การพัฒนาวิธีตรวจวัดวิตามินเครวมโดยใช้ขั้วไฟฟ้าแกรฟีนพิมพ์สกรีน

นางสาววิชชุตา เจษฎาบัณฑิต

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
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ปีการศึกษา 2559

METHOD DEVELOPMENT FOR DETERMINATION OF TOTAL VITAMIN K USING SCREEN-PRINTED GRAPHENE ELECTRODE

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A Thesis Submitted in Partial Fulfillment of the Requirements
for the Degree of Master of Science Program in Chemistry
Department of Chemistry
Faculty of Science
Chulalongkorn University
Academic Year 2016

Thesis Title	METHOD DEVELOPMENT FOR DETERMINATION OF TOTAL VITAMIN K USING SCREEN-PRINTED GRAPHENE ELECTRODE
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งานวิจัยนี้เป็นการพัฒนาวิธีใหม่ด้วยเทคนิคสแควร์เวฟแอโนดิกสทริปปิงโวลแทมเมตรีสำหรับการตรวจวัดปริมาณวิตามินเครวมในอาหารเสริมที่มีความไวสูง โดยใช้ขั้วไฟฟ้าแกรฟีนพิมพ์สกรีน ซึ่งวิธีการตรวจวัดด้วยเทคนิคนี้ประกอบด้วย 2 ขั้นตอน ขั้นแรกเป็นการให้ศักย์ไฟฟ้าทางด้านลบอย่างคงที่เป็นระยะเวลาหนึ่ง เพื่อเป็นการเพิ่มความเข้มข้น และเปลี่ยนฟอร์มของสารจากควิโนนให้เป็นไฮโดรควิโนน สำหรับขั้นที่สองเป็นการสแกนศักย์ไฟฟ้าไปทางด้านบวกเพื่อให้เกิดปฏิกิริยาออกซิเดชันสำหรับตรวจวัด ในงานวิจัยนี้มีการศึกษาพารามิเตอร์ที่เกี่ยวข้องเพื่อเพิ่มความไวในการตรวจวัด ได้แก่ พีเอชของสารละลายอิเล็กโทรไลต์ ศักย์ไฟฟ้าที่เหมาะสมในการสะสม เวลาที่เหมาะสมในการสะสม ความถี่ ขั้นศักย์ไฟฟ้า และแอมพลิจูด ภายใต้สภาวะที่เหมาะสมได้ช่วงความสัมพันธ์ระหว่างสัญญาณกระแสไฟฟ้าและความเข้มข้นของวิตามินเครวมเป็นเส้นตรงในช่วง 1 - 15 ไมโครกรัมต่อมิลลิลิตร โดยที่ค่าสัมประสิทธิ์สหสัมพันธ์อยู่ที่ 0.9904 และขีดจำกัดต่ำสุดของการตรวดวัดอยู่ที่ 0.099 ไมโครกรัมต่อมิลลิลิตร นอกจากนั้นได้มีการหาค่าร้อยละส่วนเบี่ยงเบนมาตรฐานสัมพัทธ์ของวิตามินเค 3 ความเข้มข้นที่ 5 10 และ 15 ไมโครกรัมต่อมิลลิลิตร พบว่ามีค่าเท่ากับ 3.29 3.18 และ 3.16 เปอร์เซนต์ ตามลำดับ สำหรับการประเมินประสิทธิภาพของวิธีที่พัฒนาขึ้นนั้นได้มีการนำไปประยุกต์ใช้เพื่อตรวจวัดวิตามินเครวมในตัวอย่างจริง ซึ่งพบว่าให้ค่าร้อยละการคืนกลับเป็นที่น่าพอใจอยู่ในช่วง 90.1 – 108.1 เปอร์เซนต์ อีกทั้งยังให้ผลการทดลองที่สอดคล้องกับวิธีมาตรฐานโดยมีค่าร้อยละส่วนเบี่ยงเบนมาตรฐานสัมพัทธ์ที่ยอมรับได้อยู่ในช่วง 1.7 - 6.9 เปอร์เซนต์ ดังนั้นวิธีการตรวจวัดนี้จึงเป็นวิธีทางเลือกหนึ่งสำหรับการควบคุมคุณภาพของวิตามินเคในอาหารเสริม ซึ่งให้ความไวในการตรวจวัดสูงและสามารถทำซ้ำได้

ภาควิชา เคมี ลายมือชื่อนิสิต .....

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5772146923 : MAJOR CHEMISTRY
KEYWORDS: TOTAL VITAMIN K / SCREEN-PRINTED ELECTRODE / GRAPHENE / SQWARE-WAVE ANODIC STRIPPING POTENTIAL

WHITCHUTA JESADABUNDIT: METHOD DEVELOPMENT FOR DETERMINATION OF TOTAL VITAMIN K USING SCREEN-PRINTED GRAPHENE ELECTRODE. ADVISOR: PROF. ORAWON CHAILAPAKUL, Ph.D., CO-ADVISOR: ASSOC. PROF. WEENA SIANGPROH, Ph.D., 73 pp.

In this work, a new method based on square-wave anodic stripping voltammetry (SWASV) technique using screen-printed graphene electrode was developed for a highly sensitive determination of trace total vitamin K (VK) in dietary supplement. The determination using SWASV consists of two steps. First is accumulation step at more negative potential constantly to preconcentrate and to change the substance from quinone to hydroquinone. For second step, the potential was scanned towards the positive direction for oxidation analysis. To enhance sensitivity, the various experimental parameters such as pH of electrolyte solution, accumulation potential, accumulation time, frequency, step potential, and amplitude were studied. Under the optimal conditions, the relationship between oxidative currents and concentrations of VK was linearity in the range of 1 to 15 μg mL^{-1} with a correlation coefficient of 0.9904 and limit of detection of 0.099 μg mL^{-1}. Furthermore, the relative standard deviation for three concentrations of VK at 5, 10, and 15 μg mL^{-1} were 3.29, 3.18 and 3.16 (n=10), respectively. To evaluate efficiency of the proposed method, it was applied for the determination of total vitamin K in some dietary supplements with satisfactory recovery data in the range of 90.1 – 108.1%. The results obtained were in good agreement compared to standard method. The RSD was acceptable in the range of 1.7 to 6.9%. Such high sensitivity and reproducibility, therefore, the proposed method can be used as an alternative method for quality control of VK in dietary supplement.

Department:	Chemistry	Student's Signature .....
Field of Study:	Chemistry	Advisor's Signature .....
Academic Year:	2016	Co-Advisor's Signature .....

ACKNOWLEDGEMENTS

Firstly, I would like to express my sincere appreciation to my principal advisor Prof. Dr. Orawon Chailapakul and co-advisor Assoc. Prof. Weena Siangproh for the continuous support of my Master's degree study and research with their patience, motivation and immense knowledge. Their precious guidance helped me in all the time of research and writing of this thesis. I would like to thank my thesis committee, Assoc. Prof. Vudhichai Parasuk, Assist. Prof. Suchada Chuanuwatanakul, Prof. Dr. Nattaya Ngamrojanavanich, and Dr. Amara Apilux for their direction, dedication and invaluable advice along this project.

My sincere thanks also go to Dr. Sudkate Chaiyo and Miss Siriwan Nantaphol, Ph.D. student for suggesting me in some experiments of this research. I really thanks to their all giving of knowledge, support, and encouragement. Another person that really thanks is Miss Pattarachaya Preechakasedkit, Ph.D student, for helping me to revise my writing. Without this ministering, it would not be possible to conduct this research. In particular, I am grateful to all members of Electrochemistry and Optical Spectroscopy Center of Excellence for their help, kindness and warm friendship.

For the financial support, I sincerely appreciate Science Achievement Scholarship of Thailand (SAST), Electrochemistry and Optical Spectroscopy Center of Excellence (EOSCE), Department of Chemistry, Faculty of Science, Chulalongkorn University, and The 90th Anniversary of Chulalongkorn University Fund (Ratchadaphiseksomphot Endowment Fund). I also would like to thank Department of Chemistry, Faculty of Science, Chulalongkorn University for facilities support.

Last but not the least, I must express my very profound gratitude to my family and my friends for providing me with unfailing support and continuous encouragement throughout my years of study, through the process of researching, and writing this thesis. This accomplishment would not be possible without them. Thank you.

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LIST OF ABREVIATIONS

VK	vitamin K
PK	phylloquinone or vitamin K1
MK	menaquinone or vitamin K2
A	ampere
CV	cyclic voltammetry
E	potential
L	liter
LOD	limit of detection
LOQ	limit of quantification
mg	milligram
mL	milliliter
mV	millivolt
RSD	relative standard deviation
SEM	scanning electron microscope
SD	standard deviation
SWASV	square-wave anodic stripping voltammetry
SWV	square-wave voltammetry
V	volt
µg	microgram
µL	microliter
ºC	degree celsius

CHAPTER I
INTRODUCTION

1.1 Introduction

Currently, healthcare in human acts as one of the most important factor with much growing concerned. Therefore, various kinds of supplements have been commercially available and used for the improvement or maintenance of human health. For the importance of using supplements, therefore, the amounts of ingredients in supplementary product have been controlled as suitable value for customers. The development of method for the determination of ingredient in supplementary product is thus necessary to control the accurate amount or dose of ingredient for being a good quality of product.

Among various kinds of supplements, vitamin K (VK), also known as naphthoquinone compound, is one of the ingredients found in commercial products. VK is an essential liposoluble vitamin which plays an important role in human body mechanism as calcification in bone [1, 2] and blood coagulation.[3] It is a cofactor for glutamyl carboxylase which is a catalyzed enzyme in posttranslational conversion during or after the protein biosynthesis to produce a protein called prothrombin which plays crucial role in the clotting of blood. [4-6] In the process of osteocalcin (OC) carboxylation [7], VK acts as the induced molecule for promoting the osteoblast to osteocyte transition and also preventing the osteoclastogenesis. [8] There are two predominant forms of VK in the human body: firstly, vitamin K1 (Phylloquinone, PK) is produced by leaf vegetables and algae, and secondly, vitamin K2 (Menaquinone, MK) is derived from meat, eggs, curd cheese and fermented food. Furthermore, it can be synthesized by bacteria in human intestine which employed as redox reagents in electron transport and used in oxidative phosphorylation. [9-11] Due to the important roles of VK in bone calcification and blood clotting process, therefore, the sufficient amount of VK in human body can decrease the risk of osteoporosis and extra bleeding diseases. In addition, the biological mechanism of blood clotting and bone tissues building also depends on the appropiate amount of

VK in body because it is a cofactor for the production of various vital proteins. The reference range of VK in healthy people is generally found to be between 0.2 to 3.2 ng mL^{-1} as total VK in blood. [12] Normally, a deficiency of VK occur in infants because there are no intestinal bacteria for VK synthesis, and VK can also affect to fat malabsorption syndromes, liver disease and antibiotic therapy in adults. The taking of an appropriate amount of VK is thus acted upon as an alternative way for treatment. [13]

In past decades, several analytical methods have been reported for the determination of VK such as chemiluminescence method [14], high performance liquid chromatography (HPLC) combined with ultraviolet (UV) [15, 16] , fluorescence [17-19] , electrochemistry [20, 21] , chemiluminescence [22] and mass spectrometric detection [23, 24]. However, these techniques have their own limitations such as time consuming, sophisticated instrument and need for large sample amount. To overcome these problems, the determination method providing rapid, simple, and sensitive assay is still required. Electrochemical technique has been recognized as a powerful analytical method because of its simple procedure, short analysis time, high sensitivity and selectivity. [25] For VK determination using electrochemical detection, there is just one piece of publication reported on the determination of VK using differential pulse voltammetry by a hanging mercury drop electrode. [26] The results indicated that the cathodic peak currents were greater than the anodic peak currents, and the largest peak current is observed in an acetate buffer containing 60% methanol. Although this assay provided a low detection limit, however, it requires a long time for the repetitive application of potential to increase sensitivity at low concentration level. Furthermore, a hanging mercury drop electrode is rarely suitable because of its high toxicity, time consuming and large sample volume requirement. Thus, the development of electrochemical detection to overcome the mentioned previous limitations is very necessary and interesting to obtain a new methodology as an alternative method for the determination of VK.

A screen-printed electrode (SPE) is a kind of disposable sensors based on the screen-printing technology that has many advantages such as simple operation, reliability, portability, small instrumental setups and modest cost. [27] These sensors

can be constructed by printing using various types of inks such as carbon, and graphene on the different ceramics or plastics substrates. Both the selectivity and sensitivity for the analytical procedure depend on types of printed inks used as working electrodes on the substrates. [28] Graphene is a well-known conductive material which has the excellent properties. For example, it has large surface area, excellent electrical conductivity, fast electron transportation, high thermal conductivity, excellent mechanical flexibility and good biocompatibility. [29] Due to these good features, the graphene screen-printed electrode displays a suitable sensing device for a highly sensitive electrochemical sensor. Among various electrochemical techniques, anodic stripping voltammetry or ASV is a kind of electrochemical quantitative method. This technique provides the powerful method not only for detection of some metals but also for biochemical substances. There are many advantages of ASV such as sufficient selectivity, notable sensitivity, handiness and low analysis cost which is suitable for laboratory analysis. [30] Square wave voltammetry (SWV) is commonly combined with the anodic stripping method called the square-wave anodic stripping voltammetry or SWASV because its high efficiency technique and remarkable sensitivity. Presently, it has numerous applications that prefer to use this technique in various fields including medical and food detection. The SWASV procedure is based on two steps that first is accumulation of trace analytes or called preconcentration step and then the electrochemical stripping step of the analytes accumulated on the electrode surface. [31] Due to the high performance trace level analysis, the SWASV was chosen as operated method to deal with microgram level of vitamin K in supplements.

In order to develop the measurement method to quantify the total vitamin K in pharmaceutical product such as dietary supplement, the novel method is investigated proposed by using the graphene screen-printed electrode with anodic stripping voltammetry technique. This research could act as an alternative method for both qualitative and quantitative control of VK in pharmaceutical product industry which still has few studies on this area. The benefits of this research over the previous works are new sensor devices consist of small size, requirement of small solution volume, friendly environment, simple fabrication and operation, and

have reasonable cost. Furthermore, the proposed method can reach the goal of high sensitivity in detection and can be applied for real sample analysis.

1.2 Objectives of the Research

The objectives of this research are divided in two parts as follows:

1. To develop the graphene screen-printed electrode for the determination vitamin K using SWASV
2. To apply the proposed method for the determination of vitamin K in supplementary foods

1.3 Scope of the research

The novel method for determination of total VK in supplements using square-wave anodic stripping voltammetry (SWASV) with screen-printed graphene electrode was first developed. In order to develop the method for determination of VK, the numerous processes were carried out. Firstly, the performances of electrode between screen-printed carbon and graphene screen-printed electrodes toward electrochemical behavior of VK will be investigated. For example, active surface area, and capacitance of double layer charging current were compared using cyclic voltammetry technique. For the data confirmation, the scanning electron microscopy (SEM) was operated to study the morphology of the electrode surface. To enhance sensitivity of VK determination, all experimental conditions composed of percent of ethanol in solution and pH of supporting electrolyte were optimized. Moreover, the electrochemical parameters such as accumulation potential, accumulation time, frequency, amplitude and step potential were also studied to obtain optimal conditions. For analytical performance, the different concentrations of total VK were measured to identify the linear relationship against the anodic current signal. In addition, limit of detection and limit of quantitation were also calculated. Due to the other ingredients effect in real samples on VK determination, the interferences effects were examined using the tolerance ratio method by individual spiking the interferences in VK standard solution as mass ratio. In final, the proposed method

was applied to determine VK in supplements and compared to HPLC-UV technique for validation of proposed method.

CHAPTER II
THEORY AND LITERATURE SURVEY

2.1 Vitamin K and its properties

Vitamin K is one of the fat-soluble micronutrient which is essential for human body mechanism. The general structure of vitamin K is also known as naphthoquinone compound. In natural source and human body, there are two principal forms consisting of vitamin K1 and K2. Vitamin K1 or Phylloquinone (PK) is available in green leafy vegetable and algae; and its naphthoquinone consists of methyl and a phytyl side chain. Vitamin K2, also known as Menaquinonec (MK), is regularly found in fermented food and mainly synthesized by the colon bacteria. The naphthoquinone structure of K2 also has methyl group but the difference is the composition of unsaturated side chain of isoprenoid varying in length of 1 to 14 repeats. Normally, the major form of vitamin K in food and human body is considered as PK and MK-4. [32, 33] The chemical structure of two forms is illustrated in Fig. 2.1. In biological mechanism, vitamin K has two important roles in blood coagulation [3, 4] and calcification of bone. [1, 2] In point of blood coagulation, vitamin K acts as oxidizing agent in posttranslational conversion of the protein synthesis pathway, in order to convert glutamate protein to gamma-carboxyglutamate in several proteins such as plasma clotting factors II (prothrombin), VII, IX, X and protein C. These vitamin K-dependent proteins play crucial roles in blood coagulation. [33] The reduction of vitamin K occurs in two-electron, two-proton process to form vitamin K hydroquinone, and then subsequently oxidizes back to quinone in an enzymatic process via vitamin K epoxide intermediate. [34, 35] The mechanism is shown in Fig. 2.2. In bone action, vitamin K plays an important role in maintaining bone strength by acting as cofactor for enzyme gamma carboxylase. The proteins in bone including osteocalcin (OC) and matrix Gla protein (MGP) are oxidized by vitamin K in order to convert the initial inactive form to carboxylate active form for bone mineralization and soft tissue calcification, respectively. [36] The mechanism model of action in bone is shown in Fig. 2.3.

