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METHOD DEVELOPMENT FOR DETERMINATION OF FERULIC ACID BY LAB-ON-PAPER

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A Thesis Submitted in Partial Fulfillment of the Requirements

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งานวิจัยนี้ได้ทำการพัฒนาวิธีตรวจวัดกรดเฟรูลิกด้วยอุปกรณ์ปฏิบัติการบนกระดาษร่วมกับการวิเคราะห์ด้วยสี ปฏิกิริยาการเกิดสีระหว่างกรดเฟรูลิก สารละลายโฟลินฟีนอลและสารละลายโซเดียมคาร์บอเนตจะให้สีน้ำเงิน โดยความเข้มของสีที่เกิดขึ้นจะขึ้นอยู่กับความเข้มข้นของกรดเฟรูลิก ในการตรวจวัดกรดเฟรูลิกด้วยอุปกรณ์ปฏิบัติการบนกระดาษพบว่าขีดจำกัดต่ำสุดของการตรวจวัดอยู่ที่ 7.19 ส่วนในล้านส่วน และปริมาณต่ำสุดที่ตรวจวัดได้อยู่ที่ 23.98 ส่วนในล้านส่วน โดยมีช่วงความเป็นเส้นตรงระหว่างความเข้มสีและความเข้มข้นของกรดเฟรูลิกให้ค่าสัมประสิทธิ์สูงที่ 0.9974 ในการเปรียบเทียบประสิทธิภาพการตรวจวัดกรดเฟรูลิกระหว่างการใช้อุปกรณ์ปฏิบัติการบนกระดาษกับเทคนิคโครมาโทกราฟีของเหลวสมรรถนะสูงพบว่าการตรวจวัดกรดเฟรูลิกด้วยอุปกรณ์ปฏิบัติการบนกระดาษสามารถใช้งานได้ง่าย ตรวจวัดได้รวดเร็ว ไม่เป็นพิษ นอกจากนี้อุปกรณ์ปฏิบัติการบนกระดาษสามารถนำมาประยุกต์ใช้ในการตรวจวัดตัวอย่างเครื่องสำอางได้อย่างมีประสิทธิภาพ

ภาควิชา .....เคมี..... ลายมือชื่อนิสิต .....

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In this research, the method to determine ferulic acid using lab-on-paper coupled with the colorimetric detection was developed. The colorimetric reaction between ferulic acid, folin phenol reagent, and sodium carbonate solution provided the blue color. The intensity of the color depended on the concentration of ferulic acid. The limit of detection (LOD) was found to be 7.19 ppm. The limit of quantification (LOQ) was found to be 23.98 ppm. Linear region between mean intensity and the concentration of ferulic acid was observed with a good coefficient of 0.9974. To compare the determination of ferulic acid between the lab-on-paper technique and high performance liquid chromatography, the results shows that the determination of ferulic acid by lab-on-paper is simple, fast, and non-toxic. In addition, lab-on-paper was applied to detect ferulic acid in cosmetic effectively.

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

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LIST OF ABBREVIATIONS

HPLC	High performance liquid chromatography
TLC	Thin layer chromatography
ELISA	Enzyme-linked immunosorbent assay
UV-Vis	UV-visible
ppm	Part per million
μL	Microliter
LOD	Limit of detection
LOQ	Limit of quantification
R^2	Coefficient
ROS	Reactive oxygen species
DNA	Deoxyribonucleic acid

CHAPTER I

INTRODUCTION

1.1 Introduction

Ferulic acid (3-methoxy, 4-hydroxy cinnamic acid) is a natural chemical that is found in cell walls of plants. It is found in a wide variety of plant species [1]. Ferulic acid structure is based on a benzene ring and it acts as an antioxidant by absorbing free radicals from the surrounding environment. Morever, it also acts as an anti-cancer agent. It is often used as an ingredient in various supplements that claim to slow down the aging process. In addition, this substance has many health benefits including, reduction of cholesterol in the blood, against protection the degeneration of bone, stabilization of levels of blood glucose in diabetic patients, and the relief of "hot flushing" symptoms experienced by menopausal women [2-3].

Several methods for the determination of ferulic acid have been reported including high performance liquid chromatography (HPLC) and UV-Visible spectrophotometry technique [4-5]. HPLC is a popular method for the determination of ferulic acid. However, this technique requires a high-cost equipment and toxic solvent such as acetonitrile and methanol [6]. The conventional method for the determination of ferulic acid utilizeds the reaction between ferulic acid and folin phenol reagent, and compound produced is detected by UV-Visible spectrometry. However, this technique is not suitable, because it requires high volume of sample, reagent and expensive instrument [7].

Lab-on-paper is a popular technique that is recently introduced as alternative devices for point of care testing because it has attractive features including low-cost, easy of use, low-consumption of reagent and sample, inexpensive and portable [8]. Lab-on-paper is used to detect various organic compound such as urine and saliva [9-10] and pesticides [11]. Moreover, it can be used to analyze multiple samples at the same time [12].

Lab-on-paper has the potential to be good analytical device for point of care and related applications, because it is easy to use, inexpensive and portable [13]. Many methods for fabrication of paper based devices have been reported such as photolithography [14], wax screen-printing [15], wax dipping [16] and wax printing [17]. Photolithography was the first method that was used to fabricate a paper based device. However, this method requires expensive photoresist, toxic solvent and high equipment costs such as spin coater and plasma cleaner. Wax methods do not requires expensive photoresist and toxic solvent but these methods still require skill and special equipment.

This research focuses on the development of lab-on-paper fabricated by melting wax screen-printing. This proposed method is low-cost, simple and non-toxic. This lab-on-paper was developed for ferulic acid determination. The solid wax was using hot plate melted into filter paper to form hydrophobic barrier. The influences of the widths of hydrophobic barrier and hydrophilic channel were investigated. The melting temperature and time were also examined to obtain the optimal conditions. Finally, the proposed procedure was applied to determine ferulic acid in cosmetic samples.

1.2 Objective of the research

To developed method for the determination of ferulic acid by using lab-on-paper coupled with colorimetric detection.

CHAPTER II

THEORY AND LITERATURE SURVEY

In order to understand this work, the definitions and theories of the following terms are explained : ferulic acid, optical detection, thin layer chromatography and lab-on-paper.

2.1 Ferulic acid [18 -22]

2.1.1 Properties of ferulic acid

Ferulic acid is a hydroxycinnamic acid, a type of organic compound. It is an abundant phenolic phytochemical found in plant cell wall components such as arabinoxylans as covalent side chains. It is related to trans-cinnamic acid. As a component of lignin, ferulic acid is a precursor in the manufacture of other aromatic compounds.

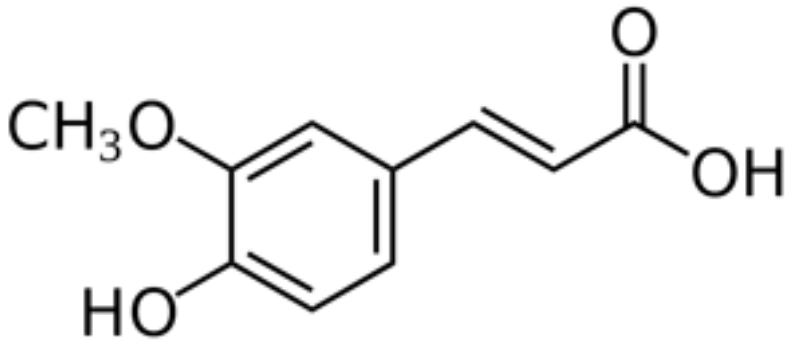

Figure 2.1 Structure of ferulic acid. [19]

Ferulic acid is found in the seeds of coffee, apple, artichoke, peanut, and orange, as well as in both seeds and cell walls of commelinid plants (such as rice, wheat, oats, and pineapple). It can be extracted from wheat bran and maize bran using concentrated alkali.

Biosynthesis of ferulic acid is by the action of the enzyme O-methyl transferase on caffeic acid. Ferulic acid, together with dihydroferulic acid, is a component of lignocellulose, serving to crosslink the lignin and polysaccharides, thereby conferring rigidity to the cell walls. It is an intermediate in the synthesis of monolignols, the monomers of lignin, and is also used for the synthesis of lignans. Ferulic acid is converted by certain strains of yeast, notably strains used in brewing of wheat beers, such as *Saccharomyces delbrueckii* (*Torulaspora_delbrueckii*), to 4-vinyl guaiacol (2-methoxy-4-vinylphenol). *Saccharomyces cerevisiae* (dry baker's yeast) and *Pseudomonas fluorescens* are also able to convert trans-ferulic acid into 2-methoxy-4-vinylphenol.

Ferulic acid, like many phenols, is an antioxidant *in vitro* in the sense that it is reactive toward free radicals such as reactive oxygen species (ROS). ROS and free radicals are implicated in DNA damage, cancer, accelerated cell aging. Animal studies and in vitro studies suggest that ferulic acid may have direct antitumor activity against breast cancer and liver cancer. Ferulic acid may have pro-apoptotic effects in cancer cells, thereby leading to their destruction. Ferulic acid may be effective for preventing cancer induced by exposure to the carcinogenic compounds benzopyrene and 4-nitroquinoline 1-oxide. Note that these are not randomized controlled trials done with human participants, and therefore, the results of these studies may not be directly applicable to human use.

2.2.2 Ferulic acid in cosmetics

Ferulic acid is a ubiquitous plant constituent found in plant cell walls, leaves and seeds. It is made from the metabolism of phenylalanine and tyrosine. It occurs primarily in seeds and leaves both in its free form and covalently linked to lignin and other biopolymers. Due to its phenolic nucleus and an extended side chain conjugation, it readily forms a resonance stabilized phenoxy radical which accounts for its potent antioxidant potential. UV absorption by ferulic acid catalyzes stable phenoxy radical formation and thereby potentiates its ability to terminate free radical chain reactions. By virtue of effectively scavenging deleterious radicals and suppressing radiation-induced oxidative reactions, ferulic acid may serve an important antioxidant function in preserving physiological integrity of cells exposed to both air and impinging UV radiation. Similar photoprotection is afforded to skin by ferulic acid dissolved in cosmetic lotions. Its addition to foods inhibits lipid peroxidation and subsequent oxidative spoilage. By the same mechanism ferulic acid may protect against various inflammatory diseases. A number of other industrial applications are based on the antioxidant potential of ferulic acid.

Ferulic acid receives significant interest in the beauty industry with regard to its skin-whitening and anti-oxidant properties. However, its use in cosmetics is limited due to pH and temperature-related instabilities. In this study, we investigated the stability of ferulic acid in eight different prototype formulae. The results confirmed that in our conditions the stability of ferulic acid is pH and temperature related. Additionally, the nature of the solvent dipropylene glycol (DPPG) showed a capacity to stabilize ferulic acid. A series of experiments was further planned for studying the mechanism of degradation of ferulic acid. In a prototype of a cosmetic medium, ferulic acid degrades first through a decarboxylation step, leading to 4-hydroxy-3-methoxystyrene. Further, ferulic acid and 4-hydroxy-3-methoxystyrene are both

involved in an additional reaction, resulting in the trans-conjugation dimer of 4-hydroxy-3-methoxystyrene. The consequences of these results in formulating ferulic acid are discussed.

2.2 Determination of total phenolic acid by folin phenol colorimetry [23-26]

Folin phenol colorimetry is based on a chemical reduction of the reagent, a mixture of tungsten and molybdenum oxides. The products of the metal oxide reduction have a blue color that exhibits a broad light absorption with a maximum at 765 nm. The intensity of light absorption at that wavelength is proportional to the concentration of phenols. The folin phenol method has been adopted as the official procedure for total phenolic levels in sample.

Folin phenol method is commonly used to analyze the Total phenolics in vegetables, Fruits and beverages. Folin phenol method, as well as how it is different. The reagent used by two methods are based on redox reactions in the reaction. Molybdotungstate ion as a reagent of Folin phenol method consists of sodium tungstate phosphomolybdic acid orthophosphoric acid and sodium bicarbonate the Folin phenol reagent consisting of sodium tungstate sodium molybdate phosphoric acid and sodium carbonate to change the color of the reaction of the ion Mo (VI), which has four. yellow. When electrons from the antioxidants. The change in the form of Mo (V), which is blue color. The following equation.

$$Na_2WO_4/Na_2MoO_4 \longrightarrow (\text{phenol-}MoW_{11}O_{40})^{4-} \quad \ldots\ldots. (1)$$

$$Mo\ (VI)\ (\text{yellow}) + e^- \longrightarrow Mo\ (V)\ (\text{blue color}) \quad \ldots\ldots. (2)$$

2.3 Optical detection [27]

Optical detection is a colorimetric assay using a scanner and a digital camera for image capturing. The image can be transmitted electronically and digitally to an off- site laboratory, which the data can be analyzed by a specialist, and the results of the analysis returned to the person administering the test. The general strategy for performing inexpensive bioassays in remote locations and for exchanging the results of the tests with off-site technicians

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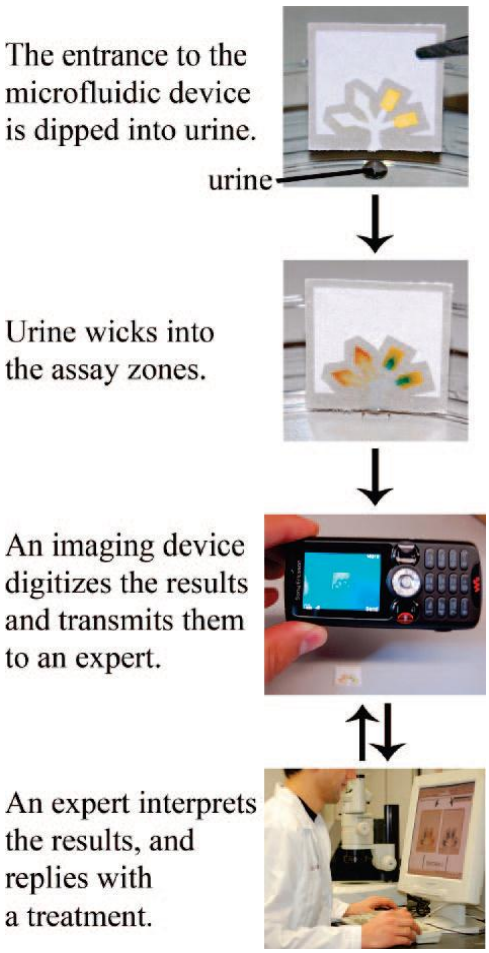


Figure 2.2 General strategy for performing inexpensive bioassays in remote locations and for exchanging the results of the tests with offsite technicians. [27]

The quantitative analysis using optical detection can be performed. Firstly, the colorimetric results are captured by camera or scanner, images are then converted into grayscale or CMYK color for increasing precision in the analysis. The test zone is selected and the mean intensity is recorded. This procedure for processing the data can be automated using general software. The advantages of this method are easy use, rapid, inexpensive and can be off-site laboratory.

