การพัฒนาตัวรับรู้ทางเคมีไฟฟ้าสำหรับการตรวจวัดสารต้านอนุมูลอิสระโดยใช้ขั้วไฟฟ้าพิมพ์สกรีน
ดัดแปรด้วยหมึกนำไฟฟ้า

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ปีการศึกษา 2557

DEVELOPMENT OF ELECTROCHEMICAL SENSOR FOR DETECTION OF ANTIOXIDANTS USING CONDUCTIVE INK MODIFIED SCREEN-PRINTED ELECTRODE

Miss Chayanee Bardpho

A Thesis Submitted in Partial Fulfillment of the Requirements
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Thesis Title	DEVELOPMENT OF ELECTROCHEMICAL SENSOR FOR DETECTION OF ANTIOXIDANTS USING CONDUCTIVE INK MODIFIED SCREEN-PRINTED ELECTRODE
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ชญานี บาตรโพธิ์ : การพัฒนาตัวรับรู้ทางเคมีไฟฟ้าสำหรับการตรวจวัดสารต้านอนุมูลอิสระโดยใช้ขั้วไฟฟ้าพิมพ์สกรีนดัดแปรด้วยหมึกนำไฟฟ้า (DEVELOPMENT OF ELECTROCHEMICAL SENSOR FOR DETECTION OF ANTIOXIDANTS USING CONDUCTIVE INK MODIFIED SCREEN-PRINTED ELECTRODE) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: ศ. ดร.อรวรรณ ชัยลภากุล, 88 หน้า.

งานวิจัยนี้ทำการพัฒนาเทคนิคอัลตราไฮเพอร์ฟอร์แมนซ์ลิควิดโครมาโตกราฟีร่วมกับขั้วไฟฟ้าดัดแปรด้วยหมึกนำไฟฟ้าผ่านการพิมพ์แบบอิงค์เจ็ทสำหรับการหาปริมาณสารต้านอนุมูลอิสระหลายชนิดอย่างรวดเร็วในคราวเดียว โดยเลือกใช้สองเทคนิคสำหรับการสร้างและดัดแปรขั้วไฟฟ้า ได้แก่ เทคนิคการพิมพ์สกรีนและเทคนิคการพิมพ์แบบอิงค์เจ็ท หมึกนำไฟฟ้าซึ่งประกอบด้วยแกรฟีนและพอลิแอนิลีนจะถูกพิมพ์ลงบนพื้นผิวหน้าขั้วไฟฟ้าแบบพิมพ์สกรีนโดยใช้เครื่องพิมพ์แบบอิงค์เจ็ท สำหรับการดัดแปรขั้วไฟฟ้านั้นได้ทำการศึกษาหาภาวะที่เหมาะสมระหว่างอัตราส่วนของแกรฟีนกับพอลิแอนิลีนและจำนวนชั้นในการพิมพ์ โดยใช้เทคนิคไซคลิกโวลแทมเมตรีตรวจวัดสารต้านอนุมูลอิสระแต่ละชนิดด้วยขั้วไฟฟ้าแบบพิมพ์สกรีนดัดแปรด้วยแกรฟีนและพอลิแอนิลีนในเซลล์เคมีไฟฟ้า พบว่าขั้วไฟฟ้าที่ดัดแปรด้วยหมึกนำไฟฟ้านั้นให้สัญญาณกระแสไฟฟ้าในการตรวจวัดสารต้านอนุมูลอิสระแต่ละชนิดเพิ่มสูงขึ้นเป็น 2 ถึง 4 เท่าเมื่อเทียบกับสัญญาณกระแสไฟฟ้าที่ได้จากขั้วไฟฟ้าที่ไม่ได้ดัดแปร ในการแยกสารต้านอนุมูลอิสระด้วยเทคนิคอัลตราไฮเพอร์ฟอร์แมนซ์ลิควิดโครมาโทกราฟีแบบเฟสผันกลับนั้นสามารถแยกสารต้านอนุมูลอิสระทั้ง 4 ชนิดออกจากกันอย่างสมบูรณ์ได้ภายในเวลา 3 นาที โดยใช้เฟสเคลื่อนที่ประกอบไปด้วยสารละลายฟอสเฟตบัฟเฟอร์ที่มีค่าพีเอช 3 และอะซิโตไนไทรล์ ในอัตราส่วน 90:10 (%v/v) เมื่อตรวจวัดที่ศักย์ไฟฟ้าเหมาะสมที่ +1.2 โวลต์ (เมื่อเทียบกับขั้วไฟฟ้าอ้างอิงซิลเวอร์/ซิลเวอร์คลอไรด์) จะได้ค่าความเป็นเส้นตรงอยู่ในช่วง 0.01 ถึง 10 ไมโครกรัมต่อมิลลิลิตร ค่าขีดจำกัดต่ำสุดของการตรวจวัดอยู่ในช่วง 1.38 ถึง 1.94 นาโนกรัมต่อมิลลิลิตร และค่าขีดจำกัดของการวิเคราะห์เชิงปริมาณ 4.59 ถึง 6.46 นาโนกรัมต่อมิลลิลิตร ดังนั้นงานวิจัยนี้ประสบความสำเร็จในการพัฒนาตัวรับรู้ทางเคมีไฟฟ้าสำหรับนำไปใช้ในการตรวจวัดปริมาณสารต้านอนุมูลอิสระหลายชนิดในตัวอย่างชา นอกจากนี้ผลที่ได้จากเทคนิคที่นำเสนอข้างต้นให้ผลการทดลองที่สอดคล้องกับเทคนิคอัลตราไฮเพอร์ฟอร์แมนซ์ลิควิดโครมาโทกราฟีร่วมกับการตรวจวัดทางแสง (UHPLC-UV) ซึ่งเป็นเทคนิคมาตรฐานของการตรวจวัด

ภาควิชา เคมี ลายมือชื่อนิสิต .....

สาขาวิชา เคมี ลายมือชื่อ อ.ที่ปรึกษาหลัก .....

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5571947923 : MAJOR CHEMISTRY

KEYWORDS: UHPLC / ELECTROCHEMICAL DETECTION / INKJET-PRINTING / GRAPHENE / POLYANILINE / ANTIOXIDANT

CHAYANEE BARDPHO: DEVELOPMENT OF ELECTROCHEMICAL SENSOR FOR DETECTION OF ANTIOXIDANTS USING CONDUCTIVE INK MODIFIED SCREEN-PRINTED ELECTRODE. ADVISOR: PROF. ORAWON CHAILAPAKUL, Ph.D., 88 pp.

A development of ultra-high performance liquid chromatography coupled with a novel inkjet-printed conductive ink-modified electrode for a fast and simultaneous determination of polyphenolic antioxidants was achieved. Two printing techniques were chosen for fabrication and modification including (i) an in-house screen-printing method and (ii) an inkjet-printing method, respectively. A conductive ink containing graphene and polyaniline nanocomposite (G-PANI) was precisely printed onto the surface of screen-printed carbon electrode (SPCE) using a dimatix inkjet material printer. For the electrode modification, optimization of significant conditions including a G-PANI ratio and a number of inkjet-printed layers was initially investigated. Using an optimized G-PANI-modified screen-printed carbon electrode (G-PANI/SPCE) in an electrochemical batch cell, cyclic voltammetric detection of individual antioxidants was studied. Compared to a bare SPCE, the G-PANI/SPCE provided higher electrochemical sensitivity with increase (2-4 times) of peak current of each antioxidant. Moreover, four antioxidants were successfully separated and determined within 3 min using a reverse phase ultra-high performance liquid chromatography (UHPLC) with a mobile phase containing phosphate buffer (pH 3) and acetonitrile (90:10, %v/v). Under an optimal detection potential at +1.2 V vs. Ag/AgCl, linear calibrations were found to be 0.01–10 μg mL^{-1} with limits of detection (S/N=3) of 1.38-1.94 ng mL^{-1} and limit of quantitation (S/N=10) of 4.59-6.46 ng mL^{-1}. Finally, this proposed method has been successfully used for the determination of antioxidants in tea samples. The results obtained from the presented method were highly good agreement with those obtained from a standard UHPLC-UV method.

Department: Chemistry Student's Signature .....

Field of Study: Chemistry Advisor's Signature .....

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LIST OF ABBREVIATIONS

ACN	acetonitrile
AE	auxiliary electrode
Ag/AgCl	silver/silver chloride
A_p	peak area current
°C	degree Celsius
C	catechin
CFA	caffeic acid
CPE	carbon-paste electrode
CV	cyclic voltammetry
CVD	chemical vapor deposition
DOD	drop-on-demand
EC	epicatechin
ECD	electrochemical detection
ECG	epicatechin gallate
EGC	epigallocatechin
EGCG	epigallocatechin gallate
E_p	peak potential
$E_{p,a}$	anodic peak potential
$E_{p,c}$	cathodic peak potential
G	graphene
GA	gallic acid
GCE	glassy carbon electrode
GO	graphene oxide
HPLC	high-performance liquid chromatography
i_p	current
K	distribution coefficient
L	liter
M	molar

NMP	N-Methyl-2-pyrrolidone
PANI	polyaniline
PEDOT	polyethylenedioxythiophene
PPy	polypyrrole
PTH	polythiophene
rGO	reduced graphene oxide
RE	reference electrode
RSD	relative standard deviation
SEM	scanning electron microscope
SD	standard deviation
SPCE	screen-printed carbon electrode
TEM	transmission electron microscopy
UHPLC	ultra-high performance liquid chromatography
V	volt
WE	working electrode
ng	nanogram
µA	microampere
µC	microcoulomb
µg	microgram
µL	microliter

CHAPTER I
INTRODUCTION

1.1 Introduction

The amount of polyphenols presented in tea exhibit antioxidant activities which have been publicized as a variety of health benefits. Antioxidants can protect human cells from damage because of free radicals. Tea consumption has linked with decreasing adverse risks such as cancer, cardiovascular arthritis, abnormalities, and pathogenic infections [1]. A tea infusion provides abundant essential compounds for a human health such as polyphenolic antioxidants, caffeine, theanine, vitamins, carbohydrates, and trace elements [2]. Various major polyphenols consist of catechin (C), epicatechin (EC), epicatechin-3-gallate (ECG), epigallocatechin (EGC), epigallocatechin-3-gallate (EGCG), gallic acid (GA), and caffeic acid (CFA). To evaluate a quality control of tea, the determination of these polyphenols containing in tea as well as in the beverages is very important in the various research fields (e.g. nutritional and epidemiological studies). Therefore, it is necessary to develop an appropriate analytical approach to determine the several phenolic compounds simultaneously in tea samples to assess their qualities and health promoting properties.

Various conventional analytical methods for separation and simultaneous determination of polyphenolic antioxidants have been broadly reported [3-6]. One of the remarkable techniques to determine these antioxidants selectively is high performance liquid chromatography (HPLC) coupled to spectrophotometry. However, the HPLC method provided the limitation in term of time-consuming which all separation and determination processes had proceeded much longer than 20 min, approximately [7-10]. To reduce the separation time for simultaneous

detection of polyphenolic antioxidants, ultra-high performance liquid chromatography (UHPLC) has become an attractive technique for high-throughput analysis due to its faster separation and higher resolution, compared to the classical HPLC technique [11, 12]. Moreover, an electrochemical detection can be applied as a detector in the UHPLC system for the sensitive determination of various electroactive antioxidants [13, 14]. Various electrode materials have been also used for measurement of these electroactive polyphenolic compounds such as metal electrode (platinum electrode [15]), glassy carbon electrode (GCE) [16], carbon-paste electrode (CPE) [17], and screen-printed carbon electrode (SPCE) [18]. Among these electrode materials, SPCEs have been paid much interest due to their significantly disposability, cost-effective method, and simplicity to couple with a thin-layer flow cell for using as the detector in the flow-based system.

To enhance the electrochemical sensitivity of working electrode, the electrode modification has been a notable issue for recent researches. Particularly, inkjet printing technology has been demonstrated as an alternative method for the electrode modification because of low cost, high production speed, selectivity, and compatibility with a wide range of substrates [19-21]. For another strategy to increase the electrode surface area in the electrochemical system, an electrode modification using a noticeable nanomaterial has played an important role to improve the electrochemical sensitivity of the sensors. Currently, graphene (G) is a prominent nanomaterial electrode modifier. It is highly useful for electroanalysis owing to its very large two-dimensional surface area, high electrical conductivity, and excellent electron transfer rate [22]. However, the disadvantage of using G to modify electrode is agglomeration. Hence, to prevent this limitation of G, polyaniline (PANI) has been also applied as an electrode modifier to improve the G distribution on the electrode surface. PANI is a multipurpose conducting polymer due to its high

electrical conductivity, simplicity to synthesize, thermal and chemical stability, and wide range of potential applications [23, 24].

According to several benefits of G, PANI and the inkjet printing technology, the conductive G-PANI ink is precisely printed onto the SPCE surface. It is an alternative way to enhance the electrochemical sensing and catalytic capabilities for an electrochemical detection of antioxidants in the UHPLC system.

1.2 Objectives

The aim of this work is to develop an inkjet-printed G-PANI modified SPCE (G-PANI/SPCE) as a new electrochemical sensor for chromatographic simultaneous determination of antioxidants in tea samples.

CHAPTER II

THEORY AND LITERATURE REVIEWS

2.1 Electrochemistry

Electrochemistry is the branch of chemistry involved with the study of reactions which charges (electrons or ions) cross the interface between two immiscible phases of matter, normally the electrode and an electrolyte solution. Electrode reaction arises at the surface of the electrode and generates a slight unbalance in the electric charges of the electrode and the solution. Charge is carried by the ions movement in the phase of electrolyte in a reaction well known as an oxidation-reduction reaction or redox reaction. Redox reaction, which occurs in electrochemical cell, is classified as (i) galvanic cell and (ii) electrolytic cell. While the reaction arises spontaneously at the electrodes and produces electrical energy in galvanic cell (Figure 2.1 (a)), the reaction in the electrolytic cell is non-spontaneously occurred by applying an electrical energy through the system (Figure 2.1 (b)) [25].

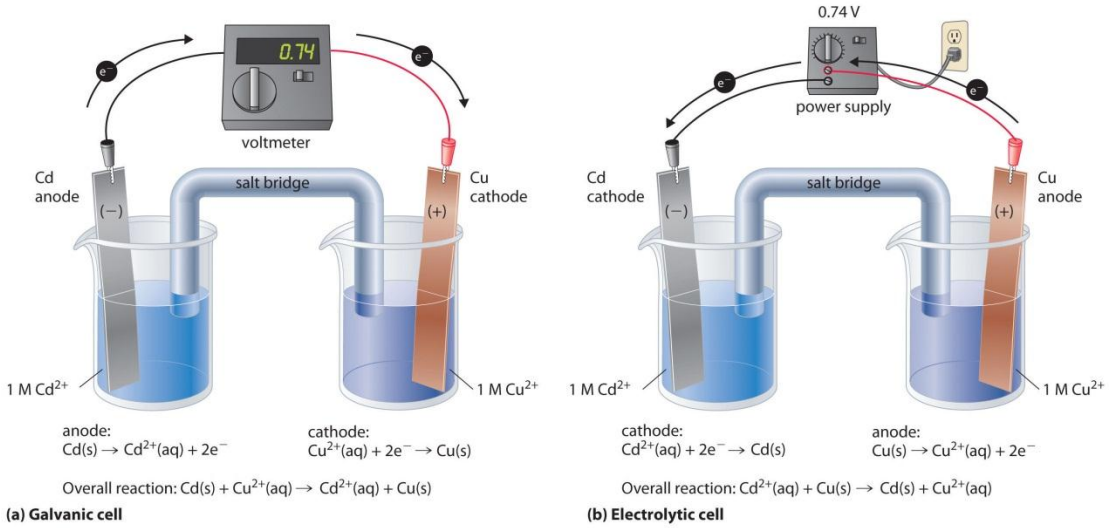

Figure 2.1 Electrochemical cell (a) galvanic cell and (b) electrolytic cell [26]

Commonly, the study of electrochemistry at working electrode in electrochemical cell is of interest. Electrochemical cell consists of three electrodes including working electrode (WE), auxiliary electrode (AE) and reference electrodes (RE). The WE represents the most important electrode since electron transfers of interested analyte occur at its surface. Typical WE materials are solid metals (i.e., Au, Pt) and carbon (i.e., graphite, graphene). In this work, screen-printed carbon electrode (SPCE) was used as a WE. Commercial carbon ink formulations are usually used for screen-printing the working electrodes, while silver-based inks are commonly employed for fabrication of the RE. Carbon ink is specifically interested for bio-sensing applications because it provides low background current and low-cost. SPCEs have been paid much attentiveness due to its significantly disposability, low cost, reliability, and ease to couple with a thin-layer flow cell for the electrochemical detector in the flow-based system [27].

2.1.1 Fundamental of electrochemistry

The redox reactions concerning the electrons transfer from one electroactive species to another take place at the surface of electrode. The transfer of electron can be represented by

$$Ox + n\ e^- \Leftrightarrow Red$$

Where Ox and Red are oxidized form and reduced form, respectively, and n represents the stoichiometric number of electrons for the redox reaction. For the law of thermodynamics, Nernst equation (2.1) exhibits that the potential of the electrode depends upon concentration of electroactive species. The electrode reaction is frequently supposed to be thermodynamically or electrochemically reversible.

$$E = E^{0'} + \frac{RT}{nF} \ln \frac{C_O}{C_R} \qquad (2.1)$$

Where E^0 is the standard potential of the redox reaction, R is the universal gas constant (8.314 J K^{-1} mol^{-1}), T is the Kelvin temperature, n is the stoichiometric number of electrons transferred for the redox reaction, F is the Faraday constant (96,487 coulombs), and C_0 and C_R are the oxidized and reduced form concentrations.

In addition, the current is conditional upon two factors as shown in Figure 2.2:

1) Mass transfer is the rate of charge transfer from the bulk of solution to the electrode.

2) Charge transfer kinetics is the rate of electrons transfer across the interface.

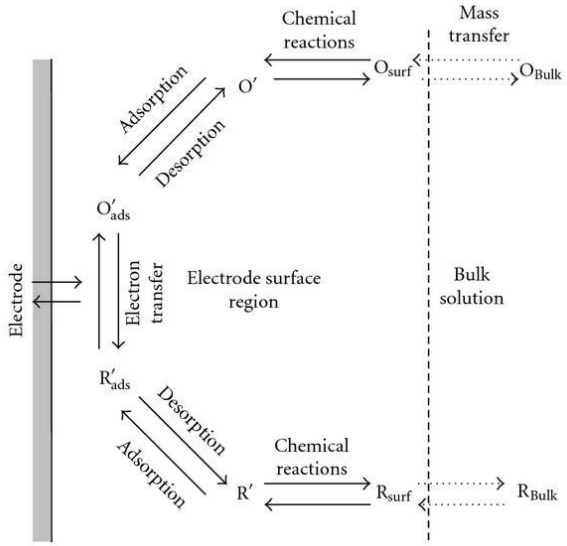

Figure 2.2 The process of electrode reaction

There are three basic processes of mass transfer: diffusion, migration and convection [28].

2.1.1.1 Diffusion process

Diffusion is the movement of molecules or ions from an area of high concentration to an area of lower concentration as shown in Figure 2.3.

