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ELECTROCHEMICAL DETECTION OF FERULIC ACID BY LAB-ON-PAPER

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A Thesis Submitted in Partial Fulfillment of the Requirements
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ปฤญจพร ทีงาม: การตรวจวัดทางเคมีไฟฟ้าของกรดเฟอรูริกด้วยอุปกรณ์ปฏิบัติการบนกระดาษ (ELECTROCHEMICAL DETECTION OF FERULIC ACID BY LAB-ON-PAPER) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: รศ.ดร. อรวรรณ ชัยลภากุล, 97 หน้า

งานวิจัยนี้ ได้พัฒนาอุปกรณ์ปฏิบัติการบนกระดาษร่วมกับการตรวจวัดทางเคมีไฟฟ้าและการตรวจวัดเชิงสี สำหรับวิเคราะห์กรดเฟอรูลิกและสารต้านอนุมูลอิสระทั้งหมดในคราวเดียวกัน ช่องไหลสารบนกระดาษถูกเตรียมด้วยเทคนิคโฟโตลิโทกราฟฟี ตัวตรวจวัดทางเคมีไฟฟ้าสำหรับวิเคราะห์กรดเฟอรูลิกถูกสร้างด้วยเทคนิคพิมพ์สกรีน การหาปริมาณกรดเฟอรูลิกตรวจวัดได้โดยใช้เทคนิคดิฟเฟอร์เรนเชียลพัลส์ ภายใต้สภาวะที่เหมาะสม พบว่า ความสูงของกระแสไฟฟ้าจากปฏิกิริยาออกซิเดชั่นมีความสัมพันธ์เป็นเส้นตรงกับความเข้มข้นของกรดเฟอรูลิกในช่วง 3 ถึง 140 มิลลิกรัมต่อลิตรที่ค่าสัมประสิทธิ์สหสัมพันธ์เท่ากับ 0.9994 ขีดจำกัดต่ำสุดของการวิเคราะห์อยู่ที่ 1 มิลลิกรัมต่อลิตร วิธีการนี้ประสบความสำเร็จในการนำมาประยุกต์ใช้ตรวจวัดกรดเฟอรูลิกในตัวอย่างเครื่องดื่มน้ำข้าวโพด โดยพบว่า เปอร์เซ็นต์การคืนกลับอยู่ในช่วง 96.85 ถึง 103.20 เปอร์เซ็นต์ นอกจากนี้ การตรวจวัดเชิงสี โดยใช้สารโฟลินซิโอคัลเต้เป็นตัวทำปฏิกิริยา ยังประสบความสำเร็จในการนำมาวิเคราะห์หาปริมาณสารต้านอนุมูลอิสระทั้งหมดได้ในคราวเดียวกัน ผลของสีที่เปลี่ยนแปลงไปในบริเวณตรวจวัด สามารถวิเคราะห์ได้โดยใช้กล้องดิจิตัล วิธีที่พัฒนาขึ้นนี้ ยังให้ผลการวิเคราะห์ที่สอดคล้องกับวิธีมาตรฐาน: ไฮเพอร์ฟอร์มานซ์ลิควิดโครมาโตการฟฟีสำหรับกรดเฟอรูลิก และยูวี-วิสสิเบิลสำหรับสารต้านอนุมูลอิสระทั้งหมด ดังนั้น วิธีนี้ จึงเป็นวิธีที่ง่าย รวดเร็ว ราคาไม่แพง และมีความว่องไวสำหรับวิเคราะห์หาปริมาณกรดเฟอรูลิกและสารต้านอนุมูลอิสระทั้งหมดในตัวอย่างอาหารชนิดต่างๆได้ในคราวเดียวกัน

สาขาวิชา ปิโตรเคมีและวิทยาศาสตร์พอลิเมอร์ ลายมือชื่อนิสิต .....

ปีการศึกษา 2554 ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์หลัก .....

5272411723: MAJOR PETROCHEMISTRY AND POLYMER SCIENCE
KEYWORDS: FERULIC ACID / LAB-ON-PAPER / DIFFERENTIAL PULSE VOLTAMMETRY / TOTAL ANTIOXIDANT CAPACITY

PRINJAPORN TEE-NGAM: ELECTROCHEMICAL DETECTION OF FERULIC ACID BY LAB-ON-PAPER. ADVISOR: ASSOC. PROF. ORAWON CHAILAPAKUL, Ph.D., 97 pp.

In this work, lab-on-paper coupled with sensitive electrochemical and simple colorimetric detection was developed to simultaneously determine ferulic acid and total antioxidant capacity. Photolithography was used to create the hydrophilic channels on filter paper. An electrochemical sensor for the determination of ferulic acid was fabricated using screen-printing technology. The quantity of ferulic acid was measured by differential pulse voltammetry. Under optimal conditions, the oxidation peak current showed a good linearity dependent on the concentration of ferulic acid in the range of 3−140 ppm with a correlation coefficient of 0.9994. The limit of detection (LOD) of the proposed method was found to be 1 ppm. This presented method was successfully applied for the determination of ferulic acid in corn cider samples. The recovery of ferulic acid ranged from 96.85 % to 103.20 %. Furthermore, the colorimetric detection was successfully employed by the Folin-Ciocalteau reagent to concurrently quantify the total antioxidant capacity. The results in their color change at the detection zone can be determined by monitoring with the digital camera method. This developed method produced results in good agreement with the standard detection methods: HPLC-UV for ferulic acid and UV-vis spectrophotometry for total antioxidant capacity. Therefore, this method can be used for simple, rapid, inexpensive and sensitive dual and simultaneous quantification of ferulic acid and total antioxidant capacity in a variety of samples.

Field of Study : Petrochemistry and Polymer Science Student's Signature .....
Academic Year : 2011 Advisor's Signature .....

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LIST OF ABBREVIATIONS

UV-vis	ultraviolet-visible
HPLC	High-Performance Liquid Chromatography
RP-HPLC	Reverse phase High-Performance Liquid Chromatography
ppm	part per million
DNA	Deoxyribonucleic aid
HDL	High Density Lipoprotein
AIDS	Acquired Immune Deficiency Syndrome
ORAC	Oxygen Radical Absorbance Capacity
FCR	Folin–Ciocalteu reagent
FA	Ferulic acid
HE	Hesperidin
CA	Cinnamic acid
CAD	Cinnamaldehyde
LOC	Lab-on-a-chip
POC	point-of-care
PDMS	polydimethylsiloxane
RPM	Revolutions per minute
GBL	Gamma-Butyrolactone
PEB	Post-Exposure-Bake
Tg	glass transition temperature
CV	Cyclic voltammetry
DPV	Differential pulse voltammetry
DDAB	Didodecyldimethylammonium bromide

NC	Nitrocellulose
WE	working electrode
RE	reference electrode
CE	counter electrode
in	inch
V	volt
mV	millivolt
cm	centimeter
s	second
^{o}C	degree Celsius
min	minute
M	molar
mM	millimolar
µM	micromolar
g	gram
mL	milliliter
µL	microliter
LOD	limit of detection
LOQ	limit of quantification
R^2	coefficient
SD	standard deviation
RSD	Relative standard deviation

CHAPTER I

INTRODUCTION

1.1 Introduction

Antioxidants are substances that protect cells from damages by reactive oxygen species known as free radicals. They are produced in oxidation reactions in cell caused by unstable molecules. Free radical damage may lead to diseases and cancer. Antioxidants are believed to be the effective nutrients in the prevention of these oxidative stress related diseases. The classes of antioxidants which are found naturally in fruits, vegetables and herbs include i.e. flavonoids, carotenoids, essential oils and phenolic acids.

Ferulic acid (4-hydroxy-3-methoxycinnamic acid) is one of ubiquitous phenolic acids in the plant kingdom. They are widely used in health foods, cosmetics and nutrition restoratives due to being pharmacologically active as an anti-microbial, anti-inflammatory, anti-arrhythmic, anti-thrombosis [1] and also having the ability to protect the radiation of ultraviolet ray. At present, the main quantitative techniques for ferulic acid detection are high–performance liquid chromatography (HPLC) [2, 3], thin-layer chromatography [4, 5], the capillary tube electrophoresis method [6] and spectrophotometry [7]. Among these methods, the detection limits obtained by selected detectors are typically low enough to detect ferulic acid in the samples; however, these assays require laborious sample preparation steps, high equipment costs and a significant amount of labor and analytical resources, which can potentially cause substantial delays in obtaining results so they are not convenient. Thus, efforts to extend the developed methodology and/or to create new devices for ferulic acid detection have been challenging. To reach these goals, Lab-on-paper device coupled with electrochemical detection was designed and developed to rapidly determine ferulic acid.

Lab-on-paper have the potential to be good alternative analytical devices for healthcare related applications because they are portable, easy to use, have a low sample volume requirement and provide rapid analysis [8, 9, 10]. Moreover, the best

concept to create lab-on-paper is the material used. They use paper which is a cellulose fiber with a high surface, easy to chemically modify, inexpensive and easy to get rid of. Photolithography is one of the most widely used fabrication methods that is used to pattern channels of hydrophilic surfaces on filter paper. In order to design the detection scheme, colorimetric detection methods were primarily used to determine multiplex analytes on lab-on-paper devices. The detection was performed by the naked eye. However, the use of the naked eye is affected by many factors including different visual perception, lighting, different cameras and optical scanners, thus, it is complicated. Recently, Dungchai et al [11] successfully solved the problem by fabricating a lab-on-paper device with electrochemical detection. The use of an electrochemical detector is an alternative detection method for many applications and has the benefits of simplicity, speed, sensitivity, low cost, portability and selectivity by selecting a suitable detection potential and electrode material [12].

As mentioned previously, ferulic acid is a potential antioxidant. Thus, the development of a new method for both quantification and evaluation of the antioxidant capacity at the same time is very important. The data obtained should be very useful for nutrition and/or for treatment in the future in case of using this ferulic acid owing to the amount of acitive antioxidant should be related to the antioxidant capacity. To simultaneously obtain the information of the antioxidant capacity corresponding to the amount of ferulic acid, we also designed another part of lab-on-paper for the determination of total antioxidant. The Folin-Ciocalteau reagent is usually used to measure phenolic compounds [13, 14]. Therefore, total soluble phenolics were simultaneously determined with the Folin-Ciocalteau reagent using the colorimetric method on lab-on-paper.

Strong analytical figures with limits of detection in the low ppm range, good sensitivity, excellent response precision and stability were observed with the electrochemical detection as well as obtained from colorimetric detection. Therefore, this innovative concept could contribute to the development of a practical, rapid, highly sensitive and accurate method for assaying ferulic acid levels in food. In addition, this work also presented the possibility of using the proposed method for the simultaneous determination of ferulic acid and total antioxidant in real samples.

1.2 Objective of the research

The two main goals of this work are as follows:

1. To develop lab-on-paper with electrochemical and colorimetric detection for the simultaneous determination of ferulic acid and total antioxidant.
2. To apply lab-on-paper with rapidity, highly sensitive and accurate method for the determination of ferulic acid and total antioxidant capacity in food sample.

1.3 Scope of the research

To achieve the research objective in this work, lab-on-paper with electrochemical and colorimetric detection was developed to determine ferulic acid and total antioxidant capacity simultaneously. Photolithography was used to create hydrophilic channels on filter paper and electrochemical detection was fabricated by the use of screen-printing technology. The influences of pH, step potential and amplitude were examined to obtain the optimal conditions. To quantify the total antioxidant capacity, the colorimetric method was performed during the ferulic acid was measured by electrochemistry. The procedure was then successfully applied to determine ferulic acid and total antioxidant in food samples.

CHAPTER II

THEORY AND LITERATURE SURVEY

2.1 Antioxidants

Antioxidants are chemicals that protect us against unstable products of metabolism, known as free radicals, which are capable of damaging cells and genetic material. As shown in Figure 2.1, the body generates free radicals as the unavoidable byproducts of turning food into energy and others are in the food and the air. Some are generated by sunlight.

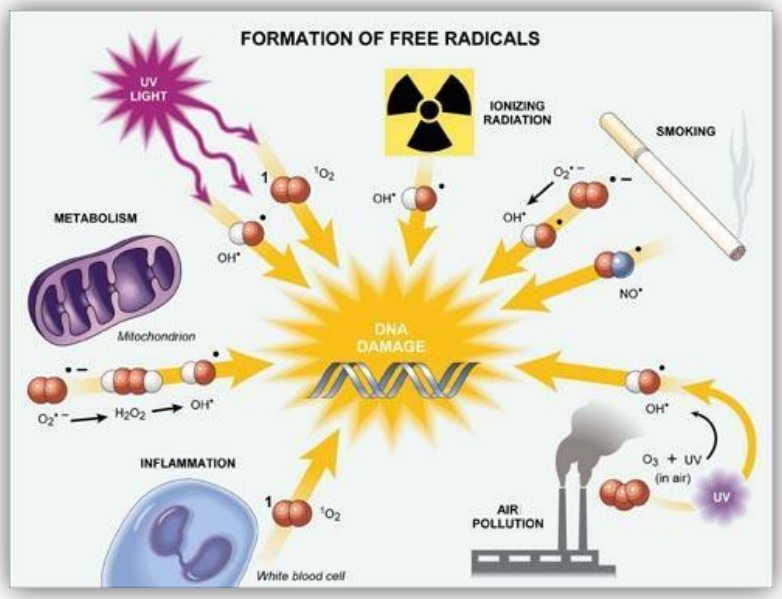


Figure 2.1 Formation of free radicals [15]

2.1.1 Mechanism of Antioxidants

Free radicals are unstable atoms or molecules characterized by at least one unpaired electron. The electrons are stolen from nearby molecules within the cell turning them into a free radical. The cells membrane is damaged by the increase in the chain reaction. Free radicals are neutralized by donating hydrogen from antioxidant. The mechanism of an antioxidant is shown in Figure 2.2.

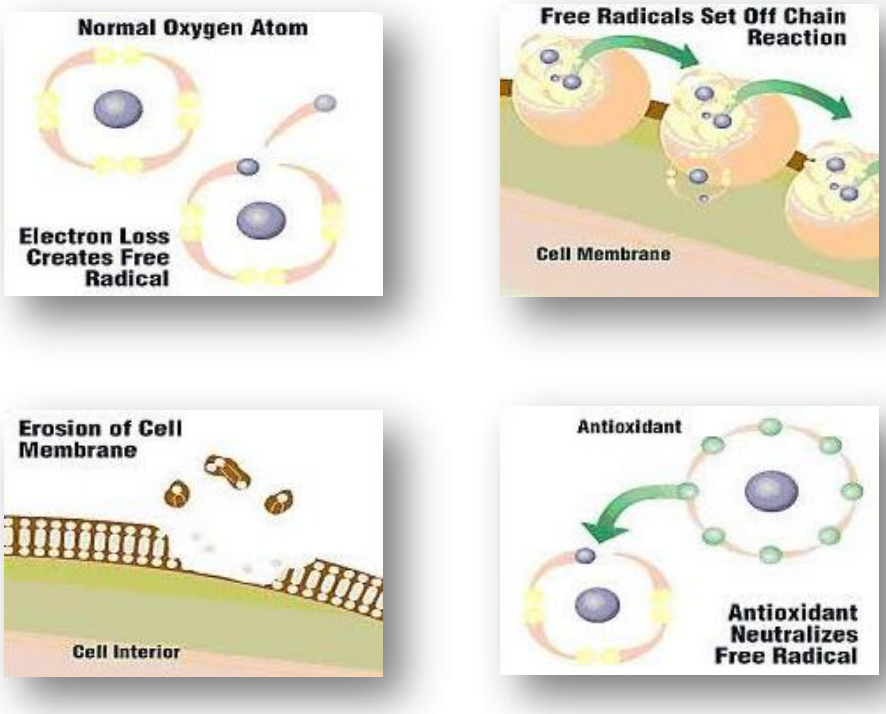


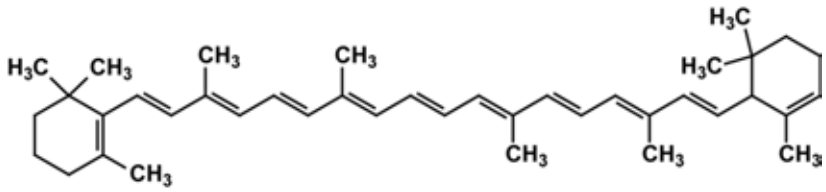
Figure 2.2 Mechanism of Antioxidants [16]

2.1.2 Health benefits of antioxidants

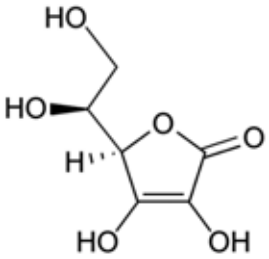
The free radicals can lead to various diseases such as heart disease, cancer, cardiovascular diseases, and cataracts and are also a leading cause for aging. In the skin, free radicals lead to the breakdown of elastin and collagen, resulting in the development of fine lines and wrinkles. Antioxidants have been investigated for the prevention of these diseases. They work by generously giving electrons to free radicals without turning into electron-scavenging substances themselves. Antioxidants are widely used in pharmacology, dietary supplements and cosmetics.

2.1.3 Classes of antioxidant

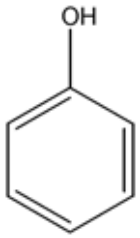
There are many different substances as is shown in Figure 2.3 that can act as antioxidants. The most familiar ones are beta-carotene, vitamins A, C, and E, lycopene and other related carotenoids. In addition, glutathione, coenzyme Q10, lipoic acid, flavonoids, phenolic compounds, polyphenols, phytoestrogens and many more act as antioxidants. Along with trace elements, such as manganese, copper, selenium, and zinc, which also have some antioxidant properties.



Beta-carotene



Vitamin C



Phenol

(The simplest phenolic compound)

Figure 2.3 Antioxidant compounds

2.2 Ferulic acid

Ferulic acid (4-hydroxy-3-methoxycinnamic acid) is one of ubiquitous phenolic compounds in the plant kingdom. The structure of ferulic acid is shown in Figure 2.4. It is naturally found at relatively high concentrations in plant cell walls, leaves and seeds. It generally has the following pharmacological functions of antioxidant, anticoagulant, antimicrobial, anti-inflammatory and anti-arrhythmic.

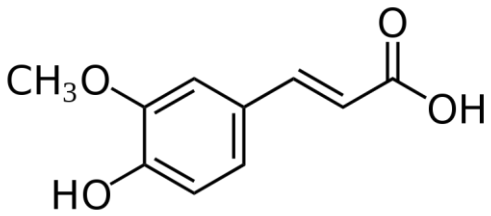


Figure 2.4 Structure of ferulic acid

2.2.1 Antioxidant activity of ferulic acid [17]

Ferulic acid is such a superior antioxidant that it is now widely used in health foods and nutrition restoratives. It forms a resonance stabilized phenoxy radical and shows high scavenging activity for superoxide, hydrogen peroxide, hydroxyl radical and nitrogen dioxide free radicals. Ferulic acid also increases the activity of enzymes that are responsible for scavenging free radicals and inhibits enzymes that catalyze the production of free radicals. Moreover, Ferulic acid can prevent peroxy nitrite-mediated oxidation of dihydrorhodamine 123 and DNA and peroxy nitrite-mediated nitration of protein-bound and free tyrosine.

2.2.2 Cholesterol-lowering activity of ferulic acid [17]

Ferulic acid has been shown to have cholesterol-lowering activity. The cholesterol-lowering activity of ferulic acid indicated that it inhibited cholesterol synthesis by competitively inhibiting the activity of hydroxymethylglutaryl CoA reductase in the liver and increasing excretion of acidic sterol. Furthermore, it was found that ferulic acid can increase the good cholesterol, HDL, and decrease triglycerides.

2.2.3 Anti-cancer effect of ferulic acid [17]

In recent years, the effects of ferulic acid on oral and large bowel carcinogenesis have been studied. Mori et al. reported the effects of ferulic acid on oral cancer in rats by adding ferulic acid to the diet after 5 weeks of exposure to 4-nitroquinoline-1-oxide (4NQO) which had been add to the drinking water. It was found that the incidences of tongue carcinomas and preneoplastic lesions were significantly lower on termination of the experiment than the group with the carcinogen alone. The results suggest that ferulic acid produces a chemopreventive activity on oral cancer.

In addition, many researchers have focused their attention on the anti-cancer activity of ferulic acid on colon and rectum cancer. Kawabata et al. examined the modifying effects of the dietary administration of ferulic acid on azoxymethane (AOM)-induced colon carcinogenesis in rats. It was found that rats which were given

ferulic acid during the initiation phase had lower incidences of colonic carcinomas than those which were given AOM alone.

