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นางสาวศุภานัน สุนันต๊ะ

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DEVELOPMENT OF MICROFLUIDIC PAPER-BASED ANALYTICAL DEVICE FOR DETERMINATION OF CREATININE IN URINE SAMPLES

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A Thesis Submitted in Partial Fulfillment of the Requirements
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งานวิจัยนี้ได้พัฒนาอุปกรณ์วิเคราะห์ของไหลจุลภาคฐานกระดาษสำหรับการตรวจวัดครีแอทินินในตัวอย่างปัสสาวะที่สะดวก ราคาถูกและ พกพาได้ วิธีการวิเคราะห์อาศัยปฏิกิริยาจาฟเฟ่ซึ่งเป็นปฏิกิริยาระหว่างครีแอทินินกับกรดพีคริกในตัวกลางที่เป็นเบสทำให้เกิดสารเชิงซ้อนครีแอทินิน-อัลคาไลน์พิเครตที่มีสีส้มซึ่งสามารถเห็นได้ชัดบนอุปกรณ์วิเคราะห์ของไหลจุลภาคฐานกระดาษ ความเข้มสีของสารเชิงซ้อนซึ่งบ่งบอกถึงความเข้มข้นของครีแอทินินถูกวัดค่าโดยใช้โปรแกรมอิมเมจเจ การศึกษาภาวะที่เหมาะสมของตัวแปรที่มีผลต่อการทดลองพบว่า ความเข้มข้นของสารละลายกรดพีคริกเท่ากับ 0.04 โมลาร์ ความเข้นข้นของสารละลายโซเดียมไฮดรอกไซด์เท่ากับ 2 โมลาร์ และ เวลาของปฏิกิริยาเท่ากับ 25 นาที เป็นภาวะที่เหมาะสม ภายใต้ภาวะที่เหมาะสมช่วงความสัมพันธ์เชิงเส้นตรงสำหรับการตรวจวัดอยู่ในช่วง 0.2-1 มิลลิโมลาร์ ขีดจำกัดในการตรวจพบคือ 0.08 มิลลิโมลาร์ และ ขีดจำกัดในการวัดเชิงปริมาณคือ 0.26 มิลลิโมลาร์ นอกจากนี้วิธีวิเคราะห์ที่พัฒนาขึ้นมีความจำเพาะต่อการตรวจวัดครีแอทินินและ มีความสามารถในการทำซ้ำที่ดี โดยค่าเบี่ยงเบนมาตรฐานสัมพัทธ์คือ 2.85, 1.10 และ 1.64 เปอร์เซนต์ สำหรับการตรวจวัดครีแอทินินที่ความเข้มข้นเท่ากับ 0.2, 0.6 และ 1 มิลลิโมลาร์ ตามลำดับ ความถูกต้องของวิธีที่พัฒนาขึ้นไม่แตกต่างอย่างมีนัยสำคัญจากวิธีมาตรฐานจาฟเฟ่โดยใช้สถิติการทดสอบทีแบบคู่ที่ค่าความเชื่อมั่น 95 เปอร์เซนต์ ท้ายที่สุดอุปกรณ์วิเคราะห์ของไหลจุลภาคฐานกระดาษที่พัฒนาขึ้นสามารถนำไปใช้ในการตรวจวัดครีแอทินินในตัวอย่างปัสสาวะ โดยมีค่าการกลับคืนอยู่ในช่วง 91.06-102.86 เปอร์เซนต์

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SUPHANAN SUNUNTA: DEVELOPMENT OF MICROFLUIDIC PAPER- BASED ANALYTICAL DEVICE FOR DETERMINATION OF CREATININE IN URINE SAMPLES. ADVISOR: ASSOC. PROF. NARONG PRAPHAIRAKSIT, Ph.D., 69 pp.

The simple, low-cost and portable microfluidic paper-based analytical devices (μPADs) for colorimetric determination of creatinine in urine samples were developed. The methodology was based on Jaffé reaction between the creatinine and picric acid in alkaline medium generating a colorimetric creatinine-alkaline picrate complex. The product exhibits an orange color which is clearly visible on μPADs. The color intensity of the complex which is indicative of the concentration of creatinine is then quantitatively determined using ImageJ software. Various experimental parameters were optimized, i.e., concentration of picric acid, concentration of sodium hydroxide, and reaction time, and the best analytical performance was achieved with 0.04 M picric acid, 2 M sodium hydroxide, and 25 minutes of reaction time. Under the optimum conditions, a wide linear range was obtained in the range of 0.2 – 1 mM with a limit of detection (LOD) and a limit of quantitation (LOQ) of 0.08 mM and 0.26 mM, respectively. Moreover, this method provided a selective determination of creatinine and showed a good reproducibility with a precision of 2.85, 1.10 and 1.64 %RSD for the detection of creatinine at 0.2, 0.6 and 1 mM, respectively. The accuracy of the proposed method was not significantly different from the standard Jaffé method by a paired t-test at 95% confidence interval. Finally, the developed devices were successfully applied for the determination of creatinine in urine samples with %recoveries in the range of 91.06-102.86%.

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

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

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LIST OF ABBREVIATIONS

AA	Ascorbic acid
ATP	Adenosine triphosphate
C	Concentration
°C	Degree Celsius
CE	Capillary electrophoresis
CKD	Chronic kidney disease
CL	Chemiluminiscence
cm	Centimeter
CV	Cyclic voltammetry
DI	Deionized
ECL	Electrochemiluminescence
EIS	Electrochemical impedance spectroscopy
FIA	Flow injection analysis
g	Gram
Glc	Glucose
HPLC	High performance liquid chromatography
ΔI	Difference of mean color intensity value
LOD	Limit of detection
LOQ	Limit of quantitation
min	Minute
mL	Milliliter
µL	Microliter

µPADs	Microfluidic paper-based analytical devices
n	Number of experiments
nm	Nanometer
PEC	Photoelectrochemistry
R^2	Coefficient of determination
RGB	Red, green and blue
rpm	Revolutions per minute
RSD	Relative standard deviation
s	Second
S	Slope
SD	Standard deviation
SWV	Square-wave voltammetry
UA	Uric acid
UV-Vis	Ultraviolet-Visible
X	Mean

CHAPTER I
INTRODUCTION

1.1 Introduction

Chronic kidney disease (CKD) has become a worldwide public health problem in the past 10 years [1]. The prevalence of CKD is high and likely to increase rapidly in the coming decades, especially in the developing countries [1, 2], with an increase of the risk factors such as diabetes, obesity, smoking, high blood pressure and cardiovascular disease [3]. The number of CKD patients is also rising sharply. Although, the symptoms in the early stage of CKD are absent, the CKD affects many parts of the body, i.e., it causes a damage of the central nervous system, a decrease of the immune response, retention of the fluids, resulting in the swelling of arms and legs, and irreversible damage of the kidneys (end-stage kidney disease, ESKD) leading to kidney transplantation [3]. Therefore, the screening tool for diagnosis of the CKD is necessary. In the CKD diagnosis, there are several tests such as blood test, urine test, which are to determine the biomarker concentration indicating CKD in blood and urine, imaging test which is to ultrasound the kidneys to evaluate their structures and sizes, and kidney biopsy which is to analyze the kidney tissue samples removed by a long and thin needle inserted through the skin to find the cause of kidney problems [3]. The two latter tests suit for the diagnosis in clinical laboratories. As for on-site diagnosis, urine test is more applicable because it is easy to collect the samples without the use of needles to draw blood samples which requires skilled users or it causes needles-stick injuries. The urine test is also suitable for people who are afraid of blood drawing and its cost is cheaper than the cost of the blood test with which medical devices are needed in the sample collection.

Creatinine is a breakdown product of creatine and phosphocreatine which are important compounds in the energy metabolism of muscle. As creatinine has no function in the body, it is eliminated from the body by kidneys through glomerular filtration and is excreted in urine at a constant rate. Abnormal concentration of creatinine in urine and serum indicates kidney malfunction [4, 5] thus is one of the key

biomarkers used for assessing the kidney function. Moreover, the concentration of creatinine in urine and serum can also indicate chronic heart failure [6], cancer [7, 8] and muscular dystrophy [5, 9]. Normal concentration of creatinine in urine ranges from 2.48 to 22.92 mM [10] depending on the individual's weight, diet and muscle mass [11].

Various detection techniques have been used for the determination of creatinine, such as, high performance liquid chromatography (HPLC) [12], capillary electrophoresis (CE) [12, 13], tandem mass spectrometry [14], flow injection analysis (FIA) [15], square-wave voltammetry (SWV) [16], electrochemical impedance spectroscopy (EIS) [17], cyclic voltammetry (CV) [18], and surface-enhanced Raman spectroscopy [19]. However, most of these techniques require expensive instruments, skilled operators and long analysis time making them unsuitable for field monitoring. Therefore, the development of simple, low-cost and portable sensors for on-site analysis of creatinine is of particular interest.

Microfluidic paper-based analytical devices (μPADs) are an alternative tool for various applications such as biochemical detection [20-22], immunological detection [23-25] and molecular detection [26-28]. When compared to other methods, the μPADs are inexpensive, easy to use, rapid, portable and disposable. Moreover, they require only small amount (micro-scale) of reagent/sample and do not require external instruments. The μPADs were first introduced by Whitesides and coworkers in 2007 [29]. A concept of the μPADs is to have hydrophilic micro-channels on the devices by creating hydrophobic barriers which direct the flow of fluids in sample zones of the devices to hydrophilic micro-channels where the reagents are immobilized, thereby the chemical reactions occur. Several fabrication methods have been used to produce the μPADs such as wax printing [30, 31], inkjet printing [32], photolithography [33], laser treatment [34] and screen-printing [35] . There are also several detection methods applicable for these μPADs, for examples, colorimetry [36, 37], electrochemistry [38], fluorescence [39] and chemiluminescence [40].

This work aims to develop the simple, low-cost and portable μPADs for the colorimetric determination of creatinine in urine samples. A wax printing was used as

the fabrication method because both wax and paper are cheap and readily available materials. Besides, it is a rapid process and organic solvents are not required making it environmental friendly. The developed μPADs consist of 2 parts; hydrophilic part and hydrophobic part. Wax pattern serves as a hydrophobic barrier through which the solution cannot penetrate and it directs the flow of solution. Consequently, solution flows in the hydrophilic part via capillary action to the detection zones. The μPADs developed in this work are based on Jaffé reaction which is widely used in clinical laboratories for creatinine detection [41]. Creatinine reacts with picric acid in alkaline medium to form creatinine-alkaline picrate complex. The product yields an orange color which is clearly visible on μPADs. The change of color is analyzed using a digital camera for quantitation. The color intensity of the colorimetric product which is related to concentration of creatinine is quantified using ImageJ software.

1.2 Objectives of the research

There are three objectives for this research;

1. To develop inexpensive, easy to use and portable paper-based analytical devices for the determination of creatinine in urine samples.

2. To develop sensitive and selective devices for the determination of creatinine in urine samples.

3. To apply the developed devices for the determination of creatinine in urine samples.

1.3 Scope of the research

The microfluidic paper-based analytical devices were designed and fabricated to obtain a suitable platform for the determination of creatinine in urine samples. Various experimental parameters were optimized to achieve the best analytical performances. Under the optimum conditions, analytical performance characteristics including range of linearity, limits of detection, limits of quantitation and reproducibility were investigated. Moreover, various interfering substances were studied for selectivity of the determination. The developed devices were then validated with the

conventional technique and finally applied for the determination of creatinine in urine samples.

CHAPTER II

THEORY AND LITERATURE REVIEWS

2.1 Metabolism of creatinine

Creatine is a molecule which requires amino acids, l-arginine, l-methionine, and glycine, for endogenously synthesis and it provides recycling of adenosine triphosphate (ATP) which is a high-energy molecule providing energy in vertebrae. In the mechanism shown in Figure 2.1, creatine is converted to phosphocreatine which is necessary for the ATP production via the enzyme creatine kinase [9].

Creatinine is a breakdown product of creatine and phosphocreatine which are important compounds in the energy metabolism of muscle. As creatinine has no function in the body, it is eliminated from the circulatory system by kidneys through glomerular filtration and is excreted in urine at a constant rate. Abnormal concentration of creatinine in urine and serum indicates kidney malfunction [4, 5] thus is one of the key biomarkers used for assessing the kidney function. Moreover, it is also indicative of chronic heart failure [6], cancer [7, 8] and muscular dystrophy [5, 9]. However, normal concentration of creatinine in urine ranges from 2.48 to 22.92 mM [10] depending on weight, diet and muscle mass [11].

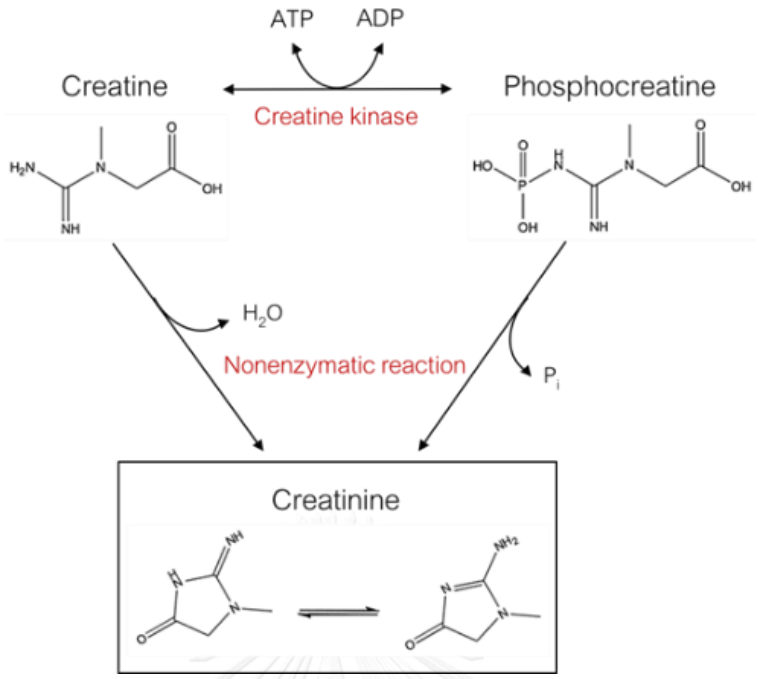


Figure 2.1 A brief process for the occurrence of creatinine. Creatine is converted to phosphocreatine via creatine kinase. Then, the breakdown of creatine and phosphocreatine generates creatinine via nonenzymatic process.