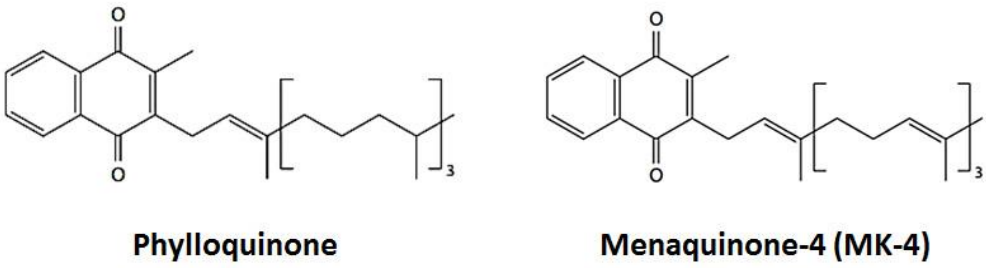


Figure 2.1 The chemical structure of vitamin K1 (Phylloquinone) and K2 (Menaquinone-4).

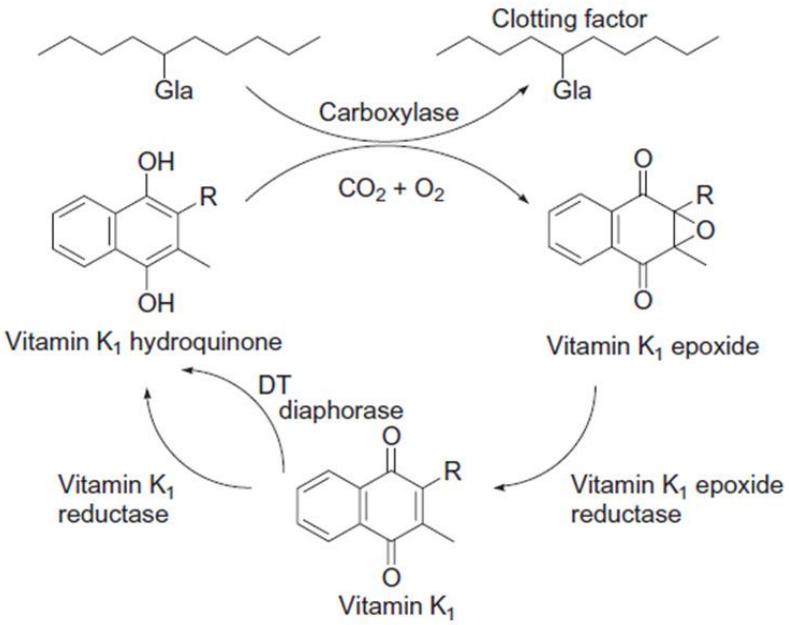


Figure 2.2 Biological reaction of vitamin K for blood clotting protein production.

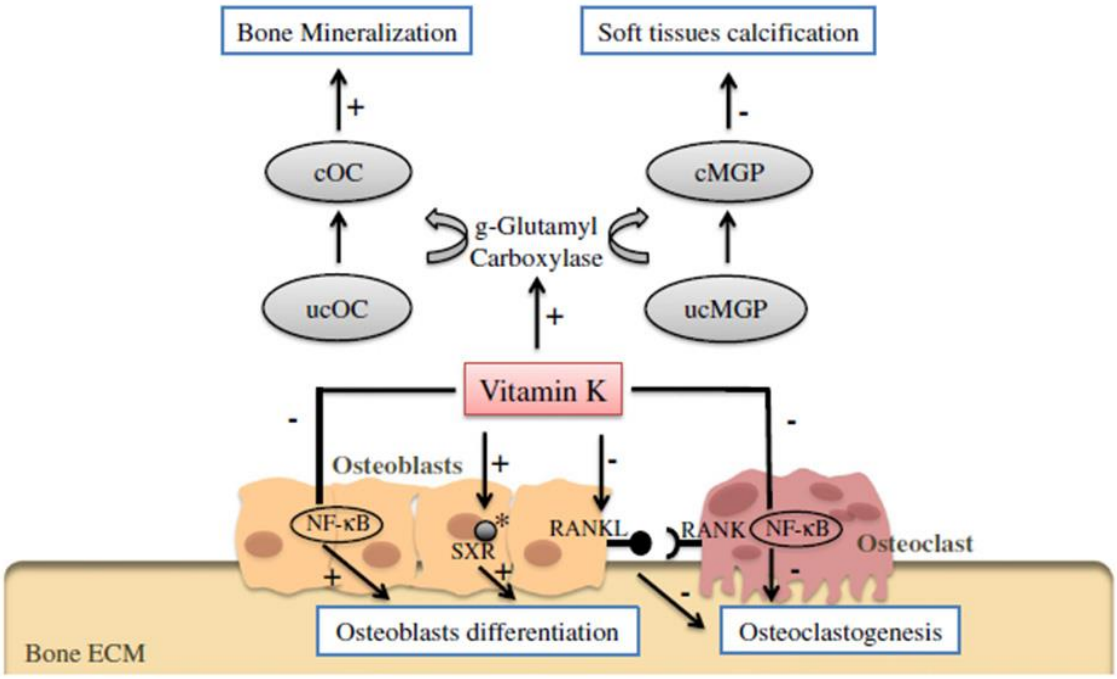


Figure 2.3 Mechanisms of action in bone: Evidence in animal models; ucOC: undercarboxylated Osteocalcin; cOC: carboxylated Osteocalcin; ucMGP: under- carboxylated Matrix Gla Protein; cMGP: carboxylated Matrix Gla Protein; NF-ΚB: nuclear factor ΚB; SXR: Steroid and Xenobiotic Receptor; RANKL: Receptor Activator of Nuclear factor Kappa B Ligand; RANK: Receptor Activator of Nuclear factor Kappa B; ECM: Extracellular matrix.

Due to the crucial biological roles of vitamin K in blood clotting and bone calcification, the deficiency of vitamin K can cause the risk of osteoporosis and extrableeding diseases. In human, vitamin K deficiency generally occurs in newborn called hemorrhagic disease. Generally, this disease occurs by poor placental transfer, absence of bacterial synthesis in the newborn's gut, low concentrations of clotting factors, and low vitamin K concentrations in human milk. Hemorrhagic disease can be prevented by intramuscular injection or oral administration. In case of adults, the deficiency of vitamin K is rarely occurred. However, this disease can cause by fat malabsorption syndromes, liver disease, and antibiotic therapy that inhibits the

synthesis of vitamin K2 in the gut by microbial. [37] In order to prevent these risks, the dietary supplement consumption is the best an alternative choice for human.

2.2 Electrochemical Technique

Electrochemical technique is considered namely as the combination between electricity and chemistry which is the measurements of electrical quantities such as current, potential, charge and their relationship to chemical parameters. In the part of electrical measurements, many beneficial applications can be provided including environmental detection, quality control for food or pharmaceutical industry, and biomedical analysis. Moreover, advance in development of electrode can be done for improving the measurement effectiveness by coupled with various type of conducting materials or specific molecules. From these attractive features, this measurement has been drawn attention as interesting chemical sensors.

In contrast to the others chemical measurements, the electrochemical processes are occurred at the interface between electrode surface and solution. The different techniques will provide their own specific signal which is used for quantitation. There are two main types of electrochemical measurement called potentiometry and voltammetry. For potentiometry, it consists of two electrodes (conducting material) and a sample solution (supporting electrolyte) as the component of electrochemical cell. On the other hand, voltammetry consists of three-electrode system in electrochemical cell. For the two electrodes, one of them responds to the target analyte called indicator or working electrode which is used for the measurement of the electricity of analyte. The second one, is called reference electrode which is used as standard electricity parameter for comparison, and the property of this electrode is constant potential (independent of the property of the solution). Therefore, electrochemical cells can be classified in two types as galvanic cell that the chemical reaction can occur simultaneously by itself to generate the energy. Another one is electrolytic cell which the reaction can occur by consumption of electricity from an external source.

Potentiometry, a static current or zero current technique which the quantitative result can obtain from the measurement of potential detected across a membrane. The different types of membrane can be used as which is specific to different ions. The regular potentiometric probes have been widely used for ion determination, such as proton, calcium, fluoride, and potassium ion in samples.

Voltammetry, a controlled-potential or static potential technique is to study electron transfer at interface between electrode and solution. Furthermore, the charge transfer of analyte can be occurred by application of a range of potential which cover standard potential or of analyte to derive the reaction, and then the quantitation of analyte can be obtained by measuring its currents. The controllable potential can force the chemical species to gain or loss an electron as reduction or oxidation, respectively. This technique can be applied to detect a lot of chemical species as electroactive species that are obvious form of their functional group such as carboxylic, hydroxyl, ketone etc. In contrast, non-electroactive compounds cannot be detected by this technique. However, the non-electroactive species can be detected by indirect determination or complexed with another electroactive compound. The voltammetry technique provide many advantages including high sensitivity and selectivity, wide linear range, low detection limit down to nanomolar level with very small sample volume (5-100μL), speciation capable detection, low cost instrumentation, portability, and wide range of electrode used. For improvement of selectivity, it can be coupled with chromatography or optical techniques.

2.2.1 Voltammetry Technique

Voltammetry is the word combining between volt, the derived unit of electrical potential and ampere which is the unit of electrical current. This technique is to study about the relationship between potential applied to the working electrode surrounded with an electrolyte containing electro-active species and quantity of current generated from substance reaction process. Electrochemical cell of voltammetry technique which specially differs from other technique is three-electrode system consisting of working, reference and counter electrode.. The

working electrode has crucial role for this technique. The interested substance will react at suitable electrical potential. The analyte will be gained or lost of electron at surface of electrode causing the flowing of current which can be measured and achieved as both qualitative and quantitative information.

The second important one is reference electrode, a well-known potential electrode which has exactly constant potential and varies owing to type and composition of that electrode. It is used to compare the potential with working electrode for measurement. The other electrode is the auxiliary or counter electrode that is the completion of electrode system. Its other benefits are to reduce the resistance of solution by placing closely with working electrode for completion the current path between them; one gets oxidation and the other one is reduction. The method for applying potential to electrode has a lot of different ways such as constant at specific potential, sweep, pulse, or staircase etc. depending on the kind of studying purpose. In this work, the voltammetric techniques used for determination of the trace level of total vitamin K in dietary supplement sample are cyclic voltammetry, square-wave voltammetry and stripping voltammetry.

2.2.1.1 Cyclic Voltammetry

Cyclic voltammetry (CV) is one of the electrochemical techniques which is widely used for achieving qualitative information of electrochemical reactions. Due to its ability, it can provide information of thermodynamics of redox processes, the kinetics of heterogeneous electron transfer reactions and on coupled chemical reactions or absorption processes. CV is usually used for the first experiment to study a rapid location of redox potentials of analytes and also used for the evaluation of the effect of media on the reaction.

Measurement process of CV includes scanning the potential linearly at working electrode in both positive and negative potential to make the oxidation and/or reduction. Then, is switching potential; it will be scanned reverse back to the baseline potential like a triangular shape potential waveform (Fig 2.4). The single or multiple cycles can be used. During the potential is sweeping, the

potentiostat measures the current of reactions resulting from applying potential which data can be observed in form of cyclic voltammogram.

Response of reversible redox reaction during a single potential scanning cycle is illustrated in Fig 2.5 assuming that only the oxidizing form (O) is initially present. When potential is scanned negatively, the oxidizing form will be started to get the electron from the electrode surface and converted to reducing form (R) at the potential approaching particular standard potential of O. The cathodic peak will be appeared and currents from this reaction are called the cathodic current. After the cathodic peak at least 90/n mV (n = number of electron), the potential scan is reversed positively, R molecules will give the electrons to electrode and turn back to O to make the anodic peak and anodic current, respectively.

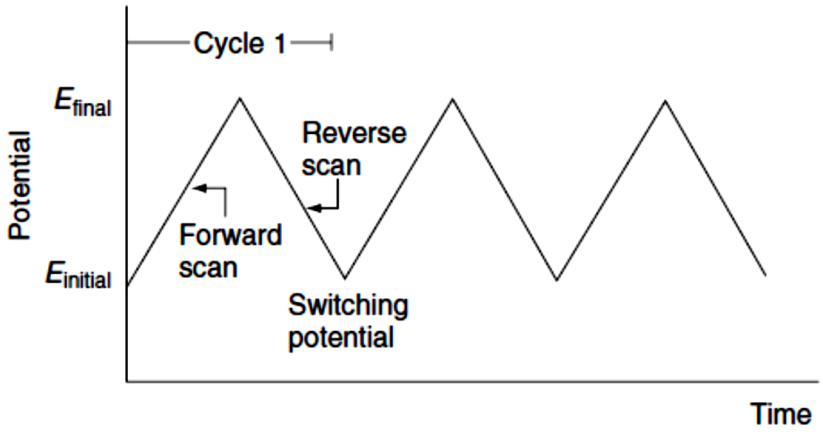


Figure 2.4 Potential-time excitation signal in a cyclic voltammetry.

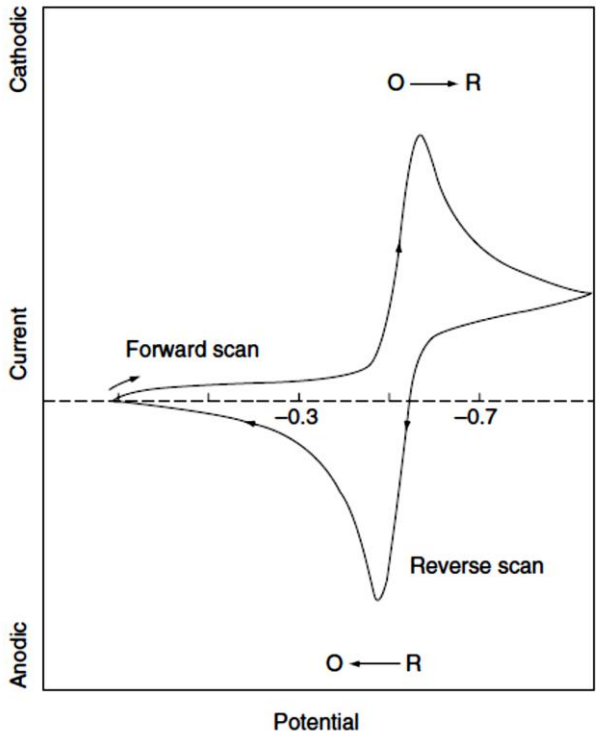


Figure 2.5 Cyclic voltammogram for a reversible O + ne- => R redox process.

2.2.1.2 Square-Wave Voltammetry

Square-wave voltammetry (SWV) is one of electrochemical techniques which is frequently used for acquiring quantitative information. The potential wave form against time consists of symmetric square-wave as forward pulse and reverse pulse which is superimposed on a base staircase potential (Fig 2.6). For data collection, the data is collected at two points which are the end of forward pulse and once at the end of reverse pulse. Then, the difference between these currents is plotted versus base staircase potential that a symmetric peak half-wave potential is finally shown as a result called square-wave voltammogram. From this voltammogram, peak height of its result can indicate the specific concentration of analyte by comparing the currents with calibration curve. The comparison of forward, reverse and different currents for reversible system was showed in Fig.2.7. SWV can provide high sensitivity because the obtained signal camefrom different currents and is coupled with discrimination of charging background current by pulsed method. Thus, the SWV provides higher current than that obtain from differential pulse

voltammetry. The low detection limit can be observed as low as 1 x 10^{-8} M or lower than this from some cases. By the way, the superior advantage of square-wave voltammetry is very fast because of its frequency provided in the range of 1 – 100 cycles per second. Therefore, high speed scan rate can be used. From that reason, analysis time is highly reduced to few minutes while the differential pulse method displays 2-3 minutes longer times than SWV. Furthermore, this technique can be used to couple with liquid chromatography and capillary electrophoresis to identify coeluting or comigration substance.

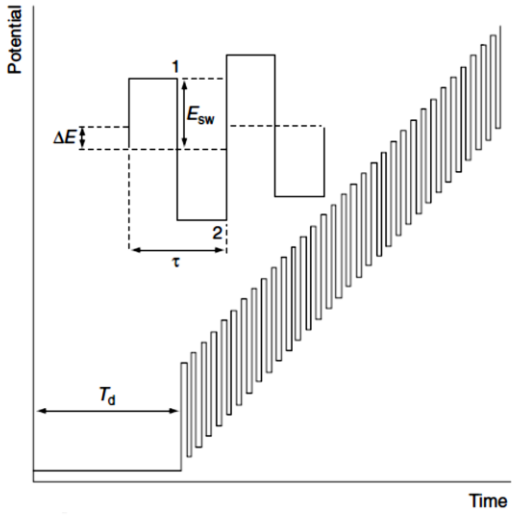


Figure 2.6 Square-wave waveform showing the amplitude Esw, step height ΔE, period T, delay time Td, and current measurement times 1 and 2.