2.2.4 Antimicrobial and anti-inflammatory activity of ferulic acid [17]

Ferulic acid has been shown to possess inhibitory activity on the growth and reproduction of viruses such as influenza virus, respiratory syncytial virus and AIDS. Ferulic acid is the active component of the rhizoma of *Cimicifuga* species, which is used frequently as an anti-inflammatory drug in Japanese Oriental medicines. Hirabayashi et al investigated that ferulic acid reduced the levels of murine interleukin-8 (IL-8) which is produced in response to the influenza virus. Sakai et al investigated that ferulic acid reduced macrophage inflammatory protein-2 (MIP-2) in a murine macrophage cell line in response to the respiratory syncytial virus (RSV) infection. These results suggest that ferulic acid might be responsible, at least in part, for the anti-inflammatory drug effect

Edeas et al [18] presented that ferulic acid and its derivatives inhibited the AIDS virus, suggesting it to be a potential chemopreventive agent for AIDS. Moreover, ferulic acid possesses a wide spectrum of antimicrobial activity. It exhibited antimicrobial activity towards Gram-positive bacteria, Gram-negative bacteria and yeasts.

2.3 Antioxidant capacity assays

Determination of antioxidants is not a straightforward process, as this is a diverse group of compounds with different reactivities to different reactive oxygen species. In food science, the oxygen radical absorbance capacity (ORAC) has become the current industry standard for assessing the antioxidant strength of whole foods, juices and food additives. Other measurement tests include the Folin-Ciocalteu reagent, and the Trolox equivalent antioxidant capacity assay.

2.3.1 Total Phenols Assay by Folin-Ciocalteu Reagent [19]

The Folin–Ciocalteu reagent (FCR) or Folin's phenol reagent -based assay is commonly known as the total phenols (or phenolic) assay and has gained popularity. FCR is a mixture of phosphomolybdate and phosphotungstate. It is used

for the colorimetric assay of phenolic and polyphenolic antioxidants and works by measuring the amount of the substance being tested needed to inhibit the oxidation of the reagent. This reagent does not only measure total phenols but it actually reacts with any reducing substance. However, this is not reflected in the name “total phenolic assay”. Various publications applied the total phenols assay by FCR and frequently found excellent linear correlations between the total phenolic profiles and the antioxidant activity. Hence, there is no different in chemistry between the two assays.

Folin–Ciocalteu reagent is typically made by mixing sodium tungstate ($Na_2WO_4 \cdot 2H_2O$), sodium molybdate ($Na_2MoO_4 \cdot 2H_2O$), concentrated hydrochloric acid , 85% phosphoric acid and water. After mixing, the mixure is boiled for 10 hours. Then, lithium sulfate ($Li_2SO_4 \cdot 4H_2O$) is added to the mixture to give an intense yellow solution. The chemical nature of the Folin–Ciocalteu reagent contains heteropolyphosphotunstates- molybdates. Possibly, sequences of reversible one- or two-electron reduction reactions lead to the blue species is $(PMoW_{11}O_{40})^{4-}$. In essence, the molybdenum is easier to be reduced in the complex and electron-transfer reaction which occurs between reductants and Mo(VI):

$$\text{Mo (VI)} + e \longrightarrow \text{Mo (V)}$$

Folin–Ciocalteu reagent reacts with Phenolic compounds only under basic conditions which are adjusted by a sodium carbonate solution. Dissociation of a phenolic proton leads to a phenolate anion, which is capable of reducing the Folin–Ciocalteu reagent. Hence, the reaction occurs through an electrontransfer mechanism. The formation of the phenolate and the Folin–Ciocalteu reagent leads to blue compounds which are independent of the structure of phenolic compounds, consequently, ruling out the possibility of coordination complexes formed between the metal center and the phenolic compounds. The total phenols assay by the Folin–Ciocalteu reagent is convenient, simple, and reproducible. As a result, a large body of data has been accumulated, and it has become a routine assay used in studying phenolic antioxidants.

2.4 Lab-on-paper

Lab-on-a-chip (LOC) is an analytical device which is suited to portable point-of-care (POC) diagnostics and on-site detection. It holds great promise for improving global health, and other applications [20, 21, 22]. Although much more simplified than conventional analytical instruments, LOC devices are still not readily accessible to average users, particularly those in developing countries because they have to be fabricated using lithography based clean-room infrastructure [23]. Moreover, they are generally made from comparatively expensive materials like silicon, glass, or plastic and have tiny pumps and valves that can be difficult to manufacture.

In the past several years, lab-on-paper was suggested as another interesting alternative analytical device. It offers great potential to be a good alternative device for healthcare and related applications because it is portable, easy to use, requires a low sample volume, provides rapid analysis and is inexpensive. Moreover, the material used for fabricating a pattern or a platform of the devices is paper, a cellulose fiber web, which provides several advantages [24] as follows;

- Inexpensive and plentiful
- Liquid can move naturally through paper without active pumping.
- Lightweight, so it is easy to store and transport.
- Can be chemically modified
- flammable and biodegradable
- flexible and compatible
- Convenient for colorimetric tests due to being usually white so it provides a strong contrast between a colored reaction and the background
- Available with a large variety of properties. For instance, filter papers with well-defined pore sizes can remove erythrocytes from blood.

The traditional lab-on-paper, paper strip tests, have been widely used and are commercially available such as a pregnancy strip tests [25] and a diabetes strip tests [26] (Figure 2.5). The concept of paper strip tests depends on the specificity between antigens and antibodies. The visible color appears when the antigen flows and binds

to the gold conjugated antibody and passed through the test line of the paper strip where it is captured by the immobilized antibody. The qualitative analysis notifying only a "yes" or "no" answer is insufficient due to the levels of analytes which are significant for diagnosis and may be linked to the degree of treatment. Hence, much effort has been directed towards the development of quantitative analysis for lab-on-paper.

(a)

(b)

Figure 2.5 Paper strip tests (a) pregnancy strip test [27] (b) diabetes strip test [28]

Whitesides et al. [8] created the first lab-on-paper device which was performed using a colorimetric assay to quantify glucose and protein concentration in artificial urine (Figure 2.6). The intensity of color was measured by digitizing in each assay zone.

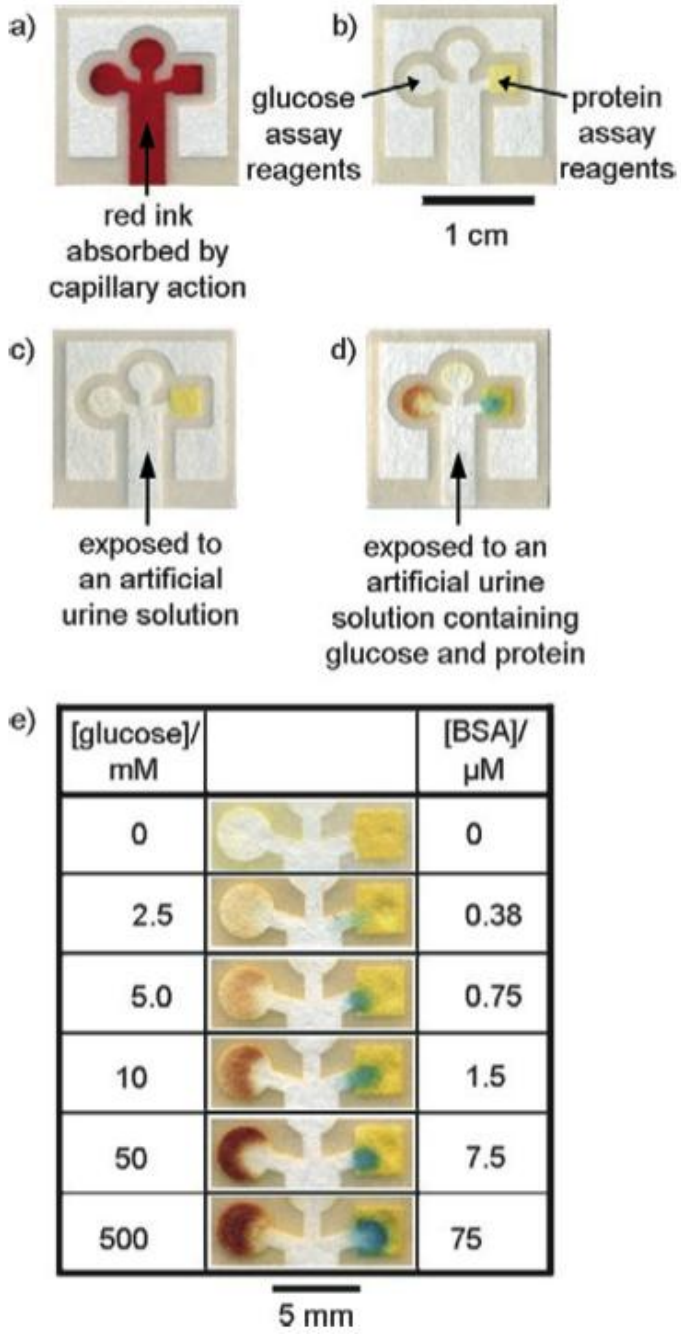


Figure 2.6 Lab-on-paper for quantifying glucose and protein concentrations in an artificial urine solution which flowed through the detection zone where the assay reagents were spotted. [8].

Due to these attractive and useful properties, a lab-on-paper device could be considered as disposable diagnostic tests which are simple and abundant enough for use in the developing world. It is suitable for portable point-of-care (POC) diagnostics and on-site detection.

There are several methods for fabricating lab-on-paper including photolithography, plotting, plasma etching, inkjet etching, cutting and wax printing. The first fabrication method of lab-on-paper was photolithography [8, 29]. SU-8, negative photoresist, is coated on the paper and polymerized by exposure to UV light through a transparency mask with a pattered design. The unexposed photoresist is removed from the paper after development. This method provided sharp edges of barrier with edge roughness of 15 μm and a minimal barrier width of 200 μm. However, it requires organic solvents to remove the unexposed photoresist and leaves an organic scum on the paper.

A plotting method was then developed. The patterns of PDMS on paper were defined by a modified desktop plotter which requires a computer to control it [30]. The hydrophilic channels from this method are clean because they are not exposed to any solvent and polymer.

Plasma etching is one of the method approaches for fabricating the hydrophilic channels on paper [31]. This method relies on plasma oxidation through a metal mask for lab-on-paper.

Inkjet etching is then reported. Polystyrene was deposited on the paper to form a hydrophobic barrier using an inkjet printer [32].

Cutting is the easiest method for fabricating lab-on-paper. A knife plotter is utilized to cut paper into patterns of hydrophilic channels. The cut patterns are encased in tape to be used for structural support of paper and facilitate manipulation of the devices [33].

The wax printing is the procedure that presently seems the best for large-volume, moderate-resolution lab-on-paper. This method has five main advantages [23]:

(i) It takes less than 5 minutes to pattern a paper.

(ii) The hydrophilic channels and test zones are never exposed to photoresists and polymers.

(iii) There is no need to use solvents.

(iv) It easily accommodates different printing colors

(v) Paper patterned by wax printing can be fed directly into an inkjet printer for delivery of reagents into the test zones of the devices.

However, wax printing has a lower resolution than photolithography. The smallest hydrophobic barriers and hydrophilic channels are 850 μm and 560 μm in width, respectively.

Table 2.1 Methods for patterning paper [30].

Method	Advantages	Disadvantages
Photolithography	A wide variety of paper up to 360 μm in width.	Channels are exposed to polymers and solvents.
Plotting	- Channels are not exposed to polymers or solvents. - Hydrophobic barriers are flexible.	Requires a customized plotter.
Plasma etching	Useful for laboratories equipped with a plasma cleaner	- Metal masks must be made for each pattern. - Cannot produce arrays of free-standing hydrophobic patterns.

Method	Advantages	Disadvantages
Inkjet etching	Reagents can be printed directly into the test zones using the printer.	Hydrophilic channels are exposed to polymers and solvents.
Cutting	Hydrophilic channels are not exposed to polymers or solvents.	Devices must be encased in tape.
Wax printing	- Rapid - requires only a commercially available printer and hot plate. - hydrophilic channels are not exposed to polymers or solvents.	The design of patterns must account for the spreading of the wax in the paper.

2.5 Photolithography

Photolithography, which means literally "light-stone-writing" in Greek, is an optical method for transferring patterns onto a substrate. It uses light to transfer a geometric pattern from a photomask, which is created by a photographic process, to a light-sensitive photoresist, which is a liquid film that can be spread out onto a substrate, exposed with a pattern and developed into a selectively placed layer for subsequent processing.. The procedure is comparable to the same process that is used in lithographic printing.

2.5.1 Photolithography Process [34, 35]

2.5.1.1 Coating

There are several methods for coating the photoresist onto a substrate. However, spin coating is virtually universal. The basic coating process is illustrated in Figure 2.7.

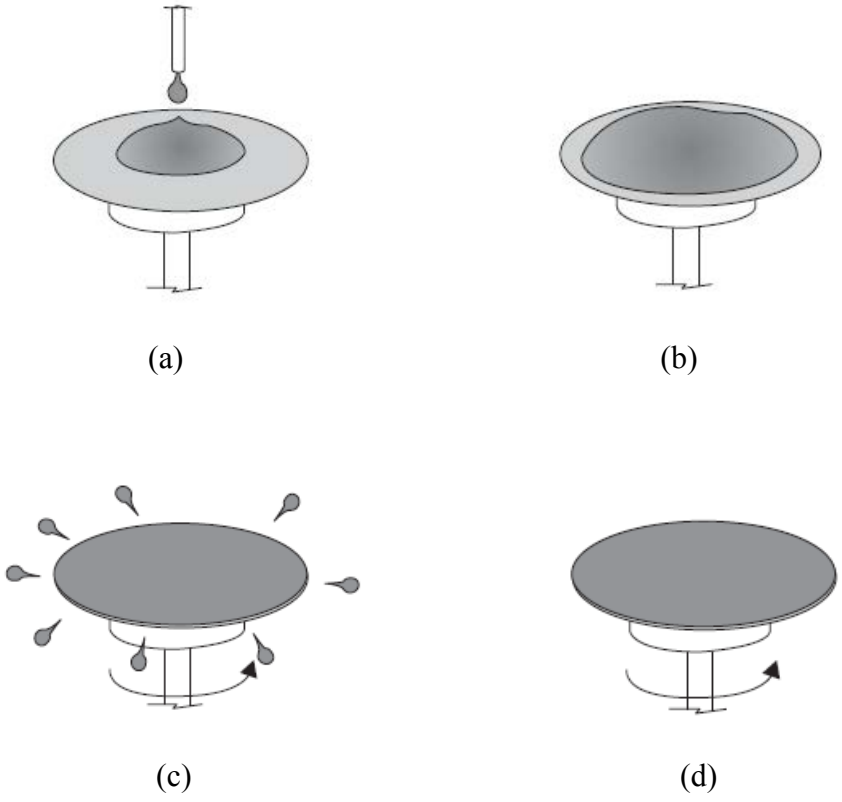


Figure 2.7 Photoresist spin-coating process [36]

The photoresist coating process begins by dispensing a controlled volume of photoresist, which depends on the substrate size, onto the center of the substrate (Figure 2.7(a)). The photoresist is firstly allowed to spread through the substrate with the rotating at a few hundred RPMs (Figure 2.7(b)). Then the spin coater rapidly ramped up to a high spin speed, regularly 1,000 to 10,000 RPMs, and throwing off excess photoresist (figure 2.7(c)). Finally, the photoresist forms a thin dry film at the high speed (Figure 2.7(d))

2.5.1.2 Pre-Bake (Soft Bake)

After coating, the photoresist still contains too much solvent for a sufficient difference to exist in developing rates between the exposed or unexposed portion of the photoresist. Therefore, a soft bake step, which shows in Figure 2.8, is employed for reducing the photoresist solvent levels from 65-85% to 10-20%. Soft bake has further benefits:

- Improves adhesion.
- Stabilizes the film and reduces the thickness of the surface.
- Drives off the residual solvent levels for improving the difference in the developing rates between the exposed or unexposed portion of the photoresist.

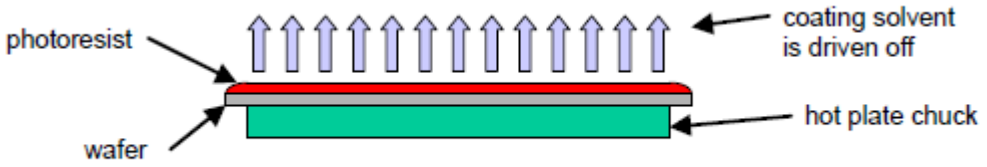


Figure 2.8 Pre-Bake process [35]

2.5.1.3 Alignment

A mask or photomask is created by a photographic process. It is aligned with the surface, so that the patterns are transferred onto the surface. The feature size is limited by the diffraction limit and depends on the size of the wavelength of light used to illuminate the mask.

2.5.1.4 Exposure

The photoresist undergoes a chemical reaction during the exposure process. In Figure 2.9, when the light strikes the surface, it can react in two ways depending on the chemical composition of the photoresist. The action of light on a positive resist causes it to become polymerised where it has been exposed to the light. A negative resist has the reverse property. The photoresist is decomposed when

exposed to UV-light. After the developing process, a negative of the mask remains as a pattern of resist.

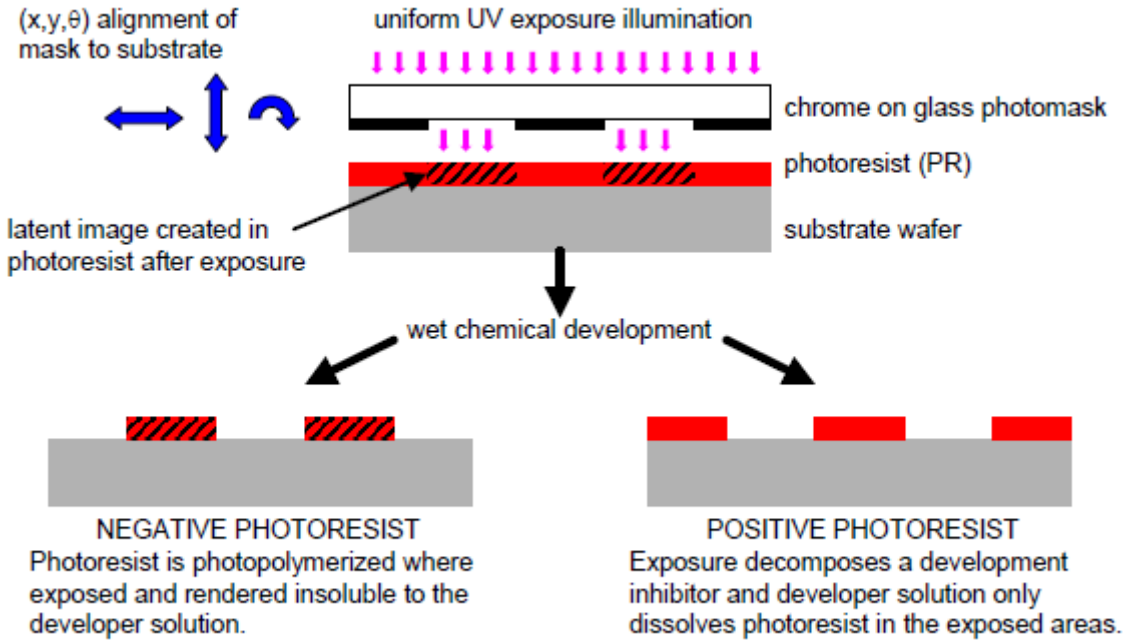


Figure 2.9 The photolithography processes [35]

2.5.1.5 Development

After exposure, development is carried out to leave behind the correct resist pattern on the surface which will serve as the photo mask that covers areas on the surface which need to be protected from chemical attack. The development process involves chemical reactions wherein unprotected parts of the resist get dissolved in the developer. A good development process has a short duration, results in minimum pattern distortion or swelling, keeps the original film thickness of protected areas intact, and recreates the intended pattern faithfully. Development is carried out either by immersion developing or spraying. It should always be followed by thorough rinsing and drying to ensure that the development action will not continue after the developer has been removed from the surface.

2.5.1.6 Post bake (Hard Bake)

This step is necessary in order to improve the adhesion of the photoresist to the surface and remove any remaining traces of the coating solvent or developer. Moreover, some shrinkage of the photoresist may occur since postbake introduces some stress into the photoresist.

2.6 Photoresist [37]

A photoresist is a light-sensitive material used to form a patterned coating on a surface. It is used in several industrial processes, such as photoengraving and photolithography. Photoresists are classified into two groups

(i) Positive resist

The exposed areas become soluble to the photoresist developer. However, the areas that are unexposed remain insoluble (Figure 2.10).