2.2 Jaffé reaction

In 1886, a colorimetric method for the detection of creatinine, known as the Jaffé reaction, was first developed by Max Jaffé [42]. Jaffé reaction is the reaction between creatinine and picric acid in alkaline condition to form a creatinine-alkaline picrate complex. The mechanism of the Jaffé reaction was proposed that picric acid was attacked by the methylene anion of creatinine at the meta position to form the Jaffé product as shown in Figure 2.2a [43]. This product yields an orange color (Figure 2.2b) which exhibits the maximum absorbance in the range of 480-520 nm as shown in Figure 2.3 [42-44].

Despite the fact that the Jaffé reaction has been discovered for many years, it is still popular and widely used as a routine detection of creatinine in urine for the estimation of kidney function in clinical laboratories [45].

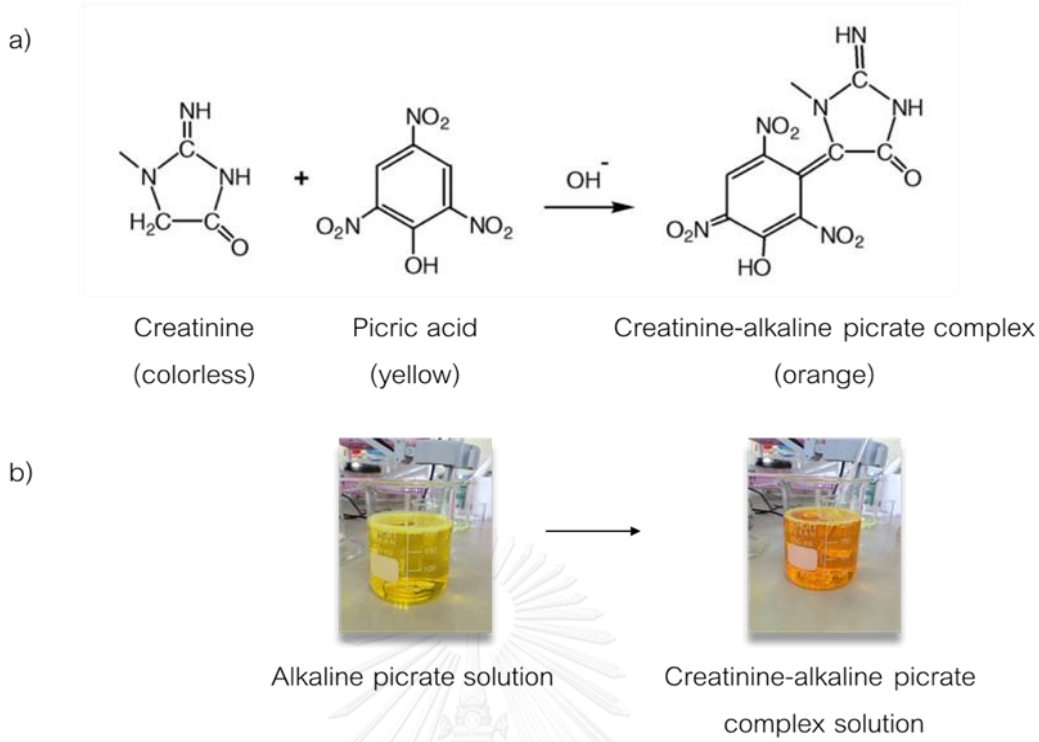


Figure 2.2 (a) Reaction scheme for the Jaffé reaction. (b) Upon the addition of creatinine into the alkaline picrate reagent, the color of the solution changes from yellow to orange.

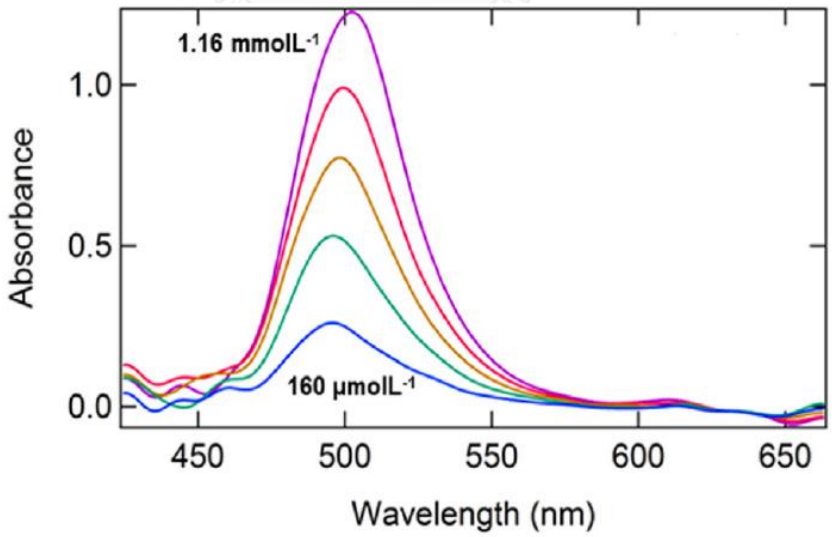


Figure 2.3 The absorption spectra of the creatinine-alkaline picrate complex with different concentrations of creatinine [5].

2.3 Microfluidic paper-based analytical devices

Microfluidic paper-based analytical devices (μPADs) are an alternative tool for various applications such as biochemical assay [20-22], immunological assay [23-25] and molecular assay [26-28]. The μPADs were pioneered by Whitesides and coworkers in 2007 [29]. They developed patterned papers as a platform for the assay of glucose and bovine serum albumin (BSA) in micro-scale of urine. These devices were fabricated using photolithography method which was used to pattern photoresist onto a hydrophilic chromatography paper to form hydrophobic walls (Figure 2.4). The patterned paper provided control of fluids moving through capillary action in hydrophilic micro-channels.

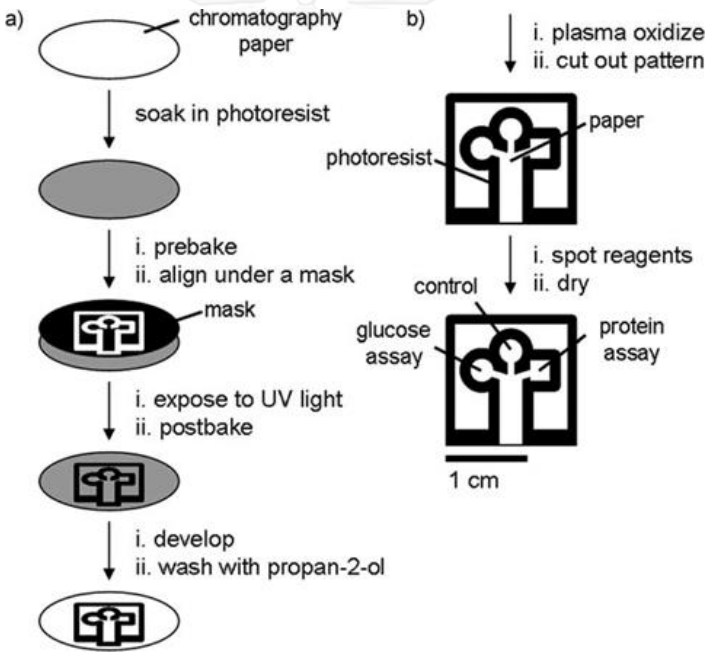


Figure 2.4 Fabrication process of the first μPADs by photolithography for bioassays: (a) SU-8 photoresist was used to pattern the hydrophobic wall onto the chromatography paper with the use of UV light and organic solvent and (b) the patterned paper was prepared for the assay of glucose and BSA [29].

Colorimetry was used as a detection technique for these assays. They investigated the color change due to the enzymatic oxidation of iodide to iodine for glucose assay and the color change due to the binding between tetrabromophenol

blue (TBPB) and proteins for protein assay. The change of color was an indicative for the presence of the analytes and associated with the concentration of these analytes. In the presence of glucose and BSA, the color changed from clear to brown and yellow to blue, respectively (Figure 2.5).

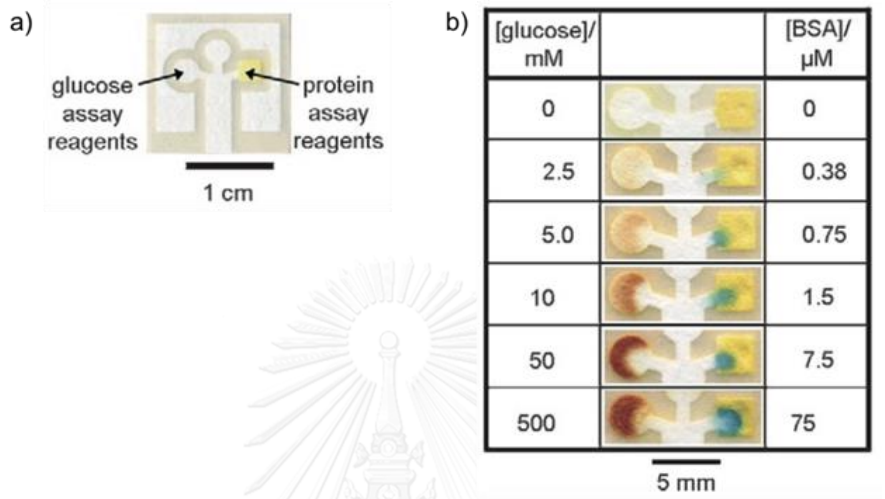


Figure 2.5 The patterned paper with photoresist for glucose and protein assay. (a) The patterned paper with test reagents on the test areas. The test areas on the left, in the middle and on the right are used for the glucose test, control test and protein test, respectively. (b) The change of color on the devices with the use of different levels of glucose and BSA in a synthetic urine [29].

In the field analysis, the particulates which can contaminate the assay did not move into the test channels and did not interfere the detection demonstrated in Figure 2.6.

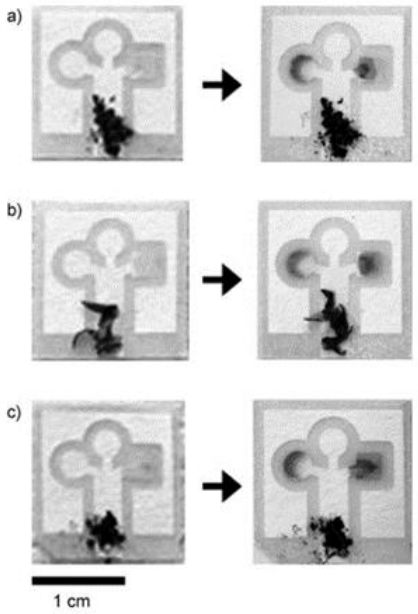


Figure 2.6 The assays with (a) dirt, (b) plant pollen and (c) graphite powder contaminated in the synthetic urine [29].

The μPADs provided various advantages including inexpensiveness, rapidness, ease of use, portability and disposability. Moreover, they require only small amount (micro-scale) of reagent/sample and do not necessarily require external instruments to enforce the fluids.

2.3.1 Types of paper for μPADs

There are several types of paper that can be used as a substrate for μPADs. The criteria for paper selection depends on the application and fabrication method of the devices [46]. Cellulose paper such as Whatman® filter is commonly used as a substrate for μPADs because it is cheap and hydrophilic which allows the penetration of fluid. Whatman No.1 filter paper is most intensively used because of the medium retention and flow rate of fluids and its compatibility with many fabrication methods [46]. Some applications or fabrication methods which require solvents that swell the cellulose fiber and restrict the pore sizes need Whatman No.4 filter paper as a substrate because it has larger pore sizes, resulting in larger surface area and faster flow rate [47]. Chromatography paper has a more uniform structure that avoids the deformation of μPAD patterns and has no additives which can interfere the assay, thus it suits for the electrochemical detection with screen-printed electrodes

[48]. Polyester which is a cellulose substrate integrated with an inorganic filter is suitable for surface chemical modification or deposition [49]. Additionally, hydrophobic nitrocellulose membrane which provides smooth and uniform pore size is suitable for the immobilization of enzymes, proteins, DNA and cells. For instance, antibodies are immobilized via electrostatic interaction between the positive charge and the negative charge of antibody carboxyl group and amino group on the membrane surface, respectively [50].

2.3.2 Fabrication methods of the μPADs

There are two main fabrication methods of the μPADs, i.e., two-dimensional (2D) and three-dimensional (3D) methods [51]. The 2D μPADs were fabricated by creating hydrophobic walls onto the hydrophilic cellulose paper so the fluid cannot penetrate and thus driven along the micro-channels through capillary action. There are several methods to fabricate the 2D μPADs including wax printing [30, 31], inkjet printing [32], flexographic printing, photolithography [33], laser treatment [34], plasma treatment, wax screen-printing, wet etching and screen-printing [35]. Here are some works using different fabrication methods. In 2008, Abe et al. [52] developed the μPADs fabricated using inkjet printing for the assay of total protein, pH and glucose in urine. Figure 2.7 illustrates the fabrication process using the inkjet printing method.

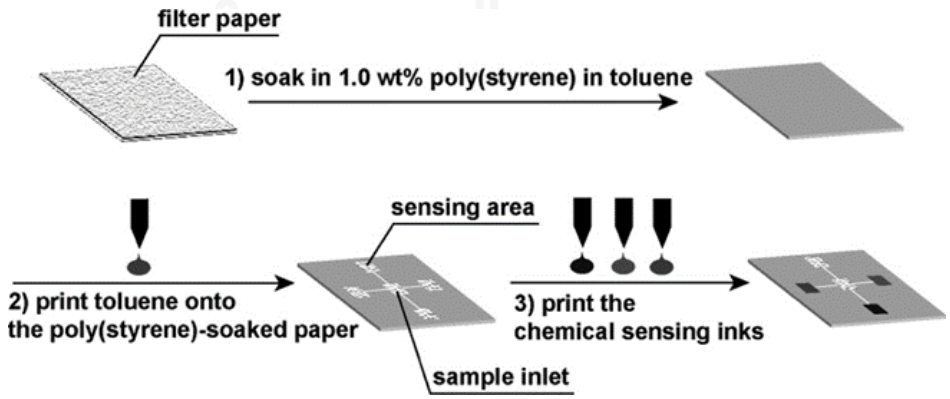


Figure 2.7 Fabrication process by inkjet printing method for multianalyte sensors. The μPAD comprises several sensing areas and a reference area, connecting to a central sample inlet area.