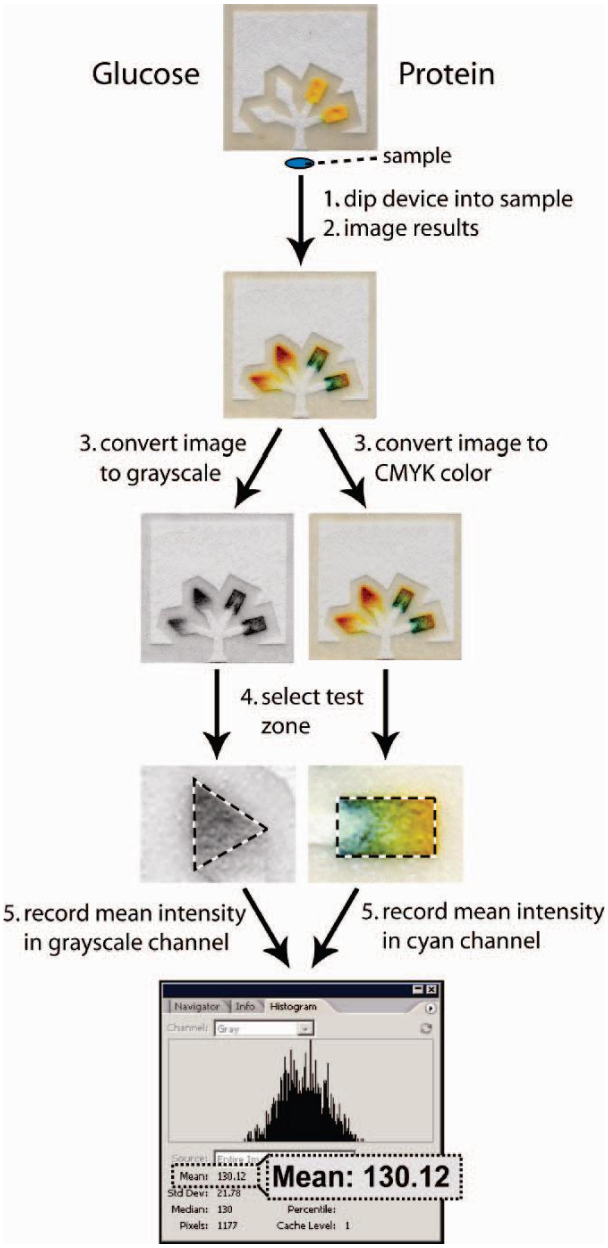


Figure 2.3 Procedure for quantifying the levels of glucose and protein in urine using image editing software (using AdobePhotoshop). [27]

2.4 Thin layer chromatography [28-30]

Thin layer chromatography (TLC) is a chromatographic technique used to separate mixtures. Thin layer chromatography is performed on a sheet of glass, plastic, or aluminum foil, which is coated with a thin layer of adsorbent material, usually silica gel, aluminium oxide, or cellulose (blotter paper). This layer of adsorbent is known as the stationary phase. Thin layer chromatographic plate is shown in Figure 2.4

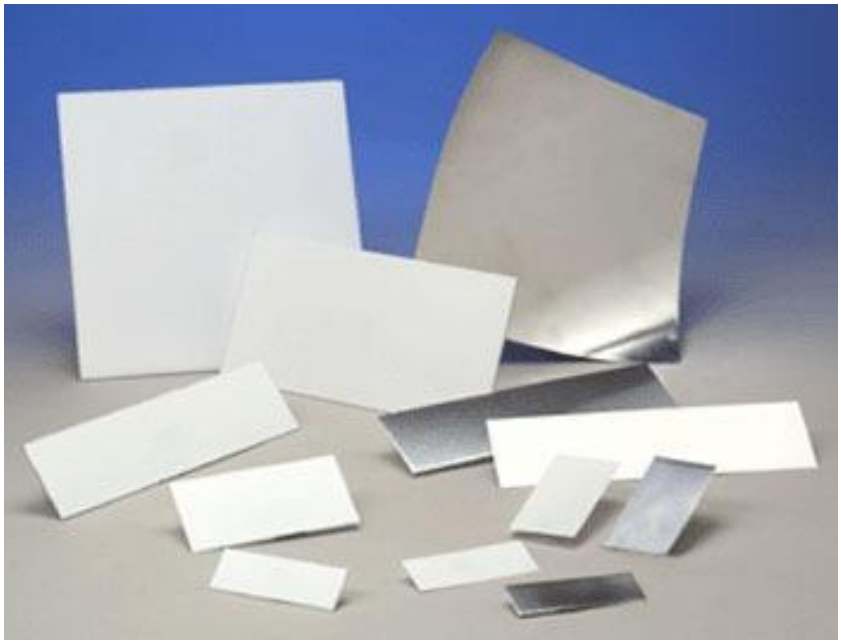


Figure 2.4 The thin layer chromatography plate. [29]

After the sample has been applied on the plate, a solvent or solvent mixture (known as the mobile phase) is drawn up via capillary action on the plate. Separation is achieved because different analytes ascend on TLC plate at different rates.

Thin layer chromatography can be used to monitor the progress of a reaction, identify compounds present in a given mixture, and determine the purity of a substance. Specific examples of these applications include: analyzing ceramides and fatty acids, detection of pesticides or insecticides in food and water, analyzing the dye

composition of fibers in forensics , assaying the radiochemical purity of radiopharmaceuticals, or identification of medicinal plants and their constituents.

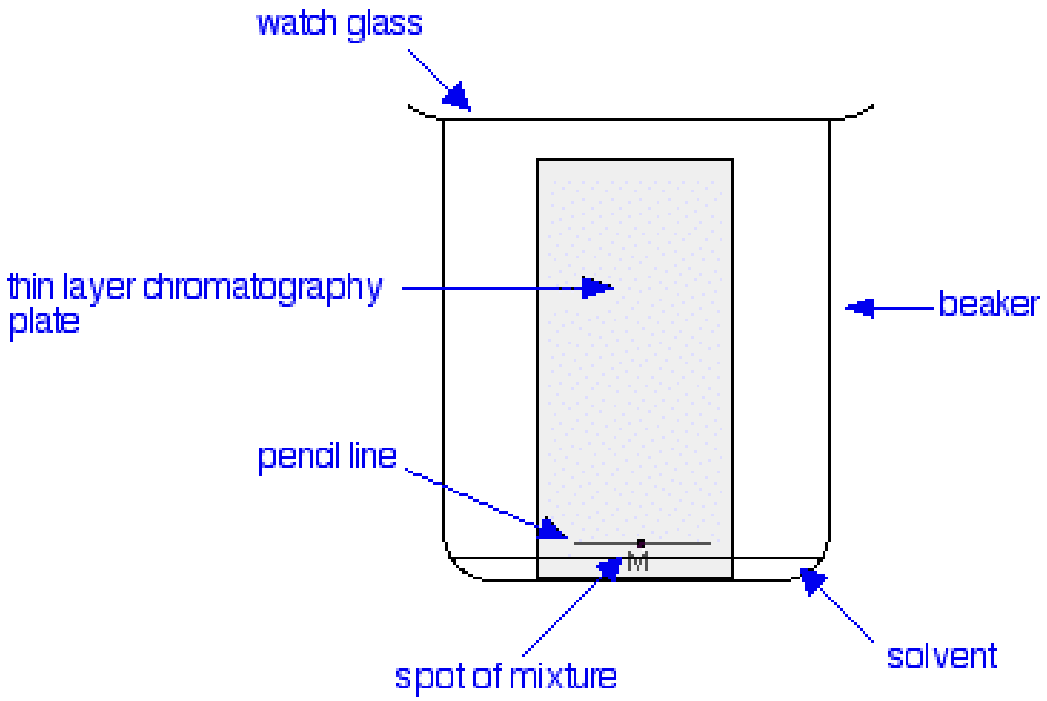


Figure 2.5 The diagram shows the chromatographic plate. [30]

2.5 Lab-on-paper [31-35]

Lab-on-paper is a device for chemical reaction occuring on the paper instead of a test tube or large equipment. This device is easy to use because the paper is lightweight, durable, difficult to break and low-cost. It relies on the principles of gravity and capillary pores in the paper pulp. The surface of paper can be prepared to be hydrophilic and hydrophobic, which causes a fluid flow in channel. This device can be used to examine blood, saliva, sweat, pH or chemicals as shown in Figure 2.6. Fludics can flow to the small openings without an external pump. General measurement is detection of the color change.

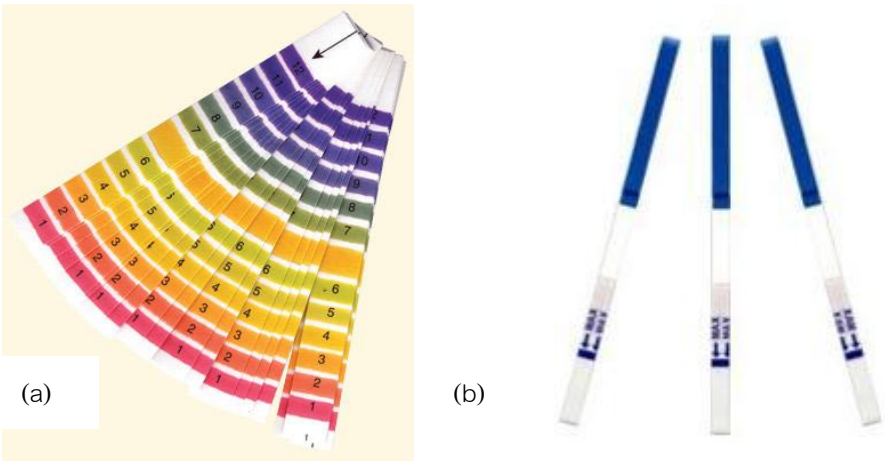


Figure 2.6 (a) Paper strip test [34] (b) Immunochromatographic test [35]

Usage of this device is similar to that used for a pregnancy test kit. The test substance is in urine. However, sometime it need to make more complex measurements, such as, detection of more than one disease simultaneously. The first examination would be by the naked eye. To be quantitative investigation, camera phones, which are cheap can be used to record the results. Then the images can be sent to a specialist in the laboratory for the diagnosis.

Lab-on-paper can be couple to a variety of detections. In this work, it can be divided into two major detections, colorimetric and electrochemical detections.

2.5.1 Lab-on-paper with colorimetric detection [36-40]

Colorimetry is technique used to quantify and describe physically the human color perception. It is similar to spectrophotometry, but is distinguished by its interest in reading spectra to the physical correlates of color perception, color values and related quantities.

Lab-on-paper can be used to detect the color on paper. This measuring of the color intensity is linearly related to the amount of compounds. It can be measured in several applications, such as medical (blood, saliva, diabetes), agriculture (pH in soil, pestisides) sample, heavy metals.

Figure 2.7 Example for lab-on-paper with colorimetric detection. [36]

2.5.2 Lab-on-paper with electrochemical detection [41]

Lab-on-paper with electrochemical detection is devices consist of micro-channels for fluid on filter paper and printed electrodes. Basic electrochemical system such as cyclic voltametry is used to investigate working electrode on paper. The component of electrochemical detection part are counter electrode (CE) working electrode (WE) and reference electrode (RE).

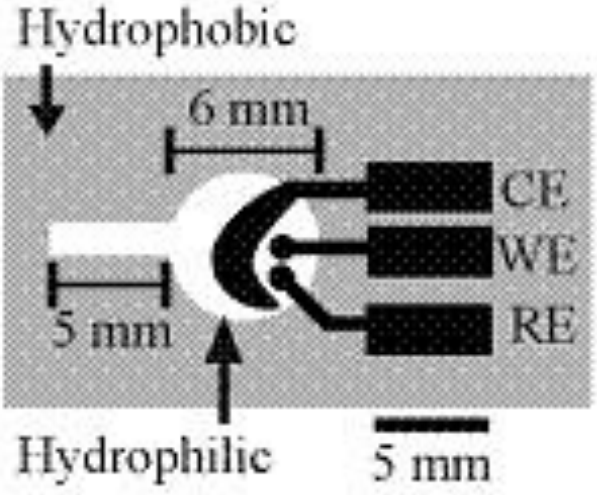


Figure 2.8 The lab-on-paper with electrochemical detection, which consists of a hydrophobic zone, hydrophilic zone, counter electrode (CE) working electrod (WE) and reference electrode (RE) [41].