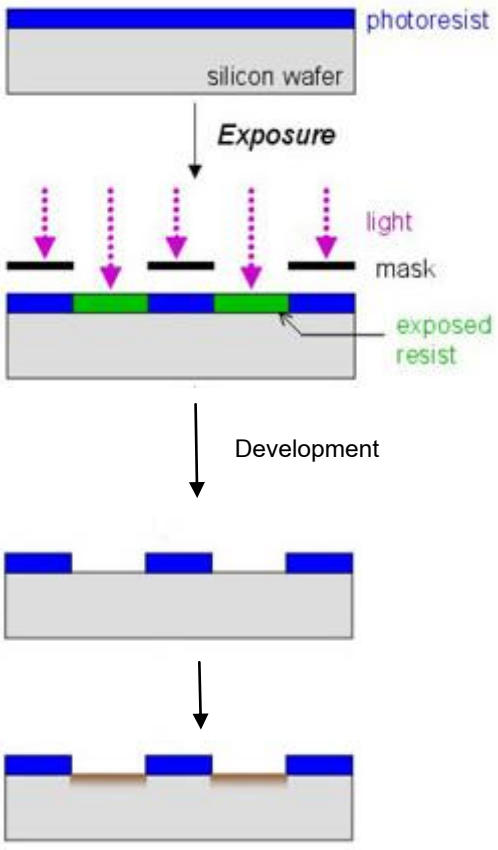


Figure 2.10 The positive photolithography processes [38]

(ii) Negative resist

The areas that are exposed to the light become insoluble since crosslinks occur during a subsequent baking step and remain after development while the unexposed areas are dissolved (Figure 2.11).

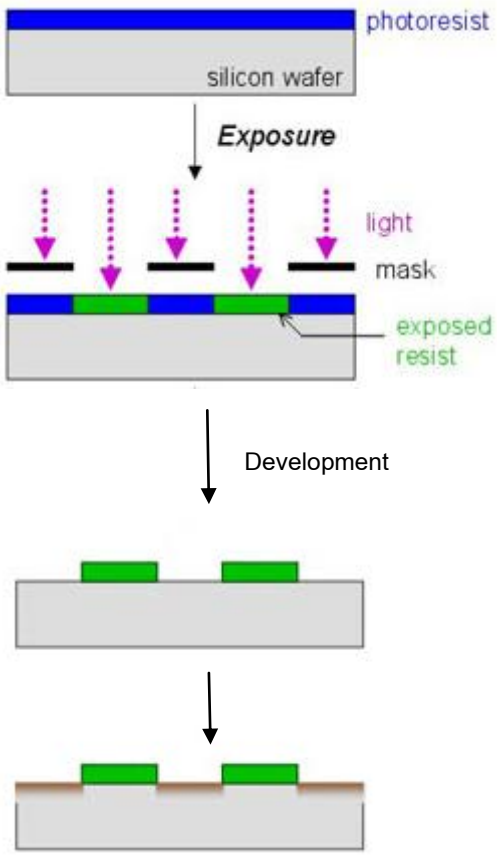


Figure 2.11 The negative photolithography processes [38]

Table 2.2 Differences between positive and negative photoresist [37]

Characteristic	Positive	Negative
Relative Cost	More Expensive	Less Expensive
Adhesion to Silicon	Fair	Excellent
Step Coverage	Better	Lower
Minimum Feature	0.5 μm and below	± 2 μm
Developer Base	Aqueous	Organic
Wet Chemical Resistance	Fair	Excellent

2.6.1 SU-8 photoresist [39, 40]

SU-8 is the common product of the negative photoresist. It contains eight polymeric epoxy groups per molecule by dissolving in an organic solvent which gives very high functionality. The molecule of SU-8 is shown in Figure 2.12. The SU-8 consists of 3 basic components:

a) An epoxy, called Epon SU-8 which is available at Shell Chemicals.

b) A solvent, called gamma-Butyrolactone (GBL) (Fig 2.13).

c) A photoacid generator taken from the family of the triarylium-sulfonium salts (Fig 2.14).

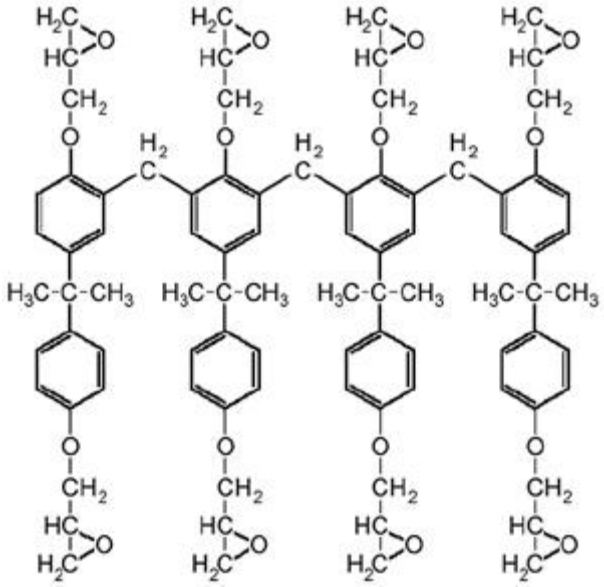


Figure 2.12 The molecule of SU-8. [41]

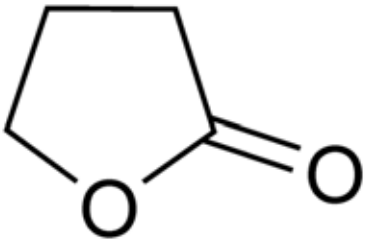


Figure 2.13 The solvent of SU-8 [42].

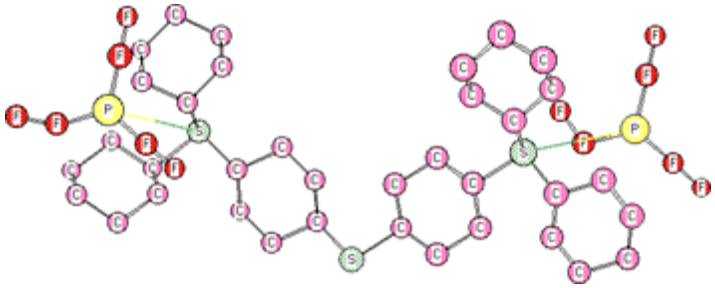


Figure 2.14 The photoacid generator of SU-8 [40].

The properties of Epon SU-8 are as follows:

- Low molecular weight
- Good solubility
- High transparency
- Glass and film formation
- Low glass transition temperature (Tg)
- Highly viscous solutions
- Ultra thick layers up to 500 μm by single coating
- Highly uniform coating
- Low edge bead
- Vertical sidewalls
- Aspect ratio > 15
- An excellent chemical resistance
- Good biocompatibility

2.6.1.1 Process cycle

The substrate is coated by a conventional spinner, with the film thickness controlled by the solids content of the epoxy solution and the spin speed. A baking stage removes excess solvent from the layer. Upon exposure to UV radiation, a strong acid is generated which causes the epoxy resin to form a ladder-like structure with a high cross-linking density when heated above a critical temperature provided in a post-exposure bake. Then, the unexposed resist is removed with a solvent in the development process.

2.6.1.2 Chemical reaction

The photoimaging mechanism of the resist during the exposure can be described by the equation below (Figure 2.15).

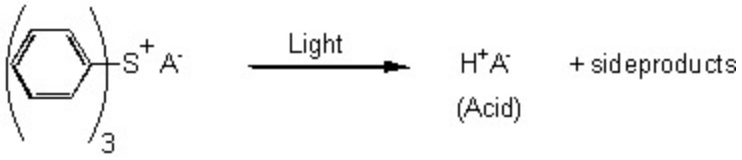


Figure 2.15 Photoimaging mechanism during the exposure [40].

For negative photoresist, photoacid (H^+A^-) is photochemically produced in the exposed photoresist film upon absorption of light, while the unexposed photoresist isn't. It acts as a catalyst in the subsequent cross-linking reaction which takes place during Post-Exposure-Bake (PEB).

The following PEB is responsible for the cross-linking mechanism in the SU-8 layer. At the ambient temperature, the reaction kinetic of the cross-linking mechanism is very slow. Therefore, baking the resist above its glass transition temperature (Tg = 55°C), which is the temperature at which the transition between solid glass and viscous fluid occurs, is necessary to increase the molecular motion and assist the cross-linking process [1]. The cross-linking reaction which is catalyzed by acid takes place in a "zipping up" fashion, where each epoxy group can react with another epoxy group, either in the same or different molecule. Cross-linking does not occur in the absence of acid, at the temperature of the PEB. Extensive cross-linking will yield a dense network since as described above each epoxy is "pre-connected" to 7 others on average. This dense network is insoluble in the developer. The reaction is illustrated in Figure 2.16.

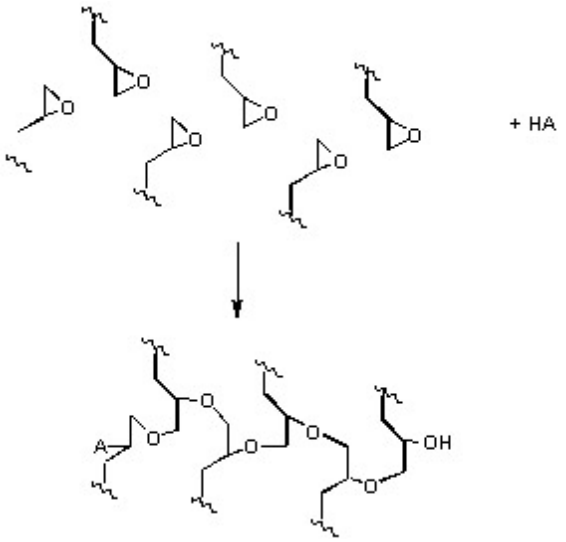


Figure 2.16 The cross link reaction during the post exposure bake [40].

The PEB must be carried out at temperatures greater than Tg in order to be effective so the resist film properties will change in several ways:

(i) Some shrinking will inevitably occur due to reduction in free volume and increase in density.

(ii) The Tg will also rise as the film becomes increasingly cross linked.

Cross-linking proceeds the film gradually until the film is solid and the cross link reaction will slow down and eventually stop. Therefore, the final Tg of the material is dependent on the PEB temperature.

2.7 Electroanalytical method [43, 44]

Electrochemistry is a branch of chemistry concerned with the interrelation of electrical and chemical effects. A large part of this field deals with the study of chemical changes caused by the passage of an electric current and the production of electrical energy by chemical reactions. In fact, the field of electrochemistry encompasses a huge array of different phenomena (e.g., electrophoresis and corrosion), devices (electrochromic displays, electro analytical sensors, batteries, and fuel cells), and technologies (the electroplating of metals and the large-scale production of aluminum and chlorine).

Scientists make electrochemical measurements on chemical systems for a variety of reasons. They may be interested in obtaining thermodynamic data about a reaction. They may want to generate an unstable intermediate such as a radical ion and study its rate of decay or its spectroscopic properties. They may seek to analyze a solution for trace amounts of metal ions or organic species. In these examples, electrochemical methods are employed as tools in the study of chemical systems in just the way that spectroscopic methods are frequently applied. There are also investigations in which the electrochemical properties of the systems themselves are of primary interest, for example, in the design of a new power source or for the electrosynthesis of some product. Many electrochemical methods have been devised. Their application requires an understanding of the fundamental principles of electrode reactions and the electrical properties of electrode-solution interfaces.

2.7.1 Cyclic votammetry

Cyclic voltammetry (CV) is a variety of potentiodynamic electrochemical measurement. It is the most widely used technique for obtaining qualitative information about electrochemical reactions. It is generally used to study the electrochemical properties of an analyte in solution. In a cyclic voltammetry experiment, the working electrode potential is ramped linearly versus time like linear sweep voltammetry. This ramping is known as the experiment's scan rate (V/s). However, cyclic voltammetry takes the experiment a step further than linear sweep voltammetry.

Cyclic voltammetry consists of scanning linearly the potential of a stationary working electrode, using a triangular potential waveform, as shown in Figure 2.17. Depending on the information sought, single or multiple cycles can be used. During the potential sweep, the potentiostat measures the current resulting from the applied potential. The resulting current-potential plot is termed a cyclic voltammogram. The cyclic voltamogram is a complicated, time-dependent function of a large number of physical and chemical parameters.

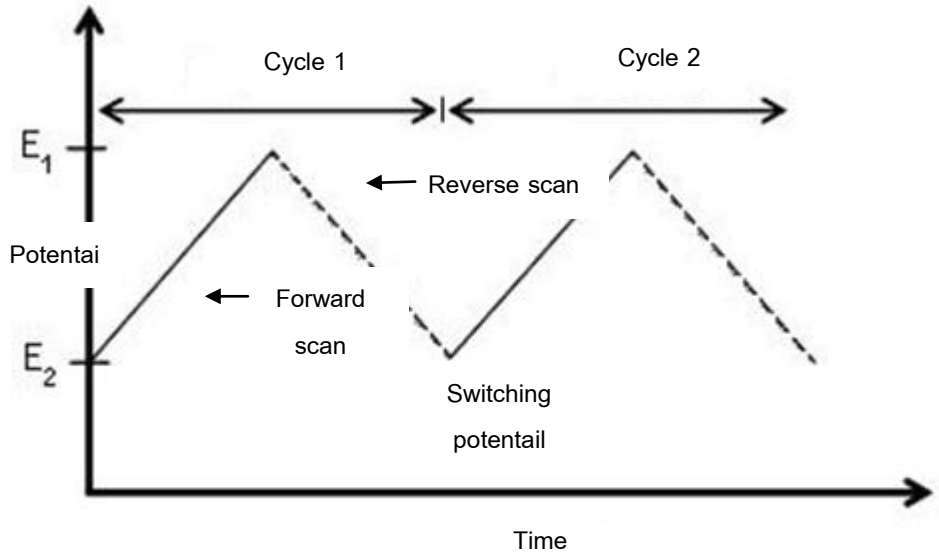


Figure 2.17 Potentail-time excitation signal in a cyclic votammetry

The potential is applied between the reference electrode and the working electrode and the current is measured between the working electrode and the counter electrode. This data is then plotted as current (i) vs. potential (E). As shown in Figure 2.18, the forward potential scan produces a current peak for any analytes that can be reduced or oxidized depending on the initial scan direction. As the potential approaches the characteristic E^0 for the redox process, the current will increase until a peak is reached. After traversing the potential region in which the reduction process takes place, the direction of the potential sweep is reversed. During the reverse scan, it will reach the potential that will reoxidize the product formed and generate a current of reverse polarity from the forward scan, resulting in the oxidation peak that will usually have a similar shape to the reduction peak. As a result, information about the redox potential and electrochemical reaction rates of the compounds is obtained.

Cyclic Voltammetry measurements were characterized by a peak potential. E_p is the potential at which the current reaches its maximum value; i_p is the value that is called the peak current. The $i_{p,a}$ and $E_{p,a}$ are the anodic peak current and anodic peak potential, respectively. The $i_{p,c}$ and $E_{p,c}$ are the cathodic peak current and cathodic peak potential, respectively.

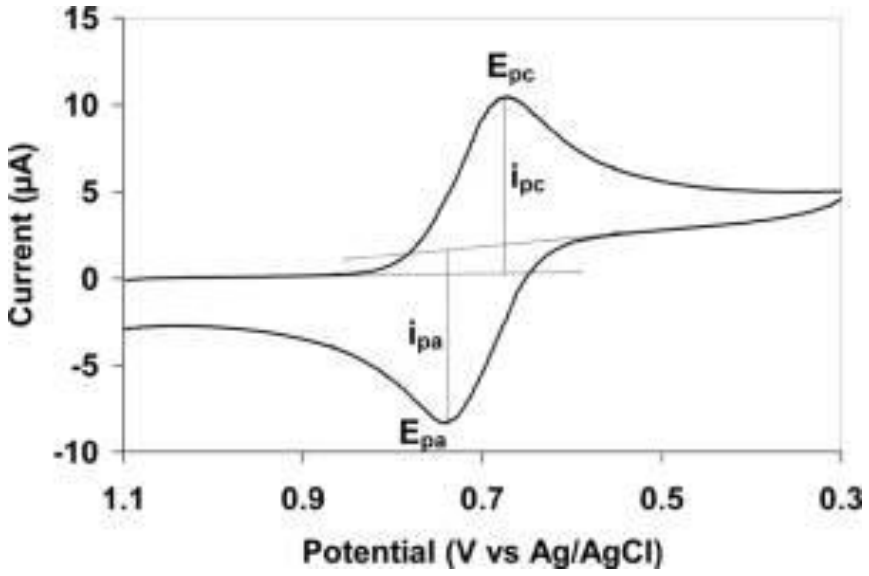


Figure 2.18 Typical reversible cyclic voltammetry with the initial sweep direction towards more positive potential [45].

For instance if the electronic transfer at the surface is fast and the current is limited by the diffusion of species to the electrode surface, then the current peak will be proportional to the square root of the scan rate. The CV experiment only samples a small portion of the solution, the material within the diffusion layer.

2.7.1.1 Reversible system

The shape of a reversible cyclic voltammogram with an electrode of fixed area is shown in Figure 2.19. The peak current for a reversible couple is given by the Randles-Sevcik equation below:

$$i_p = 2.69\times10^5 n^{3/2} A D^{1/2} C \nu^{1/2} \quad \text{at } 25°C \qquad \text{(Equation 2.1)}$$

Where n is the number of electron, A is the electrode area (in cm^2), D is diffusion coefficient (in $cm^2 \cdot s^{-1}$), C is the concentration of electroactive species (in $mol \cdot cm^3$) and ν is the potential scan rate (in $V \cdot s^{-1}$).

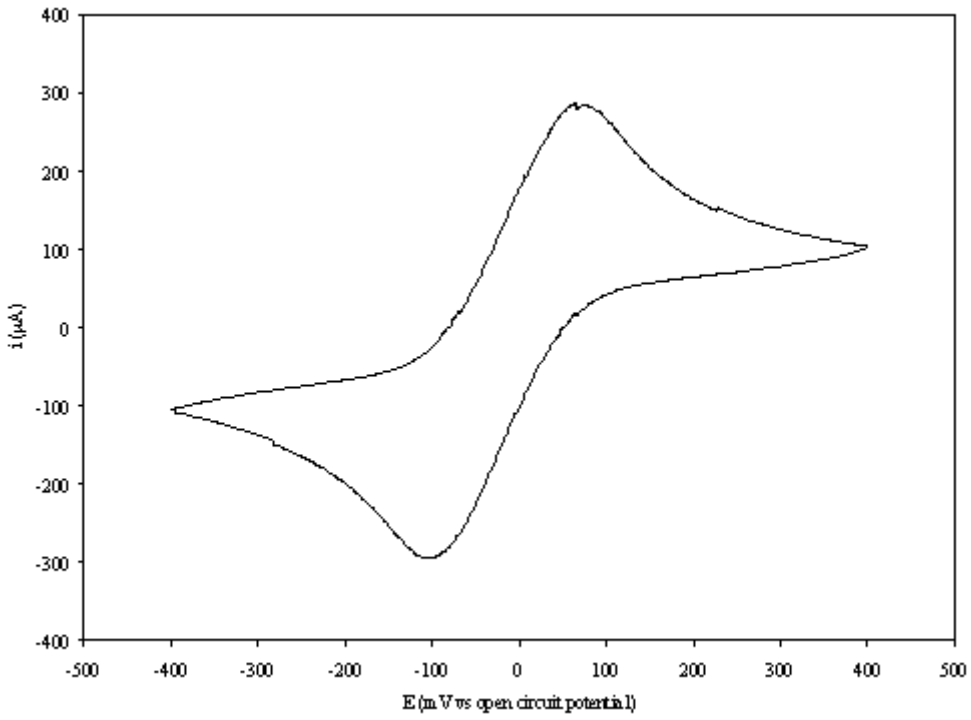


Figure 2.19 Cyclic votamogram for reversible redox process [46].

Accordingly, the current is directly proportionate to the concentration and increases with the square root of the scan rate. Such dependence on the scan rate is indicative of the electrode reaction controlled by mass transport. The reverse-to-forward peak current ratio is in unity for a simple reversible couple. This peak ratio can be strongly affected by chemical reactions coupled to the redox process. The current peaks are commonly measured by extrapolating the proceeding baseline current.

2.7.1.2 Irreversible system

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

$$i_p = (2.99\times10^5)n(\infty n_a)^{1/2}ACD^{1/2}\nu^{1/2} \quad \text{at } 25°C \qquad \text{(Equation 2.2)}$$

Where n is the number of electrons, A is the electrode area (in cm^2), D is diffusion coefficient (in $cm^2 \cdot s^{-1}$), C is the concentration of electroactive species (in $mol \cdot cm^3$) and ν is the potential scan rate (in $V \cdot s^{-1}$).

The peak current is still proportionate to the bulk concentration but will be lower in height. The peak current of the irreversible processes is about 80% of the peak for a reversible. The shape of an irreversible cyclic voltammogram with an electrode of fixed area is shown in Figure 2.20.