In 2010, Olkkonen et al. [53] employed flexographic printing as a fabrication method of μPADs as shown in Figure 2.8. Polystyrene used as printing ink was printed on the paper surface and it was allowed to move into the paper to form hydrophobic walls.

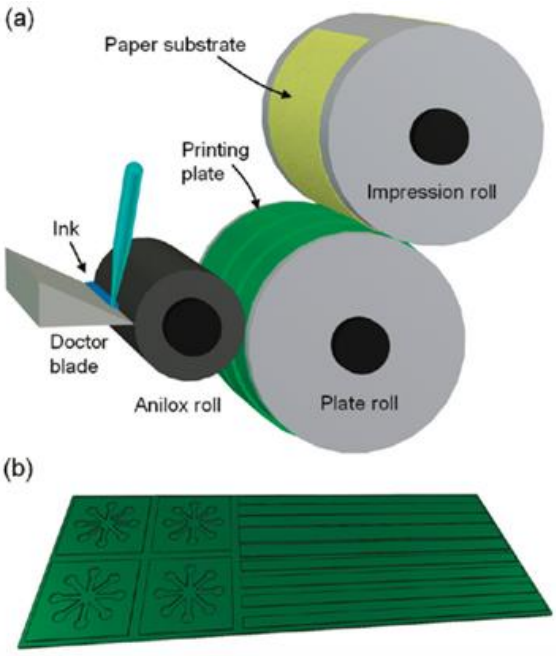


Figure 2.8 (a) Polystyrene in toluene solution used as printing ink is collected in the ink reservoir, preparing to transfer to the anilox roll. The anilox roll then speeds up and rotates to dispense the ink. The plate roll and the impression roll then start the printing process by rotation. The ink on the printing plate which contains the printing pattern shown in (b) is transferred onto the paper which is fixed on the impression roll.

In 2010, Lu et al. [30] developed the μPADs for immobilization of protein. The μPADs were fabricated in nitrocellulose (NC) membrane using wax printing. The fabrication steps were demonstrated in Figure 2.9.

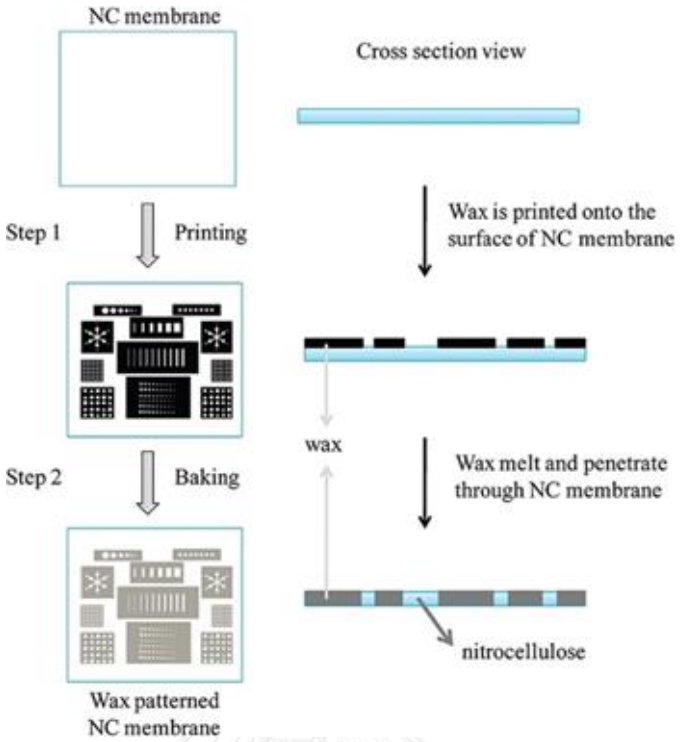


Figure 2.9 Fabrication steps for wax printing of the NC membrane-based devices for immobilization of protein. There are 2 fabrication steps. Step 1: Printing the wax onto the NC membrane surface using a wax printer. Step 2: Baking the patterned NC membrane using an oven at 125 °C for 5 min. The overall fabrication process was done within 10 min.

In 2011, Dungchai et al. [54] used wax-screen printing as a fabrication method of the μPADs for the simultaneous assay of glucose and total iron in serum. The fabrication process is shown in Figure 2.10.

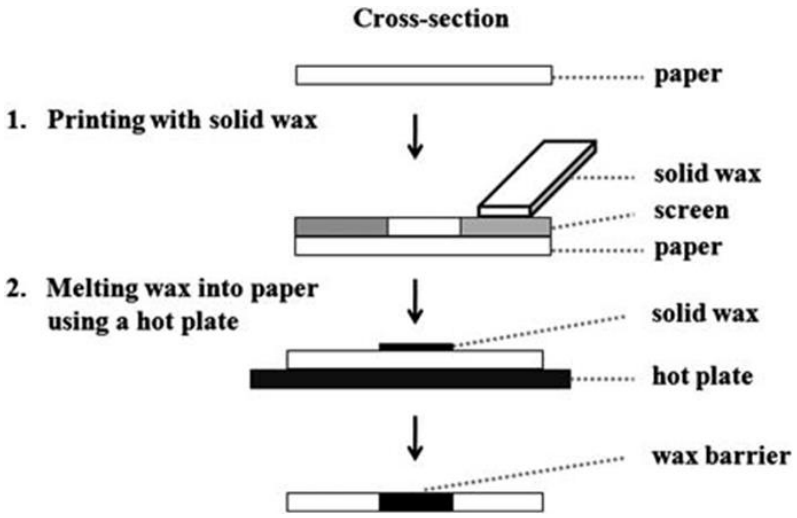


Figure 2.10 Fabrication process by wax-screen printing. There are two steps for fabrication. Step 1: printing designed patterns on the paper surface with solid wax by a screen-printing method. Step 2: melting the solid wax into the paper using a hot plate thus generating hydrophobic barriers.

In 2013, Nie et al. [55] proposed a laser cutting method to fabricate the μPADs. This fabrication process, illustrated in Figure 2.11, is to pattern hollow microstructures serving as hydrophobic barriers in paper using CO_2 laser cutting/engraving machine.

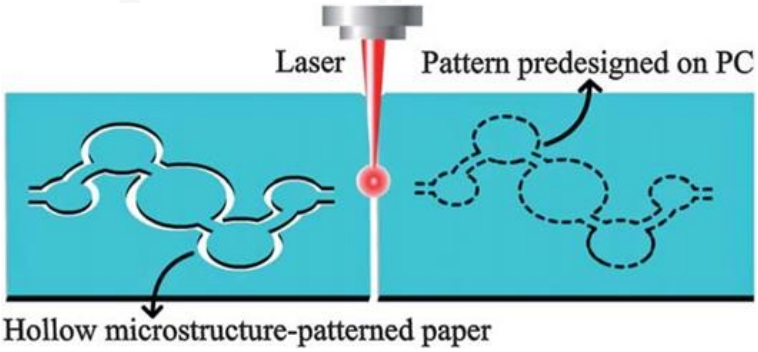


Figure 2.11 Principle of laser cutting method. This fabrication method is based on the cutting due to the heat produced by laser. The pattern-predesigned paper is burnt through its thickness.

The fabrication methods for the μPADs are summarized in Table 2.1.

Table 2.1 The main advantages and disadvantages of different fabrication methods for the μPADs [51].

Fabrication methods	Advantages	Disadvantages
Wax printing	– Simple and fast – Eco-friendly – Low-cost materials	– Requires expensive wax printers – Requires heating step
Inkjet printing	– Rapid – High resolution of patterns – Inexpensive thermal inkjet printers	– Not suitable for high throughput production
Photolithography	– Rapid – High resolution of patterns	– Requires organic solvents – Requires expensive photoresists
Flexographic printing	– Requires less amounts of samples/ reagents	– Requires more than one print of polystyrene solution
Plasma treatment	– Inexpensive patterning agent (alkyl ketene dimer)	– Gets a bigger pattern due to the over stretch of a substrate under a mask
Laser treatment	– Selective modification for surface structure	– Do not allow fluids flow laterally – Requires extra coating for fluid flow
Wet etching	– Low-cost materials	– Requires customization of printing apparatus

Fabrication methods	Advantages	Disadvantages
Wax screen-printing	– Low-cost printing screens – Eco-friendly	– Requires patterned mesh
Screen-printing	– Simple	– Low resolution of patterns – Requires other printing screens

In addition to the 2D μPADs, the 3D μPADs were fabricated by stacking different layers of paper and double-sided adhesive tape, causing the fluid to flow within the layers of paper. For instance, Martinez et al. [56] fabricated the 3D μPADs for testing of four different samples. As the fluids flowed laterally and vertically, they flowed across one another without mixing. The 3D μPAD was shown in Figure 2.12.

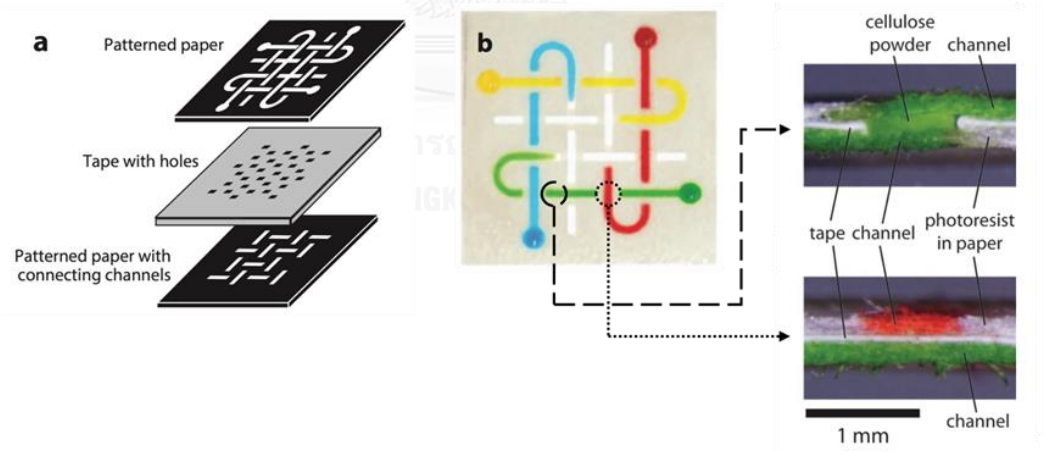


Figure 2.12 (a) The 3D μPAD consists of 3 layers; the top layer is a patterned paper with 4 channels, the middle layer is a double-sided adhesive tape containing cellulose powder-filled holes and the bottom layer is a patterned paper with connecting channels. (b) Photograph of an assembled μPAD with four dye solutions. The photograph was captured at 4 min after adding the solutions at the inlets. The right

photographs are cross sections of the 3-D μPAD displaying a channel connecting each layer of papers.

In this work, wax printing was used as a fabrication method of the μPADs because both wax and paper are inexpensive, disposable and readily available. Besides, the fabrication process is rapid, easy to operate and organic solvents are not required. Wax printing is also suitable for mass production because of a few number of steps.

2.3.3 Detection methods of the μPADs

Since its introduction, the μPADs have been applied to detect a wide range of analytes. When sample solutions in sample zones reach detection zones of the μPADs, the reactions between analytes and substrates immobilized on the μPADs occur. The signals which are developed can be detect by several techniques such as colorimetry [36, 37], electrochemistry [38], fluorescence [39], chemiluminescence (CL) [40], electrochemiluminescence (ECL) [57], photoelectrochemistry (PEC) [58], etc. A comparison of these detection techniques is displayed in Table 2.2.

Table 2.2 The main advantages and disadvantages of different detection methods of the μPADs [51].

Detection methods	Advantages	Disadvantages
Colorimetry	– Equipment-minimal – Visible to the naked eyes	– Interference from other substances – Low sensitivity
Electrochemistry	– Insensitive to ambient illumination conditions – Insensitive to impurities	-
Fluorescence	– Easy readout with a camera-equipped cellular phone	– Interference from other substances – Low sensitivity

Detection methods	Advantages	Disadvantages
Chemiluminescence	– Independent of ambient light – High sensitivity	– Requires expensive chemiluminescence reader
Electrochemilumi-nescence	– Combination of the advantages and disadvantages from the luminescence and electrochemistry	
Photoelectrochemistry	– Combination of the advantages and disadvantages from the optical methods and electrochemistry	

Colorimetry is a method in which its investigation is based on a color change, resulting from enzymatic or chemical reaction between reagents and analyte. Recently, colorimetry has been most intensively used as a detection method of the μPADs because it is simple and colorimetric signal can be read easily. When the color changes, the color intensity is recorded by scanners or digital cameras and quantitative analysis is then carried out by measuring the color intensity using image analysis software. The designs of various μPADs integrated with colorimetric detection are shown in Figure 2.13.