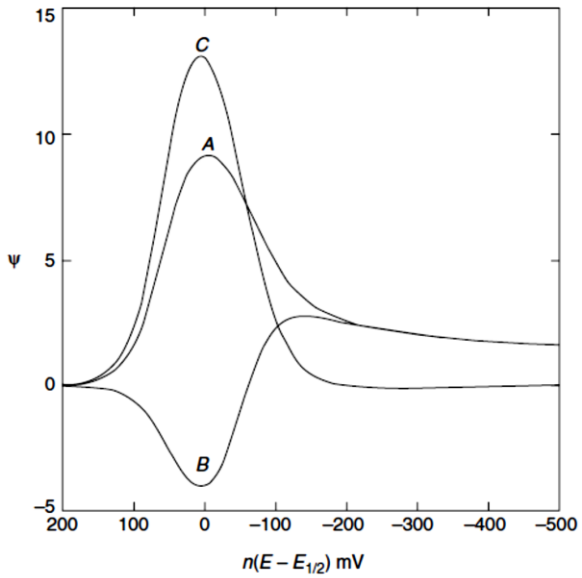


Figure 2.7 Square-wave voltammograms for reversible electron transfer: (curve A) forward current; (curve B) reverse current; (curve C) net current.

2.2.1.3 Stripping Voltammetry

Stripping analysis is an extremely sensitive electroanalytical technique which is widely used for trace metals or some organic substance detection. The high sensitivity occurs by effective preconcentration step which substances are preconcentrated onto the electrode surface before measuring by voltammetric technique. Therefore, its detection limits are 2-3 orders in magnitude lower than the conventional measurement.

There are two major steps for analytical detection. First, deposition step is related to the electrolytic deposition of a small part of substances from solution onto electrode surface for preconcentration of analytes. Anodic stripping voltammetry or ASV is one type of stripping analysis which is the most widely used form. Herein, the substances are preconcentrated by cathodic deposition at a controlled deposition potential and time. The deposited potential should be more negative about 0.3-0.5 V than standard potential for the easily reduced of analytes. In this step, mass-transfer of substance is diffusion and convection, so before starting the next step, there is a quiet period or equilibration

time to decrease the convection force and make sure the quiescent condition of the solutions.

For the second step, which is the stripping method or measurement step that involves the dissolution or stripping of the deposited substraces. Various kinds of techniques are used for stripping step, and the most general used techniques are differential pulse and square-wave voltammetry due to the fact of charging current discrimination. The procedure for anodic stripping method after preconcentration with suitable negative potential is started with scanning at the positive-going potential. Then, at the standard potential, each of substance is converted into oxidized form and stripped out from the electrode surface. The peak positions are ordered in sequence of each substance's standard potential. These identified peaks are directly proportional to concentration of substance. Thereby, several substances can be measured simultaneously by this method at low concentration level as shown in figure 2.8.

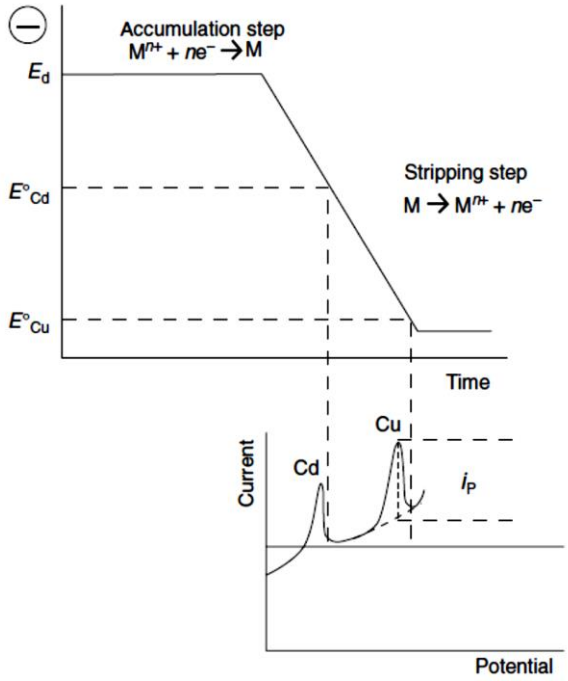


Figure 2.8 Anodic stripping voltammetry: the potential-time waveform (top), along with the resulting voltammogram (bottom).

2.2.2 Electrodes

The main instrument of electrochemical cell for controlled-potential experiment is the three electrode system including working, reference, and counter electrodes which have difference roles in current measurement. Generally, electrode provides conducting interface which allows current to flow through an electrolyte. At particular standard potential, the current of electron will be generated by oxidation or reduction process of analytes.

2.2.2.1 Screen-printed electrodes

Nowadays, there is a novel type of electrode which is easily fabricated by using screen-printed method. The different inks are chosen as conducting material e.g. carbon, graphene, graphene oxide etc. for screening on substrates such as filter paper or plastic (PVC). The selectivity and sensitivity for the analytical detection depend on the different types of ink used for screening. Based on the fabrication process, they are called namely as screen-printed electrode. The major advantages of this electrode are portability, small instrumental setups, simple to use and inexpensiveness. Hence, the application of this electrode is widely used not only for chemical detection but also for biological detection due to its versatile characteristics.

2.2.2.2 Working electrode

Working electrode is an electrode which the reaction of analytes occurs. The reaction can refer to oxidation or reduction process. Normally, this electrode should has high geometric stability such as platinum, gold, glassy carbon or graphene etc. It should provide high sensitivity and reproducible response. Therefore, the criteria for selection of materials used as working electrode are concerned to the redox behavior of the target analyte and their background current in the potential region. Furthermore, the other essential factors which should be consecutively considered are potential window, electrical conductivity, surface reproducibility, cost, availability and toxicity.

2.2.2.3 Reference electrode

Reference electrode is an electrode of known potential which is stable and reproducible. The passage of current, concentration or composition of analyte does not affect to the potential of this electrode due to its high impedance. This potential is used as a reference value for comparing with working electrode to know the exact electrical potential of analyte reactions. The constant potential of reference electrode can change owing to its type and different composition. The silver-silver chloride reference electrode (Ag/AgCl) is one type which is commonly used for three-electrode system because of its stability, low toxicity, simplicity and reasonable price comparing to other reference electrodes. So, Ag/AgCl was used as reference electrode in this work.

2.2.2.4 Counter electrode

From the two-electrode system, it has much of solution resistance from bulk solution to the electrode surface, especially for the non-aqueous system. Therefore, three-electrode system was developed instead of the two-electrode system. The adding electrode which called counter electrode or auxiliary electrode is used for completion of current path. In this arrangement, the reaction of substance occurs between working and counter electrode, one is oxidation and the other one is reduction process. The current is passed between these two electrodes for determination. The suitable property of this electrode is no affected to the behavior of the analyte and no production of any substance by electrolysis which can interfere the working electrode. Figure 2.9 shows the three-electrode system used in this work.

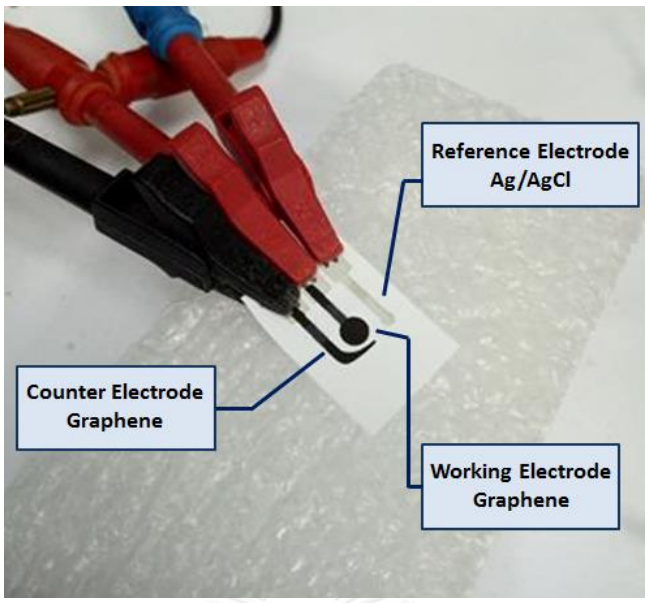


Figure 2.9 Electrochemical cell consisted of working electrode: graphene, reference electrode: silver/silver chloride, counter electrode: graphene screen-printed electrode.

2.2.2.5 Graphene

The important of allotropes of carbon have been observed for long time, but the discovery of that nanostructure of carbon was indeed breakthrough in the past decades. The first interesting material is fullerenes[38]. It was used as small cages for trapping specific atoms or molecules. Nevertheless, it is rarely used in research work because of its low chemical activity. Thus, the application is very limited. From this limitation, researchers tend toward to the use of carbon nanotube [39, 40]. It is chemically reactive substance because of alterring sp^2 carbon on the curvature of tube. It has been successfully employed for application in various works due to the versatility of design on curvature layer that directly controls its properties. However, the structure of carbon nanotube has some resemblance to the graphite because van der Waals forces cannot keep the sheet long together leading to the interesting of using graphite instead. Graphite has a structure of flat sheets of graphene which are horizontally attached to each other. It was also used in many applications but actually the chemical reaction occurred with ions or molecules is happened only the surface of graphene [41, 42]. From this

reason, many researches have been focused on the synthesis of them, and commercial product has been available.

Graphene is one type of well-known carbon structure which has a single sheet of carbon atoms arranged in the shape of six-membered ring with covalent bond, displaying a two dimensional honeycomb crystal lattice as shown in figure 2.10 [43]. It has been widely studied and applied for many purposes because of outstanding surface area, extraordinary elasticity and high electric conductivity. From this key, it is the reason why graphene has much attention since it was discovered several years ago [44-46]. Furthermore, due to its extensive efficiency on electrical, mechanical and thermal characteristics, graphene is presently used in many different applications such as sensors [47], mechanical reinforcement [48], energy storage [49, 50], and energy capture [51] etc.

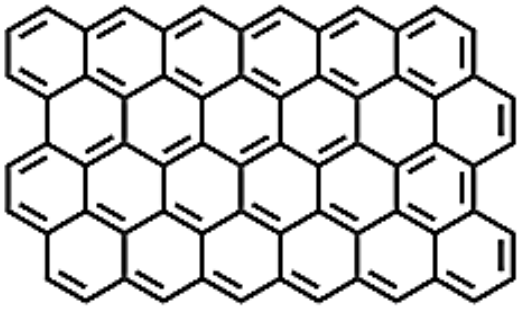


Figure 2.10 Structure of graphene.

2.3 Literature survey

The determination of vitamin K has been investigated for several decades. There are many researches about quantitative evaluation of vitamin K in different kind of samples such as foodstuff, biological samples, animal feeds and pharmaceutical products. The popular method for vitamin K determination is high performance liquid chromatography (HPLC) coupled with many types of detectors. The details of each work are described consecutively as below.

In 2003, Wakabayashi et al. [20] reported on the developed method for simultaneous determination of vitamin K1 and K2 with HPLC incorporated with

platinum catalyst reduction column and electrochemical detector in oxidation mode. Vitamin K1 and K2 were firstly separated in the reverse phase column, respectively in the condition of mixing solvent of ethanol and methanol (1:1) containing 0.025 M of sodium perchlorate as the mobile phase. The eluted vitamin K was continuously flow to postcolumn consisting of platinum agent for reducing the vitamin K, and then the electrochemical method was used to detect the oxidation process using glassy carbon as working electrode versus silver/silver chloride reference electrode. From the results, vitamin K could be separated within 80 minutes and the detection limits were in the range of 2 to 10 pg mL^{-1}. This method was also applied to determine trace level of vitamin K in human serum for investigation of physiological roles of vitamin K in bone metabolism.

In 2007, Otles et al. [16] reported the determination of vitamin K1 using HPLC coupled with reverse phase mode and UV-visible detection. The reverse phase condition consisted of acetonitrile/dichloromethane/methanol (60:20:20, v/v/v) as eluent and C18 column as stationary phase. The detection was performed using UV-vis detector at 248 nm. This proposed method was applied to three kinds of sample including olive oil, chard and human plasma. The observed content in the range of 12.7 to 18.9 µg/100 g , 65.5 to 77.5 µg/100 g , 0.22 and 0.56 ng mL^{-1} were founded, respectively.

In 2007, Ahmed et al. [52] reported the determination of vitamin K1 (Phylloquinone, PK) and K2 (Menaquinone-4, MK-4 and Menaquinone-7, MK-7) using high performance liquid chromatography with chemiluminescence detection. The vitamin K homologues were separated isocratically using ODS column within 35 min, and a mixture of imidazole–HNO_3 buffer at pH 9.0 and acetonitrile (5:95, v/v) was used as a mobile phase. After separation process, the vitamin K, which has weak fluorescence property was irradiated with on-line UV excitation source that can produce hydrogen peroxide and fluorescent product named 3,6-dihydroxyphthalic acid at the same time. The fluorescence products were determined by peroxyoxalate chemiluminescence detection. The detection limits were 3.2, 3.8 and 8.5 nM for PK, MK-4 and MK-7, respectively. Furthermore, the proposed method was successfully

applied for human serum, and the sensitivity and selectivity were sufficient for clinical and nutritional applications.

In 2010, Ducros et al. [23] proposed method for the determination of vitamin K1 in human plasma to diagnose vitamin K deficiency in patients suffering from lipid malabsorption. The method is based on HPLC coupled with tandem mass spectrometric detector. Before determination, the single-step extraction with cyclohexane was performed. Then, it was separated on a C18 column with an isocratic mobile phase. After the separation, vitamin K1 was detected by the atmospheric pressure chemical ionization (APCI) method. In this research, they also showed the results that APCI provided high sensitivity than the electrospray ionization method. The linearity between signal and concentration is in the range of 0 to 2320 ng L^{-1}, LOD and LOQ were estimated at 14 ng L^{-1} and 36 ng L^{-1}, respectively. Due to high sensitivity and selectivity of this method, it had sufficient performance to apply for real human serum of healthy volunteers and patients which were suffered from vitamin K deficiency.

In 2015, Ahmed et al. [18] reported on the use of HPLC coupled with fluorescence detection for determination of vitamin K homologues as vitamin K1 (Phylloquinone, PK) and K2 (Menaquinone-4, MK-4 and Menaquinone-7, MK-7) in human plasma to indicate the treatment of rheumatoid arthritis, osteoporosis, hepatocellular carcinoma and leukemia patients. Due to the high interfering in real samples, the efficient extraction including salting-out assisted liquid/liquid extraction (SALLE) method displayed the important role in this work. The HPLC method employed a novel fluorescence derivatization reaction using stannous chloride dissolving in acidic solution to generate high fluorescent naphthohydroquinone derivatives was proposed. Under the optimized conditions, the linear range was observed between 0.3 and 100 ng mL^{-1}, and the detection limits were found to be 0.1 to 0.17 ng mL^{-1}.

Although these above methods provided high sensitivity and selectivity, it still displayed some drawbacks such as time consuming, sophisticated instrument and large sample volume. To overcome these drawbacks, electrochemistry is one alternative technique which can provide short analysis time, simple to use, small

sample volume and efficient performance for detection. From literature survey, there are some research groups reported on the determination of vitamin K in different samples as shown below.

In 1981, Hart et al. [26] reported on the method for determination of vitamin K1 at submicrogram level by differential pulse voltammetry or DPV. The three electrode system consisted of hanging mercury drop electrode as working electrode, saturated calomel electrode as reference electrode and platinum wire as counter electrode. The highest sensitivity was obtained using acetate buffer and 60% ethanolic solution. The results showed that the electrochemical behavior of vitamin K using cyclic voltammetry technique was a reversible two-electron transfer system, and the cathodic current was higher than anodic current due to the adsorption at mercury electrode. The quantification at low concentration still had some problem of this detection method. Therefore, the measurement of VK is needed to scan repeatedly to get the higher current signal. At optimized condition, the linear range was found at 10 to 100 ng mL^{-1}.

In 1988, Vire et al. [53] described the method for determination of vitamin K3 (Menadione) which is toxic and unavailable now, using square-wave anodic stripping voltammetric technique or SWASV. The three electrode system consisted of static mercury dropping electrode as working electrode, silver-silver chloride as reference electrode and platinum wire as counter electrode. This work investigated the adsorption stripping procedure by comparing between anodic stripping and cathodic stripping one. The results were found that the anodic stripping method provided higher sensitivity than the cathodic stripping because the adsorption of reducing form was stronger. So, the step of proposed method was started with preconcentration at more negative potential for seconds and then potential was scanned towards less negative directions. After that, the effect of supporting electrolyte was studied. The high current signal occurred in acidic condition because the reducing form at preconcentration step could be supported at high proton concentration, and the highest current was observed in perchloric acid medium. For the analytical performance, the calibration curve was in the range of 2×10^{-10} to 5×10^{-7} M and the detection limit was found to be 1.3×10^{-10} M.

In 2008, Somer et al. [54] reported the method for determination of vitamin K3 in pharmaceutical product using differential pulse polarographic technique by working hanging mercury drop electrode, reference saturated calomel electrode and counter platinum wire. This work proposed two methods for direct and indirect determinations. For indirect method, they used Ti(III) as reducing agent and determined vitamin K by the evaluation of increasing Ti(IV). Both of these two methods ran in oxidation mode in acetic buffer solution. The LOD and LOQ were observed to be 7×10^{-7}M and 1.5×10^{-6} M, respectively.

These mentioned methods provided high sensitivity and short analysis time; however the researchers still used mercury as the working electrode which displayed high toxicity, difficult preparation and operation. To overcome these limitations, there was a publication reported on the use of novel electrode for vitamin K detection.