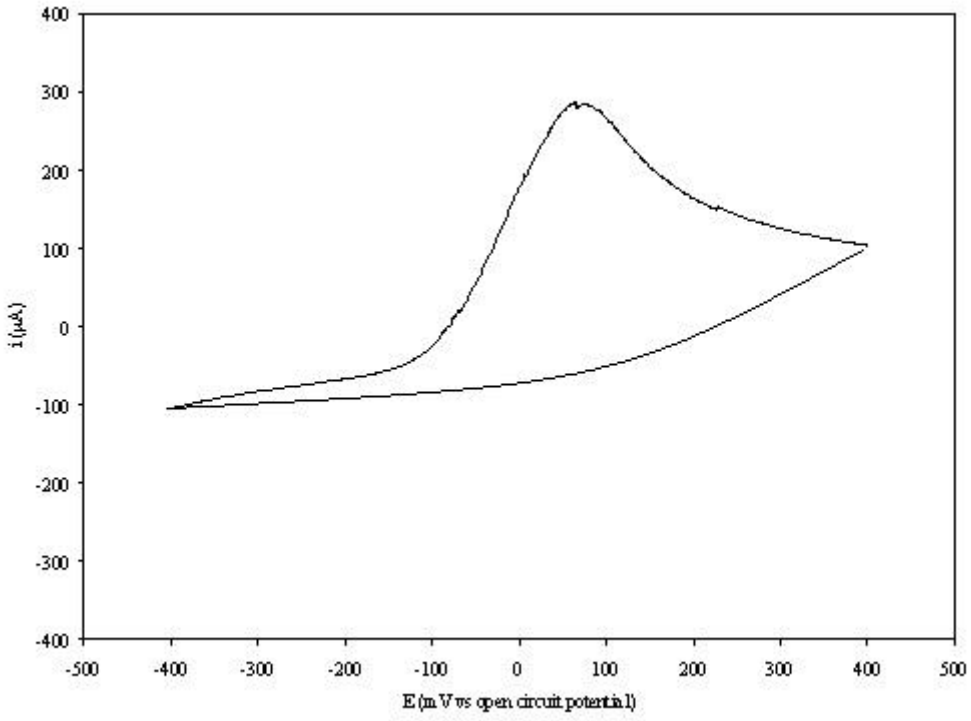


Figure 2.20 Cyclic votamogram for irreversible redox process [46].

2.7.2 Differential Pulse Voltammetry [47]

Differential Pulse Voltammetry is an extremely useful technique for measuring trace levels of organic and inorganic species. The sensitivities of differential pulse voltammetry are better than those of normal pulse voltammetry. The potential wave form for differential pulse voltammetry is shown in Figure 2.21. The important parameters for differential pulse voltammetry are as follows [48]:

(i) Pulse amplitude is the height of the potential pulse, which may or may not be constant depending upon the technique.

(ii) Pulse width is the duration of the potential pulse.

(iii) Sample period is the time at the end of the pulse during which the current is measured.

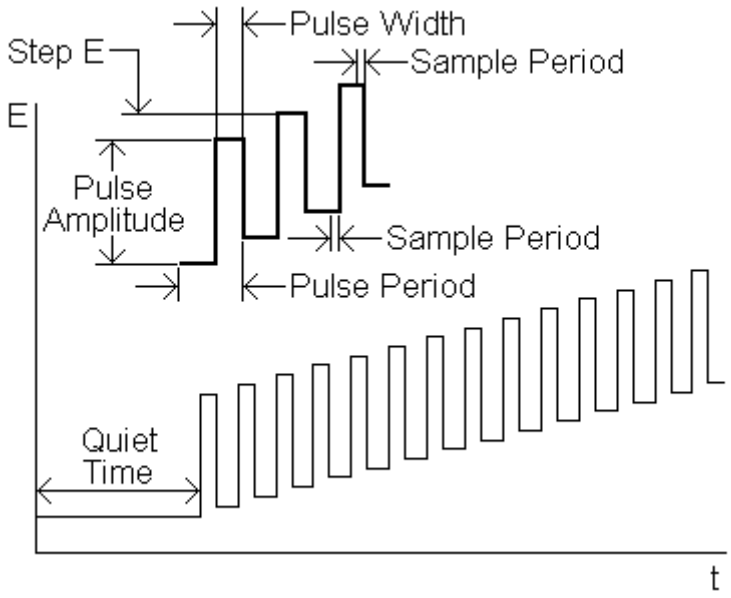


Figure 2.21 Potential for differential wave form pulse voltammetry [48]**.**

In differential pulse voltammetry, fixed magnitude pulses are applied to the working electrode at a time just before the end of the drop, as shown in Figure 2.22. The current is sampled twice, just before the pulse application and again late in the pulse life. The first current is instrumentally subtracted from the second and this current difference is plotted against the applied potential. The resulting differential pulse voltammogram consists of current peaks, the height of which is directly proportional to the concentration of the corresponding analytes.

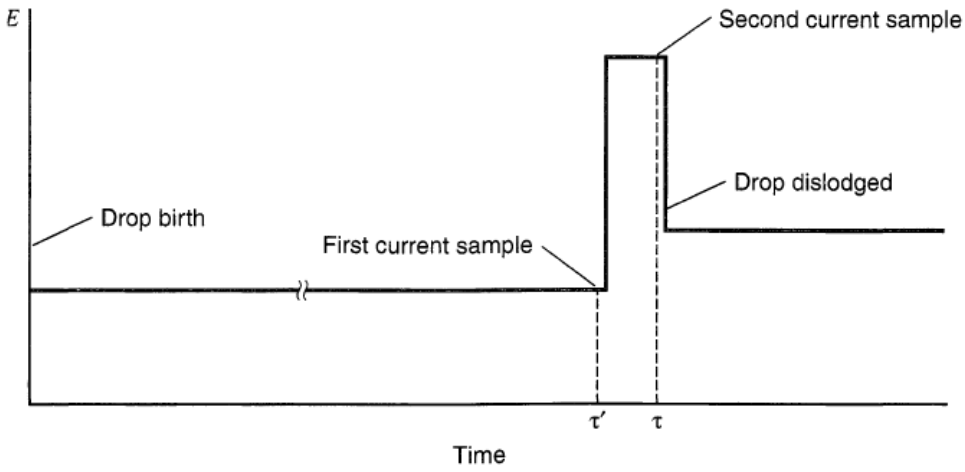


Figure 2.22 A single drop of a differential pulse voltammetry [43].

The peak-shaped response of differential pulse measurements, which are shown in Figure 2.23, results also in improved resolution between two species with similar redox potentials. Such quantitation depends not only on the corresponding peak potentials but also on the widths of the peak.

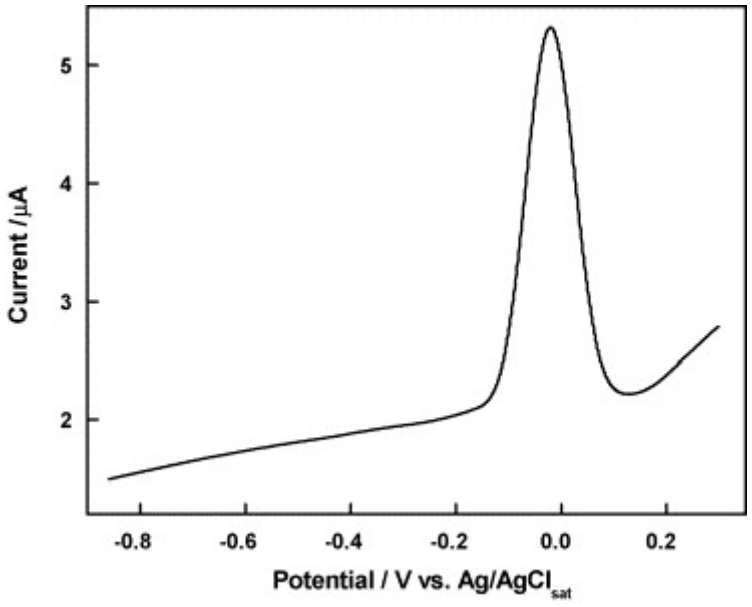


Figure 2.23 Differential pulse voltammogram

The selection of the pulse amplitude and potential scan rate usually requires a tradeoff among sensitivity, resolution and speed. For example, larger pulse amplitudes result in larger and broader peaks. Irreversible redox systems result in lower and broader current peaks compared with those predicted for reversible systems. In addition to improvements in sensitivity and resolution, the technique can provide information about the chemical form in which the analyte appears.

2.8 Colorimetric method [49]

Colorimetric method is a technique for determining the chemical compound in a solution using the change of a color reagent or a simple definition of a colorimetric method is "any technique used to measure an unknown color in reference to known colors". It can be used to evaluate any test substance that is itself colored or can react with the reagent to produce a color. In a colorimetric method, the color intensity from the reaction must be proportional to the concentration of the substance which is tested. Some reactions have limitations or variances that may give misleading results. Most limitations or variances are discussed with each particular test instruction. In the most basic colorimetric method, the reacted test sample is visually compared to a known color standard. However, matching color by the naked eye is affected by many factors including lighting conditions, different visual perception of color from one person to another and the fading of color standards. These effects limit accurate and reproducible results.

To abstain from these sources of error, a colorimeter can be used to photoelectrically measure the amount of colored light absorbed by a colored sample in reference to a colorless sample (blank). A colorimeter is generally any tool that characterizes color samples to provide an objective measure of color characteristics.

A digital camera-based colorimetric method continues to increase. It has been widely developed for the detection of various analytes such as glucose and albumin [9, 32], acetylthiocholine [8] and human IgG [50] due to its easy-to-use property. The image from the off-site can be transmitted electronically and digitally to a laboratory and the analyzed results can be returned ideally in real-time to the person administering the test. The general strategy for performing low-cost bioassays in an

outlying area and for interchanging the results of the tests with a technician is shown in Figure 2.24.

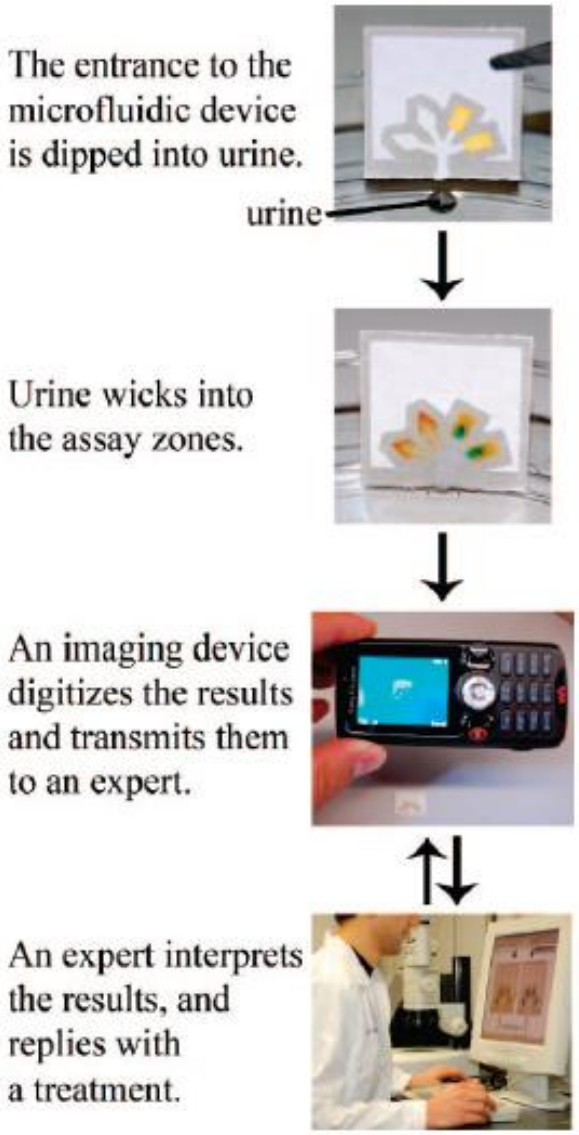


Figure 2.24 General strategies for performing inexpensive bioassays in faraway locations and for exchanging the results of the tests with an expert [32].

The procedure of the quantitative analysis is shown in Figure 2.25. Camera-based optical detection was used to record the color intensity. Firstly, a device, which is applied reagents, is dipped into the sample solution. The colorimetric results illustrated are captured by digital camera. Then, the pictures are converted into either grayscale or CMYK color for increased precision in the analysis and the test zone is selected. Finally, the mean intensity is recorded by general software such as Adobe Photoshop. The benefits of this optical method are ease of use and analysis, rapidity, inexpensive instruments and an off-site laboratory.

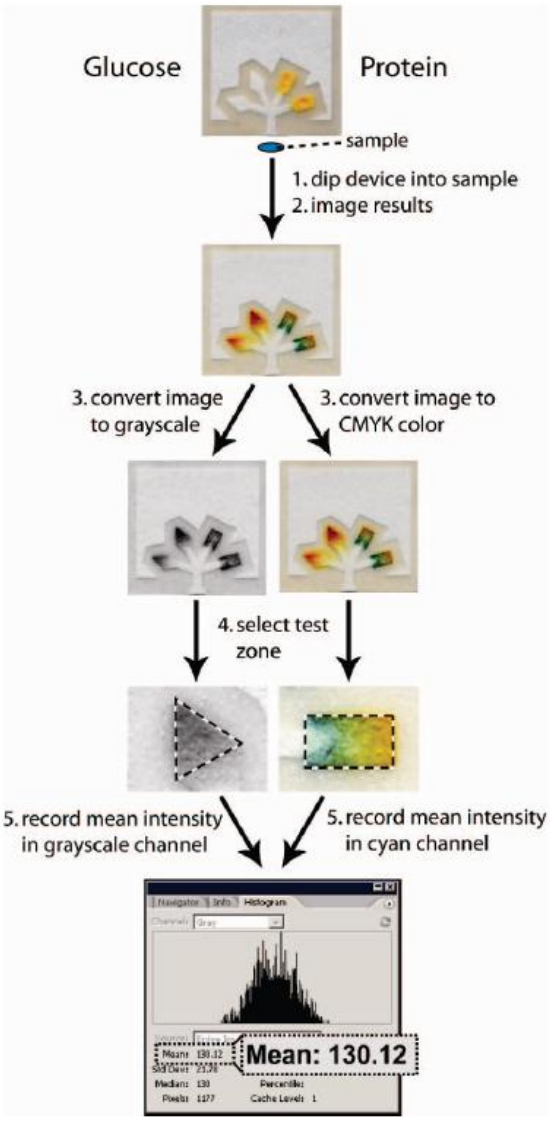


Figure 2.25 Procedure for quantifying using the Adobe Photoshop program [32].

The colorimetric method is applicable to both organic compounds and inorganic compounds. Moreover, it may be used with or without an enzymatic stage. This method is widely used in medical laboratories and for industrial purposes, e.g. the analysis of water samples in industrial water treatment.

2.9 Literature surveys

2.9.1 Conventional methods for ferulic acid detection

In 1998, Om et al. [4] reported the separation of simple phenolics and related compounds such as gallic acid, methyl gallate, pyrogallol, phloroglucinol, catechol, resorcinol, hydroquinone, catechin, epicatechin, cinnamic acid, p-coumaric acid, ferulic acid and tannic acid by thin-layer chromatography using six solvent systems. Detection was carried out using iodine vapour, ferric chloride reagent, ferric chloride-ferricyanide reagent and vanillin-sulfuric acid reagent.

In 2001, Jankovska et al. [51] introduced the determination of ferulic acid in sugar beet pulp by reverse phase high-pressure liquid chromatography (HPLC) and UV/VIS-spectroscopy. UV/Vis-spectroscopy was used to measure both non-hydrolysed and hydrolysed extracts. The HPLC estimation of ferulic acid was made in hydrolysed extracts only. It was found that the concentration of ferulic acid in sugar beet pulp was in the range of 0.3-0.9 % (m/m).

In 2003, Guo et al. [52] developed a micellar electrokinetic chromatography for determining ferulic acid and adenosine in Angelicae Radix. The contents of ferulic acid and adenosine in Angelicae Radix were determined within 20 min. Ferulic acid showed the good linearity in the range of approximately 20-320 microg.mL^{-1}. The contents of ferulic acid and adenosine in Angelicae Radix from different sources were determined.

In 2004, Hu et al. [53] presented a reversed phase high performance liquid chromatographic (RP-HPLC) method for simultaneous determination of chlorogenic acid, caffeic acid, ferulic acid, protocatechuic acid and protocatechuic aldehyde in a Chinese herb. The separation was performed on a Hypersil ODS-2 column by isocratic elution with methanol. An acetate buffer (0.2 M) was used as the mobile phase. This method showed a good linearity over the range of 2-200

microg/ml. Moreover, the developed method has reference value for the quantitative analysis of Taraxacum, Lonicera and Angelica.

In 2005, Gue et al. [54] also developed a high-performance liquid chromatographic method for the determination of gallic acid, albiflorin, paeoniflorin, ferulic acid and benzoic acid in Si-Wu decoction. All compounds were analyzed simultaneously with a Zorbox SB C-18 column by gradient elution using phosphoric acid-acetonitrile as the mobile phase. The result indicated that the content of these five compounds changed after the decocting process.

In 2006, Mabinya et al. [55] demonstrated the determination of certain phenolic compounds from plants and their chemical transformation with microorganisms or isolated enzymes. Thin layer chromatography was used for the rapid quantitative estimation of ferulic acid. Furthermore, the qualitative analysis of a number of related phenolics, some of which are transformation products of ferulic acid, is achieved on thin layer chromatographic plates by the use of different spray reagents

In 2007, Li et al. [56] developed a selective and sensitive reversed-phase high performance liquid chromatography method for the simultaneous determination of danshensu, ferulic acid, cryptotanshinone, and tanshinone IIA in rabbit plasma. The sample was prepared by liquid-liquid extraction. Chromatographic separation was successfully achieved on an Agilent HC-C(18) column using a mobile phase composed of methanol-water (from 20:80 to 80:20, v/v) containing 0.5% (v/v) glacial acetic acid. The method showed good linearity and no endogenous material interfered. The limit of quantification of ferulic acid was found to be 0.03 microg/ml. This proposed method has been successfully applied in the pharmacokinetic study and drug interaction of danshensu, ferulic acid, cryptotanshinone, and tanshinone IIA in rabbits after intravenous administration of danxiongfang.

In the same year, Li et al. [57] also fabricated L-cysteine self-assembled monolayers on the surface of gold electrode (L-Cys/Sam-Au/CME) and investigated the electrochemical behavior of ferulic acid on this modified electrode. This electrode was applied to the flow-injection irreversible biamperometric analysis. The detection limit of ferulic acid is 1.2×10^{-7} M. The advantages of this proposed method are wide linear range, high selectivity and sensitivity.

In 2008, Luo et al. [58] developed a simple, sensitive and rapid flow-injection chemiluminescence method for the determination of ferulic acid based on the chemiluminescence reaction of ferulic acid with rhodamine 6G and ceric sulfate in sulphuric acid medium. A strong chemiluminescence signal was observed when ferulic acid was injected into the acidic ceric sulfate solution in a flow-cell. The detection limit for ferulic acid was 8.7×10^{-9} mol l^{-1}. This presented method was also applied to the determination of ferulic acid in Taita Beauty Essence samples with satisfactory results.

In 2009, Ya-Ping Ding et al. [59] investigated the electrochemical behaviors of ferulic acid using the glassy carbon electrode modified with a multi-walled carbon nanotube. Cyclic voltammetry was used to quantify trace amounts of ferulic acid. The limit of detection was estimated to be 1×10^{-7} mol l^{-1}. The influences of substrate, pH and interference of coexisting substances were investigated for response properties of the electrode. This method could be used to analyze the trace amount of ferulic acid in medicaments.

In 2010, Wu et al. [60] established a HPLC method for the simultaneous determination of hesperidin (HE), ferulic acid (FA), cinnamic acid (CA) and cinnamaldehyde (CAD) in a Chinese tonic wine. After filtering the tonic wine, the filtrate was separated on a C18 column by gradient elution of acetonitrile-aqueous solution. For ferulic acid, the regression equations were linear in the range of 0.3-60.0 g/mL and the limits of detection (LOD) was found to be 0.075 g/mL. This method can be used to standardize the quality control of Chinese tonic wine to ensure accuracy and efficiency.

In the same year, Jia et al. [61] reported a simple and rapid method for the determination of ferulic acid in pharmaceutical formulations by didodecyldimethylammonium bromide (DDAB)/Nafion composite film-modified carbon paste electrode. Cyclic voltammetry was used to investigate the electrochemical behavior of ferulic acid using the proposed electrode. The oxidation peak currents of ferulic acid increase linearly with the concentration of ferulic acid in the range from 2.0×10^{-6} to 1.2×10^{-4} M. The detection limit of ferulic acid was found to be 3.9×10^{-7} M. This developed method was successfully applied to the determination of ferulic acid in pharmaceutical tablets.

The main quantitative techniques for ferulic acid detection are high–performance liquid chromatography (HPLC), thin-layer chromatography, the capillary tube electrophoresis method, spectrophotometry and electrochemistry. Among these methods, the detection limits of selected detectors are typically low enough to detect ferulic acid in samples. However, these assays require laborious sample preparation steps, high equipment costs and a significant amount of labor and analytical resources, which can potentially cause substantial delays in obtaining results.

2.9.2 Lab-on-paper devices

In 2007, Martinez et al. [8] introduced the first prototype of a paper-based device (lab-on-paper) for quantitative and multiple assays using a phone camera to obtain the results for real-time and off-site diagnosis. Photolithography is the method which is used for fabricating well-defined millimeter-sized hydrophilic channels on paper. This presented device demonstrated the ability for simple multiplexed bioassays and is attractive as a good alternatives device for use in developing countries or in clinical diagnosis because it is easy to use, portable and has no need of external power sources.

