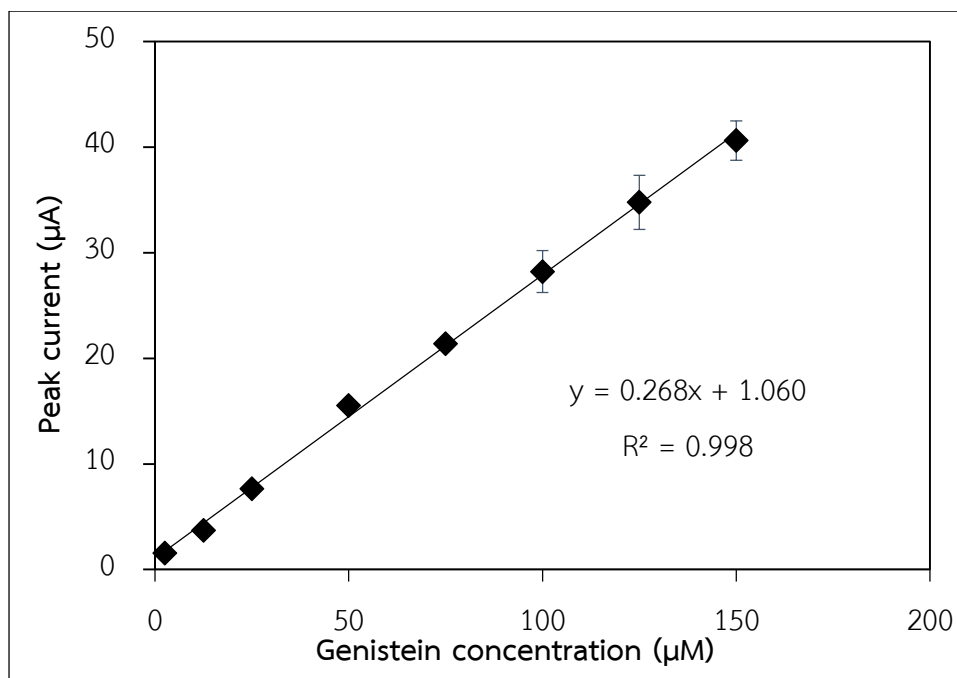
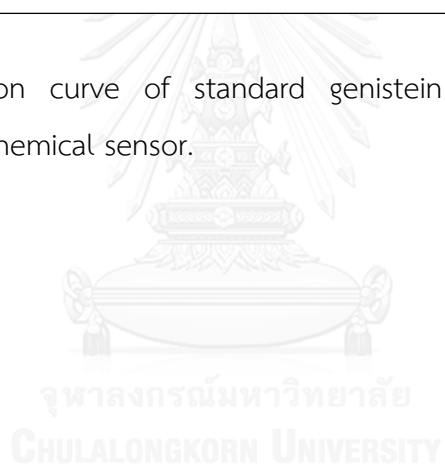


Figure 4.25 Calibration curve of standard genistein on CoPC-modified carbon electrochemical sensor.



4.2.6 Sample analysis on CoPC-modified carbon electrochemical sensor

Genistein was easily extracted out of jewel vine into ethanol under centrifugal force. This process is not only to extract genistein but also precipitate plant sediment. The supernatant can be used directly with the supporting electrolyte on the electrochemical sensor. The concentration of genistein in jewel vine was found to be 14.36 mg/g with recovery of 99.98-104.68 %. This value was close to genistein and its glycoside content reported previously [9]. Moreover, the standard addition calibration curve (Figure 4.26) provided high sensitivity as good as calibration curve in Figure 4.25 by the slightly lower slope of the curve. It means that the matrices in jewel vine sample do not significantly affect to the analysis of genistein in this process.

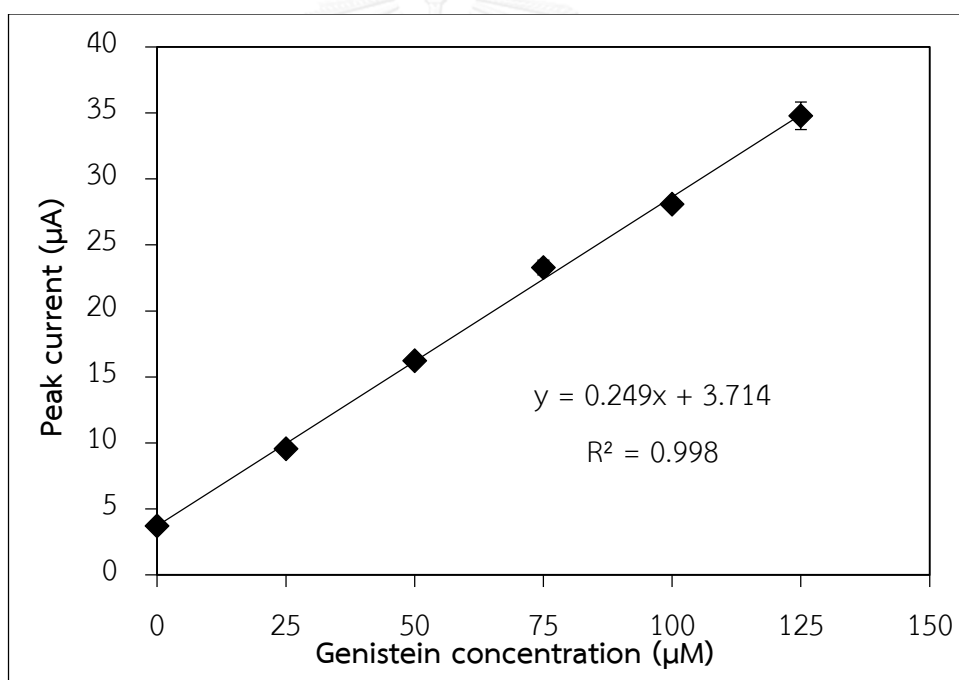


Figure 4.26 Standard addition calibration curve for accurate quantification of genistein in jewel vine on CoPC-modified carbon electrochemical sensor.

The voltammograms of genistein in jewel vine extracts analyzed by the standard addition method are shown in Figure 4.27. Genistein oxidation peak was observed at 0.7 V. The voltammogram of jewel vine extract without adding standard genistein (0 μM) showed the effective of the extraction under centrifugation force from the small amount of real sample (0.01 g). Although the other components was oxidized at 0.6 V, it did not disturb the oxidation of genistein because of the subtraction in the standard addition method.