2.6 **The wax pattern paper fluidics** [42-45]

Wax pattern paper fluidics have been reported as rapid prototyping of paper microfluidics for portable bioassay. Nitrocellulose membrane can be employed as a substrate for prototyping PDMS chip.

2.6.1 Prototyping of paper microfluidics with wax

A very simple and low-cost method to generate paper based microfluidic devices is using wax. Wax can be used as a hydrophobic barrier to direct the flow path or isolate reaction zones, which is easy to operate without external units.

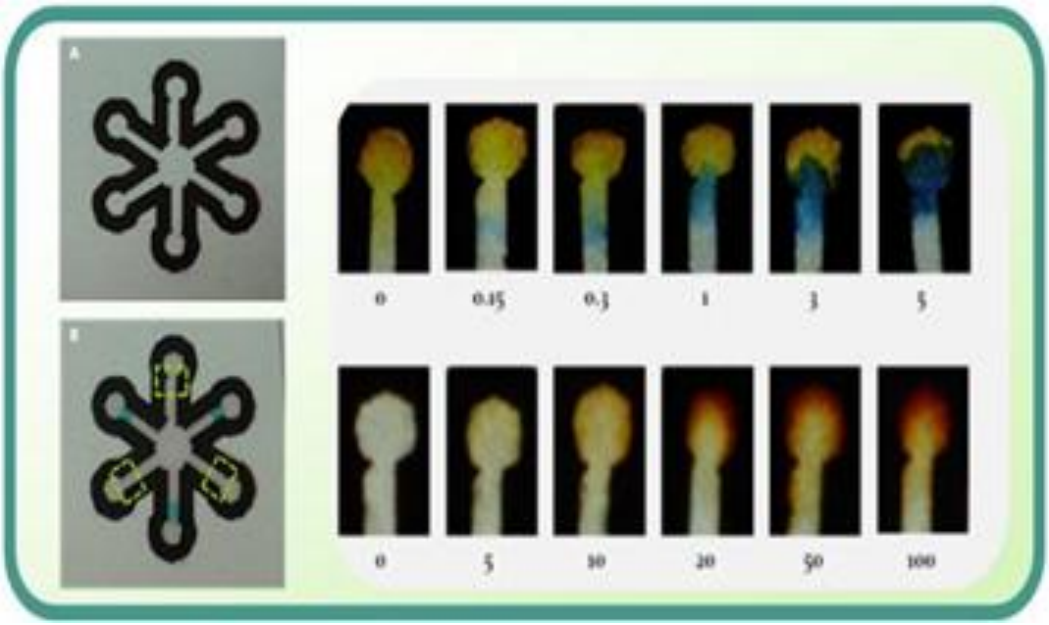


Figure 2.9 Rapid prototyping of paper microfluidics with wax for portable bioassay. [42]

2.6.2 Wax patterned nitrocellulose membrane chip

Paper-based microfluidic device can be fabricated using nitrocellulose membrane by wax printing, which can be used for protein immobilization related analysis. The fabrication process is only within 10 min including printing and baking steps. The wax-patterned nitrocellulose membrane can enhance the capabilities of paper microfluidic devices and bring new applications in this field.

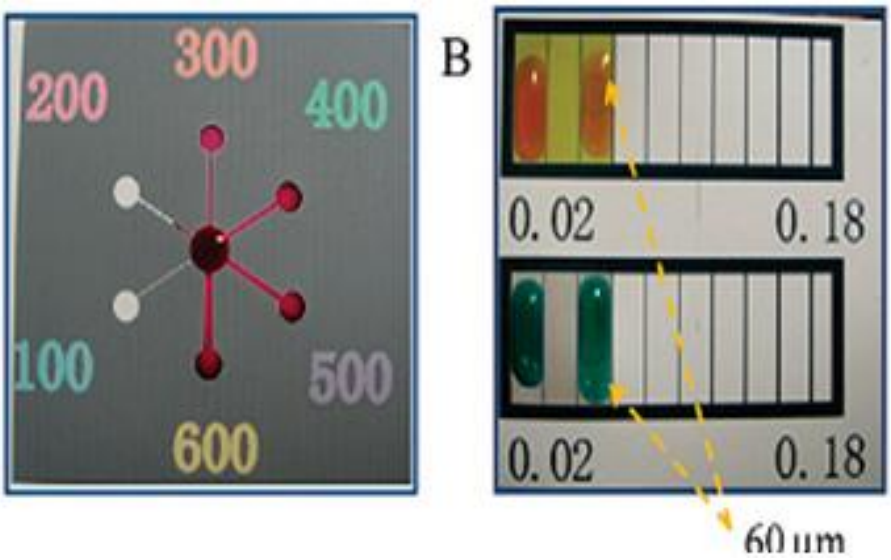


Figure 2.10 Wax Patterned Nitrocellulose Membrane Chip. [43]

2.6.3 Patterned paper as a substrate for prototyping PDMS chip

Patterned paper can be used as flexible substrate for rapidly prototyping PDMS microchip via "liquid molding". This prototyping PDMS microchip has some favorable merits including simple operation procedures, low cost and providing the potential applications in POC diagnostics.

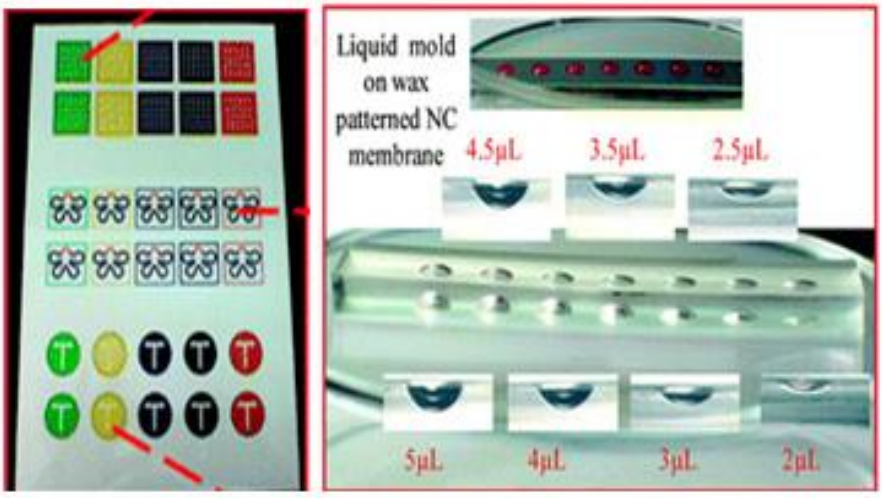


Figure 2.11 Patterned paper as a substrate for prototyping PDMS chip. [44]

2.7 Wax pattern paper fluidics

Patterning paper made for fluidic system in paper. Hydrophilic and hydrophobic zone causing a fluid flow in channel can be prepared on the surface of paper. To build the pattern into paper can be done by several ways, such as using SU-8 photoresist, wax printing, wax dipping and wax screen-printing.

2.7.1 SU-8 photoresist method [14]

SU-8 is epoxy-based negative photoresist commonly used. It is very viscous polymer that can be spun or spread over a thickness ranging from <1 micrometer up to >300 micrometer and still be processed with standard contact lithography. Its maximum absorption is for ultraviolet light with a wavelength of 365 nm. When exposed, SU-8 long molecular chains cross-link causing the solidification of the material. SU-8 series. Photoresist uses gamma butyrolactone as the primary solvent.

SU-8 was originally developed as a photoresist for the microelectronics industry, to provide a high-resolution mask for fabrication of semiconductor devices. It is now mainly used in the fabrication of microfluidics and microelectromechanical systems. It is also one of the most biocompatible materials known and is often used in bio-MEMS.

SU-8 is highly transparent in the ultraviolet region, allowing fabrication of relatively thick (hundreds of micrometers) structures with nearly vertical side walls. After exposition and developing, its highly cross-linked structure gives it high stability to chemicals and radiation damage. Cured cross-linked SU-8 shows very low levels of outgassing in a vacuum. However it is very difficult to remove, and tends to outgas in an unexposed state. The main developer for SU-8 is 1-Methoxy-2-propanol acetate.

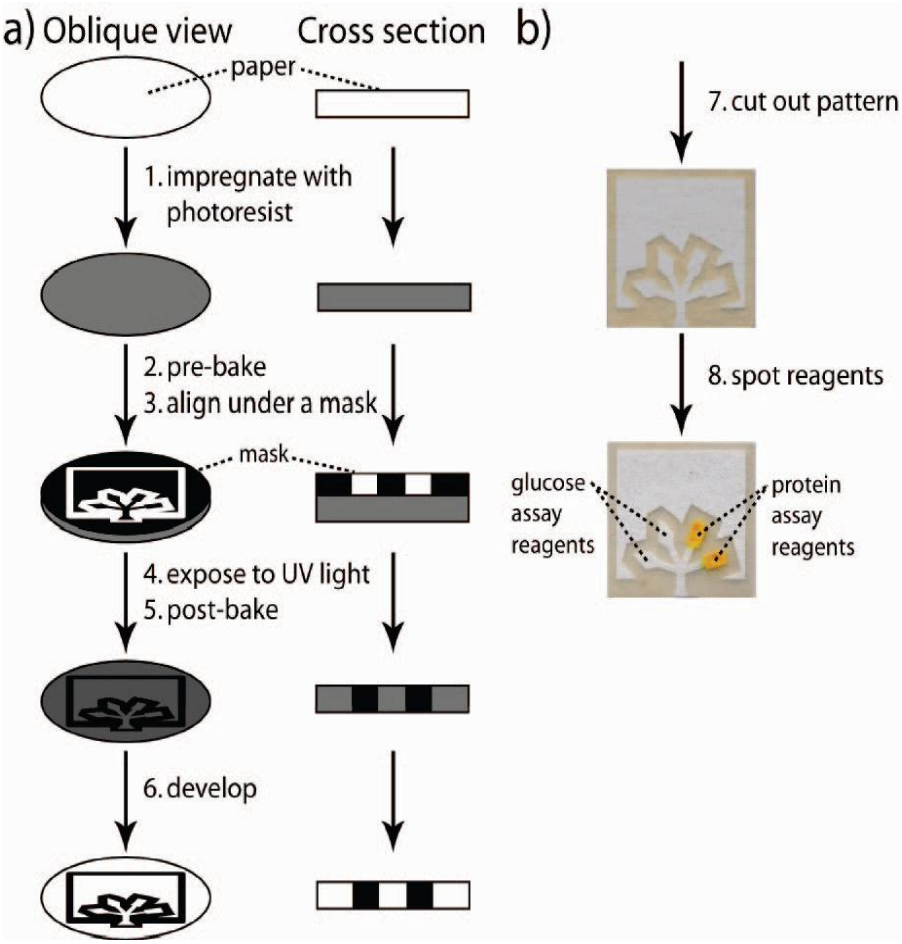


Figure 2.12 Schematic of the method for fabricating paper-based microfluidic devices. (a) Procedure for patterning paper with hydrophobic photoresist. (b) Derivatization of the device for assays. [14]

2.7.2 Wax dipping method [16]

A wax dipping method for fabricating paper-based microfluidic devices (μPADs) is reported. The iron mould for wax dipping was created by a laser cutting technique. The designed pattern was transferred onto paper by dipping an assembly mould into melted wax. The optimal melting temperature and dipping time were investigated. The optimal melting temperature was in the range of 120-130°C, and the optimal dipping time was 1s. The whole fabrication process could be finished within 1 min without the use of complicated instruments or organic solvents. The smallest hydrophilic channel that could be created by the wax dipping method was 639 ± 7 μm in size. The reproducibility of the μPAD fabrication for hydrophilic channel width of the test zone and sample zone was 1.48% and 6.30%, respectively.

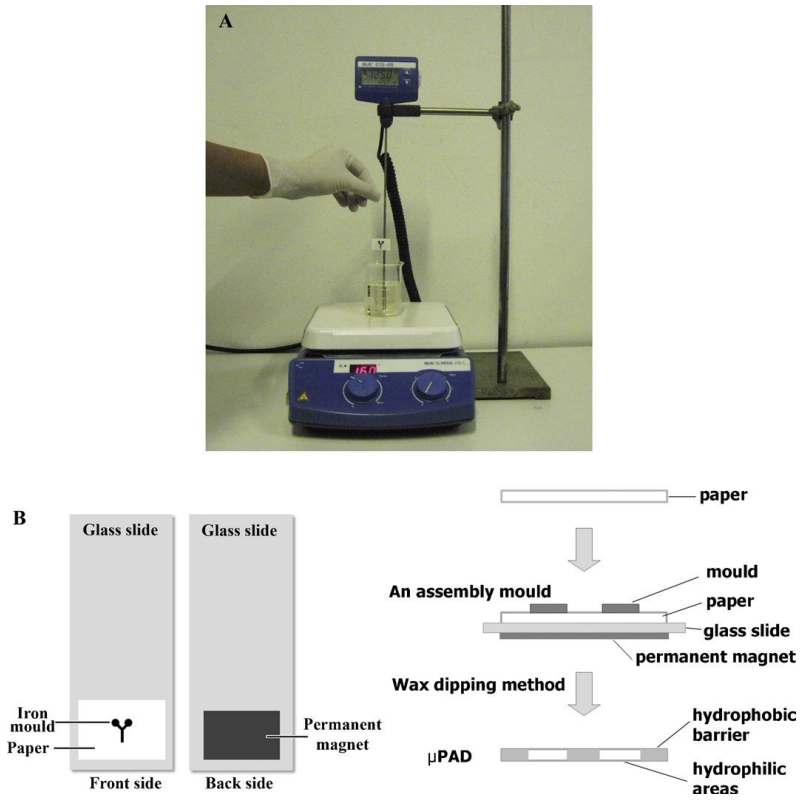


Figure 2.13 Schematic of wax dipping. [16]

2.7.3 Wax screen-printing [15]

Wax screen-printing provides a low-cost, simple and rapid method for fabricating paper-based microfluidic devices. Solid wax was rubbed through a screen onto paper filters. The printed wax was then melted into the paper to form hydrophobic barriers using only a hot plate.