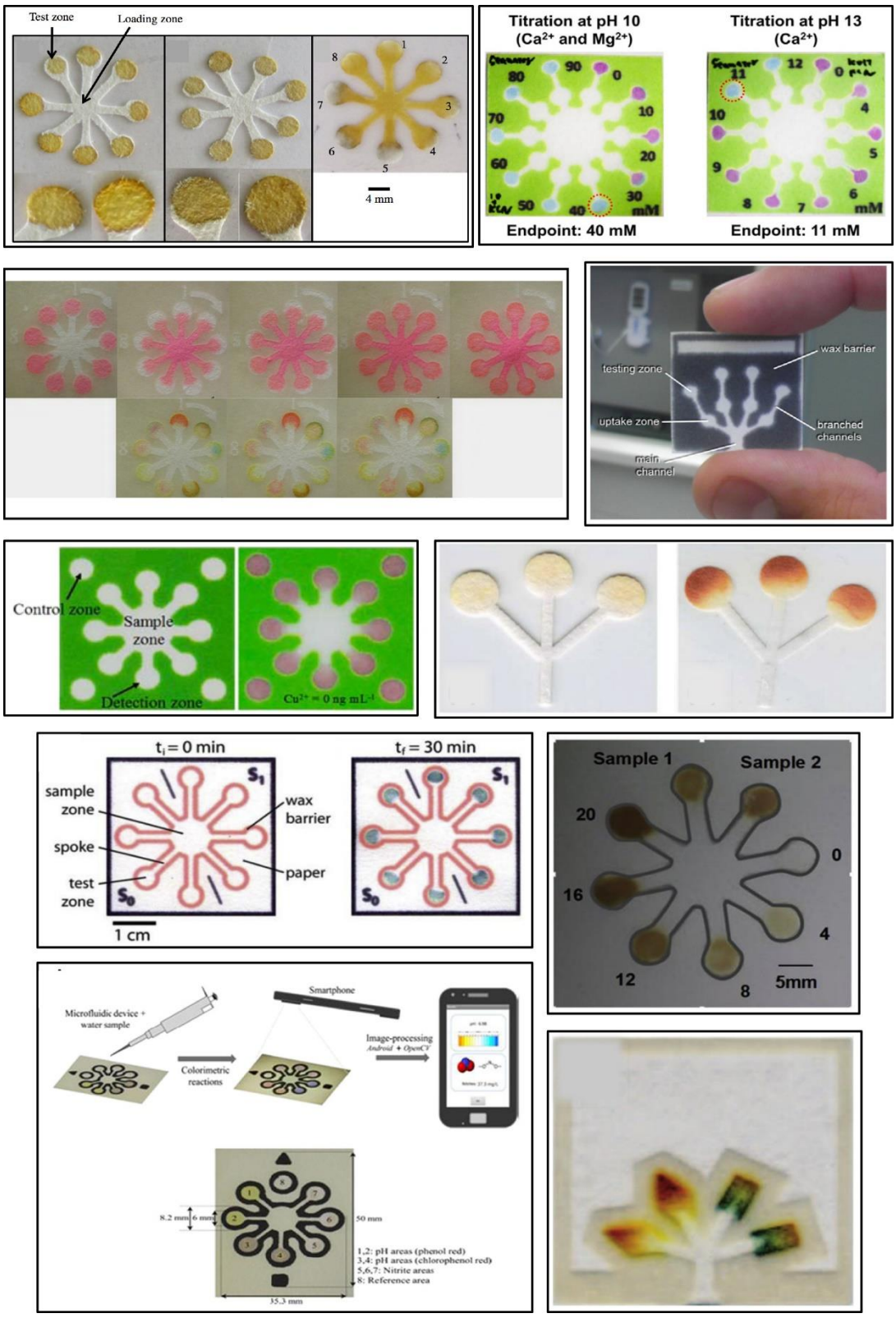


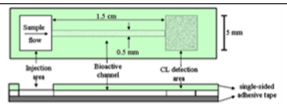
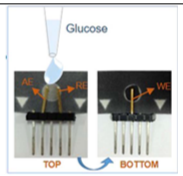
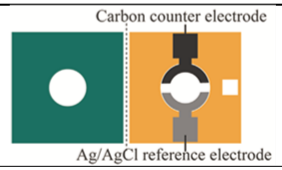
Figure 2.13 A variety of designs of μPADs integrated with colorimetric detection [59-64].

2.3.4 Applications of the μPADs

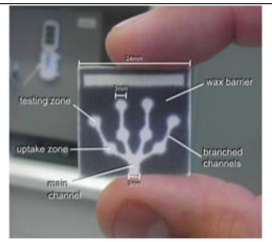
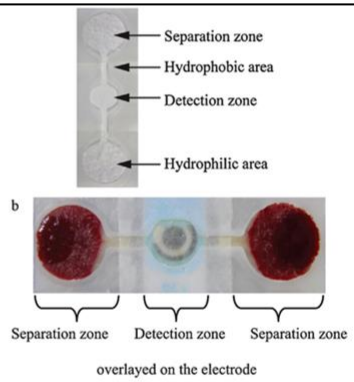
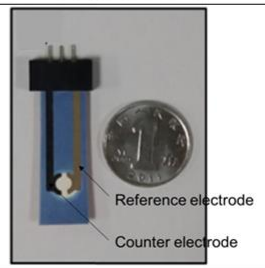
Since the μPADs were originated in 2007 [51], they have been developed and applied to test many kinds of analyte because of their portability, simplicity, inexpensiveness and ease of use. Furthermore, they are beneficial for the use of disease diagnosis with affordable price in developing countries. The μPADs have been used in many applications such as health diagnostic, environmental monitoring, biochemical analysis and food quality control. An overview of researches for each kind of applications was exemplified herein.

2.3.4.1 Health diagnosis

Table 2.3 An overview of researches for health diagnosis

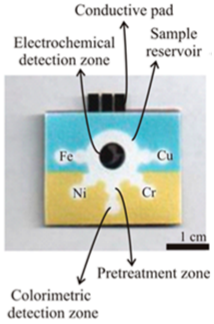
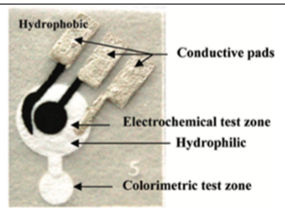
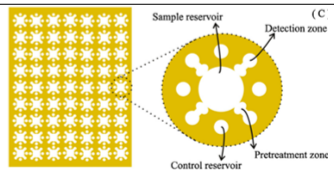
Analyte	Fabrication method	Detection method	Detection time	LOD	Linear range	Design of the device	Ref.
Uric acid	Cutting method	Chemiluminescence	-	1.9 mM	2.6-49.0 mM		[65]
Glucose	Wax printing	Electrochemistry	38 min	0.12 mM	0.3-15 mM		[66]
Breastadeno-carcinomacells	Wax printing	Electrochemilumi-nescence	-	250 cells/mL	1.0-450 x 10^7 cells/mL		[67]
Human IgG	Wax printing	Electrochemilumi-nescence	60 min	0.15 fM	1.0 fM-25 pM		[68]

An overview of researches for health diagnosis (continuous)

Analyte	Fabrication method	Detection method	Detection time	LOD	Linear range	Design of the device	Ref.
Nitrate	Wax printing	Colorimetry	15 min	10 µM	10-1000 µM		[62]
Glucose	Wax dipping	Electrochemistry	5 min	-	0-33.1 mM		[69]
Carcinoembryonic antigen (CEA)	Wax printing, screen printing	Electrochemistry	-	10 pg/mL	50 pg/mL-500 ng/mL		[70]

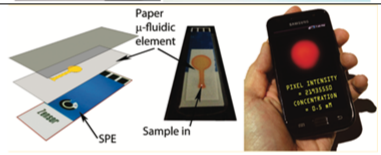
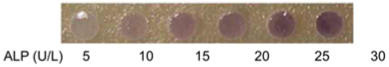
2.3.4.2 Environmental monitoring

Table 2.4 An overview of researches for environmental monitoring

Analyte	Fabrication method	Detection method	Detection time	LOD	Linear range	Design of the device	Ref.
Ni, Fe, Cu, Cr, Pb, Cd	Wax printing, screen printing	Colorimetry, electrochemistry	-	0.12 µg (Cr), 0.25 ng (Pb, Cd), 0.75 µg (Ni, Fe, Cu)	1.5-15 µg (Ni, Fe), 3.0-15 µg (Cu), 0.38-6.0 µg (Cr), 5-150 ng (Pb, Cd)		[71]
Au (III), Fe(III)	Photolithography, screen printing	Colorimetry, electrochemistry	1 min	1 ppm (Au)	1-200 ppm (Au)		[72]
Particulate Cr	Wax printing	Colorimetry	10 min	0.12 µg	0.23-3.75 µg		[73]

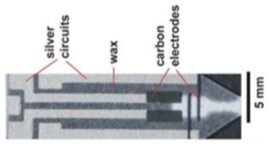
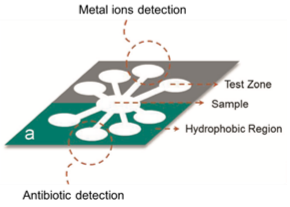
2.3.4.3 Biochemical analysis

Table 2.5 An overview of researches for biochemical analysis

Analyte	Fabrication method	Detection method	Detection time	LOD	Linear range	Design of the device	Ref.
Beta-D-galactosidase	Wax printing	Fluorescence	30 min	0.7 nM	0.7-12.0 nM		[74]
Nicotinamide adenine dinucleotide (NADH)	Inkjet printing, screen printing	Electrochemi-luminescence	-	72 µM	0.2-10 mM		[75]
Alkaline phosphatase (ALP)	Photolithography	Colorimetry	30 min	0.78 U/L	1.5-20 U/L		[76]

2.3.4.4 Food quality control

Table 2.6 An overview of researches for food quality control

Analyte	Fabrication method	Detection method	Detection time	LOD	Linear range	Design of the device	Ref.
Ethanol	Wax printing, screen printing	Electrochemistry	-	0.52 mM	0-10 mM		[77]
Ethanol	Wax printing, screen printing	Electrochemistry	-	0.2 mM	0.1-3 mM		[78]
Pentachloro-phenol	Wax-screen printing	Photoelectro-chemistry	-	4 pg/mL	0.01-100 ng/mL		[58]
Hg (II), Ag (I), antibiotic neomycin (NEO)	Wax printing	Fluorescence	10 min	121 nM (Hg (II)), 47 nM (Ag (I)), 153 nM (NEO)	0-3 µM (Hg (II)), 0-1.75 µM (Ag (I)), 0-2 µM (NEO)		[79]

2.4 Literature reviews

In 2009, Songjareon et al. [10] determined urinary creatinine using a microfluidic system integrated with a portable miniature fibre optic spectrometer. The method was based on a kinetic Jaffé reaction which is the reaction between creatinine and alkaline picrate, generating an orange-red color of creatinine-alkaline picrate complex. The reaction exhibited the absorption wavelength at 510 nm and it was investigated on poly(dimethylsiloxane) (PDMS) microchip. A linear range was in the range of 0-40 mg/L with a limit of detection of 3.3 mg/L. UV-Vis spectrophotometry based on Jaffé reaction was used to validate the proposed method.

In 2013, Tymecki et al. [80] developed a photometric device equipped with a paired emitter detector diode (PEDD) for assaying of creatinine in serum and urine using Jaffé method. The PEDD device was integrated with LED-based emitter and LED-based detector at the wavelength of 505 nm and 525 nm, respectively, and also integrated with multicommutated flow analysis (MCFA) system. The PEDD-based MCFA system for the assay of creatinine provided the determination of creatinine concentrations in submillimolar range with a limit of detection at ppm level.

In 2015, Talalak et al. [81] developed enzymatic paper-based analytical devices (enz-PADs) for colorimetric detection of creatinine in urine. The method was based on the formation of hydrogen peroxide (H_2O_2) by conversion of creatinine using creatininase, creatinase and sarcosine oxidase. The generated H_2O_2 reacts with 4-amino-phenazone and 2,4,6-triiodo-3-hydroxybenzoic acid thus generating quinoneimine which is a pink-red color. A linear range for the determination of creatinine was in the range of 2.5-25 mg/dL with a limit of detection of 2.0 mg/dL. Alkaline picrate method was used to compare to the proposed method for the analysis of creatinine. The fabrication of the enz-PADs and the procedure of creatinine assay are displayed in Figure 2.14.

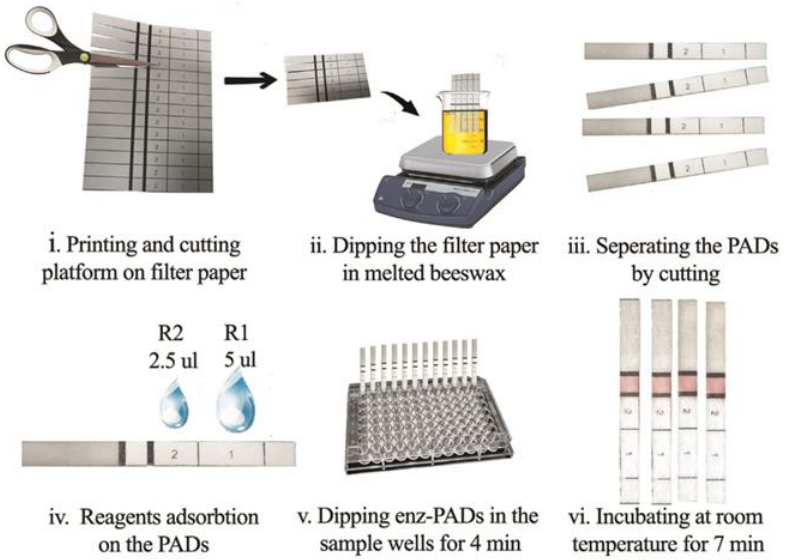


Figure 2.14 Fabrication of the enz-PADs and procedure of creatinine assay.

In 2015, Debus et al. [5] proposed two home-made platforms based on a CD Spectroscope (CDS) and Computer Screen Photo-assisted Technique (CSPT) for creatinine assay in human urine (Figure 2.15). CDS and CSPT systems for the assay of creatinine exhibited a linear range from 160 µM to 1.6 mM with limits of detection of 89 µM and 111 µM, respectively. The performance of both systems was validated using capillary electrophoresis.

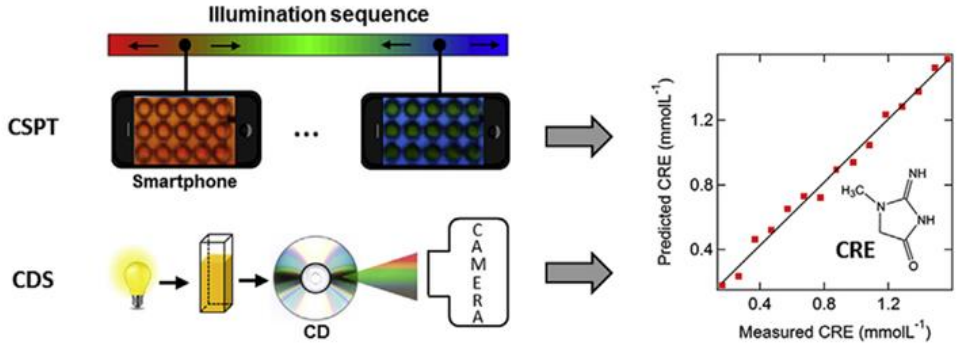


Figure 2.15 Scheme for CSPT and CDS based on Jaffé reaction.