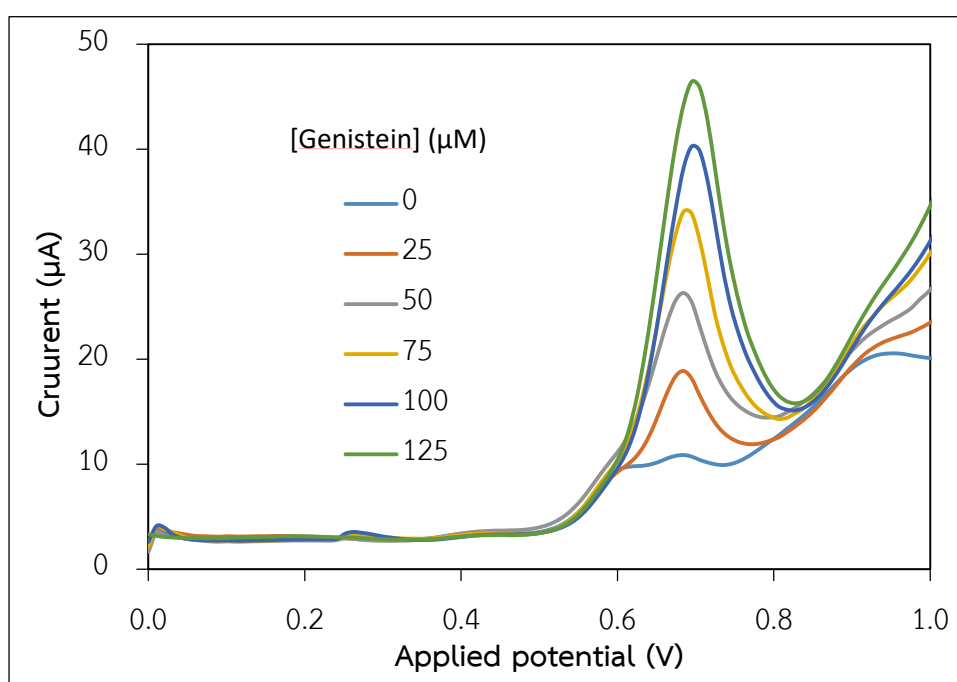


Figure 4.27 Square wave voltammograms of genistein in jewel vine extracts on CoPC-modified carbon electrochemical sensor analyzed by standard addition method.

4.3 Graphene-modified carbon electrochemical sensor

In this part, carbon graphene ink was used to screen-print as a working electrode. This commercially mixed ink was released with low resistance and high sensitive. The extraordinary properties of graphene included of very large electrical conductivity, large surface area and low cost made it was interesting to improve sensitivity of genistein. Supporting electrolyte, SWV parameters, analytical performance, and interference were also studied on this sensor.

4.3.1 Supporting electrolyte for genistein determination on graphene-modified carbon electrochemical sensor

SWV was used to find the suitable supporting electrolyte for this sensor. The oxidation of genistein was also found to provide highest current in H_2SO_4 because of acid strength and more proton generation as shown in Figure 4.28. Therefore, this acid was also selected as a supporting electrolyte for genistein determination on graphene-modified carbon electrochemical sensor.

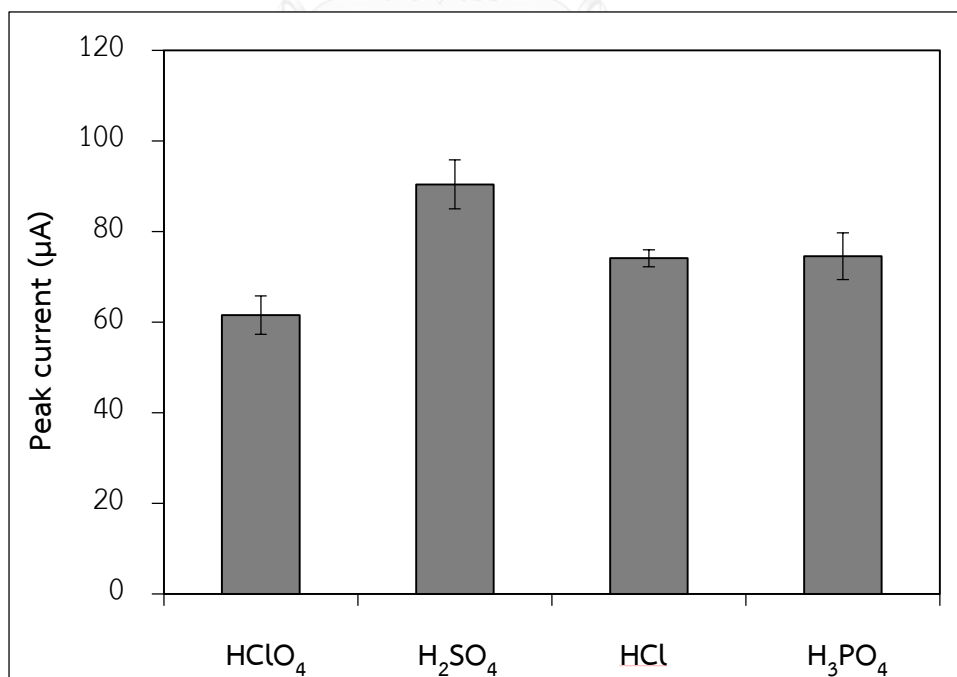


Figure 4.28 Optimization of 2 M supporting electrolyte for SWV of genistein on graphene-modified carbon electrochemical sensor.



The effect of concentration of H_2SO_4 as a supporting electrolyte on the electrode response in the range of 5 mM to 5 M was also evaluated. The relationship between the average current and H_2SO_4 concentrations was shown in Figure 4.29. The peak current of genistein increased with increasing concentrations of H_2SO_4 and reached the maximum at 2 M H_2SO_4 . The concentration of H_2SO_4 higher than 2 M can destroy the electrode surface and the excess produced hydrogen gas. The forming gas separated the electrolyte solution from the electrode limiting the electron transfer between the solvated genistein molecules and the electrode surface. Accordingly, 2 M H_2SO_4 was used as a supporting electrolyte with a good sensitivity.

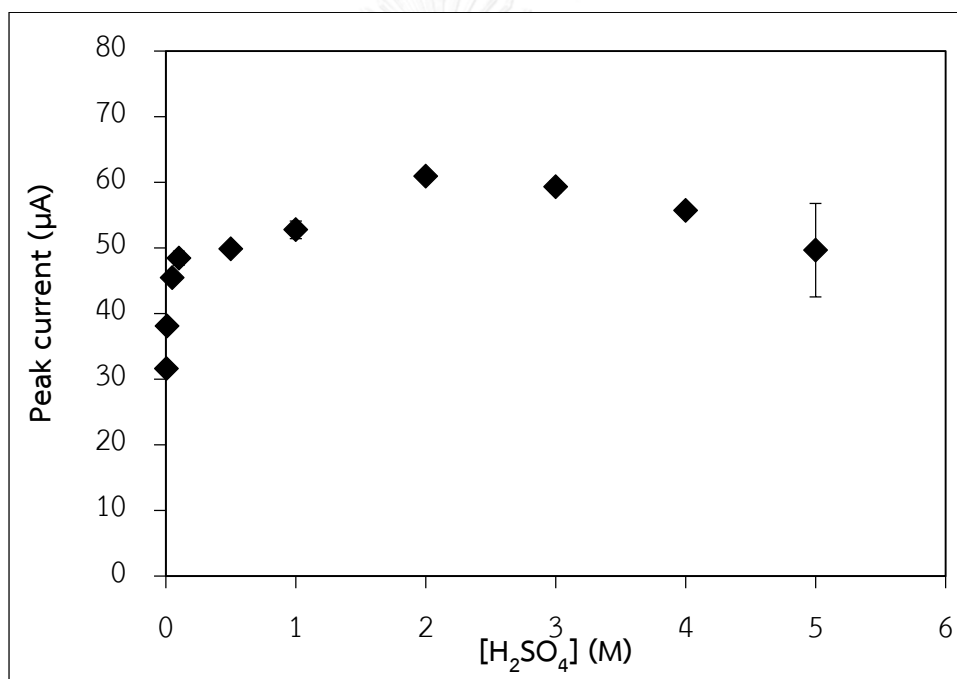


Figure 4.29 Optimization of H_2SO_4 concentration for SWV of 50 μM genistein in H_2SO_4 on graphene-modified carbon electrochemical sensor.