In addition, wax is inexpensive, can be purchased anywhere in the world, and is environmantally friendly. The wax screen-printing method is accomplished without the use of a clean room, UV lamp, organic solvent, or sophisticated instrumentation. Another advantage of our method over previous methods is that it requires only a hot plate making it ideal for fabrication of μPADs in developing countries.

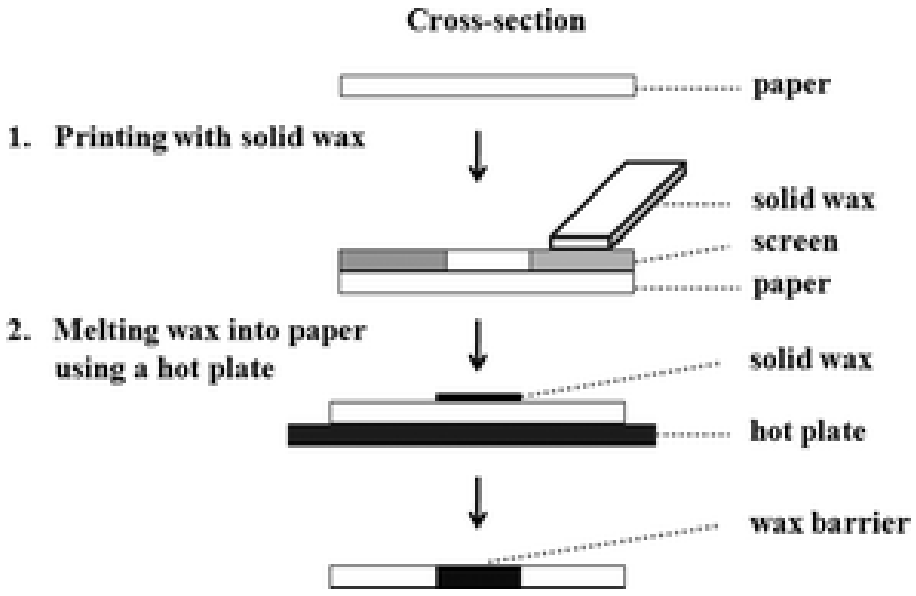


Figure 2.14 Schematic of wax screen-printing. [15]

2.8 Literature surveys

2.8.1 Lab-on-paper

Hossain *et al.* (2009) reported the bioactive paper sensors for detection of pestiside in food sample [11]. A reagentless bioactive paper-based solid-phase biosensor was developed for detection of acetylcholinestererase inhibitors, including organophosphate pestisides. The paper sensor is composed of a paper support, onto with acetylcholinestererase and a chromogenic substrate. Indophenyl acetate, were entrapped using biocompatible sol-gel derived silica inks in two different zones. The assay protocol involves introducing the sample to the sensing zone via lateral flow of a pesticide-containing solution.

In 2009, Ellerbee *et al.* used colorimetric assays in paper-based microfluidic devices by measuring the transmission of light though paper [13]. Measuring transmittance through paper represents a new method of quantitative detection that expands the potential functionality of paper-based microfluidic devices. This phototype transmittance colorimeter is in expensive, rugged and fully self-contained, and thus potentially attractive for use in resource-limited environment and developing countries.

In 2010, Cheng *et al.* proposed paper-based ELISA (P- ELISA) [46]. ELISA is widely used in biochemical analyses, these assays are typically carried out in microtiter plate or small vial. ELISA is the specificity of antibodies with high-turnover catalysis by enzymes to provide specificity and sensitivity. P-ELIZA is the combination between paper based and ELISA. It is a device for detected more rapid, require only small volumes (1-10 μm) of sample and reagent and utilizes simple equipment for detection than ELIZA.

In 2010, Hyeran noh and Scott T.Phillips report the capillary-driven flow of fluids in paper-based microfluidic devices [10]. Initial prototype of 3D paper-based microfluidic devices control the spartial distribution of fluids within a device, but provide little control over how quickly fluids move within the devices.

In 2010, Klasner *et al.* used paper-based microfluidic devices for analysis of clinical relevant analytes present in urine and saliva [9]. Paper-based microfluidic devices were used to detect urinary ketones, glucose and salivary nitrite. Paper-based devices were fabricated via photolithography in less than 3 min and were immediately ready for use for these diagnostically relevant assays. Patterned channel on filter paper were fabricated via photolithography as small as 90 μm wide with barrier as norrow as 250 μm.

In 2010, Dungchai *et al.* used multiple colorimetric indicators for paper-based microfluidic devices [12]. In thier approach, devices are designed to simultaneously quantify analytes using multiple indicators for each analyte for improving the accuracy of the assay. 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. This method was successful for quantify glucose in the range 0.5-20 mM, lactate in the range 1-25 mM and

In 2011, Wang *et al.* fabricated paper-based chemiluminescence ELISA. This modified paper device is prepare by wax screen-printing to isolate individual assay zones on cellulose [8]. Chitosan was used to modified the cellulose surface, followed by activation with glutaradhyde to covalently immobilize three different capture antibodies to detect tumor markers (from a luminol-p-iodophenol and hydrogen peroxide reaction), the group created a novel device.

2.8.2 Pattern paper

Martinez *et al.* (2007) reported the patterned paper as a platform for inexpensive, low volume, portable bioassays [14]. This paper based assay is suitable for measuring multiple samples in parallel and in a relatively short period of time. For example, in one trail, one researcher was able to run 20 different samples within 7.5 min.

In 2008, Martinez *et al.* reported simple telemedicine for developing regions camera phones and paper-based microfluidic devices for real-time, off-site diagnosis [27]. The system uses paper-based microfluidic devices for running multiple assays simultaneously. Camera phones or portable scanners are used for digitizing the intensity of color associated with each colorimetric assay, and established communications infrastructure for transferring the digital information from the assay site to an off-site laboratory for analysis by a trained medical professional. The diagnosis then can be returned directly to the healthcare provider in the field. The microfluidic devices were fabricated in paper using photolithography and were functionalized with reagents for colorimetric assays. The results of the assays were quantified by comparing the intensities of the color developed in each assay with those of calibration curves.

In 2008, Zhao *et al.* paper based bioassays using gold nanoparticle colopimetric probes [40]. The combination of paper substrates and AuNP colorimetric propes makes the final products in expensive, low-volume, portable, disposable, and easy-to-use.

In 2010, Lu *et al.* reported the fabrication and characterization of paper-based microfluidics prepared in nitrocellulose membrane by wax printing [43]. The fabrication of paper based microfluidic devices in nitrocellulose membrane by wax printing for protein immobilization related applications. The fabrication process, which can be finished within 10 min.

CHAPTER III

EXPERIMENTAL

3.1 Chemicals and reagents

3.1.1 Ferulic acid (Fluka)

3.1.2 Ascorbic acid (Fluka)

3.1.3 Folin phenol reagent (Merck)

3.1.4 Sodium carbonate (Sigma-Aldrich)

3.1.5 Wax

3.1.6 Methanol (Merck)

3.1.7 Chloroform (Fluka)

3.1.8 Formic acid (Fluka)

3.1.9 Ethyl acetate (Merck)

3.1.10 Ethanol (Merck)

3.1.11 Milli-Q water

3.1.12 1,1-diphenyl-2-picrylhydrazyl (Fluka)

3.2 Instruments and equipments

3.2.1 Filter paper (diameter 125 mm. Whatman no.1, UK)

3.2.2 Thin layer chromatographic plate (Merck)

3.2.3 Hot plate (HL instrument, USA)

3.2.4 Chromatographic tank

3.2.5 Micropipette 5 μL (Eppendorf)

3.2.6 Digital camera (Sony, Thailand)

3.2.7 Notebook with Adobe Photoshop **(**Acer, Thailand**)**

3.2.8 Beaker 5 mL

3.2.9 Volumetric flask 10 mL

3.2.10 Stop watch **(**Mini timer, Japan**)**

3.2.11 Milli-Q water system **(**Millipore, USA**)**

3.2.12 Block screen and squeegee

3.2.13 Dropper

3.2.14 Stirring rod

3.3 Preparation of solutions

3.3.1 2% Sodium carbonate solution (10 mL)

2**%** Sodium carbonate solution was prepared using 0.2 g of sodium carbonate dissolved in 10 mL of Milli-Q water.

3.3.2 4% Sodium carbonate solution (10 mL)

4**%** Sodium carbonate solution was prepared using 0.4 g of sodium carbonate dissolved in 10 mL of Milli-Q water.

3.3.3 6% Sodium carbonate solution (10 mL)

6**%** Sodium carbonate solution was prepared using 0.6 g of sodium carbonate dissolved in 10 mL of Milli-Q water.

3.3.4 7.5% Sodium carbonate solution (10 mL)

7.5**%** Sodium carbonate solution was prepared using 0.75 g of sodium carbonate dissolved in 10 mL of Milli-Q water.

3.3.5 8% Sodium carbonate solution (10 mL)

8% Sodium carbonate solution was prepared using 0.8 g of sodium carbonate dissolved in 10 mL of Milli-Q water.

3.3.6 10% Sodium carbonate solution (10 mL)

10% Sodium carbonate solution was prepared using 1 g of sodium carbonate dissolved in 10 mL of Milli-Q water.

3.3.7 12% Sodium carbonate solution (10 mL)

12% Sodium carbonate solution was prepared using 1.2 g of sodium carbonate dissolved in 10 mL of Milli-Q water.

3.3.8 14% Sodium carbonate solution (10 mL)

14% Sodium carbonate solution was prepared using 1.4 g of sodium carbonate dissolved in 10 mL of Milli-Q water.

3.3.9 16% Sodium carbonate solution (10 mL)

16% Sodium carbonate solution was prepared using 1.6 g of sodium carbonate dissolved in 10 mL of Milli-Q water.

3.3.10 1 % 1,1-diphenyl-2-picrylhydrazyl (10 mL)

1 % 1,1-diphenyl-2-picrylhydrazyl was prepared using 1 g of 1,1-diphenyl-2-picrylhydrazyl dissolved in 10 mL of ethanol.

3.3.11 400 ppm Ascorbic acid solution (10 mL)

400 ppm Ascorbic acid solution was prepared using 0.004 g of ascorbic acid dissolved in 10 mL of Milli-Q water.

3.3.12 400 ppm Ferulic acid solution (10 mL)

400 ppm Ferulic acid solution was prepared using 0.004 g of ferulic acid dissolved in 10 mL of methanol.

3.4 Preparation of paper-based devices using melting wax screen-printing

Melting wax screen-printing is a technique in which a design is generated on a screen of silk. Patterns are transferred by forcing wax melt though the mesh onto the printing surface.

In this thesis, barrier pattern for colorimetric reaction zones was designed as shown in Figure 3.1.

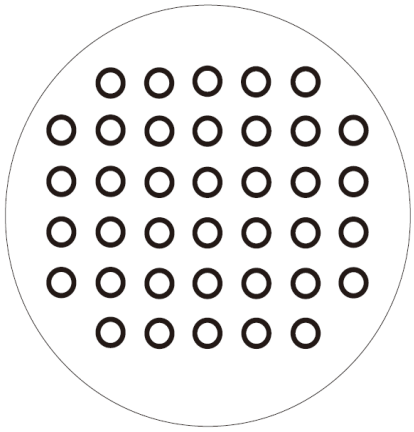


Figure 3.1 The barrier pattern for colorimetric reaction zones.

Molten wax was used as a printing material for screen-printing hydrophobic barriers on a filter paper as shown in Figure 3.2. Wax is environmentally friendly and much cheaper and easier to obtain than photoresist or PDMS. Moreover, our fabrication method is accomplished without the use of a clean room, UV lamp, organic solvents, or sophisticated instrumentation.

To fabricate paper-based device, a filter paper was placed on the hotplate, next a block screen was placed firmly onto the surface of filter paper, and solid wax was sprinkled on a screen. After the wax melts, the melting wax was squeegee for creating hydrophobic barriers on a filter paper.