In 2008, Li et al. [31] demonstrated a new method for fabricating patterns on paper surface using plasma treatment. Paper was hydrophobized and then treated by plasma in conjunction with a mask using plasma treatment. The advantages of this method are the flexibility of paper and a variety of patterns which could be easily made.

In the same year, Citterio et al. [32] presented an inkjet printing method, which required only a single printing apparatus, for the fabrication of microfluidic multianalyte chemical sensing devices made from paper. An inkjet printing device is used for the fabrication of three-dimensional hydrophilic patterns and sensing areas by printing a hydrophobic poly(styrene) layer on filter paper. Moreover, the same inkjet printing device is used to print the necessary reagents for colorimetric analytical assays into well-defined areas of the devices. The advantages of the newly presented inkjet printing method are no requirement for expensive

additional equipment and no need for curing of polymers or development of photoresists.

In 2009, Dungchai et al. [11] reported the first demonstration of electrochemical detection for paper-based microfluidic devices (lab-on-paper). For fabricating this device, photolithography was used to make microfluidic channels on filter paper and electrodes were prepared by using screen-printing technology on the patterned paper. The developed devices were then demonstrated with the determination of glucose, lactate and uric acid in biological samples using the reaction of oxidase enzyme, which produce H_2O_2, including glucose oxidase, lactate oxidase, and uricase, respectively. The determination of glucose, lactate, and uric acid in control serum samples was then performed using chronoamperometry. The quantity of glucose and lactate in the control serum samples was measured using the presribed devices were within error of the levels measured using traditional tests. This study shows the successful integration of paper-based microfluidics and electrochemical detection as an easy-to-use, inexpensive, and portable alternative for point of care monitoring.

In 2010, Dungchai et al. [62] demonstrated the devices which are designed to simultaneously quantify analytes using multiple indicators for each analyte. The use of multiple indicators for a single analyte allows for different indicator colors to be generated at different analyte concentration ranges as well as increasing the ability to better visually discriminate colors. Glucose, lactate, and uric acid in the control serum and urine samples were determined by this device to demonstrate the applicability. The results for the multi-indicator and single indicator system were compared using untrained readers to demonstrate the improvements in accuracy achieved with the new system.

In the same year, Lu et al. [63] introduced the fabrication of paper based devices (lab-on-paper) in nitrocellulose (NC) membrane by a wax-printing method for the determination of protein immobilization. The fabrication parameters such as the printing mode and the baking time and the performances of the wax-patterned NC membrane such as printing resolution, protein immobilization and sample purification capabilities were studied. The fabrication process could be achieved without the use of organic solvents and the wax-patterned NC membrane

could be produced on a large scale. This developed device would introduce new possibilities and enhance broad applications in clinical diagnosis, food safety and environmental screening.

With the obvious advantages of lab-on-paper such as being inexpensive, easy-to-use, requiring only a small volume of the sample, easy to modify chemically and providing rapid analysis, they are remarkably attractive analytical devices for healthcare related applications and developments for clinical diagnosis. Hence, the aim of this work has been set to develop a lab-on-paper device for simultaneously determin the both the antioxidant capacity and the amount of ferulic acid of the sample.

CHAPTER III

EXPERIMENTAL

This chapter has given the information of instruments and equipments, chemical, electrode preparation, sample preparation and methodology used in this present work.

3.1 Preparation of lab-on-paper

3.1.1 Instruments and equipments

The instruments and equipments for the preparation of lab-on-paper are listed in Table 3.1.

Table 3.1 List of instruments and equipments for the preparation of lab-on-paper

Instruments/equipments	Suppliers
SCS Spin coat G3P-8	Specialty Coating Systems, Inc., USA
Scanning electron microscope JSM-6400	JEOL Ltd., Japan
Intelli-ray 400, shuttered UV floodlight	Uvitron International, Inc., USA
Plasa cleaner PDC-32G	Harrick Plasma, USA
Hot air oven	Memmert, USA
Filter paper, grade 1 circles, 100 mm	Whatman, UK
Stop watch	Mini Timer, Japan

3.1.2 Chemicals

The chemicals for the preparation of lab-on-paper are listed in Table 3.2.

Table 3.2 List of chemicals for the preparation of lab-on-paper.

Chemicals	Suppliers
SU-8 3025	Micro Chem Crop., USA
SU-8 developer	Micro Chem Crop., USA
2-Propanol	Merck, Germany
Acetone	Merck, Germany

3.1.3 Methodology

The photolithography method was used to fabricate devices [8]. For the fabrication of the device, a SCS spin coater (G3P-8) was used to coat 4 g of SU-8 negative photoresist on the filter paper (No. 1, 100 mm diameter, Whatman) and baked at 95 °C for 10 min. After that, the photomask (Figure 3.1) which was designed with Adobe Illustrator software (Adobe Systems, Inc.) and fabricated by Chaiyaboon Co. (Bangkok, Thailand) was placed on the SU-8-covered paper and then irradiated with ultraviolet (UV) light (Intelli-ray 400) for 10 s followed by baking at 95 °C for 10 min. Next, the unpolymerized photoresist was removed from the paper by soaking in a SU-8 developer for 3 min and by rinsing with isopropanol. Finally, the patterned paper was dried in a hood at room temperature. The fabrication process is shown Figure 3.2.

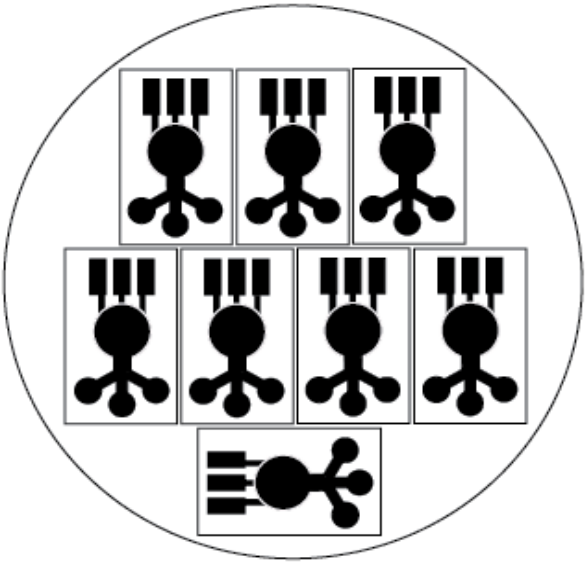


Figure 3.1 Transparent film

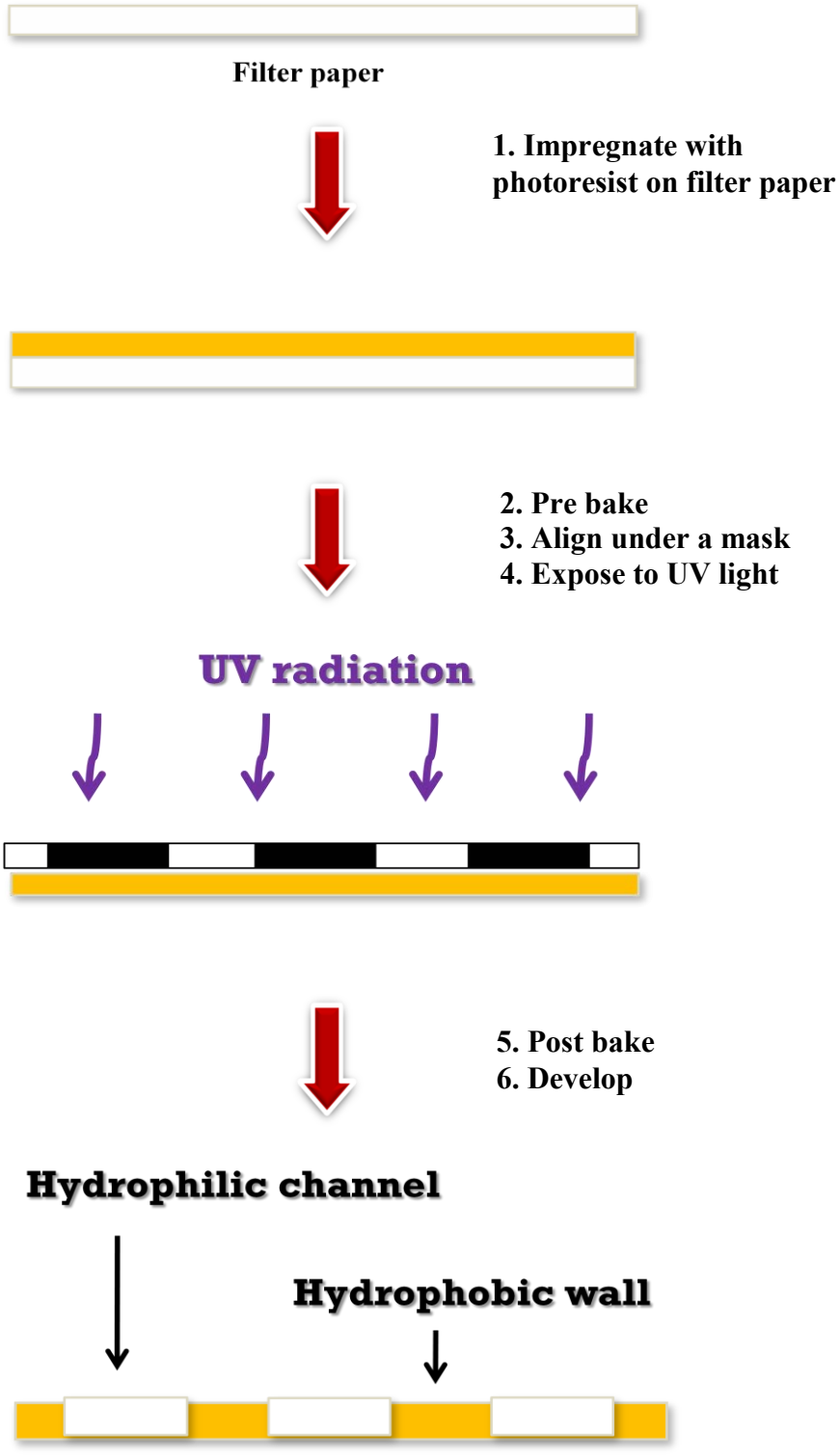


Figure 3.2 Preparation of lab-on-paper by photolithography.

3.2 Preparation of electrode

3.2.1 Instruments and equipments

The instruments and equipments involved in the preparation of electrode are listed in Table 3.3.

Table 3.3 List of instruments and equipments for the preparation of electrode.

Instruments/equipments	Suppliers
Screen-printed blocks	Chaiyaboon Co., Bangkok, Thailand
Hot air oven	Memmert, USA
Stop watch	Mini Timer, Japan

3.2.2 Chemicals

The chemicals for the preparation of electrode are listed in Table 3.4.

Table 3.4 List of chemicals for the preparation of electrode.

Chemicals	Suppliers
Carbon ink	Electrodag PF-407C, Acheson, California, USA
Silver/silver chloride ink	Electrodag 7019, Acheson, California, USA
Acetone	Merck, Germany

3.2.3 Methodology

For the electrode preparation, the screen-printed electrodes were prepared in-house. Carbon ink was used as the working electrode (WE) and the counter electrode (CE) and silver/ silver chloride ink was used as the reference electrode (RE) and conductive pads. All electrodes were screened on patterned paper and then were cured in the oven at 65 °C for 30 min. The designed paper-based microfluidics for dual electrochemical and colorimetric detection is illustrated in Figure 3.4.

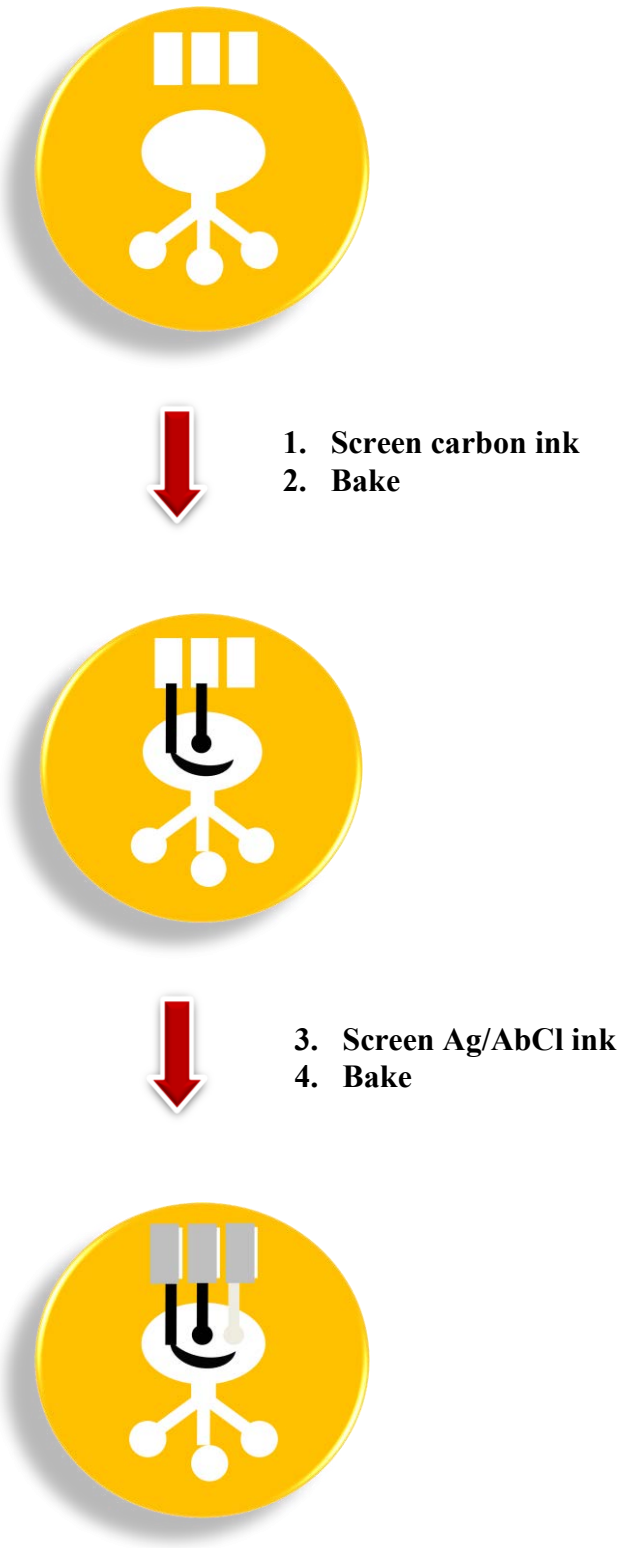


Figure 3.3 Preparation of electrode by screen-printing technology.

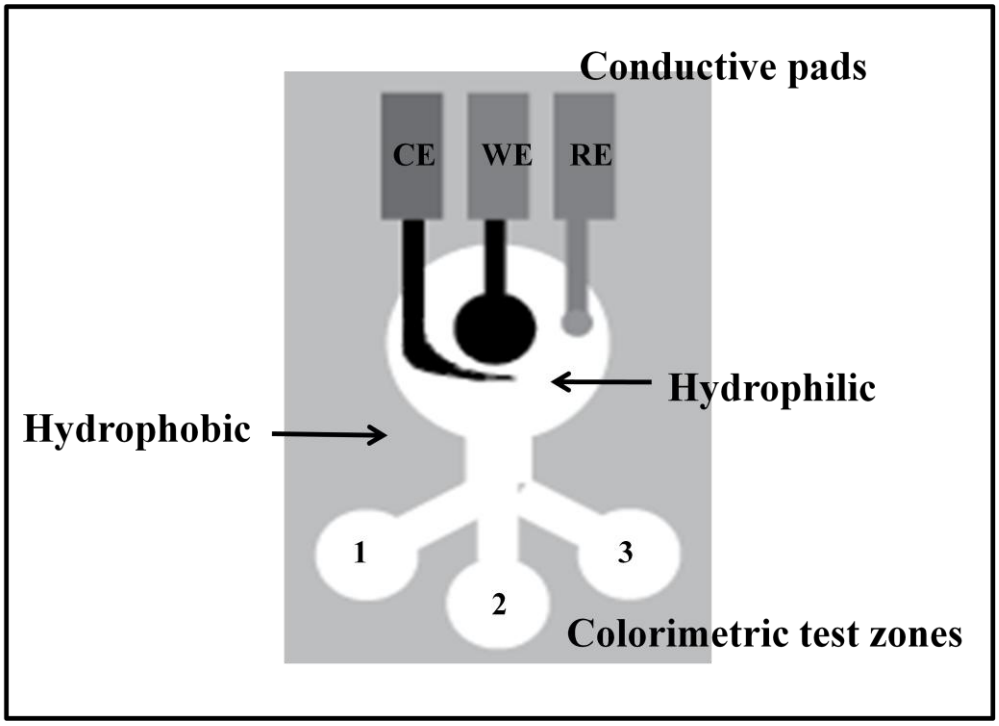


Figure 3.4 The paper-based microfluidic consisting of the electrochemical (WE, working electrode; RE, reference electrode; CE, counter electrode) and colorimetric test zones (1, 2, 3).

3.3 Chemical preparations

3.3.1 Instruments and equipments

The instruments and equipments for the chemical preparations are listed in Table 3.5.

Table 3.5 List of instruments and equipments for the chemical preparations.

Instruments/equipments	Suppliers
Milli Q Water System, R ≥ 18.2 MΩcm	Millipore, USA
Analytical balance, Mettler Toledo AB204-S	Mettler, Switzerland
pH meter, 827 pH Lab	Metrohm, Switzerland
Autopipette and tips	Eppendorf, Germany

3.3.2 Chemicals

The chemicals for the chemical preparations are listed in Table 3.6. All reagents were of analytical grade. The 18 MΩ cm^{-1} resistance water, obtained from a Millipore Milli-Q purification system, was used throughout this experiment.

Table 3.6 List of chemicals for the chemical preparations.

Chemicals	Suppliers
Sodium acetate trihydrate	Fluka, Switzerland
Acetic acid	Merck, Germany
Ferulic acid	Fluka, Switzerland
Sodium carbonate	Merck, Germany
Phosphoric acid	Merck, Germany
Sodium hydroxide	Merck, Germany

3.3.3 Methodology

3.3.3.1 Preparation of A 0.1 M acetate buffer (HAc-NaAc buffer)

A 0.1 M acetate buffer (HAc-NaAc buffer), which served as a supporting electrolyte [59], was prepared from sodium acetate trihydrate and concentrated acetic acid (100%). 3.4 g of sodium acetate trihydrate was dissolved in Milli-Q water to a final volume of 250 mL and 1.43 mL of acetic acid was diluting with 250 mL Milli-Q water. Next, mixing sodium acetate and acetic acid in appropriate ratio to obtain acetate buffer of pH 3, 4, 5, 6 and 7. 2 M phosphoric acid and NaOH was used to adjust the pH. The appropriate ratio of mixing sodium acetate and acetic acid are listed in Table 3.7.

Table 3.7 The appropriate ratio of mixing sodium acetate and acetic acid.

pH	Sodium acetate (mL)	Acetic acid (mL)
3.8	10.9	89.1
4	16.6	83.4
5	67.8	32.2
5.6	89.3	10.7

3.3.3.2 Preparation of phosphoric acid

A stock solution of 2 M phosphoric acid was prepared by diluting 13.47 mL of concentrated phosphoric acid with 100 mL Milli-Q water.

3.3.3.3 Preparation of sodium hydroxide

A stock solution of 2 M sodium hydroxide was prepared by dissolving 8 g sodium hydroxide in 100 mL Milli-Q water.

3.3.3.4 Preparation of ferulic acid

50 ppm ferulic acid solution was prepared using 0.125 g of ferulic acid dissolved in 25 mL of 0.1 M acetate buffer.

3.3.3.5 Preparation of sodium carbonate

The sodium carbonate solution was prepared by diluting concentrated sodium carbonate with Milli-Q water to a final concentration 2%, 4%, 6%, 8% and 10%.

3.4 Determination of ferulic acid

3.4.1 Instruments and equipments

The instruments and equipments for the determination of ferulic acid are listed in Table 3.8.

Table 3.8 List of instruments and equipments for the determination of ferulic acid.

Instruments/equipments	Suppliers
Potentiostat (Autolab PGSTAT 30)	Metrohm, Switzerland
Faraday cage	Copperplate, custom made

3.4.2 Methodology

A potentiostat (Autolab PGSTAT 30) was used to perform Cyclic voltammetric (CV) and differential pulse voltammetric (DPV) experiments. For voltammetric experiments, CV was performed at a scan rate of 100 mV s^{-1} and the potential was scanned from −0.2 V to 0.8 V vs Ag/AgCl. DPV was performed at pulse amplitude of 50 mV and a step potential of 7 mV in the potential range from 0 V to 1.0 V vs. Ag/AgCl.