In 2016, Zhang et al. [55] reported on the use of linear sweep voltammetry or LSV for determination of vitamin K3 in animal bloods and feedstuffs. They proposed novel working electrodes which were the glassy carbon electrode modified with poly(3,4-ethylenedioxythiophene):poly(styrene sulfonate) (PEDOT:PSS), carboxy methyl cellulose (CMC) and reduced graphene oxide on palladium (rGO@Pd) in order to provide high sensitivity. The oxidation process was selected to use in this method. The good relationship between signal and concentrations was found in the range of 4×10^{-7} M to 9×10^{-5} M and LOD was equal to 1.4×10^{-8} M.

Although the latest developed method improved the sensitivity using new modified working electrode, there was still some disadvantages in term of complication of modifier synthesis, difficult operation and the use of large sample volume. From above literature surveys, there is no report for the use of electrochemistry as the technique to determine total vitamin K in dietary supplement for quality control in pharmaceutical industry. Therefore, the high sensitivity, fast analysis time, ease of use, and small system method has been challenged to develop and set up for this work to determine total vitamin K in real samples.

Chapter III

EXPERIMENT

This chapter provides the information of chemicals used for screen – printing method to obtain the electrode and all preparation of standard solutions. The operation instruments used in this work, the electrochemical measurement and sample preparation procedure are also described.

3.1 Instruments and apparatus

The instrument including the detection device which used in all experiments are listed in Table 3.1.

Table 3.1 List of instruments and apparatus.

Instruments and apparatus	Suppliers
PGSTAT 30 instrument	Eco Chemie, The Netherlands
Screen-printing blocks	Chaiyaboon, Thailand
Analytical balance, Mettler Toledo	Mettler, Switzerland
Hot air oven	Memmert, USA
Microcentrifuge tubes	Axygen scientific, USA
Centrifuge tubes	Plusmed, Thailand
syringe	Nipro, Thailand
PTFE syringe filter (diameter 13 mm, pore size 0.45 μm)	Chromplus, Thailand
Volumetric flask and other glassware	SCHOTT, Germany
Autopipette	Eppendorf, Germany

Instruments and apparatus	Suppliers
Universal pipette tips	Plusmed, Thailand
Milli-Q water system (18 MΩ cm^{-1})	Millipore, Bedford, USA
Vortex mixer	Scientific industries, United states
Centrifuge (Universal 320R)	Hettich, Germany
pH meter	Metrohm, Switzerland

3.2 Chemicals and reagents

All chemicals and reagents used in this work were analytical grade and were used without further purification. All solutions were prepared in ultra-purified milli-Q water refined by a cartridge purification system. The chemicals and reagents are listed in Table 3.2.

Table 3.2 List of chemicals and reagents.

Chemicals	Suppliers
Vitamin K1, Phylloquinone	Sigma Aldrich, USA
Vitamin K2, Menaquinone	Sigma Aldrich, USA
Ethanol (Absolute for analysis)	Merck, Germany
Sodium dihydrogen orthophosphate (NaH_2PO_4)	BDH Analar, UK
Di-sodium hydrogen phosphate (Na_2HPO_4)	Merck, Germany
Phosphoric acid (H_3PO_4)	Merck, Germany
Sodium hydroxide (NaOH)	Merck, Germany
Calcium chloride fused grain ($CaCl_2$)	M&B, England

Chemicals	Suppliers
Magnesium Chloride ($MgCl_2$)	Merck, Germany
L-ascorbic acid	BDH Analar, UK
Vitamin D3, Colecalciferol	Supelco, USA
Vitamin E, DL-alpha tocopherol acetate	Supelco, USA
Vitamin A, Retinyl acetate	Sigma Aldrich, USA
Vitamin B2, Riboflavin	DMSC reference standard, Thailand
Potassium ferricyanide(III) ($K_3[Fe(CN)_6]$)	Sigma Aldrich, USA
Potassium nitrate (KNO_3)	Merck, Germany
Silver/silver chloride ink (Ag/AgCl)	Gwent group, UK
Carbon ink	Gwent group, UK
Carbon graphene ink	Gwent group, UK
Acetone	Merck, Germany

3.3 Chemicals and reagents preparation

3.3.1 Preparation of vitamin K standard stock and working solutions

The 2 mM stock solutions of both vitamin K1 and K2 were separately prepared in ethanol. The liquid form of 22.9 µL of vitamin K1 (MW = 450.70 g mol^{-1}, density = 0.984 g cm^{-3}) was pipetted into ethanol and then this solution was adjusted to 25 mL in volumetric flask. For the vitamin K2 in solid state, 0.0222 g of vitamin K2 (MW = 444.65 g mol^{-1}) was dissolved in ethanol and adjusted volume in the same way as vitamin K1. Both of this stock solution was stored in dry and cool place.

The working solution of vitamin K is prepared by dilution of the stock solution with solution of 60:40 of ethanol:PBS buffer at pH 3.0.

3.3.2 Preparation of 0.1 M phosphate buffer at different pH as supporting electrolyte.

The method is based on cell biology protocols from Science Gateway. The buffer solution was prepared by using two stock solutions of 0.2 M of monobasic sodium phosphate and dibasic sodium phosphate. First, 0.2 M of monobasic sodium phosphate was prepared by weighting 2.3998 g of $NaH_2PO_4 \cdot 1H_2O$ (MW = 137.99 g mol^{-1}), then dissolving in Milli-Q water and adjusting volume to 100 mL. Next, it is the preparation of 0.2 M dibasic sodium phosphate, 2.8392 g of Na_2HPO_4 anhydrous (MW = 141.96 g mol^{-1}) was weighted, dissolved in Milli-Q water and adjusted volume to 100 mL. Then, two solutions were mixed together by using 39 mL of monobasic phosphate solution and 61 mL of dibasic phosphate solution. After that, the mixture was adjusted volume to 200 mL for observing pH 7.0. Finally, the pH of this solution was adjusted to the desired value in the range of 2 to 9 using 0.1 M phosphoric acid or 0.1 M sodium hydroxide.

3.3.3 Preparation of 0.1 M H_3PO_4

The 50 mL of 0.1 M of phosphoric acid was prepared for pH adjustment of phosphate buffer. A H_3PO_4 solution was prepared by pipetting 3.38 mL of 85% v/v H_3PO_4 and diluting with Milli-Q water to final volume of 50 mL.

3.3.4 Preparation of vitamin A, B2, C, D3, E, Ca, and Mg solution for interference study

For interference effect study, 2000 μg mL^{-1} of vitamin A, B2, C, D3, E, Ca, and Mg stock solution in Milli-Q water were prepared. These solutions were diluted with solution of 60:40 of ethanol:PBS buffer at pH 3.0 in 1 mL microcentrifuge tube. This solution also contained total vitamin K at 5 μg mL^{-1}. The concentration of

interferences in the range of 5 to 50 μg mL^{-1} were observed which has 1 to 10 times compared to 5 μg mL^{-1} of total vitamin K.

For the sample preparation, the effect of NaOH concentration which was used as precipitating agent was also studied. The vitamin K solution containing Ca and Mg which was prepared via sample preparation by precipitation with difference concentrations of NaOH were determined current signal and evaluated the recovery percentage. The 6,250 μg mL^{-1} of Ca and Mg stock solutions were prepared in Milli-Q water and then diluted to 2,500 μg mL^{-1} in 1 mL microcentrifuge tubes with solution of 60:40 of ethanol:PBS buffer at pH 3.0. This concentration was 500 times higher than 5 μg mL^{-1} of total vitamin K as the exact concentration in real vitamin k sample.

3.3.5 Preparation of sodium hydroxide stock and working solutions

Stock solution of 1 M of sodium hydroxide was prepared in Milli-Q water by weighing 1.0000 g of NaOH (MW = 40.00 g mol^{-1}), then dissolving in Milli-Q water and adjusting volume to 25 mL. The working NaOH solution was prepared by diluting stock solution to various concentrations at 0.1, 0.05, 0.01, 0.005, 0.001 and 0.0005 M in 10 mL of centrifuge tube with ethanol and buffer for using as precipitating agent.

3.3.6 Preparation of 1 mM $K_3[Fe(CN)_6]$ in 0.1 M KNO_3

First, 10mM of $K_3[Fe(CN)_6$ solution was prepared, 0.0329 g of $K_3[Fe(CN)_6]$ (MW = 329.24 g mol^{-1}) was weighed into 10 mL beaker, then dissolved and adjusted to volume of 10 mL with 0.1 M KNO_3. After that, 1 mM of $K_3[Fe(CN)_6]$ was prepared by pipetting 200 μL of stock 10 mM $K_3[Fe(CN)_6$ solution and adjusting volume to 2 mL with 0.1 M KNO_3.

3.3.7 Preparation of 0.1 M KNO_3

The 0.2528 g of KNO_3 (MW = 101.10 g mol^{-1}) was weighed and dissolved in Milli-Q water. Then, the solution was adjusted the volume to 25 mL.

3.4 Fabrication of screen-printed electrode

The manual block screen-printing method was used for fabrication of the electrode in this work. The block screens have two platforms, one is for working and counter electrodes screen-printing and the other one is for reference electrode and conducting pads screen-printing as shown in figure 3.1. These platforms were designed through Adobe Illustrator program. To fabricate screen-printed electrode, the PVC substrates were firstly cut around 11 x 18 cm, then cleaned using ethanol. After that, Ag/AgCl ink was screen-printed on dried PVC substrate as first layer for reference electrode and conducting pads. The screen-printed electrode was baked at 55ºC for 30 min to dry out all solvents from the electrode surface which would interfere during measurement. The next step is to create working and counter electrodes as second layer on the same substrate consecutively using graphene ink for screen-printed graphene electrode and carbon ink for screen-printed carbon electrode. Finally, they were heated again at 55ºC for 30 min. Before measurement, the finished screen-printed electrode was attached with small piece of tape as the barrier to limit the constant contacted surface area of working electrode.

3.5 Electrochemical measurement

The electrochemical measurement was performed using PGSTAT 30 Autolab instrument. The three-electrode system was controlled the electrochemical potential by using GPES version 4.9.007 software package (Eco Chemie, The Netherlands). Screen-printed graphene electrode was cooperated for all experiment as detection device. A 50 μL of standard/sample solution was dropped cover three electrodes surface. There are two major techniques used in this work including cyclic voltammetry (CV) and square-wave anodic stripping voltammetry (SWASV). CV technique was used to study electrochemical behavior and mass transfer process.

For the condition of CV, the potential window is in the range of -1.0 to +0.2 V at scan rate of 100 mV s^{-1}. The SWASV is used for quantitative determination. The experiment is done under potential accumulation at -0.5 v for 240 s for preconcentration and altering analyte into reducing form. After this step, the stripping process is performed with scanning potential from -1.0 to +0.2 V at frequency of 14 Hz, step potential of 20 mV and pulse amplitude of 60 mV.

Figure 3.1 Screen-printing blocks for working and counter electrode (left) and silver-silver chloride reference electrode and conducting pads (right).

3.6 Electrochemical characterization

Due to the use of screen-printed graphene electrode in this work, the comparison of efficiency between screen-printed carbon and graphene electrodes were proved using cyclic voltammetric technique in the potential range of -1.0 to +0.2 V and scan rate of 100 mV s^{-1} to measure 1 mM of total vitamin K standard solution in 60% ethanolic phosphate buffer pH 3.0. The anodic peak currents (μA) of both electrodes were compared. Moreover, the measurements of 1 mM of total vitamin K by cyclic voltammetry at different scan rates from 20 to 100 mV s^{-1} were

determined for studying the mass transport behavior of analyte and the geographical characteristic was also characterized by SEM.

For the study of electrode efficiency, the conductance of these electrodes was investigated using cyclic voltammetricy measurement of 0.1 M KNO_3 in the potential range of -0.2 to +0.2 V at scan rate of 100 mV s^{-1}. From theoretical, the capacitance was calculated using equation as shown below.

$$C_d = \frac{I_{avg}}{v(A_{geometric})}$$

where I_{avg} is the average current from the forward and reverse sweep in amperes, v is the scan rate in volts per second, and $A_{geometric}$ is the geometric electrode area in square centimeters. The unit of observed capacitance value is called Farad or F.

To prove the further efficiency of screen-printed graphene electrode, the electrochemical behavior of 1 mM of $K_3[Fe(CN)_6]$ in 0.1 M KNO_3 was examined using cyclic voltammetry at different scan rate between 20 and 200 mV s^{-1}. The current signals were plotted against different square root of scan rates.

3.7 Optimization of the experimental conditions

3.7.1 Amount of ethanol in supporting electrolyte

Due to the well dissolved of vitamin K in ethanol, it is very necessary to optimize the amount of ethanol in the electrochemical media system to reduce the effect of ethanol toward to the current signal of vitamin K determination. From there, the range of 20 to 80 percentage of ethanol in PBS buffer at pH 7.4 was investigated. All experiments were done using 10 μg mL^{-1} of total vitamin K in supporting electrolyte.

3.7.2 pH of supporting electrolyte

For the preconcentration step, the formation of vitamin K was changed into reducing form. The effect of pH of supporting electrolyte was therefore studied because the concentrations of proton affect to the formation of reducing form. From that reason, the pH ranges of supporting electrolyte between 2 and 8 were studied using 10 μg mL^{-1} of total vitamin K by using the optimized ratio of ethanol and PBS of 60:40.

3.8 Optimization of the electrochemical parameter

The potential used for the accumulation of analyte is very important because it affects to the formation change of analyte from quinone to hydroquinone and also works as the preconcentration process in the same situation. The accumulation step becomes a seriously effect on the sensitivity of the determination which can change analyte state to oxidizing or reducing form by holding more positive or negative potential than its standard potential respectively. For brief identification, anodic peak was observed at -0.3 V by cyclic voltammetric technique. Therefore, the investigation of accumulation potential effect was varied in the range of -0.2 to -0.7 V and potential holded for 60 sec before less negative direction scanning.

In order to increase signal to noise ratio of the determination, the study of accumulation time is necessary as well. Difference of accumulation time is related to the different amount of analyte which can change formation. If it has excess accumulation period time, the formation changing process will be completed with high sensitivity. Thus, the time periods between 60 and 300 sec were studied for specific constant potential holding. All current signals were subtracted with background currents, and the behavior trend of its signal was investigated by plotting between currents and accumulation time.

Furthermore, the effects of frequency, step potential and amplitude were investigated. These parameters can affect to the activation and kinetic process of vitamin K redox process individually. Therefore, the ranges of 8 to 16 Hz for frequency, 5 to 25 mV for step potential, 20 to 100 mV for amplitude were examined

which all of other parameters were controlled constantly. The relationship of vitamin K current signal and different parameters were plotted to observe the best condition. All optimized electrochemical parameter experiments were done by using 10 μg mL^{-1} of total vitamin K in supporting electrolyte of ethanol and 0.1 M PBS buffer at pH 3.0 in ratio of 60:40 %.

3.9 Analytical performance

For the calibration curve, the different concentrations of total vitamin K were determined by using this developed method. Each concentration was examined for three times repeatedly, and then the average current signals (μA) were plotted against the different concentrations of total vitamin K (μg mL^{-1}) as y and x axis, respectively. After that, R^2 or correlation coefficient was calculated. The limit of detection was observed from $3\sigma/S$ which σ is the standard deviation of blank measurement for 7 times and S is the slope of calibration curve. For the limit of quantitation, the less concentration that can quantitatively detect was observed from the experiment.

3.10 Interference study

The experiment was done by measuring 5 μg mL^{-1} of vitamin K standard solution which each solution consisted of different interferences, including Ca^{2+}, Mg^{2+}, vitamin A, B2, C, D, and E in the concentrations of 5 to 50 μg mL^{-1}. In total vitamin K measurement process, the current signal was detected for three times repeatedly and the accepted current should not exceed the range of ±5 percentage.

3.11 Sample preparation and application

Real samples of three dietary supplements were purchased from drugstore in Thailand. First, a tablet was weighed and ground. For extraction, the ground powder was dissolved in solution of ethanol and 0.1 M of PBS at pH 3.0 in the ratio of 60 : 40 percentage which consisted of 0.01 M of NaOH as precipitating agent. The final

volume of 50 mL was centrifuged at 5000 rpm for 30 min. After that, the clear supernatant was filtered through 0.45 μm syringe filter to the 100 mL beaker. The solution was adjusted to pH 3.0 with 0.1 M phosphoric acid and then adjusted to the final volume of 100 mL with solution of 60:40 of ethanol : PBS buffer at pH 3.0. Furthermore, the prepared sample solution was spiked with three concentrations level of vitamin K at 4, 8, 12 μg mL^{-1} in each samples, respectively. The measurements were done repeatedly for three times. Then the recovery and RSD percentages were evaluated and compared to the results obtained from those of high performance liquid chromatography coupled with UV-Vis, which used as standard method. A t-test at the 95% confidence level with degrees of freedom of 2 was achieved.

CHAPTER IV
RESULTS AND DISCUSSION

This chapter describes the results and discussion of screen-printed electrode characterization, comparison of electrode performance, electrochemical behavior of VK, optimization of experimental conditions and SWASV electrochemical parameters. Furthermore, the analytical performances, interference study, sample preparation, and sample analysis using this proposed method are also described.

4.1 Characterization of the prepared electrodes

Due to the outstanding properties of graphene such as high electrical conductivity and extraordinary elasticity, the graphene is an attractive material that is widely used in many applications. This work is one of application using graphene as conductive media on screen-printed electrode for electron transfer measurement. In order to investigate the features of this material, the morphology was characterized using scanning electron microscopy (SEM) and compared to the screen-printed carbon electrode. The obtained images are shown in Fig. 4.1. The screen-printed carbon electrode shows a smooth surface with some of carbon sheets whereas the screen-printed graphene electrode provides more rough and porous surface with lots of graphene sheets. From these features, graphene can provide a remarkable efficiency of electrode in determination.