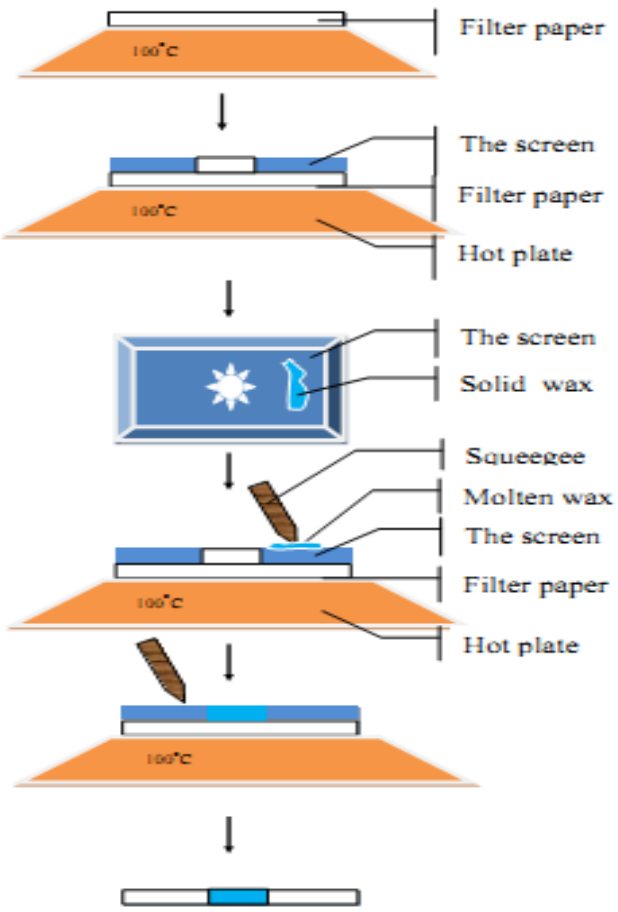


Figure 3.2 Schematic representation of melting wax screen printing for paper-based devices.

3.4.1 Optimization of temperature of wax melting

To find the optimum temperature for wax melting, 1 g of solid wax was placed on a block screen and the temperature of the hot plate was varied in the range of 40-150 °C.

3.4.2 Melting wax screen printing resolution

To determine the resolution of this method, the width of hydrophobic and hydrophilic channel were studied in the range of 100-2,000 μm and 100-2,000 μm respectively at the optimal melting temperature. After fabrication, food dye was added to the paper devices to visualize the hydrophobic and hydrophilic properties.

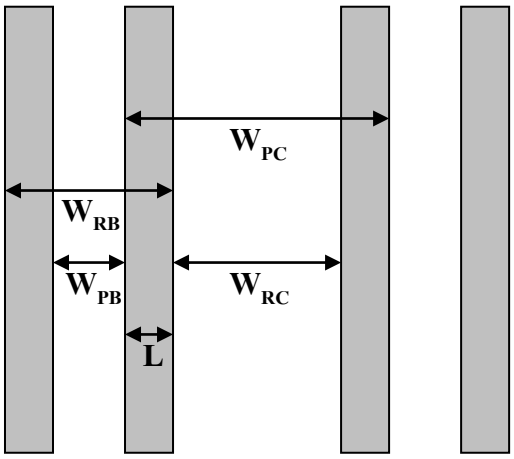


Figure 3.3 Schematic representation of the screen-printed wax in paper and definition of variables: W_{PC} is the printed width of the channel, W_{PB} is the printed width of wax hydrophobic line, W_{RC} is the resulting width of hydrophilic channel, W_{RB} is the resulting width of hydrophobic barrier after melting of wax, L is the screen-printed of wax from original edge of wax line.

3.5 Optimization of colorimetric reaction

Colorimetric reaction used in this study is the reaction between sodium carbonate solution, folin phenol reagent and ferulic acid. Typical for the classical method, the ratio used for reaction of the folin phenol reagent, sodium carbonate 7.5% and ferulic acid is 1:1:1. The reagents and ferulic acid need to be well mixed before measuring the absorbance by spectrophotometer. When this system was applied on the paper-based device (Lab-on-paper), the reagents and ferulic acid may not be mixed well as in classical method. The potential problem of this method is the incomplete reaction. Therefore, the optimal conditions for operating reaction on the filter paper were investigated.

3.5.1 Effect of sequence of solution dropping

Four sequences of solution drops are set as following:

(1**)** folin phenol reagent + Na_2CO_3 + ferulic acid

(2**)** folin phenol reagent + ferulic acid + Na_2CO_3

(3**)** Na_2CO_3 + folin phenol reagent + ferulic acid

(4**)** Na_2CO_3 + ferulic acid + folin phenol reagent

The volume of each solution was 2 μL

3.5.2 Comparison of the colorimetric reaction between the continuously drops and the drop dried solutions

After selection the sequence of solution drops in topic 3.5.1, comparison of the colorimetric reaction between the continuously drops and the drop dried solutions. The

continuously drops are the process that solution was dropped continuously without waiting for it to dry before the next drop. The drop dried system is the process that the solution was dropped and left until it dry before the next drop. The uniform of blue color at the reaction zone is the indicator of the complete reaction.

3.5.3 Effect of time between drop and drop

The time waiting for a filter paper to dry before the next drops was studied. The following time used to investigated in this topic is 10, 20, 30 and 40 minutes.

3.5.4 Effect of mixed solution before drop

To save time, the solution of folin phenol reagent and sodium carbonate was tried to mix together before use. The solution of mixed sodium carbonate solution and folin phenol reagent to reduce it was used. The solutions were dropped on paper and left them to dry at 10, 20, 30, 40 and 50 minutes.

3.5.5 Effect of concentration of sodium carbonate

In this work, the concentration of sodium carbonate was used as following: 2, 4, 6, 8, 10, 12, 14 and 16%.

3.6 Separation of ferulic acid and ascorbic acid by thin layer chromatography.

3.6.1 Effect of solvent system on the separation of ferulic acid and ascorbic acid

The TLC plate was cut into the strip (2 cm × 15 cm). TLC plate was measured from bottom up to 1 cm. Ferulic acid standard solution in methanol and ascorbic acid solution in water were applied as spots at the origin line on plate (bottom up to 1 cm) and were developed with (1) chloroform: methanol: formic acid (85: 15: 1) (2) ethyl

acetate: formic acid: water (65: 15: 20) in a pre-saturated chromatographic chamber. Developed plates were dried in a stream of hot air and was sprayed by 1% DPPH solution. Finally, spot on TLC containing of ferulic acid was visualized under the UV light (about 254 nm).

3.6.2 Separation of ferulic acid in real sample

The experiment were set up similar to the one in topic 3.6.1. The spots containing of ferulic acid were carefully cut in circle shape with a pencil. This spot of ferulic acid obtain from TLC was dissolved in methanol.

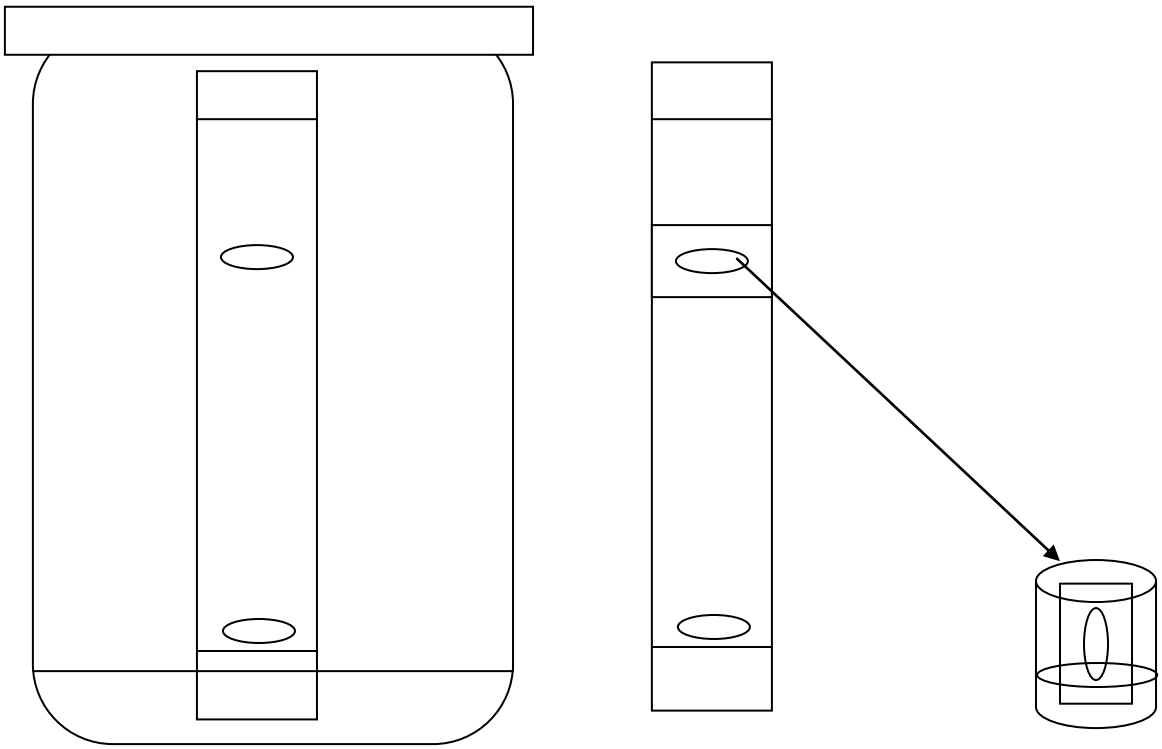


Figure 3.4 Schematic of obtaining ferulic acid from TLC plate and dissolving in methanol.

3.7 Separation and quantitative analysis of ferulic acid by coupling thin layer chromatography with lab-on-paper (TLC-LP)

There are two parts for the system of coupling thin layer chromatography with lab-on-paper. The first part obtained from dropping folin phenol reagent and 10% sodium carbonate on lab-on-paper. The second part is spot on TLC containing ferulic acid. Part 1 and part 2 are connected to obtain new devices as shown in Figure 3.5 (b). This new device was dipped into ethanol. Ethanol will drive ferulic acid to reacte with reagents on the paper-based devices. The color of test zone on the paper-based devices was captured by a digital camera. The color intensities were then analyzed using Adobe Photoshop CS3.

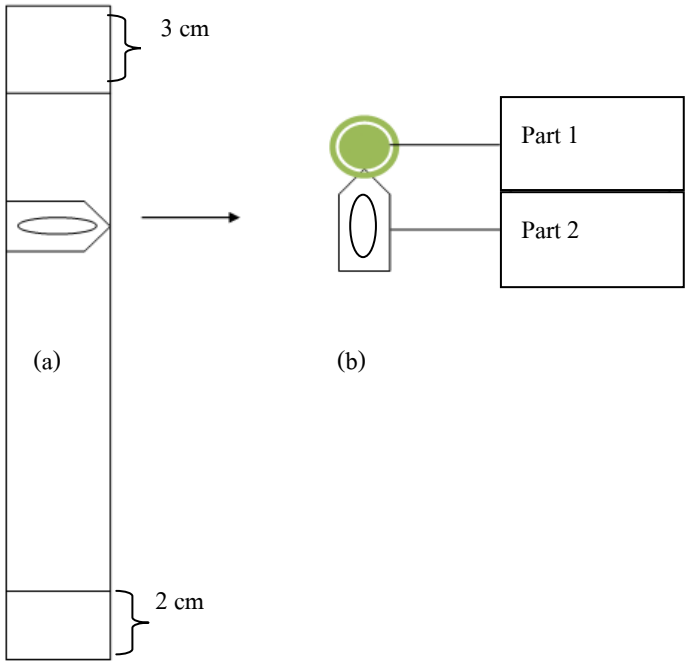


Figure 3.5 TLC (a) and TLC-LP (b) Part 1 is paper-based device, Part 2 is cut TLC plate containing of ferulic acid.

3.8 Analysis of ferulic acid in cosmetic samples

Folin phenol reagent and 10% Sodium carbonate were dropped on paper-based analytical devices, followed by ferulic acid at the reaction zone. Digital camera was used to capture the image and converted in to grayscale. Adobe Photoshop was then used to investigate the mean intensity from the histogram. Finally, the relationship between the mean intensity and the concentration of ferulic acid was obtained. The procedure of optical detection for quantifying the concentration of ferulic acid using Adobe Photoshop program was shown in Figure 3.6.

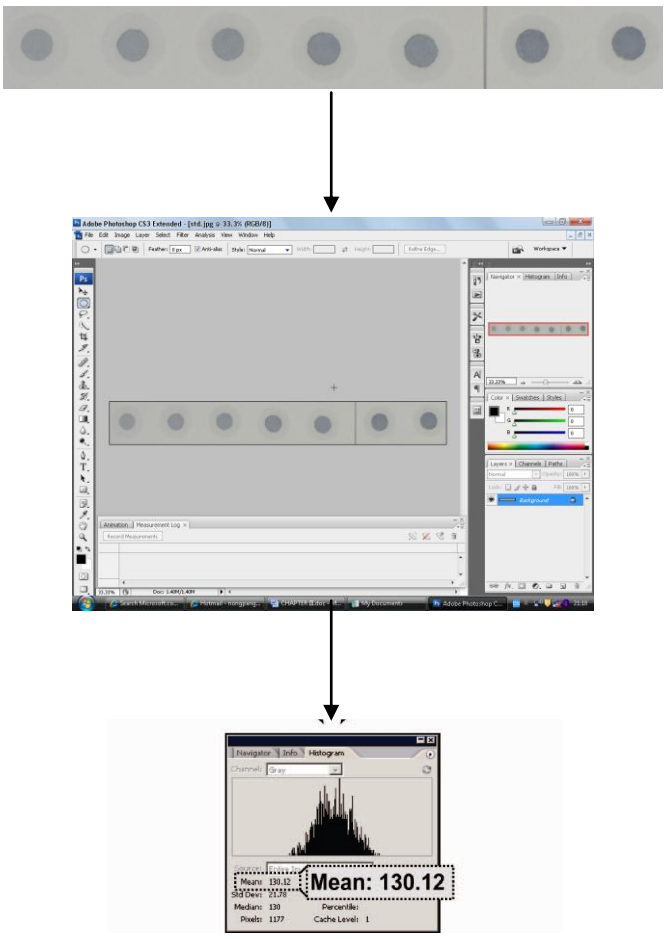


Figure 3.6 Procedure for quantifying ferulic acid using image editing software (AdobePhotoshop CS3).