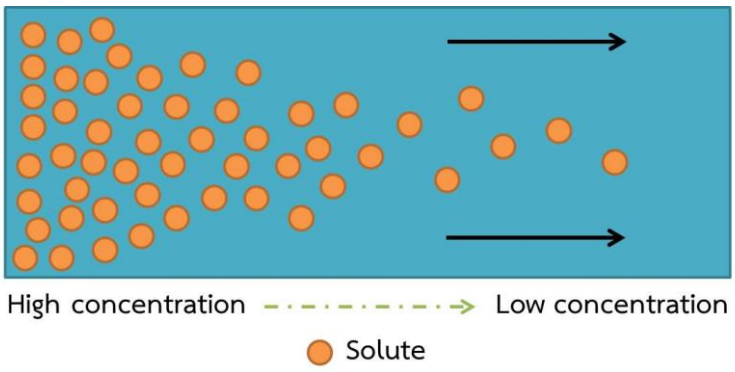

Figure 2.3 Diffusion of solutes from a different concentration

2.1.1.2 Migration process

The movement of ions or charged particles in electric field is known as migration (Figure 2.4). The increase or decrease of the applied potential at the surface of electrode in a solution containing ions affects charge migration.

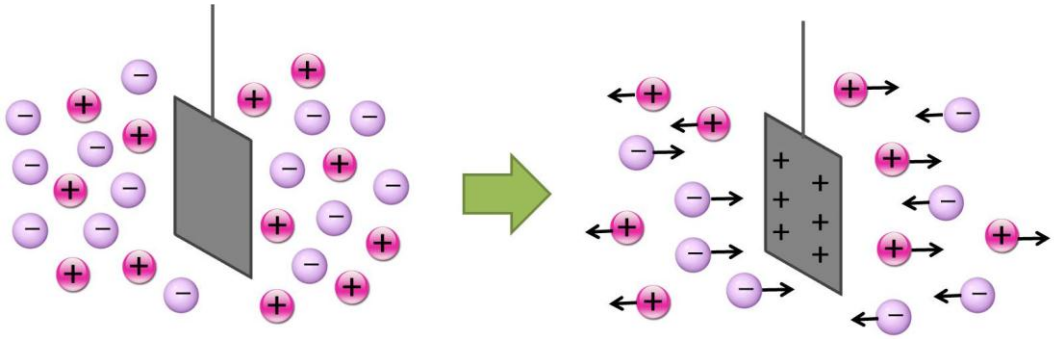

Figure 2.4 Migration of ions in the solution

2.1.1.3 Convection process

Convection is resulting from the movement of solution with external mechanical forces such as stirring, vibration, flowing or other means which is explained by hydrodynamics as shown in Figure 2.5.

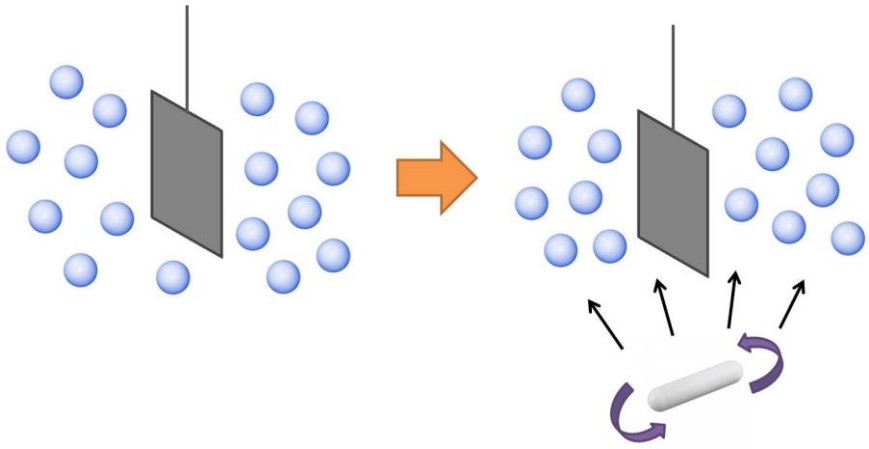

Figure 2.5 Convection of ions in the solution

2.1.2 Cyclic voltammetry

Cyclic voltammetry (CV) is a general technique for studying and comparing electrochemical properties between new electrode ideas and novel modified electrodes. This is a convenient instrument to obtain qualitative information about electron transfers processes. It is also a rapid method to estimate the number of transferred electrons, rate constants, formal reduction potentials, formation constants and reversibility of a reaction. Cyclic voltammetry experiments are performed by immersing the WE in an unstirred testing solution and then applying potential sequentially forward and reverse directions to electrode, as a function of time (shown in Figure 2.6 (a)). The height of peak current can be used to measure the analyte concentration in the bulk solution. Figure 2.6 (b) shows cyclic voltammogram. It is a plot of the current response as a function of applied potential [29, 30].

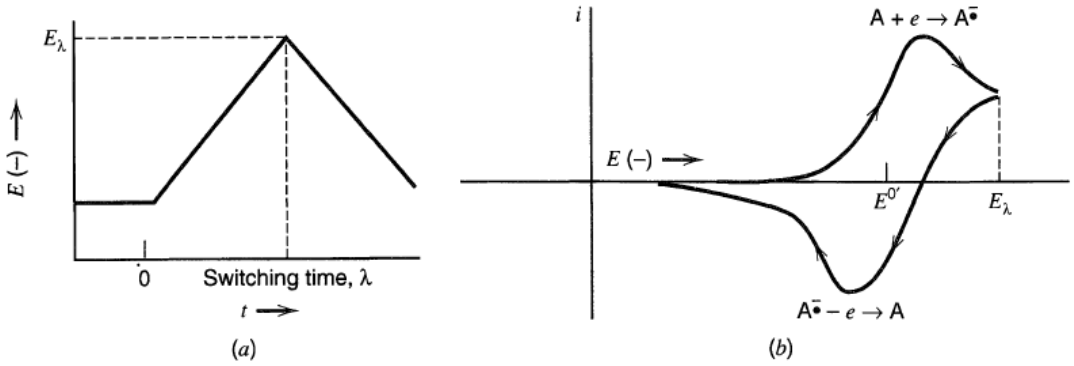

Figure 2.6 Cyclic potential sweep (a) and cyclic voltammogram (b) [25]

For the reversible reaction, n in the electrode reaction can be measured by the separation between the peak potentials (ΔE_p), which should close to 58/n mV (at 25°C). Furthermore, the reaction is fast enough to keep the concentrations of

the oxidized and reduced forms in the equilibrium with each other at the electrode surface.

Another characteristic of reversible reaction is the peak current which illustrated by Randles-Sevick equation as shown in Equation 2.2. In addition, the height of peak current of oxidation and/or reduction is proportional to the square root of the scan rate.

$$i_p = (2.69 \times 10^5) n^{3/2} A D^{1/2} C v^{1/2} \quad (2.2)$$

Where i_p is the peak current in amperes, 2.69 x 10^{-5} is a collection of constants at 25 °C, n is the number of transferred electron, A is the electrode surface area in cm^2, D is the diffusion coefficient in cm^2 sec^{-1}, v is the scan rate in mV sec^{-1}, and C is the concentration in mol cm^{-3}.

For irreversible reaction, in which the reactants convert to products but the products can't convert back to the reactants, peak current shift further apart with increasing scan rate.

2.1.3 Hydrodynamic voltammetry

Hydrodynamic voltammetry employs convection to increase the rate of mass transfer to the WE surface. The convection enhances current and sensitivity, comparing to voltammetric determinations operated in stagnant solution. The convection is motivated by stirring of the solution, movement of electrode, or flowing of the solution through WE surface at constant velocity, which is used as an electrochemical detector in the chromatographic system [31]. The scanning of the potential in hydrodynamic voltammetry occurs while the solution moves toward the electrode. Further, current is measured as a function of the potential applied to a WE. The hydrodynamic devices can be used in various techniques such as dropping

mercury electrode, rotating disc and ring disc electrode, wall jet electrode and channel electrode. For chromatographic determination, channel electrode is chosen to apply in electrochemical detector. In channel electrode, the WE is maintained in specific position, and the solution flowed through the WE surface as shown in Figure 2.7.

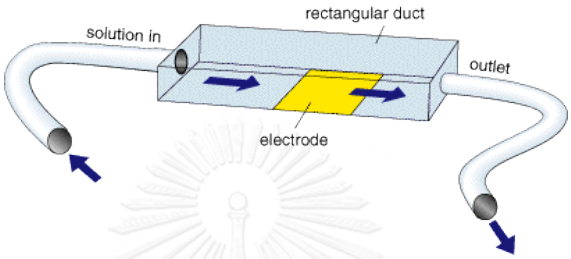

Figure 2.7 Channel electrode [31]

2.1.4 Amperometry

Amperometry is generally applied in flowing and stirring system. Current is measured at a constant applied potential as shown in the waveform in Figure 2.8. This current response is proportional to the concentration of the electroactive analyte in the solution [32].

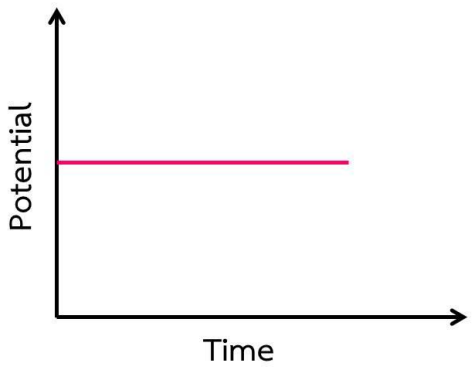

Figure 2.8 Waveform of amperometry

2.2 Inkjet-printing technique

Inkjet printing is a promising technique for patterning and creating conductive materials without the use of masks. The fluids used as inks must have the capability of being stable and accurate printing by ink-jetting. Inkjet printing has various advantages such as low cost, simplicity, flexibility, selectivity, micro-patterning capability, high production speed and compatibility with a wide range of materials and substrates. The drop-on-demand (DOD) method can precisely deposit quantities of inks on the substrates. The ink is deposited on the substrate in the droplet form by applying a short pressure pulse through a nozzle of the print head. To obtain the quality of ink-jetting, a single droplet is formed from the ejected fluid under suitable electrical conditions. For printing inks, the important physical factors are viscosity, surface tension, and density. These ink properties affect the drop formation mechanism and drop size of the inks [33-36].

Recently, inkjet printer is an attractive tool for fabrication and modification of the inexpensive and disposable bioelectronic devices such as biosensors and biochips with high resolution and precision of printing. In this work, piezoelectric Dimatix™ Materials Printer (DMP-2800) was used as shown in Figure 2.9. The pattern printing can be designed by using dimatix software or imported files from another program.

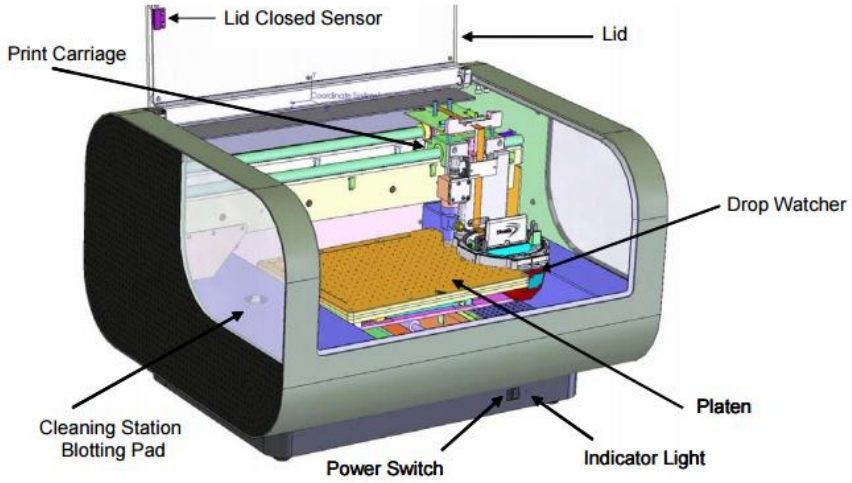

Figure 2.9 Components of Dimatix Materials Printer [37]

Furthermore, a cartridge (Figure 2.10) has 16 nozzles which can be individually controlled as well as temperature at the print head of cartridge.

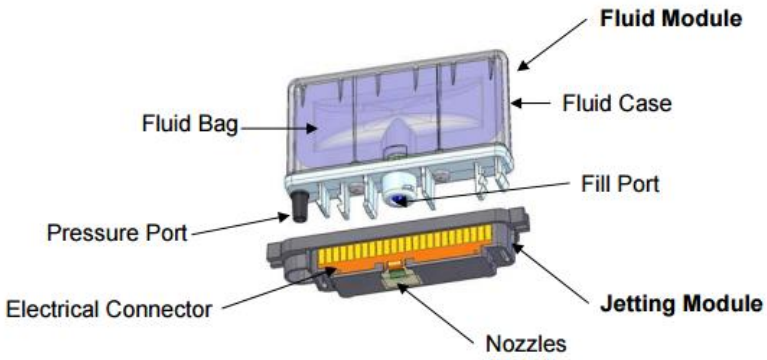

Figure 2.10 Cartridge [37]

2.3 Nanomaterials

The description of nanomaterials is materials with at least one dimension in the size of 1-100 nanometers, approximately. Nanomaterials have properties different from those of their bulk mechanisms. For example, they could enhance conductivities and improve optical or magnetic properties for sensor applications.

The four classes of nanomaterials comprise of metals or metal oxides, carbon compounds, elemental carbon, and ceramics [38]. In this work, we are interested in carbon nanomaterial namely graphene.

2.3.1 Graphene

In 2004, graphene (G) was discovered by Novoselov and Geim [39]. G is a two dimensional monolayer of single-atom thick allotrope of carbon which is packed in a sp^2-bonded carbon into honeycomb as shown in Figure 2.11. G has an outstanding electronic characteristics, good electrical conductivity, high electron transfer rate, large specific surface area, and good mechanical properties. These properties provide possibilities for highly sensitivity of electrochemical detection. In the graphene family, various materials related to graphene are known including graphene oxide (GO) and reduced graphene oxide (rGO). Moreover, graphene-based materials were obtained by several processing of graphite such as mechanical exfoliation of graphene using scotch tape, electrolytic exfoliation, chemical vapor deposition (CVD), and chemical oxidation methods.

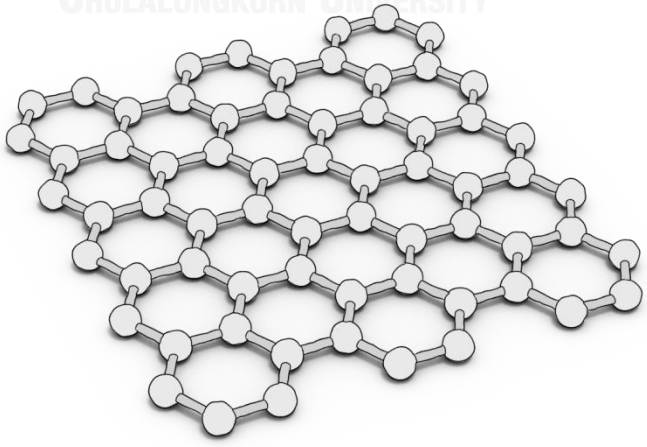

Figure 2.11 The structure of graphene [40]

Graphene is one of the most promising materials that considered being a future generation conducting material to replace traditional electrode materials. According to those properties of graphene, it demonstrated that graphene can be used with potential applications as shown in Figure 2.12 including single molecule gas detection, transparent conducting electrodes, composites, and energy storage devices (e.g. supercapacitors, solar cells, and lithium ion batteries) [41, 42].

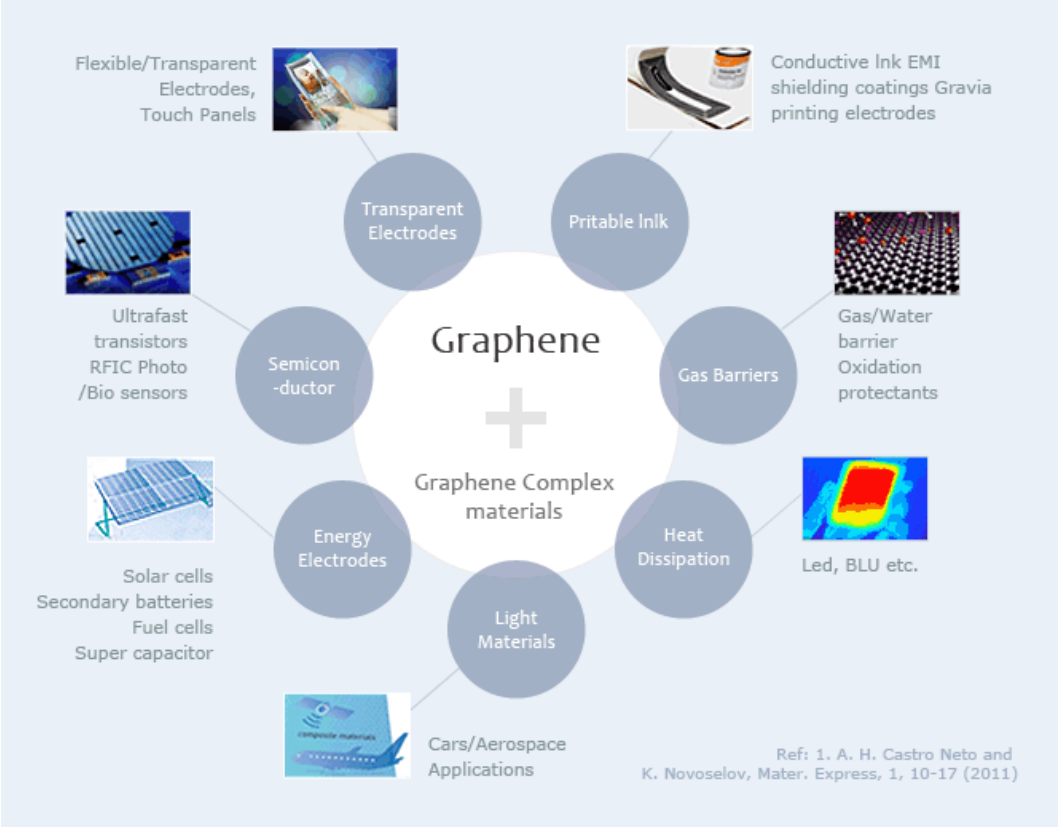

Figure 2.12 The applicatons of graphene [43]

2.3.2 Graphene and polyaniline composite

In recent years, graphene-based composites with conducting polymers, such as polyaniline (PANI) polypyrrole (PPy), polythiophene (PTH), and polyethylenedioxythiophene (PEDOT), have been considered attractive interests because of their novel electrochemical properties. Among those conducting polymers, PANI is outstanding and interesting due to its simple synthesis, high conductivity, high energy density, mechanical flexibility, environmental stability, and low cost. There are four redox states of PANI with different colors including leucoemeraldine base (LB, yellow), emeraldine base (EB, blue), emeraldine salt (ES, green), and pernigraniline base (PB, purple) as shown in Figure 2.13. The EB form of PANI can be expediently doped with acid into electrochemically active ES form with positive charges at low pH values (pH<3) [44]. Thus, the G-PANI modified electrode has verified that this composite have potential and remarkable for its application including electrochemical sensors and biosensors [45-50].