4.3.2 SWV parameters optimization on graphene-modified carbon electrochemical sensor

SWV parameters were also optimized for the genistein determination including frequency, step potential, amplitude, deposition potential and deposition time on graphene-modified carbon electrochemical sensor.

Firstly, deposition potential was studied in the range of -0.5 to 0.5 V as shown in Figure 4.30 and Figure 4.31. This parameter was expected to increase the accumulated genistein molecule on the electrode surface to improve sensitivity as mentioned in Section 4.2.4. However, the results did not affect too much to the signal improvement. The best sensitivity was obtained when the deposition potential reached 0.4 V. Therefore, the deposition potential was set at 0.4 V to optimize the deposition time.

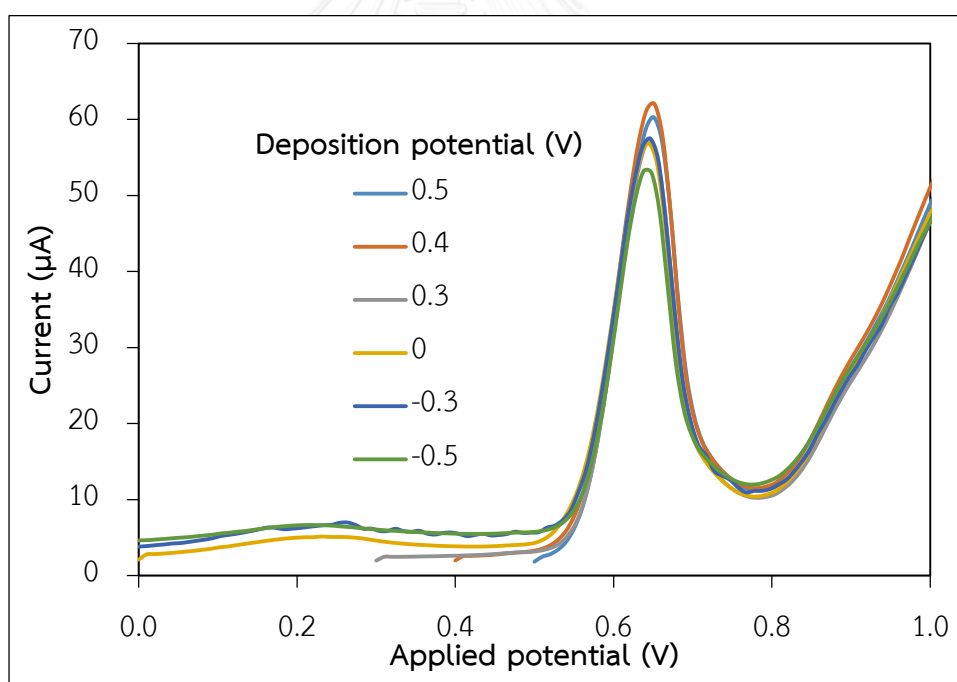


Figure 4.30 Square wave voltammograms of 5 μM genistein in 2 M H₂SO₄ on graphene-modified carbon electrochemical sensor in variation of deposition potential.

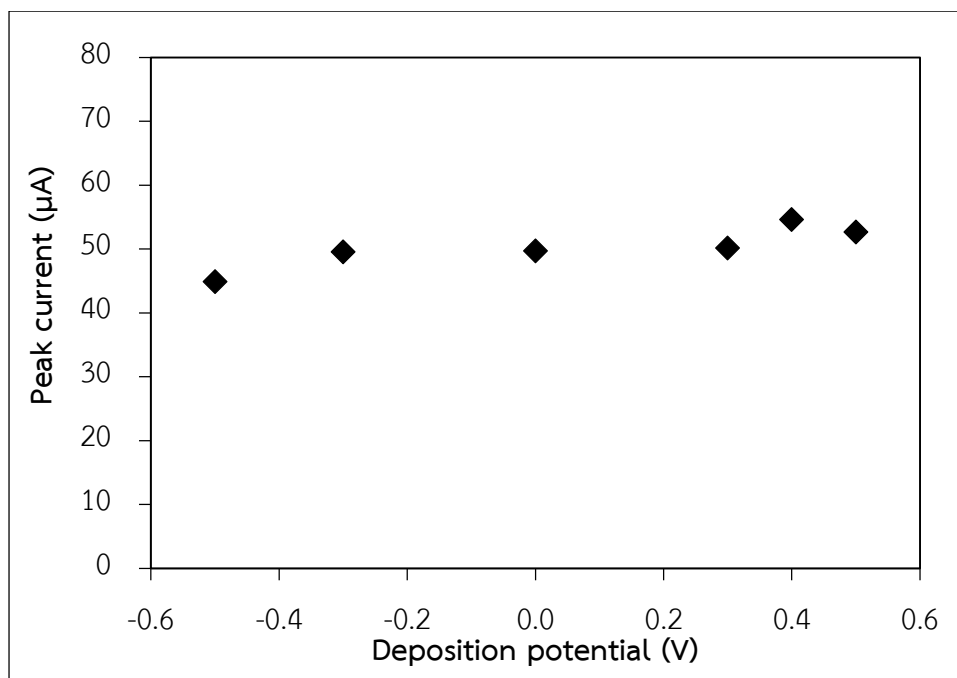


Figure 4.31 Effect of deposition potential on the SWV signal of 50 µM genistein in 2 M H₂SO₄ on graphene-modified carbon electrochemical sensor.

At 0.4 V deposition potential, deposition time was studied in the range of 0-60 s as shown in Figure 4.32 and Figure 4.33. The signal of genistein was slightly increased although 0.4 V deposition potential was held for longer time. It was not worthwhile to operate longer time. Therefore, no need to deposit the potential before measurement genistein oxidation on this sensor.