Moreover, graphene has another interesting property which can provide the high sensitivity of determination. The double layer capacitance (C_{dl}) of electrode is an kind of essential parameter for choosing an electrode material due to its effect on the sensitivity of determination. Therefore, the capacitance of a screen-printed graphene electrode was investigated and compared to a screen-printed carbon electrode with using the same fabrication procedure. For this experiment, the CV technique was used for measurement of 0.1 M KNO_3 in the potential range of -0.2 to +0.2 V at a scan rate of 100 mV s^{-1} on the different kind of electrodes. As shown in Fig .4.2, the cyclic voltammograms from two different screen-printed electrodes

exhibited as smooth background currents. From the results, it can be noticed that the current signal of the graphene electrode provided a lower background current than the carbon. In addition, the capacitance of electrodes was calculated from current at 0 V against screen-printed Ag/AgCl reference electrode using the equation below:

$$C_{dl} = \frac{I_{avg}}{v(A_{geometric})}$$

where $\mathbf{I_{avg}}$ is the average current from the forward and reverse sweep in amperes, v is the scan rate in volts per second and $\mathbf{A_{geometric}}$ is the geometric electrode area in square centimeters. The capacitances of screen-printed graphene and screen-printed carbon electrodes were evaluated at 3.45 ± 0.02 and 5.61 ± 0.05 μF cm^{-2}, respectively (n=3). The capacitance of the graphene electrode is lower than the carbon electrode due to its hydrophobicity of graphene [56]. The H-terminated of graphene has been predicted to localize at the edge [57]. As a result, the other ions in the supporting electrolyte do not accumulated on the electrode surface. In the case of carbon, its surface contains both hydrophobic and hydrophilic sites [58] which can cause a high charging layer on the electrode surface. Due to advantage of having low interfering of the capacitive layer of graphene, the graphene ink was selected as an electrode material for fabrication in order to enhance the current signal.

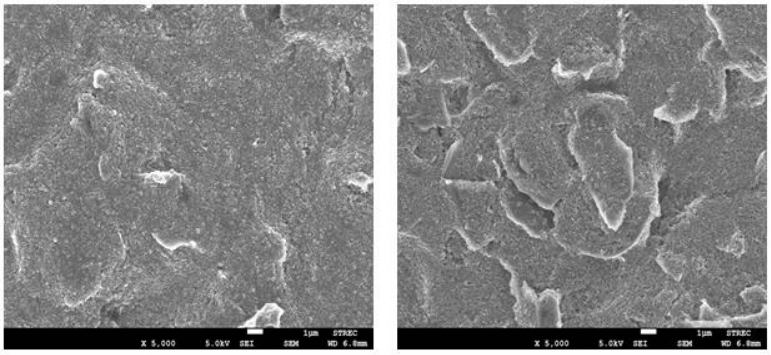


Figure 4.1 SEM images of surface morphology of screen-printed carbon (left) and graphene (right) electrodes.

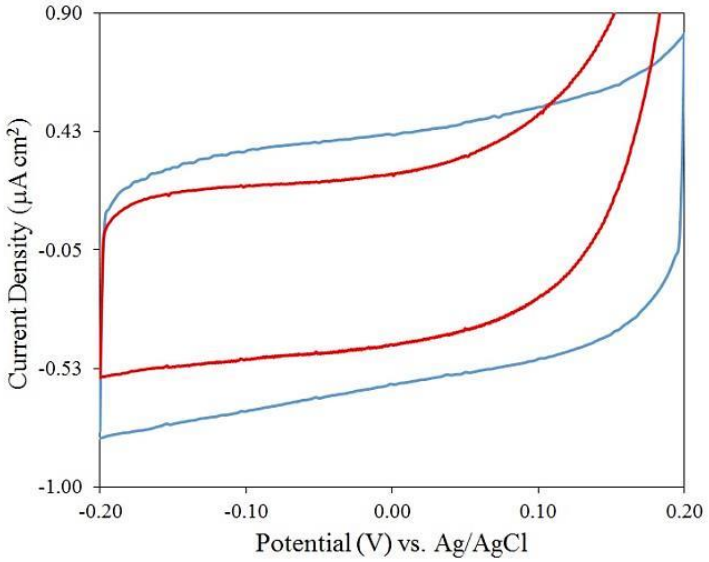


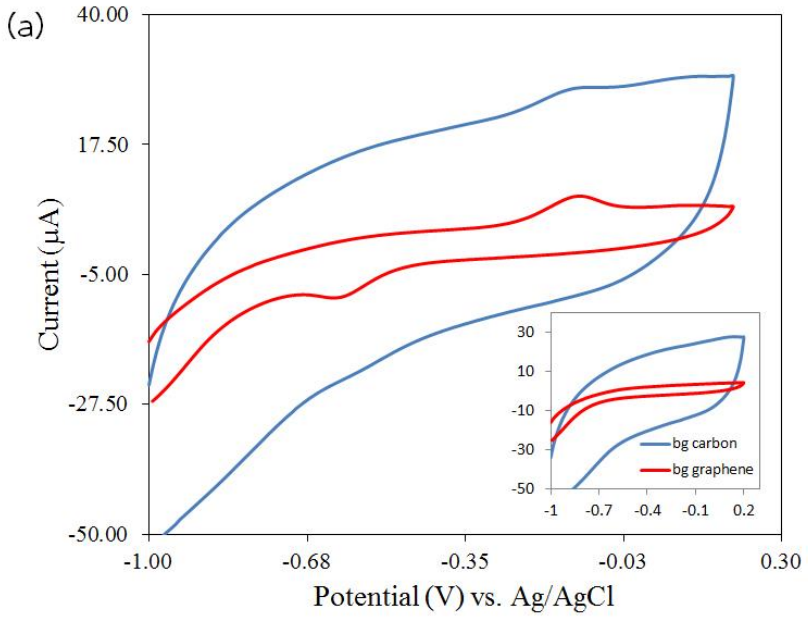
Figure 4.2 Cyclic voltammograms of 0.1 M KNO_3 on carbon (blue) and graphene (red) screen-printed electrode at scan rate of 100 mV s^{-1}.

4.2 Electrochemical behavior of VK at SPGE and influence of scan rate

In order to study the efficiency of the electrode for VK determination, the electrochemical behavior of the total VK in ethanol and phosphate buffer pH 3.0 at ratio of 60:40 was investigated on screen-printed carbon and screen-printed graphene electrodes individually using CV technique. The potential was operated in the range of -1.0 to +0.2 V at a scan rate of 100 mV s^{-1}. Moreover, the measurement of the background current from these electrodes was also performed at the same conditions. As shown in Fig. 4.3a, the cyclic voltammograms in inset displayed the results from a supporting electrolyte solution. From these results, the background current of the screen-printed graphene electrode was found to be lower than the current from the screen-printed carbon electrode due to its low capacitance as previously mentioned. In case of in the presence of VK, cyclic voltammograms obtained from screen-printed carbon and screen-printed graphene electrodes were also compared. It can be observed that the CV of the screen-printed carbon showed a broad and small anodic peak at -0.123 V, whereas, the screen-printed graphene electrode provided the defined peak and obviously high current at -0.130 V. In principle, this phenomenon is due to the less of charging double layer which leading

to the advantage of high sensitivity for total VK determination. Therefore, this material was selected to use in this work. . Furthermore, the anodic peak current was a bit higher than the cathodic one. As a result, all experiments for determination of VK were carried out under the oxidation reaction.

Because of the main goal was to develop method for determination of total VK as defined in K1 and K2. Thus, 1 mM solution of vitamin K1 and K2 were first measured separately using CV to study the electrochemical behavior and to investigate the exact location of redox potentials. The results are shown in Fig. 4.3b. From the results, the anodic peak current of K1 was observed to be around 2.12 ± 0.03 µA at the potential of -0.102 V, and K2 was 2.33 ± 0.02 µA at -0.088 V. The anodic currents and oxidation potential of both forms were not significantly different because of the similar structure with the same functional group. Hence, the developed method can be used for the determination of the total VK in dietary supplement. Furthermore, these results also indicated that VK displayed a reversible property which related to quinone-hydroquinone couple by two electrons and two protons transfer according to a previous study [59]. The mechanism of electrons and protons transfer of VK was shown in Fig. 4.4.



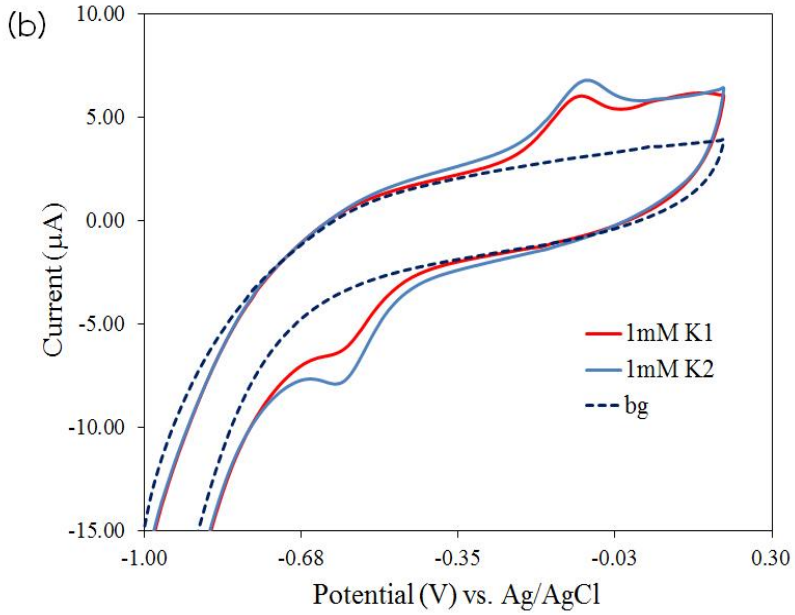
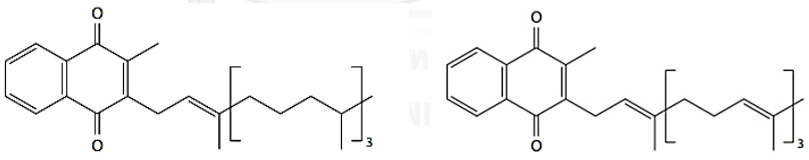


Figure 4.3 Cyclic voltammograms of (a) 1 mM of total VK at carbon (blue) and graphene (red) screen-printed electrode, comparison of background current between carbon and graphene is shown in the inset. (b) 1 mM of K1 (red) and K2 (blue) in 60:40 ratio of ethanol:PBS buffer pH 3.0 at scan rate of 100 mV s^{-1}.



Phylloquinone **Menaquinone-4 (MK-4)**

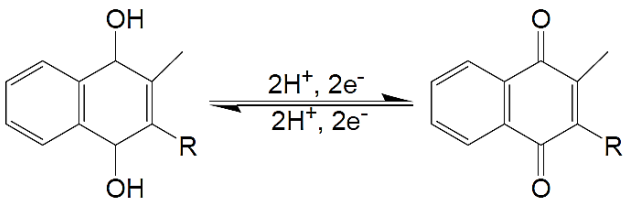


Figure 4.4 The structure of K1 (Phylloquinone) and K2 (Menaquinone-4) with electrochemical oxidation mechanism of VK at screen-printed graphene electrode.

Beside electrode characterization and electrochemical behavior, the mass transport of analyte towards the electrode surface was also investigated. The mass transfer type of VK was proved by the relationship between the scan rate and anodic current signal. In the experiment, the solution of VK was measured at different scan rate in the range of 20 to 100 mV s^{-1} on screen-printed graphene electrode. As shown in Fig. 4.5, the redox currents increased with an increasing of the scan rate. When the currents in μA were plotted against the square root of scan rate as (mV s^{-1})$^{1/2}$, the linear relationship was observed. Therefore, these results can prove that the mass transport of VK on the screen-printed graphene electrode is controlled by diffusion process.[60]

Due to the fact that the electrochemical technique for the determination of VK is one of essential topic for method development, hence, the efficiency of technique for the determination of VK was studied. Nowadays, square-wave voltammetry (SWV) is a popular technique due to its high sensitivity and short analysis time. Furthermore, it is commonly combined with the anodic stripping voltammetry so called square-wave anodic stripping voltammetry (SWASV) for trace determination. As a result, the comparison in efficiency of the two selected techniques including SWV and SWASV was investigated for VK determination. As shown in Fig. 4.6, the small and quiet broad peak at -0.121 V was obtained from the SWV technique, while the high current with a well-defined peak at -0.081 V was obtained from SWASV. When the currents of both techniques were compared together, SWASV obviously was showed to have a current signal 3.6 times higher than SWV due to SWASV contains the accumulation step at -0.5 V for 240 sec which can provide the formation change process from quinone to a hydroquinone before analysis. A reducing form of hydroquinone has stronger and more stable adsorption property than the oxidizing one; therefore, this technique can provides high sensitivity in detection as described in previous work [61]. The greater sensitivity can be observed using more negative potential for accumulation step before doing the measurement in a positive scan. Hence, the SWASV was used as suitable technique for VK determination in this work.

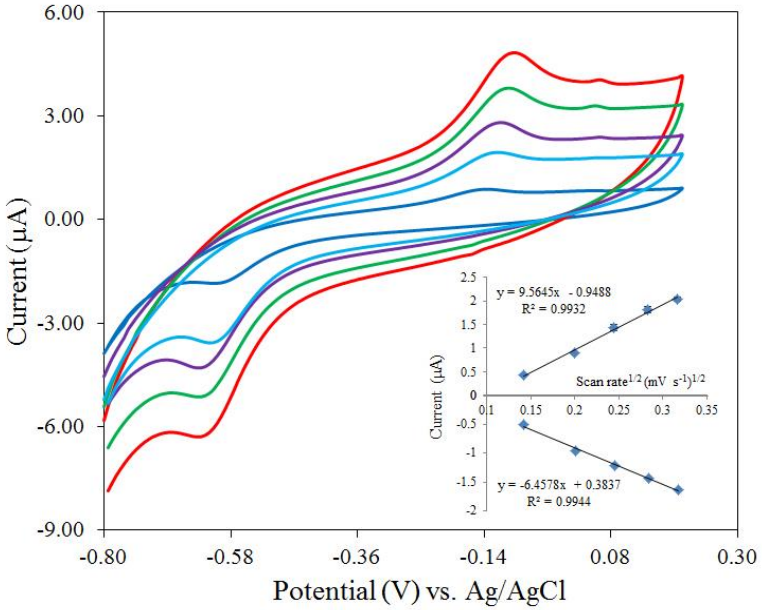


Figure 4.5 Cyclic voltammograms of 1 mM of total VK in 60:40 ratio of ethanol:PBS buffer pH 3.0 at various scan rate from 20 to 100 mV s^{-1}. The relationship between current and square root of scan rate is shown in the inset.

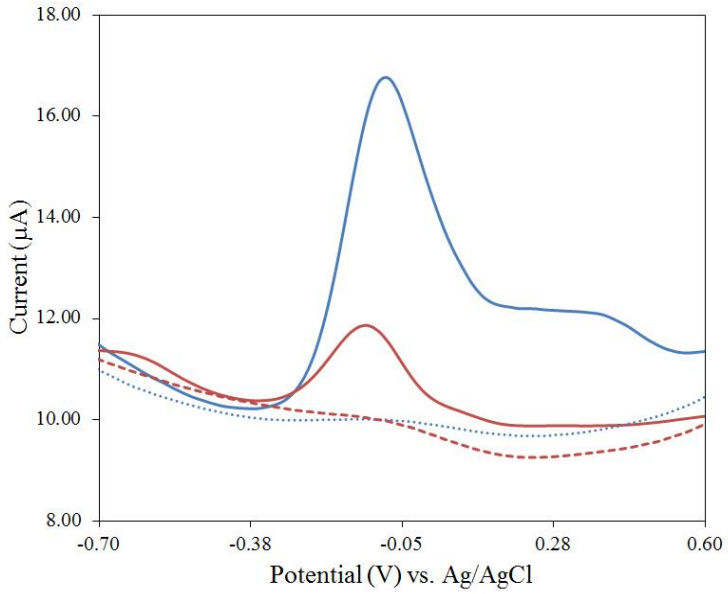


Figure 4.6 Voltammograms of 1 mM of total VK using SWV (red) and SWASV (blue) technique.

4.3 Optimization of experimental conditions

In order to develop the highly sensitive method for determination of VK in dietary supplement, variation of experimental parameters such as percentage of ethanol ratio and pH of supporting electrolyte were investigated. All experiments were carried out by measurement of 10 μg mL^{-1} of VK standard solution using the SWASV method.

4.3.1 Effect of percentage of ethanol in supporting electrolyte

The use of ethanol in supporting electrolyte is to improve the dissolution of VK. In addition, the percentage of ethanol is a great effect on the electron transfer of analyte. Therefore, the variation of ethanol content was performed by varying the percentage of ethanol in the range of 20% to 100% using CV and SWASV technique for VK measurement. The other conditions were fixing at phosphate buffer pH 7.4, accumulation potential of -0.5 V, accumulation time of 60 sec, frequency of 20 Hz step potential of 10 mV, and amplitude of 75 mV. As shown in Fig. 4.7, the anodic peak currents slightly increased with increasing percentage of ethanol from 20% to 40%. Then, the current reached the highest signal at 60% of ethanol and then rapidly decreased at 80% and 100% of ethanol because of the over amount of ethanol. These results indicated that 60% ethanol displayed an appropriate amount for well-dissolved of VK in supporting electrolyte. The cyclic voltammograms were illustrated in Fig. 4.8 and all of these results were also confirmed using SWASV method as shown in Fig. 4.9. The well-defined peak with highest current was obtained at 60% ethanol. Therefore, the condition of 60% ethanolic phosphate buffer was used in the next study.