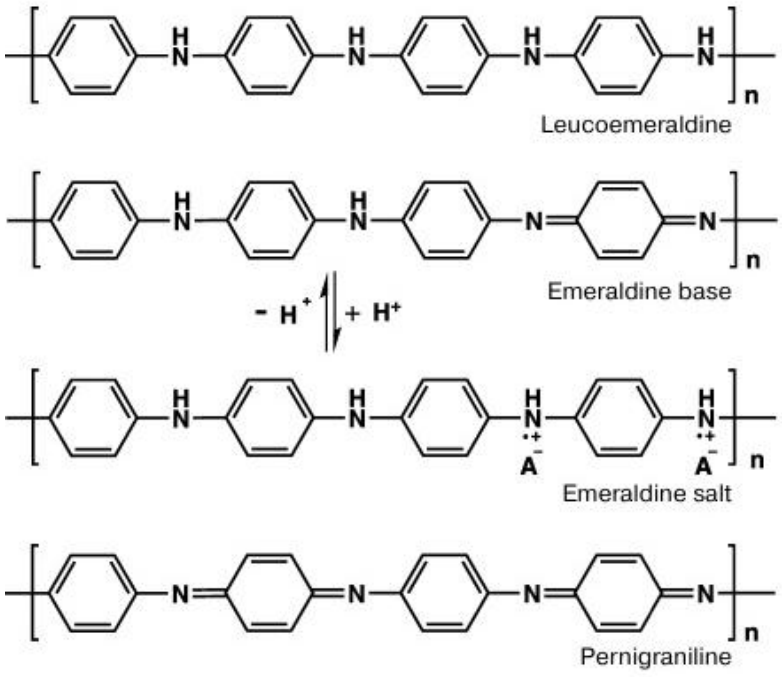

Figure 2.13 Structural formulas of PANI and its possible redox states

2.4 Chromatography

Chromatography is a separation technique which based on the partition of analytes, leading to the separation of the various analytes between two phases; stationary phase (SP) and mobile phase (MP). The MP can be a liquid, gas or supercritical fluid while the SP is a liquid film, a solid adsorbent or another solid material with excellent chemical functionality [51]. The analytes are distributed between the non-miscible phases of SP and MP. Chromatographic methods use different separation mechanisms such as adsorption, absorption (partition), permeation, and electrostatic interaction. The MP supports transportation of analyte through the column. As the analyte transportation through the chromatographic column, each analyte is retained in accordance with characteristic affinity of individual compound for the SP. The distribution of an analyte between the SP and MP in chromatography is described by the distribution coefficient (K) as shown in following equation.

$$K = \frac{C_{SP}}{C_{MP}} \tag{2.3}$$

Where C_{SP} is the analyte concentration in the SP and C_{MP} is the analyte concentration in the MP. The time passing between the sample injection and peak maximum of analyte is termed as the retention time [52].

Liquid chromatography is one of the methods in chromatography using liquid as the MP. High-performance liquid chromatography (HPLC) can be described the liquid chromatography in which the MP passes through the column, the analytes begin to separate into bands. The schematic of HPLC system is shown in Figure 2.14.

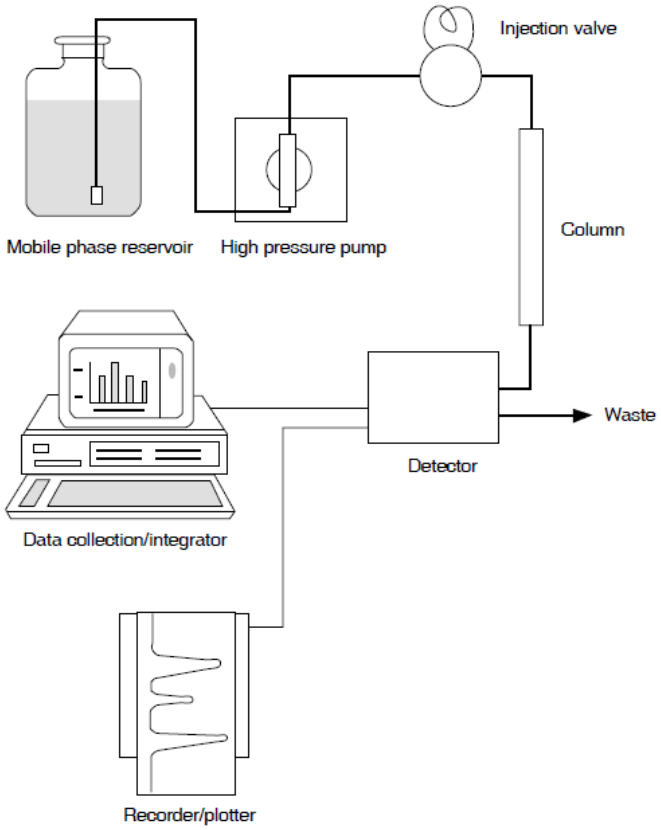

Figure 2.14 The schematic of HPLC system [53]

2.4.1 Ultra-high performance liquid chromatography

Recently, Ultra-high performance liquid chromatography (UHPLC) has become the modern HPLC platform. UHPLC, based on the employment of columns packed with sub-2-μm particles and high pressure (P > 400 bar), is ideally suited to fast chromatographic separation with short columns. UHPLC provides very high-resolution separations and excellent system performance including precision and sensitivity. Moreover, UHPLC is a green technology due to its low consuming of organic solvents and sample volume [54].

Figure 2.15 UHPC instrument

2.5 Antioxidant

An antioxidant is a substance inhibiting oxidation and deterioration of food quality. Antioxidant can preserve foods by delaying development of deterioration, rancidity, and discoloration because of lipid oxidation. Antioxidants also reduce the risk of degenerative diseases, occurring from an oxidative stress in human body. The natural antioxidants were classified into two categories: primary and secondary. Primary antioxidants are neutralized free radicals by donating hydrogen atoms or by a single electron transfer mechanism. Secondary antioxidants deactivate singlet oxygen and chelate metal ions (i.e., copper, iron). Phenolic and polyphenolic compounds are examples of secondary metabolites arising in plants [55-58].

2.5.1 Antioxidant activity

Several feasible mechanisms of polyphenol antioxidant are comprised of (i) electron and hydrogen atom transfer and (ii) the chelation of catalytic metals. The main antioxidant mechanism of phenolic compounds is the rapid donation of a hydrogen atom to active radicals (i.e., $ROO^{\bullet}$), leaving phenoxyl radicals ($PP^{\bullet}$), which

are themselves relatively stable and unreactive. The PP• intermediate also reacts with other free radicals, so terminating the propagation [59].

$$ROO^{\bullet} + PPH \longrightarrow ROOH + PP^{\bullet}$$

$$RO^{\bullet} + PPH \longrightarrow ROH + PP^{\bullet}$$

$$ROO^{\bullet} + PP^{\bullet} \longrightarrow ROOPP$$

$$RO^{\bullet} + PP^{\bullet} \longrightarrow ROPP$$

In addition, polyphenols are famous for their antioxidant properties. The antioxidant activity of polyphenols is principally ascribed to the combination of hydroxyl groups and aromatic rings. The number of hydroxyl groups is the major influence contributing to antioxidant potential [60].

2.5.2 Tea polyphenols

Tea is well-known for its antioxidant effect. A variety of antioxidant phenolic compounds in tea are responsible for this activity such as flavan-3-ols, phenolic acids, flavonols and flavones, theaflavins and thearubigins, theasinensins, and theabrownins [61, 62].

The main phenolic compounds found in tea are (+)-catechin, (–)-epicatechin (EC), (–)-epicatechin gallate (ECG), (–)-epigallocatechin (EGC), (–)-epigallocatechin gallate (EGCG), gallic acid (GA) and caffeic acid (CFA). The quantity of gallic acid in white tea is high and comparable to that of black tea. The structures of those phenolic compounds are shown in Figure 2.16.

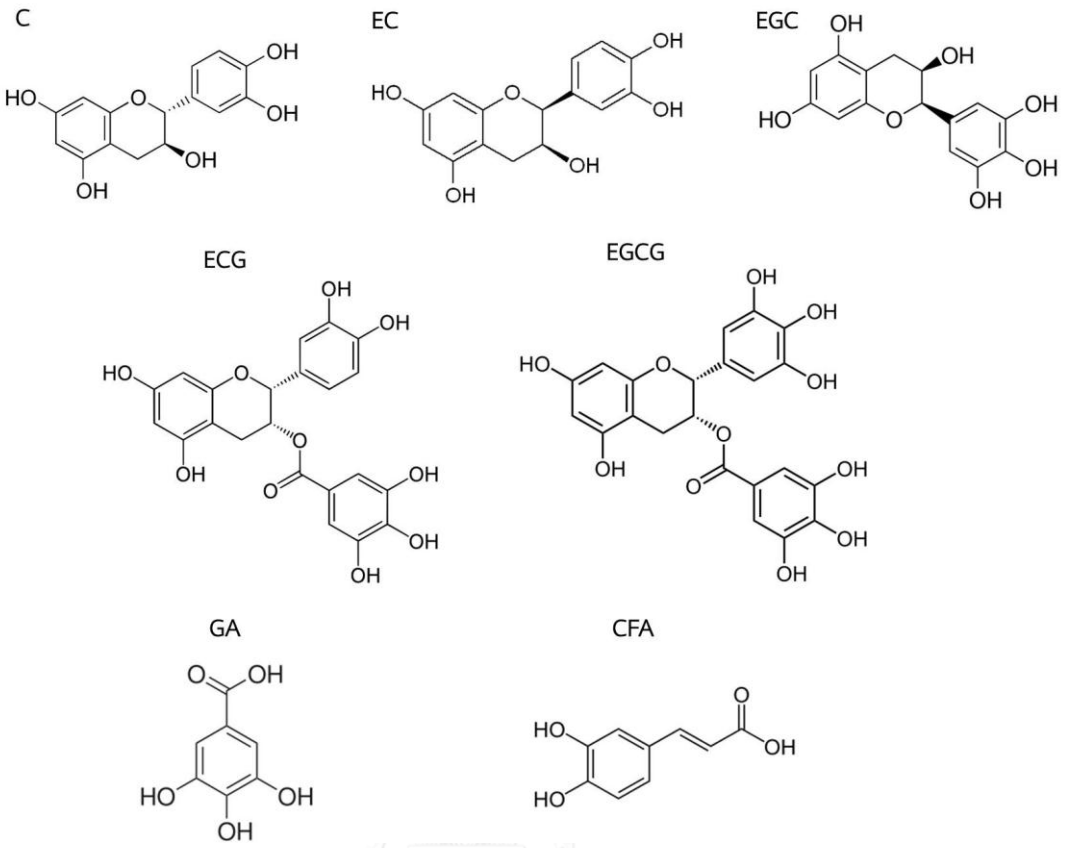

Figure 2.16 Structures of EC, EGC, ECG, EGCG, GA and CFA

The commercially available tea is distinguished in different types according to the degree of fermentation or manufacturing process:

- White tea and green tea are unfermented tea
- Oolong tea is a semi-fermented tea
- Black tea is fully fermented.

Those tea originate from the leaves of *Camellia sinensis* plant. The antioxidants in tea may help to protect against diseases like cancers (including breast, lung, skin, small intestine, pancreas and liver cancers), disease, neurological, cardiovascular, degenerative and Parkinson's diseases. Moreover, tea has anti-inflammatory and anti-aging properties.

2.5.2.1 White tea

White tea is derived mostly from newly grown buds and young leaves with tiny, silvery-white hairs, which are unexposed to sunlight and prevent chlorophyll production. For white tea production, buds and leaves are harvested only once a year in the early spring [63].

2.5.2.2 Green tea

Green tea is made from steaming the freshly harvested leaves directly for preventing the oxidation of catechins and maintaining the polyphenols in their monomeric forms. Most of the total polyphenol quantity of green tea is consisted of catechins (85%) that are water-soluble and contribute to the taste and flavor of tea [59, 63].

2.5.2.3 Oolong tea

Oolong tea contains a mixture of non-oxidized monomeric polyphenols and higher-molecular-weight theaflavins [63].

2.5.2.4 Black tea

Black tea is more oxidized and stronger in flavor than other types of tea. The polyphenols in black tea are produced from the controlled enzymatic reactions concerned in the fermentation process [63].

2.6 Literature reviews

2.6.1 Electrode modification by inkjet printing

Inkjet printing is an attractive instrument for modification of electrodes with high precision and low material waste. Several researches have been employed inkjet printing technology for modifying functional material to enhance the electrochemical performance of the printed electrode.

In 2012, Kit-Anan *et al.* [36] developed a new low-cost and disposable point-of-care paper-based electrochemical sensor using inkjet-printed PANI modified SPCE electrode for ascorbic acid detection. From the cyclic voltammetry measurement, the PANI modified electrode exhibits ten folds of peak current compared to bare graphite-paste electrode. It was found that the sensitivity of PANI modified electrode was approximately to 17.7 μA/mM in the concentration range of 30-270 μM and the limit of detection (LOD) was to 30 $\pm$ 3 μM.

In 2012, Karuwan *et al.* [35] modified SPCE with graphene-poly(3,4-ethylene-dioxythiophene):poly(styrene-sulfonate) (GP-PEDOT:PSS) by inkjet printing method. The proposed electrode is developed for salbutamol (SAL) determination with high sensitivity, a wide concentration range of 500 μM and low LOD of 1.25 μM. From cyclic voltammetric response, the oxidation peak of PEDOT:PSS modified and GP-PEDOT:PSS modified SPCEs are found to be approximately 30 and 150 times higher than bare SPCE, respectively. This inkjet-printed GP-PEDOT:PSS on SPCE is highly promising for advanced electrochemical biosensor.

In 2013, Maattanen *et al.* [64] demonstrated the suitability of the low-cost inkjet-printed three-electrode paper chip platform for various electrochemical experiments. The novel paper-based chips exhibited comparable performance to conventional electrochemical cells.

In 2013, Phongphut *et al.* [48] developed a disposable amperometric triglyceride (TG) biosensor based on Au/PEDOT-PSS nanocomposite and co-immobilized lipase, glycerol kinase and glycerol-3-phosphate oxidase inkjet-printed on the SPCE surface. From amperometric detection, the optimized electrode provided good reproducibility with wide dynamic range (0–531 mg/dL) and low LOD (7.88 mg/dL).

2.6.2 Antioxidant determination

All kinds of analytical methods have been employed for the antioxidant determination, for example, electrochemical detection method, HPLC coupled with electrochemical detection and UV detection methods. Various researches have been reported using these detection methods to measure antioxidant.

In 2003, Kotani *et al.* [65] reported determination of catechins in human plasma and in commercial canned green tea by HPLC coupled with electrochemical detection using a microbore octadecylsilica column. Under the optimal conditions, peak heights of catechins were found to be linearly in the concentration range of 2 pmol/mL to 2 nmol/mL with $r^2 > 0.999$. In this research was found that a commercial canned green tea has lower amount of catechins than in green tea leaf extraction.

In 2003, Kilmartin *et al.* [16] reported the determination of polyphenols in green, oolong and black teas and instant coffee by cyclic voltammetry using a carbon electrode. It can be seen that the higher levels of phenolic compounds were obtained in tea, prepared with a higher water temperature, for the first infusion, in the absence of milk.

In 2010, Novak *et al.* [14] studied the analysis of eight green and three black teas using square-wave voltammetry (SWV) at a glassy-carbon electrode. The content of catechins in green tea obtained by SWV was in good agreement with those obtained from reverse-phase high-performance liquid chromatography with electrochemical detection (RP-HPLC-ECD). In RP-HPLC-ECD, the separation of five catechins, namely EGC, C, EC, EGCG and ECG, was successfully separated within 16 min with LODs ranged from 23.23 to 100.7 nM and limits of quantification (LOQs) ranged from 77.43 to 335.8 nM for ECG.

In 2014, Narumi *et al.* [13] developed the ion-pair high-performance liquid chromatography with electrochemical detection (ion-pair HPLC-ECD) for simultaneous detection of tea catechins and gallic acid (GA) in human serum. The investigation of these analytes was achieved using a C18 reversed-phase column with mobile phase of phosphate buffer (pH 2.5) containing tetrahexylammonium hydrogensulfate as an ion-pair reagent. The recoveries of catechins and GA were found to be 77.3 to 93.9%. The LODs for catechins and GA in serum were found in the range of 0.4-3.1 ng mL^{-1}.

In 2014, Naldi *et al.* [66] developed the UHPC with UV detection for fast quantitation of green tea catechins and caffeine. The main six major catechins and caffeine were successfully separated with C18 analytical column (50 mm × 2.1 mm, 1.8 μm), using a mobile phase comprised of pH 2.5 triethanolamine phosphate buffer (0.1M) and acetonitrile. The recoveries of six catechins and caffeine on the commercial tea samples (Sencha, Ceylon Green and Lung Ching) ranged from 94 to 108% (n = 3). LODs were in a range of 0.1–0.4 μg mL^{-1}.

In 2015, David *et al.* [67] developed a disposable pencil graphite electrode (PGE) for determination of caffeic acid (CFA) in Turkish green, white and black teas. From differential pulse voltammetry (DPV) experiment, linearity of CFA concentration ranged from 1×10^{-7} to 3×10^{-3} M. The LOD and LOQ were 8.83×10^{-8} M and 2.94×10^{-7} M, respectively. The recoveries of CFA from Turkish green, white and black teas were 98.30%, 99.57% and 91.46%, respectively.

CHAPTER III

EXPERIMENTAL SECTION

3.1 Reagents and materials

All chemicals and reagents used in SPCE fabrication and modification and electrochemical measurements are shown in Table 3.1

Table 3.1 List of chemicals and reagents in the electrochemical experiments

Chemicals and reagents	Suppliers
Graphene (G)	A.C.S, USA
Polyaniline (PANI)	Sigma-Aldrich, USA
Camphor-10-sulfonicacid ($C_{10}H_{16}O_4S$)	Sigma-Aldrich, USA
N-Methyl-2-pyrrolidone (NMP)	Sigma-Aldrich, USA
Gallic acid (GA), 97.5-102.5% (titration)	Sigma-Aldrich, USA
(−)-Epigallocatechin (EGC), ≥ 95% (HPLC) from green tea	Sigma-Aldrich, USA
(+)-Catechin hydrate (C), ≥ 98% (HPLC)	Sigma-Aldrich, USA
Caffeic acid (CFA), ≥ 98 % (HPLC)	Sigma-Aldrich, USA
Carbon ink	Gwent group, UK
Silver/silver chloride ink	Gwent group, UK
Potassium ferricyanide ($K_3[Fe(CN)_6]$)	Sigma-Aldrich, USA
Potassium chloride (KCl)	Merck, Germany

All chemicals and reagents used in UHPLC separation and electrochemical detection (UHPLC-ECD) are shown in Table 3.2

Table 3.2 List of chemicals and reagents in the UHPLC experiments

Chemicals and reagents	Suppliers
Acetonitrile (ACN), HPLC grade	Merck, Germany
Methanol (CH_3OH), HPLC grade	Merck, Germany
Ortho-phosphoric acid (85%)	Sigma-Aldrich, USA
Potassium dihydrogen phosphate (KH_2PO_4)	Sigma-Aldrich, USA
Disodium hydrogen phosphate (Na_2HPO_4)	Sigma-Aldrich, USA
Sodium dihydrogen phosphate (NaH_2PO_4)	Sigma-Aldrich, USA

3.2 Instruments

All instruments used in this work are shown in Table 3.3.