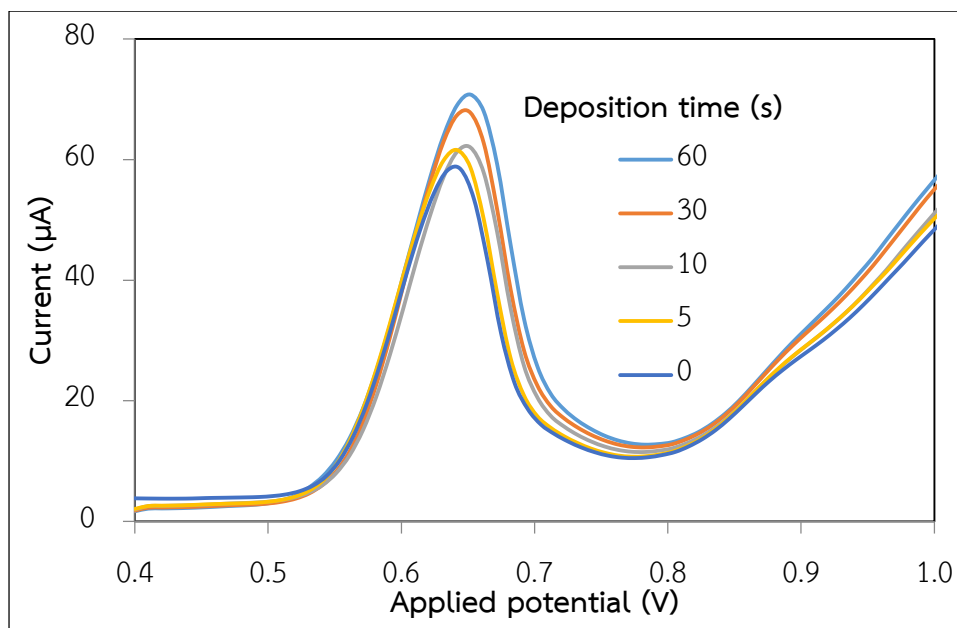


Figure 4.32 Square wave voltammogram of 50 μM genistein in 2 M H_2SO_4 on graphene-modified carbon electrochemical sensor in variation of deposition time.

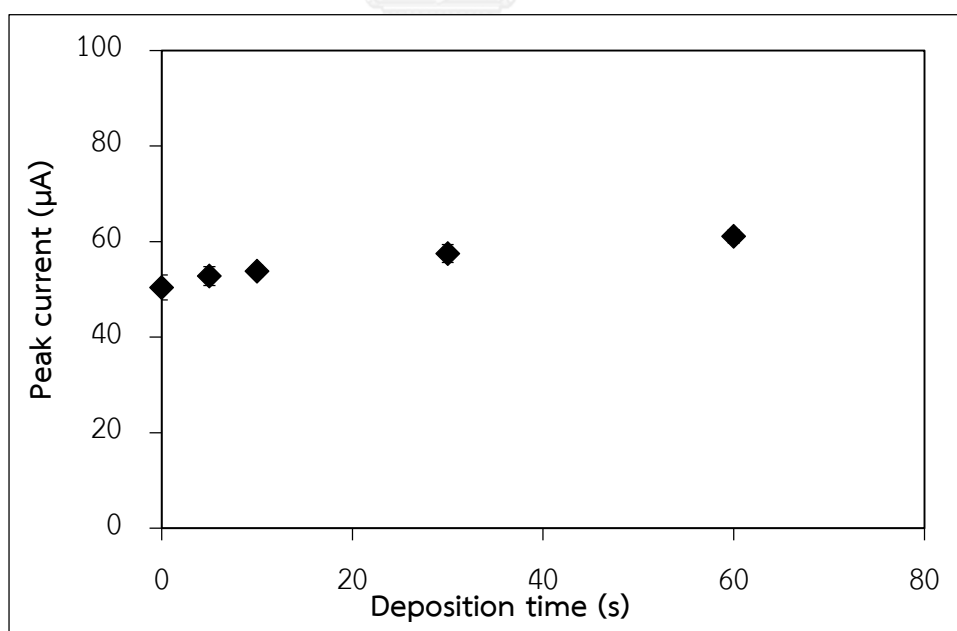


Figure 4.33 Effect of deposition time on the SWV signal of 50 μM genistein in 2 M H_2SO_4 on graphene-modified carbon electrochemical sensor

The step potential was optimized in the range of 0.001-0.012 V as shown in Figure 4.34 and Figure 4.35. As mentioned before, the increasing of this parameter affected to the increasing of scan rate (Equation 4.5). The genistein signal was enhanced when the step potential was increased because of higher scan rate. However, the poor resolution of voltammogram was found (Figure 4.34). To select the optimized value of this parameter, the peak current and the resolution of voltammogram should be compromised. Accordingly, 0.01 V was selected as the optimized value of step potential.

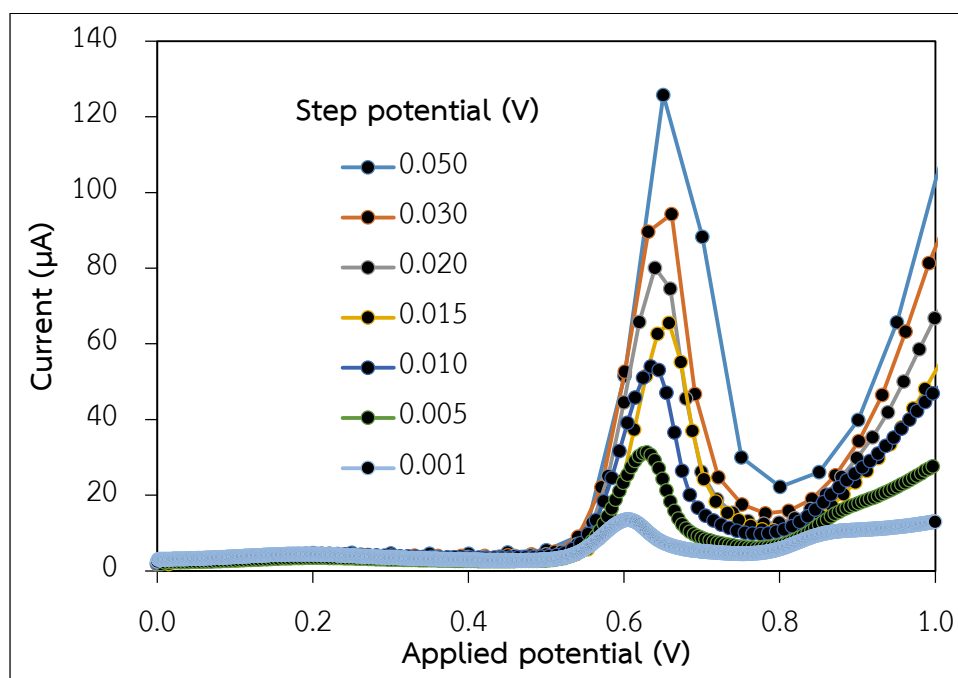


Figure 4.34 Square wave voltammograms of 50 μM genistein in 2 M H_2SO_4 on graphene-modified carbon electrochemical sensor in variation of step potential.