In 2016, Sittiwong and Unob [82] developed a paper-based platform for detection of creatinine in urine. As shown in Figure 2.16, creatinine was extracted via an ion-exchange mechanism using 3-propylsulfonic acid trimethoxysilane coated on the paper as a reagent. The extracted creatinine was then detected by Jaffé reaction,

resulting in the formation of a yellow-orange color of creatinine alkaline picrate complex. A linear range for the detection of creatinine was in the range of 10-60 mg/L with a limit of detection of 4.2 mg/L. The proposed method was validated using the spectrophotometric method.

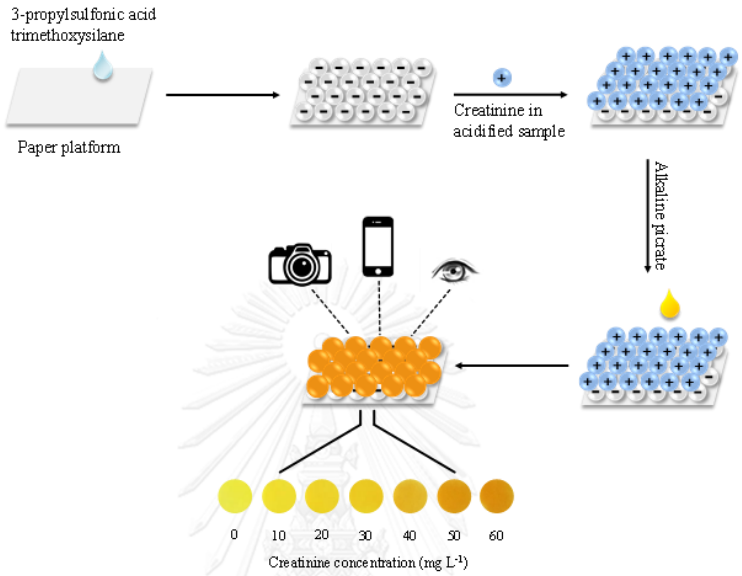


Figure 2.16 Procedure for creatinine detection using the paper platform.

CHAPTER III

EXPERIMENTAL

3.1 Chemicals and apparatus

3.1.1 Chemicals

Table 3.1 List of chemicals used in this work.

Chemicals	Suppliers
Picric acid	Sigma-Aldrich, Germany
Creatinine	
Urea	
Bovine serum albumin (BSA)	
Magnesium sulfate ($MgSO_4$)	Merck, Germany
Sodium hydroxide (NaOH)	
Potassium chloride (KCl)	
Sodium sulfate (Na_2SO_4)	
Sodium dihydrogen phosphate monohydrate ($NaH_2PO_4 \cdot H_2O$)	
Ascorbic acid (AA)	BDH, UK
Sodium oxalate ($Na_2C_2O_4$)	
Disodium hydrogen phosphate (Na_2HPO_4)	
Glucose (Glc)	Carlo Erba, France
Sodium chloride (NaCl)	
Calcium chloride ($CaCl_2$)	M&B, UK
Sodium bicarbonate ($NaHCO_3$)	
Trisodium citrate dihydrate ($Na_3C_6H_5O_7 \cdot 2H_2O$)	Fisher Scientific, UK
Ammonium chloride (NH_4Cl)	Ajax Finechem, Australia
Uric acid (UA)	Wako, Japan

3.1.2 Apparatus

Table 3.2 List of the apparatus used in this work.

Apparatus	Suppliers
UV-Visible spectrophotometer (HP HEWLETT PACKARD 8453)	Agilent, UK
Digital camera (Canon EOS 1000D)	Canon
Whatman No.1 qualitative filter paper	GE Healthcare
Wax printer (Xerox ColorQube 8570)	Xerox, Japan
Vortex mixer (MIXER UZUSIO VTX-3000L)	LMS
Balance	Mettler Toledo
Hot plate (C-MAG HS 10)	IKA
Centrifuge (Hettich UNIVERSAL 320 R)	Hettich
Centrifuge tubes	Plasmed
Microtubes	Plasmed
Micropipettes and tips	Eppendorf, Germany
Quartz cuvette	
Glasswares	

3.2 Preparations of chemicals

3.2.1 Preparation of colorimetric reagent solution

3.2.1.1 0.04 M picric acid solution

A 0.04 M picric acid solution was prepared by dissolving 0.09164 g of picric acid in 10 mL of DI water.

3.2.1.2 2 M sodium hydroxide solution

A 2 M sodium hydroxide solution was prepared by dissolving 0.80000 g of sodium hydroxide in 10 mL of DI water.

3.2.1.3 Alkaline picrate reagent solution

An alkaline picrate reagent solution was prepared by mixing the 0.04 M picric acid solution and the 2 M sodium hydroxide solution in the ratio of 1:1.

3.2.2 Preparation of creatinine solution

A 1 mM stock creatinine solution was prepared by dissolving 1.13 mg of creatinine in 10 mL of DI water.

3.2.3 Preparation of solutions for the investigation of selectivity of the creatinine determination

3.2.3.1 25 mM urea solution

A 25 mM urea solution was prepared by dissolving 7.51 mg of urea in 5 mL of DI water.

3.2.3.2 5 mM uric acid (UA) solution

A 5 mM uric acid solution was prepared by dissolving 4.20 mg of uric acid in 5 mL of DI water.

3.2.3.3 5 mM trisodium citrate ($Na_3C_6H_5O_7$) solution

A 5 mM trisodium citrate solution was prepared by dissolving 7.36 mg of trisodium citrate dihydrate in 5 mL of DI water.

3.2.3.4 5 mM potassium chloride (KCl) solution

A 5 mM potassium chloride solution was prepared by dissolving 1.86 mg of potassium chloride in 5 mL of DI water.

3.2.3.5 5 mM ammonium chloride (NH_4Cl) solution

A 5 mM ammonium chloride solution was prepared by dissolving 1.34 mg of ammonium chloride in 5 mL of DI water.

3.2.3.6 5 mM calcium chloride ($CaCl_2$) solution

A 5 mM calcium chloride solution was prepared by dissolving 2.77 mg of calcium chloride in 5 mL of DI water.

3.2.3.7 5 mM magnesium sulfate ($MgSO_4$) solution

A 5 mM magnesium sulfate solution was prepared by dissolving 3.01 g of magnesium sulfate in 5 mL of DI water.

3.2.3.8 5 mM sodium bicarbonate ($NaHCO_3$) solution

A 5 mM sodium bicarbonate solution was prepared by dissolving 2.10 mg of sodium bicarbonate in 5 mL of DI water.

3.2.3.9 5 mM sodium oxalate ($Na_2C_2O_4$) solution

A 5 mM sodium oxalate solution was prepared by dissolving 3.35 mg of sodium oxalate in 5 mL of DI water.

3.2.3.10 5 mM sodium sulfate (Na_2SO_4) solution

A 5 mM sodium sulfate solution was prepared by dissolving 3.55 mg of sodium sulfate in 5 mL of DI water.

3.2.3.11 5 mM ascorbic acid (AA) solution

A 5 mM ascorbic acid solution was prepared by dissolving 4.40 mg of ascorbic acid in 5 mL of DI water.

3.2.3.12 5 mM glucose (Glu) solution

A 5 mM glucose solution was prepared by dissolving 4.50 mg of glucose in 5 mL of DI water.

3.2.3.13 5 mM bovine serum albumin (BSA) solution

A 5 mM albumin solution was prepared by dissolving 1.66092 g of bovine serum albumin in 5 mL of DI water.

3.2.4 Preparation of artificial urine

The artificial urine solution was prepared by mixing the following substances which their concentrations are within the normal ranges of human urine. Table 3.3 shows the components and their concentrations in the artificial urine prepared in DI water.

Table 3.3 Components and their concentrations in the artificial urine [83].

Composition	Concentration (mM)
Urea	400
UA	1.00
$Na_3C_6H_5O_7 \cdot 2H_2O$	5.00
NaCl	54.00
KCl	30.00
NH_4Cl	15.00
$CaCl_2$	3.00
$MgSO_4$	2.00
$NaHCO_3$	2.00
$Na_2C_2O_4$	0.10
Na_2SO_4	9.00
$NaH_2PO_4 \cdot H_2O$	3.60
Na_2HPO_4	3.60
AA	11.50
Glc	16.75

3.3 Design and fabrication of μPADs

The μPADs (Figure 3.1) were designed using Microsoft PowerPoint. A pattern of the μPADs that is hydrophilic part comprised a sample zone and eight detection zones so as to obtain eight replicates of measurement simultaneously and hydrophobic part comprised the blue color with red, green and blue (RGB) values of 0, 153 and 255, respectively. The size of each device is approximately 2.50 x 2.50 cm and the diameters

of the sample zone and detection zone are 1.20 cm and 0.40 cm, respectively. After the design of the μPADs, they were fabricated using wax printing as fabrication method. For the fabrication process (Figure 3.2), a masking pattern was printed onto the surface of Whatman No.1 filter paper using a wax printer (Xerox ColorQube 8570). The wax-patterned paper was heated using a hot plate at 175°C for 40 s, then the wax was melted and penetrated through the paper thereby generating a hydrophobic barrier. The heated paper was subsequently covered with a transparent tape at the back side to prevent leakage of solution through the paper. This fabrication method is simple, fast and suitable for high throughput production with 48 devices per batch.

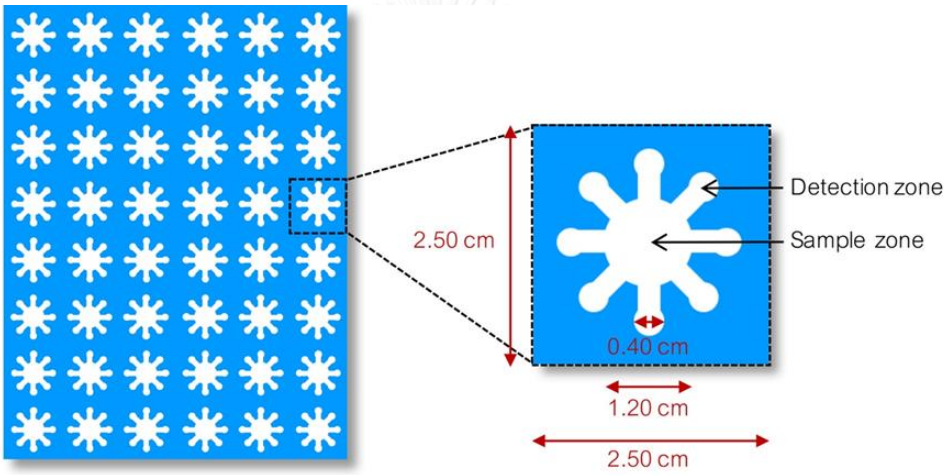


Figure 3.1 Design of the μPADs.

Figure 3.2 Fabrication process of the μPADs.

3.4 Colorimetric detection of creatinine

The experimental procedure is schematically demonstrated in Figure 3.3. To prepare the colorimetric reagent solution, 0.04 M picric acid and 2 M sodium hydroxide were mixed together (alkaline picrate reagent solution). Firstly, 1.50 μL of alkaline picrate reagent solution was dropped onto each detection zone, followed by a 50 μL

addition of creatinine standard solution onto the sample zone. The solution is then directed to the detection zones by capillary action and allowed to incubate for 25 min. Finally, the images of μPADs were captured using a digital camera (Canon EOS 1000D) in a light-controlled box. For blank test, DI water was used as a blank solution.

Figure 3.3 The experimental procedure for the colorimetric determination of creatinine. (a) Alkaline picrate reagent solution was added onto each detection zone of the μPAD. (b) Creatinine solution was then added onto the sample zone and flow to the detection zones. (c) The reaction color changed from yellow to orange.

3.5 Image processing for quantitation

To quantify the intensity of the developed color, ImageJ software was utilized. For the image processing (Figure 3.4), a color threshold was applied and hue was adjusted (100 – 255) in order to remove the wax background. The images were then converted to 8-bit and inverted; the darkness of color was proportional to the color intensity. After the inversion, the color intensity was measured at each detection zone and an average of the eight color intensity values was calculated. The average color intensity value was then subtracted by an average baseline intensity value (ΔI).