Table 3.3 List of all instruments

Instruments	Suppliers
Piezoelectric Dimatix™ Materials Printer (DMP-2800)	FUJIFILM Dimatix, USA
eDAQ potentiostat	eDAQ, Australia
CHI 1232A potentiostat	CHI Instrument, USA
UHPLC LC-20AD XR pump	Shimadzu, Japan
UHPLC SIL-20A XR autosample	Shimadzu, Japan
UHPLC CTO-20AC column oven	Shimadzu, Japan
UHPLC SPD-M20A diode array detector	Shimadzu, Japan
Silicon rubber gasket	Bioanalytical system, USA
Teflon cell gasket	Bioanalytical system, USA

Instruments	Suppliers
Teflon tubing (1/10 inch i.d.)	Upchurch, USA
PEEK tubing (0.25 mm. i.d.)	Upchurch, USA
Stainless–steel tube	Bioanalytical system, USA
Platinum wire	Bioanalytical system, USA
Silver/silver chloride electrode (Ag/AgCl)	Bioanalytical system, USA
Ultrasonic bath	Elma, Germany
Analytical balance	Mettler Toledo, Thailand
Autopipette	Eppendrof, Germany
Milli-Q water system	Merck, Germany
Hettich centrifuges	Hettich zentrifugen, Germany
Nylon membrane syringe filter (0.22 µm)	Vertical Chromatography, Thailand
Nylon membrane filter paper (0.22 µm)	Vertical Chromatography, Thailand
pH meter	Mettler Toledo, Thailand
Vortex mixer VTX-3000L	Mixer Uzusio LMS, Japan
Vacuum pump	GAST, USA

3.3 Electrode fabrication and modification

3.3.1 Electrode fabrication

A disposable screen-printed carbon electrode (SPCE) was fabricated using an in-house screen-printing method. A silver/silver chloride (Ag/AgCl) was firstly screen-printed as a conductive pad onto a polyethylene terephthalate (PET) substrate. A carbon ink was then printed to form a working electrode pattern. The

SPCEs were allowed to dry the solvent and organic residues in an oven at 65 ℃ for 30 min. Figure 3.1 shows the screen-printing procedure for fabrication of screen-printed carbon electrode (SPCE).

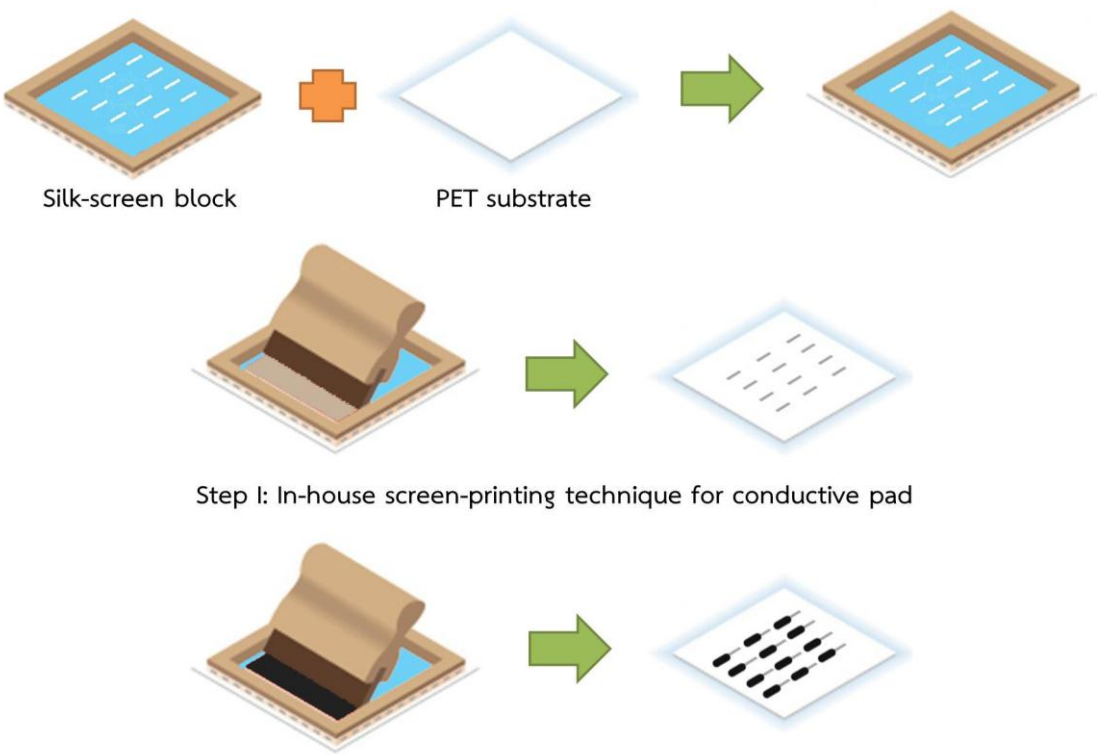

Figure 3.1 The in-house screen-printing of carbon electrode

3.3.2 Electrode modification

Initially, an electrode modifier was prepared by a simple mixing between graphene (G) and polyaniline (PANI) solutions. 10 mg of graphene powder was dispersed in 10 mL of N-Methyl-2-pyrrolidone (NMP) by sonication for 20 h. PANI solution was prepared in 10 mL of NMP by dissolving 20 mg of PANI (emeraldine base) and 25.8 mg of camphor-10-sulfonic acid ($C_{10}H_{16}O_4S$). After that, the well-mixing PANI solution was stirred for 5 h. The solution between G and PANI was mixed together to produce conductive ink by stirring for 1 h.

Next, the mixture was centrifuged at 5000 rpm for 30 min and then filtered using 0.43 μm membrane. The remaining conductive ink was ready to use for the inkjet-printing. After that, the conductive G-PANI ink was loaded into a cartridge. After the cartridge was inserted into the printer, conductive G-PANI ink was casted onto the working area of SPCE by using a piezoelectric Dimatix™ Materials Printer. The optimal inkjet printing conditions including drop spacing of 25 μm and a firing frequency of 17 V was selected for applying a small droplet size of 10 pL on the SPCE surface. To evaporate an organic solvent in the conductive ink, the modified SPCE was heated using an oven at 120 °C for 5 min. Figure 3.2 shows the electrode modification procedure of inkjet-printed G-PANI/SPCE.

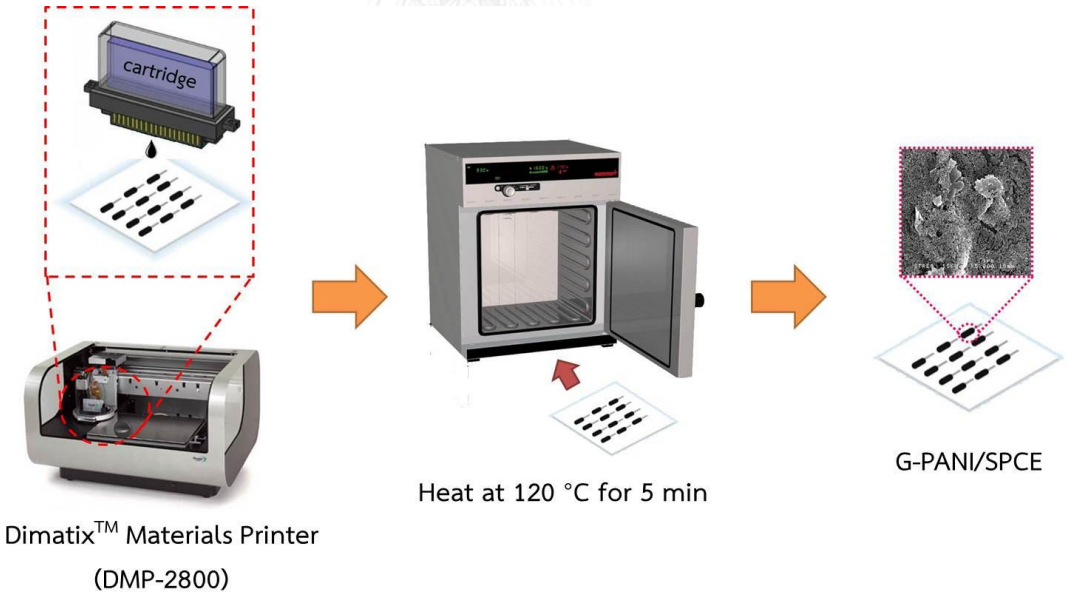

Figure 3.2 The electrode modification procedure of G-PANI/SPCE by inkjet printing

3.4 Preparation of standard stock solutions

3.4.1 Electrochemical measurement

3.4.1.1 Potassium chloride (KCl) solution

The 0.1 M KCl solution was used as a supporting electrolyte for characterization of inkjet-printed G-PANI/SPCE. The solution was prepared by dissolving 0.7455 g of KCl in Milli-Q water and adjusting to final volume of 100 mL in a volumetric flask.

3.4.1.2 Potassium ferricyanide ($K_3[Fe(CN)_6]$) solution

The G-PANI/SPCE was characterized by using 1 mM potassium ferricyanide ($K_3[Fe(CN)_6]$) solution. The solution was prepared by dissolving 8.23 mg of $K_3[Fe(CN)_6]$ in 0.1 M KCl and adjusting to a final volume of 25 mL in a volumetric flask.

3.4.1.3 Supporting electrolyte

For electrochemical study of four antioxidants, phosphate buffer was chosen as a supporting electrolyte. The 0.1 M phosphate buffer solution (pH 3) was prepared by dissolving 1.35 g of potassium dihydrogen phosphate (KH_2PO_4) in Milli-Q water to final volume of 10 mL in volumetric flask and the solution was adjusted to pH 3 with ortho-phosphoric acid. After that, the phosphate buffer (pH 3) was well mixed with acetonitrile in the ratio of 9:1.

3.4.1.4 Stock standard solutions for electrochemical measurement

The stock standard solutions of GA, EGC, and C were prepared by dissolving 10 mg of each compound in 10 mL of phosphate buffer (pH 3) while stock standard solution of CFA was prepared by dissolving 10 mg of CFA in phosphate buffer (pH 3)/methanol mixture (50:50, %v/v). Stock solutions were stored at –4 °C and resulted to be stable for a month. For cyclic voltammetry, the working standard

solutions of desired concentrations were prepared by proper dilution of these stock solutions.

3.4.2 UHPLC and electrochemical detection (UHPLC-ECD)

3.4.2.1 Stock standard solutions for UHPLC separation

The stock standard solutions of GA, EGC and C were prepared by dissolving each compound at a concentration of 1.0 mg mL^{-1} in phosphate buffer (pH 3) while CFA 1.0 mg mL^{-1} stock standard solutions were prepared in phosphate buffer (pH 3)/methanol mixture (50:50 v/v). The stock solutions were stored at −4 °C and resulted to be stable for a month. For preparation of working standard solutions, these stock standard solutions were diluted to the desired concentrations with phosphate buffer (pH 3). Finally, all solutions and solvents were filtered with 0.22 µm nylon membranes before use in UHPLC.

3.4.2.2 Preparation of mobile phase

The mobile phase consisting of 0.1 M phosphate buffer solution and acetonitrile with the ratio of 90:10 (%v/v) was chosen for chromatographic separation. The mobile phase at different pH was prepared as shown in Table 3.4. The mixture was dissolved in Milli-Q water and adjusted to the desired pH with phosphoric acid or sodium hydroxide. After that, the mixture was transferred to 500 mL volumetric flask and made up to the final volume with Milli-Q water.

Table 3.4 The preparation of phosphate buffer solution in the pH range of 2 to 7

pH	Potassium dihydrogen phosphate (KH_2PO_4) (g)	Disodium hydrogen phosphate (Na_2HPO_4)	Sodium dihydrogen phosphate (NaH_2PO_4)
2	4.8000	-	-
3	6.0900	-	-
4	6.7400	-	-
5	6.7000	0.0960	-
6	-	0.9763	5.9500
7	-	4.7200	2.3100

3.4.2.4 Preparation of tea samples

The commercial bagged teas were purchased from local supermarket in Thailand (e.g. green tea (England), white tea (England), oolong tea (China) and black tea (China)). 0.1 g of dried tea leaves were weighted and crushed with a mortar and pestle. The infusion were prepared by extraction of tea leaves with 10 mL freshly Milli-Q grade water of 80 °C, and stirred with a magnetic stirrer bar for 10 min. After that the sample solutions were sonicated for 20 min. Tea samples were filtered through paper filters, then through 0.22 µm syringe filter and diluted with Milli-Q grade water. Tea infusions were daily prepared and stored in the refrigerator to be stable over the experiment duration.

3.5 Procedure

3.5.1 Cyclic voltammetric experiment

Cyclic voltammetry (CV) was performed by using eDAQ potentiostat and home-made electrochemical cell at room temperature. The home-made electrochemical batch cell is illustrated in Figure 3.3. The working electrodes (WE) are bare SPCE and G-PANI/SPCE. The Platinum wire and silver/silver chloride (Ag/AgCl) electrode with a salt bridge were used as the auxiliary electrode (AE) and reference electrodes (RE), respectively.

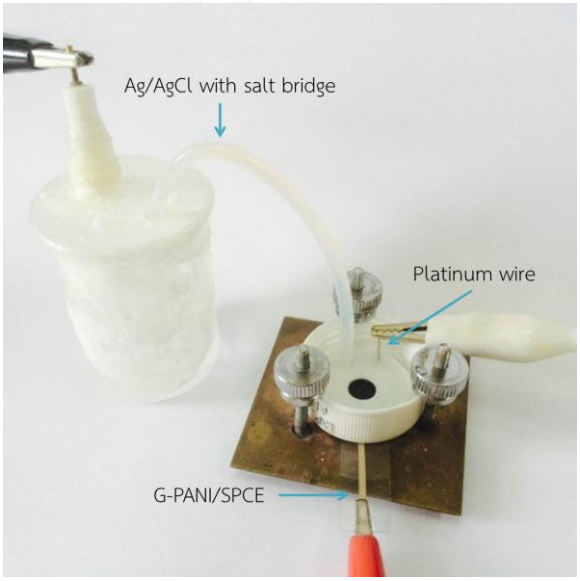

Figure 3.3 Electrochemical batch cell

3.5.1.1 Electrochemical characteristic of inkjet-printed G-PANI/SPCE

The amount of G-PANI modifier was varied for characterization of inkjet-printed G-PANI/SPCE using the standard solution of 1 mM $K_3[Fe(CN)_6]$ in 0.1 M KCl. CV was measured with the scanning potential range from -0.6 to +0.6 V at the scan rate of 100 mV s^{-1}. To study the mass transfer process of inkjet-printed

G-PANI/SPCE, the currents of 170.12 µg mL^{-1} GA were measured with varying the potential scan rates in the range of 10 to 400 mV s^{-1}.

3.5.1.2 Electrochemical behavior of antioxidants

The electrochemical behavior of antioxidants was studied by cyclic voltammetry. All CV of antioxidants were individually performed in 0.1 M phosphate buffer (pH 3):acetonitrile (9:1) at the scan rate of 100 mV s^{-1}. Cyclic voltammetric behavior of each antioxidant was investigated using different scanning potential conditions: -0.3 to +1.4 V for GA, 0 to +0.9 V for EGC, +0.6 to +1.5 V for C, and -0.2 to +1.2 V for CFA.

3.5.2 UHPLC-ECD separation

To separate the antioxidant content in tea, the UHPLC analysis was applied using the UFLCXR (Shimadzu, Japan) comprising of a 20 ADXR solvent deliver unit, an auto sampler (SIL-20A) with 0.1–100 µL loop, a thin-layer flow cell (GL Science, Japan), and an amperometric detector (CHI 1232A, CHI Instrument, USA). The chromatographic column was a Kinetex™ core-shell C18 column (50 mm x 4.6 mm i.d.; particle size, 2.6 µm, Phenomenex). The thin-layer flow cell consisted of a working G-PANI/SPCE, an auxiliary stainless steel electrode, and a reference Ag/AgCl electrode (Bioanalytical system, USA) as shown in Figure 3.4. A 1 mm thick silicon rubber gasket was used in the flow cell for limiting the surface area of modified SPCE.

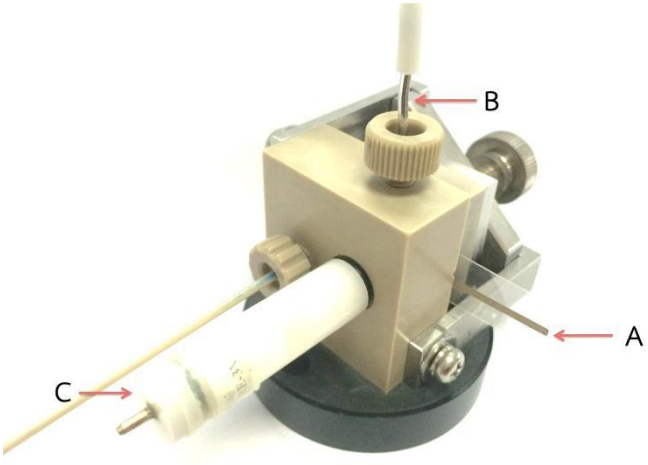

Figure 3.4 Electrochemical flow cell comprising of (A) inkjet-printed G-PANI/SPCE, (B) stainless steel tube as an auxiliary electrode and (C) silver/silver chloride (Ag/AgCl)

3.5.2.1 Mobile phase composition

The effect of mobile phase composition on the retention characteristics of four antioxidants was examined to optimize the separation performance. For simultaneous determination of GA, EGC, C and CFA, the ratios of 0.1 M phosphate buffer (pH 3) solution and acetonitrile were investigated in the range of 95:5, 90:10, 85:15 and 80:20 (%v/v) with applied detection potential of +0.7 V vs. Ag/AgCl and injection volume of 50 µL at a flow rate of 0.5 mL min^{-1}.

3.5.2.2 The effect of pH of phosphate buffer solution

The influence of pH of phosphate buffer solution in mobile phase was studied with different pH in the range of 2 to 7. The mixture of four antioxidants was measured with the mobile phase consisting of 0.1 M phosphate buffer solution and acetonitrile (90:10, %v/v) with detection potential of +1.2 V vs. Ag/AgCl and injection volume of 50 µL at a flow rate of 0.6 mL min^{-1}.

3.5.2.3 The optimization of flow rate

For complete separation of four antioxidants, the flow rates were also optimized. The flow rate values were investigated from 0.6 to 1.1 mL min^{-1} in 0.1 M phosphate buffer (pH 3) solution and acetonitrile (90:10, %v/v), injection volume of 50 µL, and applied detection potential of +1.2 V vs. Ag/AgCl.

3.5.2.4 The optimization of detection potential

The amperometric response of antioxidants determination was investigated to obtain suitable detection potential. The detection potential of four antioxidants in the amperometric detection were determined with various detection potential in the range of 0.8–1.4 V vs. Ag/AgCl in 0.1 M phosphate buffer (pH 3) solution and acetonitrile (90:10, %v/v) at a flow rate of 0.8 mL min^{-1}.

3.5.2.4 The optimal conditions for UHPLC separation with ECD

The separation of four antioxidants was carried out by UHPLC system (UFLCXR, Shimadzu, Japan) with an isocratic elution consisting of 0.1 M phosphate buffer (pH 3): ACN (90:10, %v/v). The chromatographic conditions including a Kinetex™ core-shell C18 column (50 mm x 4.6 mm i.d.; particle size, 2.6 µm, Phenomenex), injection volume of 50 µL, flow rate of 0.8 mL min^{-1}, and applied potential of + 1.2 V vs. Ag/AgCl were applied. All chromatographic separations were performed at room temperature. Table 3.5 shows the optimal chromatographic conditions.