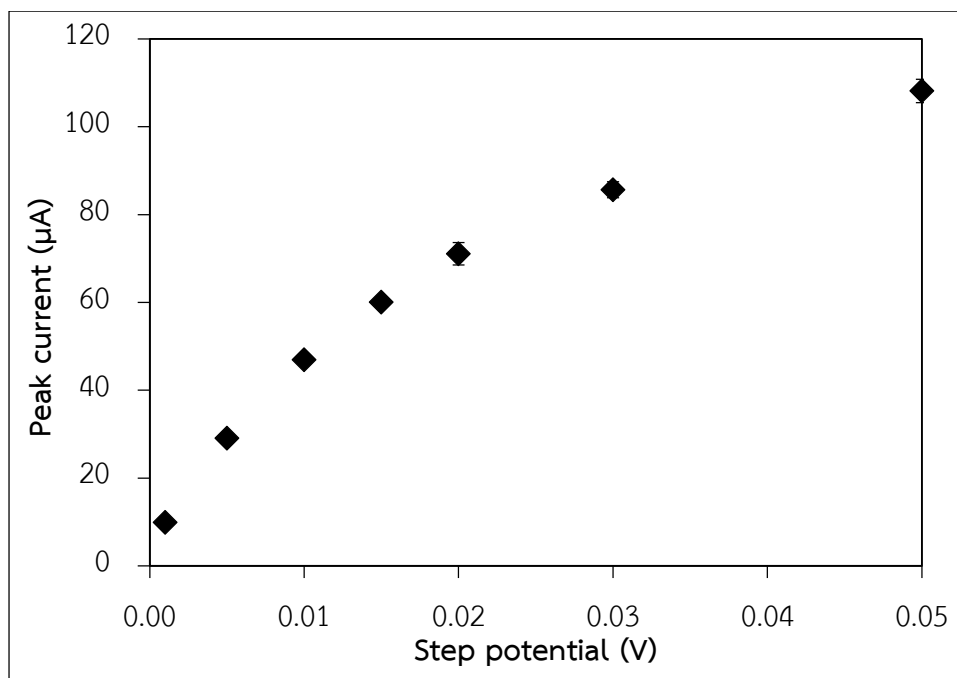


Figure 4.35 Effect of step potential on the SWV signal of 50 μM genistein in 2 M H_2SO_4 on graphene-modified carbon electrochemical sensor.

Frequency was optimized as shown in Figure 4.36 and Figure 4.37 in the range of 50-250 Hz. The genistein signal was enhanced and shifted slightly to more positive potentials when the frequency was increased because of the increasing of scan rate. The increment of genistein signal trended to be higher than the studied range, however, the upper limit of frequency that the instrument can operate was 250 Hz. Therefore, frequency was set at 250 Hz as the optimum parameter.

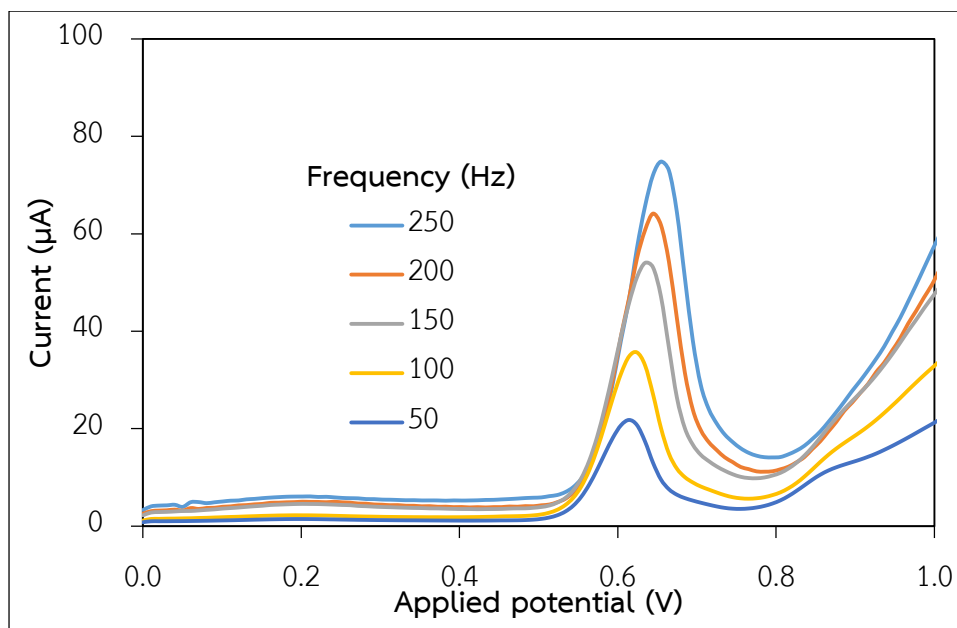


Figure 4.36 Square wave voltammograms of 50 μM genistein in 2 M H₂SO₄ on graphene-modified carbon electrochemical sensor in variation of frequency.

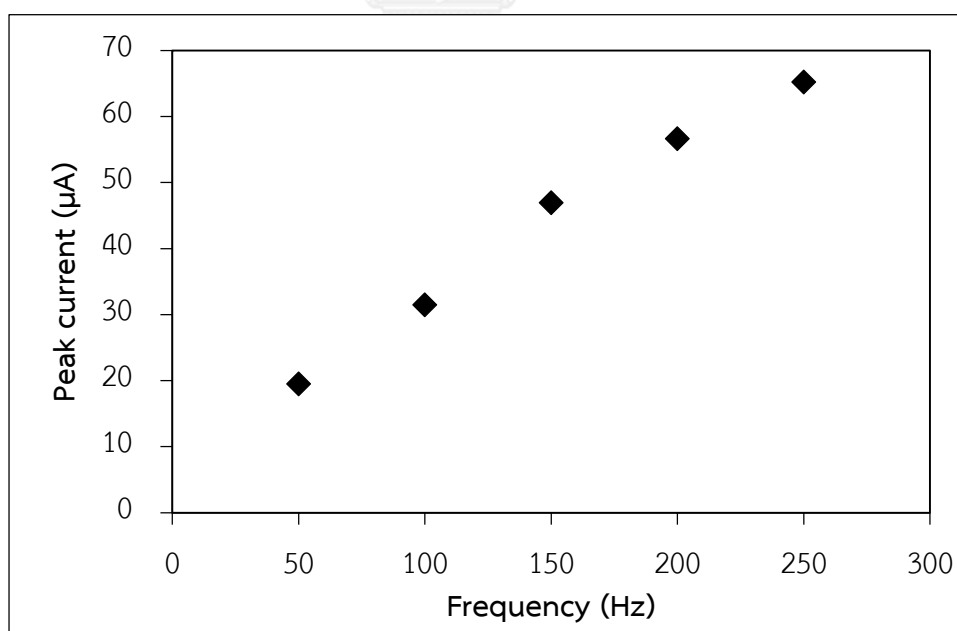


Figure 4.37 Effect of frequency on the SWV signal of 50 μM genistein in 2 M H₂SO₄ on graphene-modified carbon electrochemical sensor.

In the case of amplitude, increasing of this parameter affected to increase the peak current of genistein and the peak potential was shifted to less positive because of the larger difference between the current before and after the application of the wave as shown in Figure 4.38. Amplitude was study in the range of 0.01-0.10 V and found the suitable signal at 0.07 V (Figure 4.39).