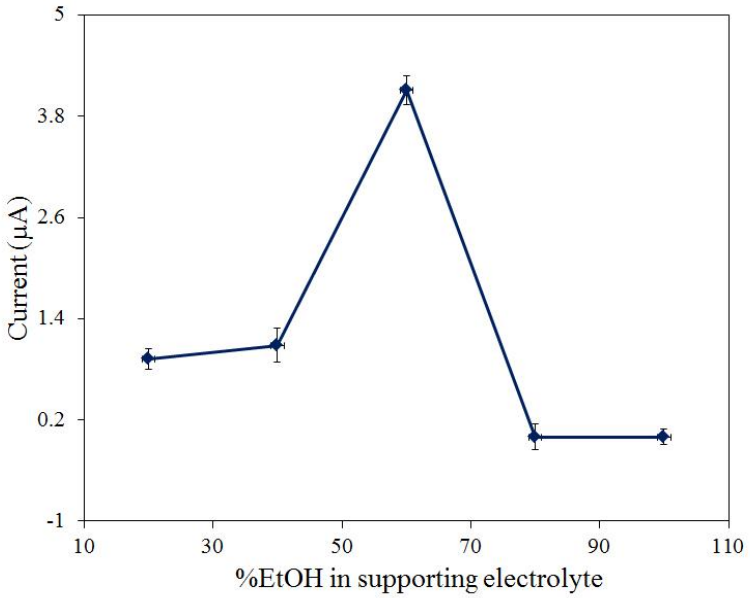


Figure 4.7 Effect of %ethanol on the peak current of 10 µg mL^{-1} of total VK.

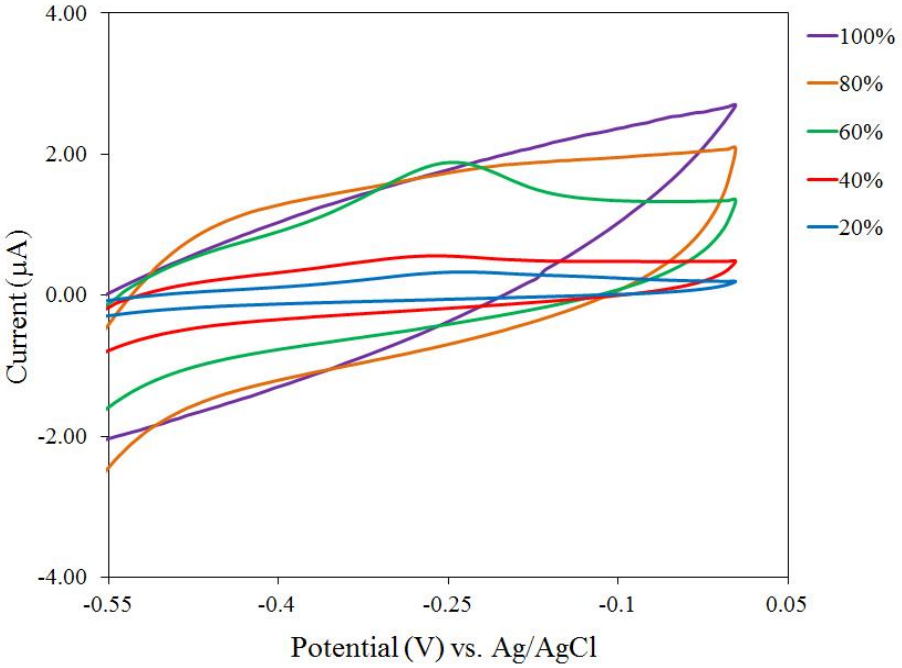


Figure 4.8 CV voltammograms of 10 µg mL^{-1} of total VK at different %ethanol (20-100%).

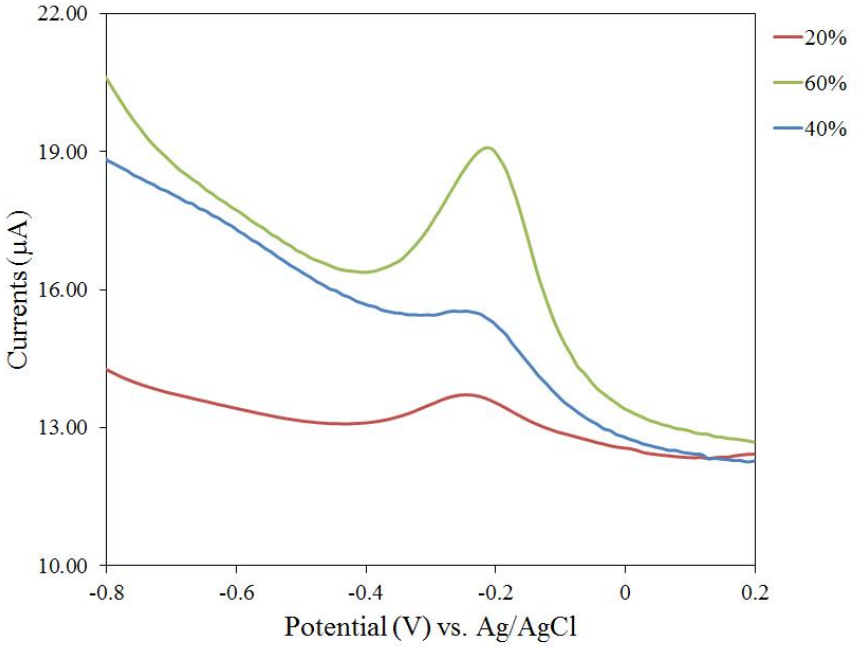


Figure 4.9 SWAS voltammograms of 10 µg mL^{-1} of total VK at different %ethanol (20-40%).

4.3.2 Effect of pH of supporting electrolyte

As the effect of pH to the formation change of analyte from quinone to hydroquinone form, VK can alter into oxidizing or reducing form depending on different pH. The formation of analyte has an influence to the adsorption property onto the electrode surface, kinetic of electron transfer, anodic peak current and sensitivity of determination. Therefore, the solutions of VK in various pHs ranged from pH 2 to pH 8 of phosphate buffer were studied using SWASV technique. The other conditions were fixing at 60% ethanolic phosphate buffer, accumulation potential of -0.5 V, accumulation time of 60 sec, frequency at 20 Hz, step potential at 10 mV and amplitude at 75 mV. The results were illustrated in Fig. 4.10. The trend line shows a rapid higher current when the pH is lower and then reach the highest current at pH 3.0 because in solution containing a high concentration of protons can provide a more reducing form of VK by protonation process. However, the current was decreased at pH 2.0 supposing that the overabundance of protons in buffer solution can accumulate and interfere with the electron transfers of analytes on the

electrode surface. As shown in Fig. 4.11, the square-wave voltammograms indicated that the anodic peak of VK quietly increased with the decreasing of pH, and the well-defined peak was observed at pH 3.0 due to the generation of reducing form. Furthermore, it can be noticed that its oxidation peak potentials shifted to negative values as the solution pH increased. The increasing of pH would promote the deprotonation of VK oxidation process. It is the reason that the oxidation peak potentials were shifted in less positive [62]. Therefore, phosphate buffer pH 3.0 was chosen as an optimal supporting electrolyte for all experiments.

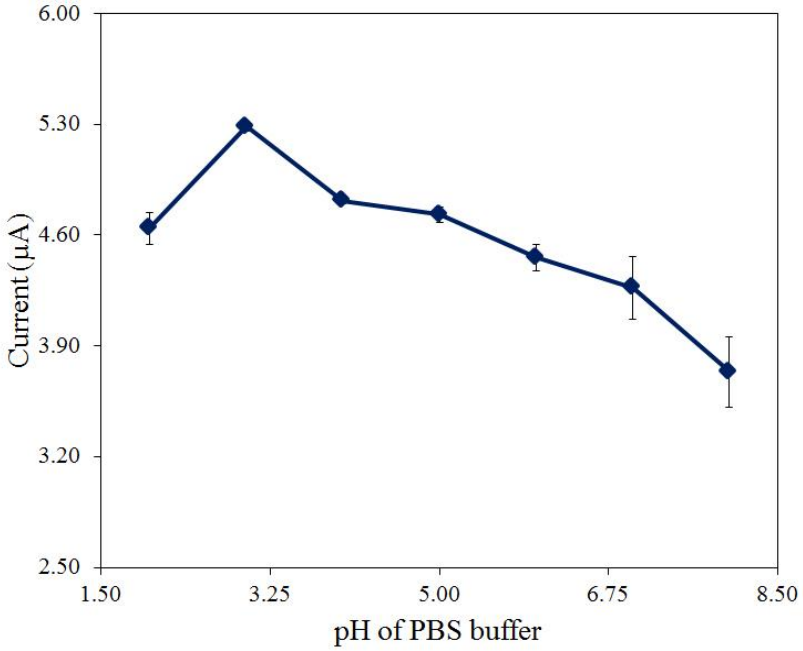


Figure 4.10 Effect of pH on the peak current of 10 µg mL^{-1} of total VK.

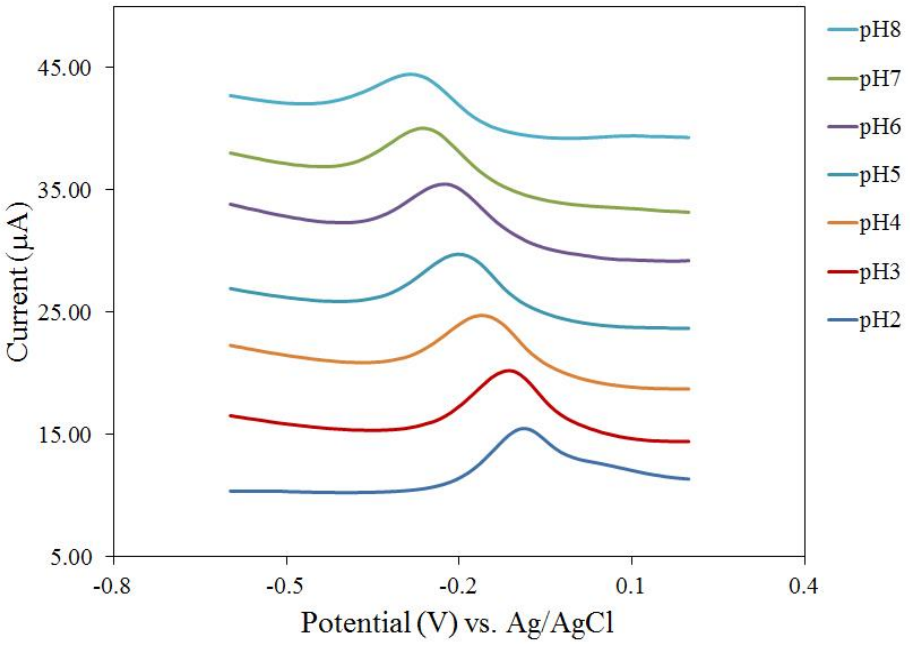


Figure 4.11 SWAS voltammograms of 10 µg mL^{-1} of total VK at different pHs (2-8).

4.4 Optimization of electrochemical parameters

In order to obtain the highly sensitive method for determination of VK in dietary supplement, electrochemical parameters such as accumulation potential, accumulation time, frequency, amplitude and step potential were further studied. All conditions were examined under the previous optimal condition using 60% ethanolic phosphate buffer pH 3.0 as supporting electrolyte.

4.4.1 Effect of accumulation potential

Accumulation potential is one of important parameters which can affect to the formation of a substance. The different accumulation potentials in the range of -0.2 V to -0.7 V were studied using 60% ethanolic phosphate buffer pH 3.0, accumulation time of 60 sec, frequency of 20 Hz, step potential of 10 mV and amplitude of 75 mV. From the result shown in Fig. 4.12, the anodic peak currents dramatically increased when the accumulation potential was raised. This behavior can be explained that the use of higher accumulation potential can provide the

sufficient power for reducing VK to hydroquinone form. This form was strongly adsorbed onto the electrode surface led to the high sensitivity. Up to the potential at -0.5 V, the currents were not significantly different because all of the quinone form was completely converted to hydroquinone. As square-wave voltammograms shown in Fig. 4.13, the anodic peak gradually increased with the increasing of accumulation potential. Between -0.5 V to -0.7 V, there was no significant difference results obtained. Therefore, the accumulation potential at -0.5 V was chosen for the next optimization.

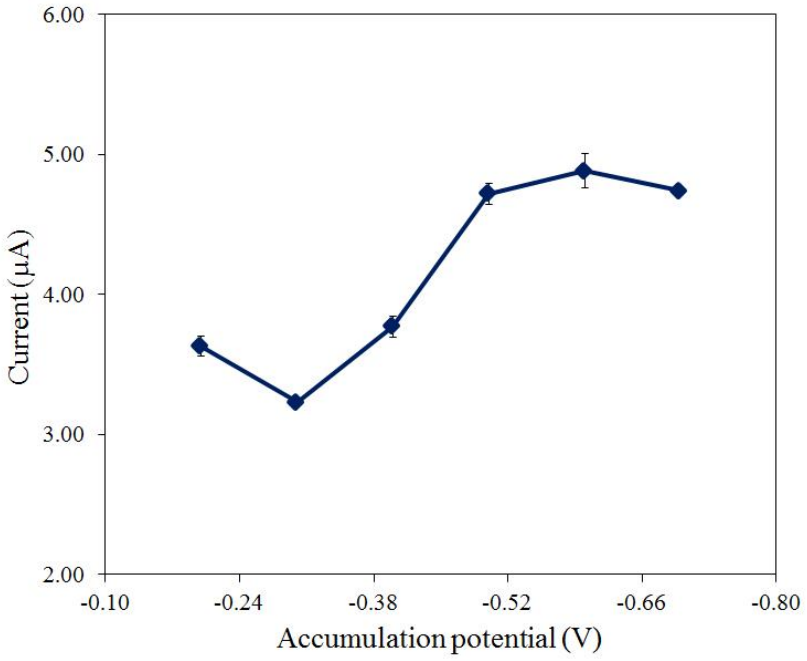


Figure 4.12 Effect of accumulation potential on the peak current of 10 μg mL^{-1} of total VK.

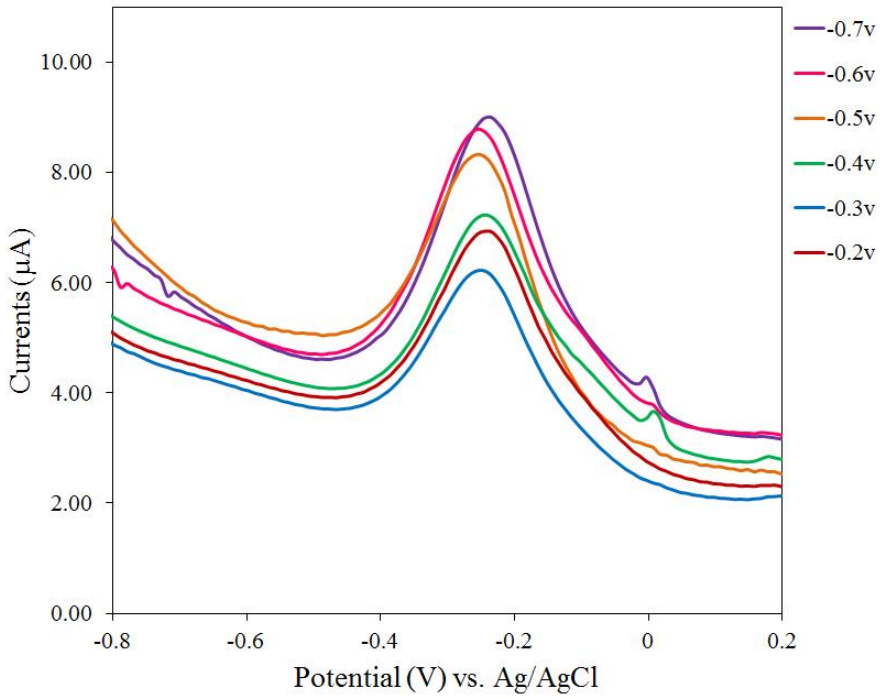


Figure 4.13 SWAS voltammograms of 10 µg mL^{-1} of total VK at accumulation potentials (-0.2 to -0.7 V).

4.4.2 Effect of accumulation time

The accumulation time is an important parameter for the development methodology to receive the high sensitivity due to its effect on the accumulation process of analyte. In this work, the different accumulation times from 60 to 300 sec were investigated by measurement of VK standard solution using SWASV. The other parameters were controlled using 60% ethanolic phosphate buffer pH 3.0, accumulation potential of -0.5 V, frequency of 20 Hz, step potential of 10 mV and amplitude of 75 mV. The obtained peak currents were subtracted with background current and presented as S/B ratio. The current signals (µA) were then plotted against the accumulation time. As the results shown in Fig. 4.14, the peak current gradually increased with the increasing of accumulation time due to there was more sufficient time for adsorption process of all VK substance. In contrast, the current decreased when the time was up to 300 sec, and the background current also increased and there was a small peak at the same VK oxidation potential. From these results, it can be explained that the decreasing of anodic peak current affected

by accumulation of other ions in supporting electrolyte which can interfere the VK determination as shown in Fig. 4.15. From Fig. 4.16, it was clearly seen that the background peak was occurred obviously at 300 sec. Hence, the accumulation time of 240 sec was selected for the next optimization.