Table 3.5 The optimal chromatographic conditions

Parameter	Optimal condition
Column	Kinetex™ core-shell C18 column (50 mm x 4.6 mm i.d.; particle size, 2.6 µm)
Mobile phase	0.1 M phosphate buffer (pH 3): ACN (90:10, %v/v)
Injection volume	50 µL
Flow rate	0.8 mL min^{-1}
Amperometric detection potential	+1.2 V vs. Ag/AgCl
Working electrode	Inkjet-printed G-PANI/SPCE
Temperature	Room temperature (~25 °C)

3.5.3 Analytical performance

3.5.3.1 Calibration curve and Linear range

Under the optimal conditions, amperometric detection of four polyphenolic antioxidants was performed in the concentration range of 0.01-10 µg mL^{-1} using inkjet-printed G-PANI/SPCE. The relationship between peak area (A_p) and concentration of polyphenolic antioxidants was plotted using linear regression analysis.

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

Limits of detection (LOD) and quantification (LOQ) were calculated from the $3SD_b/S$ and $10SD_b/S$, respectively, where SD_b is the standard deviation of blank measurement (n=10) and S is slope of the linearity. The LOD and LOQ were measured in triplicate (n=3) of a series of mixture dilutions.

3.5.3.3 Repeatability and reproducibility

To verify the repeatability of inkjet-printed G-PANI/SPCE, the solution of four antioxidants was analyzed thirty times using the same proposed electrode. The reproducibility was evaluated by analysis from ten different proposed electrodes. For the precision of intra-day and inter-day, five concentrations of four antioxidants (1, 3, 5, 7, and 9 μg mL^{-1}) were analyzed for three times within a day (intra-day) and three different days (inter-day). The relative standard deviations (%RSD) were calculated to estimate the precision and the equation of %RSD as shown in (3.1).

$$\%\text{RSD} = \frac{SD}{\overline{X}} \times 100 \quad (3.1)$$

3.5.4 Application in real samples

3.5.4.1 The simultaneous chromatographic determination of antioxidants in real samples

Four commercial bagged teas (i.e., green tea, white tea, oolong tea and black tea) were determined using the proposed UHPLC-ECD method: Quantification of GA, EGC, C and CFA were achieved by the standard addition method to examine the reliability of this proposed system.

3.5.4.2 Accuracy of analysis

To assess the accuracy of analysis, a %recovery was performed by UHPLC-ECD analysis in triplicate on measurement of each sample. Five standard solution of each antioxidant was added into the tea sample before the injection. The proposed method was also validated by the standard method of UHPLC coupled with ultraviolet detection (UHPLC–UV). A paired t-test at 95% confidential interval was applied to verify the method.

$$\%\text{Recovery} = \left(\frac{X - Y}{Z}\right) \times 100 \quad (3.2)$$

Where X is concentration of analyte found in spiked sample, Y is concentration of analyte found in blank sample and Z is standard concentration of target analyte at spiked concentration.

CHAPTER IV

Results and discussion

4.1 Characterization of G-PANI/SPCE

4.1.1 Surface morphology of G-PANI/SPCE

The G-PANI solution with a ratio of 1:2 was inkjet-printed onto the SPCE surface to form a thin layer of G-PANI. The morphology of G-PANI nanocomposite on the SPCE surface was investigated by a scanning electron microscope (SEM) and transmission electron microscopy (TEM). As shown in Figure 4.1 and 4.2, a well uniform dispersion of G-PANI nanostructure on the SPCE surface with unprecedented number of G was clearly observed. Moreover, the electron diffraction (inset of Figure 4.2) perfectly matched with the previous report [35]. From the results, it means that this modification method could lead to increase surface area of SPCE. The electrochemical sensitivity of the modified SPCE could be improved. It can be said that the inkjet-printing technique coupled with the use of G-PANI nanocomposite might be an alternative way for electrode modification method.

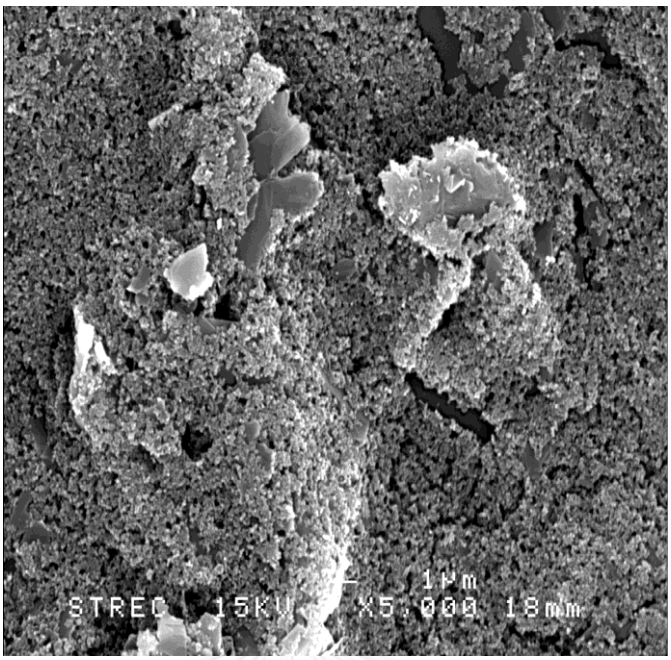

Figure 4.1 SEM image of inkjet-printed G-PANI/SPCE

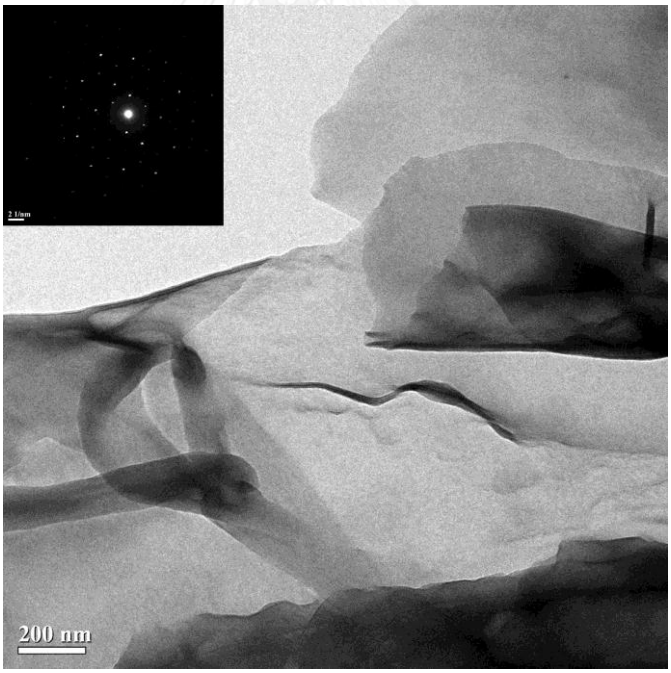

Figure 4.2 TEM image of inkjet-printed G-PANI/SPCE with electron diffraction

4.2 Electrochemical characterization of G-PANI/SPCE

4.2.1 Effect of mass ratio of G and PANI

Initially, the electrochemical characterization of developed electrode was investigated by cyclic voltammetric technique. The influence of G-PANI ratio was examined using 1 mM $K_3[Fe(CN)_6]$ in 0.1 M KCl. Figure 4.3 shows the peak currents obtained with the different G-PANI ratios. It was found that the anodic peak currents were related to the amount of G-PANI on the electrode surface. As the G-PANI ratio is higher than 1:2, the anodic peak current is reduced while the capacitive current (background current) is increased. The G-PANI ratio of 1:2 provided the highest anodic peak current response compared to other ratios. Thus, this ratio was chosen as the optimal G-PANI ratio for the electrode modification.

Next, the comparison of cyclic voltammetric behaviors of 1 mM $K_3[Fe(CN)_6]$ between bare SPCE and G-PANI/SPCE was investigated as shown Figure 4.4. Using G-PANI/SPCE, an increase of both anodic and cathodic peaks current were obtained and a decrease of potential difference ($\Delta E = E_{p,a} - E_{p,c}$) were also found. The ΔE_p of ferri/ferrocyanide measured on G-PANI/SPCE was 0.19 V vs. Ag/AgCl (red solid line). Nonetheless, ΔE_p obtained from bare SPCE was found to be 0.42 V vs. Ag/AgCl (blue solid line). These results indicated that the proposed inkjet-printed G-PANI nanocomposites can increase the electron transfer kinetics at electrode interface.

In order to study the mass transfer process of inkjet-printed G-PANI/SPCE, a variety of scan rates of GA was investigated in the range of 10 to 400 mV s^{-1} by cyclic voltammetry. A good linearity between the square root of scan rate and anodic peak current was plotted with a correlation coefficient (R^2) of 0.9950 as shown in Figure 4.5. The result can be suggested that the mass transfer at inkjet-printed G-PANI/SPCE surface was controlled by diffusion process.

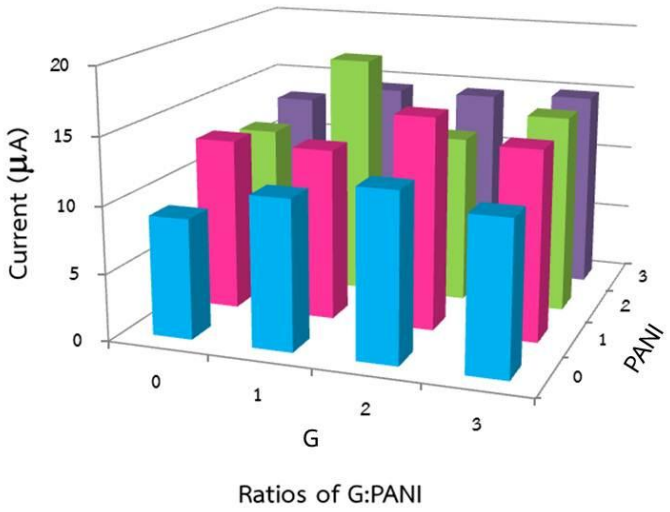

Figure 4.3 Comparison of oxidation current of the ratios between graphene and polyaniline

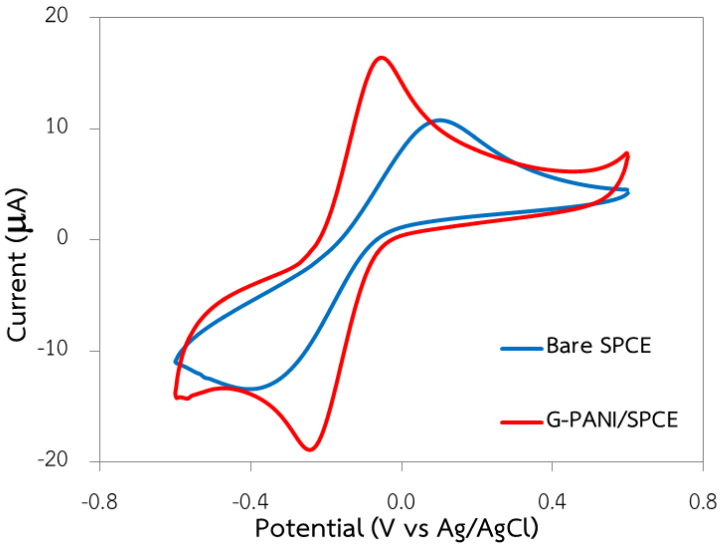

Figure 4.4 Cyclic voltammogram of 1 mM ferricyanide in 0.1 M KCl with scan rate 100 mV s^{-1} on the G-PANI/SPCE compared with bare SPCE

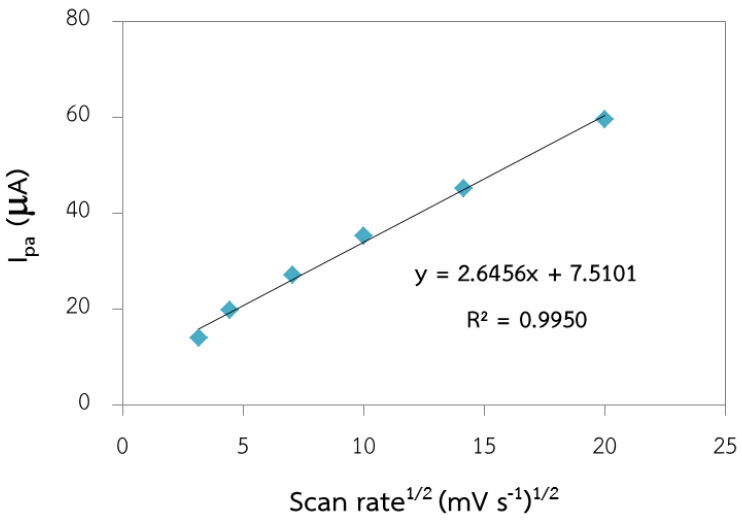

Figure 4.5 The linear relationship between square root of scan rate and anodic peak current of 1 mM GA measured on G-PANI/SPCE.

4.2.2 Effect of the number of printed layers

The electrochemical response of G-PANI/SPCE was affected by the number of inkjet-printed G-PANI layers. The number of printed layer in the range of 0-5 layers was optimized as shown in the Figure 4.6. It can be seen that, when the number of layers increased from 0 to 2 layers, the anodic peak current dramatically increased. In case of over 3 printing layers, the current significantly decreased. We believed that the excess number of printed layers of G-PANI conductive ink leads to a thicker film, which blocks the electron transfer. In addition, the decrease of an anodic peak current may be concern the aggregation of excess G amount on the SPCE surface. In this work, the optimal number of printed layers was 2 layers because it provides the highest electrochemical sensitivity and also provides the constant electrochemical response when compared to 1 layer or 3 layers. In general, the inkjet printing technique is cost effective method for electrode modification because this technique require a very small volume of electrode

modifier in the picoliter level (2-12 pL). Therefore, there is no significant difference in cost effective between 1 and 2 layers.

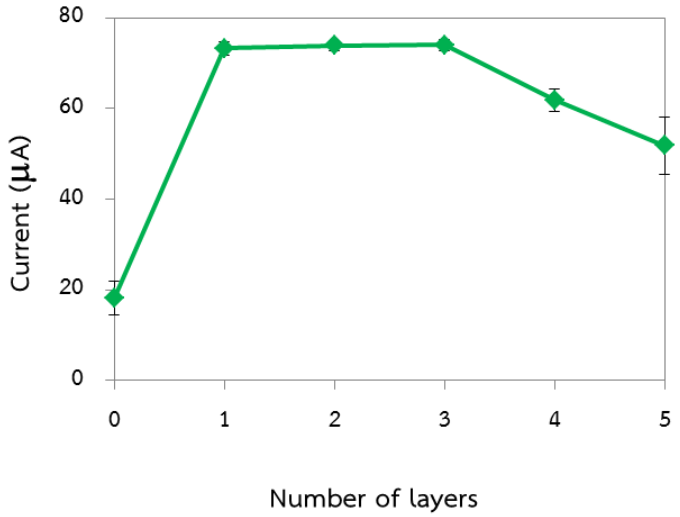

Figure 4.6 Effect of number of inkjet-printed layer on anodic peak current of 1 mM GA.

4.3 Electrochemical detection of antioxidants using G-PANI/SPCE

Cyclic voltammetry of each polyphenol antioxidants using the optimized G-PANI/SPCE at scan rate of 100 mV s^{-1} was performed. Figure 4.7, 4.8, 4.9 and 4.10, shows the cyclic voltammograms of GA, EGC, C, and CFA, respectively. The well-defined irreversible peaks of GA, EGC, and C were obtained while a clearly reversible peak of CFA was achieved. The proposed G-PANI/SPCE demonstrated a well-defined cyclic voltammograms and provided the higher current response when compared to those of bare SPCE. Moreover, the peak potentials of EGC and CFA significantly shifted to less negative potential with the potential difference of 200 mV. Also, the anodic peak potential of C slightly shifted to less negative potential with the potential difference of 50 mV approximately. It is obviously seen in Figure 4.11 that using G-PANI/SPCE can increase the peak current around 2-4 times and the shift of peak potential also enhance. This result indicated that the nanocomposite of G-PANI

exhibited the electrocatalytic activity toward the electro-oxidation of all polyphenols of interest. Furthermore, the proposed G-PANI/SPCE could be an alternative and promising choice of working electrode for sensitive antioxidants detection owing to simple fabrication, low-cost electrode material, and high-throughput modification process of G-PANI/SPCE. For the further experiments, the UHPLC separation system will be coupled with inkjet-printed G-PANI/SPCE for applying to simultaneously determine four polyphenolic antioxidants.

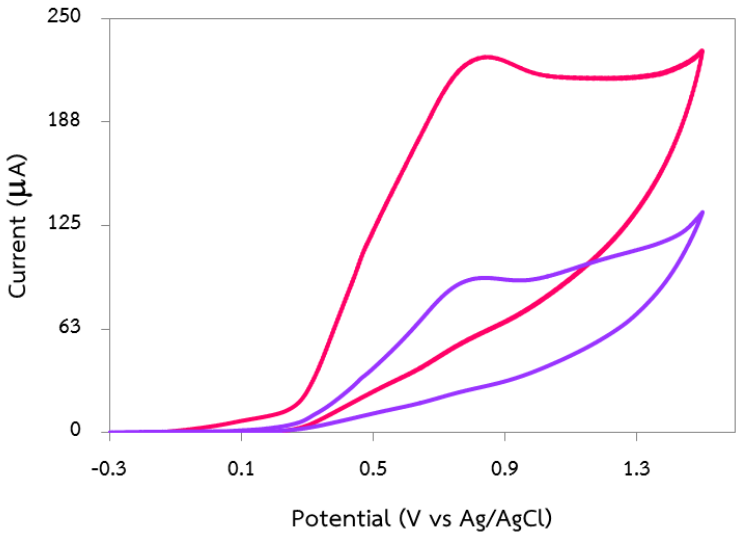

Figure 4.7 Cyclic voltammogram of 170.12 µg mL^{-1} GA measured on the G-PANI/SPCE compared to the bare SPCE in the 0.1 M phosphate solution pH 3: acetonitrile (90:10, %v/v) at a scan rate of 100 mV s^{-1}.

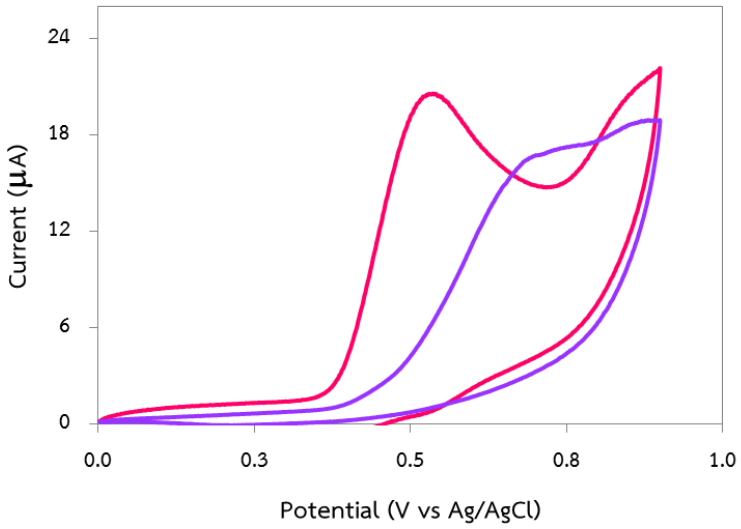

Figure 4.8 Cyclic voltammogram of 50 µg mL^{-1} EGC measured on the G-PANI/SPCE compared to the bare SPCE in the 0.1 M phosphate solution pH 3: acetonitrile (90:10, %v/v) at a scan rate of 100 mV s^{-1}.