Figure 3.5 A potentiostat (Autolab PGSTAT 30)

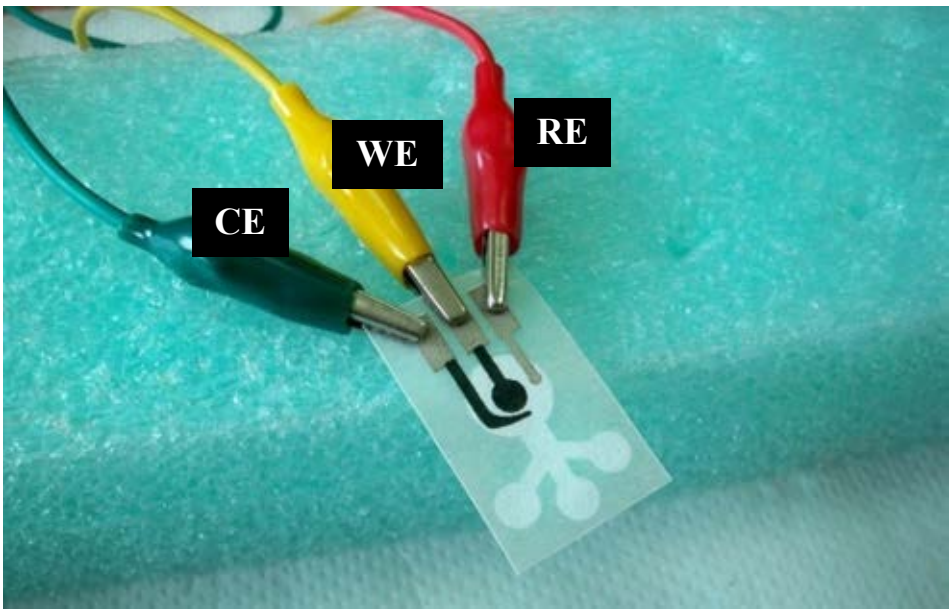


Figure 3.6 Lab-on-paper system

3.5 Determination of total antioxidant capacity using colorimetric detection

3.5.1 Instruments and equipments

The instruments and equipments for the determination of total antioxidant capacity using colorimetric detection are listed in Table 3.9.

Table 3.9 List of instruments and equipments for the determination of total antioxidant capacity using colorimetric detection.

Instruments/equipments	Suppliers
Milli Q Water System, R ≥ 18.2 MΩcm	Millipore, USA
Analytical balance, Mettler Toledo AB204-S	Mettler, Switzerland
Stop watch	Mini Timer, Japan
Autopipette and tips	Eppendorf, Germany
Digital Camera E05	Cannon, Thailand
Notebook with Adobe Photoshop	Dell, Thailand

3.5.2 Chemicals

The chemicals for the determination of total antioxidant capacity using colorimetric detection are listed in Table 3.10.

Table 3.10 List of chemicals for the determination of total antioxidant capacity using colorimetric detection.

Chemicals	Suppliers
Folin-Ciocalteau reagent	Carlo Erba, Italy
Sodium carbonate	Merck, Germany

3.5.3 Methodology

Total soluble antioxidants were determined by the colorimetric method on lab-on-paper using the Folin-Ciocalteau reagent. 2 μL of Folin-Ciocalteau reagent was applied to the colorimetric test zone. After that, 2.5 μL of 10% sodium carbonate was dipped onto this area and the material was then allowed to dry to prepare for use.

For the colorimetric method, a digital camera (EOS 1000D, Canon) was used to take the picture. Then, the picture was recorded in terms of mean intensity in the histogram using Adobe Photoshop.

1. **Take a picture of result**

2. **Select test zone**

3. **Record mean intensity**

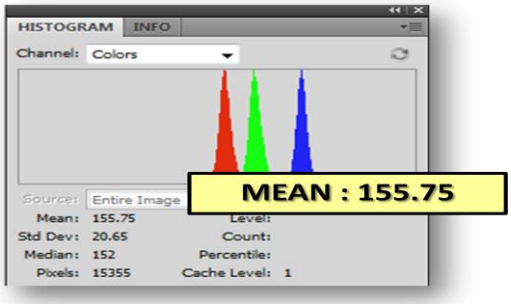


Figure 3.7 The measurement of mean intensity by Adobe Photoshop.

3.6 Analytical performance

3.6.1 Linearity

The standard solution of ferulic acid in the concentration range of 1-140 ppm was analyzed by DPV and colorimetric detection under the optimal condition. The average of triplicate measurements was used to plot calibration curve which linear range can be obtained.

3.6.2 Limit of detection (LOD)

The limit of detection (LOD) was determined statistical method from the calibration curve in the range of 1-140 ppm and calculated from $3S_{blank}$ /S where S_{blank} is the standard deviation of blank measurement (n=3) and S is the slope of the linearity or the sensitivity of the method. In this work, the LOD was obtained from experimental method which is the lowest concentration that the signal can be measured.

3.6.3 Limit of quantitation (LOQ)

The limit of quantitation (LOQ) was determined statistical method from the calibration curve in the range of 1-140 ppm and calculated from $10S_{blank}$ /S where S_{blank} is the standard deviation of blank measurement (n=10) and S is the slope of the linearity or the sensitivity of the method.

3.7 Sample analysis

3.7.1 Instruments and equipments

The instruments and equipments for sample analysis are listed in table 3.10.

Table 3.11 List of instruments and equipments for the sample analysis.

Instruments/equipments	Suppliers
Milli Q Water System, R ≥ 18.2 MΩcm	Millipore, USA
Analytical balance, Mettler Toledo AB204-S	Mettler, Switzerland
Stop watch	Mini Timer, Japan
Autopipette and tips	Eppendorf, Germany
Hot plate stirrer HS-115	HL instrument, USA
Magnetic stirring bars	SGS ICS, Switzerland
Filter paper	Whatman, UK

3.7.2 Sample preparation

Corn milk solution was prepared by weighing 5 g of commercially corn milk and then heated at 90 °C in 50 mL of Milli-Q water. After that, the samples were filtered and used for analysis. The sample of corn cider was used without any preparation. However, all of the samples were diluted once for ferulic acid and total antioxidant capacity determination.

3.7.3 Accuracy

For accuracy, the sample was spiked with standard ferulic acid at the concentration of 20 and 50 ppm. Each spiked was analyzed in triplicate runs. The accuracy is assessed in terms of recovery. The recovery is then calculated as the percentage of the measured spike of the matrix sample relative to the measured spike of the blank control, using the following formula:

$$\% \text{ Recovery} = \frac{\text{Spiked}_{\text{matrix}}}{\text{Spiked}_{\text{blank}}} \times 100$$

3.7.4 Repeatability

The repeatability was studied by ten replicate measurements of analyte solution. The repeatability is assessed in terms of the relative standard deviation (% RSD), using the following formula:

$$\% \text{ RSD} = \frac{\text{Standard deviation}}{\text{Mean}} \times 100$$

3.7.5 Comparison of the proposed method with HPLC-UV and UV-vis

Under the optimal conditions, the proposed method was applied to determination of ferulic acid and total antioxidant capacity in corn milk and corn cider sample. The results were validated by HPLC_UV method for electrochemical detection and UV-vis spectrophotometry for colorimetric detection.

CHAPTER IV

RESULTS AND DISCUSSION

In this chapter, the results and discussion obtained from the development of lab-on-paper coupled with electrochemical detection for the determination of ferulic acid were presented. The optimization of operation parameters was described. In the following sections, the simple colorimetric detection was developed to simultaneously determine ferulic acid and total antioxidant capacity. The procedure was then successfully applied to determine ferulic acid and total antioxidant in food samples and comparison with another technique.

4.1 Electrochemical Behavior of Ferulic Acid

First, cyclic voltametry was used to investigate the electrochemistry of ferulic acid on lab-on-paper. HAc-NaAc buffer solution was used as the supporting electrolyte for ferulic acid detection, because it provided the well-defined peak at carbon electrode [59]. Ferulic acid exhibited a well-defined oxidation peak during the scan of the potential toward the positive direction at carbon electrode at about 0.4 V vs. Ag/AgCl.

These results indicate that lab-on-paper coupled with the carbon electrode offers great performance and substantial sensitivity for ferulic acid detection. Therefore, the electrochemical behavior of ferulic acid can be investigated by this electrode. The cyclic voltammogram of 50 ppm feruic acid in 0.1 mol L^{-1} HAc-NaAc buffer solution (pH 5) is shown in Figure 4.1.

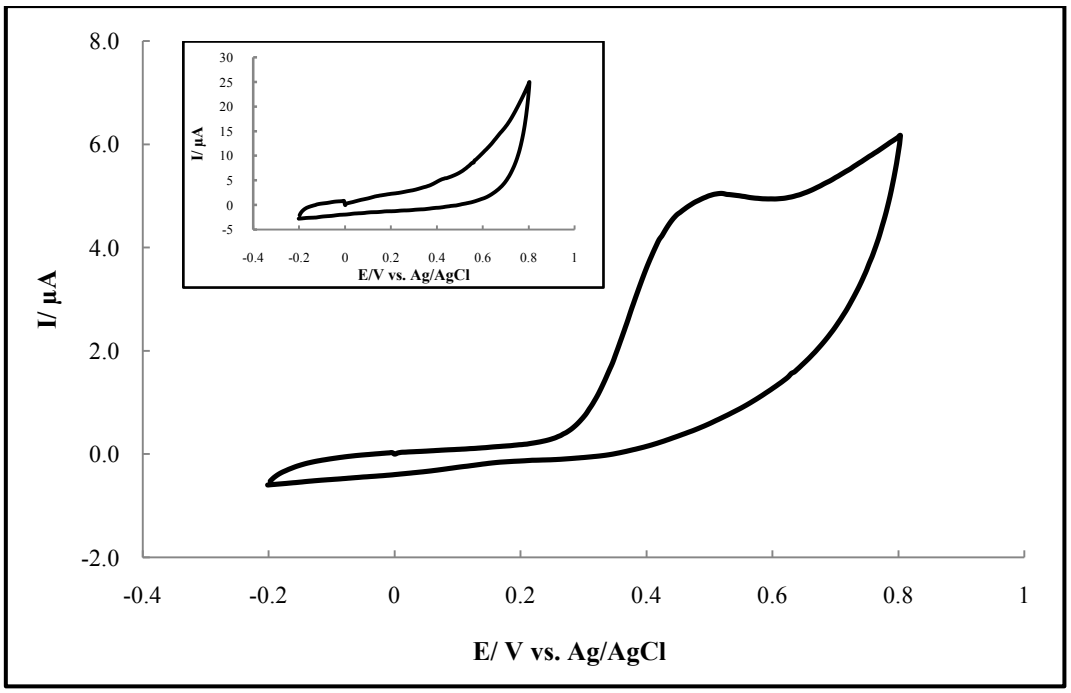


Figure 4.1 Cyclic voltammogram of 50 ppm ferulic acid in 0.1 mol L^{-1} HAc- NaAc buffer solution (pH 5). Scan rate 100 mV/s, electrode area 0.8 cm^2. Voltammograms shown are representative of at least five independent repetitions.

4.2 Optimization of the Experimental Conditions

4.2.1 Effect of pH

In any electrochemical detection, the supporting electrolyte pH had a significant impact on the ionization and redox reaction of each analyte. Therefore, the optimization of the supporting electrolyte pH was carried out for the electrochemical detection of ferulic acid. The pH values on the peak potential and oxidation current were examined in the pH range 3.0 – 7.0, as shown in Figure 4.2.

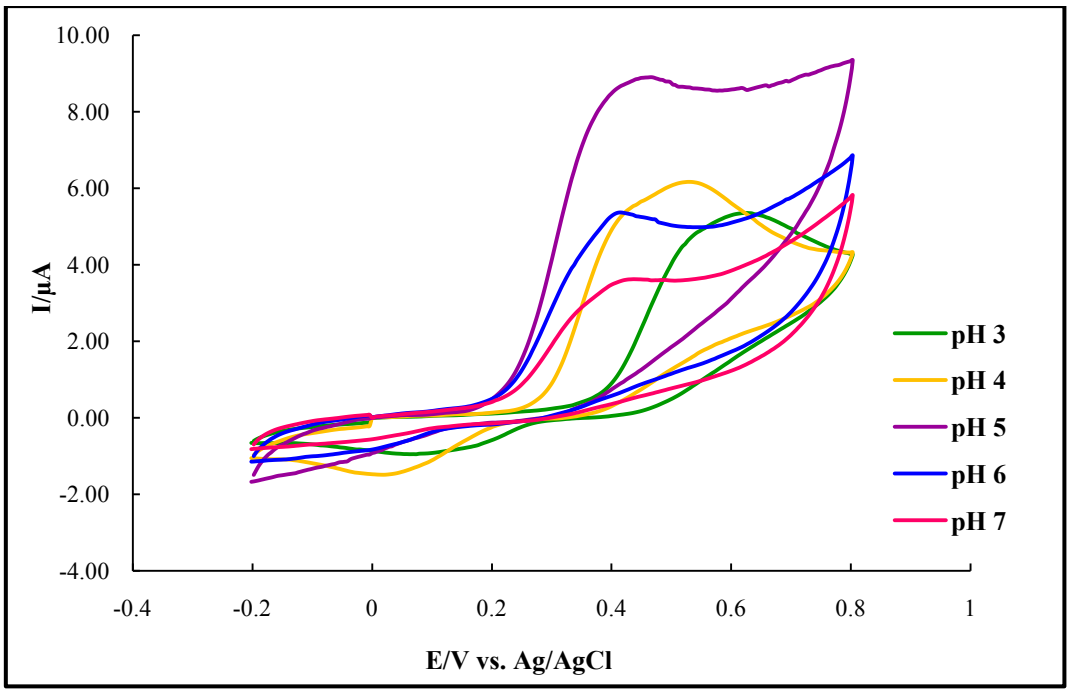


Figure 4.2 Cyclic voltammogram of 50 ppm ferulic acid in 0.1 mol L^{-1} HAc- NaAc buffer solution. Scan rate 100 mV/s. Voltammograms shown are representative of at least five independent repetitions.

All buffers contained a 0.1 mol L^{-1} acetate buffer. Figure 4.3 displays the relationship obtained from plotting between the pH values and the oxidation peaks potentials from cyclic voltammogram. As can be seen in Figure 4.3, the oxidation peak potential shifted negatively with the increase of pH. It can be observed that ferulic acid is easily oxidized with an increase of pH values since the potentials shifted negatively at a higher pH.

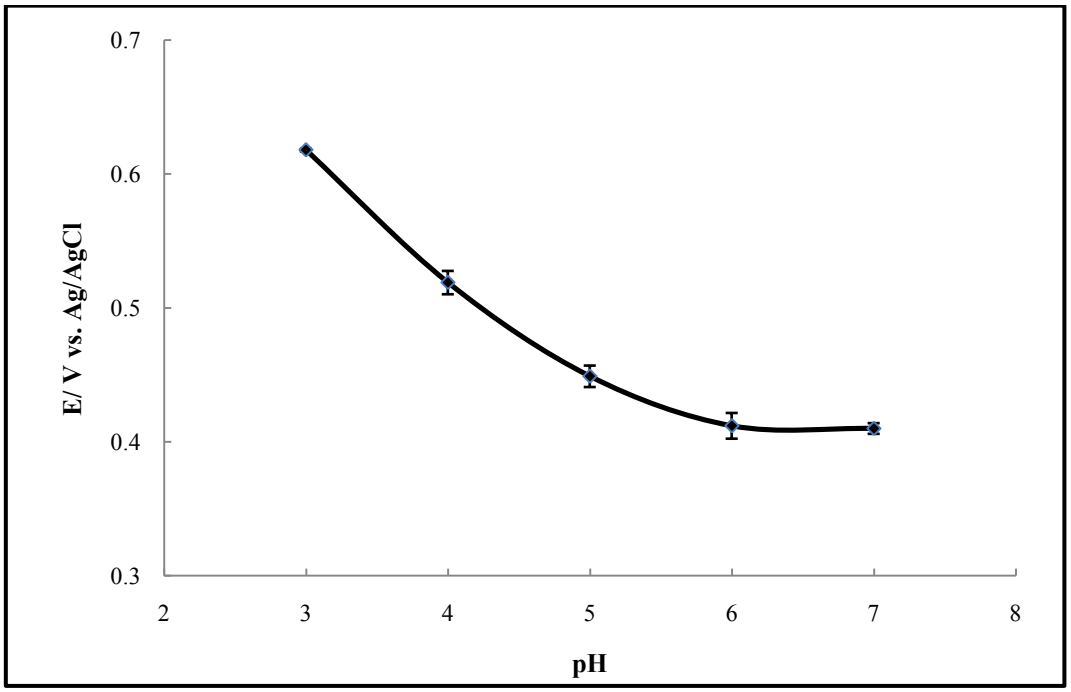


Figure 4.3 The influence of pH on the oxidation peak potential**.**

Moreover, the relationship between the oxidation peak currents and the pH values was investigated. As shown in Figure 4.4, it is evident that the oxidation peak current of ferulic acid increased sharply with increasing pH from 3.0 to 5.0 and then decreased as pH from 5.0 to 7.0. This could be the consequence of deprotonation involved in the oxidation process which was increased at higher pH values [64]. The oxygen-containing functional groups of carbon electrode can develop a negative dipolar field that interact electrostatically with the ions and dipoles in the electrolyte near the electrode surface. The molecule of ferulic acid is positively charged in strong acidic solution. When increasing pH, it changes to neutral or negatively charged. So after pH above 5.0, the electrostatic repulsion between the negative dipolar field and the negative charge of ferulate ion could be responsible for the decreased oxidation peak current with increasing pH. The results showed that a buffer pH 5.0 exhibited the highest current signal using carbon electrodes. Therefore, this buffer was selected as a suitable pH for all subsequent work.

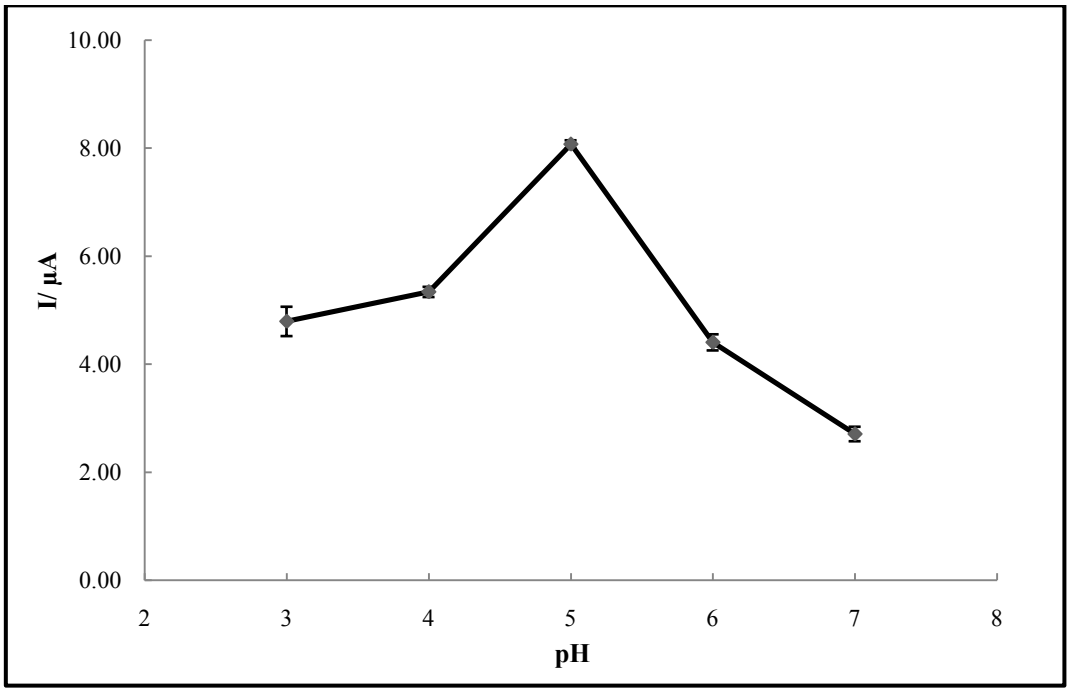


Figure 4.4 The influence of pH on the oxidation peak currents.