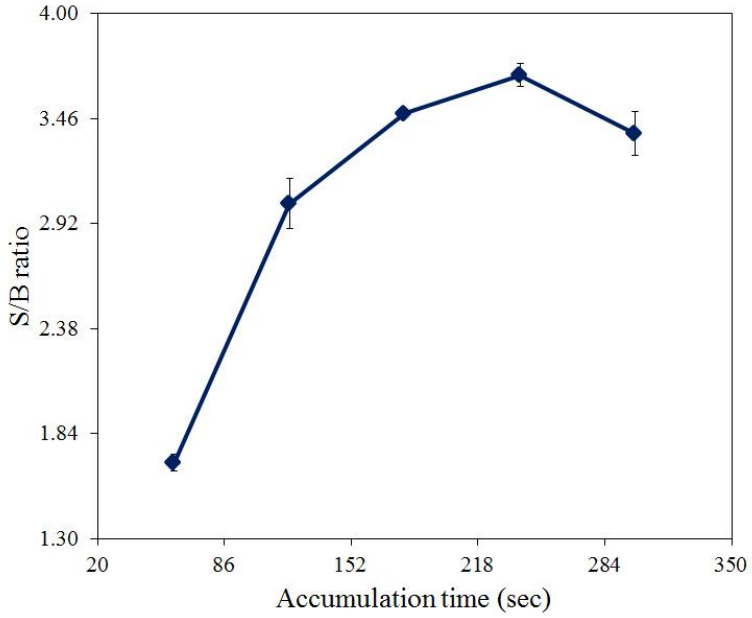


Figure 4.14 Effect of accumulation time on the peak current of 10 µg mL^{-1} of total VK.

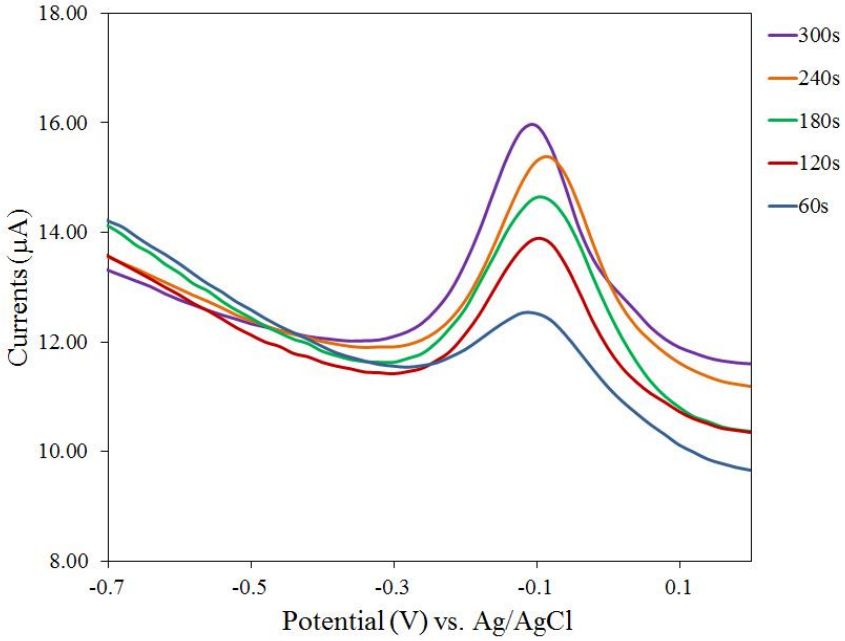


Figure 4.15 SWAS voltammograms of 10 µg mL^{-1} of total VK at different accumulation times (60-300 sec).

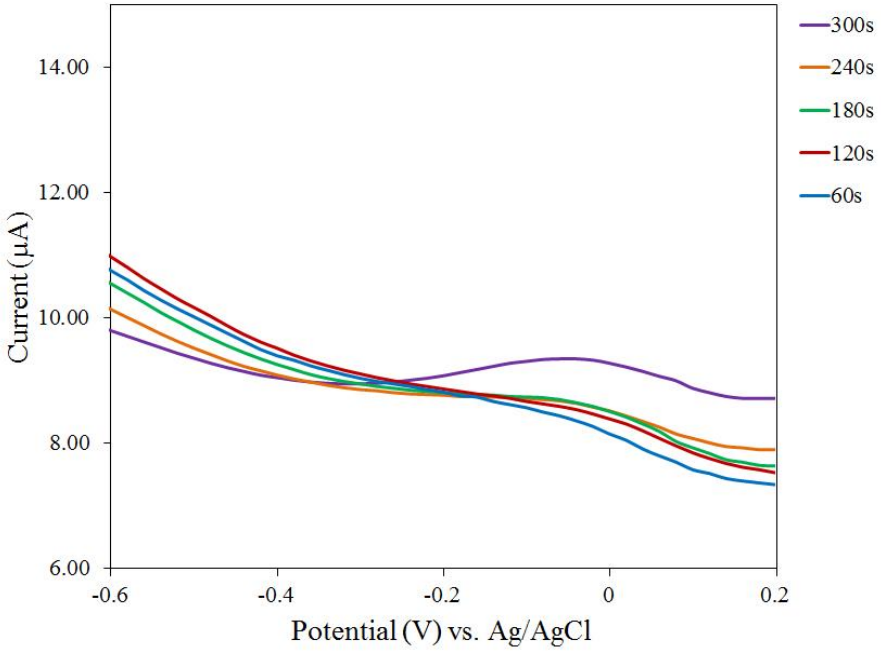


Figure 4.16 SWAS voltammograms of supporting electrolyte at different accumulation times (60-300 sec).

4.4.3 Effect of frequency

Frequency is other parameter that effect on the sensitivity of method. The range of frequency between 8 to 16 Hz was studied using SWASV technique. The previous optimal conditions were fixed at 60% ethanolic phosphate buffer pH 3.0, accumulation potential of -0.5V, accumulation time of 240 sec, step potential of 10 mV and amplitude of 75 mV. From the result illustrated in Fig. 4.17, the current signal increased when the frequency increased because of the fast potential stimulation which can cause rapid mass transfer of analyte toward to the electrode surface. Until the frequency at 14 Hz, the peak currents remained constantly because all of VK substance was completely transferred to electrode surface. The voltammograms were also shown in Fig. 4.18. Therefore, the frequency of 14 Hz was used in this work to receive the high sensitivity in detection.

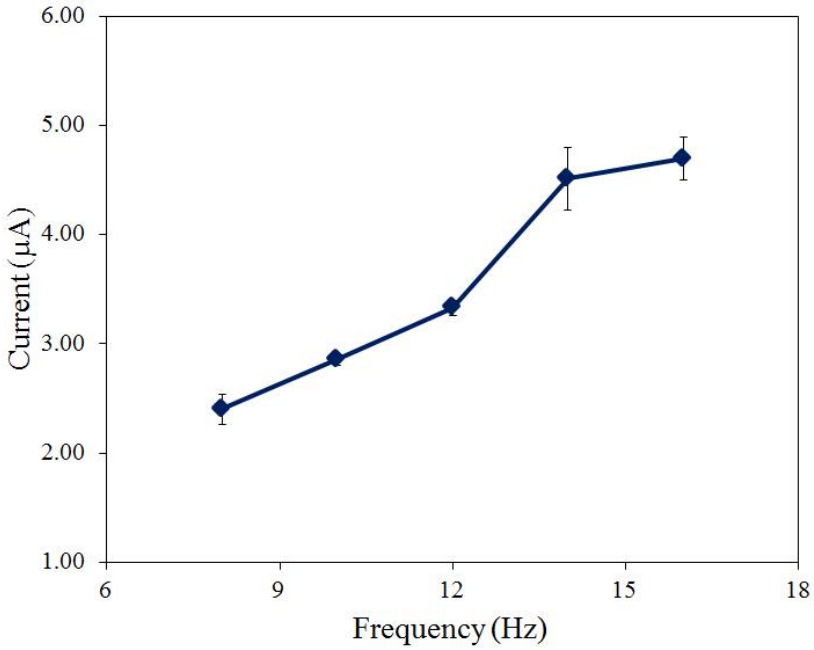


Figure 4.17 Effect of frequency on the peak current of 10 µg mL^{-1} of total VK.

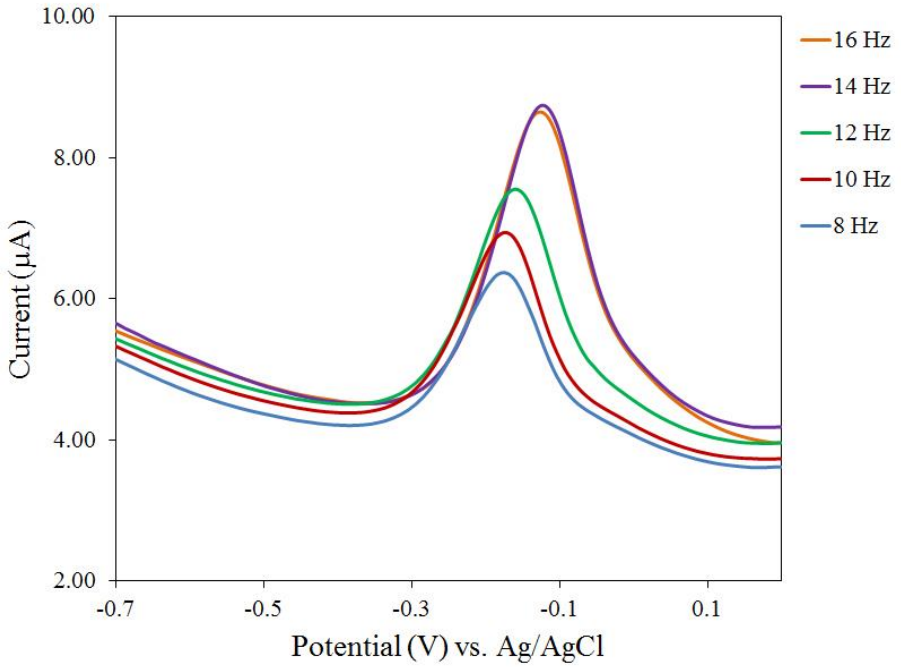


Figure 4.18 SWAS voltammograms of 10 μg mL^{-1} of total VK at different frequencies (8-16 Hz).

4.4.4 Effect of step potential

Next, the influence of step potential was investigated from 5 to 25 mV using 60% ethanolic phosphate buffer pH 3.0, accumulation potential of -0.5 V, accumulation time of 240 sec, frequency of 14 mV, and amplitude of 75 mV. In Fig. 4.19, the current signal increased with the increasing of step potential and reached the maximum value at 20 mV. Then, the current decreased immediately when the step potential was higher than 25 mV. This is due to the increasing of step potential would lower the number of points for current signal measurement leading to the lower precision. The square-wave voltammograms were shown in Fig. 4.20. As a result, the step potential of 20 mV was selected as optimal condition for the determination of VK.

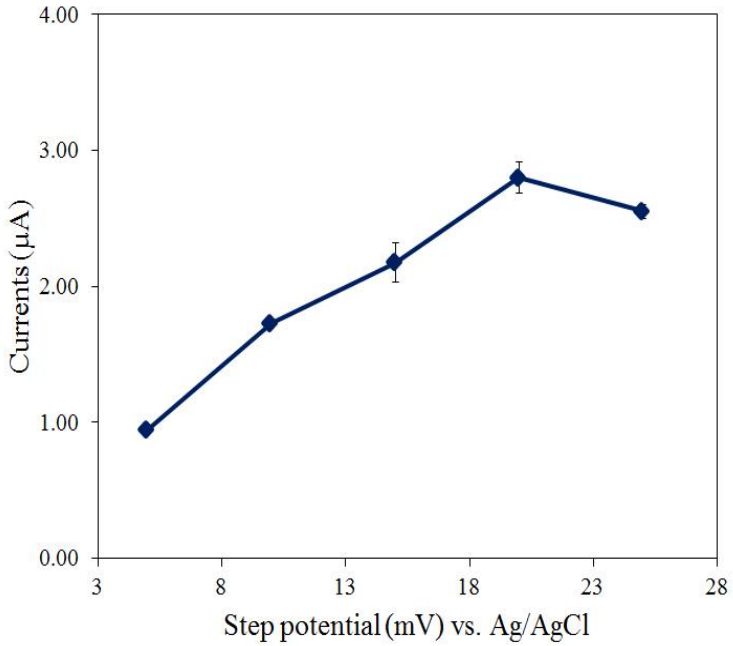


Figure 4.19 Effect of step potential on the peak current of 10 µg mL^{-1} of total VK.

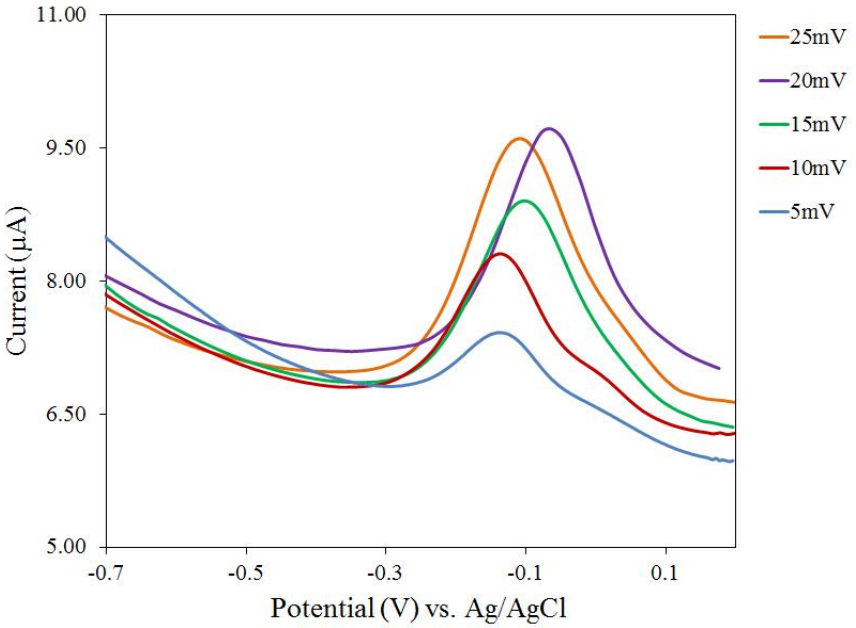


Figure 4.20 SWAS voltammograms of 10 µg mL^{-1} of total VK at different accumulation times (5-25 mV).

4.4.5 Effect of amplitude

Finally, the amplitude of square-wave voltammetry was studied over the range of 20 to 100 mV using 60% ethanolic phosphate buffer pH 3.0, accumulation potential of -0.5 V, accumulation time of 240 sec, frequency of 14 Hz, step potential of 20 mV and amplitude of 75 mV. As demonstrated in Fig. 4.21, the signals of total VK continuously increased from amplitude of 20 to 60 mV and the signals were remained constantly after 60 mV. All voltammograms were also shown in Fig.4.22. This can be explained that the amplitude of 60 mV was appropriate for electron transfer stimulation of all substances at electrode surface. Therefore, the optimal amplitude of 60 mV was used in this work.

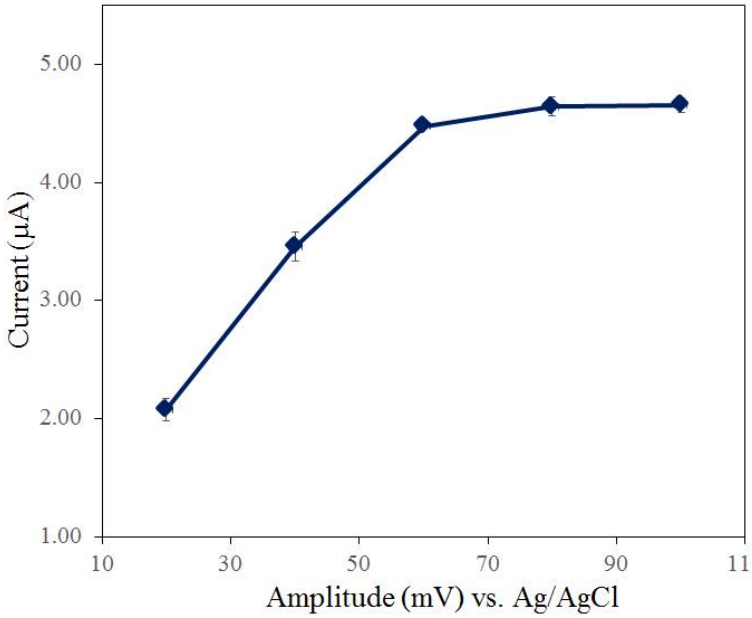


Figure 4.21 Effect of amplitude on the peak current of 10 µg mL^{-1} of total VK.