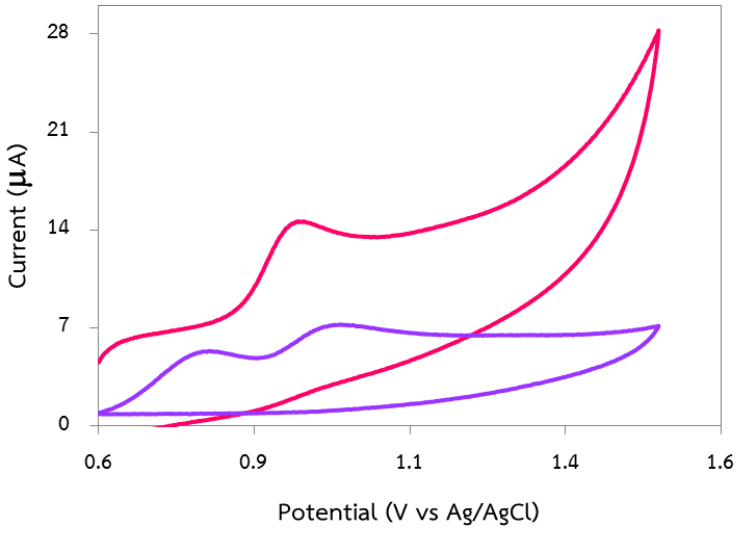

Figure 4.9 Cyclic voltammogram of 10 µg mL^{-1} C measured on the G-PANI/SPCE compared to the bare SPCE in the 0.1 M phosphate solution pH 3: acetonitrile (90:10, % v/v) at a scan rate of 100 mV s^{-1}.

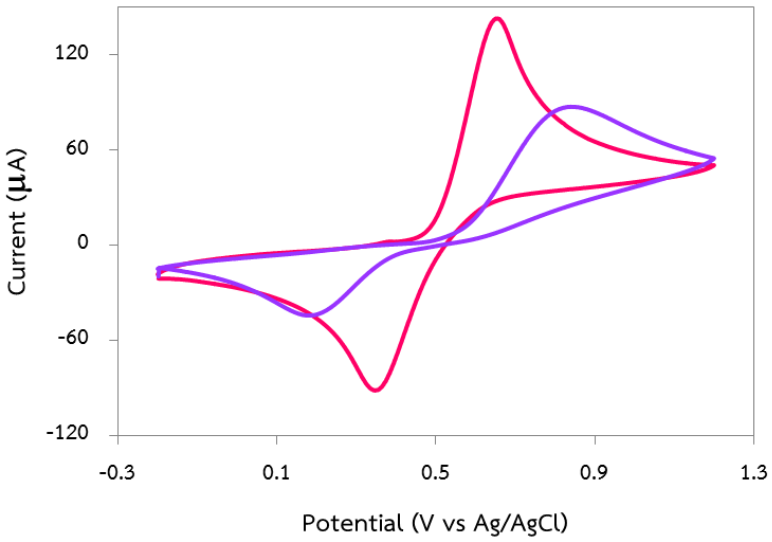

Figure 4.10 Cyclic voltammogram of 180.16 µg mL^{-1} CFA measured on the G-PANI/SPCE compared to the bare SPCE in the 0.1 M phosphate solution pH 3: acetonitrile (90:10, %v/v) at a scan rate of 100 mV s^{-1}.

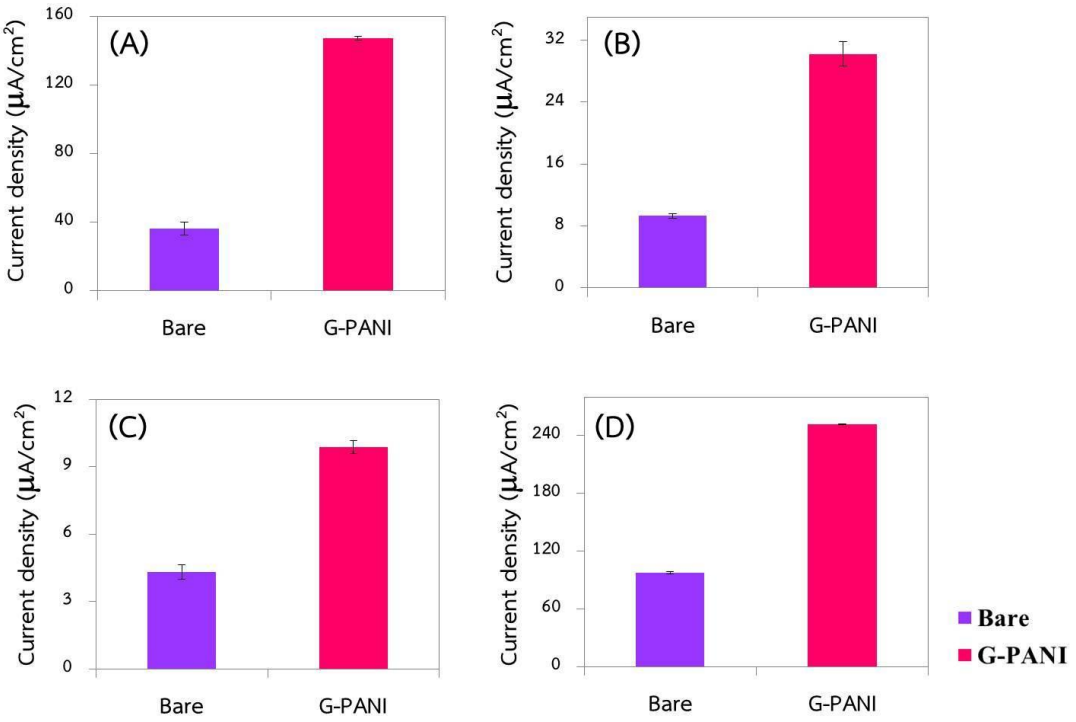

Figure 4.11 The comparison of oxidation current density of GA (A), EGC (B), C (C), and CFA (D) when compared G-PANI/SPCE with bare SPCE

4.4 Chromatographic conditions

To provide additionally selective determination of antioxidants, UHPLC systems coupled with G-PANI/SPCE was applied for the separation and simultaneous detection of polyphenolic antioxidants in tea. The reversed phase C18 column was chosen as a stationary phase for the separation of four antioxidants in the UHPLC-ECD system. The mobile phase containing 0.1 M phosphate buffer solution pH 3 and acetonitrile was selected since it offers the preferable separation and peak shapes. The detail of study will be illustrated as the following parts.

4.4.1 Effect of mobile phase composition

The effect of acetonitrile percentage used in the mobile phase on the retention characteristics of polyphenolic antioxidants was investigated. The total elution time was longer than 10 min when the percentage of acetonitrile was 5%. Moreover, for above 15% (%v/v) acetonitrile, a shorter elution time was obtained, but low resolution and sensitivity were observed. UHPLC-ECD chromatograms of a standard mixture of GA, EGC, C and CFA in different ratios of acetonitrile were shown in Figure 4.12. Finally, a 0.1 M phosphate buffer pH 3 and acetonitrile in the ratio of 90:10 (v/v) was selected as the optimal mobile phase because of resulting in the appropriate resolution of well-defined peaks with the appropriately high current response as shown in Figure 4.13.

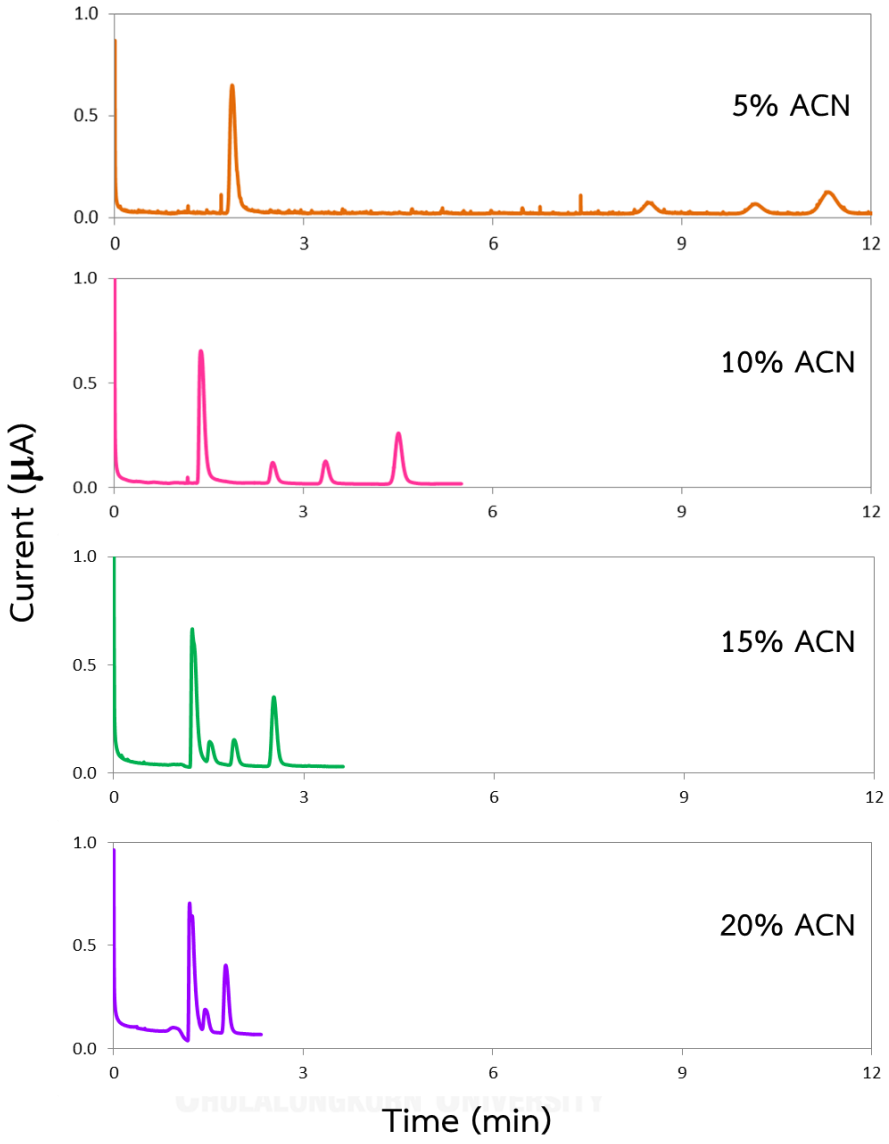

Figure 4.12 UHPLC-ECD chromatograms of a standard mixture of GA, EGC, C, and CFA at 1 µg mL^{-1} in 0.1 M phosphate buffer solution (pH 3): acetonitrile at different ratios.

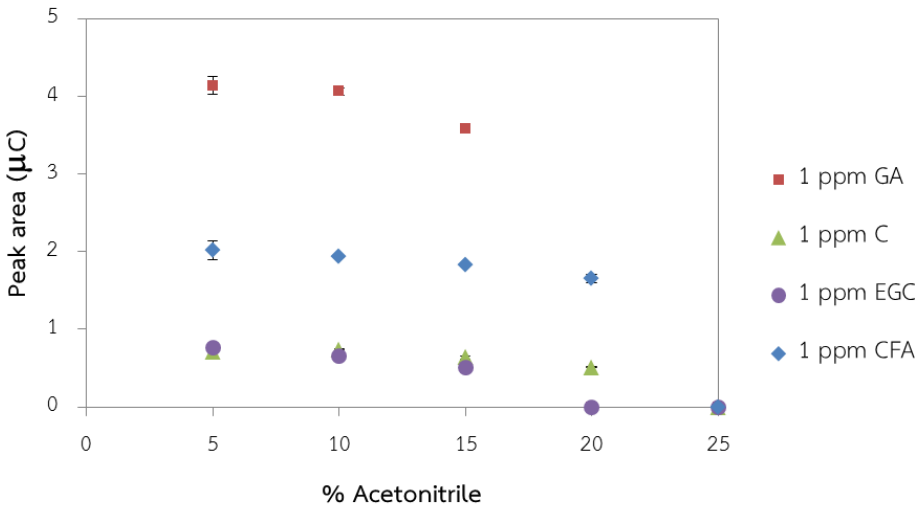

Figure 4.13 The effect of % acetonitrile on the peak area of GA, EGC, C, and CFA at 1 μg mL^{-1} in 0.1 M phosphate buffer (pH 3) solution: acetonitrile.

4.4.2 Effect of pH of phosphate buffer solution

The influence of pH of mobile phase on chromatographic separation efficiency and current response was studied. As shown in Figure 4.14, the pH of phosphate buffer solution in the range of 2 to 7 was optimized. It is evident that the current response of all polyphenol antioxidants increased with pH value from 2 to 3. Then, their current responses decreased when pH value increased more than 3. Therefore, the pH 3 was suitable pH for simultaneous detection of all four antioxidants due to high current response, well separation and well-defined peak shapes.

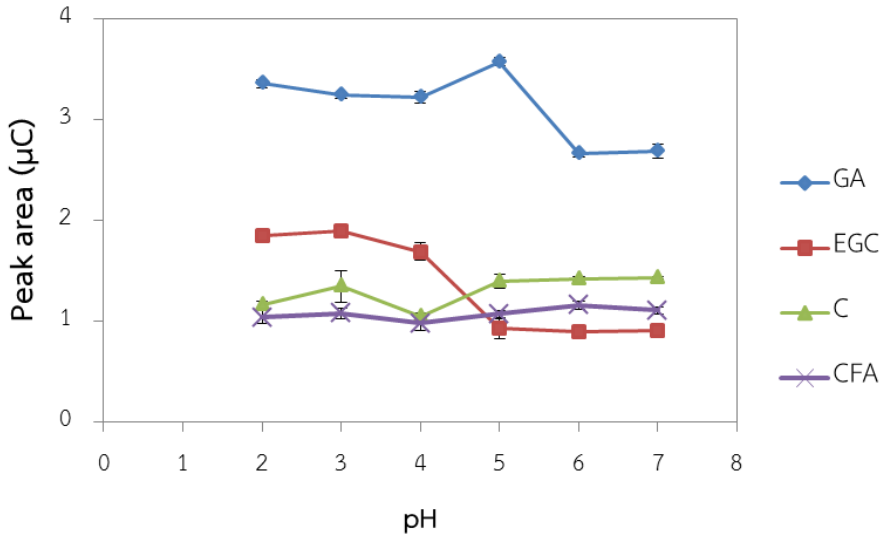

Figure 4.14 The effect of pH on current response of GA, EGC, C and CFA at 1 µg mL^{-1} in 0.1 M phosphate buffer solution:acetonitrile (90:10, %v/v). Applied potential was +0.7 V vs. Ag/AgCl.

4.4.3 Effect of flow rate

Normally, the flow rate influences on elution time. The flow rate of mobile phase was optimized in the range of 0.6 to 1.1 mL min^{-1}, and amperometric results of four antioxidants were observed. As shown in the Figure 4.15, at lower flow rate, higher current response was obtained but it might take a long time to separate antioxidants. Conversely, higher flow rate may affect the chromatographic separation that provides adequate time for interaction between analyte of interest and stationary phase. Thus, the suitable flow rate was 0.8 mL min^{-1} because it compromised between high current response and fast analysis.

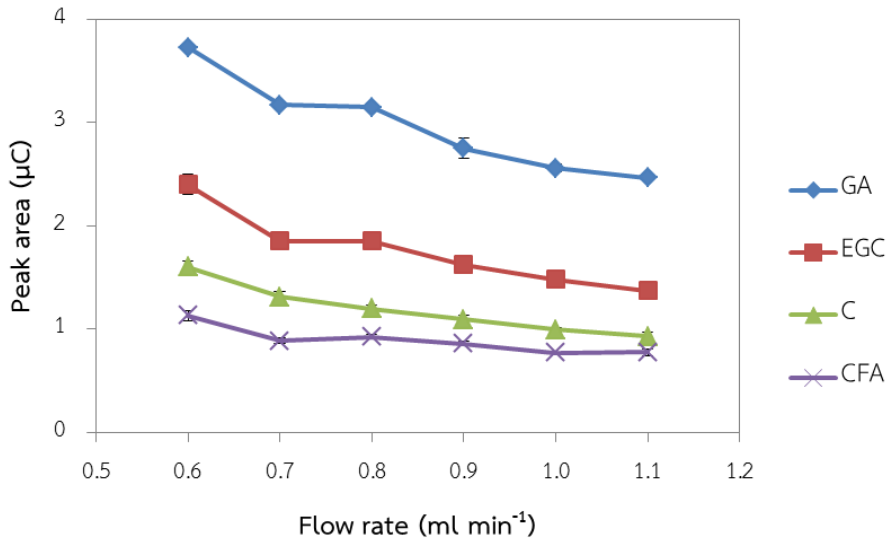

Figure 4.15 The effect of flow rate of GA, EGC, C, and CFA at 1 μg mL^{-1} in 0.1 M phosphate buffer solution (pH 3):acetonitrile (90:10, %v/v). Applied potential was +1.2 V vs. Ag/AgCl.

4.4.4 Hydrodynamic voltammetry

The detection potential affects the sensitivity and selectivity of the proposed electrode. To obtain high current signal, hydrodynamic voltammetry was employed within a detection potential ranging from 0.8–1.4 V vs. Ag/AgCl to optimize the detection potential of each polyphenolic antioxidant set for the electrochemical detection in UHPLC system as shown in the Figure. 4.16. Figure 4.17 is hydrodynamic voltammogram which exhibited the peak area (A_p) of antioxidant and signal-to-background (S/B), at each detection potentials. It was found that the S/B ratio of four antioxidants initially increased with the detection potential until the potential of +1.2 V vs. Ag/AgCl. And then, S/B ratio decreased as the detection potential increased because at high detection potential provided the high background current as well. Accordingly, the detection potential of +1.2 V was selected as optimum detection potential for amperometric detection of four polyphenolic antioxidants following their UHPLC separation.

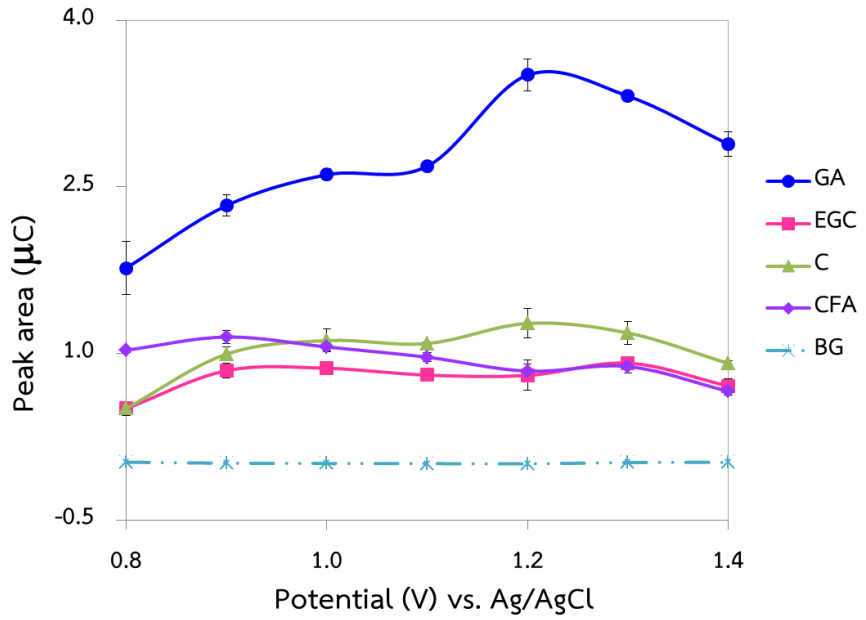

Figure 4.16 Hydrodynamic voltammetric results at the G-PANI/SPCE for a 1 µg mL^{-1} each analyte of antioxidants. Data are shown as the mean ±1 SD derived from three independent repetitions.