4.2.2 Effect of the scan rate

The influence of the scan rate was also investigated to authenticate the adsorption of ferulic acid on the carbon electrode surface. It was carried out by cyclic voltametry in the potential range from 50 to 500 mV s^{-1} (Figure 4.5). From Randles-Sevcik equation as shown in Equation 4.1, the peak current is directly proportional with the root of the scan rate.

$$i_p = (2.99\times10^5)n(\infty n_a)^{1/2}ACD^{1/2}v^{1/2} \quad \text{at } 25°C \qquad \text{(Equation 4.1)}$$

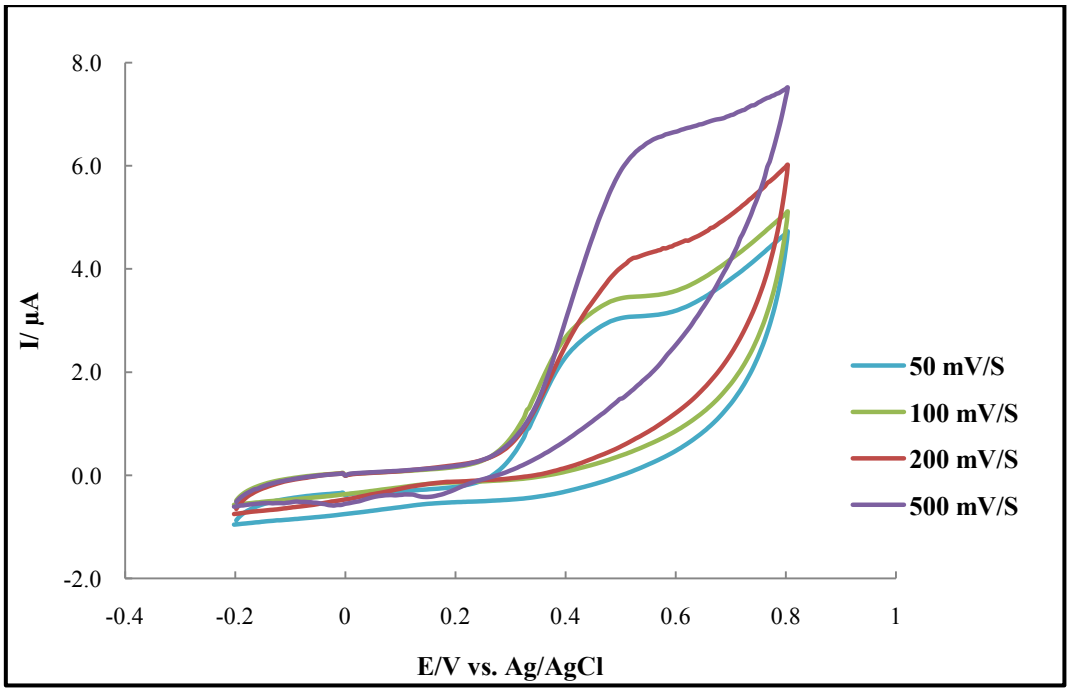


Figure 4.5 Cyclic voltammogram of 50 ppm ferulic acid in 0.1 mol L^{-1} HAc- NaAc buffer solution at different scan rate.

As Shown in Figure 4.6, the relationship between the current and the root of the scan rate was investigated. The oxidation peak currents increased linearly as the root of the scan rate increased. Hence, this confirmed that the reaction was controlled by the diffusion process and ferulic acid is slightly adsorbed on the electrode surface.

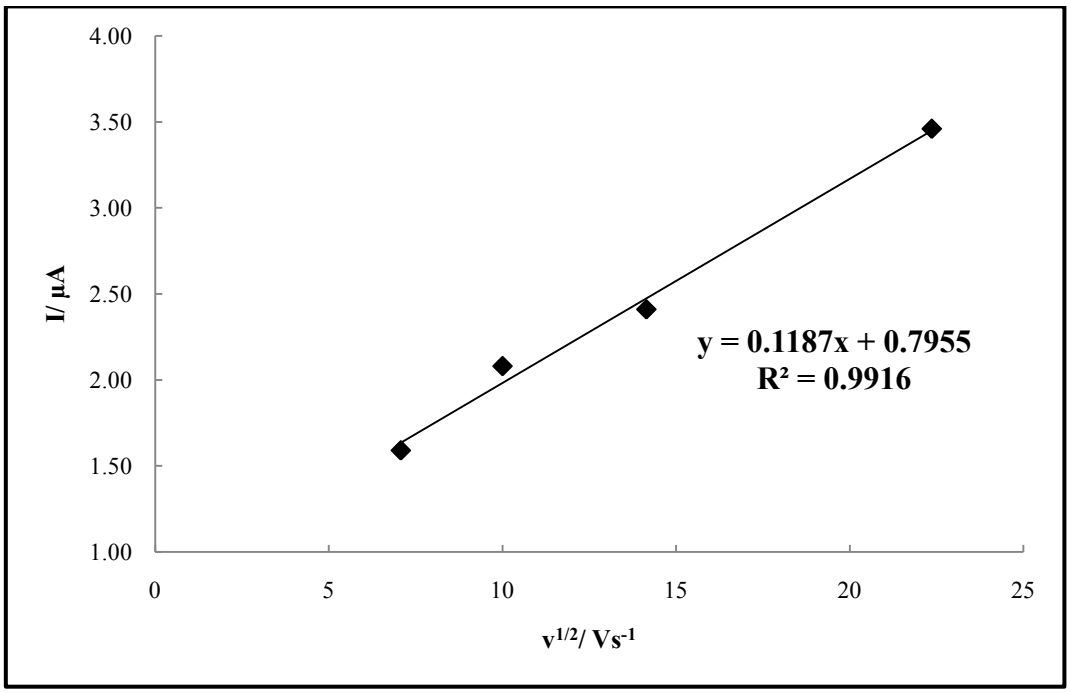


Figure 4.6 The influence of scan rate on the oxidation peak currents. Other conditions same as Figure 4.2.

4.3 Analytical performances

Differential pulse voltammetry is the typical technique used to achieve a lower detection limit compared to those obtained from cyclic voltametry. Therefore, the differential pulse voltammetry was selected as a technique used to determine ferulic acid. Figure 4.7, it can be seen that differential pulse voltammogram of ferulic acid is clearly observed.

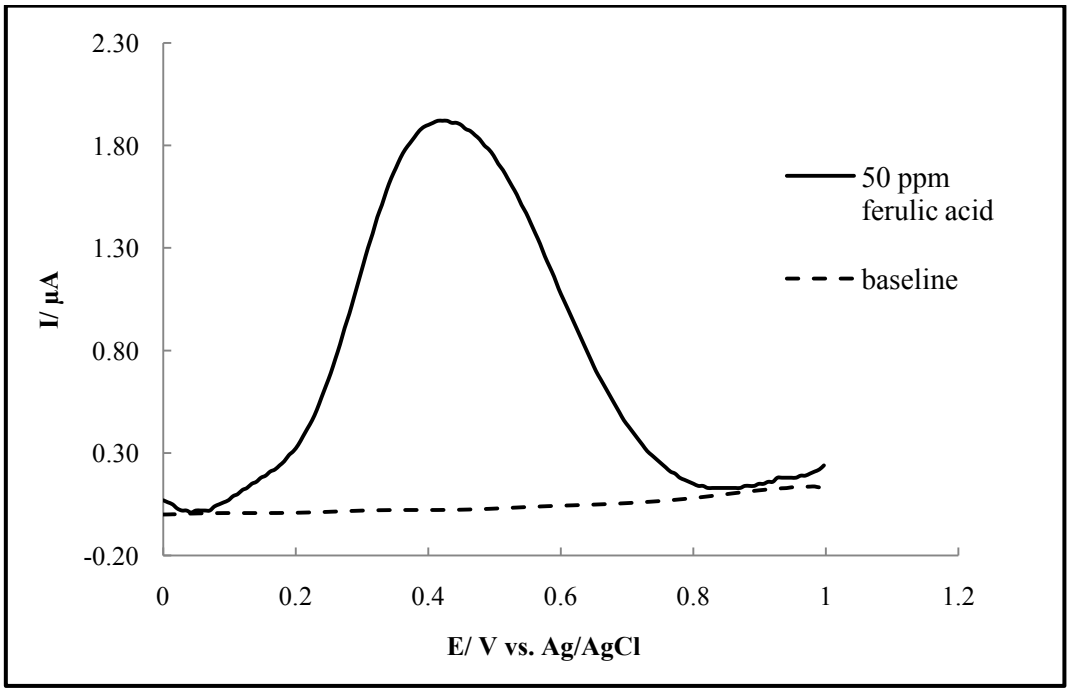


Figure 4.7 Differential pulse voltammograms of 50 ppm ferulic acid in 0.1 mol L^{-1} HAc-NaAc buffer solution (pH 5).

4.3.1 Effect of differential pulse voltammetric parameters

4.3.1.1 Effect of the pulse amplitude

To obtain the optimal conditions for the quantitative of ferulic acid, the effect of the pulse amplitude was examined. The pulse amplitude was varied from 50 mV to 250 mV. As shown in Figure 4.8, the peak currents of ferulic acid increased when pulse amplitude increased, however, the peak broadening was observed as well. Therefore, the pulse amplitude of 50 mV was chosen to trade off between the peak height and peak broadening.

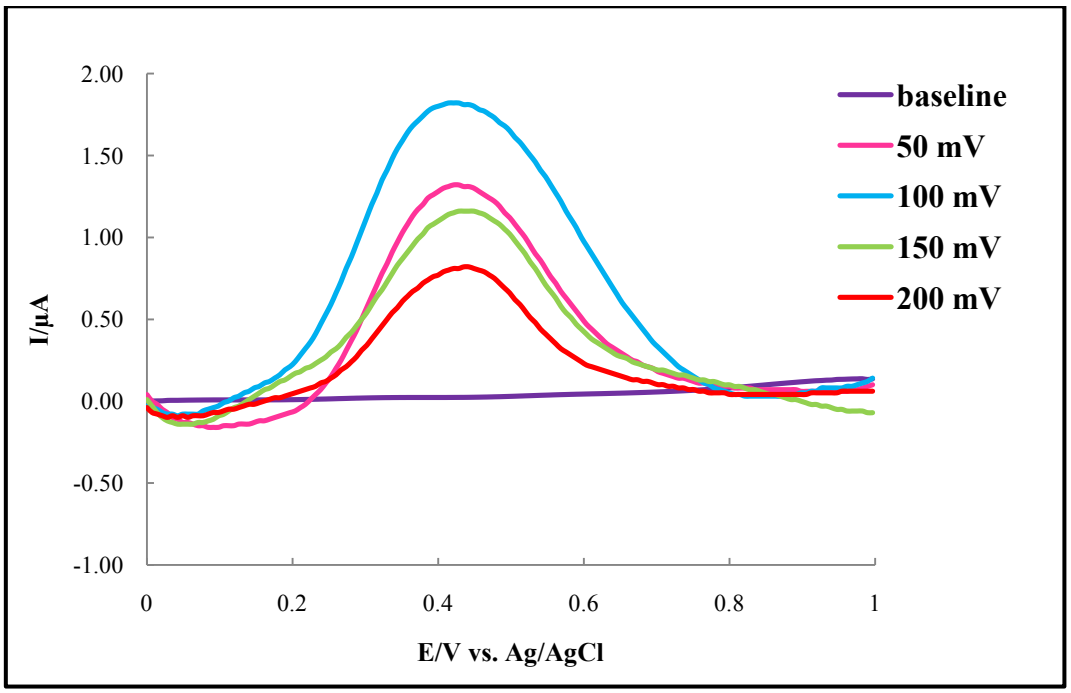


Figure 4.8 Differential pulse voltammograms of 50 ppm ferulic acid in 0.1 mol L^{-1} HAc-NaAc buffer solution (pH 5) at different pulse amplitude.

4.3.1.2 Effect of the step potential

Next, the effect of the step potential was studied. It was varied within the range of 2 mV−10 mV and the optimal value was found to be 7 mV, due to the increment in the peak intensity caused by an increment of step potential up to 7 mV and slightly decreased above 7 mV (Figure 4.8).

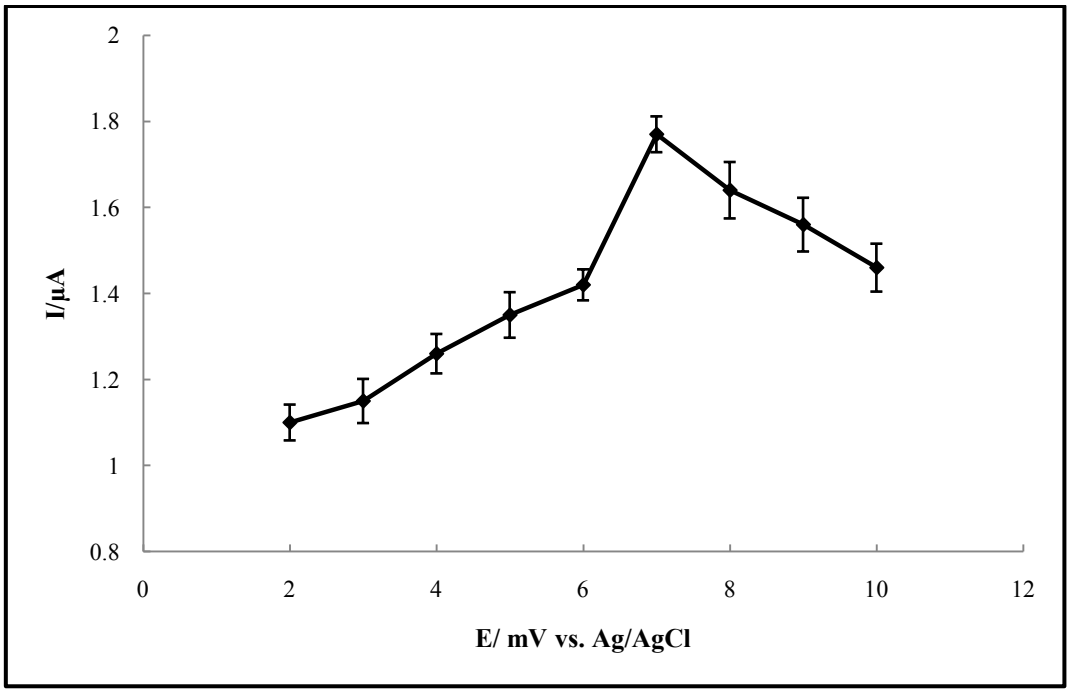


Figure 4.9 The influence of step potentail on the oxidation peak currents.

4.3.2 Calibration curves

As shown in Figure 4.9, the ferulic acid solutions were investigated by differential pulse voltammetry under optimal conditions. Defined peaks with current proportional to the analyte concentration are observed. As shown in Figure 4.10, the oxidation peak current of ferulic acid showed a linear relationship with its concentration in the range of 3 and 140 ppm (correlation coefficient of $R^2 = 0.9994$). The resulting calibration plots are linear with the sensitivity of 0.0246 μA/ppm.

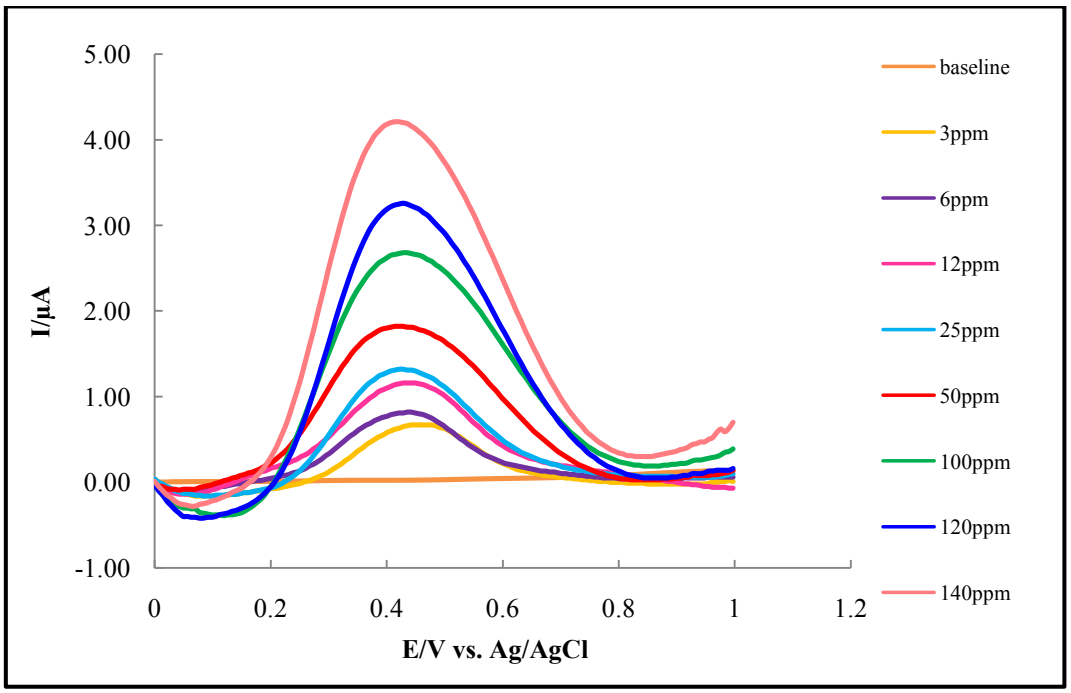


Figure 4.10 Differential pulse voltammograms of ferulic acid (3, 6, 12, 25, 50, 100, 120, 140) in 0.1 mol L^{-1} HAc- NaAc buffer solution (pH 5), measured under the optimal experimental conditions.

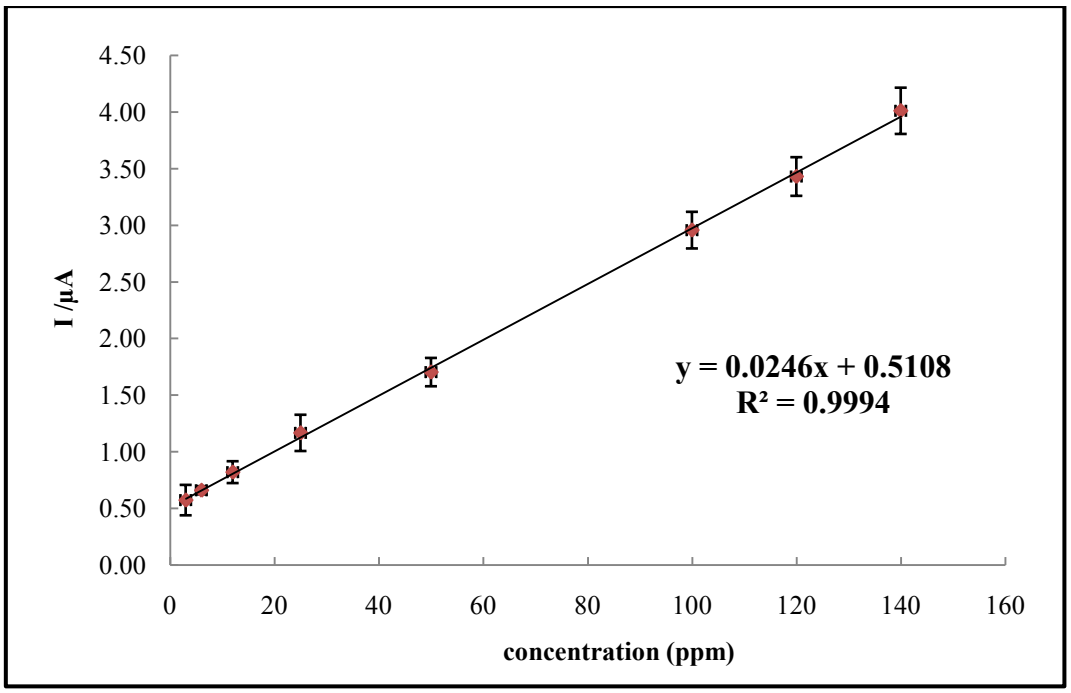


Figure 4.11 Calibration curves of ferulic acid.

4.3.3 The limit of detection (LOD) and limit of quantitation (LOQ)

The limit of detection (LOD) was obtained at concentration as low as 1 ppm based on signal-to-noise ratio (S/N) of 3 and limit of quantitation (LOQ) was also found to be 3 ppm based on signal-to-noise ratio (S/N) of 10.

4.3.4 Repeatability

In this work, the repeatability was studied by ten replicate measurements of standard ferulic acid. The developed method showed the high reproducibility in different concentrations (Table 4.1).

Table 4.1 The relative standard deviations of ferulic acid (n=10)

Concentration (ppm)	SD	%RSD
3	0.02	4.25
12	0.03	3.35
50	0.05	2.84
120	0.07	2.07

4.4 Colorimetric determination of total antioxidant capacity

4.4.1 Effect of concentration of sodium carbonate

The colorimetric detection based on the Folin-Ciocalteau Total Phenolic Assay gained popularity and was commonly used to determine the total antioxidant content. This assay actually measures the change in color when metal oxides are reduced. The Folin-Ciocalteau reagent reacted with phenolic compounds under basic conditions which were adjusted by sodium carbonate. Therefore, the effect of concentration of sodium carbonate was investigated in the concentration range of 2%-14%. After a drop of sodium carbonate was applied, the color changes were very obvious (Figure 4.11).

Mean intensity was recorded in the histogram using Adobe Photoshop. As shown in Figure 4.12, the mean intensity decreased when the concentration of sodium carbonate increased and still constant above 10%. Thus, the concentration of 10% sodium carbonate was appropriate to the determination of total antioxidant.