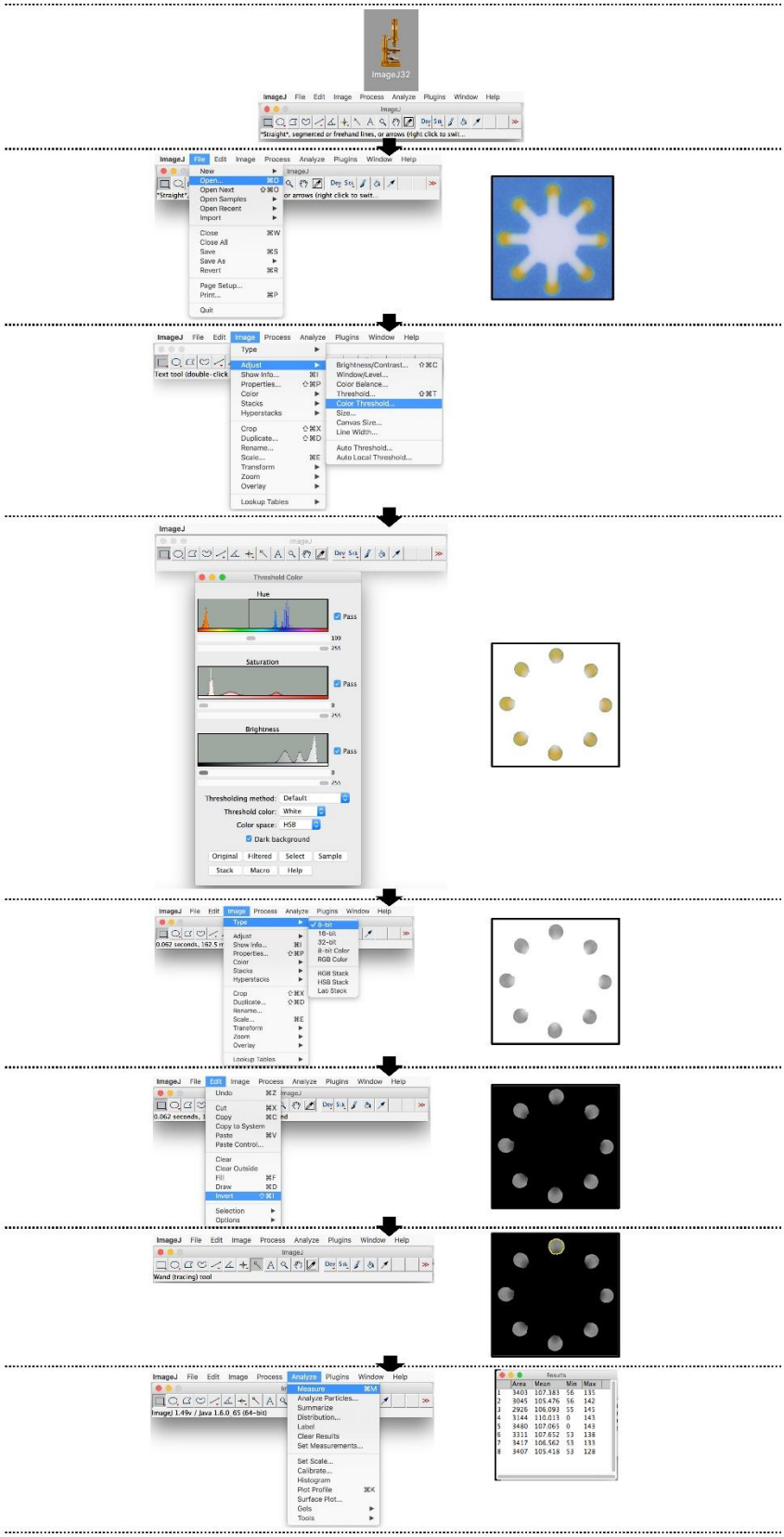


Figure 3.4 Image processing for quantitation using ImageJ software.

3.6 Optimization of concentrations of colorimetric reagent solution

Concentrations of picric acid and sodium hydroxide solution were optimized to provide the highest values of color intensity. A 0.4 mM creatinine standard solution was used as the test solution.

3.6.1 Picric acid solution

Concentrations of picric acid solution were investigated in the concentrations of 0.01, 0.02, 0.03, 0.04 and 0.05 M. The alkaline picrate reagent solutions were prepared by mixing these picric acid solutions and 2 M sodium hydroxide solutions in the ratio of 1:1 in the individual microtubes.

3.6.2 Sodium hydroxide solution

Concentrations of sodium hydroxide solution were investigated in the concentrations of 1, 2, 3, 4 and 5 M. The alkaline picrate reagent solutions were prepared by mixing these sodium hydroxide solutions and 0.04 M picric acid solutions in the ratio of 1:1 in the individual microtubes.

3.7 Optimization of ratios of picric acid to sodium hydroxide

Ratios of picric acid to sodium hydroxide were optimized to provide the highest values of color intensity. The ratios of picric acid to sodium hydroxide were investigated at 5:1, 4:1, 3:1, 2:1, 1:1. 1:2, 1:3, 1:4, 1:5, 1:6 and 1:7, and 0.4 mM creatinine standard solutions were tested.

3.8 Effect of reaction time

The effect of reaction time was studied to provide the optimal reaction time for the determination of creatinine. In this experiment, the optimal concentration of alkaline picrate reagent solution and three concentrations (0.4, 0.7 and 1 mM) of creatinine standard solutions were tested.

3.9 Analytical performance

3.9.1 Linear range, limit of detection (LOD) and limit of quantitation (LOQ)

Under the optimal conditions, a calibration curve was constructed by plotting the concentrations of creatinine standard solutions against the subtracted color mean intensity (ΔI), thus the linear range of creatinine determination was obtained. The LOD and LOQ were calculated from the following formula:

$$LOD = \frac{3SD_{blank}}{S}$$

$$LOQ = \frac{10SD_{blank}}{S}$$

Where SD_{blank} is the standard deviation from three replications of blank measurements (n=3) and S is the slope of the calibration curve.

3.9.2 Reproducibility of the μPADs

The reproducibility of μPADs was evaluated in the term of relative standard deviation (RSD) of the subtracted color mean intensity values from seven μPADs and different concentrations (0.2, 0.6 and 1 mM) of creatinine standard solution were tested. The %RSD was calculated as follows:

$$\%RSD = \frac{SD}{\bar{X}} \times 100$$

Where SD is the standard deviation of seven values of subtracted color mean intensity and X is the average of seven values of subtracted color mean intensity.

3.9.3 Selectivity

To assess the selectivity of creatinine determination, several substances commonly found in urine were investigated. Urea solution which is the main component in urine was prepared in the concentration of 25 mM (50 times of creatinine concentration used in this experiment) and UA, $Na_3C_6H_5O_7 \cdot 2H_2O$, KCl, NH_4Cl,

$CaCl_2$, $MgSO_4$, $NaHCO_3$, $Na_2C_2O_4$, Na_2SO_4, AA and Glc solutions were prepared in the concentrations of 5 mM (10 times of creatinine concentration used in this experiment) [83]. Moreover, albumin which is a protein slightly existing in urine [84] was also tested in the concentration of 5 mM (5 times of creatinine concentration used in this experiment).

Furthermore, the effect of other substances in urine on creatinine determination was also determined by comparing between the subtracted color mean intensity of 1 mM creatinine standard solution with the substances found in urine and those of 1 mM creatinine standard solution. The studied substances in the synthetic urine sample [83] comprised of 16 mM urea [85], 0.04 mM UA, 0.2 mM $Na_3C_6H_5O_7{\cdot}2H_2O$, 2.2 mM NaCl, 1.2 mM KCl, 0.6 mM NH_4Cl, 0.12 mM $CaCl_2$, 0.08 mM $MgSO_4$, 0.08 mM $NaHCO_3$, 4 µM $Na_2C_2O_4$, 0.36 mM Na_2SO_4, 0.14 mM $NaH_2PO_4{\cdot}H_2O$, 16 µM Na_2HPO_4, 0.46 mM AA [86] and 0.67 mM Glc [87].

3.10 Determination of creatinine in artificial urine samples

Prior to the analysis of real samples, the developed µPADs were applied to determine creatinine in the prepared artificial urine samples. In this experiment, creatinine standard solutions were spiked in the concentrations of 0.4, 0.6 and 0.8 mM into the artificial urine samples. The %recovery was calculated from the following formula:

$$\%\text{Recovery} = \frac{C_{\text{spiked sample}} - C_{\text{unspiked sample}}}{C_{\text{added}}} \times 100$$

Where C is the concentration of analyte in a sample.

3.11 Determination of creatinine in urine samples

To determine creatinine in urine samples, human urine samples were collected from healthy volunteers. The urine samples were centrifuged at 5000 rpm for 30 min and diluted at least 25-fold with DI water. The analytical performance of the proposed method was examined using the spiked method by spiking creatinine standard

solutions at the concentrations of 0.15, 0.30 and 0.45 mM into the urine samples. The %recovery was then calculated.

3.12 Validation of the proposed method

To validate the accuracy of the proposed method, the conventional Jaffé method was used [41, 88]. The solution containing 2.5 mL of creatinine standard solution or diluted urine samples, 1 mL of 0.04 M picric acid and 1 mL of 0.75 M sodium hydroxide was incubated at room temperature for 30 min before determination using a UV-Visible spectrophotometer at the absorbance of 490 nm.

CHAPTER IV
RESULTS AND DISCUSSION

4.1 UV-Vis absorption spectrum of the creatinine-alkaline picrate complex

Upon the addition of creatinine to the alkaline picrate reagent solution, the color of the solution changed from yellow to orange due to the interaction of the methylene anion of creatinine to the picric acid at the meta position, resulting in the formation of a creatinine-alkaline picrate complex [43]. This solution of creatinine-alkaline picrate complex exhibited a maximum absorption wavelength at 490 nm. Figure 4.1 shows the spectrum of the creatinine-alkaline picrate complex obtained from a UV-Vis spectrophotometer.

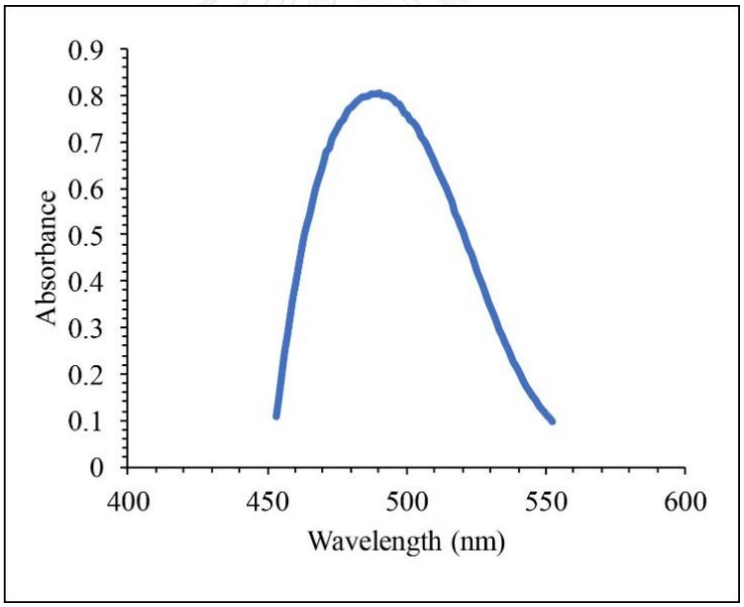


Figure 4.1 The UV-Vis absorption spectrum of the creatinine-alkaline picrate complex.

4.2 Optimization of the concentrations of colorimetric reagent solution

The concentrations of colorimetric reagent solution, i.e., picric acid and sodium hydroxide solutions were investigated. In this experiment, several μPADs were prepared by adding the mixture of 0.01, 0.02, 0.03, 0.04 and 0.05 M picric acid solution and 2 M sodium hydroxide solution onto the detection zones of individual μPADs. On the other hand, the concentration of picric acid solution was fixed (0.04 M) and the concentrations of sodium hydroxide solution were varied (1, 2, 3, 4 and 5 M). The colorimetric product was formed with the introduction of 0.4 mM creatinine. The results, demonstrated in Figure 4.2, show that the concentrations of picric acid and sodium hydroxide solution at 0.04 M and 2 M provided the highest color intensity. Therefore, these concentrations were used for the colorimetric detection of creatinine in the following experiments.

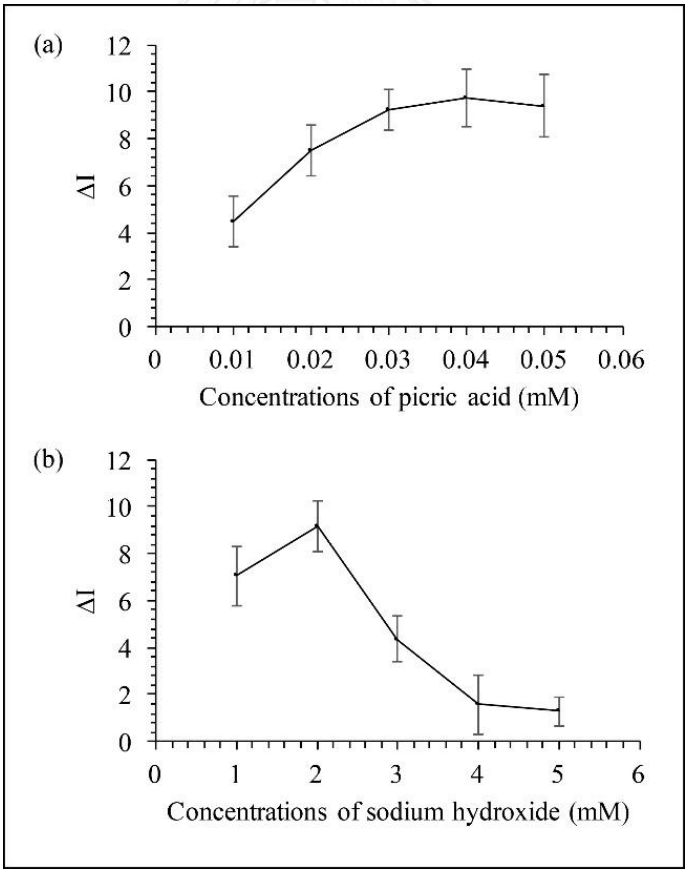


Figure 4.2 Optimization of the concentrations of (a) picric acid and (b) sodium hydroxide.

4.3 Optimization of ratios of picric acid to sodium hydroxide

The ratios of picric acid to sodium hydroxide were optimized to provide the best analytical performance. In this experiment, the optimal concentrations of picric acid and sodium hydroxide solution were used. The effect of sodium hydroxide on the assay was investigated by varying the volumes of sodium hydroxide while the volume of picric acid was constant. The results (Figure 4.3a) show that the subtracted color mean intensities (ΔI) were not significantly different with the different ratios of picric acid to sodium hydroxide because the sodium hydroxide solution was in excess. When the volumes of picric acid solution were increased and the volume of sodium hydroxide kept constant, the results (Figure 4.3b) show that the subtracted color mean intensities decreased due to a decrease in alkaline condition. Therefore, the optimal ratio of picric acid to sodium hydroxide is 1:1.