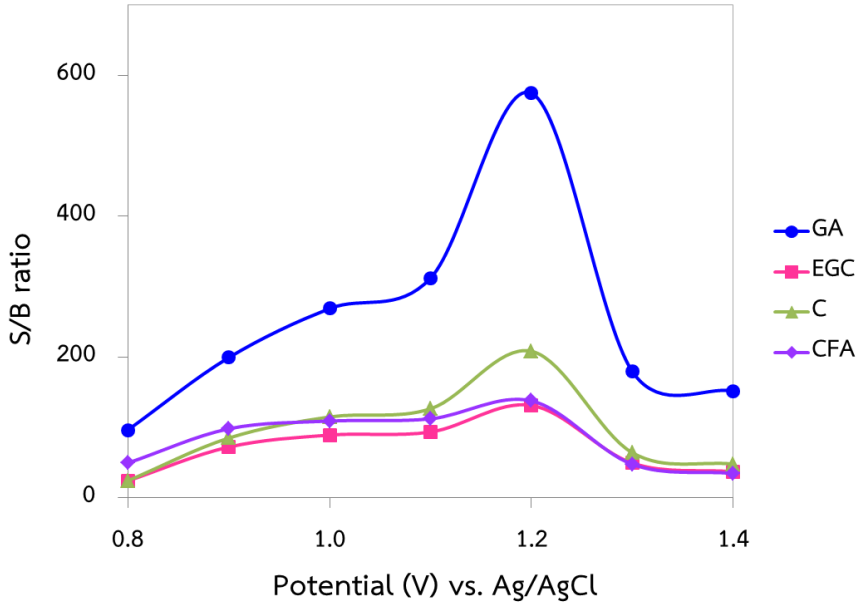

Figure 4.17 Hydrodynamic voltammogram of signal-to-background ratios at inkjet-printed G-PANI/SPCE.

4.4.5 The optimal conditions for UHPLC separation with ECD

The UHPLC-ECD conditions were optimized on the basis of elution time, peak resolution, and baseline drift in the chromatograms. For simultaneous determination of four polyphenol antioxidants, the optimal conditions were summarized in Table 4.1. Using the optimized parameters, four antioxidants were successfully separated within 3 min, and the high current responses were obtained for all antioxidants as shown in the UHPLC chromatogram; Figure 4.18. The retention time of GA, EGC, C and CFA was 0.8, 1.6, 2.2, and 2.9 min, respectively. It was found that this proposed UHPLC-ECD system offers not only rapid measurement but also high sensitivity for simultaneous determination of these antioxidants in tea.

Table 4.1 The optimal chromatographic conditions for UHPLC-ECD

Parameters	Optimal conditions
Column	Kinetex™ core-shell C18 column (50 mm x 4.6 mm i.d.; particle size, 2.6 μm)
Mobile phase	0.1 M phosphate buffer (pH 3): ACN (90:10, %v/v)
Injection volume	50 μL
Flow rate	0.8 mL min^{-1}
Amperometric detection potential	+1.2 V vs. Ag/AgCl
Working electrode	Inkjet-printed G-PANI/SPCE
Temperature	Room temperature (~25 °C)

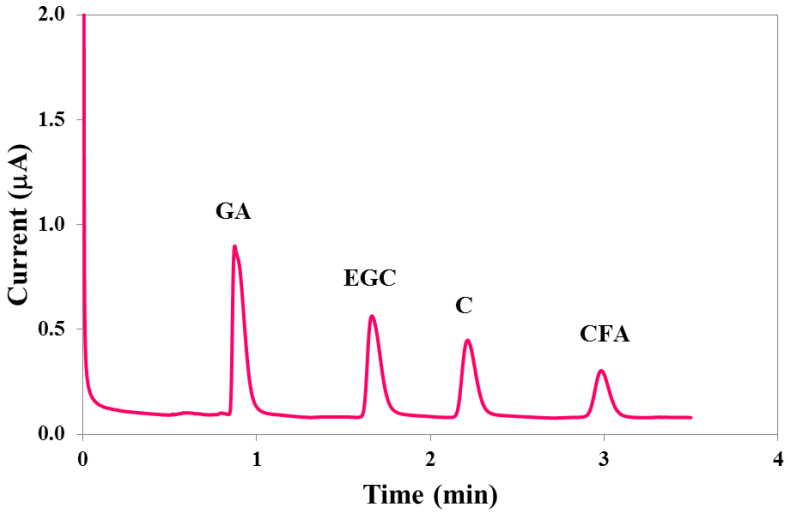

Figure 4.18 UHPLC-ECD chromatogram of 1 µg mL^{-1} GA, EGC, C, and CFA in a mobile phase of 0.1 M phosphate buffer (pH 3) solution: acetonitrile (90:10). Conditions: the optimal flow rate of 0.8 mL min^{-1}, injection volume of 50 µL and applied potential of +1.2 V vs. Ag/AgCl.

4.5 Method validation

4.5.1 Analytical performance

Linear regression analysis for the four polyphenolic antioxidants including GA, EGC, C, and CFA was achieved. The A_p obtained and concentration of each polyphenolic antioxidant were subjected to linear regression analysis as displayed in Figure 4.19, 4.20, 4.21 and 4.22. The linear calibration of GA was found to be from 0.1 to 10 µg mL^{-1} while other linear calibrations of EGC, C, and CFA were found in the range from 0.01 to 10 µg mL^{-1}. The correlation coefficients (R^2) of linearity of the plots (n = 3) is higher than 0.994.

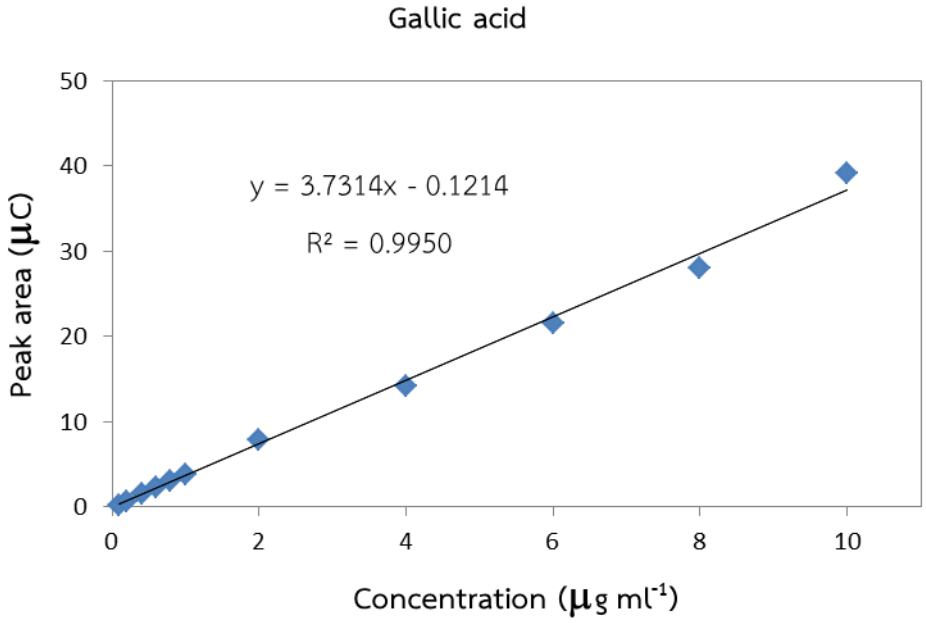

Figure 4.19 The calibration curve of GA in a range of 0.1 to 10 µg mL^{-1} from UHPLC-ECD using inkjet-printed G-PANI/SPCE.

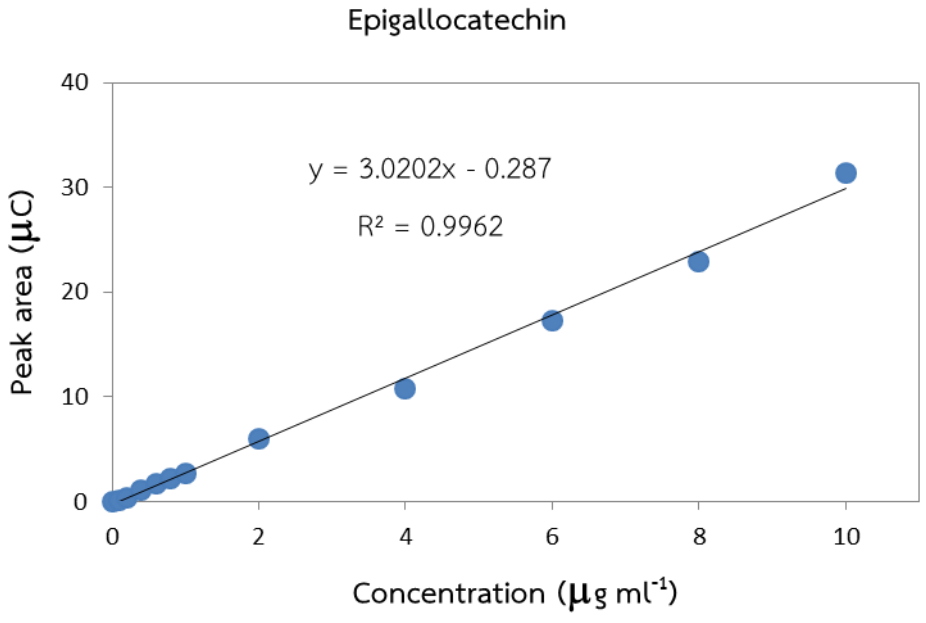

Figure 4.20 The calibration curve of EGC in a range of 0.01 to 10 µg mL^{-1} from UHPLC-ECD using inkjet-printed G-PANI/SPCE.

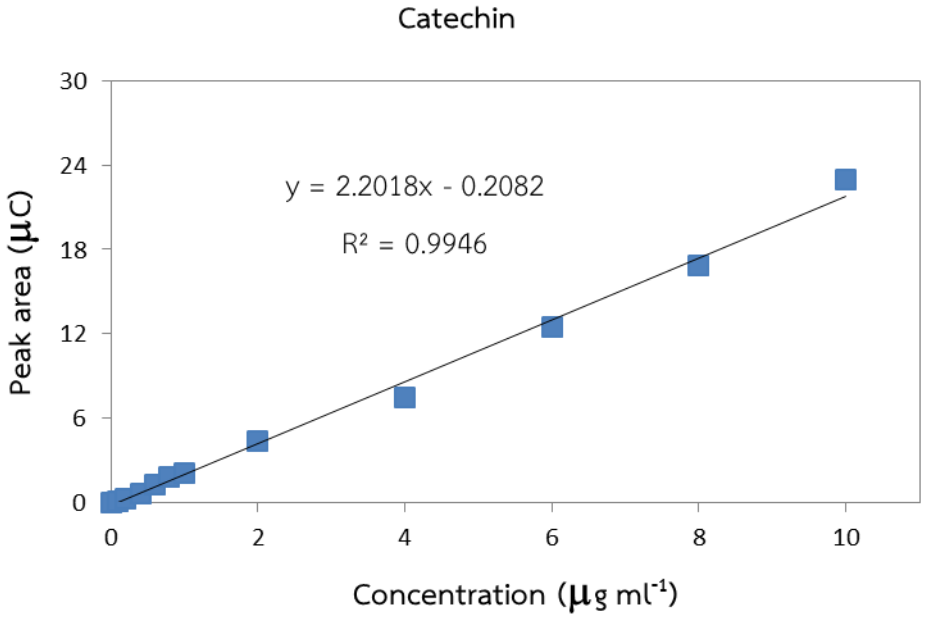

Figure 4.21 The calibration curve of C in a range of 0.01 to 10 µg mL^{-1} from UHPLC-ECD using inkjet-printed G-PANI/SPCE.

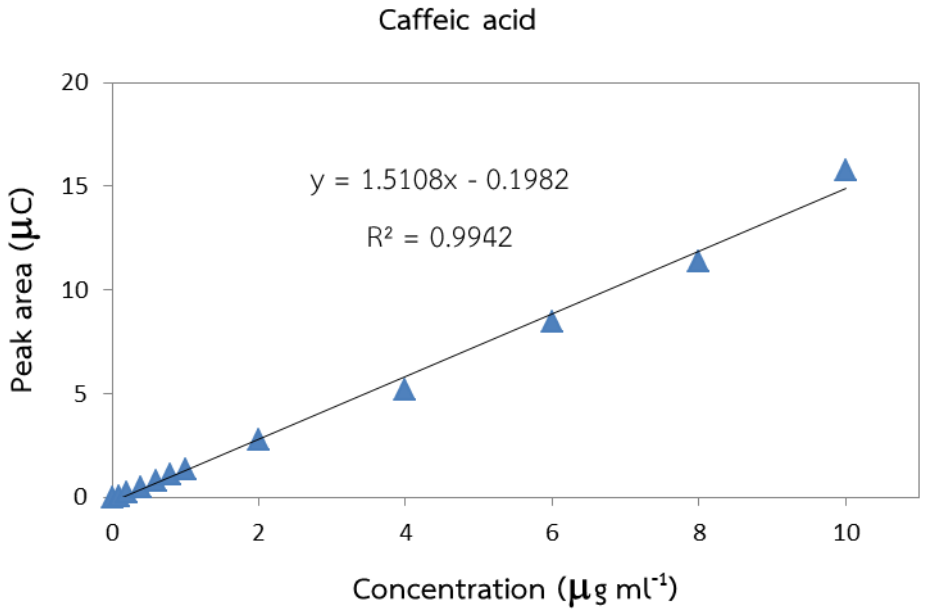

Figure 4.22 The calibration curve of CFA in a range of 0.01 to 10 µg mL^{-1} from UHPLC-ECD using inkjet-printed G-PANI/SPCE.

The sensitivity of the proposed method was evaluated by calculating the limits of detection (LOD= $3SD_b/S$) and limit of quantification (LOQ= $10SD_b/S$). LOD and LOQ were found to be in the range from 1.38 to 1.94 ng mL^{-1} and 4.59 to 6.46 ng mL^{-1}, respectively. The analytical performance of this UHPLC-ECD system (linear range, calibration equation, R^2, LOD and LOQ) were reported in Table 4.2.

Table 4.2 Linearity, Limit of detection (LOD) and Limit of quantitative (LOQ) of four antioxidants.

Analyte	Linear range (μg mL^{-1})	Calibration equations	R^2	LOD (ng mL^{-1})	LOQ (ng mL^{-1})
GA	0.1-10	y=3.7314x-0.1214	0.9950	1.38	4.59
EGC	0.01-10	y=3.0202x-0.2870	0.9962	1.80	5.97
C	0.01-10	y=2.2018x-0.2082	0.9946	1.94	6.46
CFA	0.01-10	y=1.5108x-0.1982	0.9942	1.93	6.43

4.5.2 Repeatability and reproducibility

To evaluate the precision of the method, the repeatability and reproducibility of the methodology was determined. For the intra-day precision, the repeatability of inkjet-printed G-PANI/SPCE of thirty measurements was determined on the same proposed electrode (shown in Figure 4.23) with the relative standard deviation (RSD, n=3) of the four antioxidants current response. It can be seen that the A_p obtained resembled in repeated measurements with RSDs in the range of 2.01 to 5.46% for four antioxidants. The reproducibility was also measured using ten different electrodes (shown in Figure 4.24). The results showed that the RSDs were

obtained in a range of 1.38 to 4.87% for four antioxidants, exhibiting acceptable level.

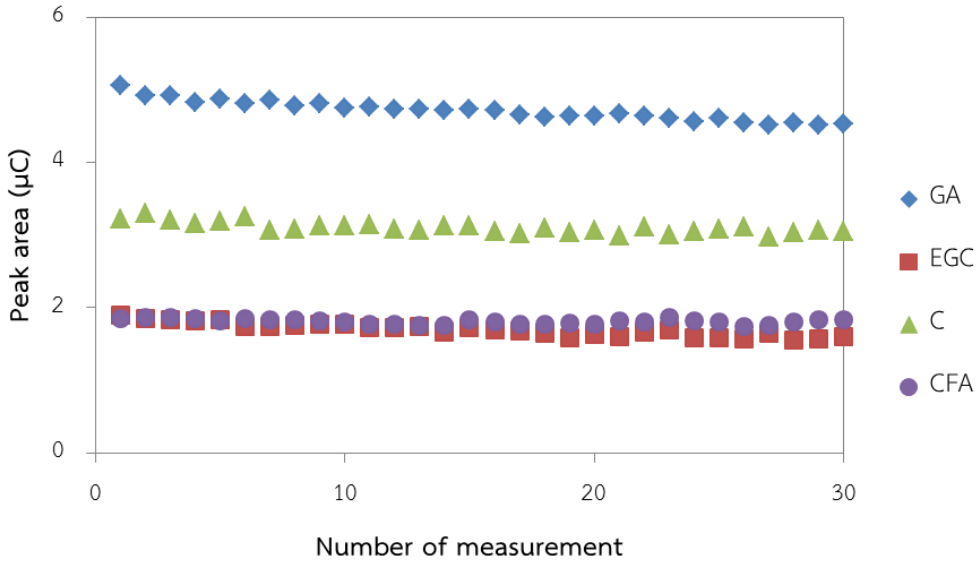

Figure 4.23 The intra-day precision of thirty measurements to study the stability of inkjet-printed G-PANI/SPCE in a mobile phase of 0.1 M phosphate buffer (pH 3) solution: acetonitrile (90:10). Conditions: the flow rate of 0.8 mL min^{-1}, injection volume of 50 µL and applied potential of +1.2 V vs. Ag/AgCl.

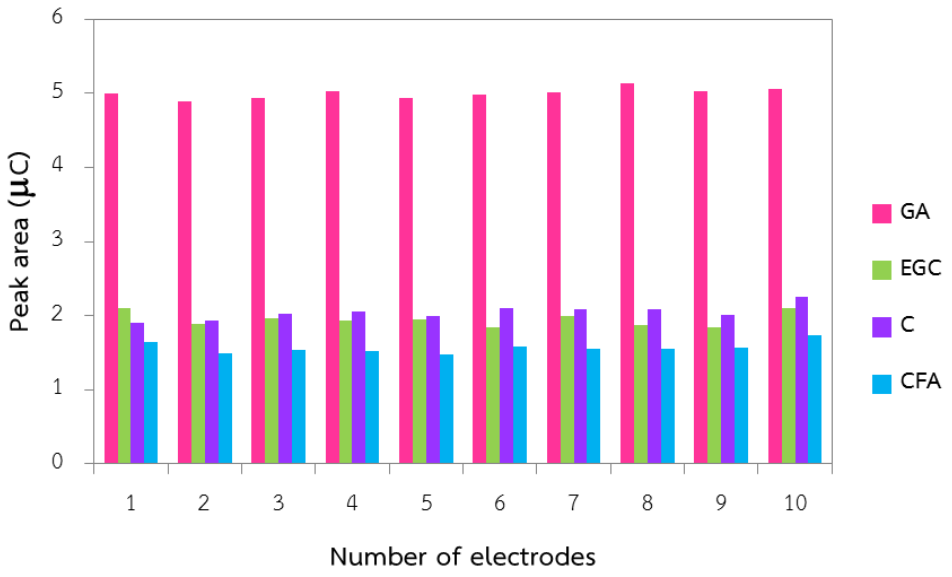

Figure 4.24 The intra-day precision of ten different electrodes measurements to study the stability of inkjet-printed G-PANI/SPCE in a mobile phase of 0.1 M phosphate buffer (pH 3) solution: acetonitrile (90:10). Conditions: the flow rate of 0.8 ml min^{-1}, injection volume of 50 µL and applied potential of +1.2 V vs. Ag/AgCl.