CHAPTER IV

RESULTS AND DISCUSSION

4.1 Preparation of paper-based devices using melting wax screen-printing

4.1.1 Optimization of temperature for wax melting

In this thesis, wax is used as materials for the preparation of paper-based devices by melting wax and then screen-printing. Typically, the melting point of wax is in the range of 40-60 °C. Therefore, to save time for preparation of paper-based devices, the best temperature for melting wax is first parameter investigated in the range from 40 °C to 160 °C by using wax 1 g.

The relationship between temperature and melting time were shown in Figure 4.1. It was found that increasing of the temperature led to the short time for melting, However, at higher temperature, the risk of paper burning also increased. To compromise the time and paper burning, the best temperature for wax melting at 100°C at 2 minute was selected as a suitable condition. In addition, there was no significant difference of results obtained at temperature higher than 100°C.

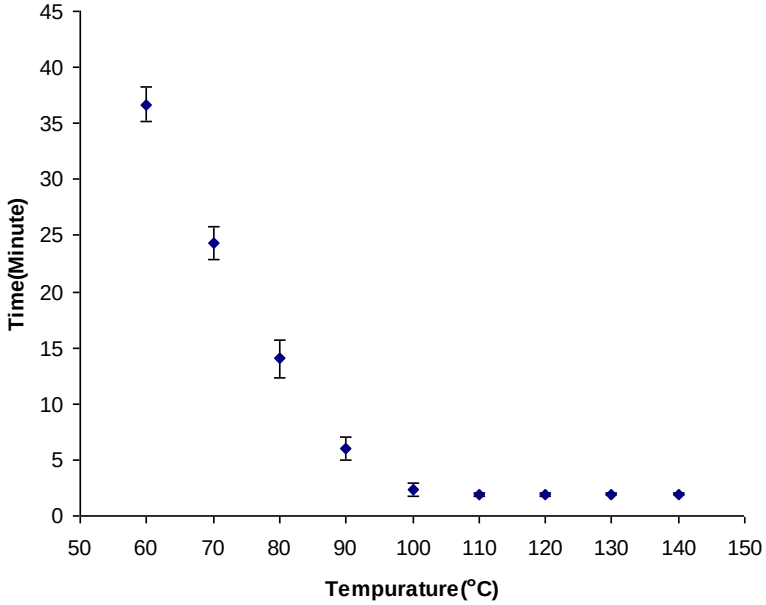
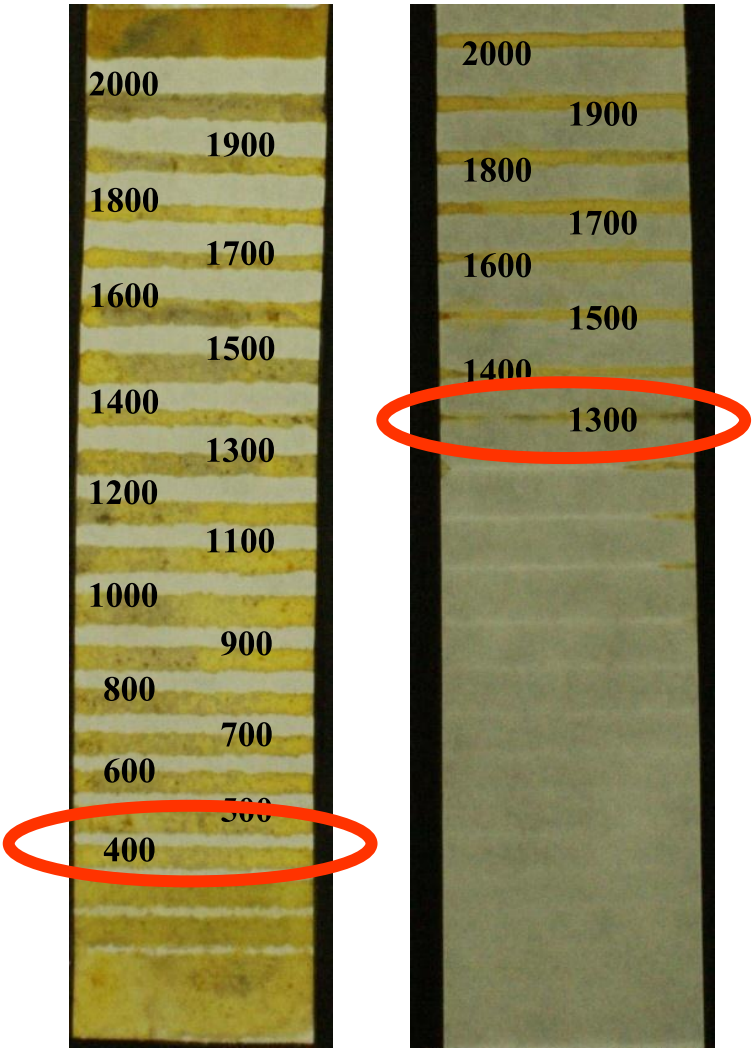


Figure 4.1 the relationship between the temperature of wax melting and time

4.1.2 Melting wax screen printing resolution

After getting the optimal temperature for wax printing, next the resolution of wax screen-printing is studied. The experiment was performed by creating the block screen witch have the width of hydrophobic barrier in the range from 100-2,000 μm. It can be seen that a minimum hydrophobic barrier of block screen at 400 μm can be used as mold for wax screen-printing. However, the width of hydrophobic barrier can be varied depended on the experimental design. After printing, the channel width is next parameter. The smallest channel width obtained from experiment was found to be 1,300 μm. In addition, from experiment, it can be said that the resolution of method was currently limited by the thickness, porosity, and orientation of paper fibers Figure 4.2 showed the results obtained from wax screen-printing on Whatman no.1 filter paper.



(a) (b)

Figure 4.2 (a) Hydrophobic barrier width (μm) (b) Hydrophilic channel width (μm)

The condition of temperature for wax melting is 100 °C. Using this optimal melting temperature, the resulting width of hydrophobic barriers (W_{RB}) was calculated using the linear equation from W_{RB}=1.1197 W_{PB}. The width of hydrophilic channels (W_{RC}) was then calculated with equation 1 and 2, where L is the length of wax spreading from the original wax line, and W_{PC} is the printed width of the channel.

$$2L = W_{RB}-W_{PB} \quad \text{.....(1)}$$

$$W_{RC} = W_{PC}-2L \quad \text{.....(2)}$$

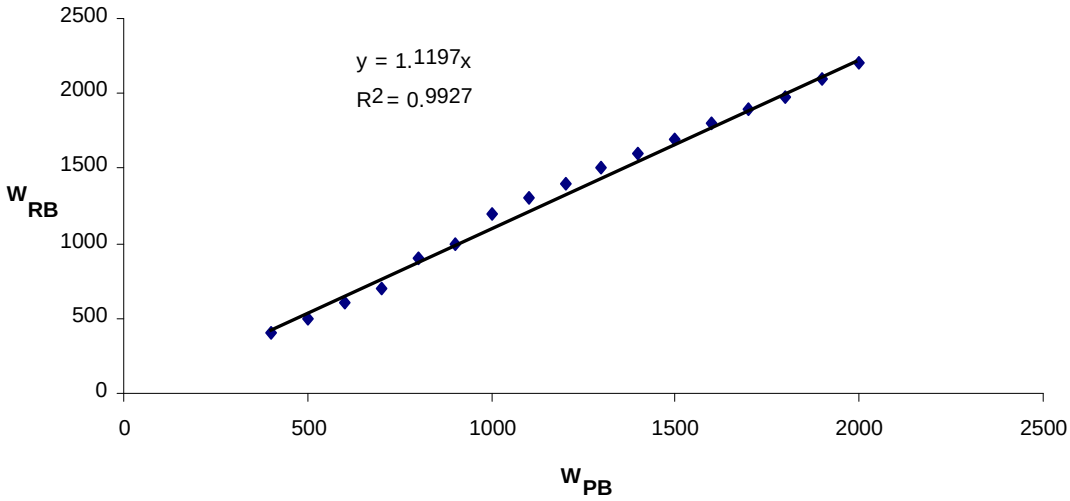


Figure 4.3 The graph plotted between the width of hydrophobic barriers (W_{RB}) with and the printed width of wax hydrophobic line (W_{PB})

4.2 Optimization of colorimetric reaction

4.2.1 Effect of sequence of solution dropping

The sequence of solution dropping was investigated. It was found that. The minimum drops of solutions onto lab-on-paper was required for the clearest blue color in reaction zone. In this work, the volume of 2 μL of each solutions was fixed and dropped on paper-based devices. Four experiments were set as following in table 4.1. It was designed to get the best results for colorimetric analysis. The results are shown in Figure 4.4.

Table 4.1 Sequence of solution dropping

Sequences			
1	**2**	**3**	**4**
Folin phenol reagent	Folin phenol reagent	Na_2CO_3	Na_2CO_3
Na_2CO_3	Ferulic acid	Folin phenol reagent	Ferulic acid
Ferulic acid	Na_2CO_3	Ferulic acid	Folin phenol reagent

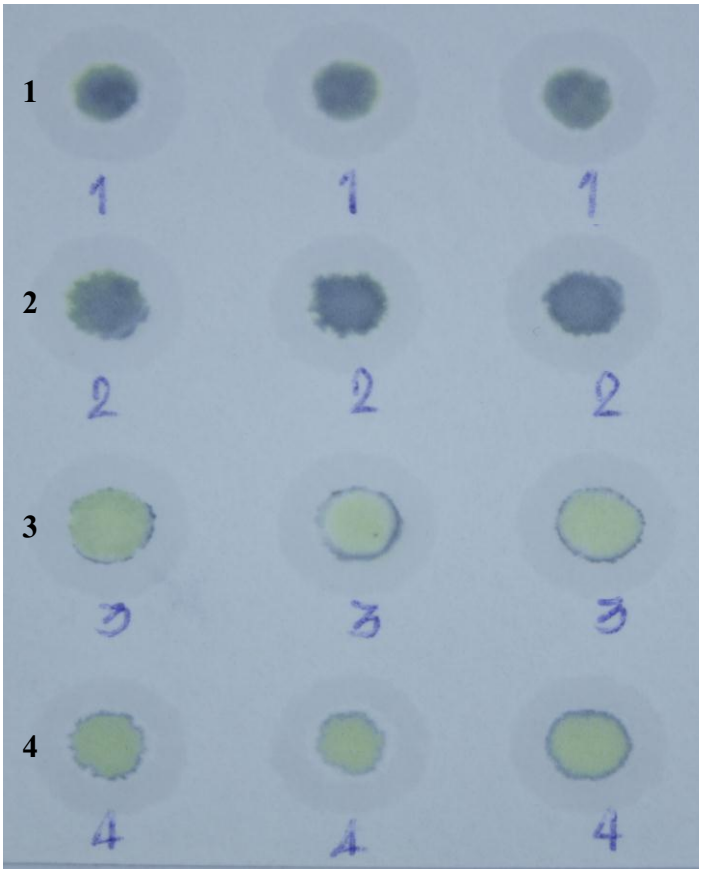


Figure 4.4 Effect of sequence of solution dropping 1, 2, 3 and 4

From the results, it was observed that the clearly blue color in the reaction zones were observed by using the sequence (1) and (2). In sequence (3) and (4), most of the reaction zones are still green color, which is the color of folin phenol reagent. It means that these sequence of solution dropping were not suitable. The process does not provide the complete reaction between reagent and analyte.

For the mechanism of reaction, normally, Mo (VI) ion in folin phenol reagent is green color. After adding the ferulic acid, the color will change from green to blue color because Mo (VI) ion was reduced by ferulic acid.

In this thesis, sequence (1) was chosen as the suitable sequence because this sequence provided the clearest blue color. Additionally, using this sequence led to the simple methodology for analysis because all reagents can be dropped onto paper before use. This means that the lab-on-paper can be completely prepared as a portable devices and ready to bring to field analysis. When the analysis was performed, only sample solution will be dropped onto the paper devices.

4.2.2 Comparison of the colorimetric reaction between the continuously drops and the drop dried solutions

Figure 4.5 illustrated the results obtained from 2 experiments. (a) is the results obtained from using the continuously drops solution on the paper and (b) is the results obtained from using the drop dried solution. It can be observed that the use of continuously drops solution showed the high variation in color at each experiment and the green of folin phenol reagent can be still observed, this means that the reaction is not complete by using this procedure. On the other hand, using the drops dried solution, the variation of color in each experiment is less than and in the reaction zone

showed the homogeneous blue color. It means that the reaction is complete. Thus, the drop dried solution technique was selected as a procedure for analysis of sample.