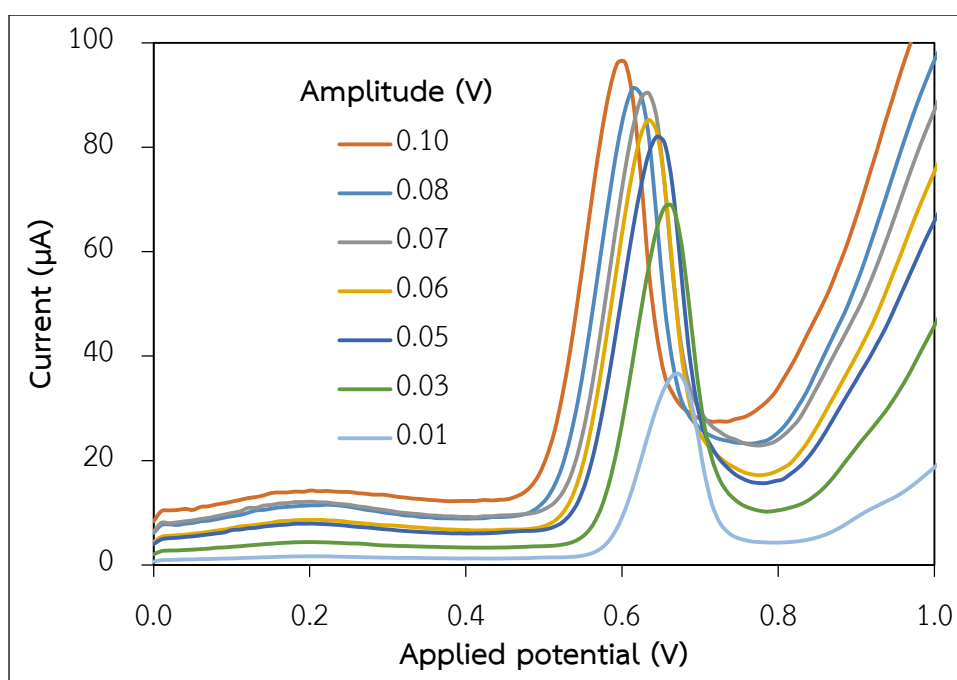


Figure 4.38 Square wave voltammograms of 50 μM genistein in 2 M H_2SO_4 on graphene-modified carbon electrochemical sensor in variation of amplitude.

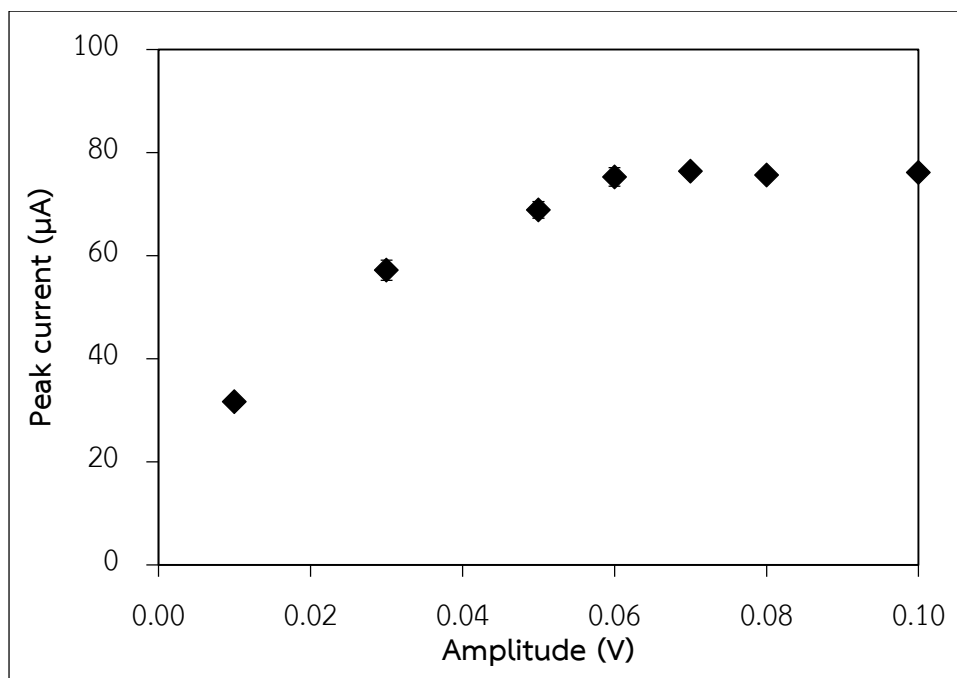


Figure 4.39 Effect of amplitude on the SWV signal of 50 μM genistein in 2 M H_2SO_4 on graphene-modified carbon electrochemical sensor.

The optimal values of the studied SWV parameters for genistein determination on graphene-modified carbon electrochemical sensor are summarized in Table 4.2.

Table 4.2 The optimized parameters of SWV for genistein determination on graphene-modified carbon electrochemical sensor

Parameters	Optimized range	Optimal value
Frequency	50 - 250 Hz	250 Hz
Step potential	0.001 - 0.05 V	0.01 V
Amplitude	0.01 - 0.10 V	0.07 V

4.3.3 The analytical performance on graphene-modified carbon electrochemical sensor

This device provide two ranges of linearity, 1-25 μM and 25-200 μM , under optimized conditions with R^2 value of 0.972 and 0.996, respectively (Figure 4.40). This sensor provided wider linear range of 1-200 μM . A little improvement of LOD and LOQ were obtained to be 1.0 μM and 1.6 μM , respectively.

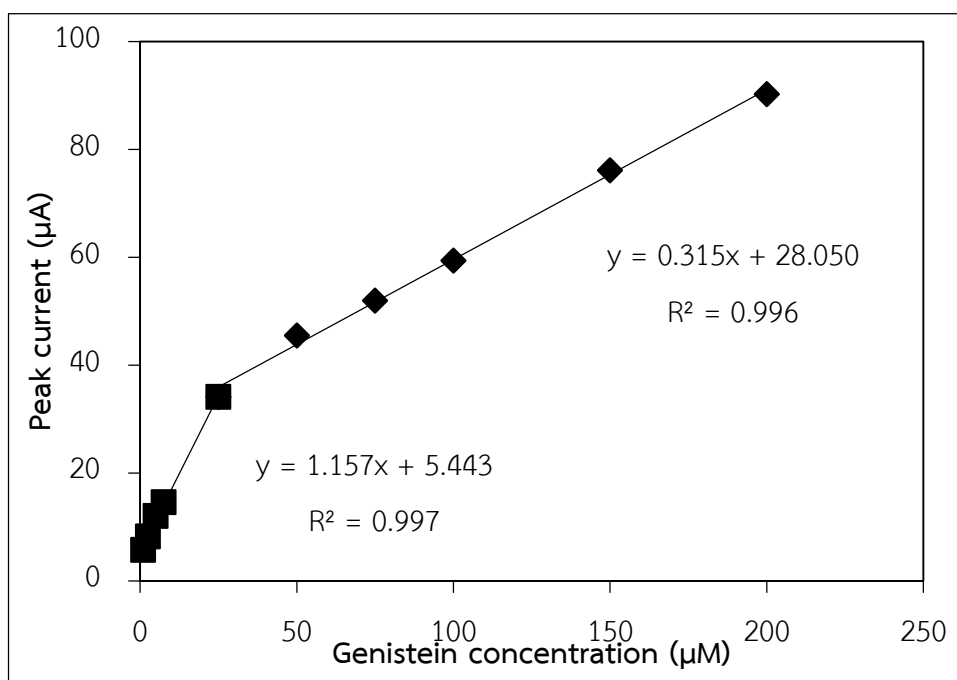


Figure 4.40 Calibration curve of standard genistein on graphene-modified carbon electrochemical sensor.