4.6 Application in real samples

4.6.1 The simultaneous separation and detection of four antioxidants in tea samples

In order to apply proposed method, the antioxidants in tea samples, i.e. black tea, white tea, oolong tea and green tea were carried out using the UHPLC-ECD system. Quantification of antioxidants was succeeded by the standard addition method to examine the reliability of this proposed system. UHPLC-ECD chromatograms of appropriately diluted black tea, white tea, oolong tea and green tea were shown in Figure 4.25. Peak identification was comprehended by retention times and verified by spiking each standard of antioxidant in the tea samples. Among the considered tea samples, black tea contain high amount of GA

(1.84 mg g^{-1}) which is possibly owing to liberation of GA from catechin gallates during fermentation of black tea. Oolong tea had the highest amount of CFA (0.68 mg g^{-1}), whereas green tea had the lowest (0.15 mg g^{-1}). In addition, white tea contained the highest content of C (2.56 mg g^{-1}), while black tea contained less (0.32 mg g^{-1}).

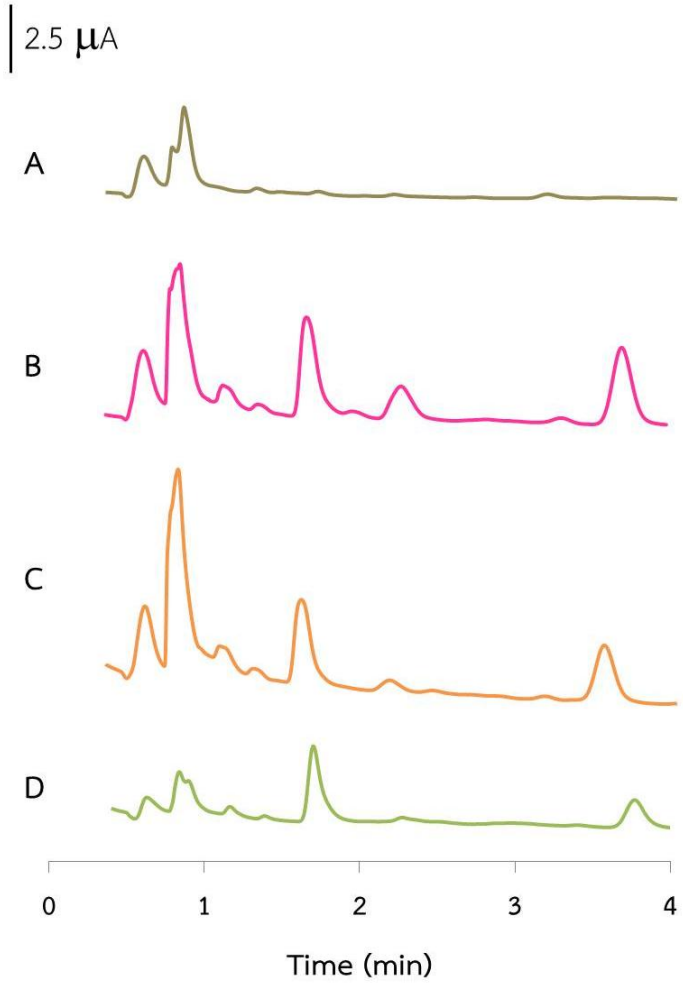

Figure 4.25 UHPLC-ECD chromatograms of (A) black tea diluted 30-fold, (B) white tea diluted 30-fold, (C) oolong tea diluted 30-fold and (D) green tea diluted 30-fold measured on inkjet-printed G-PANI/SPCE in a mobile phase of 0.1 M phosphate buffer (pH 3) solution: acetonitrile (90:10)

4.6.2 Accuracy

The accuracy was reported as the recovery percentage. The intra-day and inter-day precision and recovery for the antioxidants determination in all tea samples was illustrated in Table A3. These results indicated recovery percentages of GA, EGC, C, and CFA were in a range of 83.9–110.0% with RSDs < 5% at five concentrations of standard GA, EGC, C and CFA (1, 3, 5, 7, and 9 μg mL^{-1}) which calculated from standard addition method. The results obtained illustrated the high accuracy in determination. Therefore, this proposed method is an alternative for rapid separation and simultaneous determination of antioxidants in tea. The proposed method was also validated by the standard method using UHPLC coupled with ultraviolet detection (UHPLC–UV). A paired t-test at 95% confidential interval was achieved on the results obtained. The experimental t-value ($t_{calculated}$) of this novel method was found to be 1.7318 which is lower than critical t-value (2.1788) as shown in Table 4.3. It can be concluded that there is no significantly difference between the proposed UHPLC–ECD and the conventional UHPLC–UV methods. Hence, the proposed method is extremely suitable and can be used as an alternative assay for the detection of antioxidant with highly sensitive and rapid analysis.

Table 4.3 Content of polyphenol in tea samples

Samples	Analyte	Amount found (mg g^{-1}) (x ± SD)	
		UHPLC-ECD	UHPLC-UV
Black tea	GA	1.8442 ± 0.10	1.3073 ± 0.01
	EGC	0.0986 ± 0.02	0.1120 ± 0.01
	C	0.3215 ± 0.09	0.6047 ± 0.01
	CFA	0.4325 ± 0.04	0.4871 ± 0.01
White tea	GA	1.4032 ± 0.15	0.7030 ± 0.03
	C	2.5617 ± 0.02	2.0877 ± 0.37
	CFA	0.3851 ± 0.08	0.1703 ± 0.17
Oolong tea	GA	0.5750 ± 0.05	0.6690 ± 0.06
	C	0.6865 ± 0.06	0.5140 ± 0.57
	CFA	0.6828 ± 0.20	0.2661 ± 0.01
Green tea	GA	0.1516 ± 0.11	0.2325 ± 0.03
	C	0.4813 ± 0.15	0.5472 ± 0.04
	CFA	0.1522 ± 0.06	0.1966 ± 0.02
Paired two-tail test	t values	1.7318	
	t critical	2.1788	

CHAPTER V
CONCLUSIONS

G-PANI conductive ink was modified on SPCE by inkjet printing to create a superb performance of electrode leading to improve the electrochemical sensitivity of the antioxidants detection in the UHPLC system. From cyclic voltammetric response, an outstanding G-PANI/SPCE exhibited higher electrochemical sensitivity with increase (2-4 times) of peak current of each antioxidant, compared to bare SPCE. The optimal chromatographic conditions for simultaneous determination of antioxidants were 0.1 M phosphate buffer (pH 3) solution: acetonitrile (90:10, %v/v) and injection volume of 50 µL at flow rate of 0.8 mL min^{-1}. Four antioxidants were successfully separated and quantified within 3 min by amperometric detection with an applied potential of +1.2 V vs Ag/AgCl. The good linear calibrations of four antioxidants were found to be 0.01–10 µg mL^{-1}. The LODs (S/N=3) and LOQs (S/N=10) of four antioxidants were found to be 1.38-1.94 ng mL^{-1} and 4.59-6.46 ng mL^{-1}. The developed method was successfully applied for determination of antioxidants in commercially available tea such as white tea, green tea, oolong tea and black tea with good agreement to the UHPLC-UV. The sensitivity, accuracy, and precision of our developed method were acceptable and reliable to determine the antioxidants in real samples. Therefore, this proposed method could be a new or an alternative and rapid method for the determination of antioxidants in tea samples.

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APPENDIX

APPENDIX A

Table A1 The peak area of four antioxidants at the concentration 1 μg mL^{-1} with the various pH in 0.1 M phosphate buffer solution:ACN (90:10, %v/v)

pH	No.	Peak area (μC)			
		GA	EGC	C	CFA
2	1	3.3680	1.8480	1.1220	0.9634
	2	3.3910	1.8880	1.1580	1.0380
	3	3.3030	1.7940	1.2030	1.1200
	Mean	3.3540	1.8433	1.1610	1.0405
	SD	0.0373	0.0385	0.0331	0.0640
3	1	3.2050	1.9410	1.5570	1.1140
	2	3.2780	1.8050	1.3030	1.1110
	3	3.2420	1.9240	1.1820	1.0050
	Mean	3.2417	1.8900	1.3473	1.0767
	SD	0.0298	0.0605	0.1563	0.0507
4	1	3.2510	1.7240	1.0550	0.9699
	2	3.2730	1.7780	0.9949	0.8928
	3	3.1330	1.5670	1.0820	1.0740
	Mean	3.2190	1.6897	1.0440	0.9789
	SD	0.0615	0.0895	0.0364	0.0742

pH	No.	Peak area (μC)			
		GA	GA	GA	GA
5	1	3.9610	0.8844	1.3910	1.0620
	2	3.5860	0.8283	1.4110	1.1230
	3	3.5450	1.0650	1.3800	1.0360
	Mean	3.5713	0.9259	1.3940	1.0737
	SD	0.1872	0.1010	0.0128	0.0365
6	1	2.6180	0.8668	1.4100	1.1190
	2	2.6700	0.9008	1.4350	1.2140
	3	2.6850	0.9269	1.4110	1.1500
	Mean	2.6577	0.8982	1.4187	1.1610
	SD	0.0287	0.0246	0.0116	0.0396
7	1	2.7800	0.9042	1.4110	1.1380
	2	2.6460	0.9136	1.4450	1.1300
	3	2.6250	0.9126	1.4300	1.0530
	Mean	2.6837	0.9101	1.4287	1.1070
	SD	0.0687	0.0042	0.0139	0.0383

Table A2 The peak area of four antioxidants at the concentration 1 μg mL^{-1} with the various flow rate in 0.1 M phosphate buffer solution (pH3):ACN (90:10, %v/v)

Flow rate (mL min^{-1})	No.	Peak area (μC)			
		GA	EGC	C	CFA
0.6	1	3.7270	2.2610	1.5670	1.1040
	2	3.7530	2.4570	1.5630	1.1950
	3	3.7020	2.4880	1.6830	1.0940
	Mean	3.7273	2.4020	1.6043	1.1310
	SD	0.0208	0.1005	0.0556	0.0454
	%RSD	0.5586	4.1841	3.4687	4.0176
0.7	1	3.2060	1.8900	1.2400	0.8586
	2	3.1630	1.7820	1.3320	0.8811
	3	3.1460	1.8920	1.3710	0.9290
	Mean	3.1717	1.8547	1.3143	0.8896
	SD	0.0252	0.0514	0.0549	0.0294
	%RSD	0.7961	2.7708	4.1786	3.3002
0.8	1	3.1950	1.9450	1.2480	0.9511
	2	3.1020	1.8210	1.1890	0.8979
	3	3.1430	1.7930	1.1650	0.9249
	Mean	3.1467	1.8530	1.2007	0.9246
	SD	0.0381	0.0661	0.0349	0.0217
	%RSD	1.2094	3.5645	2.9046	2.3490

Flow rate (mL min^{-1})	No.	Peak area (μC)			
		GA	EGC	C	CFA
0.9	1	2.6090	1.6430	1.0470	0.8667
	2	2.8120	1.6060	1.0910	0.8873
	3	2.8230	1.6320	1.1450	0.8309
	Mean	2.7480	1.6270	1.0943	0.8616
	SD	0.0984	0.0155	0.0401	0.0233
	%RSD	3.5804	0.9535	3.6623	2.7044
1.0	1	2.5510	1.5160	0.9704	0.7615
	2	2.5170	1.4240	0.9971	0.7834
	3	2.6140	1.5140	1.0150	0.7666
	Mean	2.5607	1.4847	0.9942	0.7705
	SD	0.0402	0.0429	0.0183	0.0094
	%RSD	1.5693	2.8899	1.8433	1.2143
1.1	1	2.4800	1.3550	0.9499	0.7742
	2	2.4590	1.4040	0.9641	0.8168
	3	2.4670	1.3690	0.8864	0.7371
	Mean	2.4687	1.3760	0.9335	0.7760
	SD	0.0087	0.0206	0.0338	0.0326
	%RSD	0.3505	1.4976	3.6190	4.1961

Table A3 The intra-day and inter-day of the UHPLC-ECD method (n=3)

Sample	Spiked level ($\mu g\ mL^{-1}$)	Analytes	Intraday		Interday	
			Recovery (%)	RSD (%)	Recovery (%)	RSD (%)
Black tea	1.0	GA	96.6	3.9	106.8	4.4
		C	91.2	2.0	91.0	3.7
		CFA	104.1	1.8	104.8	4.7
	0.5	EGC	90.3	4.9	91.7	2.6
	3.0	GA	106.2	3.7	110.0	2.0
		C	87.9	4.4	89.7	3.0
		CFA	98.6	1.0	98.2	5.0
	1.5	EGC	92.2	5.0	93.5	4.9
	5.0	GA	104.1	4.9	107.1	1.7
		C	91.9	4.5	97.1	5.0
		CFA	99.5	1.8	99.7	4.8
	2.5	EGC	93.1	4.9	97.3	3.4
	7.0	GA	101.9	4.1	103.8	2.3
		C	98.1	4.3	98.8	3.0
		CFA	100.1	1.3	100.0	3.4
	3.5	EGC	97.0	4.9	98.3	0.6
	9.0	GA	99.9	2.9	100.9	2.7
		C	97.8	4.2	97.7	4.3

Sample	Spiked level ($\mu g\ mL^{-1}$)	Analytes	Intraday		Interday	
			Recovery (%)	RSD (%)	Recovery (%)	RSD (%)
		CFA	100.2	1.4	100.2	3.1
	4.5	EGC	98.8	4.9	98.6	3.1
White tea	1.0	GA	89.5	3.1	101.2	4.1
		C	109.4	4.8	108.8	4.9
		CFA	85.9	2.1	103.5	4.8
	3.0	GA	96.2	1.9	100.5	2.6
		C	109.8	5.0	19.7	0.2
		CFA	90.2	4.6	97.8	4.7
	5.0	GA	99.7	2.8	103.0	3.5
		C	109.0	5.0	107.9	2.3
		CFA	97.9	5.0	100.3	4.8
	7.0	GA	99.2	3.0	101.5	5.0
		C	102.7	4.4	102.8	0.4
		CFA	97.1	3.3	100.3	5.0
	9.0	GA	98.5	2.7	99.6	4.7
		C	101.6	4.8	101.5	1.4
		CFA	99.7	2.3	99.9	3.6
Oolong tea	1.0	GA	89.6	3.4	89.3	5.0
		C	109.0	5.0	109.9	4.9

Sample	Spiked level ($\mu g\ mL^{-1}$)	Analytes	Intraday		Interday	
			Recovery (%)	RSD (%)	Recovery (%)	RSD (%)
		CFA	83.9	0.6	88.9	5.0
	3.0	GA	99.9	5.0	93.6	5.0
		C	110.0	5.0	110.0	3.9
		CFA	101.4	3.0	101.4	0.2
	5.0	GA	103.2	4.9	100.5	4.0
		C	108.8	4.9	109.6	5.0
		CFA	104.9	4.8	103.3	0.9
	7.0	GA	102.5	3.6	97.9	3.8
		C	101.9	4.8	103.2	5.0
		CFA	100.4	5.0	99.8	2.2
	9.0	GA	103.5	4.6	98.6	2.7
		C	102.0	5.0	101.5	3.8
		CFA	98.3	5.0	99.1	3.4
Green tea	1.0	GA	110.0	1.6	107.3	3.1
		C	108.8	4.6	96.5	5.0
		CFA	91.4	3.6	90.0	5.0
	3.0	GA	98.7	4.6	99.4	3.8
		C	109.7	4.4	100.9	5.0
		CFA	105.7	0.8	101.9	5.0

Sample	Spiked level ($\mu g\ mL^{-1}$)	Analytes	Intraday		Interday	
			Recovery (%)	RSD (%)	Recovery (%)	RSD (%)
	5.0	GA	103.4	4.9	103.4	5.0
		C	106.2	4.4	105.3	5.0
		CFA	106.1	2.2	103.2	4.9
	7.0	GA	101.6	1.6	99.3	5.0
		C	103.0	4.9	97.0	3.3
		CFA	99.4	1.9	97.4	5.0
	9.0	GA	99.6	2.2	99.4	5.0
		C	100.0	4.8	100.1	5.0
		CFA	99.5	1.0	100.5	4.8

Table A4 The reproducibility of four antioxidants measured on different ten electrodes

Electrodes	Peak area (μC)			
	GA	EGC	C	CFA
1	5.00	2.10	1.90	1.65
2	4.90	1.89	1.94	1.50
3	4.94	1.96	2.03	1.54
4	5.03	1.94	2.05	1.53
5	4.94	1.95	2.00	1.47
6	4.98	1.85	2.11	1.58
7	5.01	2.00	2.08	1.56
8	5.14	1.88	2.08	1.55
9	5.03	1.84	2.01	1.57
10	5.06	2.11	2.25	1.73
Mean	5.00	1.95	2.05	1.57
SD	0.07	0.10	0.10	0.07
%RSD	1.38	4.87	4.73	4.75

APPENDIX B

Table B1 The acceptable values for precision from AOAC manual for peer verified methods program, VA, NOV 1993

Analyte concentration	RSD, %
100 ppm (μg mL^{-1})	± 5.3
10 ppm (μg mL^{-1})	± 7.3
1 ppm (μg mL^{-1})	± 11
100 ppb (ng mL^{-1})	± 15
10 ppb (ng mL^{-1})	± 21
1 ppb (ng mL^{-1})	± 30

Table B2 The acceptable values for accuracy from AOAC manual for peer verified methods program, VA, NOV 1993

Analyte concentration	Recovery, %
100 ppm (μg mL^{-1})	90 – 107
10 ppm (μg mL^{-1})	80 – 110
1 ppm (μg mL^{-1})	80 – 110
100 ppb (ng mL^{-1})	80 – 110
10 ppb (ng mL^{-1})	60 – 115
1 ppb (ng mL^{-1})	40 – 120

VITA

Miss Chayanee Bardpho was born on February 23, 1990 in Nakhon Ratchasima, Thailand. She graduated from Suranari Wittaya School in Nakhon Ratchasima with high school degree in 2007. She received her Bachelor's degree of Science from Thammasat University (2008-2011). After that, she has become a graduate student in the Analytical Chemistry, Department of Chemistry, Faculty of Science, Chulalongkorn University. Furthermore, she has joined the Electrochemistry and Optical Spectroscopy Research Unit (EOSRU) under the direction of Professor Dr. Orawon Chailapakul. She graduated with Master's degree in chemistry of academic year 2014 from Chulalongkorn University.

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- The International Conference on Flow Injection Analysis (ICFIA) held in Fukuoka, Japan, 30th November to 5th December 2014.
- The Pure and Applied Chemistry International Conference 2015 (PACCON2015) held in Bangkok, Thailand, 21st to 23rd January 2015.

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