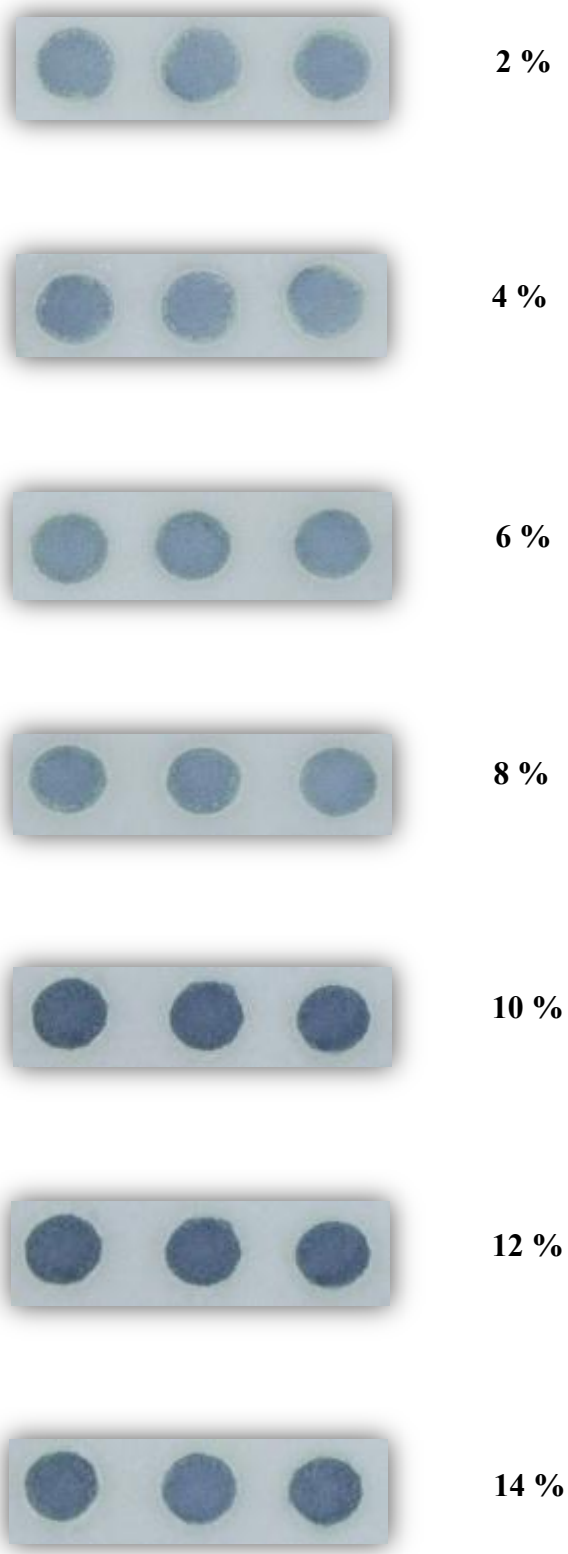


Figure 4.12 The color change at the different concentration of sodium carbonate.

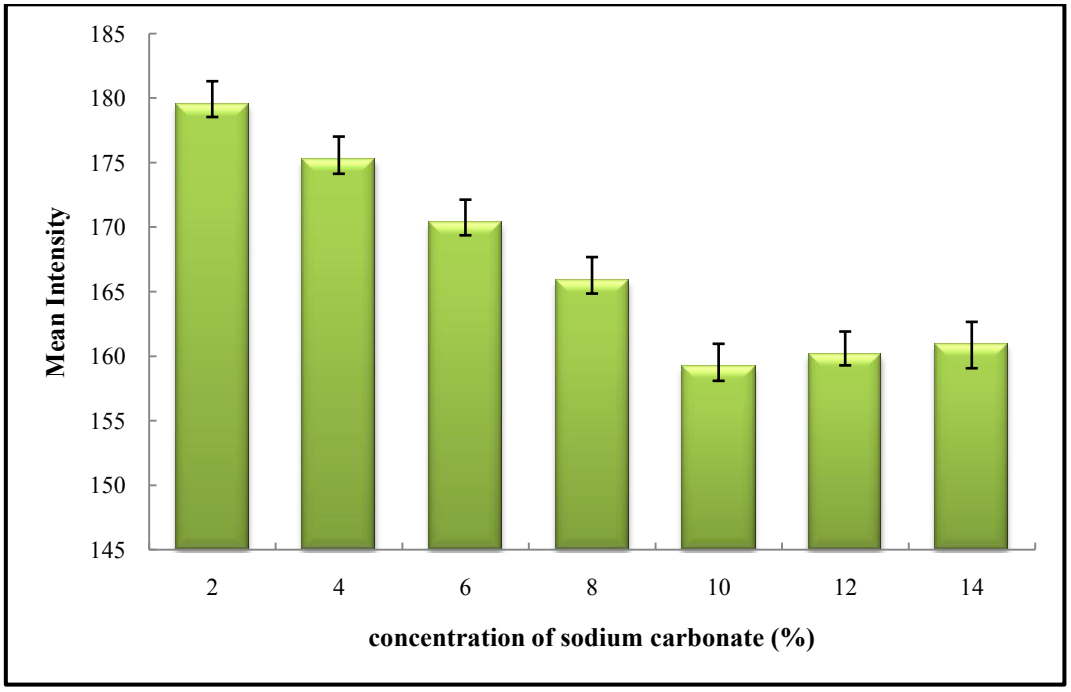


Figure 4.13 The effect of sodium carbonate on the mean intensity.

4.4.2 Calibration curves

After the concentration of sodium carbonate was achieved, the standard curve was prepared by using ferulic acid at various concentrations (1-200 ppm). Figure 4.13 showed the resulting color intensity, which is proportional to the concentration of ferulic acid and was assessed visually. The color intensity of ferulic acid from three replications was examined using adobe photoshop and the mean intensity was recorded in the histogram. By plotting the mean intensity *vs.* the ferulic acid concentration, Figure 4.14 showed a linear range between 1 and 140 ppm (correlation coefficient of $R^2 = 0.9967$).

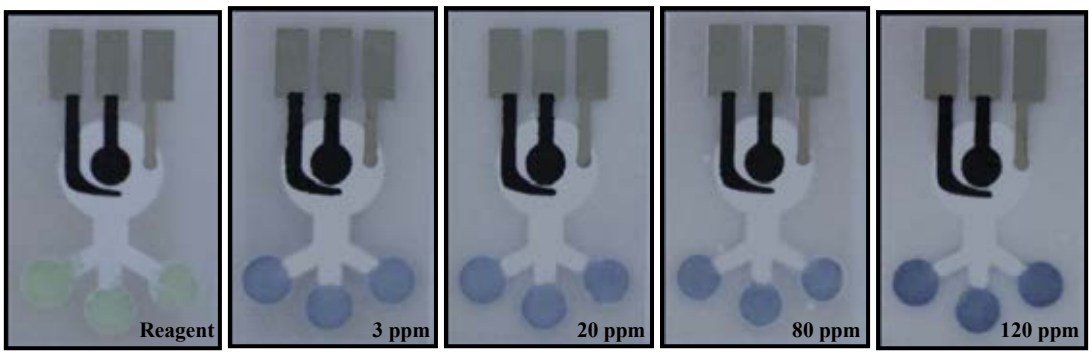


Figure 4.14 Intensity of the observed color is proportional to the concentration of ferulic acid.

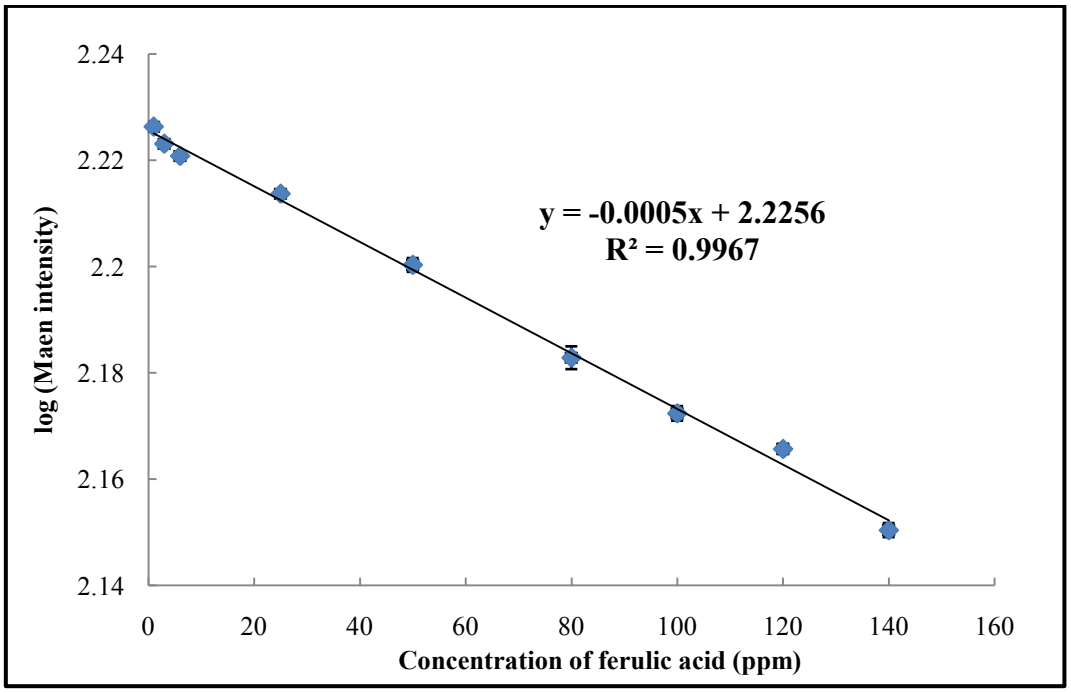


Figure 4.15 Calibration curves of ferulic acid using colorimetric detection.

4.4.3 The limit of detection (LOD) and the limit of quantitation (LOQ)

The limit of detection (LOD) was obtained at concentration as low as 1 ppm based on signal-to-noise ratio (S/N) of 3 and limit of quantitation (LOQ) was also found to be 1 ppm based on signal-to-noise ratio (S/N) of 10.

4.4.4 Repeatability

In this experiment, the relative standard deviations for ten repeating measurements of total antioxidant at different concentrations are shown in Table 4.2. This indicated that the developed method exhibited the high reproducibility.

Table 4.2 The relative standard deviations of total antioxidant (n=10)

Concentration (ppm)	SD	%RSD
5	1.80	1.08
20	1.73	1.06
80	2.34	1.52
120	3.09	2.13

4.5 Analytical application in a real sample

To verify the applicability of the proposed lab-on-paper devices and methodology developed in the present work, the target compound, which were corn cider and corn milk from local supermarkets were simultaneously determined by electrochemical and colorimetric detection using standard addition. The determination of ferulic acid and the total antioxidant capacity was performed using the same experiment conditions as previously reported. Two different samples were divided into two portions and then spiked with 20 and 50 ppm of ferulic acid, respectively. The recovery efficiencies obtained for the sample spiked with the ferulic acid standard solution using electrochemical detection are summarized in Table 4.3, which revealed a recovery in the range of 96.85 % – 99.92 % and 96.94 % –103.20 % for corn cider and corn milk, respectively.

Table 4.3 The recovery of ferulic acid (n=3)

Sample	Ferulic acid (ppm ± SD, n = 3)		% Recovery
	Added	Found	
Corn cider	0	0.24 ± 0.018	-
	20	19.61 ± 0.159	96.85
	50	50.20 ± 4.478	99.92
Corn milk	0	0.94 ± 0.086	-
	20	21.58 ± 1.180	103.20
	50	49.41 ± 4.328	96.94

For colorimetric detection, the results obtained for total antioxidant analysis in corn cider and corn milk are shown in Table 4.4, which revealed a recovery in the range of 97.60 % – 97.98 % and 97.20 % – 103.42 %, respectively.

Table 4.4 The recovery of total antioxidant (n=3)

Sample	Total antioxidant (ppm ± SD, n = 3)		% Recovery
	Added	Found	
Corn cider	0	0.50 ± 0.0006	-
	20	20.02 ± 0.0100	97.60
	50	49.49 ± 0.0544	97.98
Corn milk	0	0.58 ± 0.0009	-
	20	20.02 ± 0.0140	97.20
	50	52.29 ± 0.1045	103.42

4.6 Comparison the proposed method with HPLC-UV and UV-vis

In addition, the results were validated by HPLC-UV method for electrochemical detection [54] and UV-vis spectrophotometry for colorimetric detection [65]. The results obtained by the proposed method and HPLC-UV method and UV-vis spectrophotometry are shown in Table 4.5. and Table 4.6. It was found that the results obtained from the two methods were in good agreement and showed no significant difference between the two methods. The experimental *t-values* between the two pairs of methods were 0.948, 2.522, 0.532 for ferulic acid and 1.316, 1.160, 0.632 for total antioxidant at 0, 20 and 50 ppm, respectively (*t-critical* = 2.571). Consequently, the methods for the determination of ferulic acid and total antioxidant capacity using lab-on-paper reported here are acceptable and potentially feasible for use in real samples.

Table 4.5. Determination of ferulic acid in real samples using proposed lab-on-paper with electrochemical detection and conventional HPLC-UV

Samples	**Added**	**Ferulic acid (ppm ± SD, n = 3)**		**Proposed method**	
		Proposed method	**HPLC-UV**	**% Recovery**	**%RSD**
Corn cider	0	0.24 ± 0.018	0 ± 0.0000	0	7.68
	20	19.61 ± 1.159	18.96 ± 0.3684	96.85	5.91
	50	50.20 ± 4.478	51.01 ± 0.5326	99.92	8.92
Corn milk	0	0.94 ± 0.086	1.09 ± 0.0869	0	9.19
	20	21.58 ± 1.180	20.47 ± 0.2704	103.20	5.47
	50	49.41 ± 4.328	48.01 ± 0.2704	96.94	8.76

Table 4.6. Determination of total antioxidant in real samples using proposed lab-on-paper with colorimetric detection and conventional UV-vis spectrophotometry

Samples	**Added**	**Total antioxidant (ppm ± SD, n = 3)**		**Proposed method**	
		Proposed method	**UV-Vis**	**% Recovery**	**% RSD**
Corn cider	0	0.50 ± 0.0006	0.95 ± 0.0051	0	0.12
	20	20.02 ± 0.0100	18.79 ± 0.0051	97.60	0.05
	50	49.49 ± 0.0544	51.45 ± 0.0003	97.98	0.11
Corn milk	0	0.58 ± 0.0009	1.33 ± 0.0053	0	0.15
	20	20.02 ± 0.0140	18.23 ± 0.0002	97.20	0.07
	50	52.29 ± 0.1045	51.92 ± 0.0002	103.42	0.20

CHAPTER V

CONCLUSIONS AND FUTURE PERSPECTIVE

5.1 Conclusions

The practical, rapid, highly sensitive and accurate method for ferulic acid and total antioxidant quantitative determination was first successfully developed by lab-on-paper coupled with electrochemical and colorimetric detection. Under optimized conditions, the differential pulse voltammogram provided a well-defined oxidation peak. The calibration curve of ferulic acid showed a good linearity in the concentration range of 3 – 140 ppm with a correlation coefficient of 0.9994. The limit of detection (LOD) of the proposed method was found to be 1 ppm. To simultaneously determine total antioxidant capacity, the devices were designed and using simple colorimetric based digital camera detection. The device can detect a total antioxidant content of as low as 1 ppm, with a linear dynamic range from 1 ppm to 140 ppm.

By applying the dual detection techniques, this proposed methodology displayed a simple and rapid quantification of ferulic acid using electrochemistry and total antioxidant capacity using colorimetry. Furthermore, this method was successfully used for the quantification of ferulic acid and total antioxidant in fruit juice. In conclusion, lab-on-paper coupled with dual electrochemical and colorimetric detection provides an attractively alternative method for the both the determination of ferulic acid in food samples and the total antioxidant capacity that may also be useful for nutrition and clinical investigation of ferulic acid levels and may have a potential for various kinds of food samples.

5.2 Future perspective

In the future, lab-on-paper coupled with electrochemical and colorimetric detection can be applied for the determination of other antioxidant. Moreover, lab-on-paper could be improve sensitivity using micro and/or nanomaterial.

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APPENDIX

Table A1 The t-test distribution.

Degrees of Freedom	Probability, p		
	0.1	**0.05**	**0.01**
1	6.31	12.71	63.66
2	2.92	4.30	9.93
3	2.35	3.18	5.84
4	2.13	2.78	4.60
5	2.02	2.57	4.03
6	1.94	2.45	3.71
7	1.89	2.37	3.50
8	1.86	2.31	3.36
9	1.83	2.26	3.25
10	1.81	2.23	3.17
11	1.80	2.20	3.11
12	1.78	2.18	3.06
13	1.77	2.16	3.01
14	1.76	2.14	2.98

Table A2 The oxidation peak potential of ferulic acid at different pH.

pH	Peak potentail			Average	SD
	1	**2**	**3**		
3	0.617	0.618	0.619	0.618	0.001
4	0.525	0.509	0.523	0.519	0.009
5	0.441	0.448	0.457	0.449	0.008
6	0.404	0.411	0.423	0.412	0.010
7	0.407	0.415	0.411	0.411	0.004

Table A3 The oxidation peak currents of ferulic acid at different pH.

pH	Peak currents			Average	SD
	1	2	3		
3	4.49	4.86	5.02	4.79	0.272
4	5.27	5.31	5.45	5.34	0.094
5	8.14	8.08	7.99	8.07	0.075
6	4.24	4.47	4.52	4.41	0.149
7	2.77	2.56	2.81	2.71	0.134

Table A4 The peak currents and the step potential

Step potentail	Peak currents			Average	SD
	1	2	3		
2	1.07	1.15	1.13	1.10	0.04
3	1.10	1.20	1.17	1.15	0.05
4	1.25	1.31	1.22	1.26	0.05
5	1.29	1.37	1.39	1.35	0.05
6	1.38	1.45	1.43	1.42	0.04
7	1.74	1.76	1.82	1.77	0.04
8	1.57	1.70	1.65	1.64	0.07
9	1.49	1.61	1.58	1.56	0.06
10	1.41	1.45	1.52	1.46	0.05

Table A5 The peak currents at the different concentration of ferulic acid using electrochemical detection.

Ferulic acid (ppm)	Peak currents			Average	SD
	1	2	3		
3	0.63	0.42	0.67	0.57	0.13
6	0.70	0.63	0.65	0.66	0.04
12	0.93	0.78	0.75	0.82	0.10
25	1.33	1.01	1.16	1.17	0.16
50	1.70	1.58	1.83	1.70	0.13
100	2.81	2.93	3.13	2.96	0.16
120	3.24	3.57	3.48	3.43	0.17
140	4.08	4.17	3.78	4.01	0.20

Table A6 The relative standard deviations of ferulic acid using electrochemical detection.

	Concentration of ferulic acid (ppm)			
	3	**12**	**50**	**120**
1	0.57	0.83	1.77	3.39
2	0.61	0.85	1.74	3.46
3	0.54	0.80	1.75	3.53
4	0.60	0.83	1.68	3.31
5	0.56	0.83	1.83	3.45
6	0.55	0.85	1.69	3.51
7	0.59	0.84	1.71	3.45
8	0.57	0.89	1.69	3.56
9	0.61	0.79	1.73	3.44
10	0.57	0.82	1.67	3.49
X	0.58	0.83	1.73	3.46
SD	0.02	0.03	0.05	0.07
%RSD	4.25	3.35	2.84	2.07

Table A7 The mean intensity of the color in different concentration of sodium carbonate using digital camera.

Sodium carbonate (%)	Mean intensity			Average	SD
	1	2	3		
2	178.79	179.13	180.64	179.52	0.98
4	174.56	176.66	174.45	175.23	1.09
6	169.28	171.61	170.15	170.34	0.96
8	164.87	165.78	166.99	165.89	1.03
10	160.58	158.01	158.89	159.17	1.07
12	159.14	161.16	160.08	160.12	0.83
14	159.69	159.98	162.94	160.87	1.80

Table A8 The mean intensity of the color at different concentration of ferulic acid using colorimetric method.

Ferulic acid (ppm)	Mean intensity			Average	SD
	1	2	3		
1	2.2257	2.2265	2.2272	2.2262	0.0005
3	2.2230	2.2233	2.2230	2.2231	0.0002
6	2.2181	2.2189	2.2175	2.2181	0.0006
25	2.2126	2.2139	2.2144	2.2137	0.0008
50	2.2050	2.2074	2.2048	2.2058	0.0013
80	2.1827	2.1853	2.1805	2.1828	0.0021
100	2.1708	2.1724	2.1739	2.1724	0.0014
120	2.1645	2.1660	2.1666	2.1657	0.0010
140	2.1521	2.1500	2.1492	2.1504	0.0013

Table A9 The relative standard deviations of ferulic acid using colorimetric method.

	Concentration of ferulic acid (ppm)			
	5	**20**	**80**	**120**
1	168.15	161.76	150.89	141.57
2	167.77	163.29	156.67	145.55
3	165.31	165.89	155.42	143.87
4	166.23	166.35	151.34	148.65
5	167.79	162.33	154.23	142.11
6	164.44	161.47	152.78	149.38
7	169.45	164.55	150.33	145.63
8	166.13	164.23	155.69	140.14
9	165.37	165.39	152.81	147.35
10	163.81	162.86	156.32	146.56
X	166.45	163.81	153.64	145.08
SD	1.80	1.73	2.34	3.09
%RSD	1.08	1.06	1.52	2.13

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