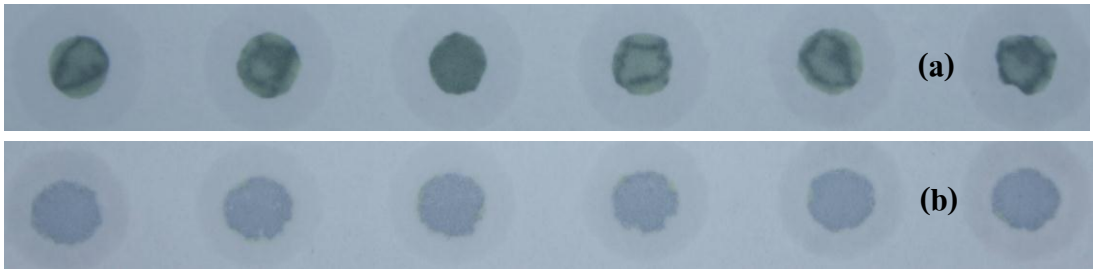


Figure 4.5 (a) The colorimetric reaction from the continuously drops solutions (b) The colorimetric reaction of the drops dried solutions

4.2.3 Effect of time between drop and drop

Effect of time between drop and drop was studied because it has effect on the blue color occurred on the reaction zone. The effect of time was studied at 10, 20, 30 and 40 minutes. When 10 and 20 minutes were selected as a time between drop and drop of solution it was found that color was not consist. It was also found that the green color of folin phenol reagent still remained after dropping sample solution. It means that these times were not enough to allow the paper dried before next drop solution.

At the time of 30 and 40 minutes, it was found that the blue color occurred uniformly representing that the reaction is complete. The intensity of the color can be measured without worrying about the non-uniformity. To compromise analysis time, 30 minutes was selected as the most suitable time for leading solution to dry before next drop.

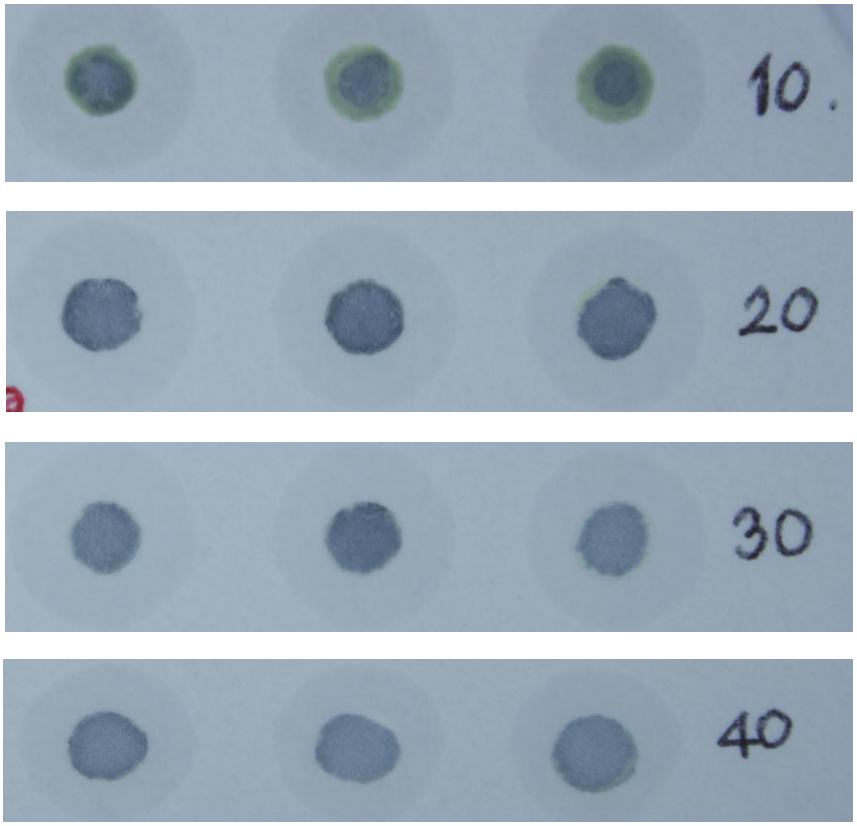


Figure 4.6 Effect of time between drop and drop at 10, 20, 30 and 40 minutes

4.2.4 Effect of mixed solution before drop

To save more analysis time, the solution of folin phenol reagent and sodium carbonate was mixed together before use. However, it was found that the color is still green even though the longer time between drop and drop was used as shown in Figure 4.7. Therefore, the concept of using mixed solution was rejected.

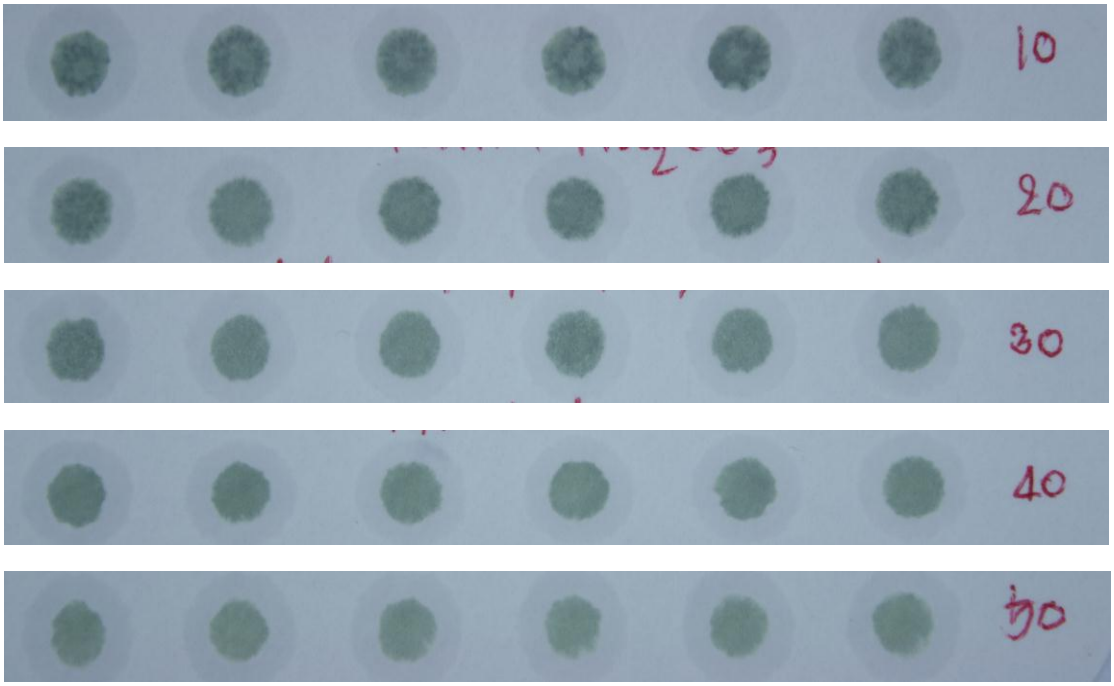


Figure 4.7 Effect of mixed solution before drop at 10, 20, 30, 40 and 50 minute

4.2.5 Effect of concentration of sodium carbonate

The use of different concentrations of sodium carbonate is affected on the completeness of reaction. The sodium carbonate concentration was investigated in the concentrations range from 2-16%. At 2-8% of sodium carbonate, the color at the reaction zone is still green indicating that the reaction is incomplete. However, at the concentrations from 10-16%, there were no green color found, and the uniform intensity of color were obtained. At 10% of sodium carbonate, it provided the highest intensity of color. Therefore, 10% of sodium carbonate was selected as the optimal concentration for all experiment.

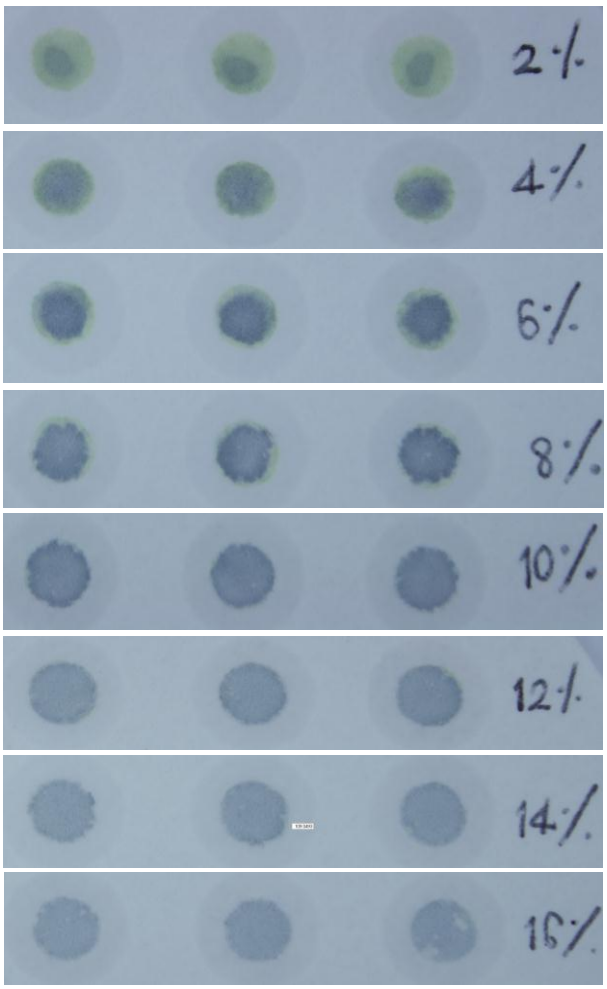


Figure 4.8 Effect of concentration of sodium carbonate at 2, 4, 6, 8, 10, 12, 14, 16 %

4.3 Separation of ferulic acid and ascorbic acid by thin layer chromatography

4.3.1 Effect of solvent system on the separation of ferulic acid and ascorbic acid

In this thesis, the separation of ascorbic acid and ferulic acid by thin layer chromatography was investigated because ascorbic acid is the important interference for ferulic acid analysis. To find the best solvent for the separation, the following solvent systems are tested.

(1) chloroform : methanol : formic acid (85 : 15 : 1)

The separation of ferulic acid from ascorbic acid by mixed. The ratio of solvent system, chloroform: methanol: formic acid is 85 : 15 : 1. The results are shown in Figure 4.9. It was found that the two analytes can be separated clearly. Table 2 summarized the R_f values of each analyte.

Figure 4.9 (a) spot of ferulic acid and ascorbic acid (b) spot of ferulic acid (c) spot of ascorbic acid

Table 4.2 The R_f value of a ferulic acid and ascorbic acid in solvent system chloroform : methanol : formic acid (85 : 15 : 1)

	R_f value		**Average R_f**
Ferulic acid	1	0.72	0.70 ± 0.01
	2	0.70	
	3	0.71	
Ascorbic acid	1	0.10	0.10
	2	0.10	
	3	0.10	

(2) ethyl acetate : formic acid : water (65 : 15: 20)

Separation of ferulic acid from ascorbic acid by solvent system is ethyl acetate: formic acid: water (65: 15: 20) found that two substances can be separated clearly as shown in Figure 4.10, however, ferulic acid is separated too fast, which made the characterization is difficult. In this thesis, this solvent system is not selected.

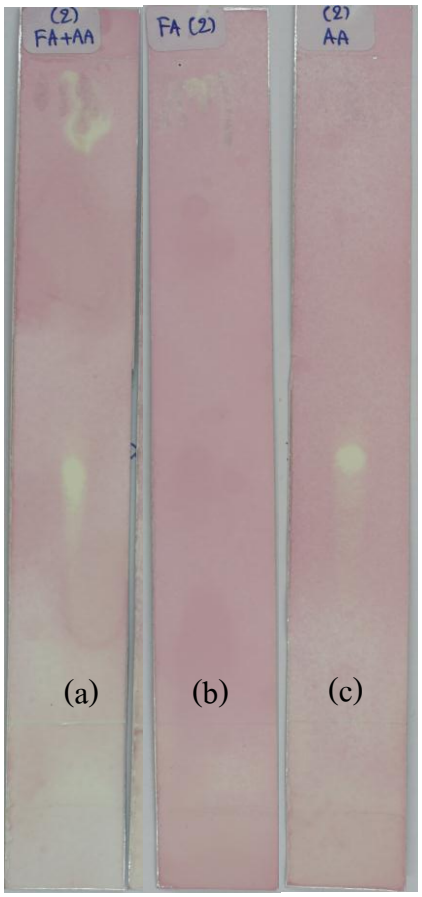


Figure 4.10 (a) spot of ferulic acid and ascorbic acid (b) spot of ferulic acid (c) spot of ascorbic acid.