4.3.4 The interferences study on graphene-modified carbon electrochemical sensor

The effect of major components, glucose, and starch, in jewel vine sample were studied. These two compounds did not cause any effect to genistein analysis as shown in Figure 4.41. Although, ascorbic acid was rarely reported component in jewel vine, it was found to be oxidized on the electrode surface at the same position as genistein. Fortunately, ethanol in the proposed system can address interference of ascorbic acid in genistein analysis process as shown in Figure 4.42.

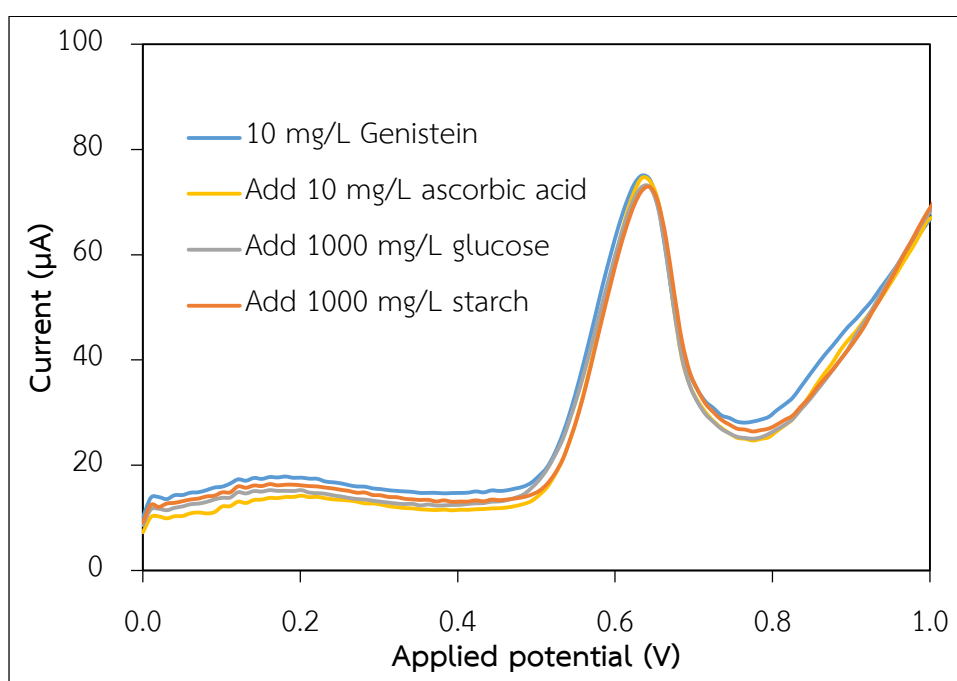


Figure 4.41 Square wave voltammograms of 10 mg/L genistein in 2 M H₂SO₄ in the presence of other components, 10 mg/L ascorbic acid, 1000 mg/L glucose, and 1000 mg/L starch.

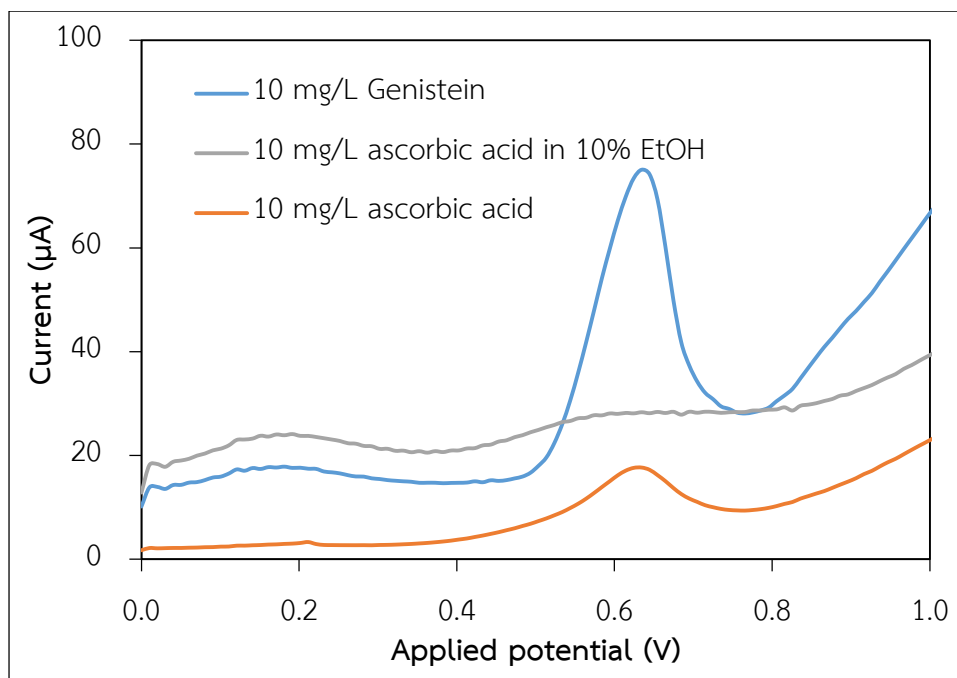


Figure 4.42 Effect of ethanol in the measurement system on the SWV signal of ascorbic acid.

CHAPTER V

CONCLUSION

5.1 Conclusion

The electrochemical sensor for genistein determination was successfully produced by the simple screen-printing technique. The effective modifying materials can be used to improve sensitivity easily. The improvement of genistein signal was illustrated in Figure 5.1. The oxidation peak of genistein on CoPC-modified carbon electrochemical sensor was high against the genistein signal on carbon electrochemical sensor. Moreover, graphene-modified carbon electrochemical sensor exhibited the highest genistein signal improvement.