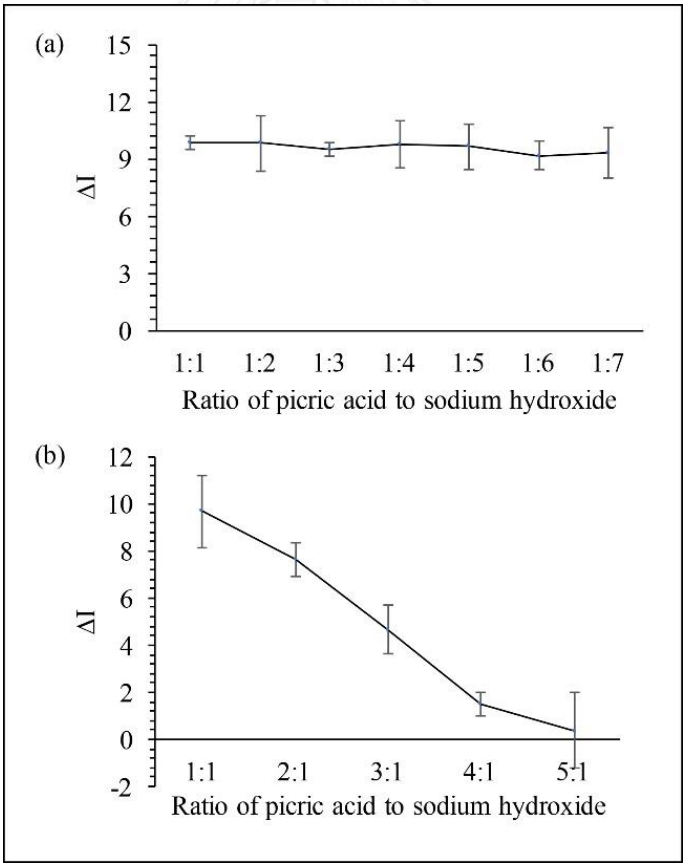


Figure 4.3 Optimization of ratios of picric acid to sodium hydroxide.

4.4 Effect of reaction time

Under the optimal concentrations of colorimetric reagent solution, the reaction time of the colorimetric reaction was investigated by adding creatinine solutions at the concentrations of 0.4, 0.7, 1 mM onto the sample zones of the prepared μPADs. The reaction time was instantly recorded when the creatinine solution reached detection zones, then the μPADs were captured using a digital camera for 30 min at 1-min intervals. As shown in Figure 4.4, the color intensity was developed in proportional with time until 24 min and was almost steady after 25 min. Accordingly, the optimal reaction time of the colorimetric reaction for creatinine determination was defined at 25 min.

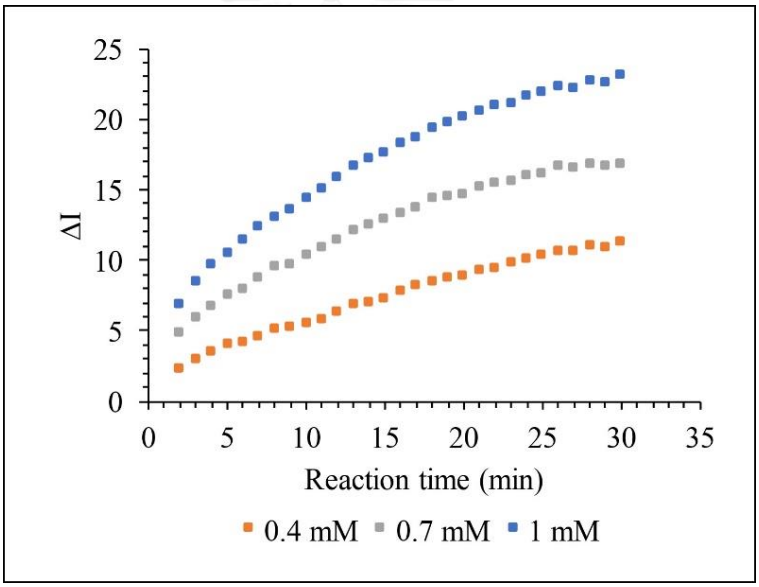


Figure 4.4 Effect of reaction time on the determination of creatinine. Three concentration levels of creatinine were studied.

The optimal conditions for the determination of creatinine are summarized in Table 4.1.

Table 4.1 Optimal conditions for the determination of creatinine.

Experimental parameter	Studied range	Optimal condition
Concentration of picric acid	0.01-0.05 M	0.04 M
Concentration of sodium hydroxide	1-5 M	2 M
Ratio of picric acid to sodium hydroxide	1:1-1:7	1:1
	1:1-5:1	
Reaction time	1-30 min	25 min

4.5 Linear range, limit of detection and limit of quantitation

Under the optimal conditions, a calibration curve (Figure 4.5) was constructed by plotting the concentrations of creatinine standard solutions against the subtracted color mean intensity. A wide linear range for the determination of creatinine was obtained in the range of 0.2-1 mM (R^2=0.99857) with a limit of detection and a limit of quantitation of 0.08 mM and 0.26 mM, respectively. The limit of detection was obtained from three times the standard deviations of the color intensity from the blank divided by the slope of the calibration curve and the limit of quantitation was obtained from ten times the standard deviations of the color intensity from the blank divided by the slope of the calibration curve. As shown in Figure 4.6, the color intensity of the creatinine-alkaline picrate complex which is clearly visible at the detection zones of the μPADs is proportional to the concentrations of creatinine.

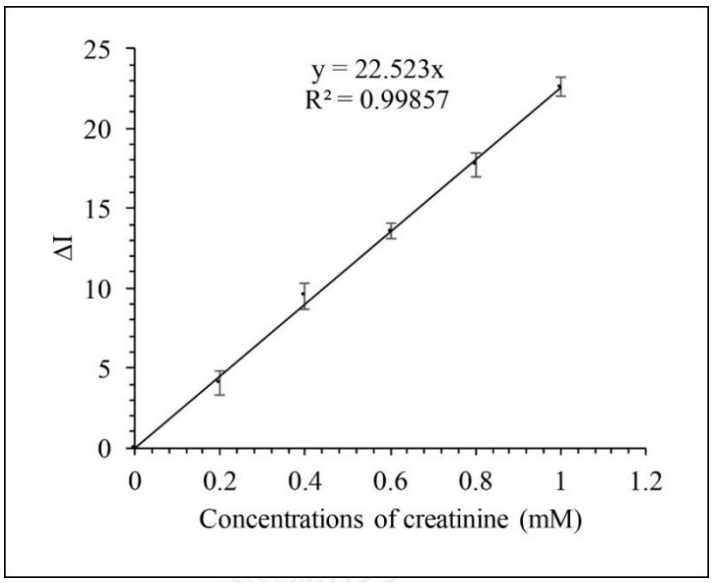


Figure 4.5 Calibration curve for the determination of creatinine. The linear range was 0.2–1 mM with the limit of detection and the limit of quantitation of 0.08 mM and 0.26 mM, respectively.

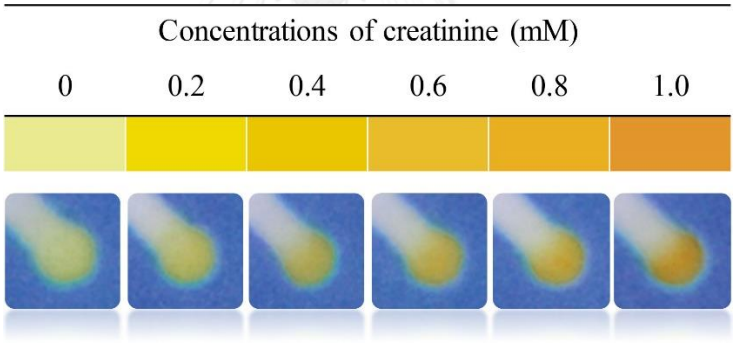


Figure 4.6 Color scheme of the Jaffé reaction with creatinine concentrations of 0.2, 0.4, 0.6, 0.8 and 1 mM. The color intensity of the creatinine-alkaline picrate complex which is clearly visible at the detection zones of the μPADs is proportional to the concentrations of creatinine.

4.6 Reproducibility of the μPADs

The reproducibility of the μPADs was evaluated in the term of relative standard deviation (RSD) of the subtracted color mean intensity values from seven μPADs and different concentrations (0.2, 0.6 and 1 mM) of creatinine standard solution were tested. The results, demonstrated in Figure 4.7, show that this method provided a good precision with %RSD of 2.85, 1.10 and 1.64% for the determination of creatinine at 0.2, 0.6 and 1 mM, respectively.

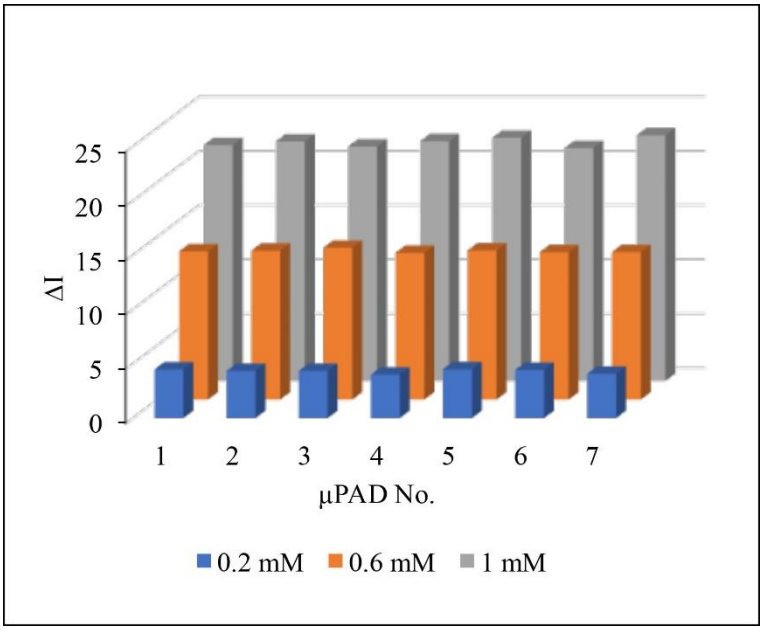


Figure 4.7 Reproducibility of the μPADs with several μPADs and different concentrations of creatinine. The %RSDs are 2.85, 1.10 and 1.64% for the determination of creatinine at 0.2, 0.6 and 1 mM, respectively.

4.7 Selectivity

To investigate the selectivity of the colorimetric determination of creatinine, various substances commonly found in urine [83] including urea, UA, $Na_3C_6H_5O_7{\cdot}2H_2O$, KCl, NH_4Cl, $CaCl_2$, $MgSO_4$, $NaHCO_3$, $Na_2C_2O_4$, Na_2SO_4, AA and Glc were tested. In addition, albumin which is a protein slightly existing in urine [84] was also tested by adding 50 μL of these solutions onto sample zones of the individual μPADs. The reaction time was instantly recorded when the solutions reached detection zones. After 25-min incubation, the μPADs were captured using a digital camera and the images were

analyzed by ImageJ software in order to quantify the color intensity. The subtracted color mean intensities of the creatinine standard and other substances were shown in Figure 4.8 and 4.9. As clearly seen, only creatinine can change the reaction color from yellow to orange. Therefore, the proposed method was evidently selective for the determination of creatinine in urine samples.

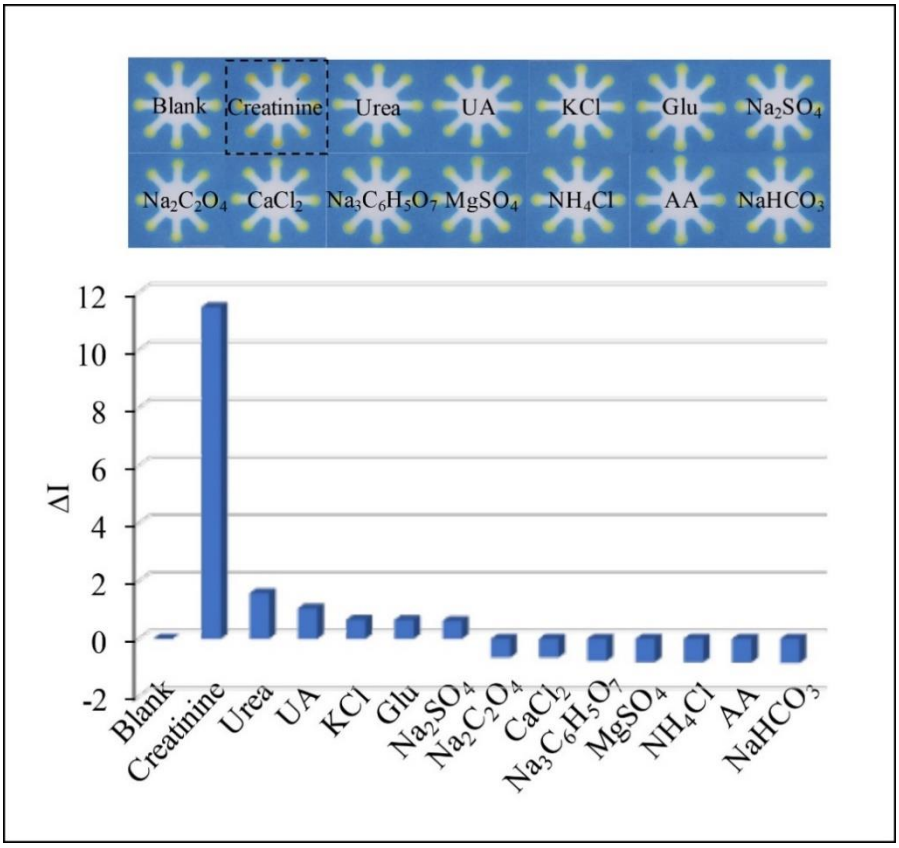


Figure 4.8 Selectivity of the determination of creatinine. 50 µL of 0.5 mM creatinine, 25 mM urea and 5 mM UA, $Na_3C_6H_5O_7{\cdot}2H_2O$, KCl, NH_4Cl, $CaCl_2$, $MgSO_4$, $NaHCO_3$, $Na_2C_2O_4$, Na_2SO_4, AA and Glc were tested. According to the photographs of the µPADs, only creatinine can change reaction color from yellow to orange.