Table 4.3 The R_f value of a ferulic acid and ascorbic acid in solvent system ethyl acetate : formic acid : water (65 : 15: 20)

	R_f value		**Average R_f**
Ferulic acid	1	0.98	0.96 ± 0.02
	2	0.95	
	3	0.96	
Ascorbic acid	1	0.37	0.38 ± 0.01
	2	0.39	
	3	0.38	

4.3.2 Separation of ferulic acid in real sample

Samples were applied as spots at the origin on plate and developed with chloroform: methanol: formic acid (85: 15: 1) in a pre-saturated chromatographic chamber. Developed plates were dried in a stream of hot air and visualized at 254 nm. As shown in figure 4.11, the spots were carefully circled with a pencil. Cut ferulic acid from TLC was dissolved with methanol for next experiment**.**

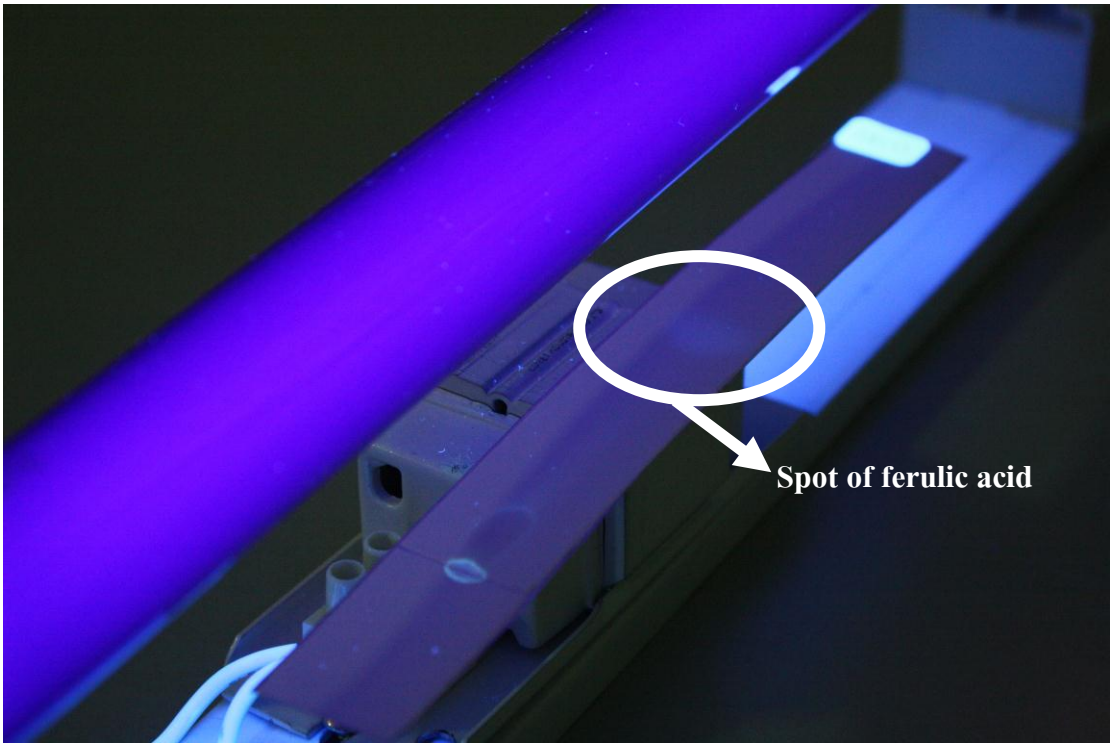


Figure 4.11 Spot of ferulic acid from developed with chloroform: methanol: formic acid (85: 15: 1)

4.4 Separation and quantitative analysis of ferulic acid by coupling thin layer chromatography with lab-on-paper (TLC-LP)

In this part, separation can be performed by using thin layer chromatography. Color at the reaction zone in part 1 is blue. Two compounds, ferulic acid and ascorbic acid provide the same color of the reaction, so separation by thin layer chromatography was performed. After separation, zone of ferulic acid was obtained on paper. Then this zone of ferulic acid was leached out by solvent and reacted with folin phenol reagent. Finally, the color at the reaction zone was captured by camera and convert to intensity by photo adobe program.

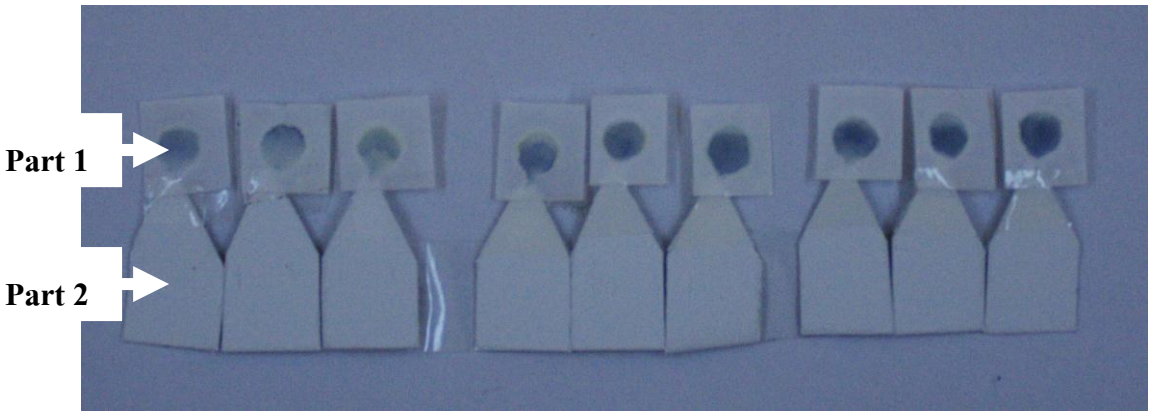


Figure 4.12 Thin layer chromatography coupled with lab-on-paper. Part 1 is lab-on-paper and Part 2 was TLC plate containing of ferulic acid.

4.5 Analytical performance

4.5.1 Calibration curve and linear range

After optimizing various experimental parameters, the analytical performance of the lab-on-paper for determination ferulic acid was investigated. Under the selected condition, plot of ferulic acid concentration and mean intensity was obtained as shown in Figure 4.16. The linear range was found to be between 20 - 140 ppm.

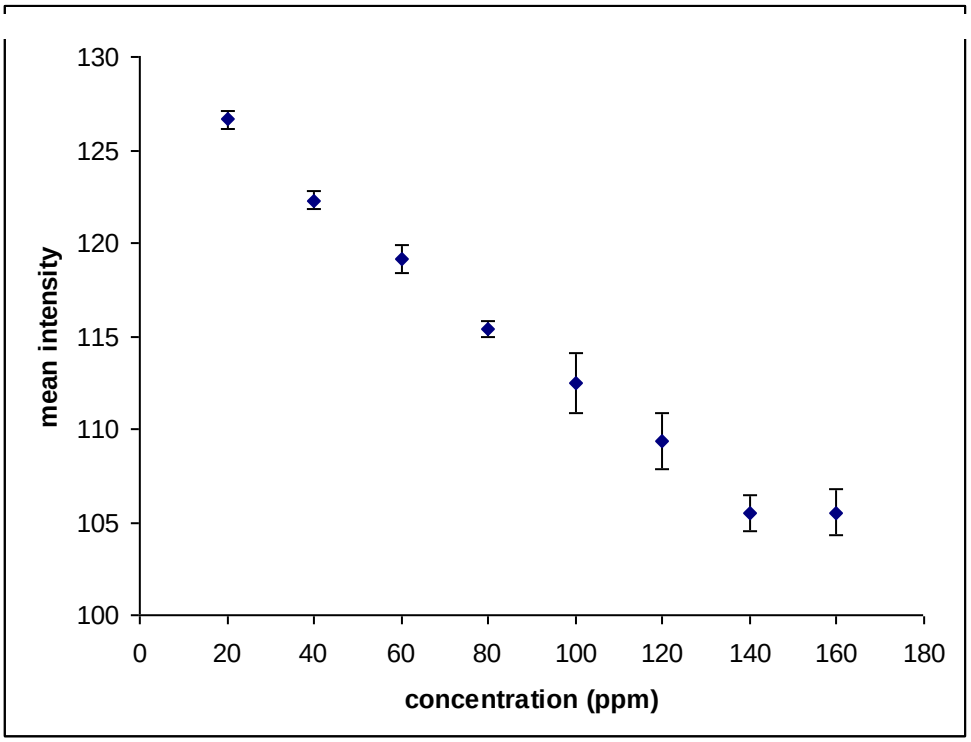


Figure 4.13 Graph shows the relationship between ferulic acid concentration and mean intensity

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

The limit of detection (LOD) and limit of quantification (LOQ) were calculate from 3SD/S and 10SD/S, where SD is standard error of the blank measurement (n=10) and S is the sensitivity of the method or the slope of linearity. LOD and LOQ were found to be 7.19 ppm and 23.98 ppm.

4.6 Analysis of ferulic acid in cosmetic samples

To verify the applicability of lab-on-paper developed in the present study, target compound, ferulic acid, in terms of cosmetic samples, was investigated. Morever, the concentration of ferulic acid found in cosmetic lab-on-paper was compared with HPLC. The results indicated that there is no significant different between using HPLC and lab-on-paper.

Using lab-on-paper, the relationship between ferulic acid concentration and blue intensity was found to be linear in the range from 20 - 140 ppm (R^2=0.9974) as shown in Figure 4.17 from this calibration curve, the concentration of ferulic acid in cosmetic samples were found as following: (1) 39.76 ppm (2) 47.40 ppm and (3) 31.19 ppm respectively.

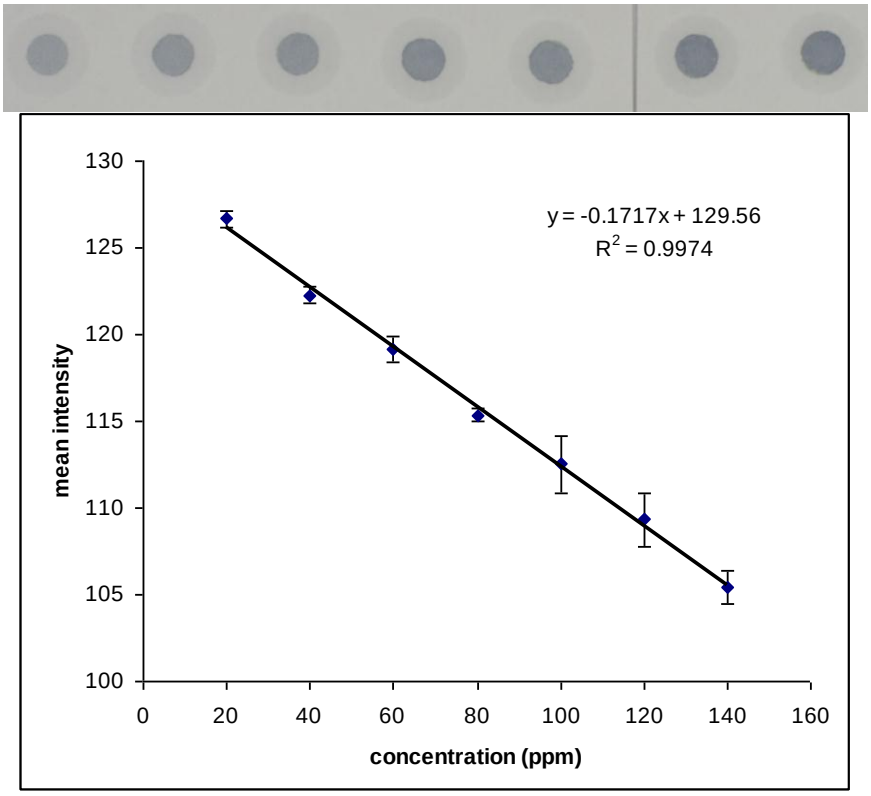


Figure 4.14 Calibration curve of ferulic acid by lab-on-paper

Using HPLC the concentration of ferulic acid in three cosmetic samples, were found as following: (1) 40.09 ppm (2) 48.76 ppm and (3) 29.11 ppm respective.

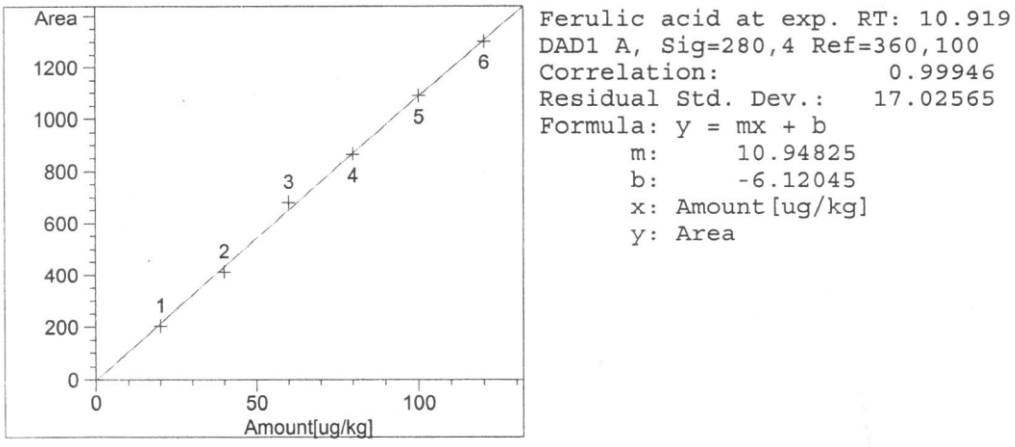


Figure 4.15 Calibration curve of ferulic acid by HPLC

Comparison of the both results, between HPLC results and lab-on-paper results.

CHAPTER V

CONCLUSION

5.1 Conclusion

In this thesis, the determination of ferulic acid by lab-on-paper coupled with colorimetric detection was developed. Material used to prepare lab-on-paper is wax by melting wax screen-printing. The best temperature for wax melting is 100 °C for 2 minute. Melting wax screen-printing created hydrophobic line on filter paper, the minimum width of hydrophobic channel and hydrophobic barrier is 1,300 and 400 μm. The area of the reaction zone has a diameter of 0.5 cm.

The colorimetric detection is a digital camera for image capturing. The image can be transmitted electronically and digitally to an off - site laboratory. The reaction between ferulic acid and folin phenol reagent, and sodium carbonate solution provide blue color on filter paper. The intensity from colorimetric detection depends on the concentration of ferulic acid.

The limit of detection (LOD) and limit of quantification (LOQ) for determination of ferulic acid were calculate from 3SD/S and 10SD/S, where SD is standard error of the blank measurement (n=10) and S is the sensitivity of the method or the slope of linearity. LOD is 7.19 ppm for determination of ferulic acid. LOQ is 23.98 ppm.

Comparison of the determination of ferulic acid between using the lab-on-paper and using high performance liquid chromatography (HPLC), it was found that the determination of ferulic acid by lab-on-paper is easier, faster and non-toxic than HPLC technique.

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