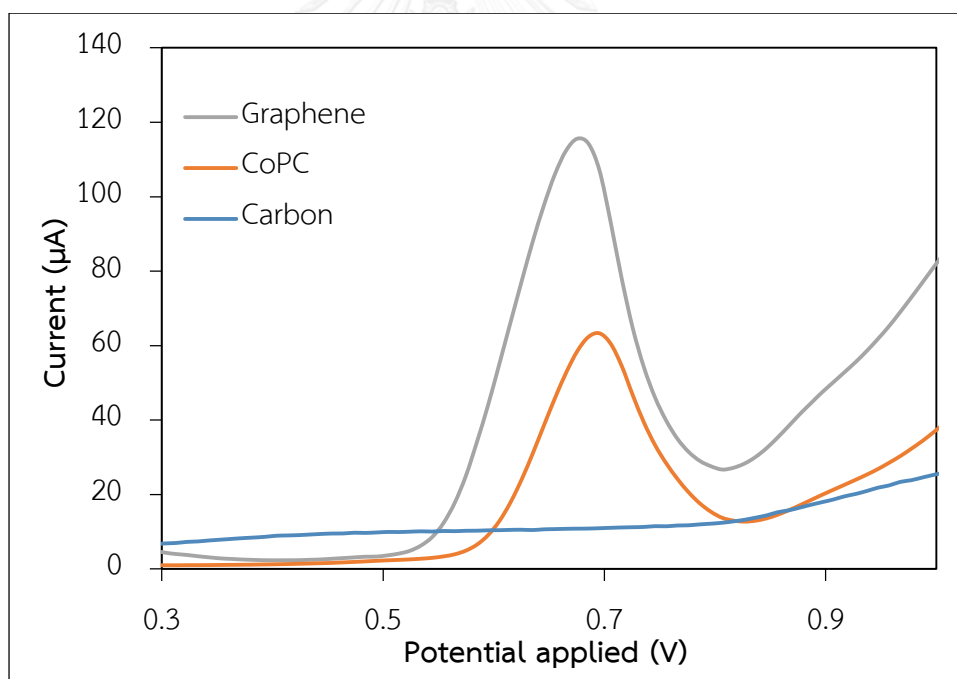


Figure 5.1 Square wave voltammograms of 0.25 mM genistein in 2 M H₂SO₄ on the electrochemical sensor with different working electrodes.

The CoPC-modified carbon electrochemical sensor provided a wide linear range of 2.5-150 μM genistein concentration. The LOD and LOQ were 1.5 μM and 5.0 μM , respectively. For the graphene-modified carbon electrochemical sensor, wider linear range of 1-200 μM genistein concentration was obtained. The lower LOD and LOQ were 1.0 μM and 1.6 μM , respectively. The analytical performance of these proposed methods and others was compared in Table 5.1. The analytical performance of the proposed methods compared with other reported methods. For genistein determination. These proposed methods provided better sensitivity than carbon disk electrode. Although the LOD was not the lowest, it is enough to measure the genistein content in the selected Thai herb, *Derris scandens*, by the proposed extraction without any preconcentration step. The wider linearity range and safer also obtained when compared with mercury electrode. Moreover, the measurement operated with only 50 μL sample. When small amount of sample was required, the usage of real sample (0.01 g) and solvent (2 mL) in extraction can be also save. In addition, SWV finished in a few second for each measurement leading to high-throughput analysis. This proposed method is promising to be an alternative method for genistein determination.

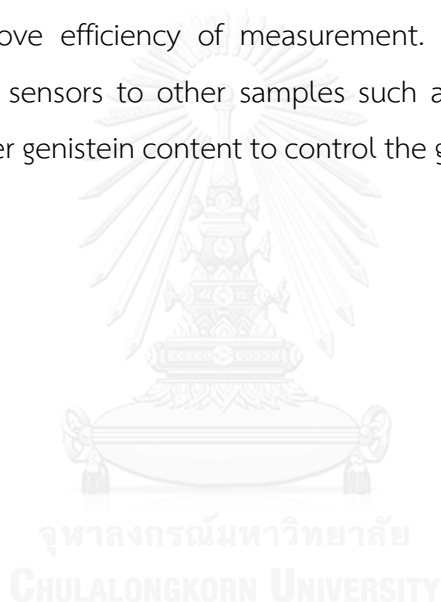
Table 5.1 The analytical performance of the proposed methods compared with other reported methods. For genistein determination.

Electrode	Linearity (μM)	LOD (μM)	LOQ (μM)	Ref.
CoPC	2.5-150	1.5	5.0	This work
Graphene	1.0-200	1.0	1.6	This work
Carbon disk	0.7-370	3.7	-	[23]
Porous graphite	1.8×10^{-3} - 3.7×10^{-1}	1.8×10^{-3}	-	[34]
HMDE	3.43×10^{-2} -1.09	3.43×10^{-2}	-	[25]
HMDE/DME*	0.2 - 50	8×10^{-2}	-	[24]

* HMDE – Hanging mercury drop electrode, DME – Dropping mercury electrode

5.2 Suggestion for future work

The developed electrochemical sensors are simple and inexpensive. These proposed methods can be used to control the amount of genistein in dietary supplement and determine the content of genistein in plant products. The production control with numerous sample is suitable because the proposed methods can operate with short analysis time. Moreover, the carbon working electrode is easy to modify by thoroughly mixing with effective modifying material. This device can be improved to be more sensitive and selective. For example, modifying working electrode with specific material or combining the produced sensor with high performance separation equipment will improve efficiency of measurement. It is leading to extend the applications of these sensors to other samples such as blood and urine which are complicated and lower genistein content to control the genistein consuming in healthy human or patient.



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APPENDIX

จุฬาลงกรณ์มหาวิทยาลัย
CHULALONGKORN UNIVERSITY

VITA

Miss Surinya Traipop was born on November 12, 1985 in Kamphaeng Phet, Thailand. She received her Bachelor's degree of Science (Chemistry) with first class honor from Naresuan University, Phitsanulok in 2007. After that, she became a Master's degree of Science (Chemistry) at the same place in 2010. Next, she accordingly becomes a Doctor of Philosophy degree in Science (Chemistry) of academic year 2016 from Chulalongkorn University. She has got a scholarship from Science Achievement Scholarship of Thailand and a research fund from The 90 th Anniversary of Chulalongkorn University (Ratchadphiseksomphot Endowment Fund)

Academic activities:

S. Traipop, O. Chailapakul, S. Chuanuwatanakul "Genistein Determination by Screen-Printed Electrochemical Sensor" Chem-CU & Okayama University Mini-Symposium, 16 January 2015, Chulalongkorn University, Bangkok , Thailand.

S. Traipop, O. Chailapakul, S. Chuanuwatanakul "Determination of Genistein Using Cobalt(II) Phthalocyanine Modified Carbon Screen-Printed Electrochemical Sensor" The 19th International Conference on Flow Injection Analysis, 30 November – 5 December 2014, ACROS Fukuoka, Japan.

S. Traipop, O. Chailapakul, S. Chuanuwatanakul "Genistein Determination by Screen-Printed Electrochemical Sensor" the 9th Congress on Science and Technology for young students, 30 May -1 June 2014, BITEC, Bangkok , Thailand .

S. Traipop, O. Chailapakul, S. Chuanuwatanakul "Electrochemical Behavior of Genistein on Screen-Printed Carbon Electrode" the 9th Mathematics and Physical Sciences Graduate Congress, 8-10 January 2014, Faculty of Science, University of Malaya, Kuala Lumpur, Malaysia.