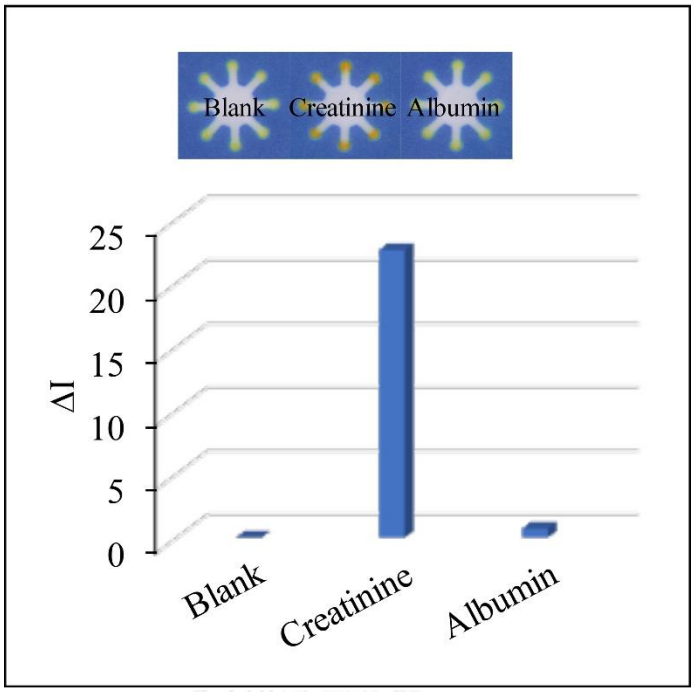


Figure 4.9 Selectivity of the determination of creatinine. 50 µL of 1 mM creatinine and 5 mM albumin were tested. According to the photographs of the µPADs, only creatinine can change reaction color from yellow to orange.

Furthermore, the effect of other substances in urine on creatinine determination was also determined by adding various contaminants commonly found in urine into 1 mM creatinine solution. The color intensity of 1 mM creatinine with interfering substances was compared to the color intensity of 1 mM creatinine. The results reported in Figure 4.10 show that the studied substances in the artificial urine sample [83] comprising of 16 mM urea [85], 0.04 mM UA, 0.2 mM $Na_3C_6H_5O_7{\cdot}2H_2O$, 2.2 mM NaCl, 1.2 mM KCl, 0.6 mM NH_4Cl, 0.12 mM $CaCl_2$, 0.08 mM $MgSO_4$, 0.08 mM $NaHCO_3$, 4 µM $Na_2C_2O_4$, 0.36 mM Na_2SO_4, 0.14 mM $NaH_2PO_4{\cdot}H_2O$, 16 µM Na_2HPO_4, 0.46 mM AA [86] and 0.67 mM Glc [87] did not significantly interfere the creatinine quantification because the difference of the color intensities was less than 5%.

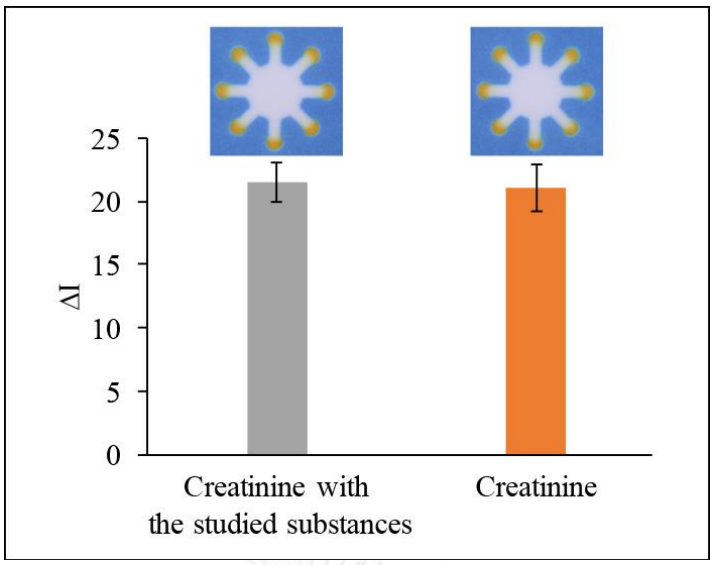


Figure 4.10 Comparison between the subtracted color mean intensity of 1 mM creatinine standard solution with the substances found in urine and those of 1 mM creatinine standard solution. The photographs show the reaction colors at the detection zones of the μPADs. The color intensities are not significantly different.

4.8 Determination of creatinine in artificial urine samples

The developed devices were applied to determine creatinine in artificial urine samples in order to show the analytical performance of the assay prior to the determination of real samples. The creatinine standard solutions were spiked in the concentrations of 0.4, 0.6 and 0.8 mM into the artificial urine samples. Table 4.2 exhibits the %recoveries and %RSDs of creatinine which are in the acceptable ranges of 97.37-100.12% and 0.86-3.16%, respectively.

Table 4.2 The recovery results of the proposed method for the determination of creatinine in artificial urine samples (n = 3).

Artificial urine	Added (mM)	Found (mM)	Recovery (%)	RSD (%)
Artificial urine 1	0	0.193 ± 0.006	-	3.16
	0.4	0.593 ± 0.015	99.96	2.46
	0.6	0.786 ± 0.010	98.21	1.22
	0.8	0.972 ± 0.008	97.37	0.86
Artificial urine 2	0	0.199 ± 0.005	-	2.51
	0.4	0.601 ± 0.010	100.12	1.59
	0.6	0.790 ± 0.009	98.40	1.10
	0.8	0.997 ± 0.009	99.66	0.95
Artificial urine 2	0	0.203 ± 0.006	-	3.01
	0.4	0.593 ± 0.013	97.68	2.16
	0.6	0.792 ± 0.010	98.24	1.31
	0.8	0.994 ± 0.014	98.94	1.38

4.9 Determination of creatinine in urine samples

To assess its applicability, the developed devices were used for the determination of creatinine in human urine samples. The analytical performance of the proposed method was examined using the spiked method by spiking creatinine standard solutions at the concentrations of 0.15, 0.30 and 0.45 mM into the urine samples. Table 4.3 exhibits the %recoveries and %RSDs of creatinine which are in the range of 91.06-102.86% and 1.27-5.77%, respectively. It was indicated that the proposed method was reliable. To demonstrate the accuracy of the proposed method, the concentrations of creatinine found in urine samples were compared to those obtained from the standard Jaffé method, i.e., UV-Vis spectrophotometry. From a paired t-test result at 95% confidence interval, the concentration results of the proposed method shown in Table 4.4 were in good agreement with those from the standard method ($t_{calculated}$ was below $t_{critical}$ (2.776)). Therefore, the proposed method is applicable for determination of creatinine in urine samples.

Table 4.3 The recovery results of the proposed method and the spectrophotometric method for the determination of creatinine in urine samples (n = 3).

Urine samples	Added (mM)	Proposed method			Spectrophotometric method		
		Found (mM)	Recovery (%)	RSD (%)	Found (mM)	Recovery (%)	RSD (%)
Sample 1	0	0.289 ± 0.012	-	4.08	0.365 ± 0.001	-	0.15
	0.15	0.441 ± 0.006	101.26	1.29	0.516 ± 0.003	100.48	0.59
	0.30	0.586 ± 0.016	98.78	2.81	0.676 ± 0.002	103.77	0.34
	0.45	0.722 ± 0.017	96.08	2.33	0.811 ± 0.002	99.14	0.19
Sample 2	0	0.336 ± 0.017	-	5.02	0.408 ± 0.008	-	1.91
	0.15	0.486 ± 0.009	100.04	1.86	0.563 ± 0.004	103.39	0.63
	0.30	0.626 ± 0.011	96.71	1.74	0.710 ± 0.003	100.80	0.42
	0.45	0.760 ± 0.044	94.19	5.77	0.862 ± 0.001	100.90	0.06
Sample 3	0	0.436 ± 0.008	-	1.81	0.451 ± 0.001	-	0.26
	0.15	0.588 ± 0.011	101.48	1.82	0.612 ± 0.002	107.40	0.31
	0.30	0.727 ± 0.012	96.98	1.71	0.751 ± 0.003	100.10	0.41
	0.45	0.846 ± 0.029	91.06	3.40	0.878 ± 0.002	94.89	0.19

The recovery results of the proposed method and the spectrophotometric method for the determination of creatinine in urine samples (n = 3) (continuous).

Urine samples	Added (mM)	Proposed method			Spectrophotometric method		
		Found (mM)	Recovery (%)	RSD (%)	Found (mM)	Recovery (%)	RSD (%)
Sample 4	0	0.363 ± 0.012	-	3.18	0.358 ± 0.003	-	0.91
	0.15	0.508 ± 0.012	96.96	2.28	0.505 ± 0.003	97.81	0.55
	0.30	0.671 ± 0.019	102.86	2.90	0.672 ± 0.005	104.52	0.67
	0.45	0.805 ± 0.021	98.37	2.60	0.816 ± 0.007	101.73	0.81
Sample 5	0	0.240 ± 0.009	-	3.90	0.247 ± 0.001	-	0.25
	0.15	0.383 ± 0.009	96.47	2.46	0.394 ± 0.001	98.04	0.18
	0.30	0.529 ± 0.007	96.89	1.27	0.546 ± 0.005	99.63	0.91
	0.45	0.673 ± 0.009	95.37	1.37	0.687 ± 0.002	97.92	0.28

Table 4.4 Concentration of creatinine in urine samples using the proposed method and the standard method (n = 3).

Urine samples	Concentration of creatinine in urine samples (mM)	
	Proposed method	Spectrophotometric method
Sample 1	9.043 ± 0.369	9.602 ± 0.014
Sample 2	10.492 ± 0.527	10.727 ± 0.205
Sample 3	13.766 ± 0.249	14.239 ± 0.037
Sample 4	9.541 ± 0.303	9.425 ± 0.086
Sample 5	6.317 ± 0.246	6.490 ± 0.016

Moreover, concentrations of creatinine in uncentrifuged urine samples were determined and compared with those in centrifuged urine samples so as to reduce the sample preparation step which is time-consuming. The results, displayed in Figure 4.11, show that there is no significant difference for each sample. Therefore, this proposed method can be done without centrifugation which can reduce time for sample preparation and suits for on-site analysis.

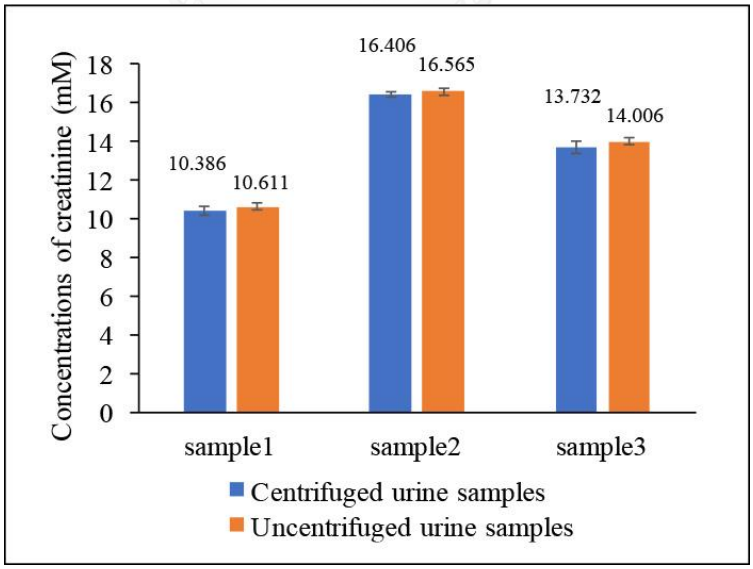


Figure 4.11 Concentrations of creatinine in centrifuged and uncentrifuged urine samples. Three urine samples were analyzed.

CHAPTER V

CONCLUSIONS

The low-cost, simple and portable μPADs for the determination of creatinine in urine samples were successfully developed. In this work, the reagent and sample solutions required only are in the micro-scale amount and the external instruments are not necessary. The fabrication process of the developed devices is rapid and easy. The materials were also eco-friendly and readily available. The μPADs detection scheme was based on a simple Jaffé reaction which gave an orange product. In the presence of creatinine, a change of color at the detection zones can be easily observed by the naked eyes or simply processed with a digital camera. Under the optimal conditions, the calibration curve was constructed and a wide linear range was obtained in the range of 0.2-1 mM (R^2=0.99857) with the limit of detection and the limit of quantitation of 0.08 mM and 0.26 mM, respectively. In addition, the proposed method provided a good reproducibility and selectivity. The accuracy of the proposed method was not significantly different from the UV-Visible spectrophotometric method by a paired t-test at 95% confidence interval. Finally, the μPADs were successfully applied for the determination of creatinine in urine samples and were demonstrated a great potential to be used for clinical laboratory.

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APPENDIX

APPENDIX A

COMPOSITION OF ARTIFICIAL URINE

Table A1 Physiological ranges of compositions of normal human urine [83].

Composition	Concentration (mM)
Urea	200
UA	1.00
Creatinine	4.00
$Na_3C_6H_5O_7$	5.00
NaCl	54.00
KCl	30.00
NH_4Cl	15.00
$CaCl_2$	3.00
$MgSO_4$	2.00
$NaHCO_3$	2.00
$Na_2C_2O_4$	0.10
Na_2SO_4	9.00
NaH_2PO_4	3.60
Na_2HPO_4	0.40

APPENDIX B

PRECISION AND ACCURACY

Table B1 Acceptable values for reproducibility obtained from AOAC guidelines for single laboratory [89].

Concentration of analyte	RSD (%)
100%	2
10%	3
1%	4
0.1%	6
0.01%	8
10 µg/g (ppm)	11
1 µg/g	16
10 µg/kg (ppb)	32

Table B2 Acceptable recovery obtained from AOAC guidelines for single laboratory [89].

Concentration of analyte	Recovery (%)
100%	98-101
10%	95-102
1%	92-105
0.10%	90-108
0.01%	85-110
10 µg/g (ppm)	80-115
1 µg/g	75-120
10 µg/kg (ppb)	70-125

VITA

Miss Suphanan Sununta was born in Chiang Mai, Thailand, on June 20, 1992. She was enrolled in the Department of Chemistry, Faculty of Science, Chulalongkorn University, Bangkok, Thailand in 2010 and received her Bachelor's degree of Science with second class honors, majoring in Chemistry in 2013. After that, she graduated with a Master's degree of Science, majoring in Chemistry (Analytical Chemistry) in academic year 2016 from Chulalongkorn University, Bangkok, Thailand.

Her proceeding paper was entitled "Determination of creatinine in urine samples using microfluidic paper-based analytical devices" (Suphanan Sununta, Poomrat Rattanarat, Orawon Chailapakul and Narong Praphairaksit) which won the SciFinder Best Paper Award in Poster Session. It is the proceedings of the Pure and Applied Chemistry International Conference 2017 (PACCON 2017) held on February 2-3, 2017 at Centra Government Complex Hotel & Convention Centre, Chaeng Watthana, Bangkok, Thailand.