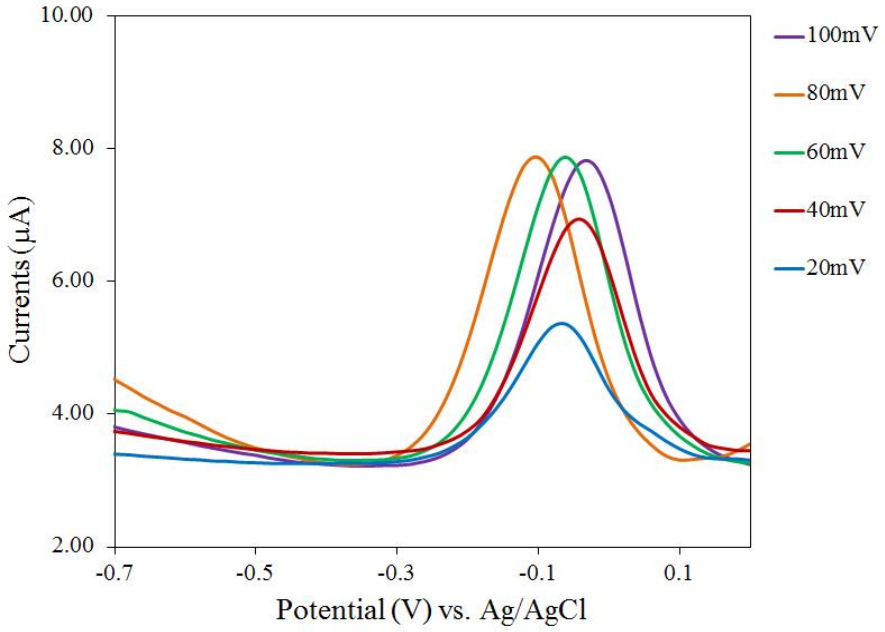
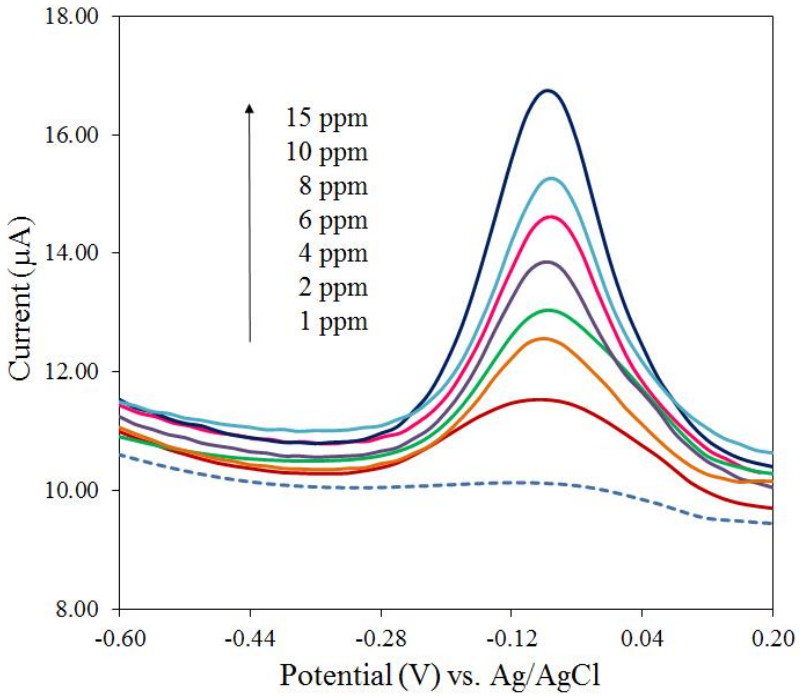


Figure 4.22 SWAS voltammograms of 10 µg mL^{-1} of total VK at different amplitudes (20-100 mV).

4.5 Analytical performance

The analytical performance of this proposed method for the determination of the total VK was evaluated with SWASV using a screen-printed graphene electrode under optimal experimental conditions. The calibration curve was constructed from different VK concentrations. The relationship between anodic peak current and concentration of analyte was then plotted, and the least square were evaluated using the linear regression method. The anodic peak current was increased with the increasing of the total VK concentration as shown in Fig. 4.23. Good linearity for the total VK determination was observed in the concentration range of 1 to 15 µg mL^{-1}. The regression equation was $y = 0.3055x + 1.5438$; $R^2 = 0.9904$. Then, the detection limit (LOD) and quantitation limit (LOQ) of the total VK determination were calculated using 3Sb/m and 10Sb/m equations (three and ten times of the standard deviation of the blank divided by slope of calibration curve), respectively. The LOD and LOQ were then found to be 0.099 µg mL^{-1} and 0.330 µg mL^{-1}, consequently. This was even though, this proposed method provided higher LOD than the previous

method that used differential pulse voltammetry with the hanging mercury drop electrode. However, it exhibits more benefits in terms of having a simple operation, nontoxicity, effective cost, small sample volume requirement and short time analysis. Therefore, this developed method acts an alternative technique for determination of total VK and can apply for dietary supplement detection. Next, the reproducibility of device was investigated by measurement of the VK at three concentrations of 5, 10 and 15 μg mL^{-1}. Each concentration was examined using ten individual electrodes under the same optimal conditions. The %RSDs was obtained at 3.29, 3.18 and 3.16, respectively and was acceptable by AOAC guidelines. (Table 1.)



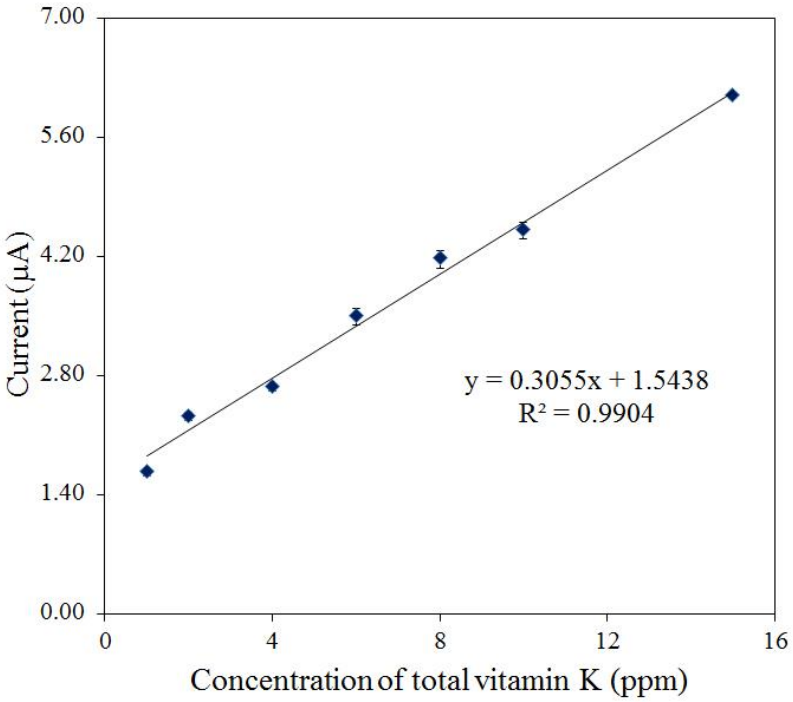


Figure 4.23 SWAS voltammograms of different concentrations of total VK (upper) and the calibration plots of current signal against the total VK concentrations (lower).

Table 4.1. Reproducibility of method using screen-printed graphene electrode. (n=10)

Concentration of VK (ppm)	Average current (μA)	%RSD
5	2.44 ± 0.08	3.29
10	3.72 ± 0.12	3.18
15	4.86 ± 0.15	3.16

4.6 Interference study

In case of the determination of the total VK in real sample as dietary supplement, the selectivity of method is necessary because there are various ingredients in dietary supplement which may be interfere the measurement process.

Therefore, other common compositions generally found in real samples such as Ca^{2+}, Mg^{2+}, vitamin A, B2, C, D and E were examined separately using tolerance ratio method with the total VK standard solution in concentration of 5 μg mL^{-1}. The tolerance limit was estimated to be within 5% of the anodic current signal. The results indicated that 5-fold mass ratio of Ca^{2+}, Mg^{2+} and vitamin E; 2-fold mass ratio of vitamin C and 0.12 fold mass ratio of vitamin D3 do not interfere with the determination of the VK as shown in Table 2.

Table 4.2. Tolerance ratio of interfering substances for the determination of 5 μg mL^{-1} of VK on screen-printed graphene electrode.

Common interferences	Tolerance ratio ($W_{interference}/W_{VK}$)
Ca^{2+}	5
Mg^{2+}	5
C	2
D3	0.12
E	5

4.7 Sample preparation

As mentioned in selectivity part, it was found the toterance ratios were not enough for applying in real sample analysis. The exact amount of interferance presented in real samples are usualy more higher than those mass ratios described above. As a result, this proposed method is not suitable for the determination of the total VK in multivitamin supplements. However, the developed method is still suitable for the determination of the total VK in supplements containing VK as the main composition. In this kind of supplement, it usually has two interferences including $Ca^{2+}$and Mg^{2+}. Therefore, the sample preparation is required before analysis. In order to overcome this problem, the simple precipitation using NaOH was used for the $Ca^{2+}$and Mg^{2+} elimination because $Ca(OH)_2$ and $Mg(OH)_2$ have limited solubility

(solubility constant, K_{sp} of Ca^{2+} = 5.5×10^{-6} and Mg^{2+} = 1.8×10^{-11}) and are in the solid residue according to the increasing of alkali concentration. [63],[64] Therefore, the concentration of NaOH used for sample preparation procedure was also investigated using the prepared mixing solution of VK and interferences (Ca^{2+} and Mg^{2+}) in the mass ratio of 500-fold following real sample limitation. The NaOH at different concentrations in the range of 0.001 to 0.1 M were added to the mixing solution. Then these mixtures were passed all preparation process as centrifugation, filtration, pH and volume adjustment, respectively. After that, the final solutions were measured using SWASV method, and the obtained current signals were calculated as recovery. As the results shown in Fig. 4.24, the recoveries were plotted against various NaOH concentrations. The result was found that the recovery of VK extremely increased over the range of 261.18 to 455.29% when the NaOH concentration is lower or more than 0.01 M. It could be explained that low concentration of NaOH was insufficient for precipitation while high concentration of NaOH was excess which can interfere the determination of total VK. The experimental results were summarized in Table 3. Therefore, 0.01 M of NaOH was used for sample preparation procedure in the proposed method.

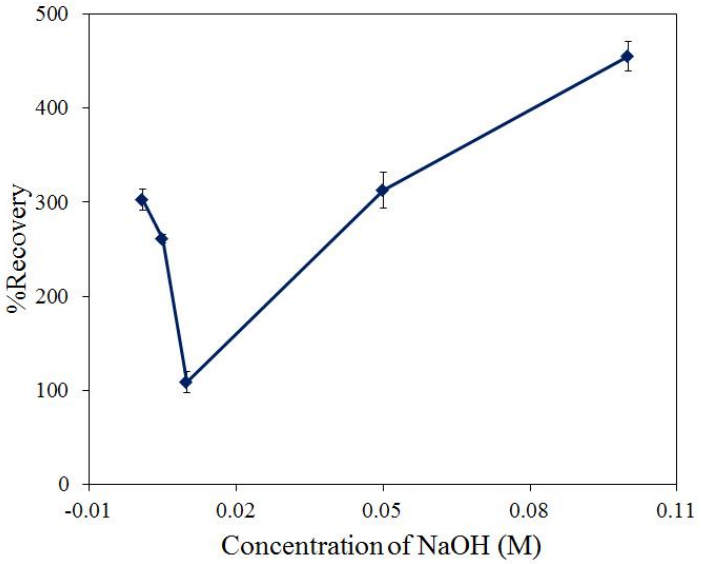


Figure 4.24 The relationship between NaOH at different concentrations and the recovery.

Table 4.3. Optimization of NaOH concentration for sample preparation using 5 μg mL^{-1} of VK and 2,500 μg mL^{-1} of Ca^{2+} and Mg^{2+} on screen-printed graphene electrode.

Conc. of NaOH (M)	Ep (V)	Ip (nA)	%Recovery
0.1	0.019	8.50	455.2885979
0.05	0.019	6.32	312.811784
0.01	0.019	3.21	108.8837971
0.005	0.019	5.53	261.1805783
0.001	0.019	6.17	302.8827059

4.8 Analytical application

In order to evaluate this proposed method for practical application, the SWASV technique was operated with a screen-printed graphene electrode for the determination of VK in dietary supplements. Three brands of dietary supplement were investigated. For accuracy and precision of method, the recovery and RSD was examined by spiking three concentrations of VK at 4, 8 and 12 μg mL^{-1}, respectively, in real sample solution before sample preparation process. Each of the concentrations was measured repeatedly three times. The results were shown in Table 4. The recovery and RSD were found to be in the range of 90.1 to 108.1 % and 0.3 to 6.6 % respectively. Results obtain can indicate the capability of this method for the determination in real sample. Furthermore, the results of this proposed method were compared with a conventional HPLC technique coupled with UV detector using non-spiked VK solution (Table 5.) The comparison exhibited a satisfactory agreement by paired t-test at the 95% confidence interval. The RSD was acceptable in the range of 1.7% to 6.9 %. Therefore, the proposed stripping method can be used as an alternative method for qualitative control in real dietary supplements.

Table 4.4. Determination of total VK in dietary supplement samples using the proposed method (n = 3).

Samples	Added ($\mu g\ mL^{-1}$)	Expected ($\mu g\ mL^{-1}$)	Found ($\mu g\ mL^{-1}$)	Recovery (%)	RSD (%)
Pharm. 1	4.0	5.0	5.1 ± 0.2	107.6	2.9
	8.0	9.0	8.4 ± 0.5	92.9	6.2
	12.0	13.0	13.8 ± 0.4	107.2	2.7
Pharm. 2	4.0	5.0	4.5 ± 0.1	92.2	0.3
	8.0	9.0	9.8 ± 0.1	108.1	0.7
	12.0	13.0	13.1 ± 0.3	102.8	2.3
Pharm. 3	4.0	5.0	4.9 ± 0.3	98.1	6.6
	8.0	9.0	8.6 ± 0.3	93.9	3.6
	12.0	13.0	11.9 ± 0.5	90.1	4.2

Table 4.5. The comparison of the proposed method and standard method for the determination of total VK in dietary supplement samples (n = 3).

Samples	Expected (μg/tablet)	Proposed method		HPLC-UV	
		Founded (μg/tablet)	%RSD	Founded (μg/tablet)	%RSD
Pharm. 1	100	93.7 ± 6.5	6.9	80.6 ± 0.1	0.1
Pharm. 2	100	80.3 ± 3.8	4.7	100.3 ± 0.4	0.4
Pharm. 3	500	507.9 ± 8.6	1.7	529.5 ± 1.27	0.2

CHAPTER V
CONCLUTIONS AND FUTURE PERSPECTIVE

5.1 Conclusions

In this research, the novel method for the determination of the total vitamin K was developed using a square-wave anodic stripping voltammetric technique on graphene screen-printed electrode. The method provides high sensitivity, simple to use, cost effectiveness, fast analysis, a low sample volume, and disposability. Furthermore, this developed method displays as being environment friendly and suits for use in the pharmaceutical industry which the pretreatment step is not necessary like previous probe. From the experiments proofed, the graphene electrode was shown to have the lower capacitance than the carbon one; hence, the charging layer accumulated on electrode surface and background current of graphene electrode are lower than carbon. Therefore, they can provide higher sensitivity for determination. Under the optimal conditions, the linear range from 1 to 15 μg mL^{-1} was observed. The limit of the detection and quantitation were achieved at 0.099 μg mL^{-1} and 1 μg mL^{-1}, respectively. The linear regression equation is $y = 0.3055x + 1.5438$; R^2 is equal to 0.9904. Moreover, for the sample preparation of dietary supplement which has Ca^{2+} and/or Mg^{2+} interfering, 0.01 M of NaOH can be used as precipitant for sample preparation in this method. Finally, the proposed method was successfully applied for dietary supplement samples and the results were compared with the high performance liquid chromatography technique cooperated with a UV-Vis detector. It was found that the results show in good agreement from both methods. There were not significantly different by paired t-test at the 95% confidence interval. The precision was presented as %RSD which observed in the range of 1.7 to 6.9 %. Therefore, this new proposed method can be used as an alternative method for qualitative control vitamin K in the pharmaceutical industry.

5.2 Future perspective

Due to the biomarker property of vitamin K which can diagnose the risk of many diseases such as osteoporosis, rheumatoid arthritis, hepatocellular carcinoma leukemia Alzheimer's disease and malabsorption disorder. [22,23] So, the method can be developed for determination of total vitamin K in biological sample i.e. human plasma, urine or blood to diagnose the risk of health. Nevertheless, the amount of vitamin K in biological sample is available in the very low level at ng mL^{-1}. Hence, the higher sensitivity method is needed to continuously develop. To overcome this problem, it has to increase electrode efficiency by using modifying material on the screen-printed electrode to obtain the higher sensitivity in detection.

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APPENDIX

VITA

Miss Whitchuta Jesadabundit was born in Bangkok, Thailand on January 11, 1992. After completing high school at Sripruetta school, Bangkok, Thailand in 2009, she got into Srinakarinwirot University, Bangkok, Thailand, and received the Bachelor's degree of Science, majoring in Chemistry on March 2014. Next, she accordingly becomes a Master's degree of Science (Analytical Chdemistry) of academic year 2017 from Chulalongkorn University.

Proceeding:

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Scholarship:

She has got a scholarship from Science achievement Scholarship of Thailand in 2010 – 2016. Moreover, she has received the 90th Anniversary of Chulalongkorn University Fund (Ratchadaphiseksomphot Endowment Fund) was granted for research fund in 2017.